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Mechanism of Aromatic Ring Cleavage of a β-Biphenylyl Ether Dimer Catalyzed by Lignin Peroxidase of *Phanerochaete chrysosporium*†

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ABSTRACT: Homogeneous lignin peroxidase (LiP) oxidized 1-(4-methoxyphenyl)-2-(2,6-dimethoxy-4-phenylphenoxy)-1,3-propanediol (I) to yield 1-(4-methoxyphenyl)-1,2,3-propanetriol (II), α-(4-methoxyphenyl)-2-oxo-1,3-dioxolane-4-methanol (III), 5-(4-methoxyphenyl)-2-oxo-1,3-dioxolane-4-methanol (IV), 4-oxo-3-phenyl-2-butenoic acid methyl ester (V), 5-hydroxy-4-phenyl-2(5*H*)-furanone (VI), 4-oxo-3-phenyl-2-pentenedioic acid dimethyl ester (VII), and 5-carbomethoxy-5-hydroxy-4-phenyl-2(5*H*)-furanone (VIII). Products II–VIII are all derived from the opening of the B aromatic ring of the substrate. Oxidation of I in $H_2{}^{18}O$ resulted in 87% and 65% incorporation of ^{18}O into the carbonyl oxygen of III and IV, respectively. Oxidation of I under $^{18}O_2$ resulted in the incorporation of two atoms of oxygen into the aldehyde (V); one of these atoms of incorporated oxygen was exchangeable with H_2O. Oxidation of I under $^{18}O_2$ also resulted in three atoms of oxygen incorporated into the ketone (VII); one of these atoms of incorporated oxygen was exchangeable with H_2O. Oxidation of I under either $^{18}O_2$ or $H_2{}^{18}O$ resulted in the incorporation of three atoms of oxygen into the lactol VI; two atoms of incorporated oxygen originate from O_2 and one from H_2O. Finally, oxidation of I under either $^{18}O_2$ or $H_2{}^{18}O$ indicated that four atoms of oxygen were incorporated into the lactol VIII; three atoms of incorporated oxygen originated from O_2 and one from H_2O. These results are explained by a mechanism involving the one-electron oxidation of the B ring of the substrate to an aryl cation radical by the H_2O_2 oxidized enzyme. This aryl cation radical intermediate undergoes nucleophilic attack by H_2O or the γ or α side-chain hydroxyls followed by coupling with O_2 to yield several possible cyclic peroxide intermediates. These intermediates undergo cleavage reactions to yield various final products. In these oxidative pathways, only the formation of the aryl cation radical is enzyme-catalyzed. Subsequent reactions appear to be nonenzymatic, accounting for the variety of ring-opened products formed.

The lignin-degrading basidiomycete *Phanerochaete chrysosporium* secretes at least two extracellular heme peroxidases during its secondary metabolic phase of growth. These enzymes, manganese peroxidase (MnP)[1] (Glenn & Gold, 1985; Paszczynski et al., 1986; Glenn et al., 1986) and lignin peroxidase (LiP) (Gold et al., 1984; Tien & Kirk, 1984; Renganathan et al., 1985), have been purified to homogeneity. The main isozymic form of lignin peroxidase is a glycoprotein with M_r 41 000, and all forms of the enzyme have a single iron protoporphyrin IX prosthetic group (Renganathan et al., 1985). Electronic absorption spectroscopy (Gold et al., 1984), EPR spectroscopy (Andersson et al., 1985), and resonance Raman spectroscopy (Andersson et al., 1985, 1987; Kuila et al., 1985) indicate that the heme iron in native lignin per-

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[1] Abbreviations: BSTFA, *N,O*-bis(trimethylsilyl)trifluoroacetamide; DMF, dimethylformamide; FT-NMR, Fourier transform nuclear magnetic resonance spectrometer; GCMS, gas chromatography–mass spectrometry; HRP, horseradish peroxidase; LiP, lignin peroxidase; MnP, manganese peroxidase; MS, mass spectrum; TMS, trimethylsilyl.

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oxidase is in the high-spin ferric state, most likely with a histidine fifth ligand. Resonance Raman data also suggest that, like most peroxidases, lignin peroxidase is pentacoordinate at room temperature (Kuila et al., 1985; Andersson et al., 1987; Dunford, 1982). However, this enzyme undergoes a temperature-dependent coordination-state equilibrium such that at ≤2 °C it is hexacoordinate (Andersson et al., 1987).

We have characterized the H_2O_2 reaction intermediates, compounds I–III of LiP, using electronic absorption spectroscopy. Like the analogous intermediates of HRP, LiP compounds I and II appear to have formal oxidation states of V and IV, respectively (Renganathan & Gold, 1986; Dunford, 1982). Recent resonance Raman studies have confirmed that the heme iron in LiP II is in the ferryl form (Andersson et al., 1987). Homogeneous LiP catalyzes the oxidation of a variety of lignin model compounds such as diarylpropanes, β-aryl ethers, and phenylpropanes (Gold et al., 1984; Tien & Kirk, 1984; Renganathan et al., 1985). A proposed mechanism that accounts for these reactions involves an initial one-electron oxidation of the aromatic substrate by lignin peroxidase compound I to form an aryl cation radical that undergoes subsequent nonenzymatic reactions to yield the final products (Kersten et al., 1985; Miki et al., 1986a; Schoemaker et al., 1985; Renganathan & Gold, 1986).

Until recently the oxidative reactions shown to be catalyzed by lignin peroxidase included side-chain cleavage (Gold et al., 1984; Tien & Kirk, 1984; Miki et al., 1986a; Hammel et al., 1985), demethoxylation (Kersten et al., 1985; Miki et al., 1986a), and intramolecular addition and rearrangements (Miki et al., 1986a,b; Kirk et al., 1986). It now has been shown that LiP can oxidize veratryl alcohol (Leisola et al., 1985) as well as β-aryl ether dimers via ring-opening reactions (Umezawa et al., 1986; Umezawa & Higuchi, 1987; Miki et al., 1987); however, the mechanism of these reactions remains unclear.

In this paper we describe the lignin peroxidase catalyzed oxidation of the novel β-biphenylyl ether dimer 1-(4-methoxyphenyl)-2-(2,6-dimethoxy-4-phenylphenoxy)-1,3-propanediol (I). Six products of ring-opening reactions are identified, and proposed pathways and mechanisms for these reactions are presented.

