# Isolation and Identification of Alfalfa (*Medicago sativa* L.) Root Saponins: Their Activity in Relation to a Fungal Bioassay

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Nine triterpene glycosides have been isolated from alfalfa (*Medicago sativa*) roots and their structures elucidated by using chromatographic, chemical, and spectroscopic techniques. Six of these saponins were identified as being glycosides of medicagenic acid, two of hederagenin, and one of soyasapogenol B. The compounds containing medicagenic acid could be further subdivided into analogues of either 3-*O*-glucoside or 3-*O*-glucuronide of medicagenic acid. The presence of the latter group has not been previously reported. Activity against the fungus *Trichoderma viride* has been determined for each of the saponins, and their structure–activity relationship is discussed with regard to a fungal bioassay routinely used for saponin determination.

## INTRODUCTION

Alfalfa saponins are naturally occurring triterpene glycosides that, due to their antifungal, hemolytic, surface, and growth-retarding activities, are widely recognized as undesirable factors of alfalfa pasture. They can be found in all alfalfa plant parts, with the highest abundance in roots (Gestetner et al., 1970; Oleszek and Jurzysta, 1986; Price et al., 1987). Of all the saponins present in roots, those with medicagenic acid as their aglycon have been shown to have the highest biological activity. However, until now this biological activity has only been evaluated for mixtures of several glycosides or unidentified compounds (Nonaka, 1986), and the biological activities of the individual compounds have not been described in detail. This is primarily because the separation of individual compounds from mixtures of saponin comprising up to 20 components is extremely difficult. Thus, in spite of the considerable progress that has been made in saponin research so far, only a few alfalfa glycosides have been separated and identified.

The compound most commonly identified in alfalfa is the 3-*O*-glucopyranoside of medicagenic acid, this having been separated by several groups in recent years (Morris et al., 1961; Timbekova and Abubakirov, 1984; Levy et al., 1986; Price et al., 1987; Oleszek et al., 1988a; Oakenfull et al., 1989). This compound exhibits high activity against both *Trichoderma viride* (Oleszek et al., 1988b) and other medically important microorganisms (Polacheck et al., 1986). The same glycoside can also be found in high amounts in *Medicago lupulina* roots (Oleszek et al., 1988a).

Other saponins, for example, the 3,28-diglucopyranoside of medicagenic acid, have been isolated from *Medicago sativa* (Timbekova and Abubakirov, 1984) and *M. lupulina* (Oleszek et al., 1988a) roots. This compound was shown to possess much reduced activity against *T. viride* (Oleszek et al., 1988b), and its activity against other microorganisms has not been extensively studied.

Recently Massiot and co-workers (Massiot et al., 1988b) have described several additional saponins from alfalfa roots. These compounds were, however, isolated as their acetyl derivatives, which, although satisfactory for structural investigations, cannot be used for biological studies since subsequent deacetylation results in cleavage of the ester-linked sugar moiety. Thus, the question of the relationship between structure and biological activity remains unanswered.

Recently, a new analytical procedure that facilitates the separation of individual compounds was described (Oleszek, 1988), and a number of alfalfa saponins were thereafter separated and identified. The present paper describes the application of this analytical procedure for the isolation and identification of several alfalfa root saponins, some of which are described here for the first time. This paper also reports the relationship between saponin structure and activity against *T. viride* and the impact these different activities may have on *T. viride* based bioassay methods.

## MATERIALS AND METHODS

**Extraction.** The dried finely powdered roots of alfalfa (var. Kleszczewska) (150 g) were extracted by refluxing for 2 h with 1.5 L of 80% ethanol. Alcohol was removed in vacuo, and the resultant brown aqueous solution was diluted with methanol to a final concentration of 35% (v/v). The solution was thoroughly mixed (5-min sonication) and centrifuged (2500*g*) for 10 min. The precipitate contained no saponins [by thin-layer chromatography (TLC)] and was discarded. The aqueous alcohol solution was applied to a C18 column (6 cm × 10 cm, 55 μm, Waters Associates, 50 g) previously preconditioned with 35% methanol. The column was washed first with 35% methanol (0.5 L) to remove sugars and phenolics (Oleszek, 1988) and then with methanol (0.5 L) to elute the saponins. Methanol was removed in vacuo to yield a yellowish powder (1.4 g).

**Isolation of Glycoside Fractions by Preparative LC.** The procedure of Oleszek (1988) was employed. The saponin powder (1.4 g) was suspended in distilled water (150 mL), the pH adjusted to 7.0 with 5% NaOH, and the suspension loaded onto a C18 (Waters Inc., 55–105 μm) packed column (2.5 cm × 40 cm), previously equilibrated with water. The column was washed first with 150 mL of distilled water (0.2 mL/min) and then with 2 L of a 0–100% linear gradient of methanol (Beckman gradient mixer). Ten-milliliter fractions were collected with a fraction collector and monitored by TLC.

Fractions showing similar patterns were combined and evaporated to dryness. Six fractions (I–VI) were thus obtained, each comprising one or two major saponins.

**High-Resolution Preparative LC.** These saponin fractions were thereafter separated by normal or reversed-phase

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0021-8561/90/1438-1810$02.50/0 

HPLC. Two types of columns were used: column A, steel, 30 cm × 1.2 cm, Lichroprep 15–25 μm C18; column B, steel, 30 cm × 1.2 cm, Lichroprep 15–25 μm Si60. Solvent systems were optimized for each fraction and consisted of methanol/water/acetic acid (75:25:0.5 v/v, fraction I; 60:40:0.5 v/v, fractions II and VI) for column A or ethyl acetate/acetic acid/water (9:2:2 v/v, fractions IV and V) and chloroform/methanol/water (7:3:1 v/v, lower layer, fraction III) for column B. Solvent was delivered with a IChF PAN (Warsaw) delivery system operating at a flow rate of 2 mL/min. Samples were made up in the required injection volume of 1.2 mL (containing approximately 100 mg of material) and injected by using a 1.2-mL sample loop with a conventional injection valve (IChF PAN). The chromatographic separation was monitored with a refractive index detector (COBRABID, Warsaw).

**Thin-Layer Chromatography (TLC).** TLC of saponins was performed on Merck silica gel 60 plates developed with (system 1) ethyl acetate/water/acetic acid (7:2:2 v/v) or (system 2) butanol/acetic acid/water (4:1:1 v/v) and on Merck C18 plates developed with (system 3) methanol/water/acetic acid (60:40:0.5 v/v). Saponins were visualized by spraying with methanol/acetic anhydride/sulfuric acid (50:5:5 v/v) followed by heating at 120 °C.

