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Towards the rational biosynthesis of substituted phenazines and phenoxazinones by laccases

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Laccases are multi-copper oxidases that oxidise a wide range of substrates including phenol and aniline derivatives, which could be further involved in coupling reactions leading to the formation of dimeric and trimeric structures. This paper describes the enzyme-mediated dimerisation of several *ortho* and *meta*, *para*-disubstituted aromatic amines into phenazine ("head-to-tail" dimers) and phenoxazinone chromophores. The redox properties of substituted aromatic amines were studied by cyclic voltammetry and the kinetic constants of CotA and *Trametes versicolor* laccases were measured for selected aromatic amines. The structure of novel enzymatically synthetised phenazine and phenoxazinone dyes using CotA laccase was assessed by NMR and MS. Overall our data show that this enzymatic green process is an efficient alternative to the classic chemical oxidation of aromatic amines and phenols, with impact in the broad field of applications of these heterocyclic compounds.

Introduction

Phenazines and phenoxazinones are heterocyclic nitrogen containing compounds that are broadly distributed in natural and synthetic products. Substituted phenazine and phenoxazinone cores are important biological active motifs of antibiotics and antibacterial agents,[1,2] anti-tumour agents,[3,4] pesticides,[5] dyestuffs,[6-8] biosensors,[9] and are the building blocks for the synthesis of organic semiconductors[10] or electrical-photochemical materials.[11] Therefore, the oxidation of amines or aminophenols into phenazines or phenoxazinones derivatives respectively, are of great interest from the biological, chemical and technological points of view.

The production of phenazines[12,13] and phenoxazinones[14] cores have been assessed through a number of chemical methods. However, most of the methods used suffer from a number of disadvantages, such as the use of organic solvents, the requirement for harsh reaction conditions and despite some progress, low production yields. The interest in enzyme-catalysed transformations is on the other hand steadily growing since many enzyme-catalysed reactions meet the criteria of green chemistry.[15] The enzyme phenoxazinone synthase is widely distributed in nature and catalyses the oxidative condensation of two molecules of substituted *ortho*-aminophenols to the phenoxazinone chromophore.[16] This enzyme is a member of the multicopper oxidase family and performs the one-electron oxidation of substrates coupled to the four-electron reduction of oxygen to water. Phenoxazinone synthases show an overall fold, structural details in the catalytic copper centres and oxidation-reduction mechanisms identical to laccases.[17] Laccase-catalysed reactions are performed in aqueous solvent systems under mild reaction pH and temperature conditions and the only waste product formed during the course of their reactions is water.[18] Therefore, laccases have drawn considerable interest among synthetic chemists in recent years and have been successfully used for the oxidation of several functional groups, such as *ortho*- and *para*-diphenols, methoxyphenols, aminophenols and lignin-related molecules.[18] Most of these transformations are based on the oxidation of activated C-H bonds to C-O bonds and of C-O bonds to C=O bonds.[18,19] Laccases can also catalyse phenolic oxidative couplings[18,20] and they can initiate the generation of *ortho*- and *para*-quinones followed by addition of different nucleophiles.[18,21]

In a previous work we reported the "green" production of dyes from *para*-substituted aromatic amines, a synthetic approach, based on the use of CotA-laccase as biocatalyst[22a] and there are also other reports in the literature related with the use of laccases for the synthesis of phenoxazinone and related heterocycles.[8,23] However, in spite of the observed chemoselectivity and the relatively good yields obtained, these studies were restricted to particular substrate substitution patterns.[8,23]

The rational production of new substituted phenazine and phenoxazinone scaffolds is highly desirable and has been mainly prevented by the absence of general methodologies for the production of diversely substituted phenazines and phenoxazines. Herein, we report the enzymatic oxidation of aromatic amines, such as *ortho*-phenylenediamines, substituted *para*-phenylenediamines and *ortho*-aminophenols, by CotA-laccase from *Bacillus* subtilis[24] for the production of substituted phenazine and phenoxazinone derivatives, extending the scope of laccase-catalysed transformations. We show that this enzymatic approach constitutes a promising method for the green production of phenazine and phenoxazinone frameworks with impact in a variety of applications.

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Results and discussion

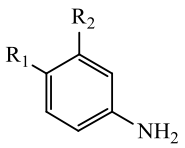
Electrochemical studies of substrates

A series of compounds derived from 1,4-phenylenediamine (1,4-PDA) and 4-aminodiphenylamine (4-ADA) were studied, together with 1,2-substituted aromatic derivatives, in order to draw trends in the type of compounds that are susceptible for efficient oxidation by laccases (**Figure 1**). It is known that the nature and position of the substituents in the amine aromatic ring affect the redox potential of the substrates and consequently the rate of electron transfer to the enzyme.[19d,22] The laccase´s activity is enhanced by the presence of electron-donating groups at the benzene ring, which by decreasing the electrochemical potential make them more easily oxidisable.[22] The pH is also a factor that plays a pivotal role in the laccase's enzymatic activity as it affects both the enzyme's performance and the redox potential of their putative substrates, i.e. their susceptibility for oxidation. Therefore, the information of the redox potential of each substrate at a particular pH is a critical parameter for predicting the performance of the enzymes under study.

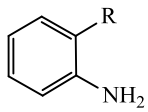
Electrochemical studies of the aromatic amines were performed by cyclic voltammetry with a platinum disk working electrode in 100 mM of Britton-Robinson (pH 4-5) or phosphate (pH 6-7) buffers.

In general the tested compounds show similar electrochemical behaviours generating well-defined irreversible oxidation peaks (E_{pa}) (**Table 1**). The absence or the presence of small reductive peaks (E_{pc}) for most of the substrates tested, indicate that the oxidation process probably generates a highly reactive quinone-diimine or quinone-imine species that undergo further non-enzymatic reactions. The only exception was for ANP, where quasi-reversible redox processes were observed, with $E_{1/2}$ in the range 0.20-0.29 V.

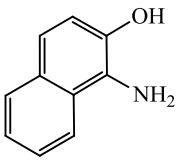
The aromatic amines displayed oxidation peaks in the potential range from 0.24 V (for ANP at pH = 7) to 0.71V (for 2-NPDA and 2-AP at pH = 4) at a scan rate of 50 mV/s. The oxidation potential is influenced by the additional presence of a substituent group (R_2) on *meta* position (see **Table 1**); the presence of an electron donor group, OH in 2,5-DAP, resulting in a lower oxidation potential and, is expected to favour the enzymatic oxidation, while the electron acceptors SO_3H (in 2,5-DABSA and 4-ADASA) and NO_2 (in 2-NPDA) groups resulted in higher oxidation potentials and a loss of reversibility of the redox processes. Moreover, the increased oxidation potentials measured for 1,2-PDA and 2-AP showed that *ortho*-substituted derivatives are more difficult to oxidise than the related *para*-substituted derivatives.



