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Synthesis and ESR studies of a novel cyclic nitrone spin trap attached to a phosphonium group-a suitable trap for mitochondria-generated ROS?

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Abstract
In this study we report the synthesis and biological application of a novel cyclic nitrone spin trap containing a phosphonium cation. This new spin trap ([4-(2-methyl-1-oxy-3, 4-dihydro-2H-pyrrole-2-carbonyloxy)-butyl]-triphenyl-phosphonium bromide, MitoBMPOBr) is a derivative of the cyclic nitrone, 5-tert-butoxycarbonyl 5-methyl-1-pyrroline *N*-oxide (BMPO). MitoBMPOBr forms radical adducts upon trapping of superoxide and hydroxyl radicals that exhibit highly distinct and characteristic EPR spectra. The stability of these adducts is comparable to those of BMPO. Because of the presence of a positively-charged phosphonium moiety, MitoBMPOBr may be suitable for trapping reactive oxygen species (ROS) in the mitochondria.

Keywords: *Spin trap, superoxide, ROS, hydroxyl radical*

Abbreviations: *BMPO, 5-tert-butoxycarbonyl 5-methyl-1-pyrroline* N-*oxide; Boc, tert-butoxycarbonyl; DEPMPO, 5-(diethoxyphosphoryl)-5-methyl-1-pyrroline* N-*oxide; DMPO, 5,5-dimethyl-1-pyrroline* N-*oxide; EMPO, 5-ethoxycarbonyl-5-methyl-pyrroline* N-*oxide; EPR, electron paramagnetic resonance; LC–MS, liquid chromatography and mass spectrometry; MitoBMPOBr, [4-(2-methyl-1-oxy-3,4-dihydro-2H-pyrrole-2-carbonyloxy)-butyl]-triphenyl-phosphonium bromide; MitoPBN, [4-[4-[[(l,l-dimethylethyl)oxidoimino]methyl]phenoxy]butyl]triphenylphosphonium bromide;* m-*CPBA,* m-*chloroperbenzoic acid; NMR, nuclear magnetic resonance; PBN,* **α**-*phenyl*-N-*tert-butyl nitrone*

Introduction

Superoxide anion and hydroxyl radicals are generated from the mitochondrial respiratory chain [1–3] in pathological conditions or in response to redox-active quinones and other drugs. The electron paramagnetic resonance (EPR)-spin trapping [4] has been used to detect transient free radicals formed during microsomal redox metabolism [5]. Spin trapping of superoxide and other ROS generated *in situ* in the mitochondria remains a major challenge. One of the major disadvantages of spin trapping in organelles and tissues is insufficient accumulation of spin traps. Most commonly used spin traps are α-phenyl-*N*-tert-butyl nitrone (PBN) [6], 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) [7], 5-tert-butoxycarbonyl 5-methyl-1-pyrroline *N*-oxide (BMPO) [8], 5-ethoxycarbonyl-5-methyl-pyrroline *N*-oxide (EMPO) [9] and 5-(diethoxyphosphoryl)-5-methyl-1-pyrroline *N*-oxide (DEPMPO) [10,11]. Recently, in an attempt to detect ROS generated in the mitochondria, a phosphonium-conjugated spin trap {[4-[4-[[(l,l-dimethylethyl) oxidoimino] methyl] phenoxy] butyl] triphenylphosphonium bromide (MitoPBN)} was developed (Scheme 1) [12,13]. In general, PBN-type nitrones do not form stable oxygen-centered radical

