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WYERONE EPOXIDE AS A PHYTOALEXIN IN *VICIA FABA* AND ITS METABOLISM BY *BOTRYTIS CINEREA* AND *B. FABAE IN VITRO*

JOHN A. HARGREAVES*, JOHN W. MANSFIELD*, DAVID T. COXON† and KEITH R. PRICE†

* Department of Biology, The University, Stirling, FK9 4LA, Scotland; † Chemistry Division, Agricultural Research Council, Food Research Institute, Colney Lane, Norwich, NR4 7UA, England

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Key Word Index—*Vicia faba*; Leguminosae; broad bean; phytoalexin; wyerone epoxide; detoxification; *Botrytis cinerea*; *Botrytis fabae*.

Abstract—Wyerone epoxide was identified as a third acetylenic phytoalexin in *Vicia faba*. The epoxide accumulated in limited lesions formed by both *Botrytis cinerea* and *B. fabae*. Products of the metabolism of wyerone epoxide by *B. cinerea* and *B. fabae* were identified as wyerol epoxide and dihydrodihydroxywyerol respectively. The metabolites were less antifungal than wyerone epoxide.

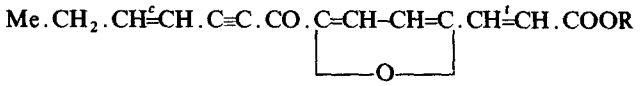
INTRODUCTION

In addition to wyerone (**1**) [1] and wyerone acid (**2**) [2] several antifungal compounds, all absent from healthy tissue, have been detected in extracts of broad bean plants infected with species of *Botrytis* [3]. In this paper we report the identification of one of the inhibitors, previously named PAI [3], as wyerone epoxide (**3**). Preliminary studies on the antifungal activity and metabolism of this phytoalexin by *Botrytis cinerea* and *B. fabae* are also discussed.

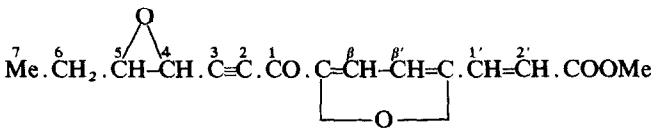
RESULTS AND DISCUSSION

Identification

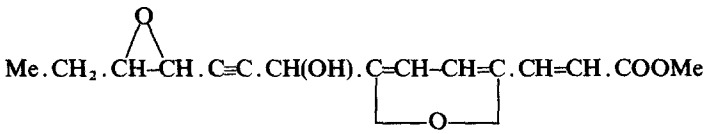
Wyerone epoxide (**3**) was detected in chromatograms of Et_2O extracts of infected tissues by its deep blue fluorescence under UV light (366 nm) and visualized as an orange spot on TLC plates after spraying with picric acid reagent [4] which is specific for epoxides. Milligram quantities of the epoxide were extracted from tissue bearing limited lesions formed by *B. cinerea* in pod cavities



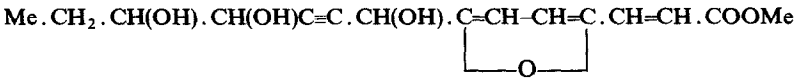
(**1**) Wyerone R=Me
(**2**) Wyerone acid R=H



(**3**) Wyerone epoxide



(**4**) Wyerol epoxide



(**5**) Dihydrodihydroxywyerol

and *B. fabae* in cotyledons six days after inoculation. After purification by PLC and crystallization from cyclohexane the phytoalexin was recrystallized (Et_2O–hexane) to give wyerone epoxide, mp 74–76°. The mass spectrum gave M^+ 274 (74%) (HRMS, found M^+ 274.0856, $C_{15}H_{14}O_5$ requires 274.0842) and prominent fragments at *m/e* 245 (31%), 243 (34%), 242 (47%) 151 (53%) and 29 (100%). UV absorption occurred in EtOH or MeOH at λ_{max} 347 nm (ε 28,100) and 238 nm (14,300). Diagnostic IR bands were detected at ν_{max} (KBr) 2220 (C≡C), 1706 (ester CO), 1640 (ketone CO). 1500 (furan) and 889 cm^{-1} (epoxide). PMR signals were observed at δ1.15 (3H, *t*, J = 7.5 Hz, 7-H), 1.76 (2H, *m*, 6-H) 3.21 (1H, *dt*, $J_{4,5}$ = 3.9, $J_{5,6\alpha}$ = $J_{5,6\beta}$ = 6.0Hz, 5-H) 3.66 (1H, *d*, $J_{4,5}$ = 3.9Hz, 4-H), 3.83 (3H, *s*, –OMe), 6.64 (1H, *d*, *J* = 16Hz, 2′–H), 6.75 (1H, *d*, *J* = 3.7Hz, β′–H) 7.37 (1H, *d*, *J* = 3.7Hz, β-H) and 7.48 (1H, *d*, *J* = 16Hz, 1′–H). The epoxide protons were very distinct in their chemical shifts and coupling constants and their assignment was confirmed by spin decoupling experiments. The signal at δ3.21 due to 5-H was reduced to a triplet *J* = 6.0Hz by irradiation of 4-H at δ3.66 and appeared as a doublet *J* = 3.9Hz when the 6-H protons were irradiated at δ1.76. The epoxide coupling constant of 3.9Hz indicated that the stereochemistry of the epoxide group was *cis*. Confirmation of the identity of wyerone epoxide was obtained by its partial synthesis from wyerone by reaction with 3-chloroperbenzoic acid.

