Saponins from Aerial Parts of Alfalfa (*Medicago sativa*)

Georges Massiot,*,† Catherine Lavaud,† Véronique Besson,† Louisette Le Men-Olivier,† and Georges Van Binst‡

Faculté de Pharmacie, Laboratoire de Pharmacognosie, 51 Rue Cognacq-Jay, 51096 Reims Cedex, France, and Fakulteit der Wetenschappen, Vrije Universiteit Brussels, Pleinlaan 2, B-1050 Brussels, Belgium

The structures of three saponins from the leaves of alfalfa, cultivar Resis, were established as 28-*O*-[β-D-xylopyranosyl-(1→4)-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranosyl]medicagenate, 28-*O*-[β-D-xylopyranosyl-(1→4)-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranosyl]-3-*O*-β-D-glucopyranosylmedicagenate, and 28-*O*-[β-D-xylopyranosyl-(1→4)-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranosyl]-3-*O*-[β-D-glucopyranosyl-(1→2)-β-D-glucopyranosyl]medicagenate; in the North American cultivar Lahontan, two saponins were isolated and their structures determined as 28-*O*-[β-D-xylopyranosyl-(1→4)-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranosyl]-3-*O*-β-D-glucuronopyranosylmedicagenate and 3-*O*-[α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranosyl-(1→2)-β-D-glucuronopyranosyl]soyasapogenol B. Structural elucidations were performed on peracetylated derivatives of the saponins by using ^1H and ^{13}C NMR with techniques such as COSY, relayed COSY, HOHAHA, ROESY, HMBC, and HMQC.

The quest for soya-independent protein sources maintains an ongoing interest in the development of more productive alfalfa cultivars (lucerne, *Medicago sativa*, Papillionaceae). Attention focuses on reduction of the level of the so-called "antifeedant" principles, among which the most notorious ones are saponins with medicagenic acid as the aglycon. Curiously, despite sporadic efforts (Morris et al., 1961; Gestetner, 1971; Timbekova and Abubakirov, 1984, 1986; Lévy et al., 1989), structural investigations on alfalfa saponins lag behind parallel efforts in other fields such as nutrition, biochemistry, and plant selection. Development of new analytical methods for the structural elucidation of saponins and related compounds has encouraged us to perform new work in this area (Massiot et al., 1986, 1988a,b). In this respect, we report here the structural elucidation of new saponins from the aerial parts of alfalfa using methods that were introduced and discussed in a previous paper on alfalfa root saponins (Massiot et al., 1988a,b).

EXPERIMENTAL PROCEDURES

General Procedures. Optical rotations were measured in a 1-dm cell on a Perkin-Elmer 241 automatic polarimeter. IR spectra were recorded on a Philips Pye Unicam SP3 300. ^1H NMR were measured at 300 MHz on Bruker AC 300 spectrometer and at 500 MHz on Bruker WM 500 spectrometer. ^{13}C NMR were obtained at 75 MHz on a Bruker AC 300 spectrometer. The AC 300 instrument was modified to allow detection in the reverse mode: HOHAHA, ROESY, HMBC, and HMQC were measured in the reverse mode, and ^1H pulses were emitted via the decoupler channel. The HOHAHA spin lock (250 ms) was generated by a MLEV-17 sequence, and the ROESY spin lock (300 ms) was a single long pulse from the decoupler.

Saponin Extraction and Purification (Resis). Dried, powdered leaves (1 kg) were boiled in 10 L of a 4:1 mixture of MeOH and water for 3 h. After cooling and filtration, MeOH was removed in vacuo and the aqueous layer was extracted three times with nBuOH (4, 2, and 2 L). The organic layers were joined and evaporated; the residue was dissolved in 1 L of MeOH. The volume of MeOH was reduced to 300 mL, and the solution was diluted with 1.5 L of ether. The precipitate was filtered and dried over P_2O_5 in vacuo. The solid residue was dissolved in 150 mL of water and dialyzed against water in seamless cellulose tubing. After 4 days, the content of the tube was freeze-dried to afford 4 g of saponins as a pale greenish solid (yield = 4 g/kg).

