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BIOTECHNOLOGICAL PRODUCTS AND PROCESS ENGINEERING

Laccase-induced C–N coupling of substituted *p*-hydroquinones with *p*-aminobenzoic acid in comparison with known chemical routes

Annett Mikolasch · Anastasia Matthies · Michael Lalk · Frieder Schauer

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Abstract Fungal laccases (benzenediol:oxygen oxidoreductase, EC 1.10.3.2) from *Pycnoporus cinnabarinus* and *Myceliophthora thermophila* were used as biocatalysts for enzymatic reaction of halogen-, alkyl-, alkoxy-, and carbonyl-substituted *p*-hydroquinones (laccase substrates) with *p*-aminobenzoic acid (no laccase substrate). During this reaction, the laccase substrate was oxidized to the corresponding quinones, which react with *p*-aminobenzoic acid by amination of the laccase substrate. The different substitutions at the hydroquinone substrates were used to prove whether the substituents influence the position of amination and product yields. The cross-coupling of methoxy-*p*-hydroquinone (alkoxylated) and 2,5-dihydroxybenzaldehyd (carbonyl-substituted) with *p*-aminobenzoic acid resulted in the formation of one monoaminated product (yield alkoxylated 52%). If monohalogen- or monoalkyl-substituted *p*-hydroquinones were used as laccase substrates, two monoaminated products (constitution isomers) were formed. The simultaneous formation of two different monoaminated products from the same hydroquinone substrate is the first report for laccase-mediated synthesis of aminated constitution isomers. Depending from the type of substituent of the hydroquinone, the positions of the two monoaminations are different. While the amination at the monoalkylated hydroquinone occurs at the 5- and 6-positions (yield 38%), the amination at monohalogenated hydroquinones was detectable at the 3- and 5-positions (yield 53%). The same product pattern could be achieved if instead of the biocatalyst laccase the chemical catalyst sodium iodate was used as the oxidant. However, the yields were partially much lower (0–45% of the yields with laccase).

Keywords Laccase · Coupling · Hydroquinones · *p*-aminobenzoic acid

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Introduction

Fungal laccases (EC 1.10.3.2) are multicopper oxidases (Thurston 1994; Yaropolov et al. 1994), which are able to transfer electrons from phenolic hydroxyl groups to molecular oxygen, resulting in unstable phenoxy radicals and water (Bollag 1992; Tatsumi et al. 1994; Heinzkill et al. 1998). Because of their high stability and wide substrate spectrum, laccases are important enzymes increasingly applied in white biotechnology. Laccases have so far been applied mainly in the fields of waste detoxification, textile dye transformation, biosensors, and applications in the food industry and in pulp bleaching (Mayer and Staples 2002; Milligan and Ghindilis 2002; Bollag 1992; Minussi et al. 2002; Rochefort et al. 2004; Mickel et al. 2003; Murugesan et al. 2006; Nagai et al. 2002). On the basis of using aqueous solutions and the mild reaction conditions, the interest for applying laccases in green chemistry grew constantly during recent years (Agematu et al. 1993; Anyanwutaku et al. 1994; Bhalerao et al. 1994; Ikeda et al. 1996, 2001; Osiadacz et al. 1999; Majcherczyk et al. 1999; Schäfer et al. 2001; Uchida et al. 2001; Hosny and

Rosazza 2002; Mikolasch and Schauer 2003; Burton 2003; Riva 2006). Most of the laccase-catalyzed syntheses of aromatic amines comprise heterologous coupling reactions performed in aqueous systems resulting in the formation of a monoaminated and/or a diaminated quinone (Niedermeyer et al. 2005; Manda et al. 2005; Mikolasch et al. 2006, 2007). The cross-couplings of 2,5-dihydroxylated arenes with amino compounds are interesting methods for C–N coupling reactions relevant for the organic chemistry and resulted in the formation of one monoaminated quinonoid product per reaction or an unclear product pattern.

To characterize the potential use of laccase-catalyzed reactions in fine chemical synthesis and to gain more basic information about the product pattern, different halogen-, alkyl-, alkoxy-, and carbonyl-substituted *p*-hydroquinones were brought to reaction with *p*-aminobenzoic acid by using fungal laccases from *Pycnoporus cinnabarinus* and *Myceliophthora thermophila*. The aim of following studies was not only the formation of new products but especially to study the influence of different substituents of laccase substrates on the position of amination in cross-coupling reactions. We show for the first time that laccase-catalyzed amination results in the simultaneous formation of two different monoaminated products (constitution isomers) if monohalogen- or monoalkyl-substituted *p*-hydroquinones were aminated with *p*-aminobenzoic acid. Furthermore, we compared the reaction course and the resulting products of laccase-catalyzed oxidative reaction with the synthesis of aminoquinones using the chemical oxidant sodium iodate (Schäfer and Aguado 1971; Pardo et al. 1979; Torres et al. 1985). Advantages of laccase-catalyzed amination are discussed.

Materials and methods

Chemicals

All *p*-hydroquinones were purchased from Sigma-Aldrich Fine Chemicals (Taufkirchen, Germany). The *p*-aminobenzoic acid was obtained from Serva Feinbiochemica (Heidelberg, Germany). All chemicals were of p.A. quality.

Enzymes

Fungal strain P. cinnabarinus SBUG-M 1044 was isolated from an oak tree in northern Germany. The white rot fungus is deposited at the strain collection of the Department of Biology of the University Greifswald (SBUG).

