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Oxidation of Phenolic Arylglycerol β-Aryl Ether Lignin Model Compounds by Manganese Peroxidase from *Phanerochaete chrysosporium*: Oxidative Cleavage of an α-Carbonyl Model Compound†

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ABSTRACT: Manganese peroxidase (MnP) oxidized 1-(3,5-dimethoxy-4-hydroxyphenyl)-2-(4-(hydroxymethyl)-2-methoxyphenoxy)-1,3-dihydroxypropane (I) in the presence of Mn^{II} and H_2O_2 to yield 1-(3,5-dimethoxy-4-hydroxyphenyl)-2-(4-(hydroxymethyl)-2-methoxyphenoxy)-1-oxo-3-hydroxypropane (II), 2,6-dimethoxy-1,4-benzoquinone (III), 2,6-dimethoxy-1,4-dihydroxybenzene (IV), 2-(4-(hydroxymethyl)-2-methoxyphenoxy)-3-hydroxypropanal (V), syringaldehyde (VI), vanillyl alcohol (VII), and vanillin (VIII). MnP oxidized II to yield 2,6-dimethoxy-1,4-benzoquinone (III), 2,6-dimethoxy-1,4-dihydroxybenzene (IV), vanillyl alcohol (VII), vanillin (VIII), syringic acid (IX), and 2-(4-(hydroxymethyl)-2-methoxyphenoxy)-3-hydroxypropanoic acid (X). A chemically prepared Mn^{III}–malonate complex catalyzed the same reactions. Oxidation of I and II in $H_2{}^{18}O$ under argon resulted in incorporation of one atom of ^{18}O into the quinone III and into the hydroquinone IV. Incorporation of one atom of oxygen from $H_2{}^{18}O$ into syringic acid (IX) and the phenoxypropanoic acid X was also observed in the oxidation of II. These results are explained by mechanisms involving the initial one-electron oxidation of I or II by enzyme-generated Mn^{III} to produce a phenoxy radical. This intermediate is further oxidized by Mn^{III} to a cyclohexadienyl cation. Loss of a proton, followed by rearrangement of the quinone methide intermediate, yields the C_α-oxo dimer II as the major product from substrate I. Alternatively, cyclohexadienyl cations are attacked by water. Subsequent alkyl–phenyl cleavage yields the hydroquinone IV and the phenoxypropanal V from I, and IV and the phenoxypropanoic acid X from II, respectively. The initial phenoxy radical also can undergo C_α–C_β bond cleavage, yielding syringaldehyde (VI) and a C_6–C_2-ether radical from I and syringic acid (IX) and the same C_6–C_2-ether radical from II. The C_6–C_2-ether radical is scavenged by O_2 or further oxidized by Mn^{III}, subsequently leading to release of vanillyl alcohol (VII). VII and IV are oxidized to vanillin (VIII) and the quinone III, respectively.

Lignin is a random, heterogeneous phenylpropanoid polymer that constitutes 15-30% of woody plant cell walls (Sarkanen & Ludwig, 1971). Since the biodegradation of the wood polysaccharides cellulose and hemicellulose is retarded by the presence of lignin (Crawford, 1981), lignin-degrading microorganisms play a key role in the Earth's carbon cycle (Crawford, 1981; Kirk & Farrell, 1987; Buswell & Odier, 1987; Gold et al., 1989). White-rot fungi are primarily responsible for the initial decomposition of lignin in wood (Buswell & Odier, 1987; Kirk & Farrell, 1987; Gold et al., 1989), which occurs via an oxidative and relatively nonspecific process (Kirk & Farrell, 1987; Gold et al., 1989; Higuchi, 1990; Schoemaker, 1990).

When cultured under ligninolytic conditions, the white-rot basidiomycete *Phanerochaete chrysosporium* produces two heme peroxidases, lignin peroxidase (LiP)[1] and manganese

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peroxidase (MnP) (Gold et al., 1984, 1989; Kuwahara et al., 1984; Tien & Kirk, 1984; Glenn & Gold, 1985; Renganathan et al., 1985; Paszczyinski et al., 1986; Buswell & Odier, 1987; Kirk & Farrell, 1987), which along with an H_2O_2-generating system (Kirk & Farrell, 1987) appear to be the major components of its lignin-degradative system. The structure and mechanism of lignin peroxidase have been studied extensively (Gold et al., 1984; Tien & Kirk, 1984; Renganathan & Gold, 1986; Miki et al., 1988) and reviewed (Kirk & Farrell, 1987; Buswell & Odier, 1987; Gold et al., 1989; Higuchi, 1990; Tien, 1987; Schoemaker, 1990). MnP has also been purified and characterized (Gold et al., 1989; Kuwahara et al., 1984; Glenn & Gold, 1985; Glenn et al., 1986; Paszcyinski et al., 1986; Wariishi et al., 1988). MnP is expressed in nitrogen-limited cultures of *P. chrysosporium* only in the presence of Mn^{II} (Brown et al., 1990, 1991). The enzyme exists as a series of isozymes (Leisola et al., 1987; Paszczyinski et al., 1986; Pribnow et al., 1989), contains one iron protoporphyrin IX prosthetic group (Glenn & Gold, 1985), and is a glycoprotein of M_r ~46000 (Kuwahara et al., 1984; Glenn & Gold, 1985; Paszczyinski et al., 1986). MnP catalyzes the H_2O_2- and Mn^{II}-dependent oxidation of a variety of organic phenols and amines (Gold et al., 1989; Glenn & Gold, 1985; Glenn et al., 1986; Paszczyinski et al., 1986; Wariishi et al., 1988).

Electronic absorption (Gold et al., 1989; Glenn & Gold, 1985; Wariishi et al., 1988), EPR and resonance Raman spectroscopic (Mino et al., 1988), and covalent modification studies (Harris et al., 1991) indicate that the heme environment of native MnP resembles that of horseradish peroxidase (Dunford & Stillman, 1976). The nucleotide sequences of cDNAs encoding MnP isozymes confirm the presence of proximal and distal histidines at the active center of the enzyme (Pribnow et al., 1989; Pease et al., 1989). In addition, spectral and kinetic evidence (Wariishi et al., 1988; Mino et al., 1988; Wariishi et al., 1989a) indicates that the H_2O_2-oxidized states (compounds I and II) and the catalytic cycle of MnP are similar to those of horseradish peroxidase and lignin peroxidase (Dunford & Stillman, 1976; Renganathan & Gold, 1986). However, completion of the MnP catalytic cycle requires Mn^{II} as shown by spectroscopic and transient-state kinetic analysis (Wariishi et al., 1988, 1989a). Most importantly, MnP oxidizes Mn^{II} to Mn^{III}, and the Mn^{III} in turn oxidizes monomeric phenols (Glenn & Gold, 1985; Glenn et al., 1986; Paszczyinski et al., 1986; Wariishi et al., 1988), phenolic lignin dimers (Wariishi et al., 1989b), and synthetic lignin (Wariishi et al., 1991) via the formation of a phenoxy radical. Chelation of the Mn^{III} by organic acids such as malonate and lactate stabilizes Mn^{III} at a high redox potential (0.9–1.2 V) (Wariishi et al., 1988; Wariishi et al., 1989a; Waters & Littler, 1965; Demmber et al., 1980). We have proposed that the freely diffusible, enzyme-generated Mn^{III}–organic acid complex would be an excellent catalyst for the oxidative depolymerization of lignin in wood (Glenn et al., 1986; Wariishi et al., 1988; 1989b, 1991) which may be partially inaccessible to enzymes such as LiP (Evans et al., 1991; Srebotnik et al., 1988).

