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

## Introduction

In response to fungal, bacterial or viral infection, numerous higher plants produce compounds (phytoalexins) with marked antifungal properties 1. 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 2, 3 and acetylenic 4, 5 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 <sup>6</sup>. Antifungal material in these extracts was detected by TLC bioassay (CHCl<sub>3</sub>: MeOH, 100: 2; Merck Kieselgel 60 F<sub>254</sub>) using *Cladosporium herbarum* Fr. as the test organism <sup>6, 7</sup>. Five inhibitory bands (*B-I*, *R<sub>F</sub>* 0.65;

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1:  $R^1 = R^3 = H$ ;  $R^2 = OH$ ;  $R^4 = OCH_3$ 

2:  $R^1 = R^2 = R^4 = OH$ ;  $R^3 = H$ 

3:  $R^1 = R^2 = R^3 = R^4 = OH$ 

4: R<sup>1</sup>=R<sup>3</sup>=R<sup>4</sup>=OH; R<sup>2</sup>=OCH<sub>3</sub> 5: R<sup>1</sup>=R<sup>3</sup>=OH; R<sup>2</sup>=R<sup>4</sup>=OCH<sub>3</sub>

6:  $R^1 = OH$ ;  $R^2 = R^3 = R^4 = OCH_3$ 

7: R1=R3=OH; R2=R4=OCH3

8:  $R^1 = OH$ ;  $R^2 = R^3 = R^4 = OCH_3$ 

9:  $R^1 = R^2 = R^3 = R^4 = OCH_3$ 

**10**:  $R^1 = R^2 = R^3 = OH$ ;  $R^4 = OCH_3$ 

11:  $R^1 = R^2 = R^3 = R^4 = OH$ 



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B-2,  $R_F$  0.43; B-3,  $R_F$  0.37; B-4  $R_F$  0.19 and B-5,  $R_F$  0.10) were associated with the inoculated tissue extract. In contrast, only one, minor, antifungal zone ( $R_F$  0.36) was evident on the control tissue chromatogram. Components of the above bands were eluted (EtOH) and further purified (see Experimental) to afford the following isoflavonoid derivatives; formononetin 1 (7-hydroxy-4'-methoxyisoflavone), genistein 2 (5,7,4'-trihydroxyisoflavone), hydroxygenistein 3 (5,7,2',4'-tetrahydroxyisoflavone) and cajanol 7 (5,2'-dihydroxy-7,4'-dimethoxyisoflavanone). Trace amounts of a sixth compound tentatively identified as 5,2'-dihydroxy-7,4'-dimethoxyisoflavone (5) were also obtained.

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 6, 8. 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 9) and AlCl<sub>3</sub> (C-5 OH group 9). Methylation (CH<sub>2</sub>N<sub>2</sub>) afforded a phenolic trimethyl ether (6) with M<sup>+</sup> 328 and a prominent fragment at m/e 297 (M<sup>+</sup> – 31) characteristic of a 2'-methoxylated isoflavone 10, 11. MS analysis of the parent phenol gave the molecular ion at m/e 286 (indicative of a tetrahydroxy isoflavone) 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'dihydroxy-4'-methoxyisoflavone; MS (rel int) M<sup>+</sup> 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 hydroxygenistein 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'-hydroxy-lated isoflavone giving M<sup>+</sup> 300 and m/e 283 (M<sup>+</sup>-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  $4^{13}$ . The neutral (MeOH) UV spectrum of 4 closely resembled that of 3; bathochromic shifts were given with NaOH and AlCl<sub>3</sub> (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 14, 15. 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 isoflavanone with monohydroxymonomethoxy substitution of both the A and Brings. 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'-methoxyisoflavanone 16). With AlCl<sub>3</sub>9, 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 OCH<sub>3</sub> to C-4') were initially assigned on biogenetic grounds and on the results of a comparative MS study involving 12 other related isoflavanones (see below and Table). Confirmation of the proposed Bring 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 isoflavanone.

