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TWO ISOPRENYLATED ISOFLAVONE PHYTOALEXINS FROM *CAJANUS CAJAN*

JAGROOP S. DAHIYA, RICHARD N. STRANGE, KEVIN G. BILYARD,* CHRISTOPHER J. COOKSEY* and PETER J. GARRATT*

Department of Botany and Microbiology, University College London, Gower Street, London WC1E 6BT, U.K.; *Department of Chemistry, University College London, 20 Gordon Street, London WC1H OAJ, U.K.

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Key Word Index—*Cajanus cajan*; Leguminosae; pigeonpea; isoprenylated isoflavones; ^{1}H NMR spectra.

Abstract—Four phytoalexins were isolated from sliced seeds of pigeonpea which had been incubated with its native microflora. Two were identified as the known pigeonpea phytoalexins, cajanin and cajanol, and the other two were characterized as new isoprenylated isoflavones.

INTRODUCTION

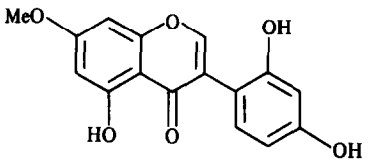
As part of our detailed investigation [1, 2] of phytoalexin accumulation in pigeonpea, we have investigated the response of sliced seeds to challenge by its native microflora [3]. Ingham [4, 5] had shown previously that etiolated stems of pigeonpea challenged with *Helminthosporium carbonum* produce four isoflavone phytoalexins, formononetin, genistein, 2′-hydroxygenistein and cajanin, and an isoflavanone, cajanol, which was also isolated from the roots. We have shown [1] that leaves challenged with *Botrytis cinerea* produce three phytoalexins, pinostrobin chalcone and two isomeric isoprenylated stilbene-2-carboxylic acids. We now report the isolation of four phytoalexins from seed, three isoflavones consisting of the previously reported cajanin [4] and two new compounds, and the isoflavanone cajanol [4, 5].

RESULTS

When imbibed seed was sliced and incubated under non-sterile conditions, antifungal compounds accumulated. Reverse-phase HPLC of extracts from this material allowed the separation of five fractions which inhibited the growth of *Cladosporium cucumerinum* [6] with retention times of 5.9, 7.0, 11.0, 14.2 and 15.6 min. The amount of material in the first fraction was small, and analytical HPLC and the ^{1}H NMR spectrum indicated that it was a complex mixture. The second fraction was solid and crystallized from methanol to give compound **1** as fawn crystals, mp 228–230°. The mass spectrum gave *m/z* 300.0640 ($C_{16}H_{12}O_6$ requires 300.0634), and there were prominent fragment ions at 299 $[M-1]^+$, 283 $[M-17]^+$, 167 $[M-133]^+$, 166 $[M-134]^+$ and 134 $[M-166]^+$. The ^{1}H NMR spectrum [$(CD_3)_2SO$] showed singlets at δ12.98, 9.38, 9.31 and 8.22, doublets at 6.97, 6.64, 6.39, 6.35 and 6.25, all of these signals each being due to one proton, and a three-proton singlet at 3.85. The electronic spectrum (methanol) showed absorption maxima at 260 nm (log ε 4.63), 285 sh (4.26) and 330 sh (3.80). These data are in accord with those reported for cajanin, previously identified as a phytoalexin of pigeonpea, and this structure is assigned to **1**.

The third fraction was a solid which was recrystallized from aqueous methanol to give compound **2** as crystals, mp 149°. The mass spectrum gave *m/z* 316.0889 ($C_{17}H_{16}O_6$ requires 316.0946), and there were prominent fragment ions at 168 $[M-148]^+$, 167 $[M-149]^+$, 150 $[M-166]^+$, 135 $[M-181]^+$ and 107 $[M-209]^+$. The ^{1}H NMR spectrum showed singlets at δ12.24 and 5.56, doublets at 6.93, 6.43, 6.08 and 6.01, double-doublets at 6.37, 4.53, 4.43 and 4.30, each of these signals being due to one proton, and three-proton singlets at 3.83 and 3.76. The electronic spectrum (methanol) had absorption maxima at 229 nm (log ε 4.5), 287 (4.49) and 335 (3.68). These data are in accord with those for cajanol [4, 5], a phytoalexin previously identified in pigeonpea. Nuclear Overhauser enhancement (NOE) experiments substantiated the revised structure [5] which was accordingly assigned to **2**.

