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NMR and EPR studies of the reaction of nucleophilic addition of (bi)sulfite to the nitrone spin trap DMPO

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The reaction of the nitrone spin trap 5,5-dimethylpyrroline-*N*-oxide (DMPO) with sodium (bi)sulfite in aqueous solutions was investigated using NMR and EPR techniques. Reversible nucleophilic addition of (bi)sulfite anions to the double bond of DMPO was observed, resulting in the formation of the hydroxylamine derivative 1-hydroxy-5,5-dimethylpyrrolidine-2-sulfonic acid, with characteristic ^{1}H and ^{13}C NMR spectra. The reaction mechanism was suggested and corresponding equilibrium constants determined. The mild oxidation of the hydroxylamine results in the formation of an EPR-detected spectrum identical with that for the DMPO adduct with sulfur trioxide anion radical. The latter demonstrates that a non-radical addition reaction of (bi)sulfite with DMPO may contribute to the EPR detection of $SO_3^{-\bullet}$ radical. This possibility must be taken into account in spin trapping analysis of sulfite radical. Copyright © 2003 John Wiley & Sons, Ltd.

KEYWORDS: NMR; ^{1}H NMR; ^{13}C NMR; EPR; free radicals; spin trapping; nitrones; sulfite-derived radicals

INTRODUCTION

Sulfites are widely used as preservatives in food industry and play an important role in metabolic processes.[1–3] The main metabolic pathway of sulfite metabolism *in vivo* is based on the function of the mitochondrial enzyme sulfite oxidase, present largely in the liver and lung tissues and responsible for a two-electron oxidation of sulfite to sulfate.[4] The formation of free radical intermediates, particularly sulfur-centered $SO_3^{-\bullet}$ and oxygen-centered $SO_4^{-\bullet}$ radicals, during sulfite metabolism via one-electron oxidation has been proposed to contribute to the mechanisms of its toxicity.[5–7] While electron paramagnetic resonance (EPR) spectroscopy provides an approach for the direct detection of $SO_3^{-\bullet}$ paramagnetic radical anion,[8] this approach is rarely possible in biological systems owing to the short lifetime and low stationary concentration of the radical.[9] Therefore, EPR spin trapping is a commonly used method for the detection of $SO_3^{-\bullet}$ radical, using mostly nitrone spin traps, such as 5,5-dimethylpyrroline-*N*-oxide (DMPO)[6,7,10–16] and its phosphorus-containing analog, 5-(diethoxy-phosphoryl)-5-methyl-pyrrolidine-*N*-oxide (DEPMPO).[17–20] However, it has been shown that some nucleophilic agents can directly react with nitrone spin traps, forming corresponding hydroxylamines.[21–23] These compounds, in turn, can be easily oxidized, forming radical adducts which are identical with those formed by genuine spin trapping, the process termed the Forrester–Hepburn mechanism.[21] Moreover, we recently observed the reaction of nucleophilic addition of (bi)sulfite [the term (bi)sulfite is generally used to refer to both sulfite and bisulfite species] to the DEPMPO spin trap, with the formation of hydroxylamine compounds, which in turn are easily oxidized to DEPMPO/$SO_3^{-\bullet}$ radical adduct.[24] Therefore, $SO_3^{-\bullet}$ detection using DEPMPO may involve non-radical addition reactions and its application requires additional controls to prove a radical mechanism of the adduct formation.

In this work, we observed a similar reaction of (bi)sulfite nucleophilic addition to another popular nitrone spin trap, DMPO. The mechanism is proposed and corresponding equilibrium constants were measured.

EXPERIMENTAL

Reagents

DMPO (5,5-dimethyl-1-pyrroline-*N*-oxide) was purchased from Sigma (St. Louis, MO, USA) and diethylenetriaminepentaacetic acid (DTPA) from Aldrich (Milwaukee, WI, USA).

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Sample preparations

Freshly prepared DMPO solution in D_2O, containing 0.2 mM DTPA, was bubbled with Ar during 1 h, then sodium sulfite was added. All solutions were titrated with HCl solution up to required pD value. For pD measurements an OP-211/1 laboratory digital pH-meter with a WTW SenTix 61 pH combination glass electrode was used. The pD value was calculated according to the equation[25] $pD = pH_{obs} + 0.4$, where pH_{obs} is the observed pH value. ^1H NMR and EPR spectra of the samples were registered. In the studies on sulfite concentration dependence, 0.2 M KCl was added to prevent variations in ionic strength.

