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ISOFLAVONOID PHYTOALEXINS FROM FUNGUS-INOCULATED LEAVES OF *APIOS TUBEROSA*

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Key Word Index—*Apios tuberosa*; Leguminosae; Papilionoideae; Phaseoleae; isoflavonoids; isoflavones; pterocarpan; phytoalexins; lanthanide shift reagent; nuclear Overhauser effect; structure elucidation; absolute configuration.

Abstract—A new phytoalexin (apiocarpin) isolated from the fungus-inoculated leaflets of *Apios tuberosa* has been identified by chemical and spectroscopic procedures as a 1,9-dihydroxylated pterocarpan possessing an angular (C-3/C-4) isopropenyldihydrofuran substituent. Apiocarpin co-occurs with the known isoflavones, genistein and 2′-hydroxygenistein.

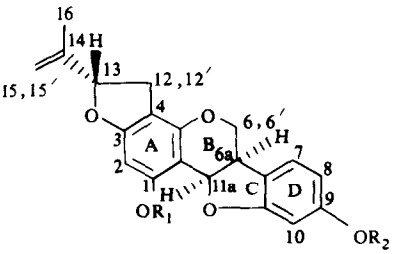
INTRODUCTION

Species belonging to the taxonomically advanced legume tribe Phaseoleae (subtribes Erythrininae, Glycininae and Phaseolinae) frequently produce complex pterocarpan phytoalexins following challenge-inoculation with micro-organisms [1, 2]. Thus, fungus-treated tissues of the cultivated soybean (*Glycine max*) characteristically accumulate anti-fungal compounds containing *gem*-dimethylchromene (glyceollins I and II), γ, γ-dimethylallyl (glyceollin IV) or isopropenyldihydrofuran (13*S*-glyceollin III, **1**) substituents [3–5]. In addition, 13*R*-**1** (canescacarpin) and the furanopterocarpan, clandestacarpin, have recently been discovered in bacteria-infiltrated leaves of *G. canescens* and *G. clandestina* respectively [6]. A variety of other inducible complex pterocarpans are also known to occur in species of related genera such as *Dolichos*, *Erythrina*, *Lablab*, *Phaseolus* and *Vigna* [1, 2].

We have now investigated the phytoalexin response of *Apios tuberosa* Moench, a perennial herb with edible tubers (potato bean, or Indian potato) native to eastern North America. In response to treatment with conidial suspensions of the fungal inducer, *Helminthosporium carbonum* [7, 8], excised leaflets of *A. tuberosa* (Phaseoleae, subtribe Erythrininae) rapidly produce genistein (5, 7, 4′-trihydroxyisoflavone, **2**), 2′-hydroxygenistein (5, 7, 2′, 4′-tetrahydroxyisoflavone, **3**) and a third, previously undescribed, phenolic compound which we have named apiocarpin. The isolation, purification and identification of apiocarpin as the complex pterocarpan **4** is described in this report.

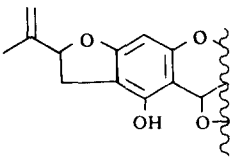
RESULTS AND DISCUSSION

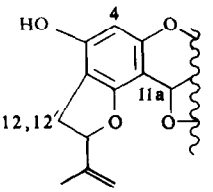
Phytoalexins **2**–**4** were initially isolated from fungus-induced leaf diffusates as outlined in the Experimental. Yields were low, however, and in later studies leaf tissues underlying the inoculum droplets were also routinely extracted as these contained **4** in comparatively large amounts. Control (water) leaf



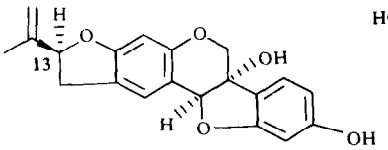
4 $R_1 = R_2 = H$

5 $R_1 = R_2 = Me$





6 **7**



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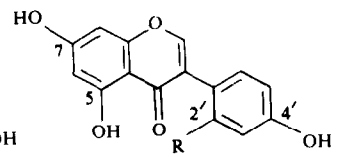

2 R = H

3 R = OH

diffusates did not contain detectable quantities of apiocarpin, or isoflavones **2** and **3**. All three *Apios* isoflavonoids proved to be fungitoxic when diffusate extracts (ethyl acetate) were tested against *Cladosporium herbarum* using the standard TLC bioassay procedure [8]. Inhibition zones produced on TLC plates by known quantities (5, 10, 25 and 50 μg) of apiocarpin were comparable in area with those given by equivalent amounts of glyceollin I [9]. In contrast, genistein and 2'-hydroxygenistein were less active; significant inhibition zones attributable to these compounds were only evident at applied levels of 50 μg, or greater. Isoflavones **2** and **3** were identified by direct comparison (UV, TLC, mass spectrometry) with authentic material previously extracted from fungus-inoculated stems of *Neonotonia* (*Glycine*) *wightii* [10].

