Zahnic Acid Tridesmoside and Other Dominant Saponins from Alfalfa (*Medicago sativa* L.) Aerial Parts

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A zahnic acid tridesmoside, 3-*O*-[β-D-glucopyranosyl(1–2)-β-D-glucopyranosyl(1–2)-β-D-glucopyranosyl]-2β,3β,16α-trihydroxyolean-12-ene-23,28-dioic acid–23-*O*-α-L-arabinopyranosyl-28-*O*-[β-D-apiofuranosyl-(1–3)-β-D-xylopyranosyl(1–4)-α-L-rhamnopyranosyl(1–2)-α-L-arabinoside], has been identified as the main saponin present in the aerial parts of alfalfa by a combination of chromatographic, chemical, and spectroscopic techniques. Moreover, four medicagenic acid glycosides and soyasaponin I have been isolated from the same source and identified. Three of the medicagenic acid glycosides are identical to those previously found in alfalfa roots, while the fourth has been identified as 2-β-hydroxy-3-β-*O*-glucuronopyranosyl 28-*O*-[α-L-rhamnopyranosyl(1–2)-α-L-arabinopyranoside]olean-12-en-23-oic acid. Biological activity was measured using the fungus *Trichoderma viride*, and hemolytic activity was assessed using a hemolytic test. The zahnic acid glycoside did not inhibit fungal growth over a wide range of concentrations and exhibited only weak hemolytic activity.

INTRODUCTION

The nutritional quality of the protein from alfalfa or derived products (leaf protein concentrates, LPC) has been significantly lowered by the presence of associated antinutritional compounds, namely saponins (Cheeke, 1980; Livingstone et al., 1979). Until recently, little information was available on the chemistry of these triterpene glycosides; this was due primarily to the complexity of their chemical structures and difficulties that were encountered in their separation. Significant progress in the separation and identification of saponins from alfalfa roots has recently been made and several structures have been published (Timbekova and Abubakirov 1984, 1985, 1986a,b; Levy et al., 1986, 1989; Massiot et al., 1988a,b; Oleszek, 1988; Timbekova et al., 1989; Oleszek et al., 1990a,b); in addition, structure-dependent hemolytic, antifungal, and allelopathic activities have been documented (Polacheck et al., 1986; Oleszek, 1990a,b; Oleszek et al., 1990a). Relatively little research has been performed on the chemistry of the saponins of alfalfa plant tops (Gestetner, 1971; Massiot et al., 1991). These papers support the general belief that the medicagenic acid glycosides possessed the most significant biological activities and hence are probably most involved in the protective mechanism of the plant. In this paper we report the isolation and structure elucidation of alfalfa-top saponins and further discuss the role of saponins in alfalfa.

MATERIALS AND METHODS

Extraction. One hundred and fifty grams of dried finely powdered alfalfa tops (leaves and stems) (var. Kleszczewska) was extracted by refluxing with 2 L of 30% methanol. The extract was filtered and applied to a C_{18} column (6 cm × 10 cm, 55 μm, Waters Associates, 50 g) preconditioned with 30% methanol. The column was washed with 30% methanol (0.2 L), and the saponins were eluted with methanol; the methanol was removed in vacuo to yield a brownish solid (4.5 g).

Isolation of Glycoside Fractions by Preparative LC. The saponin mixture was fractionated on a C_{18} column (3 cm × 30 cm, 55 μm, Waters) and eluted with a gradient formed from a water–methanol mixture as previously described (Oleszek, 1988; Oleszek et al., 1990a) to yield five fractions (I–V).

High-Resolution Preparative Liquid Chromatography (HRPLC). The saponin fractions were thereafter separated on steel columns (1.2 cm × 30 cm) filled with normal-phase packing (Lichroprep 15–25 μm Si_{60}) or reversed-phase packing (Lichroprep 15–25 μm C_{18}) as previously described (Oleszek et al., 1990a). Fractions I, II, and III were separated or purified on the C_{18} column using a methanol/water/acetic acid (60:40:05 v/v) solvent system, while fractions IV and V were separated on a Si_{60} column using ethyl acetate/acetic acid/water (10:2:2 v/v) and chloroform/methanol/water (7:3:1 v/v, lower layer), respectively.

Chromatography (TLC, Analytical HPLC) and Spectral Analysis. Saponins and sapogenins were chromatographed on Merck silica gel 60 plates developed with ethyl acetate/acetic acid/water (7:2:2 v/v) or on Merck C_{18} plates developed with methanol/water/acetic acid (60:40:0.5 v/v). Aglycons were separated on Merck C_{18} plates with methanol/water (90:10 v/v). Both intact saponins and their aglycons were visualized with methanol/acetic anhydride/sulfuric acid (50:5:5 v/v) followed by heating at 120 °C. Sugars were separated on Merck cellulose plates with benzene/butanol/pyridine/water (1:5:3:3 v/v) and visualized with a silver nitrate spray.

