JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Identification and Formation Pathway of Laccase-Mediated Oxidation Products Formed from Hydroxyphenylureas

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Hydroxyphenylureas are the first main metabolites formed in the environment from pesticide and biocide urea compounds. Because fungi release potent exocellular oxidases, we studied the ability of laccases produced by the white rot fungus, *T. versicolor*, to catalyze in vitro the transformation of five hydroxyphenylureas, to identify transformation pathways and mechanisms. Our results establish that the pH of the reaction has a strong influence on both the kinetics of the reaction and the nature of the transformation products. Structural characterization by spectroscopic methods (NMR, mass spectrometry) of eleven transformation products shows that laccase oxidizes the substrates to quinones or to polyaromatic oligomers. Slightly acidic conditions favor the formation of quinones as final transformation products. In contrast, at pH 5−6, the quinones further react with the remaining substrate in solution to give hetero-oligomers via carbon−carbon or carbon−oxygen bond formation. A reaction pathway is proposed for each of the identified products. These results demonstrate that fungal laccases could assist the transformation of hydroxyphenylureas.

KEYWORDS: Laccase; white rot fungi; *T. versicolor*; transformation pathway; hydroxyphenylurea; quinone; polyaromatic oligomers; reaction mechanism

INTRODUCTION

Phenylurea compounds have become extensively widespread in the environment since their discovery in the 1950s. Most of them are mainly used as herbicides for the selective protection of cereal crops and for the maintenance of roads and railways. Others enter the industrial processes as biocides and/or protective agents against oxidation. Nevertheless, these compounds are persistent contaminants of the environment, including soil and both surface and ground waters (*1*, *2*). As a consequence, there is an increasing interest in the biotic and abiotic degradation pathways of these chemicals (*3*). Biodegradation of phenylureas in soils is governed by many environmental factors, such as pH, temperature, and the presence of degrading micro-organisms (*4*). Only one bacterial strain, *Arthrobacter sp. N*2 (*5*), was identified as an efficient degrader of diuron [*N*-(3,4-dichlorophenyl)-*N*′,*N*′-dimethylurea] into its corresponding aniline. In soils, fungal strains transform phenylureas to N-demethylated and/or hydroxylated derivatives (*6*). Hydroxylation of the aromatic ring of phenylureas during their metabolization in higher plants has also been reported for monuron [*N*-(4-chlorophenyl)-*N*′,*N*′-dimethylurea], which is transformed to *N*′,*N*′-dimethyl-*N*-(4-chloro-2-hydroxyphenyl)urea (*7*). Of particular importance, direct and indirect photolysis of substituted phenylureas exposed to sunlight leads to several photoproducts, including those resulting from ring hydroxylation and substitution of a chlorine atom by a hydroxyl group (*8*−*14*). Functionalization by hydroxylation of the aromatic ring considerably extends the range of metabolic reactions able to further transform the pollutants (*5*−*7*).

In this context, white rot fungi, such as *T. versicolor*, offer many advantages in secreting nonspecific extracellular oxidases. Among these enzymes, laccases (EC 1.10.3.2, *p*-diphenol: dioxygen oxidoreductases) have been reported to catalyze the transformation of a wide range of xenobiotics, including phenols and chlorinated phenols. They convert these chemicals to radicals, which react to form stable polymers by coupling to each other or with humic substances in soils (*15*−*17*). Such a oxidative coupling process is a key mechanism for the formation of bound residue in the soil and for the precipitation of insoluble oligomers in waters. Because these polymers can be easily removed from water by filtration, such an enzymatic approach can be of particular interest in the decontamination of aqueous effluents (*18*).

Up to now, our work was mainly focused on the transformation of nonchlorinated derivatives by fungal laccases (*19*). In the present study, we included chlorohydroxylated phenylurea compounds, so that the hydroxylated phenylurea derivatives studied in this work are representative of the range of hydroxy-

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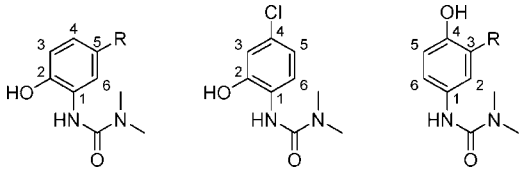
10.1021/jf060351i CCC: $33.50
Published on Web 06/15/2006

Scheme 1. Structure of Phenylurea Derivatives Used as Laccase Substrates



lated metabolites of some phenylurea herbicides found on the fields.

The potential of fungal laccases in catalyzing the transformation reactions of these pollutants as a function of the pH was investigated. In addition, we determined the chemical structures of the main products formed from nonchlorinated and chlorinated hydroxyphenylureas during the enzymatic reactions, allowing us to propose transformation pathways and reaction mechanisms. Our results better define (i) the fate of phenylureas in the environment and (ii) the potential application of laccases in decontamination efforts.

MATERIALS AND METHODS

Chemicals. Acetonitrile (HPLC grade) was obtained from Carlo Erba (Val de Reuil, France). Chemicals used as buffers and silica for chromatography (Gerudan Si 60, 40−63 μm) were purchased from VWR (Fontenay sous bois, France). Chlorzoxazone (5-chloro-2-benzoxazolone) was from Acros (Noisy le Grand, France). All other chemicals or reagents used for synthesis and assays, including 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic) acid (ABTS), were obtained from Sigma (Saint Quentin Fallavier, France).

N′,*N*′-Dimethyl-*N*-(4-hydroxyphenyl)urea (**4HF**), *N*′,*N*′-dimethyl-*N*-(2-hydroxyphenyl)urea (**2HF**), *N*′,*N*′-dimethyl-*N*-(4-chloro-2-hydroxyphenyl)urea (**2HF-4Cl**), *N*′,*N*′-dimethyl-*N*-(5-chloro-2-hydroxyphenyl)-urea (**2HF-5Cl**), and *N*′,*N*′-dimethyl-*N*-(3-chloro-4-hydroxyphenyl)urea (**4HF-3Cl**) were prepared according to procedures adapted from the literature (*19*−*21*) (see Supporting Information). The chemical structures of these compounds are shown in **Scheme 1**.