Recently, we reported the mechanism of oxidative cleavage of a free phenolic diarylpropane model compound by MnP (Wariishi et al., 1989b), strongly suggesting a role for the enzyme in the biodegradation of lignin. The arylglycerol β-aryl ether (β-O-4) is the most prevalent substructure in lignin, comprising up to 48% of the inter-unit bonds in gymnosperm (spruce) and 60% in angiosperm (birch) lignin (Sarkanen & Ludwig, 1971; Adler, 1977). In dimeric lignin models, the oxidation of the β-O-4-linked aromatic ring (B-ring) by lignin peroxidase and subsequent ring attack by water leads to the cleavage of the aryl ether bond (Kirk et al., 1986; Miki et al., 1986; Umezawa & Higuchi, 1986), resulting in the formation of free phenolic products. The likely formation of lignin fragments with phenolic arylglycerol β-aryl ether substructures during lignin degradation as well as the relatively high percentage (~15%) of phenolic residues in polymeric lignin (Sarkanen & Ludwig, 1971; Adler, 1977) prompted us to investigate the action of MnP on phenolic arylglycerol β-aryl ether model compounds.

Materials and Methods

Preparation of Compounds. 1-(3,5-Dimethoxy-4-hydroxyphenyl)-2-(4-(hydroxymethyl)-2-methoxyphenoxy)-1,3-dihydroxypropane (I) was synthesized by a modification of a previous procedure (Hosoya et al., 1980). (a) Acetosyringone in $CHCl_3$ was added to a suspension of $CuBr_2$ in ethyl acetate and refluxed for 2 h to yield bromoacetosyringone (Fieser & Fieser, 1967). MS of bromoacetosyringone (*m/z*) (TMS ether): 348 (15.6%), 346 (M^+, 15.4), 333 (17.7), 331 (15.1), 318 (16.7), 316 (16.9), 303 (15.3), 301 (16.1), 257 (5.9), 137 (12.9), 73 (100). (b) Addition of vanillin to 1.1 equivalents of sodium in dry ethanol yields sodium vanillyl alcoholate, which was used directly after evaporation of the solvent. (c) Bromoacetosyringone in DMF was added slowly to 5 equivalents of sodium vanillyl alcoholate in DMF to yield 1-(3,5-dimethoxy-4-hydroxyphenyl)-2-(4-formyl-2-methoxyphenoxy)-1-oxoethane. The product was purified on a silica gel column with hexane/ethyl acetate (2:1) as the solvent system. MS (*m/z*) (TMS ether): 418 (M^+, 9.8%), 413 (4.9), 253 (100), 209 (14.6), 151 (9.3), 73 (96.2). (d) Reaction of 1-(3,5-dimethoxy-4-hydroxyphenyl)-2-(4-formyl-2-methoxyphenoxy)-1-oxoethane with an excess of formaldehyde in the presence of diisopropylamine yielded 1-(3,5-dimethoxy-4-hydroxyphenyl)-2-(4-formyl-2-methoxyphenoxy)-1-oxo-3-hydroxypropane. MS (*m/z*) (di-TMS ether): 520 (M^+, 9.6%), 505 (2.5), 354 (8.4), 253 (100), 223 (19.3), 195 (9.6), 151 (8.4), 73 (93.0). (e) Reduction of 1-(3,5-dimethoxy-4-hydroxyphenyl)-2-(4-formyl-2-methoxyphenoxy)-1-oxo-3-hydroxypropane by $NaBH_4$ at room temperature then yielded 1-(3,5-dimethoxy-4-hydroxyphenyl)-2-(4-(hydroxymethyl)-2-methoxyphenoxy)-1,3-dihydroxypropane (I). MS (*m/z*) (tetra TMS ether): 668 (M^+, 31.2%), 653 (6.5), 353 (17.2), 327 (100), 283 (91.4), 239 (9.7), 103 (14.0), 73 (81.7).

1-(3,5-Dimethoxy-4-hydroxyphenyl)-2-(4-(hydroxymethyl)-2-methoxyphenoxy)-1-oxo-3-hydroxypropane (II) was obtained by selective reduction of the aldehyde function versus the ketone of 1-(3,5-dimethoxy-4-hydroxyphenyl)-2-(4-formyl-2-methoxyphenoxy)-1-oxo-3-hydroxypropane by $NaBH_4$ at 0 °C. II was purified by preparative TLC (benzene/acetone 1:1). MS (*m/z*) (tri TMS ether): 594 (M^+, 18.3%), 579 (2.7), 504 (2.2), 342 (16.1), 253 (100), 223 (9.2), 73 (77.9). The presence of a strong signal for the syringaldehyde fragment ion (*m/z* 253, base peak) indicated that reduction of the B-ring aldehyde and not of the α-carbonyl function had occurred.

2,6-Dimethoxy-1,4-benzoquinone (III) and 2,6-dimethoxy-1,4-dihydroxybenzene (IV) were prepared as previously reported (Wariishi et al., 1989b).

2-(4-(Hydroxymethyl)-2-methoxyphenoxy)-3-hydroxypropanal (V) and 2-(4-(hydroxymethyl)-2-methoxyphen-

[1] Abbreviations: BSTFA, *N,O*-bis(trimethylsilyl)trifluoroacetamide; DMF, *N,N*-dimethylformamide; GC, gas chromatography; GC/MS, gas chromatography/mass spectrometry; LiP, lignin peroxidase; MnP, manganese peroxidase; MS, mass spectrum; TLC, thin-layer chromatography; TMS, trimethylsilyl.

oxy)-3-hydroxypropanoic acid (X) were prepared from 2-(4-(hydroxymethyl)-2-methoxyphenoxy)-3-hydroxypropanoic acid methyl ester by reduction with diisobutylaluminum hydride in tetrahydrofuran at −70 °C (V) or basic hydrolysis (Goldsby et al., 1980) (X), respectively. 2-(4-(Hydroxymethyl)-2-methoxyphenoxy)-3-hydroxypropanoic acid methyl ester was prepared from 2-bromomalonic acid diethyl ester and 4-hydroxy-3-methoxybenzyl alcohol as described (Goldsby et al., 1980). The resulting 2-(4-(hydroxymethyl)-2-methoxyphenoxy)malonic acid diethyl ester was partially reduced by $NaBH_4$ in methanol to yield 2-(4-(hydroxymethyl)-2-methoxyphenoxy)-3-hydroxypropanoic acid methyl ester (Goldsby et al., 1980). The product was purified by preparative TLC (ethyl acetate/methanol 98:2). MS (m/z) for V (di-TMS ether): 370 (M^+, 22.1%), 340 (8.4), 280 (22.6), 225 (14.0), 209 (13.8), 191 (32.1), 103 (34.6), 73 (100). MS (m/z) for X (tri-TMS ether): 458 (M^+, 100%), 443 (12.6), 311 (11.0), 298 (24.2), 283 (18.1), 268 (9.3), 253 (3.8), 225 (29.1), 209 (20.9), 147 (32.4), 103 (34.0), 73 (83.9).

3-Methoxy-4-hydroxybenzyl alcohol (vanillyl alcohol) (VII), 3-methoxy-4-hydroxybenzaldehyde (vanillin) (VIII), 3,5-dimethoxy-4-hydroxybenzaldehyde (syringaldehyde) (VI), and 3,5-dimethoxy-4-hydroxybenzoic acid (syringic acid) (IX) were obtained from Aldrich.

