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Galloylated catechins and stilbene diglucosides in *Vitis vinifera* cell suspension cultures

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Abstract

Suspension cultures of *Vitis vinifera* were found to produce catechins and stilbenes. When cells were grown in a medium inducing polyphenol synthesis, (−)-epicatechin-3-*O*-gallate, dimeric procyanidin B-2 3′-*O*-gallate and two resveratrol diglucosides were isolated, together with a new natural compound that was identified as *cis*-resveratrol-3,4′-*O*-*β*-diglucoside by spectroscopical methods.

Keywords: *Vitis vinifera*; Vitaceae; Stilbenes; Catechins; Cell suspension culture; (−)-Epicatechin-3-*O*-gallate; Dimer procyanidin B-2 3′-*O*-gallate; Resveratrol-3,4′-*O*-β-diglucoside

1. Introduction

Grape wines contain many phenolic compounds which appear to be involved in the health benefits associated with moderate wine consumption, particularly a lower incidence of cardiovascular diseases and cancers (Renaud et al., 1999). Indeed, these compounds show a radical scavenging effect, antioxidative properties on human low-density lipoproteins (Fauconneau et al., 1997) and inhibition of human platelet aggregation (Varache-Lembège et al., 2000), which may contribute to preventing cardiovascular disorders. Among these phenolic substances, hydroxylated stilbenes have attracted much interest because relatively high quantities are found in grapes and wine, which are considered to be the most important dietary sources of these substances (Goldberg, 1995). Morever, *trans*-resveratrol (3,5,4′-trihydroxystilbene), a grapevine phytoalexin, has been reported to have cancer chemopreventive activity (Jang et al., 1997).

Using *Vitis vinifera* suspension cultures, we showed that these cells synthesize the major polyphenols found in red wine, i.e. anthocyanins, proanthocyanidins, catechins, and stilbenes (Decendit and Mérillon, 1996; Waffo-Teguo et al., 1996a,b). From these cells, we extracted four monomeric stilbene glucosides, *cis*- and *trans*-astringin and *cis*- and *trans*-resveratroloside (Waffo-Teguo et al., 1998), and have recently isolated two new dimeric stilbene glucosides, resveratrol *trans*-dehydrodimer 11-*O*-*β*-D-glucopyranoside and resveratrol *trans*-dehydrodimer 11′-*O*-*β*-D-glucopyranoside (Waffo-Teguo et al., 2001).

In the present paper, the identification of two galloylated catechins and two stilbene diglucosides in *V. vinifera* cell suspension cultures is reported.

2. Results and discussion

The cell suspension culture used in this study synthesizes many polyphenolic compounds, which were extracted by aqueous acetone. Purification of the

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EtOAc extract by four chromatographic steps (Dowex cation-exchange resin, Sephadex LH-20, Fractogel HW40 and reversed phase HPLC) yielded **1a** and **1b** as pure compounds. From the aqueous phase, three chromatographic steps (cation-exchange resin, LH-20, and RP-HPLC) yielded **2a** and **2b** as pure compounds. The structure and configuration of these compounds were deduced by spectrometric methods.

Compounds **1a** and **1b** were identified as (−)-epicatechin-3-*O*-gallate (Davies et al., 1996) and epicatechin-[4β-8]-epicatechin-3-*O*-gallate (Nonaka et al., 1983), respectively. Both these galloylated derivatives of flavan-3-ols have been previously isolated from grape seeds (Ricardo Da Silva et al., 1991a; Sun et al., 1999) and from *Fagopyrum esculentum* cell cultures (Tanaka et al., 1996). Although expected, these are newly reported for *V. vinifera* cell cultures. Compounds **1a** and **1b** were found in low concentrations (<10 mg l^{-1}) in red wines (Ricardo Da Silva et al., 1992; De Freitas, 1995). The rate of galloylation indeed is low in grape skins ($<6\%$), but it reaches 30% in grape seeds (Souquet et al., 1996). Compound **1b** is an oxygen free radical scavenger and is the most effective among the different procyanidins from grape seeds (Ricardo Da Silva et al., 1991b). Compound **1a**, also found in green tea, has been found to be a cancer chemopreventive agent (Suganuma et al., 1999).

