

The sensitivity of *Trichoderma viride* to medicagenic acid, its natural glucosides (saponins) and derivatives

WIESŁAW OLESZEK^a, KEITH R. PRICE^b, G. ROGER FENWICK^b

^a Institute of Soil Science and Plant Cultivation, 24-100 Puławy, Poland

^b AFRC Institute of Food Research, Norwich Laboratory, Norwich NR4 7UA, United Kingdom

(Received: February 19, 1988. Accepted: March 28, 1988)

Abstract

Medicagenic acid, its 3-O-glucopyranoside and 3,28-di-(0-glucopyranoside), their methyl esters and acetyl derivatives were tested for their potential to retard the growth of the fungus *T. viride*. The varying antifungal activities are discussed in relation to published data and to the applicability of *T. viride* — based biological methods for the quantification of saponins in plant material.

Key words: Medicagenic acid, glycosides, *Trichoderma viride*, fungal bioassay

INTRODUCTION

Saponins, naturally-occurring plant glycosides possessing diverse biological activities (Birk and Peri 1979) are currently attracting considerable interest (Price et al. 1987). The analysis of these compounds has, however, posed a problem for many years. Both chemical and biological methods have been developed but the lack of readily available purified saponins means that such methods are calibrated with crude extracts, the exact composition of which is generally unknown. Chemical methods may have the disadvantage of being nonspecific or may require hydrolysis or derivatization which lead to changes in the structure of the analysed species. Biological methods, for example those based upon blood — haemolysis or antifungal properties, are only applicable to certain saponins and hence cannot be applied to plants or foods in which these compounds are absent.

Of all the fungi tested, *Trichoderma viride* showed the highest sensitivity to the presence of saponins in the growth medium (Pedersen et al. 1966;

Zimmer et al. 1967, Walters 1968, Leath et al. 1972, Livingstone et al. 1977), and advantage has been taken of this sensitivity to quantify saponins in alfalfa (Zimmer et al. 1967, Jurzysta 1979). Amongst the saponins in alfalfa are those containing soyasapogenol-, medicagenic acid- and hederagenin aglycones (Price et al. 1987) and the former group are without activity towards *T. viride*. Scardavi and Elliot (1967) claimed that the surgar chain of the saponin moiety is an important factor in determining its water solubility, an essential condition for biological activity. Support for this view was recently obtained by Nonaka (1986) who showed medicagenic acid itself to have low antifungal activity towards *T. viride* as compared to its glycosides. As a result of comparing the activity of a number of saponin fractions isolated from alfalfa, this author concluded that structural features other than the aglycone were responsible for the variable growth inhibition.

Such results are however contrary to the earlier findings of Gestetner et al. (1970) and Shany et al. (1970). The former workers obtained results consistent with the biological activity of alfalfa saponins being positively correlated to the ratio of aglycone: individual sugars, and the latter authors found that alfalfa aglycones were more toxic towards *Sclerotium rolfii* than were the parent saponins. Later Assa et al. (1972) and Gestetner et al. (1971) suggested that the presence of two carboxylic acid residues and one hydroxyl group in medicagenic acid were a major, but not the only, factors determining the strong growth — impairing effects of alfalfa saponins toward fungi.

Recently the present authors have isolated and characterized saponins from *M. lupulina* roots (Oleszek et al. 1988) and the present work was carried out with these saponins, their aglycone (medicagenic acid) and its derivatives, with the hope of shedding further light on this inconsistency in the literature.

METHODS

INSTRUMENTAL ANALYSIS

Fast atom bombardment (FAB) — mass spectra were recorded on Kratos MS9/50TC spectrometer, the sample being dissolved in glycerol and bombarded with a 9KV (nominal) beam of xenon atoms produced by an Ion Tech NF atom gun. The spectra were recorded on a UV galvanometer recorder. Electron impact (EI) mass spectra were recorded on an LKB 8000 instrument, the electron beam having a nominal energy of 70 eV.

PREPARATION OF COMPOUNDS

Medicagenic acid (1), medicagenic acid-3-0-glucopyranoside (6) and medicagenic acid-3,28-di(0-glucopyranoside) (10) were available from previous studies (Oleszek et al. 1988). The remaining compounds were prepared as follows:

2,3-diacetylmedicagenic acid (3). Medicagenic acid (1, 100 mg) was dissolved in pyridine (10 cm³) and acetic anhydride (10 cm³). The mixture was left overnight at room temperature and water (30 cm³) was added. Extraction with butanol and removal of the solvent *in vacuo* provided a residue which crystallized as colourless needles (125 mg) from chloroform:methanol (1:1), mp. 215°C. EI-MS *m/z* 586, 540, 526, 248, 235, 203, 189, 133.

