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Efficient production and capture of 8-prenylnaringenin and leachianone G—biosynthetic intermediates of sophoraflavanone G—by the addition of cork tissue to cell suspension cultures of *Sophora flavescens*

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Abstract

It has previously been demonstrated that the addition of cork tissue to cell suspension cultures of *Sophora flavescens* stimulates the production of sophoraflavanone G, most of which has been recovered from the added cork tissue. In the present study, it was found that two precursors of sophoraflavanone G, 8-prenylnaringenin (sophoraflavanone B) and leachianone G, both of which have never been detected either in cultured cells or in the original plants, also accumulated in the added cork tissue. Thirteen minor flavonoids including three prenylated flavonoids, in addition to 8-prenylnaringenin and leachianone G, were isolated from the cork tissue co-incubated with *S. flavescens* cells. The new compounds flavescenones A, B and C, were determined to be (3*R*)-5, 7, 2′-trihydroxy-6-γ, γ-dimethylallyl-4′, 5′-methylenedioxyisoflavanone; 5, 7, 2′-trihydroxy-6-γ, γ-dimethylallyl-4′, 5′-methylenedioxyisoflavone and 2-[2′,4′-dihydroxy-3′-(γ-hydroxymethyl-γ-methylallyl)phenyl]-5,6-methylenedioxybenzofuran, respectively, by means of spectroscopic analyses that included 2D-NMR techniques.

Keywords: *Sophora flavescens*; Leguminosae; Cell suspension culture; Cork; Prenylated flavonoid; Sophoraflavanone G; Leachianone G; 8-Prenylnaringenin; Flavescenones A, B and C

1. Introduction

Cork is a layer of dead cells that forms a common secondary tissue located immediately below the epidermis of the root system and the trunk in higher plants, and is formed for mechanical protection against environmental stress. It plays an important role in the regulation of water and mineral storage (Kolattukudy, 1984), and in chemical defenses against pathogen attack (Kahl, 1974). Furthermore, diverse lipophilic secondary metabolites accumulate in cork tissue (Tani et al., 1985, 1986; Yamamoto et al., 1992; Hayashi et al., 1996). Commercially available cork tissue efficiently absorbs lipophilic secondary metabolites from diverse plant cell suspension cultures (Yamamoto et al., 1996, 2001b; Kirakosyan et al., 2001). Interestingly, the addition of cork tissue to culture systems increased the total amount of such final products, without affecting their cell growth. In *Sophora flavescens* cultured cells, the addition of cork tissue increased the production of sophoraflavanone G, 8-lavandulyl-2′-hydroxynaringenin (**3**), three- to five-fold higher than that of the cells cultured alone, and more than 70% of **3** was recovered from the cork tissue (see Scheme 1) (Yamamoto et al., 1996). By the simultaneous addition of cork tissue and methyl jasmonate to *S. flavescens* cultured cells, it was shown that a lavandulyl group, a unique monoterpene unit of **3,** originated from 1-deoxy-D-xylulose-5-phosphate (Yamamoto et al., 2002). The lavandulyl group was not directly transferred to the flavanone skeleton, but was formed by two dimethylallylations between which the 2′-hydroxylation occurred (Yamamoto et al., 2000, 2001a). That is, **3** is biosynthesized from naringenin via 8-prenylnaringenin (sophoraflavanone B, **1**)