The UV (ethanol) spectrum of **4** was typically pterocarpan-like, and closely resembled that of 4-methoxymedicarpin [2, 11]. However, in alkaline solution, a shoulder rather than a distinct maximum was apparent between 240 and 250 nm and this, together with the bright-orange product given by **4** on TLC plates sprayed with diazotized *p*-nitroaniline reagent, strongly suggested that, like glyceollins I–III, the compound had a hydroxyl group at C-9 and an alkyl substituent at C-3 [12].

Mass spectral analysis of apiocarpin (338[M^+]) afforded a major ion at $[M-15]^+$ but no fragments indicative of either a γ,γ-dimethylallyl substituent $[M-55/56]^+$ or a C-6a hydroxyl group $[M-18]^+$. Although significant loss of 15 mass units from the molecular ion (or a derived fragment) is observed in the mass spectrum of pterocarpans and other isoflavonoids containing a *gem*-dimethylchromene ring (e.g. phaseolin from *Phaseolus vulgaris*, and glyceollins I and II from *G. max*), such compounds invariably exhibit UV absorption above 300 nm [2] because of conjugation between the chromene ring and the aromatic chromophore. However, this feature was not associated with the neutral (ethanol) UV spectrum of apiocarpin.

Diazomethane methylation of **4** yielded a non-phenolic dimethyl ether **5** (366 $[M]^+$) whilst catalytic hydrogenation over Pd–C resulted in the rapid formation of a tetrahydro product. This latter compound had 342 $[M]^+$ and prominent mass spectral fragments at *m/z* 207 (A-ring derived ion with C-5 alkyl unit, and an additional hydroxyl group; cf. the analogous mass spectral fragment at *m/z* 191 given by the isoflavan derivatives of glyceollin I and leiocarpin [3, 13]), *m/z* 136 and 123 (B-ring derived fragment with two hydroxyl groups).

In the ^{1}H NMR spectrum of **4** (Table 1), signals attributable to the heterocyclic (B/C) ring protons (H-6/6', H-6a and H-11a) were readily identified by comparison with the spectra of 3,9-dihydroxypterocarpan, phaseollidin, and dolichins A and B [14, 15]. Chemical shift data for the D-ring protons (H-7, H-8 and H-10) were also in very close agreement with values observed for glyceollins I–III (δ 7.13 *d*, 6.36 *q* and 6.29 *d* respectively [4]), and were at slightly higher field than the A-ring protons of phaseollin and 3,9-dihydroxypterocarpan ([14] and Mulheirn, L. J., unpublished data). The ^{1}H NMR spectrum of **4** also contained signals characteristic of *gem*-benzylic (H-12/12') and methine (H-13) protons, as well as those associated with a terminal olefinic methylene group and an allylic methyl substituent. Such signals are also to be found in the ^{1}H NMR spectrum of gly-

Table 1. ^{1}H NMR spectral data for glyceollin III (**1**), apiocarpin (**4**) and its derivatives*

Proton	Glyceollin III		Apiocarpin		Apiocarpin diacetate	Apiocarpin dimethyl ether (**5**)
	δ	*J*(Hz)	δ	*J*(Hz)	δ	δ
H-1	7.27*s*	—	—	—	—	—
H-2	—	—	6.08*br s*	—	6.32	6.20
H-4	6.27*s*	—	—	—	—	—
H-6 (ax)	4.05*d*	11	3.58*t*	12, 12	3.78	3.61
H-6' (eq)	4.15*d*	11	4.25*q*	12, 5	4.40	4.27
H-6a	—	—	3.44*m*	12, 6, 5	3.72	3.48
H-7	7.23*d*	8	7.13*d*	8	7.37	7.23
H-8	6.46*q*	8, 2	6.36*q*	8, 2	6.64	6.44
H-10	6.26*d*	2	6.29*d*	2	6.54	6.37
H-11a	5.30*s*	—	5.63*d*	6	5.66	5.60
H-12	3.05†	16, 8	3.20†	16, 8	2.90	2.84
H-12'	3.42†	16, 9	3.44†	16, 8	3.29	3.23
H-13	5.26†	8	5.21†	8	5.31	5.25
H-15	4.91*br s*	—	4.87*br s*	—	4.89	4.91
H-15'	5.08*br s*	—	5.15*br s*	—	5.06	5.08
Me-16	1.77*s*	—	1.79*s*	—	1.77	1.76
OAc/OMe	—	—	—	—	{2.23	{3.75
	—	—	—	—	{2.30	{3.87

