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ScienceDirect
Phytochemistry 69 (2008) 1135–1140
PHYTOCHEMISTRY
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Glycosylation of hesperetin by plant cell cultures

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Received 22 June 2007; received in revised form 21 August 2007
Available online 26 December 2007

Abstract

The biotransformation of hesperetin by cultured cells of *Ipomoea batatas* and *Eucalyptus perriniana* was investigated. Three glycosides, hesperetin 3′-*O*-β-D-glucopyranoside (33 μg/g fr. wt of cells), hesperetin 3′,7-*O*-β-D-diglucopyranoside (217 μg/g fr. wt of cells), and hesperetin 7-*O*-[6-*O*-(β-D-glucopyranosyl)]-β-D-glucopyranoside (β-gentiobioside, 22 μg/g fr. wt of cells), together with three hitherto known glycosides, hesperetin 5-*O*-β-D-glucopyranoside (23 μg/g fr. wt of cells), hesperetin 7-*O*-β-D-glucopyranoside (57 μg/g fr. wt of cells), and hesperetin 7-*O*-[6-*O*-(α-L-rhamnopyranosyl)]-β-D-glucopyranoside (β-rutinoside, hesperidin, 13 μg/g fr. wt of cells), were isolated from cultured suspension cells of *E. perriniana* that had been treated with hesperetin. Oligosaccharide chains were regioselectively formed at the C-7 position of hesperetin to afford β-gentiobioside and β-rutinoside. On the other hand, cultured *I. batatas* cells converted hesperetin into hesperetin 3′-*O*-β-D-glucopyranoside (60 μg/g fr. wt of cells), hesperetin 5-*O*-β-D-glucopyranoside (23 μg/g fr. wt of cells), and hesperetin 7-*O*-β-D-glucopyranoside (110 μg/g fr. wt of cells).

Keywords: *Ipomoea batatas*; Convolvulaceae; *Eucalyptus perriniana*; Myrtaceae; Cultured plant cells; Biotransformation; Hesperetin; Hesperidin; Glycosides; β-Gentiobioside; β-Rutinoside

1. Introduction

Hesperetin (**1**) (5,7,3′-trihydroxyl-4′-methoxyl-flavanone), which occurs in citrus fruits and flowers, is a bioactive flavonoid (vitamin P) that has been well documented for its medicinal properties as an important Chinese traditional medicine (Formica and Regelson, 1995). Available clinical information on hesperetin (**1**) includes its effects on the blood-brain barrier, signal transduction pathway, and certain kinds of cancer (So et al., 1997; Mitsunaga et al., 2000; O'Prey et al., 2003; Youdim et al., 2003; Cooray et al., 2004). Hesperetin (**1**) barely dissolves in aqueous solution, and this low water-solubility and poor absorption after oral administration limits its further pharmacological exploitation.

Glycosylation occurs widely in plant cells and is considered to be an important method for the conversion of water-insoluble and unstable organic compounds into the corresponding water-soluble and stable compounds (Furuya et al., 1987; Tabata et al., 1988; Mastelic et al., 2004; Shimoda et al., 2006,2007a,2007b). Recently, it has been reported that the efficiency of absorption of a flavonoid glucoside, quercetin glucoside, was greater than that of quercetin itself (Hollman et al., 1995; Morand et al., 2000). In addition, glycosylation plays an important role in the activation of natural compounds such as saponins in plants (Biswas et al., 2005; Voutquenne et al., 2005; Eskander et al., 2006; Gao and Wang, 2006; Leo et al., 2006; Magid et al., 2006; Melek et al., 2007). From a physiological point of view, the glycosides of hesperetin (**1**) are both important and interesting. Hesperidin is one such hesperetin glycoside, i.e., a 7-*O*-β-rutinoside of hesperetin, and is widely used as vitamin P2, which has biologically beneficial effects such as anticancer, antimutagenic, anticarcinogenic,

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doi:10.1016/j.phytochem.2007.11.008

and cholesterol-lowering activities (Borradaile et al., 1999; Zhang et al., 2000; Garg et al., 2001; Chiba et al., 2003).

