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Karataviosides G–K, five new bisdesmosidic steroidal glycosides from the bulbs of *Allium karataviense*

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ABSTRACT

We have analyzed the steroidal glycosides in *Allium karataviense* bulbs, and isolated five new bisdesmosidic steroidal glycosides: karataviosides G–K (**1**–**5**). The structures were elucidated by extensive spectroscopic analysis, including 2D NMR and enzymatic and hydrolytic cleavage. Karatavioside G (**1**) is an entirely novel furostanol glycoside, which has an *O*-β-D-glucopyranosyl-(1 → 6)-β-D-glucopyranosyl-(1 → 6)-β-D-glucopyranosyl unit at C-26 of the aglycone. Although a variety of cholestanol glycosides have been isolated, mainly from Liliaceae and Agavaceae, karataviosides J and K (**4** and **5**) are also notable because they are the most polar cholestanol bisdesmosides discovered, in which a lycotetraose is attached to C-3 of the aglycone, and a glucose or *O*-glucosyl-(1 → 3)-glucose is attached at C-16. The isolated glycosides were also evaluated for their cytotoxic activities against cultured tumor cell lines.

1. Introduction

Allium karataviense Regel (Liliaceae) is native to central Asia, particularly Turkestan, and is one of the most widely grown dwarf species of *Allium* in rock gardens. Five steroidal glycosides, called karataviosides A, B, C, E, and F, have been isolated from the flowers of *A. karataviense* [1–4]. We have previously isolated 12 steroidal constituents, including five new compounds, from the MeOH extract of the bulbs of *A. karataviense* [5]. In this study, we report further chemical investigation of the steroidal glycoside constituents in the more polar fraction of the MeOH extract of *A. karataviense* bulbs. We isolated six new bisdesmosidic steroidal glycosides, karataviosides G–K (**1–5**), which were identified as a furostanol glycoside (karatavioside G, **1**), two spirostanol glycosides (karatavioside H and I, **2** and **3**), and two cholestanol glycosides (karataviosides K and L, **4** and **5**). The structures of the five new steroidal glycosides were determined by extensive spectroscopic analysis, including 2D NMR, and acidic or enzymatic hydrolysis. The cytotoxicity of the compounds was also evaluated against neoplastic HSC-2, HSC-3, HSC-4, and HL-60 cells.

2. Experimental

2.1. General methods

Optical rotations were measured using a JASCO P-1030 (Tokyo, Japan) automatic digital polarimeter. IR spectra were recorded on a JASCO FT-IR 620 spectrophotometer. NMR spectra were recorded on a Bruker DRX-500 spectrometer (500 MHz for ^{1}H NMR, Karlsruhe, Germany) using standard Bruker pulse programs. Chemical shifts are given as δ-value with reference to tetramethylsilane (TMS) as an internal standard. ESITOFMS data were obtained on a Waters-Micromass LCT (Manchester, U.K.) mass spectrometer. Porous-polymer polystyrene resin (Diaion HP-20, Mitsubishi-Chemical, Tokyo, Japan), silica gel (300 mesh, Fuji-Silysia Chemical, Aichi, Japan), and ODS silica gel (75 μm, Nacalai Tesque Kyoto, Japan) were used for column chromatography. TLC was carried out on precoated Silica gel 60 F_{254} (0.25 mm thick, Merck Darmstadt, Germany) and RP-18 F_{254S} (0.25 mm thick, Merck) plates, and spots were visualized by spraying with 10% H_2SO_4 aqueous solution followed by heating. HPLC was performed by using a system comprised of a CCPM pump (Tosoh, Tokyo, Japan), a CCP PX-8010 controller (Tosoh), an RI-8010 detector (Tosoh) or a Shodex OR-2 detector (Showa-Denko, Tokyo, Japan), and a Rheodyne injection port. A Capcell Pak C_{18} UG80 column (10 × 250 mm, 5 μm, Shiseido Tokyo, Japan) was employed for preparative HPLC. The following reagents were obtained from the indicated companies:

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Dulbecco's modified Eagle medium (DMEM) (Gibco Grand Island, NY, U.S.A.); fetal bovine serum (FBS) (JRH Biosciences, Lenexa, KS, U.S.A.); penicillin G sodium salt and streptomycin sulfate (Meiji-Seika, Tokyo, Japan); and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) (Sigma, St. Louis, MO, U.S.A.). All other chemicals used were of biochemical reagent grade.

2.2. Plant material

A. karataviense was purchased from a nursery in Heiwaen, Japan, and was identified by Dr. Yutaka Sashida, emeritus professor at the Tokyo University of Pharmacy and Life Sciences. A voucher specimen has been deposited in our laboratory (voucher No. 97-3-20-AK, Department of Medicinal Pharmacognosy).

2.3. Extraction and isolation

The plant material (7.0 kg fresh weight) was extracted twice with hot MeOH (13 L). The MeOH extract was concentrated under reduced pressure and the viscous concentrate (325 g) was applied to a column (Diaion HP-20, 90 × 350 mm) and successively eluted with 20% MeOH, EtOH, and EtOAc (5 L each). The collected MeOH fraction (78 g) was chromatographed on silica gel (58 × 380 mm) eluted with a stepwise gradient of $CHCl_3$–MeOH–H_2O (90:10:0, 40:10:1, 20:10:1, 1:1:0) and finally with MeOH, giving seven fractions (I–VII). Fraction VII was purified by ODS silica gel CC (60 × 280 mm) eluted with MeCN–H_2O (8:11, 3:2) into eight subfractions (VII-1 to VII-8). Fraction VII-2 was separated by CC on silica gel (28 × 310 mm) eluted with $CHCl_3$–MeOH–H_2O (7:4:1) to give **3** (29.9 mg). Fraction VII-4 was chromatographed on silica gel (43 × 340 mm) eluted with $CHCl_3$–MeOH–H_2O (7:4:1) and on ODS silica gel (30 × 325 mm) eluted with MeCN–H_2O (2:7) to afford **1** (64.9 mg) and **2** (60.0 mg). Fraction VII-6 was chromatographed on ODS silica gel (30 × 290 mm) eluted with MeCN–H_2O (1:3) and silica gel (28 × 250 mm) eluted with $CHCl_3$–MeOH–H_2O (20:10:1) to afford **4** (27.2 mg). Fraction VII-7 was applied to an ODS silica gel column (43 × 300 mm) eluted with MeCN–H_2O (1:3) to yield **5** (45.1 mg).

2.4. Karatavioside G (**1**)

Amorphous powder; $[\alpha]_D^{25}$ −60.8 (*c* 0.10, MeOH); IR (film) ν_{max} 3377 (OH), 2928 (CH) cm^{-1}; ^1H NMR (500 MHz, C_5D_5N–CD_3OD, 11:1): see Table 1; ^{13}C NMR (125 MHz, C_5D_5N–CD_3OD, 11:1): see Table 2; HR-ESITOFMS *m*/*z* 1589.6843 $[M+Na]^+$ (calcd for $C_{69}H_{114}O_{39}Na$: 1589.6835).

2.5. Enzymatic hydrolysis of **1**

Compound **1** (5.0 mg) was treated with β-D-glucosidase (10 mg, EC 3.2.1.21, Sigma) in an HOAc–NaOAc buffer (pH 5.0, 1.5 mL) at room temperature for 48 h. The crude hydrolysate was chromatographed on silica gel eluted with $CHCl_3$–MeOH–H_2O (7:4:1) to yield (25*R*)-2α-hydroxyspirost-5-en-3β-yl *O*-β-D-glucopyranosyl-(1 → 2)-[β-D-xylopyranosyl-(1 → 3)]-β-D-glucopyranosyl-(1 → 4)-β-D-galactopyranoside (**1a**; karatavioside A, 1.7 mg) [6] and a sugar fraction (0.8 mg). The sugar fraction was dissolved in H_2O (1 mL) and passed through a C18 cartridge (Sep-Pak, Waters), which was then analyzed by HPLC under the following conditions: column, UG type amino phase (Capcell Pak NH_2 UG80, Shiseido, 4.6 × 250 mm, 5 μm); solvent, MeCN–H_2O (17:3); detection, refractive index and optical rotation; flow rate, 1.0 mL/min. D-Glucose was identified by comparison of its retention time and specific rotation with that of an authentic sample: t_R = 18.2 min, positive optical rotation.