Substrate	R_1	R_2
4-ADASA	NHPh	SO_3H
2,5-DABSA	NH_2	SO_3H
2-NPDA	NH_2	NO_2
2,5-DAP	NH_2	OH



1,2-PDA R = NH_2
1,2-AP R = OH



ANP

Figure 1. Representative structures of the substituted aromatic amines.

Differences up to 200 mV were found in the substrate oxidation potentials within the pH range tested. Increasing the pH from 4 to 7 resulted in an almost linear decrease in the oxidation potential consistent with our previous results with *p*-substituted aromatic amines.[22a]

Table 1. Electrochemical data of substituted aromatic amines *vs* NHE in buffered solutions at a scan rate of 50 mV/s.

Substrate	pH=4		pH=5		pH=6		pH=7
	E_{pa} (V)	E_{pc} (V)	E_{pa}(V)	E_{pc}(V)	E_{pa} (V)	E_{pc} (V)	E_{pa} (V)
1,2-PDA	0.69	----	0.64	----	0.61	----	0.58
	----	----	0.88	----	0.88	----	0.85
2-AP	0.71	----	0.58	----	0.56	----	0.51
1,4-PDA[22a]	0.60	0.48	0.52	0.43	0.47	0.37	0.41
4-ADA[22a]	0.52	0.44	0.49	0.31	0.48	----	0.44
4-ADASA	0.62	0.53[a)]	0.59	0.45[b)]	0.58	----	0.55
	0.74	0.69	0.76	0.70[b)]	----	----	----
2,5-DABSA	0.65	----	0.63	----	0.63	----	0.52
	0.83	----	0.83	----	0.82	----	0.82
2-NPDA	0.71	----	0.69	----	0.69	----	0.64
2,5-DAP	0.41	----	0.32	----	0.30	----	----
	0.55	0.47[c)]	----	----	----	----	----
ANP	0.43	0.33[d)]	0.32[e)]	0.26	0.31	0.22[f)]	0.24[g)]

4-ADA = 4-aminodiphenylamine; [a)] ΔE = 90 mV, I_{pc}/I_{pa} = 0.5, irreversible wave; [b)] I_{pc}/I_{pa} = 0.5, irreversible wave;[c)] $E_{1/2}$ = 0.51 V, ΔE= 80 mV, I_{pc}/I_{pa} = 1.0; [d)] ΔE= 100 mV, I_{pc}/I_{pa} = 0.4, irreversible wave; [e)] $E_{1/2}$ = 0.29 V, ΔE= 60 mV, I_{pc}/I_{pa} = 1.0, [f)] $E_{1/2}$ = 0.265 V, ΔE= 90 mV, I_{pc}/I_{pa} = 1.0; [g)] $E_{1/2}$ = 0.205 V, ΔE= 70 mV, I_{pc}/I_{pa} = 1.0

Overall, the oxidation potentials measured are slightly higher or lower than the redox potential of CotA (0.55 V vs NHE[25]) and TvL enzymes (0.79 V vs NHE[26]) respectively, indicating the absence of redox constraints for their oxidation by both enzymatic systems.

Laccase-mediated oxidation of selected substrates

The biotransformation of the aromatic substrates (**Figure 1**) was initially investigated using two laccases, the bacterial CotA laccase and the commercial fungal TvL. The pH optima for selected substrates (4-ADASA, 2,5-DABSA and 2,5-DAP) were measured (data not shown) and bell-shape pH activity profiles with maximal enzymatic rates at neutral pH (around 6-7) for CotA-laccase and at slightly acidic conditions for the TvL laccase (around 4-5) were obtained.

The kinetic constants for 4-ADASA and 2,5-DABSA were measured using the two laccases (**Table 2**), showing the importance of sulfonyl side groups in decreasing the apparent K_m values of the enzymes. The specificity (k_{cat}/K_m) of CotA-laccase and TvL are comparable for the 4-ADASA substrate, however the specificity of CotA for 2,5-DABSA is one order of magnitude higher as compared with TvL. The improved specificity of CotA-laccase for both substrates combined with its optimal pH close to the neutrality encouraged us to pursue the work using the bacterial CotA-laccase.

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

CotA				
Substrate	Optimal pH	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1}.s^{-1}$)
4-ADASA	6	0.21 ± 0.12	12.6 ± 2.5	68.1 ± 24
2,5-DABSA	6	0.4 ± 0.12	31.7 ± 5	81.8 ± 10
TvL				
Substrate	Optimal pH	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1}.s^{-1}$)
4-ADASA	4	0.2 ± 0.01	13.8 ± 2.5	62.3 ± 9
2,5-DABSA	5	1.6 ± 0.3	9.6 ± 0.7	5.9 ± 0.6

Structural characterisation of the biotransformation products

At the optimal pH conditions, all the tested substrates were oxidised by CotA-laccase at a preparative scale. To estimate the yield of product formation, enzyme (1U) was added to 10 ml reaction mixtures containing 5 mmol of substrate. In the presence of enzyme, the disappearance of the substrates was completed after 24h and, in general, the main products were obtained in good to excellent isolated yields (60-90%).

The obtained products, directly as crude solids or purified using silica gel preparative chromatography, were fully characterised by IR, NMR (^{1}H, ^{13}C, COSY, HSQC, HMBC and NOESY), UV-Visible spectroscopies and mass spectrometry (**Figure 2**).

The enzymatic oxidation of *meta*,*para*-disubstituted amine derivatives (2,5-DABSA, 2-NPDA and 4-ADASA) led to the formation of phenazines with a "head-to-tail" framework. The ability of the enzyme to produce the tricyclic heterocycle core was confirmed by using 1,2-phenylenediamine as model compound. The oxidation of 1,2-PDA resulted in the formation of 2,3-diaminophenazine (**1**), a planar electron rich heterocyclic aromatic compound known by its rich organic chemistry and intense luminescence.[27] The ^{1}H NMR spectrum (CD_3OD-d_4) of **1** showed peaks in the 7.00 - 8.00 ppm region indicating the presence of six aromatic protons in a symmetrical tricyclic phenazine core consistent with data in the literature.[28] The structure was corroborated by the ESI-mass spectrum in the positive mode that exhibited a major peak at *m/z* 211 ($[M+H]^+$).

