Structure of Steroidal Saponins from Underground Parts of *Allium nutans* L.

L. S. Akhov,[†] M. M. Musienko,[†] S. Piacente,[‡] C. Pizza,[‡] and W. Oleszek*[,§]

Department of Plant Physiology, Kyiv State University, Vladimirskaya Str. 64, 252033 Kyiv, Ukraine, Dipartimento di Scienze Farmaceutiche, Universita degli Studi di Salerno, Piazza V. Emanuele 9, 84084, Salerno, Italy, and Department of Biochemistry, Institute of Soil Science and Plant Cultivation, ul. Czartoryskich 8, 24-100 Pulawy, Poland

Four steroidal glycosides including deltoside and nolinofuroside D and two novel saponins were isolated from underground parts of *Allium nutans* L. On the basis of the spectral (LSIMS and NMR) analysis, the structures of the new compounds were established as 25*R* Δ^5-spirostan 3β-ol-3-*O*-{α-L-rhamnopyranosyl(1→2)-[β-D-glucopyranosyl(1→4)]-*O*-β-D-galactopyranoside} and 25*R* Δ^5-spirostan 1β,3β-diol 1-*O*-β-D-galactopyranoside. On the basis of the extraction efficiency, the concentration of saponins was established to be about 4% of dry matter, which makes this species a good source of steroidal saponins for commercial use.

Keywords: *Allium nutans; steroidal saponins; diosgenin; ruscogenin; glycosides*

INTRODUCTION

Onions are one of the oldest cultivated plants, and they have been used in food and medicine for many centuries. Unfortunately, only a small group from more than 500 species of this genus are used by humans. However, work on the introduction of many wild species shows that these plants have similar or even more interesting properties than cultivated plants. For example, plants of *Allium nutans* possess higher levels of vitamins C and P than the cultivated onion, *Allium cepa*. A high content of sugar and very rapid growth of aerial parts make this plant an interesting species from a commercial point of view (Bulach, 1994).

There have been many reports on the steroidal saponins from *Allium* species (Hostettmann and Marston, 1995). These compounds are used in the cosmetic industry because of their emollient properties and in pharmaceuticals because of their antitumor activity (Inoue, 1995) and their ability to decrease the cholesterol level in serum (Hostettmann and Marston, 1995). In our work, we investigated the methanolic extract of the underground parts of *A. nutans* and isolated four saponins, two of which were new glycosides. *A. nutans* has not been investigated with regard to saponin occurrence, and we deemed it worthwhile to separate and establish the structures of these compounds.

MATERIALS AND METHODS

Plant Material. Saponins were extracted from the underground parts (bulbs and roots) of *A. nutans* L., grown at the Central Botanical Garden of the Ukrainian Academy of Science. Samples were collected in June 1996, oven-dried at 65 °C, and powdered.

Extraction. Samples (300 g) were refluxed twice for 2 h with 500 mL of 80% aqueous methanol. The extracts were combined, vacuum filtered, and rotary evaporated to completely remove alcohol. Extracts suspended in water were loaded on a short (6 × 10 cm, 55 μm C_{18}, Waters Assoc.) column. The column was washed with 500 mL of 30% methanol in distilled water, and then crude saponins were removed with methanol. Evaporation of the solvent yielded 12 g of a mixture of crude saponins.

Isolation of Glycoside Fractions by Preparative LC. Individual glycosides were separated by column chromatography on C_{18} (3.5 × 40 cm, 55 μm, Waters Assoc.) (Oleszek, 1988). Six grams of the mixture was loaded on the column. The column was washed first with 250 mL of distilled water (0.8 mL/min) and then with 10 L of a 30−80% v/v linear methanol gradient. The 15-mL fraction was collected with a fraction collector and monitored by TLC. Fractions showing similar patterns were combined and evaporated to dryness. This procedure gave pure compound **1** and fractions I−III containing three glycosides.

