Acta Poloniae Pharmaceutica – Drug Research, Vol. 63 No. 3 pp. 219–223, 2006 ISSN 0001–6837
Polish Pharmaceutical Society

STEROIDAL GLYCOSIDES FROM THE UNDERGROUND PARTS OF *ALLIUM URSINUM* L. AND THEIR CYTOSTATIC AND ANTIMICROBIAL ACTIVITY

DANUTA SOBOLEWSKA[1*], ZBIGNIEW JANECZKO[1], WANDA KISIEL[2], IRMA PODOLAK[1], AGNIESZKA GALANTY[1] and DANUTA TROJANOWSKA[3]

[1] Department of Pharmacognosy, Collegium Medicum, Jagiellonian University, 9 Medyczna Str., 30-688 Kraków, Poland
[2] Department of Phytochemistry, Institute of Pharmacology, Polish Academy of Sciences, 12 Smętna Str., 31-343 Kraków, Poland
[3] Department of Pharmaceutical Microbiology, Collegium Medicum, Jagiellonian University, 9 Medyczna Str., 30-688 Kraków, Poland

Abstract: The aim of this study was the isolation and structural elucidation of steroidal glycosides from the underground parts of ramson *Allium ursinum* L. The structures of the isolated compounds were established based upon chromatographic methods and 1D- and 2D-NMR, MS and IR analyses. The mixture of two steroidal saponins: (25R)-spirost-5-en-3b-ol tetrasaccharide and (25R)-spirost-5, 25(27)-dien-3b-ol tetrasaccharide, along with a 3-hydroxypregna-5,16-dien-20-one glycoside were identified. The results of *in vitro* cytotoxic activity of the mixture of spirostanol saponins against cell lines melanoma B16 and sarcoma XC and human fibroblasts HSF are also reported. The spirostanol saponins mixture was investigated to determine its *in vitro* antimicrobal activity against *Trichophyton mentagrophytes* and *Microsporum canis*.

Keywords: *Allium ursinum*, steroidal saponins, cytotoxicity

Allium ursinum L. (*Alliaceae*), commonly called ramson or wild garlic, is a perennial plant widely distributed in Europe and Asia, which develops a characteristic garlic-like smell and flavor. The aerial parts and bulbs of ramson have long been used in European traditional medicine as an antihypertensive, antiatherosclerotic, antimicrobial, antidiarrhoeal and antiphlogistic agents (1). Pharmacological studies revealed its inhibitory activity *in vitro* on 5-lipoxygenase, cyclooxygenase, angiotensin I-converting enzyme and thrombocyte aggregation (2). It was also demonstrated that chloroform and acetone/chloroform extracts might reduce serum cholesterol level primarily by inhibition of cholesterol synthesis (3). Reports on chemical composition of ramson indicated the presence of many sulfur compounds, including cysteine sulfoxides, thiosulfinates, sulfides, ajoenes, vinyldithiins, as well as flavonoids and lectins (4-6). In a search for biologically active compounds from traditional medicinal plants we isolated two spirostanol saponosides from the underground parts of *Allium ursinum*. Structure elucidation was accomplished mainly on the basis of 2D proton-proton and proton-carbon shift correlation spectroscopy. The cytotoxic and antifungal activities of these compounds are also presented. In a previous paper on ramson, we have reported the isolation and structural elucidation of a 3-hydroxypregna-5,16-dien-20-on tetraglycoside (7). Now its structure is reviewed.

EXPERIMENTAL

Materials and methods

General

IR: Specord M 80 (Carl Zeiss Jena) spectrometer. FAB-MS: Finnigan MAT 95 mass spectrometer, glycerol as the matrix, Cs ions accelerated at 13 keV. Optical rotation: Polamat-A polarimeter. NMR: Bruker AMX 500 spectrometer in C_5D_5N solution, using TMS as an internal standard. ^1H NMR spectra were recorded at 500.13 MHz and ^{13}C NMR spectra at 125.77 MHz. TLC: precoated Merck Kieselgel 60 plates with $CHCl_3$-MeOH- H_2O (23:12:2, v/v/v), $CHCl_3$-AcOH-H_2O (3:3.5:0.5, v/v/v) and $CHCl_3$-EtOAc (4:1, v/v) as mobile phases. The spots were detected by spraying with 10% H_2SO_4 followed by heating for saponins and sapogenins, and with aniline phthalate for sugars.

