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Ligninase of *Phanerochaete chrysosporium*

Mechanism of its degradation of the non-phenolic arylglycerol ß-aryl ether substructure of lignin

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This study examined the ligninase-catalysed degradation of lignin model compounds representing the arylglycerol ß-aryl ether substructure, which is the dominant one in the lignin polymer. Three dimeric model compounds were used, all methoxylated in the 3- and 4-positions of the arylglycerol ring (ring A) and having various substituents in the ß-ether-linked aromatic ring (ring B), so that competing reactions involving both rings could be compared. Studies of the products formed and the time courses of their formation showed that these model compounds are oxidized by ligninase (+ H_2O_2 + O_2) in both ring A and ring B. The major consequence with all three model compounds is oxidation of ring A, leading primarily to cleavage between $C_{(\alpha)}$ and $C_{(\beta)}$ ($C_{(\alpha)}$ being proximal to ring A), and to a lesser extent to the oxidation of the $C_{(\alpha)}$-hydroxy group to a carbonyl group. Such $C_{(\alpha)}$-oxidation deactivates ring A, leaving only ring B for attack. Studies with $C_{(\alpha)}$-carbonyl model compounds corresponding to the three basic model compounds revealed that oxidation of ring B leads in part to dealkoxylations (i.e. to cleavage of the glycerol ß-aryl ether bond and to demethoxylations), but that these are minor reactions in the model compounds most closely related to lignin. Evidence is also given that another consequence of oxidation of ring B in the $C_{(\alpha)}$-carbonyl model compounds is formation of unstable cyclohexadienone ketals, which can decompose with elimination of the ß-ether-linked aromatic ring. The mechanisms proposed for the observed reactions involve initial formation of aryl cation radicals in either ring A or ring B. The cation radical intermediate from one of the $C_{(\alpha)}$-carbonyl model compounds was identified by e.s.r. spectroscopy. The mechanisms are based on earlier studies showing that ligninase acts by oxidizing appropriately substituted aromatic nuclei to aryl cation radicals [Kersten, Tien, Kalyanaraman & Kirk (1985) J. Biol. Chem. **260,** 2609–2612; Hammel, Tien, Kalyanaraman & Kirk (1985) J. Biol. Chem. **260,** 8348–8353].

INTRODUCTION

The biochemistry of lignin degradation by wood-destroying basidiomycetes is rapidly being clarified, in large part through the use of substructure model compounds of lignin. These 'dimeric' models allow detailed studies of specific degradative reactions, which are otherwise very difficult to study in the intact lignin polymer. Lignin is complex, consisting of phenylpropane units connected via any of more than ten different C–C or C–O–C linkages (Adler, 1977). One of these, the arylglycerol ß-aryl ether or 'ß-O-4' linkage (Fig. 1), accounts for half or more of the intermonomer connections (Adler, 1977). Consequently, ß-O-4 model compounds are the single most important type for elucidating lignin biodegradative reactions.

Studies with ß-O-4 model compounds and the fungus *Phanerochaete chrysosporium* have demonstrated two modes of degradation (Umezawa *et al.,* 1983; Umezawa & Higuchi, 1985*a,b,c*). The first is initiated by oxidative cleavage between $C_{(\alpha)}$ and $C_{(\beta)}$ in the glycerol side chain, and the second, also oxidative, involves an initial cleavage of the O-$C_{(4)}$ bond in the ß-O-4 linkage (Fig. 1). Product quantification does not reveal the relative importance of

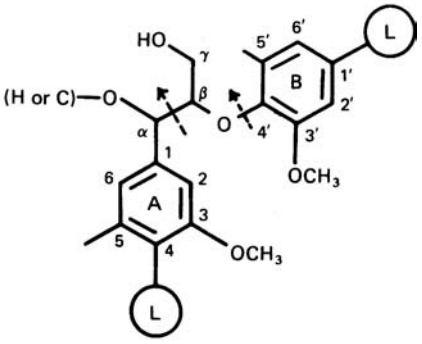

Fig. 1. Arylglycerol ß-aryl ether substructure of lignin and sites of initial cleavage in fungal degradation

The ß-O-4 linkage is the most frequent one between monomer units in lignin, accounting for 50% or more of the interunit connections (Adler, 1977). L indicates the continuation of the lignin polymeric structure. The arrows denote sites of initial cleavage in fungal degradation. Additional linkages between units, and additional methoxy groups, can occur at C-5. The numbering system illustrated is commonly used in lignin chemistry (Sarkanen & Ludwig, 1971).

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these two cleavages, because the arylglycerol product of the second cleavage is itself rapidly cleaved between $C_{(\alpha)}$ and $C_{(\beta)}$, leading to the same final products as in the first degradative mode (Enoki *et al.,* 1981; Umezawa *et al.,* 1983).

Products expected from both types of cleavage are seen on oxidation of ß-O-4 model compounds by a ligninolytic peroxidase, ligninase, secreted by *P. chrysosporium* (Gold et al., 1984). Ligninase was originally discovered on the basis of its catalysis of $C_{(\alpha)}$-$C_{(\beta)}$ cleavage (Tien & Kirk, 1983; Glenn *et al.,* 1983).

We have provided evidence recently that ligninase functions by single-electron oxidation of the aromatic nuclei to unstable cation radicals, which decompose via various reactions to yield the observed products (Kersten et al., 1985; Hammel *et al.,* 1985). Schoemaker *et al.* (1985) have suggested a similar mechanism. In the case of ß-O-4 substructures, generation of a cation radical in ring A is expected to lead mainly to $C_{(\alpha)}$-$C_{(\beta)}$ cleavage, whereas similar oxidation instead in ring B (Fig. 1) is expected to lead in part to O-$C_{(4)}$ cleavage.

Our purpose here was to assess the factors involved in these two initial cleavages catalysed by the purified ligninase in selected model compounds (Fig. 2), and to gain further insight into the mechanism involved. Results with the lignin-like model compounds indicate that $C_{(\alpha)}$-$C_{(\beta)}$. cleavage is by far the more important reaction. In addition, our findings provide information on the relationship between the structure of lignin model compounds and their reactivity to ligninase, and they reveal reactions other than the above two. The observed degradative reactions can be explained on the basis of initial formation of aryl cation radicals.

