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PAPER

Alkoxyl- and carbon-centered radicals as primary agents for degrading non-phenolic lignin-substructure model compounds

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Lignin degradation by white-rot fungi proceeds *via* free radical reaction catalyzed by oxidative enzymes and metabolites. Basidiomycetes called selective white-rot fungi degrade both phenolic and non-phenolic lignin substructures without penetration of extracellular enzymes into the cell wall. Extracellular lipid peroxidation has been proposed as a possible ligninolytic mechanism, and radical species degrading the recalcitrant non-phenolic lignin substructures have been discussed. Reactions between the non-phenolic lignin model compounds and radicals produced from azo compounds in air have previously been analysed, and peroxyl radical (PR) is postulated to be responsible for lignin degradation (Kapich *et al.*, *FEBS Lett.*, 1999, **461**, 115–119). However, because the thermolysis of azo compounds in air generates both a carbon-centred radical (CR) and a peroxyl radical (PR), we re-examined the reactivity of the three radicals alkoxyl radical (AR), CR and PR towards non-phenolic monomeric and dimeric lignin model compounds. The dimeric lignin model compound is degraded by CR produced by reaction of 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH), which under N_2 atmosphere cleaves the α–β bond in 1-(4-ethoxy-3-methoxyphenyl)-2-(2-methoxyphenoxy)-1,3-propanediol to yield 4-ethoxy-3-methoxybenzaldehyde. However, it is not degraded by the PR produced by reaction of Ce^{4+}/*tert*-BuOOH. In addition, it is degraded by AR produced by reaction of Ti^{3+}/*tert*-BuOOH. PR and AR are generated in the presence and absence of veratryl alcohol, respectively. Rapid-flow ESR analysis of the radical species demonstrates that AR but not PR reacts with the lignin model compound. Thus, AR and CR are primary agents for the degradation of non-phenolic lignin substructures.

Introduction

Lignin, a heterogeneous phenolic polymer built from phenylpropane units linked together by C–C and C–*O*–C bonds, is amongst the most abundant biopolymers on earth. Lignin in plant cell walls, associated with hemicelluloses, creates a naturally occurring composite material that imparts strength and rigidity to the plant. Growing demand exists to produce bioethanol and chemicals from lignocellulosics in response to problems of global warming and fossil-fuel depletion. For conversion of lignocellulosics to biofuels and chemicals by enzymatic saccharification and fermentation, degradation of the lignin network is necessary.

The selective white-rot fungus *Ceriporiopsis subvermispora* can potentially degrade the lignin network without significant loss of cellulose.[1–3] *C. subvermispora* degrades lignin without penetration of its extracellular enzymes into the wood cell wall. Past work has shown that this fungus secretes manganese peroxidase (MnP) and fatty acids to oxidize lipids to hydroperoxides and aldehydes.[4] The lipid peroxidation process catalyzed by MnP degrades non-phenolic β-*O*-4 lignin model compounds to release benzylic fragments.[5,6] This reaction is likely relevant to ligninolysis in sound wood, where enzymes cannot penetrate, because manganic ions produced by MnPs are diffusible oxidants in the presence of chelators, and initiate radical chain reactions from unsaturated fatty acids or hydroperoxide intermediates.[7] With respect to the radical species responsible for degradation of non-phenolic lignin model compounds by lipid peroxidation, Kapich *et al.* hypothesized that peroxyl radicals (PRs) are principal agents for the degradation of non-phenolic lignin model compounds, based on their experiments showing that radicals derived from azo compounds 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) and 4,4′-azobis(4-cyanovaleric acid) (ACVA) decomposed a non-phenolic lignin model compound.[8] However, their report did not address the possibility that initial carbon-centred radicals (CRs) from the azo compounds react directly with the lignin model compounds before reacting with molecular oxygen as follows:[9]

$$R–N{=}N–R \rightarrow 2R^{\bullet} + N_2 \quad (1)$$

$$R^{\bullet} + O_2 \rightarrow RO_2^{\bullet} \quad (2)$$

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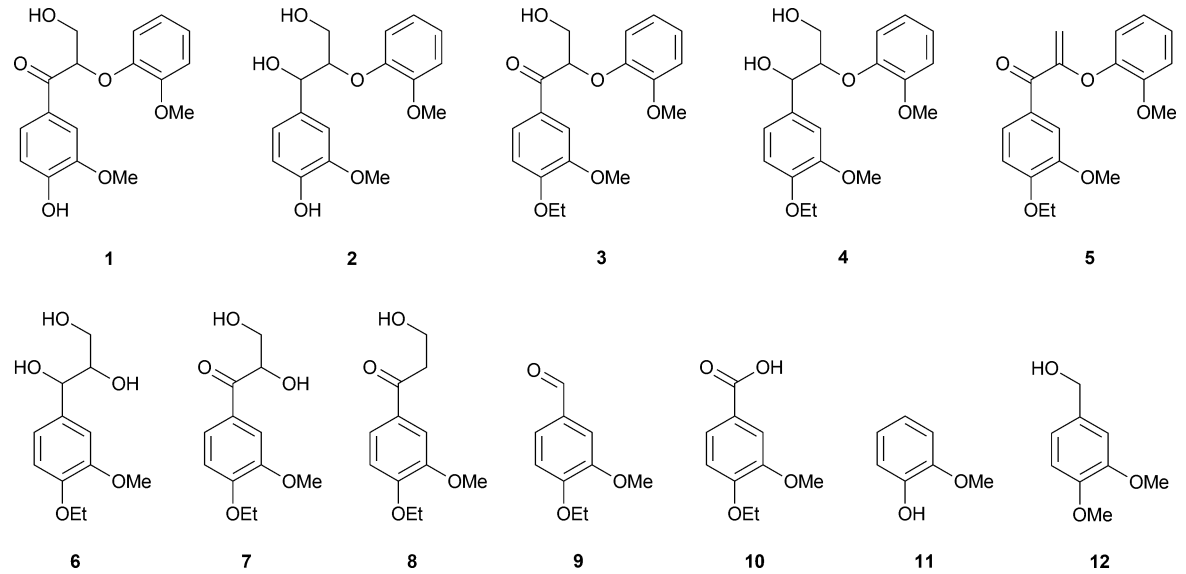

Fig. 1 Chemical structures of authentic compounds (**3**, **5–11**), the dimeric lignin model compound (**4**) and veratryl alcohol (**12**). **1** and **2** are intermediates of **3** and **4**, respectively.

Therefore, re-examination is necessary to determine the reactivity of PRs when reaction of CRs with molecular oxygen is excluded. PRs are relatively stable radicals and serve as chain-carrying radicals in lipid peroxidation.[10] This suggests that PRs function as diffusible oxidants, abstracting hydrogen from C–H bonds with small bond dissociation energies (BDEs), including the bis-allylic C–H bond, but do not play a central role in hydrogen abstraction from the recalcitrant non-phenolic lignin model compounds. To clarify the reactivity of each radical species, we generated AR, CR and PR, and analysed their reactivity towards lignin model compounds by using GC–MS and ESR, including a rapid-flow technique. Herein, we discuss these results in relation to the BDEs and energy gaps before and after reaction obtained by molecular orbital calculations.

Experimental

Materials

Acetic acid, sodium acetate, pyridine, toluene, hydrogen peroxide, 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH), 2-methoxyphenol and $H_2{}^{18}O$ were obtained from Wako Pure Chemical Industries (Osaka, Japan). $^{18}O_2$ was purchased from Shoko Company (Tokyo, Japan). Acetic anhydride and chloroform were obtained from Nacalai Tesque, Inc. (Kyoto, Japan). *tert*-Butyl hydroperoxide (*tert*-BuOOH) was a gift from NOF Corporation (Tokyo, Japan). Fluoranthene, 2,4,6-tri-*tert*-butylnitrosobenzene (BNB) and 4-ethoxy-3-methoxybenzaldehyde were obtained from Tokyo Chemical Industry Company (Tokyo, Japan). 5,5-Dimethyl-1-pyrroline-*N*-oxide (DMPO), α-(4-pyridyl-1-oxide)-*N*-*tert*-butylnitrone (4-POBN) and 3,5-dibromo-4-nitrosobenzenesulfonic acid sodium salt (DBNBS) were obtained from Labotec (Tokyo, Japan). All other chemicals used were of analytical reagent grade. Water was purified with a compact ultrapure water system (EASYpure, Barnstead, IA, USA).

