tral Al_2O_3 and crystallized from MeOH, mp 96–97°, $[\alpha]_D$ +35° (*c* 1.47, $CHCl_3$). λ_{max}^{EtOH} (nm): 231, 261 (log ε 4.510, 3.730). $\nu_{max}^{CHCl_3}$ (cm^{-1}): 3560 (OH), 1770 (γ lactone), 1600, 1510, 1460 (aromatic). PMR (ppm): 2.0–2.9 (3H, *m*, C-3, 5) 2.98 (2H, *q*, J_{AB} 14 Hz, C-6, methylene), 3.03 (1H, OH quenched by D_2O), 3.85 (12H, *s*, OMe), 4.08 (2H, *br d*, C-4 methylene), 6.5–6.9 (6H, *m*, aromatic H). MS (*m/e*): 402 (M^+), 387, 373, 345, 327, 306, 294, 278, 263, 250, 233, 219, 195, 151, 135. Found: C, 63.00; H, 6.1 $C_{22}H_{26}O_7$ requires C, 63.1: H, 6.2%.

Wikstromol triacetate. Crystallized from EtOH mp 162°, $[\alpha]_D$ +118.8° (*c* 1.56, $CHCl_3$), λ_{max}^{EtOH} (nm): 226, 274, 280 (log ε 4.04, 3.72, 3.69). ν_{max}^{KBr} (cm^{-1}), 1786 (phenolic OAc), 1770 (γ lactone), 1740 (OAc), 1600, 1510, 1460 (aromatic). PMR (ppm): 2.3 (6H, *s*, OCOMe), 2.23 (3H, *s*, OCOMe), 2.34 −3.06 (3H, *m*, C-3, 5), 3.16 (2H, *q*, J_{AB} 14 Hz, C-6 methylene), 3.85 (6H, *s*, OMe) 4.25 (2H, *br d*, C-4 methylene), 6.44–7.2 (6H, *m*, aromatic H). Ms (*m/e*): 500 (M^+), 458, 416, 374, 398, 356, 220 and 137. Found: C, 62.95; H, 5.75 $C_{26}H_{28}O_{10}$ requires C, 62.4; H, 5.6%.

Wikstromol diacetate. Colourless powder, $\nu_{max}^{CHCl_3}$ (cm^{-1}): 3450 (OH), 1760 (phenolic OAc). PMR (ppm): 2.3 (6H, *s*, OCOMe) 2.4–3.16 (3H, *m*, C-3, 5), 3.05 (2H, *q*, J_{AB} 14 Hz, C-6 methylene), 3.80 (6H, *s*, OMe), 4.09 (2H, *br d*, C-4 methylene), 6.6–7.1 (6H, *m*, aromatic). MS (*m/e*): 458 (M^+), 416, 374, 352, 323, 280, 265, 233, 210, 205, 192, 155, 149, 137 (base peak).

$LiAlH_4$ reduction of wikstromol dimethyl ether. To a soln of wikstromol diMe ether (206 mg) in THF, a suspension of $LiAlH_4$ (400 mg) in Et_2O was added and refluxed for 3 hr. The mixture was worked up and the residue (185 mg), showing one major spot on TLC (R_f 0.25, C_6H_6–MeOH, 23 : 2), was purified by chromatography over Si gel. The $CHCl_3$ eluate yielded a residue (131 mg) which crystallized from hexane –C_6H_6 (1 : 1) as colourless needles (50 mg), mp 130–131°, $[\alpha]_D$, +9° (*c*, 1.0, EtOH). λ_{max}^{EtOH} (nm): 230, 280, 290, ν_{max}^{KBr} (cm^{-1}), 3401, (OH), 1600, 1517 and 800 (aromatic). PMR (ppm): 2.05–2.68 (1H, *m*, C-3), 2.7–3.12 (2H, *m* C-5), 2.92 (2H, *s*, C-6), 3.0–3.3 (2H, *br*, *OH* quenched by D_2O), 3.52 (2H, *s*, C-1), 3.7 (2H, *br d*, C-4), 3.85 (12H, *s*, OMe), 6.68–7.00 (6H, *m*, aromatic H). MS (*m/e*): 406 M^+, 388, 357, 254, 237, 219, 189, 160, 151. Found: C, 64.8: H, 7.42 $C_{22}H_{30}O_7$ requires C, 65.20; H, 7.35%.

