Mito-DEPMPO synthesized from a novel NH_2-reactive DEPMPO spin trap: a new and improved trap for the detection of superoxide†

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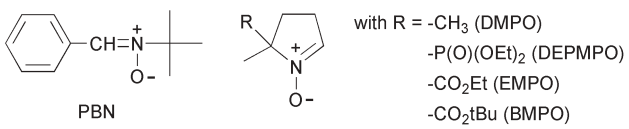
Mito-DEPMPO, a new DEPMPO analogue bearing a triphenylphosphonium group, was synthesized *via* a novel NH_2-reactive DEPMPO. The half-life of the Mito-DEPMPO superoxide adduct was estimated to be *ca.* 40 min. Using Mito-DEPMPO, reactive oxygen species generated in intact mitochondria were detected and characterized by EPR.

Reactive oxygen-centered radicals (ROR) play a key role in numerous physiological and pathological processes including aging, cancer, atherosclerosis, neurodegenerative diseases and diabetes.[1] A considerable amount of progress has been made over the past decade concerning their roles as second messengers in various physiological and pathological processes.[1,2] However, selective detection of ROR still poses major limitations, as most ROR are highly reactive species with very short lifetimes in biological systems. In the past 15 years, the EPR spin-trapping technique has developed into one of the most powerful methods for detecting ROR.[3]

Spin-trapping is a technique in which short-lived free radicals react with a nitrone spin trap to form a more persistent nitroxide spin adduct that can be conveniently detected by direct EPR. Typically, the spin adducts exhibit a characteristic hyperfine splitting pattern, enabling the structural characterization of the short-lived radical species.[4] However, there are still numerous limitations with respect to cellular applications of spin-trapping due to the instability of spin adducts, bioreduction to EPR-silent products, and compartmentalization of radical generation in cellular systems.[4] In the past decade, a significant improvement has been made with regard to enhancing the chemical stability of ROR-trapped spin adducts with the development of new spin traps such as DEPMPO,[5] EMPO[6] and BMPO[7] (Scheme 1). More recently, using β-cyclodextrin to encapsulate the DEPMPO-OOH adduct, Karoui *et al.* reported a 7-fold enhancement in *in vitro* adduct stability ($t_{1/2}$ = 96 min).[8]

Despite these improvements in spin-trap design and spin-adduct sequestration, significant challenges remain with respect to selective targeting of spin traps to increase trapping efficiency and enhance biocompatibility in biological systems. From more selective spin-trapping experiments, structural and kinetic information about the production and decay of free radicals should be obtained, as well as information on the biological significance of the trapped radicals. More precise studies will permit better knowledge of the etiology and pathogenesis of numerous diseases. Recently, few works have detailed the preparation of α-phenyl-N-*tert*-butyl nitrone (PBN)-based spin traps exhibiting lectins,[9] mitochondria,[10] cell membranes,[11] and sulfhydryl-containing peptide[12] targeting properties. However, the PBN conjugated nitrones exhibited limited spin-trapping properties toward oxygen-centered radicals. With the aim of developing an efficient spin trap that can be used as a starting block for further conjugation to relevant moieties such as targeting groups, labels or drugs, the synthesis of a DEPMPO-based spin trap bearing a reactive group for amino- and thiol-coupling in water media was performed.

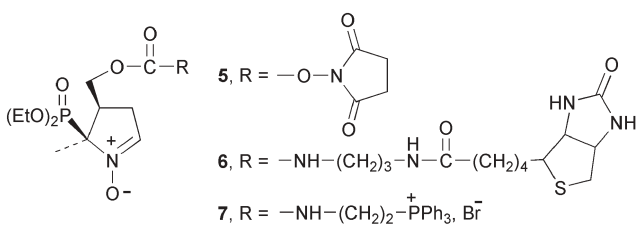


Scheme 1

We report herein the synthesis of NHS-DEPMPO, **5**, a DEPMPO analogue bearing at C4 an activated carbonate function *cis* to the phosphoryl group (Scheme 2). The facile coupling of **5** to arms involving either a biotin or a triphenylphosphonium group led to nitrones **6** and **7** (Scheme 2). Preliminary results on superoxide spin-trapping with **5** and **7** are also reported.

