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Journal of Molecular Catalysis B: Enzymatic 35 (2005) 86–92
JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC
www.elsevier.com/locate/molcatb

Laccase-induced cross-coupling of 4-aminobenzoic acid with *para*-dihydroxylated compounds 2,5-dihydroxy-*N*-(2-hydroxyethyl)-benzamide and 2,5-dihydroxybenzoic acid methyl ester

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Received 31 March 2005; received in revised form 13 May 2005; accepted 1 June 2005
Available online 5 July 2005

Abstract

A fungal laccase from *Trametes villosa* (EC 1.10.3.2 *p*-phenoloxidase) was used to mediate the oxidation and cross-coupling of two *para*-dihydroxylated benzoic acid derivatives with 4-aminobenzoic acid. The incubation of 2,5-dihydroxy-*N*-(2-hydroxyethyl)-benzamide and 4-aminobenzoic acid with laccase under oxygen conditions resulted in the formation of 2-(4′-carboxy-anilino)-*N*-(2″-hydroxyethyl)-3,6-dioxo-1,4-cyclohexadien-1-carboxamide as the main product (yield > 85%). When 2,5-dihydroxybenzoic acid methyl ester was a co-substrate of 4-aminobenzoic acid, 2-(4′-carboxy-anilino)-*N*-(2″-hydroxyethyl)-3,6-dioxo-1,4-cyclohexadien-1-carboxy methyl ester was produced (yield > 75%). Both products were N–C coupling dimers consisting of *para*-quinone and benzoic acid moieties. The formation of quinone structures in the presence of *T. villosa* laccase may be useful in pharmaceutical synthesis. Because of high product yields and low amount of by-products laccase of *T. villosa* seems to be a suitable enzyme among laccases acting at pH 5 for the synthesis of heterologous dimers.

Keywords: Laccase; Cross-coupling; Transformation; White rot fungi; *Trametes villosa*

1. Introduction

Bioactive effects of various monoaromatic compounds are closely related to their oxygen containing substituents, such as hydroxyl or keto groups. Phenolic derivatives can initiate cancer or various toxic effects by membrane disintegration or protein precipitation. However, phenolic substructures are also found among others in pharmaceuticals with analgesic, cardiovascular or antibiotic effects. The pharmacological effects of quinones are based on the structural similarity to the electron carriers of the respiration chain. Selective and gentle synthetic methods are desirable both to develop pharmaceuticals and to modify natural compounds with phenolic substructures. Chemical methods may be used for modification of phenolic substructures or their conversion to the corresponding quinone derivatives, but their use often leads to an irreversible destruction of the desired substructures. Enzymes, such as laccases, with specificity for phenolic substrates appear to be a valuable tool for controlled phenol transformation. To date, the ability of laccases to catalyze polymerization reactions have been used for pollutant detoxification, textile dye transformation, biosensing and diagnostics [1–7]. Applications in organic synthesis are limited to a few examples, including dimerization or oxidation of antibiotic compounds containing β-lactam substructures

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doi:10.1016/j.molcatb.2005.06.001

[8,9], synthesis of substituted imidazoles [10], the oxidative coupling of hydroquinone and mithramicine [11] and the oxidation of aromatic alcohols to the corresponding aldehydes [12–14]. Furthermore cross-coupling of 4-aminobenzoic acid with the *ortho*-dihydroxylated compound hydrocaffeic acid 3-(3,4-dihydroxyphenyl)-propionic acid was described [15].

In this study, a laccase from *Trametes villosa* (EC 1.10.3.2 *p*-phenoloxidase) was tested for its ability to transform also *para*-dihydroxylated benzoic acid derivatives, which were incubated with 4-aminobenzoic acid. Enzymatic reactions involving these compounds may be useful in pharmaceutical synthesis. The reaction products were isolated using high-performance liquid chromatography (HPLC) and characterized by liquid chromatography/mass spectroscopy (LC/MS) analysis and $^{1}H/^{13}C$ NMR spectroscopy.

