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FUROSTANE-TYPE STEROIDAL SAPONIN FROM *Digitalis ciliata*

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*A new furostane-type steroidal glycoside and derivative of tigogenin (**1**) was isolated from aqueous wastes from production of the cardiac drug acetyldigitoxin from leaves of* Digitalis ciliata *Trautv. (Scrophulariaceae) and characterized. The structure of the glycoside was established using physical constants, chemical transformations, and spectral data as 3-O-β-D-glucopyranosyl-(1→3) [β-D-fucopyranosyl-(1→2)-β-D-glucopyranosyl-(1→4)-β-D-galactopyranosyl-(25R)-5α-furostan-3β,22α,26-triol-26-O-β-D-glucopyranoside.*

Keywords: *Digitalis ciliata*, Scrophulariaceae, steroidal glycoside, furostane, tigogenin.

Ciliate foxglove (*Digitalis ciliata* Trautv.) (Scrophulariaceae) is widely distributed in alpine meadows of Georgia and is endemic to both the Republic and the whole Caucases region.

We reported previously on three new glycosides isolated from aqueous wastes from production of the drug acetyldigitoxin [1, 2] from leaves of ciliate foxglove. Herein we present results from a study of the chemical structure of a new furostane-type tigogenin glycoside, pentaoside **1**.

The water-soluble total steroidal glycosides were chromatographed repeatedly over a column of silica gel to isolate a mixture of two compounds with similar R_f values (**1a** and **1b**). Spots of both glycosides were colored yellowish-green on spraying chromatograms with Sannie reagent [3]; rosy red, with Erlich reagent [4]. The IR spectrum of the mixture exhibited a weak broad band at 895 cm^{-1} [5] and lacked bands for the spiro-ketal group characteristic of spirostane-type glycosides [6].

The PMR spectrum of glycoside **1b** showed a strong 3H singlet for methoxyl at δ 3.14 whereas it was missing in the spectrum of **1a**. The data were typical of furostane-type compounds. The 22-OH group is readily alkylated by lower alcohols (MeOH, EtOH) in the presence of silica gel due to its high reactivity. Therefore, the native 22-OH-furostane is practically always accompanied by its slightly less polar 22-*O*-methyl/ethyl ether.

Heating the mixture of glycosides in water for 2 h produced **1**, total acid hydrolysis of which isolated the aglycon tigogenin and a mixture of monosaccharides. TLC and GC detected D-glucose, D-galactose, and D-fucose in a 3:1:1 ratio.

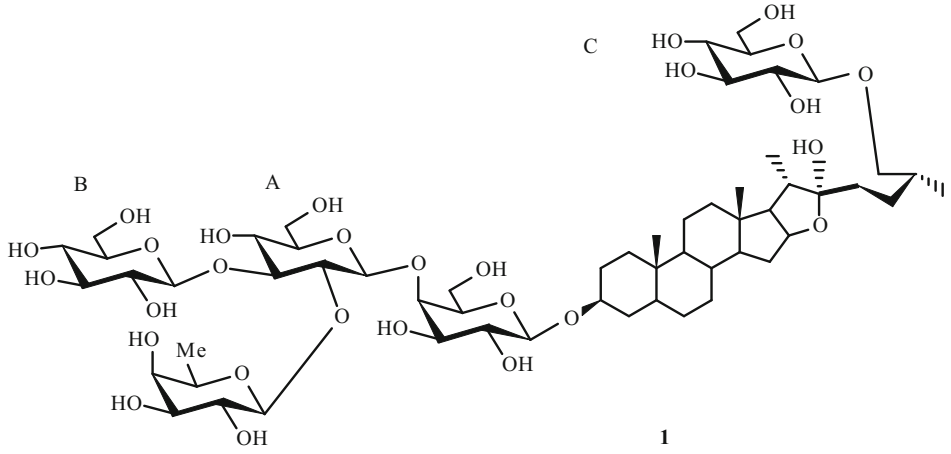

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TABLE 1. Chemical Shifts of C Atoms in Tigogenin and Glycoside **1** (Pyridine-d_5, 0 = HMDS, δ, ppm) and PMR Spectra of Sugars in Saponin **1** (δ, ppm, J/Hz, Pyridine-d_5, 0 = HMDS)