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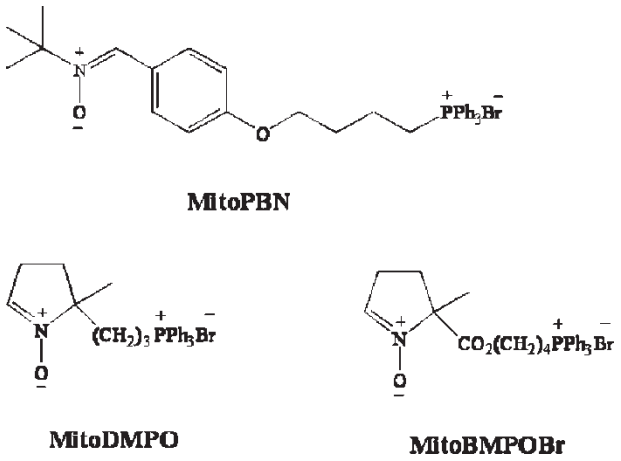
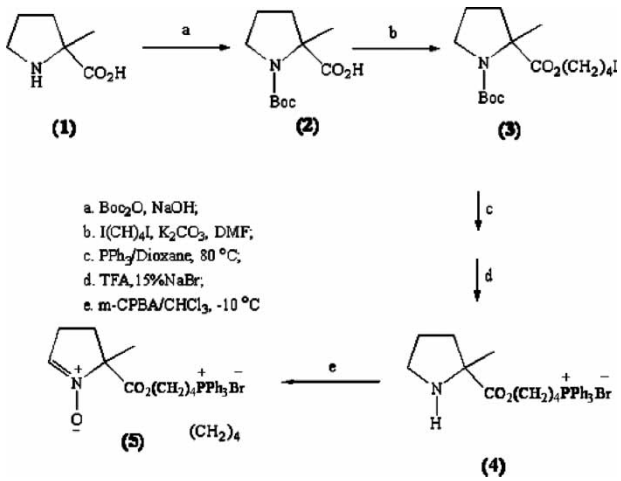


Scheme 1. Structures of phosphonium-conjugated nitrone derivatives.

adducts. These adducts also do not exhibit characteristic and distinctive ROS/EPR spectra. Typically, PBN-derived oxygen-centered adducts are extracted into organic solvents to increase their stability. However, their hyperfine couplings are strongly dependent on solvent polarity and therefore, it is easy to misassign the spin adduct structures. Murphy's group also reported a partial synthesis of a DMPO-like mitochondria-targeted spin trap, MitoDMPO [14] (scheme 1).

Herein, we report a novel phosphonium-conjugated spin trap {MitoBMPOBr, [4-(2-methyl-1-oxy-3,4-dihydro-2H-pyrrole-2-carbonyloxy)-butyl]-triphenyl-phosphonium bromide} (Scheme 2). This spin trap is similar to BMPO, a widely used spin trap that reacts with superoxide to form a BMPO-superoxide adduct with a relatively longer half-life in biological systems [8]. The phosphonium moiety in MitoBMPOBr is covalently attached to the nitrone through the butyl group. This structural feature will likely enable its accumulation into the mitochondria [13].



Scheme 2. Synthesis of MitoBMPOBr (5).

Material and methods

Materials

All chemicals were purchased from Sigma-Aldrich and used as received. ^{1}H (300 MHz) and ^{31}P (121 MHz) nuclear magnetic resonance (NMR) spectra were recorded on a Bruker AC300 (Bruker Instruments Inc., Bellirica, MA, USA) with a QNP probe for observing the proton and phosphorous nuclei. All spectral references were external. Proton spectra were referenced to TMS and phosphorous spectra were referenced to 85% phosphoric acid.

The UV spectra were obtained on a UV-1601 PC spectrophotometer (SHIMAZU Scientific Instruments, Inc.) in pure methanol. Infra red spectra of the synthesized compounds were recorded as film on FT–IR system Spectrum GX (PerkinElmer, Inc.).

The EPR spectra were recorded at room temperature on a Bruker EMX X-band spectrometer operating at 9.8 GHz and a cavity equipped with Bruker Aquax liquid sample cell. Typical spectrometer parameters were: scan range, 100 G; center field, 3505.38 G; time constant, 81.92 ms; sweep time, 41.94 s; modulation amplitude, 1.0 G; modulation frequency 100 kHz; receiver gain, 6.32×10^{4}; microwave power, 20 mW. The EPR spectra were simulated using the software developed by Dr Duling from the laboratory of Molecular Biophysics, NIEHS (Research Triangle Park, NC, USA).

Theoretical calculations were carried out for MitoBMPOBr and BMPO. The *ab initio* method with a minimum basis set of STO-3G was used, employing full geometry optimization of spin traps based on energy-lowest conformers by the semi-empirical PM3 method with HyperChem version 7.0 (Hypercube Inc.). The polka-Rebiere (conjugate gradient) algorithm method was employed for all calculations at an RMS gradient of 0.05 kcal Å^{-1} mol^{-1} and convergence criterion of 1×10^{-5} kcal mol^{-1}.