2. Experimental

2.1. Chemicals

The substrates 2,5-dihydroxy-*N*-(2-hydroxyethyl)-benzamide and 2,5-dihydroxybenzoic acid methyl ester (or compounds **1** and **4**, respectively) were bought from Sigma–Aldrich Fine Chemicals (Taufkirchen, Germany). The co-substrate 4-aminobenzoic acid (or compound **2**) was purchased from Serva Feinbiochemica & Co. (Heidelberg, Germany).

2.2. Enzyme

Extracellular laccase of *T. villosa* (EC 1.10.3.2) was obtained from Novo Nordisk (Danbury, CT, USA). Laccase activity was determined spectrophotometrically, using 2,6-dimethoxyphenol (DMP) as a substrate. One unit of laccase activity was defined as the amount of enzyme which caused a change of absorbance at 468 nm of 1 min^{-1} in 3.4 mL of 1 mM buffer solution of DMP in 0.1 M citric acid/0.2 M sodium dibasic phosphate buffer (pH 3.8). Absorbance was measured with a Model 2000 spectrophotometer (Bauch and Lomb, Rochester, NY, USA). The enzyme preparation can be used as supplied by Novo Nordisk.

2.3. Laccase-mediated coupling of 2,5-dihydroxy-N-(2-hydroxyethyl)-benzamide or 2,5-dihydroxybenzoic acid methyl ester with 4-aminobenzoic acid

Seven-milliliter aliquots of 1 mM solutions of 2,5-dihydroxy-*N*-(2-hydroxyethyl)-benzamide methyl ester or 2,5-dihydroxybenzoic acid methyl ester in 20 mM acetate buffer (pH 5) were incubated for 0–100 min with 4-aminobenzoic acid (1 mM) in the presence of *T. villosa* laccase (activity 17.45 units mL^{-1}). The incubations were carried out at room temperature with agitation at 100 rpm.

2.4. HPLC analysis

Before HPLC analysis, the reaction mixtures were passed through an HPLC grade 230 nm PolySep polypropylene filter (Osmonics, Minnetonka, MN, USA). The analysis of the filtrates was conducted on a Waters HPLC system (Milford, MA, USA) consisting of a Waters 2690 Alliance separation module, a Waters 996 photodiode array detector and a 3.9 mm × 150 mm 18 DB column of 5-μm particle size with an LC-18 guard column (Supelco, Bellefonte, PA, USA). The mobile phase at a flow rate of 1 mL min^{-1} consisting of methanol component A and an aqueous component B (0.1% phosphoric acid) was delivered at an initial ratio of 10% A to 90% B, which was brought to 100% A in 14 min. The retention times of compounds **1**–**5**, were 5.35, 3.94, 6.71, 8.8 and 7.7 min, respectively.

2.5. Procedure for the isolation of transformation products

The products were purified by solid reverse phase extraction with silicagel column (PrepSep 6 mL, 2000 mg adsorbent material, Fisher Scientific, Pittsburgh, PA, USA), charged with 12 mL of the reaction mixture. The homomolecular products of 2,5-dihydroxy-*N*-(2-hydroxyethyl)-benzamide and 2,5-dihydroxybenzoic acid methyl ester were eluted with 4 mL of a solution consisting of 30% methanol and 70% water. The cross-coupling products were eluted with 2 mL of 50% methanol and 50% water.

2.6. Characterization

The products were characterized by liquid chromatography/mass spectroscopy. MS analysis was conducted on a Quattro II mass spectrometer (Micromass, Manchester, UK) using atmospheric pressure chemical ionization (APCI) in negative ion mode. LC separation was done using a 1 mm × 150 mm BetaBasic C18 column Supelco, Bellefonte, PA, USA) with a two component solvent system delivered at 1.0 mL min^{-1} by a Shimadzu LC-10ADvp pump using the following gradient: Solvent A = 0.15% formic acid in water; Solvent B = CH_3OH; 90% A (time 0) to 100% B by 15 min, then held at 100% B by 20 min. Product **3** was additionally analyzed on an Agilent 1100 LC/MSD system (Waldbronn, Germany) with electrospray ionization under atmospheric conditions (AP-ESI). The products were also analyzed by high-resolution mass spectroscopy on a Q-TOF mass spectrometer (Q-Star Pulsar, Applied Biosystems, Forster City, CA, USA) using a nanospray source (Protana Odense, Denmark).