Penta-acetyl derivative of medicagenic acid-3-0-glucopyranoside (9). Medicagenic acid-3-0-glucopyranoside (100 mg) was acetylated as above to yield a product (135 mg), mp. 262-264°C (with browning).

Nona-acetyl derivative of medicagenic acid-3,28-di(0-glucopyranoside) (12). Medicagenic acid-3,28-di(0-glucopyranoside) (100 mg) was acetylated as above to produce 156 mg of an amorphous product, mp 246-248°C (with browning).

Medicagenic acid-23,28-dimethyl ester (4). Medicagenic acid (100 mg) was dissolved in methanol (10 cm³), cooled to 0°C and treated with an ethereal solution of diazomethane (10 cm³, in 1 cm³ portions, with stirring). The yellow solution was allowed to stand overnight and diluted with water (30 cm³). Extraction with ethyl acetate and removal of solvent afforded the diester, (105 mg), colourless needles from chloroform:methanol (1:1), mp. 226°C, EI-MS *m/z* 530, 512, 741, 267, 262, 249, 203, 189, 133.

2-methoxymedicagenic acid,23,28-dimethyl ester (5). Following the procedure of Hakomori (1964), a mixture of sodium hydride (700 mg) and dimethyl sulphoxide (25 cm³) was stirred under nitrogen for 1 hr at room temperature and 6 (200 mg) dissolved in dimethyl sulphoxide (10 cm³) was added. The solution was stirred for 1 hr and an excess of methyl iodide (10 cm³) added portionwise over 6 hr with stirring. The reaction mixture was poured into ice-water (200 cm³) and the crude product extracted with ethyl acetate. Removal of the solvent gave a yellowish-coloured solid which was chromatographed on silica gel, using benzene-acetone (20:2) as eluant. Workup of the eluate afforded a yellowish-cream, amorphous solid (214 mg), mp. 108-109°C. This product (100 mg) was dissolved in methanol (10 cm³) and 4N HCl (10 cm³) added. After refluxing for 6 hr the solution was cooled, diluted with water (50 cm³) and the methanol removed *in vacuo*. Extraction with ethyl acetate and removal of the solvent gave a crystalline product which was further purified by preparative TLC on silica gel. The plate was developed three times with benzene-acetone (20:1), the spots being visualised by spraying with water until wet. Two zones were identified, one of which yielded the desired product (25 mg), EI-MS *m/z* 544, 281, 262, 249, 203, 189, 133.

Medicagenic acid-3-0 glucopyranoside,23,28-dimethyl ester (7). Medicagenic acid-3-0-glucopyranoside (100 mg) was treated with diazomethane as above to yield the product, (102 mg), mp. 104-105°C, FAB-MS (negative ion mode) *m/z* 783 (M-H+glycerol)⁻, 691 (M-H)⁻, 677 (M-H-Me)⁻, 483 (M-H-2Me-hexose-H₂O)⁻.

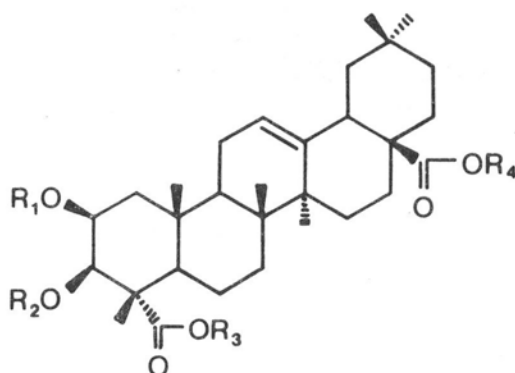
Medicagenic acid-3,28-di(0-glucopyranoside),23-methyl ester (11). Medicagenic acid-3,28-di(0-glucopyranoside), 200 mg, was methylated with diazomethane to produce 202 mg of the desired product, mp. 206-207°C,

FAB-MS (negative ion mode) m/z 1517 $(2M-H-hexose)^-$, 839 $(M-H)^-$, 677 $(M-H-hexose)^-$, 515 $(M-H-2\text{ hexose})^-$, 497 $(M-H-2\text{ hexose}-H_2O)^-$.

