a variety of matrixes when analyzed by a number of different analysts. The method has also been used on numerous other matrixes, albeit by a smaller number of analysts. It is presented as a universal method in that only minor modifications of extraction (especially soil) and cleanup steps may be required for unusual matrixes.

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Registry No. AMPA, 1066-51-9; H_2O, 7732-18-5; glyphosate, 1071-83-6.

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An Improved Procedure for the Isolation of Medicagenic Acid 3-*O*-β-D-Glucopyranoside from Alfalfa Roots and Its Antifungal Activity on Plant Pathogens

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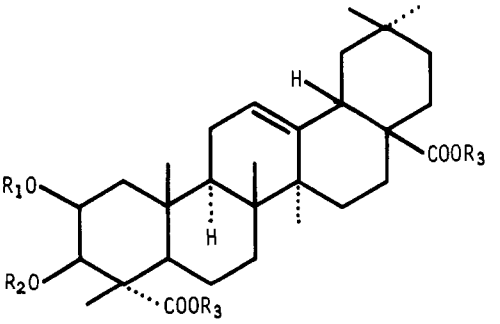
2β-Hydroxy-3β-*O*-(β-D-glucopyranosyl)-Δ^{12}-oleanene-23,28-dioic acid, known also as medicagenic acid 3-*O*-β-D-glucopyranoside, was isolated from alfalfa roots in pure form and was shown to possess potent fungistatic effects against *Trichoderma viride*, *Sclerotium rolfsii*, *Rhizopus mucco*, *Aspergillus niger*, *Phytophthora cinamommi*, and *Fusarium oxysporum* f. sp. *lycopersici*: i.e., mycelial growth inhibition of 95%, 86%, 68%, 53%, 51%, and 52%, respectively, for concentrations of 40 μg/mL and ID_{50} of 1.4, 2.3, 4.1, 1.7, 40, and 10.5 μg/mL, respectively.

INTRODUCTION

The antifungal activity, as well as other biological activities, of saponins was extensively reviewed in the literature (Kofler, 1929; Birk, 1969; Birk and Peri, 1979; Schlösser, 1983). Saponin extracts from alfalfa were shown to possess a fungistatic activity (Shani et al., 1970; Gestetner et al., 1971; Assa et al., 1972; Leath et al., 1972).

Medicagenic acid (2β,3β-dihydroxy-Δ^{12}-oleanene-23,28-dioic acid, I) was first identified by Djerassi and co-workers (Walter et al., 1955; Djerassi et al., 1957), and the corresponding 3-*O*-β-D-glucopyranoside II was first isolated from alfalfa roots and its structure determined by Morris et al. (1961) (Chart I). Although the significance of saponins derived from medicagenic acid in the antimycotic acitivity of saponin extracts from alfalfa roots was recognized in the past (Birk and Peri, 1979), a very limited study of such synthetic saponins was carried out (Gestetner et al., 1973) while none was carried out with pure native saponins presumably since alfalfa contains a large number of saponins that are difficult to separate.

Chart I



I: $R_1 = R_2 = R_3 = H$
II: $R_1 = H$, R_2 = β-D-Glc-*p*-, $R_3 = H$
III: $R_1 = R_2 = H$, $R_3 = Me$
IV: $R_1 = R_2 = Ac$, $R_3 = H$
V: $R_1 = R_2 = Ac$, $R_3 = Me$

Following screening of antimycotic compounds from alfalfa root extract, we identified a compound (G2) active against *Trichoderma viride* and *Sclerotium rolfsii*. In this work we report an improved method for the isolation of

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compound G2 and the elucidation of its structure by chemical, physical, and enzymic techniques. We also studied the antimycotic activity of compound G2 against some fungi, namely *S. rolfsii*, *Pytium aphanidermatum*, *Rhizoctonia solani*, *Rhizopus mucco*, *Aspergillus niger*, *Phytophthora cinamommi*, *Fusarium oxysporum* f. sp. *lycopesici*, and *T. viride*. These fungi (except the last) are pathogens well-known to cause agricultural damage (Agrios, 1978).

