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Synthesis and structural characterization of a novel phenoxazinone dye by use of a fungal laccase

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ABSTRACT

Laccase, isolated from *Cerrena unicolor*, is able to transform 3-amino-4-hydroxybenzensulfonic acid into a water soluble phenoxazine dye with an extinction coefficient (ε) of 8600 M^{-1} cm^{-1}. The dye has been characterized using a variety of different analytic and spectroscopic techniques like UV–vis spectroscopy, HPLC (High Performance Liquid Chromatography), ESI/MS (Electrospray Ionization Mass Spectrometry) and the following NMR experiments: ^{1}H, ^{13}C, TOCSY (Total Correlation Spectroscopy), HSQC (Heteronuclear Single Quantum Coherence), HMBC (Heteronuclear Multiple Bond Coherence) showing the structure of 2-amino-3-oxo-3H-phenoxazine-8-sulfonic acid. The advantages of the presented biocatalytic system, in alignment with chemical system to obtain Curie_22, are eco-sustainability and one step performance.

1. Introduction

Interest in the potential use of enzymes in organic synthesis has recently increased [1–3]. The application of enzymes can compete with conventional methods, especially in formation of unique compounds with novel properties that could not be easily achieved by chemical procedures. Laccases (EC 1.10.3.2) are oxidoreductases mainly produced by basidiomycete fungi. By using four Cu centres, they perform the mono-electronic oxidation of reducing substrates, coupled to the $4e^{-}$ reduction of O_2 to H_2O [4,5]. These enzymes oxidize mono, diphenols, methoxy-phenols, polyphenols, polyamines, lignins, aryl diamines [6], aminophenols as well as some inorganic ions [7].

The formed substrate radicals can undergo a broad variety of reactions and for these reasons laccases are receiving increasing attention for various industrial applications, such as pulp delignification, wood fiber modification, dye or stain bleaching, dye synthesis, synthesis of fine chemicals and contaminated water or soil remediation [8–14]. It has been reported that in basidiomycete *Pycnoporus cinnabarinus* the production of cinnabarinic acid (a red pigment, characteristic of this fungus) is a laccase-dependent process. Comprising a phenoxazine chromophore, this pigment is formed from 3-hydroxyanthranilic acid via a laccase-catalyzed oxidative process [15].

As phenoxazine compounds show interesting technological and biological properties as they have shown interesting properties not only as dyes but also as antifungal and antimicrobial agents [16] we started to explore several phenolic derivatives in order to find new substrates suitable for the preparation of novel dyes. Between all the different tested substrates, the 3-amino-4-hydroxybenzenesulfonic acid (**1**) resulted the most interesting one. The precursor (**1**) was bioconverted, in aqueous solution and at room temperature, into a new coloured compound (Curie_22) using the fungal laccase (LAC) isolated from *Cerrena unicolor* (CU355) [17].

The structure of this novel dye has been determined using UV–vis spectroscopy, HPLC (High Performance Liquid Chromatography), ESI/MS (Electrospray Ionization Mass Spectrometry) and the following NMR experiments: ^{1}H, ^{13}C, TOCSY (Total Correlation Spectroscopy), HSQC (Heteronuclear Single Quantum Coherence), HMBC (Heteronuclear Multiple Bond Coherence).

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[1] This paper is in memory of Sophie Vanhulle passed away in a tragic event while this paper was under writing. Sophie was the scientific coordinator of the EU project named SOPHIED and this paper is part of the results of that large research project. All of us are grateful to her to have leaded and inspired the research on the use of enzymes for dye biosynthesis.

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2. Experimental

2.1. Chemicals

All the solvents and reagents were purchased from Sigma–Aldrich Srl (Milan, Italy). Milli-Q quality water (Millipore, Milford, MA, USA) was used. The precursor 3-amino-4-hydroxybenzensulfonic acid (**1**) was from Fluka (Milan, Italy).

