Saponins in Alfalfa (*Medicago sativa* L.) Root and Their Structural Elucidation

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Twenty-four saponins have been identified in alfalfa roots, including 13 medicagenic acids, 2 zanhic acids, 4 hederagenins, 1 soyasapogenol A, 2 soyasapogenol B's, 1 soyasapogenol E, and 1 bayogenin glycoside. Ten of the identified compounds, including 3-*O*-[*β*-D-glucopyranosyl(1→3)-*β*-D-glucopyranosyl]-28-*O*-*β*-D-glucopyranoside medicagenate, 3-*O*-[α-L-rhamnopyranosyl(1→2)-*β*-D-glucopyranosyl-(1→2)-*β*-D-glucopyranoside] medicagenic acid, 3-*O*-[*β*-D-glucopyranosyl(1→2)-*β*-D-glucopyranosyl-(1→2)-*β*-D-glucopyranosyl]-28-*β*- D-glucopyranoside medicagenate, 3-*O*-[*β*-D-glucuronopyranosyl methyl ester]-28-*O*-[*β*-D-xylopyranosyl(1→4)-α-L-rhamnopyranosyl(1→2)-α-L-arabinopyranoside] medicagenate, 3-*O*-[α-L-rhamnopyranosyl(1→2)-*β*-D-galactopyranosyl(1→2)-*β*-D-glucuronopyranosyl]-21-*O*-α-L-rhamnopyranoside soyasapogenol A, 3-*O*-[*β*-D-glucopyranosyl(1→2)-*β*-D-glucopyranosyl(1→2)-glucopyranosyl]-28-*O*-[*β*-D-xylopyranosyl(1→4)-α-L-rhamnopyranosyl(1→2)-α-L-arabinopyranoside] medicagenate, 3-*O*-[*β*-D-glucopyranosyl(1→2)-*β*-D-glucopyranosyl(1→2)glucopyranosyl]-28-*O*-{*β*-D-xylopyranosyl(1→4)-)-[*β*-D-apiofuranosyl-(1→3)]- α-L-rhamnopyranosyl(1→2)-α-L-arabinopyranoside} medicagenate, 3-*O*-[*β*-D-glucopyranosyl(1→2)-*β*-D-glucopyranosyl(1→2)-*β*-D-glucopyranosyl]-28-*O*-[*β*-D-xylopyranosyl(1→4)-α-L-rhamnopyranosyl(1→2)-α-L-arabinopyranoside] zanhic acid, 3-*O*-[*β*-D-glucopyranosyl(1→2)-*β*-D-glucopyranosyl(1→2)-*β*-D-glucopyranosyl]-28-*O*-{*β*-D-xylopyranosyl(1→4)-[*β*-D-apiofuranoside-(1→3)]- α-L-rhamnopyranosyl(1→2)-α-L-arabinopyranoside}zanhic acid, and 3-*O*-[*β*-D-galactopyranosyl(1→2)-*β*-D-glucuronopyranosyl]-28-*O*-*β*-D-glucopyranoside bayogenin, were not reported before, and their structures were established by spectral (FAB-MS and NMR) techniques. In addition, 3-*O*-[α-L-rhamnopyranosyl(1→2)-*β*-D-galactopyranosyl(1→2)-*β*-D-glucuronopyranoside] soyasapogenol E was identified in the roots for the first time.

Keywords: *Medicago sativa; alfalfa; saponins; triterpenoid glycosides*

INTRODUCTION

Saponins occurring in alfalfa roots and aerial parts are glycosides of triterpenes, which, due to their biological activities, are regarded as antinutrients (Oleszek, 1996). Triterpene aglycons can be substituted with several functional groups that result in a number of structurally different aglycons. In alfalfa these aglycons have received trivial names such as medicagenic acid, zanhic acid, hederagenin, soyasapogenols, bayogenin, and oleanolic acid. The aglycons can be further substituted with one (monodesmosides), two (bidesmosides), or three (tridesmosides) sugar chains, which cause the saponins from different alfalfa plant parts to be multicomponent mixtures of individual glycosides. A number of dominant glycosides have been recently isolated and characterized (Massiot et al., 1988, 1991; Oleszek et al., 1990, 1992; Timbekova et al., 1996). Due to their chemical structures, saponins may show different biological activities; thus, full characterization of their mixtures is essential for understanding their biological effects (Oleszek, 1996).

None of the alfalfa parts (aerial parts, roots, and seeds) have been fully characterized to date. Thus, the aim of our present work was the chemical characterization of all saponins present even in small amounts in alfalfa roots. This resulted in the identification of a number of new saponins.

MATERIALS AND METHODS

Extraction. Finely powdered alfalfa roots [var. Radius (8 kg)] were defatted in a Soxhlet apparatus with methylene chloride and then extracted under reflux with 80% MeOH. The alcohol was evaporated, and the resulting water solution was loaded onto a C_{18} column (20 × 4.5 cm, 40 μm, J. T. Baker) preconditioned with water. The column was washed first with water and 30% MeOH, and crude saponins (40 g) were eluted with MeOH.

Isolation of Glycosides. Crude saponins (5 g) were dissolved in n-BuOH saturated with water and applied to a silica gel column (80 × 4 cm, 40 μm, J. T. Baker). The column was washed with n-BuOH saturated with water to yield eight fractions: I, 0.36 g; II, 0.59 g; III, 0.4 g; IV, 0.31 g; V, 0.33 g; VI, 0.87 g; VII, 0.66 g; and VIII, 0.36 g. These fractions containing one to five components were further purified/separated on the C_{18} column (40 × 3 cm, 40 μm, J. T. Baker) eluted with an appropriate concentration of MeOH in water (35−85%).

Chromatography and Spectral Analysis. Saponins were chromatographed on Merck silica gel ready to use plates developed with ethyl acetate/acetic acid/water (7:2:2) or chloroform/methanol/water (63:32:5) and on C_{18} plates (Merck) developed with methanol/water (7:3). Aglycons were chromatographed on silica gel developed with petroleum ether/chloroform/acetic acid (7:2:1) or benzene/methanol (9:1). Saponins were visualized with methanol/acetic anhydride/sulfuric acid (50:

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Table 1. ^{13}C NMR Chemical Shift Data of Saponins 4, 7, 10, 14, 15, 17, and 21−24 (Sapogenins)