Production of *T. versicolor* Laccase. The production and purification of laccase have been described elsewhere (*22*). Briefly, the enzyme was produced from *Trametes versicolor* cultures, induced by 2,5-xylidine in a 5-L bioreactor. The purification included two steps of chromatography, a first one on a DEAE 52 anion exchange column, and a second one on a Phenyl Sepharose (Pharmacia HiTrap) column. The purification procedure led to a homogeneous sample, as checked by electrophoresis, with a specific activity of 300 U/mg, where one unit of enzyme activity (U) is defined as the amount of enzyme that oxidizes 1 μmol of ABTS in 1 min.

Assays for laccase activity were performed by measuring the enzymatic oxidation of ABTS at 420 nm ($\epsilon = 3.6 \times 10^4$ cm^{-1} M^{-1} for the oxidation product). The reaction mixture contained 1 mM ABTS in 0.1 M Na_2HPO_4/citric acid buffer (pH 3.0) in a final volume of 1 mL. The buffer solution was saturated with air by bubbling prior to the experiment. After the enzymatic solution was added, the increase in absorbance was followed during the first 2 min, at 30 °C.

Enzymatic Reactions with Hydroxyphenylurea Derivatives. *pH Activity Profile of T. versicolor Laccase.* Determination of the optimum pH was conducted in 50 mM citrate/phosphate buffer in the range of pH 2.5−7. The chlorinated hydroxyphenylureas were solubilized at 0.1 g/L, and 0.05 U of laccase was added in a total volume of 5 mL. Samples were incubated and shaken (150 rpm) at 30 °C for 15 min. The reaction was then stopped by adding 0.5 mL of concentrated HCl. Quantitative analysis of phenylurea transformation was conducted using an HPLC system (Varian) with a reverse phase column (NovaPak C8, Waters, 2.3 mm × 150 mm). Elution was carried out at a flow rate of 1 mL min^{-1} using a solvent gradient from 100% water to 100% acetonitrile within 20 min. Detection was performed at 244 nm using a UV detector. Retention times for the hydroxyphenylureas tested ranged from 7 to 11 min. Experiments with **2HF-5Cl** were duplicated.

Time Course of Hydroxyphenylurea Transformation. Hydroxyphenylureas (1 mM) were shaken at 150 rpm at 30 °C for 2 h with a specified amount of enzyme in 10 mL of 25 mM citrate/phosphate buffer at various pH values. Incubations without enzyme served as the control. After defined periods of incubation, the reaction assays were stopped with 10% (v/v) concentrated HCl and filtered (Millipore 0.22 μm) before HPLC analysis.

Incubation with Phenylureas and Extraction of Oxidation Products. Hydroxyphenylureas (25−50 mg) were incubated with 5−10 U of laccase in 25−50 mL of aerated citrate/phosphate buffer (50 mM) at 30 °C in darkness. The extent of transformation was checked by HPLC, and the incubation stopped when the totality of the initial phenylurea was transformed. Reaction mixtures were acidified to pH 2 by addition of 1 M HCl. The reaction mixture (aqueous phase and precipitate) was treated with sodium chloride (2.5 mol·L^{-1} final concentration) and extracted with dichloromethane (25 mL) three times. The extracts were combined, dried over anhydrous sodium or magnesium sulfate, and evaporated on a vacuum evaporator at 40 °C. The residues were dissolved in 5 mL of ethyl acetate and separated on a silica column using ethyl acetate as an elution solvent. Identification of pure compounds isolated from the column was performed by mass and NMR spectroscopy.

Analytical and Spectroscopic Methods. MS and MSMS spectra were obtained using a triple quadripole instrument Nermag R 30-10 (Quad Service, Poissy, France) in electronic impact (EI) and chemical ionization (CI) modes. A Quattro LC (Micromass, Manchester, U.K.) equipped with an electrospray source ionization (ESI) (Z-spray from Micromass) was used for LC-MS determinations.

Nermag R 30-10 source conditions were set as follows: temperature, 130 °C; electron energy, 70 eV (EI) or 95 eV (CI). Samples were introduced either by gas chromatography or with a direct insertion probe for ionization by EI or by desorption chemical ionization (DCI). NH_3 was used as reagent gas at 10^{-4} Torr pressure in the source housing, and for MS-MS experiments argon was used as collision gas (4×10^{-2} Torr).

The Quattro LC electrospray source parameters were as follows: capillary, 3.25 kV; extractor, 2 V; source block temperature, 120 °C; desolvation gas, 500 L/h N_2; temperature, 400 °C. The sampling cone voltage was varied usually from 20 to 40 V. Data acquisition and processing were carried out using the software MassLynx version 4.0. The compounds were introduced by infusion (Harvard Apparatus, Holliston. MA).

^{1}H and ^{13}C NMR spectra were recorded at 293 K, on Bruker AC 200 equipment or on a Varian Mercury plus 300 instrument in deuteriochloroform ($CDCl_3$), benzene-[D_6], or DMSO-[D_6]. Chemical shifts are reported in δ ppm relative to $CHCl_3$ ($CDCl_3$) as internal reference: 7.27 ppm for ^{1}H (77.14 ppm for ^{13}C) or measured with $SiMe_4$ as internal reference following standard techniques. For the other cases, residual solvent was also used as internal standards (*23*). Coupling constants (*J*) are given in hertz (Hz). Multiplicities are recorded as s (singlet), d (doublet), t (triplet), q (quadruplet), m (multiplet), and br (broad).

RESULTS

Influence of pH on the Transformation of Chlorinated Hydroxyphenylureas by Laccase. pH-curves (**Figure 1**) exhibit the same bell shaped profile for all the chlorinated hydroxyphenylureas with a maximum between pH 4 and 5. A slight difference was observed for **2HF-5Cl**, whose pH-curve was found to be broader. In the case of **4HF-3Cl** and **2HF-4Cl**, laccase showed only residual activity at pH 7, while, with **2HF-5Cl**, the enzyme retained up to 15% of its maximal activity.

