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Melanogenesis Inhibitory and Fibroblast Proliferation Accelerating Effects of Noroleanane- and Oleanane-Type Triterpene Oligoglycosides from the Flower Buds of *Camellia japonica*[1]

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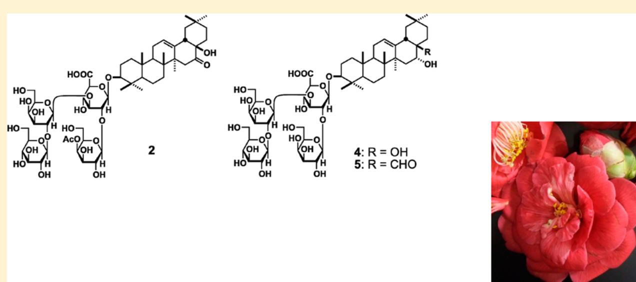
Supporting Information

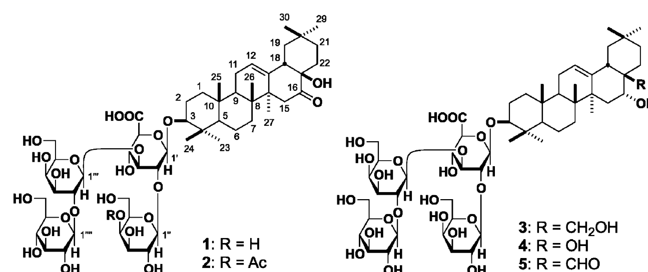
ABSTRACT: A 28-noroleanane-type triterpene oligoglycoside, camellioside E (**4**), an oleanane-type triterpene oligoglycoside, camellioside F (**5**), and the known compounds camelliosides A (**1**) and D (**3**) were isolated from a 50% EtOH extract of *Camellia japonica* flower buds from Korea. The principal constituents (**1** and **5**) significantly inhibited melanogenesis in theophylline-stimulated B16 melanoma 4A5 cells. Camellioside B (**2**), a major constituent of *C. japonica* grown in Japan, showed potent inhibition of melanogenesis [95.0 ± 1.0% (p < 0.01) at 20 μM]. The inhibitory effects of **1**, **2**, and **5** were stronger than that of the reference compound, arbutin. We believe the melanogenesis inhibitory effects of **2** and **5** are partly related to the proliferation inhibitory effects in B16 melanoma 4A5 cells. Conversely, camelliosides tended to enhance proliferation in normal human neonatal skin fibroblasts. Interestingly, camellioside B (**2**) significantly accelerated fibroblast proliferation. This biological selectivity could make camellioside B useful for treating skin disorders. Herein, we report the first scientific investigation of a triterpene that displays an inhibitory effect on melanogenesis, but that also has an enhancing effect on fibroblast proliferation.



Camellia japonica L. (Theaceae) is widely cultivated as an ornamental or garden tree in Japan. The flower buds of *C. japonica* (Camellia flowers) have been used to treat vomiting of blood and bleeding due to internal and external injury and also as an anti-inflammatory, tonic, and stomatic in Japanese folk medicine and Chinese traditional medicine. However, the chemical constituents and biological activities of the flower buds have not been characterized. Previously, we reported the isolation and structural elucidation of camelliosides A (**1**), B (**2**), C, and D (**3**), with gastroprotective and platelet aggregation effects, from flower buds of *C. japonica* collected on Izu Ōshima Island (Tokyo).[2,3] In a continuation of studies on the bioactive constituents of flower buds of *Camellia* species[4−10] we have now examined the flower buds of *C. japonica* grown on Cheju Island, located just south of the Korean peninsula. Furthermore, the inhibitory effect on melanogenesis and the proliferation-accelerating effect of their constituents and related saponins on fibroblasts were evaluated.

■ RESULTS AND DISCUSSION

A 50% ethanol extract of flower buds of *C. japonica* collected on Cheju Island, Korea,was partitioned between EtOAc−H_2O (1:1) to furnish EtOAc-soluble (2.1%) and aqueous layers. The aqueous layer was further extracted with 1-butanol (1-BuOH) to give 1-BuOH- (13.8%) and H_2O- (27.8%) soluble fractions.



