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ARTICLE

Structures of Two Novel Trimeric Stilbenes Obtained by Horseradish Peroxidase Catalyzed Biotransformation of *trans*-Resveratrol and (−)-ε-Viniferin

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Two stilbenes, *trans*-resveratrol and (−)-ε-viniferin, as well as a mixture of both, were biotransformed using horseradish peroxidase and hydrogen peroxide. Under the applied conditions *trans*-resveratrol afforded one major product, which was identified as *trans*-δ-viniferin, a resveratrol−*trans*-dehydrodimer. Large-scale biotransformation of a mixture of *trans*-resveratrol and (−)-ε-viniferin yielded two novel resveratrol trimers, resviniferin A and resviniferin B, which were obtained in a pure form after fractionation by high-speed countercurrent chromatography and final purification by preparative HPLC. Their structures were established by means of mass spectrometry and 2D NMR spectroscopic analyses, including HSQC, HMBC, COSY, and ROESY.

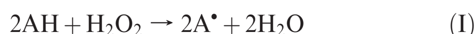
KEYWORDS: Biotransformation; horseradish peroxidase; stilbenes; high-speed countercurrent chromatography

INTRODUCTION

Stilbenes are nonflavonoid phenolics that occur in a number of plant families, the highest concentrations being found in grapes and related products. Due to their antioxidative, anticarcinogenic, and antimutagenic activities, stilbenes are considered to play a central role in the human diet. Their structural nucleus is based on a 14-carbon skeleton composed of two phenyl rings joined by an ethylene bridge. One of the most relevant stilbenes is *trans*-resveratrol (3,5,4′-trihydroxystilbene), a phytoalexin produced by grapevines in response to stress, such as fungal infection (e.g., *Botrytis cinerea*) or UV irradiation (*1*). In nature, resveratrol exists in two isomeric forms (cis and trans configurations of the olefinic bond) either as β-glycosylated derivatives, named piceids, or as the aglycone (*2*).

Stilbenes are also known to occur in oligomeric forms. Their biosynthesis includes an oxidative polymerization of the monomer resveratrol as a result of the activities of peroxidases (*3*). These enzymes utilize hydrogen peroxide to catalyze oxidative reactions and have already been used for the in vitro synthesis of stilbene oligomers (*4*, *5*).

Horseradish peroxidase (HRP) catalyzed reactions can be formulated as shown in eq I.



$$2AH + H_2O_2 \rightarrow 2A^{\bullet} + 2H_2O \qquad (I)$$

As a consequence, phenols, such as resveratrol, are oxidized by hydrogen peroxide to generate radicals, which, after diffusion from the active center of the enzyme, react with further aromatic substances to form dimeric, oligomeric, or polymeric products (*6*).

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To date, resveratrol oligomers ranging from dimer to octamer are known (*7*). Several of these, such as *trans*-α-viniferin and *trans*-ε-viniferin, show enhanced phytoalexin activity compared to the monomer (*8*). Additionally, oligomers are used as a nontoxic form of a resveratrol accumulator and can be stored by plants in nonphotosynthetic tissues. Several stilbene oligomers have been found to exhibit antibacterial, anti-HIV, anti-inflammatory, antioxidant, and antitumor activities (*5*, *9*).

Various studies have used horseradish peroxidase and hydrogen peroxide for the generation of stilbene oligomers. Langcake and Pryce (*10*) have reported the biotransformation of *trans*-resveratrol to its *trans*-dehydrodimer. Subsequently, the oligomerization of *trans*-resveratrol to pallidol, resveratrol *trans*-dehydrodimer, and leachianol F using horseradish peroxidase has been established (*3*). In addition, stilbene oligomers such as, (+)-hopeaphenol, (−)-isohopeaphenol, (−)-vitisin B, and (+)-vitisin C were derived from the biotransformation of *trans*-ε-viniferin (*11*, *12*).

The oxidative dimerization of *trans*-resveratrol and *trans*-ε-viniferin was reported by He et al. (*13*), who were able to generate (+)-davidiol A using horseradish peroxidase. Furthermore, transformations of *trans*-resveratrol with different reactants such as ferulic acid and quadrangularin A have been carried out (*14*, *15*).

For the isolation of our biotransformed stilbene oligomers the all-liquid chromatographic separation technique of high-speed countercurrent chromatography (HSCCC) has been applied. HSCCC has been successfully used for the separation and purification of various natural products and is especially suited for large-scale fractionation of complex natural extracts (*16*). The separation is based on partitioning between two immiscible solvent pairs, and up to 50000 partitioning steps per hour can be obtained. Irreversible adsorption and sample loss can be neglected (*17*).

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With regard to stilbenes, HSCCC has been applied to the isolation of *trans*-resveratrol and piceid from *Polygonum cuspidatum* using different solvent systems (*18−20*). Furthermore, HSCCC isolations of quadrangularin A and parthenocissin A from *Parthenocissus laetevirens* (*21*) and of hopeaphenol, vitisin A, and amurensin G from *Vitis chunganeniss* have been reported (*22*).

A great variety of stilbenoids are known to occur in grapevine, but the structures of many minor stilbene oligomers remain to be elucidated. Because it is rather difficult to isolate these minor constituents in sufficient amounts for subsequent structural characterization, we were searching for a more promising way to prepare them. It is known that the natural biotransformation of stilbene oligomers in grapevine can be simulated by in vitro biotransformation with horseradish peroxidase (*3*, *6*, *10−15*). Used in combination with HSCCC, larger amounts of the minor compounds can be accumulated, thus allowing their unambiguous structure determination. Using this approach, two novel minor components were isolated and characterized for the first time. As these two new compounds could occur naturally as minor constituents in grapevine extracts, the elucidation process of stilbene oligomers from grapevine extracts can be carried out more efficiently using the data reported here.

MATERIALS AND METHODS

Materials. Chemicals and solvents were obtained from the suppliers shown: acetone-d_6, 99.96% (Deutero, Kastellaun, Germany); acetone, p.a. (Roth, Karlsruhe, Germany); citric acid, anhydrous, p.a., 99.5% (Fluka, Steinheim, Germany); disodium hydrogen phosphate dihydrate p.a. (Merck, Darmstadt, Germany); horseradish peroxidase, 970 U/mg (Fluka); aqueous hydrogen peroxide, p.a., 30% (Merck); methanol-d_4, 99.96% (Deutero).