	δ									
C	**4**	**7**	**10**	**14**	**15**	**17**	**21**	**22**	**23**	**24**
1	44.7	44.6	44.6	39.7	44.5	44.6	44.7	44.5	44.8	44.7
2	71.0	70.8	70.9	27.0	70.9	71.2	71.1	70.9	70.8	70.8
3	86.9	86.6	86.6	92.3	86.0	83.1	86.6	86.3	84.6	84.9
4	53.8	53.2	53.1	44.7	53.1	43.1	53.2	53.0	53.8	53.2
5	52.8	53.1	53.1	57.2	52.9	49.0	53.2	53.0	53.3	53.2
6	21.5	21.4	21.3	19.4	21.4	18.7	21.4	21.2	21.2	21.3
7	33.0	33.1	33.0	33.8	33.1	33.5	33.8	33.1	33.2	33.3
8	40.9	40.8	40.9	41.2	40.9	40.8	41.1	40.8	41.0	40.9
9	49.0	49.0	49.0	48.0	49.0	48.0	49.0	49.0	49.0	49.0
10	37.2	37.4	37.4	37.3	37.2	37.5	37.4	37.2	37.2	37.3
11	24.5	24.6	24.6	24.9	24.5	24.7	24.6	24.5	24.8	24.6
12	123.6	123.5	123.6	123.7	123.6	123.9	123.6	123.6	124.0	123.9
13	144.8	145.2	144.8	145.0	144.7	144.9	144.7	144.7	144.7	144.7
14	43.0	43.0	43.0	42.9	42.9	43.1	42.7	42.8	42.7	43.0
15	28.6	28.7	28.7	27.0	28.8	28.7	36.4	28.8	36.4	28.8
16	23.8	24.0	23.9	28.1	23.5	24.0	74.8	23.5	74.8	23.7
17	47.9	47.6	47.9	40.0	47.6	48.0	50.2	48.0	50.2	48.2
18	42.5	42.7	42.5	44.7	42.4	42.7	42.0	42.4	42.0	42.5
19	47.1	47.2	47.2	47.8	47.0	47.2	47.6	46.9	47.5	47.1
20	31.4	31.6	31.5	37.4	31.5	31.5	31.3	31.4	31.2	31.6
21	34.7	34.6	34.8	85.5	34.7	34.9	36.4	34.7	36.3	34.8
22	33.4	33.8	33.4	79.7	33.5	33.5	33.4	33.5	33.2	33.5
23	182.0	182.5	182.5	23.4	182.2	65.2	181.2	180.9	181.2	181.2
24	14.4	13.8	13.7	64.3	13.5	14.7	13.8	13.6	14.7	14.6
25	17.1	17.1	17.1	16.2	17.0	17.5	17.2	16.9	17.2	17.2
26	17.6	17.6	17.5	17.3	17.6	17.9	17.7	17.5	17.7	17.8
27	26.3	26.4	26.3	27.0	26.2	26.4	27.3	26.2	27.3	26.4
28	178.0	181.8	178.2	22.0	177.5	178.1	177.0	177.6	176.9	177.6
29	33.3	33.5	33.4	31.8	33.5	33.5	33.4	33.3	33.2	33.4
30	23.8	24.0	23.9	21.6	23.9	24.0	25.2	23.8	25.0	24.0

5:5, v/v). Sugars were separated on Merck cellulose plates with benzene/butanol/pyridine/water (1:5:3:3) and made visible with a silver nitrate spray.

LSIMS mass spectra were recorded on a MAT 95 (Finnigan) spectrometer with glycerol as a matrix. ^{1}H and ^{13}C NMR spectra were measured on Bruker AMX-500 and DRX-600 spectrometers, and the UXNMR software package was used for NMR measurements in CD_3OD solutions. 2D experiments ^{1}H−^{1}H DQF-COSY (double quantum filtered direct chemical shift correlation spectroscopy) (Rance et al., 1983), 2D-HOHAHA (Davis and Bax, 1985; inverse detected ^{1}H−^{13}C HSQC (heteronuclear single quantum coherence) (Martin and Crouch, 1991), HMBC (heteronuclear multiple bond connectivity) (Summers et al., 1986), and ROESY (rotating-frame Overhauser enhancement spectroscopy) (Kessler et al., 1987) were obtained using UX-NMR software. The selection excitation spectra, 1D-TOCSY (Kessler et al., 1986), were acquired using waveform generator-based GAUSS shaped pulses, with mixing times ranging from 100 to 120 ms and an MLEV-17 spin-lock field of 10 kHz preceded by a 2.5 ms trim pulse.

RESULTS

Separation of crude saponin mixture on a silica gel column developed with *n*-butanol saturated with water yielded eight fractions containing one to six saponins. These were further separated/purified on a C_{18} column using methanol/water mixtures. In this way fraction I afforded one compound (**1**, 155 mg), MW 664, which was obtained in the form of needles and showed chromatographic and spectral characteristics identical to those of 3-*O*-*β*-D-glucopyranoside medicagenate (Morris, 1961; Oleszek et al., 1990).

Fraction II yielded needles from ethanol/water solution, the compound (**2**, 224 mg), MW 826, showing spectral and chromatographic characteristics identical to those of 3-*O*-*β*-D-glucopyranosyl-28-*O*-*β*-D-glucopyranoside medicagenate (Timbekova et al., 1996). The solution left after crystallization was further chromatographed on a silica gel column providing compounds **2** and **3** (4 mg), MW 766, which had the same characteristics as 3-*O*-[*β*-D-glucopyranosyl(1→2)-α-L-arabinoside hederagenin (Timbekova et al., 1996).

Fraction III consisted with six saponins, which, after separation on a C_{18} column, afforded compounds **4**−**9**.

Saponin **4** (21 mg): LSIMS (negative ion mode), *m*/*z* (rel intensity) 987 (31) [M − H]$^-$, 825 (11) [M − H − hexose]$^-$, 663 (14) [M − H − 2 hexoses]$^-$, 501 (7) [M − H − 3 hexoses]$^-$; ^{1}H NMR *δ* 5.29 (1H, t, *J* = 3.4 Hz, H-12), 4.38 (1H, ddd, *J* = 3.0, 4.0 and 4.0 Hz, H-2), 4.12 (1H, d, *J* = 4.0 Hz, H-3), 2.89 (1H, dd, *J* = 4.4 and 13.6 Hz, H-18), 1.43 (3H, s, Me-24) 1.30 (3H, s, Me-25), 1.18 (3H, s, Me-27), 0.96 (3H, s, Me-30), 0.94 (3H, s, Me-29), 0.83 (3H, s, Me-26); ^{13}C NMR data are presented in Tables 1 and 2. Acid hydrolysis revealed the presence of medicagenic acid and glucose.

Saponin **5** (4.5 mg), MW 942, showed spectral and chromatographic characteristics identical to those of 3-*O*-*β*-D-glucopyranosyl-28-*O*-[α-L-rhamnopyranosyl(1→2)-α-L-arabinopyranoside] medicagenate (Timbekova et al., 1996).

Saponin **6** (17 mg), MW 928, released on hydrolysis hederagenin, glucose, and rhamnose, and its spectral characteristics were identical with those of 3-*O*-[*β*-D-glucopyranosyl(1→2)-α-L-arabinopyranosyl]-28-*O*-*β*-D-glucopyranoside hederagenin (Timbekova et al., 1996).

Saponin **7** (5.5 mg): LSIMS (negative ion mode), *m*/*z* (rel intensity) 971 (100) [M − H]$^-$, 825 (7) [M − H − deoxyhexose]$^-$, 663 (7) [M − H − deoxyhexose − hexose]$^-$, 501 (19) [M − H − deoxyhexose − 2 hexoses]$^-$; ^{1}H NMR *δ* 5.29 (1H, t, *J* = 3.4 Hz, H-12), 4.38 (1H, ddd, *J* = 3.0, 4.0, and 4.0 Hz, H-2), 4.11 (1H, d, *J* = 4.0 Hz), 2.88 (1H, dd, *J* = 4.4 and 13.6 Hz, H-18), 1.42 (3H, s, Me-24) 1.32 (3H, s, Me-25), 1.19 (3H, s, Me-27), 0.96 (3H, s, Me-30), 0.94 (3H, s, Me-29), 0.83 (3H, s, M-26); for ^{13}C NMR see Tables 1 and 2.