The 50% EtOH extract [inhibition (%): 53.0 ± 1.7 (p < 0.01) at 100 μg/mL] and the 1-BuOH-soluble fraction [inhibition (%): 39.8 ± 1.9 (p < 0.01) at 25 μg/mL] showed inhibitory effects on melanogenesis in theophylline-stimulated B16 melanoma 4A5 cells. The 1-BuOH-soluble fraction was subjected to normal-phase and reversed-phase silica gel column chromatography and repeated HPLC to give camelliosides E (**4**, 0.076%) and F (**5**, 0.012%) together with **1** (0.21%)[2,3] and **3** (0.21%).[2,3]

Camellioside E (**4**), a colorless, amorphous powder, exhibited a positive optical rotation ($[\alpha]^{25}_D$ +11.9 in MeOH). Its IR spectrum showed absorption bands at 3450, 1718, and

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1070 cm^{-1} ascribable to OH, carboxyl, and ether components, respectively. FABMS in the positive-ion mode revealed a quasimolecular ion peak $[M + Na]^+$ at m/z 1129, from which the molecular formula $C_{54}H_{90}O_{23}$ was determined by HRMS. Acid hydrolysis of **4** with 5% aqueous H_2SO_4−1,4-dioxane yielded D-galactose, D-glucose, and D-glucuronic acid, which were identified by HPLC analysis of their tolylthiocarbamoyl thiazolidine derivatives.[11] The ^1H (pyridine-d_5) and ^{13}C NMR spectra of **4** (Table 1), which were assigned by various NMR experiments,[12] showed signals assignable to an aglycone {seven methyls [δ 0.87, 1.04, 1.12, 1.14, 1.18, 1.30, 1.83 (3H each, all s, H_3-25, 29, 24, 30, 26, 23, 27)], two methines each bearing an oxygen function [δ 3.29 (1H, dd like, J = 4.0, 10.3 Hz, H-3), 4.48 (1H, m, H-16)], and an olefinic proton [δ 5.44 (1H, s like, H-12)]} and four glycopyranosyl moieties {a β-D-glucuronopyranosyl moiety [δ 4.91 (1H, d, J = 7.5 Hz, H-1′)], a β-D-glucopyranosyl moiety [δ 5.19 (1H, d, J = 7.8 Hz H-1⁗)], and two β-D-galactopyranosyl moieties [δ 5.76 (1H, d like, J = 7.0 Hz, H-1‴), 5.81 (1H, d, J = 7.2 Hz, H-1″)]}. The carbon and proton signals in the ^1H and ^{13}C NMR spectra were superimposable on those of **1**,[2,3] except for the signals around C-16. The oligoglycoside structure at C-3 was elucidated by heteronuclear multiple bond connectivity spectroscopy (HMBC), which showed long-range correlations between the following proton and carbons: H-1′ and C-3; H-1″ and C-2′; H-1‴ and C-3′; H-1⁗ and C-2‴. The planar structure was constructed on the basis of double quantum filter correlation spectroscopy (DQF COSY) and HMBC experiments, as shown in Figure 1. Next, the relative configuration of **4** was characterized by nuclear Overhauser enhancement spectroscopy (NOESY), which showed NOE correlations between the following proton pairs: H-3α and H-5, H_3-23; H-5 and H-9, H_3-23; H-9 and H_3-27; H-15α and H-16, H_3-27; H-15β and H-16; H-16 and H-18, H_3-26; H-18 and H_3-30; H_3-24 and H_3-25; H_3-25 and H_3-26. Finally, **4** was selectively obtained by the reduction of **1** with $NaBH_4$. On the basis of these findings, the structure of **4** was characterized as shown.