*Spectra were measured in acetone-d_6 (TMS as int. standard) using either a JEOL PFT-100 spectrometer at 99.54 MHz (**1**) or a Brucker WH-360 FT spectrometer at 360 MHz (**4** and its derivatives).

†ABX multiplet.

ceollin III (**1**), its 13*R* isomer (canescacarpin), rotenone and the *Derris* isoflavonoids, glabrescin and glabrescione A [4, 6, 16], all of which possess either an angular or a linear A-ring (or D-ring in the case of rotenone) isopropenyldihydrofuran substituent. As expected, apiocarpin dimethyl ether (**5**) exhibited all the above-mentioned signals (Table 1) together with two additional three-proton (methoxy) singlets at δ 3.75 and 3.87.

The one remaining signal in the ^{1}H NMR spectrum of apiocarpin appeared as a broad singlet at δ 6.08 (δ 6.20 for the dimethyl ether **5**) and was assigned to a proton at either C-2 or C-4 of ring A which, as indicated by mass spectral examination of tetrahydro-**4**, carries both the side chain and a hydroxyl group. The high field disposition of the H-2 or H-4 proton signal is entirely in accord with the existence of a phloroglucinol, rather than a pyrogallol, oxygenation pattern in this ring; compare, for instance, the δ value of the single A-ring proton of comparable complex phloroglucinol-based pterocarpans such as edulane (δ 6.25), edulenol (δ 6.28), neorautanin (δ 6.27) and gangetin (δ 6.25) [17, 18] with that of model compounds, e.g. neoraucarpan (δ 6.92) and neoraucarpanol (δ 6.91) [17], having a pyrogallol arrangement. The proposed A-ring oxygenation pattern of apiocarpin is also indirectly supported by its co-occurrence with the 5,7-dioxygenated isoflavones genistein (**2**) and 2'-hydroxygenistein (**3**).

Although the above results permitted the ring oxygenation pattern of **4** to be deduced, they did not allow the side chain to be firmly located at either C-2 or C-4. Thus, three structures (**4**, **6** and **7**) were possible for the phytoalexin. However, **6** with the side chain arranged in a linear fashion (as in **1**) was easily eliminated on the basis of two experiments. First, apiocarpin did not afford any colouration on TLC plates sprayed with Gibbs reagent–aqueous sodium carbonate [8, 19], an indication that ring A was substituted *para* to the phenolic hydroxyl group. Secondly, nuclear Overhauser experiments on apiocarpin dimethyl ether (**5**) gave a large enhancement of the intensity of the aromatic proton signal (δ 6.20) upon irradiation of the methoxyl group at δ 3.87. This result supported the Gibbs test by confirming that the single aromatic A-ring proton was located *ortho* to the hydroxyl group, an arrangement incompatible with structure **6**.

A decision between isomers **4** and **7** was eventually reached by means of a lanthanide-shift NMR experiment. Using the reagent d_9-Eu(fod)$_3$, a marked downfield shift can be predicted for those protons spatially adjacent to hydroxyl groups. Shifts decrease with distance from the site of the hydroxyl–shift-reagent complex, and increase with reagent concentration up to a limiting value [20]. For both isomer **4** and isomer **7**, some movement of the H-2 (or H-4), H-8 and H-10 signals can be anticipated in the presence of d_9-Eu(fod)$_3$. However, only **4** should exhibit a major shift of the H-11a signal, with the side chain H-12/12' proton signals being virtually unaffected. In contrast, **7** would be expected to show a marked shift of H-12/12' but much less movement of H-11a.