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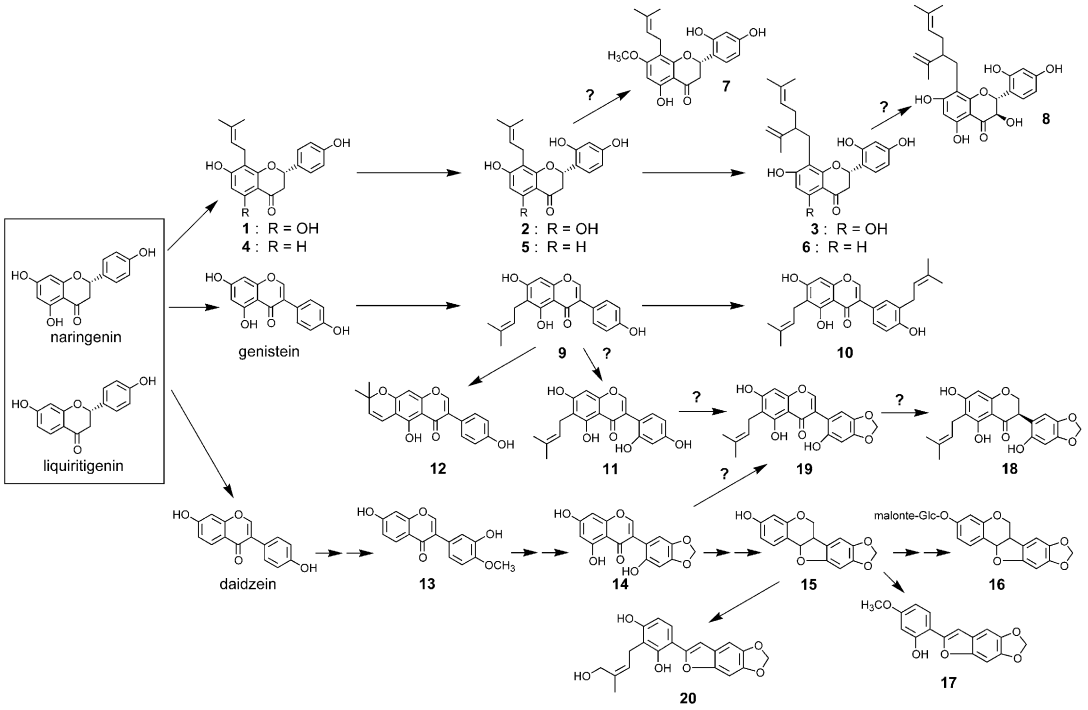

Scheme 1. Flavonoids isolated from *Sophora flavescens* cultured cells incubated with cork tissue and plausible biosynthetic routes for them.

and leachianone G (**2**) (Scheme 1). These intermediates, though, have never been identified either in cultured cells or in intact plants. However, HPLC photodiode-array analysis of the MeOH extract from the cork tissue incubated with cultured cells showed the presence of some unidentified flavanones, suggesting that cork tissue may be capable of capturing some **3**-related compounds.

This paper deals with the isolation and structure elucidation of these compounds which are barely detectable under normal culture condition. Stimulatory effects of cork tissue and methyl jasmonate on the production of these minor flavonoids in *S. flavescens* cultured cells are also reported.

2. Results and discussion

2.1. Isolation and identification of minor flavonoids accumulating in cork tissue incubated with S. flavescens cells

Cultured cells of *S. flavescens* have been shown to accumulate two 8-lavandulylated flavanones, **3** and lehmannin (**6**); a di-dimethylallylated isoflavone, lupalbigenin (**10**); and a pterocarpan glucoside, trifolirhizin 6′-*O*-malonate (**16**), as major secondary metabolites (Yamamoto et al., 1991a,b, 2002). When cells were cultivated in the presence of cork tissue, lipophilic final products **3**, **6** and **10** mainly accumulated in the cork tissue, whereas water-soluble**16** remained in the cells (Table 1). In addition to **3**, **6** and **10**, diverse unidentified flavonoids which accumulated in the cells in minute amounts were found by HPLC photodiode-array analysis in the MeOH extract of the cork tissue incubated with cells. As described in Section 3, the MeOH extract of cork tissue incubated with cells for 2 weeks was successively separated by silica gel chromatography, prep. HPLC and prep. TLC to afford three new compounds (flavescenones A–C; **18**–**20**), and 12 known flavonoids besides **3**, **6** and **10**. The known compounds were characterized as five prenylated flavanones, **1** (Delle Monache et al., 1995), **2** (Iinuma et al., 1993a), isobavachin (**4**) (Komatsu et al., 1978a), euchrenone a_7 (**5**) (Mizuno et al., 1989) and kenusanone I (**7**) (Iinuma et al., 1993b); a flavanonol, kushenol X (**8**) (Kuroyanagi et al., 1999); five isoflavones, wighteone (**9**), luteone (**11**) (Lane and Newman, 1987), alpinumisoflavone (**12**) (Martinez Olivares et al., 1982), calycosin (**13**) (Venkataratnam et al., 1987) and 2′-hydroxypseudobaptigenin (**14**) (Ingham, 1981); and a 2-arylbenzofuran, 2-(2′-hydroxy-4′-methoxyphenyl) - 5,6 - methylenedioxybenzofuran (**17**) (Komatsu et al., 1978b) by comparisons of the physical and spectral data with the respective authentic data (Scheme 1).

