Steroids 118 (2017) 41–46

Contents lists available at ScienceDirect

Steroids

journal homepage: www.elsevier.com/locate/steroids

Allimacrosides A–E, new steroidal glycosides from *Allium macrostemon* Bunge

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ARTICLE INFO

Article history:
Received 11 July 2016
Received in revised form 3 December 2016
Accepted 5 December 2016
Available online 11 December 2016

Keywords:
Allium macrostemon
Liliaceae
Steroidal glucosides
Cytotoxicity

ABSTRACT

A new pregnane-type steroidal glycoside (**1**), two new spirostane-type steroidal glycosides (**2**, **3**), and two new furostane-type steroidal glycosides (**4**, **5**), named allimacrosides A–E, together with four known compounds (**6**–**9**) were isolated from a 80% MeOH extract of *Allium macrostemon* Bunge. The identification and structural elucidation of these compounds were based on their 1D- and 2D-NMR spectra, and HR-FAB-MS data analysis. The isolated compounds were tested for cytotoxicity against four human tumor cell lines *in vitro* using the sulforhodamine B bioassay.

1. Introduction

Allium macrostemon Bunge (Liliaceae) is a perennial herb that grows in the fields and mountains of Southeast Asia. Its dried bulbs are popularly known as traditional Chinese medicine "Xiebai" and are used as treatments for several heart diseases such as thoracic pain, stenocardia, heart asthma, etc [1]. Through our search for structurally interesting compounds from *Allium* sp., we have reported the isolation of flavonoid glycosides and phenolic compounds from *A. victorialis* var. *platyphyllum* [2,3] and *A. tuberosum* [4]. A number of steroidal saponins with various biological activities, such as macrostemonosides A–S, have been isolated from the dried bulbs of *A. macrostemon* [5–9].

Continuing investigation of the steroidal glycoside constituents of the *n*-BuOH fraction of the MeOH extract has resulted in the isolation of a new pregnane-type steroidal glycoside (**1**), two new spirostane-type steroidal glycosides (**2**, **3**), and two new furostane-type steroidal glycosides (**4**, **5**), named allimacrosides A–E, together with a known pregnane-type steroidal glycoside (**6**) and three known furostane-type steroidal glycosides (**7**–**9**). The structure of the new compounds were elucidated by analysis of 1D- and 2D-NMR (^{1}H- and ^{13}C NMR, distortionless enhancement by polarization transfer (DEPT), ^{1}H-^{1}H correlated spectroscopy (COSY), heteronuclear single quantum correlation (HSQC), heteronuclear multiple bond correlation (HMBC) and nuclear Overhauser effect spectroscopy (NOESY)) data, acid hydrolysis and GC–MS. All isolated compounds (**1**–**9**) were evaluated for their cytotoxic activities against four human cancer cell lines.

2. Experimental

2.1. General experimental procedures

Optical rotations were measured on a Jasco P-1020 polarimeter in MeOH. IR spectra were recorded on a Bruker IFS-66/S FT-IR spectrometer. UV spectra were recorded using a Schimadzu UV-1601 UV–visible spectrophotometer. HR-FAB mass spectra were obtained on a JEOL JMS700 mass spectrometer. NMR spectra, including ^{1}H-^{1}H COSY, DEPT, HSQC, HMBC and NOESY experiments, were recorded on a Varian UNITY INOVA 700 NMR spectrometer operating at 700 MHz (^{1}H) and 175 MHz (^{13}C) with chemical shifts given in ppm (δ). Preparative HPLC was conducted using a Gilson 306 pump with Shodex refractive index detector and Econosil RP-C_{18} 10 μm column (250 × 10 mm). Silica gel 60 (Merck, 70–230 mesh and 230–400 mesh) and RP-C_{18} silica gel (YMG GEL ODS-A, 12 nm, S-75 μm) were used for column chromatography. TLC was performed using percolated Silica gel F_{254} plates and RP-18 F_{254s} plates (Merck). Spots were detected by TLC under UV light or by heating after spraying with 10% H_2SO_4 in C_2H_5OH (v/v). A Hewlett-Packard (HP) GC system 6890 Series equipped with a 5973 Mass Selective Detector (MSD) system was controlled by the

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http://dx.doi.org/10.1016/j.steroids.2016.12.002
0039-128X/

Enhanced ChemStation Version B.01.00 software. The capillary column used for GC was an Agilent J&W HP-5MS UI (30.0 m × 0.25 mm i.d., 0.25 μm film thickness coated 5% diphenyl 95% dimethylpolysiloxane).

2.2. Plant material

A. mactostemon was collected in Taeback, Gangwon province, Korea in April 2010, and the plant was identified by one of the authors (K. R. Lee). A voucher specimen (SKKU-NPL 1202) was deposited in the herbarium of the School of Pharmacy, Sungkyunkwan University, Suwon, Korea.