2.7. (25R)-2α-Hydroxyspirost-5-en-3β-yl O-β-D-glucopyranosyl-(1 → 2)-[β-D-xylopyranosyl-(1 → 3)]-β-D-glucopyranosyl-(1 → 4)-β-D-galactopyranoside (**1a**; karatavioside A)

An amorphous powder; ^1H NMR (500 MHz, C_5D_5N): δ_H 5.59 (1H, d, *J* = 7.8 Hz H-1‴), 5.30 (1H, br d, *J* = 4.8 Hz, H-6), 5.26 (1H, d, *J* = 7.8 Hz, H-1⁗), 5.23 (1H, d, *J* = 7.9 Hz, H-1″), 4.94 (1H, d, *J* = 7.8 Hz, H-1′), 1.13 (3H, d, *J* = 6.9 Hz, Me-21), 0.94 (3H, s, Me-19), 0.81 (3H, s, Me-18), 0.70 (3H, d, *J* = 5.5 Hz, Me-27); ^{13}C NMR (125 MHz, C_5D_5N): δ_C 45.7, 70.8, 84.5, 37.7, 140.1, 121.9, 32.2, 31.1, 50.2, 37.9, 21.2, 39.8, 40.4, 56.5, 32.2, 81.1, 62.7, 16.3, 20.4, 42.0, 15.0, 109.2, 31.8, 29.3, 30.6, 66.9, 17.3 (C-1–C-27), 103.3, 72.7, 75.1, 79.2, 75.7, 60.5 (C-1′–C-6′ of Gal), 104.6, 81.2, 87.0, 70.0, 78.1, 62.8 (C-1″–C-6″ of Glc), 104.8, 75.5, 78.4, 71.4, 78.7, 62.9 (C-1‴–C-6‴ of Glc), 104.9, 76.1, 77.6, 70.4, 67.3 (C-1⁗–C-5⁗ of Xyl).

2.7. Acid hydrolysis of **1**

A solution of **1** (10.6 mg) in 1 M HCl (dioxane–H_2O, 1:1; 2 mL) was heated at 95 °C for 1.5 h under an Ar atmosphere. After cooling, the reaction mixture was neutralized by passing it through an Amberlite column (IRA-96SB, Organo) and it was chromatographed on a column (Diaion HP-20) eluted with H_2O–MeOH (4:1) then EtOH–Me_2CO (1:1) to yield an aglycone fraction and a sugar fraction (3.7 mg). The aglycone fraction was chromatographed on silica gel (12 × 120 mm) and eluted with $CHCl_3$–MeOH (20:1) to give (25*R*)-spirost-5-ene-2α,3β-diol (**1b**, 1.3 mg) [7]. HPLC analysis of the sugar fraction under the same conditions as for the enzymatic hydrolysis showed the presence of D-galactose, t_R = 16.9 min, positive optical rotation; D-glucose, t_R = 18.2 min, positive optical rotation; and D-xylose, t_R = 10.7 min, positive optical rotation.

2.8. (25R)-Spirost-5-ene-2α,3β-diol (**1b**, yuccagenin)

An amorphous solid; ^1H NMR (400 MHz, C_5D_5N): δ_H 5.44 (1H, br d, *J* = 5.1 Hz, H-6), 3.83 (1H, m, H-3), 1.14 (3H, d, *J* = 7.0 Hz, Me-21), 1.11 (3H, s, Me-19), 0.85 (3H, s, Me-18), 0.70 (3H, d, *J* = 5.7 Hz, Me-27); ^{13}C NMR (100 MHz, C_5D_5N): δ_C 46.7, 72.7, 76.8, 40.9, 141.3, 121.3, 32.3, 31.2, 50.4, 38.6, 21.3, 39.9, 40.5, 56.6, 32.2, 81.1, 62.9, 16.4, 20.7, 42.0, 15.0, 109.3, 31.8, 29.3, 30.6, 66.9, 17.3 (C-1–C-27).

2.9. Karatavioside H (**2**)

Amorphous powder; $[\alpha]_D^{25}$ −81.9 (*c* 0.10, MeOH); IR (film) ν_{max} 3384 (OH), 2925 (CH) cm^{-1}; ^1H NMR (500 MHz, C_5D_5N): see Table 1; ^{13}C NMR (125 MHz, C_5D_5N): see Table 2; HR-ESITOFMS *m*/*z* 1411.6089 $[M+Na]^+$ (calcd for $C_{62}H_{100}O_{34}Na$: 1411.5994).

2.10. Acid hydrolysis of **2**

A solution of **2** (5.0 mg) was subjected to the acid hydrolysis conditions described for **1** to give an aglycone fraction (1.3 mg) and a sugar fraction (3.1 mg). TLC analysis of the sugar fraction showed that it contained several unidentified compounds. HPLC analysis of the sugar fraction under the same conditions as those used for **1** showed the presence of D-galactose, D-glucose, and D-xylose.

2.11. Karatavioside I (**3**)

Amorphous powder; $[\alpha]_D^{25}$ −79.8 (*c* 0.10, MeOH); IR (film) ν_{max} 3369 (OH), 2928 (CH) cm^{-1}; ^1H NMR (500 MHz, C_5D_5N): see Table 1; ^{13}C NMR (125 MHz, C_5D_5N): see Table 2; HR-ESITOFMS *m*/*z* 1445.6006 $[M+Na]^+$ (calcd for $C_{62}H_{102}O_{36}Na$: 1445.6049).

Table 1
^{1}H NMR spectral assignments for **1**–**5**.[a]

