Oligomeric compounds formed from 2,5-xylidine (2,5-dimethylaniline) are potent enhancers of laccase production in Trametes versicolor ATCC 32745

Albert Kollmann, Francois Boyer, Paul Henri Ducrot, Lucien Kerhoas, Claude Jolivalt, Isabelle Touton, Jacques Einhorn, Christian Mougin

Oligomeric compounds formed from 2,5-xylidine (2,5-dimethylaniline) are potent enhancers of laccase production in *Trametes versicolor* ATCC 32745

Albert Kollmann[1], François-Didier Boyer[1], Paul-Henri Ducrot[1], Lucien Kerhoas[1], Claude Jolivalt[2], Isabelle Touton[1], Jacques Einhorn[1] and Christian Mougin[1*]

[1]INRA, Unité de Phytopharmacie et Médiateurs Chimiques, Route de Saint-Cyr, 78026 Versailles cedex, France

[2]ENSCP, Laboratoire de Synthèse sélective organique et Produits naturels, UMR CNRS 7573, 11 rue P. et M. Curie, 75231 Paris Cedex 05, France

Short title: laccase production enhancement by oligomers formed from 2,5-xylidine

Abstract

Numerous chemicals, including the xenobiotic 2,5-xylidine, are known to induce laccase production in fungi. The present study was conducted to determine whether the metabolites formed from 2,5-xylidine by fungi could enhance laccase activity. We used purified laccases to transform the chemical, and then we separated the metabolites, identified their chemical structure and assayed their effect on enzyme activity in liquid cultures of *Trametes. versicolor*. We identified 13 oligomers formed from 2,5-xylidine. (4*E*)-4-(2,5-dimethylphenylimino)-2,5-dimethylcyclohexa-2,5-dienone at 1.25 10^{-5} M was an efficient inducer resulting in a 9-fold increase of laccase activity after 3 days of culture. Easily

synthesized in one step (67% yield), this compound could be used in fungal bioreactors to obtain a great amount of laccases for biochemical or biotechnological purposes, with a low amount of inducer.

Keywords

Enzyme induction, oxidases, *Trametes versicolor*, oligomers, xenobiotics, 2,5-xylidine

Introduction

Numerous xenobiotics, including anthropogenic or natural compounds, are known to induce laccase production in filamentous fungi belonging to different classes: ascomycetes (*Neurospora* sp.), deuteromycetes (*Botrytis* sp.) and basidiomycetes (*Pycnoporus* sp., *Trametes* sp.)(Gianfreda et al. 1999). Induction is commonly used at laboratory or industrial scales in order to obtain large amounts of enzymes for biochemical (*i.e.* structural, Bertrand et al. 2002), biotechnological (*i.e.* bioconversion and biotransformation, Jolivalt et al. 2000), environmental (*i.e.* biomarkers for ecotoxicity assessment, Mougin et al. 2002, 2003) purposes.

Several isomers of xylidines (dimethylanilines) act as laccase inducers (Soden and Dobson 2001). They have been used for more than 40 years to produce high yields of enzyme in liquid fungal cultures (Fahraeus et al. 1958; Bollag and Leonowicz 1984). In these cultures (*i.e.* the white-rot *Trametes versicolor*), laccase induction was concomitant with a red coloration of the culture medium. Compounds responsible for that colour have never been characterised. Indeed, oxidation of several poly-methylated anilines by peroxidase has been studied but as far as we know, no information is available for 2,5-xylidine (Baker et al. 1974). Previous work performed in our laboratory showed that 2,5-xylidine (2,5-dimethylaniline) was a substrate for fungal laccases (Bertrand et al. 2002) and that its enzymatic conversion led to products with a colour similar to that observed in *Trametes versicolor* cultures undertaken in presence of 2,5-xylidine.

Apart from their use in fungal cultures, xylidines are mainly used in industry as dyes. In addition, they are toxic components of rocket fuels (Kallas et al. 2003). It is thus likely that xylidines could also be transformed in soils from military bases through bacterial and fungal activity, providing an new interesting *in situ* bioremediation method.

The objectives of the present paper are (i) to isolate and identify the products formed from 2,5-xylidine by fungal laccases, (ii) to assess the potential of these transformation products to induce fungal laccase production and so possibly provide a new efficient laccase inducer.

Materials and Methods

Chemicals and reagents

2,5-Xylidine (2,5-dimethylaniline, 99 % purity, compound **1**, Figure 1) was purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France). Other chemicals and reagents were from Sigma-Aldrich or VWR (Fontenay sous Bois, France). All solvents used for chromatographic isolation were of analytical grade and came from Carlo-Erba (Val de Reuil, France).

Enzymatic transformation of 2,5-xylidine and oligomeric compounds

2,5-Xylidine was transformed using laccases produced by the white-rot fungus *Pycnoporus cinnabarinus* ss3, a monokaryotic strain originating from the parental dikaryotic strain I-937 (Herpoël et al. 2000). The strain was grown in a synthetic medium containing 35 g Glucidex 47 (dextrose), 3 g yeast extract, 0.01 mM disodium tartrate, 20 mM diammonium tartrate, 9.8 mM KH_2PO_4, 0.68 mM $CaCl_2$ $2H_2O$, 2 mM $MgSO_4$ $7H_2O$, 0.46 mM $FeSO_4$ $7H_2O$, 0.28 mM $ZnSO_4$ $7H_2O$, 0.23 mM $MnSO_4$ H_2O, 0.45 mM $CuSO_4$ $5H_20$, 1 mL of a mixed vitamin solution (Tatum et al. 1950) and 0.05% (w/v) Tween 80, per L. Culture was carried out at 30°C and stirred at 300 rpm. After 3 days, the culture was induced for laccase expression by addition of 0.5 mM ferulic acid. Culture medium (11.8 L) was collected after 11 days.

Laccases of *P. cinnabarinus* ss3 were purified as previously published (Otterbein et al. 2000). Briefly, the culture medium was first concentrated by ultrafiltration through a cellulose PLGC

membrane (molecular mass cut-off of 10000 Da). Laccases were further concentrated by a two step ammonium sulfate precipitation. In the first step, ammonium sulfate was added with stirring to a 40% (w/v) final concentration, and incubated for 1 h at 4°C. The precipitate was eliminated by a centrifugation at 17000 g for 30 min. The ammonium sulfate concentration of the supernatant was then increased to 80% (w/v) saturation, and stored overnight at 4°C. The precipitate was collected by centrifugation (17000 g for 30 min) and dissolved in 100 mL of sodium acetate buffer (0.025 M, pH 5.7). The residual ammonium sulfate was removed by dialysis. Specific laccase activity was 1.4 U/mg. At this step, lactose was added to a final concentration of 3 % (w/v), and the laccase extract was freeze-dried over a 24-h period. The resulting powder can be easily manipulated and stored for long periods without any loss of activity.

