Phytochemistry, 1979, Vol. 18, pp. 1025–1027. Pergamon Press. Printed in England.

IDENTIFICATION OF PTEROSTILBENE AS A PHYTOALEXIN FROM *VITIS VINIFERA* LEAVES

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(*Revised received* 12 *December* 1978)

Key Word Index—*Vitis vinifera*; Vitaceae; grapevine leaves; phytoalexins; *trans*-3,5-dimethoxy-4′-hydroxy stilbene; HPLC.

Abstract—An inducible antifungal compound in grapevine leaves (*Vitis vinifera* L., cv Cabernet-Sauvignon) has been identified as *trans*-pterostilbene (3,5-dimethoxy-4′-hydroxy stilbene). It is only a minor component of the phytoalexin response of *V. vinifera* but its antifungal activity is relatively high by comparison with resveratrol and the viniferins, stress metabolites which have been identified previously in grapevine. Methods for the quantitative analysis of pterostilbene, resveratrol, ε- and α-viniferins by HPLC are described.

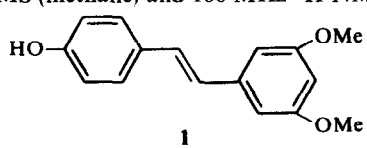
INTRODUCTION

Phytoalexins are antimicrobial compounds produced by plants in response to microbial attack and their production is widely believed to be an important disease resistance mechanism [1]. The phytoalexin response of grapevine has been shown to involve the production of the simple stilbene resveratrol (3,5,4′-trihydroxy stilbene) and a range of compounds termed the viniferins, in which resveratrol units are coupled together to produce compounds containing aryl dihydrobenzofuran units [2]. The viniferins accounted for the greater part of the fungitoxicity detectable by the *Cladosporium cucumerinum* TLC plate bioassay in extracts of UV irradiated or *Botrytis cinerea*-infected leaves [2]. However, in subsequent experiments in which phytoalexin production during infection of vine leaves by the downy mildew pathogen, *Plasmopara viticola*, was studied, the production of an additional, previously unidentified compound was detected by the above bioassay. The isolation and identification of this phytoalexin-like compound as *trans*-pterostilbene (3,5-dimethoxy-4′-hydroxy stilbene) is described here. In addition, some of the antifungal properties of pterostilbene and the circumstances in which it is produced by grapevines are described.

RESULTS AND DISCUSSION

Identity of antifungal compound with pterostilbene

The inhibitor was isolated from *P. viticola*-infected leaves by the procedure described for the isolation of pterostilbene (see Experimental). The inhibitor was identical with synthetic *trans*-pterostilbene (**1**) by comparison of TLC mobilities (Si gel, CH_2Cl_2–MeOH, 20:1), colour reactions with diazotized *p*-nitroaniline, UV spectra, GLC R_t (1% OV-101 column at 217°), GC–CI-MS (methane) and 100 MHz ^{1}H NMR.



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Antifungal activity

Pterostilbene was more fungitoxic than the viniferins (Table 1, cf. ref. [2]), having activity comparable with that of 4-hydroxy stilbene [3].

Production of pterostilbene in response to UV irradiation or fungal infection

Pterostilbene was absent from healthy grapevine leaves and was not produced in response to mechanical injury (cutting or bruising). Paradoxically, the greatest amounts of pterostilbene were detected in *P. viticola*-infected leaves (Fig. 1), the maximum concentration occurring just prior to sporulation. The slight drop in concentration subsequently coincided with the time at which the plants were returned to 100% relative humidity (RH) to encourage sporulation (see Experimental). However, the maximum concentration detected in downy mildew-infected leaves (*ca* 22 μg/g fr. wt), was relatively

