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δ-Viniferin, a Resveratrol Dehydrodimer: One of the Major Stilbenes Synthesized by Stressed Grapevine Leaves

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δ-Viniferin is a resveratrol dehydrodimer, an isomer of ϵ-viniferin. This compound has been reported as a molecule produced in vitro by the oxidative dimerization of resveratrol by plant peroxidases or fungal laccases. It was also recently identified in wines and in grape cell cultures. We have now identified this dimer by NMR, high-performance liquid chromatography−diode array detection (HPLC-DAD), and HPLC-mass spectrometry (MS) in grapevine leaves infected by *Plasmopara viticola* (downy mildew) or UV-C irradiated. Its concentration was higher than that of ϵ-viniferin and constitutes one of the most important phytoalexins derived from resveratrol.

KEYWORDS: Stilbenes; *Vitis vinifera*; resveratrol; viniferins; HPLC; LC-MS; NMR; UV spectra

INTRODUCTION

Langcake and Pryce (*1*, *2*) have demonstrated that resveratrol (3,5,4′-trihydroxystilbene) is a phytoalexin synthesized by grapevine leaf tissue following fungal infection and UV light irradiation. These authors have also identified oxidation products of resveratrol as ϵ-, α-, β-, and γ-viniferin, respectively, as a dimer, trimer, tetramer, and a more highly polymerized oligomer (*3*). Pterostilbene (3,5-dimethoxy-4′-hydroxystilbene) was also isolated from *Vitis vinifera* leaves infected by *Plasmopara viticola* (downy mildew) (*4*). This compound was later identified in healthy grapevine berries as a constitutive stilbene (*5*).

To study the oxidative dimerization of resveratrol, Langcake and Pryce (*6*) demonstrated that horseradish peroxidase oxidized resveratrol into an ϵ-viniferin mimic, the resveratrol dehydrodimer, which was not identified by these authors in grapevine leaves. This compound can also be produced, in vitro, by a laccaselike stilbene oxidase of *Botrytis cinerea* (*7*, *8*). This dimer was recently detected in wines (*9*) and in grape cell cultures (*10*) but never identified as a grapevine phytoalexin. We now describe δ-viniferin, an analogue of the resveratrol dehydrodimer, as a major resveratrol dimer with ϵ-viniferin, synthesized by *P. viticola*-infected or UV-C-irradiated grapevine leaves.

MATERIAL AND METHODS

Plant Material. Lignified canes (2 years old) of *V. vinifera* var. Gamaret were collected during the winter time in the vineyard of the Swiss Federal Agricultural Research Station for Plant Production of Changins (Switzerland). Cuttings from *V. vinifera* var. Chasselas were propagated in a climate-controlled chamber with 16 h of light at 25 °C and 8 h of dark at 18 °C. Some of them were artificially inoculated with *P. viticola* sporangia according to Gindro (*11*). Leaves were harvested for analysis 7 days after inoculation when sporangiophores of *P. viticola* were visible at their lower face. Leaves were also collected from healthy cuttings, and their undersides were exposed for 10 min to UV-C radiations (Philips TUV 30 W, 92 μW cm^{-2} at 253 and 7 nm, at 13 cm from leaves) and then maintained for 72 h in the dark in a wet chamber and removed for analysis.

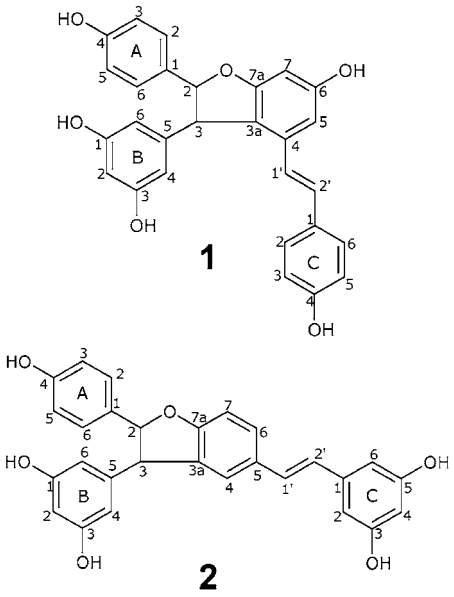

Figure 1. Structures of **1** and **2**.

