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NEW NOROLEANANE-TYPE TRITERPENE SAPONINS WITH GASTRO-PROTECTIVE EFFECT AND PLATELET AGGREGATION ACTIVITY FROM THE FLOWERS OF *CAMELLIA JAPONICA*: REVISED STRUCTURES OF CAMELLENODIOL AND CAMELLEDIONOL

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Abstract — The methanolic extract from the flowers of *Camellia japonica* was found to exhibit potent gastroprotective effect on ethanol-induced gastric mucosal lesions in rats and platelet aggregation activity. Through bioassay-guided separation, three new 28-noroleanane-type triterpene saponins, camelliosides A, B, and C, and an oleanane-type triterpene saponin, camellioside D, were isolated from the methanolic extract. The absolute stereostructures of camelliosides A—D were determined on the basis of physicochemical and chemical evidence, which included the structure revision of the triterpene part, camellenodiol and camelledionol, using X-Ray crystallographic analysis and modified Mosher's method. Camelliosides A and B showed gastroprotective and platelet aggregating effects.

The flowers of *Camellia japonica* L. (Theaceae, Japanese name "Tsubaki") have been used for treatment of blood vomiting and bleeding due to internal and external injury, and also as an antiinflammatory, tonic, and stomatic in Japanese folk medicine and Chinese traditional medicine. As chemical constituents of this natural medicine, noroleanane-type triterpene, camellenodiol, comelledionol, and maragenin II, were reported,[1] but the constituents responsible for the traditional medicinal uses have not been identified. During the course of our chemical and pharmacological studies on the bioactive saponins and glycosides,[2] we have already reported the isolation and structure elucidation of acylated polyhydroxytriterpene oligoglycosides, camelliasaponins A_1, A_2, B_1, B_2, C_1, and C_2, from the seeds of *C. japonica* and these saponins were found to inhibit alcohol absorption in rats.[3] As a continuing study, we found that the MeOH extract from the flowers of *C. japonica* showed potent gastroprotective effect on ethanol-induced gastric mucosal lesions in rats and platelet aggregation activity. Through bioassay-guided separation, we isolated three new 28-noroleanane-type triterpene saponins, camelliosides A (**1**, 0.10%, from the natural medicine), B (**2**, 0.067%), and C (**6**, 0.0052%), and an oleanane-type triterpene saponin, camellioside D (**8**, 0.0023%), together with oleanolic acid (0.0006%),[4] benzyl β-D-glucopyranoside (0.0004%),[5] methyl gallate (0.0003%),[4] (+)-catechin (0.0011%),[4] and (–)-epicatechin (0.038%).[4] This communication deals with the structure elucidation of **1**, **2**, **6** and **8**, which included the structure reinvestigation of the triterpene part, camellenodiol (**3**) and camelledionol (**4**). In addition, we describe the effects of principal saponins on gastric lesions induced by ethanol in rats and the effects on rabbit platelet aggregation.

Camellioside A (**1**),[6] colorless fine crystals from aqueous MeOH, mp 226—229°C, $[\alpha]_D^{26}$ –32.1° (c=1.8, pyridine), $C_{53}H_{84}O_{24}$, showed absorption bands ascribable to hydroxyl, carbonyl, olefin, and ether functions [IR (KBr): 3453, 2962, 1736, 1719, 1656, 1078 cm^{-1}]. Acid hydrolysis of **1** liberated D-glucuronic acid, D-galactose, and D-glucose, which were identified by GLC analysis of their trimethylsilyl thiazolidine derivatives.[7] Treatment of **1** with glycyrrhizinic acid hydrolase in 1.0 M acetate buffer (pH 4.4, 44°C) gave camellenodiol (**3**),[1] which was transformed to the 3-*O*-acetate (**3a**) and camelledionol (**4**)[1] by acetylation and oxidation, respectively. Since there has been considerable doubt as to the position of the

