Peroxyl radicals are potential agents of lignin biodegradation

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Received 16 October 1999

Abstract Past work has shown that the extracellular manganese-dependent peroxidases (MnPs) of ligninolytic fungi degrade the principal non-phenolic structures of lignin when they peroxidize unsaturated fatty acids. This reaction is likely to be relevant to ligninolysis in sound wood, where enzymes cannot penetrate, only if it employs a small, diffusible lipid radical as the proximal oxidant of lignin. Here we show that a non-phenolic β-*O*-4-linked lignin model dimer was oxidized to products indicative of hydrogen abstraction and electron transfer by three different peroxyl radical-generating systems: (a) MnP/Mn(II)/linoleic acid, (b) arachidonic acid in which peroxidation was initiated by a small amount of H_2O_2/Fe(II), and (c) the thermolysis in air of either 4,4′-azobis(4-cyanovaleric acid) or 2,2′-azobis(2-methylpropionamidine) dihydrochloride. Some quantitative differences in the product distributions were found, but these were attributable to the presence of electron-withdrawing substituents on the peroxyl radicals derived from azo precursors. Our results introduce a new hypothesis: that biogenic peroxyl radicals may be agents of lignin biodegradation.

Key words: White rot; Wood decay; Lignin biodegradation; Manganese peroxidase; Lipid peroxidation; Peroxyl radical

1. Introduction

Lignin biodegradation, an essential step in the global carbon cycle, is accomplished efficiently by only one group of organisms: the basidiomycetes that cause white rot of wood [1,2]. Some of these fungi produce extracellular lignin peroxidases that interact directly with lignin oligomers [3,4] and catalyze their cleavage via free radical mechanisms [5–8]. However, many white rot fungi evidently lack lignin peroxidases [9–12]. Moreover, it is unlikely that direct reactions between a peroxidase and lignin initiate white rot, because these fungi are able to remove lignin within the secondary cell walls of sound wood, where enzymes cannot penetrate [13,14]. These observations have led to the hypothesis that white rotters employ small, diffusible oxidants to depolymerize lignin, and that extracellular fungal enzymes may generate these reactive mediators.

To account for the extensive delignification that white rot fungi accomplish, an oxidative mediator would be expected to cleave the recalcitrant non-phenolic units that predominate in lignin [15]. We suggested earlier that manganese peroxidases (MnPs), which occur in most white rotters, may produce such an oxidant [16]. The standard MnP catalytic cycle oxidizes Mn(II) to Mn(III) chelates that are insufficiently oxidizing to degrade non-phenolic structures [17,18]. However, when unsaturated fatty acids are included in these reactions, they are peroxidized [19] and uncharacterized oxidants that can cleave non-phenolic lignin are generated [16]. If these oxidants are lipid-derived radicals, they might serve as diffusible ligninolytic mediators [16,20–22]. Here we show that this hypothesis is reasonable, because peroxyl radicals, the major propagators of lipid peroxidation, are able by themselves to cleave a non-phenolic lignin structure.

2. Materials and methods

2.1. Reagents and enzymes

An *erythro/threo* mixture of 1-(4-ethoxy-3-methoxy-*ring*-[^{14}C]phenyl)-2-(2-methoxyphenoxy)-1,3-dihydroxypropane (**1**, 0.74 mCi/mmol) was prepared by reducing 1-(4-ethoxy-3-methoxy-*ring*-[^{14}C]phenyl)-1-oxo-2-(2-methoxyphenoxy)-3-hydroxypropane (**2**) with $NaBH_4$. Model **1** was purified by high performance liquid chromatography (HPLC) as described below (Section 2.3) before use, and was more than 99% chemically and radiochemically pure.

Compound **2** was prepared from *ring*-[^{14}C]acetovanillone by the method of Landucci et al. [23]. *ring*-[^{14}C]Acetovanillone was custom synthesized by New England Nuclear. A standard of 1-(4-ethoxy-3-methoxyphenyl)-1-oxo-3-hydroxypropane (**3**) was prepared as described [24]. 4-Ethoxy-3-methoxybenzoic acid (**4**) was obtained by alkylating vanillic acid with ethyl iodide [25], and 4-ethoxy-3-methoxybenzaldehyde (**5**) was purchased from Pfaltz and Bauer. 1,2,4,5-Tetramethoxybenzene was generously contributed by P.J. Kersten.

