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Phenoxy radical detection using ^{31}P NMR spin trapping

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Recent work in our laboratory has allowed the development of quantitative ^{31}P NMR spin trapping techniques. These methods have been demonstrated to be effective tools for the detection and absolute quantification of many oxygen- and carbon-centered free radical species. Our methods rest on the fact that a free radical reacts with the nitroxide phosphorus compound, 5-diisopropoxy-phosphoryl-5-methyl-1-pyrroline-N-oxide (DIPPMPO), to form stable radical adducts, which are suitably detected and accurately quantified using ^{31}P NMR in the presence of phosphorus containing internal standards. This system was applied for the detection of phenoxy radicals, as an alternative to traditional EPR techniques. More specifically, the phenoxy radicals were produced via the oxidation of different phenols by $K_3Fe(CN)_6$. The ^{31}P NMR signals for the radical adducts of phenoxy radicals (PhO·) were assigned and found to be located at 25.2 ppm. Subsequently, this spin trapping system was applied to the oxidation of various phenols in the presence of peroxidases and 1-hydroxybenzotriazole (HBT) as a mediator: the 2,4,6-trichlorophenol and 2,4,6-tri-*tert*-butylphenol were oxidized and only phenoxy radical adducts were detected, whereas during the oxidation of 2,4-dimethylphenol and isoeugenol, other adducts were detected and related to radical delocalization. These preliminary efforts demonstrate the efficacy of our methodologies, so that a variety of radical species can now be readily detected and quantified using quantitative ^{31}P NMR spin trapping techniques. Copyright © 2009 John Wiley & Sons, Ltd.

Keywords: free radical; phenoxy radicals; phosphorous; ^{31}P NMR; spin trap

INTRODUCTION

For many years spin traps have been used to increase the stability of free radicals in order for them to be identified and detected by electron paramagnetic resonance (EPR) spectroscopy. Spin traps are highly reactive toward free radicals, thereby allowing the acquisition of abundant information on the production of such species in biological, biochemical, and chemical systems. Spin traps have been used extensively in the detection of oxygen- and carbon-centered radicals[1–4] in order to detect and quantify the involved radical species. Spin trapping entails the reaction of nitrones or nitroso spin traps (paramagnetic species) with unstable free radical systems, in order to form a more stable free radical (radical adduct), which can be detected by EPR spectroscopy or any other analytical method. Recent accounts[5] have demonstrated that phosphorus-containing spin traps give rise to radical adducts that have longer half-lives compared to other spin traps. This fact can be used to expand the capability of EPR spectroscopy. Anyway these radical adducts degrade with time, becoming diamagnetic and, therefore, EPR-undetectable. However, the presence of phosphorus within these systems allows for the use of ^{31}P nuclear magnetic resonance (NMR) spectroscopy to investigate the detailed chemistry of radical reactions in complex reaction systems. This technique was termed "NMR spin trapping" by Khramtsov *et al.*[5] The use of phosphorus-containing spin traps allows for the detection of diamagnetic products by ^{31}P NMR without the complexity of multiple signal overlap spectra usually encountered when common nuclei, such as proton or carbon, are examined. Overall, however, a possible drawback of this technique could be the reduced sensitivity of NMR compared to that of EPR. This is partly overcome by the acquisition of more NMR signals with time.[6]

Recently, a novel phosphorus compound containing nitroxide,[7] 5-diisopropoxy-phosphoryl-5-methyl-1-pyrroline-N-oxide (DIPPMPO), was investigated.[8] The new compound shows outstanding performance compared to 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) and 5-diethoxyphosphoryl-5-methyl-1-pyrroline-N-oxide (DEPMPO), toward radical analyses when used in conjugation with ^{31}P NMR. More specifically, DIPPMPO involves simple preparation and ^{31}P NMR signals for DIPPMPO/·OOH, and DIPPMPO/·OH are readily distinguishable. Moreover, DIPPMPO has a higher partition coefficient in the octanol-water system ($K_p = 2.1$) compared to DMPO (0.1) or DEPMPO (0.06), enabling the trapping experiments to be conducted in cellular or lipid-rich environments. Recent work in our laboratory has allowed the development of ^{31}P NMR spin trapping techniques for the detection and absolute quantification of many oxygen- and carbon-centered free radical species.[8]

In this study, we have applied the described spin trapping system to understand radical mechanisms that are involved in lignin chemistry. Lignin is a complex natural polymer resulting from the oxidative coupling primarily of (4-hydroxyphenyl)propanoids. The

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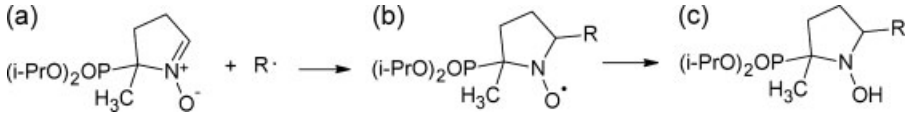


Scheme 1. The spin trap system with DIPPMPO

current theory is that the lignin polymer is formed by combinatorial like phenolic coupling reactions, via phenoxy radicals.[9] Phenolic systems are the typical substrates for laccase and peroxidase, due to their matched redox potentials.[10–12] Their oxidation proceeds through an outer-sphere electron transfer process that generates a radical cation, which after fast proton abstraction generates a phenoxy radical.[13–16]

Phenoxy radicals could be detected by EPR directly. EPR spectra of phenoxy radicals generated from lignin model compounds, have previously been reported, after oxidation of the parent compound with horseradish peroxidase (HRP) and H_2O_2.[17] Moreover, phenoxy radicals have been detected and quantified via EPR directly onto lignocellulosic materials (milled wood lignin and thermomechanical pulp) after treatment with laccase and peroxidase.[18,19] There are several examples in the literature regarding the use of spin trapping and EPR for the detection of phenoxy radicals.[20,21] For example, the oxidation of tyrosine with HRP-H_2O_2 system to form tyrosyl radicals trapped with DMPO for biomedical purposes.

