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Biotransformation of naringin and naringenin by cultured *Eucalyptus perriniana* cells

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ABSTRACT

The biotransformation of naringin and naringenin was investigated using cultured cells of *Eucalyptus perriniana*. Naringin (**1**) was converted into naringenin 7-*O*-β-D-glucopyranoside (**2**, 15%), naringenin (**3**, 1%), naringenin 5,7-*O*-β-D-diglucopyranoside (**4**, 15%), naringenin 4′,7-*O*-β-D-diglucopyranoside (**5**, 26%), naringenin 7-*O*-[6-*O*-(β-D-glucopyranosyl)]-β-D-glucopyranoside (**6**, β-gentiobioside, 5%), naringenin 7-*O*-[6-*O*-(α-L-rhamnopyranosyl)]-β-D-glucopyranoside (**7**, β-rutinoside, 3%), and 7-*O*-β-D-gentiobiosyl-4′-*O*-β-D-glucopyranosylnaringenin (**8**, 1%) by cultured cells of *E. perriniana*. On the other hand, **2** (14%), **4** (7%), **5** (13%), **6** (2%), **7** (1%), naringenin 4′-*O*-β-D-glucopyranoside (**9**, 4%), naringenin 5-*O*-β-D-glucopyranoside (**10**, 2%), and naringenin 4′,5-*O*-β-D-diglucopyranoside (**11**, 5%) were isolated from cultured *E. perriniana* cells, that had been treated with naringenin (**3**). Products, 7-*O*-β-D-gentiobiosyl-4′-*O*-β-D-glucopyranosylnaringenin (**8**) and naringenin 4′,5-*O*-β-D-diglucopyranoside (**11**), were hitherto unknown.

1. Introduction

Plant cells can conjugate sugar residues not only to endogenous metabolic intermediates but also to xenobiotics. Glycosylation reactions catalyzed by plant glycosyltransferases have diverse functions in plants, i.e., increasing the solubility and stability of the aglycones, decreasing the toxicity of xenobiotics, and activating natural compounds such as saponins. On the other hand, plant cultured suspension cells are ideal systems for studying biotransformation of exogenous organic compounds. Particularly, glycosylation is a characteristic biotransformation reaction by cultured plant cells. Recently, several papers on the biotransformation of phenolic compounds by cultured suspension cells of *Eucalyptus perriniana* reported that *E. perriniana* cell cultures had high potential to produce their β-glycosides such as β-glucoside, β-gentiobioside, and β-rutinoside of tocopherols, hesperetin, and daidzein (Shimoda et al., 2007c, 2008a,b).

Citrus flavanones, naringenin and hesperetin, occur naturally as the glycosides, naringenin 7-*O*-[2-*O*-(α-L-rhamnopyranosyl)]-β-D-glucopyranoside (naringin **1**) and hesperetin 7-*O*-[6-*O*-(α-L-rhamnopyranosyl)]-β-D-glucopyranoside (hesperidin) (Rousseff et al., 1987; Shaw et al., 1991). These glycosides as well as their aglycones have been studied in terms of their pharmaceutical properties, including anticarcinogenic, antiviral, anti-inflammatory, and anticancer activities (Borradaile et al., 1999; Zhang et al., 2000; Garg et al., 2001; Chiba et al., 2003; Miller et al., 2008; Nafisi et al., 2008; Wu et al., 2008). Earlier, Lewinsohn et al. reported that cultured cells of *Citrus paradisi* converted hesperetin into its 7-*O*-glucoside and hesperidin (Lewinsohn et al., 1986, 1989). However, little attention has been given to the biotransformation of naturally occurring glycosides such as naringin (**1**) and hesperidin by cultured plant cells. We report herein the biotransformation of naringin (**1**) with plant cell cultures of *E. perriniana* to naringenin 7-*O*-glucoside (**2**), naringenin (**3**), naringenin 4′,7-*O*-diglucoside (**5**), naringenin 5,7-*O*-diglucoside (**4**), naringenin 7-*O*-gentiobioside (**6**), naringenin 7-*O*-rutinoside (**7**), and 7-*O*-gentiobiosyl-4′-*O*-glucosylnaringenin (**8**), and biotransformation of naringenin (**3**) to its 4′-*O*-glucoside (**9**), 5-*O*-glucoside (**10**), 7-*O*-glucoside (**2**), 5,7-*O*-diglucoside (**4**), 4′,7-*O*-diglucoside (**5**), 4′,5-*O*-diglucoside (**11**), 7-*O*-gentiobioside (**6**), and 7-*O*-rutinoside (**7**), respectively.