**Analysis of Hydrolysis Products.** Each individual glycoside was hydrolyzed in 2 N HCl in 50% methanol for 8 h at 100 °C. Sugars produced were separated on Merck cellulose TLC plates with benzene/butanol/pyridine/water (1:5:3:3 v/v) and visualized by staining with silver nitrate. Aglycons were separated on Merck silica gel 60 plates developed with benzene/methanol (92:8 v/v) and were cochromatographed with appropriate standards. Alkaline hydrolysis was performed by refluxing with 5% KOH in methanol for 4 h. Prosapogenins were analyzed by TLC using solvent systems 1 and 2.

**Spectral Analyses of Glycosides.** (*a*) FAB mass spectra were recorded on a Kratos MS/50TC spectrometer. The samples were dissolved in glycerol and bombarded with a 9-kV (nominal) beam of xenon atoms, produced by an ION Tech 11NF atom gun. The spectra were recorded in both positive and negative modes on a UV galvanometer recorder.

(*b*) $^{13}C$ and $^{1}H$ NMR spectra were measured on a JEOL GX-400 spectrometer at operating frequencies of 100.4 and 399.65 MHz, respectively. The samples were examined as solutions in pyridine-$d_5$ (30 mg/0.5 mL) in 5 mm o.d. tubes at 27 °C. $^{13}C$ and $^{1}H$ chemical shifts are expressed in parts per million relative to TMS, which was added as internal reference. 2D NMR experiments (COSY and $^{13}C/^{1}H$ shift correlation) were carried out on compounds 1 and 6–8, using the phase sensitive method. A double-quantum filter was used in the COSY experiments so that all signals could be phased to pure absorption mode. A 2048 ($t_2$) × 256 ($t_1$) × 2 point data matrix was acquired for the COSY spectra with spectral widths of 3 kHz in both dimensions. The $^{13}C/^{1}H$ shift correlation spectra employed a 4096 ($t_2$, $^{13}C$) × 128 ($t_1$, $^{1}H$) × 2 data matrix with spectral widths of 11 kHz (0–110 ppm) in the $^{13}C$ dimension and 3 kHz in the $^{1}H$ dimension.

***T. viride* Bioassays.** Bioassay experiments to measure the growth in the diameter of colonies of *T. viride* in the presence of varying concentrations of isolated alfalfa root saponins were designed according to the method of Zimmer et al. (1967), as modified by Jurzysta (1979). Potato dextrose agar was made by boiling 200 g of peeled and thinly sliced potatoes in 1 L of distilled water, filtering with cheesecloth, and adding 20 g each of glucose and agar to the filtrate. The saponin (20 mg) was dissolved in 20 mL of distilled water, and aliquots from this stock were used to make test solutions of the appropriate concentrations. Each test solution (100 mL) was placed in a glass beaker and autoclaved (30 min). When hot, 33 mL of the mixture was poured into sterilized 100 × 15 mm glass Petri dishes.

After the agar had cooled and solidified, the dishes were inoculated in the center with 10-mm disks taken from the periphery of *T. viride* culture 984 (Stuteville and Skinner, 1987), grown under controlled conditions (31 °C in a dark incubator) for 72 h. After the test plates were incubated at 31 °C for 48 h, the diameter of growth along three different axes was measured and averaged. Any dishes exhibiting bacterial or fungal contamination were discarded. Each test was conducted in triplicate.

**Table I. $^{13}C$ NMR Chemical Shift Data of Saponins 1–8 (Sapogenins)**

| | δ | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| C | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
| 1 | 44.9 | 44.5 | 44.3 | 44.2 | 44.3 | 44.1 | 38.6 | 38.7 |
| 2 | 69.7 | 70.2 | 70.5 | 70.3 | 70.3 | 70.2 | 26.7 | 25.8 |
| 3 | 86.8 | 86.4 | 86.4 | 86.1 | 86.2 | 85.6 | 91.2 | 82.5 |
| 4 | 52.9 | 52.9 | 53.0 | 52.9 | 52.9 | 52.7 | 43.9 | 43.4 |
| 5 | 52.6 | 52.5 | 52.4 | 52.4 | 52.5 | 52.4 | 56.1 | 48.1 |
| 6 | 21.1 | 21.2 | 21.2 | 21.2 | 21.2 | 21.0 | 18.7 | 18.2 |
| 7 | 33.0 | 32.7 | 33.1 | 32.5 | 32.7 | 32.7 | 33.3 | 33.3 |
| 8 | 40.2 | 40.3 | 40.2 | 40.4 | 40.3 | 40.3 | 40.0 | 39.8 |
| 9 | 48.7 | 48.7 | 48.7 | 48.7 | 48.7 | 48.7 | 47.8 | 47.9 |
| 10 | 37.0 | 36.9 | 36.8 | 36.9 | 36.8 | 36.8 | 38.0 | 36.9 |
| 11 | 24.0 | 24.0 | 24.0 | 24.0 | 24.0 | 24.0 | 24.0 | 23.9 |
| 12 | 122.6 | 123.0 | 122.5 | 122.9 | 122.9 | 122.9 | 122.4 | 122.5 |
| 13 | 144.8 | 144.1 | 144.9 | 144.2 | 144.2 | 144.2 | 144.9 | 144.8 |
| 14 | 42.3 | 42.2 | 42.3 | 42.3 | 42.3 | 42.2 | 42.4 | 42.2 |
| 15 | 28.2 | 28.2 | 28.2 | 28.1 | 28.2 | 28.2 | 30.0 | 28.3 |
| 16 | 23.7 | 23.2 | 23.7 | 23.4 | 23.2 | 23.2 | 26.5 | 23.7 |
| 17 | 46.6 | 47.3 | 46.6 | 47.0 | 47.3 | 47.3 | 36.5 | 46.7 |
| 18 | 42.0 | 41.7 | 42.0 | 41.8 | 41.7 | 41.7 | 45.3 | 42.0 |
| 19 | 46.4 | 46.2 | 46.5 | 46.2 | 46.2 | 46.2 | 46.8 | 46.4 |
| 20 | 31.0 | 30.9 | 31.0 | 30.8 | 30.9 | 30.9 | 31.0 | 31.0 |
| 21 | 34.2 | 34.1 | 34.2 | 34.0 | 34.1 | 34.1 | 42.3 | 34.2 |
| 22 | 33.2 | 33.0 | 33.2 | 33.0 | 33.1 | 33.0 | 75.6 | 32.9 |
| 23 | 180.6 | 180.6 | 181.0 | 180.7 | na[a] | 180.6 | 23.1 | 65.2 |
| 24 | 14.2 | 14.1 | 14.4 | 14.2 | 14.3 | 14.2 | 63.6 | 13.4 |
| 25 | 17.0 | 16.9 | 16.9 | 16.9 | 16.9 | 16.8 | 15.9 | 16.0 |
| 26 | 17.4 | 17.4 | 17.4 | 17.5 | 17.4 | 17.4 | 17.0 | 17.4 |
| 27 | 26.2 | 26.1 | 26.3 | 26.2 | 26.1 | 26.1 | 25.7 | 26.1 |
| 28 | 180.1 | 176.2 | 180.2 | 176.4 | 176.3 | 176.3 | 21.2 | 180.2 |
| 29 | 33.2 | 33.2 | 33.3 | 33.1 | 33.2 | 33.1 | 33.3 | 33.3 |
| 30 | 23.8 | 23.7 | 23.8 | 23.7 | 23.8 | 23.7 | 28.7 | 23.8 |

