Chemistry of Natural Compounds, Vol. 31, No. 2, 1995

STEROID GLYCOSIDES OF THE SEEDS OF *Petunia hybrida*
II. THE STRUCTURES OF PETUNIOSIDES I, L, AND N

S. A. Shvets, A. M. Naibi, and P. K. Kintya

UDC 547.918+547.917

Three new steroid glycosides of the furostan series, petuniosides I, L, and N, have been isolated from the seeds of Petunia hybrida L. *Petunioside I is (25R)-5α-furostan-3β,22α,26-triol 3-O-β-*D*-galactopyranoside 26-O-β-*D*-glucopyranoside, petunioside L. (25R)-5α-furostan-3β,22α,6-triol 3-O-[O-β-*D*-glucopyranosyl-(1→4)-β-*D*-galactopyranoside] 26-O-β-*D*-glucopyranoside, and petunioside N is (25R)-5α-furostan-3β,22α,26-triol 3-O-[O-β-D-glucopyranosyl-(1→2)-O-β-D-glucopyranosyl-(1→4)-β-*D*-galactopyranoside] 26-O-β-*D*-glucopyranoside.*

We have previously reported steroid glycosides of the spirostan series that we had isolated from the seeds of the petunia *Petunia hybrida* L. [1, 2]. In the present paper we describe the results of experiments enabling us to deduce the structures of three steroid glycosides of the spirostan series that we have isolated from petunia seeds and have called petuniosides I (**1**), L (**2**), and N (**3**).

By repeated chromatography on a column of silica gel we isolated from a methanolic extract of the petunia seeds three individual compounds (**1**), (**2**), and (**3**) giving positive reactions with the Sannié [3], and Ehrlich (4) reagents, which enabled us to assign them to glycosides of the furostan series. The IR spectrum of each of them had the weak broad band at 900 cm^{-1} that is characteristic for glycosides of the furostan series [5].

The acid hydrolysis of each glycoside led to a genin which was identified from its physicochemical properties as tigogenin but, in view of the fact that the glycosides belong to the furostan series their native aglycon is in fact (25R)-5α-furostan-3β,22α,26-triol. In a hydrolysate of (**1**), by the PC and GLC of aldononitrile acetate derivatives of the sugars we detected galactose and glucose in a ratio of 1:1, and in the case of (**2**) galactose and glucose in a ratio of 1:2, and in the case of (**3**) galactose and glucose in a ratio of 1:3.

The position of attachment and the sizes of the oxide rings of the monosaccharides in each petunioside were determined by Hakomori methylation [6] followed by the methanolysis of the permethyl derivatives. The methyl glycosides were identified by TLC and GLC in the presence of authentic samples: for permethylated (**1**) we detected methyl 2,3,4,6-tetra-O-methyl-*D*-glucopyranoside (**4**) and methyl 2,3,4,6-tetra-O-methyl-*D*-galactopyranoside (**5**); for permethylated (**2**)-(**4**) and methyl 2,3,6-tri-O-methyl-*D*-galactopyranoside (**6**); and for permethylated (**3**)-(**4**), methyl-3,4,6-tri-O-methyl-*D*-glucopyranoside (**7**), and (**6**).

The sequences of the monosaccharides in the carbohydrate chains were found by partial hydrolysis. From (**1**) we obtained tigogenin and a tigogenin monoside (**8**), from (**2**), additionally, a tigogenin bioside (**9**), and from (3) we obtained (**8**), (**9**), and a tigogenin trioside (**10**).

When (**8**) was subjected to acid hydrolysis, galactose was detected in the hydrolysate. Consequently, the galactose was bound directly with the aglycon. In a hydrolysate of (**9**) we identified glucose and galactose in a ratio of 1:1, and in a hydrolysate of (**10**) glucose and galactose in a ratio of 2:1. After methylation and methanolysis of the permethylated (**8**), by GLC we detected (**5**) in the methanolysate, after the similar treatment of (**9**), we detected (**4**) and (**6**), and in the case of (**10**) we detected (**4**), (**6**), and (**7**).