2. Results and discussion

Naringin (**1**) was subjected to biotransformation by cultured cells of *E. perriniana*. Incubation of **1** with cultured *E. perriniana*

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cells for 5 days yielded seven products **2**–**8**. There were no glycoside products in the medium. The control cell cultures, which had not been treated with naringin (**1**), were subjected to the same extraction procedures and HPLC analyses, and neither naringin (**1**) nor derivatives thereof were detected in the cells and in the medium despite careful HPLC analyses. The structures of the products were elucidated through use of HRFABMS, ^{1}H and ^{13}C NMR (Table 1), H–H COSY, C–H COSY, HMBC spectroscopic analyses, and the products were identified as naringenin 7-*O*-β-D-glucopyranoside (**2**, 15%) (Hai et al., 2004), naringenin (**3**, 1%), naringenin 5,7-*O*-β-D-diglucopyranoside (**4**, 15%) (Hai et al., 2004), naringenin 4′,7-*O*-β-D-diglucopyranoside (**5**, 26%) (Liu et al., 2006), naringenin 7-*O*-[6-*O*-(β-D-glucopyranosyl)]-β-D-glucopyranoside (β-gentiobioside, **6**, 5%) (Miura et al., 1986), naringenin 7-*O*-[6-*O*-(α-L-rhamnopyranosyl)]-β-D-glucopyranoside (β-rutinoside (narirutin), **7**, 3%) (Shaw et al., 1991), and 7-*O*-β-D-gentiobiosyl-4′-*O*-β-D-glucopyranosylnaringenin (**8**, 1%), which has not been identified before.

Table 1
^{13}C chemical shifts of glycosylation products **2** and **4**–**11** in DMSO-d_6.

Product		**2**	**4**	**5**	**6**	**7**	**8**
Aglycone	2	78.5	78.4	78.5	78.3	78.5	78.5
	3	42.1	42.0	41.9	42.0	42.0	42.1
	4	196.7	196.7	196.6	196.7	196.7	196.7
	5	163.4	163.6	163.5	163.3	163.5	163.4
	6	96.2	96.0	96.0	96.0	96.0	96.0
	7	165.0	164.9	165.0	164.9	164.9	164.9
	8	95.2	95.2	95.1	95.1	95.1	95.2
	9	162.5	162.6	162.5	162.5	162.5	162.5
	10	103.2	103.1	103.2	103.1	103.2	103.2
	1′	128.3	128.3	128.3	128.3	128.2	128.3
	2′	128.1	128.2	128.3	128.1	128.2	128.2
	3′	115.1	115.1	115.1	115.0	115.1	115.1
	4′	157.0	157.0	158.3	157.0	157.0	157.2
	5′	115.1	115.1	115.1	115.0	115.1	115.1
	6′	128.1	128.2	128.3	128.1	128.2	128.2
Glc	1″	100.2	100.4	100.5	100.0	99.5	99.8
	2″	72.9	72.8	72.9	73.2	72.8	73.1
	3″	77.6	77.0	76.9	76.7	77.0	77.0
	4″	69.5	69.3	69.5	69.9	69.5	69.8
	5″	76.7	76.8	76.7	76.3	76.6	76.6
	6″	60.7	60.5	60.6	68.5	66.0	68.3
	1‴		103.7	99.7	104.1	100.6	103.9
	2‴		72.9	72.8	72.9	70.2	73.0
	3‴		77.5	77.6	76.3	70.5	76.5
	4‴		69.5	69.4	69.2	71.6	69.3
	5‴		77.5	77.6	76.0	68.2	76.1
	6‴		60.8	60.3	60.7	17.8	60.8
	1⁗						99.6
	2⁗						72.9
	3⁗						76.9
	4⁗						69.5
	5⁗						77.0
	6⁗						60.8

