Medicinal Flowers. XXXIII.[1)] Anti-hyperlipidemic and Anti-hyperglycemic Effects of Chakasaponins I–III and Structure of Chakasaponin IV from Flower Buds of Chinese Tea Plant (*Camellia sinensis*)

Hisashi Matsuda, Makoto Hamao, Seikou Nakamura, Haruka Kon'i, Megumi Murata, and Masayuki Yoshikawa*

Kyoto Pharmaceutical University; Misasagi, Yamashina-ku, Kyoto 607–8412, Japan.
Received December 28, 2011; accepted February 10, 2012; published online February 27, 2012

Effects of principal saponins, chakasaponins I–III, from the flower buds of *Camellia sinensis* cultivated in Fujian province, China on plasma triglyceride (TG) and glucose levels in olive oil or sucrose-loaded mice were examined. Chakasaponins I–III at 50 and 100 mg/kg significantly inhibited increases in plasma TG and glucose levels. Furthermore, they prevented gastric emptying, suggesting that the former inhibitory effect is partly dependent on the inhibition of gastric emptying. In addition, the chemical structure of a new acylated oleanane-type triterpene oligoglycoside, chakasaponin IV, was elucidated on the basis of chemical and physicochemical evidence.

Key words chakasaponin; *Camellia sinensis*; medicinal flower; tea plant; anti-hyperlipidemic effect; anti-hyperglydemic effect

Tea flowers, the flower buds of *Camellia* (*C.*) *sinensis* (L.) O. KUNTZE, are used as a food garnish in Japanese-style dishes (*e.g.*, botebote-cha in Shimane prefecture) or drinks in Japan (*e.g.*, hanaban-cha in Shimane and Kouchi prefectures or batabata-cha in Niigata prefecture). However, the chemical constituents and biological activities of tea flowers have not yet been characterized. Previously, we reported the isolation and structural elucidation of floratheasaponins A–C from Japanese tea flowers, the flower buds of Japanese *C. sinensis*.[2)] Floratheasaponins A–C were found to have inhibitory effects on serum triglyceride (TG) levels in olive oil-loaded mice,[2)] on ethanol- and indomethacin-induced gastric mucosal lesions in rats,[3)] and on serum glucose levels in sucrose-loaded rats.[3)] From the flower buds of Chinese tea plants cultivated in Anhui province, floratheasaponins D–I were isolated together with floratheasaponins A–C.[4)] The principal saponins, floratheasaponins A–F, were found to exhibit inhibitory effects on the release of β-hexosaminidase from RBL-2H3 cells.[4)] In the course of our studies on the saponin compositions of tea flowers cultivated in various provinces of China, we found that floratheasaponins were not contained in the tea flowers cultivated in Fujian province, from which chakasaponins I (**2**), II (**3**), III (**4**), V, and VI were isolated and their structures were determined.[5,6)] Previously, we reported inhibitory effects of chakasaponins I–III (**2**–**4**) against pancreatic lipase (*in vitro*),[5)] but no anti-hyperglycemic effects and anti-hyper glycemic effects have been reported. In this paper, we describe the effects of principal saponins (**2**–**4**) and a principal flavonol glycoside, kaempferol 3-*O*-β-D-glucopyranosyl(1→3)-α-L-rhamnopyranosyl(1→6)-β-D-glucopyranoside (**5**), on increases in plasma triglyceride and glucose levels in olive oil- or sucrose-loaded mice together with their effects on gastric emptying in mice, rat intestinal α-glucosidase (*in vitro*) and rat lens aldose reductase (*in vitro*). In addition, continuing on the structural elucidation of chakasaponins,[5,6)] the isolation and structural elucidation of a new acylated oleanane-type triterpene oligoglycoside termed chakasaponin IV (**1**) from tea flowers cultivated in Fujian province are described.

Isolation of Constituents The methanolic extract (31.1% from the dried flower buds of *C. sinensis* cultivated in Fujian province, China) was partitioned into an EtOAc–H_2O (1 : 1, v/v) mixture to furnish an EtOAc-soluble fraction (3.2%) and aqueous layer. The aqueous layer was further extracted with *n*-butanol (*n*-BuOH) to give *n*-BuOH (16.4%)- and H_2O (11.5%)-soluble fractions as reported previously.[5)] The EtOAc- and *n*-BuOH-soluble fractions were subjected to normal-phase and reversed-phase silica gel column chromatographies and repeated HPLC to give chakasaponin IV (**1**, 0.10%) together with chakasaponins I (**2**, 0.49%),[5)] II (**3**, 0.67%),[5)] and III (**4**, 0.013%),[5)] and kaempferol 3-*O*-β-D-glucopyranosyl(1→3)-α-L-rhamnopyranosyl(1→6)-β-D-glucopyranoside (**5**, 0.018%).[5,7)]

