Phytochemistry, Vol. 20, No. 6, pp. 1381–1383, 1981.
Printed in Great Britain.

0031-9422/81/061381-03 $02.00/0

TWO NOVEL STILBENE PHYTOALEXINS FROM *ARACHIS HYPOGAEA*

GRACE E. AGUAMAH,* PETER LANGCAKE,† DAVID P. LEWORTHY,† JANET A. PAGE,†
ROBERT J. PRYCE† and RICHARD N. STRANGE*

*Department of Botany and Microbiology, University College London, Gower Street, London, WC1E 6BT, U.K.; †Shell Research Limited, Shell Biosciences Laboratory, Sittingbourne Research Centre, Sittingbourne, Kent, ME9 8AG, U.K.

(*Revised received* 7 *November* 1980)

Key Word Index—*Arachis hypogaea*; Leguminosae; groundnut; phytoalexins; stilbene.

Abstract—Three phytoalexins were isolated from groundnut seeds which had been sliced and incubated for 48 hr at 25°. Two were novel isoprenylated stilbene derivatives closely related to 3,5,4′-trihydroxy-4-isopentenylstilbene which had previously been reported from the same source.

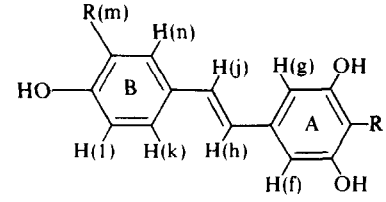
INTRODUCTION

Keen [1] found that the seeds of several plants, including groundnuts, accumulated phytoalexins when they were sliced and their natural microflora allowed to proliferate during a 3–5 day incubation period. American groundnuts synthesized two compounds which were identified as the *cis* and *trans* isomers of 3,5,4′-trihydroxy-4-isopentenylstilbene (4-isopentenylresveratrol) [2] whereas African groundnuts produced two different compounds which were identified as *cis*- and *trans*-3,5,4′-trihydroxystilbene (resveratrol) [3]. As part of a study of a leaf spot disease of groundnuts caused by *Cercospora arachidicola*, the phytoalexin-producing potential of the plant was re-investigated.

RESULTS

When imbibed American groundnuts were sliced and incubated, a microflora proliferated and phytoalexins were synthesized. Separation of EtOAc extracts by Si gel TLC and bioassay with *Cladosporium cucumerinum* [4] revealed two areas of antifungal activity. In contrast, chromatography of such extracts on Si gel columns and by HPLC led to the isolation of three antifungal compounds with retention times of 8.0 min, **1**; 9.3 min, **2** and 10.5 min, **3**. Compounds **2** and **3** co-chromatographed on Si gel TLC, explaining the occurrence of only two zones of antifungal activity in this system.

Compound **1** when dried under reduced pressure from aqueous MeCN was a microcrystalline solid which decomposed in light to yellow products. The UV spectrum (λ_{max} (MeCN/H_2O) nm; 220, 245 (sh), 310 (sh), 340, 346 (sh)) changed within minutes in daylight to give a single broad absorption maximum at 320–330 nm suggesting that it might be a hydroxylated *trans*-stilbene derivative. The chemical ionization MS (NH_4 as reagent gas) gave $(M + 1)^+ = 313$. Electron-impact MS gave $M^+ = 312.1340$ ($C_{19}H_{20}O_4$) and prominent fragments at *m/z* 297 ($M^+ - Me$) and 257 ($M^+ - Me_2CHC$), the $M^+ - 55$ fragment being indicative of an isopentenyl group. These data together with those from high-field ^{1}H NMR spectral analysis (see Table 1) suggested that the structure



Compound	R	R′
1	OH	CH_3(a), CH_3(b), CH(c)–CH(d)=CH(e)–
2	H	CH_3(a), CH_3(b), C(c)=CH(d)–CH_2(e)–
3	H	CH_3(a), CH_3(b), CH(c)–CH(d)=CH(e)–

Fig. 1. The structures of the three phytoalexins showing ^{1}H NMR assignments.

of **1** was 4-(3-methyl-but-1-enyl)-3,5,3′4′-tetrahydroxystilbene.

