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On the mechanism of biotransformation of the anthraquinonic dye Acid Blue 62 by laccases

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Abstract. We used the recombinant CotA-laccase from the bacterium *Bacillus subtilis* to investigate the biotransformation of the commercial anthraquinonic dye Acid Blue 62. Kinetics of dye biotransformation at pH 6 follows a Michaelis-Menten model. NMR and several MS techniques allowed the identification of intermediates and final products of the enzymatic biotransformation. The main final product obtained, 1-[(4-Amino-9,10-dioxo-3-sulfo-9,10-dihydroanthracen-1-yl)diazenyl]-4-cyclohexylamino-9,10-dioxo-9,10-dihydroanthracene-2-sulfonic acid, is formed through the creation of an azo link and has been previously identified as an intermediate compound in the biodegradation of Acid Blue 62 by crude fungal preparations. The identification of 1,4-diamino-9,10-dioxo-3-sulfo-9,10-dihydroanthracene-2-sulfonic acid and of cyclohexanone, in reaction mixtures with CotA-laccase and also present in reactions performed with the LAC3 laccase from the fungus *Trametes* sp. C30, suggest the occurrence of coupling reactions between the intermediate products of dye oxidation. Based on these results, we propose a mechanistic pathway for biotransformation of Acid Blue 62 by laccases. A bioassay based on the inhibitory effects of the dye and its enzymatic products on the growth of *Saccharomyces cerevisiae* shows the importance of laccases in reducing dye toxicity.

Keywords: anthraquinonic dyes, azo dye synthesis, biotransformation, biocatalysis, laccase, radical coupling

Introduction

Laccases are oxidoreductases belonging to the multicopper oxidase family of enzymes.[1] Their catalytic centres consist of three structurally and functionally distinct copper sites; T1 copper ("blue copper") is a mononuclear centre involved in the substrate oxidation, whereas T2 and T3 form a trinuclear centre involved in the dioxygen reduction to water.[2] These enzymes are useful biocatalysts for diverse biotechnological applications, owing to their relatively non-specific oxidation capacity, the lack of requirement for co-factors and the use of readily available oxygen as an electron acceptor.[3] Laccases also have uses in organic synthesis, where their typical substrates are phenols or amines, and the reaction products are dimers and oligomers derived from the coupling of reactive radical intermediates.[4] Recently, we have shown that CotA-laccase from the bacterium *Bacillus subtilis* is able to decolourise a variety of azo and anthraquinonic dyes at alkaline pH and in the absence of redox mediators.[5] We have additionally reported on the synthesis of 2,2-Bis(3'-indolyl)indoxyl, a yellow compound obtained from trimerisation of indole using the LAC3 laccase from the fungus *Trametes sp.* C30.[6]

Around 10^6 tons of synthetic dyes are produced annually, of which $1–1.5x10^5$ tons are released to the environment in wastewaters.[7] Dyes are in general stable organic pollutants, persisting in the environment, and concerns have been raised that such artificial compounds may be xenobiotic.[8] Therefore, the development of methods for their degradation have been increasingly explored.[7] Antraquinonic dyes are used either as primary or secondary dyes in commercial trichromatic dyeing formulations.[9] However limited information exists on their physicochemical or biological degradation[10-12] and even less is known on the molecular mechanism of transformation.[13,14] Nevertheless this knowledge is important, not only for the development of efficient bioremediation processes, but also for the search for

harmless dyeing compounds to be synthesized by green chemistry processes.

In this study, we report on the enzymatic biotransformation of the anthraquinonic dye Acid Blue 62 (AB62, compound **1**) into 1-[(4-amino-9,10-dioxo-3-sulfo-9,10-dihydroanthracene-1-yl)diazenyl]-4-cyclohexyl-amino-9,10-dioxo-9,10-dihydroanthracene-2-sulfonic acid) (compound **4** [14]). The enzymatic reactions were followed by UV-vis and HPLC. Based on the characterization of intermediates and final products of reaction, a possible mechanistic pathway for AB62 biotransformation through the formation of an azo link is discussed. Moreover, the toxicity of AB62 and its biotransformation products were evaluated confirming the environmental friendly character of laccase mediated processes.