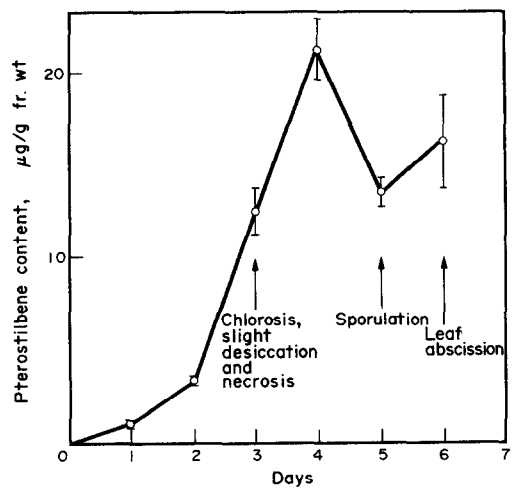

Fig. 1. Time course of pterostilbene production following inoculation of grapevine leaves with *Plasmopara viticola*. Bars show the standard errors of the means, $s\bar{x}$, for three replicates.

Table 1. Antifungal activity of pterostilbene

Test organism	TLC plate* assay	Spore† germination	Zoospore‡ release/motility	Mycelial growth† on agar
Cladosporium cucumerinum	0.5	9		
Botrytis cinerea		18		27.5
Piricularia oryzae		9		<3
Plasmopara viticola			4.5/2.3	
Pellicularia sasakii				6.3

* Minimum amount (μg) detectable by *Cladosporium cucumerinum* TLC plate assay.

† Concentrations (μg/ml) causing 50% inhibition.

‡ Concentrations (μg/ml) causing 50% inhibition of release of zoospores from sporangia of *Plasmopara viticola*, or the motility of the zoospores after their release.

low, although comparable to the concentrations of resveratrol, ε- and α-viniferins detectable in such leaves (unpublished). Lesser amounts of pterostilbene were induced by UV irradiation, a good inducing stimulus for resveratrol and the viniferins [4], the pterostilbene concentration being *ca* 9 μg/g fr. wt after 48 hr. However, in spreading lesions caused by *Botrytis cinerea* on grapevine leaves, pterostilbene was not detectable either in the blue fluorescent zone surrounding these lesions, nor in the rotted area (Table 2), although resveratrol, ε- and α-viniferins were readily detectable. Pterostilbene was not detectable in normal lignified stem tissue of grapevine, a good source of both resveratrol and ε-viniferin [2].

Pterostilbene was first isolated from the heartwood of *Pterocarpus santalinus* (the red sandalwood) by Spath and Schlager [5]. It is considered to be a common component of the heartwoods of the genus *Pterocarpus* (Leguminosae) with the exception of a few African padauks (e.g. *P. mildbreaedii* and *P. santalinoides*) [6]. The absence of pterostilbene from those woods is thought to account for the fact that they are not resistant to fungal decay. This is not the first report of the fungicidal properties of pterostilbene. It is also reported to have insecticidal and antidiabetic properties [5, 7].

In addition to the Vitaceae [2], the production of phytoalexin-like stilbenes has been described in groundnuts (*Arachis hypogaea*), where resveratrol [8] and an isoprenylated resveratrol derivative [9] were identified, and in *Pinus* species where pinosylvin and its monomethyl ether were detected [10]. This appears to be the first report, however, of the phytoalexin-like properties of pterostilbene.

Pterostilbene production would seem to be under metabolic control independent of that of the production of the viniferins. In *V. vinifera* at least, resveratrol production invariably leads to the formation of the viniferins in all situations examined so far. However, the production of the viniferins and resveratrol may occur in the absence of detectable pterostilbene production as in *B. cinerea*-infected leaves (e.g. Table 2) although this may reflect the ability of this fungus to demethylate the compound. On the basis of the present evidence it appears that pterostilbene is only a minor component of the phytoalexin response of *V. vinifera*. Thus, for example, the level of ε-viniferin can reach around 220 μg/g fr. wt within 40 hr of UV irradiation of grapevine leaves [4], while the pterostilbene content of such leaves is only *ca* 9 μg/g fr. wt by 48 hr. However, in view of the potent antifungal properties of pterostilbene and the fact that only its production in compatible plant/fungus interactions has been examined so far suggests that the possible role of pterostilbene in the disease-resistant response of grapevine should not be overlooked.

