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Laccase-Catalysed Homocoupling of Primary Aromatic Amines towards the Biosynthesis of Dyes

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Abstract: Coloured disubstituted benzoquinonimine trimeric structures are obtained as main reaction products of the oxidation of *p*-electron donor primary aromatic amines using two different laccases, CotA-laccase from *Baccilus subtilus* and TvL from *Trametes versicolor*. These orange-red to purple products, presenting high molar extinction coefficients, presumably result from oxidative homocoupling reactions, through the formation of N–C bonds at positions 2 and 5, of the laccase oxidised intermediate as showed in the proposed oxidative pathway. The product of 1,4-phenylenediamine is shown to be the trimer known as Bandrowski's base which has an established role in hair and fur dyeing. Our results also show that the occurrence and/or rates of oxidation of aromatic amines are strongly dependent on the presence of *p*-electron releasing substituents in the aromatic ring and are independent on the properties of the enzyme used. Overall our data contribute for (i) understanding key features of laccase reactivity with *p*-substituted aromatic amines and (ii) establishing enzymatic processes that lead to the synthesis of coloured bio-products under mild conditions with potential impact in the cosmetic and dye industries.

Keywords: aromatic amines; biocatalysis; dyes; laccase; oxidation; radical coupling

Introduction

Eco-friendly enzymatic bioprocesses represent an attractive and important alternative to the conventional chemical synthetic processes. The potential benefits of utilising biocatalysts, such as enzymes, compared to inorganic or organic catalysts, arise from their activity under mild conditions of temperature, pH and pressure. Moreover, higher reaction rates and specificities are usually obtained when compared with reactions using chemical catalysts.

Laccases represent an interesting group of multicopper oxidoredutive biocatalysts (EC 1.10.3.2) with great potential for industrial and environmental applications.[1,2] Laccases are widely distributed in fungi, higher plants and bacteria showing a broad spectra of low-molecular weight substrates either of natural or synthetic origin. Laccases catalyse their oxidation using dioxygen as co-substrate which is reduced to water in aqueous solutions, resulting in environmental benign and low cost reactions.[3,4] Their use in the biosynthesis of fine chemicals has been fairly explored due to the high specificity, e.g., phenolic substructures, such as *o*- and *p*-diphenols, methoxyphenols, aminophenols and lignin-related molecules.[2–6] Generally, laccases react with phenolic substrates, which loose one electron and one proton, generating the corresponding free radicals (phenoxy radicals) that are stabilised by resonance into the respective quinone structures or covalently coupled to oligo- or polymeric products. The competence of laccases to promote homo- or heteromolecular coupling reactions has been described, namely in reactions between phenols or quinonoid systems and primary amines, with the formation of new C–O, C–N or N–N products.[7–12] Aromatic amines have been mainly used as nucleophiles in phenol reactions catalysed by laccases.[8,13,14] The coupling of a typical laccase substrate, such as a substituted hydroquinone, with primary amines usually occurs as nucleophilic amination on

the aromatic ring by the substitution of hydrogen,[7,8] halides or alkyl groups.[13] The substituted quinonoid products formed are often very reactive and can undergo further amination, leading to *para*-diaminated compounds. Also, extended reaction periods, high temperatures or reactions in the presence of an excess of amine promote the formation of diaminated products.[8]

More recently, however, the use of aromatic amines as substrates for laccase was described in the oligomerisation of aniline for the production of conducting polyaniline[15] or in the polymerisation of aniline derivatives.[16]

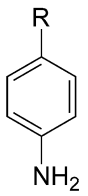
1,4-Phenylenediamine (1,4-PDA) or its substituted derivatives and 4-aminophenol (4-AP) are extensively used in the cosmetic industry as dye precursors or as oxidation bases for the permanent dyeing of furs and hair. These colourless or weakly coloured compounds give rise to coloured compounds by oxidative condensation at the highest alkaline pH range in the presence of hydrogen peroxide.[17–19] The development of less aggressive processes for the production of hair dyes is of great interest due to the involvement of direct human contact and laccases are excellent alternative candidates for the production of biocolourants under mild conditions.

Therefore, the present work focuses on the characterisation of homocoupling reactions of *p*-substituted aromatic amines using laccases. The aim is the production of biocolourants, through enzymatic reactions better tailored for oxidative synthesis. Four out of eight primary *p*-substituted aromatic amines were selected as the enzyme's substrates and orange-red to purple coloured diaminated quinonimine products were identified and characterised by NMR and mass spectrometry. The kinetic constants and conversion yields were determined and the results are discussed in relation with the properties of enzymes used: the bacterial CotA-laccase from *Bacillius subtilis* and TvL-laccase from the fungi *Trametes versicolor*. A pathway for the laccase oxidation of *p*-substituted aromatic amines is proposed.

Results and Discussion

Electrochemical Studies of Substrates

The difference in the redox potential between the oxidoreductases and their substrates is a critical parameter in determining the occurrence and/or rates of enzymatic reactions.[20–23] Therefore, the redox potential was determined by cyclic voltammetry for eight aromatic amine substrates as follows: 1,4-phenylenediamine (1,4-PDA), 4-aminodiphenylamine (4-ADA), 4-aminophenol (4-AP), 4-aminophenylacetamide (4-APA), 4-aminobenzenesulfonic acid (4-ABSA), 4-aminobenzoic acid (4-ABA), 4-aminobenzonitrile (4-ABN) and 4-nitroaniline (4-NA) (Figure 1). These measurements were performed at different pH values, since pH affects both the properties of enzymes and substrates with a strong impact in enzymatic reactivity.



1,4-PDA	R = NH_2
4-ADA	R = NHPh
4-AP	R = OH
4-APA	R = $NHCOCH_3$
4-ABA	R = COOH
4-ABSA	R = SO_3H
4-ABN	R = CN
4-NA	R = NO_2

Figure 1. Representative structures of the *p*-substituted aromatic amines.