Methods

Synthesis of N-Boc 2-methyl proline (2). Solutions containing di-tert-butyl dicarbonate (2.03 g, 9.3 mmol) in dioxane (20 ml) were slowly added to an ice-cold solution of 2-methyl proline (1 g, 7.74 mmol) in 1 N sodium hydroxide (8 ml). The two-phase mixture was stirred at 5°C for 30 min, and allowed to warm to room temperature over 3.5 h at which time TLC analysis verified the completion of the reaction. The mixture was then concentrated to half its original volume in a rotary evaporator at 35°C and acidified to pH 2–3 by the slow addition of 1 N potassium bisulfate in an ice-water bath. The reaction mixture was extracted with ethyl acetate (3×30 ml). The combined extracts were dried with $MgSO_4$ and filtered. After evaporation in vacuum, a white solid product of *N*-Boc 2-methyl proline was obtained from the filtrate (Yield: 90%).

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^{1}H-NMR(CD_3OD): δ 1.41, 1.42 (s, 9H, —C(CH_3)$_3$), 1.49, 1.51 (s, 3H, —CH_3), 1.92 (m, 3H, —CHCH_2), 2.19 (m, 1H, —CH_2—CH—CH_2), 3.47 (m, 2H, —CH_2—N).

IR (cm^{-1}): 2979.11, 2937.26, 2878.22, 1738.24, 1699.37, 1393.75, 1165.61.

*Synthesis of 2-(N-Boc 2-methyl-pyrrolidinyl) carboxylic acid 4-iodo-butyl ester (**3**)*. To a cold solution of (**2**) (1 g, 4.36 mmol) in DMF (15 ml) was added solid K_2CO_3 (0.723 g, 5.18 mmol). After stirring for 10 min in an ice-water bath, 1,4-diiodobutane (6.82 g, 21.78 mmol) was added dropwise over 30 min and the reaction mixture then was allowed to warm to room temperature over 1 h and stirred for another 3 h at room temperature. After removal of the precipitate by suction filtration, the filtrate was partitioned between ethyl acetate and water. The organic phase was washed with brine (2 × 30 ml), and dried with $MgSO_4$. The filtrate was concentrated and dried in vacuum. After separation with a silica gel flash column (CH_2C1_2 as an eluent), the sticky oil product was collected. (Yield: 95%)

^{1}H-NMR($CDCl_3$): δ 1.37, 1.41 (s, 9H, —C(CH_3)$_3$), 1.47, 1.52 (s, 3H, CH_3), 1.6–2.2 (m, 8H, CH_2), 3.2 (t, 2H, I—CH_2), 3.5 (m, 2H, N—CH_2), 4.2 (m, 2H, O—CH_2).

IR(cm^{-1}): 2976.39, 2878.22, 1739.03, 1699.49, 1393.83, 1168.70.

*Synthesis of [4-(2-methyl-pyrrolidine-2-carbonyloxy)-butyl]-triphenyl-phosphonium bromide (**4**)*. A sealed flask was charged with (**3**) (1 g, 2.43 mmol) and triphenyl phosphine (1 g, 3.81 mmol) in dioxane (10 ml) under argon. This mixture was heated to 80°C for 3 h in the dark. The resulting product [4-(*N*-Boc 2-methyl-pyrrolidine-2-carbonyloxy)-butyl]-triphenyl-phosphonium iodide was isolated as a white solid precipitate which was washed with ether, dried in vacuo, and directly deprotected by the treatment with TFA at room temperature for 3 h. After evaporation of solvents, the residue was dissolved in CH_2C1_2 and washed with saturated aqueous $NaHCO_3$ (2 × 15 ml) and aqueous NaBr (15%, 3 × 10 ml). The CH_2C1_2 layer was then dried and filtered. The colorless sticky oil product (**4**) was obtained. (Yield: 85%)

^{1}H-NMR(D_2O): δ 1.52 (s, 3H, CH_3), 1.7–2.2 (m, 8H, CH_2), 3.37 (m, 4H, P^+—CH_2 and N—CH_2), 4.29 (t, 2H, —OCH_2), 7.6–7.9 (m, 15H, ArH).

