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FUNGAL MODIFICATION OF PTEROCARPAN PHYTOALEXINS FROM *MELILOTUS ALBA* AND *TRIFOLIUM PRATENSE*

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Key Word Index—*Melilotus alba*; *Trifolium pratense*; Leguminosae; Trifolieae; clovers; pterocarpans; phytoalexin; antifungal activity; fungal metabolism; detoxification.

Abstract—Medicarpin (3-hydroxy-9-methoxypterocarpan), an isoflavonoid phytoalexin characteristic of sweetclover (*Melilotus alba*), is metabolised by three fungi, *Botrytis cinerea*, *Colletotrichum lindemuthianum* (race β) and *C. coffeanum* (isolates CCA and CCP) to afford products with decreased antifungal activity. Pterocarpan phytoalexins from red clover (*Trifolium pratense*) are similarly modified. A fourth organism, *Helminthosporium carbonum* apparently lacks this ability. Fungal-mediated tranformation principally involves hydroxylation, methylation and demethylation although ring fission has also been noted.

INTRODUCTION

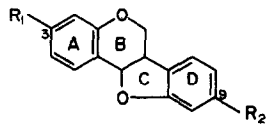
It is now well established that products with decreased antifungal activity may result from microbial metabolism of the isoflavonoid phytoalexins [1] characteristically produced by certain members of the Leguminosae (subfamily Lotoideae). This ability is generally, although not exclusively [2,3], confined to those fungi which normally are pathogenic on leguminous species. In several instances [4,5], detoxification of isoflavonoid phytoalexins appears to be an essential prerequisite for fungal pathogenicity. A variety of modification mechanisms have been described including aromatic and non-aromatic hydroxylation [6], demethylation[7], ring cleavage [8] and oxidation [5].

Leaves of white sweetclover (*Melilotus alba* Desr., tribe Trifolieae) produce one isoflavonoid phytoalexin [9] (medicarpin, **1**, 3-hydroxy-9-methoxypterocarpan) following inoculation with various non-pathogenic fungi including *Helminthosporium carbonum* Ullstrup, the agent responsible for a leaf-spot disease of *Zea mays*. In contrast, samples from leaves infected by *Colletotrichum lindemuthianum* Sacc. & Magn. (Bri. & Cav.) and *Botrytis cinerea* Fr. (two fungi pathogenic to leguminous species), contain medicarpin together with numerous fungal-mediated conversion products. This paper describes the chemical identification and antifungal activity of these and similar metabolites from red clover (*Trifolium pratense* L.; tribe Trifolieae), a species previously reported to accumulate both medicarpin and the related isoflavonoid phytoalexin, maackiain **2** (3-hydroxy-8,9-methylenedioxypterocarpan) [10].

RESULTS

(a) Melilotus alba

When detached leaflets of *M. alba* were inoculated with spore suspensions of *B. cinerea* and incubated for 48 hr the resulting diffusate [11] was found to contain eight phenolic compounds. Two of these were identified as the known isoflavonoids medicarpin **1** and vestitol **3** (7,2′-dihydroxy-4′-methoxyisoflavan) by comparison (MS, UV, TLC) with authentic material [11,12]. The others were the hitherto undescribed pterocarpans, demethylmedicarpin (3,9-dihydroxy- (**4**), 6a-hydroxymedicarpin (3,6a-dihydroxy-9-methoxy- (**5**), 6a-hydroxyisomedicarpin (3-methoxy-6a,9-dihydroxy- (**6**), 4-hydroxydemethylmedicarpin (3,4,9-trihydroxy- (**7**), 4-hydroxymedicarpin (3,4-dihydroxy-9-methoxy- (**8**) and 4-hydroxyhomopterocarpin (3,9-dimethoxy-4-hydroxy- (**9**).



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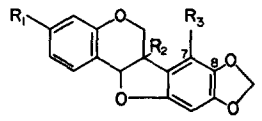

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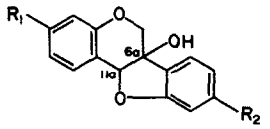

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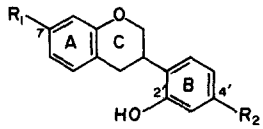

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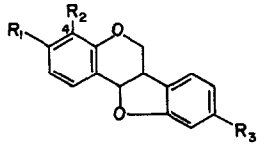

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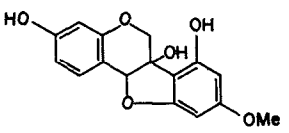


16

Studies to be reported elsewhere [9] have established that medicarpin is a phytoalexin produced when *M. alba* and 18 other *Melilotus* species are infected by the non-pathogenic fungus, *H. carbonum*. Although vestitol is also common as a leguminous phytoalexin [9] or phytoalexin precursor [13] it does not accumulate when *M. alba* is inoculated with *H. carbonum* or subjected to UV (254 nm) irradiation [9]. On this basis, vestitol and compounds **4**–**9** (none of which occur

in extracts from irradiated leaves) were considered to be medicarpin transformation products. The fungus, *Stemphylium botryosum* has previously been reported to convert phaseollin and compounds **1** and **2** to their isoflavan derivatives [8,14,15].

Compound **4** was identified by MS and UV analysis; methylation afforded a dimethyl ether indistinguishable (MS, UV, TLC) from homopterocarpin **10** (3,9-dimethoxypterocarpan). Attempts to obtain **4** by demethylation (using pyridinium chloride) of either **1** or **10** were unsuccessful owing to rapid fission of the non-aromatic pyran (B) and/or dihydrofuran (C) rings. Two products resulting from attempted demethylation of **10** were provisionally identified as the phenolic benzofuran derivatives **11** (M^+ 284, *m/e* 269; λ_{max} (EtOH) 271 and 311 nm [16]; λ_{max} (EtOH + NaOH) 328 nm) and **12** (M^+ 256; λ_{max} (EtOH) 271 and 310 nm; λ_{max} (EtOH + NaOH) 282 and 327 nm). Although two fungi can demethylate pisatin at C-3 [4,7], the present report is apparently the first to describe the C-9 demethylation of a pterocarpan phytoalexin. Recent studies [17] have provided evidence to suggest that **4** is not an intermediate in the biosynthesis of **1**.