Positions			**1**			**2**			**3**			**4**			**5**		
			δ_H		*J* (Hz)	δ_H		*J* (Hz)	δ_H		*J* (Hz)	δ_H		*J* (Hz)	δ_H		*J* (Hz)
	1	ax	1.26	t-like	12.2	1.28	dd	12.8, 12.6	2.36	dd	11.9, 11.9	1.66	m		1.65	m	
		eq	2.27	dd	12.7, 4.1	2.29	dd	12.8, 4.2	2.09	dd	11.9, 5.3	0.91	m		0.94	m	
	2		4.01	m		4.04	ddd	12.6, 9.2, 4.2	4.35	ddd	11.9, 11.9, 5.3	1.67	m		1.68	m	
			–			–			–			2.08	m		2.07	m	
	3		3.77	m		3.83	ddd	12.5, 9.2, 5.0	4.80	ddd	12.4, 11.9, 5.4	3.87	m		3.88	m	
	4	ax	2.52	t-like	12.6	2.54	t-like	12.5	2.91	dd	12.4, 12.4	2.41	br dd	11.5, 11.5	2.40	br dd	11.6, 11.6
		eq	2.68	dd	13.9, 4.9	2.69	br dd	12.5, 5.0	2.46	dd	12.4, 5.4	2.63 (2H) m			2.63 (2H) m		
	5		–			–			–			–			–		
	6		5.32	br d	3.9	5.29	br d	4.4	4.13	br s		5.23	br s		5.21	br s	
	7	a	1.85	m		1.78	m		2.16	m		1.70	m		1.66	m	
		b	1.48	m		1.42	m		1.85	m		1.36	m		1.34	m	
	8		1.48	m		1.42	m		2.21	m		1.27	m		1.25	m	
	9		0.93	m		0.93	m		1.94	m		0.82	m		0.81	m	
	10		–			–			–			–			–		
	11	ax	1.37	m		1.33	m		1.52	m		1.39 (2H)			1.36 (2H)		
		eq	1.48	m		1.44	m		1.40	m		–			–		
	12	ax	1.04	m		1.00	m		1.09	m		1.14	m		1.11	m	
		eq	1.66	m		1.59	br d	12.0	1.64	m		2.02	br d	12.4	2.01	br d	12.2
	13		–			–			–			–			–		
	14		0.99	m		0.99	m		1.23	m		0.80	m		0.79	m	
	15	a	1.93	m		1.94	m		2.07	m		2.32	ddd	14.1, 7.5, 7.5	2.27	ddd	14.2, 7.4, 7.4
		b	1.35	m		1.35	m		1.39	m		1.75	m		1.69	m	
	16		4.42	m		4.49	m		4.50	m		4.51	m		4.49	m	
	17		1.72	m		1.71	dd	7.9, 6.7	1.75	dd	7.9, 7.0	1.97	m		1.94	m	
	18		0.80	s		0.74	s		0.80	s		0.98	s		0.96	s	
	19		0.95	s		0.92	s		1.51	s		0.87	s		0.85	s	
	20		2.19	dq	6.8, 6.5	1.90	m		1.90	m		2.55	m		2.53	m	
	21		1.17	d	6.8	1.02	d	6.9	1.01	d	6.9	1.20	d	6.9	1.19	d	6.9
	22		–			–			–			4.30	m		4.24	m	
	23	a	2.00	m		1.98	dd	12.1, 10.6	1.95	dd	12.8, 12.2	1.82 (2H) m			1.80 (2H) m		
		b	1.73	m		2.66	dd	12.1, 4.6	2.62	dd	12.8, 4.6	–			–		
	24	a	1.80	m		3.98	ddd	10.6, 10.6, 4.6	3.98	ddd	12.2, 8.5, 4.6	1.94	m		1.93	m	
		b	1.33	m		–			–			1.67	m		1.67	m	
	25		1.88	m		1.99	m		1.98	m		1.66	m		1.64	m	
	26	a	4.01	m		3.53	dd	11.7, 11.4	3.51	dd	11.4, 11.4	0.94	d	6.3	0.97	d	6.3
		b	3.52	dd	9.4, 6.5	3.63	dd	11.7, 5.1	3.61	dd	11.4, 4.9	–			–		
	27		1.00	d	6.6	1.26	d	6.4	1.24	d	6.4	0.94	d	6.3	0.93	d	6.3
	OMe		3.26	s		–			–			–			–		
Gal	1′		4.87	d	7.7	4.92	d	7.6	4.78	d	7.3	4.85	d	7.8	4.88	d	7.6
	2′		4.43	t-like	8.9	4.52	t-like	8.3	4.51	dd	9.0, 7.3	4.40	dd	8.7, 7.8	4.41	dd	9.1, 7.6
	3′		4.07	m		4.12	dd	8.3, 3.3	4.13	dd	9.0, 3.1	4.09	dd	8.7, 4.2	4.11	dd	9.1, 2.6
	4′		4.53	br d	2.5	4.59	br d	3.3	4.53	m		4.54	m		4.59	br d	2.6
	5′		3.99	m		4.01	m		4.00	m		4.00	m		3.99	m	
	6′	a	4.50	br d	10.1	4.60	m		4.55	dd	10.5, 10.5	4.65	dd	9.6, 9.6	4.67	dd	9.8, 9.8
		b	4.10	m		4.18	dd	12.2, 6.0	4.06	dd	1.05, 4.2	4.16	m		4.17	m	
Glc	1″		5.14	d	7.8	5.21	d	7.9	5.20	d	7.9	5.16	d	7.8	5.17	d	7.9
	2″		4.26	t-like	8.5	4.35	dd	8.8, 7.9	4.35	dd	8.8, 7.9	4.39	dd	9.0, 7.8	4.40	dd	8.8, 7.9
	3″		4.09	t-like	8.5	4.14	dd	8.8, 8.8	4.14	dd	8.8, 8.8	4.14	dd	9.0, 9.0	4.16	m	
	4″		3.75	m		3.81	dd	8.8, 8.8	3.83	m		3.81	dd	9.0, 9.0	3.81	dd	9.4, 9.4
	5″		3.77	m		3.84	m		3.84	m		3.85	m		3.88	m	
	6″	a	4.42	br d	11.3	4.50	m		4.50	br d	10.2	4.50	br d	10.8	4.51	br d	12.4
		b	4.01	m		4.04	dd	12.0, 4.5	4.05	dd	10.2, 4.8	4.03	dd	12.0, 5.4	4.04	dd	12.4, 5.1

Glc	1‴		5.51	d	7.9	5.57	d	7.8	5.55	d	7.8	5.55	d	7.8	5.56	d	7.6
	2‴		3.95	dd	9.2, 7.9	4.04	dd	8.6, 7.8	4.04	dd	9.0, 7.8	4.05	dd	9.0, 7.8	4.06	dd	9.2, 7.6
	3‴		4.02	dd	9.2, 9.2	4.10	dd	8.6, 8.6	4.14	dd	9.0, 9.0	4.10	dd	9.0, 9.0	4.11	dd	9.2, 9.2
	4‴		4.07	dd	9.2, 9.2	4.14	dd	9.3, 8.6	4.09	m		4.20	dd	9.0, 9.0	4.21	dd	9.2, 8.9
	5‴		3.85	m		3.90	m		3.90	m		3.90	m		3.90	m	
	6‴	a	4.45	br d	12.0	4.56	dd	12.0, 2.0	4.56	br d	10.3	4.56	br d	11.4	4.56	br d	10.8
		b	4.35	dd	12.0, 5.4	4.40	dd	12.0, 5.5	4.40	dd	10.3, 6.6	4.36	m		4.37	dd	10.8, 4.5
Xyl	1‴′		5.18	d	7.8	5.25	d	7.8	5.23	d	7.8	5.21	d	7.9	5.23	d	7.9
	2‴′		3.87	dd	9.0, 7.8	3.95	dd	8.6, 7.8	3.96	dd	8.4, 7.8	3.97	dd	9.0, 7.9	3.96	dd	9.1, 7.9
	3‴′		3.99	dd	9.0, 9.0	4.08	dd	9.0, 8.6	4.08	dd	8.4, 8.4	4.10	dd	9.0, 9.0	4.09	dd	9.1, 9.1
	4‴′		4.04	m		4.11	m		4.12	m		4.13	m		4.11	m	
	5‴′	a	4.19	dd	11.2, 5.2	4.21	m		4.22	dd	10.4, 4.2	4.21	m		4.22	dd	10.1, 4.4
		b	3.61	dd	11.2, 11.2	3.66	dd	10.8, 10.8	3.66	dd	10.4, 10.4	3.65	dd	10.2, 10.2	3.67	dd	10.1, 10.1
Glc	1⁗		4.72	d	7.9	4.93	d	7.1	4.91	d	7.3	4.72	d	7.8	4.70	d	7.8
	2⁗		3.87	dd	9.1, 7.9	4.23	dd	8.8, 7.1	4.23	dd	8.8, 7.3	3.99	dd	9.0, 7.8	4.01	dd	8.8, 7.8
	3⁗		4.09	dd	9.1, 9.1	4.28	dd	9.3, 8.8	4.28	dd	8.8, 8.8	4.15	dd	9.0, 9.0	4.11	dd	8.8, 8.8
	4⁗		4.05	dd	9.1, 9.1	4.25	dd	9.3, 9.3	4.25	m		4.25	dd	9.0, 9.0	4.16	dd	8.8, 8.8
	5⁗		3.99	m		3.77	m		3.78	m		3.80	m		3.77	m	
	6⁗	a	4.78	br d	10.3	4.45	dd	11.9, 2.9	4.46	dd	11.7, 2.0	4.48	br d	10.8	4.41	br d	11.0
		b	4.26	m		4.30	m	11.9, 5.4	4.33	dd	11.7, 5.1	4.38	m		4.31	dd	11.0, 4.4
Glc	1⁗′		4.96	d	7.8	5.38	d	7.7	5.37	d	7.7				5.24	d	7.9
	2⁗′		3.88	dd	9.0, 7.8	4.11	dd	8.9, 7.7	4.12	dd	8.9, 7.7				4.05	dd	8.7, 7.9
	3⁗′		4.06	dd	9.0, 9.0	4.22	dd	8.9, 8.9	4.24	dd	8.9, 8.9				4.23	dd	8.8, 8.7
	4⁗′		4.10	dd	9.0, 9.0	4.27	dd	8.9, 8.9	4.27	dd	8.9, 8.9				4.19	dd	8.8, 8.8
	5⁗′		3.94	m		3.90	m		3.91	m					3.99	m	
	6⁗′	a	4.72	br d	11.8	4.46	dd	11.5, 2.9	4.45	dd	11.7, 3.3				4.52	dd	11.7, 2.2
		b	4.25	br d	11.8	4.39	dd	11.5, 4.5	4.39	dd	11.7, 5.0				4.31	dd	11.7, 5.5
Glc	1⁗″		4.98	d	7.8												
	2⁗″		3.94	t-like	8.4												
	3⁗″		4.12	t-like	8.4												
	4⁗″		4.11	m													
	5⁗″		3.84	m													
	6⁗″	a	4.42	br d	11.4												
		b	4.27	m													