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 isoflavanone from P. vulgaris 15) and its 2'-O-methyl ether. From an examination of several naturally-occurring and synthetic isoflavanones (Table I) it would appear that MS analysis may distinguish between derivatives with 2'-OH or 2'-OCH<sub>3</sub> 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-

Table I.	Mass	spectra	of	cajanol	(7)	and	related	isoflavanones.
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Isoflavanone		Expected major fragments					
		A	-ring	B-ring			
2'-Hydroxylated	$\mathbf{M}^{+}$	m/e	%	m/e	%		
5,2'-dihydroxy-7,4'-dimethyoxy-(7)	316	167	100	150	27		
7,2'-dihydroxy-4'-methoxy-	286	137	57	150	100		
7,2'-dihydroxy-8,9-methylenedioxy-	300	137	65	164	100		
5,7,2',4'-tetrahydroxy-	288	153	100	136	41		
5,7,2'-trihydroxy-4'-methoxy-	302	153	77	150	100		
5,2',4'-trihydroxy-6-methyl-7-methoxy-	316	181	100	136	16		
5,7,2',4'-tetrahydroxy-8-isopentenyl-	356	221	60	136	35		
2'-Methoxylated							
7-hydroxy-2',4'-dimethoxy-	300	137	_	164	100		
7,2'-dimethoxy-8,9-methylenedioxy-	328	151	8	178	100		
5,7-dihydroxy-2',4'-dimethoxy-	316	153	5	164	100		
5-hydroxy-7,2',4'-trimethoxy-	330	167	_	164	100		
5,7,2',4'-tetramethoxy-	344	181	-	164	100		
5-hydroxy-7,2',4'-trimethoxy-8-isopentenyl-	374	179		164	100		

gests that MS data may be used to assign 2'-OH or OCH $_3$  groups to new naturally-occurring isoflavanones 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 isoflavones.

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<sub>3</sub> 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<sub>3</sub> 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:  $2 \rightarrow 3 \rightarrow 4 \rightarrow 5 \rightarrow 7$ .

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  $\varepsilon=4.31$  at 288 nm for dalbergioidin,  $11^{14}$ ) 48 h after fungal inoculation was estimated to be approx.  $370 \,\mu\text{g/g}$  fr. tissue. In contrast, inoculated stems contained

much lower levels (based on  $\log \varepsilon = 4.63$  at 262 nm for  $2^8$ ) 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 cajanol 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  $\mathrm{ED}_{50}$  value of approx.  $40~\mu\mathrm{g/ml}$ . Its antifungal properties are therefore comparable with those of other isoflavonoid phytoalexins  $^{17-19}$ . Because of insufficient material  $\mathrm{ED}_{50}$  values were not obtained for the isoflavones co-occurring with cajanol. 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 Leguminosae, few isoflavanes or isoflavanes are known to act in this capacity. It is interesting that apart from cajanol, the only previously reported isoflavanene phytoalexin (5,7,2',4'-tetrahydroxy-8-isopentenylisoflavanene 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 accumulate pterocarpan (e. g. phaseollin; phaseollidin) and isoflavan (e. g. phaseollinisoflavan) phytoalexins  $^{21-23}$ , 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 isoflavanone 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 12. 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 20). The formation of simple isoflavones and an isoflavanone (but not pterocarpans or isoflavans) suggests that C. 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) for a further 6 days. At this point the etiolated 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  $^{24}$ . The stems were inoculated with droplets (5  $\mu$ l) of a conidial suspension (approx.  $5 \times 10^4$  spores/ml) of Helminthosporium carbonum Ullstrup (grown as previously described  $^6$ ) and incubated ( $22 \pm 2$  °C; approx.  $400 \ln$ ) for  $48 \ln$ . Control stems

received droplets of sterile de-ionised water. Tissues directly beneath the applied droplets were then excised and extracted with EtOH 6. The EtOH extract was reduced to dryness in vacuo (40 °C) and the residue chromatographed (Merck Kieselgel 60 F<sub>254</sub>, layer thickness, 0.25 mm) in CHCl<sub>3</sub>: MeOH (100:2) to afford five quenching bands at  $R_F$  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 25; B-2 exhibited a pale blue fluorescence. The above zones were eluted and purified (TLC) as follows, (i) B-1, n-pentane:  $Et_2O: HOAc(PEA)$  (75:25:1) gave 7 ( $R_F$  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 CHCl<sub>3</sub> (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 6, 7.

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

 $\lambda_{\rm max}$  (nm): MeOH 210, 236 sh, 249, 260 sh, 302; NaOH 208, 256, 272 sh, 337; MS (rel. int.) M+ 268 (100): m/e 267 (35), 253 (12), 149 (37), 132 (75). Fluorescent pale blue (intensifying upon exposure to NH $_3$  vapour); colour with diazotised p-nitroaniline, yellow/orange.

