Separation and Identification of Two Pterocarpanoid Phytoalexins Produced by Red Clover Leaves

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ABSTRACT

Two pterocarpanoid compounds produced by red clover (*Trifolium pratense*) leaves in response to inoculation with *Helminthosporium turcicum* were separated by thin-layer chromatography. They were identified as 3-hydroxy-9-methoxypterocarpan (medicarpin) and 3-hydroxy-8, 9-methylenedioxypterocarpan (maackiain) on the basis of ultraviolet, infra-red, and nuclear magnetic resonance spectra. The quantities of medicarpin and maackiain produced by several red clover cultivars appeared sufficient to adversely affect growth of *H. turcicum* on clover leaves.

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A number of plant species produce antifungal compounds, termed "phytoalexins", in response to infection by fungal pathogens (5). The phytoalexins isolated from the leguminous plants, peas, beans, and alfalfa have in common the pterocarpanoid ring structure (Fig. 1) (11, 12, 14). A glucoside, trifolirhizin, with the same ring structure (Fig. 1-a) was isolated from clover roots (1, 2), and Cruickshank (6) reported the production by red clover leaves of an unidentified phenolic pterocarpanoid compound which he suggested might be the aglycone of trifolirhizin, 3-hydroxy-8, 9-methylenedioxypterocarpan (Fig. 1-b). This aglycone, also known by the names maakiain (15), demethylpterocarpin, and inermin (3), was isolated by Bredenberg & Hietala (2) from clover root homogenates which had been incubated long enough to allow a glucosidase released by the cells to hydrolyze the trifolirhizin to glucose and maackiain.

Preliminary investigations in this laboratory on the phytoalexins produced by red clover leaves inoculated with *Helminthosporium turcicum* Pass. (*Trichometasphaeria turcica* Lutt.) indicated the formation of two pterocarpanoid compounds and one or more non-pterocarpanoid substances that were also inhibitory to growth of *H. turcicum*. The present study was initiated to identify the two pterocarpanoid compounds prior to studying their role in determining host specificity of pathogens of forage legumes.

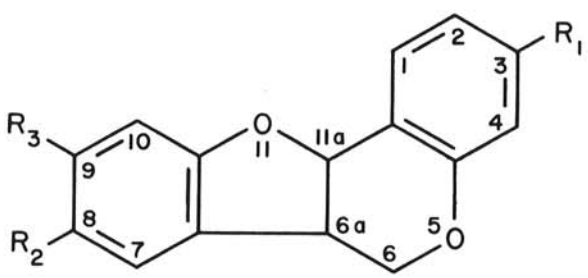

Fig. 1. The pterocarpan structure: **a)** trifolirhizin R_1 = $-OC_6H_{12}O_5$, R_2-R_3 = -O-CH_2-O-; **b)** maackiain R_1 = -OH, R_2-R_3 = -O-CH_2-O-; **c)** medicarpin R_1 = -OH, R_2 = -H, R_3 = $-OCH_3$.

MATERIALS AND METHODS.—*Isolation of pterocarpanoid compounds.*—Leaves from red clover (*Trifolium pratense* L. 'Doublecut') plants grown from seed in the greenhouse were floated on water in covered Pyrex trays; each leaflet was inoculated with 1-2 drops of a suspension of *Helminthosporium turcicum* spores (50,000 spores/ml) in 0.05% Tween 20 (polyoxylethylene sorbitan monolaurate) solution and incubated at 22-25 C (9). After 24 hr, the drops were removed by suction and replaced with drops of distilled water which were removed after a further 24-hr incubation period. The inoculated areas of each leaflet were removed with a cork borer and saved for extraction.

Pterocarpanoid compounds were removed from the pooled drops, referred to as the diffusate solution, by partitioning twice with 1 volume of CCl_4. The infected leaf tissue, in lots of 10 to 20 g, was homogenized in 10 ml 95% ethanol/g fresh weight of tissue, filtered, and washed with 5 ml 95% ethanol/g, and the ethanol solution evaporated to dryness in vacuo at 50 C. The dry residue was washed 3 times with 2 ml CCl_4/g, and the CCl_4 was partitioned 3 times with 1 volume of 0.2 N NaOH. The NaOH fraction was acidified to pH 3 with 6 N HCl, then partitioned 3 times with 0.5 volume of CCl_4. The final CCl_4 fractions of extracts and diffusates were evaporated in vacuo, and the residue was dissolved in a small volume of 95% ethanol for chromatography. This tissue extraction method was originally developed to isolate medicarpin from alfalfa tissue (Verna J. Higgins, *unpublished data*), and is unlikely to remove nonphenolic compounds.