In our recent studies on the biological glycosylation of several phenolic compounds without flavanones by cultured plant cells, we found that cultured *Eucalyptus perriniana* cells had high potential for the production of both mono- and disaccharides, β-gentiobiosides (Shimoda et al., 2006). Lewinsohn et al. reported that cultured *Citrus paradisi* cells glycosylated hesperetin (**1**) only at its 7-position to give 7-*O*-glucoside and 7-*O*-rutinoside (Lewinsohn et al., 1986,1989). On the other hand, hesperetin glycosides such as 3′-*O*-glucoside, 3′,7-*O*-diglucoside, and gentiobioside have not yet been identified. In our continuing biotransformation study with cultured plant cells, hesperetin (**1**) was chosen as a substrate. We report here the ability of plant cell cultures of *Ipomoea batatas* and *E. perriniana* to convert exogenous hesperetin into glycosides, including the 3′-*O*-glucoside, 3′,7-*O*-diglucoside, and 7-*O*-gentiobioside, which are more water-soluble.

2. Results and discussion

Hesperetin (**1**) was transformed by cultured cells of *I. batatas*. Products **2**, **3**, and **4** were obtained from the MeOH-extracts of the cells after 5 days of incubation. No products were detected in the medium. No additional conversion products were detected in the MeOH-extracts of the cells despite careful HPLC analyses. The products were identified as hesperetin 3′-*O*-β-D-glucopyranoside (**2**, 60 μg/g fr. wt of cells), hesperetin 5-*O*-β-D-glucopyranoside (**3**, 23 μg/g fr. wt of cells) (Sadykov et al., 1975), and hesperetin 7-*O*-β-D-glucopyranoside (**4**, 110 μg/g fr. wt of cells) (Del Rio et al., 1995) on the basis of analysis of their HRFABMS, ^{1}H and ^{13}C NMR (Table 1), H–H COSY, C–H COSY, HMBC, and NOE spectra. The β-glucoside product **2** has not been identified before.

The HRFABMS spectrum of **2** included a pseudomolecular ion $[M+Na]^+$ peak at m/z 487.1223, consistent with a molecular formula of $C_{22}H_{24}O_{11}$ (calcd. 487.1216 for $C_{22}H_{24}O_{11}Na$). The ^{1}H NMR spectrum of **2** showed the signal of a proton, which was attached to the anomeric carbon atom, at δ 5.06 (1H, *d*, $J = 7.6$ Hz), which suggested the presence of β-anomer in **2**. The ^{13}C NMR spectrum of **2** exhibited 22 carbon signals, including an anomeric carbon resonance at δ 99.4. The sugar component of **2** was indicated to be β-D-glucopyranose based on the chemical shifts of the carbon signals due to the sugar moiety. The linkage site of the sugar moiety was determined to be the phenolic hydroxyl group at the 3′-position of hesperetin (**1**) according to a HMBC correlation between the proton signal at δ 5.06 (H-1″) and the carbon resonance at δ 146.0 (C-3′). Thus, the structure of **2** was determined to be hesperetin 3′-*O*-β-D-glucopyranoside.

The time-course of the biotransformation of **1** with cultured *I. batatas* cells was examined. We found that the cells preferentially glucosylated the phenolic hydroxyl group at the 7-position of **1** to give 7-*O*-β-glucoside **4**. The conversion yield of the glucosylation products at 100 h decreased in the order **4**, **2**, and **3** (Fig. 1).

Table 1
^{13}C chemical shifts of the biotransformation products **2**–**7** in DMSO-d_6

Product	**2**	**3**	**4**	**5**	**6**	**7**
Aglycone						
2	78.0	78.0	78.3	78.1	78.2	78.2
3	43.2	43.1	42.0	41.9	42.1	41.9
4	196.7	199.7	196.7	196.7	196.7	196.7
5	163.2	165.2	162.6	162.6	162.7	162.7
6	95.6	94.6	96.5	96.7	95.4	95.3
7	166.3	167.4	164.9	165.0	164.9	164.8
8	94.8	93.6	95.2	95.1	96.3	96.1
9	162.4	162.5	162.6	162.4	162.2	162.2
10	101.5	105.2	103.0	103.0	103.1	103.1
1′	130.3	130.9	130.6	130.3	130.7	130.6
2′	113.6	113.8	113.9	113.6	113.9	113.9
3′	146.0	146.2	146.2	146.0	146.2	146.1
4′	148.9	147.6	147.7	148.9	147.7	147.6
5′	111.9	111.7	111.7	111.9	111.8	111.8
6′	120.5	117.4	117.6	120.5	117.6	117.7
OCH_3	55.5	55.5	55.5	55.5	55.5	55.5
Glc						
1″	99.4	104.2	99.4	99.4	99.1	99.2
2″	72.9	72.9	72.8	72.9	73.3	72.8
3″	77.6	77.6	76.1	76.8	76.7	76.0
4″	69.5	69.5	69.3	69.5	69.9	69.4
5″	77.6	77.6	76.9	76.9	76.0	75.3
6″	60.9	60.9	60.4	60.5	68.2	65.8
1‴				99.4	103.2	100.3
2‴				72.8	72.8	70.1
3‴				76.1	76.5	70.5
4‴				69.3	69.1	71.8
5‴				76.8	76.0	68.1
6‴				60.4	60.9	17.7