^1H- and ^{13}C-NMR spectra

The NMR spectra were registered on Bruker Avance 200 spectrometer in 5 mm tubes.

ESR spectroscopy

ESR spectra were measured on a Bruker EMX spectrometer in a 0.1 ml quartz tube. The instrument conditions were as follows: 50 scans, modulation amplitude 0.5 G, time constant 0.64 ms, conversion time 10.24 ms, sweep width 120 G, sweep time 10.49 s and microwave power 0.625 mW.

UV spectrophotometry

UV spectra were registered on a Shimadzu UV-1202 UV–visible spectrophotometer in quartz cuvettes of 1.0 cm thickness. The kinetic decay of ferricyanide was determined by measuring the absorption at 420 nm.

RESULTS AND DISCUSSION

Figure 1 shows the ^1H NMR spectra of DMPO (a) and the reaction mixture of 35 mM DMPO and 100 mM Na_2SO_3 (b). The main difference between the spectra is the large shift of the signal of the N=CH proton from 7.22 to 4.05 ppm, which indicates disappearance of the double bond. The doublet–doublet splitting of this proton signal [Fig. 1b] can be explained by the non-equivalence of the protons of the C^3H_2 group in the observed reaction product (P). The existence of two signals from methyl groups (1.09 and 1.21 ppm) indicates non-equivalence of the C^6H_3 and C^7H_3 groups. The ^{13}C NMR shift of C^2 (see Appendix) is in the range of a carbon atom covalently bound with two heteroatoms.

The EPR spectrum of the reaction mixture of DMPO and sulfite prepared as described in Fig. 1(b) did not show any EPR signal (data not shown). The addition of 3 mM potassium ferricyanide to the reaction mixture containing 35 mM DMPO and 100 mM Na_2SO_3 results in the appearance of an EPR spectrum (Fig. 2) with hyperfine interaction constants, $a_N = 14.5$ G and $a_H = 16.06$ G, which coincide with the parameters of the radical adduct of DMPO with $SO_3^{-\bullet}$.[6,11] While this compound can be formed by genuine spin trapping of sulfite radical, the experiment shows that oxidation of the reaction product results in the formation of the same compound.