Fatty acids were the highest quality available from Sigma and were obtained in sealed ampoules under argon. Tween 20 was the Surfact-Amps grade from Pierce Chemical Co. Lyophilized *Aspergillus niger* catalase was purchased from Calbiochem.

Recombinant *Phanerochaete chrysosporium* MnP (isozyme H4) was expressed in cultures of *Aspergillus oryzae* and was partially purified by anion-exchange chromatography on DEAE-Biogel A as described [26]. MnP activity was assayed spectrophotometrically at 469 nm by monitoring the H_2O_2- and Mn(II)-dependent oxidation of 2,6-dimethoxyphenol to 2,2′,6,6′-tetramethoxydiphenoquinone in sodium tartrate buffer at pH 4.5 and ambient temperature [18]. An extinction coefficient of 49.6 mM^{-1} cm^{-1} was used to quantitate diphenoquinone formation, and one unit of MnP activity was defined as the amount of enzyme that catalyzed the oxidation of 1 μmol of 2,6-dimethoxyphenol per minute.

2.2. Oxidations

The oxidation of model **1** during MnP-mediated lipid peroxidation was assayed under aseptic conditions by a modification of the procedure described previously [16]. Typical reaction mixtures (2.0 ml in a 6 ml scintillation vial) contained [^{14}C]**1** (0.34 mM, 1.1×10^6 dpm), $MnSO_4$ (1.0 mM), an unsaturated fatty acid (5 mM), Tween 20 (1%), and MnP (0.6 U) in sodium acetate buffer (20 mM, pH 5.0). The fatty acid and the Tween 20 were combined and emulsified before they were added to the reaction mixture. Some reactions also contained 1400 U of catalase. The reaction vials were capped loosely and were rotary-shaken at 100 rpm and 40°C.

Oxidations of model **1** during Fenton reagent-initiated lipid peroxidation were conducted in the same way, except that H_2O_2 (0.1 mM) and $FeSO_4$ (0.2 mM) were used in place of MnP and $MnSO_4$ [27].

Reactions that employed peroxyl radicals derived from azo precursors as oxidants contained [^{14}C]**1** (0.34 mM, 1.1×10^6 dpm) and 4,4′-azobis(4-cyanovaleric acid) (ACVA, 10 mM) or 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AMPD, 10 mM) in sodium acetate buffer (20 mM, pH 5). When ACVA was used, it was dissolved in the

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PII: S0014-5793(99)01432-5

sodium acetate (initial pH 8.3) to give a pH 5 solution before the lignin model was added. The reaction vials were capped loosely and were rotary-shaken at 100 rpm and 60°C.

2.3. Product analyses

Samples (0.130 ml) were taken from the reactions at intervals and were filtered through a 0.45 μm pore size nylon membrane, after which 0.100 ml of the filtrate was fractionated by reversed phase HPLC on a C18 column (Vydac 201TP104, 10 μm particle size, 250×4.6 mm). The column was eluted at 1.0 ml/min and ambient temperature with the following program: 0–5 min: $H_2O/CH_3OH/HCOOH$, 75:25:0.1. 44 min: $H_2O/CH_3OH/HCOOH$, 50:50:0.1. 46 min: $CH_3OH/HCOOH$, 100:0.1. Fractions (0.5 ml) were collected and assayed for ^{14}C by scintillation counting.

Fractions that contained products **3–5** were pooled, concentrated by rotary vacuum evaporation, redissolved in 0.050 ml of bis(trimethylsilyl)acetamide/*N,N*-dimethylformamide, 1:1, and heated briefly. Several microliters of the sample were then subjected to gas chromatography/electron impact mass spectrometry (GC/MS) at 70 eV on a Finnegan MAT GCQ gas chromatograph/mass spectrometer that was equipped with a ZB5MS (non-polar silicone polymer) capillary column (length 30 m, inner diameter 0.25 mm, film thickness 0.25 μm). The column was eluted at 100°C for 1 min, followed by a temperature gradient from 100°C to 300°C at 20°C/min. The carrier gas was helium at a flow rate of 40 cm/s. Standards of compounds **3–5** were analyzed by the same procedure.

3. Results

*3.1. Reactivity of model **1** with MnP, Mn(II) and unsaturated fatty acids*

As a preliminary step, we re-evaluated the MnP/lipid system with a β-*O*-4-linked dimer (**1**) that represents the major structure in guaiacyl (conifer) lignin [15]. Fig. 1A shows a typical time course of model **1** conversion to soluble products in the presence of MnP, Mn(II), and emulsified linoleic acid. The model was not consumed significantly in reactions that lacked any of these ingredients. Oleic, linolenic, or arachidonic acid could be used in place of linoleic acid, although the reactions were then somewhat slower (data not shown).

