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Superoxide radical trapping and spin adduct decay of 5-*tert*-butoxycarbonyl-5-methyl-1-pyrroline *N*-oxide (BocMPO †): kinetics and theoretical analysis

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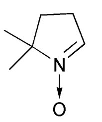
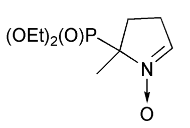
Spin-trapping of oxygen-centered radicals such as superoxide radical is increasingly becoming important in the study of biological oxidations. Superoxide spin-trapping and decay kinetics in aqueous media of the new solid nitrone 5-*tert*-butoxycarbonyl-5-methyl-1-pyrroline *N*-oxide (BocMPO) are reported and compared to those of 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) and 5-diethoxyphosphoryl-5-methyl-1-pyrroline *N*-oxide (DEPMPO). Superoxide spin-trapping by BocMPO is significantly slower than that by DEPMPO and DMPO, and the half-life of BocMPO–superoxide adduct is shorter than that of DEPMPO–superoxide adduct. The BocMPO spin-trapping ability and stability of its superoxide adducts are rationalized based on theoretical calculations.

Introduction

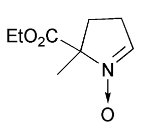
The role of superoxide radical ($O_2^{\bullet-}$) in biological oxidative stress has stimulated interest in its detection by spin trapping.[1–7] Electron paramagnetic resonance (EPR) spectroscopy has been conventionally used to detect persistent and stable radical adducts which allows identification and quantification of transient radicals in *in vivo* and *in vitro* applications through spin trapping. Spin adduct stability and their spectral uniqueness have always been a critical challenge in spin trap development.[8–10] Limitations still exist with regard to spin trap stability, efficiency, ease of handling and purification. The commonly used spin trap 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) is known to trap $O_2^{\bullet-}$ but with a half-life of only 60 s at pH 7.0,[11] while the spin trap 5-diethoxyphosphoryl-5-methyl-1-pyrroline *N*-oxide (DEPMPO) has exhibited an impressive half-life of 13 min[12,13] and 5-ethoxycarbonyl-5-methyl-1-pyrroline *N*-oxide (EMPO) a half-life of ~ 5 min in phosphate buffer of pH 7.0.[14]



DMPO



DEPMPO



EMPO

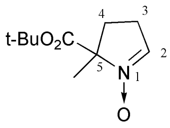
Zhao *et al.*[15] recently reported the synthesis of a new spin trap 5-*tert*-butoxycarbonyl-5-methyl-1-pyrroline *N*-oxide (BocMPO) and it was found to trap $O_2^{\bullet-}$ as well as glutathiyl radicals ($GS^{\bullet}$).

Although kinetic data for BocMPO efficiency and $O_2^{\bullet-}$ adduct stability have not been reported, a qualitative comparison of EPR spectra showed that BocMPO–$O_2^{\bullet-}$ adduct is relatively more persistent than DMPO–$O_2^{\bullet-}$ and EMPO–$O_2^{\bullet-}$ adducts. Furthermore, BocMPO is a solid which makes it easier to handle and purify from nitroxyl contaminants during synthesis than DEPMPO. We now report the determination of rate constants of formation of BocMPO–$O_2^{\bullet-}$ adduct by competitive trapping as well as its half-life in aqueous media.

Table 1 Apparent rate constants for the trapping of superoxide radical

Spin trap	k_{app}/M^{-1} s^{-1}	Competitor[a]	Ref.	r^2
DMPO	50	DEPMPO	This work	0.94
DMPO	60	DEPMPO	16	
DEPMPO	90	DMPO	17	
DEPMPO	58[b]	SOD	This work	0.99
BocMPO	7[b]	SOD	This work	0.99

[a] Measurements were done in phosphate buffer pH 7.0 using a light–riboflavin system. [b] Based on $k_{SOD} = 1 \times 10^8$ M^{-1} s^{-1} at pH 7.0 obtained from SOD–cytochrome *c* inhibition in xanthine–xanthine oxidase radical generating system.[18]



