Novel DMPO-Derived ^{13}C-Labeled Spin Traps Yield Identifiable Stable Nitroxides

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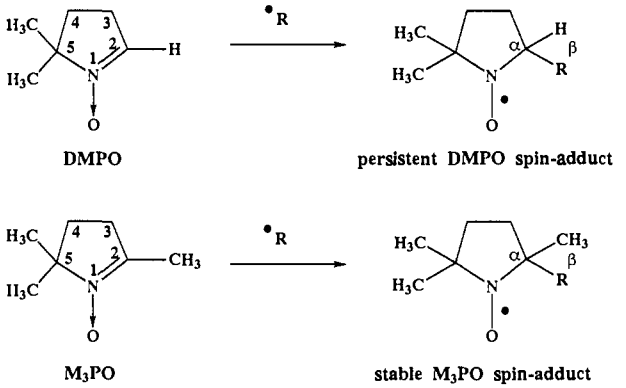
Abstract: The nitrone 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) is the most common spin trap used for studying free radicals, yet its spin adducts are rapidly and irreversibly destroyed by cells. A methyl substitution at the 2-position of DMPO results in the nitrone 2,5,5-trimethyl-1-pyrroline *N*-oxide (M_3PO). Radical addition to M_3PO is expected to produce stable spin adducts; however, they have almost the same N hyperfine splitting (hfs), and, in the absence of a β-hydrogen, different adducts are not distinguishable. To overcome this limitation, the synthesis of M_3PO labeled with ^{13}C at the nitronyl (C_2) or the 2-methyl (α or β to the aminoxyl group in the spin adduct, respectively) has been undertaken. [α-^{13}C]M_3PO was synthesized from [2-^{13}C]acetone in a three-step pathway while [β-^{13}C]M_3PO was obtained from DMPO and [^{13}C]iodomethane. For M_3PO, the nuclear magnetic moment of ^{13}C replaces that of the β-hydrogen of DMPO and provides the additional hfs necessary for spin adduct identification. Primary radicals, such as $^{\bullet}CH_3$, $^{\bullet}CO_2^-$ and $^{\bullet}OH$ were generated radiolytically, sonolytically, or enzymatically, trapped by [^{13}C]M_3PO, and gave rise to nitroxide spin adducts which were identified and their magnetic parameters determined. The [^{13}C]M_3PO spin adducts were far more stable than those of DMPO. Moreover, they were less susceptible to cellular-induced destruction. However, the superoxide adduct of M_3PO was unstable and did not persist.

Introduction

Accumulating evidence of the key role played by free radicals in chemical, biochemical, and cellular systems has prompted the search for a more direct observation of active transient species under both pathological and normal conditions. Much of the research has employed EPR spectrometry and concentrated on oxygen-centered radicals (hydroxyl, alkoxyl, peroxyl, superoxide) and carbon-centered radicals (alkyl, formate). However, the low steady-state concentration of short-lived transients and magnetic properties for hydroxyl ($^{\bullet}OH$) and superoxide ($O_2^{\bullet-}$), prevented direct detection. Therefore, an alternate indirect detection requires the use of C-nitroso and nitrone spin traps that yield secondary radicals, which persist longer, accumulate, and allow their detection and identification based on their hyperfine splitting (hfs) constants and *g*-values. The *C*-nitroso spin traps can induce toxic, carcinogenic, and mutagenic effects and are less suitable for studying biological systems.[1,2] 5,5-Dimethyl-1-pyrroline *N*-oxide (DMPO) is the most widely used spin trap for studying biological systems.[3] This spin trap effectively reacts with both oxygen-centered and carbon-centered radicals to yield persistent nitroxide spin adducts which are readily detectable and identifiable[4,5] (Scheme 1). The term "persistent" radical has been coined to describe spin adduct nitroxides having lifetimes ranging from seconds (DMPO/$^{\bullet}O_2H$)[6] to several minutes (DMPO/$^{\bullet}OH$)[7]. In contrast, when fully substituted at the α-positions, nitroxides persist indefinitely and are denoted "stable" radicals. Being paramagnetic and cell permeable, stable nitroxides, such as oxazolidinoxyl, piperidinoxyl, and pyrrolidinoxyl derivatives, are extensively used as biophysical markers to probe cellular metabolism, intracellular pH, oxygen level,[8] molecular mobility of proteins and lipids, and membrane structure.[9]

Scheme 1. DMPO, M_3PO, and Their Corresponding Spin Adducts



High concentrations of DMPO are commonly used for trapping superoxide in biological preparations[10–12] because (a) the trapping efficiency of DMPO for superoxide is extremely poor[13,14] and (b)

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it protects the spin adduct from superoxide.[15] Furthermore, most attempts to combine spin trapping by DMPO with EPR detection for studying intracellular free radicals proved unsuccessful for several possible reasons: (i) too low steady-state concentration of the intracellular radicals, (ii) site-specific generation of the radicals that renders them practically nonscavengable, (iii) insufficient level of the spin trap inside the cell, and (iv) the cell-induced rapid decay of the resulting spin adducts,[16] which is the predominant experimental constraint on the use of DMPO for detecting and studying free radicals in cellular systems. Contrary to stable nitroxides that persist inside the cells,[9] the spin adducts of DMPO (Scheme 1) are instantaneously and irreversibly destroyed.[16-18] Although there are many studies employing DMPO in cellular systems,[19,20] no valid correlation between signal intensity and flux of radicals was obtained.[21]

