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Nuclear amination catalyzed by fungal laccases: Comparison of laccase catalyzed amination with known chemical routes to aminoquinones

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Abstract

Laccases are able to initiate nuclear amination of *p*-hydroquinones with primary aromatic amines, resulting in the formation of the corresponding monoaminated and diaminated quinones. Two laccase catalyzed reactions are compared with established synthetic routes to aminoquinones, showing that formation of products from laccase catalyzed reaction is comparable with reaction using sodium iodate as oxidant. Advantages and disadvantages of laccase catalyzed amination are discussed. It is concluded that laccase catalysis is less suitable than sodium iodate oxidation for the amination of simple *p*-hydroquinones with simple amines.

Keywords: Laccase; Biotransformation; Nuclear amination; Aminoquinone; Sodium iodate; Comparison; Reaction

1. Introduction

Additions of amines to quinonoid systems are well-known reactions in organic synthesis [1]. Nitrogen addition to quinonoid compounds can occur either as nuclear amination or side-chain amination. While nuclear amination occurs by the substitution of hydrogen [2], halides [3] or alkyl groups [4] by an amine moiety, side-chain aminations [5,6] take place by the replacement of hydrogen from an alkyl substituent on the quinone ring by an amine. Steric requirements, nucleophilicity, basicity, and electronic factors determine whether nuclear or side-chain addition occurs [4,5]. Usually the quinonoid form instead of the hydroquinonoid form is observed in the final products of nuclear amination. Monoaminated quinones are often very reactive and subjected to another amination resulting in *p*-diaminated compounds. Longer reaction time, an excess of amine and a higher reaction temperature promote the formation of diaminated products [4,7].

The study of new synthetic routes to aminoquinones is of interest because a number of antineoplastic drugs in use, like mitomycin, or under development, like nakijiquinone-derivatives [8] or herbamycin-derivatives [9], contain an aminoquinone moiety. Several simple aminoquinones possess activity against a number of cancer cell-lines [10–12], antiallergic or 5-lipoxygenase inhibiting activity [12,13].

In a previous report, we showed that fungal laccases (benzenediol:oxygen oxidoreductase, EC 1.10.3.2, copper containing phenol oxidases) are able to catalyze nuclear amination of *p*-hydroquinones with primary aromatic amines in good to very good yields [7].

A deeper insight into laccase catalyzed amination is necessary if laccases are supposed to be used industrially for amination reactions. Therefore, in this study we compare the reaction course and the resulting products of laccase catalyzed reactions with established synthetic methods for the synthesis of two selected aminoquinones. Advantages and disadvantages of laccase catalyzed amination are discussed.

2. Experimental

2.1. General methods

NMR spectra were recorded at 400.13 MHz (^{1}H) and 100.11 MHz (^{13}C NMR and DEPT-135), at ambient temperature in DMSO-d_6. Spectra were referenced indirectly to tetramethylsilane via the residual ^{1}H signal of the deuterated solvent. ESR measurement was done on solid samples (sample amounts approx. 5 mg) at room temperature, an X-Band ESR spectrometer (9.5 GHz) was used.

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For reaction monitoring, samples of the reaction mixture were analyzed using an HPLC system with diode array detector. An endcapped RP_{18} column was used at a flow rate of 1.5 mL/min. A solvent system consisting of methanol (eluent A) and 0.1% (m/m) phosphoric acid (eluent B), starting from an initial ratio of 10% A and 90% B and reaching 100% A within 10.5 min, was used.

All chemicals were purchased from commercial suppliers and used as received.

2.2. Enzymes

Extracellular laccase C of *Trametes* sp. (EC 1.10.3.2) was obtained from ASA Spezialenzyme (Wolfenbüttel, Germany) and used as received (activity 1000 U/g; substrate: syringaldazine). Laccase from *Myceliophthora thermophila* (expressed in genetically modified *Aspergillus* sp.) was bought from NovoNordisk (Bagsvaerd, Denmark). It was used as received (activity 1000 U/g; substrate: syringaldazine).

2.3. Enzymatic amination procedures

Enzymatic amination of the *p*-hydroquinones with primary aromatic amines were performed as previously reported [7]. The compounds were incubated at equimolar concentrations of 2 mM (**1a**, **2**) with laccase in 10 mL of 20 mM sodium acetate buffer, pH 5 (laccase from *Trametes* sp., final activity 0.15 U mL^{-1}) or in concentrations of 2 mM (**1b**) and 10 mM (**2**) in 100 mL 10 mL of citrate–phosphate buffer (16 mM citrate, 164 mM phosphate), pH 7 (laccase from *M. thermophila*, final activity 1.0 U mL^{-1}). The reaction mixture was incubated at room temperature with agitation at 300 rpm.

