Antifungal Phytoalexins in *Phaseolus aureus* Roxb.

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Eight phytoalexins have been isolated from *Phaseolus aureus* (mung bean) seedlings after treatment with aqueous $CuCl_2$. These compounds include three isoflavones: genistein, 2′-hydroxygenistein and a compound tentatively identified as 2,3-dehydrokievitone, four isoflavanones: dalbergioidin, kievitone, cyclokievitone and 5-deoxykievitone, and the pterocarpan phaseollidin. The antifungal activities against *Cladosporium cucumerinum* were determined.

Introduction

The phytoalexins of the French bean, *Phaseolus vulgaris*, have received thorough investigation and over 20 such compounds have been isolated and characterised as isoflavonoids [1]. In contrast comparatively few studies [2] have been undertaken on the phytoalexins produced by other members of the *Phaseolus* genus despite their considerable economic importance. The mung bean, *P. aureus* Roxb., is an essential human food crop especially in S. E. Asia. To date only three isoflavonoids have been isolated from this plant: kievitone (**1**), phaseollidin (**8**) [2] and coumestrol (**9**) [3]. The present investigation reports the isolation of eight phytoalexins from *P. aureus* seedlings after treatment with $CuCl_2$.

Results and Discussion

EtOH extracts from control and $CuCl_2$-treated *P. aureus* seedlings were subjected to an antifungal TLC bioassay [4] using *Cladosporium cucumerinum*. The extract from the treated seedlings produced four inhibitory zones (Z1 R_f 0.78, Z2 R_f 0.65, Z3 R_f 0.50, Z4 R_f 0.45) whereas the control extract showed a single minor fungitoxic zone (R_f 0.50). Subsequent purification of the components of the zones yielded 8 isoflavonoids, seven of which were identified as kievitone (**1**) (360 µg/g fr. wt.), dalbergioidin (**2**) (8 µg/g fr. wt.), 5-deoxykievitone (**3**) (13 µg/g fr. wt.), genistein (**4**) (57 µg/g fr. wt.), 2′-hydroxygenistein (**5**) (16 µg/g fr. wt.), cyclokievitone (**7**) (12 µg/g fr. wt.) and phaseollidin (**8**) (20 µg/g fr. wt.) by a comparison of their UV, MS and PMR characteristics with literature values [5–11]. Because of low yields, the identification of the eighth isoflavonoid to be isolated is tentative. On the basis of its UV and MS characteristics the compound is a 2′,4′,5,7-tetrahydroxy-isoflavone which is prenylated at either position 6 or 8. Both luteone (**12**) and 2,3-dehydrokievitone (**6**), the 6 or 8-prenylated 2′,4′,5,7-tetrahydroxyisoflavones respectively, are known in nature [2]. The prenylated isoflavone from *P. aureus* resembled 2,3-dehydrokievitone rather than luteone on TLC [5] and on this basis the former structure (**6**) is preferred.

The fungitoxic activities of kievitone (**1**), dalbergioidin (**2**), genistein (**4**), 2′-hydroxygenistein (**5**) and phaseollidin (**8**) are well documented against several plant pathogens [2]. In contrast 5-deoxykievitone (**3**), cyclokievitone (**7**) and 2,3-dehydrokievitone (**6**) have been isolated only from *P. vulgaris* pod tissue after fungal inoculation [5] and no fungitoxicity data has been recorded for these compounds. Therefore the antifungal activity against the phytopathogen *Cladosporium cucumerinum* was determined for each phytoalexin isolated from *P. aureus* Roxb.