Further substitution of the nitrone as in 2,5,5-trimethyl-1-pyrroline *N*-oxide (M_3PO) should yield, upon trapping primary radicals, fully substituted adducts that are stable nitroxides rather than persistent (Scheme 1). Such adducts are anticipated to survive and be detectable inside the cell. However, without the β-hydrogen hfs, the discrimination between the various radical adducts is lost. To overcome the loss in spectral resolution, the synthesis of [^{13}C]M_3PO, labeled at the nitronyl (C_2) or the 2-methyl (α- or β-position to the nitrogen), has been undertaken. The basic rationale is that such novel spin traps will provide both the chemical *stability* and the *discriminative* magnetic features necessary for studying intracellular radicals. The use of isotopically substituted nitrones in the literature is limited to [^{15}N]- and polydeuterated DMPO, which improve the EPR sensitivity[22,23] but do not provide further chemical stability to the adducts. The use of ^{13}C enrichment in spin traps is limited to [*nitronyl*-^{13}C]-phenyl-*tert*-butylnitrone ([^{13}C]PBN)[24] and 2-phenyl-[*nitronyl*-^{13}C]DMPO,[25] although ^{13}C radical addends such as formate ($^{\bullet 13}CO_2^-$) are commonly used.[26]

The spin trapping properties of unlabeled M_3PO, although previously reported,[27] have not been studied extensively. The reason is that a large number of spin adducts of M_3PO exhibit very similar EPR spectra with $a_N \sim 15.5$ G and are hardly distinguishable.[28] Recently, its trapping efficiency for alkoxyl radicals was studied,[29] and its oxidation in the presence of ferric ions and the rate constant of its reaction with $^{\bullet}$OH were compared with those of DMPO.[30]

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Experimental Section

Materials. Catalase, hypoxanthine (HX), and xanthine oxidase (XO) (EC 1.2.3.2 xanthine, oxygen oxidoreductase) were from Calbiochem-Boehringer Co.; diethylenetriaminopentaacetic acid (DTPA) and superoxide dismutase (SOD) were obtained from Sigma; [2-^{13}C]acetone and [^{13}C]iodomethane were bought from Aldrich Chemical Co.; dimethyl sulfoxide (DMSO) was bought from Fisher. Sodium [^{13}C]formate (61% labeled) was purchased from MSD Isotopes. DMPO was kindly provided by Prof. Eli Breuer and distilled before use. The DMPO solution was purified with activated charcoal, filtered, and checked for absence of any EPR contaminating signals, and its concentration was spectrophotometrically determined using 8.00 $mM^{-1}\cdot cm^{-1}$ at 227 nm.[31] All other chemicals were prepared and used without further purification. Millipore deionized water was used throughout all experiments. Unless otherwise stated, the experiments were conducted at room temperature.

^{13}C NMR spectra (75.429 MHz) were obtained on a Varian VXR-300S (Palo Alto, CA) spectrometer using a 5-mm dual probe head, with broad-band decoupling. Spectra were obtained in deuteriochloroform and processed with a 3.0-Hz line broadening. Chemical shifts are reported in ppm using tetramethylsilane (TMS) as internal standard. ^{13}C-enriched signals were assigned as "strong" to differentiate them from ^{13}C naturally abundant signals. GC-MS spectra were determined on a Finnigan TSQ 700 instrument, using a DB-5 (30 m) column, by the Division of Identification and Forensic Science, Israel National Police, Jerusalem. Ionization was effected by electron impact (70 eV) or chemical ionization (CI) by methane. IR spectra were recorded on an Analect FTIR spectrometer (Analect Instruments FX-6160, Irvine, CA). Elemental analyses were performed by the Microanalytical Laboratory at the Hebrew University, Givat Ram, Jerusalem.

Synthesis of [α-^{13}C]M_3PO. 4-(Diethylamino)-[2-^{13}C]-2-butanone (I). Method A. To an ice-cooled solution of diiodomethane (2 mL, 25 mmol) and [2-^{13}C]acetone (1 g, 17 mmol) in 15 mL of tetrahydrofuran (THF) was added dropwise diethylamine (5.3 mL, 51 mmol) in 5 mL of THF. The mixture was heated under reflux for 3.5 h and then cooled and filtered, and the solid was washed several times with THF. The filtrate and washes were evaporated, and the residue was dissolved in 1 M HCl. Nonbasic components were extracted with petroleum ether (bp 40–60 °C). The aqueous phase was made alkaline with NaOH and saturated with K_2CO_3. Basic components were extracted with several portions of ether. The ether phase was washed with brine, dried over K_2CO_3, and evaporated to yield 2.25 g of brown liquid. This material was of sufficient purity as shown by NMR and GC-MS for use in the next step. ^{13}C NMR: δ 11.5 ($N(CH_2CH_3)_2$, s), 30.1 (C_1, d, J = 40 Hz), 41.3 (C_3, d, J = 40 Hz), 46.7 ($N(CH_2CH_3)_2$, s), 47.2 (C_4, s), 208.4 (C_2, strong s). IR (neat): ν 1360 (C—N str), 1713 (C=O str). MS (70 eV, m/z): 144 (M^+, 20), 129 (M^+-CH_3, 35), 86 (100).

Method B. A mixture of [2-^{13}C]acetone (0.75 mL, 10 mmol), paraformaldehyde (0.36 g, 12 mmol), and diethylamine hydrochloride (1.32 g, 12 mmol) in 30 mL of *N*,*N*-dimethylformamide (DMF), containing 2 drops of concentrated HCl, was maintained at 50 °C for 24 h. It was then ice-cooled, made alkaline with NaOH, and saturated with K_2CO_3. Basic components were extracted with several portions of ether. The ether phase was washed several times with water to remove DMF, dried over $MgSO_4$, and evaporated to yield 0.5 g of yellow liquid. This material was identical to the above from method A as shown by NMR.

