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Application of the new EPR spin trap 1,1,3-trimethylisoindole *N*-oxide (TMINO) in trapping HO$^{\bullet}$ and related biologically important radicals

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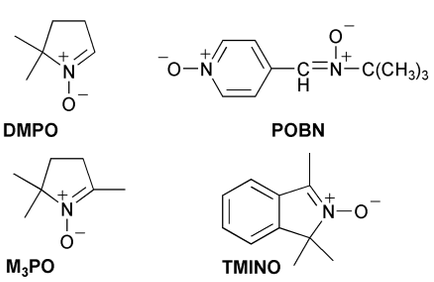
The new EPR spin trap, 1,1,3-trimethylisoindole *N*-oxide (TMINO), very efficiently scavenges several Fenton-derived carbon- and oxygen-centred radicals including hydroxyl, formyl and alkyl radicals. The adducts display good stability and narrow EPR line-widths, allowing the detection of the expected radicals as well as two-dimensional (time-resolved) EPR experiments. Trapping experiments were also undertaken with superoxide radicals (giving no EPR signals) and nitric oxide (which gave strong EPR signals attributed to the action of higher oxides of nitrogen). The selectivity of TMINO towards HO$^{\bullet}$ with respect to superoxide radicals demonstrates its potential as a useful spin-trap.

Introduction

Much of the current interest in spin trapping lies in the detection and quantification of biologically significant radicals, such as the hydroxyl radical (HO$^{\bullet}$), the superoxide radical anion ($O_2^{\bullet -}$) and nitric oxide ($^{\bullet}NO$).[1,2] The hydroxyl radical is widely believed to be the initiating species in toxic events generated by ionising radiation.[3–6] In addition, it is implicated in the biological damage associated with ischaemia/reperfusion injury.[7–10] The spin trapping of hydroxyl radicals by nitrones is often complicated by the short lifetime of the HO$^{\bullet}$ adduct, particularly for the commonly utilised nitrones DMPO[11–14] and POBN.[15] Additionally, the apparent HO$^{\bullet}$ adduct may be formed by other non-radical processes,[16–17] such as the decomposition of the $O_2^{\bullet -}$ adduct.[15]



An important strategy to avoid this problem is the development of new nitrones that exhibit specificity for HO$^{\bullet}$, especially with respect to $O_2^{\bullet -}$, and form stable or persistent adducts.[11,18–20] Substitution at the α-carbon of the nitrone precludes disproportionation of the hydroxyl radical adduct. The hydroxyl radical adduct of M_3PO for example, has been shown to be significantly more persistent than that of DMPO.[11] Most importantly, the radical adducts of M_3PO are also resistant to cellular-induced degradation. These results suggest that analogously, the isoindole-based nitrone 1,1,3-trimethylisoindole *N*-oxide (TMINO) may exhibit favourable HO$^{\bullet}$ trapping properties with regard to selectivity, reactivity and adduct stability.

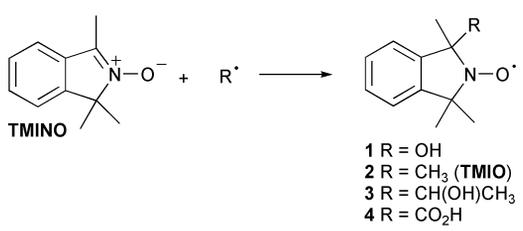
Herein, we evaluate the potential of TMINO as a useful spin trap for the detection of HO$^{\bullet}$ and $O_2^{\bullet -}$ radicals. The nitrone was used to trap hydroxyl radicals and secondary radicals generated in the presence of the known HO$^{\bullet}$ scavengers DMSO, ethanol and formate. The formation of radical adducts was monitored with two-dimensional (magnetic field *versus* time), continuous-wave EPR experiments (for example Fig. 2). To further explore the trapping ability of TMINO, $O_2^{\bullet -}$ and $^{\bullet}NO$ were also investigated. No direct EPR signals arise from spin-trapping these species, indicating that TMINO is a HO$^{\bullet}$ selective spin-trap in the presence of other reactive-oxygen species.

Results and discussion

EPR investigations

Spin-trapping the hydroxyl radical. TMINO was investigated as a spin trap for the hydroxyl radical and the secondary radicals generated by the action of HO$^{\bullet}$ on DMSO, ethanol and formate. Hydroxyl radicals were generated under standard aqueous Fenton conditions and blank reactions confirmed the stability of TMINO with respect to Fe(II) and H_2O_2.

