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A NEW ISOFLAVONE PHYTOALEXIN FROM FUNGUS-INOCULATED STEMS OF *GLYCINE WIGHTII*

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Abstract—6-Isopentenylgenistein **1** (wighteone) has been isolated as a phytoalexin from the fungus-inoculated or $CuCl_2$-treated stems of *Glycine wightii*. Plants treated with aqueous $CuCl_2$, but not those inoculated with the fungus, also accumulated genistein (**2**) and its 2′-hydroxy derivative (**3**).

INTRODUCTION

Recent evidence suggests that phytoalexins might provide useful chemotaxonomic information if applied to genera of certain plant families including the Leguminosae [1–3] and Solanaceae [4]. In view of these reports we have investigated the phytoalexins characteristically produced by members of the soybean genus (*Glycine* Willd.) which contains the taxonomically controversial species, *G. wightii* (Wight & Arn.) Verdc. [5]. The cultivated soybean, *G. max* (L.) Merr., is known to accumulate 3 isomeric 6a-hydroxylated pterocarpans [6, 7] now termed glyceollin 1, 2 and 3. We report here that *G. wightii* does not produce pterocarpan phytoalexins after fungal inoculation or treatment with aqueous $CuCl_2$; instead, this species produces 3 isoflavone derivatives, one of which is identical to an unidentified compound (LA-1) previously isolated from *Lupinus albus* L. [8, 9].

RESULTS AND DISCUSSION

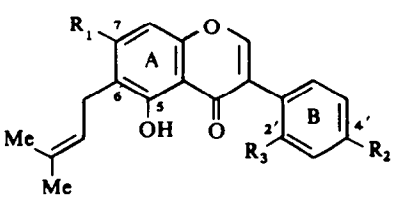
As determined by TLC bioassay [10], extracts of the fungus-inoculated stems of *G. wightii* (P.I. 275.716) contained a single antifungal compound (**1**); only traces of this substance were present in extracts of wounded but non-inoculated stems and in the tissues of plants treated with water (Table 1). Under UV light the above antifungal zone was evident as a deep purple–black fluorescent band. No other inhibitory compounds were isolated from the inoculated plants. However, exposure to aqueous $CuCl_2$ stimulated the formation of two additional deep purple fluorescent compounds (**2** and **3**) both of which were essentially absent from water-treated cuttings and fungus-inoculated stems. Compounds **2** and **3** did not inhibit the development of *Cladosporium cucumerinum* when tested by TLC bioassay [10].

Compound **2** was identified as genistein (5,7,4′-trihydroxyisoflavone) by MS, UV and TLC comparison with authentic material. Similarly, **3** gave spectral and chromatographic data indistinguishable from those of 2′-hydroxygenistein (5,7,2′,4′-tetrahydroxyisoflavone), a phytoalexin recently obtained from the grain legumes *Phaseolus vulgaris* [11] and *Cajanus cajan* [12].

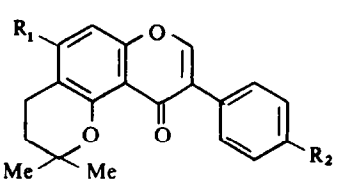
The UV and IR spectra of **1** were also consistent with its identification as a hydroxylated isoflavone. A significant MS loss of isobutene indicated that **1** (M^+ 338) possessed either an isopentenyl or dimethylchroman substituent [13]. The aromatic ring attachments (*ring A*, alkyl + two OH groups; *ring B*, single OH group) were apparent from the characteristic isoflavone-like fragmentation pattern [14, 15] which afforded ions at *m/e* 165 (A ring) and 118 (B ring). UV shifts with NaOAc and $AlCl_3$ located the A ring OH groups at C-7 and C-5 respectively [16].