Table 2. ^{13}C NMR Chemical Shifts Data of Saponins 4, 7, 10, 14, 15, 17, and 21−24 (Carbohydrates)

	δ									
C	**4**	**7**	**10**	**14**	**15**	**17**	**21**	**22**	**23**	**24**
C-1	106.3	104.4	103.7	105.4	105.1	103.7	103.8	103.6	102.0	102.1
C-2	74.6	79.0	82.4	77.4	74.5	82.4	82.4	82.2	83.5	83.2
C-3	87.1	78.8	78.0	78.4	78.0	77.0	78.1	78.0	77.7	78.0
C-4	69.6	71.3	70.0	73.9	72.9	73.1	70.1	70.0	70.2	70.2
C-5	77.6	77.6	77.8	76.9	76.3	76.1	78.0	77.9	77.9	77.9
C-6	62.1	62.3	62.2	173.5	171.3	173.0	62.2	62.0	62.2	62.1
CO_2CH_3					52.8					
C-1	105.1	102.2	103.7	102.1		105.6	103.8	103.5	103.9	103.8
C-2	75.4	80.0	84.8	78.3		73.8	84.8	84.6	84.7	84.9
C-3	77.2	78.5	77.5	76.7		74.9	77.3	77.3	77.6	77.5
C-4	71.1	71.3	70.4	71.5		70.1	71.0	70.4	71.0	70.7
C-5	72.9	77.5	77.2	76.4		78.0	77.3	77.1	77.6	77.5
C-6	62.3	62.8	62.2	62.2		62.2	62.4	62.1	62.0	62.0
C-1	95.6	102.3	105.9	102.2		95.9	105.9	105.8	106.2	106.0
C-2	73.8	72.1	76.3	72.2		74.0	76.4	76.2	76.4	75.8
C-3	78.5	72.1	77.5	72.2		78.7	77.8	77.6	77.2	77.2
C-4	71.1	74.0	70.9	73.9		71.2	71.1	70.8	71.0	70.3
C-5	78.1	69.8	78.6	69.5		78.4	78.9	78.8	79.0	79.0
C-6	62.5	17.9	62.3	17.9		62.6	62.2	62.3	62.1	62.2
C-1			95.6	104.6	93.6		94.0	93.7	93.9	93.7
C-2			73.9	72.2	75.6		75.7	75.6	75.8	76.1
C-3			78.9	72.2	70.7		71.0	70.8	71.1	70.1
C-4			70.9	74.1	66.7		67.0	66.8	66.9	66.2
C-5			78.2	70.3	63.3		63.7	63.5	62.8	62.8
C-6			62.3	17.8						
C-1					101.2		101.3	101.2	101.0	101.2
C-2					71.9		72.1	71.9	72.0	71.9
C-3					72.1		72.3	72.1	81.2	81.7
C-4					83.4		83.3	83.5	78.8	78.2
C-5					68.8		70.0	68.8	68.9	69.0
C-6					17.9		17.8	17.9	18.2	18.2
C-1					106.6		106.6	106.6	105.0	105.1
C-2					75.8		76.0	75.8	75.6	75.6
C-3					76.8		77.4	78.0	77.9	77.8
C-4					71.0		71.1	70.9	71.2	71.1
C-5					67.1		67.2	67.0	66.8	66.7
C-1									111.9	111.9
C-2									78.3	78.0
C-3									80.0	80.0
C-4									74.8	74.7
C-5									65.0	64.9
C-6										

Saponin **8** (17.5 mg), MW 898, after acid hydrolysis yielded hederagenin, glucose, and arabinose and had spectral characteristics identical to those of 3-*O*-[α-L-arabinopyranosyl(1→2)-*β*-D-glucopyranosyl(1→2)-α-L-arabinopyranoside] hederagenin (Timekova et al., 1996).

Compound **9** (13.5 mg), MW 956, afforded after hydrolysis soyasapogenol B and soyasapogenols C, D, and F as artifacts (Jurzysta, 1984). Rhamnose, galactose, and glucuronic acid were identified as sugar components. On the basis of these characteristics and spectral data, **9** was characterized as 3-*β*-*O*-[α-L-rhamnopyranosyl(1→2)-*β*-D-galactopyranosyl(1→2)-*β*-D-glucuronoside methyl ester] soyasapogenol B. This compound has been previously identified in white clover (Sakamoto et al., 1992).

Fraction IV was composed of five compounds that were successfully separated on a C_{18} column. This yielded compound **10** (7.5 mg): LSIMS (negative ion mode) *m/z* (rel intensity) 1150 (100) $[M - H]^-$, 987 (29) $[M - H -$ hexose$]^-$, 825 (17) $[M - H -$ 2 hexoses$]^-$, 663 (20) $[M - H -$ 3 hexoses$]^-$, 501 (13) $[M - H -$ 4 hexoses$]^-$; ^{1}H NMR δ 5.30 (1H, t, J = 3.4 Hz, H-12), 4.38 (1H, ddd, J = 3.0, 4.0, and 4.0 Hz, H-2), 4.12 (1H, d, J = 4.0 Hz, H-3), 2.89 (1H, dd, J = 4.4 and 13.6 Hz, H-18), 1.43 (3H, s, Me-24) 1.30 (3H, s, Me-25), 1.18 (3H, s, Me-27), 0.96 (3H, s, Me-30), 0.94 (3H, s, Me-29), 0.83 (3H, s, Me-26); for ^{13}C NMR signals see Tables 1 and 2. Acid hydrolysis yielded medicagenic acid and glucose.

Saponin **11** (10 mg), MW 1134, after hydrolysis afforded medicagenic acid, glucose, and rhamnose. Its spectral characteristics were identical to those of glycoside 3-*O*-[α-L-rhamnopyranosyl(1→2)-*β*-D-glucopyranosyl(1→2)-*β*-D-glucopyranosyl]-28-*O*-*β*-D-glucopyranoside medicagenate reported previously by Massiot et al. (1988).

Compound **12** (142 mg), MW 1074, yielded after hydrolysis medicagenic acid, glucose, xylose, rhamnose, and arabinose and was identified as 3-*O*-[*β*-D-glucopyranosyl]-28-*O*-[*β*-D-xylopyranosyl(1→4)-α-L-rhamnopyranosyl(1→2)-α-L-arabinopyranoside] medicagenate previously reported in alfalfa (Timbekova et al., 1996).

Glycoside **13** (76 mg), MW 1059, when hydrolyzed gave hederagenin, glucose, and arabinose and was on the basis of spectral characteristics identified as 3-*O*-[α-L-arabinopyranosyl(1→2)-*β*-D-glucopyranosyl(1→2)-α-L-arabinopyranosyl]-28-*O*-*β*-D-glucopyranoside medicagenate, which had been previously reported (Timbekova et al., 1996).

Saponin **16** (119 mg), MW 942, afforded on hydrolysis soyasapogenols B, C, D, and F, glucuronic acid, rhamnose, and galactose and had spectral characteristics identical to those of 3-*O*-[α-L-rhamnopyranosyl(1→2)-*β*-D-galactopyranosyl(1→2)-*β*-D-glucuronopyranoside] soyasapogenol B (trivial name soyasaponin I) (Kitagawa et al., 1988; Oleszek et al., 1988).