Materials and Methods

Enzyme. LiPII was purified from acetate-buffered agitated cultures of *P. chrysosporium* as described previously (Gold et al., 1984; Renganathan et al., 1985). The purified protein was electrophoretically homogeneous and had an RZ value of ~5.0.

Enzyme Reactions. Model compound oxidations were carried out at 37 °C for 20 min in 1 mL of sodium succinate, pH 4.5, containing LiP (5 μg), 0.02% substrate (added as a 10% solution in DMF), and H_2O_2 (100 μM).

^{18}O Incorporation Experiments. Where indicated, reaction mixtures were enriched with $H_2{}^{18}O$ (73%) and incubated under air or 100% O_2 as previously described (Renganathan et al., 1986). For experiments conducted under $^{18}O_2$, reaction vessels contained enzyme and substrate in one compartment and H_2O_2 in the other. The vessels were evacuated, flushed with argon, reevacuated, and finally equilibrated with 99% $^{18}O_2$ as described previously (Kuwahara et al., 1984). Following the reaction, mixtures were extracted with EtOAc (3 × 1 mL), dried over Na_2SO_4, evaporated with N_2, and analyzed either directly or following derivatization (BSTFA:pyridine 2:1 v/v). GCMS was performed at 70 eV on a VG Analytical 7070E mass spectrometer fitted with an HP 5790A GC and a 15-m fused silica column (DB-5, J & W Science). ^1H NMR spectra were determined with a JEOL FX90Q FT-NMR with chemical shifts expressed as parts per million (δ) downfield from an internal standard of tetramethylsilane.

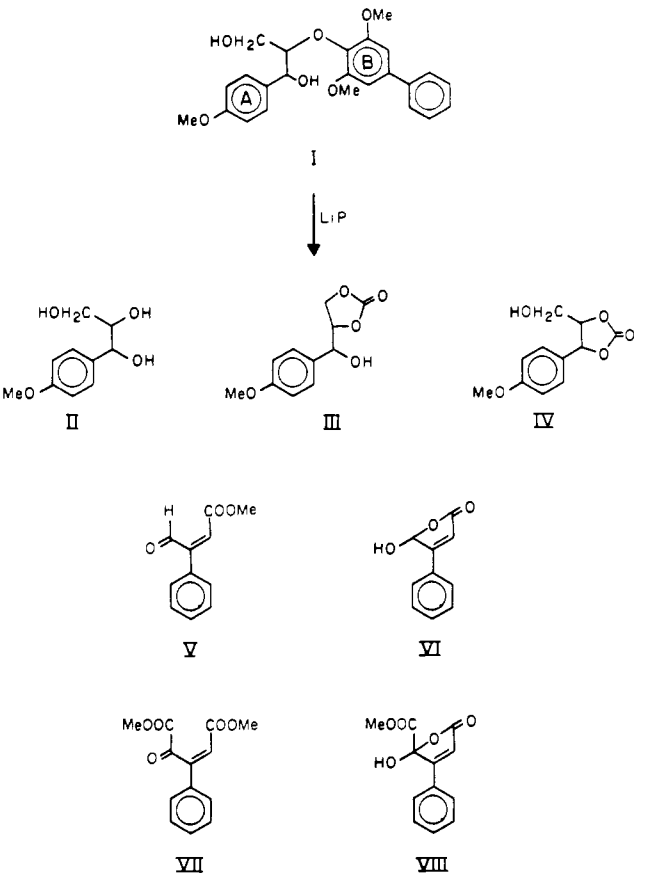

FIGURE 1: Oxidative ring cleavage of the substrate (I) by homogeneous lignin peroxidase. Reactions were conducted and products isolated and analyzed as described in the text.

Preparation of Compounds. 1-(4-Methoxyphenyl)-2-(2,6-dimethoxy-4-phenylphenoxy)-1,3-propanediol (I) was prepared as described previously (Miki et al., 1987). MS (*m/z*) (di TMS ether) 554 (M^+, 0.4), 302 (29.8), 230 (25.9), 222 (23.3), 162 (31.3), 133 (43.3).

1-(4-Methoxyphenyl)-1,2,3-propanetriol (II) was prepared from 4-methoxycinnamate by a procedure described in Enoki et al. (1981).

α-(4-Methoxyphenyl)-2-oxo-1,3-dioxolane-4-methanol (III) and 5-(4-methoxyphenyl)-2-oxo-1,3-dioxolane-4-methanol (IV) were prepared as described previously (Miki et al., 1987). III: MS (*m/z*) (mono TMS ether) 296 (M^+, 0.3), 237 (3.5), 209 (100), 135 (17), 121 (7.3), 101 (7.4). IV: MS (*m/z*) (mono TMS ether) 296 (M^+, 3.1), 206 (9.2), 162 (64.6), 135 (85), 121 (100), 103 (17.7).

4-Oxo-3-phenyl-2-butenoic Acid Methyl Ester (V). α-Methylcinnamic acid methyl ester (Va) was prepared by a Reformatsky reaction using acetophenone and methyl bromoacetate followed by dehydration using I_2 in benzene: MS (*m/z*) 176 (M^+, 0.60), 161 (5.6), 145 (92.0), 115 (100), 102 (14.2), 91 (45.1), 77 (17.9). Va and 2 equiv of SeO_2 in THF were stirred under reflux for 24 h to yield V: MS (*m/z*) 190 (M^+, 25.3), 175 (1.9), 162 (31.5), 131 (100), 115 (18.5), 103 (77.2), 91 (18.5).