Time Dependence of the Transformation of 4HF-3Cl by Laccase. The analytical method used to follow the transformation of the chlorinated hydroxyphenylurea derivatives as a function of the pH showed that pH influences the nature of the reaction products. A similar behavior was observed for all the

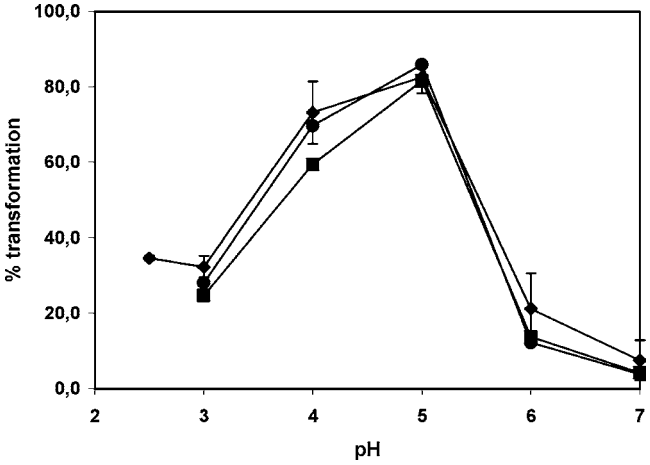


Figure 1. Influence of pH on the transformation of chlorinated hydroxyphenylureas by laccases: (◆) **2HF-5Cl**; (●) **4HF-3Cl**; (■) **2HF-4Cl**. Substrate initial concentration: 0.1 g/L in 0.1 M citrate/phosphate buffer; $T = 30$ °C. Laccase concentration: 0.011 units for **2HF-5Cl**; 0.05 units for **4HF-3Cl** and **2HF-4Cl**. Incubation duration: 15 min.

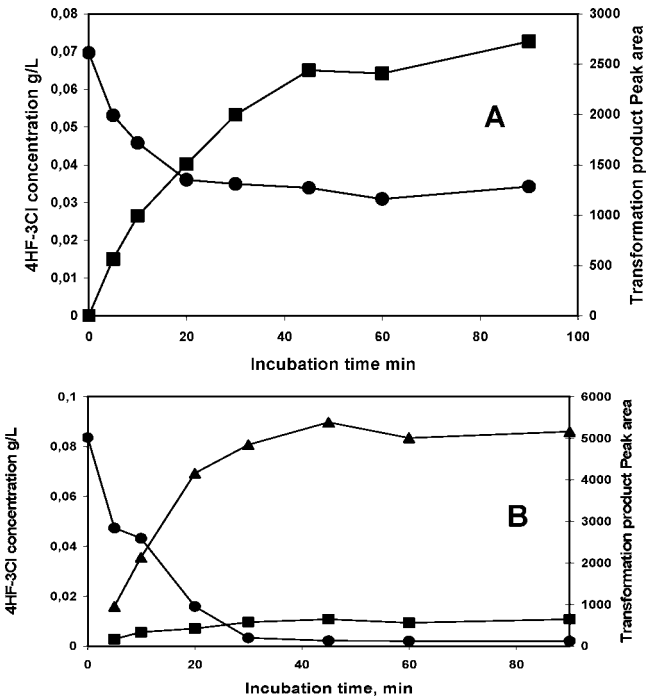


Figure 2. Time course of **4HF-3Cl** and product concentrations during the transformation of **4HF-3Cl** by *T. versicolor* laccase (0.03 units/mL) at pH 3 (**A**) and 5 (**B**): (●) **4HF-3Cl**; (■) product **I**; (▲) product **V**.

tested compounds. Consequently, transformation of **4HF-3Cl** was chosen here as a typical example. The time course of **4HF-3Cl** transformation was carried out in the presence of laccase at two pH values: 3 and 5 (**Figure 2**). At pH 3 (**Figure 2A**), the enzymatic transformation of **4HF-3Cl** led to the formation of one predominant product, **I**, whose retention time (t_R= 3.4 min) was smaller than that of **4HF-3Cl** ($t_R = 7$ min), indicating a higher polarity of the product. At pH 5 (**Figure 2B**), the transformation of **4HF-3Cl** was more rapid than that at pH 3 and led to the formation of two products, **I** and **V** ($t_R = 7.4$ min). After 45 min, 97% of the **4HF-3Cl** was transformed whereas the concentration of each of the two products reached a maximum at this time. The amount of compound **V** slightly decreased thereafter, whereas that of product **I** remained constant. The formation of both products, thus, seems to proceed via a competitive process, since product **V** did not seem to result from the further transformation of product **I**.

Transformation of all chlorinated hydroxyphenylureas displayed a similar behavior to that observed with **4HF-3Cl**: a quinonic predominant product at pH 3, more polar than its parent compound. When increasing the reaction pH, the quinonic compound concentration decreased, favoring one or more less polar oxidation products.

Identification of Products Formed from Hydroxyphenylureas by Laccase. Proposed structures for the transformation products are shown in **Tables 1**−**3**, including their ^{1}H NMR and mass spectroscopic data.

The ^{1}H NMR spectrum of compound **I** showed only one singlet (δ 6.74 ppm), suggesting a symmetric localization of hydrogen atoms on the aromatic ring. The molecular mass was found to be 108. Comparison (spectroscopic data, retention time) with commercial standards led to its identification as 1,4-benzoquinone (**Table 1**).

The ^{1}H NMR spectrum of compound **II** (**Table 1**) showed only the presence of three aromatic hydrogen atoms. According to the observed coupling constants and the multiplicity of the peaks, the signal pattern is consistent with the presence of three protons in the 3, 5, and 6 positions on the ring. From mass spectroscopy, we know that the compound retained its chlorine atom during the transformation. Together with a molecular mass of 142, this information is in agreement with the structure of 2-chloro-1,4-benzoquinone, as confirmed by comparison with the spectroscopic data of the commercial standard.

The ^{1}H NMR spectrum of compound **III** (**Table 1**) indicated the presence of a $N(CH_3)_2$ group (δ 3.08 ppm). Chemical ionization mass spectrometry indicated that the parent molecule loses its chlorine atom during the oxidation process, leading to a compound with a molecular mass of 194, which is in accordance with the structure shown in **Table 1**.

