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Triterpene Saponins from the Roots of *Medicago hybrida*

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Fourteen triterpene saponins (**1**−**14**) have been isolated from the roots of *Medicago hybrida* and their structures elucidated by FAB-MS and NMR analysis. Two of them are new compounds and were identified as hederagenin 3-*O*-[α-L-rhamnopyranosyl(1→2)-β-D-glucopyranosyl(1→2)-β-D-glucopyranosyl]-28-*O*-β-D-glucopyranoside (**7**) and oleanolic acid 3-*O*-[β-D-galactopyranosyl(1→2)-β-D-glucuronopyranosyl]-28-*O*-[α-L-rhamnopyranosyl(1→4)-β-D-glucopyranoside] (**14**). Seven saponins being mono- and bidesmosides of hederagenin (**1**, **5**, **6**, **9**), one bidesmoside of bayogenin (**2**), and two bidesmosides of 2β,3β-dihydroxyolean-12-en-23-al-28-oic acid (**11**) and oleanolic acid (**13**) are known compounds but not previously reported as saponin constituents of *Medicago*, whereas five other saponins, being mono- and bidesmosides of medicagenic acid (**3**, **4**, **8**, **10**, **12**), and one monodesmoside of hederagenin (**8**) have been previously isolated from other *Medicago* species. The presence of 2β,3β-dihydroxyolean-12-en-23-al-28-oic acid might represent an interesting intermediate in the biosynthesis of these substances.

KEYWORDS: ***Medicago hybrida*** **L.; triterpene saponins; chemical structure; FAB-MS; NMR**

INTRODUCTION

In a search for natural biopesticides, which could be less toxic to the environment than synthetic compounds, we screened roots and aerial parts of 29 *Medicago* species, as new rich sources of saponins. Results from this investigation showed that a few of the tested species, including *M. hybrida*, were very biologically active, showing higher antifungal and hemolytic activity compared to the other species under investigation. This study showed that the biological properties are related to the different saponin contents and compositions (*1*, *2*). Saponins from *Medicago* species possess fungitoxic (*3*−*8*) and insecticidal properties (*9*−*11*), and their biological activity depends on their chemical structure (*7*, *8*, *11*). *Medicago* saponins are a complex mixture of triterpenic glycosides that are derivatives of medicagenic acid, zanhic acid, hederagenin, bayogenin, and soyasapogenols, and studies on their chemical structure are particularly focused on *M. sativa* (*12*−*30*) due to its importance in animal feeding. Knowledge of the occurrence and chemical structure of saponins in other *Medicago* species is very limited, and only a few chemical investigations on *M. lupulina* (*31*), *M. polymorpha* (syn. *M. hyspida*) (*32*−*34*), *M. arabica* (*35*), *M. truncatula* (*36*, *37*), and *M. arborea* (*38*) have been previously reported.

M. hybrida (Pourret) Trautv. is a wild perennial species, endemic to the Corbier and eastern Pyrenean mountain ranges. Plants are 20−40 cm long with numerous stems, arising from the crown, prostrate to decumbent (*39*). Saponins from this species have never been extensively investigated before. The present study was undertaken to isolate and elucidate structures of saponins from roots of *M. hybrida*, which possess a very high activity both against fungi (*1*, *2*, *4*) and insects (*10*).

MATERIALS AND METHODS

Plant Material. *M. hybrida* (Pourr.) Trautv. MED 150 seeds, received from the Zentralinstitut fur Genetik und Kulturpflanzenforschung der Akademie der Wissensschaften Gatersleben (Germany), were sown in spring 2001 in Pulawy (Poland). Roots were dug up in autumn, washed with water, dried at 40 °C, ground, and used for the successive extractions.

Extraction and Isolation. The ground roots (300 g) were defatted with $CHCl_3$ in a Soxhlet apparatus for 48 h and then extracted with 95% MeOH under reflux for 24 h. This last extract was concentrated to dryness under reduced pressure, and the residue was suspended in H_2O and applied onto a 100 × 60 mm, 40−63 μm LiChroprep RP-18 column (Merck, Darmstadt, Germany) preconditioned with water. Sugars and phenolics were removed with water and with 40% MeOH. Saponins were then eluted from the column with MeOH. The solvent was evaporated in vacuo and the residue dried at 60 °C, obtaining 12 g of crude saponin mixture (4% yield).

Fractionation. Ten grams of the total saponin mixture was dissolved in *n*-BuOH saturated with water and submitted onto a 400 × 35 mm, 40−60 μm silica gel column (Merck). Fractions were eluted with *n*-BuOH saturated with water and checked by TLC on silica gel (Merck), developed with ethyl acetate/acetic acid/water (7:2:2), and RP-18, developed with 75% MeOH. Chromatograms were sprayed with 10% sulfuric acid in MeOH and heated at 130 °C. Separation on column

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chromatography gave four fractions: I (2.1 g), II (1.1 g), III (1.8 g), and IV (1.5 g).

