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SESQUITERPENE PHYTOALEXINS FROM *ULMUS GLABRA*

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Key Word Index—*Ulmus glabra*; Ulmaceae; elm; *Ceratocystis ulmi*; *Chondrostereum purpureum*; *Coriolus versicolor*; fungal infection; phytoalexin; sesquiterpene; cadinane.

Abstract—Inoculation of Wych elm (*Ulmus glabra*) with the fungi *Ceratocystis ulmi*, *Chrondrostereum purpureum* and *Coriolus versicolor* induced the formation of a series of antifungal cadinane-type sesquiterpenes.

INTRODUCTION

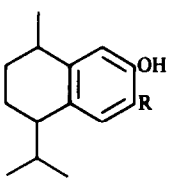
We recently reported [1] the accumulation of the phenol (−)-7-hydroxycalamenene in a narrow zone at the interface between healthy and diseased sapwood of a European lime (*Tilia europea*) infected with *Ganoderma applanatum*. As the compound was also found to have *in vitro* antifungal activity, we considered that it was probably synthesized by the tree as a phytoalexin in an attempt to contain the pathogen.

(−)-7-Hydroxycalamenene has previously been isolated as a constituent of the heartwood of several elm species [2] but there is no report of it being induced in the sapwood after fungal infection. However, the sapwood of other trees, particularly species of *Picea* and *Pinus*, has been found [3] to respond to injury and infection by producing antifungal compounds normally found only in the heartwood. The present paper reports our investigation of the occurrence of 7-hydroxycalamenene and other antifungal compounds in the sapwood of diseased elm. We used Wych elm (*Ulmus glabra*) inoculated with the fungi *Ceratocystis ulmi* (causal agent of Dutch elm disease), *Coriolus versicolor* (a white rot fungus) and *Chondrostereum purpureum* (causal agent of 'silver leaf' disease in many trees). The only previous study of elm phytoalexins, by Elgersma and Overeem, showed [4, 5] that the antifungal mansonones E and F accumulated in *Ulmus hollandica* after inoculation with spores of *Ceratocystis ulmi*.

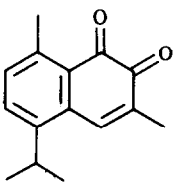
RESULTS

Ulmus glabra branches examined 2 months after inoculation with *Ceratocystis ulmi* exhibited typical symptoms of Dutch elm disease, notably severely wilted and dying leaves and a dark brown intermittent streaking of the xylem immediately beneath the bark. The staining frequently extended some 2–3 m throughout the whole length of the branch. Infections with *Chondrostereum purpureum* produced brown staining extending 10–60 cm down the branches from the inoculation dowels. In some samples there was a distinct yellow interface between the healthy sapwood and the brown stain. With *Coriolus versicolor* the fungus appeared to be restricted within a region of yellow stained wood 1–5 cm from the inoculation dowel.

TLC of ethanol extracts of the infected branches revealed, in all three treatments, compounds which were not observed in chromatograms of the extracts of healthy branches. These (**1–8**) were isolated by TLC and identified by their mass spectra and by a comparison of their UV spectra with literature data [6–9]. UV spectroscopy was also used to measure the amounts of the compounds present and the antifungal activity was assessed using a *Cladosporium cucumerinum* TLC assay [10] (Table 1). No antifungal activity was detected in extracts of the healthy sapwood.



R
1 CHO
2 Me



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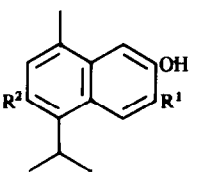
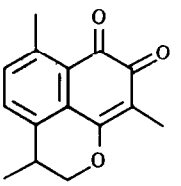


3 R^1 = CHO, R^2 = H
4 R^1 = CHO, R^2 = OMe
5 R^1 = Me , R^2 = H



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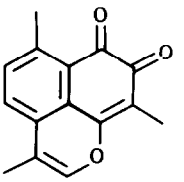

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Table 1. Induced compounds in *Ulmus glabra*

	Infection fungus	Compound 1	2	3	4	5	6	7	8
Concentration (ppm fr. wt) in infected sapwood*	*Ceratocystis ulmi*	27	244	68	16	95	127	360	222
	Coriolus versicolor	52	71	135	15	32	9	7	4
	Chondrostereum purpureum	122	84	756	31	26	7	10	5
Contribution to antifungal activity in EtOH extract†	*Ceratocystis ulmi*	−	+ + + +	+	−	+ +	+ +	+ + + + +	+ + +
	Coriolus versicolor	+	+ + +	+ + + +	+	+	−	+	+
	Chondrostereum purpureum‡	+ +	+ + +	+ + + + +	+	+	−	+	+

*Estimated by UV spectroscopy.