For ^{1}H nuclear magnetic resonance (NMR) spectroscopy, the isolated products were dried under a gentle stream of nitrogen. The NMR spectra were recorded on a Bruker DRX-400 spectrometer at ambient temperature in deuterated methanol (MeOH-d_4). The signals were referenced indirectly to tetramethylsilane via the residual ^{1}H signal of deuterated

Table 1
^{1}H NMR analysis of the substrate 2,5-dihydroxy-*N*-(2-hydroxyethyl)-benzamide (compound **1**) in d_4-methanol

Position	^{1}H shift[a]	^{1}H multiplicity	*J*-values (Hz), HH coupling
—NH—C**H**$_2$—CH$_2$—OH[b]	3.51	Triplet	$^3J = 5.7$
—NH—CH$_2$—C**H**$_2$—OH[b]	3.70	Triplet	$^3J = 5.7$
3	6.74	Doublet of doublets	$^3J_{3,4} = 8.8$; $^5J_{3,6} = 0.2$
4	6.85	Doublet of doublets	$^3J_{3,4} = 8.8$; $^4J_{4,6} = 3.0$
6	7.23	Doublet of doublets	$^4J_{4,6} = 3.0$; $^5J_{3,6} = 0.2$

[a] Referenced indirectly to TMS (0.0 ppm).
[b] Ambiguous assignments—could be reversed.

methanol at 3.31 ppm. The ^{1}H NMR- and ^{13}C NMR-spectra of compound **3** in DMSO-d_6 as solvent were acquired on a Bruker ARX-400 spectrometer. The spectra were recorded at 20 °C and calibrated using the resonances of the residual non-deuterated solvent.

2.6.1. Characterization of product of 2,5-dihydroxy-N-(2-hydroxyethyl)-benzamide and 4-aminobenzoic acid

Following the isolation and purification steps, the yield of compound **3** or 2-(4′-carboxy-anilino)-*N*-(2″-hydroxyethyl)-3,6-dioxo-1,4-cyclohexadien-1-carboxamide, a transformation product of laccase-mediated coupling between 2,5-dihydroxy-*N*-(2-hydroxyethyl)-benzamide and 4-aminobenzoic acid, was 0.126 mg mL^{-1} (>85%).

The 400 MHz ^{1}H NMR (MeOH-d_4) data are shown in Table 3; ^{1}H NMR (DMSO-d_6) and ^{13}C NMR (DMSO-d_6) data are shown in Table 4.

Comparison of ^{1}H NMR spectral data for compound **3** dissolved in MeOH-d_4 with those for compounds **1** and **2** (Tables 1 and 2) showed the presence of all protons contributed by the parent compounds except for the H-6 proton in the original 2,5-dihydroxy-*N*-(2-hydroxyethyl)-benzamide (Table 3); using MeOH-d_4 as the solvent the protons in the amino and hydroxyl groups could not be seen. Changes in the multiplicity and chemical shifts of H-3 and H-4 support the observation that 4-aminobenzoic moiety substitutes the benzamide ring at the original position 6.

The ^{1}H NMR analysis of product **3**, using DMSO-d_6 as a solvent to facilitate the observation of the amino and hydroxyl protons (Table 4), showed the loss of two protons from the hydroxyl groups at the aromatic ring of the original 2,5-dihydroxy-*N*-(2-hydroxyethyl)-benzamide. Despite a rapid conversion of the reaction product in various solvents (e.g., the red reaction product changed color to brown during 1 h at room temperature), it was possible to obtain ^{13}C NMR data that confirmed the presence of all carbons contributed by both substrates, and, along with ^{1}H NMR and mass spectral data, led to the identification of compound **3** as 2-(4′-carboxy-anilino)-*N*-(2″-hydroxyethyl)-3,6-dioxo-1,4-cyclohexadien-1-carboxamide.

The product, analyzed by LC/MS using atmospheric pressure chemical ionization (APCI) in negative ion mode showed a molecular mass of 330. This suggested coupling between the oxidized compound **1** (3,6-dioxo-cyclohexa-1,4-dien carboxylic acid (2-hydroxyethyl) amide, MW 195) and compound **2** (MW 137). The fragment ion at *m*/*z* 195 confirmed the presence of a 3,6-dioxo-cyclohexa-1,4-dien carboxylic acid (2-hydroxyethyl) amide moiety in compound **3**. The loss of —CONHCH$_2$CH$_2$O led to the fragment M^- 243 for the remainder of product **3**.