Laccase oxidation of 4-ADASA and 2,5-DABSA with the electron-withdrawing sulfonyl substituent in *meta* position relative to the amino group, resulted in the formation of phenazine coloured compounds **2** and **4** as main products. A similar phenazine derivative (**6**) was obtained from 2-NPDA, but in a lower yield. The structural identification of the phenazine skeleton (for compounds **2**, **4** and **6**) was based on observed differences in the chemical shifts of the aromatic protons. The disappearance of the singlet for H_6, present in all substrates, and the concomitant presence of resonances in the 8.01-6.38 ppm region corresponding to the two doublets for protons $H_{3,8}$ and $H_{4,9}$ confirmed the substitution at the *ortho* positions relative to the phenazine ring and the symmetry of the phenazine central core. For compound **2**, the signal at 10.75 ppm correspond to the sulfonic acid protons, while the broad signal at 6.75 ppm of compound **6** was attributed to amino groups. In contrast, no signals were found for amino or sulfonic groups for compound **4**. The assignment of the structures in both rings was confirmed by ^{1}H-^{13}C HSQC and HMBC correlations.

The molecular weights of compounds **2**, **4** and **6** were determined by negative-ion ESI mass spectrometry and the proposed structures were confirmed by tandem mass spectrometry. The full mass spectra of **2** and **4** displayed deprotonated molecular ion signals with different charges, as expected for disulfonated compounds. The mass spectra of **2** and **4** showed relative intense peaks at *m/z* 521 and 369, and at *m/z* 259.9 and 183.9 corresponding to $[M-H]^-$ and $[M-2H]^{2-}$, respectively. The MS^2 mass spectrum of the precursor ion *m/z* 521 presented fragments at *m/z* 457, 441 and 377 due to losses of 64 u (SO_2), 80 u (SO_3) and 144 u (SO_2+SO_3), respectively, which is a characteristic of aromatic disulfonates.

For compound **6**, the ESI mass spectrum presents a peak at *m/z* 299 ($[M-H]^-$) consistent with a molecular mass of 300 $g.mol^{-1}$.

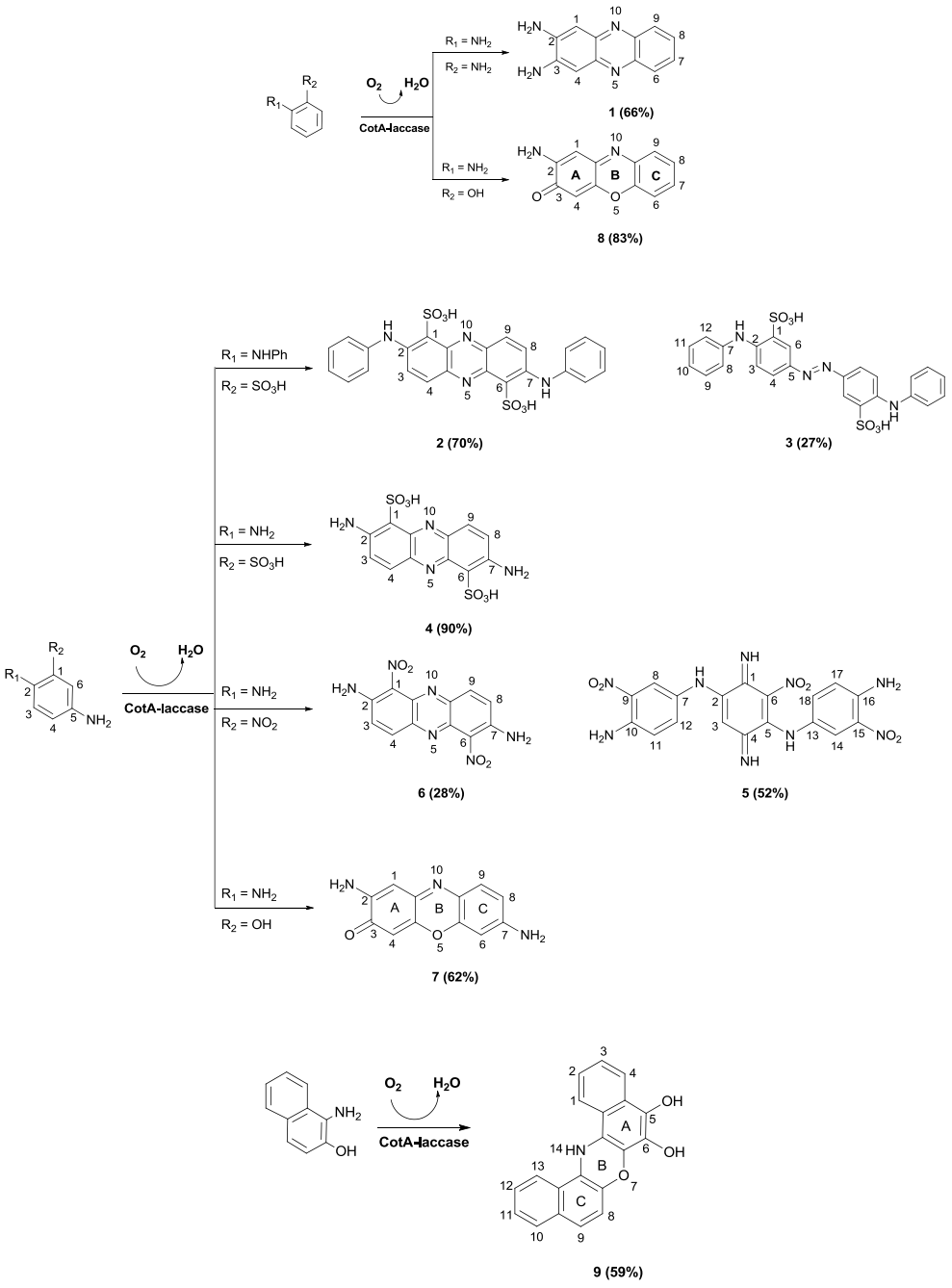


Figure 2. Structures and yields (%) of main products formed in laccase-catalysed reactions with *p*-substituted aromatic amines.

Fragmentation of the precursor ion *m/z* 299 yielded the characteristic product ions for nitro compounds,[29] at *m/z* 282, 269 and 239 attributed to $[M\text{-}H\text{-}OH]^{-\bullet}$, $[M\text{-}H\text{-}NO]^{-\bullet}$ and $[M\text{-}H\text{-}2NO]^{-}$, respectively. The fragment ion at *m/z* 239 is in agreement with the presence of two nitro groups in the structure. Compounds **2** and **4**, are water soluble, with molar extinction coefficients (ε) of 494 $M^{-1}.cm^{-1}$ and 2372 $M^{-1}.cm^{-1}$, respectively.