[a] Spectra were measured in C_5D_5N except for **1** (C_5D_5N–CD_3OD, 11:1).

Table 2
^{13}C NMR spectral assignments for **1**–**5**.[a]

Positions	1	2	3	4	5	Positions		1	2	3	4	5
1	45.4	45.7	41.3	37.6	37.6	Gal	1′	103.0	103.3	103.6	102.9	102.9
2	69.9	70.0	70.7	30.3	30.3		2′	72.3	72.6	72.6	73.2	73.3
3	84.2	84.5	83.1	78.6	78.4		3′	75.1	75.5	75.5	75.6	75.7
4	37.4	37.7	38.2	39.4	39.4		4′	79.1	79.3	79.0	79.9	80.1
5	139.9	140.0	75.3	141.0	141.0		5′	75.4	75.6	75.5	75.4	75.5
6	121.8	121.9	75.4	122.1	121.9		6′	60.3	60.5	60.4	60.8	60.7
7	31.9	32.1	35.6	32.2	32.2							
8	30.9	31.0	30.1	31.9	31.8	Glc	1″	104.3	104.6	104.7	105.1	105.3
9	50.1	50.1	45.7	50.5	50.5		2″	81.0	81.2	81.2	81.3	81.5
10	37.8	37.9	40.3	37.1	37.0		3″	86.9	86.9	86.8	87.0	86.9
11	21.0	21.1	21.5	21.3	21.2		4″	70.0	70.4	70.5	70.5	70.5
12	39.5	39.7	40.3	40.1	40.1		5″	77.3	77.5	77.5	77.6	77.7
13	40.6	40.3	40.9	42.5	42.5		6″	62.6	62.9	62.9	62.9	63.0
14	56.3	56.4	56.2	55.2	55.2							
15	32.0	32.0	32.2	37.1	37.0	Glc	1‴	104.5	104.8	104.8	104.8	104.9
16	81.2	81.5	81.6	82.8	83.0		2‴	75.7	76.0	76.1	76.2	76.3
17	64.1	62.3	62.6	58.0	58.0		3‴	78.1	78.1	78.0	77.9	77.9
18	16.1	16.3	16.7	13.7	13.6		4‴	71.0	71.3	71.3	71.0	71.1
19	20.2	20.4	18.3	19.6	19.5		5‴	77.9	78.3	78.4	78.8	78.9
20	40.4	42.1	42.1	36.0	36.1		6‴	62.3	62.7	62.8	62.5	62.5
21	16.1	14.8	14.8	12.6	12.6							
22	112.6	111.6	111.5	73.3	73.4	Xyl	1⁗	104.7	104.9	104.9	105.0	105.1
23	30.6	40.6	40.6	34.0	33.8		2⁗	74.8	75.1	75.1	75.2	75.0
24	28.0	81.8	81.7	36.8	36.9		3⁗	78.2	78.7	78.7	78.6	78.8
25	34.0	38.0	38.0	29.1	29.0		4⁗	70.4	70.7	70.9	70.9	70.9
26	75.2	65.2	65.2	23.2	23.2		5⁗	67.0	67.3	67.3	67.4	67.5
27	16.9	13.7	13.7	23.3	23.3							
OMe	47.1					Glc	1⁗′	104.6	104.2	104.2	107.0	106.6
							2⁗′	74.7	83.7	83.8	75.7	74.4
							3⁗′	77.8	78.4	78.4	78.2	89.4
							4⁗′	71.2	71.4	71.4	71.8	69.8
							5⁗′	76.9	77.8	77.8	78.7	78.0
							6⁗′	69.9	62.8	62.8	63.0	62.5
						Glc	1⁗″	105.1	106.1	106.2		106.2
							2⁗″	74.7	76.9	76.9		75.7
							3⁗″	77.9	78.3	78.3		78.4
							4⁗″	71.0	71.7	71.7		71.7
							5⁗″	76.8	78.0	78.0		78.9
							6⁗″	69.7	62.5	62.5		62.6
						Glc	1⁗‴′	105.2				
							2⁗‴′	74.8				
							3⁗‴′	78.1				
							4⁗‴′	71.2				
							5⁗‴′	78.0				
							6⁗‴′	62.3				

[a] Spectra were measured in C_5D_5N except for **1** (C_5D_5N–CD_3OD, 11:1).

2.12. Acid hydrolysis of *3*

A solution of **3** (5.0 mg) was subjected to the acid hydrolysis conditions described for **1** to give a sugar fraction (1.7 mg). HPLC analysis of the sugar fraction under the same conditions as those for **1** showed the presence of D-galactose, D-glucose, and D-xylose.

2.13. Karatavioside J (*4*)

Amorphous powder; $[\alpha]_D^{27}$ −37.0 (*c* 0.10, MeOH); IR (film) ν_{max} 3397 (OH), 2929 (CH) cm^{-1}; ^{1}H NMR (500 MHz, C_5D_5N): see Table 1; ^{13}C NMR (125 MHz, C_5D_5N): see Table 2; HR-ESITOFMS *m*/*z* 1221.5922 $[M+Na]^+$ (calcd for $C_{56}H_{94}O_{27}Na$: 1221.5880).

2.14. Acid hydrolysis of *4*

A solution of **4** (21.4 mg) was subjected to the acidic hydrolysis conditions described for **1** to yield an aglycone fraction (5.5 mg) and a sugar fraction (9.7 mg). The aglycone fraction was chromatographed on silica gel (12 × 120 mm) and eluted with $CHCl_3$–MeOH (49:1) to give (22*S*)-cholest-5-ene-3β,16β,22-triol (3.8 mg, **4a**) [8]. HPLC analysis of the sugar fraction under the same conditions as those for the enzymatic hydrolysis showed the presence of D-galactose, D-glucose, and D-xylose.

2.15. (22*S*)-Cholest-5-ene-3*β*,16*β*,22-triol (*4a*)

An amorphous solid; ^{1}H NMR (400 MHz, C_5D_5N): δ_H 5.31 (1H, br s, H-6), 3.70 (1H, m, H-3), 1.17 (1H, d, *J* = 6.8 Hz, Me-21), 0.96 (3H, s, Me-18), 0.93 (3H × 2, d, *J* = 6.3 Hz, Me-26 and Me-27), 0.82 (3H, s, Me-19); ^{13}C NMR (100 MHz, C_5D_5N): δ_C 37.2, 32.2, 72.4, 44.1, 142.2, 121.0, 21.1, 39.5, 40.6, 38.0, 21.3, 39.9, 40.5, 57.1, 40.0, 74.9, 58.0, 16.2, 20.2, 40.2, 15.1, 71.9, 33.1, 36.6, 28.4, 23.0, 22.9 (C-1–C-27).