tertiary hydroxyl group in the previous formula (**3'**) from biogenetic consideration, the structure of camellenodiol was reinvestigated. On the basis of the homo-correlation spectroscopy (H—H COSY) and heteronuclear multiple bond correlation (HMBC) experiments on **3** and **3a** and finally X-Ray crystallographic analysis[8] of **3** as shown in Figure 1, the stereostructure of camellenodiol was revised from **3'** to **3** and consequently, the structure of camelledionol (**4**) was also clarified. Furthermore, their absolute stereostructures were confirmed by the application of modified Mosher's method to the (*R*)-MTPA (**3b**) and (*S*)-MTPA (**3c**), which were prepared by the treatment of **3** with (*R*)- or (*S*)-MTPA in the presence of EDC·HCl and DMAP in CH_2Cl_2 (Figure 1).[9] The ^{1}H-NMR and ^{13}C-NMR (Table 1) spectra of **1**, which were assigned be various NMR analytical methods,[10] showed signals due to a camellenodiol moiety, a β-D-glucuronic acid moiety, two β-D-galactopyranosyl moieties, and a β-D-glucopyranosyl moiety. The tetrasaccharide structure bonding to the 3-position of **3** was characterized by HMBC experiment, which showed long-range correlations between the following protons and carbons; 1'-H and 3-C; 1''-H and 2'-C; 1'''-H and 3'-C; 1''''-H and 2'''-C (Figure 1). On the basis of above-mentioned evidence, the absolute stereostructure of **1** was elucidated as shown.

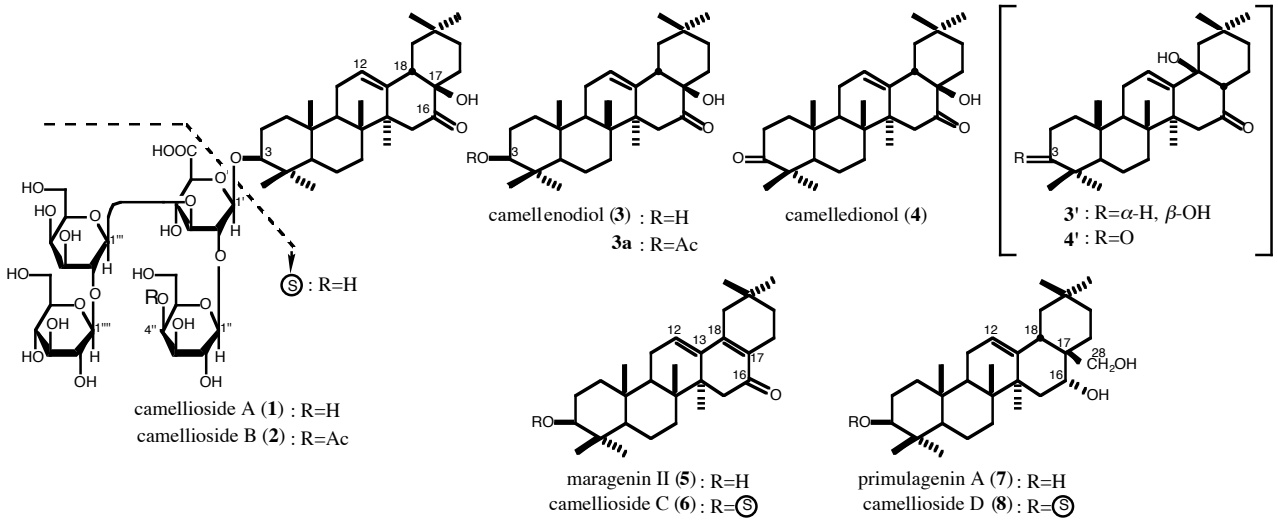

Chart 1

The ^{1}H-NMR and ^{13}C-NMR (Table 1) spectra of camellioside B (**2**)[11] were superimposable on those of **1**, except for the signals due to the acetyl group [δ 2.13 (s), δc 21.2 (q), 170.9 (s)]. Treatment of **2** with 0.1% NaOMe-MeOH liberated **1**, and HMBC experiment on **2** showed long-range correlation between the 4''-proton [δ: 5.93 (br s, 4''-H)] and acetyl carbonyl-carbon. Consequently, the structure of camellioside B (**2**) was determined as the 4''-acetyl