MATERIALS AND METHODS

Instrumentation

^{1}H-n.m.r. spectroscopy was with a Bruker (Billerica, MA, U.S.A.) WM-250 instrument; tetramethylsilane served as internal standard and [^{2}H]chloroform served as solvent. A Finnigan (San José, CA, U.S.A.) 4510 gas chromatograph–mass spectrometer with a 30 m, 0.25 μm-film-thickness DB-1 (bonded methyl silicone) fused silica capillary column (J & W Scientific, Rancho Cordova, CA, U.S.A.) was used for examination of products and synthetic samples. Electron-ionization m.s. was performed with the use of 70eV. Chemical-ionization m.s. was performed with methane as electron donor.

E.s.r. spectroscopic measurements were performed with a Varian E-109 spectrometer as previously described (Kersten *et al.,* 1985; Hammel *et al.,* 1985).

Uncorrected melting points were determined with a Thomas–Hoover capillary apparatus (Arthur H. Thomas Co., Philadelphia, PA, U.S.A.).

H.p.l.c. analyses of products were with a Gilson (Middleton, WI, U.S.A.) system 41 Gradient Analytical HPLC apparatus, with an HM/HPLC detector employing a Hewlett–Packard (Palo Alto, CA, U.S.A.) 3380A integrator and a Vydac (Hesperia, CA, U.S.A.) reverse-phase Ultrasphere ODS column.

U.v. spectroscopy was with a Pye–Unicam (Cambridge, U.K.) PU8800 UV/VIS spectrophotometer.

Production and purification of ligninase

The enzyme (referred to as H8 in Kirk *et al.,* 1986) was produced as previously described (Tien & Kirk, 1984), and was purified by h.p.l.c. in a Pharmacia (Piscataway, NJ, U.S.A.) Mono Q column (Kirk *et al.,* 1986). Activity (units μmol/min) was based on oxidation of veratryl alcohol to veratraldehyde (Kirk *et al.,* 1986).

Enzyme reactions

Unless otherwise noted, reactions were run in a total volume of 1 ml, containing 0.8 mM-H_2O_2, 0.2 mM model substrate, 0.41 unit of ligninase and 100 mM-sodium tartrate buffer, pH 3.0. The initial model substrate and H_2O_2 concentrations were saturating. The reactions were started by addition of H_2O_2. Reactions for product identification from model compound (IV) were run with four sequential (every 5 min) additions of H_2O_2 [0.1 μmol (10 μl)/addition] in a total volume of 2 ml.

Product identification

Initial identification of products was based on h.p.l.c. retention times; in all cases identification was confirmed by m.s. comparison of the compounds in the collected fractions with authentic standards. *p*-Benzoquinone was identified by g.c.–m.s. as hydroquinone diacetate after reduction and acetylation.

Product quantification

Products were resolved and quantified by h.p.l.c. with a gradient containing methanol/water : [% (v/v) methanol/time (min)] 20% /0; 20% /4; 40% /16; 40% /19; 50% /23; 50% /27; 20% /28; 20% /35. The detector response of each compound at 280 nm was determined with standardized solutions. *p*-Benzoquinone content was measured by the increase in 242 nm absorbance (ε = 18 197 M^{-1} . cm^{-1}). Product methanol was determined by isotope dilution as its 3,5-dinitrobenzoate derivative (Donnelly & Dagley, 1981). The enzymic reactions were stopped by adding 50 μl of the reaction mixture to 35 μl of 11.8 mM-NaN_3; 25 μl samples were analysed.

Lignin model compounds and chemicals

[Note: the system used in naming compounds (Fig. 2) in this section conforms to IUPAC rules; in the remaining sections, the numbering system is that used by lignin chemists, with carbon designations as in Fig. 1 (see Sarkanen & Ludwig, 1971).]

1-(3,4-Dimethoxyphenyl)-3-hydroxy-2-(2-methoxyphenoxy)propan-1-one (IV) was prepared by the method of Adler *et al.* (1 952).

1-(3,4-Dimethoxyphenyl)-3-[^{18}O]hydroxy-2-(2-methoxyphenoxy)propan-1-one was prepared by the same method used to prepare unlabelled compound (IV), by substituting [^{18}O]formaldehyde for unlabelled formaldehyde. The labelled formaldehyde was prepared by stirring a formaldehyde solution in 20% $H_2{}^{18}O$ (KOR Isotopes, Cambridge, MA, U.S.A.). The yield of the isotopic label in the 3-hydroxy group of compound (IV) was 15.6%.

1-(3,4-Dimethoxyphenyl)-2-(2-methoxyphenoxy)-propane-1,3-diol(III) was prepared by $NaBH_4$ reduction of compound (IV) in 95% (v/v) ethanol at room temperature.

1-(3,4-Dimethoxyphenyl)-3-hydroxy-2-(4-methoxyphenoxy)propan-1-one (II) was prepared by the same method used to prepare compound (IV), by substituting 4-methoxyphenol for 2-methoxyphenol. The crystalline

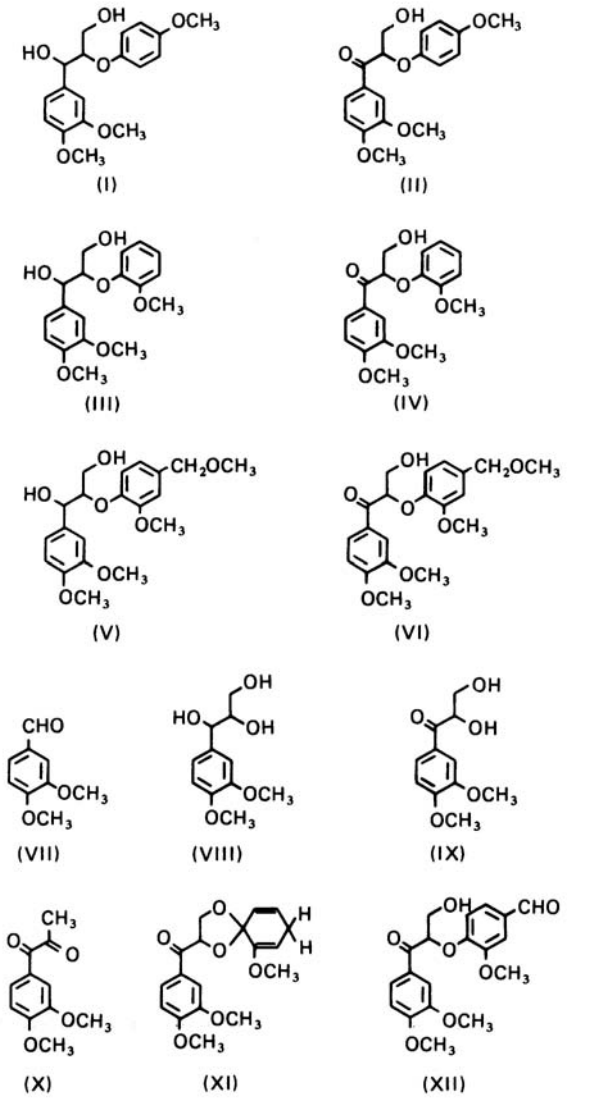


Fig. 2. Molecular formulae of model substrates and of some products of their degradation by *P. chrysosporium* ligninase