Saponins from Resis cultivar (8 g) were chromatographed on 320 mL of flash chromatography silica gel (particle size: 40–63 μm) under a pressure of 2 bar (Nonaka, 1986). The column was successively eluted with mixtures of $CHCl_3$–MeOH–H_2O [60:40:3 (4.3 L); 12:8:1 (6.6 L); 10:10:1 (1.5 L); 5:5:1 (1.3 L)]; 150-mL fractions were collected and pooled according to thin-layer chromatography. Fractions 78–91 contained compound **1**; compounds **2** and **3** were present as mixtures in fractions 43–69. Aliquot portions of these fractions were acetylated as described in Massiot et al. (1988). Samples suitable for NMR analysis were obtained by thick-layer chromatography.

Saponin Extraction and Purification (Lahontan). Powdered alfalfa leaves (Lahontan, 400 g) were boiled in 5 L of water. After cooling and filtration, the aqueous solution was extracted with 3 × 2 L of nBuOH (presaturated with water). Evaporation of the organic phase yielded a wax, which was dissolved in 5 L of MeOH; after filtration to remove the insoluble portion, the MeOH volume was reduced to 0.5 L by in vacuo evaporation, and the saponins were precipitated by means of 3 L of ether. Filtration gave a solid which was dried in vacuo over P_2O_5 (5.88 g). This crude saponin mixture was dissolved in 300 mL of water and dialyzed against pure water in seamless cellulose tubing. After 3 days, the content of the tube was freeze-dried to give 0.84 g of saponins (yield = 2 g/kg). The same yield was obtained with the hydroalcoholic process described above.

Saponins from Lahontan (7.6 g) were acetylated, and the resulting mixture was chromatographed on silica gel (500 g). The column was eluted with mixtures of $CHCl_3$–hexane–MeOH [9:1:0 (2.4 L); 90:10:1 (2 L); 90:10:2 (1.2 L); 90:10:3 (1.4 L); 90:10:4 (1.5 L); 19:2:1 (1 L); 24:0:1 (0.7 L); 19:0:1 (0.7 L); 9:0:1 (0.8 L); 1:0:1 (0.5 L)]. Fractions 80–89 yielded compound **5**, and fractions 103–110 yielded compound **4**; they were purified by thick-layer chromatography after diazomethane treatment.

Peracetylated saponin 1: ^1H NMR (300 MHz, $CDCl_3$) δ 0.77 (s, Me-26), 0.91 (s, Me-29), 0.92 (s, Me-30), 1.12 (s, Me-27), 1.26 (s, Me-25), 1.28 (d, J = 6.5 Hz, rha-6), 1.38 (s, Me-24), 2.83 (br dd, J = 12, 4 Hz, H-18), 3.37 (dd, J = 12, 8.5 Hz, xyl-5), 3.65 (t, J = 9 Hz, xyl-4), 3.72 (dd, J = 12, 3 Hz, ara-5), 3.84 (m, rha-5), 3.89 (m, ara-2), 3.93 (dd, J = 12, 5 Hz, ara-5), 4.03 (d, J = 3.5 Hz, H-3), 4.12 (dd, J = 12, 5 Hz, xyl-5), 4.28 (m, H-2), 4.66 (d, J = 7 Hz, xyl-1), 4.84 (dd, J = 8.5, 7 Hz, xyl-2), 4.95 (td, J = 8.5, 5 Hz, xyl-4), 4.95 (br s, rha-1), 5.14 (m, rha-2), 5.15 (m, rha-3), 5.15 (t, J = 8.5 Hz, xyl-3), 5.20 (m, ara-3), 5.23 (m, ara-4), 5.31 (m, H-12), 5.75 (br d, J = 3.5 Hz, ara-1); ^{13}C NMR see Table I.

Peracetylated and methylated saponin 4 was stained purple with H_2SO_4 spray (after heating of TLC plates): $[\alpha]_D$ = −3.9° ($CHCl_3$, c 0.33); IR ν_{max} (cm^{-1}) 3020–2880, 1770–1720, 1270–1210;

† Laboratoire de Pharmacognosie.
‡ Vrije Universteit Brussels.

0021-8561/91/1439-0078$02.50/0

Table I. ^{13}C Assignments for Peracetylated Compounds 1–5[a]

