Induced Isoflavonoids from Fungus-Infected Stems of Pigeon Pea
(Cajanus cajan)

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Four antifungal isoflavones [7-hydroxy-4'-methoxy-(1); 5,7,4'-trihydroxy-(2); 5,7,2',4'-tetrahydroxy-(3); 5,2',4'-trihydroxy-7-methoxy-(4)] and one isoflavanone [5,2'-dihydroxy-7,4'-dimethoxy-(5)] have been isolated from the fungus-infected stems of Cajanus cajan. A sixth compound has been provisionally identified as 5,2'-dihydroxy-7,4'-dimethoxyisoflavone (5). The structure of 5,2'-dihydroxy-7,4'-dimethoxyisoflavone (cajanol) was confirmed by synthesis from ferreirin (S''-trihydroxy''-methoxyisoflavanone).

Introduction

In response to fungal, bacterial or viral infection, numerous higher plants produce compounds (phytoalexins) with marked antifungal properties. Phytoalexins are generally considered to have a primary role in disease resistance and as such have received considerable attention in recent years. Species belonging to the Leguminosae (subfamily Lotoideae) often produce isoflavonoid phytoalexins (isoflavans and pterocarpans) although various stilbene and acetylenic derivatives have also been reported. An investigation of phytoalexin production by the agriculturally important grain-legume Cajanus cajan (L.) Millsp. has revealed several antifungal compounds including the new isoflavanone, cajanol (7). The chemical identification and antifungal properties of this and other isoflavonoids from C. cajan are described below.

Results and Discussion

Etiolated stems of C. cajan were inoculated with conidial suspensions of the non-pathogenic fungus, Helminthosporium carbonum Ullstrup and incubated for 48 h (see Experimental). Control stems were treated with de-ionised water. Tissues underlying the inoculum droplets were then excised and extracted with EtOH as previously described. Antifungal material in these extracts was detected by TLC bioassay (CHCl₃: MeOH, 100: 2; Merck Kieselgel 60 F₂₅₄) using Cladosporium herbarum Fr. as the test organism. Five inhibitory bands (B-1, Rp 0.65; 1: R₁=R₂=R₃=H; R₄=OH; R⁴=OCH₃
2: R₁=R₂=R₄=OH; R₃=H
3: R₁=R₃=R₄=OH; R₂=OCH₃
4: R₁=R₃=R₄=OH; R₂=OCH₃
5: R₁=R₃=OH; R₂=R₄=OCH₃
6: R₁=OH; R₂=R₃=R₄=OCH₃
7: R₁=R₂=OH; R₃=R₄=OCH₃
8: R₁=OH; R₂=R₃=R₄=OCH₃
9: R₁=R₂=R₄=OH; R₃=OCH₃
10: R₁=R₃=R₂=OH; R₄=OCH₃
11: R₁=R₂=R₄=OH)

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Formononetin and genistein were readily identified by UV, MS and TLC comparison with authentic specimens obtained from *Cicer arietinum* L. and *Trifolium subterraneum* L. respectively. The UV (MeOH) spectrum of compound 3 was consistent with its formulation as an isoflavone derivative and exhibited bathochromic shifts with NaOH (aromatic OH group), NaOAc (C-7 OH group) and AlCl₃ (C-5 OH group). Methylation (CH₃N₃) afforded a phenolic trimethyl ether (6) with M⁺ 328 and a prominent fragment at m/e 297 (M⁺ —31) characteristic of a 2'-hydroxy-isoflavone derivative and a major associated fragment at m/e 269 (M⁺ —17; 12). Loss of 17 mass units from the M⁺ is a feature of 4 and other isoflavones [e.g. 7,2',4'-dihydroxy-4'-methoxyisoflavone; MS (rel int) M⁺ 284(100), m/e 267(34)] with 2'-hydroxylation (Ingham, unpublished data). The fourth hydroxyl group of 3 was located at C-4' on biogenetic grounds. Structure 3 was recently confirmed by UV, MS and TLC comparison with a sample of hydroxy- genistein independently and concurrently isolated from the fungus-infected pods of *Phaseolus vulgaris* L. (French bean 12).