2.2. Enzyme

Laccase (LAC) from the white rot fungus *Cerrena unicolor* (from the Fungal Collection (FCL) of Biochemistry Department of Maria Curie-Skłodowska University, Lublin, Poland) was prepared and purified using previously described procedure [18]. Briefly, concentrated growing medium was loaded onto a High-Q Sepharose (Bio-Rad, USA) and eluted with a linear gradient from 0% to 50% NH_4SO_4 (1 M) in Tris–HCl buffer (5 mM, pH 6.5). During purification procedure LAC specimen with activity 1050 U/mg of protein was prepared. The protein concentration was measured by using the method according to Bradford [19]. Until use LAC was stored frozen at −18 °C and after thawing was used to prepare working LAC solution with a precise activity. LAC activity was determined by following the oxidation of 2,2′-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS). The reaction mixture contained LAC and 2.5 mM ABTS in 100 mM Na–tartrate buffer pH 3. Oxidation of ABTS was monitored spectrophotometrically (Varian Cary 50 Bio, USA) at λ_{max} = 414 nm (ε/dm^3 mol^{-1} cm^{-1} 36 048) and LAC activity was expressed in U ml^{-1}. One unit of LAC oxidized 1 μmol of substrate per 1 min at 25 °C.

2.3. Laccase-mediated transformation procedure

Oxidation of 5 mM precursor was carried out in presence of 1 mM Na–tartrate buffer pH 5 and LAC (activity about 1 U ml^{-1}). 150 ml of reaction mixture in 250-ml volume Erlenmeyer flask was allowed to react under orbital shaking (140 rpm) at 25 ± 3 °C. After 24-h transformation product (Curie_22) was freeze-dried and analysed its structure.

2.4. HPLC analysis (apparatus and chromatographic conditions)

The precursor **1** and Curie_22 molecules (see Scheme 1) were dissolved in dimethyl sulfoxide (DMSO) obtaining concentrations varying from 5 to 7 mM. All the solvents were of HPLC grade. The chromatography–mass spectrometry (LC–MS) was an Agilent 1100 series liquid chromatograph system (Agilent Technologies, Palo Alto, CA) including a vacuum solvent degassing unit, a binary high-pressure gradient pump and an 1100 MSD model VL benchtop mass spectrometer with API-ES interface. The Agilent 1100 series MSD single-quadrupole instrument was equipped with the orthogonal spray API-ES (Agilent Technologies, Palo Alto, CA). Nitrogen was used as nebulizer gas and drying gas (350 °C). The nebulizer gas, the drying gas, the capillary voltage, and the vaporizer temperature were set at 40 psi, 9 l min^{-1}, 3000 V and 350 °C, respectively. The LC–ESI-MS determination was performed by operating the MSD in positive ions mode. Mass spectra were acquired over the scan range *m*/*z* 100–1500 using a step size of 0.1 u. Quantitative analysis was carried out using the signal of base peak ions of various compounds.

The chromatographic separation was performed on Nucleodur® C_{18} column (250 mm × 10 mm; 5 μM) (Macherey-Nagel, Düren, Germany). The sample was injected (20 μl) after filtration with 0.45 mm Acrodisc® syringe filters (Sigma–Aldrich Srl, Milan, Italy). Detection was performed at λ_{max} = 254 nm. The separation was performed by using linear gradient elution for 60 min with a mobile phase of 0.1% (v/v) trifluoroacetic acid in water and methanol (from 95:5 to 40:60, v/v) at a flow rate of 1 ml min^{-1}.

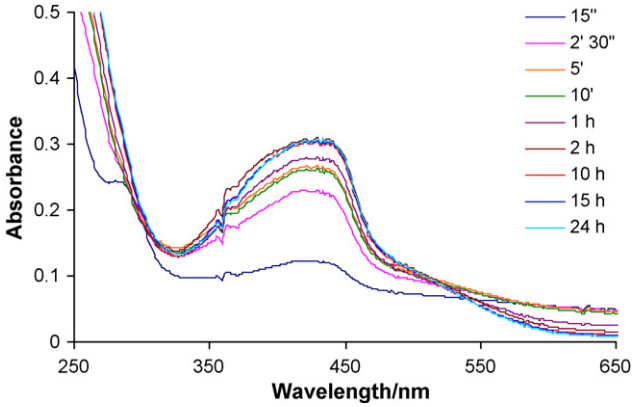

Fig. 1. UV/vis spectra recorded during 24 h bioconversion of compound **1** at 298 K with LAC. Reaction media contained substrate (1 mM), laccase (1 U ml^{-1}) in tartrate buffer pH 5.