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3,5,4'-TRIHYDROXYSTILBENE AS A PHYTOALEXIN FROM GROUNDNUTS (*ARACHIS HYPOGAEA*)

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Key Word Index—*Arachis hypogaea*; Leguminosae; groundnut; *Helminthosporium carbonum*; stilbene; phytoalexin; antifungal compound; resveratrol.

Abstract—*Cis* and *trans*-resveratrol (3,5,4'-trihydroxystilbene) have been isolated from the infected hypocotyls of *Arachis hypogaea* and implicated as phytoalexins.

INTRODUCTION

Although phytoalexin biosynthesis has been associated with infected stems [1], seeds [1] and immature pods [2] of the groundnut (*Arachis hypogaea* L.), the chemical nature of the compound or compounds involved has not been reported. As yet, no experimental data has been provided to substantiate the claim that (like many leguminous species), roots and leaves of *A. hypogaea* produce pterocarpanoid phytoalexins [3]. Other work [4] suggests that a pre-infectional antifungal compound occurs in the tissues of groundnut pods. This paper presents evidence to show that groundnut hypocotyls accumulate a mixture of *cis* and *trans*-resveratrol following infection by the non-pathogenic fungus, *Helminthosporium carbonum*.

RESULTS AND DISCUSSION

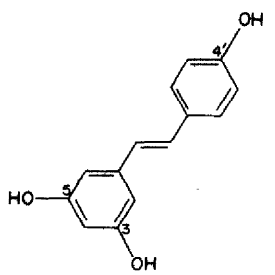
Antifungal material in hypocotyl diffusates (see Experimental) was detected by TLC ($CHCl_3$–MeOH, 100:4, Merck Si gel, F 254) bioassay [5] using *Cladosporium herbarum* as the test organism. Only one area (extending from the origin to R_f 0.11) highly inhibitory to the spore

germination of *C. herbarum* was associated with the infected hypocotyl diffusate. A minor inhibition zone was also visible at R_f 0.18. This gave an orange colouration with diazotised *p*-nitroaniline but was not examined in detail. Both antifungal zones were absent from the control diffusate chromatogram.

After elution (EtOH) and further TLC ($CHCl_3$–MeOH, 100:6, ×2), the lower inhibitory fraction was separated into two bands (B1, lower; B2, upper) by multiple development (×5) in *n*-pentane–Et_2O–HOAc (15:5:1). Although compounds B1 and B2 were both phenolic (diazotised *p*-nitroaniline, orange; Gibbs reagent, grey–black; vanillin–H_2SO_4 [6], purple–blue) and had identical MS (M^+228), their UV (EtOH) maxima were markedly different. B1 exhibited a pale-blue fluorescence (intensifying with NH_3), readily formed a trimethyl ether (M^+ 270), triacetate (M^+ 354) and dihydro-derivative (M^+ 230) and had neutral UV maxima (λ_{max}^{EtOH} (nm) 307, 322 *sh*) similar to those reported for *trans*-resveratrol **1** (3,5,4′-trihydroxystilbene) [7]. Identification of B1 as resveratrol was confirmed by comparison (UV, TLC, MS) with an authentic specimen. Compound B2 was not fluorescent and was obtained in only small quantities; this substance (λ_{max}^{EtOH} 285 nm) was formulated as *cis*-resveratrol **2** after comparison (UV, TLC) with a sample obtained by photochemical isomerisation of the *trans*-isomer [8].

Trans-resveratrol was first isolated from the roots of *Veratrum grandiflorum* (Liliaceae) [9] and has since been associated with the bark of *Nothofagus fusca* (Fagaceae) [10] and the heartwood and leaves of several *Eucalyptus* (Myrtaceae) species [7,11]. The genus *Eucalyptus* is apparently the only known natural source of the *cis*-isomer [11]. Although stilbenes related to resveratrol have been isolated from leguminous species (subfamilies Lotoideae and Caesalpinioideae), neither **1** nor **2** has previously been reported either as a phytoalexin or as an extractive from the Leguminosae. Although PC has been used to separate **1** from **2** [11], this is apparently the first report of their resolution by Si gel TLC.