NHS-DEPMPO was prepared according to a four-step synthetic sequence as described in Scheme 3 with a 25% overall yield.†

Biotin was selected because of its high binding affinity to avidin ($K_{assoc.}$ = 1.7 × 10^{15} M^{-1}) and its successful application in many biomedical assays.[13] Because of the increasing importance of the chemical biology of reactive oxygen species (ROS) in



Scheme 2

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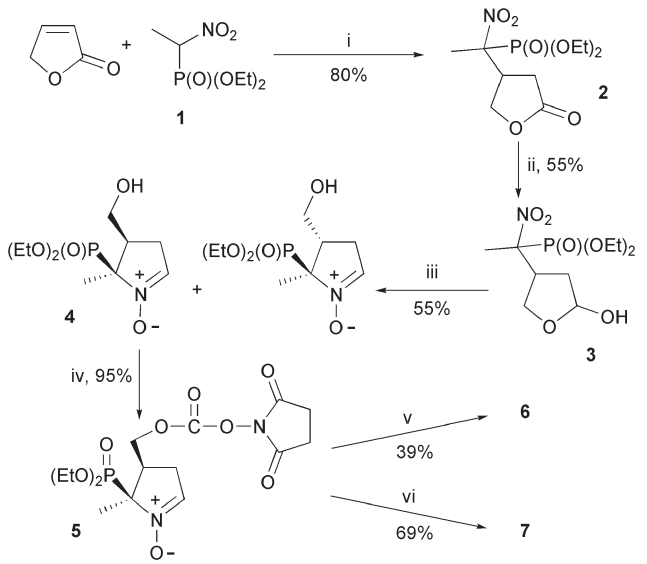
† Electronic supplementary information (ESI) available: Preparation of mitochondria, synthesis of **5**, **6** and **7**, and spectrometer settings. See DOI: 10.1039/b616076j



Scheme 3 *Reagents and conditions*: i, PBu_3, C_6H_{12}–CH_2Cl_2 (9 : 1), rt; ii, DIBAL-H, CH_2Cl_2, −78 °C; iii, Zn/NH_4Cl, H_2O/THF (10 : 1), rt; iv, DSC, Et_3N, CH_3CN, rt; v, biotinylamidopropylammonium trifluoroacetate, Et_3N, DMSO, rt; vi, (2-aminoethyl)triphenylphosphonium bromide, Et_3N, CH_2Cl_2, rt.

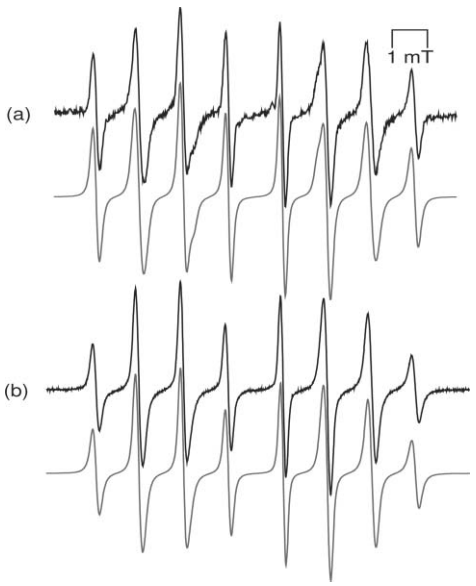


Fig. 1 Spin-trapping of superoxide radical by NHS-DEPMPO **5** and Mito-DEPMPO **7**: (a) EPR spectrum obtained after 10 min incubation of a mixture containing hypoxanthine (HX) (0.4 mM), xanthine oxidase (XO) (0.04 U mL^{-1}), diethylenetriaminepentaacetic acid (DTPA) (1 mM) and NHS-DEPMPO (20 mM) in phosphate buffer (0.1 M, pH 7.3); (b) as in (a) but with Mito-DEPMPO. The gray lines represent the computer simulations of the spectra.