Table 1. ^{13}C NMR (pyridine-d_5, 125 MHz) Data for 4 and 5

position	4 δC	5 δC	carbon	4 δC	5 δC
1	38.6	38.7	3-O-β-D-glucuronopyranosyl		
2	26.6	26.5	1′	105.8	105.7
3	89.7	89.6	2′	79.2	79.2
4	39.6	39.5	3′	84.0	83.9
5	55.9	55.8	4′	71.0	71.0
6	18.6	18.4	5′	77.0	77.0
7	33.7	33.3	6′	172.0	172.2
8	40.0	40.0	2′-O-β-D-galactopyranosyl		
9	47.3	46.9	1″	103.2	103.2
10	37.1	36.9	2″	73.9	73.9
11	23.9	23.7	3″	75.0	74.9[a]
12	122.5	123.1	4″	69.9	69.9
13	145.8	143.6	5″	76.5	76.5
14	42.0	42.0	6″	61.9	61.8
15	35.1	35.7	3′-O-β-D-galactopyranosyl		
16	77.9	73.1	1‴	101.7	101.7
17	71.4	51.4	2‴	83.4	83.4
18	48.2	40.9	3‴	75.0	75.0[a]
19	48.6	46.7	4‴	69.9	69.9
20	31.2	30.7	5‴	76.5	76.5
21	38.0	35.1	6‴	62.1	62.0
22	38.7	27.3	2‴-O-β-D-glucopyranosyl		
23	28.2	28.1	1⁗	106.7	106.7
24	16.9	16.8	2⁗	76.1	76.1
25	15.5	15.5	3⁗	78.3	78.3
26	17.9	17.4	4⁗	71.4	71.2
27	27.2	27.1	5⁗	78.5	78.5
28		205.8	6⁗	62.3	62.3
29	33.1	33.2			
30	24.8	24.2			

[a]Interchangeable.

Camellioside F (**5**), obtained as a colorless, amorphous powder with a positive optical rotation ($[\alpha]^{24}_D$ +32.7 in MeOH), showed absorption bands due to OH, aldehyde, carboxyl, and ether components in the IR spectrum. Positive-ion FABMS revealed a quasimolecular ion peak $[M + Na]^+$ at m/z 1141 from which the molecular formula $C_{54}H_{86}O_{24}$ was determined by HRMS. Acid hydrolysis of **5** yielded D-galactose, D-glucose, and D-glucuronic acid.[11] The ^1H (pyridine-d_5) and ^{13}C NMR (Table 1) spectra of **5**, which were assigned by various NMR experiments,[12] showed signals assignable to an aglycon {seven methyls [δ 0.79, 0.81, 1.00, 1.03, 1.08, 1.27, 1.74 (3H each, all s, H_3-26, 25, 29, 30, 24, 23, 27)], two methines bearing an oxygen function [δ 3.25 (1H, dd, J = 3.7, 11.5 Hz, H-3), 4.78 (1H, m, H-16)], an olefinic proton [δ 5.47 (1H, br s, H-12)], and aldehyde [δ 9.52 (1H, s, H-28)], and four glycopyranosyl moieties [δ 4.89 (1H, d, J = 7.5 Hz, H-1′), 5.18 (1H, d-like, J = 8.0 Hz H-1⁗), 5.72 (1H, d, J = 7.5 Hz, H-1‴),

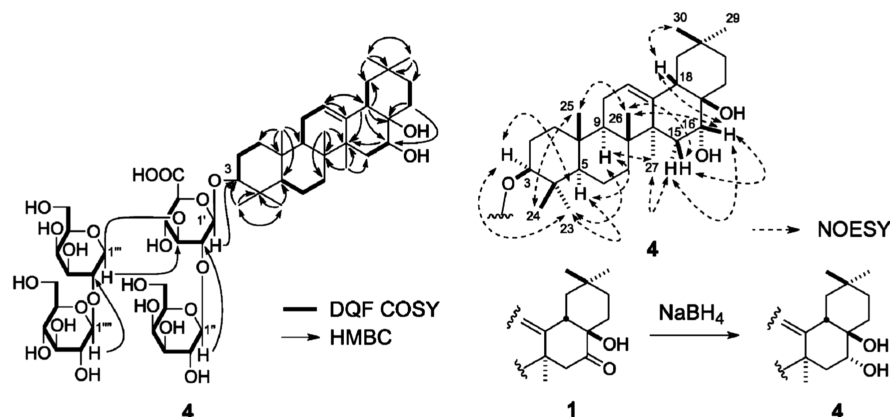

Figure 1.