Fig. 1 shows the chemical structures of the lignin model compounds and authentic compounds used in this study. 3-Hydroxy-1-(4-hydroxy-3-methoxyphenyl)-2-(2-methoxyphenoxy)-1-propanone (**1**) was synthesized from 1-(4-hydroxy-3-methoxyphenyl)ethanone *via* bromination, Williamson ether synthesis with sodium 2-methoxyphenolate and aldol condensation with methanol, as described by Hosoya *et al.* (1980).[11] 1-(4-Hydroxy-3-methoxyphenyl)-2-(2-methoxyphenoxy)-1,3-propanediol (**2**) was synthesized by reduction of **1** with $NaBH_4$. **1** and **2** were ethylated with diazoethane to give 1-(4-ethoxy-3-methoxyphenyl)-3-hydroxy-2-(2-methoxyphenoxy)-1-propanone (**3**, [MS (–TMS) *m*/*z* (%): 418 (M^+, 7.1), 268 (18.8), 179 (100), 151 (20.7), 150 (22.5), 123 (28.2), 73 (84.4)]) and 1-(4-ethoxy-3-methoxyphenyl)-2-(2-methoxyphenoxy)-1,3-propanediol (**4**, [MS (–Ac) *m*/*z* (%): 432 (M^+, 2.5), 207 (9.7), 206 (10.7), 181 (30.1), 149 (6.3), 124 (9.4), 43 (100)]), respectively. 1-(4-Ethoxy-3-methoxyphenyl)-2-(2-methoxyphenoxy)-2-propen-1-one (**5**, [MS *m*/*z* (%): 328 (M^+, 12.5), 179 (100), 151 (99.5), 149 (68.4), 135 (37.1), 123 (77.4), 77 (99.6)]) was obtained by degradation of **4** in a laccase/1-hydroxybenzotriazole system.[12] 1-(4-Ethoxy-3-methoxyphenyl)-1,2,3-propanetriol (**6**, [MS (–Ac) *m*/*z* (%): 368 (M^+, 1.9), 206 (8.3), 181 (24.7), 151 (4.9), 43 (100)]) was synthesized from 3-(4-hydroxy-3-methoxyphenyl)-2-propen-1-ol *via* ethylation by diazoethane and oxidation by OsO_4.[13] **6** was oxidized by 2,3-dichloro-5,6-dicyano-*p*-benzoquinone (DDQ) to give 1-(4-ethoxy-3-methoxyphenyl)-2,3-dihydroxy-1-propanone (**7**, [MS (–Ac) *m*/*z* (%): 324 (M^+, 4.3), 265 (2.0), 207 (8.4), 179 (29.6), 151 (21.7), 123 (9.8), 43 (100)]).[14,15] 1-(4-Ethoxy-3-methoxyphenyl)-3-hydroxy-1-propanone (**8**, [MS (–TMS) *m*/*z* (%): 296 (M^+, 28.4), 253 (19.5), 206 (50.5), 179 (20.2), 151 (16.8), 75 (100), 73 (85.8)]) was synthesized from 3-(4-hydroxy-3-methoxyphenyl)-2-propen-1-ol by ethylation with diazoethane, hydrogenation to a double bond by Pd–C/$NaBH_4$/HCl and oxidation with DDQ.[16,17] 4-Ethoxy-3-methoxybenzoic acid (**10**, [MS (–TMS) *m*/*z* (%): 268 (M^+, 40.6), 253 (92.5), 225 (37.7), 209

(92.0), 181 (51.5), 73 (100)]) was produced from vanillic acid by reaction with ethyl iodide.[18]

Instrumental analysis

GC–MS analysis was performed using a gas chromatograph–mass spectrometer (GCMS-QP5050A, Shimadzu, Kyoto, Japan) on a DB-1 column (length = 30 m; i.d = 0.25 mm; thickness = 1 μm; J&W Scientific Inc., CA, USA). Electron-impact mass spectra (EI-MS) were recorded at an ionization energy of 70 eV. The column oven temperature was maintained at 50 °C for 1 min, raised to 150 °C at a rate of 30 °C min^{-1}, maintained there for 1 min, raised to 280 °C at a rate of 5 °C min^{-1}, and maintained there.

ESR spin-trapping was peformed using a free-radical monitor (JES-FR30, JEOL, Tokyo, Japan) at room temperature under the following conditions: frequency = 9.425 GHz; centre field = 335.6 mT; sweep width = 5.0 mT; modulation width = 0.10 mT; receiver gain = 320; data points = 4096; time constant = 0.10 s; sweep time = 1.0 min; power = 4.0 mW. Rapid-flow ESR was performed using an ESR spectrometer (EMX, Bruker Biospin) equipped with a dielectric mixing resonator (ER4117D-MVT, Ibaraki, Japan) at room temperature as described in the literature[19–21] under the following conditions: frequency = 9.62 GHz; centre field = 343.0 mT; sweep width = 5.0 mT; modulation width = 0.063 mT; receiver gain = 2×10^5; data points = 512; time constant = 0.082 s; sweep time = 42 s; power = 10 mW.

Detection of lignin model compound degradation products by radicals derived from AAPH

Degradation products of lignin model compound **4** by radicals derived from AAPH were detected by GC–MS analysis. AAPH (2.7 mg), 950 μL of sodium acetate buffer (20 mM, pH 5.0) and 50 μL of the lignin model compound **4** solution (20 mM in acetone) were added to a test tube and shaken at 100 rpm and 60 °C for 3 h under N_2 or O_2 atmosphere. After the reaction, 100 μL of fluoranthene (1 mM in dichloromethane) was added and chloroform extraction was carried out. The organic layer was separated, divided into two equal fractions, transferred into separate vials and evaporated. One fraction was acetylated with 100 μL of acetic anhydride in 100 μL of pyridine at room temperature for 12 h, while the other was trimethylsilylated with 25 μL of *N*,*O*-bis(trimethylsilyl)trifluoroacetamide in 200 μL of pyridine at 60 °C for 1 h. After evaporation, 100 μL of chloroform–MeOH (1 : 1, v/v) was added to each vial and the sample was subjected to GC–MS analysis. The same experiments were carried out with $H_2{}^{18}O/N_2$ and $H_2{}^{16}O/{}^{18}O_2$ instead of $H_2{}^{16}O/N_2$ and $H_2{}^{16}O/{}^{16}O_2$. All experiments were carried out in triplicate.

Reactions of lignin model compound with Ti^{3+}/*tert*-BuOOH and Ce^{4+}/*tert*-BuOOH systems

AR and PR are produced from *tert*-BuOOH by reaction with Ti^{3+} (ref. 22) and Ce^{4+} (ref. 23) as follows:

$$Ti^{3+} + \textit{tert}\text{-BuO–OH} \rightarrow Ti^{4+} + \textit{tert}\text{-BuO}^{\bullet} + OH^{-} \quad (3)$$

$$Ce^{4+} + \textit{tert}\text{-BuOO–H} \rightarrow Ce^{3+} + \textit{tert}\text{-BuOO}^{\bullet} + H^{+} \quad (4)$$

We investigated the reactions of these unitary-radical-production systems with lignin model compound **4**. First, 50 μL of the lignin model compound **4** solution (20 mM in acetone) and 56 μL of *tert*-BuOOH (69%) were added to a test tube and diluted with 794 μL of H_2O. Then, 100 μL of $TiCl_3$ or $Ce(NH_4)_2(NO_3)_6$ (10 mM each) was added and the mixture was stirred for 5 or 10 min to produce ARs or PRs, respectively. After the reaction, 100 μL of fluoranthene (1 mM in dichloromethane) was added, chloroform extraction was carried out and the extract was evaporated. The residue was acetylated with 100 μL of acetic anhydride in 100 μL of pyridine at room temperature for 12 h. After evaporation, 100 μL of chloroform–MeOH (1 : 1, v/v) was added to the vial and the sample was subjected to GC–MS analysis. Experiments were carried out in triplicate.

