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STEROID GLYCOSIDES OF THE SEEDS OF *Nicotiana tabacum*. II. THE STRUCTURES OF NICOTIANOSIDES C AND F

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From the seeds of tobacco Nicotiana tabacum L. *we have isolated two new steroid glycosides, nicotianosides* C *and* F. *The structures of the glycosides have been shown on the basis of chemical transformations and spectral characteristics. Nicotianoside* C *is a steroid glycoside of the spirostan series and has the structure of (25S)-5α-spirostan-3β-ol 3-O-{[α-*L*-rhamnopyranosyl-(1 → 2)]-[α-*L*-rhamnopyranosyl-(1 → 4)]-β-*D*-glucopyranoside}, and nicotianoside* F *is (25S)-5α-furostan-3β,22α,26-triol 3-O-{[α-*L*-rhamnopyranosyl-(1 → 2)]-[α-*L*-rhamnopyranosyl-(1 → 4)]-β-*D*-glucopyranoside} 26-O-β-*D*-glucopyranoside.*

A preliminary analysis of the the seeds of tobacco *Nicotiana tabacum* L. for the presence of steroid glycosides showed that a methanolic extract contained glycosides of the spirostan and furostan series. In the present paper we report the isolation of two new steroid glycosides of tobacco seeds — nicotianosides C (**1**) and F (**2**) — and a proof of their structures.

By chromatography on a silica gel column of the total substances of a methanolic extract of the seeds of this plant in solvent systems with different polarities we isolated compounds (**1**) and (**2**). From its positive reaction with the Sannié reagent [1] and its negative reaction with the Ehrlich reagent [2], and also from the nature of the absorption in the IR spectrum [3], compound (**1**) was assigned to derivatives of the (25S)-spirostan series. Compound (**2**) gave positive reactions with the Sannié and Ehrlich reagents, while in the IR spectrum there was a weak broadened band at 900 cm^{-1}, which enabled us to assign it to glycosides of the furostan series.

The acid hydrolysis of (**1**) and (**2**) led to a genin (**3**), identical with an authentic specimen of neotigogenin. Since nicotianoside F (**2**) had been assigned to glycosides of the furostan series, its native aglycon was (25S)-5α-furostan-3β,22α,26-triol. In a hydrolysate of (**1**), by PC and the GLC of aldononitrile acetate derivatives, glucose and rhamnose were identified in a ratio of 1:2, and in a hydrolysate of (**2**) glucose and rhamnose in a ratio of 2:2. The ^{13}C NMR spectra of (**1**) and (**2**) corresponded to the compositions and ratios of monosaccharide residues in each of them (three signals in the region of anomeric carbon atoms in (**1**) and four such signals in (**2**)) (Table 1).

The Hakomori methylation [4] of (**1**) and (**2**), followed by methanolysis of the permethylated glycosides and analysis by GLC of the products so obtained permitted the determination of Me 2,3,4-tri-O-Me-*L*-rhamnopyranoside (**4**) and Me 3,6-di-O-Me-*D*-glucopyranoside (**5**) from (**1**), and Me 2,3,4,6-tetra-O-Me-*D*-glucopyranoside (**6**), together with (**4**) and (**5**), from (**2**). The methylation results were confirmed by the periodate oxidation of (**1**) and (**2**). In each case, glucose was detected after the acid hydrolysis of the oxidized compound.

The structures of nicotianosides C and F were also confirmed by their ^1H and ^{13}C NMR spectra. In the PMR spectrum of (**1**) (Table 2) there were the signals of three anomeric protons (with characteristic doublet splitting) in the 4.8-6.5 ppm region. On the basis of the small SSCC ($J_{1,2}$ = 1.5 Hz), signals with chemical shifts of 6.12 and 5.65 ppm were assigned to the anomeric protons of two rhamnose residues (Rha′ and Rha″). Two doublet signals of methyl groups in the high-field region of the spectrum with chemical shifts of 1.64 and 1.52 ppm and SSCCs of 6.5-7 Hz also corresponded to these residues. The positions of the signals of the other protons of these two rhamnose residues were found by the method of double homonuclear resonance in the difference variant [5], beginning from the H-1′ and H-1″ protons. The nature of their splitting and their SSCCs fully agreed with the *rhamno*- configuration of these residues. The signal of an anomeric proton with a chemical shift of 4.87

