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TWO NOVEL STILBENE-2-CARBOXYLIC ACID PHYTOALEXINS FROM *CAJANUS CAJAN*

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Key Word Index—*Cajanus cajan*; Leguminosae; pigeon pea; phytoalexins; chalcone; stilbene-2-carboxylic acid; ^{1}H NMR spectra.

Abstract—Three phytoalexins were isolated from leaves of pigeon pea which had been challenged with *Botrytis cinerea*. One was identified as pinostrobin chalcone and the other two were novel isoprenylated stilbene-2-carboxylic acids.

INTRODUCTION

Ingham [1, 2] isolated and identified five phytoalexins from the etiolated stems of pigeon pea challenged with *Helminthosporium carbonum*. Four were isoflavones, formononetin, genistein, 2′-hydroxygenistein and cajanin, and the fifth was an isoflavanone, cajanol, which was also isolated from roots. We now report the accumulation of one chalcone and two stilbene 2-carboxylic acid phytoalexins in leaves of the plant challenged with *Botrytis cinerea*.

RESULTS

Separation of ethanolic extracts of pigeon pea leaves challenged with *Botrytis cinerea* by Si gel TLC and bioassay with *Cladosporium cucumerinum* [3] gave a single zone of fungal inhibition. By contrast, reverse phase HPLC allowed the separation of three phytoalexins with retention times of 7.5 min, **1**; 14.1 min, **2**; and 16.8 min, **3**.

Compound **1** was obtained as a yellow gum which could be crystallized from chloroform as orange–red crystals, mp 148–150°. The mass spectrum gave *m/z* M^+ 270.0822 ($C_{16}H_{14}O_4$ requires 270.0892) and there were prominent fragment ions at 269 $[M-1]^+$, 193 $[M-77]^+$, 167 $[M-103]^+$ and 166 $[M-104]^+$. The ^{1}H NMR spectrum ($CDCl_3$) showed a broad one-proton singlet at δ 10.1, doublets at δ 8.04 (1H, $J = 15.6$ Hz) and 7.82 (1H, $J = 15.6$ Hz), multiplets at δ 7.70 (2H) and 7.45 (3H), and singlets at δ 5.99 (2H) and 3.81 (3H). The electronic spectra showed an absorption maximum at 342 nm (log ϵ 4.3) and the compound was labile, a new compound being formed on standing in ethanol solution for 7 days. The ^{1}H NMR spectrum of this solution showed, besides the signals for **1**, new signals at δ 12.02, 5.41 (*dd*, $J = 12.9$, 3.0 Hz), 3.05 (*dd*, $J = 16.8$, 12.9 Hz), 2.78 (*dd*, $J = 16.8$, 3.0 Hz) and 3.80 (*s*). These data are in accord with the known physical properties and chemical behaviour [4–6] of 1-(2,6-dihydroxy-4-methoxyphenyl)-3-phenyl-2-propen-1-one (pinostrobin chalcone) and we assign this structure to **1**. The change in the ^{1}H NMR spectrum on standing the solution of **1** is due to the formation of the bicyclic system **4**.

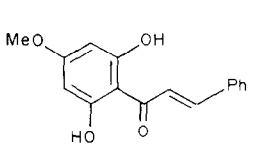
The two remaining compounds were both isolated as oils which could be crystallized from aqueous methanol. Compound **2**, pale yellow needles, mp 144–146°, showed in the mass spectrum a small *m/z* $M^+ = 338$, and there were major fragment ions at 279 $[M-59]^+$, 251 $[M-87]^+$, 206 $[M-132]^+$, 203 $[M-135]^+$, 188 $[M-150]^+$ and 162 $[M-176]^+$. The electronic spectrum had a maximum at 254 nm (log ϵ 4.30) and a shoulder at 315 nm (log ϵ 3.92). The ^{1}H NMR spectrum is shown in Table 1. The assignments are based on a comparison of the chemical shifts with those of related stilbenes and benzoic acid derivatives [7–11] and by decoupling experiments. The presence of the isoprenyl group is readily verified. The low field shift of the hydroxyl group indicates that it is adjacent to a carbonyl function [10], and in the NOE difference spectra, irradiation at the position of the methoxyl signal enhanced the signal of the H-4 proton, and irradiation at the position of the H-4 proton signal only enhanced the methoxyl proton signal. On changing to deuteriobenzene as a solvent, the H-4 and methoxyl proton signals in the ^{1}H NMR spectrum are more shielded and the isoprenyl methylene protons are deshielded, indicating that the solvent molecules are arranged over C-4, C-5 and the methoxyl group. Methylation of **2** with diazomethane gave **5**, recrystallized from petroleum (bp 60–80°), mp 94–95°. The mass spectrum showed *m/z* M^+ 352.1691 ($C_{22}H_{24}O_4$ requires 352.1673), and major fragment ions were observed at 320 $[M-32]^+$, 305 $[M-47]^+$, 277 $[M-75]^+$, 261 $[M-91]^+$, 229 $[M-123]^+$, 221 $[M-131]^+$, 220 $[M-132]^+$ and 188 $[M-164]^+$. The ^{1}H NMR spectrum showed the expected new signal at δ 3.81 (3H) and a narrow carboxyl proton signal. Decarboxylation of **5** gave **7** which has a very similar ^{1}H NMR spectrum to longistylene C, isolated and identified by delle Monache *et al.* [7]. The mass spectrum of **2** also resembled closely the mass spectrum of longistylene C [7], and all of the foregoing results are in accord with the assigned structure for **2**.