Separation of the pterocarpanoids.—Examination of the CCl_4-soluble fraction of diffusates by ultraviolet absorption spectra and by thin-layer chromatography (TLC) indicated the presence of two closely related compounds (I and II) which appeared to be phenolic pterocarpanoids. The same two compounds were present in the extracts of infected tissue. Attempts to readily separate these two compounds by TLC or crystallization were unsuccessful, but separation was obtained by repeated use of the following TLC method.

The tissue extract or diffusate material was

streaked on silica gel plates containing fluorescent indicator. We concentrated the streak in a very narrow band by allowing 95% ethanol to ascend 1-2 mm beyond the area streaked. The plate was developed 3 times in pentane:ethyl ether:acetic acid (75:25:1, v/v) (14) with the solvent allowed to move 9 cm the first time, 15 cm the second time, and 20 cm the third time. The pterocarpanoid compounds were located by exposure of a small portion of the plate to ultraviolet irradiation and that area of the plate containing pterocarpans divided into five-seven sections running parallel to the solvent front. Each section was eluted separately by centrifuging the silica gel in appropriate amounts of 95% ethanol, and the relative proportions of I and II in the eluant were determined by the ratio of the absorbance at 287 nm to the absorbance at 310 nm. One or two sections near the front edge of the eluted area normally contained only compound I, whereas one or two sections on the trailing edge contained only compound II. Sections in the middle contained both I and II in varying ratios, so these eluants were rechromatographed. Eluants containing only I or II were evaporated to dryness in vacuo, and this material (ca. 0.5-2 mg) was used to obtain infra-red (IR) and nuclear magnetic resonance (NMR) spectra.

Spectral analysis.—For IR analyses, the samples were deposited on AgCl plates or in KBr discs and the spectra recorded on a Perkin-Elmer 521 spectrometer. For NMR analyses, I and II were dissolved in deuterochloroform ($DCCl_3$), leaving some insoluble material. Spectra were obtained at 100 MHz on a Varian HA-100 spectrometer equipped with a Spectro System 100 for multiscan averaging. The spectra were the result of time-averaging multiple scans followed by data processing to enhance the resolution. In addition, IR and NMR spectra were also obtained for a sample of 3-hydroxy-8, 9-methylenedioxy-pterocarpan (maackiain) (3) supplied by W. Cocker, University of Dublin, Ireland. Ultraviolet absorption spectra were recorded on a Bausch & Lomb Spectronic 505 spectrophotometer.

Estimation of the quantity of I and II.—Estimates of the quantity and ratios of I and II produced by three red clover cultivars, Mammouth, Altaswede, and Double cut, were obtained by the diffusate method as previously described. The size of the drops of spore suspension was standardized at 0.2 ml; only one drop/leaf was added, and the drops were harvested at 24 hr. Drops of 0.05% Tween 20 solution were used on controls. The diffusates were partitioned twice with 1 volume of CCl_4, and the quantity of I or II in the CCl_4 fraction was determined from ultraviolet absorption spectra, using published extinction coefficients (15).

The sensitivity of H. turcicum to I and II.—A bioassay medium was prepared by the addition of 0.9 ml hot V-8 agar (200 ml V-8 juice, 3 g $CaCO_3$, 20 g agar, 800 ml distilled water) to 0.1 ml of 30% ethanol containing known amounts (0 to 60 μg) of I or II in 35-mm petri plates. A 3-mm mycelial disc, obtained by growing *H. turcicum* on bacterial filters (Cellulose Triacetate Metricel, pore size 0.2 μ, Gelman Instrument Co., Ann Arbour, Mich.) on the surface of V-8 agar was placed in the center of each plate, and the diameter of the growth measured after 24-, 48-, and 60-hr incubation at 25 C. ED_{50} values were determined from the results.

RESULTS.—*Identification of I and II.*—Compound I had an ultraviolet absorption spectrum (λ max 282, 287, 226, 210 nm) identical to that of 3-hydroxy-9-methoxypterocarpan (medicarpin, demethylhomopterocarpin) (4, 7, 14) (Fig. 1-c). In several TLC solvents, including chloroform, hexane, hexane:ethyl acetate:methanol (60:40:1, v/v) and the solvent described above, I had the same R_F values as medicarpin. The IR spectra of I contained lines that could be assigned to medicarpin, and a number of other lines from unidentified material. The unidentified material apparently was insoluble in $DCCl_3$, as the NMR spectrum of I was identical with that previously obtained for medicarpin (14).

