The Accumulation of Stilbene Glycosides in *Vitis vinifera* Cell Suspension Cultures

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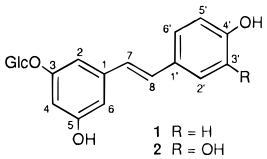
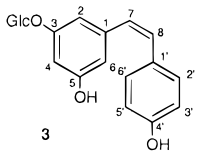
Suspension cultures of *Vitis vinifera* grown in an inducive polyphenol synthesis medium accumulated stilbene glycosides. (*E*)-piceatannol (3,5,3′,4′-tetrahydroxystilbene) 3-*O*-*β*-glucoside (**2**) and (*Z*)-resveratrol (3,5,4′-trihydroxystilbene) 3-*O*-*β*-glucoside (**3**) were isolated from these cells. Their complete structures were determined by 1D and 2D NMR techniques.

Using plant cell cultures to produce biologically active compounds has many advantages. With the help of this technique, we showed the presence of anthocyanins, proanthocyanidins, catechins, and stilbenes in *Vitis vinifera* suspension culture.[1,2] The beneficial role that wine phenolics may have in preventing cardiovascular disease is still under debate, and much work on gut absorption and pharmacokinetics remains to be done. We isolated and characterized the main polyphenols in the grape cells in order to conduct biological in vitro studies and to produce ^{13}C-labeled phenolic compounds found in red wine to solve this problem.

A great deal of interest has been focused on stilbenes. Grapes and red wine are probably the most important foodstuffs containing these substances.[3,4] Moreover, (*E*)-resveratrol may reduce human low-density lipoprotein oxidation[5] and platelet aggregation.[6] (*E*)- and (*Z*)-resveratrol and their glycosides have been characterized from wines.[3,7−9]

In this work, we report the isolation and unambiguous characterization, by NMR, of (*E*)-piceatannol 3-*O*-*β*-D-glucoside (**2**) and (*Z*)-resveratrol 3-*O*-*β*-D-glucoside (**3**) from *Vitis vinifera* cells. As far as we know, stilbene **2** has never been reported as a constituent of *Vitis vinifera* or of wines.





Three compounds, **1**−**3**, were separated from the EtOAc extract of a cell suspension of *Vitis vinifera* by a combination of chromatographic techniques. Compound **1** was identified[2] as (*E*)-piceid.[10,11] The structure and configuration for **2** were deduced by spectrometric methods. The molecular formula, $C_{20}H_{22}O_9$, was determined by a combination of mass, ^{1}H-, and ^{13}C-NMR spectra analysis. The IR spectrum showed a broad band (3376 cm^{-1}) assigned to ν_{O-H}, an intense band (1600 cm^{-1}) assigned to aromatic $\nu_{C=C}$, and a band at 1673 cm^{-1} assigned to olefinic $\nu_{C=C}$. Assignments to all proton and carbon resonances were deduced from analysis of ^{1}H−^{1}H COSY,[12] heteronuclear HMQC,[13] and HMBC[14] 2D chemical shift correlations. The most important ^{1}H−^{13}C long-range correlations (HMBC) are shown in Figure 1.

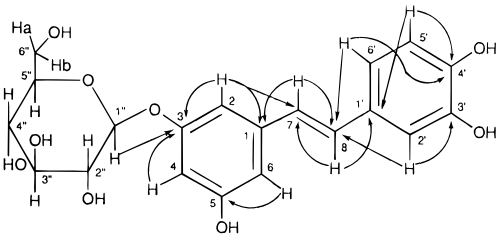


Figure 1. Important ^{1}H−^{13}C couplings observed in the HMBC NMR spectrum of **2**.