Exogenous H_2O_2 had no effect on the reaction rate (data not shown). However, catalase inhibited the initial rate by about 85%, and it inhibited the total extent of oxidation after 48 h by about 60% (Fig. 1A). Boiled catalase had no effect on the reaction. The reaction rate was unaffected by chelators of Mn(III) such as lactate or glycolate, which stimulate most other MnP-catalyzed oxidations [18] (data not shown).

Mass balance calculations done on 48 h reactions showed that about 80% of the ^{14}C originally added was available in solution for analysis, whereas the recovery was essentially quantitative in control reactions. Scintillation counting showed that the missing ^{14}C was not bound to the glassware, so we surmise that the MnP/lipid system oxidized some of the model to volatile products.

HPLC analyses showed that the remaining soluble material consisted of residual model **1**, ketone **2**, and a complex mixture of other products. Three polar cleavage products, **3–5**, were tentatively identified on the basis of their HPLC retention times. All three identifications were checked by GC/MS, and gave results that matched those obtained with authentic standards (Fig. 2A, Table 1).

*3.2. Reactivity of model **1** with peroxidizing unsaturated fatty acids*

Model **1** was oxidized when MnP and Mn(II) were replaced with other systems that promote lipid peroxidation, although the rates were slower than those obtained with the enzymic system. The highest rate was obtained with arachidonic acid that contained a small amount of Fenton reagent to initiate chain autooxidation. The initiator was required for the reaction, but clearly did not act as the proximal oxidant of **1** because arachidonic acid was also required (Fig. 1B).

Mass balance calculations showed that about 80% of the ^{14}C originally added was available for analysis after 144 h of reaction. HPLC analysis of the remaining material showed that samples oxidized with the Fenton reagent/arachidonic acid system contained essentially the same distribution of oxidation products that reactions with MnP, Mn(II) and linoleic acid did (Fig. 2B). GC/MS analysis confirmed that the cleavage products **3–5** were present (Table 1).

The approximate relative initial rates of model **1** consumption in Fenton reagent-initiated peroxidations of various unsaturated fatty acids were as follows: arachidonic, 100; linolenic, 50; linoleic, 20; oleic, 2. That is, the efficacy of fatty acids in this system correlated with their susceptibility to peroxidation [28].

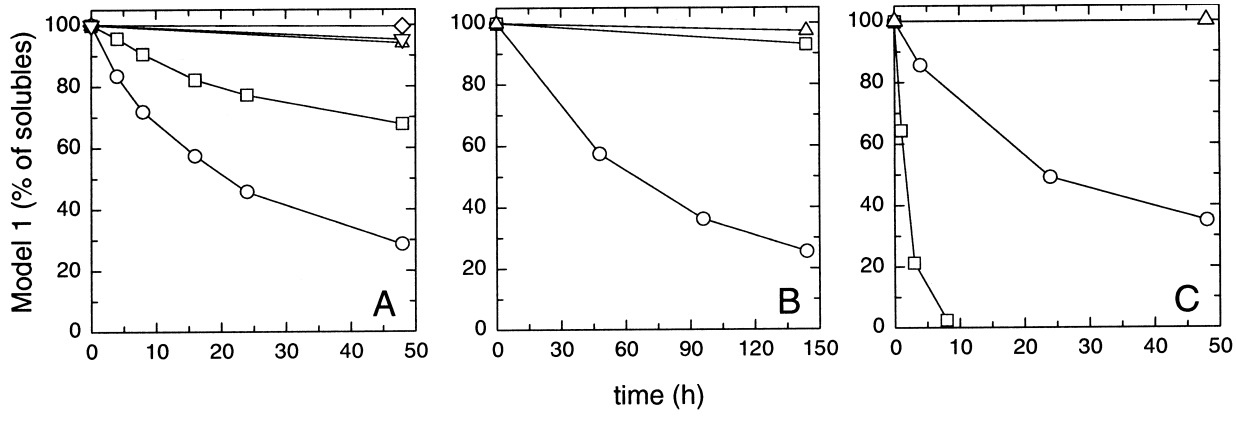

Fig. 1. Conversion of lignin model **1** to soluble products by peroxyl radical-generating systems. Samples were analyzed by HPLC as described (Section 2.3), and the data are expressed as (^{14}C in model **1**/total ^{14}C in the chromatogram)×100. A: Reactions with MnP, Mn(II), and linoleic acid: complete reaction (○), reaction minus MnP (△), reaction minus Mn(II) (▽), reaction minus linoleic acid (◇), complete reaction plus catalase (□). B: Reactions with H_2O_2, Fe(II), and arachidonic acid: complete reaction (○), reaction minus H_2O_2 and Fe(II) (□), reaction minus arachidonic acid (△). C: Reactions with azo compounds: reaction with ACVA (○), reaction with AMPD (□), reaction without azo compound (△).

