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STEROIDAL GLYCOSIDES FROM THE FLOWERS OF *Allium leucanthum*

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ABSTRACT

Furostanol and spirostanol glycosides **1** and **2** were isolated from the flowers of *Allium leucanthum*, a Caucasian endemic species that grows in Georgia. The structures were established on the base of chemical evidence and spectral analyses (¹H, ¹³C NMR, ¹H-¹H COSY, ¹H-¹³C COSY, HMBC, and HR-MS) data. Compound **1** (leucofuranoside A) was reported for the first time and was identified as 26-O-β-D-glucopyranosyl-(25R)-5α-furostane-3β,6β-diol-3-O-β-D-glucopyranosyl-(1→2)-O-β-D-xylopyranosyl-(1→3)-O-β-D-glucopyranosyl-(1→4)-β-D-galactopyranoside. Compound **2** was identified as (25R)-5α-spirostanane-3β,6β-diol-3-O-β-D-glucopyranosyl-(1→2)-β-D-glucopyranosyl-(1→4)-β-D-galactopyranoside and described for the first time in the genus *Allium*.

Keywords : *Allium leucanthum* ; Alliaceae ; spirostanol glycoside ; furostanol glycoside ; leucofuranoside A.

Saponins are an important family of glycosylated secondary metabolites that are widely distributed in the plant kingdom [1]. Besides food industrial and pharmaceutical applications, saponins are known to be the main constituents of several herbal drugs and folk medicines used worldwide [2]. Their biological activities are linked to the structure of both the aglycone and the sugar moieties. The incredibly wide chemical diversity of saponins has led to a sustained and renewed interest in these compounds.

The genus *Allium* includes up to 800 species of the world's flora. Among them, some 70 grow in the Caucasian region and 35 species are described in Georgia [3]. *Allium leucanthum* K. Koch (Alliaceae), called "whiteflower onion" in Georgia, is a Caucasian endemic species and, along with other *Allium* species, is widely used in Georgian traditional medicine as an antiseptic and antibacterial remedy. Various secondary metabolites have been identified in the genus *Allium* [4, 5]. Among them, steroidal saponins have been investigated for their antibacterial, antileishmanial, antifungal [6-9], cytotoxic [10, 11], and antioxidant activities [12]. Furthermore, steroidal saponins isolated from different species of *Allium* have shown significant cytotoxic activity [13-16],

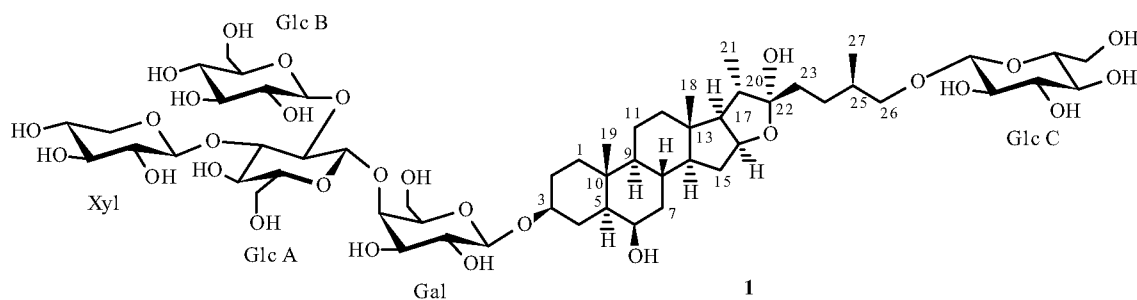
This paper describes the isolation of spirostanol and furostanol glycosides from the flowers of *Allium leucanthum* that grow in Georgia and the structural determination of two these compounds by ¹H, ¹³C nuclear magnetic resonance (NMR), ¹H-¹H correlation spectroscopy (COSY), ¹H-¹³C COSY, heteronuclear multiple bond correlation (HMBC), and high-resolution mass spectroscopy (HR-MS), and the results of hydrolytic cleavage.