Fraction V consisted of six glycosides that were separated on a C_{18} column. This gave saponins **10**, **12**, **13**, and **16** identified in fraction IV, a mixture of saponins **13** and **14**, and glycoside **15**. Column chromatography of the mixture of **13** and **14** on silica gel eluted with chloroform/methanol/water (63:32:5) yielded pure **13** and **14** (27 mg): LSIMS (negative ion mode) *m/z* (rel intensity) 1103 (93) $[M - H]^-$, 957 (15) [M − H − deoxyhexose]$^-$, 795 (8) [M − H − deoxyhexose − hexose]$^-$, 649 (6) [M − H − 2 deoxyhexose − hexose]$^-$, 473 (12) [M − H − 2 deoxyhexoses − hexose − uronic acid]$^-$; ^{1}H NMR *δ* 5.27 (1H, t, *J* = 3.4 Hz, H-12), 4.17 (1H, d, *J* = 12.5 Hz, H-24b), 3.53 (1H, d, *J* = 4.0 Hz, H-21), 3.44 (1H, d, *J* = 4.0 Hz, H-22), 3.43 (1H, dd, *J* = 11.5 and 4.0 Hz, H-1), 3.23 (1H, d, *J* = 12.5 Hz, H-24a), 2.88 (1H, dd, *J* = 4.4 and 13.6 Hz, H-18), 1.28 (3H, s, Me-23) 1.21 (3H, s, Me-27), 1.08 (3H, s, Me-30), 1.00 (3H, s, Me-26), 0.97 (3H, s, Me-28), 0.95 (3H, s, Me-29), 0.91 (3H, s, Me-25); ^{13}C NMR data, see Tables 1 and 2. This gave on hydrolysis soyasapogenol A, glucuronic acid, galactose, and rhamnose.

Saponin **15** (8.5 mg): LSIMS (negative ion mode) *m/z* (rel intensity) 1101 (94) $[M - H]^-$, 911 (32) [M − H − uronic acid]$^-$, 779 (3) [M − H − uronic acid − pentose]$^-$, 691 (17) [M − H − deoxyhexose − 2 pentoses]$^-$, 501 (30) [M − H − uronic acid − deoxyhexose − 2 pentoses]$^-$; ^{1}H NMR *δ* 5.70 (d, *J* = 7.5 Hz, H-1ara), 5.29 (1H, t, *J* = 3.4 Hz, H-12), 4.38 (1H, ddd, *J* = 3.0, 4.0, and 4.0 Hz, H-2), 4.11 (1H, d, *J* = 4.0 Hz), 2.88 (1H, dd, *J* = 4.4 and 13.6 Hz, H-18), 1.42 (3H, s, Me-24) 1.32 (3H, s, Me-25), 1.19 (3H, s, Me-27), 0.96 (3H, s, Me-30), 0.94 (3H, s, Me-29), 0.83 (3H, s, M-26); for ^{13}C NMR data, see Tables 1 and 2.

Fraction VI was suspended in boiling water and centrifuged. Precipitate was dissolved in alcohol and on TLC it was shown to be a single compound identical with saponin **16**. The supernatant contained three glycosides, which were separated on a C_{18} column to yield compounds: **17** (13 mg); LSIMS (negative ion mode) *m/z* (rel intensity) 987 (100) $[M - H]^-$, 825 (53) [M − H − hexose]$^-$, 663 (7) [M − H − 2 hexoses]$^-$, 487 (14) [M − H − 2 hexoses − uronic acid]$^-$; ^{1}H NMR *δ* 5.29 (1H, t, *J* = 3.4 Hz, H-12), 4.33 (1H, ddd, *J* = 3.0, 3.7, and 4.0 Hz, H-2), 3.78 (1H, d, *J* = 12.5 Hz, H-23b), 3.66 (1H, d, *J* = 3.7 Hz, H-3), 3.23 (1H, d, *J* = 12.5 Hz, H-23a), 2.88 (1H, dd, *J* = 4.4 and 13.6 Hz, H-18), 1.31 (3H, s, Me-25) 1.19 (3H, s, Me-27), 0.98 (3H, s, Me-24), 0.96 (3H, s, Me-30), 0.94 (3H, s, Me-29), 0.84 (3H, s, Me-26); for ^{13}C NMR data, see Tables 1 and 2.

Saponin **18** (33.5 mg), MW 1236, on the basis of its spectral characteristics, was identified as 3-*O*-[*β*-D-glucopyranosyl(1→3)-*β*-D-glucopyranosyl]-28-*O*-[*β*-D-xylopyranosyl(1→4)-α-L-rhamnopyranosyl(1→2)-α-L-arabinopyranoside] medicagenate and was previously described (Massiot et al., 1988).

Compound **19** (8 mg), MW 939, identified as 3-*O*-[α-L-rhamnopyranosyl(1→2)-*β*-D-galactopyranosyl(1→2)-*β*-D-glucuronopyranoside] soyasapogenol E. This compound, trivial name dehydrosoyasaponin I, was previously identified in alfalfa aerial parts (Kitagawa et al., 1988).

Separation of fraction VII yielded saponins: **20** (71.5 mg), MW 1087, identified as 3-*O*-*β*-D-glucuronopyranosyl-28-*O*-[*β*-D-xylopyranosyl(1→4)-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranoside] medicagenate. This compound was previously identified in alfalfa roots and aerial parts as the dominant medicagenic acid glycoside (Oleszek et al., 1990).

Saponin **21** (14 mg): LSIMS (negative ion mode) *m/z* (rel intensity) 1413 (100) $[M - H]^-$, 1251 (20) [M − H − hexose]$^-$, 1089 (14) [M − H − 2 hexoses]$^-$, 957 (32) [M − H − 2 hexoses − pentose]$^-$, 927 (13) [M − H − 3 hexoses]$^-$, 795 (13) [M − H − 3 hexoses − pentose]$^-$, 663 (11) [M − H − 3 hexoses − 2 pentoses]$^-$, 517 (14) [M − H − 3 hexoses − 2 pentoses − deoxyhexose]$^-$; ^{1}H NMR *δ* 5.32 (1H, t, *J* = 3.2 Hz, H-12), 4.53 (1H, dd, *J* = 4.0 and 11.3 Hz, H-16), 4.28 (1H, ddd, *J* = 3.0, 3.8, and 4.0 Hz, H-2), 3.66 (1H, d, *J* = 3.5 Hz, H-3), 2.99 (1H, dd, *J* = 4.4 and 13.6 Hz, H-18), 1.43 (3H, s, Me-24), 1.38 (3H, s, Me-27), 1.31 (3H, s, Me-25), 0.99 (3H, s, Me-30), 0.90 (3H, s, Me-29), 0.79 (3H, s, Me-26); for ^{13}C NMR data, see Tables 1 and 2.

