Tectorigenin, a Phytoalexin of *Centrosema haitiense* and Other *Centrosema* Species

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A phytoalexin isolated from the fungus-inoculated leaflets of *Centrosema haitiense*, *C. pubescens* (3 accessions) and *C. virginianum* has been identified as 5,7,4′-trihydroxy-6-methoxyisoflavone (tectorigenin). This compound is variously accompanied by other isoflavones including the previously described phytoalexin, cajanin (5,2′,4′-trihydroxy-7-methoxyisoflavone). Substantial quantities of aesculetin were obtained when *C. haitiense* leaves were submerged for 48 h in water or fungal spore suspensions. The taxonomic position of *Centrosema* within the legume tribe Phaseoleae is discussed.

Introduction

Although isoflavonoid phytoalexins (particularly pterocarpans and isoflavans) have been isolated from the fungus-treated tissues of many Leguminosae (subfamily Papilionoideae), comparatively few species appear to produce fungitoxic isoflavones in more than trace amounts [1]. Notable amongst these are members of the tribe Genisteae (*e. g. Laburnum anagyroides*) as well as certain Phaseoleae (*e. g. Cajanus cajan* and *Neonotonia wightii*) where simple and/or complex isoflavones frequently represent a major component of the phytoalexin response [2–4]. In other legumes, *e. g. Phaseolus vulgaris* (Phaseoleae, subtribe Phaseolinae), isoflavones generally co-occur with substantially greater quantities of pterocarpan, isoflavan or isoflavone derivatives [1]. As yet there are no reports of phytoalexin formation in *Centrosema* (Phaseoleae, subtribe Phaseolinae), a genus of approx. 35 species endemic to the American tropics and subtropics [5]. Several members of the group, *e. g. C. pubescens* ("centro") are widely grown as fodder crops. We report here the results of a recent examination of selected species of this genus.

Results and Discussion

The excised leaflets of 4 *Centrosema* species (Table I) were treated with conidial suspensions of *Helminthosporium carbonum* [6] and EtOAc soluble material extracted from the diffusates after 48 h incubation. With the exception of *C. brasilianum*, all the species produced simple phenolic isoflavones (Table I), in particular a non-fluorescent substance which exhibited UV/visible absorption maxima characteristic of those compounds having a 5,7-dihydroxy-6-methoxy substitution pattern [7]. MS analysis defined the mol. wt. as 300, and associated fragments at *m/e* 285 (M^+-Me), 257 ($M^+-Me-CO$), 167/139 (loss of Me and Me + CO respectively from a dihydroxy-monomethoxy substituted A-ring fragment [8]) and 118 (B-ring fragment with monohydroxylation [8]) suggested that the compound was probably tectorigenin (5,7,4′-trihydroxy-6-methoxyisoflavone, **1**). This provisional identification was confirmed by direct UV, MS and TLC (5 solvent systems on Si gel and cellulose) comparison with authentic material. The fungal induction of tectorigenin has not previously been described; however, this isoflavone occurs uncombined or as its 7-O-glucoside (tectoridin) in the healthy tissues of several legumes, notably species of *Baptisia* (tribe Thermopsideae) and *Dalbergia* (tribe Dalbergieae) [9–11], where it may possibly confer some degree of protection (either as a prohibitin or post-inhibitin [12]) against microbial invasion.

In addition to tectorigenin, one accession of *C. pubescens* also accumulated significant quantities of cajanin (5,2′,4′-trihydroxy-7-methoxyisoflavone, **2**), a phytoalexin previously obtained from the *H. carbonum*-inoculated stems of pigeon pea, *Cajanus cajan* (Phaseoleae, subtribe Cajaninae) [3]. Although cajanin was absent from leaf diffusates of the two other *C. pubescens* accessions, it should be noted that intraspecific variation in phytoalexin produc-

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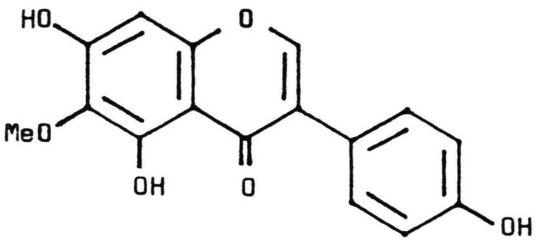
Table I. Isoflavones present in diffusates from *Helminthosporium carbonum*-inoculated leaflets of *Centrosema* species [a, b, c].