High-Resolution Preparative Liquid Chromatography (HRPLC). The saponin fractions were thereafter separated on steel columns (1.2 cm × 30 cm) filled with silica gel (Lichroprep 15−25 μm, Si_{60}) packed as previously described (Oleszek et al., 1990). Fractions II and III were separated on a Si_{60} column using methanol/chloroform (2:5 v/v) and methanol/chloroform (2:7 v/v), respectively.

Chromatography (TLC) and Spectral Analyses. Saponins were chromatographed on Merck silica gel 60 plates developed with ethyl acetate/acetic acid/water (7:2:2 v/v) and chloroform/methanol/ water (65:32:5 v/v) or on Merck C_{18} plates developed with methanol/water (60/40 v/v). Saponins were visualized with methanol/acetic anhydride/sulfuric acid (50:5:5 v/v) followed by heating at 120° C.

The NMR spectra in CD_3OD for **1**, **3**, and **4** and in d_6-DMSO for **2** were recorded with a Bruker WH-250 Spectrospin and Bruker DRX-500 using the UXNMR package. 2D experiments: ^1H-^1H DQF−COSY (double quantum filtered direct chemical shift correletion spectroscopy) (Rance at al., 1983), inverse-detected $^1H-^{13}C$ HSQC (heteronuclear single quantium coherence) (Martin and Crouch, 1991), and HMBC (heteronuclear multiple bond connectivity) (Summers et al., 1986) were obtained using UX-NMR software. Negative-ion LSIMS were registered with a MAT 95 (Finland) spectrometer,

* To whom correspondence should be addressed (e-mail WO@iung.Pulawy.pl).
[†] Kyiv State University.
[‡] Universito degli Studi di Salerno.
[§] Institute of Soil Science and Plant Cultivation.

10.1021/jf9901800 CCC: $18.00
Published on Web 07/10/1999

Table 1. ^{13}C NMR Spectral Data for Compounds 1−4

C	1	2	3	4
1	38.48	36.2	83.4	83.7
2	30.80	28.8	36.9	36.9
3	79.41	76.2	68.6	68.7
4	39.6	36.6	42.9	42.6
5	142.0	140.1	139.2	139.7
6	122.7	121.1	125.7	125.7
7	33.02	30.9	32.4	32.3
8	32.24	30.8	33.7	33.6
9	51.7	49.4	51.3	51.1
10	38.1	37.5	42.9	42.7
11	21.85	20.8	24.3	24.1
12	40.63	39.1	41.1	40.9
13	16.78	39.0	40.5	40.5
14	57.81	55.6	57.7	57.6
15	32.54	31.4	32.6	32.7
16	82.43	80.0	81.9	82.2
17	65.26	61.6	63.5	63.9
18	16.78	15.9	16.7	16.8
19	19.6	18.8	14.6	14.6
20	41.18	40.9	42.9	40.1
21	16.14	14.5	14.7	15.8
22	113	109.0	110.9	110.7
23	32.0	31.3	32.0	36.4
24	29.04	28.3	28.5	28.5
25	34.84	29.6	31.1	34.5
26	17.3	65.8	65.6	75.7
27	75.98	16.9	16.7	16.8
1′	100.5	97.9	101.9	101.9
2′	78.7	75.9	72.9	72.1
3′	77.9	75.7	74.7	74.8
4′	81.1	80.7	69.6	63.6
5′	76.3	74.3	76.0	76.0
6′	62.3	60.1	62.0	62.1
1″	104.6	103.0		104.4
2″	75.2	73.1		74.9
3″	78.2	76.3		77.8
4″	71.5	69.8		71.3
5″	77.9	76.6		77.6
6″	62.3	60.5		62.3
1‴	104.7	99.6		
2‴	75.1	70.3		
3‴	78.2	70.4		
4‴	71.8	71.7		
5‴	77.9	67.5		
6‴	62.4	17.6		
1⁗	101.9			
2⁗	72.2			
3⁗	72.5			
4⁗	74.0			
5⁗	69.7			
6⁗	17.6			

with glycerol as the matrix. EIMS were recorded with the MAT 95 spectrometer, at 70 eV.

