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Steroidal glycosides from the bulbs of *Fritillaria meleagris* and their cytotoxic activities

Yukiko Matsuo *, Daisuke Shinoda, Aina Nakamaru, Yoshihiro Mimaki *

Tokyo University of Pharmacy and Life Sciences, School of Pharmacy, 1432-1, Horinouchi, Hachioji, Tokyo 192-0392, Japan

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ABSTRACT

Steroidal glycosides (**1**–**18**), including 10 new compounds (**1**–**10**), were isolated from the bulbs of *Fritillaria meleagris* (Liliaceae). The structures of the new compounds were determined by two-dimensional (2D) NMR analysis, and by hydrolytic cleavage followed by spectroscopic and chromatographic analysis. The isolated compounds and their aglycones were evaluated for cytotoxic activity against HL-60 human promyelocytic leukemia cells and A549 human lung adenocarcinoma cells. Morphological observation and flow cytometry analysis showed that 5β-spirostanol glycoside (**2**) and a cholestane derivative (**17a**) induced apoptotic cell death in HL-60 cells through different mechanisms of action. Furthermore, the (22*R*)-spirosolanol glycoside (**11**) selectively induced apoptosis in A549 cells without affecting the caspase-3 activity level.

1. Introduction

Plants of the genus *Fritillaria* belong to the family Liliaceae, and their bulbs contain steroidal alkaloids with various biological activities such as anticholinergic, antitussive, expectorant, and anti-inflammatory effects [1–3]. *Fritillaria meleagris* L., commonly called snake's head fritillary, is a perennial plant distributed widely throughout Asia and northwestern Europe. The plant grows to a height of 30–40 cm and has flowers with a checkered pattern [4]. Only one systematic chemical characterization of *F. meleagris* has been conducted; a publication in 1958 reported the putative presence of two alkaloids by paper chromatography [5]. As part of our continuing chemical investigation of the saponin constituents of Liliaceae plants [6], a phytochemical screen of the bulbs of *F. meleagris* was performed. We report the discovery of a new steroidal alkaloid (**1**), two new spirostanol glycosides (**2**, **3**), five new furostanol glycosides (**4**–**8**), two new cholestane-type glycosides (**9**, **10**), together with eight known compounds (**11**–**18**). Extensive spectroscopic studies were conducted to determine the structures of the new steroidal glycosides, including two-dimensional (2D) NMR, and hydrolytic cleavage followed by spectroscopic and chromatographic analysis. The cytotoxicity of the isolated compounds and their aglycones was evaluated against HL-60 human promyelocytic leukemia cells and A549 human lung adenocarcinoma cells.

2. Experimental

2.1. General methods

Optical rotations were obtained using a P-1030 (Jasco, Tokyo, Japan) automatic digital polarimeter. IR spectra were recorded with a FT-IR 620 spectrophotometer (Jasco). NMR spectra (500 MHz for ^{1}H NMR) were recorded with a DRX-500 spectrometer (Bruker, Karlsruhe, Germany), using standard Bruker pulse programs. Chemical shifts are given as δ values referenced to tetramethylsilane (TMS) as an internal standard. HRESITOFMS data were obtained with an LCT mass spectrometer (Waters-Micromass, Manchester, U.K.). 5 ppm error in HRESITOFMS data has achieved the level of accuracy for formula confirmation and established the molecular formula of isolated compound. Porous-polymer polystyrene resin Diaion HP-20 (Mitsubishi-Chemical, Tokyo, Japan), BW-300 silica gel (Fuji-Silysia Chemical, Aichi, Japan), and octadecylsilanized (ODS) silica gel (Nacalai Tesque, Kyoto, Japan) were used for column chromatography (CC). TLC was carried out on precoated Silica gel 60 F_{254} (0.25 mm thick, Merck, Darmstadt, Germany) and RP_{18} $F_{254}S$ plates (0.25 mm thick, Merck), and the spots were visualized by spraying the plates with 10% H_2SO_4 in H_2O and then heating. HPLC was performed with a system composed of a CCPM pump (Tosoh, Tokyo, Japan), a CCP PX-8010 controller (Tosoh), an RI-8010 detector (Tosoh), and a Rheodyne injection port. A TSK gel ODS-100Z column (10 mm i.d. × 250 mm, 5 μm, Tosoh) was employed for the preparative HPLC. Purities of all isolated compounds were confirmed by NMR, optical rotation,

* Corresponding authors. Tel.: +81 42 676 4577; fax: +81 42 676 4579.
E-mail addresses: matsuoy@toyaku.ac.jp (Y. Matsuo), mimakyi@toyaku.ac.jp (Y. Mimaki).

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TLC, and mass spectrometry, respectively. The following materials and reagents were used for the cell cultures and the assay of cytotoxic activities: Spectra Classic microplate reader (Tecan, Salzburg, Austria); 96-well flat-bottom plate (Iwaki Glass, Chiba, Japan); JCRB 0085 HL-60 cells and JCRB 0076 A549 cells (Human Science Research Resources Bank, Osaka, Japan); fetal bovine serum (FBS) (Bio-Whittaker, Walkersville, MD, U.S.A.); 0.25% Trypsin-EDTA solution, RPMI 1640 medium, minimum essential medium (MEM), phosphate buffered saline (PBS) (Wako Pure Chemical Industries, Osaka, Japan), etoposide, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) (Sigma, St. Louis, U.S.A.); penicillin G sodium salt and streptomycin sulfate (Gibco, Grand Island, NY, U.S.A.). Paraformaldehyde (Wako Pure Chemical Industries, Osaka, Japan), Triton X-100 (Sigma), propidium iodide (PI) (Molecular Probes, Eugene, OK, U.S.A.), and ribonuclease A (RNase) (Wako Pure Chemical Industries, Osaka, Japan). All other chemicals used were of biochemical reagent grade.

2.2. Plant material

The bulbs of *F. meleagris*, which were collected in India in 2007, were obtained from Sakata-no-Tane (Kanagawa, Japan) and identified by Dr. Yutaka Sashida, professor emeritus at Tokyo University of Pharmacy and Life Sciences. A voucher specimen was deposited in our laboratory (voucher no. 07-004-FM, Department of Medicinal Pharmacognosy).

2.3. Extraction and isolation

The *F. meleagris* bulbs (fresh weight, 6.0 kg) were extracted with hot MeOH (20 L) for 12 h. After removing the solvent, the MeOH extract (300 g) was resuspended in MeOH/H_2O (3:7) to pass through a Diaion HP-20 column (50 mesh, 2000 g, 8.5 × 60 cm) and then successively eluted with MeOH/H_2O (3:7), MeOH, EtOH, and EtOAc (each 9 L). CC of the MeOH-eluted fraction (80.0 g) on silica gel (200–300 mesh, 1500 g, 8.5 × 30 cm), eluted with a stepwise gradient mixture of $CHCl_3$/MeOH/H_2O (40:10:1; 20:10:1 and 7:4:1) and finally with MeOH alone, provided five fractions (A–E). Fraction B was chromatographed on ODS silica gel (100–200 mesh, 1500 g, 8.5 × 30 cm) eluted with MeCN/H_2O (1:4; 1:3; 1:2 and 1:1) to afford **3** (19.1 mg) and **10** (8.7 mg). Fraction C was separated by an ODS silica gel column (100–200 mesh, 1500 g, 8.5 × 30 cm) eluted with MeCN/H_2O (1:4, 1:3, 1:2 and 1:1) to give **2** (14.6 mg), **13** (3.3 mg), **14** (2.1 mg), **15** (13.0 mg), **16** (3.9 mg), and **17** (37.0 mg). Fraction D was chromatographed on silica gel (200–300 mesh, 1800 g, 8.5 × 35 cm) eluted with $CHCl_3$/MeOH/H_2O (30:10:1; 20:10:1 and 7:4:1) and on ODS silica gel (100–200 mesh, 1500 g, 8.5 × 30 cm) eluted with MeCN-H_2O (1:3; 1:2 and 1:1) to afford **1** (91.8 mg), **4** (158 mg), **5** (10.1 mg), **6** (32.0 mg), **7** (9.2 mg), **8** (39.4 mg), **9** (3.5 mg), **11** (1.4 mg), **12** (8.0 mg), and **18** (1.3 mg). Isolated compounds have been attempted to crystallize but all attempts were unsuccessful. Steroidal glycosides were described as "amorphous solids" and melting point determinations should not be needed for compounds described as "amorphous solids".

2.3.1. Compound **1**

(22*S*,25*S*)-spirosol-5-en-3β-yl*O* -β-D-glucopyranosyl-(1 → 4)-*O*-[α-L-rhamnopyranosyl-(1 → 2)]-β-D-glucopyranoside (**1**): amorphous solid; $[\alpha]_D^{25}$ −64.8 (*c* 0.05, MeOH); IR ν_{max} (film) cm^{-1}: 3445 (OH), 2932 (CH); ^1H NMR (500 MHz, C_5D_5N) and ^{13}C NMR (125 MHz, C_5D_5N), see Tables 1 and 2; HRESITOFMS m/z: 884.5045 $[M + H]^+$ (calcd for $C_{45}H_{74}O_{16}N$: 884.5008).

2.3.2. Acid hydrolysis of **1**

A solution of **1** (10.0 mg) in 1 M HCl in dioxane/H_2O (1:1; 3.0 mL) was heated at 95 °C for 1 h under an Ar atmosphere. The reaction mixture was cooled, neturalized with aqueous 1 M NaOH (2.0 mL), diluted with H_2O (20 mL), and extracted with $CHCl_3$ (20 mL × 3). The $CHCl_3$ extract (7.8 mg) was purified by preparative TLC ($CHCl_3$/MeOH, 30:1) to give tomatidenol (**1a**, 2.4 mg). Steroid aglycones which were stable towards acid were obtained by the above procedures. The purity of aglycone was confirmed by NMR, optical rotation, TLC, and mass spectrometry. The aqueous residue (2.9 mg) was directly analyzed by HPLC under the following conditions that β-D-glycoside and α-D-glycoside were detected as one peak: Capcell Pak NH2 UG80 column (4.6 mm i.d. × 250 mm, 5 μm, Shiseido, Tokyo, Japan); MeCN/H_2O (85:15); detection by refractive index (RI) and optical rotation (OR); flow rate of 1.0 mL/min. D-Glucose and L-rhamnose in the aqueous residue were identified by comparing their retention times (t_R [min]) and signs of optical rotation with those of authentic samples [7,8]: L-rhamnose (7.89, negative optical rotation), and D-glucose (13.99, positive optical rotation).

2.3.3. Compound **1a**

An amorphous solid; $[\alpha]_D^{25}$ −57.2 (*c* 0.05, MeOH) (Lit. −23.5 [9]); ^{13}C NMR (125 MHz, $CDCl_3$): δ 36.7(C-1), 31.6 (C-2), 71.7 (C-3), 42.3 (C-4), 140.8 (C-5), 121.4 (C-6), 32.1 (C-7), 31.4 (C-8), 50.1 (C-9), 37.2 (C-10), 20.9 (C-11), 39.9 (C-12), 40.7 (C-13), 56.0 (C-14), 32.7 (C-15), 78.8 (C-16), 62.0 (C-17), 16.8 (C-18), 19.4 (C-19), 42.8 (C-20), 15.8 (C-21), 99.2 (C-22), 26.6 (C-23), 28.4 (C-24), 29.7 (C-25), 50.0 (C-26), 19.4 (C-27); HRESITOFMS *m/z*: 414.3374 $[M + H]^+$ (calcd for $C_{27}H_{44}NO_2$: 414.3372).

2.3.4. Compound **2**

(25*R*)-5β-spirostan-3β-yl*O*-β-D-glucopyranosyl-(1 → 4)-*O*-[α-L-rhamnopyranosyl-(1 → 2)]-β-D-glucopyranoside (**2**): an amorphous solid; $[\alpha]_D^{25}$ −60.4 (*c* 0.10, MeOH); IR ν_{max} (film) cm^{-1}: 3397 (OH), 2927 (CH); ^1H NMR (500 MHz, C_5D_5N) and ^{13}C NMR (125 MHz, C_5D_5N), see Tables 1 and 2; HRESITOFMS *m/z*: 887.4977 $[M + H]^+$ (calcd for $C_{45}H_{75}O_{17}$: 887.5004).

2.3.5. Enzymatic hydrolysis of **2**

Compound **2** (5.0 mg) was treated with naringinase (EC 232-96-4, Sigma; 554 mg) in a HOAc/KOAc buffer (pH 4.3, 5.0 mL) at room temperature for 432 h. The reaction mixture was purified by CC on silica gel ($CHCl_3$/MeOH/H_2O; 10:1:0, 7:4:1) (200–300 mesh, 100 g, 2 × 30 cm) to give smilagenin (**2a**, 0.3 mg), and a sugar fraction (2.3 mg). Acid hydrolysis of **2** with 1 M HCl have resulted in giving only D-glucose and L-rhamnose, whereas the labile aglycone decomposed under acidic conditions [10]. The preparation of the aglycone **2a** has been finally completed by enzymatic hydrolysis under the above conditions. HPLC analysis of the sugar fraction under the same conditions as those for **1** showed the presence of D-glucose (14.22, positive optical rotation) and L-rhamnose (7.98, negative optical rotation).

