Medicarpin as a Phytoalexin of the Genus *Melilotus*

John L. Ingham

Phytochemical Unit, Department of Botany, University of Reading

(Z. Naturforsch. **32 c**, 449–452 [1977]; received February 25, 1977)

Leguminosae, *Melilotus*, Sweetclover, Pterocarpan, Phytoalexin

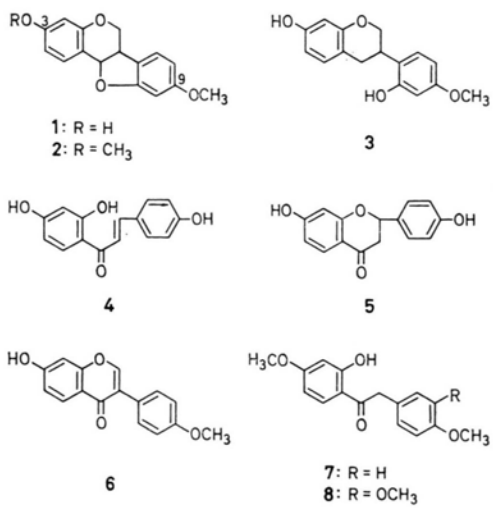
The phytoalexin medicarpin has been isolated from the fungus-inoculated leaves of 19 *Melilotus* species.

Isoflavonoid phytoalexins are produced by many species of the family Leguminosae (subfamily Lotoideae) [1, 2]. As yet, however, little effort has been made to survey phytoalexin production on a generic or tribal basis in order to provide data of taxonomic value. A recent exception involves the genus *Trigonella* where the leaves of 35 out of approx. 70 species [3] were found to produce isoflavonoid compounds following fungal inoculation [4]; at least six different phytoalexin accumulation patterns were observed and to a large extent these correlated with morphological and other chemical characters. *Trigonella* is closely related to *Melilotus* [3] and for this reason a comparative study of the phytoalexins produced by members of the latter genus has been undertaken. The results of this survey are described below.

According to a recent classification [5], the genus *Melilotus* is composed of 19 annual or biennial species (Table I) all of which are native to Europe, North Africa or Asia. Seeds of these species were grown as previously described [6] and phytoalexins isolated from detached leaflets using the drop-diffusate technique [6, 7]. Throughout this study, spore suspensions of the fungus *Helminthosporium carbonum* Ullstrup were used to induce phytoalexin formation [8]. Whenever possible two or more accessions of each *Melilotus* spp. were compared for phytoalexin production.

As shown in Table I, the isoflavonoid phytoalexin, medicarpin (**1**) (3-hydroxy-9-methoxypterocarpan) was produced by the fungus-inoculated leaves of all 19 *Melilotus* species. This substance was isolated in quantity from *M. alba* (white sweetclover) and fully characterised by a) UV, MS and TLC comparison with authentic material obtained from *Medicago sativa* [9] and *Canavalia ensiformis* [10], b) methylation (CH_2N_2) to afford homopterocarpin (**2**) and c) hydrogenation to give vestitol (**3**); authentic specimens of **2** and **3** were available for comparative purposes. Medicarpin production by the other *Melilotus* spp. was confirmed by UV and TLC comparison with the *M. alba* compound.

Large quantities of medicarpin were produced by all the *Melilotus* spp. examined (Table I). In general, its diffusate concentration ranged from 50–100 μg/ml with maximum and minimum values of 108 μg/ml (*M. dentata* 3007) and 34 μg/ml (*M. taurica* 546) respectively. The high medicarpin level associated with diffusate and leaf tissue samples from the cross, *M. polonica* × *M. alba* might well reflect the phenomenon of hybrid vigour. Diffusates from different accessions of a given species normally contained comparable amounts of **1**.



