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A TRITERPENE SAPONIN FROM *LYSIMACHIA THYRSIFLORA* L.

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Abstract: A triterpene saponoside (LTS-4) isolated from the underground parts of *Lysimachia thyrsiflora* L. was defined as 3-O-{β-D-xylopyranosyl-(1→2)-β-D-glucopyranosyl-(1→4)-[β-D-glucopyranosyl-(1→2)]-α-L-arabinopyranosyl}-cyclamiretin A. Structure assignment was performed on the basis of spectroscopic data including homo- and heteronuclear 1D and 2D NMR (COSY, TOCSY, NOESY, HETCOR, HMBC and DEPT) and FAB-MS studies. The compound was tested *in vitro* for antimicrobial and cytotoxic activity.

Keywords: *Lysimachia thyrsiflora*, *Primulaceae*, triterpene saponin, cytotoxicity.

The genus *Lysimachia* (family *Primulaceae*) comprises over a hundred species in the world. In Poland, this genus is represented by five species, including *Lysimachia thyrsiflora* L. – tufted loosestrife, however, its natural stands are scarce. Several plants of this genus have been used in traditional medicine of Europe and Asia to treat diarrhoea, fever, arthritis, and were reported to possess analgesic, antibacterial, anti-inflammatory, cytotoxic and molluscicidal properties (1). Previous chemical studies showed that triterpene saponins, benzoquinones and flavonoids were the main constituents found in this genus.

Reports on chemical composition of tufted loosestrife are limited. The presence of flavonoids and saponins was confirmed in *L. thyrsiflora* herb (2,3). Saponins were characterized as tri- and tetrasaccharides of priverogenin A. Flavonoids in whole plant were further studied by Yasukawa et al. (4) and were identified as 3-rhamnosides and 3-galactosides of isorhamnetin and quercetin.

In a search for cytotoxic compounds from Polish flora a methanol extract from the underground parts of *L. thyrsiflora* was found to show cytotoxic activity *in vitro* (95% dead cells at 80 μg/mL) (5). In this paper we report the isolation and structure elucidation of the predominant saponoside from the underground parts of this species. The compound was tested for antimicrobial and cytostatic activity *in vitro*.

EXPERIMENTAL

General procedures

Melting point was determined uncorrected using a Reichert apparatus. ^{1}H, ^{13}C and 2-D NMR spectra were recorded on a Bruker AMX 500 Spectrometer (^{1}H at 500.13 MHz, ^{13}C at 125.77 MHZ) with pyridine-d_5 as the solvent (multiplicities were determined by DEPT experiments). Chemical shifts were expressed in δ (ppm) downfield from internal TMS; coupling constants (*J*) were reported in Hz. FAB MS was obtained using a Finnigan MAT 95 mass spectrometer; glycerol as the matrix, Cs ions accelerated at 13 keV. Silica gel 60 (0.04-0.063 mm) was used for column chromatography (CC). Analytical TLC was carried out on Merck silica gel 60 aluminium plates and the spots were visualized by spraying the chromatograms with 5% H_2SO_4 in MeOH followed by heating for saponins and with aniline phthalate for sugars. Preparative TLC was carried out on Merck silica gel 60 G (0.25 mm thickness) on glass.

Plant material

The underground parts of authenticated specimens of *L. thyrsiflora* L. were collected in

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September 2004 from the Garden of Medicinal Plants at the Faculty of Pharmacy, Jagiellonian University, Kraków, Poland. A voucher specimen is deposited at the Dept. of Pharmacognosy MCJU. The plant material was air-dried and ground.

Extraction and isolation

Dried, ground plant material (100 g) was successively extracted with $CHCl_3$ and MeOH with an addition of 0.5% pyridine under reflux conditions. The MeOH extract was evaporated to dryness *in vacuo* and the resulting residue was suspended in water and eluted with n-BuOH. The process was repeated until the water phase showed no positive reaction for saponins (TLC). The n-BuOH extracts were combined and concentrated under reduced pressure. The resulting dark brown residue (5 g) was subjected to silica gel CC eluted with $CHCl_3$-MeOH-H_2O (23:12:2, v/v/v). Fractions were combined according to their TLC behavior. Fractions containing saponins were purified by preparative TLC ($CHCl_3$-MeOH-H_2O 8:7:1, v/v/v) to afford a compound denoted LTS-4 (80 mg), and minor amounts of compounds denoted LTS-7 and LTS-1.

Table 1. ^{1}H NMR and ^{13}C NMR spectral data δ (ppm) of LTS-4 (**1**) in pyridine-d_5.