2.2. Determination of three new prenylated flavonoids

The molecular formula $C_{21}H_{20}O_7$ of compound **18** was determined by HR EIMS and ^{13}C NMR spectral data. The presence of the isoflavanone skeleton was evident from the UV [λ_{max} 298, 326 (sh) nm], ^1H NMR

Table 1
Effects of cork tissues (50 mg) and methyl jasmonate (MJ, 100 μM) on production of flavonoids in *S. flavescens* cells cultured for 12 days. The means of triplicate measurement are shown

	Content (mg/20 ml medium)																	
	3	**6**	**10**	**16**	**1**	**2**	**4**[a]	**5**	**7**	**8**	**9**	**11**	**12**	**13**	**14**	**15**	**17**[b]	**19**
Control	0.45	0.11	0.17	0.69	tr[c]	0.01	0.02	tr	0.01	0.02	tr	tr	tr	0.01	tr	0.04	tr	tr
+ Cork	1.60	0.35	0.50	0.96	0.09	0.11	0.07	0.04	0.04	0.07	tr	0.02	0.05	0.04	0.02	0.08	0.01	0.04
In cork tissues (%)[d]	(60.2)	(63.6)	(21.9)	(tr)	(90.3)	(90.5)	(57.4)	(97.5)	(8.5)	(44.9)	(55.6)	(52.4)	(49.2)	(36.8)	(tr)	(tr)	(38.8)	(49.9)
+MJ	0.73	0.17	0.19	1.97	0.03	0.02	0.05	tr	0.05	0.04	tr	0.01	0.01	0.03	0.02	0.18	0.03	0.01
+Cork+MJ	2.40	0.49	0.56	2.74	0.14	0.34	0.14	0.07	0.12	0.08	0.02	0.03	0.14	0.06	0.04	0.23	0.12	0.09
In cork tissues (%)	(51.5)	(59.7)	(12.9)	(tr)	(86.4)	(81.1)	(42.5)	(93.5)	(8.1)	(22.5)	(50.8)	(18.7)	(39.7)	(51.5)	(tr)	(tr)	(21.4)	(25.4)

[a] Because of the overlapping of compounds **4** and **20**, the content of **4** is expressed as a mixed value of **4** and **20**; yielding ratio **4**:**20** (2:1).
[b] The content of **17** was expressed as a mixed value of **17** and **18**; yielding ratio **17**:**18** (3:1).
[c] Trace amount.
[d] Amount in the cork tissue are expressed as the percentage relative to the total content.

(δ 4.76 for H-2*ax*, 4.67 for H-2*eq*, 3.94 for H-3) and ^{13}C NMR (δ 69.4 for C-2, 44.8 for C-3, 196.8 for C-4) spectra. The ^{1}H NMR spectrum further showed the presence of a three one-proton aromatic singlet, three phenolic hydroxyl groups, one methylenedioxy group and one γ,γ-dimethylallyl group (Table 2). Furthermore, fragments characteristic of *retro*-Diels–Alder fragmentation (Mabry and Markham, 1975) in the EI mass spectrum indicated that **18** possessed a phloroglucinol A-ring with a γ,γ-dimethylallyl group at the C-6 or C-8 position [*m*/*z* 220 (A^+), 177 (A^+–C_3H_7), 165 (A^+–C_4H_7)] and a tetrasubstituted B-ring with hydroxyl and methylenedioxy groups at C-2′, C-4′ and C-5′ positions [*m*/*z* 164 (B^+)]. Confirmation of A- and B-ring substitution patterns was accomplished from the long-range heteronuclear couplings observed in the HMBC spectrum. Couplings of H-1″ of the γ,γ-dimethylallyl group and the chelated hydroxyl proton to the same carbon proved the latter to be C-5, and thus, that the γ,γ-dimethylallyl group is located at C-6. The

