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ISOFLAVAN PHYTOALEXINS FROM *ANTHYLLIS*, *LOTUS* AND *TETRAGONOLOBUS*

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Key Word Index—*Anthyllis*; *Lotus*; *Tetragonolobus*; *Helminthosporium carbonum*; Leguminosae; isoflavans; phytoalexins; antifungal activity; disease resistance.

Abstract—Two previously unreported phytoalexins, 7,4'dihydroxy-2'-methoxy- and 7,2',4'-trihydroxyisoflavan, have been isolated from the fungus-inoculated leaves of *Anthyllis vulneraria* and 5 *Tetragonolobus* species. Examination of *Lotus corniculatus* revealed the co-occurrence of the latter with the known isoflavans, vestitol and sativan. Only 7,2'4'-trihydroxyisoflavan and vestitol were produced by the closely related *L. uliginosus*.

INTRODUCTION

There is abundant evidence that species belonging to the Leguminosae (subfamily Lotoideae) commonly accumulate isoflavonoid phytoalexins following inoculation with fungi, bacteria or viruses [1, 2]. Although pterocarpan and isoflavan phytoalexins usually co-occur [2, 3], only examples of isoflavans (vestitol **3** and sativan **4**) have been isolated from fungus-inoculated leaves of birdsfoot trefoil (*Lotus corniculatus*) [4]. More recently, Hijwegen [5] reported that pterocarpans were produced by kidney-vetch (*Anthyllis vulneraria*) and marsh birdsfoot trefoil (*L. uliginosus*) although experimental verification of this claim was not presented. These and several species from the genus *Tetragonolobus* have recently been examined [3] and found to produce two new isoflavans for which the common names isovestitol (**1**) and demethylvestitol (**2**) are proposed. Pterocarpan phytoalexins were not obtained from any of the species examined.

RESULTS AND DISCUSSION

Detached leaflets of *Tetragonolobus maritimus* were inoculated with a conidial suspension of *Helminthosporium carbonum* and the resulting diffusate [6] collected after 48 hr incubation. Extraction and TLC purification afforded small quantities of a phenolic substance (diazotized *p*-nitroaniline, orange; Gibbs reagent, no reaction) with MS and UV (see Experimental) characteristic of a trioxygenated (dihydroxymonomethoxy) isoflavan. The A-ring (OH) and B-ring (OMe: OH) substituents of this compound (**1**) were deduced from the MS [7, 8] and assigned to C-7, 2' and 4' on biogenetic grounds [9]. Compound **1** readily formed a diacetate and dimethyl ether, the latter being indistinguishable (UV, MS, TLC) from 7,2',4'-trimethoxyisoflavan **5** [6]. Demethylation of **1** and authentic vestitol **3** also gave identical products (**2**). The above data allow **1** to be formulated as 7,4'-dihydroxy-2'-methoxyisoflavan (isovestitol): location of the single B-ring hydroxyl group at C-4' follows from the negative Gibbs test [10]. In contrast, isoflavans with a free position *para* to the 2'-hydroxyl group (e.g. vestitol, **3**) give a deep blue colouration with Gibbs reagent; no exceptions to this rule have yet been reported. Apart from its occurrence in the tribe Loteae (Table 1), isovestitol has recently been identified as a phytoalexin of *Trifolium arvense* (tribe Trifolieae) and *T. rubens* [3].

In addition to isovestitol, diffusates from *Anthyllis vulneraria* and 4 other *Tetragonolobus* species also contained appreciable quantities of a second phenolic derivative. This substance was identified as 7,2',4'-trihydroxyisoflavan **2** (demethylvestitol) by comparison (UV, MS, TLC) with the demethyl product of vestitol and by UV and MS examination of its triacetate and trimethyl ether. Unlike isovestitol, no evidence has been obtained to suggest that demethylvestitol is produced by leguminous species other than those listed in Table 1.

The identification of vestitol **3** as a phytoalexin of *T. requienii* and *Lotus uliginosus* was established by comparison with an authentic sample obtained from *L. corniculatus* [4]. Sativan **4** was isolated only from *L. corniculatus*, a previously recognized source [4].

When incorporated into agar (5–50 $\mu g\ ml^{-1}$) and tested against *H. carbonum*, compounds **1–4** all caused a marked reduction of mycelial growth. Demethylvestitol (ED_{50} *ca* 38 $\mu g\ ml^{-1}$) was the least active whilst vestitol (ED_{50} 17 $\mu g\ ml^{-1}$) and isovestitol (ED_{50} *ca* 23 $\mu g\ ml^{-1}$) were slightly more antifungal than medicarpin (ED_{50} 25 $\mu g\ ml^{-1}$) [6], a phytoalexin commonly encountered in the tribe Trifolieae [3, 11, 12]. The mycelial growth of *H. carbonum* was most affected by sativan (ED_{50} 10 $\mu g\ ml^{-1}$).

