CHEMISTRY
AN ASIAN JOURNAL
www.chemasianj.org

Accepted Article

Title: Synthesis of azobenzene dyes mediated by CotA-laccase

Authors: Maria Paula Alves Robalo, Ana Catarina Sousa, Sara Raquel Baptista, and Lígia O Martins

To be cited as: *Chem. Asian J.* 10.1002/asia.201801450

Link to VoR: http://dx.doi.org/10.1002/asia.201801450

A Journal of
ACES Asian Chemical Editorial Society

A sister journal of Angewandte Chemie and Chemistry – A European Journal

WILEY-VCH

10.1002/asia.201801450

WILEY-VCH

Synthesis of azobenzene dyes mediated by CotA-laccase

Ana Catarina Sousa[a,b], Sara R. Baptista[a], Lígia O. Martins[c] and M. Paula Robalo*[a,b]

Abstract: An eco-friendly protocol for azobenzene dyes synthesis by oxidative coupling of primary aromatic amines is reported. As efficient biocatalytic systems, CotA-laccase and CotA-laccase/ABTS (2,2´-azinobis-3-ethylbenzylthiozoline-6-sulphate) enable the oxidation of various substituted anilines, in aqueous medium, ambient atmosphere and mild reaction conditions of pH and temperature. A series of azobenzene dyes were prepared in good to excellent yields in an one-pot reaction. A mechanistic proposal for the formation of the azo derivatives is presented.
The methodology followed is experimentally simple and environmentally benign: aqueous media and mild reaction conditions. Thus, this strategy offers an alternative approach for the direct synthesis of azobenzene dyes, avoiding the harsh conditions generally required for most of the traditional synthetic methods.

Introduction

Azo dyes are one of the largest and most versatile group of colourants with an enormous commercial and industrial importance and widespread applications in several different fields such as food additives, cosmetics, pharmaceuticals, dyeing/textile industry and analytical chemistry.[1,2] At industrial level they are mainly produced through the diazotation of an aromatic primary amine and further coupling between the resultant diazonium salt and a phenol or an aromatic amine, carried out under strong acid conditions and at low temperatures and aggressive conditions.[3] Other traditional methods are based on the reduction of nitro aromatic compounds with several reducing agents[4] or involving oxidation of primary aromatic amines, using stoichiometric amounts of different chemical oxidants, such as lead tetraacetate, manganese oxide or sodium hypochlorite have been reported.[4-7] Although in general, the azo compounds are obtained in moderate to good yields, these methods suffer from several disadvantages mainly in what concern environmental issues, since excess of toxic oxidising or reducing agents are used, they are associated with the formation of by-products and the production of large volumes of down-stream waste.

In recent years, there is an increase demand to develop more sustainable synthetic protocols which had encouraged the study of alternative routes to produce this family of compounds. Several approaches have been reported involving the use of different catalysts and nanomaterials,[8-10] polymer-supported reagents for the oxidation of aromatic amines,[11] less aggressive oxidants,[12] solvent-free methods such as ultrasonic and microwave irradiation[13] or electrochemical methods.[14] Despite some progress towards a greener catalytic synthesis of aromatic azo derivatives, all the reported reactions showed limitations emphasizing the need for continuous search of alternative and cleaner methods with environmental and economic importance.

Biocatalysis has emerged as an important tool to realise swift chemical transformations in organic synthesis. The application of enzymes as biocatalysts, to substitute the classical stoichiometric chemical oxidants, represents a promising alternative in the friendly synthesis of “new green” compounds, with cost-effectiveness and environmental benefits, while simultaneously addressing the challenges of green chemistry.[15-17]

Laccases (EC 1.10.3.2, *p*-diphenol:dioxygenoxidoreductases) owing to their strong oxidation ability and operating conditions (the lack of requirement for co-factors and the use of readily available oxygen as an electron acceptor) are robust biocatalysts with potential technological interest for diverse uses in organic synthesis and biotechnological applications.[18-20] Moreover, their oxidation capabilities can be broadened by the use of redox mediators, such as the 2,2’-azinobis(3-ethylbenzothiazoline-6-sulfonate (ABTS), that act as the promoter to the required electron shuttle in the enzyme-catalysed oxidising process.[21-23] Thus, these laccase-mediator systems are widely used in the bio-oxidation of recalcitrant substrates, usually with high redox potentials, extending the range of substrates to be oxidised by laccases.

Although laccases have been extensively used in the degradation/decolourisation of textile dyes,[24-27] their use in the synthesis of azo dyes has been much less reported.[28-31] In the last years, our group have developed several eco-friendly protocols towards the synthesis of different heteroaromatic compounds using CotA-laccase from *Bacillus subtilis*, as biocatalyst in the oxidation of primary aromatic amines.[32-34] In these studies, azobenzene derivatives were obtained as secondary products, showing the feasibility of CotA-laccase to produce this type of compounds. This information prompted us to extend our studies for the formation of these compounds, using CotA-laccase and the CotA-laccase/ABTS systems.