Structure of Chakasaponin IV Chakasaponin IV (**1**) was isolated as colorless fine crystals with mp. 204.0–206.0°C (from $CHCl_3$–MeOH) and exhibited a positive optical rotation ($[\alpha]_D^{25}$ +24.6° in MeOH). Its IR spectrum showed absorption bands at 1718 and 1686 cm^{-1} ascribable to two carbonyl moieties and broad bands at 3450 and 1078 cm^{-1} suggestive of an oligoglycoside structure. Positive-ion fast atom bombardment (FAB)-MS revealed a quasimolecular ion peak at *m*/*z* 1197 $(M+Na)^+$, high-resolution (HR) FAB-MS of which revealed the molecular formula $C_{57}H_{90}O_{25}$. The ^{1}H-NMR (pyridine-d_5) and ^{13}C-NMR (Table 1) spectra of **1**, which were assigned based on results of various NMR experiments,[8)] showed signals assignable to an aglycone {seven methyls [δ 0.80, 1.00, 1.05, 1.11, 1.24, 1.26, 1.85 (3H each, all s, H_3-25, 26, 29, 24, 23, 30, 27)], a methylene and four methines with an oxygen function [δ 3.25 (1H, dd, J=3.8, 13.2 Hz, H-3), 3.60, 3.76 (1H each, both d, J=10.6 Hz, H_2-28), 4.22 (1H, d, J=4.0 Hz, H-15), 4.56 (1H, d, J=4.0 Hz, H-16), 6.16 (1H, dd, J=5.5, 11.8 Hz, H-22)], an olefinic proton [δ 5.44 (1H, br s, H-12)], and a tigloyl moiety [δ 1.41, (3H, d, J=7.0 Hz, H_3-22-*O*-Tig-4), 1.76 (3H, br s, H_3-22-*O*-Tig-5), 6.83 (1H, dq-like, H-22-*O*-Tig-3)] and four glycopyranosyl parts [δ 4.92 (1H, d, J=7.2 Hz, H-1′), 5.01 (1H, d, J=7.4 Hz, H-1‴), 5.74 (1H, d, J=7.6 Hz, H-1″), 5.77 (1H, d,

The authors declare no conflict of interest.

* To whom correspondence should be addressed. e-mail: myoshika@mb.kyoto-phu.ac.jp

Table 1. ^{13}C-NMR (125 MHz) Data for Chakasaponins IV (**1**) and **1a**

C-	**1**	**1a**	C-	**1**	**1a**
1	38.9	38.9	22-*O*-Tig		
2	26.5	26.5	1	167.8	
3	89.4	89.5	2	129.8	
4	39.5	39.5	3	136.6	
5	55.5	55.5	4	14.0	
6	18.7	18.7	5	12.2	
7	36.9	36.4	GlcA		
8	41.4	41.4	1′	105.6	105.5
9	47.1	47.1	2′	79.1	79.0
10	36.7	36.9	3′	84.0	83.9
11	23.9	23.9	4′	71.1	71.1
12	124.7	124.8	5′	76.4	76.4
13	144.5	145.0	6′	172.1	172.2
14	47.7	48.1	Gal		
15	67.5	67.3	1″	103.5	103.4
16	73.8	73.7	2″	73.3	73.3
17	45.4	44.9	3″	75.1	75.1
18	41.6	41.4	4″	70.0	70.0
19	46.9	47.3	5″	75.7	75.7
20	31.9	31.7	6″	61.9	61.9
21	41.4	45.5	Ara		
22	74.0	74.2	1‴	101.7	101.7
23	27.9	27.9	2‴	81.9	82.0
24	16.8	16.8	3‴	72.7	72.7
25	15.8	15.8	4‴	68.2	68.3
26	17.5	17.4	5‴	65.8	65.8
27	21.3	21.3	Xyl		
28	62.9	69.4	1⁗	106.8	106.8
29	33.5	33.7	2⁗	75.7	75.7
30	25.1	25.4	3⁗	78.2	78.2
			4⁗	70.7	70.7
			5⁗	67.4	67.4

Measured in pyridine-d_5.