2.5. NMR measurements

The precursor **1** and Curie_22 molecules were dissolved in $(CD_3)_2SO$ (deuterated dimethyl sulfoxide) obtaining concentrations varying from 8 to 10 mM. NMR measurements were performed either at 11.0 T with a Bruker Avance 500 MHz or at 9.4 T with a Bruker AMX 400 MHz spectrometers at controlled temperatures (±0.2 K) using a TXI (Triple Xband Inverse) or BBI (Broadband Inverse) probe. Residual water suppression was achieved by presaturation. Proton and carbon resonance assignment was obtained by means of standard homonuclear and heteronuclear 2D experiments such as ^{1}H–^{1}H TOCSY, ^{1}H–^{1}H NOESY ^{1}H–^{13}C HSQC and ^{1}H–^{13}C HMBC. Chemical shifts were referenced to internal TMS-d_4 (trimethylsilane). Spectra processing was performed on a Silicon Graphics O2 workstation using the XWINNMR 3.1 software.

3. Results and discussion

The fungal laccase (1 U ml^{-1}) reacted in batch with the precursor (5 mM) in 1 mM Na–tartrate buffer pH 5 at room temperature and as the reaction proceeded, the initial mixture changed from yellow ochre to red with the following reaction Scheme 1.

In Fig. 1 the formation of the new product, monitored using UV/vis spectroscopy, is reported. LAC-mediated transformation of

Scheme 1. Biomimetic synthesis of phenoxazinone dye.

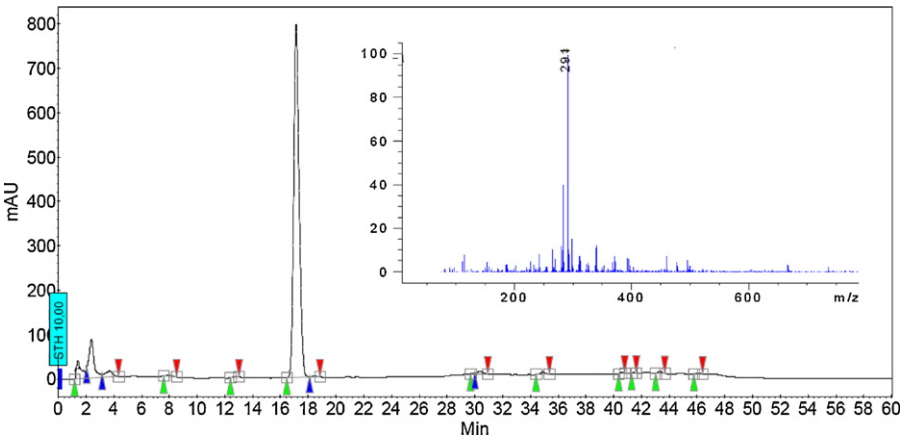

Fig. 2. HPLC chromatogram of bioconversion reaction with LAC at 300 K with 10 mM of **1**, pH = 5. Sample taken after 24 h of reaction. In the inset, chromatographic profile obtained for Curie_22 product reporting the corresponding peak molecular mass in negative ion mode *m/z* 291 [M^{-1}, 98%] is shown.

precursor was performed many times and reiteration of this process showed that it was reproducible with an yield of about 60%.

The transformation of **1** was very selective as shown by HPLC profile of the crude reaction mixture as reported in Fig. 2. Curie_22 (retention time 17.12 min in 22% MeOH/H_2O) is the main product of transformation and the LC/MS spectrum gave a negative ion mode peak at *m/z* 291 [M^{-1}].

After purification by solid phase extraction on SDB-1 column, Curie_22 was analysed by ^1H and ^{13}C NMR spectroscopy to gain additional information for the identification of its molecular structure. $(CD_3)_2SO$ was used as solvent in order to better resolve amino and hydroxyl protons.

The resulting spectra (Fig. 3, upper trace) indicated the presence of two different isomers, with a relative abundance of 70% and 30%. The signals marked with a blue star refer to the second less abundant isomer.

The recorded ^1H NMR spectra showed some differences with those of the precursor molecule (Fig. 3, lower trace). The most important variations were: (i) the disappearance of the OH broad signal at 11.0 ppm, (ii) the diverse chemical shift experienced by the three aromatic protons, as shown by the magenta arrows, and (iii) the appearance of three additional resonances at 6.80, 6.38 and 6.36 ppm, respectively marked with a red star in Fig. 3.