2.3.6. Compound **2a**

An amorphous solid; $[\alpha]_D^{25}$ −53.6 (*c* 0.05, MeOH) (Lit. −61 [11]); ^{13}C NMR (125 MHz, C_5D_5N): δ 30.6(C-1), 28.6 (C-2), 66.1 (C-3), 34.4 (C-4), 37.0 (C-5), 27.2 (C-6), 26.9 (C-7), 35.6 (C-8), 41.0 (C-9), 35.6 (C-10), 21.2 (C-11), 40.1 (C-12), 40.4 (C-13), 56.6 (C-14), 32.2 (C-15), 81.3 (C-16), 63.2 (C-17), 16.6 (C-18), 24.3 (C-19), 42.0 (C-20), 15.1 (C-21), 109.3 (C-22), 31.8 (C-23), 29.3 (C-24), 30.0 (C-25), 66.9 (C-26), 17.3 (C-27); HRESITOFMS *m/z*: 417.3382 $[M + H]^+$ (calcd for $C_{27}H_{45}O_3$: 417.3369).

Table 1
^{1}H and ^{13}C NMR chemical shift assignments for the aglycone moiety of compounds **1**–**10** in C5D5N.

Position		**1**				**2**				**3**				**4**				**5**			
		δ_H		*J* (Hz)	δ_C	δ_H		*J* (Hz)	δ_C	δ_H		*J* (Hz)	δ_C	δ_H		*J* (Hz)	δ_C	δ_H		*J* (Hz)	δ_C
1	ax	0.98	ddd	13.9, 13.9, 3.8	37.5	1.81	m		30.8	1.01	ddd	12.4, 12.4, 3.6	37.6	1.79	m		30.8	1.85	m		31.0
	eq	1.73	m			1.49	br d	11.5		1.79	br d	12.4		1.50	m			1.50	m		
2	ax	1.87	m		30.1	1.51	m		26.8	1.91	m		32.1	1.49	m		26.8	1.53	m		26.9
	eq	2.10	m			1.97	m			2.15	m			1.94	m			1.98	m		
3		3.88	m	$W_{1/2}$ = 21.7	78.1	4.21	m	$W_{1/2}$ = 21.1	76.0	3.86	m	$W_{1/2}$ = 22.5	78.1	4.21	m	$W_{1/2}$ = 19.6	76.1	4.25	m	$W_{1/2}$ = 23.9	76.2
4	ax	2.71	br dd	13.1, 12.8	38.9	1.82	m		30.9	2.75	m		39.0	1.78	m		30.9	1.85	m		31.0
	eq	2.76	br dd	13.1, 5.3		1.86	m							1.84	br d	9.8		1.91	m		
5		–			140.8	2.14	br dd	6.0, 6.0	37.1	–			140.9	2.13	br d	10.6	37.1	2.17	br dd	11.3, 3.6	37.2
6		5.30	br d	4.9	121.8	1.91	m		26.7	5.32	br d	3.7	121.8	1.91	m		26.8	1.98	m		26.9
						1.38	m							1.32	m			1.41	m		
7	ax	1.86	m		32.4	0.97	br ddd	13.4, 13.4, 2.4	26.7	1.53	m		32.4	0.97	m		26.7	1.10	m		26.9
	eq	1.49	m			1.32	m			1.92	m			1.31	m			1.31	m		
8		1.54	m		31.6	1.55	m		35.5	1.64	m		32.3	1.54	m		35.5	1.48	m		35.3
9		0.92	m		50.4	1.31	m		40.3	0.98	m		50.2	1.29	t	7.0	40.3	1.31	m		40.3
10		–			37.1	–			35.3	–			37.1	–			35.3	–			35.3
11		1.45	m		21.1	1.21	m		21.1	1.49	m		20.9	1.21	m		21.1	1.38	m		21.5
						1.36	m			1.60	m			1.35	m						
12	ax	1.11	ddd	11.5, 10.5, 4.5	40.1	1.10	m		40.3	1.50	m		32.0	1.11	m		40.4	1.17	ddd	11.9, 11.9, 3.2	40.1
	eq	1.67	br dd	11.5, 3.5		1.69	br d	13.8		2.12	m			1.75	m			1.73	m		
13		–			46.7	–			40.9	–			45.1	–			41.2	–			43.9
14		1.02	m		56.0	1.09	m		56.5	2.07	m		54.0	1.09	t	6.2	56.4	0.88	m		54.8
15	a	2.04	m		33.1	2.04	m		32.1	2.21	m		31.8	2.03	m			2.12	m		34.4
	b	1.46	m			1.40	m			1.51	m			1.39	m		32.4	1.45	m		
16		4.17	m		78.7	4.60	m		81.2	4.46	dd	7.2, 6.1	90.0	4.96	m		81.2	4.83	m		84.6
17		1.57	dd	8.2, 8.2	62.2	1.85	dd	8.3, 6.6	63.1	–			90.1	1.95	dd	7.3, 6.4	64.0	2.48	d	6.8	64.7
18		0.86	s		16.8	0.82	s		16.6	0.96	s		17.1	0.87	s		16.7	0.71	s		14.4
19		1.05	s		19.8	1.07	s		23.8	1.09	s		19.4	1.07	s		23.8	1.10	s		23.8
20		1.84	m		43.0	1.96	br dd	6.9, 6.6	42.0	2.27	m		44.7	2.23	m		40.6	–			103.6
21		1.06	d	7.2	16.2	1.14	d	6.9	15.0	1.22	d	7.0	9.7	1.32	d	6.8	16.4	1.65	s		11.8
22		–			99.4	–			109.2	–			109.8	–			110.6	–			152.4
23	ax	1.44	m		27.1	1.66	br dd	13.0, 12.5	31.8	1.90	br dd	12.1, 10.4	30.3	2.01	m		37.2	2.23	m		23.7
	eq	1.73	m			1.61	m			2.15	br d	10.4		1.95	m			2.18	m		
24		1.55	m		29.3	1.59	m		29.2	1.58	m		28.8	2.02	m		28.3	1.85	m		31.5
														1.65	m			1.49	m		
25		1.67	m		31.4	1.56	m		30.5	1.59	m		30.4	1.91	m		34.2	1.97	m		33.5
26	ax	2.94	dd	10.8, 10.8	50.6	3.51	dd	10.5, 10.5	66.9	3.50	dd	10.5, 10.5	66.7	3.92	dd	9.3, 5.6	75.2	3.96	dd	9.3, 6.8	75.0
	eq	2.83	dd	10.8, 3.8		3.59	dd	10.5, 3.0		3.52	dd	10.5, 2.9		3.61	dd	9.3, 6.0		3.62	dd	9.3, 5.5	
27		0.82	d	6.5	19.8	0.69	d	5.3	17.3	0.68	d	5.3	17.3	0.97	d	6.7	17.4	1.03	d	6.6	17.4

		6				**7**				**8**				**9**				**10**			
1	ax	0.95	m		37.5	0.99	m		37.2	0.97	m		37.5	1.01	br dd	15.6, 15.0	36.7	1.23	ddd	13.0, 13.0, 3.0	37.3
	eq	1.73	m			1.76	m			1.78	m			1.56	br dd	15.6, 4.1		1.72	br dd	13.0, 3.0	
2	ax	1.85	m		30.1	1.86	m		30.0	1.85	m		30.2	1.59	m		29.5	1.95	br dd	13.2, 13.0	31.4
	eq	2.08	m			2.10	m			2.09	m			2.05	m			2.29	m		
3		3.84	m	$W_{1/2}$ = 24.5	78.1	3.80	m	$W_{1/2}$ = 21.2	78.2	3.76	m	$W_{1/2}$=26.5	78.1	3.96	m	$W_{1/2}$ = 25.4	76.7	3.78	m	$W_{1/2}$ = 22.5	70.1
4	ax	2.70	m		38.9	2.71	m		39.0	2.69	br s		38.9	1.73	m		27.0	1.66	dd	12.7, 12.2	31.9
	eq	2.73	m											2.40	br d	13.0		2.03	br d	12.2	
5		–			140.7	–			140.8	–			140.7	2.06	dd	12.9, 2.5	56.4	2.30	m		56.9
6		5.26	br d	3.9	121.8	5.29	br s		121.8	5.26	br d	3.8	121.8	–			209.7	–			211.8
7	ax	1.89	m		32.4	1.48	m		32.1	1.49	m		32.4	1.99	dd	12.7, 12.7	46.7	2.89	dd	12.7, 12.7	42.9
	eq	1.51	m			1.87	br d	13.3		1.89	m			2.31	dd	12.7, 4.6		2.46	dd	12.7, 3.9	
8		1.53	m		32.1	1.56	m		31.7	1.55	m		32.1	1.63	m		37.8	2.35	m		40.9
9		0.97	m		50.3	0.89	m		50.4	0.96	m		50.2	1.09	m		53.7	2.20	ddd	12.0, 12.0, 4.0	46.8
10		–			37.1	–			37.1	–			37.1	–			40.9	–			40.7

11	ax	1.52	m		21.0	1.41	m		21.1	1.52	m		21.0	1.20	m		21.6	1.50	m		21.2
	eq	1.60	m			1.45	m			1.58	m			1.45	br d	9.3		1.69	br d	13.0	
12	ax	1.58	m		32.3	1.12	m		39.9	1.48	m		32.3	1.18	m		39.5	1.95	br d	13.0	33.2
	eq	1.56	m			1.73	m			2.15	m			1.85	br d	8.7		2.36	m		
13		–			45.1	–			40.8	–			45.1	–			43.1	–			49.0
14		2.03	m		53.0	1.07	m		56.6	2.01	m		53.0	1.08	m		56.0	–			83.9
15	a	2.17	m		31.9	2.03	m		32.3	2.17	m		31.9	1.37	m		24.2	2.02	m		32.4
	b	1.51	m			1.49	m			1.50	m			0.92	m						
16		4.76	t	7.1	90.4	4.95	br d	4.6	81.1	4.75	br d	4.6	90.4	1.71	m		27.6	2.43	m		21.4
														1.20	m			1.84	m		
17		–			90.8	1.94	m		63.9	–			90.8	1.73	m		52.4	2.76	dd	9.1, 9.1	50.8
18		0.99	s		17.2	0.90	s		16.4	0.99	s		17.2	0.58	s		12.3	1.35	s		17.9
19		1.08	s		19.4	1.05	s		19.3	1.06	s		19.4	0.64	s		13.0	0.81	s		13.0
20		2.50	m		43.5	2.24	m		40.7	2.49	m		43.5	2.57	m		49.4	–			76.1
21		1.36	d	7.0	10.4	1.34	d	6.9	16.4	1.36	d	6.9	10.4	1.10	d	6.8	16.7	1.52	s		22.4
22		–			111.3	–			110.7	–			111.3	–			213.0	3.86	br d	9.2	90.0
23		2.06	m		36.8	2.05	m		37.1	2.05	m		36.8	2.68	m		39.6	1.82	m		30.6
														2.52	m			1.63	m		
24	a	2.05	m		28.0	2.04	m		28.4	2.05	m		28.0	1.95	m		27.9	2.11	m		35.7
	b	1.69	m			1.69	m			1.68	m			1.64	m			1.46	m		
25		1.94	m		34.2	1.93	m		34.3	1.91	m		34.2	1.94	m		33.5	1.40	m		28.4
26	a	3.95	dd	9.4, 7.6	75.2	3.95	dd	9.2, 6.0	75.3	3.94	dd	9.3, 7.2	75.2	4.07	dd	9.6, 6.2	75.0	0.82	d	6.8	23.1
	b	3.62	dd	9.4, 6.1		3.63	dd	9.2, 7.1		3.61	dd	9.3, 5.8		3.54	dd	9.6, 5.8					
27		1.00	d	6.6	17.4	1.00	d	6.6	17.4	1.00	d	6.8	17.4	1.00	d	6.4	17.4	0.83	d	6.3	22.4

*2.3.7. Compound **3***

(25*R*)-17α-hydroxyspirost-5-en-3β-yl*O*-α-L-rhamnopyranosyl-(1 → 2)-β-D-xylopyranoside (**3**): an amorphous solid; $[\alpha]_D^{25}$ −79.7 (*c* 0.08, MeOH); IR ν_{max} (film) cm^{-1}: 3365 (OH), 2930 and 2871 (CH); ^1H NMR (500 MHz, C_5D_5N) and ^{13}C NMR (125 MHz, C_5D_5N), see Tables 1 and 2; HRESITOFMS *m/z*: 731.3961 $[M + Na]^+$ (calcd for $C_{38}H_{60}O_{12}Na$: 731.3982).

