A Revised Structure for the Phytoalexin Cajanol

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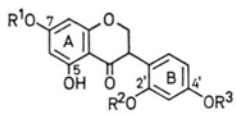
Peroxide oxidation of cajanol ethyl ether afforded the B-ring derived product, 2-methoxy-4-ethoxybenzoic acid. Formation of this compound means that the structure of cajanol, a major phytoalexin from *Cajanus cajan*, must be revised to 5,4′-dihydroxy-7,2′-dimethoxyisoflavanone.

Several isoflavonoid phytoalexins are known to accumulate in the etiolated stems of pigeon pea, *Cajanus cajan* (Leguminosae; subfamily Papilionoideae; tribe Cajaneae) following inoculation with the fungus, *Helminthosporium carbonum* [1]. These compounds have been identified [1] as the isoflavones, genistein (5,7,4′-trihydroxy-), 2′-hydroxygenistein (5,7,2′,4′-tetrahydroxy-) and cajanin (5,2′,4′-trihydroxy-7-methoxy-) and the isoflavanone, cajanol; the latter phytoalexin was originally formulated as 5,2′-dihydroxy-7,4′-dimethoxyisoflavanone (**1**).

On chromatograms sprayed with diazotised *p*-nitroaniline, cajanol (M^+ 316; $C_{17}H_{16}O_6$) immediately gives a bright orange/yellow colouration. In contrast, with Gibbs reagent [2, 3] the expected dark blue indophenol appears slowly, an observation not in accord with an unsubstituted position *para* to the proposed 2′-hydroxyl function. Thus, the 2′-hydroxy isoflavanone, ferreirin (**3**) (5,7,2′-trihydroxy-4′-methoxyisoflavanone) rapidly affords an intense blue derivative when subjected to the Gibbs test [1]. The abovementioned results suggest that in the B-ring of cajanol, the hydroxyl group may be located at C-4′ (and the methoxyl at C-2′) rather than C-2′ as previously reported [1]. The A-ring assignments (C-5 OH and C-7 OCH_3) – which were made on the basis of established spectral characteristics [4] – and the 2′,4′-oxygenation pattern of ring B are not in dispute.

Further studies on the structure of cajanol have recently been facilitated by its isolation in relatively large quantities from the $CuCl_2$-treated stems (approx. 20 mg cajanol/kg fresh wt) and roots (approx. 60 mg/kg fresh wt) of *C. cajan* (see Experimental). Ethylation of the chromatographically pure isoflavanone and subsequent H_2O_2 oxidation afforded a B-ring derived product indistinguishable by UV, MS and Co-TLC (in 5 solvent systems) from a synthetic specimen of 2-methoxy-4-ethoxybenzoic acid. Formation of this acid allows the B-ring OCH_3 and OH groups of cajanol to be unequivocally located at C-2′ and C-4′ respectively. Cajanol is thus 5,4′-dihydroxy-7,2′-dimethoxyisoflavanone (**2**) and not 5,2′-dihydroxy-7,4′-dimethoxyisoflavanone (**1**) as reported by Ingham [1]. As expected, peroxide oxidation of 5-hydroxy-7,2′-diethoxy-4′-methoxy isoflavanone (7,2′-di-O-ethylferreirin) gave 2-ethoxy-4-methoxybenzoic acid identical (UV, MS and TLC) with an authentic sample. The 2-ethoxy-4-methoxy substituted acid could be readily distinguished from the cajanol-derived isomer by comparative TLC (see Experimental).



1: $R^1=R^3=CH_3$; $R^2=H$
2: $R^1=R^2=CH_3$; $R^3=H$
3: $R^1=R^2=H$; $R^3=CH_3$
4: $R^1=R^2=R^3=H$
5: $R^1=R^3=H$; $R^2=CH_3$

Phytoalexin surveys in the Papilionoideae subfamily of the Leguminosae [5] suggest that cajanol is of exceptionally rare occurrence. Indeed, apart from *C. cajan*, this isoflavanone has only been obtained from the *H. carbonum*-inoculated hypocotyls of Florida velvet bean, *Mucuna (Stizolobium) deeringianum* (tribe Erythrineae) where it co-occurs with various other isoflavonoids including genistein, 2′-hydroxygenistein, dalbergioidin (**4**) (5,7,2′,4′-tetrahydroxyisoflavanone) and a third isoflavanone provisionally identified as 5,7,4′-trihydroxy-2′-methoxyisoflavanone (**5**) (isoferreirin). In *M. deeringianum*, the latter compound might represent the immediate biosynthetic precursor of cajanol.