Solvents for HPLC analysis included acetic acid, HPLC quality (Baker, Deventer, The Netherlands); acetonitrile, HPLC quality (Fisher Scientific, Loughborough, U.K.); methanol, HPLC quality (Fisher); and ultrapure water (NANOpure).

Solvents used for HSCCC were ethyl acetate, p.a. (Fisher); *n*-hexane (distilled, industrial quality); methanol (distilled, industrial quality); and ultrapure water (NANOpure).

trans-Resveratrol and (−)-ε-viniferin were isolated from the commercial grapevine extract of *Vitis vinifera*, Vineatrol 30 (Breko GmbH, Bremen, Germany).

Small-Scale Peroxidase Reaction of *trans*-Resveratrol and (−)-ε-Viniferin. The peroxidase reaction was carried out according to the method of Takaya et al. using optimized conditions (*3*). A suspension of horseradish peroxidase (1.33 mg of enzyme in 1 mL of McIlvaine buffer) was added to a mixture of *trans*-resveratrol (22.8 mg) dissolved in 4 mL of acetone, 4 mL of water, and 1.6 mL of McIlvaine buffer (pH 6) and stirred for 5 min at 22 °C. Aqueous hydrogen peroxide (10%, 40 μL) was added, and the solution was stirred for 45 min at 22 °C, extracted with ethyl acetate, evaporated, and lyophilized in the absence of daylight. The biotransformation of (−)-ε-viniferin (5.0 mg) was carried out with aliquots of chemicals as described above, as well as a mixture of both stilbenes (5.0 mg). For the blank experiments, educts and chemicals without application of peroxidase were used.

Large-Scale Peroxidase Reaction of *trans*-Resveratrol and (−)-ε-Viniferin. A suspension of horseradish peroxidase (6.3 mL; *c* 1 g/L) was added at 22 °C to a mixture of *trans*-resveratrol (415.7 mg) and (−)-ε-viniferin (214.3 mg) dissolved in 250 mL of an acetone/water mixture (1:1, v/v) and 50 mL of McIlvaine buffer (pH 6). Aqueous hydrogen peroxide (10%, 1.25 mL) was then added, and the solution was stirred for 45 min at 22 °C. The lyophilized product was obtained as described above.

HSCCC. A preparative high-speed countercurrent chromatograph (model 1000, Pharma-Tech Research Corp., Baltimore, MD) was equipped with three coils connected in series (total capacity of 800 mL). The two-phase solvent system was composed of *n*-hexane/ethyl acetate/methanol/water (2:3:2:3, v/v/v/v), and a HPLC pump (Biotronic BT 3020) was used to pump the solvents. The separation was carried out in the "head-to-tail" elution mode in which the upper phase acts as the stationary phase. The apparatus was rotated at a revolution speed of 850 rpm while a flow rate of 3 mL/min was used. For the separation, 630 mg of the biotransformed mixture dissolved in the upper and lower phases (1:1) was injected via an injection loop (20 mL). Elution was monitored with a Knauer K-2501 UV detector at λ = 280 nm and recorded using a Servogor 120 plotter (Georz Metrawatt). Fractions were collected with a fraction collector (Pharmacia LKB Super Frac).

TLC Analysis. Evaluation of the HSCCC fractions was performed by thin-layer chromatography on normal phase silica gel plates 60 F_{254} (Merck) with chloroform/ethyl acetate/methanol/water (25:55:5:1, v/v/v/v) as solvent system. Visualization of the stilbenes and derivatives was achieved by means of an anisaldehyde spray reagent according to the method of Stahl (*23*).

HPLC-PDA. HPLC-PDA measurements were carried out on a HPLC system from Jasco (Gross-Umstadt, Germany), consisting of a pump PU-980, a degasser DG-98050, a gradient unit LG-980-02, and a photodiode array detector MD-910. An analytical C18 column (Luna C18, 250 × 4.6 mm, 5 μm, Phenomenex, Aschaffenburg, Germany) at a flow rate of 0.8 mL/min [solvent system of 1% acetic acid (A), acetonitrile (B)] was used. The applied gradient started over 1 min at 15% B, 10 min at 24% B, 45 min at 90% B, 50 min at 100% B, and resulted in 55 min at 15% B. Each of the obtained fractions was analyzed by HPLC-PDA and HPLC-ESI-MS/MS.

HPLC-ESI-MS/MS and High-Resolution ESI-MS. A HPLC system (pump 1100 series, autosampler 1200 series) from Agilent Technologies (Böblingen, Germany) was connected with an Esquire LC-ESI-MS/MS from Bruker (Bremen, Germany). Mass spectra were recorded in the negative mode, with the capillary set at 1500 V, the end plate at −500 V, the capillary exit at −120.4 V, the dry gas at 310 °C, the gas flow at 9.0 L/min, the nebulizer at 40 psi, the target mass at *m*/*z* 500, the scan range from *m*/*z* 50 to 2200, the collision gas of helium, and a MS/MS fragmentation amplitude at 1.0 V. HPLC conditions were the same as described in the HPLC section above. High-resolution ESI-MS were recorded on a Thermo Science LTQ Orbitrap mass spectrometer.

Preparative HPLC. Fractions were purified by preparative HPLC on a Smartline system from Knauer (Smartline, pump 1000, manager 5000, detector UV K-2600, Berlin, Germany). The preparative HPLC column (Luna C18, 250 × 15.0 mm, 5 μm, Phenomenex, Aschaffenburg, Germany) was operated with a binary solvent system of ultrapure water (A) and methanol (B) at a flow rate of 4 mL/min. The gradient for the isolation of *trans*-δ-viniferin **1** was composed of 0 min at 25% B, 35 min at 45% B, and 50 min at 60% B, and the gradient for the isolation of compound **2** and **3** was 0 min at 45% B, 20 min at 53% B, and 53 min at 63% B. The fractions were monitored at λ = 280, 306, and 325 nm.

Nuclear Magnetic Resonance Spectroscopy. ^{1}H, ^{13}C, DEPT-135, ^{1}H−^{1}H −COSY, ^{1}H−^{1}H phase-sensitive ROESY, HSQC, and HMBC experiments were carried out on Bruker Avance DMX 600 or DPX 300 NMR spectrometers. Chemical shifts were referenced to the solvent signals.

trans-δ-Viniferin (*resveratrol dehydrodimer*), ***1***: amorphous powder; UV (MeOH) λ_{max} 311 nm; ESI-MS/MS *m*/*z* 453 $[M - H]^-$; MS/MS fragments *m*/*z* 435, 411, 369, 359, 347, 333, 307; ^{1}H NMR (600 MHz, methanol-d_4) and ^{13}C NMR (150 MHz, methanol-d_4) see **Table 2**.

