An EPR investigation into the reactions of alkaline hydrogen peroxide with cyanamide

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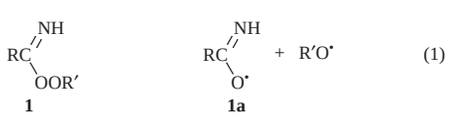
Free-radical involvement in an alkaline hydrogen peroxide/cyanamide system has been demonstrated using electron paramagnetic resonance (EPR) spectroscopy. A stable free radical is formed which shows coupling to two pairs of equivalent ^{14}N nuclei ($a_{^{14}N1}$ = 7.30, $a_{^{14}N2}$ = 2.13 G). Both hydroxyl and carbon-centered radicals have been trapped with 5,5-dimethyl-4,5-dihydro-3*H*-pyrrole *N*-oxide (DMPO) (DMPO–OH: $a_H = a_N$ = 14.9 G, DMPO–C: a_H = 24.0, a_N = 16.4 G). The presence of HOO$^\bullet$ has been inferred based on the absence of reactivity in the presence of superoxide dismutase. Involvement of superoxide and cyanamide radicals has been demonstrated by the formation of ring-opened and cyanamide coupled products obtained from reactions of alkaline hydrogen peroxide–cyanamide with substituted aromatic compounds.

Introduction

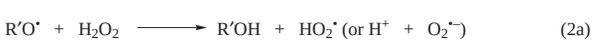
The kinetic activation of dioxygen- and hydrogen peroxide-based redox reactions is an area of intense research due to the biological and industrial importance of these reagents.[1] In biology, dioxygen and hydrogen peroxide are activated predominantly by metalloenzymes such as the cytochromes P-450, oxidases, dioxygenases and peroxidases.[2] Industrially and synthetically, reactions of dioxygen and hydrogen peroxide are often catalyzed by transition metal ions or *via* formation of peroxyacids.[1,3]

Cyanamide, H_2NCN, is a simple molecule that plays important roles in biology and industry. It has been shown to be present in interstellar gases[4] and is a potential precursor to the seminal formation of amino acids.[5] Cyanamide, in conjunction with ethanol, has been used as an alcohol deterrent drug for the treatment of alcoholism.[6] Industrially, cyanamide acting with hydrogen peroxide has been shown to promote the oxidative degradation of lignin (a constituent of wood pulp responsible for the yellowing of paper) in the bleaching of wood pulps.[7]

The mechanisms of these cyanamide–peroxide reactions are not fully understood. Sawaki and Ogata[8] suggest a radical decomposition of hydrogen peroxide induced by homolysis of peroxyimidate ion **1** [eqn. (1)]. Radical **1a** and/or hydroxyl radical then initiates the autocatalytic decomposition of hydrogen peroxide [R′ = RC(NH) or R′ = H respectively in eqn. (2a)].



(1)



(2a)

(2b)

Metal-catalyzed decomposition of *tert*-butyl and cumylhydroperoxide is also reportedly promoted by nitriles under alkaline conditions.[9] In the presence of alkyl- and aryl-nitriles, this decomposition presumably proceeds through a peroxyimidic acid-adduct, which homolytically decomposes in the presence of metal ions to the corresponding amide radical **1a** and alkoxyl radicals. These radicals then initiate the autocatalytic decomposition of the alkyl peroxide. However, in both the metal catalyzed and metal-free reactions, no direct evidence of the proposed radical species has been presented.

In the present work, the reaction of alkaline hydrogen peroxide and cyanamide is investigated using electron paramagnetic resonance (EPR) spectroscopy and spin trapping techniques allowing the direct observation and characterization of radical products associated with the reaction between cyanamide and hydrogen peroxide.[10]