The purity of the isolated compounds and their retention times (Rt) in relation to saponin standards were measured with an analytical HPLC unit (Knauer gradient system with computer control) using a 25 cm × 4.6 mm, Spherisorb 5 μm ODS-2 column (Phase Separation, Deeside, U.K.) according to the method of Oleszek et al. (1990b).

FAB mass spectra were obtained and NMR experiments were performed according to previously described procedures (Oleszek et al., 1990a). Electron impact mass spectra (EI–MS) of isolated aglycons were recorded on a Kratos MS 30 instrument with a source temperature of 200 °C and a nominal potential of 70 eV.

Gas chromatography and gas chromatography directly coupled to a mass spectrometer (GC/MS) of isolated sugars were conducted after reduction and acetylation according to the method of Blakeney et al. (1983). GC and GC/MS analyses were carried out with a capillary column (15 cm, 5 μm) loaded with

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OV225 phase and a flow rate of 8 mL min^{-1}. A gradient temperature sequence of 170 °C (5 min), 170–220 °C (12 min), 220 °C (15 min) was used with an injection temperature of 210 °C and detector at 220 °C. The mass spectrometer was a VG 301S instrument operating in EI mode with a source temperature of 210 °C and a nominal potential of 70 eV.

Analysis of Hydrolysis Products. Each individual compound was hydrolyzed with 2 N HCl in 50% methanol or with 5% KOH in methanol, and thereafter sugars, aglycons, and prosapogenins were separated and identified with TLC and/or mass spectrometry.

For saponin 3 (see below) some additional mild hydrolysis experiments were conducted. Twenty milligrams of this saponin was dissolved in 2 N HCl in 50% methanol (10 mL), and hydrolysis was performed at room temperature over a period of 4 days. Every 24 h a 2.5-mL portion of the hydrolysis mixture was passed through a Sep-Pak C_{18} cartridge (Waters Associates) preconditioned with water. The eluate from the cartridge was evaporated to dryness at 40 °C, and sugars were analyzed with TLC and, after conversion into alditol acetates, with GC and GC/MS. Prosapogenins retained on the Sep-Pak were eluted with methanol and analyzed using TLC and FAB-MS.

Biological Activity Tests. Biological activities were measured in two independent tests. Inhibitory activity against the fungus *Trichoderma viride*, culture 984 (Stuteville and Skinner, 1987), was determined according to the method of Oleszek et al. (1990a). Hemolytic activity was measured both by the hemolytic index (HI) and by hemolytic micromethods (Oleszek, 1990a).

RESULTS

Saponin Composition and Structures. The crude alfalfa-top saponin mixture was separated by preparative LC into five major fractions with the following yields: I, 65 mg; II, 201 mg; III, 367 mg; IV, 210 mg; V, 110 mg. Fraction IV comprised four components, two of which were predominant, whereas the remaining fractions each contained a single major component.

Each individual fraction was further separated and purified by HRPLC. In this manner fraction I yielded component 1 (25 mg): FAB-MS (positive ion mode) *m/z* (relative intensity) 957 (8) $[M + H]^+$, 679 (10) [M + H – pentose – deoxyhexose]$^+$, 633 (17) [M + H – pentose – deoxyhexose – HCOOH]$^+$, 503 (17) $[Ma + H]^+$, 485 (27) [Ma + H – H_2O]$^+$, 455 (100) [Ma + H – HCOOH]$^+$, (negative ion mode) 955 (100) $[M - H]^-$, 909 (5) [M – H – HCOOH]$^-$, 809 (5) [M – H – deoxyhexose]$^-$, 779 (15) [M – H – uronic acid]$^-$, 677 (28) [M – H – deoxyhexose – pentose]$^-$, 631 (16) [M – H – deoxyhexose – pentose – HCOOH]$^-$, 501 (21) $[Ma - H]^-$, 483 (8) [Ma – H – H_2O]$^-$, 455 (13) [Ma – H – HCOOH]$^-$. For ^{13}C NMR data, see Table I. Alkaline hydrolysis (8 h) yielded prosapogenin with TLC and MS characteristics identical to those of medicagenic acid 3-*O*-glucuronopyranoside (Oleszek et al., 1990a) together with arabinose and rhamnose. Acid hydrolysis (8 h) afforded medicagenic acid (by TLC), arabinose, and rhamnose. The release of uronic acid during hydrolysis could not be confirmed by TLC.