Compound **22** (14.5 mg): LSIMS (negative ion mode) *m/z* (rel intensity) 1397 (32) $[M - H]^-$, 1235 (14) [M − H − hexose]$^-$, 1073 (11) [M − H − 2 hexoses]$^-$, 941 (16) [M − H − 2 hexoses − pentose]$^-$, 911 (16) [M − H − 3 hexoses]$^-$, 779 (5) [M − H − 3 hexoses − pentose]$^-$, 647 (3) [M − H − 3 hexoses − 2 pentoses]$^-$, 501 (19) [M − H − 3 hexoses − 2 pentoses − deoxyhexose]$^-$; ^{1}H NMR *δ* 5.30 (1H, t, *J* = 3.4 Hz, H-12), 4.38 (1H, ddd, *J* = 3.0, 4.0, and 4.0 Hz, H-2), 4.12 (1H, d, *J* = 4.0 Hz, H-3), 2.89 (1H, dd, *J* = 4.4 and 13.6 Hz, H-18), 1.43 (3H, s, Me-24) 1.30 (3H, s, Me-25), 1.18 (3H, s, Me-27), 0.96 (3H, s, Me-30), 0.94 (3H, s, Me-29), 0.83 (3H, s, Me-26); for ^{13}C NMR data, see Tables 1 and 2.

Fraction VIII contained three compounds, two of which were successfuly separated. This yielded compounds **23** and **24**. **23** (11 mg): LSIMS (negative ion mode) *m/z* (rel intensity) 1543 (73) $[M - H]^-$, 1383 (5) [M − H − hexose]$^-$, 1281 (3) [M − H − 2 pentoses]$^-$, 1221 (3) [M − H − 2 hexoses]$^-$, 1135 (3) [M − H − 2 pentoses − deoxyhexose]$^-$, 1003 (12) [M − H − 3 pentoses − deoxyhexose]$^-$, 841 (3) [M − H − 3 pentoses − deoxyhexose − hexose]$^-$, 679 (3) [M − H − 3 pentoses − deoxyhexose − 2 hexoses]$^-$, 517 (6) [M − H − 3 pentoses − deoxyhexose − 3 hexoses]$^-$; ^{1}H NMR *δ* 5.32 (1H, t, *J* = 3.2 Hz, H-12), 4.53 (1H, dd, *J* = 4.0 and 11.3 Hz, H-16), 4.28 (1H, ddd, *J* = 3.0, 3.8, and 4.0 Hz, H-2), 3.66 (1H, d, *J* = 3.5 Hz, H-3), 2.99 (1H, dd, *J* = 4.4 and 13.6 Hz, H-18), 1.43 (3H, s, Me-24), 1.38 (3H, s, Me-27), 1.31 (3H, s, Me-25), 0.99 (3H, s, Me-30), 0.90 (3H, s, Me-29), 0.79 (3H, s, Me-26); for ^{13}C NMR data, see Tables 1 and 2.

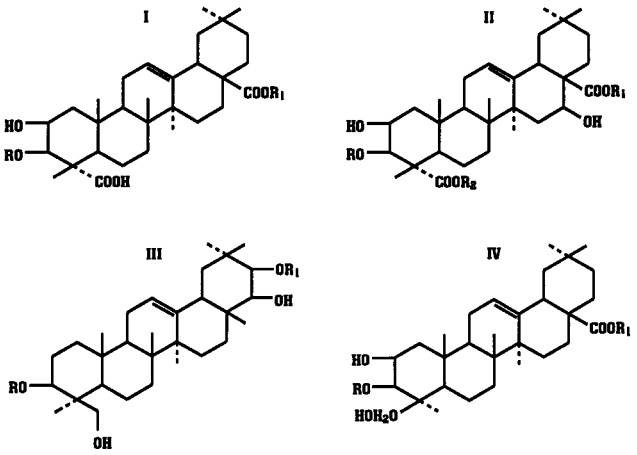
Compound **24** (3 mg): LSIMS (negative ion mode) *m/z* (rel intensity) 1529 (100) $[M - H]^-$, 1397 (12) [M − H − pentose]$^-$, 1367 (10) [M − H − hexose]$^-$, 1265 (6) [M − H − 2 pentoses]$^-$, 1205 (5) [M − H − 2 hexoses]$^-$, 1119 (4) [M − H − 2 pentoses − deoxyhexose]$^-$, 987 (26) [M − H − 3 pentoses − deoxyhexose]$^-$, 825 (4) [M − H − 3 pentoses − deoxyhexose − hexose]$^-$, 663 (9) [M − H − 3 pentoses − deoxyhexose − 2 hexoses]$^-$, 501(18) [M − H - 3 pentoses − deoxyhexose − 3 hexoses]$^-$; ^{1}H NMR *δ* 5.30 (1H, t, *J* = 3.4 Hz, H-12), 4.38 (1H, ddd, *J* = 3.0, 4.0, and 4.0 Hz, H-2), 4.12 (1H, d, *J* = 4.0 Hz, H-3), 2.89 (1H, dd, *J* = 4.4 and 13.6 Hz, H-18), 1.43 (3H,

s, Me-24) 1.30 (3H, s, Me-25), 1.18 (3H, s, Me-27), 0.96 (3H, s, Me-30), 0.94 (3H, s, Me-29), 0.83 (3H, s, Me-26); for ^{13}C NMR data, see Tables 1 and 2.

DISCUSSION

The structures of saponins **4**, **7**, **10**, **14**, **15**, **17**, and **21**−**24** have not been reported in the literature. To elucidate their full chemical characteristics, we have performed some NMR experiments analyzed the data. Thus, the ^{1}H NMR spectrum of the aglycon moiety of **10** showed signals for six tertiary methyl groups (δ 0.83, 0.94, 0.96, 1.18, 1.30, and 1.43) and signals indicative of proton linked to oxygen-bearing carbons at δ 4.12 (1H, d, J = 4.0 Hz) and δ 4.38 (1H, ddd, J = 3.0, 4.0, and 4.0 Hz). A further feature was a signal at δ 5.30 (1H, t, J = 3.4 Hz) typical of H-12 of a Δ^{12} oleanene skeleton, which was confirmed by the signals at δ 123.6 and 144.8 ascribable to C-12 and C-13 in the ^{13}C NMR spectrum (Mahato et al., 1994). A signal at δ 178.2 and the carbon resonances of rings D and E in the ^{13}C NMR spectrum suggested the occurrence of a glycosylated COOH group at C-28. A further carboxylic function (δ 182.5) was located at C-23 on the basis of the downfield shift (+14.3 ppm) exhibited by C-4 (δ 53.8) and the high-field shifts (−4.4, −4.7, −4.9) experienced, respectively, by C-3 (δ 86.6), C-5 (δ 53.1), and C-24 (δ 13.7) in comparison with the same carbon resonances in an oleanene skeleton bearing a Me-23 (Mahato et al., 1994). Also, the chemical shift of Me-24 in the ^{1}H NMR spectrum (δ 1.43) was diagnostic for a 23-COOH. Two oxygenated carbons at δ 70.9 and 86.6 were, respectively, correlated by an HSQC experiment (Martin and Crouch, 1991) to the proton resonances at δ 4.38 and 4.12. Furthermore, a DQF-COSY experiment (Rance et al., 1983) showed the sequence CH_2−CHOH (δ 4.35), allowing us to establish the occurrence of a 2β,3β-dihydroxyoleanene skeleton. On the basis of the foregoing data, the aglycon of **10** was identified as 2β,3β-dihydroxyolean-12-ene-23,28-dioic acid, known as medicagenic acid (Massiot et al., 1991). Glycosylation of the alcoholic function at C-3 and esterification of the 28-COOH group were indicated by the downfield shift (+13 ppm) and the highfield shift (−3 ppm) observed, respectively, for these carbon resonances in **10**, relative to the corresponding signals in medicagenic acid (Mahato et al., 1994).