genin carbon	1	2	3	4	5	sugar carbon	1	2	3	4	5	sugar carbon	4	5
C-1	43.0	43.5	42.9	43.0	36.7	α-L-arabinopyranose								
C-2	69.1	69.8	70.7	69.3	25.7	C-1	92.3	92.8	91.8	92.2		β-D-glucuronopyranose		
C-3	85.0	86.0	86.9	86.0	90.0	C-2	73.4	74.5	73.5	73.4		C-1	95.5	103.4
C-4	53.4	52.6	52.0	53.0	41.6	C-3	72.1	72.4	74.7	72.3		C-2	75.1	74.8
C-5	51.7	52.5	51.8	51.6	55.9	C-4	66.0	66.8	66.2	66.0		C-3	75.3	73.8
C-6	20.5	20.6	20.1	20.5	20.0	C-5	60.8	62.7	61.0	–		C-4	73.0	72.3
C-7	31.8	32.8	32.2	32.2	33.3	α-L-rhamnopyranose						C-5	75.4	74.6
C-8	39.5	40.1	39.5	39.7	38.9	C-1	97.6	98.2	97.7	98.0	98.1	C-6	–	–
C-9	48.0	48.5	49.1	48.0	47.8	C-2	69.8	71.4	69.9	70.0	69.3	CO_2CH_3	52.6	52.8
C-10	36.1	36.4	36.2	36.3	36.7	C-3	72.0	70.1	72.5	72.1	69.9			
C-11	23.4	23.4	23.0	23.5	23.6	C-4	76.1	76.9	76.2	76.6	70.8			
C-12	122.0	122.5	122.1	122.5	122.4	C-5	67.7	67.9	67.7	67.7	66.8			
C-13	143.2	143.5	143.3	143.2	143.8	C-6	17.4	17.9	17.9	17.3	17.2			
C-14	41.7	42.3	41.8	42.0	39.9	β-D-xylopyranose								
C-15	27.3	27.8	27.3	27.3	25.9	C-1	100.8	101.8	100.9	100.9				
C-16	29.7	29.5	30.0	29.7	26.9	C-2	70.7	71.3	70.7	70.7				
C-17	46.7	47.2	47.9	46.7	36.3	C-3	70.9	71.3	70.9	70.9				
C-18	41.2	41.5	41.1	41.5	44.5	C-4	69.0	69.5	69.0	69.3				
C-19	45.8	46.4	45.9	–	46.0	C-5	62.3	62.2	62.4	62.5				
C-20	30.4	31.0	30.6	30.6	30.4	β-D-glucopyranose								
C-21	33.7	34.2	33.7	33.8	38.3	C-1		101.3	102.8		100.3			
C-22	31.9	32.5	31.9	32.1	78.4	C-2		72.4	74.4		74.6			
C-23	182.0	180.5	178.6	–	23.0	C-3		72.7	69.6		70			
C-24	12.4	13.2	12.7	12.8	66.7	C-4		68.8	68.2		68.5			
C-25	16.1	16.4	16.5	16.2	15.1	C-5		72.1	71.6		72.1			
C-26	16.7	17.0	16.6	16.6	16.5	C-6		61.8	61.8		62.6			
C-27	25.6	26.0	25.7	25.8	26.0	β-D-glucopyranose								
C-28	175.4	175.8	175.5	–	21.3	C-1′			99.6					
C-29	32.8	33.1	33.0	33.0	33.6	C-2′			71.1					
C-30	23.5	23.9	23.6	23.7	27.0	C-3′			72.1					
						C-4′			68.0					
						C-5′			71.9					
						C-6′			61.7					

[a] $CDCl_3$, 75 MHz, central line of $CDCl_3$ at δ 77.0 as reference. Resonances for acetates are omitted ($\delta_{C=O}$ 170 ± 0.7; δ_{Me} 20.8 ± 0.5).