MS and UV analyses indicated that **5** and **6** were isomeric 6a-hydroxylated pterocarpans. Both compounds could be methylated to afford products identical (MS, UV, TLC) with variabilin **13** (3,9-dimethoxy-6a-hydroxypterocarpan) obtained from **10** by synthesis or fungal modification. Structure **5** was confirmed by hydrogenation which gave a product chromatographically indistinguishable from vestitol (**3**). Formation of this compound establishes unequivocally the 3-hydroxy-9-methoxy substitution pattern of **5**. On this basis, **6** can be logically formulated as the previously undescribed pterocarpan, 6a-hydroxyisomedicarpin. Hydrogenation of this compound afforded very small quantities of a phenolic derivative (provisionally identified as 7-methoxy-2',4'-dihydroxyisoflavan **14**) chromatographically similar to vestitol. Unfortunately, **14** was obtained in quantities insufficient for confirmatory MS or UV investigation. At least two fungi namely, *B. cinerea* and *C. lindemuthianum* are known to convert the pterocarpan phytoalexin, phascollin, to its 6a-hydroxy derivative [3,6].

Identification of the new trihydroxypterocarpan **7** was based on MS, UV and TLC investigation of the parent phenol and its triacetoxy and triMe derivatives. The triMe ether proved to be identical (MS, UV, TLC) with 3,4,9-trimethoxypterocarpan **15** [18], a compound also obtained by methylation of **8** and **9**. Since MS analysis clearly established **9** to be a monohydroxydimethoxypterocarpan and as the compound gave an immediate blue colouration with Gibbs reagent, the lone OH group was assigned to C-4. Data recorded for **8** also indicated a 3,4,9-trioxygenation pattern with a C-4 OH group (Gibbs reagent, deep blue). As **8** is presumably derived from medicarpin, it can most logically be formulated as 4-hydroxymedicarpin; an alternative structure namely, 3-methoxy-4,9-dihydroxypterocarpan, cannot be eliminated from available data although compound **8** appears more biogenetically acceptable.

Extracts of diffusates from *C. lindemuthianum* (race β)-inoculated leaves contained **1**, **4**, **5** and **6** together with small quantities of a third 6a-hydroxylated pterocarpan which reacted strongly to Gibbs reagent (blue). This substance was identified as the new isoflavonoid **16** (3,6a,7-trihydroxy-9-methoxypterocarpan) from its MS and UV spectra and marked polarity (R_f 0.04) in $CHCl_3$–MeOH (50:1). *Colletotrichum lindemuthianum* metabolises phaseollin to give the 6a,7-dihydroxy derivative [6] and would be expected to similarly transform medicarpin. Although the MS exhibited only a single ion (*m/e* 284) this could be derived by the facile dehydration of **16** (MW 302). Similarly, in the MS of 6a,7-dihydroxyphaseollin, the first fragment occurs at *m/e* 336 ($M^+ - H_2O$) and not at *m/e* 354 (M^+) as would normally be expected [6].

Two isolates of *C. coffeanum* Noack (CCA and CCP [19]), the casual agent of coffee berry disease and a non-pathogen of leguminous species, also converted medicarpin to its 6a-hydroxy derivative although no other transformation products were isolated from the 48 hr leaf diffusates.

Table 1. Concentration of medicarpin and other isoflavonoids in diffusates (48 hr) from fungus-inoculated and UV-irradiated leaves of *M. alba**†‡

	Compound								
	1	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**16**
Biotic inducer									
Helminthosporium carbonum	98	—	—	—	—	—	—	—	—
Botrytis cinerea	60	2	2	6	4	4	4	8	—
Colletotrichum lindemuthianum (β)	43	—	23	25	13	—	—	—	6
C. coffeanum (CCA)	38	—	—	9	—	—	—	—	—
C. coffeanum (CCP)	66	—	—	20	—	—	—	—	—
Abiotic inducer									
UV (254 nm) light	47	—	—	—	—	—	—	—	—
Control									
Aq 0.05% Tween-20	—	—	—	—	—	—	—	—	—

* Values in μg/ml diffusate. † Diffusate volumes were as follows: *H. carbonum*, 30.5 ml; *B. cinerea*, 65 ml; *C. lindemuthianum*, 4.5 ml; *C. coffeanum* (CCA), 5.5 ml; *C. coffeanum* (CCP), 4.5 ml; UV light, 3.7 ml; 0.05% Aq. Tween-20, 42 ml. ‡ Concentrations of medicarpin and vestitol are based on previously reported extinction coefficients (**1**, $\log \epsilon = 3.89$ at 287 nm [22]; **3**, $\log \epsilon = 3.62$ at 285 nm [12]). Values for other compounds are calculated either from $\log \epsilon$ for **1** (**4**, **5**, **6** and **16**) or 4-methoxymedicarpin ($\log \epsilon = 4.09$ at 284 nm [18]) (**7**, **8** and **9**).

In contrast to the aforementioned fungi, only medicarpin was present in diffusates from leaves inoculated with *H. carbonum*. No other phenolic or antifungal compounds (as determined by TLC bioassays using *Cladosporium herbarum* as the test organism [20]) were associated with samples from infected leaves. Large quantities of medicarpin, but no other substance, also accumulated after detached leaves had been briefly exposed to short-wavelength (254 nm) UV light (Table 1). This abiotic induction firmly establishes medicarpin as a product of *M. alba*; it also suggests that additional components associated with diffusates from infected leaves have arisen via the fungal-mediated transformation of medicarpin. The possibility that phytoalexin modification may reflect the activities of bacteria or other phyllosphere-inhabiting micro-organisms (which may proliferate in the applied droplets) appears very unlikely since no compounds other than medicarpin were detected when spore suspensions of *H. carbonum* were incubated on sweetclover leaves for periods ranging from 48–90 hr. Diffusates (48 hr) did not contain excessive numbers of bacteria [21] and no qualitative difference was apparent between samples from control and fungus-infected (*H. carbonum* and *B. cinerea*) leaves. Isoflavonoid compounds were absent from the control diffusates.

Isoflavonoid concentration in leaf diffusates

The relative concentration (as determined by UV spectrophotometry) of medicarpin, and its various transformation products, in diffusates from fungus-inoculated and UV-irradiated leaves is shown in Table 1. Although medicarpin was produced in response to each fungus, it accumulated to the greatest extent in diffusates from leaves infected by *H. carbonum*, an organism characterised by its apparent inability to metabolise isoflavonoid phytoalexins (Fig. 3). Table 1 clearly demonstrates that, apart from *C. coffeanum* CCA, the difference between the medicarpin level of diffusates from *H. carbonum*-infected leaves and those from leaves inoculated with *B. cinerea*, *C. coffeanum* CCP and *C. lindemuthianum*, can be largely accounted for by the presence of phytoalexin transformation products.