Table 2. Contents of pterostilbene, resveratrol, ε-viniferin and α-viniferin in grapevine leaves bearing spreading lesions caused by *Botrytis cinerea*

Sample	Amount of compound (μg/g fr. wt) Pterostilbene	Resveratrol	ε-Viniferin	α-Viniferin
Non-infected tissue	nd*	nd	nd	nd
Rotted area	nd	11.8 ± 1.2	63.1 ± 4.6	58.5 ± 18.2
Fluorescent zone	nd	90.4 ± 2.9	30.9 ± 2.0	23.4 ± 1.9

Leaf tissue was subdivided into the brown rotted area of the lesion and the blue fluorescent zone surrounding the lesion. Each result is the mean of three determinations, ± standard error, $s\bar{x}$.

* Not detectable.

EXPERIMENTAL

Extraction of pterostilbene from grapevine leaves. Leaves (cv Cabernet-Sauvignon, 200 g fr. wt) bearing sporulating lesions of *P. viticola* were extracted with EtOH (1 l.). The EtOH extract was evapd on a rotary evaporator (40°), redissolved in EtOAc (125 ml), then washed with H_2O (125 ml), NaOH (0.2 M, 125 ml) and again with H_2O (3 × 125 ml). The organic phase was chromatographed on a Si gel column (28 × 2.5 cm) slurry-packed in hexane and eluted with stepwise gradients of CH_2Cl_2 in hexane followed by MeOH in CH_2Cl_2. Fractions containing pterostilbene (eluted with 1% MeOH in CH_2Cl_2) were further purified by preparative HPLC on a column of Lichrosorb-NH_2 (10 μm particle size, E. Merck, column dimensions 20 × 0.96 cm) using 2.5% MeOH in EtOAc as eluant followed by a column of Partisil 10 (10 μm particle size, Reeve Angel, column dimensions 25 × 0.85 cm) using 1% MeOH in CH_2Cl_2 as eluant. Pterostilbene was collected as a chromatographically pure (TLC, GLC) preparation (1.8 mg).

Quantitative analysis of pterostilbene, resveratrol, ε- and α-viniferins. The tissue sample (20–200 mg fr. wt) was homogenized in a pestle and mortar with 70% MeOH (10 ml). The homogenate was clarified by centrifugation, the supernatant dried (rotary evaporator, 40°) and redissolved in EtOAc (5 ml) and aq. $NaHCO_3$ (3%, 5 ml). After mixing, the phases were separated, the organic layer washed with H_2O (5 ml) and dried under N_2. The sample was redissolved in MeOH at *ca* 1 mg fr. wt/μl MeOH. Estimates of the contents of pterostilbene, resveratrol, ε- and α-viniferins in such extracts were made by reversed phase HPLC.

For pterostilbene measurements, an appropriate amount of a quantitative internal standard of *trans*-3,4-methylenedioxy-4'-hydroxy stilbene was added to the extract before 5 μl samples (*ca* 5 mg fr. wt) were analysed on a column of Hypersil ODS (5 μm, Shandon Southern Products Ltd., Runcorn, Cheshire, 20 × 0.45 cm) using MeCN–H_2O (1:1) at 6.9 × 10^6 Pa as eluant (flow rate *ca* 1 ml/min). The eluate was monitored with a Cecil 212 UV monitor fitted with a microflow cell (10 μl) and operating at 300 nm, 0.02 A full scale deflection. R_t internal standard 6.4 min, pterostilbene 7 min. Pterostilbene content was calculated from its peak area relative to that of the internal standard

after correction for the relative detector response for the two compounds.

For measurements of resveratrol, ε- and α-viniferins, 5 μl samples of the above extract were analysed with the same HPLC system using MeCN–H_2O (7:13) as eluant, the eluate being monitored at 288 nm. R_t resveratrol 5.1 min, ε-viniferin 8 min, α-viniferin 10.2 min. Quantitative assessments were made by comparisons of peak areas with that of an external standard of ε-viniferin after applying the appropriate correction factors for the relative detector responses for each of the compounds assessed.