Analysis of radicals derived from AAPH and *tert*-BuOOH

CRs and PRs derived from AAPH under N_2 or O_2 atmosphere were analysed using ESR spin-trapping. First, 40 μL of AAPH (100 mM in 20 mM sodium acetate buffer, pH 5), 40 μL of DBNBS (100 mM in H_2O) or BNB (100 mM in methanol, ethanol or DMSO) and 320 μL of sodium acetate buffer (20 mM, pH 5) or solvent (methanol, ethanol or DMSO) were mixed in a test tube. The samples were shaken at 100 rpm and 60 °C for 5 min under N_2 or O_2 atmosphere. After reaction, the spin adducts were analysed using ESR. Although, the ESR signals of BNB adducts were accumulated 20 times, those from the other spin traps were accumulated just once.

ARs derived from *tert*-BuOOH by the reaction with Ti^{3+} were analysed using ESR spin-trapping. First, 56 μL of *tert*-BuOOH (69%) and 100 μL of 4-POBN (100 mM) were diluted with 744 μL of H_2O in a test tube. Then, 100 μL of $TiCl_3$ (10 mM) was added, the solution was mixed vigorously and the ESR spectrum was recorded immediately.

PRs derived from *tert*-BuOOH by the reaction with Ce^{4+} were analysed using ESR spin-trapping. First, 100 μL of $Ce(NH_4)_2(NO_3)_6$ (10 mM in H_2O), 56 μL of *tert*-BuOOH (69%), 100 μL of 4-POBN (100 mM) and 744 μL of H_2O were mixed in a test tube. The solution was mixed vigorously and the ESR spectrum was recorded immediately.

Analysis of radicals derived from veratryl alcohol

AR and PR derived from the two peroxides *tert*-BuOOH and H_2O_2 were analysed directly in the presence and absence of veratryl alcohol **12** by using rapid-flow ESR. Two aqueous solutions were prepared: Solution A contained 10 mM $TiCl_3$ or 6 mM of $Ce(NH_4)_2(NO_3)_6$ and 190 mM H_2SO_4; Solution B contained 0–80 mM *tert*-BuOOH or 0–90 mM H_2O_2, 0–150 mM veratryl alcohol **12** and 190 mM H_2SO_4. The H_2SO_4 was added to stabilize Ti^{3+} and to accelerate the reaction of Ce^{4+} with the peroxides. Each solution was filled into a 25 mL syringe, the syringes were set on a syringe pump and the solutions were flowed into a mixing chamber. Spectra were accumulated 30 times. Flow rates after mixing are listed in the figure legend.

Simulation of bond dissociation energy

The bond dissociation energies (BDEs) were simulated by calculating the difference in the energy before and after reaction A–H → A$^{\bullet}$ + H$^{\bullet}$, where A–H is the parent molecule and A$^{\bullet}$ is the corresponding hydrogen-abstracted radical. The BDE of

Table 1 Degradation products of non-phenolic β-*O*-4 lignin model compound **4** from reaction with AAPH-derived radicals

	Intensity ratio (%)[a]							
Sample	**3**	**4**	**5**	**6** + **7**[b]	**8**	**9**	**10**	**11**
–AAPH (N_2)	1.7	96.6						1.6
+AAPH (N_2)	6.9	42.0			3.0	41.4		6.6
–AAPH (O_2)	1.5	98.2						0.3
+AAPH (O_2)	5.7	63.7	2.7	2.6	1.1	18.6	1.8	3.6

Reactions were carried out in sodium acetate buffer (pH = 5.0) containing 5% (v/v) acetone. [AAPH] = 10 mM, [lignin model compound **4**] = 1.0 mM.[a] The values of **4**, **5**, **6**+**7**, **9** and **11** were calculated using the ratio of acetylated samples with an internal standard. The values of **3**, **8** and **10** were calculated using the ratio of trimethylsilylated samples. [b] Because **6** and **7** are difficult to separate by our GC–MS conditions, the sum of the ratio is given.

Table 2 Degradation products of non-phenolic β-*O*-4 lignin model compound **4** from reaction with metal–hydroperoxide systems

	Intensity ratio (%)[a]					
Sample	**3**	**4**	**5**	**7**	**9**	**11**
Ti^{3+}		100.0				
Ti^{3+}/*tert*-BuOOH	25.9	47.6	10.9	1.0	6.4	8.4
Ce^{4+}	2.6	92.1	0.7	0.2	4.1	0.3
Ce^{4+}/*tert*-BuOOH	2.2	97.2	0.3			0.3

[a] Reactions were carried out in water containing 5% (v/v) acetone. [lignin model compound **4**] = 1.0 mM, [*tert*-BuOOH] = 0.40 M, [Ti^{3+}] = [Ce^{4+}] = 1 mM. All values were calculated using the ratio of acetylated samples with an internal standard.

radicals such as CR and PR derived from AAPH and *tert*-BuO$^{\bullet}$ and *tert*-BuOO$^{\bullet}$ were calculated to compare their capability to abstract hydrogen. In this case, A–H is the hydrogen-added product from the radical. This simulation is based on a concept described by Wright *et al.* (2001).[24] The geometries of the closed-shell species were optimized using the AM1 method. Theoretical molecular orbital (MO) calculations for the optimized structure were performed at the B3LYP/6-311G** level of theory. For radicals, an unrestricted approach was applied. The energy of the hydrogen atom was set to its exact value, –0.50000 au (–313.75 kcal mol^{-1}). All calculations were performed using Spartan '06 software (Wavefunction Inc., Irvine, CA, USA).

Results

Analysis of lignin model compound degradation products by radicals derived from AAPH

Non-phenolic β-*O*-4 lignin model compound **4** was reacted with radicals produced by the thermolysis of AAPH under N_2 and O_2 atmospheres. Yields of the degradation products of lignin model compound **4** are shown in Table 1. The GC–MS total ion chromatogram of the degradation products from the lignin model compound **4** after reaction with AAPH-derived radicals under N_2 atmosphere is exemplified in Fig. 2. Under both N_2 and O_2 atmospheres, a major degradation product is benzaldehyde **9**. Under N_2 atmosphere, a small amount of the following additional products is produced: dimeric ketone **3**, monomeric monohydroxyl ketone **8** and guaiacol **11**. Under O_2 atmosphere, the following additional products are produced: dimeric ketone **3**, dehydrated dimeric ketone **5**, triol **6**, monomeric dihydroxyl ketone **7**, monomeric monohydroxyl ketone **8**, benzylic acid **10** and guaiacol **11**. These results indicate that the radicals derived from AAPH can selectively break the α–β bond in lignin model compound **4**, and α-oxidation and β-*O*-4 bond cleavage occur. A major difference between the reactions under N_2 and O_2 atmospheres is that benzylic acid **10** is produced from benzaldehyde **9** under O_2 atmosphere. The products formed by reaction under O_2 atmosphere are consistent with products reported to form by reaction in air.[8] Fig. 3 shows a comparison between $H_2{}^{16}O$ and $H_2{}^{18}O$ under N_2 and $^{18}O_2$ atmospheres. This result clearly indicates that the oxygen atom in water is incorporated into a carbonyl group of benzaldehyde **9** even in the presence of dissolved oxygen.