[a] na, not assigned.

## RESULTS

**Saponin Composition and Structures.** The saponin mixture was separated by preparative LC using a solvent system comprising a 0–100% gradient of methanol in water into six major fractions with the following yields: I, 26 mg; II, 461 mg; III, 120 mg; IV, 189 mg; V, 148 mg; and VI, 106 mg. All fractions consisted of one or two dominant saponin components.

To obtain individual, purified saponins, the fractions were further separated by HPLC. Thus, fraction I was run on column A, and this yielded one major component 1 (10 mg); $R_f$ 0.46 (system 1), 0.32 (system 2), 0.02 (system 3); FAB-MS, (positive ion mode) $m/z$ (rel intensity) 503 (30) $[Ma + H]^+$, 457 (61) $[Ma + H - HCOOH]^+$, 444 (100) $[Ma + H - HCOOH - H_2O]^+$, (negative ion mode) 677 (100) $[M - H]^-$, 501 (88) [M - uronic acid]$^-$, 455 (22) $[M - HCOOH]^-$, 439 (46); $^{1}H$ NMR (anomeric protons), 5.12 (d, 7.0 Hz, GlcA). For $^{13}C$ NMR data, see Tables I and II.

Acid hydrolysis (24 h) yielded medicagenic acid; uronic acid could not be confirmed by TLC. This saponin was easily soluble in water and, at a relatively low concentration, formed a jelly-like solution.

Fraction II consisted of two major components, and they were separated on column A to afford **2** and **3**. Compound **2** (70 mg): $R_f$ 0.25 (system 1), 0.17 (system 2), 0.28 (system 3); FAB-MS, (positive ion mode) 1111 (8) $[M + Na]^+$, 1089 (5) $[M + H]^+$, 957 (3) [M + H - pentose]$^+$, 913 (2) [M + H - uronic acid]$^+$, 679 (8) [M + H - 410]$^+$, 503 (12) [Ma + H]$^+$, 457 (100) $[Ma + H - HCOOH]^+$, 411 (10) [trisaccharide]$^+$, (negative ion mode) 1087 (100) $[M - H]^-$, 911 (16) [M - H - uronic acid]$^-$, 809 (3) [M - H - pentose - deoxyhexose]$^-$, 677 (21), [M - H - 410]$^-$, 501 (19) [Ma - H]$^-$, 455 (14) $[Ma - H - HCOOH]^-$, 409 (30) [trisaccharide - H]$^-$; $^{1}H$ NMR (anomeric protons) 5.12 (d, 7.0 Hz, GlcA), 6.49 (t, 2.8 Hz, Ara), 5.71 (d, 1.5 Hz, Rha), 5.12 (d, 7.0 Hz, Xyl). For $^{13}C$ NMR data, see Tables I and II.

**Table II. $^{13}C$ NMR Chemical Shift Data of Saponins 1–8 (Carbohydrates)**

| | δ | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| C | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
| | β-GlcA | β-GlcA | β-Glcp | β-Glcp | β-Glcp | β-Glcp | β-GlcA | α-Arap |
| C-1 | 105.2 | 105.6 | 105.3 | 105.4 | 105.3 | 103.0 | 105.5 | 103.4 |
| C-2 | 75.0 | 74.9 | 75.2 | 75.2 | 75.2 | 83.7 | 76.8 | 80.2 |
| C-3 | 77.7 | 77.8 | 78.1[a] | 78.4 | 78.4 | 77.9 | 78.5[a] | 72.9 |
| C-4 | 73.4 | 73.3 | 71.6 | 71.6 | 71.5 | 71.1 | 73.9 | 67.8 |
| C-5 | 76.9 | 77.2 | 78.4[a] | 78.4 | 78.4 | 78.3 | 77.7[a] | 63.9 |
| C-6 | 174.7 | 173.4 | 62.6 | 62.7 | 62.7 | 62.6 | 172.6 | |
| | | | | β-Glcp | | β-Glcp | β-Galp | β-Glcp |
| C-1 | | | | 95.8 | | 106.1 | 101.8 | 103.9 |
| C-2 | | | | 74.1 | | 77.0 | 77.8 | 83.8 |
| C-3 | | | | 78.9[a] | | 78.1 | 76.7 | 78.0 |
| C-4 | | | | 71.1 | | 71.1 | 71.2 | 71.2 |
| C-5 | | | | 79.3[a] | | 78.1 | 76.5 | 78.0 |
| C-6 | | | | 62.2 | | 62.5 | 61.6 | 62.4 |
| | | α-Arap | | | α-Arap | α-Arap | α-Rhap | α-Arap |
| C-1 | | 93.4 | | | 93.5 | 93.5 | 102.5 | 106.4 |
| C-2 | | 75.4 | | | 75.3 | 75.4 | 72.4 | 73.7 |
| C-3 | | 69.6 | | | 69.5 | 69.8 | 72.8 | 74.2 |
| C-4 | | 65.9 | | | 66.1 | 66.1 | 74.4 | 69.0 |
| C-5 | | 62.7 | | | 63.0 | 63.0 | 69.5 | 66.9 |
| C-6 | | | | | | | 19.0 | |
| | | α-Rhap | | | α-Rhap | α-Rhap | | |
| C-1 | | 101.1 | | | 101.1 | 101.1 | | |
| C-2 | | 72.7[a] | | | 72.7[a] | 72.7[a] | | |
| C-3 | | 71.9[a] | | | 71.9[a] | 71.9[a] | | |
| C-4 | | 84.2 | | | 84.3 | 84.3 | | |
| C-5 | | 68.6 | | | 68.6 | 68.6 | | |
| C-6 | | 18.4 | | | 18.4 | 18.4 | | |
| | | β-Xylp | | | β-Xylp | β-Xylp | | |
| C-1 | | 107.8 | | | 107.8 | 107.2 | | |
| C-2 | | 76.0 | | | 76.1 | 76.1 | | |
| C-3 | | 78.6 | | | 78.6 | 78.6 | | |
| C-4 | | 71.0 | | | 71.0 | 70.9 | | |
| C-5 | | 67.5 | | | 67.5 | 67.5 | | |

[a] Assignment may be reversed.