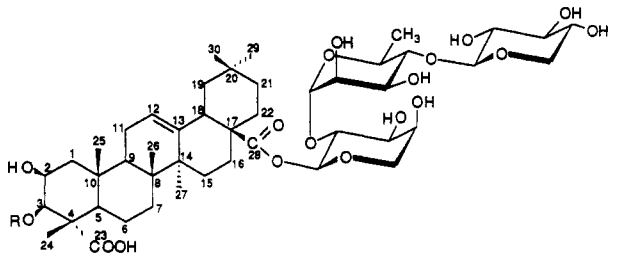
CPDMS *m/z* 1558.7 (M^+ + Na), 705.7, 550.6, 522.5, 489.2, 468.9, 356.2, 281.5, 259.4; ^1H NMR (300 MHz, $CDCl_3$) δ 0.75 (s, Me-26), 0.91 (s, Me-29), 0.92 (s, Me-30), 1.12 (s, Me-27), 1.25 (s, Me-25), 1.26 (d, *J* = 6.2 Hz, rha-6), 2.83 (br dd, *J* = 12, 4 Hz, H-18), 3.35 (dd, *J* = 11.5, 9.4 Hz, xyl-5), 3.66 (m, rha-4), 3.68 (m, ara-5), 3.70 (s, CO_2Me), 3.75 (s, CO_2Me), 3.84 (br t, *J* = 4.6 Hz, ara-2), 3.88 (dd, *J* = 10.3, 6.5 Hz, ara-5), 3.93 (dq, *J* = 6.2, 5.3 Hz, rha-5), 4.03 (d, *J* = 9.4 Hz, gln-5), 4.08 (d, *J* = 3.7 Hz, H-3), 4.13 (dd, *J* = 11.5, 5.3 Hz, xyl-5), 4.24 (m, H-2), 4.59 (d, *J* = 7.7 Hz, gln-1), 4.67 (d, *J* = 7.5 Hz, xyl-1), 4.86 (dd, *J* = 9.2, 7.5 Hz, xyl-2), 4.95 (d, *J* = 1.3 Hz, rha-1), 4.96 (td, *J* = 9.2, 5.2 Hz, xyl-4), 5.01 (dd, *J* = 9.4, 7.7 Hz, gln-2), 5.14 (t, *J* = 9.2 Hz, xyl-3), 5.14 (m, rha-3), 5.16 (m, 2 H, rha-2, ara-3), 5.18 (t, *J* = 9.4 Hz, gln-4), 5.22 (t, *J* = 9.4 Hz, gln-3), 5.23 (dt, *J* = 7, 1.6 Hz, ara-4), 5.31 (m, H-12), 5.74 (d, *J* = 4.2 Hz, ara-1); ^{13}C NMR see Table I.

Peracetylated and methylated saponin 5 was stained purple with H_2SO_4 spray (after heating): $[\alpha]_D$ = +14° ($CHCl_3$, *c* 0.29); IR ν_{max} (cm^{-1}) 3020–2880, 1780–1710; ^1H NMR (500 MHz, $CDCl_3$) δ 0.83 (s, Me-28), 0.90 (s, Me-29), 0.98 (s, Me-26), 1.01 (s, 6 H, Me-30 + Me-25), 1.13 (s, Me-27), 1.17 (s, Me-23), 1.20 (d, *J* = 6 Hz, rha-6), 1.99–2.16 (10 s, 10 $OCOCH_3$), 3.22 (dd, *J* = 11.5, 5.2 Hz, H-3), 3.68 (ddd, *J* = 9.7, 6.2, 2.7 Hz, glc-5), 3.71 (dd, *J* = 9.5, 8.0 Hz, glc-2), 3.75 (s, CO_2CH_3), 4.03 (dt, *J* = 7, 2.5 Hz, gln-2), 4.08 (dq, *J* = 9.8, 6.5 Hz, rha-5), 4.10 (m, gln-5), 4.11 (dd, *J* = 13, 2.7 Hz, glc-6), 4.30 (dd, *J* = 13, 6.2 Hz, glc-6), 4.31 (br s, 2 × H-24), 4.51 (d, *J* = 7 Hz, gln-1), 4.65 (d, *J* = 8.5 Hz, glc-1), 4.65 (t, *J* = 4.4 Hz, H-22), 4.93 (d, *J* = 1.8 Hz, rha-1), 4.94 (t, *J* = 9.7 Hz, glc-4), 5.03 (dd, *J* = 3.1, 1.9 Hz, rha-2), 5.06 (t, *J* = 9.9 Hz, rha-4), 5.18 (dd, *J* = 3.3, 9.8 Hz, rha-3), 5.18 (t, *J* = 9.5 Hz, glc-3), 5.19 (m, 2 H, gln-3 + gln-4), 5.27 (t, *J* = 3.6 Hz, H-12); ^{13}C NMR see Table I.

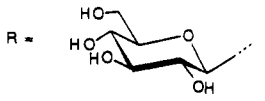
RESULTS AND DISCUSSION

Saponins were extracted from two cultivars of alfalfa grown in the INRA experimental fields at Lusignan. A highly productive but saponin-rich variety (cultivar Resis) and a saponin-poor variety (cv. Lahontan) were selected. Resis is widely cultivated in France, while Lahontan, an American cultivar, which is not resistant to lodging, is not. Nutritional properties of these cultivars were evaluated in the nutrition laboratory of INRA at Bordeaux, and it was found that their acceptance as feed by the yellow mealworm *Tenebrio molitor* was inversely proportional to the saponin and medicagenic acid levels (Pracros, 1988).