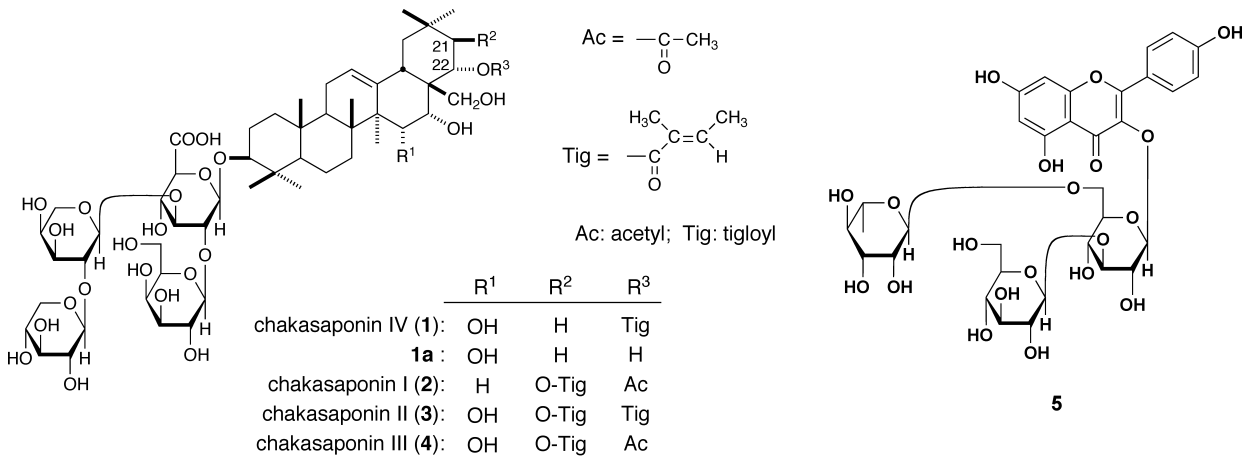

Chart 1. Chemical Structures of **1**–**5**

J=5.4 Hz H-1″)]. Treatment of **1** with 10% aqueous KOH–50% aqueous 1,4-dioxane (1 : 1) liberated desacyl-chakasaponin IV (**1a**) and an organic acid, tiglic acid, which was identified by HPLC analysis of the *p*-nitrobenzyl derivative.[9,10] The molecular formula of **1a** was determined as $C_{52}H_{84}O_{24}$ from positive-ion FAB-MS [*m/z* 1115 $(M+Na)^+$]. The ^{1}H-NMR (pyridine-δ_5) and ^{13}C-NMR (Table 1) spectra of **1a**,[8] showed signals assignable to an aglycone [δ: 0.80, 1.01, 1.06, 1.12, 1.13, 1.25, 1.85 (3H each, all s, H_3-25, 26, 29, 24, 30, 23, 27), 3.23 (1H, m, H-3), 3.71, 4.13 (1H each, both d, J=11.0 Hz, H_2-28), 4.45 (1H, m, H-15), 4.47 (1H, m, H-16), 4.62 (1H, m, H-22), 5.42 (1H, brs, H-12)] and four glycopyranosyl parts. The position of the tigloyl group in **1** was characterized on the basis of an heteronuclear multiple bond connectivity (HMBC)

experiment, in which a long-range correlation was observed between the 22-proton and tigloyl carbonyl carbon. In addition, comparison of the ^{13}C-NMR data for **1** with those for **1a** revealed acylation shifts around the 21- and 22-positions. The oligoglycoside structure at the 3-position in **1** and **1a** was elucidated by a HMBC experiment, which showed long-range correlations between the following proton and carbons: H-1′ and 3-C; H-1″ and C-2′; H-1‴ and C-3′; H-1⁗ and C-2‴. Finally, acid hydrolysis of **1a** with 5% aqueous H_2SO_4–1,4-dioxane (1 : 1, v/v) yielded a known triterpene, A_1-barrigenol,[11] as an aglycone, together with L-arabinose, D-galactose, D-glucuronic acid, and D-xylose, which were identified by GLC analysis of their trimethylsilyl thiazolidine derivatives.[12,13] On the basis of those findings, the structure of chakasaponin IV was determined to be 22-*O*-tigloyl-A_1-barrigenol 3-*O*-[*β*-D-galactopyranosyl(1→2)][*β*-D-xylopyranosyl(1→2)-*α*-L-arabinopyranosyl(1→3)]-*β*-D-glucopyranosiduronic acid (**1**).

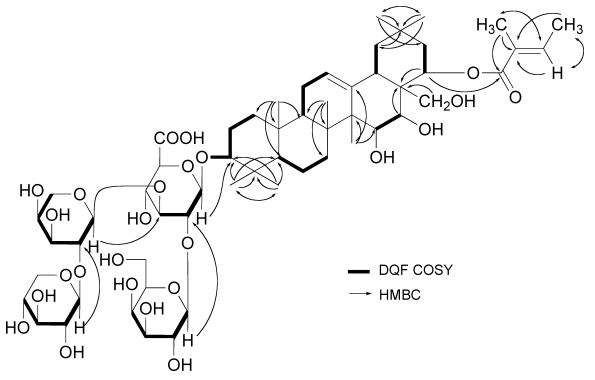

Fig. 1. DQF COSY and HMBC Correlations of **1**

Effects of 2–5 on TG Levels after Loading of Olive Oil in Mice Effects of chakasaponins (**2**–**4**) and a flavonol glycoside (**5**) on the increase in plasma triglyceride (TG) levels in olive oil-loaded mice were examined. As shown in Table 2, the chakasaponins significantly inhibited the increase at 25 and/or 50 mg/kg (*per os* (*p.o.*)), but flavonol glycoside (**5**) did not. Previously, escins from the seeds of *Aesculus turbinata* were found to have anti-hyperlipidemic effects in lipid emulsion-loaded mice.[14] The inhibitory effect of the chakasaponins in the present study was similar to that of escin IIa.

Effects of 2–5 on Glucose Levels after Loading of Sucrose in Mice Effects of **2**–**5** on the increase in plasma glucose levels in sucrose-loaded mice were examined. As shown in Table 3, the chakasaponins significantly inhibited the increase at 50 and 100 mg/kg. (*p.o.*), but the flavonol glycoside (**5**) did not. We previously reported that escin IIa inhibited the increase in glucose levels in glucose-loaded rats.[15,16] In the present study, the inhibitory effect of the chakasaponins tended to be stronger than that of escin IIa.

Effects of 2–5 on Gastric Emptying (GE) in Mice Previously, we reported that the inhibition of gastric emptying markedly reduced increase in blood glucose and TG levels in sugar and olive oil-loaded rats and mice.[16–20] Additionally, inhibitory effects on GE by chakasaponins I (**2**) and II (**3**),[21] but not **4** and **5**, on GE were reported. Therefore, effects of **2–5** on GE in mice were compared. As shown in Table 4, the chakasaponins significantly prevented GE at 50–100 mg/kg (*p.o.*) 30 min after the loading of a carboxymethyl cellulose

Table 2. Effects of Chakasaponins I–III (**2**–**4**) and Kaempferol 3-*O*-Glc(1→3)-Rha(1→6)-Glc (**5**) on Increases in Plasma TG Levels in Olive Oil-Loaded Mice

