The Structure of Desmocarpin, a Pterocarpan Phytoalexin from *Desmodium gangeticum*

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Ethyl acetate extracts of diffusates from the fungus-inoculated leaflets of *Desmodium gangeticum* have been found to contain six isoflavonoid phytoalexins including the isoflavones genistein and 2′-hydroxygenistein, and the isoflavanones dalbergioidin, diphysolone and kievitone. These known phytoalexins occur together with a new antifungal isoflavonoid (desmocarpin) for which the structure (−)-(6a*R*; 11a*R*)-1,9-dihydroxy-3-methoxypterocarpan is proposed.

Work undertaken by Purushothaman *et al.* [1, 2] has revealed that three 'complex' laevorotatory pterocarpans (gangetin, gangetinin and desmodin) occur constitutively in roots of the papilionate legume *Desmodium gangeticum* DC., a species used medicinally in parts of India and Nepal [3]. Apart from their presence in apparently healthy plants, however, it is now widely recognised that 'simple' and/or 'complex' pterocarpans may accumulate rapidly in the tissues of many papilionate legumes as a defense against invading fungi and bacteria. These and various other inducibly-formed isoflavonoids are commonly referred to as phytoalexins [4, 5], and we were anxious to determine if species of the hitherto unexamined genus *Desmodium* could respond to fungal invasion by producing one or more compounds of this type. We report here on the isoflavonoid phytoalexin response of *D. gangeticum*.

As in previous studies involving legume phytoalexins, the drop-diffusate technique [4, 5] was used to routinely isolate antifungal material from the excised, fungus (*Helminthosporium carbonum* Ullstrup)-inoculated leaflets of *D. gangeticum*. Si gel TLC ($CHCl_3$–MeOH, 20:1) of an ethyl acetate extract of the diffusate from fungus-treated leaflets afforded several compounds which reacted with diazotised *p*-nitroaniline reagent [6] to give predominantly yellow or orange colours, and with Gibbs reagent [7, 8] to give either blue or purple-blue products. Elution and further Si gel TLC of these phenolic substances as described in the Experimental section eventually yielded pure 5,7,4′-trihydroxyisoflavone (genistein, **1**), 5,7,2′,4′-tetrahydroxyisoflavone (2′-hydroxygenistein, **2**), 5,7,2′,4′-tetrahydroxyisoflavanone (dalbergioidin, **3**), 5,7,2′,4′-tetrahydroxy-6-(3,3-dimethylallyl)isoflavanone (diphysolone, **4**) and 5,7,2′,4′-tetrahydroxy-8-(3,3-dimethylallyl)isoflavanone (kievitone, **5**). Compounds **1**–**5** have all previously been found as phytoalexins in other species belonging to the subfamily Papilionoideae of the Leguminosae [4, 5, 9], and their identification was readily accomplished by UV and TLC comparison with authentic samples. Both **1** and **3** are also known to occur constitutively in two legumes (*Lespedeza cyrtobotrya*, **1** + **3** and *Ougeinia dalbergioides*, **3**) very closely allied to *D. gangeticum* [4].

As well as the above mentioned compounds, fungus-induced diffusates invariably contained substantial quantities of a new laevorotatory isoflavonoid (desmocarpin) which we have now identified as 1,9-dihydroxy-3-methoxypterocarpan (**6**). When bioassayed against *Cladosporium herbarum* Fr. [8] using the thin-layer plate procedure developed by Homans and Fuchs [10], desmocarpin (20–25 μg) gave a prominent inhibition zone (approx. 80 mm^2) similar in area to that afforded by comparable amounts of diphysolone or kievitone. Diffusates from control (H_2O-treated) leaflets were generally devoid of phytoalexin-like material, although they

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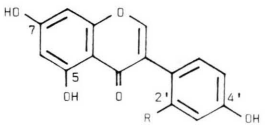
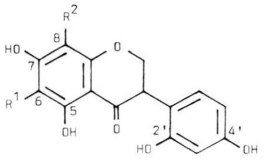
occasionally were found to contain traces (< 1 µg/ml) of a substance chromatographically indistinguishable from genistein (**1**). No evidence was obtained to indicate that the root pterocarpans gangetin, gangetinin and desmodin [1, 2] were produced as phytoalexins by the *H. carbonum*-inoculated leaflets of *D. gangeticum*.