C atom	Tigogenin	**1**	C atom	**1**	
	δ_C			δ_H	δ_C
1	37.5	37.2	Galactose*		
2	32.6	30.0	1	4.85 ($J_{1,2}$ = 7.6)	103.2 (159.0)
3	70.5	77.4	2	4.39 ($J_{2,3}$ = 9.0)	72.5
4	39.1	34.7	3	4.12 ($J_{3,4}$ = 3.2)	75.6
5	45.1	44.8	4	4.56 ($J_{4,5}$ = 1.8)	79.5
6	29.1	28.9	5	4.10 m	75.8
7	32.5	32.3	6		60.7
8	35.4	35.4	Glucose A*		
9	54.6	54.4	1	5.11 ($J_{1,2}$ = 7.6)	105.0 (162.2)
10	35.9	36.0	2	4.32 ($J_{2,3}$ = 8.6)	81.8
11	21.3	21.2	3	4.18 ($J_{3,4}$ = 8.6)	87.3
12	40.3	40.1	4	3.71 ($J_{4,5}$ = 8.7)	71.4
13	40.8	41.0	5	3.80 m	78.0
14	56.6	56.4	6		62.8
15	32.1	32.1	Glucose B*		
16	81.1	81.0	1	5.32 ($J_{1,2}$ = 7.8)	104.7 (159.4)
17	63.1	63.6	2	4.06 ($J_{2,3}$ = 8.8)	75.3
18	16.7	16.3	3	4.14 ($J_{3,4}$ = 8.8)	78.3
19	12.5	12.5	4	4.28 ($J_{4,5}$ = 9.0)	71.6
20	42.0	40.2	5	3.83 m	78.2
21	15.0	16.3	6		62.8
22	109.3	110.8	Glucose C*		
23	31.9	30.9	1	4.81 ($J_{1,2}$ = 7.5)	104.5 (157.9; 162.0)
24	29.3	28.2	2	4.03 ($J_{2,3}$ = 8.6)	75.0
25	30.6	34.1	3	4.10 ($J_{3,4}$ = 8.8)	78.5
26	66.9	75.2	4	4.23 ($J_{4,5}$ = 9.1)	71.9
27	17.2	17.3	5	3.86 m	77.8
			6		62.7
			Fucose		
			1	4.61 ($J_{1,2}$ = 7.5)	106.0 (161.4)
			2	4.24 ($J_{2,3}$ = 8.9)	71.8
			3	4.08 ($J_{3,4}$ = 3.5)	74.7
			4	4.01 m	72.8
			5	3.75 m	71.8
			6	1.52 br.s	17.0

*The resonance of H-6 could not be found in the spectrum at 300 MHz.

Enzymatic hydrolysis of glycoside **1** by β-glucosidase produced a prosapogenin that was identical to the previously isolated glycoside (25*R*),5α-spirostan-3β-ol 3-*O*-β-D-glucopyranosyl-(1→3)-[β-D-fucopyranosyl-(1→2)]-β-D-glucopyranosyl-(1→4)-β-D-galactopyranoside, data for which have been published [2].

Compound **1** was completely methylated by the Hakomori method [7] in order to elucidate the structure of the carbohydrate chain and its site of attachment. The completeness of the methylation was monitored using the disappearance of hydroxyl absorption bands in the IR spectrum. The PMR spectrum of the permethylated product exhibited five doublets in the range 4.1–5.0 ppm. These were assigned to anomeric sugar protons. The spin–spin coupling constants of these resonances (J = 7.2–8.1 Hz) were consistent with the β-configuration for all glycosidic bonds [8] and the C1-conformation for the carbohydrate oxide rings [9].