For compounds **1–4** increasing antifungal activity would appear to correlate with increasing *O*-methylation i.e. **2** < **1** ≃ **3** < **4**. Similarly, *H. carbonum* is considerably more sensitive to medicarpin than to its demethyl analogue (ED_{50} > 50 $\mu g\ ml^{-1}$) [13]. It is noteworthy that both medicarpin and demethylmedicarpin are less inhibitory to *H. carbonum* than are the derived isoflavans, vestitol and demethylvestitol. The enhanced fungitoxic properties of mono and dimethoxylated isoflavans presumably reflects their more lipophilic nature

Table 1. Isoflavan concentration in 48 hr leaf diffusates ($\mu g\ ml^{-1}$) and tissues ($\mu g\ g^{-1}$) from fungus-inoculated species of *Anthyllis*, *Lotus* and *Tetragonolobus**†‡

Species	Compound 1	2	3	4
Anthyllis vulneraria L.	58(ND)	33(ND)	—	—
Lotus corniculatus L.	—	37(TR)	115(348)	68(990)
L. uliginosus Schkuhr	—	63(TR)	85(424)	—
Tetragonolobus biflorus (Desr.) Ser.	9(143)	11(TR)	—	—
T. maritimus (L.) Roth	15(144)	TR(—)	—	—
T. palaestinus Boiss.	11(111)	20(TR)	—	—
T. purpureus Moench	14(153)	12(TR)	—	—
T. requienii (Mauri ex Sang.) Sang.	75(393)	99(46)	17(TR)	—

* First values refer to diffusate concentrations; figures in parentheses indicate leaf tissue concentrations. † Concentrations of vestitol and sativan are based on previously reported extinction coefficients (**3**, $\log \varepsilon = 3.62$ at 285 nm [4]: **4**, $\log \varepsilon = 3.62$ at 284 nm [6]). Values for **1** and **2** are based on $\log \varepsilon$ for **3**. ‡ Compounds **1–4** were absent from control diffusates and tissues. ND = Not determined. — = Not detectable.

and hence greater capacity to penetrate fungal membranes.

Whereas *T. requienii* produced **1** and **2** in substantial quantities, the 4 other *Tetragonolobus* species accumulated these compounds to a very limited degree (Table 1). Only traces of demethylvestitol were associated with *T. maritimus* and on one occasion this substance was not detected. Over several experiments, the isovestitol content of *T. maritimus* diffusates rarely exceeded 15 $\mu g\ ml^{-1}$ and was never greater than 19 $\mu g\ ml^{-1}$. Considerably higher phytoalexin levels were recorded for *A. vulneraria*, *L. uliginosus* and *L. corniculatus* although for the latter, vestitol and sativan values were somewhat lower than previously described [4]. For all 8 species (Table 1), however, the phytoalexin level of leaf tissues/diffusates 48 hr after inoculation would appear sufficient to inhibit the mycelial development of *H. carbonum*. Compounds **1–4** were not isolated from control samples. Isoflavan accumulation curves for four of the species examined are shown in Fig. 1.

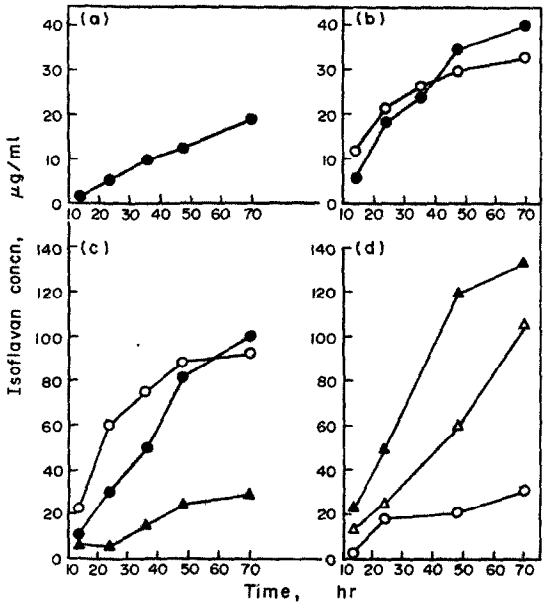
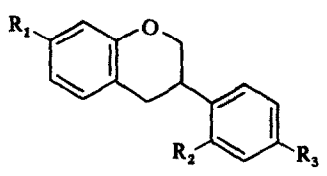


Fig. 1. Accumulation of demethylvestitol (○——○), isovestitol (●——●), vestitol(△——△) and sativan (△——△) in leaf diffusates of *T. maritimus* (a), *A. vulneraria* (b), *T. requienii* (c) and *L. corniculatus* (d)