Cultivation of P. cinnabarinus SBUG-M 1044 P. cinnabarinus was initially cultivated on malt agar plates that were incubated for 7 days at 30°C and then kept at 4°C. The liquid culture was prepared by inoculating a nitrogen-rich medium containing 5 g glucose, 1 g KH_2PO_4, 0.52 g L-asparagine, 0.5 g yeast extract, 0.5 g KCl, 0.5 g $MgSO_4 \cdot 7H_2O$, 50 mL mineral salt solution, and 50 mL $FeSO_4$ solution (0.2 $g \cdot L^{-1}$) with three 1-cm^3 agar culture fragments. The mineral salt solution contained 1 g $Ca(NO_3)_2 \cdot 4H_2O$, 0.06 g $CuSO_4 \cdot 5H_2O$, and 0.04 g $ZnSO_4 \cdot 7H_2O$ per liter (modified according to Braun-Lüllemann et al. 1997). Incubation was performed without shaking at 30°C for 7 days. A uniform inoculum was obtained by homogenization of this culture with an Ultra-Turrax homogenizer T25 (IKA Labortechnik, Staufen, Germany) at 8,000 rpm. For the production of the ligninolytic enzyme laccase, 40 mL medium inoculated with 2 mL of the homogenized preculture was incubated in 100-mL Erlenmeyer flasks for 7 days with 3,4-dimethoxybenzyl alcohol (10 mM), a known inducer of laccase. Cultures were shaken in a water bath (GFL model 1092, Burgwedel, Germany) at 30°C and 158 rpm.

Preparation of laccase from P. cinnabarinus SBUG-M 1044 P. cinnabarinus was cultivated as described above. Under these conditions, *P. cinnabarinus* produced laccase as a single extracellular enzyme with activity at a level of 500 $nmol \cdot mL^{-1} \cdot min^{-1}$ (substrate: 2,2′-amino-bis-3-ethylbenzthiazoline-6-sulfonic acid [ABTS]). The culture medium was filtered through a glass fiber filter in a Büchner funnel to separate the medium from whole cells. The cell-free culture medium was stirred with diethylaminoethyl (DEAE)-Sephacel (Sigma, Steinheim, Germany) for 1 h, and the adsorbed enzymes were eluted from the DEAE-Sephacel with 20 mM sodium acetate buffer (pH 5). This enzyme extract was desalted using a Sephadex G-25 Superfine column (Pharmacia, Freiburg, Germany). This enzyme preparation contains only isoenzymes of laccase and had an activity of 2 U mg^{-1} and was used always in sodium acetate buffer pH 5.0 because of the pH optimum around pH 5.0 (Feng et al. 1996; Eggert et al. 1996; Jonas 1998).

Laccase from M. thermophila Laccase from *M. thermophila* (expressed in genetically modified *Aspergillus sp.*) was bought from Novozymes (Bagsvaerd, Denmark). It was used as received (activity 1,000 U g^{-1}; substrate: syringaldazine) always in citrate–phosphate buffer pH 7.0 because of the pH optimum around pH 7.0 (Feng et al. 1996; Berka et al. 1997).

Measurement of laccase activity The activity of laccases was determined spectrophotometrically at 420 nm with ABTS as substrate (Bourbonnais and Paice 1990) using the method described by Jonas et al. (1998). One unit is defined as 1 $\mu mol \cdot mL^{-1} \cdot min^{-1}$.

Laccase-catalyzed amination of different substituted p-hydroquinones and quinones with p-aminobenzoic acid For

analytical scale experiments, the compounds were incubated at equimolar concentrations of 1 mM with 0.5, 1, 2, 4, or 10 U/ml of laccases (substrate: ABTS) in 1 ml of sodium acetate buffer (20 mM, pH 5.0) and citrate–phosphate buffer (18 mM citrate, 165 mM phosphate, pH 7.0) for laccases from *P. cinnabarinus* and *M. thermophila*, respectively. The reaction mixture was incubated at room temperature for 24 h.

For preparative scale, laccase of *P. cinnabarinus* (final activity 1.0 U in reaction mixture) was added to 50 mL of a solution of the compounds dissolved in sodium acetate buffer, pH 5.0. For the synthesis of monoaminated quinones, hydroquinone excess was used (2:1 mM). Products of alkyl-substituted *p*-hydroquinones were isolated 24 h after addition of the enzyme, and products of halogen-, alkoxy-, and carbonyl-substituted *p*-hydroquinones and of *p*-hydroquinone were isolated 60 min after starting the reaction.

Chemical product synthesis

Final concentrations in each reaction of *p*-hydroquinones and *p*-aminobenzoic acid **2a** were 1 mM. Final concentrations for sodium iodate were 6, 24, 50, 100, and 320 mM in sodium acetate buffer, pH 5.0, or in citrate–phosphate buffer, pH 7.0, resulting comparable oxidative reactivity as 0.5, 1, 2, 4, and 10 U activity of laccase. The reaction mixtures were incubated at room temperature for 24 h.

Isolation of products

All products were isolated from reaction mixture of preparative scale as described above.

*Product **3a*** Product **3a** (yield 32.0%) was isolated from the reaction mixture of *p*-hydroquinone **1a** and *p*-aminobenzoic acid **2a**. All isolation steps were performed by solid-phase extraction with an RP18 silica gel column (StrataC18-E, 55 μm, 70 Å, 10 g/60 ml Phenomenex, Aschaffenburg, Germany). After activation with methanol and equilibration with water, the column was loaded with 50 mL of the reaction mixture. Column was washed with 25 mL of water and 25 mL of methanol/water (10:90 *v*/*v*) to remove laccase and polar impurities from the column. **3a** was eluted with methanol/water 30:70 *v*/*v*. The red eluate was collected in fractions, which were analyzed by high-performance liquid chromatography (HPLC) for pure products. After solid-phase extraction, the product containing eluates were combined and dried under vacuum at 35°C.