5-Methyl-5-nitro-[2-^{13}C]-2-hexanone (II). 2-Nitropropane (28 mL, 0.31 mol) and the β-amino ketone I (2.2 g) were added to dry acetonitrile (50 mL), followed by dry KF (0.2 g, 3.4 mmol) and 18-crown-6 (0.24 g, 0.9 mmol). The mixture was stirred under reflux for 5 h. Following evaporation of the solvent, the mixture was partitioned between dichloromethane and 1 M HCl. The organic phase was washed with water and dried over K_2CO_3. Evaporation of dichloromethane and excess 2-nitropropane left 1.5 g of brown liquid. This material was of sufficient purity as shown by NMR and GC-MS for use in the next step. ^{13}C NMR: δ 25.8 (C_6 and 5-Me, s), 29.9 (C_1, d, J = 40 Hz), 33.9 (C_4, s), 38.1 (C_3, d, J = 40 Hz), 87.3 (C_5, s), 206.3 (C_2, strong s). IR (neat): ν 1718 (C=O str), 1538 (NO_2 asym str), 1348 (NO_2 sym str). MS (CI, m/z): 161 [$(MH)^+$, 3], 114 [$(MH)^+-HNO_2$, 100]. MS (70 eV, m/z): 114 ($M^+ - NO_2$, 30).

2,5,5-Trimethyl-[2-^{13}C]-1-pyrroline *N*-Oxide (III) ([α-^{13}C]M_3PO). A sample of II (1.4 g) was stirred with an ice-cooled solution of ammonium chloride (0.66 g) in water (18 mL) while Zn dust (1.9 g, 29 mmol) was

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added portionwise. The mixture was stirred at room temperature for 2 h, filtered, and washed several times with boiling water. The filtrate and washings were evaporated; the residue was dissolved in dichloromethane, dried over K_2CO_3, evaporated to 1.1 g of brown liquid, and distilled to give [α-^{13}C]M_3PO (0.4 g, 25% overall yield from [2-^{13}C]acetone) as a colorless liquid, bp 52–54 °C/0.4 mmHg. ^{13}C NMR: δ 13.0 (2-Me, d, J = 49 Hz), 25.4 (5-Me_2, s), 29.0 (C_3, d, J = 45 Hz), 32.2 (C_4, s), 73.1 (C_5, d, J = 11 Hz), 141.0 (C_2, strong s).[32] IR (neat): ν 1600 (C=N str), 1240 (N—O str). MS (70 eV, m/z): 128 (M^+, 100), 113 (M^+–CH_3, 35). Anal. Calcd for $C_6{}^{13}CH_{13}NO \cdot {}^1/_2H_2O$: C, 62.01; H, 10.29; N, 10.21. Found: C, 62.32; H, 10.30; N, 10.29.

Synthesis of [β-^{13}C]M_3PO. 2,2-Dimethyl-5-([^{13}C]methyl)-pyrrolidin-1-ol (IV). A solution of freshly distilled DMPO (1.2 g, 10.6 mmol) in anhydrous diethyl ether (10 mL) was added dropwise to an ice-cooled and stirred solution of [^{13}C]methylmagnesium iodide [from [^{13}C]-iodomethane (2 g, 14 mmol) and magnesium (0.34 g, 14 mmol)] in anhydrous diethyl ether (10 mL). After complete addition, the mixture was stirred at room temperature for 30 min and then ice-cooled and saturated aqueous ammonium chloride solution (15 mL) was added dropwise. After saturation with sodium chloride, the aqueous layer was extracted with ether (4 × 25 mL). The dried (anhydrous magnesium sulfate) extracts were concentrated to a yellow liquid (0.9 g). This material was of sufficient purity as shown by NMR and GC-MS for use in the next step. ^{13}C NMR: δ 19.3 (2-CH_3, s), 19.8 (5-CH_3, strong s), 26.2 (C_4, s), 27.7 (2-CH_3, s), 34.3 (C_3, s), 59.2 (C_5, d, J = 41 Hz), 63.5 (C_2, s). IR (neat): ν 3340 (br, O–H intermolec str), 1460, 1378. MS (70 eV, m/z): 144 (M^+, 20), 129 ($M^+ - CH_3$, 35), 86 (100).

5,5-Dimethyl-2-([^{13}C]methyl)-1-pyrroline *N*-Oxide (V) ([β-^{13}C]M_3PO). To a solution of **IV** (0.9 g, 7 mmol) in absolute ethanol (10 mL) were added concentrated ammonia solution (2 mL, 25%) and copper diacetate hydrate (3 mg). The reaction mixture was aerated for 3 h until a permanent bluish color was obtained and no hydroxylamine was detected by TLC (visualized by ninhydrin). After evaporation, the 5 mL-residue was saturated with sodium chloride and extracted with dichloromethane (5 × 25 mL). The extracts were washed with concentrated ammonia solution (5 mL) and saturated aqueous sodium chloride (5 mL). The dried (anhydrous magnesium sulfate) solution was concentrated and distilled to give [β-^{13}C]M_3PO (0.54 g, 40% overall yield from DMPO) as a colorless liquid, bp 56–58 °C/0.5 mmHg. ^{13}C NMR: δ 13.0 (2-Me, strong s), 25.4 (5-Me_2, s), 29.0 (C_3, s), 32.2 (C_4, s), 73.1 (C_5, s).[32] IR (neat): ν 1600 (C=N str), 1240 (N—O str). MS (70 eV, m/z): 128 (M^+, 55), 114 (100). Anal. Calcd for $C_6{}^{13}CH_{13}NO \cdot {}^3/_4H_2O$: C, 60.04; H, 10.31; N, 9.88. Found C, 60.06; H, 10.45; N, 9.84.

