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STEROIDAL GLYCOSIDES OF GITOGENIN FROM *Allium rotundum*

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Two new steroidal glycosides were isolated by fractionation of total extracted substances from inflorescences and flower stalks of Allium rotundum *(Alliaceae). The structures were determined on the basis of chemical transformations, physical constants, and spectral data as 26-*O-β-*D-glucopyranosyl-(25*R*)-5α-furostan-2α,3β,22α,26-tetraol 3-*O-β-*D-glucopyranosyl-(1→2)[β-D-xylopyranosyl-(1→3)]-β-D-glucopyranosyl-(1→4)-β-D-galactopyranoside* (**2**) *and (25*R*)-5α-spirostan-2α,3β-diol 3-*O-β-*D-glucopyranosyl-(1→3)-β-D-glucopyranosyl-(1→2)-[β-D-xylopyranosyl-(1→3)]-β-D-glucopyranosyl-(1→4)-β-D-galactopyranoside* (**3**).

Keywords: *Allium rotundum*, Alliaceae, steroidal glycoside, spirostane, furostane, gitogenin.

Plants of the genus *Allium* typically contain several groups of biologically active compounds, the principal ones of which are steroidal compounds.

Up to 40 species of this genus are indigenous to Georgia [1]. We selected the species *A. rotundum* L. (Alliaceae) for an extensive study of the chemical composition and biological activity. This species is broadly distributed throughout the whole territory of Georgia, is easily cultivated, and can supply a raw-material base on an industrial scale.

We previously isolated and characterized the main active principles, steroidal sapogenins and saponins [2, 3], phenolic compounds such as flavonoids and coumarins [4], carotinoids, and amino acids [5] from inflorescences and flower stalks of *A. rotundum* collected during flowering in the vicinity of Tbilisi (Kojori).

After preliminary work up of the total extracted substances, eight steroidal glycosides were isolated by preparative chromatography from its various fractions. The structures of five known ones were published [3]. Herein we report the isolation of yet another known glycoside **1** and determine the structures of two new glycosides **2** and **3** that were gitogenin derivatives.

Column chromatography over silica gel of enriched fractions from the BuOH extracts isolated saponins **1** and **3**; from the water-soluble extracts, saponin **2**. All three compounds gave after acid hydrolysis the aglycon gitogenin. The carbohydrate part contained according to TLC and GC D-glucose, D-galactose, and D-xylose in a 2:1:1 ratio for **1** and 3:1:1 ratio for **2** and **3**.

After Hakomori permethylation [6] and subsequent methanolysis, the hydrolysates of saponins **1**–**3** contained the same aglycon, 2-*O*-methyl ester of gitogenin. Their carbohydrate parts gave for **1** and **2** 2,3,4,6-tetra-*O*-methyl-D-glucopyranose (terminal), 2,3,4-tri-*O*-methyl-D-xylopyranose (terminal), 4,6-di-*O*-methyl-D-glucopyranose (disubstituted on C-2 and C-3), and 2,3,6-tri-*O*-methyl-D-galactopyranose (substituted on C-4). Besides the aforementioned sugars, an additional molecule of 2,4,6-tri-*O*-methyl-D-glucopyranose (substituted on C-3) was found for **3**.

Compound **1** was an amorphous powder, mp 250–252°C, $[\alpha]_D^{20}$ –69° (*c* 0.55, Py). The *m*/*z* of the molecular ion in the FAB MS of 1073 $[M + Na]^+$ corresponded to empirical formula $C_{50}H_{82}O_{23}$. The physicochemical constants; data from GC, IR, mass, ^{13}C NMR, and PMR methods; and a comparison with literature data identified **1** as the known glycoside F-gitonin, (25*R*)-5*α*-spirostan-2*α*,3*β*-diol 3-*O*-*β*-D-glucopyranosyl-(1→2)-[*β*-D-xylopyranosyl-(1→3)]-*β*-D-glucopyransoyl-(1→4)-*β*-D-galactopyranoside [7].

