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THE PRODUCTION OF RESVERATROL AND THE VINIFERINS BY GRAPEVINES IN RESPONSE TO ULTRAVIOLET IRRADIATION

PETER LANGCAKE and ROBERT J. PRYCE

Shell Research Limited, Shell Biosciences Laboratory, Sittingbourne Research Centre, Sittingbourne, Kent, ME9 8AG, England

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Key Word Index—*Vitis vinifera*; Vitaceae; grapevine; UV-irradiation; stilbenes; phytoalexins.

Abstract—The biosynthesis of resveratrol and the viniferins by grapevines in response to UV-irradiation has been studied. The action spectrum for the UV-irradiation induced production of resveratrol shows a maximum in the region 260–270 nm. This suggests that DNA is the photoreceptor for the response. It also provides an explanation for the fact that sunlight does not act as an inducer under field conditions. The results of feeding radiolabelled precursors are consistent with the proposal that resveratrol, in common with other plant stilbenes,is biosynthesised by the phenylalanine–polymalonate pathway. Direct evidence that resveratrol is the precursor of the viniferins was not obtained, but indirect evidence from time course studies and from biomimetic studies suggests that this is likely.

INTRODUCTION

The production of phytoalexins, antimicrobial compounds produced by a plant in response to infection or certain other types of physiological stimuli, is widely believed to be an important disease resistance mechanism. We have recently isolated phytoalexins from grapevine leaves. The two most important compounds appear to be ε-viniferin (**1**) and α-viniferin (**2**), but several other compounds, including the partially characterised β-viniferin (believed to be a tetramer of resveratrol) and γ-viniferin (a higher oligomer of resveratrol), are also produced [1]. These compounds, as well as their putative precursor, *trans*-resveratrol (**3**) [2], are produced in response to fungal infection or uv-irradiation. Some aspects of the biosynthesis of these compounds in response to UV-irradiation are reported here.

RESULTS AND DISCUSSION

The ability of UV-irradiation to elicit phytoalexins and resveratrol production raises two questions. Why is sunlight not effective as an eliciting agent in field-grown plants and what is the photoreceptor for this response? To investigate these questions an action spectrum for the biosynthesis of resveratrol in grapevine leaf discs was determined (Fig. 1). The action spectrum clearly shows a maximum in the region 260–270 nm. At wavelengths above 300–310 nm, little or no resveratrol production occurred. Sunlight is deficient in radiation of wavelengths below 300–310 nm [10] and this probably explains why the biosynthesis of resveratrol is not induced by sunlight in field-grown plants. Moreover, we have found that although irradiation of the abaxial surface of vine leaves leads to the formation of resveratrol (and of the viniferins), comparable irradiation of the adaxial (upper) surface does not induce resveratrol biosynthesis.

The current hypothesis on the mechanism of induction of phytoalexins (in leguminous plants) supposes that in normal tissue, the genes involved are repressed. Agents which induce phytoalexin production are thought to derepress these genes or promote their transcription, for example, by causing a conformational change in the DNA [3,4]. Hadwiger and Schwochau [5] have implied that DNA itself is the photoreceptor for the UV-irradiation induced response. The similarity between the action spectrum obtained here and the absorption spectrum for DNA is consistent with this hypothesis.

We have emphasised previously [2] the similarities between the production of the isoflavanoid/pterocarpan phytoalexins in leguminous plants and the production of resveratrol in grapevines. The stilbenes and the isoflavanoids/pterocarpans are believed to be biosynthesised by the phenylalanine-polymalonate pathway [6,7]. To test this we studied the incorporation of various radiolabelled precursors into resveratrol following UV-irradiation in both leaves and immature grape berries. As judged by the isotope dilution values, incorporations of ^{14}C-labelled acetate, malonate, phenylalanine and tyrosine were considerably greater with

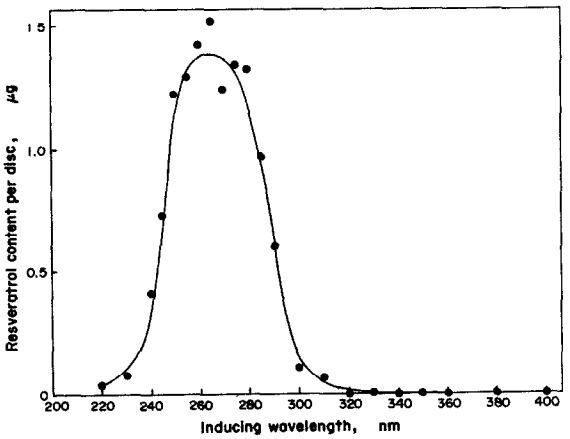


Fig. 1. Action spectrum for the UV-radiation induction of resveratrol synthesis in grapevine leaf discs.