Enzyme. Manganese peroxidase isozyme 1 (MnP1) was purified from the extracellular medium of acetate-buffered agitated cultures of *P. chrysosporium* strain OGC101 (Alic et al., 1987) as previously reported (Glenn & Gold, 1985; Wariishi et al., 1989a). The purified enzyme was electrophoretically homogeneous.

Enzyme Reactions. Model compound oxidations were typically carried out at 25 °C for 2 min under air in 1 mL of 50 mM malonate, pH 4.5, containing MnP (1 μg), substrate (0.2 mM), and $MnSO_4$ (0.4 mM). Reactions were initiated by adding H_2O_2 (1 mM final concentration).

^{18}O-Incorporation Experiments. Reaction vessels contained enzyme, $MnSO_4$ and substrate in 50 mM malonate, pH 4.5, in one compartment and H_2O_2 in 50 mM malonate, pH 4.5, in the other. The vessels were evacuated and flushed with scrubbed argon three times and equilibrated with $^{18}O_2$ (95%, Monsanto Research Corp.) as described (Kuwahara et al., 1984; Renganathan et al., 1986) after which the contents were mixed. For experiments conducted in $H_2{}^{18}O$, reaction mixtures were enriched with $H_2{}^{18}O$ (74%) and incubated under argon as described (Wariishi et al., 1989b; Renganathan et al., 1986). $H_2{}^{18}O$ (98%) was obtained from Isotec Inc., Miamisburg, OH.

Oxidation of Substrates I and II by Mn^{III}–Malonate Complex. Mn^{III}–malonate complex (10 mM stock solution) was prepared by dissolving Mn^{III}–acetate (Aldrich) in 0.5 M malonate buffer, pH 4.5, immediately prior to use as described (Wariishi et al., 1988). Reaction mixtures contained substrate (0.2 mM) and Mn^{III} (0.4 mM) in 50 mM malonate, pH 4.5. Reactions were carried out for 5 min at room temperature under air and analyzed immediately.

Product Analysis. Upon completion of the reaction, the reaction mixture was acidified with dilute hydrochloric acid to pH 3 and extracted three times with an equal volume of $CHCl_3$. The combined organic phase was dried over Na_2SO_4, and the solvent was evaporated with N_2 and analyzed after derivatization with BSTFA/pyridine (2:1 v/v). GC/MS was performed at 70 eV on a VG Analytical 7070E mass spectrometer fitted with an HP 5790A GC and a 30-m fused silica column DB-5, J&W Scientific. GC analysis was performed on a similar GC and column. The temperature gradient was programmed at 10 °C/min from 80 to 320 °C. Products were identified by comparison of their retention times and mass spectra with those of chemically synthesized standards. Product formation was quantitated by a GC/flame ionization detector using calibration curves of authentic synthetic standards. 2-Methoxynaphthalene was used as an internal standard.

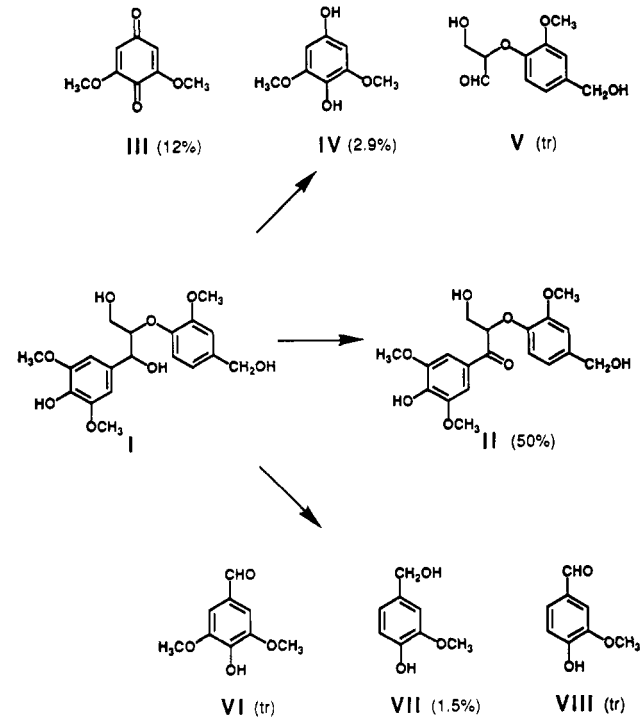

FIGURE 1: Products obtained from the oxidation of I by manganese peroxidase. Yields are in parentheses (tr = trace). Reactions were conducted and analyzed as described in the text.

Degradation of the Dimeric Substrates I and II in Stationary Cultures of P. chrysosporium. The organism was grown from a conidial inoculum at 38 °C in stationary culture as described (Enoki et al., 1981; Gold et al., 1982). The medium used was as described (Enoki et al., 1981; Kirk et al., 1978) with 2% glucose, 1.2 mM ammonium tartrate, and 20 mM sodium 2,2-dimethylsuccinate, pH 4.5, as the carbon source, nitrogen source, and buffer, respectively. Cultures were incubated under air for 3 days, after which they were purged with 99.9% O_2 every 3 days. On day 6, the substrate was added to the cultures to a final concentration of 60 μM, and after the indicated time intervals, the mycelial mat and the extracellular medium were extracted with ethyl acetate at pH ~2.0. The organic layer was washed with NaCl-saturated water at pH 2, dried over Na_2SO_4, and evaporated under reduced pressure. The products were analyzed after trimethylsilylation on GC and GC/MS. The products were quantitated as described above.

Results

Enzyme Reactions. Oxidation of the phenolic arylglycerol β-aryl ether I by MnP under aerobic and anaerobic conditions yielded the dimeric ketone II, 2,6-dimethoxy-1,4-benzoquinone III, 2,6-dimethoxyhydroquinone IV, the phenoxypropanal V, syringaldehyde (VI), vanillyl alcohol (VII), and vanillin (VIII) (Figure 1). Under the conditions used, oxidation of the benzylic hydroxyl group of I to an α-carbonyl was the predominant reaction with the formation of 50 mol % of the ketone II. The yields of the quinone III (12 mol %), the hydroquinone IV (2.9 mol %), and vanillyl alcohol (VII), (1.5 mol%) were considerably less. The phenoxypropanal V, syr-

Table I: Mass Spectra and GC Retention Times of Substrates and Products[a]

product	GC retention time (min)	mass spectrum m/z (rel intensity)
I (tetra-TMS ether)	17.7	668 (M^+, 3.2%), 653 (6.5), 353 (17.2), 327 (100), 283 (91.4), 239 (9.7), 103 (14.0), 73 (81.7)
II (tri-TMS ether)	18.8	594 (M^+, 18.3%), 579 (2.7), 504 (2.2), 342 (16.1), 253 (100), 223 (9.2), 73 (77.9)
III	7.3	170 (M^+ + 2, 12.5%), 168 (M^+, 59.8), 125 (17.1), 80 (50.0), 69 (100)
IV (di-TMS ether)	8.5	314 (M^+, 80.4%), 299 (18.5), 284 (100), 73 (74.5)
V (di-TMS ether)	11.9	370 (M^+, 22.1%), 355 (2.4), 340 (8.4), 280 (22.6), 225 (14.0), 209 (13.8), 191 (32.1), 103 (34.6), 73 (100)
VI (TMS ether)	8.7	254 (M^+, 52.7%), 239 (68.7), 224 (100), 209 (11.0), 153 (20.3), 73 (93.4)
VII (di-TMS ether)	8.0	298 (M^+, 100%), 283 (42.4), 268 (37.5), 209 (84.8), 179 (34.8), 117 (35.9), 73 (92.4)
VIII (TMS ether)	6.9	224 (M^+, 62.0%), 209 (75.0), 194 (100), 137 (10.3), 73 (80.9)
IX (di-TMS ether)	10.4	342 (M^+, 59.5%), 327 (54.8), 312 (45.2), 297 (26.2), 253 (19.0), 73 (100)
X (tri-TMS ether)	13.0	458 (M^+, 100%), 443 (12.6), 311 (11.0), 298 (24.2), 283 (18.1), 268 (9.3), 253 (3.8), 225 (29.1), 209 (20.9), 147 (32.4), 103 (34.0), 73 (83.9)

[a] Products were analyzed as described in the text. Retention times and mass spectra of reaction products were identical to those of chemically synthesized standards.