Dried and powdered flowers of *Allium leucanthum* (500 g) were extracted twice with hot MeOH-H₂O (8:2, v/v). After removal of solvent, the extract was suspended in water and then extracted with *n*-BuOH. The *n*-BuOH extract was chromatographed over Diaion HP-20, using MeOH-H₂O as the eluent in gradient conditions and EtOAc. The spirostanol fraction was collected in MeOH-H₂O (7:3, v/v), and the furostanol fraction in MeOH-H₂O (5:5, v/v). The spirostanol and furostanol fractions were subjected to column chromatography (CC) on silica

gel to give compounds 1 and 2.

TABLE 1. ^1H (400 MHz) and ^{13}C (100 MHz) NMR Data of Compound 1 (pyridine- d_5 - D_2O , 10:1, δ , ppm)

C atom	δ_{C} (DEPT)	δ_{H}	C atom	δ_{C} (DEPT)	δ_{H}
Aglycone			Glc-A		
1	38.8 (CH ₂)	1.53, 0.89	1	104.4 (CH)	5.15
2	29.9 (CH ₂)	2.13, 1.77	2	80.8 (CH)	4.32
3	78.4 (CH)	4.08	3	86.9 (CH)	4.12
4	32.4 (CH ₂)	2.27 2.13	4	69.9 (CH)	3.77
5	47.8 (CH)	1.07	5	77.1 (CH)	3.85
6	70.8 (CH)	4.00	6	62.4 (CH ₂)	4.50, 4.06
7	40.1 (CH ₂)	1.76, 1.14	Glc-B		
8	30.4 (CH)	2.17	1	104.2 (CH)	5.58
9	54.5 (CH)	0.63	2	75.6 (CH)	4.06
10	35.9 (C)	-	3	77.4 (CH)	4.21
11	20.9 (CH ₂)	1.42	4	70.8 (CH)	4.20
12	40.4 (CH ₂)	2.06, 1.14	5	78.2 (CH)	3.96
13	41.2 (C)	-	6	62.0 (CH ₂)	4.50, 4.39
14	56.1 (CH)	1.11	Xyl		
15	32.3 (CH ₂)	2.10, 1.50	1	104.6 (CH)	5.19
16	81.2 (CH)	4.99	2	74.7 (CH)	3.95
17	63.4 (CH)	2.01	3	77.9 (CH)	4.13
18	16.7 (Me)	0.89	4	70.4 (CH)	4.13
19	15.9 (Me)	1.21	5	66.9 (CH ₂)	4.26, 3.71
20	40.4 (CH)	2.25	Glc-C		
21	16.2 (Me)	1.36	1	104.4 (CH)	4.81
22	110.9 (C)	-	2	74.8 (CH)	4.03
23	36.7 (CH ₂)	2.07	3	77.9 (CH)	4.29
24	28.1 (CH ₂)	2.04, 1.65	4	71.3 (CH)	4.15
25	34.1 (CH)	1.96	5	78.0 (CH)	3.97
26	75.3 (CH ₂)	3.98, 3.67	6	62.4 (CH ₂)	4.52, 4.31
27	17.3 (Me)	1.03			
Gal					
1	102.1 (CH)	5.01			
2	72.7 (CH)	4.44			
3	75.1 (CH)	4.20			
4	79.7 (CH)	4.61			
5	75.1 (CH)	4.09			
6	60.7 (CH ₂)	4.63, 4.28			



