

Isolation of the Phytoalexin Medica­rpin from Leaflets of *Arachis hypogaea* and Related Species of the Tribe Aeschynomeneae

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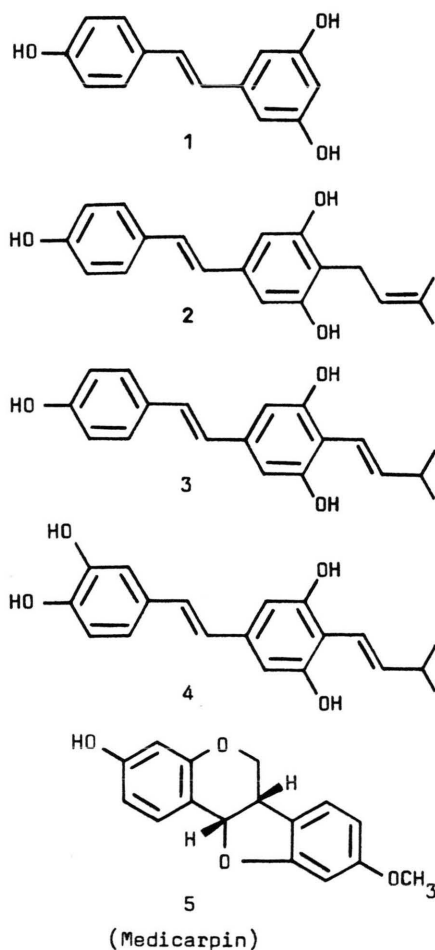
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Arachis hypogaea, Groundnut, Isoflavonoid, Phytoalexin, (+)-Medica­rpin

A phytoalexin produced by the leaflets of seven cultivars of *Arachis hypogaea* (groundnut) after natural infection by *Cercospora arachidicola* or *Phoma arachidicola* has been characterised as the isoflavonoid (+)-medica­rpin (3-hydroxy-9-methoxypterocarpan). Treatment of excised groundnut leaflets with an aqueous solution of CuSO₄ or with a spore suspension of the fungus *Helminthosporium carbonum* has also been found to stimulate medica­rpin biosynthesis.

Introduction

It has recently been shown that the fungus-infected cotyledons and etiolated hypocotyls of the cultivated groundnut, *Arachis hypogaea* L. (Leguminosae – Papilionoideae; tribe Aeschynomeneae) variously accumulate the phenolic stilbene phytoalexins **1–4** [1–3]. In addition, Cole [4] reported that extracts of groundnut leaves naturally-infected with the leaf spot fungus *Cercospora arachidicola* Hori contained a compound which inhibited the growth of another groundnut leaf pathogen, *Phoma arachidicola* Marasas, Pauer & Boerema (the fungus responsible for the web blotch disease), when tested using the thin-layer plate bioassay procedure developed by Homans and Fuchs [5]. This unidentified antifungal substance could not be detected in extracts of healthy, fungus-free leaves. We have now re-examined the response of groundnut leaves to infection by *C. arachidicola* and *P. arachidicola* and have found that both fungi can bring about phytoalexin accumulation. In this paper we describe the isolation and purification of the *Cercospora*- and *Phoma*-induced phytoalexin, and its identification as the phenolic dextrorotatory isoflavonoid, medica­rpin **5** (3-hydroxy-9-methoxypterocarpan).



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Experimental Methods, Results and Discussion

Six long-season cultivars of *A. hypogaea* (Egret, P84/5/244, P84/5/256, 6/11/11, Flamingo and 60/66) and one short-season cultivar (Jacana) were grown in heavy clay soil at the Henderson Research Station, 28 km N.E. of Harare, Zimbabwe. Leaflets (2 replicates/sample) naturally-infected with either *C. arachidicola* or *P. arachidicola* were collected after about 14, 16, 18 and 20 weeks growth in the case of the long-season cultivars, and after approx. 8, 10, 12 and 14 weeks growth for the single short-season cultivar. Sample sizes varied according to the number of infected leaflets observed on each cultivar but normally were within the range 10–50 g.

Medicarpin was extracted from *Cercospora*- and *Phoma*-infected leaflets with 60% aqueous MeOH (15 ml/g fresh tissue) using the facilitated diffusion technique [6]. After approx. 4 h agitation, the MeOH extract was decanted and concentrated to a quarter bulk under reduced pressure to remove the solvent. The remaining aqueous phase was shaken ($\times 3$) with equal volumes of EtOAc, and the combined organic fractions for each extract were dried (24 h) over anhydrous Na_2SO_4 before being reduced to dryness *in vacuo* (40 °C). The oily residue was then taken up in $\text{CH}_3\text{CN}-\text{H}_2\text{O}$ (1:1) prior to semi-preparative HPLC on a column of Spherisorb ODS, 10 μm (25 \times 1 cm i.d.; solvent system, $\text{CH}_3\text{CN}-\text{H}_2\text{O}$ 1:1) as reported earlier [2]. Any material in the eluant which absorbed UV light at 290 nm was routinely tested for antifungal activity against the growth of *Cladosporium cucumerinum* Ell. & Arth. on TLC plates [5, 7]. Medicarpin (**5**) eluted from the semi-preparative column with a retention time of 7.2 min. Quantification of **5** was performed by HPLC on a column of Spherisorb ODS, 10 μm (25 \times 0.46 cm i.d.) with butyrophene as the internal standard [2].