Table 2
^{1}H NMR and ^{13}C NMR spectral data of compounds **18** and **19**

Position	**18**		**19**	
	^{13}C NMR	^{1}H NMR	^{13}C NMR	^{1}H NMR
2	69.4	4.76 (*dd*, 11.8, 3.3) 4.67 (*dd*, 11.8, 3.8)	156.8	7.92 (*s*)
3	44.8	3.94 (*t*, 3.3)	121.8	
4	196.8		181.9	
5	162.0		160.3	
6	107.4		111.1	
7	164.9		162.9	
8	95.6	5.96 (br *s*)	93.9	6.45 (br *s*)
9	160.8		156.8	
10	101.4		105.8	
1′	114.6		112.7	
2′	150.2		151.7	
3′	100.2	6.50 (*s*)	99.7	6.58 (*s*)
4′	148.0		149.5	
5′	142.1		141.4	
6′	106.5	6.95 (*s*)	108.4	6.62 (*s*)
1″	21.1	3.28 (*d*, 7.0)	22.0	3.46 (*d*, 6.6)
2″	121.1	5.19 (*t*-like *m*, 7.0)	123.0	5.25 (t-like *m*, 6.6)
3″	135.8		131.8	
4″	25.8	1.72 (*s*)	25.8	1.76 (*s*)
5″	17.9	1.78 (*s*)	17.9	1.83 (*s*)
OCH_2O	101.3	5.86 (*d*, 12.0)	101.7	5.94 (*s*)
OH		12.00 (*s*, chelated) 6.35, 7.59 (br, *s* each)		13.19 (*s*, chelated) 6.52, 7.51 (br, *s* each)

Values are in ppm (δ_H and δ_C). Compounds **18** and **19** were measured in $CDCl_3$. Assignments were confirmed by COSY, HMQC and HMBC experiments. Figures in parentheses are coupling constants (*J*) in Hz.

methylenedioxy protons exhibited coupling with two low-field aromatic quaternary carbons (C-4′ and C-5′), and H-3 also exhibited coupling with C-2′, thereby confirming that the methylenedioxy group is located between C-4′ and C-5′, and that a hydroxyl group is attached to C-2′ (Fig. 1A). The absolute stereochemistry at C-3 was assigned as 3*R* by the observation of a positive Cotton effect at 355 nm in the CD spectrum (Yahara et al., 1989; Galeffi et al., 1997). Therefore, the structure of **18** was characterized as (3*R*)-5,7,2′-trihydroxy-6-γ,γ–dimethylallyl-4′,5′-methylenedioxyisoflavanone, and the compound was named flavescenone A.

Compound **19** exhibited a molecular ion peak at *m*/*z* 382 in the EIMS analysis. HR EIMS analysis revealed the molecular formula to be $C_{21}H_{18}O_7$, which is two protons less than that of **18**. The UV absorption pattern and a sharp singlet signal at δ 7.92 in the ^{1}H NMR spectrum suggested **19** as an isoflavone derivative (Mabry et al., 1970; Markham and Mabry, 1975). By comparing ^{13}C and ^{1}H NMR spectral data with those of **18** (Table 2), as well as by the EIMS analysis, the structure of**19** was established to be 5, 7, 2′-trihydroxy-6-γ,γ–dimethylallyl-4′, 5′-methylenedioxyisoflavone, and the compound was named flavescenone B.