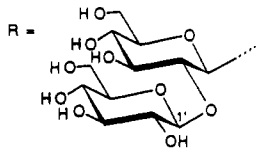
The extraction procedure previously employed (Massiot et al., 1988a,b) (primary extraction with methanol–water, partition between butanol and water, and precipitation with ether) yielded 14 and 21 g/kg of crude saponins from Lahontan and Resis, respectively. At this stage, the saponins were contaminated with lower molecular weight impurities such as free sugars, and after dialysis, yields of saponins dropped to 2 and 4 g/kg, respectively. Saponins from the Lahontan cultivar were freed from their salts by H^+ ion exchange (IRN 77), peracetylated (Ac_2O, DMAP), methylated with diazomethane, and subjected to chromatography. Saponins from the Resis cultivar were separated by flash chromatography and then peracetylated for identification purposes. Three saponins from the Resis cultivar were identified as 28-*O*-[β-D-xylopyranosyl-(1→4)-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranosyl]medicagenate (**1**), 28-*O*-[β-D-xylopyranosyl-(1→4)-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranosyl]-3-*O*-β-D-glucopyranosylmedicagenate (**2**), and 28-*O*-[β-D-xylopyranosyl-(1→4)-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranosyl]-3-*O*-[β-D-glucopyranosyl-(1→2)-β-D-glucopyranosyl]medicagenate (**3**). From the Lahontan cultivar, two saponins were identified as 28-*O*-[β-D-xylopyranosyl(1→4)-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranosyl]-3-*O*-β-D-glucuronopyranosylmedica-



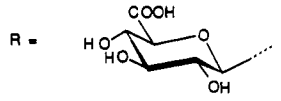
R = H 1



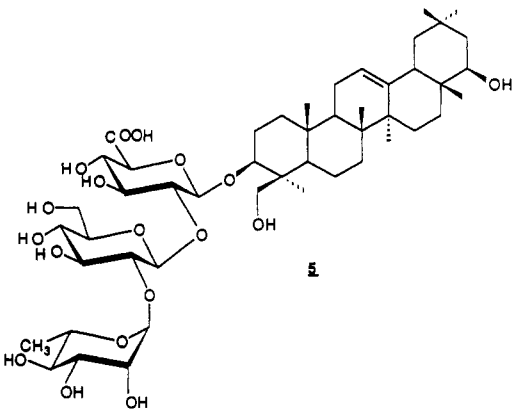
2



3



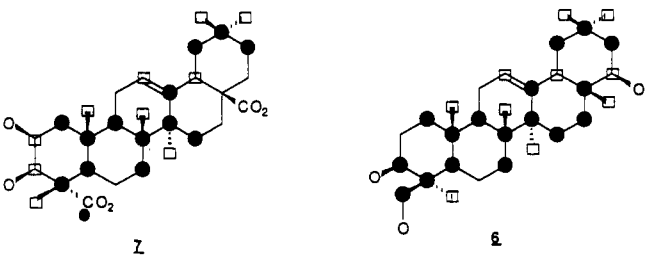
4



5

genate (4) and3-*O*-[α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranosyl-(1→2)-β-D-glucuronopyranosyl]soyasapogenol B (5).

Genin identification was based on high-field ^1H and ^{13}C NMR and on previous knowledge of alfalfa sapogenins (Massiot et al., 1988b). The presence of soyasapogenol B in structure 5 was identified by its seven angular methyl groups and deshielded H-24 and the presence of medicagenic acid in 1–4 by its six angular methyl groups and deshielded H-2. The assignments of the ^{13}C NMR spectra of soyasapogenol B 6 in 5 and of medicagenic acid 7 in compounds 2 and 4 are based on the novel ^1H–^{13}C correlation experiments in the reverse mode: HMQC for ^1J (Müller, 1979) and HMBC for ^2J and ^3J (Bax and Summers, 1986). Interestingly, these experiments allow assignments of most of the signals of the triterpene by observation of correlations with the angular methyl protons (Figure 1). Methyl 27 is the only methyl that shows correlation with an olefinic nonprotonated carbon signal; it shows a correlation with a single methylene C-15 and with two quaternary carbon atoms C-8 and C-14, which are not definitively assigned. These latter two resonances allow recognition of CH_3-26 which also give cross peaks with a methine C-9 and a methylene C-7. Methine C-9, in turn, allows identification of Me-25 and thence location of a quaternary carbon atom C-10, of a methine C-5, and