Results and Discussion

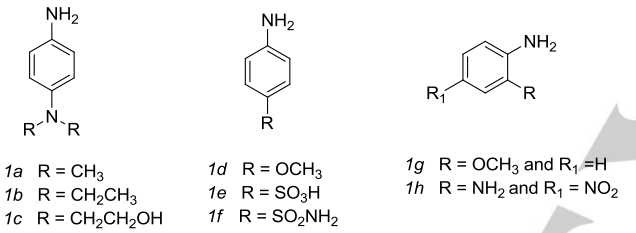
A set of potential *para*- and *ortho*-substituted aromatic amines (Figure 1) were first tested in their ability to act as substrates of CotA-laccase. The oxidation of simple model

[a] Prof. Dr.M. P. Robalo*, Prof. Dr. A.C. Sousa, Msc S. R. Baptista
Área Departamental de Engenharia Química
Instituto Superior de Engenharia de Lisboa, Instituto Politécnico de Lisboa
Rua Conselheiro Emídio Navarro, 1, 1959-007 Lisboa, Portugal
* E-mail: mprobalo@deq.isel.ipl.pt
[b] Prof. Dr.M. P. Robalo, Prof. Dr. A.C. Sousa
Centro de Química Estrutural
Instituto Superior Técnico, Universidade de Lisboa
Av. Rovisco Pais, 1049-001 Lisboa, Portugal
[c] Prof. Dr. L. O. Martins
Instituto de Tecnologia Química e Biológica
Universidade Nova de Lisboa
Av. da República, 2780-150 Oeiras, Portugal

Supporting information for this article is given via a link at the end of the document.

aromatic amines has been used as a simple approach to identify the substrate's structural features that are important for the success of the enzyme-catalysed redox reactions as well as to understand the correspondent mechanistic pathways.[32-36]

The electronic nature of the substituents and their positions relative to the reactive amino group in the aromatic ring of the substrates showed to be decisive in the formation of products with different structures. Several different cores, such as dimers, trimers, phenoxazines, phenoxazinones and carbazoles were obtained using CotA-laccase as mediator, being their formation delimited by the balance between the substrate's ability to generate a stable radical intermediate, promoted by laccase, and its nucleophilicity as a partner in the sequential coupling reaction. The presence of electron-withdrawing groups in the aromatic ring was related with the formation of less stable radical intermediates and the formation of azo compounds as secondary products.[32] Thus, the presence of electron-withdrawing groups or electron-donor groups such as disubstituted amines and alkoxy groups, which could restrain the benzoquinonediimine formation, was considered in the choice of substrates.



1a R = CH_3
1b R = CH_2CH_3
1c R = CH_2CH_2OH
1d R = OCH_3
1e R = SO_3H
1f R = SO_2NH_2
1g R = OCH_3 and R_1 =H
1h R = NH_2 and R_1 = NO_2

Figure 1. Representative structures of substrates, namely: *N,N*-dimethyl-1,4-phenylenediamine (***1a***), *N,N*-diethyl-1,4-phenylene-diamine (***1b***), *N,N*-bis(2-hydroxyethyl)-1,4-phenylenediamine sulfate (***1c***), 4-methoxyaniline (***1d***), 4-aminobenzenesulfonic acid (***1e***), 4-aminobenzenesulfonamide (***1f***), 2-methoxyaniline (***1g***) and 4-nitrobenzene-1,2-diamine (***1h***).

Since the enzymatic mechanism relies on an oxidation-reduction process, the chemical and electrochemical properties of the substrates are important issues for the success of the enzyme-catalysed reaction. Table 1 shows the substrate's acidity constant (p*K*a) values[37] and the electrochemical data (*vs* NHE) determined by cyclic voltammetry (CV).

The pH of the reaction medium can also be a critical parameter since both enzyme activity and substrate ability for electron abstraction are pH dependent. All the substrates show acidity constants below 6 (Table 1), the optimum pH determined for the CotA-laccase mediated reactions with aromatic amines. Therefore, at pH 6 the aromatic amines tested are predominantly in the neutral forms in solution and prone to be oxidised by the enzyme.

Under the experimental conditions used, the studied aromatic amines showed one or two quasi-reversible or irreversible oxidation processes in the potential range 0.41 V to 1.18 V. At pH 6, only three (***1a-c***) of the eight aromatic amines exhibited an

Table 1. pK_a and electrochemical data *vs.* NHE of 1x10^{-3}M of aromatic amines in 1:9 ethanol:phosphate buffered solutions (20 mM phosphate buffer, pH6) at a scan rate of 100 mVs^{-1}.

Substrate	pK_a in H_2O[37]	E_{pa} (V)	E_{pc} (V)
1a	2.38	0.41[a] 0.52[b]	0.36[a] 0.44[b]
1b	2.38	0.47 0.76	0.24 --
1c	--	0.42 0.60	0.34 0.51
1d	5.29	0.76	0.32
1e	2.93-3.32	1.18	--
1f	2.09-2.36	[c]	--
1g	4.39–4.49	0.83	--
1h	--	0.91	--

[a] $E_{1/2}$=0.38, quasi-reversible wave, ΔE = 50 mV. [b] $E_{1/2}$=0.48, quasi-reversible wave, ΔE = 80 mV. [c] Not detected. Potencial out of the experimental range.

E_{pa} value lower or similar to the redox potential of CotA-laccase (0.535 V *vs* NHE)[38] and are therefore expected to be promptly oxidised by the enzyme. Our previous results with *para*-substituted and *ortho,para*-disubstituted aromatic amines showed that the electronic nature and the position of the substituent groups are a condition for the success of the laccase mediated oxidation.[32,33]

A colourimetric activity screening was performed for all the substrates (5 mM) at different pH values (20 mM phosphate buffer, pH 6, 7 and 8) in the presence of CotA-laccase (1 UmL^{-1}) at 37 °C. For the water insoluble substrates, a small amount of ethanol was used to solubilise the amines, representing 10 % of the total reaction volume. The time course of the reactions was also monitored by thin layer chromatography (TLC).

CotA-laccase was able to oxidise substrates ***1a-d*** and ***1g***, yielding intense coloured products (from orange to purple) in a short period of time (1.5-2h), as expected from the electrochemical data and pH 6 was found to be the optimum pH value for CotA-laccase activity. For substrates ***1e***, ***1f*** and ***1h***, with higher E_{pa} values than CotA-laccase (0.535 V *vs* NHE), no oxidation products were detected even after more than 48h of incubation.