Compound **2** was obtained as a microcrystalline substance on evaporation of the solvent. The UV spectrum (λ_{max} (MeCN/H_2O) nm; 220, 295 (sh), 307, 324, 340 (sh)) was similar to that of resveratrol (λ_{max} (EtOH): 281, 297 (sh), 306, 320, 336 (sh)) and strongly suggested that it was a hydroxylated *trans*-stilbene derivative. The

Table 1. 360 MHz ^{1}H NMR data for groundnut phytoalexins

	Compound 1			Compound 2			Compound 3		
Assignment	Shift	Multiplicity	J (Hz)	Shift	Multiplicity	J (Hz)	Shift	Multiplicity	J (Hz)
(a)	1.09	(d)	7	1.65*	(s)		1.10	(d)	7
(b)	—			1.78*	(s)		—		
(c)	2.41	(m)		—			2.40	(m)	
(d)	6.68	(m)		5.30	(t)	~6	6.68	(m)	
(e)	6.68	(m)		3.36	(d)	~6	6.68	(m)	
(f)	6.58	(s)		6.58	(s)		6.60	(s)	
(g)	6.58	(s)		6.58	(s)		6.60	(s)	
(h)	6.76†	(d)	16	6.82†	(d)	16	6.82†	(d)	16
(j)	6.89†	(d)	16	6.91†	(d)	16	6.94†	(d)	16
(k)	6.89	(dd)	2/8	7.38	(d)	9	7.39	(d)	8
(l)	6.80	(d)	8	6.82	(d)	9	6.82	(d)	8
(m)	—			6.82	(d)	9	6.82	(d)	8
(n)	7.04	(d)	2	7.38	(d)	9	7.39	(d)	8

*,†Assignments may be interchanged.
Multiplicities: s = singlet, d = doublet, t = triplet, m = multiplet.

spectrum changed on exposure of the solution to weak daylight for a few minutes to give a single absorption maximum at *ca* 290 nm, typical of the corresponding *cis*-stilbene. The electron-impact MS of **2** gave *m/z* (rel. int.): 296 (M^+) (29.8), 281 (7.8), 279 (5.7), 241 (29.9), 44 (100), the M − 55 fragment as with **1** indicating an isopentenyl group. These data, together with those of high-field ^{1}H NMR spectral analysis (see Table 1), confirmed **2** as 4-(3-methyl-but-2-enyl)-3,5,4′-trihydroxystilbene (4-isopentenylresveratrol), the compound previously identified by Keen and Ingham [2] from groundnuts.

The stability of **3** was similar to that of **1** and it, too, decomposed to yellow products on solvent evaporation. The UV spectrum resembled that of **1** (λ_{max} (MeCN/H_2O) nm; 219, 241 (sh), 327 (sh), 331, 346 (sh), 364 (sh)) and also changed rapidly in daylight to give a single broad absorption maximum at 305–310 nm. The electron-impact MS, however, gave a spectrum very similar to **2**, M^+ being 296 and prominent fragments occurring at *m/z* 281, 279 and 241. Accurate mass determinations gave M^+ = 296.1238 ($C_{19}H_{20}O_3$), 281.1038 ($C_{18}H_{17}O_3$ = M − Me) and 241.0911 ($C_{15}H_{13}O_3$ = M − Me_2CHC), the M − 55 fragment as with the other two compounds suggesting the presence of an isopentenyl group. These data, together with those from high-field ^{1}H NMR spectral analysis (see Table 1), suggested the structure of **3** as 4-(3-methyl-but-1-enyl)-3,5,4′-trihydroxystilbene.