Fraction II consisted of one major component, **2** (60 mg), exhibiting a molecular weight of 1088 mu; on acid hydrolysis this compound afforded medicagenic acid, arabinose, rhamnose, and xylose. Alkaline hydrolysis produced a prosapogenin identical (TLC and FAB-MS data) with medicagenic acid 3-*O*-glucuronopyranoside. The TLC, FAB-MS, and ^{13}C NMR spectral data of the intact saponin were identical with those of 3-β-*O*-(glucuronopyranosyl) 28-*O*-[β-D-xylopyranosyl(1–4)-α-L-rhamnopyranosyl(1–2)-α-L-arabinoside] medicagenic acid (3-GlcA,28-Ara-Rha-Xyl Ma) (Oleszek et al., 1990a).

Fraction III provided a single compound, **3** (210 mg): FAB-MS (positive ion mode) 1717 (22) $[M - K]^+$, 1701 (18) $[M + Na]^+$, 1585 (100) [M + K – pentose]$^+$, 1569 (64) [M + Na – pentose]$^+$, 1453 (45) [M + K – 2pentoses]$^+$, 1021 (54), 1005 (64) [M + H – 4pentoses – deoxyhexose]$^+$, 975 (82), 959 (64) [M + H – 4pentoses – deoxyhexose – HCOOH]$^+$, (negative ion mode) 1677 (17) $[M - H]^-$, 1545 (73) [M – H – pentose]$^-$, 1413 (23) [M – H – 2pentoses]$^-$, 1383 (13) [M – H – pentose – hexose]$^-$, 1281 (3) [M – H – 3pentoses]$^-$, 1135 (3) [M – H – 3pentoses – deoxyhexose]$^-$, 1003 (17) [M – H – 4pentoses – deoxyhexose]$^-$, 957 (20) [M – H – 4pentoses – deoxyhexose – HCOOH]$^-$, 841 (3) [M – H – 4pentoses – deoxyhexose – hexose]$^-$, 679 (3) [M – H – 4pentoses – deoxyhexose – 2hexoses]$^-$, 517 (13) [Za – H]$^-$, 499 (13) [Za – H – H_2O]$^-$, 469 (33), 455 (100), 409 (33) [trisaccharide – H]$^-$, 383 (66), 337 (40). For ^{13}C NMR data, see Table I.

Alkaline hydrolysis of **3** yielded prosapogenin **3a**, FAB-MS (positive ion mode) 1043 (31) $[M + K]^+$, 1027 (4) $[M + Na]^+$, 1005 (6) $[M + H]^+$, 959 (2) [M + H – HCOOH]$^+$, 881 (12) [M + K – hexose]$^+$, 865 (4) [M + Na – hexose]$^+$, 843 (2) [M + H – hexose]$^+$, 775 (12), 591 (51), 499 (100), (negative ion mode) 1003 (100) $[M - H]^-$, 957 (7) [M – H – HCOOH]$^-$, 841 (28) [M – H – hexose]$^-$, 679 (8) [M – H – 2hexoses]$^-$, 517 (28) $[Za - H]^-$, 499 (10) [Ma – H – H_2O]$^-$, 485 (4) [3hexoses – H]$^-$, 483 (3), 471 (14) [Za – H – HCOOH]$^-$, 455 (66). For ^{13}C NMR data, see Table I.

Acid hydrolysis of **3** afforded aglycon **3b** (R_f value on RP 18 of 0.35; higher than that of the medicagenic acid standard, R_f 0.17). Mass spectra of **3b** gave the following ions: EI-MS 470, 456, 408, 394, 264, 246, 201, 187, 173; CI-MS 456 (95), 441 (25), 411 (100), 393 (53), 377 (27), 202 (43), 188 (100), 173 (70). For ^{13}C NMR of **3b** data, see Table I.