Fungal spores in liquid nutrient were incubated at 25 °C with each compound at a series of concentrations. After 3 days the mycelium was well established in the controls and at this time, the lowest concentration of phytoalexin at which no mycelial development was observed was designated the Minimum Inhibitory Concentration (MIC) in µg/ml. Results are recorded in Table I. Continued monitoring for a further 98 h indicated no mycelial development in any of the incubates containing

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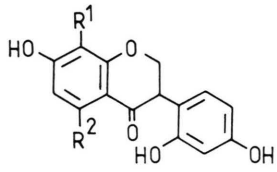

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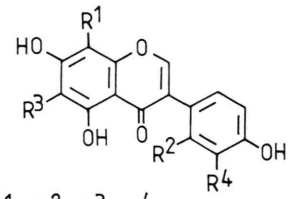


4 $R^1 = R^2 = R^3 = R^4 = H$

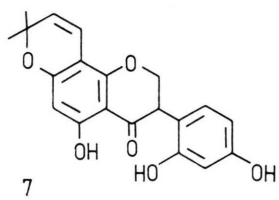
5 $R^1 = R^3 = R^4 = H$, $R^2 = OH$

6 $R^1 = CH_2CH=C(CH_3)_2$, $R^2 = OH$, $R^3 = R^4 = H$

11 $R^1 = R^2 = R^4 = H$, $R^3 = CH_2CH=C(CH_3)_2$

12 $R^1 = R^4 = H$, $R^2 = OH$, $R^3 = CH_2CH=C(CH_3)_2$

13 $R^1 = R^3 = H$, $R^2 = OH$, $R^4 = CH_2CH=C(CH_3)_2$



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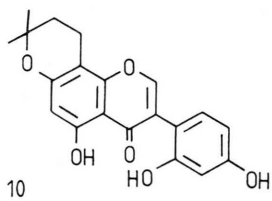

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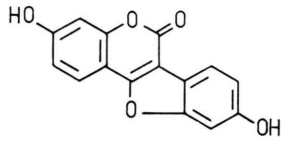

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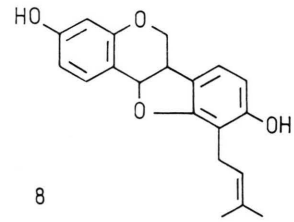

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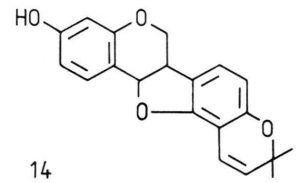

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phytoalexins at their MIC concentrations. The levels of activity of kievitone, dalbergioidin, genistein, 2′-hydroxygenistein and phaseollidin against *C. cucumerinum* are of the same orders as their activities aginst other plant pathogens [2]. It has been observed previously [13–15] that the prenylated isoflavanone kievitone and the prenylated isoflavones wighteone (**11**) and luteone (**12**) are more active than their non-prenylated analogues dalbergioidin, genistein and 2′-hydroxygenistein respectively, and it has been suggested that this reflects the greater lipid-solubility of the prenylated derivatives. Enhanced activity in prenyl derivatives has been confirmed in the present study in which kievitone (MIC 25–50 µg/ml) was considerably more inhibiting than dalbergioidin (MIC 75–100 µg/ml). We have also found that phaseoluteone (**13**) a 3′-prenylated isoflavone produced by *P. vulgaris* is more fungitoxic against *C. cucumerinum* than 2′-hydroxygenistein (unpublished work). 5-Deoxykievitone (MIC 10–25 µg/ml) was the most fungitoxic phytoalexin tested. The greater activity of this compound compared with kievitone possibly also results from its increased lipophilicity. The high activity of 5-deoxykievitone is unusual for an isoflavanone [2] and deserves further investigation against a broader spectrum of plant pathogens. Cyclokievitone was active at 100 µg/ml which because of lack of material was the only concentration tested. The yield of 2,3-dehydrokievitone was too low for biological testing.

Woodward [5, 12] has proposed that two biosynthetic pathways leading to kievitone operate in *P. vulgaris*. One pathway would proceed from genistein to 2′-hydroxygenistein to dalbergioidin to kievitone whereas the second pathway would

Table I. Antifungal activity of *P. aureus* phytoalexins against *Cladosporium cucumerinum* spore germination.

