Medicinal Flowers. XXXVI.[1] Acylated Oleanane-Type Triterpene Saponins with Inhibitory Effects on Melanogenesis from the Flower Buds of Chinese *Camellia japonica*

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Four acylated oleanane-type triterpene oligoglycosides, sanchakasaponins E–H, were isolated from the flower buds of *Camellia japonica* cultivated in Yunnan province, China, together with four known triterpene oligoglycosides. The chemical structures of the new triterpene oligoglycosides were elucidated on the basis of chemical and physicochemical evidence. The inhibitory effects of the triterpene oligoglycoside constituents on melanogenesis in theophylline-stimulated B16 melanoma 4A5 cells were investigated.

Key words *Camellia japonica*; sanchakasaponin; triterpene oligoglycoside; melanogenesis inhibitor; medicinal flower

The Theaceae plant, *Camellia* (*C.*) *japonica* L., has been widely cultivated as an ornamental in Japan, China, and Taiwan. The flower buds of *C. japonica* have been used for the treatment of blood stagnation, 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. In the course of characterizing the flower buds of *Camellia* spiecies,[1–9] we have reported the isolation and structural elucidation of camelliosides A–F and sanchakasaponins A–D from the flower buds of Japanese, Chinese, and Korean *C. japonica* as well as their gastroprotective, platelet-aggregating, and inhibitory effects on melanogenesis.[10,11] Our continuing search led us to the additional isolation of four new acylated oleanane-type triterpene oligoglycosides, sanchakasaponins E (**1**), F (**2**), G (**3**), and H (**4**), from the flower buds of *C. japonica* cultivated in Yunnan province, China, together with four known triterpene oligoglycosides. In addition, the inhibitory effects of the saponin constituents on melanogenesis in theophylline-stimulated B16 melanoma 4A5 cells were investigated. In this paper, we describe the isolation, structural elucidation, and inhibitory effects on melanogenesis in theophylline-stimulated B16 melanoma 4A5 cells of the triterpene oligoglycoside constituents.

The methanolic extract (10.0% from the dried flower buds of *C. japonica* cultivated in Yunnan province, China) was partitioned into an EtOAc–H_2O (1 : 1, v/v) mixture to furnish an EtOAc-soluble fraction (2.14%) and aqueous layer. The aqueous layer was further extracted with 1-butanol to give 1-butanol- (5.69%) and H_2O- (2.12%) soluble fractions as described previously.[1] The 1-butanol-soluble fraction was subjected to normal- and reversed-phase silica-gel column chromatographies and repeated HPLC to give four new acylated oleanane-type triterpene oligoglycosides, sanchakasaponin E (**1**, 0.0021%), F (**2**, 0.0057%), G (**3**, 0.0036%) and H (**4**, 0.0034%) together with four known compounds, yuchasaponin A (**5**, 0.0034%),[8] sasanquasaponins I (**6**, 0.0080%)[9] and III (**7**, 0.017%),[9] and ternstoemiaside C (**8**, 0.0050%).[12]

Structure of Sanchakasaponins E–H (1–4) Sanchakasaponin E (**1**) was isolated as a colorless amorphous powder with negative optical rotation ($[\alpha]_D^{25}$ −38.8° in MeOH). Its IR spectrum showed absorption bands at 3350, 1730, 1713, 1695, and 1050 cm^{-1} due to hydroxyl, ester, α,β-unsaturated ester, carboxyl and ether components. FAB-MS in the positive-ion mode revealed a quasimolecular ion peak $[M+Na]^+$ at *m/z* 1283 from which the molecular formula $C_{61}H_{96}O_{27}$ was determined by high-resolution (HR)-MS. Treatment of **1** with a 10% aqueous KOH–1,4-dioxane (1 : 1, v/v) mixture yielded a desacyl-derivative [desacyl-jegosaponin],[13] together with angelic acid. The angelic acid was identified from the retention time in a HPLC analysis of its *p*-nitrobenzyl ester.[9] Acid hydrolysis of **1** with 5% aqueous H_2SO_4-1,4-dioxane yielded barringtogenol C[14] and monosaccharides (D-glucose, D-galactose, L-rhamnose, and D-glucuronic acid), which were identified by HPLC of their tolylthiocarbamoyl thiazolidine derivatives.[15] The ^{1}H-NMR (pyridine-d_5) and ^{13}C-NMR (Table 1) spectra of **1**, which were assigned by various NMR experiments,[16] showed signals assignable to a desacyl-jegosaponin component: seven methyls [δ 0.76, 0.82, 1.05, 1.08, 1.16, 1.30, 1.90 (all s, H_3-25, 26, 24, 29, 23, 30, 27)], a methylene [δ 3.37, 3.62 (both d, J=10.4 Hz, H_2-28)] and four methines with an oxygen function [δ 3.14 (dd, J=3.8, 11.8 Hz, H-3), 4.44 (m, H-16), 6.21 (d, J=10.0 Hz, H-22), 6.62 (d, J=10.0 Hz, H-21)], an olefin [δ 5.36 (br s, H-12)], and four glycopyranosyl moieties {β-D-glucuronopyranosyl [δ 4.87 (d like, J=7.0 Hz, H-1′)], β-D-glucopyranosyl [δ 5.92 (d, J=7.5 Hz, H-1″)], β-D-galactopyranosyl [δ 6.22 (d like, J=7.0 Hz, H-1‴)], and α-L-rhamnopyranosyl [δ 6.26 (br s, H-1⁗)]} together with an angeloyl group [δ 2.01 (s, H_3-5⁗), 2.10 (d, J=7.2 Hz, H_3-4⁗), 5.96 (q, J=7.0 Hz, H-3⁗)] and an acetyl group [δ 2.15 (s, H-2⁗)]. The positions of the acyl groups and the structure of the oligoglycoside moiety were confirmed based on double quantum filter correlation spectroscopy (DQF COSY) and heteronuclear multiple bond connectivity (HMBC) spectroscopy. Long-range correlations were observed between the following proton and carbon pairs: H-1′ and C-3; H-1″ and C-2′; H-1‴ and C-3′; H-1⁗ and C-2‴; H-21 and C-1⁗′; H-22 and C-1⁗″. From all this evidence, the chemical structure of sanchakasaponin E was determined to be 21-*O*-angeloyl-22-*O*-acetyl-barringtogenol C 3-*O*-[β-D-glucopyranosyl(1→2)][α-L-rhamnopyranosyl(1→2)-β-D-galactopyranosyl(1→3)]-β-D-glucuronopyranoside (**1**).