The enzymatic cleavage of (**1**) with β-glucosidase led to a spirostanol analog coinciding in its physicochemical constants with (**8**), while from (**2**), we obtained a progenin identical with (**9**), and from (**3**) we obtained (**10**).

The furanostanol structures (**1**), (**2**), and (**3**) were confirmed by reduction with sodium tetrahydroborate in aqueous solution. After the acid hydrolysis of the reduced compounds and chromatographic purification of the aglycon obtained,

Institute of Genetics, Academy of Sciences of the Republic of Moldova, 277002, Kishinev, ul. Desnaya, 20. Translated from Khimiya Prirodnykh Soedinenii, No. 2, pp. 247-252, March-April, 1995. Original article submitted August 29, 1994.

0009-3130/95/3102-0203$12.50

TABLE 1. Chemical Shifts of the ^{13}C Atoms of Petunioside N (**3**) (δ, ppm, 0 — TMS, C_5D_5N)

C-atom	δ	C-atom	δ	C-atom	δ
		Aglycon			
1	37.4	11	21.1	21	16.0
2	29.9	12	39.8	22	110.8
3	83.8	13	40.7	23	36.8
4	34.8	14	56.5	24	28.1
5	45.1	15	32.2	25	34.0
6	28.3	16	80.7	26	75.1
7	32.2	17	63.4	27	17.2
8	31.6	18	16.3	O—CH_3	49.9
9	50.3	19	19.2		
10	36.9	20	40.4		
		Sugars			
Galactose		Glucose		Glucose	
1	103.65	1	105.0	1″	106.7
2	72.9	2	85.5	2″	76.6
3	75.7	3	78.4	3″	77.8
4	80.5	4	71.0	4″	72.0
5	75.75	5	78.9	5″	78.1
6	60.8	6	63.3	6″	62.2
		1′	106.7		
		2′	76.5		
		3′	77.9		
		4′	71.9		
		5′	78.2		
		6′	62.3		

dihydrotigogenin was obtained in each case. This indicates that the monosaccharide residues were attached at C-3 and C-26 of the aglycon while the readily reduced OH group at C-22 was not glycosylated.

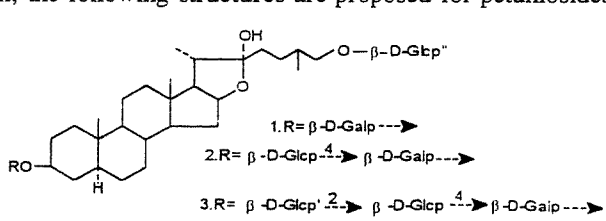
The attachment of a glucose molecule in the C-26 position of the genin in each glycoside was confirmed experimentally. For this purpose, compounds (**1-3**) were subjected to oxidative cleavage by Marker's method [7]. The peracetylated compounds (**1-3**) were converted by boiling in acetic acid into the $\Delta^{20(22)}$ compounds and were then treated with CrO_3 in glacial acetic acid at room temperature. The resulting ketones were cleaved with KOH in tertiary butyl alcohol, as a result of which the two component parts of the glycoside were obtained in each case: a glycoside of 3β-hydroxy-5α-pregn-16-en-20-one and the product of the side chain of the steroid component of the glycoside in which, after acetylation and methylation with diazomethane, the tetraacetate of the glucoside of methyl δ-hydroxy-γ-methyl-*n*-valerate was identified.

The mass spectrum of this compound showed the characteristic peaks for acetylated glucose, together with which fragments appeared having *m/z* 129 ($C_7H_{13}O_2$) and 97 (129 − MeOH]$^+$ which are characteristic for the acid residue [7]. The configurations of the glycosidic centers of the monosaccharides were determined from the molecular rotation differences of the glycosides and the progenins in accordance with Klyne's rule [8]. The structure of petunioside N was also confirmed by its ^{13}C NMR spectrum, where the signals of four anomeric C atoms were found in the 100-105 ppm region.