When hydroxyl radicals were generated in the presence of TMINO, a single adduct exhibiting a strong three-line EPR signal with excellent signal to noise was obtained. The nitrogen hyperfine constant ($A/g\beta$ = 14.95 G) is consistent with a relatively electronegative substituent and the spectrum was attributed to the hydroxyl radical adduct of TMINO (**1**) (Scheme 1).



Scheme 1 Formation of the radical adducts of TMINO.

Time-resolved, two-dimensional EPR experiments were conducted to determine the stability of the hydroxyl radical adduct. By fitting a single exponential to the time slice corresponding to the maximum intensity of the central resonance of the two-dimensional data sets, the half life of the adduct was found to be greater than or equal to 813 s for each of the HO$^{\bullet}$

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Table 1 X-band EPR characteristics of the radical adducts of TMINO

Radical Source	Radical adduct	%	g_{av}	$A_{av}(N)/g\beta$ [a]
H_2O_2/Fe^{2+}	HO-TMINO (**1**)	100	2.00567	14.95
H_2O_2/Fe^{2+}/DMSO	H_3C-TMINO (**2**; TMIO)	100	2.00578	14.34
H_2O_2/Fe^{2+}/EtOH	HO-TMINO (**1**)	57 [b]	2.00567	14.90
	$CH_3(OH)CH$-TMINO (**3**)	43 [b]	2.00570	14.15
H_2O_2/Fe^{2+}/EtOH	HO-TMINO (**1**)	92 [c]	2.00567	14.72
	$CH_3(OH)CH$-TMINO (**3**)	8 [c]	2.00570	14.14
$H_2O_2/Fe^{2+}/HCO_2^-$	HO-TMINO (**1**)	22 [b,d]	2.00567	14.91
	HO_2C-TMINO (**4**)	78 [b,d]	2.00549	15.50
"$^{\bullet}$NO" [e] (in benzene)	**6**	31 [b]	2.00578	14.23
	7	69 [b]	2.00595	12.75
"$^{\bullet}$NO" [e] (in benzene)	**6**	25 [f]	2.00578	14.17
	7	75 [f]	2.00595	12.86

[a] Units Gauss. [b] After approximately 1 min. [c] After 25 min. [d] After 1 h. [e] Probably contaminated with higher oxides of nitrogen. [f] After 1 h 25 min.