Reactions of lignin model compound with Ti^{3+}/*tert*-BuOOH and Ce^{4+}/*tert*-BuOOH systems

The lignin model compound **4** was reacted with the Ti^{3+}/*tert*-BuOOH and Ce^{4+}/*tert*-BuOOH systems.

Reaction with the Ti^{3+}/*tert*-BuOOH system degrades the lignin model compound **4** to give dimeric ketone **3**, dehydrated dimeric ketone **5**, monomeric dihydroxyl ketone **7**, benzaldehyde **9** and guaiacol **11** (Table 2). However, without *tert*-BuOOH, the lignin model compound **4** is not decomposed. Thus, non-phenolic β-*O*-4 lignin model compound **4** is decomposed by the alkoxyl radical (*tert*-BuO$^{\bullet}$).

tert-BuOOH is known to react with Ce^{4+} to give *tert*-BuOO$^{\bullet}$. Reaction with the Ce^{4+}/*tert*-BuOOH system degrades the lignin model compound **4** to give dimeric ketone **3**, dehydrated dimeric ketone **5** and guaiacol **11**. However, without *tert*-BuOOH, the lignin model compound **4** produces dimeric ketone **3**, dehydrated dimeric ketone **5** and guaiacol **11** at intensities almost the same

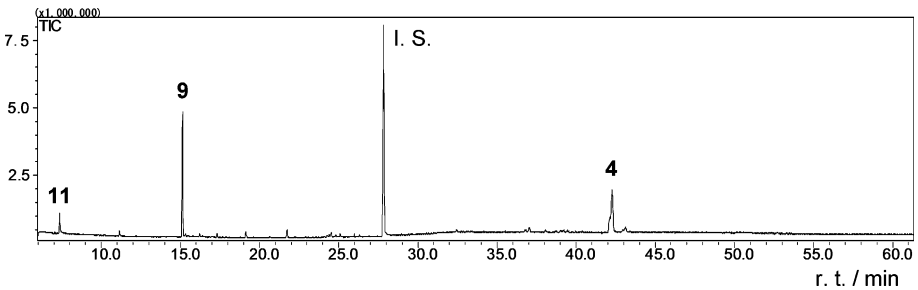

Fig. 2 GC–MS total ion chromatogram of the degradation products from the lignin model compound (**4**) after reaction with AAPH-derived radicals under N_2 atmosphere.

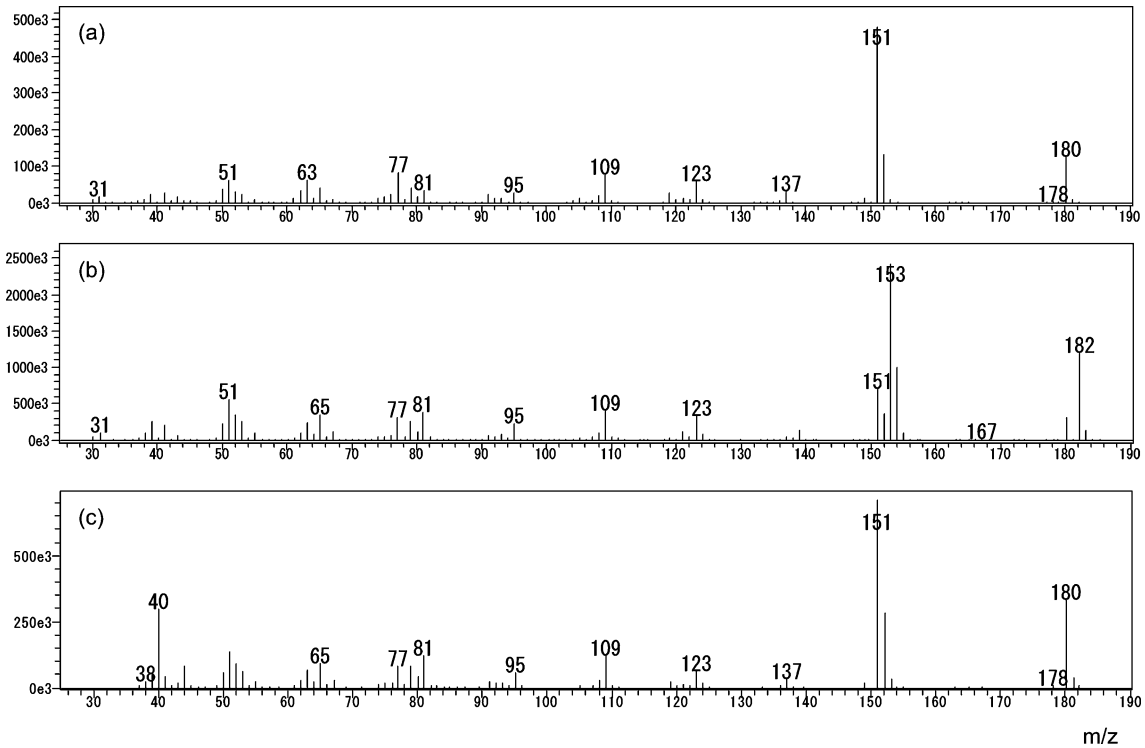

Fig. 3 Mass spectra of **9** produced from lignin model compound **4** by the AAPH-derived radical under various reaction conditions: (a) $H_2{}^{16}O$ solvent under N_2 atmosphere; (b) $H_2{}^{18}O$ solvent under N_2 atmosphere; (c) $H_2{}^{16}O$ solvent under $^{18}O_2$ atmosphere. Other reaction conditions were as listed in Table 1.

as for the complete reaction system with *tert*-BuOOH. A minor difference between the two reaction systems is that, without *tert*-BuOOH, monomeric dihydroxyl ketone **7** and benzaldehyde **9** are produced. Thus, although Ce^{4+} reacts directly with the lignin model compound **4**, it reacts more readily with *tert*-BuOOH, producing PRs, which are unreactive towards the lignin model compound **4**.

5 is thought to be produced by deacetylation of the acetyl derivative of benzyl ketone **3** at elevated temperature based on GC–MS analysis.[17] In the present study, **3** is produced in the reactions of AAPH under both O_2 and N_2 atmospheres, but **5** is produced only in the reaction under O_2 atmosphere (Table 1), suggesting that **5** is produced during radical reaction of the lignin model compound **4**.

Analysis of radicals derived from AAPH and peroxides

Fig. 4 shows the ESR spectra of DBNBS spin adducts produced by reaction with AAPH-derived radicals under N_2 and O_2 atmospheres. Under the N_2 atmosphere, only a triplet signal (1 : 1 : 1) is evident. However, under the O_2 atmosphere, both a triplet signal and a sextet signal (1 : 3 : 3 : 3 : 3 : 1) are evident, and the triplet signal is lower in intensity than that under N_2 atmosphere. Therefore, we conclude that the triplet signals with hfsc a(N) = 1.28 mT originate from the CR adduct.

