JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Triterpene Saponins from Barrel Medic (*Medicago truncatula*) Aerial Parts

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Triterpene saponins from *Medicago truncatula* aerial parts have been separated and their structures determined by the extensive use of 1D- and 2D-NMR experiments including $^{1}H-^{1}H$ (DQF-COSY, 1D-TOCSY) and $^{1}H-^{13}C$ (HSQC, HMBC) spectroscopy along with ESIMS. Fifteen individual compounds were isolated that included seven medicagenic acid and eight zanhic acid glycosides. Additionally, two soyasapogenol B and soyasapogenol E glycosides were identified by MS/MS and TLC. Four medicagenic acid glycosides (**5**, **11**, **12**, **14**) and eight zanhic acid glycosides (**1**−**4**, **6**−**9**) are reported here for the first time. The common feature of *M. truncatula* aerial part saponins is the (1→3) linkage between the two glucose units at C-3 of medicagenic and zanhic acids, which is different from that found in alfalfa (*Medicago sativa*), where this linkage was always (1→2). This may suggest differences in glucosyltransferases between these two *Medicago* species.

KEYWORDS: Barrel medic; *Medicago truncatula*; triterpene saponins

INTRODUCTION

Saponins are glycosylated plant secondary metabolites found in many major food crops. They consist of a triterpenoid or steroid aglycon attached to various numbers of sugar side chains. Due to their chemical structures, saponins may show different biological activities: they may show toxicity to insect (*1*−*3*), they may reduce digestibility of protein in ruminants, and they may express antinutritional properties (*4*). Many have potent antifungal properties (*5*). Certain saponins have also been recognized as potent anticancer agents (*6*).

Barrel medic (*Medicago truncatula* Gaertn.) is closely related to alfalfa (*Medicago sativa* L.) and has been chosen as a model legume because of its prolific nature, small diploid genome, self-fertilization, ease of genetic transformation, and rapid regeneration time (*7*). Genes from *M. truncatula* share very high sequence identity to the corresponding genes from alfalfa and appear to be arranged on chromosomes in an order similar to those of other legumes. These features make *M. truncatula* an excellent model for studying biological aspects that are unique to legumes (*7*). *M. truncatula* has been targeted in a functional genomics projects that incorporate profiling of gene expression (transcriptome), protein expression (proteome), and metabolite expression (metabolome) to better understand the biological processes associated with legumes and their interaction with environment (*8*). Profiling and identification of a large variety of natural products from *M. truncatula*, including saponins, is crucial to metabolomics and functional genomics (*9*, *10*).

In alfalfa, the primary saponins are the glycosides of medicagenic acid, zanhic acid, hederagenin, bayogenin, and soyasapogenols (*4*, *5*, *11*, *12*). It was also shown that chromatographic profiles of saponins in aerial parts of different cultivars of *M. sativa* are similar (*13*). However, little is known about the saponin profile of *M. truncatula*. The only work was performed on roots of this species using an LC-MS/MS technique by Huhman and Sumner (*10*). Thus, the aim of our present work was the chemical characterization of all saponins present in *M. truncatula* aerial parts. This resulted in the chemical characterization of dominant saponins and in the identification of a number of new compounds.

MATERIALS AND METHODS

Spectroscopic Analysis. Optical rotations were obtained in MeOH at 20 °C on a Jasco P-1020 spectropolarimeter (Jasco Inc., Easton, MD). ESI-MS was performed on a Finnigan LC-Q Deca ion trap mass spectrometer (Thermo Electron Corp., Bellefonte, PA) scanned from 150 to 1200 Da. The mass spectroscopic data were acquired and processed using Xcalibur software (negative and positive modes). Samples were dissolved in MeOH and infused in the ESI source by using a syringe pump at a flow rate of 3 μL/min. The capillary voltage was 5 V, the spray voltage 5 kV, and the tube lens offset 50 V. The capillary temperature was 220 °C.

Exact masses were measured by a Voyager DE mass spectrometer (Applied Biosystems, Foster City, CA). Samples were analyzed by matrix-assisted laser desorption ionization (MALDI) mass spectrometry.

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10.1021/jf048178i CCC: $30.25
Published on Web 02/18/2005

A mixture of analyte solution and α-cyano-4-hydroxycinnamic acid (Sigma) was applied to the metallic sample plate and dried. Mass calibration was performed with the ions from ACTH (fragment 18−39) at 2465.1989 Da and angiotensin III at 931.5154 Da as internal standard.

NMR spectra in CD_3OD were obtained using a Bruker DRX-600 spectrometer (Bruker BioSpin GmbH, Rheinstetten, Germany), operating at 599.19 MHz for ^1H and at 150.86 MHz for ^{13}C. Two-dimensional (2D) experiments, $^1H-^1H$ DQF-COSY (double filtered direct chemical shift correlation spectroscopy), inverse detected $^1H-^{13}C$ HSQC (heteronuclear single quantum coherence), and HMBC (heteronuclear multiple bond connectivity), were obtained using UXNMR software. Selective excitation spectra, 1D-TOCSY, were acquired using waveform generator-based Gauss-shaped pulses, the mixing time ranging from 100 to 120 ms and a MLEV-17 spin-lock field of 10 kHz preceded by a 2.5 ms trim pulse.

Plant Material. Seeds of *M. truncatula* (A17 Jemalong) were obtained from Dr. XianZhi He, The Samuel Roberts Noble Foundation, Ardmore, OK, where a voucher specimen is deposited, and from Dr. J. M. Prosperi, INRA-SGAP Montpellier, France. *M. truncatula* was cultivated in an experimental field of the Institute of Soil Science and Plant Cultivation in Pulawy, Poland. Plants were harvested at the beginning of flowering. The plant material was dried, finely powdered, and used for the successive extraction.

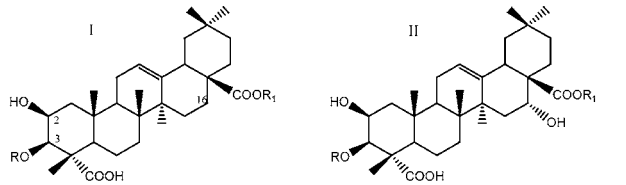
Extraction. *M. truncatula* aerial parts that had been powdered (300 g) were extracted with 80% MeOH at room temperature. After 72 h, the extract was filtered and the residues were extracted two times with 80% MeOH by boiling for 2 h. The extracts were combined, and the solvent was removed under reduced pressure.

Purification. The crude extract (20 g) was suspended in water, and the solution was applied to a 6 cm × 10 cm, 40−63 μm LiChroprep RP-18 glass column previously preconditioned with water. The column was washed first with water to remove sugars and then with 40% MeOH to elute phenolics. Total saponins were eluted with 70% MeOH (*14*).

Fractionation and Separation. Crude saponins powder (2 g) was suspended in distilled water and loaded onto a 3 cm × 40 cm, 40−63 μm LiChroprep RP-18 column (Millipore Corp., Bedford, MA). The column was washed with a 0−100% linear gradient of MeOH in water (Linear Gradient Former, Beckman Instrument Inc., Palo Alto, CA). Ten milliliter fractions were collected and checked by TLC on silica gel (Si 60 F_{254} 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. The fractions showing similar profiles were combined. Column chromatography gave 13 fractions, each comprising three to five major saponins.

Single saponins were separated from each fraction by silica gel (Si60, Merck) chromatography on a 20 cm × 1 cm, 15−25 μm, LiChroprep eluted with ethyl acetate/acetic acid/water (9:2:2). In this way 15 (**1**−**15**) pure compounds were isolated (**Figure 1**).