Total acid hydrolysis of the permethylate produced the aglycon, the physical constants and spectral data of which coincided with those for an authentic sample of tigogenin, and a collection of methylated sugars, which were identified in combination with authentic samples by TLC and GC as 2,3,4,6-tetra-*O*-methyl-D-glucopyranose; 2,3,4-tri-*O*-methyl-D-fucopyranose; 4,6-di-*O*-methyl-D-glucopyranose; and 2,3,6-tri-*O*-methyl-D-galactopyraonse.

The GC data agreed well with the spectral data. Thus, the FAB mass spectrum of saponin **1** had key peaks with *m*/*z* values 1251 $[M + Na]^+$, 1105 $[M + Na - deoxyhexose]^+$, 1089 $[M + Na - hexose]^+$, and 439 $[M + Na - tetraose + glucose]^+$.

The structure of the carbohydrate chain and the site of attachment of the sugar units to the aglycon of glycoside **1** were established using PMR and ^{13}C NMR data (Table 1).

The PMR spectrum of the carbohydrate part of the glycoside was interpreted using ordinary and difference versions of selective homonuclear double resonance. The measured SSCC agreed well with the fact that the carbohydrate part of saponin **1** contained three molecules of D-glucose, one D-galactose, and one D-fucose whereas their values for the anomeric protons corresponded to the β-configuration for the glycosidic bonds of all sugars [10].

Conclusions drawn from the GC results about the structure of the carbohydrate chain were consistent with experiments involving the nuclear Overhauser effect, which could establish correlations of through-space couplings between protons through three (vicinal) and four bonds. Thus, pre-irradiation of fucose anomeric proton H-1 (δ 4.61) strengthened two resonances for fucose H-2 (δ 4.24) and glucose A H-2 (δ 4.32) by 6.3 and 8.1%, respectively. Pre-irradiation of glucose B H-1 (δ 5.32) caused the same response for glucose B H-2 (δ 4.06) and glucose A H-3 (δ 4.18). Pre-irradiation of glucose A H-1 (δ 5.11) intensified glucose A H-2 (δ 4.32) and galactose H-4 (δ 4.56). Thus, glucose B and fucose glycosylated glucose A at C-3 and C-2, respectively; glucose A, galactose at C-4.

The ^{13}C NMR spectrum (Table 1) taken with full spin–spin decoupling with protons contained 57 lines, of which 27 belonged to the aglycon and 30, to the sugars. Five resonances were observed for anomeric C atoms; five strong resonances, for methyls at strong-field, of which four belonged to tigogenin whereas the fifth came from a deoxyhexose (fucose).

A comparison of chemical shifts for C atoms of glycoside **1** in the aglycon (C-3 and C-26) and the carbohydrate chain (galactose C-4 and glucose A C-2 and C-3) with literature values for tigotenin [11] and carbohydrates [11, 12] showed that they all experienced a paramagnetic shift of the order of 5.8-9.0 ppm, i.e., were glycosylation centers. This agreed fully with the GC and PMR data presented above. Furthermore, the heteronuclear SSCC in ^{13}C spectra that were taken without decoupling confirmed that the OR group on C-1 of all sugars had the equatorial orientation, i.e., all glycosidic bonds with the C1-conformation of their oxide rings had the β-configuration [13].

Thus, the results confirmed that glycoside **1** was 3-*O*-β-D-glucopyranosyl-(1→3)[β-D-fucopryanosyl-(1→2)]-β-D-glucopyrnaosyl-(1→4)-β-D-galactopyranosyl-(25*R*),5α-furostan-3β,22α,26-triol-26-*O*-β-D-glucopyranoside.

EXPERIMENTAL

General comments have been published [1, 2]. We used solvent systems $CHCl_3$:MeOH:H_2O (65:22:4, 1a; 65:35:8, 1b); $CHCl_3$:MeOH (10:1, 2a; 50:1, 2b); and $CHCl_3$:MeOH:Me_2CO:H_2O (3:3:3:1, 3).

Isolation of Glycoside 1. Preliminary processing of total extracted substances has been described in detail [1].