		9	**10**	**11**
Aglycone	2	78.5	78.4	78.4
	3	42.2	42.1	42.1
	4	196.6	196.7	196.6
	5	163.5	163.7	163.6
	6	96.0	96.0	96.0
	7	164.9	164.8	164.8
	8	95.0	95.0	95.1
	9	162.6	162.5	162.6
	10	103.1	103.2	103.1
	1′	128.3	128.2	128.2
	2′	128.3	128.1	128.1
	3′	115.1	115.2	115.2
	4′	157.5	157.0	157.2
	5′	115.1	115.2	115.2
	6′	128.3	128.1	128.1
Glc	1″	99.5	103.5	99.2
	2″	72.8	72.9	72.7
	3″	77.3	77.6	77.6
	4″	69.5	69.7	69.3
	5″	77.5	77.4	77.5
	6″	60.6	60.4	60.4
	1‴			103.7
	2‴			72.7
	3‴			77.3
	4‴			69.6
	5‴			77.3
	6‴			60.5
	1⁗			
	2⁗			
	3⁗			
	4⁗			
	5⁗			
	6⁗			

The HRFABMS spectrum of **8**, which showed a $[M+Na]^+$ peak at m/z 781.2170, suggested the formula of $C_{33}H_{42}O_{20}$ (calcd. 781.2167 for $C_{33}H_{42}O_{20}Na$), indicating the incorporation of three hexoses in the molecule. The ^{1}H NMR spectrum of compound **8** showed signals for anomeric protons at δ 4.25 (1H, *d*, *J* = 8.0 Hz), 4.90 (1H, *d*, *J* = 8.0 Hz), and 5.06 (1H, *d*, *J* = 7.6 Hz), suggesting the presence of three β-anomers. The ^{13}C NMR spectrum of **8** included anomeric carbon signals at δ 99.6, 99.8, and 103.9. On the basis of the chemical shifts of the carbon resonances due to the sugar moiety, the sugar component of **8** was determined as β-D-glucopyranose. HMBC correlations between proton and carbon resonances, δ 5.06 (H-1⁗)/δ 157.2 (C-4′), δ 4.90 (H-1‴)/δ 164.9 (C-7), and δ 4.25 (H-1‴)/δ 68.3 (C-6″), established that glucosyl residues were attached at the 4′- and 7-positions of naringenin (**3**), and that the pair of β-D-glucopyranosyl residues at C-7 position was 1,6-linked. Thus, compound **8** was 7-*O*-β-D-gentiobiosyl-4′-*O*-β-D-glucopyranosylnaringenin.

Fig. 1 shows the time-course of the biotransformation of **1** with cultured *E. perriniana* cells. It was found that formation of 7-*O*-β-glucoside **2** first occurred, followed by production of di- and trisaccharides **4**–**8**. Very small amounts of naringenin (**3**) were found during the incubation period. These findings suggested that naringenin 7-*O*-β-D-glucopyranoside (**2**), which had been formed by hydrolysis of **1** with *E. perriniana* cells, was glucosylated to afford di- and trisaccharides **4**–**8**, as shown in Scheme 1.

Next, naringenin (**3**) was transformed by the same biotransformation system with cultured *E. perriniana* cells as the case of naringin (**1**). After incubation, products **2** (14%), **4** (7%), **5** (13%), **6** (2%), **7**