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TABLE 1. PMR Spectra of the Carbohydrate Part of **2** and **3** (Py-d_5, δ, ppm, J/Hz, 0 = HMDS)

Atom	Compound **2**				
	Gal	Glc 1	Glc 2	Glc 3	Xyl
H-1	4.95 (d, J = 7.7)	5.19 (d, J = 8.1)	5.52 (d, J = 8.0)	4.83 (d, J = 7.8)	5.22 (d, J = 8.0)
H-2	4.52 m	4.32 (dd, J = 8.1; 8.8)	4.03 m	4.01 (dd, J = 7.8, 8.7)	3.90 m
H-3	4.14 m	4.13 m	4.10 m	4.12 m	4.09 m
H-4	4.55 m	3.76 (dd, J = 9.6; 9.6)	4.05 m	4.23 m	4.07 m
H-5	4.01 m	3.84 (ddd, J = 9.6, 7.2, 2.3)	3.89 (ddd, J = 8.0, 5.8, 2.3)	3.87 m	
H-6	4.18 m; 4.59 m	4.04 m; 4.48 m	4.38 m; 4.51 m	4.20 m; 4.42 m	
H-5α					3.64 (dd, J = 10.8; 10.0)
H-5β					4.18 (dd, J = 4.8; 10.8)
Atom	Compound **3**				
	Gal	Glc 1	Glc 2	Glc 3	Xyl
H-1	4.93 (d, J = 7.6)	5.19 (d, J = 7.9)	5.54 (d, J = 7.0)	5.07 (d, J = 7.9)	5.14 (d, J = 7.6)
H-2	4.49 m	4.30 m	4.05 m	3.98 m	3.92 m
H-3	4.11 m	4.09 m	4.07 m	4.12 m	4.04 m
H-4	4.58 (d, J = 3.1)	3.78 m	3.91 m	4.09 m	4.08 m
H-5	4.01 m	3.81 m	3.79 m	3.84 m	
H-6	4.19 m; 4.58 m	4.03 m; 4.47 m	4.25 m; 4.40 m	4.22 m; 4.45 m	
H-5α					3.62 (dd, J = 10.8; 10.4)
H-5β					4.18 m

