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TRANS-RESVERATROL-3-*O*-β-GLUCOSIDE (PICEID) IN CELL SUSPENSION CULTURES OF *VITIS VINIFERA*

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Key Word Index—*Vitis vinifera*; Vitaceae; grape; stilbenes; piceid; resveratrol glucoside; catechins; cell suspension culture.

Abstract—Suspension cultures of *Vitis vinifera* were found to produce catechins and stilbenes. When the cells were grown in a polyphenol-inducing medium, a stilbene glucoside (piceid) was isolated. By using spectroscopic methods, the structure was determined as *trans*-resveratrol-3-*O*-β-glucoside.

INTRODUCTION

Recently, we reported on the presence of anthocyanins, proanthocyanidins and catechins in a *Vitis vinifera* cell suspension culture [1]. Characterization of the main polyphenols in these grape cells is being undertaken in order to produce isotopically ^{13}C-labelled phenolic substances of the type found in red wine for studies on the absorption, *in vivo* metabolism, and pharmacokinetics of wine phenolics. The antioxidant properties of these compounds [2–5] may provide an explanation for the 'French paradox', i.e. the epidemiological evidence showing that in France, the consumption of red wine may counteract the effects of saturated fats and reduce the risk of coronary heart disease [6].

In the present paper, the identification of catechins and piceid, a stilbene glucoside, in *V. vinifera* cell suspension cultures is reported.

RESULTS AND DISCUSSION

The cell suspension culture used in this study synthesizes many polyphenolic compounds which were extracted by aqueous acetone. Rapid chromatographic (TLC) estimation showed that the catechins were predominantly in the Et_2O extract of the initial aqueous residue, although the EtOAc extract contained some procyanidin oligomers and an unexpected compound (**1a**) which was predominant. This compound produced a violet colouration upon spraying with anisaldehyde reagent on TLC plates.

Catechin and epicatechin were purified from the Et_2O extract by semi-preparative C18 reverse phase HPLC. The spectroscopic and chromatographic data of these native compounds were found to be identical with those of authentic samples [7, 8].

From the EtOAc extract, three chromatographic steps (cation-exchange resin, LH20 and C18 HPLC) yielded **1a** as a pure compound. The structure and configuration for **1a** were deduced by spectrometric methods. The molecular formula, $C_{20}H_{22}O_8$, was determined by a combination of mass, and ^{1}H and ^{13}C NMR spectral analyses.

The IR spectrum (KBr) showed a broad band (3335 cm^{-1}) assigned to ν_{O-H} and an intense band (1597 cm^{-1}) assigned to aromatic $\nu_{C=C}$. The assignments to all proton and carbon resonances were deduced from analysis of the ^{1}H-^{1}H COSY [9] and heteronuclear HMQC [10] and HMBC [11] 2D chemical shift correlations. The ^{13}C NMR data for compound **1a** are reported in Table 1, and compared with the literature [12].

The ^{1}H NMR spectrum shows the presence of two sets of signals. The former, between δ 3 and 5, is assigned to glycosyl protons. This is consistent with the ^{13}C NMR spectrum which shows six signals characteristic of a β glucose unit. The latter set, between δ 6.3 and 7.5, is constituted by three systems of olefinic and aromatic protons. At δ 7.35 and 6.76, two doublets (J = 8.5 Hz) are assigned to an AA′XX′ system of a 1-4 disubstituted aromatic ring; three broad singlets at δ 6.44, 6.61 and 6.78 are assigned to three *meta* related protons of a 1, 3, 5 trisubstituted aromatic ring and two doublets (δ 6.84 and 7.00) with a large coupling constant (J = 16.3 Hz) show a *trans* olefinic proton system. These signals are consistent with a *trans* stilbene system substituted by a glucoside. Moreover, the coupling constant (J = 7.1 Hz) and chemical shift (δ 4.88) are indicative of the β configuration of the glucosyl bond.

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Table 1. ^{13}C NMR data of compound **1a**

C	δ	δ [12]*
1	141.4	140.9
2	107.0	106.2
3	160.5	160.2
4	104.1	103.9
5	159.6	159.4
6	108.4	108.2
7	126.7	126.5
8	130.0	129.9
1′	130.3	130.0
2′, 6′	128.9	128.9
3′, 5′	116.5	116.4
4′	158.5	158.2
Glucose		
1″	102.4	102.1
2″	75.0	74.8
3″	78.1	78.1
4″	71.5	71.5
5″	78.3	77.8
6″	62.6	62.8

*Assignments reported by Mattivi *et al.*

Interpretation of the NMR (^1H and ^{13}C) spectra of the peracetylated derivative of **1a** indicates that it is a hexaacetate whose spectra have all of the skeletal characteristics of the native product.

