Spin Trapping with 5-Methyl-5-phenylpyrroline *N*-Oxide. A Replacement for 5,5-Dimethylpyrroline *N*-Oxide

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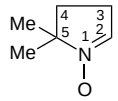
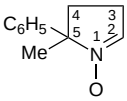
Received August 1, 1996

5,5-Dimethylpyrroline *N*-oxide (DMPO)[1] has become the most commonly used spin trap in biological systems to date. This is because the hydroxyl radical spin adduct gives a simple and distinctive EPR spectrum[2] consisting of four peaks with relative intensity 1:2:2:1, and the superoxide/hydroperoxyl radical spin adduct gives a different but also distinctive multiline EPR spectrum[2] composed of 6 major peaks plus additional partially resolved doublets. These unique signatures are readily recognizable in the presence of each other and thus provide a convenient approach to the study of systems where both the hydroxyl and superoxide/hydroperoxyl radicals are produced simultaneously. Moreover, since EPR spectroscopy is a very sensitive technique the spin trapping method is unequaled in its capability to detect low concentrations of these two species when *in situ* experiments can be arranged. It has been shown that DMPO is better than cytochrome *c* in detecting superoxide,[3,4] and alternate methods for detecting hydroxyl radical by salicylate or phenylalanine hydroxylation[5] also bring along with them problems associated with metabolic production of similar derivatives. Cytochrome *c* and salicylate are the "benchmark" methods for detecting superoxide and hydroxyl radicals, respectively.

However, a major concern in the use of DMPO is the presence of artifacts some of which appear to be due to compounds also produced in the synthesis of DMPO.[6] Small molecular weight nitrones are very reactive molecules and self-reaction is usually prevented by attaching substituents with sterically hindering groups or conjugation.[7,8] DMPO is relatively unprotected and contains no aryl groups. The melting point is low, and the colorless crystalline state is difficult to achieve. Most preparations result in a liquid which acquires a yellow color on standing. This product often contains (a) an EPR signal which confounds the analysis of an EPR spectrum,[6] (b) a toxic compound detrimental to the longevity of cells,[9] and (c) a "dormant" compound (perhaps a hydroxylamine) which exposes itself to EPR spectroscopy only after free radicals are formed.[10] For best results pure DMPO must be kept under N_2 in the dark and at dry ice temperature even during shipping, and used immediately after warming to room temperature.



DMPO
5,5-dimethylpyrroline *N*-oxide



MPPO
5-methyl-5-phenylpyrroline *N*-oxide

We have synthesized a DMPO replacement which gives similar hydroxyl radical and superoxide/hydroperoxyl radical spin adduct signatures as DMPO but which can be readily prepared in pure form and appears to be stable under normal shelf-life conditions indefinitely. Here we describe the first synthesis of 5-methyl-5-phenylpyrroline *N*-oxide (MPPO). Derivatives of DMPO for the purpose of spin trapping have been synthesized before, but all of them suffer from serious drawbacks ranging from complex EPR spectra of spin adducts to expensive pathways for synthesis. Moreover, simply placing a phenyl or methyl group in the 3- or 4-position leaves the 3- or 4-tertiary carbon−hydrogen bond vulnerable to reaction with the same radicals the nitronyl function is designed to trap. MPPO has the carbon position on carbon-5 protected with a methyl group.

The prime reason for seeking improvements in spin trapping methodology is for use in biological applications.[11] Although *in vivo* detection of free radicals by magnetic resonance is making progress,[12] there is still a problem with sensitivity. The sensitivity of these methods is directly dependent on the number of EPR lines attributed to any spin adduct. Thus, the most desirable spin traps are those which give the least number of EPR hyperfine lines and which produce the most persistent spin adducts with no artifacts.[6]

The approach we have used to synthesize MPPO is a textbook preparation of synthon I, namely, 2-methylpyrroline *N*-oxide by Michael addition of nitromethane to methyl vinyl ketone followed by reductive cyclization with zinc:

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(1) Janzen, E. G.; Liu, J. I.-P. *J. Magn. Reson.* **1973**, *9*, 510.
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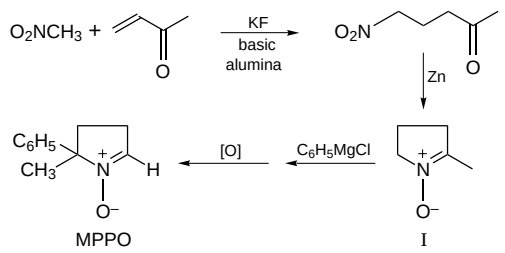
S0022-3263(96)01476-4 CCC: $14.00



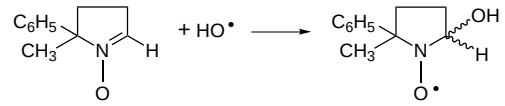
Addition of phenyl magnesium chloride to I followed by oxidation of the produced hydroxylamine with cupric acetate provides MPPO in an overall yield of 28%. With a melting point of 68 °C, MPPO is easier to purify than DMPO (mp 25−30 °C).

A key point in the synthesis of MPPO is the temperature of Grignard addition to I.

If addition is performed at room temperature most EPR spectra of MPPO spin adducts contain a major impurity signal consisting of a 14 G triplet. This artifact is completely removed by low-temperature addition of Grignard, and final purification of MPPO by sublimation.

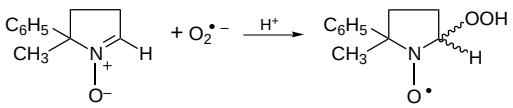
The importance of removing *all* EPR detectable impurities or precursors to EPR detectable impurities cannot be underestimated. Most literature references to the preparation of nitrones describe methods of synthesis which are rarely tested by EPR. Persistent nitroxides are always produced in these schemes and never removed because the concentrations are too low to influence the outcome of the usual organic synthesis. EPR spectrometers have high sensitivity, and good spin adduct spectra can be obtained from 1×10^{-7} M solutions of 0.5 mL volume samples. If the nitrone is typically 10−100 mM in concentraion a 0.001% EPR active impurity can give a spectrum intensity equal to that of the spin adduct of interest. Commercial sources of DMPO have failed to recognize this problem, and hence the large number of reported artifactual determinations of false spin trapping results.[6]

Typical hydroxyl radical generating systems produced a 1:2:2:1 EPR spectrum (since $a_N = a_{\beta-H}$) assigned to the HO• adduct of MPPO (see Figure 1).



At this time we do not know whether the 1:2:2:1 pattern is due to a mixture of diastereoisomeric spin adducts where each isomer happens to give the same hyperfine splitting constants, and hence the same EPR spectral pattern, or whether only one isomer (hydroxyl group *cis* or *trans* to the phenyl group) is formed. For usual hydroxyl radical spin trapping experiments where only the question of detection is considered, knowledge of the stereochemistry of addition is not necessary.

The addition of superoxide to MPPO produces an EPR spectrum very similar to that of DMPO (see Figure 2).



In this case careful inspection indicates the presence of two EPR spectra. The stronger spectrum has EPR parameters very similar to those of DMPO: $a_N = 14.0$, $a_{\beta-H} = 12.1$, $a_{\gamma-H} = 1.1$ G. The weaker spectrum has a smaller β-H hyperfine splitting: $a_N = 14.1$, $a_{\beta-H} = 7.6$, $a_{\gamma-H} = 0.7$ G.

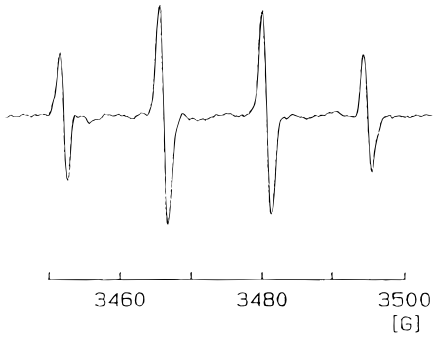


Figure 1. EPR spectrum of the hydroxyl radical spin adduct of MPPO produced in water from photolysis of 1% H_2O_2 in the presence of 50 mM MPPO.