product melted at 105.5–107 °C. ^{1}H-n.m.r. (δ, p.p.m.): 3.73 (3H, s, Ar-OC*H*$_3$), 3.89 (3H, s, Ar-OC*H*$_3$), 3.95 (3H, s, Ar-OC*H*$_3$), 4.11 (2H, m, -C$_{(3)}$*H*$_2$), 5.42 (1H, dd, -C$_{(2)}$ *H*, *J* = 4.4 and 6.2 Hz), 6.76-6.90 (5H, m, Ar-*H*), 7.56 (1H, d, Ar-*H*$_2$, *J* = 2.0 Hz) and 7.74 (1H, dd, Ar-*H*, *J* = 2.1 and 8.5 Hz). M.s. [*m/z* (%): 332 (*M*$^+$, 2.1), 314 (1.2), 302 (7.2), 165 (100), 151 (4.0) and 137 (2.8).

1-(3,4-Dimethoxyphenyl)-2-(4-methoxyphenoxy)-propane-l,3diol (I) was prepared by $NaBH_4$ reduction of compound (II) in 95% (v/v) ethanol at room temperature, yielding a colourless oil. ^{1}H-n.m.r. (δ, p.p.m.): 3.51–3.81 (2H, m, -C$_{(3)}$*H*$_2$), 3.76 (3H, s, Ar-OC*H*$_3$), 3.86 (6H, s, Ar-OC*H*$_3$), 4.21-4.24 (1H, m, -C$_{(2)}$*H*), 4.95 (1H, d, -C$_{(1)}$*H*, J = 6.4 Hz) and 6.78-6.98 (7H, m, Ar-*H*). M.s. [*m/z* (%)]: 334 (*M*$^+$, 5.1), 210 (2), 181 (3.9), 167 (16.6), 150 (100), 139 (11.8), 124 (16.4) and 109 (5.8).

1-(3,4-DimethoxyphenyI)-2-(2-methoxy-4-methoxy-methylphenoxy)propan-1-one (VI) was prepared by the same method used to prepare compound (IV), by substituting vanillyl alcohol methyl ether for 2-methoxy-phenol. Vanillyl alcohol methyl ether was prepared from vanillin by (*a*) benzylation with benzyl chloride, (*b*) $NaBH_4$-reduction, (*c*) methylation with iodomethane, and (d) hydrogenation over Pd/C.

1-(3,4-Dimethoxyphenyl)-2-(2-methoxy-4-methoxy-methylphenoxy)propane-1,3-diol (V), was prepared by $NaBH_4$ reduction ofcompound (VI) in 95% (v/v) ethanol at room temperature.

1-(3,4-Dimethoxyphenyl)propane-1,2,3-triol (VIII) was prepared from 3,4-dimethoxycinnamic acid (Aldrich) by (a) methylation with diazomethane in methanol, (*b*) reduction with $LiAlH_4$ in diethyl ether, and (*c*) oxidation with OsO_4 in ether.

1-(3,4-Dimethoxyphenyl)-2,3dihydroxypropan-1-one (IX) was obtained by oxidation of compound (VIII) with 2,3-dichloro-5,6-dicyanobenzoquinone (Aldrich) in dioxan.

Structures of all synthesized compounds were confirmed by ^{1}H n.m.r. and m.s. All other compounds were obtained from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.).

RESULTS

We first examined the ligninase-catalysed degradation of model substrates (I) and (II), which have *p*-dialkoxy substitution in ring B. Previous observations (P. J. Kersten, M. Tien, B. Kalyanaraman & T. K. Kirk, unpublished work) had indicated that rings with *p*-dialkoxy substituents are more readily oxidized by the ligninase than those with *o*-dialkoxy substituents. We confirmed this observation in initial experiments with *p*-and *o*-dimethoxybenzenes, showing that the former is much more rapidly oxidized. Approximate turnover numbers at pH 3.0 are 5.1 s^{-1} and 1.0 s^{-1} respectively. The experiments with model substrates (I) and (II) therefore facilitated study of the consequences of ready oxidation in ring B. Those studies were followed by investigations of increasingly more lignin-like model substrates: (III) and (IV), and then (V) and (VI). The model substrates in each pair differ only in the oxidation state of C$_{(a)}$.

Model substrates (I) and (II)

Model compound (I) was readily oxidized by the ligninase system (ligninase/H_2O_2/O_2) with almost quantitative formation of veratraldehyde (VII); the rate of formation of veratraldehyde was slightly lower than the rate of disappearance of compound (I) (Fig. 3*a*). In addition to veratraldehyde, small amounts (< 2% each) of veratrylglycerol (VIII) and 1-(3,4dimethoxyphenyl)-2,3-dihydroxypropane-1-one (IX) were also produced (Fig. 3*a*). Additional identified products included *p*-benzoquinone, methanol and compound (II), all present in trace amounts. No major products were detected from the B ring of compound (I).