Aqueous wastes from production of the drug acetyldigitoxin were condensed. Steroidal saponins were extracted repeatedly with *n*-BuOH. The resulting extracts were evaporated to a resinous state and suspended in water with vigorous stirring. The precipitate that was insoluble in water was separated by decantation of the supernatant solution. The liquid was again extracted with *n*-BuOH. The solvent was evaporated. The solid was dissolved in EtOH. Glycosides were precipitated with acetone and dried. Chromatography of the resulting total glycosides using systems 1a and 1b produced a mixture of glycoside **1** and its 22-*O*-methyl ether. Boiling the mixture in water for 2 h produced chromatographically homogeneous glycoside **1** (0.38 g), 0.008% calculated for the weight of air-dried raw material.

3-*O*-β-D-Glucopyranosyl-(1→3)[β-D-fucopyranosyl-(1→2)]-β-D-glucopyranosyl-(1→4)-β-D-galactopyranosyl-(25*R*),5α-furostan-3β,22α,26-triol-26-*O*-β-D-glucopyranoside (1). Amorphous powder, cream color, mp 212–215°C, $[\alpha]_D^{20}$ –53.6° (*c* 0.45, $CHCl_3$:MeOH, 1:1). FAB-MS (*m*/*z*, %): 1251 (37) $[M + Na]^+$, 1105 (74) $[M + Na - deoxyhexose]^+$, 1089 (100) $[M + Na - hexose]^+$, 439 (49) $[M + Na - tetraose + glucose]^+$. IR spectrum (KBr, ν, cm^{-1}): 3500–3360 (OH), 2940 (CH), 895 (weak broad band). PMR spectrum (CD_3OD, δ, ppm, J/Hz): 0.84 (3H, s, Me-18), 0.89 (3H, s, Me-19), 0.98 (3H, d, J = 6.6, Me-27), 1.02 (3H, d, J = 6.9), 3.29 (1H, m, H-26b), 3.41 (1H, m, H-26a), 3.68 (1H, m, H-3), 4.39 (1H, m, H-16).

Acid Hydrolysis. A solution of glycoside **1** (55 mg) in H_2SO_4 (1 M) and EtOH (50%) was refluxed for 2 h and diluted with water. The resulting precipitate was separated by filtration and purified by recrystallization from MeOH to

produce colorless crystals (28 mg), $C_{27}H_{44}O_3$, mp 198–200°C, $[\alpha]_D^{20}$ –48.4° (*c* 0.52, $CHCl_3$). IR spectrum (KBr, ν, cm^{-1}): 3400 (OH), 980, 920, 900, 860 (intensity of band at 900–920, 25*R*-type spiroketal). The ^{13}C NMR spectrum [11] and joint TLC (system 2a) with an authentic sample identified the aglycon as tigogenin. TLC (system 3) of the condensed aqueous hydrolysate detected D-glucose, D-galactose, and D-fucose with R_f 0.32, 0.30, and 0.36, respectively.

Methylation of Compound 1. Glycoside **1** (110 mg) was dissolved in DMSO (10 mL), treated slowly with NaH (100 mg), stirred vigorously (1 h), treated slowly with CH_3I (1.5 mL), stirred another 3 h, poured into water (0.1 L), and extracted with $CHCl_3$ (5 × 10 mL). The combined $CHCl_3$ extracts were treated with sodium thiosulfate and evaporated to dryness. The solid was chromatographed over a column of silica gel (system 2b). Recrystallization from MeOH afforded the permethylated product (82 mg) that was subjected to acid hydrolysis as described above. The precipitated aglycon was separated and identified as tigogenin.

The aqueous solution of methylated sugars was refluxed for 6 h, neutralized with EDE-10P anion-exchanger, and evaporated to dryness. GC identified the methylglycosides described above in the discussion.

Enzymatic Hydrolysis of Glycoside 1. A solution of glycoside **1** (65 mg) in water was treated with β-glucosidase (30 mg) and left at room temperature for 10 h. The resulting precipitate was filtered off, washed with water, dried, and chromatographed over a column of silica gel (system 1a). Recrystallization from EtOH afforded a glycoside (37 mg) that had physical constants, IR and PMR spectra, and TLC behavior that identified it as the native glycoside reported previously [2].

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