Resviniferin A, ***2***: amorphous powder; UV (MeOH) λ_{max} 323 nm; ESI-MS/MS *m*/*z* 679 $[M - H]^-$; MS/MS fragments *m*/*z* 661, 643, 621, 585, 573, 451, 439, 345, 333; HR-ESI-MS *m*/*z* 681.2111 $[M + H]^+$ (calcd for $[C_{42}H_{32}O_9 + H]^+$ 681.2119); ^{1}H NMR (600 MHz, acetone-d_6) and ^{13}C NMR (150 MHz, acetone-d_6) see **Table 2**.

Resviniferin B, ***3***: amorphous powder; UV (MeOH) λ_{max} 331 nm; ESI-MS/MS *m*/*z* 679 $[M - H]^-$; MS/MS fragments *m*/*z* 661, 585, 573, 451, 359, 345, 333; HR-ESI-MS *m*/*z* 681.2099 $[M + H]^+$ (calcd for $[C_{42}H_{32}O_9 + H]^+$ 681.2119); ^{1}H NMR (600 MHz, acetone-d_6) and ^{13}C NMR (150 MHz, acetone-d_6) see **Table 2**.

RESULTS AND DISCUSSION

Small-Scale Peroxidase Reaction of *trans*-Resveratrol and (−)-ε-Viniferin. In this preliminary study *trans*-resveratrol and (−)-ε-viniferin were used for the reactions with horseradish peroxidase and hydrogen peroxide on a small scale. The conversion was analyzed by HPLC-PDA and HPLC-ESI-MS/MS. All given

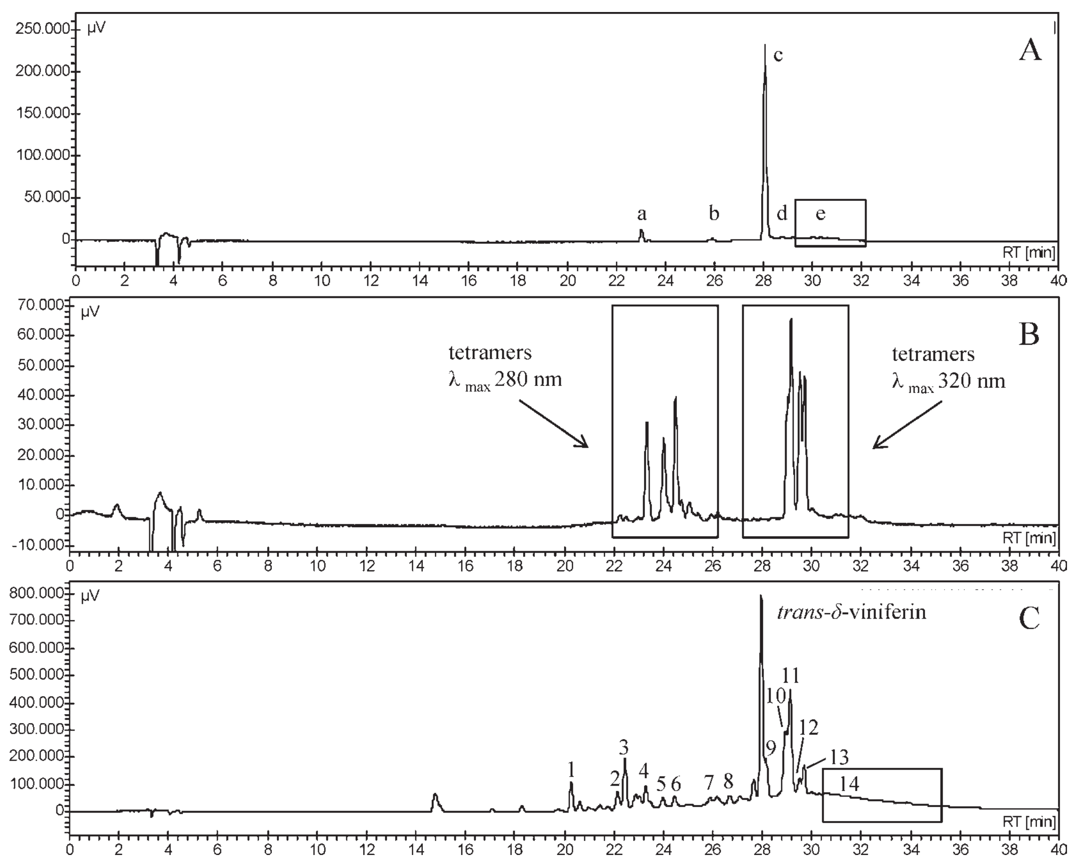


Figure 1. HPLC-PDA chromatograms of biotransformed stilbene oligomers from small-scale experiments: (**A**) chromatogram of biotransformed *trans*-resveratrol at 306 nm [*trans*-resveratrol (peak a), trimer A (peak b), *trans*-δ-viniferin (peak c), trimer B (peak d), and polymeric compounds (peak e)]; (**B**) chromatogram of biotransformed (−)-ε-viniferin at 280 nm [tetramers (λ_{max} 280 nm) and tetramers (λ_{max} 320 nm)]; (**C**) chromatogram of biotransformed mixture of *trans*-resveratrol and (−)-ε-viniferin at 280 nm [dimer (peak 1), trimers (peaks 2−5), tetramer (peak 6), trimer (peak 7), (−)-ε-viniferin (peak 8), *trans*-δ-viniferin, trimers (peaks 9−11), tetramers (peaks 12 and 13), and polymeric compounds (peak 14)].

contents of the compounds were determined by HPLC-PDA at 306 nm. Under optimized conditions 95% of *trans*-resveratrol (peak a, **Figure 1A**) was biotransformed to 83% of its *trans*-dehydrodimer *trans*-δ-viniferin (peak c). In addition, two resveratrol trimers [trimer A (peak b), 1%; trimer B (peak d), 2%] were detected with molecular ions at m/z 679 $[M - H]^-$ as well as 9% of polymeric compounds (peak e). Using preparative HPLC, 2.2 mg of *trans*-δ-viniferin could be purified. The structure was established using data from ESI-MS/MS, ^{1}H NMR, and ^{13}C NMR measurements according to the data of Pezet et al. (*24*) and Huang et al. (*25*). In **Figure 2** a possible biogenetic pathway for *trans*-δ-viniferin generated by horseradish peroxidase is presented.