There was no evidence to suggest that any *Melilotus* species accumulated the other pterocarpan (maackiain) [4] or isoflavan (vestitol, sativan or isosativan) [5, 6, 11, 12] phytoalexins isolated from either *Trigonella* or the allied genus *Medicago*. Trace quantities of the chalcone, isoliquiritigenin (**4**) and the flavanone, liquiritigenin (**5**) were occasionally detected (Table I). Only *M. italica* produced measurable quantities of formononetin (**6**), despite the probability that this isoflavone functions (together with **4**) as a medicarpin precursor [13]. Measurable amounts of **1** were obtained from the control diffusates of only 6 accessions: *M. dentata* 3007 (2 μg/ml), *M. infesta* 61-98 (3 μg/ml), *M. italica* 58-256 (2 μg/ml), *M. speciosa* 59-51 (4 μg/ml), *M. sulcata* ssp. *segetalis* 2159 (4 μg/ml), and *M. polonica* × *M. alba* (7 μg/ml). Medicarpin was not obtained from the control tissues of any *Melilotus* spp.

Requests for reprints should be sent to J. L. Ingham, Department of Botany, University of Reading, *Reading RG6 2AS*, England.

Table I. Isoflavonoid and flavonoid compounds in 48 h leaf diffusates (μg/ml) and tissues (μg/g) from *Helminthosporium carbonum*-inoculated species of *Melilotus*.

Species	Source	Diffusate MD	F	LQ	IQ	Tissue MD
Subgenus Melilotus						
**M. alba* Desr.	Hall	89	—	—	—	809
**M. alba* Desr.	Cluj	83	ND	ND	ND	ND
**M. alba* Desr.	Local	88	ND	ND	ND	ND
**M. altissima* Thuill.	Brandon 58-259	90	ND	ND	ND	ND
**M. altissima* Thuill.	Brandon 58-262	98	—	2	—	842
M. dentata (W.K.) Pers.	Brandon 59-57	76	ND	ND	ND	ND
M. dentata (W.K.) Pers.	Brandon 3007	108	—	4	1	666
**M. hirsuta* Lipsky	Brandon 58-44	52	—	3	—	401
**M. officinalis* (L.) Lam.	Cluj	64	—	—	—	658
**M. officinalis* (L.) Lam.	Local	71	ND	ND	ND	ND
**M. polonica* (L.) Desr.	Brandon 3446	82	—	6	—	613
**M. suaveolens* Ledeb.	Brandon 223	58	—	1	—	490
**M. taurica* (M.B.) Ser.	Brandon 60-56	59	—	5	—	475
**M. taurica* (M.B.) Ser.	Brandon 546	34	ND	ND	ND	ND
**M. wolgica* Poir.	Brandon 59-50	59	ND	ND	ND	ND
**M. wolgica* Poir.	Brandon 60-11	47	—	9	1	529
Subgenus Micromelilotus						
**M. elegans* Salzm.	Brandon 61-134	87	—	TR	TR	698
**M. indica* (L.) All.	Brandon 58-197	48	—	—	TR	530
**M. indica* (L.) All.	Brandon 58-204	56	ND	ND	ND	ND
**M. indica* (L.) All.	Oulu	64	ND	ND	ND	ND
**M. infesta* Guss.	Brandon 61-98	70	—	2	—	617
**M. infesta* Guss.	Brandon 62-9	59	ND	ND	ND	ND
**M. italica* (L.) Lam.	Brandon 58-256	65	ND	ND	ND	ND
**M. italica* (L.) Lam.	Brandon 856	50	4	1	—	499
**M. macrocarpa* Coss. & Dur.	Brandon 61-97	52	—	—	—	577
M. messanensis (L.) All.	Brandon 58-195	83	—	—	—	648
M. messanensis (L.) All.	Brandon 524	76	ND	ND	ND	ND
**M. neapolitana* Ten.	Brandon 58-245	41	ND	ND	ND	ND
**M. neapolitana* Ten.	Brandon 3217	65	—	6	5	624
**M. speciosa* Dur.	Brandon 59-51	83	—	2	—	719
**M. speciosa* Dur.	Brandon 536	80	ND	ND	ND	ND
M. sulcata Desf. ssp. *brachystachys* Maire	Brandon 58-263	86	ND	ND	ND	ND
M. sulcata Desf. ssp. *brachystachys* Maire	Brandon 862	88	—	—	—	560
M. sulcata Desf. ssp. *segetalis* (Brot.) Maire	Brandon 535	67	ND	ND	ND	ND
M. sulcata Desf. ssp. *segetalis* (Brot.) Maire	Brandon 863	95	ND	ND	ND	ND
M. sulcata Desf. ssp. *segetalis* (Brot.) Maire	Brandon 2159	65	—	1	TR	509
Hybrid						
**M. polonica* (L.) Desr. × *M. alba* Desr.	Brandon	133	ND	ND	ND	1139