*Compound **1**:* amorphous powder (4.8 mg); $[\alpha]_D^{20}$ −17.97° (MeOH, *c* 0.1); ESI-MS, 1143 $[M + Na]^+$, 981 $[M + Na - hexose]^+$, 865 $[M + Na - deoxyhexose - pentose]^-$, 703 $[M + Na - deoxyhexose - pentose - hexose]^+$; HRMALDIMS, *m/z* $[M + Na]^+$ calcd for $C_{53}H_{84}O_{25}Na$, 1143.5199, found, 1143.5187; ^1H and ^{13}C NMR data of the aglycon moiety superimposable on those reported for compound **8**. ^1H NMR data of the sugar portion: δ 4.43 (d, *J* = 7.5 Hz, H-1 GlcI), 3.44 (dd, *J* = 7.5 and 9.0 Hz, H-2 GlcI), 3.55 (dd, *J* = 9.0 and 9.1 Hz, H-3 GlcI), 3.49 (dd, *J* = 9.1 and 9.1 Hz, H-4 GlcI), 3.30 (m, H-5 GlcI), 3.74 (dd, *J* = 5.0 and 12.0 Hz, H-6_a GlcI), 3.83 (dd, *J* = 3.5 and 12.0 Hz, H-6_b GlcI), 4.59 (d, *J* = 7.5 Hz, H-1 GlcII), 3.28 (dd, *J* = 7.5 and 9.1 Hz, H-2 GlcII), 3.40 (dd, *J* = 9.1 and 9.1 Hz, H-3 GlcII), 3.30 (dd, *J* = 9.1 and 9.1 Hz, H-4 GlcII), 3.33 (m, H-5 GlcII), 3.65 (dd, *J* = 5.0 and 12.0 Hz, H-6_a GlcII), 3.89 (dd, *J* = 2.0 and 12.0 Hz, H-6_b GlcII), 5.77 (d, *J* = 2.6 Hz, H-1 Ara), 3.79 (dd, *J* = 2.6 and 8.0 Hz, H-2 Ara), 3.93 (dd, *J* = 4.0 and 8.0 Hz, H-3 Ara), 3.87 (m, H-4 Ara), 3.49 (dd, *J* = 3.0 and 12.0 Hz, H-5_a Ara), 3.95 (dd, *J* = 2.0 and 12.0 Hz, H-5_b Ara), 4.97 (d, *J* = 1.5 Hz, H-1 Rha), 3.83 (dd, *J* = 1.5 and 2.5 Hz, H-2 Rha), 3.65 (dd, *J* = 2.5 and 9.0 Hz, H-3 Rha), 3.40 (dd, *J* = 9.0 and 9.0 Hz, H-4 Rha), 3.67 (m, H-5 Rha), 1.30 (d, *J* = 6.5 Hz, H-6 Rha). For ^{13}C NMR data of the sugar portion, see **Table 1**.



Compound	Aglycon	R	R_1
1	II	β-Glc-(1→3)-β-Glc	α-Rha-(1→2)-α-Ara
2	II	β-Glc-(1→3)-β-Glc	β-Api-(1→3)-α-Rha-(1→2)-α-Ara
3	II	β-Glc-(1→3)-β-Glc	β-Xyl-(1→4)-α-Rha-(1→2)-α-Ara α-Ara-(1→3)┘
4	II	β-Glc-(1→3)-β-Glc	α-Ara-(1→3)-α-Rha-(1→2)-α-Ara
5	I	β-GlcA	β-Glc
6	II	β-Glc-(1→3)-β-Glc	β-Xyl-(1→4)-α-Rha-(1→2)-α-Ara β-Api-(1→3)┘
7	II	β-Glc-(1→3)-β-Glc	β-Xyl-(1→4)-α-Rha-(1→2)-α-Ara
8	II	β-Glc	β-Xyl-(1→4)-α-Rha-(1→2)-α-Ara
9	II	β-Glc-(1→3)-β-Glc	α-Rha-[4-Ac]-(1→2)-α-Ara
10	I	β-GlcA	β-Xyl-(1→4)-α-Rha-(1→2)-α-Ara
11	I	β-Glc-(1→3)-β-Glc	α-Rha-(1→2)-α-Ara
12	I	β-Glc(1→3)-β-Glc	β-Xyl-(1→4)-α-Rha-(1→2)-α-Ara α-Ara-(1→3)┘
13	I	β-Glc-(1→3)-β-Glc	β-Xyl-(1→4)-α-Rha-(1→2)-α-Ara
14	I	β-Glc-(1→3)-β-Glc	β-Xyl-(1→4)-α-Rha-(1→2)-α-Ara β-Api-(1→3)┘
15	I	β-Glc	β-Xyl-(1→4)-α-Rha-(1→2)-α-Ara

Figure 1. Chemical formulas of identified compounds (I, medicagenic acid; II, zanhic acid).

*Compound **2**:* amorphous powder (2.1 mg); $[\alpha]_D^{20}$ −12.05° (MeOH, *c* 0.1); ESI-MS, 1275 $[M + Na]^+$, 865 $[M + Na - deoxyhexose - 2 pentoses]^+$, 703 $[M + Na - deoxyhexose - 2 pentoses - hexose]^+$; HRMALDIMS, *m/z* $[M + Na]^+$ calcd for $C_{58}H_{92}O_{29}Na$, 1275.5622, found, 1275.5615; ^1H and ^{13}C NMR data of the aglycon moiety superimposable on those reported for compound **8**. ^1H NMR data of the sugar portion: δ 4.45 (d, *J* = 7.5 Hz, H-1 GlcI), 3.44 (dd, *J* = 7.5 and 9.0 Hz, H-2 GlcI), 3.57 (dd, *J* = 9.0 and 9.1 Hz, H-3 GlcI), 3.49 (dd, *J* = 9.1 and 9.1 Hz, H-4 GlcI), 3.30 (m, H-5 GlcI), 3.74 (dd, *J* = 5.0 and 12.0 Hz, H-6_a GlcI), 3.83 (dd, *J* = 3.5 and 12.0 Hz, H-6_b GlcI), 4.59 (d, *J* = 7.5 Hz, H-1 GlcII), 3.28 (dd, *J* = 7.5 and 9.1 Hz, H-2 GlcII), 3.39 (dd, *J* = 9.1 and 9.1 Hz, H-3 GlcII), 3.29 (dd, *J* = 9.1 and 9.1 Hz, H-4 GlcII), 3.32 (m, H-5 GlcII), 3.65 (dd, *J* = 5.0 and 12.0 Hz, H-6_a GlcII), 3.90 (dd, *J* = 2.0 and 12.0 Hz, H-6_b GlcII), 5.72 (d, *J* = 2.6 Hz, H-1 Ara), 3.80 (dd, *J* = 2.6 and 8.0 Hz, H-2 Ara), 3.93 (dd, *J* = 4.0 and 8.0 Hz, H-3 Ara), 3.89 (m, H-4 Ara), 3.52 (dd, *J* = 3.0 and 12.0 Hz, H-5_a Ara), 3.95 (dd, 2.0 and 12.0 Hz, H-5_b Ara), 4.98 (d, *J* = 1.5 Hz, H-1 Rha), 4.00 (dd, *J* = 1.5 and 2.5 Hz, H-2 Rha), 3.68 (dd, *J* = 2.5 and 9.0 Hz, H-3 Rha), 3.52 (dd, *J* = 9.0 and 9.0 Hz, H-4 Rha), 3.63 (m, H-5 Rha), 1.30 (d, *J* = 6.5 Hz, H-6 Rha), 5.20 (d, *J* = 3.7 Hz, H-1 Api), 4.02 (d, *J* = 3.7 Hz, H-2 Api), 3.78 (d, *J* = 10.0 Hz, H-4_a Api), 4.09 (d, *J* = 10.0 Hz, H-4_b Api), 3.63 (br s, H-5 Api). For ^{13}C NMR data of the sugar portion, see **Table 1**.