2,5-Xylidine (50 mM) was incubated with 10 unit of laccase at 30°C in darkness under shaking in 500 mL of 100 mM citrate/phosphate buffer pH 5. A negative control without laccase was performed in parallel. After a 5-day incubation, the medium was extracted 3-times with 150 mL dichloromethane. The extracts were pooled together and concentrated (5 mL) under reduced pressure for sequential preparative chromatography.

A similar protocol was used to assess the transformation of purified oligomers by laccases. Each compound (10 mM in 100 µL acetone) was incubated in the presence of enzyme during 7 days. Remaining compounds were extracted by dichloromethane and quantified by HPLC.

Fungal assays for laccase induction

Trametes versicolor ATCC 32745 (a strain of Biosafety level 2 according to the Directive 2000/54 of the European Communities) was grown on a culture medium described by Lesage-Meesen *et al.* (1996), containing maltose and ammonium tartrate as carbon and nitrogen sources. Final copper concentration in the medium was 30 µM. A mycelial mat on agar plugs

(10 mm diameter) was inoculated into 10 mL of the culture medium in a 150-mL Erlenmeyer flask. Cultivation was carried out statically in the dark at 25°C. After 4 days of incubation, concentrated solutions of xenobiotics in acetone (100 µL solution per Erlenmeyer flask) were added to the cultures at different concentrations as described in the text. Aliquots were then assayed for laccase activity after a further incubation period (zero to seven days).

Laccase activity measurements

Laccase activity was determined by monitoring the slope of the increase in A_{420} due to the oxidation of 2 mM and 2,2'-azinobis-(3-ethylbenzthiazoline-6-sulfonic) acid (ABTS) (ε_{420} 36 000 M^{-1} cm^{-1}) in citrate-phosphate buffer (0.1M, pH 3) at 30°C (Wolfenden and Wilson 1982). Solutions of enzyme were added in a final volume of 1 mL assay media. The buffer solution was saturated with air by bubbling prior to the experiment. One unit of enzyme activity is defined as the amount of enzyme that oxidizes one µmol ABTS in one min.

Analytical methods

High Performance Liquid Chromatography (HPLC) analyses were performed on a Waters apparatus comprising a 600 solvent delivery system and a 990 diode array detector. Nuclear Magnetic Resonance (NMR) data (^{1}H: 300 MHz; ^{13}C: 75.5 MHz) were recorded on a Varian Mercury 300 instrument. All NMR spectra were recorded in deuteriochloroform ($CDCl_3$) except in some cases in C_6D_6 as indicated in the text. Chemical shifts are reported in δ ppm relative to $CHCl_3$ ($CDCl_3$) as internal reference: 7.27 ppm for ^{1}H (77.14 ppm for ^{13}C) or C_6H_6 (C_6D_6) 7.15 ppm for ^{1}H (128.62 ppm for ^{13}C). Coupling constants (*J*) are given in Hertz (Hz). Multiplicities are recorded as s (singlet), d (doublet), t (triplet), q (quadruplet) and m (multiplet) and br (broad). Mass Spectrometry (MS) and tandem Mass Spectrometry (MS/MS) experiments were performed on a Quattro LC™ mass spectrometer with a Z-

spray^{TM} ElectroSpray Ionization (ESI) source (Micromass, Manchester, UK). The ESI source parameters were: capillary 3.25 kV (positive mode), extractor 2 V, source block temperature 120°C, desolvatation gas temperature 400°C. Argon was used for collision-induced dissociation at a pressure of 3.5 10^{-3} torr and collision energy was 45 eV as standard conditions. The sampling cone voltage was set in the 20-50 V range to optimize the parent ion. Data acquisition and processing were carried out using software MassLynx version 4.0. Compounds were introduced using an HPLC apparatus Alliance 2695 equipped with an autosampler (2.5 µL injected) and a 2487 dual lambda absorbance detector set at 300 nm (Waters, Milford, MA) or by infusion (Harvard Apparatus, Holliston, MA). The separation of the compounds by HPLC was achieved using a column Uptisphere ODB (150 x 2 mm, 5 µm, Interchrom, Montluçon, France) with a 10 x 2.1 mm precolumn, and a mobile phase as following: water/acetonitrile 90/10 (v/v) during the first 5 min, then a linear gradient up to 100% acetonitrile at 30 min, followed by a 10 min stage at 100% acetonitrile. Each solvent was acidified with 0.2% acetic acid and the flow rate was 200 µL/min. The effluent from the UV detector was introduced without any split into the mass spectrometer. Infrared spectra (IR) were obtained on a Nicolet Avatar 320 FT-IR and are reported in terms of frequency of absorption (ν, cm^{-1}). UV spectra were obtained on a Beckman DU640B spectrophotometer. Melting points, recorded on a Buchi 510 are uncorrected.

Isolation procedures of 2,5-xylidine transformation products

Half of the concentrated residue (approx. 900 mg dry weight) dissolved in dichloromethane was subjected to column chromatography (3 cm *i.d.* x 30 cm) loaded with Silica gel 40 (35-70 mesh), and eluted with 4 successive mixtures of dichloromethane/ethyl acetate (100:0 / 90:10 / 50:50 / 0:100, 500 ml each) to obtain 15 colored fractions. These fractions represented 70% of initial material. Afterwards, each fraction (21 to 128 mg) was concentrated to dryness

under reduced pressure, dissolved in dichloromethane, and subjected to preparative HPLC on a Supelcosil LC-Si column (21.2 mm *i.d.* x 25 cm) eluted with a ternary mixture of *n*-hexane/dichloromethane/ethyl acetate (97:3:0 to 0:95:5 in 100 min) at the flow rate of 16 ml/min. Fractions from successive runs were pooled according to their retention times and UV-Vis spectra (230-650 nm). The combined fractions were then concentrated to dryness, dissolved in acetonitrile, and analysed through a second preparative HPLC on a Kromasil C18 column (20 mm *i.d.* x 25 cm) eluted with a gradient of water/acetonitrile (15:85 to 0:100 in 60 min) at 16 ml/min. Finally, the fractions corresponding to the purified compounds were concentrated under vacuum and the resulting aqueous phase was extracted 3-time with dichloromethane. After evaporation of the solvent, the samples were used for spectrometric identification.

In parallel, all the steps were tested by thin layer chromatography carried out on E Merck Ref 5554 precoated silica gel 60F 254 plates, and eluted with dichloromethane/ethyl acetate (95:5).