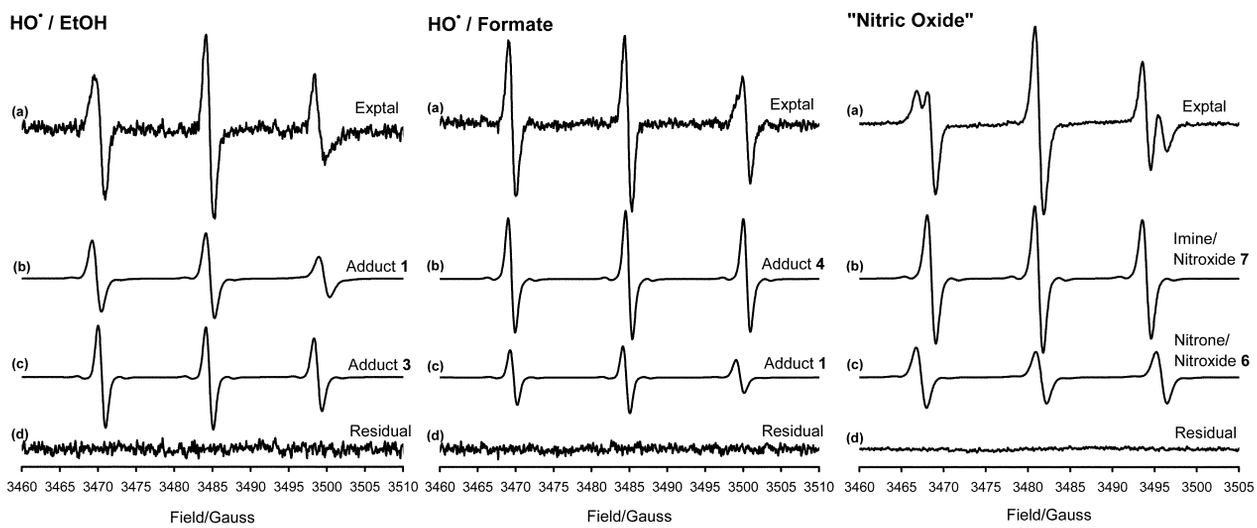


Fig. 1 EPR spectra of the radical adducts of TMINO. The radical source for each set of spectra is listed at the top left. For each set of spectra: (a) experimental spectrum recorded immediately after commencement of the reaction; (b, c) computer simulations of the constituent radical adducts. Relative weightings are listed in Table 1; (d) the residual obtained by subtracting spectrum a − (b + c).

trapping experiments. Notably, in most cases the EPR signal exhibited essentially no degradation over the duration of the experiment (approximately 1 h). The variation in observed $t_{1/2}$ values was attributed to reaction with varying amounts of adventitious oxidisable impurities.

When TMINO was exposed to aqueous Fenton conditions in the presence of DMSO, the capture of methyl radicals resulted in the formation of the known isoindoline nitroxide 1,1,3,3-tetramethylisoindolin-2-yloxyl (TMIO; **2**) (Scheme 1). The EPR spectrum of the single adduct exhibited a nitrogen hyperfine coupling ($A/g\beta$) which is consistent with that of TMIO in water (Table 1) and the intensity of the two-dimensional spectrum was essentially stable over the period of observation.

The exposure of TMINO to aqueous Fenton conditions in the presence of ethanol was expected to result in the formation of nitroxide **3** through the capture of α-hydroxyethyl radicals (Scheme 1). One-dimensional EPR spectra were recorded at the commencement of the reaction and after 25 minutes. Deconvolution of the experimental spectra using EWVoigtN [21] indicated the presence of two radical adducts (Fig. 1). These were identified as the HO$^{\bullet}$ adduct (**1**) observed previously and the α-hydroxyethyl adduct of TMINO (**3**) according to the observed nitrogen hyperfine couplings (Table 1). Scaling information indicated that the ratio of the HO$^{\bullet}$ adduct to the α-hydroxyethyl adduct increased from 1.35 : 1 to 11.5 : 1 over the first 25 minutes, suggesting that adduct **3** is less stable than **1** under these conditions. The acquisition of time-resolved EPR spectra was restricted by insufficient signal to noise after 25 minutes.

Exposure of TMINO to aqueous Fenton conditions in the presence of sodium formate gave a very persistent experimental spectrum which, upon deconvolution, also indicated the formation of two radical adducts (Fig. 1). These were identified as the HO$^{\bullet}$ adduct (**1**) and expected formyl adduct (**4**) of TMINO, on the basis of their nitrogen hyperfine couplings (Scheme 1). The large nitrogen hyperfine interaction of **4** ($A/g\beta$ = 15.50 G) is typical of nitroxides with electron withdrawing substituents proximate to the radical moiety. A two-dimensional EPR spectrum of the reaction mixture showed a composite three-line nitroxide signal which was essentially stable over the duration of the experiment (approximately 1 hour). Scaling information indicated that the formyl and HO$^{\bullet}$ adducts were present in a constant ratio of 3.55 : 1 respectively over this period of observation.

Spin trapping of superoxide. When TMINO was exposed to superoxide generated from a standard xanthine oxidase system, using either xanthine or hypoxanthine as the enzyme substrate, EPR spectroscopy failed to indicate the presence of new nitroxide species. The absence of an EPR signal attributable to the superoxide adduct of TMINO may be due to the inability of the nitrone to trap the radical anion. Equally, the superoxide adduct may be rapidly destroyed, converted to the corresponding hydroxylamine or oxoammonium cation as part of a dismutation cycle and consequently not reaching a detectable steady-state concentration. The destruction of spin adducts by superoxide has been reported for the analogous nitrone M_3PO [22] and also DMPO.[23]