The accumulation of medicarpin in the *Cercospora*- or *Phoma*-infected leaflets of the long-season groundnut cultivars Egret, 6/11/11 and Flamingo, and the short-season cultivar Jacana, is shown in Fig. 1 (A–D). As expected, medicarpin concentrations tended to rise dramatically as the growing season progressed and the intensity of infection increased. Phytoalexin accumulation was particularly marked in the *Phoma*-infected leaf tissues of Egret and Flamingo, and had reached levels of approx. 2 and 5.5 mg/g fresh wt. respectively when the final samples were collected after 20 weeks growth (Fig. 1A, C). In con-

trast, however, there was apparently little change in medicarpin levels between weeks 16 and 20 when Egret leaflets were infected with *C. arachidicola* (Fig. 1A) despite the fact that the percent disease (assessed as previously described [8]) increased from 5 to 15% over the same period. A somewhat compa-

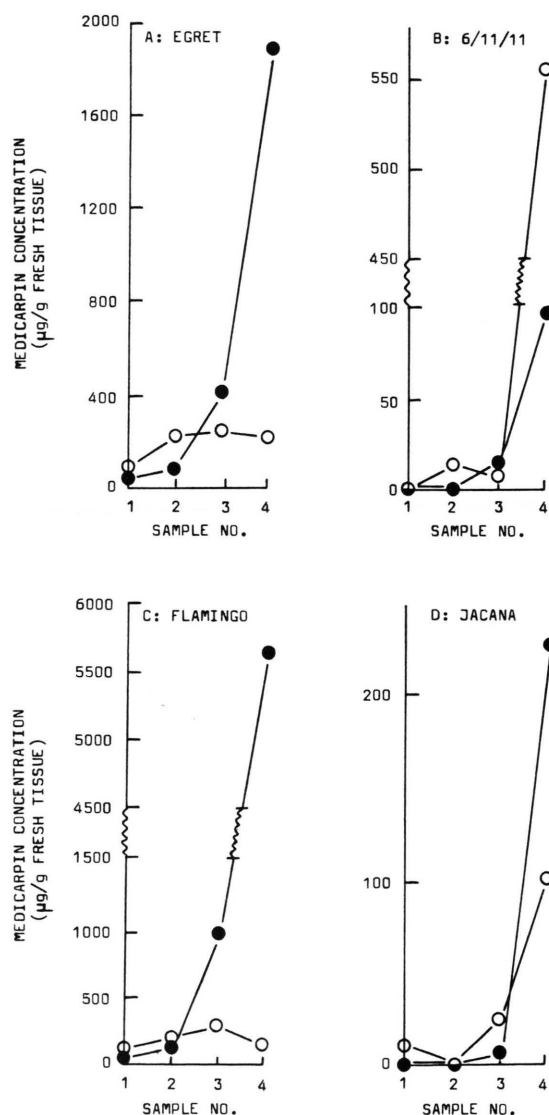


Fig. 1. Accumulation of medicarpin ($\mu\text{g/g}$ fresh tissue) in the leaflets of four groundnut (*A. hypogaea*) cultivars infected with *Cercospora arachidicola* (○—○) or *Phoma arachidicola* (●—●). Leaflet samples (designated 1–4) were collected at 14 (= sample 1), 16 (= 2), 18 (= 3) and 20 (= 4) weeks after planting except those from the short-season cultivar, Jacana, which were harvested after 8, 10, 12 and 14 weeks growth.

able medicarpin accumulation curve was obtained after infection of Flamingo leaflets by *C. arachidicola* (Fig. 1C). In addition to Egret, 6/11/11, Flamingo and Jacana, medicarpin was also produced (although in generally much smaller quantities by the leaflets of three other long-season cultivars (P 84/5/244, P 84/5/256 and 60/66) examined during the course of the present study. Medicago concentrations recorded for the final (week 20) samples were as follows: P 84/5/244, (15 µg/g fresh tissue, *Cercospora*-infected; 110 µg/g, *Phoma*-infected), P 84/5/256 (45 µg/g, *Phoma*-infected), and 60/66 (75 µg/g, *Cercospora*-infected). Little or no medicarpin was isolated at any time from leaflets of P 84/5/256 and 60/66 infected with *C. arachidicola* and *P. arachidicola* respectively.