Compound **2** was easily soluble in water and methanol. Acid hydrolysis (8 h) yielded medicagenic acid, xylose, rhamnose, and arabinose. Again no uronic acid was detected. However, TLC analysis of prosapogenins showed the presence of a compound with chromatographic characteristics identical with those of compound **1**. Moreover, alkaline hydrolysis (16 h) of **2** afforded the prosapogenin with TLC behavior and FAB-MS identical with those for compound **1**.

Also obtained from fraction II was compound **3** (40 mg): $R_f$ 0.69 (system 1), 0.63 (system 2), 0.02 (system 3); FAB-MS, (positive ion mode) 665 (10) $[M + H]^+$, 503 (15) $[Ma + H]^+$, 457 (85) $[Ma + H - HCOOH]^+$, 441 (100) $[Ma + H - HCOOH - H_2O]^+$, (negative ion mode) 663 (42) $[M - H]^-$, 501 (15) [M - H - hexose]$^-$, 455 (11) $[Ma + H - HCOOH]^-$, 439 (38) $[Ma + H - HCOOH - H_2O]$; $^1H$ NMR (anomeric protons) 5.14 (d, 7.3 Hz, Glc). For $^{13}C$ NMR data, see Tables I and II.

Acid hydrolysis (8 h) afforded medicagenic acid and glucose. Compound **3** was easily soluble in methanol and moderately soluble in water.

Fraction III provided one main compound which was purified on column B. This yielded **4** (20 mg), which was crystallized from an ethanol/water mixture, providing needles: mp 267–268 °C; $R_f$ 0.4 (system 1), 0.6 (system 2), 0.3 (system 3); FAB-MS, (positive ion mode) 849 (78) $[M + Na]^+$, 665 (17) [M + H - hexose]$^+$, 503 (19) [M + H - 2 hexoses]$^+$, 457 (100) $[Ma + H - HCOOH]^+$, (negative ion mode) 825 (100) $[M - H]^-$, 663 (93) [M - H - hexose]$^-$, 501 (23) $[Ma - H]^-$; $^1H$ NMR (anomeric protons) 5.11 (d, 7.6 Hz, Glc), 6.31 (d, 7.9 Hz, Glc). For $^{13}C$ NMR data, see Tables I and II.

Acid hydrolysis (8 h) afforded medicagenic acid and glucose. Alkaline hydrolysis was completed successfully overnight at room temperature and yielded glucose and a prosapogenin with TLC and FAB-MS parameters identical with those found for compound **3**.

Fraction IV consisted of two major components that were successfully separated with column B. This provided compounds **5** and **6**.

Compound **5** (30 mg): $R_f$ 0.25 (system 1), 0.17 (system 2), 0.28 (system 3); FAB-MS (positive ion mode) 1097 (29) $[Ma + Na]^+$, 1075 (6) $[M + H]^+$, 943 (3) [M + H - pentose]$^+$, 665 (7) [M + H - 409]$^+$, 503 (19) $[Ma + H]^+$, 457 (100) $[Ma + H - HCOOH]^+$, 439 (90) $[Ma + H - HCOOH - H_2O]^+$, 411 (58) [trisaccharide]$^+$, (negative ion mode) 1073 (100) $[M - H]^-$, 911 (9) [M - H - hexose]$^-$, 941 (2) [M - H - pentose]$^-$, 663 (29) [M - H - 409]$^-$, 501 (14) $[Ma - H]^-$, 439 (95) $[Ma - H - HCOOH - H_2O]^-$, 409 (2) [trisaccharide - H]$^-$; $^1H$ NMR (anomeric protons) 5.12, (d, 7 Hz, Glc), 6.49 (t, 2.9 Hz, Ara), 5.77 (d, 1.6 Hz, Rha), 5.12 (d, 7 Hz, Xyl). For $^{13}C$ NMR data, see Tables I and II.

Acid hydrolysis (8 h) afforded medicagenic acid, glucose, xylose, rhamnose, and arabinose. Alkaline hydrolysis (24 h, 90 °C) produced a prosapogenin identical (TLC, FAB-MS) with compound **3**.

Compound **6** (10 mg): $R_f$ 0.23 (system 1), 0.15 (system 2), 0.31 (system 3); FAB-MS, (positive ion mode) 1237 (16) $[M + H]^+$, 1075 (3) $[M + H - \text{hexose}]^+$, 913 (4) $[M + H - 2 \text{ hexoses}]^+$, 827 (4) $[Ma + H - 411]^+$, 503 (27) $[Ma + H]^+$, 457 (100) $[Ma + H - COOH]^+$, 439 (54) $[Ma - HCOOH - H_2O]^+$, 411 (40) [trisaccharide + H]$^+$, (negative ion mode) 1235 (100) $[M - H]^-$, 1103 (18) $[M - H - \text{pentose}]^-$, 1073 (11) $[M - H - \text{hexose}]^-$, 911 (10) $[M - H - 2 \text{ hexoses}]^-$, 825 (18) $[M - H - 409]^-$, 409 (4) [trisaccharide - H]$^-$; $^1$H NMR (anomeric protons) 5.12 (d, 7.0 Hz, Glc), 5.22 (d, 7.6 Hz, Glc), 6.49 (t, 2.4 Hz, Ara), 5.76 (d, 1.7 Hz, Rha), 5.12 (d, 7 Hz, Xyl). For $^{13}$C NMR data, see Tables I and II.

Acid hydrolysis (8 h) afforded medicagenic acid, glucose, xylose, rhamnose, and arabinose. Alkaline hydrolysis yielded a prosapogenin with TLC characteristics different from those of compound **3**, which on acid hydrolysis afforded medicagenic acid and glucose.