Separation. Individual saponins were separated from the fractions by means of reversed-phase chromatography on a 400 × 25 mm, 40−63 μm, LiChroprep RP-18 column (Merck), eluted with aqueous MeOH. Fraction I eluted with 60% MeOH afforded saponin **1** (340 mg) and saponin **2** (47 mg); further elution with 65% MeOH gave saponins **3** (280 mg) and **4** (250 mg). Fraction II eluted with 65% MeOH yielded saponins **5** (57 mg) and **6** (75 mg). After elution with 70% MeOH, saponin **7** (330 mg) and saponin **8** (20 mg) were obtained. Fraction III eluted with 60% MeOH gave saponin **9** (630 mg) and saponin **10** (54 mg); 65% MeOH allowed saponin **11** (140 mg) to be obtained. Fraction IV eluted with 50% MeOH gave saponin **12** (160 mg), and elution with 55% MeOH gave saponin **13** (180 mg) and then saponin **14** (57 mg).

HPLC Analyses. All of the fractions obtained by reversed-phase chromatography were analyzed by HPLC using a Perkin-Elmer (Norwalk, CT) chromatograph equipped with an LC250 binary pump and a DAD 235 detector. Separation was performed on a 250 × 4.6 mm i.d., 5 μm, Discovery C18 column (Supelco, Bellefonte, PA) with the following mobile phase: solvent A, CH_3CN/0.05% CF_3COOH; solvent B, H_2O/1% MeOH/0.05% CF_3COOH. Chromatographic runs were carried out under gradient elution from 25% (5 min isocratic condition) to 90% of solvent A in 50 min. Ten microliters of methanolic solutions (1 mg/mL) of samples **1**−**14** was injected. Saponins were eluted at 1.0 mL/min, and detection was by UV monitoring at 215 nm.

Hydrolysis of Saponins. Saponin crude mixture (5 mg) and each individual pure saponin (2−3 mg) were hydrolyzed with 10 mL of 2 N HCl in 50% acqueous methanol under reflux for 8 h, and thereafter sugars and aglycones were separated and identified with TLC, GC, and GC-MS methods. From the hydrolysis reaction mixture, methanol was removed under vacuum and sapogenins were extracted with ethyl acetate (3 × 5 mL) and dried. The aqueous solutions were freeze-dried and used for sugar determination.

To obtain a quantity of the new aglycone of saponin **11**, 35 mg of this saponin was hydrolyzed with 50 mL of the acidic solution. After solvent extraction, 15 mg of pure aglycone was obtained (75% yield). ^1H and ^{13}C spectra of this sapogenin were recorded by dissolving the sample in pyridine-d_5.

Sapogenin Analyses. Sapogenins were separated on Merck silica gel 60 plates developed with petroleum ether/chloroform/acetic acid (7:2:1) or benzene/methanol (9:1). Spots were visualized by spraying with methanol/acetic anhydride/sulfuric acid (50:5:5 v/v) followed by heating at 120 °C. GC-FID and GC-MS of sapogenins were recorded after their methylation and acetylation/silylation (*40*). The sapogenin samples were dissolved in MeOH (0.3 mL) and treated with CH_2N_2. These solutions were divided in two subsamples, and the solvent was eliminated under a stream of N_2. Acetylation was performed by using 0.2 mL of pyridine/acetic anhydride/4-(dimethylamino)pyridine (1:1:0.1) and, after one night of stirring, water was added and methylated-peracetylated compounds were extracted with ethyl acetate (3 × 1 mL). The solution was dried over anhydrous Na_2SO_4, concentrated under a stream of N_2, and used for GC-FID and GC-MS analyses. Silylation was performed on the methylated sapogenin subsamples using 0.2 mL of a mixture of pyridine/hexamethyldisilazane/chlorotrimethylsilane (2:1:1) at 70 °C for 10 min. Samples were diluted with isooctane and used for GC-FID and GC-MS analyses. GC-FID analyses of both methylated-acetylated and methylated-silylated sapogenins were carried out using a Perkin-Elmer (Norwalk, CT) model 8500 GC equipped with a 30 m × 0.32 mm i.d., 0.25 μm DB-5 capillary column. Injector and detector temperatures were set at 350 °C, and the oven temperature program was as follows: 90 °C for 5 min, increased at 20 °C/min to 250 °C for 1 min and then increased at 4 °C/min to 350 °C for 15 min. Samples (1 μL) were injected in the splitless mode. Helium was the carrier gas with a head pressure of 12.2 psi. GC-MS analyses were carried out using a Perkin-Elmer Clarus 500 GC equipped with a MS detector and a 30 m × 0.25 mm i.d., 0.25 μm Elite-5MS capillary column using the same chromatographic conditions as for GC-FID. Mass spectra were acquired over a 50−850 amu range at 1 scan/s with an ionizing electron energy of 70 eV. The transfer line temperature was 300 °C, and the carrier gas was He at 1.2 mL/min. Retention times and MS spectra were compared to those of previously identified sapogenins. The aglycone of saponin **11** was also characterized by NMR experiments.