A comparison of the spectral characteristics with literature information [9] confirmed the structure of the aglycon component as (25R)-5α-furostan-3β,22α,26-triol and showed substitution at the C-3 atom (downfield shift from 70.85 to 78.1 ppm) and at the C-26 atom (downfield shift of the signal of the methylene carbon atom by 8.3 ppm).

In the ^{13}C NMR spectrum of the carbohydrate moiety of (**3**), chemical shifts of the C-4 carbon atom of galactose and of the C-2 carbon atom of glucose were observed (downfield shifts from 71.9 to 80.5 ppm and from 76.5 to 85.6 ppm, respectively), which showed substitution at the C-4 atom of galactose and the C-2 atom of glucose. The assignment of the signals of the C-atoms of the terminal glucose residues was made by the use of literature information on the chemical shifts of terminal glucose residues in various glycosides [10].

On the basis of the facts given, the following structures are proposed for petuniosides I, L, and N:



EXPERIMENTAL

The separation of the total steroid glycosides and the purification of the aglycons and methyl derivatives were carried out on columns filled with silica gels L 40/100, 100/160, and 100/250 μm. The following solvent systems were used: 1) chloroform—methanol (4:1); 2) chloroform—methanol—water (65:35:10, lower layer); 3) chloroform—methanol—water (65:35:5); 4) benzene—diethyl ether (7:3); 5) benzene—ethanol (9:1); and 6) *n*-butyl alcohol—benzene—pyridine—water (5:1:3:3, upper layer).

We used FN-3 paper for PC, and Silufol plates for TLC. Thin-layer chromatograms were revealed with the Sannié reagent (a 1% solution of aniline in ethyl alcohol) followed by treatment with sulfuric acid, and with the Ehrlich reagent (1% alcoholic solution of *p*-dimethylaminobenzaldehyde). A solution of aniline phthalate was used to reveal the sugars.

Melting points were determined on a Boëtius stage. Specific rotations were measured on a Zeiss polarimeter. IR spectra were taken on a Specord 74-IR spectrophotometer. Mass spectra were recorded on a MKh 1320 instrument. The gas–liquid chromatography of the aldononitrile acetate derivatives of the sugars and methylated methyl glycosides was carried out on a Chrom-5 chromatograph with a flame-ionization detector, using a glass column 2.4 m long filled with 5% of E-60 on Chromaton NAW-HMDS. The carrier gas was helium.

The temperature for the chromatography of the sugar aldononitrile acetates was 180-230°C with programming of the temperature at 3°C per minute, and the temperature for chromatographing the methylated methyl glycosides was 140°C. The rate of flow of the carrier gas was 45 ml/min. ^{13}C NMR spectra were taken on a Bruker AM-300 instrument at a working frequency of 75 MHz in deuteropyridine at 70°C.

Isolation of the Steroid Glycosides. Air-dry petunia seeds (0.5 kg) were ground, defatted with chloroform, and extracted by 70% aqueous ethyl alcohol (3 × 2 liters) with heating. The extract obtained was evaporated to an aqueous residue, which was treated with butyl alcohol. The butanolic extracts were concentrated and the total glycosides were precipitated from solution with acetone. The precipitate was filtered off and dried. Yield 12 g.

The total glycosides isolated were chromatographed repeatedly on columns of silica gel using solvent systems 1, 2, and 3. This gave 180 mg of petunioside I (**1**), mp 176°C $[\alpha]_D^{20}-68°$ (s 1.0; CH_3OH), 225 mg of petunioside L (**2**), mp 187-189°C, $[\alpha]_D^{20}-75°$ (s 1.0; H_2O); and 286 mg of petunioside N (**3**), mp 192-193°C, $[\alpha]_D^{20}-89°$ (s 1.0; H_2O).