2.3. Extraction and isolation

Dried whole plants of *A. mactostemon* (1.5 kg) were extracted with 80% MeOH three times at room temperature and evaporated under reduced pressure to give a residue (210.0 g), which was dissolved in water (800 ml) and partitioned with solvent to give *n*-hexane (10.0 g), $CHCl_3$ (5.5 g), EtOAc (1.9 g), and *n*-BuOH (12.2 g) soluble layers. The *n*-BuOH-soluble layer (12.2 g) was chromatographed on a silica gel column (diameter × height: 5.5 × 35.0 cm, 500.0 g) with a $CHCl_3$-MeOH-H_2O (20:10:1 to 2:3:1) to give 10 fractions (B1-B10) based on a TLC analysis. Fraction B5 (1.3 g) was separated on a RP-C_{18} silica gel open column (2.5 × 33.0 cm, 80.0 g), eluting with 60% aqueous MeOH to give five subfractions (B51-B55). Subfraction B54 (140 mg) was separated on Lobar A®-column eluted with $CHCl_3$-MeOH-H_2O (30:10:1) to give two subfractions (B541-B542). Subfraction B542 (120 mg) was separated on RP-C_{18} Lobar A®-column (40% aqueous MeCN) to give two subfractions (B5421-B5422). Subfraction B5422 (40 mg) was purified by RP-C_{18} semi-prep. HPLC (35% aqueous MeCN) to afford compounds **1** (13 mg, t_R = 18.8 min) and **6** (9 mg, t_R = 21.8 min). Subfraction B5421 (70 mg) was purified by RP-C_{18} semi-prep. HPLC (25% aqueous MeCN) to afford compounds **7** (18 mg, t_R = 21.5 min) and **8** (36 mg, t_R = 25.0 min). Subfraction B541 (20 mg) was purified by RP-C_{18} semi-prep. HPLC (25% MeCN) to afford compound **9** (14 mg, t_R = 13.2 min). Fraction B7 (2.6 g) was separately chromatographed on a Diaion HP-20 column (3.0 × 30.0 cm, 100.0 g) eluting with a gradient solvent system of 100% H_2O and 100% MeOH, yielding subfractions B71 and B72. Subfraction B72 (1.5 g) was separated on an RP-C_{18} silica gel open column (2.5 × 30.0 cm, 80 g), eluting with 40% aqueous MeOH to give six subfractions (B721-B726). Subfraction 724 (95 mg) was purified by RP-C_{18} semi-prep. HPLC (23% aqueous MeCN) to afford compound **4** (10 mg, t_R = 16.2 min). Subfraction 725 (40 mg) was purified by RP-C_{18} semi-prep. HPLC (30% aqueous MeCN) to afford compounds **3** (10 mg, t_R = 11.8 min), **2** (7 mg, t_R = 13.7 min), and **5** (3 mg, t_R = 15.7 min).

2.3.1. Allimacroside A (**1**)

White amorphous powder; $[\alpha]_D^{25}$-48.4 (MeOH); UV (MeOH) λ_{max}: 239 nm; IR (KBr) ν_{max}: 3385, 2934, 1664, 1371, 1070 cm^{-1}; ^1H NMR (C_5D_5N, 700 MHz) data for aglycone moiety, see Table 1; ^1H NMR data for sugar moiety: H-1′ [4.87, d (7.7)], H-2′ (4.38, m), H-3′ (4.08, m), H-4′ [4.57, d (3.2)], H-5′ [3.95, dd, (9.0, 5.6)], H-6′ (4.65, m; 4.15, m), H-1″ [5.14, d (8.0)], H-2″ (4.38, m), H-3″ (4.20, m), H-4″ (3.83, m), H-5″ (3.84, m), H-6″ [4.48, d (10.5); 3.99, m], H-1‴ [5.58, d (7.7)], H-2‴ (4.05, m), H-3‴ (4.13, m), H-4‴ (4.14, m), H-5‴ (4.03, m), H-6‴ (4.56, m; 4.26, m), H-1⁗ [5.29, d (8.0)], H-2⁗ (4.04, m), H-3⁗ (4.18, m), H-4⁗ (4.23, m), H-5⁗ (3.86, m), H-6⁗ (4.53, m; 4.36, m); ^{13}C NMR (C_5D_5N, 175 MHz) see Table 2; HR-FAB-MS *m*/*z* 985.4251 $[M+Na]^+$ (calcd. for $C_{45}H_{70}NaO_{22}$: 985.4251).

Pregna-5,16-dien-3β-ol-20-one (**1a**): Colorless gum; $[\alpha]_D^{25}$-27.3 (MeOH); ^1H NMR ($CDCl_3$, 700 MHz): δ_H 6.64 (1H, t, *J* = 3.0 Hz, H-16), 5.32 (1H, d, *J* = 5.2 Hz, H-6), 3.47 (1H, m, H-3), 2.19 (3H, s, H-21), 0.92 (3H, s H-19), 0.87 (3H, s, H-18). HR-FAB-MS *m*/*z* 315.2319 $[M+H]^+$ (calcd. for $C_{21}H_{31}O_2$: 315.2319).

2.3.2. Allimacroside B (**2**)

Colorless gum; $[\alpha]_D^{25}$-32.0 (MeOH); IR (KBr) ν_{max}: 3385, 2927, 1563, 1512, 1451, 1371, 1075, 895 cm^{-1}; ^1H NMR (C_5D_5N, 700 MHz) data for aglycone moiety, see Table 1; ^1H NMR data for sugar moiety: H-1′ [4.86, d (7.5)], H-2′ (4.42, m), H-3′ (4.10, m), H-4′ [4.58, d (2.8)], H-5′ [3.95, dd (8.7, 5.7)], H-6′ (4.68, m; 4.20, m), H-1″ [5.13, d (8.0)], H-2″ (4.37, m), H-3″ (4.19, m), H-4″ (3.77, m), H-5″ (3.82, m), H-6″ (4.45, m; 3.98, m), H-1‴ [5.56, d (7.7)], H-2‴ (4.05, m), H-3‴ (4.12, m), H-4‴ (4.13, m), H-5‴ (4.02, m), H-6‴ (4.52, m; 4.25, m), H-1⁗ [5.29, d (8.0)], H-2⁗ (4.05, m), H-3⁗ (3.85, m), H-4⁗ (4.23, m), H-5⁗ (3.85, m), H-6⁗ (4.52, m; 4.37, m), H-1′′′′′ [4.90, d (7.7)], H-2′′′′′ (4.05, m), H-3′′′′′ (4.18, m), H-4′′′′′ (4.26, m), H-5′′′′′ (3.84, m), H-6′′′′′ (4.51, m; 4.35, m); ^{13}C NMR (C_5D_5N, 175 MHz) see Table 2; HR-FAB-MS *m*/*z* 1265.5778 $[M+Na]^+$ (calcd. for $C_{57}H_{94}NaO_{29}$: 1265.5773).

