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## *Brief Communication*

# THE SPIN TRAPPING OF SUPEROXIDE WITH $M_4PO$ (3,3,5,5-Tetramethylpyrroline-*N*-oxide)

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**Abstract**—The spin trap 3,3,5,5-tetramethylpyrroline-*N*-oxide ($M_4PO$) reacts with superoxide to produce a short-lived spin adduct ($M_4PO/\cdot OOH$, $A_N$ = 14.0 G, $A_H$ = 6.5 G) that decays by a first-order process ($t_{1/2} \approx$ 35 s at pH 7.4). This short lifetime may limit its usefulness in studies of superoxide.

**Keywords**—Spin trapping, Superoxide, Free radicals, Hydroxyl radical, 3,3,5,5-Tetramethylpyrroline-*N*-oxide ($M_4PO$), Hydrated electron

## INTRODUCTION

Since the demonstration by Janzen and Liu[1] that DMPO is an effective spin trap, it has been used extensively in the biochemical/biomedical realm for the study of free radical metabolites. A great number of studies have dealt with the spin trapping of superoxide and/or hydroxyl free radicals by DMPO.[2] Unfortunately, the spin trapping of superoxide by DMPO has limitations due to the short lifetime of DMPO/·OOH ($t_{1/2} \approx$ 60 s, pH 7)[3] as well as the complicating reactions due to the presence of adventitious catalytic metals.[4] However, Janzen et al.[5] have reported that the spin adducts of the related nitrone $M_4PO$ are longer-lived than for DMPO. This would be a great advantage in the spin trapping of superoxide. We report in this communication that the previously reported hyperfine splittings for the superoxide spin adduct of $M_4PO$ ($M_4PO/\cdot OOH$) are in error, and, unfortunately, that the lifetime of $M_4PO/\cdot OOH$ is actually shorter than that of DMPO/·OOH, making it perhaps less useful than DMPO in studies of superoxide.

## MATERIALS AND METHODS

$M_4PO$, hypoxanthine, and xanthine oxidase were from Sigma. $M_4PO$ can develop a colored impurity much like that observed with DMPO.[3] This impurity appears to be a carbon-centered radical having $A_N$ = 16.03 G and $A_H$ = 24.50 G. As with DMPO, treatment with activated charcoal[3] was used, when needed, to remove this impurity. The concentration of the purified aqueous solution of $M_4PO$ was determined using $\epsilon_{228} = 1.01 \times 10^4\ M^{-1}cm^{-1}$.

The lifetime of $M_4PO/\cdot OOH$ was determined with the riboflavin-DETAPAC system as in ref. 3. Briefly, a solution containing 60 $\mu$M riboflavin, 100 mM $M_4PO$, and 1 mM DETAPAC in 50 mM pH 7.4 phosphate buffer was illuminated briefly, using a slide projector, directly in the ESR cavity. After cessation of illumination the height of the low field $M_4PO/\cdot OOH$ ESR signal was followed as a function of time. The X.O./hypoxanthine system was used to generate superoxide as in ref. 4. The Fenton system used 1 mM $H_2O_2$, 1 mM ferrous ammonium sulfate, and 50 mM $M_4PO$ in 50 mM pH 7.4 phosphate buffer to generate ·OH. When present, formate and DMSO were at 100 mM.

The photoionization of chlorpromazine, using a $N_2$-saturated solution for $e^-_{aq}$(H·) and $N_2O$-saturated solution for ·OH, was used to generate the $M_4PO/\cdot H$ and $M_4PO/\cdot OH$ spin adducts. One flash from a PRA FP-1000 flash photolysis system was used to irradiate the sample, which was then immediately examined by ESR. The photolysis solutions contained 200 $\mu$M $M_4PO$ and 29 $\mu$M CPZ in $N_2O$- or $N_2$-bubbled 100 mM pH 6.0 phosphate buffer.

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Adventitious metals were removed from the phosphate buffers with chelating resin (Sigma). In the demetaled buffer the loss of ascorbate was 0.3% or less in the standard test[6] indicating effective removal of catalytic metals. ESR spectra were recorded using either a Varian E-104 or Bruker ESP-300 spectrometer.

