# A Further Investigation of Phytoalexin Formation in the Genus *Trifolium*

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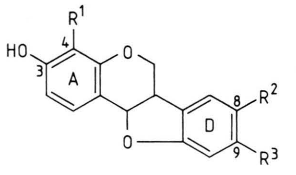
*Trifolium,* Isoflavonoids, Pterocarpans, Isoflavans, Stilbene, Phytoalexins

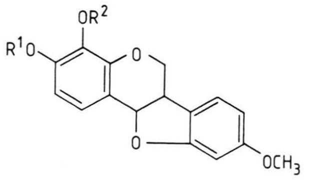
An earlier study of phytoalexin formation in the genus *Trifolium* has now been extended to include a further 61 species and subspecies. Using the drop-diffusate method, isoflavonoid phytoalexins were isolated from the fungus-inoculated leaflets of 55 accessions, whilst four others produced the stilbene derivative resveratrol. Phytoalexins could not be obtained from the leaflets of two species, *T. billardieri* and *T. grandiflorum*. The pterocarpan medicarpin was the most commonly encountered phytoalexin, occurring alone or in various combinations with the known *Trifolium* isoflavonoids maackiain (pterocarpan), vestitol, isovestitol, sativan, isosativan and arvensan (all isoflavans). Two additional pterocarpans, 4-methoxymedicarpin from *T. cherleri* and *T. pallescens,* and 4-hydroxyhomopterocarpin also from *T. pallescens,* were recognized for the first time as *Trifolium* phytoalexins. Re-examination of *T. aureum* revealed that this species produced resveratrol as a phytoalexin and not the isoflavan vestitol as previously described.

## Introduction

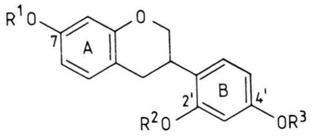
In reference [1], it was reported that isoflavonoid phytoalexins were typically produced by the fungus-inoculated leaflets of more than 40 species belonging to the genus *Trifolium* (Leguminosae-Papilionoideae; tribe Trifolieae). Only two yellow-flowered species (*T. campestre* and *T. dubium*) from the section Chronosemium responded differently by accumulating large quantities of the stilbene resveratrol (3,5,4′-trihydroxystilbene, **11**). Although medicarpin (3-hydroxy-9-methoxypterocarpan, **1**) was the most common *Trifolium* phytoalexin, it frequently co-occurred with other isoflavonoids such as the pterocarpan maackiain (3-hydroxy-8,9-methylenedioxypterocarpan, **2**), and the isoflavans vestitol (7,2′-dihydroxy-4′-methoxyisoflavan, **3**), isovestitol (7,4′-dihydroxy-2′-methoxyisoflavan, **4**), sativan (7-hydroxy-2′,4′-dimethoxyisoflavan, **5**), isosativan (7,4′-dimethoxy-2′-hydroxyisoflavan, **6**) and arvensan (7,2′-dimethoxy-4′-hydroxyisoflavan, **7**). Many of these compounds have also been recognized as phytoalexins in other genera of the tribe Trifolieae (*e.g.* **1** in *Melilotus* [2], **1** and **2** in *Ononis* [3], **1, 2, 3** and **5** in *Trigonella* [4], and **1, 4, 5** and **6** in *Medicago* [5]). Apart from medicarpin (**1**) and maackiain (**2**), a third pterocarpan, 4-methoxymaackiain (3-hydroxy-4-methoxy-8,9-methylenedioxypterocarpan, **10**) is known to occur as a minor phytoalexin in the leaflets of *T. hybridum* [1, 6].