2.3.3. Allimacroside C (**3**)

Colorless gum; $[\alpha]_D^{25}$-28.0 (MeOH); IR (KBr) ν_{max}: 3385, 2928, 1549, 1452, 1434, 1371, 1305, 1074, 896 cm^{-1}; ^1H NMR (C_5D_5N, 700 MHz) data for aglycone moiety, see Table 1; ^1H NMR data for sugar moiety: H-1′ [4.87, d (7.7)], H-2′ [4.43, t (8.4)], H-3′ (4.07, m), H-4′ [4.57, d (2.6)], H-5′ [3.95, dd (8.7, 5.7)], H-6′ (4.67, m; 4.17, m), H-1″ [5.14, d (8.0)], H-2″ (4.35, m), H-3″ (4.21, m), H-4″ (3.81, m), H-5″ (3.83, m), H-6″ (4.47, m; 3.99, m), H-1‴ [5.57, d (8.0)], H-2‴ (4.05, m), H-3‴ (4.13, m), H-4‴ (4.14, m), H-5‴ (4.03, m), H-6‴ (4.53, m; 4.26, m), H-1⁗ [5.29, d (8.0)], H-2⁗ (4.06, m), H-3⁗ (3.86, m), H-4⁗ (4.24, m), H-5⁗ (3.86, m), H-6⁗ (4.52, m; 4.37, m), H-1′′′′′ [4.92, d (8.0)], H-2′′′′′ (4.06, m), H-3′′′′′ (4.19, m), H-4′′′′′ (4.27, m), H-5′′′′′ (3.85, m), H-6′′′′′ (4.52, m; 4.36, m); ^{13}C NMR (C_5D_5N, 175 MHz) see Table 2; HR-FAB-MS *m*/*z* 1263.5621 $[M+Na]^+$ (calcd. for $C_{57}H_{92}NaO_{29}$: 1263.5622).

2.3.4. Allimacroside D (**4**)

Colorless gum; $[\alpha]_D^{25}$-23.3 (MeOH); IR (KBr) ν_{max}: 3385, 2931, 1549, 1452, 1377, 1161, 1077 cm^{-1}; ^1H NMR (C_5D_5N, 700 MHz) data for aglycone moiety, see Table 1; ^1H NMR data for sugar moiety: H-1′ [4.92, d (7.5)], H-2′ (4.53, m), H-3′ (4.11, m), H-4′ (4.58, m), H-5′ (4.02, m), H-6′ (4.61, m; 4.16, m), H-1″ [5.17, d (8.0)], H-2″ (4.32, m), H-3″ (4.17, m), H-4″ (3.82, m), H-5″ (3.82, m), H-6″ (4.47, m; 4.00, m), H-1‴ [5.57, d (8.0)], H-2‴ (4.03, m), H-3‴ (4.15, m), H-4‴ (4.13, m), H-5‴ (3.87, m), H-6‴ (4.54, m; 4.40, m), H-1⁗ [5.29, d (7.7)], H-2⁗ (4.03, m), H-3⁗ (4.18, m), H-4⁗ (4.23, m), H-5⁗ (3.93, m), H-6⁗ (4.53, m; 4.26, m), H-1′′′′′ [4.81, d (7.7)], H-2′′′′′ (4.02, m), H-3′′′′′ (4.23, m), H-4′′′′′ (4.14, m), H-5′′′′′ (4.03, m), H-6′′′′′ (4.54, m; 4.38, m); ^{13}C NMR (C_5D_5N, 175 MHz) see Table 2; HR-FAB-MS *m*/*z* 1281.5728 $[M+Na]^+$ (calcd. for $C_{57}H_{94}NaO_{30}$: 1281.5722).

2.3.5. Allimacroside E (**5**)

Colorless gum; $[\alpha]_D^{25}$-14.0 (MeOH); IR (KBr) ν_{max}: 3385, 2930, 1549, 1450, 1369, 1306, 1161, 1076 cm^{-1}; ^1H NMR (C_5D_5N, 700 MHz) data for aglycone moiety, see Table 1; ^1H NMR data for sugar moiety: H-1′ [4.87, d (7.5)], H-2′ (4.43, m), H-3′ (4.09, m), H-4′ [4.57, d (2.8)], H-5′ (3.95, m), H-6′ (4.67, m; 4.16, m), H-1″ [5.14, d (8.0)], H-2″ (4.37, m), H-3″ (4.20, m), H-4″ (3.83, m), H-5″ (3.84, m), H-6″ (4.48, m; 4.00, m), H-1‴ [5.57, d (7.7)], H-2‴ (4.06, m), H-3‴ (4.12, m), H-4‴ (4.14, m), H-5‴ (3.86, m), H-6‴ (4.53, m; 4.37, m), H-1⁗ [5.29, d (8.0)], H-2⁗ (4.05, m), H-3⁗ (4.19, m), H-4⁗ (4.23, m), H-5⁗ (3.95, m), H-6⁗ (4.53, m; 4.26, m), H-1′′′′′ [4.86, d (7.5)], H-2′′′′′ (4.05, m), H-3′′′′′ (4.25, m), H-4′′′′′ (4.25, m), H-5′′′′′ (4.03, m), H-6′′′′′ (4.58, m; 4.40, m); ^{13}C NMR

Table 1
^{1}H-NMR data (700 MHz) for the aglycone moieties of compounds **1**–**5**[a] (δ in ppm, C_5D_5N, J in Hz).