7 6

Figure 1. HMQC (squares) and HMBC (dots) assignments for the ^{13}C resonances of medicagenic acid (7) and soyasapogenol B (6).

of a methylene C-1. C-5 revealed Me-24 which, in turn, led to recognition of C-3, C-4, and C-23 (a carbonyl in the case of 7; in compound 6, configuration of C-4 is different and C-24 is a methylene and C-23, an angular methyl). The pair of geminal methyls Me-29 and Me-30 are easily distinguished because they share three correlations: quaternary C-20 and methylenes 19 and 21, which are easily singled out by observation of correlations with H-18 in 7 and H-22 in 6. Figure 1 summarizes these assignments symbolized by dots for HMBC-based assignments and squares for HMQC-based assignments (24 assignments for medicagenic acid; 27 assignments for soyasapogenol B). Moreover, it completes the assignments of quaternary carbon resonances of soyasapogenol B (Baxter et al., 1990).

Arabinose Configuration and Conformation in Compounds 1–4. Saponins 2 and 3 are known compounds that were previously identified in alfalfa roots (Massiot et al., 1988a,b); saponin 2 is probably identical with medicoside J (Timbekova and Abubakirov, 1986). Discrepancies regarding the proposed anomeric configurations of L-arabinose in medicoside J and in saponin 2 lead us to question our original assignment and review the problem again. In peracetylated 2, spin networks were identified by COSY, relayed COSY, and HOHAHA experiments; HMBC and HMQC experiments allowed unambiguous assignments of most of the carbon resonances, but limitations were imposed by severe overlapping among the sugar proton resonances. Important carbon resonances which are definitively assigned are those of the anomeric carbons: 101.3 ppm for glucose, 101.8 ppm for xylose, 98.2 ppm for rhamnose, and 92.8 ppm for arabinose. The HMBC experiment also permitted sequencing of all the elements of the molecule, and positioning of the ester residue on the same acid function in 1–4: Me-24 gives a ^3J correlation with a carboxylate carbon at δ 180.5, while the anomeric proton of the arabinose yields a correlation with another carboxylate carbon at δ 175.8. The problem of the configuration of arabinopyranoses in esters of hindered triterpene carboxylic acids has been discussed at length by Tori using ^1H and ^{13}C NMR arguments (Ishii et al., 1981). Particularly puzzling is the fact that in α-L-arabinosyl esters the $^3J_{H\text{-}1\text{-}H\text{-}2}$ couplings vary from 2.8 to 6.2 Hz depending on the equilibrium between $^1C_4$ and $^4C_1$ conformations. A value of 2.3 Hz is measured in pentaacetyl-β-L-arabinopyranose in the more stable $^4C_1$ conformation. In our case $J_{H\text{-}1\text{-}H\text{-}2}$ was found to be 3.5 Hz in peracetylated 1, 6 Hz in peracetylated 2, 4 Hz in 2, and 4.2 Hz in peracetylated 4, suggesting the α-L rather than the β-L configuration. Definitive proof was obtained here through Overhauser effect enhancements. Owing to difficulties in measuring NOE at 300 MHz in medium-sized molecules, rotating frame experiments (ROESY) were performed (Bothner-by et al., 1984; Penders et al., 1989). In every compound that was examined (peracetylated 1–4, 2, and 4), ROEs were found between arabinose H-1 and H-3, indicating α-L-arabinoses in $^4C_1$ conformation

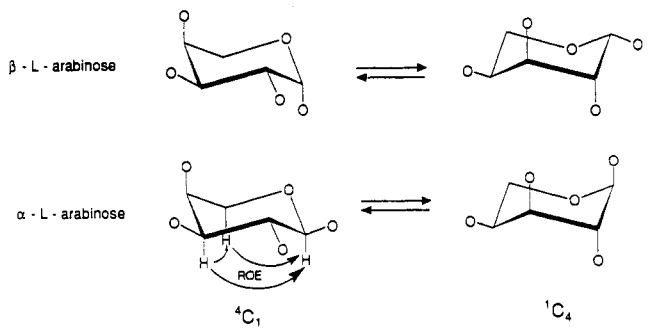


Figure 2. $^4C_1$, $^1C_4$ equilibria for α- and β-arabinoses.

but not ruling out the presence of some $^1C_4$ conformation isomer (Figure 2). The ROESY experiment also afforded confirmation of the β-D-glucose and β-D-xylose configurations (H-1–H-5 ROEs); α-L-rhamnose configuration is deduced from H-1–H-5 long-range coupling (COSY-LR) (Massiot et al., 1990). Sequential information is provided by genin H-3 to glucose H-1 ROE, by rhamnose H-1 to arabinose H-2 ROE, and by xylose H-1 to rhamnose H-4 ROE. The structure of the triterpenoid moieties (medicagenic acid for 1–4 and soyasapogenol for 5) and the positions of attachment of the sugars were established as described above.