## RESULTS AND DISCUSSION

### *Hydroxyl radical and hydrogen atom*

To verify the assignment of the $M_4PO/\cdot OH$ spin adduct hyperfine splittings (Table 1), we generated $M_4PO/\cdot OH$ in a unique way, that is, via the photoionization of chlorpromazine:[7]

$$CPZ \xrightarrow{UV} CPZ^{+}\cdot + e_{aq}^{-}$$

In nitrogen-saturated solutions we would expect:

$$M_4PO + e_{aq}^{-} + H^{+} \longrightarrow M_4PO/\cdot H$$

and this was observed (Table 1), parallel to our observations with DMPO.[7] However, in $N_2O$-saturated solution we would expect:

$$e_{aq}^{-} + N_2O + H^{+} \longrightarrow \cdot OH + N_2$$

$$M_4PO + \cdot OH \longrightarrow M_4PO/\cdot OH$$

Indeed, when $N_2$ is replaced with $N_2O$, the $M_4PO/\cdot H$ adduct is completely suppressed and we observed only an $M_4PO$ adduct with hyperfine splitting constants consistent with those previously assigned to $M_4PO/\cdot OH$, Table 1. We observed the same adduct when a Fenton system or an iron supplemented X.O./hypoxanthine system was used to generate $M_4PO/\cdot OH$, Table 1. These results verify the previous assignment for $M_4PO/\cdot OH$.[5]

### *Methyl radical*

When ·OH reacts with DMSO the methyl radical is produced, which can then be spin trapped. This set of reactions and DMPO have been used as an indicator of ·OH production.[8] When using the Fenton system to generate ·OH in the presence of 100 mM DMSO (nitrogen-purged solution), the intensity of $M_4PO/\cdot OH$ was significantly decreased and a new six-line spectrum arose that we attribute to $M_4PO/\cdot CH_3$, Table 1. If the spin trapping solution was air-saturated, rather than $N_2$-purged, the methyl radical will react with oxygen yielding $CH_3OO\cdot$, which can be spin trapped. Indeed, in air-saturated solution we observed no $M_4PO/\cdot CH_3$, but rather a six-line spectrum that we attribute to $M_4PO/\cdot OOCH_3$, Table 1. Both $M_4PO/\cdot CH_3$ and $M_4PO/\cdot OOCH_3$ appear to be relatively stable as only a modest loss of intensity ($< \approx 15\%$ over 30 min) was observed.

### *Superoxide*

Janzen et al.[5] have made a tentative assignment for the hyperfine splitting constants of $M_4PO/\cdot OOH$ (see

Table 1. Hyperfine Splitting for Spin Adducts of $M_4PO$

| Radical | Solvent | $A^N$/G | $A^H$/G | How Produced | Ref. |
|---|---|---|---|---|---|
| $H\cdot$ ($e_{aq}^{-} + H^{+}$) | W(P6.0)* | 16.6 | 21.8 (2) | CPZ + UV light + $N_2$ | Here |
| $H\cdot$ | MeOH | 15.56 | 19.8 (2) | n-$Bu_3SnH$ | Ref. 5 |
| ·C? | W(P7.4) | 16.03 | 24.50 | Impurity in the $M_4PO$ | Here |
| $\cdot CH_3$ | W(P7.4) | 16.74 | 27.23 | Fenton + DMSO + $N_2$ | Here |
| $\cdot CH_3$ | W | 16.60 | 27.00 | 30% $H_2O_2$ + DMSO + UV light | Ref. 5 |
| $CO_2^{-}$ | W(P7.4) | 15.81 | 19.99 | Fenton + formate | Here |
| $CO_2^{-}$ | W | 15.71 | 19.85 | DBPO + formate | Ref. 5 |
| $\cdot OOCH_3$ | W(P7.4) | 14.63 | 9.11 | Fenton + DMSO + air | Here |
| ·OH | W(P6.0) | 15.3 | 16.8 | CPZ + UV light + $N_2O$ | Here |
| ·OH | W(P7.4) | 15.30 | 16.90 | Fenton system | Here |
| ·OH | W(P7.0) | 15.30 | 16.85 | Riboflavin-DETAPAC | Here |
| ·OH | W(P6.0) | 15.29 | 16.81 | Peroxydisulfate | Ref. 5 |
| ·OH | W(P6.0) | 15.28 | 16.73 | 1% $H_2O_2$ + UV light | Ref. 5 |
| ·OH | W(P7.8) | 15.3 | 16.5 | X.O./hypoxanthine | Ref. 11 |
| ·OOH | W(P7.4) | 14.03 | 6.44 | X.O./hypoxanthine | Here |
| ·OOH | W(P7.0) | 14.01 | 6.65 | Riboflavin-DETAPAC | Here |
| ·OOH | W(P7.8) | 13.7 | 7.5 | X.O./hypoxanthine | Ref. 11 |
| ·OOH† | W(P6) | 15.67 | 20.01 | 1% $H_2O_2$ + UV light | Ref. 5 |
| ·OOH‡ | W | 15.7 | 20.0 | | Ref. 9 |
| ·OOH‡ | W | 15.7 | 10.0 | | Ref. 10 |