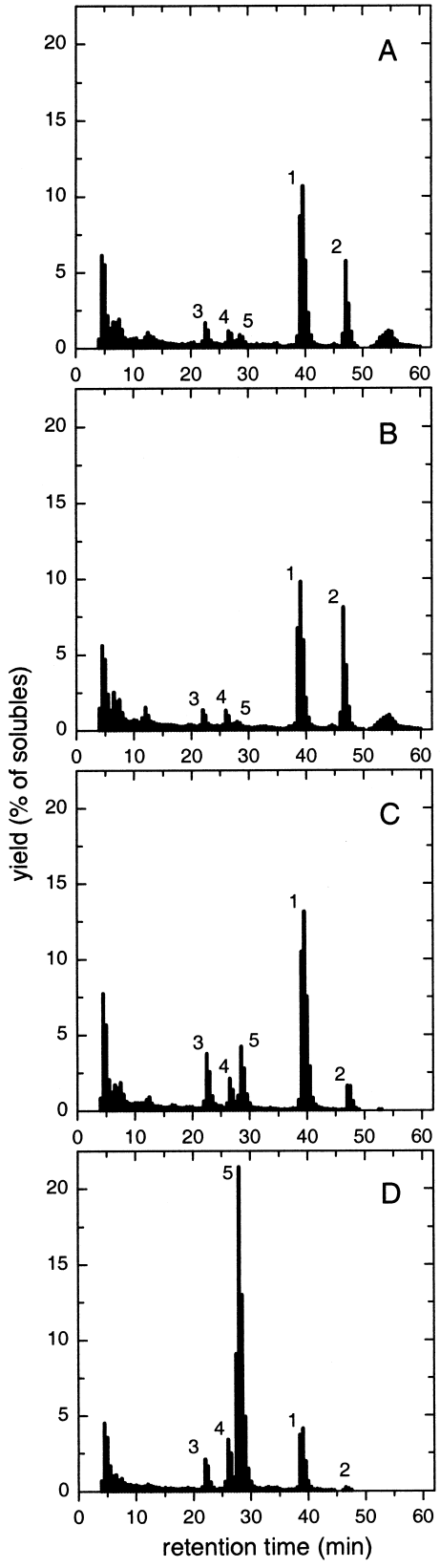

Fig. 2. HPLC radiochromatograms of products obtained from model **1** by peroxyl radical-generating systems. A: With MnP, Mn(II), and linoleic acid at 48 h. B: With H_2O_2, Fe(II), and arachidonic acid at 144 h. C: With ACVA at 48 h. D: With AMPD at 3 h. Peaks identified as compounds **1–5** (see Figs. 3 and 4) are labeled.

3.3. Reactivity of model *1* with peroxyl radicals derived from azo compounds

Model **1** was oxidized when it was heated with azo compounds that yield peroxyl radicals upon thermolysis. Treatment with ACVA at 60°C oxidized the model at approximately the same rate that the MnP/lipid system did (Fig. 1C). About 85% of the ^{14}C remained in solution after 48 h. HPLC and GC/MS analysis of the remaining material showed a product distribution generally similar to that obtained with the two lipid peroxidation systems. However, ACVA cleaved model **1** to products **3–5** more efficiently than the lipid peroxidation systems did, and it gave a lower yield of the uncleaved ketone **2** (Fig. 2C, Table 1).

The azo compound AMPD was even more reactive with model **1**. Thermolysis at 60°C resulted in a rate of model consumption about 10 times that observed with ACVA (Fig. 1C). Unlike the other peroxyl radical-generating systems we investigated, AMPD thermolysis oxidized little of the model to volatile products – over 90% of the ^{14}C remained in solution – and it also gave a negligible yield of the uncleaved ketone **2**. Instead, the radicals derived from AMPD acted as markedly selective oxidants, in that they cleaved model **1** predominantly between C_α and C_β to give the benzaldehyde **5** (Fig. 2D, Table 1).