Table 1. Incorporation of radiolabel into resveratrol by immature grape berries after feeding various radioactive potential precursors

Precursor fed	Compound fed		Product isolated				
	Amount fed (μCi)	Specific activity (μCi/μmol)	Resveratrol isolated (μmol)	Specific activity (μCi/μmol)	% Incorporation	Isotope* dilution	Experiment number
2-^{14}C-malonate	4.45	17	0.014	1.5×10^{-1}	0.049	340	(i)
U-^{14}C-tyrosine	5.66	475	0.009	4.7×10^{-3}	0.0008	101000	
2-^{14}C-acetate	4.96	58	0.124	1.42×10^{-2}	0.036	12000	(ii)
U-^{14}C-phenylalanine	4.34	513	0.139	1.01×10^{-1}	0.324	5000	

* Specific activity of radiolabel fed divided by specific activity of resveratrol isolated, allowing for incorporation of 3 molecules of either acetate or malonate into one molecule of resveratrol.

Table 2. Incorporation of radiolabel into resveratrol by grapevine leaves after feeding various radioactive potential precursors

Precursor fed	Compound fed		Product isolated			
	Amount fed (μCi)	Specific activity (μCi/μmol)	Resveratrol isolated (μmol)	Specific activity (μCi/μmol)	% Incorporation	Isotope* dilution
U-^{14}C-phenylalanine	1.60	513	0.14	4.8×10^{-3}	0.041	106900
U-^{14}C-tyrosine	2.67	475	0.11	9.7×10^{-4}	0.004	489700
2-^{14}C-malonate	2.06	17	0.21	4.0×10^{-2}	0.414	1300
2-^{14}C-acetate	4.02	58	0.23	3.3×10^{-3}	0.018	52700

* Specific activity of radiolabel fed divided by specific activity of resveratrol isolated, allowing for incorporation of 3 molecules of either acetate or malonate into one molecule of resveratrol.

berries (Table 1) than with leaves (Table 2). However, the amounts of resveratrol formed in berries were generally rather lower than in leaves. In all experiments, the incorporation of tyrosine was very poor. Phenylalanine and acetate both functioned as good precursors while the incorporation of malonate was exceptionally good. Both the percentage incorporation and the isotope dilution values obtained by feeding radioactive precursors to intact plant tissues will be influenced by various factors such as the ease with which the precursor reaches the site of synthesis, the intracellular pool sizes of non-labelled precursors and alternative metabolic pathways. Nevertheless, the results are consistent with the operation of the phenylalanine–polymalonate pathway in the biosynthesis of resveratrol.

In some experiments, the radiolabelled resveratrol isolated was methylated and degraded by permanganate oxidation [8]. The distribution of radioactivity between the two fragments of the molecule, 4-methoxybenzoic acid and 3,5-dimethoxybenzoic acid, was then determined. The results (Table 3) are also consistent with the operation of the phenylalanine–polymalonate pathway.

Table 3. Distribution of radioactivity within the resveratrol molecule after feeding radioactive precursors. Radiolabelled resveratrol was isolated, then methylated and subjected to permanganate oxidation. Distribution of radioactivity between the products, 4-methoxybenzoic acid and 3,5-dimethoxybenzoic acid, was then determined. Figures in parenthesis give the distribution of radiolabel which is predicted by the phenylalanine–polymalonate pathway

Precursor fed	% Radiolabel in:	
	4-Methoxy ring	3,5-Dimethoxy ring
U-^{14}C-phenylalanine	80 (78)	20 (22)
2-^{14}C-malonate	1 (0)	99 (100)