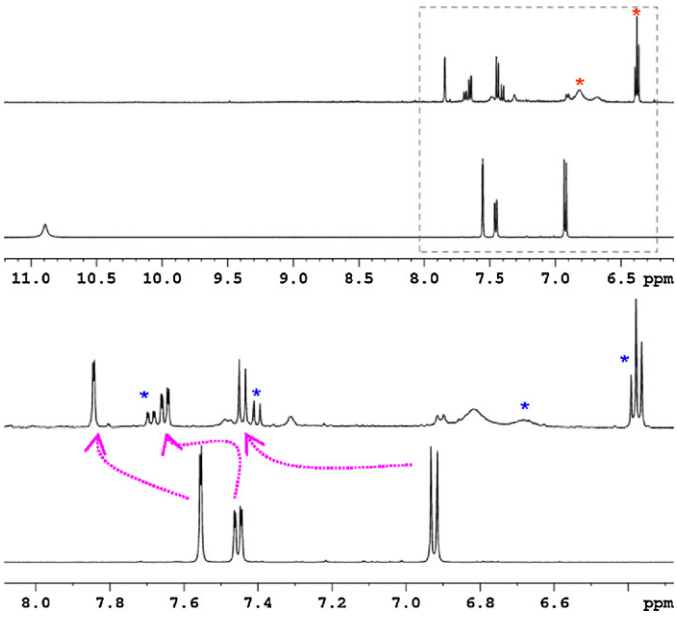

Fig. 3. ^1H 1D NMR spectrum of precursor (lower trace) **1** and product in $(CD_3)_2SO$ (upper trace), *T* = 300 K. The region in the frame, with an enlarged view, is shown. The red stars indicate the new signals in product molecule, the blue ones indicate the signals corresponding to the less abundant isomer. The magenta arrows show the shift exhibited by the **1** aromatic protons. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

Moreover the three aromatic signals at 7.83, 7.64 and 7.43 ppm possess identical multiplicity of those of **1** suggesting the occurrence of very similar spin system patterns. On the contrary the large chemical shift variations 0.28 (H2), 0.52 (H5) and 0.20 (H6) indicated a substantial change of their chemical environments with a particular rearrangements nearby H5 proton.

The analysis of 2D ^1H–^1H COSY and TOCSY experiments confirmed the previous assignment but did not show the occurrence of any further correlations useful to assign the signals at 6.80, 6.38 and 6.36 ppm.

The ^{13}C NMR analysis of Curie_22 (Fig. 4A and B, upper traces) and precursor (Fig. 4A and B, lower traces) carbon spectra was also performed. The two traces largely differ, in particular many more signals appear in the spectra belonging to Curie_22: (i) a signal at 180.1 ppm appeared indicating the presence of C=O group, (ii) in the 150–139 ppm region five-six sharp signals were evident, in respect to the only two present in the **1** molecule and (iii) two new ^{13}C resonances are present at 103.4 and 98.4 ppm.

In order to understand the molecular structure of Curie_22 molecule and to assign all the ^1H and ^{13}C signals ^1H–^{13}C HSQC and ^1H–^{13}C HMBC experiments were performed. Such experiments are very effective since they allow to correlate to each proton a carbon signal, to link the different spin systems and to assign the quaternary carbons.

In particular it was found that the ^1H signals at 6.38 and 6.36 ppm directly correlate to the two ^{13}C signals at 103.4 and 98.4 ppm which were assigned as CH motifs (Fig. 5A). Moreover also the carbonyl at 180.1 ppm and the other three quaternary carbon at 148.8, 148.4 and 147.3 ppm showed a long range correlation with the two protons at 6.38 and 6.36 ppm (Fig. 5B and C). All these findings suggested that all those nuclei were correlated at least with a $^3J_{CH}$ and that they, belong to a six carbon ring. The existence of an additional six carbon ring was found since all the other missing six carbon resonances were correlated to the aromatic protons at 7.83, 7.64 and 7.43 ppm, as illustrated in Fig. 5C.

All these results have allowed to perform the assignment of each resonances, reported in Table 1 which fit unambiguously with the proposed molecular structure.

On the other hand, Curie_22 is present as a mixture of isomers, as indicated by the double resonances for H6 and H7. A possible structure consistent with the spectral properties can be the isomeric 2-amino-3-oxo-3H-phenoxazine-7-sulfonic acid.