Aglycone	^{1}H mult. (*J* Hz)	^{13}C	DEPT moiety	Sugar	^{1}H mult. (*J* Hz)	^{13}C	DEPT
				Arabinose (A)			
1	0.88, 1.64	39.23	CH_2				
2	1.82, 2.01	26.58	CH_2	A1	4.80 *d* (5.8)	104.72	CH
3	3.17 *dd* (11.0, 4.2)	89.16	CH	A2	4.53	79.73	CH
4	-	39.76	C	A3	4.27	73.30	CH
5	0.69 *d* (11.5)	55.74	CH	A4	4.25	78.67	CH
6	1.30, 1.43	17.95	CH_2	A5	3.70, 4.65	64.35	CH_2
7	1.22, 1.52	34.37	CH_2	Glucose (G) terminal			
8	-	42.56	C	G1	5.48 *d* (7.8)	104.83	CH
9	1.28	50.45	CH	G2	4.07	76.05	CH
10	-	44.04	C	G3	4.19	77.53	CH
11	1.46, 1.73	19.16	CH_2	G4	4.30	71.12	CH
12	1.58, 1.97	32.35	CH_2	G5	4.02	77.77	CH
13	-	86.50	C	G6	4.40, 4.57	63.06	CH_2
14	-	44.62	C	Glucose (G`) inner			
15	1.50, 2.21 *dd* (14.5, 5.0)	36.78	CH_2	G`1	4.99 *d* (7.8)	104.21	CH
16	4.22	76.92	CH	G`2	3.90	85.27	CH
17	-	45.76	C	G`3	3.80	77.99	CH
18	1.40	53.34	CH	G`4	4.19	71.90	CH
19	2.12, 2.85 *t* (14.0, 13.0)	33.40	CH_2	G`5	4.29	78.28	CH
20	-	48.32	C	G`6	4.29, 4.45	62.35	CH_2
21	2.09, 2.55	30.50	CH_2	Xylose (X)			
22	1.46, 2.12	32.69	CH_2	X1	4.93 *d* (7.1)	107.57	CH
23	1.23 *s*	28.10	CH_3	X2	4.02	76.20	CH
24	1.08 *s*	16.64	CH_3	X3	4.26	78.20	CH
25	0.81 *s*	16.38	CH_3	X4	4.13	70.73	CH
26	1.29 *s*	18.54	CH_3	X5	3.74,4.56	67.45	CH_2
27	1.55 *s*	19.80	CH_3				
28	3.19 *q*, 3.56 *d* (7.6)	77.67	CH_2				
29	1.02 *s*	24.13	CH_3				
30	9.63 *s*	207.69	CH				

Assignments based on COSY, TOCSY, HETCOR and HMBC experiments.

Table 2. Selected data from NOESY experiments of LTS-4 (**1**) in pyridine-d_5.

proton-proton connectivities between:	
3.17 (H-3 aglycone)	0.69 (H-5 aglycone)
3.17 (H-3 aglycone)	1.23 (H-23 aglycone)
4.22 (H-16 aglycone)	1.29 (H-26 aglycone)
4.22 (H-16 aglycone)	3.19 (H-28a aglycone)
3.17 (H-3 aglycone)	4.80 (H-1 arabinose)
4.25 (H-4 arabinose)	4.99 (H-1 inner glucose)
4.53 (H-2 arabinose)	5.48 (H-1 terminal glucose)
3.90 (H-2 inner glucose)	4.93 (H-1 xylose)

LTS-4 (**1**). Powder, m.p. 262-267°C, (lit. (6) 262-263°C); positive ion mode FAB-MS m/z: 1083 $[M + Na]^+$, negative ion mode FAB-MS m/z: 1059 $[M - H]^-$, 927 $[M - H -$ pentose$]^-$, 897 $[M - H -$ hexose$]^-$, 765 $[M - H -$ (pentose + hexose)$]^-$; ^1H and ^{13}C NMR data are given in Table 1.

Hydrolysis: total acid hydrolysis was performed on a TLC plate with gaseous HCl for 25 min according to procedure described in (7).

Antifungal and antibacterial screening

Compound **1** was tested by the conventional paper disc diffusion method against *Candida albicans, C. glabrata, C. parapsilosis, Aspergillus niger, Staphylococcus aureus, Pseudomonas aeruginosa*, *Trichophyton mentagrophytes* and *Microsporum canis*. Strain suspensions in 0.85% NaCl (100 µL) from 24 h incubation were applied to Petri dishes with Sabourand agar medium. Concentrations up to 400 µg/mL were tested and growth inhibition zones were observed after 24 h incubation at 37°C. Control experiments without tested compound were carried out as well.

