Flavonoid and Isoflavonoid Compounds from Leaves of Sainfoin (*Onobrychis vicii folia*)

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The 5-deoxyisoflavones, afrormosin and formononetin have been isolated from healthy leaves of *Onobrychis viciifolia*. In addition to these compounds, several other flavonoid (isoliquiritigenin; liquiritigenin; garbanzol) and isoflavonoid (medicarpin; vestitol) derivatives including the new isoflavanone, vestitone (7,2′-dihydroxy-4′-methoxyisoflavanone), are present in leaves inoculated with the fungus *Helminthosporium carbonum*.

Isoflavonoid compounds are frequently isolated from species belonging to the Leguminosae (subfamily Papilionoideae) where they normally occur constitutively in non-living heartwood or in the healthy, undamaged tissues of leaves and roots [1]. Occasionally, however, isoflavonoids may accumulate in living cells as a defensive response following invasion by fungi or other micro-organisms [2]. These induced compounds, which are termed phytoalexins, have already been obtained from numerous temperate and tropical legumes [2, 3]. The present paper describes the isolation of both induced and constitutive isoflavonoids from leaves of sainfoin (*Onobrychis viciifolia* Scop., tribe Hedysareae), a perennial forage legume extensively cultivated on calcareous soils in Europe. No isoflavonoid compounds have yet been obtained from either *O. viciifolia* or any other member of the tribe Hedysareae.

Detached leaflets were inoculated with spore suspensions of *Helminthosporium carbonum* Ullstrup and the resulting diffusate extracted and chromatographed ($CHCl_3$: MeOH, 50 : 1) as previously described [4, 5] to afford diazotised *p*-nitroaniline positive bands at R_F 0.50 (*B1*), 0.22 (*B2*), 0.16 (*B3*), 0.10 (*B4*) and 0.07-origin (*B5*). TLC bioassays using *Cladosporium herbarum* Fr. [6] indicated that fungitoxic activity was associated only with *B1* and *B3*. All zones were eluted (EtOH) and their components purified by single- or multiple-development TLC in *n*-pentane : Et_2O : HOAc (PEA) (see Experimental) to afford small quantities of the previously unreported isoflavonoid, 7,2′-dihydroxy-4′-methoxyisoflavanone (**1**) (*vestitone*) in addition to the following known compounds, 3-hydroxy-9-methoxypterocarpan (**2**) (medicarpin), 6,4′-dimethoxy-7-hydroxyisoflavone (**3**) (afrormosin), 7-hydroxy-4′-methoxyisoflavone (**4**) (formononetin), 7,2′-dihydroxy-4′-methoxyisoflavan (**5**) (vestitol), 2′,4′,4-trihydroxychalcone (**6**) (isoliquiritigenin) and 7,4′-dihydroxyflavanone (**7**) (liquiritigenin). Traces of a pale-yellow fluorescent compound having UV maxima essentially identical to those of 3,7,4′-trihydroxyflavanone (**8**) (garbanzol) were also isolated from leaf diffusates, although this provisional identification has still to be confirmed. Garbanzol has previously been obtained only from chickpeas, *Cicer arietinum* (tribe Vicieae) [7]. Compounds **2**–**7** were identified by direct comparison (UV, TLC) with authentic samples. Diffusates from leaves treated with de-ionised H_2O contained variable amounts of **3** and **4** but not compounds **1**, **2** or **5**–**8**.

The MS of **1** (see Experimental) was typical of a 2′-hydroxylated isoflavanone [5], exhibiting a small molecular ion at *m/e* 286 (corresponding to $C_{16}H_{14}O_5$) and lacking the M^+-1 peak which is reported to characterise flavanone derivatives [8], *cf.* liquiritigenin (**7**) M^+ 256 (100%), 255 (70) and its 4′-O-methyl ether, M^+ 270 (100), 269 (50) [3]. The isoflavanone nature of **1** was also apparent from its UV (MeOH) spectrum; pronounced bathochromic shifts were evident in the presence of NaOH (57 nm; aromatic OH) and NaOAc (58 nm; C-7 OH) but not $AlCl_3$ (absence of C-5 OH). Acetylation and methylation gave respectively a diacetate and a diMe ether whilst hydrogenation at room temp. as previously described [9] afforded vestitol (**5**) indistinguishable (MS, UV, TLC) from an authentic specimen. Compound **1** is thus 7,2′-dihydroxy-4′-methoxyisoflavanone, a structure recently confirmed by comparison with synthetic material [10].