The biotransformation of (−)-ε-viniferin yielded four major components showing a UV maximum at 320 nm (**Figure 1B**) and molecular ions at m/z 905 $[M - H]^-$. The latter indicates that these are resveratrol tetramers generated by dimerization of *trans*-ε-viniferin radicals. All four tetramers exhibited a similar MS/MS fragmentation with three larger fragments at m/z 811 $[M - H - C_6H_5 - OH]^-$, Δ94, 799 $[M - H - C_7H_5 - OH]^-$, Δ106, and 451 $[M - H - \text{dimer}]^-$, Δ454, and a variable smaller fragment.

Three further minor components with a UV maximum at 280 nm and molecular ions at m/z 905 $[M - H]^-$ were also identified as resveratrol tetramers. In contrast to the major components, the MS/MS fragmentation gave the same fragments for all minor components at m/z 811, 717, 611, 451, and 357. The only differences between these stilbenes were the variable intensities of the MS/MS fragments and different retention times. In **Figure 3** the generation of radical intermediates from (−)-ε-viniferin by horseradish peroxidase and hydrogen peroxide according to the scheme of Takaya et al. (*12*) is outlined. Such radicals can combine to give various different stilbene oligomers, such as (+)-vitisin A, which would explain the diversity of tetramers detected in the HPLC chromatogram.

In further experiments, the biotransformation of a mixture of *trans*-resveratrol and (−)-ε-viniferin was investigated. Chromatogram **C** of **Figure 1** presents the complexity of the conversion product. One of the major compounds with a molecular ion at m/z 453 $[M - H]^-$ is related to *trans*-δ-viniferin. On the basis of their molecular masses, further stilbene oligomers were detected as a resveratrol dimer (peak 1), resveratrol trimers (peaks 2−5, 7, 9−11), resveratrol tetramers (peaks 6, 12, 13), and the remaining *trans*-ε-viniferin (peak 8). Polymeric transformation products (peak 14) were detected at later retention times. All blank tests yielded no transformation products.

Under optimized reaction conditions the conversion rate in each transformation was >95%. These small-scale experiments confirmed that horseradish peroxidase catalyzed reaction of *trans*-resveratrol and *trans*-ε-viniferin gives rise to a large variety of different stilbene oligomers. Therefore, a large-scale conversion was carried out to allow isolation of minor components.

Large-Scale Peroxidase Reaction and HSCCC Separation. For the preparative formation of resveratrol oligomers, a mixture of *trans*-resveratrol and (−)-ε-viniferin was converted by horseradish peroxidase as described above. The oligomeric fraction was separated by HSCCC using a solvent system composed of *n*-hexane/ethyl acetate/methanol/water (2:3:2:3, v/v/v/v). The fractionation of the stilbene oligomers was visualized by TLC. HSCCC separation yielded nine fractions. Using HPLC-PDA

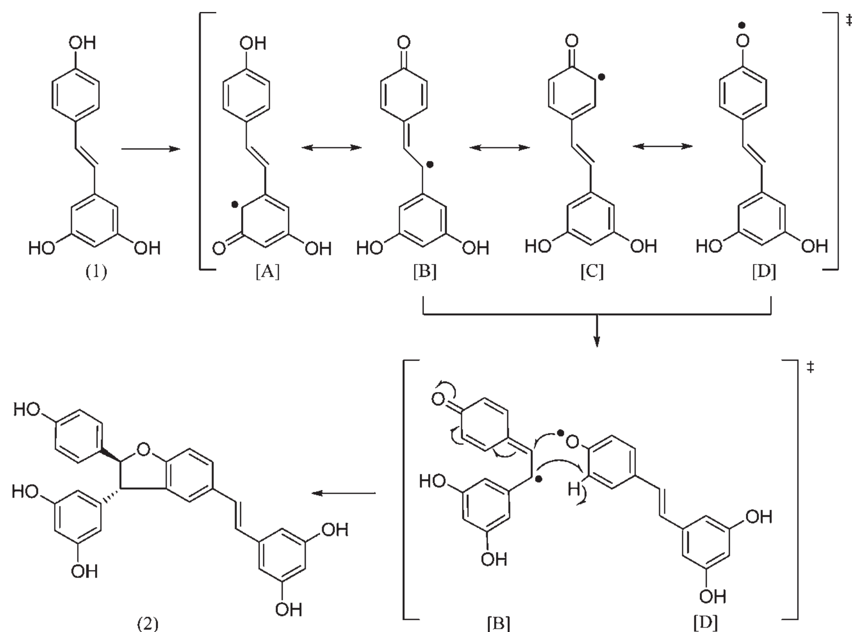


Figure 2. Hypothetical biogenetic pathway of *trans*-δ-viniferin catalyzed by horseradish peroxidase: resveratrol (1), possible radical intermediates [A−D], and *trans*-δ-viniferin (2).

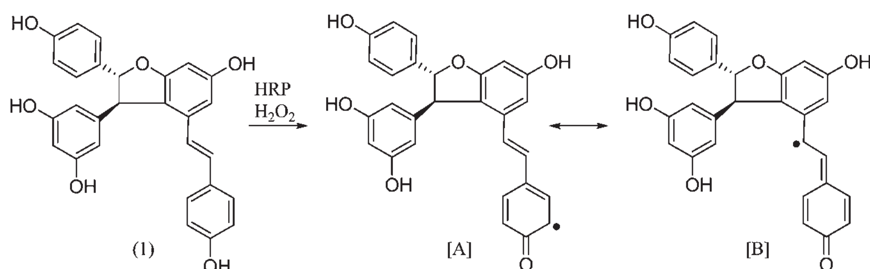


Figure 3. Generation of radical intermediates from (−)-ε-viniferin by horseradish peroxidase and hydrogen peroxide according to Takaya et al. (*12*): (−)-ε-viniferin (1) and radical intermediates [A and B].

and HPLC-ESI-MS/MS analyses more than 20 different stilbene oligomers were detected, and the major components of each fraction are shown in **Table 1**.

Fractions 1 and 2 were composed primarily of polymeric components. Their polar nature resulted in their elution directly after the breakthrough of mobile phase. In fraction 3 a resveratrol trimer and a resveratrol tetramer were detected as major components. However, due to the small amount of material (6.8 mg), a further purification was not carried out. A resveratrol tetramer was enriched in fraction 4, and the fraction size of 50.8 mg allowed preparative amounts of this stilbene to be isolated. Fraction 5 consisted of various stilbene oligomers showing similar retention times.

