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Acylated sucroses and acylated quinic acids analogs from the flower buds of *Prunus mume* and their inhibitory effect on melanogenesis ☆

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ABSTRACT

The methanolic extract from the flower buds of *Prunus mume*, cultivated in Zhejiang Province, China, showed an inhibitory effect on melanogenesis in theophylline-stimulated B16 melanoma 4A5 cells. From the methanolic extract, five acylated sucroses, mumeoses A–E, and three acylated quinic acid analogs, 5-*O*-(*E*)-*p*-coumaroylquinic acid ethyl ester, and mumeic acid-A and its methyl ester, were isolated together with 13 known compounds. The chemical structures of the compounds were elucidated on the basis of chemical and physicochemical evidence. Inhibitory effects of the isolated compounds on melanogenesis in theophylline-stimulated B16 melanoma 4A5 cells were also investigated. Acylated quinic acid analogs substantially inhibited melanogenesis. In particular, 5-*O*-(*E*)-feruloylquinic acid methyl ester exhibited a potent inhibitory effect [inhibition (%): 21.5 ± 1.0 ($P < 0.01$) at 0.1 μM]. Moreover, its biological effect was much stronger than that of the reference compound, arbutin [inhibition (%): 10.6 ± 0.6 ($P < 0.01$) at 10 μM]. Interestingly, the obtained acylated quinic acid analogs displaying melanogenesis inhibitory activity showed no cytotoxicity [cell viability >97% at 10 μM]. It is concluded that acylated quinic acid analogs are promising therapeutic agents for the treatment of skin disorders.

1. Introduction

Prunus (P.) *mume* SIEB. et ZUCC. (Rosaceae) has been widely cultivated as an ornamental plant in Japan, Taiwan, China, and Korea, and its fruit has been used as a food garnish (pickled ume) and drink (ume brandy). In addition, the flowers, immature fruit, leaves, branches, seeds, and roots of *P. mume* have been exploited as traditional Chinese medicines. In particular, the flowers have been prescribed for treatment of skin disorders and eye pain and for detoxification, stomachic, expectorant, and sedative purposes. Previous studies have focused on the constituents from *P. mume* and their associated bioactivities [e.g., anticancer activity (Jeong et al., 2006), effect on adrenocorticotropic hormone and catecholamine levels in plasma (Ina et al., 2004), radical scavenging activity (Matsuda et al., 2003), inhibitory activity on squalene synthase (Choi et al., 2007) and inhibitory activity on aldose reductase and platelet aggregation (Yoshikawa et al., 2002)].

Identifying inhibitors of melanin production derived from natural medicines is of interest (Fujimoto et al., 2012; Matsuda et al., 2009; Nakamura et al., 2010, 2012a,b; Nakashima et al., 2010). As a continuation of our studies on inhibitors of melanogenesis derived from medicinal flowers, it was found that a methanolic (MeOH) extract from flower buds of Chinese *P. mume* showed inhibitory effects on melanogenesis. From the MeOH extract, five new acylated sucroses, mumeoses A (**1**), B (**2**), C (**3**), D (**4**), and E (**5**), and three new acylated quinic acid analogs, 5-*O*-(*E*)-*p*-coumaroylquinic acid ethyl ester (**6**), mumeic acid-A (**7**), and mumeic acid-A methyl ester (**8**) were isolated, together with 13 known compounds (Fig. 1). In this paper, the isolation and structural elucidation of **1**–**8** are described, as well as inhibitory effects of acylated quinic acid analogs on melanogenesis in theophylline-stimulated B16 melanoma 4A5 cells.

2. Results and discussion

2.1. Isolation of compounds from the flower buds of P. mume

A MeOH extract of the dried flower buds (30.4%) of *P. mume* (cultivated in Zhejiang Province, China) showed melanogenesis inhibitory activity [inhibition (%): 31.1 ± 1.1 ($P < 0.01$) at 100 μg/mL]. The MeOH extract was partitioned into an EtOAc–H_2O (1:1, v/v) mixture to furnish an EtOAc-soluble fraction (6.6%) and an aqueous layer. The latter was further extracted with 1-butanol to give 1-butanol- (7.5%) and H_2O- (13.0%) soluble fractions. The

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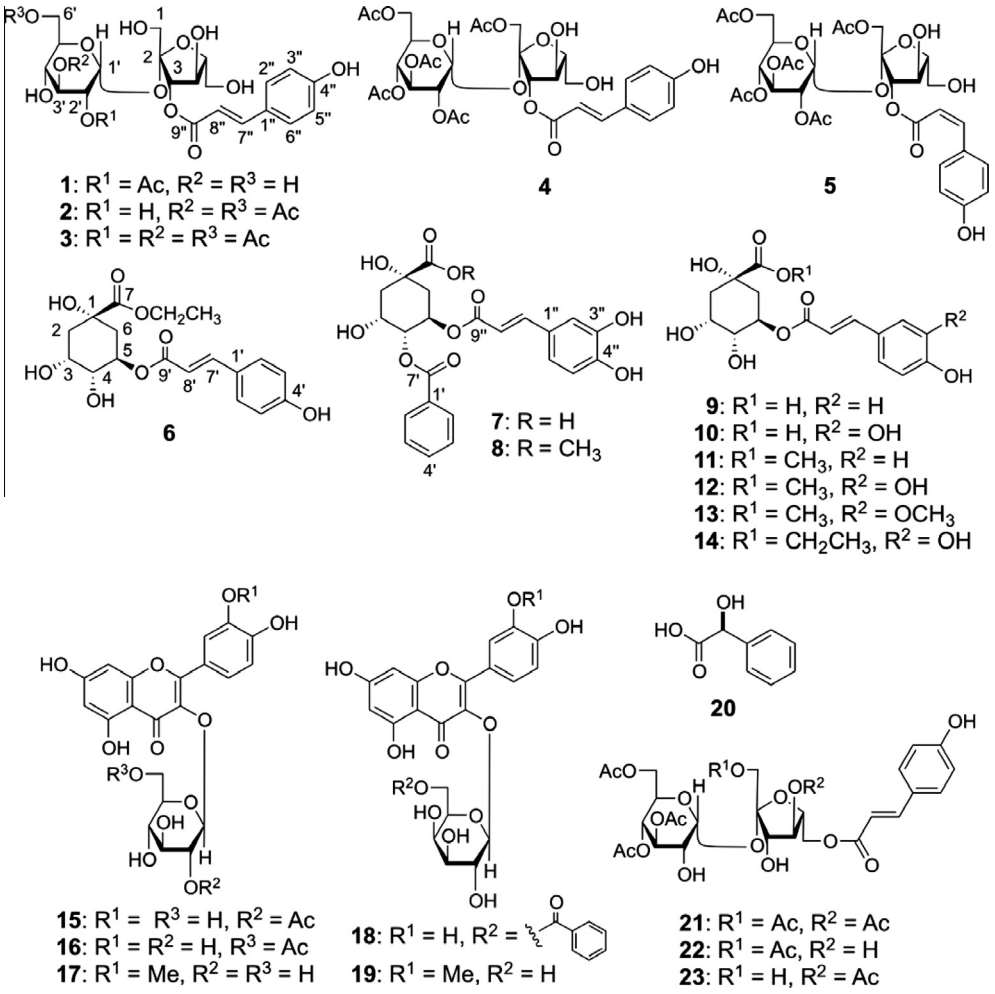

Fig. 1. Structures of compounds isolated from the flower buds of *P. mume*.

1-butanol- and the EtOAc-soluble fractions were found to have significant inhibitory effects on melanogenesis [inhibition (%): 27.5 ± 6.8 ($P < 0.01$), 41.5 ± 1.9 ($P < 0.01$), respectively at 100 μg/mL], but the H_2O-soluble fraction showed no detectable effect even at 100 μg/mL. The 1-butanol- and the EtOAc-soluble fraction were then subjected to normal- and reversed-phase silica-gel column chromatography and repeated HPLC. From the 1-butanol-soluble fraction, three new acylated sucroses, mumeose A (**1**, 0.00032%), B (**2**, 0.0010%), and C (**3**, 0.00050%), and a new acylated quinic acid analog, 5-*O*-(*E*)-*p*-coumaroylquinic acid ethyl ester (**6**, 0.0031%), were isolated together with eight known compounds, 5-*O*-(*E*)-*p*-coumaroyl quinic acid (**9**, 0.015%) (Parejo et al., 2004), chlorogenic acid (**10**, 0.11%) (Yoshikawa et al., 1999), 5-*O*-(*E*)-*p*-coumaroyl quinic acid methyl ester (**11**, 0.0013%) (Jaiswal and Kuhnert, 2011), chlorogenic acid methyl ester (**12**, 0.11%) (Zhu et al., 2005), 5-*O*-(*E*)-feruloylquinic acid methyl ester (**13**, 0.0013%) (Smarrito et al., 2008), chlorogenic acid ethyl ester (**14**, 0.038%) (Abe and Marumo, 1972), quercetin 3′-*O*-(2″-*O*-acetyl)-β-D-glucopyranoside (**15**, 0.0011%) (Machida et al., 2009), and D-mandelic acid (**20**, 0.047%). From the EtOAc-soluble fraction, two new acylated sucroses, mumeoses D (**4**, 0.00047%) and E (**5**, 0.00023%) were isolated, as well as two new acylated quinic acid analogs, mumeic acid-A (**7**, 0.0039%) and mumeic acid-A methyl ester (**8**, 0.0034%), together with seven known compounds, 5-*O*-(*E*)-*p*-coumaroylquinic acid methyl ester (**11**, 0.0014%), chlorogenic acid methyl ester (**12**, 0.016%), chlorogenic acid ethyl ester (**14**, 0.0027%), quercetin 3′-*O*-(2″-*O*-acetyl)-β-D-glucopyranoside (**15**, 0.0016%), quercetin 3-*O*-(6″-*O*-acetyl)-β-D-glucopyranoside (**16**, 0.0010%) (Wang et al., 2008), isorhamnetin 3-*O*-β-D-glucopyranoside (**17**, 0.017%) (Beck and Häberlein, 1999), quercetin 3-*O*-(6″-*O*-benzoyl)-β-D-galactopyranoside (**18**, 0.00059%) (Singh et al., 2009), isorhamnetin 3-*O*-β-D-galactopyranoside (**19**, 0.0006%) (Hsich et al., 2004), and quercetin (0.0068%). In this case, acylated sucroses, prunoses I (**21**), II (**22**), III (**23**), which were isolated from the flower buds of Japanese *P. mume* (Yoshikawa et al., 2002; Matsuda et al., 2003), were not detected in the flower buds of Chinese *P. mume.*