The concentration of compounds **1**–**8** in control and fungus-induced diffusates from leaves of *O. viciifolia* is shown in Table I. Although medicarpin, afrormosin and formononetin were also present in diffusates from 3 additional *Onobrychis* spp., only 2 of these apparently produced vestitol (Table I). The concentration of medicarpin and vestitol in leaf tissues underlying the inoculum droplets was deter-

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Table I. Concentration (μg/ml)[a,b] of flavonoid and isoflavonoid compounds in diffusates (48 h) from leaves of *O. viciifolia* and 3 other *Onobrychis* species.

Species	Compound							
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**
O. viciifolia Scop.	3(−)	39(−)	12(TR-10)	13(TR-8)	25(−)	5(−)	6(−)	4(−)
O. arenaria (Kit.) DC.	ND	8(−)	10(10)	6(4)	−	ND	ND	ND
O. montana DC.	ND	10(−)	14(16)	8(5)	15(−)	ND	ND	ND
O. tanaitica Sprengel	ND	13(−)	10(6)	6(6)	18(−)	ND	ND	ND

ND, not determined; TR, trace; −, not detectable.
[a] Concentrations of compounds **1**−**7** are based on previously reported extinction coefficients (**1** [10]; **2/4/6/7** [17]; **3** [16]; **5** [11]). The value given for **8** is based on log ε for **7**.
[b] Data in parentheses refer to control diffusates.

mined as follows. Ethanolic leaf extracts [11] were chromatographed (Et_2O : *n*-hexane, 3 : 1) and zones corresponding to markers of **2** (approx. R_F 0.66) and **5** (approx. R_F 0.61) eluted with EtOH. The medicarpin thus obtained was purified by TLC in $CHCl_3$ (×3); further purification of vestitol was unnecessary. Although both compounds were readily isolated from *H. carbonum*-inoculated leaves (**2**, 135 μg/g fr wt; **5**, 98 μg/g) they could not be detected in control extracts.

Quantitative determination of afrormosin and formononetin by the above procedure was complicated because both isoflavones co-chromatographed with dense chlorophyll bands (**3**, approx. R_F 0.33; **4**, approx. R_F 0.43). This difficulty was overcome by use of a technique involving base/acid partition. Leaf extracts (EtOH) were reduced to dryness (*in vacuo*, 40°), the residue dissolved in CCl_4 (60 ml) and the resulting solution shaken (×4) with equal vol. aq NaOH (0.2 N). The pooled, pale-yellow, NaOH fractions were then acidified (pH 3, 2 N HCl), extracted (×4) with 0.5 vol CCl_4 and the organic phase reduced to dryness prior to TLC ($CHCl_3$: MeOH, 50 : 1). The isoflavones were eluted and purified as described for *B1* and *B2* in the Experimental section. Afrormosin and formononetin were isolated from both the fungus-inoculated (**3**, 118 μg/g fr wt; **4**, 36 μg/g) and water-treated (**3**, 127 μg/g; **4**, 49 μg/g) leaves. Medicarpin was isolated from inoculated leaves in quantities (140 μg/g) comparable with those obtained following direct TLC of leaf extracts. As shown in Fig. 1, both **2** and **5** accumulated rapidly in *H. carbonum*-induced diffusates whereas the levels of **3** and **4** were relatively stable over the 70 h incubation period. The above results confirm that afrormosin and formononetin are constitutive in leaves of sainfoin whilst medicarpin and vestitol are induced.

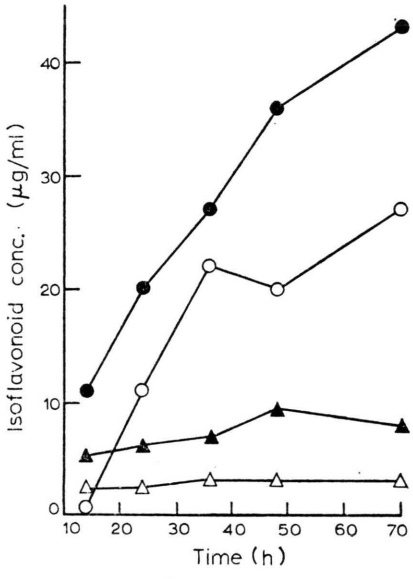

Fig. 1. Accumulation of medicarpin (●−●), vestitol (○−○), afrormosin (▲−▲) and formononetin (△−△) in diffusates from *H. carbonum*-inoculated leaves of *O. viciifolia*.

The antifungal properties of medicarpin and vestitol (both of which are well-known legume phytoalexins [2]) have been reported elsewhere [4, 6, 11]; in contrast **3**, **6** and **7** ($ED_{50} \gg 50$ μg/ml) and **4** (ED_{50} 250 − 350 μg/ml) were essentially inactive when tested against the mycelial growth of *H. carbonum* [6, 12]. Although synthetic vestitone (15 μg) gave a significant inhibition zone (39 mm²) in a TLC bioassay against *C. herbarum*, its very low diffusate concentration (Table I) suggests that it functions principally as a direct or indirect precursor of vestitol [*i.e.* (**6** → **4** → ?) → **1** → **5** or **1** → ? → **2** → **5**] rather than as a resistance factor of *O. viciifolia*.