In the biotransformation of 4-ADASA by CotA-laccase, a secondary azo product, compound **3**, was formed and isolated through preparative chromatography. The ^{1}H NMR spectrum showed a singlet at 8.15 ppm and two doublets at 7.75 and 7.36 ppm, with characteristic *meta* coupling constants, attributed to protons of H_6, H_4 and H_3, respectively. This is indicative that compound **3** has a similar structural pattern to the substrate. In addition, the shift of all aromatic signals for low field is consistent with the presence of a symmetrical structure connected by a N=N bond. Furthermore, the respective mass spectrum shows peaks at *m/z* 523 ($[M\text{-}H]^{-}$), 545 ($[M\text{-}2H+Na]^{-}$) and 260.9 ($[M\text{-}2H]^{2-}$) which are consistent with a

molecular mass of 524 g.mol^{-1}. MS^2 spectrum of the $[M-H]^-$ ion at *m/z* 523 was characterised by the loss of SO_3 resulting in an abundant fragment ion with *m/z* 443, which further led to product ions with *m/z* 415 (rearrangement loss of N_2 from the group azo) and 247 (cleavage of C-N bound at the central group azo) in the MS^3 experiment. The formation of azo compounds was previously observed as laccase biotransformation products derived from *para*-substituted aromatic amines[22] or the anthraquinonic Acid Blue 62 dye. [30,31]

The biotransformation of 2-NPDA led also to the formation of two products, the previuosly described yellow phenazine **6** and an additional dark red product (**5**), with a 2,5-diaminated 1,4-benzoquinonediimine skeleton, resulting from the sequential self-coupling of the 2-NPDA intermediate (**Figure 2**) at positions 2 and 5. The multiplicity and resonances of the signals found in ^{1}H and ^{13}C-NMR spectra are in accordance with previously reported data for similar benzoquinonediimine structures.[22a] The coupling must be performed through the amino group in *meta* position to the nitro group, as suggested by the correlations found in the NOESY-NMR spectrum between NH_2 protons and H_{11} and H_{17}. The full ESI mass spectrum of **5** shows a base peak at *m/z* 452 ($[M-H]^-$) which is in agreement with a molecular mass of 453 g.mol^{-1}. Fragmentation of the precursor ion *m/z* 452 generated a predominant product ion with *m/z* 405 attributed to the loss of HNO_2.

Substitution of the *meta* electron-withdrawing groups by an hydroxyl group led to the formation of a phenoxazine derivative, which highlights the potential use of laccases for the preparation of compounds based on structural variations of the 2-aminophenoxazin-3-one scaffold.

The conversion of 2,5-DAP yielded a purple product **7** for which the specific pattern of a phenoxazine ring was identified. Downfield shielding was observed for all the proton signals of this heterocycle relative to their respective parent compound. Furthermore, the three aromatic signals at 7.44 (H_9), 6.77 (H_8) and 6.63 ppm (H_6) possess identical multiplicity as those of the substrate 2,5-DAP, suggesting the occurrence of a very similar spin system pattern for ring C. The presence of two singlets for protons H_1 and H_4 define the A ring. In the ^{13}C spectrum, resonances at low field regions, namely, 181.5 and 151.2 ppm, characteristic of carbonyl and imine quaternary carbons (C_3 and C_{11}) confirmed the presence of a quinone-imine ring. The ^{1}H-^{13}C HMBC spectrum also provided crucial information for the attribution of the quaternary carbons and in particular confirmed the substitution at C_7 (ring C). The connectivities between H_6 and H_8 with C_7 together with the coupling constants of the protons H_6, H_8 and H_9 support the substitution pattern of ring C. For ring A, the substitution pattern was proposed based in the correlations between H_1 and C_2, C_{11} and C_{12} and between H_4 and C_3, C_{11} and C_{12}.

The proposed structure **7** was corroborated by ESI/MS data. Full scan spectrum in positive-ion mode exhibits two major peaks at *m/z* 228 ($[M+H]^+$) and 250 ($[M+Na]^+$). MS^2 spectrum of protonated molecule showed a peak at *m/z* 200 ($[M+H-CO]^+$) due to the characteristic loss of a CO molecule (-28 u). Additionally the IR spectrum with a band at 1649 cm^{-1}, assigned to the carbonyl group, and without the wide band at ν=3200 to 2500 cm^{-1}, typical of intramolecular hydrogen bonds of the phenolic group, corroborates the formation of the quinone-imine central ring.

The synthesis of phenoxazine derivatives using laccases and *ortho*-aminophenols substrates was also confirmed through the use of 2-AP and ANP as model compounds. Laccase-mediated conversion of 2-AP yielded the phenoxazine **8**, a brown product with identical physicochemical features to the 2-amino-3H-phenoxazin-3-one.[32] The structure was confirmed in ESI-MS in positive-ion mode, which produced two main ions with *m/z* 213 ($[M+H]^+$) and 235 ($[M+Na]^+$). The precursor ion *m/z* 213 exhibited a prominent fragment at *m/z* 185 corresponding to the loss of a CO molecule.

The oxidation of ANP yields also a phenoxazine compound (**9**) for which the presence of six aromatic signals (four doublets and two triplets) in the region 7.25 – 8.42 ppm in the ^{1}H NMR spectrum showed identical multiplicity as those of the substrate ANP, suggesting the occurrence of very similar spin system pattern for naphthalene ring C. The presence of two pairs of related doublets and triplets for protons H_1 to H_4 define the naphthalene A ring. Although many attempts were made to detect the quaternary carbons on the ^{13}C spectrum, these were not observed, by reasons that are not clear at the present time.

The ESI mass spectra of **9** show intense peaks at *m/z* 316 and 338, and at *m/z* 314 assigned to the protonated molecule $[M+H]^+$ and its sodiated adduct $[M+Na]^+$, and to the deprotonated molecule $[M-H]^-$, respectively. The even nominal mass found for the (de)protonated ions dictates an odd number of nitrogen atoms in the structure. In order to confirm the proposed structure CID experiments were performed. The major fragmentation channel observed in the MS^2 spectrum of **9** is due to the loss of 28 u (CO) yielding ion at *m/z* 288. Further fragmentation of these ion given rise to ions at *m/z* 270 (loss of a water molecule) and 260 (loss of a CO molecule), and other low-abundance products, as summarized in the fragmentation routes proposed for **9** (see Supplementary information).