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TABLE 1. Chemical Shifts of the ^{13}C Carbon Atoms of Neotigogenin (**3**), Nicotianoside C (**1**), and Nicotianoside F (**2**) (δ, ppm, 0 — TMS, C_5D_5N)

C–atom	Compound			C–atom	Compound	
	3	1	2		1	2
1	37.0	37.1	37.0	Glc		
2	31.5	29.9	30.2	1	100.0	100.0
3	71.3	78.0	78.6	2	79.0	78.4
4	38.1	34.3	33.9	3	77.4	77.1
5	44.6	44.8	44.5	4	78.4	78.1
6	28.6	28.9	28.6	5	77.1	76.9
7	32.2	31.9	32.1	6	61.6	61.6
8	35.0	34.8	35.1	Rha′		
9	54.3	54.1	54.3	1	102.3	102.4
10	35.6	35.3	35.3	2	72.6	72.9
11	21.0	21.2	21.0	3	72.8	73.0
12	40.2	40.4	40.1	4	74.2	74.2
13	40.6	40.6	40.3	5	70.6	70.6
14	56.6	56.5	56.8	6	18.6	18.7
15	31.9	32.1	32.0	Rha″		
16	80.9	80.6	81.0	1″	103.0	103.1
17	62.4	61.9	62.2	2″	72.6	72.7
18	16.5	16.4	16.5	3″	72.8	72.9
19	12.1	12.0	12.2	4″	74.0	74.0
20	42.1	42.3	42.1	5″	69.7	69.7
21	14.3	14.6	14.3	6″	18.8	18.8
22	109.3	109.2	112.2	Glc″		
23	27.0	27.1	35.4	1″		105.2
24	25.9	25.9	28.3	2″		75.4
25	26.2	26.1	36.3	3″		78.7
26	65.1	65.1	71.8	4″		71.9
27	16.3	16.2	17.2	5″		78.6
O–CH_3	–	–	49.8	6″		63.0

TABLE 2. Chemical Shifts (δ, ppm, 0 — TMS, C_5D_5N) and SSCCs (J, Hz) of the Protons of Nicotianoside C (**1**)

Protons of the aglycon and the sugars of (1)	δ, ppm, J, Hz	Protons of the aglycon and the sugars of (1)	δ, ppm, J, Hz
H–3	3.82 m		L–Rha′
CH_3–18	0.71 s	H–1	6.12 d $J_{1.2}=1.5$
CH_3–19	0.76 s	H–2	4.62 dd $J_{2.3}=3.5$
CH_3–21	1.06 d $J_{20.21}=7.0$	H–3	4.42 dd $J_{3.4}=9.5$
CH_3–27	1.03 d $J_{25.27}=7.0$	H–4	4.18 t $J_{4.5}=9.5$
H–26A	3.62 m $J_{26A.26B}=11.0$	H–5	4.72 dq $J_{5.6}=6.5$
H–26B	3.30 br.d	H–6	1.64 d $J_{6.5}=6.5$
	D–Glc		*L*–Rha″
H–1	4.82 d $J_{1.2}=7.5$	H–1″	5.65 $J_{1.2}=1.5$
H–2	4.0 t $J_{2.3}=7.3$	H–2″	4.50 $J_{2.3}=3.5$
H–3	4.06 dd $J_{3.4}=9.8$	H–3″	4.40 $J_{3.4}=9.5$
H–4	4.38 t $J_{4.5}=10.0$	H–4″	4.19 $J_{4.5}=9.5$
H–5	3.5 m	H–5″	4.73 $J_{5.6}=7.0$
H–6A	3.9 dd $J_{6A.AB}=12.5$	H–6″	1.52 $J_{6.5}=7.0$

ppm and a large SSCC ($J_{1,2}$ = 7.5 Hz) undoubtedly belonged to the remaining glucose residue and determined the configuration of its glycosidic bond. The nature of the splittings of the signals of the other protons of this residue also confirmed its *gluco*-configuration.

The other signals in the high-field region of the spectrum (0.5-2.0 ppm) were assigned to the methyl groups at C-18 and C-19 (two singlets) and C-21 and C-27 (both with the characteristic doublet splitting, J = 7 Hz, with the proton on the neighboring C-20 or C-25 atom). Also found were the signals of the H-3 proton (3.82 ppm, characteristic doublet–doublet splitting) and of the H-26A,B protons (3.62 and 3.30 ppm, $J_{26A,26B}$ = 11 Hz). All this confirmed the steroid nature of the aglycon, which was assigned to the spiro- series, while the close values of the chemical shifts of the C-21-CH_3 and C-27-CH_3 protons showed that the steroid was a derivative of the (25S)-neo series [6].