*2.3.8. Acid hydrolysis of **3***

A solution of **3** (8.0 mg) in 0.5 M HCl in dioxane/H_2O (1:1, 2.0 mL) was heated at 95 °C for 2 h under an Ar atmosphere. The reaction mixture was cooled, and then neturalized with an Amberlite IRA-96SB column (Organo, Tokyo, Japan) (16–50 mesh, 100 g, 2.0 × 30 cm). The mixture was then eluted through a Diaion HP-20 column (MeOH/H_2O, 2:3; MeOH; EtOH/Me_2CO, 1:1) (50 mesh, 50 g, 2.0 × 15 cm). The fraction eluted with EtOH/Me_2CO (1:1) (5.3 mg) was purified by CC on silica gel ($CHCl_3$/MeOH, 15:1) (200–300 mesh, 50 g, 2 × 18 cm) to give pennogenin (**3a**, 1.9 mg). HPLC analysis of the fraction eluted with MeOH/H_2O (2:3) (2.7 mg) under the same conditions as those used for **1** showed the presence of L-rhamnose (7.70, negative optical rotation) and D-xylose (9.13, positive optical rotation).

*2.3.9. Compound **3a***

An amorphous solid; $[\alpha]_D^{25}$ −87.3 (*c* 0.08, MeOH) (Lit. −74.1 [12]); ^{13}C NMR (125 MHz, C_5D_5N): δ 37.8(C-1), 32.4 (C-2), 71.3 (C-3), 43.5 (C-4), 141.9 (C-5), 121.0 (C-6), 32.1 (C-7), 32.4 (C-8), 50.3 (C-9), 37.0 (C-10), 21.0 (C-11), 32.6 (C-12), 44.8 (C-13), 53.1 (C-14), 31.8 (C-15), 90.0 (C-16), 90.1 (C-17), 17.2 (C-18), 19.6 (C-19), 45.2 (C-20), 9.7 (C-21), 109.8 (C-22), 32.1 (C-23), 28.8 (C-24), 30.2 (C-25), 66.7 (C-26), 17.3 (C-27); HRESITOFMS *m/z*: 431.3162 $[M + H]^+$ (calcd for $C_{27}H_{43}O_4$: 431.3161).

*2.3.10. Compound **4***

(25*R*)-26-[(β-D-glucopyranosyl)oxy]-22α-hydroxy-5β-furostan-3β-yl*O*-β-D-glucopyranosyl-(1 → 4)-*O*-[α-L-rhamnopyranosyl-(1 → 2)]-β-D-glucopyranoside (**4**): an amorphous solid; $[\alpha]_D^{25}$ −43.7 (*c* 0.26, MeOH); IR ν_{max} (film) cm^{-1}: 3396 (OH), 2929 (CH); ^1H NMR (500 MHz, C_5D_5N) and ^{13}C NMR (125 MHz, C_5D_5N), see Tables 1 and 2; HRESITOFMS *m/z*: 1089.5491 $[M + Na]^+$ (calcd for $C_{51}H_{86}O_{23}Na$: 1089.5458).

*2.3.11. Acid hydrolysis of **4***

A solution of **4** (20.0 mg) was subjected to the acid hydrolysis described for **3** to give **2a** (1.9 mg) and a sugar fraction (5.6 mg). HPLC analysis of the sugar fraction under the same conditions as those used for 1 showed the presence of D-glucose (13.91, positive optical rotation) and L-rhamnose (7.98, negative optical rotation).

*2.3.12. Enzymatic hydrolysis of **4***

Compound **4** (15.0 mg) was treated with β-D-glucosidase (EC 232-589-7, Sigma) (25.0 mg) in HOAc/NaOAc buffer (pH 5.0, 3.0 mL) at room temperature for 23 h. The reaction mixture was purified by CC on silica gel ($CHCl_3$/MeOH/H_2O, 30:10:1; 7:4:1) (200–300 mesh, 100 g, 2 × 20 cm) to yield **2** (10.4 mg) and D-glucose (13.97, positive optical rotation).

*2.3.13. Compound **5***

(25*R*)-26-[(β-D-glucopyranosyl)oxy]-5β-furost-20(22)-en-3β-yl *O*-β-D-glucopyranosyl-(1 → 4)-*O*-[α-L-rhamnopyranosyl-(1 → 2)]-β-D-glucopyranoside (**5**); an amorphous solid; $[\alpha]_D^{25}$ −31.7 (*c* 0.29, MeOH); IR ν_{max} (film) cm^{-1}: 3445 (OH), 2928 (CH); ^1H NMR (500 MHz, C_5D_5N) and ^{13}C NMR (125 MHz, C_5D_5N), see Tables 1 and 2; HRESITOFMS *m/z*: 1049.5518 $[M + H]^+$ (calcd for $C_{51}H_{85}O_{22}$: 1049.5533).

Table 2
^{1}H and ^{13}C NMR chemical shift assignments for the sugar moieties of compounds **1**–**10** in C5D5N.

1						**2**						**3**					
Position			δ_H		J (Hz)	δ_C	Position		δ_H		J (Hz)	δ_C	Position		δ_H		J (Hz)	δ_C
Glc (I)	1′		4.95 d		6.8	100.0	Glc (I)	1′	4.80 d		7.3	101.9	Xyl	1′	4.86 d		7.0	101.2
	2′		4.22 dd		8.8, 6.8	77.3		2′	4.25 dd		8.4, 7.3	76.6		2′	4.19 dd		7.8, 7.0	77.9
	3′		4.23 dd		9.1, 8.8	77.7		3′	4.23 dd		8.8, 8.4	78.0		3′	4.15 dd		8.0, 7.8	79.6
	4′		4.21 dd		9.1, 8.8	82.0		4′	4.20 dd		8.8, 8.8	82.2		4′	4.13 m			71.4
	5′		3.85 m			76.2		5′	3.81 m			76.2		5′	a 4.28 br d		10.6	67.0
	6′	a	4.52 br d		11.0	61.9		6′	a 4.50 br d		12.2	62.0			b 3.61 dd		10.6, 9.3	
		b	4.46 br d		11.0				b 4.44 br d		12.2							
Rha	1″		6.25 br s			101.8	Rha	1″	6.34 br s			101.3	Rha	1″	6.35 br s			102.2
	2″		4.74 br d		1.8	72.4		2″	4.71 br s			72.3		2″	4.80 br s			72.5
	3″		4.58 dd		9.3, 3.2	72.8		3″	4.53 dd		9.3, 3.0	72.6		3″	4.61 br d		9.3	72.8
	4″		4.34 dd		9.3, 9.3	74.1		4″	4.31 dd		9.5, 9.3	74.0		4″	4.34 dd		9.3, 9.3	74.1
	5″		4.94 m			69.5		5″	4.76 m			69.4		5″	4.95 m			69.5
	6″		1.76 d		6.2	18.6		6″	1.72 d		6.1	18.7		6″	1.76 d		5.8	18.6
Glc (II)	1‴		5.13 d		7.9	105.2	Glc (II)	1‴	5.11 d		7.9	105.1						
	2‴		4.06 dd		8.4, 7.9	74.9		2‴	4.05 dd		8.5, 7.9	74.9						
	3‴		4.22 dd		9.1, 8.4	78.3		3‴	4.22 dd		9.3, 8.5	78.3						
	4‴		4.27 dd		9.1, 9.1	71.2		4‴	4.24 dd		9.3, 9.3	71.2						
	5‴		3.98 m			78.5		5‴	3.95 m			78.4						
	6‴	a	4.46 dd		11.0, 2.1	62.0		6‴	a 4.44 br d		12.2	62.0						
		b	4.33 dd		11.0, 4.8				b 4.30 br d		12.2							

4						**5**					
Position			δ_H		J (Hz)	δ_C	Position		δ_H		J (Hz)	δ_C
Glc (I)	1′		4.78 d		7.8	101.9	Glc (I)	1′	4.82 d		7.5	102.0
	2′		4.20 dd		8.8, 7.8	76.1		2′	4.28 dd		8.2, 7.5	76.0
	3′		4.21 dd		8.8, 8.8	76.5		3′	4.21 dd		9.0, 8.2	76.5
	4′		4.19 dd		9.2, 8.8	82.1		4′	4.22 dd		9.0, 9.0	82.3
	5′		3.79 m			76.0		5′	3.83 m			76.1
	6′	a	4.48 dd		11.1, 2.9	61.9		6′	a 4.52 m			62.1
		b	4.42 br d		11.1				b 4.47 m			
Rha	1″		6.33 br s			101.3	Rha	1″	6.38 br s			101.3
	2″		4.68 br s			72.2		2″	4.72 br s			72.4
	3″		4.50 dd		8.8, 3.1	72.6		3″	4.56 dd		9.3, 2.7	72.7
	4″		4.30 dd		9.0, 8.8	73.9		4″	4.33 dd		9.3, 9.3	74.1
	5″		4.75 m			69.3		5″	4.81 m			69.4
	6″		1.71 d		6.1	18.7		6″	1.74 d		6.3	18.8
Glc (II)	1‴		5.10 d		7.8	105.1	Glc (II)	1‴	5.13 d		7.9	105.2
	2‴		4.02 dd		8.3, 7.8	74.8		2‴	4.06 dd		8.6, 7.9	75.0
	3‴		4.20 dd		9.1, 8.3	78.2		3‴	4.26 dd		8.8, 8.6	78.1
	4‴		4.21 dd		9.1, 9.1	71.1		4‴	4.29 dd		8.8, 8.8	71.2
	5‴		3.94 m			78.3		5‴	4.74 m			78.3
	6‴	a	4.41 dd		12.5, 2.4			6‴	a 4.45 br d		13.2	62.1
		b	4.29 br d		12.5				b 4.31 m			
Glc (III)	1⁗		4.78 d		7.8	104.8	Glc (III)	1⁗	4.85 d		7.8	104.9
	2⁗		4.00 dd		8.0, 7.8	75.1		2⁗	4.05 dd		8.6, 7.8	75.2
	3⁗		4.20 dd		8.8, 8.0	78.5		3⁗	4.24 dd		8.8, 8.6	78.6
	4⁗		4.18 dd		9.2, 8.8	71.6		4⁗	4.25 dd		8.8, 8.8	71.7
	5⁗		3.90 m			78.4		5⁗	3.95 m			78.5
	6⁗	a	4.51 m			62.7		6⁗	a 4.57 dd		11.8, 2.7	62.9
		b	4.35 m						b 4.41 dd		11.8, 5.0	

6							**7**							**8**						**9**							**10**				
Position			δ_H		J (Hz)	δ_C	Position		δ_H		J (Hz)	δ_C		Position		δ_H		J (Hz)	δ_C	Position		δ_H		J (Hz)	δ_C		Position		δ_H		J (Hz)	δ_C
Glc (I)	1′		4.93 d		7.7	100.0	Xyl	1′	4.83 d		7.7	100.8		Xyl	1′	4.80 d		7.6	100.8	Glc (I)	1′	5.06 d		7.7	102.2		Glc	1′	4.96 d		7.6	106.4
	2′		4.22 dd		8.0, 7.7	77.3		2′	4.19 m			77.3			2′	4.17 m			77.3		2′	4.07 dd		8.6, 7.7	75.2			2′	4.06 dd		8.8, 7.6	75.4
	3′		4.23 dd		8.8, 8.8	77.7		3′	4.23 m			77.4			3′	4.19 m			77.4		3′	4.25 dd		8.8, 8.6	78.6			3′	4.24 dd		9.0, 8.8	78.5
	4′		4.22 dd		8.8, 8.8	82.0		4′	4.24 m			79.3			4′	4.18 m			79.3		4′	4.15 dd		9.0, 8.8	71.7			4′	4.29 dd		9.0, 9.0	71.5
	5′		3.85 m			76.1		5′	a 4.38 brd		10.0	64.3			5′	a 4.36 br d		11.6	64.3		5′	4.10 ddd		9.0, 5.3, 1.6	77.3			5′	4.30 m			78.4
	6′	a	4.51 dd		11.8, 3.4	61.9			b 3.63 dd		10.0, 9.2					b 3.60 dd		11.6, 9.3		6′	a 4.88 dd		11.4, 1.6	70.3			6′	a 4.51 br d		10.7	62.4
		b	4.44 dd		11.8, 5.5																b 4.34 dd		11.4, 5.3					b 4.35 brd		10.7	
Rha	1″		6.23 br s			101.8	Rha	1″	6.29 br s			102.2		Rha	1″	6.25 br s			102.1	Glc (II)	1″	5.13 d		7.8	105.5							
	2″		4.73 br s			72.4		2″	4.77 br s			72.4			2″	4.75 br d		3.0	72.4		2″	4.07 dd		8.6, 7.8	75.4							
	3″		4.57 dd		9.3, 3.3	72.7		3″	4.57 br d		9.4	72.7			3″	4.56 dd		9.4, 3.0	72.7		3″	4.25 dd		8.8, 8.6	78.6							
	4″		4.33 dd		9.3, 9.3	74.1		4″	4.35 dd		9.4, 9.4	74.1			4″	4.33 dd		9.4, 9.4	74.1		4″	4.27 dd		8.8,8.8	71.9							