*2.2. Structures of mumeoses A–E (**1**–**5**), 5-O-(E)-p-coumaroylquinic acid ethyl ester (**6**), mumeic acid-A (**7**), and mumeic acid-A methyl ester (**8**)*

Mumeose A (**1**) was isolated as a white amorphous powder with positive optical rotation (**1**: $[\alpha]_D^{15}$ +114.8, in MeOH). Its IR spectrum showed absorption bands at 3400, 1730, 1697, 1603, 1515, and 1033 cm^{-1} due to hydroxy, ester, α,β-unsaturated ester, aromatic ring, and ether functions. FABMS in the positive-ion mode gave a quasimolecular ion peak ($[M+Na]^+$) at *m/z* 553, from which the molecular formula $C_{23}H_{30}O_{14}$ was determined by high-resolution (HR) MS. Treatment of **1** with a 10% aqueous KOH-1,4-dioxane (1:1, v/v) mixture yielded D-sucrose, which was identified by comparison of the retention time and optical rotation (t_R: 19.8 min with positive rotation) with that of an authentic sample on

reversed-phase HPLC analysis using an optical rotation detector. Acid hydrolysis of **1** with a 5% aqueous H_2SO_4-1,4-dioxane yielded D-glucose and fructose together with (*E*)-*p*-coumaric acid. D-Glucose was changed to the tolylthiocarbamoyl thiazolidine derivative by reaction with L-cysteine methyl ester and *o*-torylisothiocyanate and identified by comparison of its retention time (t_R: 19.7 min) with that of authentic sample on reversed-phase HPLC analysis (Tanaka et al., 2007). (*E*)-*p*-Coumaric acid was also identified by comparison of its retention time (t_R: 17.1 min) with that of an authentic sample on reversed-phase HPLC analysis. The ^{1}H NMR (methanol-d_4) and ^{13}C NMR (Table 1) spectra of **1**, which were assigned by various NMR experiments (DEPT, DQF COSY, HMQC and HMBC spectra), showed signals assignable to an acetyl group [δ 2.07 (s, CH_3CO–)] and a (*E*)-*p*-coumaroyl group [δ 6.43 (d, J = 16.2 Hz, H-8″), 6.81 (d, J = 8.6 Hz, H-3″, 5″), 7.51 (d, J = 8.6 Hz, H-2″, 6″), 7.70 (d, J = 16.2 Hz, H-7″)] and a sucrose moiety [δ 3.43 (m, H-4′), 3.45, 3.61 (both d-like, H_2-1), 3.74 (m, H-6′a), 3.78 (m, H_2-6, 3′), 3.89 (m, H-5, 5′, 6′b), 4.36 (dd, J = 8.3, 8.3 Hz, H-4), 4.56 (dd, J = 3.7, 10.1 Hz, H-2′), 5.47 (d, J = 8.3 Hz, H-3), 5.55 (d, J = 3.7 Hz, H-1′)]. The linkage between two monosaccharides, as well as the positions of the acetyl group and (*E*)-*p*-coumaroyl moiety, were confirmed based on heteronuclear multiple bond connectivity (HMBC) spectroscopy. Namely, long-range correlations were observed between the following proton and carbon pairs: H-3 and C-9″, H-1′ and C-2, H-2′ and an acetyl carbonyl carbon. On the basis of this evidence, the chemical structure of mumeose A (**1**) was determined to be 2′-*O*-acetyl-3-*O*-(*E*)-*p*-coumaroylsucrose.

Mumeoses B (**2**), C (**3**), and D (**4**), which were obtained as white amorphous powders with positive optical rotations (**2**: $[\alpha]_D^{15}$ +46.9, **3**: $[\alpha]_D^{15}$ +54.0, **4**: $[\alpha]_D^{15}$ +6.0, in MeOH), showed absorption bands due to hydroxy, ester, α,β-unsaturated ester, and ether functionalities in their IR spectra. Their molecular formulas ($C_{25}H_{32}O_{15}$ of **2**, $C_{27}H_{34}O_{16}$ of **3**, $C_{31}H_{38}O_{18}$ of **4**) were determined from the quasimolecular ion peaks (*m*/*z* 595 $[M+Na]^+$ for **2**, 637 $[M+Na]^+$ for **3**, 721 $[M+Na]^+$ for **4**) in the positive-ion FABMS and by HRMS measurement. Basic hydrolysis of **2**–**4** with a 10% aqueous KOH-1,4-dioxane (1:1, v/v) mixture yielded D-sucrose. Acid hydrolysis of **2**–**4** with a 5% aqueous H_2SO_4-1,4-dioxane yielded D-glucose and fructose together with (*E*)-*p*-coumaric acid, as well as **1**. The ^{1}H NMR (methanol-d_4) and ^{13}C NMR (Table 1) spectra of **2**, which were assigned by various NMR experiments, showed signals assignable to two acetyl groups [δ 1.98, 2.08 (s, CH_3CO– × 2)], a (*E*)-*p*-coumaroyl group [δ 6.43 (d, J = 15.8 Hz, H-8″), 7.71 (d, J = 15.8 Hz, H-7″), 7.53 (d, J = 8.6 Hz, H-2″, 6″), 6.61 (d, J = 8.6 Hz, H-3″, 5″)] and a sucrose moiety [δ 3.46 (dd, J = 9.4, 9.4, H-4′), 3.59 (m, H_2-1, H-2′), 3.83 (m, H_2-6), 3.94 (ddd, J = 3.1, 3.1, 7.9 Hz, H-5), 4.18 (m, H-5′, 6′a), 4.34 (dd, J = 7.9, 7.9 Hz, H-4), 4.46 (m H-6′b), 5.18 (dd, J = 9.4, 9.4, H-3′), 5.49 (d, J = 7.9 Hz, H-3), 5.50 (d, J = 3.1 Hz, H-1′)]. The proton and carbon signals in the ^{1}H and ^{13}C NMR (Table 1) spectra of **2** were superimposable on those of **1**, except for the resonances around the glucose part of **2**. The linkage between two monosaccharides, as well as the positions of two acetyl groups and a (*E*)-*p*-coumaroyl moiety, were confirmed based on HMBC spectroscopy. Namely, long-range correlations were observed between the following proton and carbon pairs: H-3

Table 1
^{1}H and ^{13}C NMR (125 MHz) spectroscopic data for **1**–**5** in CD_3OD (δ in ppm, J in Hz).

