Supporting Information

Insights into the Homocoupling Reaction of 4-Methylamino Benzoic Acid Mediated by *Trametes Versicolor* Laccase

Andrea Martorana, Caterina Bernini, Daniela Valensin, Adalgisa Sinicropi, Rebecca Pogni, Riccardo Basosi, Maria Camilla Baratto

Table of Contents:

1) Experimental Section

Continuous-wave (CW) X-band ESR measurements were carried out on a Bruker E500 ELEXSYS Series using the Bruker ER 4122 SHQE cavity at 298° K. The experimental conditions were: 9.45GHz microwave frequency, 0.2mT modulation amplitude, 2mW microwave power, 100KHz modulation frequency and 80dB gain.

UV-Vis spectra were recorded on a Hewlett Packard 8453 spectrometer using a quartz cuvette of 0.5 mL. The spectra were performed maintaining the same molar ratio used for EPR spectroscopy with a final concentration of 1 μM laccase and 0.15 mM precursor.

NMR spectra were performed either at 14.1 T with a Bruker Avance 600 MHz spectrometer at controlled temperatures (± 0.2 K) using a SEI (SElective Inverse) probe. Proton and carbon resonance assignment was obtained by means of standard homonuclear and eteronuclear 2D experiments such as ^{1}H-^{1}H COSY, ^{1}H-^{1}H NOESY, ^{1}H-^{13}C HSQC and ^{1}H-^{13}C HMBC. Spectra processing was performed on a Silicon Graphics O2 workstation using the XWINNMR 3.6 software.

MS spectra were performed on LC/MSD chromatography system, Agilent 110 series, equipped with UV detector. Analysis conditions: 95% of MeOH and 5% of H_2O, 0.4 mL/min of flow rate, ESI ionization, 9 L/min of nitrogen flow, temperature 350°C, atomization pressure 40 psi.

IR spectrum was recorded on a Perkin-Elmer FT-IR 1725X spectrophotometer in a OTTL cell.

Laccase form *Trametes Versicolor* (26.0 U/mg protein), and 4-methylamino benzoic acid (97%) were obtained from Sigma and used without further purification.
An aqueous solution 15mM of 4-methylamino benzoic acid (0.0747 mmol in 5 mL) was added to an acetate buffer solution (1.0M at pH 4.5) 100 μM of laccase from *Trametes Versicolor* (0.1 μmol in 5 mL). The mixture becomes purple immediately and was incubated overnight at room temperature. To completely remove the presence of laccase from the product, the solution was centrifuged at 4000 rpm with the Centricon Plus-20 centrifugal filter (NMWL 10,000 Dal) (Amicon, Millipore) at 25° C for 20 minutes. The eluate was recovered and dried in vacuum.
For the immobilization of laccase 100 mg of Eupergit C and 20 UI of laccase in 1 ml of acetate binding buffer (0.1 M pH 4.5) were used. The reaction mixture was vortexed for 2 minutes and rocked for 24 h at room temperature. At the end of coupling period, the mixture was filtered and washed several times at first with water and then with 1.0 M NaCl until no activity was detected in the washings. Finally the solid was resuspended in binding buffer and gently packed in a column. The precursor solution was introduced in the column, passed through solid phase and eluted drop by drop. During coupling reaction the colour of Eupergit C changes from white to purple and the product was collected.

The product was analysed by $^{1}H/^{13}C$ NMR without further purification. δ_H (600 MHz, $CDCl_3$, Me_4Si) 3.71 (t, $J_{1,3}$ 5 Hz, 2H, CH_2); δ 3.66 (s, 2H, CH_2); δ 3.59 (t, $J_{1,3}$ 5Hz, 2H, CH_2); δ 3.33 (s, 1H). ^{13}C NMR (150 MHz, $CDCl_3$) 72.7; 70.3; 61.6. ES/MS *m/z*: 481, 437 (44), 393 (44), 349 (44), 305 (44), 261 (44), 217 (44). FT-IR (chloroform) ν_{max}/cm^{-1} 3446, 2929, 1119, 1065.

2) UV-Vis Spectra

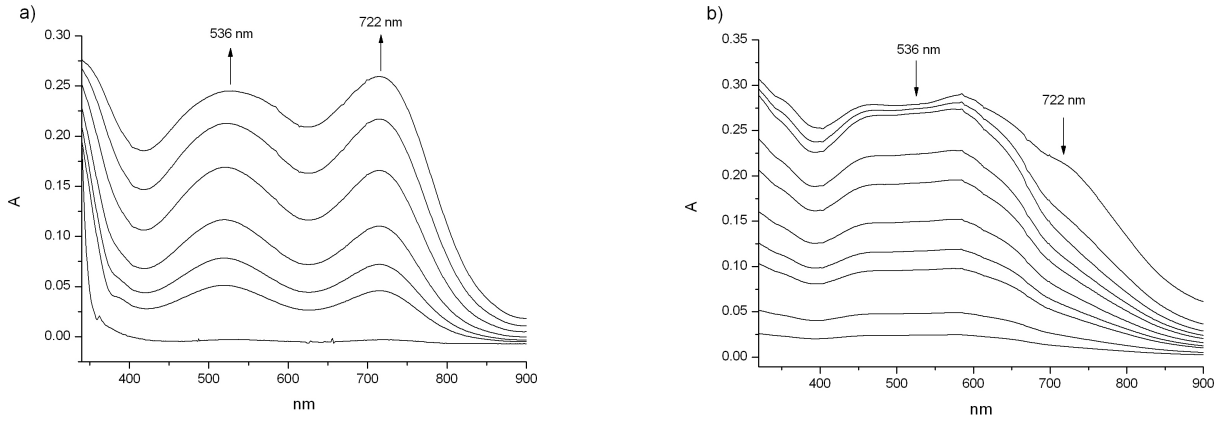


Figure S1. Kinetic trend of laccase-precursor system: a) detection in the first 30 minutes of reaction, two bands at 722 and 536 nm appear corresponding to cation radical dimer **2**·+ a and neutral radical dimer **2**· respectively; b) detection from 30 minutes to 24 hours, the band at 722 nm disappears quickly after 30 minutes while the band at 536 nm decreases slowly to zero within 24 h.