The AP-ESI measurement of compound **3** showed a peak at *m*/*z* 353 $[M + Na]^+$ in positive mode and a peak at *m*/*z* 329 $[M - H]^-$ in negative mode.

The investigation of product **3** by high-resolution mass spectroscopy in positive ion mode showed a peak $[M + H]^+$ at *m*/*z* 331.0930 as compared to 330.0852 that was calculated $[M]^+$ of $C_{16}H_{14}N_2O_6$.

Table 2
^{1}H NMR analysis of the substrate 4-aminobenzoic acid (compound **2**) in d_4-methanol

Position	^{1}H shift[a]	^{1}H multiplicity	*J*-values (Hz), HH coupling
2,6[b]	6.63	Doublet[c]	$^3J = 8.9$
3,5[b]	7.74	Doublet[c]	$^3J = 8.9$

[a] Referenced indirectly to TMS (0.0 ppm).
[b] Assignments based on larger change in ^{1}H shift for downfield resonance in reactions.
[c] Doublets show weak long range coupling due to magnetic inequivalence.

Table 3
^{1}H NMR analysis of the reaction product (compound **3**, in methanol-d_4) formed through laccase-mediated coupling of 2,5-dihydroxy-*N*-(2-hydroxyethyl)-benzamide (compound **1**) and 4-aminobenzoic acid (compound **2**)

Position	^{1}H shift[a]	^{1}H multiplicity	*J*-values (Hz), HH coupling
—NH—C**H**$_2$—CH$_2$—OH[b]	3.27	Triplet	$^3J = 5.7$
—NH—CH$_2$—C**H**$_2$—OH[b]	3.58	Triplet	$^3J = 5.7$
5	6.72	Doublet	$^3J_{3,4} = 8.9$
4	6.97	Doublet	$^3J_{3,4} = 8.9$
3′,5′[c]	7.84	Doublet[d]	$^3J = 9.4$
2′,6′[c]	6.73	Doublet[d]	$^3J = 9.4$

[a] Referenced indirectly to TMS (0.0 ppm).
[b] Ambiguous assignments—could be reversed.
[c] The primed numbers refer to the 4-aminobenzoic acid moiety. Assignments based on larger change in ^{1}H shift for downfield resonance in this reaction product. See text for more information.
[d] Doublets show weak long range coupling due to magnetic inequivalence.

Table 4
^{1}H NMR and ^{13}C NMR analyses of the reaction product (compound **3**, in DMSO-d_6) formed through laccase-mediated coupling of 2,5-dihydroxy-*N*-(2-hydroxyethyl)-benzamide (compound **1**) and 4-aminobenzoic acid (compound **2**)

Position	^{1}H shift[a]	^{1}H multiplicity	*J*-values (Hz), HH coupling	^{13}C[b]
1	–	–	–	105.5
6	–	–	–	184.2
–NH–**C**H_2–CH_2–OH	3.17	Triplet	$^{3}J = 5.7$	41.2
–NH–CH_2–**C**H_2–OH	3.41	Triplet	$^{3}J = 5.6$	59.3
5	6.76	Doublet	$^{3}J_{3,4} = 9.3$	133.8
4	6.81	Doublet	$^{3}J_{3,4} = 9.5$	138.9
3	–	–	–	182.9
2	–	–	–	148.6
7	–	–	–	166.3
1′	–	–	–	143.4
2′,6′	7.30	Doublet	$^{3}J = 7.9$	123.3
3′,5′	7.84	Doublet	$^{3}J = 8.5$	129.7
4′	–	–	–	127.2
7′	–	–	–	166.7
–N**H**–CH_2–CH_2–OH	9.14	Triplet	$^{3}J = 5.3$	–
–CH_6–N**H**–CH_6–	12.42	Singlet	–	–
–NH–CH_2–CH_2–O**H**	4.77	Singlet	–	–
–COO**H**	12.79	Singlet	–	–

[a] Referenced indirectly to TMS (0.0 ppm).
[b] Calibrated on the resonances of the residual non-deuterated solvent.