BocMPO

Results and discussion

Kinetics of superoxide radical adduct formation

Table 1 shows the apparent rate constants for spin trapping of superoxide derived from competition experiments. The field position of the lowest peak for each measured spin trap has a standard deviation of only ± 0.10 G based on 12 randomly selected spectra. For DEPMPO spectra, the peak height ratio between the lowest and the adjacent higher-field peaks (where no overlap with competitor spin trap occurs) was found to be 0.81 ± 0.03 allowing reliable measurements of kinetic data. Peak height intensities of the low-field peaks for BocMPO and

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† The acronym BocMPO will be used instead of BMPO to distinguish it from the BMPO which has already been assigned for 5-*n*-butyl-5-methyl-1-pyrroline *N*-oxide (M. J. Turner and G. M. Rosen, *J. Med. Chem.*, 1986, **29**, 2439).

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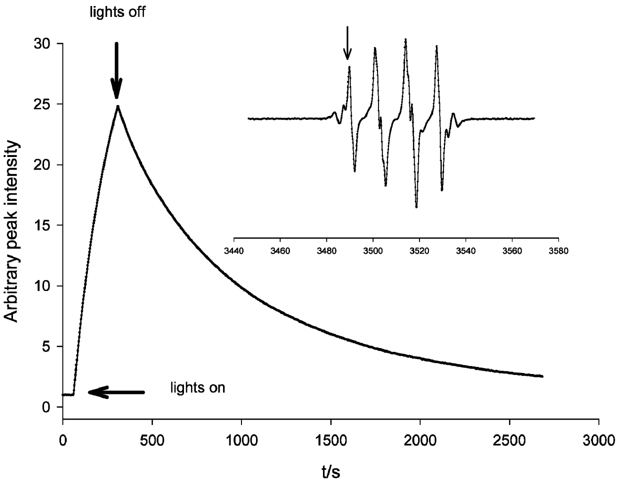


Fig. 1 Low-field peak time scan of 100 mM BocMPO before and after 4 min of irradiation in phosphate buffer, pH 5.6 containing 100 µM DETPA and 100 µM riboflavin.

DEPMPO were normalized and plotted against time. As presented in Fig. 1, no signal was observed before irradiation, while peak formation occurs when the light source is turned on. Peak height intensity begins to rise as a function of time and plateaus over a certain period of time, then decreases when the light source is turned off. This same behaviour was also observed for DEPMPO. During irradiation in competition experiments, the initial rate of adduct formation was linear for the first 60 s. Data were plotted using the equation

$$\frac{V}{v} = 1 + \frac{k_c[\mathrm{C}]}{k_{\mathrm{DEPMPO}}[\mathrm{DEPMPO}]}$$

where V and v are initial rates of superoxide formation in the absence and presence of a competitor trap C [superoxide dismutase (SOD) or a nitrone], respectively; k_c and k_{DEPMPO} are the second-order rate constants of the competitor spin trap and DEPMPO, respectively; [C] and [DEPMPO] are concentrations. The apparent rate constant of 50 M^{-1} s^{-1} of DMPO–OOH formation which is reasonably close to the previously reported value[17] was estimated using $k_{\mathrm{DEPMPO}} = 58$ M^{-1} s^{-1} from a competition experiment with SOD. However, competition of DMPO with SOD did not allow measurement of the apparent rate constant due to the fast decomposition of superoxide adduct to the hydroxyl adduct. Competitive trapping by BocMPO with DEPMPO did not show significant change in DEPMPO rate of adduct formation even up to 400 mM/50 mM (BocMPO/DEPMPO) concentrations. Competition experiments with SOD (Fig. 2) gave k_{BocMPO} and k_{DEPMPO} values of 7 and 58 M^{-1} s^{-1}, respectively, consistent with the observations made during competitive trapping of DEPMPO with BocMPO or DMPO. These values suggest that BocMPO is relatively slow in trapping superoxide radicals compared to DMPO and DEPMPO.