5-Hydroxy-4-phenyl-2(5H)-furanone (VI). α-Methylcinnamic acid ethyl ester (VIa) was prepared by a Reformatsky reaction using acetophenone and ethyl bromoacetate followed by dehydration using I_2 in benzene. VIa: ^1H NMR ($CDCl_3$) δ 1.31 (3 H, t, *J* = 7.0 Hz), 2.57 (3 H, s), 4.22 (2 H, q, *J* = 7.0 Hz), 6.13 (1 H, s), 7.2–7.6 (5 H, m). VIa and

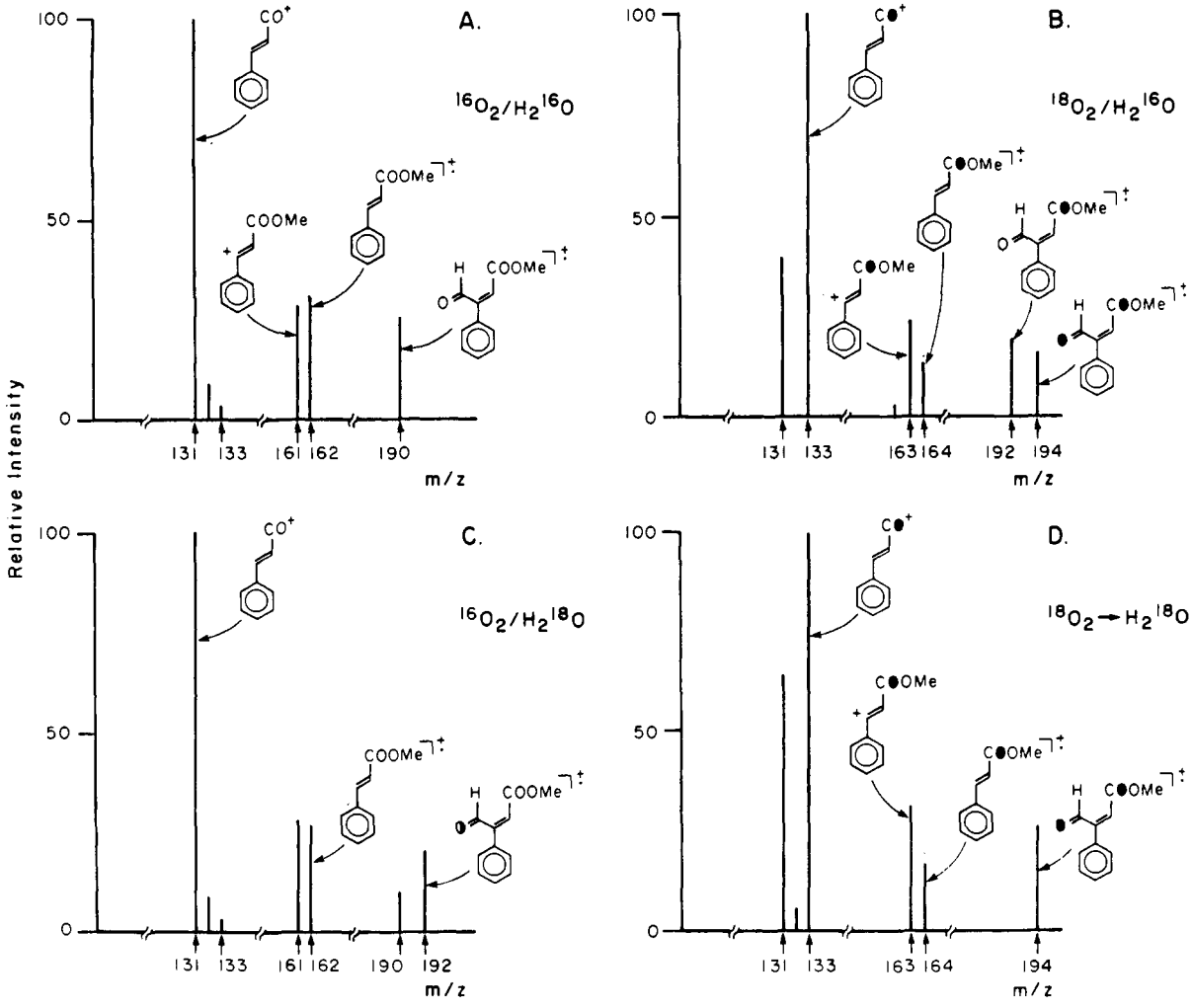

FIGURE 2: Portion of the MS of the aldehyde (V) showing the molecular ion and several key fragment ion regions. (A) Product formed under $^{16}O_2$ and $H_2{}^{16}O$. (B) Product formed under $^{18}O_2$ and in $H_2{}^{16}O$. (C) Product formed under $^{16}O_2$ but in $H_2{}^{18}O$. (D) Product formed under $^{18}O_2$ and reincubated in $H_2{}^{18}O$ after extraction. (●) Oxygen derived from O_2; (◑) oxygen derived from H_2O.

1 equiv of SeO_2 in acetic acid:H_2O (90:10) were stirred at 120 °C for 16 h to yield the lactol VI: ^1H NMR (acetone-d_6) δ 6.42 (1 H, s), 6.58 (1 H, s), 7.2–7.8 (5 H, m); ^1H NMR (monoacetate) ($CDCl_3$) δ 2.15 (3 H, s), 6.46 (1 H, s), 7.46 (1 H, s), 7.5 (5 H, m); MS (mono TMS ether) (m/z) 248 (M^+, 1.3), 233 (26.6), 205 (46.0), 102 (100), 77 (25.3), 75 (21.3).

4-Oxo-3-phenyl-2-pentenedioic Acid Dimethyl Ester (VII). β-Bromophenylpropionic acid methyl ester, obtained by the bromination of methyl phenylproprionate with HBr, was added to methyl glyoxalate thioacetal carbanion (Cregge et al., 1973). The resultant addition product was treated with *N*-bromosuccinimide at room temperature for 5 min and purified over a silica gel column to yield VII: ^1H NMR ($CDCl_3$) δ 3.78 (3 H, s), 3.92 (3 H, s), 6.44 (1 H, s), 7.2–7.5 (5 H, m); MS (m/z) 248 (M^+, 3.0), 189 (100), 161 (72.4), 115 (37.5), 102 (37.5).

5-Carbomethoxy-5-hydroxy-4-phenyl-2(5H)-furanone (VIII) was prepared by a described procedure (Miki et al., 1987): ^1H NMR δ ($CDCl_3$) 3.82 (3 H, s), 6.53 (1 H, s), 7.3–7.6 (5 H, m), 5.3 (1 H); MS (m/z) (mono TMS ether) 306 (M^+, 2.7), 291 (14.5), 247 (78.7), 219 (8.2), 102 (39.4), 89 (39.9), 73 (100).