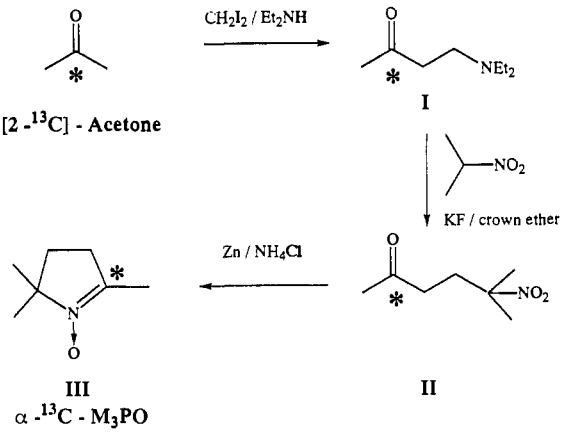
EPR Measurements. Samples were drawn into a gas-permeable, 0.8-mm-i.d. Teflon capillary. The capillary was inserted into a quartz tube and then placed within the EPR cavity. During the experiment, the sample within the spectrometer cavity was flushed with either air or N_2 without disturbing the sample. The EPR spectra were recorded on a Varian E9 X-band spectrometer, operating at 9.45 GHz, 100 kHz modulation frequency, 1 G modulation amplitude, and 10–20 mW microwave power. To study the decay kinetics of the spin adduct, the magnetic field was kept constant while the intensity of the EPR signal was followed. The concentrations of the spin adducts produced were quantitated using known concentrations of 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPOL) as a calibration standard. Spectral identification was further confirmed by simulating the EPR spectra based on the derived hfs assuming isotropic and coinciding g and A tensors.

Sonolysis Experiments. Each sample of 100 μL was drawn into a 10-cm-long gas-permeable Teflon capillary. One or several such capillaries were immersed in water inside a glass cell, which was placed in the center of a sonication bath (Bransonic 1200; 50 kHz frequency, 80.5 W input power). Argon was bubbled for 15 min through the water before and during sonication. The water level in the glass cell was adjusted to the same level of the water in the sonication bath, and the temperature was maintained at 25 °C. The sonication time was 15–30 min to achieve optimal spin adduct yield.[33]

Radiation Experiments. Before irradiation, 200-μL samples were gassed for 10–15 min with either N_2 or N_2O. The samples were irradiated at room temperature using a ^{60}Co γ-ray source at a dose rate of 11 Gy/min. The samples were scanned for EPR signal right after irradiation.

Chemiluminescence (CL) Experiments. Superoxide radicals were generated using aerated 40 mM phosphate buffer, pH 7, containing 2 mM HX, 50 μM DTPA, 0.1 mM to 0.3 mM lucigenin, 260 U/mL catalase, 10 mM linolenic acid, and 20 mU/mL XO at 25 °C, with or without gentle stirring. The experiments were started by adding XO, followed by additions of varying DMPO or M_3PO concentrations (5–50 mM). The rate of CL emission was measured by photon counting using a luminometer (Lumac/3M Biocounter M2010).[34,35]

Scheme 2. Synthetic Route for [α-^{13}C]M_3PO Spin Trap



Cells. The human lung A549 and breast MCF-7 adenocarcinoma cell lines were used to study the cellular persistence of the M_3PO spin adducts. Logarithmic-phase A549 human lung cells were harvested from monolayers shortly before the experiment, washed several times, suspended in RPMI medium, and let to settle down. The medium above the packed cells was discarded, and aliquots were taken and mixed with radiolytically preformed spin adducts. The suspension was placed in an aerated gas-permeable Teflon capillary, and the decay of the EPR signal of the spin adduct was monitored.

Results

Synthesis. [α-^{13}C]M_3PO was synthesized from [2-^{13}C]acetone according to Scheme 2. First, acetone was converted to the β-amino ketone I either by a classic Mannich aminomethylation with paraformaldehyde and diethylammonium chloride in a dipolar aprotic solvent[36,37] or, alternatively, by a modified reaction in which formaldehyde was replaced by diiodomethane.[38] β-Amino ketones as their quaternary salts and β-chloroketones are suitable precursors for α,β-unsaturated ketones. Compound I underwent elimination to methyl vinyl ketone, which *in situ* reacted with 2-nitropropane to give II in a Michael addition reaction catalyzed by potassium fluoride/crown ether in acetonitrile,[37,39] instead of the usual aqueous Triton B catalyst.[30,40] This strategy is advantageous since it avoids the preparation and purification of methyl vinyl ketone, which is unstable and tends to polymerize on standing. The γ-nitroketone II was reduced by zinc in aqueous ammonium chloride to the hydroxylamine intermediate and subsequently underwent cyclization to the nitrone [α-^{13}C]M_3PO (III).[27,30]

[β-^{13}C]M_3PO was synthesized according to Scheme 3 from DMPO by a Grignard reaction with [^{13}C]methylmagnesium iodide, which was generated from [^{13}C]iodomethane, giving the corresponding hydroxylamine **IV**. After catalytic oxidation of IV by copper(II), the final product [β-^{13}C]M_3PO (V) was obtained.[41]