Medicagenic acid-3-O-glucopyranoside,28-methyl ester (8). The above diester (11, 100 mg) was dissolved in 5% KOH in methanol (30 cm^3) and refluxed for 2 hr. Distilled water (30 cm^3) was added and the methanol removed *in vacuo*. The aqueous solution was acidified to pH 5 with HCl and extracted with butanol. Evaporation of butanol gave the desired product, FAB-MS (negative ion mode) m/z 677 $(M-H)^-$, 515 $(M-H-hexose)^-$, 497 $(M-H-hexose-H_2O)^-$.

FUNGAL BIOASSAY

The antifungal activity of the individual products was determined using the fungus *Trichoderma viride*, isolated by Zimmer et al. (1967). The method of Zimmer et al. (1967), as modified by Jurzysta (1979) was employed. Where the individual compounds were insoluble in water, they were dissolved at the



- 1: $R_1 = R_2 = R_3 = R_4 = H$
- 2: $R_1 = R_2 = H, R_3 = R_4 = Na$
- 3: $R_1 = R_2 = Ac, R_3 = R_4 = H$
- 4: $R_1 = R_2 = H, R_3 = R_4 = Me$
- 5: $R_2 = H, R_1 = R_3 = R_4 = Me$
- 6: $R_1 = H, R_2 = \beta\text{-D-Glc-p}, R_3 = R_4 = H$
- 7: $R_1 = H, R_2 = \beta\text{-D-Glc-p}, R_3 = R_4 = CH_3$
- 8: $R_1 = H, R_2 = \beta\text{-D-Glc-p}, R_3 = H, R_4 = CH_3$
- 9: $R_1 = Ac, R_2 = 4\text{ Ac } \beta\text{-D-Glc-p}, R_3 = R_4 = H$
- 10: $R_1 = H, R_2 = \beta\text{-D-Glc-p}, R_3 = H, R_4 = \beta\text{-D-Glc-p}$
- 11: $R_1 = H, R_2 = \beta\text{-D-Glc-p}, R_3 = CH_3, R_4 = \beta\text{-D-Glc-p}$
- 12: $R_1 = Ac, R_2 = 4\text{ Ac } \beta\text{-D-Glc-p}, R_3 = H, R_4 = 4\text{ Ac } \beta\text{-D-Glc-p}$

Fig. 1. Schematic diagrams of compounds tested for activity against *Trichoderma viride* growth

appropriate concentration in ethanol and as such were added to the test solution. To avoid any influence of ethanol on *T. viride* growth, a preliminary test was conducted on the inhibition of the fungus by this solvent. When ethanol was used in this manner, the maximum amount of ethanol added to the culture medium was 0.25 cm^3 (Fig. 2) and an equal volume of alcohol was added to the control plates.

RESULTS AND DISCUSSION

Medicagenic acid, its 3- and 3,28-glucosides and their methyl and acetyl derivatives (Fig. 1) were examined for their harmful effect on *T. viride* growth, the latter being shown diagrammatically in Fig. 3. The importance of the

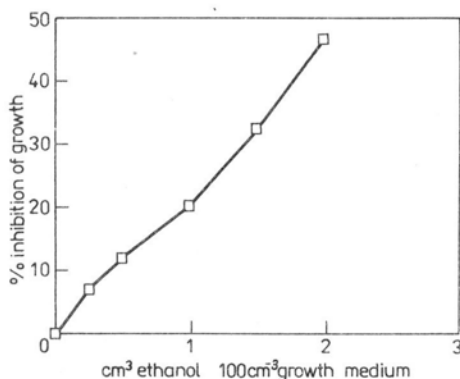


Fig. 2. Inhibition of *T. viride* growth by adding ethanol to the growth medium

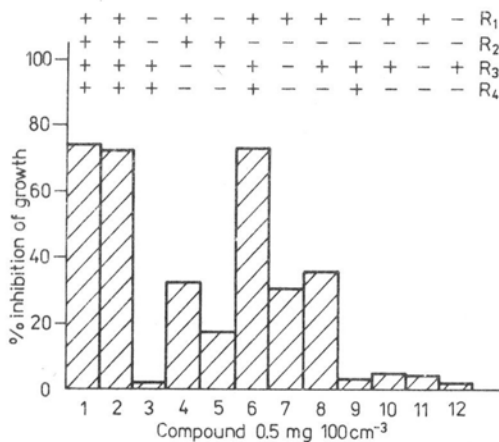


Fig. 3. Inhibition of *T. viride* growth by medicagenic acid, medicagenic acid-3-O-glucopyranoside, medicagenic acid-3,28-di(O-glucopyranoside) and their derivatives at a concentration of $0.5 \text{ mg } 100 \text{ cm}^{-3}$

solubility of the compounds in the medium is shown in Figs. 4 and 5. The disodium salt of medicagenic acid (2), which readily dissolved in water, inhibited the growth of *T. viride* to a greater degree than the less soluble, neutral compound. However, when the latter was dissolved in a small volume of ethanol before addition to the growth medium the activity was found to be similar to that of the salt.