RESULTS

As shown in Figure 1, the β-biphenylyl ether I was oxidized by lignin peroxidase under aerobic conditions to yield the triol II and six ring-opened products. The triol II, the cyclic carbonates III and IV, and the lactol VIII were identified previously in our laboratory as products of the LiP-catalyzed oxidation of I (Miki et al., 1987). Three additional products have now been identified from their GC retention times and GCMS spectra as the aldehyde (V) [MS (m/z) 190 (M^+, 25.3)], the lactol VI [MS (mono TMS ether) (m/z) 248 (M^+, 1.3)], and the ketone (VII) [MS (m/z) 248 (M^+, 3.0)]. No products were obtained when the reactions were conducted in the absence of H_2O_2 or enzyme. Ring-opened products were not obtained when the reaction was conducted in the absence of oxygen.

Figure 2 and Table IC show the molecular ion and key fragment ion peaks for the aldehyde (V) formed under a variety of conditions. V formed under $^{16}O_2$ and in $H_2{}^{16}O$ exhibited a molecular ion peak at m/z 190 and key fragment ion peaks at 162 and 131. When V was formed under $^{18}O_2$ and reincubated after extraction in $H_2{}^{18}O$, two atoms of ^{18}O were incorporated. One atom of ^{18}O was incorporated at the aldehydic position and the other at the acid carbonyl position. When V was formed under $^{18}O_2$ and $H_2{}^{16}O$, one atom of ^{18}O remained incorporated at the acid carbonyl position. Finally, when V was formed in $H_2{}^{18}O$, one atom of ^{18}O was incorporated in the aldehydic position. These results indicate that during the reaction two atoms of oxygen from O_2 are incorporated into V at the aldehydic and acid carbonyl positions but that the aldehydic oxygen is readily exchangeable with H_2O.

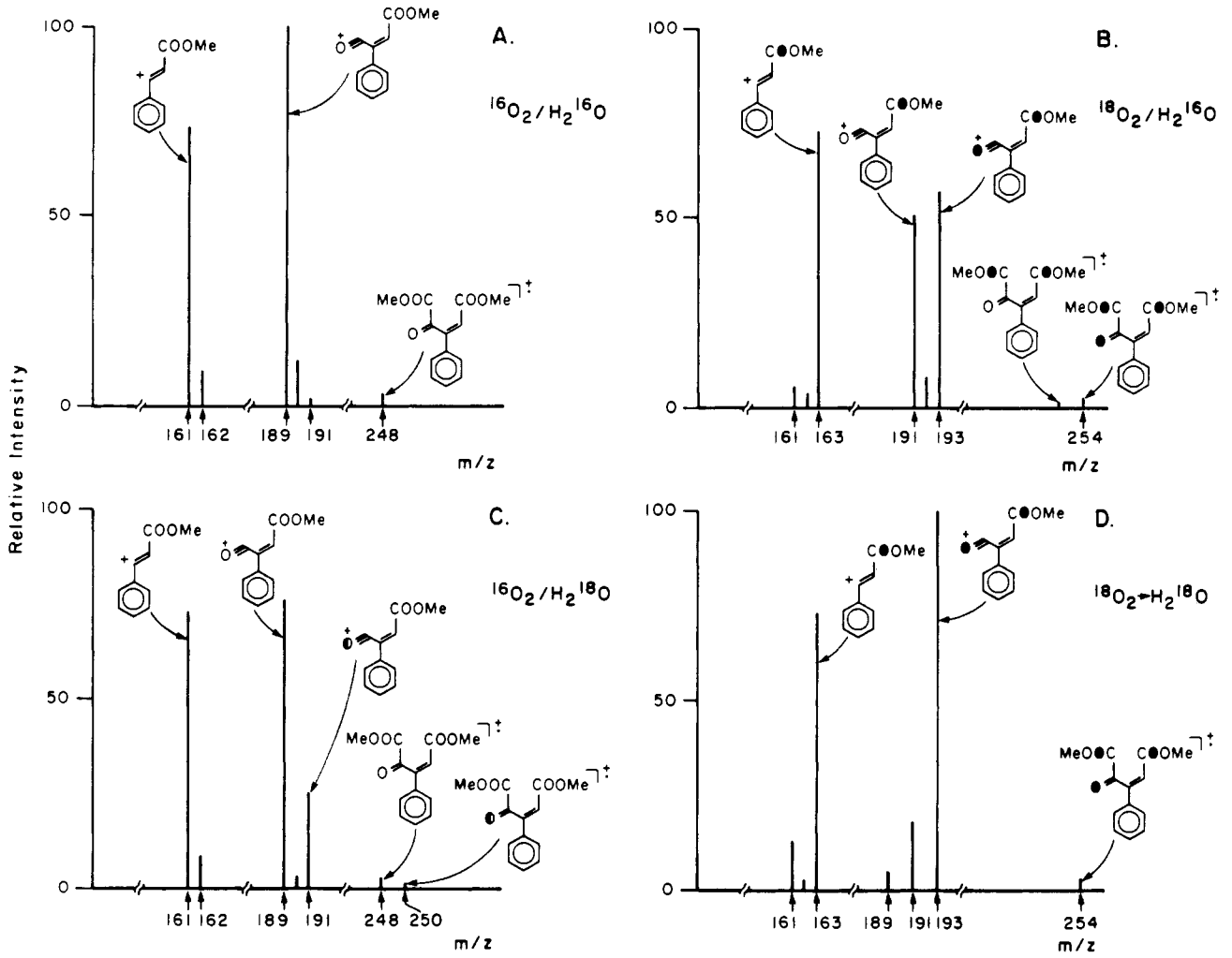

FIGURE 3: Portion of the MS of the ketone (VII) showing the molecular ion region and several key fragment ion regions. (A) Product formed under $^{16}O_2$ and $H_2{}^{16}O$. (B) Product formed under $^{18}O_2$ and in $H_2{}^{16}O$. (C) Product formed under $^{16}O_2$ but in $H_2{}^{18}O$. (D) Product formed under $^{18}O_2$ and reincubated in $H_2{}^{18}O$ after extraction. (●) Oxygen derived from O_2; (◑) oxygen derived from H_2O.