Position		**1**	J (Hz)	**2**	J (Hz)	**3**	J (Hz)	**4**	J (Hz)	**5**	J (Hz)
		δ_H		δ_H		δ_H		δ_H		δ_H	
1	a	1.65 m		1.47 m		1.64 m		2.30 dd	12.8, 4.4	1.64 m	
	b	0.90 m		0.75 m		0.93 m		1.29 m		0.93 m	
2	a	2.07 m		2.01 m		2.08 m		4.06 m		2.08 m	
	b	1.67 m		1.57 m		1.68 m		–		1.68 m	
3		3.86 m		3.90 m		3.85 m		3.82 m		3.88 m	
4	a	2.65 dd	13.3, 2.4	1.75 m		2.63 dd	13.2, 2.1	2.69 dd	14.0, 5.2	2.63 dd	13.6, 2.4
	b	2.41 t-like	12.2	1.75 m		2.40 t-like	12.4	2.54 t-like	12.4	2.39 t-like	12.4
5		–		0.85 m		–		–		–	
6	a	5.30 o[b]		1.06 m		5.26 br d	4.1	5.28 br s		5.26 br d	4.1
	b	–		1.06 m		–		–		–	
7	a	1.84 m		1.45 m		1.79 m		1.80 m		1.77 m	
	b	1.52 m		0.73 m		1.42 m		1.45 m		1.41 m	
8		1.50 m		1.37 m		1.45 m		1.47 m		1.42 m	
9		0.90 m		0.45 m		0.83 m		0.93 m		0.79 m	
10		–		–		–		–		–	
11	a	1.46 m		1.33 m		1.38 m		1.46 m		1.33 m	
	b	1.46 m		1.12 m		1.32 m		1.38 m		1.33 m	
12	a	2.59 m		1.57 m		1.61 m		1.69 m		1.79 m	
	b	1.35 m		0.97 m		1.03 m		1.07 m		1.08 m	
13		–		–		–		–		–	
14		1.28 td	11.5, 6.6	0.96 m		0.99 m		1.01 m		0.87 m	
15	a	2.12 ddd	16.9, 6.6, 3.4	2.08 m		1.94 m		1.97 m		1.97 m	
	b	1.87 m		1.31 m		1.35 m		1.41 m		1.37 m	
16		6.59 dd	3.1, 1.8	4.49 m		4.50 m		4.93 m		4.94 m	
17		–		1.72 m		1.72 m		1.91 m		2.06 m	
18		0.91 s		0.72 s		0.73 s		0.86 s		0.83 s	
19		0.87 s		0.60 s		0.85 s		0.93 s		0.85 s	
20		–		1.90 m		1.91 m		2.20 m		–	
21		2.23 s		1.03 d	6.9	1.05 d	6.9	1.31 d	6.9	1.38 s	
22				–		–		–		–	
23	a			2.66 dd	12.9, 4.7	2.67 dd	13.0, 4.8	2.04 m		4.32 m	
	b			1.95 dd	12.9, 10.7	1.96 dd	13.0, 10.7	2.00 m		–	
24	a			4.02 ddd	10.7, 10.7, 4.7	4.03 ddd	10.7, 10.7, 4.8	2.04 m		2.46 dt	14.0, 6.2
	b			–		–		1.67 m		2.22 dt	14.3, 7.9
25				1.88 m		1.90 m		1.90 m		2.10 m	
26	a			3.62 dd	11.3, 4.7	3.63 dd	11.3, 4.9	3.93 m		4.00 m	
	b			3.56 dd	11.4, 11.3	3.57 dd	11.4, 11.3	3.60 m		3.70 dd	9.5, 6.0
27				1.13 d	6.5	1.14 d	6.5	0.97 d	6.7	1.08 d	6.7
OMe										3.15 s	

[a] Assignments are based on DEPT, HSQC, COSY, HMBC, and NOESY experiments.
[b] Signal pattern unclear due to overlapping.

(C_5D_5N, 175 MHz) see Table 2; HR-FAB-MS m/z 1277.5778 $[M+Na]^+$ (calcd. for $C_{58}H_{94}NaO_{29}$: 1277.5773).

2.4. Acid hydrolysis of compounds *1*–*5* and sugar determination

Each compound (2.5 mg) was dissolved in 2 mL of 15% HCl. The solution was heated at 80 °C for 2 h. The hydrolysate was extracted with CH_2Cl_2, and the aqueous layer was neutralized using an Amberlite IRA-67 column to yield the sugar. The sugar acquired from the hydrolysis was dissolved in anhydrous pyridine (0.1 mL), and 2.0 mg of L-cysteine methyl ester hydrochloride was added. The mixture was stirred at 60 °C for 1.5 h and trimethylsilylated through adding 0.1 mL of 1-trimethylsilylimidazole for 2 h. The mixture was partitioned with *n*-hexane and H_2O (0.3 mL each), and the *n*-hexane layer (1.0 μL) was analyzed through GC/MS. Identification of D-galactose (20.093 min) and D-glucose (22.103 min) were detected in each case by co-injection of the hydrolysate with standard silylated sugars.

2.5. Cytotoxicity test

A sulforhodamine B bioassay (SRB) was used to determine the cytotoxicity of the compounds. The cytotoxic activity of each compound against four cultured human tumor cells was examined *in vitro* at the Korea Research Institute of Chemical Technology. The tumor cell lines were A549 (non small cell lung adenocarcinoma), SK-OV-3 (ovarian cancer cells), SK-MEL-2 (skin melanoma), and HCT15 (colon cancer cells) [10]. Etoposide was used as the positive control. IC_{50} values for the cytotoxicity of etoposide were 1.02, 1.74, 0.21, and 1.93 μM to A549, SK-OV-3, SK-MEL-2, and HCT15 cells, respectively.