Compound **IV** was the major product obtained by oxidation of **4HF** at pH 5. The NMR spectrum (**Table 2**) indicated the presence of a $N(CH_3)_2$ group and of 2 × 2 aromatic protons. The MS fragmentation pattern (EI spectrum) gave a *m*/*z* of 178 with fragments 134 and 106 representative of the successive fragmentations of the substituted urea group on the parent molecule. On the basis of these large similarities with the parent molecule, together with published ^{1}H NMR data on acetyl *p*-benzoquinone imine (*24*), it was concluded that compound **IV** was the 1,1-dimethyl-3-(4-oxocyclohexa-2,5-dienylidene)-urea resulting from the oxidation of **4HF**.

Compound **V** was obtained from transformation of **4HF-3Cl**. From ^{1}H NMR spectrum analysis (**Table 2**), it can be assumed that the molecule retained the dimethylated substituted phenylurea motif. The aromatic hydrogen substitution pattern on the ^{1}H NMR spectrum is very similar to that observed for **II**. Data from mass spectrometry indicated a molecular mass of 212, i.e., loss of two mass units compared to the parent molecule and the presence of one chlorine atom. The ^{13}C spectrum showed three CH (140.92, 134.04, 127.79) and two quaternary carbons (179.02 and 161.96), whose chemical shift is very close to that observed for a 2-chloro-1,4-quinonimine cycle, confirming the structure of **V** as (3*E*)-3-(3-chloro-4-oxocyclohexa-2,5-dienylidene)-1,1-dimethylurea. It was noticed that the ^{13}C NMR and ^{1}H NMR spectra contained additional peaks (around 40% of the total intensity) assigned to the corresponding (3*Z*) isomeric form of the molecule.

The oxidation products formed during the transformation of *ortho*-hydroxylated phenylureas **2HF** and **2HF-4Cl** at pH 5 were

Table 1. ^{1}H NMR and Mass Spectroscopic Data for Hydroxyphenylurea Transformation Products at pH 3

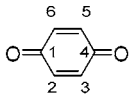
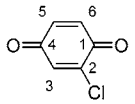
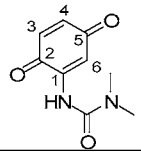
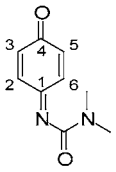
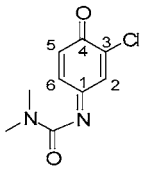
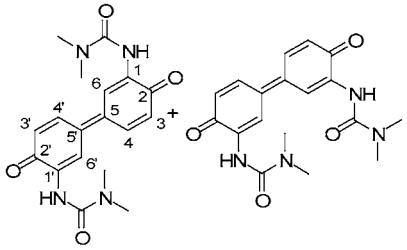
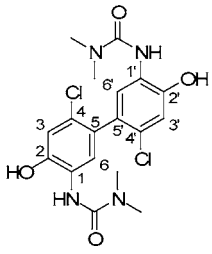
Parent molecule	Product identification number		^{1}H NMR (300MHz) Solvent $CDCl_3$ Position	δ_H(multiplicity, J) (200 MHz)	MS *m/z* (%)
4HF	**I**		2, 3, 5, 6	6.74 (1H, s)	CI (CH_4) 108 (100) [M^+]
4HF-3Cl	**II**		3 6 5	7.03 (1H, d, 2.4) 6.94 (1H, d, 10) 6.83 (1H, dd, 10, 2.4)	EI MS (70 eV) 142 (70) [M^-]
2HF-5Cl	**III**		3, 4 6 NH $N(CH_3)_2$	6.74 (2H, m) 7.38 (1H, d, 2.1) 7.58 (1H, s) 3.08 (6H, s)	CI (NH_3) 212 (100) [MNH_4]$^+$ 195 (90) [MH]$^-$

Table 2. ^{1}H NMR and Mass Spectroscopic Data for Hydroxyphenylurea Transformation Products at pH 5

Parent molecule	Product		^{1}H NMR (300 MHz) Solvent $CDCl_3$ Position	δ_H(multiplicity, J) (200 MHz)	MS *m/z* (%)
4HF	**IV**		2, 6 3, 5 $N(CH_3)_2$	6.65 (2H, d, 10) 7.16 (2H, d, 10) 3.12 (6H, s)	EI (70eV) 178 [M^+] (25) 134 [M- N $(CH_3)_2^-$] (14) 106 [M- CON $(CH_3)_2^-$] (10) 72 (100) 44 (18)
4HF-3Cl	**V**		2 5 6 $N(CH_3)_2$	7.38 (1H, d, 2.4) 6.81 (1H, d. 10.7) 7.17 (1H, d, 2.4, 10.7) 3.11(6H, s)	ESI 232 (30) [MNa]$^+$ 213 (100) [MH]$^+$
2HF	**VI** and **VIbis**		3, 3' 4, 4' 6, 6' NH $N(CH_3)_2$	6.65 (2H, d, 9) 8.06 (2H, dd, , 9, 2) 8.74 (2H, d, 2) 7.9 (2H, s) 3.10 (12H, s)	CI (NH_3) negative mode 355 (100) [M - H]$^-$ 310 (15) [(M – H) - 45]$^-$ 265 (5) [(M – H) - 2 × 45]$^-$ 376 (15) [MNH_4]$^-$
2HF-4Cl	**VII**		3, 3' 6, 6' NH $N(CH_3)_2$	7.04 (2H, s) 7.60 (2H, s) 7.89 (2H, s) 3.04 (12H, s)	APCI 427 (80) [MH]$^+$ 382 (100)[MH^+ - $NH(CH_3)_2$] 338 (40) [MH^- - $NH(CH_3)_2$ - $N(CH_3)_2$]

identified as dimeric species resulting from a carbon−carbon coupling of the parent molecules (**Table 2**). Mass spectrometry analysis (positive and negative modes) of the **2HF** transformation extract indicated a molecular mass of 356 and exhibited fragments at (M − 45) and (M − 2 × 45) in the negative mode, which is representative of two successive losses of the dimethylated amine fragment. ^{1}H NMR spectrum resulted from the superposition of the signals of two regioisomers, compound **VI** (more than 80%) and compound **VIbis**. The ^{1}H NMR spectrum of the major isomer, **VI**, was representative of a symmetrical dimer. The signal assignment is consistent with the presence of a dimethylated phenylurea fragment and of three aromatic protons in the 3, 4, and 6 positions. ^{13}C NMR confirmed the presence of the urea carbonyl (154.71 ppm) and indicated the presence of one aromatic quinone (180.81 ppm), together with two quaternary carbons (138.62, 135.17) and three CH (134.12, 128.16, 111.26) on each aromatic cycle. These results are in agreement with the structures of **VI** and **VIbis** proposed in

