

# Mechanism of enzymatic degradation of the azo dye Orange II determined by ex situ $^1\text{H}$ nuclear magnetic resonance and electrospray ionization-ion trap mass spectrometry

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## Abstract

Removal of azo dye effluents generated by textile photography industries is a main issue in wastewater treatment. Enzymatic treatment of dyes appears to be one of the most efficient processes for their degradation. The elucidation of degradation pathways is of special interest considering health and environmental priorities. Ex situ nuclear magnetic resonance (NMR) spectroscopy and electrospray ionization (ESI)-ion trap mass spectrometry performed directly on incubation medium have been used for the first time to follow kinetics of sulfonated azo dye Orange II enzymatic degradation. Nine transformation products were identified using these complementary analyses performed ex situ without any prior treatment. Three types of cleavage are proposed for the degradation pathway: (i) a symmetrical splitting of the azo linkage that leads to the formation of 4-aminobenzenesulfonate (and 1-amino-2-naphthol, not detected); (ii) an asymmetrical cleavage on the naphthalene side that generates 1,2-naphthoquinone and 4-diazoniumbenzenesulfonate as products, with the latter one being transformed into 4-hydroxybenzenesulfonate; and (iii) a third degradation pathway that leads to 2-naphthol and 4-hydroxybenzenesulfonate. Moreover, three other intermediates have been identified. This study, which constitutes the first concomitant use of  $^1\text{H}$  NMR spectroscopy and ESI-ion trap mass spectrometry in this field, illustrates the indubitable interest of the ex situ approach.

**Keywords:**  $^1\text{H}$  NMR spectroscopy; Electrospray ionisation-ion trap mass spectrometry; Azo dye; Orange II; Manganese peroxidase

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Color removal in effluents generated by the textile industry is a main issue in wastewater treatment [1]. Dyes in these effluents are compounds with very low biodegradability. These wastewaters commonly are treated using physico-chemical methods such as coagulation, adsorption, and oxidation with ozone. All of these pro-

cesses are very expensive and generate large volumes of sludge, and in certain cases they need chemical additives that, in turn, can be hazardous for the environment. On the other hand, conventional biological treatments using activated sludge have been found to be ineffective for the removal of dyes from wastewater [2-4]. Anaerobic processes are more adequate for the treatment of these compounds [5,6].

White rot fungi can degrade many complex compounds due to the extracellular enzymes they produce.

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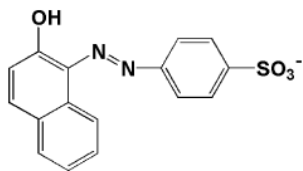


Fig. 1. Chemical structure of Orange II azo dye.

Most of them are oxidases and peroxidases with a very high oxidative capacity [7–9]. It has been suggested that these enzymes could oxidize the dye structures to form compounds with lower molecular weight and lower toxicity. Among these enzymes, manganese peroxidase (MnP)<sup>1</sup> and lignin peroxidase (LiP) are most frequently applied to dye degradation [10–13]. Previous studies have demonstrated that MnP, with its cofactors in the adequate concentration, can degrade azo dyes such as Orange II, reaching decolorization of 90% in very short periods of time (e.g., 10 min) [14].

The molecular structure of Orange II is characterized by the presence of an azo group (Fig. 1). The initial step of biotransformation of an azo compound usually involves a reductive cleavage of the azo bond, which under anaerobic conditions leads to the formation of aromatic amines known to be carcinogenic [15]. These amines are more hydrophobic and, thus, may easily cross the cell membranes, consequently being more toxic than the original dye. In the case of Orange II, an anaerobic reduction by azoreductase leads to sulfanilate and 1-amino-2-naphthol [16]. Therefore, by anaerobic treatment of Orange II, toxicity increases nearly 100-fold [17]. One potential advantage of the enzymatic degradation of Orange II might be the lower toxicity of the generated oxidized effluent compared with the one obtained after an anaerobic treatment. However, a detailed study of the metabolic degradation pathway and identification of the enzymatic degradation products are necessary to explain the effluent toxicity. Thus, the elucidation of the Orange II transformation mechanism is of special interest considering environmental priorities.

Several studies have been carried out to determine the mechanism for peroxidase-catalyzed azo dye degradation. Spadaro and Renganathan [18] studied the degradation mechanism of Disperse Yellow 3 naphthol analog by high-performance liquid chromatography (HPLC)

<sup>1</sup> Abbreviations used: MnP, manganese peroxidase; LiP, lignin peroxidase; HPLC, high-performance liquid chromatography; GC–MS, gas chromatography–mass spectrometry; LC–MS, liquid chromatography–mass spectrometry; FAB–MS, fast atom bombardment–mass spectrometry; ESI–MS, electrospray ionisation–mass spectrometry; <sup>1</sup>H NMR, <sup>1</sup>H nuclear magnetic resonance; MS, mass spectrometry; 1D, one-dimensional; 2D, two-dimensional; TSPd<sub>4</sub>, tetradeuterated sodium trimethylsilylpropionate; HMBC, heteronuclear multiple bond connectivity; TOCSY, total correlation spectroscopy; DMP, 2,6-dimethoxyphenol; DFT, density functional theory.

and gas chromatography–mass spectrometry (GC–MS). They described the release of the azo linkage as molecular nitrogen and the formation of two major final compounds: 1,2-naphthoquinone and acetanilide. If the mechanism of the Orange II degradation were similar to that, the final products of its degradation should be 1,2-naphthoquinone and benzenesulfonate. However, Goszczynski et al. [19] suggested two different mechanisms for the degradation of sulfonated azo dyes. The first is based on a symmetrical cleavage of the azo group and results in the formation of quinone monoimine and nitroso derivatives as direct oxidation products, and the second is an asymmetrical cleavage and yields quinone and phenyl diazene derivatives. These compounds may finally undergo various spontaneous reactions that result in the formation of secondary products. The analytical techniques used were liquid chromatography–mass spectrometry (LC–MS) and GC–MS after extraction and derivatization of the samples. The analog of Orange II degradation should lead to the secondary products 1-amino-2-naphthol and sulfanilate for the symmetric pathway and 1,2-naphthoquinone, benzenesulfonate, and 4-hydroxybenzenesulfonate for the asymmetric one. Finally, Chivukula et al. [20] characterized 1,2-naphthoquinone and 4-sulfophenylhydroperoxide as final products of Orange II degradation using HPLC, GC–MS, fast atom bombardment–mass spectrometry (FAB–MS), and electrospray ionization mass spectrometry (ESI–MS).

In the current work, *ex situ* and *in situ* <sup>1</sup>H nuclear magnetic resonance (<sup>1</sup>H NMR) and ESI-ion trap mass spectrometry (MS) were applied concomitantly to identify the intermediates and final products of the enzymatic degradation of Orange II by MnP. NMR spectroscopy offers some advantages for this purpose in comparison with the other techniques used previously [21–24]. First, NMR is a nondestructive technique. Second, NMR does not require any treatment of the samples. Third, NMR spectroscopy is both qualitative and quantitative, and it allows the determination of concentrations without the need for commercial or synthetic standards. Fourth, one-dimensional (1D) and two-dimensional (2D) spectra can be exploited for the identification of the compounds. Fifth, NMR is a rapid technique that is very suitable for the identification of unstable compounds. Nevertheless, because of an intrinsic low sensitivity (detection limit ~1 μM), the results should be confirmed by highly sensitive analytical methods such as MS [25]. Thus, ESI-ion trap MS has been used. Indeed ion trap analyzer is particularly suitable for studying metabolism of low-molecular weight compounds. Moreover, it allows the acquisition of MS<sup>n</sup> spectra that may produce invaluable structural information data when combined with in-source fragmentation.

Finally, in this article, we combine NMR, ESI–MS, and HPLC techniques, aiming to identify and quantify

both intermediates and final products. Based on our results, we propose a new pathway for Orange II degradation by MnP. Moreover, the ecotoxicity of Orange II and some of its identified degradation products is evaluated through their effect on *Photobacterium phosphoreum* (Microtox bioassay).