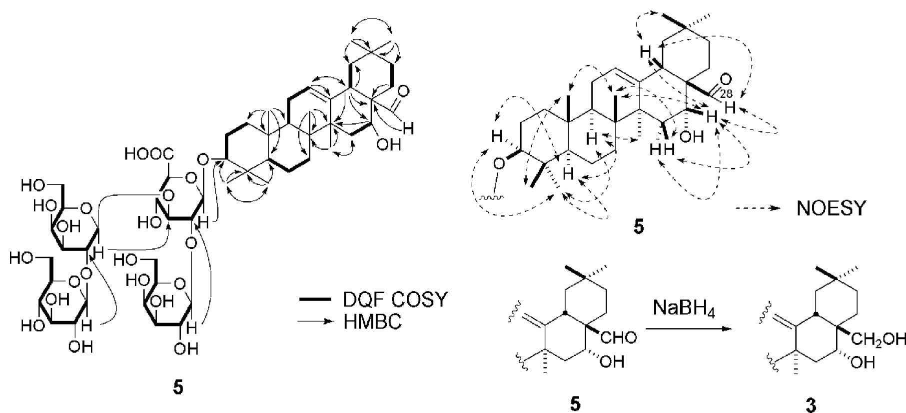

Figure 2.

5.78 (1H, d, J = 7.7 Hz, H-1″)]. The carbon and proton signals in the ^{1}H and ^{13}C NMR spectra were superimposable on those of **3**, except for the signals around the 17-position. The planar structure was constructed on the basis of DQF COSY and HMBC experiments as shown in Figure 2. Next, the relative configuration of **5** was characterized by NOESY, which showed NOE correlations between the following proton pairs: H-3α and H-5, H_3-23; H-5 and H-9, H_3-23; H-9 and H_3-27; H-15α and H-16; H-15β and H-16; H-16 and H_3-26, H-28; H-18 and H-28, H_3-30; H_3-24 and H_3-25; H_3-25 and H_3-26. Finally, **3** was derived from **5** by reduction with $NaBH_4$. On the basis of these findings, the structure of **5** was characterized as shown.

Melanin pigmentation is a major defense mechanism against the deleterious effects of ultraviolet rays and is principally responsible for determining skin color. 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, we have examined the inhibitory effects of several diarylheptanoids, flavonoids, and sterol glycosides in theophylline-stimulated B16 melanoma 4A5 cells.[13−15] As a continuation of these studies, the inhibitory effects of constituents from the flowers buds of *C. japonica* on melanogenesis were examined. The principal constituents, **1** and **5**, significantly inhibited melanogenesis in B16 melanoma 4A5 cells (Table 2). In addition, **2**,[2,3] which was a major constituent isolated from *C. japonica* on Izu Ōshima Island in Japan by us, had a significant inhibitory effect on melanogenesis [inhibition (%): 95.0 ± 1.0 (p < 0.01) at 20 μM]. The inhibitory effects of **1**, **2**, and **5** were stronger than that of the reference compound, arbutin [inhibition (%): 13.3 ± 2.1 (p < 0.05) at 20 μM]. Furthermore, **2** [inhibition (%): 49.0 ± 4.6 (p < 0.01) at 20 μM] and **5** [inhibition (%): 52.8 ± 9.7 (p < 0.01) at 20 μM] were found to inhibit proliferation in B16 melanoma 4A5 cells (Table 3). These results suggested that the inhibitory effects of **2** and **5** on melanogenesis were partly related to their inhibitory action against the proliferation of B16 melanoma 4A5 cells. Next, the effect of camelliosides on fibroblast proliferation was examined by using the WST-8 assay. The results of these studies were also confirmed by determining changes in the protein content of the cells (Table 4). Fibroblasts are the main cells responsible for producing collagen and the extracellular matrix. As such, fibroblasts play a crucial role in maintaining the structural integrity of animal tissues. Furthermore, enhancers of fibroblast proliferation are thought to be important in the wound-healing process.[16−18] Camelliosides tend to enhance the proliferation of normal human neonatal skin fibroblasts. Notably, **2** significantly accelerated fibroblast proliferation [acceleration (% of control): 132.1 ± 6.9 (p < 0.05) at 20 μM in the WST-8 assay, 130.0 ± 4.0 (p < 0.05) at 10 μM in the BCA protein assay]. In contrast, sasanquasaponins I (**6**),[19] II (**7**),[19] and III (**8**)[19] and yuchasaponin A (**9**),[10] which were previously isolated by us as major constituents from the flower buds of Camellia species (*C. sasanqua* and *C. oleifera*), with acyl groups at the 21- and/or 22-positions, showed proliferation inhibitory effects at 10 μM in fibroblasts (Figure 3). With regard to structural requirements, the presence of acyl groups at the 21- and/or 22-positions was suggested to be unfavorable for fibroblast proliferation-accelerating effects. Conversely, **2** significantly accelerated proliferation in normal human neonatal skin fibroblasts. This