It was possible to isolate 4 and 8 mg of two resveratrol trimers (resviniferin A, **2**; resviniferin B, **3**) from fraction 6 with purities of > 96% (HPLC-PDA, λ_{max} = 306 nm) by preparative HPLC. Structure determination of these substances was carried out by ESI-MS/MS and 2D NMR spectroscopic analysis.

Fraction 7 consisted of a mixture of the dehydrodimer *trans*-δ-viniferin and a resveratrol trimer with a molecular ion of *m*/*z* 679 $[M - H]^-$. In fraction 8 the same dimer was present as the major compound at a purity of > 87%. Due to the fraction size of 28.3 mg, fraction 8 represents a good source for the isolation of *trans*-δ-viniferin in a purity higher than in the case of the biotransformation with *trans*-resveratrol. Fraction 9 contained *trans*-δ-viniferin in addition to various other stilbene oligomers.

Structural Elucidation of the Trimeric Compounds. The structures of components **1**, **2**, and **3** (**Figure 4**) were confirmed as *trans*-δ-viniferin and the newly identified resviniferin A and resviniferin B, respectively, by means of electrospray ionization mass spectrometry (ESI-MS/MS), ^{1}H, ^{13}C, DEPT-NMR, and 2D NMR spectroscopic analysis, including HSQC, HMBC, COSY, and ROESY. The data of *trans*-δ-viniferin **1** were in agreement with previously published data (*24*, *25*).

Resviniferin A (**2**) was obtained as a pale white amorphous powder, with a high-resolution molecular ion at *m*/*z* 681.2111 $[M + H]^+$ corresponding to a resveratrol trimer. The ^{1}H NMR spectrum in acetone-d_6 showed two sets of AA′XX′ type ortho-coupled aromatic hydrogens at δ 6.67 and 6.61 (each 2H, d, *J* = 8.6 Hz) for ring A_1, 7.27 and 6.91 (each 2H, d, *J* = 8.5 and 8.4 Hz) for ring C_1, two sets of AX_2 type meta-coupled aromatic hydrogens at δ 6.49 (2H, d, *J* = 2.1 Hz) and 6.25 (1H, m) for ring B_2, 6.14 (2H, d, *J* = 2.1 Hz) and 6.25 (1H, m) for ring C_2, one set of meta-coupled aromatic hydrogens at δ 6.33 and 6.27 (each 1H, d, *J* = 2.0 and 2.1 Hz) for ring A_2, one set of ABX type ortho−meta-coupled aromatic hydrogens at δ 7.11 (1H, br s), 6.87 (1H, d, *J* = 8.3 Hz), and 7.36 (1H, dd, *J* = 8.3 and 1.5 Hz) for ring B_1, two sets of aliphatic hydrogens at δ 5.60 (1H, d, *J* = 4.9 Hz, H-7a) and 4.38 (1H, d, *J* = 4.8 Hz, H-8a), δ 5.43 (1H, d, *J* = 4.4 Hz, H-7c) and 4.58 (1H, d, *J* = 4.4 Hz, H-8c), and two coupled doublets at δ 6.91 and 6.73 (each 1H, d, *J* = 16.8 and 16.3 Hz, H-7b, H-8b) for a trans-configured double bond. The correlations of the ring

Table 1. Characterization of Fractions Achieved by HSCCC

fraction	amount (mg)	$[M - H]^-$ *m/z*	resveratrol oligomers
1	341.9		polymeric compounds
		679	three trimers
		905	two tetramers
2	29.2		polymeric compounds
		453	dimer
		679	trimer
3	6.8	679	trimer
		905	tetramer
4	50.8	905	tetramer
5	25.6	679	two trimers
		905	two tetramers
6	38.8	679	resviniferin A
		679	resviniferin B
		905	tetramer
7	9.9	453	*trans*-δ-viniferin
		679	trimer
8	28.3	227	*trans*-resveratrol
		453	*trans*-δ-viniferin
		679	trimer
9	11.6	453	*trans*-δ-viniferin

systems, double bond, and aliphatic hydrogens were confirmed from correlations in the 2D $^1H-^1H$ COSY spectrum of **2**. The ^{13}C NMR spectrum of **2** exhibited four aliphatic carbons at δ 91.2, 52.3, 94.0, and 56.9 (C-7a, C-8a, C-7c, C-8c), besides 38 aromatic and olefinic carbons between δ 102.4 and 162.4. All protonated carbons were assigned from the HSQC spectrum. The ^1H and ^{13}C NMR data are shown in **Table 2**. Supporting evidence for the proposed structure of **2** was obtained from HMBC measurements. Important CH long-range correlations from the HMBC spectrum (**Figure 5**), over two or three bonds, were found between H-8a and eight carbon signals [δ 132.3 (C-1a), 91.2 (C-7a), 142.2 (C-9a), 119.8 (C-10a), 106.7 (C-14a), 123.7 (C-2b), 133.0 (C-3b), 159.7 (C-4b)]. Similar correlations were assigned to hydrogen H-8c [δ 134.3 (C-1c), 94.0 (C-7c), 147.4 (C-9c), 106.8 (C-10c,14c), 142.2 (C-9a), 119.8 (C-10a), 162.4 (C-11a)]. The nature and number of correlations indicated the central position of protons H-8a and H-8c in the structure. Both hydrogens are at positions connecting the monomer units of ring systems A, B, and C. Furthermore, correlations between H-10b and the carbon signal δ 127.1 (C-8b) in addition to H-7b and C-6b (δ 128.3) verified the connection of the olefinic system and the two aromatic rings B_1 and B_2.