^{31}P-NMR(D_2O): δ 23.67.

LC–MS: 446.2 [(M—Br)$^+$] (single peak in LC).

IR(cm^{-1}): 3446.26, 1717.15, 1439.66, 1113.70, 750.23, 723.81, 691.10, 531.86, 506.91.

*Synthesis of [4-(2-Methyl-1-oxy-3,4-dihydro-2H-pyrrole-2-carbonyloxy)-butyl]-triphenyl-phosphonium bromide(**5**) (MitoBMPOBr)*. A flask equipped with a pressure equalized addition funnel was charged with (**4**) (1 g, 1.90 mmol) in 100 ml $CHCl_3$. The solution of m-chloroperbenzoic acid (m-CPBA) (0.94 g, 3.81 mmol) in 50 ml $CHCl_3$ was added dropwise to the flask through addition funnel at −10°C over 1 h and stirred for another 30 min at the same temperature. Then the reaction mixture was washed with saturated aqueous $NaHCO_3$ (2 × 15 ml) and saturated aqueous NaBr (2 × 10 ml), respectively. The dried and filtered organic layer was concentrated in a rotary evaporator. After flash column purification (silica gel, 15% CH_2C1_2 in methanol), the MitoBMPOBr (**5**) was obtained (30% yield).

^{1}H-NMR(D_2O): δ 1.54 (s, 3H, CH_3), 1.75 (m, 2H, CH_2), 1.91 (m, 2H, CH_2), 2.2 (m, 1H, CH_2), 2.4 (m, 1H, CH_2), 2.6 (m, 1H, CH_2), 2.75 (m, 1H, CH_2), 3.34 (m, 2H, P^+—CH_2), 4.24 (m, 2H, —OCH_2), 7.19 (t, 1H, HC═N), 7.6–7.9 (m, 15H, ArH).

^{31}P-NMR(D_2O): δ 23.66.

LC–MS: 460.2 [(M—Br)$^+$] (single peak in LC).

IR(cm^{-1}): 3411.23, 1738.56, 1586.45, 1438.65, 1113.27, 750.23, 724.12, 692.57, 532.00, 506.91.

UV: 226 nm (ϵ = 25,245 $M^{-1}cm^{-1}$).

Results and discussion

MitoBMPOBr (**5**) was synthesized by a five-step method as illustrated in Scheme 2. First, 2-methyl proline (**1**) was protected by Boc group [15]; then, the iodobutyl group was connected to the Boc protected 2-methyl proline [15–17]; in the following steps, the phosphonium group was introduced by reaction of triphenyl phosphine with (**3**) in dioxane. After deprotection and anion exchange, compound (**4**) was prepared; finally, the MitoBMPOBr (**5**) was obtained after the oxidization of the compound (**4**) by *m*-CPBA in chloroform at −10°C.

Trapping of superoxide anion and hydroxyl radicals by MitoBMPOBr

The addition of xanthine oxidase to an aerobic solution containing xanthine (0.5 mM), MitoBMPOBr (25 mM) and 100 μM DTPA in phosphate buffer (100 mM, pH 7.4) yielded an ESR spectrum attributable to the MitoBMPOBr—OOH adduct (Figure 1(a)). Addition of SOD (250 U/ml) to the above incubation mixture totally abrogated formation of the MitoBMPOBr—OOH adduct. This confirmed that the spectrum (Figure 1(a)) arose from trapping of $O_2^{\cdot-}$. The EPR spectra corresponding to that of the MitoBMPOBr—OH adduct, formed from decomposition of MitoBMPOBr—OOH, was not detected under these conditions (Figure 1(a)).