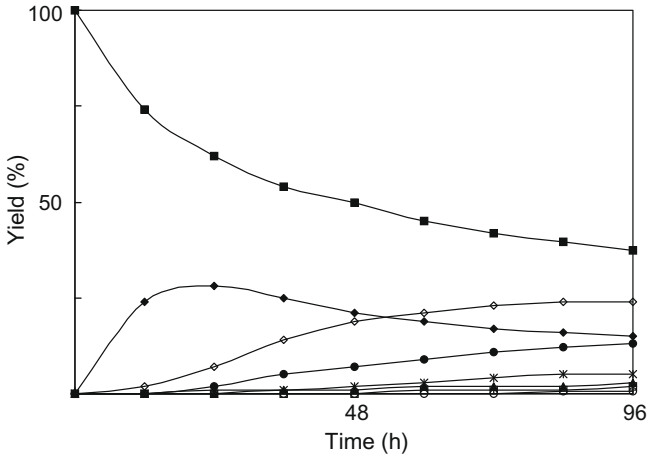
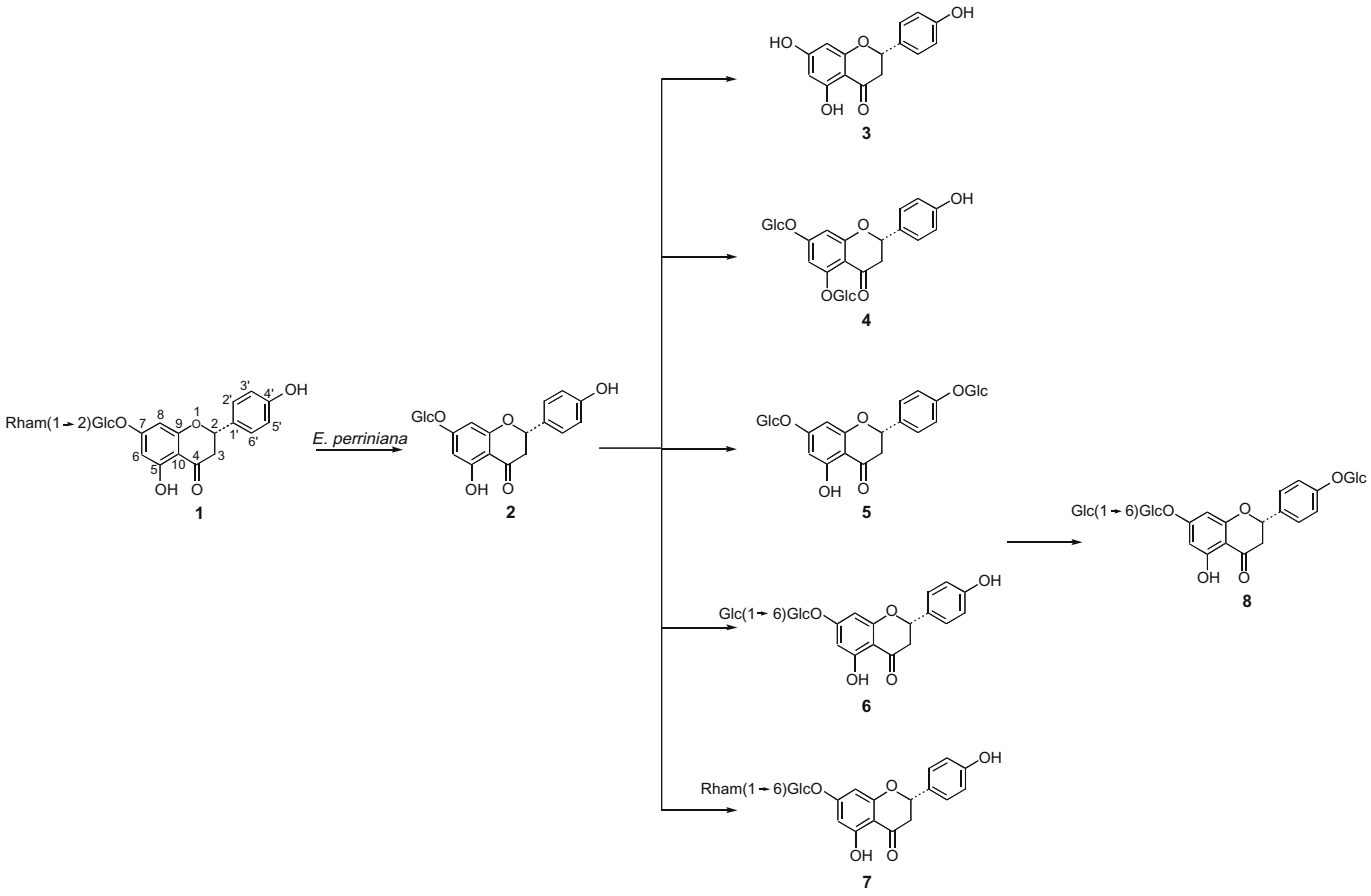


Fig. 1. Time-course of biotransformation of naringin (**1**) by cultured cells of *E. perriniana*. Yield is expressed as a percentage relative to the total amount of all reaction products. Yields of **1** (■), **2** (♦), **3** (○), **4** (●), **5** (◇), **6** (∗), **7** (▲), and **8** (□) are plotted.