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

 $\lambda_{\rm max}$  (nm): MeOH 208, 262, 295 sh; NaOH 219, 276; NaOAc 272, 328; Borate 262; AlCl<sub>3</sub> 273, 312 sh, 366; HCl 273, 312 sh, 366; MS (rel. int.) M<sup>+</sup> 270(100): m/e 269(23), 153(51), 152(29), 135(7), 124(19), 118(32). Diazotised  $p\text{-nitro-aniline, orange/yellow; colour with Gibbs reagent $^6$, deep blue.}$ 

Hydroxygenistein  $\mathbf{3}$  (5,7,2',4'-tetrahydroxyiso-flavone)

 $\lambda_{\rm max}$  (nm): MeOH 209, 261, 288 sh; NaOH 274; NaOAc 270; Borate 260; AlCl<sub>3</sub> 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 ( $R_F$  0.73, CHCl<sub>3</sub>: CCl<sub>4</sub>, 3:1);  $\lambda_{\rm max}$  (nm) MeOH 210, 260, 285 sh; MS (rel. int.) M+ 328 (100): m/e 327 (10), 313 (M+ CH<sub>3</sub>; 6), 297 (M+ OCH<sub>3</sub>; 33), 167 (36), 162 (21). Diazotised p-nitroaniline, yellow/orange; Gibbs reagent, blue.

Cajanin 4 (5,2'-4'-trihydroxy-7-methoxyisoflavone)

 $\lambda_{\text{max}}$  (nm): MeOH 216, 260, 288, 330 sh; NaOH 272; AlCl<sub>3</sub> 268, 306 sh; HCl 269, 306 sh; addition of NaOAc and Borate did not affect the MeOH spectrum; MS (rel. int.) M<sup>+</sup> 300 (100): m/e 299 (14), 283 (M<sup>+</sup> – OH; 27), 167 (86), 166 (18), 151 (31), 150 (28), 134 (46). Diazotised *p*-nitroaniline, orange/yellow; Gibbs reagent, deep blue. *Dimethylether* ( $R_F$  0.73, CHCl<sub>3</sub>: CCl<sub>4</sub>, 3:1); UV and MS as given for trimethyl ether of **3**.

# 5,2'-dihydroxy-7,4'-dimethyoxyisoflavone 5

 $\lambda_{\rm max}$  (nm): MeOH 211, 260, 285 sh; NaOH 276; AlCl<sub>3</sub> 271, 314 sh, 366; HCl 272, 314 sh, 366; 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'-dimethoxyiso-flavanone)

 $\lambda_{\rm max}$  (nm): MeOH 217, 229, 287; NaOH 218, 245 sh, 287, 358; AlCl<sub>3</sub> 266 sh, 274, 283 sh, 310, 368; HCl 266 sh, 274, 283 sh, 309, 365; addition of NaOAc and Borate did not affect the MeOH spectrum; MS (rel. int.) M<sup>+</sup> 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, CHCl<sub>3</sub>);  $\lambda_{\rm max}$  (nm): MeOH 218, 229, 287; NaOH 218, 254 sh, 287, 358; AlCl<sub>3</sub> 266 sh, 274, 283 sh, 310, 370; HCl 266 sh, 274, 283 sh, 309, 369; addition of NaOAc and Borate did not affect the MeOH spectrum; MS (rel. int.) M<sup>+</sup> 330 (23): m/e 165 (14), 164 (100), 149 (30), 135 (7), 121 (14). Diazotised p-nitroaniline, yellow/orange; Gibbs re-

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agent, deep blue. Dimethyl ether **9** ( $R_F$  0.94, CHCl<sub>3</sub>);  $\lambda_{\rm max}$  (nm): MeOH 210, 228, 284; addition of NaOH, NaOAc, Borate, AlCl<sub>3</sub> and HCl did not affect the neutral spectrum; MS (rel. int.) M<sup>+</sup> 344(12): m/e 180(6), 165(12), 164(100), 149 (28), 135(7), 121(12).

# Ferreirin 10 (5,7,2'-trihydroxy-4'-methoxyiso-flavanone)

 $\lambda_{\rm max}$  (nm): MeOH 212, 225, 288, 327 sh; NaOH 214, 244 sh, 323; NaOAc 253 sh, 287 sh, 325; Borate 288, 328 sh; AlCl<sub>3</sub> 274 sh, 310, 370; HCl 274 sh, 309, 369; MS (rel. int.) M<sup>+</sup> 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 Me<sub>2</sub>CO (2 ml), K<sub>2</sub>CO<sub>3</sub> (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 (C<sub>6</sub>H<sub>6</sub>: 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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