* Correspondence to: Dr D. Sobolewska, Department of Pharmacognosy, Collegium Medicum, Jagiellonian University, 9 Medyczna Str., 30–688 Kraków, Poland. E–mail: dsobolew@cm–uj.krakow.pl

GLC: Chromatograph N-504; glass column (0.3 x 220 cm) filled with Chromosorb W-AW-DMCS (2% HI-EFF-3BP).

Plant material

The underground parts of *Allium ursinum* were collected in September in Czaslaw, Poland. A voucher specimen was deposited in the Herbarium of the Department of Pharmacognosy, Collegium Medicum, Jagiellonian University.

Extraction and isolation

Air-dried powdered underground parts (578 g) of *Allium ursinum* were extracted with hot methanol (3 x 1 L MeOH; 3h), and concentrated to dryness. The dry residue (32 g) was dissolved in water and partitioned successively with n-hexane and n-BuOH. The butanol-soluble portion was subjected to chromatographic separation on a silica gel column. The column was eluted with $CHCl_3$-MeOH-H_2O (23:12:2) to yield compounds **1** (21 mg) and **2** (10 mg).

Compound 1. Colorless needles, m.p. 212-216°C, $[\alpha]_D^{24}$ = -92.5° (c 0.1; MeOH), IR (KBr) ν cm-1: 3420, 1049 (OH); 880, 900, 921 (C-22-spiro), ^{13}C-NMR (pyridine – d_5) δ (ppm): see Table 1. ^{1}H NMR (pyridine – d_5) δ (ppm): 6.38 (1H, br *s*, Rha $_1$), 5.81 (1H, br *s*, Rha $_2$), 6.26 (1H, br *s*, Rha $_3$), 5.32 (1H, H-6), 4.92 (1H, Glc), 4.53 (1H, H-16), 1.76 (3H, d, *J* = 6.1 Hz), 1.59 (3H x 2, d, *J* = 5.7 Hz), 1.04 (3H, s, Me-19), 0.82 (3H, s, Me-18).
Positive ion FAB-MS (**1a**) *m/z* 1014.5 $[M+H]^+$, 868.1 = $[M + H – 147]^+$, 721.9 = $[M + H – 147\text{-}146]^+$, 413.6 = $[M + H – 2 \times 147 – 146 – 161]^+$,
(**1b**) *m/z* 1013.0 $[M+H]^+$, 866.5 = $[M + H – 147]^+$, 720.5 = $[M + H – 147\text{-}146]^+$, 412.0 = $[M + H – 2 \times 147 – 146 – 161]^+$.

Acid hydrolysis of 1

Compound 1 (3 mg) was hydrolyzed with 5% methanolic HCl (5 mL) for 4 h and diluted with water (5 mL). Methanol was evaporated under reduced pressure and the water phase was partitioned with diethyl ether (3 x 5 mL). The ether layers containing the aglycones were concentrated to dryness. Prior to analysis the resulting dry residue was dissolved in chloroform.

Cytotoxic assay

The cytotoxic activity of 1 (in DMSO) was tested against murine cancer cell lines melanoma B16 and sarcoma XC and human fibroblasts HSF which were grown in MEM medium supplemented with 10% heat inactivated calf serum and a mixture of antibiotics (37°C, 5% CO_2). Cells were transferred into 24-well microtiter plates (density $1{,}5\times10^4$ cells/well) and preincubated for 24 h to allow stabilization. The compound was added to the wells at different concentrations (1 to 12 μg mL-1). The cells were incubated for 24 or 48 h. The viability of the cells was tested using trypan blue dye. Cytotoxic activity was measured as a percentage of dead cells.

