Phytochemistry, 1976, Vol. 15, pp. 1411–1412. Pergamon Press. Printed in England.

6a-HYDROXYPTEROCARPANS FROM RED CLOVER

JOHN N. BILTON, JULIAN R. DEBNAM and IAN M. SMITH
Depts of Chemistry and Botany, Imperial College of Science and Technology, London SW7, England

(*Received* 24 *February* 1976)

Key Word Index—*Trifolium pratense*; Leguminosae; red clover; antifungal pterocarpans; fungal mediated hydroxylation.

Abstract—Pisatin and homopisatin have been identified as antifungal compounds in red clover infected with either *Botrytis cinerea*, a non-pathogen, or *Sclerotinia trifoliorum*, a clover pathogen. In addition 6a-hydroxymaackiain and 6a-hydroxymedicarpin have been detected. *In vitro* experiments have established that *Sclerotinia* can convert both maackiain and medicarpin to their respective 6a-hydroxylated products.

INTRODUCTION

Red clover (*Trifolium pratense*. L.) is known to produce the pterocarpans maackiain (**1**) and medicarpin (**2**) in response to fungal infection [1] and to contain isoflavones [2] and coumestans [3]. Work on the relationship between isoflavanoids in red clover and disease resistance [4] confirmed the presence of isoflavones and maackiain/medicarpin, but also revealed some antifungal substances which could not immediately be identified with compounds previously reported. This report describes the characterisation of these antifungal compounds and shows that two of these products can arise by fungal mediated hydroxylation of maackiain and medicarpin.

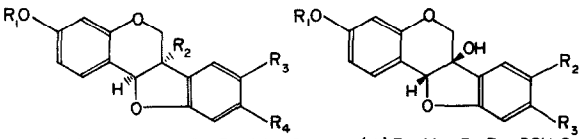
RESULTS AND DISCUSSION

Red clover leaves infected with a non-pathogen, *Botrytis cinerea Fr*, afforded an extract which exhibited a new zone of fungal inhibition during the TLC bioassay procedure [5]. This fraction could be separated into two components by careful multiple elution chromatography with Et_2O–petrol mixtures on Si gel plates. Both components contained no free phenolic OH groups since their UV spectra showed no base shift. They were extremely acid labile, giving products which showed a characteristic coumestrol type of fluorescence under UV light.

The major compound, λ_{max}^{EtOH} nm: 282, 287, and 310, gave an intense orange–brown colour reaction when a TLC spot of the sample was exposed to UV light. The MS [*m/e* 314 (M^+,$C_{17}H_{14}O_6$), 299 (M^+-Me), 296 (M^+-H_2O), 295 (M^+-H_2O-H), 286, 285, 269, 255, 177 and 163] was indistinguishable from that of an authentic sample of pisatin (**4**) run under the same conditions. Thus the identity of the compound was established. The pronounced photolability of pisatin has been noted by Perrin and Bottomley [6].

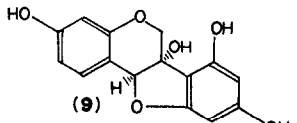
The second compound, slightly less polar on TLC than pisatin, was closely related to homopterocarpin (**3**), having a λ_{max}^{EtOH} nm: 282 and 287, and a MS *m/e* 300 (M^+,$C_{17}H_{16}O_5$), 285 (M^+-Me), 282 (M^+-H_2O), 281 (M^+-H_2O-H), 272, 271, 255 and 241. Metastable defocussing on the fragment ions revealed a decomposition pattern similar to that of pisatin. Acid catalysed dehydration of the sample and subsequent autoxidation of the resulting pterocarpen afforded the dimethoxycoumestan (**13**), MS *m/e* 296 (M^+,$C_{17}H_{12}O_5$), identified by comparison with an authentic sample prepared by treatment of coumestrol with ethereal CH_2N_2. The parent compound was therefore identified as the pterocarpan homopisatin(variabilin) (**5**) that has been briefly noted by two groups of workers [7, 8]. Racemic homopisatin has been synthesised from homopterocarpin [9].

The pisatin/homopisatin mixtures were initially detected in extracts from clover leaves infected with *Sclerotinia*, but subsequent work was performed on tissue inoculated with *Botrytis* since this produced larger amounts of these substances.