Figure 3 and Table ID show the molecular ion and key fragment ion peaks for the ketone (VII) formed under a variety of conditions. VII formed under $^{16}O_2$ and $H_2{}^{16}O$ exhibited a molecular ion peak at m/z 248 and key fragment ions at 189 and 161. When VII was produced under $^{18}O_2$ and reincubated after extraction with $H_2{}^{18}O$, three atoms of ^{18}O were incorporated. One atom of oxygen was incorporated at the ketone position, and two atoms of oxygen were incorporated at each of the acid carbonyl positions. When VII was formed under $^{18}O_2$ and $H_2{}^{16}O$, one atom of oxygen was incorporated into each of the acid carbonyl positions, and approximately 60 atom % incorporation was found at the ketone position. Finally, when VII was formed in $H_2{}^{18}O$, approximately 60 atom % incorporation was found at the ketone position. All of these results indicate that three atoms of oxygen from O_2 are incorporated into the ketone (VII) and that the oxygen incorporated into the ketone position is apparently slowly exchangeable with water.

The relative intensities of the molecular ion region of the mass spectra for the six ring-opened products are shown in Table I. Cleavage of I in $H_2{}^{18}O$ (73% enriched) resulted in 87 atom % incorporation of oxygen into the cyclic carbonate III (Table IA). Analysis of the mass spectral fragment ion pattern (data not shown) suggested that the ^{18}O atom was incorporated into the carbonyl oxygen. In contrast, when the cyclic carbonate was formed under $^{18}O_2$, approximately 15% oxygen incorporation was observed. When the cyclic carbonate IV was formed in $H_2{}^{18}O$, approximately 65 atom % ^{18}O incorporation was found in the carbonyl position. In addition, when the reaction was conducted under $^{18}O_2$, approximately 26 atom % incorporation was observed, confirming our earlier work (Miki et al., 1987).

Cleavage of I under $^{18}O_2$ resulted in the incorporation of two atoms of oxygen into the lactol VI ($M^+ + 4 = 252$) (Table I). When the reaction was conducted in $H_2{}^{18}O$, one atom of oxygen was apparently incorporated into the lactol VI. Thus, a total of three atoms of oxygen are incorporated into VI during the reaction: Two atoms originate from O_2, and one originates from H_2O.

Figure 4 and Table IF show the molecular ion and key fragment ions for the lactol VIII formed under a variety of conditions. VIII formed under $^{16}O_2$ and $H_2{}^{16}O$ exhibited a molecular ion at m/z 234 and key fragment ions at 175 and 147. When VIII was formed under $^{18}O_2$, three atoms of ^{18}O were incorporated ($M^+ + 6 = 240$). Fragment ions at (175 + 4) = 179 and at (147 + 2) = 149 suggested that one atom of oxygen was incorporated into the acid carbonyl position, and a second atom of oxygen is incorporated into the ketone position. Incorporation of the third atom of oxygen was apparently distributed between the hydroxyl and lactol positions (see below). The presence of ions at m/z 238 and 177 suggested that at least one of the incorporated oxygen atoms (probably the hydroxyl) was slowly exchangeable with H_2O. When VIII was formed in $H_2{}^{18}O$, approximately one atom of

Table I: Relative Intensities (%) of Molecular Ion Region of Mass Spectra of Products Formed by the Enzymatic Oxidation of the β-Biphenylyl Ether Dimer I

(A) Cyclic Carbonate III

m/z	$^{18}O_2$	$H_2{}^{18}O$	$^{16}O_2/H_2{}^{16}O$
296	100	56.6	100
298	23.0	100	5.5

(B) Cyclic Carbonate IV

m/z	$^{18}O_2$	$H_2{}^{18}O$	$^{16}O_2/H_2{}^{16}O$
296	100	100	100
298	40.8	92.0	5.5

(C) Aldehyde (V)

m/z	$^{18}O_2$	$H_2{}^{18}O$	$^{18}O_2/H_2{}^{18}O$[a]	$^{16}O_2/H_2{}^{16}O$
190	0	49.0	0	100
192	100	100	0–	1.3
194	78.9	1.3	100	0

(D) Ketone (VII)

m/z	$^{18}O_2$	$H_2{}^{18}O$	$^{18}O_2/H_2{}^{18}O$[a]	$^{16}O_2/H_2{}^{16}O$
248	0	100	0	100
250	0	66.7	0	2.0
252	80.6	1.1	0	0
254	100	0	100	0

(E) Lactol VI[b]

m/z	$^{18}O_2$	$H_2{}^{18}O$	$^{16}O_2/H_2{}^{16}O$
248	0	50.2	100
250	100	100	4.9
252	82.4	20.5	0

(F) Lactol VIII

m/z	$^{18}O_2$	$H_2{}^{18}O$	$^{16}O_2/H_2{}^{16}O$
234	0	100	100
236	0	88.9	1.9
238	47.5	26.3	0
240	100	0	0

[a] Reincubation in $H_2{}^{18}O$ after extraction. [b] Analyzed after trimethylsilylation.

^{18}O was incorporated. As discussed below, this incorporation was probably distributed between the hydroxyl and lactol positions.

Discussion

Lignin peroxidase, a heme-containing, H_2O_2-requiring enzyme, was first purified from the extracellular medium of ligninolytic cultures of the white rot basidiomycete *P. chrysosporium* (Gold et al., 1984; Tien & Kirk, 19884; Renganathan et al., 1985). Recently, we have characterized the oxidized states of LiP as being similar to compounds I and II of HRP (Renganathan & Gold, 1986; Dunford, 1982). LiPI and LiPII apparently contain 2 and 1 oxidizing equiv over the ferric resting state of the enzyme and normally the reduction of LiPI to the native enzyme proceeds via two single-electron steps (Renganathan & Gold, 1986). LiPI oxidizes a variety of lignin model compounds, and these various oxidations have been discussed in terms of an aryl cation radical intermediate (Kersten et al., 1985; Schoemaker et al., 1985; Miki et al., 1986a). The involvement of an aryl cation radical in the LiP-catalyzed oxidation of dimethoxybenzene has been demonstrated by EPR (Kersten et al., 1985). Electron-donating groups appear to be necessary for the formation and/or stabilization of the aryl cation radical (Walling et al., 1984), and our recent studies (unpublished) show that LiP dehydrogenates only those benzyl alcohols with at least two alkoxy substitutions on the ring. These observations led us to study the mechanism of LiP-catalyzed oxidation of β-aryl ether dimers under anaerobic conditions (Miki et al., 1986a) and to conclude that the number of alkoxy substituents on each aromatic ring has a profound influence on the pathway of oxidation.