## Materials and methods

### Chemicals

Orange II, benzenesulfonate, sulfanilate, 2-naphthol, and glucose were obtained from Sigma. 4-Hydroxybenzenesulfonate and 1-amino-2-naphthol were purchased from Aldrich. 1,2-Naphthoquinone was obtained from Janssen Chimica. Tetradeuterated sodium trimethylsilylpropionate (TSPd<sub>4</sub>) was purchased from Eurisotop (Saint Aubin, France).

### Synthesis of diazonium salt

Sulfuric acid (2.2 g) was dissolved in 20 ml of a 10% sodium carbonate solution. After the complete solubilization and cooling in an ice bath, 1.0 g of NaNO<sub>2</sub> was added. This solution was mixed with another one containing 10 g of ice and 3 ml of concentrated hydrochloric acid [26]. <sup>1</sup>H and <sup>1</sup>H-<sup>13</sup>C heteronuclear multiple bond connectivity (HMBC) NMR spectra were performed on this crude solution without any purification. However, pH adjustment (to 5) was necessary to compare the spectra with those of enzymatic reactions. NMR data were consistent with those described previously [27] but differed slightly due to pH effect ( $\delta$  <sup>1</sup>H: 8.30 [2H], 8.72 [2H];  $\delta$  <sup>13</sup>C: 150 [Cquat], 133 [2C], 129 [2C], 116 [Cquat]).

### Synthesis of 4-nitrosobenzenesulfonate

Assays of 4-nitrosobenzenesulfonate were performed according to [28–30] but were adapted for benzenesulfonate derivatives.

### Enzyme production

MnP was secreted by the white rot fungus *Bjerkandera* sp. strain BOS55, which was isolated and identified as described previously [31]. The enzyme was produced in a culture medium consisting of cheese whey [32], in a 10-L fermenter (Braun-Biotech International, Melsungen, Germany) equipped with pH, redox, temperature and pO<sub>2</sub> sensors, and an on-line data collection and acquisition system [33]. Crude enzyme was concentrated by ultrafiltration using an acrylic miniset Filtron (Pall) with 10-kDa molecular mass cutoff-type membranes. Before use, it was centrifuged for 10 min at 20,000g to remove polysaccharides.

### Degradation assays

Degradation assays were performed, at room temperature in 100-ml Erlenmeyer flasks containing 50 ml of the reaction medium. This mixture consisted of: MnP with initial activity of 200 U L<sup>-1</sup>, 1 mM oxalate, 15 μM Mn<sup>2+</sup>, and 1.5-mM solutions of Orange II, benzenesulfonate, sulfanilate, or 4-hydroxybenzenesulfonate. The pH was adjusted to 4.5. The reaction started with the addition of H<sub>2</sub>O<sub>2</sub> using a peristaltic pump at a flow rate of 27.2 μmol L<sup>-1</sup> min<sup>-1</sup>. Controls were run in parallel without the addition of H<sub>2</sub>O<sub>2</sub>. A blank control was made without substrate but with MnP and the continuous addition of H<sub>2</sub>O<sub>2</sub>.

For in situ enzymatic assays (performed directly in the NMR magnet), H<sub>2</sub>O<sub>2</sub> was generated in situ by a glucose oxidase (Boehringer Mannheim, Mannheim, Germany). The initial activity was set up to 1 U in final volume (NMR tube content: 600 μl) to avoid “overproduction” of H<sub>2</sub>O<sub>2</sub> that would have inactivated MnP. Glucose (2.8 mM) was used as substrate.

### <sup>1</sup>H NMR spectroscopy

#### Preparation of NMR samples

Samples were collected every 10 min. No harvesting or filtration was done. The crude samples (540 μl) were supplemented with 60 μl of a 5-mM solution of TSPd<sub>4</sub> in D<sub>2</sub>O. TSPd<sub>4</sub> constituted a reference for chemical shifts (0 ppm) and quantification. D<sub>2</sub>O was used for locking and shimming.

#### <sup>1</sup>H NMR spectra

<sup>1</sup>H NMR was performed at 25 °C at 500 MHz on an Avance 500 Bruker spectrometer (Bruker Biospin, Rheinstetten, Germany) equipped with a triple-resonance (<sup>1</sup>H, <sup>13</sup>C, <sup>15</sup>N) inverse probe with 5-mm-diameter tubes containing 600 μl of sample, with water being suppressed by a classical two phase-shifted pulse saturation sequence. This solvent suppression gave more accurate results in terms of quantification than did those observed on standards using the classical double-pulsed field gradient echo sequence WATERGATE. Approximately 256 scans were collected (90° pulse, 7 μs; saturation pulse, 3 s; relaxation delay, 1 s; acquisition time, 4,679 s; 65,536 data points). A 1-Hz exponential line-broadening filter was applied before Fourier transformation, and a baseline correction was performed on spectra before integration using Bruker software. Under these conditions, the limit of detection and quantification were within the range of 1 and 10 μM, respectively.

#### Quantification of metabolites

The concentration of metabolites was calculated as follows:  $[m] = \{(9A_0 \times [TSPd_4]) / (b \times A_{ref})\} \times 1.1$ , where

$[m]$  is the concentration of metabolite  $m$ ,  $A_0$  is the area of metabolite  $m$  resonance in the  $^1\text{H}$  NMR spectrum,  $[\text{TSPd}_4]$  is the concentration of the reference,  $A_{\text{ref}}$  is the area of reference resonance in the  $^1\text{H}$  NMR spectrum,  $b$  is the number of protons of metabolite  $m$  in the signal integrated, and 9 is the number of protons resonating at 0 ppm. The 1.1 factor came from dilution of  $\text{TSPd}_4$ .

#### *$^1\text{H}$ 2D NMR experiments*

Two-dimensional phase-sensitive total correlation spectroscopy (TOCSY) experiments with water resonance suppression by a WATERGATE sequence (put at the end of the sequence) were used to assign all members of a coupled spin network. Spectral widths were adjusted in both dimensions to encompass all  $^1\text{H}$  signals of interest. Two "mixing times" (corresponding to several cycles of MLEV-17 spinlock sequence) were tested: 30 and 90 ms. The responses of eight scans for each of 256  $t_1$  increments were acquired. Zero filling in  $t_1$  and sine window function in both dimensions were applied prior to 2D Fourier transformation.

#### *MS analysis*

Mass spectrometry experiments were performed in positive and negative modes on an Esquire LC-ion trap mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with an orthogonal geometry ESI source. Nitrogen was used as the drying ( $3\text{ L min}^{-1}$ ) and nebulizing (6 psi) gas at  $300^\circ\text{C}$ . In the negative mode, the spray shield was set to 4.0 kV and the capillary cap was set to 4.5 kV. The voltages applied to the metal glass capillary interface and to skimmer 1 were  $-50$  and  $-20\text{ V}$ , respectively. In the positive mode, voltages were switched. The instrument was calibrated in positive and negative ion modes using a tuning mixture obtained from Agilent Technologies (Wilmington, DE, USA). Scanning was performed from  $m/z$  50 to 400 in the standard resolution mode at a scan rate of  $13\text{ kDa s}^{-1}$ . Each MS spectrum was recorded by averaging 10 spectra. For MS/MS experiments, the precursor ions were isolated by ion scan frequencies to eject all other ions from the trap. The precursor ions were fragmented by applying a resonance frequency on the end cap electrodes matching the frequency of the selected ions. Fragmentation occurred in the ion trap due to collision with Helium buffer gas (pressure  $7 \times 10^{-6}\text{ mbar}$ ).

All samples were prepared in a water:acetonitrile mixture (50:50). Before analysis, each sample was diluted 1/100. For commercial compounds, approximately  $10\text{-pmol}\mu\text{l}^{-1}$  solutions were used. Solutions were introduced into the mass spectrometer by continuous infusion using a syringe pump (Cole Parmer Instrument, Vernon Hills, IL, USA) at a flow rate of  $180\mu\text{l h}^{-1}$ .