From the results presented in Table 2, it is clear that there is major deshielding of H-11a and H-10, whereas H-2 and H-8 move considerably less. Significantly, only minimal shifts were observed with respect to the side chain signals (H-12/12'), although movement of H-6/6' indicated that some chelation had probably occurred between the reagent and the oxygen atom of ring B. This type of binding may also explain why the H-10 shift is somewhat larger than that of other aromatic protons (H-2 and H-8) *ortho* to the phenolic hydroxyl groups; the reagent attached at C-1 may interact weakly with the furanoid oxygen of ring C thus producing an effect on H-10 which is additive to that resulting from the adjacent Eu complex at C-9. The above results permit apiocarpin to be correctly formulated as **4**.

Apiocarpin has a large negative optical rotation at 589 nm (sodium lamp) and, in conjunction with its CD

Table 2. Europium shift data for apiocarpin (**4**)*

Proton	Chemical shift of apiocarpin (δ)	Chemical shift increment (ppm) on addition of d_9-Eu(fod)$_3$ (mg)		
		2.46 mg	3.63 mg	5.64 mg
H-2	6.36	0.06	0.12	0.24
H-6 (ax)	3.42	0.09	0.19	0.38
H-6'(eq)	3.89	0.07	0.14	0.29
H-6a	2.96	0.07	0.14	0.38
H-7	6.60	0.04	0.08	0.18
H-8	6.21	0.09	0.19	0.39
H-10	6.20	0.19	0.39	0.59
H-11a	5.28	0.33	0.68	0.99
H-12	2.89	0.03	0.06	0.12
H-12'	3.03	0.01	0.03	0.08
H-13	4.95	0.02	0.03	0.06
H-15	4.72	0.01	0.01	0.03
H-15'	5.01	0.02	0.03	0.05
Me-16	1.53	0.03	0.04	0.07

*Determined in C_6D_6 at 360 MHz using a Brucker WH-360 FT spectrometer.

maxima [$[\theta]_{235}$ −45100, $[\theta]_{288}$ +10140 (EtOH)], can thus be assigned the 6a*R*; 11a*R* absolute configuration [2, 6]. Moreover, the CD spectrum of the osmate ester of **4** [15] showed a positive Cotton effect at 475 nm ($[\theta]_{max}$ *ca* +6000) similar to that observed earlier for glyceollin III (**1**), but opposite to that of rotenone ($[\theta]_{max}$ −5800)[4]. The absolute stereochemistry of apiocarpin is therefore fully and unequivocally defined as 6a*R*: 11a*R*; 13*S*.

After 48 hr incubation, the concentration of **4** (ϵ = 5300 at 286 nm) in *H. carbonum*-induced leaf diffusates ranged from 8 to 14 μg/ml; corresponding values for genistein (**2**) and 2′-hydroxygenistein (**3**) were 6–12 μg/ml and 18–26 μg/ml respectively. Leaf tissues directly below the inoculum droplets contained apiocarpin at levels between 186 and *ca* 270 μg/g fr. wt. Isoflavonoids **2–4** were also isolated from diffusates (**2**, 8–11 μg/ml; **3**, 20–29 μg/ml; **4**, 10–20 μg/ml) and underlying tissues (**4**, 190–300 μg/g fr. wt) following treatment of detached *Apios* leaflets with droplets of aqueous copper sulphate (0.15 g $CuSO_4$/100 ml deionized H_2O).

EXPERIMENTAL

All TLC separations were undertaken using pre-coated, glass-backed plates (Merck Si gel 60, F-254; layer thickness, 0.25 mm). The optical rotation of **4** was determined (in MeOH) at 20° on a Perkin-Elmer Model 141 polarimeter. CD spectra were measured in CH_2Cl_2 using a Cary 61 spectropolarimeter. Formation of the osmate ester of **4** was carried out as described elsewhere [15].

Plant material. Fresh leaves of *Apios tuberosa* Moench were collected from a plant growing at the Royal Botanic Gardens, Kew, U.K. A voucher specimen has been deposited in the Department of Botany Herbarium, University of Reading.