Position	**1**		**2**		**3**		**4**		**5**	
	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H
Sucrose part										
1	63.1	3.45 d like 3.61 d like	65.3	3.59 m	64.5	3.45 d (11.6) 3.60 d (11.6)	66.3	4.09 d (11.7) 4.21 d (11.7)	66.0	4.20 m
2	105.2	–	104.9	–	105.4	–	103.9	–	103.8	–
3	78.9	5.47 d (8.3)	79.5	5.49 d (7.9)	78.6	5.53 d (8.6)	79.8	5.38 d (7.8)	79.2	5.37 d (8.0)
4	73.6	4.36 dd (8.3, 8.3)	74.1	4.34 dd (7.9, 7.9)	73.9	4.32 dd (8.6, 8.6)	73.9	4.31 dd (7.8, 7.8)	73.4	4.25 dd (8.0, 8.0)
5	83.9	3.89 m	84.2	3.94 ddd (3.1, 3.1, 7.9)	84.2	3.92 m	84.5	3.93 m	84.4	3.89 m
6	64.7	3.78 m	63.7	3.83 m	63.7	3.81 m	63.4	3.78 m	63.2	3.76 m
1′	90.9	5.55 d (3.7)	92.9	5.50 d (3.1)	90.5	5.62 d (3.7)	90.6	5.68 d (3.6)	90.3	5.65 d (3.9)
2′	74.6	4.56 dd (3.7, 10.1)	71.3	3.59 m	72.2	4.73 dd (3.7, 10.1)	71.6	4.88 m	71.6	4.84 m
3′	72.2	3.78 m	76.9	5.18 dd (9.4, 9.4)	73.7	5.32 dd (10.1, 10.1)	71.3	5.39 dd (10.1, 10.1)	71.3	5.34 dd (9.9, 9.9)
4′	71.3	3.43 m	69.8	3.46 dd (9.4, 9.4)	69.7	3.56 dd (10.1, 10.1)	70.0	4.98 dd (10.1, 10.1)	69.8	4.99 m
5′	74.2	3.89 m	72.1	4.18 m	71.8	4.17 m	69.7	4.34 m	69.6	4.14 m
6′	62.3	3.74 m 3.89 m	65.0	4.18 m 4.46 m	64.6	4.20 m 4.47 m	63.6	4.18 m	63.3	4.09 m
p-Coumaroyl part										
1″	127.2	–	127.3	–	127.2	–	127.1	–	127.5	–
2″,6″	131.5	7.51 d (8.6)	131.5	7.53 d (8.6)	131.6	7.53 d (8.6)	131.6	7.53 d (8.6)	134.0	7.67 d (8.6)
3″,5″	116.8	6.81 d (8.6)	116.7	6.61 d (8.6)	116.8	6.79 d (8.6)	116.8	6.81 d (8.6)	115.9	6.76 d (8.6)
4″	161.5		161.3		161.4		161.6		160.4	
7″	147.6	7.70 d (16.2)	147.6	7.71 d (15.8)	147.7	7.71 d (15.8)	148.0	7.74 d (16.1)	146.7	6.99 d (13.0)
8″	114.5	6.43 d (16.2)	114.7	6.43 d (15.8)	114.5	6.44 d (15.8)	114.2	6.43 d (16.1)	115.4	5.89 d (13.0)
9″	168.5	–	168.6	–	168.6	–	168.2	–	167.3	–
Acyl part										
CH_3COO–	172.7	–	172.8	–	172.0	–	171.3	–	171.3	–
			172.8	–	172.1	–	171.5	–	171.6	–
					172.7	–	171.8	–	171.8	–
							172.0	–	172.1	–
							172.4	–	172.4	–
CH_3COO–	21.0	2.07 s	20.8	1.98 s	20.8[a]	2.00[b] s	20.4[c]	1.83[d] s	20.6[e]	1.96[f] s
			21.1	2.08 s	20.8[a]	2.02[b] s	20.5[c]	1.93[d] s	20.6[e]	2.00[f] s
					20.9[a]	2.07[b] s	20.6[c]	2.04[d] s	20.6[e]	2.02[f] s
							20.7[c]	2.06[d] s	20.7[e]	2.05[f] s
							20.7[c]	2.09[d] s	20.7[e]	2.08[f] s

[a–f] Interchangeable.

and C-9″, H-1′ and C-2, H-3′, 6′ and two acetyl carbonyl carbons. The ^{1}H NMR (methanol-d_4) and ^{13}C NMR (Table 1) spectra of **3** and **4** showed signals assignable to three acetyl groups [δ 2.00, 2.02, 2.07 (s, CH_3CO– × 3) for **3**] and five acetyl groups [δ 1.83, 1.93, 2.04, 2.06, 2.09 (s, CH_3CO– × 5) for **4**], respectively, together with a (*E*)-*p*-coumaroyl group and a sucrose moiety. The proton and carbon resonances in the ^{1}H and ^{13}C NMR (Table 1) spectra of **3** were superimposable on those of **1**, except for the signals around the glucose part of **3**. The ^{1}H and ^{13}C NMR chemical shift values of the 3′ and 6′ positions of the glucose part on **3** were observed at lower fields compared with those of **1**. By acetylation of one or two hydroxy groups on the sugar moiety, the ^{1}H and ^{13}C chemical shift values of the neighboring sugar-skeleton protons and carbon of the connected acetyl groups were generally shifted downfield. Therefore, **3** was suggested to possess two acetyl groups at the 3′ and 6′ positions of **1**. In addition, since the ^{1}H and ^{13}C NMR chemical shift values of the 1 position of **4** were observed at the lower fields compared with those of **3**, the existence of an acetyl group at the 1 position of the fructose part of **4** was suggested. Next, positions of acetyl groups of **3** and **4** were confirmed based on HMBC spectroscopy. Namely, long-range correlations were observed between the following proton and carbon pairs [H-2′, 3′, 6′ and three acetyl carbonyl carbons for **3**, H-1, 2′, 3′, 4′, 6′ and five acetyl carbonyl carbons for **4**]. Consequently, the chemical structures of mumeoses B (**2**), C (**3**), and D (**4**) were characterized to be 3′,6′-di-*O*-acetyl-3-*O*-(*E*)-*p*-coumaroylsucrose, 2′,3′,6′-tri-*O*-acetyl-3-*O*-(*E*)-*p*-coumaroylsucrose, and 1,2′,3′,4′,6′-penta-*O*-acetyl-3-*O*-(*E*)-*p*-coumaroylsucrose, respectively.

Mumeose E (**5**), obtained as a white amorphous powder with a negative optical rotation ($[\alpha]_D^{15}$ −30.7 in MeOH), showed absorption bands due to hydroxy, ester, α,β-unsaturated ester, and ether functionalities in the IR spectrum. Its positive FABMS showed a quasimolecular ion peak at *m*/*z* 721 [M+Na]$^+$ and a molecular formula $C_{31}H_{38}O_{18}$ was determined by HRMS measurement. Basic hydrolysis of **5** yielded D-sucrose and acid hydrolysis of **5** yielded D-glucose, fructose, and (*Z*)-*p*-coumaric acid, respectively. (*Z*)-*p*-Coumaric acid was identified by comparison of its retention time (t_R: 18.5 min) with that of an authentic sample on reversed-phase HPLC analysis. The ^{1}H NMR (methanol-d_4) and ^{13}C NMR (Table 1) spectra of **5** showed signals assignable to five acetyl groups [δ 1.96, 2.00, 2.02, 2.05, 2.08 (s, CH_3CO– × 5)] and a (*Z*)-*p*-coumaroyl group [δ 5.89 (d, *J* = 13.0 Hz, H-8″), 6.76 (d, *J* = 8.6 Hz, H-3″, 5″), 6.99 (d, *J* = 13.0 Hz, H-7″), 7.67 (d, *J* = 8.6 Hz, H-2″, 6″)] together with a sucrose moiety. Comparison of the NMR spectroscopic data for **5** with those for **4** led us to confirm the structure of **5** to be the *cis*–*trans* isomer of **4**. Consequently, the chemical structure of mumeose E (**5**) was characterized to be 1,2′,3′,4′,6′-penta-*O*-acetyl-3-*O*-(*Z*)-*p*-coumaroylsucrose.

5-*O*-(*E*)-*p*-Coumaroylquinic acid ethyl ester (**6**) was isolated as a white amorphous powder with negative optical rotation ($[\alpha]_D^{15}$ −8.9 in MeOH). Its IR spectrum showed absorption bands at 3400, 1720, 1690, 1603 and 1515 cm^{-1} due to hydroxy, ester, α,β-unsaturated ester, and aromatic ring moieties. The EIMS of **6** showed a molecular ion peak at *m*/*z* 366 [M]$^+$ and the molecular formula $C_{18}H_{22}O_8$ was determined by HRMS measurement. Treatment of **6** with a 5% aqueous H_2SO_4-1,4-dioxane yielded D-(−)-quinic acid and (*E*)-*p*-coumaric acid. D-(−)-Quinic acid was identified by comparison of the analytical data (NMR and MS spectra and optical rotation) with that of an authentic sample. (*E*)-*p*-Coumaric acid was identified by comparison of its retention time (t_R: 17.1 min) with that of an authentic sample on reversed-phase HPLC analysis. The ^{1}H NMR (methanol-d_4) and ^{13}C NMR (Table 1) spectra of **6** showed signals assignable to a D-(−)-quinoyl moiety [δ 2.00 (m, H_2-2), 2.18 (m, H_2-6), 3.72 (dd, *J* = 3.1, 7.6 Hz, H-4), 4.10 (m, H-3), 5.28 (ddd, *J* = 4.6, 7.6, 7.6 Hz, H-5)], a (*E*)-*p*-coumaroyl group [δ 6.28 (d, *J* = 15.8 Hz, H-8′), 7.52 (d, *J* = 15.8 Hz, H-7′), 7.44 (d, *J* = 8.6 Hz, H-2′, 6′), 6.54 (d, *J* = 8.6 Hz, H-3′, 5′)] and an ethyl ester part [δ 1.23 (t, *J* = 7.0 Hz, OCH_2CH_3), 4.13 (q, *J* = 7.0 Hz, OCH_2CH_3)]. The proton and carbon resonances in the ^{1}H and ^{13}C NMR spectra of **6** were superimposable on those of **11**, except for the signals around the ethyl ester part of **6**. The positions of the (*E*)-*p*-coumaroyl group and ethyl ester part were confirmed based on DQF COSY and HMBC spectroscopy. Namely, long-range correlations were observed between the following proton and carbon pairs: H-5 and C-9′, OCH_2CH_3 and C-7. Consequently, the chemical structure of **6** was determined to be 5-*O*-(*E*)-*p*-coumaroylquinic acid ethyl ester.

