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TEPHROCARPIN, A PTEROCARPAN PHYTOALEXIN FROM *TEPHROSIA BIDWILLI* AND A STRUCTURE PROPOSAL FOR ACANTHOCARPAN

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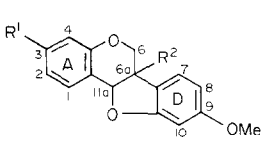
Abstract—The isoflavonoids (−)-6a*R*; 11a*R*-maackiain, (−)-6a*S*; 11a*S*-pisatin and (−)-6a*R*; 11a*R*-4-methoxymaackiain have been isolated as phytoalexins from fungus-treated leaflets of *Tephrosia bidwilli*. They co-occur with two additional fungitoxic isoflavonoids identified as (−)-6a*S*; 11a*S*-3, 6a-dihydroxy-4-methoxy-8, 9-methylenedioxypterocarpan (tephrocarpin) and (−)-6a*S*; 11a*S*-6a-hydroxy-3,4:8,9-dimethylenedioxypterocarpan. The latter compound is indistinguishable from a partially-formulated phytoalexin (acanthocarpan) recently discovered in leaflets of *Caragana acanthophylla*.

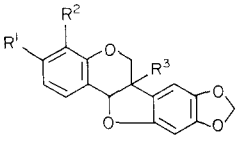
INTRODUCTION

Previous phytochemical studies involving the large (over 400 species) pantropical genus *Tephrosia* (Leguminosae; subfamily Papilionoideae; tribe Tephrosieae) have led to the isolation and identification of numerous isoflavonoid (isoflavone, rotenoid and coumestan) derivatives, some of which (e.g. rotenone from *T. virginiana* and other *Tephrosia* spp.) possess pronounced insecticidal and fish-poisoning properties [1]. Certain isoflavonoids—particularly pterocarpans and isoflavans—are also toxic to micro-organisms, their biosynthesis in the tissues of papilionoid legumes frequently occurring as a consequence of fungal or bacterial invasion. *De novo* formation of defensive anti-microbial chemicals (phytoalexins [2]) is a characteristic feature of many Leguminosae [3], although as yet such compounds do not appear to have been recognized in *Tephrosia*.

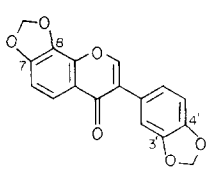
Recently, however, we examined a range of *Tephrosia* spp. and found that in every case detached leaflets readily produced pterocarpan phytoalexins following inoculation with spore suspensions of the fungus, *Helminthosporium carbonum*. For example, *T. purpurea* and *T. villosa* both accumulated the known isoflavonoids medicarpin (**1**), variabilin (**2**), maackiain (3-hydroxy-8, 9-methylenedioxypterocarpan, **3**) and pisatin (3-methoxy-6a-hydroxy-8, 9-methylenedioxypterocarpan, **4**). Pterocarpans **3** and **4** were also isolated from leaflets of *T. grandiflora*, *T. virginiana* and *T. bidwilli*, but the latter species (which is indigenous to Australia) additionally produced 4-methoxymaackiain (**5**), acanthocarpan (a partly-formulated pterocarpan phytoalexin originally obtained from the leguminous shrub, *Caragana acanthophylla*; tribe Galegeae [4]), and a hitherto undescribed isoflavonoid for which we propose the com-



1 R^1 = OH, R^2 = H
2 R^1 = OMe, R^2 = OH



3 R^1 = OH, R^2 = R^3 = H
4 R^1 = OMe, R^2 = H, R^3 = OH
5 R^1 = OH, R^2 = OMe, R^3 = H
6 R^1 = R^3 = OH, R^2 = OMe
7 R^1 = R^2 = O − CH_2 − O, R^3 = OH



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mon name tephrocarpin. This paper reports the identification of tephrocarpin as 3,6a-dihydroxy-4-methoxy-8, 9-methylenedioxypterocarpan (**6**) and presents evidence to show that acanthocarpan is 6a-hydroxy-3,4:8,9-dimethylenedioxypterocarpan (**7**).