Table 2. Inhibitory Effects of Compounds 1−5 on Melanogenesis in B16 Melanoma 4A5 Cells

	inhibition (%) for melanogenesis[a,b]		
sample	5 μM	10 μM	20 μM
1	12.1 ± 2.9	36.0 ± 2.5**	74.9 ± 1.1**
2	33.1 ± 1.8**	64.6 ± 1.5**	95.0 ± 1.0**
3	0.3 ± 1.8	−6.2 ± 3.0	8.3 ± 2.8
4	21.9 ± 1.7**	16.7 ± 3.7**	23.3 ± 2.3**
5	−19.7 ± 1.5	16.3 ± 2.6**	91.6 ± 3.1**
sample	10 μM	20 μM	40 μM
arbutin[c]	−0.1 ± 3.5	13.3 ± 2.1*	18.0 ± 1.5**

[a]Each value represents the mean ± SEM (n = 4). [b]Significantly different from the control, *p < 0.05, **p < 0.01. [c]Reference compound.

Table 3. Inhibitory Effects of Compounds 1−5 on Proliferation in B16 Melanoma 4A5 Cells

	inhibition of proliferation (%)[a,b]		
sample	5 μM	10 μM	20 μM
1	−3.8 ± 2.3	5.1 ± 2.3	19.7 ± 1.4**
2	11.9 ± 6.8	26.0 ± 13.0	49.0 ± 4.6**
3	16.1 ± 5.9	16.3 ± 9.4	29.5 ± 2.0
4	1.9 ± 8.2	4.3 ± 6.4	13.3 ± 6.2
5	17.4 ± 16.5	55.0 ± 11.7**	52.8 ± 9.7**

[a]Each value represents the mean ± SEM (n = 4). [b]Significantly different from the control, **p < 0.01.

Table 4. Effects of Saponins 1−8 on the Formation of WST-8 Formazan and the Determination of Protein Content in Normal Human Neonatal Skin Fibroblasts

sample	conc (μM)	WST-8 formazan/well (% of control)[a,b]	protein content/well (% of control)[a,b]
1	5.0	114.6 ± 7.8	120.8 ± 6.0
	10.0	114.3 ± 10.6	126.9 ± 6.7
	20.0	113.6 ± 8.5	113.8 ± 7.0
2	5.0	111.5 ± 0.7	122.3 ± 5.3
	10.0	121.5 ± 12.7	130.0 ± 4.0*
	20.0	132.1 ± 6.9*	130.1 ± 5.0*
3	5.0	107.6 ± 5.3	110.7 ± 9.3
	10.0	98.6 ± 4.7	113.1 ± 11.7
	20.0	108.6 ± 6.7	94.7 ± 4.8
4	5.0	108.3 ± 7.0	113.9 ± 2.6
	10.0	110.0 ± 2.5	110.6 ± 6.5
	20.0	116.2 ± 3.4	114.9 ± 5.6
5	5.0	102.1 ± 6.9	120.4 ± 1.7
	10.0	102.5 ± 8.2	120.3 ± 6.1
	20.0	102.2 ± 1.6	110.0 ± 7.0
6	10.0	34.9 ± 0.9**	71.5 ± 1.4**
	50.0	16.4 ± 0.1**	44.8 ± 1.0**
7	10.0	16.8 ± 0.1**	53.9 ± 2.0**
	50.0	16.5 ± 0.2**	48.9 ± 1.3**
8	10.0	16.6 ± 0.2**	48.6 ± 1.9**
	50.0	16.8 ± 0.2**	48.8 ± 2.0**
9	10.0	16.6 ± 0.0**	45.3 ± 1.3**
	50.0	16.8 ± 0.1**	49.4 ± 2.1**
reference	5.0	109.2 ± 6.3	112.8 ± 4.5
compound[c]	10.0	123.7 ± 8.3	109.6 ± 5.8
	20.0	122.8 ± 8.9	117.4 ± 5.7

[a]Each value represents the mean ± SEM (n = 4). [b]Significantly different from the control, $*p < 0.05$, $**p < 0.01$. [c]Reference compound: L-ascorbic acid phospate magnesium salt n-hydrate.