Lotus corniculatus and *L. uliginosus* are closely related and are difficult or impossible to distinguish on the basis of their morphology [14] or leaf and petal flavonoid patterns (Harborne, J. B., personal communication). Data presented in Table 1 indicate that these species can be readily separated by reference to their leaf phytoalexins. Thus, *L. corniculatus* accumulates substantial quantities of compounds **2–4** whereas *L. uliginosus* is apparently unable to produce even trace quantities of the latter. The present study has also provided some tentative chemical support for the delimitation of *Tetragonolobus* from *Lotus*, two genera which have occasionally been regarded as synonymous [15]. Thus, isovestitol was produced uniformly in *Tetragonolobus* but was not observed in samples from the two *Lotus* species. Similarly, sativan was absent from *Tetragonolobus* whilst vestitol (a major phytoalexin of both *L. corniculatus* and *L. uliginosus*) was also poorly represented in this genus. The leaf phenolic pattern of *T. maritimus* also distinguishes this species from several members of the genus *Lotus* [16].

Despite repeated examination, no evidence has been obtained for production of pterocarpans by members of the tribe Loteae. This contradicts a previous report [5] and is surprising since pterocarpans have been proposed as biosynthetic precursors of 2′-oxygenated isoflavans [17]. Moreover, recent surveys [3, 18] of genera in the Trifolieae indicate that isoflavans almost invariably



1: $R_1 = R_3 = OH$: $R_2 = OMe$
2: $R_1 = R_2 = R_3 = OH$
3: $R_1 = R_2 = OH$: $R_3 = OMe$
4: $R_1 = OH$: $R_2 = R_3 = OMe$
5: $R_1 = R_2 = R_3 = OMe$

co-occur with the pterocarpans from which they can be directly or indirectly derived. In the Loteae, however, this situation may not apply since medicarpin and demethylmedicarpin (the logical precursors of **2** and **3** respectively) were not detected. Nevertheless, for species listed in Table 1 the terminal stages of phytoalexin biosynthesis would appear to involve straightforward methylation reactions, e.g. **2** → **1** (*A. vulneraria*) and **2** → **3** → **4** (*L. corniculatus*). For *T. requienii*, the biosynthetic pathway presumably branches at demethylvestitol to afford the isomeric isoflavans **1** and **3**; C-2′ methylation appears to be the favoured route as **3** accumulates to only a limited extent. Whilst the precursor of demethylvestitol is not immediately apparent, this substance could arise via (a) reduction of an isoflavone, isoflavanone or isoflavene or (b) reduction/ring fission of a dehydropterocarpan. It is interesting, therefore, that *H. carbonum*-inoculated leaflets of *T. maritimus* have been found to produce small quantities of a substance indistinguishable (UV, TLC) from synthetic 3,9-dihydroxydehydropterocarpan (Ingham, J. L., unpublished data). Accumulation of this compound provides some circumstantial support for its involvement in isoflavan biosynthesis. Another compound provisionally identified as a dihydroxy-monomethoxy 2-arylbenzofuran (cf. vignafuran [19]) has also been obtained from *T. maritimus* (Ingham J. L., unpublished data). This substance appears to be a phytoalexin and, like compound **2**, might well be derived from a dehydropterocarpan precursor.

EXPERIMENTAL

MS were determined at 100 μA, 70 eV and 8 kV (source temp. *ca* 180°: probe temp. *ca* 30°).

Plant material. Leaves of *Anthyllis vulneraria* L., *Lotus corniculatus* L., *L. uliginosus* Schkuhr and *Tetragonolobus maritimus* (L.) Roth, were collected from established plants growing at the University of Reading Botanic Garden. Seeds of *T. biflorus* (Desr.) Ser., *T. palaestinus* Boiss., *T. purpureus* Moench and *T. requienii* (Mauri ex Sang.) Sang. were obtained from various European institutes [3] and grown as previously described [20] for *ca* 10 weeks prior to leaflet excision and fungal inoculation.