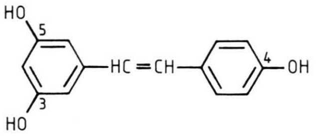
**1:** R$^1$ = R$^2$ = H; R$^3$ = OCH$_3$
**2:** R$^1$ = H; R$^2$ = R$^3$ = O − CH$_2$ − O
**10:** R$^1$ = OCH$_3$; R$^2$ = R$^3$ = O − CH$_2$ − O



**8:** R$^1$ = CH$_3$; R$^2$ = H
**9:** R$^1$ = H; R$^2$ = CH$_3$
**12:** R$^1$ = R$^2$ = CH$_3$



**3:** R$^1$ = R$^2$ = H; R$^3$ = CH$_3$
**4:** R$^1$ = R$^3$ = H; R$^2$ = CH$_3$
**5:** R$^1$ = H; R$^2$ = R$^3$ = CH$_3$
**6:** R$^1$ = R$^3$ = CH$_3$; R$^2$ = H
**7:** R$^1$ = R$^2$ = CH$_3$; R$^3$ = H



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The variety of isoflavonoids already obtained from *Trifolium* leaflets [1, 6, 7] suggested that a further investigation might reveal previously unrecognized phytoalexins, and at the same time provide additional sources of compounds **1–7, 10** and **11.** The present paper describes the isolation of phytoalexins from 59 *Trifolium* species and subspecies, only one of which *(T. aureum)* was included in the previous survey [1]. Fungus-inoculated leaflets were found to produce either resveratrol (yellow-flowered species from the section Chronosemium) or medicarpin, with the latter compound often being variously accompanied by isoflavonoids **2–7.** The *T. hybridum* phytoalexin 4-methoxymaackiain (**10**) was not detected. However, both *T. cherleri* and *T. pallescens* yielded the new phytoalexin 4-methoxymedicarpin (3-hydroxy-4,9-dimethoxypterocarpan, **9**) whilst small quantities of the isomer 4-hydroxyhomopterocarpin (3,9-dimethoxy-4-hydroxypterocarpan, **8**) were also isolated from *T. pallescens.*

## Results and Discussion

Phytoalexins were obtained from fungus *(Helminthosporium carbonum)* inoculated *Trifolium* leaflets using the drop-diffusate technique as previously described [8]. Corresponding control leaflets were treated with droplets of deionized water. Phytoalexins were extracted from the resulting 48 h diffusates [8] by shaking with ethyl acetate (see Experimental section).

Silica gel thin-layer chromatography (Si gel TLC) of these extracts revealed that phytoalexins were produced by 59 out of the 61 (97%) *Trifolium* species examined (Table I). Diffusates from 55 fungus-treated species (93%) contained medicarpin (**1**) whereas the stilbene resveratrol (**11,** 4 species, 7%) was of comparatively rare occurrence. Resveratrol was never associated with medicarpin or with any other isoflavonoid phytoalexin(s). Except for occasional traces of medicarpin and/or the corresponding isoflavan vestitol (**3**), compounds **1–9** and **11** were absent from control diffusates. Two out of the 61 accessions (3%) listed in Table I (*T. billardieri* and *T. grandiflorum*) failed to accumulate any detectable phytoalexins under the conditions employed. Several species included in the earlier survey (*e.g. T. badium* and its close ally *T. spadiceum*) also failed to produce either isoflavonoid or stilbene phytoalexins following treatment with *H. carbonum* [1].

Medicarpin was the only phytoalexin detected in diffusates from 13 out of the 55 (24%) isoflavonoid-producing *Trifolium* species (Table I). In diffusates from the remaining 42 accessions (76%), medicarpin occurred in various combinations with up to four other isoflavonoids. However, with the exception of the pterocarpan maackiain (**2**) from 19 species (**1 + 2** only in 9 species) and the isoflavan vestitol (**3**) from 31 species (**1 + 3** only in 15 species), these compounds were, as noted previously [1], of comparatively rare occurrence (Table I). Thus, the accumulation of isovestitol (**4**) was restricted to *T. dalmaticum, T. mutabile* and *T. stellatum* subsp. *xanthinum,* whilst sativan (**5**) and isosativan (**6**) were isolated together from 3 species, with each accumulating separately in the diffusates from a further 4 and 2 species respectively. Arvensan (**7**) was isolated only from *T. dalmaticum* and *T. stellatum* subsp. *xanthinum.* No further sources of the *T. hybridum* phytoalexin 4-methoxymaackiain (**10**) [1, 6] were discovered.