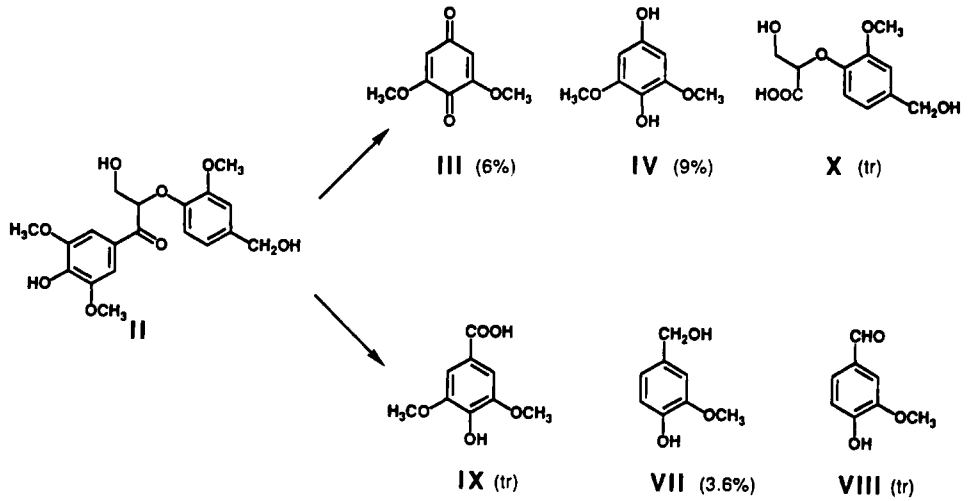

FIGURE 2: Products obtained from the oxidation of II by manganese peroxidase. Yields are in parentheses (tr = trace). Reactions were conducted and analyzed as described in the text.

ingaldehyde (VI), and vanillin (VIII) were observed in trace amounts. All products were identified by comparing their GC retention times and mass spectra with those of chemically prepared standards (Table I). The same product pattern was obtained when the reaction was conducted under either aerobic or anaerobic conditions. No products were obtained when either enzyme, H_2O_2, or Mn^{II} was omitted from the reaction mixture. MnP also oxidized vanillyl alcohol (VII) to vanillin (VIII) (data not shown) suggesting that the vanillin produced during the MnP oxidation of I was generated from vanillyl alcohol.

As shown in Figure 2, MnP readily oxidized the ketone II generating 2,6-dimethoxybenzoquinone III (6%), 2,6-dimethoxyhydroquinone IV (9%), and vanillyl alcohol (VII) (3.6%). Vanillin (VIII), syringic acid (IX), and the phenoxypropionic acid X were obtained in trace amounts. No products were obtained when the ketone II was incubated in the absence of either enzyme, H_2O_2, or Mn^{II}.

^{18}O-Incorporation Studies. When the substrate I was incubated with MnP under $^{18}O_2$ in $H_2{}^{16}O$, no incorporation of labeled oxygen into any of the products was observed. When the reaction was conducted in $H_2{}^{18}O$ under argon, the hydroquinone IV incorporated one (60.0 atom %, M^+ = 316 m/z) or two (38.7 atom %, M^+ = 318 m/z) atoms of ^{18}O (Table II, section A). Similarly, the quinone III incorporated one (57.4 atom %, M^+ = 170 m/z) or two (41.7 atom %, M^+ = 172 m/z) atoms of ^{18}O. The phenoxypropanal V incorporated 54.0 atom % and the syringaldehyde VI incorporated 84.7 atom % of ^{18}O when formed under argon in $H_2{}^{18}O$. No oxygen incorporation was observed in compounds II, VII, and VIII. When incubated in $H_2{}^{18}O$-containing (88% enriched) malonate buffer for 10 min (with no MnP, H_2O_2, and Mn^{II} added), hydroquinone IV and syringaldehyde VI showed incorporation of one atom of labeled oxygen. In addition, when extracted reaction products from the oxidation of I conducted under $Ar/H_2{}^{18}O$ were reincubated in malonate buffer in $H_2{}^{16}O$ for 10 min, incorporation of only one atom of ^{18}O was observed in III (70.2 atom %) and IV (59.2 atom %) (Table II, section A). Under these conditions, the incorporation of a second atom of labeled oxygen was strongly suppressed for product IV (4.8 atom %) and disappeared for III. These results suggest that during the oxidation of substrate I only one atom of ^{18}O from $H_2{}^{18}O$ is incorporated into product IV and its oxidation product III due to the direct attack of water on reaction intermediates. The incorporation of a second atom of ^{18}O in III and IV was a consequence of hydroxyl exchange with water. Therefore, the total incorporation of one ^{18}O from water amounted to 99.1% for the quinone III (m/z 170 plus 172, taking the isotope peaks into account) (Table II, section A); the corresponding number for the hydroquinone IV (m/z 316 plus 318) was 98.7%.

Incorporation of ^{18}O into the phenoxypropanal V and syringaldehyde (VI) also decreased sharply during the $H_2{}^{16}O$ reincubation experiment, suggesting that the incorporation of ^{18}O from $H_2{}^{18}O$ into the aldehydes V and VI stemmed from a similar exchange process. Exchange of labeled oxygen with water in aldehydes has been observed previously (Miki et al., 1988).

Table II: Relative Intensities (%) of Molecular Ion Region of Mass Spectra of Products Formed by Enzymatic Oxidation

product[f]	m/z	$^{18}O_2$[a]	$H_2{}^{18}O$[b]	$H_2{}^{18}O/H_2{}^{16}O$[c]	$H_2{}^{16}O$
A. Enzymatic Oxidation of the Arylglycerol β-Aryl Ether I					
II	594	100	100	100	100
	596	6.6	8.3	8.7	8.3
III	168	100	47.2	71.5	100
	170	6.5	100	100	8.8
	172	0	78.4	8.9	0
IV	314	100	42.6	88.2	100
	316	8.7	100	100	10.8
	318	0	72.4[e]	18.2	0
V	370	100	100	100	100
	372	37.6	84.7[e]	9.7	10.9
VI	254	nd[d]	54.9	nd[d,e]	100
	256	nd[d]	100[e]	nd[d,e]	5.2
B. Enzymatic Oxidation of the Aryl-C_α-oxoglycerol β-Aryl Ether II					
III	168	100	32.1	100	
	170	12.5	100	12.8	
	172	0	36.2	0	
IV	314	100	36.8	100	
	316	10.3	100	12.5	
	318	0	23.1[e]	0	
IX	342	nd[d]	100	100	
	344	nd[d]	72.7	0	
X	458	100	100	100	
	460	15.4	80.8	9.2	

[a] Reaction under 95% ^{18}O-enriched O_2. [b] Reaction in 74% $H_2{}^{18}O$ containing buffer under argon. [c] As footnote *b*, followed by reincubation of extracted products in $H_2{}^{16}O$-containing buffer. [d] Not determined because only a trace of the product was detectable. [e] See text for discussion of oxygen exchange with H_2O. [f] Products were isolated and analyzed as described in the text.