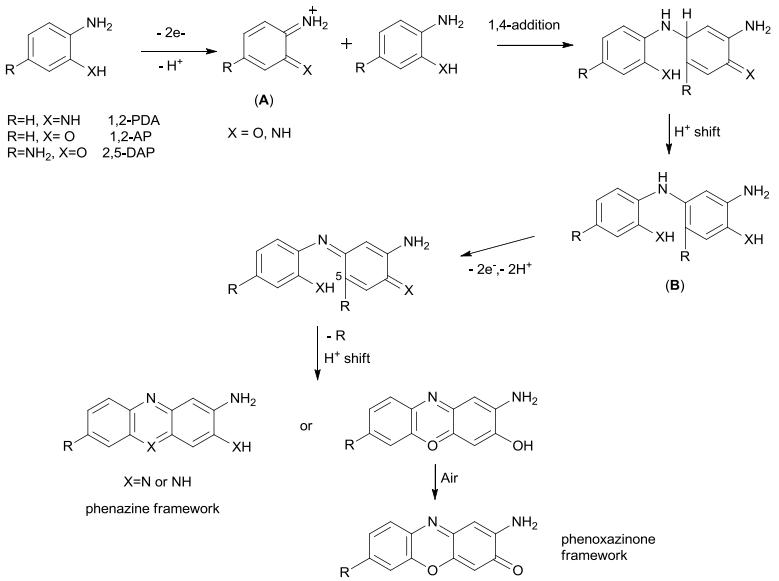
The structural characterization of the orange compound **9** and the MS results in particular, indicated the presence of two hydroxyl groups (naphthalene ring A). This suggests the hydrolysis of the imine group in the final reduced phenoxazine intermediate which could occurs during the oxidation or the purification processes, as previously described by Bruyneel *et al.*[8c]

Proposed pathway for laccase's oxidation of substituted aromatic substrates

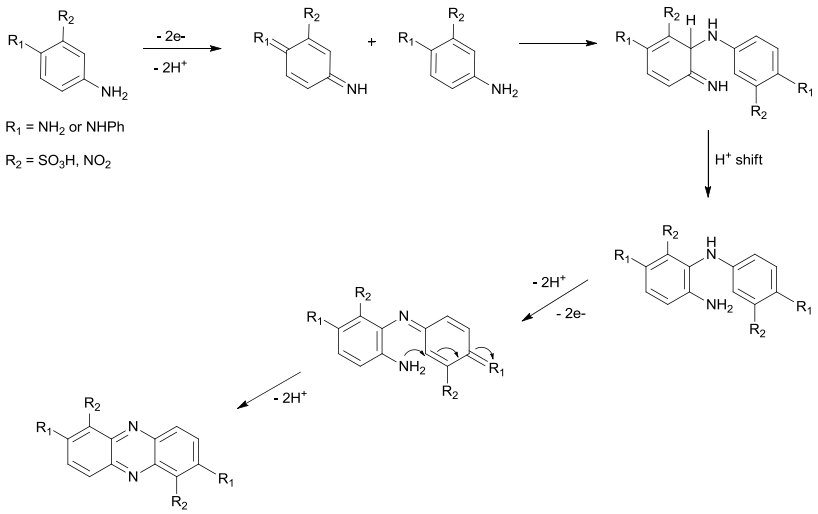
An enzymatic mechanistic pathway for the production of substituted heterocycles with phenazine or phenoxazine scaffolds is proposed based in our experimental results and in previous results on the oxidative cross-coupling of substituted *o*-aminophenols[33,34] (**Scheme 1**).

The *ortho*-diamine or *ortho*-aminophenol precursor is enzymatically processed through two successive one-electron oxidations, generating the *ortho*-quinone-diimine or *ortho*-quinone-imine intermediates (**A**). These species are further involved in a 1,4-conjugated Michael addition, on its most electrophilic carbon atom (in *para* position to the quinonic group), together with a nucleophilic partner (the amino group of another substrate molecule). This second step, followed by a proton shift, yields the first coupling intermediate (**B**). The second 2e$^-$ oxidation is also expected to be mediated by the laccase as suggested by Bruyneel *et al.*[33] Intramolecular Michael

ortho-substituted amines



meta, *para*-disubstituted amines



Scheme 1. Proposed oxidative pathway for the formation of the phenazine and phenoxazinone frameworks from laccase and substituted aromatic amines.

addition of the amino group (or phenol) to the C5 atom leads to the aminophenazine or fully reduced aminophenoxazine, which is spontaneously air-oxidised to produce the desired heterocycle.[33,34]

This intramolecular Michael addition might bring to the displacement of an amino group in the 2,5-DAP, within the formation of the phenoxazine moiety.[8d] The loss of the NH_2 group in one of the two substrate molecules is consistent with similar observations on the formation of the phenoxazine core accompanied by the loss of COOH or CHO groups, previously reported by Suzuki *et al.*[35] and Hughes *et al.*[36]

For the *m,p*-disubstituted aromatic amines (2,5-DABSA, 2-NPDA and 4-ADASA), the first benzoquinonediimine intermediate is formed through laccase oxidation in a similar manner as described above. However, the sequential non-enzymatic step followed a different pattern, where the intermediate and the nucleophilic amino group of another substrate molecule are involved. In contrast to the *ortho* disubstituted derivatives, the nucleophilic attack at the *para* position relative to the amino group is hampered by the presence of a substituent. Instead, the nucleophilic attack occurs at the *ortho* position, adjacent to the sulfonyl or nitro side groups. The subsequent proton shift aromatises the first oxidised intermediate as above. A $2e^-$ oxidation followed by an intramolecular Michael addition of the aromatic amino group again yields a tricyclic phenazine framework.

Experimental

General procedures

All reagents and solvents are commercially available (Sigma-Aldrich Co) and were used without further purification. The products characterisation was performed by 1D-NMR (^{1}H,^{13}C) and 2D-NMR (COSY, HSQC, HMBC and NOESY) spectra, obtained in DMSO-d_6, D_2O, CD_3CN-d_3 or CD_3OD-d_4 on a Bruker Advance 400MHz spectrometer. Chemical shifts are reported in ppm relative to the solvent peaks and coupling constants (*J*) are reported in Hertz. FTIR spectra were obtained in KBr pellets on a Bruker Vertex 70 FT-IR spectrometer.

ESI-MS spectra were acquired on a 500-MS LC ion trap mass spectrometer (Varian, Inc., Palo Alto, CA), operated in the positive and negative ion modes. The optimised operating parameters were: ion spray voltage: ±5 kV; capillary voltage: 20 V and RF loading: 90%. Nitrogen was used as nebulizing and drying gas, at pressures of 25 and 10 psi, respectively; the drying gas temperature was set at 300 ºC. The spectra were recorded in the range 100 – 1000 Da. Spectra typically correspond to the average of 20–35 scans. Tandem mass spectra were obtained with an isolation window of 2.0 Da, excitation energy values between 1.2 and 2.2 V, and an excitation time of 10 ms (CID up to MS^3).