Compound **20** ($C_{20}H_{18}O_6$ by HREIMS) exhibited a UV spectrum typical of a 2-arylbenzofuran derivative (Komatsu et al., 1978b). The ^{1}H NMR spectrum of **20** was closely related to that of **17**. However, instead of the ABX type of proton signals arising from the B-ring as well as a methoxyl signal for **17**, two *ortho*-coupled aromatic proton signals (δ 7.40, 6.53) and two phenolic hydroxy proton signals (δ 8.62, 7.75) were observed for **20**. Moreover, the ^{1}H and ^{13}C NMR spectra of **20** exhibited a set of resonances assignable to a γ-hydroxymethyl-γ-methylallyl group attached at the B-ring. The (2″*Z*) configuration of the double bond in the γ-hydroxy-methyl-γ-methylallyl group was determined by the difference NOE experiment, in which irradiation of H-2″ or H-4″ enhanced both H-1″ and H-5″. The appearance of the two oxygen-bearing aromatic carbons at lower fields of δ 153.7 and 157.2 indicated that it could not be *ortho*-dihydroxy substitution (Agrawal, 1989), revealing that the remaining two phenolic hydroxyl and one γ-hydroxymethyl-γ-methylallyl groups were located at C-2′, C-4′ and C-3′, respectively. A HMBC experiment (Fig. 1B) enabled compound **20** to be finally designated as 2-[2′, 4′-dihydroxy-3′-(γ-hydroxymethyl-γ-methylallyl) phenyl]-5,6-methylenedioxybenzofuran, and named flavescenone C.

2.3. Effects of cork tissue and methyl jasmonate on the production of minor flavonoids

The contents of flavonoids in *S. flavescens* cells cultured in the presence of cork tissue (50 mg/flask) and/or methyl jasmonate (100 μM) are shown in Table 1. As previously reported, the amounts of major flavonoids **3**, **6**, **10** and **16** increased by addition of either cork tissue or methyl jasmonate (Yamamoto et al., 1996, 2001b, 2002). When cork tissue and methyl jasmonate were added simultaneously to the medium, the amounts of the major flavonoids were further increased. The accumulation of lipophilic flavonoids **3**, **6** and **10** was stimulated much more by addition of cork tissue than by addition of methyl jasmonate, and most of the flavonoids were captured by the co-incubated cork tissue. On the other hand, methyl jasmonate strongly increased the accumulated amounts of water-soluble **16** (more than 3-fold that in the control cultures) while cork tissue increased production of **16** only 1.5-fold. Minor flavonoids demonstrated in the present study, some of which were thought to be intermediates of the final products (Scheme 1), were barely detectable in normal cultured cells. As for the major lipophilic final products, the amounts of minor flavonoids increased by addition of cork tissue and/or methyl jasmonate. The production of the intermediates of **3** (**1** and especially **2**), both of which have never been identified either in *S. flavescens* cultured cells or in the original plants, were dominantly stimulated by the addition of cork tissue. In the presence of cork tissue and methyl jasmonate, the amount of **2** was about 16-fold and 3-fold that of control cells, respectively, while simultaneous addition of cork tissue and methyl jasmonate increased the amount of **2** up to

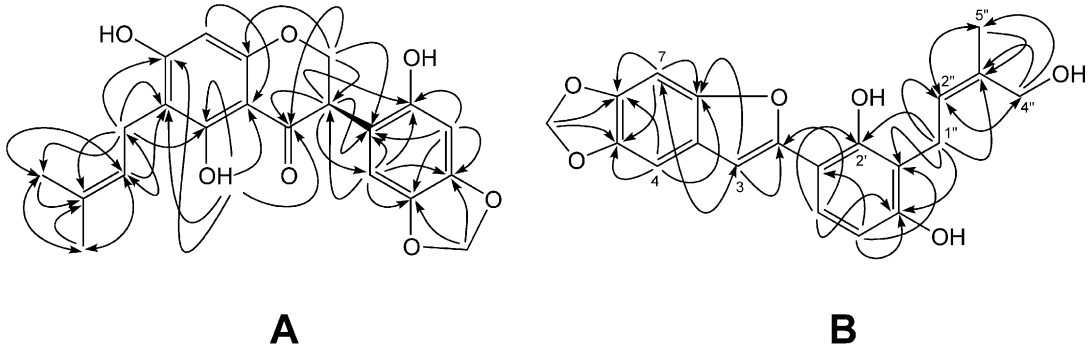

Fig. 1. ^{13}C–^{1}H long-range correlations in the HMBC spectra of compounds **18** (A) and **20** (B).

about 50-fold that of control cells. Analogous stimulatory effects were also observed for **1**, as well as for 5-deoxy prenylated flavanones **4** and **5**, both of which were the intermediates of **6**.

