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Stimulating the production of homoisoflavonoids in cell suspension cultures of *Caesalpinia pulcherrima* using cork tissue

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Abstract

It has previously been demonstrated that cork tissue increases the efficiency of the production of lipophilic secondary metabolites in diverse plant cell suspension cultures. In the present study, three new homoisoflavonoids – named dihydrobonducellin, 2′-methoxydihydrobonducellin, and 2′-methoxybonducellin – and bonducellin and isobonducellin were isolated from *Caesalpinia pulcherrima* cultured cells coincubated with cork tissue. Cork tissue increased the production of 2′-methoxybonducellin by about 7-fold relative to control cells, and more than 80% of the product was recoverable from the cork tissue. When cork tissue and methyl jasmonate or yeast extract were added simultaneously to the medium, the amount of 2′-methoxybonducellin produced increased further. The production of the other four homoisoflavonoids was enhanced by variable amounts. Our results indicate that the addition of cork tissue would be an effective technique for investigating formation of secondary metabolites that usually accumulate only in trace amounts.

Keywords: *Caesalpinia pulcherrima*; Leguminosae; Cell suspension culture; Cork tissue; Homoisoflavonoids; Dihydrobonducellin; 2′-Methoxydihydrobonducellin; 2′-Methoxybonducellin

1. Introduction

Caesalpinia pulcherrima Swartz is a leguminous, perennial large shrub or small tree that is widely distributed in the tropics. Their stems are used as abortifacients and emmenagogues, whereas the fruits are used as a cure for dysentery (Quisimbing, 1951). Previous studies on this plant have isolated several homoisoflavonoids, flavonoids, and diterpenoids, some of which have antimicrobial and antitumor properties (McPherson et al., 1983; Che et al., 1986; Ragasa et al., 2002; Srinivas et al., 2003). Our preliminary work on cell cultures of this plant has demonstrated that the addition of cork tissue stimulates the production of homoisoflavonoids, most of which is recoverable from the added cork tissue (Yamamoto et al., 2001). However, the structures of those homoisoflavonoids had not been determined. Here we report the isolation and structural characterization of five homoisoflavonoids including three new ones obtained from *C. pulcherrima* cultured cells by the addition of cork tissue and methyl jasmonate/yeast extract. The stimulatory effects of cork tissue, methyl jasmonate, and yeast extract on the production of these homoisoflavonoids in cultured cells are also demonstrated.

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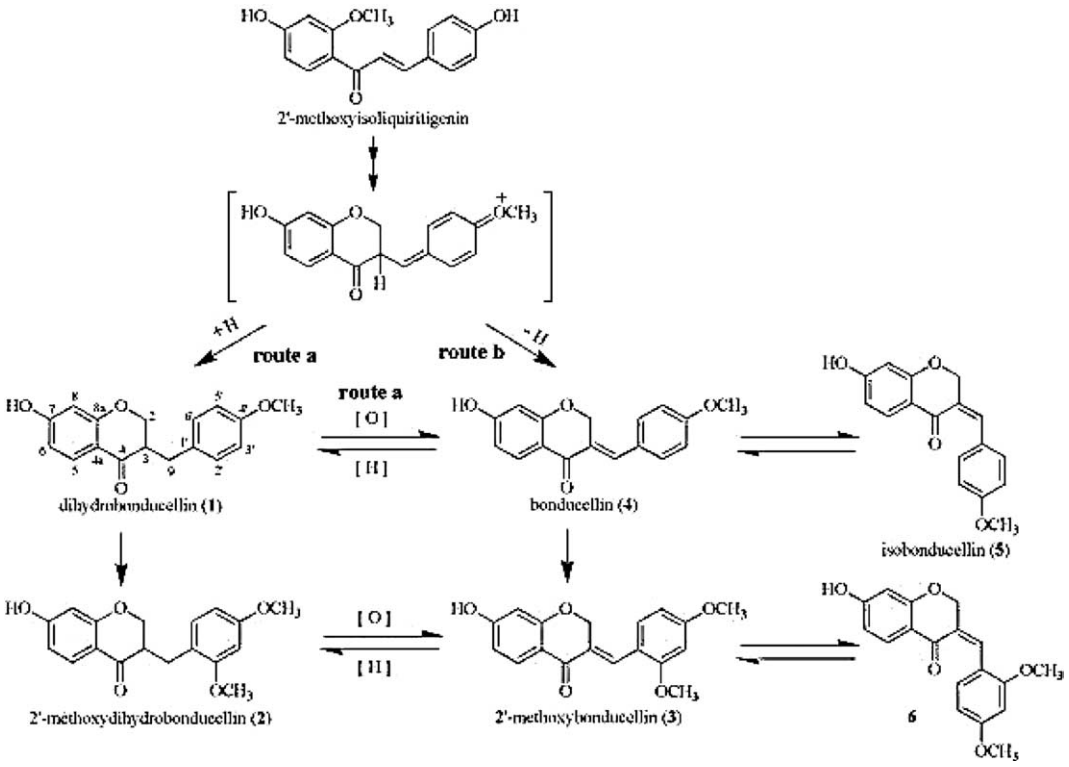
2. Results and discussion