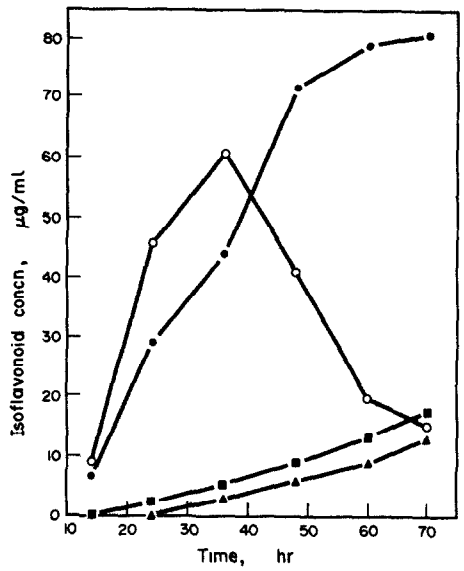


Fig. 1. Accumulation (μg/ml) of medicarpin (○), 6a-hydroxymedicarpin (■) and 6a-hydroxyisomedicarpin (▲) in diffusates from *B. cinerea*-infected leaves of *M. alba*. The *H. carbonum*/medicarpin accumulation curve (●) is also shown.

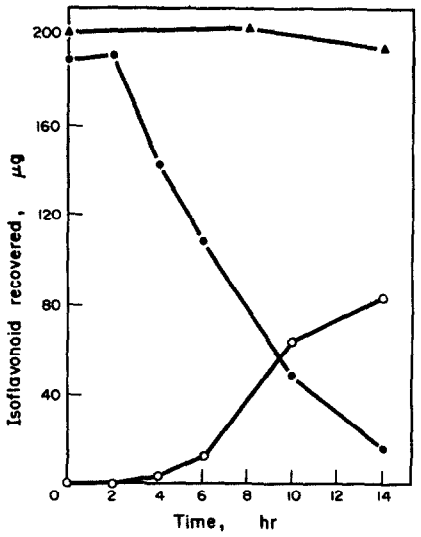


Fig. 3. Recovery (μg) of medicarpin (●) and 6a-hydroxymedicarpin (○) from shake cultures of *B. cinerea*. Recovery of medicarpin from cultures of *H. carbonum* (▲) is also shown.

Accumulation of medicarpin and its transformation products

Detached sweetclover leaflets were inoculated with spore suspensions of *H. carbonum*, *B. cinerea* or *Colletotrichum coffeanum* (CCP) and diffusates removed at intervals of 14, 24, 36, 48, 60 and 70 hr thereafter; control samples were collected at 14 and 70 hr only. Medicarpin was produced rapidly by the *H. carbonum*-infected leaves (Fig. 1) and accumulated steadily throughout the entire experimental period; maximum production occurred 14 hr (7 μg/ml) to 48 hr (72 μg/ml) after inoculation. Beyond this point, production gradually declined with the final (70 hr) sample containing little medicarpin additional to that of the 48 hr diffusate. Phytoalexin biosynthesis was not associated with the water-inoculated leaves.

In diffusates from leaves affected by *B. cinerea*, medicarpin reached a maximum concentration (61 μg/ml) 36 hr after inoculation (Fig. 1). Thereafter (and in direct contrast to the *H. carbonum*-accumulation curve) its diffusate level rapidly declined and by 70 hr was little greater (16 μg/ml) than that recorded for the 14 hr sample (9 μg/ml). This marked decrease coincided with the appearance of 6a-hydroxymedicarpin (first visible after 24 hr) and 6a-hydroxyisomedicarpin (visible after 36 hr) as well as **7**, **8** and **9** (36 hr onwards) although accumulation curves were not determined for these latter three compounds.

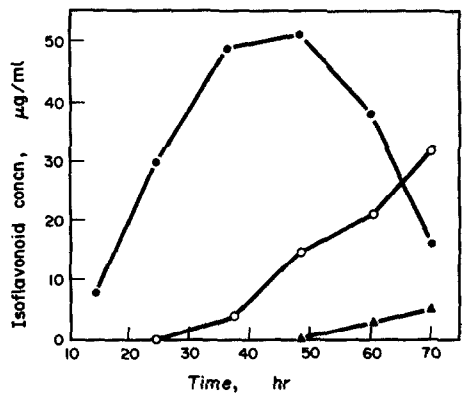


Fig. 2. Accumulation (μg/ml) of medicarpin (●), 6a-hydroxymedicarpin (○) and 6a,7-dihydroxymedicarpin (▲) in diffusates from *C. coffeanum* (CCP)-infected leaves of *M. alba*.

The *C. coffeanum* (CCP)/medicarpin curve also resembled that of *B. cinerea* (Fig. 2) although the concentration decline did not occur until *ca* 50 hr after inoculation. 6a-hydroxymedicarpin was evident from 36 hr onwards; in contrast to an earlier study (Table 1), very small quantities of 3,6a,7-trihydroxy-9-methoxypterocarpan were also encountered although this compound was isolated only from the 60 and 70 hr diffusates. The presence of **16** in the 60 hr but not the 48 hr diffusates suggests that *C. coffeanum* requires long exposure to 6a-hydroxymedicarpin before producing enzymes capable of further metabolizing this compound.

Antifungal activity of medicarpin and compounds **3–6**

Mycelial growth tests indicated that medicarpin was highly inhibitory to *H. carbonum* (ED_{50} 25 μg/ml) but much less active against *B. cinerea* (ED_{50} 66 μg/ml). The latter fungus also appears to be relatively insensitive to the effects of phaseollin (ED_{50} > 50 μg/ml) [23] and pisatin (ED_{50} *ca* 100 μg/ml) [24]. However, three medicarpin transformation products namely, 6a-hydroxymedicarpin, 6a-hydroxyisomedicarpin and demethylmedicarpin were essentially inactive against *B. cinerea* having ED_{50} values well in excess of 100 μg/ml. Other studies have demonstrated that phaseollin is considerably more inhibitory to *B. cinerea* than its 6a-hydroxy derivative [3]; the low antifungal activity of the 6a-hydroxylated pterocarpan, pisatin, has already been noted [24]. In

contrast, the isoflavan vestitol (3) had an ED_{50} value (58 μg/ml) comparable with that of medicarpin. Higgins [15] found that both maackiain (2) and the fungus-derived isoflavan were equally inhibitory to the germ tube growth of *Stemphylium botryosum*. Although conversion to the isoflavan cannot be regarded as detoxification *per se*, studies with maackiain/*S. botryosum* [15] suggest that this initial step is the first in a series leading to the formation of non-toxic products. Rapid detoxification of vestitol might explain why this compound does not accumulate in *B. cinerea*-induced diffusates (Table 1).

