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Isolation and Structural Determination of Triterpenoid Glycosides from the Aerial Parts of Alsike Clover (*Trifolium hybridum* L.)

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Supporting Information

ABSTRACT: Five azukisapogenol glycosides (**1**−**5**) have been isolated from the aerial parts of alsike clover (*Trifolium hybridum* L.), and their structures were elucidated by combined spectroscopic, spectrometric (1D and 2D NMR; HRESIMS, ESI−MS/MS), and chemical methods. Three of them are new compounds and were identified as 3-*O*-[-α-L-arabinopyranosyl(1→2)]-β-D-glucuronopyranosyl azukisapogenol (**1**), 3-*O*-[-β-D-glucuronopyranosyl(1→2)-β-D-glucuronopyranosyl]-29-*O*-β-D-glucopyranosyl azukisapogenol (**2**), and 3-*O*-[-α-L-arabinopyranosyl(1→2)-β-D-glucuronopyranosyl]-29-*O*-β-D-glucopyranosyl azukisapogenol (**3**). The remaining two (**4**, **5**) are known compounds but have not been previously described as saponins constituents of the genus *Trifolium*. Also, azukisapogenol is reported here as a triterpenoid aglycone for the first time in this genus. Finally, the main chemotaxonomic features that may be recognized as specific of *Trifolium* species were discussed.

KEYWORDS: *alsike clover, Trifolium hybridum L., triterpenoid saponins, azukisapogenol, structural elucidation, HRESIMS, ESI−MS/MS, NMR*

INTRODUCTION

Alsike clover (*Trifolium hybridum* L., Fabaceae) is a perennial plant, which has been widely cultivated as forage, cover crops, and for erosion control. It is distributed worldwide, especially in Europe, North and South America, Northwest and South of Africa, and Western Asia.[1]

Apart from its potential interest as forage crop, it has been also of interest for its content of saponins. This group of secondary metabolites is well-known to possess a broad spectrum of biological properties, such as molluscicidal, anti-inflammatory, antifungal, hemolytic, cytotoxic, and antitumor.[2] Saponins have also been used as food and feed additives, providing benefits to human and animal health, and performance. The beneficial effects of saponins, such as the defaunation of microorganisms in ruminants, have been shown.[3] In addition, several human feeding studies suggest that the consumption of soybean and other legumes, which contain soyasaponins, has hypocholesterolemic activity and may be beneficial in diabetes control.[4]

As indicated by several studies on the species of the genus *Trifolium*, such as *T. incarnatum*,[5] *T. repens*,[6,7] *T. alexandrinum*,[8] and *T. resupinatum*,[9] the saponins in these plants are the mixture of triterpenic pentacyclic glycosides being most often derivatives of soyasapogenol A, B, C, and E. These have as a common feature the hydroxymethyl group at C-24 and a double bond between C-12 and C-13. The sugars portion consists of one or three units, where a β-D-glucuronic acid moiety is linked at the C-3 position of the aglycone. The bidesmosidic saponins that have been also identified usually had a diglucoside linkage at C-22.

The qualitative studies of saponins in the seeds of *T. hybridum* and 56 other species of *Trifolium*[10] confirmed the presence of glycosides of soyasapogenol B in all tested species, as well as 22-*O*-glucosylated or 22-*O*-diglucosylated derivatives of soyasaponin I in some of them. Phytochemical analysis of the aerial parts of a number of species of *Trifolium* proved the presence of phenolic acids, clovamides, and flavonoids in *T. hybridum*.[11] However, the occurrence of saponins in the aerial parts of this plant has not been investigated so far.

Therefore, the aim of the current investigation was to isolate and elucidate the chemical structures of the saponins in aerial parts of *T. hybridum*, and additionally to contribute to the chemotaxonomic knowledge in the genus *Trifolium*.

MATERIALS AND METHODS

Plant Material. Seeds of authenticated material of *T. hybridum* were provided by Genebank, Zentralinstitute für Pflanzen-genetik and Kulturpflanzenforschung (Gatersleben, Germany). Seeds were planted (1 m × 1 m plots) in an experimental field of the Institute of Soil Science and Plant Cultivation in Puławy, Poland. The plants were harvested at the beginning of flowering, lyophilized, finely powdered, and used for the successive extraction.

Extraction and Purification. The dried and finely powdered *T. hybridum* aerial parts (leaves, stems, and flowers, 300 g) were defatted with $CHCl_3$ in a Soxhlet apparatus (fats made up 3.8% of dried material). Defatted material (288 g) was then extracted with 70%

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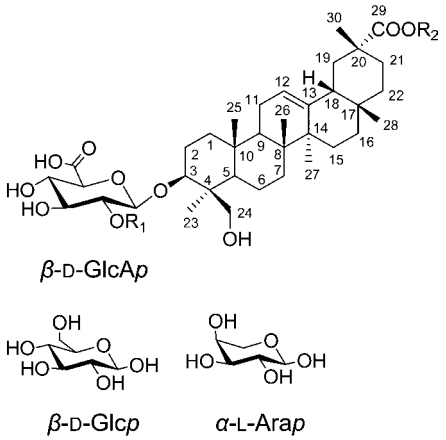
MeOH with reflux for 2 h. The solvent was removed under reduced pressure, and the residue was resuspended in water. The solution was applied onto a 10 cm × 6 cm, 40−63 μm LiChroprep RP-18 glass column (Merck, Darmstadt, Germany), previously conditioned with water. The column was washed first with water and then with 40% MeOH to remove sugars and phenolics. Total saponins were eluted with 80% MeOH and dried under vacuum; 11.7 g (4.1%, of defatted material) of crude saponins was obtained.