Compound **3**, pale yellow needles, mp 150–165° dec., showed in the mass spectrum a *m/z* $M^+ = 338$, and there were major fragment ions at 320 $[M-18]^+$,

Table 1. 200 MHz ^{1}H NMR spectral data for **2** and **3**

2				**3**			
Chemical shift(δ)			Intensity, multiplicity [J(Hz)]	Chemical shift (δ)		Intensity multiplicity [J(Hz)]	Assignment
$CDCl_3$	$(CD_3)_2CO$	C_6D_6		$(CD_3)_2CO$	C_6D_6		
11.4	12.0	11.6	1H, *br s*	12.5	—	1H, *br s*	OH-3
7.49–7.26	7.54–7.33	7.39–7.06	5H, *m*	7.2–7.6	7.2–7.6	5H, *m*	H-10–H-14
7.34	*	*	1H, *d*, J = 16.6	8.01	7.96	1H, *d*, J = 16.2	H-7
6.40	6.38	6.37	1H, *d*, J = 16.6	7.01	6.88	1H, *d*, J = 16.2	H-8
				6.86	6.50	1H, *s*	H-6
6.45	6.51	6.39	1H, *s*	—	—	—	H-4
5.17	5.12	5.34	1H, *t* of septets J = 6.7, 1.3†	5.15	5.66	1H, *t* of septets J = 7.6, *ca* 1†	H-16
3.87	3.92	3.17	3H, *s*	3.98	3.28	3H, *s*	Me O-5
3.33	3.58	3.50	2H, *d*, J = 6.7	3.35	3.78	2H, *d*, J = 7.6	H-15
1.68	1.65	1.65	3H, *d*, J = 1.3†	1.77	1.73	3H, *d*, J = 1.3†	H-(18, 19)
1.56	1.56	1.54	3H, *d*, J = 1.3†	1.64	1.90	3H, *d*, J = 1.3†	H-(18, 19)

*Signal in C_6H_5 resonance band.
†Observed with resolution enhancement.



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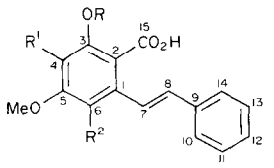
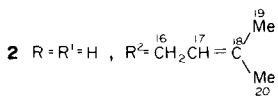


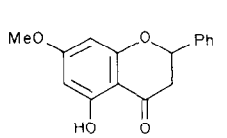


2 R = R¹ = H, R² = $CH_2CH=C(Me)_2$

3 R = R² = H, R¹ = $CH_2CH=CMe_2$

5 R = Me, R¹ = H, R² = $CH_2CH=CMe_2$

6 R = Me, R² = H, R¹ = $CH_2CH=CMe_2$



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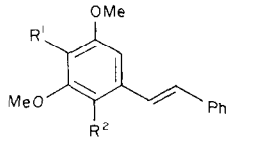