*W (water) P (phosphate), pH 6.0.

†Tenative assignment in ref. 5 that is in error. We believe this is most likely $CO_2^{-}$ (or perhaps ·CHO) from the formalin in the commercial buffer used.

‡These quotations also appear to be in error.

Table 1). We believe these quotations are in error. Using the riboflavin/DETAPAC system and the X.O./hypoxanthine system, we have assigned quite different hyperfine splitting constants based on the parallel results observed in each system; specificity was assured by the ability of SOD to inhibit the signal. Our hyperfine splittings for $M_4PO$/·OOH are similar to those reported by Rosen and Turner[11] (see Table 1 and Fig. 1). This assignment is not confused by the $M_4PO$/·OH spin adduct because it was generated by a means that does not produce superoxide (see above).

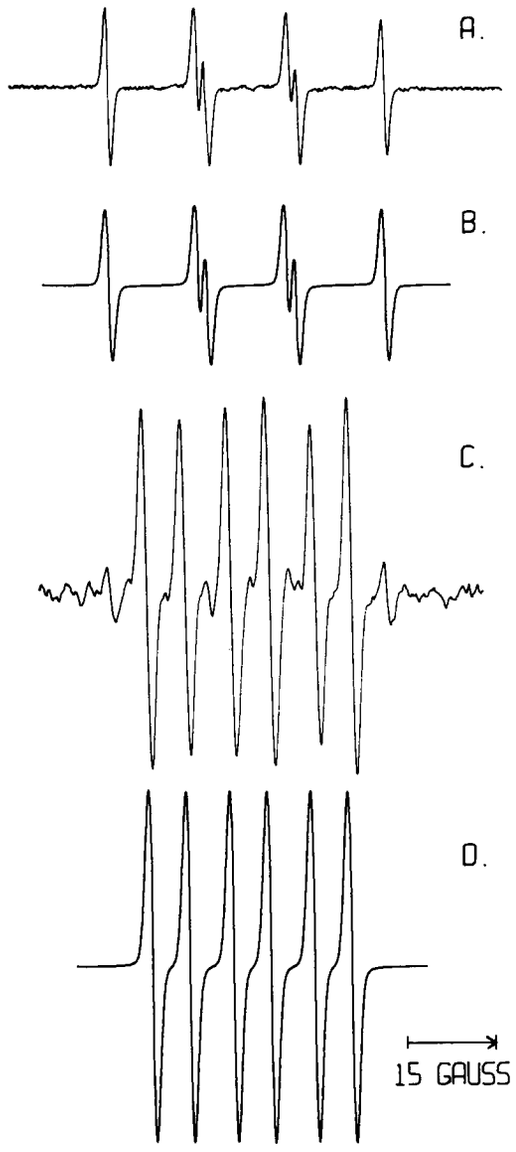


Fig. 1. ESR Spectra of $M_4PO$ adducts. (A) ESR spectrum of $M_4PO$/·OH produced by the Fenton system. (B) Simulation of $M_4PO$/·OH using $A_N$ = 15.30 G and $A_H$ = 16.90 G. (C) Spectrum of $M_4PO$/·OOH produced by the X.O.(12 mU/ml)/hypoxanthine (0.5 mM) system in air-saturated pH 7.4 phosphate buffer with 100 $\mu$M DETAPAC and 100 mM $M_4PO$. This signal was suppressed by SOD (50 U/ml), however catalase at 200 U/ml had no effect. A small amount of $M_4PO$/·OH can also be seen in this spectrum. (D) Simulation of $M_4PO$/·OOH using $A_N$ = 14.03 G and $A_H$ = 6.44 G. The Varian E-104 instrument settings were: Scan, 25 G/min; power, 20 mW; modulation amplitude, 1 G; time constant, 0.13 s (for A), 1 s (for C); receiver gain, 1.6 × $10^3$ (for A), 5 × $10^4$ (for C).