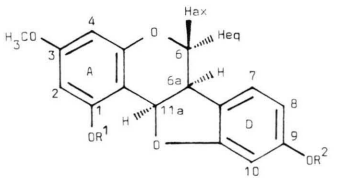
The identity of desmocarpin ($[M]^+$ 286) as a monomethoxylated pterocarpan was immediately evident from its ^{1}H NMR spectrum. This revealed a single 3H methoxyl resonance at δ 3.72, and signals at δ 5.62d, 4.21dd, 3.54t and 3.45m attributable respectively to the heterocyclic ring protons H-11a, H-6eq, H-6ax and H-6a. Virtually coincident δ



1: R = H
2: R = OH

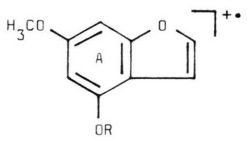
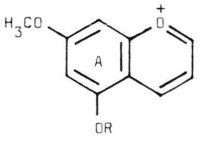
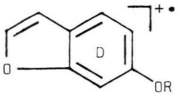
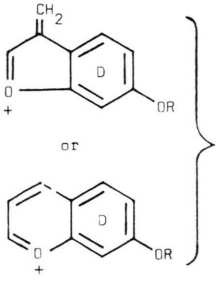


3: $R^1 = R^2 = H$
4: $R^1 = CH_2-CH=C(CH_3)_2$; $R^2 = H$
5: $R^1 = H$; $R^2 = CH_2-CH=C(CH_3)_2$



6: $R^1 = R^2 = H$
7: $R^1 = R^2 = CH_3$

Table I. Possible structures of some minor fragments observed in the mass spectrum of desmocarpin and its dimethyl ether [22, 23].

Fragment[a]	Desmocarpin (**6**)	Desmocarpin dimethyl ether (**7**)
	**a**: R = H (*m/z* 177)	**a′**: R = CH_3 (*m/z* 191)
	**b**: R = H (*m/z* 164)	**b′**: R = CH_3 (*m/z* 178)
	**c**: R = H (*m/z* 147)	**c′**: R = CH_3 (*m/z* 161)
	**d**: R = H (*m/z* 134)	**d′**: R = CH_3 (*m/z* 148)

[a] Information on the abundance of each ion fragment is given in the Experimental section.

values have also been reported for the corresponding protons of apiocarpin [11], a 1,9-dihydroxylated pterocarpan phytoalexin produced by *Apios tuberosa*. The aromatic (A/D) ring protons of **6** appeared as a pair of *meta*-coupled doublets (δ 5.98 and 6.17; $J = 2.3$ Hz; H-2 and H-4), and as an ABX system ($J = 8.0$ and 2.2 Hz), the latter being characterised by chemical shift values identical with those earlier assigned to H-7 (δ 7.13), H-8 (δ 6.36) and H-10 (δ 6.29) of apiocarpin [11].

In addition to the molecular ion (*m/z* 286) and a prominent fragment at $M^+ - 15$ (*m/z* 271), the MS of desmocarpin exhibited signals of low intensity at *m/z* 177 (**a**), 164 (**b**), 147 (**c**) and 134 (**d**) (Table I) which suggested that one of the aromatic rings (considered to be D from the preceding ^{1}H NMR chemical shift data) was monohydroxylated (fragment ions **c** and **d**), and that the other possessed both an OH and an OCH_3 substituent (fragment ions **a** and **b**). Exactly comparable minor fragments (**a′** – **d′**) at *m/z* 191/178 and *m/z* 161/148 were also present in the MS of the non-phenolic dimethyl ether ($[M]^+$ 314; **7**) resulting from treatment of **6** with diazomethane [12]. Because pterocarpans are invariably oxygenated at C-3 and C-9 [4], it follows that desmocarpin must possess a C-9 OH group (cf. apiocarpin [11]) with the remaining *meta*-related substituents residing on ring A.

The 1-hydroxy-3-methoxy oxygenation pattern assigned to **6** was preferred over the isomeric 1-methoxy-3-hydroxy arrangement found in the *Psophocarpus* phytoalexin 1-methoxyphaseollidin [13] for two reasons. First, desmocarpin gave a dark blue colour on TLC plates sprayed with Gibbs reagent [7, 8], a result which indicated the presence of an unsubstituted position *para* to the phenolic A-ring OH group. Secondly, an NOE difference experiment showed that irradiation of the methoxyl group (δ 3.72) caused enhancement of both the H-2 and H-4 signals. No other protons were affected. When considered together, the above observations allow the OCH_3 group to be unambiguously located at C-3, and thus desmocarpin is 1,9-dihydroxy-3-methoxypterocarpan (**6**). Desmocarpin is strongly laevorotatory and therefore possesses the 6a*R*; 11a*R* absolute configuration [5].

The concentration of each phytoalexin in 48 h fungus-induced diffusates was determined spectrophotometrically using extinction coefficients previously published for genistein ($\varepsilon = 42700$ at 262 nm [14], compounds **1** and **2**), dalbergioidin ($\varepsilon = 20420$ at 288 nm [15]), kievitone ($\varepsilon = 16600$ at 293 nm [16], compounds **4** and **5**) and melilotocarpan B (4,9-dihydroxy-3-methoxypterocarpan, $\varepsilon = 5888$ at 283 nm [17], compound **6**). The resulting values indicate that dalbergioidin (58 µg/ml diffusate) and desmocarpin (40 µg/ml) are the major phytoalexins produced by *D. gangeticum*, these being followed in decreasing order of abundance by kievitone (24 µg/ml), 2′-hydroxygenistein (14 µg/ml), diphysolone (11 µg/ml) and genistein (8 µg/ml).