Fig. 5 shows the ESR accumulation spectra of BNB spin adducts produced by the AAPH-derived radicals under N_2 atmosphere in 90% (v/v) of various solvents. Fig. 5a, for methanol solvent, shows triple quintet and quartet signals from 0.5 to 20.5 min. The quartet signals disappear from 21 to 41 min. For the triple quintet signals,

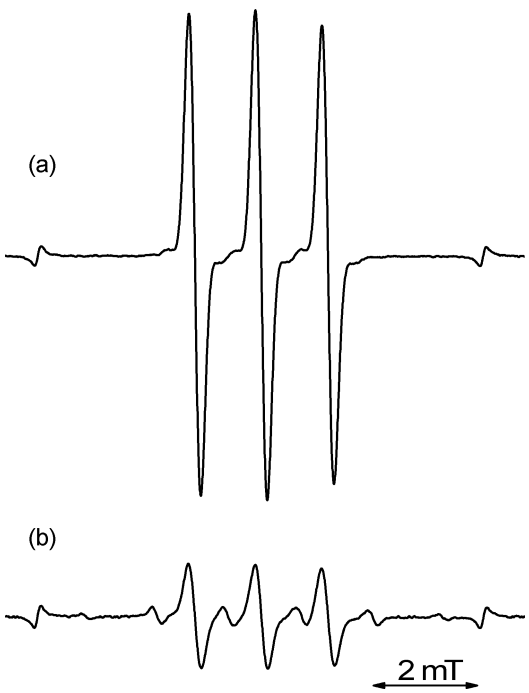

Fig. 4 ESR spectra of DBNBS spin adducts produced by reaction with AAPH-derived radicals in 18 mM sodium acetate buffer (pH 5) under various atmospheres: (a) N_2 atmosphere; (b) O_2 atmosphere. [AAPH] = [DBNBS] = 10 mM.

hfsc a(N) = a(H_β) = 1.38 mT; a(H_{meta}) = 0.08 mT originates from the hydroxymethyl radical adduct produced by hydrogen abstraction

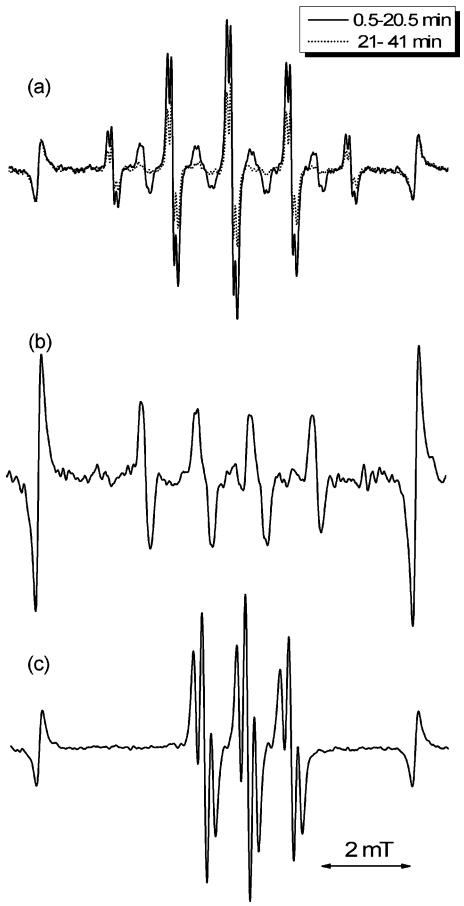

Fig. 5 ESR spectra of BNB spin adducts produced by reaction with AAPH-derived radicals under N_2 atmosphere in 90% (v/v) of various solvents: (a) methanol; (b) ethanol; (c) DMSO. [AAPH] = [BNB] = 10 mM.

from methanol by CR derived from AAPH. Fig. 5b, for ethanol solvent, shows the presence of a similar α-hydroxyethyl radical adduct, with hfsc a(N) = 1.41 mT and $a(H_\beta)$ = 1.34 mT. Fig. 5c, for DMSO solvent, shows triple triplet signals, with hfsc a(N) = 1.01 mT and $a(H_{meta})$ = 0.20 mT. This radical is assigned to a CR adduct due to the small a(N) value because BNB reportedly traps bulky radicals such as tertiary CR to give anilino (not nitroxide) spin adducts with small a(N) values.[25,26] Under O_2 atmosphere, no ESR signals are observed for all three solvents. BNB is known to be a strong spin-trapping agent for CR,[27] which explains why ESR spin adducts are detected under N_2 but not O_2 atmosphere.

Fig. 6 shows the ESR spectra of 4-POBN spin adducts produced by the reaction of *tert*-BuOOH with Ti^{3+} and Ce^{4+} in the presence of 4-POBN. Reaction of the Ti^{3+}/*tert*-BuOOH system gives an AR adduct (a(N) = 1.63, a(H) = 0.29); while the reaction of the Ce^{4+}/*tert*-BuOOH system gives a PR adduct (a(N) = 1.51 mT, a(H) = 0.23 mT). The hfsc values are identical to those previously reported.[28,29]

Radicals derived from veratryl alcohol

Radicals generated by the reaction of Ti^{3+} with H_2O_2 or *tert*-BuOOH in the presence and absence of veratryl alcohol **12** were analysed directly using rapid-flow ESR.

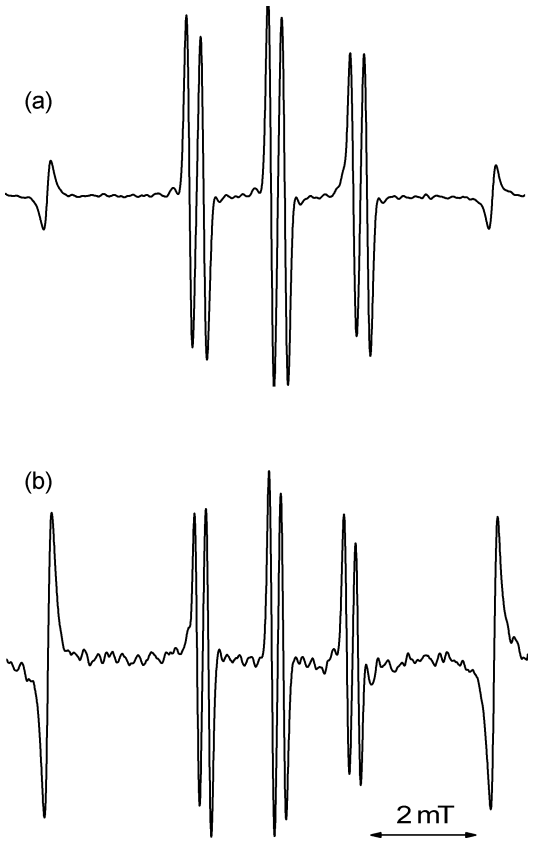

Fig. 6 ESR spectra of 4-POBN spin adducts produced in various *tert*-BuOOH systems: (a) Ti^{3+}/*tert*-BuOOH system; (b) Ce^{4+}/*tert*-BuOOH system. [*tert*-BuOOH] = 0.40 M; [4-POBN] = 10 mM; $[Ti^{3+}] = [Ce^{4+}]$ = 1 mM.

Reaction of Ti^{3+} with H_2O_2 gives hydroxyl radicals (HO•) according to eqn (5):[22]

$$Ti^{3+} + HO–OH \rightarrow Ti^{4+} + HO^{\bullet} + OH^{-} \quad (5)$$

Fig. 7 shows the ESR spectra of various Ti^{3+} reaction systems. In Fig. 7a, reaction with H_2O_2 causes signals from hydroxyl radicals (HO•) to appear. In Fig. 7b, reaction with H_2O_2 in the presence of veratryl alcohol **12** causes signals from HO• to disappear and a set of new complex signals to appear; the new signals are assigned to radicals derived from hydroxylated veratryl alcohol. The spectral pattern differs from those previously reported for the same reaction.[30] In Fig. 7c, reaction with *tert*-BuOOH causes signals from *tert*-BuO• and $H_3C^{\bullet}$ to appear. Reaction of Ti^{3+} with *tert*-BuOOH is known to give *tert*-BuO•, according to eqn (3). However, the alkoxyl radical readily decomposes by β-scission to give a ketone and a CR—that is, *tert*-BuO• decomposes by β-scission to give a methyl radical, according to eqn (6):[22]

$$tert\text{-}BuO^{\bullet} \rightarrow CH_3COCH_3 + H_3C^{\bullet} \quad (6)$$

In Fig. 7d, reaction with *tert*-BuOOH in the presence of veratryl alcohol **12** causes signals from *tert*-BuO• to disappear and signals from $H_3C^{\bullet}$ to grow slightly smaller, indicating that *tert*-BuO• preferentially reacts with veratryl alcohol **12**. Unlike for the Ti^{3+}/H_2O_2 system, radicals derived from the substrate are not directly detected.