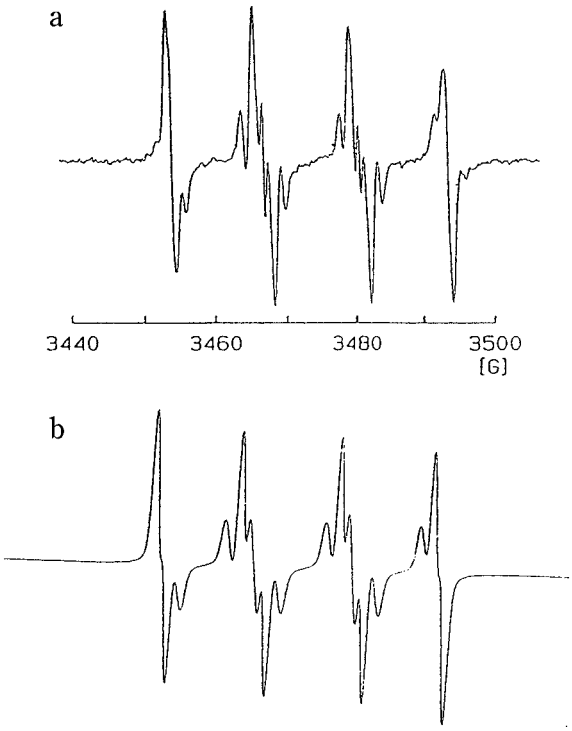


Figure 2. (a) EPR spectrum of hydroperoxyl/superoxide radical spin adduct of MPPO produced in water from photolysis of 30% H_2O_2 in the presence of 50 mM MPPO. (b) Computer simulation of the hydroperoxyl/superoxide radical spin adduct shown in a. Parameters: scan width 100 G; (component A) line width 1.1 G, relative amount 0.81, $a_N = 14$ G, $a_{\beta-H} = 12.1$ G, $a_{\gamma-H} = 1.1$ G; (component B) line width 1.2 G, relative amount 0.19, $a_N = 14.1$ G, $a_{\beta-H} = 7.6$ G, $a_{\gamma-H} = 0.7$ G.

In response to a reviewer's comment it is fair to say that the possible presence of diastereomeric spin adduct spectra does complicate the issue. The spectra are more complex and some sensitivity is lost. However, for some problems this feature may serve as an advantage in sorting out real spin trapping events from imposter spin adduct products. Other spin adduct spectra of MPPO have been described and illustrated elsewhere.[13]

Preliminary *in vivo* studies in the rat show that MPPO is successful at trapping trichloromethyl radicals produced in the liver metabolism of carbon tetrachloride. When $^{13}CCl_4$ is used the spectrum shown in Figure 3 is

(13) Janzen, E. G.; Sankuratri, N.; Kotake, Y. *J. Magn. Reson. B.* **1996**, *111*, 254−261.

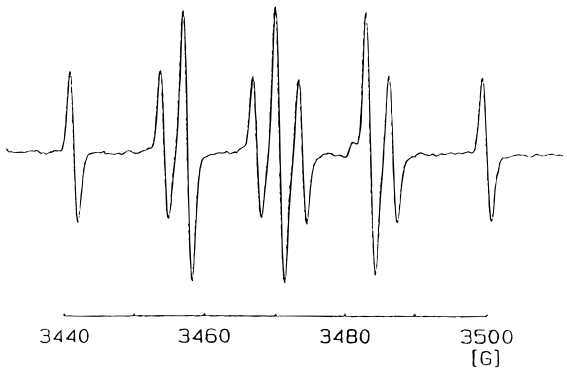


Figure 3. EPR spectrum in $CHCl_3$ obtained from Folch extraction ($CHCl_3/CH_3OH$ 2:1) of homogenized liver from a rat (226 g) subjected to $^{13}CCl_4$ (66.9 μL) and MPPO (27 mg) in 1 mL each of phosphate buffer (pH 7.5) and corn oil suspension by intraperitoneal injection after 1 h. The β-H and β-^{13}C hyperfine splittings are accidently equal: $a_N = 13.0$, $a_{\beta-H} = 16.3$, $a_{\beta-^{13}C} = 16.3$ in $CHCl_3$.