*Compound **3**:* amorphous powder (3 mg); $[\alpha]_D^{20}$ −14.6° (MeOH, *c* 0.1); ESI-MS, 1407 $[M + Na]^+$, 1275 $[M + Na - pentose]^+$, 1245 $[M + Na - hexose]^+$, 1083 $[M + Na - 2 hexoses]^+$, 865 $[M + Na - 3 pentoses - deoxyhexose]^+$; HRMALDIMS, *m/z* $[M + Na]^+$ calcd for $C_{63}H_{100}O_{33}Na$, 1407.6045, found, 1407.6035; ^1H and ^{13}C NMR data of the aglycon moiety superimposable on those reported for compound **8**. ^1H NMR data of the sugar portion: δ 4.42 (d, *J* = 7.5 Hz, H-1 GlcI), 3.44 (dd, *J* = 7.5 and 9.0 Hz, H-2 GlcI), 3.55 (dd, *J* = 9.0 and 9.1 Hz, H-3 GlcI), 3.49 (dd, *J* = 9.1 and 9.1 Hz, H-4 GlcI), 3.29 (m, H-5 GlcI), 3.73 (dd, *J* = 5.0 and 12.0 Hz, H-6_a GlcI), 3.83 (dd, *J* = 3.5 and 12.0 Hz, H-6_b GlcI), 4.59, (d, *J* = 7.5 Hz, H-1 GlcII), 3.28

Table 1. ^{13}C NMR Data of the Sugar Portions of Zanhic Acid Glycosides **1**–**4**, **8**, and **9**[a]

	δ					
C	**1**	**2**	**3**	**4**	**8**	**9**
	glucose I	glucose I	glucose I	glucose I	glucose	glucose I
C-1	104.6	104.6	104.9	104.7	104.8	104.8
C-2	74.7	74.8	74.7	74.7	75.8	74.5
C-3	87.4[b]	87.3	87.5	87.5	77.7	87.2
C-4	69.6	69.7	69.7	69.7	71.0	69.5
C-5	77.8	77.9	78.5	77.9	78.2	77.3
C-6	62.2	62.2	62.2	62.2	62.2	62.0
	glucose II	glucose II	glucose II	glucose II		glucose II
C-1	105.4	105.2	105.4	105.3		105.8
C-2	75.6	75.6	75.6	75.6		75.4
C-3	77.9	77.9	77.9	77.9		77.7
C-4	71.6	71.7	71.6	71.6		71.4
C-5	78.2	78.2	78.2	78.2		77.8
C-6	62.7	62.6	62.6	62.6		62.5
	arabinose	arabinose	arabinose I	arabinose I	arabinose	arabinose
C-1	93.8	93.8	94.1	93.9	93.9	94.2
C-2	76.3	76.0	75.5	75.9	75.6	76.1
C-3	70.2	70.5	70.4	70.6	71.0	71.1
C-4	66.2	66.4	66.9	66.7	66.9	66.8
C-5	62.7	63.0	63.3	63.2	63.2	63.7
	rhamnose	rhamnose	rhamnose	rhamnose	rhamnose	rhamnose
C-1	101.9	101.9	101.0	101.3	101.4	101.6
C-2	72.3	72.3	72.2	72.0	72.1	72.3
C-3	72.0	80.5	82.6	82.5	72.1	70.0
C-4	73.8	72.8	78.4	72.8	83.3	75.2
C-5	70.5	70.4	69.1	70.2	68.9	68.2
C-6	17.9	17.9	18.3	18.1	17.9	17.8
4-COMe						172.7
4-COMe						21.1
		apiose	arabinose II	arabinose II	xylose	
C-1		112.6	106.3	106.6	106.4	
C-2		78.3	73.0	73.0	75.4	
C-3		80.0	74.6	74.1	77.7	
C-4		74.9	70.0	69.7	70.9	
C-5		65.2	67.2	67.1	66.9	
			xylose			
C-1			105.2			
C-2			75.9			
C-3			77.9			
C-4			71.4			
C-5			67.0			

[a] Assignment based on DQF-COSY, 1D-TOCSY, HSQC, and HMBC experiments. [b] Substituted carbons are underlined.

(dd, J = 7.5 and 9.1 Hz, H-2 GlcII), 3.39 (dd, J = 9.1 and 9.1 Hz, H-3 GlcII), 3.30 (dd, J = 9.1 and 9.1 Hz, H-4 GlcII), 3.34 (m, H-5 GlcII), 3.65 (dd, J = 5.0 and 12.0 Hz, H-6$_a$ GlcII), 3.89 (dd, J = 2.0 and 12.0 Hz, H-6$_b$ GlcII), 5.67 (d, J = 2.6 Hz, H-1 AraI), 3.80 (dd, J = 2.6 and 8.0 Hz, H-2 AraI), 3.92 (dd, J = 4.0 and 8.0 Hz, H-3 AraI), 3.86 (m, H-4 AraI), 3.52 (dd, J = 3.0 and 12.0 Hz, H-5$_a$ AraI), 3.94 (dd, J = 2.0 and 12.0 Hz, H-5$_b$ AraI), 5.02 (d, J = 1.5 Hz, H-1 Rha), 4.07 (dd, J = 1.5 and 2.5 Hz, H-2 Rha), 3.91 (dd, J = 2.5 and 9.0 Hz, H-3 Rha), 3.71 (dd, J = 9.0 and 9.0 Hz, H-4 Rha), 3.77 (m, H-5 Rha), 1.28 (d, J = 6.5 Hz, H-6 Rha), 4.54 (d, J = 7.4 Hz, H-1 AraII), 3.64 (dd, J = 7.4 and 9.0 Hz, H-2 AraII), 3.50 (dd, J = 3.9 and 9.0 Hz, H-3 AraII), 3.82 (m, H-4 AraII), 3.58 (dd, J = 2.0 and 11.5 Hz, H-5$_a$ AraII), 3.89 (dd, J = 4.0 and 11.5 Hz, H-5$_b$ AraII), 4.74 (d, J = 7.8 Hz, H-1 Xyl), 3.12 (dd, J = 7.8 and 9.0 Hz, H-2 Xyl), 3.30 (dd, J = 9.0 and 9.0 Hz, H-3 Xyl), 3.50 (m, H-4 Xyl), 3.19 (dd, J = 11.0 and 11.0 Hz, H-5$_a$ Xyl), 3.86 (dd, J = 11.0 and 5.0 Hz, H-5$_b$ Xyl). For ^{13}C NMR data of the sugar portion, see **Table 1**.

Compound 4: amorphous powder (15 mg); $[\alpha]_D^{20}$ −14.18° (MeOH, *c* 0.1); ESI-MS, 1275 [M + Na]$^+$, 1143 [M + Na − pentose]$^+$, 951 [M + Na − 2 hexoses]$^+$, 865 [M + Na − 2 pentoses − deoxyhexose]$^+$, 703 [M + Na − 2 pentoses − deoxyhexose − hexose]$^+$; HRMALDIMS, *m/z* [M + Na]$^+$ calcd for $C_{58}H_{92}O_{29}Na$, 1275.5622, found, 1275.5613; ^{1}H and ^{13}C NMR data of the aglycon moiety superimposable on those reported for compound **8**. ^{1}H NMR data of the sugar portion: δ 4.43 (d, J = 7.5 Hz, H-1 GlcI), 3.44 (dd, J = 7.5 and 9.0 Hz, H-2 GlcI), 3.55 (dd, J = 9.0 and 9.1 Hz, H-3 GlcI), 3.49 (dd, J = 9.1 and 9.1 Hz, H-4 GlcI), 3.30 (m, H-5 GlcI), 3.75 (dd, J = 5.0 and 12.0 Hz, H-6$_a$ GlcI), 3.84 (dd, J = 3.5 and 12.0 Hz, H-6$_b$ GlcI), 4.59 (d, J = 7.5 Hz, H-1 GlcII), 3.28 (dd, J = 7.5 and 9.1 Hz, H-2 GlcII), 3.39 (dd, J = 9.1 and 9.1 Hz, H-3 GlcII), 3.30 (dd, J = 9.1 and 9.1 Hz, H-4 GlcII), 3.33 (m, H-5 GlcII), 3.66 (dd, J = 5.0 and 12.0 Hz, H-6$_a$ GlcII), 3.90 (dd, J = 2.0 and 12.0 Hz, H-6$_b$ GlcII), 5.72 (d, J = 2.6 Hz, H-1 AraI), 3.82 (dd, J = 2.6 and 8.0 Hz, H-2 AraI), 3.93 (dd, J = 4.0 and 8.0 Hz, H-3 AraI), 3.87 (m, H-4 AraI), 3.53 (dd, J = 3.0 and 12.0 Hz, H-5$_a$ AraI), 3.96 (dd, J = 2.0 and 12.0 Hz, H-5$_b$ AraI), 5.03 (d, J = 1.5 Hz, H-1 Rha), 4.08 (dd, J = 1.5 and 2.5 Hz, H-2 Rha), 3.72 (dd, J = 2.5 and 9.0 Hz, H-3 Rha), 3.58 (dd, J = 9.0 and 9.0 Hz, H-4 Rha), 3.74 (m, H-5 Rha), 1.29 (d, J = 6.5 Hz, H-6 Rha), 4.48 (d, J = 7.4 Hz, H-1 AraII), 3.66 (dd, J = 7.4 and 9.0 Hz, H-2 AraII), 3.56 (dd, J = 3.9 and 9.0 Hz, H-3 AraII), 3.83 (m, H-4 AraII), 3.59 (dd, J = 2.0 and 11.5 Hz, H-5$_a$ AraII), 3.89 (dd, J = 4.0 and 11.5 Hz, H-5$_b$ AraII); for ^{13}C NMR data of the sugar portion, see **Table 1**.