Analyses of Sugars and Determination of Absolute Configuration. Sugars were separated on Merck cellulose plates with benzene/butanol/pyridine/water (1:5:3:3) and made visible with a silver nitrate spray (3% in water solution). The determination of sugar absolute configurations was carried out by GC-FID using a 30 m × 0.32 mm i.d., 0.25 μm Chirasil-Val column (Alltech, Deerfield, IL). Sugar samples were suspended in MeOH (0.5 mL) and treated with CH_2N_2. Solvent was eliminated under a stream of N_2, the residue dissolved in 1-(trimethylsilyl)imidazole (Tris-Z) and pyridine (1:1, 0.3 mL), and the solution stirred at 60 °C for 5 min. After the solution had been dried under N_2, the residue was separated by water and CH_2Cl_2 (1 mL, 1:1). The organic layer was used for GC analyses. The temperature gradient system for the oven was as follows: 60 °C for 3 min, raised to 200 °C at 5 °C/min; injector and detector temperatures were set at 200 and 250 °C, respectively. Helium was the carrier gas with a head pressure of 12 psi; samples (0.2 μL) were injected in the splitless mode. Authentic reference compounds from Sigma-Aldrich (Milano, Italy), treated in the same way as reported for samples, were used for sugar identification. Co-injection of each hydrolysate with the standards gave single peaks. Sugar identification was also carried out by GC-MS as described in Tava et al. (*40*).

NMR Analyses. ^1H and ^{13}C NMR were measured on a Bruker AV-300 spectrometer at the operating frequencies of 300.13 and 75.13 MHz, respectively. The samples were examined as solutions in pyridine-d_5 (10 mg/0.5 mL) in 5 mm tubes at 25 °C. ^1H and ^{13}C chemical shifts were expressed in parts per million relative to pyridine signals at 7.2 and 123.5 ppm, respectively. 2D NMR experiments (H,H DQF−COSY; H,H TOCSY; H,C HSQC; H,C HMBC) were carried out on all compounds using the phase sensitive method. On the basis of 2D NMR analyses, assignments of ^1H and ^{13}C signals were obtained.

FAB-MS Analyses. FAB-MS spectra were conducted on a Finnigan MAT spectrometer. The samples were homogeneously mixed with glycerol and bombarded with 13-kV cesium-beam atoms. Mass spectra were recorded in the range of *m*/*z* 100−1500 by scanning the magnetic field in 10 s with mass resolution 1000.

Melting points were determined using a Büchi (Flawil, Switzerland) apparatus. Optical rotations were measured on a Jasco model P-1020 polarimeter. IR spectra were recorded in KBr with a Spectrum 2000 instrument. Elemental analyses were carried out on a Carlo Erba instrument. Spectroscopic data here reported equally refer to all of the analyzed saponins: IR ν_{max}^{KBr} cm^{-1}, 3100−3600 (large, OH), 2950 (CH), 1730 (C═O), 1640 (C═C), 1000−1200 (large, C−O). Molecular formulas obtained by elemental analysis of saponins were in agreement with data obtained by FAB-MS analysis.

RESULTS AND DISCUSSION

The crude saponins obtained from *M. hybrida* roots (4% yield) were fractionated by a combination of silica gel and RP-18 open column chromatography to afford 14 saponins (**1**−**14**) in a pure form as confirmed by HPLC analyses. Investigations performed by TLC, GC, and GC-MS on the aglycone composition of the total saponin mixture revealed the presence of hederagenin as the main compound (35% of the total genins), followed by medicagenic acid (19%), oleanolic acid (17%), and bayogenin (10%), together with a new sapogenin (2%), further identified as 2β,3β-dihydroxyolean-12-en-23-al-28-oic acid.

To elucidate the chemical structures of saponins **1**−**14**, spectroscopic and chemical techniques were employed. The TLC analyses of hydrolysis products of single saponins showed five types of aglycones. R_f values and GC-MS and NMR data, compared to that of reference compounds, revealed the presence of hederagenin in saponins **1** and **5**−**9** and medicagenic acid in compounds **3**, **4**, **10**, and **12**. The aglycone moiety of saponins **13** and **14** was identified as oleanolic acid, the aglycone of saponin **2** as bayogenin, and the aglycone moiety of saponin **11** as 2β,3β-dihydroxyolean-12-en-23-al-28-oic acid.

Table 1. ^{13}C NMR Data (δ) of the Aglycone Moieties of Saponins **7**, **11**, and **14**

C	7	11	14	C	7	11	14
1	38.62	45.07	38.82	16	23.79	24.02	23.60
2	28.23	67.73	26.45	17	46.95	46.93	47.43
3	84.31	84.35	90.09	18	41.70	41.71	41.68
4	43.31	54.41	39.68	19	46.12	46.12	46.94
5	48.41	47.78	55.82	20	30.70	30.72	30.85
6	18.31	20.15	18.36	21	32.50	32.65	33.08
7	33.94	33.94	34.49	22	32.83	32.50	32.22
8	39.88	40.25	41.02	23	65.99	206.25	28.19
9	48.07	48.37	49.85	24	13.25	11.28	16.22
10	36.88	36.44	36.92	25	16.07	17.14	14.89
11	23.36	23.32	21.08	26	17.49	17.60	16.44
12	122.80	122.79	122.75	27	26.00	26.10	26.14
13	144.12	144.07	144.51	28	176.42	176.53	176.48
14	42.09	42.30	42.69	29	33.05	33.10	33.12
15	25.53	28.08	25.97	30	23.61	23.63	23.66

Table 2. ^{13}C NMR Data (δ) of the Saccharide Moieties of Saponins **7**, **11**, and **14**