RESULTS AND DISCUSSION

Phytoalexins were isolated from excised, *H. carbonum*-inoculated *T. bidwilli* leaflets using the drop-diffusate technique [2, 5]. Extracts (ethyl acetate) of 48 hr diffusates were chromatographed as described in the Experimental to yield five isoflavonoids, all of which proved to be highly fungitoxic when tested (TLC plate bioassay [6, 7]) against spore germination of *Cladosporium herbarum*. Three of these phytoalexins were subsequently identified as maackiain (**3**), pisatin (**4**) and 4-methoxymaackiain (**5**) by direct UV and Si gel TLC comparison with authentic material [4, 8, 9]. As expected, the above-mentioned compounds were not produced by excised *Tephrosia* leaflets treated only with de-ionized water.

The new phytoalexin, tephrocarpin (**6**), had M^+ 330, and UV maxima (EtOH and EtOH + NaOH; see Experimental for details) strikingly similar to those of **5**. An absorption peak at 257 nm in the alkali spectrum of **6** (cf. pterocarpans **3** [10] and **5**) provided good evidence for aromatic hydroxylation at C-3 [11], whilst rapid dehydration in acidic medium (EtOH + conc. HCl) to yield anhydrotephrocarpin (UV λ_{max} 339 and 357 nm) established the presence of a non-aromatic, tertiary (C-6a) hydroxyl group [3, 12]. A purple–pink (TLC) Hansen reaction [13] revealed the methylenedioxy unit which was provisionally assigned to C-8/C-9 by analogy with phytoalexins **3–5** and in view of the intense UV (EtOH) absorption peak at 310 nm [3]. Tephrocarpin did not afford any colour on TLC plates sprayed with Gibbs reagent-aqueous sodium carbonate [7, 14].

When compared with the ^{1}H NMR spectrum of **5** (Table 1), that of tephrocarpin differed in two notable respects: firstly the H-6a resonance was absent; and secondly, the C-6 protons both appeared with similar chemical shifts. These differences are indicative of a C-6a hydroxyl substituent (cf. pisatin spectrum, Table 1) and hence up-hold the UV data discussed above. It is also clear from Table 1 that the A/D-ring substitution pattern of tephrocarpin is the same as in pterocarpan **5**. Furthermore, assignment of the methylenedioxy group to ring D, rather than to ring A, is fully supported by the chemical shifts of the methylenedioxy protons which are in very close agreement with those of **4** and **5**. In contrast, an A-ring methylenedioxy unit situated at C-3/C-4 would be expected to exhibit a quite different chemical shift pattern (see acanthocarpan spectrum, Table 1). Tephrocarpin is thus 3,6a-dihydroxy-4-methoxy-8, 9-methylenedioxypterocarpan (**6**).

The fifth *Tephrosia* pterocarpan (**7**) was indistinguishable (UV, MS, TLC) from the partially-characterized *Caragana* phytoalexin, acanthocarpan [4]. This non-phenolic, methylenedioxy-substituted compound (M^+ 328) contains an acid-labile C-6a hydroxyl group and, moreover, is spectroscopically (UV in EtOH) comparable with both **5** and **6**. These facts suggest that (as in 4-methoxymaackiain