Model compound (II), which differs from compound (I) in having a carbonyl group instead of a hydroxy group at C$_{(a)}$, was also readily oxidized by the ligninase system (Fig. 3b). Three major products were identified: compound (IX) (50% conversion), *p*-benzoquinone, and methanol. A fourth major product, eluted just after compound (II) from the h.p.l.c. column, was not identified, but presumably was analogous to a product formed from analogue model compound (IV), discussed below. Together, product (IX) and this unknown probably accounted for over 90 mol% of products from

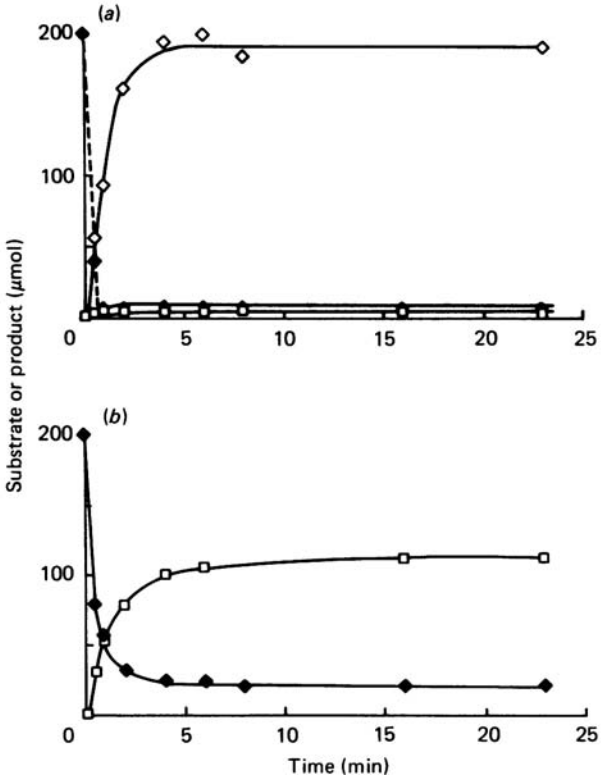


Fig. 3. Time course of oxidation of model compounds (I) (*a*) and (II)(*b*) by ligninase/H_2O_2/O_2, and of product formation

Residual starting materials and products were determined periodically on samples by h.p.l.c. Symbols: ◆, model compound (I) or (II); ◇, veratraldehyde (VII); ▲, veratrylglycerol (VIII) ; □, 1-(3,4dimethoxyphenyl)-2,3-dihydroxypropane-1-one (IX). Methanol and *p*-benzoquinone were also major products from model compound (II) (see the text).

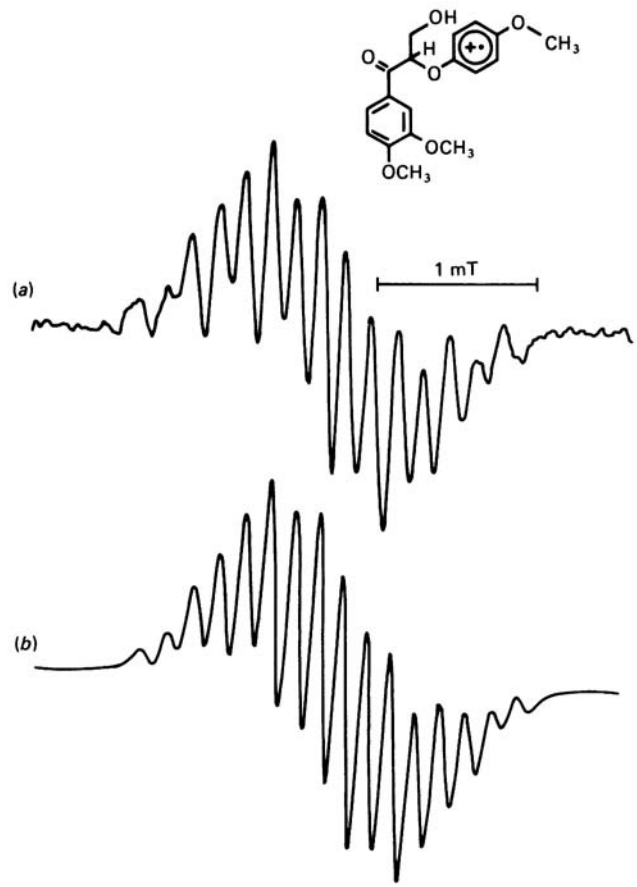


Fig. 4. E.s.r. spectrum of reaction mixture obtained during oxidation of model compound (II) by ligninase

Reaction and instrument parameters are described in the Materials and methods section. The structure shows the cation radical responsible for the signal. (*a*) shows the e.s.r. spectrum actually obtained, and (*b*) is a computer simulation.

ring A. The *p*-benzoquinone and methanol were measured only at the end of the reaction; both were present in approx. 50 mol%.

Model compound (II) was oxidized to an intermediate that exhibited an e.s.r. signal at room temperature in the absence of spin-trapping agents (Fig. 4*a*), as shown previously for certain methoxybenzenes (Kersten *et al.,* 1985). The e.s.r. spectrum (Fig. 4*a*) was attributed to the corresponding radical cation of compound (II), on the basis of computer simulation (Fig. 46). The magnetic parameters for the compound (II) radical are as follows: a^{Hortho} = 295 μT (2.95 G) (2H); a^{Hmeta} = 160 μT (1.60 G) (2H); a^{H} = 335 μT (3.345 G) (3H); a^{H} = 445 μT (4.45 G) (1H). Only the trans form (the more stable) of the radical cation is considered for this spectral simulation, although a small contribution from the cis form cannot be ruled out.

Model substrates (III) and (IV)

Model compound (III), more lignin-related than compound (I) because the methoxy group in ring B is ortho to the glycerol ether linkage (see Fig. 1), was also rapidly oxidized by the ligninase system, although not as fast as was compound (I). As seen with compound (I), veratraldehyde (VII) was the major ligninase oxidation product. A second major product was the $C_{(\alpha)}$-oxidized product, compound (IV); in addition, small amounts of veratrylglycerol (VIII) and compound (IX) were formed (Fig. 5*a*). Methanol was detected but was a minor product. No products from ring B were observed.