Spectrometric identification of isolated compounds

Compound **2**, (4*E*)-4-(2,5-dimethylphenylimino)-2,5-dimethylcyclohexa-2,5-dienone. Orange solid. M.p. 71-72°C (Et_2O). – ^{1}H NMR: δ = 7.13 (d, J = 7.5 Hz, 1 H, H-3'), 6.92 (br d, J = 7.5 Hz, 1 H, H-4'), 6.71 (q, J = 1.5 Hz, 1 H, H-3), 6.55 (q, J = 1.2 Hz, 1 H, H-6'), 6.40 (br s, 1 H, H-6), 2.27 (s, 3 H, Me-3'), 2.23 (d, J = 1.2 Hz, 3 H, Me-5), 2.00 (s, 3 H, Me-1'), 1.87 (d, J = 1.5 Hz, 3 H, Me-5). – ^{13}C NMR: δ = 188.3 (s), 155.9 (s), 149.4 (s), 148.9 (s), 141.3 (s), 135.9 (s), 130.8 (d), 130.5 (d), 126.0 (d), 125.6 (d), 125.3 (s), 119.1 (d), 21.1 (q), 17.7 (q), 17.6 (q), 15.8 (q). – ESI MS *m/z* (%) 240 (MH^+, 100). – IR (neat, cm^{-1}): 2920, 1654, 1646, 1633, 1606, 1496, 1262. – UV spectrum (CH_3CN): λ_{max} 277 nm ($\log_{10}\varepsilon$ 4.12); λ_{max} 575

nm ($\log_{10}\varepsilon$ 3.05). For this compound **2**, the *E*-stereochemistry of the imine was established by 2D NMR COSY spectrum and nOe difference spectroscopy.

Compound **3**. Yellow-orange solid. M.p. 152-153°C (EtOAc/Et_2O). – ^{1}H NMR: δ = 7.12 (d, *J* = 7.8 Hz, 2 H), 6.89 (d, *J* = 7.8 Hz, 2 H), 6.54 (q, *J* = 0.9 Hz, 2 H), 6.47 (s, 2 H), 2.34 (s, 6 H), 2.16 (d, *J* = 0.9 Hz, 6 H), 2.07 (s, 6 H). – ^{13}C NMR: δ = 158.6 (s), 149.5 (s), 143.7 (s), 135.9 (s), 130.3 (d), 125.1 (d), 122.8 (d), 119.4 (d), 21.1 (q), 17.9 (q), 17.6 (q). – ESI MS *m/z* (%) 343 (MH^+, 100). Product ions [343]: 327, 313, 222, 208, 120. – IR (neat, cm^{-1}): 2920, 1598, 1581, 1495, 1430, 1376, 1267. – UV spectrum (CH_3CN): λ_{max} 304 nm ($\log_{10}\varepsilon$ 4.48); λ_{max} 455 nm ($\log_{10}\varepsilon$ 3.56).

Compound **4**. Blue-purple oil. – ^{1}H NMR: δ = 7.11 (d, *J* = 7.5 Hz, 1 H), 6.94 (q, *J* = 1.5 Hz, 1 H), 6.90 (s, 1 H), 6.86 (s, 1 H), 6.78 (d, *J* = 7.5 Hz, 1 H), 6.55 (s, 1 H), 6.50 (s, 1 H), 2.30-2.29 (m, 6 H), 2.25 (s, 3 H), 2.24 (s, 3 H), 2.13 (s, 3 H), 1.99 (d, *J* = 1.5 Hz, 3 H). – ^{13}C NMR: δ = 188.5 (s), 156.6 (s), 149.7 (s), 142.6 (s), 141.7 (s), 140.9 (s), 140.6 (s), 136.7 (s), 130.9 (d), 130.3 (d), 129.9 (s), 125.8 (d), 125.1 (s), 125.0 (s), 122.8 (d), 122.1 (d), 120.3 (d), 119.8 (d), 21.3 (q), 18.0 (q), 17.8 (q), 17.5 (q, 2 C), 16.0 (q). – ESI MS *m/z* (%) 359 (MH^+, 100), 344 (60), 239 (40). Product ions [359]: 343, 239, 224, 135. – IR (neat, cm^{-1}): 3366, 3018, 2920, 2858, 1641, 1618, 1579, 1499, 1313, 1263. – UV spectrum (CH_3CN): λ_{max} 289 nm ($\log_{10}\varepsilon$ 4.39); λ_{max} 571 nm ($\log_{10}\varepsilon$ 3.83).

Compound **5**. Dark-orange oil. – ^{1}H NMR: δ = 6.99 (s, 1 H), 6.86-6.84 (m, 2 H), 6.71 (s, 1 H), 6.56 (q, *J* = 1.2 Hz, 1 H), 6.47 (s, 1 H), 2.31 (d, *J* = 1.2 Hz, 3 H), 2.22 (s, 3 H), 2.10 (s, 3 H), 2.05 (s, 3 H), 2.02 (s, 3 H), 1.98 (d, *J* = 1.2 Hz, 3 H). – ^{13}C NMR: δ = 188.4 (s), 157.8 (s), 149.5 (s), 147.6 (s), 141.7 (s), 141.2 (s), 139.0 (s), 134.7 (s), 134.3 (s), 133.1 (s), 132.3 (d), 131.8 (d), 130.8 (d), 125.7 (d), 120.8 (s), 119.8 (d), 117.5 (d), 19.6 (q), 19.4 (q), 17.8 (q), 17.6 (q), 17.1 (q), 15.9 (q). – ESI MS *m/z* (%) 359 (MH^+, 100). Product ions [359]: 343, 329, 312,

~~238~~, 223, 208, 193. – IR (neat, cm^{-1}): 3460, 3369, 3008, 2921, 1645, 1627, 1605, 1571, 1582, 1496, 1268. – UV spectrum (CH_3CN): λ_{max} 282 nm ($\log_{10}\varepsilon$ 4.40); λ_{max} 495 nm ($\log_{10}\varepsilon$ 3.41).

Compound **6**. Red oil. – ^{1}H NMR (C_6D_6): δ = 7.08 (s, *J* = 7.5 Hz, 1 H), 6.94-6.89 (br s, 1 H), 6.82 (d, *J* = 7.5 Hz, 1 H), 6.71 (q, *J* = 1.2 Hz, 1 H), 6.58 (s, 1 H), 6.46 (s, 1 H), 6.38-6.34 (br s, 1 H), 3.60-3.10 (vbr s, 2 H, NH_2), 2.23 (s, 3 H), 2.15 (s, 3 H), 2.14 (s, 3 H), 2.12 (s, 3 H), 2.06 (d, *J* = 1.2 Hz, 3 H), 1.84 (s, 3 H). – ^{13}C NMR: δ = 158.5 (s), 149.0 (br s), 143.8 (br s), 142.3 (br s), 135.9 (s), 130.4 (d), 125.0 (d), 124.8 (d), 123.0 (d), 121.9 (d), 119.7 (d), 117.8 (br s), 21.1 (q), 18.1 (q), 18.0 (q), 17.8 (q), 17.6 (q), 17.2 (q) 6 carbons are missing. – ESI MS *m/z* (%) 358 (MH^+, 100), 343 (5). Product ions [358]: 342, 328, 237, 223, 135. – IR (neat, cm^{-1}): 3500-2900 (br), 1598, 1497, 1303. – UV spectrum (CH_3CN): λ_{max} 300 nm ($\log_{10}\varepsilon$ 4.47); λ_{max} 515 nm ($\log_{10}\varepsilon$ 3.84).