Induction, isolation and purification of compounds **1–4**. (*a*) *Leaf diffusates.* Detached leaflets were inoculated with a conidial suspension of *Helminthosporium carbonum* Ullstrup [21] and incubated as previously described [13]; control leaflets received droplets of de-ionized H_2O. Diffusate extracts (EtOAc [13]) were reduced to dryness *in vacuo* (40°) and the residue then chromatographed (TLC, Merck Kieselgel 60, F_{254}) in $CHCl_3$–MeOH (50:1) to afford compounds **1** (*ca* R_f 0.47), **2** (0.03), **3** (0.45) and **4** (0.65). Isovestitol and vestitol (or, for *T. requienii*, a combined isovestitol/vestitol zone) were eluted (EtOH) and purified to homogeneity by TLC in *n*-pentane–Et_2O–HOAc (75:25:3, ×3). In this system **1** (*lower*) and **3** (*upper*) were obtained as well separated bands. Eluates of **2** and **4** were further purified using $CHCl_3$–MeOH (5:1) (**2**: R_f 0.54) or *n*-pentane–Et_2O–HOAc (75:25:1) (**4**: R_f 0.38). Compounds **1–4** were absent from the control diffusates. (*b*) *Leaf tissues.* After removal of the diffusates, leaf tissues directly underlying the inoculum or H_2O droplets were excised and macerated in EtOH (*ca* 10 ml/0.2 g fr. tissue). Macerates were centrifuged and the supernatant decanted: the residual pellet was then further extracted (×2 or 3) with EtOH. The bulked supernatants were reduced to dryness (*in vacuo*, 40°) prior to Si gel TLC (Et_2O–*n*-hexane, 3:1). Isoflavan zones were located at *ca* R_f 0.71 (**1**), 0.22 (**2**), 0.76 (**3**) and 0.80 (**4**). Each compound was eluted and purified as described above.

7,4′-dihydroxy-2′-methoxyisoflavan (**1**). Diazotized *p*-nitroaniline, orange: λ_{max}^{EtOH} (nm) 209, 225*sh*, 282, 285*sh*: $\lambda_{max}^{EtOH+NaOH}$ (nm) 219, 243, 295: MS *m/e* (rel. int.) 273(7), 272(M^+; 33), 151(12), 150(100), 149(22), 148(5), 147(7), 138(18), 137(31), 136(5), 135(16), 134(5), 123(15), 121(18), 109(11), 107(20). *Dimethyl ether* (CH_2N_2) (R_f 0.88, $CHCl_3$): λ_{max}^{EtOH} (nm) 209, 226, 280, 284, 289*sh*: MS *m/e* (rel int) 301(9), 300(M^+: 41), 165(14), 164(100), 152(27),151(45), 150(11), 149(68), 148(8), 137(14), 136(9), 135(7), 121(45). *Diacetate* (C_5H_5N–Ac_2O–HOAc) (R_f 0.53, $CHCl_3$): λ_{max}^{EtOH} (nm) 209, 224*sh*, 275*sh*, 280: MS *m/e* (rel. int.) 357(3), 356(M^+: 8), 315(8), 314(35), 273(10), 272(40), 151(18), 150(100), other fragments as given for **1**.

7,2′,4′-trihydroxyisoflavan (**2**). Diazotized *p*-nitroaniline, orange/yellow; Gibbs reagent, purple/blue: λ_{max}^{EtOH} (nm) 209, 225*sh*, 283; $\lambda_{max}^{EtOH+NaOH}$ (nm) 219, 244*sh*, 297; MS *m/e* (rel. int.) 259(10), 258(M^+: 61), 149(38), 137(16), 136(100), 135(63), 134(26), 123(81), 111(36), 109(31), 107(22). *Trimethyl ether*: TLC, UV and MS as given for dimethyl ether of **1**. *Triacetate* (R_f 0.51, $CHCl_3$): λ_{max}^{EtOH} (nm) 212, 275, 283: MS *m/e* (rel. int.) 385(4), 384(M^+: 9), 343(11), 342(25), 301(7), 300(27), 259(9), 258(19), 137(28), 136(100), other fragments as given for **2**.

7,2′-dihydroxy-4′-methoxyisoflavan (**3**). Diazotized *p*-nitroaniline, yellow: Gibbs reagent, deep blue; UV and MS as lit. [13]. Dimethyl ether: TLC, UV and MS as given for diMe ether of **1**. Diacetate (R_f 0.51, $CHCl_3$): λ_{max}^{EtOH} (nm) 208, 225, 276, 282: MS *m/e* (rel. int.) 357(2), 356(M^+: 7), 315(2), 314(10), 273(2), 279(9), 271(3), 151(11), 150(100), other fragments as lit. [13]. This was demethylated with pyridinium chloride to yield **2**.

7-hydroxy-2′,4′-dimethoxyisoflavan (**4**). Diazotized *p*-nitroaniline, yellow: UV as lit. [6]; MS *m/e* (rel. int.) 287(11), 286 (M^+: 52), 165(14), 164(100), 153(6), 152(33), 151(74), 150(8), 149(36), 147(12), 135(10), 134(6), 122(11), 121(44), 119(10), 115(6), 108(10), 107(16). Monomethyl ether; TLC, UV and MS as given for diMe ether of **1**. *Monoacetate* (R_f 0.87, $CHCl_3$): λ_{max}^{EtOH} (nm) 208, 227*sh*, 278, 284: MS *m/e* (rel. int.) 329(5), 328 (M^+; 18), 287(3), 286(13), 165(13), 164(100), other fragments as given for **4**.

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