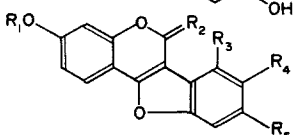


(**1**) R_1=H, R_2=H, R_3,R_4=OCH_2O
(**2**) R_1=H, R_2=H, R_3=H R_4=OMe
(**3**) R_1=CH_3, R_2=H, R_3=H, R_4=OMe
(**6**) R_1=H, R_2=OH, R_3,R_4=OCH_2O
(**7**) R_1=H, R_2=OH, R_3=H, R_4=OMe
(**8**) R_1=H, R_2=OH, R_3=H, R_4=OH

(**4**) R_1=Me, R_2,R_3=OCH_2O
(**5**) R_1=Me, R_2=H, R_3=OMe



(**9**)



(**10**) R_1=H, R_2=O, R_3=OH, R_4=H, R_5=OH
(**11**) R_1=H, R_2=O, R_3=H, R_4,R_5=OCH_2O
(**12**) R_1=H, R_2=H_2, R_3=H, R_4,R_5=OCH_2O
(**13**) R_1=Me, R_2=O, R_3=H, R_4=H, R_5=OMe

Clover tissue infected with *Sclerotinia trifoliorum* Erikss. also yielded a new zone of fungal inhibition by the bioassay procedure, and this corresponded to a mixture of two phenolic substances. They were photolabile, giving reddish spots on TLC plates exposed to UV light, and were acid sensitive in a manner similar to pisatin. These substances were not detected in clover inoculated with *Botytis*. The major, more polar compound on TLC had a λ_{max}^{EtOH} nm: 282, 287 and 310, and MS *m/e* 300 (M^+,$C_{16}H_{12}O_6$), 282 (M^+-H_2O), 281 (M^+-H_2O-H), 272, 271, 255 ($C_{15}H_{11}O_4$), 241 ($C_{14}H_9O_4$) and 163 ($C_9H_7O_3$). An FT PMR (100 HHz, $CDCl_3$, TMS) of the sample revealed an aromatic substitution pattern like that of maackiain [10]. The C–6 methylene protons appeared as an AB quartet at δ 4.12 and δ 3.93 (J = 12 Hz) and the C-11a proton was a singlet at δ 5.20. The methylenedioxy protons occurred as an AB quartet centred at δ 5.86 (J = 1.5 Hz). However, the spectrum had been run in a solvent which contained sufficient acid to catalyse the dehydration of the sample, so that additional peaks were present corresponding to the pterocarpen anhydrosophorol (**12**). The methylenedioxy protons collapsing to a singlet at δ 5.93 and the C–6 protons to a singlet at δ 5.44. The dehydrated sample gave a MS which contained two M^+, anhydrosophorol *m/e* 282 (M^+,$C_{16}H_{10}O_5$), 281 (M^+-H) and its autoxidation product medicagol (**11**) at *m/e* 296 (M^+,$C_{16}H_8O_6$). The parent phenol was thus identified as 6a-hydroxymaackiain (**6**). A similar compound has been reported as a fungal metabolite of (+)pisatin [11, 12]. However the two products are presumably enantiomeric (see later).

The second phenolic component had a λ_{max}^{EtOH} nm: 282 and 287, and MS *m/e* 286 (M^+,$C_{16}H_{14}O_5$), 271 (M^+-Me), 268 (M^+-H_2O), 267 (M^+-H_2O-H), 258, 257, 253, 241 and 227. The similarity of the MS to that of the other 6a-hydroxypterocarpans indicated that the compound was 6a-hydroxymedicarpin (**7**), a previously unreported pterocarpan.

The occurrence of maackiain/medicarpin and 6a-hydroxymaackiain/6a-hydroxymedicarpin in infected clover tissue suggested that there might be a fungal mediated link between these two types of pterocarpan. It was found that (−)maackiain and (−)medicarpin were indeed metabolised by *Sclerotinia in vitro* to give 6a-hydroxylated products identical to those isolated from the infected plant tissue. If the hydroxylation proceeds in a manner analogous to that of the phaseollin/6a-hydroxyphaseollin transformation [13] then the products would have the 6aS, 11aS configuration. An assignment of configuration for the pisatin/homopisatin samples can only be inferred, in the absence of rotation data, by comparison with other legume systems in which the 6aR, 11aR configuration is known to occur. It is worth noting that (+)6a-hydroxymaackiain can be derived by a demethylation route from (+) pisatin utilizing *Fusarium* [12]. It is not known whether *Sclerotinia* can perform this transformation in our system. The presence of pisatin/homopisatin could also be the result of a fungus mediated conversion of maackiain/medicarpin, but it is worth noting that the former compounds accumulated in *Botrytis* lesions in which the 6a-hydroxy compounds were not detected.