The aromatic amines show their oxidation processes in the potential range 0.40 to 1.30 V at a scan rate of 100 mV s^{-1} (Table 1) and the electrochemical behaviour showed to be dependent on the electronic nature of the *p*-substituent group. The aromatic amines with *p*-electron-withdrawing groups (4-ABA, 4-ABSA, 4-ABN and 4-NA) were characterised by well-defined irreversible oxidation peaks (E_{pa}) in the pH range. The absence of reduction peaks (E_{pc}) indicates that the electrochemically generated species are not stable and rapidly decay to non-reducible counterparts. Compounds with *p*-electron-donor groups, in particular 1,4-PDA and 4-ADA, presented quasi-reversible redox processes at lower pHs (4 and 5), which become irreversible for higher pHs.

Independently of the electronic nature of the *p*-substituent, the oxidation potentials decrease as the pH increases and differences up to 230 mV (for 4-AP) were obtained. Based on the obtained results, the amines can be split in two groups: the first group comprising compounds with *p*-electron-donor groups, showing oxidation potentials between 0.40 and 0.87 V (1,4-PDA, 4-ADA, 4-AP and 4-APA) and more prone to be oxidised, and the second group comprising substrates harbouring *p*-electron-withdrawing groups, with significantly higher oxidation potentials, between 1.07 and 1.30 V (4-ABA, 4-ABSA, 4-ABN and 4-NA). Considering the redox potential of the T1 copper centres of CotA and TvL laccases, 0.55 V and 0.79 V (*vs.* NHE)[24,25] respectively, only the first group of compounds is expected to be oxidised by the laccase systems.

Enzymatic Oxidation of *p*-Substituted Aromatic Amines

The first indication of reaction between *p*-substituted aromatic amines and laccases is the formation of

Table 1. Electrochemical data of *p*-substituted aromatic amines *vs.* NHE in buffered solutions at a scan rate of 100 mV s^{-1}.

Substrate	pH 4			pH 5		pH 6		pH 7
	E_{pa} (V)	E_{pc} (V)	$E_{1/2}$ (V)	E_{pa} (V)	E_{pc} (V)	E_{pa} (V)	E_{pc} (V)	E_{pa} (V)
1,4-PDA	0.60	0.48	0.54[a]	0.52[b]	0.43	0.47	0.37[c]	0.41
4-ADA	0.52	0.44	0.48[d]	0.49	0.31	0.48	–	0.44
4-AP	0.63	0.43	–	0.53	0.32	0.48	0.26	0.40[e]
4-APA	0.87	–	–	0.83	–	0.82	–	0.79
4-ABA	–[f]	–[f]	–	–	–	1.20	–	1.07
4-ABSA	1.22	–	–	–	–	1.18	–	1.16
4-ABN	–[f]	–[f]	–	–	–	1.26	–	1.23
4-NA	–[f]	–[f]	–	–	–	1.30	–	1.21

[a] $\Delta E = 120$ mV, $I_{pc}/I_{pa} = 1.0$.
[b] $E_{1/2} = 0.475$ V, quasi-reversible wave, $\Delta E = 90$ mV, $I_{pc}/I_{pa} = 0.9$.
[c] $\Delta E = 100$ mV, $I_{pc}/I_{pa} = 0.5$, irreversible wave.
[d] $\Delta E = 80$ mV, $I_{pc}/I_{pa} = 1.0$.
[e] $E_{pc} = 0.23$ V.
[f] Not detected. Potentials out of the experimental range.

colour when the enzyme was added to reaction mixtures. According to our prediction, the aromatic amines with *p*-electron-withdrawing groups were not oxidised, while the compounds bearing *p*-electron-donor groups were all oxidised by both laccases, highlighting the need for a good match between the redox features of the enzyme and the substrate. We have tested the optimal pH for the four substrates selected (1,4-PDA, 4-ADA, 4-AP and 4-APA) and have concluded that, as shown previously to occur with phenolic substrates,[26] TvL shows maximal rates at pH 4–5, whereas CotA shows a preference for pH values close to neutrality, pH 5–7 (data not shown). This difference relates to the presence of a negatively charged residue close to the active site in TvL and also in other fungal laccases, proposed to have a role in facilitating the formation and stabilisation at low pH values of the radical intermediate formed during the catalytic reaction.[27–29] In the case of CotA, or any bacterial laccase identified so far, no negatively charged residue in the vicinity of the substrate binding site[30] is present and the efficiency of the oxidation of phenols or aromatic amines rely mostly on the protonation/deprotonation state equilibria of the substrates themselves.[26,31]

The kinetic constants of CotA and TvL laccases for the selected substrates were determined at the respective optimal pH values (Table 2). CotA in general exhibited higher specificity (k_{cat}/K_M) values than TvL. No major differences in K_M are observed except for 4-APA where 4-fold higher K_M values were calculated as compared with the other substrates. In contrast, the oxidation rates (k_{cat}) showed major differences; the electron-richer amines 1,4-PDA, 4-ADA and 4-AP are oxidised faster, while the 4-APA reacts slower similarly to what was previously observed for the oxidation of different phenolic substrates.[23,26,32]

The parameter k_{cat} is reflective of the enzymatic rate-limiting step, assumed to be the substrate oxidation and accordingly, the k_{cat} values followed the trend of redox potential of the substrates.

The higher redox potential of TvL would appear to favour an increased reaction rate, as the electron-transfer rate (k_{ET}) is a major component of the parameter k_{cat}. However, the oxidation rates for the substrates tested are lower for TvL, except for 4-ADA, as compared with CotA (Table 2). According to the Marcus theory, two additional factors apart from the redox potential (E^o) affect the k_{ET} in proteins: the donor-acceptor electronic coupling (H_{AD}), where the exact geometry of the protein matrix has an important role and the reorganisation energy (λ) which also depends on the structure and dynamics of the protein.[33] In the present case, the reasons for the small

Table 2. Kinetic parameters of oxidation reactions catalysed by CotA and TvL laccases. Reactions were performed at the optimal pH (Britton–Robinson buffer) for each substrate.