Key: MD, medicarpin; F, formononetin; LQ, liquiritigenin; IQ, isoliquiritigenin; ND, not determined; TR, trace; —, not detectable. Species marked * release coumarin upon tissue maceration.

Concentration of diffusate components based on the following extinction coefficients, i) *medicarpin:* $\varepsilon = 7.762$ at 287 nm [19], ii) *formononetin* $\varepsilon = 29.510$ at 250 nm [20], iii) *liquiritigenin:* $\varepsilon = 14.790$ at 275 nm [21] and iv) *isoliquiritigenin:* $\varepsilon = 30.900$ at 370.5 nm [22].

Key to seed/plant sources: Brandon, Research Station, Canada Department of Agriculture, Brandon, Manitoba, Canada (source designations of Brandon seed samples are also given); Cluj, Botanic Garden, University of Cluj-Napoca, Roumania; Hall, Robson Quality Seeds Inc., Hall, New York, U.S.A.; Local, Leaves collected from locally established wild plants; Oulu, Botanic Garden, University of Oulu, Finland.

In addition to compounds **1** and **4** – **6**, the diffusates from several species (*M. indica* 58-197; *M. italica* 856; *M. neapolitana* 3217; *M. polonica* 3446; *M. speciosa* 59-51; *M. wolgica* 60-11) contained small quantities of a phenolic compound (R_F approx. 0.77 in $CHCl_3$: MeOH, 50 : 1; *cf.* **1**, R_F approx. 0.60) which gave an orange colouration with diazotised *p*-nitroaniline. This substance (termed MN-1 because of its initial isolation from fungus-induced diffusates of *M. neapolitana*) did not exhibit antifungal activity in a TLC bioassay against spore germination of *Cladosporium herbarum* Fr.[8]. Although not fully identified, MN-1 was provisionally formulated as a deoxybenzoin-like derivative after UV and MS comparison with the deoxybenzoins (**7** and **8**) produced from 7,4′-dimethoxy and 7,4′,5′-trimethoxyisoflavone (see Experimental). The neutral spectrum (MeOH) of MN-1 was unaffected by NaOAc thereby suggesting the presence of a methoxyl substituent at the position corresponding to C-7 of flavonoids[14]; daidzein deoxybenzoin (which is hydroxylated at this position) gives a 56 nm bathochromic shift with NaOAc[12].

In terms of its phytoalexin production, *Melilotus* clearly exhibits a more uniform response than does the related genus *Trigonella*[4]. However, there is a definite link between these genera in that a group of 13 morphologically '*Melilotus*-like' *Trigonella* species are also characterised by their exclusive production of medicarpin[4, 12]; moreover, the majority of these *Trigonella* species also release coumarin upon tissue maceration[4, 12, 15], a feature associated with 15 out of the 19 *Melilotus* species listed in Table I. In contrast, 11 other *Trigonella* species (termed '*Medicago*-like')[12] do not release coumarin and are characterised by the formation, not only of medicarpin, but also of the isoflavans vestitol and sativan, two phytoalexins absent from *Melilotus* but commonly encountered in the taxonomically related genus *Medicago*[3, 12]. The strong chemical similarities between a) *Melilotus* and the '*Melilotus*-like' *Trigonella* group and b) *Medicago* and the '*Medicago*-like' members of *Trigonella*, suggests that taxonomically *Trigonella* may occupy a position intermediate between *Melilotus* on the one hand and *Medicago* on the other. A detailed account of the phytoalexins characteristic of *Medicago* is in preparation and will be published elsewhere.