Fraction V included one dominant component that was separated on column A. This yielded compound **7** (10 mg): $R_f$ 0.26 (system 1), 0.28 (system 2), 0.22 (system 3); FAB-MS, (positive ion mode) 943 (37) $[M + H]^+$, 797 (14) $[M + H - \text{deoxyhexose}]^+$, 635 (9) $[M + H - \text{deoxyhexose} - \text{hexose}]^+$, 441 (100) $[M + H - \text{deoxyhexose} - \text{hexose} - \text{uronic acid} - H_2O]^+$, 423 (100) $[M + H - \text{deoxyhexose} - \text{hexose} - \text{uronic acid} - 2H_2O]^+$, (negative ion mode) 941 (100) $[M - H]^-$, 795 (24) $[M - H - \text{deoxyhexose}]^-$, 633 (28) $[M - H - \text{deoxyhexose} - \text{hexose}]^-$, 439 (6) $[M - H - \text{deoxyhexose} - \text{hexose} - \text{uronic acid} - H_2O]^-$; $^1$H NMR (anomeric protons) 4.99 (d, 7 Hz, GlcA), 5.79 (d, 7.3 Hz, Gal), 6.29 (d, 1.7 Hz, Rha). For $^{13}$C NMR data, see Tables I and II.

Fraction VI consisted of two components with very similar polarities and was successfully separated on column B to yield compounds **8** and **9**.

Compound **8** (15 mg): $R_f$ 0.42 (system 1), 0.22 (system 2); FAB-MS, (positive ion mode) 921 (100) $[M + Na]^+$, 899 (100) $[M + H]^+$, 767 (40) $[M + H - \text{pentose}]^+$, 605 (12) $[M + H - \text{pentose} - \text{hexose}]^+$, (negative ion mode) 897 (100) $[M - H]^-$, 765 (35) $[M - H - \text{pentose}]^-$, 603 (50) $[M - H - \text{pentose} - \text{hexose}]^-$, 471 (36) $[M - H - 2 \text{ pentoses} - \text{hexose}]^-$; $^1$H NMR (anomeric protons) 5.32 (d, 4.9 Hz, Ara), 5.28 (d, 7.3 Hz, Glc), 5.31 (d, 6.4 Hz, Ara). For $^{13}$C NMR data, see Tables I and II.

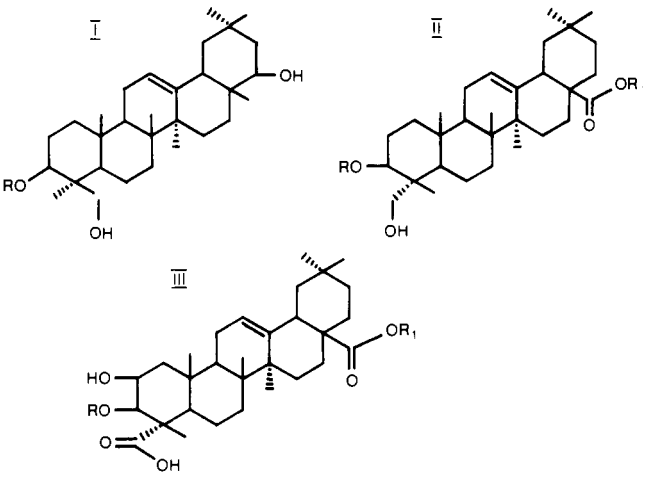
Acid hydrolysis afforded hederagenin, glucose, and arabinose, while alkaline hydrolysis (8 h) did not change the product.

Compound **9** (3 mg): $R_f$ 0.45 (system 1), 0.25 (system 2); FAB-MS, (positive ion mode) 789 (21) $[M + Na]^+$, 767 (100) $[M + H]^+$, 605 (8) $[M + H - \text{hexose}]^+$, (negative ion mode) 765 (100) $[M - H]^-$, 603 (85) $[M - H - \text{hexose}]^-$, 471 (61) $[M - H - \text{hexose} - \text{pentose}]^-$.

Acid hydrolysis (8 h) produced hederagenin, glucose, and arabinose, while alkaline hydrolysis (8 h) yielded a chromatographically unchanged product.

These data are consistent with the structures shown in Figure 1 for compounds **1–9**.

**Antifungal Activities.** Antifungal activities of the separated compounds are given in Figures 2 and 3, and their inhibitory activities, which show the weight (milligrams) of saponins in growth medium (100 mL) that is able to inhibit fungus growth by 50% ($IA_{50}$ values), are given in Table III. The highest activity was found for the 3-*O*-glucopyranoside of medicagenic acid (**3**) (Figure 2), and fungal growth was completely retarded at a concentration of 0.21 mg/100 mL. Bisdesmoside analogues of this compound showed lower activities which do not fully correlate to the number of sugars attached to the aglycon. Thus, the 3,28-diglucoside of medicagenic acid (**4**) has an $IA_{50}$ value of 3.30, much higher than that for **5**, $IA_{50}$ 1.35, but similar to that found for **6**, $IA_{50}$ 3.50.




| Compounds | Aglycone | R | $R_1$ |
|---|---|---|---|
| 1. | III | GlcA | H |
| 2. | III | GlcA | Ara-Rha-Xyl |
| 3. | III | Glc | H |
| 4. | III | Glc | Glc |
| 5. | III | Glc | Ara-Rha-Xyl |
| 6. | III | Glc-Glc | Ara-Rha-Xyl |
| 7. | I | GlcA-Gal-Rha | — |
| 8. | II | Ara-Glc-Ara | H |
| 9. | II | Ara-Glc | H |

**Figure 1.** Structures of compounds **1–9**.

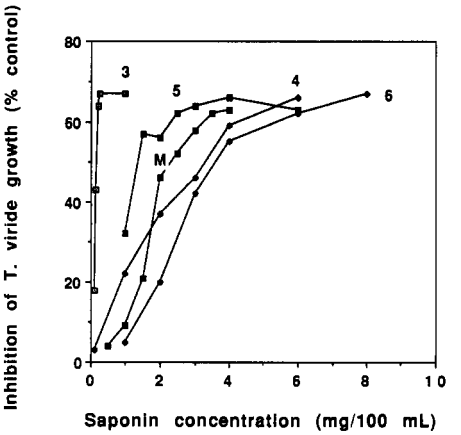



**Figure 2.** Inhibition of *T. viride* growth by alfalfa root saponins: (3) 3-Glc medicagenic acid; (4) 3,28-diglucoside medicagenic acid; (5) 3-Glc, 28-Ara-Rha-Xyl medicagenic acid; (6) 3-Glc-Glc, 28-Ara-Rha-Xyl medicagenic acid; (M) cholesterol-precipitable alfalfa root saponin mixture.

The structural analogue of compound **3** (**1**) also showed a very high $IA_{50}$ value of 0.91, but its corresponding bisdesmoside (**2**) had an $IA_{50}$ value of 4.75 and was the compound with least activity (Figure 3).