	CotA					TvL			
Substrate	Optimal pH	K_M [mM]	k_{cat} [s^{-1}]	k_{cat}/K_M [mM^{-1}s^{-1}]	Substrate	Optimal pH	K_M [mM]	k_{cat} [s^{-1}]	k_{cat}/K_M [mM^{-1}s^{-1}]
1,4-PDA	7	0.6 ± 0.1	14.3 ± 0.5	22.6 ± 4.1	1,4-PDA	5	1.3 ± 0.1	6.0 ± 0.1	4.8 ± 0.2
4-ADA	5	0.4 ± 0.1	14.3 ± 0.3	35.0 ± 4.0	4-ADA	4	1.4 ± 0.2	29.5 ± 2.0	21.3 ± 1.0
4-AP	6	0.5 ± 0.2	2.2 ± 0.2	4.6 ± 0.8	4-AP	5	0.5 ± 0.1	0.8 ± 0.1	1.6 ± 0.4
4-APA	7	1.3 ± 0.1	0.20 ± 0.04	0.10 ± 0.02	4-APA	5	3.9 ± 0.4	0.4 ± 0.1	0.10 ± 0.04

differences on the overall rate of reactions of the two enzymes must rely in the electronic tunnelling and/or the reorganisation energy of the two electron transfer processes (from the substrate to the T1 centre and from this to the trinuclear site) in TvL and CotA. However, none of these possibilities was investigated here.

The conversion yields of CotA for the different *p*-substituted aromatic amines after 24 h of reaction are presented in Table 3. The orange-red to purple reaction products, with high molar extinction coefficients, were isolated by solid-phase extraction (products soluble in buffer solution) or filtration (insoluble products) in amounts considered sufficient for structural characterisation. Higher conversion yields were obtained for 4-ADA 1,4-PDA and 4-AP when compared with 4-APA, in agreement with the kinetic data. Identical products and similar conversion yields were obtained for the TvL enzyme (data not shown).

Table 3. Products formed after 24 h of reaction using 5 mM substrate, 1 U mL^{-1} CotA laccase, at 37 °C in methanol:buffer (1:10).

Substrate	pH	Reaction products (Yield [%])[a]	Colour	λ [nm] (ε [M^{-1} cm^{-1}])
1,4-PDA	7	**1b** (90)	dark purple	510 (3,467)
	8	**1a** (95)	orange	450 (3,408)[b]
4-ADA	6	**2** (97)	violet	570 (1,046)[c]
4-AP	6	**5** (75)	brown	400 (9,551)[c]
4-APA	7	**3** (31)	orange	360 (8,335)
		4 (20)	yellow	–

[a] Isolated yields.
[b] Acetone:buffer (1:10).
[c] Methanol.

Overall our results suggest that the electronic nature of the *p*-substituent plays a crucial role in the oxidation efficiency of aromatic amines by laccase.

Identification of the Biotransformation Products

The oxidation of 1,4-PDA, 4-ADA and 4- APA, i.e., those bearing amine or amide electron-releasing groups, result in the formation of trimers with a 1,4-substituted-2,5-benzoquinonediimine skeleton (**1**–**3**, Figure 2). The oxidation of 4-AP, with a hydroxy group results in the formation of the trimeric structure **5**, with a 1,4-benzoquinonemonoimine skeleton substituted at positions 2 and 5.

The aromatic nature of the benzoquinonediiminic structures (**1**, **2** and **3**) was confirmed by the presence of multiple signals in the range 5.5–7.8 ppm and 90-160 ppm in both ^{1}H and ^{13}C NMR spectra, respectively. The central iminic skeleton defined at C-2 and C-5 positions was identified, in all cases, by the presence of characteristic singlets for protons H-3 and H-6 in the aromatic region (5.68–7.64 ppm) and the corresponding carbon signals in the 90–100 ppm range in the ^{13}C NMR spectra. The multiplicity of these protons (H-3, H-6) and their correlation with carbons C-2 and C-5 (153–156 ppm), in the ^{1}H-^{13}C HMBC spectra, confirmed the attributed quinonoid character of the structures. For compound **1a**, the ^{1}H NMR data were in accordance with the literature values.[34]

The oxidation product of 1,4-PDA, the trimer 1,4-diamino-2,5-benzoquinonediimine (**1**) was isolated in two forms **1a** and **1b** (Figure 2), showing distinct colours (orange and dark purple, respectively) and distinct water solubilities. We have analysed both forms by UV-Vis spectrophotometry and have observed a reversible interconversion upon pH change in accordance with the pK_a of the trimer.[35] The two absorption bands, near 335 and 450 nm observed for **1a** (Figure 3), arise from π-π* electronic transitions within the benzoquinonoid ring and are shifted to 350 and 540 nm, respectively in form **1b**. This red-shift, more pronounced for the lowest energy band, may result from the delocalisation of the conjugated π-system and the shift values found are within the range of related benzoquinonediimine derivatives.[35,36] Therefore, the appearances of **1a** and **1b** are explained by the protonation/deprotonation at the iminic nitrogens and formation of dicationic cyanine subunits stabilised by intramolecular delocalisation in **1b**.

This trimer is known as the Bandrowski's base and is traditionally obtained from 1,4-PDA oxidation in aqueous ferrocyanide solution[35] or by permanent hair colouration processes with alkaline hydrogen peroxide.[18] Taking this into account, the present work shows an alternative bioprocess for the production of this product at high yields (90 to 95%) and milder conditions.