#### *HPLC analysis*

LC analyses were performed on an HP 1090 M Series II Agilent ChemStation HPLC (Agilent Technologies) equipped with a diode array detector. A  $25\text{-}\mu\text{l}$  sample volume was introduced through a LiChrospher 100 RP-18 column ( $4\text{ mm i.d.} \times 250\text{ mm length}$ ) containing  $5\mu\text{m}$  packed particles (Merck KgaA, Darmstadt, Germany). The mobile phase contained 30% acetonitrile and 70%  $0.03\text{ M}$  ammonium carbonate and was adjusted to pH 7.9. The flow rate was  $0.8\text{ ml min}^{-1}$  at isocratic conditions and  $30^\circ\text{C}$ . Chromatograms were obtained at a  $231\text{-nm}$  wavelength.

#### *Enzymatic activities*

MnP activity was determined by monitoring the oxidation of 2,6-dimethoxyphenol (DMP) to cerulignone. These measurements were carried out at  $30^\circ\text{C}$  and at a wavelength of  $468\text{ nm}$  with a Shimadzu UV-1603 spectrophotometer (Shimadzu, Kyoto, Japan). The reaction mixture contained a final concentration of  $50\text{ mM}$  sodium malonate, pH 4.5,  $1\text{ mM}$  DMP,  $1\text{ mM}$   $\text{MnSO}_4$ , and up to  $600\mu\text{l}$  of supernatant in a total volume of  $1\text{ ml}$ . The reaction started with the addition of  $0.4\text{ mM}$   $\text{H}_2\text{O}_2$  [31]. The molar extinction coefficient of the cerulignone was  $49,600\text{ M}^{-1}\text{ cm}^{-1}$  [34]. One unit of activity is defined as the amount of enzyme that is required to obtain  $1\mu\text{mol}$  of the oxidized product per minute.

#### *Toxicity tests*

Toxic responses of nine samples using the Microtox toxicity test (Azur Environmental, Newark, DE, USA) were determined. This test uses a luminescent marine bacterium, *P. phosphoreum*, as the bioassay organism. It exposes bacteria to samples, which are diluted in Milli-Q water if necessary, and measures the toxic effect on the organism. Indeed, the reduction of light output is usually proportional to the concentration of the contaminants present. Thus, in the current experiments, two values of  $\text{EC}_{50}$  were calculated: after 5 and 15 min of exposure to the samples at  $15^\circ\text{C}$ . The order of October 13, 1989, which develops the Methods of Characterization of Toxic and Dangerous Wastes, establishes that wastewater is toxic if it presents an  $\text{EC}_{50}$  equal to or less than  $3000\text{ ppm}$ .

## **Results and discussion**

#### *Enzymatic degradation of Orange II*

Our aim was to analyze the crude supernatants by  $^1\text{H}$  NMR spectroscopy and ESI-MS *ex situ* without any treatment.

### Identification of the degradation compounds by NMR

Fig. 2 represents the NMR spectra of the degradation samples from 0 to 6 h. In the 1D  $^1\text{H}$  NMR spectrum obtained at 0 min, only signals belonging to Orange II are visible. It may be noticed that chemical shifts of several Orange II signals are deshielded while the concentration decreases. This concentration dependence of chemical shifts is not unusual for such functionalized aromatic compounds. Signals of naphthalene and benzenesulfonic moieties are quite well resolved, but slightly broadened, compared with pure solution of Orange II. This might be due to the presence of  $15\ \mu\text{M}$  paramagnetic  $\text{Mn}^{2+}$ .

After 10 min, the signals of Orange II decreased while many new resonances appeared. An integration profile, as well as 2D homonuclear  $^1\text{H}$  TOCSY patterns, allowed us to identify several spin networks and, thus, several transformation products. Both 30- and 90-ms mixing times were used for the 2D TOCSY experiments to give a total correlation of all the protons of a chain with one another. Nevertheless, because of the overlapping of many signals, some protons could not be clearly distinguished.

A first spin network was constituted at least by signals resonating at 6.42, 7.57, 7.65, 7.71, and 8.03 ppm as shown by 1D (Fig. 2) and 2D TOCSY spectra of the 20-min sample (data not shown). Many signals were overlapped with those of Orange II and could only be deduced from TOCSY experiments. These resonances were assigned to 1,2-naphthoquinone 1 protons, as shown by comparison with the commercial compound spectrum recorded in similar conditions. 1,2-naphtho-

quinone signals increased with time from 10 to 60 min in a range from 25 to  $142\ \mu\text{M}$  (Fig. 3). Then its concentration decreased slowly until approximately  $50\ \mu\text{M}$ , probably due to an "abiotic" decomposition.

A second spin network was constituted by two resonances at 6.95 and 7.64 ppm (Fig. 2). These signals, which appeared after 10 min, belong to 4-hydroxybenzenesulfonate 2. Their  $^1\text{H}$  chemical shifts appeared to be independent of the concentration that increased to reach approximately  $271\ \mu\text{M}$  at 60 min (Fig. 3). Then its concentration decreased to reach a plateau of approximately  $50\ \mu\text{M}$ .

A third product with two resonances at 6.89 and 7.58 ppm (Fig. 2) was identified as 4-aminobenzenesulfonate 3 (or sulfanilate). The small variation of chemical shifts ( $<0.05\ \text{ppm}$ ) observed on standards for concentra-

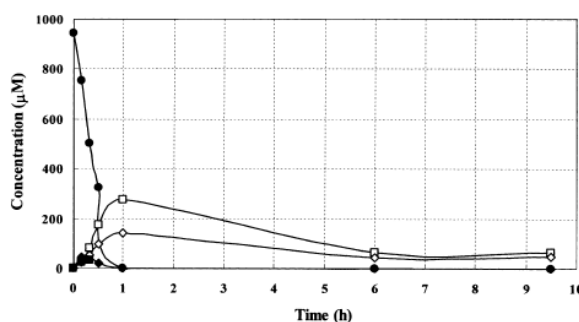


Fig. 3. Time courses of Orange II degradation product concentration obtained from NMR data. ●, Orange II; □, 4-hydroxybenzenesulfonate; ◆, 1,2-naphthoquinone; ■, sulfanilate; ◇, and 4-diazoniumbenzenesulfonate.

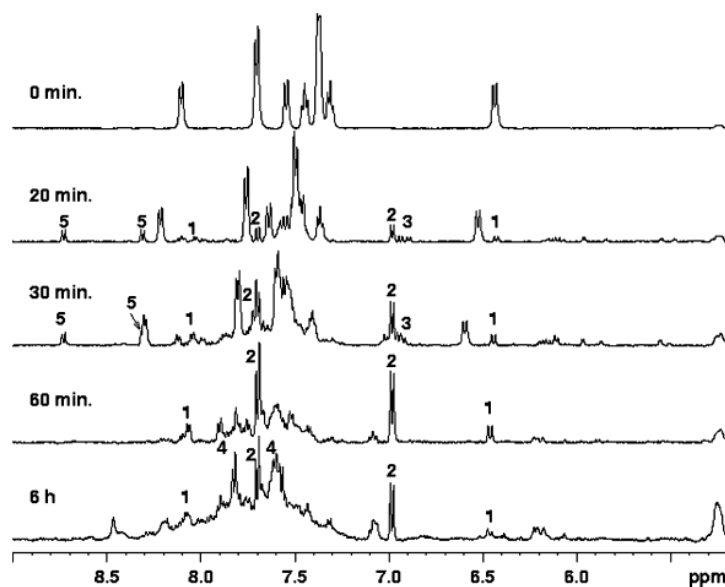


Fig. 2. Ex situ  $^1\text{H}$  NMR spectra of the samples taken after 0, 20, 30, and 60 min and 6 h of incubation of Orange II with MnP: (1) 1,2-naphthoquinone; (2) 4-hydroxybenzenesulfonate; (3) 4-aminobenzenesulfonate; (4) benzenesulfonate; and (5) 4-diazoniumbenzenesulfonate.

tions between 0.1 and 1.5 mM did not affect the spectra interpretation. Sulfanilate concentration reached approximately 30  $\mu$ M from 10 min and then decreased slightly until 60 min below the detection limit (Fig. 3).