Results and Discussion

Monitoring AB62 biotransformation by UV-vis and HPLC

Transformation of AB62 by CotA-laccase resulted in a decrease in the intensity of the dye absorption bands, at λ_{max} = 600 and 630 nm, along with an increase in absorption around 500 nm due to the formation of reddish biotransformation products (data not shown). The time course of AB62 biotransformation was additionally monitored by HPLC (Figure 1). In the first minutes of reaction, a peak with a R_t of 13 min (formed at a rate of 1.7 ± 0.2 μmol.min^{-1}.mg^{-1}, Figure 1 insert) was detected in chromatograms. However, as the reaction proceed, this peak decreased (at a rate of 0.5 ± 0.1 μmol.min^{-1}.mg^{-1}) concomitant with the appearance of a major peak at 50 min (at a rate of 0.9 ± 0.3 μmol.min^{-1}.mg^{-1}). These observations are consistent with the initial formation of an intermediate product (compound **2**, DAAS, see Scheme 1) before its consumption in further reactions. A more detailed kinetic analysis was performed and as can be seen in Figure 2, we observed an increase in the dye consumption rates with increasing dye concentration for values up to 1 mM, and above this, the rates become independent of the dye concentration. Therefore, the enzyme steady state kinetic constants for AB62 were calculated according to the Michaelis-Menten model: V_{max} of 1.3 ± 0.9 μmol.min^{-1}.mg^{-1}, k_{cat} of 1.4 ± 0.6 s^{-1} and K_m of 296 ± 49 μM. The catalytic efficiency (k_{cat}/K_m) of CotA laccase towards AB62 oxidation (4.7 x 10^3 s^{-1}M^{-1}) is 2-3 orders of magnitude lower than that of fungal laccases.[11] Nevertheless, the lower catalytic efficiency towards AB62 oxidation proved to be advantageous for the detection and purification of the intermediate **2** (DAAS) in reactions with this enzyme.

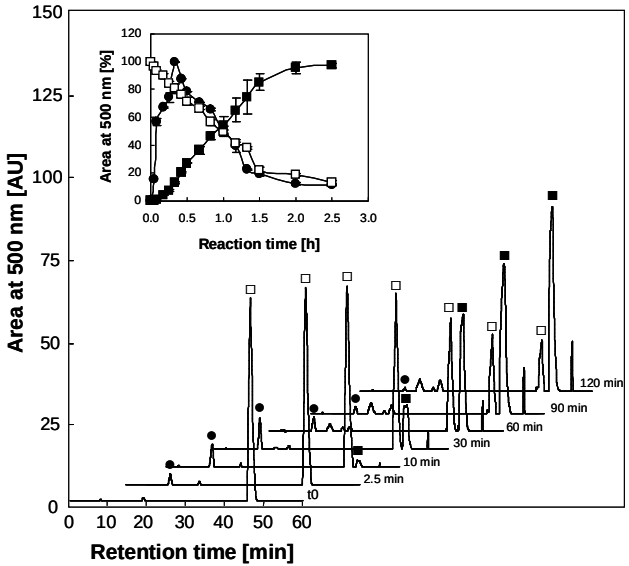


Figure 1. Time course for AB62 biotransformation by CotA-laccase as monitored by HPLC. **Insert** HPLC peak areas at 500 nm versus time of the reaction**.** [□, AB62 and products: ●, R_t 13 min and, ■R_t 50 min].

Identification of AB62 biotransformation products

The biotransformation products of AB62 were identified in the enzymatic reaction mixtures and after purification using NMR, MS/MS^n, LC-MS and GC-MS analysis. The ^{1}H NMR spectrum of purified intermediate product with an R_t of 13 min is shown in Figure 3A (data summarised in the experimental section). In the chemical shift region of the aromatic protons, the ^{1}H NMR spectrum of compound **2** shows a double triplet and a double doublet (two protons each) centered at 7.75 and 8.30 ppm, typical for the ring A protons (H6, H7 and H5, H8 respectively) of