Hydroxyl radical was generated using the Fenton system (H_2O_2 and Fe^{2+}). In the presence of MitoBMPOBr (25 mM), an intense ESR signal was detected that was persistent for several hours

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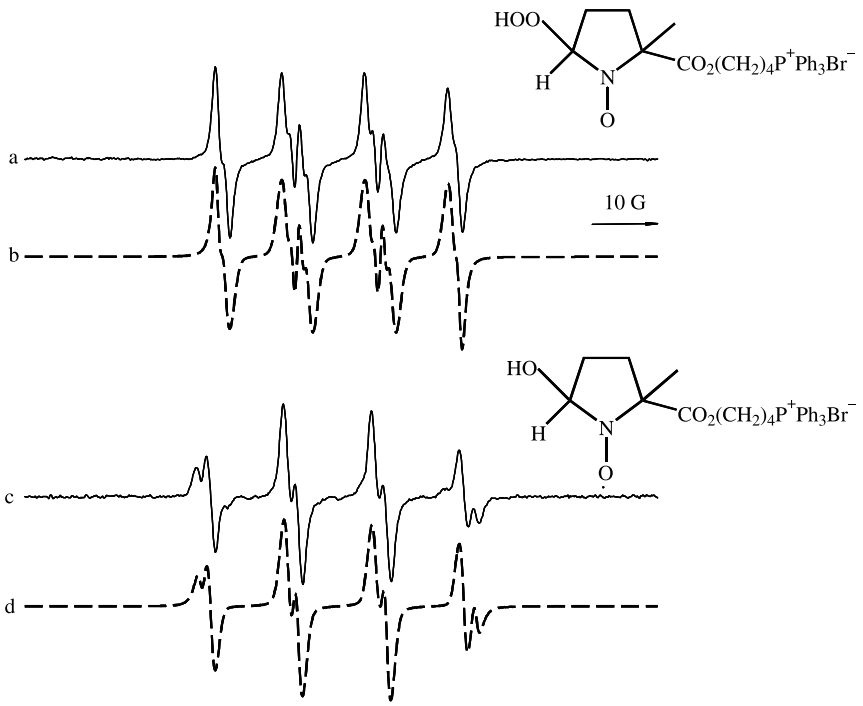

Figure 1. (a) EPR spectrum of MitoBMPOBr—OOH. Spectra were obtained in phosphate buffer (100 mM, pH 7.4) containing MitoBMPOBr (25 mM), catalase (300 U/ml), xanthine (0.5 mM), xanthine oxidase (25 mU/ml) and DTPA(100 μM); (b) computer simulation the EPR shown in (a); (c) EPR spectrum of MitoBMPOBr—OH. Spectra were obtained in phosphate buffer (100 mM, pH 7.4) containing MitoBMPOBr (25 mM), H_2O_2 (5 mM) and $FeSO_4$ (5 mM); (d) computer simulation of the spectrum shown in (c).

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(Figure 1(b)). This ESR spectrum was assigned to the MitoBMPOBr—OH adduct.

Based on the computer simulations of the spectra (Figure 1(c),(d)), the spin adducts responsible for Figure 1(a),(b) were assigned to the corresponding superoxide and hydroxyl spin adducts, i.e. the superimposition of two diastereoisomers of the MitoBMPOBr adducts (Table I).

Kinetics decay of the superoxide adduct

The stability of the superoxide adduct was determined as follows: incubation mixtures contained MitoBMPOBr (25 mM), xanthine (0.5 mM), xanthine oxidase (25 mU/ml) and catalase (300 U/ml). After 5 min, SOD (250 U/ml) was added to dismutate superoxide anion formed in this system. Figure 2 shows the relative decay kinetics of MitoBMPOBr and BMPO superoxide adducts (peak intensities were normalized). The half-life data were calculated on basis of first-order rate constant ($r^2 > 0.99$) (Table I).

The half-life of MitoBMPOBr–OOH (10.2 min) is much longer than that of the *n*BuMPO–OOH (1.1 min), but it is less than that of the BMPO–OOH (15.7 min) (Table I). This suggests that conjugation to

Table I. ESR hyperfine splitting constants of MitoBMPOBr adducts.