The trace accumulation of biosynthetic intermediates makes the elucidation of the biosynthetic route of plant secondary metabolites difficult. Some elicitors, as well as jasmonic acid and its methyl ester, have been applied for the identification of such trace intermediates. In cell cultures of *Rauwolfia serpentina*×*Rhozya struca* somatic hybrid, the addition of methyl jasmonate causes dramatic changes to indole alkaloid biosynthesis, and diverse minor alkaloids have been identified (Sheludko et al., 1999). Such stimulatory effects, however, have been generally temporary, and cultivation of cells with elicitors for a longer period has resulted in decreases of the products, presumably owing to catabolism to unknown compounds (Kessmann and Barz, 1986; Gunia et al., 1991; Rijhwani and Shanks, 1998). Furthermore, inhibition of cell growth or cell death has been observed when larger amounts of elicitors are added (Creelman and Mullet, 1995). In contrast to such stress inducers, cork tissue stimulates the production of lipophilic secondary metabolites without any inhibitory effect on cell growth, making it possible to subculture the cells by incubating with cork tissue (Yamamoto et al., 1996). In *S. flavescens* cell cultures, the inclusion of cork tissue increased not only the amounts of final products (**3**, **6** and **10**) but also those of their intermediates (**1**, **2**, **4**, **5** and **9**), most of which were easily isolated from the co-incubated cork tissue. Moreover, the stimulatory effect of cork tissue on the production of prenylated flavonoids was superior to that of methyl jasmonate. The synergistic stimulatory effect on the production of lipophilic flavonoids induced by simultaneous addition of cork tissue and methyl jasmonate, and the different responses between when cork tissue or methyl jasmonate were used on the production of the water-soluble **16,** strongly suggest that the stimulatory effects of cork tissue and methyl jasmonate could be regulated in different ways. The mechanisms underlying the stimulatory effects of cork tissue require further investigation, but cork tissue might not only stimulate the activation of enzyme(s) involved in the biosynthesis of prenylated flavonoids, but also dominantly absorb the compounds produced. Preliminary work has shown that polysaccharides in the hemicellulose B fraction from cork tissue stimulated production of **3** (Yamamoto et al., 2001a). In differentiated plants, cork tissue act as the accumulation sites of the lipophilic secondary metabolites (Tani et al., 1985, 1986; Yamamoto et al., 1992; Hayashi et al., 1996). The incubation of cork tissue with dedifferentiated cells might reflect the close interaction between sites of biosynthesis and accumulation in natural conditions. In the present study, we demonstrated that the addition of cork tissue was another effective technique for the enhancement of levels of lipophilic secondary metabolites including their intermediates, and for investigating complex secondary metabolism cultured in plant cells.

The identification of compounds **1** and **2** from cork tissue co-incubated with *S. flavescens* cells strongly supports our proposed biosynthetic route to **3** (Scheme 1) deduced from enzymic studies of naringenin8-dimethylallyltransferase (Yamamoto et al., 2000), 8-prenylnaringenin 2′-hydroxylase (Yamamoto et al., 2001a) and uncharacterized dimethylallyltransferase which gives **3** as the final product (Yamamoto et al., 2000). However the mechanisms underlying the efficient absorption of **1** and **2** into the cork tissue, and how they are transported, remains to be clarified. To elucidate the regulatory mechanisms governing the stimulatory effects of cork tissue, enzymic studies involving the lavandulyl-group-forming enzyme leachianone G 2″-dimethylallyltransferase using **2** isolated in the present study are now in progress.