In order to circumvent this limitation and promote the oxidation of these last substrates, we explored the use of a CotA-laccase/mediator catalytic system, where ABTS, a water soluble compound, was employed as redox mediator.[21,23] The ABTS is oxidised by CotA-laccase to the $ABTS^{\bullet+}$ radical cation, which oxidises the substrate (Figure 2), extending the range of possible substrates oxidised by the enzyme.

The oxidation of substrates ***1e***, ***1f*** and ***1h*** with the catalytic CotA-laccase/ABTS system were conducted using 1U.mL^{-1} of CotA-laccase and two different ABTS concentrations (1 mM and 5 mM), leading to similar results: the formation of coloured products for substrate ***1h*** at pH 6, while substrates ***1e*** and ***1f*** remained unchanged. The presence of strong withdrawing groups in ***1e*** and ***1f*** could be responsible for the amino group deactivation, which hamper its enzymatic oxidation.

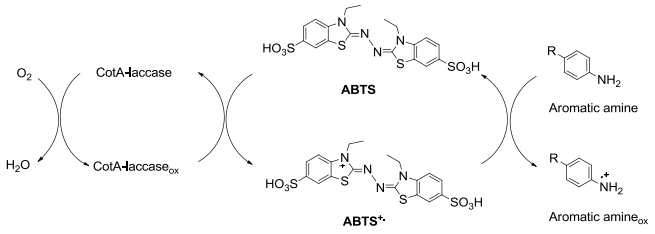


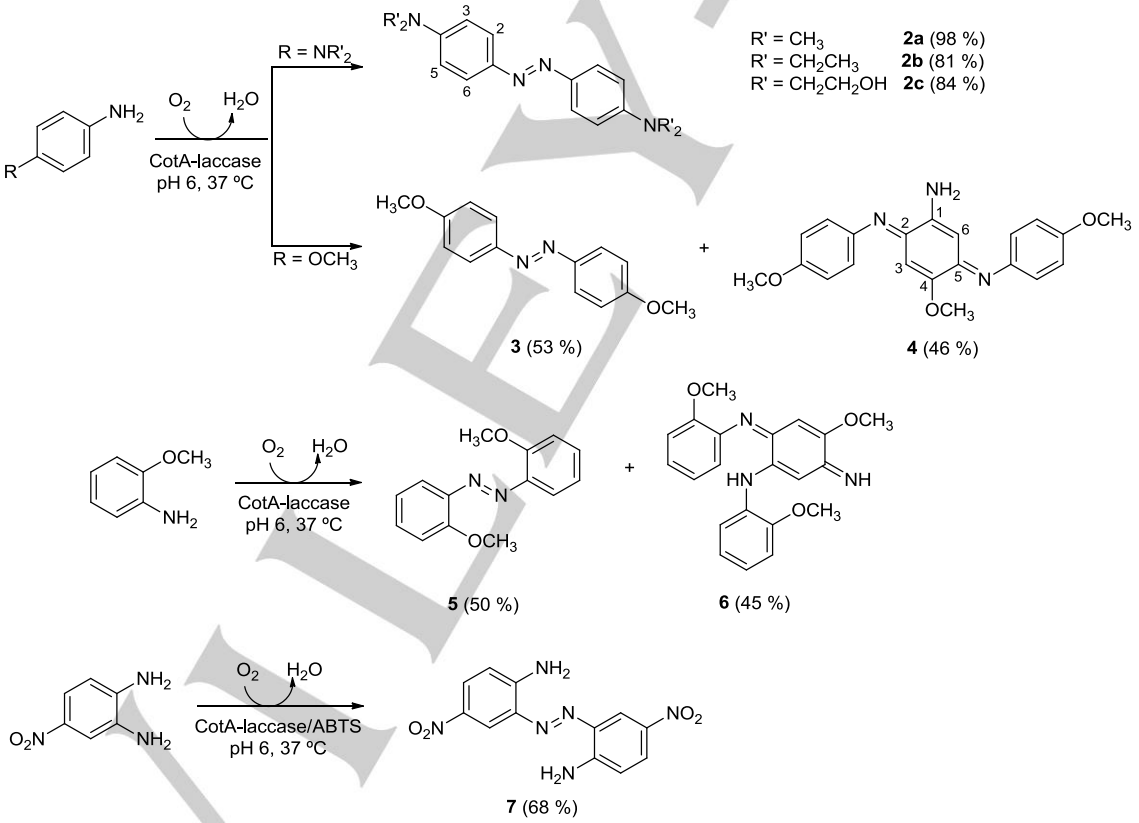
Figure 2. The catalytic cycle of CotA-laccase/ABTS oxidising system

The reactions with substrates ***1a-d***, ***1g*** and ***1h*** (5 mM) were scaled-up at the experimental optimised conditions (1:9 ethanol: 20 mM phosphate buffer, pH 6, 37 °C). A change from colourless to coloured media were observed and after 2h of reaction the oxidation products were isolated at moderate to excellent yields as summarised in scheme 1.