†*Cladosporium* TLC bioassay. Visual assessment of inhibition resulting from each compound. Extremes of scale are: +, spot just detectable; + + + + +, large intense zone of inhibition.

‡*Chondrostereum purpureum* infections also produced two unknown compounds with lower R_fs than **1**–**8**, both with inhibition rating + + +.

DISCUSSION

Sesquiterpenes **1**–**6** have all been previously reported as heartwood constituents of various elm species but are now shown to be also induced in fungus-infected sapwood of *Ulmus glabra*. Included among the compounds are 7-hydroxycalamenene (**2**) and the mansonones E (**7**) and F (**8**). The latter were previously isolated from *Ulmus hollandica* following inoculation with *Ceratocystis ulmi* [4, 5]. No other induced antifungal compounds were detected in the earlier experiments but it has been suggested [11] that use of boiling ethanol in the extraction procedures may have caused unstable substances to decompose or to undergo further reactions. The present work has used much milder isolation procedures.

Table 1 illustrates quantitative differences in the compounds produced as a result of the three types of infection. *Ceratocystis ulmi* induced high levels of the mansonones E (**7**) and F (**8**) and 7-hydroxycalamenene (**2**). In contrast, the two basidiomycetes induced very little of the mansonones but a considerable amount of the yellow-coloured aldehyde 7-hydroxycadalenal (**3**) which is almost certainly responsible for the yellow colour interface between healthy and diseased sapwood observed in *Chondrostereum purpureum* infections. 7-Hydroxycadalenal has previously been found in the outer heartwood of slippery elm (*Ulmus rubra*) where it produces yellow stains which can be a problem in furniture manufacture [7].

The extracts of all three types of infected wood produced several antifungal zones on TLC (Table 1). The most intense zone produced by *Ceratocystis ulmi* was due to mansonone E (**7**) while with *Chondrostereum purpureum* and *Coriolus versicolor* 7-hydroxycadalenal (**3**) gave the largest antifungal zone. 7-Hydroxycalamenene (**2**) produced a substantial zone in all cases. The levels of activity observed with this assay generally corresponded well with the amounts of compounds present as measured by UV spectroscopy (Table 1).

The role of these tree phytoalexins in disease resistance remains to be determined. It is also uncertain why their relative amounts vary after infection with different pathogens although similar variations have been observed with phytoalexins in herbaceous plants, particularly French bean and potato [12]. The mansonones E (**7**) and F (**8**) have previously [4] been found to have little *in vitro* activity against *Ceratocystis ulmi* and in our experiments the rapid growth rate of the pathogen and early onset of disease symptoms would suggest that there was little effective resistance. Little is known about natural infections of elm with fungi such as *Chrondrostereum purpureum* and *Coriolus versicolor* but it is possible that the induced antifungal compounds may at least retard the progress of infection.

EXPERIMENTAL

Plant material and inoculation procedures. Ulmus glabra trees, *ca* 5 m tall and 10 years old, were used. Six separate branches, 2–3 m long, were treated with each fungus. An isolate of *Chondrosteum purpureum* was obtained from diseased wood of perry pear and grown on 5% malt agar. Infection dowels were prepared by soaking 5 × 1 cm cylindrical lengths of wood in 10% malt extract soln, autoclaving and transferring to actively growing cultures of *Chrondrostereum purpureum*. They were incubated in darkness for 4 weeks at 25° after which the dowels were thoroughly penetrated by fungal mycelium. The ends of 3–5 cm diameter branches of *Ulmus glabra* were severed, the exposed wood drilled to a depth of 5 cm and the fungus-impregnated dowels inserted into the holes.