3. Results and discussion

Compound **1** was isolated as a white amorphous powder. The molecular formula was determined to be $C_{45}H_{70}O_{22}$ from the molecular ion peak $[M+Na]^+$ at m/z 985.4251 (calcd. for $C_{45}H_{70}NaO_{22}$: 985.4251) in the positive-ion high resolution (HR)-fast atom bombardment (FAB)-MS. The IR spectrum showed characteristic absorptions for α,β-unsaturated ketone (1664 cm^{-1}), hydroxyl (3385 cm^{-1}), and glycosidic linkage (1000–1160 cm^{-1}) [11]. The ^{1}H NMR spectrum of **1** (Table 1) displayed the signals of two olefinic protons at δ_H 6.59 (dd, J = 3.1, 1.8 Hz, H-16) and 5.30 (overlap, H-6), an oxymethine proton at δ_H 3.86 (m, H-3), and three methyl singlet signals at δ_H 2.23 (s, H-21), 0.91 (s, H-18), and 0.87 (s, H-19) of aglycone, and four anomeric protons at 4.87 (d, J = 7.7 Hz, H-1′), 5.14 (d, J = 8.0 Hz, H-1″), 5.58 (d, J = 7.7 Hz, H-1‴), and 5.29 (d, J = 8.0 Hz, H-1⁗) of four sugar moieties. The ^{13}C NMR spectrum (Table 2) showed a total of 45 carbon signals, of which 21 carbons were assigned to the aglycone and the remaining 24 carbons to

Table 2
^{13}C NMR data (175 MHz) for compounds **1–5**[a] (δ in ppm, C_5D_5N).

Position	1	2	3	4	5	Position	1	2	3	4	5
1	38.6	36.8	38.7	47.0	37.2	Gal 1′	104.0	102.2	104.0	104.6	102.5
2	31.4	29.7	31.4	71.3	29.9	2′	74.5	73.0	74.5	73.9	72.9
3	79.4	77.1	79.3	85.7	77.9	3′	76.8	75.4	77.0	76.8	75.3
4	40.5	34.6	40.5	39.0	39.0	4′	81.5	80.0	81.5	80.9	80.0
5	142.7	44.4	142.2	141.3	140.8	5′	76.5	75.1	76.6	76.6	75.0
6	122.6	28.7	122.9	123.2	121.3	6′	61.8	60.4	61.8	61.8	60.3
7	33.0	32.1	33.5	33.5	31.7	Glc 1″	106.4	104.9	106.4	105.9	104.9
8	31.6	34.9	32.8	32.3	30.7	2″	82.7	81.3	82.8	82.6	81.2
9	52.0	54.1	51.5	51.5	49.7	3″	89.8	88.3	89.7	89.9	88.8
10	38.3	35.4	38.3	39.2	36.7	4″	72.1	70.6	72.1	72.1	70.6
11	22.2	21.0	22.3	22.4	20.3	5″	78.8	77.3	78.8	78.8	77.3
12	36.4	39.8	41.0	41.1	38.9	6″	64.3	62.8	64.3	64.3	62.8
13	47.5	40.5	41.7	42.0	40.0	Glc 1‴	106.2	104.7	106.2	106.1	104.7
14	57.7	56.1	57.9	57.7	56.4	2‴	77.4	75.9	77.4	77.3	75.9
15	33.6	31.7	33.3	33.7	33.2	3‴	79.1	77.7	79.1	79.5	77.6
16	146.0	81.3	82.7	82.4	83.7	4‴	72.8	71.3	72.9	72.5	71.4
17	156.5	62.3	63.7	65.1	66.4	5‴	79.9	78.4	80.0	79.7	78.3
18	17.2	16.4	17.6	17.7	13.4	6‴	63.6	62.1	63.6	63.8	62.1
19	20.5	12.0	20.6	21.7	19.1	Glc 1⁗	105.8	104.3	105.9	105.8	104.3
20	197.6	41.9	43.4	42.0	82.1	2⁗	76.6	75.1	76.5	76.9	75.0
21	28.4	14.6	16.4	17.7	15.0	3⁗	79.9	78.4	80.0	79.9	78.4
22		113.7	112.9	111.9	157.0	4⁗	72.2	70.7	72.2	73.0	70.7
23		40.6	42.1	38.5	96.0	5⁗	79.9	77.8	79.9	79.8	78.4
24		81.2	82.8	29.6	29.4	6⁗	63.6	62.1	63.6	63.6	62.1
25		38.0	39.5	35.5	34.7	Glc 1⁗′		106.2	107.7	106.2	104.7
26		64.9	66.4	76.6	75.0	2⁗′		75.3	76.8	76.5	75.0
27		13.3	14.8	18.7	17.3	3⁗′		78.4	80.0	80.0	78.4
OMe					48.6	4⁗′		71.5	73.0	72.9	71.5
						5⁗′		78.4	79.3	79.9	78.4
						6⁗′		62.6	64.1	64.1	62.6

[a] Assignments are based on DEPT, HSQC, and HMBC experiments.