In the case of 4-APA, two products are formed, the trimer **3**[37] and an additional yellow azo product (**4**)[38] resulting from the coupling of the enzymatic oxidised 4-APA intermediates (Figure 2). The ^{1}H NMR spectrum of **4** showed a multiplet centered at 7.80 ppm indicating the formation of a symmetric structure and a low field shift for all the aromatic protons, consistent with the formation of an N=N bond. Furthermore, the respective mass spectrum shows peaks at $m/z = 295$ and 297 in negative and positive modes, respectively, as well as a major peak at $m/z = 319$ ($[M+Na]^+$) which are consistent with a molecular mass of 296 g mol^{-1}. Fragmentation of the positive ion ($m/z = 297$) generated the major ions with m/z of 255, 162 and 134 (base peak), which can be explained by cleavage of the C–N amide bound ($m/z = 255$) and by the cleavage at the central azo group followed by loss of

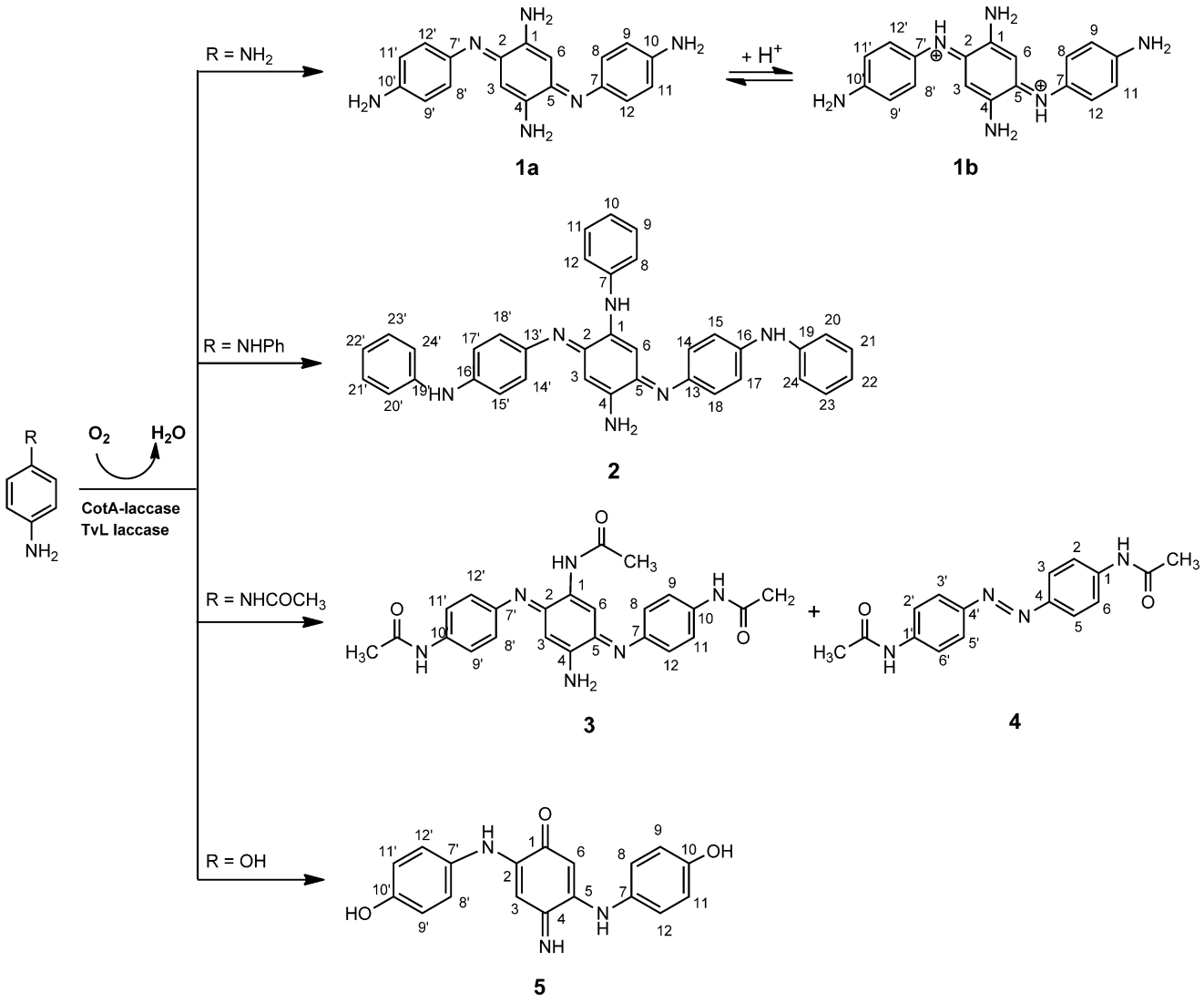


Figure 2. Structures of main products formed in laccase reactions with *p*-substituted aromatic amines.

N_2, a typical fragmentation of aromatic azo compounds.[39]

The formation of an azo compound as product of the biotransformation of aromatic amines was previously observed by Vanhulle et al.[10] in the degradation of the anthraquinonic dye, Acid Blue 62 by laccases. We showed later that this azo product is formed through the creation of an azo bond by coupling between the amine radical intermediates of the enzymatic reaction.[11]

The oxidation of 4-AP leads to formation of a brown solid (**5**) with a 2,5-diaminated 1,4-quinonemonoiminic structure. The multiplicity of H-3 and H-6 protons, the presence of two signals at low field, 177.6 and 161.6 ppm (characteristic from C=O and C=N bonds respectively), in the ^{13}C NMR spectrum and the HMBC correlations between the H-3, H-6 and the carbonyl signal at 177.6 ppm, are indicative of the diaminated 1,4-quinonemonoiminic structure. The presence of the hydroxy group on C-1, from which a quinonic structure could be easily obtained, should be the responsible for the formation of the 1,4-benzoquinonemonoimine skeleton. This trimeric structure is different from the one reported before for 4-AP oxidation[40,41] leading to the formation of the 1,4-substituted-2,5-benzoquinonediimine trimer.

Proposed Pathway for the Oxidation of *p*-Substituted Aromatic Amines by Laccases

Electron-donor *p*-substituted aromatic amines can be used as substrates for laccases with their oxidation being initiated by one electron abstraction by the T1 Cu(II) ion of laccases. This leads to the formation of aminyl radicals in a pathway similar to the one previously described for the oxidation of phenol derivatives.[1,4,6,42]

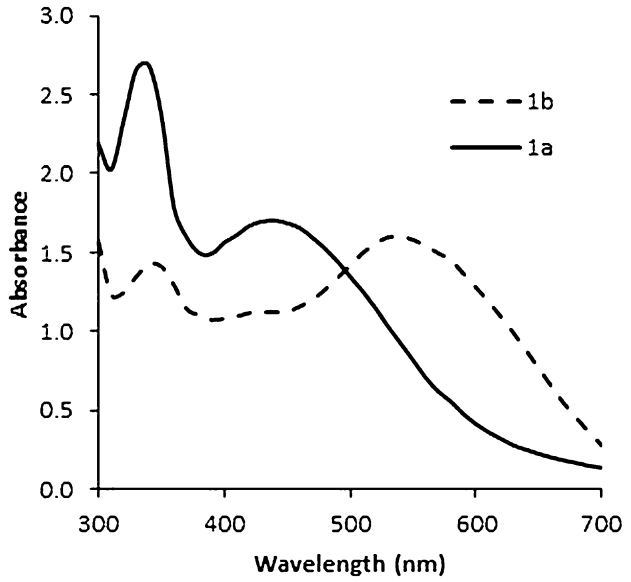


Figure 3. UV-Vis spectra of forms **1a** and **1b** (5×10^{-4} M) in Britton–Robinson buffer (pH 4 or 10) with 10% of $(CH_3)_2CO$.

