

## Flavonoid and Isoflavonoid Compounds from Leaves of Sainfoin (*Onobrychis viciifolia*)

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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 ( $\text{CHCl}_3$ :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<sub>2</sub>O: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 Viciae) [7]. Compounds 2–7 were identified by direct comparison (UV, TLC) with authentic samples. Diffusates from leaves treated with de-ionised H<sub>2</sub>O 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<sub>16</sub>H<sub>14</sub>O<sub>5</sub>) and lacking the M<sup>+</sup>-1 peak which is reported to characterise flavanone derivatives [8], cf. liquiritigenin (7) M<sup>+</sup> 256 (100%), 255 (70) and its 4'-O-methyl ether, M<sup>+</sup> 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<sub>3</sub> (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 ( $\mu\text{g/ml}$ )<sup>a,b</sup> 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    |
| <i>O. viciifolia</i> Scop.    | 3(—)     | 39(—) | 12(TR-10) | 13(TR-8) | 25(—) | 5(—) | 6(—) | 4(—) |
| <i>O. arenaria</i> (Kit.) DC. | ND       | 8(—)  | 10(10)    | 6(4)     | —     | ND   | ND   | ND   |
| <i>O. montana</i> DC.         | ND       | 10(—) | 14(16)    | 8(5)     | 15(—) | ND   | ND   | ND   |
| <i>O. tanaitica</i> Sprengel  | ND       | 13(—) | 10(6)     | 6(6)     | 18(—) | ND   | ND   | ND   |

ND, not determined; TR, trace; —, not detectable.

<sup>a</sup> 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  $\log \epsilon$  for 7.

<sup>b</sup> Data in parentheses refer to control diffusates.

mined as follows. Ethanolic leaf extracts [11] were chromatographed ( $\text{Et}_2\text{O} : n\text{-hexane}$ , 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  $\text{CHCl}_3$  ( $\times 3$ ); further purification of vestitol was unnecessary. Although both compounds were readily isolated from *H. carbonum*-inoculated leaves (2, 135  $\mu\text{g/g}$  fr wt; 5, 98  $\mu\text{g/g}$ ) 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  $\text{CCl}_4$  (60 ml) and the resulting solution shaken ( $\times 4$ ) with equal vol. aq NaOH (0.2 N). The pooled, pale-yellow, NaOH fractions were then acidified (pH 3, 2 N HCl), extracted ( $\times 4$ ) with 0.5 vol  $\text{CCl}_4$  and the organic phase reduced to dryness prior to TLC ( $\text{CHCl}_3 : \text{MeOH}$ , 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  $\mu\text{g/g}$  fr wt; 4, 36  $\mu\text{g/g}$ ) and water-treated (3, 127  $\mu\text{g/g}$ ; 4, 49  $\mu\text{g/g}$ ) leaves. Medicarpin was isolated from inoculated leaves in quantities (140  $\mu\text{g/g}$ ) 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.

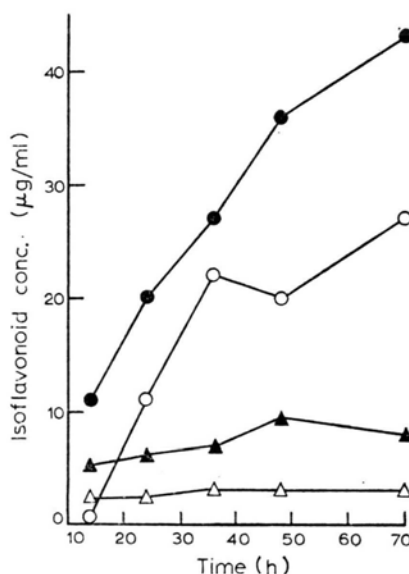


Fig. 1. Accumulation of medicarpin (●—●), vestitol (○—○), afrormosin (▲—▲) and formononetin (△—△) in diffusates from *H. carbonum*-inoculated leaves of *O. viciifolia*.

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 ( $\text{ED}_{50} \gg 50 \mu\text{g/ml}$ ) and 4 ( $\text{ED}_{50}$  250–350  $\mu\text{g/ml}$ ) were essentially inactive when tested against the mycelial growth of *H. carbonum* [6, 12]. Although synthetic vestitone (15  $\mu\text{g}$ ) gave a significant inhibition zone (39  $\text{mm}^2$ ) 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  $\rightarrow$  4  $\rightarrow$  ?)  $\rightarrow$  1  $\rightarrow$  5 or 1  $\rightarrow$  ?  $\rightarrow$  2  $\rightarrow$  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,  $\times 3$ ) **5** (upper zone) and **1** (lower zone), iv) **B4** PEA (75:25:3,  $\times 4$ ) **6** (upper zone) and **7** (lower zone) and v) **B5** PEA (75:25:3,  $\times 4$ ) **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<sub>2</sub>O) ( $R_F$  0.31, CHCl<sub>3</sub>)  $\lambda_{max}$  (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<sub>3</sub>)  $\lambda_{max}$  (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<sub>2</sub>N<sub>2</sub>) 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<sub>3</sub> ( $\times 8 - \times 10$ ) (**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])  $\lambda_{max}$  (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<sub>3</sub> 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<sub>3</sub>:CCl<sub>4</sub>, 3:1)  $\lambda_{max}$  (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<sub>3</sub>)  $\lambda_{max}$  (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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