Phytoalexins from *Dolichos biflorus*

Noel T. Keen

Department of Plant Pathology, University of California, Riverside, California 92521, USA

and

John L. Ingham

Phytochemical Unit, Department of Botany, University of Reading, Reading RG6 2AS, England

Z. Naturforsch. **35 c**, 923–926 (1980); received June 30, 1980

Leguminosae, *Dolichos*, Isoflavonoids, Phytoalexins, Structure Elucidation

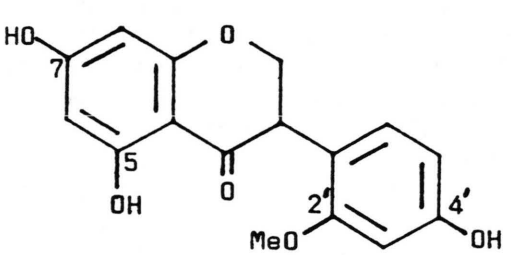
Genistein, 2′-hydroxygenistein, dalbergioidin, kievitone and phaseollidin have been found to accumulate in leaves and stems of *Dolichos biflorus* (horsegram) following inoculation with the non-pathogens *Pseudomonas pisi* and *Phytophthora megasperma* f. sp. *glycinea*, respectively. They are accompanied 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′-tetrahydroxyisoflavanone, **4**), kievitone (5,7,2′,4′-tetrahydroxy-8-isopentenylisoflavanone, **5**), phaseollidin (3,9-dihydroxy-10-isopentenylpterocarpan, **6**), coumestrol (3,9-dihydroxycoumestan, **7**) and psoralidin (3,9-dihydroxy-2-isopentenylcoumestan, **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 H_2O-infiltrated (control) leaves. These pre-infectional coumestans, as well as compounds **1**–**6** were also obtained from



Isoferreirin (1)

Reprint requests to Professor N. T. Keen or Dr. J. L. Ingham.

0341-0382/80/1100-0923 $ 01.00/0

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* isoflavonoids 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 Erythrininae), 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 isoflavonoids. 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 (×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, ×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 (×3–5) in CM (**1**, upper zone; **2**, lower zone); and iii) B4, CM (95:5, ×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:Et_2O: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.

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

Acknowledgements

Financial support was provided by the National Science Foundation (grant PCM 7724346 to N.T.K.) and the Science Research Council (to J.L.I.). We thank R. W. Butters (Tate and Lyle Ltd.) for MS analyses.

[1] J. L. Ingham, Z. Naturforsch. **32 c,** 1018 (1977).
[2] J. L. Ingham, Proc. Int. Legume Conf. Kew 1978, in press.
[3] N. W. Preston, Phytochemistry **16,** 2044 (1977).
[4] J. A. Lackey, Botan. J. Linn. Soc. **74,** 163 (1977).
[5] J. L. Ingham, Z. Naturforsch. **31 c,** 504 (1976).
[6] L. E. Powell, Plant Physiol. **39,** 836 (1964).
[7] T. J. Mabry, K. R. Markham, and M. B. Thomas, The Systematic Identification of Flavonoids, Springer, New York 1970.
[8] J. L. Ingham, Z. Naturforsch. **34 c,** 159 (1979).
[9] J. L. Ingham, N. T. Keen, and T. Hymowitz, Phytochemistry **16,** 1943 (1977).
[10] J. G. Wyman and H. D. VanEtten, Phytopathology **68,** 583 (1978).
[11] N. T. Keen and B. W. Kennedy, Physiol. Plant Pathol. **4,** 173 (1974).
[12] F. M. Lyon and R. K. S. Wood, Physiol. Plant Pathol. **6,** 117 (1975).
[13] R. Biggs, Aust. J. Chem. **28,** 1389 (1975).
[14] T. L. Kuan and D. C. Erwin, Phytopathology, in press.
[15] N. T. Keen, J. J. Sims, D. C. Erwin, E. Rice, and J. E. Partridge, Phytopathology **61,** 1084 (1971).
[16] N. T. Keen, Phytopathology **68,** 1239 (1978).
[17] B. M. Lund and G. D. Lyon, J. Chromatogr. **110,** 193 (1975).