Despite the presence of bulky substituents in both DEPMPO and BocMPO, the difference in their reactivity towards superoxide radical poses some questions regarding what other factors can influence trapping efficiency. Superoxides by virtue of the oxygen electronegativity are nucleophilic and are known to react with slightly polarized functional groups such as the carbon centers in carbonyl compounds.[1] The chemistry of nitrones mimics that of carbonyl compounds in a variety of ways in which the 2-C is analogous to the carbonyl carbon.[19] It is logical to assume that the reactivity of nitrones towards superoxide radicals can be regulated by the electrophilicity of 2-C. The degree of 2-C electrophilicity can be influenced by the substituents attached to the 5-C position, and therefore theoretical studies were carried out to correlate atomic charge on 2-C with reactivity. Mulliken atomic charges are used to estimate atomic charges and since these values have an approximate relation to the experimental, four different methods of calculation were used. A consistent trend can be seen in Table 2 from the values collected using both semi-empirical and *ab initio* methods. Decreasing positive charge on 2-C in the order of $-PO(OEt)_2 > -CF_3 > -CO_2t\text{-Bu} > -CH_3/-C(CH_3)_3$ is observed. In comparison with the hypothetical compounds with electron withdrawing $-CF_3$ and electron donating $-C(CH_3)_3$ groups, $-PO(OEt)_2$ has the strongest electron-withdrawing effect which significantly decreases the electronegativity of 2-C. The $-CO_2t$-Bu substituent however decreases the 2-C electronegativity much more than the $-CH_3$ and $-t$-Bu groups, obviously due to the electron-withdrawing property of most carboxy groups. The relatively high reactivity of DMPO towards $O_2^{\bullet -}$ could not be correlated with the calculated high negative charge on 2-C but it should be noted however that reported[20] rate constants for superoxide trapping by DMPO have values ranging from 1.2 to 310 M^{-1} s^{-1} in only a narrow pH range of 6.4–7.8.

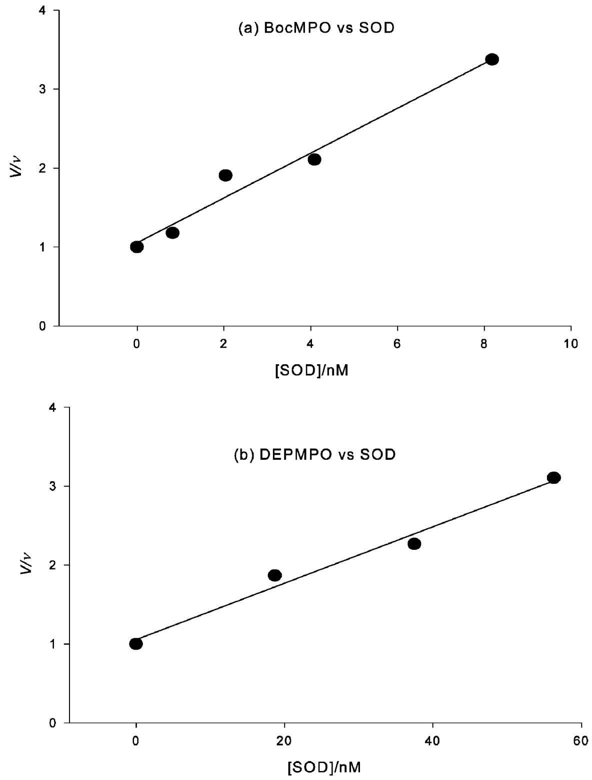
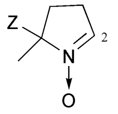
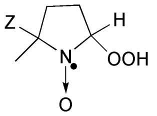


Fig. 2 Plots of *V*/*v* for the competitive superoxide trapping of (a) BocMPO (50 mM) and (b) DEPMPO (46.7 mM) with various amounts of SOD; *V* and *v* correspond to the initial rates of superoxide adduct formation in the absence and presence of SOD, respectively. Superoxide trapping was generated in phosphate buffer, pH 7.0 containing 100 µM DETPA and 25 µM riboflavin.