Mumeic acid-A (**7**) and mumeic acid-A methyl ester (**8**), which were obtained as white amorphous powders with negative optical rotations, showed absorption bands due to hydroxy, benzoyl, α,β-unsaturated ester, and aromatic ring moieties in their IR spectra. The molecular formulas, $C_{23}H_{22}O_{10}$ and $C_{24}H_{24}O_{10}$, were determined by HRMS. Acid hydrolysis of **7** and **8** with a 5% aqueous H_2SO_4-1,4-dioxane yielded D-(−)-quinic acid, benzoic acid, and (*E*)-*p*-caffeic acid. D-(−)-Quinic acid was identified by comparison of the analytical data with that of the authentic sample as well as **6**. Benzoic acid and (*E*)-*p*-caffeic acid were identified by comparison of their retention times (t_R: 23.0 min for benzoic acid, t_R: 12.1 min for (*E*)-*p*-caffeic acid) with those of authentic samples on reversed-phase HPLC analysis. The ^{1}H NMR (methanol-d_4) and ^{13}C NMR (Table 1) spectra of **7** showed signals assignable to a D-(−)-quinoyl moiety [δ 2.11 (m, H-2a), 2.26 (m, H-2b, H_2-6), 4.43 (m, H-3), 5.20 (br d, *J* = 8.3, H-4), 5.71 (m, H-5)], a benzoyl group [δ 7.37 (dd, *J* = 7.5, 7.5 Hz, H-3′, 5′), 7.50 (m, H-4′), 7.99 (d, *J* = 7.5 Hz, H-2′, 6′)] and a (*E*)-*p*-caffeoyl group [δ 6.09 (d, *J* = 15.8 Hz, H-8″), 7.43 (d, *J* = 15.8 Hz, H-7″), 6.91 (br s, H-2″), 6.68 (d, *J* = 8.1 Hz, H-5″), 6.81 (br d, *J* = 8.1 Hz, H-6″)]. The proton and carbon resonances of the quinic acid part in the ^{1}H and ^{13}C NMR spectra of **7** were superimposable on those of 4,5-*O*-*trans*-*p*-dicaffeoyl-D-quinic acid (Shi et al., 2007). The positions of a (*E*)-*p*-caffeoyl group and a benzoyl group in **7** were also confirmed by HMBC experiments, which showed long-range correlations between the following proton and carbon pairs; H-4 and C-7′, H-5 and C-9″. The ^{1}H NMR (methanol-d_4) and ^{13}C NMR spectra of **8** showed signals assignable to a methyl ester part [δ 3.72 (s, C(=O)OCH_3)] together with a D-(−)-quinoyl moiety, a benzoyl group, and a (*E*)-*p*-caffeoyl group. This result and the detailed DQF COSY and HMBC experiments led us to confirm the structure of **8** to be the methyl ester derivative of **7**. Consequently, the chemical structures of mumeic acid-A (**7**) and mumeic acid-A methyl ester (**8**) were 4-*O*-benzoylchlorogenic acid and 4-*O*-benzoylchrologenic acid methyl ester.

2.3. Inhibitory effects of the compounds on melanogenesis in B16 melanoma 4A5 cells

Melanin production, which is principally responsible for skin color, is a major defense mechanism against harmful ultraviolet rays in sunlight. However, excess production of melanin after long periods of exposure to the sun can cause dermatological disorders such as melasma, freckles, postinflammatory melanoderma, and solar lentigines. To develop inhibitors of melanogenesis, the inhibitory effects of several diarylheptanoids, flavonoids, sterol glycosides, and acylated triterpene glycosides were examined in theophylline-stimulated B16 melanoma 4A5 cells (Fujimoto et al., 2012; Matsuda et al., 2009; Nakashima et al., 2010; Nakamura et al., 2010, 2012a,b). As a continuation of these studies, the inhibitory effects of constituents from the flowers buds of *P. mume* on melanogenesis were examined. Among the isolates, acylated quinic acid analogs **6**–**14** significantly inhibited melanogenesis (Table 3). Indeed, **6**–**14** each displayed greater potency for inhibiting melanogenesis than that of the reference compound, arbutin (Fujimoto

et al., 2012). Particularly, 5-*O*-(*E*)-feruloylquinic acid methyl ester (**13**) exhibited a potent inhibitory effect on melanogenesis [inhibition (%): 21.5 ± 1.0 ($P < 0.01$) at 0.1 μM]. Next, the cytotoxic effects of the constituents on B16 melanoma 4A5 cells were investigated. Many of the compounds displaying melanogenesis inhibitory effects, such as arbutin, diarylheptanoids, flavonoids and saponins, have cytotoxic action at concentrations greater than 10 μM (Fujimoto et al., 2012; Matsuda et al., 2009; Nakashima et al., 2010; Nakamura et al., 2012a,b). Interestingly, acylated quinic acid analogs **6**–**14** showed potent inhibitory effects on melanogenesis, but displayed no cytotoxicity [cell viability >97% at 10 μM]. Therefore, acylated quinic acid analogs are promising therapeutic agents for the treatment of skin disorders. By contrast, acylated sucroses **1**–**5**, flavonol glycosides **15**–**19**, and D-mandelic acid (**20**) had no inhibitory effects on melanogenesis at 1–100 μM.

2.4. HPLC profile comparison of acylated quinic acid analogs *6*, *8*, *11*–*14*

In the present study, nine acylated quinic acid analogs including four methyl esters (**8**, **11**–**13**) and two ethyl esters (**6** and **14**) were isolated from the MeOH extract of flower buds of *P. mume*. In order to establish whether these methyl- or ethyl esters were artifacts generated during separation procedures, the HPLC profiles of the 1-butanol- and EtOAc-soluble fractions from the MeOH-extract were compared with those fractions from the MeCN/water (1:1, v/v)-extract of the flower buds of *P. mume*. The 1-butanol- and EtOAc-soluble fractions of MeCN/water (1:1, v/v)-extract were prepared without using methanol nor ethanol. A portion of the 1-butanol-soluble and EtOAc-soluble fractions of the MeOH- and MeCN/water (1:1, v/v)-extracts were dissolved in MeCN and subjected to reversed-phase HPLC. Acylated quinic acid analogs (**6**, **8**–**14**) isolated from the MeOH extract were detected in the MeCN/water (1:1, v/v) extract by comparing their retention times with those of authentic samples. In addition, the HPLC analysis of 1-butanol- and EtOAc-soluble fractions of MeOH- and the MeCN/water (1:1, v/v) extracts showed comparable HPLC profiles. Based on these results, the methyl- and ethyl ester derivatives of quinic acid (**6**, **8**, **11**–**14**), which were isolated from a MeOH extract of flower buds of *P. mume*, seem to be genuine natural products.

3. Conclusion

Five new acylated sucroses, mumeoses A (**1**), B (**2**), C (**3**), D (**4**) and E (**5**), and three new acylated quinic acid analogs, 5-*O*-(*E*)-*p*-coumaroylquinic acid ethyl ester (**6**), and mumeic acid-A (**7**) and its methyl ester (**8**), were isolated from the flower buds of *P. mume* cultivated in Zhejiang Province, China. Acylated quinic acid analogs **6**–**14** significantly inhibited melanogenesis without inducing cytotoxicity. Further structure activity relationship studies and elucidation of the inhibitory mechanism are warranted.

4. Experimental

4.1. General experimental procedures

The following instruments were used to obtain physical data: specific rotations, a Horiba SEPA-300 digital polarimeter (l = 5 cm); IR spectra, a Thermo Electron Nexus 470; FABMS and HRFABMS, a JEOL JMS-SX 102A mass spectrometer; ^{1}H NMR spectra, JEOL JNM-LA 500 (500 MHz) spectrometer; ^{13}C NMR spectra, JEOL JNM-LA 500 (125 MHz) spectrometer; HPLC, a Shimadzu SPD-10AVP UV–VIS detector. COSMOSIL 5C18-MS-II [4.6 mm I.D. – 250 mm (particle size: 5 μm) and 20 mm I.D. – 250 mm (particle size: 5 μm)] and 5C18-AR-II [4.6 mm I.D. – 250 mm (particle size: 5 μm) and 20 mm I.D. – 250 mm (particle size: 5 μm)] columns were used for analytical and preparative purposes.