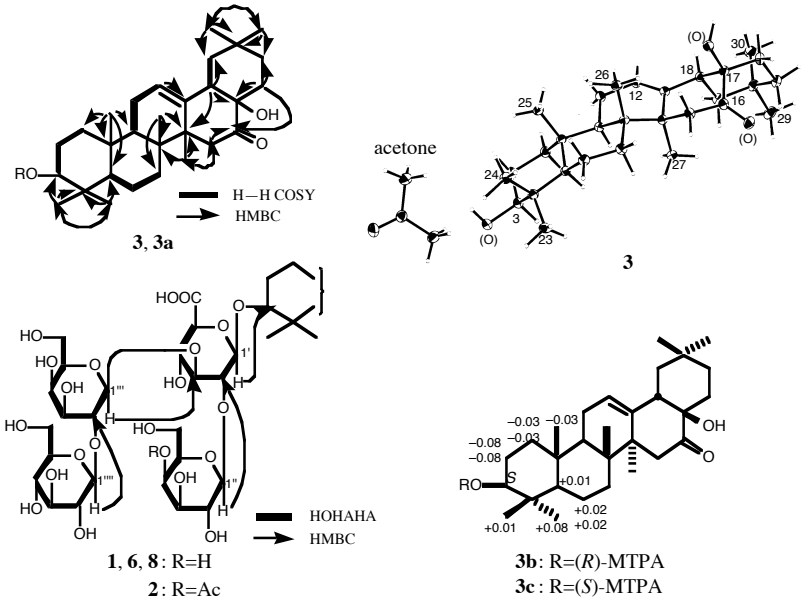

Figure 1. HMBC Correlations, X-Ray Crystallographic analysis, and Modified Mosher's Method

Camellioside C (**6**)[12] liberated maragenin II (**5**)[13] on enzymatic hydrolysis with glycyrrhizinic acid hydrolase, while D-glucuronic acid, D-galactose, and D-glucose were obtained on acid hydrolysis.[7] The ^{1}H-NMR and ^{13}C-NMR (Table 1) spectra of **6** showed signals assignable to a meragenin II part together with a β-D-glucuronic acid, two β-D-galactopyranosyl, and a β-D-glucopyranosyl moieties. The oligoglycoside structure of **6** was elucidated by HMBC experiment as shown in Figure 1. On the basis of this evidence, the structure of camellioside C (**6**) was determined as shown.

Camellioside D (**8**)[14] liberated primulagenin A (**7**)[15] on the enzymatic hydrolysis. The component monosaccharides of **8** were clarified to be same as those of **1**, **2**, and **6** by acid hydrolysis. The proton and carbon signals due to the tetraglycoside moiety in the ^{1}H-NMR and ^{13}C-NMR (Table 1) spectra of **8** were found to be superimposable on those of **1** and **6**. The oligoglycoside structure of **8** was confirmed by HMBC experiment (Figure 1). This evidence led us to determine the structure of camellioside D (**8**) to be as shown.

Table 1. ^{13}C-NMR Data for Camelliosides A (**1**), B (**2**), C (**6**), and D (**8**)