*Products **3b$_1$**, **3b$_2$*** Products $\mathbf{3b_1}$ and $\mathbf{3b_2}$ together (yield together 38.9%) were isolated from the reaction mixture of 2-methyl-*p*-hydroquinone **1b** and *p*-aminobenzoic acid **2a** by the same isolation steps as described for **3a**.

*Product **3c*** Product **3c** (yield 6.7%) was isolated from the reaction mixture of 2-*ter*t-butyl-*p*-hydroquinone **1c** and *p*-aminobenzoic acid **2a** by the same isolation steps as described for **3a** with one exception. **3c** was eluted with methanol/water 50:50 *v*/*v*.

*Product **3d*** Product **3d** (yield 51,9%) precipitated from the reaction mixture of 2-methoxy-*p*-hydroquinone **1d** with *p*-aminobenzoic acid **2a** as a red solid. The red precipitate was washed three times with 50 mL of water and dried on the atmosphere.

*Products **3g$_1$**, **3g$_2$*** Product $\mathbf{3g_1}$ (yield 38.17%) and $\mathbf{3g_2}$ (yield 15.1%) were isolated from the reaction mixture of 2-chloro-*p*-hydroquinone **1g** and *p*-aminobenzoic acid **2a** by the same isolation steps as described for **3a** until the elution of the product from the RP18 silica gel column. $\mathbf{3g_1}$ and $\mathbf{3g_2}$ together were eluated from the column with 100% methanol. This eluate was evaporated to a smaller volume of 10 ml. $\mathbf{3g_1}$ and $\mathbf{3g_2}$ were separated by preparative HPLC (Merk-HITATCHI, column: LiChroCART® 125-4 RP 18e; 5 μm [Merck, Darmstadt, Germany]). One RP18 silica gel column was loaded with the fractions of separated product $\mathbf{3g_1}$, and an other RP18 silica gel column was loaded with the fractions of separated product $\mathbf{3g_2}$ to remove the products from the water phase. The products were eluated with 100% methanol. The methanol eluates with pure products were dried under vacuum at 35°C.

*Product **3h*** Product **3h** (yield 38.45%) was isolated from the reaction mixture of 2,6-dichloro-*p*-hydroquinone **1h** and *p*-aminobenzoic acid **2a** by the same isolation steps as described for **3a** with one exception. **3h** was eluted with 100% methanol.

For all products, yields have not been optimized.

Characterization of products

Analytical high-performance liquid chromatography For routine analysis, samples of the incubation mixture were analyzed using an HPLC system LC-10ATvP (Shimadzu, Germany) consisting of an FcV-10ATvP pump, a SPD-M10AvP photodiode array detector (200–595 nm), and a SCL-10AvP control unit controlled by VPClass 5.0. The separation of the substances was achieved on an end-capped, 5-μm, LiChroCart 125-4 RP 18 column (Merck). A solvent system consisting of phosphoric acid, 0.1% pH 2 (eluent A), and methanol (eluent B), starting from an initial ratio of 90% A and 10% B and reaching 100% B within 14 min, was used at a flow rate of 1 mL/min.

Mass spectrometry The products were characterized by mass spectrometry (MS) and liquid chromatography/MS (LC/MS) using a Bruker-Daltoniks micrOTOF instrument (Bremen, Germany; ionization method: electrospray ionization [ESI], dry and nebulizer gas: nitrogen, software: HyStar).

Nuclear magnetic resonance The nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Avance 600 instrument (Rheinstetten, Germany) at 600 MHz.

3a *(2-[4′-(Carboxyphenyl)amino]-1,4-benzoquinone)* Synthesis and isolation are as described above. The characteristics are as follows: Red precipitate. Yield 32.0%. R_f (HPLC) 9.56 min, UV-Vis (MeOH) λ_{max} 201, 285, 497 nm.^{1}H NMR (methanol-d_4) δ 8.07(d, ^{3}J=8.6 Hz, 2H, H3′, H5′), 7.41 (d, ^{3}J=8.5 Hz, 2H, H2′, H6′), 6.80 (d, ^{3}J= 10.1 Hz, 1H, H6), 6.73 (dd, ^{3}J=10.1 Hz, ^{4}J=2.5 Hz, 1H, H5), 6.22 (d, ^{4}J=2.5 Hz, 1H, H3). ^{13}C NMR δ 187.0 (C4), 182.5 (C1), 170.2 (C7′), 143.5 (C2), 142.0 (C1′), 138.5 (C5), 131.3 (C3′, C5′), 128.2 (C4′), 121.5 (C2′, C6′). Heteronuclear multiple bond correlations (HMBC): data shown in supporting material. Atmospheric pressure ESI (AP-ESI): neg. mode $[M-H]^-$ 242.046063 *m/z* (calculated 242.04585 *m/z*).

