

Phytoalexins from *Dolichos biflorus*

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Genistein, 2'-hydroxygenistein, dalbergioidin, kievitone and phaseollidin have been found to accumulate in leaves and stems of *Dolichos biflorus* (horsegram) following inoculation with the non-pathogens *Pseudomonas pisi* and *Phytophthora megasperma* f. sp. *glycinea*, respectively. They are accompanied by isoferreirin (5,7,4'-trihydroxy-2'-methoxyisoflavanone), a compound not previously reported as a natural product. Coumestrol and psoralidin occur constitutively in *Dolichos* leaves and stems.

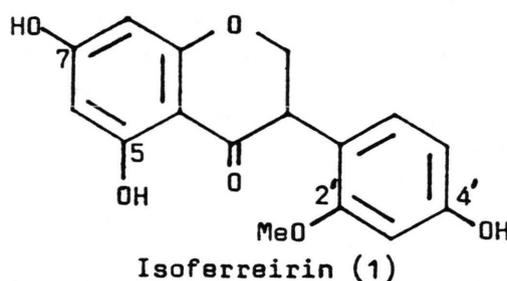
Introduction

A variety of isoflavonoid phytoalexins have been isolated from papilionate grain legumes such as *Lablab purpureus* (syn. *L. niger*), *Phaseolus vulgaris*, *Psophocarpus tetragonolobus* and *Vigna unguiculata* [1–3], all of which belong to the widely distributed and taxonomically advanced tribe Phaseoleae (subtribe Phaseolinae) [4]. As part of a continuing phytochemical study of the Leguminosae we have examined the phytoalexins produced by horsegram, *Dolichos biflorus* L. (Phaseoleae, subtribe Phaseolinae), a pulse extensively grown in parts of southern India. This paper describes the isolation and identification of eight induced and constitutive *Dolichos* isoflavonoids including a previously unknown isoflavanone (5,7,4'-trihydroxy-2'-methoxyisoflavanone) for which we suggest the trivial name *isoferreirin*.

Results and Discussion

Extracts of *Pseudomonas pisi*-infiltrated *Dolichos* leaves were chromatographed (see Experimental) to afford four fluorescence-quenching zones (B1–B4), three of which (B1, B3 and B4) were associated with pronounced antifungal activity as judged by

TLC bioassays against *Cladosporium cucumerinum*; only B3 and B4 were antibacterial when similarly bioassayed using *P. pisi* as the test organism. All four zones were eluted, and after further TLC purification (see Experimental) yielded isoferreirin (5,7,4'-trihydroxy-2'-methoxyisoflavanone, **1**) together with the known isoflavonoids, genistein (5,7,4'-trihydroxyisoflavone, **2**), 2'-hydroxygenistein (5,7,2',4'-tetrahydroxyisoflavone, **3**), dalbergioidin (5,7,2',4'-tetrahydroxyisoflavone, **4**), kievitone (5,7,2',4'-tetrahydroxy-8-isopentenylisoflavone, **5**), phaseollidin (3,9-dihydroxy-10-isopentenylpterocarpan, **6**), coumestrol (3,9-dihydroxycoumestran, **7**) and psoralidin (3,9-dihydroxy-2-isopentenylcoumestran, **8**). Compounds **2–8** were identified by UV, MS and TLC (4 or 5 solvent systems) comparison with authentic material. Only coumestrol and psoralidin were isolated from H₂O-infiltrated (control) leaves. These pre-infectional coumestans, as well as compounds **1–6** were also obtained from



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Dolichos stems inoculated with the fungus *Phytophthora megasperma* f. sp. *glycinea*.

MS analysis of the new isoflavanone (isoferreirin, **1**) revealed a low intensity parent ion (m/e 302; $C_{16}H_{14}O_6$) and associated major fragments at m/e 153 (base peak), 150, 135 and 107. Together with UV data (see Experimental), the MS fragmentation pattern strongly suggested that **1** was an isoflavanone derivative [5], this possibility being subsequently confirmed by CH_2N_2 methylation [6] to yield a phenolic diMe ether (M^+ 330) identical (UV, MS, TLC) with 5-hydroxy-7,2',4'-trimethoxyisoflavanone prepared from authentic ferreirin (5,7,2'-trihydroxy-4'-methoxyisoflavanone, **9**). As **1** separated from **9** upon Si gel TLC in $CHCl_3$:MeOH (25:1) (**1**, R_F 0.46; **9**, R_F 0.54), it was provisionally identified as a ferreirin isomer. Two OH groups were readily assigned to ring A (C-5 and C-7) on the basis of bathochromic shifts (see Experimental) observed when $AlCl_3$ in MeOH, or solid anhyd. NaOAc were added to methanolic solutions of **1** [7]. A-ring dihydroxylation and a B-ring substituted with single OH and OMe groups could also be deduced from the major MS ions at m/e 153 and 150 respectively [5].