	1	**2**	**6**	**8**		**1**	**2**	**6**	**8**
C-1	38.5	38.7	38.9	38.9	GlcA-1'	105.5	105.8	105.6	105.7
C-2	26.4	26.7	26.6	26.6	-2'	79.0	78.5	79.1	79.3
C-3	89.5	89.9	89.5	89.5	-3'	83.7	84.8	84.0	84.6
C-4	39.4	39.8	39.6	39.6	-4'	70.9	71.2	71.0	71.1
C-5	55.7	55.9	55.8	55.9	-5'	76.9	77.1	77.0	77.0
C-6	18.3	18.6	18.4	18.6	-6'	171.9	171.7	171.7	171.8
C-7	33.2	33.5	33.8	33.3					
C-8	40.1	40.3	39.0	40.1	Gal-1''	103.0	102.8	103.1	103.3
C-9	46.9	47.1	46.3	47.1	-2''	73.7	73.2	73.9	74.0
C-10	36.8	37.1	36.8	36.9	-3''	74.7	74.0	74.9	75.0
C-11	23.8	24.1	24.4	23.9	-4''	69.8	72.0	69.9	70.0
C-12	124.1	124.2	127.3	122.4	-5''	76.3	75.1	76.4	76.4
C-13	142.3	142.6	139.2	145.2	-6''	61.8	61.4	61.9	61.8
C-14	48.1	48.4	45.1	42.1					
C-15	43.3	43.5	40.4	34.9	4''-Ac		21.2		
C-16	214.9	214.8	199.1	74.3			170.9		
C-17	76.3	76.5	129.0	41.1					
C-18	52.7	53.0	146.3	42.6	Gal-1'''	101.4	101.9	101.6	101.8
C-19	48.2	48.3	44.5	48.4	-2'''	83.0	83.5	83.2	83.5
C-20	30.9	31.1	29.2	31.3	-3'''	74.7	75.1	74.9	75.0
C-21	37.2	37.4	34.7	37.2	-4'''	69.8	69.9	69.9	69.9
C-22	31.5	31.8	21.3	30.4	-5'''	76.3	76.5	76.4	76.4
C-23	28.1	28.2	28.1	28.2	-6'''	61.9	62.5	62.1	62.1
C-24	16.7	16.9	16.9	16.9					
C-25	15.3	15.5	15.6	15.8	Glc-1''''	106.2	106.8	106.5	106.7
C-26	17.7	17.9	18.0	17.1	-2''''	75.8	76.2	76.0	76.1
C-27	27.0	27.3	23.3	27.4	-3''''	78.1	78.5	78.3	78.4
C-28				70.2	-4''''	71.0	71.4	71.2	71.2
C-29	32.6	32.9	28.6	33.5	-5''''	78.2	78.5	78.4	78.5
C-30	23.6	23.9	28.2	24.9	-6''''	62.2	61.8	62.4	62.4

Measured in C_5D_5N at 68 MHz.

GlcA: β-D-glucopyranosiduronic acid; Gal: β-D-galactopyranosyl; Glc: β-D-glucopyranosyl

The MeOH extract was found to cause potent protective activity against ethanol-induced gastric lesions in rats[16] after a single oral administration of 200 mg/kg dose. Among the fractions from the MeOH extract, the *n*-BuOH-soluble fraction (saponin fraction) showed the gastroprotective effect at a lower dose. The principal saponins, camelliosides A (**1**) and B (**2**), from the *n*-BuOH-soluble fraction inhibited the gastric lesions in a dose-dependent manner (5—20 mg/kg) and their inhibitory effects were stronger than that of a reference compound, omeprazole.

Table 2. Effects of the MeOH Extract, AcOEt-, *n*-BuOH, and H_2O-Soluble Fractions from the Flowers of *C. japonica*, Camelliosides A (**1**) and B (**2**), and Omeprazole on Gastric Lesions Induced by EtOH in Rats

Treatment	Dose (mg/kg, p.o.)	*N*	Gastric lesions	
			Lesion index (mm)	Inhibition (%)
Control	—	12	115.8±8.7	—
MeOH ext.	100	8	71.3±12.6	38.4
	200	8	11.0±5.3**	90.5
AcOEt-soluble fraction	50	8	101.8±27.1	12.1
n-BuOH-soluble fraction	25	8	58.7±15.8**	49.3
	50	8	37.6±13.0**	67.6
H_2O-soluble fraction	50	8	149.6±12.1	–29.2
Omeprazole	50	8	9.5±6.1**	91.8
Control	—	9	141.4±9.0	—
Camellioside A (**1**)	5	6	93.8±13.3	33.7
	10	6	85.1±30.2	39.8
	20	6	32.0±10.6**	77.3
Camellioside B (**2**)	5	6	92.1±20.0	34.9
	10	6	63.1±20.6**	55.4
	20	6	11.6±3.1**	91.8
Control	—	7	119.1±10.1	—
Omeprazole	20	7	57.8±12.7**	51.4
	50	6	5.5±3.0**	95.4

Each value represents the mean±S.E.
Significantly different from the control group, *p>0.05, **p>0.01.