RESULTS

Saponins Composition and Structures. The methanolic extract of the underground parts of *A. nutans* was subjected to a reverse-phase C_{18} column chromatography to give compound **1** and fractions I−III. Fraction II was composed of two components, whereas the remaining fractions each contained a single major glycoside. Further purification with HRPLC yielded three individual saponins with the following characteristics.

Compound **1** (4.3 g) was obtained as a white, amorphous powder: LSIMS (negative-ion mode) *m/z* 1065 [M − H]$^-$, 903 [M − H − hexose]$^-$, 755 [M − H − hexose − deoxyhexose]$^-$, 594 [M − H − 2 hexoses − deoxyhexose]$^-$, 433 [M − H − 3 hexoses − deoxyhexose]$^-$. For ^{13}C NMR data, see Table 1.

Compound **2** (0.6 g) was crystallized from chloroform−methanol−water (65:32:5). LSIMS (negative-ion mode) *m/z* 883 [M − H]$^-$, 737 [M − H − deoxyhexose]$^-$, 721 [M − H − hexose]$^-$, 575 [M − H − hexose − deoxyhexose]$^-$, 413 [M − H − 2 hexoses − deoxyhexose]$^-$. ^{1}H NMR δ 3.50 (H-3, m), 5.35 (H-6, br d, J = 5.4 Hz), 4.28 (H-16, m), 0.75 (Me-18, s), 0.98 (Me-19, s), 0.90 (Me-21, d, J = 6.5 Hz), 0.75 (Me-27, d, J = 6.5 Hz). For ^{13}C NMR data, see Table 1. For ^{1}H NMR values of the sugar portion, see Table 2.

Compound **3** (32 mg). LSIMS (negative-ion mode) *m/z* 591 [M − H], 413 [M − H − hexose − OH]. ^{1}H NMR δ 3.53 (H-1, dd, J = 3.5, 11.5 Hz), 3.45 (H-3, m), 5.59 (H-6, br d, J = 5.4 Hz), 4.42 (H-16, m), 0.85 (Me-18, s), 1.12 (Me-19, s), 1.09 (Me-21, d, J = 6.5 Hz), 0.85 (Me-27, d, J = 6.5 Hz). For ^{13}C NMR data, see Table 1. For ^{1}H NMR values of the sugar portion, see Table 2.

Compound **4** (28 mg). LSIMS (negative-ion) *m/z* 771 [M − H]$^-$, 609 [M − H − hexose]$^-$, 447 [M − H − 2 hexoses]$^-$. ^{1}H NMR δ 3.52 (H-1, dd, J = 3.5, 11.5 Hz), 3.43 (H-3, m), 5.57 (H-6, br d, J = 5.4 Hz), 4.39 (H-16, m), 0.87 (Me-18, s), 1.13 (Me-19, s), 1.02 (Me-21, d, J = 6.5 Hz), 0.98 (Me-27, d, J = 6.5 Hz). For ^{13}C NMR data, see Table 1. For ^{1}H NMR values of the sugar portion, see Table 2.

DISCUSSION

Extraction of the underground parts of *A. nutans* with aqueous methanol followed by purification on a C_{18} short column yielded crude saponins with an efficiency of 4% of dry matter. This efficiency shows that *A. nutans* can be recognized as a rich source of steroidal saponins for commercial use. The mixture of crude saponins consisted of four glycosides, as evaluated by TLC, with compound **1** making up about 87% and compound **2** 12% of the total. Spectral analysis of compound **1** showed the molecular weight to be 1064. Degradation patterns indicated the presence of three hexoses and one deoxyhexose, which after hydrolysis and TLC analysis of sugars were identified as glucose and rhamnose. The presence of these four sugars was confirmed also by ^{13}C NMR. Other characteristics of the NMR spectra of compound **1** were identical to those published for the compound with the trivial name deltoside, 25*R* Δ^5-furosten 3β,22α,26 triol 26-*O*-β-D-glucopyranosyl, 3-*O*-{α-L-rhamnopyranosyl(1→2)−[β-D-glucopyranosyl(1→4)]-*O*-β-D-glucopyranoside} reported before (Agrawal, 1985).