The objective of this paper is focused at developing a spin trapping ^{31}P NMR based technique that uses DIPPMPO for the detection of phenoxy oxygen- and carbon-centered radicals involved in the oxidation of complex phenolic mixtures.

RESULTS AND DISCUSSION

Scheme 1 shows the DIPPMPO spin trap system. The radical reacts with the spin trap DIPPMPO (a) to form the adduct (b), which is EPR detectable. The diamagnetic species (b) decays to the paramagnetic species (c). The adduct (c) is ^{31}P NMR detectable and the chemical shift of the phosphorus atom could be related to the nature of the radical being trapped R·.

Trapping oxygen-centered radicals

·OH and HO_2· represent the most important oxygen-centered radical species involved in biological processes. In this paper, oxygen-centered radicals (·OH and HO_2·) have been generated and trapped with DIPPMPO. The radicals have been detected by ^{31}P NMR and characterized with gas chromatography-mass spectroscopy (GC-MS). The use of MS allowed for an understanding of the detailed radical adduct structure.

Table 1 shows the ^{31}P NMR chemical shifts for the adducts of the oxygen-centered radicals under examination. The ·OH adducts showed a peak at 25.2 ppm, while the HO_2· adduct showed signals at 16.9 and 17.1 ppm. It is likely that these signals correspond to the two diastereomeric forms of the reaction product with DIPPMPO.

Table 1. ^{31}P NMR signals for DIPPMPO reaction adducts of oxygen-centered radicals

Species	Generating system	Chemical shift (ppm)
DIPPMPO	—	22.2
DIPPMPO/·OH	$Fe^{2+} + H_2O_2 +$ DTPA	25.3
DIPPMPO/·OOH	$H_2O_2 + O_2 + OH^-$	16.9/17.1

The structures of the hydroxyl and hydroperoxy radical adducts were elucidated by carrying out GC-MS analyses of the freeze-dried samples. Table 2 shows the mass spectra of the DIPPMPO and the hydroxy and hydroperoxy radical adducts of DIPPMPO.

The mass spectrum of DIPPMPO shows a peak at *m/z* 263 that could be attributed to the molecule [M^+], and other major fragments at *m/z* 221 and 179 that relate to the loss of isopropyl group (42 u). The main peak at *m/z* 98 has been related to the loss of the diisopropyl(oxido)phosphoranyl radical ·P(O)(O-C_3H_7)$_2$ of 165 u. The mass spectrum of ·OH adducts (nitrone) exhibits a similar fragmentation pathway with the peak at *m/z* 279 related to the molecule [M^+], and the peak at *m/z* 237 and 195 related to the losses of the isopropyl groups (42 u). Similarly, the main peak at *m/z* 114 has been related to the loss of the radical ·P(O)(O-C_3H_7)$_2$. For the ·OOH adducts the major peak at 254 *m/z* corresponds to the loss of one isopropyl group. The molecular peak [M^+] at *m/z* 296 and the peak related to the loss of the second isopropyl group (*m/z* 212) are also present.

Trapping of carbon-centered radicals

As previously reported,[8] carbon-centered radicals (methyl ·CH_3, hydroxymethyl ·CH_2OH, hydroxyethyl ·CH(OH)CH_3, acyl ·C(O)CH_3) were generated and trapped with DIPPMPO. The radicals were detected by ^{31}P NMR. Table 3 shows the chemical shifts of the different carbon-centered adducts.

Trapping phenoxy radicals

Phenols are the typical substrates for laccase and peroxidase enzymes, due to their matched redox potentials.[10–12] Electron

Table 2. Fragment ions for DIPPMPO reaction adducts of oxygen-centered radicals

Species	Mass spectrum (*m/z*)
DIPPMPO	263 (5), 221 (23), 179 (25), 162 (13), 144 (10), 98 (100), 82 (20), 80 (21).
DIPPMPO/·OH	279 (5), 237 (7), 195 (16), 156 (14), 123 (10), 114 (100), 98 (8), 86 (13).
DIPPMPO/·OOH	296 (10), 254 (100), 212 (11), 143 (3), 112 (2).