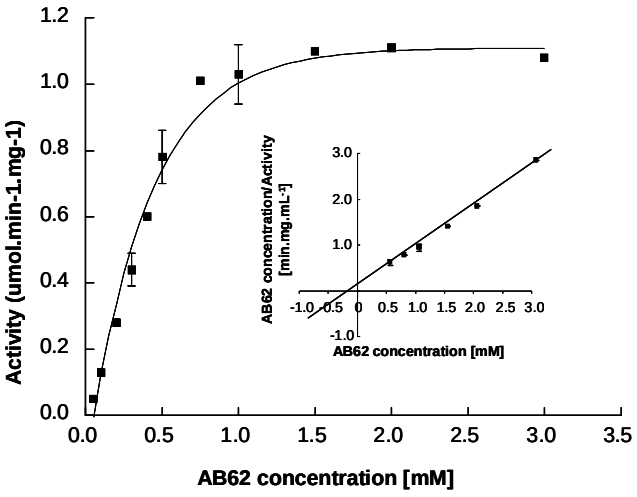


Figure 2. Non-hyperbolic saturation kinetics of CotA-laccase with AB62. **Insert:** Hannes plot.

an anthraquinone skeleton. Moreover, the singlet at 7.80 ppm, which corresponds to one proton, could be assigned to the ring C proton H3 and proves that the ring C is tri-substituted in positions 1, 2 and 4. No further signals are observed in the aliphatic region

revealing the absence of the cyclohexyl group initially present in the structure of AB62. The mass spectrum of this compound exhibit peaks with *m/z* 317 and 319 in negative and positive ionization modes, respectively (Figure 3B), leading to a molecular mass of 318. Fragmentation of the peak with a *m/z* of 319 produced two ions with *m/z* of 237 and 255 that could be formed through the loss of SO_3H (81 amu) and SO_2 (64 amu) groups. Therefore, this compound, that results most likely from the loss of the cyclohexyl moiety of **1** through enzymatic oxidation, can be identified as 1,4-diamino-9,10-dioxo-3-sulfo-9,10-dihydroanthracene-2-sulfonic acid (DAAS, **2**). This compound is also an intermediate in the photocatalytic degradation of Acid Blue 25, a structurally similar anthraquinonic dye.[16]

The ^{1}H NMR spectrum obtained for the purified product with *Rt* of 50 min contains signals in two different regions (data summarised in the experimental section). The chemical shifts found in the 1-4 ppm region are within the expected shift range for the cyclohexyl moiety, whereas the chemical shifts in the low-field aromatic region (7-9 ppm) are consistent with the presence of two slightly different anthraquinonic skeletons. The ^{13}C NMR spectrum shows signals in agreement with the ^{1}H NMR data. The mass spectrum shows peaks at *m/z* 713 and 715 in negative and positive mode, respectively. Fragmentation of the negative ion generated the major ions with *m/z* of 605, 384 and 302, and minor ions with *m/z* 633, 525, 317. The formation of ions with *m/z* of 384 and 302 can be explained by cleavage of the carbon-nitrogen bond adjacent to the central azo linkage. Minor ions result from the loss of the cyclohexyl and SO_3H (*m/z* 633, 525) or from the cleavage of the azo bond (*m/z* 317).[15] From these results, we concluded that the compound is 1-[(4-amino-9,10-dioxo-3-sulfo-9,10-ihydroanthracene-1-yl)diazenyl]-4-cyclohexyl-amino-9,10-dioxo-9,10-dihydroanthracene-2-sulfonic acid) (**4**) previously identified by Vanhulle et al.[14]

LC-MS analysis of the total reaction mixtures after 2h revealed the presence of 9 compounds: DAAS (**2**), compound **4** and compounds with *m/z* of 248, 576, 604, 622, 633, 715, 734 and 797 (Table 1). The structures for those are proposed (except for molecular ion *m/z* 622), based in the molecular ion and mass spectrometric fragmentation ions (compounds **5-10**, Scheme 1). The mass ion *m/z* 633 (**5**) should result from the coupling of two oxidised DAAS molecules (**2A**) through formation of an azo bond. Indeed, the dimer with *m/z* of 633 was identified by LC-MS as the main product of CotA reaction using compound **2** (1 mM) as substrate (at a slow rate of 0.19 ± 0.02 $\mu mol.min^{-1}.mg^{-1}$). Compounds **8** and **9** should result from further degradation of this dimer as can be seen on Scheme1. The ion *m/z* 797 (compound **6**), results from the coupling of two molecules of **1** and the ion at *m/z* 734