To determine the relative stereochemistry at C-7a/C-8a and C-7c/C-8c of **2**, NOE as well as ROESY measurements have been carried out. There is a considerable amount of ^1H NMR data published for related structures in which rings A_1, A_2, and B_1 are present in which the former two rings are in a trans configuration about the C-7a/C-8a bond of the 2,3-dihydrofuran system (*11*, *25*−*27*). In many of these the magnitude of the vicinal coupling constant between H-7a and H-8a has been determined and shows values between 4.0 and 10.0 Hz. This indicates that the magnitude of this coupling is not a reliable guide to assess the configuration. In compounds with no substituents on rings A_1, A_2, and B_1 ortho to the five-membered ring, larger values are observed for the coupling constants, whereas the presence of substituents results in smaller values. Presumably, this arises from a change in the conformational preference of this ring system with a subsequent decrease in the vicinal coupling. As a

Table 2. ^1H and ^{13}C NMR Data of *trans*-δ-Viniferin (**1**), Resviniferin A (**2**), and Resviniferin B (**3**)

	1[a]		**2**[b]		**3**[b]	
position	^1H	^{13}C	^1H	^{13}C	^1H	^{13}C
1a		132.4		132.3		132.6
2(6)a	7.07 d (8.5)	128.9	6.67 d (8.6)	127.4	7.19 d (8.5)[1c]	128.0[1]
3(5)a	6.68 d (8.5)	116.3	6.61 d (8.6)	115.9	6.82 d (8.6)[2]	116.1
4a		158.8		157.7		158.2[3]
7a	5.29 d (8.4)	94.9	5.60 d (4.9)	91.2	5.41 d (7.7)	93.9
8a	4.30 d (8.4)	58.8	4.38 d (4.8)	52.3	4.39 d (7.7)	57.7
9a		145.4		142.2		145.2
10a	6.02 d (2.2)	107.7		119.8	6.16 d (2.2)	107.3
11a		160.0		162.4		159.6
12a	6.08 t (2.2)	102.5	6.33 d (2.0)	96.4	6.26 d (2.2)	102.3
13a		160.0		160.3		159.6
14a	6.02 d (2.2)	107.7	6.27 d (2.1)	106.7	6.16 d (2.2)	107.3
1b		132.4		131.7		131.8[4]
2b	7.09 br s	124.2	7.11 br s	123.7	6.99 br s	125.0
3b		132.8		133.0		131.9[4]
4b		161.0		159.7		160.6
5b	6.75 d (8.3)	110.4	6.87 d (8.3)	110.4	6.76 d (8.4)	110.2
6b	7.27 dd (8.2, 1.6)	128.9	7.36 dd (8.3, 1.5)	128.3	7.20 m	127.7
7b	6.88 d (16.0)	129.4	6.91 d (16.8)	129.1	6.93 d (16.3)	130.3
8b	6.69 d (16.0)	127.5	6.73 d (16.3)	127.1	6.72 d (16.3)	124.2
9b		141.2		140.7		136.4
10b	6.32 d (2.1)	105.8	6.49 d (2.1)	105.7		120.0
11b		159.7		159.5		162.4
12b	6.03 t (2.2)	102.7	6.25 m	102.7	6.32 d (2.0)	96.8
13b		159.7		159.5		159.7
14b	6.32 d (2.1)	105.8	6.49 d (2.1)	105.7	6.70 d (2.1)	104.4
1c				134.3		133.8
2(6)c			7.27 d (8.5)	127.8	7.20 d (8.5)[1]	128.5[1]
3(5)c			6.91 d (8.4)	116.4	6.83 d (8.5)[2]	116.1
4c				158.1		158.4[3]
7c			5.43 d (4.4)	94.0	5.42 d (5.4)	93.9
8c			4.58 d (4.4)	56.9	4.46 d (5.5)	57.1
9c				147.4		147.3
10(14)c			6.14 d (2.1)	106.8	6.22 m	107.0
11(13)c				160.1		159.7
12c			6.25 m	102.4	6.22 m	102.1

[a] Measured in CD_3OD. [b] Measured in CD_3COCD_3. [c] [1,2,3,4], shifts are interchangeable.

consequence one must resort to a careful assessment of the NOE data in which a trans orientation of the aryl pair (A_1/ A_2) present in the 2,3-dihydrofuran system is deduced from NOEs between H-7a and the ortho H(s) of ring A_2 and H-8a and the ortho H(s) of ring A_1 (*27*, *28*).

The experimental ROESY spectrum of **2** is shown in **Figure 6**, and the details relevant for the determination of the relative stereochemistry of the two 2,3-dihydrofurans are summarized in **Figure 7**. In both cases the observed NOEs are compatible with trans configurations of the C-7 and C-8 of these systems. In addition, the significant NOE interactions of H-7a and H-8a as well as H-7c and H-8c indicate the non-pseudoaxial position of both vicinal hydrogen pairs and subsequent small coupling constants. The experimental NOEs observed here for **2** are very similar to those reported for *trans*-diptoindonesin B, an oligostilbene natural product found in the tree bark of *Dryobalanops oblongifolia* (*29*). However, compound **2** and *trans*-diptoindonesin B exhibited significant differences of the carbon chemical shifts (> 0.6 ppm) in methanol-d_4 of C-7a, C-8a, C-9a, C-10a, C-3b, C-1c, C-2c, C-6c, and C-8c. The chemical shifts of these carbons are affected by the particular configuration of H-7a/H-8a and H-7c/H-8c and indicate an alternative stereochemistry of the two 2,3-dihydrofurans compared to *trans*-diptoindonesin B. Thus, resviniferin A is a novel stereoisomer of *trans*-diptoindonesin B; the absolute stereochemistry of both compounds can be

clarified only by X-ray crystallographic analysis. Currently this is not possible as these compounds do not crystallize.

Resviniferin B (**3**) was obtained as a pale white amorphous powder and exhibited a high-resolution molecular ion of m/z 681.2099 $[M + H]^+$. **Table 2** comprises the ^{1}H and ^{13}C NMR data of **3**. The ^{1}H NMR spectrum in acetone-d_6 showed the presence of two sets of AA′XX′ type ortho-coupled aromatic hydrogens at δ 7.19 and 6.82 (each 2H, d, J = 8.5 and 8.6 Hz) for ring A_1, 7.20 and 6.83 (each 2H, d, J = 8.5 Hz) for ring C_1, two sets of AX_2 type meta-coupled aromatic hydrogens at δ 6.16 (2H, d, J = 2.2 Hz) and 6.26 (1H, d, J = 2.2 Hz) for ring A_2, 6.22 (2H, m) and 6.22 (1H, m) for ring C_2, one set of meta-coupled aromatic hydrogens at δ 6.32 and 6.70 (each 1H, d, J = 2.0 and 2.1 Hz) for ring B_2, one set of ABX type ortho−meta-coupled aromatic hydrogens at δ 6.99 (1H, br s), 6.76 (1H, d, J = 8.4 Hz), and 7.20 (1H, m) for ring B_1, two sets of aliphatic hydrogens at δ 5.41 (1H, d, J = 7.7 Hz, H-7a) and 4.39 (1H, d, J = 7.7 Hz, H-8a) and δ 5.42 (1H, d, J = 5.4 Hz, H-7c) and 4.46 (1H, d, J = 5.5 Hz, H-8c), and two coupled doublets at δ 6.93 and 6.72 (each 1H, d, J = 16.3 Hz, H-7b, H-8b) for a trans configured double bond. The ^{13}C NMR spectrum of **3** showed 4 aliphatic carbons at δ 93.9, 57.7, 93.9, and 57.1 (C-7a, C-8a, C-7c, C-8c), as well as 38 aromatic and olefinic carbons between δ 102.1 and 162.4. Furthermore, all protonated carbons were assigned from the HSQC spectrum.

