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Metabolism of Stilbene Phytoalexins by *Botrytis cinerea* : 1. Characterization of a Resveratrol Dehydrodimer.

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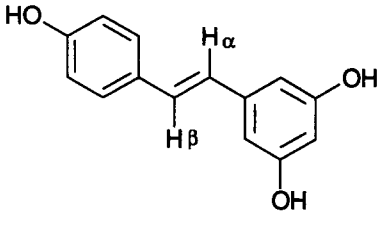
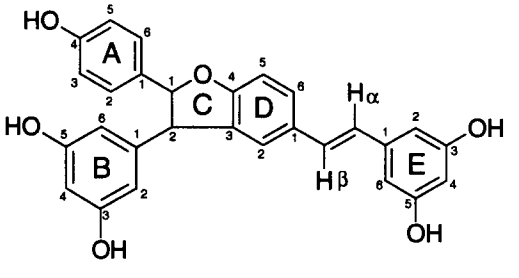
Abstract : Resveratrol, a grapevine phytoalexin, is metabolized by a laccase-like stilbene-oxidase of *Botrytis cinerea*, the causal organism for grey mould. Characterization of one major metabolite formed during this degradation process as a resveratrol dehydrodimer allowed us to precize the reaction mechanism of this enzyme on stilbenes. © 1998 Elsevier Science Ltd. All rights reserved.

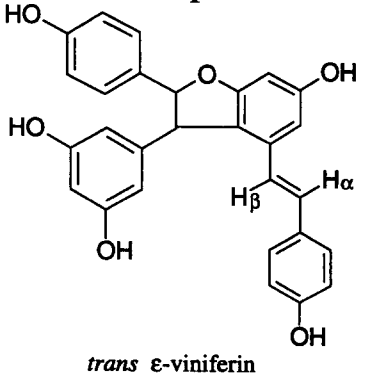
Previously published results have shown that grapevines synthesize natural antimicrobial compounds in response to fungal infection[1]. These compounds which are referred as phytoalexins[2] belong to the family of stilbenes[3], the major constituents of which are resveratrol (*trans* 3,5,4' tri-hydroxystilbene) **1** and its dehydrodimer, ε-viniferin **2**[4].



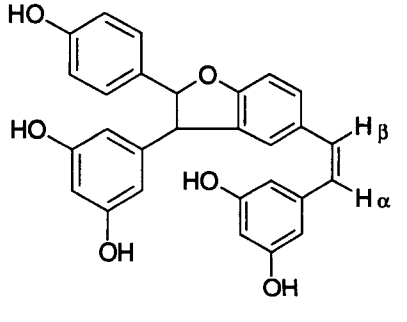
trans - resveratrol
1



resveratrol *trans* - dehydrodimer
3



trans ε-viniferin
2



resveratrol *cis*-dehydrodimer
4

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In 1991, it was described that resveratrol is metabolized by a laccase-like stilbene oxidase[5] produced by *Botrytis cinerea*, the causal organism for grey mould[6]. Recently, we have isolated and characterized unambiguously the resveratrol metabolite **3**[16] and its isomer **4**[17]. We describe here our results in this area.

Compound **3** was isolated after incubation of resveratrol in culture filtrates of *B. cinerea* and purified by T.L.C. and H.P.L.C[14,15]. As the resveratrol metabolite **3** was obtained as an oil, X-ray crystallography on this compound was impossible, and the absolute stereostructure of **3** was not determined. Spectral data (U.V. and fluorimetry) showed that this metabolite has absorption maxima beetween 308-336 nm and 281-313 nm and a high blue fluorescence, characteristics of *trans*-stilbenes[7,8].

High resolution E.I.M.S. examination (70eV) of **3** ($M^{+\cdot}$ calc. 454.14164, found 454.14322) allowed us to determine that its molecular formula was $C_{28}H_{22}O_6$. Low resolution G.C.-M.S. results of derivatized trimethylsilylether of **3** ($M^{+\cdot}$ = 814) demonstrated the presence of five hydroxy groups in **3**.

^{1}H-NMR and ^{13}C-NMR spectral data suggested that **3** is a dehydrodimer of **1** $C_{14}H_{12}O_3$ with a dihydrobenzofuran structure. Upon the addition of 1N sodium hydroxide, the absorption maximum of the *trans*-stilbene chromophore did not shift to a higher wavelength. This indicates the absence of a phenolic group in the 4-position of the stilbene moiety ; hence dimerization of **1** by laccases or peroxidases[9,13] requires the hydroxy group in the 4-position of one resveratrol unit.