Scheme 1 shows the proposed pathway that leads to the formation of the disubstituted benzoquinonediimine trimers. The initial step of the laccase oxidation process should be the abstraction of an electron followed by deprotonation of the substrate. The end products are expected to be two short-lived intermediates: an aminium cation radical ($\mathbf{A}^{+\bullet}$) or a neutral radical species ($\mathbf{A}^{\bullet}$), the aminyl radical, in accordance with species previously detected in the course of the chemical oxidation of 1,4-PDA by ferricyanide[19] and as intermediates of the amine's oxidation by cytochrome P450 enzyme.[43,44] Thus, electron-donor substituents in the *p*-position, by stabilising the radical cation, are expected to lower the transition state energy and speed up the enzymatic oxidation, whereas electron-withdrawing substituents do retard it.

Therefore, starting from the radical intermediates, which are also susceptible to sequential self-conjugation, the reaction proceeds through the formation of the benzoquinonediimine intermediate and N–C coupling in the activated *ortho* position (C-5) to the amino group to form a homomolecular dimeric structure. After the first coupling, the second addition on the aromatic ring will be performed in the *para* position (C-2) relative to the first covalent C–N bond site and the central ring is stabilised by resonance.

According to this pathway, the stability of the radicals seems to be of major importance for the catalytic efficiency and the presence of electron-donating substituents on the aromatic ring is found to be a key factor for this stability.

Conclusions

Herein, we characterise the ability of laccase to generate colour "*in situ*" with potential application in, e.g., hair permanent dyeing from originally non-coloured aromatic amines. The use of oxidoreductive enzymes represents a convenient and eco-friendly way of synthesising new molecules of complex structure. Therefore, laccases can be used in alternative bioprocesses for the cosmetic and dye industries.

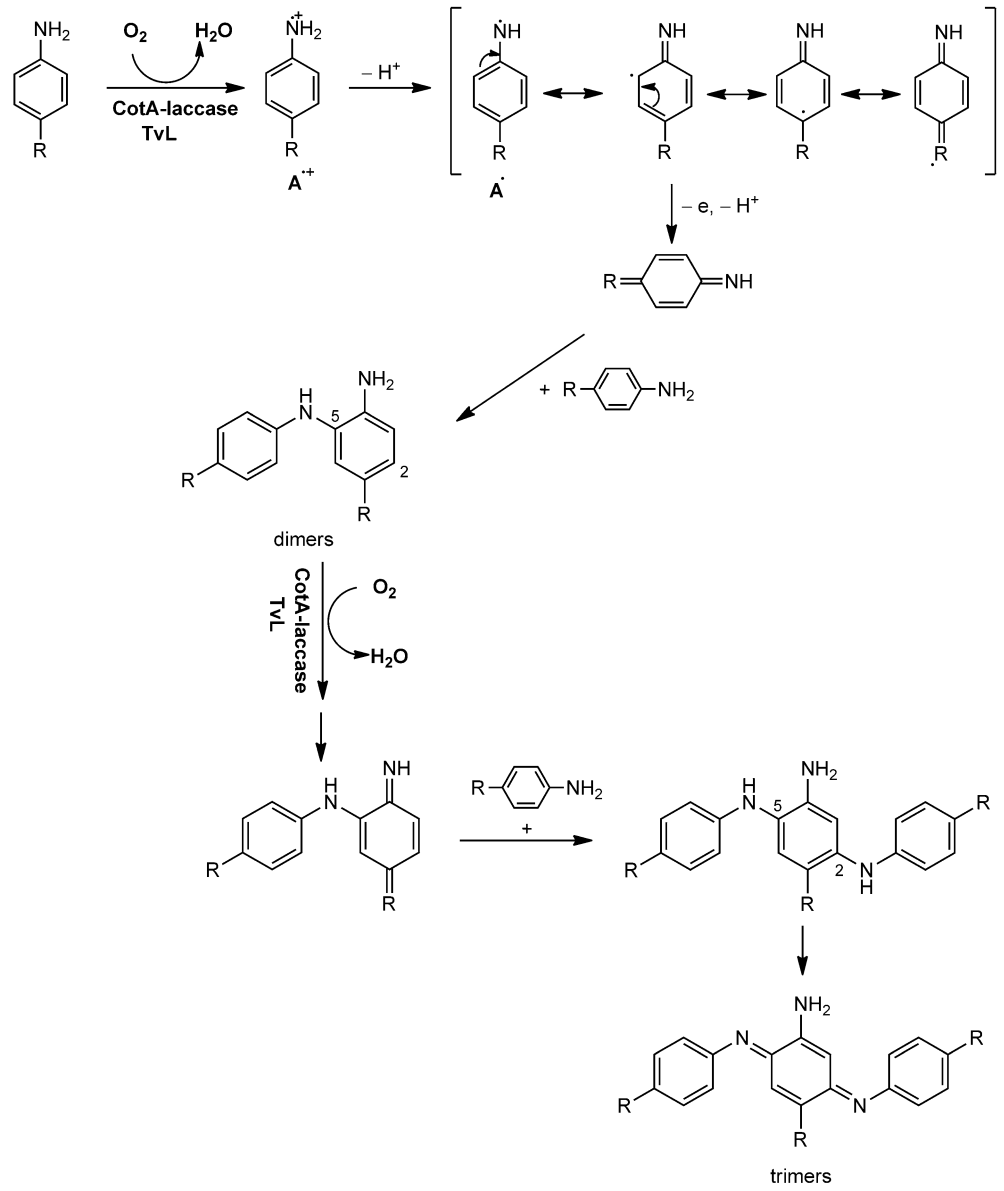
Coloured homocoupling products from 1,4-PDA, 4-ADA, 4-APA with trimeric 1,4-substituted-2,5-benzoquinonediimine structures were unambiguously identified and characterised, together with a trimeric 1,4-quinonemonoimine structure for 4-AP, where the presence of the hydroxy group allowing easy oxidation to quinone, is responsible for the formation of the substituted 1,4-quinonemonoimine trimer.