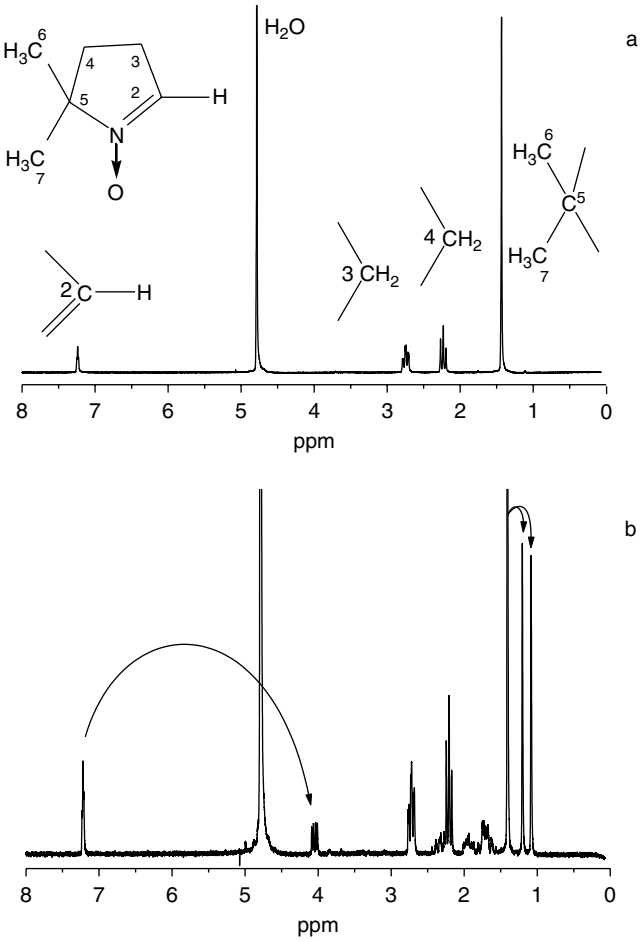


Figure 1. (a) ^1H NMR spectrum of DMPO in D_2O. (b) ^1H NMR spectrum of the reaction mixture containing 35 mM DMPO, 100 mM Na_2SO_3 and 0.2 mM DTPA. A solution of DMPO and DTPA in D_2O was bubbled with Ar for 1 h. After bubbling, solid Na_2SO_3 was added. The mixture was titrated with HCl to $pH_{obs} = 7.1$ and then the NMR spectrum was registered. The concentration of the product, P, was determined from the integral intensity of the C^6H_3 and C^7H_3 peaks. The arrows point to the shift of the signal of the N=CH proton from 7.22 ppm in the parent nitrone to 4.05 ppm in the product, and to appearance of two signals, at 1.09 and 1.21 ppm, from non-equivalent C^6H_3 and C^7H_3 groups in the product.

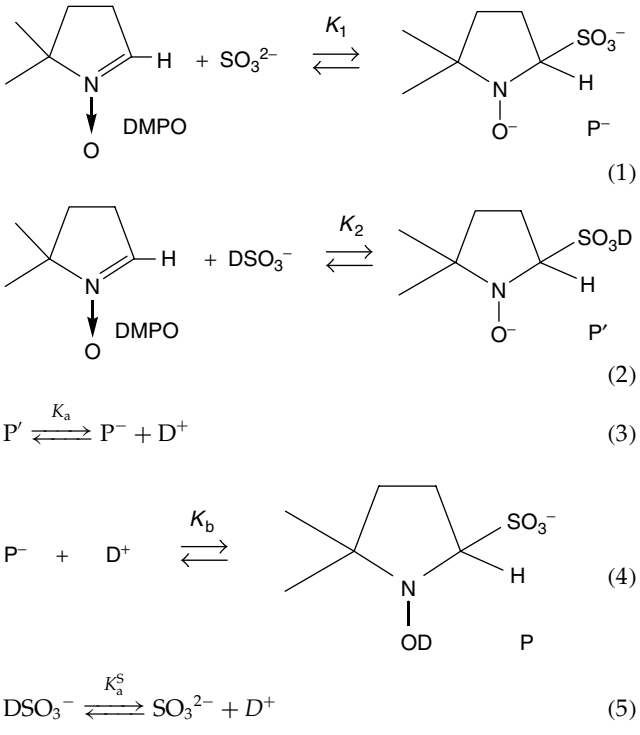
Figure 3 demonstrates the changes in the ^1H NMR spectrum of the reaction mixture containing 37 mM DMPO and 100 mM sodium sulfite after addition of benzaldehyde, which removes (bi)sulfite from reaction mixture. This leads to the disappearance of the signals of the reaction product P with a corresponding increase in the intensity of the DMPO peaks. Therefore, the reaction of DMPO with sodium sulfite is reversible, again in agreement with a similar conclusion for the DEPMPO spin trap.[24]

The strong dependence of the concentration of the reaction product P on the acidity of the solution was observed with decreases in [P] at higher pH_{obs} (Table 1).

Based on all the data above, a mechanism of the reaction of DMPO with (bi)sulfite is proposed similar to that for the reaction of DEPMPO with (bi)sulfite,[24] and is shown in

Figure 2. EPR spectrum of reaction mixture containing DMPO, 35 mM, sodium sulfite, 100 mM, and oxidant, $K_3Fe(CN)_6$, 3 mM in D_2O, 0.2 mM DTPA, $pH_{obs} = 7.1$. Spectrometer settings were as follows: microwave power, 0.625 mW; modulation amplitude, 0.5 G; sweep time, 10.49 s; number of scans, 50. Computer simulation of the spectrum gives the parameters $a_N = 14.5$ G and $a_H = 16.06$ G.

Eqns (1)–(5).



The reaction scheme considers three different ionization states of the DMPO adduct with sulfite, P^-, P' and P. The hydroxylamine derivative, P (1-hydroxy-5,5-dimethylpyrrolidine-2-sulfonic acid anion), is the predominant form ($pK_b \approx 10^{12}$–10^{14} l mol^{-1},[26] and degree of dissociation of sulfonic group, $\alpha \approx 1$[27]).