Compound 5: amorphous powder (3.2 mg); $[\alpha]_D^{20}$ −25.29° (MeOH, *c* 0.1); ESI-MS, 863 [M + Na]$^+$, 687 [M + Na − uronic acid]$^+$, 525 [M + Na − uronic acid-glucose]$^+$; HRMALDIMS, *m/z* [M + Na]$^+$ calcd for $C_{42}H_{64}O_{17}Na$, 863.4041, found, 863.4033. ^{1}H NMR data of the aglycon moiety: δ 5.30 (t, J = 3.4 Hz, H-12), 4.38 (ddd, J = 3.0, 4.0 and 4.0 Hz, H-2), 4.12 (d, J = 4.0 Hz, H-3), 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 of the aglycon moiety: δ 44.7 (C-1), 71.0 (C-2), 86.2 (C-3), 53.8 (C-4), 52.8 (C-5), 21.6 (C-6), 33.2 (C-7), 40.9 (C-8), 49.0 (C-9), 37.2 (C-10), 24.5 (C-11), 123.6 (C-12), 144.8 (C-13), 43.3 (C-14), 28.6 (C-15), 23.8 (C-16), 47.9 (C-17), 42.5 (C-18), 47.1 (C-19), 31.2 (C-20), 34.5 (C-21), 33.4 (C-22), 182.0 (C-23), 14.4 (C-24), 17.2 (C-25), 17.6 (C-26), 26.3 (C-27), 178.0 (C-28), 33.3 (C-29), 23.8 (C-30). ^{1}H NMR data of the sugar portion: δ 4.40 (d, J = 7.5 Hz, H-1 GlcA), 3.29 (dd, J = 7.5 and 9.0 Hz, H-2 GlcA), 3.42 (dd, J = 9.0 and 9.0 Hz, H-3 GlcA), 3.42 (dd, J = 9.0 and 9.0 Hz, H-4 GlcA), 3.60 (d, J = 9.0 Hz, H-5 GlcA), 5.41 (d, J = 7.5 Hz, H-1 Glc), 3.34 (dd, J = 7.5 and 9.1 Hz, H-2 Glc), 3.38 (dd, J = 9.1 and 9.1 Hz, H-3 Glc), 3.37 (dd, J = 9.1 and 9.1 Hz, H-4 Glc), 3.42 (m, H-5 Glc), 3.70 (dd, J = 5.0 and 12.0 Hz, H-6$_a$ Glc), 3.84 (dd, J = 2.0 and 12.0 Hz, H-6$_b$ Glc); for ^{13}C NMR data of the sugar portion, see **Table 2**.

Compound 6: amorphous powder (11.5 mg); $[\alpha]_D^{20}$ −22.54° (MeOH, *c* 0.1); ESI-MS (negative ion mode), 1383 [M − H]$^-$; HRMALDIMS, *m/z* [M + Na]$^+$ calcd for $C_{63}H_{100}O_{33}Na$, 1407.6045, found, 1407.6036; ^{1}H and ^{13}C NMR data of the aglycon moiety superimposable on those reported for compound **8**; ^{1}H and ^{13}C NMR data of the sugar portion superimposable on those reported for compound **14**.

Compound 7: amorphous powder (10.6 mg); $[\alpha]_D^{20}$ −29.50° (MeOH, *c* 0.1); ESI-MS, 1251 [M − H]$^-$, 841 [M − H − deoxyhexose − 2 pentoses]$^-$; HRMALDIMS, *m/z* [M + Na]$^+$ calcd for $C_{58}H_{92}O_{29}Na$, 1275.5622, found, 1275.5617; ^{1}H and ^{13}C NMR data of the aglycon moiety superimposable on those reported for compound **8**; ^{1}H and ^{13}C NMR data of the sugar portion superimposable on those reported for compound **13**.

Compound 8: amorphous powder (1.1 mg); $[\alpha]_D^{20}$ −20.06° (MeOH, *c* 0.1); ESI-MS, 1089 [M − H]$^-$, 957 [M − H − pentose]$^-$, 679 [M − H − deoxyhexose − 2 pentoses]$^-$; HRMALDIMS, *m/z* [M + Na]$^+$ calcd for $C_{52}H_{82}O_{24}Na$, 1113.5094, found, 1113.5089. ^{1}H NMR data of the aglycon moiety: δ 5.31 (t, J = 3.4 Hz, H-12), 4.53 (br m, $W_{1/2}$ = 7.0 Hz, H-16) 4.35 (ddd, J = 3.0, 3.8 and 4.0 Hz, H-2), 4.13 (d, J = 4.0 Hz, H-3), 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). ^{13}C NMR of the aglycon moiety: δ 44.7 (C-1), 71.1 (C-2), 86.0 (C-3), 53.2 (C-4), 53.2 (C-5), 21.4 (C-6), 33.8 (C-7), 41.1 (C-8), 49.0 (C-9), 37.4 (C-10), 24.6 (C-11), 123.6 (C-12), 144.7 (C-13), 42.7 (C-14), 36.4 (C-15), 74.8 (C-16), 50.2 (C-17), 42.0 (C-18), 47.6 (C-19), 31.3 (C-20), 36.4 (C-21), 33.4 (C-22), 181.8 (C-23), 13.9 (C-24), 17.2 (C-25), 17.7 (C-26), 27.5 (C-27), 177.0 (C-28), 33.4 (C-29), 25.2 (C-30). ^{1}H NMR data of the sugar portion: δ 4.45 (d, J = 7.5 Hz, H-1 Glc), 3.24 (dd, J = 7.5 and 9.0 Hz, H-2 Glc), 3.38 (dd, J = 9.0 and 9.1 Hz, H-3 Glc), 3.37 (dd, J = 9.1 and 9.1 Hz, H-4 Glc), 3.32 (m, H-5 Glc), 3.70 (dd, J = 5.0 and 12.0 Hz, H-6$_a$ Glc), 3.83 (dd, J = 3,5 and 12.0 Hz, H-6$_b$ Glc), 5.66 (d, J = 2.6 Hz, H-1 Ara), 3.82 (dd, J = 2.6 and 8.0 Hz, H-2 Ara), 3.90 (dd, J = 4.0 and 8.0 Hz, H-3 Ara), 3.86 (m, H-4 Ara), 3.52 (dd, J = 3.0 and 12.0 Hz, H-5$_a$ Ara), 3.93 (dd,

Table 2. ^{13}C NMR Data of the Sugar Portions of Medicagenic Acid Glycosides **5**, **11**, **12**, and **14**[a]