The following experimental materials were used for chromatography: normal-phase silica gel column chromatography (cc), Silica gel BW-200 (Fuji Silysia Chemical Ltd., 150–350 mesh); reversed-phase silica gel cc, Chromatorex ODS DM1020T (Fuji Silysia Chemical Ltd., 100–200 mesh); TLC, precoated TLC plates with Silica gel 60F_{254} (Merck, 0.25 mm) (ordinary phase) and Silica gel RP-18 F_{254S} (Merck, 0.25 mm) (reversed phase); reversed-phase HPTLC, precoated TLC plates with Silica gel RP-18 WF_{254S} (Merck, 0.25 mm). Detection was achieved by spraying with 1% $Ce(SO_4)_2$–10% aqueous H_2SO_4 followed by heating.

4.2. Plant material

Dried flower buds of *P. mume* cultivated in Zhejiang Province, China, were commercial products purchased from Tochimoto Tenkaido Co. Ltd. (Osaka, Japan) in April 2011. The botanical identification was undertaken by one of the authors (M.Y.). A voucher of the plant is on file in our laboratory (KPU Medicinal Flower-PM 2011).

4.3. Extraction and isolation

Dried flower buds (2.0 kg) were extracted with MeOH (10 L × 3) under conditions of reflux for 3 h. Evaporation of the solvents under reduced pressure provided a MeOH extract (608.0 g, 30.4%). An aliquot of the MeOH extract (486.4 g) was suspended in distilled H_2O (20 L), and partitioned with EtOAc (20 L × 3) to furnish an EtOAc-soluble fraction (106.0 g, 6.6%) and an aqueous phase. The latter was further partitioned with 1-butanol (10 L × 3) to give a 1-butanol-soluble fraction (119.3 g, 7.5%) and an H_2O-soluble fraction (256.0 g, 16.0%). An aliquot of the 1-butanol-soluble fraction (80.0 g) was subjected to normal phase silica gel cc [Silica gel BW-200 (2.4 kg, 100 mm I.D. – 180 mm), Hexane → $CHCl_3$–MeOH (50:1 → 20:1 → 10:1 → 5:1 → 10:3, v/v) → MeOH] to give seven fractions [Fr.B1 (450 mg), Fr.B2 (960 mg), Fr.B3 (850 mg), Fr.B4 (1.37 g), Fr.B5 (4.70 g), Fr.B6 (7.24 g), Fr.B7 (41.4 g)]. Fraction B5 (4.70 g) was further separated by reversed phase silica gel cc [Chromatorex ODS DM1020T (132 g, 30 mm I.D. – 80 mm), MeOH–H_2O (20:80 → 30:70 → 40:60 → 50:50 → 60:40, v/v) → MeOH] to give 10 fractions [Fr.B5-1, Fr.B5-2 (730 mg), Fr.B5-3, Fr.B5-4 (260 mg), Fr.B5-5, Fr.B5-6, Fr.B5-7, Fr.B5-8, Fr.B5-9 (170 mg), Fr.B5-10]. Fraction B5-2 (108 mg) was purified by HPLC [COSMOSIL 5C18-MS-II (preparative column), H_2O–MeOH–AcOH (750:250:3, v/v/v)] to give **20** (68 mg). Fraction B5-4 (260 mg) was purified by HPLC [COSMOSIL 5C18-MS-II (preparative column), H_2O–MeOH–AcOH (700:300:3, v/v/v)] to give **10** (129 mg). Fr.B5-9 (170 mg) was purified by HPLC [COSMOSIL 5C18-AR-II (preparative column), H_2O–MeOH–AcOH (650:350:3, v/v/v)] to give two fractions [Fr.B5-9-1 (61 mg), Fr.B5-9-2 (39 mg)]. Fraction B5-9-1 (61 mg) was purified by HPLC [COSMOSIL 5C18-AR-II (preparative column), H_2O–MeOH–AcOH (650:350:3, v/v/v)] to give **11** (14 mg) and **13** (14 mg). Fraction B5-9-2 (39 mg) was purified by HPLC [COSMOSIL 5C18-MS-II (preparative column), H_2O–MeCN–AcOH (800:200:3, v/v/v)] to give **3** (5.3 mg). Fraction B6 (7.24 g) was further separated by normal phase silica gel cc [Silica gel BW-200 (230 g, 40 mm I.D. – 110 mm), $CHCl_3$–EtOAc (1:1, v/v) → $CHCl_3$–EtOAc–2-propanol–MeOH (20:20:1:1 → 15:15:1:1 → 10:10:1:1 → 5:5:1:1 → 1:1:1:1, v/v) → MeOH] to give five fractions [Fr.B6-1, Fr.B6-2 (1.56 g), Fr.B6-3 (3.83 g), Fr.B6-4 (920 mg), Fr.B6-5]. Fraction B6-2 (1.56 g) was purified by reversed phase silica gel cc [Chromatorex ODS DM1020T (66 g, 20 mm I.D. – 90 mm), MeOH–H_2O (20:80 → 30:70 → 40:60 → 50:50 → 60:40, v/v) → MeOH] to give seven fractions [Fr.B6-2-1, Fr.B6-2-2 (368 mg), Fr.B6-2-3 (63 mg), Fr.B6-2-4 (840 mg), Fr.B6-2-5 (62 mg), Fr.B6-2-6, Fr.B6-2-7]. Fraction B6-2-2 (37 mg) was purified by HPLC