The authors declare no conflict of interest.

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Table 1. ^{13}C-NMR (125 MHz) Spectroscopic Data for Compounds **1**–**4** in C_5D_5N

Position	1	2	3	4	Position	1	2	3	4
1	38.7	39.0	38.7	38.8	3-*O*-*β*-D-Glucuronopyranosyl				
2	26.4	26.5	26.4	26.5	1′	105.4	105.5	105.4	105.4
3	89.7	89.8	89.6	89.8	2′	79.4	79.4	79.4	79.7
4	39.7	39.7	39.7	39.7	3′	82.7	82.5	82.7	83.1
5	55.7	55.6	55.7	55.8	4′	71.3	71.3	71.2	71.1
6	18.5	18.9	18.5	18.5	5′	77.0	77.1	77.0	77.1
7	33.1	36.8	33.1	33.1	6′	172.4	172.5	172.2	172.3
8	40.1	41.5	41.7	41.7	2′-*O*-*β*-D-Glucopyranosyl				
9	46.9	47.1	46.9	46.9	1″	102.7	102.7	102.7	
10	36.7	37.0	36.8	36.8	2″	76.3	76.3	76.3	
11	23.8	24.0	23.8	23.8	3″	78.5	78.5	78.5	
12	123.1	125.5	123.1	123.1	4″	72.6	72.6	72.6	
13	142.8	143.7	143.8	143.7	5″	78.2	78.3	78.2	
14	41.7	47.8	40.1	40.1	6″	63.6	63.7	63.6	
15	34.7	67.6	35.2	35.2	2′-*O*-*β*-D-Galactopyranosyl				
16	68.0	73.4	70.0	70.2	1″				103.7
17	44.6	48.4	45.1	44.9	2″				73.5
18	40.0	41.0	40.9	40.9	3″				75.3
19	47.2	46.9	47.5	47.5	4″				70.3
20	36.3	36.4	32.1	32.1	5″				76.9
21	78.9	78.7	41.8	41.8	6″				62.9
22	74.4	73.7	73.1	73.0	3′-*O*-*β*-D-Galactopyranosyl				
23	16.7	16.8	16.8	16.9	1‴	101.4	101.3	101.4	101.5
24	28.0	27.9	28.0	28.2	2‴	76.5	76.5	76.5	77.0
25	15.6	15.8	15.7	15.7	3‴	75.7	76.1	76.1	76.1
26	16.9	17.6	16.9	16.9	4‴	71.2	71.4	71.3	70.9
27	27.5	21.3	27.6	27.6	5‴	77.5	77.5	77.5	77.3
28	63.8	63.2	63.6	63.7	6‴	61.9	62.1	61.9	61.9
29	20.2	20.3	25.2	25.2	3′-*O*-*β*-D-Galactopyranosyl				
30	29.5	29.6	33.5	33.5	1⁗	102.4	102.4	102.4	102.7
1⁗	167.8	168.2	167.9	168.0	2⁗	72.6	72.6	72.6	72.6
2⁗	129.0	129.2	130.0	129.6	3⁗	72.6	72.7	72.7	72.8
3⁗	137.1	136.6	136.3	136.5	4⁗	73.9	73.9	73.9	74.0
4⁗	15.9	16.0	14.1	15.9	5⁗	69.8	69.9	69.8	69.9
5⁗	21.0	21.1	12.3	21.0	6⁗	18.3	18.3	18.3	18.4
1⁗′	170.0	167.8	—	—					
2⁗′	22.1	129.0	—	—					
31⁗′	—	137.5	—	—					
41⁗′	—	15.8	—	—					
51⁗′	—	20.7	—	—					