C	5	11	12	14
	glucuronic acid	glucose I	glucose I	glucose I
C-1	105.2	104.6	104.4	104.6
C-2	75.2	74.7	74.6	74.8
C-3	77.8	87.2	87.0	87.2
C-4	73.6	69.5	69.5	69.5
C-5	76.3	77.7	78.5	77.9
C-6	173.0	62.0	62.1	62.0
		glucose II	glucose II	glucose II
C-1		105.3	105.0	105.3
C-2		75.6	75.0	75.6
C-3		77.7	77.8	77.8
C-4		71.5	71.5	71.4
C-5		77.8	78.2	77.9
C-6		62.6	62.6	62.6
	glucose	arabinose	arabinose I	arabinose
C-1	96.5	93.5	93.5	93.5
C-2	73.9	76.2	75.5	76.2
C-3	78.8	70.0	70.4	69.9
C-4	71.1	66.0	66.7	66.3
C-5	77.8	62.3	63.3	62.5
C-6	62.4			
		rhamnose	rhamnose	rhamnose
C-1		101.8	100.7	101.3
C-2		72.2	72.0	71.8
C-3		72.0	82.2	81.6
C-4		73.6	78.9	78.2
C-5		70.5	68.9	69.0
C-6		17.8	18.3	18.0
			arabinose II	apiose
C-1	C-1		106.1	112.2
C-2	C-2		72.8	78.1
C-3	C-3		74.4	80.0
C-4	C-4		69.9	74.9
C-5	C-5		67.2	64.8
			xylose	xylose
C-1	C-1		104.8	105.3
C-2	C-2		75.7	75.5
C-3	C-3		77.9	77.9
C-4	C-4		71.2	71.2
C-5	C-5		66.7	66.9

[a] Assignment based on DQF-COSY, 1D-TOCSY, HSQC, and HMBC experiments. [b] Substituted carbons are underlined.

J = 2.0 and 12.0 Hz, H-5$_b$ Ara), 5.06 (d, J = 1.5 Hz, H-1 Rha), 3.87 (dd, J = 1.5 and 2.5 Hz, H-2 Rha), 3.87 (dd, J = 2.5 and 9.0 Hz, H-3 Rha), 3.59, (dd, J = 9.0 and 9.0 Hz, H-4 Rha), 3.72 (m, H-5 Rha), 1.32 (d, J = 6.5 Hz, H-6 Rha), 4.53 (d, J = 7.8 Hz, H-1 Xyl), 3.22 (dd, J = 7.8 and 9.0 Hz, H-2 Xyl), 3.30 (dd, J = 9.0 and 9.0 Hz, H-3 Xyl), 3.52 (m, H-4 Xyl), 3.20 (dd, J = 11.0 and 11.0 Hz, H-5$_a$ Xyl), 3.88 (dd, J = 11.0 and 5.0 Hz, H-5$_b$ Xyl); for ^{13}C NMR data of the sugar portion, see **Table 1**.

*Compound **9**:* amorphous powder (28.7 mg); $[\alpha]_D^{20}$ −17.81° (MeOH, *c* 0.1); ESI-MS, 1161 [M − H]$^-$, 1119 [M − H − COCH$_2$]$^-$, 841 [M − H − COCH$_2$ − deoxyhexose − pentose]$^-$; HRMALDIMS, *m*/*z* [M + Na]$^+$ calcd for $C_{55}H_{86}O_{26}Na$, 1185.5305, found, 1185.5292; ^{1}H and ^{13}C NMR data of the aglycon moiety superimposable on those reported for compound **8**. ^{1}H NMR data of the sugar portion: δ 4.45 (d, J = 7.5 Hz, H-1 GlcI), 3.45 (dd, J = 7.5 and 9.0 Hz, H-2 GlcI), 3.57 (dd, J = 9.0 and 9.1 Hz, H-3 GlcI), 3.50 (dd, J = 9.1 and 9.1 Hz, H-4 GlcI), 3.32 (m, H-5 GlcI), 3.75 (dd, J = 5.0 and 12.0 Hz, H-6$_a$ GlcI), 3.85 (dd, J = 3.5 and 12.0 Hz, H-6$_b$ GlcI), 4.61 (d, J = 7.5 Hz, H-1 GlcII), 3.29 (dd, J = 7.5 and 9.1 Hz, H-2 GlcII), 3.41 (dd, J = 9.1 and 9.1 Hz, H-3 GlcII), 3.30 (dd, J = 9.1 and 9.1 Hz, H-4 GlcII), 3.33 (m, H-5 GlcII), 3.67 (dd, J = 5.0 and 12.0 Hz, H-6$_a$ GlcII), 3. 91 (dd, J = 2.0 and 12.0 Hz, H-6$_b$ GlcII), 5.68 (d, J = 2.6 Hz, H-1 Ara), 3.82 (dd, J = 2.6 and 8.0 Hz, H-2 Ara), 3.92 (dd, J = 4.0 and 8.0 Hz, H-3 Ara), 3.87 (m, H-4 Ara), 3.55 (dd, J = 3.0 and 12.0 Hz, H-5$_a$ Ara), 3.95 (dd, J = 2.0 and 12.0 Hz, H-5$_b$ Ara), 5.12 (d, J = 1.5 Hz, H-1 Rha), 3.91 (dd, J = 1.5 and 2.5 Hz, H-2 Rha), 3.81 (dd, J = 2.5 and 9.0 Hz, H-3 Rha), 4.95 (dd, J = 9.0 and 9.0 Hz, H-4 Rha), 3.83 (m, H-5 Rha), 1.18 (d, J = 6.5 Hz, H-6 Rha), 2.11 (s, COCH$_3$-4); for ^{13}C NMR data of the sugar portion, see **Table 1**.

*Compound **10**:* amorphous powder (11.2 mg) with spectroscopic characteristics similar to those previously published (*5*).

*Compound **11**:* amorphous powder (6.4 mg); $[\alpha]_D^{20}$ −0.22° (MeOH, *c* 0.1); ESI-MS, 1103 [M − H]$^-$; HRMALDIMS, *m*/*z* [M + Na]$^+$ calcd for $C_{53}H_{84}O_{24}Na$, 1127.5250, found, 1127.5244; ^{1}H and ^{13}C NMR data of the aglycon moiety superimposable on those reported for compound **5**. ^{1}H NMR data of the sugar portion: δ 4.46 (d, J = 7.5 Hz, H-1 GlcI), 3.45 (dd, J = 7.5 and 9.0 Hz, H-2 GlcI), 3.57 (dd, J = 9.0 and 9.1 Hz, H-3 GlcI), 3.49 (dd, J = 9.1 and 9.1 Hz, H-4 GlcI), 3.32 (m, H-5 GlcI), 3.74 (dd, J = 5.0 and 12.0 Hz, H-6$_a$ GlcI), 3.84 (dd, J = 3.5 and 12.0 Hz, H-6$_b$ GlcI), 4.61 (d, J = 7.5 Hz, H-1 GlcII), 3.28 (dd, J = 7.5 and 9.1 Hz, H-2 GlcII), 3.41 (dd, J = 9.1 and 9.1 Hz, H-3 GlcII), 3.30 (dd, J = 9.1 and 9.1 Hz, H-4 GlcII), 3.33 (m, H-5 GlcII), 3.67 (dd, J = 5.0 and 12.0 Hz, H-6$_a$ GlcII), 3.91 (dd, J = 2.0 and 12.0 Hz, H-6$_b$ GlcII), 5.83 (d, J = 2.6 Hz, H-1 Ara), 3.83 (dd, J = 2.6 and 8.0 Hz, H-2 Ara), 3.96 (dd, J = 4.0 and 8.0 Hz, H-3 Ara), 3.90 (m, H-4 Ara), 3.50 (dd, J = 3.0 and 12.0 Hz, H-5$_a$ Ara), 3.96 (dd, J = 2.0 and 12.0 Hz, H-5$_b$ Ara), 4.98 (d, J = 1.5 Hz, H-1 Rha), 3.86 (dd, J = 1.5 and 2.5 Hz, H-2 Rha), 3.67 (dd, J = 2.5 and 9.0 Hz, H-3 Rha), 3.42 (dd, J = 9.0 and 9.0 Hz, H-4 Rha), 3.70 (m, H-5 Rha), 1.29 (d, J = 6.5 Hz, H-6 Rha); for ^{13}C NMR data of the sugar portion, see **Table 2**.