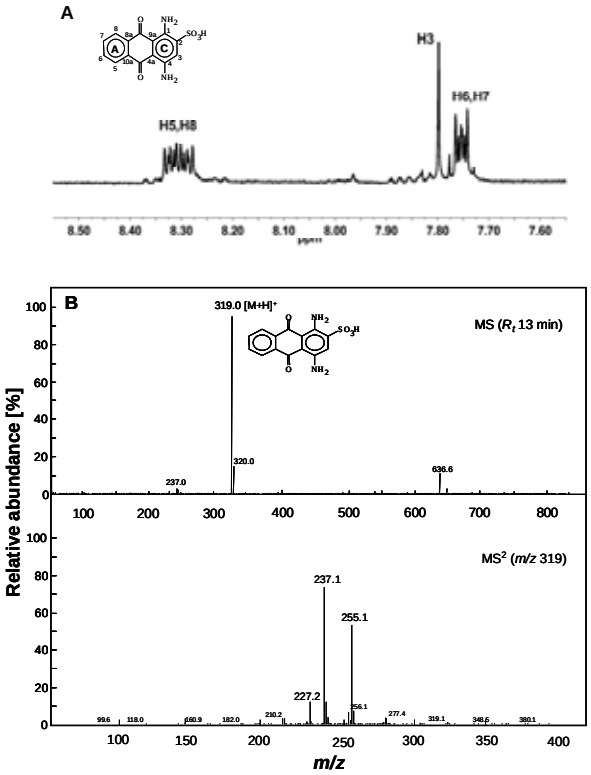


Figure 3. ^{1}H NMR (**A**) and MS/MS^n (**B**) spectra of the purified 1,4-Diamino-9,10-dioxo-3-sulfo-9,10-dihydroanthracene-2-sulfonic acid (**2**).

(compound **7**), corresponds to the sodium adduct of **4** $[m/z\ 715 + Na]^+$. Incubation of **4** (M = 714), the main product of the reaction with CotA-laccase in different assay conditions did not result in any transformation of the compound in contrast to that observed in the study of Vanhulle et al.[14]

We simultaneously analysed the products of AB62 biotransformation by using LAC3 laccase from the fungus *Trametes sp.* C30. This enzyme presents higher rates of oxidation as compared CotA-laccase.[17,18] Etheral extracts of reaction mixtures analysed by TLC upon appropriate staining, revealed the presence of a single compound of medium polarity. A comparison with standards led to the conclusion that cyclohexanone (**3**) could correspond to this compound, a proposition which was later confirmed by GC-MS analysis. In the course of the LAC3 catalysed dimerisation process of AB62, products **3** and **4** accumulates approximately in equal amounts (comparable yields) and at about the same rate(comparable kinetics). The LC-MS analysis of mixtures after 10 min of AB62 reaction by LAC3 revealed the presence of 4 compounds with *m/z* of 319, 633, 702 and 715 in positive mode. The ions *m/z* of 319, 633 and 715 could be assigned to structures **2**, **5** and **4** respectively, in accordance with

Table 1. LC-MS^n data for AB62 biotransformation products by CotA-laccase after 2h of reaction.

m/z	MS^n product ions

R_t (min)	(+)	(-)	MS^2	MS^3
13	319	317	237, 255	-
16	633	631	317, 441, 469, 523, 551	-
19	248		148, 230	-
24	576	574	414, 496	-
26	734	732	306, 388, 416, 653	-
28	604	602	522, 540	-
45	401	399	319	237, 255
50	715	713	302, 317, 384, 525, 605, 633	-
54	622		603	406, 469, 491, 521, 585
	797	795	335, 399, 715, 779	

the results obtained for the biotransformation with CotA-laccase. The ion *m/z* of 702 could result from loss of a NH_2 group in the hydrazine intermediate, which results in turn, from the coupling of two radicals **2A**, just before the final oxidation step leading to the final product **4** (see Scheme 2).

Proposed mechanism of AB62 biotransformation by laccases

Based on the intermediates and products identified with the two different laccases, we propose a mechanistic pathway for the biotransformation of AB62 (Scheme 2). Two routes are possible as the enzyme can catalyse a single-electron oxidation, either from the primary or the secondary amines of compound **1**, forming the reactive species **1A** and **1B**, respectively. Radical **1B** can be sequentially oxidized into an imine, which would hydrolyse through the breakdown of the N-C bond and the loss of cyclohexanone (**3**), followed by a proton abstraction. This produces compound **2** (DAAS), the first intermediate in this pathway. This route is supported by the identification of intermediate (**2**) and product (**3**) over the course of enzymatic AB62 transformations. Subsequently, intermediate **2** could be further oxidized and the resulting radical form (**2A**) should lead to the main product of the biotransformation process, the azo dimer (**4**). This dimer should result from the radical coupling of **2A** with one molecule of oxidized dye (compound **1A**), leading to the formation of an hydrazine, followed by an oxidative step that could also be catalysed by laccases. To a very low extent, the dimerisation of single radicals **2A** and **1A** should end in the formation of compounds **5** and **6** by a similar process as described for **4**. In all case, the formation of an azo bond occurs. The final product **4** was identified by NMR and MS techniques as described previously. The formation (at low concentrations) of the dimeric compounds **5** and **6** was supported by the $LC\text{-}MS^n$ analysis and by the presence of products **8** and **9** in reaction mixtures (Scheme 1).

