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# Biotransformation of resveratrol: synthesis of *trans*-dehydrodimers catalyzed by laccases from *Myceliophtora thermophyla* and from *Trametes pubescens*

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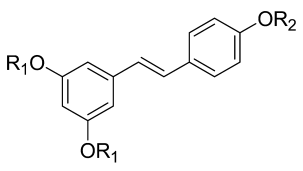
**Abstract**—Laccases from different sources have been used, for the first time, for the selective oxidation of the stilbenic phytoalexin *trans*-resveratrol (3,5,4′-trihydroxystilbene, **1a**) on a preparative scale. Specifically, the enzymes from *Myceliophtora thermophyla* and from *Trametes pubescens* gave the dehydrodimer **2a** in 31 and 18% isolated yields, respectively. These results compare favorably with the reported data for the chemically catalyzed dimerization of **1a** (18% yields). The antioxidant properties of **2a** have also been investigated.


## 1. Introduction

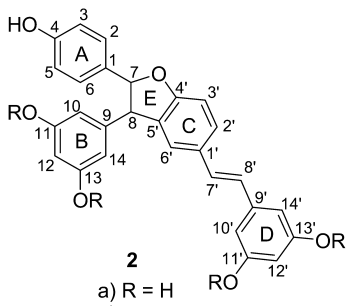
*trans*-Resveratrol (3,5,4′-trihydroxystilbene, **1a**) is a stilbenic phytoalexin produced by plants via a metabolic sequence induced in response to biotic or abiotic stress factors.[1] It has been found in a multitude of dietary plants, such as pines, legumes and grapevines: thus, relatively high concentrations of this compound are present in grape juice and in red wine.[2] Resveratrol is one of the phenolic compounds present in wine that could be responsible for the decrease in coronary heart disease observed among wine drinkers (French paradox).[3] Growing evidence suggest that resveratrol plays a role in the prevention of carcinogenesis.[4] In addition to resveratrol, its oligomers, the so-called 'viniferins', have also been found in plants[5] as a result of infection or stress. Oligomeric stilbenes are reported to exhibit a wide-array of biological activities, such as antimicrobial, anti-HIV, anti-inflammatory;[5] they are also reported to be potentially important cancer chemoprotective agents, being able to inhibit cellular events associated with carcinogenesis.[5,6] Due to their occurrence in various edible vegetables and fruits, these compounds may represent nutritionally important constituents.

Despite that, as many of these compounds are exclusively obtained by extraction from natural sources, the studies of their biological properties are limited by their very scarce availability.

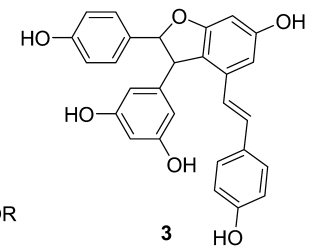
Few synthetic approaches to the oxidative coupling of resveratrol have been reported. By treating **1a** with 2,2-diphenyl-1-picrylhydrazyl free radical (DPPH) a major




**1**
a) $R_1 = R_2 = H$
b) $R_1 = Ac$; $R_2 = H$
c) $R_1 = H$; $R_2 = CH_3$




**2**
a) R = H
b) R = Ac




**3**

*Keywords*: Resveratrol; Laccase; Polyphenols; Biotransformation.
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product was isolated in 18% yield and identified as trans-resveratrol dehydrodimer **2a**.[7] On the other hand, the treatment of **1a** with $FeCl_3$ failed to give **2a**, affording the isomeric dimer ε-viniferin (**3**) as the sole product.[8]

Different studies have shown that resveratrol is transformed in vivo (for instance by incubating **1a** with *Botrytis cinerea* cultures) into various oxidized products (dimers), such as **2a**.[9,10] On the other hand, to the best of our knowledge, there are only four reports on the in vitro biocatalyzed oxidation of **1a**: the first two describe a horseradish peroxidase–hydrogen peroxide promoted dimerization of **1a** to **2a**,[11] the third reports on the oxidative degradation of **1a** by action of a soybean lipoxygenase (products structures not identified),[12] while the fourth describes the performances of a purified laccase isolated from a *B. cinerea* strain.[13] The latter reaction was run on analytical scale, monitored by HPLC and the formed product was identified as ε-viniferin **3** simply by comparison with the HPLC elution time of an authentic sample.