The MS of 4 was typical of a simple 2'-hydroxylated isoflavone giving M⁺ 300 and m/e 283 (M⁺ —17), 167 and 134. The latter ions can be formulated as dioxygenated fragments derived respectively from the A-ring (methoxyl; hydroxyl) and B-ring (two hydroxyls) of 4. The neutral (MeOH) UV spectrum of 4 closely resembled that of 3; bathochromic shifts were given with NaOH and AlCl₃ (C-5 OH) but not with NaOAc (absence of C-7 OH). Methylation afforded a product identical (UV, MS, TLC) with 5-hydroxy-7,2',4'-trimethoxyisoflavone obtained from 3. Formation of this compound establishes the 5,7,2',4'-oxygenation pattern of 4 and allows the single A-ring methoxyl group to be located at C-7 and the two B-ring hydroxyls at C-2' and 4'. The common name *cajanin* is proposed for compound 4.

Compound 7 exhibited a UV (MeOH) spectrum characteristic of an isoflavanone derivative. The MS was uncomplicated affording a small molecular ion (m/e 316) together with major fragments at m/e 167 and 150. These ions can be respectively derived from an isoflavone with monohydroxy-monomethoxy substitution of both the A and B-rings. Methylation gave a monomethyl (8) and a dimethyl (9) ether; these were identical (UV, MS, TLC) with products resulting from the methylation of ferreirin (10) (5,7,2'-trihydroxy-4'-methoxyisoflavone). With AlCl₃, the UV (MeOH) spectrum of 7 exhibited a 23 nm bathochromic shift (C-5 OH) thereby unequivocally locating the A-ring methoxyl at C-7. The B-ring substituents (OH to C-2' and OCH₃ to C-4') were initially assigned on biogenetic grounds and on the results of a comparative MS study involving 12 other related isoflavonones (see below and Table). Confirmation of the proposed B-ring oxygenation pattern was provided by selective (C-7) methylation of ferreirin (10) to yield small quantities of a compound indistinguishable (UV, MS, TLC) from 7. The common name *cajanol* is suggested for this hitherto undescribed isoflavone.

The MS of cajanol was characterised by major fragments at m/e 167 (13; A-ring derived) and 150 (14; B-ring). In contrast, a fragment corresponding to 13 was not observed in the MS of 2'-O-methylcajanol (8) which afforded only the expected B-ring ion at m/e 164. A comparable situation has recently been noted for kievitone (a 2'-hydroxylated isoflavone from *P. vulgaris*) and its 2'-O-methyl ether. From an examination of several naturally-occurring and synthetic isoflavonones (Table 1) it would appear that MS analysis may distinguish between derivatives with 2'-OH or 2'-OCH₃ groups. For the latter, a major A-ring fragment (corresponding to 13) was either not observed or was of very low intensity. Cajanol clearly belongs to the C-2' (OH) category. The above information is presented here because (i) it provides confirmation of structure 7 for cajanol and (2) it sug-
suggests that MS data may be used to assign 2'-OH or OCH$_3$ groups to new naturally-occurring isoflavonones which have been isolated in quantities insufficient for detailed spectroscopic and degradative analysis and for which unambiguous synthesis is impracticable. Isoflavanones listed in Table I did not afford MS ions at M$^+$ — 17 or M$^+$ — 31 characteristic of 2'-OH or 2'-OCH$_3$ substituted iso-

The isoflavone character of compound 5 was evident from its UV (MeOH) spectrum which was virtually superimposable on that of cajanin (4). Bathochromic shifts were given with NaOH and AlCl$_3$ but not with NaOAc. Methylation yielded traces of a product identical (UV, TLC) with the trimethoxyisoflavone 6. Compound 5 separated with difficulty from formononetin (1), a feature which suggests that both isoflavones may have the same OH: OCH$_3$ ratio (1:1). In the absence of confirmatory MS data 5 has been provisionally formulated as 5,2'-dihydroxy-7,4'-dimethoxyisoflavone (4'-O-methylcajanin). Although the isomeric structure 5,4'-dihydroxy-7,2'-dimethoxyisoflavone cannot be entirely excluded, 5 is more biologically acceptable since it then falls within the biosynthetic sequence: 2 → 3 → 4 → 5 → 7.