The ^{1}H-NMR spectrum of **2** showed two sets of signals. The former, between *δ* 3 and 5, was assigned to glycosyl protons, consistent with the ^{13}C-NMR spectrum, which showed six signals characteristic of a *β*-D-glucose unit. The latter set, between *δ* 6 and 7.5, consisted of three systems of olefinic and aromatic protons. At *δ* 7.02, 6.88, and 6.77, respectively, two doublets and one doublet-doublet (J = 2, J = 8.1 Hz) were assigned to an AMX system of a 1,3,4-trisubstituted aromatic ring; one broad singlet at *δ* 6.63, one doublet at *δ* 6.80 (J = 1.8 Hz), and one triplet at *δ* 6.78 were assigned to three *meta*-related protons of a 1,3,5-trisubstituted aromatic ring; and two doublets (*δ* 6.82 and 6.97) with a large coupling constant (J = 16.2 Hz) indicated a *trans* olefinic proton system. These signals were consistent with a hydroxylated *trans* stilbene system substituted by a glucoside. Moreover, one doublet at *δ* 4.92 (J = 7.1 Hz) was indicative of the *β* configuration of the glucosyl bond. Due to the precise position of the glucosyl unit, it cannot be bonded to 4′ because there was a long-range coupling (HMBC) between the anomeric proton and C-3 and because the three *meta* aromatic protons were all non-equivalent. Compound **2** was concluded to be (*E*)-3,5,3′,4′-tetrahydroxystilbene 3-*O*-*β*-D-glucoside (piceatannol 3-*O*-*β*-D-glucoside). This compound was discovered in the leaves of *Eucalyptus dundasii* and is also called astringin.[15]

The structure of **3** was elucidated by 1D and 2D NMR experiments. The ^{13}C-NMR data were unambiguously assigned on the basis of HMQC and HMBC spectra; our results are similar to those previously reported by Mattivi *et al.*[3] and indicate that compound **3** is (*Z*)-piceid or (*Z*)-resveratrol 3-*O*-*β*-D-glucoside.

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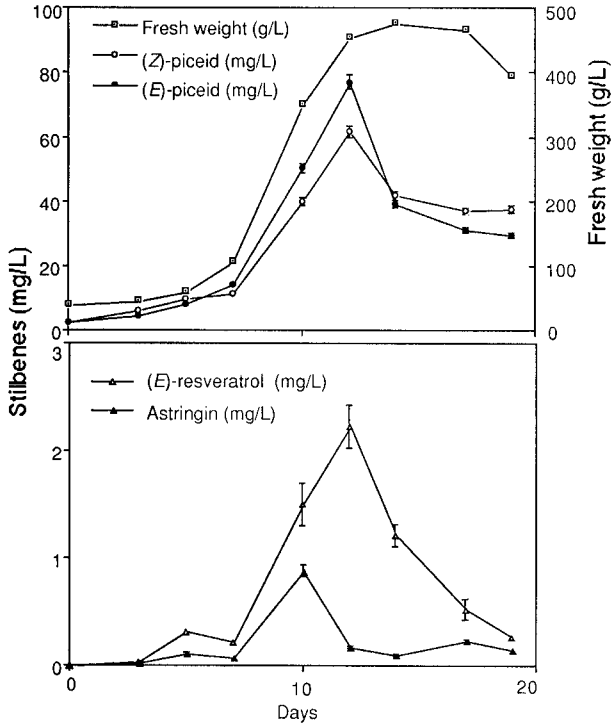

Figure 2. Time courses of cell growth and stilbene production in grape cell suspension culture.

To determine the optimal period for the production of stilbenes by grape cells in the induction medium, cultures were analyzed throughout a 19-day period (Figure 2). Maximal production of **1** (77 mg/L) and **3** (62 mg/L) occurred on day 12, and that of **2** (0.9 mg/L, based on **1** as standard) on day 10. (*E*)-Resveratrol, identified by co-chromatography with an authentic standard using HPLC analysis, peaked on day 12. Under these conditions, stilbene production and growth seem to be coupled, as also has been seen for anthocyanins and condensed tannins.[1]

Our data show that suspension cultures of *Vitis vinifera* accumulate high levels of these stilbenes, which can be isolated for biological study of the exact role of each phenolic compound. For example, the biological properties of the (*Z*)-isomers are little known,[9] and we can obtain (*Z*)-resveratrol by enzymatic hydrolysis of **3**. Moreover, **3** seems to occur naturally in grape cells (extraction and purification being performed in the dark). Stilbenes are certainly stored within the cells in the form of glycosides. Also, stilbene glycosides **1** and **3** are present in significant amounts in grape berries and red wines.[3, 9] On the other hand, astringin (**2**) has not been reported from any component of *Vitis vinifera*. Further investigations are being undertaken to characterize this substance in red wine, which has been shown recently with its aglycon in the bark of *Picea* species.[16,17] Piceatannol is a known inhibitor of protein-tyrosine kinases[18] and is active as an antileukemic agent.[16]