Despite the failure to detect 4-methoxymaackiain, both *T. cherleri* and *T. pallescens* produced 4-oxygenated pterocarpans not previously reported as *Trifolium* phytoalexins. Medicarpin was also isolated from the diffusates of these *Trifolium* species. The first new phytoalexin ($M^+$ 300; colour with diazotized *p*-nitroaniline reagent [9], orange) was readily identified as 3,9-dimethoxy-4-hydroxypterocarpan (4-hydroxyhomopterocarpin, **8**) by UV, MS and Si gel TLC comparison with an authentic sample [8]. Apart from its formation by *T. pallescens,* small quantities of 4-hydroxyhomopterocarpin (approx. 2 µg/ml diffusate) have also been isolated together with other isoflavonoids (*e.g.* **1, 2, 3** and **10** [1]) from the *H. carbonum*-inoculated leaflets of *T. hybridum* [10].

In addition to **8,** *T. pallescens* produced an isomeric 4-oxygenated pterocarpan ($M^+$ 300; colour with diazotized *p*-nitroaniline reagent [9], yellow) likewise found in diffusates from *T. cherleri.* Upon methylation, the compound afforded a product indistinguishable (UV, MS, Si gel TLC) from authentic 3,4,9,-trimethoxypterocarpan (**12**) [8], whilst formation of a non-phenolic monoacetate confirmed the presence of one aromatic hydroxyl group. This was assigned to C-3 (ring A), as in

Table I. Occurrence of isoflavonoid and stilbene phytoalexins in diffusates from the *Helminthosporium carbonum*-inoculated leaflets of *Trifolium* species.

| | | Phytoalexin concentration [µg/ml][a,b,c,d] | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *Trifolium* species[e] | Seed source[f] | Resveratrol (**11**) | Medicarpin (**1**) | Maackiain (**2**) | 4-Methoxymedicarpin (**9**) | 4-Hydroxyhomopterocarpin (**8**) | Vestitol (**3**) | Isovestitol (**4**) | Sativan (**5**) | Isosativan (**6**) | Arvensan (**7**) |
| *T. africanum* Ser. | K | – | 57 | 4 | – | – | – | – | – | – | – |
| *T. angulatum* Waldst. & Kit. | D | – | 8 | 17 | – | – | 72 | – | 53 | – | – |
| *T. argutum* Banks & Sol. | K | – | 36 | – | – | – | 69 | – | – | – | – |
| *T. aureum* Pollich | Kw | 50 | – | – | – | – | – | – | – | – | – |
| *T. bivonae* Guss. | K | – | 109 | – | – | – | 4 | – | – | – | – |
| *T. bocconei* Savi | Cm | – | 131 | 8 | – | – | – | – | – | – | – |
| *T. bocconei* Savi subsp. *tenuifolium* (Ten.) Griseb. | Kw | – | 32 | 12 | – | – | – | – | – | – | – |
| *T. boissieri* Guss. ex Soyer-Willemet & Godr. | K | 32 | – | – | – | – | – | – | – | – | – |
| *T. bullatum* Boiss. & Hausskn. | Ta | – | 3 | 1 | – | – | 51 | – | – | 42 | – |
| *T. burchellianum* Ser. | C | – | 146 | – | – | – | 22 | – | – | – | – |
| *T. burchellianum* Ser. subsp. *johnstonii* (Oliv.) Gillett | K | – | 223 | – | – | – | 26 | – | – | – | – |
| *T. cherleri* L. | A | – | 119 | – | 12 | – | – | – | – | – | – |
| *T. clusii* Godr. & Gren. | Ta | – | 24 | 2 | – | – | 146 | – | 8 | 30 | – |
| *T. congestum* Guss. | P | – | 23 | 20 | – | – | – | – | – | – | – |
| *T. dalmaticum* Vis. | K | – | 68 | 15 | – | – | 12 | 29 | – | – | 32 |
| *T. dichroanthum* Boiss. | A | – | 8 | – | – | – | – | – | – | – | – |
| *T. echinatum* M.B. | Ct | – | 53 | – | – | – | 14 | – | – | – | – |
| *T. eriosphaerum* Boiss. | Ta | – | 39 | – | – | – | – | – | – | – | – |
| *T. fucatum* Lindl. | Co | – | 86 | – | – | – | – | – | – | – | – |
| *T. glanduliferum* Boiss. | H | – | 62 | – | – | – | – | – | – | – | – |
| *T. hirtum* All. | Cm | – | 189 | – | – | – | – | – | – | – | – |
| *T. lupinaster* L. | V | – | 127 | – | – | – | 39 | 15 | 37 | – | – |
| *T. masaiense* Gillett | C | – | 39 | – | – | – | tr | – | – | – | – |
| *T. medium* L. subsp. *banaticum* (Heuff.) Hendryck | A | – | 20 | 8 | – | – | 32 | – | n.d. | – | – |
| *T. medium* L. subsp. *sarosiense* (Hazsl.) Simonkai | A | – | 24 | 11 | – | – | 17 | – | n.d. | – | – |
| *T. micranthum* Viv. | N | 42 | – | – | – | – | – | – | – | – | – |
| *T. miegeanum* Maire | Cm | – | 24 | 4 | – | – | 15 | – | – | – | – |
| *T. mutabile* Portenschl. | Ct | – | 5 | – | – | – | 106 | – | 19 | 26 | – |
| *T. obscurum* Savi | B | – | 40 | – | – | – | 18 | – | – | – | – |
| *T. palaestinum* Boiss. | Ta | – | 11 | – | – | – | – | – | – | – | – |
| *T. pallescens* Schreb. | G | – | 32 | – | 3 | 3 | – | – | – | – | – |
| *T. pallidum* Waldst. & Kit. | Kw | – | 62 | 59 | – | – | – | – | – | – | – |
| *T. parnassi* Boiss. & Sprun. | Bd | – | 40 | – | – | – | – | – | – | – | – |
| *T. parryi* A. Gray | Ca | – | 85 | – | – | – | – | – | – | – | – |
| *T. patens* Schreb. | Cn | 17 | – | – | – | – | – | – | – | – | – |
| *T. patulum* Tausch | Kw | – | 29 | 23 | – | – | tr | – | – | – | – |
| *T. pauciflorum* D'Urv | K | – | 50 | 6 | – | – | – | – | – | – | – |
| *T. phleoides* Pourr. ex Willd. | A | – | 4 | 32 | – | – | – | – | – | – | – |
| *T. pignantii* Brogn. & Bory | Sk | – | 35 | 35 | – | – | 2 | – | – | – | – |
| *T. pilulare* Boiss. | Ta | – | 31 | – | – | – | – | – | – | – | – |
| *T. plebeium* Boiss. | K | – | 40 | – | – | – | – | – | – | – | – |
| *T. retusum* L. | Co | – | 43 | – | – | – | 37 | – | – | – | – |
| *T. rueppellianum* Fresen. | K | – | 102 | – | – | – | 37 | – | – | – | – |