The conversion of maackiain and medicarpin to 6a-hydroxy derivatives is the first step in their degradation by *Sclerotinia*. and the appearance of these compounds in rotted tissue suggests that the hydroxylation occurs *in vivo*. With respect to the further degradation of these compounds, it is of interest to compare the 7-hydroxylation of 6a-hydroxyphaseollin [13] with the occurrence of the trihydroxycoumestan (**10**) in diseased white clover [3]. The latter compound may be the product of the later stages of a detoxification sequence of the type:- (8)→(9)→→(10). We have observed more polar fungal metabolites being formed from the 6a-hydroxypterocarpans *in vitro* and these are under investigation.

EXPERIMENTAL

Details of the plant pathology, isolation and bioassay techniques have been reported elsewhere [4]. Final purification of the pterocarpans was performed on Si gel plates and eluting with either Et_2O-petrol or Me_2CO-petrol mixtures. In general it was found that very small amounts of the pterocarpans could be visualized on TLC by exposure to an intense UV source for a short time. Structural information could be obtained from these results, since the colour reactions of the 6a-hydroxy pterocarpans, in particular, reflected the effect of the C–3 substituents on the absorption maxima of the corresponding isoflavylium pseudobases [6, 15]. MS were run at 70 eV and 100 μA trap current using a ceramic probe, with the source at the lowest possible temp. The M^+ and (M^+-H_2O) ion intensities showed a dependence upon source temp, indicating that some thermolytic loss of H_2O was occurring at higher temps. Second field free metastable ions were detected by running the spectra at 18 eV and operating at maximum gain. First field free transitions were observed by the standard defocussing technique. The PMR spectrum of 6a-hydroxymaackiain was run on a 100 MHz instrument operating with a digitization rate of 0.5 Hz/point. (−)Maackiain was produced by natural enzymic hydrolyxis of trifolirhizin in clover roots. (−)Medicarpin was isolated from sliced jack bean cotyledons inoculated with *Botrytis cinerea* [14].

REFERENCES

1. Higgins, V. J. and Smith, D. G. (1972) *Phytopathology* **62**, 235.
2. Virtanen, A. I. and Hietala, P. K. (1958) *Acta Chem. Scand.* **12**, 579. Pope, G. S. and Wright, H. G. (1954) *Chem. Ind.* 1019. Bredenberg, J. B. (1961) *Suomen Kemistilehti* B34, 23.
3. Wong, E. and Latch, G. C. M. (1971) *Phytochemistry* **10**, 466. Livingston, A. L., Bickoff, E. M., Lundin, R. E. and Jurd, L. (1964) *Tetrahedron* **20**, 1963.
4. Debnam, J. R. and Smith, I. M. (1976) *Physiol. Plant. Pathol.* (in preparation).
5. Bailey, J. A. and Burden, R. S. (1973) *Physiol. Plant. Pathol.* **3**, 171.
6. Perrin, D. R. and Bottomley, W. (1962) *J. Am. Chem. Soc.* **84**, 1919.
7. Cruickshank, I. A. M. and Perrin, D. R. (1969) *Phytochemistry* **8**, 971.
8. Ollis, W. D. and Kurosawa, K. (1968) *Recent Advan. Phytochemistry* **1**, 357.
9. Bevan, C. W. L., Birch, A. J., Moore, B. and Mukerjee, S. K. (1964) *J. Chem. Soc.* 5991.
10. Pachler, K. G. R. and Underwood, W. G. E. (1967) *Tetrahedron* **23**, 1817.
11. Van't Land, B. G., Wiersma Van Duin, E. D. and Fuchs, A. (1975) *Acta Botan. Neerl.* **24**, 251.
12. Van Etten, H. D., Pueppke, S. G. and Kelsey, T. C. (1975) *Phytochemistry* **14**, 1103.
13. Burden, R. S., Bailey, J. A. and Vincent, G. C. (1974) *Phytochemistry* **13**, 1789.
14. Keen, N. T. (1969) *Phytopathology* **62**, 1365.
15. Baker, W. (1929) *J. Chem. Soc.* 1593.