Compound **7**. Red oil. – ^{1}H NMR: δ = 7.14 (d, *J* = 7.8 Hz, 1 H), 6.91 (br d, *J* = 7.8 Hz, 1 H), 6.86 (q, *J* = 1.2 Hz, 1 H), 6.67 (q, *J* = 0.6 Hz, 1 H), 6.61 (s, 1 H), 6.59 (q, *J* = 0.6 Hz, 1 H), 6.57 (q, *J* = 1.2 Hz, 1 H), 6.52 (s, 1 H), 6.51 (s, 1 H), 2.35 (s, 3 H), 2.32 (d, *J* = 1.2 Hz, 3 H), 2.23 (br s, 3 H), 2.21 (br s, 3 H), 2.15 (s, 3 H), 2.13 (s, 3 H), 2.09 (s, 3 H), 1.98 (d, *J* = 1.2 Hz, 3 H). – ^{13}C NMR: δ=188.4 (s), 158.9 (s), 158.6 (s), 157.8 (s), 149.4 (s), 147.4 (s), 145.7 (s), 144.0 (s), 143.6 (s), 141.1 (s), 135.9 (s), 130.7 (d), 130.4 (d), 130.1 (s), 127.6 (s), 126.7 (s), 125.6 (d), 125.2 (d), 122.9 (d), 122.7 (d), 121.2 (d), 121.1 (d), 119.4 (d), 21.1 (q), 18.1 (q), 18.0 (q), 17.8 (q), 17.7 (q, 2 C), 17.6 (q), 16.0 (q). – ESI MS *m/z* (%) 498 (MNa^+, 10), 476 (MH^+, 100). Product ions [476]: 460, 355, 341, 327, 253, 238, 222. – IR (neat, cm^{-1}): 2919, 1644, 1631, 1598, 1579. – UV spectrum (CH_3CN): λ_{max} 298 nm ($\log_{10}\varepsilon$ 4.26); λ_{max} 523 nm ($\log_{10}\varepsilon$ 3.61).

Compound **8**. Red oil. – ^{1}H NMR (C_6D_6): δ = 7.31 (s, 1 H), 7.30 (s, 1 H), 7.08 (s, 1 H), 7.04 (q, *J* = 1.2 Hz, 1 H), 6.87 (s, 1 H), 6.63 (s, 1 H), 6.48 (s, 1 H), 6.46 (s, 1 H), 2.40 (s, 3 H), 2.36 (s, 3 H), 2.30 (s, 6 H), 2.29 (s, 3 H), 2.25 (d, *J* = 1.2 Hz, 3 H), 2.02 (s, 3 H), 1.96 (s,

3 H). – ^{13}C NMR: δ = 159.6 (s), 158.3 (s), 149.8 (s), 145.2 (s), 144.6 (s), 143.6 (s), 143.4 (s), 142.1 (s), 139.1 (s), 135.1 (s), 133.3 (d), 132.7 (s), 132.6 (d), 130.2 (s), 126.3 (s), 123.7 (d), 122.9 (d), 122.5 (d), 120.9 (d), 120.2 (s), 120.0 (s), 117.9 (d), 117.0 (d), 20.4 (q, 2 CH_3), 18.6 (q, 3 CH_3), 18.4 (q), 17.4 (q, 2 CH_3). – ESI MS *m/z* (%) 477 (MH^+, 100). Product ions [477]: 461, 447, 237, 223, 135. – IR (neat, cm^{-1}): 3455, 3372, 3218, 3006, 2919, 1624, 1598, 1572, 1500, 1260. – UV spectrum (CH_3CN): λ_{max} 300 nm ($\log_{10}\varepsilon$ 4.36); λ_{max} 519 nm ($\log_{10}\varepsilon$ 3.74).

Compound **9**. Blue oil. – ^{1}H NMR: δ = 6.96 (br s, 2 H), 6.95 (s, 2 H), 6.89 (s, 1 H), 6.80 (s, 1 H), 6.55 (q, *J* = 1.2 Hz, 1 H), 6.50 (s, 1 H), 2.30 (d, *J* = 1.2 Hz, 3 H), 2.28 (s, 6 H), 2.25 (s, 3 H), 2.16 (s, 3 H), 2.05 (s, 3 H), 2.02 (s, 3 H), 2.00 (d, *J* = 1.2 Hz, 3 H). – ^{13}C NMR: δ = 188.5 (s), 156.5 (s), 149.7 (s), 142.3 (s), 141.1 (s), 140.4 (s), 140.2 (s), 135.5 (s), 135.0 (s), 134.9 (s), 132.5 (d), 132.2 (d), 130.2 (d), 125.8 (d), 125.4 (s), 124.3 (s), 122.2 (d), 120.6 (d), 119.6 (d), 118.5 (d), 19.8 (q), 19.6 (q), 18.1 (q), 17.9 (q), 17.5 (q), 17.4 (q), 17.2 (q), 16.0 (q). – ESI MS *m/z* (%) 478 (MH^+, 100). Product ions [478]: 462, 341, 238. – IR (neat, cm^{-1}): 3378, 2921, 1638, 1617, 1493, 1395, 1261. – UV spectrum (CH_3CN): λ_{max} 289 nm ($\log_{10}\varepsilon$ 3.55); λ_{max} 574 nm ($\log_{10}\varepsilon$ 2.91).