The B-ring substitution pattern (C-2' OMe and C-4' OH) of **1** was proved unequivocally in the following manner. Ethylation with diethyl sulphate gave 7,4'-di-O-ethylisoferreirin (**10**) admixed with a small quantity of the corresponding 7-O-ethyl derivative (**11**). After TLC purification, the diethylated isoflavanone was degraded using the alkali-peroxide procedure [8] to yield a B-ring derived product indistinguishable (UV, MS, TLC) from 2-methoxy-4-ethoxybenzoic acid. Formation of this compound allows the structure of isoferreirin to be firmly defined as 5,7,4'-trihydroxy-2'-methoxyisoflavanone. 2-Ethoxy-4-methoxybenzoic acid would have been obtained if the C-2' and C-4' substituents of **1** were reversed as in ferreirin [8].

TLC bioassays (*Cladosporium cucumerinum*) of the induced *Dolichos* isoflavanoids indicated that phaseollidin was the most fungitoxic being followed by kievitone, dalbergioidin and isoferreirin respectively. Genistein and 2'-hydroxygenistein were inactive, thereby supporting the results of an earlier study [9]. Against *P. pisi*, only kievitone and dalbergioidin exhibited strong antibacterial properties. Other workers have also found that kievitone is inhibitory to various *Pseudomonas* species including

P. pisi ([10] and S. S. Gnanamanickam, pers. commun.). Although coumestrol is known to inhibit *P. phaseolicola* and *P. glycinea* [11, 12] neither it nor psoralidin have yet been tested against *P. pisi*.

Isoflavonoids **2–6** have all previously been isolated as phytoalexins from species belonging to the Phaseoleae [1–3, 5, 13] and as such their formation by *D. biflorus* – a member of the same legume tribe – is not particularly surprising. It is noteworthy, however, that isoflavan derivatives are apparently not produced by *D. biflorus* despite the fact that several compounds of this type have been obtained from *Phaseolus vulgaris*, *Lablab purpureus* and *Vigna unguiculata* [1, 2]. Similarly, the present study failed to reveal any phaseollin (a well-known phytoalexin of *P. vulgaris*) although its presumed biosynthetic precursor, phaseollidin accumulated in significant amounts. Although isoferreirin has been provisionally identified as a phytoalexin in *Helminthosporium carbonum*-inoculated hypocotyls of Florida velvet bean, *Stizolobium deeringianum* (Phaseoleae, subtribe Erythrinae), it could not be isolated from this source in amounts sufficient for comprehensive structural analysis ([8] and J. L. Ingham, unpublished data). Direct comparison (UV, MS, TLC) of the *Stizolobium* compound and *Dolichos* isoferreirin has now confirmed that they are identical.

Experimental

Plant and fungal material. Seeds of *Dolichos biflorus* L. (obtained from J. L. Hudson, Seedsman, Redwood City, California) were glasshouse grown (22°–30°C without supplemental lighting) in 10 cm pots containing a sand/peat moss mixture. Cultures of *Pseudomonas pisi* (supplied by R. Goodman, Dept. of Plant Pathology, University of Missouri, Columbia, Missouri, USA) and *Phytophthora megasperma* f. sp. *glycinea* (race 1, isolate P 1139) [14] were maintained on nutrient agar and V-8 juice agar, respectively [11, 15].

Induction and extraction of Dolichos isoflavanoids. Leaves from 3–4 week-old *Dolichos* plants were infiltrated with H_2O suspensions of *P. pisi* (8×10^7 cells/ml) using a hand sprayer [16]. After a further 2–4 days in the glasshouse, the leaves were harvested and immediately extracted (approx. 7 h) with 40% EtOH as described elsewhere [16]. The extracts

were concentrated *in vacuo* (40 °C) and then shaken ($\times 2$) with approx. equal vols of EtOAc. The pooled organic fractions were dried (anhyd. MgSO_4), evaporated, and the residue transferred to vials with peroxide-free Et_2O . Following removal of solvent and re-dissolution in EtOAc, the extracts were chromatographed (Si gel TLC, GF-254, layer thickness 0.375 mm) in hexanes*:EtOAc:MeOH (HEM, 60:40:1) to afford fluorescence-quenching bands at R_F 0.49 (B1), 0.41 (B2), 0.31 (B3) and 0.21 (B4). These zones were eluted (Me_2CO) and their components further purified (Si gel TLC) as follows, i) B1/B2, CHCl_3 : Me_2CO : NH_4OH (65:35:1) gave **6** (R_F 0.58) and **8** (R_F 0.13) respectively; ii) B3, CHCl_3 :MeOH (CM, 95:5, $\times 3$) gave **5** (lower zone), **7** (intermediate zone) and **1 + 2** (upper zone), the latter partially resolved isoflavonoids being eluted together prior to complete separation by multiple development ($\times 3-5$) in CM (**1**, upper zone; **2**, lower zone); and iii) B4, CM (95:5, $\times 3$) gave **3** (upper zone) and **4** (lower zone).