2.6.2. Characterization of product of 2,5-dihydroxybenzoic acid methyl ester and 4-aminobenzoic acid

The yield of compound **5** or 2-(4′-carboxy-anilino)-*N*-(2″-hydroxyethyl)-3,6-dioxo-1,4-cyclohexadien-1-carboxy methyl ester was 0.169 mg mL^{-1} (>75%). The 400 MHz ^{1}H NMR (MeOH-d_4) data are shown in Table 5.

The molecular mass of compound **5** was 301, according to the cross-coupling of the quinone derivative of 2,5-dihydroxybenzoic acid methyl ester (166) and 4-aminobenzoic acid (137). The ion peak at *m/z* 270 ($M^- - 31$, OCH_3) indicated the loss of a methoxy group. Furthermore, the fragment ions *m/z* 243 ($M^- - 58$, –CO_2/CH_3/+H) and *m/z* 166 ($M^- - 135$, –N–C_6H_4–COOH) were detectable. High-resolution mass analysis of compound **5** in positive ion mode showed a peak $[M+H]^+$ at 302.0613, and 301.0586 was calculated for $[M]^+$ of $C_{15}H_{11}NO_6$.

Table 5
^{1}H NMR analysis of the reaction product (compound **5**, in methanol-d_4) formed through laccase-mediated coupling of 2,5-dihydroxybenzoic acid methyl ester (compound **4**) and 4-aminobenzoic acid (compound **2**)

Position	^{1}H shift[a]	^{1}H multiplicity	*J*-values (Hz), HH coupling
–CO–OC**H**$_3$	3.71	Singlet	–
5	6.72	Doublet	$^{3}J_{3,4} = 8.9$
4	7.03	Doublet	$^{3}J_{3,4} = 8.9$
3′,5′[b]	7.79	Doublet[c]	$^{3}J_{3,4} = 8.8$
2′,6′[b]	6.63	Doublet[c]	$^{3}J_{3,4} = 8.8$

[a] Referenced indirectly to TMS (0.0 ppm).
[b] The primed numbers refer to the 4-aminobenzoic acid moiety. Assignments based on larger change in ^{1}H shift for downfield resonance in this reaction product.
[c] Doublets show weak long range coupling due to magnetic inequivalence.

3. Results

3.1. Cross-coupling of 2,5-dihydroxy-N-(2-hydroxyethyl)-benzamide and 4-aminobenzoic acid

The reaction involving *T. villosa* laccase (17.45 2,6-dimethoxyphenol (DMP) units mL^{-1}) and the test substrates 2,5-dihydroxy-*N*-(2-hydroxyethyl)-benzamide and 4-aminobenzoic acid (compounds **1** and **2**) resulted in the formation of one main cross-coupling product **3** (Fig. 1) as determined by HPLC analysis. As the reaction proceeded, the initially colorless mixture changed to red.

The analysis of the reaction mixture by HPLC showed a gradual decrease in the concentration of each of the substrates. At the same time, an accumulation of the reaction product **3** (retention time = 6.71 min) was observed (Fig. 2). After 80 min of incubation, 2,5-dihydroxy-*N*-(2-hydroxyethyl)-benzamide was no longer present in the reaction mixture, whereas 4-aminobenzoic acid was still detectable. A small amount of 2,5-dihydroxy-*N*-(2-hydroxyethyl)-benzamide (<5%) was transformed by laccase to homomolecular products and consequently was not available for the cross-coupling reaction with 4-aminobenzoic acid. Prolonging the incubation time beyond 80 min yielded small quantities of a second product, which was not characterized in this study. The main product **3** was purified by solid reverse phase extraction (yield > 85%) for LC/MS and ^{1}H and ^{13}C NMR analyses.