A fourth characteristic pattern, at 7.56 and 7.82 ppm, was observed both in 1D (Fig. 2) and 2D TOCSY spectra. The corresponding signals belong to benzenesulfonate 4, as shown by comparison with the standard. Its quantification was not possible by *ex situ* NMR due to the overlapping of many signals. Nevertheless, the relative high intensity of these signals at the end of the kinetic showed the importance of this compound. Indeed, it may be produced by several routes, as discussed subsequently.

From 10 to 30 min, several signals in the range 5.30–6.20 ppm appeared. These signals disappeared rapidly at room temperature. They could correspond to unstable alkene compounds resulting from ring openings of different aromatic products. These signals, as well as signals mentioned above, were not present in control samples, meaning that they are not produced by MnP lysis. For example, two “doublets” at 6.11 and 6.16 ppm and a broad signal at 6.06 ppm were observed on the 20-min spectrum. The apparent coupling constants for the doublets are approximately 10 Hz. The 6.16-ppm resonance is correlated with a signal at 7.54 ppm, as revealed by the corresponding 2D TOCSY spectrum (data not shown).

Two doublets at 8.30 and 8.72 ppm required special attention because they seem to belong to an unstable compound (Fig. 2). Indeed, after 60 min at room temperature (even without action of the enzyme), these doublets disappeared. These resonances do not appear to belong to a greater spin network, as revealed by 2D TOCSY spectra, thereby indicating a possible symmetrical aromatic compound. Moreover, such deshielded resonances let us envisage the presence of a highly polar group such as nitro. No match was found with 4-nitrobenzenesulfonate, as shown on the commercial compound spectrum. In fact, the high instability of the compound observed in our samples let us envisage the presence of 4-diazoniumbenzenesulfonate 5. Indeed, this diazonium salt must be a key step in the degradation pathway of azo dyes, but it has never been identified regardless of the analytical method used, although it has been suggested in several published studies [19,20]. The presence of this compound and its quantification are crucial to confirm or disconfirm the presence of an asymmetrical cleavage of the azo bond. So, this compound was synthesized near 0 °C, as described in [26], and the spectra of the crude reaction medium was recorded as a function of temperature, pH, concentration, and time (data not shown). As expected,  $^1\text{H}$  NMR spectra may be recorded within 5 min to avoid thermal decomposition of the diazonium. Taking these experimental conditions into account, the spectrum of the synthetic product showed the presence of two doublets at 8.30 and

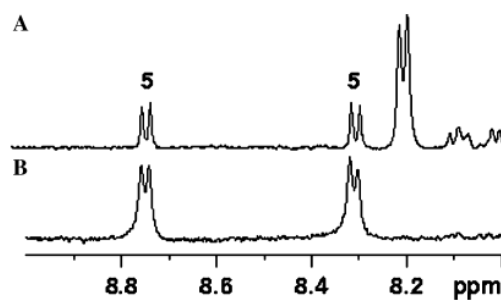


Fig. 4. Comparison of  $^1\text{H}$  NMR spectra of 15 min *in situ* experiment (A) with synthetic 4-diazoniumbenzenesulfonate salt (B).

8.72 ppm, identical to those observed in the kinetic samples (Fig. 4). This constitutes the first clear evidence of diazonium formation during Orange II degradation. The hydrolysis of diazonium salt into 4-hydroxybenzenesulfonate and benzenesulfonate is well established [35] and may constitute a source in the formation of these products during the degradation process. Moreover,  $^1\text{H}$  NMR spectra of synthetic 4-diazoniumbenzenesulfonate recorded at 25 °C after 1 and 4 h showed the appearance of benzenesulfonate and 4-hydroxybenzenesulfonate, respectively, thereby confirming this mechanism. Several unidentified products also appeared after several hours during this decomposition. To appreciate the importance of this “diazonium route,” it would be necessary to quantify the corresponding signals. However, because of thermal decomposition during spectra analysis ( $\sim 30$  min), the quantification was not precise. Still, we found that diazonium reached roughly 10% of Orange II degradation (Fig. 3). To go further in this quantification, we developed an enzymatic degradation of Orange II where  $\text{H}_2\text{O}_2$  was produced *in situ* by a glucose oxidase. This approach was used for a purely analytical goal. This *in situ* production of  $\text{H}_2\text{O}_2$  avoided perturbation of NMR signal detection due to the contribution of a turbulent flow of liquid. The *in situ*  $^1\text{H}$  NMR spectra were recorded at 12 °C to limit thermal decomposition and to avoid the loss of enzyme activities. Indeed, it should be noted that decreasing the temperature to 6 °C affected the enzymatic activities dramatically. In these conditions (12 °C), the Orange II degradation rate was limited to approximately 70% (due to the lack of agitation). On the other hand, the stability of the diazonium was greatly enhanced at this temperature in comparison with 25 °C. In fact, in these conditions, the diazonium production reached approximately 20% of Orange II transformation, indicating the importance of this route. Convinced that the enzymatic degradation pathway may be different in these conditions (e.g., lower temperature, different amount of  $\text{H}_2\text{O}_2$ ), we nevertheless observed the formation of several products identical to those found in the batch kinetic. It validated our quantitative approach and reinforced the role of the 4-diazoniumbenzenesulfonate in the enzymatic mechanism.

On the 20-min 2D TOCSY spectrum (30 ms mixing time), another well-resolved doublet resonating at 6.93 ppm is correlated to a doublet at 7.82 ppm (data not shown); this symmetrical aromatic compound has not yet been identified. 4-Nitrosobenzenesulfonate and 4-sulfophenylhydroperoxide were proposed (in [19] and [20], respectively) as possible intermediates or end products during azo dye transformation. The hypothetical  $^1\text{H}$  chemical shifts of the nitroso derivative could be consistent with those observed in the spectra [29]. Unfortunately, assays of 4-nitrosobenzenesulfonate synthesis were not completely successful because no product has been isolated as a pure compound, regardless of the method used [28–30]. However, it was possible to identify the products present in the different crude reaction mixtures by NMR ( $^1\text{H}$ ,  $^1\text{H}$ - $^{13}\text{C}$ , and  $^1\text{H}$ - $^{15}\text{N}$  HMBC experiments) and ESI-ion trap MS. These identifications were facilitated by the comparison of several synthesis methods and by the use of several concentrations and pH values for the analyzed solutions. Among the compounds identified, 4-nitrosobenzenesulfonate gave an AB system with two resonances at 8.07 and 8.10 ppm. In the degradation samples, it was not possible to identify these resonances due to the presence of many overlapped signals. However, it should be noted that C-nitrosoaromatic compounds can form dimers in solution [36]. In fact, whatever the synthesis method used, dimers of 4-nitrosobenzenesulfonate appeared to be preponderant in aqueous solutions, as confirmed by the analysis of the charge states in ESI-MS spectra and the  $^{15}\text{N}$  chemical shifts. This suggests that even if 4-nitrosobenzenesulfonate was generated during Orange II degradation, it would probably not have been produced at a high level due to its dimerization. Consequently, NMR might not be sensitive enough to detect it.

So far as 4-sulfophenylhydroperoxide is concerned, no clear data are reported in the literature for such aromatic compound synthesis and analysis. Thus, to assign the remaining NMR signals observed in the Orange II degradation spectra and to go further in the plausible involvement of 4-sulfophenylhydroperoxide as a transformation product, quantum calculations of  $^1\text{H}$  NMR

chemical shifts [37,38] were envisaged as a complementary tool to elucidate the structure of presumed benzenesulfonate derivatives. The calculations were carried out with the Gaussian 03 software [39]. Geometry of each identified and hypothetical structure was optimized within the density functional theory (DFT) with B3LYP functional in the 6-31G(d) basis set. The resulting optimized geometries were used as a starting point for GIAO NMR chemical shift calculations at the 6-311+G(2d,p) level. The preliminary results are summarized in Table 1. Because of an obvious lack of chemical shift averaging during calculations (calculations performed in a vacuum), no ideal symmetry was observed in terms of  $^1\text{H}$  chemical shifts except for 4-nitrosobenzenesulfonate. Nevertheless, it should be noted that even if the chemical shifts of the protons close to the sulfonate group are overestimated (by  $\sim 0.3$  ppm), the effect of the para function seems to be rather precise. So far as 4-hydroxy or 4-hydroperoxy is concerned, several para substituent orientations were envisaged; the values presented in Table 1 correspond to averaged values of each isomer. In this way, it appeared that 4-sulfophenylhydroperoxide is a quite realistic hypothesis.