The detailed 360 MHz ^{1}H NMR spectral assignments for **1**–**3** are given in Table 1. The resonance signals at ~6.8 ppm and ~6.9 ppm (H(h) and H(j)) which are coupled to each other (*J* = 16 Hz) have chemical shifts characteristic of the double bond protons of a *trans*-stilbene. The aromatic substitution patterns were deduced from chemical shifts and spin decoupling experiments. Thus, all three compounds have two equivalent *meta*-protons in ring A (H(f) and H(g) and **2** and **3** have ring B with 1–4-disubstitution whereas **1** has 1,3,4-trisubstitution. The side chain structures were deduced from the results of double resonance experiments. In **1** and **3**, the methyl resonance signal appeared as a doublet (integrating to 6 protons) at 1.09 ppm, characteristic of a Me_2CH moiety. Irradiation of the multiplet at 2.4 ppm collapsed the methyl resonance signals and the olefinic signal at 6.68 ppm, establishing the side chain as $Me_2CH-CH=CH-$. In **2** the methyl resonances appeared as singlets in a region of the spectrum suggesting $Me_2C=$ i.e. 1.65 and 1.78 ppm; the olefinic proton at 5.3 ppm appeared as a triplet coupled to the methylene group at 3.36 ppm which was a doublet. The side chain structure was therefore $Me_2C=CH-CH_2$.

Analytical HPLC of EtOAc extracts with valerophenone as internal standard indicated that **1** was the predominant phytoalexin and accumulated to concentrations of 3.69 mg/g dry wt of sliced, imbibed seeds, whereas the concentrations of **2** and **3** reached 1.16 and 0.95 mg/g, respectively.

DISCUSSION

Leguminous plants are now known to synthesize many phytoalexins in response to microbial challenge or conditions of stress [5, 6]. Most are isoflavanoids or closely related to them (e.g. 2-arylbenzofurans [7]) although furanoacetylenes have been found in *Vicia* [8, 9] and *Lens* spp. [10]. Stilbenes, which are closely related to isoflavanoids in terms of biosynthesis [11], have been recognized as phytoalexins in groundnuts [2, 3] but in the present study two new compounds (**1**, **3**) are reported. Compound **1** was the major constituent in sliced peanut seeds (3.69 mg/g dry wt seed), while **2** (previously reported by Keen and Ingham [2]) and **3** were present in concentrations of 1.16 and 0.95 mg/g dry wt of seed, respectively.

Examples of isoprenylated derivatives of phenolic phytoalexins include kievitone and phaseollidin, both from French bean. In some other phytoalexins the isoprenyl group is cyclized with an adjacent phenolic hydroxyl group to produce various fused ring structures such as those found in glyceollins I, II and III from soyabean [12]. In these examples, isoprenylation increased the fungitoxicity of the corresponding non-prenylated compound which has low activity [13]. Keen

and Bruegger have ascribed this increased fungitoxicity to an increase in lipophilicity and therefore presumably greater affinity for membranes, the usual site of phytoalexin action [14]. In support of this contention, Kuhn and Smith [15] found that hydration of the isopentenyl side chain of kievitone decreased fungitoxicity. It will be interesting to compare the fungitoxicity of the compounds reported in this paper with resveratrol which, despite its identification as a phytoalexin of African groundnuts [3], appears to have low activity [16].

In their studies of groundnuts, Ingham [3] and Keen and Ingham [2] claimed that both the *cis* and *trans* isomers of the stilbene derivatives co-occurred naturally. By contrast, the stilbene phytoalexins isolated in the present study all appeared to be in the *trans* configuration as shown by both UV and ^{1}H NMR spectral analysis. Similarly, Langcake and Pryce [17] failed to detect the *cis* isomers of resveratrol or ε-viniferin as natural products of *Vitis vinifera*. *Cis–trans* isomerization of the 3-methyl-but-1-enyl side chain in **1** and **3** was not detected although thermodynamically the *trans* isomer would be highly favoured.

EXPERIMENTAL

Elicitation and extraction of phytoalexins. Groundnut seeds contained in their shells and originating from the U.S.A. (cv unknown) were purchased locally. The seeds (500 g) were shelled, soaked in H_2O overnight to allow imbibition to occur and cut into slices 1–2 mm thick before incubating in the dark at 25°. After 48 hr, by which time the natural microflora had proliferated, the slices were extracted by homogenizing in 95% EtOH (3 l.). From this point onwards all samples were protected from light as far as possible. The homogenate was filtered, evapd under red. pres. at <40° to a small vol., partitioned 3× against equal vols of petrol (b.p. 40–60°) and the petrol fraction discarded. The remaining aq. fraction was partitioned 3× against EtOAc and the combined EtOAc phases dried.

