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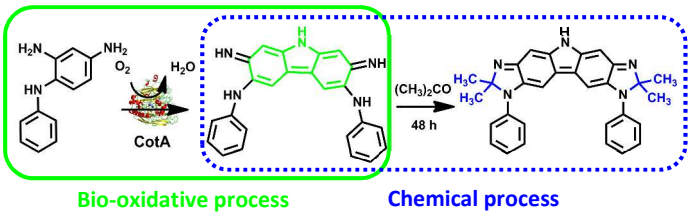

The bacterial CotA laccase mediates the oxidation of 2,4-diaminophenyldiamine leading to the formation of a benzocarbazole derivative.

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An enzymatic route to a benzocarbazole framework using bacterial CotA laccase

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The CotA laccase-catalysed oxidation of the *meta,para*-disubstituted arylamine 2,4-diaminophenyldiamine delivers, under mild reaction conditions, a benzocarbazole derivative (1) (74% yield), a key structural motif of a diverse range of applications. This work extends the scope of aromatic frameworks obtained using these enzymes and represents a new efficient and clean method to construct in one step C-C and C-N bonds.

Carbazole and its fused aromatic systems are well known to display important photophysical properties and a wide range of attractive biological activities[1,2] from antimicrobial, anticancer to anti-inflammatory properties.[3-5] Furthermore, carbazoles are important building blocks for the synthesis of functional materials such as luminescent polymers or photovoltaic devices.[6,7]

Considering that benzannulated and heteroannulated carbazole analogues are not found in abundance from natural sources and are important molecular structural motifs with a diverse range of applications, the development of new synthetic approaches for these heterocyclic ring systems is highly desired.

A plethora of synthetic methods were developed for the construction of carbazoles,[8] including direct oxidative cyclisation reactions starting from diarylamines,[9,10] using several chemical catalytic versions and alternative oxidisers.[11-15]

In spite of the variety of available methods, they all show common limitations, such as moderate yields and a limited scope for the transformations.

Although carbazole and other heterocyclic nitrogen-containing compounds can be transformed by cultures of bacteria and fungi to produce a variety of new derivatives, many of them useful for the synthesis of new candidate drugs,[16,17] not much is known about enzyme-catalysed oxidations leading to the formation of carbazole derived products.

Enzyme-catalysed reactions are of increasing importance in organic synthesis as they allow a multitude of selective and efficient reactions in aqueous systems at room temperature. Laccases (benzenediol:oxygen oxidoredutase, EC 1.10.3.2), a group of multi-copper oxidases ubiquitous in nature, show versatile biochemical properties and are known to catalyse the oxidation of a wide range of structurally diverse aromatic compounds under mild conditions.[18,19] These enzymes frequently exhibit high oxidative selectivity in aqueous solution reactions providing a unique green chemistry alternative for organic synthesis.[19,20]

It is well established that these "ideally green" enzymes work using aerial oxygen and produce water as the only by-product in a catalytic cycle during which four substrate molecules are oxidised. The oxidation of substrates leads to free-radical intermediates, which are known to participate in a variety of nonenzymatic reactions.[18-21]

We have previously reported on the enzymatic oxidation of *para*-substituted amines, using the bacterial CotA laccase from *Bacillus subtilis*, to produce coupling trimers[19a] and of *meta,para*-disubstituted amines leading to different frameworks, including phenazines and phenoxazinones[19b].

Herein, we report the biotransformation of a *meta,para*-disubstituted arylamine 2,4–diaminodiphenylamine (2,4-DADPA) (**figure 1**) using the CotA-laccase, leading to a benzocarbazole derivative through an intramolecular oxidative coupling step, in a reaction that extends the scope of known aromatic frameworks which can be obtained using laccases.

The efficiency of the oxidation of substrates by a laccase depends on several factors, such as the difference between the redox potentials of the laccase and of the substrate and the pH of the reaction medium; this affects not only the catalytic activity of laccase, but

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also the chemical properties of the substrates, including their redox potential. Therefore, the electrochemical behaviour of the target substrate 2,4-DADPA was studied by cyclic voltammetry with a platinum disk working electrode in Britton-Robinson or phosphate buffers in the pH range 4-7. 2,4-DADPA generates well-defined irreversible oxidation peaks (E_{pa}) in the range 0.42 – 0.35V at a scan rate of 50 mV/s for all the pH tested (4-7). The absence of reductive peaks (E_{pc}) indicates that the oxidation process probably generate a quinone-diimine species which is highly reactive and undergo further chemical reactions.