High resolution mass measurements were performed on a MicrOTOF equipped with an ESI source (Bruker, Daltonics) at Unidade de Spectrometria, Universidade de Santiago de Compostela.

The UV-Visible spectra of the substrates and the molar extinction coefficients were obtained in a Nicolet Evolution 300 and an Agilent Technologies Cary 60 spectrophotometers.

Recombinant CotA-laccase from *Bacillus subtilis* (1U defined as the amount of enzyme that transformed 1µmol of ABTS per min at 37°C) was produced and purified as described previously.[20e,24] *Trametes versicolor* laccase (TvL, specific activity 20 $U.mg^{-1}$; 1U 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 -18ºC prior to use.

Electrochemical measurements

The redox potentials for all aromatic amines and aminophenols were measured by cyclic voltammetry using an EG&G Princeton Applied Research Model 273A 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 1:9 MeOH: 100 mM buffer (phosphate buffer for pH ≥ 6 and Britton-Robinson (B&R) buffer for pH 4-5) solutions 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 50 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).

Enzymatic assays

The effect of pH on the oxidation of 2,5-DABSA, 4-ADASA, 2,5-DAP (1mM) by CotA-laccase and TvL was performed in B&R buffer (pH 3-10; 100mM phosphoric acid, 100 mM boric acid and 100 mM acetic mixture with 0.5 M NaOH to the desired pH) by measuring the initial rate of substrate oxidation at 460 nm. The kinetic parameters for the aromatic amine substrates (0.05-5 mM) were monitored at 37 ºC 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[24] using bovine serum albumin as a standard.

CotA laccase mediated reactions: general procedure

Preparative scale reactions were performed under the following conditions: in a 25 ml round-bottom flask, the aromatic substrate 4-ADASA, 2,5-DABSA, 2-NPDA, 1,2-PDA, 2,5-DAP, 2-AP, ANP (0.05 mmol) dissolved in 1 ml of methanol was added to 9 ml of 100 mM phosphate buffer (pH 6-7). Then, the laccase (1 $U.ml^{-1}$) was added and the reaction mixture was stirred at 37 ºC in aerobic conditions. The conversion was followed by thin layer chromatography (TLC) on aluminium sheet Silicagel 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. Whenever necessary the crude residues were purified by preparative chromatography.

Oxidation of 1,2-Phenylenediamine (1,2-PDA)

At pH 7, a dark yellow solid was collected by filtration, washed with diethyl ether and dried under vacuum to afford compound **1**.

1 : yellow-brownish solid; yield: 3.2 mg (0.0152 mmol, 66 %); FTIR (KBr) ν(cm^{-1}): 3433, 3335, 3177, 1642, 1493, 1467, 1406, 1336, 1261, 1224, 1101; ^{1}H NMR (CD_3OD-d_4): δ (ppm) = 7.95 (m, 2H, H6, H9), 7.62 (m, 2H, H7, H8), 7.03 (s, 2H, H1, H4); $^{13}C\{H\}$ NMR (CD_3OD-d_4): δ (ppm) = 146.4 (C2, C3), 143.7 (C11, C12), 141.2 (C13, C14), 128.5 (C7, C8), 128.1 (C6, C9) and 103.2 (C1,C4); UV/Vis: 440 nm, ε=18387 $M^{-1}.cm^{-1}$ (MeOH); ESI-MS positive mode: *m/z* = 211.1 $[M+H]^+$.

Oxidation of 4-Aminodiphenylamine-2-sulfonic acid (4-ADASA)

At pH 6, a brown solution was obtained and after solvent evaporation, the residue was extracted with methanol. The final residue was purified by silicagel column chromatography (mobile phase: MeOH:CH_2Cl_2 1:3) to afford two fractions. The first eluted pink fraction and the second yellow fraction were dried under vacuum to afford compounds **2** and **3**, respectively.

2*:* Pink solid; yield: 9.1 mg (0.017 mmol, 70%); FTIR (KBr) ν(cm^{-1}): 3436, 2925, 1627, 1597,1545, 1509, 1420, 1386, 1331, 1198, 1173, 1112, 1043, 823, 793, 796, 710, 625; ^{1}H NMR (DMSO-d_6): δ(ppm)= 10.75 (s, 2H, SO_3H), 7.98 (d, 2H, *J*=9.2Hz, H4, H9), 7.70

(d, 2H, *J*=9.2 Hz, H3, H8), 7.40 (t, 4H, *J*=7.8 Hz, H17, H17', H19, H19'), 7.22 (d, 4H, *J*=7.8 Hz, H16, H16', H20, H20'), 7.17 (t, 2H, *J*=7.2Hz, H18, H18'), ^{13}C{H} NMR (DMSO-d_6): δ(ppm)= 141.2 (C2, C7), 141.1 (C15, C15'), 139.7 (C11, C13), 138.1 (C12, C14), 131.4 (C4, C9), 129.6 (C17, C17', C19, C19'), 123.2 (C18, C18'), 122.9 (C3, C8), 121.4 (C16, C16', C20, C20'), 120.2 (C3, C8); UV/Vis: 460 nm, ε= 494 $M^{-1}.cm^{-1}$ (MeOH); ESI-MS negative mode: *m/z* 521.1 $[M-H]^-$, *m/z* 259.9 $[M-2H]^{2-}$; MS^2 (*m/z*, (relative intensity)): 457 (100%) $[(M-H)-SO_2]^-$, 441 (45%) $[(M-H)-SO_3]^-$, 377 (10%) $[(M-H) - (SO_2+SO_3)]^-$; HR-ESI/MS: *m/z* calcd. for $C_{24}H_{17}N_4O_6S_2$ $[M-H]^-$: 521.0595; found 521.0574.