When the ketone II was oxidized by MnP under argon in $H_2{}^{18}O$, one (77.0 mol %) or two atoms (18.8 mol %) of ^{18}O were incorporated into the quinone III (Table II, section B). For the hydroquinone IV, the ^{18}O incorporation was 80.6 atom % and 9.0 atom %, respectively. On the basis of exchange reactions of compounds III and IV with water as outlined above, the incorporation of only one atom of ^{18}O into products III and IV each was attributable to the direct attack of water during the reaction sequence. Accordingly, the total incorporated of one ^{18}O from water was calculated to be 95.8% for the quinone III (*m/z* 170 plus 172) and 89.6% for the hydroquinone IV (*m/z* 316 plus 318) (Table II, section B), respectively. Syringic acid (IX) incorporated 56.9 atom %, and the phenoxypropionic acid X incorporated 53.5 atom % of ^{18}O when the reaction was run under argon/$H_2{}^{18}O$. No ^{18}O exchange occurred when the acids IX or X were incubated in $H_2{}^{18}O$-containing malonate buffer for 10 min. Vanillyl alcohol (VII) and vanillin (VIII) did not incorporate labeled oxygen. No labeled oxygen was incorporated into any of the products when the oxidation of ketone II was conducted under $^{18}O_2$.

Oxidation by Mn^{III} Complex. Both substrates I and II were oxidized by chemically prepared Mn^{III}–malonate complex. The products formed were identical to those produced by the enzyme reactions. Addition of 2 equivalents of Mn^{III} to the substrate I resulted in its oxidation to the ketone II with a yield of approximately 80%.

Degradation of Dimeric Model Compounds I and II in Cultures of P. chrysosporium. Incubation of I in ligninolytic cultures of *P. chrysosporium* resulted in a 62.5% decrease in substrate concentration within 1 day; after 3 days 87.5% of the substrate was degraded (Figure 3). Formation of the C_α-oxo-product II was observed shortly after the addition of substrate I (Figure 3) and reached its maximum after 6 h of incubation. At this point, the sum of substrate I and oxidation

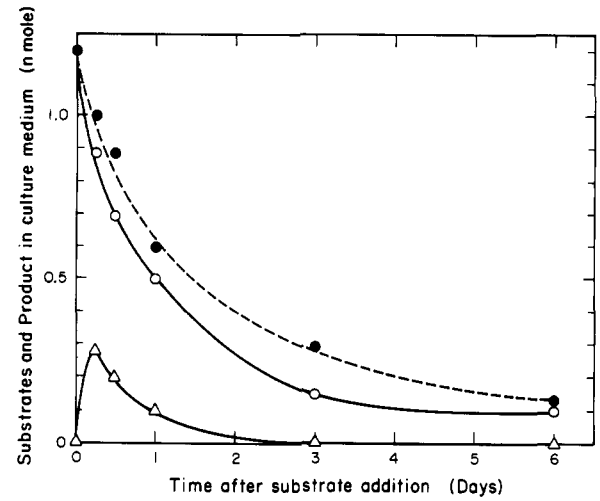

FIGURE 3: Degradation of substrates I and II in stationary cultures of *P. chrysosporium*. Addition of I (○) to cultures resulted in decrease in substrate concentration and formation of the C_α-oxo-compound II (△). Addition of II (●) to cultures also resulted in a rapid decrease in substrate concentration. Stationary cultures of *P. chrysosporium* were grown and degradation experiments were performed as described in the text.

product II accounted for 98% of the initial substrate. Similarly, a 50% decrease in substrate concentration within one day was observed when the C_α-oxo-compound II was added to ligninolytic cultures of *P. chrysosporium*; after 3 days, 76% of the substrate was degraded (Figure 3). Twelve hours after addition of the substrate II to the cultures, the benzoquinone III, the hydroquinone IV, vanillyl alcohol (VII), syringic acid (IX), and the phenoxypropionic acid X were identified in culture extracts. These compounds were further metabolized and could not be detected in extracts after an additional day of incubation. Neither reduction of II to I nor formation of the phenoxypropanal V or syringaldehyde (VI) from II were observed.

Discussion

Our previous studies demonstrated that MnP catalyzes the oxidative cleavage of a free phenolic diarylpropane lignin substructure (Wariishi et al., 1989b) as well as the in vitro depolymerization of syringyl and guaiacyl synthetic lignin (Wariishi et al., 1991). Since the arylglycerol β-aryl ether substructure is the most prevalent in native lignin, a study of the oxidation of the phenolic arylglycerol β-aryl ether I by MnP was undertaken. A recent study showed that a MnP from *Pleurotus ostreatus* oxidatively cleaved a phenolic arylglycerol β-aryl ether; however, no mechanism for this reaction was provided (Kofujita et al., 1991). The presence of a methoxy group at the 5-position of ring A in I (Figure 1) prevents the formation of side products via radical coupling reactions.

The phenolic arylglycerol β-aryl ether I was readily oxidized by MnP to produce a variety of products via C_α-oxidation, alkyl–phenyl cleavage, and C_α–C_β cleavage (Figures 1 and 2). Product yields suggest that C_α-oxidation, resulting in the formation of the ketone II is the major initial reaction. In contrast, initial alkyl–phenyl and C_α–C_β cleavage may be relatively minor pathways in the degradation of I. Alkyl–phenyl cleavage leads to the formation of the benzoquinone III, the hydroquinone IV, and the phenoxypropanal V. C_α–C_β cleavage generates syringaldehyde (VI) from the A-ring; however, the B-ring counterparts could not be detected. Both vanillyl alcohol (VII) and vanillin (VIII) also were identified as reaction products of I. Vanillyl alcohol (VII) is oxidized by Mn^{III} to form vanillin (VIII), suggesting that the vanillin