The structure of the oligosaccharide unit was determined by 2D-NMR spectroscopy. A DQF-COSY experiment allowed the sequential assignment of most resonances for each sugar unit, starting from the well-isolated anomeric proton signals at δ 4.50, 4.65, 4.76, and 5.41. Nevertheless, on the basis of only DQF-COSY not all proton resonances could be successfully determined with confidence. Complete assignments of all the proton resonances in each sugar unit were achieved by a combination of DQF-COSY and 2D-HOHAHA results (Davis and Bax, 1985). A 2D-HOHAHA experiment was used to resolve the overlapped spectra of oligosaccharides into a subset of individual monosaccharide spectra. Thus, the shifts of the sugar resonances were atrributable to four β-D-glucopyranosyl units. An HSQC experiment that correlated all proton resonances with those of the corresponding carbon allowed the assignments of the interglycosidic linkages by comparison of the observed carbon chemical shifts with those of the corresponding methylpyranosides (Breitmaier and Voelter, 1989). On the basis of the HSQC experiment it was possible to deduce that the two β-D-glucopyranosyl units with the anomeric proton signals at δ 4.50 (gluI) and δ 4.76 (gluII) were substituted at C-2 and the two β-D-glucopyranosyl units with the anomeric proton signals at δ 4.65 (gluIII) and δ 5.41 (gluIV) were terminal. Chemical shifts of H-1$_{gluIV}$ (δ 5.41) and C-1$_{gluIV}$ (δ 95.6) indicated this sugar to be involved in an ester linkage with the C-28 carboxylic group. Because from the HSQC results this sugar unit was shown to be terminal, it could be easily determined that the sugar portion at C-28 was made up of a single sugar unit. In the HMBC experiment (Summers et al., 1986) a cross-peak due to long-range correlations between C-3 (δ 86.6) of the aglycon and H-1$_{gluI}$ (δ 4.50) indicated that this sugar unit was linked at C-3 of the aglycon. Similarly, cross-peaks were observed between C-2$_{gluI}$ (δ 82.4) and H-1$_{gluII}$ (δ 4.76), C-2$_{gluII}$ (δ 84.8), and H-1$_{gluIII}$ (δ 4.65).



Comp.	Aglycon	R	R1
4	I	Glc(1→3)-Glc	Glc
7	I	Rha(1→2)-Glc(1→2)-Glc	H
10	I	Glc(1→2)-Glc(1→2)-Glc	Glc
14	III	Rha(1→2)-Gal(1→2)-GlcA	Rha
15	I	GlcA-CH_3	Xyl(1→4)-Rha(1→2)-Ara
17	IV	Gal(1→2)-GlcA	Glc
21	II	Glc(1→2)-Glc(1→2)-Glc	Xyl(1→4)-Rha(1→2)-Ara
22	I	Glc(1→2)-Glc(1→2)-Glc	Xyl(1→4)-Rha(1→2)-Ara
23	II	Glc(1→2)-Glc(1→2)-Glc	Xyl(1→4)-Rha(1→2)-Ara Api(1→3)⌋
24	I	Glc(1→2)-Glc(1→2)-Glc	Xyl(1→4)-Rha(1→2)-Ara Api(1→3)⌋

Figure 1. Chemical formulas of newly identified compounds (I, medicagenic acid; II, zanhic acid; III, soyasapogenol A; IV, bayogenin).

On the basis of these findings, compound **10** was established to be 3-*O*-[β-D-glucopyranosyl(1→2)-β-D-glucopyranosyl(1→2)-β-D-glucopyranosyl]-28-β-D-glucopyranoside medicagenate (Figure 1).

Compounds **4**, **7**, **22**, and **24** are further medicagenic acid derivatives. In the ^{13}C NMR spectrum of compound **7** the signal at δ 181.8 (C-28) indicated that the 28-COOH function of medicagenic acid was free. For the sugar portion, the one-dimensional ^{1}H NMR spectrum showed characteristic signals of three anomeric protons at δ 4.46 (d, J = 7.5 Hz), δ 4.82 (d, J = 7.5 Hz), and δ 5.22 (d, J = 1.5 Hz) and one methyl doublet at δ 1.32 (d, J = 6.5 Hz). On the basis of the 2D-NMR analysis (2D-HOHAHA, DQF-COSY, HSQC), which allowed the assignment of all the ^{1}H and ^{13}C NMR signals, a trisaccharide chain made up of two β-D-glucose units substituted at C-2 and a terminal α-L-rhamnose were deduced (Table 1). The structure of the trisaccharide chain was confirmed by the connectivities between

H-1_{gluI} (δ 4.46) and C-3_{agl} (δ 86.6), H-1_{gluII} (δ 4.82) and C-2_{gluI} (δ 79.0), and H-1_{rha} (δ 5.22) and C-2_{gluII} (δ 80.0). Thus, compound **7** was deduced to be 3-*O*-[α-L-rhamnopyranosyl(1→2)-β-D-glucopyranosyl(1→2)-β-D-glucopyranoside medicagenate.

In the same way 2D-NMR experiments indicated that the sugar portion of compound **4** was characterized by a β-D-glucopyranosyl unit linked at C-28 (H-1_{gluIII} = δ 5.41) and a disaccharide chain made up of two β-D-glucopyranosyl unit linked at C-3. In this case a ^{13}C NMR glycosylation shift was observed for C-3 (δ 87.1) of the glucopyranosyl unit linked at C-3 of the aglycone (Table 1). Thus, **4** could be determined as 3-*O*-[β-D-glucopyranosyl(1→3)-β-D-glucopyranosyl]-28-*O*-β-D-glucopyranoside medicagenate (Figure 1).

Compounds **22** and **24** possess, respectively, sugar chains made up of six and seven sugars. With regard to the sugar portion of **22** in the ^{1}H NMR spectrum, six anomeric proton signals (δ 4.52, d, J = 7.5 Hz; δ 4.53, d, J = 7.5 Hz; δ 4.67, d, J = 7.5 Hz; δ 4.77, d, J = 7.5 Hz; δ 5.07, d, J = 1.5 Hz; and δ 5.67, d, J = 3.7 Hz) and one methyl doublet (δ 1.31, d, J = 6.5 Hz), suggesting the occurrence of one deoxyhexose unit, were well-resolved signals. The other sugar signals were overlapped in the region between δ 3.21 and 3.97. The structures of the oligosaccharide moieties were deduced using 1D-TOCSY and 2D NMR experiments. Because of the selectivity of the multistep coherence transfer, the 1D-TOCSY method (Kessler et al., 1986) allowed the subspectrum of a single monosaccharide unit to be extracted from the crowded overlapped region. The isolated anomeric proton signals resonating in an uncrowded region of the spectrum (between δ 4.52 and 5.67) were the starting point for the 1D-TOCSY experiments. Selected 1D-TOCSY obtained by irradiation of each anomeric proton signal yielded the subspectrum of each sugar residue with high digital resolution. Each subspectrum contained the scalar-coupled protons within each sugar residue. In some cases, because of the small coupling constants, the distribution of magnetization around the spin system was impeded. For this reason, for example, it was possible to identify only three protons (δ 3.92, 3.85, and 3.57) coupled to the anomeric signal at δ 5.67 in the case of arabinose. In the case of the 6-deoxyhexose, an easier identification of all of the proton signals was accomplished by recording 1D-TOCSY experiments, by irradiating the methyl doublet. Because in the TOCSY method both direct and relayed connectivities occur, we also recorded a DQF-COSY spectrum. The results of 1D-TOCSY and DQF-COSY experiments allowed the sequential assignments of all proton resonances to the individual monosaccharides. Thus, the shifts of sugar resonances were attributable to an L-arabinopyranosyl (δ H-1_{ara} = 5.67), an α-L-rhamnopyranosyl (δ H-1_{rha} = 5.07), β-D-xylopyranosyl (δ H-1_{xyl} = 4.52), and three β-D-glucopyranosyl (δ H-1_{gluI} = 4.53; δ H-1_{gluII} = 4.77; and δ H-1_{gluIII} = 4.67) units. In the case of the arabinopyranosyl unit, the J_{H1-H2} coupling constant (3.7 Hz) has been reported not to be diagnostic on its own, owing to the high conformational mobility of arabinopyranosides ($^4C_1 \leftrightarrow {}^1C_4$). As we reported in previous works (De Tommasi et al., 1993), evidence of L-arabinopyranoside was obtained from the ROESY (Kessler et al., 1987) spectrum, which showed NOE from C-1_{ara} to C-2_{ara}, C-3_{ara}, and C-5_{ara} as expected for α-L-arabinopyranoside in rapid $^4C_1 \leftrightarrow {}^1C_4$ conformational exchange.