3*:* yellow solid; yield: 3.5 mg (0.007 mmol, 27%); FTIR (KBr) ν(cm^{-1}): 2925, 2855, 1636, 1535, 1468, 1321, 1084, 1024, 934, 538; ^{1}H NMR (CD_3OD-d_4), δ(ppm) = 8.38 (d, 2H, *J*=2.4Hz, H6, H6'), 7.81 (dd, 2H, *J*=9.2Hz, *J*=2.4Hz, H4, H4'), 7.37 (d, 2H, *J*=9.2Hz, H3, H3'), 7.35 (t, 2H, *J*=7.6Hz, H9, H11, H9', H11'), 7.27 (d, 4H, *J*=7.6Hz, H8, H12, H8', H12'), 7.08 (t, 2H, *J*=7.6Hz, H10, H10'). ^{13}C{H} NMR (CD_3OD-d_4): δ(ppm) = 145.8 (C5), 145.0 (C7), 142.2 (C2), 131.5 (C1),130.48 (C9, C9', C11, C11'), 126.3 (C4), 124.6 (C6), 124.32 (C10), 122.44 (C8, C8', C12, C12'), 115.24 (C3); ESI-MS negative mode: *m/z* 523.1 $[M-H]^-$; *m/z* 545.1 $[M-2H+Na]^-$ and *m/ z* 260.9 $[M-2H]^{2-}$, MS^2 (*m/z*, (relative intensity)): 459 (20%) $[(M-H)-SO_2]^-$, 443 (100%) $[(M-H)-SO_3]^-$; MS^3 (*m/z*, (relative intensity)): 415 (15%) $[(M-H)-SO_3-N_2]^-$, 247 (100%) $[C_{12}H_9NO_3S]^{-\bullet}$; HR-ESI/MS: *m/z* calcd. for $C_{24}H_{19}N_4O_6S_2$ $[M-H]^-$: 523.0746; found 523.0712.

Oxidation of 2,5-Diaminobenzene sulphonic acid (2,5-DABSA)

At pH 6, a dark magenta solution was obtained and after solvent evaporation, the final residue affords a dark magenta solid **4**.

4: Dark magenta solid; yield: 8.0 mg (0.02 mmol, 90%); FTIR (KBr) ν(cm^{-1}): 3435, 1631, 1498, 1439, 1316, 1186, 1128, 1046, 960, 877, 799, 710, 682, 648; ^{1}H NMR (D_2O)[a]: δ(ppm) = 7.04 (d, 2H, *J*=9.5Hz, H4, H9); 6.38 (d, 2H, *J*=9.5Hz, H3, H8); ^{13}C{H} NMR (D_2O)[a]: δ(ppm) = 146.6 (C2, C7); 139.5 (C11, C13); 137.9 (C12, C14); 132.9 (C4, C9); 127.2 (C3, C8); 111.3 (C1, C6); UV/Vis: 520 nm, ε=2372 $M^{-1}.cm^{-1}$ (H_2O); ESI-MS negative mode: *m/z* 369.1 $[M-H]^-$, *m/z* 183.9 $[M-2H]^{2-}$; HR-ESI/MS: *m/z* calcd. for $C_{12}H_9N_4O_6S_2$ $[M-H]^-$: 368.9964; found 368.9958.

[a] referenced to $(CD_3)_2CO$-d_6 solvent.

Oxidation of 2-nitro-1,4-phenylenediamine (2-NPDA)

At pH 6, the dark red solid was collected by filtration and was purified by Silicagel column chromatography (mobile phase: $C_4H_8O_2$:$CH_3(CH_2)_4CH_3$ 3:1) to afford two fractions. The first eluted red fraction and the second yellow fraction were dried under vacuum to afford compounds **5** and **6**, respectively.

5*:* dark red solid; yield: 3.9 mg (0.008 mmol, 52%); FTIR (KBr) ν(cm^{-1}): 3469, 3354, 2958, 2923, 2853, 1721, 1629, 1568, 1508, 1469, 1431, 1337, 1243, 1162, 1083, 821; ^{1}H NMR (CD_3OD-d_4) δ(ppm) = 7.71 (d, 1H, *J*=2.4Hz, H8), 7.56 (d, 1H, *J*=2.4Hz, H14), 7.19 (dd, 1H, *J*=2.4Hz and *J*=9.2Hz, H12), 7.11 (dd, 1H, *J*=2.4Hz and *J*=8.8Hz, H18), 7.04 (d, 1H, *J*=8.8Hz, H11), 6.93 (d, 1H, *J*=8.8Hz, H17), 5.95 (s, 1H, H3); ^{1}H NMR (CD_3CN-d_3): δ(ppm) = 8.58 (sbr, 1H, NH), 8.25 (sbr, 1H, NH), 7.69 (s, 1H, H8), 7.59 (s, 1H, H14), 7.20 (d, 1H, *J*=9.2Hz, H12), 7.12 (d, 1H, *J*=8.8Hz, H18), 7.04 (d, 1H, *J*=8.8Hz, H11), 6.93 (d, 1H, *J*=8.8Hz, H17), 6.56 (sbr, 4H, NH_2), 5.96 (s, 1H, H3), 5.84 (sbr, 2H, NH iminic ring). ^{13}C{H} NMR (CD_3OD-d_4): δ(ppm) = 153.0 (C1), 152.8 (C4), 152.7 (C2), 145.3 (C10), 144.9 (C16), 140.5 (C7), 139.9 (C13), 132.6 (C12), 132.2 (C15), 132.0 (C9), 130.5 (C18), 120.8 (C11), 120.6 (C17), 119.7 (C5), 118.3 (C6), 118.0 (C8), 115.6 (C14), 91.9 (C3); UV/Vis: 450 nm, ε=6626 $M^{-1}.cm^{-1}$ (MeOH); ESI-MS negative mode: *m/z* 452.2 $[M-H]^-$; MS^2 (*m/z*, (relative intensity)): 405 (100%) $[(M-H)-HNO_2]^-$; HR-ESI/MS: *m/z* calcd. for $C_{18}H_{16}N_9O_4$ $[M-H]^-$: 452.1073; found 452.1067.

6*:* yellow solid; yield: 2.1 mg (0.007mmol, 28%); FTIR (KBr) ν(cm^{-1}): 3418, 2924, 2853, 1641, 1522, 1384, 1101; ^{1}H NMR (CD_3CN-d_3): δ(ppm) = 7.91 (d, 2H, *J*=9.6Hz, H4, H9), 7.46 (d, 2H, *J*=9.6Hz, H3, H8) and 6.75 (sbr, 4H, NH_2);); UV/Vis: 350 nm, ε=3998 $M^{-1}.cm^{-1}$ (MeOH); ESI-MS negative mode: *m/z* 299.1 $[M-H]^-$; MS^2 (*m/z*, (relative intensity)): 282 (100%) $[(M-H)-OH^{\bullet}]^{-\bullet}$, 269 (42%) $[(M-H)-NO^{\bullet}]^{-\bullet}$, 239 (25%) $[(M-H)-2xNO^{\bullet}]^-$; HR-ESI/MS: *m/z* calcd. for $C_{12}H_7N_6O4_6$ $[M-H]^-$: 299.0534; found 299.0521.