C	7	11	14
	3-Glc (I)	3-GlucAc (I)	3-GlucAc (I)
1	103.73	103.81	104.97
2	**79.40**	74.44	**81.97**
3	78.87	78.15	78.29
4	71.61	73.26	73.51
5	77.99	75.86	77.42
6	62.70	176.42	174.89
	Glc (II)		Gal (II)
1	101.88		101.73
2	**79.52**		73.27
3	78.40		75.20
4	71.04		70.49
5	77.72		76.52
6	62.55		62.38
	Rha (III)		
1	102.63		
2	72.21		
3	72.56		
4	74.19		
5	69.88		
6	18.91		
	28-Glc (IV)	28-Glc (II)	28-Glc (III)
1	95.71	95.73	95.74
2	74.19	74.09	74.77
3	78.85	78.84	76.41
4	71.86	71.05	**78.41**
5	79.31	79.30	76.78
6	62.16	62.16	62.07
			Rha (IV)
1			102.61
2			72.23
3			72.62
4			73.91
5			70.12
6			18.79

Sugar moieties were identified by means of TLC, comparing their R_f values with those of reference compounds. The absolute configuration of the sugar residues was obtained from GC analysis on a chiral column.

The assignment of all ^1H and ^{13}C signals was obtained on the basis of 2D NMR experiments. The structure elucidation of all saponins was performed by analyzing NMR and FAB-MS data. The molecular weights were obtained from elemental analyses and FAB-MS spectra and evaluated from NMR data in which all carbons were revealed. FAB-MS fragmentation ions were also used to establish the sugar chain in the molecule. ^{13}C NMR chemical shift data are reported in **Tables 1** and **2**; ^1H NMR data are reported in **Tables 3** and **4**. The chemical structures of identified saponins **1**−**14** are presented in **Figure 1**. All of the monosaccharides were determined to be in their pyranose form from their ^{13}C NMR data. Sugar configurations were also establish by comparison of their spectroscopic data with literature values (*41*, *42*).

Compound **1**, 340 mg, MW 634, released hederagenin and glucose after acid hydrolysis. Its spectroscopic characteristics were identical to those of hederagenin 3-*O*-β-D-glucopyranosyl, previously identified in *Hedera nepalensis* (*43*).

Compound **2**, 47 mg, MW 812, after hydrolysis gave bayogenin and glucose and had spectroscopic characteristics identical to those of bayogenin 3-*O*-β-D-glucopyranosyl-28-*O*-β-D-glucopyranoside, previously identified in *Aster batangensis* (*44*).

Compound **3**, 280 mg, MW 664, revealed after acid hydrolysis the presence of medicagenic acid and glucose. Its chromatographic and spectroscopic characteristics were identical to those of medicagenic acid 3-*O*-β-D-glucopyranosyl, previously identified in *M. sativa* (*30*) and *M. lupulina* (*31*).

Compound **4**, 250 mg, MW 826, after acid hydrolysis gave medicagenic acid and glucose and had chromatographic and spectroscopic characteristics identical to those of medicagenic acid 3-*O*-β-D-glucopyranosyl-28-*O*-β-D-glucopyranoside, previously identified in *M. sativa* (*30*) and *M. lupulina* (*31*).

Compound **5**, 57 mg, was isolated as an amorphous solid: mp 215−217 °C, $[\alpha]_D^{25}$ 10.40° (MeOH, *c* 0.6). The molecular formula was estimated as $C_{43}H_{68}O_{15}$ (MW 824). Acid hydrolysis of this saponin gave hederagenin, glucose and glucuronic acid in the ratio 1:1. FAB-MS, *m*/*z* (relative intensity) 809 (5) [M − Me]$^-$, 661 (100) [M − H − hexose]$^-$, 471 (10) [M − H − hexose − glucuronate]$^-$. The ^1H NMR spectrum of this compound showed the presence of two anomeric at δ 6.41 and 5.28, confirmed in the ^{13}C NMR spectrum by the anomeric carbon signals at δ 106.41 and 95.72. From a comparison of the ^{13}C spectrum of saponin **5** with that of hederagenin, glycosylation shifts at C-3 and C-28 indicated that this compound is a 3,28-bidesmoside of hederagenin. The HMBC experiments clearly show a long-range correlation between C-3 (δ 82.21) and the anomeric proton at δ 5.28 and between C-28 (δ 176.41) and the anomeric proton at δ 6.41. DQF-COSY and TOCSY experiments allowed the sequential assignment of resonances for each sugar unit, starting from the anomeric proton signals: the shifts of the sugar resonances were attributed to a β-D-glucopyranoside (anomeric proton at δ 6.41) and a β-D-glucuronopyranoside (anomeric proton at δ 5.28). NMR data of compound **5** are superimposed to those of saponin **9**, except for the presence of an additional singlet signal at δ 3.76 in the ^1H NMR spectrum that correlates with the carboxylic group of glucuronic acid at δ 170.79 in the ^{13}C spectrum. This clearly demonstrates this function is esterified as methyl ester. The presence of the O−CH_3 signal at δ 51.95 in the ^{13}C NMR spectrum was also shown. In the FAB-MS spectra no molecular ion (MW 824) was observed; the first peak detected was *m*/*z* 809 (5% relative intensity), which corresponds to the loss of a methyl group from the molecule. The base peak was detected at *m*/*z* 661, which corresponds to the loss of the hexose unit, from which the successive loss of the methyl glucuronate unit gave the peak at *m*/*z* 471 (10% relative intensity) that corresponds to hederagenin. On the basis of these findings, the structure of this compound was established to be hederagenin 3-*O*-β-D-glucuronopyranosyl methyl ester-28-*O*-β-D-glucopy-