Apart from medicarpin, only vestitol (ED_{50} 17 μg/ml) and demethylmedicarpin were tested against the mycelial growth of *H. carbonum*. Like *B. cinerea*, this fungus was more sensitive to medicarpin than to its demethyl analogue (ED_{50} > 50 μg/ml). On chromatograms sprayed with *C. herbarum* [20], inhibition zones attributable to medicarpin were visibly greater than those associated with comparable amounts of **4**, **5** and **6**. The antifungal properties of **7**, **8**, **9** and **16** were not determined.

In vitro *production of 6a-hydroxymedicarpin*

Preliminary experiments indicated that medicarpin (at a concn of *ca* 10 μg/ml medium) disappeared within 12 hr when incubated in shake culture with *B. cinerea*. Culture extracts provided no evidence to suggest that medicarpin had been superficially adsorbed onto the fungal mycelium or that it was present (in an unaltered form) within the hyphal strands. When these experiments were repeated with larger quantities of medicarpin (*ca* 20 μg/ml), it was found that disappearance of the phytoalexin was paralleled by the accumulation of 6a-hydroxymedicarpin (Fig. 3). In contrast, medicarpin was recovered unchanged from shake cultures of *H. carbonum*, from flasks containing phytoalexin but no fungus and from flasks containing **1** and autoclaved mycelium (15 psi/20 min) of *B. cinerea*. Phenolic material was not associated with cultures of *B. cinerea* growing in the absence of medicarpin. A short, but definite, lag-phase (*ca* 2 hr) prior to medicarpin detoxification may reflect the production, by *B. cinerea*, of an inducible phytoalexin-transforming enzyme (or enzymes) as suggested for other fungi [15,25]. Surprisingly, no evidence was obtained for the accumulation of medicarpin conversion products other than **5**. It should be appreciated, however, that 6a-hydroxymedicarpin was produced under conditions very different from those of the *B. cinerea/M. alba* interaction. In the latter instance, the fungus is exposed to continual medicarpin production and is essentially bathed in a concentrated solution (or suspension) of **1** (and its conversion products) for periods of 48 hr or longer.

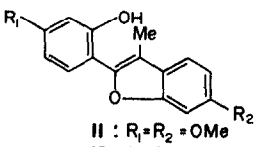
(b) *Trifolium pratense*

Diffusates (48 hr) from *H. carbonum*-infected leaves contained substantial quantities of medicarpin (45 μg/ml) and maackian **2** (42 μg/ml based on log ε = 3.83 at 310 nm [26]). The identity of both compounds was established by comparison (UV, TLC) with authentic material. Neither phytoalexin was associated with the control diffusates. A TLC bioassay [20] gave no indication of other antifungal material in samples from the infected leaves. Diffusates from UV-irradiated leaves contained small quantities of both **1** (19 μg/ml) and **2** (14 μg/ml).

Medicarpin and maackiain also accumulated when *T. pratense* was inoculated with *B. cinerea*, *C. lindemuthianum* and *C. coffeanum* (CCP) although the diffusate concentration of each phytoalexin was low (*ca* 10 μg/ml) in comparison with the values recorded for samples from the *H. carbonum*-infected leaves. This feature is consistent with fungal-mediated pterocarpan metabolism.

In addition to **1** (11 μg/ml) and **2** (8 μg/ml), *C. coffeanum*-induced diffusates (60 hr) were found to contain other phenolic material. A fraction with R_f 0.16 ($CHCl_3$–MeOH; 50:1) was identified as a mixture of 6a-hydroxymedicarpin (**5**) and 6a-hydroxymaackiain **17** (3,6a-dihydroxy-8,9-methylenedioxypterocarpan) after UV and MS analysis and TLC against authentic **5** and **17**, the latter compound being obtained by fungal demethylation of pisatin [7]. Dehydration (conc HCl) afforded a product having UV maxima (340 and 357 nm) identical with those of anhydrosophorol (3-hydroxy-8,9-methylenedioxypterocarp-6a-ene). Diffusate concentrations of 6a-hydroxymedicarpin (based on log ε for **1** [22]) and 6a-hydroxymaackiain (log ε = 3.87 at 309 nm [7]) were 75 and 46 μg/ml respectively.

A second phenolic fraction (R_f 0.05, $CHCl_3$–MeOH; 50:1) gave a positive Gibbs test (blue) and had MS and UV spectra consistent with its formulation as a mixture of 6a,7-dihydroxymedicarpin (**16**) and the hitherto undescribed pterocarpan, 6a,7-dihydroxymaackiain **18** (3,6a,7-trihydroxy-8,9-methylenedioxypterocarpan). Like **16** obtained from *M. alba*, MS analysis of the above mixture did not reveal the expected M^+ at *m/e* 302 (**16**) or *m/e* 316 (**18**); only fragments corresponding to the dehydroderivatives were apparent. Applying log ε for **1** [22] and **17** [7] respectively gave the following diffusate concentrations, (a) 6a,7-dihydroxymedicarpin, 7 μg/ml and (b) 6a,7-dihydroxymaackiain, 5 μg/ml. Attempts to separate **5** from **17** and **16** from **18** were unsuccessful.



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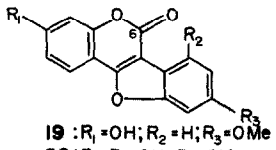

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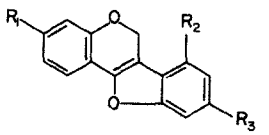

21 : R_1=OH; R_2=H; R_3=OMe
22 : R_1=R_2=OH; R_3=OMe

DISCUSSION

The demonstration that *B. cinerea*, *C. lindemuthianum* and *C. coffeanum* possess the ability to directly 6a-hydroxylate the pterocarpan nucleus is interesting for it immediately suggests that further detoxification may proceed via 6a/11a-dehydration and oxidation of the resulting pterocarp-6a-ene to afford a coumestan (6-oxo-pterocarp-6a-ene). Although some dehydropterocarpans exhibit antifungal activity (H. D. Van Etten, *pers. commun.*), coumestans apparently do not. The above process may either be directly influenced by fungal enzymes or, alternatively, may result indirectly from processes associ-

ated with pathogenesis. For example, *B. cinerea* is known to produce oxalic acid [27], a compound which presumably lowers the pH of colonised tissues to levels optimal for the activity of a fungal pectin methylesterase [27]. In turn, this enzyme releases pectic acid which may contribute to the pH decrease. Under these circumstances it is conceivable that compounds such as 6a-hydroxymedicarpin and 6a,7-dihydroxymedicarpin might dehydrate, the resulting anhydro-derivatives then undergoing autoxidation [28] to yield the inactive coumestan analogues.