Next, hesperetin (**1**) was biotransformed using cultured cells of *E. perriniana* by the same procedure as in the biotransformation with *I. batatas*. After a 5-days incubation period, products **2** (33 μg/g fr. wt of cells), **3** (23 μg/g fr. wt of cells), **4** (57 μg/g fr. wt of cells), **5** (217 μg/g fr. wt

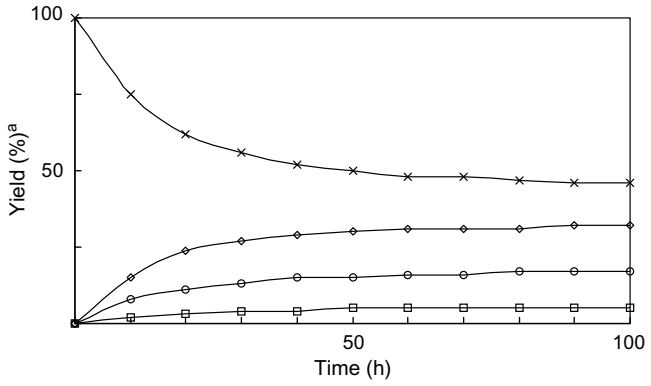


Fig. 1. Time-course of the biotransformation of hesperetin (**1**) by cultured cells of *I. batatas*. [a]Yield is expressed as a percentage relative to the total amount of all reaction products. Yields of **1** (×), **2** (○), **3** (□), and **4** (◇) are plotted.