Compound 1 was obtained as an amorphous solid: $[\alpha]_{\text{D}}^{24}$ -45.3° (c 0.35, MeOH). High-resolution (HR)-electrospray ionization (ESI)-time of flight (TOF)-MS of 1 showed an $[\text{M} + \text{Na}]^+$ peak at m/z 1253.5773 corresponding to the empirical molecular formula $\text{C}_{56}\text{H}_{64}\text{O}_{29}$. The ^1H NMR spectrum of 1 (Table 1) showed characteristic proton signals due to two tertiary methyls at δ 0.89 (3H, s) and 1.21 (3H, s), two secondary methyls at δ 1.03 (3H, d, $J = 6.5$ Hz) and 1.36 (3H, d, $J = 6.8$ Hz), and five anomeric protons at δ 5.01 (1H, d, $J = 7.6$ Hz), 5.15 (1H, d, $J = 7.8$ Hz), 5.58 (1H, d, $J = 7.9$ Hz), 5.19 (1H, d, $J = 7.8$ Hz), and 4.81 (1H, d, $J = 7.8$ Hz). The ^{13}C NMR spectrum showed 56 peaks: 29 for the sugar moieties, including five anomeric carbons at δ 102.1, 104.4, 104.2, 104.6, and 104.4, and 27 for the aglycone part (Table 1). The ^1H NMR data, an acetylic carbon

signal at δ_C 110.9 in the ^{13}C NMR spectrum, and a positive color reaction in Ehrlich's test indicated **1** to be a furostanol saponin. Enzymatic hydrolysis of **1** produced a spirostanol saponin, identified by comparison with reference samples as (25*R*)-5 α -spirostane-3 β ,6 β -diol-3-*O*- β -D-glucopyranosyl-(1 \rightarrow 2)-[β -D-xylopyranosyl-(1 \rightarrow 3)]- β -D-glucopyranosyl-(1 \rightarrow 4)- β -D-galactopyranoside [4] and glucose. Chiral GC-FID analyses of the monosaccharides obtained after acid hydrolysis of **1** confirmed the D conformation of galactose, glucose, and xylose. As is usual with naturally occurring furostanol glycosides, one glucosyl group was shown to be linked to the C-26 hydroxyl group of the aglycone by an HMBC correlation of the anomeric proton at δ 4.81 (3H, d, J = 7.8 Hz) with C-26 of the aglycone at δ_C 75.3. Thus, **1** was identified as 26-*O*- β -D-glucopyranosyl-(25*R*)-5 α -furostane-3 β ,-6 β -diol-3-*O*- β -D-glucopyranosyl-(1 \rightarrow 2)-*O*- β -D-xylopyranosyl-(1 \rightarrow 3)-*O*- β -D-glucopyranosyl-(1 \rightarrow 4)- β -D-galactopyranoside and was named leucofuroside A.