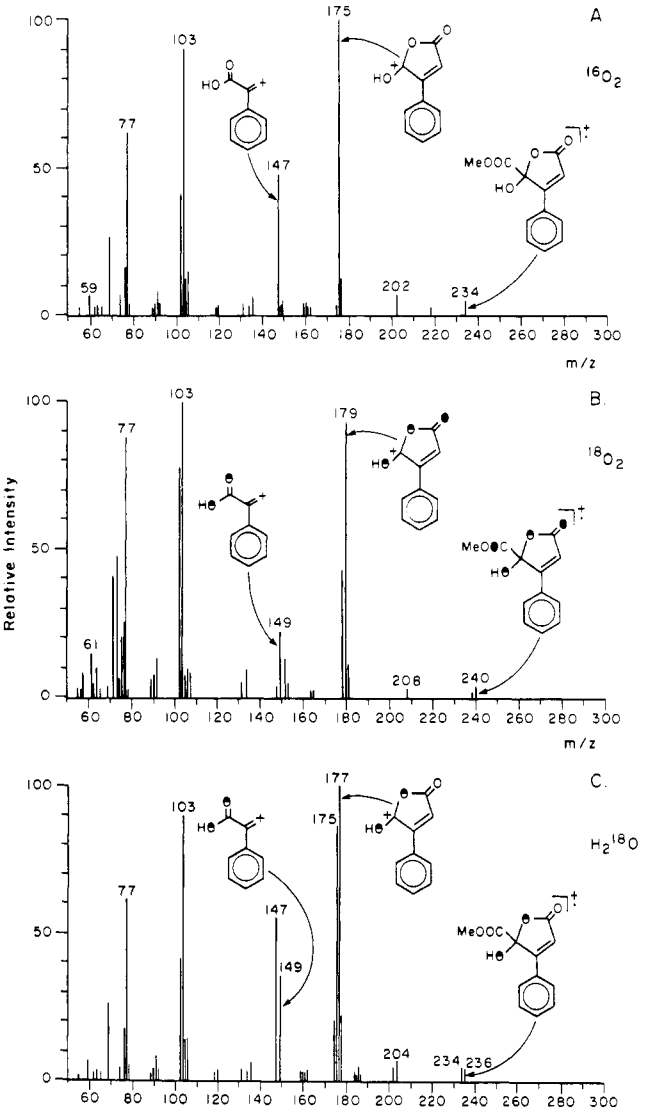

FIGURE 4: Portion of the MS of the lactol VIII showing the molecular ion region and several key fragment ion regions. (A) Product formed under $^{16}O_2$. (B) Product formed under $^{18}O_2$. (C) Product formed in $H_2{}^{18}O$. (⊖) One O atom is incorporated into either of the positions indicated, and these possibilities are not distinguished by the fragmentation pattern; (●) Oxygen derived from O_2.

Earlier studies have shown that under aerobic conditions cultures of *P. chrysosporium* oxidize both lignin (Chen et al., 1983; Terasawa et al., 1983) and lignin model compounds (Umezawa & Higuchi, 1985) via pathways involving ring-opening reactions. Recently, it has been shown that lignin peroxidase from *P. chrysosporium* is responsible for the catalysis of these ring-opening reactions (Umezawa et al., 1986; Umezawa & Higuchi, 1987; Miki et al., 1987). In order to better understand the mechanism of these ring-opening reactions, we designed a β-aryl ether dimer (I) that would yield, upon oxidation, readily identifiable B-ring-opened products. Since the A ring has only one electron-donating alkoxy group, it will not readily form an aryl cation radical. The B ring of the substrate, with three alkoxy groups, readily forms an aryl cation radical, and the phenyl ring substituent on the B ring facilitates in the isolation of ring-opened fragments.

The β-biphenylyl ether dimer (I) is oxidized by LiP to yield the triol (II) and six ring-opened products (Figure 1). Identification of these products, the ^{18}O incorporation data, and other currently available evidence (Renganathan & Gold, 1986; Hammel et al., 1985; Umezawa & Higuchi, 1987) have allowed us to propose mechanisms for the LiP-catalyzed

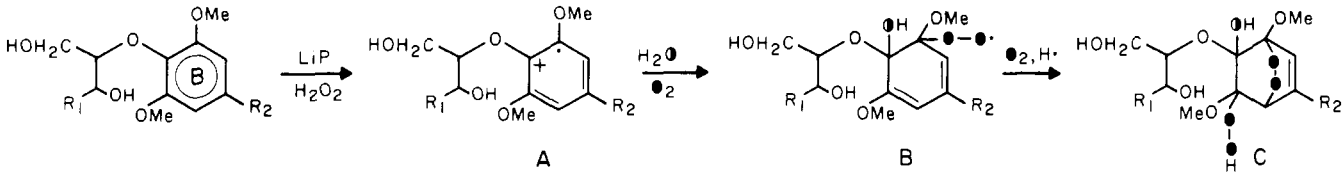

FIGURE 5: Proposed pathway for the first three steps in the oxidation of the substrate I by lignin peroxidase.