Fractionation and Separation. Crude saponins powder (2 g) was suspended in 30% MeOH and loaded onto a 40 cm × 3 cm, 40−63 μm LiChroprep RP-18 column (Millipore Corp., Bedford, MA). The column was washed with a 30−100% step gradient (steps of 10%) of MeOH in water. Ten milliliter fractions were collected and checked by TLC on Si 60 F254 silica gel (Merck, Warsaw, Poland), developed with ethyl acetate/acetic acid/water (7:2:2). Chromatograms were sprayed with Liebermann−Burchard reagent and heated at 130 °C. Fractions showing similar profiles were combined. Column chromatography gave 22 fractions, from F6 to F22, each comprising two to four major saponins.

Single saponins were separated from each fraction by Si 60 silica gel (Merck) chromatography on a 20 cm × 1 cm column, 15−25 μm, LiChroprep eluted with ethyl acetate/acetic acid/water (9:2:2).[12] In this way, five (**1**−**5**) pure compounds were isolated (Figure 1).



Compound	R_1	R_2
1	α-L-Arap	H
2	β-D-GlcAp	β-D-Glcp
3	α-L-Arap	β-D-Glcp
4	H	H
5	H	β-D-Glcp

Figure 1. Structures of saponins **1**−**5** isolated from *T. hybridum* aerial parts.

Mass Spectra. Exact masses of the new saponins were measured by an UPLC-QTOF ESI (Waters Synapt G2, Manchester, UK) high resolution mass spectrometer (HRMS). The samples were dissolved with methanol and analyzed. Mass spectra were recorded in positive ion mode with the range of m/z 100−2000, mass resolution of 20 000, and acceleration voltage of 0.7 kV.

The fragmentation patterns of the saponins were obtained using a Thermo LCQ Advantage Max ion-trap mass spectrometer. The samples were dissolved in MeOH/H_2O (8:2) at the concentration of 100 ppm and injected by direct infusion at a flow rate of 5 μL/min with a syringe pump. The spectrometer was operated in the negative electrospray mode with the following parameters: spray voltage 3.9 kV, capillary voltage −47 V, tube lens offset −60 V, capillary temperature 240 °C. Nitrogen sheath and auxiliary gas flows were of 65 and 10 arbitrary units, respectively. Full scan spectra were acquired over the range of m/z 150−2000. Automated MS/MS was performed by isolating the molecular ion using an isolation width of m/z 1.0, fragmentation amplitude of 1.0 V, threshold set at 100, and ion charge control on, with maximum acquiring time set at 250 ms.

NMR Measurements. All experiments were performed on a Varian INOVA-600 spectrometer equipped with 5 mm ^{1}H {^{15}N−^{31}P} PFG high-field inverse detection z-gradient probe. ^{1}H (599.78 MHz) and ^{13}C (150.83 MHz) NMR spectra were recorded in pyridine-d_5 or pyridine-d_5/D_2O (9:1) at 25 °C. Chemical shifts are given on the δ scale and were referenced to residual pyridine, δ_H 8.70, 7.55, 7.18 and δ_C 149.84, 135.50, 123.48. Varian pulse sequences using gradient were applied, and all 2D spectra, except for HMBC, were recorded in the phase-sensitive mode. The 1D TOCSY and 1D ROESY spectra were recorded using a PFG selective excitation. For 1D TOCSY, the MLEV17, mixing time was set with an acquisition array of 15, 30, 55, 70, 100, 150, 200 ms and for 1D ROESY, a 200 ms spin lock mixing time. On the basis of 1D and 2D NMR analysis, assignments of ^{1}H and ^{13}C signals were obtained.

Acid Hydrolysis of Saponins. Compounds **1**−**5** (each 2 mg) were treated with 1 N HCl (1 mL) at 80 °C for 3 h. After cooling, the solvent was eliminated with a stream of N_2, dry residue was suspended in water, and aglycones were extracted with ethyl acetate (3 × 2 mL). The aqueous layer, containing sugars, was neutralized with Amberlite IRA-400 (OH^- form). For each samples, both the organic and the aqueous solution were dried under N_2 and stored for the subsequent analyses.