Although the following point is discussed in more detail subsequently, it should be pointed out that the amount of compounds formed is approximately 30% of degraded Orange II, as shown by NMR (Fig. 3). It is clear that many other broadened signals appear from 60 min onward. They might correspond to rather insoluble aromatic compounds resulting from parallel oxidative reaction or from the biotic or abiotic decomposition of the “metabolites” described above. MS will certainly play an important role in their identification.

#### *Identification of the degradation compounds by ESI-ion trap MS*

To go further in the understanding of the Orange II degradation mechanism, we have examined the ESI-MS behavior of Orange II and its degradation products at different times of incubation with MnP. This analysis was performed on the supernatants without any treatment as for NMR analysis.

Table 1  
Calculated (GIAO DFT B3LYP/6-311+G[2d,p]) and experimental  $^1\text{H}$  chemical shifts (ppm) of various benzenesulfonates

Transformation product	H2		H6		H3		H5	
	Calc.	Exp.	Calc.	Exp.	Calc.	Exp.	Calc.	Exp.
4-H	8.16	7.82	8.41	7.82	7.16	7.56	7.18	7.56
4-OH	8.19	7.64	8.17	7.64	6.88	6.95	6.62	6.95
4-NH <sub>2</sub>	7.86		8.16		6.24		6.28	
4 <sup>+</sup> NH <sub>3</sub>	9.10	7.58	8.36	7.58	6.77	6.89	6.84	6.89
4-NO <sub>2</sub>	8.33	8.38	8.33	8.38	8.34	8.02	8.34	8.02
4-Diazonium	8.66	8.72	8.92	8.72	8.16	8.30	7.96	8.30
4-Hydroperoxy	8.04		8.21		6.03		6.17	

Note. Calc., calculated; Exp., experimental.

Because of its strong acidity, the sulfonic group is completely dissociated in aqueous solution at the reaction pH. Hence, negative ion mode MS analysis is more sensitive than positive ion mode for Orange II and its degradation products containing sulfonic groups, as reported in the literature for ESI-MS [40].

Mass spectrum obtained with the monosulfonated dye Orange II exhibited the  $[M-H]^-$  peak as the only observed ion (Fig. 5). Typical fragments ( $m/z$  247, 171, and 156) of this compound were observed as a result of in-source fragmentation, and their relative intensities increased when increasing voltages were applied to skimmer 1 ( $-50$  V) and capillary exit ( $-130$  V). Fragmentation of  $m/z$  327 produced five ions, the major one with  $m/z$  171 and the minor ones with  $m/z$  263, 247, 156, and 107 (Fig. 5). The mechanism proposed for the formation of  $m/z$  171 corresponds to the cleavage of the azo bond. This fragment is an odd electron ion, and its formation from an even electron  $[M-H]^-$  ion is due to collision with helium atoms in the ion trap. This fragmentation pathway has been established by Bruins and colleagues [41] for Acid Red 88, another monosulfonated azo dye. In the  $MS^3$  spectrum ( $327 > 171 > \text{products}$ ) of Orange II, the loss of  $SO_2$  (64 amu) leads to the  $m/z$  107 ion, and this confirms (in an unambiguous way) the presence of the sulfonate group in the  $m/z$  171 fragment. Thus, the loss of  $SO_2$  during fragmentation in negative mode can be used as a specific marker for selective monitoring of

the presence of a sulfonate group in degradation products.

Using negative mode ESI-MS, seven Orange II degradation products were identified: 4-hydroxybenzenesulfonate ( $m/z$  173), 4-aminobenzenesulfonate ( $m/z$  172), benzenesulfonate ( $m/z$  157), 4-sulfophenylhydroperoxide ( $m/z$  189), 2-naphthol ( $m/z$  143), 1-diazo-2-naphthol ( $m/z$  171), and 4-nitrosobenzenesulfonate ( $m/z$  186) (Figs. 6A and B).

For the first three products, the presence of a sulfonate group was confirmed by the loss of  $SO_2$  (64 amu) during fragmentation ( $m/z$  109 for 4-hydroxybenzenesulfonate,  $m/z$  108 for 4-aminobenzenesulfonate, and  $m/z$  93 for benzenesulfonate). 4-Hydroxybenzenesulfonate was detected from the beginning of the incubation, and its relative intensity increased regularly to reach a maximum at 60 min. The same profile was observed for benzenesulfonate, although the increase was less significant. On the other hand, the relative intensity of 4-aminobenzenesulfonate was weak and constant for the first 30 min, and it increased significantly from 60 min to the end of the incubation.

A small peak at  $m/z$  189 was visible and reached a maximum intensity at 60 min (Fig. 6); it may be assigned to 4-sulfophenylhydroperoxide 6, as suggested by Chivukula et al. [20]. The authors have reported that this compound is stable at ambient temperature but that it decomposes at temperatures above  $60^\circ\text{C}$ , and this could

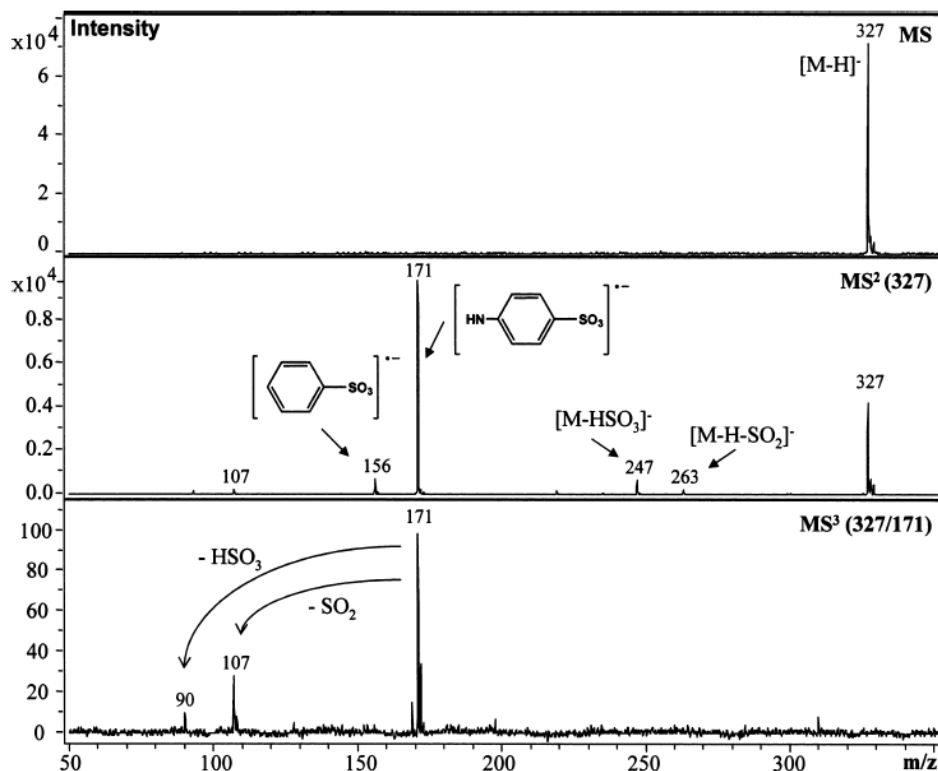


Fig. 5. ESI-ion trap mass spectra of Orange II ( $m/z$  327) and its  $MS^n$  fragmentations recorded in negative mode.