Antimycotic evaluation

Evaluation of pure **1** was performed following the conventional disc assay procedure for activity against *Trichophyton mentagrophytes* and *Microsporum canis*. The tested strains were grown on Sabourand broth. Paper disc with the solution of **1** (2-400 μg/mL) was put in the middle of the plate with the tested strain (10^6 cells/mL). The incubation was carried out at 27°C for 5-7 days. Growth inhibition zone was observed after this time.

RESULTS AND DISCUSSION

The dry MeOH extract of bulbs and roots of *Allium ursinum* was partitioned successively between n-hexane, n-butanol and water. The n-BuOH-soluble portion was subjected to silica gel column chromatography to afford **1** and **2.**

Compound **1** was obtained as colorless needles; mp. 212-216°C, $[\alpha]_D^{24}$ = -92.5° (c 0.1; MeOH). It responded positively to the Liebermann-Burchard and negatively to the Ehrlich reagent. On acid hydrolysis with 5% HCl in MeOH, **1** afforded diosgenin which was identified by cochromatography (TLC) with an authentic sample. The sugar residues present in the acidic hydrolysate of **1** were identified as rhamnose and glucose in the ratio 3:1 based on TLC and GLC analysis. This was further confirmed by FAB-MS fragmentation ions. The IR spectrum of **1** showed a strong absorption at 3420 and 1049 cm-1 due to OH groups and an olefinic group at 1629.8 cm-1; moreover bands characteristic of a spirostane ring at 880, 900, 921 cm-1 were also seen.

Positive ion FAB-MS spectrum of **1** displayed, however, two quasi-molecular ion peaks at 1014.5 $[M+H]^+$ and 1013.0 $[M+H]^+$, thus indicating the presence of two compounds differing by two mass units, with molecular formulae $C_{51}H_{82}O_{20}$ and $C_{51}H_{80}O_{20}$, respectively. These compounds, which were denoted as **1a** and **1b**, could not be separated by column chromatography. Therefore, they were analyzed as a mixture by means of one- and two-dimensional NMR spectroscopy. The nature of the sugar residues and the sequence of the saccharide chains of **1a** and **1b** were determined using a combi-

nation of ^{1}H-NMR, ^{13}C-NMR, DEPT 135^{o}, ^{1}H-^{1}H COSY, HETCOR and HMBC. The ^{1}H-NMR spectrum displayed signals of four anomeric protons at δ 4.92, 5.81, 6.26 and 6.38 ppm and of three secondary methyl groups characteristic of 6-deoxypentose at 1.76, 1.59 and 1.59 ppm.

The sugar spin system was assigned on the basis of ^{1}H-^{1}H-COSY and HETCOR. The position of interglycosidic linkages were determined using the HMBC spectrum. The significant glycosylation shifts showed that two terminal rhamnoses were linked to the C-2 position of the glucopyranose and C-4 position of the inner rhamnopyranose. The α configuration of the rhamnopyranosyl units were established from signals ascribable to their C_5 data (70.43 ppm, 69.57 ppm and 68.35 ppm). This was further confirmed by the three-bond coupled strong HMBC correlations from the anomeric proton to the C-3 and C-5 carbons (8). The absence of ^{13}C glycosylation shift for two rhamnosyl residues suggested that they were the terminal units. The FAB-MS spectrum (positive ions) of **1** exhibited fragment ions at *m/z* 868.1 = $[M + H - 147]^{+}$, 721.9 = $[M + H - 147\text{-}146]^{+}$, 413.6 = $[M + H - 2 \times 147 - 146 - 161]^{+}$ and 866.5 = $[M + H - 147]^{+}$, 720.5 = $[M + H - 147\text{-}146]^{+}$, 412.0 = $[M + H - 2 \times 147 - 146 - 161]^{+}$ supporting the sugar sequence of **1a** and **1b**, respectively.

The spectroscopic results suggested that these compounds must be monodesmosidic saponins. The signal ascribable to C-3 shifted downfield by approximately +7 ppm and the signals due to C-2 and C-4 shifted to higher field by -1 ppm and -3 ppm, respectively, indicating that the sugar chain was bound to C-3 position of the aglycones.