Species	Seed Source	Isoflavone(s) Detected	Concentration µg/ml
C. brasilianum Benth.	Hl (CPI 40061)	None	–
C. haitiense Urb. & Ekman	Hl (CPI 46540)	Tectorigenin	38
		Afrormosin	2
		Glycitein	< 2
		6,7,4′-Trihydroxyisoflavone	TR
C. pubescens Benth.	Hl	Tectorigenin	16
		Afrormosin	2
		Formononetin	2
C. pubescens Benth.	Gm	Tectorigenin	13
		Afrormosin	1
		Formononetin	< 1
		Glycitein	TR
C. pubescens Benth.	Jn	Tectorigenin	14
		Cajanin	23
C. virginianum (L.) Benth.	Hl (CPI 40054)	Tectorigenin	26
		Formononetin	TR

TR = trace (insufficient for UV quantification); identified by TLC comparison with authentic material.
[a] Isoflavone concentrations were determined spectrophotometrically using the following extinction coefficients: i) $\varepsilon = 30000$ for tectorigenin (at 268 nm) and cajanin (260 nm); and ii) $\varepsilon = 22400$ for afrormosin, glycitein (both at approx. 258 nm) and formononetin (250 nm) [21, 22].
[b] Seed sources: Gm = S. S. Gnanamanickam, Centre for Advanced Studies in Botany, University of Madras, India; Hl = E. F. Henzell, The Cunningham Laboratory, C. S. I. R. O., St. Lucia, Queensland, Australia (plant introduction (CPI) numbers are in parentheses); Jn = D. H. Janzen, Department of Biology, University of Pennsylvania, Philadelphia, Pennsylvania, USA (collected in the Santa Rosa National Park, Costa Rica).
[c] *C. haitiense* = *C. schottii* (R. J. Clements and R. J. Williams, *pers. commun.*).



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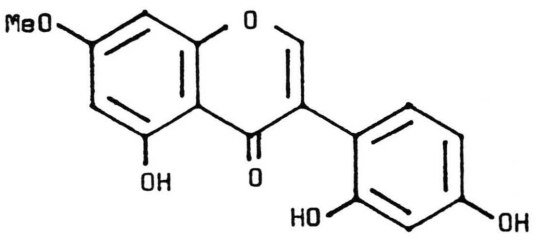

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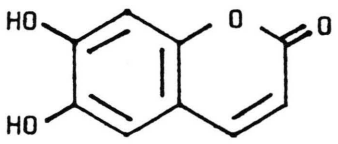

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tion has recently been described in legumes such as *Arachis hypogaea* [13, 14] and *Vigna unguiculata* [15]. As misidentification of the Costa Rican accession of *C. pubescens* has been excluded (D. H. Janzen, *pers. commun*), its differing phytoalexin response presumably reflects genuine chemical variability in this very widely distributed species (D. H. Janzen and R. J. Clements, *pers. commun*).

Apart from compounds **1** and **2**, leaf diffusates variously contained other known isoflavones with 7,4′(formononetin) or 6,7,4′(afrormosin, glycitein and 6,7,4′-trihydroxyisoflavone) oxygenation patterns. All were present at low concentrations (Table I), and upon TLC biassay against *Cladosporium herbarum* [16] only tectorigenin and cajanin were associated with regions of antifungal activity. None of the above isoflavones was detected in control (H_2O) diffusates. All attempts to isolate tectorigenin, cajanin or other antifungal material from diffusates of *C. brasilianum* were unsuccessful.

With *C. haitiense*, an attempt was also made to increase the yield of tectorigenin by immersing leaflets in a dense spore suspension of *H. carbonum*. However, examination of the resulting diffusate revealed not tectorigenin, but large quantities (5 mg/0.75 g dry wt. leaves) of a colourless, crystalline compound (M^+ 178; Diacetate, M^+ 262) indistinguishable (UV, TLC) from authentic aesculetin (6,7-dihydroxycoumarin, **3**). The same compound was obtained in comparable amounts when *C. haitiense* leaflets were similarly submerged in deionised H_2O. Although aesculetin does not occur naturally in the leaves of *C. haitiense*, its 7-O-glucoside (aesculin) can be isolated in high yield (6.5% on a dry wt. basis) by extraction with aqueous MeOH. Hydrolysis of aesculin to aesculetin occurs

readily upon tissue damage, and formation of the latter compound as described above may well reflect a stress response of the immersed leaflets. TLC bioassays (*C. herbarum*) indicated that neither aesculin nor aesculetin possessed antifungal activity.