Results and discussion

EPR spectroscopy was used to characterize the radical species generated during the reaction of hydrogen peroxide with cyanamide under alkaline conditions. After a short induction period (~1 minute depending on the pH) the solution turns yellow generating a single EPR active species **2**. The EPR spectrum of **2** is a pentet-of-pentets centered at g = 2.01 as shown in Fig. 1A. For the cyanamide system, the pentet-of-pentets splitting pattern may arise from coupling of the electron spin to two equivalent ^{14}N nuclei and four equivalent ^{1}H nuclei (expected peak intensity ratio, 1 : 3 : 5 : 3 : 1), or coupling to two inequivalent pairs of ^{14}N nuclei (expected peak intensity ratio, 1 : 2 : 3 : 2 : 1). Fig. 1B shows the absorption spectrum of the data in 1A (fine solid line), as well as the simulations using ^{14}N–^{1}H coupling (dotted line) and exclusively ^{14}N coupling (bold line). The arrows in Fig. 1B show the inadequacy of the ^{14}N–^{1}H-coupling model. Likewise, carrying out the reaction in D_2O had no effect on the splitting pattern or spectral shape, further indicating no involvement of ^{1}H nuclei. Thus the spectrum is best fit by coupling to two inequivalent pairs of ^{14}N nuclei: $a_{^{14}N1}$ = 7.30 and $a_{^{14}N2}$ = 2.13 G, as shown in the simulated spectrum C.

The nitrogen coupling was confirmed using ^{15}N-labeled cyanamide. Analogous to the ^{14}N cyanamide EPR signal, the doubly labeled ^{15}N-cyanamide ($^{15}NH_2C^{15}N$) EPR signal, Fig. 2 (bottom spectra), shows the expected triplet-of-triplets. The spectrum is nicely simulated using g = 2.01, $a_{^{15}N1}$ = 10.3 and $a_{^{15}N2}$ = 2.98 G (top spectra). The larger ^{15}N coupling constants are as expected based on $|\gamma_{^{15}N}/\gamma_{^{14}N}|$ = 1.40.[10]

The EPR spectrum obtained from the reaction of alkaline hydrogen peroxide and the amino-labeled ^{15}N-cyanamide ($^{15}NH_2C^{14}N$) shown in Fig. 3 (bottom spectra), can be simulated using the EPR parameters of the singly labeled ^{14}N and ^{15}N derivatives of Figs. 1 and 2. The simulated spectrum

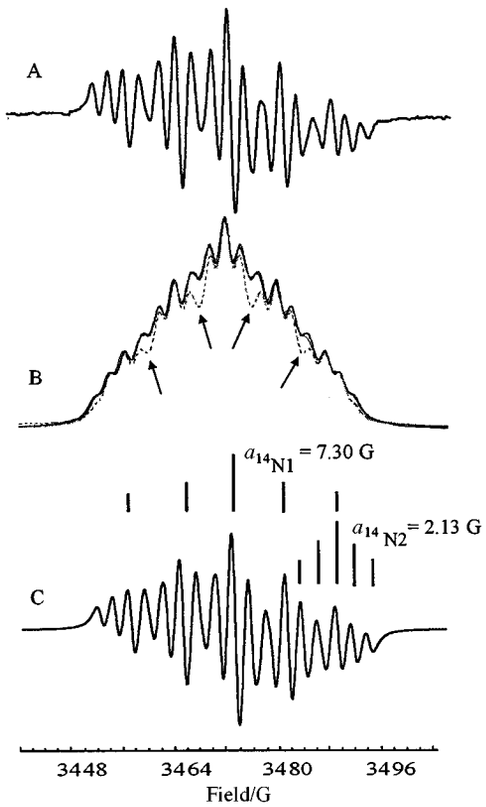


Fig. 1 EPR spectrum obtained upon mixing cyanamide and hydrogen peroxide at pH 8 to 12 (spectra A) and the corresponding absorption spectrum (spectra B, first integral) for the experimental spectrum (solid line) and simulated spectra using two equivalent ^{14}N and four equivalent ^{1}H nuclei (dotted line), or two inequivalent pairs of ^{14}N nuclei (bold line). Simulated spectrum (spectra C). The stick diagram shows coupling to two equivalent pairs of ^{14}N nuclei: ($a_{^{14}N1}$ = 7.30; $a_{^{14}N2}$ = 2.13 G). For clarity, only one manifold of the smaller coupling pattern is shown.

Large *a*	^{14}N | ^{14}N	^{15}N | ^{15}N	^{14}N | ^{15}N
Small *a*	^{15}N | ^{15}N	^{14}N | ^{14}N	^{15}N | ^{14}N

Scheme 1

indicates a 1:1:1 mixture of three isotopomers of **2**. As depicted in Scheme 1, the first has two equivalent ^{14}N with $a_{^{14}N}$ = 7.30 G and two equivalent ^{15}N with $a_{^{15}N}$ = 2.98 G. The second isotopomer has two equivalent ^{15}N with $a_{^{15}N}$ = 10.3 G and two equivalent ^{14}N with $a_{^{14}N}$ = 2.13 G. The third has two inequivalent ^{15}N nuclei ($a_{^{15}N1}$ = 10.3 G; $a_{^{15}N2}$ = 2.98 G) and two inequivalent ^{14}N nuclei ($a_{^{14}N1}$ = 7.30; $a_{^{14}N2}$ = 2.13 G).