As a part of our general interest in biocatalysis[14] and, more specifically, in enzyme-catalyzed carbon–carbon bond formation,[15] we have recently started an investigation on the synthetic potential of laccases. In this paper we report on the preparative-scale oxidation of *trans*-resveratrol and of some of its derivatives by action of two laccases, isolated from *Mycelyopthora thermophyla* and from *Trametes pubescens*, respectively.

## 2. Results and discussion

Laccases are oxidoreductases (the so-called 'blue oxidases'), widely distributed in fungi and in some bacteria and higher plants and able to catalyze the oxidation of various phenolic compounds at the expense of molecular oxygen.[16] The active site of these enzymes consists of a metallic cluster containing four copper atoms, all of them being involved in the redox process via a radical cyclic mechanism.[17] The oxidation of a specific phenolic derivative depends on the redox potential difference between the target compound and the so called 'T1'-copper. Laccases show an exceptional substrate versatility and therefore are potentially suitable biocatalysts for the mild oxidation of organic compounds. So far, the main limitation to their use has been their scarce availability. However, mainly to satisfy the demand for new 'green' processes by the textile and pulp and paper industries, some of these enzymes have recently been cloned and overexpressed and are becoming commercially available.[18]

Literature reports on the synthetic applications of laccases can be divided into two groups. In the first one, these enzymes are used to oxidize a suitable chemical compound (i.e., Tempo), which in turn acts as a 'mediator' for the oxidation of the synthetic target;[19] one example is the laccase-mediated production of aldehydes from the corresponding alcohols.[20]

The second group of reports describes the direct oxidation of phenolic derivatives, such as estradiol[21] and penicillin X,[22] to give dimers and higher oligomers. The main drawback of these biotransformations is the extensive polymerization that may arise from the radical mechanism of the oxidative process,[23] producing a complex mixture of poly-phenolic oligomers.

At variance, this has not been the case with *trans*-resveratrol. Following an optimized protocol for the use of laccases in organic solvents,[24] resveratrol (a compound that is poorly soluble in aqueous buffer solutions) has been dissolved in *n*-BuOH (pre-saturated with 25 mM phosphate buffer, pH 6.5) and submitted to the action of the laccase from *Myceliophtora thermophyla* supported on glass beads. The suspension has been shaken at 45 °C for 4 days and the formation of a more polar product has been observed by TLC. Similarly to resveratrol, this compound exhibits an intense purple color under UV irradiation (254 nm) on TLC plates: this information indicates that the new molecule maintains the extended conjugation of the stilbene system. The product has been isolated by flash chromatography in 31% yield and identified as the *trans*-dehydrodimer **2a** by EI-MS and extensive mono- and bi-dimensional NMR analysis.

The EI-MS shows a molecular ion peak at *m/z* 454, corresponding to the structure of a dehydrodimer of resveratrol (*m/z* calcd for $C_{28}H_{22}O_6$ is 454), while $^1H$ and $^{13}C$ NMR data, reported in Table 1, suggest the substituted dihydrobenzofurane structure **2a**, in accordance with the values reported in literature.[10b] To unambiguously assign the relative stereochemistry of the stereogenic centers of C-7 and C-8, a detailed NMR study has been performed.

Initial inspection of the $^1H$ NMR spectrum of **2a** in DMSO-$d_6$ has shown the presence of three different types of aromatic OH's at 9.14, 9.17 and 9.48 ppm (integral: 2H, 2H and 1H, respectively) suggesting a penta-phenolic structure. Additionally, the $^1H$ NMR spectrum exhibits signals due to one 4-hydroxybenzene moiety (multiplet at 6.85 ppm, AA′ system, 2H, and 7.24 ppm, BB′ system, 2H), to two 3,5-dihydroxybenzene moieties (doublet at 6.20 ppm, 2H; triplet at 6.26 ppm, 1H; triplet at 6.29 ppm, 1H; doublet at 6.54 ppm, 2H), to two *trans* olefinic protons (doublets at 6.90 and 7.06 ppm), and to two aliphatic protons (doublets at 4.47 and 5.45 ppm).