Standards. Pure *trans*-resveratrol was obtained from Sigma Chemical Co. (St. Louis, MO). *Trans*-piceid (*trans*-resveratrol 3-*O*-β-D-

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glucoside) was extracted from *Polygonum cuspidatum* and purified according to Waterhouse and Lamuela-Raventos (*12*). *trans*-ϵ-Viniferin was extracted from lignified canes of Gamaret (100 g FW), sliced into small pieces, and then homogenized in 1 L of MeOH−H_2O (7:3, v/v) for 10 min (Omni-mixer homogenizer, Omni International, U.S.A.). After it was filtered and evaporated with MeOH under vacuum, the aqueous residue was adjusted to 100 mL with distilled water and extracted three times with 100 mL of EtOAc. Further purifications on a preparative low-pressure C18 column (Lichroprep, Merck) and a preparative high-performance liquid chromatography (HPLC) column (Lichrospher, Merck) using mixtures of MeOH and water yielded 30 mg of partially pure *trans*-ϵ-viniferin. Final purification was made on 20 cm × 20 cm, 2 mm thick silica thin-layer chromatography (TLC) preparative plates (KH Merck), developed with $CHCl_3$−MeOH (8:1, v/v). The UV (366 nm) fluorescent band at R_f 0.25 was harvested, and pure *trans*-ϵ-viniferin (**Figure 1**, **1**) (23 mg) eluted with MeOH. The structure, confirmed by ^{1}H NMR (400 MHz, CD_3OD) and ^{13}C NMR (100 MHz, CD_3OD), was in accordance with Lin et al. (*13*) and by MS (ESI) (*m*/*z* 453.1 $[M - H]^-$; 435 (MS^2) $[M - H - H_2O]^-$).

trans-δ-Viniferin (synonym to *trans*-resveratrol dehydrodimer) was enzymatically synthesized from resveratrol, and the stilbene oxidase of *B. cinerea* was purified as described by Pezet (*14*). Ten milliliters of an acetone solution of resveratrol (5 mg/mL) and 2.5 mL of an enzyme solution (79.5 μg of proteins) were added to 1 L of distilled water and stirred at 30 °C for 30 min. The enzymatic reaction mixture was then extracted with EtOAc. This extract was purified by semi-preparative HPLC on C18 and by preparative TLC as for ϵ-viniferin. The UV (366 nm) fluorescent band at R_f 0.25 was harvested, and pure *trans*-δ-viniferin (**Figure 1**, **2**) (16.6 mg) eluted with MeOH. The structure was confirmed by ^{1}H NMR (400 MHz, CD_3OD): 8.54 (brs, OH); 7.37 (dd, J = 8.2 et 1.6 Hz, H-6); 7.17 (s, H-4); 7.16 (d, J = 8.5 Hz, H-2,6A); 6.96 (d, J = 16.2 Hz, H-2′); 6.84 (d, J = 8.3 Hz, H-7); 6.78 (d, J = 16.2 Hz, H-1′); 6.77 (d, J = 8.6 Hz, H-3,5A); 6.42 (d, J = 2.2 Hz, H-2,6C); 6.18 (t, J = 2.2 Hz, H-2B); 6.13 (t, J = 2.2 Hz, H-4C); 6.12 (d, J = 2.2, H-4,6B); 5.38 (d, J = 8.4 Hz, H-2); 4.39 (d, J = 8.3 Hz, H-3) and ^{13}C NMR (CD_3OD, 100 MHz): 161.05 (C-7a); 159.98 (C-1,3B); 159.67 (C-3,5C); 158.77 (C-4A); 145.39 (C-5B); 141.19 (C-1C); 132.87, 132.43 et 132.37 (C-3a, C-1A, C-5); 129.39 (C-2′); 128.68 (C-2,6A, C-6); 127.48 (C-1′); 124.18 (C-4); 116.34 (C-3,5A); 110.38 (C-7); 107.75 (C-4,6B); 105.82 (C-2,6C); 102.74 (C-4C); 102.50 (C-2B); 94.94 (C-2); 58.0 (C-3) was in accordance to Breuil et al. (*9*) and by SM (ESI) (*m*/*z* 453 $[M - H]^-$, 435 (MS^2) $[M - H - H_2O]^-$).

trans-Pterostilbene was synthesized according to Pezet and Pont (*5*) and Perret (*15*). ′H NMR, *J trans*: 16.4 Hz. *cis* Isomers of each stilbene were obtained by sunlight exposition, for 10 min, of a methanolic standard solution (500 ng/mL for each stilbene except for *trans*-δ-viniferin at 300 ng/mL).