Assessment of Acid Blue 62 and enzymatic products environmental fate

The toxicity of synthetic dyes as well as of their bioconversion products presents a high environmental concern.[7,8] Thus, in this study, a yeast-based bioassay was used to compare the potential toxicity of AB62 and products. The use of the eukaryotic model *Saccharomyces cerevisiae* could provide meaningful clues of potential cytotoxicity of these compounds for mammals or aquatic eukaryotic organisms.[19] Our results show that AB62 causes a significant inhibitory effect on yeast growth (Figure 4) and values of LOEC and IC_{50} of around 7 and 420 µM (3 and 177 mg/L) respectively, were estimated.

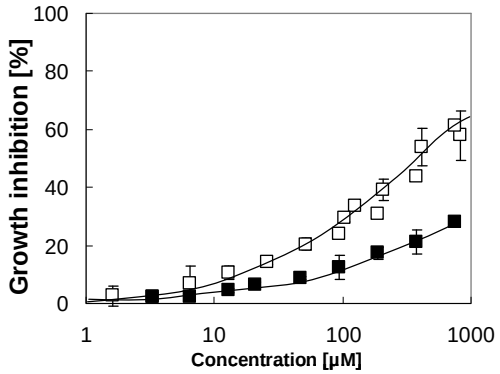
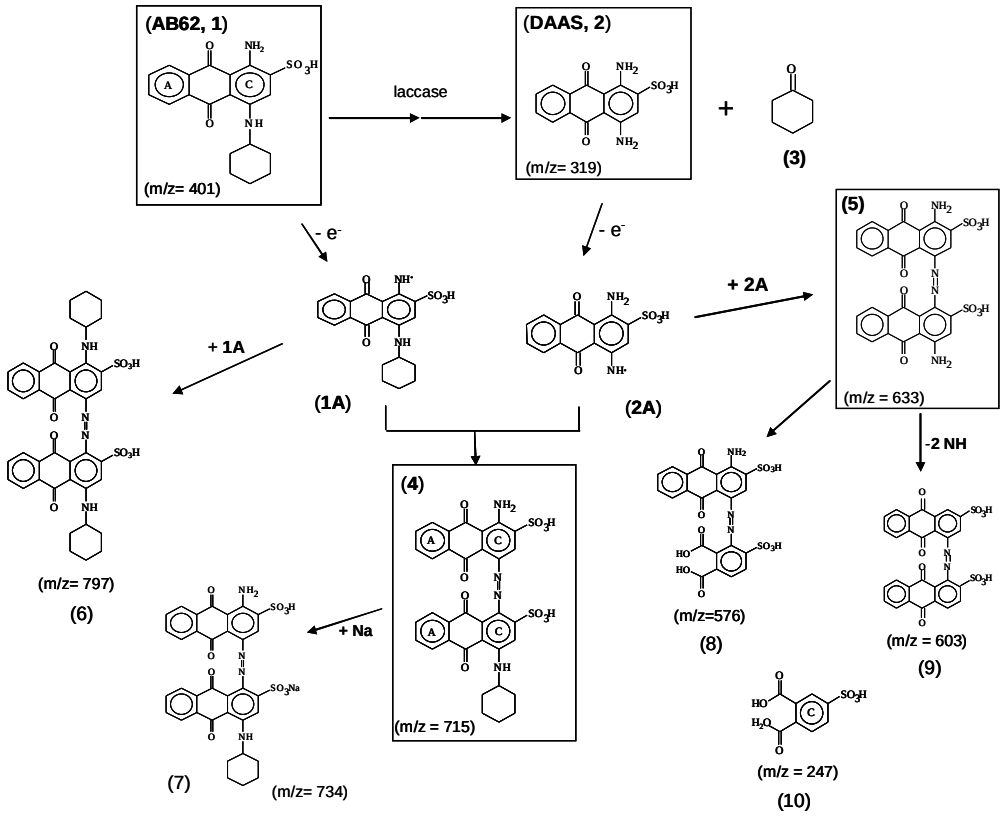
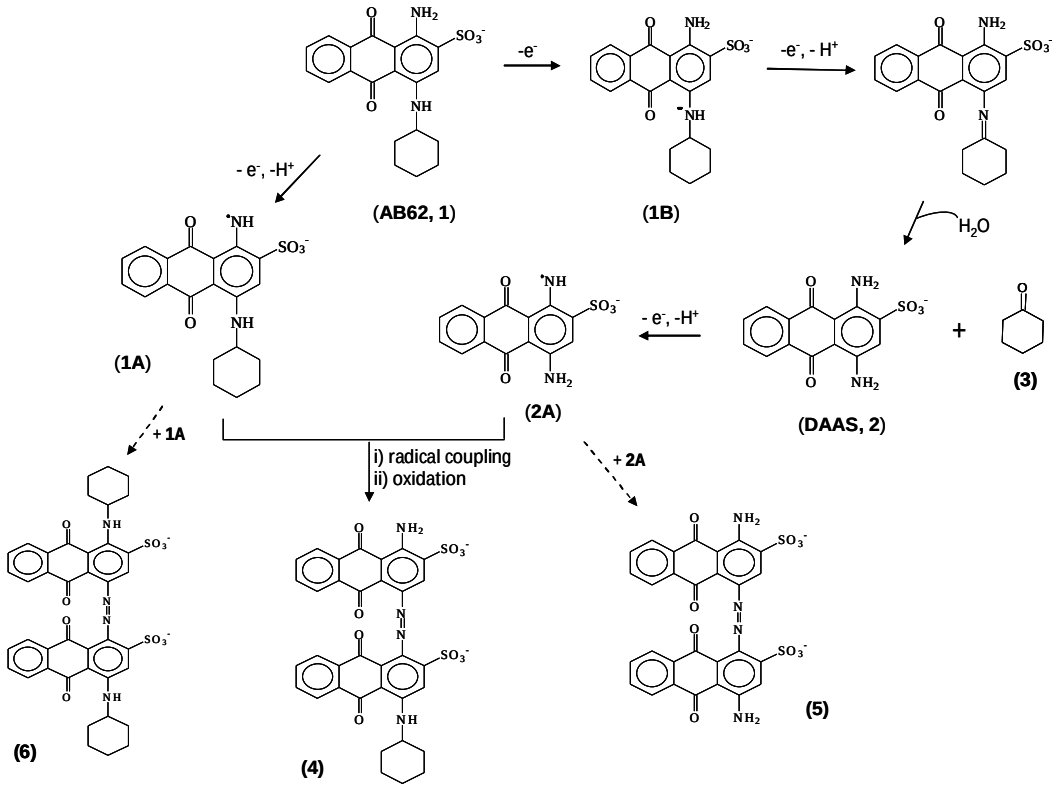


Figure 4. Toxicity of AB62 (**1**, □) and compound **4** (■) as determined by the inhibitory effects to *S. cerevisae* BY4741 growth.

The IC_{50} is well above the expected dye concentrations in the environment but is within the same order of magnitude of the typical dye concentration in spent dye baths.[7] The mixture containing AB62 biotransformation products after 2 h of reaction with CotA-laccase was significantly less toxic to the yeast cell population, resulting in 28.5 ± 4.8 % of inhibition of growth as compared with 66.3 ± 2.2% inhibition with the untreated dye solution. Consistent with the reduced overall toxicity of AB62 solution, we show that compound 4, the reddish azo product that accumulates during the biotransformation reaction, is significantly less toxic to the yeast (LOEC ~ 45 µM, $IC_{50} > 750$ µM) than the parent molecule (Figure 4). Moreover, compound **4** is at least 5-fold less toxic to yeast than the azo dye SOG (IC_{50} ~ 150 µM [5]).



Scheme 1. Identified products of AB62 biotransformation by CotA-laccase. The ions *m/z* of 319, 633 and 715 were also identified in reaction mixtures with LAC3 from *Trametes* sp. C30.



Scheme 2. Proposed mechanism of AB62 biotransformation by laccases.