The comparison of these results with the related 4-aminodiphenylamine (4-ADA)[19a] in similar experimental conditions, showed that 2,4-DADPA has a lower oxidation potential and a less reversible redox process and consequently is more prone to be oxidised by the enzyme. Increasing the pH from 4 to 7 resulted in an almost linear decrease of the oxidation potential, consistent with our previous results with mono and disubstituted aromatic amines.[17] The oxidation potential of 2,4-DADPA (0.35 – 0.42V) is lower than the redox potential for CotA[22] (0.55 V vs NHE), which means the absence of redox constraints for the reaction to occur. The pH profile of CotA for 2,4-DADPA was determined and a bell shaped curve was obtained (data not shown) with a maximal activity at pH 6.

The kinetic parameters of CotA laccase for 2,4-DADPA were determined at pH 6. The calculated K_m and k_{cat} values are 0.8 ± 0.2 mM and 1.3 ± 0.1 s^{-1}, respectively. These values show that CotA presents a significantly decreased catalytic specificity (k_{cat}/K_m) towards 2,4-DADPA oxidation by one order of magnitude (1.5 ± 0.1 $mM^{-1}.s^{-1}$) when compared with its specificity to oxidise the related substrate 4-ADA.[19a]

After the optimisation of the reaction conditions, the enzymatic synthesis was carried out using 5 mM of 2,4-DADPA in the presence of 1 $U.mL^{-1}$ of CotA laccase at 37ºC, in an aqueous buffered solution at pH=6 and under atmospheric conditions for 24h. The reaction was monitored by TLC and a dark purple solid (**1**) was obtained with 74% isolated yield and was fully characterised by IR, NMR (^1H, ^{13}C, COSY, HSQC and HMBC) spectroscopies and Mass Spectrometry. In acetone, the biotransformation product **1** was totally transformed in compound **2** (**Figure 1**).

The enzymatic oxidation of 2,4-DADPA led to the formation of a carbazole based skeleton (**1**), which was a totally unexpected result since laccase oxidation of the related compounds 4-aminodiphenylamine-2-sulfonic acid (4-ADASA), 2,5-diaminobenzene sulphonic acid (2,5-DABSA) and 2,5-diaminophenol (2,5-DAP) result in the synthesis of phenazines or phenoxazinone frameworks respectively.[19b] Apparently the substitution of the *meta* electron-withdrawing sulfonic group by an amino group leads to the formation of a carbazole derivative, which highlights the potential use of CotA-laccase for the preparation of compounds based on structural variations of the carbazole scaffold.

The structural identification of the carbazole heterocylic core (for compound **1**) was based on differences observed in the chemical shifts of the aromatic protons. A downfield shielding was detected for all the proton signals of this heterocycle relative to their respective parent compound. The disappearance of the doublets for H_3 and H_4 and the singlet for H_6 present in the substrate and the presence of resonances at 7.51 and 6.81 ppm, as two different singlets attributed to protons $H_{1,8}$ and $H_{4,5}$ respectively, in compound **1** confirmed the substitution positions and also the symmetry of the carbazole central core. All the other aromatic protons were identified by resonances in the aromatic region δ 7.07-6.61 ppm. The amine and imine protons were also identified by three broad singlets at δ = 9.56, 6.42 and 4.44 ppm. In the ^{13}C spectrum, resonances at high field regions, namely, 118.5 and 96.7 ppm, attributed to $C_{1,8}$ and $C_{4,5}$ respectively and a signal at low-field (δ = 143.7 ppm), characteristic of imine quaternary carbons ($C_{2,7}$), confirmed the presence of the quinone-imine positions. The assignment of the structure was further established by the ^1H-^{13}C HMBC and HSQC correlations. The proposed structure was corroborated by the full ESI-mass spectrum in the positive mode that exhibited a major peak at *m/z* 378 ($[M+H]^+$). Fragmentation of the precursor ion *m/z* 378 generated a predominant product ion with *m/z* 286 attributed to $[M+H-(C_6H_6N^{\bullet})]^{+\bullet}$ in agreement with the loss of one arylamine radical unit.