Six days after inoculation yields of 179 and 92.7 μg wyerone epoxide/g. fr. wt were obtained from cotyledon tissue bearing limited lesions caused by *Botrytis fabae* or *B. cinerea* respectively, and 68.5 μg/g. fr. wt from pod seed cavities inoculated with *B. cinerea*. The phytoalexin was not detected in spreading lesions caused by *B. fabae* in pod endocarp or in tissues inoculated with water alone.

Metabolism and antifungal activity

Incubation of wyerone epoxide with germinating conidia or growing mycelium of *B. cinerea* or *B. fabae* led to the equally rapid disappearance of the phytoalexin (monitored at λ_{max} 347 nm) and appearance of compounds absorbing at 310 nm. Analysis of the incubation mixtures by TLC (hexane, 60–80°: acetone, 2:1) showed that *B. cinerea* and *B. fabae* metabolized the epoxide (R_f 0.54) to products with λ_{max} 310 nm at R_f 0.42 (**4**) and 0.13 (**5**) respectively. Results of time course studies on the metabolism of the phytoalexin by germinating conidia suggested that both fungi converted the epoxide to (**4**) but only *B. fabae* subsequently metabolized (**4**) to the second product (**5**).

Three days after the addition of wyerone epoxide to mycelial cultures of both species of *Botrytis* milligram quantities of the metabolites were recovered and subjected to spectral analyses. The molecular formula of the metabolite produced by *Botrytis cinerea* as determined by mass spectrometry was $C_{15}H_{16}O_5$ (M^+ 276.0972) and it was identified as wyerol epoxide (**4**) on the basis of the following evidence. The UV spectrum was similar to that of wyerol [1,5] with λ_{max} 310 nm (ε 20,000). The PMR shifts of the furan protons δ6.53 (1H, *d*, β-H) and 6.60 (1H, *d*, β′-H) and the presence of a methine proton δ5.53 (1H, br s, 1-H) also indicated that the ketone function had disappeared. IR absorption was present at 3570, 3350 (-OH) and 868 cm^{-1} (epoxide). PMR signals due to the epoxide protons were at δ3.09 (1H, *dt*, 5-H) and 3.54 (1H, *dd*, 4-H). 5-H appeared as a double triplet as in wyerone epoxide but 4-H showed further long range coupling to 1-H ($J_{1,4}$ ~ 1Hz) giving rise to a double doublet in the spectrum of wyerol epoxide. Wyerol epoxide was visualised on thin layer chromatograms as an orange spot on a yellow background after treatment with picric acid spray reagent for epoxides [4].

The more polar metabolite produced by *Botrytis fabae* was visualised on chromatograms as a deep pink spot on a white background after treatment with lead tetraacetate–rosaniline reagent [4]. It had a molecular formula $C_{15}H_{18}O_6$ as determined by MS (M^+ 294.1107) and was identified as 4,5-dihydro-4,5-dihydroxywyerol (**5**), from the following spectral data. The UV spectrum of (**5**) was virtually identical to that of wyerol epoxide (**4**) with λ_{max} 310 nm (ε 18,000). The PMR spectrum of dihydrodihydroxywyerol showed that the epoxide protons had disappeared and new multiplets centred at δ3.65 and 4.3 had appeared. Above δ5 the PMR spectrum of (**5**) was virtually identical to that of (**4**). The IR spectrum of (**5**) showed very strong hydroxyl adsorption at 3380 cm^{-1} with a shoulder at 3560 cm^{-1}. This information together with the lead tetraacetate-rosaniline confirmatory colour test for a vicinal diol was strong evidence in favour of the 4,5-dihydro-4,5-dihydroxywyerol structure (**5**).

The ED_{50}s for activity of wyerone epoxide against germ tube growth by *B. cinerea* and *B. fabae* were 6.4 and 16.0 μg/ml respectively. The metabolites were less antifungal, but wyerol epoxide was more active against *B. fabae* (ED_{50} 38.5 μg/ml) than *B. cinerea* (ED_{50} 583 μg/ml by extrapolation). Dihydrodihydroxywyerol did not inhibit germ tube growth at the highest concentration tested (100 μg/ml).