Table I. Continued.

| *Trifolium* species[e] | Seed source[f] | Phytoalexin concentration [μg/ml][a,b,c,d]<br>Resveratrol (**11**) | Medicarpin (**1**) | Maackiain (**2**) | 4-Methoxymedicarpin (**9**) | 4-Hydroxyhomopterocarpin (**8**) | Vestitol (**3**) | Isovestitol (**4**) | Sativan (**5**) | Isosativan (**6**) | Arvensan (**7**) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *T. salmoneum* Mout. | Ta | – | 11 | – | – | – | – | – | – | – | – |
| *T. saxatile* All. | G | – | 3 | 45 | – | – | – | – | – | – | – |
| *T. semipilosum* Fresen. cultivar "Safari" | C | – | 66 | – | – | – | – | – | – | – | – |
| *T. stellatum* L. subsp. *xanthinum* (Freyn) Bald. | Kw | – | 32 | – | – | – | 60 | 25 | – | – | 31 |
| *T. subterraneum* L. subsp. *brachycalycinum* Katzn. & Morley | K | – | 64 | – | – | – | 28 | – | – | 3 | – |
| *T. subterraneum* L. subsp. *yanninicum* (Katzn. & Morley) Zoh. | K | – | 59 | – | – | – | 27 | – | – | – | – |
| *T. sylvaticum* Gérand ex Loisel. | Kw | – | 52 | – | – | – | 12 | – | tr | – | – |
| *T. thalii* Vill. | Sm | – | 100 | – | – | – | tr | – | – | – | – |
| *T. trichocephalum* M.B. | S | – | 22 | 3 | – | – | – | – | – | – | – |
| *T. tridentatum* Lindl. | Bc | – | 81 | – | – | – | 5 | – | – | – | – |
| *T. tumens* Stev. ex M.B. | A | – | 69 | – | – | – | 83 | – | – | – | – |
| *T. uniflorum* L. | Kw | – | 80 | – | – | – | – | – | – | – | – |
| *T. usambarense* Taub. | C | – | 101 | – | – | – | 11 | – | – | – | – |
| *T. vavilovii* Eig | Ta | – | 37 | 2 | – | – | 14 | – | 6 | – | – |
| *T. vesiculosum* Savi | Sn | – | 34 | – | – | – | 151 | – | 37 | 15 | – |
| *T. wormskioldii* Lehm. | Bc | – | 131 | – | – | – | 7 | – | – | – | – |