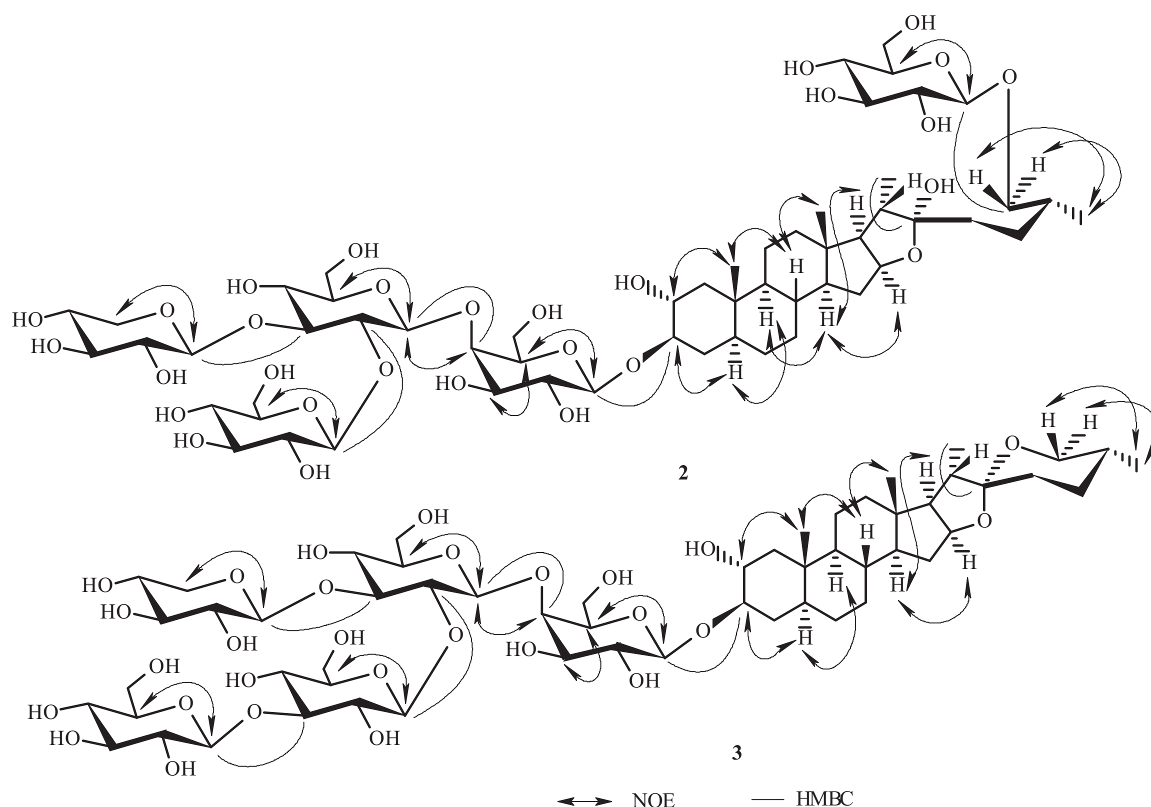

Fig. 1. Structures of **2** and **3** and key correlations in their HMBC and NOE spectra.

Compound **2** was an amorphous powder that gave two spots on TLC plates with a small difference in the R_f values ($\Delta R_f \sim 0.1$). The IR spectrum lacked absorption bands for a spiroketal group. One broad band was observed at 890 cm^{-1}. Ehrlich reagent [8] gave a rosy red color. According to these characteristics, **2** was assigned as a furostane glycoside. In fact, its enzymatic hydrolysis by β-glucosidase produced a prosapogenin that was identical to F-gitonin, i.e., **2** was the furostanol analog of F-gitonin.

TABLE 2. ^{13}C NMR Chemical Shifts and Heteronuclear Constants ^{1}J (CH) of **2** and **3** and Gitogenin (Py-d_5, δ, ppm, J/Hz, 0 = HMDS)

C atom	Genin ($CDCl_3$)	Aglycon		Sugar		**2**	**3**	^{1}J (CH)
		2	**3**					
1	45.0	45.8	45.8	Gal 1	1	102.8	103.1	160.4
2	73.0	70.7	70.5		2	72.2	72.5	
3	76.4	85.3	85.2		3	75.2	75.5	
4	35.6	33.5	33.5		4	79.1	79.1	
5	44.8	44.8	44.7		5	75.2	75.5	
6	27.8	28.2	28.2		6	60.2	60.6	
7	31.8	32.1	32.1	Glc 1	1	104.3	104.3	162.6
8	34.4	34.6	34.6		2	81.0	80.6	
9	54.3	54.5	54.4		3	86.9	87.0	
10	37.6	36.9	36.9		4	70.1	70.3	
11	21.2	21.4	21.5		5	77.2	77.5	
12	39.9	40.0	40.1		6	62.7	63.0	
13	40.6	41.1	40.8	Glc 2	1	104.5	103.9	163.7
14	56.1	56.3	56.3		2	75.9	74.6	
15	31.4	32.3	32.3		3	77.0	87.8	
16	80.8	81.4	81.1		4	71.0	69.6	
17	62.1	64.3	63.0		5	77.2	77.7	
18	16.5	16.3	16.6		6	62.6	62.5	
19	13.6	13.7	13.5	Glc 3	1	105.0	105.4	160.9/163.0
20	41.5	40.4	42.0		2	75.2	75.6	
21	14.5	16.0	15.0		3	78.4	78.0	
22	109.2	110.7	109.2		4	71.8	71.5	
23	32.0	30.8	31.8		5	78.6	78.4	
24	28.8	28.1	29.3		6	63.0	62.4	
25	30.3	34.4	30.6	Xyl	1	104.6	104.8	161.2
26	66.8	74.7	66.9		2	74.9	75.1	
27	17.1	17.2	17.3		3	78.2	78.4	
					4	70.2	70.5	
					5	67.1	67.2	

This was confirmed by the peak for the molecular ion and the fragmentation of the carbohydrate part in its FAB MS (*m/z*): 1253 $[M + Na]^+$, 1121 $[M + Na – pentose]^+$, 1091 $[M + Na – hexose]^+$, 797 $[M + Na – (pentose + 2 hexoses)]^+$, 635 $[M + Na – (pentose + 3 hexoses)]^+$, 455 $[M + Na – (pentose + 4 hexoses + H_2O)]^+$.