Fig. 8 shows the ESR spectra of various Ce^{4+} reaction systems. In Fig. 8a, reaction with H_2O_2 gives a broad ESR signal assigned

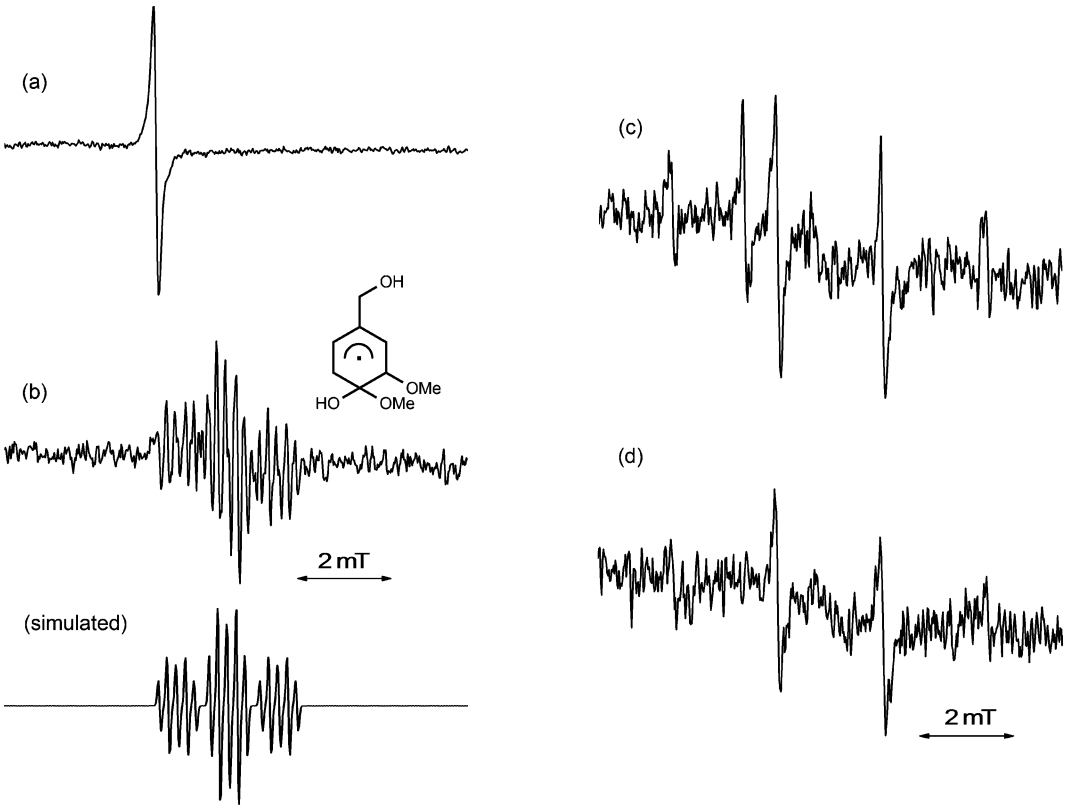

Fig. 7 ESR spectra of various Ti^{3+} reaction systems (VA = veratryl alcohol **12**): (a) Ti^{3+}/H_2O_2 system; (b) Ti^{3+}/H_2O_2/VA system; (c) Ti^{3+}/*tert*-BuOOH system; (d) Ti^{3+}/*tert*-BuOOH/VA system. The concentration of each reagent after mixing was as follows: [Ti^{3+}] = 5 mM; [H_2O_2] = 45 mM; [*tert*-BuOOH] = 40 mM; [VA] = 75 mM; [H_2SO_4] = 190 mM. The flow rates after mixing were as follows: (a) and (b), 20 mL min^{-1}; (c) and (d), 0.1 mL min^{-1}. The assigned radical structure is shown in (b).

to the hydroperoxyl radical (HOO•), which is produced according to eqn (7):[31]

$$Ce^{4+} + HOO–H \rightarrow Ce^{3+} + HOO^{\bullet} + H^{+} \quad (7)$$

In Fig. 8b, reaction with H_2O_2 in the presence of veratryl alcohol **12** causes no spectral change, indicating that the hydroperoxyl radical is unreactive towards veratryl alcohol **12**. However, when peroxides are omitted from the reaction system, Ce^{4+} reacts directly with veratryl alcohol **12** to give veratryl alcohol cation radical (eqn (8), where VA = veratryl alcohol **12**), whose ESR spectrum is identical to that previously reported for veratryl alcohol cation radical produced by the same reaction.[32]

$$Ce^{4+} + VA \rightarrow Ce^{3+} + VA^{+\bullet} \quad (8)$$

In Fig. 8c, reaction with *tert*-BuOOH gives a broad signal attributed to *tert*-BuOO•. When reactions are performed at different concentrations of *tert*-BuOOH and fixed concentrations of Ce^{4+} and veratryl alcohol **12**, the spectra change with the concentration of *tert*-BuOOH. Without *tert*-BuOOH, the spectra are identical to that for the veratryl cation radical. Signals from the veratryl cation radical decrease and new signals from *tert*-BuOO• increase with increasing concentration of *tert*-BuOOH. Thus, in the presence of *tert*-BuOOH and veratryl alcohol **12**, Ce^{4+} preferentially reacts with *tert*-BuOOH to give *tert*-BuOO• (eqn (4)), but signals from *tert*-BuOO• increase due to low reactivity towards veratryl alcohol **12**. This result is in accordance with degradation of lignin model compound **4** by the radical systems (Table 2).

Calculation of the bond dissociation energy

The BDEs of **4** were calculated by the MO method (Table 3). We first calculated the BDEs of the C–H bonds in linoleic acid to verify the accuracy of the simulation. Values for the bonds at the bis-allyl and allyl positions were calculated to be 76.1 and 87.5 kcal mol^{-1}, respectively, in good agreement with values previously reported.[33] We then simulated the BDEs of the C–H bonds in four stereoisomers of the lignin model compound **4**. For each isomer, the value for the bond at the benzyl position is the smallest. Values are smaller for the *erythro* isomer than for the *threo* isomer. Energy differences for the molecules before and after hydrogen abstraction at the benzyl position by the three radicals, AR, CR and PR, were calculated to be in the order AR > CR > PR (Table 4).

Discussion

In studies of white rot, oxidative enzymes capable of decomposing non-phenolic β-*O*-4 lignin model compounds are of major concern. Lignin peroxidases (LiPs) and versatile peroxidases (VPs) are target enzymes that decompose the recalcitrant structure. However, a majority of white-rot fungi do not secrete LiPs and VPs, and most laccases (Lccs) and manganese peroxidases (MnPs)

Table 3 Bond dissociation energies of the C–H bond in non-phenolic β-*O*-4 lignin model compound 4

	BDE/kcal mol^{-1}			
	erythro		*threo*	
Hydrogen type	α-*R*, β-*S*	α-*S*, β-*R*	α-*R*, β-*R*	α-*S*, β-*S*
α-*H*	85.1	85.3	92.3	92.5
β-*H*	105.5	105.5	106.5	106.6
γ-*H*	101.7	101.9	101.3	101.5
–OC*H*$_3$	103.5, 104.5	104.4, 105.0	103.5, 104.4	103.6, 104.5
–OC*H*$_2$CH$_3$	103.5	102.2	103.6	103.4
–OCH$_2$C*H*$_3$	108.8	108.0	108.7	108.8
ring-*H*	117.2–121.1	117.3–119.5	117.3–119.5	117.2–120.0

Table 4 Changes in potential of radicals after hydrogen abstraction

Radical	–BDE/kcal mol^{-1}
AAPH (-C$^{\bullet}$)	–93.8
AAPH (-COO$^{\bullet}$)	–87.5
tert-BuO$^{\bullet}$	–105.1
tert-BuOO$^{\bullet}$	–86.5

cannot directly oxidize the non-phenolic β-*O*-4 lignin model compounds. Rather, co-oxidation of mediators and lipids by these enzymes decomposes the lignin model compounds.