The addition of metal chelating agents (diethylenetriaminepentamethylenepentaphosphonic acid, DTMPA, or ethylenediaminetetraacetic acid, EDTA) had no effect on the reaction and spectra obtained, confirming that free metal ions are not involved in the reaction.

Radical **2** is readily formed at pH 8–12. However, the decomposition of **2** was found to be pH dependent following the order pH 12 > pH 10 > pH 8. The concentration of the radical **2** was quantified (±15%) using a standard curve obtained for Fremy's salt.[9a] The maximum amount of **2** observed corresponds to approximately 25% of the hydrogen peroxide consumed. At pH 8 and in the presence of excess cyanamide, radical **2** is remarkably stable. Even after several months, the signal is readily observable. Addition of more hydrogen peroxide results in rapid loss of signal suggesting that peroxide is responsible for the decomposition of **2** to EPR silent diamagnetic products. In agreement with this conclusion, no EPR signal was observed in the presence of excess hydrogen peroxide.

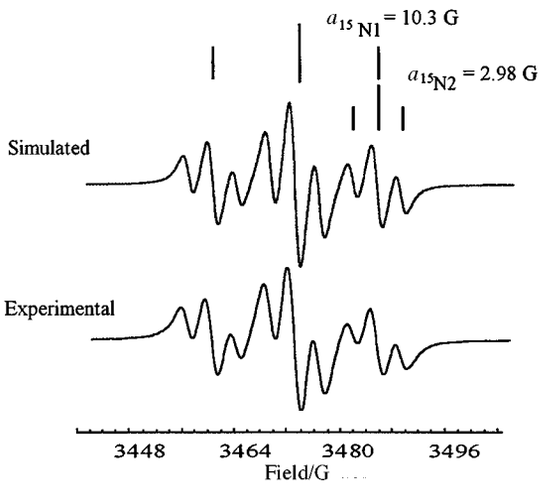


Fig. 2 EPR spectra obtained from the reaction of hydrogen peroxide and ^{15}N-cyanamide ($^{15}NH_2C^{15}N$) at pH 10 (bottom). Top spectra ($a_{^{15}N1}$ = 10.3; $a_{^{15}N2}$ = 2.98 G) is a computer simulation of the experimental data.

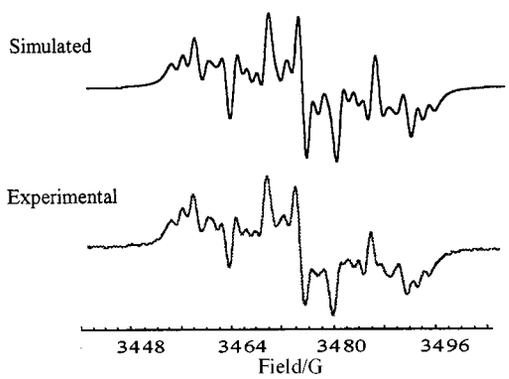


Fig. 3 EPR spectra obtained from the reaction of hydrogen peroxide and ^{15}N-cyanamide ($^{15}NH_2CN^{14}$) at pH 10 (bottom spectrum). Computer simulation (top spectra) reveals three different isotopically labeled compounds [(1) $a_{^{15}N1}$ = 10.28; $a_{^{15}N2}$ = 2.98 G; (2) $a_{^{14}N1}$ = 7.30; $a_{^{15}N2}$ = 2.98 G; and (3) $a_{^{15}N1}$ = 10.28; $a_{^{15}N2}$ = 2.98; $a_{^{14}N3}$ = 7.30; $a_{^{14}N4}$ = 2.13 G].