Conclusions

The enzymatic biotransformation process of AB62 was examined by kinetic studies and HPLC, NMR and MS analyses, leading to the identification of reaction intermediates and final products. In a previous study on the biodegradation process of AB62 by crude fungal enzyme preparations, compound **4** is described to be an intermediate product transiently formed before its degradation.[14] In this work we have unambiguously identified the two main final reaction products (1-[4-Amino-9,10-dioxo-3-sulfo-9,10-dihydroanthracen-1-yl)diazenyl]-4-cyclohexylamino-9,10-dioxo-9,10-dihydroanthracene-2-sulfonic acid (**4**), cyclohexanone (**3**)) and a major intermediate (1,4- diamino-9,10-dioxo-3-sulfo-9,10-dihydroanthracene-2-sulfonic acid (**2**)). The mechanistic scheme that can be drawn corresponds to a two-step sequence of oxidation reactions dominated by the loss of the cyclohexyl substituent of the amino group on AB62 and the further coupling of AB62 and DAAS radicals. We show that the enzymatic biotransformation process is effective in reducing the toxicity of solutions containing the dye AB62. This study demonstrates the potential use of laccases in the oxidative degradation of anthraquinonic dyes in the environment. It also gives insights over the biosynthesis of azo dye compounds with low toxicological properties.

Experimental section

Enzymatic biotransformation of AB62

CotA-laccase activity towards Acid Blue 62 dye (AB62, Yorkshire Europe, Belgium, 97% purity) was measured using 1 mM of dye and 1 $U.mL^{-1}$ of CotA in 20 mM sodium phosphate buffer, pH 6.[18] Reactions were conducted at 37 ºC, with shaking (180 rpm) and time-points samples were withdrawn, diluted and analyzed by spectrophotometry and HPLC. The decrease in the dye concentration was monitored at 630 nm (λ_{max} of AB62). The effect of pH on the enzymatic activity (pH 4-10) was determined in Britton-Robinson buffer (100 mM phosphoric acid, 100 mM boric acid and 100 mM acetic acid mixture titrated to the desired pH with 0.5 M NaOH). Kinetic parameters were determined from Lineweaver-Burk plots. The enzymatic transformation of AB62 (6.6 mM) by LAC3 from *Trametes sp*. C30 (2 $U.mL^{-1}$) [17] was performed under dioxygen over-pressure at 2 bar, in Schlenk tubes, with a final volume of 10 mL in 0.1 M acetate buffer, pH 5, and at 30ºC. All enzymatic assays were performed at least in triplicate.

Spectroscopic analysis

The UV-visible absorption spectra were obtained at room temperature using a Nicolet Evolution 300 spectrophotometer from Thermo Industries. ^{1}H and ^{13}C NMR spectra were obtained at room temperature with an Avance Bruker 400 spectrometer in MeOD-d^4 solvent. The ^{1}H and ^{13}C chemical shifts are reported in part per million (ppm) using the solvent signal as internal reference. Spectral assignments follow the numbering scheme shown in Figure 3A. Two dimensional HH COSY and HMQC experiments were used to assign all members of the coupled spin network.

HPLC analysis

HPLC analyses were performed in a HPLC Merck Hitachi with a diode-array system, using a reverse phase C-18 column (250 x 4 mm length, 5 µM particle size and pore of 100 Å, from LiChrospher 100 RP-18, Merck). Samples were diluted 1:1 in acetonitrile (ACN, 99.9%, Lab-SCAN, Dublin, Ireland) and centrifuged before injection (50 µL). Compounds were eluted with a linear gradient of a mobile phase from 20 to 40 % of ACN plus 0.1 % (v/v) of trifluoroacetic acid (TFA), over 50 min, and maintained isocratic at 40 % for 10 min. The flow rate was 1 $mL.min^{-1}$ and temperature 40ºC.