We previously demonstrated that radical reactions of copper complexes with organic hydroperoxides produce free radicals and depolymerize a synthetic non-phenolic lignin polymer at ambient temperature in aqueous media.[34] The radical reactions delignify both hardwood and softwood, resulting in fibre separation without apparent morphological changes in the cell wall thickness, as observed in selective white rot.[35] This suggests the potential role of free radicals as a strong lignin oxidizing agent in selective white rot, where lignin decomposes at sites far from enzymes. Lipid peroxidation by MnP also generates free radicals even at sites far from enzymes, if chelators for manganese ions are involved because chelated Mn^{3+} initiates radical chain reactions in both unsaturated fatty acids and lipid hydroperoxides.[7] A major concern in these radical reactions is what type of radical species initially oxidizes the recalcitrant structure of lignin. Kapich *et al.* analysed radicals by means of thermolysis of azo compounds and concluded that PR is responsible for the degradation of non-phenolic lignin.[8] However, CR involvement in the degradation cannot be excluded because the researchers carried out the thermolysis reactions only under aerobic atmosphere, where CR competitively reacts with molecular oxygen and the lignin model compounds.

To determine the relative reactivities of the radical species, we generated ARs, CRs and PRs and analysed the reactivity of each towards non-phenolic lignin model compounds by using ESR and GC–MS.

To determine the relative reactivity of CR, we derived CR from AAPH (by thermolysis under N_2 atmosphere). We found that the radical cleaves the α–β bond of the lignin model compound **4** to produce 3-ethoxy-4-methoxybenzaldehyde **9** (Table 1). Baiocco *et al.* reported that, in reactions of laccase with benzyl alcohol in the presence of mediator compounds, both the hydrogen abstraction at the benzyl position [radical hydrogen-atom-transfer (HAT) route] and electron abstraction from a benzene ring [electron-transfer (ET) route] occur, producing benzaldehyde.[36] Our ESR spin-trapping experiments show that CR derived from AAPH also reacts with methanol and ethanol to abstract a hydrogen atom from the α-carbon (Fig. 5). Because CR is known to serve as a hydrogen acceptor rather than an electron acceptor,[37–39] it is reasonable that the CR derived from AAPH abstracts a hydrogen atom from the benzyl position of the lignin model compound **4** to produce a benzyl radical. Experiments of the lignin model compound **4** degradation by thermolysis of AAPH under O_2 atmosphere give a fragmentation pattern that closely resembles that under anaerobic atmosphere, except for the generation of benzoic acid **10** under O_2 atmosphere, which can be ascribed to

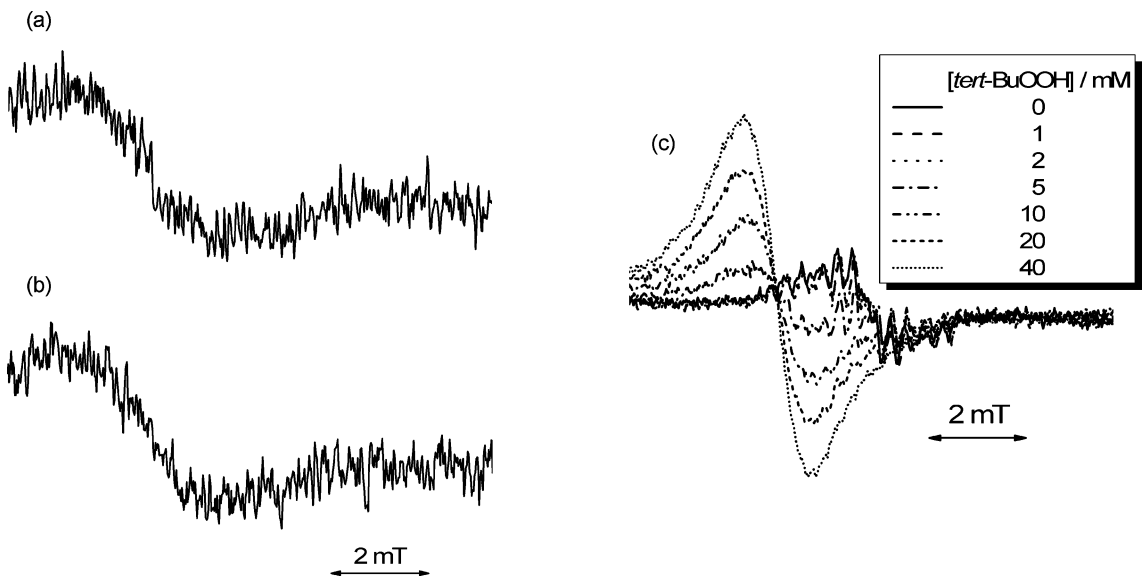

Fig. 8 ESR spectra of various Ce^{4+} reaction systems (VA = veratryl alcohol **12**): (a) Ce^{4+}/H_2O_2 system; (b) Ce^{4+}/H_2O_2/VA system; (c) Ce^{4+}/*tert*-BuOOH/VA system. The concentration of each reagent after mixing was as follows: [Ce^{4+}] = 3 mM; [H_2O_2] = 45 mM; [*tert*-BuOOH] = 40 mM; [VA] = 75 mM; [H_2SO_4] = 190 mM. The flow rates after mixing were 0.1 mL min^{-1}.

