Isoflavonoid Phytoalexins from Leaves of *Trifolium arvense*

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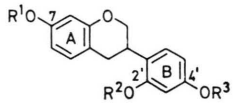
(Z. Naturforsch. **32 c**, 446–448 [1977]; received February 25, 1977)

Leguminosae, *Trifolium*, Isoflavans, Pterocarpans, Phytoalexins

In response to fungal inoculation the leaves of *Trifolium arvense* accumulate the known isoflavonoid phytoalexins, medicarpin, maackiain, sativan, vestitol, and isovestitol. A previously undescribed isoflavan derivative (arvensan; 7,2′-dimethoxy-4′-hydroxyisoflavan) is also produced by this species. The identification and synthesis of arvensan is described.

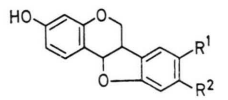
Numerous isoflavonoid phytoalexins [1] have been isolated from species belonging to the family Leguminosae (subfamily Lotoideae) and implicated as factors in disease resistance [2, 3]. These compounds are often pterocarpan or isoflavan in nature [2, 4, 5] although isoflavone [6] and isoflavanone [6, 7] phytoalexins have also been described. A recent comprehensive survey of genera (*e. g. Melilotus*, *Medicago*, *Trigonella*, and *Trifolium*) within the tribe Trifolieae has revealed that the leaves of many species accumulate isoflavan and pterocarpan derivatives following inoculation with conidial suspensions of the fungus, *Helminthosporium carbonum* Ullstrup [3, 4]. This paper reports the isolation of a new phytoalexin from hare's-foot clover (*Trifolium arvense* L.) and presents evidence to support its identification as 7,2′-dimethoxy-4′-hydroxyisoflavan (**1**).

Phytoalexins were obtained from the *H. carbonum*-inoculated leaflets of *T. arvense* using the drop-diffusate technique [5]. TLC ($CHCl_3$: MeOH, 50 : 1) of diffusate extracts [4, 5] gave two broad phenolic bands at R_F 0.59 – 0.66 (termed B-1) and R_F 0.43 – 0.47 (B-2). These were eluted with EtOH and the solvent removed *in vacuo* (40 °C). The B-1 fraction was then further chromatographed ($CHCl_3$, ×5) to afford, i) a mixture of medicarpin and maackiain (**2**) and (**3**) (*lower zone*), ii) sativan (**4**) (*intermediate zone*) and iii) an upper band (compound **1**) which gave a bright orange colouration when sprayed with diazotised *p*-nitroaniline; compounds **2** – **4** are all characterised by the intense yellow colour of their diazo derivatives. Medicarpin and maackiain were eventually resolved by TLC (×3) in *n*-pentane : Et_2O : HOAc (75 : 25 : 3) [4].



1: $R^1=R^2=CH_3$; $R^3=H$
4: $R^1=H$; $R^2=R^3=CH_3$
5: $R^1=R^2=H$; $R^3=CH_3$
6: $R^1=R^3=H$; $R^2=CH_3$
7: $R^1=R^3=CH_3$; $R^2=H$
8: $R^1=R^2=R^3=CH_3$

Upon multiple development (×3) in this latter system, B-2 also separated into two components namely, vestitol (**5**) (*upper zone*) and isovestitol (**6**) (*lower zone*). All the above compounds were chromatographically homogeneous and were absent from extracts of the control leaf diffusate [5]. The identification of compounds **2** – **6** was based on a UV and TLC (5 solvent systems) comparison with authentic material and on the colours formed after spraying developed chromatograms with Gibbs reagent [8] and diazotised *p*-nitroaniline.



2: $R^1=H$; $R^2=OCH_3$
3: $R^1=R^2=O-CH_2-O$

Although medicarpin, vestitol and sativan are common as phytoalexins in members of the tribe Trifolieae [3], maackiain is encountered less frequently and as yet has been associated with only two genera (*Trigonella* and *Trifolium* [4, 9, 10]) of this tribe. Isovestitol has previously been described as a phytoalexin of *Anthyllis vulneraria* and five *Tetragonolobus* species [11] (tribe Loteae) but in the Trifolieae it is of exceptionally rare occurrence [3]. However, in addition to *T. arvense*, isovestitol has recently been isolated (together with **2**, **3**, **4**, and **5**) from *T. rubens* [3]. There was no evidence to suggest that *T. arvense* produced either 4-methoxymaackiain or isosativan (**7**), two isoflavonoid phytoalexins obtained from the leaves of alsike clover (*T. hybridum*) [4].