[a] n.d. = not determined; tr = trace constituent; – = not detected.

[b] Phytoalexin concentrations (μg/ml diffusate) were determined spectrophotometrically after Si gel TLC purification (see Experimental section) using the following extinction coefficients: **1,** log ε = 3.90 at 287 nm; **2,** log ε = 3.83 at 310 nm; **3** (and **4**), log ε = 3.62 at 285 nm; **5,** log ε = 3.62 at 284 nm; **6,** log ε = 3.71 at 285 nm; **7,** log ε = 3.77 at 280–282 nm (for lit. sources see [1]); **9** (and **8**), log ε = 4.09 at 284 nm [20]; **11,** log ε = 4.45 at 305 nm [21]. Apart from occasional traces of medicarpin (**1**) and vestitol (**3**), compounds **1–9** and **11** were not detected in diffusates from control (water-treated) leaflets.

[c] No evidence was obtained to suggest that isoflavonoid (pterocarpan/isoflavan) or stilbene phytoalexins were produced by the fungus-inoculated leaflets of either *T. billardieri* Spreng. (seed source, Co) or *T. grandiflorum* Schreb. (seed source, Kw).

[d] Resveratrol from *T. aureum* could be separated into the *cis-* (approx. 10 μg/ml diffusate) and *trans-* (approx. 40 μg/ml diffusate) isomers by Si gel TLC in PEAM (see Experimental section, and [18]).

[e] The names applied to the *Trifolium* species listed in Table I closely follow those used by Zohary and Heller [22].

[f] Seed sources: A = Institute of Genetics and Plant Breeding, Aschersleben, East Germany; B = Bordeaux Botanic Garden, France; Bc = University of British Columbia Botanic Garden, Vancouver, Canada; Bd = Berlin-Dahlem Botanic Garden, Berlin; C = The Cunningham Laboratory, C.S.I.R.O., St. Lucia, Queensland, Australia; Ca = The Cary Arboretum, Millbrook, New York, U.S.A.; Cm = Coimbra Botanic Garden, Portugal; Cn = University of Caen Botanic Garden, France; Co = Copenhagen Botanic Garden, Denmark; Ct = Catania Botanic Garden, Italy; D = Debrecen Botanic Garden, Hungary; G = Geneva Botanic Garden, Switzerland; H = The Hebrew University of Jerusalem Botanic Garden, Jerusalem, Israel; K = S. Katznelson, The Volcani Institute of Agricultural Research, Nve-Ya'ar Experimental Station, Haifa, Israel; Kw = Royal Botanic Gardens, Kew, England; N = Nantes Botanic Garden, France; P = Palermo Botanic Garden, Italy; S = Stavropol Botanic Garden, U.S.S.R.; Sk = University of Skopje Botanic Garden, Yugoslavia; Sm = "La Jaysinia" Alpine Botanic Garden, Samoëns, France; Sn = University of Siena Botanic Garden, Italy; Ta = University of Tel-Aviv Botanic Garden, Israel; V = Versailles Botanic Garden, France.

medicarpin, from the UV (MeOH + NaOH) maximum at 254 nm [11]. Since the 3,4,9-oxygenation pattern has been established, it follows that the underivatized monohydroxy phytoalexin must be methylated at C-4 (ring A) and C-9 (ring D). The compound is thus 3-hydroxy-4,9-dimethoxypterocarpan (4-methoxymedicarpin, **9**). UV, MS and Si gel TLC comparison of the *Trifolium*-derived phytoalexin with 4-methoxymedicarpin obtained by DDQ oxidation of synthetic 7,2′-dihydroxy-8,4′-dimethoxyisoflavan [12] confirmed that the two compounds were identical. Apart from its formation as a phytoalexin in *Trifolium* leaflets, 4-methoxymedicarpin has recently been reported to occur in the roots of *T. repens* [13].