2.1. Isolation and identification of homoisoflavonoids accumulated in C. pulcherrima cells and coincubated cork tissue

Cell suspension cultures of C. pulcherrima were established in MS liquid medium (Murashige and Skoog, 1962) containing 10 μM 2,4-dichlorophenoxyacetic acid and 1 μM 6-benzylaminopurine. As described in Section 3, the *n*-BuOH fraction from the MeOH extract of the fresh cells with added cork tissue was successively separated with silica gel and Toyopearl HW-40C chromatographies, preparative HPLC and preparative TLC to afford three new compounds (**1**–**3**) and two known ones (Scheme 1). The known compounds were identified as bonducellin (**4**) and its isomer, isobonducellin (**5**), based on a comparison of their spectroscopic data with those in the literature (McPherson et al., 1983; Srinivas et al., 2003). In MeOH or Me_2CO, **4** was easily isomerized to **5**, and at the equilibrated state the ratio of **4** and **5** was about 2:3, as revealed by HPLC and ^{1}H NMR analyses.

2.2. Structural determination of three new homoisoflavonoids

Compound **1** was obtained as a white amorphous powder. HR EIMS showed a quasi-molecular peak at *m*/*z* 284.1036 ([M]$^+$, calculated 284.3116), corresponding to the molecular formula $C_{17}H_{16}O_4$, including ten degrees of unsaturation. The ^{13}C NMR spectrum of **1** showed the presence of a carbonyl group (*δ* 193.0), a methoxy group (*δ* 55.3), a methylene (*δ* 31.8), an oxygen-bearing methylene (*δ* 68.2), a methine (*δ* 47.6), and 12 aromatic carbons (7 tertiary, 2 quaternary, and 3 quaternary with oxygen-attached carbons). The ^{1}H NMR spectrum exhibited two sets of double-doublet methylene signals [*δ* 4.31 (*dd*, *J* = 4.0, 11.2 Hz) and 4.13 (*dd*, *J* = 7.5, 11.2 Hz) for H-2, and *δ* 3.16 (*dd*, *J* = 4.2, 13.8 Hz) and 2.64 (*dd*, *J* = 10.8, 13.8 Hz) for H-9] and a double-double–double-doublet signal [*δ* 2.77 (*dddd*, *J* = 4.0, 4.2, 7.5, 10.8 Hz) for H-3], characteristic of the 3-benzylchroman-4-one skeleton of **1**. In addition, an aromatic ABX coupling system [*δ* 7.82 (*d*, *J* = 8.0 Hz), 6.52 (*dd*, *J* = 2.2, 8.0 Hz), 6.37 (*d*, *J* = 2.2 Hz)] and an aromatic AA′BB′ coupling system [*δ* 7.13 (2H, *d*, *J* = 8.4 Hz) and 6.84 (2H, *d*, *J* = 8.4 Hz)] arising from rings A and B were observed in the ^{1}H NMR spectrum, respectively. These observations suggested that **1** was a homoisoflavonoid with a 1,3,4-trisubstituted ring A and a *para*-disubstituted ring B. The EIMS showed two fragment peaks at *m*/*z* 137 and *m*/*z* 148 attributable to a *retro*-Diels–Alder reaction, and a base peak at *m*/*z* 121 corresponding to a methoxybenzyl/tropylium ion, suggesting that the methoxy group was present at C-4′ and that the hydroxyl group was attached to C-7 (Heller and Tamm, 1981).