Compound **10**. Orange oil. – ^{1}H NMR: δ = 7.59 (s, 1 H), 7.45 (s, 1 H), 7.23 (d, *J* = 8.1 Hz, 1 H), 7.16 (d, *J* = 8.1 Hz, 1 H), 7.13 (d, *J* = 7.8 Hz, 1 H), 6.86 (d, *J* = 7.8 Hz, 1 H), 6.62 (q, *J* = 0.6 Hz, 1 H), 6.58-6.56 (m, 2 H), 6.48 (s, 1 H), 2.75 (s, 3 H), 2.72 (s, 3 H), 2.40 (s, 3 H), 2.35 (s, 3 H), 2.18 (s, 9 H), 2.13 (s, 3 H), 2.08 (s, 3 H). – ^{13}C NMR: δ = 158.8 (s), 158.6 (s), 152.4 (s), 151.3 (s), 149.4 (s), 148.2 (s), 144.3 (s), 143.5 (s), 136.8 (s), 136.1 (s), 135.9 (s), 134.7 (s), 131.2 (d, 2 **C**H), 130.4 (d), 126.6 (s), 125.2 (d), 123.1 (d), 122.8 (d), 120.9 (d), 119.4 (d), 118.0 (d), 116.5 (d), 21.2 (q), 21.1 (q), 18.0 (q), 17.9 (q, 2 C), 17.6 (q), 17.5 (q), 17.4 (q). – ESI MS *m/z* (%) 475 (MH^+, 100), 343 (95). Product ions [475]: 459, 354, 341, 327, 311, 252, 237, 222, 207. – IR (neat, cm^{-1}): 2920, 1597, 1582, 1496, 1268. – UV spectrum (CH_3CN): λ_{max} 300 nm ($\log_{10}\varepsilon$ 4.42); λ_{max} 365 nm ($\log_{10}\varepsilon$ 4.48),); λ_{max} 465 nm ($\log_{10}\varepsilon$ 4.03).

Compound **11**. Purple-blue oil. – ^{1}H NMR: δ = 6.96-6.91 (m, 6 H), 6.55 (q, *J* = 1.2 Hz, 1 H), 6.51 (s, 1 H), 2.34 (s, 3 H), 2.30 (d, *J* = 1.2 Hz, 3 H), 2.28 (s, 3 H), 2.25 (s, 3 H), 2.16 (s, 3 H), 2.06 (s, 3 H), 2.00 (s, 3 H), 1.99 (d, *J* = 1.2 Hz, 3 H). – ESI MS *m/z* (%) 478 (MH^+, 100). Product ions [478]: 462, 341, 253, 238, 224. – IR (neat, cm^{-1}): 3363, 2920, 1652, 1639, 1621, 1605, 1575, 1496. – UV spectrum (CH_3CN): λ_{max} 266 nm ($\log_{10}\varepsilon$ 3.25); λ_{max} 288 nm ($\log_{10}\varepsilon$ 3.29); λ_{max} 570 nm ($\log_{10}\varepsilon$ 2.48).

Compound **12**. Orange oil. – ^{1}H NMR: δ = 7.23 12 (d, *J* = 7.8 Hz, 1 H), 7.00-6.88 (m, 3 H), 6.66 (q, *J* = 0.6 Hz, 1 H), 6.56 (q, *J* = 0.6 Hz, 1 H), 6.54 (s, 1 H), 6.48 (s, 1 H), 2.34 (s, 3 H), 2.33 (s, 3 H), 2.20 (s, 3 H), 2.17 (s, 3 H), 2.08 (s, 3 H), 2.07 (s, 3 H), 2.05 (s, 6 H). – ^{13}C NMR: δ=158.7 (s), 149.5 (s), 148.3 (s), 143.75 (s), 143.71 (s), 137.5 (s), 135.9 (s), 135.2 (s), 134.1 (s), 132.2 (d), 131.8 (d), 130.4 (d), 125.4 (s), 125.1 (d), 122.85 (d), 122.79 (d), 120.0 (d), 119.5 (d), 21.2 (q), 19.7 (q), 19.6 (q), 18.1 (q), 18.0 (q), 17.6 (q, 2C), 17.3 (q). – ESI MS *m/z* (%) 462 (MH^+, 50), 252 (32), 231 (100). Product ions [462]: 446, 432, ~~355~~, 341, 327, 239, 222. – IR (neat, cm^{-1}): 3360, 1598, 1580, 1487, 1266. – UV spectrum (CH_3CN): λ_{max} 306 nm ($\log_{10}\varepsilon$ 4.11); λ_{max} 469 nm ($\log_{10}\varepsilon$ 3.19).

Compound **13**. Red oil. – ^{1}H NMR: δ = 6.98 (s, 1 H), 6.88 (s, 2 H), 6.70 (s, 1 H), 6.68 (s, 1 H), 6.66 (s, 1 H), 6.60 (s, 1 H), 6.57 (s, 1 H), 6.53 (s, 1 H), 6.52 (s, 1 H), 2.33 (s, 3 H), 2.21 (s, 9 H), 2.16 (s, 3 H), 2.12 (s, 3 H), 2.09 (s, 3 H), 2.06 (s, 3 H), 2.03 (s, 3 H), 1.99 (s, 3 H). – ^{13}C NMR: δ = 188.4 (s), 159.0 (s), 158.6 (s), 157.8 (s), 149.5 (s), 148.2 (s), 147.6 (s), 145.6 (s), 144.1 (s), 143.5 (s), 142.3 (s), 141.1 (s), 138.1 (s), 134.8 (s), 134.3 (s), 133.1 (s), 132.2 (d), 131.9 (d), 130.7 (d), 127.6 (s), 126.6 (s), 125.7 (d), 125.3 (s), 123.0 (d), 122.6 (d), 121.21 (d), 121.15 (d), 120.4 (s), 119.9 (d), 117.1 (d), 19.7 (q), 19.6 (q), 18.1 (q), 18.0 (q), 17.8 (q), 17.7 (2 C, q), 17.6 (q), 17.0 (q), 16.0 (q). – ESI MS *m/z* (%) 595 (MH^+, 25), 318 (50), 298 (100). Product ions [595]: 579, 565, 460, 355, 341, 253, 239. – IR (neat, cm^{-1}): 3385, 3007,

2954, 2920, 1652, 1642, 1628, 1598, 1576, 1487, 1440, 1376, 1263. – UV spectrum (CH_3CN): λ_{max} 211 nm ($\log_{10}\varepsilon$ 4.88); λ_{max} 282 nm ($\log_{10}\varepsilon$ 4.94); λ_{max} 520 nm ($\log_{10}\varepsilon$ 4.09).

Compound **14**. Blue oil. – ^{1}H NMR (C_6D_6): δ = 7.06 (d, *J* = 7.8 Hz, 1 H), 6.94 (s, 1 H), 6.84-6.82 (m, 3 H), 6.80 (s, 1 H), 6.78 (s, 1 H), 6.74 (s, 1 H), 6.45 (d, *J* = 1.2 Hz, 1 H), 6.43 (s, 1 H), 2.13-2.07 (30 H). – ESI MS m/z (%) 595 (MH^+, 100), 481 (10), 413 (12), 342 (5). Product ions [595]: 579, 565, 474, 460, 341, 253, 238. – IR (neat, cm^{-1}): 2956, 2918, 1644, 1627, 1604, 1573, 1496. – UV spectrum (CH_3CN): λ_{max} 204 nm ($\log_{10}\varepsilon$ 4.20); λ_{max} 303 nm ($\log_{10}\varepsilon$ 3.93); λ_{max} 554 nm ($\log_{10}\varepsilon$ 3.38).