According to Eqns (1)–(5) the product of equilibrium constants, $K' = K_1K_bK_a^S = K_2K_bK_a$, can be calculated from experimentally measured parameters:

$$K' = K_1K_bK_a^S = K_2K_bK_a = \frac{K_a^S + [D^+]}{[D^+]} \frac{[P]}{([Na_2SO_3]_0 - [P])[DMPO]} \quad (6)$$

where [P] and [DMPO] are NMR-detected equilibrium concentrations of the reaction product P and DMPO spin trap, respectively; $[Na_2SO_3]_0$ is the initial sodium sulfite concentration; K_a^S is the acidity constant for the DSO_3^- anion; and K_a and K_b are acidity and basicity constants for corresponding hydroxylamine compounds in D_2O. Equation (6) explains the strong dependence of the product concentration on pH_{obs} (Table 1). Fitting of the experimental values to Eqn (6) shows good agreement between [P] values obtained experimentally and those calculated using $pK_a^S = 7.6$ and $K' = 18$ l mol^{-1} (see Table 1, $[D^+]$ values were calculated from pH_{obs} as described in the Experimental section). The observed difference between pK_a^S values of the HSO_3^- anion in H_2O (7.2)[28] and DSO_3^- in D_2O (7.6 ± 0.2) is in good agreement with the fact that for the most ionizable groups, pK values for ionization with loss of a deuteron in D_2O are higher by ~0.4–0.6 units than those for ionization of the same groups with loss of proton in H_2O.[29]

The value of K' (being a product of the equilibrium constants) must be independent of variations in the concentrations of D^+, DMPO and sulfite. This is in agreement with the data shown in Table 1. The values of K' were calculated using $pK_a(DSO_3^-) = 7.6$ and are in the range 18 ± 2 l mol^{-1}.

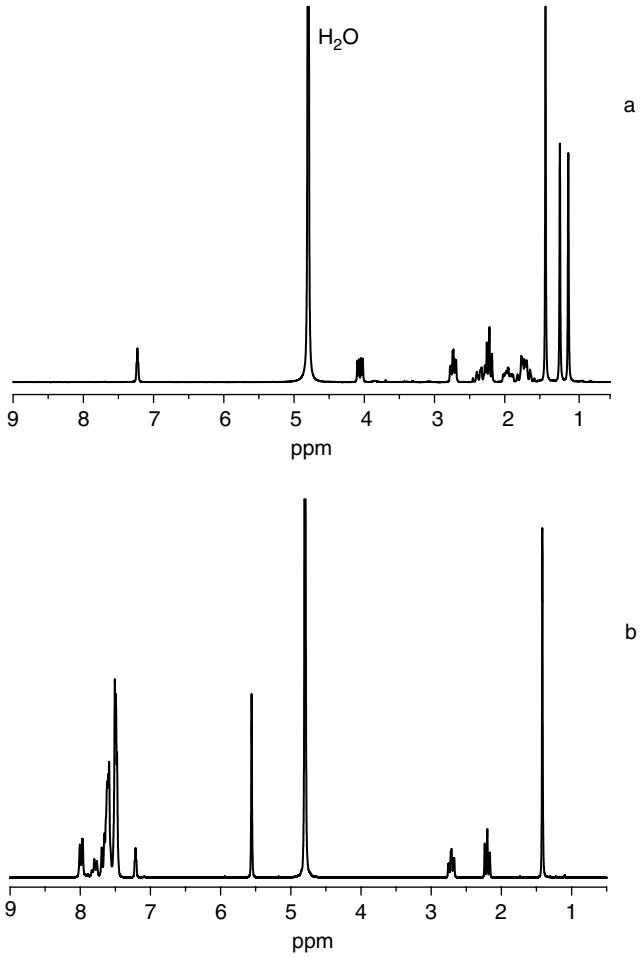


Figure 3. (a) ^{1}H NMR spectrum of reaction mixture containing 37 mM DMPO and 100 mM sodium sulfite in D_2O at $pH_{obs} = 6.62$ [cf. Fig. 1(b)]. (b) The same as (a) after addition of 190 mM benzaldehyde and titration of the reaction mixture to $pH_{obs} = 6.6$.