Experimental

Mass and UV spectra were determined as previously described[16].

Extraction and purification of **1**. Leaf diffusates were extracted (×3) with equal volumes of EtOAc. After bulking and removal of solvent (*in vacuo*; 40 °C) the residue was chromatographed (Si gel[16]; $CHCl_3$: MeOH, 50 : 1) to afford **1** at approx. R_F 0.60. Compounds MN-1, **6** and a joint **4**/**5** zone were located at approx. R_F 0.77, 0.24 and 0.10 respectively. Additional purification of **1**, **6** and MN-1 proved unnecessary. Compounds **4** and **5** were resolved by TLC in *n*-pentane : Et_2O : HOAc (75 : 25 : 3, ×3) (**4**, *lower zone;* **5** *upper yellow zone*). Final purification of **5** was by TLC on cellulose F (Merck) using H_2O as the developing solvent (**5** remains at the origin). Tissues underlying the inoculum droplets were excised[17] and extracted with EtOH[17]. TLC of the extract (Et_2O : *n*-hexane, 3 : 1) gave a deeply quenching, faint green band at approx. R_F 0.67. This was eluted (EtOH) and rechromatographed in $CHCl_3$ to afford **1** (R_F 0.45) and, for most *Melilotus* spp. (Table I) a band (R_F 0.85) attributable to coumarin formed by enzymic hydrolysis of coumarinyl glucoside[15] during the extraction process. Coumarin was firmly identified by UV, MS and TLC comparison with an authentic sample; crystallisation (aq. EtOH) gave colourless needles, mp. and mmp. 67 – 69 °C.

3-Hydroxy-9-methoxypterocarpan **1**. MS and UV as lit.[9, 10]. *Dimethyl ether* **2** (CH_2N_2) MS and UV as lit.[6]. *Hydrogenation of* **1**. Compound **1** (3.5 mg), EtOH (7 ml), H_2SO_4 (25%; 1 drop) and Pd-C (10%; 5 mg) were shaken in an atmosphere of H_2 at 80 °C for 3 h. Removal of solvent and catalyst followed by TLC ($CHCl_3$: MeOH, 50 : 1) gave **3** (R_F 0.24) identical (MS, UV, TLC) with an authentic sample. Crystallisation (aq. MeOH) gave colourless needles, mp. 154 – 156 °C (lit. 156 °C)[18].

Compounds **4**, **5** and **6**. UV maxima as lit.[14]; all were indistinguishable (TLC) from authentic specimens.

Compound MN-1. Colour with diazotised *p*-nitroaniline, bright orange; λ_{max} (nm) *MeOH*: 216, 229sh, 240sh, 279, 318; *NaOH*: 218, 245sh, 279, 354; *NaOAc*: 279, 318; *NaOAc* + *Borate*: 279, 315; $AlCl_3$: 284sh, 307, 363; $AlCl_3$ + *HCl*: 275sh, 284, 304, 362; MS (rel. int.) 284 (M^+?; 13), 151(53), 149(19), 121(100).