Culture of grape cells in vitro is done under sterile conditions. It is interesting to note that stilbene synthesis can be induced in grape cells without fungal infection. The presence of these molecules in wines, therefore, is not necessarily related to the use of grapes parasitized by *Botrytis cinerea*, the causal organism for the gray mold. Indeed, abiotic stresses such as UV light can induce the stilbene synthase activity in members of the Vitaceae.[19]

Experimental Section

General Experimental Procedures. UV spectra were measured in MeOH using a Hitachi U-2000 spectrophotometer. IR spectra were obtained on a KBr disk using a Shimadzu IR-470 spectrophotometer. NMR spectra were performed with a Bruker AMX-500 spectrometer. FABMS were recorded using glycerol as matrix, in positive-ion mode.

Cell Culture. Cell suspension cultures of *Vitis vinifera* (L.) cv Gamay Fréaux var. Teinturier (Vitaceae) were maintained as previously described.[1] The maintenance medium (MM) contained B5 macroelements,[20] microelements,[21] and vitamins[22] and was supplemented with 58 mM sucrose, 250 mg/L casein hydrolysate, 0.54 μM 1-naphthaleneacetic acid, and 0.93 μM kinetin. Experiments were performed by inoculating a 7-day-old cell suspension into an induction medium (IM_1) at a 1:8 (v/v) ratio, for one transfer.[1] IM_1 was the same as MM, but contained 2 mM $(NH_4)_2SO_4$, 2.2 mM NaH_2PO_4, 2 mM $MgSO_4$, and 175 mM sucrose. Cells were harvested at different times by filtration under partial vacuum (nylon cloth, 30 μm), rapidly washed with cold distilled H_2O, weighed, and then frozen until analysis.

Extraction, Isolation, and Identification of the Stilbene Compounds. Frozen cells (600 g), harvested at day 12, were homogenized with Me_2CO-H_2O (6:4) (2 × 960 mL). The extract was concentrated *in vacuo*, and the resulting aqueous extract (500 mL) was extracted with EtOAc (4 × 500 mL). The EtOAc extract (630 mg) was dissolved in $MeOH-H_2O$ (1:1) (1 mL) and chromatographed on a cation-exchange resin column (2.6 × 51 cm) eluted with H_2O followed by $MeOH-H_2O$ (1:1). Fractions were monitored by TLC as already described[23] and grouped as either A (H_2O, 1 L) or B ($MeOH-H_2O$ 1:1, 1.5 L). Fraction B (350 mg) was then chromatographed on a Sephadex LH-20 column (1.5 × 60 cm) and eluted using a step gradient of MeOH in H_2O and a flow rate of 1.5 mL min^{-1}: fraction 1 (H_2O, 150 mL); fractions 2−4, ($MeOH-H_2O$ 1:4, 1.2 L); fraction 5 ($MeOH-H_2O$ 3:7, 1 L); fraction 6 ($MeOH-H_2O$ 2:3, 400 mL); fractions 7−9 ($MeOH-H_2O$ 1:1, 1.2 L), and fraction 10 (MeOH, 600 mL). Compounds **1** [(*E*)-resveratrol 3-*O*-β-glucoside (45 mg)] and **2** [(*E*)-piceatannol 3-*O*-β-glucoside (8 mg)] in fraction 5 were obtained by preparative HPLC (t_R 49.65 and 38.24 min) on an Ultrasep RP18 (6 μm) reversed-phase C_{18} column (12.8 mm i.d. × 250 mm) with a column guard. The elution program, at 3 mL min^{-1}, was 100% A (0−30 min) and 100% B (30−110 min), each with $MeOH-H_2O-TFA$ (A = 35:165:0.005, B = 55:145:0.005). The chromatogram was monitored at 286 and 306 nm using a UV detector. Compound **3**, (*Z*)-resveratrol 3-*O*-β-glucoside (35 mg), in fraction 3 was purified by preparative HPLC (t_R 80.89 min); conditions were the same as above.