HPLC and LC-MS Analysis. Quantitative analysis of stilbenes by HPLC was carried out, using a Ginkotek system consisting of a

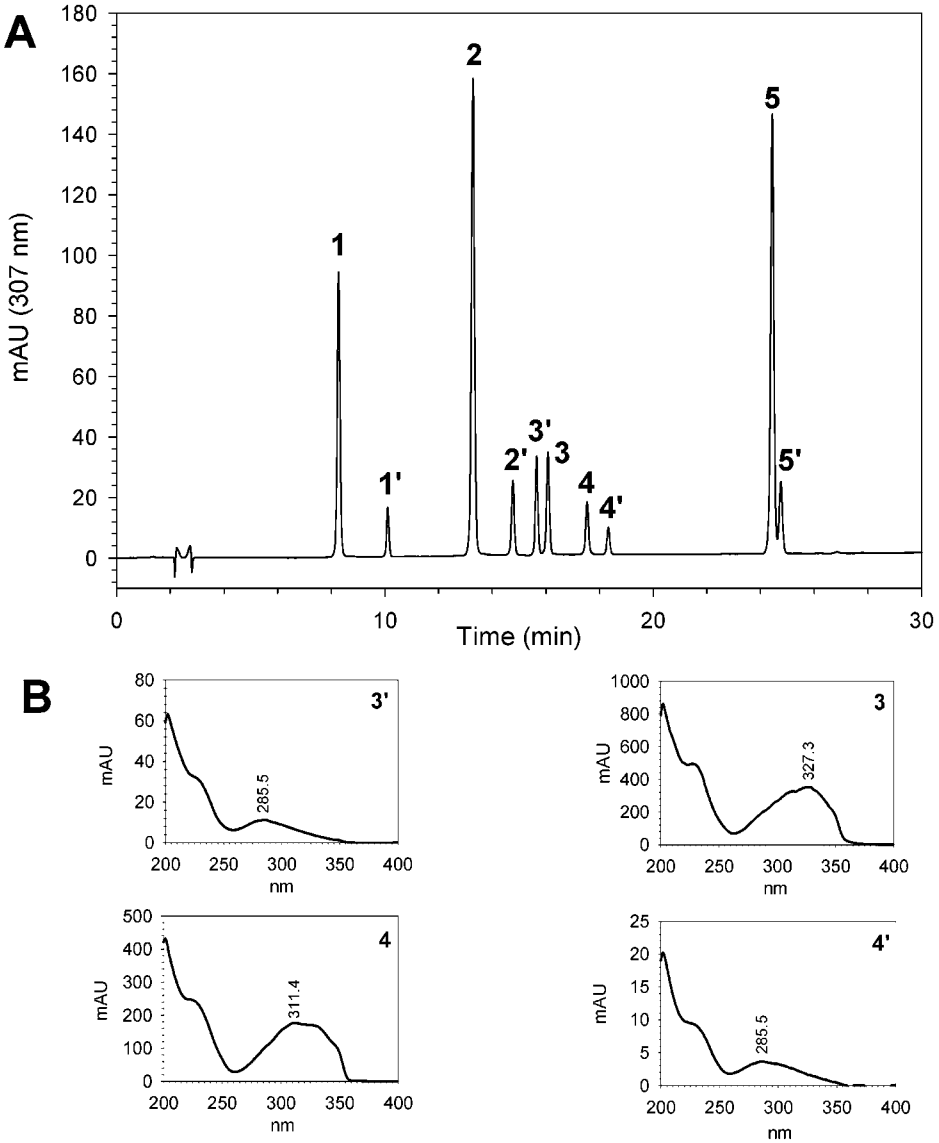

Figure 2. (A) HPLC chromatogram of pure *trans*- and *cis*- stilbenes. (B) UV spectra of *cis*-ϵ-viniferin (**3′**), *trans*-ϵ-viniferin (**3**), *trans*-δ-viniferin (**4**), and *cis*-δ-viniferin (**4′**).

quaternary pump (Ginkotek model 480), an automatic solvent degasser (Supelco Mobile Phase Degassing System 55020), an autosampler (Ginkotek Gina 50), a diode array detector (Ginkotek UVD 340S), and the software Chromeleon (Dionex), and qualitative analysis was performed by HPLC-MS using an Agilent 1100 series LC/MSD trap in ESI mode. These two LC systems were equipped with a 250 mm × 4.6 mm i.d., 5 μm, C18 Lichrospher column (Merck). The solvent system consisted of 1 min isocratic 20% acetonitrile in water, 30 min linear gradient from 20 to 75% acetonitrile in water, 2 min linear gradient from 75 to 100% acetonitrile, 3 min isocratic 100% acetonitrile, 1 min linear gradient from 100 to 20% acetonitrile in water, and 4 min isocratic 20% acetonitrile in water delivered at a flow rate of 1 mL/min. The column temperature was 25 °C for HPLC-MS system and room temperature for HPLC.