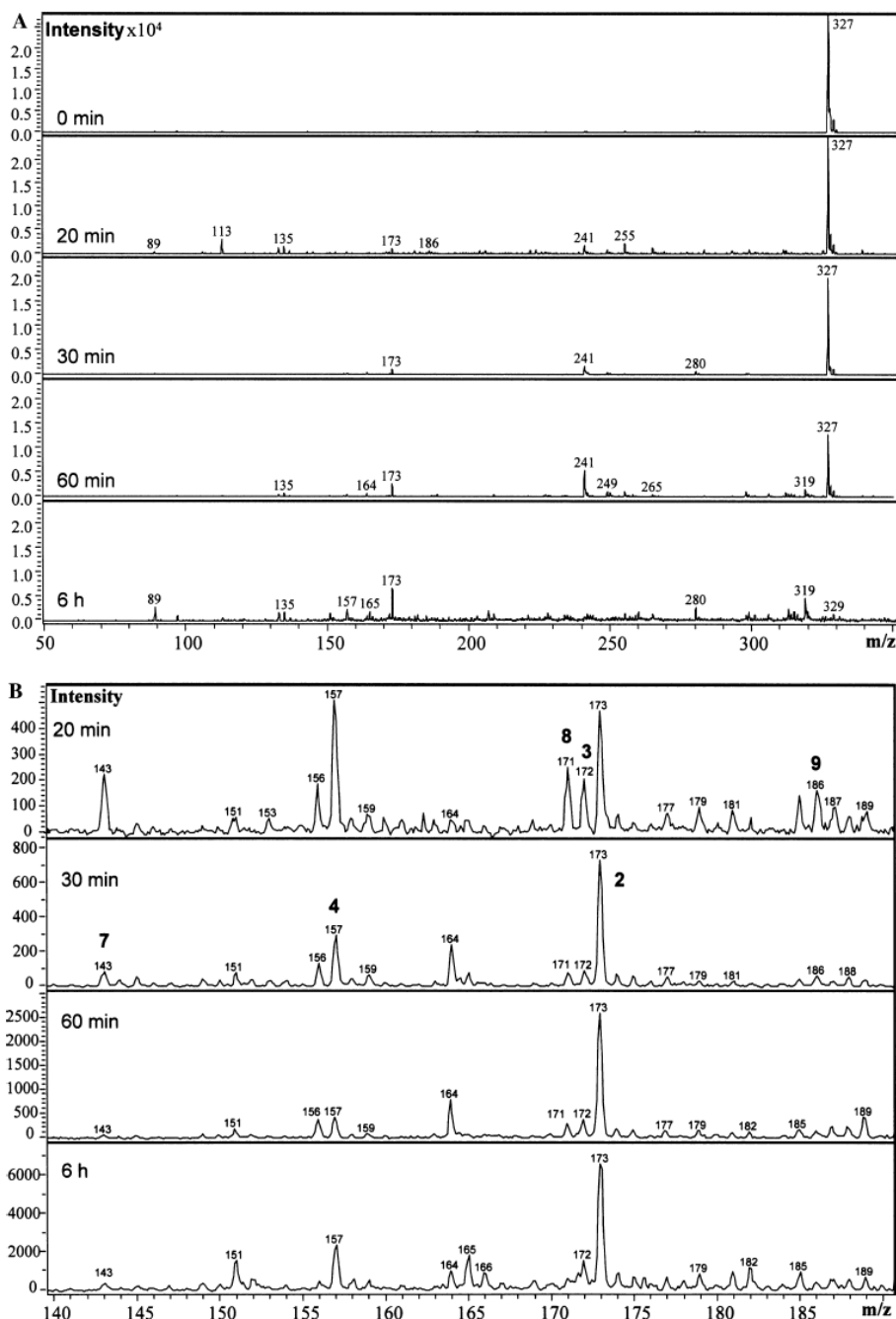


Fig. 6. (A) Ex situ ESI mass spectra of the samples taken after 0, 20, 30, and 60 min and 6 h of incubation of Orange II with MnP. (B) Expanded region from  $m/z$  140 to 192 for 20, 30, and 60 min and 6 h of reaction.

explain the low intensity of the corresponding signal. Because of its low intensity and the presence of several ions of close mass, it was not possible to obtain a correct MS/MS spectrum. So, this assignment was confirmed by in-source fragmentation performed in a limited range ( $m/z$  50–200) to avoid higher  $m/z$  ion fragmentation. This

experiment showed the disappearance of the  $m/z$  189 peak, whereas the intensity of the  $m/z$  173 and 157 peaks increased. These peaks resulted from the loss of one and two oxygen atoms, respectively, from the molecular ion. Chivukula et al. proposed that 4-sulfophenylhydroperoxide might be one of the two identified end products of

Orange II degradation. This point is discussed subsequently.

An ion at  $m/z$  143 was observed all along the kinetic at a relative constant level. It was assigned to the molecular ion of 2-naphthol **7** (Fig. 6). This is the first time that this compound has been proposed as an Orange II transformation product. 2-Naphthol was not clearly identified by  $^1\text{H}$  NMR analysis due to its low concentration in the samples ( $<10\ \mu\text{M}$ ) and the overlapping of its signals with those of many other compounds. However, its presence was clearly confirmed by HPLC analysis, as explained below.

A peak at  $m/z$  171 was observed after 10 min (data not shown) at a relatively low intensity that increased to reach a maximum at approximately 60 min. To confirm that this peak did not originate from an Orange II fragment (see above),  $\text{MS}^n$  experiments were performed; the  $m/z$  107 Orange II characteristic  $\text{MS}^3$  fragment was not observed, and the  $m/z$  171 peak was assigned to 1-diazo-2-naphthol **8** (Fig. 6). Its presence is consistent with that of 2-naphthol after the loss of  $\text{N}_2$ . In addition, 1-diazo-2-naphthol hydrolysis may yield to 1,2-dihydroxynaphthalene with a characteristic molecular ion at  $m/z$  159 in negative mode. Such a peak was effectively observed on the MS spectra in negative mode but at too low a level to accredit these results.

After 10 min of incubation with MnP (data not shown), a peak at  $m/z$  186 was observed. It could be attributed to 4-nitrosobenzenesulfonate **9** (Fig. 6). This compound present in the reaction mixture at the beginning of the kinetic seems to decrease rapidly. Actually, the nitroso group could easily be reduced to an amine-generating sulfanilate ( $m/z$  172).

Positive ionization mode was also tested to detect positively charged species (data not shown). Only one

compound, 1,2-naphthoquinone, was identified in these conditions due to its unambiguous  $m/z$  159 ion. It appeared at 10 min (data not shown), and its maximum relative intensity was reached at 20 min. The MS/MS analysis of protonated 1,2-naphthoquinone  $[\text{M} + \text{H}]^+$  ( $m/z$  159) showed a single peak at  $m/z$  131 corresponding to the loss of CO (28 amu). This fragmentation was also observed with the commercial product.

1-Amino-2-naphthol might be a potential degradation product [16]. The commercial compound was detected only in positive mode, showing an  $[\text{M} + \text{H}]^+$  ion at  $m/z$  160. This compound was not detected in our study regardless of the ionization conditions used.

#### Identification of the degradation compounds by HPLC

HPLC analyses were performed to confirm the presence of the low concentrated degradation products. For this purpose, commercial standards were used as calibration standards. Elution times and UV spectra were compared with those determined with the degraded samples. Thus, the identities of three compounds were confirmed. Fig. 7 presents a chromatogram of a 1-h enzymatic degradation sample. The Orange II was eluted at approximately 6.93 min, but the retention time varied slightly depending on the concentration. Actually, the UV signal corresponding to Orange II decreased and its retention time increased slightly from sample to sample. It should be notified that two peaks (1.59 and 1.86 min) appeared on HPLC chromatograms. They were also present in the enzymatic crude. The peak at 2.01 min (Fig. 7, compound **2**) was identified as 4-hydroxybenzenesulfonate, and its concentration decreased with time to reach  $80\ \mu\text{M}$  at 8 h.