Wooden chips 10 mm long × 5 mm in diameter were soaked overnight in malt extract, sterilized and incubated for 3 weeks with an actively growing culture of *Coriolus versicolor* in potato dextrose agar. They were then used to inoculate branches by inserting into small holes drilled into the side of each branch, 1 m from the trunk.

An aggressive strain of *Ceratocystis ulmi* was obtained from Dr. J. W. Gibbs of the Forestry Commission. This was propagated by shaking a spore suspension in sterile Tehnernoffs medium for 3 days at 25°, giving dense suspensions which contained predominently yeast-type spores (*ca* 5×10^7 spores per ml). Branches were inoculated by cutting through the bark with a razor blade and placing the spore suspension onto the exposed xylem. The wounds were then sealed with adhesive tape.

Extraction and isolation. Infected branches were cut and removed from the trees 8 weeks after inoculation. The bark of *Ceratocystis ulmi* diseased branches was removed and the brown stained wood immediately below was shaved off with a razor blade. Branches with *Coriolus versicolor* and *Chondrostereum*

purpureum infections were cut open longitudinally. The stained wood extending from the dowels was planed, care being taken to include in the sample the wood adjacent to healthy sapwood at the limits of the infection. Shavings were also taken of the sapwood of uninoculated healthy branches.

Samples of wood (10 g) were extracted with EtOH (100 ml) at ambient temp. for 2 weeks. After filtration the EtOH was evapd and the residue dissolved in MeOH (5 ml). Aliquots (10–50 μl) were applied to Merck silica gel F 254 TLC plates, the plates developed in $CHCl_3$ or hexane–Me_2CO (4:1) and then sprayed with a spore suspension of *Cladosporium cucumerinum* [10]. Isolation of the sesquiterpenes **1–8** was by TLC first in $CHCl_3$ (R_f values 0.66, 0.38, 0.66, 0.64, 0.37, 0.43, 0.32, 0.20, respectively) followed by re-chromatography in hexane–Me_2CO (4:1) (R_f values 0.56, 0.30, 0.45, 0.40, 0.24, 0.29, 0.14, 0.06, respectively). Detection was by colour (compounds **2, 3, 6, 7** and **8**), colour with diazotized *p*-nitroaniline (positive reaction with **1–5**) and colour with 2,4-dinitrophenylhydrazine (positive reaction with **1, 3, 4, 6** and **7**). Low resolution MS were obtained on a Finnigan 4021 MS-DS.

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REFERENCES

1. Burden, R. S. and Kemp, M. S. (1983) *Phytochemistry* **22**, 1039.
2. Rowe, J. W., Seikel, M. K., Roy, D. W. and Jorgensen, E. (1972) *Phytochemistry* **11**, 2513.
3. Kuć, J. and Shain, L. (1977) in *Antifungal Compounds* (Siegel, M. R. and Sisler, H. D., eds.), Vol. 2, p. 497. Marcel Decker, New York.
4. Overeem, J. C. and Elgersma, D. M. (1970) *Phytochemistry* **9**, 1949.
5. Elgersma, D. M. and Overeem, J. C. (1971) *Neth. J. Plant Pathol.* **77**, 168.
6. Marini Bettolo, G. B., Casinovi, C. G. and Galeffi, C. (1965) *Tetrahedron Letters* 4857.
7. Fracheboud, M., Rowe, J. W., Scott, R. W., Fanega, S. M., Buhl, A. J. and Toda, J. K. (1968) *Forest Prod. J.* **18**, 37.
8. Fallagher, M. J. and Sutherland, M. D. (1965) *Aust. J. Chem.* **18**, 1111.
9. Rowe, J. W. and Toda, J. K. (1969) *Chem. Ind.* 922.
10. Bailey, J. A. and Burden, R. S. (1973) *Physiol. Plant Pathol.* **3**, 171.
11. Bell, A. A. and Mace, M. E. (1981) in *Fungal Wilt Diseases of Plants* (Mace, M. E., Bell, A. A. and Beckman, C. H., eds.), p. 461. Academic Press, London.
12. Bailey, J. A. (1982) in *Phytoalexins* (Bailey, J. A. and Mansfield, J. W., eds.), p. 291. Blackie, Glasgow.