Experimental

Mass and UV spectra were determined as previously described [1]. All chromatographic separations were undertaken using Merck, pre-coated Si gel TLC plates (F 254; layer thickness, 0.25 mm).

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Induction of cajanol

Locally purchased seeds of pigeon pea *(Cajanus cajan* [L.] Millsp.) were germinated as previously described [1], sown in moist vermiculite and grown (darkness, 24 °C) for 15 – 20 days. The etiolated stems (200 – 300 cm) were then cut into short (approx. 10 cm) lengths and placed in clear plastic boxes containing $CuCl_2 \cdot 2\,H_2O$ (5×10^{-3} M) in 0.05% aqueous Tween 20 to a depth of 3 – 5 mm. Roots of *C. cajan* were washed with de-ionised H_2O to remove the vermiculite and then immersed in aqueous $CuCl_2$ as described above. Stems and roots were incubated [1] for 5 or 6 days prior to extraction of cajanol.

Isolation and purification of cajanol

After incubation, the faintly brown stems (approx. 50 g) were macerated in warm MeOH (60 °C; 300 ml), filtered by suction and the fibrous mat re-extracted. The combined filtrates were reduced (*in vacuo*, 40 °C) to about 15 ml, diluted with de-ionised H_2O (250 ml) and shaken (×3) with equal volumes of EtOAc. The pooled organic fractions were reduced to dryness and the residue chromatographed in $CHCl_3$: MeOH (50 : 1) to afford cajanol as a brown fluorescent band at R_F 0.60. This zone was eluted (MeOH) and re-chromatographed (*n*-pentane : Et_2O : HOAc, 75 : 25 : 3, ×3) to give the pure isoflavanone (1 – 1.5 mg). Root tissues (approx. 150 g) were treated as described above except that 600 ml MeOH were used for the initial extraction. Final yields of cajanol varied from 8 to 11 mg. Only traces of cajanol were obtained from stems and roots incubated in aqueous Tween 20.

5,4′-dihydroxy-7,2′-dimethoxyisoflavanone (**1**) *(cajanol)*

λ_{max} (nm): MeOH 214 (100%), 229 (94%), 287 (85%), 336 sh (13%); NaOH 215 (100%), 244 (43%), 287 (38%), 356 (24%); NaOAc, Borate and $AlCl_3$ as lit. [1]; MS as lit. [1] *4′-acetoxy derivative* (Py-Ac_2O) (R_F 0.80, $CHCl_3$). λ_{max} (nm): MeOH 215 (100%), 229 (84%), 288 (78%), 334 sh (10%); $AlCl_3$ 312, 368; MS (rel. int.) 358 (M^+; 8), 316 (7), 192 (16), 168 (7), 167 (95), 166 (15), 151 (8), 150 (100), 135 (32), 107 (22).

Ethylation of cajanol

Cajanol (10 mg), dry Me_2CO (5 ml), anhydrous K_2CO_3 (1 g) and diethyl sulphate (15 µl) were refluxed (approx. 60 °C) for 2 h. After removal of K_2CO_3 (by centrifugation) and Me_2CO (*in vacuo*, 40 °C), the residue was chromatographed ($CHCl_3$) to give impure 5-hydroxy-7,2′-dimethoxy-4′-ethoxyisoflavanone (4′-O-ethylcajanol) at R_F 0.71. Elution and additional TLC in *n*-pentane : Et_2O : HOAc (75 : 25 : 1) afforded about 9 mg of the pure isoflavanone (R_F 0.59).

4′-O-ethylcajanol

λ_{max} (nm): MeOH 212 (100%), 229 (90%), 259 sh (34%), 286 (81%), 338 sh (13%); NaOH 214 (100%), 247 sh (24%), 287 (25%), 356 (6%); $AlCl_3$ 272, 310, 368; $AlCl_3$ + HCl 270 sh, 307, 366; addition of NaOAc and Borate did not affect the MeOH spectrum; MS (rel. int.) 344 (M^+; 11), 179 (23), 178 (100), 165 (3), 163 (5), 150 (5), 149 (7), 135 (12), 107 (8). Diazotised *p*-nitroaniline, yellow/orange; Gibbs reagent, dark blue (colour developing over several min); Fluorescent brown under long wavelength UV light.