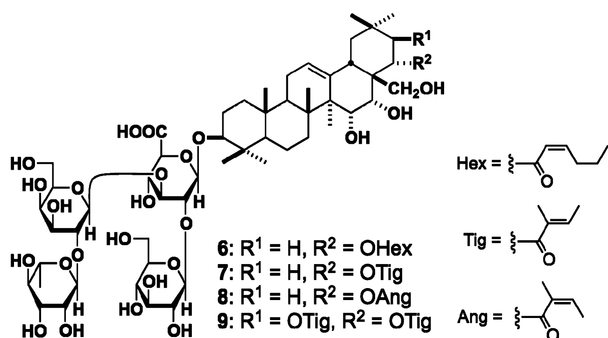

Figure 3.

cell selectivity could make **2** useful for treating skin disorders. To the best of our knowledge, this is the first scientific investigation of a triterpene with an inhibitory effect on melanogenesis, but enhancing effect on fibroblast proliferation. Further elucidation of the mechanism involved and examinations in vivo are warranted.

■ EXPERIMENTAL SECTION

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 Shimadzu FTIR-8100 spectrometer; FABMS and HRFABMS, a JEOL JMS-SX 102A mass spectrometer; ^{1}H NMR spectra, a JEOL JNM-LA 500 (500 MHz); ^{13}C NMR spectra, a JEOL JNM-LA (125 MHz) spectrometer with tetramethylsilane as an internal standard; and HPLC, Shimadzu RID-6A refractive index and SPD-10Avp UV−vis detectors. COSMOSIL 5C$_{18}$-MS-II {[250 × 4.6 mm i.d. (5 μm) for analytical purposes] and [250 × 20 mm i.d. (5 μm) for preparative purposes], Nacalai Tesque} columns were used. The following experimental conditions were used for chromatography: ordinary-phase silica gel column chromatography (CC), silica gel BW-200 (Fuji Silysia Chemical, Ltd., 150−350 mesh); reverse-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) (reverse 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.

Plant Material. Flower buds of *Camellia japonica* were collected on Cheju Island, Korea, in 2008. 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-2008-1).

Extraction and Isolation. Dry flower buds of *C. japonica* (1.0 kg) were extracted three times with a mixed solution of ethanol (5 L × 3) and water (5 L × 3) for three hours under reflux. Evaporation of the solvent under reduced pressure provided a 50% ethanol extract (444 g, 44.4%). A portion (440 g) of the extract was partitioned using an EtOAc (4 L × 5)−H_2O (4 L) mixture to furnish an EtOAc-soluble fraction (21.0 g, 2.1%) and aqueous phase, which was extracted with 1-BuOH (4 L x× 5) to give 1-BuOH- (137.9 g, 13.8%) and H_2O- (278.0 g, 27.8%) soluble fractions. A part of the 1-BuOH-soluble fraction (137.5 g) was subjected to normal-phase silica gel CC [3.4 kg, $CHCl_3$ → $CHCl_3$−MeOH−H_2O (14:6:1 → 7:3:1 → 6:4:1 → 5:5:1 → MeOH)] to give 12 fractions (1−12). Fraction 6 (12.8 g) was subjected to reversed-phase silica gel CC [400 g, MeOH−H_2O (30:70 → 40:60 → 50:50 → 60:40 → 70:30 → MeOH)] to give nine fractions (6-1 to 6-9). A part of fraction 6-4 (105 mg) was separated by HPLC [MeOH−H_2O in 1% AcOH (70:30)] to give **1** (18.7 mg), **3** (17.3 mg), and **4** (24.2 mg). A part of fraction 6-5 (75 mg) was separated by HPLC [MeOH−H_2O in 1% AcOH (70:30)] to give **1** (25.9 mg) and **3** (26.1 mg). A part of fraction 6-6 (45 mg) was separated by HPLC [MeOH−H_2O in 1% AcOH (80:20)] to give **5** (9.5 mg).