Table 1. ^{1}H NMR data for *Tephrosia bidwilli* pterocarpans (**4–7**)*

	Pisatin (**4**)	4-Methoxymaackiain (**5**)	Tephrocarpin (**6**)	Acanthocarpan (**7**)
H-1	7.36*d*, 1H (J = 8.5 Hz)	7.05*d*, 1H (J = 8.5 Hz)†	7.04*d*, 1H (J = 8.5 Hz)†	7.00*d*, 1H (J = 8.5 Hz)†
H-2	6.63*q*, 1H (J = 8.5, 2.5 Hz)	6.59*d*, 1H (J = 8.5 Hz)	6.59*d*, 1H (J = 8.5 Hz)	6.61*d* 1H (J = 8.5 Hz)
H-4	6.39*d*, 1H (J = 2.5 Hz)	—	—	—
H-6ax/eq	4.11*s*, 2H	*ca* 3.65*m*, 1H, H-6*ax*‡ 4.37*m*, 1H, H-6*eq*‡	4.13*s*, 4.15*s*, 2H	4.16*s*, 2H
H-6a	—	*ca* 3.65*m*, 1H	—	—
H-7	6.89*s*, 1H	6.89*s*, 1H§	6.90*s*, 1H	6.91*s*, 1H
H-10	6.35*s*, 1H	6.39*s*, 1H§	6.35*s*, 1H	6.36*s*, 1H
H-11a	5.29*s*, 1H	5.50*m*, 1H	5.27*s*, 1H	5.30*s*, 1H
O–CH_2–O (A-ring)	—	—	—	5.92*d*, 6.00*d*, 2H (J = 1.1 Hz)
O–CH_2–O D–ring)	5.92*d*, 5.94*d*, 2H (J = 0.9 Hz)	5.92, 5.93, 2H	5.92*d*, 5.94*d*, 2H (J = 1.0 Hz)	5.94, 5.95, 2H
OMe	3.75*s*, 3H	3.76*s*, 3H	3.74*s*, 3H	—

*Solvent, $(CD_3)_2CO$; chemical shifts are expressed as δ values (TMS reference); figures in parentheses refer to coupling constants.

†Long-range coupling (J = *ca* 0.5 Hz) with H-11a also observed.

‡Assignments based on those given by Woodward [19] for synthetic 3,9-dihydroxypterocarpan.

§Assigned on the basis of expected substituent effects, and the broader signal width of H-7 due to long-range coupling with H-6a.

and tephrocarpin) the aromatic rings of acanthocarpan are oxygenated at positions 3/4 and 8/9, a view confirmed by comparison of the ^{1}H NMR data for **5–7** (Table 1). The acanthocarpan ^{1}H NMR spectrum also revealed two methylenedioxy groups. These must be sited at C-3/C-4 and C-8/C-9 and interestingly can be distinguished by their chemical shifts. Thus, the 8/9-methylenedioxy groups in compounds **4–7** give rise to two very close proton resonances at *ca* δ 5.93 which may or may not exhibit 1 Hz coupling. On the other hand, the 3/4-methylenedioxy attachment of **7** appears as two clearly distinct proton resonances (δ 5.92 and 6.00) with 1.1 Hz coupling. Acanthocarpan is therefore defined as 6a-hydroxy-3,4:8,9-dimethylenedioxypterocarpan (**7**).

The *T. bidwilli* pterocarpans all give large $[\alpha]_D$ values and can thus be assigned the following absolute configurations [11]; compounds **3** and **5** (6a*R*; 11a*R*), and compounds **4**, **6** and **7** (6a*S*; 11a*S*). Although (+)-6a*R*; 11a*R*-pisatin is known to be a major phytoalexin of *Pisum sativum* [12] and some *Lathyrus* species (e.g. *L. sativus*; [Ingham, J. L. and Markham, K. R. unpublished results]), the (−)-enantiomer described above has not previously been discovered as a natural product.

This paper reports for the first time that, like many Papilionoideae [1, 3], fungus-treated *Tephrosia* spp. possess the capacity to produce pterocarpan phytoalexins. One of these—acanthocarpan (**7**) from *T. bidwilli*—is especially notable because it is as yet the only characterized pterocarpan with methylenedioxy substitution of both rings A and D. Interestingly, however, the apparently healthy roots of *T. maxima* contain a closely related substance, namely maximaisoflavone A (7,8:3′,4′-dimethylenedioxyisoflavone, **8** [15]), and this suggests that other comparable isoflavonoids might well occur elsewhere in *Tephrosia*. The precise biosynthetic origin of acanthocarpan remains to be established although clearly it could arise either indirectly from 4-methoxymaackiain (**5**) or directly from tephrocarpin (**6**), since phenols with *ortho*-methoxylation are known to be precursors of their methylenedioxy analogues [16]. Apart from *T. bidwilli*, **7** has recently been encountered as a leaf phytoalexin in *T. rosea* (a species also of Australian origin) where it co-occurs with both **4** and **5**.