Table 3. ^{1}H NMR Data (δ) of the Aglycone Moieties of Saponins **7**, **11**, and **14**[a]

proton	7	11	14
H-1a	1.08, 1H, m	1.68, 1H, m	1.10, 1H, m
H-1b	1.52, 1H, m	2.61, 1H, m	1.49, 1H, m
H-2	1.18–1.22, 2H, m	4.51, 1H, m	1.15–1.25, 2H, m
H-3	4.38, 1H, dd (14.0, 4.0)	4.18, 1H, d (4.0)	4.30, 1H, dd (14.0, 4.0)
H-5	1.58, 1H, dd (12.0, 4.0)	1.55, 1H, m	1.53, 1H, m
H-6	1.18 and 1.71, 2H, m	1.12 and 1.65, 2H, m	1.18 and 1.65, 2H, m
H-7	1.15 and 1.35, 2H, m	1.15 and 1.40, 2H, m	1.16 and 1.41, 2H, m
H-9	1.75, 1H, m	1.83, 1H, m	1.68, 1H, m
H-11	1.90–2.15, 2H, m	2.00–2.15, 2H, m	1.85–2.10, 2H, m
H-12	5.52, 1H, t (3.0)	5.55, 1H, t (3.0)	5.52, 1H, t (3.0)
H-15	2.00 and 2.35, 2H, m	1.15 and 2.25, 2H, m	1.80 and 2.15, 2H, m
H-16	1.90–2.10, 2H, m	2.10–2.22, 2H, m	1.95–2.20, 2H, m
H-18	3.25, 1H, dd (14.0, 4.0)	3.28, 1H, dd (14.0, 4.0)	3.30, 1H, dd (14.0, 4.0)
H-19	1.31 and 1.78, 2H, m	1.35 and 1.70, 2H, m	1.27 and 1.75, 2H, m
H-21	1.80–1.95, 2H, m	1.80–1.90, 2H, m	1.80–1.90, 2H, m
H-22	1.35–1.45, 2H, m	1.75–1.85, 2H, m	1.35–1.46, 2H, m
H-23a	3.70, 1H, bd (12.0)	9.82, 1H, s	
H-23b	4.39, 1H, bd (12.0)		
Me-23			1.30, 3H, s
Me-24	1.18, 3H, s	1.71, 3H, s	0.85, 3H, s
Me-25	0.98, 3H, s	1.62, 3H, s	1.15, 3H, s
Me-26	1.22, 3H, s	1.18, 3H, s	1.10, 3H, s
Me-27	1.26, 3H, s	1.36, 3H, s	1.25, 3H, s
Me-29	0.97, 3H, s	0.99, 3H, s	0.95, 3H, s
Me-30	0.94, 3H, s	0.96, 3H, s	0.91, 3H, s

[a] Assignments were established by HSQC, DQF-COSY, and TOCSY spectra. *J* values (in hertz) are given in parentheses.

ranoside. This compound is probably an artifact originating from saponin **9** during extraction (*45*).

Compound **6**, 75 mg, MW 662, revealed after acid hydrolysis the presence of hederagenin and glucuronic acid. Its spectroscopic characteristics were identical to those of hederagenin 3-*O*-β-D-glucuronopyranosyl methyl ester, previously identified in *Hedera nepalensis* (*43*) and *Aralia armata* (*46*).

Compound **7**, 330 mg, was isolated as an amorphous solid: mp 224−225 °C, $[\alpha]_D^{25}$ −4.12 ° (MeOH, *c* 1.0). The molecular formula was estimated as $C_{54}H_{88}O_{23}$ (MW 1104). Acid hydrolysis of this saponin gave hederagenin and glucose and rhamnose in the ratio of 3:1. FAB-MS, *m*/*z* (relative intensity) 1103 (100) $[M - H]^-$, 941 (99) $[M - H -$ hexose$]^-$, 795 (25) $[M - H -$ hexose − rhamnose$]^-$, 633 (45) $[M - H -$ 2 hexoses − rhamnose$]^-$, 471 (20) $[M - H -$ 3 hexoses − rhamnose$]^-$; NMR data are reported in **Tables 1**−**4**. The ^{13}C NMR spectrum of **7** showed the presence of four anomeric signals at δ 103.73, 102.63, 101.88, and 95.71. Glycosylation of the alcoholic function at C-3 and esterification of the 28-COOH group were indicated by the downfield shift and the highfield shift observed, respectively, for these carbon resonances, relative to the corresponding signals in hederagenin. The structure of saccharide units was determined by 2D NMR spectroscopy. DQF-COSY and TOCSY experiments allowed the sequential assignment of resonances for each sugar unit, starting from the well-isolated anomeric proton signals at δ 6.42, 5.91, 5.22, and 4.98 in the ^{1}H NMR spectra. The complete assignments of all proton resonances in each sugar were achieved. In the HMBC experiment the anomeric signal at δ 5.22 showed long-range correlation with C-3 at δ 84.31, indicating that glucose is the first sugar directly linked to the triterpenic structure at C-3. The second sugar of the chain is glucose. The position of this second glucose unit was indicated in the HMBC experiment in which the two anomeric protons at δ 5.22 (H-$1_{Glu\ I}$) and 4.98 (H-$1_{Glu\ II}$) give a correlation with the same carbon (C-$2_{Glu\ I}$) at δ 79.40. The third sugar of the chain is rhamnose, and its anomeric proton

