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ISOLATION, STRUCTURE DETERMINATION, SYNTHESIS, AND ANTIFUNGAL ACTIVITY OF A NEW NATIVE ALFALFA-ROOT SAPONIN*

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ABSTRACT

An alfalfa-root saponin (compound F) was isolated and its chemical structure determined as 3β-[*O*-α-D-glucopyranosyl-(1→4)-β-D-glucopyranosyl]-2β-hydroxy-Δ^{12}-oleanene-23,28-dioic acid (3β-medicagenic acid β-maltoside). It was synthesized *via* a modified Koenigs–Knorr reaction employing light-sensitive esters as protective groups for the aglycon. The saponin has antimycotic activity against some plant pathogenic fungi similar to that of its aglycon and a previously isolated homologous saponin, the percent inhibition being 90, 57, 67, 89, 62, and 68 for *Sclerotium rolfsii, Fusarium oxysporum* f. sp. *lycopersici, Rhizoctonia solani, Trichoderma viride, Aspergillus niger,* and *Pythium aphanidermatum,* respectively, at a concentration of 40 μg/mL.

INTRODUCTION

Medicagenic acid (**1**) is believed to be the major aglycon contributing to antifungal activity of alfalfa-root saponin extracts[1,2]. Earlier, in the process of screening for antimycotic compounds in such extracts, an improved method was described for the isolation of 3β-(*O*-β-D-glucopyranosyl)-2β-hydroxy-Δ^{12}-oleanene-23,28-dioic acid[3] (3β-medicagenic acid β-D-glucopyranoside, **2**). This compound demonstrated considerable antimycotic activity against both plant pathogenic fungi[3] and medically important yeasts[4]. In the same process, yet another compound (compound F, **3**), apparently a saponin derived from medicagenic acid, was also shown to possess antifungal activity against *Trichoderma viride*. Modification of the isolation procedure afforded this compound (**3**) in pure form, although in yields significantly

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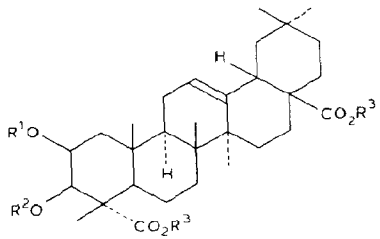
lower than those of compound **2** and, furthermore, the yields were frequently subject to changes. In some cases, only trace amounts of this native saponin **3** were present.

Upon establishing the chemical structure of compound **3**, a partial synthesis of this compound was undertaken starting from the corresponding aglycon (**1**) that, in some cases, comprises almost 1% of dry alfalfa roots. The synthesis involved a modified Koenigs–Knorr procedure and the protection of carboxyl functions of the aglycon as the light sensitive 2-nitrobenzyl esters[5–8]. It should be noted that saponin **3** was claimed to have been synthesized[2], although neither a detailed procedure nor physical data for this saponin were reported.

Since medicagenic acid is believed to be the major aglycon contributing to antifungal activity of alfalfa-root saponins[1,2], it is of interest to compare the activity of compound **3** with that of the aglycon **1** and the homologous saponin **2**.



1 $R^1 = R^2 = R^3 = H$
2 $R^1 = R^3 = H$, $R^2 = \beta$-D-glucopyranosyl
3 $R^1 = R^3 = H$, $R^2 = O$-α-D-glucopyranosyl-(1→4)-β-D-glucopyranosyl
4 $R^1 = R^2 = H$, $R^3 = 2$-nitrobenzyl
5 $R^1 = H$, $R^2 = O$-(2,3,4,6-tetra-O-acetyl-α-D-glucopyranosyl)-(1→4)-2,3,4-tri-O-acetyl-β-D-glucopyranosyl, $R^3 = 2$-nitrobenzyl
6 $R^1 = Ac$, $R^2 = O$-(2,3,4,6-tetra-O-acetyl-α-D-glucopyranosyl)-(1→4)-2,3,4-tri-O-acetyl-β-D-glucopyranosyl, $R^3 = 2$-nitrobenzyl
7 $R^1 = R^3 = Me$, $R^2 = H$