Considering the yields of products formation, we can conclude that the formation of azo compounds was favoured for anilines with *para*-tertiary amino groups (***1a-c***), with yields above 80 % and for the *ortho*,*para*-substituted substrate (***1h***) with a yield around 70 %. The methoxy-substituted anilines (***1d*** and ***1g***) lead to the formation of a predominant azo product at lower yields (50-53 %) and secondary products with benzoquinonimine structures, like those characterised in previous studies.[32]

Overall the results obtained indicate that the electronic character of the substituent groups and their relative position to the amino group determine the reaction course and the products type formed, supporting the results found in our previous studies.[32-34]

The proposed structures were based in NMR and ESI-MS data analysis and data in the literature.[9,10,14] The ^{1}H-NMR spectra of the products ***2a-c*** and ***3*** showed similar patterns, characterised by the presence of two doublets, shifted downfield when compared with the corresponding substrates (***1a-d***). The observed signals show the coherent multiplicity and resonances for aliphatic (δ = 1.23 to 4.33 ppm), methoxy (3.89 ppm) and aromatic protons (6.76 to 7.88 ppm). ^{13}C-NMR signals showed concordant results, with a low-field shift resonance for C_1 up to δ



Scheme 1. Structures and yields (%) of products obtained from the CotA-laccase and CotA-laccase/ABTS mediated reactions

= 140 ppm. Furthermore, the electrospray ionisation first-order full mass spectra of azo compounds ***2a-c*** and ***3*** exhibited the respective protonated molecules $[M+H]^+$ at *m/z* 269, 325, 389 and 243. The respective fragmentation patterns observed in the MS^2 spectra are consistent with the proposed structures showing the characteristic fragment ions due to the losses of coherent radical ions and N_2 fragment (see experimental section).

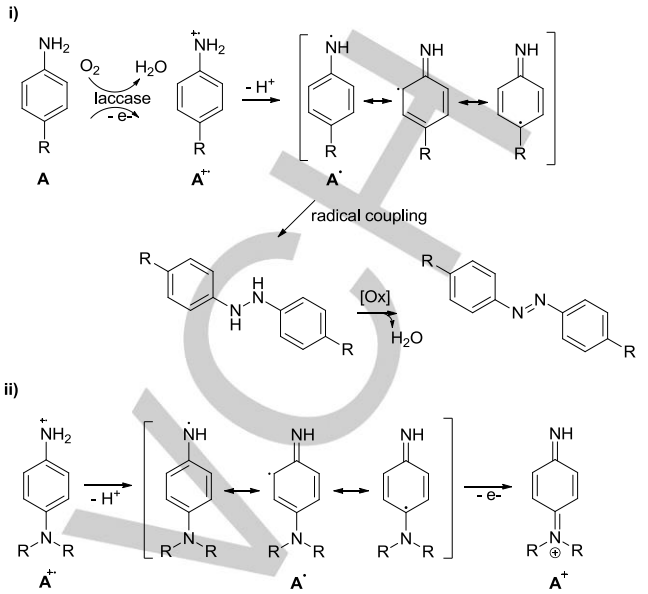
Compound ***4***, the secondary oxidation product was identified as a benzoquinonediimine trimer, in agreement to those previously reported.[32] ^{1}H and ^{13}C-NMR spectra of ***4***, are entirely consistent with the central iminic skeleton showing the presence of characteristic singlets for protons H_3 and H_6 in the range 5.76 – 6.07 ppm and the correspondent carbon signals in the 90.6 - 91.6 ppm range. Furthermore the presence of three methoxy groups was confirmed by the three singlets in the ^{1}H-NMR spectra with resonances at δ = 3.15, 3.78 and 3.82 ppm. The structure was corroborated by the ESI mass spectrum in positive mode that exhibits a main peak at *m/z* 364 ($[M+H]^+$) which generated in the MS^2 fragmentation spectrum three fragments with *m/z* 349, 333 and 318 due to the losses of methyl, methoxy and both radicals.

For the *ortho*-substituted amine ***1g*****,** the formation of two products, the azo compound ***5*** and the benzoquinone-diimine trimer ***6***, was confirmed by the spectroscopic and MS data, in agreement with the observed in the oxidation of the *para* isomer.

The ^{1}H-NMR spectra of the azo product ***7*** is characterised by the presence of one singlet and two doublets, shifted downfield when compared with the precursor ***1h***. The proposed structure is also based on the analysis of the first-order negative ion ESI mass spectrum that exhibit the respective *m/z* ion $[M-H]^-$ at 301, whose fragmentation generates in the MS^2 spectrum the *m/z* ion at 273, due to the loss of N_2 fragment.

The results outlined prompted us to propose a reaction pathway for the formation of azo compounds (Scheme 2i). The enzyme promotes the formation of a short-lived intermediate, the aminium cationic radical ($A^{\bullet+}$), which is subsequently deprotonated leading to the formation of the aminyl neutral radical ($A^{\bullet}$). The radical coupling of two species ($A^{\bullet}$) forms the covalent HN-NH bond, which suffers subsequent dehydrogenation to give the respective azo compound. Due to the presence of the tertiary amine group (***1a-c***) in the *para*-position, we assume that the formation of the aminyl neutral radical ($A^{\bullet}$) prevails over the formation of the benzoquinone-diimine intermediate (A^+) (Scheme 2ii).

Thus, the species A^+ is more likely unstable than $A^{\bullet}$ and the radical mechanism overcomes the nucleophilic coupling reactions described previously,[32] leading to the formation of the benzoquinone-diimine trimers. For the reactions where two products were obtained (***1d*** and ***1g***), we propose that both intermediates ($A^{\bullet}$ and A^+) are formed and competitive pathways take place, with the consequent formation of the two different structures.



Scheme 2. i) Proposed pathway for the formation of azo compounds mediated by CotA-laccase; ii) Formation of the benzoquinone-diimine intermediate in tertiary aromatic amines.