Structural Determination of Novel Compounds 1, 4, and 5. The ^{13}C NMR spectrum of peracetylated 1 showed that it is a simple derivative of medicagenic acid with only three anomeric carbon signals at δ 100.8, 97.6 and 92.3. Analysis of the COSY spectrum of peracetylated 1 readily revealed the presence of a terminal β-D-xylopyranose, a C-4 branched α-L-rhamnopyranose, and a C-2 branched α-L-arabinopyranose. Sequencing of these elements was performed with a ROESY experiment leading to the above-mentioned structural assignments described above. The peracetylated saponin 4 yielded a dimethyl ester after diazomethane treatment. Analysis of the COSY and ROESY spectra of this ester permitted identification of the xylose 1→4 rhamnose 1→2 arabinose chain of sugars. A fourth sugar, exhibiting an anomeric proton doublet at δ 4.59 (J = 7.7 Hz), was identified as a terminal uronic acid. The resonance of H-5 of this sugar as a doublet with J = 9.4 Hz (δ 4.03) allowed its identification as a β-D-glucuronopyranosyl acid, located on O-3 of the genin as a consequence of a ROE between anomeric H-1 and genin H-3.

Saponin 5 consisted of soyasapogenol B substituted by sugars on C-3 since positions C-22 and C-24 were acylated. The ^1H NMR spectrum of peracetylated 5 showed a three-proton singlet at δ 3.75 for a methyl ester introduced by the diazomethane treatment which was performed prior to separation. While three anomeric carbon atoms were detected at δ 98.1, 100.3 and 103.4, only two anomeric protons are observable at δ 4.65 (d, J = 8.7 Hz) and 4.51 (d, J = 7 Hz). Analysis of a COSY and of a relayed COSY experiment allowed identification of the first anomeric proton as H-1 of a 2-substituted β-D-glucopyranosyl residue. The second anomeric proton was part of a 2-substituted uronic acid. Even at 500 MHz, this five-spin system was not easily analyzed owing to superimposition and strong coupling of H-3 and H-4. The third sugar was a terminal rhamnose, analysis of the proton spectrum of which was performed with the methyl three-proton doublet at δ 1.15 (J = 6 Hz) as starter. Final identification of the uronic acid as a β-D-glucuronopyranosyl acid relied on a ROESY experiment, which showed H-1 to H-5 magnetization transfer. This experiment allowed sequencing of the saponin as rhamnose 1→2 glucose 1→2 glucuronic acid 1→3 soyasapogenol B (ROEs were observed between glucuronic acid H-1 and genin H-3, between glucose H-1 and glucuronic acid H-2, and between rhamnose H-1 and glucose H-2).

CONCLUSION

To the best of our knowledge, saponins 1, 4, and 5 are described here for the first time and saponin 5 is only the second saponin with a soyasapogenol B genin ever isolated from alfalfa. It differs from soyasaponin I, originally found in soya (Kitagawa et al., 1976) and recently isolated from alfalfa seeds (Besson, 1989), by having a glucose instead of a galactose residue. The isolation and identification of three saponins from the Resis cultivar and of two saponins from the Lahontan cultivar must be considered as preliminary work. Saponin mixtures from the aerial parts of these taxa are far more complex than the root saponin mixtures (Massiot et al., 1988a,b). Some components in the mixtures, such as 1, are highly unstable, for reasons that are not clear at the moment, and this contributes to the difficulty of purifying alfalfa leaf saponins. Work is in progress in our laboratory to separate new saponins from alfalfa with a view to evaluation of their properties in relation to the antifeedant problem.

ACKNOWLEDGMENT

We thank CNRS, INRA, and the Region Champagne-Ardenne for generous support of this work. We express special gratitude to Dr. J. M. Nuzillard for help with HOHAHA, ROESY, HMBC, and HMQC experiments.

LITERATURE CITED

Bax, A.; Summers, M. F. ^1H and ^{13}C Assignments from Sensitivity Enhanced Detection of Heteronuclear Multiple-Bond Connectivity by 2D Multiple Quantum NMR. *J. Am. Chem. Soc.* **1986**, *108*, 2093–2094.