	5″		4.92	m		69.4		5″		4.89	m		69.6		5″		4.88	m		69.5		5″		3.96	m		78.5
	6″		1.75	d	6.1	18.7		6″		1.76	d	6.2	18.7		6″		1.75	d	6.2	18.6		6″	a	4.53	br d	11.3	62.8
																							b	4.40	br d	11.3	
Glc (II)	1‴		5.12	d	7.8	105.2	Glc (I)	1‴		5.01	d	7.8	104.0	Glc (I)	1‴		4.98	d	7.8	103.9							
	2‴		4.04	dd	8.3, 7.8	74.9		2‴		4.00	dd	8.4, 7.8	74.6		2‴		3.98	dd	8.4, 7.8	74.6	Glc (III)	1‴		4.78	d	7.8	104.9
	3‴		4.22	dd	8.8, 8.3	78.2		3‴		4.23	dd	8.8, 8.4	78.4		3‴		4.18	dd	8.8, 8.4	78.4		2‴		4.00	dd	8.6, 7.8	75.1
	4‴		4.26	dd	8.8, 8.8	71.2		4‴		4.24	dd	8.8, 8.8	71.5		4‴		4.20	dd	8.8, 8.8	71.4		3‴		4.19	dd	8.8, 8.6	78.6
	5‴		3.97	m		78.4		5‴		3.98	m		78.2		5‴		3.96	m		78.2		4‴		4.28	dd	8.8, 8.8	71.8
	6‴	a	4.45	br d	11.0	62.1		6‴	a	4.53	br d	12.7	62.4		6‴	a	4.50	br d	11.5	62.3		5‴		4.03	m		78.5
		b	4.32	brd	11.0				b	4.34	br d	12.7				b	4.31	brd	11.5			6‴	a	4.63	br d	11.0	63.0
																							b	4.43	dd	11.0, 5.6	
Glc (III)	1⁗		4.81	d	7.7	104.9	Glc (II)	1‴’		4.82	d	7.7	104.9	Glc (II)	1⁗		4.81	d	7.6	104.9							
	2⁗		4.02	dd	8.1,7.7	75.2		2⁗		4.03	dd	8.2, 7.7	75.2		2⁗		4.01	dd	8.2, 7.6	75.1							
	3⁗		4.22	dd	8.8, 8.1	78.6		3⁗		4.22	dd	8.8, 8.2	78.6		3⁗		4.22	dd	8.8, 8.2	78.6							
	4⁗		4.21	dd	8.8, 8.8	71.7		4⁗		4.25	dd	8.8, 8.8	71.7		4⁗		4.21	dd	8.8, 8.8	71.7							
	5⁗		3.95	m		78.4		5⁗		3.96	m		78.3		5⁗		3.94	m		78.5							
	6⁗	a	4.54	br d	11.8	62.8		6⁗	a	4.56	br d	12.5, 4.5	62.8		6⁗	a	4.53	br d	11.8	62.8							
		b	4.37	br d	11.8				b	4.38	br d	12.5				b	4.36	br d	11.8								

*2.3.14. Acetylation of **5***

A mixture of **5** (2.0 mg) and Ac_2O (1.0 mL) in pyridine (1.0 mL) was stirred at room temperature for 24 h. After the excess Ac_2O was decomposed by H_2O (10 mL), the reaction mixture was extracted with EtOAc (10 mL × 3). The EtOAc extract was purified by preparative TLC (hexane/Me_2CO, 1:1) to yield **5b** (1.3 mg).

*2.3.15. Preparation of **5b** from **4***

A mixture of **4** (10.0 mg) and Ac_2O (2.0 mL) in pyridine (2.0 mL) was stirred at 110 °C for 3 h. After the excess Ac_2O was decomposed by H_2O (10 mL), the reaction mixture was extracted with EtOAc (10 mL × 3). The EtOAc extract was purified by preparative TLC (hexane/Me_2CO, 1:1) to yield **5b** (1.0 mg).

*2.3.16. Compound **5b***

An amorphous solid; $[\alpha]_D^{25}$ −12.8 (*c* 0.05, MeOH); IR ν_{max} (film) cm^{-1}: 2926 (OH), 1749 (C=O); ^1H NMR (500 MHz, C_5D_5N): δ 3.71 (1H, m, $W_{1/2}$ = 28.5 Hz, H-3), 3.49 (1H, dd, *J* = 9.6, 5.7 Hz, H-26a), 2.21, 2.20, 2.16, 2.12, 2.06 × 2, 2.04 × 2, 2.03 × 2, 2.02, 2.00 × 2 (each 3H, s, Ac × 13), 1.68 (3H, s, Me-21), 1.47 (3H, d, *J* = 6.2 Hz, Rha-6), 1.07 (3H, s, Me-18 or Me-19), 0.95 (3H, d, *J* = 6.6 Hz, Me-27), 0.79 (3H, s, Me-18 or Me-19); HRESITOFMS *m/z*: 1617.6672 $[M + Na]^+$ (calcd for $C_{77}H_{110}O_{35}Na$: 1617.6725).

*2.3.17. Compound **6***

(25*R*)-26-[(β-D-glucopyranosyl)oxy]-17α,22α-dihydroxyfurost-5-en-3β-yl *O*-β-D-glucopyranosyl-(1 → 4)-*O*-[α-L-rhamnopyranosyl-(1 → 2)]-β-D-glucopyranoside (**6**); an amorphous solid; $[\alpha]_D^{25}$ −57.1 (*c* 0.25, MeOH); IR ν_{max} (film) cm^{-1}: 3446 (OH), 2949 (CH); ^1H NMR (500 MHz, C_5D_5N) and ^{13}C NMR (125 MHz, C_5D_5N), see Tables 1 and 2; HRESITOFMS *m/z*: 1103.5288 $[M + Na]^+$ (calcd for $C_{51}H_{84}O_{24}Na$: 1103.5250).

*2.3.18. Enzymatic hydrolysis of **6***

A solution of **6** (5.0 mg) was hydrolyzed with naringinase (16.5 mg) under the same conditions described for **2** to yield **3a** (0.4 mg) and a sugar fraction (2.7 mg). HPLC analysis of the sugar fraction under the same conditions as those used for **1** showed the presence of D-glucose (14.02, positive optical rotation) and L-rhamnose (7.81, negative optical rotation).

A solution of **6** (2.0 mg) with β-D-glucosidase (8.0 mg) was subjected to the enzymatic hydrolysis described for **4** to yield **14** (1.0 mg) and a sugar fraction (0.5 mg). HPLC analysis of the sugar fraction under the same conditions as those used for **1** showed the presence of D-glucose (14.04, positive optical rotation).

*2.3.19. Compound **7***

(25*R*)-26-[(β-D-glucopyranosyl)oxy]-22α-hydroxyfurost-5-en-3β-yl *O*-β-D-glucopyranosyl-(1 → 4)-*O*-[α-L-rhamnopyranosyl-(1 → 2)]-β-D-xylopyranoside (**7**); an amorphous solid; $[\alpha]_D^{25}$ −35.7 (*c* 0.42, MeOH); IR ν_{max} (film) cm^{-1}: 3445 (OH), 2931 (CH); ^1H NMR (500 MHz, C_5D_5N) and ^{13}C NMR (125 MHz, C_5D_5N), see Tables 1 and 2; HRESITOFMS *m/z*: 1057.5205 $[M + Na]^+$ (calcd for $C_{50}H_{82}O_{22}Na$: 1057.5195).

*2.3.20. Enzymatic hydrolysis of **7***

A solution of **7** (5.0 mg) was hydrolyzed with naringinase (100 mg) under the same conditions described for **2** to yield diosgenin (**7a**, 0.4 mg) and a sugar fraction (1.7 mg). HPLC analysis of the sugar fraction under the same conditions as those used for **1** showed the presence of D-glucose (14.12, positive optical rotation), L-rhamnose (7.56, negative optical rotation), and D-xylose (9.39, positive optical rotation).

*2.3.21. Compound **7a***

An amorphous solid; $[\alpha]_D^{25}$ −135.9 (*c* 0.05, MeOH) (Lit. −123 [13]); ^{13}C NMR (125 MHz, C_5D_5N): δ 37.8(C-1), 31.7 (C-2), 71.3 (C-3), 42.0 (C-4), 142.0 (C-5), 121.0 (C-6), 32.1 (C-7), 31.8 (C-8), 50.4 (C-9), 37.0 (C-10), 21.2 (C-11), 39.9 (C-12), 40.5 (C-13), 56.7 (C-14), 32.3 (C-15), 81.1 (C-16), 62.9 (C-17), 16.4 (C-18), 19.6 (C-19), 40.5 (C-20), 15.0 (C-21), 109.3 (C-22), 31.7 (C-23), 29.2 (C-24), 30.2 (C-25), 66.9 (C-26), 17.3 (C-27); HRESITOFMS *m/z*: 415.3221 $[M + H]^+$ (calcd for $C_{27}H_{43}O_3$: 415.3212).

*2.3.22. Compound **8***

(25*R*)-26-[(β-D-glucopyranosyl)oxy]-17α,22α-dihydroxyfurost-5-en-3β-yl *O*-β-D-glucopyranosyl-(1 → 4)-*O*-[α-L-rhamnopyranosyl-(1 → 2)]-β-D-xylopyranoside (**8**); an amorphous solid; $[\alpha]_D^{25}$ −62.3 (*c* 0.25, MeOH); IR ν_{max} (film) cm^{-1}: 3445 (OH), 2934 (CH); ^1H NMR (500 MHz, C_5D_5N) and ^{13}C NMR (125 MHz, C_5D_5N), see Tables 1 and 2; HRESITOFMS *m/z*: 1073.5187 $[M + Na]^+$ (calcd for $C_{50}H_{82}O_{23}Na$: 1073.5145).

*2.3.23. Enzymatic hydrolysis of **8***

A solution of **7** (5.0 mg) was hydrolyzed with naringinase (18.0 mg) under the same conditions described for **2** to yield **3a** (1.5 mg) and a sugar fraction (1.7 mg). HPLC analysis of the sugar fraction under the same conditions as those used for **1** showed the presence of D-glucose (13.95, positive optical rotation), L-rhamnose (7.62, negative optical rotation), and D-xylose (9.16, positive optical rotation).

*2.3.24. Compound **9***

(25*R*)-3β-[(*O*-β-D-glucopyranosyl-(1 → 6)-β-D-glucopyranosyl)oxy]-26-[(β-D-glucopyranosyl)oxy]-5α-cholestane-6,22-dione (**9**); an amorphous solid; $[\alpha]_D^{25}$ −43.4 (*c* 0.18, MeOH); IR ν_{max} (film) cm^{-1}: 3364 (OH), 2932 and 2872 (CH), 1707 and 1648 (C=O); ^1H NMR (500 MHz, C_5D_5N) and ^{13}C NMR (125 MHz, C_5D_5N), see Tables 1 and 2; HRESITOFMS *m/z*: 941.4745 $[M + Na]^+$ (calcd for $C_{45}H_{74}O_{19}Na$: 941.4722).

*2.3.25. Enzymatic hydrolysis of **9***

A solution of **9** (3.0 mg) was hydrolyzed with naringinase (20.0 mg) under the conditions described for **2** to yield **17a** (0.8 mg) and a sugar fraction (1.4 mg). HPLC analysis of the sugar fraction under the same conditions as those used for **1** showed the presence of D-glucose (14.01, positive optical rotation).

*2.3.26. Compound **17a***

An amorphous solid; $[\alpha]_D^{25}$ −34.5 (*c* 0.03, MeOH) (Lit. −16.3 [14]); IR ν_{max} (film) cm^{-1}: 2940 (CH), 1705 (C=O); ^{13}C NMR (125 MHz, C_5D_5N): δ 37.0 (C-1), 31.8 (C-2), 70.0 (C-3), 27.7 (C-4), 56.9 (C-5), 210.2 (C-6), 46.8 (C-7), 37.9 (C-8), 53.8 (C-9), 40.9 (C-10), 21.7 (C-11), 39.6 (C-12), 43.2 (C-13), 56.1 (C-14), 24.4 (C-15), 27.7 (C-16), 52.4 (C-17), 12.3 (C-18), 13.3 (C-19), 49.4 (C-20), 16.7 (C-21), 214.0 (C-22), 39.9 (C-23), 31.3 (C-24), 36.2 (C-25), 67.4 (C-26), 17.2 (C-27); HRESITOFMS *m/z*: 433.3327 $[M + H]^+$ (calcd for $C_{27}H_{45}O_4$: 433.3318).

*2.3.27. Compound **10***

(20*R*,22*R*)-22-[(β-D-glucopyranosyl)oxy]-3β,14α,20-trihydoxy-5α-cholestan-6-one (**10**): an amorphous solid; $[\alpha]_D^{25}$ −6.3 (*c* 0.03, MeOH); IR ν_{max} (film) cm^{-1}: 3420 (OH), 2950 and 2869 (CH), 1698 (C=O); ^1H-NMR (500 MHz, C_5D_5N) and ^{13}C-NMR (125 MHz, C_5D_5N), see Tables 1 and 2; HRESITOFMS *m/z*: 635.3788 $[M + Na]^+$ (calcd for $C_{33}H_{56}O_{10}Na$: 635.3771).

*2.3.28. Enzymatic hydrolysis of **10***

A solution of **10** (5.0 mg) was hydrolyzed with naringinase (30.0 mg) under the conditions described for **2** to yield tenuifoliol

(**10a**, 2.8 mg) and a sugar fraction (1.7 mg). HPLC analysis of the sugar fraction under the same conditions as those used for **1** showed the presence of D-glucose (13.89, positive optical rotation).