2.16. Preparation of (S)-MTPA ester and (R)-MTPA ester from *4a*

A solution of **4a** (1.9 mg) in CH_2Cl_2 (1 mL) was treated with (*S*)-MTPA (15 mg) in the presence of DCC (15 mg) and 4-DMAP (15 mg). After the reaction mixture was stirred at room

temperature for 15 h, it was purified by silica gel CC eluted with hexane–EtOAc (5:1) to give the (*S*)-MTPA ester **4b** (1.8 mg). The (*R*)-MTPA ester **4c** (1.9 mg) was also obtained from **4a** through the same procedure. (*S*)-MTPA ester **4b**: ^{1}H NMR (500 MHz, $CDCl_3$) δ_H: 7.52–7.32 (10H, aromatic protons), 5.37 (1H, br dd, J = 7.0, 7.0 Hz, H-22), 5.36 (1H, br d, J = 6.1 Hz, H-6), 4.82 (1H, m, $W_{1/2}$ = 23.1 Hz, H-3), 4.34 (1H, m, H-16), 3.50 and 3.45 (each 3H, s, OMe), 2.13 (1H, m, H-20), 1.59 (1H, m, H-23a), 1.46 (1H, m, H-23b), 1.44 (1H, m, H-25), 0.94 (3H, s, Me-18), 0.88 (3H, d, J = 6.9 Hz, Me-21), 0.80 (3H, s, Me-19), 0.77 (3H, d, J = 6.6 Hz, Me-26), 0.75 (3H, d, J = 6.6 Hz, Me-27). (*R*)-MTPA ester **4c**: ^{1}H NMR (500 MHz, $CDCl_3$) δ_H: 7.53–7.32 (10H, aromatic protons), 5.34 (1H, br dd, J = 6.9, 6.9 Hz, H-22), 5.34 (1H, br d, J = 6.0 Hz, H-6), 4.82 (1H, m, $W_{1/2}$ = 22.1 Hz, H-3), 4.27 (1H, m, H-16), 3.51 and 3.50 (each 3H, s, OMe), 2.09 (1H, m, H-20), 1.66 (1H, m, H-23a), 1.53 (1H, m, H-23b), 1.47 (1H, m, H-25), 0.92 (3H, s, Me-18), 0.82 (3H, d, J = 6.9 Hz, Me-21), 0.80 (3H, d, J = 6.5 Hz, Me-26), 0.79 (3H, s, Me-19), 0.78 (3H, d, J = 6.5 Hz, Me-27).

2.17. Karatavioside K (*5*)

Amorphous powder; $[\alpha]_D^{27}$ −43.0 (*c* 0.10, MeOH); IR (film) ν_{max} 3400 (OH), 2930 (CH) cm^{-1}; ^{1}H NMR (500 MHz, C_5D_5N): see Table 1; ^{13}C NMR (125 MHz, C_5D_5N): see Table 2; HR-ESITOFMS *m*/*z* 1383.6398 $[M+Na]^+$ (calcd for $C_{62}H_{104}O_{32}Na$: 1383.6408).

2.18. Acid hydrolysis of *5*

A solution of **5** (10.2 mg) was subjected to acidic hydrolysis as described for **1** to yield an aglycone fraction (2.2 mg) and a sugar fraction (4.9 mg). The aglycone fraction was chromatographed on silica gel (12 × 120 mm) and eluted with $CHCl_3$–MeOH (49:1) to give **4a** (1.1 mg). HPLC analysis of the sugar fraction under the same conditions as in the case of enzymatic hydrolysis showed the presence of D-galactose, D-glucose, and D-xylose.

2.19. Assay for cytotoxic activity

The methodology for the assays using HSC-2, HSC-3, HSC-4, and HL-60 cells has been described previously [9]. In brief, the cells were incubated at 37 °C for 48 h in DMEM supplemented with 10% heat-inactivated FBS, except for HL-60 cells which were cultured in RPMI-1640 containing 10% FBS. Cell viability was assessed using the MTT method for all except HL-60 cells in which case the trypan blue exclusion procedure was used.

3. Results and discussion

Fresh *A. karataviense* bulbs (7.0 kg) were extracted with hot MeOH. The MeOH extract was passed through a porous polymer polystyrene resin column (Diaion HP-20), and the MeOH-eluted fraction was separated by column chromatography (CC) using silica gel and octadecylsilanized (ODS) silica gel, to afford karataviosides G (**1**, 64.9 mg), H (**2**, 60.0 mg), I (**3**, 29.9 mg), J (**4**, 27.2 mg), and K (**5**, 45.1 mg) (Fig. 1).