Reactions were carried out at least three times.

2.4. Established amination procedures

If not stated otherwise, reaction mixtures were incubated in the dark at room temperature with agitation at 300 rpm. Reactions were carried out at least twice. Final concentrations in each reaction of **1a** and **2** were 2 mM, in reactions of **1b** and **2** were 2 and 10 mM, respectively. Final concentrations for cupric acetate were 2 mM, for sodium iodate 6 mM, and for silver (I) oxide 8 mM.

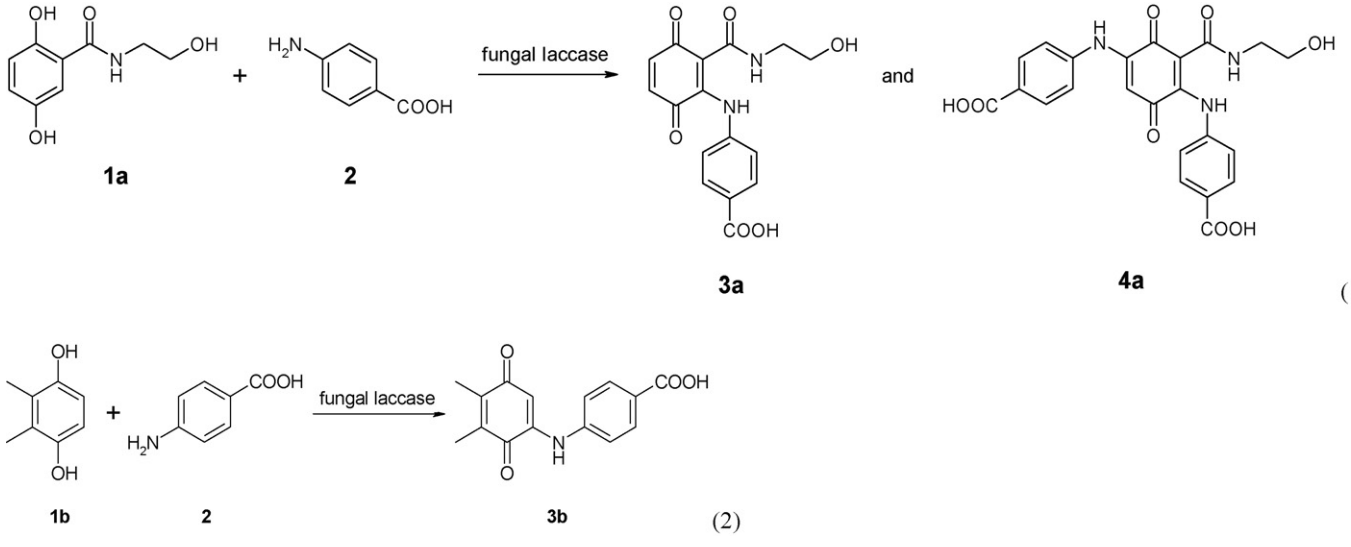
- *Reaction in methanol* [14]. **1a** and **2** or **1b** and **2** were dissolved in 10 mL of methanol.
- *Reaction using cupric acetate* [15,16]. **2** and cupric acetate were dissolved in 6 mL of methanol. To this solution, 4 mL of a solution of **1a** or **1b** in methanol was added drop wise.
- *Reaction using sodium iodate* [2,17]. **1a** and **2** or **1b** and **2** were dissolved in 3.33 mL of methanol and 5.66 mL of water. A solution of sodium iodate in 1 mL of water was added.
- *Reaction using silver (I) oxide* [18]. **1a** and **2** or **1b** and **2** were dissolved in 20 mL of diethyl ether under argon atmosphere. After addition of 200 mg $MgSO_4$ and Ag_2O the reaction mixture was refluxed.

3. Results and discussion

As reported before [7], laccase catalyzed aminations of 2,5-dihydroxybenzoic acid derivatives and of alkylated *p*-hydroquinones with primary aromatic amines differ in a considerable extent. Therefore, for this study one product from each reaction class was chosen for further investigation. Reactions examined here are shown in Eqs. (1) and (2) (Scheme 1).

3.1. Comparison of laccase catalyzed reaction with established synthesis

Methods compared with laccase catalyzed amination included reaction in methanol with [15,16] or without cupric acetate [14], reaction in methanol/water using sodium iodate [2,17] and reaction in diethyl ether using silver (I) oxide as oxidant [18]. The scope of the comparison comprised the reaction rate, the purity of the products, the stability of the products in the reaction medium, and the yields of the respective products.