3b₁ *(5-[4′-(Carboxyphenyl)amino]-2-methyl-1,4-benzoquinone)* Synthesis and isolation are as described above. The characteristics are as follows: Red precipitate. Yield together with **3b₂** 38.9%. R_f (HPLC) 10.86 min, UV-Vis (MeOH) λ_{max} 201, 283, 488 nm. ^{1}H NMR (acetonitrile-d_3) δ 8.00 (d, ^{3}J=8.6 Hz, 2H, H3′, H5′), 7.38 (d, ^{3}J=8.6 Hz, 2H, H2′, H6′), 6.22 (s, 1H, H6), 6.18 (d(s), ^{4}J=2.8 Hz, 1H, H3), 2.06 (d, ^{4}J=2.8 Hz, 1H, H7), 2.04 (s, 2H, H7). ^{13}C NMR δ 185.0 (C1), 184.3 (C4), 167.1 (C7′), 143.9 (C1′), 143.0 (C5), 135.0 (C2) 132.0 (C3′, C5′), 124.6 (C2′, C6′) 103.0 (C3), 103.2 (C6), 15.2 (C7). HMBC: data shown in supporting material. AP-ESI: neg. mode $[M-H]^-$ 256.057324 *m/z* (calculated 256.06155 m/z). AP-ESI: pos. mode $[M+H^+]$ 258.07495 *m/z* (calculated 258.07605 *m/z*).

3b₂ *(6-[4′-(Carboxyphenyl)amino]-2-methyl-1,4-benzoquinone)* Synthesis and isolation are as described above. The characteristics are as follows: Red precipitate. Yield together with **3b₁** 38.9%. R_f (HPLC) 11.11 min, UV-Vis (MeOH) λ_{max} 201, 283, 496 nm. ^{1}H NMR (acetonitrile-d_3) δ 8.00 (d, ^{3}J=8.6 Hz, 2H, H3′, H5′), 7.38 (d, ^{3}J=8.6 Hz, 2H, H2′, H6′), 6.62 (m, J=1.9 Hz, J=2.8 Hz, 1H, H3), 6.52 (m, J=1.9 Hz, 1H, H5), 2.05 (d, J=2.8 Hz, 1H, H7), 2.02 (d (s), J=1.9 Hz, 2H, H7). ^{13}C NMR δ 187.7 (C1), 167.1 (C7′), 149.7 (C6), 143.9 (C1′), 132.0 (C3′, C5′), 130.7 (C2), 124.6 (C2′, C6′), 100.1 (C3), 100.4 (C5), 16.1 (C7). HMBC: data shown in supporting material. AP-ESI: neg. mode $[M-H]^-$ 256.057324 *m/z* (calculated 256.06155 *m/z*). AP-ESI: pos. mode $[M+H^+]$ 258.07495 *m/z* (calculated 258.07605 *m/z*).

3c *(6-[4′-(Carboxyphenyl)amino]-2-tert-butyl-1,4-benzoquinone)* Synthesis and isolation are as described above. The characteristics are as follows: Black precipitate. Yield 6.7%. R_f (HPLC) 11.96 min, UV-Vis (MeOH) λ_{max} 201, 282, 495 nm. ^{1}H NMR (methanol-d_4) δ 8.04 (d, ^{3}J=8.4 Hz, 2H, H3′, H5′), 7.37 (d, ^{3}J=8.4 Hz, 2H, H2′, H6′), 6.51 (d (s), ^{4}J=1.8 Hz, 1H, H3), 6.13 (d(s), ^{4}J=1.8 Hz, 1H, H5), 1.33 (s, 9H, H8). ^{13}C NMR δ 182.8 (C1), 168.9 (C7′), 152.4 (C2), 142.2 (C1′), 133.0 (C3), 130.6 (C3′, C5′), 128.3 (C4′), 121.4 (C2′, C6′), 99.7 (C5), 34.9 (C7), 27.9 (C8). HMBC: data shown in supporting material. AP-ESI: neg. mode $[M-H]^-$ 298.105948 *m/z* (calculated 298.10845 *m/z*).

3d *(5-[4′-(Carboxyphenyl)amino]-2-methoxy-1,4-benzoquinone)* Synthesis and isolation are as described above. The characteristics are as follows: Red precipitate. Yield 51.9%. R_f (HPLC) 9.58 min, UV-Vis (MeOH) λ_{max} 201, 279, 499 nm. ^{1}H NMR (DMSO-d_6) δ 9.26 (s, 1H, NH), 7.94 (d, ^{3}J=8.6 Hz, 2H, H5′, H3′), 7.46 (d, ^{3}J=8.6 Hz, 2H, H6′, H2′), 6.08 (s, 1H, H3), 5.9 (s, 1H, H6), 3.81 (s, 3H, H7). ^{13}C NMR δ 183.3 (C4), 180.3 (C1), 167.1 (C7′), 161.2 (C2), 144.1 (C5), 142.5 (C1′), 131.3 (C3′, C5′), 127.2 (C4′), 122.7 (C2′, C6′), 104.8 (C3), 100.2 (C6). HMBC: data shown in supporting material. AP-ESI: neg. mode $[M-H]^-$ 272.054910 *m/z* (calculated 272.056398 *m/z*).

3f₁ *(3-[4′-(Carboxyphenyl)amino]-2-bromo-1,4-benzoquinone)* The characteristics are as follows: R_f (HPLC) 9.44 min, UV-Vis (MeOH) λ_{max} 201, 221, 288, 515 nm. LC/MS *m/z* AP-ESI: pos. mode; $[M+H]^+$ 322.0 and 324.0 *m/z* (calculated 322.12 *m/z*).

3f₂ *(5-[4′-(Carboxyphenyl)amino]-2-bromo-1,4-benzoquinone)* The characteristics are as follows: R_f (HPLC) 10.91 min, UV-Vis (MeOH) λ_{max} 201, 289, 510 nm. LC/MS *m/z* AP-ESI: pos. mode $[M+H]^+$ 322.0 and 324.0 *m/z* (calculated 322.12 *m/z*), $[M+Na]^+$ 344.0 and 346.0 *m/z*, $[M+H–COOH]^+$ 277.0 and 279.0 *m/z*, $[2M+H]^+$ 644.9 *m/z*, $[2M+Na]^+$ 666.9 *m/z*.