Scheme 1. Homoisoflavonoids (compounds **1**–**5**) isolated from *C. pulcherrima* cultured cells coincubated with cork tissue.

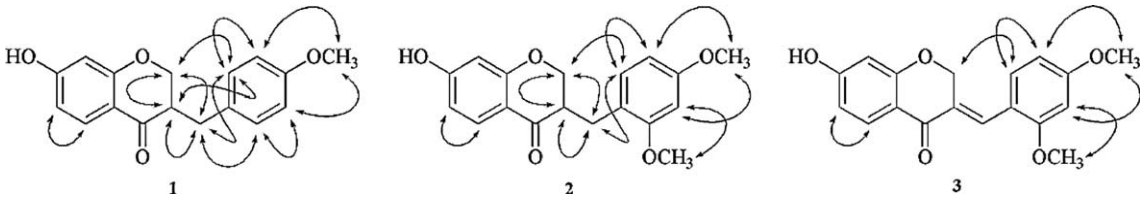


Fig. 1. Selected NOE correlations in compounds **1**–**3**.

This result was further confirmed by the NOESY experiment (Fig. 1). Therefore, compound **1** was established as 7-hydroxy-3-(4-methoxybenzyl)chroman-4-one, and named dihydrobonducellin.

Compound **2** exhibited a molecular ion peak at *m*/*z* 314 in the EIMS analysis (at 30 mass units higher than that of **1**), and HR EIMS analysis revealed that its molecular formula was $C_{18}H_{18}O_5$. The ^{1}H NMR spectrum of **2** was very similar to that of **1**, suggesting that **2** also possessed a 3-benzylchroman-4-one skeleton. However, instead of the aromatic AA′BB′ coupling system attributed to the *para*-disubstituted B-ring in **1**, another ABX coupling system in ring B was observed in the ^{1}H NMR spectrum of **2** along with two methoxy groups [δ 3.78, 3.81 (each 3H, *s*)] (Table 1). Based on the fragmentation in the EIMS, in which two fragment peaks at *m*/*z* 137 and *m*/*z* 178 attributable to a *retro*-Diels–Alder reaction and the base peak at *m*/*z* 151 corresponding to a dimethoxybenzyl/tropylium ion were evident, it was suggested that the two methoxy groups were connected at ring B. The locations of two methoxy groups at C-2′ and C-4′ were further confirmed by the NOESY experiment (Fig. 1), which showed correlations between one methoxy group (δ 3.81) and H-3′, and between another one (δ 3.78) and both H-3′ and H-5′. Thus, compound **2** was identified as 7-hydroxy-3-(2,4-dimethoxybenzyl)chroman-4-one, and named 2′-methoxydihydrobonducellin.

Compound **3** exhibited a molecular ion peak at *m*/*z* 312 in the EIMS analysis, and HR EIMS analysis revealed that its molecular formula was $C_{18}H_{16}O_5$. Comparison of the ^{1}H NMR spectra of **3** and **4** suggested that **3** also possessed a **3**-benzylidenechroman-4-one skeleton. But, similar to **1** and **2**, an ABX coupling system in place of the AA′BB′ coupling system, and two methoxy groups [δ 3.89, 3.91 (each 3H, *s*)] were observed in the ^{1}H NMR spectrum of **3** (Table 1). The existence of two fragment peaks at *m*/*z* 151 and *m*/*z* 175 in the EIMS spectrum of **3** as well as the correlations observed in the NOESY experiment (Fig. 1) indicated that two methoxy groups in **3** were also placed at C-2′ and C-4′. The *E*-geometry of the double bond at C-3 and C-9 in **3** was clearly indicated by the characteristic ^4J-coupling of the methylene protons (δ 5.28, *d*, J = 1.6 Hz) at C-2 and the vinylic proton (δ 7.87, *d*, J = 1.6 Hz) at C-9, since the *Z*-geometry positions the H-9 away from the anisotropic region of the carbonyl group and makes this proton resonate at a higher field (Böhler and Tamra, 1967; Heller and Tamm, 1981). Therefore, the structure of **3** was characterized as 7-hydroxy-3-(2,4-dimethoxybenzylidene)chroman-4-one, and named 2′-methoxybonducellin. Similar to **4**, **3** was gradually isomerized to its *Z*-isomer 6 (Scheme 1) in the solvent; in the ^{1}H NMR spectrum, the signals assigned to H-2 (δ 4.99), H-6′ (δ 8.15), and H-9 (δ 7.16) of the *Z*-isomer **(6)** were observed after repeated analyses.