Although resveratrol has been presumed to be the precursor of ε-viniferin and the other vine phytoalexins [1], direct evidence that this is the case has not, as yet, been obtained. Attempts have been made to introduce resveratrol (and its triacetyl derivative) into vine leaves in the hope that the leaves will convert the compound to one or more of the viniferins. These were unsuccessful, probably because of the difficulties encountered in

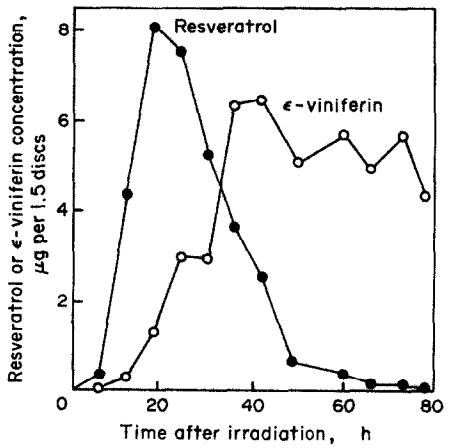
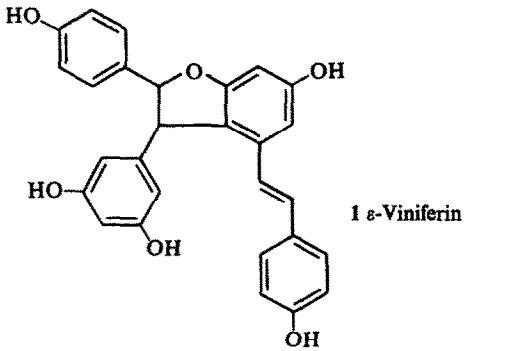


Fig. 2. Time course of production of resveratrol (●) and ε-viniferin (○) in grapevine leaf discs following UV irradiation.

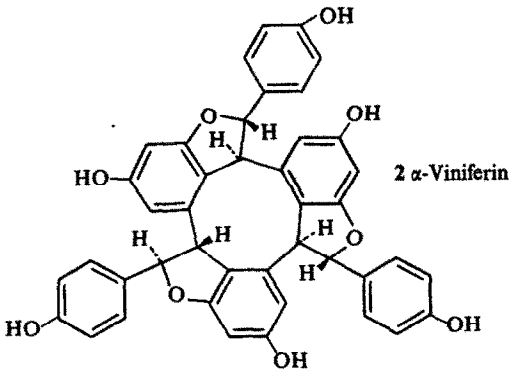
obtaining uptake of resveratrol by the leaf. Biomimetic studies in which various 4-hydroxylated stilbenes, including resveratrol have been subjected to oxidative dimerization with horseradish peroxidase/H_2O_2 [1,9] support the hypothesis that resveratrol is the precursor of the viniferins, but it may be significant that the resveratrol dimer (4) formed under these circumstances, although analogous in structure to ε-viniferin (1), was not identical with it. Attempts to obtain ε-viniferin from resveratrol using crude enzyme extracts of vine leaves (both UV-irradiated and non-irradiated) have so far been unsuccessful.

Indirect evidence that resveratrol may be the biosynthetic precursor of ε-viniferin has come from a study of the time-course of production of these two compounds in leaf discs following UV-irradiation (Fig. 2). In several experiments, resveratrol was consistently produced very rapidly following UV-irradiation, rising to a maximum at 18 hr and decreasing thereafter. In the same experiments, the production of ε-viniferin was slower than that of resveratrol and a plateau was reached about 36 hr after irradiation, at which time the concentration of resveratrol had declined considerably, presumably because of its conversion to ε-viniferin.

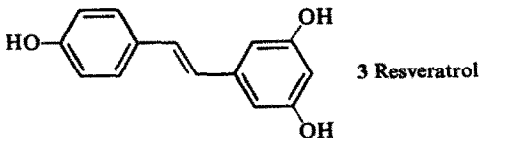
In these experiments in which leaf discs were UV-irradiated, the production of the trimeric compound α-viniferin (2) was not detected. However, in experiments in which the time course of production of resveratrol, ε-viniferin and α-viniferin during infection of intact plants by *Plasmopara viticola* (the downy mildew pathogen of grapevine) was measured, these compounds were produced sequentially. Thus the monomer, resveratrol, was first detected, followed by the dimer, ε-viniferin, and then the trimer, α-viniferin. This sequence of events is that which would be expected if the viniferins are produced by the sequential coupling of resveratrol units.