Scheme 1. Biotransformation of naringin (**1**) by cultured cells of *E. perriniana*.

(1%), naringenin 4′-*O*-β-D-glucopyranoside (**9**, 4%) (Ko et al., 2006), and naringenin 5-*O*-β-D-glucopyranoside (**10**, 2%) (Ogundaini et al., 1996), and **11** (5%) were isolated from the MeOH-extracts of the cells. Product **11** was identified as naringenin 4′,5-*O*-β-D-diglucopyranoside, which has not been identified before.

The HRFABMS spectrum of product **11** showed a pseudomolecular ion $[M+Na]^+$ peak at *m*/*z* 619.1641 consistent with a molecular formula of $C_{27}H_{32}O_{15}$ (calcd. 619.1639 for $C_{27}H_{32}O_{15}Na$), suggesting that two new hexoses were introduced to the substrate. The

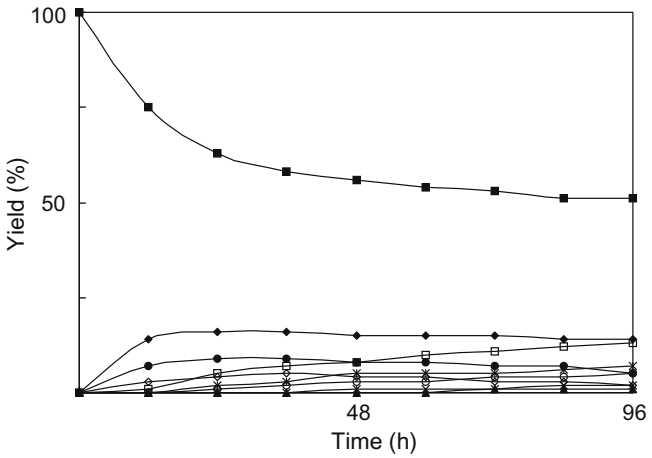


Fig. 2. Time-course of biotransformation of naringenin (**3**) by cultured cells of *E. perriniana*. Yield is expressed as a percentage relative to the total amount of all reaction products. Yields of **2** (♦), **3** (■), **4** (∗), **5** (□), **6** (x), **7** (▲), **9** (●), **10** (◇), and **11** (○) are plotted.