Table 4. ^{1}H NMR Data (δ) of the Monosaccharide Moieties of Saponins **7**, **11**, and **14**[a]

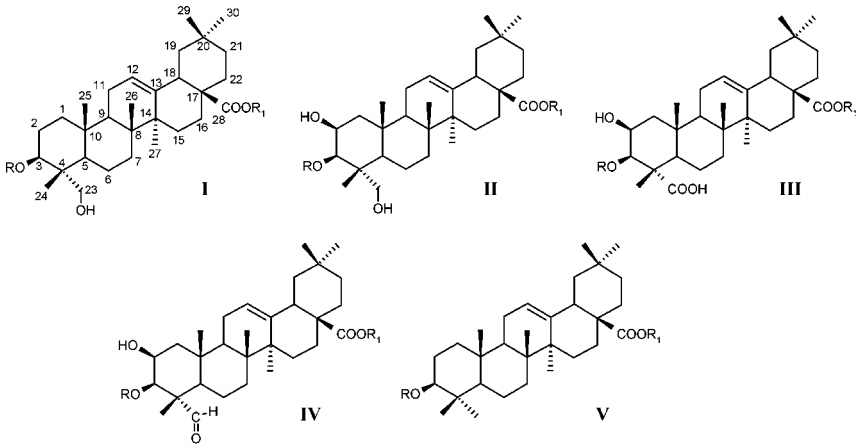
C	7	11	14
	3-Glc (I)	3-GlucAc(I)	3-GlucAc(I)
1	5.22, d (7.5)	4.89, d (7.0)	4.82, d (7.0)
2	4.08	4.29	4.18
3	4.20	4.25	4.22
4	4.28	4.05	4.01
5	3.95	4.35	4.27
6	4.40, 4.55		
	Glc (II)		Gal (II)
1	4.98, d (7.5)		5.61, d (7.0)
2	4.60		4.55
3	4.32		4.07
4	4.21		4.40
5	3.92		4.01
6	4.40, 4.55		4.30, 4.65
	Rha (III)		
1	5.91, br s		
2	4.87		
3	4.74		
4	4.42		
5	5.08		
6	1.88, d (6.0)		
	28-Glc (IV)	28-Glc (II)	28-Glc (III)
1	6.42, d (7.5)	6.37, d (7.5)	6.45, d (7.5)
2	4.30	4.27	4.23
3	4.43	4.32	4.28
4	4.45	4.38	4.34
5	4.12	4.11	4.64
6	4.40, 4.55	4.45, 4.55	4.42, 4.55
			Rha (IV)
1			5.80, br s
2			4.62
3			4.50
4			4.25
5			4.88
6			1.78, d (6.0)

[a] Assignments were established by HSQC, DQF-COSY, and TOCSY spectra. *J* values (in hertz) are given in parentheses.

(H-$1_{Rha\ III}$) at δ 5.91 gave a correlation with C-$2_{Glu\ II}$ at δ 79.52. The C-28 at δ 176.42 gave a clear correlation in the HMBC experiment with the anomeric proton at δ 6.42 (H-$1_{Glu\ IV}$). These findings were also extrapolated from the FAB-MS spectrum. The molecular ion $[M - H]^-$ at *m*/*z* 1103 corresponds to hederagenin plus three hexoses plus rhamnose. The peak at *m*/*z* 941 corresponds to the loss of a hexose sugar. The other peaks observed were *m*/*z* 795 $[M - H -$ hexose − rhamnose$]^-$, 633 $[M - H -$ 2 hexoses − rhamnose$]^-$, and 471 $[M - H -$ 3 hexoses − rhamnose$]^-$, which corresponds to hederagenin. On the basis of these data saponin **7** was established to be hederagenin 3-*O*-[α-L-rhamnopyranosyl(1→2)-β-D-glucopyranosyl(1→2)-β-D-glucopyranosyl]-28-*O*-β-D-glucopyranoside.

Compound **8**, 20 mg, MW 766, released hederagenin and glucose and arabinose in the ratio of 1:1 after acid hydrolysis. Its chromatographic and spectroscopic characteristics were identical to those of hederagenin 3-*O*-[β-D-glucopyranosyl-(1→2)-α-L-arabinopyranosyl], previously identified in *M. sativa* (*30*) and *M. arabica* (*35*).

Compund **9**, 630 mg, MW 810, released hederagenin and glucuronic acid and glucose in the ratio of 1:1 after acid hydrolysis. Its spectroscopic characteristics were identical to those of hederagenin 3-*O*-β-D-glucuronopyranosyl-28-*O*-β-D-glucopyranoside, previously identified in *Chenopodium quinoa* (*47*).