Compound	MIC [μg/ml]
Kievitone	25– 50
Dalbergioidin	75–100
5-Deoxykievitone	10– 25
Genistein	50– 75
2′-Hydroxygenistein	75–100
Cyclokievitone	≦ 100
Phaseollidin	50– 75

diverge at 2′-hydroxygenistein by prenylation to produce 2,3-dehydrokievitone. It is conceivable that both of these alternative pathways also operate in *P. aureus*. It is interesting to note that whereas in *P. vulgaris* pod tissue following fungal inoculation, the level of dalbergioidin far exceeds the level of 2,3-dehydrokievitone (Ratio approx. 6 to 1) [5, 12], in *P. aureus* seedlings following $CuCl_2$ treatment, the levels of these two phytoalexins are very similar. This difference may reflect a preference for one of the pathways in *P. vulgaris*. Cyclokievitone is presumably a metabolite of kievitone in both *P. vulgaris* and *P. aureus*. Its synthesis in *P. vulgaris* pods following fungal inoculation could be controlled by either host or pathogen enzymes. Since it occurs in *P. aureus* seedlings after $CuCl_2$ treatment its production is likely to result from host metabolism.

The taxonomy of the Leguminosae is extremely complicated! According to Maréchal *et al.* [16] *P. aureus* Roxb. is a synonym for *Vigna radiata* var. *radiata* L. Ingham [17] has reported that *V. radiata* produces phaseollin (**14**) as a phytoalexin although in the present study this compound could not be detected in *P. aureus* Roxb. It seems likely that our plants were different from those used by Ingham. Phaseollin is a major phytoalexin of *P. vulgaris* following both biotic [6, 8, 18] and abiotic [19] induction, and the compound has also been reported to occur as a phytoalexin in *P. leucanthus* and *P. lunatus* [17]. Thus *P. aureus* Roxb. is the only *Phaseolus* species studied to date which does not produce phaseollin as a major phytoalexin.

Experimental

Plant material and induction of phytoalexin synthesis

Seeds of *P. aureus* Roxb. (obtained from Thomson and Morgan Ltd.) were surface sterilised by washing successively in EtOH, dilute detergent (Teepol) and 0.5% NaClO solution. The seeds were allowed to germinate in running H_2O at approx. 20 °C and were transferred to moist sterilised vermiculite when radicles were about 1 cm long. The seedlings were grown on in darkness at 25 °C for 11 days. Phytoalexin synthesis was induced in test seedlings by immersing the roots in aq. $CuCl_2$ (3×10^{-3} M) for 19 h. The roots were then rinsed and immersed in H_2O at 25 °C for a further 4 days. Control seedlings had their roots immersed in H_2O throughout.

Extraction and purification of phytoalexins

Induced and control seedlings were macerated in cold EtOH repeatedly until all pigment was extracted. The alcoholic extracts were pooled, evaporated to dryness in vacuo and the residue taken up in H_2O and extracted with EtOAc (X4). The combined organic fractions were concentrated in vacuo and aliquots of the induced and control extracts were chromatographed on Si gel GF_{254} TLC plates in Hexane–EtOAc–MeOH (6–4–1). Developed chromatograms were air-dried to remove traces of solvent and then treated in one of two ways. To detect antifungal compounds the chromatogram was sprayed with a dense spore suspension (ca. 10^6 spores/ml) of *Cladosporium cucumerinum* in a liquid nutrient medium (Czapek Dox). Chromatograms were then incubated for 3–5 days at 25 °C in darkness under moist conditions. To detect induced phenolic compounds, other developed chromatograms were sprayed with Stahl's Fast Blue B Salt reagent [20]. Intense purple or scarlet colours were observed on these plates at R_f values equivalent to those of the inhibitory zones on the spore-sprayed plates. It was therefore possible to use the Fast Blue B salt reagent in the subsequent purification of components of the inhibitory zones by TLC in the following solvent systems. Z1 in Hexane–Me_2CO (2–1) then $CHCl_3$–MeOH (25–1) yielded (**8**); Z2 in Hexane–Me_2CO (2–1) then $CHCl_3$–MeOH (25–1) yielded (**4, 6, 7**); Z3 in $CHCl_3$-propan-2-ol (9–1) then $CHCl_3$–MeOH (8–2) yielded (**1, 3**); Z4 in $CHCl_3$-propan-2-ol (9–1) then $CHCl_3$–MeOH (8–2) yielded (**2, 5**). The minor fungitoxic zone in the control extract contained only kievitone.