The fourth model substrate examined, compound (IV), is a poor ligninase substrate compared with compounds (I), (II) and (III). Nevertheless, it is slowly oxidized (Fig. 5*b*), with formation of a single major product which was eluted after compound (IV) from the h.p.l.c. column. This product is relatively unstable, being almost completely degraded simply on evaporation of solvent, as indicated by re-chromatography of the residue. The u.v. spectrum of the product (taken before solvent evaporation after isolation by h.p.l.c.) is similar to that of compound (IV), with maxima at 276 nm and 306 nm [compound (IV): 279 nm and 309 nm); the A_{276}/A_{306} ratio is also similar to the compound (IV) ratio A_{279}/A_{309} (1.4:1 compared with 1.3:1)]. The u.v.-spectral data therefore suggest structural similarity with compound (IV). Examination of the product (i.e. of the residue following solvent evaporation after h.p.l.c. separation) by chemical-ionization m.s. revealed three prominent peaks [*m/z* (% of base peak = 21 664]: 125 (75), 209 (100) and 333 (36). Examination of this residue by g.c.–m.s. revealed guaiacol (*o*-methoxyphenol) and 1-(3,4-dimethoxy-

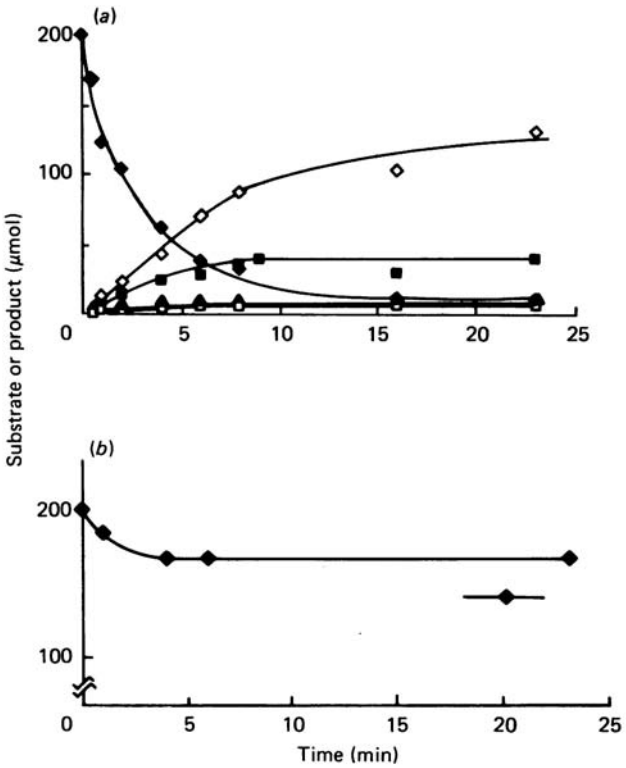
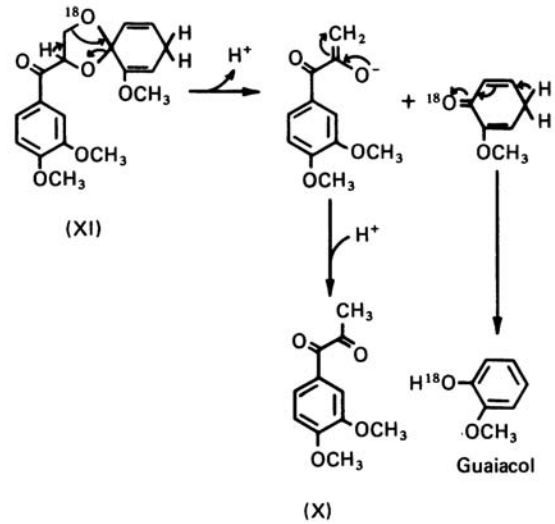


Fig. 5. Time course of oxidation of model compounds (III) (*a*) and (IV) (*b*) by ligninase, and of product formation

Products were quantified by h.p.l.c. Symbols in (a): ◆, model compound (III); ■, compound model (IV); ◇, veratraldehyde (VII); ▲, veratrylglycerol (VIII); □, 1-(3,4-dimethoxyphenyl)-2,3-dihydroxypropane-1-one (IX). Symbol in (*b*): ◆, compound model (IV). The lower datum point in (*b*) is from incubation with sequential peroxide addition (see the Materials and methods section). The only product observed by h.p.l.c. from model compound (IV) was identified as compound (XI) (see the text). Traces of methanol were detected by g.c.–m.s. from both compounds (III) and (IV).

phenyl)propane-1,2-dione (X); both were identified by electron-ionization m.s. comparison with authentic compounds. These two compounds were eluted much earlier than the unknown product from the h.p.l.c. column, and therefore are decomposition products of it. The $M + 1$ parent ions of these compounds account for the *m/z* 125 and *m/z* 209 peaks in the chemical-ionization m.s. spectrum of the residue. The peak at *m/z* 333 is presumed to be the $M + 1$ peak for the parent unknown, indicating an M_r of 332, which is the same as that of starting material. On the basis of these observations, we propose the structure (XI) for the unknown product; its decomposition to guaiacol and the diketone (X) is illustrated in Scheme 1. Evidence that the structure and the decomposition route are essentially correct was obtained with compound (IV) labelled with ^{18}O in the $C_{(\gamma)}$-hydroxy group. The unknown formed on oxidation of this model by the ligninase system yielded ^{18}O-labelled guaiacol (72% isotope retention), as illustrated in Scheme 1. The fact that ^{18}O retention was not 100% is probably due to isotope exchange with $H_2{}^{16}O$ in one of the decomposition intermediates; we detected no decomposition products other than compound (X) and guaiacol.



Scheme 1. Possible mechanism for the decomposition of cyclohexadienone ketal (XI)

Cyclohexadienone ketal (XI) is the suspected structure of the major ligninase-catalysed oxidation product of model compound (IV). The compound is unstable, decomposing spontaneously to guaiacol and diketone (X) (see the text). A possible mechanism for that decomposition is illustrated. When model compound (IV) was labelled with ^{18}O at $C_{(\gamma)}$ ^{18}O was found in product guaiacol as shown.

Model substrates (V) and (VI)

Although model compounds (III) and (IV) more accurately represent lignin than do model compounds (I) and (II), they do not have the alkyl substituent at $C_{(1)}$ in ring B that is found in lignin (see Fig. 1). Consequently, we examined two additional model substrates, compounds (V) and (VI), which have a methoxymethyl group at $C_{(1)}$. Model compound (V), like compounds (I) and (III), was readily oxidized by ligninase, more rapidly than compound (III), but somewhat more slowly than compound (I). Veratraldehyde (VII) was the dominant product, accounting for slightly over 50 mol% of ring A of compound (V); product (VI) was also identified, being present in approx. 23 mol%. Dicarbonyl product (XII) was present in trace amounts, as were veratrylglycerol (VIII) and compound (IX).

Model compound (VI) was also oxidized by the ligninase system, at about the same rate as was compound (IV); the only identified product was the dicarbonyl compound (XII), which is not itself a substrate for ligninase. Another product, which was eluted from the h.p.l.c. column just after compound (VI), is presumed to be analogous to product (XI) from compound (IV).