The types of glycosidic bonds and of the carbohydrate part of the glycoside followed from a two-dimensional ROESY experiment (nuclear Overhauser effects in a rotating system of coordinates) [7]. Thus, in the ROESY spectrum of (**1**) there were

cross-peaks with δ 4.87 and 3.83 ppm, showing the spatial propinquity of the H-1 proton of Glc and H-3 of the aglycon and unambiguously determining the monosaccharide linked to the aglycon, cross-peaks with δ 6.16 and 4.05 ppm, showing the linkage of the Rha′ residue with the C-2-OH group of the Glc residue, and cross-peaks with δ 5.65 and 4.42 ppm, confirming a 1→4 bond between Rha″ and Glc.

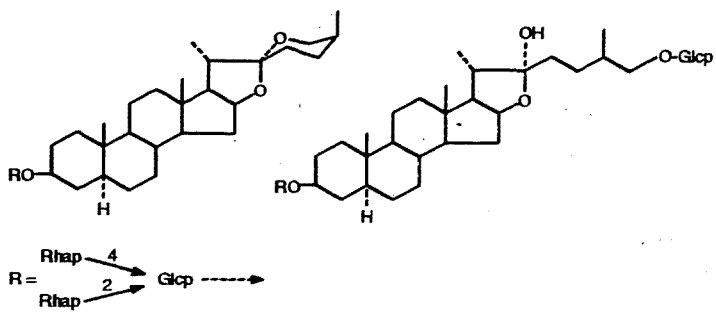
The signals of three anomeric C atoms were also found in the 100-105 ppm region of the ^{13}C NMR spectrum of nicotianoside C (see Table 1). The assignment of the signals of the C atoms of the terminal rhamnose residues was made with the use of literature information for unsubstituted methyl α-rhamnopyranoside [8] and for the chemical shifts of terminal rhamnose residues in various glycosides [9]. The signals of the C atoms of a 2,4-disubstituted β-glucose were assigned with the use of known α- and β-effects of glycosylation [10]. The signals of the C-atoms of the aglycon moiety of the glycoside agreed well with literature results for neotigogenin [11]. A positive effect on C-3 (6.7 ppm) in the aglycon and negative β-effects on C-2 and C-4 confirmed the position of attachment of the carbohydrate chain to the aglycon.

Thus, nicotianoside C is (25S)-5α-spirostan-3β-ol 3-O-{[O-α-*L*-rhamnopyranosyl-(1→2)]-[O-α-*L*-rhamnopyranosyl-(1→4)]-β-*D*-glucopyranoside}.

The ^{13}C NMR spectrum of nicotianoside F (see Table 1) was broadly similar to the spectrum of nicotianoside C. However, in the region of anomeric C atoms there was one additional signal with δ 105.2 ppm. Moreover, in the spectral region of 60-80 ppm, as compared with the spectrum of (**1**), there were five additional signals. From their chemical shifts, they were unambiguously assigned to a terminal β-glucose residue. The other signals in the 60-80 ppm region coincided completely with those for (**1**).

In the low-field region of the spectrum of (**2**), as compared with the spectrum of (**1**), the chemical shifts of the C-23, C-24, C-25, and C-27 atoms were shifted downfield, which is characteristic for steroids of the furostan series. The chemical shift of the signal of the C-26 atom (downfield shift from 70.3 to 75.4 ppm) corresponds to a glycosylated form of a furostan. Thus, nicotianoside F contains two carbohydrate chains — one at the C-3 atom of the aglycon, as in (**1**), and the other, consisting of a single glucose residue, at the C-26 atom.

On the basis of the facts given above, nicotianoside F has the following structure: (25S)-5α-furostan-3β,22α,26-triol 3-O-{[O-α-*L*-rhamnopyranosyl-(1→2)]-[O-α-*L*-rhamnopyranosyl-(1→4)]-β-*D*-glucopyranoside} 26-O-β-*D*-glucopyranoside.