Acid hydrolysis of **3** also released monosaccharides, which were identified by TLC as glucose (R_f 0.17), arabinose (R_f 0.29), xylose (R_f 0.35), and rhamnose (R_f 0.5), together with an unknown component (R_f 0.81). These monosaccharides were also analyzed by GC/MS after conversion into alditol acetates. The GC/MS revealed

Table I. ^{13}C NMR Chemical Shift Data of Saponins[a]

genin carbon	1	3	3a	3b	sugars (anomeric carbon) 1	3	3a
1	44.5	44.2	44.1	45.2	β-BlcA	β-Glcp	β-Glcp
2	70.3	70.3	70.2	71.7	105.6	102.5	102.7
3	86.4	85.5	85.7	76.0			
4	52.9	52.7	52.7	54.0	α-Arap	β-Glcp	β-Glcp
5	52.5	52.7	52.7	52.4	93.5	104.5	104.5
6	21.2	20.9	20.9	20.9	21.6		
7	32.7	33.3	33.3	33.5	α-Rhap	β-Glcp	β-Glcp
8	40.3	40.4	40.3	40.4	101.1	106.5	106.6
9	48.7	47.9	47.9	48.0			
10	36.9	36.8	36.9	37.1		α-Arap[b]	
11	24.0	24.0	24.0	24.1		93.6	
12	123.0	122.0	122.3	122.4			
13	144.1	144.5	145.2	145.2		α-Rhap	
14	42.2	42.1	42.2	42.3		101.1	
15	28.2	36.0	36.1	36.1*			
16	23.2	75.9	74.7	74.7		β-Xylp	
17	47.3	49.5	48.9	48.9		105.0	
18	41.7	41.2	41.4	41.5			
19	46.2	47.1	47.3	47.3		β-Apif	
20	30.9	30.9	31.1	31.1		111.7	
21	34.1	36.0	36.2	36.2*			
22	33.0	32.1	32.9	32.9			
23	180.6	176.0	180.7	181.0		α-Arap[c]	
24	14.1	14.3	14.1	13.8		93.3	
25	16.9	17.1	17.0	17.1			
26	17.4	17.4	17.4	17.5			
27	26.1	27.2	27.2	27.3			
28	176.3	175.9	180.0	180.1			
29	33.2	33.3	33.3	33.3			
30	23.7	24.8	23.0	24.8			

[a] In d_5-pyridine, 27 °C. [b] At C-28. [c] At C-23 (see text).

the presence of rhamnose (R_t = 37.8 min), arabinose (R_t = 41 min), xylose (R_t = 44.3 min), and glucose (R_t = 63.5 min) with the molar ratio of 1:2:1:3. In the GC/MS spectrum the unknown component exhibited R_t = 46.7 min and an EI-MS spectrum 217 (10), 187 (100), 175 (38), 145 (50), 127 (13), 115 (52), 103 (68), 86 (40), 74 (26), 61 (38). Acid hydrolysis of **3a** afforded an aglycon possessing the same TLC characteristics as compound **3b**, together with glucose. Some further chemical experiments were performed on **3** to establish the location of sugars in the saponin molecule. Thus, treatment of **3** with diazomethane gave an unchanged product. Mild acid hydrolysis of **3** after 48 h afforded one sugar component (R_f 0.81), identical to the unknown monosaccharide (above) and a mixture of **3** and prosapogenin **3c**. Compound **3c** gave an unchanged product when treated with diazomethane and produced the following FAB mass spectrum: (positive ion mode) 1585 (10) $[M + K]^+$, 1569 (7) $[M + Na]^+$, 1453 (50) $[M + K - \text{pentose}]^+$, 1439 (25) $[M + Na - \text{pentose}]^+$, 1291 (5) $[M + K - \text{pentose} - \text{hexose}]^+$, 1275 (5) $[M + Na - \text{pentose} - \text{hexose}]^+$, 1027 (10), 997 (10), 981 (12), 501 (50), 473 (75) $[Za + H - HCOOH]^+$, 455 (100) $[Za + H - HCOOH - OH]^+$, (negative ion mode) 1545 (30) $[M - H]^-$, 1413 (100) $[M - H - \text{pentose}]^-$, 1281 (9) $[M - H - 2\text{pentoses}]^-$, 1251 (25) $[M - H - \text{pentose} - \text{hexose}]^-$, 1163 (15) $[M - H - \text{pentose} - \text{hexose} - HCOOH]^-$, 1135 (10) $[M - H - \text{trisaccharide}]^-$, 1089 (15) $[M - H - \text{pentose} - 2\text{hexoses}]^-$, 1003 (25) $[M - H - \text{pentose} - \text{trisaccharide}]^-$, 957 (30) $[M - H - 2\text{pentoses} - 2\text{hexoses}]^-$, 927 (15) $[M - H - \text{pentose} - 3\text{hexoses}]^-$, 851 (10), 795 (10), 517 (20) $[Za - H]^-$, 499 (15) $[Za - H - H_2O]^-$, 471 (25) $[Za - HCOOH]^-$, 455 (95) $[Za - H - HCOOH - OH]^-$, 409 (10) $[\text{trisaccharide} - H]^-$, 383 (60), 337 (60).