Compound **2** was isolated as an amorphous solid: $[\alpha]_D^{24}$ -34.0° (c 0.10, MeOH). HR-ESI-TOF-MS of **2** showed a pseudomolecular ion peak at m/z 941.4716 $[\text{M} + \text{Na}]^+$, corresponding to the empirical molecular formula $\text{C}_{45}\text{H}_{74}\text{O}_{19}$, which was also deduced by analysis of its ^{13}C NMR and distortionless enhancement by polarization transfer (DEPT) spectral data. The ^1H NMR spectrum of **2** showed characteristic proton signals due to two tertiary methyls at δ 0.82 (3H, s) and 1.05 (3H, s), two secondary methyls at δ 0.79 (3H, d, J = 6.4 Hz) and 0.95 (3H, d, J = 7.0 Hz), and three anomeric protons at δ 4.39 (1H, d, J = 7.2 Hz), 4.53 (1H, d, J = 7.2 Hz), and 4.66 (1H, d, J = 7.8 Hz). The ^{13}C NMR spectrum showed 45 peaks: 18 for the sugar moieties, including three anomeric carbons at δ 102.5, 104.8, and 106.2, and 27 for the aglycone part. Acid hydrolysis of **2** with 1 M HCl in dioxane- H_2O (1:1, v/v) produced steroidal sapogenin, glucose, and galactose. Chiral gas chromatography flame ionization detector (GC-FID) analyses of the monosaccharides confirmed their D-configuration. The physical and spectral data allowed the identification of the sapogenin as (25*R*)-5 α -spirostane-3 β ,-6 β -diol (β -chlorogenin) [17], suggesting **2** to be a β -chlorogenin triglycoside. ^1H - ^1H shift COSY allowed the sequential assignments from H-1 to H₂-6 of each monosaccharide, including identification of their multiple patterns and coupling constants. These sugar linkages were confirmed by long-range correlations on the HMBC spectrum. The ^1H and ^{13}C NMR signals indicated the presence of a terminal β -D-glucopyranosyl unit (Glc B) [δ_{H} 4.66 (d, J = 7.8 Hz); δ_{C} 106.2, 76.3, 77.5, 70.7, 78.7, 61.9], a C-2 substituted β -D-glucopyranosyl unit (Glc A) [δ_{H} 4.53 (d, J = 7.2 Hz); δ_{C} 104.8, 84.9, 78.1, 71.7, 77.8, 63.2], and a C-4 substituted β -D-galactopyranosyl unit (Gal) [δ_{H} 4.39 (d, J = 7.2 Hz); δ_{C} 102.5, 73.2, 75.1, 80.5, 75.6, 60.8]. In the HMBC spectrum, correlations were observed from δ 4.66 (H-1 of Glc B) to δ 84.9 (C-2 of Glc A), δ 4.53 (H-1 of Glc A) to δ 80.5 (C-4 of Gal), and δ 4.39 (H-1 of Gal) to 79.8 (C-3 of the aglycone). Thus, compound **2** was identified as (25*R*)-5 α -spirostane-3 β ,6 β -diol-*O*- β -D-glucopyranosyl-(1 \rightarrow 2)-*O*- β -D-glucopyranosyl-(1 \rightarrow 4)- β -D-galactopyranoside, recently isolated and characterized by Akihito et al. as compound **2** [18]. The mentioned compound was described for the first time in the genus *Allium*.

EXPERIMENTAL

General. Spectra were recorded on a Braker DRX-500 and a Braker Avance 400 instrument. ^1H and ^{13}C NMR chemical shifts in ppm were referenced with the residual solvent (CD_3OD) signals (δ_{H} 3.31 and δ_{C} 49.0) or with TMS as internal standard (for pyridine- d_5 - H_2O). High-resolution electrospray ionization mass spectrometry was conducted in the positive mode on an Applied Biosystems/MDS Sciex QSTAR XL QqTOF MS system. The flowers were dried by microwave irradiation oven Pr KS-22E, 850 W, 2450 MHz. GC-FID analysis was performed on an Agilent 7890A series equipped with an InertCap Chiramix column (30 m \times 0.25 mm \times 0.25 μm). The temperature program was 120°C for 1 min, followed by a rise of 4°C/min to 180°C, which was maintained for 120 min. Optical rotation $[\alpha]_D^{25}$ was measured on an Autopol IV polarimeter. For CC, silica gel 60 (40-63 μm , Merck) and Diaion HP20 resin (Mitsubishi) were used. Thin-layer chromatography (TLC) analysis of saponins was performed on silica gel 60 F254 plates (Merck) and eluted with CH_2Cl_2 -MeOH- H_2O (26:14:3 v/v/v). Spots were detected by spraying the plates with vanillin-sulfuric acid (in EtOH) reagent, followed by heating at 110°C (spirostanols were colored yellow, furostanols dark green, and they gave a positive color reaction in Ehrlich's test).

Plant Material. The flowers of *Allium leucanthum* K. Koch were collected in the Dmanisi region of Georgia (June 2005) and identified by Prof. Jumber Kuchukhidze. A voucher specimen is kept in the Department of Pharmacognosy and Botany Faculty of Pharmacy, Tbilisi State Medical University, Tbilisi, Georgia (flowers No. AL 0605).