EXPERIMENTAL SECTION

Instruments. IR spectra were recorded on a Nicolet MX-S FTIR; MS (electron impact) were recorded on a VG 7035 mass spectrometer; ^1H NMR spectra were recorded with a Bruker HFX-10 (90-MHz) instrument; and ^{13}C NMR were recorded with a Bruker WP-200 sy (200-MHz) instrument; GC were run on a Packard gas chromatograph; HPLC separations were performed on a SP-8000 liquid chromatograph equipped with a Merck RSIL-C 18-HL (50 × 1 cm) column; optical rotations were measured with a Bendix polarimeter.

TLC. TLC analyses were carried out on Merck silica gel 60 F_{254} plates, developed with EtOAc–H_2O–AcOH (7:2:2, v/v) in the instance of saponin preparation and with EtOAc–$CHCl_3$ (4:1, v/v) for the preparation of medicagenic acid and its derivatives. HPTLC analyses were performed on Merck RP-18 F_{254} plates and developed with MeOH–H_2O (4:1, v/v). Staining in all cases involved spraying with H_2SO_4 (5% v/v in EtOH) and heating for 10 min at 100 °C.

Extraction and Purification. Alfalfa (*Medicago sativa* L. Gilboa variety from Ram ranch, Beer Sheva) root flour (200 g) was extracted with EtOH 80% (v/v, 2.5 L) in water for 16 h at 60 °C. The solid was then filtered and the ethanol removed under reduced pressure. The resulting aqueous solution was twice extracted with ether (400-mL portions), which was subsequently removed under reduced pressure while the extract was adsorbed onto silica gel (Merck, 70–230 mesh, 6 g) that was applied and chromatographed (2-g portions) on the same adsorbent (150 g) with EtOAc–H_2O–AcOH (7:2:2, v/v, 350 mL). Fractions containing a typical blue spot at R_f 0.75 (in later experiments authentic G2 was used as a marker) were pooled, and the solvents were evaporated while the extract was adsorbed onto silica gel (Merck, 70–230 mesh, 2 g). This fraction (C1, 2 g) was rechromatographed on the same adsorbent (100 g) with EtOAc–MeOH (4:1, v/v, 200 mL) followed by EtOAc–H_2O AcOH (7:2:2, v/v, 300 mL). Fractions containing the typical blue spot on TLC (R_f 0.75) were pooled, solvents were evaporated, and the residue was resuspended in water (5 mL) and lyophilized to yield about 80 mg of G.

Preparation G was further purified to yield G2 by applying C1 onto a flash chromatography column (Merck, silica gel 60, 230–400 mesh, 15 g) eluted successively with EtOAc–MeOH [4:1, 2:1, and 1:1 (v/v, each fraction 250 mL)] at a flow rate of 10 mL/min. Fractions containing G (TLC) were pooled and adsorbed onto silanized silica (Merck, silica gel 60, silanized 70–230 mesh, 1.5 g) while solvents were removed under reduced pressure. This fraction (C2, 1.5 g) was chromatographed on the same adsorbent (15 g) with 250-mL portions of MeOH–H_2O [45:55, 50:50, and 55:45 (v/v) successively]. Fractions containing G (TLC) were pooled and partially evaporated, and the residual mostly aqueous solution was lyophilized to yield G1 (55 mg). The last preparation (G1, 20 mg in 1 mL of MeOH) was applied to a preparative HPLC column eluted with a MeOH–H_2O gradient ranging from 70% to 85% over 60 min. Fractions (4-mL each) were monitored by TLC; those containing G1 were further monitored by HPTLC. Fractions containing a sole blue spot at R_f 0.28 after staining were pooled, concentrated under reduced pressure, and lyophilized to yield a white amorphous solid—G2 [16 mg starting from 20 mg of G1; mp 251–254 °C; $[\alpha]^{30}_D$ +70.6° (EtOH)] that eventually proved to be medicagenic acid 3-*O*-β-D-glucopyranoside (II) [lit. mp 253–254 °C; $[\alpha]_D$ +70° (Morris et al. (1961)].