Cytotoxicity screening

Cytotoxic activity was tested on murine cancer cell line sarcoma XC. Cells were grown in Modified Eagle's Medium (MEM) with antibiotics, supplemented with 10% calf serum, transferred into 24-well microtiter plates (density 1.5×10^4/well) and preincubated for 24 h (37°C, 5% CO_2). Test solutions prepared in MeOH were diluted with MEM to obtain appropriate concentrations (2 – 20 µg/mL) which were added to the wells. After 48 h of incubation the viability of cells was determined using trypan blue dye in comparison to the controls to which MeOH or MEM were added. Colchicin (Fluka) was used as a cytotoxic reference compound. Cytotoxicity was measured as a percentage of dead cells by microscopic examination.

RESULTS AND DISCUSSION

The underground parts of *L. thyrsiflora* were extracted successively with $CHCl_3$ and MeOH with an addition of 0.5% pyridine to avoid cleavage of an epoxy bridge often present in primulaceous saponins (8). The MeOH extract was partitioned between n-BuOH and water and the n-BuOH soluble fraction was subjected to repeated column and preparative chromatography, as described in experimental part, to afford compound **1** denoted as LTS-4. Acid hydrolysis of **1** on a TLC plate confirmed the presence of glucose, arabinose and xylose. Results of

Table 3. Selected long-range couplings (δ, ppm) observed in HMBC experiments of LTS-4 (**1**) in pyridine-d_5.

proton	carbon
3.17 (H-3 aglycone)	28.10 (C-23 aglycone), 16.64 (C-24 aglycone) 104.72 (C-1 arabinose)
0.69 (H-5 aglycone)	16.38 (C-25 aglycone)
1.50 (H-15 aglycone)	19.80 (C-27 aglycone), 76.92 (C-16 aglycone)
2.85 (H-19 aglycone)	24.13 (C-29 aglycone)207.69 (C-30 aglycone)
1.23 (H-23 aglycone)	89.16 (C-3 aglycone), 16.64 (C-24 aglycone)
1.08 (H-24 aglycone)	89.16 (C-3 aglycone), 28.10 (C-23 aglycone)
0.81 (H-25 aglycone)	55.74 (C-5 aglycone), 18.54 (C-26 aglycone)
1.29 (H-26 aglycone)	50.45 (C-9 aglycone)
4.80 (H-1 arabinose)	89.16 (C-3 aglycone)
4.99 (H-1 inner glucose)	78.67 (C-4 arabinose)
5.48 (H-1 terminal glucose)	79.73 (C-2 arabinose)
4.93 (H-1 xylose)	85.27 (C-2 inner glucose)

negative ion mode FAB MS, ^{13}C NMR and DEPT NMR analysis indicated a molecular formula $C_{52}H_{84}O_{22}$.

^{1}H NMR showed signals of six tertiary methyl groups at δ 0.81, 1.02, 1.08, 1.23, 1.29 and 1.55 ppm, the corresponding methyl carbons were assigned upon HETCOR, at δ 16.38, 24.13, 16.64, 28.10, 18.54 and 19.80 ppm. Moreover, in the lower field region of the ^{1}H NMR spectrum a proton signal due to the formyl group at δ 9.63 ppm and signals of four sugar anomeric protons at δ 5.48 (*d*, 7.8), 4.99 (*d*, 7.8), 4.93 (*d*, 7.1) and 4.80 (*d*, 5.8) ppm were seen.

Among the 30 carbons of the triterpene aglycone in the ^{13}C NMR spectrum, as was disclosed by the DEPT experiment, six were methyls, eleven were methylenes, six were methines and seven were quarternary carbons including one oxygen-bearing methylene (δ 77.67 ppm), three oxygen-bearing methines (δ 76.92, 89.16, 207.69 ppm) and one oxygen-bearing quaternary carbon (δ 86.50 ppm). The structural assignment was initiated from the long-range coupling networks observed between methyl protons and the adjacent carbons from the HMBC experiment. This analysis showed that the aglycone of **1** had an oleanane skeleton with an epoxy bridge between C-13 (δ 86.50 ppm) and C-28 (δ 77.67 ppm), a formyl group at C-30 (δ 207.69 ppm) and a hydroxyl at C-16 (δ 76.92 ppm). The α configuration of the hydroxyl group at C-16 was evident from the chemical shift in comparison to literature data (16αOH: ca. δ 77 ppm; 16βOH: ca. δ 74 ppm) (6, 9, 10) and from NOESY results, as spatial proximities were observed between H-16 and H-28 (see Table 2) (11). The orientation of the hydroxyl at C-3 could be deduced from the spatial proximities observed between H-3 (δ 3.17 ppm) and H-23 (δ 1.23 ppm) and H-3 and H-5 (δ 0.69 ppm) (see Table 2) and from the *J* value of H-3 (*dd*, *J* = 11 and 4.2 Hz) (11, 12). The above data obtained for the aglycone part of LTS-4 corresponded well with cyclamiretin A (3β,16α-dihydroxy-13β,28-epoxy-30-oleanal), what was further confirmed by comparison of NMR data with the literature (6, 13).