RESULTS AND DISCUSSION

During the isolation process of compound **2** from alfalfa roots, an additional antifungal component (**3**) was detected by a bioassay on t.l.c.[3] and subsequently isolated by a modification of the same procedure. Compound **3** proved to be a derivative of medicagenic acid, on the basis that both acid hydrolysis and extensive enzymic digestion liberated medicagenic acid as the sole aglycon. Furthermore, quantitative sugar analysis[9,10] suggested the presence of a disaccharide composed of two D-glucose residues as the sugar component of **3**. This finding was further supported by methylation analysis[11] which suggested also a (1→4)-linkage between the two residues. Since α-D-glucosidase but not β-D-glucosidase released D-glucose from **3**, and the aglycon was liberated only by a mixture of both enzymes, it can be assumed that the disaccharide consists of an α-D-glucopyranosyl group followed by

a β-D-glucopyranosyl residue. Thus, the structure of **3** is proposed as the 3-*O*-β-*malto* derivative of medicagenic acid, namely 3β-[*O*-α-D-glucopyranosyl-(1→4)-β-D-glucopyranosyl]-2β-hydroxy-Δ^{12}-oleanene-23,28-dioic acid.

In contrast to the isolation of **2**, compound **3** was isolated only in a very low yields and, thus, a partial synthesis was devised. The aglycon (**1**) is available, in approximately 1% yield[1] from dry alfalfa roots after a brief acid hydrolysis of a saponin extract. The two carboxyl functions of the aglycon (**1**) were protected as the light-sensitive 2-nitrobenzyl esters to yield **4** possessing u.v. and i.r. absorbances typical for an aromatic nitro group, and ^{1}H-n.m.r. signals compatible with the proposed structure. Compound **4** was treated with 2,3,6-tri-*O*-acetyl-4-*O*-(2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl)-α-D-glucopyranosyl bromide in a modified Koenigs–Knorr reaction to yield **5** (46% yield). The ^{1}H-n.m.r. spectrum of the compound had the expected acetate and aromatic signals. The doublet (δ 5.41, *J*

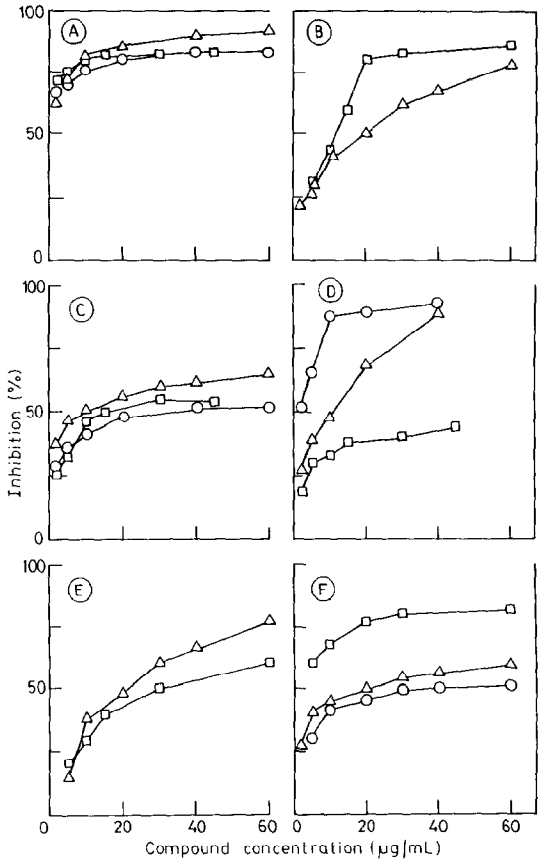


Fig. 1. Growth inhibition of some plant pathogenic fungi by compound **3** (△). The inhibition is compared, in the same experiment, with that of compound **1** (□) and compound **2** (○) (*cf.* refs. 3 and 13). (A) *S. rolfsii*, (B) *R. solani*, (C) *A. niger*, (D) *T. viride*, (E) *P. aphanidermatum*, and (F) *F. oxysporum*. For experimental details, see Table I.