Fraction IV consisted of four glycosides, the two most abundant of which were isolated and identified. Compound 4 (10 mg) showed TLC, HPLC, and FAB-MS characteristics, and prosapogenins and sugars obtained after hydrolysis, identical to those obtained for 3-*O*-β-D-glucopyranosyl 28-*O*-[β-D-xylopyranosyl(1–4)-α-L-rhamnopyranosyl(1–2)-α-L-arabinoside] medicagenic acid (Oleszek et al., 1990a).

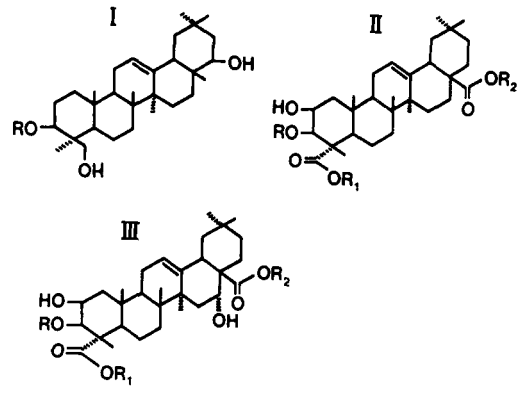
Compound **5** (12 mg) was identified using methods similar to those for **4**, above, as 3-*O*-[β-D-glucopyranosyl-(1–2)-β-D-glucopyranosyl] 28-*O*-[β-D-xylopyranosyl(1–4)-α-L-rhamnopyranosyl(1–2)-α-L-arabinoside] medicagenic acid (Oleszek et al., 1990a).

Fraction V afforded two compounds, one of which, **6** (20 mg), yielded soyasapogenol B and glucuronic acid, galactose, and rhamnose on hydrolysis. Its spectral characteristics were identical to that of soyasaponin I (Oleszek et al., 1990a).

These data for compounds 1–6 are consistent with the structures shown in Figure 1.

Biological Activity. The inhibitory activities of the major saponins against *T. viride* are shown in Figure 2; no inhibitory activity was found for the zahnic acid tridesmoside, **3**. Over the concentration range 1–5 mg/100 mL of growth medium, compound **3** slightly stimulated fungus growth. At concentrations higher than 8 mg/100 mL some inhibition was observed, although this was not statistically significant. The monodesmoside of zahnic acid, **3a**, obtained from alkaline hydrolysis of **3** did, however, show antifungal activity, and fungal growth was completely retarded at a concentration of 10 mg/100 mL (IA_{50} 7 mg/100 mL).

The bisdesmoside of medicagenic acid **2**, the second most abundant saponin in alfalfa tops, inhibited *T. viride* most strongly and showed an IA_{50} value of 4.85 mg/100 mL.



Compound	Aglycon	R	R_1	R_2
1.	II	-GlcA	H	-Ara-Rha
2.	II	-GlcA	H	-Ara-Rha-Xyl
3.	III	-Glc-Glc-Glc	-Ara	-Ara-Rha-Xyl-Api
3a.	III	-Glc-Glc-Glc	H	H
3c.	III	-Glc-Glc-Glc	-Ara	-Ara-Rha-Xyl
4.	II	-Glc	H	-Ara-Rha-Xyl
5.	II	-Glc-Glc	H	-Ara-Rha-Xyl
6.	I	-GlcA-Gal-Rha	-	-

Figure 1. Structures of compounds 1–6.

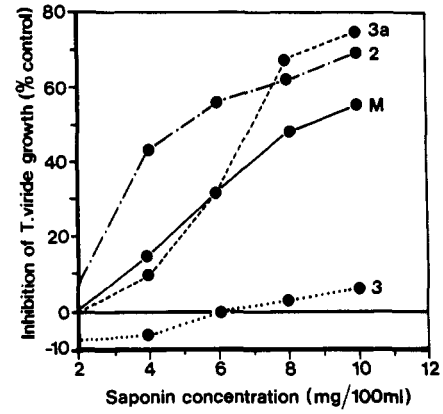


Figure 2. Inhibition of *T. viride* growth by alfalfa top saponins: (2) 3-GlcA,28-Ara-Rha-Xyl medicagenic acid; (3) 3-Glc-Glc-Glc,-23-Ara,28-Ara-Rha-Xyl-Api zahnic acid; (3a) 3-Glc-Glc-Glc zahnic acid; (M) cholesterol-precipitable alfalfa top saponin mixture.