A detailed analysis of the COSY and HMQC correlation data allowed us to assign every proton with its associated carbon and to verify the structural formula **3** . The pentaphenolic structure was confirmed by ^{1}H-NMR data at δ 8.2 p.p.m. (hydroxyl groups). *Trans*-ethylenic protons at δ 7.08 (Hβ) and δ 6.92 (Hα) were determined by COSY and HMQC correlations. The proton at δ 7.45 (H-6D) showed COSY correlation with the proton at δ 7.28 (H-2D) and with the proton at δ 6.89 (H-5D) allowing us to reject the dimer structure **2** . This was further confirmed by protons at δ 6.30 (H-4B, triplet) and δ 6.28 (H-4E, triplet) in **3** showing, respectively, COSY correlations with the protons at δ 6.21 (H-2B, H-6B) and δ 6.55 (H-2E, H-6E).

The carbon at δ 93.57 showed correlation with the proton at δ 5.49 (H-1C). Similarly the proton at δ 4.49 (H-2C) and the carbon at δ 57.15 were correlated. Proton (H-1C) and proton (H-2C) showed correlations, respectively, with (H-2A, H-6A) and with (H-2B, H-6B). Hence the relative position of the aromatic protons of the rings (A, B) was determined. Data obtained by Langcake and Pryce (1977)[9] and by Hölscher and Schneider (1996)[10], allowed us to assign quaternary carbons at δ 140.28 to the (1E) position, at δ 144.73 to (1B) ring, at δ 157.88 to the (4A) C-OH function, at δ 159.00 to the (3E, 5E) C-OH functions and at δ 159.22 to the (3B,5B) C-OH functions. All these results obtained by COSY and HMQC correlations permitted to determine the chemical structure of the benzenic rings (labelled A, B, D, E) and their relative position to the C-ring.

^{1}H-NMR data and COSY correlations of compound **4** obtained by photoisomerisation of **3**, showed protons engaged in a *cis*-binding. Moreover, the UV spectrum of **4** is very similar to that of *cis*-resveratrol[8], thus indicating that light-induced isomerization of the *trans*-stilbene moiety in the dehydrodimer **3** leads to the *cis*-isomer.

The chemical skeleton of rings (A, B, C, D, E) was conserved, as indicated by ^{1}H-NMR and COSY correlations. The data obtained with **3** and **4** allowed us to determine unambiguously the dehydrodimeric structure of the resveratrol metabolite produced by laccase of *B. cinerea* . This enzyme is a *p*- and *o*-diphenol-oxidase[11,12]. Our results suggest that resveratrol undergoes an oxidative dimerization process during its degradation by *B. cinerea* analogous to that of the formation of **2** in grapevines by peroxidases[9,13], and that the coupling of the two resveratrol units involved the phenolic group situated in the 4-position of the stilbene moiety. Thus the oxidation mechanism of resveratrol by laccase of *B. cinerea* resulted in the dimerization of *p*-hydroxy substituted stilbene units.

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REFERENCES AND NOTES

1. Langcake, P.; Pryce, R.J., *Physiol. Plant Pathol.*, **1976**, *9*, 77-86.
2. Adrian, M.; Jeandet P.; Veneau, J.; Weston, L.A.; Bessis, R., *J. Chem. Ecol.*, **1997**, *23*, 1689-1702.
3. Stoessl, A., Biosynthesis of phytoalexins, in : *Phytoalexins*, Bailey, J.A.; Mansfield, J.W., Eds.; Blackie : Glasgow and London, **1982**, pp. 133-180.
4. Langcake, P.; Pryce, R.J., *Experientia*, **1977a**, *33*, 151-152.
5. Pezet, R.; Pont, V.; Hoang-Van, K., *Physiol. Mol. Plant Pathol.*, **1991**, *39*, 441-450.
6. Sbaghi, M.; Jeandet, P.; Bessis, R.; Leroux, P., *Plant Pathol.*, **1996**, *45*, 139 -144.
7. Jeandet, P.; Breuil, A.C.; Adrian, M.; Weston, L.A.; Debord, S.; Meunier, P.; Maume, G.; Bessis, R., *Anal. Chem.*, **1997**, in press.
8. Hillis, W.E.; Ishikura, N., *J. Chromatogr.*, **1968**, *32*, 323-336.
9. Langcake, P.; Pryce, R.J., *J.C.S. Chem. Comm.*, **1977b**, 208-210.
10. Hölscher, D.; Schneider, R., *Phytochemistry*, **1996**, *43*, 471-473.
11. Mayer, A. M., *Phytochemistry*, **1987**, *26*, 11-20.
12. Mayer, A.M.; Harel, E., *Phytochemistry*, **1979**, *18*, 193-215.
13. Pedreno, M.A.; Morales, M.; Calderon, A.A.; Zapata, J.M.; Ros Barcelo, A., A *trans*-Resveratrol oxidizing basic peroxydase isoenzyme from *Vitis vinifera*, in : *Plant peroxidases : Biochemistry and Physiology*, Obinger, C.; Burger, U.; Ebermann, R.; Penel, C.; Greppin, H., Eds; University of Genova, **1996**, pp. 338-344.