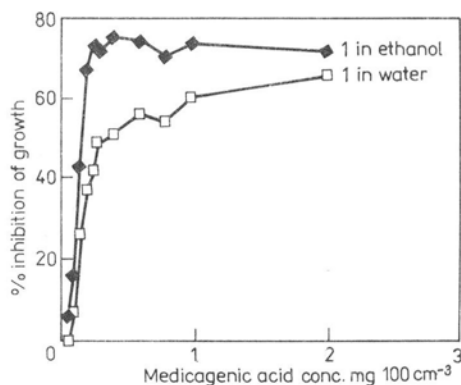


Fig. 4. Effect of medicagenic acid on *T. viride* growth. 1-in ethanol — medicagenic acid dissolved in ethanol prior to adding into growth medium. 1-in water — medicagenic acid added directly into growth medium

The detrimental effect of medicagenic acid (1) on fungal growth was very obvious. The complete inhibition of growth occurred at a concentration of $0.22 \text{ mg } 100 \text{ cm}^{-3}$, significantly lower than that reported by Nonaka (1986), who found that 0.21 mg medicagenic acid per 100 cm^3 inhibited the growth of *T. viride* by only 32%. The latter result is, however, consistent with the data obtained on the effect of medicagenic acid without initial solubilization (Fig. 4). Medicagenic acid-3-0-glucopyranoside (6) exhibited a similar activity to medicagenic acid, completely retarding fungal growth at a concentration of $0.2 \text{ mg } 100 \text{ cm}^{-3}$ (Fig. 5). This finding is similar to that found by Levi et al. (1986).

In contrast, the bisdesmoside, (10), medicagenic acid-3,28-di-(0-glucopyranoside) retarded growth to much lower degree than the above compounds (Fig. 6). Complete inhibition was observed at a concentration of $5 \text{ mg } 100 \text{ cm}^{-3}$, over twenty times higher than found for (1) or (6). The sharp drop in biological activity following the binding of a glucose moiety to the C-28 carboxylic acid group may be due to the blockage of an important active site. To examine this possibility in more detail a number of derivatives were prepared and subjected to bioassay (Figs. 5, 6).

In all cases blockage of the carboxylic acid groups (R_3 , R_4 in compounds 4, 5, 7, 11) led to impaired growth, so that 55-60% of the growth was maintained even at concentrations of $10 \text{ mg } 100 \text{ cm}^{-3}$. Blocking of the -OH groups in the aglycone (R_1 , R_2 in compounds 3, 9, 12) or the sugar moieties (compounds 9 and 12) produced a very significant lowering of antifungal activity. For

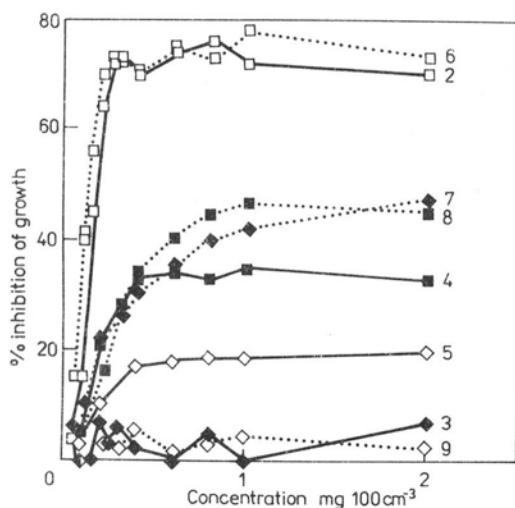


Fig. 5. Inhibition of *T. viride* growth by different concentrations of medicagenic acid, medicagenic acid-3-0-glucopyranoside and their derivatives