Spin adduct	Diastereomers	Hyperfine splitting constants (G)			$t_{1/2}$(min)
		A_N	A_H^β	A_H^γ	
MitoBMPOBr–OOH	51.9	13.1	9.6	0.1	10.2
	48.1	13.1	11.9	0.2	
MitoBMPOBr–OH	66.8	13.8	11.99	–	–
	33.2	14.86	13.4	–	–
MitoBMPOBr–CO_2	100	14.6	16.9	–	–
MitoBMPOBr–SO_3	100	13.50	14.6	–	–
MitoBMPOBr–CH_2OH	100	12.2	6.1	–	–
MitoBMPOBr–CH_3	100	14.99	21.8	–	–
BMPO–CH_3	100	15.3	21.7	–	–
BMPO–OOH[a]	55	13.4	12.1	–	15.7
	45	13.4	9.4	–	

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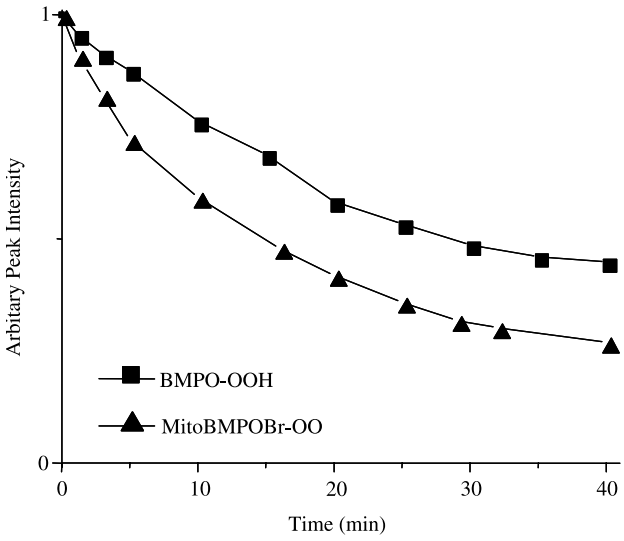

Figure 2. Kinetics decay of MitoBMPOBr and BMPO superoxide adducts. Spin traps (25 mM) were mixed with catalase (300 U/ml), xanthine (0.5 mM), and xanthine oxidase (25 mU/ml) in phosphate buffer (100 mM, pH 7.4) containing 100 μM DTPA.

the phosphonium cation can greatly increase the stability of superoxide adduct. Previous reports indicate that the electron withdrawal ability and steric hindrance of C5-substituent group contribute to the stability of the superoxide spin adducts [18,19].

Trapping of other radicals

Both carbon-centered and sulfur-centered radicals were also trapped by MitoBMPOBr. The hyperfine coupling constants are listed in Table I. These radicals shown in Table I were generated by adding methanol, sodium formate, potassium sulfite, and DMSO to the Fenton reaction system in the presence of MitoBMPOBr (25 mM) (Figure 3(a)–(c)). Except for the methyl adduct, all other adducts consist of one of diastereomers (Figure 4).

The beta hydrogen coupling constants of $CO_2^{\cdot-}$ and $SO_3^{\cdot-}$ adducts were slightly higher than the nitrogen coupling constants. Spin trapping experiments in the Fenton system with DMSO yielded a more complicated spectrum, which was attributed to the superimposition of both MitoBMPOBr—OH (81%) and MitoBMPOBr—CH_3 (19%) adducts (Figure 4(a),(b)). This might be due to the steric hindrance for trapping of methyl radical as compared to the hydroxyl radical. One would expect that the spin-trapping efficiency with negatively-charged radicals ($CO_2^{\cdot-}$ and $SO_3^{\cdot-}$) should be greater than with neutral carbon-centered radicals. The *ab initio* geometry optimization of MitoBMPOBr revealed that the bulky phosphonium group is on one side of the nitrone ring which subsequently increases the steric hindrance for trapping. The distance between