High activity was also shown by 3-*O*-Ara-Glc-Ara hederagenin (**8**) ($IA_{50}$ = 2.25). Its analogue, 3-*O*-Ara-Glc hederagenin, was isolated in trace amounts and could not be tested against *T. viride*. Figures 2 and 3, for comparative purposes, also include data obtained for the mixture of alfalfa root saponins (M) isolated by the cholesterol precipitation method (Oleszek and Jurzysta, 1986). This mixture showed an activity of $IA_{50}$ = 2.35, close to the average value of all the saponins tested.

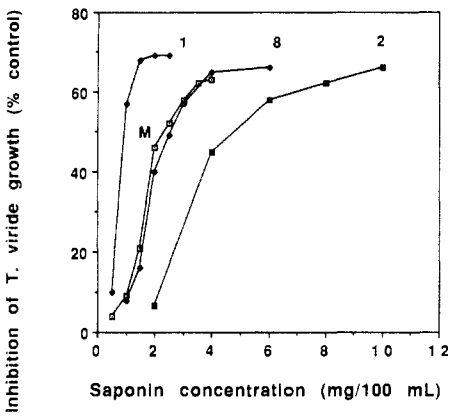



**Figure 3.** Inhibition of *T. viride* growth by alfalfa root saponins: (1) 3-GlcA medicagenic acid; (2) 3-GlcA,28-Ara-Rha-Xyl medicagenic acid; (8) 3-Ara-Glc-Ara hederagenin; (M) cholesterol-precipitable alfalfa root saponin mixture.

**Table III. Values of Alfalfa Root Saponins against *T. viride***

| compound | $IA_{50}$ value, mg/100 mL |
|---|---|
| 3-Glc Ma[a] | 0.16 |
| 3-Glc, 28-Ara-Rha-Xyl Ma | 1.35 |
| 3-Glc, 28-Glc Ma | 3.30 |
| 3-Glc-Glc, 28-Ara-Rha-Xyl Ma | 3.50 |
| 3-GlcA Ma | 0.91 |
| 3-GlcA, 28-Ara-Rha-Xyl Ma | 4.75 |
| 3-Ara-Glc-Ara hederagenin | 2.55 |
| alfalfa root saponin mixture | 2.35 |

[a] Ma, medicagenic acid.

## DISCUSSION

Reversed-phase column chromatography with a methanol/water gradient solvent system has been shown to be a powerful tool in separating multicomponent saponin mixtures into simple subfractions. These subfractions have been further separated and purified with HRPLC in either normal or reversed-phase modes and yielded several, high-purity, naturally occurring alfalfa root saponins. Their chemical structures were determined by means of chemical and spectral analyses.

$^{13}C$ nuclear magnetic resonance data for the sapogenins and the sugar units of the saponins are given in Tables I and II, respectively. The assignments of the $^{13}C$ NMR resonances for the sapogenins were based on detailed 2D NMR experiments on medicagenic acid, hederagenin, and soyasapogenol B. These experiments, including NOESY and COLOC (Ernst et al., 1987), made a complete assignment possible including all the methyl and quaternary carbons. As discussed below, compounds **1–6** were found to be derivatives of medicagenic acid and compounds **7** and **8** derivatives of soyasapogenol B and hederagenin, respectively. All the saponins had sugar residues attached at O-3 of the aglycon unit since the chemical shift of C-3 was found to be about 10 ppm higher in all the compounds than in the parent sapogenins. In addition, compounds **2** and **4–6** had sugars attached by an ester linkage to the C-28 of the carboxylic acid group. This was shown by the characteristic change in $^{13}C$ chemical shift of C-28 from 180.2 (free carboxylic group) to 176.3 ppm (esterified carboxylic group). In the case of medicagenic acid derivatives it is not immediately obvious whether these sugar units are attached at C-28 or at C-23 of the carboxylic acid group. Various methods of resolving this problem were discussed by Massiot et al. (1988b), who showed that the sugars were linked at C-28. It can be seen from Table I that in compounds **2** and **4–6** the chemical shift of C-17 (adjacent to C-28) is 47.3 ppm, whereas in **1** and **3** (and in medicagenic acid itself) it is 46.6 ppm. By contrast the chemical shift of C-4 (adjacent to C-23) shows little variation in **1–6**. Therefore, it appears that the chemical shift of C-17 can be used as a simple indicator of esterification at C-28 in addition to the methods proposed by Massiot et al. (1988b). The presence of sugar units at C-28 also affects the $^{13}C$ chemical shifts of C-12 and C-13, with C-12 showing an increase of 0.4 ppm and C-13 showing a decrease of 0.7 ppm when the carboxylic group is esterified.

Compound **1** was shown by FAB mass spectrometry to have a molecular weight of 678 mu. Spectra in both the negative and positive ion modes showed strong ions of $m/z$ 501 and 503, respectively, indicating the presence of medicagenic acid ($M_r$ 502). The presence of medicagenic acid was also confirmed by acid hydrolysis of this compound. Thus, the loss of 176 mu from the molecular ion was related to the uronic acid molecule. This was further supported by the $^{13}C$ NMR spectrum, which showed the presence of three carboxyl carbons. The other resonance signals were in good agreement with those obtained for medicagenic acid and glucuronic acid. Observation of the anomeric carbon signal at 105.2 ppm and a coupling, $J_{12}$, of 7 Hz ($^1H$ spectrum) shows that the glucuronic acid exists in the $\beta$-pyranoside form (Gorin and Mazurek, 1975). Considering all these data, **1** can be identified as 2-$\beta$-hydroxy-3-$\beta$-*O*-(glucuronopyranosyl)-$\Delta^{12}$-oleane-23,28-dioic acid (medicagenic acid 3-*O*-$\beta$-glucuronopyranoside). This compound has been previously found in hydrolysis products of saponins isolated from *Herniaria glabra* (Klein et al., 1982) and also in hydrolysis products of alfalfa leaf saponins (Massiot et al., 1988a). This has, however, not been reported so far as a naturally occurring compound in alfalfa and has not been found as prosapogenin present in the products of hydrolysis of alfalfa root saponins. Thus, its presence in alfalfa roots is reported here for the first time. One reason for this is the difficulty of separating this compound from plant material by using a silica gel support since its chromatographic characteristics are similar to those of 3,28-diglucopyranoside medicagenic acid. The method reported here allows easy separation since the compound can be eluted from the column with water, allowing other compounds to be retained on the column.