Sanchakasaponin F (**2**), obtained as a colorless amorphous powder with a negative optical rotation ($[\alpha]_D^{25}$ −12.3° in MeOH), showed absorption bands in the IR spectrum due to hydroxyl, *α*,*β*-unsaturated ester, carboxyl and ether functions. FAB-MS in the positive-ion mode revealed a quasimolecular ion peak $[M+Na]^+$ at *m*/*z* 1339 from which the molecular formula $C_{64}H_{100}O_{28}$ was determined by HR-MS. Alkaline hydrolysis of **2** provided a desacyl-derivative [desacyl-yuchasaponin A],[8] together with angelic acid. The angelic acid was identified from retention time in a HPLC analysis of its *p*-nitrobenzyl ester.[9] Acid hydrolysis of **2** yielded R_1-barrigenol[17] and monosaccharides (D-glucose, D-galactose, L-rhamnose, and D-glucuronic acid), which were identified by HPLC of their tolylthiocarbamoyl thiazolidine derivatives.[15] The ^{1}H-NMR (pyridine-d_5) and ^{13}C-NMR (Table 1) spectra of **2**, which were assigned by various NMR experiments,[16] showed signals assignable to a desacyl-yuchasaponin A component: seven methyls [*δ* 0.78, 0.98, 1.05, 1.08, 1.11, 1.31, 1.84 (all s, H_3-25, 26, 24, 29, 23, 30, 27)], a methylene [*δ* 3.48, 3.74 (both d, *J*=10.2 Hz, H_2-28)] and five methines with an oxygen function [*δ* 3.18 (dd-like, H-3), 4.21 (m, H-15), 4.45 (m, H-16), 6.31 (d, *J*=10.4 Hz, H-22), 6.69 (d, *J*=10.4 Hz, H-21)], and four glycopyranosyl moieties {*β*-D-glucuronopyranosyl [*δ* 4.82 (d-like, *J*=7.0 Hz, H-1′)], *β*-D-glucopyranosyl [*δ* 5.91 (d-like, *J*=7.0 Hz, H-1″)], *β*-D-galactopyranosyl [*δ* 6.20 (d-like, *J*=7.0 Hz, H-1‴)], and *α*-L-rhamnopyranosyl [*δ* 6.23 (brs, H-1⁗)]} together with two angeloyl groups [*δ* 1.73 (s, H_3-5⁗′), 1.95 (d, *J*=7.1 Hz, H_3-4⁗′), 2.00 (s, H_3-5⁗′), 2.08 (d, *J*=7.1 Hz, H_3-4⁗′), 5.77 (d, *J*=7.1 Hz, H-3⁗′), 5.94 (d, *J*=7.1 Hz, H-3⁗′)]. The positions of the acyl groups and the structure of the oligoglycoside moiety were confirmed based on DQF COSY and HMBC experiments. Long-range correlations were observed between the following proton and carbon pairs: H-1′ and C-3; H-1″ and C-2′; H-1‴ and C-3′; H-1⁗ and C-2‴; H-21 and C-1⁗′; H-22 and C-1⁗′. On the basis of all this evidence, the chemical structure of sanchakasaponin F

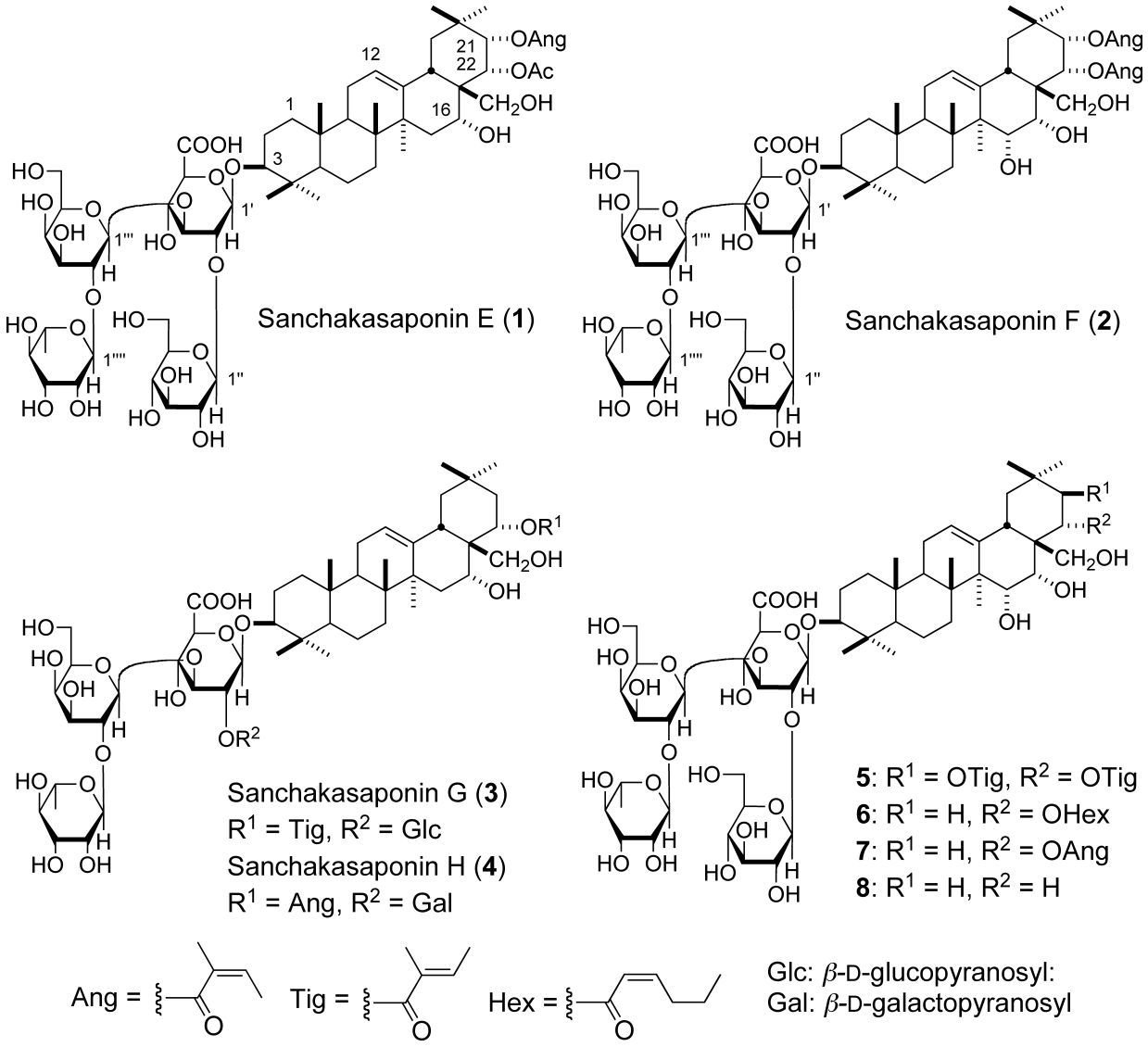

Fig. 1. Structure of Saponin Constituents Isolated from Flower Buds of Chinese *C. japonica*

was determined as 21,22-*O*-di-angeloyl-R_1-barrigenol 3-*O*-[*β*-D-glucopyranosyl(1→2)][*α*-L-rhamnopyranosyl(1→2)-*β*-D-galactopyranosyl(1→3)]-*β*-D-glucuronopyranoside (**2**).