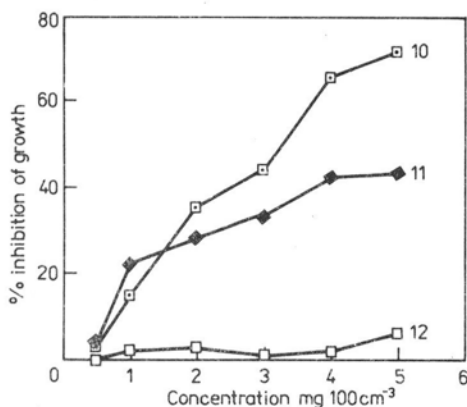


Fig. 6. Inhibition of *T. viride* growth by different concentrations of medicagenic acid-3,28-di(0-glucopyranoside) and its methyl and acetyl derivatives

example, 2,3-diacetylmedicagenic acid (3) at a concentration of $6 \text{ mg } 100 \text{ cm}^{-3}$ inhibited fungal growth by 40%, similar to that found for medicagenic acid with both carboxylic acid groups estrified (4). The blocking of both aglycone and sugar -OH groups (compounds 9 and 12) reduced the inhibition of *T. viride* to 5% (at concentrations of 2 and $5 \text{ mg } 100 \text{ cm}^{-3}$, respectively).

The results presented here indicate that the antifungal activity of medicagenic acid and its derivatives against *T. viride* is markedly dependent upon free $-\text{CO}_2\text{H}$ and $-\text{OH}$ groups. The above data are broadly in agreement with the findings of Gestetner et. al. (1970) except in terms of evaluating the

relative effects on growth impairing activity of the -OH and -CO₂H substituents. Gestetner et al. (1970) estimated that both functional groups were equally important in this respect, although it must be noted that these workers used a different organism (*Sclerotium rolfsii*) and examined a single, high, concentration of the chemical(s). In contrast the present work has examined the antifungal effects over a range of concentrations and enables differences between functional groups to be better identified.

Examination of Fig. 3 clearly shows that, for the aglycones and monodesmosides (1-9), the maximum activity is associated with free R₁, R₂ (-OH) and R₃, R₄ (-CO₂H) groups. It is also pertinent to consider the relative biological activities of medicagenic acid, hederagenin and soyasapogenol B, the structural relationships between which are shown in Fig. 7. Compared with the former, the antifungal activity of hederagenin is much reduced (Shany et al. 1970, Gestetner et al. 1971) whilst that of soyasapogenol B is negligible, and the same trend is evident in their growth impairing effects toward *Tribolium castaneum* larvae. These activities are consistent with the structural requirements discussed above.

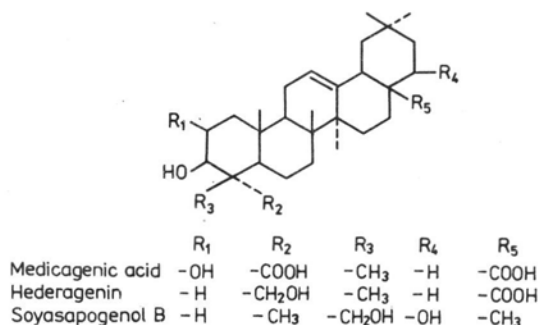


Fig. 7. Structural relationship between medicagenic acid, hederagenin and soyasapogenol B

In alfalfa crop there is clear evidence that the ratio of saponins containing soyasapogenol- and medicagenic acid aglycones varies greatly with the part of the plant examined (seed, root, stem, leaf) and hence, the biological activity of extracts derived therefore will also vary (Price et al. 1987). It is also highly probable that the relative quantities of particular glycosides may vary under different environmental conditions. It is thus important both that the part of the plant used to provide materials examined for their biological or clinical effects be specified and that methods of analysis of individual saponins be developed. As has been demonstrated in this paper the analysis of the medicagenic acid aglycones would give no indication of biological activity, since the potent monoglucoside (6) and slightly active diglucoside (10) would be included in the figure obtained and therefore methods based on the *T. viride* assay can not be used to obtain reliable quantitative data.

Acknowledgement

This work was done under RPBR-22 program. The authors are grateful for the assistance of the Mass Spectroscopy Group, IFRN and to MAFF under whose funding part of this work was conducted.

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Wrażliwość Trichoderma viride na kwas medikagenowy — na jego naturalne glukozydy (saponiny) i na związki pochodne

Streszczenie

Badano wpływ kwasu medikagenowego, jego 3-0-glukopiranozydu i 3,28-di(0-glukopiranozydu) oraz ich pochodnych metylowych i acetylowych na wzrost grzyba *Trichoderma viride*. Stwierdzono duże różnice w aktywności poszczególnych związków i dane te są dyskutowane z danymi literatury. Na podstawie uzyskanych wyników podkreślono małą precyzję metod biologicznych, opartych na reakcji wzrostowej *T. viride*, używanych do ilościowego oznaczania zawartości saponin w materiale roślinnym.