mitochondrial diseases,[14] it appeared that accumulating a spin trap within mitochondria will increase the mechanistic understanding of the role of ROS. Murphy *et al.*[10] developed a novel approach by which PBN, conjugated to the triphenylphosphonium cation (Mito-PBN), was selectively targeted to mitochondria. Mito-PBN was shown to be more potent than 'non-targeted' PBN in inhibiting mitochondrial oxidation. Although the conjugation to a triphenylphosphonium cation helps 'target' PBN to mitochondria, the Mito-PBN oxy-radical adducts are still as unstable as the PBN oxy-radical adducts. The EPR detection and characterization of Mito-PBN adducts are still ambiguous.

Spin-trapping experiments coupled with EPR detection were performed in a phosphate buffer solution (pH 7.3) using NHS-DEPMPO **5** as the spin trap. Trapping superoxide using two different generating systems (Hypoxanthine/Xanthine Oxidase and KO_2/18-C-6 systems)[15] led to the detection of a persistent signal shown in Fig. 1(a). Using the HX/XO system, the signal was cancelled when SOD (600 U mL^{-1}) was previously added to the trapping reaction mixture. Moreover, when glutathione peroxidase (10 U mL^{-1}) and reduced glutathione (1.2 mM) were added, only the signal of the hydroxyl radical spin adduct, NHS-DEPMPO/OH, was observed. Based on these additional experiments, the signal shown in Fig. 1(a) can be unambiguously assigned to the NHS-DEPMPO/OOH spin adduct (Table 1). The eight-line spectrum of NHS-DEPMPO/OOH is clearly less complex than the one observed with DEPMPO/OOH. With NHS-DEPMPO it is reasonable to assume that trapping of superoxide is stereospecific, and occurs on the less hindered face of the pyrroline *N*-oxide ring, yielding only the *trans* form of the NHS-DEPMPO/OOH adduct. The spectrum of this adduct was easily calculated with the ROKI program,[16] assuming a chemical exchange between the two conformers in a 5.6 : 4.4 ratio (Table 1). These results are in agreement with those obtained with *cis* 4-PhDEPMPO, a DEPMPO analogue bearing a phenyl group in the 4-position.[15]

The superoxide spin-trapping experiments were also conducted with Mito-DEPMPO and very similar spectra were obtained as shown in Fig. 1(b). No superoxide adduct was detected when SOD was added to the trapping reaction mixture. In the presence of glutathione peroxidase (10 U mL^{-1}) and reduced glutathione (1.2 mM) only the signal due to the hydroxyl radical spin adduct, Mito-DEPMPO/OH, was observed. The hyperfine splitting constant's (hfsc's) parameters obtained by simulation exhibited very close values to those of NHS-DEPMPO/OOH (Table 1). The half-life times of Mito-DEPMPO/OOH and DEPMPO/OOH were determined following the same experimental procedure, *i.e.* the monitoring of their EPR signal decay.[15] Surprisingly, the half-life of the Mito-DEPMPO/OOH adduct was estimated to be *ca.* 40 min which is 2.5 and 45 times more than those reported for the

Table 1 Simulated EPR hyperfine splitting constants for radical adducts of Mito-DEPMPO and NHS-DEPMPO

Adducts	Generating system	Diastereoisomer	Conformer	k/s^{-1} [a]	a_P/mT	a_N/mT	a_H/mT	a_{H_γ}/mT[b]
5-OOH	HX/XO, phosphate buffer	*trans* (100%)	T_1 (56%)	1.38×10^7	5.224	1.327	1.066	0.039, 0.019,
			T_2 (44%)		5.347	1.252	1.285	0.074, 0.019, 0.048 (2), 0.083
7-OOH	HX/XO, phosphate buffer	*trans* (100%)	T_1 (69.7%)	1.16×10^7	5.327	1.279	1.237	0.05 (3), 0.048 (2), 0.044, 0.016
			T_2 (30.3%)		5.201	1.297	1.013	

[a] Exchange rate constants in s^{-1}. [b] Number of equivalent protons are given in parentheses.