Treatment	Dose (mg/kg, *p.o.*)	*n*	Plasma TG (mg/100 mL)		
			2.0 h	4.0 h	6.0 h
Normal	—	6	115.5±12.4**	126.7±9.0**	122.9±10.4**
Control	—	8	440.5±45.2	359.4±43.2	267.8±37.8
Chakasaponin I (**2**)	25	6	435.7±67.4	296.8±45.2	197.7±26.6
	50	6	284.2±9.6**	347.5±27.2	257.7±34.4
Normal	—	6	152.4±13.5**	149.8±15.0**	118.5±14.7*
Control	—	8	553.8±49.8	522.7±44.0	259.8±50.3
Chakasaponin II (**3**)	25	6	431.8±49.8**	436.9±63.3	240.2±24.1
	50	6	249.5±31.1**	416.7±71.2	390.0±73.9
Normal	—	6	124.0±8.4**	94.7±8.3**	87.0±11.3**
Control	—	8	407.2±73.0	385.1±71.4	207.8±36.1
Chakasaponin III (**4**)	25	6	394.1±81.4	300.5±67.2	184.8±36.8
	50	6	214.4±62.7*	314.3±88.2	255.5±60.0
Normal	—	6	164.8±20.1**	128.0±13.5**	103.2±12.0*
Control	—	8	418.1±54.5	398.6±42.1	231.6±44.0
5	25	6	469.5±86.6	395.5±114.9	213.6±64.1
	50	6	383.9±41.8	444.6±46.7	191.4±33.3
Normal	—	7	91.9±9.4**	97.3±7.4**	90.6±9.4**
Control	—	9	440.3±60.2	393.2±60.1	263.3±45.0
Orlistat	5	7	371.3±41.5	297.0±67.4	171.9±24.9
	10	7	203.8±52.1**	160.4±47.7**	129.1±16.6**
Normal	—	6	114.9±18.1**	110.4±12.0**	134.8±7.7*
Control	—	8	546.7±59.4	375.9±66.2	271.5±62.5
Escin IIa	50	8	464.2±45.3	244.5±38.5	310.9±91.5
	100	8	308.9±61.4*	285.8±46.9	270.2±44.4

Values represent the mean±S.E.M. Significantly different from the control group, *$p<0.05$, **$p<0.01$.

Table 3. Effects of Chakasaponins I–III (**2**–**4**) and Kaempferol 3-*O*- Glc(1→3)-Rha(1→6)-Glc (**5**) on Increases in Plasma Glucose Levels in Sucrose-Loaded Mice

Treatment	Dose (mg/kg, *p.o.*)	*n*	Plasma glucose (mg/100 mL)		
			0.5 h	1.0 h	2.0 h
Normal	—	6	128.5±5.3**	149.4±2.6**	121.7±5.3**
Control	—	8	226.9±8.2	194.2±6.2	144.3±3.0
Chakasaponin I (**2**)	50	6	199.6±8.7**	213.7±9.4	161.7±6.5
	100	6	196.8±8.2**	200.1±5.9	161.6±6.6
Normal	—	6	116.5±7.9**	132.7±10.2**	117.5±8.0**
Control	—	8	221.5±14.4	200.3±10.5	138.2±4.6
Chakasaponin II (**3**)	50	6	185.7±9.9**	179.3±5.3	148.4±2.4
	100	5	178.5±8.3**	187.3±10.7	150.9±3.6
Normal	—	6	126.5±4.0**	135.4±3.9**	124.2±3.9**
Control	—	9	238.7±5.9	207.3±9.7	141.2±3.5
Chakasaponin III (**4**)	50	6	215.9±10.6**	198.0±6.3	157.4±5.9*
	100	6	189.0±10.2**	187.6±6.8*	160.0±3.6**
Normal	—	6	124.1±8.7**	133.8±7.2**	125.9±6.6**
Control	—	8	241.7±13.4	217.5±10.3	167.2±3.9
5	50	6	270.5±16.1	207.9±11.4	157.9±7.8
	100	6	243.7±18.0	225.0±16.7	159.5±7.9
Normal	—	6	124.8±7.3**	143.0±5.4**	131.8±6.4**
Control	—	9	218.7±4.0	208.9±6.8	163.7±3.7
Acarbose	10	6	162.4±11.7**	183.8±3.8*	151.5±6.3
	20	6	153.8±10.2**	185.4±8.1*	152.8±3.8
Normal	—	6	122.2±6.3**	123.2±4.9**	112.9±5.1**
Control	—	9	263.3±16.2	235.1±10.7	168.9±7.6
Escin IIa	50	6	254.2±14.0	224.2±10.4	166.6±7.2
	100	6	206.4±12.9**	205.0±7.5**	175.9±11.1

Values represent the mean±S.E.M. Significantly different from the control group, *$p<0.05$, **$p<0.01$.

sodium salt (CMC-Na) solution, but the flavonol glycoside (**5**) had no such effect, similar to the results obtained with olive oil- and sucrose-loaded mice. We previously reported that escin IIa inhibited GE in mice and this contributed to the anti-hyperglycemic effect in glucose-loaded rats and mice.[16,22–24] In the present study, the inhibitory effect of the chakasaponins tended to be stronger than that of escin IIa. These findings suggest that the inhibition of GE is involved in the anti-hyperglycemic and anti-hyperlipidemic effects.

Effects of 2–5 on the Activities of Rat Intestinal α-Glucosidase and Rat Lens Aldose Reductase Next, inhibitory effects of **2**–**5** on the activities of rat intestinal α-glucosidase (maltase and sucrase) were examined, since **2**–**4** significantly reduced plasma glucose levels in sucrose-loaded mice. Compounds **2**–**5** did not inhibit the enzymatic activity less than 14.6% at 400 μM. Aldose reductase has been reported to catalyze the reduction of glucose to sorbitol as a key enzyme in the polyol pathway. Sorbitol does not readily diffuse across cell membranes, and its intracellular accumulation has been implicated in chronic complications of diabetes such as cataracts.[25] Therefore, the inhibitory effects of **2**–**5** on the activity of rat lens aldose reductase were examined. The chakasaponins (**2**–**4**) had little effect (inhibition at 100 μM: 3.0%, 5.1%, 18.0%, respectively), but **5** had a moderate effect with an IC_{50} value of 20.8 μM.