The comparison of proton–proton spin systems and couplings of the ^{1}H NMR-spectra and mass spectra of compound **3** with those of compounds **1** and **2** showed structural similarities, which led to the identification of

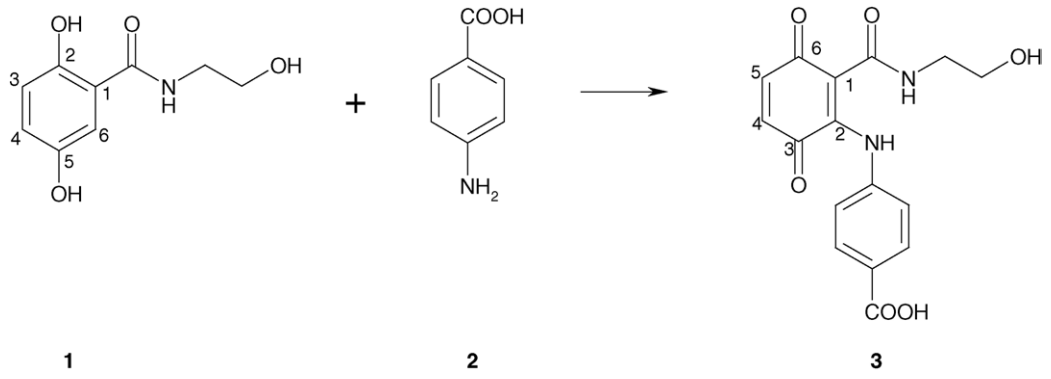


Fig. 1. Reaction pathway scheme for laccase-mediated cross-coupling of 2,5-dihydroxy-*N*-(2-hydroxyethyl)-benzamide (compound **1**) and 4-aminobenzoic acid (compound **2**), yielding the product 2-(4′-carboxy-anilino)-*N*-(2″-hydroxyethyl)-3,6-dioxo-1,4-cyclohexadien-1-carboxamide (compound **3**).

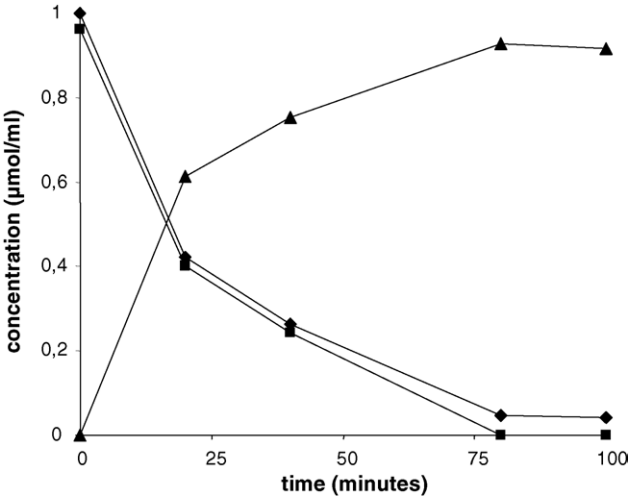


Fig. 2. Kinetics of laccase-mediated substrate disappearance: 2,5-dihydroxy-*N*-(2-hydroxyethyl)-benzamide (■; compound **1**), 4-aminobenzoic acid (♦; compound **2**), and product formation: 2-(4′-carboxy-anilino)-*N*-(2″-hydroxyethyl)-3,6-dioxo-1,4-cyclohexadien-1-carboxamide (▲; compound **3**).

compound **3** as 2-(4′-carboxy-anilino)-*N*-(2″-hydroxyethyl)-3,6-dioxo-1,4-cyclohexadien-1-carboxamide (Fig. 1). The outcome of ^{1}H NMR analysis was verified by ^{13}C NMR spectroscopy.

3.2. Cross-coupling of 2,5-dihydroxybenzoic acid methyl ester and 4-aminobenzoic acid

When 2,5-dihydroxybenzoic acid methyl ester (compound **4**) was mixed with 4-aminobenzoic acid (compound **2**) and incubated with the laccase (17.45 DMP units mL^{-1}), the initially colorless mixture changed to red and one cross-coupling product **5** was formed (Fig. 3) as the two substrates disappeared (Fig. 4).

The disappearance of the substrates and simultaneous accumulation of **5**, were monitored by HPLC; the retention time of **5** was 7.7 min. The yield of **5**, following an 80-min incubation was about 75%, which was sufficient for spectrometric characterization. At the end of incubation, 4-aminobenzoic acid was still detectable, but 2,5-dihydroxybenzoic acid methyl ester was completely used. A small amount of **4** (3%) was transformed by the laccase to homomolecular products, thus escaping cross-coupling with 4-aminobenzoic acid.