3g₁ *(3-[4′-(Carboxyphenyl)amino]-2-chloro-1,4-benzoquinone)* Synthesis and isolation are as described above. The characteristics are as follows: Black precipitate. Yield 38.17%. R_f (HPLC) 9.58 min, UV-Vis (MeOH) λ_{max} 201, 223, 287, 513 nm. ^{1}H NMR (methanol-d_4) δ 7.96 (d, ^{3}J=8.4 Hz, 2H, H3′, H5′), 7.09 (d, ^{3}J=8.4 Hz, 2H, H2′, H6′), 6.95 (d, ^{3}J= 10.1 Hz, 1H, H6), 6.85 (d, ^{3}J=10.1 Hz, 1H, H5). ^{13}C NMR δ 181.9 (C4), 179.8 (C1), 172.1 (C7′), 142.6 (C1′), 140.9 (C3), 137.5 (C6), 133.3 (C5), 129.5 (C3′, C5′), 127.2 (C4′),

122.3 (C2′, C6′), 113.8 (C2). HMBC: data shown in supporting material. AP-ESI: neg. mode $[M-H]^-$ 276.015621 *m/z* (theoretical value 276.006910 *m/z*).

3g₂ *(5-[4′-(Carboxyphenyl)amino]-2-chloro-1,4-benzoquinone)* Synthesis and isolation are as described above. The characteristics are as follows: Black precipitate. Yield 15.1%. R_f (HPLC) 10.86 min, UV-Vis (MeOH) λ_{max} 201, 285, 513 nm. ^{1}H NMR (methanol-d_4) δ 8.14 (d, ^{3}J=8.4 Hz, 2H, H3′, H5′), 7.42 (d, ^{3}J=8.4 Hz, 2H, H2′, H6′), 7.09 (s, 1H, H3), 6.29 (s, 1H, H6). ^{13}C NMR δ 181.3 (C4), 178.5 (C1), 169.5 (C7′), 146.1 (C2), 145.2 (C5), 141.5 (C1′) 131.2 (C3′, C5′), 130.8 (C3), 128.3 (C4′), 122.5 (C2′, C-6′), 100.1 (C6). HMBC: data shown in supporting material. AP-ESI: neg. mode $[M-H]^-$ 276.010965 *m/z* (theoretical value 276.006910 *m/z*).

3h *(3-[4′-(Carboxyphenyl)amino]-2,6-dichloro-1,4-benzoquinone)* Synthesis and isolation are as described above. The characteristics are as follows: Black–violet precipitate. Yield 38.45%. R_f (HPLC) 11.34 min, UV-Vis (MeOH) λ_{max} 201, 225, 284, 525 nm. ^{1}H NMR (acetonitrile-d_3) δ 7.98 (s, 1H, NH), 7.95 (d, ^{3}J=8.6 Hz, 2H, H3′, H5′), 7.12 (d, ^{3}J= 8.6 Hz, 2H, H2′, H6′), 7.09 (s, 1H, H5). ^{13}C NMR δ 180.9, 177.2, 173.6, 146.4, 143.2, 141.8, 131.8, 130.8, 127.1, 124.0, 114.5. AP-ESI: neg. mode $[M-H]^-$ 309.965546 *m/z* (calculated 309.9685 m/z). AP-ESI: pos. mode $[M+H^+]$ 311.982417 *m/z* (calculated 311.98245 *m/z*).

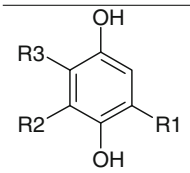
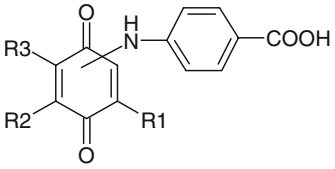
3i *(3-[4′-(Carboxyphenyl)amino]-2,5-dichloro-1,4-benzoquinone)* The characteristics are as follows: R_f (HPLC) 10.97 min, UV-Vis (MeOH) λ_{max} 201, 287, 525 nm. LC/MS *m/z* AP-ESI: pos. mode $[M+H]^+$ 312.0 and 314.0 *m/z* (calculated 312.12 *m/z*), $[M+Na]^+$ 334.0 *m/z*, $[M+H-Cl]^+$ 278.0 *m/z*, $[M+H-COOH]^+$ 267.0 *m/z*, $[M+H-Cl-COOH]^+$ 233.0 m/z.

3j *(3-[4′-(Carboxyphenyl)amino]-2-acetyl-1,4-benzoquinone)* The characteristics are as follows: R_f (HPLC) 9.64 min, UV-vis (MeOH) λ_{max} 201, 263, 483 nm. LC/MS *m/z* AP-ESI: pos. mode $[M+H]^+$ 286.1 *m/z* (calculated 285.26 *m/z*), $[M+Na]^+$ 308.1 *m/z*, $[M+H-H_2O]^+$ 268.1 *m/z*, $[M+H-COOH]^+$ 241.0 *m/z*.