Screening. Alfalfa root extract in 80% (v/v) EtOH in water (50 mL) was passed through a charcoal (Darco G-60)–Celite 545 (1:1, w/w, 40 g) column eluted with EtOH (600 mL). The EtOH was evaporated and the residue resuspended in water (50 mL), twice extracted with EtOAc (50 mL each portion), and washed with water (60 mL). The solvent was concentrated to about 5 mL and applied (0.5 mL) on a TLC plate (8-cm line, 1.5 mL). After the plate was developed and dried, a 3-cm strip was cut and stained, while the residual strip was sprayed with a suspension of *T. viride* spores in sporulating medium (D-glucose 1 g, $NaNO_3$ 1 g, KCl 0.25 g, $MgSO_4 \cdot 7H_2O$ 0.25 g, KH_2PO_4 0.5 g, $FeSO_4 \cdot 7H_2O$ 5 mg, and sucrose 15 g in 1 L of water) and incubated in a humid cell for 72 h at room temperature. Antifungal activity was assigned to the strips where no sporulation of the fungus was detected (most notably R_f 0.75 coinciding with that of G2 and two additional weaker bands at R_f 0.7 and 0.65, respectively).

Fungistatic Activity in Culture. All fungi cultures were grown in 85-mm Petri dishes on 15 mL of solid medium prepared by the method of Joham (1943). Samples of G2 were added to the medium to produce the saponin concentration desired, while a medium devoid of any saponin served as control. The media were then autoclaved for 20 min at 120 °C. Fungistatic activity was determined by a modification of the method of Falck (1907): a 5-mm disk of the fungus culture (36-h-old culture for *R. mucco* and *R. solani*, 72-h-old culture for *S. rolfsii and T. viride*, or 96-h-old culture for *F. oxysporum* f. sp. *lycopersici*, *P. cinamommi*, *A. niger*, and *P. aphanidermatum*) was inoculated onto a plate containing the appropriate concentration of G2 (2–80 μg/mL, five replicates for each concentration). Incubation was carried out at 26 ± 1 °C, and the diameter of mycelial growth was measured at time intervals calculated to assure at least four measurements before the mycelium in the control dishes reached the end of the dish (31–120 h).

Calculations. The area of mycelial growth for each case was calculated from the diameter measured, and two-way analysis of variance (ANOVA) for time and concentration was performed on these data. When an interaction of time × concentration occurred, one-way ANOVAs were performed as post hoc test. The mean area of five replicates of each concentration at a given time was divided by the corresponding value of the control and multiplied by 100 to yield the percent growth in each case. Substraction of percent growth from 100 gave the percent inhibitation.

Structure Determination. (*1*) *Medicagenic Acid and Derivatives.* Medicagenic acid (I) was prepared by a modification of the method of Gestetner et al. (1966). Sapogenin extract from 100 g of alfalfa root flour was chromatographed (Merck, silica gel 60, 70–230 mesh, 100 g) with EtOAc–$CHCl_3$ (4:1, v/v, 400 mL) followed by EtOAc (300 mL), and medicagenic acid containing fractions (TLC, yellow spot after staining, R_f 0.35) were concentrated and lyophilized (75 mg). Compounds III–V, products of methylation (diazomethane) and acetylation (pyridine, Ac_2O), identified by TLC (R_f values of 0.62, 0.65, and 0.8 for III–V, respectively) possessed melting points close to those of Djerassi et al. (1957, supplementary table I). IR,

Table II. ^{13}C NMR Data of Compounds I and II[a]

carbon no.	I	II	carbon no.	I	II
1	46.50	46.72	19	46.70	48.81
2	71.72	71.27	20	26.32	26.37
3	76.01	86.10	21	33.05	33.12
4	45.10	46.52	22	28.30	28.31
5	52.41	52.55	23	180.81	180.36
6	21.65	23.89	24	13.87	14.33
7	33.26	33.88	25	17.47	17.51
8	40.30	40.26	26	17.07	16.91
9	48.90	48.77	27	24.10	24.06
10	37.05	37.02	28	181.15	180.95
11	23.72	23.72	29	31.06	31.07
12	122.63	122.62	30	23.87	23.89
13	144.90	144.96	1[b]		105.45
14	42.06	42.07	2[b]		75.25
15	34.26	34.32	3[b]		78.42
16	33.26	33.82	4[b]		70.36
17	54.05	52.94	5[b]		78.26
18	42.38	42.38	6[b]		62.70

[a] δ values, 200 MHz, pyridine d_5. Assignment based on Ishii et al. (1984) and Tschesche et al. (1980). Some of these assignments may require reversal. [b] Glucose.

MS, and ^1H NMR spectra (supplementary material) are also in accordance with the proposed structures. This was further supported by the ^{13}C NMR spectrum of medicagenic acid (Table II).