Compound **1** formed a diacetate and a triacetate derivative but only a phenolic diMe ether (**4**) since the C-5 OH is presumably strongly chelated with the carbonyl group; as **4** gave a blue–grey colouration with Gibbs reagent [3, 17] the isopentenyl unit can be assigned to C-6 rather than C-8 as in the isoflavanone phytoalexin, kievitone [18]. This view was supported by acidic cyclisation of **4** to yield the expected non-phenolic chroman derivative **6**. Cyclisation of **1** afforded *ca* equivalent amounts of two phenolic compounds identified by MS and UV as the chroman derivatives **7** (α-isomer) and **8** (β-isomer). Formation of **7** and **8** unequivocally locates the A ring isopentenyl group of **1** at C-6.

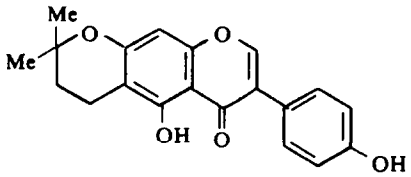
The B-ring OH group of **1** was initially assigned to



1: $R_1 = R_2 = OH$; $R_3 = H$
4: $R_1 = R_2 = OMe$; $R_3 = H$
5: $R_1 = R_2 = R_3 = OH$



6: $R_1 = R_2 = OMe$
7: $R_1 = R_2 = OH$



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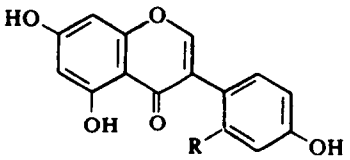
C-2′ on the basis of an observed but weak MS ion corresponding to $M^+ - 17$ [12]. However, H_2O_2 oxidation of the diMe ether (4) gave a product chromatographically inseparable from *p*-methoxybenzoic acid; formation of this compound allows the B ring oxygen function to be placed at C-4′, a feature biogenetically consistent with the production of 2 and 3 by *G. wightii*. The MS of numerous 2′-methoxylated isoflavones have



2: R = H
3: R = OH

been reported to exhibit intense ions at $M^+ - 31$ [12, 19–21]. However, for both the diMe ether (4) and its chroman derivative (6), ions at $M^+ - 31$ were either not observed or were of extremely low intensity (see Experimental). Weak $M^+ - 31$ ions associated with the MS of isoflavones lacking 2′-methoxylation have been reported on several occasions [19, 22]. From the above data 1 can be formulated as 5,7,4′-trihydroxy-6-isopentenylisoflavone for which the common name *wighteone* is proposed. Wighteone is identical to an antifungal compound (LA-1) previously obtained from the healthy leaves and fungus-inoculated hypocotyls of white lupin, *Lupinus albus* [8, 9].

Quantitative measurement of the isoflavones from *G. wightii* (Table 1) confirmed that whereas inoculation with *Phytophthora megasperma* Drechs. var. *sojae* Hildb. led to a marked accumulation of 1, compounds 2 and 3 were either absent or present in only trace amounts. In contrast, cuttings treated with aqueous $CuCl_2$ produced wighteone together with significant quantities of genistein and 2′-hydroxygenistein. To determine if production of 1, 2 and 3 was a characteristic feature of *G. wightii*, 7 accessions of varied geographical origin (see *Experimental*) were extracted following fungal inoculation or treatment with $CuCl_2$. All produced 1, 2 and 3 in response to $CuCl_2$ but only 1 in response to *P. megasperma* var. *sojae*. These data (and similar results for *G. max* [23] and *Phaseolus lunatus* [24]) are consistent with the hypothesis [23] that certain plants capable of a multiple phytoalexin defence response may preferentially accumulate those compounds which are most inhibitory to a particular invading organism whilst at the same time exhibiting a less specific response to abiotic elicitors such as $CuCl_2$.

Table 1. Accumulation of isoflavones in tissues of *G. wightii* (P.I. 275.761) following inoculation with *P. megasperma* var. *sojae* or treatment with aqueous $CuCl_2$*

Compound	Fungus inoculated		$CuCl_2$ treated	
	Control (wounded only)	Inoculated	Control (H_2O)	Treated
Wighteone (1)	14	900	18	400
Genistein (2)	<5	<5	<5	560
2′-Hydroxygenistein (3)	<5	<5	<5	340

* All concentrations in μg/g fr. tissue. Values for wighteone are based on log ε = 4.45 at 266 nm for luteone (5) [25]; values for genistein and 2′-hydroxygenistein are based on log ε = 4.63 at 262 nm for 2 [12].