Sanchakasaponins G (**3**) and H (**4**), each obtained as a colorless amorphous powder with a negative optical rotation (**3**: $[\alpha]_D^{25}$ −27.7°, **4**: $[\alpha]_D^{25}$ −12.8° in MeOH), showed absorption bands in their IR spectra due to hydroxyl, *α*,*β*-unsaturated ester, carboxyl, and ether functions. The same molecular formula $C_{59}H_{94}O_{25}$ was determined for both compounds from a quasimolecular ion peak (*m*/*z* 1225 $[M+Na]^+$) in positive-ion FAB-MS and by HR-MS measurements. Alkaline treatment yielded a desacyl-derivative [ternstroemiaside A[12] from **3** and **4a** from **4**] together with organic acids (tiglic acid from **3**; angelic acid from **4**), which were identified by HPLC of their *p*-nitrobenzyl derivatives.[9] Acid hydrolysis yielded camelliagenin A[18–20] and monosaccharides (D-glucose, D-galactose, L-rhamnose, and D-glucuronic acid from **3**; D-galactose, L-rhamnose, and D-glucuronic acid from **4**), which were identified by HPLC of their tolylthiocarbamoyl thiazolidine derivatives.[15] The ^{1}H-NMR (pyridine-d_5) and ^{13}C-NMR (Table 1) spectra of **3**[16] showed signals assignable to a ternstroemiaside A component: seven methyls [*δ* 0.78, 0.86, 1.06, 1.07, 1.17, 1.29, 1.87 (all s, H_3-25, 26, 24, 29, 23, 30, 27)], a methylene [*δ* 3.54, 3.62 (both d, *J*=10.4 Hz, H_2-28)] and three methines with an oxygen function [*δ* 3.19 (dd-like, H-3), 4.67 (m, H-16), 6.19 (m, H-22)], and four glycopyranosyl moieties {*β*-D-glucuronopyranosyl [*δ* 4.88 (d-like, *J*=7.0 Hz, H-1′)], *β*-D-glucopyranosyl [*δ* 5.92 (d-like, *J*=7.3 Hz, H-1″)], *β*-D-galactopyranosyl [*δ* 6.22 (d-like, *J*=7.0 Hz, H-1‴)], and *α*-L-rhamnopyranosyl [*δ* 6.27 (br s, H-1⁗)]} together with tigloyl groups [*δ* 1.54 (d, *J*=7.1 Hz, H_3-4⁗), 1.87 (s, H_3-5⁗), 6.99 (q, *J*=7.1 Hz, H-3⁗)]. On the other hand, the proton and carbon signals in the ^{1}H- and ^{13}C-NMR (Table 1) spectra[16] of **4** were superimposable on those of **3**, except for the signals due to the 22-acyl group and the glycopyranosyl moieties of **3**. The ^{1}H-NMR (pyridine-d_5) and ^{13}C-NMR spectra of **4** showed signals assignable to an angeloyl group [*δ* 1.93 (s, H_3-5⁗), 2.07 (d, *J*=7.0 Hz, H_3-4⁗), 5.90 (q, *J*=7.0 Hz, H-3⁗)] and four glycopyranosyl moieties {*β*-D-glucuronopyranosyl [*δ* 4.90 (d-like, *J*=7.0 Hz, H-1′)], two *β*-D-galactopyranosyl [*δ* 5.77 (d, *J*=7.3 Hz, H-1″), 6.14 (d, *J*=7.6 Hz, H-1‴)], and *α*-L-rhamnopyranosyl [*δ* 6.24 (br s, H-1⁗)]} together with an camelliagenin A component. The positions of the

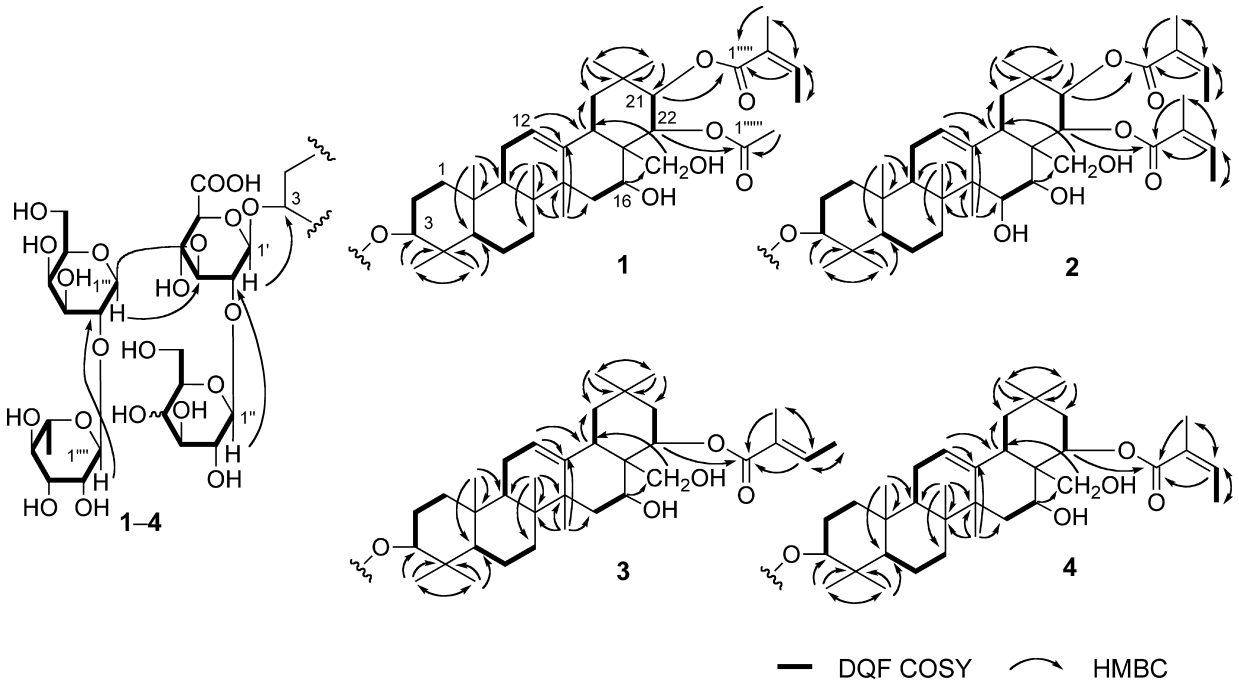

Fig. 2. Important 2D-NMR Correlations of **1**–**4**

Table 2. Inhibitory Effects of Constituents on Melanogenesis in B16 Melanoma 4A5 Cells