Usually, such stability in aqueous systems is observed for radicals of aminoxyls such as DMPO (5,5-dimethyl-4,5-dihydro-3*H*-pyrrole *N*-oxide); however, the larger of the two ^{14}N coupling constants ($a_{^{14}N1}$ = 7.30 G) is considerably smaller than is expected for aminoxyl radicals (10 < a_N < 20 G).[10b] In addition, the hydroxyurea radical (H_2N–CO–$NHO^{\bullet}$) shows coupling to both the hydrogen and nitrogen (a_N = 8.0, a_H = 11.6 G and g = 2.006)[11] and is not consistent with the observed spectra, which do not show ^{1}H coupling. Therefore, **2** must be derived from an initial cyanamide radical, $(H_2NCN)^{\bullet}$,[12] which then combines with a second molecule of cyanamide. Since hydrogen peroxide can act as either an oxidant or a reductant,[13] the radicals that are present in the reaction mixture—either radical anion or radical cation—will depend on the direction of the electron transfer. Symons and co-workers have found that the radical anion prepared by γ-irradiation of cyanamide readily abstracts a proton from the matrix to yield a neutral radical with a large proton coupling constant [$a_H \approx 70$ G, eqn. (3)].[14]

$$H_2N{-}C{\equiv}N + e^- \xrightarrow{H^+} \underset{H}{\overset{H_2N}{\diagdown}}\!\!C{=}\dot{N} \qquad (3)$$

a_H = 70 G

We do not observe such a spectrum; thus, it is unlikely that $(H_2NCN)^{\bullet}$ results from an initial reduction of cyanamide. Once

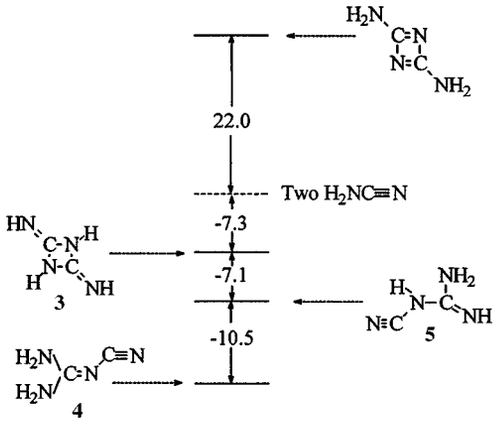


Fig. 4 Relative energies (kcal mol^{-1}) of the dimers of cyanamide.

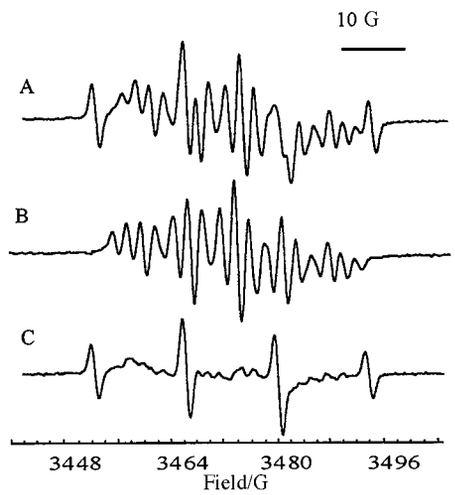


Fig. 5 EPR spectra obtained at 3 minutes, A, and 30 minutes, B, after the addition of hydrogen peroxide to a pH 10 solution of cyanamide and DMPO. Spectrum C is the difference spectrum obtained from A − B, and is diagnostic of DMPO-trapped hydroxyl radical, DMPO-OH.

generated $(H_2NCN)^{\bullet}$ can react with cyanamide to produce the radical dimer **2**. In aqueous alkaline solution, cyanamide

$$(H_2NCN)^{\bullet} + H_2NCN \longrightarrow (H_4N_4C_2)^{\bullet} \qquad (4)$$

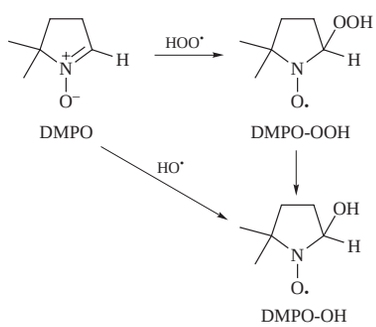
readily forms stable dimers and trimers.[15] Molecular orbital calculations have shown that a cyclic diazetidine, structure **3**, and two acyclic dimeric cyanamide compounds, **4** and **5**, are more stable than cyanamide.[16]