Wyerone epoxide (**3**) has been identified as a third component of the multiple phytoalexin response of *Vicia faba* to fungal infection by *Botrytis* [3]. The accumulation of the epoxide to antifungal concentrations in limited lesions indicates that it may play an important part in the inhibition of fungal growth within infected tissues. Wyerone epoxide is more active than wyerone against both *B. cinerea* and *B. fabae* but slightly less inhibitory than wyerone acid under the bioassay conditions employed [5,6]. Wyerone epoxide like other wyerone derivatives, except wyerol epoxide, is more active against *B. cinerea* than *B. fabae* [2, 5, 6]. Although the major metabolite of the phytoalexin produced by *B. fabae* (dihydrodihydroxywyerol, **5**) is less antifungal than that produced by *B. cinerea* (wyerol epoxide, **4**) it is unlikely that the differential sensitivity of these fungi to wyerone epoxide is associated with detoxification. Whether or not the phytoalexin is metabolized by *Botrytis in vivo* remains to be determined.

EXPERIMENTAL

PLC was carried out on 2 mm thick layers of Merck Si gel GF_{254} (type 60) and TLC on 0.25 mm precoated plates (Si gel 60 F_{254} Merck 5715). Unless otherwise stated, all PMR data are for $CDCl_3$ soln using TMS as internal standard while IR and UV measurements were in $CHCl_3$ and EtOH respectively.

Isolation of wyerone epoxide (**3**). Tissue bearing limited lesions formed by *B. cinerea* in pod seed cavities [7] (2.5 kg) and by *B. fabae* in cotyledons [5] (15 kg) was collected 6 days after inoculation, extracted with Et_2O and extracts subjected to PLC as described for the isolation of wyerone [5]. Plates were developed in hexane (br 60–80°)–Me_2CO (2:1) and after

drying in $CHCl_3$–petrol (br 60–80°) (2:1). The epoxide was detected under UV light (366 nm) as a deep blue fluorescing band at R_f 0.78; wyerone ran to R_f 0.86. After elution the epoxide was futher purified by PLC in $CHCl_3$ (containing 2% EtOH) followed by $CHCl_3$–petrol (br 60°–80°). Wyerone epoxide was recovered from final R_f 0.65 and after the removal of contaminating chlorophyll from pod endocarp with activated charcoal was crystallized from cyclohexane. Yields of 16 and 52 mg of the crystalline product were recovered from pod and cotyledon tissues respectively. Concentrations of the epoxide in 4 g samples of inoculated tissues were determined by UV spectrophotometry (λ_{max} 347, ϵ 28,100) following TLC of ether extracts prepared as previously described [5]. After development in hexane–Me_2CO and $CHCl_3$–petrol as above wyerone epoxide was eluted in MeOH from R_f 0.53.

Synthesis of wyerone epoxide from wyerone. Wyerone (20 mg) and 3-chloroperbenzoic acid (30 mg) were dissolved in $CDCl_3$ (0.5 ml) in a 5 mm diam. NMR tube. The tube was then heated (water bath) at 55° and the reaction monitored by PMR until complete disappearance of *cis* olefinic protons of wyerone had occurred. Work up with aq $NaHCO_3$ soln to remove excess reagent and 3-chlorobenzoic acid followed by PLC gave wyerone epoxide (9 mg) identical with the natural product (UV IR, PMR, MS).

Metabolism of wyerone epoxide by Botrytis. All incubations were carried out in synthetic pod nutrient soln (SPN) [5] in the dark at 18°. Wyerone epoxide was added in MeOH to SPN to give the required concentration of phytoalexin, final MeOH concentration was 0.5%. For time course studies 5 ml aliquots of wyerone epoxide soln (10 μg/ml SPN) were incubated with conidia (10^5/ml) in 100 ml conical flasks. At intervals (18, 30, 42 hr) after inoculation UV absorption spectra of bathing solns were recorded and the contents of triplicate flasks each extracted 3 × with equal vols of Et_2O. Ether extracts were subjected to TLC. There was no loss of wyerone epoxide in the absence of fungi. For the isolation of milligram quantities of the metabolites (**4**) and (**5**) mycelia of *B. fabae* or *B. cinerea* were incubated in wyerone epoxide solns (19 μg/ml SPN) as previously described for the production of wyerol from wyerone [5]. Three days after inoculation incubation mixtures were extracted with Et_2O and extracts from cultures of *B. cinerea* or *B. fabae* each separated on PLC plates developed in hexane (br 60–80°)–Me_2CO (2:1). Metabolites were detected as absorbing bands under UV light (254 nm) and eluted in $CHCl_3$ and Et_2O. Wyerol epoxide (4) R_f 0.49, 7 mg and dihydrodihydroxywyerol (5) R_f 0.27, 5.7 mg were recovered from the metabolism of 11 mg of wyerone epoxide by *B. cinerea* and *B. fabae* respectively.

Bioassays. Antifungal activity was assayed as previously described [5].

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