Figure 2, for comparative purposes, also includes data obtained for the mixture of the alfalfa-top saponins (M) isolated by the cholesterol precipitation method and routinely used as a calibrating standard for biological tests used for saponin determination (Jurzysta et al., 1984). This mixture showed IA_{50} 8.8 mg/100 mL, higher than that found for compounds **2** and **3a**. The hemolytic index value obtained for zahnic acid tridesmoside, **3**, was 2000—much lower than that found for the medicagenic acid bisdesmoside, **2**, and saponin mixture M, 3581 and 3900, respectively. Similarly, compound **3** could not be detected with the hemolytic micromethod test when present in the solution at a concentration lower than 2 mg/mL.

At a concentration of 2.5 mg/mL saponin **3** produced ring width of 6 mm × 12, whereas these values were 34.8 and 37.4 for compounds **2** and M, respectively.

DISCUSSION

Separation of alfalfa-top saponins on a C_{18} column, using the procedure previously applied to alfalfa-root saponins (Oleszek et al., 1990a), yielded five fractions each consisting of one or two main compounds. Six individual pure compounds were separated from these fractions, and their structures were elucidated with spectral and chemical techniques.

Thus, compound 1 possessed a molecular weight of 956 mu. Detailed examination of the FAB mass spectrum showed the loss of uronic acid from the molecular ion, indicated by an abundant ion of 779 mu in the negative ion mode. Moreover, ions of 679 and 677 mu in the positive and negative ion spectra, respectively, correspond to the loss of pentose–deoxyhexose fragment, and an ion of 809 mu in the negative ion mode indicated that the deoxyhexose was terminal. The abundant ions at 501 and 503 mu correspond to medicagenic acid (Oleszek et al., 1990a). The presence of three sugar units in the molecule was also confirmed by the ^{13}C NMR anomeric carbon signals at 105.6, 93.4, and 101.1 ppm, corresponding to glucuronic acid, arabinose, and rhamnose, respectively. These data were further supported by analysis of the hydrolysis products where 3-*O*-glucuronopyranoside medicagenic acid was identified following alkaline hydrolysis, while the presence of medicagenic acid, arabinose, and rhamnose was confirmed after acid hydrolysis. On the basis of these findings saponin 1 was identified as 2-β-hydroxy-3-β-*O*-(glucuronopyranosyl) 28-*O*-[α-L-rhamnopyranosyl(1–2)-α-L-arabinopyranoside] Δ^{12}-olean-23-oic acid (3-GlcA,28-Ara-Rha Ma).

Thus, compound 1 is a structural dexylo analogue of 3-GlcA,28-Ara-Rha-Xyl medicagenic acid, the most abundant component of alfalfa-root saponins (Oleszek et al., 1990a,b). Its presence in alfalfa has not previously been reported. A similar compound, the dexylo analogue of 3-Glc,28-Ara-Rha-Xyl medicagenic acid, was previously identified in alfalfa roots by Timbekova et al. (1989).

Saponin 2 showed chemical and spectral characteristics identical to these of 3-GlcA,28-Ara-Rha-Xyl medicagenic acid. This compound was previously identified in alfalfa roots (Oleszek et al., 1990a), where it occurred as the most abundant compound (Oleszek et al., 1990b). HPLC also showed that 3-GlcA,28-Ara-Rha-Xyl medicagenic acid comprised over 90% of the total saponin mixture separated from alfalfa tops by cholesterol precipitation (Oleszek, 1991). Its presence in the aerial parts of var. Lahontan, but not in var. Resis, has been recently reported (Massiot et al., 1991).

Saponin 3 (210 mg) was readily isolated and was present at amounts several times higher than those of the other identified glycosides. Mass spectra showed a molecular weight of 1678 mu, the largest of any saponin identified in alfalfa so far. When hydrolyzed in alkaline solution saponin **3a** was formed, having a molecular weight of 1004; acid hydrolysis and spectral examination indicated the formation of three molecules of glucose and the aglycon **3b**. The molecular weight of this aglycon was 518 mu, 16 units higher than the MW of medicagenic acid and suggestive of a hydroxy derivative of this aglycon. This in turn suggested that the aglycon **3b** might be zahnic acid, originally identified in *Zahna golugensis* (Dimbi et al., 1984) and later confirmed in an aglycon mixture derived from alfalfa-top saponins (Massiot et al., 1988). To support this identity, aglycon **3b** was chromatographed on C_{18} plates and had R_f values higher than that of medicagenic acid. This confirmed the higher polarity of **3b** as compared to medicagenic acid and agrees with previous findings (Massiot et al., 1988). Final evidence for the structure was obtained from spectral analysis. Thus, the retro-Diels–Alder MS fragmentation afforded several peaks, one of which (264 mu) proved the location of the OH group in the C/D ring fragment of the aglycon molecule.