four sugar moieties. A comparison of the NMR spectral findings of **1** with literature data revealed that the aglycone pair of **1** was identical to that of pregna-5,16-dien-3β-ol-20-one [12]. The remaining 24 carbon signals were supposed to be the sugar moiety (R_1), which was identical to that of solanigroside B (**6**) [13]. Starting from these four anomeric protons, the exact identity of the monosaccharides and the sequence of the tetrasaccharide chain were also determined by analysis of a combination of DEPT, ^{1}H-^{1}H COSY, HSQC, and HMBC spectra. The connectivity of the four sugars was mainly based on the HMBC correlations: H-1′ (δ_H 4.87, d, J = 7.7 Hz,) with C-3 (δ_C 79.4) of the aglycone, H-1″ (δ_H 5.14, d, J = 8.0 Hz) with C-4′ (δ_C 81.5), H-1‴ (δ_H 5.58, d, J = 7.7 Hz) with C-2″ (δ_C 82.7), and H-1⁗ (5.29, d, J = 8.0 Hz) with C-3″ (δ_C 89.8). Acid hydrolysis of **1** afforded the aglycone pregna-5,16-dien-3β-ol-20-one (**1a**) and two kinds of sugars, D-glucose and D-galactose, which were identified by GC analysis after derivatization [14]. The identification of the aglycone was confirmed by ^{1}H NMR, optical rotation, and HR-FAB-MS analyses [15]. The relative stereochemistry of the aglycone was corroborated by the NOESY cross-peaks of H-1$_{ax}$/H-3, H-8/H$_3$-18 and H$_3$-19, H-9/H-14. The anomeric configuration of D-glucose and D-galactose was determined to be β form, respectively, on the basis of the J value of the anomeric proton in D-glucose (8.0 Hz) and D-galactose (7.7 Hz). Thus, the structure of **1** was established as pregna-5,16-dien-3β-ol-20-one-3-*O*-β-D-glucopyranosyl(1→2)-[β-D-glucopyranosyl(1→3)]-β-D-glucopyranosyl(1→4)-β-D-galactopyranoside, named allimacroside A (Fig. 1).

Compound **2** was isolated as a colorless gum. The molecular formula was determined to be $C_{57}H_{94}O_{29}$ from the molecular ion peak $[M+Na]^+$ at m/z 1265.5778 (calcd. for $C_{57}H_{94}NaO_{29}$: 1265.5773) in the positive-ion HR-FAB-MS. The ^{1}H NMR spectrum of **2** showed signals for four steroidal methyl groups at δ_H 1.13 (d, J = 6.5 Hz, H-27), 1.03 (d, J = 6.9 Hz, H-21), 0.72 (s, H-18), and 0.60 (s, H-19). The ^{13}C NMR spectrum showed a total of 57 carbon signals, of which 27 carbons were assigned to the aglycone and the remaining 30 carbons were assigned to the sugar moieties. A comparison of the NMR spectral data of **2** with literature data revealed that the aglycone pair of **2** was identical to that of (24*S*,25*S*)-5α-spirostane-3β,24-diol [14]. The remaining 30 carbon signals were supposed to be 5 sugars, one D-galactose and four D-glucose moieties. The anomeric configuration of D-glucose was determined by J values at δ_H 5.56 (d, J = 7.7 Hz, H-1‴), 5.29 (d, J = 8.0 Hz, H-1⁗), 5.13 (d, J = 8.0 Hz, H-1″), 4.90 (d, J = 8.0 Hz, H-1⁗′) as all β forms, D-galactose by J values at δ_H 4.86 (d, J = 8.0 Hz, H-1′) as β form, and the sequence of sugars in the oligosaccharide chain was deduced by the HMBC correlations between H-1⁗′/C-24, and H-1′/C-3. Acid hydrolysis of **2** gave D-galactose and D-glucose in a ratio of 1:4, while the aglycone was decomposed under acidic conditions. Identification of D-galactose and D-glucose was performed by GC analysis. The relative stereochemistry of the aglycone was corroborated by cross-peaks of H-14/H-5, H-9, H-16, and H-17 and H-21/H-17 in the NOESY spectrum. The chemical shifts of C-5 (δ_C 44.4), C-9 (δ_C 54.1) and C-19 (δ_C 12.0) were consistent with those of the 5αH configuration [16–18]. The proton multiplicity of H-24, with J values of 10.7 Hz (H-24/H-23ax), 10.7 Hz (H-24/H-25), 4.7 Hz (H-24/H-23 eq), and NOE correlations between H-23ax and H-20/H-25, and between H-26ax and H-24/H-16 in the phase-sensitive NOESY spectrum were consistent with the 22α, 24*S*, and 25*S* configurations (Fig. 2). Thus, the structure of **2** was established as (24*S*,25*S*)-24-[(β-D-glucopyranosyl)oxy]-5α-spirostan-3β-yl-*O*-β-D-glucopyranosyl(1→2)-[β-D-glucopyranosyl(1→3)]-β-D-glucopyranosyl(1→4)-β-D-galactopyranoside, named allimacroside B (Fig. 1).

Compound **3** was isolated as a colorless gum. The molecular formula was determined to be $C_{57}H_{92}O_{29}$ from the molecular ion peak $[M+Na]^+$ at m/z 1263.5621 (calcd. for $C_{57}H_{92}NaO_{29}$: 1263.5622) in the positive-ion HR-FAB-MS. The ^{1}H NMR spectrum of **3** showed signals for four steroidal methyl groups at δ_H 1.14 (d, J = 6.5 Hz, H-27), 1.05 (d, J = 6.9 Hz, H-21), 0.85 (s, H-19), and 0.73 (s, H-18),