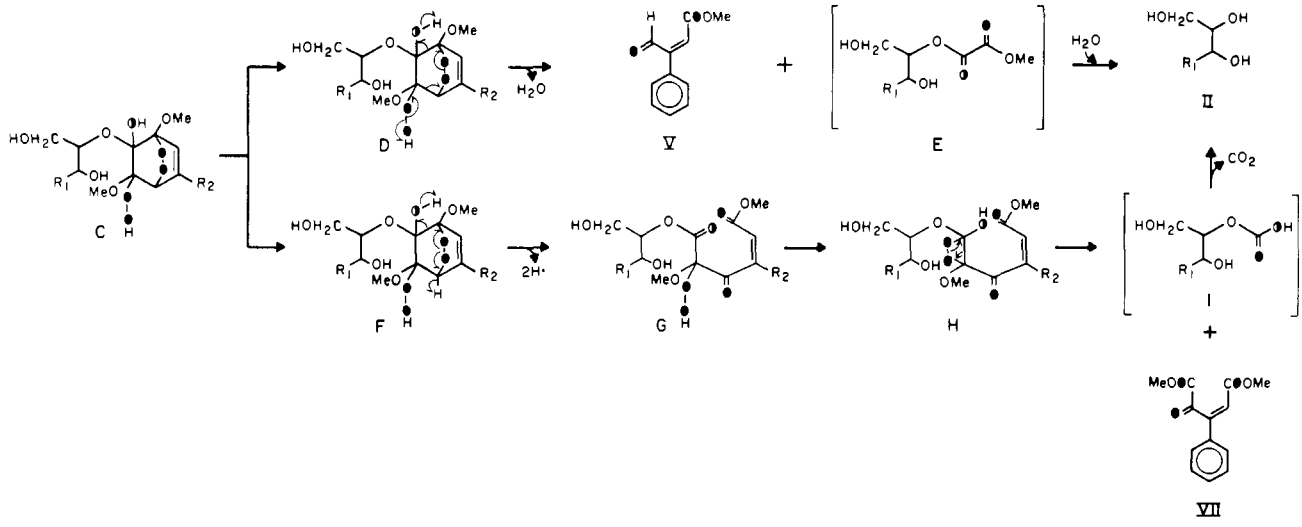

FIGURE 6: Two pathways proposed for the breakdown of the cyclic peroxide intermediate C.

aromatic ring opening of I. These results can be explained in terms of the LiP compound I oxidation of ring B of the substrate to form an aryl cation radical A (Figure 5). The aryl cation radical can undergo nucleophilic addition at the cation site and coupling with the O_2 at the radical site to form the hydroxylated peroxide radical intermediate B (Figure 5). The coupling of ground-state triplet oxygen to cation radicals generated by the oxidation of alkylated olefins or dienes is known to yield dioxetanes (Nelson, 1987). In these enzyme reactions conducted in aqueous medium, both water and O_2 might be expected to add to the cation radical (Umezawa & Higuchi, 1987). In the next step, intermediate B cyclizes to a cyclic peroxide derivative, and the resulting carbon-centered radical is scavenged by O_2, yielding another peroxide radical, which probably abstracts a hydrogen radical from either the enzyme or the solvent to produce intermediate C (Figure 5). The coupling of O_2 with cyclic peroxides has been reported (Porter et al., 1976). Alternatively, as recently postulated (Haemmerli et al., 1987), the carbon-centered radical might couple with a hydroperoxide radical produced during the reaction to yield the intermediate C.

Formation of several of the identified ring-cleavage products can be explained by mechanisms involving homolytic cleavage of either the cyclic peroxide or hydroperoxide O–O bonds of intermediate C (Figure 6). Cleavage initiated at the hydroperoxide D (Figure 6) yields the methyl oxalate ester E, the aldehyde (V), and water. Two atoms of molecular oxygen are incorporated into the aldehyde and acid carbonyl positions of V (Figure 2) as predicted by this pathway. The methyl oxalate ester E is probably hydrolyzed to yield the final product (II). A methyl oxalate derivative analogous to E has been identified as a product of the LiP-catalyzed oxidation of an α,β-ethoxylated β-aryl ether dimer (Umezawa et al., 1986).

In an alternative pathway, bond cleavage initiated between the cyclic peroxide oxygens of F (Figure 6) would generate the hydroperoxide intermediate G. The hydroperoxide can cyclize to yield the dioxetane H (Figure 6), which is unstable and would break down to yield the carbonic ester I and the ketone VII. The carbonic ester is readily hydrolyzed to yield II. Three atoms of molecular oxygen are incorporated into the ketone and two acid carbonyl positions of VII (Figure 3) as predicted by this pathway. A related mechanism involving dioxetane formation has been proposed for the HRP-catalyzed oxidation of isobutyraldehyde to triplet acetone (Baader et al., 1985).

We previously showed that lignin peroxidase can catalyze the rearrangement of dimeric as well as monomeric β-aryl ethers to the corresponding γ-aryl ether under anaerobic conditions (Miki et al., 1986a,b; Hammerich & Parker, 1984). This rearrangement involves a nucleophilic attack of the γ-hydroxyl on the C-4 carbon of the B-ring cation radical, resulting in a cyclohexadiene radical with a spirodioxolane structure. Subsequent cleavage of the dioxolane generates a rearranged ether. Later, we proposed (Miki et al., 1987) that under aerobic conditions the cyclohexadiene radical would be scavenged by molecular oxygen, leading to aromatic ring cleavage and formation of the cyclic carbonates III and IV. Recently, the incorporation of ^{18}O from $H_2{}^{18}O$ into the cyclic carbonate has been observed (Umezawa & Higuchi, 1987), and this has been confirmed in the present study (Table I). The mechanism presented in Figure 7 explains these observations. This mechanism is similar to one recently proposed (Umezawa & Higuchi, 1987). In this pathway the B-ring cation radical is converted to a cyclohexadiene radical via attack of the γ-hydroxyl on the C-4 carbon of the B ring. The cyclohexadiene radical couples with oxygen to produce the peroxide radical J. The intermediate J cyclizes to form a cyclic peroxide radical and couples with oxygen. The resulting peroxide radical abstracts a hydrogen radical from the enzyme or solvent to generate the intermediate K. These steps are similar to those postulated in the formation of C (Figure 5). The lone-pair electrons of K assist in the heterolytic cleavage of the dioxetane bridge with the uptake of a proton, yielding L (Figure 7). The oxonium ion generated, L, is neutralized by the nucleophilic attack of water to yield M. Homolytic cleavage of the hydroperoxide M yields IX and the cyclic