Sample	Inhibition (%) of melanogenesis				IC_{50} (μM)
Conc. (μM)	0 μM	1 μM	3 μM	10 μM	
Sanchakasaponin E (**1**)[a]	0.0±0.7	13.3±2.5**	69.2±2.6**	94.8±0.5**	2.1
Sanchakasaponin F (**2**)[a]	0.0±1.5	16.7±2.4**	51.4±2.9**	91.7±0.4**	2.9
Sanchakasaponin H (**4**)[a]	0.0±9.0	0.4±7.7	15.9±6.4*	87.2±1.6**	4.7
Yuchasaponin A (**5**)[a]	0.0±4.1	22.1±5.3**	56.4±6.1**	98.8±0.3**	2.5
Sasanquasaponin I (**6**)[b]	0.0±4.4	–5.7±4.3	51.1±3.2**	96.3±0.3**	3.0
Sasanquasaponin III (**7**)[b]	0.0±1.8	12.5±1.1*	59.8±9.9**	98.7±0.3**	2.5
Ternstoemiaside C (**8**)[b]	0.0±3.0	9.8±5.4	13.6±1.4*	14.7±4.6*	>10
	Inhibition (%) of melanogenesis				
Conc. (μM)	0 μM	30 μM	100 μM	300 μM	—
Arbutin[b]	0.0±1.4	20.4±0.5**	38.1±0.9**	61.5±0.6**	174

Each value represents the mean±S.E.M. (N=4). Significantly different from the control, *p<0.05, **p<0.01. *a*) The cell viability for **1**, **2**, **4**, and **5** at 10 μM is less than 27% (cytotoxic effects were observed). *b*) The cell viability for **6**, **7**, **8** and arbutin at 10 μM is more than 70%.

acyl groups and the structure of the oligoglycoside moieties of **3** and **4** were confirmed on the basis of DQF COSY and HMBC experiments (Fig. 2). From this evidence, the chemical structure of sanchakasaponin G and H was determined as 22-*O*-tigloyl-camelliagenin A 3-*O*-[β-D-glucopyranosyl(1→2)][α-L-rhamnopyranosyl(1→2)-β-D-galactopyranosyl(1→3)]-β-D-glucuronopyranoside (**3**) and 22-*O*-angeloyl-camelliagenin A 3-*O*-[β-D-galactopyranosyl(1→2)][α-L-rhamnopyranosyl(1→2)-β-D-galactopyranosyl(1→3)]-β-D-glucuronopyranoside (**4**), respectively.

Melanogenesis Inhibitory Effects of Triterpene Oligoglycoside Constituents Melanin production is principally responsible for skin color, and melanin pigmentation is a major defense mechanism against UV rays from the sun. 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 diaryheptanoids, flavonoids, and sterol glycosides in theophylline-stimulated B16 melanoma 4A5 cells.[21–23] As a continuation of these studies, the inhibitory effects of constituents from the flowers buds of *C. japonica* on melanogenesis were examined. Among the isolates, sasanquasaponins I (**6**, IC_{50}=3.0 μM) and II (**7**, IC_{50}=2.5 μM) with an acyl group at the 22-position substantially inhibited melanogenesis without cytotoxic effects. The effects of **6** and **7** were stronger than that of a reference compound, arbutin (IC_{50}=174 μM).[22,23] Sanchakasaponins E (**1**) and F (**2**) and yuchasaponin A (**5**) with two acyl groups at the 21- and 22-positions also inhibited melanogenesis, but had cytotoxic effects at 3 μM.

In conclusion, four new acylated oleanane-type triterpene saponins, sasanquasaponins E–H (**1**–**4**), were isolated from the flower buds of *C. japonica* cultivated in Yunnan province, China, and their chemical structures were elucidated on the basis of chemical and physicochemical evidence. In addition, the isolated constituents, sasanquasaponins I (**6**) and II (**7**) with an acyl group at the 22-position, substantially inhibited

melanogenesis without cytotoxic effects.

Experimental

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; FAB-MS and HR-FAB-MS, 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 (250×4.6 mm i.d. and 250×20 mm i.d.) and 5C18-AR-II (250×4.6 mm i.d.) columns were used for analytical and preparative purposes.

The following materials were used for chromatography: normal-phase silica gel column chromatography, Silica gel BW-200 (Fuji Silysia Chemical, Ltd., 150–350 mesh); reversed-phase silica gel column chromatography, 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.

Plant Material The dried flower buds of *C. japonica* cultivated in Yunnan province, China, were purchased from Tochimoto Tenkaido Co., Ltd. (Osaka, Japan) in May 2010, and identified by one of authors (M.Y.). A voucher specimen is on file in our laboratory.

