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STEROIDAL GLYCOSIDES FROM *Digitalis ciliata* LEAVES

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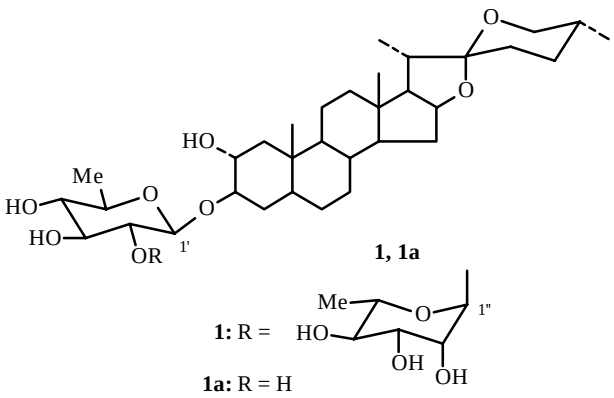
Two new steroidal glycosides of the spirostane and furostane classes, derivatives of gitogenin, were found in wastes from producing acetyldigitoxin preparation from Digitalis ciliata *Trautv. (Scrophulariaceae). The structures of the glucosides were established using physical constants, chemical transformations, and IR, mass, and NMR spectra.*

Key words: *Digitalis ciliata*, Scrophulariaceae, new steroidal glycosides, spirostane, furostane, gitogenin.

Among plants of the genus *Digitalis* (Scrophulariaceae) growing in Georgia, the species *D. ciliata* is indigeneous to the Caucuses [1]. It contains raw material that is rich in cardenolide cardiac glycosides and biosynthesizes glycosides of almost all genins typical of this genus: digitoxigenin, gitoxigenin, digoxigenin, gitaloxigenin, and diginatigenin [2]. Triterpenes, steroidal glycosides, carotinoids, and other compounds were also observed in *D. ciliata*. Its extracts exhibited cardiotonic and fungicidal activity [3]. It has been proposed as a source of acetyldigitoxin, which is a preparation effective for cardiac insufficiency [4].

Herein we present results from physical chemical and spectral research on two new steroidal glycosides **1** and **2** that were isolated from aqueous mother liquors remaining after separation of acetyldigitoxin fractions.



Compound **1** was identified as a 25*R*-spirostane [6] based on a positive color reaction with Sannie-Lapin reagent [5] and characteristic absorptions in the IR spectrum.

Acid hydrolysis of **1** produced the aglycon, which was identified by physical chemical constants and spectral (IR, mass, NMR) data as 25*R*,5α-spirostan-2α,3β-diol, or gitogenin. TLC of the carbohydrate part of the hydrolysate detected L-rhamnose and D-quinovose.

The carbohydrate part of the molecule was a biose as indicated unambiguously by its FAB mass spectrum in which peaks for the molecular ion at *m*/*z* 747 $[M + Na]^+$ and at *m*/*z* 601 $[M + Na - \text{deoxyhexose}]^+$ and *m*/*z* 455 $[M + Na - \text{biose}]^+$ were observed.

The ^{13}C NMR spectrum obtained with complete proton decoupling contained 39 lines (Table 1). Of these, 3 were quaternary; 20, methine; 10, methylene; and 6, methyl according to the DEPT spectrum.

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TABLE 1. Chemical Shifts (δ, ppm) of C Atoms in **1**, **1a**, and **2** (Pyridine-d_5, HMDS = 0)

C atom	Compound		C atom	Compound			^{1}J (CH)/Hz
	1	**2**		**1**	**1a***	**2**	
1	45.9	45.8	D-Quinovose				
2	70.8	70.6	1′	104.4	105.9	104.5	160.2
3	85.8	85.7	2′	80.3	76.0	80.5	
4	33.9	33.8	3′	77.4	77.8	77.4	
5	44.8	44.8	4′	77.1	77.5	77.2	
6	28.2	28.2	5′	73.0	73.0	73.1	
7	32.1	32.1	6′	18.3	18.2	18.3	
8	34.6	34.7	L-Rhamnose				
9	54.5	54.5	1″	102.4		102.3	170.8
10	37.0	36.9	2″	72.6		72.5	
11	21.6	21.4	3″	72.8		72.8	
12	40.1	40.0	4″	74.1		74.1	
13	40.7	41.0	5″	69.6		69.5	
14	56.4	56.3	6″	18.6		18.6	
15	32.2	32.1	D-Glucose				
16	81.2	81.0	1‴			105.0	157.4
17	63.0	63.5	2‴			75.2	
18	16.7	16.3	3‴			78.5	
19	13.5	13.5	4‴			71.6	
20	42.0	40.3	5‴			78.2	
21	15.0	16.4	6‴			63.0	
22	109.2	110.7					
23	31.7	30.8					
24	29.4	28.1					
25	30.6	34.0					
26	66.9	75.1					
27	17.3	17.2					

*Chemical shifts of the aglycon agree with those of **1** within experimental uncertainty and are therefore not given.