Inspection of the various dimeric compounds for cyanamide reveal that only the cyclic diazetidine structure **3** in Fig. 4, has the spectroscopically required C_2 symmetry (two pairs of inequivalent nitrogen nuclei observed by EPR). Furthermore, neither electrochemical oxidation nor addition of alkaline hydrogen peroxide to dicyandiamide, **5**, results in any observable EPR signals. Thus, we conclude that **2** is the *radical cation* of the diazetidine **3**. It is surprising that ^{1}H coupling is not observed; however, it may be too small to be observed in the rather broad lines of the EPR spectrum of **2** (W_{pp} ~1.3 G). Several stable alkyl- and aryl-substituted diazetidines have been prepared and one has been structurally characterized.[17]

The stability of radical **2** may be due to delocalization of the unpaired electron within the π-framework of the diazetidine. At pH 10 and 12, **2** decomposes to become EPR silent. This decomposition is probably due to nucleophilic attack of HOO^- or $HNCN^-$ at the electrophilic carbon of the diazetidine.[17b] In fact in the presence of excess cyanamide the only product detected by GCMS was melamine.[18] However despite several attempts, we have not been able to definitively characterize **2** by mass spectrometry.[19] We are currently attempting to isolate **2**, and derivatives thereof, to more fully characterize the structural and electronic properties of this novel species.

The spin trap DMPO was used to trap and further characterize the radical products. Fig. 5 presents the EPR data collected 3 minutes (spectrum A) and 30 minutes (spectrum B) after the addition of hydrogen peroxide to a pH 10 solution of cyanamide and DMPO. Two species are present in spectrum A—*untrapped* radical **2** and a DMPO-trapped, oxygen-centered radical. This oxygen-centered radical signal is more clearly seen in spectrum C, which is obtained by spectral subtraction (spectrum A − spectrum B). The splitting pattern shown ($a_N = a_H = 14.9$ G) is identical to the pattern reported for the hydroxyl radical adduct of DMPO, namely DMPO–OH.[20] The DMPO–OH adduct, which is first to form, is quickly concealed by the intense signal of **2**.[21]

It is known that DMPO trapped hydroxyl radical can arise *via* two reaction pathways; direct trapping of hydroxyl radical, or decomposition of the DMPO perhydroxyl spin adduct (DMPO–OOH *vide infra*).[22]

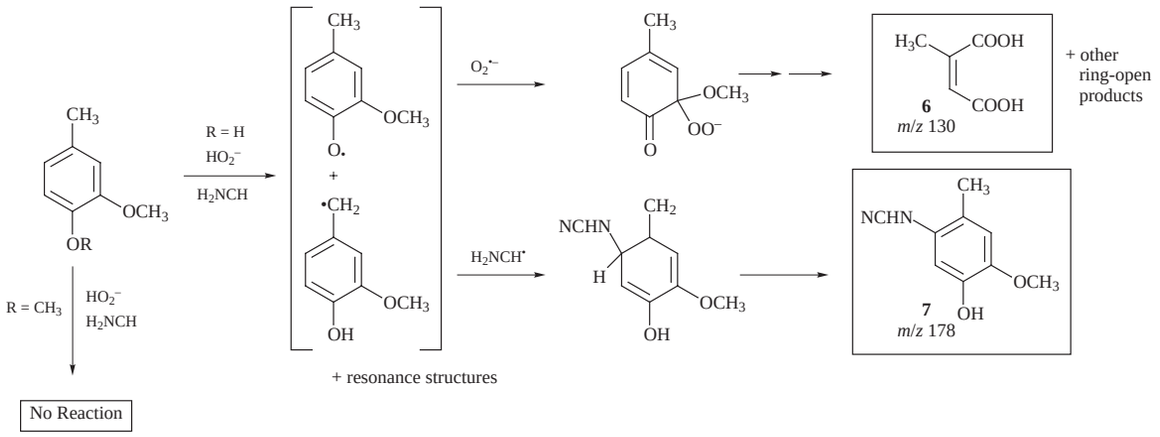


Scheme 2

The potential involvement of superoxide ($O_2^{\bullet -}$) or perhydroxyl radical ($HOO^{\bullet}$) was evaluated by including superoxide dismutase (SOD) in the reaction mixture at pH 9.[23] In the presence of SOD the formation of **2** was completely inhibited. However, radical **2** was readily observed in the presence of bovine serum albumin (BSA), indicating that the protein itself was not responsible for the SOD-based suppression of the formation of **2**. These results strongly suggest that $O_2^{\bullet -}$ or $HOO^{\bullet}$ is essential for the formation of **2** ($HO_2^{\bullet} \rightleftharpoons O_2^{\bullet -} + H^+$; $pK_a = 4.8$). Although superoxide radical has neither been trapped nor directly observed by EPR spectroscopy, this is not surprising since, as discussed above, DMPO–OOH is unstable and decomposes to DMPO–OH within minutes of formation ($t_{\frac{1}{2}}$ ~ 60 s at 25 °C and pH 4.5).[24]