Synthesis of compound **2**, (4*E*)-4-(2,5-dimethylphenylimino)-2,5-dimethylcyclohexa-2,5-dienone

We used a method similar to that previously described by Dibattista et al. (2002). To a solution of 2,5-dimethylaniline (4.2 mL, 33.03 mmol) in benzene (90 mL) at 10°C under argon was added dropwise a solution of $TiCl_4$ (5.5 mL, 1 M in CH_2Cl_2, 5.5 mmol). The resultant solution was stirred for 10 minutes at 10°C and a solution of 2,5-dimethyl-*p*-benzoquinone (1.5 g, 11.01 mmol) in benzene was added at 10°C. The reaction mixture was warmed to room temperature, stirred for 5 h diluted with Et_2O (150 mL). The organic phase was washed with aqueous saturated sodium bicarbonate solution (100 mL), aqueous HCl (10%) (100 mL), brine (100 mL), dried ($MgSO_4$) and concentrated. The residue was purified by chromatography on silica gel (EtOAc/cyclohexane 0:100 to 5:95) to give 1.78 g of compound **2** (67%, 7.44 mmol) as an orange solid.

Results

Isolation and identification of compounds formed from 2,5-xylidine

After the different steps of preparative chromatography, 2,5-xylidine and thirteen transformation products were purified from the incubation medium with laccases of *P. cinnabarinus*. One dimeric (compound **2**), 4 trimeric (compounds **3**-**6**), 6 tetrameric (compounds **7**-**12**) and 2 pentameric (compounds **13**,**14**) forms were purified and identified from the organic extracts (Figure 1). Their structures were established through UV, LC-ESI-MS, LC-ESI-MS/MS and NMR analysis. Almost all compounds were identified using a combination of data issuing from these techniques. The structure determination of the isomeric pentamers was particularly delicate. Indeed, compound **14** could be assigned by careful comparison of the NMR data with those of compound **7**, a tetramer analogue and probable precursor. However, a detailed comparative MS/MS study of the decomposition of the MH^+ ions of isomers **13** and **14** in function of the collision energy was necessary to identify **13** unambiguously (and to differentiate it from another potent isomeric form, containing the same cyclic internal subunits but ordered differently). In the case of **13**, the most decisive diagnostic products ions resulting from the decomposition of the selected parent ion MH^+ are those corresponding formally to successive losses of CH_4 and the bicyclic radical H_2N-$C_6H_2(CH_3)_2$-$C_6H_2(CH_3)_2$° (at m/z 355) or O=$C_6H_2(CH_3)_2$=N-$C_6H_2(CH_3)_2$° (at m/z 341). These two ions are accompanied by complementary cations respectively at m/z 239 and 253.

The amount of products recovered from 1 g initial 2,5-xylidine varied according to the compounds from 7 to 65 mg per g of 2,5-xylidine, without a clear relationship with the size of the molecules (Figure 1). All the compounds purified from the dichloromethane extract represented 36% of the initial amount of the parent compound. It is likely that a part of 2,5-xylidine was transformed into higher-molecular weight compounds (or eventually into hydrophilic compounds) that were not extracted by the solvent.

Additional transformation experiments of 2,5-xylidine were performed using purified laccases from *T. versicolor*. Most of the transformation products of 2,5-xylidine identified with laccase from *P. cinnabarinus* were also formed in the presence of laccase from *T. versicolor*.

Effects of transformation products of 2,5-xylidine on laccase activity

We evaluated the potential of individual oligomers (**2** to **12**) formed from 2,5-xylidine to enhance fungal laccase production in liquid cultures of *T. versicolor* after 72-hour treatments (Figure 2). Compounds **13** and **14** have not been assayed because of their very low aqueous solubility. Compounds **3**, **10** and **12** had no or only minor effects. All other compounds diversely induced laccases, with a concentration-dependent relationship. The most active compounds were **2**, **4** and **6**. They resulted in a 6 to 9-fold enhancement of laccase activity with respect to untreated cultures (negative controls) and in a 2 to 3-fold enhancement with respect to 2,5-xylidine (compound **1**) used as a positive control. No toxic effects of oligomers (**2** to **12**) have been noticed on fungal growth (data not shown).

In order to assess the potential of the oligomers to induce laccase production during the first hours of treatment, we measured the time-course of laccase induction on 4-day cultures spiked with selected compounds, each at $1.25\ 10^{-5}$ M. Laccase activity was measured every 4 hours during the initial 28-hour period (Figure 3A). As evidenced above, compounds **3** and **12** failed in increasing enzyme yields. All other compounds were efficient from the beginning of the incubations, with an activity higher than that due to the parent compound **1** (2,5-xylidine). The dimer **2** was the most efficient inducer. Then, long-term incubations have been performed to investigate the maintenance of the effects (Figure 3B). The results were similar with a plateau concerning compound **2** between 72 and 144 hours.

Proposal for a new inducer of fungal laccases

We found that the most potent inducer in the present study was the compound **2**, (4*E*)-4-(2,5-dimethylphenylimino)-2,5-dimethylcyclohexa-2,5-dienone, whose efficiency was around 3-fold higher than that of the reference compound 2,5-xylidine (**1)** at low concentration (around 10^{-5} M, Figure 3 and Table 1). At ten-fold higher concentrations, levels of induction were quite identical for the two compounds 72 hours after spiking of the culture medium. Similar concentrations of 2,5-xylidine are classically used in many laboratories to obtain significant amounts of enzymes.

Discussion

Enzymatic transformation of 2,5-xylidine, a well-known inducer of fungal laccases, led to the formation of numerous oligomers by oxidative coupling. We isolated, purified and identified 13 of them. The formation of these oligomers could be explained by an iterative mechanism similar to that published by Baker et al. (1974). As purified oligomeric transformation products were not further transformed by purified laccases in the absence of added 2,5-xylidine (data not shown), it can be assumed that each n-mer compound results from the reaction between a 2,5-xylidine radical formed by laccase on a (n-1)-mer compound. In addition, it can be assumed that intermediates can combine with 2,5-dimethylbenzoquinone likely produced *in-situ* to yield Schiff bases.

We assessed the potential of 11 oligomers in inducing laccase activity in cultures of *T. versicolor*. They diversely enhanced laccase activity, with a time and concentration-dependent relationship. The effect of the most efficient compounds reached a plateau, which was probably due to the medium depletion in key components required for active laccase biosynthesis. Laccases are multicopper oxidases, which combine the four-electron reduction of dioxygen to water with the one-electron oxidation of four reducing substrate molecules. As

a consequence, copper is a key component for laccase activity. At high concentrations in the culture media (above 500 µM), the metal is known to stimulate the expression of laccase genes (Baldrian and Gabriel 2002; Soden and Dobson 2001). At lower amounts, it positively affects the activity and the stability of the formed enzymes. Because of its low concentration in our experiments (30 µM), it is likely that copper does not act as an inducer. By contrast, the medium can be rapidly depleted in copper, thus limiting the production of laccase in an active and stable form.