The ^{13}C NMR assignments for **3b** (Table I) were obtained from a combination of 2D NMR experiments (C/H shift correlation and COSY) and by comparison with the assignments for medicagenic acid based saponins (Oleszek et al., 1990a). The shift of the signal for C-16, from 23.2 ppm in medicagenic acid to 74.7 ppm in aglycon **3b**, demonstrated the position of substitution of the OH group to be C-16 (Tori et al., 1976). Further confirmation that the OH group is at C-16 (rather than C-15, C-21, or C-22) was obtained from the COSY spectrum. The proton at 5.23 ppm (correlated with the ^{13}C resonance at 74.7 ppm) is coupled to two methylene protons (1.71 and 2.38 ppm). The COSY spectrum shows a long-range coupling (^4J) between the axial methyl protons (H-27, 1.83 ppm) and the axial methylene proton at 1.71 ppm. Therefore, the two methylene protons must be H-15 and the signals at 5.23/74.7 ppm must arise from H-16 and C-16. The signal given by H-16 is a triplet (J = 3.5 Hz) showing that H-16 is equatorial. Hence, aglycon **3b** is the 16-OH derivative of medicagenic acid, 2β,3β,16α-trihydroxy-Δ^{12}-oleane-23,28-dioic acid (zahnic acid) (Dimbi et al., 1984).

Considering the above findings, prosapogenin **3a** was shown to be the monodesmoside of zahnic acid having three molecules of glucose, and, following closer examination of the ^{13}C NMR spectra, this was identified as 3-*O*-[glucopyranoside(1–2)glucopyranoside(1–2)glucopyranoside] zahnic acid.

Mass spectrometry and ^{13}C NMR spectral data of the intact saponin **3** indicated the presence of eight sugars in the molecule. Besides three glucose molecules at the 3-O position, this saponin contained five other sugars including four pentoses and a deoxyhexose. On acid hydrolysis it was shown that the five sugars included arabinose, xylose, and rhamnose in the molar ratio of 2:1:1 and an unidentified sugar component. These five sugars were ester linked to the aglycon either in one or in two chains. Treatment of **3** with diazomethane yielded an unchanged product, indicating the absence of free COOH groups. Moreover, ^{13}C NMR spectra showed that both carboxyl groups of zahnic acid were esterified (176.0 and 175.9 ppm for C-23 and C-28, respectively). Thus, compound **3** must have sugar components attached to both carboxyl groups of zahnic acid.

Mild hydrolysis of saponin **3** after 48 h produced prosapogenin **3c** and the unidentified sugar component. The treatment of **3c** with diazomethane again gave an unchanged product, indicating no free COOH groups, and showed the unknown sugar to be attached as a terminal component in one of the ester-linked sugar chains. In the negative mode mass spectrum of **3c**, strong ions at 1413 and 1281 mu indicate the loss of one and two pentoses from the parent molecule, respectively. At the same time a strong ion of 409 mu was found, and this was similar to an ion present in the spectra of medicagenic acid bisdesmosides, corresponding to trisaccharide xylose–rhamnose–arabinose (Oleszek et al., 1990a). The same ion, but of enhanced intensity, was found also in the mass spectrum of the intact saponin **3**. Thus, it was concluded that saponin **3c** contained two ester-linked chains: Ara–Rha–Xyl located at C-28 and Ara at C-23.

The unknown sugar component released from hydrolysis of **3** when chromatographed on TLC had the highest R_f value of all sugars detected. Its EI mass spectrum consisted of strong ions of 217, 187, 175, 145, and 103 mu. Both TLC characteristics and MS data were in good agreement with the data obtained previously for apiose (Brillouet et al., 1989). Further proof for the identity of this sugar was found from the examination of ^{13}C NMR data of saponin **3**. The chemical shift signal of 111.7 ppm found in this spectrum is characteristic for the anomeric carbon of apiose (Ishii et al., 1984).

Tentative assignment of the ^{13}C NMR spectrum of 3 suggested terminal apiose as a part of a sequence of sugar units identical to that found at the C-28 position in platycodin D, the main saponin from the roots of *Platycodon grandiflorum* (Ishii et al., 1984).