Therefore, a *trans* resveratrol glucoside structure is proposed for **1a**. Due to the precise position of the glucosyl unit, it cannot be bonded to 4′ because there is long range coupling (HMBC) between the anomeric proton and C-3 and because the three *meta* aromatic protons are all non-equivalent. Therefore, only one position is in good agreement with the above data and **1a** is, therefore, resveratrol-3-*O*-β-glucoside. Our spectroscopic data are consistent with those previously given in the literature for piceid (**1a**) [12, 13]. This compound has also been discovered in leaves of *Eucalyptus* [14] and in roots of *Polygonum cuspidatum* which are used in Asia as a treatment for atherosclerosis [15]. It has also been described as a stress compound produced by *Veratrum grandifolium* leaves when treated with cupric chloride [16].

In this study, **1a** production by grape cells was examined in IM1 medium which induces anthocyanin and condensed tannin accumulation [1], within a culture period of 17 days (results not shown). Optimal accumulation of **1a** (quantified by HPLC) occurs between day 10 and day 12, i.e. at the end of the growth phase. The piceid content is similar to the highest level found in berry skins of *Pinot noir* [17], i.e. about 200 $\mu g\ g^{-1}$ dry wt.

This study demonstrates for the first time that piceid, catechin and epicatechin are present in significant amounts in grape cell suspension cultures.

Recent reports [12, 17–21] describe the presence of resveratrol and/or piceid isomers in grape berries and red wines. In some wines, piceid is the predominant resveratrol derivative [19]. There is continuing interest in resveratrol glycosides, since the aglycone has been found to inhibit the copper-catalysed oxidation of human low density lipoproteins [4] and the aggregation of platelets [20]. The former can also be hydrolysed by glycosidases in the human gastro-intestinal tract [20].

EXPERIMENTAL

Cell culture. Cell suspension cultures of *V. vinifera* (L.) cv Gamay Fréaux var. Teinturier were established and maintained as described previously [1]. The maintenance medium (MM) contained B5 macroelements [22], microelements [23] and vitamins [24] supplemented with 58 mM sucrose, 250 mg l^{-1} casein hydrolysate, 0.54 μM 1-naphthaleneacetic acid and 0.93 μM kinetin. Experiments were performed by subculturing the cells for one transfer at a dilution rate 1.25/10 (v/v) in induction medium (IM1) [1]. This was similar to 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 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 piceid (**1a**). Frozen cells (340 g) were harvested at day 12 and homogenized with Me_2CO–H_2O (3:2) (2 × 500 ml). The extract was concd *in vacuo* and the resulting aq. extract (150 ml) was extracted with Et_2O (5 × 100 ml) and EtOAc (4 × 150 ml), successively. The EtOAc extract (1 g) was dissolved in MeOH–H_2O (1:1) (1 ml) and chromatographed on a cation-exchange resin column (1.5 × 60 cm) eluted with H_2O (600 ml) then MeOH–H_2O (1:1) (900 ml) at 1 ml min^{-1}; the second fr. was purified on a Sephadex LH-20 column (1.5 × 60 cm) and eluted using a step gradient of MeOH in H_2O at a flow rate of 0.8 ml min^{-1}. The fr. eluted with MeOH–H_2O (7:13) contained a major compound which, on TLC (silica gel), developed in toluene–Me_2CO–HCO_2H (3:6:1), gave a blue fluorescent spot under UV light (366 nm) (R_f 0.41) and a violet colouration after spraying with anisaldehyde reagent. This fr. (50 mg) was purified by HPLC on an Ultrasep RP18 (6 μm) reversed-phase C_{18} column (8 mm i.d. × 250 mm) with column guard. The elution prog. at 3 ml min^{-1} was 100%A (0–30 min) and 100%B (30–110 min), each MeOH–H_2O–TFA (A = 35:165:0.005, B = 55:145:0.005). The chromatogram was monitored at 280 nm using a UV detector. The major peak (R_t 60.65 min) yielded pure piceid (compound **1a**, 15 mg).