Fractionation of extracts. Samples were fractionated on a Si gel column (25 × 2.5 cm) and by prep. HPLC (Prep LC/System 500 Waters Associates) on the same adsorbent (30 × 6 cm) with petrol (b.p. 40–60°/EtOAc (7:3) as mobile phase in both systems. Active fractions were film-evapd and dissolved in MeCN–H_2O (1:1) before introduction into another HPLC apparatus. This consisted of a column (25 × 1.0 cm i.d.) of Hypersil ODS, a Pye–Unicam LC-UV detector set at 335 nm and a Tekman potentiometric chart recorder. Solvent (MeCN–H_2O, (1:1)) was pumped through the system by an Altex pump at 4 ml/min (*ca* 70 kg/cm^2) and samples (2 ml) were introduced by means of an Altex sample injection valve. In order to minimize degradation, UV spectra of pure samples of the phytoalexins were recorded in the HPLC solvent immediately after collection, as well as after exposure to daylight for 5–10 min.

^{1}H NMR spectra. Spectra were obtained on a Bruker WH 360 spectrometer system operating in the FT mode. Typically 24 scans were accumulated with a 30° pulse, 2 sec relaxation delay, 4 Hz spectral width with 16 K data points. The centre of the Me_2CO resonance signal was assigned as 2.051 ppm.

Quantitative estimation of the phytoalexins. Samples (20 μl) containing the phytoalexins were chromatographed by reverse-phase HPLC as above but with the difference that an analytical column (25 × 0.46 cm i.d.) rather than a semi-preparative column was used and valerophenone was included as an internal standard. Concns of the phytoalexins were calculated by measuring the peak areas and correcting for the detector response to the phytoalexins and the internal standard.

Acknowledgements—We wish to thank the Science Research Council for a grant for the purchase of HPLC. One of us (G.E.A.) also acknowledges with thanks a scholarship from the River State Scholarship Board of Nigeria.

REFERENCES

1. Keen, N. T. (1975) *Phytopathology* **65,** 91.
2. Keen, N. T. and Ingham, J. L. (1976) *Phytochemistry* **15,** 1794.
3. Ingham, J. L. (1976) *Phytochemistry* **15,** 1791.
4. Bailey, J. A. and Burden, R. S. (1973) *Physiol. Plant Pathol.* **3,** 171.
5. Harborne, J. B., Boulter, D. and Turner, B. L. (1971) *Chemotaxonomy of the Leguminosae.* Academic Press, London.
6. Harborne, J. B. and Ingham, J. L. (1978) *Phytochemical Society of Europe Symposia Series* 15, p. 343.
7. Martin, M. and Dewick, P. M. (1979) *Phytochemistry* **18,** 1309.
8. Letcher, R. M., Widdowson, D. A., Deverall, B. J. and Mansfield, J. W. (1970) *Phytochemistry* **9,** 249.
9. Hargreaves, J. A., Mansfield, J. W. and Rossall, S. (1977) *Physiol. Plant Pathol.* **11,** 227.
10. Robeson, D. J. (1978) *Phytochemistry* **17,** 80.
11. Langcake, P. and Pryce, R. J. (1977) *Phytochemistry* **16,** 1193.
12. Lyne, R. L., Mulheirn, L. J. and Leworthy, D. P. (1976) *J. Chem. Soc. Chem. Commun.* 497.
13. Lyne, R. L. and Mulheirn, L. J. (1978) *Tetrahedron Letters* 3127.
14. Keen, N. T. and Bruegger, B. (1977) *Am. Chem. Soc. Symp. Ser.* 62, 1.
15. Kuhn, P. J. and Smith, D. A. (1979) *Physiol. Plant Pathol.* **14,** 179.
16. Langcake, P. and Pryce, R. J. (1976) *Physiol. Plant Pathol.* **9,** 77.
17. Langcake, P. and Pryce, R. J. (1977) *Experientia* **33,** 151.