Compound **2a** was obtained as a white, amorphous powder. Its molecular formula, $C_{20}H_{32}O_{13}$ was established by the high resolution FAB$^+$ mass spectrum m/z 575.1762 $[M+Na]^+$ (calc. for $C_{20}H_{32}O_{13}Na$, 575.1741). The data of 1D and 2D NMR spectra are consistent with a resveratrol diglucoside structure. The ^{1}H NMR spectrum of **2a** showed two sets of signals. The former, between δ 4.9 and 3.1, is assigned to glycosyl protons. This is in agreement with the ^{13}C NMR spectrum which shows characteristic signals of two glucose units each linked with an aromatic cycle. Two doublets at δ 4.90 and 4.61 corresponding to protons linked to carbon atoms δ 102.4 and δ 102.5 with $J=7.5$ and 7.2 Hz, respectively, indicated two anomeric protons of two glycosidic parts. The latter set, between δ 7.2 and 6.3, is composed of three systems of two olefinic and seven aromatic protons. At δ 7.17 and 6.95, two doublets ($J=8.5$ Hz) are assigned to an AA'XX' system of a 1–4 disubstituted aromatic ring. Three broad singlets at 6.47, 6.38 and 6.36 are assigned to three *meta* related protons of a 1,3,5-trisubstituted aromatic ring, and two doublets ($J=12$ Hz) at δ 6.51 and 6.43 show a *cis* olefinic system. All these signals are consistent with a *cis* resveratrol unit linked with two glycosidic units. The NOE correlations between each anomeric proton and the two aromatic protons, δ 4.61 (H-1″) with δ 6.46 (H-2) and δ 6.38 (H-4) then δ 4.90 (H-1‴) with δ 6.97 (H-3′) and (H-5′), indicated the 3 and 4′ position of a glycosidic linkage. Moreover, 2D HMBC experiments showed correlations between δ 4.61 (H-1″) and δ 160.1 (C-3) and between δ 4.90 (H-1‴) and δ 158.2 (C-4′), thus confirming the positions of the glycosidic units. Assignment of H-7 and H-8 positions on the olefinic bond was performed by HMBC correlations with carbons C-1, C-2, C-6 and C-1′, C-2′, C-6′ respectively. The UV spectrum with γ_{max} 284 nm was consistent with a *cis* stilbenoid structure. These data indicate that this new compound is *cis*-resveratrol-3,4′-*O*-β-diglucoside, which has never previously been isolated or synthetized (Fig. 1).

Compound **2b** was identified as mulberroside E, the *trans*-resveratrol-3,4′ diglucoside, isolated from cells of *Morus alba* (Hano et al., 1997) and from roots of *Veratrum taliense* (Zhou et al., 2001).

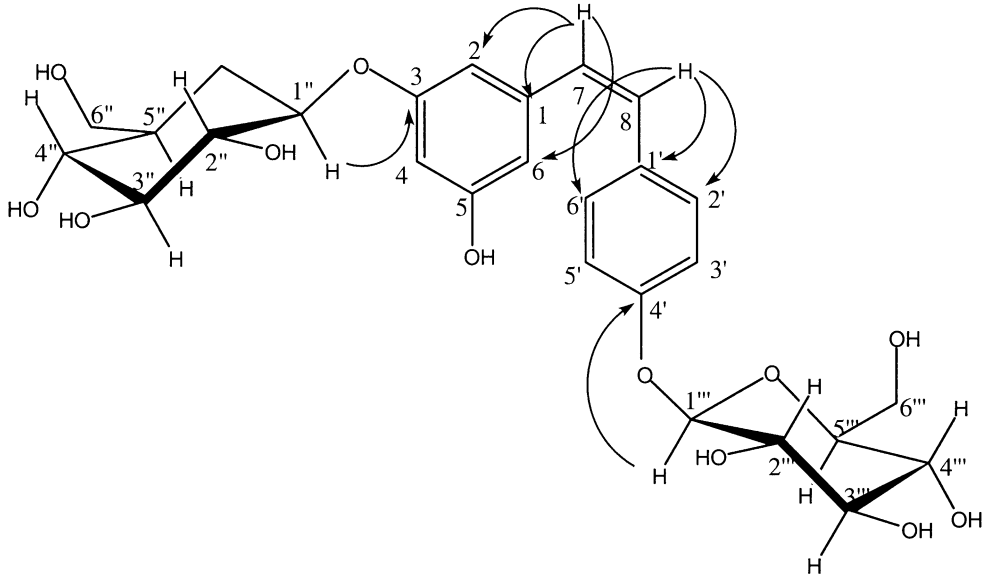

Fig. 1. Resveratrol diglucoside (**2a**) isolated from *Vitis vinifera* cell cultures. Arrows indicate HMBC correlations.