Compound	Aglycone	R	R_1
1	**I**	β-D-Glc	H
2	**II**	β-D-Glc	β-D-Glc
3	**III**	β-D-Glc	H
4	**III**	β-D-Glc	β-D-Glc
5	**I**	β-D-GlcA Me	β-D-Glc
6	**I**	β-D-GlcA Me	H
7	**I**	α-L-Rha(1→2)-β-D-Glc(1→2)-β-D-Glc	β-D-Glc
8	**I**	β-D-Glc(1→2)-α-L-Ara	H
9	**I**	β-D-GlcA	β-D-Glc
10	**III**	β-D-Glc(1→2)-β-D-Glc	β-D-Glc
11	**IV**	β-D-GlcA	β-D-Glc
12	**III**	β-D-GlcA	β-D-Glc
13	**V**	β-D-Gal(1→2)-β-D-GlcA	β-D-Glc
14	**V**	β-D-Gal(1→2)-β-D-GlcA	α-L-Rha(1→4)-β-D-Glc

Figure 1. Structures of saponins **1**–**14** (I, hederagenin; II, bayogenin; III, medicagenic acid; IV, 2β,3β-dihydroxyolean-12-en-23-al-28-oic acid; V, oleanolic acid).

Compound **10**, 54 mg, MW 988, released medicagenic acid and glucose after acid hydrolysis. Its chromatographic and spectroscopic characteristics were identical to those of medicagenic acid 3-*O*-[β-D-glucopyranosyl(1→2)-β-D-glucopyranosyl]-28-*O*-β-D-glucopyranoside, previously identified in *M. sativa* (*30*).

Compound **11**, 140 mg, was isolated as an amorphous solid: mp 228−230 °C, $[\alpha]_D^{25}$ 33.51° (MeOH, *c* 1.0). The molecular formula was estimated as $C_{42}H_{64}O_{16}$ (MW 824). Acid hydrolysis of this saponin gave 2β,3β-dihydroxyolean-12-en-23-al-28-oic acid and glucose and glucuronic acid in the ratio 1:1. FAB-MS, *m*/*z* (relative intensity) 823 (100) $[M - H]^-$, 661 (16) $[M - H -$ hexose$]^-$, 485 (17) $[M - H -$ hexose − uronic$]^-$; NMR data are reported in **Tables 1**−**4**. The triterpenic structure of the aglycone moiety was established on the basis of NMR and GC-MS data as previously reported (*38*). Saponin **11** showed two anomeric signals at δ 103.81 and 95.73 in the ^{13}C NMR spectrum and at δ 6.37 and 4.89 in the ^1H NMR spectrum. The signal of the carboxylic group at δ 176.53 indicates the presence of sugar linked in this position. The ^1H NMR chemical shift observed at δ 6.37 correlated with the carbon at δ 95.73 is characteristic of glucose linked at the C-28 position. In the HMBC experiment a correlation between the anomeric proton at δ 6.37 and this carboxylic group was found. In HMBC spectra a clear correlation between C-3 (δ 84.35) and the anomeric proton of glucuronic acid (δ 4.89) was observed. The presence of the aldehydic group on the aglycone moiety was confirmed by the signal at δ 206.25 in the ^{13}C NMR spectrum and by the singlet signal at δ 9.82 in the ^1H NMR spectrum of saponin **11**. The structure of this saponin was also confirmed by FAB-MS. Loss of the hexose unit from the molecular ion $[M - H]^-$ *m*/*z* 823 gave the ion at *m*/*z* 661. Loss of the glucuronic unit gave the corresponding aglycone at *m*/*z* 485. On the basis of these data saponin **11** was established to be 2β,3β-dihydroxyolean-12-en-23-al-28-oic acid 3-*O*-β-D-glucuronopyranosyl-28-*O*-β-D-glucopyranoside. The same saponin has been previously reported as a constituent of *Spinacia oleracea* (*48*), its chemical characterization being based only on ^1H NMR. Full structural identification (^1H, ^{13}C, melting point, $[\alpha]^{25}_D$, FAB-MS) has been instead accomplished for saponin **11** from *M. hybrida*, and data are reported here for the first time.

Compound **12**, 160 mg, MW 840, released medicagenic acid and glucuronic acid and glucose in the ratio of 1:1 after acid hydrolysis. Its chromatographic and spectroscopic characteristics were identical to those of medicagenic acid 3-*O*-β-D-glucuronopyranosyl-28-*O*-β-D-glucopyranoside, previously identified in *M. truncatula* (*37*).

Compound **13**, 180 mg, MW 956, released oleanolic acid and glucuronic acid, glucose, and galactose in the ratio of 1:1:1 after acid hydrolysis. Its spectroscopic characteristics were identical to those of oleanolic acid 3-*O*-[β-D-glucuronopyranosyl(1→2)-

β-D-galactopyranosyl]-28-*O*-β-D-glucopyranoside, previously identified in *Tetrapanax papiriferum* (*49*).