In conclusion, principal saponins, chakasaponins I–III (**2**–**4**) from the flower buds of *Camellia sinensis* cultivated in Fujian province, China significantly inhibited increases in plasma TG and glucose levels in olive oil or sucrose-loaded mice at 50 and 100 mg/kg, but did not effect the activity of rat intestinal α-glucosidase or rat lens aldose reductase. Furthermore, inhibition of GE was involved in the anti-hyperglycemic and anti-hyperlipidemic effects of **1**–**3**. In addition, the chemical structure of a new acylated oleanane-type triterpene

Table 4. Effects of Chakasaponins I–III (**2**–**4**) and Kaempferol 3-*O*-Glc(1→3)-Rha(1→6)-Glc (**5**) on Gastric Emptying in Mice

Treatment	Dose (mg/kg, *p.o.*)	*n*	Gastric emptying (%)
Control	—	9	75.0±4.1
Chakasaponin I (**2**)	25	7	68.5±5.0
	50	7	50.2±3.0**
	100	7	37.9±1.4**
Control	—	10	73.1±2.1
Chakasaponin II (**3**)	25	7	65.2±3.9
	50	7	56.9±2.7**
	100	7	40.5±6.7**
Control	—	10	76.8±2.4
Chakasaponin III (**4**)	25	7	70.7±1.9
	50	7	52.0±2.7**
	100	7	39.8±1.7**
Control	—	7	81.4±4.7
5	50	6	90.5±1.3
	100	6	87.6±2.0
Control		8	86.2±1.3
Escin IIa	25	6	81.7±6.5
	50	6	69.6±3.0*
	100	6	53.8±4.0**

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

oligoglycoside, chakasaponin IV, was elucidated on the basis of chemical and physicochemical evidence.

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, Shimadzu FTIR-8100 spectrometer; FAB-MS and high resolution (HR)-FAB-MS, a JEOL JMS-SX 102A mass spectrometer; ^{1}H-NMR spectra, JEOL JNM-LA 500 (500 MHz) and JEOL JNM-ECA 600 (600 MHz) spectrometers; ^{13}C-NMR spectra, JEOL JNM-LA (125 MHz) and JEOL JNM-ECA 600 (150 MHz) spectrometers with tetramethylsilane as an internal standard; and HPLC detector, Shimadzu RID-6A refractive index and SPD-10A*vp* UV-VIS detectors. HPLC column, 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, Japan} and Develosil C30-UG-5 {[250×4.6 mm i.d. (5 μm) for analytical purposes] and [250×20 mm i.d. (5 μm) for preparative purposes], Nomura Chemical, Japan} columns were used.

The following materials were used for chromatography: ordinary-phase silica gel column chromatography, Silica gel BW-200 (Fuji Silysia Chemical, Ltd., Japan, 150–350 mesh); reverse-phase silica gel column chromatography, Chromatorex ODS DM1020T (Fuji Silysia Chemical, Ltd., Japan, 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$_2$SO$_4$ followed by heating.

Plant Material The flower buds of *Camellia sinensis*, cultivated in Fujian province, China, were collected in 2006. The botanical identification was undertaken by one of the authors (M. Y.). A voucher of the plant is on file in our laboratory (2006. China-06F).

Isolation of Chakasaponin IV The dried flower buds of *C. sinensis* (1.5 kg, Fujian province, China) were extracted three times with MeOH (15 L×3) for three hours under reflux. Evaporation of the solvent under reduced pressure provided a MeOH extract (467 g, 31.1%). A portion (450 g) of the MeOH extract was partitioned into an EtOAc (2 L×3)/H$_2$O (2 L) mixture to furnish an EtOAc-soluble fraction (46 g, 3.2%) and aqueous phase, which was extracted with *n*-BuOH (2 L×3) to give *n*-BuOH (237 g, 16.4%)- and H$_2$O (166 g, 11.5%)-soluble fractions as reported previously.[5] A part of the *n*-BuOH-soluble fraction (142 g) was subjected to normal-phase silica gel column chromatography [3 kg, CHCl$_3$→CHCl$_3$: MeOH : H$_2$O (30 : 10 : 1→10 : 3 : 1→7 : 3 : 1→6 : 4 : 1)→MeOH] to give five fractions [Fr. 1, Fr. 2, Fr. 3, Fr. 4 (115.0 g), Fr. 5]. Fr. 4 (115 g) was subjected to reversed-phase silica gel column chromatography [530 g, MeOH : H$_2$O (10 : 90→30 : 70→50 : 50→70 : 30→MeOH)] to give nine fractions [Fr. 4-1, Fr. 4-2, Fr. 4-3, Fr. 4-4, Fr. 4-5, Fr. 4-6 (12.5 g), Fr. 4-7, Fr. 4-8, and Fr. 4-9]. Fraction 4-6 (12.5 g) was subjected to reversed-phase silica gel column chromatography [370 g, MeOH : H$_2$O (50 : 50→60 : 40→MeOH) to give five fractions [Fr. 4-6-1, Fr. 4-6-2, Fr. 4-6-3 (6.2 g), Fr. 4-6-4, Fr. 4-6-5]. A part of Fr. 4-6-3 (1.0 g) was further separated by HPLC [MeOH–H$_2$O (65 : 35, v/v)] to give chakasaponin IV (**1**, 14.2 mg).

The procedure used to isolate compounds **2**–**5** is described in ref. 5.