Extraction and Isolation. Dried and powdered flowers of *Allium leucanthum* (500 g) were extracted twice with hot MeOH- H_2O (8:2, v/v, 5 L). After evaporation of the solvent, the residue (79 g) was suspended in water and the saponins were extracted with *n*-BuOH. The *n*-BuOH extract (32 g) was chromatographed over Diaion HP-20, using MeOH- H_2O as eluent in gradient conditions (0 \rightarrow 100%) and EtOAc. The spirostanol fraction (14.5 g) was

collected in MeOH-H₂O (7:3, v/v), and the furostanol fraction (5.9 g) in MeOH-H₂O (5:5, v/v). The furostanol saponins were subjected to CC on silica gel and eluted with CH₂Cl₂-MeOH-H₂O (40:12:2, v/v/v) to give compound **1** (32 mg). The spirostanol saponins were subjected to CC on silica gel and eluted with CH₂Cl₂-MeOH-H₂O (45:14:2, v/v/v) to give compound **2** (25 mg).

Compound 1. Amorphous solid, $[\alpha]_D^{24}$ -45.3° (c 0.35, MeOH). HR-ESI-TOS-MS [m/z 1230.588, $[M + H]^+$, calcd for C₅₆H₉₄O₂₉Na, 1253.577]. ¹H NMR (400 MHz) and ¹³C NMR (100 MHz) spectroscopic data are shown in Table 1.

Enzymatic Hydrolysis. A solution of **1** (10 mg) was treated with β-D-glycosidase (EC, Sigma, USA) (10 mg) in AcOH-AcONa buffer (pH 5; 10 mL) at room temperature for 12 h. The reaction mixture was chromatographed on Diaion HP-20 eluted with Me₂CO-EtOH (3:2, v/v) and on silica gel eluted with CH₂Cl₂-MeOH-H₂O (45:14:2, v/v/v) to give (25*R*)-5α-spirostan-3β,6β-diol-3-*O*-β-D-glucopyranosyl-(1→2)-*O*-β-D-xylopyranosyl-(1→3)-*O*-β-D-glucopyranosyl-(1→4)-β-D-galactopyranoside (6 mg) and a sugar fraction (2 mg). The sugar fraction was analyzed on silica gel TLC by comparison with standard sugars in a CH₂Cl₂-MeOH-H₂O (50:25:5, v/v/v) solvent system and further developed using an orthophosphoric acid solution of 5% naphthoresorsinol in EtOH, followed by heating at 110°C to give glucose.

Acid Hydrolysis. A solution of **1** (10 mg) in 1 M HCl (dioxane-H₂O, 1:1, v/v; 3 mL) was heated at 100°C for 2 h under an Ar atmosphere. The reaction mixture was neutralized with *N,N*-dioctylmethylamine (10% in CHCl₃) and was chromatographed on silica gel eluted with CHCl₃-MeOH (9:1, v/v) to give β-chlorogenin (3.3 mg) and a sugar fraction (5.8 mg). The sugar fraction was analyzed by silica gel TLC and comparison with standard sugars in a CH₂Cl₂-MeOH-H₂O (50:25:5, v/v/v) solvent system; the monosaccharides were identified by TLC in a CH₂Cl₂-CH₃OH-H₂O (50:25:5, v/v/v) solvent system and further developed using an orthophosphoric acid solution of 5% naphthoresorsinol in EtOH, followed by heating at 110°C to give galactose, glucose, and xylose.

Determination of Stereochemistry of Monosaccharide. The aqueous phase (after acid hydrolysis) was evaporated and dried, and the residue containing monosaccharides was acetylated with acetic anhydride (1 mL) in pyridine (1 mL) for 24 h. The monosaccharide acetates were extracted with EtOAc, and the organic fraction was treated with water, followed by a saturated solution of NaHCO₃. The sample obtained was analyzed by GC-FID on a chiral column for comparison with authentic samples of monosaccharide acetates. The retention indices of monosaccharide acetates were compared with those of authentic samples. D-Galactose, D-glucose, and D-xylose were identified for compound **1**.

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