Scheme 1.

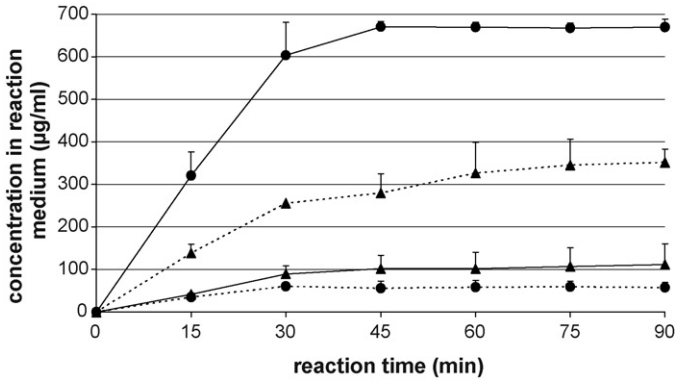


Fig. 1. Comparison of product formation for **3a** (●) and **4a** (▲) in equimolar reactions (2 mM) using laccase from *Trametes* sp. (solid lines) or cupric acetate (dashed lines).

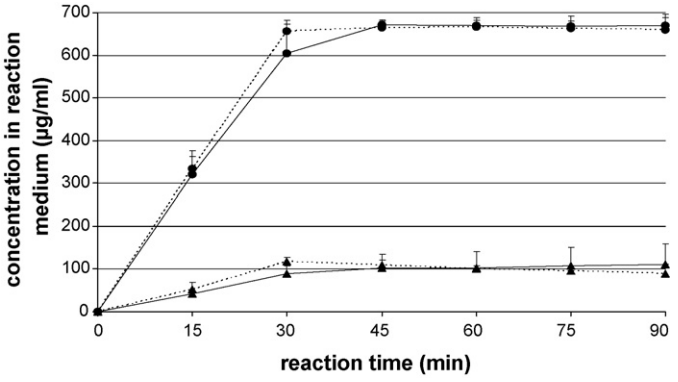


Fig. 2. Comparison of product formation for **3a** (●) and **4a** (▲) in equimolar reactions (2 mM) using laccase from *Trametes* sp. (solid lines) or sodium iodate (dashed lines).

3.2. Amination of 2,5-dihydroxy-N-(2-hydroxyethyl)-benzamide with 4-aminobenzoic acid

Reaction of **1a** and **2** in methanol gave no products within 24 h (data not shown).

Reaction in methanol using cupric acetate as oxidant resulted in the formation of both products (Fig. 1). Compared with laccase catalyzed reaction, after 90 min the accumulated amount of **3a** is very low, but formation of **4a** shows a higher yield (Table 1). However, even after 24 h **1a** and **2** still were not consumed completely, and the amounts of **4a** accumulated were lower than in the laccase catalyzed reaction using amine excess. A high number of uncharacterized by-products was formed in significant amounts in addition to **3a** and **4a** (Fig. 3). This reaction was not comparable with laccase catalysis regarding the reaction rates, the amounts of products formed and the purity of the products.

Reaction in diethyl ether using silver (I) oxide as oxidant had a different course, as well. Among other not identified by-products, **3a** and **4a** were formed in lower yields over the 90 min reaction time, leaving high amounts of educts unconverted (Table 1, Fig. 3).

Reaction of **1a** and **2** in methanol/water using sodium iodate was comparable to laccase catalyzed reactions. A sodium iodate concentration of 6 mM was equal to the used laccase activity of 0.15 U mL^{-1} concerning the formation rate of **3a** and **4a** (Fig. 2). Two further uncharacterized by-products were formed in very low quantities (Fig. 3), but relative product formation of **3a** and **4a** were nonetheless high (Table 1). The products formed were identical regarding their HPLC retention time, UV–vis, ^{1}H and ^{13}C NMR spectra. In both reactions, the stability of the products in solution was comparable over a period of 45 min after maximum product formation. As formation of both products was comparable, this reaction was investigated further.

Increased amounts of laccase or sodium iodate resulted in much faster reactions. Ten-fold increase reduced the time for complete reaction from 45 min to less than 5 min in both cases. Even at oxidant concentrations this high, no additional by-products could be detected.