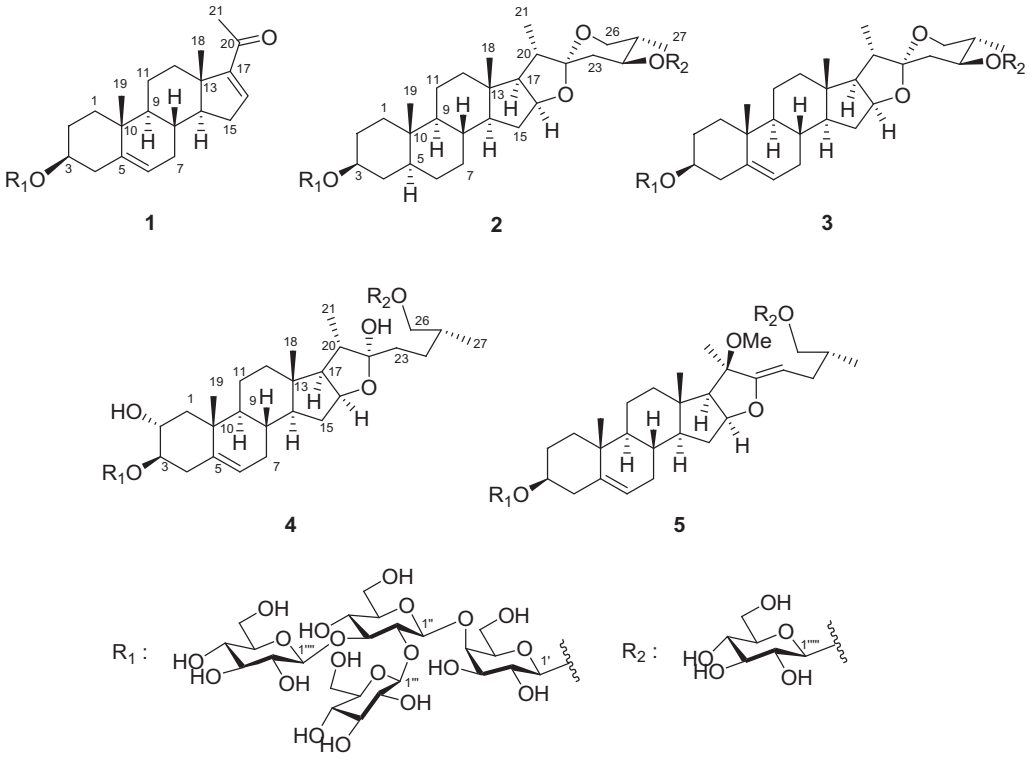

Fig. 1. Structures of compounds **1**–**5**.

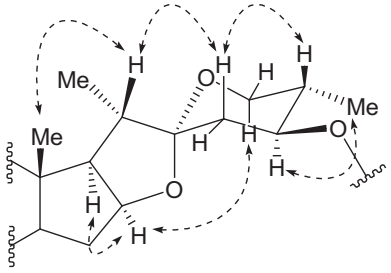

Fig. 2. Important NOE correlations of compound **2**.

as well as signals for five anomeric protons at δ_H 5.57 (d, J = 8.0 Hz, H-1‴), 5.29 (d, J = 8.0 Hz, H-1⁗), 5.14 (d, J = 8.0 Hz, H-1″), 4.92 (d, J = 8.0 Hz, H-1⁗′), and 4.87 (d, J = 7.7 Hz, H-1′), and an olefinic proton signal at δ_H 5.26 (br d, J = 4.1 Hz, H-6) (Table 1). The ^{13}C NMR spectrum showed a total of 57 carbon signals, of which 27 carbons were assigned to the aglycone and the remaining 30 carbons were assigned to the sugar moieties. The ^{1}H NMR spectra of **3** were very similar to those of compound **2** except for an additional double bond at δ_H 5.26 (br d, J = 4.1 Hz, H-6) and δ_C 142.2 (C-5) and 122.9 (C-6). A comparison of the NMR spectral data of **3** with those in the literature revealed that the aglycone of **3** was identical to that of (3β,24S,25S)-spirost-5-ene-3,24-diol [19]. The relative stereochemistry of the aglycone was confirmed by cross-peaks of H-14/H-9, H-16, and H-17 and H-21/H-17 in the NOESY spectrum. The sugar units were the same with those in the compound **2**. Acid hydrolysis of **3** gave D-galactose and D-glucose in a ratio of 1:4, while the aglycone was decomposed under acidic conditions. Thus, the structure of **3** was established as (24S,25S)-24-[(β-D-glucopyranosyl)oxy]-spirost-5-ene-3β,24-diol-3-O-β-D-glucopyranosyl(1→2)-[β-D-glucopyranosyl(1→3)]-β-D-glucopyranosyl(1→4)-β-D-galactopyranoside, named allimacroside C (Fig. 1).

Compound **4** was isolated as a colorless gum. The molecular formula was determined to be $C_{57}H_{94}O_{30}$ from the molecular ion peak $[M+Na]^+$ at m/z 1281.5728 (calcd. for $C_{57}H_{94}NaO_{30}$: 1281.5722) in the positive-ion HR-FAB-MS. The ^{1}H NMR spectrum of **4** showed signals for four steroidal methyl groups at δ_H 1.31 (d, J = 6.9 Hz, H-21), 0.97 (d, J = 6.7 Hz, H-27), 0.93 (s, H-19), and 0.86 (s, H-18), as well as signals for five anomeric protons at δ_H 5.57 (d, J = 7.7 Hz, H-1‴), 5.29 (d, J = 8.0 Hz, H-1⁗), 5.17 (d, J = 8.0 Hz, H-1″), 4.92 (d, J = 7.5 Hz, H-1′), and 4.86 (d, J = 7.5 Hz, H-1⁗′), and an olefinic proton signal at δ_H 5.28 (brs, H-6) (Table 1). The ^{13}C NMR spectrum of **4** had signals at δ 141.3 and 123.2, indicating the presence of an olefinic bond. A comparison of the NMR spectral findings of **4** with literature data revealed that the aglycone pair of **4** was identical to that of fistulosaponin F [20]. The position of OH group at C-2 was confirmed by the HMBC correlations between C-2 and H-1b (δ_H 1.29, m) and H-4a (δ_H 2.69, dd, J = 14.0, 5.2 Hz), and α-form of OH group by the NOESY cross-peak of H-2 with H-19 [20]. The stereochemistry of the OH group at C-22 was assigned as α-orientation by comparison of NMR data with other same skeleton derivatives [20,21]. The 25R form was determined by the chemical shifts of C-23 (δ_C 38.5), C-24 (δ_C 29.6), C-25 (δ_C 35.5), C-26 (δ_C 76.6) and C-27 (δ_C 18.7) [7,20–22]. This was also confirmed by the difference between the chemical shifts of two proton signals of H-26 [22]. The relative stereochemistry of the aglycone was corroborated by the NOESY cross-peaks of H-14/H-9, H-16, and H-17 and H-21/H-17. Among the 57 carbon signals in the ^{13}C NMR spectrum, 27 signals were assigned to the aglycone and the remaining 30 signals to five hexose units. In the HMBC experiment, long-range correlations between the anomeric proton H-1⁗′ and C-26 of the aglycone, and between the anomeric proton H-1′ and C-3 of the aglycone, allowed us to identify C-26 and C-3 as the glucosidic linkage sites. Acid hydrolysis of **4** gave D-galactose and D-glucose in a ratio of 1:4, while the aglycone was decomposed under acidic conditions. Thus, the structure of **4** was established as (25R)-26-O-β-D-glucopyranosyl-5-enefurostan-2α,3β,22α,26-