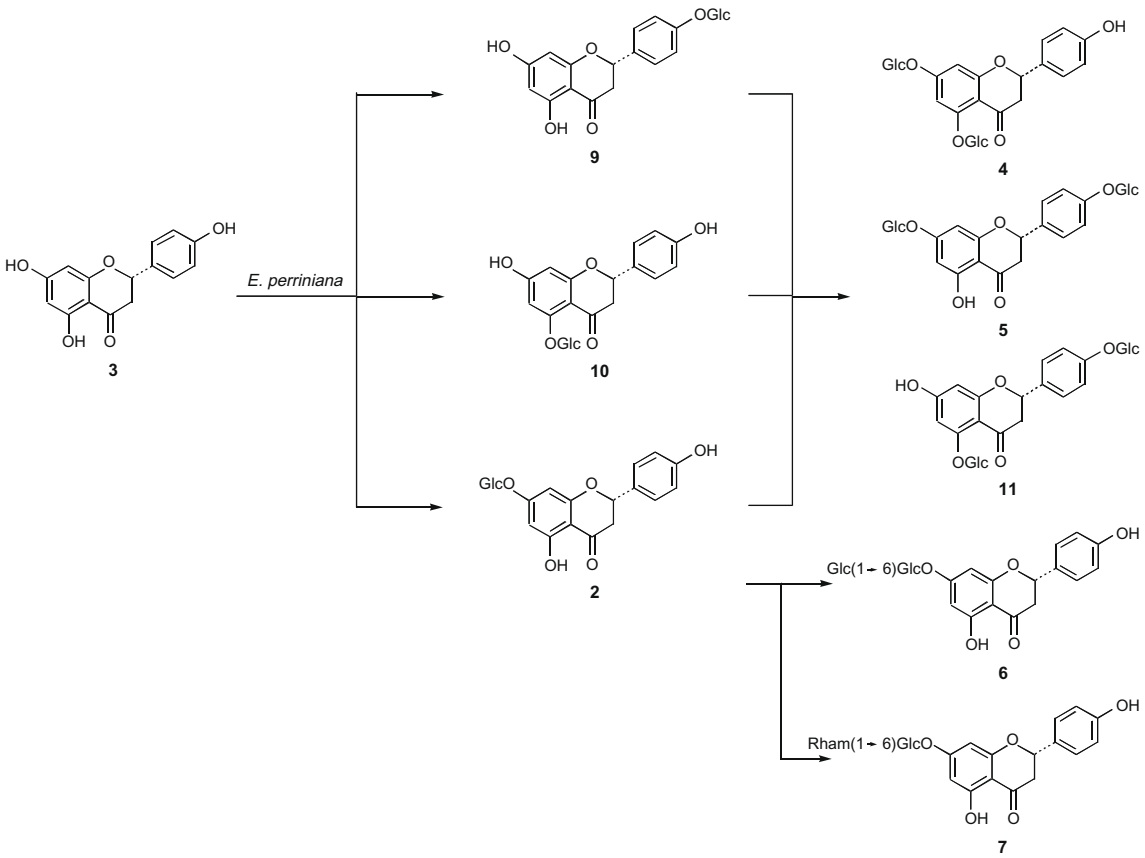
^{1}H NMR spectrum of **11** displayed two anomeric proton signals at δ 4.97 (1H, *d*, *J* = 7.6 Hz) and 5.05 (1H, *d*, *J* = 8.0 Hz). The sugar component in product **11** was determined to be β-D-glucopyranose, according to the chemical shifts of the sugar carbon resonances. The ^{13}C NMR spectrum of **11** showed two anomeric carbon resonances at δ 99.4 and 103.7, as the HMBC spectrum of **11** exhibited interactions between the anomeric proton at the 1″-position (δ 5.05) and the carbon at the 4′-position (δ 157.2), as well as between the anomeric proton at 1‴-position (δ 4.97) and the carbon at 5-position (δ 163.6). These findings established that the glucopyranosyl residues were attached to the phenolic hydroxyl group at the C-4′ and C-5 positions of naringenin (**3**). Thus, structure of compound **11** was determined to be naringenin 4′,5-*O*-β-D-diglucopyranoside.

A time-course experiment was carried out to investigate the ability of cultured cells of *E. perriniana* to convert **3** (Fig. 2). The biotransformation pathway of naringenin (**3**) is shown in Scheme 2.

3. Conclusion

The results of this study established that naringin (**1**) was converted into six glycosides of naringenin and into a small amount of naringenin (**3**) by cultured *E. perriniana* cells. It is postulated that five glycosides, i.e., naringenin 4′,7-*O*-β-D-diglucopyranoside (**5**), naringenin 5,7-*O*-β-D-diglucopyranoside (**4**), naringenin 7-*O*-β-D-gentiobioside, naringenin 7-*O*-β-D-rutinoside, and 7-*O*-β-D-gentiobiosyl-4′-*O*-β-D-glucopyranosylnaringenin (**8**), were generated from naringenin 7-*O*-β-D-glucoside (**2**), which had been produced by hydrolysis of naringin, not from naringenin.

On the other hand, naringenin (**3**) was converted into eight glycosides, i.e., naringenin 4′-*O*-β-D-glucopyranoside (**9**), naringe-



Scheme 2. Biotransformation of naringenin (**3**) by cultured cells of *E. perriniana*.