Results

A number of different substituted *p*-hydroquinones **1a** to **1j** (Table 1) were subjected to laccase-catalyzed transformation with *p*-aminobenzoic acid **2a**. Resulting from these

Table 1 *p*-Hydroquinones used as substrates in laccase-catalyzed reaction with *p*-aminobenzoic acid **2a** and synthesized products

***p*-Hydroquinones**		**Monoaminated products**
		
Un-substituted	**1a** R1 = R2 = R3 = H	**3a**
Alkyl-subsituted	**1b** R1 = R3 = H, R2 = CH_3	**$3b_1$, $3b_2$**
	1c R1 = R3 = H, R2 = $C(CH_3)_3$	**3c**
Alkoxy-subsituted	**1d** R1 = R3 = H, R2 = OCH_3	**3d**
	1e R1 = R2 = OCH_3, R3 = H	0[b]
Halogen-substituted	**1f** R1 = R3 = H, R2 = Br	**$3f_1$, $3f_2$**
	1g R1 = R3 = H, R2 = Cl	**$3g_1$, $3g_2$**
	1h[a] R1 = R2 = Cl, R3 = H	**3h**
	1i R1 = R3 = Cl, R2 = H	**3i**
Carbonyl-substituted	**1j** R1 = R3 = H, R2 = $COCH_3$	**3j**

[a] Quinonoid substrate because hydroquinone was not available
[b] No product formed

reactions, 12 N–C coupling dimers consisting of *p*-quinone and aminobenzoic acid moieties could easily be detected by HPLC by using a diode array detector. With exception of **1e**, all substrates were rapidly transformed by laccases independent of the laccase source (substrate utilization 100% within 1h **1a**–**1d** and **1f**–**1h**, 100% within 3h **1i**–**1j**, determined by HPLC).

Using *p*-hydroquinone (**1a**) or monoalkylated derivates (**1b**, **1c**) in equimolar reactions, the substrates were oxidized to the corresponding quinones as described for various *p*-hydroquinones (Niedermeyer et al. 2008; Leontievsky et al. 2001; Liu et al. 1981). The corresponding quinones (the first transformation products of the laccase mediated reaction) of **1a**, **1b**, and **1c** were detectable for a long time by HPLC/UV-Vis, and the yields of monoaminated products (**3a**, **3b$_1$**, **3b$_2$**, **3c**) in solution were comparatively low (Fig. 1). Aminations of monohalogenated *p*-hydroquinones with *p*-aminobenzoic acid were faster than the reactions of *p*-hydroquinone or monoalkylated derivates. Furthermore, the yields of monoaminated products (**3g$_1$**, **3g$_2$**) were higher if monohalogenated *p*-hydroquinones were used as laccase substrates. When dihalogenated *p*-hydroquinones were used, a large amount of products were formed. Methoxy-*p*-hydroquinone reacted very rapidly to one monoaminated product in quantitative yield, whereas 2,6-dimethoxy-*p*-hydroquinone was not transformed.

Structural characterization of monoaminated main products

Mass spectral and NMR analyses of the products **3a**, **3c**, **3d**, **3h**, **3i**, and **3j** yielded structural data attributed to the coupling of one *p*-hydroquinone (**1a**, **1c**, **1d**, **1h**, **1i**, **1j**) with one molecule *p*-aminobenzoic acid accompanied by loss of four hydrogen atoms. The structural data of **3a**, **3c**, **3d**, **3h**, **3i**, and **3j** are described in "Materials and methods" and led to the identification of the products as carboxyphenylamino-1,4-benzoquinones.

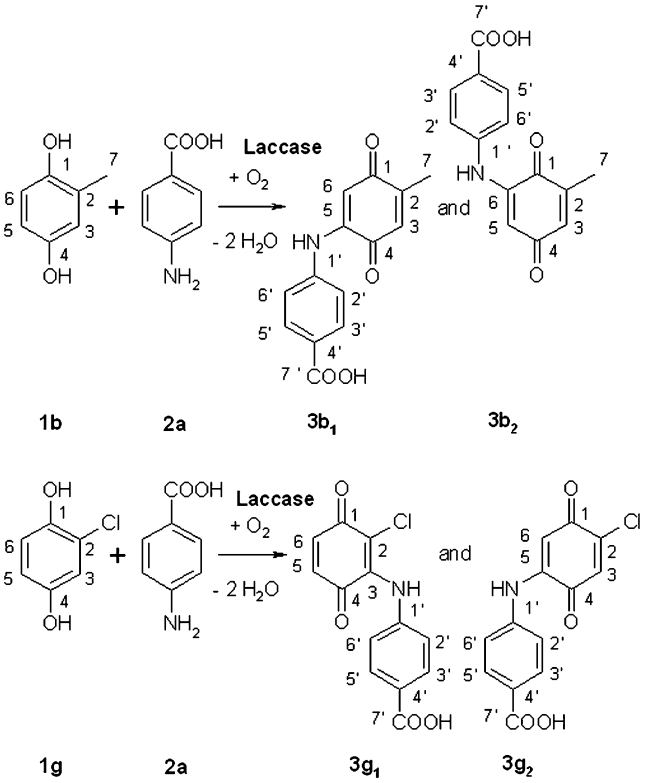


Fig. 2 Principal product patterns of monoaminated products (**3b$_1$**, **3b$_2$** and **3g$_1$**, **3g$_2$**) in equimolar laccase-catalyzed reactions (1 mM) of *p*-hydroquinones (**1b**, **1g**) with *p*-aminobenzoic acid (**2a**)

Laccase-catalyzed amination of methyl-*p*-hydroquinone and chloro-*p*-hydroquinone with *p*-aminobenzoic acid resulted in the formation of **3b$_1$**, **3b$_2$** and **3g$_1$**, **3g$_2$** (Fig. 2), respectively. Whereas **3b$_1$** (t_R=10.86 min) and **3b$_2$** (t_R=11.11 min) showed nearly the same retention times in the HPLC and could not be separated from each other, **3g$_1$** (t_R=9.58 min) and **3g$_2$** (t_R=10.86 min) were isolated as pure substances with distinct chromatographic behavior. After separation of the products **3g$_1$** and **3g$_2$** as pure