The PMR spectrum exhibited singlets for two methyls bound to quaternary C atoms, doublets for four methyls bound to tertiary C atoms, and signals for two anomeric protons (see Experimental).

Partial acid hydrolysis produced prosapogenin **1a**. TLC of the hydrolysate detected rhamnose. According to several experiments [7], the α-glycosylation effect of rhamnose (axial hydroxyl on C-1) is much less that for sugars with an equatorial hydroxyl and less than +5.0 ppm. By taking this into account and comparing chemical shifts (CS) of the sugars in **1** and **1a**, the signal at δ 80.6 could be assigned tentatively to quinovose C-2′. Furthermore, a significant negative β-effect was observed and shifted the anomeric C of quinovose to strong field by 1.5 ppm. This also is possible only for substitution at C-2′.

In order to confirm this hypothesis, **1** was methylated by the Hakomori method [8]. After methanolysis of the permethyl product, TLC of the carbohydrate part detected 2,3,4-tri-*O*-methylrhamnopyranose and 3,4-di-*O*-methylquinovopyranose. Thus, the biose of **1** consisted of a terminal rhamnose bound to quinovose through C-2′.

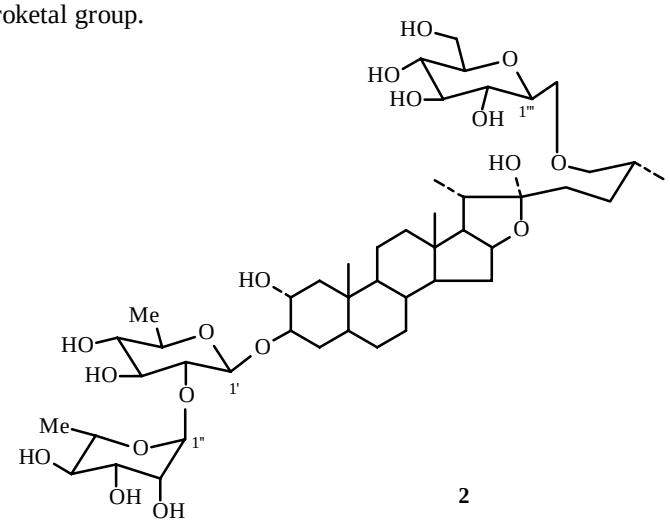
The SSCC of anomeric protons (J = 7.4 Hz, quinovose) and the broad singlet (rhamnose) that were observed in the PMR spectrum corresponded to vicinal, diaxial, and diequatorial constants. Furthermore, ^{13}C NMR spectra without decoupling but with retention of the nuclear Overhauser effect (NOE) showed heteronuclear SSCC ^{1}J(CH) = 160.2 Hz (quinovose) and ^{1}J(CH) = 170.8 Hz (rhamnose). According to the literature [9], these values unambiguously indicated the β-configuration for the anomeric C of D-quinovose and the α-configuration of L-rhamnose.

The attachment site of the carbohydrate chain to the aglycon was determined by comparing the CS of **1** with those of gitogenin [7, 10]. Atom C-3 of the genin experienced the largest weak-field shift (+9.1 ppm) whereas C-2 and C-4 shifted to strong field by 2.3 and 3.3 ppm, respectively. The attachment of the sugar chain to aglycon C-3 was also consistent with the

NOE experiment. Preirradiation of the anomeric quinovose proton produced a strengthening of almost 6% (by difference) of the multiplet at δ 3.87, which was assigned to aglycon H-3.

Thus, the results indicated that **1** was (25*R*),5α-spirostan-2α,3β-diol 3-*O*-α-L-rhamnopyranosyl-(1→2)-β-D-quinovopyranoside.

Compound **2** was identified as a furostane from the nature of the color with Ehrlich reagent [11] and the lack in the IR spectrum of bands for a spiroketal group.



2

Acid hydrolysis produced the aglycon, which was identified by the method described above as gitogenin. TLC of the hydrolysate detected L-rhamnose, D-quinovose, and D-glucose.