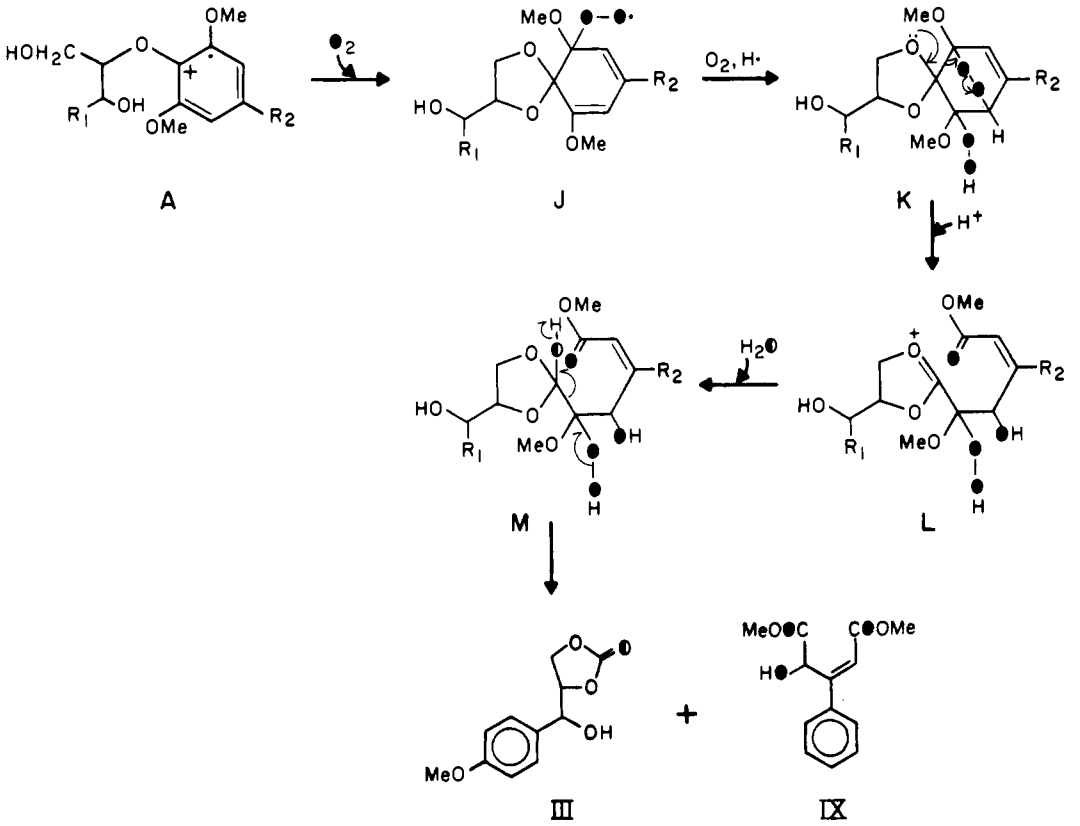

FIGURE 7: Intramolecular addition followed by radical coupling, radical cleavage, and attack by water leading to the formation of the cyclic carbonate III.

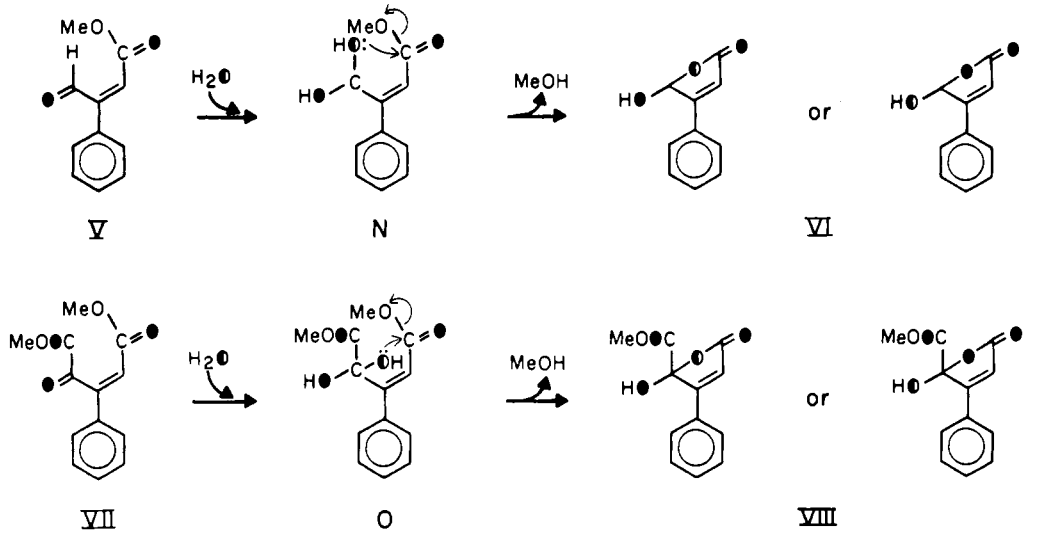

FIGURE 8: Nonenzymatic conversion of the aldehyde (V) and the ketone (VII) to the lactol VI and the lactol VIII, respectively. This conversion results in the distribution of the label between the lactol and hydroxyl positions.

carbonate with one atom of oxygen incorporated from H_2O as observed (Table I). A product corresponding to IX has been observed in our chromatograms but as yet has not been identified conclusively.

The lactol products VI and VII are probably derived from the corresponding methyl esters V and VII. These enzyme reactions were performed at pH 4.5, where the carbonyl groups of the aldehyde and ketone can exist in the hydrated form. The hydroxyl groups in intermediates N and O are equivalent, one arising from molecular O_2 and the other from water. Nucleophilic displacement of a methoxyl group (N or O, Figure 8) by the hydroxyl would yield methanol and the lactols VI and VIII, which have two possible ^{18}O labeling patterns.

In summary, we have obtained evidence for several pathways involved in the aromatic ring opening of the β-biphenylyl ether dimer I catalyzed by lignin peroxidase. All of these pathways are initiated by a one-electron oxidation of the substrate by lignin peroxidase compound I to form an aryl cation radical. This aryl cation radical undergoes a variety of nonenzymatic reactions including nucleophilic substitutions by H_2O or intramolecular hydroxyls or coupling with O_2, leading to unstable peroxy radical intermediates that break down via radical cleavages, yielding the ring-cleaved products. The nature of the products is greatly influenced by the substituents on the aromatic rings of the substrate (Miki et al., 1986a) and by the stability of the reaction intermediates. The variety of side-chain-cleaved and ring-opened products obtained from the lignin peroxidase oxidation of simple β-aryl ether dimers

reported here and previously (Miki et al., 1986a,b; Kirk et al., 1986; Umezawa & Higuchi, 1987) parallels the diversity of products obtained from the *P. chrysosporium* oxidation of lignin (Chen et al., 1983; Terasawa et al., 1983). This is further evidence that lignin peroxidase plays a major role in the oxidation of lignin by this organism.

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