Conclusions

In summary, a new sustainable biocatalytic protocol for the synthesis of azo dyes is proposed, using an efficient biocatalyst CotA-laccase, working in aqueous media and mild reaction conditions. The complete oxidation of colourless aromatic amines was reached in the first 2h of reaction, under atmospheric conditions. The laccase/ABTS system showed to be a useful alternative for the formation of azo compounds in the cases where the redox constraints seem to be the only responsible for the absence of reaction. This approach can be used for extend the scope of aromatic amines to be oxidised by this procedure. The formation of these structures broadens the set of structures that can be obtained by the CotA-laccase approach, which showed up to be a useful and efficient alternative to the conventional chemical routes and a greener solution for the synthesis of azobenzene dyes.

Experimental Section

1. Materials and instrumentation: All reagents and solvents were from analytical grade, acquired from commercial suppliers and used without further purification. Thin-layer chromatography was carried out on TLC aluminum sheets coated with silica gel 60 F_{254} (Merck). Column chromatographies were carried out with Silicagel Fischer 60A (200 micron) and compounds eluted with the appropriate eluent systems.

The products characterization was performed by 1D-NMR (^{1}H,^{13}C) and 2D-NMR (COSY, HSQC and HMBC) spectra, obtained on a Bruker Avance (400MHz) spectrometer with a 5 mm probe in $CDCl_3$, MeOD-d_4, $(CD_3)_2CO$-d_6 and DMSO-d_6 as solvents. Chemical shifts are reported in ppm relative to the solvent peaks and coupling constants (*J*) are reported in Hertz. Resonance and structural assignments were based on the

analysis of coupling patterns, including the $^{13}C-^{1}H$ coupling profiles obtained in bidimensional heteronuclear multiple bond correlation (HMBC) and heteronuclear single quantum coherence (HSQC) experiments, performed with standard pulse programs. LRESI mass spectrum and tandem mass experiments were carried out on a LCQ Fleet mass spectrometer operated in the ESI positive/negative ion modes (Thermo Scientific). The optimized parameters were as following: ion spray voltage, ±4.5 kV; capillary voltage, +16 and -20 V; tube lens offset, -63 and +82 V; sheath gas (N_2), 80 arbitrary units; auxiliary gas, 5 arbitrary units; capillary temperature, 250 °C. The spectra were recorded in the range 100 – 1000 Da. Spectrum typically corresponds to the average of 20–35 scans. High Resolution ESI(+/-) mass spectra were obtained on a QTOF Impact II™ mass spectrometer (Bruker Daltonics, GMBH; Germany), operating in the high-resolution ion mode**.** Calibration of the TOF analyser was performed with a 10 mM sodium formate calibrant solution. Data was processed using Data Analysis 4.2 software (Bruker Daltonics, Bremen, Germany).

The redox potentials of all substrates were measured by cyclic voltammetry using an EG&G Princeton Applied Research (PAR) Model 273A potentiostat/ galvanostat monitored with a personal computer loaded with Electrochemistry PowerSuite v2.51 software from PAR. Cyclic voltammograms were carried out using 1 mM solutions of the substrates in 9:1 phosphate buffer pH 6 (20 mM):Ethanol, in a three-electrode configuration cell with an home-made platinum-disk working electrode (1.0 mm diameter), a platinum wire counter electrode and an Ag/AgCl reference electrode (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. 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).

Recombinant CotA-laccase from *Bacillus subtilis* was produced and purified as previously described.[39,25] Activity was determined by following the 2,2-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid monitored spectrophotometrically at 420 nm (1U defined as the amount of enzyme which oxidizes 1 µmol of ABTS per min at 37 °C). The purified solution of CotA-laccase was stored frozen at -18 °C prior to use. The protein concentration was measured by using the Bradford assay using bovine serum albumin as a standard. [39]

The screening and optimization conditions were conducted using microplates (96-well) at pH values 6, 7 and 8 (phosphate buffer 20 mM) with 1 UmL^{-1} of CotA-laccase and 5 mM of each substrate per well. For substrates (***1e,f*** and ***1h***), the same procedure was performed with 1 and 5 mM of ABTS. Plates were left at room temperature, under shake conditions and reactions were monitored by TLC. For water insoluble substrates, 10% of ethanol was used.

2. General procedure for the oxidative reactions mediated by CotA-laccase: Substrates ***1a-d*** and ***1g***: 5 mM of each substrate were dissolved in 1:9 mL (ethanol: 20 mM phosphate buffer at pH 6) and 1 $U.mL^{-1}$ of CotA-laccase solution was added. Reactions were incubated at 37 °C with stirring in the presence of air, monitored by TLC and stopped when oxidation was complete.

3. General procedure for the oxidative reactions mediated by CotA-laccase/ABTS system: Substrates ***1e,f*** and ***1h***: 5 mM of each substrate were dissolved in 1:9 mL (ethanol: 20 mM phosphate buffer at pH 6) and 1UmL^{-1} of CotA-laccase solution was added followed by the addition of 1mM of ABTS. Reactions were incubated at 37 °C with stirring in the presence of air, monitored by TLC and stopped when oxidation was complete or after 48h.

For the soluble products, the final solutions were concentrated in vacuum and purified, when required, by column chromatography (silica gel 60 Merck) with the adequate eluent system. In the case of insoluble products, the final aqueous solution was filtered, and the residue purified by the same method.

3.1. Oxidation of *N, N*-dimethyl-1,4-phenylenediamine (*1a*): After 2h, the solvent was removed under reduced pressure and the residue washed with methanol and isolated as a pure compound (***2a***).