The treatment of fungal cultures with the oligomers formed from 2,5-xylidine resulted in an increased laccase activity. At the level of gene transcription, it has been demonstrated previously that xylidine was an inducer of laccases in *P. sajor-casu* (Soden and Dobson 2001). In our case, after extraction of the mRNAs from cultures of *T. versicolor*, synthesis of cDNAs by RT-PCR and relative quantification of genes by real time PCR, we confirmed these results, also showing time- and dose-response relationships. We demonstrated also that compound **2** enhanced the expression of laccase genes (Jolivalt et al. unpublished data).

High amounts of 2,5-xylidine are necessary to significantly induce the production of laccases because the molecule itself is a substrate for laccases (Bertrand 2002) and so is transformed during the culture time course. By contrast, compound **2,** which is not a substrate of laccases, could be useful as an inducer in biotechnological applications aiming at producing a great amount of laccases with a low amount of inducer. As a consequence, we developed a protocol for its easy synthesis in one step (67% yield) at 1-g scale from commercially available 2,5-dimethyl-*p*-benzoquinone and 2,5-xylidine. This protocol enables sufficient production of compound **2** for laboratory experiments.

In summary, the present study provides new results concerning the potential of the family of xylidines and related compounds to induce laccases in *T. versicolor*.

Acknowledgment

We are very grateful to Dr M. Asther (UBCF, INRA Luminy) for the generous gift of laccase purified from *P. cinnabarinus* ss3.

References

Baker PB, Saunders C (1974) Studies in peroxidase action-XXIII. The oxidation of poly-methylated anilines. Tetrahedron 30: 337-341 and references cited therein

Baldrian P and Gabriel J (2002) Copper and cadmium increase laccase activity in *Pleurotus ostreatus*. FEMS Microbiol Letters 206: 69-74

Bertrand T, Jolivalt C, Briozzo P, Caminade E, Joly N, Madzak C and Mougin C (2002) Crystal structure of a four-copper laccase complexed with an arylamine: insights into substrate recognition and correlation with kinetics. Biochemistry 41: 7325-7333

Bollag J-M and Leonowicz A (1984) Comparative studies of extracellular fungal laccases. Appl Environ Microbiol 48: 849-854

Dibattista J, Schmidt BM, Buyle Padias A, Hall HK Jr (2002) Substituent effects on the polycondensation of quinines with aromatic amines to form poly(quinone imine)s. J Polymer Sci A: Polymer Chem 40: 43-54

Fahraeus G, Tullander V and Ljunggren H (1958) Production of high laccase yields in cultures of fungi. Physiol Plant 11: 631-643

Gianfreda L, Xu F and Bollag J-M (1999) Laccases: a useful group of oxidoreductive enzymes. Bioremed J 3: 1-25

Herpoël I, Moukha S, Lesage-Meesen L, Sigoillot J-C and Asther M (2000) Selection of *Pycnoporus cinnabarinus* strains for laccase production. FEMS Microbiol Lett 183: 301-306

Jolivalt C, Brenon S, Caminade E, Mougin C, Pontié M (20000) Immobilization of laccase from *Trametes versicolor* on a modified PVDF microfiltration membrane: characterization of the grafted support and application in removing a phenylurea pesticide wastewater. J Membrane Sci 180: 103-113

Kallas J, Reinik J, Jakobsson K, Kirso U (2003) Physico-chemical methods for rocket fuel polluted groundwater treatment. Proceedings of the 4th European Meeting on Environmental Chemistry, Plymouth, England, p. 187

Lesage-Meesen L, Delattre M, Haon M, Thibault J-F, Colonna Ceccaldi B, Brunerie P, Asther M (1996) A two-step bioconversion process for vanillin production from ferulic acid combining *Aspergillus niger* and *Pycnoporus cinnabarinus*. J Biotechnol 50: 107-113

Mougin C, Kollmann A and Jolivalt C (2002) Enhanced production of laccase in the fungus *Trametes versicolor* by the ddition of xenobiotics. Biotechnol Lett 24: 139-142

Mougin C, Jolivalt C, Briozzo P, Madzak C (2003) Fungal laccases: from structure-activity studies to environmental applications. Environ Chem Lett 1: 145-148

Otterbein L, Record E, Chereau D, Herpoël I, Asther M and Moukha S (2000) Isolation of a new laccase isoform from the white-rot fungi *Pycnoporus cinnabarinus* strain ss3. Can J Microbiol 46: 759-763

Soden DM and Dobson ADW (2001) Differential regulation of laccase gene expression in *Pleurotus sajor-caju*. Microbiology 147: 1755-1763

Tatum EL, Barrat RW, Fries N and Bonner D (1950) Biochemical mutant strains of *Neurospora* produced by physical and chemical treatment. Am J Bot 37: 38-46

Wolfenden BS, Wilson RL (1982) Radical cations as a reference chromogens in kinetics studies of one electron transfer reactions: pulse radiolysis studies of 2,2'-azinobis-(3-ethylbenzthiazoline-6-sulphonate). J Chem Soc Perkin Trans II 805-812

Table 1. Laccase activity measured in cultures of *T. versicolor* grown for 72 hours in the presence of inducers at various concentrations. Data are means ± standard errors (n=3).

Treatment	Laccase activity (U/mL)		
	10^{-5} M	5 10^{-5} M	5 10^{-4} M
Control	0.26 ± 0.10	0.26 ± 0.10	0.26 ± 0.10
2,5-Xylidine (**1**)	2.14 +- 0.12	5.35 ± 1.17	5.60 ± 0.18
Compound **2**	6.61 ± 1.89	6.71 ± 1.13	n.m.

n.m.: not measured

Figure 1. Structure of compounds purified from incubation media containing 2,5-xylidine and fungal laccase. Yields are under brackets.