*Compound **12**:* amorphous powder (19.8 mg); $[\alpha]_D^{20}$ −15.37° (MeOH, *c* 0.1); ESI-MS, 1367 [M − H]$^-$, 1235 [M − H − pentose]$^-$, 1205 [M − H − hexose]$^-$; HRMALDIMS, *m*/*z* [M + Na]$^+$ calcd for $C_{63}H_{100}O_{32}Na$, 1391.6095, found 1391.6076; ^{1}H and ^{13}C NMR data of the aglycon moiety superimposable on those reported for compound **5**. ^{1}H NMR data of the sugar portion: δ 4.45 (d, J = 7.5 Hz, H-1 GlcI), 3.45 (dd, J = 7.5 and 9.0 Hz, H-2 GlcI), 3.57 (dd, J = 9.0 and 9.1 Hz, H-3 GlcI), 3.49 (dd, J = 9.1 and 9.1 Hz, H-4 GlcI), 3.29 (m, H-5 GlcI), 3.79 (dd, J = 5.0 and 12.0 Hz, H-6$_a$ GlcI), 3.84 (dd, J = 3.5 and 12.0 Hz, H-6$_b$ GlcI), 4.60 (d, J = 7.5 Hz, H-1 GlcII), 3.29 (dd, J = 7.5 and 9.1 Hz, H-2 GlcII), 3.41 (dd, J = 9.1 and 9.1 Hz, H-3 GlcII), 3.80 (dd, J = 9.1 and 9.1 Hz, H-4 GlcII), 3.34 (m, H-5 GlcII), 3.67 (dd, J = 5.0 and 12.0 Hz, H-6$_a$ GlcII), 3.90 (dd, J = 2.0 and 12.0 Hz, H-6$_b$ GlcII), 5.72 (d, J = 2.6 Hz, H-1 AraI), 3.84 (dd, J = 2.6 and 8.0 Hz, H-2 AraI), 3.95 (dd, J = 4.0 and 8.0 Hz, H-3 AraI), 3.87 (m, H-4 AraI), 3.52 (dd, J = 3.0 and 12.0 Hz, H-5$_a$ AraI), 3.95 (dd, J = 2.0 and 12.0 Hz, H-5$_b$ AraI), 5.06 (d, J = 1.5 Hz, H-1 Rha), 4.09 (dd, J = 1.5 and 2.5 Hz, H-2 Rha), 3.92 (dd, J = 2.5 and 9.0 Hz, H-3 Rha), 3.71 (dd, J = 9.0 and 9.0 Hz, H-4 Rha), 3.79 (m, H-5 Rha), 1.29 (d, J = 6.5 Hz, H-6 Rha), 4.53 (d, J = 7.4 Hz, H-1 AraII), 3.65 (dd, J = 7.4 and 9.0 Hz, H-2 AraII), 3.52 (dd, J = 3.9 and 9.0 Hz, H-3 AraII), 3.84 (m, H-4 AraII), 3.60 (dd, J = 2.0 and 11.5 Hz, H-5$_a$ AraII), 3.90 (dd, J = 4.0 and 11.5 Hz, H-5$_b$ AraII), 4.71 (d, J = 7.8 Hz, H-1 Xyl), 3.12 (dd, J = 7.8 and 9.0 Hz, H-2 Xyl), 3.30 (dd, J = 9.0 and 9.0 Hz, H-3 Xyl), 3.51 (m, H-4 Xyl), 3.19 (dd, J = 11.0 and 11.0 Hz, H-5$_a$ Xyl), 3.87 (dd, J = 11.0 and 5.0 Hz, H-5$_b$ Xyl); for ^{13}C NMR data of the sugar portion, see **Table 2**.

*Compound **13**:* amorphous powder (28.8 mg) with spectroscopic characteristics similar to those previously published (*19*).

*Compound **14**:* amorphous powder (8.7 mg); $[\alpha]_D^{20}$ −20.08° (MeOH, *c* 0.1); ESI-MS, 1367 [M − H]$^-$, 825 [M − H − 3 pentoses − deoxyhexose]$^-$; HRMALDIMS, *m*/*z* [M + Na]$^+$ calcd for $C_{63}H_{100}O_{32}$-Na, 1391.6095, found, 1391.6086; ^{1}H and ^{13}C NMR data of the aglycon moiety superimposable on those reported for compound **5**. ^{1}H NMR of the sugar portion: δ 4.46 (d, J = 7.5 Hz, H-1 GlcI), 3.44 (dd, J = 7.5 and 9.0 Hz, H-2 GlcI), 3.57 (dd, J = 9.0 and 9.1 Hz, H-3 GlcI), 3.50 (dd, J = 9.1 and 9.1 Hz, H-4 GlcI), 3.32 (m, H-5 GlcI), 3.75 (dd, J = 5.0 and 12.0 Hz, H-6$_a$ GlcI), 3.85 (dd, J = 3.5 and 12.0 Hz, H-6$_b$ GlcI), 4.61 (d, J = 7.5 Hz, H-1 GlcII), 3.28 (dd, J = 7.5 and 9.1 Hz, H-2 GlcII), 3.41 (dd, J = 9.1 and 9.1 Hz, H-3 GlcII), 3.30 (dd, J = 9.1 and 9.1 Hz, H-4 GlcII), 3.33 (m, H-5 GlcII), 3.67 (dd, J = 5.0 and 12.0 Hz, H-6$_a$ GlcII), 3.90 (dd, J = 2.0 and 12.0 Hz, H-6$_b$ GlcII), 5.79 (d, J = 2.6 Hz, H-1 Ara), 3.82 (dd, J = 2.6 and 8.0 Hz, H-2 Ara), 3.96 (dd, J = 4.0 and 8.0 Hz, H-3 Ara), 3.90 (m, H-4 Ara), 3.51 (dd, J = 3.0 and 12.0 Hz, H-5$_a$ Ara), 3.95 (dd, J = 2.0 and 12.0 Hz, H-5$_b$ Ara), 4.98 (d, J = 1.5 Hz, H-1 Rha), 4.03 (dd, J = 1.5 and 2.5 Hz, H-2

Rha), 3.91 (dd, $J = 2.5$ and 9.0 Hz, H-3 Rha), 3.72 (dd, $J = 9.0$ and 9.0 Hz, H-4 Rha), 3.77 (m, H-5 Rha), 1.29 (d, $J = 6.5$ Hz, H-6 Rha), 5.29 (d, $J = 3.7$ Hz, H-1 Api), 4.07 (d, $J = 3.7$ Hz, H-2 Api), 3.79 (d, $J = 10.0$ Hz, H-4_a Api), 4.13 (d, $J = 10.0$ Hz, H-4_b Api), 3.62 (br s, H-5 Api), 4.65 (d, $J = 7.8$ Hz, H-1 Xyl), 3.18 (dd, $J = 7.8$ and 9.0 Hz, H-2 Xyl), 3.30 (dd, $J = 9.0$ and 9.0 Hz, H-3 Xyl), 3.52 (m, H-4 Xyl), 3.18 (dd, $J = 11.0$ and 11.0 Hz, H-5_a Xyl), 3.88 (dd, $J = 11.0$ and 5.0 Hz, H-5_b Xyl); for ^{13}C NMR data of the sugar portion, see **Table 2**.

*Compound **15**:* amorphous powder (2.7 mg) with spectroscopic characteristics similar to those previously published (*5*).

RESULTS AND DISCUSSION

Preliminary TLC comparison of saponin fractions from aerial parts of *M. sativa* and *M. truncatula* showed distinct differences between the two species especially in the more polar (lower R_f values) region. Spraying with Lieberman−Burchard reagent indicated that both species contained medicagenic acid glycosides (blue or greenish spots) and zanhic acid glycosides (green-yellow spots). Separation of *M. truncatula* saponins on an RP18 column in a gradient of water/methanol resulted in a number of fractions consisting of a few glycosides with very similar polarities. Thus, it was necessary to further purify these fractions on a silica gel column using acidified solvent suppressing ion formation on the column. As a result, 15 dominant saponins were isolated from the aerial parts of *M. truncatula*.

Compounds **5** and **10**−**15** in the aglycon part showed similar NMR characteristics. The MS and NMR characteristics of the aglycon of saponin **5** were consistent with the data reported previously for 2β,3β-dihydroxyolean-12-ene-23,28-dioic acid, known by the trivial name of medicagenic acid (*5*, *16*).