(2) *Compound G2 (II).* G2 was hydrolyzed (Tsukamoto et al., 1956) and neutralized with $CaCO_3$; the sugars were qualitatively determined by descending paper chromatography (Whatman No. 3MM paper, *n*-BuOH–pyridine–benzene–water, 5:3:3:1, v/v, upper phase) and stained with silver nitrate (Sharon and Jeanloz, 1960). Glucose, the sole sugar component, was quantitated by the phenol–sulfuric acid test (Dubois et al., 1956; 24% sugar, w/w) and by glucose oxidase–peroxidase test (Raabo and Terkildsen, 1960; 26% glucose, w/w). The anomeric conformation of the glucosidic linkage was determined by subjecting compound G2 to hydrolysis by either α-glucosidase (Sigma, EC 3.2.1.20, from yeast) or β-glucosidase (Sigma, EC 3.2.1.21, from almonds) (a 5 mg/mL suspension of G2 with 10 U/mL enzyme incubated at 37 °C), followed by glucose determination. Glucose was liberated by β-glucosidase (12%, w/w, after 1 h), while none was liberated with α-glucosidase after 5 h. Methylation analysis (Harris et al., 1984) yielded a single peak (RT 10.2 min) coinciding with that of 2,3,4,6-tetra-*O*-methyl-1,5-diacetoxyglucitol. Hydrolysis also afforded the aglycon of G2 that cochromatographed with I; it was derivatized to compounds possessing melting points similar to those of III–V (supplementary Table I) and spectral data very close to those of the later compounds.

RESULTS AND DISCUSSION

Since alfalfa, and particularly its roots, was known to possess a significant antifungal activity (Birk and Peri, 1979), we undertook the screening and isolation of major antimycotic components from alfalfa root extracts. Screening, employing *T. viride*—a particularly sensitive fungus (Zimmer et al., 1967)—demonstrated the presence of a major antimycotic compound (G2) accompanied by two minor ones. Assuming nothing on the structure of this compound, it was isolated and purified by column chromatography and HPLC.

Alfalfa roots (100 g) were extracted with EtOH (80%, v/v in water) and subjected to column chromatography (silica gel, silanized silica and preparative HPLC), yielding pure compound G2 (100–110 mg). The purification sequence of G2 was accompanied by a bioassay with a culture of *S. rolfsii*. Thus, the initial preparation (G) gave mycelial

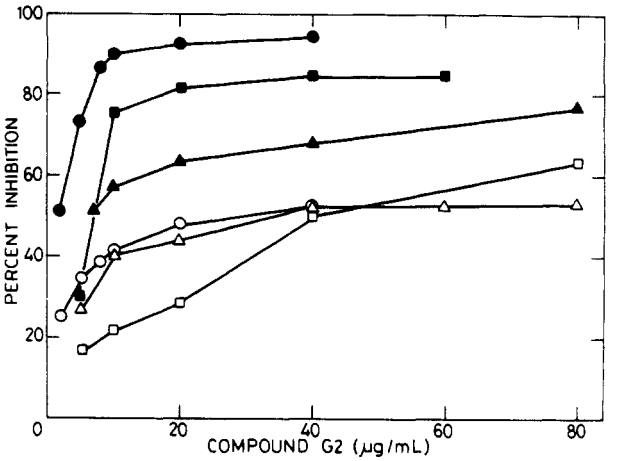

Figure 1. Maximal inhibition of several fungi by compound G2: ●, *T. viride* (54 h); ■, *S. rolfsii* (53 h); ▲ *R. mucco* (31 h); ○, *A. niger* (120 h); △, *F. oxysporum* (120 h); □, *P. cinammomi* (120 h).

Table III. Concentration of Compound G2 Responsible for 50% of Maximal Inhibition (ID_{50}) for Different Fungi

fungus	ID_{50}, μg/mL	fungus	ID_{50}, μg/mL
T. viride	1.4	*A. niger*	1.7
S. rolfsii	2.27	*P. cinamommi*	40.0
R. mucco	4.1	*F. oxysporum*	10.5

growth inhibition of 62% at 40 μg/mL and a concentration producing 50% of maximal inhibition (ID_{50}) of 5.3 μg/mL; the HPLC-purified preparation (G2) inhibited mycelial growth by 86% at 40 μg/mL with ID_{50} of 2.3 μg/mL.