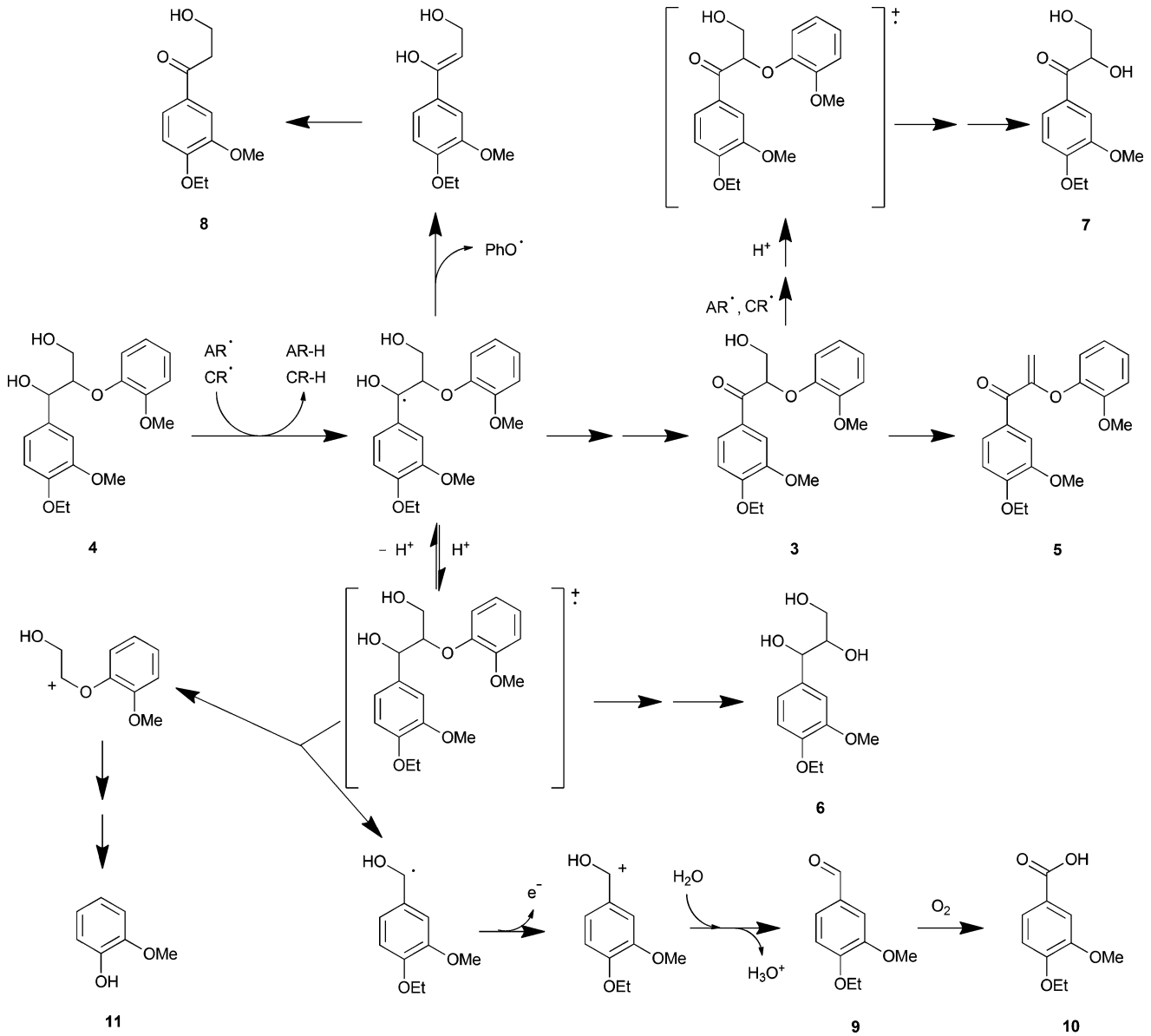

Fig. 9 Proposed pathway for degradation of lignin model compound **4** by free radicals.

autoxidation of benzaldehyde **9** (Table 1). Thus, CR derived from AAPH initially reacts with the lignin model compound **4**.

To determine the relative reactivity of PR, we derived PR from the Ce^{4+}/*tert*-BuOOH reaction system in the presence of the lignin model compound **4**. The lignin model compound **4** remains stable under this condition (Table 2). PR is known to generate di-*tert*-butyl peroxide and *tert*-BuO• or singlet oxygen, according to the following equations:[40]

$$2\ \textit{tert}\text{-BuOO}^{\bullet} \rightarrow \textit{tert}\text{-BuOO}(\textit{tert}\text{-Bu}) + O_2 \quad (9)$$

$$2\ \textit{tert}\text{-BuOO}^{\bullet} \rightarrow 2\ \textit{tert}\text{-BuO}^{\bullet} + O_2 \quad (10)$$

In addition, *tert*-BuO• is known to decay by β-scission into $H_3C^{\bullet}$ and acetone (eqn (6)) in accordance with the rate constant for β-scission in the solvent system used.[41] This means that three radicals—*tert*-BuOO•, *tert*-BuO• and $H_3C^{\bullet}$—can be produced if the chain reactions proceed. Fig. 8 shows, however, that the product of the Russel reaction, *tert*-BuO• (eqn (10)), and its β-scission product $H_3C^{\bullet}$, are not detected by ESR. Therefore, the contributions of secondary AR and methyl radical are negligible; the first reaction governs the overall radical species accessible to the lignin model compounds, and PR is unreactive towards non-phenolic lignin model compounds. Also, rapid-flow ESR experiments clearly demonstrate that *tert*-BuOO• and HOO• do not oxidize the non-phenolic lignin monomer, veratryl alcohol **12** (Fig. 8), therefore, PR derived from *tert*-BuOOH is unreactive towards non-phenolic lignin model compounds.

To determine the relative reactivity of AR, we derived AR from the Ti^{3+}/*tert*-BuOOH reaction system and found that it degrades non-phenolic lignin model compound **4** (Table 2). We also found that AR is consumed by reaction with veratryl alcohol **12** (Fig. 7). $H_3C^{\bullet}$ is also produced in this reaction, but the decrease in its signal intensity is small. Thus, AR derived from *tert*-BuOOH reacts with non-phenolic monomeric and dimeric lignin model compounds.

These results are in agreement with earlier findings that the oxidation reactivity of the three radicals is in the order AR > CR ≫ PR. For example, Koppenol reported that the values of reduction potential E^0 for RO$^{\bullet}$/ROH, ROO$^{\bullet}$/ROOH, C$^{\bullet}$H$_2$OH/CH$_3$OH and CH$_3$C$^{\bullet}$HOH/CH$_3$CH$_2$OH are +1.6, +1.0, +1.2 and +1.0 V, respectively.[42] Our experiments show that AR derived from *tert*-BuOOH and CR derived from AAPH react with the lignin model compound **4**, but PR derived from *tert*-BuOOH is unreactive towards the dimer and veratryl alcohol **12** (E^0 = 1.4 V).[43] The differential reactivity of radicals in abstracting hydrogen at the benzyl position of lignin model compound **4** is supported by BDE calculations (Table 4), which show that the oxidation reactivity is in the order AR > CR ≫ PR.

Finally, we propose a pathway for degradation of lignin model compound **4** by radicals initiated by hydrogen abstraction at the benzyl position (Fig. 9). Two reaction routes have been proposed for the initial reaction of the lignin model compound **4** with AR and CR: (1) electron abstraction from the aromatic ring and (2) hydrogen abstraction at the benzyl position.[8] The major reaction products are benzaldehyde **9** produced by C_α–C_β cleavage from the first route, and benzyl ketone **3** produced by reaction of the benzyl radical intermediate with molecular oxygen and subsequent release of hydroperoxy radical from the second route. The benzyl radical, an initial product of the lignin model compound **4** by hydrogen abstraction, can be transformed to the aryl cation radical intermediate upon elimination of a proton. This complicates our understanding of the initial reaction. However, because CR and AR react not as electron acceptors but as hydrogen acceptors,[37–39] it is plausible that the initial reaction starts with hydrogen abstraction.

By comparing $H_2{}^{16}O$ and $H_2{}^{18}O$ under N_2 and $^{18}O_2$ atmospheres (Fig. 3), it is clearly evident that ^{18}O in water is incorporated into a carbonyl group of benzaldehyde **9** even in the presence of dissolved oxygen. Fig. 9 shows the following possible pathway sequence that explains this finding: (1) formation of an aryl cation radical, (2) Cα–Cβ cleavage, (3) formation of a benzyl radical and cation intermediate and (4) nucleophilic attack of water. This sequence is consistent with reported reactions of the aryl cation radical.[44,45] Thus, we correctly propose that the major radical species responsible for the degradation of non-phenolic lignin model compound **4** in lipid peroxidation are AR and CR, both of which abstract hydrogen from the benzyl position. Although AR and CR are important for lignin degradation, we point out that PR and the aryl radical may also play important roles in propagating radical chain reactions in lipid peroxidation.

Conclusion

We studied lignin biodegradation using white-rot fungi by investigating the reactivities of free radicals towards non-phenolic lignin model compounds. Spin-trapping ESR and GC–MS analysis show that carbon-centred radicals (CRs) derived from 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) decompose the β-*O*-4 dimeric lignin model compound with accompanying α–β bond cleavage. Reactions producing alkoxyl radical (AR) from *tert*-BuOOH oxidize the α-position of the dimeric lignin model compound and cleave the β-*O*-4 bond, but reactivity is less than for reactions producing CR. The lower reactivity of the AR-producing system can be explained by β-scission of AR because AR exhibits the highest reactivity towards veratryl alcohol, as determined by rapid-flow ESR, and by the finding that energy differences between the lignin model compound before and after hydrogen abstraction at the benzyl position by AR, PR and CR are in the order AR > CR > PR. In addition, PR derived from *tert*-BuOOH is not reactive towards the lignin model compound, as expected from bond dissociation energy (BDE) simulation. Incorporation of the ^{18}O in $H_2{}^{18}O$ into the benzaldehyde derivative occurs even in the presence of dissolved oxygen. This result can be explained by the following pathway sequence: (1) formation of an aryl cation radical, (2) Cα–Cβ cleavage, (3) formation of a benzyl radical and cation intermediate and (4) nucleophilic attack of water. Thus, in free-radical-mediated lignin degradation, CR- and AR-producing systems are important for initiating hydrogen abstraction from lignin model compounds, although the role of PR as a chain-carrying radical should also be considered.

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