Table 1
Concentration of the products **3a** and **4a** in the reaction media after 90 min reaction time in µg/ml using the reaction initiators laccase, cupric acetate, silver (I) oxide and sodium iodate

	Laccase	Cupric acetate	Silver (I) oxide	Sodium iodate
3a	670.0	57.6	156.1	660.0
4a	111.5	351.9	11.8	88.3

3.3. Amination of 2,3-dimethylhydroquinone with 4-aminobenzoic acid

As amination of alkylated *p*-hydroquinones takes much more time than amination of 2,5-dihydroxybenzoic acid derivatives [7], for this reaction the amounts of formed aminoquinone after 24 h were compared.

Like in the case of the amination of **1a** with **2**, reactions in methanol with and without cupric acetate or reactions using silver (I) oxide were unlike laccase catalyzed reactions. No significant amount of amination product **3b** was formed within 24 h in either of these reactions. Again, reactions using sodium iodate as oxidant were comparable to laccase catalysis regarding the amount of product formed and the purity of the product (data not shown). However, the reaction proved to be highly dependent on the pH of the reaction medium. Quinone formation from **1b** due to sodium iodate oxidation is very fast at pH 5 and very slow at pH 7. Hence reaction in water:methanol (1:1, pH approx. 5) did result in only small amounts of amination product if the pH was not adjusted to pH 7 by addition of buffer after the rapid quinone formation. If the pH of the reaction medium was controlled from the beginning on by the same buffer we used in laccase catalyzed reactions (pH 7.4), after 24 h the same amount of product **3b** was formed, although initial quinone formation was much slower. The fact that aminoquinone formation shows a pH dependency has been described before [19].

Laccase catalyzed formation of phenoxy-radicals has been proposed to be the initial mechanism of laccase catalyzed coupling reactions [20,21]. Therefore, it was highly interesting to investigate whether the resulting products in laccase initiated oxidative aminations retained a radical character. ESR