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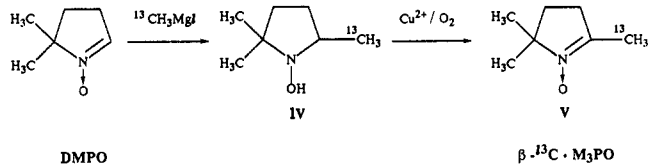
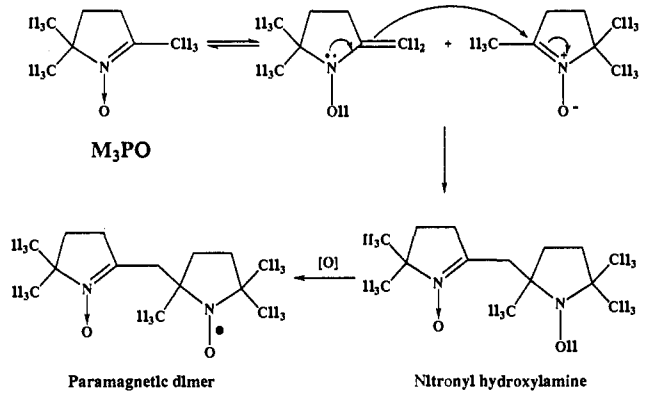
Scheme 3. Synthetic Route for [β-^{13}C]M_3PO Spin Trap



Scheme 4. Proposed Mechanism for the Paramagnetic Impurity Formation[42]



Like DMPO, both [α-^{13}C]- and [β-^{13}C]M_3PO are hygroscopic, and photoirradiation might form paramagnetic contaminants. Consequently, [α-^{13}C]- and [β-^{13}C]M_3PO were protected from light and stored at −20 °C, either neat or diluted in water.

Spin-Trapping and EPR Measurements. A. Carbon-Centered Radicals. (i) Paramagnetic Contaminant. A thoroughly purified solution of either [α-13C]- or [β-^{13}C]M_3PO contained paramagnetic contaminants, as reported earlier[42,43] (Scheme 4). When a solution of 0.1 M phosphate buffer, pH 7, containing either [α-^{13}C]M_3PO or [β-^{13}C]M_3PO (40 mM) was scanned for EPR signal, six-line and nine-line signals were observed, respectively (Figure 1). Because the six-line signal observed with [α-^{13}C]M_3PO has hfs similar to those of the spin adduct [α-^{13}C]M_3PO/•OH (see below), it could not be unequivocally attributed to the dimeric impurity. However, in the case of the [β-^{13}C]M_3PO, the nine-line signal could be readily attributed to the previously reported dimeric paramagnetic impurity (Scheme 4). The nine-line spectrum exhibited a primary nitrogen hfs (a_N) and two identical carbon hfs ($a_{C\text{-}13}$) as described in Table 1 (one $a_{C\text{-}13}$ for each monomer). This nine-line spectrum confirms that the main impurity of M_3PO is not chemically identical to M_3PO/•OH. Fresh distilled samples of [β-^{13}C]M_3PO showed as low as 10 ppm of the paramagnetic dimeric impurity, but its concentration increased up to 0.1% in old samples.

(ii) Formate Radical Adduct. When an N_2O-saturated 0.1 M phosphate buffer, pH 7, containing 0.1 M sodium formate and 40 mM of either [α-^{13}C]- or [β-^{13}C]M_3PO was γ-irradiated and monitored for EPR signal, the spectra shown in Figure 2a and c were observed. The signal intensity increased with the increase of the deposited dose. The spectra exhibited a primary nitrogen hfs (a_N) and a carbon hfs ($a_{C\text{-}13}$), as summarized in Table 1. The value of a_N agrees with that previously published for unlabeled M_3PO.[30] With N_2O and in the absence of oxygen, the predominant radical is $^{\bullet}CO_2^-$, which yields the spin adduct [^{13}C]M_3PO/$^{\bullet}CO_2^-$. To ensure that the observed signal indeed results from the formate radical adduct of [^{13}C]M_3PO, the experiment has been repeated using ^{13}C-labeled formate (61% abundance). Figure 2b displays a typical EPR spectrum, which can be analyzed as a superposition of two different species: one has a six-line spectrum and the second has a 12-line spectrum. The six-line spectrum is attributable to [α-^{13}C]M_3PO/$^{\bullet}$$^{12}CO_2^-$, and the 12-line spectrum corresponds to [α-^{13}C]M_3PO/$^{\bullet}$$^{13}CO_2^-$. The 12-line spectrum is characterized by a primary nitrogen hfs (a_N) and two ^{13}C hfs constants ($a_{\alpha\text{-}C\text{-}13}$ and $a_{\beta\text{-}C\text{-}13}$) corresponding to α-^{13}C-M_3PO and $^{\bullet}$$^{13}CO_2^-$, respectively (Table 1).

Because formate radical is a reducing species, it can be expected to reduce part of the spin adducts during irradiation to the corresponding hydroxylamine. Indeed, after the irradiation was complete a gradual increase of the signal intensity of [^{13}C]M_3PO/$^{\bullet}CO_2^-$ was observed during the measurement within the EPR

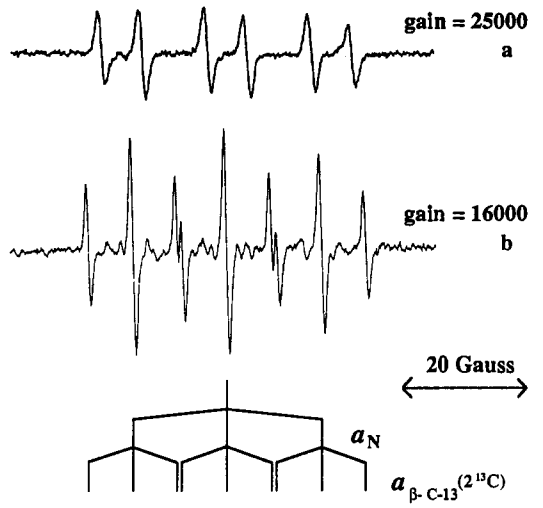


Figure 1. EPR spectra of [^{13}C]M_3PO paramagnetic contaminant. The EPR signal of un-irradiated 0.1 M aerobic phosphate buffer, pH 7, containing (a) 40 mM [α-^{13}C]M_3PO or (b) 40 mM [β-^{13}C]M_3PO. The instrument settings were 100 kHz modulation frequency, 1 G modulation amplitude, and 20 mW incident microwave power.