The fourth fraction was a gum, compound **3**, which we were unable to crystallize. The mass spectrum gave *m/z* 352.0945 ($C_{20}H_{16}O_6$ requires 352.0946), and there were prominent fragment ions at 337 $[M-15]^+$, 335 $[M-17]^+$, 203 $[M-149]^+$, 195 $[M-157]^+$ and 167 $[M-185]^+$. The ^{1}H NMR spectrum [$(CD_3)_2SO$] showed signals at δ13.25 (1H), 9.36 (1H), 9.28 (1H), 8.19 (1H) and 6.58 (1H), doublets at 6.96 (1H, J = 8.5 Hz) and 6.34 (1H, J = 2.1 Hz), double-doublets at 6.25 (1H, J = 8.2, 2.2 Hz), 5.43 (1H, J = 9.6, 7.2 Hz) and 2.90 (1H, J = 15.6, 7.3 Hz), multiplets at 5.07 (1H) and 4.92 (1H), and a broad singlet at 1.70 (3H). In $(CD_3)_2CO$ as solvent a new signal could be observed at δ3.42 (*dd*, 1H, J = 15.6, 9.8 Hz) which had been obscured by water in the $(CD_3)_2SO$ solvent spectrum. The multiplets were also resolved into a septet at δ5.13 (J = 0.9 Hz) and a quintet of doublets at 4.94 (J = 1.5, 0.9 Hz). Compound **3** is, from these spectral data, an isoflavone with a cyclized isoprenyl side chain, but the position and orientation of this side chain cannot be deduced from these data. Consequently, compound **3** was methylated to give the dimethoxy derivative **5**. The ^{1}H NMR spectrum ($CDCl_3$) of **5** showed signals at δ7.83 (*s*, 1H), 7.38 (*d*, 1H, J = 8.4 Hz), 6.62 (*dd*, 1H, J = 8.4, 2.2 Hz), 6.61 (*d*, 1H, J = 2.2 Hz), 6.39 (*s*, 1H), 5.39 (*dd*, 1H, J = 9.2, 7.2 Hz), 5.10 (*br s*, 1H), 4.96 (*br s*, 1H), 3.85 (*s*, 3H), 3.79 (*s*, 3H), 3.42 (*dd*, 1H, J = 16.4, 9.2 Hz), 3.05 (*dd*, 1H, J



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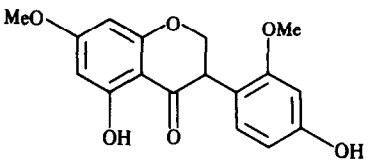

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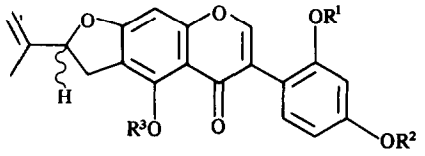

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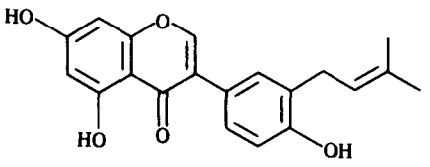
5 $R^1 = R^2 = Me, R^3 = H$
6 $R^1 = R^2 = R^3 = Me$



4

= 16.4, 7.2 Hz) and 1.77 (*br s*, 3H). A NOE experiment revealed that irradiation at the position of the δ3.85 signal enhanced the signals at δ6.62 and 6.61 while irradiation at the position of the δ3.79 signal enhanced only the signal at δ6.61. Clearly the two methoxyl groups are in a 1', 3' position on the C-ring with the 2', 4' and 5' positions unsubstituted.

Further methylation of **5** gave the trimethoxy derivative **6** which was purified by HPLC. The ^{1}H NMR spectrum ($CDCl_3$) showed signals at δ7.76 (*s*, 1H), 7.2 (*d*, 1H), 6.60 (*s*, 1H), 6.54 (*d*, 1H), 5.3 (*m*, 1H), 5.12 (*br s*, 1H), 4.96 (*br s*, 1H), 3.93 (*s*, 3H), 3.84 (*s*, 3H), 3.77 (*s*, 3H), 3.48 (*dd*, 1H, J = 16.1, 9.3 Hz), 3.11 (*dd*, 1H, J = 16.2, 7.6 Hz) and 1.78 (*br s*, 3H). When the spectrum was taken in C_6D_6 as solvent, considerable chemical shift changes were observed. The spectrum then showed signals at δ7.38 (*s*), 7.29 (*d*, J = 8.3 Hz), 6.51 (*d*, J = 2.3 Hz), 6.45 (*dd*, J = 8.3, 2.4 Hz), 6.16 (*s*), 4.95 (*br s*), 4.74 (*dd*, J = 9.4, 7.7 Hz), 4.73 (*br s*), 3.85 (*s*), 3.39 (*s*), 3.29 (*s*), 2.92 (*dd*, J = 15.8, 9.4 Hz), 2.73 (*dd*, J = 15.8, 7.7 Hz) and 1.48 (*br s*).

The small shift (−0.08 ppm) of the signal of the new methoxyl group on changing the solvent from $CDCl_3$ to C_6D_6 compared to the larger shifts of the two other methoxyl groups (−0.45, −0.48 ppm) indicates that the sites next to the new methoxyl group are substituted. This was supported by NOE experiments made with the sample in C_6D_6, where irradiation at the position of the δ3.29 methoxyl group enhanced the signal at 6.51, and irradiation at the position of the 3.39 methoxyl group enhanced both the signals at 6.5 and 6.45, whereas irradiation at the position of the 3.85 methoxyl group did not enhance any signal. From these results we assign structure **3** to this compound, the stereochemistry of the junction of the 2-propenyl group to the dihydrofuran ring not being established.