The phytoalexin data presented in Table I are interesting because of the uncertain allocation of *Centrosema* to the subtribe Phaseolinae [17]. Studies undertaken at Reading University (J. L. Ingham, unpublished data) indicate that subtribes of the Phaseoleae fall into two broad categories depending on whether their constituent genera are characterised by the accumulation of simple or predominantly complex isoflavonoid phytoalexins. Thus, in the Phaseolinae, *Centrosema* is clearly atypical as species belonging to other genera examined (*e. g. Dolichos, Lablab, Phaseolus, Psophocarpus* and *Vigna*) [1, 18] invariably produce phytoalexins with prenyl or dimethylchromen attachments. Although simple isoflavones (*e. g.* 2′-hydroxygenistein) do occur in some Phaseolinae, it is significant that as yet neither cajanin nor tectorigenin has been detected in this subtribe. *Centrosema* is also unusual in at least one other chemical respect, namely the occurrence in seeds of the non-protein amino acid canavanine, a compound otherwise absent from the Phaseolinae [17]. In contrast, the subtribes Cajaninae and Diocleinae (excluding *Pachyrrhizus* [19]) are characterised by formation of simple isoflavonoids with cajanin (but not tectorigenin) appearing in a number of genera including *Dioclea, Cajanus, Canavalia* and *Rhynchosia* ([3] and J. L. Ingham, unpublished data). Canavanine is likewise of almost universal occurrence in Diocleinae [17]. The above data indicate that *Centrosema* is misplaced within the Phaseolinae, and additionally suggest the possibility of a chemical link between this genus and members of the Diocleinae and/or Cajaninae. It is satisfying to note that our results are in broad agreement with the findings of recent, independent study by Lackey [20] who, on the basis of chromosome counts, proposed that *Centrosema* (and certain other controversial genera such as *Clitoria*) should be transferred from the Phaseolinae to a new morphologically well-defined subtribe termed the Clitoriinae.

Experimental

Plant material. Seeds were obtained from the sources listed in Table I. Voucher specimens of all *Centrosema* accessions have been deposited in the University of Reading Herbarium.

Isolation of Centrosema isoflavones. Si gel TLC ($CHCl_3$:MeOH, 50:3 layer thickness 0.25 mm) of diffusate extracts (EtOAc) variously afforded isoflavone bands at R_F 0.70 (afrormosin), 0.46 (formononetin), 0.36 (cajanin), 0.35 (tectorigenin), 0.23 (glycitein) and 0.10 (6,7,4′-trihydroxyisoflavone). For the Costa Rican accession of *C. pubescens*, cajanin and tectorigenin were eluted (MeOH) together, and subsequently resolved by TLC in either C_6H_6:MeOH, 9:1 (**1**, R_F 0.43; **2**, R_F 0.34) or *n*-pentane:Et_2O:glacial HOAc, 75:25:3, ×3 (**1**, upper zone; **2**, lower zone). Isoflavones from all other sources were quantified without additional purification.

Isolation of aesculin and aesculetin. One dimensional PC (H_2O:glacial HOAc, 20:3) of leaf extracts (70% MeOH) gave aesculin as a purple/white fluorescent (UV) band at high R_F. Freshly harvested leaves were also macerated in H_2O and incubated for 1 h (room temp.). Upon 1D PC (H_2O:glacial HOAc, 20:3) this extract yielded aesculetin as a blue fluorescent (UV) zone located well below a marker of the unhydrolysed glucoside. EtOAc extracts of submerged leaf diffusates were chromatographed (TLC) using $CHCl_3$:MeOH, 50:3 (aesculetin, R_F 0.0–0.10).

Spectroscopic data. a) Isoflavones. UV maxima as lit. [3, 7, 21, 22]. MS (rel. int.): tectorigenin, 301 (17), 300 (M^+; 95), 299 (8), 286 (9), 285 (54), 283 (11), 282 (42), 258 (14), 257 (100), 254 (14), 167 (5), 153 (7), 150 (9), 139 (22), 119 (13), 118 (18); cajanin, as lit. [3]. The other *Centrosema* isoflavones were not isolated in quantities sufficient for MS analysis, b) Aesculetin. λ_{max} (nm) MeOH 215, 231, 260, 300, 351; NaOMe 245, 277 sh, 400; NaOAc 240 sh, 270 sh, 310 sh, 377; NaOAc + H_3BO_3 241, 270, 311, 373. MS (rel. int.) 179 (6), 178 (M^+; 68), 150 (100), 133 (10). *Diacetate* (Py-Ac_2O). MS (rel. int.) 262 (M^+; 3), 220 (11), 179 (10), 178 (100), 177 (4), 150 (31).

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Note added in proof: Recent studies (by J. L. I.) of 4 additional *Centrosema* species have revealed the following isoflavone phytoalexins. i) *C. pascuorum:* tectorigenin (20 µg/ml diffusate) + cajanin (16 µg/ml), ii) *C. plumieri* (2 accessions): tectorigenin (29 and 34 µg/ml respectively), iii) *C. sagittatum:* tectorigenin (19 µg/ml), and iv) *C. schiedeanum* cv. "Belalto": tectorigenin (12 µg/ml). Traces of afrormosin and formononetin were present in the fungus-induced diffusates of every species except *C. pascuorum.* A minor antifungal isoflavone (approx. 4 µg/ml) obtained from both accessions of *C. plumieri* has been provisionally identified as 6-hydroxygenistein (5,6,7,4′-tetrahydroxyisoflavone).