DISCUSSION

Our earlier work with ligninase and selected lignin model compounds (Kersten *et al.*, 1985; Hammel *et al.*, 1985) indicated that the enzyme oxidizes its lignin-like substrates by one electron to form aryl cation radicals. These radicals, depending on the substituents on the ring, undergo a variety of reactions. In the present study, we, have examined some of the relationships between the substituents on the aromatic nuclei in ß-O-4 models and their reactions after ligninase oxidation. The nature and

pattern of the substituents on the aromatic nuclei also determine their reactivity to the ligninase, presumably reflecting their oxidation potentials and their steric properties. We have observed that strong electron-withdrawing substituents such as $C_{(\alpha)}$-carbonyl groups tend to inactivate aromatic nuclei. In contrast, methoxy groups tend to activate. Presumably this involves in part resonance stabilization of the cation radical by the lone pairs of electrons on the oxygen atom. Favourable positioning of the methoxy groups also affects reactivity, presumably through charge distribution of the cation radical. Thus *p*-dimethoxybenzene is a better substrate than *o*-dimethoxybenzene. The nature and pattern of the substituents also affect the subsequent reactions of the cation radical. Alkyl side chains containing $C_{(\beta)}$-hydroxy groups and $C_{(\beta)}$–O– undergo preferential $C_{(\alpha)}$-$C_{(\beta)}$ cleavage. Our results show that a less preferred reaction is oxidation of the benzylic hydroxy group to a carbonyl group. In the absence of alkyl side chains, alkoxy groups on the aromatic ring can undergo dealkoxylation. Here again, the positioning of the substituents is expected to influence greatly the nature of the decomposition reactions (Kersten *et al.,* 1985).

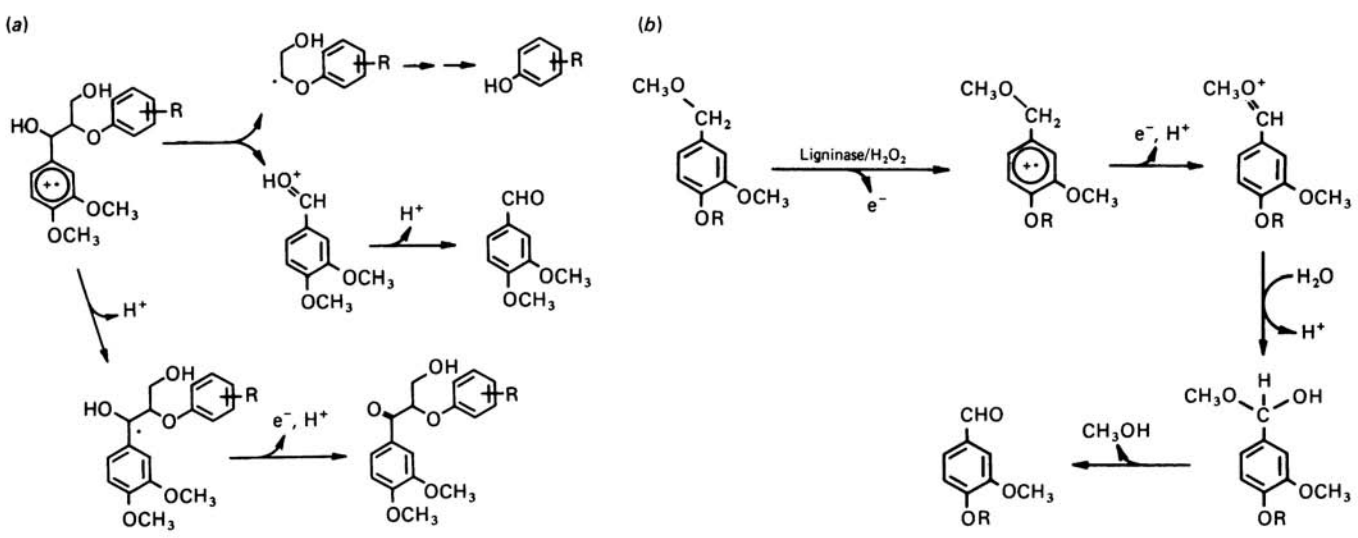
Our results are consistent with the results obtained by Snook & Hamilton (1974), who studied the one-electron oxidation of phenylalkanols to aryl cation radicals. The products found from such oxidations are similar to those found in the ligninase-catalysed oxidation of analogous substrates. On the basis of the relationships described above, predictions can be made about the reactions of the aromatic nuclei in the lignin polymer. Our results in the present study of ß-O-4 model compounds can be interpreted within the framework of the above view of ligninase action. Four types of reactions have been observed in this work: cleavage between $C_{(\alpha)}$ and $C_{(\beta)}$, oxidation of $C_{(\alpha)}$, dealkoxylation of aryl alkoxy substituents, and formation of unstable products, probably cyclohexadienone ketals.

$C_{(\alpha)}$-$C_{(\beta)}$ cleavage

A mechanism for $C_{(\alpha)}$-$C_{(\beta)}$ cleavage via a cation radical intermediate can be formulated as illustrated in Scheme 2(*a*). The cleavage results in formation of an aromatic aldehyde from ring A, and a $C_{(\beta)}$-centred radical from ring B. Formation of $C_{(\beta)}$-centred radicals accompanying $C_{(\alpha)}$-$C_{(\beta)}$ cleavage on ligninase oxidation has been established with ß-1 (1,2-diarylpropane-1,3-diol) model compounds; under aerobic conditions those radicals add molecular O_2, leading eventually to formation of a hydroxy group at $C_{(\beta)}$ (Hammel *et al.,* 1985). Earlier studies indicated that $C_{(\beta)}$-hydroxylation also accompanies ligninase-catalysed $C_{(\alpha)}$-$C_{(\beta)}$ cleavage in ß-O-4 model compounds (Tien & Kirk, 1984), which presumably could also occur via addition of molecular O_2 to a $C_{(\beta)}$-centred radical. Scheme 2(*a*) thus is deduced from our results with lignin model compounds of both the ß-1 and ß-O-4 types, as well as from the results obtained by Snook & Hamilton (1974).

The unstable hemiacetal formed by $C_{(\beta)}$-hydroxylation decomposes to yield glycolaldehyde and a phenol from ring B (Tien & Kirk, 1984). Thus *p*-methoxyphenol is formed from ligninase-catalysed $C_{(\alpha)}$–$C_{(\beta)}$ cleavage of model compound (I), and *o*-methoxyphenol from model compound (III). These phenolic products are expected to be oxidatively polymerized by ligninase (Tien & Kirk, 1984); such oxidation probably explains why these phenolic products from ring B of these model compounds were not detected.