Table 1
^{1}H NMR spectral data of compounds **1**–**4** measured in acetone-d_6 [300 MHz, δ, multiplicity, coupling constants (Hz) in parentheses]

	1	**2**	**3**	**4**
H-2a	4.31 (*dd*, 4.1, 11.2)	4.29 (*dd*, 4.5, 11.5)	5.28 (2H, *d*, 1.6)	5.35 (2H, *d*, 1.6)
H-2b	4.13 (*dd*, 7.8, 11.2)	4.13 (*dd*,9.0, 11.5)		
H-3	2.77 (*dddd*, 4.0, 4.2, 7.5, 10.8)	2.89 (*dddd*, 9.5, 9.0, 5.0, 4.5)		
H-5	7.82 (*d*, 8.0)	7.71 (*d*, 9.0)	7.84 (*d*, 8.7)	7.85 (*d*, 8.5)
H-6	6.52 (*dd*, 2.2, 8.0)	6.55 (*dd*, 2.5, 9.0)	6.61 (*dd*, 2.4, 8.7)	6.62 (*dd*, 2.2, 8.0)
H-8	6.37 (*d*, 2.2)	6.34 (*d*, 2.5)	6.38 (*d*, 2.4)	6.40 (*d*, 2.2)
H-9	3.16 (*dd*, 4.2, 13.8)	3.21 (*dd*, 5.0, 14.0)	7.87 (2H, *d*, 1.6)	7.69 (2H, *d* ,1.6)
	2.64 (*dd*, 10.8, 13.8)	2.54 (*dd*, 9.5, 14.0)		
H-2′	7.13 (*d*,8.4)			7.42 (*d*, 8.4)
H-3′	6.84 (*d*, 8.4)	6.54 (*d*, 1.5)	6.68 (*d*, 2.4)	7.07 (*d*, 4.4)
H-5′	6.84 (*d*, 8.4)	6.46 (*dd*,1.5,8.5)	6.62 (*dd*, 2.4, 8.7)	7.07 (*d*, 8.4)
H-6′	7.13 (*d*, 8.4)	7.07 (*d*, 8.5)	7.13 (*d*, 8.7)	7.42 (*d*, 8.4)
OCH_3-2′		3.81 (*s*)	3.91 (*s*)	
OCH_3-4′	3.78 (*s*)	3.78 (*s*)	3.89 (*s*)	3.88 (*s*)
OH-7	9.38 (*br s*)	9.32 (*br s*)	9.44 (*br s*)	10.12 (*br s*)

2.3. Effects of cork tissue, methyl jasmonate, and yeast extract on the production of homoisoflavonoids

In a preliminary experiment, the elicitors methyl jasmonate and yeast extract were screened at various doses to optimize their concentrations for maximum homoisoflavonoid accumulation (Fig. 2). The production of compounds **1**–**6** was evaluated by quantitative HPLC analysis as described in Section 3. Since **3** and **4** are easily isomerized to their geometric isomers, the summations of **3** and **6**, and of **4** and **5** are shown as **3** and **4**, respectively, in the figures. As shown in Fig. 2, the control cells mainly produced a small amount of **3**, and **2** was not detected. When 0.3 mM methyl jasmonate was added, the contents of **1**, **3**, and **4** increased 4-, 3-, and 2-fold compared to the control cells, respectively. Methyl jasmonate at a higher concentration (1 mM) caused cell death, although a small amount of **2** was induced. Yeast extract produced maximum amounts of **1**, **3**, and **4** at a concentration of 0.05% (w/v). *C. pulcherrima* cells seem to be rather sensitive to this elicitor compared to other cell cultures, since cell growth was strongly inhibited even in 0.15% yeast extract, and **2** accumulated in place of other metabolites (especially **3**).