Table 1. Equilibrium concentrations of the product, P, and spin trap, DMPO, measured by ^{1}H NMR spectroscopy 1 h after the mixing of argon-bubbled solutions of 19 mM DMPO and 63 mM sodium sulfite in D_2O, 0.2 mM DTPA at 23 °C and various pH_{obs}

pH_{obs}	[P] (mM)	$[P]^a_{calc}$ (mM)	[DMPO] (mM)	$\log[K' (l\ mol^{-1})]^a$
8.07 ± 0.05	2.0 ± 0.1	2.0	17.0 ± 1.7	1.20 ± 0.23
7.61 ± 0.05	4.0 ± 0.2	4.1	15.0 ± 1.5	1.21 ± 0.20
6.90 ± 0.05	6.7 ± 0.3	6.8	12.3 ± 1.2	1.24 ± 0.13
6.60 ± 0.05	8.6 ± 0.4	8.5	10.5 ± 1.0	1.28 ± 0.07
6.10 ± 0.05	9.2 ± 0.5	9.3	9.7 ± 1.0	1.29 ± 0.03
5.61 ± 0.05	9.6 ± 0.5	9.6	9.4 ± 1.0	1.30 ± 0.02
5.09 ± 0.05	9.6 ± 0.5	9.7	9.4 ± 1.0	1.29 ± 0.01
4.64 ± 0.05	9.7 ± 0.5	9.7	9.3 ± 1.0	1.30 ± 0.01

[a] Calculated parameters were obtained by the fitting experimental data to Eqn (6).

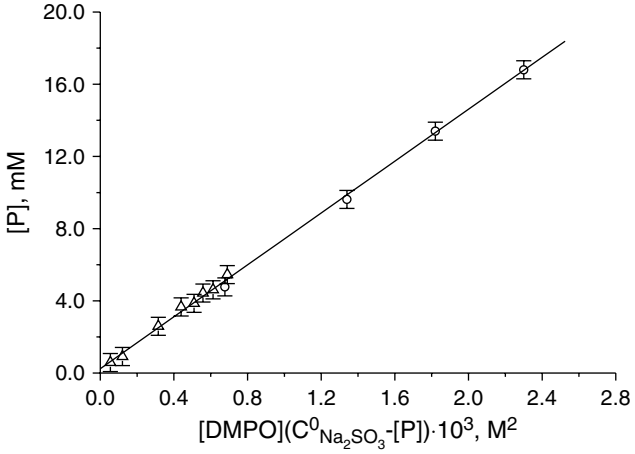


Figure 4. Dependences of the equilibrium product concentration, [P], on initial spin trap and sulfite concentrations. (△) Dependence of [P] on sulfite concentration; solution of 20 mM DMPO, 0.2 mM DTPA and 0.2 M KCl in D_2O was bubbled with Ar for 1 h and solid sodium sulfite was then added; (○) Dependence of [P] on DMPO concentration; solution of DMPO, 0.2 mM DTPA in D_2O was bubbled with Ar for 1 h and solid sodium sulfite, 100 mM, was then added. The samples were titrated with HCl to $pH_{obs} = 7.1$ and then ^{1}H NMR spectra were registered. The concentration of the product, [P], was determined from the integral intensity of the C^6H_3 and C^7H_3 peaks.

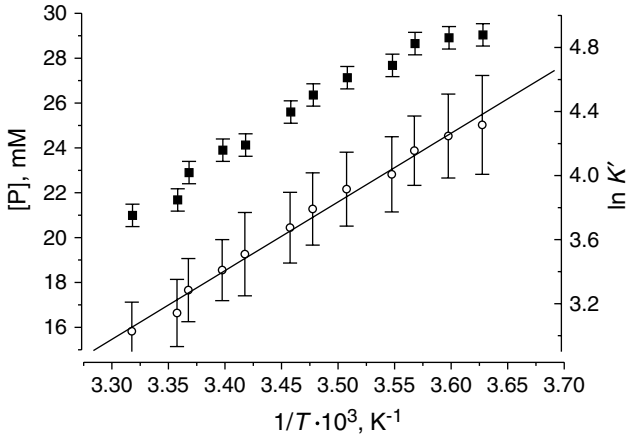


Figure 5. Dependences of equilibrium product concentration, [P] (■, left side), and constant K' (○, right side) on inverse of temperature. The experimental concentrations were as follows: DMPO, 37 mM; Na_2SO_3, 100 mM; DTPA, 0.2 mM; $pH_{obs} = 6.62$. The linear approximation for K' represents fitting of the experimental data to the equation $\ln K' = A + B(1/T)$, yielding parameters $A = -11.5 \pm 0.5$ and $B = (7.37 \pm 0.13) \times 10^3$ K.