Table 1. ^{13}C NMR chemical shift assignments of compounds **1a** and **1b.**

	Aglycones		***Sugar moiety***
^{13}C	**1a chemical shift (δ ppm)**	**1b chemical shift (δ ppm)**	**1a and 1b chemical shift (δ ppm)**
C–1	37.55	37.55	***Glc***
C–2	30.21	30.21	***C–1*** 100.41
C–3	78.04	78.04	***C–2*** **78.16**
C–4	39.03	39.03	***C–3*** 77.77
C–5	140.88	140.88	***C–4*** **77.90**
C–6	121.82	121.82	***C–5*** 77.04
C–7	32.35	32.35	***C–6*** 61.31
C–8	31.74	31.74	***Rha*** $_1$ (1→2) Glc (terminal)
C–9	50.36	50.36	***C–1*** 102.20
C–10	37.19	37.19	***C–2*** 72.52
C–11	21.15	21.15	***C–3*** 72.90
C–12	39.91	39.85	***C–4*** **74.05**
C–13	40.51	40.51	***C–5*** 69.57
C–14	56.69	56.69	***C–6*** 18.67
C–15	32.25	32.21	***Rha*** $_2$ (1→4) Glc (inner)
C–16	81.15	81.15	***C–1*** 102.28
C–17	62.96	62.96	***C–2*** 72.90
C–18	16.36	16.36	***C–3*** 73.29
C–19	19.44	19.44	***C–4*** **80.45**
C–20	42.02	41.91	***C–5*** 68.39
C–21	15.05	15.01	***C–6*** 18.45
C–22	109.30	109.48	***Rha*** $_3$ (1→4) Rha $_2$ (terminal)
C–23	31.88	29.0	***C–1*** 103.31
C–24	29.31	33.26	***C–2*** 72.66
C–25	30.64	144.48	***C–3*** 72.90
C–26	66.91	65.05	***C–4*** 74.18
C–27	17.35	108.73	***C–5*** 70.42

Moreover, the 1D- and 2D-NMR spectra indicated that compounds **1a** and **1b** had identical sugar moieties, but different aglycones (see Table 1). These two sapogenins were overlapped to produce a single spot on the TLC plate, which was identified as diosgenin. The ^{1}H-NMR spectrum showed signals characteristic of four steroidal methyl groups; two appeared as singlets at δ 0.82 ppm (3H, *s*, Me-18) and 1.04 ppm (3H, *s*, Me-19), and the other two as doublets at δ 1.09 ppm (*d*; *J*=6.8) and 0.69 ppm (*d*; *J*=5.2). The quaternary carbon atom C-5 (140.88 ppm) and the methine carbon atom C-6 (121.82 ppm) were easily assigned based on ^{13}C-NMR and DEPT 135^{o} spectra. The ^{13}C chemical shifts of **1b** were almost identical to those of **1a** except for the chemical shifts of F-ring and C-20. The ketal carbon signal at δ 109.3 ppm (**1a**) and 109.5 ppm (**1b**) were assigned to the spiroketal carbon atom (C-22) with two oxygens attached. A feature differing **1b** from **1a** was the substitution of the C-25 – methyl group by the exocyclic methylene group. Two carbonyl resonances at δ 144.5 ppm and d 108.7 ppm were assigned to carbon atoms C-25 and C-27 of **1b**, respectively (9). Four signals at δ 41.9, 29.0, 33.3 and 65.0 ppm were assigned to the C-20, C-23, C-24 and C-26 positions, respectively. The ^{13}C-NMR and ^{1}H-NMR spectra implied that the 27-Me orientation of **1a** was equatorial. The obtained data confirmed that the aglycone of **1a** was diosgenin – (25R)-spirost-5-en-3b-ol, while the sapogenin of **1b** was (25R)- spirost-5, 25(27)-dien-3b-ol.