2a: Brownish solid; yield: 6.7 mg (0.025 mmol, 98 %); ^{1}H NMR (DMSO-d_6) δ(ppm) = 7.68 (d, 4H, *J* = 9,1 Hz, $H_{2,2'}$, $H_{6,6'}$), 6.79 (d, 4H, *J* = 9.2 Hz, $H_{3,3'}$ $H_{5,5'}$), 3.01 (s, 12H, CH_3); $^{13}C\{H\}$ NMR (DMSO-d_6) δ(ppm) = 151.6 ($C_{4,4'}$), 143.2 ($C_{1,1'}$), 123.7 ($C_{2,2'}$, $C_{6,6'}$), 111.8 ($C_{3,3'}$, $C_{5,5'}$), CH_3[a]; ESI-MS positive mode: 269 $[M+H]^+$ and *m/z* 281 $[M+Na]^+$; MS^2 (*m/z*): 254, 241, 148, 120 and 105. ESI-HRMS: *m/z* calcd. for $[C_{16}H_{20}N_4+H]^+$: 269.1750; found 269.1761.

[a] Under the solvent signal.

3.2. Oxidation of *N, N*-diethyl-1,4-phenylenediamine (*1b*): After 2h, the dark blue solution was concentrated under reduced pressure and the residue was purified by column chromatography with a mixture of methanol:chloroform 1:4, yielding the pure compound (***2b***).

2b: dark blue solid; yield: 6.5 mg (0.020 mmol, 81 %); ^{1}H NMR (MeOD-d_4) δ(ppm) = 7.69 (d, 4H, *J* = 9.2 Hz, $H_{2,2'}$, $H_{6,6'}$), 6.76 (d, 4H, *J* = 9.2 Hz, $H_{3,3'}$ $H_{5,5'}$), 3.47 (q, 8H, CH_2) and 1.27 (s, 12H, CH_3); $^{13}C\{H\}$ NMR (MeOD-d_4) δ(ppm) = 149.3 ($C_{4,4'}$), 144.0 ($C_{1,1'}$), 124.9 ($C_{2,2'}$, $C_{6,6'}$), 112.8 ($C_{3,3'}$, $C_{5,5'}$), 45.3 (CH_2) and 12.7 (CH_3); ESI-MS positive mode: 325 $[M+H]^+$; MS^2 (*m/z*): 310, 296 and 148; ESI-HRMS: *m/z* calcd. for $[C_{20}H_{28}N_4+H]^+$: 325.2387; found 325.2387.

3.3. Oxidation of *N,N*-bis(2-hydroxyethyl)-1,4-phenylenediamine sulfate (***1c***): After 2h, the solvent was removed under reduced pressure and the residue was purified over a column of silica gel eluting with a mixture of methanol:chloroform 1:4, yielding the pure compound (***2c***).

2c: dark purple solid; yield: 8.1 mg (0.021 mmol, 84 %); ^{1}H NMR (DMSO-d_6) δ(ppm) = 7.63 (d, 4H, *J* = 9.2 Hz, $H_{2,2'}$, $H_{6,6'}$), 6.78 (d, 4H, *J* = 9.2 Hz, $H_{3,3'}$, $H_{5,5'}$), 4.33 (t, 4H, *J* = 13.2 Hz, CH_2) and 4.12 (t, 4H, *J* = 13.2 Hz, CH_2); $^{13}C\{H\}$ NMR (DMSO-d_6) δ(ppm) = 127.2 ($C_{2,2'}$, $C_{6,6'}$), 123.7 ($C_{3,3'}$, $C_{5,5'}$), 61.4 (CH_2), 59.4 (CH_2). ESI-MS positive mode: 389 $[M+H]^+$; MS^2 (*m/z*): 344; MS^3 (*m/z*): 313. ESI-HRMS: *m/z* calcd. for $[C_{20}H_{28}N_4O_4+H]^+$: 389.2163; found 389.2183.

3.4. Oxidation of 4-methoxyaniline (*1d*): After 2h, a light orange solution with a dark orange precipitate was formed. The precipitate was collected and purified over a column of silica gel eluting with a mixture of ethyl acetate:hexane 3:1 to afford two pure brownish compounds (***3*** and ***4***).

3: Red brownish solid; yield: 3.2 mg (0.013 mmol, 53 %); ^{1}H NMR (Acetone-d_6) δ(ppm) = 7.88 (d, 4H, *J* = 8.8 Hz, $H_{3,5}$, $H_{3,5'}$), 7.08 (d, 4H, *J*=8.8 Hz, $H_{2,6}$, $H_{2',6'}$), 3.89 (s, 6H, CH_3); $^{13}C\{H\}$ NMR (Acetone-d_6) δ(ppm) = 162,8 ($C_{1,1'}$), 147,8 ($C_{4,4'}$), 125,3 ($C_{2,2'}$, $C_{6,6'}$), 115,1 ($C_{3,3'}$, $C_{5,5'}$), 55,7 (CH_3). ESI-MS positive mode: 243 $[M+H]^+$. MS^2 (*m/z*): 228, 215 and 136. ESI-HRMS: *m/z* calcd. for $[C_{14}H_{14}N_2O_2+H]^+$: 243.1129; found 243.1128.

4: Brownish solid; yield: 2.8 mg (0.008 mmol, 46 %); ^{1}H NMR (Acetone-d_6) δ(ppm) = 6.98-6.87 (m, 8H,$H_{8,9,11,12}$, $H_{8',9',11',12'}$), 6.07 (s, 1H, H_3), 5.76 (s, 1H, H_6), 3.82 (s, 3H, CH_3), 3.78 (s, 3H, CH_3), 3.75 (s, 3H, CH_3); $^{13}C\{H\}$ NMR (Acetone-d_6) δ(ppm) = 162.8, 157.4 and 157.3 (C_2 and C_5), 147.7, 126.3, 125.1, 124.5, 123.7, 123.3, 123.1., 115.5, 115.3, 115.2, 114.9, 91.8 and 90.6 (C_3 and C_6), 56.0 (CH_3), 55.7 (CH_3) and 55.6 (CH_3);

ESI-MS positive mode: 364 $[M+H]^+]^+$; MS^2 (*m/z*): 349, 333 and 318. ESI-HRMS: *m/z* calcd. for $[C_{21}H_{21}N_3O_3+H]^+$: 364.1646; found 364.1656.