TABLE I

ID_{50}[a] OF MEDICAGENIC ACID DERIVATIVES WITH SOME PLANT PATHOGENIC FUNGI

Fungus	*Incubation time (h)*	*Compound*		
		1[b]	**2**[c]	**3**
S. rolfsii	75	0.9	2.3	1.8
R. solani	35	10	>80	8.0
T. viride	75	3.2	1.4	9.8
A. niger	150	5.2	1.7	1.4
F. oxysporum	150	1.4	10.5	3.2
P. aphanidermatum	35	11.5	>80	13.5

[a]Compound concentration (μg/mL) producing half of the maximal percent inhibition, in Joham medium[14], at an incubation temperature of 26 $\pm 1°$; values are the mean of 3 replicates which did not vary by more than 2%. [b]Data from ref. 13. [c]Data from ref. 3.

4.1 Hz) was assigned to H-1′ of the 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl group already present in the bromide derivative, whereas that at δ 4.57 (J 8.0 Hz) was attributed to H-1 of the β-D-glucopyranosyl residue, suggesting a β-maltosyl heptaacetate residue. In order to better establish the structure of **5**, native compound **3** was esterified and acetylated to yield **6**. Comparison of the ^{1}H-n.m.r. spectra of **5** and **6** showed that, apart from the expected additional acetyl group (s, δ 2.02, 3 H) in **6**, the spectra were almost identical. Irradiation of **5**, followed by deacetylation in methanolic sodium methoxide afforded a saponin identical to native **3** in an overall yield (from the aglycon) of approximately 26%. Thus, about 400 mg of **3** could be obtained synthetically from **1**, isolated from 200 g of alfalfa root flour, whereas a maximum of 40 mg of **3** could be isolated directly from an equal amount of the same flour.

In the synthesis of compound **2**, Morris and Tankersley[12] assumed the regiospecificity of the Koenigs–Knorr reaction, and no attempt was made to protect OH-2 of medicagenic acid. They argued that this group being axial and part of a bulky aglycon is not reactive. Later, we established (by ^{13}C-n.m.r.) that compound **2** is indeed a 3-*O*-β-D-glucopyranosyl derivative of medicagenic acid[3]. In the present synthesis of **5**, compound **4** had free OH-2(a) and OH-3(e), and it was also assumed that regiospecificity will yield a substitution at OH-3. That this has been the case was shown by subjecting both **2** and **3** to methylation followed by hydrolysis. Both compounds yielded **7** possessing free OH-3 as expected.

Since both **1** and **2** showed antifungal activity[3,13], a detailed study of the antifungal activity of **3** against some plant pathogens was undertaken. The results (Fig. 1, Table I) suggest that **3** has an antifungal activity comparable with that of **1** and **2**. In the case of *Aspergillus niger* and *Sclerotium rolfsii,* changes in the sugar component are of marginal importance. In all other cases, however, the data strongly suggest that the presence of the sugar component and its nature affect the antifungal activity (Fig. 1). These modifications may alter the hydrophilicity of the

compounds, thus affecting the transport to the site of action, or if enzymic hydrolysis of the compound is required for biological activity[15], the changes may influence the binding and catalytic rates of the enzymes required.

EXPERIMENAL

General methods. — Melting points were determined with a Büchi 510 apparatus. Optical rotations were measured with a Bendix polarimeter. ^{1}H-N.m.r. spectra were recorded, for solution in (^{2}H)chloroform, on a Bruker WH 300 (300 MHz) instrument; i.r. spectra, for potassium bromide discs, wih a Nicolet MX-S FTIR spectrophotometer; u.v. spectra with a Bauch and Lomb spectronic 2000 instrument; and colorimetric measurements with a Zeiss PMQ II instrument. Photolyis was carried out in a RPR-100 apparatus (Rayonet, The Southern New England Ultraviolet Company, Hamden, CT 06514) with RPR 3500Å lamps in Pyrex glassware. Gas chromatographies were performed with a Packard model 824 instrument, equipped with a 3% OV on Carbowax AW (60–80 mesh) column[11]. Phenol–H_2SO_4 test[9] (total sugar) was carried out with D-glucose standards.