The structure of **2** was studied further using PMR and ^{13}C NMR. Lines were assigned by interpreting data obtained from COSY, HOHAHA, HMQC [9], and HMBC [10] spectra (Tables 1 and 2, Fig. 1). Thus, interpretation of HOHAHA spectra together with HMQC spectra enabled resonances for protons of the five sugar units (three glucoses and one each of xylose and galactose) to be clearly identified. The SSCC of anomeric protons fell in the range 7.0–8.1 Hz. Therefore, all sugar units had the *β*-configuration of the glycosidic bonds and adopted the pyranose form [11]. This was consistent with a correlation between H-1 and H-5 of each sugar unit in the NOESY spectrum [12].

GC data for the structure of the sugar chain were confirmed by HMBC spectra in which cross-peaks for correlations of through-space couplings between Gal H-1 (δ 4.94) and aglycon C-3 (δ 85.3); Glc-1 H-1 (δ 5.19) and Gal C-4 (δ 79.1); Glc-2 H-1 (δ 5.54) and Glc-1 C-2 (δ 8.10); Xyl H-1 (δ 5.22) and C-3 (δ 86.9); and Glc-3 H-1 (δ 4.83) and aglycon C-26 (δ 74.7) were observed.

Thus, **2** was 26-*O*-*β*-D-glucopyranosyl-(25*R*)-5*α*-furostan-2*α*,3*β*,22*α*,26-tetraol 3-*O*-*β*-D-glucopyranosyl-(1→2)[*β*-D-xylopyranosyl-(1→3)]-*β*-D-glucopyranosyl-(1→4)-*β*-D-galactopyranoside.

The IR spectrum of compound **3** had a set of absorption bands that was characteristic of a spiroketal group and gave a yellow color with Sannie reagent [13]. This allowed it to be assigned as a spirostane glycoside. Results of GC analysis agreed well with data from the fragmentation of the carbohydrate chain in its FAB MS (*m/z*): 1235 $[M + Na]^+$, 1103 $[M + Na – pentose]^+$, 1073 $[M + Na – hexose]^+$, 911 $[M + Na – 2 hexoses]^+$, 617 $[M + Na – (pentose + 3 hexoses)]^+$, 455 $[M + Na – (pentose + 4 hexoses)]^+$.

Assignments of PMR and ^{13}C NMR spectra were made using the aforementioned programs. Through-space couplings between Gal H-1 (δ 4.93) and aglycon C-3 (δ 85.2); Glc-1 H-1 (δ 5.19) and Gal C-4 (δ 79.1); Glc-2 H-1 (δ 5.54) and Glc-1 C-2 (δ 80.6); Xyl H-1 (δ 5.14) and Glc-1 C-3 (δ 87.0); Glc-3 H-1 (δ 5.07) and Glc-2 C-3 (δ 87.8) were observed according to HMBC spectra, i.e., the structure of the carbohydrate chain corresponded to that shown in Fig. 1. The measured homonuclear SSCC of anomeric protons and heteronuclear SSCC of anomeric C atoms indicated unambiguously that the glycoside bonds of all sugars had the *β*-configuration with the C1 conformation of their oxide rings [11, 14]. Thus, **3** was (25*R*)-5*α*-spirostan-2*α*,3*β*-diol 3-*O*-*β*-D-glucopyranosyl-(1→3)-*β*-D-glucopyranosyl-(1→2)-[*β*-D-xylopyranosyl-(1→3)]-*β*-D-glucopyranosyl-(1→4)-*β*-D-galactopyranoside.

EXPERIMENTAL

General Comments. TLC used Kieselgel $60F_{254}$ (Merck) and Silufol UV-254 plates; column chromatography, KSK silica gel (particle size <63 and 63–100 μm). We used solvent systems 1) $CHCl_3$:MeOH:H_2O (65:15:2, a; 65:22:4, b; 65:35:8, c) and 2) $CHCl_3$:MeOH (10:1, a; 50:1, b).