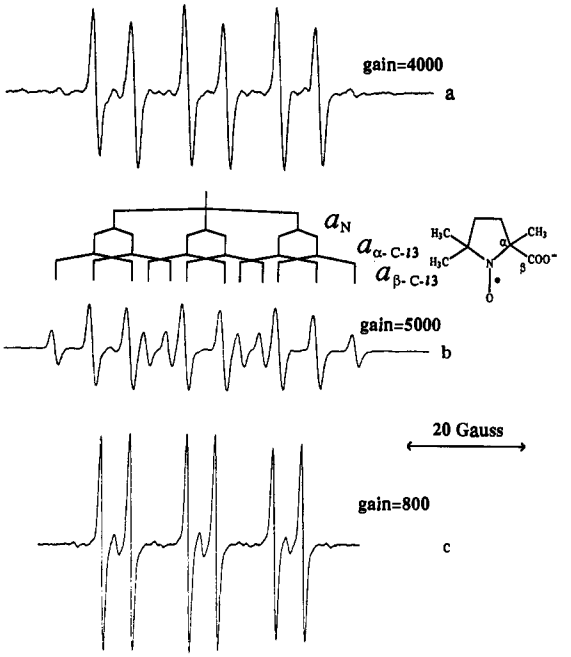


Figure 2. EPR spectra of [α-^{13}C]- and [β-^{13}C]M_3PO spin adducts of formate radical. The EPR signals were observed following γ-irradiation of N_2O-saturated 0.1 M phosphate buffer, pH 7, containing (a) 40 mM [α-^{13}C]M_3PO and 0.1 M sodium [^{12}C]formate, (b) 40 mM [α-^{13}C]M_3PO and 0.1 M sodium [^{13}C]formate (61%), or (c) 40 mM [β-^{13}C]M_3PO and 0.1 M sodium [^{12}C]formate. The instrument settings were 100 kHz modulation frequency, 1 G modulation amplitude, and 20 mW incident microwave power.

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Table 1. Hyperfine Splitting Constants for [α-^{13}C]- and [β-^{13}C]M_3PO Spin Adducts

radical addend	experimental conditions	[α-^{13}C]M_3PO		[β-^{13}C]M_3PO		a_N reported for [^{12}C]M_3PO
		a_N (G)	$a_{C\text{-}13}$ (G)	a_N (G)	$a_{C\text{-}13}$ (G)	
$^{\bullet}CO_2^-$	γ-irradiation, 0.1 M formate[a]	15.87	6.71	15.35	5.14	15.8[30]
$^{\bullet}OH$	sonolysis	15.87[b]	6.00[b]			15.9[c]
$^{\bullet}OH$	γ-irradiation	15.87[b]	6.00[b]	14.76	4.30	15.2[30]
$^{\bullet}CH_3$	γ-irradiation, 0.1 M DMSO	16.41	6.12[d]	15.88	7.18[d]	16.3[30]
e	untreated spin trap	15.80	6.00	15.30	7.25 (2^{13}C)	15.84[28]

[a] Upon irradiation of sodium [^{13}C]formate, an extra hfs constant ($a_{\beta\text{-}C\text{-}13}$ = 13.08 G) was obtained. [b] Due to the overlap with the contaminant, there is high uncertainty in the hfs values. [c] This signal was previously assigned to $M_3PO/^{\bullet}H$[30]; however, the lack of the β-hydrogen hfs constant supports the new assignment. [d] These hfs constants were reported from ^{13}C-satellites in toluene as $a_{\alpha\text{-}C\text{-}13}$ = 5.6 G and $a_{\beta\text{-}C\text{-}13}$ = 6.9G.[55] [e] Paramagnetic dimeric contaminant.

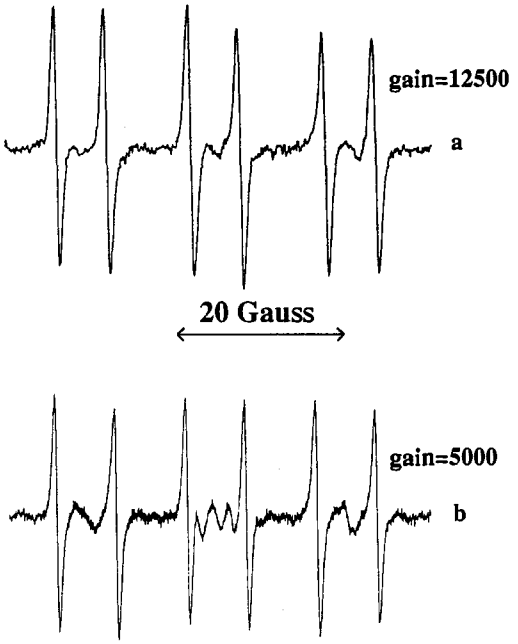

Figure 3. EPR spectra of [α-^{13}C]- and [β-^{13}C]M_3PO spin adducts of methyl radical. The EPR signal of the methyl adduct was observed following γ-irradiation of N_2O-saturated 0.1 M phosphate buffer, pH 7, containing 0.1 M DMSO and (a) 40 mM [α-^{13}C]M_3PO or (b) 40 mM [β-^{13}C]M_3PO. The instrument settings were 100 kHz modulation frequency, 1 G modulation amplitude, and 20 mW incident microwave power.