Chakasaponin IV (**1**): Colorless fine crystals from CHCl$_3$–MeOH; mp 204.0–206.0°C; $[\alpha]_D^{25}$ +24.6° (c=0.89, MeOH); IR (KBr) ν_{max} 3450, 2962, 1718, 1686, 1078 cm^{-1}; ^{1}H-NMR (500 MHz, pyridine-d_5) δ: 0.80, 1.00, 1.05, 1.11, 1.24, 1.26, 1.85 (3H each, all s, H$_3$-25, 26, 29, 24, 23, 30, 27), 1.41 (3H, d, J=7.0 Hz, H$_3$-22-*O*-Tig-4), 1.76 (3H, br s, H$_3$-22-*O*-Tig-5), 3.25 (1H, dd, J=3.8, 13.2 Hz, H-3), 3.60, 3.76 (1H each, both d, J=10.6 Hz, H$_2$-28), 4.22 (1H, d, J=4.0 Hz, H-15), 4.56 (1H, d, J=4.0 Hz, H-16), 4.92 (1H, d, J=7.2 Hz, H-1′), 5.01 (1H, d, J=7.4 Hz, H-1‴), 5.44 (1H, br s, H-12), 5.74 (1H, d, J=7.6 Hz, H-1″), 5.77 (1H, d, J=5.4 Hz H-1‴), 6.16 (1H, dd, J=5.5, 11.8 Hz, H-22), 6.83 (1H, dq-like, H-22-*O*-Tig-3); ^{13}C-NMR (125 MHz, pyridine-d_5) δ_C: given in Table 1; positive-ion FAB-MS: m/z 1197 (M+Na)$^+$; HR-FAB-MS: m/z 1197.5676 [Calcd for C$_{57}$H$_{90}$O$_{25}$Na (M+Na)$^+$: 1197.5669].

Alkaline Hydrolysis of Chakasaponin IV (1) A solution of chakasaponin IV (**1**, 12 mg) in 50% aqueous 1,4-dioxane (0.5 mL) was treated with 10% aqueous KOH (0.5 mL) and stirred at 37°C for 1 h. The 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 reaction product. A part of the reaction product was dissolved in (CH$_2$)$_2$Cl$_2$ (2.0 mL) and the solution was treated with *p*-nitrobenzyl-*N*,*N*′-diisopropylisourea (10 mg), then stirred at 80°C for 1 h. The reaction solution was subjected to HPLC [column: YMC-Pack ODS-A, 250×4.6 mm i.d.; mobile phase: MeCN–H$_2$O (50 : 50, v/v); detection: UV (254 nm); flow rate: 1.0 mL/min] to identify the *p*-nitrobenzyl ester of tiglic acid (t_R 35.1 min) from **1**. The rest of the reaction product was subjected to normal-phase silica gel column chromatography [50 mg, CHCl$_3$–MeOH–H$_2$O (10 : 3 : 1 lower layer→6 : 4 : 1)] to give desacyl-chakasaponin IV (**1a**, 10.0 mg).

Desacyl-chakasaponin IV (**1a**): Colorless fine crystals from CHCl$_3$–MeOH; mp 211.0–213.0°C; $[\alpha]_D^{26}$ +38.7° (c=0.33, MeOH); ^{1}H-NMR (600 MHz, pyridine-d_5) δ: 0.80, 1.01, 1.06, 1.12, 1.13, 1.25, 1.85 (3H each, all s, H$_3$-25, 26, 29, 24, 30, 23, 27), 3.23 (1H, m, H-3), 3.71, 4.13 (1H each, both d, J=11.0 Hz, H$_2$-28), 4.45 (1H, m, H-15), 4.47 (1H, m, H-16), 4.62 (1H, m, H-22), 4.90 (1H, d, J=7.2 Hz, H-1′), 5.00 (1H, d, J=7.4 Hz, H-1‴), 5.42 (1H, br s, H-12), 5.70 (1H, d, J=7.4 Hz, H-1′), 5.77 (1H, d, J=5.2 Hz H-1‴); ^{13}C-NMR (150 MHz, pyridine-d_5) δ_C: given in Table 1; positive-ion FAB-MS: m/z 1115 (M+Na)$^+$; HR-FAB-MS: m/z 1115.5245 [Calcd for C$_{52}$H$_{84}$O$_{24}$Na (M+Na)$^+$: 1115.5250].

Acid Hydrolysis of Desacyl-Chakasaponin IV (1a) A solution of **1a** (2.0 mg) in 5% aq. H$_2$SO$_4$–1,4-dioxane (1 : 1, v/v, 1.0 mL) was heated under reflux for 2 h. After cooling, the reaction mixture was neutralized with Amberlite IRA-400 (OH$^-$ form) and the resin was removed by filtration. Evaporation of the solvent from the filtrate under reduced pressure gave a product, which was applied to a Sep-Pack C$_{18}$ cartridge with H$_2$O and MeOH. The MeOH eluate was identified as A$_1$-barrigenol. The H$_2$O eluate was concentrated and the residue was treated with L-cysteine methylester hydrochloride (1.0 mg) in pyridine (0.1 mL) at 60°C for 1 h. After the reaction, the solution was treated with *N*,*O*-bis(trimethylsilyl)trefluoroacetamide (0.1 mL) at 60° for 1 h. The supernatant was then subjected to GLC to identify the derivatives of D-glucuronic acid (i), D-galactose (ii), L-arabinose (iii) and D-xylose (iv). (*Supelco*

STB-1 [30 m×0.25 mm i.d.] capillary column; 230°C column temperature; N_2 carrier gas; t_R, (i) 26.5 min, (ii) 25.6 min, (iii) 15.1 min, (iv) 19.3 min).