HPLC detection of stilbenes was at 307 nm, and calibration curves were calculated for each *cis*- and *trans*-stilbene on the basis of seven different concentrations from 0.5 to 1000 ng (injection volume 10 μL). UV spectra were made from 200 to 400 nm using the diode array on line detection. Molar extinction coefficients (ϵ) at 307 nm in MeOH are for ϵ-viniferin, 17.857, and for δ-viniferin, 13.181.

Sample Preparation. Three pieces of Chasselas leaves were cut in three different *P. viticola* sporulating zones (20.4 mg/73.96 mm^2; 18.38 mg/66.63 mm^2; 11.22 mg/40.68 mm^2) and separately crushed into 300 μL of MeOH placed into a glass tube and then centrifuged (ALC Microcentrifugette 4214, Kontron, 14 000 rpm). Aliquots (10−30 μL) of the supernatant were directly injected into the HPLC or HPLC-MS systems. Three pieces were cut in three distinct parts of one UV-C Chasselas irradiated leaves (15.16 mg/54.96 mm^2; 20.10 mg/72.87 mm^2; 17.16 mg/62.21 mm^2), and samples were prepared for analysis as described above. The surfaces of leaf pieces were measured using a stereoscopic lens equipped with a digital camera (Leica DC 100) and the software Leica IM 1000.

RESULTS AND DISCUSSION

If the *trans*-resveratrol is considered as a nonantifungal stilbene (*6*), the viniferins are toxic for the fungal parasites (*16*) and could be considered as true grapevine phytoalexins. Woody parts of grapevines contained stilbenes (*17*) and particularly large amounts of ϵ-viniferin (**1**) as constitutive compounds. However, its isomer δ-viniferin (**2**) is absent from lignified grapevine canes (result not shown). This woody material is suitable for extraction and purification of ϵ-viniferin. Once pure, this dimer of resveratrol was characterized by NMR and MS. Its retention time on a C18 chromatographic column and its UV spectrum obtained by diode array detection were shown in **Figure 2A**,**B**.

δ-Viniferin was easily synthesized by horseradish peroxidase (*6*) or by fungal laccases (*8*, *9*) from synthetic resveratrol. Pure δ-viniferin was also obtained by enzymatic biosynthesis and chemically characterized as it was done for its isomer (**Figure 2A**,**B**). These two dimers of *trans*-resveratrol, *trans*-resveratrol itself and its glycoside, and *trans*-pterostilbene constitute a pool of stilbene standards useful for the elaboration of an efficient HPLC separation method and further qualitative and quantitative analysis in plants. The analytical method proposed here was adapted from Pezet et al. (*18*) and is similar to that described by Jeandet et al. (*19*). Light exposition of a *trans*-stilbene standard solution has been shown to partially photoisomerize stilbenes into *cis* forms (*19*). Both *cis* and *trans* standard stilbenes were well-separated by HPLC (**Figure 2A**). UV spectra of *trans* and *cis* isomers of ϵ- and δ-viniferin were obtained by diode array detection, and with their retention times, these compounds were easily characterized in a chromatographic separation (**Figure 2B**). In contrast to the other stilbenes analyzed, *cis*-ϵ-viniferin has a shorter retention time than its *trans* form. According to the high sensitivity of modern diode array detectors, stilbenes in both *trans* and *cis* isomers can be detected at 307 nm even at concentrations as low as 0.5 ng per injection volume. If a higher sensitivity is required, a fluorimetric detector at 330 nm for the excitation and 374 nm for the emission wavelength can be coupled to the diode array detector (*1*, *18*).