Chromatography. — Descending paper chromatography was performed on Whatman No. 3MM paper. The paper was developed descending with 3:5:1:3 pyridine–butanol–benzene–water (upper phase, all solvents v/v). The sugars were detected with the $AgNO_3$ reagent[16]. T.l.c. was performed on precoated Silica Gel 60 F_{254} sheets (E. Merck, D-6600 Darmstadt, F.R.G.), and h.p.t.l.c. on precoated RP-18 F_{254} sheets (E. Merck). In both cases, compounds were detected by viewing under u.v. light or by spraying with H_2SO_4. Column chromatography was performed on Silica Gel 60 (0.063–0.2 mm, Merck). L.c. separations were performed with a Varian LC-5000 liquid chromatograph equipped with a preparative Merck RSIL-C-18-HL column (50 × 1 cm). Separations in both cases were monitored by t.l.c.

Enzymes. — The enzymes used were α-D-glucosidase (EC 3.2.1.20) from yeast and β-D-glucosidase (emulsin, EC 3.2.1.21) from almonds, both purchased from Sigma Chemical Co., St. Louis, MO 63118.

3β-[O-α-D-Glucopyranosyl-(1→4)-β-D-glucopyranosyl]-2β-hydroxy-Δ^{12}-oleanene-23,28-dioic acid (**3**). — (*a*) The isolation was accomplished by a modification of a general method previously described[3,4]. Alfalfa (*Medicago sativa* L. Gilboa variety from the RAM ranch Beer Sheva) root flour (200 g) was extracted with aqueous ethanol (80% 2.5 L) for 16 h at 60°. The solid was filtered off 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 adsorbing the extract onto silica gel (6 g); portions (2 g each) were chromatographed on the same adsorbent (150 g) with 7:2:2 ethyl acetate–water–acetic acid (500 mL). Fractions showing the typical blue spot (compound F) on t.l.c. plates (same solvent, R_F 0.6) after spraying were pooled and evaporated while adsorbing the partially purified compound on silica gel (3 g). This

preparation was further purified by flash chromatography (Merck, Silica Gel 60, 230–400 mesh, 15 g) and successive elution with 4:1, 2:1, and 1:1 ethyl acetate–methanol (each fraction 250 mL) at a flow rate of 10 mL/min. Fractions containing compound F were pooled and applied (20–40-mg samples) to a h.p.l.c. column eluted with 62% aqueous methanol. Fractions (4 mL each) were monitored by t.l.c., and those containing compound F were further monitored by h.p.t.l.c. in 62% aqueous methanol; a single blue spot at R_F 0.48 indicated a pure compound. These fractions were pooled, concentrated under reduced pressure, and lyophilized to yield a white amorphous solid, m.p. 160–162°, $[\alpha]_D^{27}$ +113° (*c* 0.5, water).

(*b*) Compound **5** (200 mg) was dissolved in absolute methanol (200 mL) and irradiated for 16 h. Sodium methoxide in methanol (0.1M, 50 mL) was subsequently added and the solution was kept for 16 h at 4°. After neutralization (HCl) and evaporation, the residue was applied to a silica gel column eluted with 7:2:2 ethyl acetate–acetic acid–water (0.5 L). Fractions containing compound F (t.l.c.) were pooled, evaporated, redissolved in methanol (3 mL), and furher purified by h.p.l.c. (1 mL portions) in 62% aqueous methanol. Fractions showing a blue spot after spraying on h.p.t.l.c. in the same solvent (R_F 0.48) were pooled and concentrated, and the resulting solution was lyophilized to yield white amorphous **3** (183 mg, 91.5% yield), m.p. 161–163°, $[\alpha]_D^{24}$ +113° (*c* 0.5, water).

Anal. calc. for total sugar[9,10] (glucose): 43.58%. Found: 43.22%.