nin 5-*O*-β-D-glucopyranoside (**10**), naringenin 7-*O*-β-D-glucopyranoside (**2**), naringenin 4′,5-*O*-β-D-diglucopyranoside (**11**), naringenin 4′,7-*O*-β-D-diglucopyranoside (**5**), naringenin 5,7-*O*-β-D-diglucopyranoside (**4**), naringenin 7-*O*-β-D-gentiobioside (**6**), and naringenin 7-*O*-β-D-rutinoside (**7**). This is the first description of production of naringenin 7-*O*-β-D-rutinoside by biotransformation of naringin (**1**) and naringenin (**3**) with cultured *E. perriniana* cells. No formation of naringenin 4′-*O*-β-D-glucopyranoside (**9**), naringenin 5-*O*-β-D-glucopyranoside (**10**), and naringenin 4′,5-*O*-β-D-diglucopyranoside (**11**) was found in the case of the biotransformation of naringin (**1**) by *E. perriniana* cells. These findings also indicated that naringenin 7-*O*-β-D-glucopyranoside (**2**) was converted into other five glycosides **4**–**8** in the biotransformation of naringin (**1**). Several studies on the biotransformation of flavanones by plant cell cultures have been reported so far (for example, Lewinsohn et al., 1986, 1989; Shimoda et al., 2008a). However, there are no reports on the biotransformation of both flavanone glycosides and their aglycone flavanones to clarify the biotransformation pathways of flavanone glycosides. It would be useful for investigation of metabolism of flavanone glycosides by cultured plant cells to compare the biotransformation pathway of flavanone glycosides with that of aglycone flavanones.

Recently, we reported the glycosylation of hesperetin to hesperidin, i.e., hesperetin 7-*O*-β-rutinoside, by cultured cells of *E. perriniana* (Shimoda et al., 2008a). The results obtained here suggested that cultured cells of *E. perriniana* can glycosylate flavanones such as hesperetin and naringenin (**3**) to the corresponding 7-*O*-β-rutinosides. The present biotransformation system is useful to convert flavanone glycosides to other glucosyl conjugates of flavanones. Further studies on the physiological properties of flavanone glycosides are now in progress.

4. Experimental

4.1. General

Naringin (**1**) and naringenin (**3**) were purchased from Aldrich Chemical Co. ^{1}H and ^{13}C NMR, H–H COSY, C–H COSY, and HMBC spectra were measured using a Varian XL-400 spectrometer in DMSO-d_6 solution, whereas HRFABMS was performed using a JEOL MStation JMS-700 spectrometer. HPLC utilised a YMC-Pack R&D ODS column (150 × 30 mm) [solvent: CH_3CN–H_2O (3:17, v/v); detection: UV (280 nm); flow rate: 1.0 ml/min].

4.2. Plant material and culture conditions

Cultured cells of *E. perriniana* have been cultivated for over 20 years in our laboratory (Furuya et al., 1987), and were used in this study. Cultured *E. perriniana* cells were cultivated at 4-week intervals on solid medium (MS medium; 100 ml in a 300-ml conical flask) containing 10 mM 2,4-dichlorophenoxyacetic acid and 1% agar (adjusted to pH 5.7) in the dark. A solid culture was changed to a suspension culture by transferring the solid cells to liquid medium (100 ml) in a 300-ml conical flask.

4.3. Biotransformation of naringin (*1*) and naringenin (*3*) by cultured *E. perriniana* cells and isolation of products

Substrate (a total amount of 1.5 mmol), naringin (**1**) or naringenin (**3**), was individually administered to the 10 flasks (0.15 mmol/flask) containing the cultured suspension cells and the flasks were incubated at 25 °C on a rotary shaker (120 rpm) in the dark according to the previously reported methods (Shimoda et al., 2007a,b,c,

2008a,b). After a 5-day incubation period, the cells and medium were separated by filtration with suction. The filtered medium was extracted with EtOAc. The medium was further extracted with *n*-BuOH. The *n*-BuOH fraction was analyzed by HPLC. The cells were extracted with MeOH for 12 h and then sonicated for 5 min. The MeOH fraction was concentrated, and partitioned between H_2O and EtOAc. The EtOAc fractions were combined, concentrated, and analyzed by HPLC. The H_2O fraction was applied to a Diaion HP-20 column, which was washed with H_2O and then eluted with MeOH. The MeOH eluate was subjected to preparative HPLC to isolate products.

The time-course experiment was carried out according to the previously reported procedures (Shimoda et al., 2007a,b,7c, 2008a,b).