These diglucosides of stilbenes are uncommon compounds. The known *trans*-isomer mulberroside E is a dose-dependent inhibitor of xanthine oxidase just as piceid and resveratrol (Zhou et al., 2001), so the activity of the *cis* isomer can now be investigated. Like piceids and resveratrols, it will be necessary to search for them in wine and grape juice. More recently, other stilbene diglucosides, α-viniferin and pallidol diglucosides, have been obtained from white wine in small quantities of about 1–3 mg per 100 l (Baderschneider and Winterhalter, 2000). It now appears that many stilbene derivatives occur in grapevine and wine. Some of them have recently been isolated from wine, such as *trans*-astringin, where they are present in significant amounts, e.g. a mean concentration of 10 mg l^{-1} in red wines (Ribeiro de Lima et al., 1999).

This study demonstrates that grape cell suspension cultures are able to accumulate significant amounts of galloylated flavan-3-ol and complex stilbene derivatives which will now be used in biological activity tests.

3. Experimental

3.1. General procedures

Mass spectra were recorded with VG Autospec-Q in the FAB^+ mode. NMR spectra were recorded at 303 K on a 500 MHz Bruker spectrometer using CD_3OD as a solvent. UV spectra were recorded with a Hitachi U 2000 spectrophotometer. HPLC purifications were carried out on a Gilson gradient system equipped with an UV-vis detector Kontron model 430. IR spectra were recorded with a Bomen MB 100 spectrophotometer. Optical rotations were conducted on a Perkin-Elmer polarimeter.

3.2. Cell culture

Cell suspension cultures of *V. vinifera* (L.) cv Gamay Fréaux var. Teinturier (Vitaceae) were maintained as described previously (Decendit and Mérillon, 1996) in a culture medium containing B5 macroelements (Gamborg et al., 1968), microelements (Murashige and Skoog, 1962), vitamins (Morel, 1970) and supplemented with 58 mM sucrose, 250 mg l^{-1} casein hydrolysate, 0.54 μM 1-naphthaleneacetic acid and 0.93 μM kinetin.

For experimental purposes, we inoculated a 7-day-old cell suspension into an induction medium (IM1) at a 1:8 (v/v) ratio in 1-l flasks or a 10-l bioreactor. This was similar to the maintenance medium, but contained 2 mM $(NH_4)_2SO_4$, 2.2 mM NaH_2PO_4, 2 mM $MgSO_4$ and 175 mM sucrose (Decendit and Mérillon, 1996). Methyl jasmonate (25 μM) was added at day 6 or 7 to induce polyphenol accumulation (Krisa et al., 1999). Cells were harvested at day 12 or 14 by vacuum filtration and were rapidly washed with cold distilled water, weighed and stored at −20 °C until analysis.

3.3. Extraction and isolation

Frozen cells (900 g, fr wt) were homogenized with Me_2CO–H_2O (6:4). The extract was concentrated in vacuo and the resulting aq. extract was extracted with EtOAc. The EtOAc extract was dissolved in MeOH–H_2O (1:1) and chromatographed on a cation-exchange resin (Dowex 50WX4, 200–400 mesh, H form, Supelco) column (25 mm i.d.×600 mm) eluted with H_2O, then MeOH–H_2O (75:25). The second fraction was purified on a Sephadex LH-20 column (25 mm i.d.×500 mm) and eluted using a step gradient of MeOH in H_2O. The fraction eluted with 100% MeOH was further purified on Fractogel TSK HW-40 (F) (Merck) eluted with 100% MeOH, resulting in two main fractions. These fractions were purified by semipreparative HPLC on an Ultrasep RP18 (6 μm) (Bischoff) reversed phase C_{18} column (12.8 mm i.d.×250 mm) with guard column, with binary gradient eluant (solvent A, H_2O; B, acetonitrile). The elution prog. at 3 ml min^{-1} was 10% B (0–10 min), 10–30% B (10–42 min), 30–100% B (42–50 min), 100% B (50–55 min), 100–10% B (55–60 min) and 10% B (60–65 min). The chromatogram was monitored at 280 nm. Two major peaks yielded pure (−)-epicatechin-3-*O*-gallate (compound **1a**, R_t 21.9 min, 29 mg) and procyanidin B-2 3′-*O*-gallate (compound **1b**, R_t 16.7 min, 17.5 mg).