The assignments have been made on the basis of $^1H$, $^{13}C$-inverse detected single-quantum (HSQC)[25] and multiple-bond (HMBC)[26] correlation experiments. The long-range correlation between H-2,6 at 7.24 ppm and C-7 at 94.72 ppm, and between H-10,14 at 6.20 ppm and C-8 at 58.52 ppm allows to establish that the A ring is bonded to C-7 whereas the B ring is bonded to C-8. The low, but detectable, observed correlation between H-7 at 5.45 and C-4′ at 161.3 ppm confirms the presence of the fused oxygenated five-member ring and is in favor of a dihedral angle H-7–C-7–O–C-4′ close to 90°.

The value of $^3J$(H-7,H-8) measured from $^1H$ NMR spectrum (7.97 Hz) suggests a predominant conformation with the two H-7 and H-8 protons in a pseudo *trans*-axial arrangement, whereas the $^3J$(H-7′,H-8′) is 16.33 Hz, as expected for a double bond with a *trans* configuration. The distinction

**Table 1**. $^{1}H$ and $^{13}C$ NMR data for compounds **2a** and **2b**

| | $\delta_H$ (mult., *J* Hz) | | | $\delta_C$ | |
|---|---|---|---|---|---|
| | 2a[a,b] | 2b[c] | | 2a[b] | 2b[c] |
| | | | C-1 | 133.28 | 129.47 |
| H-2,6 | 7.24 (BB′ multiplet) | 7.20 (BB′ multiplet) | C-2,6 | 129.23 | 127.89 |
| H-3,5 | 6.85 (AA′ multiplet) | 6.76 (AA′ multiplet) | C-3,5 | 116.85 | 115.29 |
| | | | C-4 | 159.10 | 157.65 |
| H-7 | 5.45 (d, 7.96) | 5.54 (d, 8.13) | C-7 | 94.72 | 92.08 |
| H-8 | 4.47 (d, 7.96) | 4.76 (d, 8.13) | C-8 | 58.52 | 54.80 |
| | | | C-9 | 145.91 | 143.80 |
| H-10,14 | 6.20 (d, 2.16) | 6.92 (d, 2.10) | C-10,14 | 108.11 | 118.74 |
| | | | C-11,13 | 160.44 | 151.08 |
| H-12 | 6.29 (t, 2.16) | 6.95 (t, 2.10) | C-12 | 103.08 | 114.79 |
| | | | C-1′ | 132.43 | 130.08 |
| H-2′ | 7.43 (dd, 8.26, 1.63) | 7.47 (dd, 8.45, 1.36) | C-2′ | 129.29 | 128.24 |
| H-3′ | 6.87 (d, 8.26) | 6.94 (d, 8.45) | C-3′ | 110.81 | 109.67 |
| | | | C-4′ | 161.30 | 159.14 |
| | | | C-5′ | 132.81 | 130.76 |
| H-6′ | 7.26 (broad s) | 7.24 (broad s) | C-6′ | 124.62 | 123.07 |
| H-7′ | 7.06 (d, 16.33) | 7.26 (d, 16.35) | C-7′ | 129.80 | 130.24 |
| H-8′ | 6.90 (d, 16.33) | 7.02 (d, 16.35) | C-8′ | 127.92 | 124.07 |
| | | | C-9′ | 141.46 | 139.79 |
| H-10′,14′ | 6.54 (d, 2.10) | 7.21 (d, 2.10) | C-10′,14′ | 106.42 | 116.73 |
| | | | C-11′,13′ | 160.22 | 151.02 |
| H-12′ | 6.26 (t, 2.10) | 6.82 (t, 2.10) | C-12′ | 103.43 | 114.29 |
| 11,13-$CH_3$ | | 2.22 | 11,13-$CH_3$ | | 20.74 |
| 11′,13′-$CH_3$ | | 2.26 | 11′,13′-$CH_3$ | | 20.74 |
| | | | 11,13-C=O | | 168.80 |
| | | | 11′,13′-C=O | | 168.90 |
| OH | | 9.53 | | | |

[a] Single broad signal due to hydroxy protons was found at 8.20.
[b] Aceton-$d_6$
[c] DMSO-$d_6$.

between the ethylenic protons has been made through the analysis of the H,C long-range correlation found in the HMBC spectrum: the signal of H-7′ at 7.06 ppm shows a correlation with the carbon signal at 124.62 ppm, directly bonded to H-6′ (i.e., C-6′), whereas the signal due to H-8′ at 6.90 ppm is correlated to the carbon signal at 106.42 ppm, directly bonded to H-10′, 14′ (i.e., C-10′,14′).