Compound **1** exhibited a mass spectral fragmentation pattern entirely consistent with its identification as a trisubstituted (dimethoxy-monohydroxy)

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isoflavan [12, 13]. In fact, the mass and UV (EtOH) spectral data recorded for **1** (see Experimental) closely resembled those of 7,4′-dimethoxy-2′-hydroxyisoflavan **7** (isosativan) [4]. The molecular ion was evident at *m/e* 286 and was accompanied by three major fragments at *m/e* 150 (*a*), 149 (*b*) and 137 (*c*; base peak). These ions allow the aromatic ring substituents (which biogenetically should be at C-7, 2′ and 4′) to be assigned as follows, i) *A-RING*: C-7 (OCH_3; fragment *b*) and ii) *B-RING*: C-2′/C-4′ (OCH_3/OH; fragment *a* and *c*). As **1** did not react to Gibbs reagent [8, 14] (*cf.* isosativan, deep blue [4]) the single B-ring hydroxyl group must be located at C-4′ rather than at C-2′. The above oxygenation pattern was confirmed by methylation (with diazomethane [15]) to afford a monomethyl ether identical (UV, MS, TLC) with 7,2′,4′-trimethoxyisoflavan (**8**) similarly prepared from vestitol (**5**). Compound **1** is thus 7,2′-dimethoxy-4′-hydroxyisoflavan; this substance (which has not previously been described as either a natural or synthetic product) has been named *arvensan* after the source plant, *Trifolium arvense*.

Arvensan has been synthesised *via* 7,4′-dibenzyloxy-2′-methoxyisoflavone [16] itself produced from the corresponding 2′-acetoxychalcone by oxidative rearrangement using thallium (III) nitrate trihydrate [17]. Debenzylation of the above isoflavone followed by selective methylation and hydrogenation gave a product chromatographically and spectrally indistinguishable from natural arvensan. As noted earlier, arvensan gives a deep orange colouration when sprayed with diazotised *p*-nitroaniline. This feature is characteristic of several isoflavans [3, 18] (*e. g.* **6**) which possess at C-4′ hydroxyl group. In contrast, compounds with methoxylation at C-4′ often give a bright yellow colour (*e. g.* **4** and **5**) with diazotised *p*-nitroaniline.

Im TLC bioassays against spore germination of *Cladosporium herbarum* Fr. [8] pronounced antifungal zones were associated with applied arvensan levels of 10, 20, and 30 μg. When tested against the mycelial growth of *H. carbonum* [8] arvensan had an ED_{50} value of about 30 μg/ml; its fungitoxic activity is thus of approximately the same order as that of medicarpin (ED_{50} 25 μg/ml) and maackiain (ED_{50} 33 μg/ml) [8]. The other isoflavans from *T. arvense* appear slightly more inhibitory than arvensan (*cf.* **4**, ED_{50} 10 μg/ml; **5**, ED_{50} 17 μg/ml; **6**, ED_{50} 23 μg/ml). Nevertheless, the high arvensan level (50 μg/ml) associated with *H. carbonum*-induced diffusates suggests that formation of this compound may contribute significantly to disease resistance. Substantial quantities of the other isoflavonoid phytoalexins (**2** – **6**) were also produced by *T. arvense* (**2**, 65 μg/ml; **3**, 63 μg/ml; **4**, 105 μg/ml; **5**, 65 μg/ml; **6**, 123 μg/ml).

The formation, by *T. arvense*, of several structurally similar pterocarpans and isoflavans suggests that in the plant these compounds may be biosynthetically related. In *Medicago sativa* there is labelling evidence to support the view that sativan is derived *via* methylation of vestitol and that vestitol and medicarpin can be interconverted [19]. However, the latter two compounds may originate simultaneously from a common intermediate [19]. Although arvensan may be derived by methylation of isovestitol, no logical demethyl precursor of the latter compound was isolated from *T. arvense*. In the genus *Tetragonolobus*, isovestitol co-occurs with 7,2′,4′-trihydroxyisoflavan (demethylvestitol [11]), a substance which could be readily converted to comcound **5** by methylation at C-2′. However, repeated investigation has failed to reveal the production of demethylvestitol by *T. arvense*; it is conceivable, therefore, that isovestitol may originate by host-plant demethylation of sativan. No evidence was obtained to suggest that *T. arvense* accumulated methylenedioxy substituted isoflavans related to maackiain.