Cajanol (7) appears to be the primary antifungal compound produced by stems of pigeon pea. Although the extinction coefficient for 7 could not be determined, its concentration (based on log $\varepsilon$ = 4.63 at 262 nm for 2$^9$) of compounds 2 — 5 namely, 105 $\mu$g/g (2), 37 $\mu$g/g (3), 74 $\mu$g/g (4), and 22 $\mu$g/g (5). Nevertheless, whilst these isoflavones may principally function as cajanolin precursors, their total in vivo concentration (approx. 240 $\mu$g/g) may also be sufficient to significantly influence the disease resistance of C. cajan. Only traces of genistein (2) were isolated from stems treated with de-ionised water; compounds 1, 3 — 5 and 7 were not detected.

When incorporated into agar and tested against the mycelial growth of H. carbonum, cajanol exhibited an ED$^{50}$ value of approx. 40 $\mu$g/ml. Its antifungal properties are therefore comparable with those of other isoflavonoid phytoalexins$^{17-19}$. Because of insufficient material ED$^{50}$ values were not obtained for the isoflavones co-occurring with caja-
nol. However, the antifungal properties of formononetin (1) and genistein (2) have been reported elsewhere$^6,20$.

Although pterocarpan and isoflavan derivatives commonly occur as phytoalexins in the Legumino-
sae, few isoflavones or isoflavonanes are known to act in this capacity. It is interesting that apart from cajanol, the only previously reported isoflavone phytoalexin (5,7,2',4'-tetrahydroxy-8-isopentenyliso-

flavone or kievitone$^{15}$) has been isolated from species of Phaseolus and Vigna$^{15,21}$, two genera taxonomically related to Cajanus. However, whereas P. vulgaris and V. unguiculata (L.) Walp. also ac-
cumulate pterocarpian (e. g. phaseollin; phaseoll-
din) and isoflavan (e. g. phaseollinisoflavan) phyto-
alexins$^{21-23}$, representatives of these isoflavonoid
groups were not obtained from the fungus-inoculated stems of *C. cajan*. Nor was there evidence to suggest that pigeon peas produced kievitone or any other prenylated isoflavonone phytoalexin.

The chemical similarity between *C. cajan* and *P. vulgaris* is further supported by the isolation of genistein (2) and hydroxygenistein (3) from infected tissues of both species. In the Leguminosae, compounds 1–5 appear to be the only reported induced isoflavones although a preliminary investigation (Ingham, unpublished data) has provided some evidence for isoflavone accumulation in the fungus-infected hypocotyls of hyacinth bean (*Lablab niger* Medik. cultivar 'Rongai'). Moreover, *Glycine wightii* Grah. apparently produces a novel 4'-deoxy isoflavone following inoculation with the fungus, *Phytophthora megasperma* var. *sojae* (N. T. Keen, personal communication). Again it is noteworthy that both *Lablab* and *Glycine* are close taxonomic relatives of *Cajanus*. Outside the Leguminosae, an isoflavone phytoalexin (betavulgarin) has been reported from sugar beet (*Fam. Chenopodiaceae*). The formation of simple isoflavones and an isoflavone phytoalexin following inoculation with the fungus, *G. sojae* apparently produces a novel 4'-deoxy isoflavone phytoalexin. The chemical similarity between *C. cajan* and *P. vulgaris* is chemically less advanced than related species such as *Glycine max*, *V. unguiculata*, and *Cajanus cajan* is chemically less advanced than related species such as *P. vulgaris* and *V. unguiculata*.

**Experimental**

MS were determined on an AEI MS-12 instrument (heated direct inlet system) linked to an online DS-30 computer. UV spectra were recorded in MeOH using a Pye-Unicam SP 1800 spectrophotometer fitted with a Unicam AR-25 recorder.

**Induction, isolation and purification of compounds 1–5 and 7**

Locally purchased seeds of pigeon pea (*Cajanus cajan* (L.) Millsp.) were germinated (4 days in darkness at 24 °C) between moist paper in sealed plastic boxes. Seedlings were then transferred to a light potting compost and grown (darkness, 24 °C) between moist paper in sealed plastic boxes. Seedlings were then transferred to a light potting compost and grown (darkness, 24 °C) between moist paper in sealed plastic boxes. Seedlings were then transferred to a light potting compost and grown (darkness, 24 °C) between moist paper in sealed plastic boxes. Seedlings were then transferred to a light potting compost and grown (darkness, 24 °C) between moist paper in sealed plastic boxes.