(E)-Astringin 2: UV λ_{max} (log ϵ) 218 (4.33), 306 (4.21), 326 (4.31) nm; IR, ν_{max} (cm^{-1}) 3376 (O−H), 1673, 1600 (C=C); ^1H NMR (CD_3OD, 500.13 MHz) δ 7.02 (1H, d, J = 2 Hz, H-2′), 6.97 (1H, d, J = 16.2 Hz, H-8), 6.88 (1H, dd, J = 2 Hz, J = 8.1 Hz, H-6′), 6.82 (1H, d, J = 16.2 Hz, H-7), 6.80 (1H, br s, H-2), 6.77 (1H, d, J = 8.1 Hz, H-5′), 6.63 (1H, br s, H-6), 6.48 (1H, br s, H-4), 4.92 (1H,

d, J = 7.1 Hz, Glc H-1″); ^{13}C NMR (CD_3OD, 125.77 MHz) δ 160.46 (C-3), 159.55 (C-5), 146.62 (C-3′), 146.49 (C-4′), 141.42 (C-1), 130.98 (C-1′), 130.30 (C-8), 126.67 (C-7), 120.32 (C-6′), 116.44 (C-5′), 108.35 (C-6), 107.08 (C-2), 104.12 (C-4), 102,43 (C-1″), 78.23 (C-5″), 78.06 (C-3″), 74.96 (C-2″), 71.49 (C-4″), 62.60 (C-6″); FABMS, m/z $[MH]^+$ 407.

(*Z*)-Piceid 3: UV λ_{max} (log ϵ) 216 (4.14), 291 (3.91) nm; IR ν_{max} (cm^{-1}) 3350 (O−H), 2850 (C−H), 1600 (C═C); ^{1}H NMR (CD_3OD, 500.13 MHz) δ 7.08 (2H, d, J = 8.5 Hz, H-2′, H-6′), 6.65 (2H, d, J = 8.7 Hz, H-3′, H-5′), 6.39 (1H, br s, H-6), 6.46 (1H, d, J = 12.1 Hz, H-8), 6.51 (1H, br s, H-2), 6.39 (1H, br s, H-4), 6.35 (1H, d, J = 12.2 Hz, H-7), 4.69 (1H, d, J = 7.2 Hz, Glc H-1″); ^{13}C NMR (CD_3OD, 125.77 MHz) δ 160.07 (C-3), 159.30 (C-5), 157.75 (C-4′), 141.07 (C-1), 131.39 (C-2′, C-6′), 131.35 (C-8), 129.91 (C-1′), 129.11 (C-7), 116.03 (C-3′, C-5′), 111.12 (C-6), 109.20 (C-2), 103.93 (C-4), 102.31 (C-1″), 77.95 (C-3″), 77.80 (C-5″), 74.81 (C-2″), 71.07 (C-4″), 62.24 (C-6″); FABMS, m/z $[MH]^+$ 391.

Quantification of Stilbenes. Freeze-dried cells (50 mg) were extracted with MeOH (5 mL) overnight at +4 °C. The resulting MeOH solution was evaporated to dryness *in vacuo*. The extract was chromatographed on a cation-exchange resin column (6 mm × 40 mm) and eluted with 75% (v/v) aqueous MeOH to obtain stilbenes. Analysis of stilbenes (**1**−**3**) was performed by HPLC on an Ultrasep RP18 (4 μm) reversed-phase C18 column (4 mm i.d. × 250 mm). Solvents used for the separation were: C, H_2O adjusted to pH 2.4 with acetic acid; D, 20%C with 80% MeCN.[24] The elution program at 1 mL/min was as follows: 0 min, 18% D in C; 11.5 min, 18% D in C; 18.5 min, 23% D in C; 22.5 min, 24.5% D in C; and 28.5 min, 31% D in C. The eluate was monitored at 286 nm for **3** (t_R 25 min) and at 306 nm for **1** and **2** (t_R 16 and 9 min, respectively). Stilbene contents were estimated from a calibration curve that was prepared with standards of **1** and **3** (purified from cultured grape cells), respectively. All extractions and measurements were done in triplicate.

For both standards the response was linear from 10 mg/L (*Z*-piceid) or 3 mg/L (*E*-piceid) to 500 mg/L, by injection of 100-μL samples (correlation coefficient r = 0.999). With the method described above, we could not quantify levels lower than 5 (for **3**) and 2 μg/g dry wt (for **1** and **2**). (*E*)-Resveratrol was estimated from a calibration curve of authentic standard (Sigma) with the same HPLC method (t_R 29 min).

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