Identification of Aglycones. All saponins yielded the same aglycone, which was identified as azukisapogenol by comparison of its physical data ($[\alpha]_D$, melting point, ^{1}H NMR, ^{13}C NMR, and MS) with reported values.[13]

Determination of Absolute Configurations of Sugars. To determine the absolute configuration of monosaccharide constituents of isolated compounds **1**−**5**, the method of Tanaka et al.[14] was used with slight modifications. Sugars of each sample were dissolved in pyridine (0.5 mL) containing L-cysteine methyl ester hydrochloride (2 mg) and heated at 60 °C for 1 h; *o*-tolyl isothiocyanate (2 μL) was then added, and the mixture was heated at 60 °C for 1 h. Each reaction mixture was directly analyzed by reversed-phase HPLC using a model 616 pump, a 996 photodiode array detector, and a 717-plus autosampler (Waters, Milford, MA). The column used was a 250 mm × 4 mm i.d., 5 μm, Eurospher 100 C18 (Knauer, Berlin, Germany); mobile phase MeCN−H_2O (25:75, v/v) containing 50 mM H_3PO_4; detection UV (250 nm); flow rate 0.8 mL/min; column temperature 35 °C. The HPLC column was washed with MeOH after each injection.

The derivatives of monosaccharides L-arabinose, D-glucuronic acid in **1**, D-glucuronic acid, D-glucose in **2**, L-arabinose, D-glucose, D-glucuronic acid in **3**, D-glucuronic acid in **4**, and D-glucose, D-glucuronic acid in **5** were identified by comparison of their retention times with those of authentic samples (Sigma-Aldrich, Steinheim, Germany) treated in the same way as described above (R_t: D-glucose 14.57 min, L-glucose 13.33 min, D-arabinose 17.55 min, L-arabinose 16.63 min, D-glucuronic acid 14.48 min, L-glucuronic acid 13.95 min).

Melting points were determined using a Büchi (Uster, Switzerland) apparatus. Optical rotations were measured on a Perkin-Elmer 241 polarimeter.

Chemical Data of Saponins 1−5. Saponin **1**: $[\alpha]^{20}_D$ +11.0 (MeOH, *c* 0.1). HRESIMS, m/z 803.4201 $[M + Na]^+$ calcd for $C_{41}H_{64}O_{14}Na$, 803.4194. ESI−MS (negative ion mode), m/z (relative intensity) 779.4 (100%) $[M - H]^-$, which fragmented in the MS/MS giving 629 (100%) $[(M - H) - 150(Ara + H_2O)]^-$, 471 (29%) $[(M - H) - 150(Ara + H_2O) - 158(GlcA-H_2O)]^-$. ^{1}H and ^{13}C NMR spectra are reported in Tables 1 and 2.

Saponin **2**: $[\alpha]^{20}_D$ −6 (MeOH/H_2O, 1:1, *c* 0.3). HRESIMS, m/z 1009.4611 $[M + Na]^+$ calcd for $C_{48}H_{74}O_{21}Na$, 1009.4620. ESI−MS (negative ion mode), m/z (relative intensity) 985.5 (100%) $[M - H]^-$, which fragmented in the MS/MS giving 823.3 (100%) $[(M - H) - 162(Glc)]^-$, 647.4 (2%) $[(M - H) - 162(Glc) - 176(GlcA)]^-$, 471.5 (2%) $[(M - H) - 162(Glc) - 176(GlcA) - 176(GlcA)]^-$, 351.1 (54%) $[(GlcA(1\rightarrow2)GlcA - H_2O) - H]^-$. ^{1}H and ^{13}C NMR spectra are reported in Tables 1 and 2.

Table 1. NMR Spectroscopic Data (600 MHz) for the Aglycones of Compounds 1−3[a]

	1[b]		2[b]		3[c]	
carbon	δ_C, type	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)
1	38.6, CH_2	1.30 m	38.8	1.36 m	37.5	1.25 m
		0.74 m		0.82 m		0.68 m
2	26.7, CH_2	2.23 m	26.6	2.23 m	25.3	2.33 m
		1.91 dddd (13.5, 12.9, 12.1, 3.8)		1.95 m		1.76 m
3	90.4, CH	3.36 dd (12.1, 4.5)	90.0	3.47 dd (11.5, 4.5)	89.3	3.33 dd (11.9, 4.1)
4	44.0, C		44.0		42.6	
5	56.0, CH	0.76 m	56.2	0.78 dd (6.9, 2.2)	55.0	0.62 m
6	18.6, CH_2	1.53 m	19.0	1.61 m	17.3	1.35 m
		1.28 m		1.41 m		1.00 m
7	33.0, CH_2	1.35 m	33.1	1.33 m	31.8	1.17 m
		1.20 m		1.17 m		1.00 m
8	40.0, C		39.9		38.7	
9	47.6, CH	1.49 m	47.6	1.42 m	46.4	1.24 m
10	36.5, C		36.5		35.2	
11	24.0, CH_2	1.75 m (2H)	23.9	1.71 m (2H)	22.8	1.54 m (2H)
12	122.9, CH	5.22 t (3.7)	123.0	5.12 t (3.8)	121.9	4.98 t (3.8)
13	144.5, C		144.2		143.1	
14	41.8, C		41.7		40.6	
15	26.4, CH_2	1.71 m	26.3	1.64 m	25.1	1.48 m
		0.92 m		0.84 m		0.69 m
16	27.2, CH_2	2.13 m	27.0	1.94 ddd (13.6, 13.3, 4.5)	25.8	1.78 m
		0.84 brd (12.6)		0.72 m		0.60 m
17	32.7, C		32.6		31.4	
18	46.4, CH	2.11 m	46.2	2.01 dd (13.5, 4.3)	45.0	1.87 dd (13.6, 2.9)
19	41.5, CH_2	2.54 dd (13.7, 13.7)	40.6	2.43 dd (13.8, 13.5)	39.3	2.26 dd (13.6, 13.8)
		1.68 m		1.61 m		1.44 m
20	42.8, C		43.1		42.0	
21	29.9, CH_2	2.25 m	29.5	1.59 m	28.2	1.89 m
		1.70 m		1.22 m		1.45 m
22	36.5, CH_2	1.53 m	36.0	1.43 m	34.8	1.27 m
		1.36 m		1.24 m		1.11 m
23	22.5, CH_3	1.26 s	22.8	1.41 s	21.4	1.15 s
24	63.3, CH_2	4.29 d (11.5)	63.3	4.35 d (11.6)	62.1	4.10 d (11.7)
		3.36 d (11.5)		3.63 d (11.6)		3.21 d (11.7)
25	15.6, CH_3	0.71 s	15.5	0.76 s	14.4	0.51 s
26	16.8, CH_3	0.88 s	16.7	0.84 s	15.6	0.66 s
27	26.0, CH_3	1.22 s	25.9	1.08 s	24.8	0.93 s
28	28.3, CH_3	0.91 s	28.2	0.84 s	27.0	0.70 s
29	181.2, C		177.7		177.5	
30	19.9, CH_3	1.46 s	19.5	1.36 s	18.3	1.24 s