Total energies E_{tot} and heats of formation ΔH_f were calculated for both nitrone spin traps (Table 3) and their respective superoxide adducts (Table 2). For nitrone spin traps, a general trend in order of decreasing E_{tot} for all calculations was observed: $-PO(OEt)_2 > -CF_3 > -CO_2t\text{-Bu} > -CH_3/-C(CH_3)_3$ while heats of formation ΔH_f are in the order of: $-CF_3/-PO(OEt)_2 > -CO_2t\text{-Bu} > -CH_3/-C(CH_3)_3$. The order of decreasing ΔH_f for their respective superoxide spin adducts is: $-CF_3/-PO(OEt)_2 > -CO_2t\text{-Bu} > -CH_3/-C(CH_3)_3$. A *trans* conformation was used in the calculation, since two stereogenic centers are present (except for DMPO) upon trapping of the superoxide radical. Differences in total energies $\Delta E_{\mathrm{tot}} = E_{\mathrm{tot}}$ (spin adduct) − E_{tot} (spin trap) for all compounds are almost the same with average values of $\Delta E_{\mathrm{tot}} = -15{,}050$ kcal mol^{-1} for AM1 and $-15{,}150$ kcal mol^{-1} for MNDO.

Table 2 Net atomic charges (Mulliken) of 2-C in theoretically optimized nitrone spin traps. Total energies (E_{tot} in kcal mol^{-1}) and heats of formation (ΔH_f in kcal mol^{-1}) of the superoxide spin adducts[a]

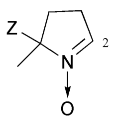




	Net charge of 2-C				E_{tot} (ΔE_{tot})[b]		ΔH_f ($\Delta\Delta H_f$)[c]	
Z	AM1	PM3	MNDO	*ab initio*	AM1	MNDO	AM1	MNDO
–PO(OEt)$_2$ (DEPMPO)	−0.2322	−0.5486	−0.1299	0.0228	−84631.4 (−15055.4)	−85633.8 (−15140.2)	−214.1 (−40.3)	−152.3 (−33.4)
–CO$_2$*t*-Bu (BocMPO)	−0.2788	−0.5586	−0.1676	0.0126	−76875.5 (−15048.2)	−77211.8 (−15145.2)	−110.5 (−35.0)	−100.1 (−38.7)
–CH$_3$ (DMPO)	−0.2960	−0.5661	−0.1819	0.0026	−48377.2 (−15055.1)	−48601.9 (−15147.7)	−29.1 (−32.7)	−28.0 (−41.2)
–CF$_3$	−0.2673	−0.5418	−0.1483	0.0131	−81004.7 (−15050.7)	−80795.6 (−15150.3)	−177.2 (−37.2)	−167.7 (−43.7)
–C(CH$_3$)$_3$	−0.2957	−0.5685	−0.1829	0.0090	−59145.7 (−15048.0)	−59399.8 (−15148.0)	−36.0 (−34.7)	−17.8 (−41.5)

[a] See experimental section for the calculation procedure employed. [b] $\Delta E_{tot} = E_{tot}$ (spin adduct) − E_{tot} (spin trap). [c] $\Delta\Delta H_f = \Delta H_f$ (spin adduct) − ΔH_f (spin trap).