Table 3. ^{1}H NMR and Mass Spectroscopic Data for **2HF-5Cl** Transformation Products at pH 6.5

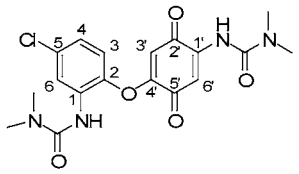
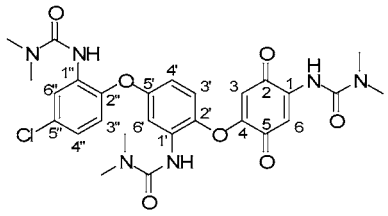
Product		^{1}H NMR		ESI MS
		Position	δ_H(multiplicity, J)	*m/z* (%)
			Solvent: C_6D_6	
VIII		3	7.00 (1H, d. 9)	
		4	6.86 (1H, dd, , 2.4, 9)	429 (100) $[MNa]^-$
		6	7.07 (1H, d. 2.4)	407 (5) $[MH]^+$
		3'	6.02 (1H, s)	
		6'	8.01 (1H, s)	
		NH	8.11 (2H, s)	
		$N(CH_3)_2$	2.58 (6H, s)	
			2.25 (6H, s)	
			Solvent: $CDCl_3$	
IX		3	5.97 (1H, s)	623 (10) $[MK]^+$
		6	6.94 (1H, s)	607 (100) $[MNa]^-$
		3'	6.81 (1H, d, 9)	585 (20) $[MH]^+$
		4'	6.77 (1H, dd, 3, 9)	562 (15) $[MNa^+ -$
		6'	7.12 (1H, d, 3)	$NH(CH_3)_2]$
		3"	7.21 (1H, d, 9)	540 (30) $[MH^+ -$
		4"	6.92 (1H, dd, 2.5, 9)	$NH(CH_3)_2]$
		6"	7.27 (1H, d. 2.5)	495 (5) $[MH^+ - 2$
		NH	8.38 (1H, s)	$\times NH(CH_3)_2]$
			8.19 (1H, s)	451 (2) $[MH^+ - 2$
			8.02 (1H, s)	$\times NH(CH_3)_2 -$
		$N(CH_3)_2$	3.14 (6H, s)	$N(CH_3)_2]$
			3.07 (6H, s)	
			3.00 (6H, s)	

Table 2, with the *E* conformation being assigned to the major product (compound **VI**), which is sterically favored.

Because of the poor chromatographic separation of the crude extract after **2HF-4Cl** transformation at pH 5, identification was based on the mass spectrometry analysis of fractions collected from HPLC. The purest fraction contained, together with unreacted parent **2HF-4Cl**, one major product compound (**VII**). Mass spectra indicated a molecular mass of 426 for **VII**, including two chlorine atoms. Two successive losses of a dimethylated amine in the fragmentation pattern could be attributed to the presence of two urea groups on **VII**. The ^{1}H NMR spectrum contained four singlets (**Table 2**). The signal at δ 7.89 ppm disappeared in the presence of D_2O and so can be assigned to NH, by analogy with the chemical shift of the parent molecule **2HF-4Cl** (δ 7.79 ppm). Two singlets were assigned to aromatic hydrogen atoms in the *para* position. To account for the molecular mass, compound **VII** was thus identified as the dimeric hydroxyl compound, derived from the coupling of the parent phenylurea in the *para* position, with reference to the hydroxyl group.

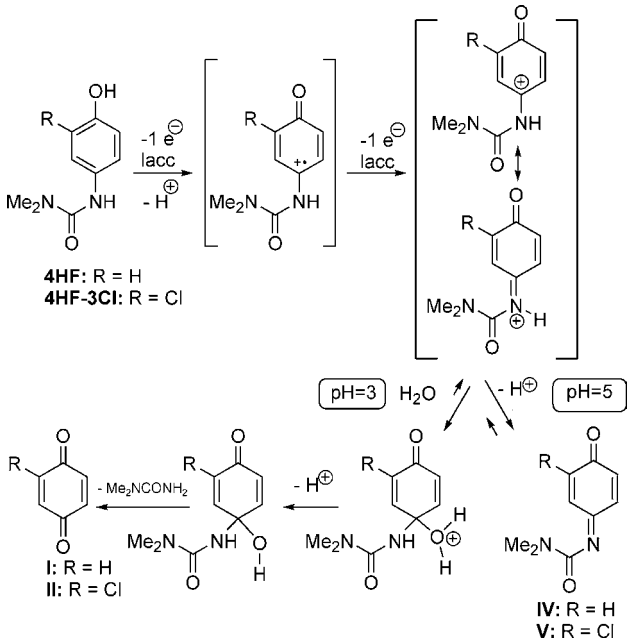
At pH 5, oxidation of **2HF-5Cl** resulted in the formation of 2,5-benzoquinone **II** as a final product. Increasing the pH to pH 6.5 allowed shifting to the formation of three different products, identified as chlorzoxazone and compounds **VIII** and **IX** (**Table 3**). They were recovered from three successive fractions collected from the silica column. In addition to a distinctive R_f, each compound was characterized by a different color. The fraction with the higher R_f (0.8, ethyl acetate), chlozoxazone, was yellow, the intermediate fraction (R_f 0.61, ethyl acetate, product **VIII**) was orange, and the most retained fraction (R_f 0.32, ethyl acetate, product **IX**) was pink. Chlorzoxazone was identified by comparison with an authentic sample (spectroscopic data and retention time). From MS data, the molecular mass of product **VIII** was 406, which corresponds to a dimeric oligomer derived from **2HF-5Cl**, and the molecule contains one chlorine atom. ^{13}C NMR indicated the presence of 12 aromatic carbons in compound **VIII**, which confirmed the oligomerization extent. ^{13}C NMR showed typical signals for quinones in the range of 180 ppm. Compound **VIII** contained two carbonyls from a urea group in the range of 150 ppm, showing that each aromatic cycle kept its urea moiety. The ^{1}H NMR signal at δ 8.11 ppm disappeared in the presence of D_2O and was thus assigned to NH protons. On the basis of the assignment of the two ^{1}H NMR singlet protons in 2-acetamido-5-ethoxy-1,4-benzoquinone (*25*), it was hypothesized that **VIII** could contain two hydrogens in the *para* position on a disubstituted 1,4-benzoquinone (δ 8.01 and 6.02 ppm in C_6D_6). The ^{1}H NMR multiplets and coupling constants of the aromatic protons of **VIII** are representative of a 1, 2, 5 aromatic substitution pattern. A NOE experiment showed correlation between H3 and H3′ protons, which is consistent with assigning H3, H4, H6 on one hand and H3′, H6′ on the other hand to two different aromatic cycles.