The relative stereochemistry of C-7 and C-8 carbons has been proposed on the basis of the analysis of the NOESY[27] NMR spectrum (400 ms mixing time) and of the computer-aided energy-minimized stereostructure obtained. The NOESY spectrum clearly shows the absence of NOE in-phase cross-peaks between H-7 and H-8, and the presence of positive in-phase cross peaks between H-7 and H-10,14 (of comparable intensity with respect to those between H-7 and H-2,6) and between H-8 and H-2,6 (of comparable intensity with respect to those between H-8 and H-10,14), thus confirming that H-7 and H-8 are in a prevalent *trans*-axial arrangement. The remaining NOE observed (i.e., between H-10,14 and H-6′) is in accordance with the conclusions drawn from the H,C correlation experiments. According to the modified Karplus-equation reported by Haasnoot,[28] the $^{3}J$(H-7,H-8) value found for the compound **2a** (7.96 Hz) would result in a dihedral angle of about −140°, which is in good accordance with that found from the computer-aided calculation for the *trans*-dia-stereoisomer (−141°). In this way the isolated product has been unambiguously identified as the compound **2a**. The same product could be isolated, albeit in a lower yield (18%), when the bio-catalytic oxidation of **1a** was performed using a laccase from *Trametes pubescens* in a biphasic system acetate buffer—AcOEt.[24]

The structure of **2a** indicates that the dimerization reaction proceeds exclusively through the phenoxy radical derived from the 4′-OH of **1a**. As further evidence, it has been found that the 4′-*O*-methyl resveratrol derivative **1c** is unreactive under the same reaction conditions, whereas the 3,5-diacetylated derivative **1b** is oxidized to give the expected *trans*-dehydrodimer **2b**, whose structure has been assigned by $^{1}H$ and $^{13}C$ NMR analysis using HMBC, NOESY and ROESY experiments (Table 1).

The antioxidant activity of **2a** has been determined by the DPPH reduction method,[29] by plotting, as indicated in the Section 4, the remaining percentage of DPPH as a function of the molar ratios of **2a** over DPPH. The resveratrol dimer reacts with DPPH, reaching a steady state after about 3 h. An $EC_{50}$ of 5.2±0.4 (mmol **2a**/mmol DPPH) has been determined, which is slightly greater than that observed for resveratrol [4.1±0.3 (mmol/mmol DPPH)] and comparable with that of its biologically active analogue pterostilbene [5.0±0.4 (mmol/mmol DPPH)].[4c]

## 3. Conclusions

In this paper we have shown the usefulness of isolated laccases for the synthesis of the resveratrol dimer **2a** in good yields and under very mild reaction conditions (atmospheric air, enzyme, solvent). To our knowledge, this is the first report on the preparative-scale oxidative dimerization of **1a** catalyzed by this group of oxidative enzymes. Due to the wide-array of biological activity exhibited by resveratrol oligomers, the compounds synthesized may serve as lead for the development of new drugs and as nutraceuticals. Furthermore, the bio-oxidation of resveratrol, affording dimeric product(s), is interesting not only from a chemical point of view but it has an utmost importance, for example, for the wine-making industry. The use of enzyme preparations in the wine-making industry to improve the quality of wine is well established:[30] for example, the color stabilization by removal of excess phenols using laccase has been proposed. Immobilization of enzymes for their use in wine making is another interesting possibility.

As regards the antioxidant activity of **2a**, this dimer is able to scavenge DPPH radical with an $EC_{50}$ value comparable with that observed for resveratrol and its analogues, disclosing a preliminary positive information on the bioactivity of **2a**. Being easily scaled up, this simple and selective biotransformation can afford the dimeric product(s) in sufficient amounts to allow the evaluation of the biological activity of compound **2a** and of other resveratrol derivatives (i.e., **2b**): in particular, the protection against lipid peroxidation, the effects on cell proliferation and the ability to inhibit replicative DNA polymerase are under investigation in our laboratory and will be reported in due course.