$C_{(\alpha)}$-$C_{(\beta)}$ cleavage was by far the predominant reaction of ligninase action on model compounds (I), (III) and (V). The most readily oxidized was model compound (I), which gave veratraldehyde (VII) in almost quantitative yield. With this model, $C_{(\alpha)}$-$C_{(\beta)}$ cleavage occurred in two ways: as a primary reaction, and as a secondary one. Both aromatic nuclei in compound (I) can be easily oxidized by ligninase, on the basis of results with simpler compounds having similar substituents on the aromatic nuclei. Thus ring A is analogous to veratryl alcohol, and ring B is analogous to 1,4-dimethoxybenzene. From ring A oxidation, veratraldehyde would be formed directly by $C_{(\alpha)}$-$C_{(\beta)}$ cleavage. From ring B oxidation, it would be formed indirectly by O-$C_{(4)}$ cleavage followed by subsequent $C_{(\alpha)}$-$C_{(\beta)}$ cleavage in the ring A product, veratrylglycerol (VIII). If compound (I) had been



Scheme 2. (a) Possible mechanisms for $C_{(\alpha)}$-$C_{(\beta)}$ cleavage, $C_{(\alpha)}$ oxidation and (*b*) $C_{(\alpha)}$-methoxy-group demethoxylation via cation radical intermediates

oxidized only in ring A, veratraldehyde should have been formed at the same rate that compound (I) disappeared. The facts that veratraldehyde formation was somewhat slower and that the final stoichiometry showed that nearly 1 mol of veratraldehyde is formed/mol of compound (I) oxidized indicate that a portion of the veratraldehyde was formed via the intermediate veratrylglycerol; detection of veratrylglycerol as a minor product supports this interpretation.

Results with model compound (II) indicate that $C_{(a)}$-carbonyl groups deactivate the aromatic nucleus, in accord with the observation that veratraldehyde, compound (IX) and compound (XII) are not oxidized by ligninase. Thus the $C_{(a)}$-carbonyl group in model compound (II), as in model compounds (IV) and (VI), precludes oxidation of ring A, leaving only ring B as a substrate for ligninase. Importantly, the ready oxidation of compound (II), with formation of product (IX) via O-$C_{(4)}$ cleavage, shows clearly that a $C_{(a)}$-carbonyl group in ring A does not prevent attack on ring B in the ß-O-4 structure. This finding indicates that the low rate of oxidation of the more lignin-like $C_{(a)}$-carbonyl-bearing compounds (IV) and (VI) to product (IX) (which is not oxidized further) reflects a relative lack of susceptibility of ring B in those models. Results with compound (VI) indicate that oxidation of $C_{(a)}$ in ring B is favoured over O-$C_{(4)}$ cleavage, even when the $C_{(a)}$-hydroxy group is etherified, as it is in many of the units of lignin (Adler, 1977).

$C_{(a)}$ oxidation

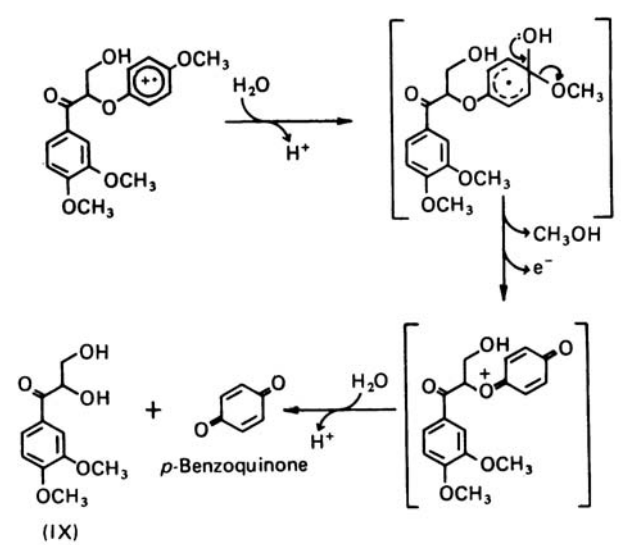
A mechanism for $C_{(a)}$-oxidation via a cation radical intermediate is illustrated in Scheme 2(*a*). We presume that the second electron is removed by the ligninase, perhaps without release of the intermediate cation radical. The mechanism shown in Scheme 2(*b*) explains the formation of a $C_{(a)}$-carbonyl group from the methoxymethyl group in model compound (VI). In that case (sequential) two-electron oxidation results in formation of a cation at $C_{(a)}$; water addition with proton elimination yields an unstable hemiacetal, which decomposes with loss of methanol (Scheme 2*b*). The mechanism in Scheme 2(*b*) suggests that alkyl and aryl substituents at $C_{(a)}$ in lignin, i.e. linkages through oxygen atoms to hemicelluloses and to other units of the lignin polymer (see Adler, 1977), are cleaved by ligninase. Previous studies had demonstrated $C_{(a)}$-$C_{(ß)}$ cleavage in a ß-1 model compound containing a methoxy group at C,, by whole cultures of *P. chrysosporium* (Kirk & Nakatsubo, 1983).

Our results, showing that $C_{(a)}$-oxidation is one consequence of ligninase action, are in accord with investigations of the chemistry of lignin degradation by whole cultures of fungi. Studies of the polymeric and low-M_r degradation products formed from lignin during degradation by white-rot fungi, including *P. chrysosporium,* have shown that $C_{(a)}$-carbonyl groups are prominent (Hata, 1966; Kirk & Chang, 1975; Ellwardt *et al.,* 1981; for review see Chen & Chang, 1985). Because the carbonyl groups render the aromatic nuclei resistant to oxidation by ligninase, it seems evident that other enzymes are involved in further degradation of the $C_{(a)}$-carbonyl-bearing structures. Ligninolytic cultures of *P. chrysosporium* apparently are not able to reduce such $C_{(a)}$-carbonyl groups (Fenn & Kirk, 1984), so that reduction, followed by $C_{(a)}$-$C_{(ß)}$ cleavage, is unlikely. Chemical and spectroscopic characterization of the polymeric degradation products of lignin resulting from attack by white-rot fungi has provided indirect evidence for the existence of extracellular enzymes that demeth-(ox)ylate, hydroxylate and cleave aromatic rings in the polymer (Kirk & Chang, 1975; Ellwardt *et al.,* 1981; Chua *et al.,* 1982; see Kirk & Shimada, 1985). Such reactions might provide alternative modes for the degradation of carbonyl-bearing structures. As mentioned below, Huynh & Crawford (1985) have reported an extracellular demethoxylating enzyme from *P. chrysosporium.*