*2.3.29. Compound **10a***

An amorphous solid; $[\alpha]_D^{25}$ 6.22 (*c* 0.14, MeOH) (Lit. 16.8 [15]); IR ν_{max} (film) cm^{-1}: 3446 (OH), 2926 (CH), 1693 (C=O); ^{13}C NMR (125 MHz, C_5D_5N): δ 37.3(C-1), 31.4 (C-2), 70.1 (C-3), 31.9 (C-4), 56.9 (C-5), 211.8 (C-6), 42.9 (C-7), 41.0 (C-8), 46.8 (C-9), 40.8 (C-10), 21.3 (C-11), 33.0 (C-12), 48.9 (C-13), 83.9 (C-14), 32.4 (C-15), 21.6 (C-16), 50.2 (C-17), 17.8 (C-18), 13.0 (C-19), 76.8 (C-20), 21.2 (C-21), 76.9 (C-22), 30.3 (C-23), 37.2 (C-24), 28.2 (C-25), 22.4 (C-26), 23.2 (C-27); HRESITOFMS *m/z*: 451.3428 $[M+H]^+$ (calcd for $C_{27}H_{47}O_5$: 451.3424).

*2.3.30. Compound **11***

An amorphous solid; $[\alpha]_D^{25}$ −104.3 (*c* 0.04, MeOH) (Lit. −86.5 [16]); ^{13}C NMR (125 MHz, C_5D_5N), see Table 3; HRESITOFMS *m/z*: 722.4482 $[M+H]^+$ (calcd for $C_{39}H_{64}NO_{11}$: 722.4479).

*2.3.31. Compound **12***

An amorphous solid; $[\alpha]_D^{25}$ −36.5 (*c* 0.36, MeOH) (Lit. −36.5 [14]); ^{13}C NMR (125 MHz, C_5D_5N), see Table 3; HRESITOFMS *m/z*: 722.4495 $[M+H]^+$ (calcd for $C_{39}H_{64}NO_{11}$: 722.4479).

*2.3.32. Compound **13***

An amorphous solid; $[\alpha]_D^{25}$ −93.8 (*c* 0.11, MeOH) (Lit. −136.0 [18]); ^{13}C NMR (125 MHz, C_5D_5N), see Table 3; HRESITOFMS *m/z*: 739.4292 $[M+H]^+$ (calcd for $C_{39}H_{63}O_{13}$: 739.4269).

*2.3.33. Compound **14***

An amorphous solid; $[\alpha]_D^{25}$ −62.4 (*c* 0.11, MeOH); ^1H-NMR (500 MHz, C_5D_5N): δ 5.28 (1H, br d, *J* = 4.9 Hz, H-6), 3.84 (1H, m, $W_{1/2}$ = 27.5 Hz, H-3), 3.51 (2H, br d, *J* = 9.8 Hz, H-26), 1.23 (3H, d, *J* = 7.2 Hz, Me-21), 1.09 (3H, s, Me-19), 0.97 (3H, s, Me-18), 0.69 (3H, d, *J* = 5.8 Hz, Me-27), 6.26 (1H, br s, H-1″ of Rha), 5.13 (1H, d, *J* = 7.9 Hz, H-1‴ of Glc (II)), 4.97 (1H, d, *J* = 7.7 Hz, H-1′ of Glc (I)), 4.75 (1H, br s, H-2″ of Rha), 4.58 (1H, dd, *J* = 9.2, 3.0 Hz, H-3″ of Rha), 4.06 (dd, *J* = 8.5, 7.9 Hz, H-2‴ of Glc (II)), 1.76 (3H, d,

Table 3
^{13}C-NMR (125 MHz, C_5D_5N) spectral assignments for **11**–**18**.

Position	11	12	13	14	15	16	17	18
1	37.5	37.5	37.6	37.5	37.5	36.7	36.7	37.7
2	30.2	30.2	30.2	30.1	30.1	39.4	29.4	30.4
3	78.3	78.3	78.2	78.1	78.1	76.2	76.7	78.0
4	39.0	39.0	39.0	38.9	38.9	26.5	27.0	39.1
5	140.9	140.9	140.8	140.8	140.8	56.5	56.3	140.9
6	121.8	121.8	121.8	121.8	121.8	209.4	209.8	121.8
7	32.4	32.4	32.4	32.4	32.2	46.7	46.6	32.3
8	31.6	31.6	31.6	31.8	31.7	37.3	37.7	32.1
9	50.3	50.4	50.2	50.2	50.3	53.7	53.7	50.3
10	37.2	37.2	37.2	37.2	37.1	41.0	40.8	37.2
11	21.2	21.1	20.9	21.0	21.1	21.5	21.5	21.0
12	40.1	40.1	31.8	32.0	39.8	39.6	39.5	32.0
13	40.6	40.7	45.1	44.8	40.5	40.9	43.1	45.1
14	56.7	56.0	53.0	53.0	56.7	56.3	55.9	53.0
15	32.6	33.1	32.1	32.1	31.4	31.7	24.2	32.5
16	78.8	78.7	90.0	90.1	81.4	80.8	27.6	90.5
17	63.5	62.2	90.1	90.2	62.9	62.8	52.3	90.8
18	16.5	16.8	17.1	17.1	16.3	16.4	12.2	17.2
19	19.4	19.4	19.4	19.4	19.4	13.1	13.0	19.4
20	41.6	43.0	44.8	45.1	42.0	41.9	49.3	43.6
21	15.7	16.2	9.7	9.7	15.0	14.9	16.6	10.5
22	98.4	99.4	109.8	109.8	111.8	109.2	213.9	111.4
23	34.7	27.1	30.0	30.0	32.3	31.8	39.6	36.9
24	31.1	29.3	28.8	28.8	29.3	29.2	27.8	28.0
25	31.6	31.4	30.4	30.4	35.5	30.6	33.4	34.3
26	48.1	50.6	66.7	66.7	103.1	66.9	74.9	75.2
27	19.8	19.8	17.3	17.3	16.7	17.3	17.3	17.5
OMe					55.6			

Position	11	12	Position	13	14	15	16	Position	17	Position	18
Glc (I)	100.4	100.4	Glc (I)	100.3	100.0	100.0	100.0	Glc (I)	102.1	Glc (I)	101.3
	77.8	77.9		77.8	77.3	77.3	78.2		75.3		77.9
	79.7	79.6		79.6	77.7	77.7	79.5		78.6		79.6
	71.8	71.8		71.8	82.0	82.0	72.0		71.7		71.8
	77.8	77.9		77.9	76.2	76.2	78.4		78.5		77.9
	62.7	62.7		62.7	61.9	61.9	62.8		62.8		62.8
Rha	102.1	102.1	Rha	102.1	101.8	101.8	102.2	Glc (II)	104.9	Rha	102.1
	72.6	72.6		72.6	72.5	72.4	72.5		75.1		72.6
	72.9	72.8		72.8	72.8	72.8	72.8		78.6		72.8
	74.2	74.2		74.2	74.2	74.1	74.1		71.8		74.2
	69.5	69.5		69.5	69.5	69.5	69.5		78.5		69.5
	18.7	18.7		18.7	18.7	18.6	18.7		63.0		18.7
			Glc (II)		105.2	105.2				Glc (II)	105.0
					75.0	75.0					75.2
					78.3	78.3					78.6
					71.2	71.2					71.4
					78.5	78.5					78.5
					62.1	62.1					62.8

J = 6.3 Hz, H-6″ of Rha); HRESITOFMS m/z: 923.4651 $[MH + Na]^+$ (calcd for $C_{45}H_{72}O_{18}Na$: 923.4616).

2.3.34. Compound **15**

An amorphous solid; $[\alpha]_D^{25}$ −60.1 (c 0.59, MeOH) (Lit. −95.9 [19]); ^{13}C NMR (125 MHz, C_5D_5N), see Table 3; HRESITOFMS m/z: 937.4803 $[M + Na]^+$ (calcd for $C_{46}H_{74}O_{18}Na$:937.4773).

2.3.35. Compound **16**

An amorphous solid; $[\alpha]_D^{25}$ −96.9 (c 0.15, MeOH) IR ν_{max} (film) cm^{-1}: 3410 (OH), 2926 (CH), 1708 (C=O); ^1H-NMR (500 MHz, C_5D_5N): δ 4.01 (1H, m, $W_{1/2}$ = 18.5 Hz, H-3), 3.58 (1H, dd, J = 10.7, 3.7 Hz, H-26a), 3.49 (1H, dd, J = 10.7, 10.7 Hz, H-26b), 2.38 (1H, dd, J = 12.9, 4.4 Hz, H-7 eq), 1.13 (3H, d, J = 6.9 Hz, Me-21), 1.13 (3H, d, J = 6.9 Hz, Me-21), 1.03 (1H, ddd, J = 13.6, 13.6, 3.8 Hz, H-1), 0.78 (3H, s, Me-18), 0.77 (3H, s, Me-19), 0.69 (3H, d, J = 5.9 Hz, Me-27), 6.34 (1H, br s, H-1″ of Rha), 4.65 (1H, dd, J = 9.2, 3.0 Hz, H-3″ of Rha), 1.78 (3H, d, J = 6.2 Hz, H-6″ of Rha); HRESITOFMS m/z: 761.4092 $[M + Na]^+$ (calcd for $C_{39}H_{62}O_{13}Na$: 761.4088).

2.3.36. Compound **17**

An amorphous solid; $[\alpha]_D^{25}$ −40.3 (c 0.10, MeOH) (Lit. −42.4 [20]); IR ν_{max} (film) cm^{-1}: 3420 (OH), 2939 (CH), 1699 (C=O); ^{13}C NMR (125 MHz, C_5D_5N), see Table 3; HRESITOFMS m/z: 779.4130 $[M + Na]^+$ (calcd for $C_{39}H_{64}O_{14}Na$: 779.4194).

2.3.37. Compound **18**

An amorphous solid; $[\alpha]_D^{25}$ −109.7 (c 0.08, MeOH) (Lit. −85.2 [18]); ^{13}C NMR (125 MHz, C_5D_5N), see Table 3; HRESITOFMS m/z: 871.4699 $[M + H–OH–OCH_3]^+$ (calcd for $C_{44}H_{71}O_{17}$: 871.4691).

2.4. Cell culture assays

The growth of HL-60 and A549 cells was measured with an MTT assay according to a previously reported method [21].

2.4.1. Assay for caspases-3, -8, and -9 activation

The activities of caspases-3, -8, and -9 in HL-60 cells were measured by using a commercially available kit (Apopcyto Caspases-3, -8, and -9 Colorimetric Assay Kit, Medical and Biological Laboratories, Aichi, Japan) as previously described [21]. A549 cells (2×10^6) were treated with each compound for 12 and for 24 h, in separate experiments, and the cells were centrifuged and collected. The cell lysate (50 μL, equivalent to 200 μg of protein) was mixed with portions of reaction buffer (50 μL) containing the substrates for caspases-3, -8, and -9 (DEVD-p-nitroanilide (pNA), IETD-pNA, and LEHD-pNA). After incubation for 5 h at 37 °C, the absorbance at 405 nm of the liberated pNA chromophore was measured using a microplate reader. The activities of caspases-3, -8, and -9 were evaluated in triplicate.

2.4.2. Cell cycle analysis by flow cytometry

HL-60 cells (3×10^6) were treated with each compound for 12 and for 24 h, in separate experiments, washed with PBS, and fixed with 1% paraformaldehyde at 0 °C for 10 min and 70% EtOH at −20 °C overnight. The cells were treated with 0.25% Triton X-100 and were stained with PI for 20 min. Analysis of the cell cycle distribution was performed using a flow cytometer (FACSCanto II, BD Biosciences, USA).

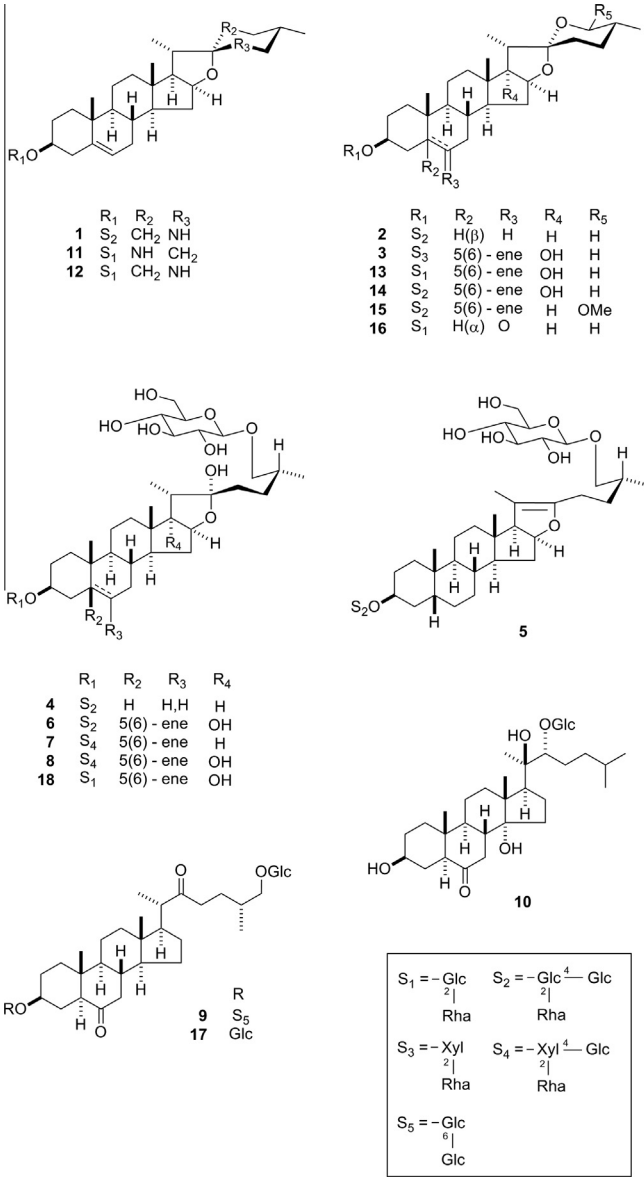

Fig. 1. Steroidal glycosides isolated from *Fritillaria meleagris*.