The isolation of compound **5** was performed by solid phase extraction. Mass and ^{1}H NMR spectrometry indicated that compound **5** was a product of cross-coupling of the quinone derivative of compound **4** and compound **2**, and was designated as 2-(4′-carboxy-anilino)-*N*-(2″-hydroxyethyl)-3,6-dioxo-1,4-cyclohexadien-1-carboxy methyl ester (Fig. 3).

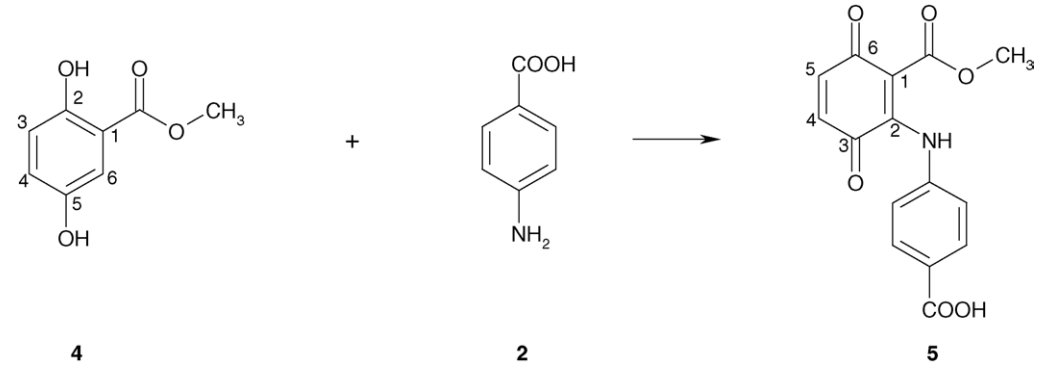
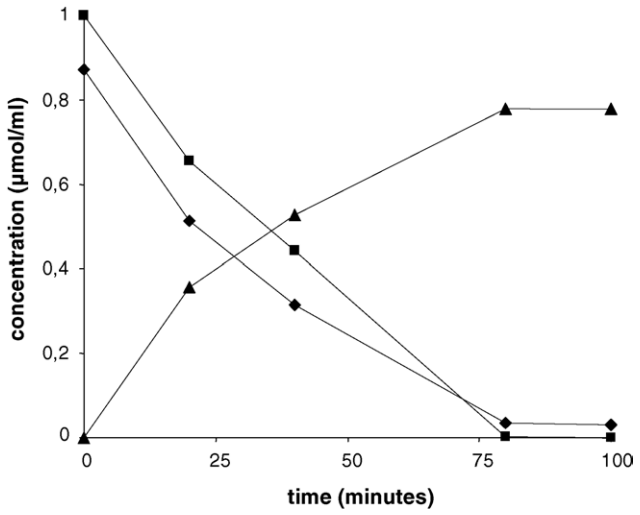


Fig. 3. Reaction pathway scheme for laccase-mediated cross-coupling of 2,5-dihydroxybenzoic acid methyl ester (compound **4**) and 4-aminobenzoic acid (compound **2**), yielding the product 2-(4′-carboxy-anilino)-*N*-(2″-hydroxyethyl)-3,6-dioxo-1,4-cyclohexadien-1-carboxy methyl ester (compound **5**).



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4. Discussion

The fungal laccase from *T. villosa* was able to mediate the cross-coupling of two *para*-dihydroxylated benzoic acid derivatives with 4-aminobenzoic acid in a similar way as described for *ortho*-dihydroxylated substrates like hydrocaffeic acid [15] The reaction showed strong regioselectivity. The transformation products were formed via N–C coupling of 4-aminobenzoic to C6-position of oxidized compounds **1** or **4**. This implies the formation of a stable semiquinone radical at this position, which is favoured because of steric protection, and mesomeric effect of the carbonyl group at the side chain.

In the absence of a co-substrate, compounds **1** and **4** were transformed to relatively stable homomolecular products (data not shown), which, to date, have not been identified. The substrate reactions did not seem to produce stable benzoquinone derivatives. Homomolecular product formation was also observed for *ortho*-dihydroxylated compounds [15,16]. No homomolecular product formation, however, was detected for 4-aminobenzoic acid.