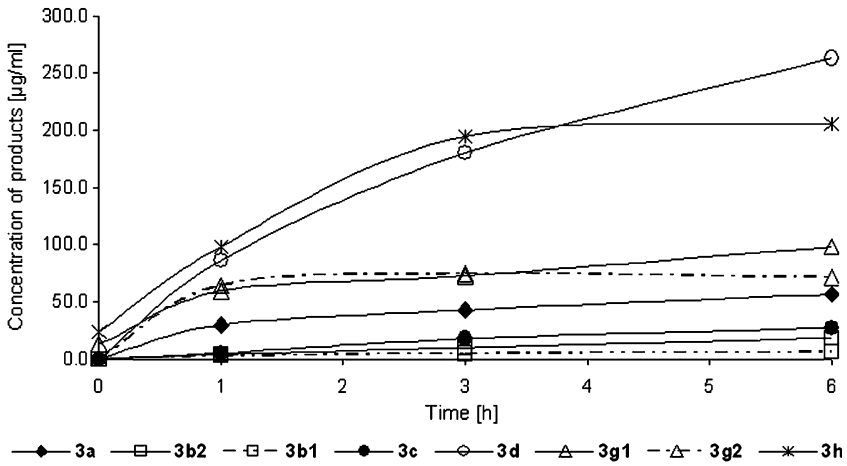


Fig. 1 Reaction course of product formation (analyzed with HPLC) for **3a**, **3b$_1$**, **3b$_2$**, **3c**, **3d**, **3g$_1$**, **3g$_2$**, and **3h** in equimolar reactions (1 mM), using laccase from *P. cinnabarinus*

precipitates, mass spectral and NMR spectral data showed that **3g$_1$** is aminated at the C3 position of the quinone ring and **3g$_2$** is aminated at the C5 position of the quinone ring.

Although the products **3b$_1$** and **3b$_2$** could only be obtained as a mixture, mass spectral and NMR analyses led to the identification of **3b$_1$** (minor product) as a product which is aminated at the C5 position of the quinone ring and of **3b$_2$** (major product) as a product which is aminated at the C6 position as described for chemical aminations of methyl-*p*-quinone with primary aromatic amines (Yogo et al. 1991; Kallmayer and Tappe 1986).

Comparison of laccase-catalyzed reaction with different laccase sources and with established synthesis Using laccase from *P. cinnabarinus*, the monoaminated products were the main products sometime accompanied by one or more diaminated products (consisting of *para*-quinone and two aminobenzoic acid moieties) in low rates (data not shown). The yields of monoaminated products were nearly independent of the activity of laccase from *P. cinnabarinus* (Fig. 3a). In contrast to the laccase of *P. cinnabarinus*, higher activity of laccase from *M. thermophila* results in higher amount of formed products (Fig. 3b). So, the kind of laccase did influence the quantity of monoaminated products but not the product pattern.

Compared with the laccase-catalyzed reaction (100% relative yield), after 1 h, the accumulated amounts of **3a**, **3g$_1$**, and **3g$_2$** from the reactions with sodium iodate as the oxidant is low at a low concentration of sodium iodate (0–45% relative yield, Fig. 3). With increasing concentration of laccases and sodium iodate, the relative yields of the synthetic method with sodium iodate increased, but a higher concentration of sodium iodate as 320 mM is impossible, because 320 mM means saturated solution. In contrast to sodium iodate, the activity of laccase can be much higher without any problem. The accumulated amounts of **3b$_1$** and **3b$_2$** were very low with every method tested, but they were somewhat higher with laccases than with sodium iodate.

Discussion

Most of the *p*-hydroquinonoid substrates were readily utilized by laccase of *P. cinnabarinus* and of *M. thermophila* independent of source of laccases and were oxidized to the corresponding quinones as described for various *p*-