Fig. 3 shows the effects of cork tissue, methyl jasmonate, and yeast extract on the production of homoisoflavonoids. Similar to our preliminary result (Yamamoto et al., 2001), cork tissue at 50 mg/flask increased the production of compound **3** about 7-fold relative to control cells, while the amounts of **1** and **4** were enhanced 4- and 3-fold, respectively, and more than 80% of these metabolites were recoverable from the cork tissue. The stimulatory effect of cork tissue on the production of **3** was about 3-fold higher than that of methyl jasmonate (0.3 mM) or yeast extract (0.05%) used alone. When cork tissue and methyl jasmonate were simultaneously added to the medium, the amounts of **1**, **3**, and **4** were further increased to 6-, 10-, and 5-fold those of control cells, respectively. Similar stimulatory effects on these metabolites were also observed when both cork tissue and yeast extract were present in the medium.

Homoisoflavonoids are very restricted in their distribution, being reported only from several genera in Liliaceae and Hyacinthaceae (Kouno et al., 1973; Heller and Tamm, 1981), *Dracaena* species (Machala et al., 2001), *Caesalpinia* species (Purushothaman et al., 1982; McPherson et al., 1983; Namikoshi et al., 1987a,b), *Hoffmanosseggia intricata* (Wall et al., 1989), and *Pterocarpus marsupium* (Jain et al., 1997). The production of homoisoflavonoids in plant cell cultures has not been reported previously. In the present study, we demonstrated that cultured cells of *C. pulcherrima* produced the 3-benzylidenechroman-4-one and 3-benzylchroman-4-one types of homoisoflavonoids. Only small amounts were produced in the control cultures, but this was greatly increased by the addition of methyl jasmonate, yeast extract, and/or cork tissue. Among these additives, cork tissue had the greatest effect on the production and its inhibitory effect on cell growth was negligible, which is consistent with previous reports (Yamamoto et al., 2001; Zhao et al., 2003). This indicates that the addition of cork tissue would be an effective technique for investigating formation of secondary metabolites that usually accumulate only in trace amounts.

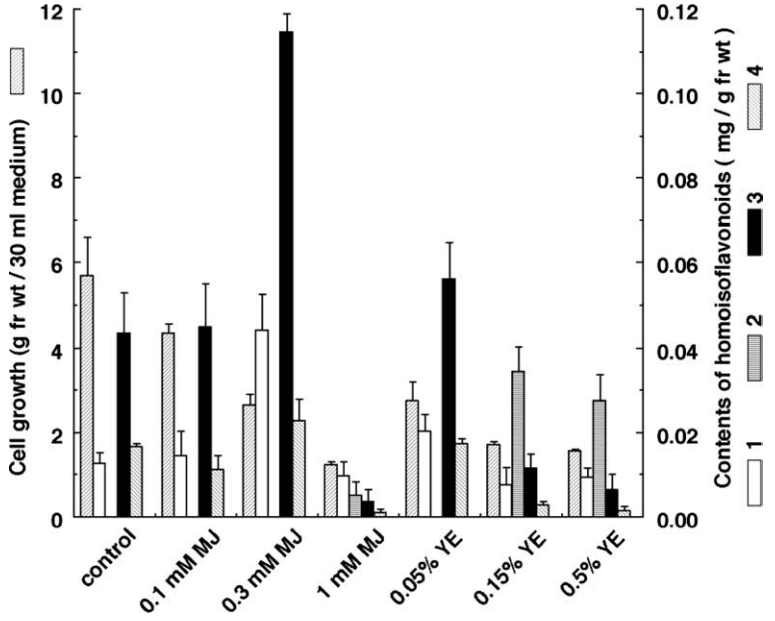


Fig. 2. Effects of methyl jasmonate (MJ) and yeast extract (YE) on the production of homoisoflavonoids by *C. pulcherrima* cultured cells. Since **3** and **4** are easily isomerized to their geometric isomers **6** and **5**, the summations of **3** and **6**, and of **4** and **5** are shown as **3** and **4**, respectively. Values are the means of triplicate determinations; error bars are SEs.