Thin-layer plate bioassays [14, 15] against the growth of *Cladosporium herbarum* indicated that 4-hydroxyhomopterocarpin (25 μg) and 4-methoxymedicarpin (25 μg) possessed antifungal activity similar to that of medicarpin (**1**).

Although no isoflavans derived from maackiain (**2**) or pterocarpans **8** and **9** were isolated from *Trifolium* diffusates, the isoflavan (vestitol, **3**) corresponding to medicarpin (**1**) was regularly encountered. In diffusates from 11 *Trifolium* species (Table I), vestitol was variously accompanied by the related isoflavans isovestitol (**4**), sativan (**5**), isosativan (**6**) and arvensan (**7**). Isoflavans **5** and **6** presumably originate by O-methylation (at C-2′ and C-7 respectively) of vestitol (**3**) since the former compounds invariably co-occur with **3** in *Trifolium* and other genera of the Trifolieae (*Medicago* [5] and *Trigonella* [4]). As shown in Table I, isovestitol and arvensan are also accompanied by vestitol although neither can be directly derived from **3.** Whilst arvensan (**7**) is probably formed from isovestitol (**4**) by 7-O-methylation (*cf.* **3**→**6**), the biosynthetic precursor of **4** in *Trifolium* remains obscure. In *Tetragonolobus* and allied genera (*e.g. Lotus* and *Hosackia*) of the tribe Loteae, both vestitol and isovestitol generally appear to arise from demethylvestitol (7,2′,4′-trihydroxyisoflavan) [10, 16], but the latter compound was never detected in *Trifolium* diffusates. However, isovestitol frequently co-occurs with sativan (Table I, and ref. [1]), an observation which suggests that **4** might possibly arise from **5** by selective 4′-O-demethylation.

In the previous survey [1] only *T. campestre* and *T. dubium* (section Chronosemium) were reported to produce the stilbene phytoalexin resveratrol (**11**). A third member of the section *(T. aureum)* was listed as being a source of vestitol (**3**). Three further members of the section Chronosemium (*T. boissieri, T. micranthum* and *T. patens*) have now also been found to accumulate resveratrol (Table I), and in view of the anomalous response of *T. aureum* [1], it was decided that this species should be re-examined. Using plants grown from a new batch of verified seeds, it was found that *T. aureum* rapidly produced resveratrol and not the isoflavan vestitol as stated earlier [1]. All the resveratrol-producing *Trifolium* species so far recognized are yellow-flowered, a feature unusual in the genus where white, cream or various shades of red are the predominant flower colours. The accumulation of resveratrol (rather than one or more isoflavonoids) in diffusates from these yellow-flowered species suggests that the section Chronosemium may occupy a taxonomically isolated position within *Trifolium.* Resveratrol does not yet appear to have been recognized as a phytoalexin in other genera of the tribe Trifolieae [2–5, 17] although it is known to accumulate in the fungus-inoculated hypocotyls of *Arachis hypogaea* (tribe Aeschynomeneae) [18].

## Experimental

All the *Trifolium* accessions were raised in a greenhouse (min. temp. 20 °C) from verified seeds (see Table I for details of seed sources). A minimum day-length of 16 h was maintained by a bank of overhead fluorescent tubes giving an initial light intensity of approx. 6500 lx at or near soil level. Leaflets for inoculation were collected after 3–6 months growth. Cultures of the fungus *Helminthosporium carbonum* were grown as described elsewhere [19]. Si gel TLC separations were carried out on Merck pre-coated, glass-backed plates (F-254, layer thickness 0.25 mm) using one or more of the following solvent systems: a) chloroform, b) chloroform–methanol, CM (10:1 or 50:1), c) *n*-pentane–diethyl ether–glacial acetic acid, PEA (75:25:1 or 75:25:3), and d) PEA–methanol, PEAM (75:25:6:1). Solvent equilibration times varied from 25–45 min.