Structure determination of compound **3**. — Compound **3** (isolated from alfalfa) was hydrolysed[17], the acid neutralized with $CaCO_3$, and the identity of the sugars monitored by paper chromatography. D-Glucose, the only hydrolysis product, was determined quantitatively by both the phenol–H_2SO_4 test[9] (42.9%) and the D-glucose oxidase–peroxidase test[10] (calc.: 43.58%, found: 43.41%). In order to determine the anomeric configuration of the glucosidic linkages, **3** was subjected to hydrolysis by either α-D-glucosidase, β-D-glucosidase, or a mixture of both enzymes (compound **3**, 5 mg/mL; 100/mL of each enzyme in water, pH 5.6, incubated at 37°), followed by the D-glucose oxidase–peroxidase test. D-Glucose was liberated by α-D-glucosidase (11%; 1 h) and with a mixture of α-D- and β-D-glucosidase (15%; 1 h), whereas none was liberated by β-D-glucosidase alone (5 h). Incubation with the mixture of enzymes also afforded the aglycon, a spot of which coincided in t.l.c. (3:1 v/v, chloroform–ethyl acetate) with that of an authentic sample of medicagenic acid. Methylation analysis[11] yielded two peaks (R_F 10.2 and 22.7 min, respectively) corresponding to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl- and 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl-D-glucitol, respectively.

Di-(2-nitrobenzyl) 2β,3β-dihydroxy-Δ¹²-oleanene-23,28-dioate (**4**). — Compound **1** (1.04 g, 2 mmol) was dissolved in *N,N*-dimethylformamide (30 mL), 2-nitrobenzyl bromide (1 g, 4.65 mmol) was added, followed by triethylamine (0.6 mL, 5.8 mmol), and the solution was kept for 16 h at room temperature in the dark (compare ref. 18). Water (40 mL) was added and the resulting suspension was twice extracted with cold ethyl acetate (50 mL portions). The combined organic layer was twice washed with 1.5M HCl (50-mL portions), 0.5M Na_2CO_3 (50-mL

portions), and water (50 mL), and then dried (Na_2SO_4) and evaporated. The residue was applied to a silica gel column (100 g, 3 cm in diameter) and eluted with the following mixtures of ethyl acetate–chloroform: 1:1 (0.2 L), 2:1 (0.4 L), and 3:1 (0.4 L). The eluate was monitored by t.l.c. in 3:2 ethyl acetate–chloroform and fractions containing a u.v.-absorbing spot (R_F 0.45) were pooled, evaporated, resuspended in water, and lyophilized to yield a white, amorphous product (1.07 g, 68.5% yield), m.p. 87–89°; $[\alpha]_D^{25}$ +62° (*c* 1, chloroform); ν_{max}^{KBr} 3501 (OH), 1728 (CO), and 1530 cm^{-1} (NO_2); $\lambda_{max}^{CHCl_3}$ 260 nm (ε 13460); ^{1}H-n.m.r.: δ 8.11 (q, 2 H, *J* 2.9 Hz, arom.), 7.69 (s, 4 H, arom.), 7.64 (q, 2 H, *J* 2.9 Hz, arom.), 5.58, 5.54, 5.49 and 5.46 (d, total 4 H, $J_{a,b}$ 14.1 Hz, benzylic CH_2), 5.26 (t, 1 H, *J* 2.0 Hz, HC=C) and additional chemical shifts typical to medicagenic acid.

Anal. Calc. for $C_{44}H_{56}N_2O_{10}$: N, 3.63. Found: N, 3.72.