Bioassay Methods. Animals Male ddY mice weighing about 25–30 g were purchased from Kiwa Laboratory Animal Co., Ltd., Wakayama, Japan. The animals were housed at a constant temperature of 23±2°C and fed a standard laboratory chow (MF, Oriental Yeast Co., Ltd., Tokyo, Japan). The animals were fasted for 20–24 h prior to the beginning of the experiment, but were allowed free access to tap water. All of the experiments were performed with conscious mice unless otherwise noted. The experimental protocol was approved by the Experimental Animal Research Committee at Kyoto Pharmaceutical University.

Effect on Plasma Triglyceride (TG) Levels in Olive Oil-Treated Mice The experiments were performed as described in our previous report.[20] Each test sample was administered orally to fasted mice and olive oil (5 mL/kg) was administered *p.o.* 30 min thereafter. Blood samples (*ca.* 0.1 mL) were collected into a polyethylene tube (1.5 mL) containing heparin (5 units/tube) from the infraorbital venosus plexus, 2, 4, and 6 h after the olive oil treatment. Plasma TG levels were determined by an enzymatic method using Triglyceride E test Wako (Wako Pure Chemical Industries Ltd., Osaka, Japan).

Effect on Plasma Glucose Levels in Sucrose-Loaded Mice The experiments were performed as described previously.[26] Each test sample was administered orally to fasted mice, and a 20 (w/v) % sucrose solution (10 mL/kg) was administered *p.o.* 30 min thereafter. Blood samples (*ca.* 0.1 mL) were collected into a polyethylene tube (1.5 mL) containing heparin (5 units/tube) from the infraorbital venosus plexus, 0.5, 1, and 2 h after the sucrose loading. After the centrifugation of blood samples, plasma glucose levels were determined enzymatically using Glucose CII test Wako (Wako Pure Chemical Industries Ltd., Osaka, Japan). An intestinal α-glucosidase inhibitor, acarbose, was used as a reference compound.

Gastric Emptying (GE) in Mice The rate of GE was determined using phenol red.[20] Briefly, a solution of 1.5% carboxymethyl cellulose sodium salt (CMC-Na) containing 0.05% phenol red as a marker was given intragastrically (0.3 mL/mouse) to 20–24 h-fasted mice. Thirty minutes later, the mice were sacrificed by cervical dislocation under ether anesthesia. The abdominal cavity was opened, the gastroesophageal junction and pylorus were clamped, and the stomach was removed, weighed, placed in 10 mL of 0.1 M NaOH, and homogenized. The suspension was allowed to settle for 1 h at room temperature, and 5 mL of the supernatant was added to 0.5 mL of 20% trichloroacetic acid (w/v) and centrifuged at 3000 rpm for 20 min. Next, 4 mL of supernatant was mixed with 4 mL of 0.5 M NaOH, and the amount of phenol red was determined from the absorbance at 560 nm. The test sample was given orally 30 min prior to the administration of the CMC-Na solution. Gastric emptying (%) in the 30-min period was calculated according to the following equation:

$$\text{gastric emptying (\%)} = 1 - \frac{\text{amount of phenol red in test sample-treated group}}{\text{amount of phenol red administered}} \times 100$$

Effect on Rat Intestinal α-Glucosidase The experiments were performed as described in our previous report.[20,26] The rat small intestinal brush border membrane fraction was prepared and its suspension in 0.1 M maleate buffer (pH 6.0) was used as the small intestinal α-glucosidase of maltase, sucrase, isomaltase, and trehalase. The enzyme suspension was diluted to hydrolyse maltose and sucrose to produce *ca.* 0.30 and *ca.* 0.15 μmol/tube of D-glucose in the following reaction. The substrate (maltose: 37 mM, sucrose: 37 mM), test compound and enzyme in 0.1 M maleate buffer (pH 6.0, 0.1 mL) were incubated together at 37°C. After 30 min, 0.4 mL of water was added to the test tube, and the tube was immediately immersed in boiling water for 2 min to stop the reaction, then cooled with water. The glucose concentration was determined by an enzymatic method. Each test sample was dissolved in dimethyl sulfoxide (DMSO). Measurements were performed in duplicate, and the IC_{50} was determined graphically. An intestinal α-glucosidase inhibitor, acarbose (IC_{50} for maltase and sucrase of 2.0 and 1.7 μM, respectively),[20] was used as a reference compound.

Effect on Rat Lens Aldose Reductase The experiments were performed as described previously.[20,26] The supernatant fluid of rat lens homogenate was used as a crude enzyme. The enzyme suspension was diluted to produce *ca.* 10 nmol/tube of nicotinamide adenine dinucleotide phosphate (NADP) in the following reaction. The incubation mixture contained 135 mM phosphate buffer (pH 7.0), 100 mM Li_2SO_4, 0.03 mM reduced nicotinamide adenine dinucleotide phosphate (NADPH), 1 mM DL-glyceraldehyde as a substrate, and 100 μL of enzyme fraction, with 25 μL of sample solution, in a total volume of 0.5 mL. The reaction was initiated by the addition of NADPH at 30°C. After 30 min, the reaction was stopped by the addition of 150 μL of 0.5 M HCl. Then, 0.5 mL of 6 M NaOH containing 10 mM imidazole was added, and the solution was heated at 60°C for 20 min to convert NADP to a fluorescent product. Fluorescence was measured using a fluorescence microplate reader (FLUOstar OPTIMA, BMG Labtechnologies) at an excitation wavelength of 360 nm and an emission wavelength of 460 nm. Each test sample was dissolved in DMSO. Measurements were performed in duplicate, and the IC_{50} was determined graphically. An aldose reductase inhibitor, epalrestat (IC_{50}=0.072 μM),[20] was used as a reference compound.