Table 3. ^{31}P NMR signals for DIPPMPO reaction adducts of carbon-centered radicals

Species	Generating system	Chemical shift (ppm)
DIPPMPO	—	22.2
DIPPMPO/·CH_3	DMSO + 3% H_2O_2 + UV light	23.1
DIPPMPO/·CH_2OH	Methanol + 3% H_2O_2 + UV light	22.6
DIPPMPO/·$CH(OH)CH_3$	Ethanol + 3% H_2O_2 + UV light	27.3
DIPPMPO/·$C(O)CH_3$	Acetone + UV light	30.2

Table 4. ^{31}P NMR signals for DIPPMPO reaction adducts of different phenols

Substrates	Generating system	Chemical shift (ppm)
—	—	22.2
—	$K_3Fe(CN)_6$	22.2
—	HRP-H_2O_2	22.2
—	HRP-HBT-H_2O_2	17.9/23.5
2,4,6-trichlorophenol	$K_3Fe(CN)_6$	25.1
2,4,6-trichlorophenol	HRP-H_2O_2	25.1
2,4,6-tributylphenol	$K_3Fe(CN)_6$	25.2
2,4,6-tributylphenol	HRP-H_2O_2	22.2
2,4,6-tributylphenol	HRP-HBT-H_2O_2	25.2
2,4-dimethylphenol	HRP-H_2O_2	25.2/27.0
Isoeugenol	HRP-H_2O_2	17.5/25.2/27.0

abstraction and subsequent deprotonation gives rise to phenoxy radicals, which play a pivotal role in lignin chemistry.[12–16] Overall spin trapping systems could be powerful tools to understand the radical intermediates involved in lignin chemistry.

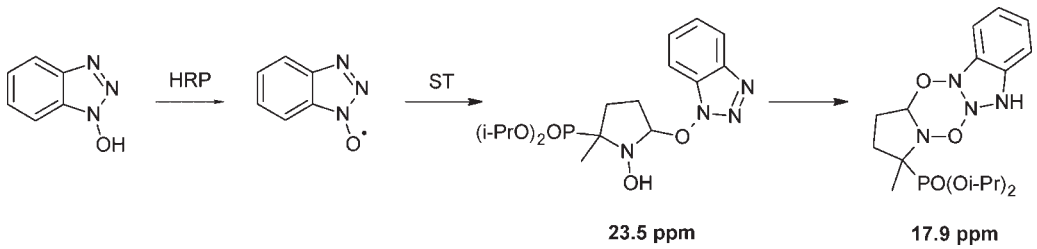
In Table 4 our data on spin trap experiments with DIPPMPO and different phenols are shown.

There are different oxidation systems for the generation of phenoxy radicals. Initially, the phenoxy radicals were produced by hexacyanoferrate(III) in a biphasic system of benzene–water. This oxidation system is simple and well known: during this reaction the O—H bond is homolytically cleaved by the donation of an electron to a powerful one-electron acceptor as a transition-metal ion in a high-valence state, to generate the phenoxy radicals. This system was applied to the oxidation of 2,4,6-trichlorophenol and 2,4,6-tri-*tert*-butylphenol in the presence of DIPPMPO. At the onset of our investigation, we used such phenols because the positions of the radical delocalization were blocked by the groups in the 2, 4, and 6 positions and the formation of oligomeric products, formed via coupling of oxygen- to carbon-centered radicals, was a minor reaction. In the absence of substrate, no reaction occurred between the spin trap DIPPMPO and $K_3Fe(CN)_6$. As such the ^{31}P NMR showed only a peak at 22.2 ppm related to the native spin trap. However, in the presence of substrates, the ^{31}P NMR showed the formation of one radical adduct with a chemical shift at 25.1 ppm for the 2,4,6-trichlorophenol and 25.2 ppm for the 2,4,6-tri-*tert*-butylphenol. This signal was thus assigned to the adducts of the phenoxy radical with DIPPMPO.

Similar results were obtained when we used HRP with H_2O_2 as oxidation system in a buffered solution (pH 4.5) with the addition of a small amount of dimethylformamide (DMF) to overcome the problem of substrate insolubility. In the absence of substrate, no reaction occurred between the spin trap DIPPMPO and HRP, and the ^{31}P NMR showed only a peak at 22.2 ppm related to the native spin trap. The ^{31}P NMR spectra showed a single peak at 25.1 ppm for the 2,4,6-trichlorophenol and 25.2 ppm for the 2,4,6-tri-*tert*-butylphenol, as previously obtained in the presence of $K_3Fe(CN)_6$.

In the case of 2,4,6-tri-*tert*-butylphenol oxidation and in the presence of only HRP and H_2O_2, the substrate was recovered unchanged (99%) and no signal in the ^{31}P NMR spectrum was observed. The bulky butyl groups in positions 2 and 6 hindered the approach of the enzyme to the phenolic group. However, in the presence of an oxidation mediator of small size, such as 1-hydroxybenzotriazole (HBT), finally the signal of the phenoxy radical adduct was observed. The >N—O· species generated from the interaction of HBT by HRP, and in view of its small size and matching value of bond dissociation energy (BDE) (85 kcal/mol for HBT *vs.* 84–87 kcal/mol for phenols),[22] allowed the abstraction of a H-atom from the O—H bond of 2,4,6-tri-*tert*-butylphenol and afforded the corresponding phenoxy radical. It is important to note that during the oxidation of HBT with HRP and H_2O_2 in the absence of the substrate, we were able to detect two adducts at 23.5 (doublet) and 17.9 ppm and traces of OH· and HOO· adducts at 25.3 and 16.9–17.0 ppm. We interpreted these results as a consequence of adduct formation of the HBT radical with DIPPMO, and the structure of these radical adducts is actually under investigation (Scheme 2).