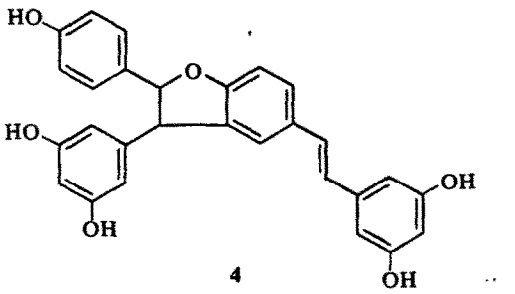
1 ε-Viniferin



2 α-Viniferin



3 Resveratrol



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EXPERIMENTAL

All extraction procedures were carried out in subdued light to discourage *trans* to *cis*-isomerization of both resveratrol and ε-viniferin. All experiments were carried out on two or more occasions.

Action spectrum of resveratrol biosynthesis. Discs (14 mm in diameter) from healthy, fully expanded leaves of greenhouse grown vine plants (*Vitis vinifera* L. var. Cabernet-Sauvignon) were exposed to light of various wavelengths using the light source (xenon arc) and grating diffractometer from an Aminco–Bowman spectrofluorometer. To compensate for the fact that the intensity of light generated by this source varies with wavelength, its spectral energy was measured using a Y.S.I. Kettering model 65-A radiometer. Exposure times for the discs were then adjusted so as to provide the same amount of energy as a 10 min exposure at 250 nm (570 J m^{-2}). After irradiation, discs were incubated on moist filter paper in the dark at 26° for 20 hr. Resveratrol content of the discs was then measured by GLC as described previously [2].

Incorporation of radiolabelled precursor into resveratrol. (a) *Leaves.* Radioactive precursors were made up in 2 ml H_2O and into each soln was placed the petiole (freshly cut under H_2O) of 2 young fully expanded grapevine leaves (var. Sultana). These were allowed to transpire in a gentle airstream for *ca* 2 hr, then UV-irradiated from the underside for 15 min using a Camag TLC inspection lamp (254 nm, 12 cm distant). Leaves were left in the soln 18 hr, topping up with H_2O where necessary. All leaves took up half the soln or more although there were large differences between leaves in the amounts taken up. Resveratrol was isolated from the leaves 20 hr after irradiation. (b) *Immature grape berries.* Berries from greenhouse-grown plants (var Cabernet-Sauvignon) were cut in half longitudinally and placed on moist filter paper, cut surface uppermost, in petri dishes. To the cut surface of each half, an aq. soln of the radioactive potential precursor (5 μl, *ca* 0.5 μCi) was applied. Berries were then UV-irradiated (Camag TLC inspection lamp, 254 nm, 17 cm distant, 10 mins) and incubated at 26° in the dark for 18 hr. Resveratrol was isolated from both leaves (*ca* 3 g fr. wt) and berries (approx 8 g fr. wt) as follows. The fresh material was ground in a pestle and mortar with sand and 15 ml of either MeOH (for berries) or 70% MeOH (for leaves). The homogenate was clarified by centrifugation and the supernatant conc to *ca* 4 ml. The concentrate was extracted with EtOAc (5 ml), the organic phase washed with 3% $NaHCO_3$ (5 ml), then H_2O (5 ml) and applied to the origin of a paper chromatogram (Whatman 3 MM). This was developed for *ca* 40 cm with 50% MeOH (descending) and the bright blue fluorescent band at R_f *ca* 0.25 corresponding with *trans*-resveratrol was eluted with Me_2CO. The Me_2CO extract was dried, redissolved in EtOAc (50 μl) and applied as a single injection to an HPLC column of Partisil-10 (Reeve Angel,

250 × 9.5 mm) operating at 26.5 kg cm^{-2} using 5% MeOH in CH_2Cl_2 as eluant. The eluate was monitored using a Cecil 212 UV monitor at 310 nm. Resveratrol was eluted as a chromatographically pure (TLC, GLC [2]) preparation. The resveratrol content of this preparation was estimated by GLC using a quantitative internal standard of octacosane as described previously [2]. Radioactivity was measured in toluene–Triton X100–butylPBD (21·11..14 g) scintillation fluid using an Intertechnique scintillation spectrometer.