tetraol-3-*O*-*β*-D-glucopyranosyl(1→2)-[*β*-D-glucopyranosyl(1→3)]-*β*-D-glucopyranosyl(1→4)-*β*-D-galactopyranoside, named allimacroside D (Fig. 1).

Compound **5** was isolated as a colorless gum. The molecular formula was determined to be $C_{58}H_{94}O_{29}$ from the molecular ion peak $[M+Na]^+$ at *m/z* 1277.5778 (calcd. for $C_{57}H_{94}NaO_{30}$: 1277.5773) in the positive-ion HR-FAB-MS. The ^1H NMR spectrum of **5** showed signals for five steroidal methyl groups at δ_H 1.38 (s, H-21), 1.08 (d, *J* = 6.7 Hz, H-27), 0.85 (s, H-19), 0.83 (s, H-18), and 3.15 (s, OMe) as well as signals for five anomeric protons at δ_H 5.57 (d, *J* = 7.7 Hz, H-1‴), 5.29 (d, *J* = 8.0 Hz, H-1⁗), 5.14 (d, *J* = 8.0 Hz, H-1″), 4.97 (d, *J* = 7.5 Hz, H-1′), and 4.86 (d, *J* = 8.0 Hz, H-1⁗′). The ^{13}C NMR spectrum (Table 2) showed a total of 58 carbon signals, of which 28 carbons were assigned to the aglycone and the remaining 30 carbons to the sugar moieties. Comparison of the ^{13}C NMR spectroscopic signals of the aglycone moiety of **5** with literature values and an extensive HMQC and HMBC data analysis, showed that the aglycone of **5** was 20-methoxyl-furosta-5,22-dien-3*β*,26-diol [23]. The relative stereochemistry of the aglycone was corroborated by the cross-peaks of H-14/H-9, H-16, and H-17 in the NOESY spectrum. The *β*-configuration of methoxy group at C-20 was determined by cross-peak between H-21 (δ_H 1.38) and H-17 (δ_H 2.06) in the NOESY spectrum. Comparison of ^1H NMR, ^{13}C NMR, COSY, and HMBC spectrum for the sugar moieties of **5** with those of **4** suggested the same sugar chains. Acid hydrolysis of **5** gave D-galactose and D-glucose in a ratio of 1:4, while the aglycone was decomposed under acidic conditions. Thus, the structure of **5** was established as 26-*O*-*β*-D-glucopyranosyl-20*β*-methoxyl-25(*R*)-furostan-5,22(23)-dien-3*β*,26-diol-3-*O*-*β*-D-glucopyranosyl(1→2)-[*β*-D-glucopyranosyl(1→3)]-*β*-D-glucopyranosyl(1→4)-*β*-D-galactopyranoside, named allimacroside E (Fig. 1).

Additionally, four known compounds were isolated. By comparing their NMR spectroscopic data with those in the literature, their structures were determined to be solanigroside B (**6**) [13], macrostemonoside O (**7**) [8], (25*R*)-26-*O*-*β*-D-glucopyranosyl-22-hydroxy-5*β*-furost-3*β*-26-diol-3-*O*-*β*-D-glucopyranosyl-(1″→2′)-*β*-D-galactopyranoside (**8**) [24], and ceparoside B (**9**) [25].

The cytotoxic activities of the isolated compounds (**1**–**9**) were evaluated by determining their inhibitory effects on human tumor cell lines (A549, SK-OV-3, SK-MEL-2, and HCT15) *in vitro* using the sulforhodamine B (SRB) assay. Compound **3** showed moderate cytotoxicity against SK-MEL-2 cell with IC_{50} values of 23.43 μM. Also, Compound **6** showed moderate cytotoxicity against A549 and SK-MEL-2 cells with IC_{50} values of 23.68 and 14.27 μM, respectively. But the other compounds were inactive (IC_{50}: >30.0 μM).

In summary, five new steroidal saponins, allimacrosides A–E (**1**–**5**), together with four known compounds (**6**–**9**) were isolated from the MeOH extract of *A. macrostemon*. Their structures were determined by the spectral data, including 2D NMR spectra. All the isolated compounds were evaluated their cytotoxic activities against A549, SK-OV-3, SK-MEL-2, and HCT-15 cell lines. Compounds **3** and **6** showed moderate cytotoxic activity against the SK-MEL-2 cells, with IC_{50} values of 23.43 and 14.27 μM, respectively. However, the other compounds were inactive against the four human tumor cell lines.

Acknowledgements

This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2012R1A5A2A28671860). We thank the Korea Basic Science Institute (KBSI) for the MS spectral measurements.

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.2016.12.002.

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