The stems were excised and cut into short (approx. 10 cm) lengths; the cut ends were sealed with petroleum jelly and the prepared stems then placed on glass slides over moist paper in enclosed plastic boxes. The stems were inoculated with droplets (5 μl) of a conidial suspension (approx. 5 × 10⁴ spores/ml) of *Helminthosporium carbonum* Ullstrup (grown as previously described) and incubated (22 ± 2 °C; approx. 400 lx) for 48 h. Control stems received droplets of sterile de-ionised water. Tissues directly beneath the applied droplets were then excised and extracted with EtOH. The EtOH extract was reduced to dryness in vacuo (40 °C) and the residue chromatographed (Merck Kieselgel 60 F₂₅₄, layer thickness, 0.25 mm) in CHCl₃:MeOH (100:2) to afford five quenching bands at Rₚ 0.65 (B-1), 0.43 (B-2), 0.37 (B-3), 0.19 (B-4) and 0.10 (B-5). All 5 zones gave an orange/yellow colouration with diazotised p-nitroaniline; B-2 exhibited a pale blue fluorescence. The above zones were eluted and purified (TLC) as follows, (i) *B*-1, *n*-pentane: Et₂O:HOAc (PEA) (75:25:1) gave 7 (*Rₚ* 0.21), (ii) *B*-2, PEA (75:25:1, 4X) 5 (upper zone) and 1 (lower zone), (iii) B-3, PEA (75:25:1, 4X) 4, (iv) *B*-4, PEA (75:25:3, 2X) followed by CHCl₃ (4X) 2 and (v) *B*-5, PEA (75:25:6, 3X) 3. All the above compounds were homogeneous when chromatographed in additional solvent systems. TLC examination of control extracts gave small quantities of 2. Compounds 1, 3–5 and 7 were not detected. Chromatogram bioassays were undertaken as previously described.

**Formononetin 1 (7-hydroxy-4'-methoxyisoflavone)**

λₓᵧ (nm): MeOH 210, 236 sh, 249, 260 sh, 302; NaOH 208, 256, 272, 337; MS (rel. int.) M+ 268 (100); m/e 267 (35), 253 (12), 149 (37), 132 (75). Fluorescent pale blue (intensifying upon exposure to NH₃ vapour); colour with diazotised p-nitroaniline, yellow/orange.

**Genistein 2 (5,7,4'-trihydroxyisoflavone)**

λₓᵧ (nm): MeOH 208, 262, 295 sh; NaOH 219, 276; NaOAc 272, 328; Borate 262; AlCl₃ 273, 312 sh, 366; HCl 273, 312 sh, 366; MS (rel. int.) M+ 270 (100); m/e 269 (23), 153 (51), 152 (29), 135 (7), 124 (19), 118 (32). Diazotised p-nitroaniline, orange/yellow; colour with Gibbs reagent, deep blue.

**Hydroxygenistein 3 (5,7,2',4'-tetrahydroxyisoflavone)**

λₓᵧ (nm): MeOH 209, 261, 288 sh; NaOH 274; NaOAc 270; Borate 260; AlCl₃ 267, 310 sh, 361; HCl 267, 310 sh, 361; MS (rel. int.) M+ 286 (100); m/e 285 (19), 269 (M+ - OH; 30), 153 (58), 152 (21), 134 (36). Diazotised p-nitroaniline, orange/yellow; Gibbs reagent, blue. Trimethyl ether 6 (*Rₚ* 0.73, CHCl₃:CCl₄, 3:1); λₓᵧ (nm) MeOH 210, 260, 285 sh; MS (rel. int.) M+ 328 (100); m/e 327 (10), 313 (M+ - CH₃; 6), 297 (M+ - OCH₃; 33), 167 (36), 162 (21). Diazotised p-nitroaniline, yellow/orange; Gibbs reagent, blue.
Cajanin 4 (5,2'-4'-trihydroxy-7-methoxyisoflavone)
\[ \lambda_{max} (\text{nm}): \text{MeOH} 216, 260, 288, 330 \text{ sh}; \text{NaOH}\]
272; AlCl₃ 268, 306 sh; HCl 269, 306 sh; addition of NaOAc and Borate did not affect the MeOH spectrum; \( \text{MS (rel. int.) } M^+ 300(100): m/e 299(14), 283(M^-OH); 27, 167(86), 166(18), 151(31), 150(28), 134(46). \) Diazotised p-nitroaniline, orange/yellow; Gibbs reagent, deep blue. *Dimethyl ether* (\( R_F 0.73, \text{CHCl}_3: \text{C}_2\text{H}_5\text{OH} 3:1 \)) ; UV and MS as given for trimethyl ether of 3.