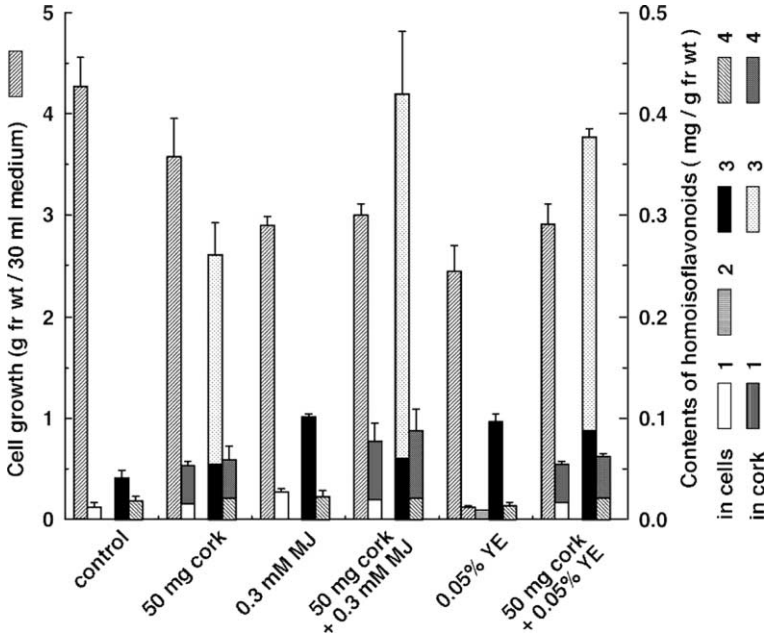


Fig. 3. Effects of cork tissue and MJ/YE on the production of homoisoflavonoids by *C. pulcherrima* cultured cells. Cork tissue (50 mg) and/or MJ or YE were added to the medium. Values are the means of triplicate determinations; error bars are SEs.

Dewick (1975) suggested that homoisoflavonoids are derivatives of flavonoids, and formed from 2′-methoxychalcone, the 2′-methoxy carbon of which are incorporated into ring C during cyclization (Scheme 1). It is postulated that the 3-benzylidenechroman-4-one skeleton is formed by the addition of a hydride ion and the successive oxidation (route a) or the direct loss of a proton (route b). The efficient trapping of **1** by the addition of cork tissue suggests that in *C. pulcherrima* cells, **4** is biosynthesized from chalcone precursor via route a, since the cork tissue has been demonstrated to efficiently trap the intermediate of sophoraflavanone G in *Sophora flavescens* cell cultures (Zhao et al., 2003).

3. Experimental

3.1. General

Cork sheets (2 mm thickness, Dainaga Cork, Osaka, Japan) were washed successively with $CHCl_3$, MeOH, and H_2O, as reported previously (Yamamoto et al., 1996). Methyl jasmonate and yeast extract were purchased from Tokyo Kasei (Tokyo, Japan). ^1H and ^{13}C NMR spectra were recorded in Me_2CO-d_6 with spectrometers (Varian Unity plus 500 and Varian Gemini 300) operating at 500 and 300 MHz for ^1H NMR, and 75 MHz for ^{13}C NMR, respectively. Mass spectra were recorded on a JEOL JMS DX-303 spectrometer. UV spectra were measured with a UV-1600 visible spectrophotometer. Preparative HPLC was performed using a Hikarisil C18 column (20 × 250 mm, Shodex, Tokyo, Japan) at room temperature, with a CH_3CN/H_2O solvent system containing 0.1% HCOOH at a flow rate of 5 ml min^{-1}, whilst monitoring the absorbance at 272 nm. Column chromatographies were performed with silica gel (60 N, 60-210 mesh, Kanto Kagaku, Tokyo, Japan) and Toyopearl HW-40C, (Tosoh). TLC and preparative TLC were performed on Kieselgel 60 F_{254} plates (Merck).