3.4. Electron transfer from methoxylated aromatic rings by AMPD-derived radicals

Because of its high reactivity, we were able to test the AMPD system for its ability to oxidize a lignin-related structure by electron transfer. When 1,2,4,5-tetramethoxybenzene (5 mM) was heated with AMPD (10 mM) in 50 mM phosphate buffer at pH 3, the result was a yellow color that faded slowly over several minutes. The absorption spectrum of the solution exactly matched that of the 1,2,4,5-tetramethoxybenzene cation radical, with $\lambda_{max} = 450$ nm [29]. ACVA did not give this result when it was heated with 1,2,4,5-tetramethoxybenzene.

4. Discussion

Our results show that peroxidizing fatty acids oxidized a non-phenolic β-*O*-4-linked lignin structure, irrespective of whether MnP was used to promote the peroxidation. It follows that direct contact between the enzyme and the lignin structure is not essential in the MnP/lipid system. That is, small, diffusible agents derived from peroxidizing fatty acids must be able by themselves to act as proximal oxidants of lignin. This property of the MnP/lipid system may allow it to initiate ligninolysis in intact wood, where enzymes cannot penetrate [13,14].

Peroxyl radicals (ROO•) are the principal oxidants that propagate lipid peroxidation [28,30]. Therefore, it is significant that the thermolysis of azo compounds in air, a relatively clean source of peroxyl radicals [28], oxidized the β-*O*-4 structure to products that were also generated by the lipid peroxidation systems. These results lead us to propose that the ligninolytic oxidants generated by the MnP/lipid system are peroxyl radicals. Indeed, the azo compound data suggest that any extracellular fungal metabolite could mediate ligninolysis if it can be oxidized to a diffusible peroxyl radical.

We think that other oxidants known to occur during lipid peroxidation are less likely to degrade lignin. Some transition

Table 1
HPLC and GC/MS data on oxidation products derived from model **1**

Product	HPLC peak retention time (min)	Yield (% of total soluble ^{14}C)				GC peak retention time (min)	Principal ions in mass spectrum (*m/z*)
		MnP/Mn(II)/ linoleic acid (48 h)	Peroxidizing arachidonic acid (144 h)	ACVA (48 h)	AMPD (3 h)		
3	22.0–22.5	3.8	3.5	8.9	4.9	8.6[a]	296 (M^+), 281, 253, 223, 206, 179, 151[a]
4	26.0–26.5	2.5	2.6	3.9	6.7	7.2[a]	268 (M^+), 253, 225, 209, 181, 151[a]
5	28.0–28.5	3.0	1.8	9.8	50.9	5.9	180 (M^+), 152, 151
2	46.5–47.0	11.1	15.8	4.0	0.6	–[b]	–[b]

[a]Of trimethylsilyl derivatives.
[b]Not determined.

metal species cleave lipid hydroperoxides reductively to give highly reactive alkoxyl radicals (RO•), but because these undergo rapid intramolecular epoxidation or β-scission [30], intermolecular reactions with lignin are probably infrequent. The bimolecular reaction of peroxyl radicals generates singlet oxygen ($^1O_2$) [28], but previous work showed that this oxidant did not cleave a non-phenolic lignin model compound [31]. We have not excluded the possibility that the MnP/lipid system generates hydroperoxyl radicals (HOO•), which are approximately as reactive as peroxyl radicals [32], but scavenging of this species by the Mn(II) in the system [28] would be expected to reduce its ligninolytic efficiency.

A peroxyl radical could oxidize structure **1** by abstracting its benzylic hydrogen at C_α. This reaction is analogous to the allylic H-abstraction reactions that propagate fatty acid peroxidation [28,30]. Most of the resulting benzylic radicals are expected to add O_2 and then eliminate HOO•, thus yielding the uncleaved ketone **2**. However, these benzylic radicals can also eliminate phenoxy radicals to give the cleaved ketone **3** [24,33] (Fig. 3).

Alternatively, a peroxyl radical could oxidize **1** by electron transfer from an aromatic ring, as lignin peroxidases do [7,8]. The ability of AMPD-derived peroxyl radicals to oxidize 1,2,4,5-methoxybenzene to its cation radical suggests that this mechanism is reasonable. The cation radical of **1** can deprotonate to give a benzylic radical, thus giving the same products that the H-abstraction pathway does, but previous results [34] indicate that C_α-C_β cleavage to give the benzaldehyde **5** will be the major reaction. Not all of the **5** thus formed is expected to persist in the reaction, because the MnP/lipid system oxidizes benzaldehydes, probably via H-abstraction, to benzoic acids such as **4** [35] (Fig. 4).