From the HSQC experiment the absence of any ^{13}C NMR glycosylation shift for the β-D-glucopyranosyl unit with H-1 at δ 4.67 and the β-D-xylopyranosyl unit suggested that these sugars were terminal units. Glycosylation shifts were observed for C-2_{gluI} (δ 82.2), C-2_{gluII} (δ 84.6), C-2_{ara} (δ 75.6), and C-4_{rha} (δ 83.5) (Table 2). Chemical shifts of H-1_{ara} (δ 5.67) and C-1_{ara} (δ 93.7) indicated that this sugar unit was involved in an ester linkage with the C-28 carboxylic group (Oleszek et al., 1992).

The positions of the sugar residues were unambiguously defined by the HMBC experiment. Cross-peaks due to long-range correlation between C-3 (δ 86.6) of the aglycon and H-1_{gluI} (δ 4.53), C-2_{gluI} (δ 82.2) and H-1_{gluII} (δ 4.77), C-2_{gluII} (δ 84.6) and H-1 of the terminal glucose (δ 4.67) allowed us to deduce, by linkage to C-3 of the aglycon, the identical saccharide chain as in **10**. Similarly, the sequence of the trisaccharide chain at C-28 was indicated by the cross-peaks between C-2_{ara} (δ 75.6) and H-1_{rha} (δ 5.07) and C-4_{rha} (δ 83.5) and H-1_{xyl} (δ 4.52). Thus, the terminal xylose was linked at C-4 of rhamnose, which in turn was attached at C-2 of arabinose. A cross-peak between H-1 of arabinose (δ 5.67) and the ^{13}C NMR resonance of the 28-COOH group (δ 178.2) provided further evidence for an ester linkage between the trisaccharide chain and the aglycon. On the basis of these findings, compound **22** was established to be 3-*O*-[β-D-glucopyranosyl(1→2)-β-D-glucopyranosyl-(1→2)-β-D-glucopyranosyl]-28-*O*-[β-D-xylopyranosyl(1→4)-α-L-rhamnopyranosyl(1→2)-α-L-arabinopyranoside] medicagenate (Figure 1).

The 2D-NMR analysis of the sugar portion of **24** and comparison with that of **22** revealed the same trisaccharide chain linked at C-3 of the aglycon and the occurrence of an additional β-D-apiofuranosyl unit (H-1_{api} = δ 5.29) (De Tommasi et al., 1993) in the saccharide chain at C-28. The HSQC experiment allowed us to deduce the same glycosylation sites as in **22** and a further glycosylation shift on C-3 of the α-L-rhamnopyranosyl unit. On the basis of these results it was possible to establish the presence of a nodal rhamnopyranosyl residue glycosylated at C-3 and C-4 (Zhang et al., 1995). In good agreement with this evidence were the ^{1}H and ^{13}C resonances of the rhamnopyranosyl unit, which showed some differences from those reported for **22**. In particular, it is to be noted the high-field shift exhibited by C-4_{rha} in the case of a C-3, C-4 branched rhamnose (**24**) if compared to the corresponding carbon in a rhamnose unit only glycosidated at C-4 (**22**) (Table 1). In the HMBC experiment key correlation peaks through glycosidic linkages showed the same sugar sequence as in **22**. Furthermore, the correlation between the signals at δ 5.29 (H-1_{api}) and δ 81.7 (C-3_{rha}) proved the linkage of the β-D-apiofuranosyl to C-3 of the nodal rhamnose unit. Thus, compound **24** was defined as 3-*O*-[β-D-glucopyranosyl(1→2)-β-D-glucopyranosyl(1→2)-β-D-glucopyranosyl]-28-*O*-{β-D-xylopyranosyl(1→4)-[β-D-apiofuranosyl-(1→3)]- α-L-rhamnopyranosyl(1→2)-α-L-arabinopyranoside} medicagenate.

The analysis of ^{1}H and ^{13}C NMR data of compounds **21** and **23** suggested the occurrence of the same aglycon in the two compounds and sugar portions, which matched very closely, respectively, those observed in **22** and **24**. The main differences between the aglycon of **21** and **23** and medicagenic acid were the downfield shifts of C-16 (δ 74.8) and C-15 (δ 36.4) in the ^{13}C NMR spectrum and the downfield shift of the axial methyl group at C-14

(Me-27, δ 1.38) in the ^{1}H NMR spectrum, implying an additional hydroxyl group at C-16 in **21** and **23**. This hypothesis was unambiguously confirmed by the HMBC spectrum, which showed cross-peaks between the proton signal at δ 4.53 and C-14, C-15, C-17, C-18, and C-22. The 16α configuration of the hydroxyl group was evident from the chemical shift and the small J values of H-16 (δ 4.51, br m), characteristic of an equatorial proton. Thus, the aglycon of **21** and **23** was identified as 2β,3β,-16α-trihydroxyoleane-23,28-dioic acid known as zanhic acid (Oleszek et al., 1992), and compounds **21** and **23** were identified as 3-*O*-[β-D-glucopyranosyl(1→2)-β-D-glucopyranosyl(1→2)-β-D-glucopyranosyl]-28-*O*-[β-D-xylopyranosyl(1→4)-α-L-rhamnopyranosyl(1→2)-α-L-arabinopyranoside] zanhic acid and 3-*O*-[β-D-glucopyranosyl(1→2)-β-D-glucipyranosyl(1→2)-β-D-glucopyranosyl]-28-*O*-{β-D-xylopyranosyl(1→4)-[β-D-apiofuranosyl-(1→3)]-α-L-rhamnopyranosyl(1→2)-α-L-arabinopyranoside} zanhic acid, respectively (Figure 1).