EXPERIMENTAL

Plant material. Seeds of *Tephrosia bidwilli* Benth. were supplied by the Curator, King's Park and Botanic Garden, Perth, Australia. Plants were grown (20–24°) in a greenhouse without supplementary lighting, leaflets for fungus inoculation being collected at varying intervals over a period of *ca* 18 months.

Isolation and purification of pterocarpans **3–7**. Si gel TLC (Merck, F-254, layer thickness 0.25 mm) of *H. carbonum*-induced diffusate extracts (EtOAc) using $CHCl_3$–MeOH 50:1 (5 hr equilibration at 20°) gave prominent fluorescence-quenching zones at R_f 0.81 (**5**), 0.73 (**4**+**7**), 0.61 (**3**) and 0.44 (**6**). After elution (EtOH), the components of these bands were purified (Si gel TLC) as follows: **3**, *n*-pentane–Et_2O-glacial HOAc (PEA) (75:25:1), ×3; **4**+**7**, PEA (75:25:3), ×2 gave **4** (upper zone) and **7** (lower zone); **5**, PEA (75:25:1) (R_f 0.46); and **6**, PEA (75:25:3), ×4. Further TLC of **6** (C_6H_6–MeOH, 9:1, R_f 0.40) was also occasionally required. Compounds **3–7** were all homogeneous when chromatographed in several additional TLC solvent systems. Fungus-induced diffusates contained substantial quantities of 4-methoxymaackiain (*ca* 35 μ g/ml) and acanthocarpan (*ca* 30 μ g/ml based on $\log \epsilon = 4.11$ at 308 nm for **5** [17]), but much smaller amounts of maackiain (*ca* 5 μg/ml), pisatin (*ca* 5 μg/ml) and tephrocarpin (*ca* 15 μg/ml based on $\log \epsilon$ for **5** [17]). The phytoalexins were not produced following treatment of detached *T. bidwilli* leaflets with droplets of deionized H_2O.

Maackiain (**3**). Diazotized *p*-nitroaniline reaction, yellow; Hansen reaction, purple-pink. UV and MS data as lit. [10, 18]. $[\alpha]_{589nm}$ −212° (*ca* 0.07 mg in 1 ml MeOH).

Pisatin (**4**). Hansen reaction, purple-pink. UV and MS data as lit. [12, 18]. $[\alpha]_{589nm}$ −258° (*ca* 0.17 mg in 1 ml MeOH).

4-Methoxymaackiain (**5**). Diazotized *p*-nitroaniline reaction, yellow; Hansen reaction, purple-pink. UV λ_{max}^{EtOH} nm: 214 (100), 236 sh (42), 273 sh (7), 283 sh (11), 311 (35); $\lambda_{max}^{EtOH+NaOH}$ nm: 212, 256, 295 sh, 311. MS: 314 $[M]^+$ (100). $[\alpha]_{589nm}$ −201° (*ca* 0.76 mg in 1 ml MeOH).

Tephrocarpin (**6**). Diazotized *p*-nitroaniline reaction, yellow; Hansen reaction, purple-pink. UV λ_{max}^{EtOH} nm: 212 (100), 240 sh (23), 273 sh (6), 284 sh (9), 310 (23); $\lambda_{max}^{EtOH+NaOH}$ nm: 214, 257, 295 sh, 311; $\lambda_{max}^{EtOH+conc.HCl}$ nm: principal pterocarpene maxima at 339 and 357. MS: 330 $[M]^+$ (100). $[\alpha]_{589 \text{ and } 365 nm}$ −267° and −667° respectively (*ca* 0.04 mg in 1 ml MeOH).

Acanthocarpan (**7**). Hansen reaction, purple-pink. UV λ_{max}^{EtOH} nm: 212 (100), 244 sh (30), 276 sh (9), 286 sh (12), 309 (27); $\lambda_{max}^{EtOH+conc.HCl}$ nm: principal pterocarpene maxima at 339 and 355. MS: 328 $[M]^+$ (100). $[\alpha]_{589nm}$ −259° (*ca* 0.23 mg in 1 ml MeOH).

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