Saponin **14**, 57 mg, was isolated as an amorphous solid: mp 231−234 °C, $[\alpha]_D^{25}$ 12.14° (MeOH, *c* 1.0). The molecular formula was estimated as $C_{54}H_{86}O_{23}$ (MW 1102). After acid hydrolysis, saponin **14** gave oleanolic acid and glucuronic acid, glucose, galactose, and rhamnose in the ratio of 1:1:1:1. FAB-MS, *m*/*z* (relative intensity) 1101 (100) $[M - H]^-$, 955 (70) $[M - H -$ rhamnose$]^-$, 939 (75) $[M - H -$ hexose$]^-$, 793 (95) $[M - H -$ rhamnose $-$ hexose$]^-$, 631 (20) $[M - H -$ rhamnose $- 2$ hexoses$]^-$, 455 (45) $[M - H -$ rhamnose $- 2$ hexoses $-$ uronic$]^-$. NMR data are reported in **Tables 1**−**4**. Four anomeric signals were evident at δ 104.97, 102.61, 101.73, and 95.74 in the ^{13}C NMR spectrum and at δ 6.45, 5.80, 5.61, and 4.82 in the ^1H NMR spectrum. From DQF-COSY and TOCSY experiments, the shifts of the sugar resonances were attributed to a β-glucuronopyranoside (H-$1_{GluAc\ I}$, anomeric proton at δ 4.82), β-galactopyranoside (H-$1_{Gal\ II}$, anomeric proton at δ 5.61), β-glucopyranoside (H-$1_{Glu\ III}$, anomeric proton at δ 6.45), and α-rhamnopyranoside (H-$1_{Rha\ IV}$, anomeric proton at δ 5.80). 2D NMR experiments indicated the presence of a disaccharide chain made up of one β-glucuronopyranose directly linked to the C-3 of aglycone and a β-galactopyranoside linked at C-2 of glucuronic acid. In HMBC spectra a correlation between C-3 (δ 90.09) and the anomeric proton of glucuronic acid was observed. The position of galactose was also indicated in the HMBC experiment: the two anomeric carbons at δ 104.97 (C-$1_{GluAc\ I}$) and 101.73 (C-$1_{Gal\ II}$) gave a correlation with the same proton at δ 4.18 (H-$2_{GluAc\ I}$) of glucuronic acid. The C-28 carboxylic group at δ 176.48 indicates the presence of sugar linked in this position. The ^1H NMR chemical shift observed at δ 6.45 correlated with the carbon at δ 95.74 is characteristic of glucose linked at the C-28 position. In the HMBC experiment a correlation between the anomeric proton at δ 6.45 (H-$1_{Glc\ III}$) and this carboxylic group was found. The sugar linked to glucose was determined as rhamnose. The position of this sugar was indicated in the HMBC spectra in which a correlation between the signal at δ 5.80 (H-$1_{Rha\ IV}$) and the signal at δ 78.41 (C-$4_{Glu\ III}$) was observed. The structure of saponin **14** was also confirmed by FAB-MS. The molecular ion $[M - H]^-$ *m*/*z* 1101 represents the base peak. Loss of rhamnose gave *m*/*z* 955, and loss of a hexose unit gave *m*/*z* 939. The peak at *m*/*z* 793 represents $[M - H -$ rhamnose $-$ hexose$]^-$ from which the loss of a hexose unit gave *m*/*z* 631. From this last peak, the loss of a glucuronic unit giving the corresponding aglycone oleanolic acid (*m*/*z* 455) confirms that glucuronic acid is directly linked to the triterpene. On the basis of these data saponin **14** was established to be oleanolic acid 3-*O*-[β-D-galactopyranosyl-(1→2)-β-D-glucuronopyranosyl]-28-*O*-[α-L-rhamnopyranosyl-(1→4)-β-D-glucopyranoside.

The 14 triterpene saponins isolated from the roots of *M. hybrida* are glycosides of five different aglycones: hederagenin, detected in saponins **1** and **5**−**9**; medicagenic acid, detected in saponins **3**, **4**, **10**, and **12**; oleanolic acid, detected in saponins **13** and **14**; bayogenin detected in saponin **2**; and 2β,3β-dihydroxyolean-12-en-23-al-28-oic acid, found in saponin **11**. In this species no glycosides of zanhic acid were found.

A very interesting feature of the saponins isolated from the roots of *M. hybrida* is the presence of the aldehydic group at the C-23 position in the aglycone of saponin **11**. The same compound has been characterized as a sapogenin moiety in the saponins isolated from *M. arborea* leaves (*38*). This metabolite might represent an intermediate in *Medicago* saponin oxidative biosynthetic steps leading from a methyl group to the corresponding carboxylic acid function (*50*, *51*). The following aglycone moieties identified in *M. hybrida* are representative of oxidative products at C-3 from alcohol to acid: bayogenin (II, **Figure 1**), 2β,3β-dihydroxyolean-12-en-23-al-28-oic acid (IV), and medicagenic acid (III). All of the cited compounds possess the same stereochemistry (2β,3β) in the triterpene skeleton. Medicagenic acid (III) may originate from bayogenin, by subsequent oxidative enzymatic steps involving the formation of the corresponding C-23 aldehyde-substituted compound (IV). Additionally, hederagenin (I) might originate from oleanolic acid (V) by a selective oxidative demethylation at C-23 and, in turn, hederagenin can be a substrate for the production of bayogenin (II), with a mechanism involving a stereochemically specific enzyme able to insert the hydroxyl group in the 2β position of the triterpene skeleton. Although these mechanisms must be demonstrated, the suggested hypothesis can give an idea of the biosynthesis of these substances.

All of the identified saponins from *M. hybrida*, except compound **8**, have glucose or glucuronic acid as the first sugar at the C-3 position and, when present, glucose at the C-28 position. This suggests a high enzymatic selectivity for the sugar position independent of the genin involved. A shorter sugar chain, made up of two sugars, is present in compounds **8**, **10**, **13**, and **14**, and only one three-sugar chain saponin (**7**) was detected. As in the case of *M. arabica* (*35*), the presence of a high concentration of saponins with relatively small amount of sugars in their structure can explain the high fungitoxic and insecticidal activity of the total saponin mixture from *M. hybrida* root.

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