Alternatively, in the presence of 2,4,6-tri-*tert*-butylphenol, we were able to detect only a signal at 25.2 ppm related to the phenoxy radical, with traces of residual HOO· adducts. It is known



Scheme 2. Mechanism of trapping HBT radical generated by HRP/H_2O_2 with DIPPMPO

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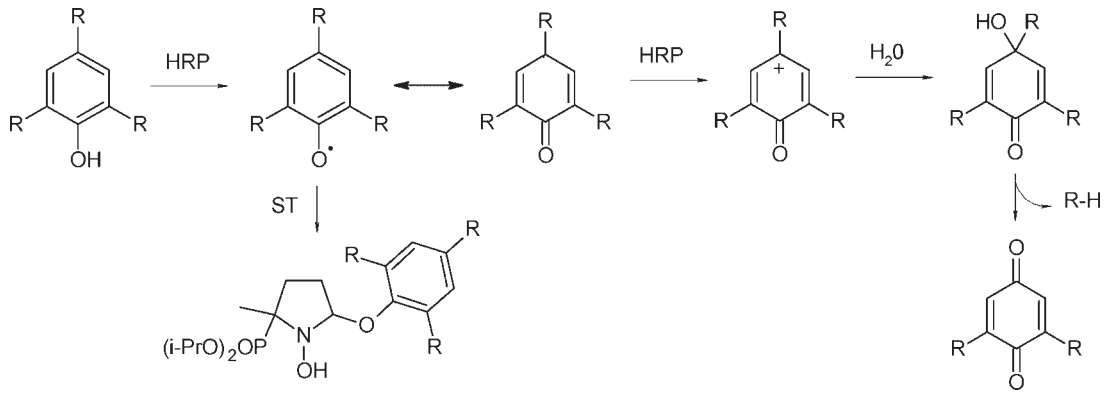


Scheme 3. Phenoxy radical adduct formation in presence of DIPPMPO

that the rate of hydrogen abstraction by the HBT radical is a fast reaction ($k_H = 66\,M^{-1}s^{-1}$ at 25 °C in acetonitrile for phenol).[23] The reaction of adduct formation of the HBT radical with DIMMPO should be slower than the H-abstraction reaction from 2,4,6-tri-*tert*-butylphenol.

These data are in good agreement with earlier literature studies related to the oxidation of phenolic compounds by the laccase-mediator system.[22] In Scheme 3 the adduct formation reaction is shown. In our work, it became possible using GC-MS to detect the quinone (the main product of the oxidation) in accordance with the report of Ferrari.[24]

In Fig. 1 the ^{31}P NMR spectra of the oxidation of 2,4,6-trichlorophenol and 2,4,6-tri-*tert*-butylphenol in the presence of DIPPMPO is shown.

Due to the proximity of the chemical shifts of the HO· and the phenoxy radical adducts with DIPPMPO (25.3 and 25.2 ppm