EXPERIMENTAL

For chromatography we used silica gels 11/160 and 40/100 μm, Silufol plates, and FM-3 paper, and the solvent systems 1) chloroform–methanol (4:1); 2) chloroform–ethanol–water (65:30:3); 3) benzene–ethanol (9:1); 4) benzene–diethyl ether (7:3); and 5) butanol–benzene–pyridine–water (5:1:3:3 — upper layer). For revealing the substances we used the Sannié reagent (1% alcoholic solution of vanillin), the Ehrlich reagent (1% alcoholic solution of *p*-dimethylaminobenzaldehyde), a solution of aniline phthalate, and concentrated sulfuric acid.

GLC analysis was conducted on a Chrom-5 instrument, using for the sugar derivatives a column 2.4 m long filled with 5% of XE-60 on Chromaton N-AW-HMDS. The carrier gas was helium. The temperature for chromatographing the sugar aldononitrile derivatives was 180-230°C with a rate of rise of 3°C per minute, while the temperature for chromatographing the methylated methyl glycosides was 140°C at a rate of flow of carier gas of 45 ml/min.

Melting points were determined on a Boëtius stage, specific rotations on a Zeiss polarimeter, IR spectra on a Specord 71-IR spectrophotometer, mass spectra on a MKh-1303 instrument, PMR spectra on Bruker AM-300 and WM-250 instruments using solutions in pyridine-d_5 at 70°C, and ^{13}C NMR spectra on an AM-300 instrument (working frequency 75 MHz) under analogous conditions.

Isolation of the Steroid Glycosides. Air-dry tobacco seeds (0.2 kg) were ground and extracted with chloroform–methanol (2:1). The chloroform–methanolic extract was diluted with water and allowed to separate. The aqueous methanolic extract so obtained was evaporated to a dry residue which was then chromatographed repeatedly on a column of silica gel, using for elution solvent systems 1 and 2 successively. In this way we isolated 1420 mg of nicotianoside C, mp 268-269°C, $[\alpha]_D^{22}$ −73° (*c* 1.0; CH_3OH), and 2310 mg of nicotianoside F, mp 181-182°C, $[\alpha]_D^{20}$ −75° (*c* 1.0; H_2O).

Acid Hydrolysis of Nicotianosides C and F. Each glycoside (100 mg) was hydrolyzed with 2.5% sulfuric acid at 110°C for 8 h in sealed tubes, after which the reaction mixture was diluted with water. The solution of the hydrolysate was extracted with diethyl ether (4 × 20 ml). The ethereal extract was concentrated and chromatographed on a column of silica gel in system 4. For each nicotianoside the aglycon was identified as neotigogenin, mp 203°C, $[\alpha]_D^{20}$ −75° (*c* 1.0; $CHCl_3$), $[M]^+$ 416. The IR spectrum (3360, 960, 918 > 985, 860, 835 cm^{-1}) and the ^{13}C NMR spectrum of the neotigogenin agreed with those given in the literature [11].

Methylation of the Nicotianosides and Methanolysis of Their Permethylated Derivatives. Any one of the glycosides (50 mg) was dissolved in 15 ml of methylsulfonyl anion (prepared from 700 mg of NaH and 30 ml of DMSO) and was permethylated at 50°C for 1 h in an atmosphere of argon. The reaction mixture was treated with 20 ml of CH_3I and was left at room temperature for 12 h in the dark, after which it was diluted with water and extracted with chloroform. The resulting chloroform extract was washed with saturated $Na_2S_2O_3$ solution and with water and was concentrated in vacuum. The permethylated glycoside derivatives so obtained were chromatographed on a column of silica gel in system 3.

Each of the permethylated glycosides was subjected to methanolysis with 72% $HClO_4$ in methanol (1:10) at 110°C for 6 h. The the precipitate that had deposited was filtered off and the solution was neutralized with ion-exchange resin and evaporated, and the residue was analyzed by TLC and GLC in the presence of markers, as a result of which the methyl glycosides mentioned in the discussion were identified.

Periodate Oxidation. A solution of 25 mg of nicotianoside C in 10 ml of methanol and a solution of 30 mg of nicotianoside F in 15 ml of water were each treated with 15 ml of a 2% solution of $NaIO_4$. The mixture was held at room temperature for 48 h and then a few drops of ethylene glycol was added, and after 1 h the mixture was extracted with butan-1-ol. The butanolic extract was evaporated and hydrolyzed with 2.5% H_2SO_4 at 110°C for 8 h. By chromatography on paper (system 5), glucose was identified in each hydrolysate.

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