Isosativan: an Isoflavan Phytoalexin from *Trifolium hybridum* and other *Trifolium* Species

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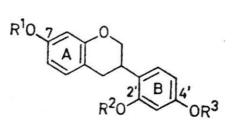
An isoflavonoid phytoalexin isolated from the fungus-infected leaves of *Trifolium hybridum* has been identified as 7,4′-dimethoxy-2′-hydroxyisoflavan.

The disease resistance of many higher plants may depend on the post-infectional accumulation of antifungal compounds called phytoalexins [1]. In general, species of the Leguminosae (subfamily Lotoideae) produce isoflavonoid phytoalexins (pterocarpans and isoflavans) although one exception (the furanoacetylene, wyerone acid from *Vicia faba*) has been reported [2]. During a phytochemical survey of the genus *Trifolium*, it was found that in addition to known compounds, the fungus-infected leaves of alsike clover (*T. hybridum* L.) produced an isoflavonoid not previously described as a phytoalexin. From the evidence presented below, this compound has been formulated as vestitol-7-O-methyl ether (**1**).

Phytoalexins were isolated from the detached leaves of *T. hybridum* using the drop-diffusate technique as previously described [3]. Conidial suspensions of the non-pathogenic fungus, *Helminthosporium carbonum* Ullstrup served as the phytoalexin inducer. Diffusates from infected leaves were extracted with EtOAc and the organic fractions bulked and reduced to dryness. TLC ($CHCl_3$: MeOH, 100 : 2, Merck, Si-gel F_{254}, 0.25 mm) of the residue afforded three major phenolic zones at R_F 0.14 (Band 1), 0.47 (Band 2) and 0.55 (Band 3); a minor zone (Band 4) was also apparent at approx. R_F 0.66. The Band 1 component was purified in *n*-pentane : Et_2O : HOAc (PEA) (75 : 25 : 3, 3 X) to afford the known isoflavan phytoalexin, vestitol [4]



1: $R^1=R^3=CH_3$; $R^2=H$
2: $R^1=R^2=H$; $R^3=CH_3$
3: $R^1=H$; $R^2=R^3=CH_3$
4: $R^1=R^2=R^3=CH_3$

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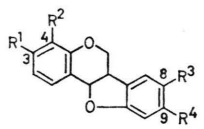
(**2**). Purification of Band 2 ($CHCl_3$, 3 X) gave sativan [3] (**3**) together with a lower phenolic fraction which separated in PEA (75 : 25 : 3, 3 X) to give the pterocarpans medicarpin (**5**) (upper zone) and maackiain (**6**) [5] (lower zone). Identification



5: $R^1=OH$; $R^2=R^3=H$; $R^4=OCH_3$
6: $R^1=OH$; $R^2=H$; $R^3=R^4=O-CH_2-O$
7: $R^1=OH$; $R^2=OCH_3$; $R^3=R^4=O-CH_2-O$
8: $R^1=R^4=OCH_3$; $R^2=R^3=H$

of compounds **2** – **6** was based on a UV and TLC comparison with authentic material. TLC of Band 4 (PEA, 75 : 25 : 1) gave small quantities of a compound (R_F 0.37) identified (MS and UV) as 4-methoxymaackiain (**7**). Control diffusates [3] contained only traces of **2**, **5** and **6**.