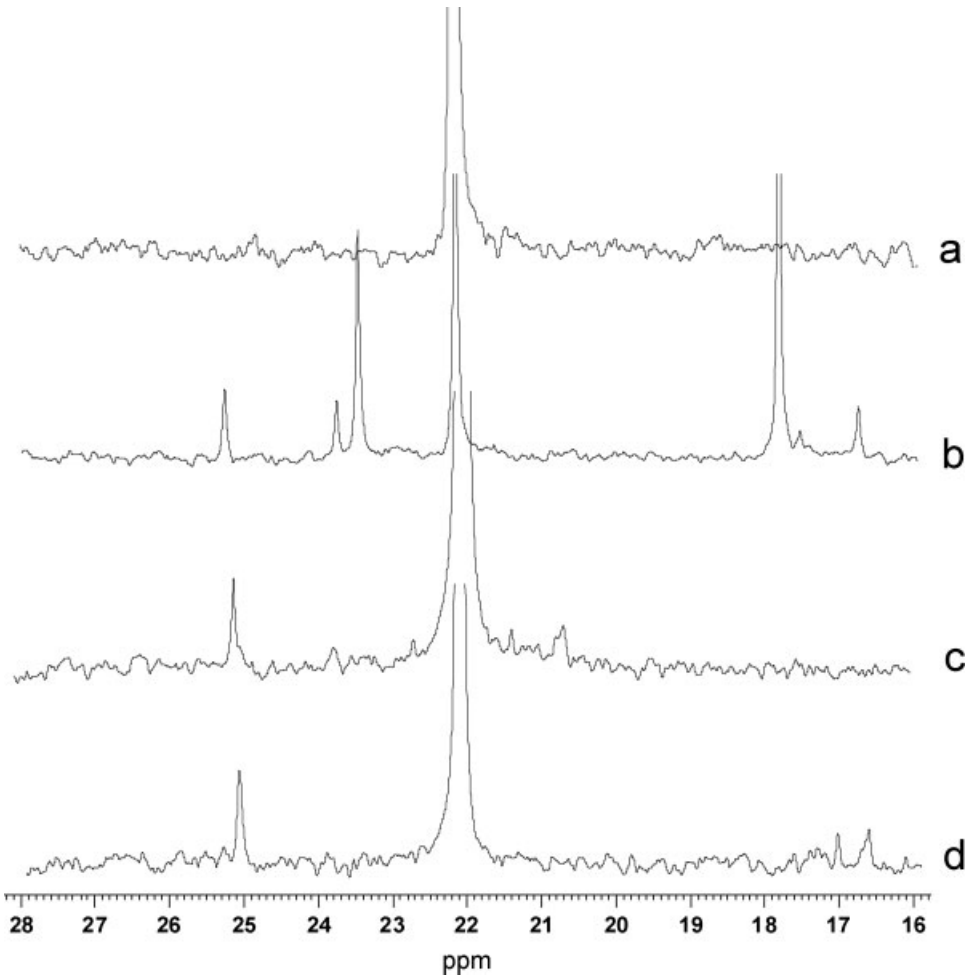


Figure 1. ^{31}P NMR spectra of: (a) DIPPMPO; (b) DIPPMPO and HBT in presence of HRP/H_2O_2; (c) DIPPMPO and 2,4,6-trichlorophenol in presence of HRP/H_2O_2; (d) DIPPMPO and 2,4,6-tri-*tert*-buthylphenol in presence of HBT and HRP/H_2O_2. The spectra of phenoxy radical adducts generated by $K_3Fe(CN)_6$ were not reported but are similar to spectrum c and d

respectively), mass spectroscopy was used to conclusively determine that the signal observed at 25.2 ppm corresponds to the structure shown in Scheme 3. Unfortunately, GC-MS did not reveal any species that possessed the correspondent molecular weight of the phenoxy or the HO· radical adduct. It is likely that during the MS analyses, the structure readily fragmented. However, the data seem to confirm the nature of the adduct as a phenoxy radical trapped with DIPPMPO. Similar efforts confirming our work are apparent in the literature.[20,21,25] Gunther and coworker[25] trapped the phenoxy radical of tyrosine with the HRP system. More specifically, an experiment with tyrosine ^{13}C and ^{17}O labeled, showed that the tyrosine radical adduct DMPO/·O-Tyr was formed by trapping phenolic oxygen, demonstrating that the tyrosine-derived radical is a phenoxyl radical.

With the present spin trap system we were able to detect the phenoxy radical adduct using ^{31}P NMR. Analogous EPR techniques have shown that for example, in the case of 2,4,6-trichlorophenol, EPR permits the detection of the phenoxy radical at low concentrations in the absence of spin trap.[26] Such EPR spectra were characterized by a 1:2:1 triplet arising by coupling of the unpaired electron density with the *meta* protons ($a^{H3} = a^{H5} = 2.35$ G). These data indicate that HRP catalyzes the hydroperoxide-dependent oxidation of the trichlorophenol to the corresponding trichlorophenoxyl radical.

However, in the case of different phenols, the DIPPMPO spin trap system shows interesting properties when compared and contrasted to the EPR method described above. For example, our work using the DIPPMPO spin trap in the reaction of phenols, such as 2,4-dimethyl phenol and isoeugenol (2-methoxy-4-(1-propenyl)phenol), the obtained data demonstrated significant advantage of this technique with respect to EPR. More specifically, EPR possesses limitations such as fast signal decay and spectral complexity. The issue of signal stability could be overcome by the use of spin trap, however, the spectra still show extreme complexity.

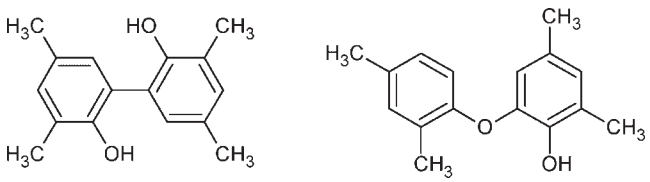
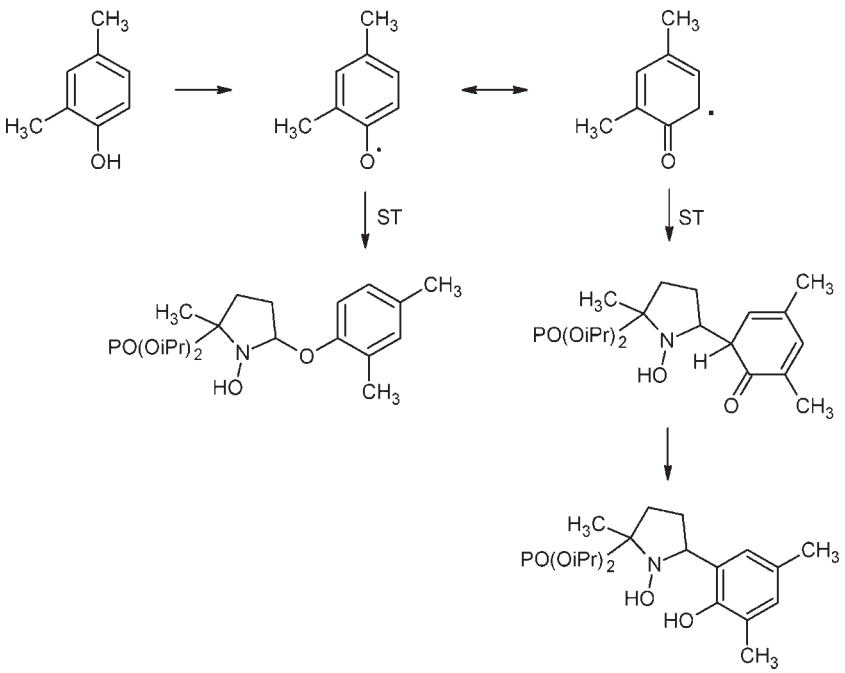