3) Computations

Table S1. TD-DFT computed absorption maxima λ_{max} (nm), excitation energies E_{exc} (eV) and oscillator strengths (f_{osc}) for **2**·+ and **2**· species:

	2·+			**2**·		
	λ_{max}	E_{exc}(eV)	f_{osc}	λ_{max}	E_{exc}(eV)	f_{osc}
Experimental	722	1.72		536	2.32	
B3LYP/6-31G*	679.41	1.82	0.38	569.59	2.18	0.19
B3LYP/6-31+G*	682.97	1.82	0.39	582.55	2.13	0.21

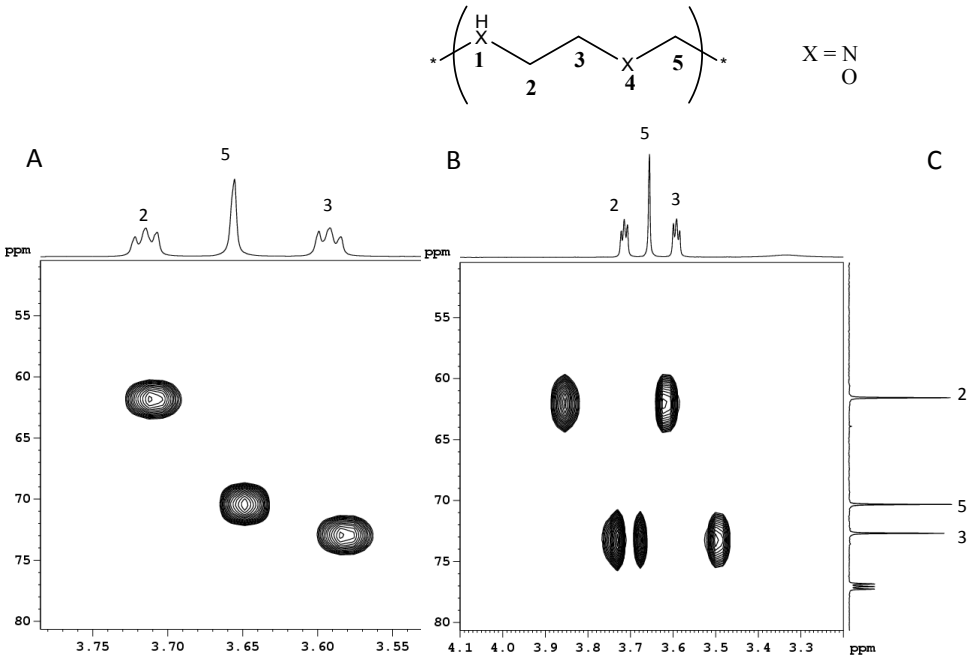
The difference in energy between computed and experimental date is in the range of 0.1-0.2 eV which is well within the accepted accuracy of TDDFT excitation energies calculations for medium to large size organic molecules (see for example, Richard R. M. et al., J. Chem. Theory Comput. 2011, 7, 1296–1306 and De Angelis F. et al., Nanotechnology, 2008, 19, 424002).

Table S2. B3LYP/EPR-II computed Mulliken π-spin densities for **2**·+ and **2**· species:

Molecular position	**π-spin densities**	
	2·+	**2**·
N7	0.32	0.22
C1	0.13	0.07
C2	-0.06	-0.03
C3	0.11	0.07
C4	-0.05	-0.04
C5	0.10	0.07
C6	-0.06	-0.03
N7’	0.31	0.47
C1’	0.12	0.12
C2’	-0.05	-0.05
C3’	0.10	0.12
C4’	-0.05	-0.09
C5’	0.10	0.13
C6’	-0.05	-0.06

4) NMR assignments

Both ^{1}H and ^{13}C NMR spectra showed that the obtained product had a purity higher than 90%. The combined use of 1D and 2D NMR spectroscopy, together with MS experiments, allowed to assign the structure reported below to the product obtained from the reaction between Laccase and 4-methylamino benzoic acid. In particular the ^{1}H-^{13}C HSQC and HMBC spectra (Figures S2A and S2B respectively) allowed to obtain the chemical shift assignment reported in Figure S2C.



	^{1}H (ppm)	^{13}C (ppm)
-XH (1)	3.33	
$-CH_2-$ (2)	3.71	61.6
$-CH_2-$ (3)	3.59	72.7
-X- (4)		
$-CH_2-$ (5)	3.66	70.3

Figure S2. Selected regions of ^{1}H-^{13}C HSQC (A) and ^{1}H-^{13}C HMBC (B) spectra and chemical shift assignment (C) of the product obtained from the reaction between laccase and 4-methylamino benzoic acid.