Further purification of Band 3 (PEA, 75 : 25 : 1, R_F 0.63; $CHCl_3$, R_F 0.37) afforded a phenolic compound **1** which reacted to both diazotised *p*-nitroaniline [6] (yellow) and Gibbs reagent [7] (deep blue). UV maxima [nm] recorded for this compound were, 1. *EtOH*: 214, 227 sh, 281, 284 and 289 sh and 2. *EtOH* + *NaOH*: 219, 245 sh, 286 sh, 291 and 300 sh. The MS was typical of a simple isoflavan [8] and gave a molecular ion at *m/e* 286 (corresponding to $C_{17}H_{18}O_4$) and prominent fragments at *m/e* 151, 150 (base), 149, 148, 138 and 137. The ions at *m/e* 150 and 137 can be obtained by fragmentation of an isoflavan with monomethoxymonohydroxy substitution of the B-ring; the ion at *m/e* 149 can be formulated as a monomethoxylated fragment derived from ring-A. Since naturally occurring isoflavans are oxygenated at C-7, 2′ and 4′, the above compound was formulated as 7,4′-dimethoxy-2′-hydroxyisoflavan (**1**) (vestitol-7-O-methyl ether). The phenolic hydroxyl group was located at C-2′ (rather than C-4′) from the positive (blue) Gibbs reaction [7]. Although **1** has been extracted from the wood of *Dalbergia ecastophyllum* [9], it has not previously been associated with herbaceous plant tissues. Nor has **1** been assigned a common name; in view of its isomeric relationship to the isoflavan phytoalexin, sativan [3] (**3**), the trivial name *Isosativan* would seem appropriate.

Structure **1** for isosativan was confirmed by acetylation, methylation and synthesis, since data (UV, MS) reported for the *Dalbergia* metabolite [9] differ slightly from those noted above. Acetylation and TLC purification ($CHCl_3$, R_F 0.49) gave a monoacetate ($\lambda_{max.}^{EtOH}$ nm: 215, 226, 280 sh, 283 and 289) with M^+ 328 and fragments at *m/e* 286

(M-42), 151, 150 (base), 149, 148 and 137. The ion at M-42 is indicative of an aromatic hydroxyl group [10]. When methylated with diazomethane, isosativan afforded a monomethyl ether ($CHCl_3$, R_F 0.90) ($\lambda_{max.}^{EtOH}$ nm: 211, 226, 280, 284 and 289 sh; M^+ 300, *m/e* 165, 164 (base), 152, 151, 149 and 121) indistinguishable (UV, MS and TLC) from a sample of the trimethoxyisoflavan **4**. Formation of this compound establishes unequivocally the 7,2′,4′-oxygenation pattern of isosativan.

The structure of isosativan was finally verified by synthesis from homopterocarpin (**8**). Crystalline **8** (5 mg), glacial HOAc (3 ml) and 10% Pd-C (5 mg) were hydrogenated at 80 °C for 25 min. After removal of catalyst and solvent, the residue was chromatographed in $CHCl_3$: MeOH (100 : 2) to afford dihydrohomopterocarpin (**1**). This compound was found to be identical (UV, MS and TLC) with isosativan.

When bioassayed against the mycelial growth of *H. carbonum*, the antifungal activity of isosativan (ED_{50} 16 μg/ml) was found to be comparable with that of the related isoflavans, vestitol (ED_{50} 17 μg/ml) and sativan (ED_{50} 10 μg/ml). Although leaf diffusates from *T. hybridum* contain isosativan (10 μg/ml) in relatively small quantities (*cf.* **2**, 170 μg/ml; **3**, 8 μg/ml; **5**, 70 μg/ml; **6**, 56 μg/ml; **7**, 2 μg/ml) there seems little doubt that this compound functions as a resistance factor.

As well as *T. hybridum*, isosativan is produced by *T. subterraneum* L., *T. spumosum* L., *T. scabrum* L., *T. stellatum* L. and *T. tomentosum*. These species also accumulate vestitol from which isosativan can be derived. For *T. hybridum*, the terminal stages of phytoalexin biosynthesis presumably involve conversion of medicarpin (**5**) to vestitol (**2**) and methylation (at C-7 or 2′) of the latter compound to give either isosativan (**1**) or sativan (**3**). The chemically 'advanced' pterocarpan maackiain (**6**) is apparently produced by a route which does not require the participation of medicarpin [11]. From a phytochemical comparison with over 50 other *Trifolium* species (J. L. Ingham, unpublished data), it appears that in terms of isoflavonoid production, *T. hybridum* is one of the more chemically evolved members of the genus. The results of the abovementioned survey will be published elsewhere.

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