Complete Acid Hydrolysis of (1-3). Compounds (**1**) (40 mg), (**2**) (50 mg), and (**3**) (80 mg) were hydrolyzed with 2.5% sulfuric acid in sealed tubes at 110°C for 8 h, and the reaction mixtures were diluted with water and extracted with diethyl ether. The extracts obtained were evaporated and chromatographed on columns of silica gel in system 4. The aglycon isolated in each case was identified as tigogenin, mp 202-203°C, $[\alpha]_D^{20}-65°$ (s 1.0; $CHCl_3$), $[M]^+$ 416.

IR spectrum: 3300, 3020, 968, 920<900, 856 cm^{-1} (characteristic absorptions for a spiroketal chain of the (25R)-series). After the extraction of the aglycon, the aqueous hydrolysate of each glycoside was neutralized with anion-exchange resin (HCO_3^- form), and concentrated to a syrupy residue. By PC in system 6 and GLC of the aldononitrile sugars [11], in the hydrolysate of (**1**) galactose and glucose were identified in a ratio of 1:1, in the case of (**2**), galactose and glucose (1:2), and in the case of (**3**) galactose and glucose (1:3).

The Methylation of the Petuniosides and Their Progenins. A solution of 40 mg of a petunioside or a progenin in 10 ml of methylsulfonyl anions (prepared from 700 mg of NaH and 30 ml of dimethyl sulfoxide) was stirred at 50°C in an atmosphere of argon for an hour. In each case, methyl iodide (10 ml) was added to the reaction mixture and it was left in the dark for 12 h. Then it was diluted with water and extracted with chloroform, washed with $Na_2S_2O_3$ solution and with water, evaporated to dryness, and chromatographed on a column with elution by system 5. The permethylate isolated in each case was subjected to methanolysis with 72% $HClO_4$ in methanol (1:10) at 100°C for 6 h. The methyl glycosides mentioned in the discussion were identified by GLC in the methanolysis products.

Partial Acid Hydrolysis of the Petuniosides. Solutions of 100 mg each of compounds (**1**), (**2**), and (**3**) in 20 ml of 1% H_2SO_4 in methanol were heated in the water bath at 80°C for 2 h. The course of the reaction was monitored every 20 min by TLC in systems 1 and 2. The reaction mixture was diluted with water and extracted with butanol (3 × 20 ml). The butanolic extract was separated, and the residue was chromatographed on a column of silica gel in system 1. From (**1**) was obtained progenin (**8**) (37 mg), mp 273-274°C, $[\alpha]_D^{20}-64°$ (s 1.0; CH_3OH), from (**2**) were obtained (**8**) (30 mg) and (**9**) (28 mg), mp 246°C, $[\alpha]_D^{20}-70°$ (s 1.0; CH_3OH), and from (**3**) were obtained (**8**), (**9**), and (**10**) mp 258°C, $[\alpha]_D^{20}-68°$ (s 1.0; Py).

Enzymatic Hydrolysis of the Petuniosides. In 50 ml of water in each case, 100-mg samples of compound (**1-3**) were incubated with the β-glucosidase from the stomach of the grape snail *Helix pomatia* at 37°C for 24 h. The course of the reaction was monitored by TLC in systems 1 and 2. After the end of the reaction, in each case the hydrolysate was extracted

with butyl alcohol, and the extract was concentrated in vacuum to a dry residue. The reaction products were separated on columns of silica gel in system 1. Compound (**1**) gave a progenin identical in its physicochemical constants with (**8**), while compound (**2**), gave (**9**), and compound (**3**) gave (**10**).