[a]The assignments were based on $^1H-^1H$ COSY, 2D-TOCSY, HSQC, HSQC-TOCSY, and HMBC experiments. [b]Acquired in pyridine-d_5. [c]Acquired in pyridine-d_5/D_2O (9:1).

Saponin **3**: $[\alpha]^{20}_D$ +26 (MeOH/H_2O, 1:1, *c* 0.1). HRESIMS, *m/z* 965.4709 $[M + Na]^+$ calcd for $C_{47}H_{74}O_{19}Na$, 965.4722. ESI−MS (negative ion mode), *m/z* (relative intensity) 941.4 (100%) $[M - H]^-$, which fragmented in the MS/MS giving 779.4 (100%) $[(M - H) - 162(Glc)]^-$, 629.3 (3%) $[(M - H) - 162(Glc) - 150(Ara + H_2O)]^-$, 471.3 (1%) $[(M - H) - 162(Glc) - 150(Ara + H_2O) - 158(GlcA - H_2O)]^-$. ^1H and ^{13}C NMR spectra are reported in Tables 1 and 2.

Saponin **4**: ESI−MS (negative ion mode), *m/z* (relative intensity) 647.3 (100%) $[M(C_{36}H_{56}O_{10}) - H]^-$, which fragmented in the MS/MS giving 471.4 (100%) $[(M - H) - 176(GlcA)]^-$.

Saponin **5**: ESI−MS (negative ion mode), *m/z* (relative intensity) 809.3 (100%) $[M(C_{42}H_{66}O_{15}) - H]^-$, which fragmented in the MS/MS giving 647.4 (100%) $[(M - H) - 162(Glc)]^-$, 471 (3%) $[(M - H) - 162(Glc) - 176(GlcA)]^-$.

RESULTS AND DISCUSSION

Structure Elucidation. Crude saponins were fractionated by reverse-phase (RP-18) column chromatography in a gradient of water/methanol to yield a number of fractions, which then were further purified on a silica gel column using acidified solvent to suppress ion formation on the column. As a result, the saponins **1**−**5** were obtained in pure forms (Figure 1), out of which **1**−**3** are reported for the first time in this study. To elucidate the chemical structures of the new compounds, combined NMR, HRESIMS, and ESI−MS/MS techniques were employed. The assignments of all ^{13}C and ^1H signals were performed on the basis of 2D NMR experiments (HSQC, HMBC, DQF-COSY, TOCSY, ROESY, HSQC-TOCSY) and selective excitation methods (1D-ROESY and 1D-TOCSY acquired "in array") and are reported in Tables 1 and 2. The exact molecular weights were obtained by

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Table 2. NMR Spectroscopic Data (600 MHz) for the Sugar Units of Compounds 1−3[a]