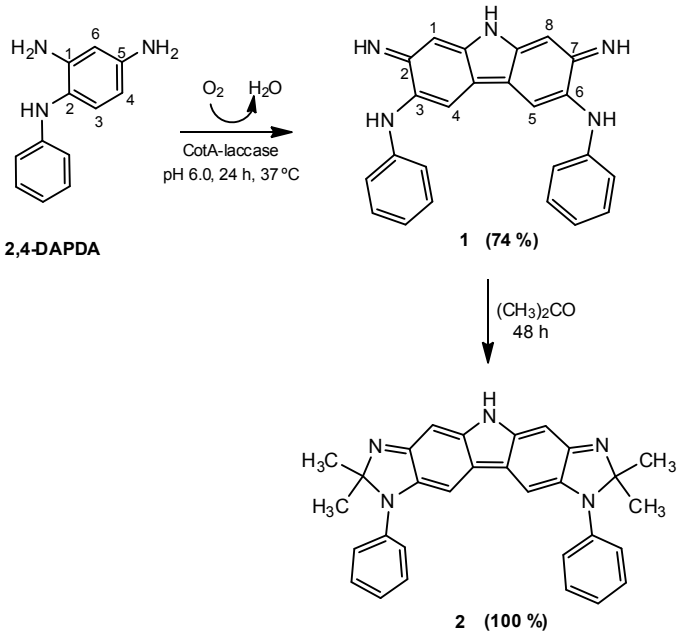


Figure 1 – Structures and yields (%) of the product formed in the laccase-catalysed reaction with 2,4-DADPA (**1**) and its reaction product with acetone (**2**).

Upon dissolution of compound **1** in acetone, a colour modification was observed from the original dark purple to green. This change was followed by ^1H NMR (Figure **2**), being both compounds visible after 24h and a complete conversion from **1** to product **2** observed at 48h. The formation of the disubstituted imizadole rings involving the acetone and the imino and secondary amino groups induce a considerable shielding effect on both $H_{1,8}$ and $H_{4,5}$ protons while the other aromatic protons are deshielded. The ^{13}C shift signals follow the same trend and the 2D-NMR experiments confirm the structure.

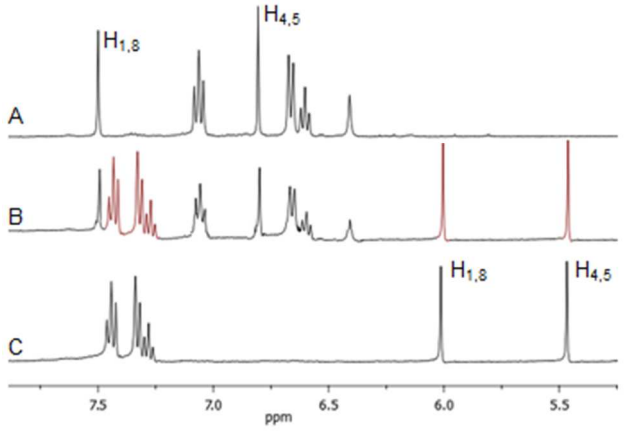


Figure 2 - Evolution in time of ^1H-NMR spectra in acetone-d^6; A: Initial time; B: after 24h; C: after 48h

Furthermore, the structural assignment of compound **2** was additionally supported by single X-ray diffraction analysis[23] (**figure 3**). Crystals were obtained by slow evaporation of a cold saturated acetone solution. The most noticeable feature of this molecule is the fragment of five heterocyclic rings that form a totally planar sequence where the highest deviation from the planar conformation is shown by atom C1 (mean plane deviation of 0.0371 Å). The two phenyl rings bonded to N2 and N5, have different orientations towards the least-square plane of the main structure of the molecule formed by the carbazole fragment. Wherein C21-C26 ring has almost an orthogonal orientation (dihedral angle is 81.2º), the plane formed by C51-C56 phenyl ring is slightly twisted from the plane of main structure with a dihedral angle of 45.3º.