Baxter, R. L.; Price, K. R.; Fenwick, G. R. Sapogenin structure: Analysis of the ^{13}C and ^1H NMR spectra of soyasapogenol B. *J. Nat. Prod.* **1990**, *53*, 298–302.

Besson, V. Ph.D. Thesis, Reims, 1989.

Bothner-by, A. A.; Stephens, R. L.; Lee, J. M.; Warren, C. D.; Jeanloz, R. W. Structure Determination of a Tetrasaccharide by Transient Nuclear Overhauser Effects in the Rotating Frame. *J. Am. Chem. Soc.* **1984**, *106*, 811–813.

Gestetner, B. Structure of a Saponin from Lucerne. *Phytochemistry* **1971**, *10*, 2221–2223.

Ishii, H.; Kitagawa, I.; Matsushita, K.; Shirakawa, K.; Tori, K.; Tozyo, T.; Yoshimura, Y. The Configuration and Conformation of the Arabinose Moiety in Platycodins, Saponins isolated from Platycodon Grandiflorum, and Mi-saponins from Madhuca Longifolia based on Carbon-13 and Hydrogen-1 NMR Spectroscopic Evidence: The Total Structures of the saponins. *Tetrahedron Lett.* **1981**, *22*, 1529–1532.

Kitagawa, I.; Yoshikawa, M.; Yosioka, I. Structures of three Soyabean Saponins: Soyasaponin I, Soyasaponin II and Soyasaponin III. *Chem. Pharm. Bull.* **1976**, *24*, 121–129.

Lévy, M.; Zehavi, U.; Naim, M.; Polacheck, I. Isolation, Structure Determination, Synthesis and Antifungal Activity of a new native Alfalfa-Root Saponin. *Carbohydr. Res.* **1989**, 115–123.

Massiot, G.; Lavaud, C.; Guillaume, D.; Le Men-Olivier, L.; Van Binst, G. Identification and Sequencing of Sugars in Saponins Using 2D ^1H NMR. *J. Chem. Soc., Chem. Commun.* **1986**, 1485–1487.

Massiot, G.; Lavaud, C.; Le Men-Olivier, L.; Van Binst, G.; Miller, S. P. F.; Fales, H. M. Structural Elucidation of Alfalfa Root Saponins by Mass Spectrometry and Nuclear Magnetic Resonance Analysis. *J. Chem. Soc., Perkin Trans. 1* **1988a**, 3071–3079.

Massiot, G.; Lavaud, C.; Guillaume, D.; Le Men-Olivier, L. Reinvestigation of the Sapogenins and Prosapogenins from Alfalfa (Medicago sativa). *J. Agric. Food Chem.* **1988b**, *36*, 902–909.

Massiot, G.; Lavaud, C.; Besson, V. *Bull. Soc. Chim. Fr.* **1990**, 440–445.

Morris, R. J.; Dye, W. B.; Gisler, P. S. Isolation, Purification and Structural Identity of an Alfalfa Root Saponin. *J. Org. Chem.* **1961**, *26*, 1241–1243.

Müller, L. Sensitivity Enhanced Detection of Weak Nuclei using Heteronuclear Multiple Quantum Coherence. *J. Am. Chem. Soc.* **1979**, *101*, 4481–4484.

Nonaka, M. Variable sensitivity of Trichoderma viride to Medicago sativa saponins. *Phytochemistry* **1986**, *25*, 73–75.

Penders, A.; Delaude, C.; Pepermans, H.; Van Binst, G. Identification and Sequencing of Sugars in Acetylated Saponin of Blighia welwitschii by NMR Spectroscopy. *Carbohydr. Res.* **1989**, *190*, 109–120.

Pracros, P. *Agronomie* **1988**, *8*, 793–799.

Timbekova, A. M.; Abubakirov, N. K. Triterpene Glycosides of Alfalfa. *Khim. Prir. Soedin.* **1984**, 451–453; **1986**, 607–609; **1986**, 614–616.

Received for review March 15, 1990. Revised manuscript received June 29, 1990. Accepted July 11, 1990.

Registry No. 1, 129570-95-2; 1 peracetylated, 129570-96-3; 2, 120039-30-7; 3, 120039-31-8; 4, 128192-15-4; 4 peracetylated and methylated, 129592-69-4; 5, 129592-68-3; 5 peracetylated and methylated, 129618-61-7.