Apart from its accumulation in response to *C. arachidicola* and *P. arachidicola*, medicarpin has also been isolated (by means of the drop-diffusate technique) from the excised leaflets of an unnamed groundnut cultivar following treatment with droplets of aqueous CuSO₄ [9] or spore suspensions of the non-pathogenic fungus, *Helminthosporium carbonum* Ullstrup [7, 10]. EtOAc extracts of 5-day, fungus- or CuSO₄-induced diffusates were chromatographed (Merck Si gel TLC, F-254, layer thickness 0.25 mm) in CHCl₃-MeOH (50:1) to give impure **5** as a band at approx. *R_F* 0.55 (colour with diazotised *p*-nitroaniline reagent, yellow). The material in this zone was eluted (MeOH) and further chromatographed (Si gel TLC), first in *n*-pentane-Et₂O-glacial HOAc (75:25:1, ×2) and then, after elution, in benzene-MeOH (9:1; approx. *R_F* 0.62) to afford pure medicarpin. On average, diffusates from *H. carbonum*- and CuSO₄-treated leaflets contained medicarpin at a concentration of 9 and 6 µg/ml respectively (based on UV quantification using log ε = 3.90 at 287 nm [11]). Leaf tissues immediately beneath the droplets were also removed and thoroughly extracted with EtOH. Si gel TLC of these extracts as described above yielded small but readily detectable quantities of medicarpin (about 50 and 80 µg/g fresh tissue from *H. carbonum*- and CuSO₄-treated leaflets respectively). Medicago was not detected when *Arachis* leaflets were treated only with de-ionised H₂O (control).

Identification of the groundnut leaf phytoalexin as medicarpin (**5**) was confirmed by a UV and TLC comparison with material previously isolated from *Melilotus alba* [10]. The MS of the *Arachis* compound gave a molecular ion at *m/z* 270 (100%; cor-

responding to C₁₆H₁₄O₄), whilst its ¹H NMR spectrum (acetone-d₆; 360 MHz; TMS reference): δ 7.31 (d, 1H, *J* = 8.4 Hz, H-1), 7.22 (d, 1H, *J* = 8.1 Hz, H-7), 6.55 (dd, 1H, *J* = 8.4 & 2.5 Hz, H-2), 6.44 (dd, 1H, *J* = 8.1 & 2.3 Hz, H-8), 6.37 (d, 1H, *J* = 2.2 Hz, H-10), 6.34 (d, 1H, *J* = 2.5 Hz, H-4), 5.50 (br d, 1H, H-11a), 4.25 (m, 1H, H-6eq), 3.73 (s, 3H, OCH₃) and 3.59 (m, 2H, H-6ax/H-6a) proved to be indistinguishable from that of authentic medicarpin determined under the same conditions. Optical activity measurements indicated that *Arachis*-derived medicarpin was dextrorotatory ([α]_{589 nm} = +193°; 0.1 mg in 1 ml MeOH), and the compound can therefore be assigned the 6a*S*; 11a*S* absolute configuration depicted in **5**.

Although several stilbene phytoalexins (**1–4**) have recently been isolated from *A. hypogaea* [1–3], the production of medicarpin, a highly fungitoxic isoflavonoid (pterocarpan), by fungus-infected or

Table I. Occurrence of medicarpin (**5**) and other pterocarpan phytoalexins in nine genera belonging to the tribe Aeschynomeneae^a.

Subtribe and Genus	Compound ^b				
	Medicago	Maackiain	Nissicarpin	Fruticarpin	Nissolicarpin
Aeschynomeneinae					
<i>Aeschynomene</i>	+	+	–	–	–
<i>Geissaspis</i>	+	–	–	–	–
<i>Humularia</i>	+	+	–	–	–
<i>Kotschya</i>	+	+	–	–	–
<i>Smithia</i>	+	+	–	–	–
Ormocarpinae					
<i>Nissolia</i>	–	–	+	+	+
Poiretiinae					
<i>Zornia</i>	+	+	–	–	–
Stylosanthinae					
<i>Arachis</i> ^c	+	–	–	–	–
<i>Stylosanthes</i>	+	–	–	–	–

Key: + = Present; – = Not detected

^a Classification according to Rudd [14].

^b Medicago, 3-hydroxy-9-methoxypterocarpan; Maackiain, 3-hydroxy-8,9-methylenedioxypterocarpan; Nissicarpin, 3,7-dihydroxy-9-methoxypterocarpan; Fruticarpin, 7-hydroxy-3,9-dimethoxypterocarpan; Nissolicarpin, 3,7-dihydroxy-2,9-dimethoxypterocarpan.

^c Data from the present study. Four stilbene phytoalexins are also known to be produced by *A. hypogaea* [1–3].

CuSO₄-treated groundnut leaves has not been reported elsewhere. However, the ability to form medicarpin brings *A. hypogaea* more into line with other members of the tribe Aeschynomeneae where surveys undertaken by one of us (J.L.I.) have revealed the widespread occurrence of pterocarpin phytoalexins including compound **5** (Table I). In several aeschynomeneoid legumes, medicarpin is accompanied by the related pterocarpin maackiain, but the latter compound is apparently absent from both *Arachis* and *Stylosanthes*, the only two genera of subtribe Stylosanthinae so far examined for phytoalexin production. Finally, whilst *Nissolia* (sub-

tribe Ormocarpaceae) is atypical in that medicarpin and maackiain are replaced, at least in *N. fruticosa*, as leaf phytoalexins by nissicarpin, fruticarpin and nissolicarpin [12], it is worth noting that these three pterocarpanes are (like *Arachis* medicarpin) strongly dextrorotatory, a feature unusual amongst the generally laevorotatory 6aH-pterocarpin phytoalexins [13].

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