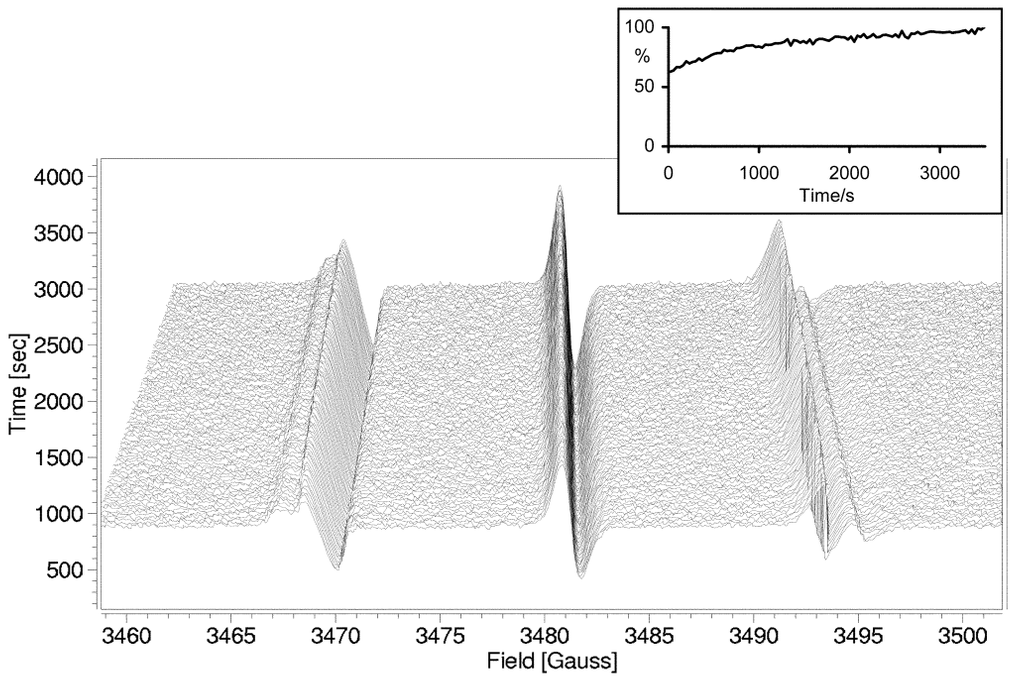


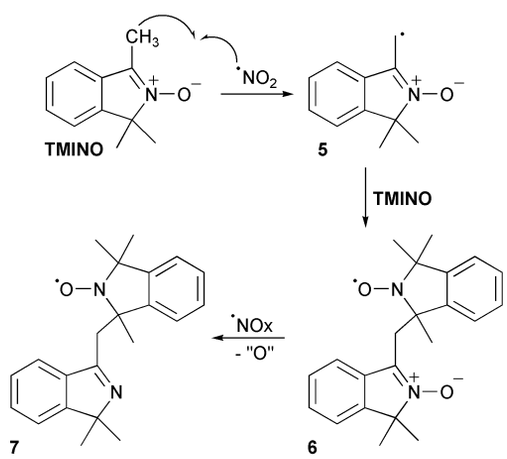
Fig. 2 Two-dimensional (magnetic field *versus* time) EPR experiment ($\nu = 9.78027$ GHz) obtained after injecting a benzene solution of TMINO into an EPR tube pre-charged with $^{\bullet}$NO. The insert shows the time dependence of the central resonance of the composite spectrum.

Treatment of TMINO with nitric oxide. TMINO solutions were treated with nitric oxide generated by the slow reaction of nitric acid with copper powder in a thoroughly deoxygenated system. When benzene solutions of the nitrone were treated with nitric oxide for either 20 minutes or 2 hours, strong EPR spectra were obtained. The solution treated for 20 min gave an experimental spectrum which was deconvoluted to confirm the presence of two nitroxide species ($A/g\beta$ = 12.93 and 14.22 G). The solution treated with $^{\bullet}$NO for 2 hours exhibited a similar EPR spectrum, although the EPR linewidths were considerably larger (spectra not shown). It was immediately obvious that neither of the observed nitroxides was the $^{\bullet}$NO adduct of TMINO, due to the absence of an additional nitrogen hyperfine coupling. Aqueous solutions of TMINO failed to give an EPR signal upon treatment with $^{\bullet}$NO.

The same nitroxides were observed when a sealed EPR tube that had been pre-charged with nitric oxide, was injected with a benzene solution of the nitrone. Fig. 1 shows the initial EPR spectrum of this system. In consecutive time-resolved, two dimensional EPR experiments the signal intensity of the composite spectrum increased over the first 30 minutes and then remained constant for the following 80 minutes (Fig. 2). Deconvolution indicated that the relative ratios of the two nitroxides varied over this period (Table 1).