Although no other antifungal compounds were isolated from *G. wightii* P.I. 275.761, 230.325 or 224.980, a minor inhibitory fraction that migrated with genistein (in *n*-hexane–EtOAc–MeOH, 60:40:1) was detected in extracts of the other $CuCl_2$ or fungus-treated accessions. This unidentified substance was chromatographically distinct from the prenylated isoflavone, luteone (5) [8, 25]. Although wighteone may well be derived from genistein, there was no evidence to suggest that 2′-hydroxy genistein was similarly converted to luteone by *G. wightii*.

In the TLC bioassay, wighteone (detection minimum 1 to 3 μg) exhibited high antifungal activity against *Cladosporium cucumerinum*. In contrast, neither genistein nor 2′-hydroxygenistein were active even at an applied level of 200 μg. Although genistein has been shown to have antifungal properties [26, 27], the present study suggests that it is much less active than the 6-isopentenyl analogue, a feature which presumably reflects the greater lipid solubility of the latter compound. Luteone (the 6-isopentenyl derivative of 2′-hydroxygenistein) is also highly fungitoxic [8, 25].

There is considerable taxonomic evidence to indicate that *G. wightii* should be removed from the genus *Glycine* [28]; indeed, Lackey [29] has recently proposed that *G. wightii* should be renamed *Neonotonia wightii* nov. sp. Data from the present study are consistent with this conclusion since *G. wightii* produces large quantities of the isoflavone phytoalexin, wighteone (1) whereas the cultivated soybean (*G. max*) accumulates antifungal pterocarpan derivatives [6]. Furthermore, our unpublished observations have shown that 5 additional *Glycine* species (whose continued inclusion in the genus *Glycine* is supported by other evidence) [28] also produce pterocarpan analogues although these are structurally distinct from glyceollin, the phytoalexin of *G. max*.

EXPERIMENTAL

Plant material. Seeds of *Glycine wightii* (P.I. 275.761) (originally collected in Brazil) were grown as previously described [7].

Compounds 1–3 were isolated principally from this accession; the same isoflavones were also obtained from *G. wightii* P.I. 230.325 (South Africa), 224.980 (Rhodesia), 339.699 (Australia), 259.545 (Brazil) and 230.323 (Tanzania) and from an unnumbered Kenyan acquisition (our code: No. 0015). Seeds of *Lupinus albus* (supplied by H. Offutt, University of Arkansas or available from previous work [8]) were grown as described for *G. wightii*.

Isoflavone induction, isolation and purification. Stems (*G. wightii*) or hypocotyls (*L. albus*) were wound-inoculated with mycelium of the fungus *Phytophthora megasperma* var. *sojae* and incubated for 48–72 hr [7]; alternatively, cuttings of *G. wightii* were placed in H_2O (control) or 1 mM $CuCl_2$ (abiotic induction) for 4–5 days. After incubation, fungus-inoculated tissues were excised and extracted with 80% EtOH. These extracts were filtered by suction, conc (*in vacuo*, 40°) and then partitioned (×3) against equal vols of EtOAc. The organic fraction was either chromatographed directly (TLC, Si gel GF_{254}, layer thickness, 0.375 mm) or the isoflavones extracted into aq. K_2CO_3 (5%). After acidification (to *ca* pH 4 with 4N HCl) the isoflavones were re-extracted with EtOAc prior to TLC in *n*-hexane–EtOAc–MeOH (60:40:1). Entire control or $CuCl_2$-treated cuttings were also extracted as described above. 3 major phenolic compounds (**1**, R_f 0.35; **2**, R_f 0.26 and **3**, R_f 0.17) were obtained from cuttings of *G wightii* treated with aq. $CuCl_2$. All were eluted (Me_2CO) and rechromatographed in $CHCl_3$–Me_2CO–18 M NH_4OH (50:50:1) to afford **1** (R_f 0.55), **2** (R_f 0.15) and **3** (R_f 0.05). Prior to UV and MS examination all 3 compounds were purified as follows: **1**, $CHCl_3$–MeOH (100:4) (R_f 0.37), **2** and **3**, *n*-pentane–Et_2O–MeOH–HOAc (75:25:6:6) (R_f 0.28 and 0.17 resp). Only traces of compounds 1–3 were obtained from H_2O-treated cuttings; fungus-inoculated tissues contained **1** but very little **2** or **3**.