Furthermore, we examined the rabbit platelet aggregation *in vitro*. As a result, **1** and **2** concentration-dependently caused the aggregation of the platelets (10—100 μg/mL) (Table 3).[17,18] The gastroprotective and platelet aggregating effects of this natural medicine and the principle saponin constituents may be important evidence substantiating the traditional effect of this natural medicine such as the treatment effects of blood vomiting and bleeding and antiinflammatory and stomatic effects.

Table 3. Platelet Aggregating Activities of Camelliosides A (**1**) and B (**2**)

Treatment	Concentration (μg/mL)	Aggregation (% of control)
Camellioside A (**1**)	1	15
	10	16
	50	47
	100	83
Camellioside B (**2**)	1	17
	10	13
	50	43
	100	104

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4 These constituents were identified by comparison of their physical data with those of authentic samples.

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6 **1**: High-resolution positive-ion FAB-MS: Calcd for $C_{53}H_{84}O_{24}Na$ $(M+Na)^+$: 1127.5250. Found: 1127.5244. ^{1}H-NMR (C_5D_5N) δ: 0.87, 0.90, 0.98, 1.10, 1.27, 1.34, 1.37 (all s, 25, 29, 30, 24, 26, 23, and 27-H_3), 3.10 (dd, *J*=3.5, 14.5 Hz, 18-H), 3.27 (dd, *J*=4.5, 11.8 Hz, 3-H), 4.90 (d, *J*=7.4 Hz, 1'-H), 5.18 (d, *J*=7.7 Hz, 1''''-H), 5.49 (br s, 12-H), 5.70 (d, *J*=7.6 Hz, 1'''-H), 5.76 (d, *J*=7.6 Hz, 1''-H). Positive-ion FAB-MS: *m/z* 1127 $(M+Na)^+$. Negative-ion FAB-MS: *m/z* 1103 $(M-H)^-$, 941 $(M-C_6H_{11}O_5)^-$, 779 $(M-C_{12}H_{21}O_{10})^-$.

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8 Crystal data for **3**: $C_{29}H_{46}O_3\cdot(CH_3)_2CO$, crystal dimensions: 0.25 × 0.18 × 0.30 mm; crystal system: orthorhombic; lattice type: primitive; lattice parameters: a=13.237(2) Å, b=28.911(2) Å, c=7.649(2) Å, V=2927.2(8) Å; space group: $P2_12_12_1$ (#19); Z value: 4; D_{calc}: 1.136 g/cm^3; F_{000}: 1104.00; μ(CuKα): 5.65 cm^{-1}; diffractometer: Rigaku AFC7R (rotating anode); radiation: CuKα (λ=1.54178 Å) graphite monochromated; structure solution: TEXSAN (direct methods: SAPI91); residuals: R=0.081, Rw=0.140, R1=0.046; goodness of fit indicator: 1.38.

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10 The ^{1}H- and ^{13}C-NMR spectra of new compounds were assigned on the basis of homo- and hetero-correlation spectroscopy (^{1}H–^{1}H, ^{13}C–^{1}H COSY), homo- and hetero-nuclear Hartmamm-Hahn spectroscopy (^{1}H–^{1}H, ^{13}C–^{1}H HOHAHA) and heteronuclear multiple bond correlation (HMBC) experiments.

11 **2**: colorless fine crystals from aqueous MeOH, mp 221—224°C, $[\alpha]_D^{23}$ –30.0° (*c*=0.8, pyridine). High-resolution positive-ion FAB-MS: Calcd for $C_{55}H_{86}O_{25}Na$ $(M+Na)^+$: 1169.5356. Found: 1169.5352. IR (KBr): 3453, 2962, 1744, 1726, 1655, 1380, 1078 cm^{-1}. ^{1}H-NMR (C_5D_5N) δ: 0.87, 0.90, 0.98, 1.10, 1.27, 1.34, 1.37 (all s, 25, 29, 30, 24, 26, 27, and 23-H_3), 2.13 (s, –OCOCH_3), 3.08 (dd-like, 18-H), 3.30 (dd, *J*=3.5, 11.0 Hz, 3-H), 4.89 (d, *J*=7.6 Hz, 1'-H), 5.16 (d, *J*=7.6 Hz, 1''''-H), 5.45 (br s, 12-H), 5.70 (d, *J*=7.6 Hz, 1'''-H), 5.87 (d-like, 1''-H). Positive-ion FAB-MS: *m/z* 1169 $(M+Na)^+$. Negative-ion FAB-MS: *m/z* 1145 $(M-H)^-$, 983 $(M-C_6H_{11}O_5)^-$, 941 $(M-C_8H_{13}O_6)^-$, 821 $(M-C_{12}H_{21}O_{10})^-$.