While the identities of the observed nitroxides remain speculative, the EPR spectrum of one component corresponds with that of the paramagnetic impurity detected in nitrone solutions that had been stored under benchtop conditions or exposed to oxygen, compound **6**.[24] The formation of nitrone–nitroxide **6** from the action of nitric oxide is difficult to justify, as nitric oxide is relatively long-lived and non-aggressive. In the presence of trace amounts of adventitious oxygen however, or through the slow dismutation of nitric oxide, small amounts of $^{\bullet}NO_2$ and higher oxides of nitrogen may be formed. These higher oxides of nitrogen are known to be much more reactive. For example, $^{\bullet}NO_2$ is known to react rapidly with chelotropic spin traps at 500 times the rate of $^{\bullet}$NO,[25] and abstract hydrogen from olefins.[26]

We propose that **6** may be formed by a radical process initiated by hydrogen abstraction, as shown in Scheme 2. Delocalised nitroxide radicals such as **5** are known to be reactive due to the high spin density on the α-carbon. Spin



Scheme 2 Proposed radical formation of nitroxides **6** and **7**.

trapping of this species by further TMINO would give rise to the proposed nitrone–nitroxide **6**. Nitroxide **5** must be short-lived and present in low steady state concentrations, as it is not observed by EPR. The spectrum of this compound would be characterised by a large, well-resolved proton hyperfine coupling from the methylene protons, due to delocalisation of the unpaired spin.

The second nitroxide would form through the subsequent reaction of **6** with $^{\bullet}$NO or higher oxides of nitrogen. The EPR spectrum of this compound exhibits a smaller nitrogen hyperfine interaction (average $A/g\beta$ = 12.89 G) than that attributed to nitrone–nitroxide **6**, consistent with a less electron-withdrawing substituent. Deoxygenation of **6** could give imine–nitroxide **7**, *via* a process that has some literature precedent.[27]

That the nitroxides observed in the presence of nitric oxide are formed *via* the action of higher oxides of nitrogen and not directly from $^{\bullet}$NO itself was confirmed when a benzene solution of TMINO was injected into an EPR tube containing nitric oxide deliberately contaminated with air. The brown coloration of the gas confirmed the presence of NO_x species. EPR spectra were recorded periodically, with the intensity of the composite spectrum increasing over the first 23 minutes and then remaining relatively constant. The experimental solution

gave strong EPR spectra and deconvolution indicated the presence of the nitroxide species observed previously. The intensity of the EPR signal confirmed that the nitroxides were present in greater concentrations than in previous reactions, further supporting the role of NO_x species in their formation.

Conclusion

From these studies we conclude that the novel isoindole nitrone TMINO is a suitable trap for the direct detection of carbon- and oxygen-centred radicals, including the hydroxyl radical. Significantly, the trap is selective for the HO$^{\bullet}$ radical with respect to superoxide, as no detectable paramagnetic species were formed upon exposure to the latter. TMINO is particularly useful for detecting secondary radicals produced by the action of HO$^{\bullet}$ on DMSO and formate, forming stable isoindoline nitroxides. Interestingly, in addition to the expected radical adducts, the HO$^{\bullet}$ adduct was also observed when TMINO was exposed to HO$^{\bullet}$ in the presence of ethanol and formate.

One of the prime advantages of this new nitrone spin trap is the stability of the adducts (isoindoline nitroxides) formed by spin-trapping. Isoindoline nitroxides possess some advantages over other classes of nitroxides and this is reflected by the stability and longevity of the paramagnetic compounds generated by spin trapping with TMINO. The EPR characteristics obtained from spectral fitting for the observed radical adducts of TMINO are listed in Table 1.

Experimental

General

X-band (9 GHz) EPR spectra were obtained using a Bruker Elexsys E500 multifrequency continuous wave EPR spectrometer equipped with an EIP 548B microwave frequency counter and a Bruker ER035M gaussmeter for microwave frequency and magnetic field calibration. A conventional X-band rectangular TE_{102} microwave cavity was utilised for all spectra measured in this study. EPR spectra were simulated using XSophe[28,29] in conjunction with Xepr[30] software.

1,1,3-Trimethylisoindole *N*-oxide (TMINO) was synthesised from 1,1,3,3-tetramethylisoindolin-2-yloxyl (TMIO; **2**) as described previously.[24] Xanthine oxidase (Grade I from buttermilk in 2.3 M $(NH_4)_2SO_4$ containing 1 mM sodium salicylate) was purchased from Sigma and used as supplied.