On the basis of the foregoing data the structures of the two compounds are proposed to be: (25R)-spirost-5-en-3b-ol 3-O-α-rhamnopyranosyl-(1→4)-α-rhamnopyranosyl-(1→4)- [a-rhamnopyranosyl-(1→2)]-glucopyranoside (**1a)** and (25R)-spirost-5,25(27)-dien-3β-ol 3-O-α-rhamnopyranosyl-(1→4)-α-rham-

Table 2. ^{13}C NMR chemical shift assignments of sugar moiety of compound **2**.

Sugar moiety ^{13}**C (δ ppm)**
Glc
C–1 100.40
C–2 ***78.14***
C–3 77.76
C–4 ***77.97***
C–5 77.05
C–6 61.24
Rha $_{1}$ (1→2) Glc (terminal)
C–1 102.14
C–2 72.54
C–3 72.88
C–4 74.04
C–5 69.57
C–6 18.66
Rha $_{2}$ (1→4) Glc (inner)
C–1 102.25
C–2 72.93
C–3 73.30
C–4 ***80.43***
C–5 68.35
C–6 18.45
Rha $_{3}$ (1→4) Rha $_{2}$ (terminal)
C–1 103.30
C–2 72.68
C–3 72.93
C–4 74.16
C–5 70.43
C–6 18.91

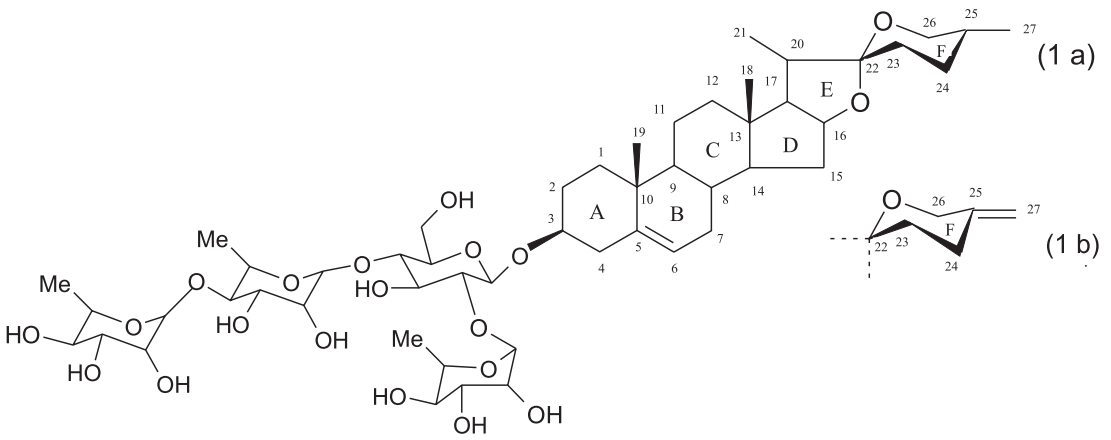

Figure 1. The structures of **1a** and **1b.**

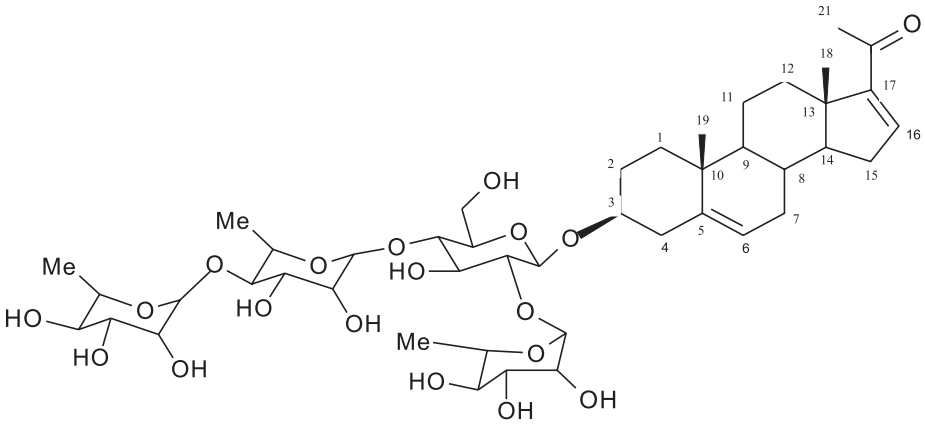

Figure 2. The structure of **2.**

nopyranosyl-(1→4)- [α-rhamnopyranosyl-(1→2)]-glucopyranoside (**1b**). A survey of the literature showed that the compound identical with **1a** was identified in *Disocorea cayenensis* (10) and *Paris polyphylla* var. *yunnanensis* (11).

