

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

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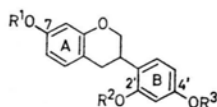
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Leguminosae, *Trifolium*, Isoflavan, Pterocarpin, Phytoalexin

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 phytoalexins¹. In general, species of the Leguminosae (subfamily Lotoideae) produce isoflavonoid phytoalexins (pterocarpan and isoflavans) although one exception (the furanoacetylene, wyerone acid from *Vicia faba*) has been reported². 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**).

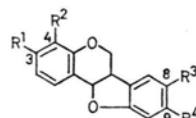
Phytoalexins were isolated from the detached leaves of *T. hybridum* using the drop-diffusate technique as previously described³. Conidial suspensions of the non-pathogenic fungus, *Helminthosporium carbonum* Ullstrup served as the phytoalexin inducer. Diffusates from infected leaves were extracted with EtOAc and the organic fractions bulked and reduced to dryness. TLC (CHCl₃:MeOH, 100:2, Merck, Si-gel F₂₅₄, 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:Et₂O:HOAc (PEA) (75:25:3, 3 X) to afford the known isoflavan phytoalexin, vestitol⁴



- 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 (CHCl₃, 3 X) gave sativan³ (**3**) together with a lower phenolic fraction which separated in PEA (75:25:3, 3 X) to give the pterocarpan 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 diffusates³ contained only traces of **2**, **5** and **6**.

Further purification of Band 3 (PEA, 75:25:1, *R_F* 0.63; CHCl₃, *R_F* 0.37) afforded a phenolic compound **1** which reacted to both diazotised *p*-nitroaniline⁶ (yellow) and Gibbs reagent⁷ (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 isoflavan⁸ and gave a molecular ion at *m/e* 286 (corresponding to C₁₇H₁₈O₄) 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 reaction⁷. 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, sativan³ (**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* metabolite⁹ differ slightly from those noted above. Acetylation and TLC purification (CHCl₃, *R_F* 0.49) gave a monoacetate ($\lambda_{\text{max}}^{\text{EtOH}}$ nm: 215, 226, 280 sh, 283 and 289) with *M*⁺ 328 and fragments at *m/e* 286



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(M-42), 151, 150 (base), 149, 148 and 137. The ion at M-42 is indicative of an aromatic hydroxyl group¹⁰. When methylated with diazomethane, isosativan afforded a monomethyl ether (CHCl_3 , R_F 0.90) ($\lambda_{\text{max}}^{\text{EtOH}}$ 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 $\mu\text{g/ml}$) was found to be comparable with that of the related isoflavans, vestitol (ED_{50} 17 $\mu\text{g/ml}$) and sativan (ED_{50} 10 $\mu\text{g/ml}$). Although leaf diffusates from *T. hybridum* contain isosativan (10

$\mu\text{g/ml}$) in relatively small quantities (cf. **2**, 170 $\mu\text{g/ml}$; **3**, 8 $\mu\text{g/ml}$; **5**, 70 $\mu\text{g/ml}$; **6**, 56 $\mu\text{g/ml}$; **7**, 2 $\mu\text{g/ml}$) 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 medicarpin¹¹. 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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