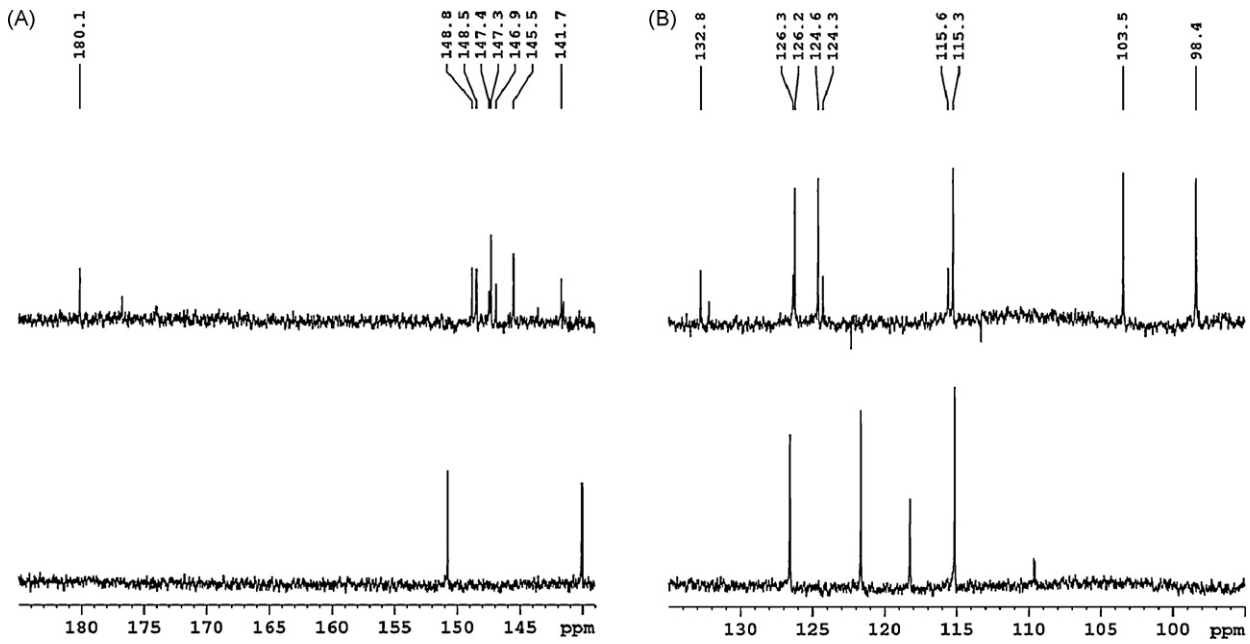

Fig. 4. ^{13}C 1D NMR spectrum of precursor (lower traces) and product (upper traces) in $(CD_3)_2SO$ $T = 300$ K. (A) 185–139 ppm region; (B) 135–95 ppm region.

3-Amino-4-hydroxybenzensulfonic acid (**1**) behaved as a good substrate for laccase and produced a new dense ochre dye derived from oxidative dimerization of the precursor. This novel dye has interesting properties being water soluble, with an extinction coefficient (ε) of 8600 M^{-1} cm^{-1} reduced toxicity, not mutagenic and good dyeing properties both on cotton and wool as supports. Laccases perform the mono-electronic oxidation of a reducing substrate at the T1 copper site. The oxidation of substrate is governed by the Marcus outer sphere mechanism [20]. Hydroxyl and amino groups are the preferred oxidation sites by the enzyme. It is possible to follow the catalytic mechanism identifying the radical species formed on the substrate molecule by using the EPR technique. This was successfully achieved in the analysis of two different well-known substrates, the ABTS (2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid)) and violuric acid, an N–OH synthetic mediator. These two molecules act as synthetic mediators and are characterized by good affinity for the T1 site and formation of long lived radical species [21]. A stable cationic radical was identified for ABTS while in the case of violuric acid, the formation of a neutral radical species was detected [21]. Considering these achievements we can hypotisize that also in our case the first event is the phenoxyl radical formation. The fact that a radical species cannot be detected in this case, might be due, to the possible disproportionation of the radical to the corresponding quinone imine species. The subsequent reaction of another molecule of 3-amino-4-hydroxybenzensulfonic acid with the quinone imine might bring to the displacement of the sulfonic group para to the carbonyl group with the formation of the phenoxazinone moiety [22]. As it was earlier reported in literature the treatment of a quinone imine in acetone with 2-aminophenol resulted in the formation of 2-aminophenoxazinone [23]. This suggests that the steps subsequent to the first oxidation of the substrate do not involve enzymatic catalysis. Similar reactions with the formation of the quinone imine intermediate were found in the oxidation of 2-aminophenol to 2-aminophenoxazinone by tyrosinase [24] and for the formation of the phenoxazi-