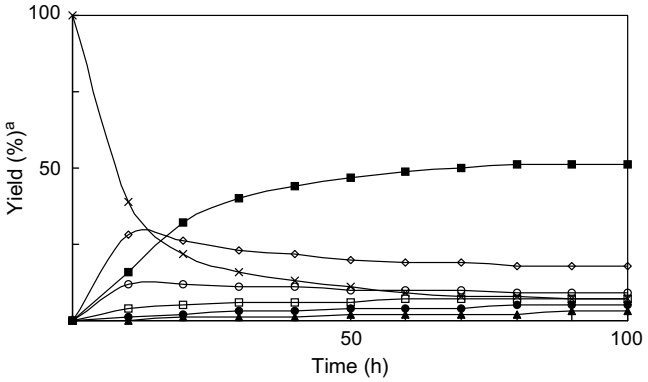


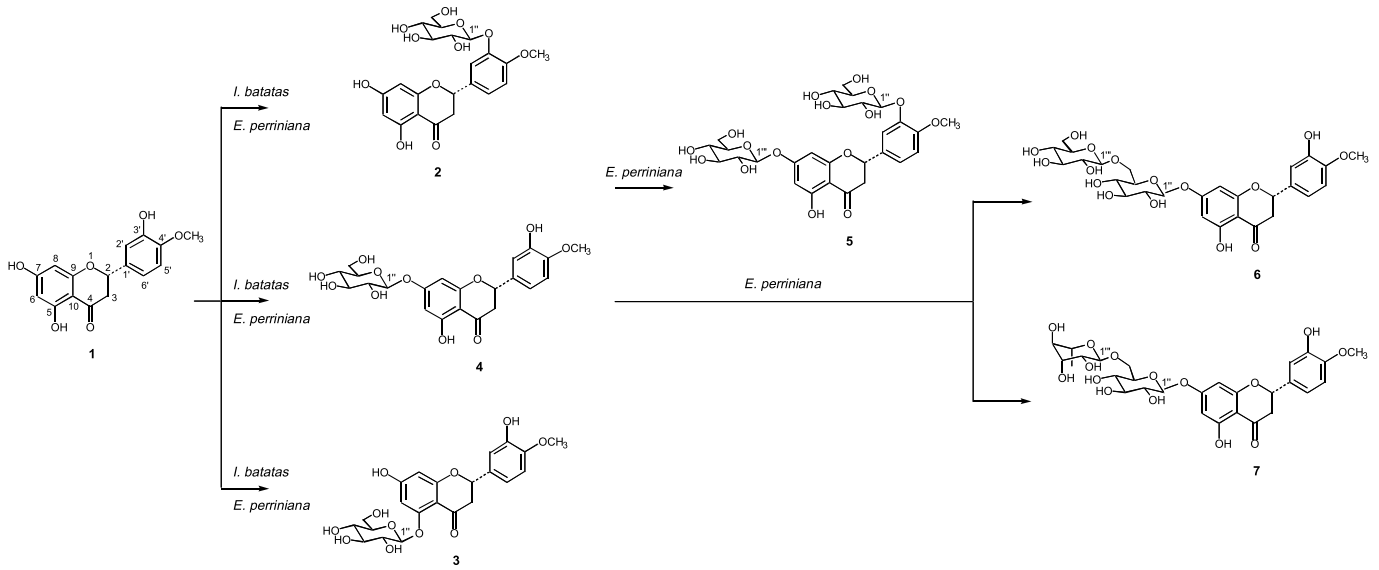
Fig. 2. Time-course of the biotransformation of hesperetin (**1**) by cultured cells of *E. perriniana*. [a]Yield is expressed as a percentage relative to the total amount of all reaction products. Yields of **1** (×), **2** (○), **3** (□), **4** (◇), **5** (■), **6** (●), and **7** (▲) are plotted.

of cells), **6** (22 μg/g fr. wt of cells), and **7** (13 μg/g fr. wt of cells) were obtained from the MeOH-extracts of the cells. The products **5**, **6**, and **7** were identified as hesperetin 3′,7-*O*-β-D-diglucopyranoside (**5**), hesperetin 7-*O*-[6-*O*-(β-D-glucopyranosyl)]-β-D-glucopyranoside (β-gentiobioside, **6**), and hesperetin 7-*O*-[6-*O*-(α-L-rhamnopyranosyl)]-β-D-glucopyranoside (β-rutinoside, hesperidin, **7**) (Rousseff et al., 1987). The disaccharide products **5** and **6** have not been identified before.

The HRFABMS spectrum of product **5** showed a pseudomolecular ion $[M+Na]^+$ peak at *m/z* 649.1751, which suggested a molecular formula of $C_{28}H_{34}O_{16}$ (calcd. 649.1745 for $C_{28}H_{34}O_{16}Na$). The ^1H NMR spectrum of **5** included two signals of anomeric protons at δ 4.97 (1H, *d*, $J = 7.6$ Hz) and δ 5.05 (1H, *d*, $J = 8.0$ Hz), indicating the presence of two β-anomers in the sugar moiety. The ^{13}C NMR spectroscopic data of **5** included an anomeric carbon signal at δ 99.4, corresponding to C-1″ and C-1‴. The HMBC spectrum included correlations between the anomeric proton signal at δ 5.05 (H-1″) and the carbon resonance at δ 146.0 (C-3′), and between the anomeric proton signal at δ 4.97 (H-1‴) and the carbon resonance at δ 165.0 (C-7). This result confirms that β-D-glucopyranosyl residues were attached to the phenolic hydroxyl groups at C-3′ and C-7 of hesperetin (**1**), respectively. Thus, compound **5** was identified as hesperetin 3′,7-*O*-β-D-diglucopyranoside.