## 4. Experimental

### 4.1. Materials and methods

Laccase from *Myceliophtora thermophyla* was from Novozymes A/S, Denmark, while the laccase from *Trametes pubescens* was provided by Professor Haltrich.[31] Compounds **1b**[32] and **1c**[4c] were synthesized following the procedures reported in the literature. TLC: precoated silica gel 60 $F_{254}$ plates (Merck). Flash chromatography: silica gel 60 (70–230 mesh, Merck). Mass spectra were acquired with a combined HPLC particle beam MS-engine. NMR spectra were recorded in aceton-$d_6$ or DMSO-$d_6$ solutions at 400 MHz and the chemical shift reported are in ppm relative to tetramethylsilane as external standard. Energy minimized stereostructure of **2a** was obtained by MM+ calculation using HyperChem 5.0 molecular modeling program. Antiradical activity by DPPH: spectrophotometric data were acquired using a UV/VIS spectrometer.

### 4.2. Adsorption of the laccase from *M. thermophyla* on glass beads

*M. thermophyla* laccase was dialyzed against 25 mM phosphate buffer pH 6.5 and lyophilized. The enzyme was dissolved in the same buffer (60 mg/ml) and the solution was dropped onto glass beads (1 ml/3 g). The slurry was mixed and left to dry at room temperature with occasional mixing for nearly 2 days.

**4.2.1. Oxidation of resveratrol catalyzed by *M. thermophyla* laccase.** Resveratrol (100 mg) was dissolved in 6 ml of *n*-butanol saturated with 25 mM phosphate buffer, pH 6.5. 200 mg (120 U) of *M. thermophyla* laccase supported onto glass beads was added and the suspension was shaken at 45 °C for 4 days. The enzyme and a little amount of a brown precipitate were separated by filtration, the solvent was evaporated and the crude residue was purified by flash chromatography (eluent: petrol 40–60 °C/ethyl acetate/methanol=6:4:0.5) to give 31 mg of **2a** (31% yield). Pale yellow amorphous solid. $^1$H NMR (acetone-$d_6$, TMS): 4.47 (d, $J$=8.0 Hz, 1H, H-8), 5.45 (d, $J$=8.0 Hz, 1H, H-7), 6.20 (d, $J$=2.2 Hz, 2H, H-10,14), 6.26 (t, $J$=2.1 Hz, 1H, H-12′), 6.29 (t, $J$=2.2 Hz, 1H, H-12), 6.54 (d, $J$=2.1 Hz, 2H, H-10′,14′), 6.85 (AA′ multiplet, 2H, H-3,5), 6.87 (d, $J$=8.3 Hz, 1H, H-3′), 6.90 (d, $J$=16.3 Hz, 1H, H-8′), 7.06 (d, $J$=16.3 Hz, 1H, H-7′), 7.24 (BB′ multiplet, 2H, H-2,6), 7.26 (broad s, 1H, H-6′), 7.43 (dd, $J$=8.3, 1.6 Hz, 1H, H-2′). $^{13}$C NMR (acetone-$d_6$, TMS): 58.52, 94.72, 103.08, 103.43, 106.42, 108.11, 110.81, 116.85, 124.62, 127.92, 129.23, 129.29, 129.80, 132.43, 132.81, 133.28, 141.46, 145.91, 159.10, 160.22, 160.44, 161.30.

MS (EI, 70 eV): $m/z$ (%)=454 (39, $M^+$); 228 (100); 179 (93); 123 (82).

Anal. calcd for $C_{28}H_{22}O_6$: C, 74.00%; H, 4.88%; O, 21.12%. Found C, 74.06; H, 4.93.