From MS data, **IX** has a mass of 584, corresponding to a trimer of its parent molecule. It contains only one chlorine atom. Two successive losses of 45 units followed by loss of fragments of 44 units indicate that each of the aromatic cycles of **IX** bears the urea group of the parent molecule **2HF-5Cl**, which is confirmed by three ^{13}C NMR signals in the range of urea carbonyl (155.85, 154.09, 153.25 ppm) as well as the three singlets (8.38, 8.19, 8.02 ppm), corresponding to NH in the ^{1}H NMR spectrum (**Table 3**). The ^{13}C NMR spectrum also showed the presence of 18 aromatic carbons, including two quinonic carbons (182.47, 181.08 ppm). Both ^{1}H NMR singlets (6.94 and 5.97 ppm in $CDCl_3$) are supposed to be representative of two aromatic protons in the *para* position on a disubstituted 1,4-benzoquinone, as encountered in compound **VIII**. The six remaining ^{1}H NMR signals can be attributed to the three protons of each of the two aromatic cycles in the 3, 4, 6 positions on each cycle. The highest chemical shifts were assigned to the chlorinated ring, in accordance with related aromatic values (*35*). All results led to the identification of **IX** as drawn in **Table 3**.

DISCUSSION

Study of the oxidative transformation of hydroxyphenylurea derivatives by laccase from *T. versicolor* clearly demonstrated the influence of the pH of the incubation mixture. First, the extent of degradation of the substrates greatly depended on the pH. The activity curve exhibited a bell shaped profile with a

Scheme 2. Possible Reaction Mechanism in the Laccase-Catalyzed Oxidation of **4HF** and **4HF-3Cl**



maximum between pH 4 and 5 for all three chlorinated hydroxyphenylureas tested. Such behavior is representative of phenolic substrates and was previously observed, among numerous examples, with syringic and vanillic acids (*27*), sinapic acid (*28*), and 2,4,6-trichlorophenol (*29*). Xu (*30*) postulated that this bell shape profile is a consequence of two opposite effects: the ascending part of the curve is generated by the redox potential difference between the reducing substrate and the type 1 copper of laccase, which increases with the pH, whereas the descending part results from the binding of an hydroxide anion to the type 2/type 3 coppers of laccase, which inhibits the activity at higher pH.

For all the tested phenylureas, the *para*-quinonic derivative was obtained as the predominant product at pH 3. At pH 5, the amount of 2-chlorobenzoquinone in the incubation mixture was only 20% of that at pH 3 (**Figure 2**). An earlier report of our group in regard to the transformation products of **4HF** as a result of *T. versicolor* oxidation (*19*) led to a similar conclusion: at pH 3, the only products observed during the transformation of HF derivatives were *p*-benzoquinones. The formation of quinones in laccase-catalyzed reactions has been observed before at low pH for the enzymatic oxidation of vanillic and syringic acids (*27*) or chlorophenolic compounds (*29*). Recently, Niedermeyer (*26*) reported that amination of alkylated *p*-hydroquinones with aromatic amines using laccases proceeded through the intermediary of the formation of the corresponding quinone.

Scheme 2 suggests a possible mechanism for the formation of a *p*-benzoquinone from **4HF** and **4HF-3Cl**. First, a laccase-catalyzed one-electron oxidation generates a phenoxy radical by removal of hydrogen and one electron from the phenoxy group of the parent hydroxyphenyl. A second enzymatic oxidation is then assumed to proceed, leading to the formation of a carbocation intermediate at the 4 position, favored due to a possible stabilization by resonance via the imine form of the molecule. At higher pH, the carbocation can lose a proton to form an imine which becomes the major final product of the reaction. In slightly acidic conditions, hydrolysis of the cationic group in the *para* position proceeds via a nucleophilic addition of water, leading to the formation of the corresponding *p*-benzoquinone. Such a mechanism involving two different pathways with a common intermediate species, i.e. the carbocation, is consistent with the experimental evidence that at a given pH the concomitant formation of the imine and the *p*-benzoquinone is observed.

At higher pH, **2HF** and **2HF-4Cl**, which are not substituted at the *para* position, do not lead to the formation of an imine but to dimeric compounds. As is the case in the previous mechanism, two successive laccase-mediated oxidations are leading to a cationic intermediate (**Scheme 3**). In a third step, the nucleophilic addition of the parent molecule at the carbocation leads to the formation of a carbon−carbon coupled dimer. Once the cation is generated, coupling is completed without further involvement of the enzyme. Preferential coupling at the C4 position might be due to less sterical hindrance of the corresponding cation, which is stabilized due to the presence of an urea group in the *meta* position.