Compound **2** was crystallized from chloroform−methanol−water solution as plates. The molecular weight of this compound was established by LSIMS as 884 ($C_{42}H_{76}O_{19}$). Three doublet signals due to anomeric protons (δ 4.52, 5.08, and 4.26), two tertiary methyl signals (δ 0.75 and 0.98), and two secondary methyl signals (δ 0.90 and 0.75) were observed in the ^{1}H NMR spectrum. The ^{13}C NMR spectrum of **2** showed a total of 27 carbons for the aglycone including 4 methyl, 10 methylene, and 9 methine groups. Eighteen additional carbons belonged to sugar units. The signals of C-23, C-24, C-25, and C-26 (31.3, 28.3, 29.6, and 65.8 ppm, respectively) and also of C-5 and C-6 (140.1 and 121.1 ppm) allowed the identification of diosgenin as the aglycone of **2** (Agrawal, 1985). Regarding the sugar portion, selected ID-TOCSY (total correlation spectroscopy) spectra obtained by irradiaiting the three anomeric proton signals yielded the subspectrum of each sugar residue with high digital resolution. The results of ID-TOCSY combined with those of a DQF−COSY experiment allowed us to identify the three sugar units

Table 2. ^{1}H NMR Spectral Data of Sugars for Compounds 2−4

	2	3	4
1′	4.52, d, J = 7.5	4.30, d, J = 7.8	4.30, d, J = 7.8
2′	3.25, dd, J = 7.5, 9.0	3.52, dd, J = 7.8, 9.0	3.50, dd, J = 7.8, 9.0
3′	3.53, dd, J = 4.0, 9.0	3.48, dd, J = 4.2, 9.0	3.49, dd, J = 4.5, 9.0
4′	3.30, dd, J = 2.0, 4.0	3.87, dd, J = 4.2, 2.0	3.86, dd, J = 2.0, 4.5
5′	3.28, ddd, J = 2.0, 3.0, 5.0	3.47, ddd, J = 2.0, 3.5, 5.0	3.47, ddd, J = 2.0, 3.5, 4.5
6′	3.58, dd, J = 5.0, 12.0	3.47, dd, J = 5.0,12.0	3.75, dd, J = 4.5, 12.0
	3.75, dd, J = 3.0, 12,0	3.78, J = 3.5,12.0	3.78, J = 3.5, 12.0
1″	4.26, d, J = 7.5		4.27, d, J = 7.5
2″	3.00, dd, J = 7.5, 9.0		3.20, dd, J = 7.5, 9.0
3″	3.18, dd, J = 9.0, 9.0		3.38, dd, J = 9.0, 9.0
4″	3.10, dd, J = 9.0, 9.0		3.30, dd, J = 9.0, 9.0
5″	3.22, ddd, J = 2.5, 5.0, 9.0		3.28, ddd, J = 2.5, 4.5, 9.0
6″	3.42, dd, J = 5.0, 12.0		3.69, dd, J = 4.5, 9.0
	3.71, dd, J = 2.5, 12.0		3.88, dd, J = 2.5, 9.0
1‴	5.08, d, J = 1.5		
2‴	3.63, dd, J = 1.5, 2.5		
3‴	3.42, dd, J = 2.5, 9.0		
4‴	3.22, J = 9.0, 9.0		
5‴	4.00, m		
6‴	1.15, d, J = 6.5		

as β-D-galactopyranosyl (δ 4.52), α-L-rhamnopyranosyl (δ 5.08), and β-D-glucopyranosyl (δ 4.26). From the HSQS experiment, the absence of any ^{13}C NMR glycosidation shift for the β-D-glucopyranosyl and the α-L-rhamnopyranosyl residues suggested that those sugars were terminal. 2,4-disubstituted β-D-galactopyranose was deducted from the signals at δ 75.9 and 80.7 ascribable, respectively, to C-2 and C-4 of the β-D-galactopyranosyl unit. Cross-peaks due to long-range correlation between C-3 (δ 76.2) of the aglycone and H-1 gal (δ 4.52), C-2 gal (δ 75.9) and H-1 rha (δ 5.08), and C-4 gal (δ 80.7) and H-1 glu (δ 4.26) allowed us to identify compound **2** as 25*R* Δ^5-spirostan-3β-ol 3-*O*-{α-L-rhamnopyranosyl(1→2)-[β-D-glucopyranosyl(1→4)]-*O*-β-D-galactopyranoside}.