**4.2.2. Oxidation of resveratrol catalyzed by *T. pubescens* laccase.** Resveratrol (100 mg) was dissolved in 4 ml of ethyl acetate, while the laccase (110 U) was dissolved in 4 ml of 20 mM acetate buffer, pH 4.5. The biphasic system was shaken at room temperature for 24 h. The organic solvent was evaporated and the crude residue was purified by flash chromatography (eluent: petrol 40–60 °C/ethyl acetate/methanol=6:4:0.5) to give 18 mg of **2a** (18% yield).

**4.2.3. Attempted oxidation of 4′-*O*-methyl-resveratrol (1c) by *M. thermophyla* laccase.** 4′-*O*-Methyl-resveratrol (**1c**, 3 mg) was dissolved in 0.5 ml of *n*-butanol saturated with 25 mM phosphate buffer, pH 6.5. 50 mg (30 U) of *M. thermophyla* laccase supported onto glass beads were added and the system was shaken at 45 °C. No reaction was observed after 48 h.

**4.2.4. Oxidation of 3,5-di-*O*-acetyl-resveratrol (1b) by *M. thermophyla* laccase.** 3,5-Di-*O*-acetyl-resveratrol (**1b**, 35 mg) was dissolved in 8 ml of *n*-butanol saturated with 25 mM phosphate buffer, pH 6.5. 150 mg (90 U) of the *M. thermophyla* laccase supported onto glass beads was added and the system was shaken at 45 °C for 4 days. The enzyme and a little amount of a brown precipitate were separated by filtration, the solvent was evaporated and the crude residue was purified by flash chromatography (eluent: petrol 40–60 °C/ethyl acetate/methanol=75:25:5) to give 9 mg of **2b** (26% yield). Pale yellow oil. $^1$H NMR (acetone-$d_6$): 2.22 (s, 3H, $CH_3$), 2.26 (s, 3H, $CH_3$), 4.76 (d, $J$=8.1 Hz, 1H, H-8), 5.54 (d, $J$=8.1 Hz, 1H, H-7), 6.76 (AA′ multiplet,

2H, H-3,5), 6.82 (t, $J$=2.1 Hz, 1H, H-12′), 6.92 (d, $J$=2.1 Hz, 2H, H-10,14), 6.94 (d, $J$=8.5 Hz, 1H, H-3′), 6.95 (t, $J$=2.1 Hz, 1H, H-12), 7.02 (d, $J$=16.4 Hz, 1H, H-8′), 7.20 (BB′ multiplet, 2H, H-2,6), 7.21 (d, $J$=2.1 Hz, 2H, H-10′,14′), 7.24 (broad s, 1H, H-6′), 7.26 (d, $J$=16.4 Hz, 1H, H-7′), 7.47 (dd, $J$=8.5, 1.4 Hz, 1H, H-2′), 9.53 (s, 1H, OH). $^{13}$C NMR (acetone-$d_6$): 20.74 (2C), 54.80, 92.08, 109.67, 114.29, 114.79, 115.29, 116.73, 118.74, 123.07, 124.07, 127.89, 128.24, 129.47, 130.08, 130.24, 130.76, 139.79, 143.80, 151.02, 151.08, 157.65, 159.14, 168.80, 168.90.

MS (EI, 70 eV): $m/z$ (%)=622 (33, $M^+$); 580 (36); 538 (27); 496 (15); 454 (7); 270 (33); 228 (69); 221 (86); 179 (100); 123 (77).

Anal. calcd for $C_{36}H_{30}O_{10}$: C, 69.44%; H, 4.86%; O, 25.70%. Found C, 69.47; H, 4.91.

### 4.3. Antiradical activity

The antioxidant activity of **2a** was determined using DPPH as a free radical, following the method described by Berset,[29] using different concentrations of **2a** (0.069, 0.137, 0.296, 0.468 mM). A methanolic solution of **2a** (100 μl) was added to 3.9 ml of a 0.06 mM DPPH methanolic solution. The decrease in absorbance at 515 nm was evaluated until the reaction reached a plateau (about 3 h). The percentage of residual DPPH at the steady state was reported onto a graph as a function of the molar ratio of antioxidant to DPPH. Antiradical activity was defined as the amount of antioxidant necessary to decrease the initial DPPH concentration by 50% (efficient concentration).

## 5. Supplementary material

NMR and mass spectra of **2a** and **2b**, and energy minimized stereostructure of **2a** are available on request.

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