In contrast to **2**, **3** contained ring systems A and C, which exhibited identical chemical structures with very similar chemical shifts. Therefore, an exact assignment of the hydrogens H-2a, H-6a (δ 7.19) and H-2c, H-6c (δ 7.20) as well as H-3a, H-5a

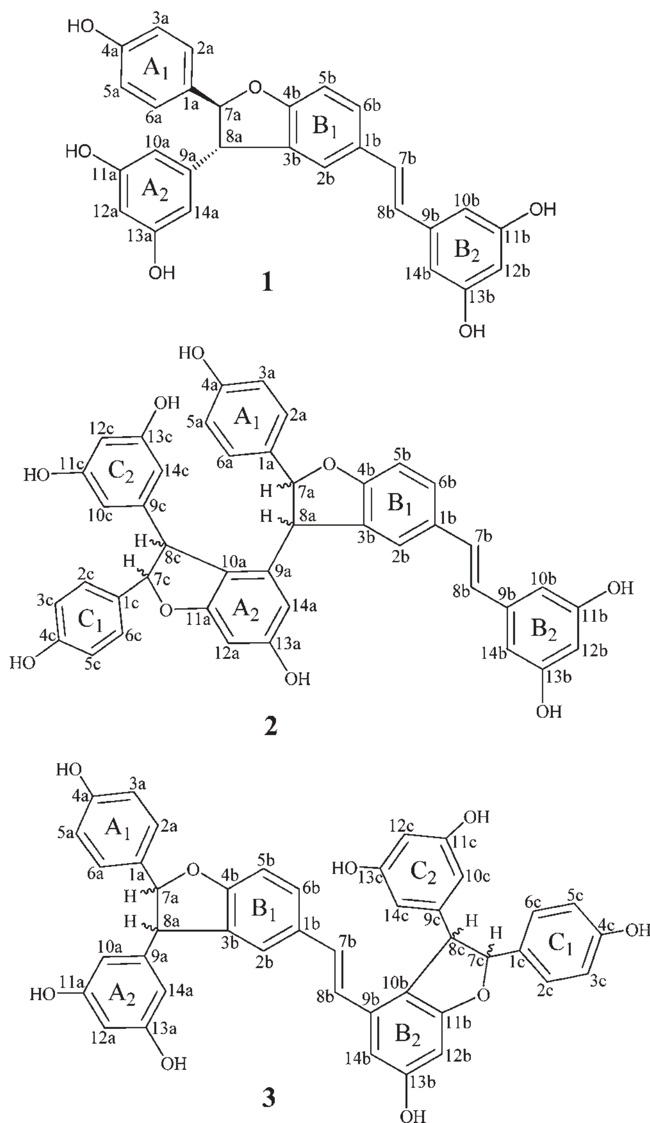


Figure 4. Structures of *trans*-δ-viniferin (**1**), resviniferin A (**2**), and resviniferin B (**3**).

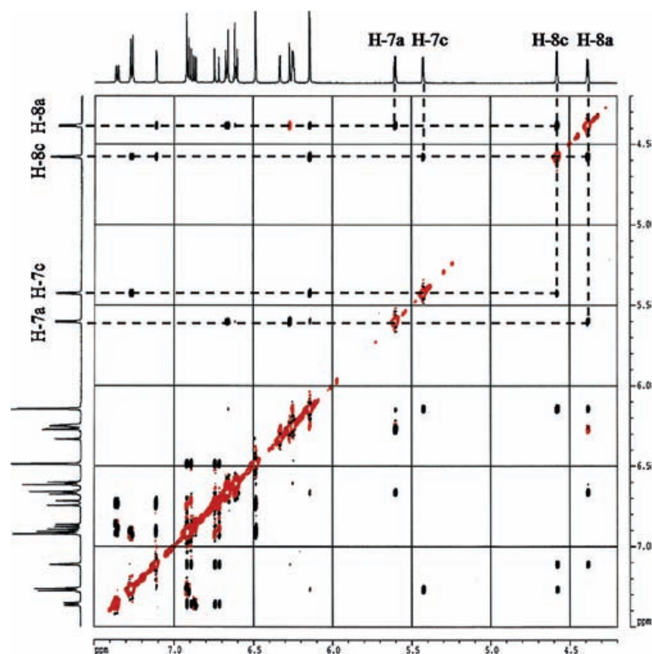
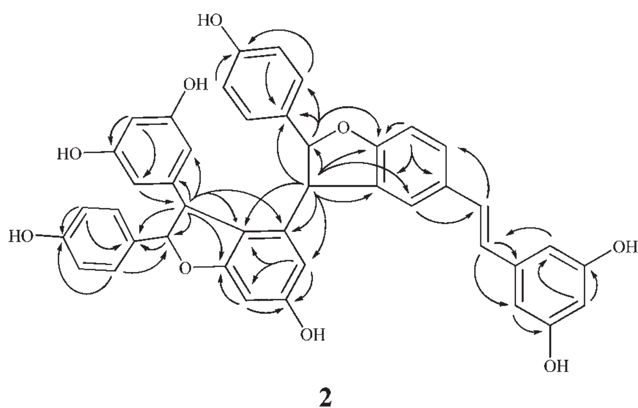


Figure 6. Phase-sensitive ROESY spectrum of **2**.



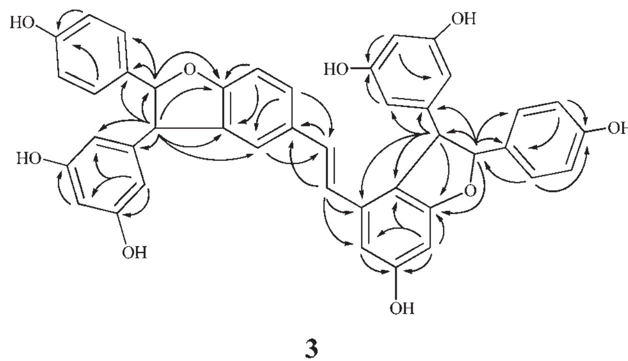


Figure 5. Selected HMBC correlations of **2** and **3**.