Table 3 Total energies (E_{tot} in kcal mol^{-1}) and heats of formation (ΔH_f in kcal mol^{-1}) in theoretically optimized nitrone spin traps[a]



	E_{tot}				ΔH_f		
Z	AM1	PM3	MNDO	*ab initio*	AM1	PM3	MNDO
–PO(OEt)$_2$ (DEPMPO)	−69577.9	−64630.3	−70493.6	−648228.0	−173.8	−170.3	−118.6
–CO$_2$*t*-Bu (BocMPO)	−61827.3	−57407.3	−62066.6	−413635.6	−75.5	−92.8	−61.4
–CH$_3$ (DMPO)	−33329.1	−30794.3	−33454.2	−224864.7	6.9	−9.3	−12.1
–CF$_3$	−65954.3	−60205.3	−65645.3	−408334.9	−140.0	−155.6	−124.0
–C(CH$_3$)$_3$	−44097.8	−41134.5	−44251.8	−297486.5	−1.3	−20.2	23.7

[a] See experimental section for the calculation procedure employed.

The $\Delta\Delta H_f = \Delta H_f$ (spin adduct) − ΔH_f (spin trap) values range from 32.7–40.3 and 33.4–43.7 kcal mol^{-1} using AM1 and MNDO, respectively. There is a reversal in order of increasing $\Delta\Delta H_f$ going from the AM1 to the MNDO method, but nevertheless, the significant differences in values for $\Delta\Delta H_f$ indicate the presence of a substituent effect during spin adduct formation.

Kinetics of superoxide radical adduct decay

Decay rates of BocMPO– and DEPMPO–$O_2^{\bullet -}$ adducts (see Fig. 3) are significantly slower than that of DMPO–$O_2^{\bullet -}$ adduct. These adducts were still persistent even after the light source had been shut off for 45 min while DMPO adduct peak intensity plateaued after less than 5 min. Decay plots were analyzed up to about 45 min. Peak intensities were normalized and plotted. The presence of carbon-centered radical was very minimal with about less than 10% formed after 4 min of irradiation and did not interfere with the low field peaks of BocMPO and DEPMPO (C-centered radical adduct: a_N = 14.93 G, a_H = 21.73 G; for BocMPO–$O_2^{\bullet -}$ adduct: 55% a_N = 13.41 G, a_H = 11.95 G and 45% a_N = 13.37 G, a_H = 9.43 G) based on spectral simulation. The Guggenheim method was employed in data analysis since final peak intensity at infinity time I_∞ is not known. Analyses of decay plots of BocMPO–$O_2^{\bullet -}$ adduct show that the first-order plot begins to deviate from linearity after 6.5 min, while the second-order plot also deviates from linearity. This behaviour is more pronounced for BocMPO–$O_2^{\bullet -}$ adduct at pH 8.2 and for DEPMPO–$O_2^{\bullet -}$ adduct at pH 7.0.

A complex mechanism is proposed in which BocMPO–$O_2^{\bullet -}$ adduct undergoes a pseudo-first order decay and disproportionation of the spin adducts to diamagnetic species. Therefore parallel first- and second-order reactions with a differential rate equation (1) of

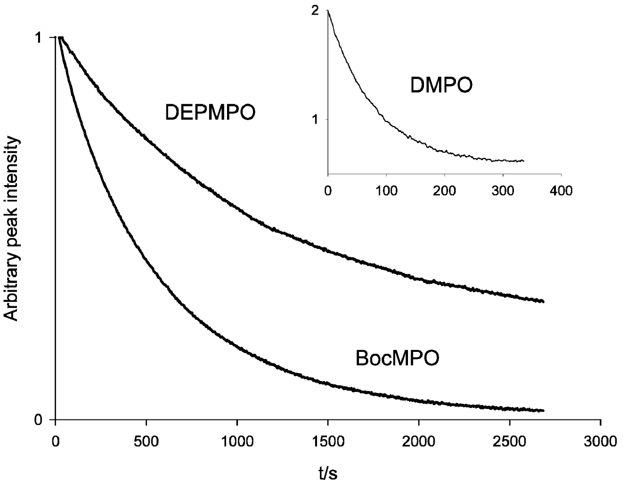


Fig. 3 Comparison of decay rates of superoxide adducts of 100 mM DEPMPO, BocMPO and 200 mM DMPO in phosphate buffer, pH 7.0 after 5 min of irradiation using light–(0.1 mM) riboflavin radical generating system.