1-Amino-2-naphthol and 1,2-naphthoquinone gave similar UV spectra and chromatograms showing two

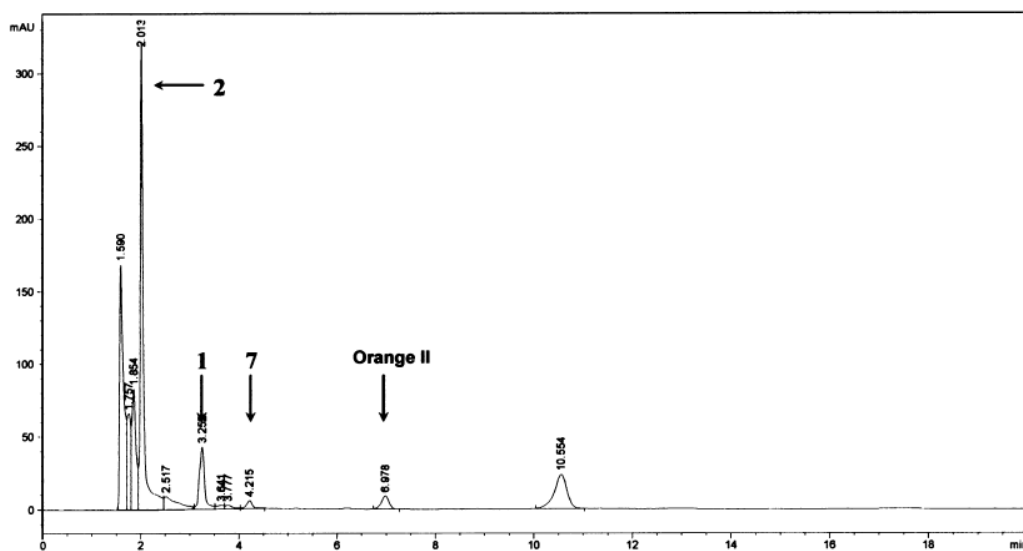


Fig. 7. HPLC chromatogram of the 60-min sample: (1) 1,2-naphthoquinone; (2) 4-hydroxybenzenesulfonate; (7) 2-naphthol.

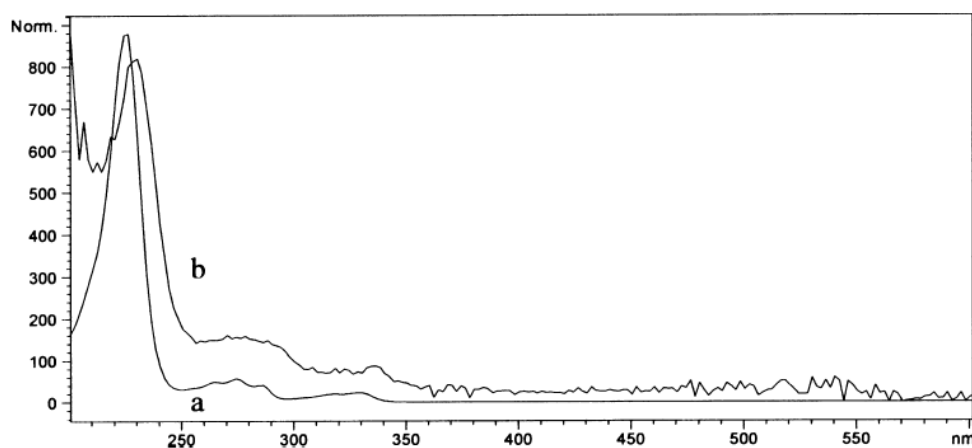


Fig. 8. UV spectra of commercial 2-naphthol (a) and of 4.215-min HPLC peak (b) in the 60-min sample.

main peaks (3.23 and 10.88 min) and several small signals. It is well known that these compounds are very unstable and may be degraded into the same products. Moreover, a quinone/hydroquinone-type equilibrium may be concerned. Regardless, 1,2-naphthoquinone peaks presented higher area than did those of 1-amino-2-naphthol at the same concentration. And on the 60-min chromatogram (Fig. 7, compound 1), the 3.23-min signal area reached a level that is not compatible with 1-amino-2-naphthol. Thus, this peak was assigned to 1,2-naphthoquinone, in agreement with NMR and ESI-MS results.

The 4.21-min signal observed on the 60-min chromatogram (Fig. 7, compound 7) was assigned to 2-naphthol. This result, confirmed by the corresponding UV spectra (Fig. 8), is consistent with results obtained with ESI-MS. Besides, it should be noted that the concentration of this compound was less than 10  $\mu\text{M}$ . This could explain why this compound was not detected by ex situ NMR.

The time course of Orange II and the degradation products by HPLC confirmed the NMR data; that is, the identified products represent only 30% of the degraded Orange II, considering a stoichiometric relationship of 1:1 with respect to 1,2-naphthoquinone and 4-hydroxybenzenesulfonate. On the one hand, this imbalance might be due to a parallel production of other compounds originating from different degradation pathways or from the natural decomposition of some intermediates. On the other hand, the imbalance might be due to the decomposition or enzymatic degradation of these compounds themselves. However, it might also be due to various kinetic factors.

#### *Enzymatic degradation of the intermediates*

Because of the observed production of benzenesulfonate and the disappearance of 4-hydroxybenzenesulfonate, we supposed that those products could be further

degraded by the enzymatic system. So, the degradation of sulfanilate, 4-hydroxybenzenesulfonate, and benzenesulfonate by MnP was analyzed by NMR and HPLC. Benzenesulfonate was not degraded, but the other two compounds reached degradation percentages of 70 and 90%, respectively, after 8 h. Many signals were visible on the corresponding  $^1\text{H}$  NMR spectra, but their very low intensity did not allow us to identify them. HPLC analysis showed the appearance of several peaks, but we were not able to identify them on the sole basis of their UV spectra. It should be noticed that a peak at 1.76 min increased during sulfanilate and 4-hydroxybenzenesulfonate degradation.

#### *Toxicity of the crude extracts and identified transformation products*

Identifying products formed during wastewater treatment is essential to determine their potential toxicity and then to modify the process. Thus, the toxicity of some metabolites identified was analyzed using the Microtox normalized test. As shown in Table 2, no toxic effect was observed with the Microtox test for Orange II, the effluent of the enzymatic degradation, 4-hydroxybenzenesulfonate, and benzenesulfonate in the range of tested concentrations. Nevertheless, 1,2-naphthoquinone and 2-naphthol were found to be toxic in these conditions.

#### *Proposed mechanism*

Several azo dye degradation mechanisms by microorganisms or isolated enzymes have been proposed previously [16,18–20]. Two main analytical strategies were used by the authors to propose these pathways: extraction of supernatants followed by HPLC and GC-MS (electronic impact) analyses or samples analysis by MS (ESI-MS and FAB-MS techniques) and/or HPLC-MS or GC-MS after extraction and derivatization.

Table 2  
Microtox normalized toxicity tests of nine samples

Sample	Dilution factor	EC <sub>50</sub> (5) (ppm)	EC <sub>50</sub> (5) (equitox/m <sup>3</sup> ) <sup>a</sup>	EC <sub>50</sub> (15) (ppm)	EC <sub>50</sub> (15) (equitox/m <sup>3</sup> ) <sup>a</sup>	Toxicity
A	100	3,471	288	3478	287	No toxic
B	2	61,599	16	40,718	25	No toxic
C	2	356,320	3	354,882	3	No toxic
D	1	847,790	1	n.d.	—	n.d.
E	10	n.c.	—	n.c.	—	n.c.
F	1	n.d.	—	n.d.	—	n.d.
G	10,000	20	50,505	12	82,645	Toxic
H	1000	212	4717	207	4831	Toxic
I	20	n.c.	—	n.c.	—	n.c.

Note. A, 10 mM Orange II; B, 100 ppm Orange II, 1 mM oxalate, 33 μM Mn<sup>2+</sup>; C, enzymatic degradation effluent; D, 10 mM 4-hydroxybenzenesulfonate; E, 10 mM sulfanilate; F, 10 mM benzenesulfonate; G, 10 mM 1,2-naphthoquinone in acetonitrile; H, 10 mM 2-naphthol in acetonitrile; I, acetonitrile; n.c., no conclusive results in the conditions of the assay; n.d., no detectable level of toxicity with this methodology.

<sup>a</sup> Equivalence between percentage of dilution and equitox/m<sup>3</sup> is established on the basis of the definition of the regulation AFNOR T 90-301, attachment B, page 8: number equitox/m<sup>3</sup> = (1/EC<sub>50</sub>)\*100.