The rates of reactions, conversion yields and products formed are independent of the enzymatic system used. The enzyme's efficiency is mostly dependent on the redox features of substrates and consequently on the electronic nature of the *p*-substituent. Our data show that the presence of a *p*-electron-donor group converts recalcitrant amine molecules into laccase substrates by increasing the electronic density on the amine group.

Experimental Section

General Procedures

All reagents and solvents are commercially available (Sigma–Aldrich Co) and were used without further purification. The UV-visible spectra of compounds **1a**, **1b**, **2**, **3** and **5** were obtained in B&R buffer or methanol in a Cecil Instruments CE2041 spectrophotometer. The molar extinction coefficients for compounds (**1**, **2**, **3** and **5**) were determined in the concentration range (**1***:* 3×10^{-5}–4.5×10^{-4} M, **2***:* 1×10^{-4}–5.5×10^{-4} M, **3**: 3×10^{-5}–4.5×10^{-4} M and **5***:* 3×10^{-5}–2.5×10^{-4} M) in a Nicolet Evolution 300 spectrophotometer. The product characterisation was performed by 1D NMR (^1H,^{13}C) and 2D NMR (COSY, HSQC, HMBC and NOESY) spectra, obtained in DMSO-d_6, $(CD_3)_2CO$, or CD_3OD on a Bruker Advance 400 MHz spectrometer. Chemical shifts are reported in ppm relative to the solvent peaks and coupling constants (*J*) are reported in Hertz. FT-IR spectra were obtained in KBr pellets on a Bruker Vertex 70 FT-IR spectrometer. Mass spectra (MS) were recorded on a 500-MS LC ion trap mass spectrometer (Varian, Inc., Palo Alto, CA), operated in the positive and negative electrospray ionisation (ESI) modes. The optimised operating parameters were: ion spray voltage: ±5 kV; capillary voltage: 20 V and RF loading: 90%. HR-MS (EI) were recorded on a Micromass Autospec at Unidade de Spectrometría, Universidade de Santiago de Compostella. Recombinant CotA-laccase from *Bacillus subtilis* (1 U mL^{-1} defined as the amount of enzyme that transformed 1 μmol of ABTS per



Scheme 1. A proposed oxidative pathway for the formation of the disubstituted benzoquinonediimine trimers from laccase and aromatic amines.

min at 37 °C) was produced and purified as described previously.[31,45] *Trametes versicolor* laccase (TvL, specific activity 20 U mg^{-1}; 1 U mL^{-1} defined as the amount of enzyme that transformed 1 μmol of catechol per min) was purchased from Sigma–Aldrich. Both laccases were stored frozen at −18 °C prior to use.

Electrochemical Measurements

The redox potentials for *p*-substituted aromatic amines were measured by cyclic voltammetry using an EG&G Princeton Applied Research Model 273 A potentiostat/galvanostat monitored with a personal computer loaded with Electrochemistry PowerSuite v2.51 software from Princeton Applied Research. Cyclic voltammograms were obtained using 1 mM of compounds in 1:10 MeOH:buffer (phosphate buffer for pH ≥ 6 and Britton–Robinson (B&R) buffer (pH 4–10; 100 mM phosphoric acid, 100 mM boric acid and 100 mM acetic mixture with 0.5 M NaOH to the desired pH) for pH 4 and 5, using a three-electrode configuration cell with an home-made platinum-disk working electrode (1.0 mm diameter), a platinum wire counter electrode and a silver/silver chloride reference electrode (Ag/AgCl) (purchased from Radiometer analytical, SAS, France). The potential was scanned from −0.7 to +1.2 V at a scan rate of 100 mV s^{-1}. All measurements were done at room temperature and the solutions were deaerated with dinitrogen before use. The measured potentials were corrected by +0.197 V to the normal hydrogen electrode (NHE).

Enzymatic Assays

The effect of pH on the oxidation of 1,4-PDA, 4-ADA, 4-AP and 4-APA (5 mM) by CotA-laccase was performed in

Britton–Robinson (B&R) buffer by measuring the initial rate of product formation: 510 nm ($\varepsilon = 3{,}467\,M^{-1}cm^{-1}$), 570 nm ($\varepsilon = 1{,}046\,M^{-1}cm^{-1}$), 400 nm ($\varepsilon = 9{,}551\,M^{-1}cm^{-1}$) and 360 nm ($\varepsilon = 8{,}335\,M^{-1}cm^{-1}$), respectively. The kinetic parameters for the aromatic amine substrates (0.05–5 mM) were monitored at 37 °C at the optimum pH for each substrate. Kinetic constants (K_M and k_{cat}) were fitted directly to the Michaelis–Menten equation (OriginLab software, Northampton, MS, USA). All enzymatic assays were performed at least in triplicate. The protein concentration was measured by using the Bradford assay[45] using bovine serum albumin as a standard.

The laccase biotransformations of *p*-substituted aromatic amines (at a final concentration of 5 mM) were first followed by colour appearance using microplates (96-well) during 24 h at 37 °C in B&R buffer (1:10, MeOH:buffer) with 1 $U\,mL^{-1}$ of enzyme. A control without enzyme was also prepared to check for the auto-oxidation of substrates. Reactions that resulted in colour formation were selected for scale-up.