3.5. Oxidation of 2-methoxyaniline (*1g*): After 2h, the reddish solution was concentrated under reduce pressure and the residue was purified by column chromatography, eluting with a mixture of ethyl acetate:hexane 3:1 to afford two brown solids (***5*** and ***6***).

5: Brown solid; yield: 3.0 mg (0.013 mmol, 50 %); ^{1}H NMR ($CDCl_3$) δ(ppm) = 6.97 (d, 2H, *J*=8.4 Hz, $H_{6,6'}$), 6.88 (d, 2H, J=8.4 Hz, $H_{3,3'}$), 6.78 (t, 2H, *J*=8.8 Hz, $H_{5,5'}$), 6.73 (t, 2H, *J*=8.8 Hz, $H_{4,4'}$) and 3.85 (s, 6H, CH_3). ^{13}C{H} NMR ($CDCl_3$) δ(ppm) =152.6 (C_2), 139.5 (C_1), 130.4 (C_4), 121.7 (C_5), 120.7 (C_6), 112.1 (C_3) and 56.7 (CH_3). ESI-MS positive mode: 243 $[M+H]^+$; MS^2 (*m/z*): 228, 215, 136 and 120. ESI-HRMS: *m/z* calcd. for $[C_{14}H_{14}N_2O_2+H]^+$: 243.1129; found 243.1128.

6: Brown solid; yield: 2.7 mg (0.0074 mmol, 45 %); ^{1}H NMR ($CDCl_3$) δ(ppm) = 7.68 (d, 1H, *J*=8.0 Hz, H_{17}), 7.22 (s, 1H, H_4), 7.16 (t, 1H, J=8.0 Hz), 7.05 (m, 3H), 7.01 (d, 2H, J=8.0 Hz), 6.88 (s, 1H, H_1), 3.91 (3H, CH_3), 3.85 (3H, CH_3) and 3.77 (3H, CH_3). ^{13}C{H} NMR ($CDCl_3$) δ(ppm) = 159.9, 153.2, 153.1, 151.9, 149.8, 136.9, 127.9, 125.5, 122.8, 122.1, 121.7, 112.3, 111.6, 97.7 (C_1), 91.9 (C_4), 56.7(CH_3), 56.1(CH_3) and 55.9 (CH_3).ESI-MS positive mode: 364 $[M+H]^+$; ESI-HRMS: *m/z* calcd. for $[C_{21}H_{21}N_3O_3+H]^+$: 364.1646; found 364.1656.

3.6. Oxidation of 4-nitrobenzene-1,2-diamine (*1h*): After 2h, the brownish mixture was extracted with ethyl acetate, the solvent removed under reduce pressure and the residue was purified by column chromatography with a mixture of methanol:chloroform 1:4, yielding the compound (***7***).

7: Dark yellow solid; yield: 5.1 mg (0.0169 mmol, 68 %); ^{1}H-RMN (Acetone-d_6) δ(ppm) = 8.65 (s, 2H, $H_{3,3'}$), 8.07 (d, 2H, *J*=9.2 Hz, $H_{5,5'}$) and 7.06 (d, 2H, *J*=9.2 Hz, $H_{6,6'}$). ^{13}C{H} RMN (Acetone-d_6) δ(ppm) = 151.5 ($C_{1,1'}$), 138.5 ($C_{2,2'}$), 135.1 ($C_{4,4'}$), 127.7 ($C_{5,5'}$), 120.9 ($C_{3,3'}$) and 117.5 ($C_{6,6'}$). ESI-MS negative mode: 301 $[M-H]^-$; MS^2 (*m/z*): 273; ESI-HRMS: *m/z* calcd. for $[C_{12}H_{10}N_6O_4-H]^-$: 301.0691; found 301.0690.

Acknowledgements

Funding: This work was supported by the FCT – Fundação para a Ciência e Tecnologia [Projecto Estratégico UID/QUI/00100/ 2013,PTDC/BBB-EBB/0122/2014, RECI/QEQQIN/0189/2012 and Projeto Infraestruturas 022125] and Research Unit GREEN-it "Bioresources for Sustainability" at ITQB [UID/Multi/ 04551/20139]. We gratefully acknowledge to Dr. M. Conceição Oliveira for the helpful discussion about ESI-MS results and the IST-UL NMR and MS Networks for facilities.