Because products **3–5** all occurred in every system we investigated, we conclude that peroxyl radicals probably oxidize β-*O*-4 structures by both H-abstraction and electron transfer. Although the product distributions varied with different peroxyl radical-generating systems, the trends we observed are consistent with previous observations on the reactivity of these oxidants. As the substituents on peroxyl radicals become more electron-withdrawing, the reactivity of the radicals increases and they tend to oxidize donors more by electron transfer than by H-abstraction in aqueous media [36,37]. Thus, fatty acid-derived peroxyl radicals (with alkyl substituents) gave the lowest extent of C_α-C_β cleavage, ACVA-derived radicals (with –CN substituents) gave a greater extent, and AMPD-derived radicals (with $=NH_2^+$ substituents) operated almost entirely by this route (Fig. 2).

Some other features of the lipid peroxidation systems may also contribute to the relatively low extents of lignin model cleavage they give. The non-polar environment in lipid bilayers probably favors H-abstraction over electron transfer when peroxyl radicals oxidize donors [36,37]. In addition, the higher solubility of O_2 in lipids as opposed to water [28] may favor the oxygen addition reactions that convert **1** to uncleaved ketones such as **2**. However, these limitations are not inconsistent with a ligninolytic role for lipid peroxidation in vivo, because we previously observed that two white rot fungi oxidized a polymeric lignin model much more exten-

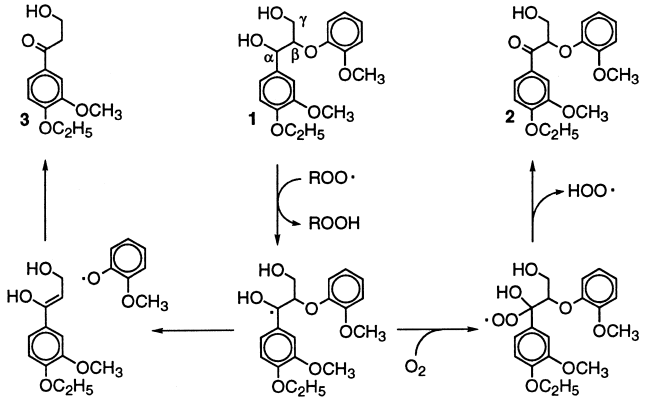

Fig. 3. Proposed pathways for the oxidation of model **1** to compounds **2** and **3** by peroxyl radicals.

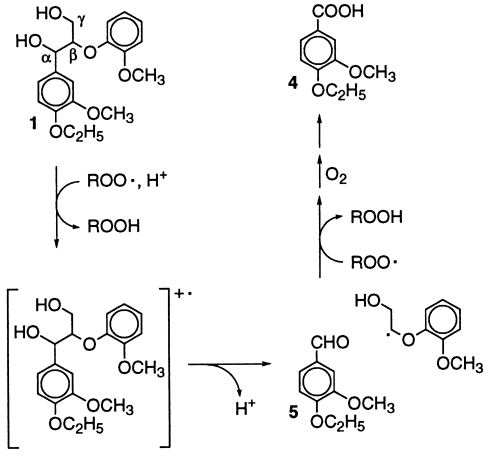

Fig. 4. Proposed pathways for the oxidation of model **1** to compounds **4** and **5** by peroxyl radicals.

sively to an uncleaved ketone than they did via C_α-C_β cleavage [38].

Some questions remain unresolved. First, we do not yet know the mechanism by which MnP promotes fatty acid peroxidation. The simplest explanation is that MnP turnover through a standard peroxidase cycle results in the oxidation of Mn(II) to Mn(III), which in turn oxidizes fatty acid hydroperoxides to chain-propagating peroxyl radicals. However, this picture is probably incomplete, because the MnP/lipid system is not completely dependent on H_2O_2 (Fig. 1A). This result suggests that other, uncharacterized oxidants react with MnP in the system. Second, it is unclear why the system operates more efficiently with linoleic acid than it does with more readily peroxidizable fatty acids. One possibility, which requires further investigation, is that MnP may react directly with some product of linoleic acid peroxidation.

Acknowledgements: We thank R. Pettersen for the mass spectrometric analyses. The use of trade names herein does not constitute official endorsement of any product by the U.S. Department of Agriculture. This work was supported by U.S. Department of Energy Grant DE-FG02-94ER20140 (to K.E.H.).

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