The NMR data of **2** were in full agreement with the signals of the pregnadienolone tetraglycoside previously isolated by us from *Allium ursinum* (7). The ^{13}C-NMR spectrum due to the sugar chain of **2** was almost superimposable on that of **1** suggesting that the tetrasaccharide sequence of **2** was the same as that of **1**. The structure of sugar residue now was revised (see Table 2). On the basis of spectroscopic evidence the structure of compound **2** was determined as 3-O-α-rhamnopyranosyl-(1→4)-α- rhamnopyranosyl-(1→4) -[α-rhamnopyranosyl-(1→2)]-glucopyranoside 3-hydroxy-pregna-5, 16-dien-20-one.

Cytotoxic activity of **1** (a mixture of **1a** and **1b**) on melanoma B16, sarcoma XC and human fibroblasts HSF was evaluated. Compound **1** was found active against murine melanoma B16 and sarcoma XC. The compound exhibited 100% effect at 2 μg mL^{-1} on both strains. It showed no activity towards human fibroblasts HSF at concentrations below 3 μg mL^{-1}. With regard to antifungal properties against *Trichophyton mentagrophytes* and *Microsporum canis* the saponin was active at concentration of 400 μg mL^{-1}. Our previous studies have shown the activity of compound **1** against *Candida glabrata* and *Candida parapsilosis* (MIC values 200 mg mL^{-1} and 250 mg mL^{-1}, respectively) [12]. Compound **1** did not show any toxicity against *Pseudomonas aeruginosa* and *Aspergillus niger* at concentrations up to 400 mg mL^{-1}, in the disk assay study. This low fungistatic activity is most probably due to a relatively long sugar chain. Dioscin, the analogue of **1a** which possesses shorter sugar chain (-1 Rha) showed stronger activity (between 12.5 and 25 mg mL^{-1}) against *Candida* [10].

REFERENCES

1. Treben M.: Apteka Pana Boga. Porady i praktyka stosowania ziół leczniczych. Natur-Produkt TOM-MARK, Warszawa 1992.
2 Sendl A., Elbl G., Steinke B., Redl K., Breu W., Wagner H.: Planta Med. 58, (1992).
3 Sendl A., Schliack M., Loeser R., Stanislaus F., Wagner H.: Atherosclerosis 94, 79 (1992).
4 Wagner H., Sendl A.: Dtsch. Apoth. Ztg 33, 1809 (1990).
5 Carotenuto A., De Feo V., Fattorusso E., Lanzotti V., Magno S., Cicala C.: Phytochem. 41, 531 (1996).
6 Kaku H., Goldstein I.J., Van-Damme E.J., Peumans W.J.: Carbohydr. Res. 229, 347 (1992).
7 Janeczko Z., Sobolewska D., Podolak I.: Acta Pol. Pharm. Drug Res. 57, 131 (2000).
8 Yokosuka A., Mimaki Y., Sashida Y.: J. Nat. Prod. 65, 1293 (2002).
9 Agrawal P.K., Jain D.C., Gupta R.K., Thakur R.S.: Phytochem. 24, 2479 (1985).
10 Sautour M., Mitaine-Offer A-C., Miyamoto T., Dongmo A., Lacaille-Dubois M-A. Planta Med. 70, 90 (2004).
11 Matsuda H., Pongpiriyadacha Y., Morikawa T., Kishi A., Kataoka S., Yoshikawa M.: Bioorg. Med. Chem. Lett. 13, 1101 (2003).
12 Sobolewska D., Janeczko Z., Galanty A., Trojanowska D.: 3rd International Symposium on Natural Drugs. Proceedings. Naples, 2-4 October 2003.

Received: 31.03.2006