Synthesis of 7-O-methylformononetin deoxybenzoin **7**. 7,4′-Dimethoxyisoflavone (1.5 mg) in EtOH (2 ml) was heated (10 min; 70 °C; N_2 atmos.) with aq. NaOH (4%; 3 ml). After dilution (H_2O; 20 ml) and acidification (2 N HCl) to pH 3, the soln was extracted (×3) with EtOAc. Si gel TLC of the extract ($CHCl_3$) gave the required deoxybenzoin at R_F 0.76; this was further purified by TLC in *n*-pentane : Et_2O : HOAc (75 : 25 : 1, R_F

0.69). Colour with diazotised *p*-nitroaniline, bright orange; λ_{max} (nm) *MeOH*: 215, 228sh, 238sh, 276, 318; *NaOH*: 219, 245sh, 277, 355; *NaOAc*: 276, 318; *NaOAc + Borate*: 276, 318; $AlCl_3$: 275sh, 302, 361; $AlCl_3$ + *HCl*: 275sh, 283, 297, 360; MS (rel. int.) 272(8), 152(11), 151(100), 149(4), 121(9).

Synthesis of cabreuvin deoxybenzoin **8**. 7,4′,5′-Trimethoxyisoflavone (1 mg) was treated with aq. NaOH as described above. Work up and TLC ($CHCl_3$) afforded cabreuvin deoxybenzoin at R_F 0.64. Colour with diazotised *p*-nitroaniline, bright orange; λ_{max} (nm) *MeOH*: 216, 230sh, 278, 319; *NaOH*: 220, 245sh, 278, 356; MS (rel. int.) 302(10), 165(11), 152(10), 151(100).

The author thanks R. W. Butters (Tate and Lyle Ltd.) for MS analyses and G. A. Stevenson (Canadian Department of Agriculture) for supplying seeds of the Brandon accessions of *Melilotus*.

[1] H. D. VanEtten and S. G. Pueppke, Biochemical Aspects of Plant-Parasite Relationships (J. Friend and D. R. Threlfall, ed.), p. 239, Academic Press, London 1976.
[2] J. L. Ingham, Botan. Rev. **38**, 343 [1972].
[3] J. Hutchinson, The Genera of Flowering Plants **1**, 456, Clarendon Press, Oxford 1964.
[4] J. L. Ingham and J. B. Harborne, Nature **260**, 241 [1976].
[5] G. A. Stevenson, Can. J. Plant Sci. **49**, 1 [1969].
[6] J. L. Ingham, Phytochemistry **15**, 1489 [1976].
[7] J. L. Ingham and R. L. Millar, Nature **242**, 125 [1973].
[8] J. L. Ingham, Phytopathol. Z. **87**, 353 [1976].
[9] D. G. Smith, A. G. McInnes, V. J. Higgins, and R. L. Millar, Physiol. Plant Pathol. **1**, 41 [1971].
[10] N. T. Keen, Phytopathology **62**, 1365 [1972].
[11] J. L. Ingham, Z. Naturforsch. **31 c**, 331 [1976].
[12] J. L. Ingham, Ph. D. thesis, University of Reading, U.K. 1976.
[13] P. M. Dewick, Phytochemistry **14**, 979 [1975].
[14] T. J. Mabry, K. R. Markham, and M. B. Thomas, The Systematic Identification of Flavonoids, Springer, New York 1970.
[15] H. J. Gorz and F. A. Haskins, Crop Science **4**, 193 [1964].
[16] J. L. Ingham, Z. Naturforsch. **31 c**, 504 [1976].
[17] J. L. Ingham, Phytochemistry, in press.
[18] K. Kurosawa, W. D. Ollis, B. T. Redman, I. O. Sutherland, O. R. Gottlieb, and H. Magalhães Alves, J. Chem. Soc. Chem. Comm. **1968**, 1265.
[19] S. H. Harper, A. D. Kemp, and W. G. E. Underwood, Chem. Ind. **1965**, 562.
[20] W. D. Ollis, The Chemistry of Flavonoid Compounds (T. A. Geissman, ed.), p. 353, Pergamon Press, London 1962.
[21] D. M. X. Donnelly, J. C. Thompson, W. B. Whalley, and S. Ahmad, J. Chem. Soc. Perkin I **1973**, 1737.
[22] R. G. Cooke and I. D. Rae, Aust. J. Chem. **17**, 379 [1964].