3.2. Cell cultures

The establishment and subculturing of cell suspension cultures of *C. pulcherrima* were described previously (Yamamoto et al., 2001). For the experiment reported here, cells (3 g) were inoculated in MS liquid medium (30 ml) containing 10 μM 2,4-dichlorophenoxyacetic acid, 1 μM 6-benzylaminopurine, and additive(s). Cork tissue (50 mg/flask) and/or yeast extract (0.05%, w/v) was added to the medium, and its pH was adjusted to 6.4 before autoclaving. Filter-sterilized 0.3 M methyl jasmonate solution in DMSO (30 μl) was aseptically added to the medium (final concentration 0.3 mM) after autoclaving. Cultivation was performed for 21 days on a rotary shaker at 100 rpm in darkness at 25 °C. Three replicates were used each experiment, which was repeated at least twice.

3.3. Extraction and isolation

After culturing for 21 days, cells and cork tissue were harvested together by filtration. The mixture (398 g,

fresh weight) were extracted three times with MeOH by ultrasonication at room temperature for 90 min to give an extract that was suspended in H_2O and then partitioned successively with *n*-BuOH. After concentration in vacuo, the *n*-BuOH extract (3.8 g) was subjected to silica gel CC(hexane: Me_2CO, 5:1–1:1). Compound **4** was recrystallized from MeOH as yellow needles (12 mg). Other fractions were applied to Toyopearl HW-40C (MeOH), and then were separated by preparative HPLC (37% CH_3CN) and/or preparative TLC ($CHCl_3$–Me_2CO, 12:1) to afford **1** (3.5 mg), **2** (1.5 mg), **3** (1.5 mg), and **5** (2.2 mg).

3.3.1. Dihydrobonducellin (*1*)

White amorphous powder, UV λ_{max}^{MeOH} nm (log ε): 312sh (3.81), 276 (4.09); HR EIMS (*m*/*z*): 284.1036 ($[M]^+$) for $C_{17}H_{16}O_4$ (calc., 284.3116). EIMS (*m*/*z*): 284, 253, 163, 148, 137, 121. ^{13}C NMR (75 MHz, Me_2CO-d_6): δ 193.0 (C-4), 167.9 (C-7), 163.6 (C-8a), 158.3 (C-4′), 130.2 (C-1′), 130.1 (C-2′, C-6′), 128.8 (C-5), 114.5 (C-6), 114.1 (C-3′, C-5′), 103.0 (C-4a, C-8), 68.2 (C-2), 55.3 (OCH_3), 47.6 (C-3), 31.8 (C-9). For ^1H NMR data, see in Table 1.

3.3.2. 2′-Methoxydihydrobonducellin (*2*)

A white amorphous powder, UV λ_{max}^{MeOH} nm (log ε): 312sh (3.39), 277 (3.63). HR EIMS (*m*/*z*): 314.1136 ($[M]^+$) for $C_{18}H_{18}O_5$ (calc., 314.3379). EIMS (*m*/*z*): 314, 299, 283, 178, 163, 151, 137. For ^1H NMR data, see in Table 1.

3.3.3. 2′-Methoxybonducellin (*3*)

A yellow amorphous powder, UV λ_{max}^{MeOH} nm (log ε): 364 (3.72), 305sh (3.57). HR EIMS (*m*/*z*): 312.1424 ($[M]^+$) for $C_{18}H_{16}O_5$ (calc., 312.3320). EIMS (*m*/*z*): 312, 297, 281, 175, 151, 137. For ^1H NMR data, see in Table 1.

3.4. Quantitative analysis of homoisoflavonoids

Cells and coincubated cork tissue were harvested separately, and then extracted with MeOH (10 ml) for 90 min by ultrasonication at room temperature. As an internal standard, 0.5 mg of alizarin dissolved in 0.5 ml of Me_2CO was added to each extract. After centrifugation, each supernatant was subjected to reversed-phase HPLC. HPLC analyses for compounds **1**–**6** were performed on Capcellpack C18 AG-120A column (4.6×250 mm, Shiseido, Tokyo, Japan) with 1% HOAc containing 37% CH_3CN in 40 min, at a flow rate of 1.0 ml min^{-1} (oven temperature, 40 °C). The quantities of homoisoflavonoids were calculated from the peak area at 272 nm for **1** and **2**, and at 350 nm for **3**–**6** recorded using a Chromatopac C-R4A (Shimadzu, Kyoto, Japan).

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