GC was performed in a Chrom-5 instrument. Monosaccharides were chromatographed as trimethylsilyl ethers of methylglycosides on a column (3 m × 4 mm) packed with N-AW chromaton containing SE-30 silicone phase (5%) thermostatted at 190°C with He carrier gas at flow rate 45 mL/min.

Methylglycosides of methylated sugars were prepared by refluxing (4 h) methyl ethers in anhydrous MeOH containing HCl (5%). The resulting products were chromatographed over a column (1.2 m × 3 mm) with cellite containing 1,4-polybutanediol succinate (20%) thermostatted at 160°C with He carrier gas at flow rate 50 mL/min.

Mass spectra were taken in a Kratos MS 50 RF instrument in glycerin matrix. IR spectra were recorded in KBr on a UR-20 instrument. PMR and ^{13}C NMR spectra were obtained from Py-d_5 solutions with HMDS internal standard on an AMX-500 instrument (Bruker) at operating frequency 500.11 MHz for ^1H and 125.76 MHz for ^{13}C.

Isolation of Glycosides. Ground air-dried aerial parts of the plant (1.0 kg) were macerated for 1.5 h each first with Et_2O and then with EtOAc. The resulting material was extracted with MeOH (70°) in a Soxhlet apparatus for 2 h. The resulting extracts were filtered, evaporated to a resinous consistency, and suspended in H_2O with vigorous stirring. The water-insoluble precipitate was separated by decantation of the supernatant liquid. Then, the liquid was extracted with *n*-BuOH. The extract was condensed. The precipitate was dissolved in EtOH. Glycosides were precipitated by acetone and dried. The resulting total glycosides were chromatographed over a column of silica gel using systems 1b and 1c to afford a mixture of **2** and its 22-*O*-methyl ether. Refluxing the mixture in H_2O (2 h) produced chromatographically homogeneous glycoside **2** (0.38 g, 0.038% calculated per weight of air-dried raw material).

The insoluble precipitate was chromatographed over a column of silica gel (systems 1a and 1b). Fractions containing chromatographically homogeneous glycoside **3** were collected. Fractions containing more than one compound were combined and rechromatographed using the same systems. The yield of compound **3** was 0.47 g (0.047% calculated per weight of air-dried raw material).

26-*O*-*β*-D-Glucopyranosyl-(25*R*)-5*α*-furostan-2*α*,3*β*,22*α*,26-tetraol 3-*O*-*β*-D-glucopyranosyl-(1→2)-[*β*-D-xylopyranosyl-(1→3)]-*β*-D-glucopyranosyl-(1→4)-*β*-D-galactopyranoside (2). Amorphous powder, cream color, $C_{56}H_{94}O_{29}$, mp 195–198°C, $[\alpha]_D^{20}$ –59.8° (*c* 0.45, Py). Data for the FAB MS are given in the text. IR spectrum (KBr, ν_{max}, cm^{-1}): 3600–3450 (OH), 890.

PMR spectrum (Py-d_5, δ, ppm, J/Hz): 4.51 (1H, q, J = 6.9, H-16), 4.03 (1H, ddd, J = 10.6, 7.8, 5.3, H-2), 3.87 (1H, ddd, J = 11.1, 8.5, 5.3, H-3), 3.59 (1H, dd, J = 10.4, 3.1, H-26a), 3.50 (1H, dd, J = 10.4, 10.4, H-26b), 1.18 (3H, d, J = 6.9, Me-21), 1.01 (3H, d, J = 6.6, Me-27), 0.92 (3H, s, Me-19), 0.80 (3H, s, Me-18).