Karatavioside G (**1**) was obtained as an amorphous solid and its molecular formula was $C_{69}H_{114}O_{39}$ based on the high-resolution electrospray ionization time-of-flight mass spectrometry (HR-ESI-TOFMS) (*m*/*z* 1589.6843 $[M+Na]^+$) and ^{13}C NMR data. The ^{1}H NMR spectrum showed signals for four steroid methyl groups at δ_H 1.17 (3H, d, J = 6.8 Hz), 1.00 (3H, d, J = 6.6 Hz), 0.95 (3H, s), and 0.80 (3H, s), a methoxy group at δ_H 3.26 (3H, s, OMe), an olefinic proton at δ_H 5.32 (1H, br d, J = 3.9 Hz), and seven anomeric protons at δ_H 5.51 (1H, d, J = 7.9 Hz), 5.18 (1H, d, J = 7.8 Hz), 5.14 (1H, d, J = 7.8 Hz), 4.98 (1H, d, J = 7.8 Hz), 4.96 (1H, d, J = 7.8 Hz), 4.87 (1H, d, J = 7.7 Hz), and 4.72 (1H, d, J = 7.9 Hz) (Table 1). These ^{1}H NMR data, together with an acetalic carbon signal at δ_H 112.6 in the ^{13}C NMR spectrum, and a positive color reaction with Ehrlich's reagent, suggested that **1** was a 22α-methoxyfurostanol saponin [10]. Enzymatic hydrolysis of **1** with β-D-glucosidase gave (25*R*)-2α-hydroxyspirost-5-en-3β-yl *O*-β-D-glucopyranosyl-(1 → 2)-[β-D-xylopyranosyl-(1 → 3)]-β-D-glucopyranosyl-(1 → 4)-β-D-galactopyranoside (3-*O*-β-lycotetraoside) (karatavioside A: **1a**) [6] and D-glucose. Acid hydrolysis of **1** with 1 M HCl in dioxane–H_2O (1:1) yielded a sapogenin identified as (25*R*)-spirost-5-ene-2α,3β-diol (yuccagenin: **1b**) [7], D-galactose, D-glucose, and D-xylose. Identification of the monosaccharides was carried out by direct HPLC analysis of the hydrolysate, using a combination of refractive index (RI) and optical rotation (OR) detectors. The spectral features and chemical evidence suggested that **1** was the furostanol glycoside of **1a**, in which the sugar moieties were attached to both the C-3 and C-26 hydroxy groups, as is often the case in naturally occurring 3,26-dihydroxy furostanol glycosides. However, the sugar residue attached to C-26 was structurally significant because it was assumed to be composed of three monosaccharides. The presence of seven sugar units in **1** caused the sugar proton signals to overlap almost completely, which meant they could not be fully assigned in a straightforward way using conventional 2D NMR methods, such as ^{1}H–^{1}H COSY, 2D TOCSY, and HMQC spectra. Analysis of the 1D TOCSY spectra followed by interpreting the ^{1}H–^{1}H COSY, HSQC–TOCSY, and HMQC spectra allowed the ^{1}H and ^{13}C NMR signals of each sugar to be assigned. The ^{1}H NMR subspectra of the individual monosaccharide units were obtained by using selective irradiation of easily identifiable anomeric proton signals in a series of 1D TOCSY experiments [11,12]. The subsequent analysis of the ^{1}H-^{1}H COSY spectrum resulted in the sequential assignment of all the proton resonances of the seven monosaccharide units, including identification of their signal multiplet patterns and coupling constants (Table 1). The proton resonances were correlated with those of the corresponding one-bond coupled carbons in the HMQC and HSQC–TOCSY spectra, allowing the unambiguous assignment of the carbon shifts (Table 2). The carbon chemical shifts were compared with those reported in the literature, taking into account the effect of the *O*-glycosylation shift, which indicated that **1** contained a terminal β-D-glucopyranosyl unit (Glc⁗′) and two 6-substituted β-D-glucopyranosyl units (Glc⁗, Glc⁗′) in addition to a β-lycotetraosyl unit linked to C-3 of the aglycone. The β-orientation of the anomeric centers of the three D-glucopyranosyl units was consistent with the relatively large $^3J_{H\text{-}1,H\text{-}2(ax)}$ values of their anomeric protons (7.8–7.9 Hz). In the HMBC spectrum of **1** (Fig. 2), correlation peaks were observed between δ_H 4.98 (H-1 of Glc⁗′) and δ_C 69.7 (C-6 of Glc⁗′), δ_H 4.96 (H-1 of Glc⁗′) and δ_C 69.9 (C-6 of Glc⁗), and between δ_H 4.72 (H-1 of Glc⁗) and δ_C 75.2 (C-26 of the aglycone). HMBC correlations were also detected between δ_H 5.18 (H-1 of Xyl‴′) and δ_C 86.9 (C-3 of Glc″), δ_H 5.51 (H-1 of Glc‴) and δ_C 81.0 (C-2 of Glc″), δ_H 5.14 (H-1 of Glc″) and δ_C 79.1 (C-4 of Glc′), and between δ_H 4.87 (H-1 of Gal′) and δ_C 84.2 (C-3 of the aglycone). HMBC correlations were also detected between δ_H 5.18 (H-1 of Xyl‴′) and δ_C 86.9 (C-3 of Glc″), δ_H 5.51 (H-1 of Glc‴) and δ_C 81.0 (C-2 of Glc″), δ_H 5.14 (H-1 of Glc″) and δ_C 79.1 (C-4 of Glc′), and between δ_H 4.87 (H-1 of Gal′) and δ_C 84.2 (C-3 of the aglycone). All of these data were consistent with the structure (25*R*)-26-[(*O*-β-D-glucopyranosyl-(1 → 6)-β-D-glucopyranosyl-(1 → 6)-β-D-glucopyranosyl)oxy]-2α-hydroxy-22α-methoxyfurost-5-en-3β-yl *O*-β-D-glucopyranosyl-(1 → 2)-[β-D-xylopyranosyl-(1 → 3)]-β-D-glucopyranosyl-(1 → 4)-β-D-galactopyranoside, which was assigned to **1**.

Karatavioside H (**2**) had the molecular formula $C_{62}H_{100}O_{34}$ according to the HR-ESITOFMS (*m*/*z* 1411.6089 $[M+Na]^+$) and ^{13}C NMR spectral data. The ^{1}H NMR spectrum of **2** showed signals for four typical steroid methyl groups at δ_H 1.26 (d, J = 6.4 Hz),

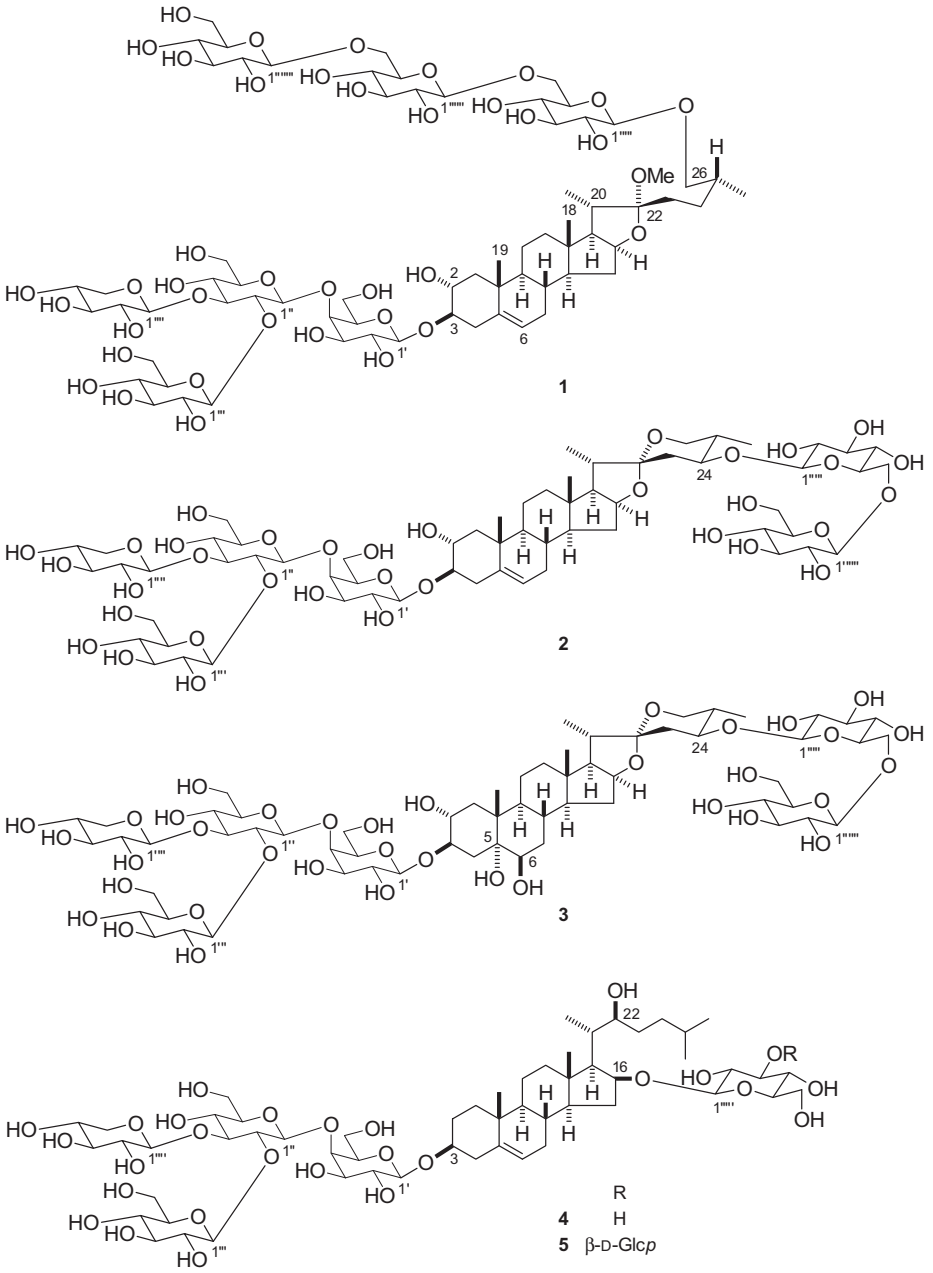


Fig. 1. Structures of **1**–**5**.