Equation (6) predicts that the dependences of the NMR-detected product P on the initial concentrations of sodium sulfite and DMPO should be linear in coordinates of [P] versus $([DMPO]_0 - [P])([Na_2SO_3]_0 - [P])$. To confirm this statement, the dependences of the P yield on the initial concentrations of DMPO and sulfite were studied at $pH_{obs} = 7.1$ using ^{1}H NMR spectroscopy and the results are shown in Fig. 4. The observed linearization of the experimental dependences in the performed coordinates further supports the reaction scheme.

Figure 5 represents the temperature dependence of the experimentally measured concentration of the product P and constant K' calculated using Eqn (6). The dependence of $\ln K'$ on $1/T$ is in good agreement with linear fit, $\ln K' = A + B(1/T)$, with parameters $A = -11.5 \pm 0.5$ and $B = (7.37 \pm 0.13) \times 10^3$ K. Taking into account Eqn (6), we obtain:

$$\ln K_1 = \ln K' - \ln K_b K_a^S = -\frac{\Delta_{r1}H}{RT} + \frac{\Delta_{r1}S}{R}$$
$$\ln K_2 = \ln K' - \ln K_b K_a = -\frac{\Delta_{r2}H}{RT} + \frac{\Delta_{r2}S}{R} \quad (7)$$

where $\Delta_{r1}H$, $\Delta_{r2}H$ and $\Delta_{r1}S$, $\Delta_{r2}S$ are the standard enthalpies and entropies of reactions (1) and (2), respectively. These thermodynamic characteristics can be evaluated using the fitting parameters A and B:

$$\Delta_{r1}H = \Delta_{r2}H = -36.3 \pm 1.1 \text{ kJ}$$
$$\Delta_{r1}S = -95.5 \pm 4.7 \text{ J K}^{-1} - R \ln K_b K'_a \quad (8)$$
$$\Delta_{r2}S = -95.5 \pm 4.7 \text{ J K}^{-1} - R \ln K_b K_a^S$$

In the presence of an oxidizing agent, sulfite and DMPO, two processes, both genuine spin trapping and

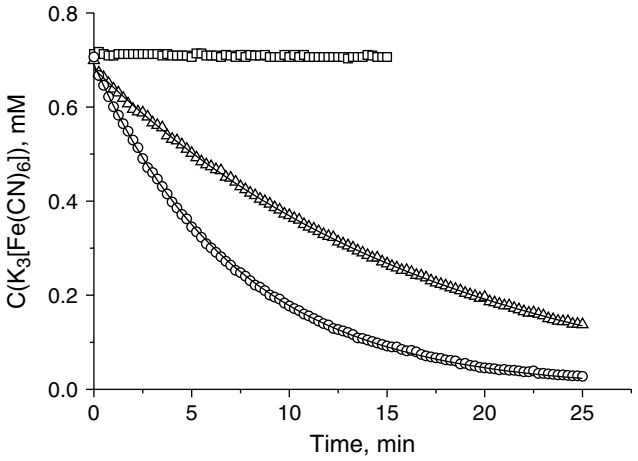


Figure 6. Decay of $K_3[Fe(CN)_6]$ in the reaction with sulfite in the presence and absence of DMPO in argon-bubbled phosphate buffer, pH 7.0 (0.5 mM DTPA). (□) 0.71 mM $K_3[Fe(CN)_6]$ and 14.3 mM DMPO; (△) 0.71 mM $K_3[Fe(CN)_6]$ and 4.84 mM Na_2SO_3; (○) 0.71 mM $K_3[Fe(CN)_6]$, 4.84 mM Na_2SO_3 and 14.3 mM DMPO. The absorption of ferricyanide at 420 nm was measured. Solid lines represent exponential fitting of the data yielding the characteristic time of the accumulation of the ferricyanide decay, $\tau_1 = 17.53 \pm 0.18$ min in the absence (△) and $\tau_2 = 7.37 \pm 0.02$ min in the presence (○) of DMPO.