Summing up the above considerations, saponin 3 was identified as 3-*O*-[β-D-glucopyranosyl(1–2)-β-D-glucopyranosyl(1–2)-β-D-glucopyranosyl]-2β,3β,16α-trihydroxyolean-12-ene-23,28-dioic acid–23-*O*-α-L-arabinopyranosyl 28-*O*-[β-D-apiofuranosyl(1–3)-β-D-xylopyranosyl(1–4)-α-L-rhamnopyranosyl(1–2)-α-L-arabinopyranoside]. This compound has not been reported previously, and this is the first information on the natural occurrence of a zahnic acid tridesmoside in alfalfa. For a long time it was thought that medicagenic acid glycosides are the dominant saponins of alfalfa tops and that they are accompanied by much smaller amounts of hederagenin and soyasapogenol B glycosides (Gestetner et al., 1970; Jurzysta, 1982). Livingstone (1959) reported a novel aglycon from alfalfa and gave it a trivial name, lucernic acid. However, until recently these findings were unable to be reproduced. Recently, Massiot et al. (1988) identified zahnic acid in alfalfa tops and suggested the similarity of its 28-13 lactone to lucernic acid. They also found a glycoside and a glucuronide of zahnic acid in a prosapogenin mixture obtained from the hydrolysis of alfalfa-top saponins. However, in a paper on the identification of saponins from alfalfa varieties Lahontan and Resis they did not describe the presence of any natural zahnic acid-containing saponin. The present data show that zahnic acid glycosides are indeed naturally present in alfalfa tops and that, at least in some varieties, they are the main saponin component.

Compounds 4–6 were present in low quantities. According to their spectral, TLC, analytical HPLC, and chemical characteristics they were identical to 3-Glc,28-Ara-Rha-Xyl and 3-Glc-Glc,28-Ara-Rha-Xyl medicagenic acid and soyasaponin I standards, respectively, isolated from alfalfa roots (Oleszek et al., 1990a,b). Compounds 4 and 5 were identified previously in alfalfa var. Resis but not in var. Lahontan (Massiot et al., 1991).

Some tests of biological activity were performed on the major saponins isolated. These showed that the zahnic acid tridesmoside, in a wide range of concentrations, did not inhibit *T. viride* growth. This saponin also had a HI value much lower than that of any medicagenic acid mono- and bisdesmosides and, moreover, showed very weak hemolysis in the microhemolytic tests. Such low biological activity may suggest that this compound is not of great importance in alfalfa. However, when the compound was hydrolyzed in alkaline solution, the antifungal activity increased considerably to the levels found for 3-GlcA-28-Ara-Rha-Xyl medicagenic acid and the mixture of saponins obtained from alfalfa tops by cholesterol precipitation. Thus, deblocking of the COOH groups of the tridesmoside in an alkaline environment generates a compound with enhanced biological activity.

These findings suggest two important consequences concerning the presence of zahnic acid tridesmosides in alfalfa. First of all, these glycosides cannot be assayed and quantified with *T. viride*, the biological test most often used for saponin determination (Zimmer et al., 1967; Livingstone et al., 1977; Jurzysta, 1979). Similarly, the low hemolytic activity indicates that relatively high concentrations of this compound in plant material would be needed for its detection by the microhemolytic method. Thus, biological tests on which a great deal of published work is based are not suitable for the quantification of zahnic acid tridesmoside. New methodology, therefore, is required for this purpose.

Second, a preliminary taste trial with laboratory staff volunteers, using pure saponins isolated from alfalfa aerial parts, showed that zahnic acid tridesmoside is the most bitter and throat-irritating compound of all compounds tested. If similar effects are found in animals, the palatability of an alfalfa-based diet may be lowered and may adversely affect feed intake. Cheeke (1980) recorded effects on feed intake to be one of the main, if not the major, mechanisms by which legume saponins exerted their growth-depressing effects. Moreover, as observed during the present work, the zahnic acid tridesmoside easily degrades in both alkaline and acidic environment into its monodesmoside, which possesses considerable biological activity. Such conditions can be found in the animal gastrointestinal tract. Hence, the nonactive tridesmoside when eaten may break down into an active component, which could influence rumen microflora and/or various nutrient digestion and absorption processes (Gee et al., 1989; Johnson et al., 1986; Lu and Jorgensen 1987; Lu et al., 1987). As has been shown in several instances with individual glycosides of medicagenic acid, a compound having high activity in one test was not necessarily active in any other and vice versa (Oleszek, 1990b). Thus, the behavior and the activity of the zahnic acid tridesmoside in the digestive tract cannot be predicted.

All of these considerations clearly demonstrate that the alfalfa saponin question cannot be regarded only in terms of the presence of biologically active medicagenic acid glycosides. These potential consequences arising from the occurrence of zahnic acid tridesmoside indicate that more research is needed, including work on nutritional aspects and development of determination techniques.

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