1.02 (d, J = 6.9 Hz), 0.92 (s), and 0.74 (s), an olefinic proton at δ 5.29 (br d, J = 4.4 Hz), and six anomeric protons at δ_H 5.57 (d, J = 7.8 Hz), 5.38 (d, J = 7.7 Hz), 5.25 (d, J = 7.8 Hz), 5.21 (d, J = 7.9 Hz), 4.93 (d, J = 7.1 Hz), and 4.92 (d, J = 7.6 Hz). An acetalic carbon signal characteristic of spirostanol steroids was observed at δ_C 111.6 (C) in the ^{13}C NMR spectrum. Acid hydrolysis of **2** afforded D-galactose, D-glucose, and D-xylose, whereas the labile spirostanol aglycone decomposed under the acidic conditions. Signals for the four methine protons attached to the oxygen-bearing carbons were observed at δ_H 4.49 (m), 4.04 (ddd, J = 12.6, 9.2, 4.2 Hz), 3.98 (ddd, J = 10.6, 10.6, 4.6 Hz), and 3.83 (ddd, J = 12.6, 9.2, 5.0 Hz), which were assigned to H-16, H-2, H-24, and H-3, respectively. The proton spin-coupling constants indicated the 2α, 3β, 24*S*, and 25*S* configurations. These spectroscopic and chemical properties, and the comparison of the ^{1}H and ^{13}C NMR spectra of **2** with those of **1** and a reference compound [13] revealed that the aglycone was (24*S*,25*S*)-spirost-5-ene-2α,3β,24-triol and that a lycotetraosyl group was attached at C-3 of the aglycone. In addition to the ^{1}H and ^{13}C NMR signals arising from the lycotetraosyl unit, the ^{1}H-^{1}H COSY, 1D-selective TOCSY, and HMQC spectra contained signals which arose from a 2-substituted β-D-glucopyranosyl unit [$\delta_{H\text{-}1}$ 4.93 (1H, d, J = 7.1 Hz); δ_C 104.2, 83.7, 78.4, 71.4, 77.8, 62.8] and a terminal β-D-glucopyranosyl unit [$\delta_{H\text{-}1}$ 5.38 (1H, d, J = 7.7 Hz); δ_C 106.1, 76.9, 78.3, 71.7, 78.0, 62.5] in **3** (Tables 1 and 2). In the HMBC spectrum, long-range correlations were observed between δ_H 5.38 (H-1 of Glc$''''$) and δ_C 83.7 (C-2 of Glc$''''$), and between δ_H 4.93 (H-1 of Glc$''''$) and δ_C 81.8 (C-24 of the aglycone), which were consistent with an *O*-β-D-glucopyranosyl-(1 → 2)-β-D-glucopyranosyl linkage to C-24 of the aglycone. Further HMBC correlations between δ_H 5.25 (H-1 of Xyl$''''$) and δ_C 86.9 (C-3 of Glc$''$), δ_H 5.57 (H-1 of Glc$'''$) and δ_C 81.2 (C-2 of Glc$''$), δ_H 5.21 (H-1 of Glc$''$) and δ_C 79.3 (C-4 of Gal′), and between δ_H 4.92 (H-1 of Gal′) and δ_C

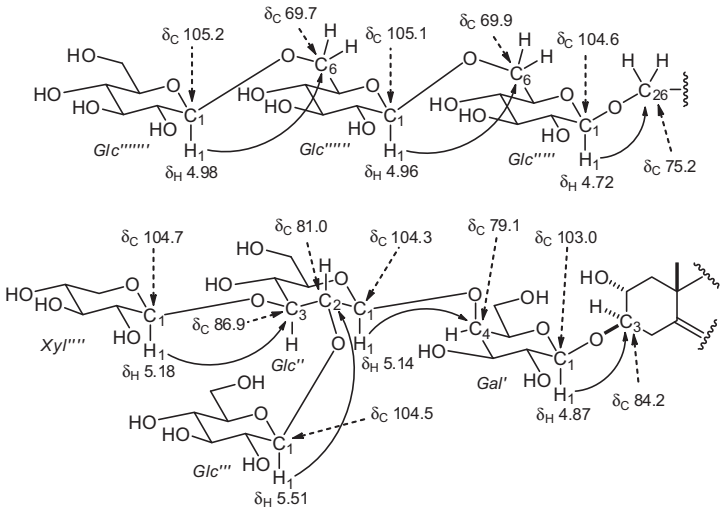


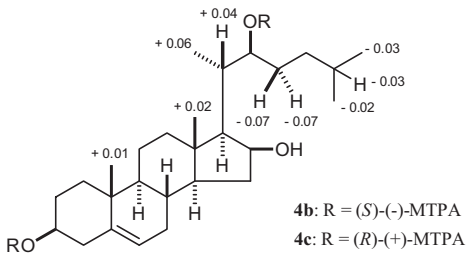
Fig. 2. HMBC correlations of the sugar moieties of **1**.

84.5 (C-3 of the aglycone) supported the presence of a lycotetraosyl group attached to C-3 of the aglycone. Thus, **2** was assigned as (24*S*,25*S*)-24-[(*O*-β-D-glucopyranosyl-(1 → 2)-β-D-glucopyranosyl) oxy]-2α-hydroxyspirost-5-en-3β-yl *O*-β-D-glucopyranosyl-(1 → 2)-[β-D-xylopyranosyl-(1 → 3)]-β-D-glucopyranosyl-(1 → 4)-β-D-galactopyranoside.

Karatavioside I (**3**) had the molecular formula $C_{62}H_{102}O_{36}$ according to the HR-ESITOFMS (*m*/*z* 1445.6006 [M+Na]$^+$) and ^{13}C NMR spectral data. The spectral features of **3** were analogous to those of **2**; signals corresponding to a lycotetraosyl group attached to C-3 of the aglycone and an *O*-β-D-glucopyranosyl-(1 → 2)-β-D-glucopyranosyl group linked to C-24 of the aglycone were identified. However, the ^{1}H and ^{13}C NMR signals from the double bond between C-5 and C-6 observed in **2** were replaced by an oxymethine proton at δ_H 4.13 (br s, H-6) and carbons bearing an oxygen atom at δ_C 75.3 (C-5) and 75.4 (C-6) in **3**, implying that **3** was produced by the saturation of the 5(6)-double bond of **2** and the simultaneous introduction of hydroxy groups at C-5 and C-6. Furthermore, the H-3 and Me-19 signals were shifted significantly downfield by 0.97 ppm and 0.59 ppm, respectively, when the ^{1}H NMR spectrum of **3** was compared with that of **2**. These spectral data and comparing the ^{1}H and ^{13}C NMR spectra of **3** with those of a previously reported compound [5], revealed that the aglycone of **3** was (24*S*,25*S*)-5α-spirostan-2α,3β,5,6β,24-pentol and that the C-3 and C-24 hydroxy groups of the aglycone were replaced with a lycotetraosyloxy unit and an (*O*-β-D-glucopyranosyl-(1 → 2)-β-D-glucopyranosyl)oxy unit, respectively. Therefore, **3** was concluded to be (24*S*,25*S*)-24-[(*O*-β-D-glucopyranosyl-(1 → 2)-β-D-glucopyranosyl)oxy]-2α,5α,6β-trihydroxyspirostan-3β-yl *O*-β-D-glucopyranosyl-(1 → 2)-[β-D-xylopyranosyl-(1 → 3)]-β-D-glucopyranosyl-(1 → 4)-β-D-galactopyranoside.