(25*R*)-5*α*-Spirostan-2*α*,3*β*-diol 3-*O*-*β*-D-glucopyranosyl-(1→3)-*β*-D-glucopyranosyl-(1→2)-[*β*-D-xylopyranosyl-(1→3)]-*β*-D-glucopyranosyl-(1→4)-*β*-D-galactopyranoside (3). Amorphous yellowish powder, $C_{56}H_{92}O_{28}$, mp 271–273°C, $[\alpha]_D^{20}$ –51° (*c* 0.49, Py). Data for the FAB MS are given in the text. IR spectrum (KBr, ν_{max}, cm^{-1}): 3600–3400 (OH), 982, 924, 898, 862 (intensity 898 > 924 = spiroketal of 25-*R*-series).

PMR spectrum (Py-d_5, δ, ppm, J/Hz): 4.54 (1H, q, J = 7.0, H-16), 4.07 (1H, ddd, J = 10.6, 7.8, 5.3, H-2), 3.88 (1H, ddd, J = 11.2, 8.7, 5.4, H-3), 3.58 (1H, dd, J = 10.4, 3.1, H-26a), 3.50 (1H, dd, J = 10.4, 10.4, H-26b), 1.14 (3H, d, J = 7.0, Me-21), 0.90 (3H, s, Me-19), 0.82 (3H, s, Me-18), 0.70 (3H, d, J = 5.4, Me-27).

Acid Hydrolysis. Compounds **1**–**3** (100 mg each) were dissolved separately in aqueous MeOH (15 ml, 50%) containing conc. H_2SO_4 (0.6 mL), refluxed for 8 h, cooled, and diluted with H_2O. The resulting precipitates were separated by filtration and purified by recrystallization from MeOH to afford colorless crystals (40.7 mg, 34.2, and 34.8) of the aglycon, mp 267–270°C (dec), $[\alpha]_D^{20}$ –63.5° (*c* 0.4, $CHCl_3$), EI-MS (*m/z*): 432 $[M]^+$, $[C_{27}H_{44}O_4]^+$, 417, 318, 289, 139. IR spectrum (KBr, ν_{max}, cm^{-1}): 3450 (OH), 990, 960, 925, 905, 875 (intensity 905 > 925 = spiroketal of 25*R*-series).

PMR spectrum ($CDCl_3$, δ, ppm, J/Hz): 4.36 (1H, m, H-16), 3.32–3.62 (4H, m, H-2, H-3, H-26a,b), 0.95 (3H, d, J = 6.6, Me-21), 0.85 (3H, s, Me-19), 0.78 (3H, d, J = 6.4, Me-27), 0.76 (3H, s, Me-18). Table 2 presents the ^{13}C NMR data. A comparison of the results with the literature [15] identified the aglycon as gitogenin.

Enzymatic Hydrolysis. Glycoside **3** (70 mg) was dissolved in H_2O (30 mL), treated with *β*-glucosidase (10 mg), left at room temperature for 10 h, and extracted (×3) with *n*-BuOH. The organic layer was evaporated. The enzymolysis products were chromatographed over a column of silica gel using system 1c to afford a spirostane glycoside (35.8 mg). Its physicochemical constants, ^{13}C NMR spectrum, and chromatographic mobility compared with an authentic sample identified **1** as F-gitonin.

Methanolysis of Permethylated Products. Solutions of permethylated products of glycosides **1-3** (60 mg) in aqueous MeOH (40 mL, 60%) containing H_2SO_4 (5%) were hydrolyzed at 100°C for 8 h. The hydrolysates were diluted with H_2O (150 mL) and evaporated to the original volume. The resulting precipitates were recrystallized from acetone to afford 34.2 mg (from **1**), 31.8 mg (from **2**), and 32.1 mg (from **3**) of compounds, $C_{28}H_{46}O_4$, mp 220–223°C, $[\alpha]_D^{20}$ –119.8° (*c* 0.6, $CHCl_3$). IR spectrum (KBr, ν_{max}, cm^{-1}): 3500 (OH), 980, 926, 901, 864 (intensity 901 > 926 = spiroketal chain of 25*R*-series), $[M + Na]^+$ 469. A comparison with the literature [7] showed that these compounds were identical to the 2-*O*-methyl ether of gitogenin.

The aqueous part of the hydrolysate was cooled, neutralized with anion exchanger (HCO_3^--form), and concentrated. GC with authentic samples identified the methylglycosides mentioned above for glycosides **1**–**3**.

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