Interestingly, homomolecular products were also formed in the heteromolecular reaction mixtures. Nevertheless, cross-coupling seems to prevail in the homomolecular reaction, and the accumulated homomolecular products remained below 5%. On the other hand, these homomolecular transformations can considerably diminish the yield of the desired cross-coupling products. For instance, in the presence of 4-aminobenzoic acid, up to 20% of hydrocaffeic acid was transformed to undesirable products [15], and up to 50% of syringic acid was lost to undesirable products in the presence of 3,4-chloroaniline [15].

Most of the dihydroxylated substrates tested to date for laccase-mediated cross-coupling with 4-aminobenzoic acid had similar reaction pathways. They retained their *ortho*- or *para*-dihydroxyaryl substructures [11,17] and acquired an additional amino substituent [17]. In contrast, the reaction products obtained in this study ended up with assuming quinone substructures as a result of cross-coupling. Spectrometric data implied that the products were formed via semiquinone intermediates of the diphenolic substrates that underwent C–N coupling with 4-aminobenzoic acid through 1,4-nucleophilic addition of the latter to the quinoid rings followed by further oxidation to the quinonoid system. Most of aminobenzoquinones were synthesized chemically under strong conditions, for example, with sodium periodate [18], with peracetic acid or under high temperatures [19]. In this study, we show the enzymatic derivatization of a *p*-hydroquinone by use of laccase. Laccase derived products with quinonoid substructures were also described by Niedermeyer et al. [20]. The work compares reactions with laccases of different pH value (*Trametes spec*., pH 5 and *Myceliophthora thermophila*, pH 7). The results suggest that laccases acting at pH 5 seemed to be more useful for the reactions with 2,5-dihydroxybenzoic acid derivatives and laccases with pH 7 optimum seemed to be more suitable for transforming alkylated *p*-hydroquinones. However, the differences could also be an effect of pH value and not of different sources of laccase. In order to find the most suitable enzyme for specific reaction laccases acting with the same pH value should be compared. Thus, using laccase of *T. villosa* (pH 5) enabled increase of the yield of aminated product of 2,5-dihydroxy-*N*-(2-hydroxyethyl)-benzamide and 4-aminobenzoic acid from 70% (*Trametes spec*., also pH 5) [20] to 85%. Also comparison of formation of diaminated by-products using different laccases at the same pH value (pH 5) shows differences in the ratio of product to diaminated by-product: *Trametes spec*. 7:1 [20]; *T. villosa* 17:1, *Pycnoporus cinnabarinus* 13:1 (data not shown; enzyme preparation according to [15]). Consequently, formation of the by-product could be minimized with laccase of *T. villosa*.

The high stability of laccase in solution as well as the mild reaction conditions used in laccase catalyzed reactions make this enzyme attractive for fine chemical synthesis. A scale up of the reaction to hike up available amount of products is possible. For that purpose it is necessary to increase the experimental volume. Higher concentration of starting compounds result only in increasing accumulation of several by-products. Laccase-mediated formation of quinones was also determined for other *ortho*-dihydroxylated [21] and *para*-dihydroxylated [22] substrates. The chemical structure and the functional groups of quinonoids contribute their physico–chemical properties, which are important for a lot of biological processes. Natural benzoquinone derivatives like Vitamin K and plastoquinone are well-known [19]. Furthermore quinonoids such as anthracyclines, lapachols, etc. are beside chloroethyl containing compounds very frequent among clinically approved anticancer drugs in the USA [23]. In the past, it was paid a lot of attention also to synthetic derivatives of quinones like aminobenzoquinones [19,24].

Although phenolic substrates do not retain the hydroxyl groups, the above-discussed coupling reactions with laccase may be useful in pharmaceutical syntheses. Common antibiotics, such as mitomycin (benzoquinone) and actinorhodin (naphthoquinone) are quinone derivatives of hydroxylated aromatics. Also tetracycline and anthracycline are antibiotics with quinone structures. Furthermore quinone derivatives like doxorubicin are known as efficient anti-cancerogenics. The concept of laccase application for the synthesis of pharmaceuticals with quinone substructures will be pursued in further studies.

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