The fungistatic activity of compound G2 was tested against several plant pathogen fungi. While no effect of G2 on the growth of *P. aphanidermatum* and *R. solani* was detected at concentrations below 80 μg/mL, there is a pronounced effect on the growth of *S. rolfsii*, *T. viride*, *R. mucco*, *A. niger*, *P. cinamommi*, and *F. oxyporum* f. sp. *lycopersici*. Growth curves for these fungi were plotted for both the control and various concentrations of G2 (data not shown) in order to assure that measurements were taken in the quasi-logarithmic phase of fungus growth. There were significant main effects of G2 concentration ($P < 0.001$) and time ($P < 0.001$) for the mycelial growth of all fungi. An interaction between time and concentration was also observed. Post hoc one-way ANOVAs performed separately for each concentration across all time periods and at each time across all concentrations resulted in a highly significant effect ($P < 0.001$). Percent inhibition was then calculated, and the maximum percent inhibition was plotted vs. compound G2 concentration (Figure 1), resulting in, as expected, saturation curves. The fungus most sensitive to G2 is *T. viride*. This finding is supported by the use of this fungus in an established bioassay for saponin determination (Zimmer et al., 1967). In view of the limited arsenal of fungistats available against *F. oxysporum* and *P. cinamommi* it is pertinent to note the effect of compound G2 on these fungi. The fungistatic activity of compound G2 against plant pathogens (Figure 1) is in all probability important to the plant itself and has potential use as a special purpose fungistat. Subsequently, ID_{50} values for each fungus are summarized (Table III). It is interesting that, in most cases, the percent inhibition of G2 is higher and the ID_{50} lower than with *Styrax officinalis* saponin A, which was recently studied by us (Zehavi et al., 1986).

Following the study of fungistatic activity, the structure of compound G2 was elucidated. As detailed in the Experimental Section, compound II was shown to be a β-D-

glucopyranoside of medicagenic acid by unambiguous means. Since only indirect evidence supported the original assignment of the 3-O substitution over the 2-O substitution in compound II (Morris and Tankersley, 1963), additional ^{13}C NMR study was undertaken (Table II). The shift of C-3 from δ 76.01 to 86.10 with only a minor change at C-2 supports 3-O substitution, and the peak at δ 105.45 is a further illustration of the β anomeric linkage in compound II.

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Registry No. I, 599-07-5; II, 49792-23-6; III, 5159-36-4; IV, 104693-46-1; V, 22471-97-2.

Supplementary Material Available: Melting points (Table I) and spectral data for compounds I and III–V (2 pages). Ordering information is given on any current masthead page.

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Production of Fusarin C by *Fusarium spp.*

Jeffrey M. Farber* and Gregory W. Sanders

Various Canadian species of *Fusarium* were studied for their ability to produce fusarin C. On corn, all 10 *Fusarium graminearum* strains produced fusarin C at levels much higher than those reported for South African strains. Besides *F. graminearum*, five other species also produced the mycotoxin (*Fusarium avenaceum, Fusarium culmorum, Fusarium poae, Fusarium sambucinum, Fusarium sporotrichioides*). Experiments done with *F. graminearum* growing in liquid culture demonstrated that aeration, temperature, and pH played critical roles in the biosynthesis of fusarin C. Optimal production occurred in 100 mL of glucose–yeast extract–peptone medium (pH 6.0), which was shaken at 100 rpm and incubated at 28 °C.

INTRODUCTION

Fusarin C, a highly mutagenic and potentially carcinogenic compound (Gelderblom et al., 1984a,b; Marasas et al., 1984a; Cheng et al., 1985), was first isolated from extracts of a North American strain of *Fusarium moniliforme* Sheldon growing on cracked corn (Wiebe and Bjeldanes, 1981). This mycotoxin was later found to be produced as well by South African strains of *F. moniliforme* (Gelderblom et al., 1983, 1984b). The structure of the compound has been elucidated (Figure 1; Gelderblom et al., 1984a; Gaddamidi et al., 1985) and its biosynthetic pathway partially worked out (Steyn and Vleggaar, 1985).

Fusarin C has been found to occur naturally in both hand-selected visibly *Fusarium*-infected and healthy-looking corn kernels in South Africa (Gelderblom, 1984b)

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