Product **6** gave a $[M+Na]^+$ peak at *m/z* 649.1750 in the HRFABMS spectrum, which suggested a molecular formula of $C_{28}H_{34}O_{16}$ (calcd. 649.1745 for $C_{28}H_{34}O_{16}Na$). The ^1H NMR spectrum of **6** showed two anomeric proton signals at δ 4.17 (1H, *d*, $J = 8.0$ Hz) and 4.99 (1H, *d*, $J = 8.0$ Hz). Based on the chemical shifts of the sugar carbon signals, the sugar component in **6** was determined to be β-D-glucopyranose. The ^{13}C NMR chemical shift of C-6″ was comparatively shifted downfield to δ 68.2. HMBC correlations were observed between the anomeric proton signal at δ 4.99 (H-1″) and the carbon resonance at δ 164.9 (C-7), and between the anomeric proton signal at δ 4.17 (H-1‴) and the carbon resonance at δ 68.2 (C-6″). These data confirm that the inner glucopyranosyl residue was attached to the phenolic hydroxyl group at C-7 of hesperetin (**1**), and that the pair of β-D-glucopyranosyl residues was 1,6-linked. Thus, compound **6** was identified as hesperetin 7-*O*-[6-*O*-(β-D-glucopyranosyl)]-β-D-glucopyranoside.

A time-course experiment was used to investigate the biotransformation pathway of **1** by cultured cells of *E. perriniana*. As shown in Fig. 2, mono-glucoside products **2**, **3**, and **4** were produced at an early stage of incubation, whereas disaccharides **5**, **6**, and **7** were accumulated predominantly after 20 h of incubation. On the other hand, **2** and **4** were each converted to **5** by cultured *E. perriniana* cells. In addition, **4** was glycosylated to **6** and **7** when **4** was



Scheme 1. Glycosylation of hesperetin (**1**) by cultured cells of *I. batatas* and *E. perriniana*.

used as the substrate. These findings indicated that disaccharide products were formed from the corresponding mono-glucosides, as shown in Scheme 1. Glucosylation occurred preferentially at the 7-position of hesperetin (**1**) rather than at the 3′- and 5-positions as in the case of biotransformation with *I. batatas*.

3. Conclusions

Cultured cells of *I. batatas* converted hesperetin (**1**) into three mono-glucoside products, hesperetin 3′-*O*-β-D-glucopyranoside (**2**), hesperetin 5-*O*-β-D-glucopyranoside (**3**), and hesperetin 7-*O*-β-D-glucopyranoside (**4**). On the other hand, biotransformation by cultured cells of *E. perriniana* also gave disaccharide products, i.e., hesperetin 3′,7-*O*-β-D-diglucopyranoside (**5**), hesperetin 7-*O*-[6-*O*-(β-D-glucopyranosyl)]-β-D-glucopyranoside (β-gentiobioside) (**6**), and hesperetin 7-*O*-[6-*O*-(α-L-rhamnopyranosyl)]-β-D-glucopyranoside (**7**) (β-rutinoside, hesperidin). The glycosylation ability and activity of *E. perriniana* toward hesperetin were much higher than those of *I. batatas*.

Hesperetin 5-*O*-β-D-glucopyranoside (**3**), hesperetin 7-*O*-β-D-glucopyranoside (**4**), and hesperidin (**7**) have been reported previously. The 5-*O*-β-glucoside (**3**) has been found in the roots and flowers of *Persica vulgaris* (Sadykov et al., 1975). The 7-*O*-β-glucoside (**4**) has been isolated from the fruits of tangero Nova, a mandarin hybrid (*Citrus reticulate* B) × tangero orlando (*C. reticulate* × *C. paradisi* Macf.) (Del Rio et al., 1995). The 7-*O*-β-rutinoside (hesperidin) (**7**) is contained in *Citrus* species, such as oranges (*Citrus sinensis*), lemons (*Citrus limon*), and mandarins (*Citrus reticulata*) (Rousseff et al., 1987). Hesperetin 3′-*O*-β-D-glucopyranoside (**2**), hesperetin 3′,7-*O*-β-D-diglucopyranoside (**5**), and hesperetin 7-*O*-β-gentiobioside (**6**) were three new compounds.

Recently, it has been reported that hesperetin (**1**) was demethylated at the 4′-position to yield eriodictyol by rat liver microsomes (Breinholt et al., 2002). The present results showed that the metabolism of hesperetin (**1**) in cultured plant cells was quite different from that in rat liver microsomes.