Isolation of apiocarpin and isoflavones **2** *and* **3**. Phytoalexins were initially obtained from excised *A. tuberosa* leaflets by means of the drop-diffusate technique [7, 8]. Si gel TLC ($CHCl_3$–MeOH, 50:1) of diffusate extracts (EtOAc) gave apiocarpin (**4**) (R_f 0.26), genistein (**2**) (R_f 0.17) and 2′-hydroxygenistein (**3**) (R_f 0.10). All three isoflavonoids were eluted (EtOH) and then re-chromatographed in *n*-pentane–Et_2O–HOAc, (75:25:3, ×3) followed, in the case of **3** and **4**, by C_6H_6–MeOH, (9:1, ×3) prior to spectroscopic examination. Because leaf diffusates contained only small quantities of **4**, this phytoalexin was also isolated from leaf tissues underlying the inoculum droplets. In a typical experiment, excised tissues (1500 inoculation sites ≡ *ca* 5 g fr. material) were exhaustively extracted with EtOH (5 × 100 ml). The soln obtained following vacuum filtration of the extract was reduced (*in vacuo*, 40°) to *ca* 20 ml, H_2O (150 ml) was then added, and the vol. again reduced to between 20 and 40 ml. After a final addition of H_2O (100 ml), the soln was shaken with EtOAc (3 × 200 ml) and the bulked organic fractions reduced to dryness. Si gel TLC ($CHCl_3$–MeOH, 100:12) of the residue yielded impure apiocarpin (R_f 0.66) well separated from the major leaf pigments which ran close to the solvent front; zones attributable to isoflavones **2** and **3** were located at R_f 0.59 and 0.49 respectively. Apiocarpin was eluted (EtOH) and then re-chromatographed as described above. Extraction of equivalent quantities of control (H_2O-treated) leaf material failed to reveal even traces of compounds **2–4**.

Genistein (**2**) *and 2′-hydroxygenistein* (**3**). UV and MS data as lit. [21].

Apiocarpin (**4**). Diazotized *p*-nitroaniline, orange; Gibbs reagent, no reaction. UV λ_{max}^{EtOH} nm: 214 (100%), 238 sh (30%), 286 (21%; ϵ = 6300), 293 sh (16%); $\lambda_{max}^{EtOH+NaOH}$ nm: 212, 246 sh, 298. MS *m/z* (rel. int.): 339 (19), 338 $[M]^+$ (78), 337 (13), 336 (7), 324 (24), 323 (100), 321 (7), 189 (7), 161 (8), 147 (5). High resolution MS: $[M]^+$ 338.1150 ($C_{20}H_{18}O_5$). ^1H NMR data, see Table 1. $[\alpha]_{589\,nm}$ − 180° (*ca* 0.15 mg in 1 ml MeOH). Di Me ether (CH_2N_2)(R_f 0.84: $CHCl_3$–CCl_4, 3:1), UV λ_{max}^{EtOH} nm: 214 (100%) 246 sh (19%), 285 (13%), 293 sh (9%); the EtOH spectrum was unaffected by addition of aq. NaOH. MS *m/z* (rel. int.): 367 (18), 366 $[M]^+$ (100), 365 (15), 352 (25), 351 (86), 336 (8), 203 (13), 162 (7), 161 (13), 151 (8), 149 (22), 137 (12). ^1H NMR data, see Table 1.

Tetrahydroapiocarpin (*apiocarpin isoflavan*). H_2 (generated by the action of conc. HCl on As-free Zn granules) was passed (45 min; room temp.) through a stirred soln of **4** (*ca* 1 mg) in HOAc (3 ml) containing 10% Pd-C (5 mg). After removal of catalyst and solvent, the residue was chromatographed ($CHCl_3$–MeOH, 100:12) to afford the tetrahydro derivative (*ca* 0.5 mg; R_f 0.35). Diazotized *p*-nitroaniline, orange; Gibbs reagent, purple–blue. UV λ_{max}^{EtOH} nm: 212 (100%), 236 sh (36%), 282 (15%), 287 sh (13%); UV $\lambda_{max}^{EtOH+NaOH}$ nm: 212, 242 sh, 291. MS *m/z* (rel. int.): 343 (6), 342 $[M]^+$ (29), 208 (9), 207 (100), 206 (33), 191 (21), 167 (6), 163 (24), 151 (57), 149 (17), 139 (8), 136 (28), 135 (8), 125 (8), 123 (32).

NMR experiments. NMR shift experiments were performed in dry C_6D_6 using d_9-tris(heptafluoro-2,2-dimethyl-3,5-dioctanedionato)europium [d_9-Eu(fod)$_3$] as the shift reagent. Apiocarpin (*ca* 1.1 mg) was treated with increasing amounts of the reagent as shown in Table 2; spectra were run (at 29°) 10–20 min after each addition of d_9-Eu(fod)$_3$. Nuclear Overhauser effect experiments were carried out on apiocarpin dimethyl ether (**5**) (*ca* 1 mg) in a sealed NMR tube containing degassed Me_2CO-d_6.

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