3. Experimental

3.1. General

Cork sheets (2-mm thick, Dainaga Cork, Osaka, Japan) were washed successively with $CHCl_3$, MeOH, and H_2O, as reported previously (Yamamoto et al., 1996). Methyl jasmonate was purchased from Tokyo Kasei (Tokyo, Japan). ^1H and ^{13}C NMR spectra were recorded in CD_3COCD_3 and $CDCl_3$ using Varian Unity plus 500 and Varian Gemini 300 spectrometers operating at 500 MHz and 300 MHz for ^1H, and 125 MHz and 75 MHz for ^{13}C, respectively. MS were recorded on a JEOL JMS DX-303 spectrometer. UV and CD spectra were measured with a UV-1600 visible spectrophotometer and a J-725N spectrometer, respectively. Prep. HPLC was carried out using a Hikarisil C18 column (20×250 mm, Shodex, Japan) at room temperature, with a MeOH/H_2O solvent system containing 0.1% HCOOH, at a flow rate of 5 ml min^{-1}, whilst monitoring the absorbance at 294 nm. CC was performed with Silica Gel 60 N (60-210 mesh, Kanto Kagaku, Tokyo, Japan) and TOYOPEARL HW-40C (Tosoh, Tokyo, Japan). TLC and prep. TLC were performed on Kieselgel 60 F_{254} plates (Merck).

3.2. Plant material and culture method

The establishment and subculturing of cell suspension cultures of *S. flavescens* has been described previously (Yamamoto, et al., 1996). In each experiment, cells (1 g) were inoculated in MS liquid medium (20 ml) (Murashige and Skoog, 1962) containing 1 μM 2,4-D, 1 μM kinetin and additive(s), and cultured for 12 days on a

rotary shaker at a speed of 100 rpm at 23 °C in the dark. Cork tissue (50 mg/flask) was added to the medium before autoclaving. Filter-sterilized 100 mM methyl jasmonate solution in DMSO (20 μl) was aseptically added to the medium (final conc. 100 μM) after autoclaving.

3.3. Extraction and isolation

The co-incubated cork tissue (11 g) collected from 220 flasks (650 g fresh cells) was extracted three times with 2 l MeOH by ultrasonication at room temperature for 90 min. The MeOH extract (2.13 g) after concentration was subjected to silica gel chromatography using hexane–acetone (5:1–2:1) to yield Frs. 1–7 in increasing order of polarity. Frs. 1, 2, 4, 6 and 7 were respectively separated by prep. HPLC (55–70% MeOH) and/or prep. TLC (hexane–acetone, 3:1 or $CHCl_3$–MeOH, 20:1–40:1) to afford **12** (2.8 mg) and **17** (30 mg) from fr. 1; **19** (2 mg) from fr. 2; **20** (2 mg), **4** (4 mg) and **11** (2.2 mg) from fr. 4; **5** (8 mg), **8** (2 mg), **13** (3.5 mg) and **14** (2 mg) from fr. 6; and **7** (1 mg) from fr. 7. Fr. 3 was subjected to repeated chromatography on silica gel with $CHCl_3$–MeOH (40:1–20:1) to yield a mixture of two compounds, which was further purified by prep. HPLC (70% MeOH) and prep. TLC ($CHCl_3$–MeOH, 15:1) to give **19** (5 mg), **18** (18 mg) and **1** (3 mg). Fr. 5 was aplied to a TOYOPEARL HW-40 column eluted with EtOH to yield a crude fr. of **2**, which was further purified by prep. HPLC (60% MeOH) to afford **2** (93 mg).

3.3.1. Flavescenone A (*18*)

Off-white amorphous powder, UVλ_{max}^{MeOH} nm (log ε): 326sh (3.85), 298 (4.48), 234sh (4.44), 214 (4.55); CD (MeOH): $[\theta]_{355}+1085$, $[\theta]_{309}-820$, $[\theta]_{291}+6103$; HR EIMS (*m*/*z*): 384.1211 ($[M]^+$) for $C_{21}H_{20}O_7$ (calc., 384.3848). EIMS (*m*/*z*): 384, 329, 311, 284, 256, 221, 220, 205, 190, 177, 165, 164, 163, 149. For ^1H and ^{13}C NMR spectral data, see Table 2.