Earlier, it has been reported that cultured cells of *C. paradisi* glycosylated hesperetin (**1**) only at its 7-position to give the 7-*O*-glucoside and the 7-*O*-rutinoside (Lewinsohn et al., 1986,1989). Compared to the biotransformation with *C. paradisi*, cultured cells of *E. perriniana* showed high potential for the glycosylation of hesperetin to give six products, three of which were new. Although we recently reported that cultured *E. perriniana* cells glycosylated phenolic compounds, thymol, carvacrol, and eugenol, to give the corresponding disaccharides, β-gentiobiosides, the regioselectivity of *E. perriniana* for disaccharide formation was unclear (Shimoda et al., 2006). We should emphasize that oligosaccharide chains were formed only at the C-7 position of hesperetin (**1**) to give β-gentiobioside and β-rutinoside, hesperidin, by *E. perriniana*. Thus, the ability and regioselectivity of cultured *E. perriniana* cells for the glycosylation of hesperetin (**1**) were elucidated for the first time. This is also the first report on the production of hesperidin (**1**) by plant cell culture without *Citrus* cell culture.

In general, flavonoids are not necessarily most active within the living body, since they are poorly absorbed from the intestine or rapidly eliminated. It has been reported that the glycosylation of the flavonoid aglycone influences its absorption. In the case of quercetin glucoside, the efficiency of absorption was higher than that for quercetin itself (Hollman et al., 1995; Morand et al., 2000). Hollman et al. elucidated the mechanism by which glucosylation facilitates quercetin absorption: the glucosides could be transported into enterocytes by the glucose transporter SGLT1 and could then be hydrolyzed inside the cells by cytosolic β-glucosidase (Hollman et al., 1995). The therapeutic value of hesperetin glycosides obtained here is of pharmacological interest. Studies on the physiological and pharmacological properties of hesperetin glycosides are now in progress.

4. Experimental

4.1. General

Hesperetin (**1**) used as a substrate was purchased from Aldrich Chemical Co. The ^{1}H and ^{13}C NMR, H–H COSY, C–H COSY, NOE, and HMBC spectra were measured using a Varian XL-400 spectrometer in DMSO-d_6 solution and the chemical shifts were expressed in δ (ppm) with reference to TMS. HRFABMS was performed using a JEOL MStation JMS-700 spectrometer. HPLC was performed with 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; retention times for substrate and products: **1**: 61.0 min, **2**: 17.5 min, **3**: 18.2 min, **4**: 16.1 min, **5**: 4.9 min, **6**: 8.5 min, **7**: 9.5 min].

4.2. Plant material and culture conditions

Cell culture of *E. perriniana*, which is an efficient biocatalyst to produce both mono- and disaccharides, was induced in our laboratory, and has been cultivated for over 20 years. *I. batatas* cell culture was induced in our laboratory and has been cultivated for 15 years. These cell cultures were used in the experiments. Cultured cells were subcultured 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 suspension culture was started by transferring the cultured cells to 100 ml of liquid medium in a 300-ml conical flask.

4.3. Biotransformation of hesperetin (*1*) by cultured plant cells

A total of 1.5 mmol of hesperetin (**1**) was added to the 10 flasks (0.15 mmol/flask) containing the suspension cultured cells and the cultures were incubated at 25 °C on a rotary shaker (120 rpm) in the dark. After 5 days, the cells were separated from the medium by filtration with suction. The filtered medium (ca. 80 ml) was extracted with EtOAc (100 ml × 3). The medium was further extracted with *n*-BuOH (100 ml × 3). The *n*-BuOH fraction was analyzed by HPLC. The cells were extracted with MeOH (100 ml × 3) for 12 h and then sonicated for 5 min. The MeOH fraction was conc. and partitioned between H_2O (30 ml) and EtOAc (40 ml × 3). The EtOAc fractions were combined and analyzed by HPLC. The H_2O fraction was applied to a Diaion HP-20 column and the column was washed with H_2O and then eluted with MeOH. The MeOH eluate was subjected to preparative HPLC to give products.

4.4. Product identification

The structures of the products were determined on the basis of analysis of their HRFABMS, ^1H and ^{13}C NMR, H–H COSY, C–H COSY, NOE, and HMBC spectra.