Compound **2**, whose presence in alfalfa roots was tentatively reported (Oleszek 1988), was shown to have a molecular weight of 1088 mu by FAB-MS: negative ion mode produced a spectrum containing ions at $m/z$ 1087, 911, 809, 677, 501, and 409 corresponding to the (M – H) ion and sequential losses of uronic acid (176 mu), pentose plus deoxyhexose (278 mu), and trisaccharide (410 mu). This also included an ion $m/z$ 501 which corresponded to the deprotonated medicagenic acid molecule. The high abundance of ion $m/z$ 409 was assigned to the trisaccharide moiety comprising arabinose, rhamnose, and xylose since these three sugars were found to be present in the acid hydrolysis products in equimolar proportions. Alkaline hydrolysis of compound **2** yielded a product identical with 3-*O*-glucuronopyranoside medicagenic acid (**1**). Thus, it was concluded that the trisaccharide was linked via an ester linkage. The $^{13}C$ NMR spectrum contained four anomeric carbons at 105.6, 93.4, 101.1, and 107.8 ppm, corresponding to glucuronic acid, arabinose, rhamnose, and xylose, respectively. A group of resonance signals in the region of 60–85 ppm was identical with that found for the glucuronic acid residue in compound **1**, and the remaining signals in this region were the same as those previously reported (Ishii et al.,

1984) for the trisaccharide unit β-D-Xylp(1→4)α-L-Rhap-(1→2)α-L-Arap linked to C-28 in deapioplatycodin D. The chemical shifts of H-1(Ara) and C-1(Ara), which are 6.51 and 93.4 ppm, respectively, show that arabinose is connected to the aglycon via an ester linkage. The H-1-(Ara) resonance was not a simple doublet because the chemical shifts of H-2 and H-3(Ara) were almost identical, giving rise to second-order effects. However, the coupling constant $J_{12}$ of 2.8 Hz, obtained by spectral analysis, shows that the α-L-Ara residue has the $^1C_4$ conformation (Ishii et al., 1981). Compound **2** can therefore be identified as 2-β-hydroxy-3-β-*O*-(glucuronopyranosyl)-28-*O*-[β-D-xylopyranosyl(1→4)-α-L-rhamnopyranosyl(1→2)-α-L-arabinoside)-$\Delta^{12}$-olean-23-oic acid (3-GlcA,28-Ara-Rha-Xyl medicagenic acid). This compound was found to be the most abundant in alfalfa roots, and its structure has not been previously reported.

Acid hydrolysis of compound **3** revealed glucose and medicagenic acid, and from examination of the mass spectrum it was evident that the molecule contained only one glucose moiety. The $^{13}C$ NMR spectrum was consistent with that obtained for medicagenic acid with a glucose attached at the 3-O position. The presence of this compound in alfalfa roots has been previously reported (Morris et al., 1961; Levy et al., 1986), and it has also been reported as the main component in saponin mixtures isolated from *M. lupulina* roots (Oleszek et al., 1988a).

Compound **4** resembled compound **3** in that acid hydrolysis yielded glucose and medicagenic acid. However, the mass spectrum showed the molecular weight to be 826 mu, which suggested the presence of two glucose sugars in the molecule. Abundant ions in the negative ion mode spectrum of $m/z$ 663 and 501 corresponded to the sequential loss of one and two glucoses, respectively. Alkaline hydrolysis of saponin **4** produced compound **3**, which indicated that one of the glucoses was ester linked. This was also confirmed by the NMR spectra which showed one anomeric carbon at 105.4 ppm (identical with compound **3**) and the second anomeric carbon at 95.8 ppm and corresponding H-1 at 6.31 ppm which is characteristic for the β-anomeric linkage of an esterified (C-28) glucose unit (Kizu et al., 1985). Thus, saponin **4** was unequivocally identified as medicagenic acid 3,28-di(*O*-glucupyranoside). This saponin was previously identified in alfalfa roots (Timbekova and Abubakirov, 1984; Massiot et al., 1988b) and was also found in *M. lupulina* roots (Oleszek et al., 1988a). It is of interest to note that in this study compound **4** was the only medicagenic acid glycoside that could be successfully crystallized.

Compound **5** was shown to have a molecular weight of 1076 mu and after acid hydrolysis yielded medicagenic acid, glucose, arabinose, rhamnose, and xylose. Alkaline hydrolysis produced a compound identical with compound **3**, which indicated that the arabinose, rhamnose, and xylose were attached via an ester linkage. This was further supported by the presence of strong ions $m/z$ 665 and 663 (positive and negative ion mode, respectively) that corresponded to the loss of a trisaccharide moiety and by the ions $m/z$ 411 and 409 corresponding to a trisaccharide unit of the same mass to that found in compound **2**. The $^{13}C$ NMR spectrum contained four anomeric carbons at 105.3, 93.5, 101.1, and 107.8 ppm corresponding to glucose, arabinose, rhamnose, and xylose, respectively. The other resonance signals corresponding to sugar carbons were identical with those obtained for glucose (cf. compound **3**) and (1,2)-linked arabinose, (1,4)-linked rhamnose, and terminal xylose as found for compound **2**. Thus, compound **5** was concluded to be 3-β-*O*-(glucopyranosyl)-28-*O*-[β-D-xylopyranosyl(1→4)-α-L-rhamnopyranosyl-(1→2)-α-L-arabinosyl]medicagenic acid. This is in good agreement with the previous findings of Massiot et al. (1988b), who detected the same compound in alfalfa roots using acetylation of the saponin mixture as an aid to the separation of the individual saponins.

Compound **6** had a molecular weight of 1236. Both positive and negative spectra showed the loss of a trisaccharide moiety from the molecule ($m/z$ 827 and 825, respectively) and strong ions of $m/z$ 411 and 409 corresponding to the trisaccharide itself, as was described for **2** and **5** above. Strong ions of $m/z$ 1073 and 911 in the negative ion spectrum indicated the loss of one and two hexoses from the molecule. Moreover, an ion, $m/z$ 1103, suggested the loss of a terminal pentose which was comparable to the loss of xylose from the Ara-Rha-Xyl sugar chain found in compounds **2** and **5**. This suggested that the two hexoses were attached at the O-3 position and was further supported by the analysis of the hydrolysis products. The alkaline hydrolysis of compound **6** produced a sapogenin that had TLC characteristics different from those of compound **3** but which on acid hydrolysis yielded medicagenic acid and glucose. The $^{13}C$ NMR spectrum of **6** showed the presence of both an inner and a terminal glucose with three other sugars, namely, arabinose, rhamnose, and xylose, having chemical shifts identical with those in compounds **2** and **5**. The increase in chemical shift for C-2 of the inner glucose unit to 8.37 ppm (cf. **5**) showed that this residue was linked to the terminal glucose unit at position O-2. Considering all these data, compound **6** was identified as 3-*O*-[β-D-glucopyranosyl(1→2)-β-D-glucopyranoside]-28-*O*-[β-D-xylopyranosyl(1→4)-α-L-rhamnopyranosyl(1→2)-α-L-arabinopyranoside] medicagenic acid. The occurrence of this compound in alfalfa roots was recently reported (Massiot et al., 1988b).