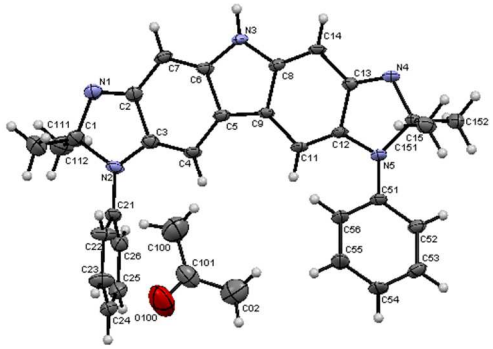


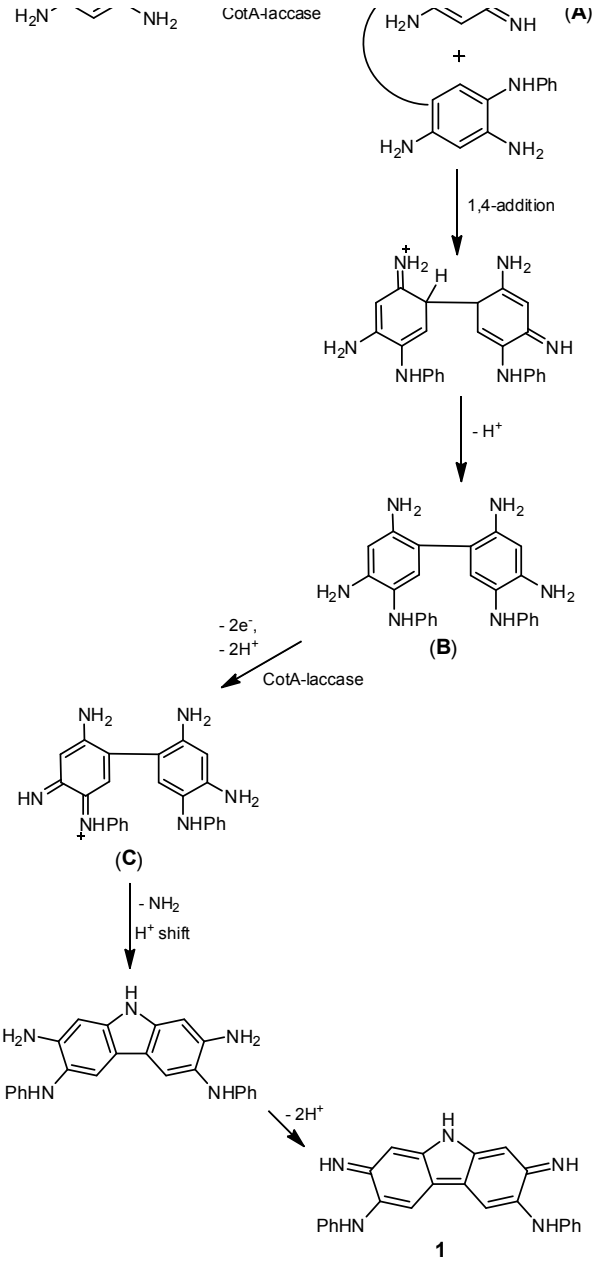
Figure 3 – Molecular diagram (Mercury[24]) showing crystallographic labeling scheme for compound **2**. Thermal ellipsoids are drawn at the 50% probability level.

Scheme 1 shows the proposed pathway for the formation of the carbazole based framework considering the enzymatically produced active species and the previous literature reports,[19b,25,26] The first step is the in-situ generation of the *ortho*-benzoquinonediimine intermediate (**A**) catalysed by laccase and following a similar pathway as previously described for *ortho*- or *para*-substituted amines and *meta,para*-disubstituted aryl amines.[19] This intermediate further reacts with nucleophilic agents such as the aromatic amine present in solution via 1,4-addition reaction. The nucleophilic attack is carried out at the C4 position activated by the presence of the two amino groups in the *ortho* and *para* positions, leading to the formation of a new C-C bond and a homomolecular dimeric structure. This second step, followed by a proton loss, yields the first coupling intermediate (**B**). This non-isolable product underwent a subsequent oxidation probably mediated by laccase to give (**C**), which further undergo intramolecular 1,4-addition reaction to form the benzocarbazole derivative **1**. The target product was obtained by the displacement of an amino group of one of the two substrate molecules in accordance with results reported before.[19b,27]

Conclusions

In summary, CotA-laccase was used to catalyse the oxidative coupling of a *meta,para*-disubstituted arylamine, 2,4-DADPA, leading to the synthesis of an unexpected product, a symmetric benzocarbazole derivative, considering the previous observed formation of phenazine and/or phenoxazinone frameworks from related *meta,para*-disubstituted arylamines, using the same enzyme. The dissolution of this enzymatic product in acetone led to the formation of a new compound where two new disubstituted imidazole rings are formed due to a condensation reaction between the benzocarbazole and acetone.