The fifth fraction was an oil, compound **4**, which we could not crystallize. The mass spectrum gave *m/z* 338.1155 ($C_{20}H_{18}O_5$ requires 338.1155), and there were prominent fragment ions at 323 $[M-15]^+$, 309 $[M-29]^+$, 283 $[M-55]^+$, 253 $[M-85]^+$, 153 $[M-185]^+$ and 131 $[M-209]^+$. The ^{1}H NMR spectrum $[(CD_3)_2SO]$ showed signals at δ12.94 (*s*, 1H) 10.88 (*s*, 1H), 9.50 (*s*, 1H), 8.26 (*s*, 1H), 7.19 (*br s*, 1H), 6.80 (*d*, 1H, J = 8.1 Hz), 6.36 (*d*, 1H, J = 2.1 Hz), 6.19 (*d*, 1H, J = 2.1 Hz), 5.27 (*br t*, 1H, J = 6.7 Hz), 3.21 (*d*, 2H, J = 6.8 Hz) and 1.65 (*s*, 6H). The spectrum taken in $(CD_3)_2CO$ showed considerable shifts for some of the protons and, in particular, the δ7.19 signal was resolved as a doublet shifted to δ7.33 (J = 2.2 Hz), and the six-proton singlet became two broad three-proton singlets at δ1.72 and 1.70. These data show that **4** is an isoflavone with an isoprenyl side chain on ring C adjacent to the hydroxyl group, and the structure shown for **4** is assigned.

DISCUSSION

We have shown previously that the leaves of pigeonpea accumulate a chalcone and two isomeric isoprenylated stilbene-2-carboxylic acids [1]. In contrast, Ingham found that the etiolated hypocotyls of the plant synthesize flavanoid phytoalexins [4, 5]. The seed phytoalexins are also flavanoid and two, the isoflavone cajanin and the isoflavanone cajanol, have now been identified in both tissues.

The two new phytoalexins are both isoprenylated isoflavones, the isoprenyl group in **3** being cyclized to form a 2-propenyldihydrofuran. Compound **3** is related to luteone, previously reported from *Argyrocytisus battandieri*, *Hardenbergia violacea*, *Laburnum anagyroides* and *Lupinus albus* [7], having the same hydroxylation pattern but cyclization having occurred between the 7-hydroxyl group and the isoprenyl side chain in **3**, followed by proton loss. The pattern of cyclization to give a 2-propenyldihydrofuran ring, although well known in pterocarpans, e.g. glyceollin III [8], has not been previously reported in isoflavone phytoalexins.

Compound **4** has not been previously isolated as a natural product but it has been synthesized [9] and our data are in accord with those reported. It is isomeric with wighteone, a phytoalexin found in *Argyrocytisus battandieri*, *Laburnum anagyroides*, *Lupinus albus* and *Neonotonia wightii* [7], differing from it in the position of

the isoprenyl side chain, which is at C-6 in wighteone, and it is closely related to licoisoflavone A, a phytoalexin isolated from *Hardenbergia violacea* and *Phaseolus vulgaris*, which is further hydroxylated at C-2′ [7].

EXPERIMENTAL

Elicitation and extraction of phytoalexins. Pigeonpea seeds were soaked in H_2O for 4 hr before being cut into slices *ca* 2 mm thick. After incubation under moist conditions for 72 hr, the material was homogenized in 70% MeOH. The homogenate was filtered and the filtrate evapd under red. pres. at < 40° and partitioned 3 × against petrol and 3 × against EtOAc. The combined EtOAc phases were dried and dissolved in MeCN–H_2O (1:1).

Fractionation of extracts. Samples (1 ml) were injected into an HPLC instrument consisting of an Altex pump and injection valve, a column (25 × 1.0 cm i.d.) of Hypersil ODS, a Pye Unicam LC-UV detector set at 290 nm, and a Tekman potentiometer chart recorder. Active fractions were defined originally by their ability to inhibit *Cladosporium cucumerinum* in the TLC assay [6] and subsequently by their retention time and absorption of light at 290 nm.

Methylation of **3**. A mixture of **3** (7.9 mg), K_2CO_3 (140 mg) and Me_2SO_4 (87 mg) in Me_2CO (10 ml) was heated to reflux for 8 hr. The resulting mixture was filtered and the filtrate evapd to give **5**, 7.2 mg, as a brown solid. Compound **6** was prepared similarly, except that the amount of Me_2SO_4 was increased (200 mg) and the amount of solvent decreased (1.0 ml). Compound **6** was purified by HPLC [25 cm × 4.5 mm (i.d.), Partisil 5 column, 0.5% MeOH in CH_2Cl_2, detector at 254 nm].

^{1}H NMR *spectra.* Spectra were obtained on a Varian XL-200 spectrometer using TMS as internal standard. NOE expts were performed by the subtraction of two spectra which differed only in the value of the homonuclear decoupling frequency.

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