The structure of the sugar units was based on complete assignment of all proton resonances in each sugar unit achieved by a combination of DQF-COSY and 1D-TOCSY experiments. The ^{1}H NMR spectrum of compound **5** showed for the sugar portion two anomeric proton signals at δ 5.41 and 4.40. 1D-TOCSY experiments obtained by irradiating selectively the signal at δ 5.41 showed the spin system of a glucose unit, whereas 1D-TOCSY experiments obtained by irradiating the signal at δ 4.40 suggested the occurrence of a glucuronic unit. The chemical shifts of H-1_{glc} (δ 5.41) and C-1_{glc} (δ 96.5), identical with our previous data (*12*), indicated that this sugar was linked to the C-28 carboxylic group. In the HMBC experiment a cross-peak due to long-range correlation between C-3 (δ 86.6) of the aglycon and H-1_{glcA} (δ 4.40) indicated that this sugar unit was linked at C-3 of the aglycon. Thus, the compound was identified as 3-*O*-β-glucuronopyranosyl-28-*O*-β-glucopyranoside medicagenic acid. To the best of our knowledge this compound has not been previously identified in any plant species.

^{1}H and ^{13}C NMR and MS (1088 mu) data of compound **10** were superimposable on those reported for 3-*O*-β-glucuronopyranosyl-28-*O*-[β-xylopyranosyl-(1→4)-α-rhamnopyranosyl-(1→2)-α-arabinopyranoside] medicagenic acid previously identified in alfalfa roots (*5*) and also in alfalfa aerial parts (*11*, *17*) as the dominant medicagenic acid glycoside (*18*).

Similar characteristics were found for compound **15**, which showed a quasi-molecular ion at *m*/*z* 1073 [M − H]$^-$ and also four anomeric carbons like compound **10**, but glucuronic acid was replaced by glucose (δ 104.5 and δ 4.37). Thus, **15** was identified as 3-*O*-β-glucopyranosyl-28-*O*-[β-xylopyranosyl-(1→4)-α-rhamnopyranosyl-(1→2)-α-arabinopyranoside] medicagenic acid, previously reported in alfalfa roots (*5*, *16*) and alfalfa aerial parts (*11*, *17*).

^{1}H and ^{13}C NMR and MS (1236 mu) data of compound **13** allowed us to identify this compound as 3-*O*-[β-glucopyranosyl-(1→3)-β-glucopyranosyl]-28-*O*-[β-xylopyranosyl-(1→4)-α-rhamnopyranosyl-(1→2)-α-arabinopyranoside] medicagenic acid, known as albesoside-A, previously isolated from the roots of *Aster albescens* (*19*) but never reported in *Medicago* species. This compound is similar to a glycoside identified previously in alfalfa roots and aerial parts, but differs from it only for the linkage of the disaccharide at C-3, which is (1→3) and not (1→2).

The quasi-molecular ion of compound **11** was recorded at *m*/*z* 1103 [M − H]$^-$, which corresponded to saponin **13** without one terminal pentose. Analysis of the NMR data of **11** in comparison with those of **13** clearly showed that **11** differed from **13** only in the absence of the terminal xylopyranosyl unit. Again, (1→3) linkage of two glucoses occurred as suggested by the downfield shift exhibited by C-3_{glcI} (δ 87.2) and on the basis of the long-range correlation in the HMBC spectrum between the signal at δ 4.61 (H−1_{glcII}) and the signal at δ 87.2 (C-3_{glcI}). Thus, saponin **11** was identified as 3-*O*-[β-glucopyranosyl-(1→3)-β-glucopyranosyl]-28-*O*-[α-rhamnopyranosyl-(1→2)-α-arabinopyranoside] medicagenic acid, never reported before.

Compounds **12** and **14** showed identical quasi-molecular ions at *m*/*z* 1367 [M − H]$^-$, which was 132 mu higher than that found for saponin **13**, indicating the occurrence of one extra pentose in the molecule. The ^{13}C NMR spectrum of **12** showed, in addition to the aglycon signals, 33 signals ascribable to a sugar portion made up of two hexose units, one 6-deoxyhexose, and three pentose units (**Table 2**). In the ^{1}H NMR spectra six anomeric proton signals resonated at δ 5.72 (d, $J = 2.6$), 5.06 (d, $J = 1.5$), 4.71 (d, $J = 7.8$), 4.60 (d, $J = 7.5$), 4.53 (d, $J = 7.4$), and 4.45 (d, $J = 7.5$). A methyl doublet ascribable to Me-6 of the deoxyhexose sugar at δ 1.29 (d, $J = 6.5$) was also evident. Structure elucidation of the sugar portion was achieved by 1D-TOCSY, DQF-COSY, HSQC, and HMBC experiments (**Table 2**). The 1D-TOCSY subspectra obtained by irradiating the anomeric proton signals at δ 4.60 and 4.45 showed the typical spin system of β-glucose units, whereas the subspectrum obtained by irradiating the signals at δ 4.71 allowed us to establish this proton as belonging to a β-xylose unit. In the case of the 6-deoxyhexose (H-1 = δ 5.06), an easier identification of an α-rhamnose unit was obtained by recording the 1D-TOCSY experiments also irradiating the methyl doublet at δ 1.29. The 1D-TOCSY spectrum obtained by irradiating the signal at δ 5.72 showed two signals (δ 3.95 and 3.84), whereas that resulting from the irradiation of the anomeric proton at δ 4.53 showed three signals (δ 3.84, 3.65, and 3.52). In both cases the nature of the two sugars could not be deduced only on the basis of 1D-TOCSY experiments but was accomplished by the HSQC experiment, which correlated each hydrogen signal to the corresponding carbon signal, allowing us to identify the two sugars as a 2-substituted α-arabinopyranose (δ 5.72) and a terminal α-arabinopyranose (δ 4.53). Furthermore, the HSQC experiment allowed the identification of the other sugars as a terminal β-glucopyranose (H-1 = δ 4.60), a terminal β-xylopyranose (H-1 = δ 4.71), a 3-substituted β-glucopyranose (H-1 = δ 4.45), and a 3,4-disubstituted α-rhamnopyranose (H-1 = δ 5.06). The sugar sequence was deduced from the HMBC spectrum, in which long-range correlations were observed from H-1_{glcI} (δ 4.45) to C-3 of the aglycon (δ 86.2), H-1_{glcII} (δ 4.60) to C-3_{glcI} (δ 87.0), H-1_{araI} (δ 5.72) to C-28 of the aglycon (δ 178.2), H-1_{rha} (δ 5.06) to C-2_{araI} (δ 75.5), from H-1_{araII} (δ 4.53) to C-3_{rha} (δ 82.2), and from H-1_{xyl} (δ 4.71) to C-4_{rha} (δ 78.9). Thus, the structure of **12** was identified as the new 3-*O*-[β-glucopyranosyl-(1→3)-β-glucopyranosyl]-28-*O*-{β-xylopyrano-

syl-(1→4)-[α-arabinopyranosyl-(1→3)]-α-rhamnopyranosyl-(1→2)-α-arabinopyranoside} medicagenic acid.

NMR data of **14** in comparison with those of **12** suggested that the difference between the two compounds should be confined to the substitution of the terminal α-L-arabinopyranose unit occurring in **12** with a *β*-apiofuranose unit in **14** (**Table 2**). Thus, the structure of compound **14** was defined as the new 3-*O*-[*β*-glucopyranosyl-(1→3)-*β*-glucopyranosyl]-28-*O*-{*β*-xylopyranosyl-(1→4)-[*β*-apiofuranosyl-(1→3)]-α-rhamnopyranosyl-(1→2)-α-arabinopyranoside} medicagenic acid.