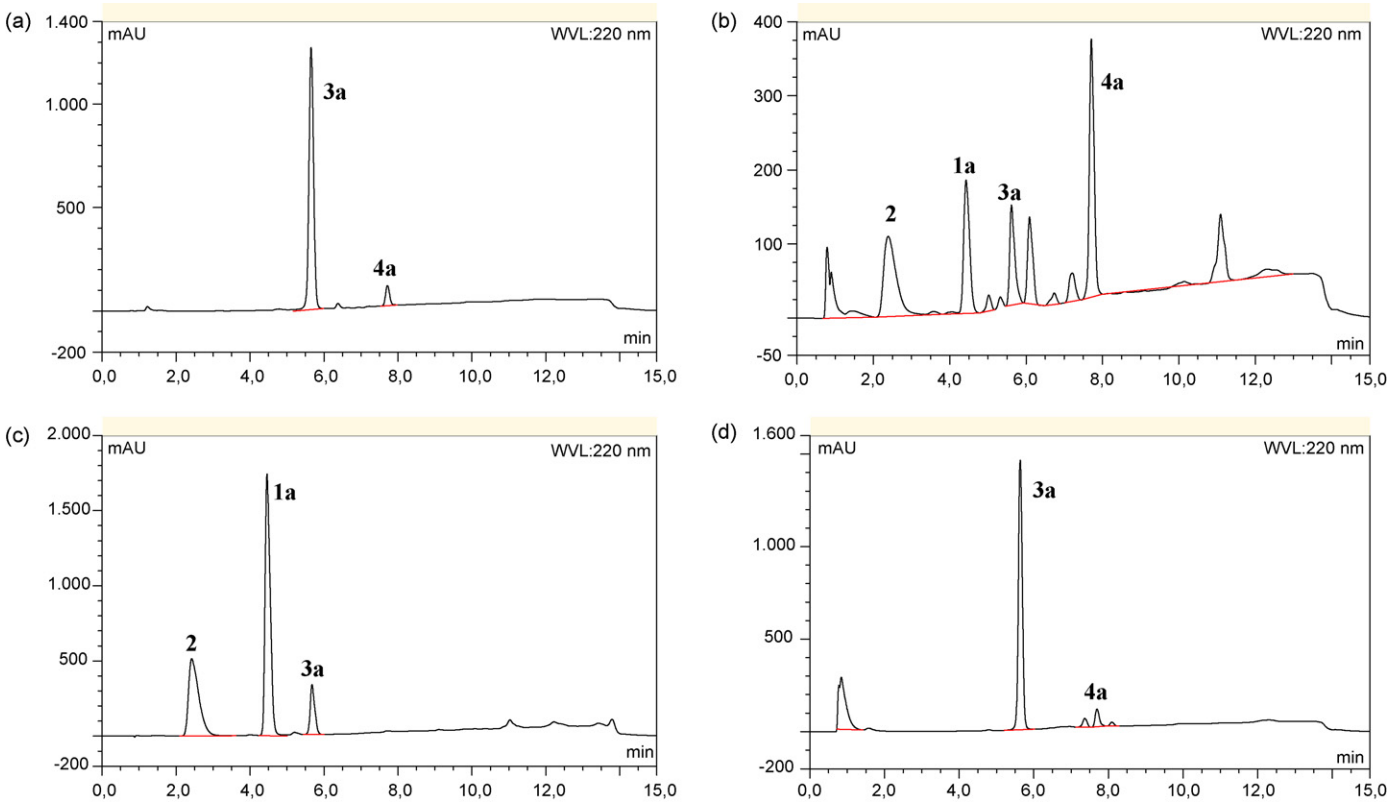


Fig. 3. HPLC chromatograms of the described reactions after 90 min reaction time. (a) Laccase; (b) cupric acetate; (c) silver (I) oxide; (d) sodium iodate.

measurements showed this to be not the case. The two products **3a** and **4a** of neither laccase catalyzed amination nor amination using sodium iodate as oxidant showed significant amounts of free radicals. In most samples, no signals could be observed. In few samples a signal with very low signal-to-noise ratio was detected, indicating a radical concentration below 1% of the sample amount. As only insignificant traces of radicals could be detected in the products, and as amination of quinones with amines is known to be easily accomplished without additional reagents [10,22–25], we think that the role of laccases in nuclear amination of hydroquinones lies solely in the initial oxidation of the hydroquinones to the corresponding quinones. Copper(II), acting as electron acceptor in laccase catalyzed reactions [26], is a one-electron oxidant, so a free-radical mechanism could be expected for the reaction. However, as laccases usually contain four copper(II) atoms, two directly subsequent one-electron oxidation steps could occur, making the direct oxidation of the hydroquinones to the corresponding quinones likely. Formation of quinones or quinonemethides has been proposed before to be the initial step in other addition reactions catalyzed by laccases [27–29].

Comparison of laccase catalyzed amination with established methods of quinonamine synthesis showed that reactions using sodium iodate as oxidant are leading to the same products in the same amounts and rates. In the case of **1a** and **2**, both amination methods resulted in the formation of only two products, whereas the other tested methods using cupric acetate or silver (I) oxide as oxidant either led to the formation of a high number of uncharacterized compounds, resulting in low yields of mono- and diaminated products, or to no product formation, respectively.

Detailed comparison of the reactions using laccase or sodium iodate showed that they are comparable regarding reaction rate, purity of the products, stability of the products in the reaction medium, and yields. However, amination using sodium iodate as oxidant has some advantages over laccase catalyzed reactions.

First, the avoidance of buffer salts and proteins in the reaction medium can simplify the accomplishment of the reaction and the isolation of the products. Second, even though laccases are quite stable dried and in solution, sodium iodate is easier to handle, more stable, and cheaper. However, these laccase catalyzed reactions can possibly be optimized so that the enzymes could be used in lower, catalytic quantities. Sodium iodate on the other hand will always be required in a stoichiometric amount. The possibilities of laccase immobilization and reuse after the reaction do not compensate these disadvantages, as immobilization is more complicated and activity of the immobilized enzymes dwindles [30]. Third, laccase catalyzed reactions depend on the presence of sufficient amounts of oxygen in solution. This problem, being acute especially in closed spaces like reaction reactors and high volumes, has been overcome in reactors using an integrated bubble-free aeration system [31]. Sodium iodate does not need additional oxygen to act as oxidant, so that closed reaction vessels do not pose a problem.

An advantage of laccase catalysis can be that it is a catalytic oxidation which, in principle, produces water as the sole by-product, and therefore could be ecologically friendlier. A further advantage of laccase catalysis could be the amination

of educts bearing a substituent susceptible to oxidation besides the *p*-hydroquinonoid structure, which would be oxidized by sodium iodate but not by the more selective laccases. However, as there are examples for laccases oxidizing non-phenolic structures [32–34], this advantage of laccases over sodium iodate is not ensured and still has to be investigated.

4. Conclusion

Laccases are enzymes of great interest for the use in fine chemical synthesis. However, it is shown here that laccase catalyzed amination of simple *p*-hydroquinones with simple primary aromatic amines can be accomplished more conveniently using the well-known chemical oxidant sodium iodate. It is likely that *p*-quinone formation is the initial step in laccase initiated nuclear amination reactions, and that the role of laccases in the reactions described here solely lies in laccases being an oxidant oxidizing *p*-hydroquinones.

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