General Procedure for CotA-Laccase Oxidation of the *p*-Substituted Aromatic Amines

Preparative scale reactions were performed under the following conditions: in a 100-mL round-bottom flask, the aromatic amine 1,4-PDA, 4-ADA, 4-APA or 4-AP (0.025 mmol) dissolved in 5 mL of methanol was added to 45 mL of 100 mM phosphate buffer (pH 5–7). Then, the laccase (1 $U\,mL^{-1}$) was added and the reaction mixture was stirred at 37 °C under aerobic conditions. The conversion was followed by thin layer chromatography (TLC) on aluminium sheet silica gel 60 F_{254} (Merck). After 24 h, the insoluble products were separated by filtration and dried. For soluble products, the solvent was evaporated under reduced pressure and the products were isolated by solid-phase extraction with methanol and solvent evaporation. The crude residues were purified by preparative column chromatography with silica gel Fischer 60 A (200 micron).

Oxidation of 1,4-Phenylenediamine (1,4-PDA)

The final reaction colour showed to be pH dependent. At pH 8, an orange solution with a solid residue was observed. After filtration, an orange solid (**1a**) was obtained as a pure compound. For pH 7 a dark purple solution was obtained and the final residue was purified by silica gel column chromatography (mobile phase: MeOH:$CHCl_3$ 1:3) to afford a dark purple solid (**1b**).

1a: (neutral form), orange solid; yield: 25.2 mg (0.079 mmol, 95%); FT-IR (KBr): ν = 3461 (N–H), 3417 (N–H), 3335, 3033, 1604, 1544, 1501, 1415, 1322, 1275, 1236, 1208, 1166, 1127, 1008, 939, 857, 834, 728, 691, 607, 560, 534 cm^{-1}; ^{1}H NMR (CD_3OD): δ = 6.76 (m, 8H, H-8, H-12, H-8′, H-12′, H-9, H-11, H-9′, H-11′), 5.79 (s, 2H, H-3, H-6); ^{13}C NMR (CD_3OD): δ = 156.0 (C-2, C-5), 123.9 (C-8, C-12, C-8′, C-12′), 117.1 (C-9, C-11 and C-9′, C-11′), 93.4 (C-3, C-6), resonances for C-1, C-4, C-7, C-7′ and C-10, C-10′ were not detected in CD_3OD; ^{1}H NMR [$(CD_3)_2CO$]: δ = 6.67 (m, 8H, H-8, H-12, H-8′, H-12′, H-9, H-11, H-9′, H-11′), 5.79 (s, 2H, H-3, H-6), 5.69 (s, 4H, NH_2, aromatic ring), 4.52 (s, 4H, NH_2, iminic ring); ^{13}C NMR [$(CD_3)_2CO$]: δ = 154.7 (C-2, C-5), 149.3 (C-1, C-4), 145.7 (C-7, C-7′), 141.9 (C-10, C-10′), 123.3 (C-8, C-12, C-8′, C-12′), 115.5 (C-9, C-11, C-9′, C-11′), 92.3 (C-3,C-6); MS-ESI (positive mode): *m/z* = 319, $[M+H]^+$; HR-MS(EI): *m/z* = 318.1592, calcd. for $C_{18}H_{18}N_6$: 318.1593.

1b: (protonated form), dark purple solid; yield: 24.0 mg (0.075 mmol, 90%); FT-IR (KBr): ν = 3463 (N–H), 3418 (N–H), 3337 (N–H), 3210, 1601, 1541, 1502, 1435, 1403, 1277, 1239, 1166, 1077, 940, 834, 817, 565, 539, 518, 502 cm^{-1}; ^{1}H NMR (CD_3OD): δ = 6.95 (d, 4H, *J* = 8.7 Hz, H-8, H-12, H-8′, H-12′), 6.78 (d, 4H, *J* = 8.7 Hz, H-9, H-11, H-9′, H-11′), 6.02 (s, 2H, H-3, H-6); ^{13}C NMR (CD_3OD): δ = 148.3 (C-7, C-7′), 125.9 (C-8, C-12 and C-8′, C-12′), 116.6 (C-9, C-11 and C-9′, C-11′), 94.2 (C-3, C-6), resonances for C-1–C-4, C-2–C-5, and C-10, C-10′ were not detected in CD_3OD.

Oxidation of 4-Aminodiphenylamine (4-ADA)

At pH 6, the dark red residue was collected by filtration and purified by silica gel column chromatography [mobile phase: $(CH_3)_2CO$:C_6H_{14} 1:2] to afford a violet solid (**2**).

2: violet solid; yield: 44.0 mg (0.08 mmol, 97%); FT-IR (KBr): ν = 3488 (N-H), 3369 (N-H), 3279, 3029, 1595, 1548, 1505, 1444, 1401, 1315, 1237, 1213, 1171, 1112, 1076, 1027, 997, 879, 831, 748, 693, 518, 500 cm^{-1}; ^{1}H NMR (DMSO-d_6): δ = 8.45 (s, 1H, NH), 8.13 (s, 2H, NH), 7.33–7.19 (m, 8H, H-8, H-9, H-11, H-12, H-21, H-21′, H-23, H.23′), 7.19–6.95 (m, 9H, H-10, H-15 H-15′, H-17, H-17′, H-20, H-20′, H-24, H-24′), 6.88 (dd, 4H, *J* = 8.0 Hz, H-14, H-14′, H-18, H-18′), 6.79 (t, 2H, *J* = 7.2 Hz, H-22, H-22′), 6.31 (s, 2H, NH_2), 6.17 (s, 1H, H-3), 5.83 (s, 1H, H-6); ^{13}C NMR (DMSO-d_6): δ = 153.6 (C-5), 153.3 (C-2), 148.9 (C-1), 144.0 (C-7, C-19, C-19′), 143.9 (C-4), 143.9 and 142.8 (C-13, C-13′), 139.5 and 139.3 (C-16, C-16′), 129.1 (C-21, C-21′, C-23, C-23′), 126.0 (C-9, C-11), 123.0 (C-10), 122.5 and 122.3 (C-14, C-14′, C-18, C-18′), 121.6 (C-8, C-12), 119.2 (C-22, C-22′), 117.9 (C-15, C-15′, C-17, C1-7′), 116.0 (C-20, C-20′, C-24, C-24′), 91.2 (C-3), 90.7 (C-6); MS-ESI (positive mode): *m/z* = 547, $[M+H]^+$; (negative mode) *m/z* = 545, $[M-H]^-$; HR-MS (EI): *m/z* = 546.2534, calcd. for $C_{36}H_{30}N_6$: 546.2532.