Antifungal bioassays. Cladosporium cucumerinum *bioassay on Si gel TLC plates.* This was carried out as described in ref. [11].

Mycelial growth on agar. This was carried out as described previously [3]. The growth medium was Czapek Dox Agar (Oxoid Ltd.).

Spore germination tests. Pterostilbene was added to sterilized malt extract peptone medium (2% malt extract, 0.5% peptone) as an EtOH soln to give a final EtOH concn of 1%. Two-fold dilution steps were used, the highest concn tested being 100 μg/ml. Drops (30 μl) of these solns were applied to wells of microscope cavity slides and to each drop a washed spore suspension (3 μl) of the appropriate test organism at 10^6 spores/ml in malt extract peptone medium was added. Slides were incubated over moist filter paper in Petri dishes at 25° for *ca* 17 hr before percentage germination was assessed. For each concn two replicate slide cavities were used.

Effects on zoospores of P. viticola. Freshly collected sporangia from sporulating lesions of *P. viticola* on a vine leaf were washed and resuspended in H_2O at *ca* 5×10^4/ml. This suspension was dispensed in 1 ml aliquots, to each of which was added either 5 μl Me_2CO or 5 μl of an Me_2CO soln of pterostilbene. Two-fold dilution steps of test compound were used, the highest concn tested being 50 μg/ml. After mixing, duplicate drops (30 μl) from each concn of test compound were transferred to wells of microscope cavity slides. These were incubated over moist filter paper in Petri dishes at 25°. After 3 hr, slides were scored for effects against release of zoospores from the sporangia or for the motility of the zoospores which had been released, as described previously [3].

Pterostilbene production in response to UV irradiation or fungal infection. UV irradiation. Detached grapevine leaves were laid, abaxial surface uppermost, on moist filter paper in plastic trays, then irradiated using a Camag TLC inspection lamp (254 nm, filter removed, total incident energy 3.7 W/m^2, 5 min duration). Trays were then enclosed in polythene bags and incubated at 25° in the dark. Samples, each consisting of 3 discs (14 mm dia), were cut from the leaves at random using a cork borer at the appropriate time intervals. Samples were stored at −20° before extraction and HPLC analysis.

P. viticola *infection.* Leaves of young grapevine plants, *ca* 30 cm high, were inoculated uniformly and heavily on their abaxial surfaces with a fresh sporangial suspension in H_2O. Plants were maintained at 100% RH in the greenhouse for 24 hr, then returned to normal greenhouse conditions (*ca* 23°). Four days after inoculation, plants were returned to 100% RH conditions to encourage sporulation of the pathogen. Under these conditions, confluent sporulation on the undersurfaces of inoculated leaves occurred. At the appropriate time intervals, samples, each consisting of 3 discs (14 mm dia), were cut at random from the 3 uppermost inoculated leaves using a cork borer. Samples were stored at −20° before extraction and HPLC analysis.

B. cinerea *infection.* Young, fully expanded grapevine leaves were detached and laid on moist filter paper in plastic trays. Leaves were inoculated with 4 drops (20 μl each) of a spore suspension of *B. cinerea* as described previously [3]. After 3 days, spreading lesions had developed *ca* 15 mm in dia. Tissue was divided into two types, (a) the brown, rotted area of the lesion and (b) the blue fluorescent zone (5–10 mm wide) surrounding the rotted area [3]. All tissue of the same type from a single leaf was pooled, and there were 3 replicate leaves. Tissue was weighed, then stored at −20° before extraction and HPLC analysis. In all cases, analyses of samples of the appropriate control tissues were conducted.

Acknowledgements—We are grateful to Mr. J. Wellby for carrying out the MS and to Mr. B. Armitage for synthesizing pterostilbene and 3,4-methylenedioxy-4′-hydroxy stilbene.

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