Compound II had an ultraviolet absorption spectrum with maxima at 310, 287, 282, 226, and 210 nm. In 0.05 N NaOH, the maxima were 249 and 300-302 nm with an inflection at 310 nm. Several known pterocarpanoid compounds have similar ultraviolet absorption spectra, but of these, only maackiain, because it is a phenolic compound, has a similar change in the spectrum when in alkali solution (2). Although amounts of purified II were too small to attempt crystallization, on several occasions crystals of II formed on the clover leaf beneath the drop. The melting point of these crystals was in the range of 178-181 C. Reporting melting points for maackiain are 180-181 (13, 15, 16), 175-177 (8), 179-180 (3), and 179.5-180 C (2). The IR spectrum of II was similar to that for maackiain. The NMR spectrum of II was identical to that of the maackiain sample obtained from W. Cocker. The spectrum of maackiain was similar to that reported for pterocarpin (10). The chemical shifts of the aromatic protons on the ring bearing the methoxyl substituent in pterocarpin differ slightly (0.1 ppm) from those for the corresponding protons in maackiain (where the substituent is an hydroxyl group). Otherwise the spectra of the two compounds in $DCCl_3$ are essentially the same.

Quantity of I and II.—In diffusates (24- and 48-hr drops combined), obtained in six experiments using the cultivar Doublecut without standardizing the drop size, the ratio of maackiain to medicarpin ranged from 1.0:0.65 to 1:1.1, and the concentrations ranged from 6 to 35 μg/ml of medicarpin and 10 to 47 μg/ml of maackiain. Extracts of two lots of infected leaf tissue accumulated from these diffusate experiments contained (i) 174 μg maackiain/g fresh weight of tissue, 70 μg medicarpin/g; and (ii) 155 μg maackiain/g, 120 μg medicarpin/g. Extracts of healthy leaf tissue contained < 2 μg maackiain/g and < 3 μg medicarpin/g.

Based on the average of two experiments in which a drop size of 0.2 ml was used, diffusates obtained after 24 hr on the cultivars Mammouth, Altaswede, and Doublecut contained 31, 24, and 34 μg/ml maackiain

and 26, 26, and 33 μg/ml medicarpin, respectively. Diffusates obtained using only 0.05% Tween 20 solution did not contain detectable levels of maackiain or medicarpin.

ED_{50} values.—In the bioassays, the growth of *H. turcicum* during the first 24-hr period was similar in all treatments, but differences between treatments were obvious after 48-hr incubation. This lack of inhibition in the early stage of growth was first interpreted as being caused by the initial growth of mycelium arising primarily from nutrients in the inoculum disc. Later, the discovery that medicarpin (and probably maackiain) is strongly adsorbed to Metricel filters suggested that the two compounds may have been removed from the medium in the immediate area of the inoculum disc by adsorption to the piece of filter transferred with the inoculum. Because of this lack of inhibition in the first 24 hr of growth, ED_{50} values were determined using the amount of radial growth of mycelium occurring between 24 and 60 hr. Under these conditions, the level of medicarpin or maackiain required to inhibit growth of *H. turcicum* by 50% was in the range of 35 to 45 μg/ml. Bioassays done using crystalline medicarpin obtained from alfalfa (14) gave ED_{50} values in this same range, indicating that any impurities in the medicarpin samples obtained from clover did not affect the bioassay.

DISCUSSION.—The evidence presented indicates that the two pterocarpanoids produced by red clover in response to infection by *H. turcicum* are 3-hydroxy-9-methoxypterocarpan (medicarpin) (Fig. 1-c) and 3-hydroxy-8, 9-methylenedioxypterocarpan (maackiain) (Fig. 1-b). Similar results were recently obtained by Cruickshank & Perrin (*personal communication*), who tentatively identified two compounds produced by red clover as medicarpin and maackiain. The presence of maackiain in infected red clover is not unexpected, as it was previously shown (2) that trifolirhizin in clover roots could readily be converted to maackiain. The presence of medicarpin in conjunction with maackiain is more surprising, although medicarpin is produced by alfalfa (*Medicago sativa*) (14), which like *Trifolium* belongs to the subtribe Trifolieae. The fact that red clover and alfalfa have at least one phytoalexin in common may partly explain the ability of certain pathogens to be pathogenic on both of these hosts.

The combined amount of maackiain and medicarpin occurring in diffusate solutions and in the inoculated leaf tissue when compared to the ED_{50} of these two compounds in bioassays against *H. turcicum*, appeared to be sufficient to adversely affect further development of that fungus on clover leaves. Although the extraction procedure used for the leaf tissue is probably considerably less than 100% efficient, the combined amount of the two compounds was as high as 275 μg/g fresh wt of infected tissue. This value is in excess of the level of these pterocarpanoids that one would expect to completely stop growth of *H. turcicum* in the tissue. Undoubtedly, the size of the inoculum drop will affect the level of compounds in both the diffusate and the tissue. In these experiments, the drop size used was as large as was technically possible, so maximum dilution of the compounds should have occurred.

The use of the described TLC method to separate medicarpin and maackiain, although inefficient because of the repetitions required, is the first reported method of separating these two compounds that does not require converting them to their acetate or methyl derivatives (7, 8). The use of the multiscan averaging technique to obtain NMR spectra made it possible to identify the two compounds using samples of less than 2 mg, thus eliminating the time-consuming task of accumulating and extracting large quantities of diffusates and diseased tissue.

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