In addition to the *Onobrychis* species listed in Table I, several other members of the tribe Hedysareae have recently been found to produce phytoalexins [3]. They include *Hedysarum boutignyanum* (**2** and **5**), *H. coronarium* and *H. boreale* (both **2**, **5** and sativan [4]) and the Mediterranean shrub, *Ebenus cretica* (**2** and maackiain [13]). Formononetin was the only constitutive isoflavone isolated from these species. The structure of an isoflavanone-like phytoalexin produced by leaves of *Alhagi pseudalhagi* is currently under investigation [3].

Experimental

Mass and UV spectra were determined as previously described [5].

Plant material. Seeds of *Onobrychis viciifolia* (obtained from Miln Marsters Ltd., Chester, England) were grown as previously described [14] for approx. 6 months prior to leaflet inoculation. Leaves of *O. arenaria, O. montana* and *O. tanaitica* were collected from established plants growing at the University of Reading Botanic Garden.

Purification of B1 – B5. Eluates (EtOH) of each band were reduced to dryness and rechromatographed as follows, i) *B1* PEA (75 : 25 : 1) gave **2** (R_F 0.61) and **3** (R_F 0.22), ii) *B2* PEA (75 : 25 : 1), **4** (R_F 0.12), iii) *B3* PEA (75 : 25 : 3, ×3) **5** (upper zone) and **1** (lower zone), iv) *B4* PEA (75 : 25 : 3, ×4) **6** (upper zone) and **7** (lower zone) and v) *B5* PEA (75 : 25 : 3, ×4) **8** (lower zone) accompanied by traces of a flavanone- or isoflavanone-like derivative (upper zone) which could not be identified.

Compounds **2 – 8**. UV as lit [14, 15] MS (rel. int.) **2/4/5** as lit. [5, 14]; **3**, M^+ 298 (100): *m/e* 297 (36), 283 (18), 166 (45), 132 (32). *Acetate of* **3** (Py-Ac_2O) (R_F 0.31, $CHCl_3$) λ_{max} (nm) MeOH 209, 254, 324; MS (rel. int.) M^+ 340 (39): *m/e* 299 (17), 298 (100), 297 (33). *Acetate of* **4** (R_F 0.66, $CHCl_3$) λ_{max} (nm) MeOH 210, 255, 305 sh; MS (rel. int.) M^+ 310 (78): *m/e* 269 (18), 268 (100), 267 (27). *MonoMe ethers of* **2** *and* **5** (CH_2N_2) MS, UV and TLC as lit. [11, 14] Liquiritigenin (**7**) could be distinguished from its isomer, dihydrodaidzein (**9**) (7,4′-dihydroxyisoflavanone) (kindly supplied by W. Barz) by TLC in $CHCl_3$ (×8 – ×10) (**7**, upper zone; **9**, lower zone).

7,2′-dihydroxy-4′-methoxyisoflavanone (**1**) (*vestitone*). Colour with diazotised *p*-nitroaniline, bright yellow; colour with Gibbs reagent, deep blue (*cf.* vestitol [14]) λ_{max} (nm) MeOH 212 (100%), 230 sh (70%), 277 (68%), 311 (33%); NaOH 218 (100%), 245 sh (38%), 295 sh (25%), 334 (48%); NaOAc 255, 280, 286 sh, 335; Borate 278, 312 sh; addition of $AlCl_3$ did not affect the MeOH spectrum. MS (rel. int.) M^+ 286 (37): *m/e* 268 (6), 151 (10), 150 (100), 149 (10), 137 (57), 121 (5). *DiMe ether* (R_F 0.41, $CHCl_3 : CCl_4$, 3 : 1) λ_{max} (nm) MeOH 212, 230, 274, 312; MS (rel. int.) M^+ 314 (10): *m/e* 165 (11), 164 (100), 163 (2), 150 (5), 149 (29), 135 (9), 121 (12). *Diacetate* (R_F 0.39, $CHCl_3$) λ_{max} (nm) MeOH 216, 225 sh, 257, 282 sh, 316; MS (rel. int.) M^+ 370 (1): *m/e* 329 (2), 328 (10), 311 (3), 310 (11), 287 (1), 286 (5), 285 (2), 269 (2), 268 (12), 151 (22), 150 (100), 149 (10), 137 (34), 121 (8).

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