Karatavioside J (**4**) had the molecular formula $C_{56}H_{94}O_{27}$ based on the HR-ESITOFMS data (*m*/*z* 1221.5922 [M+Na]$^+$). The ^{1}H NMR spectrum showed signals for five anomeric protons at δ_H 5.55 (d, *J* = 7.8 Hz), 5.21 (d, *J* = 7.9 Hz), 5.16 (d, *J* = 7.8 Hz), 4.85 (d, *J* = 7.8 Hz), and 4.72 (d, *J* = 7.8 Hz), along with signals for five steroid methyl groups of the cholestane type at δ_H 1.17 (d, *J* = 6.8 Hz), 0.93 (s), 0.88 (d, *J* = 6.1 Hz), 0.87 (d, *J* = 6.1 Hz), and 0.82 (s). Compound **4** was a glycoside consisting of seven carbohydrate moieties bound to a cholesterol derivative, and acid hydrolysis of **4** yielded the aglycone (**4a**), and D-galactose, D-glucose, and D-xylose as the carbohydrate moieties. Compound **4a** was identified as (22*S*)-cholest-5-ene-3β,16β,22-triol [8]; however, the absolute configuration at C-22 of the reference compound was previously determined by the comparison of its NMR data with those of (22*S*)-16β,22-dihydroxycholest-4-en-3-one [8]. To confirm the absolute configuration at C-22, we used the modified Mosher's method to analyze **4a**. Treatment of **4a** with (*S*)- or (*R*)-α-methoxy-α-trifluoromethylphenylacetic acid (MTPA) in the presence of *N*,*N*′-dicyclohexylcarbodiimide (DCC) and 4-dimethylaminopyridine (4-DMAP) afforded the (*S*)-MTPA ester **4b** and (*R*)-MTPA ester **4c**, respectively. The chemical shift difference values (Δδ) between **4b** and **4c** shown in Fig. 3 indicate that the absolute configuration at C-22 of **4a** was *S*. Analysis of the ^{1}H-^{1}H COSY, 1D-selective TOCSY, HMQC, and HMBC spectra of **4** indicated that a lycotetraosyl moiety was attached to C-3 of the aglycone, as in the other glycosides isolated in this study. Furthermore, six signals corresponding to a terminal β-D-glucopyranosyl unit (Glc'''') were observed at δ_C 107.0 (CH), 75.7 (CH), 78.2 (CH), 71.8 (CH), 78.7 (CH), and 63.0 (CH_2). The anomeric proton signal due to the terminal glucosyl group at δ_H 4.72 showed an HMBC correlation with C-16 at δ_C 82.8. Thus, the structure of **4** was determined to be (22*S*)-16β-[(β-D-glucopyranosyl)oxy]-22-hydroxycholest-5-en-3β-yl *O*-β-D-glucopyranosyl-(1 → 2)-[β-D-xylopyranosyl-(1 → 3)]-β-D-glucopyranosyl-(1 → 4)-β-D-galactopyranoside.

Karatavioside K (**5**) had the molecular formula $C_{62}H_{104}O_{32}$ based on the HR-ESITOFMS data (*m*/*z* 1383.6398 [M+Na]$^+$). The molecular formula was larger than that of **4** by $C_6H_{10}O_5$, which corresponded to one hexose unit. The ^{1}H NMR spectrum of **5** showed six anomeric protons signals at δ_H 5.56 (d, *J* = 7.6 Hz), 5.24 (d, *J* = 7.9 Hz), 5.23 (d, *J* = 7.9 Hz), 5.17 (d, *J* = 7.9 Hz), 4.88 (d, *J* = 7.6 Hz), and 4.70 (d, *J* = 7.8 Hz), and five steroid methyl group signals at δ_H 1.19 (d,



$\Delta\delta$ (Hz) = $\Delta\delta_{(S)\text{-MTPA}}$-$\delta_{(R)\text{-MTPA}}$ ($CDCl_3$, 500 MHz)

Fig. 3. Chemical shifts differences between (*S*)-MTPA ester (**4b**) and (*R*)-MTPA ester (**4c**) of **4a**.

Table 3
Cytotoxic activities of **1**–**5**, **1a**, etoposide, and melphalan against malignant cells.

Compounds	CC_{50} (μM)[a]			
	HSC-2	HSC-3	HSC-4	HL-60
1	27	>80	>80	>80
1a	1.4	2.4	1.5	2.2
2	>80	>80	>80	>80
3	35	>80	>80	>80
4	60	>80	>80	27
5	>80	>80	>80	>80
Etoposide	24	n.d.[b]	n.d.	0.3
Melphalan	13	25	32	1.4

[a] Data represent the mean of three experiments performed in triplicate.
[b] Not determined.

J = 6.9 Hz), 0.97 (d, *J* = 6.3 Hz), 0.96 (3H, s), 0.93 (d, *J* = 6.3 Hz), and 0.85 (3H, s, Me-19). Acid hydrolysis of **5** yielded **4a**, D-galactose, D-glucose, and D-xylose. In the ^{13}C NMR spectrum of **5**, the signals corresponding to C-3 and C-16 of the aglycone residue were observed at δ 78.4 and 83.0, respectively, indicating that the sugar linkages were at C-3 and C-16, as they were in **4**. When the ^{13}C NMR spectrum of **5** was compared with that of **4**, six additional signals corresponding to a terminal β-D-glucopyranosyl unit (Glc$^{''''''}$) were observed at δ_C 106.2, 75.7, 78.4, 71.7, 78.9, and 62.6, and the signals from C-3 of the β-D-glucopyranosyl residue (Glc$^{''''}$) attached to C-16 and its neighboring carbons were different, whereas all other signals were very similar. In the HMBC spectrum, correlation peaks were observed between δ_H 5.24 (H-1 of Glc$^{''''''}$) and δ_C 89.4 (C-3 of Glc$^{''''}$), and between δ_H 4.70 (H-1 of Glc$^{''''}$) and δ_C 83.0 (C-16 of the aglycone), indicating the β-D-glucopyranosyl-(1 → 3)-*O*-β-D-glucopyranose linkage to C-16 of the aglycone. Accordingly, **6** was determined to be (22*S*)-16β-[(*O*-β-D-glucopyranosyl-(1 → 3)-β-D-glucopyranosyl)oxy]-22-hydroxycholest-5-en-3β-yl *O*-β-D-glucopyranosyl-(1 → 2)-[β-D-xylopyranosyl-(1 → 3)]-β-D-glucopyranosyl-(1 → 4)-β-D-galactopyranoside.

Karatavioside G–K (**1**–**5**) and karatavioside A (**1a**) were evaluated for their cytotoxic activity against HSC-2, HSC-3, and HSC-4 human oral squamous cell carcinoma and HL-60 human promyelocytic leukemia cell lines (Table 3). Etoposide or melphalan were used as positive controls. Karatavioside A (**1a**), the spirostanol glycoside corresponding to **1** with lycotetraose, showed relatively potent cytotoxicity against HSC-2, HSC-3, HSC-4 and HL-60 cells. As reported in our previous papers, some spirostanol lycotetraosides exhibited cytotoxicity against cultured tumor cells [14–16]. Karatavisides G, I, and J (**1**, **3**, and **4**) exhibited moderate to weak cytotoxic activity against HSC-2 and/or HL-60 cells.

Karatavisides G–K (**1**–**5**) are new bisdesmosidic steroidal glycosides with up to seven monoglycosides, and have a common tetraglycoside (lycotetraose) at the C-3 hydroxy group of the aglycone. Furostanol glycosides are a well-known class of steroidal glycosides, which are precursors of spirostanol glycosides. Naturally occurring 22,26-hydroxyfurostanols exclusively exist in the glycoside form, and most of them bear a β-D-glucopyranosyl unit at C-26 [17], which prevents cyclization and formation of the steroid F ring as seen in spirostanols [18]. A small number of furostanols with a rhamnosyl or diglucosyl unit at C-26 have been isolated from plants [19–22]. The structure of karatavioside G (**1**) is unique because it is the first example of a naturally occurring furostanol glycoside with a triglucosyl unit at C-26 of the aglycone, although the biosynthetic pathway remains unclear. Several bisdesmosidic spirostanol glycosides have been extensively isolated from higher plants; however, **3** and **4** are first 3,24-*O*-bisdesmosidic spirostanol glycosides with a 24-*O*-diglycosyl unit. Among a variety of cholestanol glycosides isolated from Liliaceae and Agavaceae plants, karataviosides J and K (**4** and **5**) are the most polar naturally occurring cholestanol bisdesmosides discovered and have sugar units composed of five and six monosaccharides, respectively.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.steroids.2014.09.010.

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