[COSMOSIL 5C18-MS-II (preparative column), H_2O–MeOH–AcOH (700:300:3, v/v/v)] to give **9** (15 mg) and **10** (14 mg). Fraction B6-2-3 (63 mg) was purified by HPLC [COSMOSIL 5C18-MS-II (preparative column), H_2O–MeOH–AcOH (700:300:3, v/v/v)] to give **9** (18 mg) and **10** (5.6 mg), and **12** (16 mg). Fraction B6-2-4 (20 mg) was purified by HPLC [COSMOSIL 5C18-MS-II (preparative column), H_2O–MeOH–AcOH (650:350:3, v/v/v)] to give **12** (8.7 mg) and **14** (9.5 mg). Fraction B6-2-5 (62 mg) was purified by HPLC [COSMOSIL 5C18-MS-II (preparative column), H_2O–MeOH–AcOH (550:450:3, v/v/v)] to give **6** (33 mg) and **14** (8.4 mg). Fraction B6-3 (3.83 g) was purified by reversed phase silica gel cc [Chromatorex ODS DM1020T (132 g, 25 mm I.D. – 110 mm), MeOH–H_2O (20:80 → 30:70 → 40:60 → 50:50 → 60:40, v/v) → MeOH] to give nine fractions [Fr.B6-3-1 (186 mg), Fr.B6-3-2 (2.47 g), Fr.B6-3-3, Fr.B6-3-4 (520 mg), Fr.B6-3-5 (158 mg), Fr.B6-3-6, Fr.B6-3-7, Fr.B6-3-8 (51 mg), Fr.B6-3-9]. Fraction B6-3-1 (186 mg) was purified by HPLC [COSMOSIL 5C18-MS-II (preparative column), H_2O–MeOH–AcOH (800:200:3, v/v/v)] to give **10** (17 mg). Fraction B6-3-2 (100 mg) was purified by HPLC [COSMOSIL 5C18-MS-II (preparative column), H_2O–MeOH–AcOH (700:300:3, v/v/v)] to give **10** (28 mg) and **12** (24 mg). Fraction B6-3-4 (26 mg) was purified by HPLC [COSMOSIL 5C18-MS-II (preparative column), H_2O–MeOH–AcOH (700:300:3, v/v/v)] to give **12** (8.6 mg). Fraction B6-3-5 (158 mg) was purified by HPLC [COSMOSIL 5C18-MS-II (preparative), H_2O–MeOH–AcOH (650:350:3, v/v/v)] to give **9** (4.0 mg) and **12** (10 mg). Fraction B6-3-8 (51 mg) was purified by HPLC [COSMOSIL 5C18-MS-II (preparative column), H_2O–MeOH–AcOH (650:350:3, v/v/v)] to give **15** (12 mg) and **17** (30 mg). Fraction B6-4 (910 mg) was purified by reversed phase silica gel cc [33 g, MeOH–H_2O (20:80 → 30:70 → 40:60 → 50:50 → 60:40, v/v) → MeOH] to give six fractions [Fr.B6-4-1, Fr.B6-4-2, Fr.B6-4-3 (366 mg), Fr.B6-4-4 (175 mg), Fr.B6-4-5 (48 mg), Fr.B6-4-6]. Fraction B6-4-3 (37 mg) was purified by HPLC [COSMOSIL 5C18-MS-II (preparative column), H_2O–MeOH–AcOH (700:300:3, v/v/v)] to give **9** (6.2 mg) and **10** (15 mg). Fraction B6-4-4 (175 mg) was purified by HPLC [COSMOSIL 5C18-MS-II (preparative column), H_2O–MeOH–AcOH (700:300:3, v/v/v)] to give **1** (3.4 mg), **2** (11 mg), **9** (13 mg), **10** (18 mg), and **12** (8.1 mg). Fraction B6-4-5 (48 mg) was purified by HPLC [COSMOSIL 5C18-MS-II (preparative column), H_2O–MeOH–AcOH (650:350:3, v/v/v)] to give **14** (11 mg). A part of the EtOAc-soluble fraction (80 g) was subjected to normal phase silica gel cc [Silica gel BW-200 (2.4 kg, 100 mm I.D. – 180 mm), *n*-hexane → *n*-hexane–EtOAc (5:1 → 3:1, v/v) → $CHCl_3$–MeOH (100:1 → 50:1 → 20:1 → 10:1 → 4:1 → 2:1, v/v) → MeOH] to give six fractions [Fr.E1 (15.9 g), Fr.E2 (4.13 g), Fr.E3 (9.73 g), Fr.E4 (6.39 g), Fr.E5 (20.6 g), Fr.E6 (13.3 g)]. Fraction E4 (6.39 g) was further separated by reversed phase silica gel cc [Chromatorex ODS DM1020T (200 g, 30 mm I.D. – 120 mm), MeOH–H_2O (30:70 → 40:60 → 50:50 → 60:40, v/v) → MeOH] to give five fractions [Fr.E4-1, Fr.E4-2, Fr.E4-3, Fr.E4-4 (750 mg), Fr.E4-5 (1.82 g)]. Fraction E4-4 (750 mg) was further separated by normal phase silica gel cc [Silica gel BW-200 (17 g, 15 mm I.D. – 60 mm), *n*-hexane → *n*-hexane–EtOAc (10:1 → 5:1 → 2:1 → 1:1 → 1:2, v/v) → EtOAc → MeOH] to give three fractions [Fr.E4-4-1, Fr.E4-4-2 (596 mg), Fr.E4-4-3]. Fraction E4-4-2 (596 mg) was purified by HPLC [COSMOSIL 5C18-MS-II (preparative column), H_2O–MeOH–AcOH (700:300:3, v/v/v) and H_2O–MeCN–MeOH–AcOH (650:300:50:3, v/v/v/v)] to give **4** (5.6 mg) and **5** (2.8 mg). Fraction E5 (20.6 g) was separated by normal phase silica gel cc [Silica gel BW-200 (720 g, 50 mm I.D. – 210 mm), $CHCl_3$ → $CHCl_3$–MeOH (50:1 → 20:1 → 15:1 → 10:1 → 5:1, v/v) → MeOH] to give seven fractions [Fr.E5-1, Fr.E5-2, Fr.E5-3, Fr.E5-4 (7.18 g), Fr.E5-5 (3.56 g), Fr.E5-6 (3.80 g), Fr.E5-7 (1.29 g)]. Fraction E5-4 (7.18 g) was further separated by reversed phase silica gel cc [Chromatorex ODS DM1020T (200 g, 30 mm I.D. – 120 mm), MeOH–H_2O (20:80 → 30:70 → 40:60 → 50:50 → 60:40 → 80:20 → 90:10, v/v) → MeOH] to give nine fractions [Fr.E5-4-1, Fr.E5-4-2, Fr.E5-4-3, Fr.E5-4-4 (220 mg), Fr.E5-4-5 (162 mg), Fr. E5-4-6, Fr.E5-4-7, Fr.E5-4-8 (538 mg), Fr.E5-4-9 (170 mg)]. Fraction E5-4-4 (220 mg) was purified by HPLC [COSMOSIL 5C18-MS-II (preparative column), H_2O–MeCN–AcOH (800:200:3, v/v/v)] to give **12** (25 mg). Fraction E5-4-5 (162 mg) was purified by HPLC [COSMOSIL 5C18-AR-II (preparative column), H_2O–MeCN–MeOH–AcOH (700:150:150:3, v/v/v/v)] to give **11** (17 mg) and **14** (32 mg). Fraction E5-4-8 (538 mg) was purified by HPLC [COSMOSIL 5C18-MS-II (preparative column), H_2O–MeCN–AcOH (700:300:3, v/v/v)] to give quercetin (68 mg). Fraction E5-4-9 (170 mg) was purified by HPLC [COSMOSIL 5C18-MS-II (preparative column), H_2O–MeCN–AcOH (700:300:3, v/v/v)] to give **8** (12 mg) and quercetin (14 mg). Fraction E5-5 (3.6 g) was further separated by reversed phase silica gel cc [Chromatorex ODS DM1020T (120 g, 25 mm I.D. – 100 mm), MeOH–H_2O (20:80 → 30:70 → 40:60 → 50:50 → 60:40 → 80:20 → 90:10, v/v) → MeOH] to give nine fractions [Fr.E5-5-1, Fr.E5-5-2, Fr.E5-5-3 (326 mg), Fr.E5-5-4, Fr.E5-5-5, Fr.E5-5-6 (152 mg), Fr.E5-5-7, Fr.E5-5-8 (114 mg), Fr.E5-5-9]. Fraction E5-5-3 (326 mg) was purified by HPLC [COSMOSIL 5C18-MS-II (preparative column), H_2O–MeCN–AcOH (800:200:3, v/v/v)] to give **12** (193 mg). Fraction E5-5-6 (152 mg) was purified by HPLC [COSMOSIL 5C18-MS-II (preparative column), H_2O–MeCN–AcOH (700:300:3, v/v/v)] to give **7** (47 mg). Fraction E5-5-8 (114 mg) was purified by HPLC [COSMOSIL 5C18-MS-II (preparative column), H_2O–MeCN–AcOH (700:300:3, v/v/v)] to give **8** (30 mg) and **18** (7.1 mg). Fraction E5-6 (3.80 g) was separated by reversed phase silica gel cc [Chromatorex ODS DM1020T (123 g, 25 mm I.D. – 100 mm), MeOH–H_2O (20:80 → 30:70 → 40:60 → 50:50 → 60:40 → 80:20, v/v) → MeOH] to give seven fractions [Fr.E5-6-1, Fr.E5-6-2, Fr.E5-6-3 (214 mg), Fr.E5-6-4 (187 mg), Fr.E5-6-5, Fr.E5-6-6, Fr.E5-6-7]. Fraction E5-6-3 (214 mg) was purified by HPLC [COSMOSIL 5C18-MS-II (preparative column), H_2O–MeCN–AcOH (800:200:3, v/v/v)] to give **16** (12 mg), **17** (108 mg), and **19** (6.6 mg). Fraction E5-6-4 (187 mg) was purified by HPLC [COSMOSIL 5C18-MS-II (preparative column), H_2O–MeCN–AcOH (750:250:3, v/v/v)] to give **15** (69 mg) and **17** (19 mg).

4.4. Mumeose A (*1*)

White amorphous powder (3.4 mg, 0.00032%); $[\alpha]_D^{15}$ +114.8 (*c* 0.16, MeOH); UV [MeOH, nm (logε)]: 229 (4.22), 315 (4.40); IR (ATR): ν_{max} 3400, 1730, 1697, 1603, 1515, 1448, 1369, 1233, 1162, and 1033 cm^{-1}; for ^1H and ^{13}C NMR spectroscopic data, see Table 1; positive-ion FABMS: *m*/*z* 533 $[M+Na]^+$; HRFABMS: *m*/*z* 553.1573 (Calcd for $C_{23}H_{30}O_{14}Na$ $[M+Na]^+$: *m*/*z* 553.1533).

4.5. Mumeose B (*2*)

White amorphous powder (11 mg, 0.0010%); $[\alpha]_D^{15}$ +46.9 (*c* 0.33, MeOH) UV [MeOH, nm (logε)]: 228 (4.34), 316 (4.56); IR (ATR): ν_{max} 3400, 1730, 1704, 1604, 1515, 1450, 1371, 1238, 1167, and 1034 cm^{-1}; for ^1H and ^{13}C NMR spectroscopic data, see Table 1; positive-ion FABMS: *m*/*z* 595 $[M+Na]^+$; HRFABMS: *m*/*z* 595.1636 (Calcd for $C_{25}H_{32}O_{15}Na$ $[M+Na]^+$: *m*/*z* 595.1639).

4.6. Mumeose C (*3*)

White amorphous powder (5.3 mg, 0.00050%); $[\alpha]_D^{15}$ +54.0 (*c* 0.39, MeOH); UV [MeOH, nm (logε)]: 228 (4.30), 316 (4.55); IR (ATR): ν_{max} 3400, 1730, 1713, 1604, 1515, 1443, 1369, 1226, 1162, and 1038 cm^{-1}; for ^1H and ^{13}C NMR spectroscopic data, see Table 1; positive-ion FABMS: *m*/*z* 637 $[M+Na]^+$; HRFABMS: *m*/*z* 637.1747 (Calcd for $C_{27}H_{34}O_{16}Na$ $[M+Na]^+$: *m*/*z* 637.1745).

4.7. Mumeose D (**4**)

White amorphous powder (5.6 mg, 0.00047%); $[\alpha]_D^{15}$ +6.0 (*c* 0.21, MeOH); UV [MeOH, nm (log ε)]: 229 (4.24), 316 (4.39); IR (ATR): ν_{max} 3400, 1742, 1717, 1604, 1516, 1437, 1368, 1220, 1158, and 1033 cm^{-1}; for ^1H and ^{13}C NMR spectroscopic data, see Table 1; positive-ion FABMS: *m*/*z* 721 $[M+Na]^+$; HRFABMS: *m*/*z* 721.1948 (Calcd for $C_{31}H_{38}O_{18}Na$ $[M+Na]^+$: *m*/*z* 721.1956).