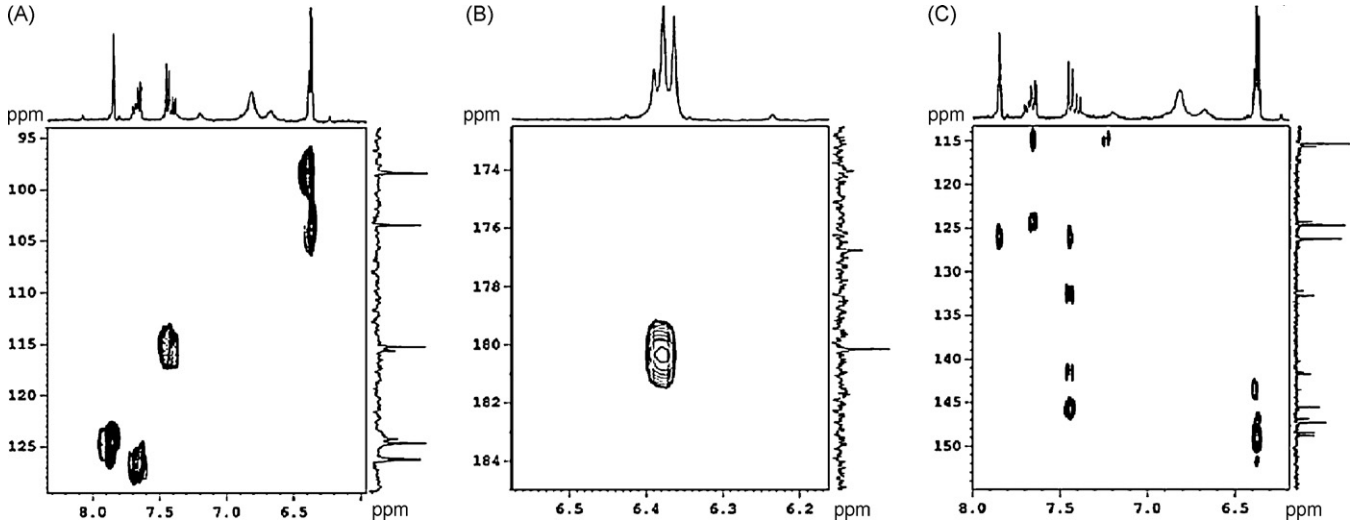

Fig. 5. (A) ^{1}H–^{13}C HSQC spectrum (B) and (C) different regions of ^{1}H–^{13}C HMBC spectrum of Curie_22 in $(CD_3)_2SO$ $T = 300$ K.

Table 1
^{1}H and ^{13}C chemical shift (ppm) and J_{HH} (Hz) of precursor and product molecules in $(CD_3)_2SO$ at 300 K. Chemical shift are referred to TMS.

Type	^{1}H δ (ppm)	J_{HH} (Hz)	^{13}C δ (ppm)
Precursor			
1	–	–	118.2
2	7.55	(d) 1.8	121.6
3	–	–	140.0
4	–	–	150.7
5	6.92	(d) 8.4	115.1
6	7.45	(dd) 8.4;1.8	126.6
–OH	10.9	(bs)	
Curie_22			
1	6.36	(s)	103.4
2	–	–	147.3
3	–	–	180.1
4	6.38	(s)	98.4
5a	–	–	148.5
6	7.44	(d) 8.4	115.2
6a	–	–	141.7
7	7.65	(dd) 8.4;1.8	126.2
8	–		132.7
9	7.84	(d) 1.8	124.6
9a	–	–	145.5
10a	–	–	148.8
$–NH_2$	6.82	(bs)	

none chromophore of actinomycin by phenoxazinone synthase [25].

4. Conclusions

In this paper the one step bioconversion of 3-amino-4-hydroxybenzensulfonic acid into a compound of phenoxazine structure is reported. The water soluble dye has been unambiguously characterized using a variety of analytical and spectroscopic techniques. The use of oxidative enzymes like laccase, represents a convenient and ecofriendly way for the synthesis of new molecules of complex structure.

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