7 R¹ = H, R² = $CH_2CH=CMe_2$

8 R¹ = $CH_2CH=CMe_2$, R² = H

305 $[M-33]^+$, 294 $[M-44]^+$, 279 $[M-59]^+$ and 239 $[M-99]^+$. The electronic spectrum had absorption maxima at 313 nm (log ϵ 4.37) and 254 nm (log ϵ 4.40). The ^{1}H NMR spectrum is shown in Table 1. Again the presence of an isoprenyl group is apparent and this is also in accord with the mass spectral fragmentation pattern. The low field hydroxyl signals again indicates that the hydroxyl and carboxyl groups are adjacent. In the NOE difference spectra, irradiation at the position of the methoxyl signal caused enhancement of the H-6 proton signal, while on irradiation at the position of the H-6 proton both the methoxyl and the olefinic proton signals are enhanced. On changing to deuteriobenzene as solvent, the H-6 and methoxyl protons are shielded and the methylene group of the isoprenyl side chain is deshielded, the shifts to H-6 and the methylene group being greater than the comparable shifts in **2**. Methylation of **3** with diazomethane gave **6**, recrystallized from petroleum (bp 60–80°), mp 95–97°. The mass spectrum showed *m/z* M^+ 352.1667 ($C_{22}H_{24}O_4$ requires 352.1673), and major fragment ions were observed at 337 $[M-15]^+$, 321 $[M-31]^+$, 320 $[M-32]^+$, 306 $[M-46]^+$, 305 $[M-47]^+$, 278 $[M-74]^+$, 277 $[M-75]^+$ and 265 $[M-87]^+$. The ^{1}H NMR spectrum showed a new signal at δ 3.85 and the carboxyl proton signal had narrowed. Decarboxylation of **6** gave **8**, which was identical in all observed respects with the data reported for the methylated derivative of longistylene A [7, 9]. The mass spectral fragmentation pattern of **3** also closely resembled that of longistylene A and all of these results are in accord with the assigned structure for **3**.

DISCUSSION

Pinostrobin chalcone (**1**) has been described from the fern *Onychium auratum* [6] and from other sources [4, 5], but it has not previously been recognized as a phytoalexin. The corresponding flavanone **4** is widely distributed in *Pinus* spp. [4]. Stilbene phytoalexins have been identified in several legume species [12–15] as well as in members of the Vitacae [16], Pinaceae [17] and Malvaceae [18], but this appears to be the first report of a stilbene-2-carboxylic acid phytoalexin. Stilbene-2-carboxylic acids are rare in plants, and only two such compounds appear to be known, hydrangeic acid and gaylussacin [19].

Chalcones and stilbenes are synthesized by the shikimic and polymalonic acid route but diverge in the manner in which the acetate groups from malonyl CoA cyclize [20]. Pigeon pea is unusual in that both types of cyclization appear to occur and that the stilbene 2-carboxylic acids so formed are not decarboxylated.

Isoprenylation is frequently found in phytoalexins from the Leguminosae and in some instances has been shown to be essential for antifungal activity [21]. In the leaf phytoalexins of pigeon pea, substitution occurs at either of the two unsubstituted positions on the activated phenyl ring, whereas in *Hardenbergia violacea* the two phytoalexins (licoisoflavone A and luteone) [22] are substituted at activated positions on different rings. Substitution at both activated positions in pigeon pea may be precluded by steric factors or the resulting compound may have no antifungal activity and would consequently have escaped attention in this study. All three compounds isolated had the thermodynamically favoured *trans*-configuration and none of the *cis*-compounds were observed. In earlier reports, both isomers [12, 14] or only the *trans*-isomers [15, 23] have been observed when stilbenes were isolated from other sources.

EXPERIMENTAL

Elicitation and extraction of phytoalexins. Pigeon pea seeds (cv Prabhat) were planted in John Innes No. 1 compost and grown in the greenhouse until they were 5–6 weeks old. Leaves were detached and wounded with a hypodermic needle in 20 intercostal regions before applying a spore suspension (20 μl wound; 5.6×10^6 spores/ml). The inoculated leaves were incubated at high humidity for 48 hr in the dark at 25°. After incubation they were extracted with 95% EtOH by the facilitated diffusion technique [24]. The solvent was removed under red. pres. below 40° and the residue dissolved in MeCN–H_2O–HOAc (65:35:1).

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 310 nm and a Tehman potentiometric chart recorder. Active fractions, recognized initially by bioassay on TLC with *C. cucumerinum* [3] and subsequently by retention time and absorption of light at 310 nm, were collected and dried.

The fraction containing **1** was crystallized from $CHCl_3$, and those containing **2** and **3** were crystallized from aq. MeOH.

Methylation of 2 and 3. Excess CH_2N_2 was distilled in Et_2O into a soln of the compound in Et_2O and the mixture was allowed to stand in the dark for 24 hr. The Et_2O was removed in a stream of N_2 and the residue crystallized from petrol (bp 60–80°), 2% Et_2O.

Decarboxylation. The dimethoxy compounds were mixed with Cu powder and quinoline (3 × wt of reactant) and heated at 190–210° for 1.5 hr. The cooled product was dissolved in Et_2O (5 ml), the Et_2O soln filtered, the filtrate washed with 1 M HCl (2 ml (2 ml), 10% Na_2CO_3 (2 ml), dried ($MgSO_4$) and the solvent removed to give the product.

^{1}H *NMR spectra.* Spectra were obtained on a Varian XL-200 spectrometer operating in the FT mode using Me_4Si as int. 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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