5,7,4'-Trihydroxy-6-isopentenylisoflavone **1** (*wighteone*). Diazotised *p*-nitroaniline, orange–yellow; Gibbs reagent, blue. λ_{max}^{EtOH} 215, 268 nm; NaOH 277 nm; NaOAc 270 nm; $AlCl_3$ 271 nm; IR ($CHCl_3$) cm^{-1} 1730 (C=O), 3450 (OH); MS [30] *m/e* (rel. int.) 339 (10), 338 (M^+; 55; $C_{20}H_{18}O_5$), 337 (6), 323 (15; $C_{19}H_{15}O_5$), 321 (16), 296 (18; $C_{16}C^{13}H_{11}O_5$), 295 (67; $C_{17}H_{11}O_5$), 284 (16; $C_{15}C^{13}H_{11}O_3$), 283 (100; $C_{16}H_{11}O_5$), 165 (7), 118 (6; C_8H_6O). *DiMe ether* **4** (Me_2SO_4–K_2CO_3–Me_2CO) (R_f 0.35, CCl_4–$CHCl_3$, 4:1). Diazotised *p*-nitroaniline, orange–brown; Gibbs reagent, blue–grey. λ_{max}^{EtOH} 268 nm; NaOH 272 nm; $AlCl_3$ 270 nm; MS [30] *m/e* (rel. int.) 367 (11), 366 (M^+; 48; $C_{22}H_{22}O_5$), 351 (18; $C_{21}H_{19}O_5$), 335 (4; $C_{21}H_{19}O_4$) 324 (23; $C_{18}C^{13}H_{15}O_5$), 323 (100; $C_{19}H_{15}O_5$), 312 (17; $C_{17}C^{13}H_{15}O_5$), 311 (93; $C_{18}H_{15}O_5$), 281 (9), 132 (7; C_9H_8O). *7,4'-Diacetoxy derivative* (Py–Ac_2O) (R_f 0.36, $CHCl_3$). λ_{max}^{EtOH} 212, 258, 333 nm; MS *m/e* (rel. int.) 423 (8), 422 (M^+; 27), 381 (10), 380 (41), 379 (23), 367 (8), 365 (7), 363 (5), 339 (5), 338 (25), other fragments as given for **1**. *Triacetate* (R_f 0.30, *n*-hexane–EtOAc–MeOH, 60:40:1) λ_{max}^{EtOH} 212, 258, 333 nm; MS *m/e* (rel. int.) 465 (1), 464 (M^+; 4), 422 (34), 380 (89), 338 (57), other fragments as given for **1**.

5,7,4'-Trihydroxyisoflavone **2** (*genistein*). Diazotised *p*-nitroaniline, orange–yellow; Gibbs reagent, blue; UV and MS as lit. [11, 12, 16]. *7,4'-Diacetoxy derivative* (R_f 0.57, $CHCl_3$). λ_{max}^{EtOH} 208, 255, 327 nm; MS *m/e* (rel. int.) 355 (6), 354 (M^+; 23), 313 (13), 312 (45), 271 (14), 270 (100), other fragments as lit. [11, 12].