The FAB mass spectrum contained peaks for the molecular ion at *m*/*z* 927 $[M + Na]^+$ and fragments at *m*/*z* 781 $[M + Na - deoxyhexose]^+$, *m*/*z* 765 $[M + Na - hexose]^+$, and *m*/*z* 635 $[M + Na - biose]^+$. Enzymatic hydrolysis of **2** by β-glucosidase produced the prosapogenin, the structure of which was identical to the native glycoside **1** according to all physical chemical constants and spectral data (IR, NMR, mass). TLC of the hydrolysate detected D-glucose. Therefore, **2** was considered to be a furostanol analog of **1**. This was confirmed by the ^{13}C NMR spectrum, the assignment of which was based on the literature [7].

The $^1J(CH)$ SSCC of D-glucose (157.4 Hz) and CS of C-26 at δ 75.1 ppm indicated that the D-glucose was bound to the C-26 hydroxyl through a β-glycoside bond [9]. The SSCC of the glucose anomeric proton (7.8 Hz) was also consistent with this type of bond. The SSCC of the sugars in the biose were analogous to those of the biose of **1**.

Therefore, **2** was 3-*O*-α-L-rhamnopyranosyl-(1→2)-β-D-quinovopyranosyl-(22ξ,25*R*),5α-furostan-2α,3β,22α,26-tetraol 26-*O*-β-D-glucopyranoside.

EXPERIMENTAL

General Comments. TLC was performed on fixed layers of KSK silica gel (<63 μm) containing gypsum (10%) and on Silufol UV 254 plates. Column chromatography used KSK silica gel (<63 μm, 63-100 μm). The following solvent systems were used: $CHCl_3$:CH_3OH:H_2O (65:15:2, 1; 65:22:4, 2; 65:35:8, 3). Spirostane steroids were detected using Sannie—Lapin reagent [5]; furostane steroids, Ehrlich reagent [11]; sugar derivatives, *o*-toluidinesalicylate.

GC was performed in a Chrom-5 chromatograph on a column (1.2 × 3 mm) with cellite containing 1,4-polybutanediolsuccinate (20%), thermostat temperature 160°C, He carrier gas, and flow rate 50 mL/min. Mass spectra were recorded on a Kratos MS 50 RF instrument in a glycerine matrix; IR spectra, on a UR-20 instrument in KBr; PMR and ^{13}C NMR spectra, on a WM-250 (Bruker) instrument in pyridine-d_5 with HMDS internal standard.

Isolation of Glycosides 1 and 2. Wastes from production of acetyldigitoxin from *D. ciliata* contained steroidal saponins, glycosides of the starting material. These were isolated from the aqueous mother liquor remaining after separation of the acetyldigitoxin fractions by removing remaining cardenolides with an alcohol:chloroform mixture (1:2 and 1:3). Steroidal saponins were extracted repeatedly with *n*-butanol. The butanol extracts were evaporated to a resinous consistency and

suspended in portions in water with vigorous stirring. The solid that was insoluble in water was separated by decantation of the supernatant liquid, which was extracted with *n*-butanol. The solution was evaporated to dryness. The solid was dissolved in ethanol. Glycosides were precipitated by acetone.

The solid that was insoluble in water was chromatographed over a column with silica gel (systems 1 and 2). Fractions containing chromatographically homogeneous glycoside **1** (0.45 g) were collected.

Chromatography of the water-soluble compounds using systems 2 and 3 produced a mixture of **2** and its 22-*O*-methyl ether. Boiling the mixture in water (3 h) produced chromatographically homogeneous **2** (0.34 g). The overall yields calculated per air-dried raw material were 0.009% (**1**) and 0.007% (**2**).

Glycoside 1. Amorphous powder, $[\alpha]_D^{25}$ -68° ($CHCl_3$:CH_3OH, 1:1, *c* 0.20). FAB MS (*m*/*z*, %): 747 (20) $[M + Na]^+$, 601 (100) $[M + Na$ - deoxyhexose$]^+$, 455 (53) $[M + Na$ - biose$]^+$. IR spectrum (ν, KBr, cm^{-1}): 3420 (OH), 2940 (CH), 980, 950, 920, 900 (spiroketal), 865, 820.