3. Results and discussion

3.1. Structural elucidation

The bulbs of *F. meleagris* (6.0 kg of fresh weight) were extracted with hot MeOH. The MeOH extract (300 g) was passed through a Diaion HP-20 column, and the MeOH-eluted fraction (80.0 g), in which steroidal glycosides were enriched, was subjected to CC using silica gel and ODS silica gel, and to reversed-phase preparative HPLC, giving compounds **1**–**18** (Fig. 1). Compounds **11**–**18** were identified as (22*R*,25*R*)- spirosol-5-en-3β-yl *O*-α-L-rhamnopyranosyl-(1 → 2)-β-D-glucopyranoside (**11**) [16], (22*S*,25*S*)-spirosol-5-en-3β-yl *O*-α-L-rhamnopyranosyl-(1 → 2)-β-D-glucopyranoside (**12**) [22,23], (25*R*)-17α-hydroxy-spirost-5-en-3β-yl *O*-α-L-rhamnopyranosyl-(1 → 2)-β-D-glucopyranoside (**13**) [24], (25*R*)-17α-hydroxyspirost-5-en-3β-yl *O*-β-D-glucopyranosyl-(1 → 4)-*O*-[α-L-rhamnopyranosyl-(1 → 2)]-β-D-glucopyranoside (**14**) [17], (25*R*,26*R*)-26-methoxyspirost-5-en-3β-yl *O*-β-D-glucopyranosyl-(1 → 4)-*O*-[α-L-rhamnopyranosyl-(1 → 2)]-β-D-glucopyranoside (**15**) [25], (25*R*)-3β-[(*O*-α-L-rhamnopyranosyl-(1 → 2)-β-D-gluco-

pyranosyl)oxy]-5α-spirostan-6-one (**16**) [26], (25*R*)-3β-[(β-D-glucopyranosyl)oxy]-26-[(β-D-glucopyranosyl)oxy]-5α-cholestane-6,22-dione (**17**) [20], and (25*R*)-26-[(β-D-glucopyranosyl)oxy]-17α,22α-dihydroxyfurost-5-en-3β-yl *O*-α-L-rhamnopyranosyl-(1 → 2)-β-D-glucopyranoside (**18**) [24], respectively, by comparison of their physical and spectroscopic data with literature values.

Compound **1** was obtained as an amorphous solid, $[\alpha]_D^{25}$ −64.8 in MeOH, and showed a positive color reaction in the Dragendorff test on TLC, which indicates the presence of an alkaloid. The molecular formula of **1** was assigned as $C_{45}H_{74}O_{16}N$ based on the HRESI-TOFMS (*m/z* 884.5045 $[M+H]^+$, calcd. 884.5008), and the ^{13}C NMR data (45 carbon signals). The IR spectrum of **1** suggested the presence of hydroxy groups (3445 cm^{-1}). The ^1H NMR spectrum showed two singlet signals for tertiary methyl groups at δ_H 1.05 and 0.86 (each s), and three doublet signals for secondary methyl groups at δ_H 1.76 (d, *J* = 6.2 Hz), 1.06 (d, *J* = 7.2 Hz), and 0.82 (d, *J* = 6.5 Hz). The signal at δ_H 1.76 was assigned to the methyl group of 6-deoxyhexose. The above spectral properties, together with a quaternary carbon signal at δ_C 99.4 (C) and a pair of olefinic carbon signals at δ_C 140.8 (C) and 121.8 (CH) in the ^{13}C NMR spectrum, and a broad doublet olefinic proton signal at δ_H 5.30 (br d, *J* = 4.9 Hz) in the ^1H NMR spectrum suggested that the aglycone of **1** was a spirosol-5-ene derivative. Furthermore, the ^1H and ^{13}C NMR spectra of **1**, contained signals for three anomeric protons at δ_H 6.25 (br s), 5.13 (d, *J* = 7.9 Hz), and 4.95 (d, *J* = 6.8 Hz), and the corresponding carbon signals at δ_C 105.2 (CH), 101.8 (CH), and 100.0 (CH). Acid hydrolysis of **1** with 1 M HCl in dioxane/H_2O (1:1) gave (22*S*,25*S*)-spirosol-5-en-3β-ol (tomatidenol, **1a**) [9] as the aglycone, and D-glucose and L-rhamnose as the carbohydrate moieties. The NOE correlations between Me-21 (δ_H 1.06) and H_2-23 (δ_H 1.73 and 1.44), and H-23 eq (δ_H 1.73) and H-16 (δ_H 4.17)/H-17 (δ_H 1.57) in the NOESY spectrum of **1** were consistent with the 22*S* configuration of the aglycone. The monosaccharides and their absolute configurations were identified by direct HPLC analysis of the hydrolysate. The ^1H–^1H COSY and the HMQC spectra of **1** suggested that the triglycoside attached to the C-3 hydroxy group, which was composed of a C-2 and C-4 disubstituted β-D-glucopyranosyl unit (Glc (I)), a terminal α-L-rhamnopyranosyl unit (Rha), and a terminal β-D-glucopyranosyl unit (Glc (II)), was the same as that of the known compounds **14** and **15**. This was determined by HMBC correlations between the H-1″ proton of Rha at δ_H 6.25 and C-2′ of Glc (I) at δ_C 77.3; the H-1‴ proton of Glc (II) at δ_H 5.13 and C-4′ of Glc (I) at δ_C 82.0; and between the H-1′ proton of Glc (I) at δ_H 4.95 and C-3 of the aglycone at δ_C 78.1. Thus, **1** was assigned as (22*S*,25*S*)-spirosol-5-en-3β-yl*O*-β-D-glucopyranosyl-(1 → 4)-*O*-[α-L-rhamnopyranosyl-(1 → 2)]-β-D-glucopyranoside.

The HRESITOFMS data (*m/z* 887.4977 $[M+H]^+$, calcd. 887.5004) showed that **2** had a molecular formula of $C_{45}H_{74}O_{17}$. The ^1H NMR spectrum of **2** showed signals for four steroidal methyl groups at δ_H 1.14 (d, *J* = 6.9 Hz), 1.07 (s), 0.82 (s), and 0.69 (d, *J* = 5.3 Hz), and for three anomeric protons at δ_H 6.34 (br s), 5.11 (d, *J* = 7.9 Hz), and 4.80 (d, *J* = 7.3 Hz). The ^{13}C NMR spectrum of **2** showed signals which were assigned to an acetalic carbon (δ_C 109.2), four methyl groups (δ_C 23.8, 17.3, 16.6, and 15.0), and three anomeric carbons (δ_C 105.1, 101.9, and 101.3). These NMR data suggested that **2** was a spirostanol glycoside. Enzymatic hydrolysis of **2** with naringinase gave (25*R*)-5β-spirostan-3β-ol (smilagenin, **2a**) [27], D-glucose, and L-rhamnose. The NOE correlations between H-5 (δ_H 2.14) and Me-19 (δ_H 1.07), and Me-19 and H-8 (δ_H 1.55) in the NOESY spectrum of **2** provided evidence for the steroidal aglycone A/B cis (5β) ring function. Analysis of the ^1H NMR, ^{13}C NMR, HMQC, and HMBC spectra of **2** indicated that the triglycoside linked to C-3 of the aglycone was the same as that of **1**. Based on the above data, **2** was assigned as (25*R*)-5β-spirostan-3β-yl*O*-β-D-glucopyranosyl-(1 → 4)-*O*-[α-L-rhamnopyranosyl-(1 → 2)]-β-D-glucopyranoside.

The ^1H NMR spectrum of **3** ($C_{38}H_{60}O_{12}$) displayed signals for four steroidal methyl groups at δ_H 1.22 (d, *J* = 7.0 Hz), 1.09 (s), 0.96 (s), and 0.68 (d, *J* = 5.3 Hz), and two anomeric protons at δ_H 6.35 (br s) and 4.86 (d, *J* = 7.0 Hz), which suggested that it was a steroidal diglycoside closely related to **13**. Acid hydrolysis of **3** with 0.5 M HCl in dioxane/H_2O (1:1) furnished (25*R*)-17α-hydroxyspirost-5-en-3β-ol (pennogenin, **3a**), [27] L-rhamnose, and D-xylose. The results of acid hydrolysis of **3** and the comparison of the ^1H and ^{13}C NMR spectra with those of **13** implied that the inner monosaccharide constituent of **3** was different from that of **13**. Instead of the signals for a 2-substituted glucopyranosyl moiety, five signals were observed, which could be assigned to a 2-substituted β-D-xylopyranosyl residue (Xyl) [$\delta_{H\text{-}1}$ 4.86 (d, *J* = 7.0 Hz); δ_C 101.2 (CH), 77.9 (CH), 79.6 (CH), 71.4 (CH), and 67.0 (CH_2)]. In the HMBC spectrum of **3**, the H-1″ proton of Rha at δ_H 6.35 showed a long-range correlation with the C-2′ of Xyl at δ_C 77.9, of which H-1′ at δ_H 4.86 exhibited a correlation with the C-3 of the aglycone at δ_C 78.1. Accordingly, 3 was assigned as (25*R*)-17α-hydroxyspirost-5-en-3β-yl *O*-α-L-rhamnopyranosyl-(1 → 2)-β-D-xylopyranoside.

The HRESITOFMS data (m/z 1089.5491 $[M+Na]^+$, calcd. 1089.5458) showed **4** had a molecular formula of $C_{51}H_{86}O_{23}$. The ^1H NMR spectrum of **4** showed signals for two tertiary methyl groups at δ_H 1.07 and 0.87 (each s), two secondary methyl groups at δ_H 1.32 (d, *J* = 6.8 Hz), and 0.97 (d, *J* = 6.7 Hz), and for four anomeric protons at δ_H 6.33 (br s), 5.10 (d, *J* = 7.8 Hz), and 4.78 (d, 2H, *J* = 7.8 Hz). In addition, an acetalic carbon signal at δ 110.6 and a positive color reaction in Ehrlich's test, suggested that **4** was a furostanol glycoside with four monosaccharides. Enzymatic hydrolysis of **4** with β-D-glucosidase yielded **2** and D-glucose, whereas acid hydrolysis of **4** with 0.5 M HCl in dioxane/H_2O (1:1) gave the corresponding spirostanol sapogenin **2a**, D-glucose, and L-rhamnose. The HMBC spectrum of **4** showed a long range correlation between H-1⁗ of Glc (III) at δ_H 4.78 (d, *J* = 7.8 Hz) and C-26 of the aglycone at δ_C 75.2, which is typical of naturally occurring furostanol glycosides. The absolute configuration of the C-22 hydroxy group of **4** was established as C-22α based on the NOE correlations between the signals of the H-20 proton at δ 2.23 and the H_2-23 protons at δ_H 2.01 and 1.95. The ^{13}C NMR signals of C-22 and its neighboring carbons of **4** were similar to those of a reported 22α-hydroxyl furostanol glycoside, which also supported the assignment of the absolute configuration [28]. Accordingly, the structure of **4** was assigned as (25*R*)-26-[(β-D-glucopyranosyl)oxy]-22α-hydroxy-5β-furostan-3β-yl *O*-β-D-glucopyranosyl-(1 → 4)-*O*-[α-L-rhamnopyranosyl-(1 → 2)]-β-D-glucopyranoside.