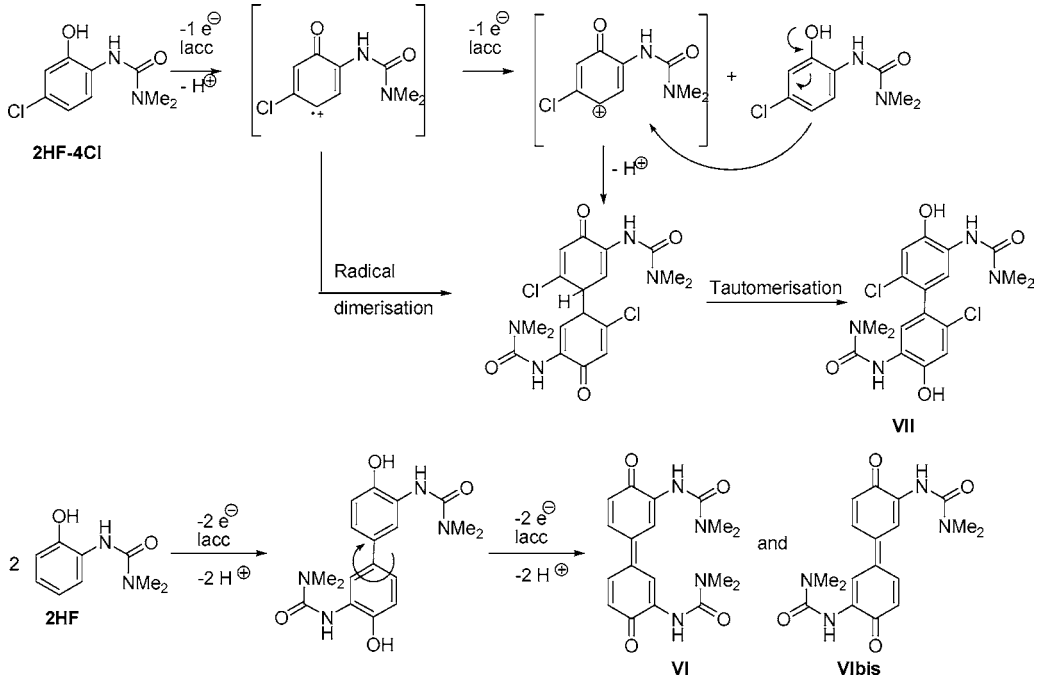
An alternative mechanism involving a laccase-catalyzed one-electron oxidation of the substrate (**2HF** or **2HF-4Cl**) to form a phenoxy radical and its subsequent dimerization cannot be ruled out (**Scheme 3**, radical dimerization route). In that case, assuming that the preferred entry of a new substituent is at the position *ortho* or *para* to the hydroxyl group, with the *meta* position being unreactive, three isomers of dimers with C−C bonds are expected to be formed from **2HF** and **2HF-4Cl**, as derived from the reaction between two radicals. However, only one of these, the 5,5-di(2-hydroxyphenylurea) dimer could be identified, which is consistent with the cation formation route rather than the radical dimerization one.

There are only a few examples of laccase-catalyzed formation of a C−C bond in the literature since the pioneering work of Bollag et al. (*32*−*33*). However, similar preferential coupling in the *para* position to the hydroxyl group was reported for sinapic acid (*28*), although 21 dimeric products could be expected if a random coupling took place. Recently, Ciecholewski et al. (*34*) performed the oxidative dimerization of salicylic esters with laccase in an attempt to provide a new route for the synthesis of functionalized biaryls. Selective carbon−carbon bond formation was observed at the *ortho* position to the hydroxyl group of the salycilate, likely because it was the only unsubstituted position on the ring.

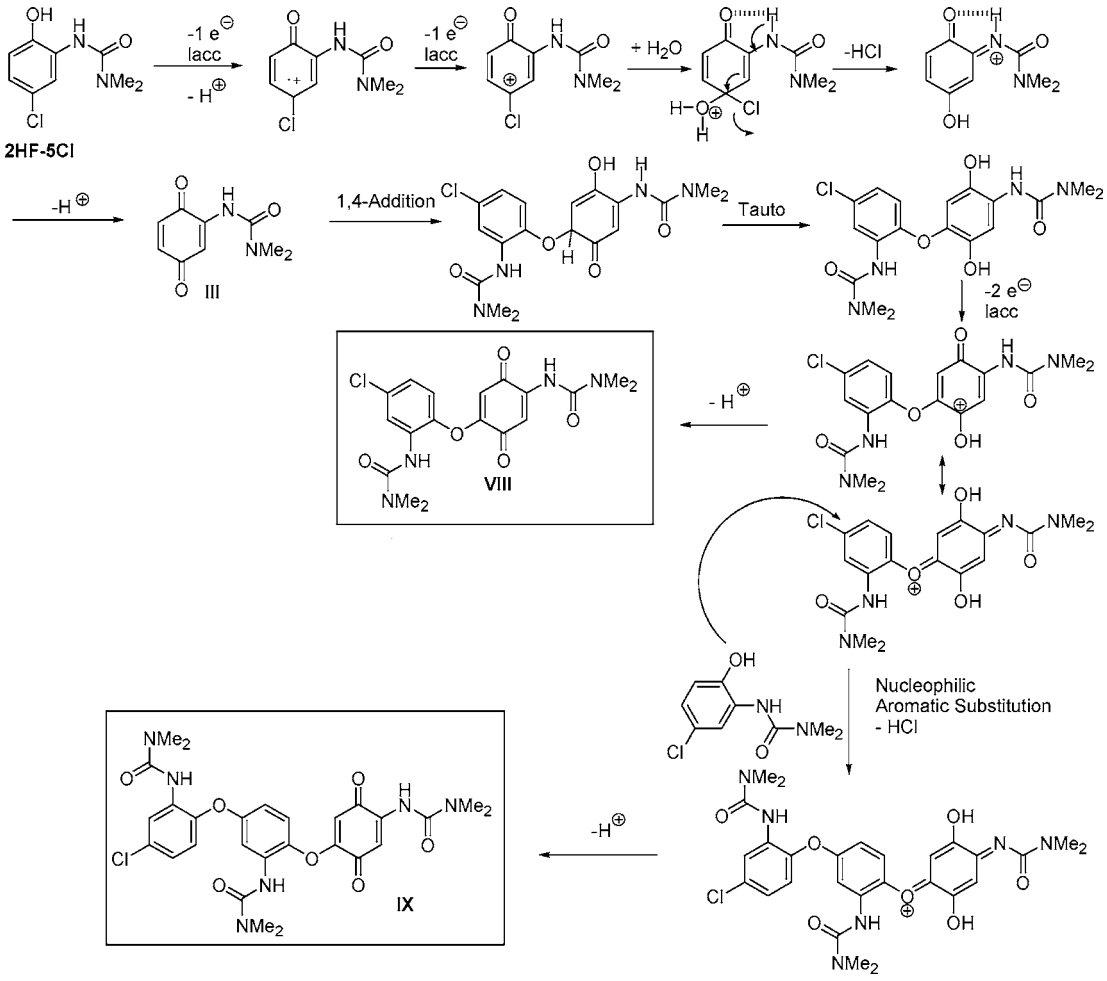
Dimers formed by coupling via C−C bond formation remained phenolic compounds, thus likely to be potential substrates for *T. versicolor* laccase, which is known for its weak specificity. Tridimensional structure studies (*22*) have shown that the enzyme possesses a large cavity (around 10 Å) at the reducing substrate active site. In the case of **2HF**, it can thus be suggested that a second biocatalytic oxidation takes place following the coupling step, leading to the corresponding quinones **VI** and **VIbis**. However, no quinonic compound was found from **2HF-4Cl** oxidation. The reason that the **2HF** phenolic dimer is more readily oxidable than the corresponding **2HF-4Cl** one is most likely because of the electron-withdrawing property of the chlorine, reducing the electron density at the phenoxy group, thus making **2HF-4Cl** more difficult to be further oxidized. It should be noted that a dimeric quinonic compound was previously found as the final predominant product of enzymatic oxidation of sinapic acid (*28*).