4.8. Mumeose E (**5**)

White amorphous powder (2.8 mg, 0.00023%); $[\alpha]_D^{15}$ −30.7° (*c* 0.13, MeOH); UV [MeOH, nm (log ε)]: 229 (4.20), 314 (4.42); IR (ATR): ν_{max} 3400, 1740, 1717, 1604, 1515, 1456, 1368, 1220, 1152, and 1035 cm^{-1}; for ^1H and ^{13}C NMR spectroscopic data, see Table 1; positive-ion FABMS: *m*/*z* 721 $[M+Na]^+$; HRFABMS: *m*/*z* 721.1959 (Calcd for $C_{31}H_{38}O_{18}Na$ $[M+Na]^+$: *m*/*z* 721.1956).

4.9. 5-O-(E)-p-Coumaroyl quinic acid ethyl ester (**6**)

White amorphous powder (33 mg, 0.0031%); $[\alpha]_D^{15}$ −8.9° (*c* = 0.29, MeOH); UV [MeOH, nm (log ε)]: 228 (4.34), 314 (4.56); IR (ATR): ν_{max} 3400, 1720, 1690, 1603, 1515, 1444, 1166, and 1079 cm^{-1}; for ^1H and ^{13}C NMR spectroscopic data, see Table 2; EIMS: *m*/*z* 366 $[M]^+$; HREIMS: *m*/*z* 366.1308 (Calcd for $C_{18}H_{22}O_8$ $[M]^+$: *m*/*z* 366.1314).

4.10. Mumeic acid-A (**7**)

White amorphous powder (47 mg, 0.0039%); $[\alpha]_D^{15}$ −82.9 (*c* 0.22, MeOH); UV [MeOH, nm (log ε)]: 232 (4.28), 329 (4.41); IR (ATR): ν_{max} 3400, 1720, 1703, 1695, 1599, 1510, 1455, 1150, and 1109 cm^{-1}; for ^1H and ^{13}C NMR spectroscopic data, see Table 1; positive-ion EIMS: *m*/*z* 458 $[M]^+$; HREIMS: *m*/*z* 458.1204 (Calcd for $C_{23}H_{22}O_{10}$ $[M]^+$: *m*/*z* 458.1212).

4.11. Mumeic acid-A methyl ester (**8**)

White amorphous powder (42 mg, 0.0034%); $[\alpha]_D^{15}$ −11.9 (*c* 0.81, MeOH); UV [MeOH, nm (log ε)]: 232 (4.20), 328 (4.48). IR (ATR): ν_{max} 3400, 1730, 1716, 1698, 1601, 1508, 1456, 1160, and 1110 cm^{-1}; for ^1H and ^{13}C NMR spectroscopic data, see Table 2; positive-ion FABMS: *m*/*z* 495 $[M+Na]^+$; HRFABMS: *m*/*z* 495.1264 (Calcd for $C_{24}H_{24}O_{10}Na$ $[M+Na]^+$: *m*/*z* 495.1262).

4.12. Acid hydrolysis of **1**–**8**

Compounds **1**–**8** (**1**–**5**:1.5 mg, **6**–**8**:10 mg) were dissolved in 5% aqueous H_2SO_4–1,4-dioxane (1:1, v/v, **1**–**5**: 2.0 mL, **6**–**8**:10.0 mL), and each solution was heated at 90 °C for 3 h, and neutralized with Amberlite IRA-400 (OH^- form). After drying *in vacuo*, a small aliquot of the residue was dissolved in H_2O–MeOH (1:1, v/v) and analyzed by reversed-phase HPLC to identify (*E*)- or (*Z*)-*p*-coumaric, (*E*)-*p*-caffeic and benzoic acids, respectively, [column: COSMOSIL 5C18-MS-II, 4.6 mm I.D. – 250 mm; mobile phase A: H_2O–AcOH (1000:3, v/v), B: MeCN–AcOH (1000:3, v/v), Linear gradient: mobile phase A–B (90:10 → 72:28, v/v, in 24.0 min); detection: UV (300 nm); flow rate: 1.0 mL/min; column temperature: ambient]. (*E*)-*p*-Coumaric acid (from **1**–**4**, **6**), (*Z*)-*p*-coumaric acid (from **5**), (*E*)-*p*-caffeic acid (from **7** and **8**) and benzoic acid (from **7** and **8**) were identified by comparison of their retention times with those of authentic samples [(*E*)-*p*-caffeic acid: t_R = 12.1 min, (*E*)-*p*-coumaric acid: t_R = 17.1 min, (*Z*)-*p*-coumaric acid: t_R = 18.5 min, benzoic acid: t_R = 23.0 min]. In addition, the remaining parts of residue from **1**–**5**, from which glucose and fructose were identified by TLC (ordinary phase) [$CHCl_3$–MeOH–H_2O (30:15:3, v/v/v)]], were dissolved in pyridine (0.1 mL) containing L-cysteine methyl ester hydrochloride (0.5 mg) and heated at 60 °C for 1 h. *o*-torylisothiocyanate (0.5 mg) in pyridine (0.1 mL) was added to the mixture and heated at 60 °C for 1 h. The reaction mixture was analyzed by reversed-phase HPLC [column: COSMOSIL 5C18-AR-II, 4.6 mm I.D. – 250 mm; mobile phase: MeCN–0.05 M H_3PO_4 (23: 77, v/v); detection: UV (250 nm); flow rate: 0.8 mL/min; col-

Table 2
^1H and ^{13}C NMR (125 MHz) spectroscopic data for **6**–**8** in CD_3OD (δ in ppm, *J* in Hz).

Position	**6**			**7**		**8**	
	δ_C	δ_H		δ_C	δ_H	δ_C	δ_H
Quinic acid part			*Quinic acid part*				
1	75.8	–	1	76.2	–	75.8	–
2	38.0	2.00 m	2	38.4	2.11, 2.26 m	38.4	2.30 m
3	70.5	4.10 m	3	69.3	4.43 m	68.5	4.43 m
4	72.8	3.72 dd (3.1, 7.6)	4	76.7	5.20 br d (8.3)	75.8	5.24 br d (7.8)
5	72.2	5.28 ddd (4.6, 7.6, 7.6)	5	68.8	5.71 m	68.9	5.64 m
6	37.9	2.18 m	6	39.5	2.26 m	38.7	2.26 m
7	175.0	–	7	177.0	–	175.2	–
p-Coumaroyl part			*Benzoyl part*				
1′	127.1	–	1′	131.1	–	131.1	–
2′,6′	116.9	7.44 d (8.6)	2′,6′	130.8	7.99 d (7.5)	130.8	8.05 d (7.3)
3′,5′	131.2	6.54 d (8.6)	3′,5′	129.5	7.37 dd (7.5, 7.5)	129.5	7.44 dd (7.3, 7.3)
4′	161.4	–	4′	134.4	7.50 m	134.4	7.57 m
5′	146.8	7.52 d (15.8)	7′	167.5	–	167.4	–
6′	115.2	6.28 d (15.8)	*p-Caffeoyl part*				
7′	168.3	–	1″	127.6	–	127.5	–
OCH_2CH_3	62.5	4.13 q (7.0)	2″	115.2	6.91 br s	115.1	6.97 br s
OCH_2CH_3	14.3	1.23 t (7.0)	3″	146.8	–	146.8	–
			4″	149.6	–	149.8	–
			5″	116.4	6.68 d (8.1)	116.5	6.74 d (7.5)
			6″	123.0	6.81 br d (8.1)	123.1	6.89 br d (7.5)
			7″	147.5	7.43 (15.8)	147.6	7.47 d (15.8)
			8″	114.6	6.09 d (15.8)	114.5	6.13 d (15.8)
			9″	168.2	–	167.8	–
			$C(O)OCH_3$			53.1	3.72 s

Table 3
Inhibitory effects of compounds **6**–**14** isolated from flower buds of *P. mume* on melanogenesis in B16 melanoma 4A5 cells.