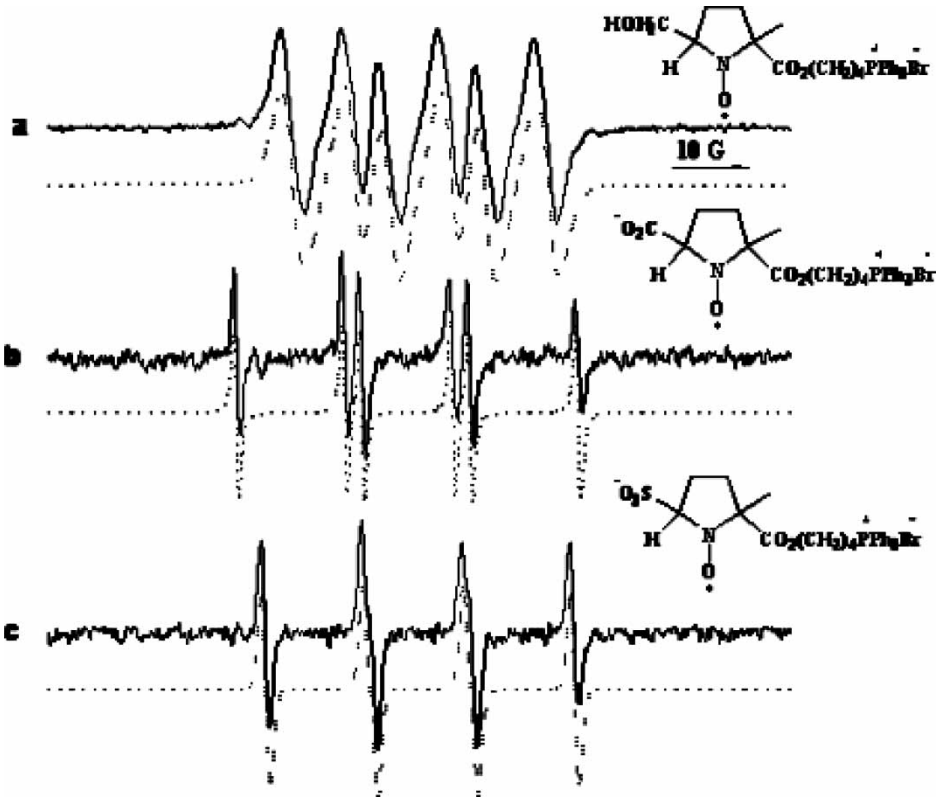

Figure 3. (a) MitoBMPOBr–CH_2OH; (b) MitoBMPOBr–$CO_2^{\cdot-}$; (c) MitoBMPOBr–$SO_3^{\cdot-}$. Spectra were obtained in phosphate buffer (100 mM, pH 7.4) containing MitoBMPOBr (25 mM), H_2O_2 (5 mM) and FeSO4 (5 mM) in the presence of methanol, sodium formate and potassium sulfite, respectively.

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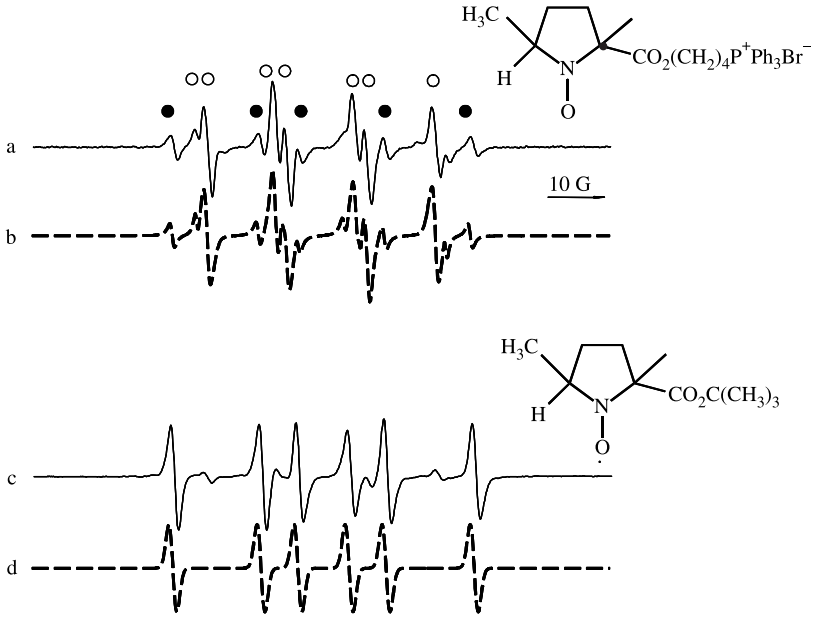