H_2O_2 oxidation of 4′-O-ethylcajanol

The above compound (2 mg), aqueous KOH (10%; 3 ml), EtOH (0.5 ml) and H_2O_2 (30%; 0.2 ml added in 20 µl portions over 1 h) were stirred (50 °C ± 2 °C) for 90 min. The mixture was then acidified (2 N HCl; pH 3), extracted (×3) with equal volumes EtOAc and the organic fractions pooled and reduced to dryness. TLC ($CHCl_3$: MeOH, 50 : 1) of the residue gave a broad, fluorescence-quenching band (R_F 0.44) opposite the marker of authentic 2-methoxy-4-ethoxybenzoic acid (*cf.* authentic 2-ethoxy-4-methoxybenzoic acid, R_F 0.56). Traces of unchanged 4′-O-ethylcajanol were detected (diazotised *p*-nitroaniline) at R_F 0.90. Further TLC purification (*n*-pentane : Et_2O : HOAc, 75 : 25 : 3) of the R_F 0.44 fraction gave 2-methoxy-4-ethoxybenzoic acid (R_F 0.32; *cf.* 2-ethoxy-4-methoxybenzoic acid, R_F 0.46) indistinguishable (UV, MS, TLC) from synthetic material.

2-methoxy-4-ethoxybenzoic acid

λ_{max} (nm) EtOH: 214 (100%), 254 (81%), 289 (46%); MS (rel. int.) 196 (M^+; 70), 179 (5), 167 (7), 151 (65), 139 (37), 138 (11), 123 (8), 122 (25), 121 (100), 108 (10). The acid derived from 4′-O-ethylcajanol co-chromatographed with authentic material in the following solvents: (a) $CHCl_3$: CCl_4 (3 : 1), R_F 0.11 (b) C_6H_6 : MeOH (9 : 1), R_F 0.27

and (c) Et_2O : *n*-hexane (3 : 1), R_F 0.20. Corresponding R_F values for 2-ethoxy-4-methoxybenzoic acid were 0.23, 0.43 and 0.36.

Ethylation of ferreirin

Ferreirin (12 mg) was ethylated using the procedure described for cajanol. TLC purification ($CHCl_3$: CCl_4, 3 : 1) of the reaction products gave about 10 mg of 5-hydroxy-7,2′-diethoxy-4′-methoxyisoflavanone (7,2′-di-O-ethylferreirin) (R_F 0.25) together with small quantities (about 1.5 mg) of 5,2′-dihydroxy-7-ethoxy-4′-methoxyisoflavanone (7-O-ethylferreirin) (R_F 0.06).

7-O-ethylferreirin

λ_{max} (nm): MeOH 215 (100%), 229 sh (95%), 287 (90%), 340 sh (15%); NaOH 218 (100%), 245 sh (53%), 289 (61%), 352 (34%); $AlCl_3$ 274 sh, 311, 368; $AlCl_3$ + HCl 274 sh, 309, 368; addition of NaOAc and Borate did not affect the MeOH spectrum; MS (rel. int.) 330 (M^+; 24), 182 (8), 181 (100), 178 (27), 163 (10), 153 (16), 151 (7), 150 (47), 149 (11), 148 (5), 124 (5), 121 (5). Diazotised *p*-nitroaniline, bright yellow; Gibbs reagent, deep blue (colour developing rapidly); Fluorescent orange/brown under long wavelength UV light.

7,2′-di-O-ethylferreirin

λ_{max} (nm): MeOH 214 (100%), 228 (93%), 287 (87%), 338 sh (16%); NaOH 216 (100%), 246 sh (52%), 287 (68%), 357 (23%); $AlCl_3$ 274 sh, 312, 370; $AlCl_3$ + HCl 274 sh, 309, 370; addition of NaOAc and Borate did not affect the MeOH spectrum; MS (rel. int.) 358 (M^+; 15), 179 (13), 178 (100), 177 (5), 165 (6), 164 (5), 163 (56), 150 (15), 149 (32), 135 (5), 121 (5). Diazotised *p*-nitroaniline, pale yellow; Gibbs reagent, weak blue; Fluorescent orange/brown under long wavelength UV light.

H_2O_2 oxidation of 7,2′-di-O-ethylferreirin

The above isoflavanone (5 mg) was treated with H_2O_2 as described for 4′-O-ethylcajanol. TLC purification of the product (see oxidation of 4′-O-ethylcajanol for solvents and R_F values) gave 2-ethoxy-4-methoxybenzoic acid indistinguishable (UV, MS, TLC) from an authentic sample.

2-ethoxy-4-methoxybenzoic acid

λ_{max} (nm): EtOH 215 (100%), 254 (78%), 289 (35%); MS (rel. int.) 196 (M^+; 33), 163 (20), 152 (7), 151 (19), 150 (100), 135 (6), 122 (48), 107 (23). For comparative TLC data see 2-methoxy-4-ethoxybenzoic acid.

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