$$-\frac{d[BA]}{dt} = k_1[BA] + 2k_2[BA]^2 \qquad (1)$$

were employed, where [BA] is the concentration of BocMPO–$O_2^{\bullet -}$ adduct, k_1 and k_2 are the first- and second-order rate constants, respectively. A coefficient of 2 was included in

Table 4 First-order approximation half-lives of nitrone–superoxide adducts

Spin trap	pH	$k_1/10^4\text{s}^{-1}$	$k_2[\text{BA}]_0/10^4\text{s}^{-1}$	$t_{1/2}/\text{s}^a$
BocMPO	5.6	7.70	4.5	897
	7.0	13.5	2.5	513
	8.2	14.4	3.2	481
DMPO	7.0	129.0		54
DEPMPO	7.0	8.13	2.2	852

[a] Based on the first-order rate constant and values are mean averages of 3 measurements. Standard deviation is 5–10% of the actual values.

the second-order expression to avoid ambiguity and to denote that two BocMPO–$O_2^{\bullet-}$ adducts undergo disproportionation reaction.

Integration of variables yields eqn. (2), where $[\text{BA}]_0$ is the initial spin adduct concentration.

$$[\text{BA}] = \frac{[\text{BA}]_0 k_1}{[k_1 + 2k_2[\text{BA}]_0]\text{e}^{k_1 t} - 2k_2[\text{BA}]_0} \qquad (2)$$

The decay plot was linear during 25–35 min of the decay using a pseudo first-order approximation. The resulting k_1 was used to fit eqn. (2) and to determine $k_2[\text{BA}]_0$. This method of determining k_1 was not applicable to the DEPMPO–$O_2^{\bullet-}$ adduct since only less than 70% of the initial spin adduct concentration had decomposed over the period of 45 min. Instead, a simultaneous fit of k_1 and $k_2[\text{BA}]_0$ was employed using eqn. (1). The k_1 for DMPO–$O_2^{\bullet-}$ was calculated based on a pseudo-first order model. Values for k_1 and $k_2[\text{BA}]_0$ are presented in Table 4. First-order decay of DEPMPO–$O_2^{\bullet-}$ adduct is pH-dependent which shows that decay is slowest in acidic conditions. Similar behaviour has been observed for the superoxyl adducts of DMPO[11] and DEPMPO[12] at various pH. The half-lives of 852 and 54 s for DEPMPO–$O_2^{\bullet-}$ and DMPO–$O_2^{\bullet-}$ adducts are of the same magnitude as the half-lives reported previously.[12,17,21]

These differences in spin adduct stability can be correlated with the calculated relative total energies E_{tot} and heats of formation ΔH_{f} (Table 3) of the superoxide spin adducts. The order of decreasing E_{tot} and ΔH_{f} is evident going from electron-withdrawing to electron-donating substituents. Although the actual mechanism of decay is not clear at the moment, the presence of electron-withdrawing substituents can significantly enhance the half-lives of superoxide spin adducts.

Conclusion

Superoxide radical trapping by BocMPO is significantly slower compared to DMPO and DEPMPO. The half-life of the $O_2^{\bullet-}$ adduct of BocMPO is much longer than that of DMPO and shorter than that of DEPMPO. These characteristics are consistent with theoretical calculations, indicating that the electron-withdrawing characteristic of the substituents at 5-C is important in enhancing the electronegativity of 2-C. Moreover, the large negative total energy of the minimized structure of the radical adducts of BocMPO and DEPMPO relative to DMPO may provide reasonable predictions on the stability of the spin adduct formed. Theoretical analysis on spin traps and their corresponding spin adducts can be a useful tool in the future design of nitrone spin traps.