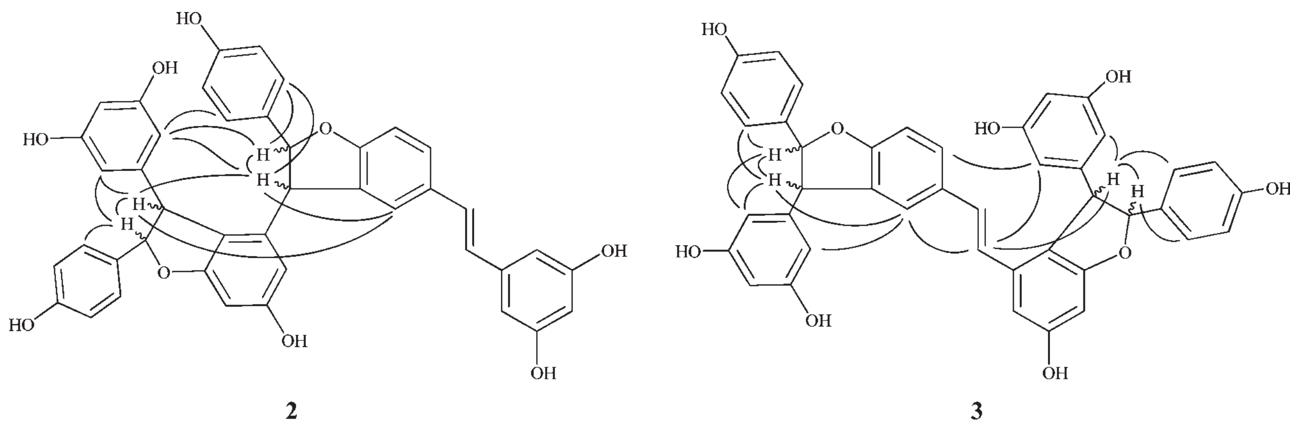


Figure 7. Selected NOE interactions of **2** and **3**.

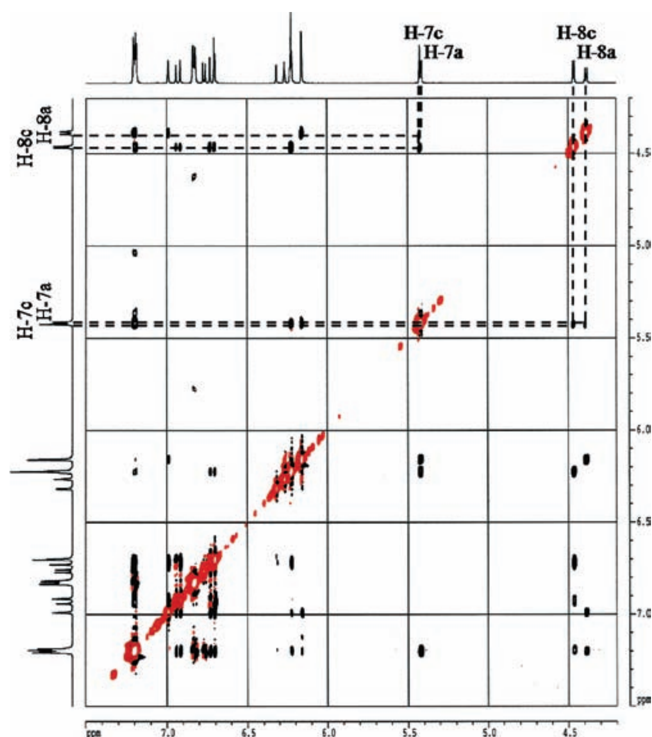


Figure 8. Phase-sensitive ROESY spectrum of **3**.

(δ 6.82) and H-3c, H-5c (δ 6.83) was not possible. In **Table 2** interchangeable shifts are marked with numerals.

In the HMBC spectrum significant correlations (**Figure 5**) were observed between H-2b and the carbon signals δ 130.3 (C-7b), H-8b, δ 131.8 (C-1b) and H-8b, and δ 104.4 (C-14b), indicating the presence of a stilbene skeleton (ring B_1-C-7b-C-8b-ring B_2). Further important CH long-range correlations were found between H-8a and seven carbon signals [δ 132.6 (C-1a), 93.9 (C-7a), 145.2 (C-9a), 107.3 (C-10a,14a), 125.0 (C-2b), 131.9 (C-3b), 160.6 (C-4b)]. Similar correlations were assigned to hydrogen H-8c [δ 133.8 (C-1c), 93.9 (C-7c), 147.3 (C-9c), 107.0 (C-10,14c), 136.4 (C-9b), 120.0 (C-10b), 162.4 (C-11b)]. The CH long-range correlations of H-8a and H-8c defined the connections between the three monomers composed of ring systems A, B, and C.

The relative configuration of **3** was established on the basis of NOE experiments. ROESY interactions between H-7a and H-8a and between H-7c and H-8c again indicated a trans orientation of both hydrogen pairs. **Figure 8** shows the ROESY spectrum of **3** with interactions of the four aliphatic hydrogens. In contrast to **2**, no interactions between H-8a and H-8c were detected. This is not surprising as the two furan rings are far apart in the linear structure. Further NOE interactions (**Figure 7**) between, for example, H-8a/H-2b, H-2b/H-8b, H-8b/H-8c, and H-8b/H-14c, confirmed the proposed structure of **3**. Again, the absolute stereochemistry of **3** can be clarified only by X-ray structural analysis.

Both trimers could be synthesized by horseradish peroxidase as products of three *trans*-resveratrol units or a dimerization of *trans*-resveratrol and (−)-ε-viniferin. In the latter case, the hypothetical biogenetic pathway for **2** should proceed via the intermediate [B] of (−)-ε-viniferin (**Figure 3**) or intermediate [A] for **3**.

Biotransformation of *trans*-resveratrol and *trans*-ε-viniferin using horseradish peroxidase permits access to a large variety of different stilbene oligomers. Application of HSCCC for selective fractionation of biotransformed stilbene oligomers gave further access to two related minor components. The isolation and structure determination of these two resveratrol trimers, exhibiting trans-configured aliphatic hydrogen pairs, are described here for the first time. As these two new compounds may occur naturally as minor constituents in grapevine extract, our reference data provide a means of detecting the presence of these stilbene oligomers in future investigations of such extracts.

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