Figure 1c shows a typical $M_4PO$/·OOH spectrum generated by our X.O./hypoxanthine system. Note that a small amount of $M_4PO$/·OH is also present. Both signals are suppressed when SOD (50 units/ml) is included in the incubation. However, catalase (200 units/ml) does not affect the intensity of either signal. Thus, it appears that the $M_4PO$/·OH signal is not produced from the $H_2O_2$ generated by the X.O./hypoxanthine system, but rather it is a breakdown product of $M_4PO$/·OOH, parallel to what is often observed with DMPO/·OOH.[8]

Although Janzen et al.[5] report that $M_4PO$ spin adducts are in general longer-lived than DMPO spin adducts, this is unfortunately not the case for $M_4PO$/·OOH. Using the riboflavin-DETAPAC system,[3] we have observed that $M_4PO$/·OOH decays via a first-order process with $t_{1/2} \approx 35$ s at pH 7.4 (see Fig. 2). This lifetime is less than the $t_{1/2} \approx 50$ s observed for DMPO/·OOH at pH 7.4.[3] Furthermore, Rosen and Turner have reported that the rate of the reaction of $O_2^-/HO_2\cdot$ with $M_4PO$ is ten-fold slower than with DMPO.[11] Thus, the use of $M_4PO$ in superoxide studies

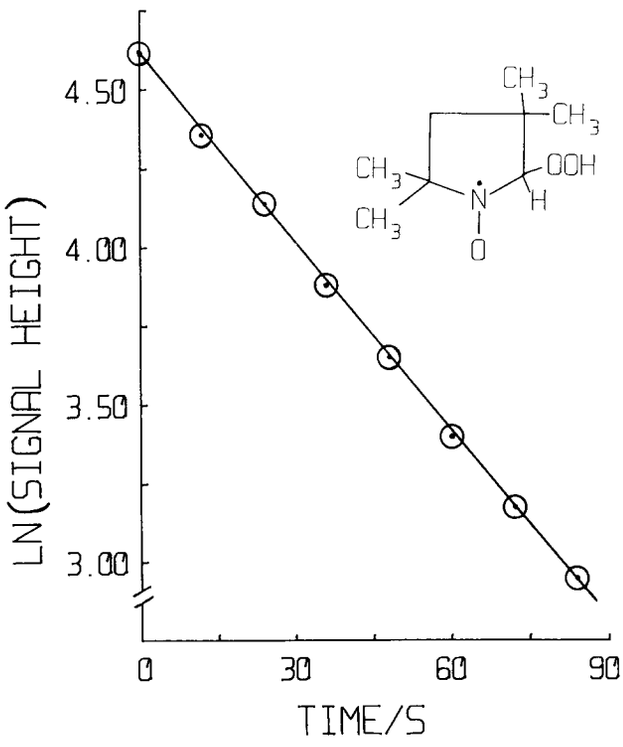


Fig. 2. First-order decay of $M_4PO$/·OOH generated using the riboflavin-DETAPAC system. See Materials and Methods. This curve demonstrates a first-order process with $T_{1/2}$ = 34.8 s, that is, $k$ = 1.99 × $10^{-2}$ $s^{-1}$.

will in general have more taxing limitations than presented by the use of DMPO. However, the increased lipophilicity of $M_4PO$ relative DMPO[11] could offer some potential advantage to $M_4PO$ usage when trying to spin trap superoxide at intracellular locations.

These results demonstrate that the $M_4PO/\cdot OOH$ spin adduct is even more fleeting than the DMPO/·OOH adduct. This shorter lifetime may limit its usefulness in studies of superoxide formation. In addition we have provided the appropriate hyperfine splittings for $M_4PO/\cdot OOH$ to aid researchers using $M_4PO$.

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## ABBREVIATIONS

CPZ—chlorpromazine
DBPO—di-*tert*-butylperoxalate
DETAPAC—diethylenetriaminepentaacetic acid
DMPO—dimethylpyrroline-*N*-oxide
DMSO—dimethylsulfoxide
$M_4PO$—3,3,5,5-tetramethylpyrroline-*N*-oxide
MeOH—methyl alcohol
SOD—superoxide dismutase
X.O.—xanthine oxidase