oxidation of hydroxylamine P, may take place, resulting in the formation of the same radical adduct, DMPO–$SO_3^{-\bullet}$. To elucidate the contribution of these two pathways in the formation of the DMPO-$SO_3^{-\bullet}$ adduct (detected by EPR in the presence of potassium ferricyanide, Fig. 2), we measured the kinetics of the reduction of $K_3[Fe(CN)_6]$ by sulfite in argon-bubbled phosphate buffer, pH 7.0, in the presence and absence of DMPO (Fig. 6). Figure 6 shows that addition of DMPO significantly increases the rate of $K_3[Fe(CN)_6]$ reduction, which can be explained by the additional reaction of ferricyanide with the hydroxylamine P. The experimental kinetics in the absence and presence of DMPO show exponential curves with characteristic times $\tau_1 = 17.53 \pm 0.18$ min and $\tau_2 = 7.37 \pm 0.02$ min, respectively. Assuming that the formation of the product P in the reaction of DMPO with sulfite is much faster than its oxidation, we obtain

$$\frac{dC(Ox)}{dt} = -k_{f1}[Na_2SO_3]C(Ox) - k_{f2}[P]C(Ox) \qquad (9)$$

where $C(Ox)$ is the concentration of $K_3[Fe(CN)_6]$ and k_{f1} and k_{f2} are the rate constants of ferricyanide reactions with sulfite and product P, respectively. Taking into account that $[P] << [Na_2SO_3]$, [DMPO] {$[P] = 0.77$ mM from the estimate using Eqn (6) with $[D^+]$ substituted by $[H^+]$ and $K' \approx 18\,l\,mol^{-1}$}, we obtain the solution of the Eqn (9) in the exponential form:

$$C(Ox) = C_0(Ox)\exp\{-t(k_{f1}[Na_2SO_3] + k_{f2}[P])\} \qquad (10)$$

This approximation allows the estimation of the values of the rate constants of ferricyanide reduction by sulfite and hydroxylamine P as $k_{f1} = 0.2\,l\,mol^{-1}\,s^{-1}$ and $k_{f2} = 1.7\,l\,mol^{-1}\,s^{-1}$, respectively.

CONCLUSION

The reversible nucleophilic addition of (bi)sulfite to the double bond of the nitrone spin trap DMPO resulting in the formation of a hydroxylamine compound was observed. The assignment of the product and establishment of the mechanism of its formation were based on EPR and NMR data, and corresponding thermodynamic parameters of the reactions were obtained. Mild oxidation of the observed hydroxylamine results in the formation of nitroxide radical which is identical with the paramagnetic adduct formed by genuine EPR spin trapping of $SO_3^{-\bullet}$ radical by the DMPO spin trap. The results presented in this paper show the necessity of more careful analysis of $SO_3^{-\bullet}$ radical spin trapping data obtained in solutions containing sulfite, spin trap and an oxidant. Note that the redox potentials for sulfite anions are $E(SO_3^-/SO_3^{2-}) = 0.63$ V, $E(SO_3^-/HSO_3^-) = 0.84$ V vs NHE.[29] The redox potential values of the various hydroxylamines are in the range 0.3–1 V.[30,31] Therefore it is expected that the sulfite-oxidizing agent may oxidize the hydroxylamine product, P, interfering with genuine the EPR spin trapping experiment as was shown for ferricyanide.

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APPENDIX

Parameters of NMR spectra of 1-hydroxy-5,5-dimethylpyrrolidine-2-sulfonic acid (P)

C^{13} NMR

C^2: d, 79.35 ppm, J(C,H) = 149 Hz. C^3: 21.65 ppm, 134 Hz < J(C,H) < 136 Hz. C^4: t, 32.90 ppm, 132 Hz < J(C,H) < 138 Hz. C^5: 73.76 ppm. C^6: q, 24.63 ppm, 127 Hz < J(C,H) < 125 Hz. C^7: q, 17.06 ppm, 125 Hz < J(C,H) < 127 Hz.

^1H NMR

C^2H: d-d, 4.05 ppm. C^3H_2, C^4H_2: m, 1.5–2.5 ppm; C^6H_3, C^7H_3: 1.21 ppm, 1.09 ppm.