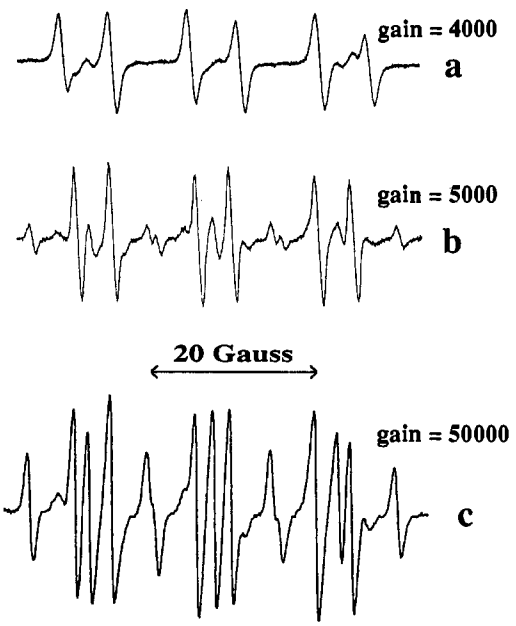

Figure 4. EPR spectra of [α-^{13}C]- and [β-^{13}C]M_3PO spin adducts of hydroxyl radical obtained by sonolysis, by radiation, or enzymatically. The EPR signals were observed (a) following sonolysis of argon-saturated 40 mM aqueous [α-^{13}C]M_3PO, (b) following γ-irradiation of N_2O-saturated 0.1 M phosphate buffer, pH 7, containing 40 mM [β-^{13}C]M_3PO, and (c) employing aerated 0.1 M phosphate buffer, pH 7, containing 4 mM HX, 0.4 U/mL XO, 40 mM [β-^{13}C]M_3PO, 50 μM iron(II), 25 μM EDTA, and 30 U/mL SOD. The instrument settings were 100 kHz modulation frequency, 1 G modulation amplitude, and 20 mW incident microwave power.

instrument, which indicates reoxidation by oxygen of the hydroxylamine to the spin adduct.

(iii) Methyl Radical. Irradiation of a solution containing 0.1 M DMSO and either [α-^{13}C]- or [β-^{13}C]M_3PO gave rise to a six-line signal (Figure 3), attributable to [α-^{13}C]- and [β-^{13}C]$M_3PO/^{\bullet}CH_3$ spin adducts (Table 1).

B. Oxygen-Centered Radicals. (i) Hydroxyl Radical. An aqueous solution of 40 mM [α-^{13}C]M_3PO was sonolyzed under argon for 25 min and scanned for EPR signal. The spectrum observed was a superposition of the contaminant dimer and a new signal having similar hfs constants (Figure 4a). The two signals could not be resolved (Table 1). The new signal is attributable to [α-^{13}C]$M_3PO/^{\bullet}OH$.

A similar signal was obtained when [α-^{13}C]$M_3PO/^{\bullet}OH$ was generated radiolytically by γ-irradiation for 20 min of N_2O-saturated phosphate buffer containing 40 mM [α-^{13}C]M_3PO (Table 1).

Because of the poor distinction between [α-^{13}C]$M_3PO/^{\bullet}OH$ and the dimeric contaminant labeled at the α-position, the radiation experiment was repeated with [β-^{13}C]M_3PO. In this case, the hfs values of [β-^{13}C]$M_3PO/^{\bullet}OH$ (Table 1) are different from those of the contaminant labeled at the β-position (see Figures 1b and 4b).

As a further confirmation, $^{\bullet}OH$ was generated enzymatically using the hypoxanthine/xanthine oxidase (HX/XO) reaction, superoxide dismutase (SOD), iron(II), and ethylenediaminetetraacetic acid (EDTA).[19] The HX/XO reaction was used as a superoxide generating system.[44] In the presence of SOD and iron(II)/EDTA, genuine $^{\bullet}OH$ radicals were generated via the Fenton reaction. The reaction was performed at room temperature in aerated 0.1 M phosphate buffer, pH 7, containing 4 mM HX, 50 μM iron(II), 25 μM EDTA, 0.4 U/mL XO, and 40 mM [β-^{13}C]M_3PO in a gas-permeable Teflon capillary (see Methods) placed inside the EPR cavity. The use of 4 mM HX and a steady supply of air around the sample ensured continuous $O_2^{\bullet-}$ flux for a long duration. In the presence of SOD, a signal with hfs constants similar to those of [β-^{13}C]$M_3PO/^{\bullet}OH$ obtained radiolytically was observed (Figure 4c and Table 1).

When the experiment was repeated in the absence of SOD, the [β-^{13}C]$M_3PO/^{\bullet}OH$ signal was not detected, which suggests a rapid superoxide-induced destruction of $M_3PO/^{\bullet}OH$ similar to what was observed for the DMPO/$^{\bullet}$OH adduct.[15]

(ii) Superoxide Reaction with [^{13}C]M_3PO. Upon exposure of [α-^{13}C]- or [β-^{13}C]M_3PO to HX/XO, the intensity of the impurity signal increased. This increase was attributed to the oxidation of the dimeric nitronyl hydroxylamine by superoxide[42] (Scheme 4). No signal attributable to [^{13}C]$M_3PO/O_2^{\bullet-}$ was detected. The lack of [^{13}C]$M_3PO/O_2^{\bullet-}$ signal could result either from a

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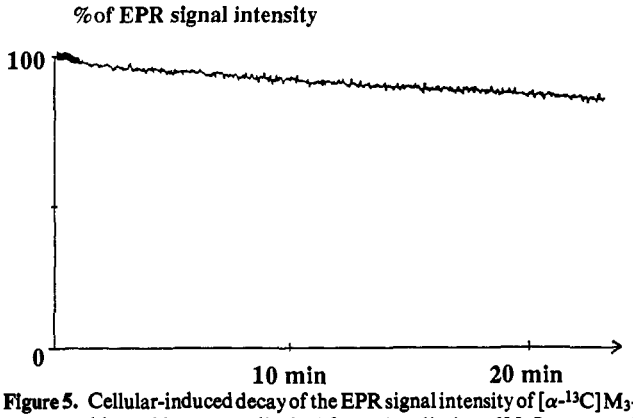