Kievitone (**1**) yield 360 μg/g fr.wt. UV MeOH λ_{max} nm: 210, 293, 340; MS *m/e* (rel. intens.):

356 (88) M^+, 338 (12), 221 (60), 205 (36), 192 (35), 177 (39), 165 (100), 136 (39); PMR 250 MHz $(CD_3)_2$ CO: δ6.96 (1 H, d, J = 8.3 Hz, C-6′), 86.45 (1 H, d, J = 2.3 Hz, C-3′), 86.34 (1 H, d, d, J = 8.3 Hz, 2.3 Hz, C-5′), δ6.04 (1 H, s, C-6), δ5.21 (1 H, br. t., J = 7.1 Hz, C-2″), δ4.67–δ4.04 (3 H, m, C-2a, 2b, 3), δ3.24 (2 H, d, J = 6.9 Hz, C-1″), δ1.74 (3 H, s, Me), δ1.64 (3 H, s, Me).

Dalbergioidin (**2**) yield 8 µg/g fr. wt. UV MeOH λ_{max} nm: 201, 227 sh, 289; MS *m/e* (rel. intens.): 288 (66) M^+, 153 (100), 136 (75); PMR 250 MHz $(CD_3)_2CO$: 86.94 (1 H, d, J = 8.6 Hz, C-6′), δ6.45 (1 H, d, J = 2.4 Hz, C-3′), δ6.34 (1 H, d, d, J = 8.3 Hz, 2.7 Hz, C-5′), δ5.96 (1 H, s, C-6?), δ5.95 (1 H, s, C-8?), δ4.66–δ4.05 (3 H, m, C-2a, 2b, 3).

5-Deoxykievitone (**3**) yield 13 µg/g fr. wt. UV MeOH λ_{max} nm: 201, 223 sh, 287; MS *m/e* (rel. intens.): 340 (52) M^+, 205 (84) 203 (16), 176 (32), 161 (32) 149 (100), 136 (50); PMR 400 MHz $CDCl_3$: δ7.72 (1 H, d, J = 8.6 Hz, C-5), δ7.38 (1 H, d, J = 8.4 Hz, C-6′), δ6.51 (1 H, J = 2.4 Hz, C-3′), δ6.39 (1 H, d, d, J = 8.4 Hz, 2.4 Hz, C-5′), δ5.26 (1 H br. t., $J \cong$ 7.2 Hz, C-2″), δ5.01 (1 H, d, d, $J \cong$ 12.0 Hz, 3.9 Hz, C-2a), δ4.81 (1 H, d, d, $J \cong$ 12.0 Hz, 4.8 Hz, C-3), δ3.91 (1 H, t, $J \cong$ 3.6 Hz, C-3), δ3.45 (1 H, br. d, $J \cong$ 7.4 Hz, C-1″), δ1.86 (3 H, s, Me), δ1.78 (3 H, s, Me).

Genistein (**4**) yield 57 µ g/g fr. wt. UV MeOH λ_{max} nm: 208, 262, 330 sh; MS *m/e* (rel. intens.): 270 (100) M^+, 268 (39), 153 (57), 12 (30), 124 (17), 118 (26); PMR 400 MHz $(CD_3)_2CO$: δ8.17 (1 H, s, C-2). δ7.46 (2 H, d, d, J = 8.8 Hz, 2.3 Hz, C-2′, C-6′), δ6.91 (2 H, d, d, J = 8.8 Hz, 2.3 Hz, C-3′, C-5′), δ6.42 (1 H, d, J = 2.3 Hz, C-8), δ6.29 (1 H, d, J = 2.3 Hz, C-6).