Experimental

Nitrones

DEPMPO (OxisResearch, Oregon) and DMPO (Dojindo Labs, Japan) were used. The spin trap BocMPO was prepared based on the procedure described by Zhao *et al.*[15] All materials were used as purchased from Aldrich without further purification. A minor procedural modification was performed since addition of *tert*-butyl 2-nitropropionate to sodium ethoxide solution produced a thick slurry to which excess ethanol was added such that the mixture could be mechanically stirred. After 4 h of stirring the mixture was acidified with acetic acid until the cloudy mixture turned clear. Recrystallization in absolute ethanol yielded white crystals (1.1 g, 43% yield based on 3.5 g of dioxolane), mp 94–95 °C; δ_{H} (200 MHz; CDCl_3; Me_4Si) 1.49 (9 H, s, *t*-Bu), 1.67 (3 H, s, C(5)Me), 2.10–2.16 and 2.51–2.59 (2 H, m, C(4)H), 2.80–2.64 (2 H, m, C(3)H), 6.98 (1 H, s, C(2)H).

EPR Measurements

EPR measurements were carried out on a Bruker EMX X-band spectrometer with HS resonator at room temperature. General instrument settings are as follows unless otherwise noted: microwave power, 10 mW; modulation amplitutude, 1.0 G; receiver gain 3.17–3.56 × 10^5; for field sweep: scan time, 168 s; time constant, 328 ms; for time scan: scan time 335–2680 s; time constant, 1310 ms. Measurements were performed in an AquaX flow-through sample cell.

Competitive spin trapping

All kinetic experiments were performed using Dulbelcco's phosphate buffered saline (Gibco) containing 0.1 mM diethylenetriaminepentaacetic acid (DETPA). Superoxide dismutase (SOD) (Sigma) is from bovine erythrocytes with activity of 3820 units/mg protein. All solutions were bubbled with oxygen gas prior to the addition of riboflavin. The sample cell was irradiated with a 150 W light source positioned 12 cm away from the sample cavity. DEPMPO was used as a spin trap in competition with DMPO and BocMPO. In a typical competitive spin trapping experiment, 200 μL of the nitrone mixture contains a constant 100 mM DEPMPO, 0–300 mM DMPO or BocMPO and 25 μM riboflavin. For competition involving SOD, concentrations ranging from 2–10 nM and 20–60 nM SOD (based on m.w. of 32,000 D)[22,23] were used against BocMPO and DEPMPO, respectively. The mixture was transferred to a 1 mL syringe and injected into the AquaX sample cell. Kinetic measurments began when the power source was turned on. Growth of the low-field peak was monitored as a function of time, over a period of 168 s. All data were taken from duplicate or triplicate measurements.

Decay kinetics

Buffer pHs were adjusted using 1 M solutions of NaOH or HCl. In a typical decay kinetic study, 0.4 mL contained 100 mM of the nitrone and 0.1 mM riboflavin. The mixture was injected into the sample cell and irradiated for 4 min. The low-field peak decay was monitored as a function of time over a period of 2680 s after the light power source was turned off.

Theoretical calculations

Theoretical calculations were carried out using a HyperChem version 6.0 program (Hypercube Inc., Gainesville, FL). Atomic net charge and total energies were estimated using AM1, PM3 and MNDO semi-empirical quantum chemistry methods. Additional estimates were performed at the *ab initio* level with a minimum basis set of STO-3G.[24] The Polak–Rebiere (conjugate gradient) algorithm method was employed for all calculations at an RMS gradient of 0.1 kcal Å^{-1} mol^{-1} and convergence criterion of 0.01 kcal mol^{-1}. Singlet and doublet state calculations were performed on nitrone spin traps and their corresponding superoxide spin adducts, respectively. Net charges were compared as well as the total potential energy of each nitrone. Cartesian coordinates of minimized structures of both the nitrones and their corresponding superoxide spin adducts can be obtained from the authors.

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