The attachment of the sugar chain was indicated by the low field shift of C-3 (δ 89.16). FAB MS fragmentation pattern suggested that the chain was branched: $[M - H]^-$ ion at m/z 1059 and fragments corresponding to an independent loss of a pentose unit $[(M - H) - 132]^-$ at m/z 927, and a hexose unit $[(M - H) - 162]^-$ at m/z 897 and to a loss of a pentose-hexose unit $[(M - H) - (132 + 162)]^-$ at m/z 765.

The nature of the monosaccharides and the sequence of a tetrasaccharide chain was determined by a combination of TOCSY, COSY, NOESY, DEPT, HMBC and HETCOR. Starting from the anomeric protons of each sugar the hydrogens within each spin system were identified using COSY and TOCSY, the corresponding ^{13}C resonances were assigned by HETCOR and further confirmed by HMBC (Table 1). The pyranose forms of all monosaccharides were determined from their ^{13}C data as compared to published methyl glycosides (14, 15). The β anomeric configuration of two glucoses and of xylose was evident from their coupling constants (7-8 Hz) (Table 1). According to the literature the *J* value of arabinopyranosides is not diagnostic on its own due to rapid conformational mobility ($^{4}C_1$ and $^{1}C_4$) and orientation should be established based on the NOE effects (9, 12). Thus, the coupling constant (5.8 Hz) observed for arabinopyranosyl unit, together with NOE connectivities between H-1, H-3 and H-5 confirmed an α orientation.

Comparison of ^{13}C shifts of individual sugar units with model compounds suggested that xylose and one of the glucoses were terminal units whereas arabinose was the branched centre. The interglycosidic linkages were established from HMBC and NOESY experiments. In HMBC a cross peak was seen between the signals at 4.80 ppm (H-1 of arabinose) and 89.16 ppm (C-3 of the aglycone) which confirmed the attachment position of the sugar chain. Other key cross peaks were observed between: H-1 of inner glucose and C-4 arabinose; H-1 of terminal glucose and C-2 arabinose; H-1 of xylose and C-2 inner glucose (Table 3). The same conclusion was drawn from NOESY experiment (Table 2).

From the above evidence the structure of LTS-4 (**1**) was elucidated as:
3-O-{β-D-xylopyranosyl-(1→2)-β-D-glucopyranosyl-(1→4)-[β-D-glucopyranosyl-(1→2)]-α-L-arabinopyranosyl}-cyclamiretin A.

Survey of the available literature revealed that compounds of such structure were previously isolated from *Ardisia crispa* and *A. crenata* (ardisiacrispin A) and from *Cyclamen sp.* (desglucocyclamin I) (6, 9, 16, 17). Ardisiacrispin A was shown to have utero-contracting activity (16). Data on antimicrobial properties of saponins prompted us to test the activity of LTS-4 against some bacterial and fungal strains, however, it was found inactive at concentrations up to 400 μg mL^{-1}. The results of preliminary cytotoxicity screening of crude extracts from *L. thyrsiflora* indicated that methanol extract from the underground parts of the plant was most active against target cells (sarcoma XC) with 95% dead cells at 80 μg mL^{-1} and the bioactivity-guided frac-

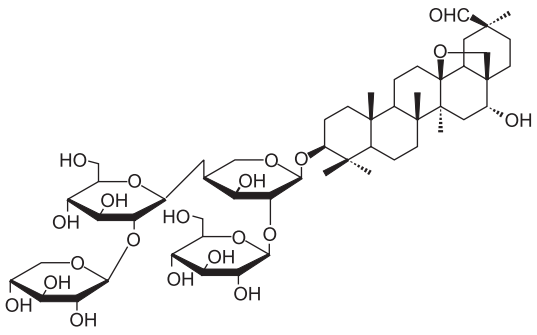

Figure 1. The structure of LTS-4 (**1**).

tionation indicated that saponins may be responsible for this effect (5). We decided to test the cytotoxicity of LTS-4 (**1**), which was the predominant saponin constituent, along with two minor saponins also isolated from the methanolic extract (LTS-1 and LTS-7). ED_{50} values for LTS-4, LTS-7 and LTS-1 were 4.5, 6.0 and 7.0 μg mL^{-1}, respectively. Colchicin, which was used as a reference substance had ED_{50} 2.5 μg mL^{-1}. These results confirmed the assumption that saponin constituents were responsible for the overall cytotoxic action of the methanolic extract. It is worth noting that compound LTS-1, which is more polar, has much lower cytotoxic activity in comparison with the two less polar saponins. Compound LTS-4, described in this paper, had the highest cytotoxic activity, therefore, it was also tested against murine and human melanoma cell lines and human skin fibroblasts. Its effects on the cell viability, proliferation and morphology were studied and these results are discussed in more detail in a separate report (submitted).

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