The spectroscopic data for **5** ($C_{51}H_{84}O_{22}$) suggested it was a furostanol glycoside, with a similar structure to **4**. However, the molecular formula of **5** was H_2O smaller than that of **4** and the ^{13}C NMR spectrum of **5** indicated the presence of an olefinic functionality [δ_C 152.4 (C) and 103.6 (C)]. Furthermore, the Me-21 doublet signal observed at δ_H 1.32 (d, *J* = 6.8 Hz) and the H-17 signal at δ_H 1.95 (dd, *J* = 7.3, 6.4 Hz) in the ^1H NMR spectrum of **4** were replaced by a deshielded methyl singlet signal at δ_H 1.65 (Me-21) and a doublet signal at δ_H 2.48 (H-17, d, *J* = 6.8 Hz), respectively, in the spectrum of **5**. These data suggested that **5** was the $\Delta^{20(22)}$-pseudo-furostanol glycoside of **4**. This was confirmed by the fact that the tridecaacetate of **5** (**5b**) was the same as the product obtained by treating **4** with Ac_2O in pyridine at 110 °C for 3 h, during which the dehydration occurred between C-20 and C-22, and all the hydroxy groups were acetylated. The structure of **5** was assigned as (25*R*)-26-[(β-D-glucopyranosyl)oxy]-5β-furost-20(22)-en-3β-yl *O*-β-D-glucopyranosyl-(1 → 4)-*O*-[α-L-rhamnopyranosyl-(1 → 2)]-β-D-glucopyranoside.

The HRESITOFMS data (*m/z* 1103.5288 $[M+Na]^+$, calcd. 1103.5250) showed that the molecular formula of **6** was $C_{51}H_{84}O_{24}$, and its spectroscopic data showed that it was the 26-(β-D-glucopyranosyl)oxy-22α-hydroxyfurostanol glycoside of **14**.

Enzymatic hydrolysis of **6** with β-D-glucosidase gave **14** and D-glucose, whereas enzymatic hydrolysis of **6** using naringinase gave **3a** as the corresponding spirostanol sapogenin, D-glucose, and L-rhamnose. The HMBC correlations of **6** confirmed that the triglycoside linked to C-3 of the aglycone was the same as that of **14**, and that one β-D-glucopyranosyl unit was attached to C-26. Thus, the structure of **6** was assigned as (25*R*)-26-[(β-D-glucopyranosyl)oxy]-17α,22α-dihydroxyfurost-5-en-3β-yl-β-D-glucopyranosyl-(1 → 4)-*O*-[α-L-rhamnopyranosyl-(1 → 2)]-β-D-glucopyranoside.

Compound **7** ($C_{50}H_{82}O_{22}$) appeared to be a furostanol saponin related to **6**. The ^{1}H NMR spectrum displayed signals for two tertiary methyl groups at δ_H 1.05 and 0.90 (each s), two secondary methyl groups at δ_H 1.34 (d, J = 6.9 Hz) and 1.00 (d, J = 6.6 Hz), and for four anomeric protons at δ_H 6.29 (br s), 5.01 (d, J = 7.8 Hz), 4.83 (d, J = 7.7 Hz), and 4.82 (d, J = 7.7 Hz). Enzymatic hydrolysis of **7** with naringinase gave (25*R*)-spirost-5-en-3β-ol (diosgenin, **7a**) [27], D-glucose, L-rhamnose, and D-xylose. The ^{1}H–^{1}H COSY and HMQC correlations indicated that **7** contains a C-2 and C-4 disubstituted β-D-xylopyranosyl moiety [$\delta_{H\text{-}1}$ 4.83 (1H, d, J = 7.7 Hz); δ_C 100.8, 77.3, 77.4, 79.3, 64.3 (C-1′ to C-6′)], a terminal α-L-rhamnopyranosyl moiety [$\delta_{H\text{-}1}$ 6.29 (1H, br s); δ_C 102.2, 72.4, 72.7, 74.1, 69.6, 18.7 (C-1″ to C-6″)], and two terminal β-D-glucopyranosyl moieties [$\delta_{H\text{-}1}$ 5.01 (1H, d, J = 7.8 Hz); δ_C 104.0, 74.6, 78.4, 71.5, 78.2, 62.4 (C-1‴ to C-6‴) (Glc (I)); $\delta_{H\text{-}1}$ 4.82 (1H, d, J = 7.7 Hz); δ_C 104.9, 75.2, 78.6, 71.7, 78.3, 62.8 (C-1⁗ to C-6⁗) (Glc (II))]. In the HMBC spectrum, long-range correlations were observed between H-1″ of Rha at δ_H 6.29 and C-2′ of Xyl at δ_C 77.3; H-1‴ of Glc (I) at δ_H 5.01 and C-4′ of Xyl at δ_C 79.3; H-1′ of Xyl at δ_H 4.83 and C-3 of the aglycone at δ_C 78.2; and between H-1⁗ of Glc (II) at δ_H 4.82 and C-26 of the aglycone at δ_C 75.3. The NOE correlations between the H-20 proton at δ_H 2.24 and the H_2-23 protons at δ_H 2.05 (2H) were consistent with the C-22α configuration. Based on these data, the structure of **7** was assigned as (25*R*)-26-[(β-D-glucopyranosyl)oxy]-22α-hydroxyfurost-5-en-3β-yl *O*-β-D-glucopyranosyl-(1 → 4)-*O*-[α-L-rhamnopyranosyl-(1 → 2)]-β-D-xylopyranoside.

Compound **8** ($C_{50}H_{82}O_{23}$) showed spectral features similar to those of **7**. However, the molecular formula of **8** was one oxygen atom in excess of **7** and significant differences were observed in the signals for ring D (C-13 to C-17). When the ^{1}H and ^{13}C NMR spectra of **8** were compared with those of **7**, the signal for the C-17 carbon at δ_C 63.9 (CH) was displaced by the downfield-shifted quaternary carbon at δ_C 90.8 (C). Enzymatic hydrolysis of **8** with naringinase gave **3a**, D-glucose, L-rhamnose, and D-xylose. These data indicated a hydroxy group was located at C-17α in **8** and allowed the structure of **8** to be assigned as (25*R*)-26-[(β-D-glucopyranosyl)oxy]-17α,22α-dihydroxyfurost-5-en-3β-yl *O*-β-D-glucopyranosyl-(1 → 4)-*O*-[α-L-rhamnopyranosyl-(1 → 2)]-β-D-xylopyranoside.

The molecular formula of **9** ($C_{45}H_{74}O_{19}$) was $C_6H_{10}O_5$ in excess of **17**, which corresponded to a hexosyl unit. The ^{1}H and ^{13}C NMR data for **9** were analogous to those of **17**; the cholestane skeleton had two carbonyl groups and two β-D-glucopyranosyl moieties [Glc (I) and Glc (II)]. However, unlike **17**, **9** had signals which could be assigned to one more β-D-glucopyranosyl residue (Glc (III)). Enzymatic hydrolysis of **9** with naringinase gave (25*R*)-3β,26-dihydroxy-5α-cholestane-6,22-dione (**17a**) [14] and D-glucose. In the HMBC spectrum, long-range correlations were observed between H-1″ of Glc (II) at δ_H 5.13 and C-6′ of Glc (I) at δ_C 70.3; H-1′ of Glc (I) at δ_H 5.06 and C-3 of the aglycone at δ_C 76.7; and between H-1‴ of Glc (III) at δ_H 4.78 and C-26 of the aglycone at δ_C 75.0. The anomeric configuration of the Glc (III) unit was assigned as β from the relatively large J value of the anomeric proton (7.8 Hz). Thus, the structure of **9** was (25*R*)-3β-[(*O*-β-D-glucopyranosyl-(1 → 6)-β-D-glucopyranosyl)oxy]-26-[(β-D-glucopyranosyl)oxy]-5α-cholestane-6,22-dione.

The HRESITOFMS data showed that **10** had a molecular formula of $C_{33}H_{56}O_{10}$. The IR spectrum of **10** showed a prominent carbonyl absorption at 1698 cm^{-1} and the ^{1}H NMR spectrum exhibited singlet signals for three tertiary methyl groups at δ_H 1.52, 1.35, and 0.81 (each s), two secondary methyl groups at δ_H 0.83 (d, J = 6.3 Hz), and 0.82 (d, J = 6.8 Hz), and an anomeric proton at δ_H 4.96 (d, J = 7.6 Hz). These spectral features suggested that **10** was also a cholestane glycoside with a carbonyl group. Enzymatic hydrolysis of **10** with naringinase gave (20*R*,22*R*)-3β,20,22-trihydoxy-5α-cholestan-6-one (tenuifoliol, **10a**) [15,29] and D-glucose. In the HMBC spectrum of **10**, H-1′ of the β-D-glucopyranosyl unit at δ_H 4.96 (d, J = 7.6 Hz) showed a long-range correlation with C-22 of the cholestane aglycone at δ_C 90.0. The structure of **10** was identified as (20*R*,22*R*)-22-[(β-D-glucopyranosyl)oxy]-3β,14α,20-trihydoxy-5α-cholestan-6-one.

A variety of steroidal glycosides (**1**–**17**) were isolated from the bulbs of *F. meleagris*, and they were classified as steroidal alkaloid glycosides of (**1**, **11**, and **12**), spirostanol derivatives (**2**, **3**, and **13**–**16**), furostanol derivatives (**4**–**7**, **8**, and **18**), pseudo-furostanol derivatives (**5**), and cholestane derivatives (**9** and **17**). Compounds **1**–**10** are new naturally occurring steroidal glycosides, and **3**, **7**, and **8** are rare types of steroidal glycosides which contain an inner β-D-xylopyranosyl moiety directly attached to the C-3 hydroxy group of the aglycone.

3.2. Cytotoxic activity

The isolated compounds **1**–**18** were evaluated for their cytotoxic activities against HL-60 and A549 cells using a modified MTT assay method (Table 4). Compounds **4** and **13**–**15** exhibited cytotoxic activities against both HL-60 and A549 cells with IC_{50} values ranging from 3.8 ± 0.25 μM to 6.8 ± 0.25 μM. Etoposide and cisplatin were used as the positive controls, and had IC_{50} values of 0.3 ± 0.01 μM and 4.8 ± 0.15 μM against HL-60 cells, and

Table 4
Cytotoxic activities of **1**–**18**, **1a**, **2a**, **3a**, **7a**, **10a**, and **17a** against HL-60 and A549 cells.

Compounds	IC_{50} (μM)[a]	
	HL-60	A549
1	5.0 ± 0.16	>10
1a	>10	>10
2	5.7 ± 0.10	>10
2a	>10	>10
3	>10	>10
3a	>10	>10
4	3.8 ± 0.25	6.8 ± 0.25
5	>10	7.6 ± 0.24
6	>10	4.5 ± 0.05
7	>10	>10
7a	>10	>10
8	>10	>10
9	>10	>10
10	>10	>10
10a	>10	>10
11	>10	7.9 ± 0.16
12	4.4 ± 0.92	>10
13	6.1 ± 0.04	6.5 ± 0.22
14	6.8 ± 0.05	4.4 ± 0.09
15	4.7 ± 0.09	5.2 ± 0.02
16	>10	>10
17	>10	>10
17a	7.8 ± 0.09	>10
18	>10	>10
Etoposide	0.3 ± 0.01	4.8 ± 0.15
Cisplatin	1.1 ± 0.01	2.4 ± 0.06

[a] Data are represented the mean value ± S.E.M. of three experiments performed in triplicate.