*Camellioside E (**4**):* colorless, amorphous powder; $[\alpha]^{25}_{D}$ +11.9 (c 0.45, MeOH); IR (KBr) ν_{max} 3450, 2918, 1718, 1070 cm^{-1}; ^{1}H NMR (pyridine-d_5, 500 MHz,) δ 0.79 (1H, br d like, J = 12.0, H-5), 0.87, 1.04, 1.12, 1.14, 1.18, 1.30, 1.83 (3H each, all s, H$_3$-25, 29, 24, 30, 26, 23, 27), 1.63 (1H, br d like, J = 10.9, H-15α), 1.76 (1H, dd like, J = 8.6, 8.6, H-9), 2.23 (1H, m, H-22β), 2.33 (1H, m, H-22α), 2.74 (1H, br d like, J = 10.9, H-15β), 2.83 (1H, m, H-18), 3.29 (1H, dd like, J = 4.0, 10.3 Hz, H-3), 4.48 (1H, m, H-16), 4.91 (1H, d, J = 7.5 Hz, H-1′), 5.19 (1H, d, J = 7.8 Hz H-1⁗), 5.44 (1H, s like, H-12), 5.76 (1H, d like, J = 7.0 Hz, H-1‴), 5.81 (1H, d, J = 7.2 Hz, H-1″); ^{13}C NMR (pyridine-d_5, 125 MHz) δ given in Table 1; positive-ion FABMS m/z 1129 [M + Na]$^+$; HRFABMS m/z 1129.5774 (calcd for $C_{54}H_{90}O_{23}Na$ [M + Na]$^+$, 1129.5771).

*Camellioside F (**5**):* colorless, amorphous powder; $[\alpha]^{24}_{D}$ +32.7 (c 0.77, MeOH); IR (KBr) ν_{max} 3450, 2914, 1720, 1718, 1070 cm^{-1}; ^{1}H NMR (pyridine-d_5, 500 MHz) δ 0.72 (1H, d like, J = 11.8, H-5), 0.79, 0.81, 1.00, 1.03, 1.08, 1.27, 1.74 (3H each, all s, H$_3$-26, 25, 29, 30, 24, 23, 27), 1.66 (1H, m, H-15a), 1.62 (1H, m, H-9), 2.03 (1H, m, H-15b), 2.87 (1H, m, H-18), 3.25 (1H, dd, J = 3.7, 11.5 Hz, H-3), 4.78 (1H, m, H-16), 4.89 (1H, d, J = 7.5 Hz, H-1′), 5.18 (1H, d like, J = 8.0 Hz H-1⁗), 5.47 (1H, br s, H-12), 5.72 (1H, d, J = 7.5 Hz, H-1‴), 5.78 (1H, d, J = 7.7 Hz, H-1″), 9.52 (1H, s, H-28); ^{13}C NMR (pyridine-d_5, 125 MHz) δ given in Table 1; positive-ion FABMS m/z 1141 [M + Na]$^+$; HRFABMS m/z 1141.5402 (calcd for $C_{54}H_{86}O_{24}Na$ [M + Na]$^+$, 1141.5407).

Acid Hydrolysis and Monosaccharide Composition of Camelliosides A and F. To determine the absolute configurations of constituent monosaccharides of **4** and **5**, the reported method[11] by Tanaka et al. was used with slight modifications as follows. Compounds **4** and **5** (each 1.0 mg) were dissolved in 5% aqueous H_2SO_4−1,4-dioxane (1:1, v/v, 1.0 mL), and each solution was heated

at 90 °C for 3 h. After extraction three times with EtOAc, the aqueous layer was neutralized with Amberlite IRA-400 (OH^- form). After drying in vacuo, the residue was dissolved in pyridine (0.1 mL) containing L-cysteine methyl ester hydrochloride (0.5 mg) and heated at 60 °C for 1 h. A solution of *o*-tolylisothiocyanate (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 (Nacalai Tesque), 250 × 4.6 mm i.d. (5 μm); mobile phase: MeCN−H_2O in 1% AcOH (18:82, v/v); detection: UV (250 nm); flow rate: 0.8 mL/min; column temperature: 35 °C] to identify the derivatives of constituent monosaccharides (D-galactose, D-glucose, and D-glucuronic acid) in **4** and **5** by comparison of their retention times with those of authentic samples (t_R: D-galactose 40.0 min, L-galactose 42.5 min, D-glucose 47.0 min, L-glucose 42.7 min, D-glucuronic acid 49.0 min, L-glucuronic acid 44.0 min).