5,7,2',4'-Tetrahydroxyisoflavone **3** (*2'-hydroxygenistein*). Diazotised *p*-nitroaniline, orange–yellow; Gibbs reagent, blue; UV and MS as lit. [11, 12]. *Tetraacetate* (R_f 0.15, *n*-hexane–EtOH–MeOH, 60:40:1). λ_{max}^{EtOH} 243, 300 nm; MS *m/e* (rel. int.) 455 (2), 454 (M^+; 6), 412 (9), 370 (14), 354 (12), 352 (14), 328 (50), 312 (15), 310 (10), 286 (48), 153 (100), other fragments as lit. [11, 12].

Peroxide oxidation of **4**. H_2O_2 (30%) was added dropwise in 4 portions (0.2 ml, 0.1 ml, 0.1 ml and 0.2 ml at intervals of 1 hr) to a stirred soln of **4** (*ca* 2 mg) in EtOH (2 ml) and aq. KOH (15%; 0.5 ml) at room temp. The soln was stirred 18 hr at room temp., acidified (2N HCl) and extracted (×4) with equal vols of EtOAc. The EtOAc fractions were combined and reduced to dryness (*in vacuo*, 40°) and the residue chromatographed in *n*-pentane–Et_2O–HOAc (75:25:3) to afford small quantities of a product (R_f 0.84) chromatographically indistinguishable from authentic *p*-methoxybenzoic acid (cf. *o*-methoxybenzoic acid, R_f 0.38). Both compounds also had identical R_f values when chromatographed in HOAc–$CHCl_3$ (3:1, R_f 0.90), C_6H_6–MeOH (45:2, R_f 0.14) and $CHCl_3$–MeOH (50:1, R_f 0.24); in the latter system the oxidation product of **4** readily separated from *m*-methoxybenzoic acid (R_f 0.37). Oxidation of biochanin A (5,7-dihydroxy-4'-methoxyisoflavone [3]) by the above procedure also gave *p*-methoxybenzoic acid.

Acidic cyclisation of **4**. **4** (*ca* 600 μg), HOAc (1 ml) and H_2SO_4 (25%; 1 drop) were stirred for 30 min and then allowed to stand at room temp. for 14 hr. The soln was then diluted (H_2O; 25 ml) and extracted with EtOAc (3 × 15 ml). After removal of EtOAc (*in vacuo*, 40°) the residue was chromatographed in CCl_4–$CHCl_3$ (4:1) to afford the chroman **6**, R_f 0.03. This was eluted and rechromatographed (*n*-pentane–Et_2O–HOAc, 75:25:2, R_f 0.24) prior to UV and MS.

5,6-Dimethylchroman-7,4'-dimethoxyisoflavone (**6**). Diazotised *p*-nitroaniline and Gibbs reagent, no reaction; λ_{max}^{MeOH} 210, 263, 320 (*sh*) nm; NaOH–$AlCl_3$ no change; MS *m/e* (rel. int.) 367 (19), 366 (76), 351 (10), 335 (10), 324 (5), 323 (32), 312 (23), 311 (100), 281 (15), 132 (15).

Acidic cyclisation of **1**. **1** (*ca* 1.2 mg) was treated with HOAc and H_2SO_4 as described for **4**. Work up and TLC (*n*-pentane–Et_2O–HOAc, 75:25:6) afforded two phenolic bands at R_f 0.11 (**7**) and 0.54 (**8**).

5,6-Dimethylchroman-7,4'-dihydroxyisoflavone **7** (*α-isowighteone*). Diazotised *p*-nitroaniline, yellow–orange; Gibbs reagent, no reaction; λ_{max}^{MeOH} 262 nm; NaOH 273 nm; NaOAc 268 nm; NaOAc + borate 262 nm; MS *m/e* (rel. int.) 339 (43), 338 (61), 295 (30), 284 (21), 283 (100), 282 (21).

5,4'-Dihydroxy-6,7-dimethylchromanisoflavone **8** (*β-isowighteone*). Diazotised *p*-nitroaniline, yellow–orange; Gibbs reagent, blue; λ_{max}^{MeOH} 266 nm; NaOH 279 nm; NaOAc and NaOAc + borate, no change; MS *m/e* (rel. int.) 339 (17), 338 (89), 295 (38), 284 (17), 283 (100), 282 (34).

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