Keywords: Dyes • Laccase • Biocatalysis • Green synthesis

[1] K. Hunger, Industrial Dyes: Chemistry, Properties, Applications, Wiley-VCH: Weinheim, 2003.
[2] H. S.Freeman, A. T. Peters, Colorants for Non-textile Applications, Elsevier Science: New York, 2000.
[3] W. Herbst, K. Hunger, Industrial Organic Pigments: Production, Properties, Applications, Wiley-VCH: Weinheim, 1997.
[4] E. Merino, *Chem. Soc. Rev.* **2011**, 40, 3835-3853 and references therein.
[5] E. Baer, A. L. Tosoni, *J. Am. Chem. Soc.* **1956**, 78, 2857-2858.
[6] H. K. Hombrecher, K. Lüdtke, *Tetrahedron* **1993**, 49, 9489-9494.
[7] J. Burdon, C. J. Morton, D. F. Thomas, *J. Chem. Soc.* **1965**, 2621-2627.
[8] A. Grirrane, A. Corma, H. Garcia, *Science* **2008**, 322(5908), 1661-1664.
[9] J. Wang, L. Hu, X. Cao, J. Lu, X Li, H. Gu, *RSC Adv.* **2013**, 3, 4899-4902.
[10] J. Kim, J. H. Park, Y. K. Chung, K. H. Park, *Adv. Synth. Catal.* **2012**, 354, 2412-2418.
[11] M. Caldarelli, I. R. Baxendale, S. V. Ley, *Green Chem.* **2000**, 2, 43-45.
[12] W. Lu, C. Xi, *Tetrahedron Lett.* **2008**, 49(25), 4011-4015.
[13] Z. Wu, B. Ondruschka, G. Cravotto, D. Garella, J. Asgari, *Synth. Commun.* **2008**, 38(15), 2619-2624.
[14] D. Nematollahi, Z. Zohdijamil, H. Salehzadeh, *J. Electroanal. Chem.* **2014**, 720-721, 156-161.
[15] C. M. Clouthier, J. N. Pelletier, *Chem. Soc. Rev.* **2012**, 41, 1585–1605.
[16] F. Hollmann, I. W. C. E. Arends, K. Buehler, A. Schallmey, B. Bühler, *Green Chem.* **2011**, 13, 226-265.
[17] R. A. Sheldon, J. M. Woodley, *Chem. Rev.* **2018**, 118, 801−838.
[18] D. M. Mate, M. Alcalde, *Microb. Biotechnol.* **2017**, 10, 1457–1467 and references therein.
[19] M. Mogharabi, M. Faramarzi, A. *Adv. Synth. Catal.* **2014**, 356(14-15), 897-927 and references therein.
[20] M. D. Cannatelli, A. J. Ragauskas, *Chem. Rec.* **2017**, 17, 122–140.
[21] B. Branchi, C. Galli, P. Gentili, *Org. Biomol. Chem.* **2005**, 3, 2604-2614.
[22] R. Bournbonnais, D. Leech, M. G Paice, *Biochim. Biophys. Acta* **1998**, 1397, 381-390.
[23] I. Bibi, H.N. Bhatti, M. Asgher, *Biochem. Eng. J.* **2011**, 56, 225-231.
[24] M. Bozic, V. Kokol, *Dyes Pigm.* **2008**, 76, 299-309.
[25] L. Pereira, A. V. Coelho, C. A. Viegas, M. M. C. Santos, M. P. Robalo, L. O. Martins, *J. Biotechnol.* **2009**, 139(1), 68–77.
[26] P. Zucca, G. Cocco, F. Sollai, E. Sanjust, *Biocatalysis* **2015**, 1, 82–108.
[27] S. Sen, S. Raut, P. Bandyopadhyay, S. Raut, *Fungal Biol Rev.* **2016**, 30, 112-133 and references therein.
[28] L. Pereira, A. V. Coelho, C. A. Viegas, C. Ganachaud, G. Lacazio, T. Tron, M. P. Robalo, L. O. Martins, *Adv. Synth. Catal.* **2009**, 351, 1857-1865.
[29] E. Enaud, M. Trovaslet, F. Bruyneel, L. Billottet, R. Karaaslan, M. E. Sener, P. Coppens, A. Casas, I. J. Jaeger, C. Hafner, R. C. A. Onderwater, A. M. Corbisier, J. Marchand-Brynaert, S. Vanhulle, *Dyes & Pigments* **2010**, 85, 99-108.
[30] J. Polak, A. Jarosz-Wilkolazka, A. Szuster-Ciesielska, K. Wlizlo, M. Kopycinska, J. Sojka-Ledakowicz, J. Lichawska-Olczyk, *J. Clean Prod.* **2016**, 112, 4265-4272.
[31] J. Polak, A. Jarosz-Wilkolazka, *Process Biochem.* **2012**, 47, 1295–1307 and references therein.
[32] A. C. Sousa, L. O. Martins, M. P. Robalo *Adv. Synth. Catal.* **2013**, 355(14-15), 2908-2917.
[33] A.C. Sousa, M. C. Oliveira, L. O. Martins, M. P. Robalo, *Green Chem.* **2014**, 16(9), 4127-4136.
[34] A. C. Sousa, M. C. Oliveira, L. O. Martins, M. P. Robalo, *Adv. Synth. Catal.* **2018**, 360, 575 – 583.
[35] A. C. Sousa, M. F. M. M. Piedade, L. O. Martins, M.P. Robalo, *Green Chem.* **2016**, 18, 6063–6070.
[36] A. C. Sousa, M. F. M. M. Piedade, L. O. Martins, M. P. Robalo, *Green Chem.* **2015**, 17, 1429–1433.
[37] Internet Bond-energy Databank (iBonD) Home http://ibond.chem.tsinghua.edu.cn or http://ibond.nankai.edu.cn.
[38] P. Durão, Z. Chen, A. Fernandes, P. Hildebrandt, D.H. Murgida, S. Todorovic, M. M. Pereira, E. P. Melo, L.O. Martins, *J. Biol. Inorg. Chem.* **2008**, 13, 183–193.
[39] L. O. Martins, C. M. Soares, M. M. Pereira, M.M., M. Teixeira, G. H. Jones, A. O. Henriques, *J. Biol. Chem.* **2002**, *277*, 18849-18859.

10.1002/asia.201801450

10.1002/asia.201801450

FULL PAPER

*Ana Catarina Sousa, Sara R. Baptista, Lígia O. Martins and M. Paula Robalo**

Page No. – Page No.

Synthesis of azobenzene dyes mediated by CotA-laccase

A simple and green approach for the synthesis of azo dyes has been developed. CotA-laccase, an eco-friendly catalyst, was used to promote the oxidative coupling of substituted aromatic amines towards the azobenzene dyes formation, in moderate to excellent yields, and working in mild conditions and short reaction times.