Compound **7** gave soyasapogenols B–D and F and rhamnose, galactose, and glucuronic acid on acid hydrolysis, which is consistent with the presence of soyasapogenol B, together with the formation of the remaining soyasapogenols as artifacts of hydrolysis (Jurzysta, 1978; Price et al., 1986). The negative ion mode mass spectrum showed ions $m/z$ 941, 759, and 663 corresponding to the molecule ion and sequential losses of rhamnose and galactose, respectively. These data as well as close examination of the $^{13}C$ NMR spectrum proved this compound to be identical with the saponin known as soyasaponin I, which is widely distributed in nature (Price et al., 1987). It has, however, not been previously reported in alfalfa roots. The $^{13}C$ NMR assignments for compound **7**, as given in Table II, are based on 2D NMR experiments and differ slightly from those given previously by Yoshikawa et al. (1985).

Fraction VI contained two glycosides, one of which, compound **8**, was dominant and the other, compound **9** occurred only in trace amounts. These two compounds, however, were closely related in their structures as both of them on acid hydrolysis yielded arabinose, glucose, and hederagenin, whereas alkaline hydrolysis produced unchanged products. This suggested that both compounds were monodesmosides substituted at the O-3 position. Compound **9** produced a mass spectrum with ions of $m/z$ 765, 603, and 471 (negative ion mode) that corresponded to the molecular ion and subsequent losses of glucose and arabinose, respectively. This suggested that **9** was the 3-*O*-arabinoglucoside of hederagenin. The $^{13}C$ NMR spectrum was not obtained because of insufficient material. Compound **8** had a molecular ion 132 mu higher than that of compound **9**. Since similar sugars were found in the hydrolysis products of both compounds, it could be

calculated that compound 8 had one arabinose unit more than compound 9. The extra arabinose was shown to be a terminal sugar as indicated by the presence of ion $m/z$ 765 in the mass spectrum of compound 8. Detailed assignment of the $^{13}C$ NMR spectrum using 2D NMR experiments revealed the presence of an inner and a terminal arabinose and an inner glucose. The glycosidic shift of carbon C-2 in inner arabinose and glucose indicated the site of the sugar linkages. The terminal α-L-Ara residue was in the $^4C_1$ conformation ($J_{12}$ = 6.4 Hz), and this conformation also predominated for the (1,2)-linked α-L-Ara unit ($J_{12}$ = 4.9 Hz) (Ishii et al., 1981). The chemical shifts of H-2 and H-3 of the latter residue were almost identical, so that the signal for H-1 appeared as a triplet rather than a doublet. Thus, compound 8 was identified as 3-*O*-[α-L-arabinopyranosyl(1→2)-β-D-glucopyranosyl-(1→2)-α-L-arabinopyranoside] hederagenin and compound 9 its dearabinoside analogue. The presence of these compounds in alfalfa roots has been previously reported (Timbekova and Abubakirov, 1984).

The data presented here clearly show that alfalfa root saponins consist of medicagenic acid, hederagenin, and soyasapogenol B glycosides and is in good agreement with previous findings (Gestetner et al., 1970; Oleszek and Jurzysta, 1986). Whereas hederagenin and soyasapogenol B glycosides were found as monodesmosidic structures, the medicagenic acid glycosides could be found both in monodesmosidic and bisdesmosidic forms, with the latter being the most abundant. Moreover, it can be recognized that the medicagenic acid glycosides can be divided into two distinct groups which are substituted at the O-3 positions of the sapogenin either with glucose or glucuronic acid. The glucuronic acid substituted medicagenic acid glycosides have not been previously reported in alfalfa roots.

The activities of the individual, purified saponins against *T. viride* varied strongly. The highest activity found was for 3-*O*-glucopyranoside medicagenic acid (3), this result supporting previous findings (Levy et al., 1986; Oleszek et al., 1988; Polacheck et al., 1986). The bisdesmosidic analogues were less active, the activity gradually declining as the number of sugar residues in the saponin molecule increased. A similar trend was found for the 3-*O*-glucuronide of medicagenic acid (1) and its bisdesmoside (2). However, both were much less active than their analogues with glucose linked at the 3-O position. The exception was the 3,28-diglucopyranoside of medicagenic acid, and this result remains to be explained. Hederagenin, not previously reported to be active against *T. viride*, had similar activity to that of the mixture of cholesterol-precipitable alfalfa root saponins. This saponin mixture was used previously as the calibrating standard for saponin determination in alfalfa roots (Oleszek and Jurzysta, 1986) by the *T. viride* test.

The test conducted here has been widely used for saponin determination in alfalfa plant material (Zimmer et al., 1967; Jurzysta, 1979), and since the composition of individual alfalfa saponins in most alfalfa materials remains so far unknown, this method has usually been calibrated with undefined saponin mixtures (Jurzysta et al., 1984). Relative levels of the individual components in these mixtures may vary depending on the plant part used and also on the climatic or agronomic conditions. Comparison of the curves calculated for individual saponins in this study shows that when determination of saponins is made by using the saponin mixture as a standard, the final result can be strongly influenced by a quantitative variation of the mixture components.

Thus, when plant tissue rich in monodesmosidic saponins is analyzed, the final result may be significantly overestimated; conversely, if bisdesmosides are dominant, the final value will be underestimated. The data thus obtained are highly imprecise, and this fact strongly indicates that methods for the separation and determination of *individual* saponins are in urgent need of development.

ACKNOWLEDGMENT

We are grateful to Ms B. Ciarkowska and Mrs. B. Wojewodka for excellent technical assistance in isolating the compounds and to the IFRN Mass Spectrometry Group for the FAB mass spectra.

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Received for review December 21, 1989. Accepted May 15, 1990.

**Registry No.** **1**, 84161-90-0; **2**, 128192-15-4; **3**, 49792-23-6; **4**, 106074-96-8; **5**, 120039-30-7; **6**, 120039-31-8; **7**, 51330-27-9; **8**, 102349-43-9; **9**, 128192-16-5.