14. Incubation of resveratrol (1) in culture filtrates of *B. cinerea* : 12 mg of resveratrol (Sigma, St Louis, MO) in 68 mL of absolute ethanol were added to 720 mL of 15-d-old culture filtrates (pH 5.2) of *B. cinerea* obtained as described in Sbaghi *et al* [6]. Resveratrol and its major metabolite (3) were extracted with ethyl acetate (v : v). Organic phases were concentrated under vacuum, at 35°C. The dried extract was then dissolved in MeOH before pre-purification by TLC.
15. Purification procedure : Compound (3) was pre-purified by preparative TLC using reversed-phase material RP_{18} (RP 18F254S, Merck, Darmstadt, Germany) in MeOH / water 7:3 (v : v). Observation of the TLC plates under long wavelength UV-light (366 nm) revealed the presence of two compounds with a blue fluorescence characteristics of the *trans*-stilbenes[1]. The first compound (Rf = 0.66) was identified as the non-degraded resveratrol, the second compound (Rf = 0.53) corresponding to the resveratrol metabolite (3). Bands corresponding to compound (3) were collected and eluted in ethyl acetate. The *cis* and the *trans* isomers of (3) were then separated by semi-preparative HPLC using an Ultrabase C18 reversed-phase column (5µm, 250x4 mm) with a mobile phase of 40% acetonitrile / 60% water at a flow rate of 4 mL / min. Detection was at 308 nm.
16. Resveratrol *trans*-dehydrodimer (3) : EI-MS m/z 454.14322 ($M^{+\cdot}$) ; Trimethylsilylether : GC-MS = 814 $(M)^{+\cdot}$; UV λmax MeOH 307.7 nm ; ^{1}H-NMR (500MHz, C_3D_6O) δ 4.49 (d, *J* = 8.0 Hz, H-2C), 5.49 (d, *J* = 8.0 Hz, H-1C), 6.21 (d, *J* = 2.0 Hz , H-2B, H-6B), 6.28 (t, *J* = 2.1 Hz, H-4E), 6.30 (t, *J* = 2.1 Hz, H-4B), 6.55 (d, *J* = 2.1 Hz, H-2E, H-6E), 6.87 (d, *J* = 8.8 Hz, H-3A, H-5A), 6.89 (d, *J* = 8.4 Hz, H-5D), 6.92 (d, *J* = 16.4 Hz, H-α), 7.08 (d, *J* = 16.2 Hz, H–β), 7.26 (d, *J* = 8.7 Hz, H-2A, H-6A), 7.28 (brs, H-2D), 7.45 (dd, *J* = 1.5 and 8.3 Hz, H-6D), 8.20 (brs, integration : 5H) ; ^{13}C-NMR (125 MHz, C_3D_6O) ∂ 57.15 (C-2C), 93.57 (C-1C), 106.86 (C-2B, C-6B), 101.79 (C-4B), 102.12 (C-4E), 105.15 (C-2E, C-6E), 109.86 (C-5D), 115.62 (C-3A, C-5A), 123.44 (C-2D), 126.7 (C-α), 128.09 (C-2A, C-6A), 128.60 (C-β), 128.14 (C-6D), 131.26, 131.67 and 132.05 (C-1D, C-1A, C-3D), 140.28 (C-1E), 144.73 (C-1B), 157.88 (C-4A), 159.00 (C-3E, C-5E), 159.22 (C-3B, C-5B), 160.13 (C-4D).
17. Resveratrol *cis*-dehydrodimer (4) : Trimethylsilylether : GC-MS = 814 ($M^{+\cdot}$) ; UV λmax MeOH 281 nm ; ^{1}H-NMR (500MHz, C_3D_6O) : δ 4.43 (d, *J* = 8.4 Hz, H-2C), 5.38 (d, *J* = 8.6 Hz, H-1C), 6.14 (d, *J* = 2.2 Hz, H-2B, H-6B), 6.23 (t, *J* = 2.1 Hz, H-4E), 6.25 (t, *J* = 2.2 Hz, H-4B), 6.33 (d, *J* = 2.0 Hz, H-2E, H-6E), 6.36 (d, *J* = 12.4 Hz, Hα), 6.50 (d, *J* = 12.1 Hz, Hβ), 6.77 (d, *J* = 8.3 Hz, H-5D), 6.87 (d, *J* = 8.8 Hz, H-3A, H-5A), 6.96 (brs, H-2D), 7.22 (d, *J* = 8.8 Hz, H-6D), 7.23 (d, *J* = 8.8 Hz, H-2A, H-5A).