At pH 13, radical **2** is not formed and two new radicals are trapped with DMPO. Fig. 6A presents the spectrum of the reaction mixture. Fig. 6B is the simulation of one component of this mixture, and provides the parameters $g = 2.01$, $a_{^{14}N} = 16.3$ and $a_H = 23.5$ G. These values are consistent with a trapped carbon-centered radical.[25] This DMPO adduct is potentially a DMPO-trapped cyanamide radical (**2**) in which the cyanamide carbon adds to the β-position of DMPO. Fig. 6C is the simulation of the second component of spectrum 6A ($g = 2.01$, $a_{^{14}N} = 16.4$ G) and is consistent with the loss of the β-hydrogen as previously seen by Janzen and co-workers.[26]

The presence of both superoxide and cyanamide radicals was evident from the reaction of creosol (R = H, Scheme 3) with alkaline hydrogen peroxide in the presence of cyanamide. Under the reaction conditions (see Experimental section), approximately 70% of the creosol was converted to oxidation products in 30 minutes. Numerous degradation products resulted and were identified as ring opened diacids (*i.e.* methylmaleic acid, **6**) and a cyanamide radical coupling product, **7**. These results strongly suggest the involvement of perhydroxyl/



Scheme 3

superoxide and cyanamide radicals in these reactions (Scheme 3). The initial step in Scheme 3 is hydrogen atom abstraction from creosol. This occurs readily with both HO$^{\bullet}$ and HOO$^{\bullet}$. However, as shown by Gierer and co-workers, H-atom abstraction from methylveratrole occurs only with HO$^{\bullet}$ *not* HOO$^{\bullet}$.[27] Since no degradation of methylveratrole occurred in the presence of H_2NCN–H_2O_2, it can be concluded that HO$^{\bullet}$ is not important in the H_2NCN–H_2O_2 reaction system.

It is known that aromatic ring-cleavage occurs from the reaction of perhydroxyl radicals and phenol derivatives.[28] The hydroperoxylcyclohexadienone intermediate, which results from the coupling of phenoxyl and perhydroxyl radicals, undergoes intramolecular nucleophilic attack and subsequent ring opening (upper pathway Scheme 3). As expected, methylveratrole (R = CH_3, Scheme 3) did not react with the cyanamide-hydrogen peroxide system and was recovered quantitatively.[29]

Conclusions

The work presented here provides the first direct evidence that several radical species are produced during the reaction between alkaline hydrogen peroxide and cyanamide. A stable cyanamide-based radical, **2**, has been characterized by EPR spectroscopy and we have proposed that **2** is the cation radical of the diazetidine compound **3**. Superoxide (or perhydroxyl) radicals are essential for the observed chemistry as indicated by studies with SOD and DMPO trapping experiments. DMPO trapping has also implicated the formation of a carbon-centered radical that is most likely associated with the initial cyanamide oxidation product, (H_2NCN)$^{\bullet}$.

Experimental

Materials

Cyanamide, 5,5-dimethyl-4,5-dihydro-3*H*-pyrrole *N*-oxide (DMPO), and potassium nitrosodisulfonate (Fremy's salt) were obtained from Aldrich and used without further purification. Bovine serum albumin (BSA) and superoxide dismutase (SOD) (4400 units mg^{-1}) were purchased from Sigma Chemical. Milli-pore grade de-ionized water and potassium hydroxide were purchased from Fluka Chemicals. Diethylenetriaminepentamethylenepentaphosphonic acid (DTMPA) and ethylenediaminetetraacetic acid (EDTA) were supplied by Buchman Chemicals.