EPR spin-trapping studies

The purity of the nitrone stock solution with respect to paramagnetic contaminants was assessed by EPR spectroscopy at the commencement of each set of experiments. In the event that the nitrone showed evidence of noticeable levels of paramagnetic contamination, the material was repurified as described previously.[24]

Reaction mixtures for the spin trapping experiments were prepared in glass sample vials and drawn with a syringe, *via* teflon tubing, into either a quartz capillary or a Bruker AquaX cell within the EPR resonant cavity.

Spin trapping the hydroxyl radical. Standard aqueous Fenton conditions were utilised for the production of HO$^{\bullet}$ radicals. All stock solutions were prepared with ultra-pure water, deoxygenated by bubbling with argon, and stored under argon. Reaction mixtures were prepared in glass sample vials and initiated by the addition of Fe(II) solution. The samples were vortexed and immediately introduced into the spectrometer resonant cavity. One-dimensional and time-resolved, two-dimensional continuous wave EPR spectra were recorded for each sample.

Blank reactions confirmed the stability of TMINO towards Fe(II) and H_2O_2. No EPR signal was observed from a mixture of TMINO (100 mM; 500 μl), H_2O_2 (8.8 mM; 34 μl) and H_2O (500 μl). Similarly, a mixture of TMINO (100 mM; 250 μl) and $FeSO_4$ (0.3 mM; 250 μl) was found to be EPR silent.

The spin trapping of HO$^{\bullet}$ radicals by TMINO was investigated by the addition of $FeSO_4$ (0.3 mM; 500 μl) to a mixture of the nitrone (100 mM; 466 μl) and H_2O_2 (8.8 mM; 34 μl).

Spin trapping the methyl, formyl and α-hydroxyethyl radicals. Standard aqueous Fenton conditions in the presence of the HO$^{\bullet}$ scavengers DMSO, ethanol and sodium formate, were used to generate the methyl, α-hydroxyethyl and formyl radicals respectively. Reaction mixtures were prepared as per the trapping of HO$^{\bullet}$, but with the inclusion of 5% w/v of the HO$^{\bullet}$ scavenger.

Spin trapping the superoxide radical anion. Superoxide was generated using xanthine oxidase with hypoxanthine or xanthine as the enzyme substrate. Reaction mixtures were prepared to contain final reagent concentrations of 50 mM TMINO, 0.4 mM hypoxanthine (or xanthine) and 0.4 unit cm^{-3} xanthine oxidase. Reagents were prepared in either ultra-pure water or phosphate buffer (pH 7.4), with parallel reactions being performed in each medium.

Typically, hypoxanthine (4 mM; 50 μl) was added to a mixture of xanthine oxidase (28 mg protein cm^{-3}; 0.69 unit mg^{-1} protein; 10 μl) and TMINO (10 mg cm^{-3}, 57 mM; 440 μl) in either ultra-pure water or phosphate buffer. The reagents were mixed in a glass sample vial and vigorously vortexed to saturate with air. The sample was then immediately introduced into a quartz capillary within the spectrometer resonant cavity as described above.

Treatment of TMINO with nitric oxide. Nitric oxide was generated chemically by the slow reaction of HNO_3 (8 M) with copper powder and purified by two consecutive scrubbings with 5 M NaOH. The reaction apparatus was repeatedly evacuated and flushed with UHP argon prior to use in order to exclude O_2. All solutions were prepared from degassed water and thoroughly deoxygenated by argon bubbling and freeze–thaw cycles immediately before use.

Nitrone solutions were treated with nitric oxide according to one of several techniques. Benzene solutions of the nitrone (10 mg cm^{-3}, 57 mM; 1 cm^3) were treated with a continuous stream of nitric oxide for either 20 or 120 min. Aqueous solutions were treated for 120 min. The reaction mixtures were transferred under argon into an EPR tube and stored on ice prior to measurement of their EPR spectra.

Alternately, an EPR tube equipped with a rubber septum was carefully degassed, pre-charged with nitric oxide, and the nitrone solution added by syringe immediately prior to spectroscopy. In addition to standard one-dimensional spectra, two-dimensional (time-resolved) spectra were also recorded for such samples.

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