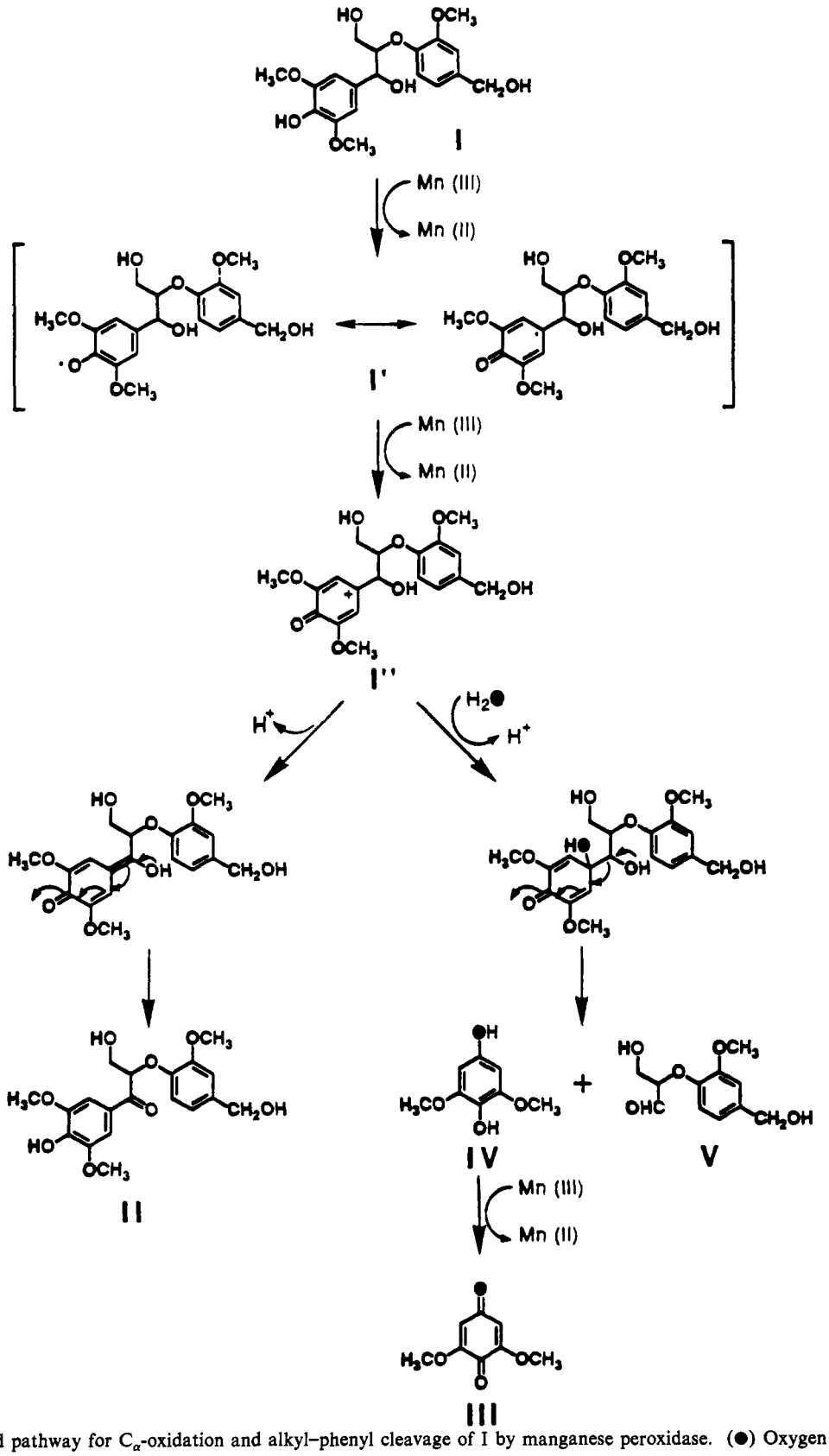

FIGURE 4: Proposed pathway for C_α-oxidation and alkyl–phenyl cleavage of I by manganese peroxidase. (●) Oxygen derived from H_2O.

observed in the oxidation of I arose as an oxidation product of vanillyl alcohol. Substrate I is oxidized by chemically prepared Mn^{III} to produce identical products, indicating that enzyme-generated Mn^{III} in turn oxidized the phenolic substrate. Identification of these oxidation products in conjunction with the results of ^{18}O-incorporation studies suggests three different mechanisms for the MnP-catalyzed oxidative cleavage of I.

Mechanism of C_α-Oxidation of I. Enzyme-generated Mn^{III} abstracts an electron from the phenol yielding the cyclohexadienyl radical I′, which is subsequently oxidized by Mn^{III} to give the corresponding cation I″ (Figure 4). The oxidation of a benzylic radical to a benzyl cation by Mn^{III} has been reported (Heiba et al., 1969). Loss of an α-proton results in the formation of an uncharged quinone methide intermediate that rearranges to yield the phenolic ketone II. In support of

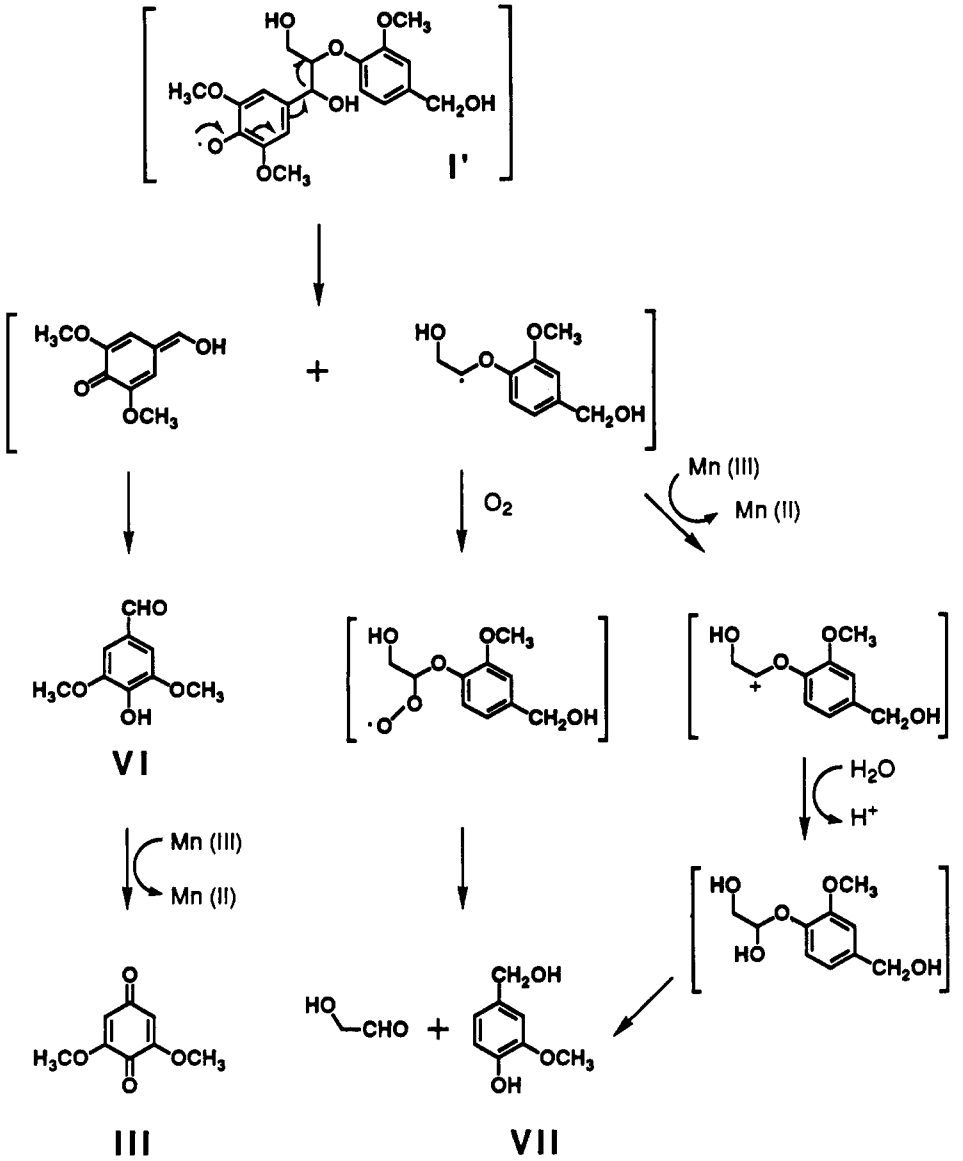

FIGURE 5: Proposed pathway for C_α–C_β cleavage of I by manganese peroxidase.

this mechanism, no labeled oxygen from molecular oxygen or $H_2{}^{18}O$ was incorporated into II (Table II, section A). This mechanism is identical to that proposed previously for the C_α oxidation of a free phenolic diarylpropane model compound by MnP (Wariishi et al., 1989b). Similar mechanisms have been proposed for the C_α-oxidation of phenolic dimers by laccase (Kawai et al., 1988).