Procedures

Reactions of cyanamide and hydrogen peroxide were carried out as follows: To an aqueous solution of cyanamide (0.2–2.0 M, pH 8–12) was added an aqueous solution of hydrogen peroxide (0.1–10-fold excess, same pH). The solution was mixed for 30 seconds and then transferred to a quartz EPR flat cell. The pH was maintained by addition of 1 M NaOH. The metal chelating agent DTMPA or EDTA was added to some solutions to ascertain the influence of metal ions on the system. For spin trapping experiments, DMPO (usually a 10-fold excess relative to cyanamide) was added to the cyanamide solution prior to hydrogen peroxide addition. Enzymes were pH adjusted and added (500 to 1000 units) prior to hydrogen peroxide addition. The concentration of hydrogen peroxide was determined by thiosulfate titration of iodine liberated from the reaction between hydrogen peroxide and potassium iodide.

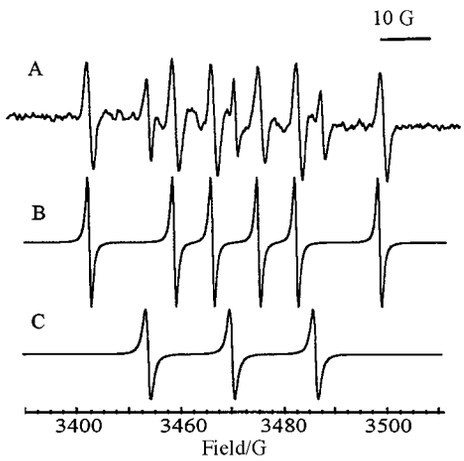


Fig. 6 EPR spectra obtained from the reaction of hydrogen peroxide and cyanamide at pH 13 in the presence of DMPO (spectrum A). Spectra B ($a_{^{14}N}$ = 16.3; $a_{^{14}H}$ = 23.5 G) and C ($a_{^{14}N}$ = 16.4 G) present computer simulations of the two species that compose spectrum A.

The oxidation of creosol and methylveratrole with cyanamide activated hydrogen peroxide was carried out as follows: To an aqueous solution of creosol (5.0 mg, 0.036 mmol, pH 10) and H_2NCN (7.6 mg, 0.18 mmol) was added H_2O_2 (0.18 mmol, 10 μL 30% H_2O_2). The mixture was stirred at room temperature for 30 minutes. Approximately 70% of creosol was consumed over this time period. The reaction mixture was neutralized to pH 6 with 10% H_2SO_4 and extracted with ethyl acetate (3 × 10 mL). The organic phases were collected, dried with Na_2SO_4 and the solvent removed under vacuum. The aqueous phase was then acidified to pH 2, extracted, dried and collected. The acidic fraction products were then methylated with diazomethane and analyzed by GCMS. The GCMS data for the numerous dicarboxylic acids were compared with those of known compounds; however, the yield of any single ring-opened product was low.

3-Hydroxy-4-methoxy-6-methyl-*N*-methylaniline **7**. Yellow solid. *m*/*z* (high resolution) EI(+): M_{sample} 178.0744 ($M_{theoretical}$ 178.0742; Δ*M* 1 ppm), (low resolution) EI, 178 (M^+,100), 149

(11), 135 (34), 121 (5), 107 (12), 77 (4), 65 (16). Elemental composition ($C_9H_{10}N_2O_2$). δ_H (DMSO) 2.276 (s, 3H), 3.870 (s, 3H), 6.498 (s, 1H), 6.656 (s, 1H), 7.320 (s, 1H). δ_C (DMSO) 21.470 (CH_3), 55.882 (OCH_3), 108.8 (CH), 115.8 (CH), 120.5 (CN), 133.4 (CH), 134.1 (C–CH_3), 142.5 (C–OH), 145.2 (C–OCH_3).

Methylmaleic acid dimethyl ester **6**. *m*/*z* (low resolution) EI, 158 (M^+), 127 (M − 31,100), 99 (20), 85 (2), 68 (8), 59 (28), 53 (5).

Instrumentation

GCMS data were obtained by the North Carolina State University Mass Spectrometry Facility using an HP5985 instrument. EPR spectra were obtained on an IBM ER200D instrument operating at X-band (~9.45 GHz). Micowave frequency was measured with a Hewlett Packard 5350B frequency counter and the field at $g = 2.0037$ was calibrated with diphenylpicrylhydrazyl (DPPH). All spectra were collected at ambient temperature (~25 °C) using a quartz flat cell. Microwave power was 2–20 milliwatts and field modulation was 0.1–1 G_{pp}. Analog EPR data were digitized using EPRWare and spectra were simulated using EWSim (Scientific Software Solutions).[30] The double integrals of the derivative data were obtained using the EPRWare data manipulation software.

Acknowledgements

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