It is interesting, therefore, that coumestans characteristically accumulate when the leaf tissues of certain leguminous species (e.g. alfalfa and white clover) are infected by pathogenic fungi. These are the precise conditions under which phytoalexin detoxification would be expected to occur. For instance, white clover (*Trifolium repens*) leaflets infected by *Pseudopeziza trifolii* contain high levels of 3-hydroxy-9-methoxycoumestan (**19**) and trifoliol **20** (3,7-dihydroxy-9-methoxycoumestan) [29] both of which could theoretically arise via fungal detoxification of medicarpin (e.g. **1** → **5** → **21** → **19**; **1** → **5** → **16** → **22** → **20**), a compound produced in quantity (> 100 μg/ml diffusate) by the *H. carbonum*-infected leaves [9]. Chromatograms of diffusate extracts from leaves of *M. alba* inoculated with *B. cinerea* or *C. lindemuthianum* (but not *H. carbonum*) have been found to exhibit blue fluorescent regions at or near the origin ($CHCl_3$–MeOH, 50:1). Although no compounds have been firmly identified, this feature is characteristic of several coumestans (e.g. **19** and **20** [30]) and may well indicate their presence in leaf diffusates. It should be noted, however, that whilst the above theory is attractive from several aspects it seems unlikely that phytoalexin detoxification is the only factor influencing the coumestan content of forage legumes. Aphid infestation, physiogenic leaf-spotting and growth conditions have all been reported to increase leaf coumestan levels [30] although none would be expected to stimulate phytoalexin biosynthesis.

The fungal 6a-hydroxylation of **1** and **2** suggests that in leguminous species a comparable plant-mediated transformation may lead to phytoalexins such as pisatin. In the fungus-infected leaves of *Pisum sativum*, pisatin could be produced from maackiain [31] (itself possibly derived from 7,3′-dihydroxy-4′-methoxyisoflavone, P. M. Dewick, *personal communication*) either by (*a*) direct 6a-hydroxylation and subsequent C-3 methylation or (*b*) methylation (to afford pterocarpin, 3-methoxy-8,9-methylenedioxypterocarpan) immediately prior to 6a-hydroxylation. Preliminary data (D. Robeson, *personal communication*) suggest that traces of 6a-hydroxymaackiain (**17**) may accumulate when leaflets of *P. sativum* (and certain pisatin-producing *Lathyrus* species) are inoculated with the non-pathogenic fungus, *Helminthosporium sativum*; although fungal C-3 demethylation of pisatin cannot be completely excluded, the tentative identification of **17** as a diffusate component provides at least some circumstantial support for one (*a*) of the above mentioned biosynthetic routes.

In addition to 6a-hydroxylation, *B. cinerea* can apparently *ortho*-hydroxylate the medicarpin molecule in a manner comparable with bacterial transformation of the flavanonol, taxifolin [32,33]. For taxifolin, further oxidation leads to A-ring fission and eventual molecular cleavage. *B. cinerea* may similarly degrade medicarpin although evidence suggests that C-3 methylation (8 → 9) or C-9 demethylation (8 → 7) can also occur. The fungal methylation of isoflavonoid derivatives has not been previously reported. As *B cinerea* can apparently methylate pterocarpans at C-3, it is surprising that neither **10** or **13** were isolated from the 48 hr diffusates. Chromatogram bioassays indicate that both compounds have little or no antifungal activity against the spore germination of *C. herbarum* [9]. Although *B. cinerea* oxidises the non-isoflavonoid phytoalexin, capsidiol, to afford the ketonic detoxification product capsenone [34], it is apparently unable to similarly transform **1**; there was no evidence to suggest that 48 hr diffusates from *M. alba* contained a medicarpin analogue corresponding to 1a-hydroxyphaseollone [5,25], a keto-substituted phaseollin detoxification product of *Fusarium solani* f. sp. *phaseoli*.

The present study has also provided evidence to suggest that demethylmedicarpin (**4**) is identical with CP-1, a medicarpin metabolite of undetermined constitution previously obtained when leaflets of *Medicago sativa* were inoculated with the tomato pathogen, *Colletotrichum phomoides* [2]. Like **4** (which is produced in quantity by *C. lindemuthianum*, Table 1), CP-1 reacts strongly with diazotised *p*-nitroaniline (orange) and exhibits UV maxima (EtOH) similar to those of **1**; as expected CP-1 also has a low R_f value (0.12) in *n*-pentane-Et_2O–HOAc (75:25:1) (cf. **4**, R_f 0.10) and is apparently stable in this acidic solvent system thereby arguing against its possible formulation as 6a-hydroxyisomedicarpin (**6**).

Two fungi namely, *Fusarium solani* f. sp. *pisi* and *Ascochyta pisi* have been reported to effect the C-3 demethylation of pisatin [4,7]. However, no evidence for production of demethylmedicarpin was obtained when leaves of *M. alba* were inoculated with conidial suspensions of either *A. pisi* race 1 or race 2 although both stimulated marked accumulation of **1** [9]. As pisatin demethylation appears to determine the pathogenicity of *A. pisi* to pea [4], the inability of this fungus to modify **1** probably explains both its sensitivity to this phytoalexin (ED_{50} *ca* 30 μg/ml, race 2) and its non-pathogenic nature on *M. alba*. The C-3 and C-9 demethylation of pterocarpan phytoalexins presumably involves different and highly specific methyl transferase enzymes.

Finally, in the Leguminose (as in other plant families) phytoalexin metabolism is undoubtedly an important prerequisite for colonisation by certain fungi; however, that this feature is not invaribly linked to pathogenicity is demonstrated by the ability of several non-pathogenic organisms (e.g. *C. coffeanum*) to modify the pterocarpan molecule. These fungi may well be highly sensitive to both the phytoalexin and its modification product(s). Although several metabolites associated with diffusates of *B. cinerea* (a fungus normally non-pathogenic to *M. alba*) have only slight antifungal properties, it is possible that the total isoflavonoid level of affected tissues may still present an environment unfavourable for successful mycelial development. Other fungi pathogenic to *M. alba*. (e.g. *Stagnospora meliloti*) apparently do not metabolise medicarpin [9]; here, pathogenicity may be related to factors such as the insensitivity of mycelial growth to *in vivo* phytoalexin levels.

EXPERIMENTAL

MS were determined using a heated direct inlet system (source temp 180°; probe temp 30°) at 100 μA, 70 eV and 8 kV.

Plant material. Melilotus alba Desr. cv Arctic and *Trifolium pratense* L. cv Altaswede were grown from seed (20°, *ca*

6500 lx) for 12 months prior to use. Leaf material was removed from established plants at intervals of *ca* 7 weeks to encourage new growth.