Mechanism of Alkyl–Phenyl Cleavage of I. Alternatively, the cation intermediate I″ (Figure 4) can be attacked by water yielding a hydroxy-substituted cyclohexadienone intermediate which undergoes alkyl–phenyl bond cleavage to yield the hydroquinone IV and the phenoxy-substituted propanal V. Under these reaction conditions, Mn^{III} oxidizes the hydroquinone IV to the benzoquinone III (Wariishi et al., 1989b). As predicted by this pathway, one atom of ^{18}O from $H_2{}^{18}O$ was incorporated into both the hydroquinone IV and benzoquinone III, no incorporation of ^{18}O from $H_2{}^{18}O$ occurs during the formation of the phenoxypropanal V, and no incorporation of ^{18}O from $^{18}O_2$ into the products is observed.

Mechanism of the C_α–C_β Cleavage of the Arylglycerol β-Aryl Ether I. In this mechanism the phenoxy radical I′ undergoes homolytic cleavage, giving rise to a quinone methide and a C_6–C_2 ether radical (Figure 5). Rearrangement of the quinone methide results in the formation of syringaldehyde (VI) (Wariishi et al., 1989b). Syringaldehyde (VI), in turn, would be oxidized by Mn^{III}, generating 2,6-dimethoxy-1,4-benzoquinone III (Wariishi et al., 1989b); thus, it probably is a transient species and is observed only in trace amounts. The observed incorporation of one atom of ^{18}O from water into the benzoquinone III is predicted by this pathway and is in accordance with our earlier results (Wariishi et al., 1989b). The C_6–C_2 ether radical would be scavenged by O_2 under aerobic conditions to form a peroxy radical (Figure 5) which, after abstraction of a hydrogen atom and hydrolysis, would yield vanillyl alcohol (VII) and glycoaldehyde. In the absence of oxygen, the C_6–C_2 ether radical would be oxidized by Mn^{III} to a carbonium ion (Wariishi et al., 1989b) which would be attacked by H_2O to form a hemiacetal after loss of a proton. Hydrolysis of the hemiacetal would yield the same products. Under aerobic conditions, oxygen scavenging and oxidation of the C_6–C_2 ether radical are probably competing reactions (Wariishi et al., 1989b).

Importantly, the free phenolic aryl-C_α-oxoglycerol β-aryl ether II undergoes oxidative cleavage catalyzed by MnP, resulting in the formation of 2,6-dimethoxy-1,4-benzoquinone (III), 2,6-dimethoxy-1,4-hydroxybenzene (IV), vanillyl alcohol (VII), vanillin (VIII), syringic acid (IX), and the phenoxypropionic acid X (Figure 2). The quinone III and the hy-

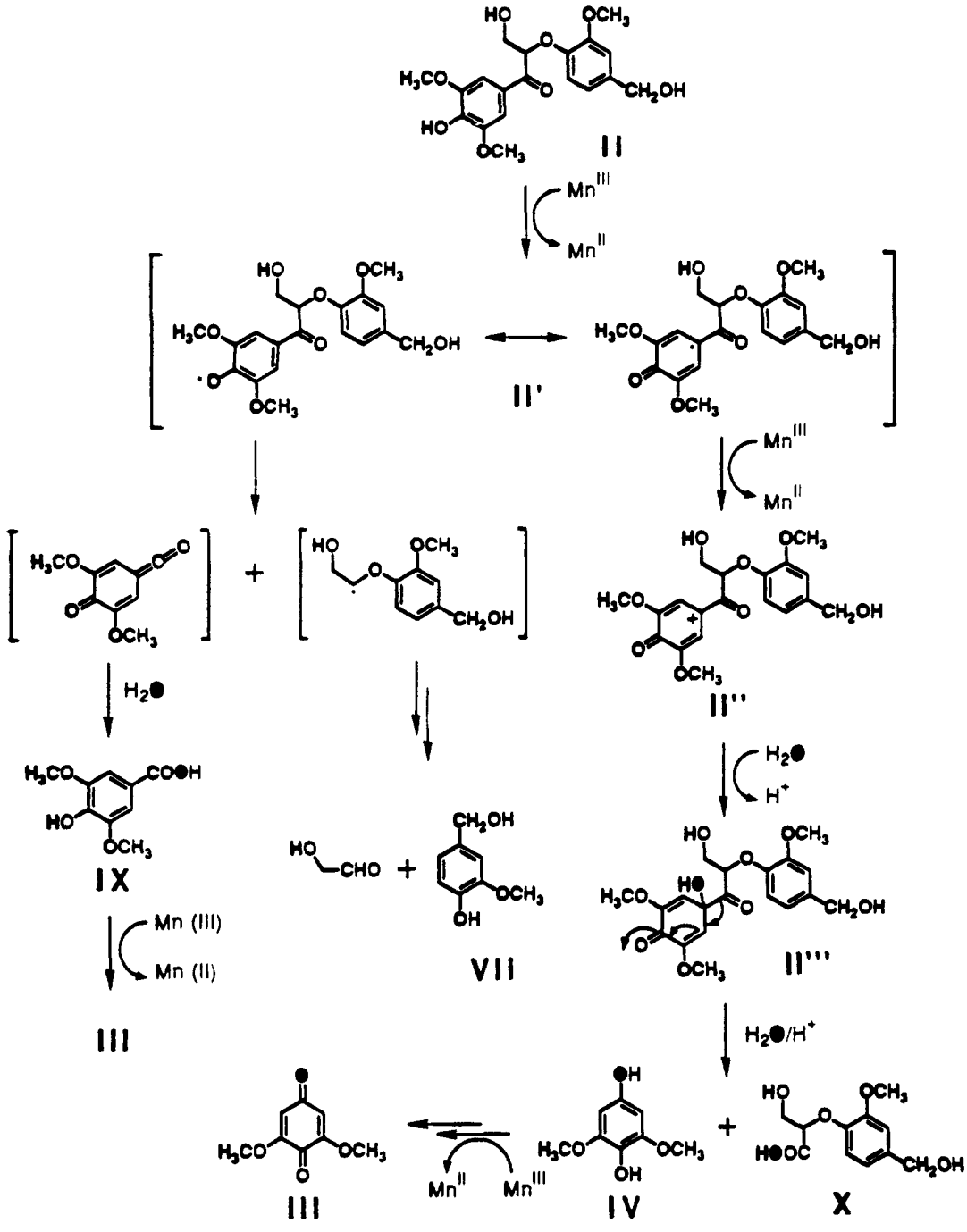

FIGURE 6: Proposed pathway for C_α–C_β cleavage and alkyl–phenyl cleavage of II by manganese peroxidase. (●) Oxygen derived from H_2O.

droquinone IV are the major products formed, together accounting for more than 80% of the quantifiable products. However, product quantification does not reveal the relative importance of alkyl–phenyl and C_α–C_β cleavage, because syringic acid from the latter cleavage is itself rapidly oxidized by Mn^{III}, resulting in decarboxylation and formation of the benzoquinone III as in the alkyl–phenyl cleavage pathway (Wariishi et al., 1989b). The observed accumulation of II in the course of the oxidation of I indicates that II may be a less favorable substrate for MnP-generated Mn^{III} perhaps owing to reduced solubility. Nevertheless, incubation of II with MnP results in a decrease in substrate concentration comparable with that of I, typically exceeding 90% in 10 min. Chemically prepared Mn^{III} generates the same products from substrate II. The results of ^{18}O-incorporation studies suggest two mechanisms for the MnP-catalyzed oxidation of II (Figure 6).