Figure 4. (a) EPR spectrum of MitoBMPOBr (25 mM) in the Fenton reaction system [H_2O_2 (5 mM), $FeSO_4$ (5 mM)] with the presence of DMSO. The EPR spectrum of MitoBMPOBr–CH_3 (closed circles) and the EPR spectrum of MitoBMPOBr–OH (open circles) are shown; (b) computer simulation of the spectrum shown in (a); (c) EPR spectrum of the BMPO-methyl adduct obtained under the same conditions as shown in (a); (d) computer simulation of the BMPO–CH_3 adduct.

the phosphorus atom of phosphonium and the oxygen atom of nitrone is only 3.45 Å (Figure 5). This suggests a strong positive field around the nitrone ring. This particular feature is probably responsible for the faster trapping reaction with the anion radicals. Furthermore, the Mulliken charge of two-position carbon of MitoBMPOBr is 0.0608, which is much higher than in BMPO (0.0099). This coincides with ^{1}H-NMR data of C-2 hydrogen (e.g.) 7.19 versus 6.95 in MitoBMPOBr and BMPO. The higher value of the Mulliken charge in MitoBMPOBr also indicates a higher rate of spin trapping free radicals.

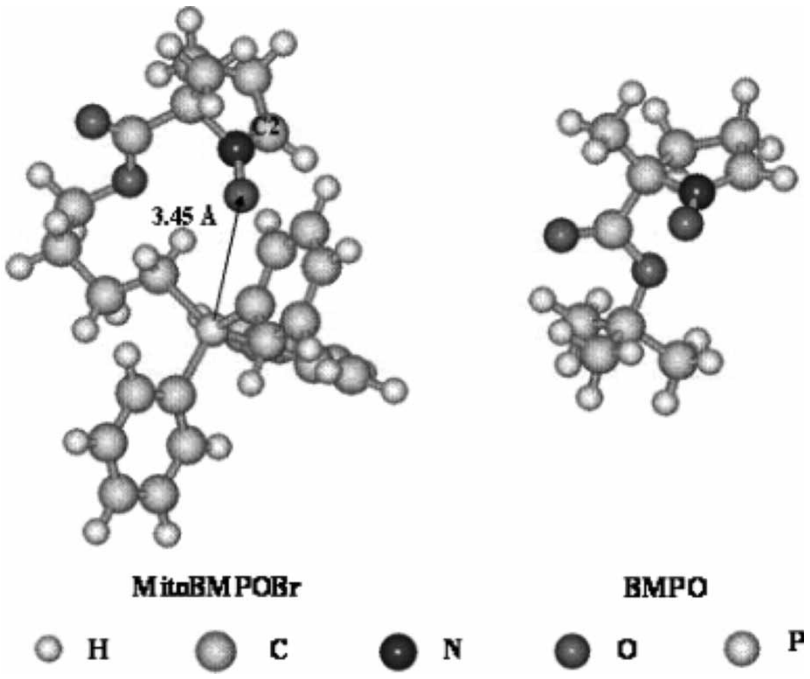

Figure 5. Energy-minimized conformers of Mito-BMPOBr and BMPO.

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Preferential uptake of phosphonium-derivative into mitochondria

Recently, Murphy and co-workers synthesized mitochondria-targeted antioxidants, MitoQ and Mito-Vit-E [20]. These compounds are phosphonium conjugated derivatives of parent antioxidants, co-enzyme Q and alpha-tocopherol. Dhanasekaran et al. prepared a mito-carboxy proxyl (Mito-CP), a nitroxide that localizes in the mitochondria [21]. These mitochondria-targeted antioxidants were considerably more effective in inhibiting peroxide-induced mitochondrial oxidant formation and oxidative damage and apoptosis in endothelial cells treated with glucose/glucose oxidase. Therefore, it appears that irrespective of the antioxidant structure, attachment to the phosphonium, conjugation to the phosphonium group makes them more accessible to the mitochondria.

Conclusions

In this study we report the synthesis of a new phosphonium-conjugated spin trap, MitoBMPOBr, that forms a superoxide adduct with a relatively longer half-life. EPR parameters of spin adducts, especially those of superoxide and hydroxyl adducts, are distinctly different and highly characteristic. This hydrophilic spin trap, by virtue of its cationic nature, may be ideal for trapping mitochondrial superoxide and hydroxyl radicals using the EPR spectroscopy.

Acknowledgements

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