	1[b]		2[b]		3[c]	
carbon	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)
	β-D-GlcA		β-D-GlcA		β-D-GlcA	
1′	105.2	4.91 d (7.7)	104.7	5.02 d (7.2)	103.2	4.68 d (8.0)
2′	78.6	4.40 dd (7.7, 9.0)	81.1	4.37 dd (7.2, 9.0)	79.0	4.03 dd (8.0, 8.0)
3′	78.0	4.34 dd (9.0, 9.0)	78.0	4.39 dd (9.0, 8.2)	76.7	4.17 dd (8.0, 9.1)
4′	73.5	4.54 dd (9.0, 9.6)	73.2	4.54 dd (8.2, 9.7)	71.9	4.14 dd (9.1, 9.7)
5′	77.6	4.58 d (9.6)	77.6	4.61 d (9.7)	76.7	4.18 d (9.7)
6′	172.7		172.3		171.9	
	α-L-Ara		β-D-GlcA		α-L-Ara	
1″	104.8	5.48 d (7.5)	105.1	5.74 d (7.2)	103.5	5.10 d (7.7)
2″	73.6	4.43 dd (7.5, 9.4)	75.7	4.27 dd (7.2, 9.2)	71.7	4.21 dd (7.7, 9.5)
3″	74.9	4.04 dd (9.4, 3.4)	77.8	4.28 dd (9.2, 4.9)	73.2	3.87 dd (9.5, 3.5)
4″	70.3	4.13 m	73.2	4.55 m	68.7	4.03 m
5″	67.5	4.23 d (12.3)	77.7	4.55 m	66.1	4.12 d (12.3)
		3.66 d (12.3)				3.50 d (12.3)
6″			170.0			
			β-D-Glc		β-D-Glc	
1‴			96.0	6.37 d (8.1)	94.5	6.06 d (8.2)
2‴			74.3	4.19 dd (8.1, 9.0)	72.6	4.00 dd (8.2, 8.7)
3‴			78.7	4.27 dd (9.0, 8.9)	76.7	4.13 dd (8.7, 9.2)
4‴			71.0	4.34 dd (8.9, 9.7)	69.5	4.03 dd (9.2, 9.5)
5‴			79.5	4.04 ddd (9.7, 4.5, 2.6)	77.7	3.87 m
6‴			62.1	4.45 d (12.0, 2.6)	60.6	4.23 d (12.3)
				4.37 dd (12.0, 4.5)		4.08 dd (12.3, 5.1)

[a]The assignments were based on ^{1}H−^{1}H COSY, 1D-TOCSY, 1D-ROESY, HSQC, HSQC-TOCSY, and HMBC experiments. [b]Acquired in pyridine-d_5. [c]Acquired in pyridine-d_5/D_2O (9:1).

HRESIMS and the fragmentation ions by means of ESI−MS/MS, which were also used to establish sugar chain in the molecules. The absolute configurations of the sugar residues of each compound were determined after hydrolysis using the method of Tanaka et al.[14] with slight modifications, and the aglycones moieties were confirmed by comparison of their physical data with those already reported in the literature.

Compound **1** (6 mg) was isolated as an amorphous white solid. The HRESI mass spectrum supported a molecular formula of $C_{41}H_{64}O_{14}$. The ESI−MS showed a molecular ion peak at m/z 779 $[M - H]^-$, and the MS/MS spectrum of this ion showed the fragment ion peaks at m/z 629 $[(M - H) - 150]^-$, corresponding to the loss of a pentose unit plus water, and m/z 471 $[(M - H) - 150 - 158]^-$, due to further loss of a hexosyluronic acid unit less water. The triterpenoid glycosidic structure for this compound was also confirmed by NMR spectra (Tables 1 and 2). The ^{1}H NMR spectrum (in pyridine-d_5) showed for the aglycone moiety (Table 1) signals of six tertiary methyl groups at δ 0.71, 0.88, 0.91, 1.22, 1.26, and 1.46, which correlate in the HSQC experiment with the carbon signals at δ 15.6, 16.8, 28.3, 26.0, 22.5, and 19.9, respectively. An additional feature was the signal at δ 5.22 (t, J = 3.7 Hz) typical of H-12 of the Δ^{12}-oleanene skeleton, which was confirmed by the presence in the ^{13}C NMR spectrum of the olefinic signals at δ144.5 and 122.9 attributable to C-13 and C-12. The occurrence of a carboxylic group was suggested for the signal at δ 181.2 in the ^{13}C NMR spectrum, which was assigned as C-29 due to the HMBC correlation throughout three bonds with the methyl group at δ 1.46 (C-30). Its equatorial orientation was also shown by 2D ROESY spectrum, in which a correlation between the signal at δ 2.11 (H-18) and the C-30 methyl group was clearly observed (Figure 2). A signal

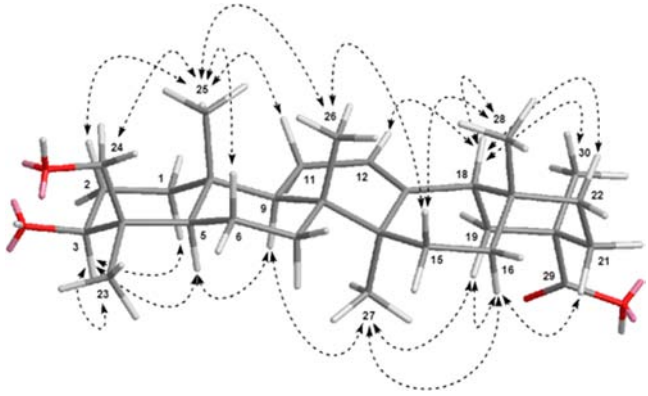

Figure 2. Main correlations observed in 2D ROESY spectrum for aglycone moiety of **1**, azukisapogenol.