Certain *trans*-stilbenes undergo photochemical isomerisation to yield the corresponding *cis*-isomers [8]. However, both **1** and **2** were isolated from diffusates which had been induced and chromatographed in a darkened laboratory. A sample of authentic **1** was unaltered after being subjected to identical procedures. This indicates that **2** is a product of *A. hypogaea* and not an artifact produced by exposure of **1** either to daylight or to the short-wavelength (254 nm) UV light used for chromatogram inspection. Chemical evidence has also been provided to suggest that both *cis* and *trans*-3,5,-dimethoxystilbene occur naturally in the bark of *Pinus banksiana*



1 *trans*-resveratrol
2 *cis*-isomer

[12]. However, for *A. hypogaea*, as for other species [11,12], production of the *trans*-stilbene appears to be favoured.

In diffusates from infected hypocotyls, **1** attained a concentration (based on $\epsilon = 4.45$ at 305 nm [7]) 24 hr after inoculation of 32–44 μg/ml. Although ϵ for **2** could not be located, its diffusate concentrations were estimated ($\epsilon = 4.04$ at 277 nm for *cis*-3,5-dimethoxystilbene [12]) to be *ca* 6–11 μg/ml. Thus **2** comprises some 20–25% of the total stilbene content of infected diffusates. Hathway [11] reported that in the heartwood of *Eucalyptus* spp., the concentration of **1** was 10–20 times greater than that of **2**; for *P. banksiana*, the *cis*–*trans* ratio of the related compound, 3,5-dimethoxystilbene, is *ca* 1:6 [12].

Although the total (*cis* + *trans*) stilbene content of diffusates from infected groundnut hypocotyls ranged from 38–55 μg/ml, neither **1** nor **2** was isolated from the underlying tissues in quantities suitable for UV determination. Moreover, all attempts to isolate **1** and **2** from the detached and fungus-infected leaves of either greenhouse-grown or 12-day-old etiolated groundnut plants [13] were unsuccessful. This suggests that for *A. hypogaea*, disease resistance may depend on factors additional to phytoalexin formation. It is noteworthy, however, that no biochromatographic [5] data has been obtained to support the suggestion [4] that groundnut tissues contain a pre-infectional antifungal compound.

When incorporated into agar and tested against the mycelial growth of *H. carbonum*, *trans*-resveratrol (ED_{50} *ca* 50 μg/ml) was less inhibitory than the isoflavonoid phytoalexins, medicarpin (ED_{50} 25 μg/ml) and sativan (ED_{50} 10 μg/ml) [13]. On biochromatograms, the inhibition zones given by **1** (5, 10 and 15 μg) were much less extensive than those for corresponding levels of medicarpin. The ED_{50} value of *cis*-resveratrol was not determined. However, against spore germination of *Monilinia fructicola* both *cis* and *trans*-3,5-dimethoxy-3′-hydroxystilbene were reported to have similar antifungal activity [14]. The inhibitory properties of the resveratrol isomers might therefore be comparable and may reflect their ability to inactivate enzymes with sulphydryl groups [15].

Previously, stilbene phytoalexins have been associated only with the Gymnospermae. For instance, both pinosylvin and its 5-*O*-methyl ether accumulate in the diseased sapwood of *Pinus taeda* [16], *P. radiata* [17] and *P. resinosa* [18] where they may restrict the development of invading fungi. The present work has involved a study of a Spanish–Valencia groundnut of West African origin and has revealed only *cis* and *trans*-resveratrol. In contrast, the *cis* and *trans*-isomers of 4-isopentenylresveratrol (but not resveratrol itself) have been found as phytoalexins in several North American varieties (Spanish-Valencia and Virginia) of *A. hypogaea* [19]. Whilst this varietal difference is unusual, it is plausible in view of the disjunct nature of the cultivars concerned. Moreover, several cowpea (*Vigna unguiculata*) lines accumulate the pterocarpan phytoalexin, phaseollidin whereas others produce the isoflavan, 2′-*O*-methylphaseollidinisoflavan [20]. Although the minor inhibition zone (R_f 0.18) evident on biochromatograms might reflect traces of 4-isopentenylresveratrol, this possibility was not investigated.

Despite repeated attempts, no evidence has been obtained to suggest that hypocotyls or leaves of *A. hypogaea* produce isoflavonoid phytoalexins [3]. Prior to this

investigation, the furanoacetylenes wyerone and wyerone acid (from *Vicia faba*) were the only non-isoflavonoid phytoalexins associated with the Leguminosae [21,22]. The isolation of resveratrol from *A. hypogaea* and, more recently, from the *H. carbonum*-infected leaves of *Trifolium dubium* and *T. campestre* (Lotoideae, Trifolieae), provides further evidence of chemical anomalies within the Leguminosae. However, a comparative study of phytoalexins from several leguminous tribes (e.g. Trifolieae, Loteae and Vicieae) (J. L. Ingham, *unpublished observations*) suggests that non-isoflavonoid phytoalexins are of very rare occurrence.