Because of the different analytical methods used, two main routes were proposed in the literature: a symmetrical azo bond cleavage [16] and an asymmetrical [18,20] azo bond cleavage. Some authors suggested the coexistence of the two pathways after identification of one or two “end products” [19]. Nevertheless, there was no clear evidence of the implication of one method or the other.

With the approach proposed in our work, it is obvious that both symmetrical and asymmetrical azo bond cleavages take place. The concomitant analyses by ex situ NMR and ESI-MS let us propose new routes for Orange II degradation, including three new “metabolites” (Fig. 9).

The presence of 4-nitrosobenzenesulfonate and sulfanilate in the reaction mixture indicated a symmetrical cleavage of the azo linkage even if 1-amino-2-naphthol was not observed. Indeed, reduction of nitrosobenzene derivatives may occur electrochemically, by hydrated electrons, or by chemical-reducing agents [42]. This reduction proceeds in two steps: reduction to hydroxylamine and then to amine. In addition, it should be noted that nitrosobenzene derivatives may be the target of many nucleophiles, leading to many potential degradation products. In other respects, sulfanilate was rapidly transformed by MnP in our conditions. The same phenomenon was observed when sulfanilate was used in a degradation study with a whole culture of *Phanerochaete chrysosporium* [43] and with ligninase [19].

Formation of 1,2-naphthoquinone and 4-diazoniumbenzenesulfonate (observed for the first time during Orange II degradation) was compatible with an asymmetrical cleavage near the naphthalene moiety. This diazonium derivative may result from a spontaneous oxidation of phenylhydrazinesulfonic acid (not observed), as suggested by Goszczynski et al. [19]. Hydrolysis of the diazonium salt produces the 4-hydroxybenzenesulfonate, and its reduction produces the benzenesulfonate. In our study, both were observed by NMR and ESI-MS. As

reported by Chivukula et al. [20], the phenyl radical generated by elimination of molecular nitrogen from the diazonium compound can be scavenged by oxygen to yield the corresponding 4-sulfophenylhydroperoxide. The authors identified this compound on the basis of FAB analysis, but they did not observe it using ESI. Chivukula et al. suggested that it was transformed in the source to 4-hydroxybenzenesulfonate and benzenesulfonate without emphasizing the importance of these two compounds in the mechanism, as was clearly evidenced by <sup>1</sup>H NMR in our study. Our ESI-MS results clearly indicate that 4-hydroxybenzenesulfonate and benzenesulfonate are formed since the beginning of the degradation. These observations are in agreement with the presence of 4-sulfophenylhydroperoxide in our samples, as shown by ESI-MS.

The third simultaneous degradation pathway is a second asymmetrical cleavage near the phenyl moiety. This pathway leads to the formation of 4-hydroxybenzenesulfonate and 1-diazo-2-naphthol, the latter of which may be transformed, in turn, into 2-naphthol by loss of N<sub>2</sub>.

## Conclusion

The approach used in this study allows direct identification of nine compounds during Orange II enzymatic degradation. Moreover, we have shown that both symmetrical and asymmetrical azo bond cleavages occurred in our enzymatic system. The use of ex situ and in situ NMR was crucial to get the real fingerprint of the samples. This noninvasive method performed directly on the incubation medium allows us to identify 4-diazoniumbenzenesulfonate as a key product in the enzymatic degradation pathway. This compound has never been identified previously due to its high thermal instability. Quantification of degradation products was also possible insofar as signals were not overlapped and the concentra-

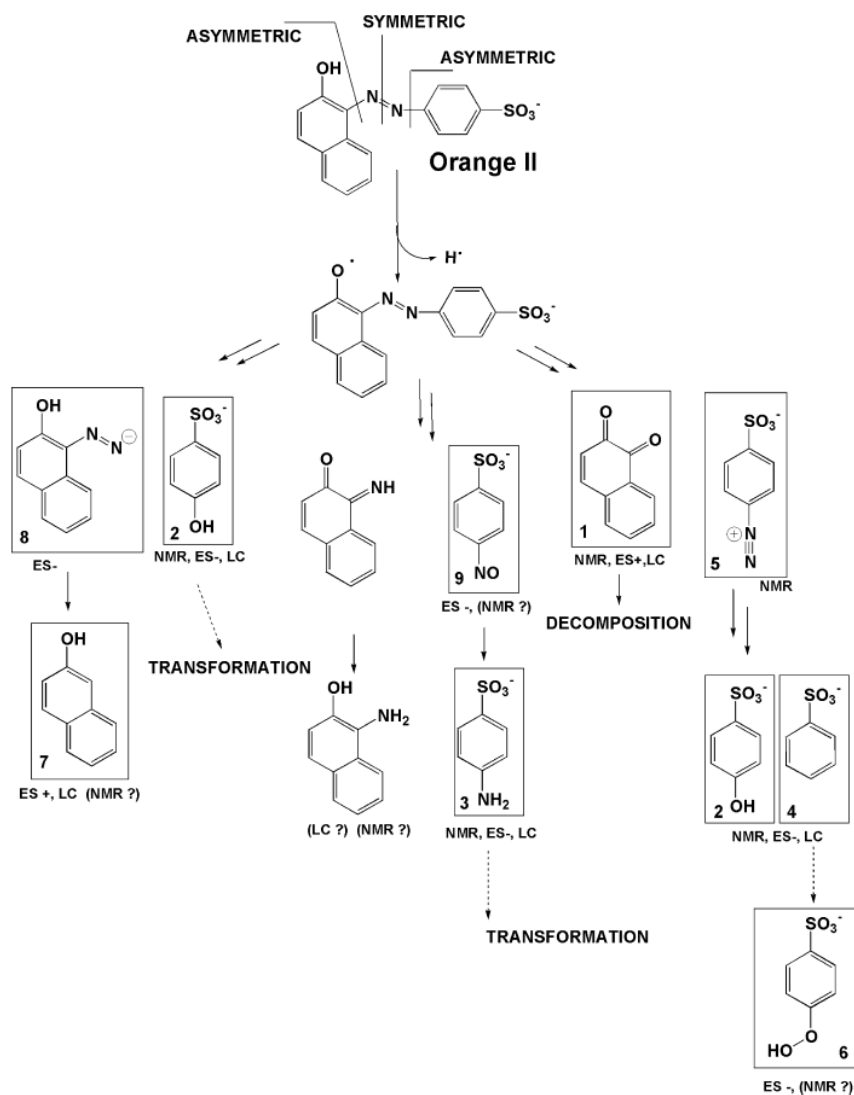


Fig. 9. Proposed degradation pathway of Orange II by MnP: (1) 1,2-naphthoquinone; (2) 4-hydroxybenzenesulfonate; (3) 4-aminobenzenesulfonate; (4) benzenesulfonate; (5) 4-diazoniumbenzenesulfonate; (6) 4-sulfophenylhydroperoxide; (7) 2-naphthol; (8) 1-diazo-2-naphthol; and (9) 4-nitrosobenzenesulfonate.

tion was not less than 10  $\mu$ M. Despite the complexity brought about by the ex situ approach, we have used ESI-ion trap MS to confirm NMR data. All compounds observed in <sup>1</sup>H NMR spectra were found in MS analysis, with the exception of diazonium salt. Moreover, compounds not observed in NMR spectra for sensitivity reasons have been observed by MS, for example, 1-diazo-2-naphthol and 2-naphthol. 2-Naphthol, the presence of which was also confirmed by HPLC, had never been described previously as an azo dye degradation product, but it is a key point in the enzymatic mechanism.

The enzymatic process used in this work allows a complete and rapid degradation of Orange II. By analyzing several degradation products identified in the crude samples, we have shown that at least two of them were

toxic (using the Microtox test). Taking this fact into account, work is in progress to develop a process allowing total mineralization of azo dyes.

Finally, the contribution of the ex situ approach in such an application and the complementarity of NMR and MS analyses are undeniable and can easily be extended to other azo dye studies. In addition, NMR and MS spectra collected during this work may be used as a spectral data bank for future studies.

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