*Di-(2-nitrobenzyl) 3β-[O-(2,3,4,6-tetra-*O*-acetyl-α-*D*-glucopyranosyl)-(1→4)-*O*-(2,3,6-tri-*O*-acetyl-β-*D*-glucopyranosyl)]-2β-hydroxy-*Δ^{12}*-oleanene-23,28-dioate* (**5**). — Ag_2CO_3 (275 mg, 1 mmol) and $AgClO_4$ (20 mg, 0.1 mmol) were added to **4** (385 mg, 0.5 mmol) in dry benzene (60 mL). The mixture was boiled under reflux in the dark for 3 h while 25 mL of solvent was removed through a side arm. After cooling under N_2, dry molecular sieve 4A (2 g) was added and stirring was continued for 30 min, after which a solution of 2,3,6-tri-*O*-acetyl-4-*O*-(2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl)-α-D-glucopyranosyl bromide[19] (700 mg, 1 mmol) in dry benzene (30 mL) was added. The reaction was monitored by t.l.c. in 3:2 chloroform–ethyl acetate, disappearance of **4** indicating completion. After filtration through a Celite filter, the solution was twice washed with 1% NaCl (50-mL portions), dried (Na_2SO_4), and evaporated. The residue was applied to a silica gel column (100 g, 3 cm in diameter) and eluted with the following mixtures of ethyl acetate–chloroform: 1:2 (0.3 L), 1:1 (0.4 L), and 2:1 (0.3 L). Fractions containing a u.v.-absorbing spot (t.l.c., same solvent, R_F 0.65) were pooled, evaporated, resuspended in water, and lyophilized to yield white, amorphous **5** (345 mg, 46% yield), m.p. 76–78°, $[\alpha]_D^{24}$ +52° (*c* 1, chloroform); $\lambda_{max}^{CHCl_3}$ 259 nm (ε 12860); ^{1}H-n.m.r.: δ 8.12 (q, 2 H, *J* 2.9 Hz, arom.), 7.62 (s, 4 H, arom.), 7.50 (q, 2 H, *J* 2.9 Hz, arom.), 5.57, 5.53, 5.48, and 5.45 (d, total 4 H, $J_{a,b}$ 14.2 Hz, 2 $C_6H_5CH_2$), 5.41 (d, 1 H, $J_{1',2'}$ 4.1 Hz, H-1′), 5.38 (t, 1 H, $J_{3',4'}$ 9.3, $J_{4',5'}$ 9.3 Hz, H-4′), 5.29 (1 H, t, *J* 2.0 Hz, HC=C), 5.23 (t, 1 H, $J_{2',3'}$ 9.1 Hz, H-3′), 5.09 (t, 1 H, $J_{3,4}$ 9.1, $J_{2,3}$ 9.0 Hz, H-3), 5.01 (dd, 1 H, H-2′), 4.95 (t, 1 H, $J_{1,2}$ 8.0 Hz, H-2), 4.57 (d, 1 H, H-1), 4.17 (dd, 1 H, $J_{6'a,6'b}$ 12.2, $J_{5',6a'}$ 3.4 Hz, H-6′a), 4.08 (dd, 1 H, $J_{6a,6b}$ 12.0, $J_{5,6b}$ 4.0 Hz, H-6b), 3.97 (dd, 1 H, H-6′b), 3.90 (t, 1 H, $J_{4,5}$ 9.0 Hz, H-4), 3.82 (dd, 1 H, H-6b), 3.77 (m, 1 H, H-5′), 3.45 (m, 1 H, H-5), 2.16, 2.13, 2.06, and 2.04 (s, total 21 H, 7 OAc), and chemical shifts typical for medicagenic acid.

Anal. Calc. for total sugar[9]: 25.90%. Found: 26.22%.

*Di-(2-nitrobenzyl) 2β-acetoxy-3β-*O*-[2,3,4,6-tetra-*O*-acetyl-α-*D*-glucopyranosyl)-(1→4)-*O*-(2,3,6-tri-*O*-acetyl-β-*D*-glucopyranosyl)]-*Δ^{12}*-oleanene-23,28-dioate* (**6**). — The di-(2-nitrobenzyl) ester of **3** was obtained from the natural product (17