Compound **3** was obtained as an amorphous solid. The elemental analysis confirmed the molecular formula of **3** as $C_{33}H_{52}O_9$ by the secondary-ion mass spectrum (*m*/*z* 591 [M − H]$^-$). The ^{1}H NMR spectrum showed an anomeric proton signal at δ 4.30, two tertiary methyl signals (δ 0.85 and 1.12), and two secondary methyl signals (δ 1.09 and 0.85). The ^{13}C NMR spectrum exhibited for the aglycone a total of 27 carbons including four methyls, nine methylenes, and nine methines.

A comparison of the ^{13}C NMR spectrum of **3** with the spectrum of **2** showed that **3** has a similar type of aglycone, except for a β-hydroxyl group at C-1. Thus, the aglycone of **3** was identified as ruscogenin (25*R* Δ^5-spirostan-1β,3β-diol). The higher deshielding of C-1 if compared to unglycosylated models allowed us to conclude that sugar was linked to the C-1 position. On the basis of NMR analysis, the sugar unit was identified as β-D-galactose. From the above data, compound **3** was identified as 25*R* Δ^5-spirostan-1β,3β-diol 1-*O*-β-D-galactopyranoside.

Compound **4** was obtained as a white, amorphous powder. Its molecular formula $C_{39}H_{64}O_{15}$ was confirmed by the mass spectrum (*m*/*z* 771 [M − H]$^-$). Two doublet signals due to anomeric protons (δ 4.30 and 4.27), two tertiary methyl signals (δ 0.87 and 1.13), and two secondary methyl signals (δ 1.02 and 0.98) were observed in the ^{1}H NMR spectrum. The ^{13}C NMR spectrum exhibited a total of 27 carbons, except for the sugar moiety: four methyl, nine methylene, and nine methine. The signal assignable to C-26 (75.7 ppm) and C-25 (34.5 ppm) allowed us to conclude that **4** was the furostanol

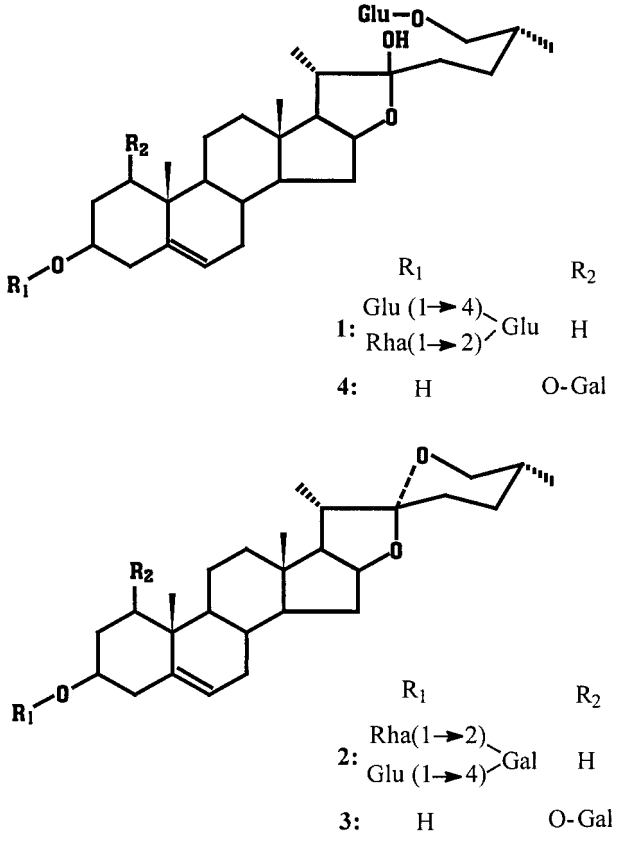

Figure 1. Chemical formulas of identified saponins **1**−**4**.