Figure 5. Cellular-induced decay of the EPR signal intensity of [α-^{13}C]M_3PO spin adduct of formate radical. After γ-irradiation of N_2O-saturated 0.1 M phosphate buffer, pH 7, containing 40 mM [α-^{13}C]M_3PO and 0.1 M sodium formate, the solution was mixed witlr a suspension of packed A549 human cells (approximately 1:1) at room temperature, and the time dependence of the intensity of the EPR signal was monitored. The instrument settings were 100 kHz modulation frequency, 1 G modulation amplitude, and 20 mW incident microwave power.

failure of superoxide to add to M_3PO or from a fast destruction of the spin adduct by superoxide, like in the case of M_3PO/$^{\bullet}$OH.

(iii) Chemiluminescence Assay. In order to assess the reaction of M_3PO with superoxide, the lucigenin-dependent chemiluminescence assay for superoxide was utilized.[34,45] To an HX/XO reaction in the presence of 250 μM lucigenin and 10 mM linolenic acid[35] were added varying concentrations of DMPO or M_3PO. The inhibition of chemiluminescence was measured, and the competition of DMPO and M_3PO for superoxide was evaluated.[34] The reactivity of M_3PO toward superoxide was of the same order as the value recently reported for DMPO[46] (data not shown). The previously determined values of 10 $M^{-1}\cdot s^{-1}$ for DMPO and 7 $M^{-1}\cdot s^{-1}$ for M_3PO[13] were based on erroneous assignment of the dimeric impurity of M_3PO, which was taken as $M_3PO/O_2^{\bullet-}$.

C. Spin Adduct Stability. The spin adducts [^{13}C]$M_3PO/^{\bullet}CH_3$, [^{13}C]$M_3PO/^{\bullet}CO_2^-$, and [^{13}C]$M_3PO/^{\bullet}$OH, obtained by trapping of radiolytically generated radicals, persisted over days. To examine the spin adduct stability in the presence of cells, a solution of either [^{13}C]$M_3PO/^{\bullet}CH_3$ or [^{13}C]$M_3PO/^{\bullet}CO_2^-$, previously produced by radiation, was mixed with a suspension of packed MCF-7 or A549 human cells, and the intensity of the EPR signal was followed. A slow spin loss was observed, where the adduct exhibited a half-life longer than 2 h (Figure 5), in contrast to DMPO/$^{\bullet}$OH, which was destroyed within several minutes in the presence of cells.[16]

Discussion

Spin Adduct Stability. Although the advantage of labeling DMPO with ^{13}C has been considered previously,[47] so far ^{13}C-labeling was employed primarily in the radical addends.[26,48-53] The present results demonstrate the potential of ^{13}C-labeled M_3PO in trapping both carbon-centered and oxygen-centered free radicals to yield identifiable spin adducts which are practically stable rather than persistent radicals. Most importantly, they are relatively stable in cellular systems. Consequently, [^{13}C]M_3PO can be used to trap and report on intracellular free radicals.

[α-^{13}C]M_3PO vs [β-^{13}C]M_3PO. Although the introduction of an α-^{13}C in the M_3PO molecule resulted in a second hfs, the range of $a_{\alpha\text{-C-}13}$ for different carbon- and oxygen-centered adducts was only from 6 to 7 G. Such a narrow range makes the discrimination between different adducts difficult. For example, it does not allow the distinction between the dimeric paramagnetic impurity and $M_3PO/^{\bullet}$OH, where a high level of impurity can mask the spin adduct. From these results we can conclude that different addends do not extert significantly different influences on the hfs of the ^{13}C at the α-position. The conclusion is also supported by work on [*nitronyl*-^{13}C]PBN,[24,54] on ^{13}C satellites of pyrrolidine nitroxides,[55] and 2-phenyl-[*nitronyl*-^{13}C]DMPO.[25] We therefore introduced the ^{13}C at the 2-methyl. The rationale for using a β-^{13}C labeling is that the 2-[^{13}C]methyl, like the β-H of DMPO, was expected to provide a hfs more sensitive than the hfs of the α-nitronyl to different addends. This premise is supported by ENDOR studies on PBN,[54] which also show a more significant variation of $a_{\beta\text{-C-}13}$ than of $a_{\alpha\text{-C-}13}$, and studies on ^{13}C satellites of nitroxides.[55] In our work, $a_{\beta\text{-C-}13}$ ranged from 4 to 7 G. The second advantage of [β-^{13}C]M_3PO is that because the dimeric impurity has a nine-line signal, it does not superimpose with the signals of the spin adducts and therefore allows further improvement in spectral discrimination (Figure 4c).

[^{13}C]M_3PO Trapping of Superoxide. The trapping efficiency of superoxide by M_3PO is of the same order of magnitude as that of DMPO, based on its inhibitory effect on the chemiluminescence generated in a superoxide–lucigenin system.[34] Therefore, the failure to detect [^{13}C]$M_3PO/^{\bullet}O_2H$ most probably derives from a rapid destruction of the spin adduct by superoxide.[15] In other words, as in the case of DMPO, the intensity of the spin-adduct signal does not truly reflect the steady-state concentration or the production rate of superoxide.

In conclusion, the newly synthesized [^{13}C]M_3PO spin traps, especially [β-^{13}C]M_3PO, offer additional possibilities for studying free radicals by spin trapping coupled with EPR detection. Particularly, it may prove useful for trapping and probing intracellular radicals other than superoxide.

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