Finally, it was found that the oligomers **VIII** and **IX** resulting from the coupling of aromatic rings via an ether bond were obtained from the laccase-catalyzed oxidation of **2HF-5Cl** at pH 6.5. The proposed pathway (**Scheme 4**) includes the laccase-catalyzed formation of the corresponding *p*-quinone as the first step, followed by a subsequent nonenzymatic addition at the *para* position to the urea substituent via the hydroxyl group of

Scheme 3. Possible Reaction Mechanism in the Laccase-Catalyzed Oxidation of **2HF** and **2HF-4Cl** at pH 5



Scheme 4. Possible Reaction Mechanism in the Laccase-Catalyzed Oxidation of **2HF-5Cl** at pH 6.5



the parent **2HF-5Cl**. The presence of the *p*-quinone in the reaction medium is of course a prerequisite in this pathway and is supported by the experimental observation that the quinone formation is the major transformation route of **2HF-5Cl,** even at pH 5. Although limited at similar pH for the other hydroxyphenylureas such as **4HF** or **2HF**, the presence of *p*-quinone in the reaction medium can be explained by the more electrophilic character of the carbocation formed after the two-electron

oxidation of **2HF-5Cl,** due to the presence of chlorine, favoring the subsequent addition of water. A second argument is that, thanks to the hydrogen bond with the hydrogen of the urea amine, the delocalization of the nonbinding doublet of its nitrogen atom is favored, enhancing the leaving character of the chlorine atom (**Scheme 4**).

However, quinone formation is only the first step of the **2HF-5Cl** transformation pathway. As it is known that at pH 6.5 the transformation of the substrate is very low, the quinone is in the presence of a rather high concentration of unreacted substrate in solution, which is likely to undergo a nucleophilic addition on the quinone. A similar mechanism of addition on *para*-quinone was recently described for the synthesis of fungal laccase-catalyzed aminoquinones, resulting from the amination of *p*-hydroquinones with primary aromatic amines (*26*) at an *ortho* position to the quinoic carbonyl, as observed in the present work.

Compound **IX** resulted from a nucleophilic substitution on the aromatic ring taking place *para* to the first C−O coupling site. One interesting point to note is the release of chlorine ions as a result of the coupling reaction. Chlorine atoms were released if they happened to be attached to carbon atoms engaged in a coupling or nucleophilic addition reaction. A similar observation was reported for laccase-catalyzed transformation of chlorophenols (*15*).

Conclusion. The hydroxyphenylurea derivatives studied in this work are metabolites or photochemical degradation products formed from phenylurea compounds widespread in the environment, including soils and waters. Functionalization of their aromatic ring through hydroxylation makes phenylureas potential substrates for oxidative enzymes and thus for a further degradation. This paper offers the first data on both the transformation mechanisms and the structure of the metabolites formed by transformation with laccase from *T. versicolor*, an oxidase produced in many soil fungi.

We have shown that laccase-mediated transformation of hydroxyphenylureas led to quinones at pH 3. By contrast, at higher pH values, these compounds are further transformed into oligomers by chemical processes. The polymerization process is of special importance for the environmental fate of HF derivatives. First, when the oxidation coupling reaction occurs at the chlorinated site of the substrate, the induced dehalogenation contributes to the overall detoxification effect since it is generally recognized that toxicity decreases after dechlorination. Second, as we postulated that the mechanism of covalent bond formation involves the addition of a nucleophile, leading to the formation of heterodimers, the same mechanism is likely to take place between quinones resulting from the two electron oxidation of hydroxyphenylureas and humic acids present in soils. Such a polymerization process of binding to humic acid has been previously reported for chlorophenols (*15*). Potentially toxic quinones formed from laccase-catalyzed oxidation of HF derivatives are thus likely to be bound to the organic matter of soils and thus to lose their bioavailability and, as a consequence, their ecotoxicity.

ACKNOWLEDGMENT

We thank Dr. Debrauwer (UMR 1089 Xénobiotiques, INRA-ENVT, Toulouse) for acquisition of the APCI mass spectrum of compound **VII** and Dr. David Tepfer for the careful reading of the manuscript.

Supporting Information Available: Synthesis procedure and spectroscopic data (^{1}H and ^{13}C NMR, mass spectrometry) of compounds **2HF**, **4HF**, **4HF-3Cl**, **2HF-4Cl**, and **2HF-5Cl** and compounds **I**−**IX**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Received for review February 6, 2006. Revised manuscript received May 11, 2006. Accepted May 11, 2006.

JF060351I