Compound **1a**. $[\alpha]_D^{25} = -60°$ (MeOH; *c* 0.5); IR ν_{max}^{KBr} cm^{-1}: 3335 (O-H), 2920 and 2895 (C-H), 1598 (C=C); UV λ_{max}^{MeOH} nm (log ε): 305 (4.44); FAB-MS (positive ion mode, glycerol matrix), *m/z*: 391 $[M + H]^+$; ^1H NMR [500.13 MHz, CD_3OD]: δ 7.35 (2H, *d*, $J_{2',3'} = 8.5$ Hz, $J_{5',6'} = 8.5$ Hz, H-2′, H-6′), 7.00 (1H, *d*, $J_{7,8} = 16.3$ Hz, H-8), 6.84 (1H, *d*, H-7), 6.78 (1H, *br s*, H-2), 6.76 (2H, *d*, H-3′, H-5′), 6.61 (1H, *br s*, H-6), 6.44 (1H, *br s*, H-4), 4.88 (1H, *d*, $J_{1'',2''} = 7.1$ Hz, Glc

H-1″), 3.92 (1H, *dd*, $J_{5'',6a''}$ = 1.5 Hz, $J_{6a'',6b''}$ = 12 Hz, Glc H-6a″), 3.70 (1H, *dd*, $J_{5'',6b''}$ = 5.8 Hz, Glc H-6b″), 3.48–3.38 (4H, *m*, Glc H-2″, H-3″, H-4″, H-5″); ^{13}C NMR (125.77 MHz, CD_3OD): Table 1.

Acetylation of compound **1a**. Compound **1a** (8 mg) was acetylated using the method described in ref. [8]. The resulting peracetates were separated by prep. TLC which was performed on silica gel using $CHCl_3$–EtOAc–C_6H_{12} (4:1:1). The spot at (R_f 0.43) was eluted with $CHCl_3$–CH_3OH (7:3) to yield pure piceid peracetate (15 mg). $[\alpha]_D^{22} = -11°2$ ($CHCl_3$; *c* 0.64); IR(film) $\nu_{max}^{CHCl_3}$ cm^{-1}: 2929 (C-H), 1753 (C=O), 1596 (C=C), 1218 (C-O); UV $\lambda_{max}^{CHCl_3}$ nm (log ε): 301 (4.25); FAB-MS (positive ion mode, glycerol matrix), *m/z* (rel. int.): 665 $[M + Na]^+$ (3), 643 $[M + H]^+$ (7, 2), 642 $[M]^+$ (14), 331 (100); ^{1}H NMR [500.13 MHz, $CDCl_3$]: δ 7.49 (2H, *d*, $J_{2',3'}$ = 8.4 Hz, $J_{5',6'}$ = 8.4 Hz, H-2′, H-6′), 7.10 (2H, *d*, H-3′, H-5′), 7.05 (1H, *d*, $J_{7,8}$ = 16.3 Hz, H-8), 6.98 (1H, *br s*, H-2), 6.97 (1H, *br s*, H-6), 6.95 (1H, *d*, H-7), 6.66 (1H, *br s*, H-4), 5.30 (1H, *m*, Glc H-3″), 5.28 (1H, *m*, Glc H-2″), 5.17 (1H, *t*, $J_{4'',5''} = J_{3'',4''}$ = 8.5 Hz, Glc H-4″), 5.14 (1H, *d*, $J_{1'',2''}$ = 7.00 Hz, Glc H-1″), 4.28 (1H, *dd*, $J_{5'',6a''}$ = 1.5 Hz, $J_{6a'',6b''}$ = 12 Hz, Glc H-6a″), 4.18 (1H, *dd*, $J_{5'',6b''}$ = 5.6 Hz, Glc H-6b″), 3.92 (1H, *ddd*, Glc H-5″); ^{13}C NMR (125.77 MHz, $CDCl_3$): δ 170.4, 170.1, 169.3, 169.2, 169.2 and 169.0 (C=O), 157.5 (C-3), 151.6(C-5), 150.4 (C-4′), 139.7 (C-1), 134.4 (C-1′), 129.5 (C-8), 127.5 (C-2′, C-6′), 127.4 (C-7), 121.8 (C-3′, C-5′), 114.4 (C-6), 112.6 (C-2), 109.6 (C-4), 98.9 (C-1″), 72.7(C-3″), 72.1 (C-5″), 71.1 (C-2″), 68.3 (C-4″), 62.0 (C-6″), 21.0 (2 Me), 20.6 (1 Me), 20.5 (3 Me).

Isolation of catechins. The Et_2O extract (250 mg) of the initial water residue was dissolved in MeOH–H_2O (1:1) (1 ml) and subjected to CC over a cation-exchange resin (1.5 × 60 cm), eluted with H_2O (210 ml), then MeOH–H_2O (1:1) (900 ml). This fr. was purified by semi-prep. HPLC on the same column and with identical conditions as for the EtOAc extract. Three compounds **1b** (R_t 18.80 min), **2b** (R_t 38.73 min) and **3b** (R_t 60.65 min) were isolated. Compounds **1b** (2 mg) and **2b** (3 mg) were catechin and epicatechin respectively, **3b** and **1a** were identical to each other.

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