5,2'-dihydroxy-7,4'-dimethoxyisoflavone 5
\[ \lambda_{max} (\text{nm}): \text{MeOH} 211, 260, 285 \text{ sh}; \text{NaOH} 276; \text{AlCl}_3 271, 314 sh, 366; \text{HCl} 272, 314 sh, 366; \text{addition of NaOAc and Borate did not affect the MeOH spectrum. Diazotised p-nitroaniline, yellow/orange; Gibbs reagent, blue.}

Cajanol 7 (5,2'-dihydroxy-7,4'-dimethoxyisoflavanone)
\[ \lambda_{max} (\text{nm}): \text{MeOH} 217, 229, 287; \text{NaOH} 218, 245 sh, 287, 358; \text{AlCl}_3 266 sh, 274, 283 sh, 310, 368; \text{HCl} 266 sh, 274, 283 sh, 309, 365; \text{addition of NaOAc and Borate did not affect the MeOH spectrum. Diazotised p-nitroaniline, yellow/orange; Gibbs reagent, blue.}

Monomethyl ether 8 (\( R_F 0.83, \text{CHCl}_3 \)); \[ \lambda_{max} (\text{nm}): \text{MeOH} 218, 229, 287; \text{NaOH} 218, 254 sh, 287, 358; \text{AlCl}_3 266 sh, 274, 283 sh, 310, 370; \text{HCl} 266 sh, 274, 283 sh, 309, 369; \text{addition of NaOAc and Borate did not affect the MeOH spectrum; MS (rel. int.) } M^+ 316(18): m/e 168(10), 167(100), 150(27), 137(4), 135(15), 107(9). \) Diazotised p-nitroaniline, yellow/orange; Gibbs reagent, deep blue. *Monomethyl ether* 8 (\( R_F 0.83, \text{CHCl}_3 \)); \[ \lambda_{max} (\text{nm}): \text{MeOH} 218, 229, 287; \text{NaOH} 218, 254 sh, 287, 358; \text{AlCl}_3 266 sh, 274, 283 sh, 310, 370; \text{HCl} 266 sh, 274, 283 sh, 309, 369; \text{addition of NaOAc and Borate did not affect the MeOH spectrum; MS (rel. int.) } M^+ 316(18): m/e 168(10), 167(100), 150(27), 137(4), 135(15), 107(9). \) Diazotised p-nitroaniline, yellow/orange; Gibbs reagent, deep blue.

Dimethyl ether 9 (\( R_F 0.94, \text{CHCl}_3 \)); \[ \lambda_{max} (\text{nm}): \text{MeOH} 210, 228, 284; \text{addition of NaOH, NaOAc, Borate, AlCl₃ and HCl did not affect the neutral spectrum; MS (rel. int.) } M^+ 344(12): m/e 180(6), 165(12), 164(100), 149(28), 135(7), 121(12). \) *Ferreirin* 10 (5,7,2'-trihydroxy-4'-methoxyisoflavone)
\[ \lambda_{max} (\text{nm}): \text{MeOH} 212, 225, 288, 327 sh; \text{NaOH} 214, 244 sh, 323; \text{NaOAc} 253 sh, 287 sh, 325; \text{Borate} 288, 328 sh; \text{AlCl}_3 274 sh, 310, 370; \text{HCl} 274 sh, 309, 369; \text{MS (rel. int.) } M^+ 302(28): m/e 153(71), 151(14), 150(100), 149(10), 135(7), 121(14), 107(16). \) Diazotised p-nitroaniline, yellow/orange; Gibbs reagent, deep blue. *Dimethyl and trimethyl ethers* ; UV and MS as given for monomethyl and dimethyl ethers of 7 respectively.

Selective methylation of ferreirin
Ferreirin (5 mg), dry Me₂CO (2 ml), K₂CO₃ (1 g) and dimethyl sulphate (1 molar equivalent) were stirred under reflux (approx. 60 °C) for 1 h. After filtration and removal of solvent, the product was purified by Si gel TLC (C₆H₆:EtOAc:MeOH:petrol, 6:4:1:3) to afford small quantities of a compound (approx. \( R_F 0.73 \)) indistinguishable (MS, UV, TLC) from cajanol (7).

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2. J. L. Ingham, Phytochemistry, in press.