EXPERIMENTAL

MS were determined on an AEI MS12 instrument (heated direct inlet system) linked to a DS-30 computer.

Isolation of **1** *and* **2**. Groundnut (*Arachis hypogaea* L. cv Spanish–Valencia) seeds were interspersed with slices of unpeeled apple fruit and germinated (5 days in darkness at 24°) between moist paper towels in sealed plastic boxes. Seedlings were then transferred to a sandy loam; after a further 6 days (darkness, 24°) the etiolated hypocotyls (2–4 cm) were excised and treated as previously described [23]. Prepared tissues were inoculated with droplets (10 μl) of a conidial suspension (*ca* 5 × 10^4 spores/ml) of *Helminthosporium carbonum* Ullstrup and incubated (22°, *ca* 400 lx) for 24 hr. Control tissues received de-ionised H_2O. Diffusates [13] were then collected, diluted with EtOH (5 ml) and reduced to dryness (*in vacuo*, 40°) prior to Si gel TLC. For the dark induction of **1** and **2**, incubation units containing inoculated hypocotyls were covered with black polyethylene and placed in a cupboard (21°) for 24 hr. At no time was experimental material exposed to daylight or to the fluorescent laboratory lights. Diffusates from detached leaflets were obtained and extracted as previously described [13].

Trans-*resveratrol* (**1**). λ_{max}^{EtOH} (nm) 210, 220*sh*, 238*sh*, 296*sh*, 307, 322*sh*, 340*sh*; $\lambda_{max}^{EtOH+NaOH}$ (nm) 211, 248*sh*, 320*sh*, 347: MS (rel int) M^+ 228(100), 227(12), 211(5), 181(8). Methylation (CH_2N_2) afforded a trimethyl ether (R_f 0.84, $CHCl_3$); λ_{max}^{EtOH} (nm) 208, 228*sh*, 283*sh*, 293, 310*sh*, 326*sh*; MS (rel int) M^+ 270(100), 269(6), 255(3). Acetylation (C_5H_5N–Ac_2O–HOAc) gave a triacetate (R_f 0.54, $CHCl_3$); λ_{max}^{EtOH} (nm) 212, 228*sh*, 288*sh*, 297, 311*sh*, 326*sh*; MS (rel int) M^+ 354(25), 313(13), 312(63), 271(10), 270(53), 229(20), 228(100), 227(15), 211(8), 181(17).

Hydrogenation of **1**. A mixture of **1** (*ca* 1 mg) and 10% Pd–C (3 mg) in MeOH (3 ml) was shaken in an atmosphere of H_2 (20°) for 30 min. After removal of catalyst and solvent, the residue was chromatographed (Si gel TLC, $CHCl_3$–MeOH, 100:4) to afford 3,5,4′-trihydroxydihydrostilbene (diazotised *p*-nitroaniline, yellow–orange; Gibbs reagent, purple–blue) as a non-fluorescent band (R_f 0.11). λ_{max}^{EtOH} (nm) 212, 224, 280; $\lambda_{max}^{EtOH+NaOH}$ (nm) 218, 242, 294; MS (rel int) M^+ 230(15), 107(100).

Cis-*resveratrol* (**2**). λ_{max}^{EtOH} (nm) 210, 220*sh*, 285; $\lambda_{max}^{EtOH+NaOH}$ (nm) 215, 230*sh*, 247*sh*, 302; MS (rel int) M^+ 228(100), 227(12), 211(4), 181(8). *Trans–cis* isomerisation of **1** was undertaken as follows. Authentic **1** (200–300 μg) was applied (EtOH) to a Si gel TLC plate and irradiated (254 nm, 90 min). The chromatogram was then developed in *n*-pentane–Et_2O–HOAc (15:15:1, × 5) to give **2** identical (UV, TLC) with the *Arachis* metabolite.

Mycelial growth test. **1** was incorporated into Czapek–Dox agar at concentrations of 5–50 μg/ml. The medium (0.2 ml) was dispensed into Beltsville dishes (2 replicates/concentration), a plug of *H. carbonum* mycelium placed centrally on the agar and the dishes incubated (darkness, 25°) for 48 hr. The % inhibition of radial mycelial growth for each treatment (relative to that of the control) was then determined.

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