12 **6**: colorless fine crystals from aqueous MeOH, mp 224—227°C, $[\alpha]_D^{26}$ –15.8° (*c*=0.1, MeOH). High-resolution positive-ion FAB-MS: Calcd for $C_{53}H_{82}O_{23}Na$ $(M+Na)^+$: 1109.5145. Found: 1109.5137. UV [MeOH, nm, (log ε)]: 299 (4.0). IR (KBr): 3453, 2926, 1736, 1665, 1655, 1078 cm^{-1}. ^{1}H-NMR (C_5D_5N) δ: 0.84, 0.87, 0.88, 0.90, 1.10, 1.20, 1.30 (all s, 25, 26, 30, 29, 24, 27, and 23-H_3), 3.30 (dd, *J*=4.5, 11.7 Hz, 3-H), 4.90 (d, *J*=7.4 Hz, 1'-H), 5.17 (d, *J*=8.1 Hz, 1''''-H), 5.69 (d, *J*=7.6 Hz, 1'''-H), 5.76 (d, *J*=7.7 Hz, 1''-H), 6.07 (br s, 12-H). Positive-ion FAB-MS: *m/z* 1109 $(M+Na)^+$, 1087 $(M+H)^+$. Negative-ion FAB-MS: *m/z* 1085 $(M-H)^-$, 923 $(M-C_6H_{11}O_5)^-$, 761 $(M-C_{12}H_{21}O_{10})^-$.

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14 **8**: colorless fine crystals from aqueous MeOH, mp 222—225°C, $[\alpha]_D^{26}$ –3.2° (*c*=0.1, MeOH). High-resolution positive-ion FAB-MS: Calcd for $C_{54}H_{88}O_{24}Na$ $(M+Na)^+$: 1143.5563. Found: 1143.5538. IR (KBr): 3453, 2926, 1719, 1655, 1078 cm^{-1}. ^{1}H-NMR (C_5D_5N) δ: 0.85, 0.94, 1.05, 1.09, 1.12, 1.30, 1.81 (all s, 25, 26, 29, 24, 30, 23, and 27-H_3), 2.47 (dd-like, 18-H), 3.32 (dd, *J*=4.3, 11.9 Hz, 3-H), 4.59 (br s, 16-H), 4.92 (d, *J*=7.3 Hz, 1'-H), 5.18 (d, *J*=7.9 Hz, 1''''-H), 5.37 (br s, 12-H), 5.64 (d, *J*=7.6 Hz, 1'''-H), 5.76 (d, *J*=7.6 Hz, 1''-H). Positive-ion FAB-MS: *m/z* 1143 $(M+Na)^+$. Negative-ion FAB-MS: *m/z* 1119 $(M-H)^-$, 957 $(M-C_6H_{11}O_5)^-$, 795 $(M-C_{12}H_{21}O_{10})^-$.

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18 Wash rabbit platelets were prepared as described previously.[17] The platelets suspension (5 × 10^5 cells/μL) in 225 μL of an assay buffer (137 mM NaCl, 2.7 mM KCl, 3.8 mM HEPES, pH 7.4) and 25 μL of 12 mM $CaCl_2$/saline were mixed and incubated at 37ºC for 1 min. One μL of test sample dissolved in DMSO was added to 24 μL of the assay buffer, and the solution was dropped into the platelets suspension with stirring. Aggregation responses were recorded by an aggregometer (Hema Tracer 1, Nikko Bioscience, Japan). The aggregation (%) was calibrated with the platelets suspension (0% transmission) and the assay buffer (100% transmission).