In some experiments, stems of *D. biflorus* were wound-inoculated with mycelium of *P. megasperma* f. sp. *glycinea* as described elsewhere [15]. After incubation (48 h; 25 °C), the stems were harvested and triturated (Sorvall Omni-Mixer) in 80% EtOH [15]. The filtered extract was concentrated, shaken with EtOAc and the organic phase then processed as outlined for bacteria-treated leaves.

5,7,4'-Trihydroxy-2'-methoxyisoflavanone (1) (isoferreirin). Diazotised *p*-nitroaniline, orange; Gibbs reagent, deep blue λ_{max} (nm) MeOH 212 (100%), 229 (96%), 289 (92%), 330 sh (37%); NaOH 214, 244, 324; NaOAc 254 sh, 286 sh, 325 (addition of solid H_3BO_3 regenerated the MeOH spectrum); AlCl_3 275, 286, 310, 365. MS (rel. int.) 303 (4), 302 (M^+ ; 20), 154 (6), 153 (100), 151 (5), 150 (49), 149 (10), 137 (5), 135 (44), 107 (27). **7,4'-Di-O-methyl ether** (CH_2N_2 ; R_F 0.67, CHCl_3 : CCl_4 , 3:1). Diazotised *p*-nitroaniline, orange/yellow; Gibbs reagent, blue. λ_{max} (nm) MeOH 214 (100%), 229 (85%), 287 (71%), 330 sh (13%); NaOH 214, 246 sh, 287, 357; AlCl_3 274, 284 sh, 310, 365; the MeOH spectrum was unaffected by addition of NaOAc. MS (rel. int.)

331 (2), 330 (M^+ ; 14), 165 (11), 164 (100), 151 (4), 150 (5), 149 (56), 121 (30).

Ethylation of isoferreirin. Pure isoferreirin (1.5 mg) was ethylated with diethyl sulphate [8] and the products chromatographed (Si gel TLC, layer thickness 0.25 mm) in CHCl_3 : CCl_4 (3:1) to give 7,4'-di-O-ethylisoferreirin **10** (R_F 0.72; approx. 1.2 mg) and 7-O-ethylisoferreirin **11** (R_F 0.05; approx. 0.2 mg). Both compounds were eluted (MeOH) and rechromatographed in either *n*-pentane:Et₂O:glacial HOAc (PEA, 75:25:3) (**11**, R_F 0.37) or PEA + CCl_4 (75:25:3:25) (**10**, R_F 0.71) prior to UV and mass spectroscopy.

5-Hydroxy-7,4'-diethoxy-2'-methoxyisoflavanone (10). Diazotised *p*-nitroaniline, orange; Gibbs reagent, Prussian blue. λ_{max} (nm) MeOH 213 (100%), 229 (81%), 287 (70%), 330 sh (13%); NaOH 218, 245 sh, 288, 357; AlCl_3 274, 284 sh, 311, 364; the MeOH spectrum was unaffected by addition of NaOAc. MS (rel. int.) 359 (3), 358 (M^+ ; 10), 179 (13), 178 (100), 163 (23), 150 (8), 135 (27), 107 (22).

5,4'-Dihydroxy-7-ethoxy-2'-methoxyisoflavanone (11). Diazotised *p*-nitroaniline, orange; Gibbs reagent, Prussian blue. λ_{max} (nm) MeOH 212 (100%), 229 (81%), 287 (70%), 330 sh (13%); NaOH 215, 244, 289, 355; AlCl_3 274, 284, 309, 364; the MeOH spectrum was unaffected by addition of NaOAc. MS (rel. int.) 331 (3), 330 (M^+ ; 13), 182 (9), 181 (100), 153 (19), 150 (18), 149 (8), 135 (26), 107 (21).

Bioassays. Fungitoxic material was located on TLC plates using the *Cladosporium cucumerinum* bioassay procedure [15]; detection of antibacterial compounds involved a modification of the technique described by Lund and Lyon [17]. After development in HEM, the TLC plates were dried (5 min; 110 °C), sprayed with a suspension of *P. pisi* (approx. 10^9 cells/ml) in trypticase soy broth and incubated (25 °C) for 16 h. The plates were then partially dried (2–10 min; room temp.) and sprayed with aesculin reagent [17]. Inhibition zones were apparent after incubation for 1–3 h at 25 °C.

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* A commercial mixture of hexane isomers (bp 68–70 °C).

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