Extraction and Isolation The dried flower buds (4.0 kg) were extracted three times with methanol under reflux for 3 h. Evaporation of the solvent under reduced pressure provided a MeOH extract (398 g, 10.0%). A part of the MeOH extract (236 g) was partitioned into an EtOAc–H_2O (1 : 1, v/v) mixture to furnish an EtOAc-soluble fraction (50.8 g, 2.14%) and an aqueous phase. The aqueous phase was further extracted with 1-butanol to give a 1-butanol-soluble fraction (135 g, 5.69%) and an H_2O-soluble fraction (50.3 g, 2.12%). A part of the 1-butanol-soluble fraction (104 g) was subjected to normal phase silica gel column chromatography [3.90 kg, $CHCl_3$–EtOAc–isopropanol–MeOH (3 : 3 : 1 : 1→2 : 2 : 1 : 1→1 : 1 : 1 : 1, v/v/v/v)→$CHCl_3$–EtOAc–isopropanol–MeOH–H_2O (10 : 10 : 10 : 10 : 1→5 : 5 : 5 : 5 : 1→5 : 5 : 10 : 10 : 4, v/v/v/v/v)→MeOH] to give six fractions [Fr.1 (3.67 g), Fr.2 (13.7 g), Fr.3 (10.7 g), Fr.4 (5.70 g), Fr.5 (24.6 g), Fr.6 (0.56 g)]. Fraction 5 (24.6 g) was further separated by reversed phase silica gel column chromatography [750 g, MeCN–H_2O (10 : 90→15 : 85→20 : 80→25 : 75→30 : 70→40 : 60→50 : 50→60 : 40, v/v)→MeOH] to give eight fractions [Fr.5-1, Fr.5-2, Fr.5-3, Fr.5-4, Fr.5-5, Fr.5-6 (2.10 g), Fr.5-7 (17.9 g), Fr.5-8]. Fraction 5-6 (2.10 g) was further separated by reversed phase silica gel column chromatography [60 g, MeOH–H_2O (50 : 50, v/v)→MeOH] to give four fractions [Fr.5-6-1, Fr.5-6-2, Fr.5-6-3 (630 mg), Fr.5-6-4 (364 mg)]. Fraction 5-6-3 (630 mg) was purified by HPLC [H_2O–MeOH–MeCN–AcOH (300 : 525 : 175 : 3, v/v/v/v), COSMOSIL 5C18-MS-II] to give **8** (58.9 mg). Fr.5-6-4 (364 mg) was purified by HPLC [H_2O–MeOH–MeCN–AcOH (450 : 275 : 275 : 3, v/v/v/v), COSMOSIL 5C18-MS-II] to give **8** (33.7 mg). Fraction 5-7 (17.9 g) was further separated by reversed phase silica gel column chromatography [600 g, MeOH–H_2O (55 : 45→70 : 30, v/v)→MeOH] to give three fractions [Fr.5-7-1, Fr.5-7-2 (2.62 g), Fr.5-7-3 (7.00 g)]. A part of fraction 5-7-2 (1.62 g) was purified by HPLC [H_2O–MeCN–AcOH (650 : 350 : 3, v/v/v), COSMOSIL 5C18-MS-II] to give **7** (194 mg). A part of fraction 5-7-3 (2.40 g) was further separated by HPLC [H_2O–MeOH–MeCN–AcOH (300 : 575 : 175 : 3, v/v/v/v), COSMOSIL 5C18-MS-II] to give 10 fractions [Fr.5-7-3-1, Fr.5-7-3-2, Fr.5-7-3-3, Fr.5-7-3-4 (145 mg), Fr.5-7-3-5 (48.2 mg), Fr.5-7-3-6, Fr.5-7-3-7 (113 mg), Fr.5-7-3-8 (127 mg), Fr.5-7-3-9, Fr.5-7-3-10 (68.4 mg)]. Fraction 5-7-3-4 (145 mg) was purified by HPLC [H_2O–MeCN–AcOH (550 : 450 : 3, v/v/v), COSMOSIL 5C18-MS-II] to give **1** (13.2 mg) and **3** (19.2 mg). Fraction 5-7-3-5 (48.2 mg) was purified by HPLC [H_2O–MeCN–AcOH (550 : 450 : 3, v/v/v), COSMOSIL 5C18-MS-II] to give **4** (18.0 mg). Fraction 5-7-3-7 (113 mg) was purified by HPLC [H_2O–MeCN–AcOH (550 : 450 : 3, v/v/v), COSMOSIL 5C18-MS-II] to give **6** (50.4 mg). Fraction 5-7-3-8 (127 mg) was purified by HPLC [H_2O–MeCN–AcOH (550 : 450 : 3, v/v/v), COSMOSIL 5C18-MS-II] to give **5** (21.4 mg). Fraction 5-7-3-10 (68.8 mg) was purified by HPLC [H_2O–MeOH–MeCN–AcOH (250 : 562 : 188 : 3, v/v/v/v), COSMOSIL 5C18-MS-II] to give **2** (36.0 mg). The known saponins were identified by comparison of their physical data ($[\alpha]_D$, ^{1}H-NMR, ^{13}C-NMR, and MS) with reported values.

Sanchakasaponin E (**1**): A colorless amorphous powder; $[\alpha]_D^{25}$ −38.8° (c=0.26, MeOH); IR attenuated total reflectance (ATR): ν_{max} 3350, 2910, 1730, 1713, 1695, and 1050 cm^{-1}; ^{1}H-NMR (pyridine-d_5, 500 MHz) δ: 0.76, 0.82, 1.05, 1.08, 1.16, 1.30, 1.90 (3H each, all s, H_3-25, 26, 24, 29, 23, 30, 27), 1.42 (3H, d, J=6.0 Hz, H-6‴), 2.01 (3H, s, H-5⁗), 2.10 (3H, d, J=7.2 Hz, H-4⁗), 2.15 (3H, s, H-2⁗), 3.14 (3H, dd, J=3.8, 11.8 Hz, H-3), 3.37 (1H, d, J=10.4 Hz, H-28a), 3.62 (1H, d, J=10.4 Hz, H-28b), 4.44 (1H, m, H-16), 4.87 (1H, d like, J=7.0 Hz, H-1′), 5.36 (1H, brs, H-12), 5.92 (1H, d, J=7.5 Hz, H-1″), 5.96 (1H, q, J=7.2 Hz, H-3⁗), 6.21 (1H, d, J=10.0 Hz, H-22), 6.22 (d like, J=7.0 Hz, H-1‴), 6.26 (1H, brs, H-1⁗), 6.62 (1H, d, J=10.0 Hz, H-21); ^{13}C-NMR: given in Table 1; positive-ion FAB-MS: m/z 1283 $[M+Na]^+$; high-resolution positive-ion FAB-MS: m/z 1283.6037 (Calcd for $C_{61}H_{96}O_{27}Na$ $[M+Na]^+$: m/z 1283.6037).