Oxidation of 4-Aminophenylacetamide (4-APA)

At pH 7, an orange solid that was collected by filtration and purified by silica gel column chromatography [mobile phase: $(CH_3)_2CO$:C_6H_{14} 1:1] to afford two fractions. The first eluted orange fraction and the yellow fraction were dried under vacuum to afford compounds (**3**) and (**4**), respectively.

3: orange solid; yield: 11.5 mg (0.026 mmol, 32%); FT-IR (KBr): ν = 3439 (N–H), 3371 (N–H), 2925, 2853, 1666 (C=O), 1604, 1503, 1405, 1371, 1315, 1261, 1206, 1109, 1016, 843, 728, 597, 505 cm^{-1}; ^{1}H NMR (DMSO-d_6): δ = 9.98 (NH aromatic rings), 9.92 (NH aromatic rings), 9.44 (s, 1H, NH iminic ring), 7.64 (s, 1H, H-6), 7.62 (d, 4H, *J* = 8.8 Hz, H-9, H-9′, H-11, H-11′), 6.83 (d, 4H, *J* = 8.8 Hz, H-8, H-8′, H-12, H-12′), 6.37 (s, 2H, NH_2 iminic ring), 5.68 (s, 1H, H-3), 2.09 (s, 3H, CH_3), 2.06 (s, 3H, CH_3), 2.05 (s, 3H, CH_3); ^{13}C NMR (DMSO-d_6): δ = 169.2 (C=O), 168.1 (C=O), 167.9 (C=O), 154.1 (C-2), 153.3 (C-5), 148.0 (C-4), 145.3 and 144.7 (C-7, C-7′), 138.1 (C-1), 136.3 and 135.5 (C-10, C-10′), 121.2 (C-8, C-12), 120.9 (C-8′, C-12′), 119.6 (C-9, C-11), 119.4 (C-9′, C-11′), 103.3 (C-6), 90.5 (C-3), 24.6 (CH_3), 23.97 (CH_3), 23.9 (CH_3); UV/Vis [CH_3OH:B&R pH 6 (1:10)]: λ = 250 (ε =

11,170 $M^{-1}cm^{-1}$). 360 (ε = 8,335 $M^{-1}cm^{-1}$) 430 nm (shoulder); MS-ESI (positive mode): *m/z* = 467, $[M+Na]^+$, 445, $[M+H]^+$; MS-ESI: (negative mode) *m/z* = 443 $[M-H]^-$; HR-MS (EI): *m/z* = 444.1910, calcd. for $C_{24}H_{24}N_6O_3$; 444.1910.

4: yellow solid; yield: 7.2 mg (0.024 mmol, 20%); FT-IR (KBr): ν = 3489, 3361 (N–H), 3326 (N–H), 3192, 3071, 2924, 2853, 1733, 1662 (C=O), 1603, 1543 (NO_2), 1501, 1466, 1410, 1370, 1325, 1306, 1271, 1157, 1113, 1075, 1041, 1021, 963, 846, 727, 648, 607, 548 cm^{-1}; ^{1}H NMR (DMSO-d_6): δ = 10.26 (s, NH); 7.83–7.79 (m, 8H, H-2, H-2′, H-3, H-3′, H-5, H-5′, H-6, H-6′), 2.09 (s, 3H, CH_3); ^{13}C NMR (DMSO-d_6): δ = 169.2 (C=O), 148.0 (C-1, C-1′), 142.4 (C-4, C-4′), 123.8 (C-2, C-2′, C-6, C-6′), 119.6 (C-3, C-3′, C-5, C-5′), 24.6 (CH_3); MS-ESI (positive mode): *m/z* = 319 $[M+Na]^+$, 297 $[M+H]^+$, 255, 162, 134 (MS^2); ME-ESI (negative mode): *m/z* = 295 $[M-H]^-$; HR-MS (EI): *m/z* = 296.1274, calcd. for $C_{16}H_{16}N_4O_2$: 296.1273.

Oxidation of 4-Aminophenol (4-AP)

At pH 6, a dark brown residue that was collected by filtration and purified by Silicagel chromatography [mobile phase: $(CH_3)_2CO$:C_6H_{14} (1:1)] to afford a brown solid (**5**).

5: brown solid; yield: 19.9 mg (0.062 mmol, 75%); FT-IR (KBr): ν = 3181 (OH), 1607 (C=O), 1564, 1510, 1372, 1251, 1168, 1064, 867, 822, 564, 510 cm^{-1}; ^{1}H NMR (DMSO-d_6): δ = 7.09 (d, 2H, *J* = 8.8 Hz, H-8, H-12), 7.06 (d, 2H, *J* = 8.8 Hz, H-8′, H-12′), 6.79 (d, 2H, *J* = 8.4 Hz, H-9, H-11), 6.78 (d, 2H, *J* = 8.8 Hz, H-9′, H-11′), 5.91 (s, 1H, H-3), 5.48 (s, 1H, H-6); ^{13}C NMR (DMSO-d_6): δ = 177.6 (C-1), 161.6 (C-4), 154.8 (C-10), 154.5 (C-10′), 151.1 (C-5), 143.3 (C-2), 131.3 (C-7′), 130.1 (C-7), 125.1 (C-8, C-12), 124.6 (C-8′, C-12′), 115.5 (C-9, C-11), 115.3 (C-9′, C-11′), 94.5 (C-6), 93.3 (C-3); MS-ESI (positive mode): *m/z* = 322, $[M+H]^+$; ME-ESI (negative mode): *m/z* = 320, $[M-H]^-$; HR-MS (EI): *m/z* = 321.1109, calcd. for $C_{18}H_{15}N_3O_3$: 321.1113.

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