Fungal material. *Helminthosporium carbonum* Ullstrup and *Botrytis cinerea* Fr. were maintained on V-8 vegetable juice agar [35]; *Colletotrichum lindemuthianum* (Sacc. & Magn.) Bri. & Cav. (race β) and *C. coffeanum* Noack (isolates CCA and CCP) were cultured on a glucose-neopeptone medium [36]. All fungi were incubated 21° ± 2°) under illumination (12 hr/day) from two white fluorescent tubes and a single near UV 'Black–Light' (max emission *ca* 365 nm) to promote sporulation. Cultures were used after 18–25 days incubation.

In vivo *production of phytoalexins and fungal conversion products.* Detached leaflets were inoculated with conidial suspension of *H. carbonum* and *B. cinerea* (*ca* 5×10^4 spores/ml) or the *Colletotrichum* spp. (*ca* 2.5×10^5 spores/ml) [11] and incubated (22°, *ca* 400 lx) for 48 or 60 hr. Control leaves received droplets of 0.05% aq. Tween-20 [11]. Resulting diffusates [11] were extracted with EtOAc (×4) and organic fractions reduced to dryness (*in vacuo*, 40°) prior to Si gel F_{254} TLC ($CHCl_3$–MeOH; 50:1). *A. Melilotus alba*: Five phenolic bands (*B-1*, R_f 0.74; *B-2*, R_f 0.47; *B-3*, R_f 0.30; *B-4*, R_f 0.19 and *B-5*, R_f 0.15) were obtained by TLC of diffusates from *B. cinerea*-infected leaves. *B-4* separated into two phenolic zones (*B-4.1*, R_f 0.68: *B-4.2*, R_f 0.56) in Et_2O–*n*-hexane (3:1). The above bands were purified as follows, (i) *B-1*, $CHCl_3$–CCl_4 (3:1) gave **9** (R_f 0.34), (ii) *B-2*, *n*-pentane–Et_2O–HOAc (75:25:1), **1** (R_f 0.44), (iii) *B-3*, C_6H_6–MeOH (9:1), **8** (R_f 0.28), (iv) *B-4.1*, $CHCl_3$–MeOH (50:1), **4** (R_f 0.18) and **3** (R_f 0.22), (v) *B-4.2*, $CHCl_3$–MeOH (50:1, ×5) **5** (*upper zone*) and **6** (*lower zone*), (vi) *B-5*, *n*-pentane–Et_2O–HOAc (75:25:4, ×2) **7**. TLC of diffusates from *C. lindemuthianum* and *C. coffeanum*-infected leaves gave **1**, **4**, **5**, **6** (purified as above) and **16** (R_f 0.04) which was purified in $CHCl_3$–MeOH (20:3, R_f 0.46). All of these compounds were homogenous when chromatographed in additional solvent systems.

B. Trifolium pratense. Si gel TLC ($CHCl_3$–MeOH, 50:1) of diffusates from *C. coffeanum*-inoculated leaves gave phenolic bands at R_f 0.61 (*B-1*, compounds **1** and **2**), R_f 0.16 (*B-2*, **5** + **17**) and R_f 0.05 (*B-3*, **16** + **18**). These bands were purified as follows, (i) *B-1*, $CHCl_3$ (×3) followed by resolution of **1** and **2** using *n*-pentane–Et_2O–HOAc (75:25:3, ×3), (ii) *B-2*, $CHCl_3$ (×4) and (iii) *B-3*, C_6H_6–MeOH (9:1, ×8).

Phytoalexin induction by UV irradiation. Detached leaflets [11] were irradiated (30 min, 254 nm) from a distance of *ca* 5 cm. Treated leaves were then inoculated with droplets of 0.5% aq Tween-20 and incubated (see above) for 48 hr prior to diffusate extraction and TLC purification as described for *M. alba* and *T. pratense.*

Bioassays. TLC bioassays and mycelial growth tests were undertaken as previously described [37]. For the latter, each isoflavonoid compound was incorporated into agar at concn from 10–50 μg/ml (**4**) or 10–100 μg/ml (**1**, **3**, **5**, **6**). The % inhibition of radial mycelial growth for each treatment (relative to that of the control) was determined after *ca* 48 hr.

In vitro *production of 6a-hydroxymedicarpin* (**5**) *and variabilin* (**13**). Spores of *H. carbonum* and *B. cinerea* from 18-day-old cultures (see *Fungal material*) were suspended in modified Czapek-Dox (Oxoid) liquid medium (adjusted to pH 6 with Pi buffer), filtered through sterile cheese-cloth (3 layers) and adjusted to a concn of *ca* 5×10^4 spores/ml with additional medium. The suspension was placed in 25 ml conical flasks (10 ml/flask) and pre-incubated (24 hr; 24°) on a waterbath-shaker (*ca* 100 strokes/min). Medicarpin in EtOH was then added to the flasks (final concn *ca* 20 μg **1**/ml medium) and these incubated as above for 0–14 hr periods (see Fig. 3). At each time interval 2 flasks were removed, their contents pooled and the medium separated from fungal mycelium by centrifugation. Fungal pellets were resuspended in H_2O (5 ml) and centrifugation repeated. Bulked supernatants were extracted (EtOAc, 4 × 20 ml) and the organic phase reduced to dryness prior to Si gel TLC (Et_2O–*n*-hexane, 3:1). Fungal mycelium was macerated in EtOH (2 × 5 ml) and then centrifuged. Ethanolic supernatants were bulked, reduced to dryness and chromatographed as described above. **1** and **5** were visible as quenching bands (**1**, R_f 0.65; **5**, R_f 0.38). An ethanolic soln of homopterocarpin (**10**) was added to cultures of *B. cinerea* (final concn *ca* 30 μg/ml medium) and these incubated for 10 hr. Extraction and TLC purification ($CHCl_3$) afforded variabilin (R_f 0.36) together with unchanged **10** (R_f 0.69).

3-hydroxy-9-methoxypterocarpan (**1**). Diazotised *p*-nitroaniline, yellow; λ_{max}^{EtOH} (nm) 213, 228 *sh*, 282, 287; $\lambda_{max}^{EtOH + NaOH}$ (nm) 217, 248, 288, 293 *sh*; MS *m/e* (rel int) M^+ 270 (100), 269 (40), 255 (33), 227 (5), 226 (8), 197 (10), 181 (10), 161 (16), 149 (19), 148 (31), 147 (23), 134 (12). *Monoacetate* (C_5H_5N–Ac_2O–HOAc) (R_f 0.89, $CHCl_3$); λ_{max}^{EtOH} (nm) 212, 286, 292 *sh*; MS *m/e* (rel int) M^+ 312 (33), 271 (17), 270 (100). *Monomethyl ether* (CH_2N_2) (R_f 0.77, $CHCl_3$); λ_{max}^{EtOH} (nm) 209, 228 *sh*, 281 *sh*, 286, 292 *sh*; MS *m/e* (rel int) M^+ 284 (100), 283 (75), 269 (15), 161 (34), 149 (19), 147 (45); α_D^{22} −214° (2 mg in 1 ml MeOH).