Aryl dealkoxylation

A mechanism for dealkoxylation of aromatic alkyl ethers was postulated in connection with our studies of the action of ligninase on alkoxybenzenes (Kersten *et al.,* 1985). There it was shown that methanol is a product from the methoxybenzenes, and that ethanol is produced from 1,4-diethoxybenzene. Thus we postulate that the dealkoxylations observed here, including both demeth-oxylation and O-$C_{(4)}$ cleavage, occur via a similar mechanism. Scheme 3 illustrates a possible mechanism for formation of product (IX), *p*-benzoquinone and methanol on ligninase oxidation of model compound (II).

Formation of a small amount of methanol from model compounds (111) and (IV) reveals that ligninase probably is responsible for at least some of the methoxy-group loss that accompanies lignin degradation by *P. chrysosporium* (see Chen & Chang, 1985). Ander & Eriksson (1985) have shown that methanol is the product of the methoxy-group loss that accompanies lignin degradation by this fungus. The role of ligninase in the demethoxylation of lignin is not yet known, and other enzymes might well be involved. Huynh & Crawford (1985) have reported aryl demeth-(ox)ylase activity in the culture filtrate of ligninolytic cultures of *P. chrysosporium.* They used 2-methoxy-3-phenylbenzoic acid as substrate; whether the enzyme affects lignin or model compounds that more closely resemble lignin substructures was not determined.



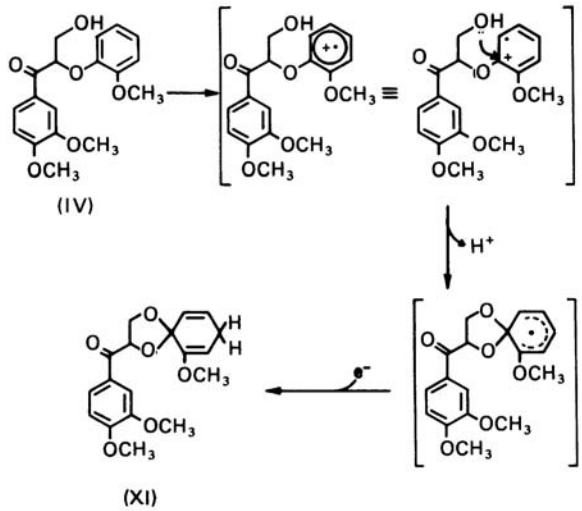
Scheme 3. Possible mechanism for formation of product (IX), *p*-benzoquinone and methanol from model compound (II) via initial formation of a cation radical in ring B

Even though O–$C_{(4)}$ cleavage in lignin-like model compounds is not a favoured consequence of ligninase action, it does occur to some extent, as shown by the results with model compounds (III)-(VI). Gold *et al.* (1984) have also reported that ligninase (which they refer to as 'diarylpropane oxygenase') oxidizes a *β*-O-4 model compound analogous to compound (III) in part to the phenylglycerol product, although they did not indicate that cleavage was O-$C_{(4)}$. Enoki et al. (1981) and Umezawa *et al.* (1983) showed that *β*-O-4 model compounds analogous to compounds (III) and (V) are degraded in part via the phenylglycerol products by intact cells of *P. chrysosporium.* In an elegant series of experiments involving isotope labelling, Umezawa & Higuchi (1985*a,b,c*) have proved that the phenylglycerol products formed in the intact cultures arise via O-$C_{(4)}$ cleavage and not via $C_{(\beta)}$-O cleavage. Our results are in accord with theirs, and indicate that ligninase is responsible for the O-$C_{(4)}$ cleavage. Our results, indicating that the yield of the phenylglycerol products of cleavage of *β*-O-4 models is quite low, are in accord with the results obtained by Enoki et al. (1981) with intact cultures.

Probable formation of cyclohexadienone ketals

Scheme 4 gives a possible mechanism for formation of suspected product (XI) from model compound (IV) via a cation radical in ring B. It is not known how the single-electron reduction of the radical could take place after cyclization via the $C_{(\gamma)}$-oxygen atom, but disproportionation between two radicals is one possibility, as is abstraction of a hydrogen atom from another molecule.

The h.p.l.c. results indicated that products analogous to compound (XI) are also formed from the other $C_{(\alpha)}$-carbonyl-containing model compounds (II) and (IV), suggesting that formation of such unstable cyclohexadienone ketals may be important in the action of ligninase on certain *β*-O-4 structures during lignin degradation. However, oxidation of the lignin-like $C_{(\alpha)}$-carbonyl model compounds (IV) and (VI) was slow and incomplete with the isolated enzyme.



Scheme 4. Possible mechanism for formation of product (XI) from model compound (IV) via initial formation of a cation radical in ring B

Umezawa & Higuchi (1985*d*) reported migration of the phenoxy moiety from $C_{(\beta)}$ to $C_{(\gamma)}$ during degradation of a model compound similar to compound (II) in whole cultures of P. chrysosporium. They suggested that the migration occurs via a cyclic radical intermediate analogous to that shown in Scheme 4. Their results therefore suggest that such cyclization occurs even when there is no $C_{(\alpha)}$-carbonyl group in the substrate.

CONCLUSIONS

Our results indicate that the major consequence of ligninase-catalysed oxidation of *β*-O-4 substructure model compounds is cleavage between $C_{(\alpha)}$ and $C_{(\beta)}$. $C_{(\alpha)}$-oxidation is much less frequent, and aryl dealkoxyl ation is a relatively minor reaction. A fourth consequence of ligninase oxidation observed here is formation of an unstable cyclohexadienone ketal from the $C_{(\beta)}$ ether-linked aromatic ring in structures with $C_{(\alpha)}$-carbonyl groups. All of the reactions except $C_{(\alpha)}$ oxidation result in cleavage of the ether linkage between $C_{(\beta)}$ and the aromatic ring. The various reactions reported here can be explained on the basis of initial formation of aryl cation radicals.

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