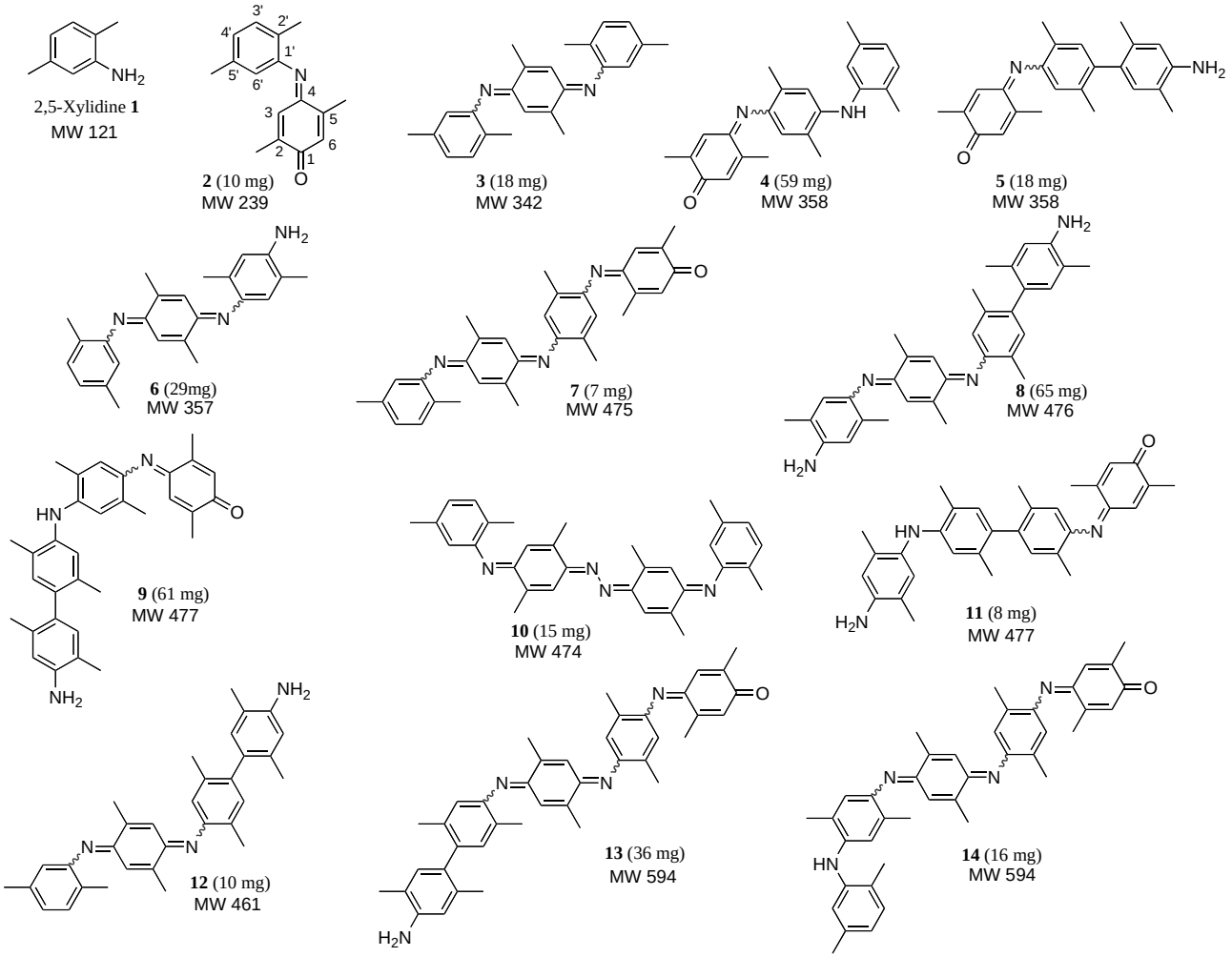

Figure 2. Concentration dependent laccase activity in liquid cultures of *T. versicolor* incubated during 72 hours in the presence of selected oligomers formed from 2,5-xylidine. Oligomer concentrations were: black, 0.31 10^{-5} M; light grey, 0.62 10^{-5} M and dark grey, 1.25 10^{-5} M. The numbers of compounds refer to those of the structures shown in Figure 1. Bars represent the standard error of each mean (n = 3).

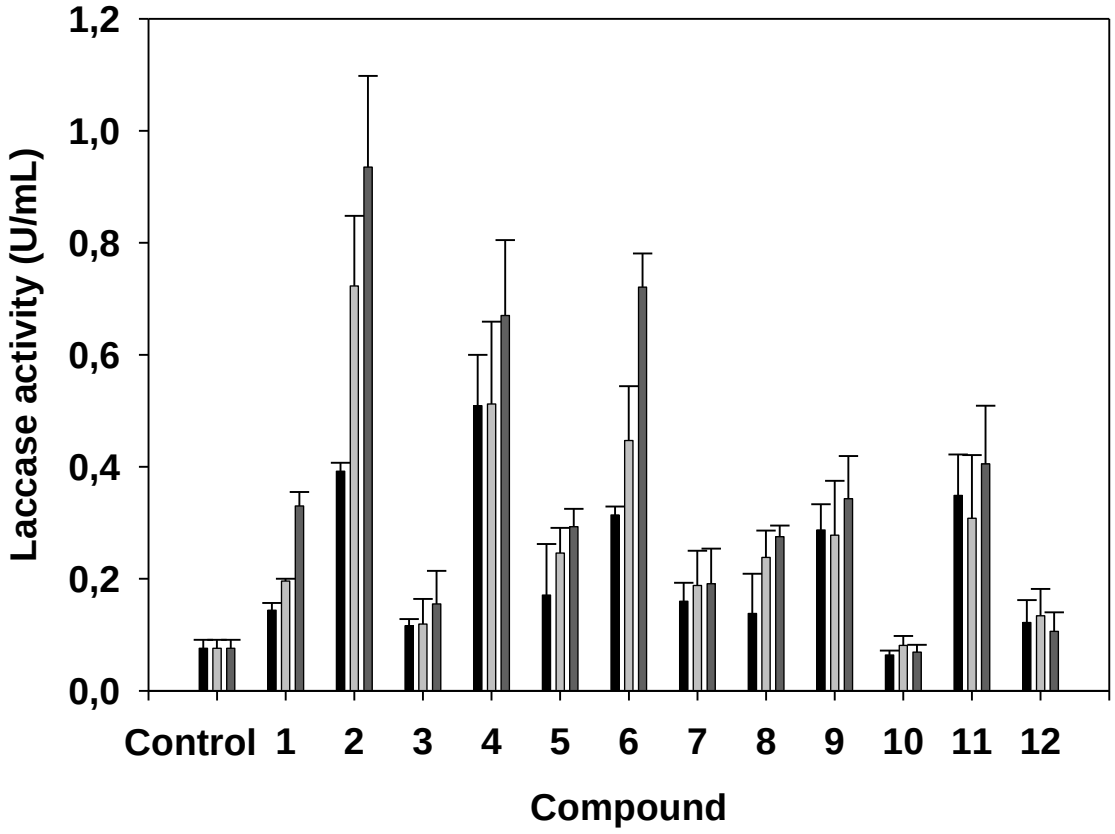

Figure 3. Time-course of laccase activity in liquid cultures of *T. versicolor* incubated during 28 hours (A) and 144 hours (B) in the presence of selected oligomers formed from 2,5-xylidine. Oligomer concentration was 1.25 10^{-5} M. The numbers of compounds refer to those of the structures shown in Figure 1. Bars represent the standard error of each mean (n = 4).