7,2'-Dihydroxy-4'-methoxyisoflavan (**3**). Diazotised *p*-nitroaniline, yellow; Gibbs reagent, deep blue; λ_{max}^{EtOH} (nm) 210, 226 *sh*, 281, 285; $\lambda_{max}^{EtOH + NaOH}$ (nm) 218, 241 *sh*, 295; MS *m/e* (rel int) M^+ 272 (31), 151 (12), 150 (100), 149 (15), 138 (17), 137 (32), 135 (15), 121 (16), 109 (12), 107 (15).

3,9-Dihydroxypterocarpan (**4**). Diazotised *p*-nitroaniline, orange; λ_{max}^{EtOH} (nm) 211, 228 *sh*, 283 *sh*, 288, 294 *sh*; $\lambda_{max}^{EtOH + NaOH}$ (nm) 215, 248, 297; MS *m/e* (rel int) M^+ 256 (100), 255 (58), 149 (16), 148 (24), 134 (23). *DiMe ether* (R_f 0.90, $CHCl_3$); UV and MS as given for monomethyl ether of **1**.

3,6a-Dihydroxy-9-methoxypterocarpan (**5**). Diazotised *p*-nitroaniline, yellow; λ_{max}^{EtOH} (nm) 214, 229 *sh*, 282, 286, 292 *sh*; $\lambda_{max}^{EtOH + NaOH}$ (nm) 215, 250, 288, 292 *sh*; $\lambda_{max}^{EtOH + HCl}$ (nm) 211, 231, 251 *sh*, 282 sh, 289, 335, 352; MS *m/e* (rel int) M^+ 286 (100), 271 (21), 268 (26: $M^+ - H_2O$), 267 (15), 259 (10), 258 (50), 257 (12), 253 (7), 241 (35), 229 (12), 227 (30). *Monomethyl ether* (R_f 0.33, $CHCl_3$); λ_{max}^{EtOH} (nm) 212, 228 *sh*, 281, 286, 291 *sh*; $\lambda_{max}^{EtOH + HCl}$ (nm) 210, 231, 251 *sh*, 280 *sh*, 288, 325 *sh*, 335, 352; MS *m/e* (rel int) M^+ 300 (100), 284 (16), 285 (57), 282 (29: $M^+ - H_2O$), 281 (17), 272 (41), 257 (17), 255 (25), 241 (28), 151 (40), 150 (22), 149 (74), 148 (14).

Hydrogenation of **5**. 6a-hydroxymedicarpin (*ca* 250 μg), EtOH (2 ml), HCO_2H (2 ml), HOAc (1 ml) and 10% Pd–C (*ca* 2 mg) were shaken in an atmosphere of H_2 at 60° for 20 min. After removal of catalyst and solvent the residue was chromatographed ($CHCl_3$–MeOH; 100:2) to afford **3** (R_f 0.30). Diazotised *p*-nitroaniline, yellow; Gibbs reagent, deep blue.

3-Methoxy-6a,9-dihydroxyterocarpan (**6**). Diazotised *p*-nitroaniline, orange; λ_{max}^{EtOH} (nm) 213, 228 *sh*, 282, 286, 292 *sh*; $\lambda_{max}^{EtOH + NaOH}$ (nm) 216, 248 *sh*, 281 *sh*, 288, 297 *sh*; $\lambda_{max}^{EtOH + HCl}$ (nm) 212, 231, 251 *sh*, 281 *sh*, 288, 336, 353; MS *m/e* (rel int) M^+ 286 (8), 271 (12), 269 (17), 268 (92: $M^+ - H_2O$), 267 (100), 258 (10), 253 (8), 252 (7). *Monomethyl ether* (R_f 0.33, $CHCl_3$); UV and MS as given for **5**.

Hydrogenation of **6**. 6a-hydroxyisomedicarpin (*ca* 250 μg) was hydrogenated as described for **5**. TLC gave a small quantity of **14** (R_f 0.29, $CHCl_3$–MeOH; 50:1). Diazotised *p*-nitroaniline, orange; Gibbs reagent, blue.

3,4,9-Trihydroxypterocarpan (**7**). Diazotised *p*-nitroaniline, yellow brown. Gibbs reagent, blue/grey; λ_{max}^{EtOH} (nm) 216, 284, 293 *sh*, $\lambda_{max}^{EtOH + NaOH}$ (nm) 222, 247 *sh*, 300; MS *m/e* (rel int) M^+ 272 (100). *TriMe ether* (R_f 0.86, $CHCl_3$); λ_{max}^{EtOH} (nm) 216, 230 *sh*, 284, 291 *sh*; MS *m/e* (rel int) M^+ 314 (100), 313 (11), 299 (23); *Triacetate* (R_f 0.73, $CHCl_3$); λ_{max}^{EtOH} (nm) 213, 280; MS *m/e* (rel int) M^+ 398 (9), 356 (26), 315 (9), 314 (81), 273 (10), 272 (100).

3,4-Dihydroxy-9-methoxypterocarpan (**8**). Diazotised *p*-nitroaniline, orange; Gibbs reagent, deep blue; λ_{max}^{EtOH} (nm) 219, 229 *sh*, 285, 292 *sh*; $\lambda_{max}^{EtOH + NaOH}$ (nm) 222, 246, 295; MS *m/e* (rel int) M^+ 286 (100), 285 (31), 271 (31). *DiMe ether* (R_f 0.86, $CHCl_3$); UV and MS as given for triMe ether of **7**. *Diacetate* (R_f 0.57 $CHCl_3$); λ_{max}^{EtOH} (nm) 216, 230 *sh*, 282, 288 *sh*; MS *m/e* (rel int) M^+ 370 (18), 329 (14), 328 (85), 287 (16), 286 (100), 285 (13), 271 (15).

3,9-Dimethoxy-4-hydroxypterocarpan (**9**). Diazotised *p*-nitro-

aniline, orange; Gibbs reagent, deep blue; λ_{max}^{EtOH} (nm) 216, 285, 292 *sh*; $\lambda_{max}^{EtOH + NaOH}$ (nm) 224, 260, 286, 293 *sh*; MS *m/e* (rel int) M^+ 300 (100), 299 (14), 285 (29). *Mono Me ether* (R_f 0.77, $CHCl_3$); UV and MS as given for triMe ether of 7. *Monoacetate* (R_f 0.48, $CHCl_3$); λ_{max}^{EtOH} (nm) 212, 230 *sh*, 282, 292 *sh*; MS *m/e* (rel int) M^+ 342 (37), 301 (13), 300 (100), 299 (11), 285 (18).