After extraction with EtOAc, the aq. phase was chromatographed on a cation-exchange resin column rinsed with H_2O, then eluted with MeOH–H_2O (75:25). The latter was purified on a Sephadex LH-20 column and eluted with 100% EtOH, resulting in two main fractions. The first fraction was finally purified by semipreparative HPLC using the same C1 8 column. The elution prog. at 2 ml min^{-1} was 10–23% B (0–20 min), 23–100% B (20–21 min), 100% B (21–31 min), 100–10% B (31–32 min) and 10% B (32–42 min) with A = H_2O and B = MeCN. The chromatogram was monitored at 286 and 306 nm. Two peaks yielded pure resveratrol diglucosides, *trans*-3,5,4′-trihydroxystilbene-3,4′-di-*O*-β-glucopyranoside (compound **2b**, R_t 8.85 min, 4.9 mg) and *cis*-3,5,4′-trihydroxystilbene-3,4′-di-*O*-β-glucopyranoside (compound **2a**, R_t 17.67 min, 11.5 mg). Extracts were constantly protected from light to avoid *trans–cis* isomerization.

3.4. cis-Resveratrol-3,4′-O-β-diglucoside (*2a*)

A white amorphous powder, $[\alpha]_D^{25} = -42$ (*c* 1, MeOH). IR ν_{max}^{KBr} cm^{-1}: 3335 (O–H), 2920 and 2895 (C–H), 1598 (C=C). UV λ_{max}^{MeOH} nm (log ε): 282 (4.14). FABHRMS (positive ion mode, glycerol matrix), *m*/*z*: 575.176210 $[M+Na]^+$, calc. for $C_{20}H_{32}O_{13}Na$, *m*/*z*: 575.174061. MS FAB^+ *m*/*z* (rel. int.): 575 $[M+Na]^+$

(23), 553 $[M+H]^+$ (17), 391 $[M-161]^+$ (100). ^1H NMR (500.13 MHz, CD_3OD): δ 7.17 (2H, *d*, $J=8.5$ Hz, H-2′, H-6′), 6.96 (2H, *d*, H-3′, H-5′), 6.50 (1H, *d*, $J_{7,8}=11.9$ Hz, H-8), 6.46 (1H, *br s*, H-2), 6.45 (1H, *d*, H-7), 6.39 (1H, *br s*, H-4), 6.37 (1H, *br s*, H-6), 4.90 (1H, *d*, $J_{1''',2'''}=7.5$ Hz, H-1‴), 4.61 (1H *d*, $J_{1'',2''}=7.2$ Hz, H-1″), 3.88 (1H, *dd*, $J_{5''',6a'''}=2.2$ Hz, $J_{6a''',6b'''}=11.9$ Hz, H-6a‴), 3.72 (1H, *dd*, $J_{5''',6b'''}=2.5$ Hz, $J_{6a''',6b'''}=11.9$ Hz, H-6b‴), 3.68 (1H, *dd*, $J_{5'',6a''}=5{,}6$ Hz, $J_{6a'',6b''}=11.9$ Hz, H-6a″), 3.66 (1H, *dd*, $J_{5'',6b''}=4.7$ Hz, $J_{6a'',6b''}=11.9$ Hz, H-6b″), 3.48 to 3.15 (8H, *m*, H-2″, H-2‴, H-3″, H-3‴, H-4″, H-4‴, H-5″, H-5‴). ^{13}C NMR (125.77 MHz, CD_3OD): δ 160.1 (C-3), 159.5 (C-5), 158.2 (C-4′), 140.8 (C-1), 133.0 (C-1′), 131.3 (C-2′, C-6′), 130.9 (C-8), 130.4 (C-7), 117.6 (C-3′, C-5′), 111.4 (C-6), 109.3 (C-2), 104.4 (C-4), 102.4 (C-1″, C-1‴), 78.2 (C-5″, C-5‴), 77.9 (C-3″, C-3‴), 75.0 (C-2″, C-2‴), 71.5 (C-4″, C-4‴), 62.6 (C-6″, C-6‴).

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