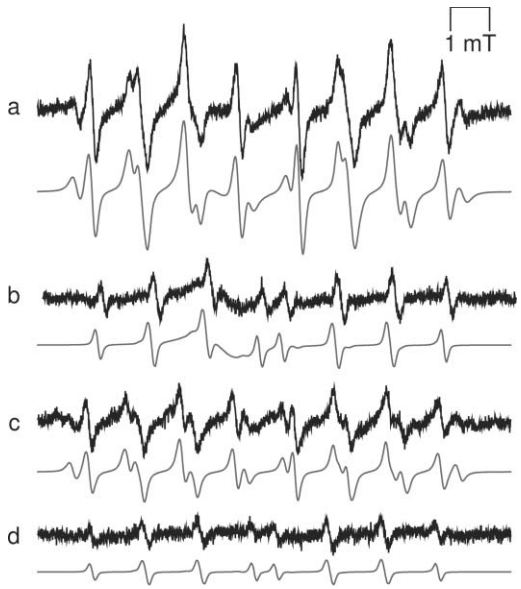


Fig. 2 Spin-trapping from intact mitochondria: (a) EPR spectrum obtained in the presence of isolated mitochondria (200 μg of proteins) with Mito-DEPMPO **7** (50 mM) in phosphate buffer, pH 7.3 after 20 min of incubation and then adding succinate (100 μM) at 37 °C; (b) as in (a) but with DEPMPO (50 mM); (c) as in (a) but without succinate; (d) as in (b) but without succinate. The gray lines represent the computer simulations of the spectra.

DEPMPO/OOH and DMPO/OOH adducts respectively. Moreover, the initial concentration of Mito-DEPMPO/OOH is significantly higher than that of DEPMPO/OOH, suggesting a higher affinity of the superoxide anions for the Mito-DEPMPO trap. Further work will be reported in due course to confirm the role of the positively charged triphenylphosphonium group that would facilitate the electrostatic guidance of the anionic superoxide radical towards the spin trap, leading to an increase in the superoxide trapping rate. It is noteworthy that many studies on Cu, Zn-superoxide dismutase (Cu, Zn-SOD) have revealed that cationic residues close to the active site play a key role in enhancing the dismutase activity.[17]

Preliminary experiments were performed with isolated mitochondria[18] from RAW 264.7 cells in the presence of Mito-DEPMPO **7** or DEPMPO (Fig. 2). In the presence of Mito-DEPMPO, we observed a mixture of superoxide (45%), hydroxyl (18%) and alkyl adducts (35%) when succinate was added for the activation of the Electron Transport Chain (ETC). However, in the presence of DEPMPO, we did not detect any superoxide adduct; only a weak signal from the hydroxyl adduct was observed [Fig. 2(b)]. In the absence of succinate, a significant decrease for both spectra was observed suggesting that the trapping of ROS is succinate-dependent and that they come from isolated mitochondria [Fig. 2(c) and 2(d)]. These results indicate that Mito-DEPMPO and not DEPMPO is able to trap superoxide formed in mitochondria.

In summary, NHS-DEPMPO is a versatile precursor for the preparation and targeting of a site-directed DEPMPO-based spin trap. Owing to its increased affinity for superoxide anions, Mito-DEPMPO may become the trap of choice for detecting and quantifying superoxide anions generated in biological systems. To our knowledge, this is the first experiment where ROR were detected from intact mitochondria by using the EPR spin-trapping technique.

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