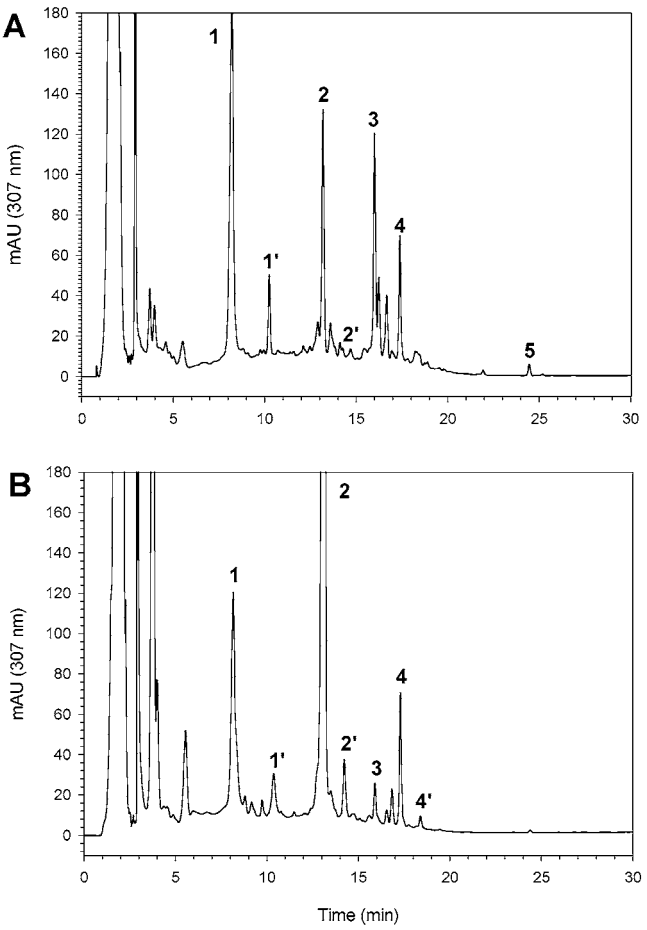

Figure 3. HPLC chromatograms of extracts of (A) *P. viticola*-infected grapevine leaf (var. Chasselas) and of (B) UV-irradiated grapevine leaf (var. Chasselas).

Table 1. Concentrations of Stilbenes Measured in *P. viticola*-Infected Leaves, UV-C-Irradiated Leaves, and Healthy Leaves of Grapevine var. Chasselas

	mg/g fresh weight[a]		
stilbenes peak no.	+ *P. viticola*[b]	+ UV irradiation[c]	control
1, *trans*-piceid	64.93 ± 0.95	18.74 ± 3.69	*d*
1′, *cis*-piceid	12.85 ± 1.34	1.69 ± 0.11	*d*
2, *trans*-resveratrol	49.97 ± 8.48	337.53 ± 27.47	0.28 ± 0.02
2′, *cis*-resveratrol	6.45 ± 2.09	0.32 ± 0.09	*d*
3, *trans*-ϵ-viniferin	86.81 ± 18.12	8.63 ± 1.45	*d*
3′, *cis*-ϵ-viniferin	7.31 ± 1.08	*d*	*d*
4, *trans*-δ-viniferin	155.68 ± 20.36	165.71 ± 18.42	2.04 ± 0.43
4′, *cis*-δ-viniferin	*d*	3.42 ± 0.17	*d*
5, *trans*-pterostilbene	5.14 ± 0.46	*d*	*d*
5′, *cis*-pterostilbene	*d*	*d*	*d*

[a] Mean of three replicates ± standard deviation. [b] Analysis made 7 days after inoculation. [c] Analysis made 72 h after UV irradiation. [d] No peak detected for this stilbene.

In grapevine leaves and berries, *trans*-resveratrol and its derivatives are induced by biotic elicitation as fungal parasites (*16*, *20*), fungal exoenzymes (*21*), or abiotic elicitations as UV irradiation (*2*), aluminum chloride (*22*), or fungicide-containing fosetyl-Al (*23*).