Figure 2. Products of radical coupling of 2,4-dimethylphenol oxidation with HRP

In our work, when we examined the oxidation of the enumerated phenols with HRP/H_2O_2, the DIPPMPO spin trapping system was silent since no radical adducts were detected. However, GC-MS analyses of the reaction medium showed the presence of dimers related to radical coupling. Apparently, the phenoxy radicals and the relative delocalized structures reacted faster through radical coupling to form dimers than with DIPPMPO to form adducts. To overcome this problem we modified the mode and rate of addition of the reactant since earlier studies in lignin chemistry have shown that the rate of addition of the monomeric precursors is a crucial factor in determining whether there is radical coupling or not. If the reactants are added in a batch mode, the method of addition is termed Zulauf (ZL), while if the reactants are added in a slow and continuous way, the method is termed Zutropf (ZT). The differences between the ZT and ZL methods are the different concentrations of the monomeric radicals at the beginning of the polymerization reaction. In the case of the ZL method, the concentration of monomeric radicals is high, because of the fast addition. On the contrary during the ZT method, the radical concentration is low, because of the slow addition and coupling between two monomers is less frequent.

As mentioned above, during the oxidation of 2,4-dimethylphenol and isoeugenol under ZL conditions, no adducts were detected by ^{31}P NMR. However, during the oxidation of these phenols under ZT conditions the adducts were readily detected via ^{31}P NMR.



Scheme 4. Adduct formation from the oxidation of 2,4-dimethylphenol in the presence of DIPPMPO

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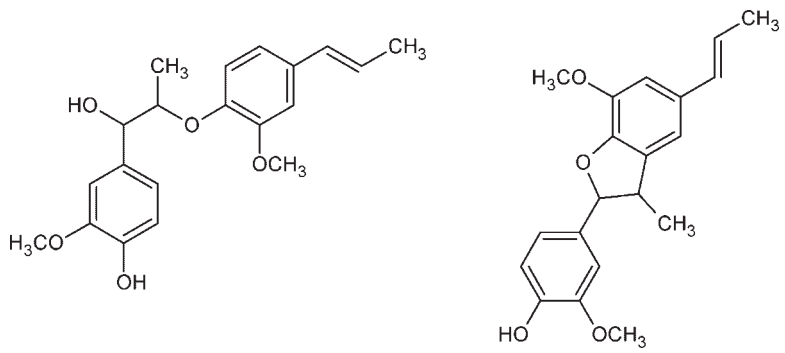


Figure 3. Products of radical coupling of isoeugenol oxidation with HRP

When 2,4-dimethylphenol was used, the dimers due to biphenyl 5–5′ and ether bond 4-O-5 coupling structures were detected *vis-a-vis* GC-MS as shown in Fig. 2, while the ^{31}P NMR spectrum showed two different signals at 25.2 and 27.0 ppm. The signal at 25.2 ppm was assigned to the phenoxy radical adduct, on the basis of the previous measurements while the signal at 27.0 ppm was assigned to the ortho adducts in the fifth position (Scheme 4).

During the oxidation of isoeugenol with HRP and H_2O_2 the reaction products were the dimers generated by radical coupling reactions. These dimers were identified as the dilignols emerging from the β-5 and β-O-4 structures (Fig. 3).

Using ^{31}P NMR in the presence of DIPPMPO we observed the formation of three different adducts at 27.0, 25.2, and 17.5 ppm, respectively. The signal at 27.0 ppm was assigned to the *ortho* adduct and the signal at 25.2 ppm to the phenoxy radical adduct, on the basis of our previous measurements. The signal at 17.5 ppm was assigned to the radical delocalized in the β position. Figure 4 shows the ^{31}P NMR spectrum obtained from the oxidation of enumerated phenols with HRP in the presence of DIPPMPO and the signals are all in accordance with the previous discussion.

However, more information is needed to further understand and substantiate the nature of some of these adducts and their