present in ^{1}H NMR at δ 3.36 and its correlation in HSQC experiment with the carbon resonance at δ 90.4 suggested the presence of a secondary alcoholic group. The location of this hydroxyl group was determined to be at 3β (equatorial), by means of analysis of the value of coupling constants of H-3 (dd, J = 12.1, 4.5 Hz), which indicate both ^3J (axial−axial) and ^3J (axial−equatorial) coupling with H-2$_{ax}$ (δ 1.91) and H-2$_{eq}$ (δ 2.23), respectively. At the same time, the typical 24-hydroxymethyl group of the aglycone was deduced by the ^{1}H NMR signals at δ 3.36 (d, J = 11.5 Hz) and δ 4.29 (d, J = 11.5 Hz), which showed a cross peak in HSQC experiment with the carbon resonance signal at δ 63.3, as well as in HMBC spectrum with carbon signals at δ 22.5 (C-23, methyl group) and δ 90.4 (C-3), being consistent with the data reported in the literature.[6,8,15] The NOEs observed in 2D ROESY spectrum (Figure 2) were used to establish the three-dimensional disposition of the rest of substituents in the aglycone, as well as the relative configurations of chiral centers. Therefore, the structure of aglycone of **1** was elucidated as (20*R*,4*S*)-3β,24-dihydroxy-12-en-olean-29-oic acid, named azukisapogenol, and previously identified as a constituent of saponins in *Vigna angulariz*,[13] *Oxytropis glabra*,[16] and *Oxytropis myriophylla*.[17] However, it is reported here for the first time in the genus *Trifolium*. This result was confirmed after hydrolysis of **1**, which yielded an aglycone identified as azukisapogenol by $[\alpha]_D$, melting point, ^{1}H NMR, ^{13}C NMR, and MS data as compared to those reported in the literature.[13,16,17]

In addition, ^{1}H and ^{13}C NMR spectra of **1** showed the presence of two anomeric protons at δ 4.91, 5.48 and carbons at δ 105.2, 104.8 (Table 2). The individual sugar units were identified by a combination of DQF-COSY, 1D TOCSY, and 1D ROESY experiments. 1D TOCSY and 1D ROESY spectra

were acquired from selective excitation of each anomeric proton and were in particular very helpful. Selective TOCSY experiment for signal at δ 4.91 revealed a typical spin system of β-glucuronic acid (Figure 3A); while for the signal at δ 5.48

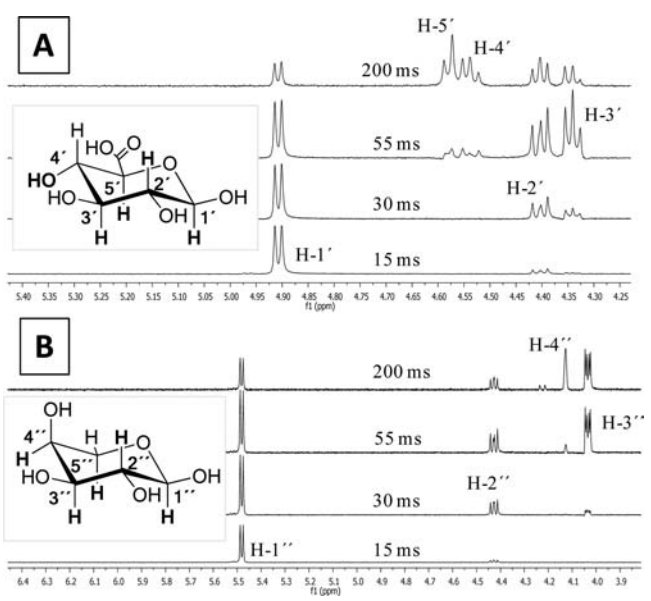

Figure 3. 1D TOCSY experiments acquired "in array". Shown subspectra corresponding to 15, 30, 55, and 200 ms, obtained by irradiation of anomeric protons of **1** at (A) δ 4.91 (β-glucuronic acid) and (B) δ 5.48 (α-arabinose).

(Figure 3B), it allowed one to observe correlations for H-1″/H-2″ and H-2″/H-3″, and a relatively small coupling constant of H-3″/H-4″. This evidence together with the 1D ROESY correlations of H-1″/H-3″ and H-1″/H-5″ indicated the presence of α-arabinose as a sugar unit. Analysis of the HSQC and HSQC-TOCSY experiments allowed the assignment of the signals in ^{13}C NMR spectrum corresponding to both sugar units. The sequence of sugars chain and connection with the aglycone then were determined by the employment of the HMBC experiment. Thus, the anomeric signal at δ 4.91 (H-1_{GlcA}) showed a long-range correlation with the signal at δ 90.4 (C-3), indicating that the glucuronic acid is directly linked to the triterpenic aglycone at C-3, which was confirmed by the observed correlation in 1D ROESY between δ 4.91 (H-1_{GlcA}) and δ 3.36 (H-3). The chain Ara(1→2)GlcA was also shown by HMBC experiment, where a clear cross peak between the signal at δ 5.48 (H-1_{Ara}) and δ 78.6 (C-2_{GlcA}) was observed, and confirmed by 1D ROESY correlation between δ 5.48 (H-1_{Ara}) and δ 4.40 (H-2_{GlcA}). To verify the nature of the sugar units and to determine their absolute configurations, the sugars obtained after acid hydrolysis of **1** were converted into the thiazolizine derivatives to arylthiocarbamate using L-cysteine methyl ester and *o*-tolylisothiocyanate. The reaction mixture then was analyzed by C18 HPLC, and the retention times (R_t) were compared to values obtained for derivatives of each D- or L-authentic sugars. The peaks at 14.45 and 16.59 min coincided with derivatives of D-glucuronic acid and L-arabinose (R_t of D-glucuronic acid, 14.48; R_t of L-arabinose, 16.63), respectively. Finally, the structure of this saponin was elucidated as 3-*O*-[α-L-arabinopyranosyl(1→2)]-β-D-glucuronopyranosyl azukisapogenol, which represents a new identified natural compound.