Our analysis was performed on grapevine leaves infected by

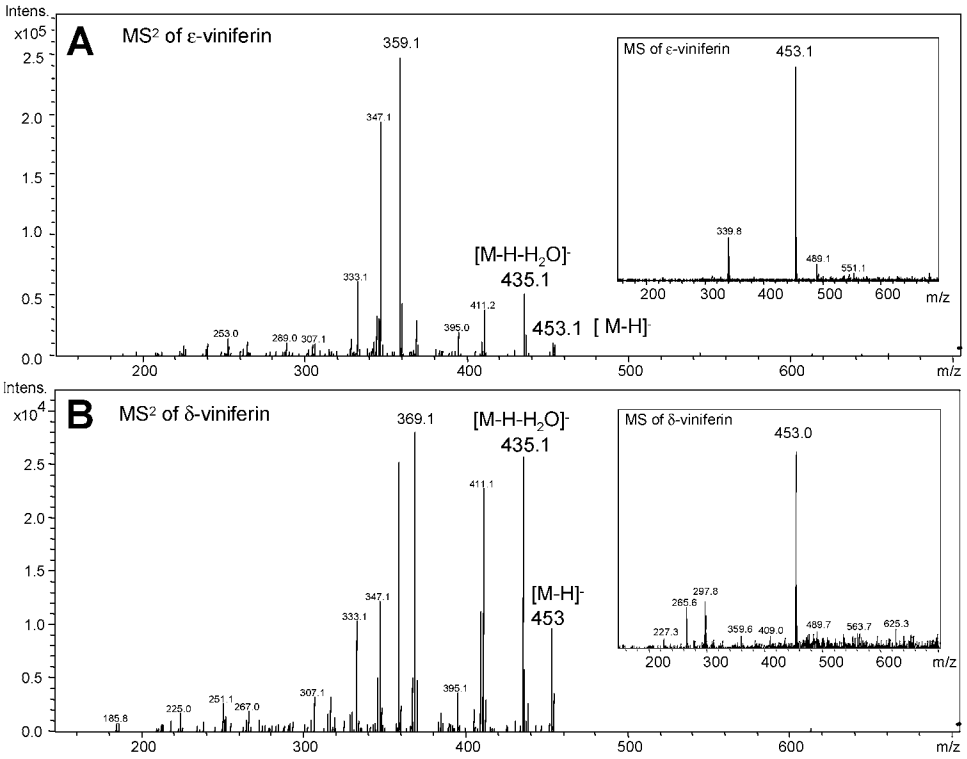

Figure 4. Mass spectra of (A) peaks of **3** (*trans*-ϵ-viniferin) and (B) peaks of **4** (*trans*-δ-viniferin) obtained by HPLC-MS of *P. viticola* and UV-irradiated grapevine leaves extracts (var. Chasselas).

downy mildew (*P. viticola*) or submitted to UV irradiation. Many authors described sophisticated and time-consuming stilbene extraction methods requiring large quantities of plant material (*20*, *24*). However, stilbenes can be efficiently extracted from small leaf pieces with a low volume of MeOH (1−100 mg fresh weight in 100−500 μL). Methanolic extracts were directly injected after clarification by centrifugation. In several experiments, stilbenes were detected in less than 1 μg (fresh weight) of stressed grapevine leaves (results not shown). Such extracts were separated by HPLC to detect by UV (DAD, 307 nm) the stilbenes synthesized by *P. viticola* infected grapevine leaves and by HPLC-MS to confirm their identity. *trans*- and *cis*-Piceid, *trans*-resveratrol, *trans*-ϵ- and *trans*-δ-viniferins, and pterostilbene were detected (**Figure 3A**). MS has confirmed that peaks 3 (ϵ-viniferin, **1**) and 4 (δ-viniferin, **2**) were *trans*-resveratrol dimers (m/z = 453 $[M - H]^-$) (**Figure 4A**,**B**). UV spectra obtained by diode array detection and retention times are in a complete accordance with those of viniferin standards (**Figure 2A**,**B**).

After 72 h of incubation, UV-irradiated leaves synthesized the same stilbenes. However, some differences were observed as follows: *cis*-δ-viniferin was detected, and pterostilbene was absent in these extracts (**Figure 3B**).

Seven days after inoculation with *P. viticola*, δ-viniferin was the most abundant stilbene detected. In UV-irradiated leaves, resveratrol exhibited the highest concentration. However, the δ-viniferin concentration was almost the same in both samples (**Table 1**). The resveratrol trimer, α-viniferin, described by Pryce and Langcake (*25*), was never detected in our grapevine leaf samples.

trans-Resveratrol is the primary phytoalexin produced by a stilbene synthase induced by a stress as described in a recent review (*26*). Viniferins resulted from an oxidative dimerization of resveratrol carried out by supposed grapevine cell peroxidases (*27*). These authors identified a *trans*-resveratrol oxidizing basic peroxidase and proposed a mechanism for the synthesis of ϵ- and δ-viniferins to explain the presence of these two isomers in stressed grapevine leaves.

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