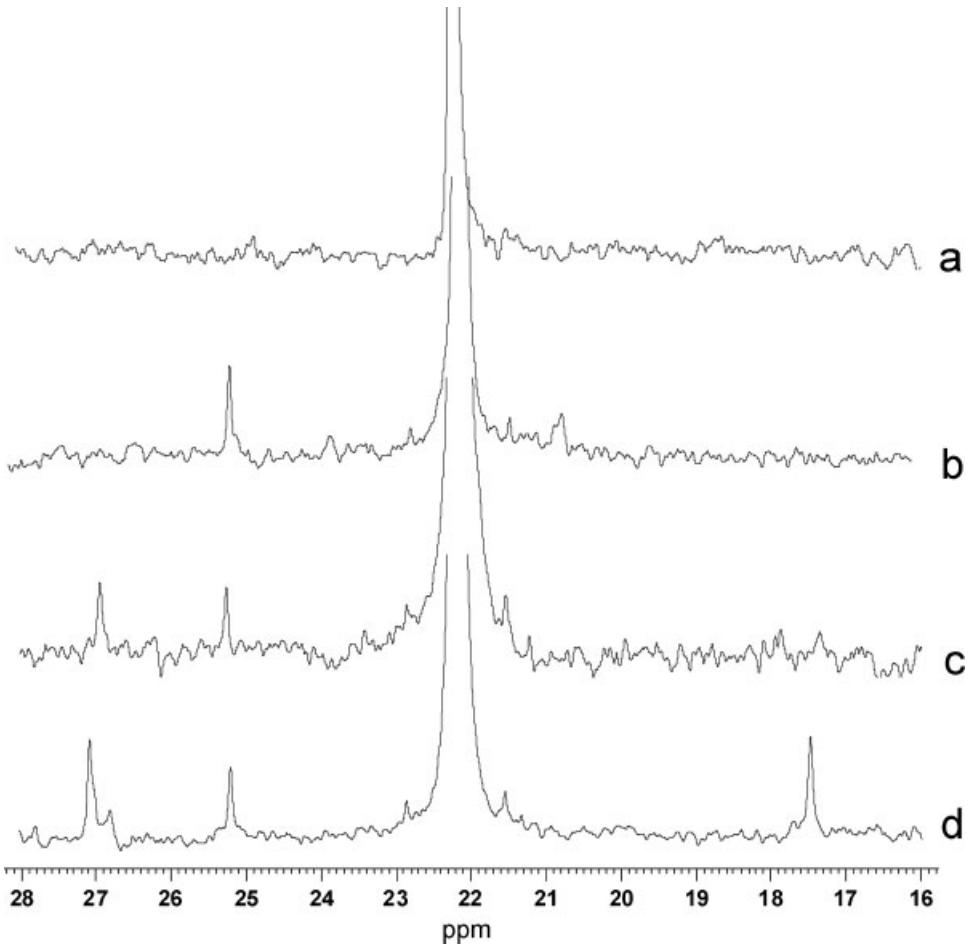
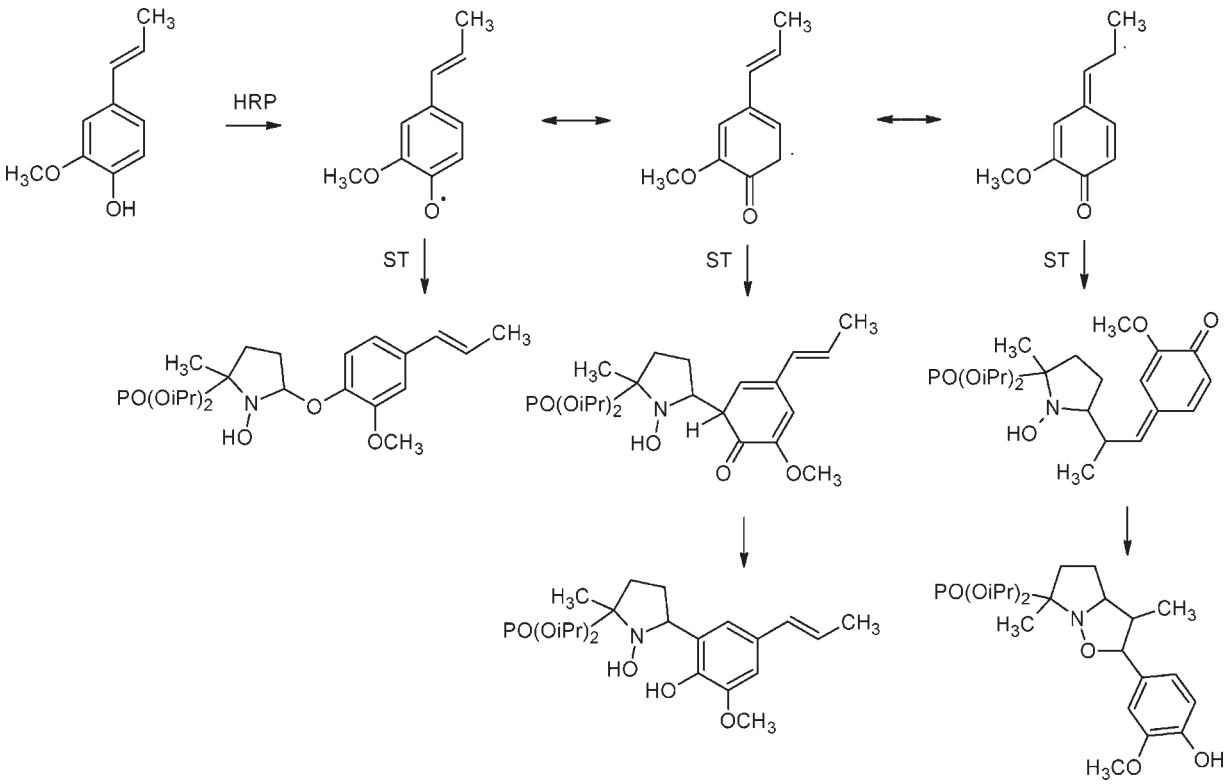


Figure 4. ^{31}P NMR spectra of: (a) DIPPMPO; (b) DIPPMPO and 2,4,6-trichlorophenol; (c) DIPPMPO and 2,4-dimethyl phenol; (d) DIPPMPO and isoeugenol. The oxidation system was HRP/H_2O_2



Scheme 5. Adduct formation from oxidation of isoeugenol in presence of DIPPMPO

structures. For example, the proposed formation of the cyclic compound, (Scheme 5) similar to the benzofurans encountered during lignin biosynthesis, is currently under investigation in our laboratory.