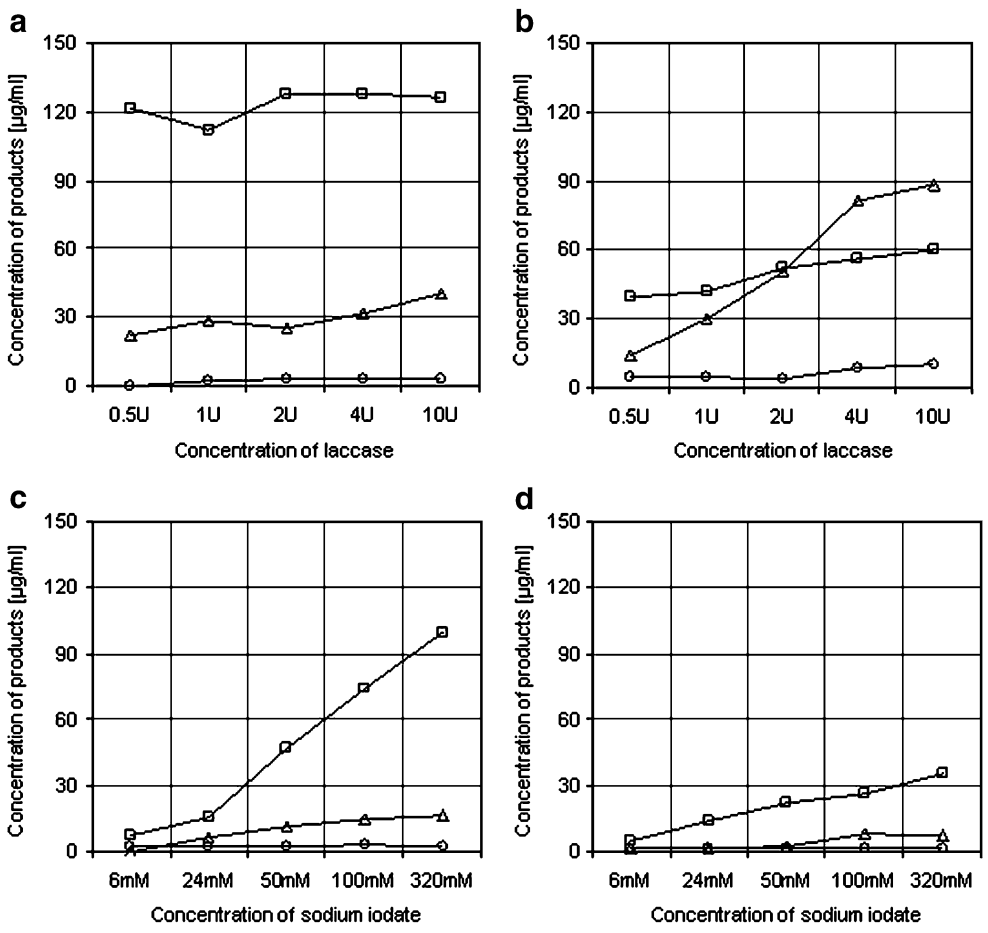


Fig. 3 Comparison of product formation (analyzed with HPLC) for **3a** (*triangles*), **3b$_1$** and **3b$_2$** together (*circles*), **3g$_1$** and **3g$_2$** together (*squares*) in equimolar reactions (1 mM) using laccase from *P. cinnabarinus* pH 5.0 (**a**), laccase from *M. thermophila* pH 7.0 (**b**), sodium iodate pH 5.0 (**c**), and pH 7.0 (**d**). Final concentrations for sodium iodate were 6, 24, 50, 100, and 320 mM, resulting comparable oxidative reactivity as 0.5, 1, 2, 4, and 10 U activity of laccase

hydroquinones (Leontievsky et al. 2001; Liu et al. 1981; Nakamura 1960; Brown 1967). Interestingly, **1e** was not transformed into the quinone under the reaction conditions used due to the fact that **1e** was not a substrate of laccases and of sodium iodate, whereas **1d** reacted very rapidly to the corresponding quinone. The fact that **1e** is not a substrate of laccases and of sodium iodate and did not react to the corresponding quinone explains the lack of amination products because di- and trimethoxylated *p*-quinones could be chemically aminated (Schäfer et al. 1971).

Structural characterization of halogen-, alkyl-, alkoxy-, and carbonyl-substituted reaction products showed that in the course of laccase-catalyzed reactions, one monoaminated oxidative coupling product or two constitution isomers were formed by amination of *p*-hydroquinones. The position of the amination was dependent on the character, position, and number of the substituents on the substrate. Using monomethyl-*p*-hydroquinone and monohalogenated *p*-hydroquinones, the amination resulted in the formation of two constitution isomers in every case. Whereas in previous experiments, the products of the laccase-catalyzed reaction of monomethyl-*p*-hydroquinone could not be isolated (Niedermeyer et al. 2005), now we could show that two products were produced. **$3b_1$** is aminated at the C5 position of the quinone ring, and **$3b_2$** is aminated at the C6 position. Using monohalogenated *p*-hydroquinones, the amination took place at C3 and C5. Consequently, the position of amination depends on electronic and steric effects of the substituents. In summary, we could show that for laccase-catalyzed reactions of alkyl-substituted *p*-hydroquinones with *p*-aminobenzoic acid, the order of reactivity is as follows: C6>C5>>C3 (position C3 did not show any reactivity in enzymatic reaction), according to the order of Brunmark and Cadenas (1989) for chemical reactions.

Laccase-catalyzed amination at the aromatic ring system of carbonyl-substituted *p*-hydroquinones (2,5-dihydroxyacetophenon, 2,5-dihydroxybenzoic acid derivatives) led to one monoaminated product with C–N coupling at the *ortho*-position (C3) to the carbonylic groups as described for aminations with primary aromatic amines (Manda et al. 2005; Niedermeyer et al. 2005), with amino acids (Manda et al. 2006), or with amino-β-lactams (Mikolasch et al. 2006, 2007). In summary, we could show that for the laccase-catalyzed reaction of carbonyl-substituted *p*-hydroquinones with *p*-aminobenzoic acid, the order of reactivity is as follows: C3>>C6>C5 (position C5 and C6 did not show any reactivity in enzymatic reaction), according to the order of Brunmark and Cadenas (1989) for chemical reactions.

To further determine the potential use of laccase-catalyzed reactions in fine chemical synthesis, we compared reactions catalyzed by laccase of *P. cinnabarinus* and of *M. thermophila* with an established synthetic method using sodium iodate as the oxidant (Schäfer and Aguado 1971; Pardo et al. 1979; Torres et al. 1985).

If we compare the accumulated amounts of monoaminated products for one reaction, catalyses with laccases cause better yields than with sodium iodate that means that laccase is the better oxidant for the reaction of halogen-, alkyl-, alkoxy-, and carbonyl-substituted *p*-hydroquinones with *p*-aminobenzoic acid. Laccase from *P. cinnabarinus* (pH 5.0) should be applied for the aminations of halogen-, alkoxy-, and carbonyl-substituted *p*-hydroquinones, whereas for alkyl-substituted *p*-hydroquinones, the laccase from *M. thermophila* seems to be more effective. Derived from all results, laccase-induced C–N coupling has some advances over reactions using sodium iodate as the oxidant.

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