PMR spectrum (250 MHz, δ, ppm, J/Hz): 0.72 (3H, d, J = 6.4, Me-27), 0.83 (3H, s, Me-18), 0.91 (3H, s, Me-19), 1.14 (3H, d, J = 7.0, Me-21), 1.54 (3H, d, J = 6.1, Me-6′), 1.60 (3H, d, J = 6.2, Me-6″), 3.55 (2H, m, H-26), 3.87 (1H, m, H-3), 4.56 (1H, q, J = 7.0, H-16), 4.88 (1H, d, J = 7.4, H-1′), 6.24 (1H, br.s, H-1″).

Glycoside 2. Amorphous powder, $[\alpha]_D^{25}$ -61° ($CHCl_3$:CH_3OH, 1:1, *c* 0.40). FAB MS (*m*/*z*, %): 927 (43) $[M + Na]^+$, 781 (90) $[M + Na$ - deoxyhexose$]^+$, 765 (100) $[M + Na$ - hexose$]^+$, 635 (52) $[M + Na$ - biose$]^+$. IR spectrum (ν, KBr, cm^{-1}): 3500-3350 (OH), 2930 (CH), 905 (weak broad band).

PMR spectrum (δ, ppm, J/Hz): 0.80 (3H, s, Me-18), 0.88 (3H, Me-19), 1.02 (3H, d, J = 6.5, Me-27), 1.16 (3H, d, J = 6.7, Me-21), 1.52 (3H, d, J = 6.1, Me-6′), 1.62 (3H, d, J = 6.2, Me-6″), 4.82 (1H, d, J = 7.8, H-1‴), 4.90 (1H, d, J = 7.4, H-1′), 6.21 (1H, br.s, H-1″).

Acid Hydrolysis. Glycosides **1** and **2** (150 mg each) were dissolved in aqueous CH_3OH (50 mL, 50%) containing conc. H_2SO_4 (2 mL) and heated at boiling for 8 h. The resulting preipitate was filtered off and recrystallized from ethanol to afford aglycon (48 mg, **1**; 42 mg, **2**).

Gitogenin, amorphous powder, $[\alpha]_D^{23}$ -78° ($CHCl_3$, *c* 0.2). FAB MS (*m*/*z*, %): 455 $[M + Na]^+$, 440, 341, 312, 162. IR spectrum (ν, KBr, cm^{-1}): 3430 (OH), 2935 and 2875 (CH), 1050, 975, 950, 920, 895, 865.

PMR spectrum (δ, ppm, J/Hz): 0.70 (3H, d, J = 6.2, Me-27), 0.83 (3H, s, Me-18), 0.88 (3H, s, Me-19), 1.14 (3H, d, J = 7.0, Me-21), 3.50-3.58 (2H, m, H-26), 3.86 (1H, m, H-3), 4.04 (1H, m, H-2), 4.56 (1H, q, J = 6.8, H-16). TLC of the carbohydrate part of the hydrolysate after separation using system 3 identified in the presence of authentic samples quinovose and rhamnose (R_f 0.53 and 0.57) for **1** and quinovose, rhamnose, and glucose (R_f 0.53, 0.57, 0.39) for **2**.

Methylation of Compound 1. Glycoside **1** (100 mg) was methylated with methyl iodide in DMF in the presence of NaH by the Hakomori method [8]. The extent of methylation was monitored by TLC (one spot), IR spectroscopy (lack of signals for hydroxyls), and PMR spectroscopy (appearance of additional signals at δ 3.40-3.60 for six methoxyls). The resulting permethylated product (82 mg) was subjected to total acid hydrolysis as described above. The precipitated aglycon was separated. The aqueous solution of methylated sugars was boiled for another 5 h, after which the mixture was neutralized with anion exchanger EDE-10P and evaporated to dryness. GC detected 2,3,4-tri-*O*-methyl-L-rhamnose (terminal) and 3,4-di-*O*-methyl-D-quinovose (C-2′ substituted).

Partial Acid Hydrolysis. Glycoside **1** (100 mg) was heated at 80°C in HCl:CH_3OH (0.1 m, 10 mL) for 1 h, neutralized with KOH:CH_3OH (3%), washed with water, and evaporated to dryness. The solid was chromatographed over a column using system 1 to afford **1a** (45 mg).

Enzymatic Hydrolysis of Compound 2. A solution of **2** (100 mg) in water was treated with β-glucosidase (50 mg) and left at room temperature for 8 h. The resulting solid was filtered off, washed with water, dried, chromatographed over a column using systems 1 and 2, and recrystallized from ethanol to afford a glycoside (57 mg) that was identified using IR, PMR, and ^{13}C NMR spectra as native glycoside **1**.

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