CONCLUSIONS

These experiments have shown the power of the proposed DIPPMPO spin trapping system to understand the chemistry of different phenols under oxidative conditions. This study demonstrated the ability of the DIPPMPO spin trapping system to readily visualize and study the various delocalized forms of phenoxy radicals. Using a nitroso spin trap such as MNP (2-methyl-2-nitrosopropane) and 2,3,5,6-tetramethyl-1-nitrosobenzene, the radical adducts during phenolic oxidations were mainly in the *ortho* and *para* positions.[25,27,28] Furthermore, using a nitroxide spin trap such as DMPO, leads to the formation of a C—O bond between the spin trap and the phenol.[20,25] In our work no such limitations were observed since we were able to detect the different adducts related to the different canonical forms of the radicals. Overall, these preliminary data formed the foundation for a targeted understanding of the nature, identity, and mechanisms of radical activity in a variety of biomolecular processes.

EXPERIMENTAL

Synthesis of DIPPMPO

DIPPMPO was synthesized according to a modified two-step procedure,[7] in which a catalytic amount of the Lewis acid, boron trifluoride diethyl etherate, was added to shorten the reaction time of formation of diisopropyl-(2-methyl-1-pyrrolidin-2-yl) phosphonate from 12 to 3 days in high yield (96%). It was then oxidized with H_2O_2 using catalytic amounts of Na_2WO_4. The ^{31}P NMR spectra showed a single resonance at 22.2 ppm, in agreement with the literature. The chemical shifts as well as the multiplicities for the proton resonances were: δ_H (400.13 MHz; $CDCl_3$; Me_4Si) 6.822 (1H, q, $J_{H,P}$ 2.8, $J_{H,H}$ 2.8, HC = N), 4.81 (1H, d sept., $J_{H,P}$ 0.6, $J_{H,H}$ 6.3, $OCHMe_2$), 4.72 (1H, d sept., $J_{H,P}$ 1.1, $J_{H,H}$ 6.3, $OCHMe_2$), 2.65–2.81 (2H, m, CH_2), 2.44–2.55 (1H, m, CH_2), 1.92–2.08 (1H, m, CH_2), 1.60 (3H, d, $J_{H,H}$ 14.79, CH_3), 1.306 (3H, dd, $J_{H,P}$ 0.24, $J_{H,H}$ 6.3, CH_3), 1.286 (3H, d, $J_{H,H}$ 6.3, CH_3), 1.282 (3H, d, $J_{H,H}$ 6.3, CH_3), 1.277 (3H, d, $J_{H,H}$ 6.3, CH_3). EI Mass Spectrum (*m/z*): 263 (5), 221 (23), 179 (25), 162 (13), 144 (10), 98 (100), 82 (20), 80 (21). The spin trap was stored under argon at −78 °C.

Oxidation of phenol by $K_3Fe(CN)_6$

A 5 mM solution of 2,4,6-trichlorophenol or 2,4,6-tri-*tert*-butyl phenol in the presence of 1 mM of DIPPMPO in 100 mL of benzene was stirred with 100 mL water solution of 7.5 mM of $K_3Fe(CN)_6$ at RT for 4 h under argon. The organic solution (750 μL) was diluted with 250 μL $CDCl_3$ in the presence of $Cr(acac)_3$ as relaxation agent and trimethylphosphate as internal standard, for ^{31}P NMR analyses.

Oxidation of phenol by HRP

A solution of 10 mM of different phenols in 3 mL of dimethylformamide and buffer solution, pH 4.5 (1:1) with 10 mM of H_2O_2 was added over a period of 4 h by a microsyringe pump in 3 mL solution of HRP (200 U/g), HBT 0.5 mM (where necessary), and DIPPMPO 2 mM in buffer solution pH 4.5. For ^{31}P NMR, 750 μL samples were added to 250 μL of D_2O with chromium chloride as relaxation agent and trimethylphosphate as internal standard.

^{31}P NMR spectra

^{31}P NMR spectra were acquired on a Bruker-300 spectrometer (operating at 121.49 MHz). The chemical shifts reported are relative to external orthophosphoric acid (85%). All spectra were acquired with proton decoupling. The total number of scans for all experiments was 256–1024 with an acquisition time of 1.60 s. Trimethylphosphate was used as the internal standard for quantification and was added to the sample prior to measurement. The relaxation time (T_1) of the internal standard was measured and was determined to be approximately 13.5 s. In order to decrease the relaxation time, a relaxation agent (chromium chloride or chromium acetylacetonate) was added to the mixture. With the addition of relaxation agent (30–35 mmol/L) to the samples prior to NMR measurement, the relaxation time of the phosphorus nuclei was decreased to 200 ms. $5T_1$ was used for the pulse delay.

Gas chromatography-mass spectrometry (GC-MS)

Freeze-dried samples were solubilized in about 10 mL of hexane. Structural analyses were performed by injecting 2 μL of the extracted sample in a Hewlett Packard 5972 mass spectrometer (EI 70 eV) interfaced to a Hewlett Packard 5890-A gas chromatograph. Chromatographic separation was performed using a DB-5 30 m·0.25 mm fused silica capillary column (J. and W. Scientific Agilent Technologies). Chromatographic conditions: initial temperature 60 °C, 2 min isothermal, 10 °C/min up to 200 °C, 6 °C/min up to 280 °C, 20 min isothermal. Carrier gas: He (purity 99.995%), constant flow 1.0 mL/min.

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