Compound **2** (16 mg) was isolated as an amorphous white solid; the molecular formula was determined as $C_{48}H_{74}O_{21}$. Its ESI−MS showed a molecular ion peak at *m/z* 985 $[M - H]^-$. Other fragmentation ion peaks were observed at *m/z* 823 $[(M - H) - 162]^-$, 647 $[(M - H) - 162 - 176]^-$, and 471 $[(M - H) - 162 - 176 - 176]^-$, corresponding to the successive loss of one hexose residue and two hexosyluronic acid moieties, respectively. ESI−MS/MS also showed a peak at *m/z* 351, which corresponded to two hexosyluronic acid units obtained by the breaking of glycosidic bond with the aglycone and losing of a molecule of water. Assignments of all NMR signals of the aglycone portion of **2** were performed from extensive 2D NMR analysis. The ^{1}H and ^{13}C NMR chemical shifts (in pyridine-d_5) of the aglycone moiety of **2** (Table 1) were similar to those of **1**, indicating also the presence of azukisapogenol. Only slight differences were detected between them, mainly in the carbon resonance signal of carboxylic group at C-29 (δ 177.7), which was found to be 3.5 ppm shifted to higher field than that of **1**, due to a glycosidic esterification. The ^{1}H NMR spectrum of **2** showed signals for three anomeric protons at δ 5.02, 5.74, and 6.37, which had correlations in the HSQC spectrum with the anomeric carbon signals at δ 104.7, 105.1, and 96.0, respectively. The nature of sugar units was determined using the same procedure as described above for **1**. In this way, the signals at δ 5.02 and δ 5.74 were identified as β-glucuronic acid and δ 6.37 as β-glucose. The connections of sugars to the aglycone were suggested by HMBC experiment, where long-range correlations between the anomeric signal at δ 5.02 (H-$1_{GlcA'}$) and signal at δ 90.0 (C-3), as well as between signal at δ 6.37 (H-1_{Glc}) and signal at δ 177.7 (C-29), were observed, and confirmed the presence of a bidesmosidic saponin. The chain GlcA″(1→2)GlcA′ was also shown by a cross peak between the signal at δ 5.74 (H-$1_{GlcA''}$) and δ 81.1 (C-$2_{GlcA'}$) observed in HMBC experiment, and confirmed by a ROESY correlation between δ 5.74 (H-$1_{GlcA''}$) and δ 4.37 (H-$2_{GlcA'}$). The acid hydrolysis of **2** and subsequent HPLC analysis of sugar units confirmed the presence of D-glucose and D-glucuronic acid; the peaks at 14.69 and 14.45 min were coincident with the derivative of D-glucose and D-glucuronic acid (R_t of D-glucose, 14.57; R_t of D-glucuronic acid, 14.48), respectively. This compound was elucidated as 3-*O*-[-β-D-glucuronopyranosyl-(1→2)-β-D-glucuronopyranosyl]-29-*O*-β-D-glucopyranosyl azukisapogenol and represents a new bidesmosidic natural saponin.

Compound **3** (9 mg) was isolated as an amorphous white solid; the molecular formula was determined as $C_{47}H_{74}O_{19}$. Its ESI−MS displayed a molecular ion peak at *m/z* 941 $[M - H]^-$. The MS/MS of this ion showed a pattern similar to that in **1**, with the fragmentation ion peaks at *m/z* 779 $[(M - H) - 162]^-$, 629 $[(M - H) - 162 - 150]^-$, and 471 $[(M - H) - 162 - 150 - 158]^-$, corresponding to the successive loss of an hexose, a pentose plus water, and an hexosyluronic acid less water, respectively. The ^{1}H NMR spectrum of **3** (in pyridine-d_5/D_2O, 9:1) showed for the aglycone moiety (Table 1) signals of six tertiary methyl groups at δ 0.51, 0.66, 0.70, 0.93, 1.15, and 1.24, which correlate in the HSQC experiment with the carbon signals at δ 14.4, 15.6, 27.0, 24.8, 21.4, and 18.3, respectively. The Δ^{12}-oleanane skeleton was suggested due to the presence of an olefinic signal in ^{1}H NMR at δ 4.98 (t, J = 3.8 Hz), as well as the ^{13}C NMR signals at δ 143.1 and 121.9, assigned as C-13 and C-12, respectively. After a comprehensive analysis of 2D NMR experiments, the presence of a carboxylic group at C-29, a hydroxyl group at C-3, and 24-hydroxymethyl group was also

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established. Therefore, it suggested that the aglycone of **3** was the same as that in **1** and **2**.