The ^{1}H NMR spectrum of **17** showed for the aglycon moiety six methyl singlets (δ 0.84, 0.94, 0.96, 0.98, 1.19, and 1.31) and two doublets at δ 3.23 (1H, d, J = 12.5 Hz) and δ 3.78 (1H, d, J = 12.5 Hz, overlapping with the sugar signals and later revealed by DQF-COSY). Further evidence was signals at δ 3.66 (1H, d, J = 3.7 Hz, overlapping with the sugar signals and later revealed by DQF-COSY) and δ 4.33 (1H, ddd, J = 3.0, 3.7, and 4.0 Hz) and the typical signal of H-12 of a Δ^{12}-oleanene skeleton at δ 5.29 (1H, t, J = 3.4 Hz). ^{13}C NMR signals showed a close similarity to those reported for the 2β,3β,23-trihydroxyolean-12-en-28-oic acid, known as bayogenin (Mahato et al., 1994). From a comparison of the ^{13}C NMR spectrum of **17** with that of bayogenin, glycosylation shifts at C-2 (−1.0 ppm), C-3 (+9.9 ppm), and C-28 (−3.7 ppm) indicated that **17** was a 3,28-bidesmoside of bayogenin. For the sugar portion, three anomeric proton signals at δ 4.65, 4.69, and 5.42 were evident in the ^{1}H NMR spectrum. 2D-NMR analysis allowed us to deduce that the signal at δ 5.42 belonged to a terminal β-D-glucopyranosyl unit linked acylglycosidically to the 28-COOH function, as confirmed by the correlation peak through glycosidic linkage between the signal at δ 5.42 (H-1$_{glu}$) and the signal at δ 178.1 (C-28). On the basis of the DQF-COSY and 2D-HOHAHA results the spin systems correlated to the anomeric proton signals at δ 4.65 and 4.69 were, respectively, attributed to a β-D-glucuronopyranosyl unit and a β-D-galactopyranosyl unit. The attachment of the β-D-galactopyranosyl unit at C-2 of the β-D-glucuronopyranosyl unit and of this sugar unit to C-3 of the aglycon was subsequently confirmed by the HMBC correlations observed between H-1$_{gal}$ (δ 4.69) and C-2$_{gluA}$ (δ 82.4) and H-1$_{gluA}$ (δ 4.65) and C-3$_{agl}$ (δ 83.1). Hence, compound **17** was identified as 3-*O*-[β-D-galactopyranosyl(1→2)-β-D-glucuronopyranosyl]-28-*O*-β-D-glucopyranoside bayogenin (Figure 1).

The ^{1}H NMR spectrum of **14** showed, for the aglycon moiety, signals for six tertiary methyl groups at δ 0.91, 0.95, 0.97, 1.00, 1.08, 1.21, and 1.28. In the lower field region, together with the olefinic proton signal at δ 5.27 ascribable to H-12, a pair of geminal protons at δ 3.23 (d, J = 12.5 Hz) and δ 4.17 (d, J = 12.5 Hz) were evident. The DQF-COSY revealed, for the aglycon moiety, the signal of H-3 at δ 3.43 (dd, J = 4.0 and 11.5 Hz) and two additional signals at δ 3.44 (d, J = 4.0 Hz) and δ 3.53 (d, J = 4.0 Hz), which coupled to each other and did not show further couplings. The ^{13}C NMR spectrum of the aglycon portion featured signals indicating the 3β,21β,22β,24-tetrahydroxy-12-ene triterpene, known as soyasapogenol A (Mahato et al., 1994), occurring as the aglycon of some soyasaponins (Kitagawa et al., 1988). Glycosylation shifts were observed for C-3 (δ 92.3) and C-21 (δ 85.5), which appeared downfield shifted if compared to the corresponding signals in soyasapogenol A. The ^{1}H NMR spectrum showed for the sugar portion the occurrence of four anomeric proton signals at δ 4.48, 4.80, 4.92, and 5.18 and two methyl doubletes at δ 1.28 and 1.30. 1D-TOCSY experiments obtained by irradiating each anomeric proton signal and the two doublets at δ 1.28 and 1.30 were recorded. On the basis of the DQF-COSY results, the 1D-TOCSY subspectra of the four-monosaccharide unit could be easily interpreted. In fact, signals in both experiments displayed full coupling information, which helped assignments and allowed identification of the multiplet patterns of one β-D-glucuronopyranosyl unit, one β-D-galactopyranosyl unit, and two rhamnopyranosyl units. From the HSQC experiment the locations of the interglycosidic linkages at C-2 (δ 77.8) of the β-D-glucuronopyranosyl unit and at C-2 (δ 78.3) of the β-D-galactopyranosyl unit were deduced. HMBC correlations between the anomeric protons and the carbon sites of glycosylation (H-1$_{gluA}$ and C-3$_{agl}$, H-1$_{gal}$ and C-2$_{gluA}$, H-1$_{rhaI}$ and C-2$_{gal}$, and H-1$_{rhaII}$ and C-21) allowed us to establish the occurrence of an α-L-rhamnopyranosyl unit at C-21 and an α-L-rhamnopyranosyl (1→2) β-D-galactopyranosyl (1→2) β-D-glucuronopyranosyl chain linked at C-3. Whereas the presence of an α-L-rhamnopyranosyl unit at C-21 is a very unusual finding, the sugar chain linked at C-3 is typical of the soyasaponins (Kitagawa et al., 1988; Mohamed et al., 1995). It is to be noted that the chemical shift of C-2$_{gluA}$ is high-field shifted if compared to the corresponding carbon in compound **17**, but it is in good agreement with the corresponding value in soyasaponins showing, linked at C-3 of different soyasapogenols, the same sugar chain as in **14** (Mohamed et al., 1995).

On the basis of the foregoing evidence the structure attributed to compound **14** was identified as 3-*O*-[α-L-rhamnopyranosyl(1→2)-β-D-galactopyranosyl(1→2)-β-D-glucuronopyranosyl]-21-*O*-α-L-rhamnopyranoside soyasapogenol A (Figure 1).

The concentration evaluated on the isolation efficiency of novel saponins was not high. Each of them occurred in alfalfa roots in the concentration not exceeding 2% in total saponins isolated. The most dominant were the compounds **1** (15.2% of total), **2** (22%), **12** (14%), **13** (7.4%), **16** (11.6%), and **20** 7%), which remained in good agreement with the previous findings obtained with an HPLC method for the Boja variety (Nowacka and Oleszek, 1994). An interesting finding was the isolation of complex zanhic acid glycosides form alfalfa roots. These compounds were never identified in roots before; they were isolated from alfalfa tops, where in some instances they can make up to 0.5% in dry matter (Oleszek et al., 1992). It is not established if the site of zanhic acid synthesis is the alfalfa roots, from which they are being transported to the tops, or if the opposite situation takes place.

From the comparison of medicagenic acid glycosides it is clear that position 3-O of medicagenic acid can be exclusively glycosylated with glucose or glucuronic acid. The second sugar in this chain is also glucose, and then the third sugar unit can be either glucose or rhamnose.

Substiution at C28 can be done also either with glucose, which is in such a case the only sugar present in this position, or with arabinose, and next being always rhamnose and terminal xylose. In some cases of the most complex structures, apiose, which rarely occurs in nature, can also be found. Similar glycosylation patterns can be also found in zanhic acid glycosides (**21** and **23**).

It is clear that enzymes responsible for glycosylation can use as acceptor both zanhic and medicagenic acids. It is not known so far if the two aglycons are synthesized independently before glycosylation or if medicagenic acid is being oxidized to zanhic acid after glycosylation. This remains to be clarified.

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