Experimental

Mass and UV spectra were determined as previously described [6]. TLC separations were undertaken using precoated, glass-backed plates (Merck Kieselgel 60 F_{254}, layer thickness, 0.25 mm).

Plant material. Seeds of *Trifolium arvense* L. (obtained from the Botanic Garden, Klagenfurt, Austria) were grown as previously described [20]. Leaflets for fungal inoculation [5] were collected when the plants were 6 – 9 months old.

Fungal material. Cultural conditions, preparation of spore suspensions and source of *Helminthosporium carbonum* Ullstrup have been reported elsewhere [8].

7,2′-Dimethoxy-4′-hydroxyisoflavan (**1**) (*arvensan*). Colour with diazotised *p*-nitroaniline, orange; no colour was observed with Gibbs reagent. λ_{max} *EtOH* (nm) 205 (log ε 4.70), 225 (4.17), 280 – 282 (3.77), 287sh (3.71); *EtOH* + *NaOH* (nm) 217, 244sh, 285, 290, 298sh; MS rel. int.) 287 (15), 286(M^+; 89), 151(40), 150(84), 149(35), 148(30), 138(27), 137(100), 135(42), 121(45), 107(41). *Monomethyl ether* **8** (R_F 0.93, $CHCl_3$) UV and MS as lit. [4, 11]. *Monoacetate* (R_F 0.16, CCl_4 : $CHCl_3$, 4 : 1); λ_{max} *EtOH* (nm) 213, 225sh, 275sh, 280, 289sh; MS (rel. int.) 329(6), 328(M^+; 40), 286(23), 192(21), 151(11), 150 (100), 149(42), 148(22), 138(13), 137(52), 135 (29), 121(14), 119(11), 107(18).

Synthesis of arvensan. a) *7,4′-Dihydroxy-2′-methoxyisoflavone.* This intermediate was synthesised as previously described [17].

b) *7,2′-Dimethoxy-4′-hydroxyisoflavone.* The preceeding isoflavone (150 mg in DMF (20 ml) was stirred (60 °C) for 1 h with K_2CO_3 (2 g) and CH_3I (75 mg). The mixture was then poured into H_2O and extracted with EtOAc (×6). After removal of EtOAc (*in vacuo*, 40 °C) the residue was crystallised from MeOH to give the desired product (108 mg). λ_{max} *MeOH* (nm) 211, 240, 248, 287, 306sh; *MeOH* + *NaOH* (nm) 211, 240, 247sh, 281, 291sh, 305sh; MS (rel. int.) 299(23), 298 (M^+; 100), 297(18), 281(15), 269(10), 268(11), 267(65), 152(6), 151(77), 149(13(, 148(10), 147 (27), 146(7), 119(9), 107(6), 105(7); mp. 212 – 217 °C. Colour with diazotised *p*-nitroaniline, orange.

c) (±)*-7,7′-Dimethoxy-4′-hydroxyisoflavon* (**1**). The above isoflavone (87 mg), HOAc (20 ml) and Pd-C (10%; 80 mg) were shaken overnight in an atmosphere of H_2. After filtration and removal of solvent, the product was chromatographed (C_6H_6 : EtOAc : MeOH : petrol, 6 : 4 : 1 : 3) to give an oil which solidified upon treatment with a little aq. MeOH (yield, 55 mg). UV and MS as reported for the natural product; mp. 111 – 115 °C. The synthetic and natural isoflavans had identical R_F values in $CHCl_3$: MeOH (50 : 1, R_F 0.65), C_6H_6 : MeOH (9 : 1, R_F 0.80), $CHCl_3$ (R_F 0.20), Et_2O : *n*-hexane (3 : 1, R_F 0.83), and *n*-pentane : Et_2O : HOAc (75 : 25 : 1, R_F 0.48).

The authors thank R. W. Butters (Tate and Lyle Ltd.) for MS analyses and the Science Research Council for financial support (to J. L. I.).

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