A pathway for the biotransformation of 2,4-DADPA is proposed. Overall our results show that the enzyme-catalysed oxidative process forms the basis of a simple and environmentally benign method for the synthesis of carbazole derivatives, extending simultaneously the scope of different heterocyclic scaffolds obtained through the use of these enzymes.



Scheme 1 – Proposed oxidative pathway for the carbazole synthesis assisted by laccases

Experimental section

CotA laccase assay

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.[28,29] The oxidation of ABTS was followed by an absorbance increase at 420 nm (ε= 3.6 x10^4 M^{-1}.cm^{-1}). The protein concentration was measured using the Bradford assay[30] and bovine serum albumin as a standard.

Biotransformation of 2,4-DADPA

A solution of 10.0 mg (0.05 mmol) of 2,4-DADPA in methanol (1 ml) was added to a stirred solution (9 ml) of phosphate buffer (100 mM, pH 6). The reaction was started by adding a preparation of laccase (1U.mL^{-1}) and was incubated at 37 ºC, for 24h in aerobic conditions. The conversion was followed by thin layer chromatography (TLC) on aluminium sheet Silicagel 60 F254 (Merck). After 24 h, the insoluble dark purple product was separated by filtration, washed with diethyl ether and dried under vacuum to afford **1** as a pure compound.

Compound **1**: dark purple solid. Isolated yield: 7.0 mg (0.019 mmol, 74%); FTIR (KBr) $\nu_{máx}$/cm^{-1}: 3347,1631, 1598, 1495, 1448, 1308, 1240, 1155, 1073, 853, 735, 695, cm^{-1} ; ^{1}H NMR (acetone-d^6) δ/ppm= 9.56 (bs, NH), 7.51 (s, 2H, H1,8), 7.07 (t, 4H, 7.8Hz, H16,18, H16',18'), 6.81 (s, 2H, H4,5) 6.67 (d, 4H, 8.0Hz, H15,19, H15',19'), 6.61 (t, 2H, 8.0Hz, H17,17'), 6.42 (bs, NH), 4.44 (bs, NH). ^{13}C{H} NMR (acetone-d^6) δ/ppm= 149.34 (C14,14'), 143.68 (C2,7), 140.91 (C10,13), 129.68 (C16,18 and C16',18'), 121.90 (C3,6), 118.47 (C1,8), 118.07 (C17,17'), 116.61 (C11,12), 114.58 (C15,19 and C15',19'), 96.68 (C4,5); UV/Vis: 460 nm, ε=6321 M^{-1}.cm^{-1} (MeOH:B&R pH 6); ESI-MS positive mode: *m/z* = 378.1 $[M+H]^+$; MS^2 *m/z* (relative intensity): 286.2 (27%) $[M+H-(C_6H_6N^{\bullet})]^{+\bullet}$. HR- ESI/MS: *m/z* calcd. for $C_{24}H_{20}N_5$ $[M+H]^+$: 378.1716; found: 378.1707.

Compound **2**: green solid, 100% of conversion from (**1**) after 48h. ^{1}H NMR (acetone-d^6) δ/ppm= 7.44 (t, 4H, 7.6Hz, H16,18 and H16',18'), 7.33 (d, 4H, 7.6Hz, H15,19 and H15',19'), 7.28 (t, 2H, 7.8Hz, H17,17'), 6.01 (s, 2H, H1,8), 5.47 (s, 2H, H4,5), 2.09 (s, 6H, CH_3), 2.07 (s, 6H, CH_3); ^{13}C{H} NMR (acetone-d^6) δ/ppm= 161.53 (C2,7), 153.32 (C10,13), 144.64 (C3,6), 140.91 (C14,14'), 130.53 (C16,18 and C16',18'), 127.95 (C15,19 and C15',19'), 127.38 (C17,17'), 125.68 (C11,12), 93.99 (C1,8), 86.62 (C4,5).

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