Purification and identification of reaction products

Two products of AB62 biotransformation by CotA-laccase as monitored by HPLC with R_t at 13 and 50 min were purified using a disposable solid phase extraction RP18 tubes (Supelclean LC-18, SUPELCO, Bellefonte, PA, USA). The compound with R_t 13 min was purified after 30 min of reaction at pH 8, and the compound with R_t 50 min after 2h of reaction at pH 6. The reactions were stopped with ACN (1:1) and samples liophilized and ressuspended in 0.1% TFA (v/v). Elution of the compounds was performed by a gradient of increasing ACN concentrations in 0.1% TFA. The resulting purified fractions were analysed by MS and NMR as follows: 1,4-Diamino-9,10-dioxo-3-sulfo-9,10-dihydroanthracene-2-sulfonic acid (DAAS, **2**), HPLC R_t: 13 min; violet colour (λ_{max} = 560 nm, ε_{560} = 92 $M^{-1}.cm^{-1}$); ^{1}H NMR (δ (400 MHz, MeOD): 7.75 (dt, 2H, J = 7.2; 2.4 Hz), 7.80 (s,1H), 8.30 (dd, 2H, J= 6.8; 1.2 Hz); ^{13}C NMR (δ (400 MHz, MeOD): 127.9, 128.2, 129.5, 133.0, 134.4, 134.5, 135.0, 147.5, 152.6, 181.7, 185.1.

1-[(4-Amino-9,10-dioxo-3-sulfo-9,10-dihydroanthracen-1-yl)diazenyl]-4-cyclohexylamino-9,10-dioxo-9,10-dihydroanthracene-2-sulfonic acid (**4**), HPLC R_t: 50 min; red colour (λ_{max} = 500 nm, ε_{560} = 769 $M^{-1}.cm^{-1}$) ^{1}H NMR (δ (400 MHz, MeOD): 1.44 (m, 1H), 1.55 (q, 4H), 1.71 (m, 1H), 1.84 (m, 2H), 2.14 (m, 2H), 3.76 (m, 1H), 7.71 (t, 2H, J= 7.6 Hz), 7.80 (m, 2H, J= 7.2 Hz), 7.88 (s, 1H), 7.98 (dt, 2H, J= 6.4; 0.8 Hz), 8.24 (t, 2H, J= 8.4 Hz), 8.62 (s, 1H); ^{13}C NMR (δ (400 MHz, MeOD): 25.4, 25.5, 26.9, 33.9, 34.0, 52.3, 114.4, 118.3, 127.3, 127.6, 127.8, 127.9, 132.1, 133.2, 134.5, 134.7, 134.8, 135.3, 135.5, 135.8, 136.2, 143.5, 145.4, 150.9, 152.0, 185.7, 185.9, 186.4, 186.5. MS (ESI): *m/z* (negative mode): 713 ($[M-H]^-$), 633, 605, 525, 384, 317, 302.

LC-ESI-ion trap analysis

The ESI-ion trap MS system was a LCQ ion trap mass spectrometer (Thermofinnigan) equipped with electrospray source and run by Xcalibur (Thermofinnigan) version 1.3 software. The HPLC separation was performed using the conditions described above. The following conditions were used in experiments with ESI source in positive and negative mode: temperature of the heated capillary, 350 ºC; source voltage 4.5 kV. Nitrogen was used as sheath gas and auxiliary gas. The sheath and auxiliary gas flow rates were 80 and 20 arbitrary units, respectively. HPLC-MS was performed in the full scan mode from *m/z* 50 to 2,000. All the fragmentation experiments were done with 50% collision energy.

GC-MS analysis

The LAC3 reaction was stopped at times 0, 20, 40 and 60 minutes and the mixture extracted with diethyl ether (v/v) containing 5 mg of 2-cyclohexenone as an internal standard. The organic phase was first dried over sodium sulfate and then analysed by GC-MS using an HP-6890 GC apparatus coupled to an HP-5973 mass spectrometer. The column was a DBWAX (30 m x 0.25 mm, 0.25 μm) and He was the carrier gas delivered at a 1 $mL.min^{-1}$. flow rate. The following program was used for the elution: 40°C for 2 min then rising to 150°C at 10°C/min and hold for 10 minutes. GC-MS analysis of CotA reactions were performed at the Analytical Laboratory, Analytic Services Unit, IBET, Portugal.

Toxicity analysis

A previously described microplate toxicity assay based on the inhibitory effects on the growth of a cell population of *S. cerevisiae* BY4741 (at pH 6.5) was used with minor adaptations.[10, 18] The lowest-observed-effect-concentration (LOEC) is defined as the lowest concentration of the dye that affected yeast growth by around 5% and IC_{50} is the concentration at which toxic effects produce 50% inhibition of growth. Data reported are average values with standard deviations of results from at least three independent toxicity tests (with three replicas).

Acknowledgments

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