2′-Hydroxygenistein (**5**) yield 16 µg/g fr. wt. UV MeOH λ_{max} nm: 203, 262, 288 sh; MS *m/e* (rel. intens.): 286 (100) M^+, 285 (17), 269 (31), 153 (77), 134 (46); PMR 250 MHz $(CD_3)_2CO$: δ8.17 (1 H, s, C-2), δ7.11 (1 H, d, J = 8.5 Hz, C-6′), δ6.47 (1 H, d, J = 2.5 Hz, C-3′), δ6.45 (1 H, d, J = 2.0 Hz, C-8), δ6.43 (1 H, d, d, J = 8.5 Hz, 2.5 Hz, C-5′), δ6.30 (1 H, d, J = 2.0 Hz, C-6).

2,3-Dehydrokievitone (**6**) yield 16 µg/g fr. wt. UV MeOH λ_{max} nm: 264, 287 sh, 335 sh; MS *m/e* (rel. intens.): 354 (70) M^+, 353 (5), 339 (20), 337 (15), 311 (100), 299 (65), 298 (10), 219 (15), 205 (25), 177 (35), 165 (65), 161 (15), 153 (55), 135 (20), 134 (50). Treatment of **6** with glacial HOAc/25%/ H_2SO_4aq produced a single isomer, possibly cyclo-2,3-dehydrokievitone (**10**) UV MeOH λ_{max} nm: 265 nm.

Cyclokievitone (**7**) yield 12 µg/g fr. wt. UV MeOH λ_{max} nm: 270, 295 sh, 355; MS *m/e* (rel. intens.): 354 (46) M^+, 339 (100), 321 (68), 217 (45), 203 (65); PMR 250 MHz $(CD_3)_2CO$: δ6.94 (1 H, d, J = 8.3 Hz, C-6′), δ6.53 (1 H, d, J = 9.6 Hz, C-1″), δ6.45 (1 H, d, J = 2.3 Hz, C-3′), δ6.37 (1 H, d, d, J = 8.4 Hz, 2.3 Hz, C-5′), δ5.89 (1 H, s, C-6, δ5.62 (1 H, d, J = 10.1 Hz, C-2″), δ4.68 (1 H, t, J = 11.0 Hz, C-2a), δ4.55 (1 H, d, d, J = 11.1 Hz, 6.2 Hz, C-2b, δ4.29 (1 H, d, d, J = 11.3 Hz, 6.3 Hz, C-3), δ1.44 (3 H, s, Me), δ1.43 (3 H, s, Me).

Phaseollidin (**8**) yield 20 µg/g fr. wt. UV MeOH λ_{max} nm: 214, 230 sh, 281, 287; MS *m/e* (rel. intens.): 324 (100) M^+, 323 (10), 309 (15), 269 (40), 268 (85), 267 (37); PMR 250 MHz $CDCl_3$: δ7.41 (1 H, d, J = 8.5 Hz, C-1), δ6.95 (1 H, d, J = 8.0 Hz, C-7), δ6.56 (1 H, d, d, J = 8.3 Hz, 2.3 Hz, C-2), δ6.42 (1 H, d, J = 2.3 Hz, C-4), δ6.38 (1 H, d, J = 8.3 Hz, C-8), δ5.45 (1 H, br. d., J = 6.4 Hz, C-11a), δ5.35 (1 H, br. t., J = 7.5 Hz, C-13), δ4.23 (1 H, m, C-6 eq.), δ3.64 (1 H, m, C-6ax), δ3.52 (1 H, m, C-6a), δ3.36 (2 H, d, J = 7.5 Hz, C-12), δ1.80 (3 H, s, Me), δ1.74 (3 H, s, Me).

Determination of fungitoxicity

0.2% solutions of each phytoalexin in EtOH were prepared and aliquots were dispensed into multi-well assay trays so as to give quantities of 0, 10 µg, 25 µg, 50 µg, 75 µg and 100 µg of the phytoalexin per well. The volume in each well was brought to 50 µL by the addition of further EtOH and then 950 µL of a spore suspension containing *C. cucumerinum* (ca. 2×10^6 spores/ml) in Czapek Dox liquid nutrient was added to each well. Each compound except cyclokievitone was tested at each concentration in quadruplicate. The assay trays were then incubated at 25 °C in the dark and 3 days later the MIC was determined for each compound.

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