The ^{1}H and ^{13}C NMR spectra of **3** showed also the presence of three anomeric protons at δ 4.68, 5.10, and 6.06, and carbons at δ 103.2, 103.5, and 94.5 (Table 2), which were identified in the same way as in **1** and **2** as β-glucuronic acid, α-arabinose, and β-glucose, respectively. Their absolute configurations were determined as D-glucuronic acid, L-arabinose, and D-glucose. By means of HMBC experiment, their connectivity was established. The anomeric signal at δ 4.68 (H-1_{GlcA}) showed a long-range correlation with the signal at δ 89.3 (C-3), while a cross peak between the signal at δ 6.06 (H-1_{Glc}) and the signal at δ 177.5 (C-29) indicated, as in **2**, that it is a bidesmosidic saponin. The Ara(1→2)GlcA linkage was also established by HMBC experiment, where a clear cross peak between the signal at δ 5.10 (H-1_{Ara}) and δ 79.0 (C-2_{GlcA}) was observed, and confirmed by a ROESY correlation between δ 5.10 (H-1_{Ara}) and δ 4.03 (H-2_{GlcA}). Consequently, this compound was elucidated as 3-*O*-[-α-L-arabinopyranosyl(1→2)-β-D-glucuronopyranosyl]-29-*O*-β-D-glucopyranosyl azukisapogenol and represents a new bidesmosidic natural saponin.

For compound **4**, 7 mg, MW 648, its ESI−MS/MS indicated the presence of only one hexosyluronic acid unit, which was identified after hydrolysis as β-D-glucuronic acid. It had spectroscopic characteristics identical to those of 3-*O*-β-D-glucuronopyranosyl azukisapogenol (myrioside B), which was previously reported from *O. myriophylla*.[17]

For compound **5**, 3 mg, MW 810, its ESI−MS/MS showed the loss of hexose unit (β-D-glucose) and hexosyluronic acid unit (β-D-glucuronic acid). Spectroscopic characteristics of this saponin were consistent with those of 3-*O*-[-β-D-glucuronopyranosyl]-29-*O*-β-D-glucopyranosyl azukisapogenol (myrioside C), which was previously described in *O. myriophylla*.[17]

In the present work, three new triterpenoid saponins 3-*O*-[-α-L-arabinopyranosyl(1→2)]-β-D-glucuronopyranosyl azukisapogenol, 3-*O*-[-β-D-glucuronopyranosyl(1→2)-β-D-glucuronopyranosyl]-29-*O*-β-D-glucopyranosyl azukisapogenol, and 3-*O*-[-α-L-arabinopyranosyl(1→2)-β-D-glucuronopyranosyl]-29-*O*-β-D-glucopyranosyl azukisapogenol, from the aerial parts of *T. hybridum*, have been isolated and their structures elucidated. Additionally, two known triterpenoid saponins, myrioside B and C, were also isolated. An interesting feature of those saponins is the identification of azukisapogenol as a new aglycone for saponins of *Trifolium* species. The most common aglycones isolated so far within this genus have been soyasapogenol A, B, and E, and their glycosylated derivatives.[5−9] However, azukisapogenol was isolated for the first time from *Vigna angulariz*[13] and subsequently found in *Oxytropis glabra*[16] and *Oxytropis myriophylla*.[17] On the other hand, in this investigation, soyasapogenols were not confirmed in the aerial parts (leaves, steams, and flowers) of *T. hybridum*, while as previously reported they were constituents of saponins occurring in the seeds.[10] This difference of contents of specific triterpenoid saponins in different parts of the plant may suggest a specially differentiated biosynthetic pathway, perhaps as a result of different oxidation steps of β-amyrin and/or different biological functions. A comparable conclusion was proposed by Huhman et al.[18] during the studies of aerial and subterranean parts of *Medicago truncatula*, species also belonging to the Fabaceae family.

Concerning the sugar portion, all of the isolated compounds of the β-D-glucuronic acid as monosaccharide unit were directly linked at the C-3 of the aglycone moiety, similarly to the saponins previously reported from other *Trifolium* species.[5−9] So it could be recognized as a chemotaxonomic feature in the *Trifolium* genus. However, this evidence is not conclusive, because an exhaustive phytochemical research in all organs of the plant has not been carried out in the present work. In addition, α-L-arabinose and β-D-glucuronic acid were eventually found linked 1→2 to β-D-glucuronic acid. Bidesmosidic saponins are characterized by the presence of a β-D-glucose linked at C-29, as a glycosidic esterification.

On the basis of our results in the investigation of *T. hybridum*, we are studying the saponins composition in other species of this genus for a better understanding of their metabolism and biological roles in these plants.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

NMR spectra (^{1}H and ^{13}C NMR, HSQC, HMBC, ROESY, and DQF-COSY) for the new compounds (**1**−**3**), including TOCSY spectra for compounds **2** and **3**. ESI−MS/MS spectra of compounds **1**−**3**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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