3,6a,7-Trihydroxy-9-methoxpterocarpan (**16**). Diazotised *p*-nitroaniline, yellow-orange; Gibbs reagent, blue; λ_{max}^{EtOH} (nm) 211, 227 *sh*, 282, 287, 292 *sh*; $\lambda_{max}^{EtOH + NaOH}$ (nm) 217, 247, 296; $\lambda_{max}^{EtOH + HCl}$ (nm) 212, 230 *sh*, 251 *sh*, 280 *sh*, 288, 326 *sh*, 335, 352; MS *m/e* (rel int) 284 (100: $M^+ - H_2O$).

3-Hydroxy-8,9-methylenedioxypterocarpan (**2**). Diazotised *p*-nitroaniline, yellow; λ_{max}^{EtOH} (nm) 209, 232 *sh*, 282, 287, 311; $\lambda_{max}^{EtOH + NaOH}$ (nm) 218, 252, 301, 311 *sh*; MS *m/e* (rel int) M^+ 284 (100), 283 (27), 175 (10), 162 (15), 151 (10), 149 (23), 147 (7), 134 (14).

3,6a-Dihydroxy-9-methoxypterocarpan/3,6a-dihydroxy-8,9-methylenedioxypterocarpan (**5** + **17**). Diazotised *p*-nitroaniline, yellow–orange; λ_{max}^{EtOH} (nm) 213, 228 *sh*, 281, 286, 309; $\lambda_{max}^{EtOH + NaOH}$ (nm) 223, 252, 288 *sh*, 293, 315 *sh*; $\lambda_{max}^{EtOH + HCl}$ (nm) 213, 231 *sh*, 251 *sh*, 282 *sh*, 287, 340, 357; MS *m/e* M^+ 300/286, *m/e* 282 (M^+ 300 − H_2O), 281, 272, 271, 268 (M^+ 286 − H_2O), 267, 258, 257, 255, 243, 242, 241, 227, 163.

3,6a,7-Trihydroxy-9-methoxypterocarpan/3,6a,7-trihydroxy-8,9-methylenedioxypterocarpan (**16** + **18**). Diazotised *p*-nitroaniline, orange; Gibbs reagent, blue; λ_{max}^{EtOH} (nm) 211, 227 *sh*, 281, 286, 306; $\lambda_{max}^{EtOH + NaOH}$ (nm) 214, 250, 287–291, 311 *sh*; $\lambda_{max}^{EtOH + HCl}$ (nm) 212, 230 *sh*, 248 *sh*, 286, 325 *sh*, 333, 341, 349; MS *m/e* 298 (M^+ 316 − H_2O), 284 (M^+ 302 − H_2O).

Synthesis of 3,9-dimethoxy-6a-hydroxypterocarpan (**13**). Homopterocarpin (25 mg), EtOH (10 ml) and conc HCl (0.1 ml) were refluxed for 5 min. The reaction mixture was diluted with H_2O (50 ml), reduced (*in vacuo*, 40°) to 10 ml and the above process then repeated with a further quantity (100 ml) of H_2O. The final aq suspension (*ca* 10 ml) was diluted (40 ml H_2O), extracted with Et_2O (4 × 50 ml) and the organic fractions pooled and reduced to dryness. Si gel PLC (0.5 mm, $CHCl_3$–MeOH; 50:1) gave two major fractions at R_f 0.82 (*B-1*) and R_f 0.64 (*B-2*). These were chromatographed in *n*-pentane–Et_2OH–HOAc (75:25:1) to afford: (i) *2-(2-hydroxy-4-methoxyphenyl)-5-methoxy-3-methylbenzofuran* **11** (*B-1*, R_f 0.45; diazotised *p*-nitroaniline, orange–brown; Gibbs reagent, blue; λ_{max}^{EtOH} (nm) 214, 227 *sh*, 271 311; $\lambda_{max}^{EtOH + NaOH}$ (nm) 211, 250 *sh*, 273 *sh*, 322; MS *m/e* (rel int) M^+ 284 (100), 269 (81). *Monoacetate* (R_f 0.54, $CHCl_3$; λ_{max}^{EtOH} (nm) 212, 228 *sh*, 271, 309; MS *m/e* (rel int) M^+ 326 (21), 284 (100), 269 (74)) and (ii) *7,4′-dimethoxy-2′-hydroxyisoflav-3-ene* (*B-2*, R_f 0.42; diazotised *p*-nitroaniline, yellow; Gibbs reagent blue; λ_{max}^{EtOH} (nm) 220, 244 *sh*, 327; $\lambda_{max}^{EtOH + NaOH}$ (nm) 215, 342; MS *m/e* (rel int) M^+ 284 (100) 283 (20), 270 (20), 269 (70)). Acetylation and TLC purification gave the 2′-acetoxyisoflav-3-ene derivative (R_f 0.84, $CHCl_3$; λ_{max}^{EtOH} (nm) 216, 241, 302 *sh*, 320; MS *m/e* (rel int) M^+ 326 (26), 285 (20), 284 (100), 283 (40), 270 (12), 269 (46). This was slowly added to OsO_4 (40 mg) [16] in Et_2O (6 ml) and the soln left for 18 hr (9 hr at 0°; 9 hr at 20°) before pptn of the osmate ester [16]. After centrifugation the osmate ester pellet was dissolved in $CHCl_3$ (20 ml) and shaken with aq Na_2CO_3 [16]. $CHCl_3$ was then removed *in vacuo* and the residue chromatographed ($CHCl_3$–MeOH; 50:1) to afford impure **13** (R_f 0.77). Final purification (Et_2O–*n*-hexane; 3:1) gave (±)-variabilin (*ca* 1.5 mg) (R_f 0.72) identical (MS, UV, TLC) with the monoMe ether of 6a-hydroxymedicarpin (**5**). Variabilin was accompanied by small quantities of a phenolic compound R_f 0.50, Et_2O–*n*-hexane; 3:1) provisionally identified as the new isoflavanone, 7,4′-dimethoxy-2′-hydroxyisoflavanone (diazotised *p*-nitroaniline, yellow; Gibbs reagent, blue; λ_{max}^{EtOH} (nm) 215, 228 *sh*, 275, 312; $\lambda_{max}^{EtOH + NaOH}$ (nm) 219, 232 *sh*, 278, 312 *sh*; M^+ 300).

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