Degradation of resveratrol. Radioactive resveratrol isolated as above was methylated with an excess CH_2N_2 in Et_2O–MeOH for 18 hr at room temp. The products were dried under N_2 and resveratrol trimethyl ether was purified by HPLC as above using MeOH–CH_2Cl_2–petrol (bp 60–80°) (1.25:98.75:400). Non-labelled resveratrol trimethyl ether was added to the purified sample as carrier and the sample was dissolved in 0.5 ml Me_2CO. To this was added aq. $KMnO_4$ soln (0.5 ml, 10 mg ml^{-1}), and 1 hr later a further 0.5 ml $KMnO_4$. After a total of 1.5 hr at room temp. the reaction mixture was acidified (4 drops M HCl) and extracted with EtOAc (2 ml). The EtOAc extract was washed and applied to a Si gel TLC plate. After development with CH_2Cl_2–MeOH (4:1), the reaction products (4-methoxy- and 3,5-dimethoxy-benzoic acids) were located under UV light, eluted with MeOH and radiocounted. No attempt was made to correct for losses on chromatography.

Time course of production of resveratrol and ε-viniferin in grapevine leaves. Leaves (var. Cabernet-Sauvignon) were detached and UV-irradiated on the abaxial surface for 5 min. (Camag TLC inspection lamp. 254 nm, 17 cm distant). Discs (14 mm in diameter) were then cut at random from the interveinal areas of the leaves and incubated on moist filter paper in the dark at 25° in petri dishes. At the appropriate time intervals two samples, each of 5 discs, were collected and stored at −20°. Samples were extracted by grinding in a pestle and mortar with sand and 70% MeOH (10 ml). The homogenate was clarified by centrifugation, the supernatant was dried on a rotary evaporator and redissolved in water (5 ml) and EtOAc (5 ml). After mixing thoroughly, the phases were separated and the organic phase was washed with $NaHCO_3$ (3%, 5 ml), then H_2O (2 × 5 ml). The organic phase was evaporated to dryness in N_2, then redissolved in EtOAc (100 μl). Samples (2 μl, i.e. equivalent to 0.1 leaf discs) were examined by HPLC on a column of modified Partisil-5 (Reeve Angel, prepared by directly reacting the silica with 3-aminopropyltriethoxysilane and silylating residual-OH groups with trimethylchlorosilane, column dimensions 100 × 4.5 mm) operating at 53 kg cm^{-2} using 15% MeOH in EtOAc as eluant. The eluate was monitored at 288 nm with a Cecil 212 UV monitor. This system allows the simultaneous separation and quantitation of resveratrol, ε-viniferin and α-viniferin. Resveratrol concentration was also measured by GLC using a quantitative internal standard of octacosane as described previously [2]. There was good agreement between the methods in their estimates of the resveratrol concentration.

REFERENCES

1. Langcake, P. and Pryce, R. J. (1977) *Experientia* **33**, 151.
2. Langcake, P. and Pryce, R. J. (1976) *Physiol. Plant Pathol.* **9**, 77.
3. Hadwiger, L. A. and Schwochau, M. E. (1969) *Phytopathology* **59**, 223.
4. Hadwiger, L. A., Jafri, A., von Broembsen, S. and Eddy, R. (1974) *Plant Physiol.* **53**, 52.
5. Hadwiger, L. A. and Schwochau, M. E. (1971) *Plant Physiol.* **47**, 588.
6. Money, T. (1973) Specialist Periodical Report, Biosynthesis, Vol. 2. p. 183. The Chemical Society, London.
7. Pryce, R. J. (1972) *Phytochemistry* **11**, 1355.
8. von Rudloff, E. and Jorgensen, E. (1963) *Phytochemistry* **2**, 297.
9. Langcake, P. and Pryce, R. J. (1977) *J. Chem. Soc. Chem. Commun.* in press.
10. Gates, D. M. (1966) *Science* **151**, 523.