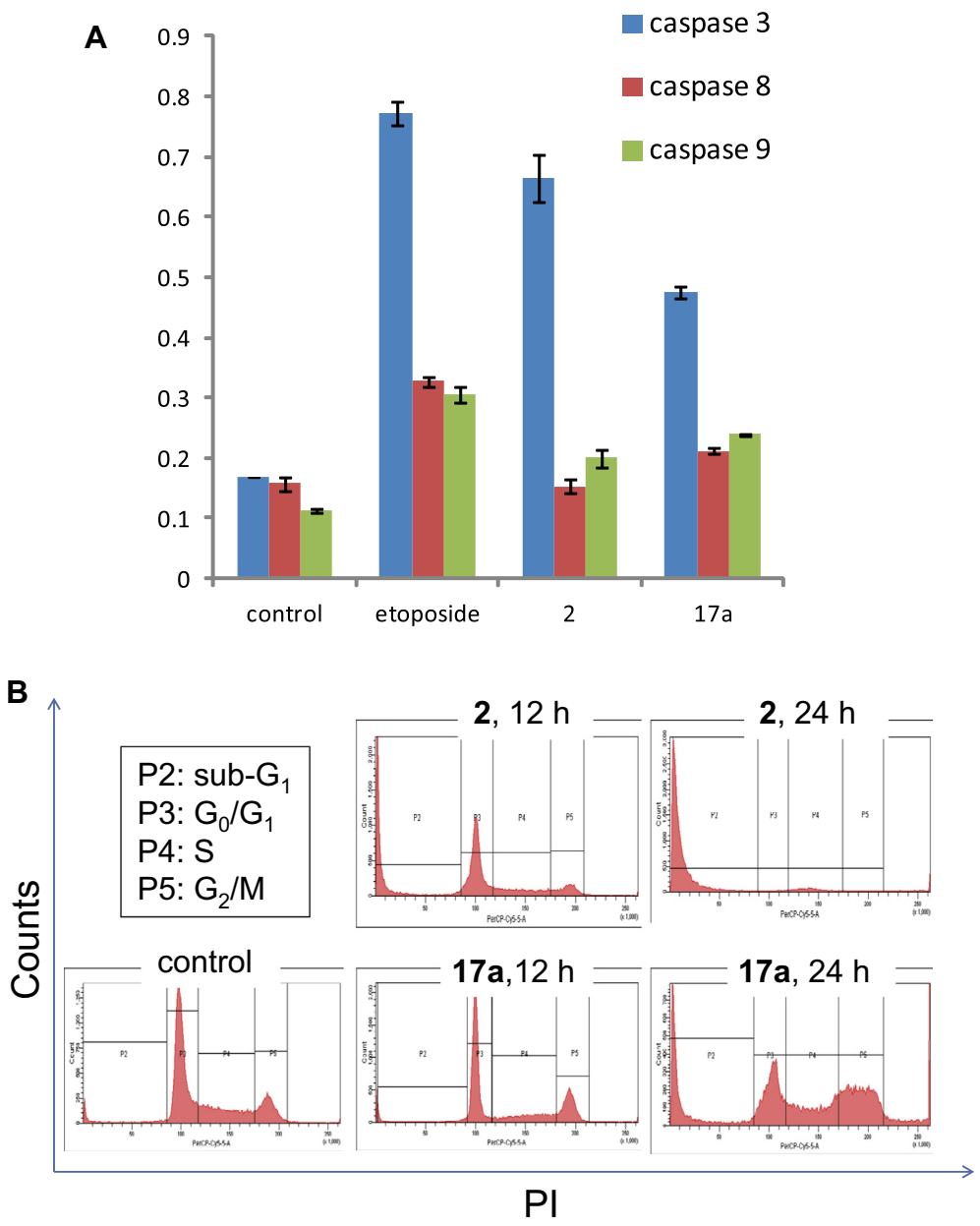

Fig. 2. (A) Caspases-3, -8, and -9 activities in **2**, **17a**, or etoposide-treated HL-60 cell lysates. HL-60 cells were incubated with 20 μg/mL of **2**, **17a**, or etoposide at 37 °C for 17 h. Each value represents the mean ± standard error of triplicate measurements. (B) The cell populations in HL-60 cells stained with propidium iodide (PI) determined by flow cytometry (FACS). HL-60 cells were incubated with 20 μg/mL of **2**, **17a** or etoposide for 12 and 24 h, respectively. The experiments were performed in triplicate.

1.1 ± 0.01 μM and 2.4 ± 0.06 μM against A549 cells, respectively. The (22*S*)-spirosol glycosides, (**1** and **12**) only showed cytotoxic activities against HL-60 cells with respective IC_{50} values of 5.0 ± 0.16 and 4.4 ± 0.92 μM, whereas the (22*R*)-spirosol glycoside (**11**) was selectively cytotoxic to A549 cells with an IC_{50} value of 7.9 ± 0.16 μM. Interestingly, the absolute configuration of C-22 contributed to the selective cytotoxicity of the spirosol glycosides. The comparison of the cytotoxic activities of **6**, **13**, and **14** with those of **3**, **7**, and **8**, showed that replacing the β-D-glucopyranosyl unit at C-3 of the aglycone with the β-D-xylopyranosyl unit diminished the cytotoxic activities of **6**, **13**, and **14**. This showed that the β-D-glucopyranosyl moiety at C-3 in the cytotoxic glycosides plays an important role in their activity. Morphological observation of the cultured tumor cells stained with DAPI suggested that **2** and **17a** induced apoptosis in HL-60 cells, and **11** induced apoptosis in 549 cells (data not shown). The activation of caspase by **1**, **11**, and **17a** was evaluated. Although no significant activation of caspases-8 and -9 were observed, caspase-3 was activated when the HL-60 cells were treated with **2** and **17a** at a sample concentration of 20 μg/mL for 17 h (Fig. 2). Furthermore, the cell cycle distribution of HL-60 cells treated with **2** and **17a** for **12** and 24 h was analyzed using flow cytometry (Table 5). The sub-G1 population of HL-60 cells, which was quantified with the apoptosis index, was 4.4 ± 0.46% in the vehicle control. When HL-60 cells were cultured with **2** (20 μg/mL) for **12** and 24 h, the sub-G1 population increased to 36.0 ± 0.60% and 88.1 ± 0.95%, respectively. This implied that the inhibition of growth by **2** was mediated by the time-dependent induction of apoptosis, rather than cell cycle arrest in HL-60 cells. In contrast, the sub-G1 peak appeared after HL-60 cells were treated with **17a** continuously for 24 h, and the G2/M phase cell population also increased to 27.7 ± 0.05%. This showed that **17a** simultaneously arrested HL-60 cell proliferation in the G2/M

Table 5
Effects of **2** and **17a** on cell cycle distribution of HL-60.[a]

	% sub G0/G1	% G0/G1	% *S*	% G2-M
Control	4.4 ± 0.46	51.5 ± 0.73	26.4 ± 0.87	16.4 ± 1.77
2, 12 h	36.0 ± 0.60	32.3 ± 5.82	13.6 ± 0.83	9.7 ± 0.99
2, 24 h	88.1 ± 0.95	1.6 ± 0.45	6.7 ± 0.85	1.1 ± 0.01
17a, 12 h	6.0 ± 1.10	46.3 ± 2.30	24.2 ± 4.00	19.9 ± 3.60
17a, 24 h	17.9 ± 1.30	22.7 ± 1.60	18.3 ± 1.42	27.7 ± 0.05
Etoposide	42.9 ± 4.20	33.9 ± 1.14	18.5 ± 3.23	4.2 ± 1.08

Data are represented the mean value ± S.E.M. of three experiments performed in triplicate.
[a] The cell cycle distribution was determined by flow cytometry.

Table 6
Effects of **11** on cell cycle distribution of A549.[a].

	% sub G0/G1	% G0/G1	% *S*	% G2-M
Control	0.63 ± 0.38	67.2 ± 2.77	14.3 ± 0.65	14.4 ± 1.30
11, 24 h	19.4 ± 1.83	63.7 ± 2.23	10.7 ± 0.43	4.8 ± 0.45
Etoposide	15.6 ± 2.20	58.3 ± 1.05	14.7 ± 0.70	10.8 ± 1.35

Date are represented the mean value ± S.E.M. of three experiments.
Performed in triplicate.
[a] The cell cycle distribution was determined by flow cytometry.

phase, and that **2** and **17a** induced apoptotic cell death in HL-60 cells through a different mechanism of action. Finally, **11** was selectively cytotoxic to A549 cells, but was not cytotoxic to HL-60 cells. Treatment of A549 cells with **11** (20 μg/mL) for 24 h increased the sub-G1 phase cells, and induced apoptotic cell death without affecting the caspase-3 activity level (Table 6). Although the cytotoxic potency of **2**, **11**, and **17a** was not significantly greater than that of the etoposide and cisplatin positive controls, it is notable that they appeared to induce apoptotic cell death in cultured tumor cells through different mechanisms of action. Compound **11** has shown a different mechanism of cytotoxicity, and that **11** might be considered as a lead compound for further preclinical studies in cancer chemotherapy. More detailed studies of their cytotoxicity are in progress.

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Appendix Supplementary. data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.steroids.2013.02.012.

References

[1] Li H-J, Jiang Y, Li P. Characterizing distribution of steroidal alkaloids in *Fritillaria* spp. and related compound formulas by liquid chromatography–mass spectrometry combined with hierarchical cluster analysis. J Chromatogr A 2009;1216:2142–9.
[2] Rhaman UA, Akhtar NM, Choudhary IM, Tsuda Y, Sener B, Khalid A, et al. New steroidal alkaloids from *Fritillaria imperialis* and their cholinesterase inhibiting activities. Chem Pharm Bull 2002;50:1013–6.
[3] Wang D, Wang S, Chen X, Xu X, Zhu J, Nie L, et al. Antitussive, expectorant and anti-inflammatory activities of four alkaloids isolated from bulbs of *Fritillaria wabuensis*. J Ethnopharmacol 2012;139:189–93.
[4] Marija P, Angelina S, Sladana J, Milana T. Somatic embryogenesis and bulblet regeneration in snakehead fritillary (*Fritillaria meleagris* L.). Afr J Biotechnol 2011;10:16181–8.
[5] Bauer S, Masler L, Orszagh S, Mokry J, Tomko J. Alkaloids from *Fritillaria meleagris*. Chem Zvesti 1958;12:584–6.
[6] Matsuo Y, Watanabe K, Mimaki Y. New steroidal glycosides from rhizomes of *Clintonia udensis*. Biosci Biotechnol Biochem 2008;72:1714–21.
[7] Nakamura S, Chen G, Nakashima S, Matsuda H, Pei Y, Yoshikawa M. Brazilian natural medicines. IV. New noroleanane-type triterpene and ecdysterone-type sterol glycosides and melanogenesis inhibitors from the roots of Pfaffia glomerata. Chem Pharm Bull 2010;58:690–5.
[8] Mimaki Y, Matsuo Y, Watanabe K, Sakagami H. Furostanol glycosides from the rhizomes of Helleborus orientalis. J Nat Med 2010;64:452–9.
[9] Alphonse WW, Sumesh CC, Gerald M, Udo E, Wilson MN. Bioactive steroidal alkaloid glycosides from *Solanum aculeastrum*. Phytochemistry 2002;59:79–84.
[10] Higuchi R, Kitamura Y, Komori T. Thermal degradation of glycosides, I degradation of typical triterpenoid and steroid glycosides. Liebigs Ann Chem 1986:638–46.
[11] Askew FA, Farmer SN, Kon GAR. Sapogenins part I. The sapogenins of Sarsaparilla root. J Chem Soc 1936:1399–403.
[12] Glebko LI, Syromolotova NN, Strigina LI, Gorovoi PG. Spectrophotometric determination of a spirostane aglycone – Pennogenin- in *Polygonatum stephyllum*. Chem Nat Compd 1982;18:307–10.
[13] Maisashvili MR, Eristavi LI, Gvazava LN, Gugunishvili DM. Steroidal sapogenins from *Allium rotundum*. Chem Nat Compd 2007;43:756–7.
[14] Shimomura H, Sashida Y, Mimaki Y. Studies on the chemical constituents of *Lilium henryi* Baker. Chem Pharm Bull 1988;36:2430–46.
[15] Mimaki Y, Sashida Y, Shimomura H. Lipid and steroidal constituents of *Lilium auratum* var. Platyphyllum and *L. tenuifolium*. Phytochemistry 1989;28:3453–8.
[16] Mimaki Y, Sashida Y. Steroidal saponins and alkaloids from the bulbs of *Lilium brownii* var. colchestri. Chem Pharm Bull 1990;38:3055–9.
[17] Chen CX, Zhou J. Steroid saponins of aerial parts of *Paris polyphylla* var. *yunnanensis*. Acta Botan Yunnan 1990;12:323–9.
[18] Nohara Y, Miyahara K, Kawasaki T. Steroid saponins and sapogenins of underground parts of *Trillium kamtschaticum* pall. II pennogenin- and krytptogenin 3-*O*-glycosides and related compounds. Chem Pharm Bull 1975;23:872–85.
[19] Mimaki Y, Sashida Y. Steroidal and phenolic constituents of *Lilium speciosum*. Phytochemistry 1991;30:937–40.
[20] Kitajima J, Komori T, Kawasaki T. Studies on the constituents of crude drug "Fritillariae Bulbs". V. New sterol glycosides from aerial parts of *Fritillaria thunbergii* MIQ. Yakugaku Zasshi 1982;102:1009–15.
[21] Matsuo Y, Mimaki Y. Lignans from *Santalum album* and their cytotoxic activities. Chem Pharm Bull 2010;58:587–90.
[22] Roensch H, Schreiber K. Solanum-alkaloide—LXXII: Über γ1-und δ-solamarin, zwei neue tomatidenol-glykoside aus *Solanum dulcamara* L. Phytochemistry 1966;5:1227–33.
[23] Weissenberg M. Isolation of solasodine and other steroidal alkaloids and sapogenins by direct hydrolysis-extraction of *Solanum* plants or glycosides therefrom. Phytochemistry 2001;58:501–8.
[24] Ono M, Takamura C, Sugita F, Masuoka C, Yoshimitsu H, Ikeda T, Nohara T. Two new steroid glycosides and a new sesquiterpenoid glycoside from the underground parts of *Trillium kamtschaticum*. Chem Pharm Bull 2007;55:551–6.
[25] Mimaki Y, Satou T, Kuroda M, Sashida Y, Hatakeyama Y. New steroidal constituents from the bulbs of *Lilium candidum*. Chem Pharm Bull 1998;46:1829–32.
[26] Abbas FA. Steroidal saponins from *Solanum unguiculatum* (A.) Rich. Bull Fac Pharm Cairo Univ 2000;38:147–53.
[27] Agrawal PK, Jain DC, Gupta RK, Thakur RS. Carbon-13 NMR spectroscopy of steroidal sapogenins and steroidal saponins. Phytochemistry 1985;24:2479–96.
[28] Fattorusso E, Iorizzi M, Lanzotti V, Taglialatela-Scafati O. Chemical composition of Shallot (*Allium ascalonicum Hort.*). J Agric Food Chem 2002;50:5686–90.
[29] Mimaki Y, Ishibashi N, Ori N, Sashida Y. Steroidal glycosides from the bulbs of *Lilium dauricum*. Phytochemistry 1992;31:1753–8.