obtained from a chloroform extraction of homogenized liver. Because the hydrogen and β-^{13}C hyperfine splitting constant in the spin adduct are acccidentally equal the pattern becomes a 1:2:1 triplet of 1:1:1 triplets. It should be noted that DMPO is ineffective in trapping radicals from rat liver metabolism of CCl_4.[14]

Experimental Section

2-Methylpyrroline *N*-Oxide (2-MPO). To nitromethane (50 mL, 0.9 mol) and methyl vinyl ketone (10 mL, 0.1 mol) in 500 mL of dry THF was added 20 g of KF/basic alumina containing 3.5 mol equiv KF/g of alumina with vigorous stirring for 1.5 h. After the solution was filtered and washed with ethyl acetate, rotoevaporated, and distilled, 13.2 g (83% yield) of a colorless oil (5-nitro-2-pentanone) was isolated (bp = 37 °C at 0.03 Torr) and used in the next step. 5-Nitro-2-pentanone (10 g, 0.07 mol) and ammonium chloride (4.4 g, 0.82 mol) in 40 mL of water were cooled to −10 °C and treated with zinc dust added in small portions over 3 h with vigorous stirring (temperature less than 5 °C). After this solution was stirred (30 min) and filtered, washed with methanol, concentrated, extracted with $CHCl_3$, dried with $MgSO_4$, and rotoevaporated, the nitrone was isolated by distillation [(bp 56 °C at 0.02 Torr; 4.62 g (61% yield)]. ^{1}H-NMR (300 MHz, $CDCl_3$): δ 3.91 (2H, br t, J = 6.5 Hz, H-5), 2.69 (2H, br t, J = 6.7 Hz, H-3), 2.13 and 2.07 (2H, AB q, J = 7.7 Hz, H-4), 2.01 ppm (3H, s, methyl). ^{13}C-NMR (75 MHz, $CDCl_3$): δ 143.6 (C-2), 60.6 (C-5), 31.4, 15.0, 10.8 ppm.

5-Methyl-5-phenylpyrroline *N*-Oxide (MPPO). To a precooled (−20 °C) solution of a phenylmagnesium chloride (25 mL, 0.075 mol) in dry ether was added 2-MPO (4.0 g, 0.04 mol) in ether. After stirring, the reaction was quenched with wet ether followed by NH_4Cl solution. The $Mg(OH)_2$ was filtered and washed with chloroform, and the solvent was evaporated under reduced pressure to yield a yellow oil (4.1 g, 57%). This oil was treated with copper acetate (520 mg) in methanol (15 mL) containing aqueous NH_4OH and bubbled with air until the solution remained blue. After evaporation and dissolution in chloroform, the solution was chromatographed on a silica column and evaporated as a waxy solid. MPPO was isolated and sublimed at 40 °C (0.02 Torr), yield 2 g (28%) mp 68 °C. IR (thin film): ν_{max} 1573, 1501, 1447, 1375, 1276, 1233, 1193, 1072, 1025, 822, 765 cm^{-1}. ^{1}H-NMR (300 MHz, $CDCl_3$): δ 7.30 (5H, m, aromatic), 7.16 (1H, t, J = 2.4 Hz, H-2), 2.61 (2H, m, H-3), 2.50 and 2.36 ppm (2H, m, H-4). ^{13}C-NMR (75 MHz, $CDCl_3$): δ 141.7 (C-2), 133.9, 128.8, 127.7, 125.3, 79.2, 36.6, 24.6, 24.3 ppm. Anal. Calcd. for $C_{11}H_{13}NO$: C, 75.50; H, 7.50; N, 8.00. Found: C, 75.51; H, 7.56; N, 7.89.

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(14) Results from unpublished experiments with DMPO indicate variable outcomes in the *in vivo* metabolism of CCl_4 in the rat (work with Dr. J. L. Poyer and Ms. M. S. West in these laboratories). When DMPO is used, usually no spin adduct is obtained. Very occasionally a weak EPR signal is recorded but this result is not reproducable.