Compounds **1**−**4** and **6**−**9** showed the same NMR features for the aglycon portion. The ^{1}H NMR spectrum of compound **8** for the aglycon showed signals for six tertiary methyls at *δ* 0.79 (3H, s, Me-26), 0.90 (3H, s, Me-29), 0.99 (3H, s, Me-30), 1.31 (3H, s, Me-25), 1.38 (3H, s, Me-27), and 1.43 (3H, s, Me-24) and signals indicative of a proton linked to oxygen-bearing carbons at *δ* 4.53 (1H, br m, $W_{1/2}$ = 7.0 Hz, H-16), 4.35 (1H, ddd, J = 3.0, 3.8 and 4.0 Hz, H-2), and 4.13 (1H, d, J = 4.0 Hz, H-3). The main difference between the aglycon of **1**−**4** and **6**−**9** and medicagenic acid was the occurrence of a further secondary alcoholic function at C-16 (*δ* 74.8). Thus, this aglycon was identified as 2*β*,3*β*,16α-trihydroxyolean-12-ene-23,28-dioic acid, known by the trivial name of zanhic acid (*5, 16*).

The MS data of **8** showed a quasi-molecular ion at *m*/*z* 1089 [M − H]$^-$. The ^{1}H and ^{13}C NMR data of the sugar portion of **8** were superimposable on those found for saponin **15**. Thus, compound **8** was identified as 3-*O*-*β*-glucopyranosyl-28-*O*-[*β*-xylopyranosyl-(1→4)-α-rhamnopyranosyl-(1→2)-α-arabinopyranoside] zanhic acid.

Compounds **2**, **4**, and **7** showed an identical molecular mass of 1252 mu. The NMR data for the sugar portion of **7** showed that it was the same as observed in compound **13**. Thus, **7** was identified as the new 3-*O*-[*β*-glucopyranosyl-(1→3)-*β*-glucopyranosyl]-28-*O*-[*β*-xylopyranosyl-(1→4)-α-rhamnopyranosyl-(1→2)-α-arabinopyranoside] zanhic acid. Analysis of the NMR data of the sugar portion of **2** clearly suggested the occurrence of the usual glc-(1→3)-glc disaccharide at C-3 of the aglycon and of a trisaccharide portion at C-28. On the basis of 2D NMR analysis the three sugars were identified as α-arabinopyranose (H-1 = *δ* 5.72), α-rhamnopyranose (H-1 = *δ* 4.98), and *β*-apiofuranose (H-1 = *δ* 5.20). The sugar sequence, also in this case, was deduced from the HMBC spectrum in which long-range correlations were observed from H-1$_{ara}$ (*δ* 5.72) to C-28 of the aglycon (*δ* 177.0), H-1$_{rha}$ (*δ* 4.98) to C-2$_{ara}$ (*δ* 76.0) and from H-1$_{api}$ (*δ* 5.20) to C-3$_{rha}$ (*δ* 80.5). Thus, compound **2** was identified as the new 3-*O*-[*β*-glucopyranosyl-(1→3)-*β*-glucopyranosyl]-28-*O*-[*β*-apiofuranosyl-(1→3)-α-rhamnopyranosyl-(1→2)-α-arabinopyranoside] zanhic acid.

NMR data of **4** in comparison with those of **2** suggested that **4** differed from **2** for the substitution of the terminal *β*-apiofuranose unit occurring in **2** with an α-arabinopyranose unit in **4** (**Table 2**). Thus, the structure of compound **4** was identified as 3-*O*-[*β*-glucopyranosyl-(1→3)-*β*-glucopyranosyl]-28-*O*-[α-arabinopyranosyl-(1→3)-α-rhamnopyranosyl-(1→2)-α-arabinopyranoside] zanhic acid.

The MS spectrum of compound **1** showed a quasi-molecular ion at *m*/*z* 1143 [M + Na]$^+$, which was 132 mu lower than that of **2**, **4**, and **7**. ^{1}H and ^{13}C NMR data of the sugar portion of **1** showed that it was the same as observed in compound **11**. Thus, the structure of **1** was defined as the new 3-*O*-[*β*-glucopyranosyl-(1→3)-*β*-glucopyranosyl]-28-*O*-[α-rhamnopyranosyl-(1→2)-α-arabinopyranoside] zanhic acid.

The MS spectrum of compound **9** showed a quasi-molecular ion at *m*/*z* 1161 [M − H]$^-$, which was just 42 mu higher than that found for saponin **1**. Analysis of ^{1}H and ^{13}C NMR data clearly suggested that compound **9** differed from **1** only for the occurrence of an acetylation at C-4 of the rhamnose unit. This feature was proved by the downfield shift of H-4$_{rha}$ (*δ* 4.95 in **9** vs *δ* 3.40 in **1**) and C-4$_{rha}$ (*δ* 75.2 in **9** vs 73.8 in **1**) (**Table 1**). Thus, compound **9** was identified as the new 3-*O*-[*β*-glucopyranosyl-(1→3)-*β*-glucopyranosyl]-28-*O*-[4-*O*-acetyl-α-rhamnopyranosyl-(1→2)-α-arabinopyranoside] zanhic acid.

Compounds **3** and **6** showed a molecular mass of 1384 mu. The analysis of ^{1}H and ^{13}C NMR data of compounds **3** and **6** suggested the occurrence of the same sugar portion, which matched very closely those observed in **12** and **14**, respectively. Thus, compound **3** was identified as the new 3-*O*-[*β*-glucopyranosyl-(1→3)-*β*-glucopyranosyl]-28-*O*-{*β*-xylopyranosyl-(1→4)-[α-arabinopyranosyl-(1→3)]-α-rhamnopyranosyl-(1→2)-α-arabinopyranoside} zanhic acid and compound **6** as the new 3-*O*-[*β*-glucopyranosyl-(1→3)-*β*-glucopyranosyl]-28-*O*-{*β*-xylopyranosyl-(1→4)-[*β*-apiofuranosyl-(1→3)]-α-rhamnopyranosyl-(1→2)-α-arabinopyranoside} zanhic acid.

On the basis of TLC, the last fraction from the RP 18 column contained only two compounds. They showed very similar retention factor, and their separation by column chromatography was not successful. Thus, the mixture was analyzed by ESI-MS/MS. This revealed that the two compounds had quasi-molecular ions at *m*/*z* 941 and 939 mu [M − H]$^-$, which corresponded to soyasaponin I and Rha-Gal-GlcA-soyasapogenol E, respectively (*10*). This finding was further supported by cochromatography of the mixture with authentic standards in two solvent systems.

The structural data obtained in this research show that, with regard to saponin aglycon structure, dominant saponins of *M. truncatula* are similar to those found in alfalfa (*M. sativa*). Both species contain medicagenic acid, zanhic acid, and soyasapogenol glycosides. Two of medicagenic acid glycosides, that is, 3-*O*-*β*-glucuronopyranosyl-28-*O*-[*β*-xylopyranosyl-(1→4)-α-rhamnopyranosyl-(1→2)-α-arabinopyranoside] medicagenic acid and 3-*O*-*β*-glucopyranosyl-28-*O*-[*β*-xylopyranosyl-(1→4)-α-rhamnopyranosyl-(1→2)-α-arabinopyranoside] medicagenic acid, are identical in both species. The differences occur when a second glucose is attached at the glucose unit in position C-3. In alfalfa both for medicagenic acid and zanhic glycosides, the glucose−glucose linkage was always (1→2), whereas in *M. truncatula* the linkage was always (1→3). Another evident difference is the number of glucoses in the chain linked to C-3 of the aglycon. In *M. truncatula* only one or just two glucose units occur, whereas in *M. sativa* one, two, or three glucose units can be present, but usually with (1→2) linkage. Only in one case, in 3-*O*-[*β*-glucopyranosyl-(1→3)-*β*-glucopyranosyl]-28-*O*-*β*-glucopyranoside medicagenic acid, isolated in the roots of *M. sativa* (*12*), was a glucose-(1→3)-glucose linkage observed. This may suggest that *M. sativa* possesses both (1→2) and (1→3) specific glucosyltransferases, whereas in *M. truncatula* only (1→3) specific glucosyltransferase is present.

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Received for review November 2, 2004. Revised manuscript received January 11, 2005. Accepted January 12, 2005. This work was financed by the Polish Committee for Science as 2004-2007 Project 2 P06A 037 27.

JF048178I