Oxidation of Petuniosides I, L, and N. Each of the petuniosides (300 mg) was acetylated and the peracetates obtained were heated in glacial acetic acid with stirring at 120°C for 1.5 h. After chromatographic purification, the $\Delta^{20(22)}$ derivatives of the glycosides were obtained and these were each dissolved in 10 ml of glacial acetic acid in solution to which 100 mg of sodium acetate was added. A solution of 200 mg of chromium trioxide in 5 ml of 80% acetic acid and 2 ml of water was added dropwise over 20 min at 15°C to each of the solutions obtained, and stirring was continued for an hour at room temperature. The oxidized products (110 mg) were hydrolyzed with a solution of 1.2 g of KOH in 5 ml of *tert*-butyl alcohol and 2 ml of water in a current of nitrogen with constant stirring at 30°C for 4 h, and then the reaction was continued at room temperature for another 30 min. As a result of oxidation, in each case the side-chain of the steroid moiety of the glycoside yielded the same product — δ-hydroxy-γ-methyl-*n*-valeric acid glucoside, the structure of which was shown by mass spectrometry after its acetylation and methylation with diazomethane. Mass spectrum: *m/z* 331, 243, 242, 200, 169, 157, 145, 141, 109, and 98 (characteristic for acetylated glucose) and the peaks of fragments of the acid moiety with *m/z* 129 ($C_7H_{13}O_3$) and 97 (which agrees with literature information [7]).

The other component part, the 3β-hydroxy-5α-pregn-16-en-20-one glycoside (20 mg), was hydrolyzed with 3 ml of 4 M HCl in 3 ml of C_6H_6 at 80°C for 3 h. The pregnenolone was acetylated in 4 ml of a 2:3 mixture of Ac_2O and pyridine and, after chromatographic purification, 3β-acetoxy-5α-pregn-16-en-20-one was identified (mp 161°C, $[M]^+$ 358, IR spectrum, cm^{-1}: 1724, 1662 (characteristic for a Δ^{16}-20-one), 956, 918, 895, 820 cm^{-1} [12].

Reduction of the Petuniosides. A solution of 50 mg of one of the petuniosides in 30 ml of water was treated with 300 mg of $NaBH_4$, and the mixture was left at room temperature for 24 h and was then hydrolyzed with 3 N HCl (50 ml) by heating for 6 h. Each hydrolysate was extracted with chloroform. Preparative chromatography on silica gel in the solvent system C_6H_6—$(CH_3)_2O$ (20:1) led to the isolation of dihydrotigogenin (mp 165-166°C, mass spectrum: *m/z* 418 $[M]^+$, 400, 344, 341, 313, 273, 255, and 144) [12].

REFERENCES

1. P. K. Kintia [Kintya], S. A. Shvets, and A. M. Naibi, 5th Symposium on the Analysis of Steroids. Summaries of Papers, Szombathely (1993), p. 25.
2. P. K. Kintia [Kintya], M. A. Naibi, and S. A. Shvets, 16th Meeting of the International Study Group for Steroid Hormones. Abstracts Book, Vienna (1993), p. 76.
3. C. Sannié and H. Lapin, Bull. Soc. Chim. France, **19**, 1080 (1952).
4. S. Kiyosawa and H. Masakaru, Chem. Pharm. Bull., 1162 (1968).
5. R. Tschesche, B. T. Tyoa, G. Wulff, et al., Tetrahedron Lett., No. 49, 5141 (1968).
6. S. Hakamori, J. Biochem. (Tokyo), **55**, 205 (1964).
7. R. Tschesche, G. Ludke, and G. Wulff, Chem. Ber., **102**, 1253 (1969).
8. W. Klyne, Biochem. J., **47**, No. 4, xli (1950).
9. P. K. Agrawal, D. C. Jain, R. K. Gupta, and R. S. Thakur, Phytochemistry, **24**, 2479 (1985).
10. S. Chen and J. Snyder, J. Org. Chem., **54**, 3679 (1989).
11. V. V. Krokhmalyuk, V. Ya. Chirva, and P. K. Kintya , Izv. Akad. Nauk SSSR, Ser. Biol. i Khim. Nauk, No. 1, 85 (1975).
12. S. C. Sharma, R. Chand, O. P. Sati, et al., Phytochemistry, **25**, 1241 (1983).