Statistics Values are expressed as mean±S.E.M. For the statistical analysis, a one-way analysis of variance followed by William's test for dose-dependent effects was used.

Acknowledgements This research was supported by the Academic Frontier Project and a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan, and Hoh-ansha Foundation, and Japan-China Medical Association.

References and Notes

1) Part XXXII: Morikawa T., Li X., Nishida E., Nakamura S., Ninomiya K., Matsuda H., Hamao M., Muraoka O., Hayakawa T., Yoshikawa M., *Chem. Pharm. Bull.*, **59**, 889–895 (2011).
2) Yoshikawa M., Morikawa T., Yamamoto K., Kato Y., Nagatomo A., Matsuda H., *J. Nat. Prod.*, **68**, 1360–1365 (2005).
3) Yoshikawa M., Wang T., Sugimoto S., Nakamura S., Nagatomo A., Matsuda H., Harima S., *Yakugaku Zasshi*, **128**, 141–151 (2008).
4) Yoshikawa M., Nakamura S., Kato Y., Matsuhira K., Matsuda H., *Chem. Pharm. Bull.*, **55**, 598–605 (2007).

5) Yoshikawa M., Sugimoto S., Kato Y., Nakamura S., Wang T., Yamashita C., Matsuda H., *Chem. Biodivers.*, **5**, 903–915 (2009).
6) Yoshikawa M., Sugimoto S., Nakamura S., Matsuda H., *Chem. Pharm. Bull.*, **56**, 1297–1303 (2008).
7) Wada S., He P., Hashimoto I., Watanabe N., Sugiyama K., *Biosci. Biotechnol. Biochem.*, **64**, 2262–2265 (2000).
8) The ^{1}H- and ^{13}C-NMR spectra of **1** were assigned with the aid of distortionless enhancement by polarization transfer (DEPT), DQF COSY, heteronuclear multiple quantum coherence spectroscopy (HMQC), and HMBC experiments.
9) Yoshikawa M., Murakami T., Yoshizumi S., Murakami N., Yamahara J., Matsuda H., *Chem. Pharm. Bull.*, **44**, 1899–1907 (1996).
10) Matsuda H., Nakamura S., Fujimoto K., Moriuchi R., Kimura Y., Ikoma N., Hata Y., Muraoka O., Yoshikawa M., *Chem. Pharm. Bull.*, **58**, 1617–1621 (2010).
11) Ito S., Ogino T., Sugiyama H., Kodama M., *Tetrahedron Lett.*, **24**, 2289–2294 (1967).
12) Hara S., Okabe H., Mihashi K., *Chem. Pharm. Bull.*, **34**, 1843–1845 (1986).
13) Nakamura S., Murakami T., Nakamura J., Kobayashi H., Matsuda H., Yoshikawa M., *Chem. Pharm. Bull.*, **54**, 1545–1550 (2006).
14) Hu J. N., Zhu X. M., Han L. K., Saito M., Sun Y. S., Yoshikawa M., Kimura Y., Zheng Y. N., *Chem. Pharm. Bull.*, **56**, 12–16 (2008).
15) Yoshikawa M., Murakami T., Matsuda H., Yamahara J., Murakami N., Kitagawa I., *Chem. Pharm. Bull.*, **44**, 1454–1464 (1996).
16) Matsuda H., Murakami T., Li. Y., Yamahara J., Yoshikawa M., *Bioorg. Med. Chem.*, **6**, 1019–1023 (1998).
17) Yoshikawa M., Matsuda H., *Biofactors*, **13**, 231–237 (2000).
18) Yoshikawa M., Nakamura S., Ozaki K., Kumahara A., Morikawa T., Matsuda H., *J. Nat. Prod.*, **70**, 210–214 (2007).
19) Shimoda H., Ninomiya K., Nishida N., Yoshino T., Morikawa T., Matsuda H., Yoshikawa M., *Bioorg. Med. Chem. Lett.*, **13**, 223–228 (2003).
20) Matsuda H., Asao Y., Nakamura S., Hamao M., Sugimoto S., Hongo M., Pongpiriyadacha Y., Yoshikawa M., *Chem. Pharm. Bull.*, **57**, 487–494 (2009).
21) Hamao M., Matsuda H., Nakamura S., Nakashima S., Semura S., Maekubo S., Wakasugi S., Yoshikawa M., *Bioorg. Med. Chem.*, **19**, 6033–6041 (2011).
22) Matsuda H., Li Y., Murakami T., Yamahara J., Yoshikawa M., *Eur. J. Pharmacol.*, **368**, 237–243 (1999).
23) Matsuda H., Li Y., Yoshikawa M., *Life Sci.*, **66**, PL41–PL46 (2000).
24) Matsuda H., Li Y., Yoshikawa M., *Life Sci.*, **67**, 2921–2927 (2000).
25) Terashima H., Hama K., Yamamoto R., Tsuboshima M., Kikkawa R., Hatanaka I., Shigeta Y., *J. Pharmacol. Exp. Ther.*, **229**, 226–230 (1984).
26) Morikawa T., Chaipech S., Matsuda H., Hamao M., Umeda Y., Sato H., Tamura H., Kon'i H., Ninomiya K., Yoshikawa M., Pongpiriyadacha Y., Hayakawa T., Muraoka O., *Bioorg. Med. Chem.*, **20**, 832–840 (2012).