Reduction Reaction of Camelliosides A (1) and F (5). A solution of **1** (15.3 mg) in THF (2 mL) was treated with $NaBH_4$ (4 mg) and stirred at 37 °C for 1 h. The reaction mixture was neutralized with a 1% AcOH aqueous solution and evaporated under reduced pressure. The reaction product was subjected to HPLC [CH_3CN−H_2O in 1% AcOH (60:40)] to give **4** (8.2 mg). By the same method, **5** (15.0 mg) was transformed into **3** (6.2 mg). Compounds **3** and **4** were identified by comparison of their physical data ([$\alpha]_D$, ^{1}H NMR, ^{13}C NMR, and MS) with those of natural forms of **3** and **4**, respectively.

Reagents for Bioassays. Dulbecco's modified Eagle's medium (DMEM, 1 or 4.5 g/L glucose) was purchased from Sigma-Aldrich (St. Louis, MO, USA); fetal bovine serum (FBS), penicillin, amphotericin B, and streptomycin were purchased from Gibco (Invitrogen, Carlsbad, CA, USA); the Cell Counting Kit-8 was from Dojindo Lab. (Kumamoto, Japan); the Pierce BCA protein assay kit was from Thermo Fisher Scientific Inc. (MA, USA), and the other chemicals were purchased from Wako Pure Chemical Co., Ltd. (Osaka, Japan).

Cell Culture. Murine B16 melanoma 4A5 cells (RCB0557)[20] were obtained from Riken Cell Bank (Tsukuba, Japan) and grown in DMEM (4.5 g/L glucose) supplemented with 10% FBS, penicillin (100 units/mL), amphotericin B (0.25 mg/mL), and streptomycin (100 μg/mL) at 37 °C in 5% CO_2/air. Normal human neonatal skin fibroblasts (NB1RGB)[21,22] were obtained from TOYOBO and grown in DMEM (1 g/L glucose) supplemented with 10% FBS, penicillin (100 units/mL), streptomycin (100 μg/mL), and amphotericin B (0.25 mg/mL) at 37 °C in 5% CO_2/air. The murine B16 melanoma 4A5 cells and human neonatal fibroblasts 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.

Melanogenesis. Screening for melanogensis using B16 melanoma 4A5 cells was performed as described previously[23] with slight modifications. The melanoma cells were seeded into 24-well multiplates (2.0 × 10^4 cells/400 μL/well). After 24 h of culture, a test compound and 1 mM theophylline were added. The cells were then incubated for 72 h, harvested by incubating with PBS containing 1 mM EDTA and 0.25% trypsin, and washed with PBS. The cells were treated with 1 M NaOH (120 μL/tube, 80 °C, 30 min) to yield a lysate. An aliquot (100 μL) of the lysate was then transferred to a 96-well microplate, and the optical density of each well was 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%. Inhibition (%) was calculated using the following formula, where *A* and *B* indicate the optical density of vehicle- and test compound-treated groups, respectively.

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

Viability of Melanoma Cells. Cell viability was assessed as in our previous report[23] with slight modifications. Murine B16 melanoma 4A5 cells were seeded into 96-well microplates (5.0 × 10^4 cells/100 μL/well) and incubated for 24 h. After 72 h incubation with 1 mM theophylline and a test compound, the cells were washed with PBS. Then, 100 μL of DMEM (4.5 g/L glucose) and 10 μL of WST-8 solution (Cell Counting Kit-8) were added to each well. After 2 h further 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%. Inhibition of proliferation (%) was calculated using the following formula, where *A* and *B* indicate the optical density of vehicle- and test compound-treated groups, respectively.

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

Viability of Fibroblasts. The normal human neonatal skin fibroblasts were seeded into 96-well microplates (5.0 × 10^4 cells/100 μL/well). After 48 h of incubation with a test compound, the same method (WST-8 cell proliferation assay) as above was used. For the determination of protein content, however, the BCA method (BCA protein assay kit) was also used.

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

■ ASSOCIATED CONTENT

Supporting Information

NMR spectra [^{1}H (solvent: CD_3OD, pyridine-d_5), ^{13}C NMR (solvent: pyridine-d_5), DQF COSY, HMQC, and HMBC] for the new compounds are available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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