Spectral data for new biotransformation products are as follows:

*Hesperetin 3′-O-β-D-glucopyranoside (**2**)*: HRFABMS: calcd for $C_{22}H_{24}O_{11}Na$ $[M+Na]^+$ *m/z* 487.1216, found 487.1223; ^1H NMR (400 MHz, DMSO-d_6): δ 2.77 (1H, *dd*, $J = 17.8$, 2.8 Hz, H-3a), 3.15–3.82 (6H, *m*, H-2″, 3″, 4″, 5″, 6″), 3.32 (1H, *dd*, $J = 17.8$, 12.8 Hz, H-3b), 3.78 (3H, *s*, OCH_3), 5.06 (1H, *d*, $J = 7.6$ Hz, H-1″), 5.49 (1H, *dd*, $J = 12.6$, 2.8 Hz, H-2), 5.89 (1H, *d*, $J = 2.8$ Hz, H-6), 5.91 (1H, *d*, $J = 2.8$ Hz, H-8), 7.03 (1H, *dd*, $J = 8.4$, 2.0 Hz, H-6′), 7.09 (1H, *d*, $J = 8.4$ Hz, H-5′), 7.25 (1H, *d*, $J = 2.0$ Hz, H-2′); for ^{13}C NMR (100 MHz, DMSO-d_6) spectra, see Table 1.

*Hesperetin 3′,7-O-β-D-diglucopyranoside (**5**)*: HRFABMS: calcd for $C_{28}H_{34}O_{16}Na$ $[M+Na]^+$ *m/z* 649.1745, found 649.1751; ^1H NMR (400 MHz, DMSO-d_6): δ 2.78 (1H, *dd*, $J = 17.8$, 2.8 Hz, H-3a), 3.17–3.65 (12H, *m*, H-2″, 2‴, 3″, 3‴, 4″, 4‴, 5″, 5‴, 6″, 6‴), 3.32 (1H, *dd*, $J = 17.8$, 12.8 Hz, H-3b), 3.78 (3H, *s*, OCH_3), 4.97 (1H, *d*, $J = 7.6$ Hz, H-1‴), 5.05 (1H, *d*, $J = 8.0$ Hz, H-1″), 5.52 (1H, *dd*, $J = 12.8$, 2.8 Hz, H-2), 6.14 (1H, *d*, $J = 2.8$ Hz, H-6), 6.18 (1H, *d*, $J = 2.8$ Hz, H-8), 7.03 (1H, *dd*, $J = 8.4$, 2.0 Hz, H-6′), 7.09 (1H, *d*, $J = 8.4$ Hz, H-5′), 7.25 (1H, *d*, $J = 1.8$ Hz, H-2′); for ^{13}C NMR (100 MHz, DMSO-d_6) spectra, see Table 1.

*Hesperetin 7-O-[6-O-(β-D-glucopyranosyl)]-β-D-glucopyranoside (**6**)*: HRFABMS: calcd for $C_{28}H_{34}O_{16}Na$ $[M+Na]^+$ *m/z* 649.1745, found 649.1750; ^1H NMR (400 MHz, DMSO-d_6): δ 2.78 (1H, *dd*, $J = 17.8$, 2.8 Hz, H-3a), 3.00–3.99 (12H, *m*, H-2″, 2‴, 3″, 3‴, 4″, 4‴, 5″, 5‴, 6″, 6‴), 3.32 (1H, *dd*, $J = 17.8$, 12.8 Hz, H-3b), 3.78 (3H, *s*, OCH_3), 4.17 (1H, *d*, $J = 8.0$ Hz, H-1‴), 4.99 (1H, *d*, $J = 8.0$ Hz, H-1″), 5.49 (1H, *dd*, $J = 12.8$, 2.8 Hz, H-2), 6.17 (1H, *d*, $J = 2.8$ Hz, H-6), 6.22 (1H, *d*, $J = 2.8$ Hz, H-8), 6.88 (1H, *dd*, $J = 8.4$, 1.8 Hz, H-6′), 6.94 (2H, *m*, H-2′, 5′); for ^{13}C NMR (100 MHz, DMSO-d_6) spectra, see Table 1.

4.5. Time-course experiments

Fifty grams (fr. wt) of the suspension cells was individually partitioned into each of 10 flasks containing (MS medium) 100 ml. Substrate (0.1 mmol) was individually administered to each flask (1 mmol/l), and the mixtures were incubated on a rotary shaker at 25 °C. At a regular time interval (10 h), one of the flasks was taken out from the rotary shaker, and the cells and medium were separated by filtration. The extraction and analytical procedures were the same as those described in Section 4.3.

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