analogue of **3**. Thus, **4** was identified as 25*R* Δ^5-furostan-1β,3β,22α,26-tetraol 1-*O*-β-D-galactopyranosyl, 26-*O*-β-D-glucopyranoside. A similar compound was isolated from *Nolina microcarpa S. Walt* (Shevchuk, 1991). The structures of the four identified compounds are presented in Figure 1.

The glycosides of diosgenin and ruscogenin commonly occur in the genus *Allium* (Kintya, 1975). The differences, however, can be found between glycoside profiles due to the place of sugar substitution to the aglycone and because of different sugar chain compositions. With regard to *A. nutans*, two identified glycosides (**1, 4**) are the same as those previously reported and two of them (**2**, **3**) are novel compounds, not found in other species. Saponin **3** was reported previosly not as a natural

compound but as a saponin obtained after enzymatic hydrolysis of *Nolina microcarpa S. Walt* (Shevchuk, 1991).

LITERATURE CITED

Agrawal, P. K.; Iain, D. S.; Gupta, R. K.; Thakur, R. S. Carbon-13 NMR spectroscopy of steroidal sapogenins and steroidal saponins. *Phytochemistry* **1985**, 24, 2479−2496.

Bulach, P. E. Onions of flora of Middle Asia and their culture in Ukraine. *Kiev. Nauk. Dumka.* **1994**, 124 (in Russian).

Hostettmann, K.; Marston, A. *Saponins*; Cambridge University Press: Cambridge, 1995; p 522.

Inoue, T.; Mimaki, Y.; Sashida, Y.; Nishino, A.; Satomi, Y.; Nishino, H. Steroidal glycosides from *Allium macleanii* and *A. senences*, and their inhibitory activity on tumor promoter-induced phospholipid metabolism of Hela cells. *Phytochemistry* **1995**, *40*, 521−525.

Kintya, P. K.; Lazuryevskiy, G. V. Spirostane steroidal glycosides. *Kishenev. Shtiica.* **1975**, 146 (in Russian).

Martin, G. E.; Crouch, R. C. Inverse detected two-dimensional NMR methods application in natural products chemistry. *J. Nat. Prod.* **1991**, *54*, 1−70.

Oleszek, W. Solid-phase extraction-fractionation of alfalfa saponins. *J. Sci. Food Agric.* **1988**, *44*, 43−49.

Oleszek, W.; Price, K. R.; Colquhoun, I. J.; Jurzysta, M.; Ploszynski, M.; Fenwick, G.R. Isolation and identification of alfalfa (*Medicago sativa*) root saponins: their activity in relation to a fungual bioassay. *J. Agric. Food Chem.* **1990**, *38*, 1810−1817.

Rance, M.; Sorenson, O. W.; Bodenhausen, G.; Ernst, R. R.; Wuthrich, K. Improved spectral resolution in COSY ^{1}H NMR spectra of proteins via double quantum filtering. *Biochem. Biophys. Res. Commun.* **1983**, *117*, 479−485.

Shevchuk,; Vollerner, Y. S.; Shashkov, A. S.; Chirva, V. Y. Spirostane and furostane steroids of *Nolina microcarpa*. *Khim. Prir. Soedin.* **1991**, *5*, 678−686 (In Russian).

Summers, M. F.; Marzilli, L. G.; Bax, A. Complete ^{1}H and ^{13}C assignments of coenzyme through the use of new two-dimensional NMR experiments. *J. Agric. Food Chem.* **1986**, *108*, 4285−4294.

Received for review February 22, 1999. Revised manuscript received May 28, 1999. Accepted May 31, 1999. L.S.A. thanks the Institute of Soil Science and Plant Cultivation, Pulawy, Poland, which provided a grant for this investigation.

JF9901800