4.4. Identification of biotransformation products

Structures of products were elucidated on the basis of spectroscopic techniques including HRFABMS, ^{1}H and ^{13}C NMR, H–H COSY, C–H COSY, and HMBC spectroscopic analyses.

Spectral data for new compounds are as follows.

7-*O*-β-D-Gentiobiosyl-4′-*O*-β-D-glucopyranosylnaringenin (**8**): $[\alpha]_D^{25}$ −59.2 (*c* 0.09, MeOH); HRFABMS: *m*/*z* 781.2170 $[M+Na]^+$; ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.72 (1H, *dd*, *J* = 17.3, 3.0 Hz, H-3a), 3.17 (1H, *m*, H-3b), 3.25–3.81 (18H, *m*, H-2″, 2‴, 2⁗, 3″, 3‴, 3⁗, 4″, 4‴, 4⁗, 5″, 5‴, 5⁗, 6″, 6‴, 6⁗), 4.25 (1H, *d*, *J* = 8.0 Hz, H-1‴), 4.90 (1H, *d*, *J* = 8.0 Hz, H-1″), 5.06 (1H, *d*, *J* = 7.6 Hz, H-1⁗), 5.48 (1H, *dd*, *J* = 12.6, 3.0 Hz, H-2), 6.10 (2H, *m*, H-6, 8), 6.90 (2H, *d*, *J* = 8.4 Hz, H-3′, 5′), 7.40 (2H, *d*, *J* = 8.4 Hz, H-2′, 6′); ^{13}C NMR (100 MHz, DMSO-d_6): δ 42.1 (C-3), 60.8 (C-6‴, C-6⁗), 68.3 (C-6″), 69.3 (C-4‴), 69.5 (C-4⁗), 69.8 (C-4″), 72.9 (C-2⁗), 73.0 (C-2‴), 73.1 (C-2″), 76.1 (C-5‴), 76.5 (C-3‴), 76.6 (C-5″), 76.9 (C-3⁗), 77.0 (C-3″, C-5⁗), 78.5 (C-2), 95.2 (C-8), 96.0 (C-6), 99.6 (C-1⁗), 99.8 (C-1″), 103.2 (C-10), 103.9 (C-1‴), 115.1 (C-3′, C-5′), 128.2 (C-2′, C-6′), 128.3 (C-1′), 157.2 (C-4′), 162.5 (C-9), 163.4 (C-5), 164.9 (C-7), 196.7 (C-4).

Naringenin 4′,5-*O*-β-D-diglucopyranoside (**11**): $[\alpha]_D^{25}$ −35.1 (*c* 0.15, MeOH); HRFABMS: *m*/*z* 619.1641 $[M+Na]^+$; ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.73 (1H, *dd*, *J* = 17.8, 2.8 Hz, H-3a), 3.15 (1H, *m*, H-3b), 3.20–3.55 (12H, *m*, H-2″, 2‴, 3″, 3‴, 4″, 4‴, 5″, 5‴, 6″, 6‴), 4.97 (1H, *d*, *J* = 7.6 Hz, H-1‴), 5.05 (1H, *d*, *J* = 8.0 Hz, H-1″), 5.51 (1H, *dd*, *J* = 12.6, 2.8 Hz, H-2), 6.11 (2H, *m*, H-6, 8), 6.90 (2H, *d*, *J* = 8.4 Hz, H-3′, 5′), 7.41 (2H, *d*, *J* = 8.4 Hz, H-2′, 6′); ^{13}C NMR (100 MHz, DMSO-d_6): δ 42.1 (C-3), 60.4 (C-6″), 60.5 (C-6‴), 69.3 (C-4″), 69.6 (C-4‴), 72.7 (C-2″, C-2‴), 77.3 (C-3‴, C-5‴), 77.5 (C-5″), 77.6 (C-3″), 78.4 (C-2), 95.1 (C-8), 96.0 (C-6), 99.2 (C-1″), 103.1 (C-10), 103.7 (C-1‴), 115.2 (C-3′, C-5′), 128.1 (C-2′, C-6′), 128.2 (C-1′), 157.2 (C-4′), 162.6 (C-9), 163.6 (C-5), 164.8 (C-7), 196.6 (C-4).

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