Sanchakasaponin F (**2**): A colorless amorphous powder; $[\alpha]_D^{25}$ −12.3° (c=0.92, MeOH); IR(ATR): ν_{max} 3350, 2914, 1720, 1695, and 1050 cm^{-1}; ^{1}H-NMR (pyridine-d_5, 500 MHz) δ: 0.78, 0.98, 1.05, 1.08, 1.11, 1.31, 1.84 (3H each, all s, H_3-25, 26, 24, 29, 23, 30, 27), 1.41 (3H, d like, H-6‴), 1.73 (3H, s, H-5⁗), 1.95 (3H, d, J=7.1 Hz, H-4⁗), 2.00 (3H, s, H-5⁗), 2.08 (3H, d, J=7.1 Hz,, H-4⁗), 3.18 (3H, dd-like, H-3), 3.48 (1H, d, J=10.2 Hz, H-28a), 3.74 (1H, d, J=10.2 Hz, H-28b), 4.21 (1H, m, H-15), 4.45 (1H, m, H-16), 4.82 (1H, d like, J=7.0 Hz, H-1′), 5.49 (brs, H-12), 5.77 (1H, d, J=7.1 Hz, H-3⁗), 5.91 (1H, d-like, J=7.0 Hz, H-1″), 5.94 (1H, d, J=7.1 Hz, H-3⁗), 6.20 (1H, d-like, J=7.0 Hz, H-1‴), 6.23 (1H, brs, H-1⁗), 6.31 (1H, d, J=10.4 Hz, H-22), 6.69 (1H, d, J=10.4 Hz, H-21); ^{13}C-NMR: given in Table 1; positive-ion FAB-MS: m/z 1339 $[M+Na]^+$; high-resolution positive-ion FAB-MS: m/z 1339.6296 (Calcd for $C_{64}H_{100}O_{28}Na$ $[M+Na]^+$: m/z 1339.6293).

Sanchakasaponin G (**3**): A colorless amorphous powder; $[\alpha]_D^{25}$ −27.7° (c=0.30, MeOH); IR(ATR): ν_{max} 3350, 2906, 1720, 1698, and 1050 cm^{-1}; ^{1}H-NMR (pyridine-d_5, 500 MHz) δ: 0.76, 0.86, 1.06, 1.07, 1.17, 1.29, 1.87 (all s, H_3-25, 26, 24, 29, 23, 30, 27), 1.44 (3H, d, J=5.8 Hz, H-6⁗), 1.54 (3H, d,

J=7.1 Hz, H-4‴″), 1.87 (3H, s, H-5‴″), 3.19 (3H, dd-like, H-3), 3.54 (1H, d, J=10.4 Hz, H-28a), 3.62 (1H, d, J=10.4 Hz, H-28b), 4.67 (1H, m, H-16), 4.88 (d-like, J=7.0 Hz, H-1′), 5.37 (1H, brs, H-12), 5.92 (1H, d-like, J=7.3 Hz, H-1″), 6.19 (1H, m, H-22), 6.22 (1H, d-like, J=7.0 Hz, H-1‴), 6.27 (1H, brs, H-1⁗), 6.99 (1H, q, J=7.1 Hz, H-3‴″); ^{13}C-NMR: given in Table 1; positive-ion FAB-MS: m/z 1225 $[M+Na]^+$; high-resolution positive-ion FAB-MS: m/z 1225.5969 (Calcd for $C_{59}H_{94}O_{25}Na$ $[M+Na]^+$: m/z 1225.5976).

Sanchakasaponin H (**4**): A colorless amorphous powder; $[\alpha]_D^{25}$ −12.8° (c=0.40, MeOH); IR(ATR): ν_{max} 3350, 2918, 1720, 1700, and 1050 cm^{-1}; ^{1}H-NMR (pyridine-d_5, 500 MHz) δ: 0.76, 0.83, 1.03, 1.16, 1.25, 1.27, 1.85 (3H each, all s, H_3-25, 26, 24, 29, 23, 30, 27), 1.43 (3H, d, J=5.8 Hz, H-6⁗), 1.93 (3H, s, H-5‴″), 2.07 (3H, d, J=7.0 Hz, H-4‴″), 3.20 (3H, dd, J=3.1, 11.6 Hz, H-3), 3.54 (1H, d, J=10.7 Hz, H-28a), 3.68 (1H, d, J=10.7 Hz, H-28b), 4.62 (1H, m, H-16), 4.90 (1H, d-like, J=7.0 Hz, H-1′), 5.34 (1H, brs, H-12), 5.77 (1H, d, J=7.3 Hz, H-1″), 5.90 (1H, q, J=7.0 Hz, H-3‴″), 6.14 (1H, d, J=7.6 Hz, H-1‴), 6.21 (1H, dd, J=5.7, 11.9 Hz, H-22), 6.24 (1H, brs, H-1⁗); ^{13}C-NMR: given in Table 1; positive-ion FAB-MS: m/z 1225 $[M+Na]^+$; high-resolution positive-ion FAB-MS: m/z 1225.5983 (Calcd for $C_{59}H_{94}O_{25}Na$ $[M+Na]^+$: m/z 1225.5976).