Sample (μM)	Inhibition (%)					
	Control	0.1	0.3	1	3	10
6	0.0 ± 6.7	14.3 ± 1.0	21.1 ± 2.8**	43.3 ± 1.0**	51.1 ± 2.8**	61.2 ± 2.6**
7	0.0 ± 1.4	19.1 ± 0.4	23.3 ± 4.7*	36.4 ± 8.4**	43.3 ± 4.7**	47.4 ± 3.9**
8	0.0 ± 5.9	3.1 ± 2.2	13.2 ± 6.6	23.9 ± 2.6**	34.2 ± 1.6**	27.2 ± 0.6**
9	0.0 ± 5.2	9.1 ± 1.4	18.5 ± 2.4**	39.1 ± 4.8**	43.9 ± 2.2**	47.1 ± 2.4**
10	0.0 ± 6.5	15.4 ± 3.1	38.1 ± 2.6**	56.4 ± 2.9**	58.7 ± 3.5**	58.1 ± 2.9**
11	0.0 ± 6.5	5.5 ± 1.6	14.3 ± 6.2	26.5 ± 1.7**	34.1 ± 2.4**	47.6 ± 4.1**
12	0.0 ± 8.3	11.5 ± 3.1	25.0 ± 3.1**	43.6 ± 3.9**	53.0 ± 2.3**	57.9 ± 2.9**
13	0.0 ± 2.1	21.5 ± 1.0**	22.4 ± 0.3**	38.9 ± 1.0**	52.4 ± 3.6**	63.8 ± 1.8**
14	0.0 ± 5.9	6.0 ± 1.8	21.3 ± 1.1**	44.0 ± 3.8**	42.3 ± 6.1**	51.9 ± 3.0**

Sample (μM)	Inhibition (%)			
	Control	10	30	100
Arbutin[a]	0.0 ± 1.4	10.6 ± 0.6**	20.4 ± 0.5**	38.1 ± 0.9**

Each value represents the mean ± S.E.M. ($n = 4$).
Significantly different from the control, *$P < 0.05$, **$P < 0.01$.
Compounds, **1**–**5**, **15**–**20**, had no inhibitory effects on melanogenesis at 1–100 μM.
The cell viabilities at 10 μM of compounds **6**–**14** are more than 97.4%.
The cell viabilities at 3 μM are more than 95.6%.
[a] Reference compound (Fujimoto et al., 2012).

umn temperature: 35 °C] to identify the derivatives of constituent monosaccharides in **1**–**5** by comparison of their retention times with those of authentic samples (t_R: D-glucose; 19.7 min) (Tanaka et al., 2007). On the other hand, the remaining parts of residue from **6**–**8** were dissolved in water, and purified by HPLC [H_2O–MeOH–AcOH (980: 20:3, v/v/v), COSMOSIL 5C18-PAQ] to give D-(−)-quinic acid (**6**: 2.5 mg, **7**: 2.0 mg, **8**: 2.1 mg), which was identified by comparison of their analytical data (NMR and MS spectra and optical rotation) with that of the authentic sample.

4.13. Alkaline hydrolysis of *1*–*5*

Compounds **1**–**5** (1.5 mg) were individually treated with a 10% aqueous KOH–1,4-dioxane (1:1, v/v, 1.0 mL) and stirred at 37 °C for 24 h. Each reaction mixture was neutralized with Dowex HCR W2 (H^+ form) and the resin was removed by filtration. Evaporation of the solvent from the filtrate under reduced pressure yielded a crude product. Each crude product was dissolved in H_2O and applied to normal-phase HPLC [column: YMC-Pack Polyamine II, 4.6 mm I.D. – 250 mm; mobile phase: MeCN–H_2O (3:1, v/v); detection: optical rotation [Shodex OR-2 (Showa Denko Co. Ltd., Tokyo, Japan); flow rate: 1.0 mL/min; column temperature: ambient] to identify D-sucrose as a constituent of **1**–**5** by comparison of their retention times and optical rotation with that of authentic sample (t_R = 19.8 min with positive rotation).

4.14. HPLC profile comparison

Dried flower buds (40.0 g) were extracted with MeCN/H_2O (800 mL × 3, 1:1, v/v) under reflux for 3 h. Evaporation of the solvent under reduced pressure provided a MeCN/H_2O extract (16.2 g, 40.5%). The MeCN/H_2O extract (16.2 g) was suspended in distilled H_2O (500 mL), and partitioned with EtOAc (500 mL × 3) to furnish an EtOAc-soluble fraction (1.8 g, 4.5%). The aqueous phase was further extracted with 1-butanol (500 mL × 3) to give a 1-butanol-soluble fraction (10.3 g, 25.8%) and an H_2O-soluble fraction (4.2 g, 10.5%). An aliquot of the 1-butanol-soluble fraction of the MeOH- and MeCN/H_2O-extracts was dissolved in MeCN (2.0 mg/mL) and subjected to reversed-phase HPLC [column: Inertsil ODS-3, 3.0 mm I.D. – 150 mm; mobile phase A: H_2O–AcOH (1000:3, v/v), B: MeCN–AcOH (1000:3, v/v), gradient: mobile phase A–B (0.0–10.0 min: linear gradient with 90–10 → 82–18, 10.0–30.0 min: isocratic with 82–18, 30.0–70.0 min: linear gradient with 82–18 → 0–100); detection: UV (265 nm); flow rate: 0.45 mL/min; column temperature: 25 °C]. Acylated quinic acid analogs (**6**, **8**–**14**) isolated from the 1-butanol-soluble fraction of the MeOH extract were also detected in the 1-butanol-soluble fraction of the MeCN/H_2O extract by comparing their retention times with those of authentic samples (t_R **6**: 42.6 min, **9**: 17.0 min, **10**: 12.0 min, **11**: 27.0 min, **12**: 17.7 min, **13**: 26.4 min, **14**: 27.8 min). Furthermore, a portion of the EtOAc-soluble fraction of the MeOH- and MeCN/water-extracts was dissolved in MeCN (1.0 mg/mL) and subjected to reversed-phase HPLC [column: Inertsil ODS-3, 3.0 mm I.D. – 150 mm; mobile phase A: H_2O–AcOH (1000:3, v/v), B: MeCN–AcOH (1000:3, v/v), gradient: mobile phase A–B (0.0–45.0 min: linear gradient with 80–20 → 50–50); detection: UV (241 nm); flow rate: 0.45 mL/min; column temperature: 25 °C]. Compound **8** isolated from the EtOAc-soluble fraction of the MeOH extract was identified in that of the MeCN/water extract (t_R **8**: 22.6 min). In addition, the HPLC analysis of the 1-butanol- and EtOAc-soluble fractions of the MeOH- and MeCN/H_2O-extracts showed comparable HPLC profiles.

4.15. Reagents for bioassays

D-Glucopyranose, sucrose, D-(−)-quinic acid, benzoic acid, (*E*)-*p*-caffeic acid and (*E*)-*p*-coumaric acid were purchased from Tokyo Chemical Industry. (*Z*)-*p*-Coumaric acid was prepared from (*E*)-*p*-coumaric acid by UV irradiation according to the reported procedure (Kort et al., 1996). Dulbecco's modified Eagle's medium (DMEM, 4500 mg/L glucose) was purchased from Sigma–Aldrich (St. Louis, MO, USA); fetal bovine serum (FBS), penicillin, and streptomycin were purchased from Gibco (Invitrogen, Carlsbad, CA, USA); the Cell Counting Kit-8™ was from Dojindo Lab. (Kumamoto, Japan); and the other chemicals were purchased from Wako Pure Chemical Co. Ltd. (Osaka, Japan).

4.16. Cell culture

Murine B16 melanoma 4A5 cells (RCB0557) were obtained from Riken Cell Bank (Tsukuba, Japan), and grown in DMEM supplemented with 10% FBS, penicillin (100 U/mL), and streptomycin

(100 μg/mL) at 37 °C in 5% CO2/air. The cells were harvested by incubation in phosphate-buffered saline (PBS) containing 1 mM EDTA and 0.25% trypsin for ca. 5 min at 37 °C and used for the subsequent bioassays.

4.17. Melanogenesis

Melanoma cells (2.0×10^4 cells/400 μL/well) were seeded into 24-well multiplates. After 24 h of culture, a test compound and theophylline 1 mM were added and incubated for 72 h. Cells were harvested by incubating with PBS containing 1 mM EDTA and 0.25% trypsin, and cells were washed with PBS. Cells were treated with NaOH 1 M (120 μL/tube, 80 °C, 30 min) to yield a lysate. An aliquot (100 μL) of this was transferred to a 96-well microplate, and the optical density of each well measured with a microplate reader (Model 550, Bio-Rad Laboratories) at 405 nm (reference: 655 nm). The test compound was dissolved in DMSO, and the final concentration of DMSO in the medium was 0.1%. The production of melanin was corrected based on cell viability. Inhibition (%) was calculated using the following formula, and IC_{50} values were determined graphically.

$$\text{Inhibition } (\%) = [(A - B)/A]/(C/100) \times 100$$

where *A* and *B* indicate the optical density of vehicle- and test compound-treated groups, respectively, and *C* indicates cell viability (%).

4.18. Cell viability

The melanoma cells (5.0×10^3 cells/100 μL/well) were seeded into 96-well microplates and incubated for 24 h. After 70 h incubation with theophylline 1 mM and a test compound, 10 μL of WST-8 solution (Cell Counting Kit-8™) was added to each well. After a further 2 h in culture, the optical density of the water-soluble formazan produced by the cells was measured with a microplate reader (Model 550, Bio-Rad Laboratories, Hercules, CA, USA) at 450 nm (reference: 655 nm). The test compound was dissolved in dimethylsulfoxide (DMSO), and the final concentration of DMSO in the medium was 0.1%. Cell viability (%) was calculated using the following formula.

$$[\text{Cell viability } (\%) = B/A \times 100]$$

where *A* and *B* indicate the optical density of vehicle- and test compound-treated groups, respectively.

4.19. Statistical analyses

Values are expressed as the mean ± S.E.M. A one-way analysis of variance followed by Dunnett's test was used for statistical analyses.

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