Mechanism of C_α–C_β Cleavage of the Aryl-C_α-oxoglycerol β-Aryl Ether II. One-electron oxidation of II by enzyme-generated Mn^{III} results in the formation of the phenoxy radical II′. Homolytic cleavage of the phenoxy radical II′ would result in an uncharged ketene quinone methide, which can take up water to form syringic acid (IX) (Figure 6). IX has been shown (Wariishi et al., 1989b) to be readily oxidized by MnP yielding the benzoquinone III. Thus, as with syringaldehyde (VI), syringic acid (IX) is probably a transient product and is isolated in trace amounts. The corresponding B-ring counterpart from C_α–C_β cleavage of II is a C_6–C_2 ether radical identical to the one formed in the C_α–C_β cleavage of I; reaction with molecular oxygen or water, followed by hydrolysis as proposed above would yield the observed product vanillyl alcohol (VII) (Figure 6).

Mechanism of Alkyl–Phenyl Cleavage of the Aryl-C_α-oxoglycerol β-Aryl Ether II. By analogy to substrate I, II is

oxidized by enzyme-generated Mn^{III} to the phenoxy radical II′ and further to the cation intermediate II″ (Figure 6). Attack by water yields the tertiary alcohol intermediate II‴, which would readily cleave at the alkyl–phenyl bond after nucleophilic attack by water, resulting in the hydroquinone IV and the phenoxypropionic acid X. The former can be oxidized by Mn^{III} to the benzoquinone III as discussed above. In support of the pathway depicted in Figure 5, products III, IV, and X showed 77.0 atom %, 80.6 atom %, and 53.5 atom % ^{18}O incorporation, respectively, when the reactions are conducted under argon/$H_2{}^{18}O$.

Fungal Metabolism of I and II by P. chrysosporium. Stationary cultures of *P. chrysosporium* degrade the model compounds I and II efficiently. Addition of I or II to ligninolytic cultures results in a decrease of substrate concentration within the first 24 h of 62.5% and 50%, respectively (Figure 3). During the first 6 h of incubation, substrate I is quantitatively oxidized to the C_α-oxo-compound II, indicating that in ligninolytic cultures the degradation of I proceeds mainly via C_α-oxidation. Thus, findings from both in vivo and in vitro experiments strongly suggest that the degradation of substrate I proceeds mainly via C_α-oxidation. Six to twelve hours after the addition of II, culture extracts contain the same products as observed in enzymatic oxidations of II. No evidence for reduction of II to I was obtained as reported previously for a non-phenolic C_α-oxo dimer (Fenn & Kirk, 1984).

This is the first report of the oxidative cleavage of a phenolic C_α-oxo-lignin model by MnP. A benzylic C_α-oxo group withdraws electron density from the aromatic system, therefore deactivating the A-ring toward oxidation by LiP (Kirk et al., 1986). In contrast, our results demonstrate that MnP is capable of oxidizing a phenolic C_α-oxo-substituted substructure. The observed difference in reactivity of LiP and MnP toward oxidation of C_α-oxo-substituted substrates can be explained by their different modes of electron abstraction. LiP oxidizes the aromatic ring directly to generate a cation radical, whereas MnP oxidizes the phenol to the phenoxy radical. Our results suggest that a C_α-oxo substituent on a phenolic, dimethoxy-substituted aromatic ring does not affect significantly the oxidation potential of the phenol group. In a recent investigation of a closely related syringyl-C_α-oxoglycerol β-guaiacyl ether model compound by laccase from *Coriolis versicolor*, only C_α–C_β cleavage of the substrate was observed (Higuchi, 1990). No alkyl–phenyl cleavage of the C_α carbonyl substrate was reported. Although no detailed reaction mechanism for the observed products was given, comparison of those results with our findings may suggest an intrinsic difference between the oxidizing capabilities of laccase and MnP. In the MnP reaction, in addition to C_α–C_β cleavage, the C_α-oxo-substituted phenoxy radicals can be further oxidized by Mn^{III} to their corresponding cations, leading to alkyl–phenyl cleavage with direct formation of hydroquinones and benzoquinones. Since *P. chrysosporium* effectively degrades quinones and hydroquinones (Schoemaker, 1990; Tuor et al., 1990; Valli & Gold, 1991), this unique feature may be important for the degradation of lignin by this white-rot fungus.

In summary, both the phenolic arylglycerol β-aryl ether I and the aryl-C_α-oxoglycerol β-aryl ether II are oxidatively degraded by MnP via similar mechanisms. The mechanisms proposed for the degradation of the substrates are initiated by a one-electron oxidation to generate a phenoxy radical. The radical species can either undergo homolytic C_α–C_β cleavage or can be oxidized by a second equivalent of Mn^{III} to a cation. The predominant formation of the C_α-oxo-compound II from substrate I suggests that further oxidation of the initially formed radical I′ by Mn^{III} is more favorable than C_α–C_β cleavage. Initial enzymatic oxidations are followed by a variety of nonenzymatic reactions such as loss of a proton, attack of hydroxy anion, or isomerization and re-aromatization reactions leading to the observed products. Oxidative C_α–C_β cleavage and alkyl–phenyl cleavage of both substrate I and II occur with incorporation of oxygen from water.

The mechanism for the cleavage of C_α-oxo-compound II suggests an important pathway for the degradation of polymeric lignin. Analysis of spruce lignin suggests a frequency of 15–30 free phenolic hydroxyl groups and 20 carbonyl groups per 100 phenylpropane units (Sarkanen & Ludwig, 1971). The occurrence of phenolic, C_α-oxo-substituted substructures in native lignin and in partially degraded lignin is therefore likely. Our results indicate that MnP can directly oxidize these substructures leading to the depolymerization of lignin. The free diffusion of the Mn^{III}–organic acid complex would favor such reactions (Wariishi et al., 1989b, 1991).

LiP and MnP apparently constitute a synergistic degradation system for lignin. LiP is capable of oxidizing non-phenolic aromatic rings, and MnP attacks phenolic C_α-hydroxy- and C_α-oxo-substructures in lignin. Initial reactions catalyzed by these enzyme would generate both phenolic and non-phenolic fragments. The formation of C_α-oxo-substituted structures would not necessarily hamper the degradative process; LiP can oxidize an adjacent B-ring in C_α-oxo-substituted structures with subsequent β-ether bond cleavage (Kirk et al., 1986), whereas MnP could directly attack phenolic C_α-oxo-substructures. Importantly, these results suggest that new enzymes capable of cleaving C_α-oxo-substructures need not be hypothesized.

Further studies of the degradation of C_α-oxo-substituted lignin and model compounds by MnP and LiP are in progress.

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