Alkaline Hydrolysis of Sanchakasaponins Solutions of sanchakasaponins E–H (**1**–**4**, each 4.0 mg) were treated with 10% aqueous KOH–1,4-dioxane (1 : 1, v/v, 1.0 mL) and stirred at 37°C for 6 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, most of which was subjected to reversed-phase silica gel solid-phase extraction (Sep-Pak-C18, Waters Co., Ltd.) [H_2O→MeOH] to give desacyl-saponins (desacyl-jegosaponin from **1**, desacyl-yuchasaponin from **2**, ternstroemiaside A from **3**, and **4a** from **4**). Their desacyl-saponins were identified by comparison of their ^{1}H- and ^{13}C-NMR data with authentic samples or reported values.

4a: Colorless amorphous powder; ^{1}H-NMR (pyridine-d_5, 500 MHz) δ_H: 0.77, 0.88, 1.05, 1.17, 1.26, 1.27, 1.85 (all s, H_3-25, 26, 24, 29, 23, 30, 27), 1.44 (3H, d, J=6.1 Hz, H-6⁗), 3.24 (3H, dd, J=4.0, 11.6 Hz, H-3), 3.67 (2H, m, H_2-28), 4.05 (1H, m, H-22), 4.59 (1H, m, H-16), 4.89 (1H, d-like, H-1′), 5.33 (brs, H-12), 5.79 (1H, d, J =7.7 Hz, H-1″), 6.18 (1H, d, J=7.9 Hz, H-1‴), 6.26 (1H, brs, H-1⁗); ^{13}C-NMR (pyridine-d_5, 500 MHz) δ_C: 15.7, 16.9×2, 18.3×2, 23.8, 25.5, 26.5, 27.5, 28.1, 31.8, 33.2, 33.8, 34.7, 36.8, 38.9, 39.8, 40.1, 41.7, 42.2, 44.3, 44.9, 47.0, 47.9, 55.8, 62.0, 62.9, 68.4, 69.9, 70.3, 71.0, 71.2×2, 72.6×2, 72.8, 73.5, 74.0, 75.3, 76.1, 77.0, 77.1×2, 77.4, 79.8, 83.1, 89.9, 101.5, 102.7, 103.6, 105.4, 123.1, 144.3, 172.2; positive-ion FAB-MS: m/z 1143 $[M+Na]^+$.

Next, a part of the crude product (each 1.0 mg) obtained from **1**–**4** was dissolved in $(CH_2)_2Cl_2$ (0.5 mL), respectively. The solution was treated with p-nitrobenzyl-N,N'-diisopropylisourea (4 mg), then stirred at 80°C for 1 h. The reaction solution was subjected to HPLC [column: COSMOSIL 5C18-MS-II, 250×4.6 mm i.d.; mobile phase: MeCN–H_2O (50 : 50, v/v); detection: UV (254 nm); flow rate: 1.0 mL/min; column temperature: room temperature] to identify the p-nitrobenzyl esters of tiglic acid (t_R: 20.9 min, from **3**) and angelic acid (t_R: 23.0 min, from **1**, **2** and **4**).

Acid Hydrolysis and Monosaccharide Composition of Sanchakasaponins Compounds **1**–**4** (1.0–2.0 mg) were dissolved in 5% aqueous H_2SO_4–1,4-dioxane (1 : 1, v/v, 2.0 mL), and each solution was heated at 90°C for 3 h. After extraction three times with EtOAc, the EtOAc fraction was purified by normal-phase silica gel column chromatography [$CHCl_3$: MeOH : H_2O [10 : 3 : 1, lower phase] to give barringtogenol C (from **1**), R_1-barrigenol (from **2**), and camelliagenin A (from **3** and **4**), which were identified by comparison of their physical data with authentic samples and reported values. 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. *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, 250×4.6 mm i.d.; mobile phase: MeCN–0.05 M H_3PO_4 (77 : 23, v/v); detection: UV (250 nm); flow rate: 0.8 mL/min; column temperature: 35°C] to identify the derivatives of constituent monosaccharides in sanchakasaponins E–H (**1**−**4**) by comparison of their retention times with those of authentic samples (t_R: D-glucose; 19.7 min, D-galactose; 17.0 min, L-rhamnose; 33.7 min and D-glucuronic acid; 20.4 min).

Reagents for Bioassays Dulbecco's modified Eagle's medium (DMEM, 4.5 g/L glucose) was purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.); fetal bovine serum (FBS), penicillin, and streptomycin were purchased from Gibco (Invitrogen, Carlsbad, CA, U.S.A.); the Cell Counting Kit-8^{TM} was from Dojindo Lab. (Kumamoto, Japan); and the other chemicals were purchased from Wako Pure Chemical Co., Ltd. (Osaka, Japan).

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 units/mL), and streptomycin (100 μg/mL) at 37°C in 5% CO_2/air. The cells were harvested by incubation in phosphate-buffered saline (PBS) containing 1 mM ethylenediaminetetraacetic acid (EDTA) and 0.25% trypsin for *ca.* 5 min at 37°C and used for the subsequent bioassays.

Melanogenesis The 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 the cells incubated for 72 h. The cells were harvested by incubating with PBS containing 1 mM EDTA and 0.25% trypsin, and then washed with PBS. The cells were treated with NaOH 1 M (120 μL/tube, 80°C, 30 min) to yield a lysate. An aliquot (100 μL) of the lysate was 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 dimethyl sulfoxide (DMSO). 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.

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

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

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 of incubation with theophylline 1 mM and a test compound, 10 μL of WST-8 solution (Cell Counting Kit-8^{TM}) 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, U.S.A.) at 450 nm (reference: 655 nm). The test compound was dissolved in DMSO. The final concentration of DMSO in the medium was 0.1%. Cell viability (%) was calculated using the following formula.

$$\text{cell viability (\%)} = A / B \times 100$$

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

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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