Oxidation of 2,5-Diaminophenol.2HCl (2,5-DAP)

At pH 7, the dark solid was collected by filtration, washed with diethyl ether and dried under vacuum to afford compound **7**.

7: purple solid; yield: 3.2 mg (0.014 mmol, 62%); FTIR (KBr) ν(cm^{-1}): 3427, 3334, 3212, 1649, 1571, 1504, 1481, 1424, 1326, 1236, 1204, 1128, 840, 810, 705; ^{1}H NMR (CD_3OD-d_4): δ(ppm)= 7.44 (d, 1H, *J* =8.8Hz, H9), 6.77 (dd, 1H, *J*=2.4 Hz and *J*=8.8 Hz, H8), 6.63 (sd, 1H, *J*=2.4 Hz, H6), 6.48 (s, 1H, H1), 6.33 (s, 1H, H4); ^{13}C{H} NMR (CD_3OD-d_4): δ(ppm)= 181.5 (C3) 153.6 (C13), 151.2 (C11), 147.2 (C12), 146.8 (C14), 143.5 (C2), 130.3 (C9), 127.9 (C7), 115.1 (C8), 103.9 (C1), 101.3 (C4), 99.3 (C6); UV/Vis: 520 nm, ε=8950 $M^{-1}.cm^{-1}$ (MeOH); 460 nm, ε=5455 $M^{-1}.cm^{-1}$ (MeOH). ESI-MS positive mode: *m/z* 228.1 $[M+H]^+$, *m/z* 250.0 $[M+Na]^+$; MS^2 (*m/z*, (relative intensity)): 200 (100%) $[(M+H)-CO]^+$. HR-ESI/MS: *m/z* calcd. for $C_{12}H_{10}N_3O_2$ $[M+H]^+$: 228.0773; found: 228.0762.

Oxidation of 2-Aminophenol (2-AP)

At pH 7, the brown solid was collected by filtration, washed with diethyl ether and dried under vacuum to afford compound **8**.

8: brown solid; yield: 4.6 mg (0.021 mmol, 83%); FTIR (KBr) ν(cm^{-1}): 3412, 3305, 1657, 1587, 1573, 1495, 1470, 1467, 1425, 1401, 1291, 1273, 1204, 1173, 1147, 1116, 894, 844, 762, 584; ^{1}H NMR (CD_2Cl_2-d_2): δ(ppm)= 7.74 (d, 1H, *J*=8.4Hz, H6), 7.45-7.37 (m, 3H, H7, H8, H9), 6.47 (s, 1H, H4), 6.36 (s, 1H, H1); ^{13}C{H} NMR (CD_2Cl_2-d_2): δ(ppm)= 180.6 (C3), 149.8 (C14), 149.2 (C2), 146.2 (C13), 143.1 (C11), 134.4 (C12), 129.9 (C9), 129.1 (C6), 125.6 (C8), 116.3 (C7), 104.2 (C1), 101.1 (C4); UV/Vis: 430 nm, ε=9095 $M^{-1}.cm^{-1}$ (MeOH); ESI-MS positive mode: *m/z* 213.1 $[M+H]^+$, *m/z*

235.0 $[M+Na]^+$; MS^2 (*m/z*, (relative intensity)): 185 (100%) $[(M+H)-CO]^+$.

Oxidation of 1-Amino-2-naphthol (ANP)

At pH 7, the final residue was purified by silica gel preparative chromatography (mobile phase: $C_4H_8O_2$:$CH_3(CH_2)CH_3$; 3:1) to afford a dark orange solid **9**.

9: dark orange solid; yield: 4.5 mg (0.014 mmol, 59 %); FTIR (KBr) ν (cm^{-1}): 3442, 1634, 1524, 1467, 1383, 1278, 1083; ^1H NMR (CD_3OD): δ(ppm)= 8.42 (d, 1H, 7.5Hz, H13), 8.17 (d, 1H, 8.0Hz, H4), 7.90-7.78 (m, 3H, H10, H12, H1), 7.75 (t, 1H, 7.5Hz, H11), 7.63 (d, 1H, 8.5Hz, H8), 7.44 (t, 1H, 7.8Hz, H2), 7.33 (t, 1H, 7.8Hz, H3), 7.26 (d, 1H, 9.0Hz, H9); $^{13}C\{H\}$ NMR (CD_3OD-d_4) δ(ppm)= 135.3 (C13), 133.1 (C4), 132.7 (C10), 130.1 (C12), 129.3 (C1), 129.1 (C11), 128.2 (C8), 125.2 (C2), 122.4 (C3), 119.7 (C9); ESI-MS positive and negative mode: *m/z* 316 $[M+H]^+$; *m/z* 338 $[M+Na]^+$ and *m/z* 314 $[M-H]^-$; MS^2 (*m/z*, (relative intensity)): 298 (5%) $[(M+H)-H_2O]^+$, 288 (100%) $[(M+H)-CO]^+$, 246 (10%) $[(M+H)-C_3H_2O_3]^+$, 158 (10%) $[C_{10}H_8NO]^+$; MS^3 (*m/z*, (relative intensity)): 270 (100%) $[(M+H)-CO-H_2O]^+$, 260 (7.5%) $[(M+H)-CO-CO]^+$, 158 (10%) $[C_{10}H_8NO]^+$, 115 (8%) $[C_9H_7]^+$; HR-ESI/MS: *m/z* calcd. for $C_{20}H_{12}NO_3$ $[M-H]^-$: 314.0823; found 314.0817.

Conclusions

The efficiency of CotA-laccase to mediate coupling reactions was demonstrated using several *meta*,*para*-disubstituted phenylamines, *ortho*-phenylenediamines and substituted *ortho*-aminophenols as substrates leading to the synthesis of a diversity of phenazine and phenoxazinone frameworks. These were obtained in good to excellent yields and the identification of the products provided a basis for the proposal of a pathway for the laccase catalysed oxidation of substituted aromatic substrates. The laccase mediated oxidation of a given *ortho*-substituted amine or *meta*,*para*-disubstituted amine generate directly *ortho*-quinonediimine (or *ortho*-quinoneimine) or *para*-quinonediimine intermediates, respectively. These highly electrophilic species then reacts with a nucleophilic partner following the chemical rules governing the classical 1,4-conjugated Michael addition.

Overall these results show that the enzyme-catalysed oxidative sequence, a green chemistry process, constitutes a valuable alternative to the chemical oxidative coupling of aromatic amines and phenolic precursors providing a promising approach for the rational synthesis of different heterocyclic scaffolds.

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Table of contents:

The laccase-catalysed oxidative coupling of substituted aromatic amines is described, extending the scope of laccases towards the production of phenazine and phenoxazinone derivatives.