3.3.2. Flavescenone B (*19*)

Off-white amorphous powder, UVλ_{max}^{MeOH} nm (log ϵ): 305 (4.35), 266 (4.47), 218 (4.59). HR EIMS (*m*/*z*): 382.1022 ($[M]^+$) for $C_{21}H_{18}O_7$ (calc., 382.3690). EIMS (*m*/*z*): 382, 339, 327, 310, 282, 221, 220, 205, 177, 165, 162, 149. For ^1H and ^{13}C NMR spectral data, see Table 2.

3.3.3. Flavescenone C (*20*)

Off-white amorphous powder, UVλ_{max}^{MeOH} nm (log ϵ): 343 (4.36), 329 (4.39), 283 (4.02), 218 (4.32). HR EIMS (*m*/*z*): 354.1134 ($[M]^+$) for $C_{20}H_{18}O_6$ (calc., 354.3586). EIMS (*m*/*z*): 354 $[M]^+$, 336 $[M–H_2O]^+$, 321 $[M–H_2O–CH_3]^+$, 313, 272, 267, 236, 212, 198, 187, 186, 171, 169, 163,162, 150, 147. ^1H NMR (500 MHz, Me_2CO-d_6) δ: 8.62, 7.75 (1H each, br *s*, OH×2), 7.40 (1H, *d*, *J* = 8.7 Hz, H-6′), 7.05 (1H, *s*, H-4), 6.99 (1H, *d*, *J* = 1 Hz, H-3), 6.95 (1H, *d*, *J* = 1 Hz, H-7), 6.53 (1H, *d*, *J* = 8.7 Hz, H-5′), 5.95 (2H, *s*,-OCH_2O-), 5.47 (1H, t-like *m*, *J* = 7.2 Hz, H-2″), 3.86 (2H, br *s*, H-4″), 3.59 (1H, br *s*, OH), 3.47 (2H, *d*, *J* = 7.2 Hz, H-1″), 1.75 (3H, *s*, CH_3-5″). NOE: irradiation at δ 5.47 (H-2″) or 3.86 (H-4″), both produced enhancement at δ 3.47 (H-1″) and 1.75 (H-5″). ^{13}C NMR (125 MHz, Me_2CO-d_6) δ: 157.2 (C-4′), 155.0 (C-2), 153.7 (C-2′), 149.7 (C-8), 146.6 (C-6), 145.5 (C-5), 136.6 (C-3″), 125.6 (C-6′), 123.7 (C-9), 123.0 (C-2″), 116.5 (C-3′), 111.3 (C-1′), 108.8 (C-5′), 103.8 (C-3), 102.2 (OCH_2O), 99.9 (C-7), 94.0 (C-4), 68.3 (C-4″), 22.5 (C-1″), 13.9 (C-5″).

3.4. Quantitative analysis of flavonoids

Cells and co-incubated cork tissue were harvested separately, and were extracted with MeOH (10 ml) for 90 min by ultrasonication at room temperature. As an internal standard, 1-naphthalene acetic acid (0.5 mg) dissolved in MeOH (1 ml) was added to each extract. After centrifugation, each supernatant was subjected to reversed phase HPLC. HPLC analyses for **3**, **6**, **7**, **10**, **13**, **14**, **16** and **19** were carried out on a Capcellpack C18 AG-120A column (4.6×250 mm, Shiseido, Japan) with 1% AcOH containing an MeCN–H_2O gradient of 10–30% MeCN in 40 min, then to 80% MeCN in another 50 min at a flow rate of 0.9 ml min^{-1} (oven temp, 40 °C). Quantitative analyses of other flavonoids were carried out on the same column with 1% AcOH containing a MeOH–H_2O gradient of 50–80% MeOH in 60 min, then to 100% MeOH in another 10 min at the same flow rate. The quantities of all flavonoids were calculated from the peak area at 294 nm recorded using Chromatopac C-R4A (Shimadzu, Kyoto, Japan).

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