Experimental

Plant and fungus material

Plants of *Desmodium gangeticum* DC. were raised [18] from seeds supplied by the Forest Research Institute, Dehra Dun, India. Leaflets for phytoalexin studies were harvested at regular intervals [18] after the plants reached an age of approx. 12 weeks, all flower heads being periodically removed to encourage leaf production. Cultures of *H. carbonum* Ullstrup and *C. herbarum* Fr. were maintained as reported elsewhere [8].

Isolation and purification of Desmodium phytoalexins

Droplets of *H. carbonum* spore suspension [19] were applied to the lower surface of excised *D. gangeticum* leaflets and then incubated [20] for 48 h. The resulting faintly yellow, cloudy diffusate was extracted (× 3) with equal volumes of EtOAc, and the combined organic fractions were reduced to dryness at 40 °C using a rotary evaporator. Si gel TLC (Merck, F-254, layer thickness 0.25 mm) of the residue in $CHCl_3$–MeOH (20:1) gave desmocarpin **6** (R_F 0.34), genistein **1** (R_F 0.29), 2′-hydroxygenistein **2** + diphysolone **4** (R_F 0.19), kievitone **5** (R_F 0.16) and dalbergioidin **3** (R_F 0.12). After elution with MeOH (4 × 2.5 ml), the above compounds were purified by Si gel TLC as follows: **6**, *n*-pentane–diethyl ether–glacial acetic acid (PEA), 75:25:6 (R_F 0.19), and **1**, **2** + **4**, **3** and **5**, PEA, 75:25:6, × 3. Phytoalexins **2** (lower zone) and **4** (upper zone) were readily separated by multiple development in the PEA system. A final TLC run in benzene–MeOH, 9:1 was sometimes required in order to completely free **2** (R_F 0.21) and **6** (R_F 0.30) from various very minor non-flavonoid contaminants. Compounds **1**–**5** were identified by UV and

TLC comparison with samples previously obtained from *Lupinus albus* (**1** and **2** [18]), *Dolichos biflorus* (**3** and **5** [21]) and *Diphysa robinioides* (**4** [9]). TLC examination of extracts of diffusates from H_2O-treated leaflets occasionally revealed traces of genistein but compounds **2**–**6** were not detected.

1,9-Dihydroxy-3-methoxypterocarpan (**6**) *(desmocarpin)*

Colour with diazotised *p*-nitroaniline reagent [6], orange; colour with Gibbs reagent/aqueous Na_2CO_3 [7, 8], dark blue. UV: λ_{max}, nm: MeOH 212 (100%), 236 sh (24%), 284–288 plateau (11%) or occasionally 286 (11%), 293 sh (10%); λ_{max}, nm: MeOH + NaOH 209, 245 sh, 294. MS: *m/z* 287 (18%), 286 ($[M]^+$; 100%), 285 (28%), 271 ($M^+ - 15$; 18%), 226 (10%), 211 (12%), 177 (7%; **a**), 167 (14%), 164 (9%; **b**), 149 (50%), 147 (7%; **c**), 134 (8%; **d**). ^1H NMR (acetone-d_6; 250 MHz; TMS reference): δ 7.13 (1H, d, J = 8.0 Hz, H-7), 6.36 (1H, dd, J = 8.0, 2.2 Hz, H-8), 6.29 (1H, d, J = 2.2 Hz, H-10), 6.17 (1H, d, J = 2.3 Hz, H-4), 5.98 (1H, d, J = 2.3 Hz, H-2), 5.62 (1H, d, J = 6.4 Hz, H-11a), 4.21 (1H, approx. dd, J = 10.5, 4.6 Hz, H-6eq), 3.72 (3H, s, $OC\underline{H}_3$), 3.54 (1H, approx. t, J = 10.5 Hz, H-6ax), 3.45 (1H, m, H-6a). $[\alpha]_{589\,nm} - 250°$ (approx. 850 µg, based on ε = 5888 at 283 nm for melilotocarpan B [17], in 1 ml of MeOH). *Dimethyl ether* (**7**) (CH_2N_2; R_F 0.77 in $CHCl_3$–CCl_4, 2:1). Compound **7** did not give a colour on TLC plates treated with either diazotised *p*-nitroaniline reagent or Gibbs reagent/aqueous Na_2CO_3. UV: λ_{max}, nm: 211 (100%), 235 sh (24%), 283–287 plateau (9%) or 285 (9%), 292 sh (7%). The MeOH spectrum of **7** was unaffected by aqueous NaOH. MS: *m/z* 315 (21%), 314 ($[M]^+$; 100%), 313 (27%), 312 (9%), 300 (7%), 299 ($M^+ - 15$; 17%), 191 (9%; **a′**), 178 (15%; **b′**), 162 (11%), 161 (10%; **c′**), 148 (17%; **d′**).

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