mg, 20 μmol) in the procedure described for **1** but without chromatographic purification. The resulting, nonisolated ester was further acetylated (1:1 pyridine–acetic anhydride, 30 mL, 16 h, 4°). After the addition of cold water (40 mL), the mixture was extracted with ethyl acetate (50 mL) washed with 1.5M HCl (3 portions, 40 mL each), followed by 5% aqueous Na_2CO_3 (3 portions, 40 mL each), and finally with water (40 mL). Evaporation gave a syrup that was applied to a silica gel column (10 g, 1 cm diam.) eluted with 3:2 chloroform–ethyl acetate. Fractions showing an u.v. absorbing spot (t.l.c., same solvent, R_F 0.68) were pooled, evaporated, resuspended in water, and lyophilized to yield **6** (15 mg, 11 μmol, 54% yield), white amorphous solid, m.p. 71–73°, $[\alpha]_D^{23}$ +49° (*c* 1, chloroform); $\lambda_{max}^{CHCl_3}$ (chloroform) 258 nm (ε 13410); ^{1}H-n.m.r.: δ 8.11 (q, 2 H, *J* 2.9 Hz, arom.), 7.64 (s, 4 H, arom.), 7.50 (q, 2 H, *J* 2.9 Hz, arom.), 5.57, 5.52, 5.48, and 5.45 (d, total 4 H, $J_{a,b}$ 14.1 Hz, 2 benzene CH_2), 5.43 (d, 1 H, $J_{1',2'}$ 4.0 Hz, H-1′), 5.38 (t, 1 H, $J_{3',4'}$ 9.3, $J_{4',5'}$ 9.2 Hz, H-4′), 5.27 (t, 1 H, *J* 2.0 Hz, HC=C), 5.23 (t, 1 H, $J_{2',3'}$ 9.1 Hz, H-3′), 5.11 (t, 1 H, $J_{3,4}$ 9.0, $J_{2,3}$ 9.0 Hz, H-3), 5.03 (dd, 1 H, H-2′), 4.92 (t, 1 H, $J_{1,2}$ 8.0 Hz, H-2), 4.54 (d, 1 H, H-1), 4.19 (dd, 1 H, $J_{6'a,6'b}$ 12.0, $J_{5',6a'}$ 3.3 Hz, H-6′a), 4.07 (dd, 1 H, $J_{6a,6b}$ 12.1, $J_{5,6a}$ 4.0 Hz, H-6a), 3.97 (dd, 1 H, H-6′b), 3.92 (t, 1 H, $J_{4,5}$ 9.0 Hz, H-4), 3.81 (dd, 1 H, H-6b), 3.76 (m, 1 H, H-5′), 3.45 (m, 1 H, H-5), 2.17, 2.15, 2.05, 2.04, and 2.02 (s, total 24 H, 8 OAc), and chemical shifts typical to medicagenic acid.

Anal. Calc. for total sugar[9]: 24.92%. Found: 25.12%.

Dimethyl 3-β-hydroxy-2-O-methyl-Δ^{12}-oleanene-23,28-dioate (**7**). — This compound was prepared as described by Levy *et al.*[13] starting from **2**. Compound **2** or **3** (32 or 42 mg, respectively; 0.5 mmol) was dissolved in dry dimethyl sulfoxide (1 mL), and 2M sodium methylsulfinyl carbanion in dimethyl sulfoxide (0.5 mL) was added and the solution cooled to ~10°. After addition of methyl iodide (0.3 mL), the mixture was allowed to reach room temperature during a period of 30 min, after which water (2 mL) was added and the solution twice extracted with chloroform (2-mL portions). After evaporation of the solvents, the resulting (nonisolated) product was suspended in 2M trifluoroacetic acid (3 mL) and heated under reflux for 2 h. After evaporation and removal *in vacuo* of traces of acid, the residue was applied to a silica gel column (10 g, 1 cm diam.) in 1:2 ethyl acetate–chloroform (150 mL). The column was monitored by t.l.c. in the same solvent and fractions containing a reddish spot (R_F 0.7) were pooled, evaporated, resuspended in water, and lyophilized to yield **7** (~12 mg, 20 μmol), white amorphous product; ^{1}H-n.m.r.: δ 3.32 (s, 3 H, CH_3O), 3.62 (s, 3 H, CO_2CH_3), 3.71 (s, 3 H, CO_2CH_3), and chemical shifts typical to medicagenic acid.

Determination of antifungal activity. — The fungistatic activity of **3** was determined and calculated as previously described[1,20]. The fungal strains were kindly provided by the Department of Phytopathology, Faculty of Agriculture, The Hebrew University of Jerusalem.

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