### *Isolation and purification of Trifolium phytoalexins*

Phytoalexins were isolated from *H. carbonum*-inoculated *Trifolium* leaflets by means of the drop-diffusate technique [8]. Control leaflets were treated with droplets of deionized water. After 48 h incubation [8], the diffusates (5–15 ml) were collected and shaken (× 3) with equal volumes of ethyl acetate. These ethyl acetate extracts were reduced to dryness (*in vacuo*, 40 °C), the residues generally being chromatographed in CM (50:1; all species except those producing resveratrol **11**) to variously yield 4-hydroxyhomopterocarpin **8** (approx. $R_F$ 0.71), 4-methoxymedicarpin **9** ($R_F$ 0.68), isosativan **6** ($R_F$ 0.64), medicarpin **1** + maackiain **2**, sativan **5** and arvensan **7** (all $R_F$ 0.56–0.61), and vestitol **3** + isovestitol **4** (both at $R_F$ 0.23–0.27). After elution (methanol, 2 × 5 ml), mixtures of isoflavonoids (**1 + 2; 1 + 2 + 5; 1 + 2 + 7; 3 + 4**) were separated by multiple development (× 3– × 5) Si gel TLC in chloroform or PEA (75:25:3) as previously described [6, 7]. Extracts of *T. hybridum* diffusates were treated as reported in ref. [6] except that 4-hydroxyhomopterocarpin (**8**; approx. $R_F$ 0.71) and 4-methoxymaackiain (**10**; approx. $R_F$ 0.68) were eluted jointly, before being separated by Si gel TLC in PEA (75:25:1). In this system, **8** was located at $R_F$ 0.18, and **10** at $R_F$ 0.38. Ethyl acetate extracts of diffusates from *T. aureum, T. boissieri, T. micranthum* and *T. patens* were chromatographed in CM (10:1) to give resveratrol (**11**) at $R_F$ 0.06. In PEAM, resveratrol from *T. aureum* separated into the *cis* ($R_F$ 0.22) and *trans* ($R_F$ 0.14) isomers [18].

### *Physico-chemical properties of Trifolium phytoalexins* **1–8** *and* **11**

UV and MS data, and colours given on Si gel thin-layer plates sprayed with Gibbs reagent/aqueous $Na_2CO_3$ [15] and/or diazotized *p*-nitroaniline reagent [9] were as previously reported for resveratrol **11** [18], medicarpin **1** [8], maackiain **2** [8], 4-hydroxyhomopterocarpin **8** [8], vestitol **3** [8, 16], isovestitol **4** [16], sativan **5** [16, 20], isosativan **6** [6] and arvensan **7** [7].

### *3-Hydroxy-4,9-dimethoxypterocarpan* **9** *(4-methoxymedicarpin)*

Colour given (TLC plates) with diazotized *p*-nitroaniline reagent [9], yellow. Gibbs test response [15], (–). UV: $\lambda_{max}$, nm: MeOH 211, 234 sh, 285, 292 sh; +NaOH 218, 256, 287, 293 sh. MS (rel. int.): *m/z* 300 ($M^+$; 100; $C_{17}H_{16}O_5$), 299 (12), 285 ($M^+-15$; 25), 284 (4), 161 (10), 151 (6), 149 (13), 148 (21), 137 (16), 136 (5), 135 (6), 133 (6), 125 (6), 123 (8), 121 (8).

*Monomethyl ether* **12** ($CH_2N_2$; $R_F$ 0.85 in chloroform). UV and MS data as given for 3,4,9-trimethoxypterocarpan [8]. *Monoacetate* (Py–$Ac_2O$; $R_F$ 0.80 in chloroform). UV: $\lambda_{max}$, nm: MeOH 211, 234 sh, 284, 291 sh. MS (rel. int.): *m/z* 342 ($M^+$; 9; $C_{19}H_{18}O_6$), 300 ($M^+-42$; 100), 285 ($M^+-42-15$; 25), 284 (6), 267 (5), 164 (5), 161 (18), 152 (5), 151 (7), 149 (8), 148 (24), 139 (5), 137 (6), 133 (8).

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