Free Radical Research, November 2006; 40(11): 1166–1172

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Synthesis and characterization of a practically better DEPMPO-type spin trap, 5-(2,2-dimethyl-1,3-propoxy cyclophosphoryl)-5-methyl-1-pyrroline *N*-oxide (CYPMPO)

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Accepted by B. Kalyanaraman

(Received 5 June 2006; in revised form 29 June 2006)

Abstract

5-(2,2-Dimethyl-1,3-propoxy cyclophosphoryl)-5-methyl-1-pyrroline *N*-oxide (CYPMPO), a new cyclic DEPMPO-type nitrone was evaluated for spin-trapping capabilities toward hydroxyl and superoxide radicals. CYPMPO is colorless crystalline and freely soluble in water. Both the solid and diluted aqueous solution did not develop electron spin resonance (ESR) signal for at least 1 month at ambient conditions. CYPMPO can spin-trap superoxide and hydroxyl radicals in both chemical and biological systems, and the ESR spectra are readily assignable. Half life for the superoxide adduct of CYPMPO produced in UV-illuminated hydrogen peroxide solution was approximately 15 min, and in biological systems such as hypoxanthine (HX)/xanthine oxidase (XOD) the half-life of the superoxide adduct was approximately 50 min. In UV-illuminated hydrogen peroxide solution, there was no conversion from the superoxide adduct to the hydroxyl adduct. Although overall spin-trapping capabilities of CYPMPO are similar to DEPMPO, its high melting point, low hygroscopic property, and the long shelf-life would be highly advantageous for the practical use.

Keywords: *Spin trap, DMPO, DEPMPO, CYPMPO, superoxide*

Abbreviations: *DMPO, 5,5-dimethyl-1-pyrroline* N*-oxide; DEPMPO, 5-(diethoxyphosphoryl)-5-methyl-pyrroline* N*-oxide; CYPMPO, 5-(2,2-dimethyl-1,3-propoxy cyclophosphoryl)-5-methyl-1-pyrroline* N*-oxide; ESR, electron spin resonance; HFSC, hyperfine splitting constants; PMA, phorbol myristate acetate*

Introduction

Since its inception nearly 38 years ago [1,2], spin trapping technique has become an indispensable tool for research in free radical chemistry and biology. Because it is believed that oxygen radicals play a crucial role in biological oxidative damage, the essential requirements of spin trapping compounds (spin traps) must be their high ability of trapping and stabilizing oxygen radicals, such as superoxide and hydroxyl radicals. Although there have been several useful spin traps for the identification and quantification of biological oxygen radicals, DMPO (Figure 1 left) has been most

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ISSN 1071-5762 print/ISSN 1029-2470 online © 2006 Informa UK Ltd.
DOI: 10.1080/10715760600883254

frequently used [3,4]. However, it is regrettable that its short shelf-life, poor availability of the pure compound, and the short lifetime of the superoxide adduct seem to discourage and repel investigators from its wider use.

Summarizing the drawbacks of DMPO as a biological spin trap: (1) DMPO is difficult to handle because the melting point is 35°C [3]; (2) neat DMPO or its aqueous solution readily develop free radical impurities at ambient conditions [5]; (3) DMPO superoxide adduct is unstable (half life at pH7.4 is less than 1 min) [6,7]; and (4) DMPO superoxide adduct decomposes into hydroxyl adduct in the presence of metal ions [8]. In the attempt to overcome these drawbacks, several DMPO-analogs have been synthesized and tested. In those studies, structural modification of DMPO has been made by: (1) introducing one or more methyl groups into 3- or 4-position of pyrroline ring [9]; (2) replacing 5-methyl group with the higher alkyl, alkyloxy or carbamoyl group [10–12]; and (3) replacing one 5-methyl group with a substituted phosphoryl group [13–15]. Some of these spin traps have achieved considerable improvement in the stability of the superoxide adduct as compared to DMPO, and have become commercially available. For example, in the case of DEPMPO (Figure 1 center), some drawbacks of DMPO, such as poor stability of the spin trap itself and the superoxide adduct are improved. Thus, the use of DEPMPO has made it possible the first *in vivo* whole-body electron spin resonance (ESR) detection of spin adducts in mice [16]. However, neat DEPMPO is a very hygroscopic oil [13] and its handling and purification requires extra effort.

We report here the synthesis and characterization of a new DEPMPO-type spin trap, 5-(2,2-dimethyl-1,3-propoxy cyclophosphoryl)-5-methyl-1-pyrroline *N*-oxide (CYPMPO, IUPAC name: 2-(5,5-dimethyl-2-oxo-2λ^5-[1,3,2]dioxaphosphinan-2-yl)-2-methyl-3,4-dihydro-2*H*-pyrrole 1-oxide) (Figure 1, right). The structure of CYPMPO is similar to DEPMPO but the melting point is 126°C, therefore, CYPMPO is slightly hygroscopic colorless crystalline at room temperature. Moreover, diluted CYPMPO aqueous solution can be stored at ambient conditions for at least one month and no free radical impurities were developed. The stability of the hydroxyl and superoxide adducts was the same as or better than DEPMPO. Overall, we believe CYPMPO is more practically useful spin trap than DMPO and DEPMPO, and its use would expand the versatility of spin trapping technique in biological oxygen radical research.

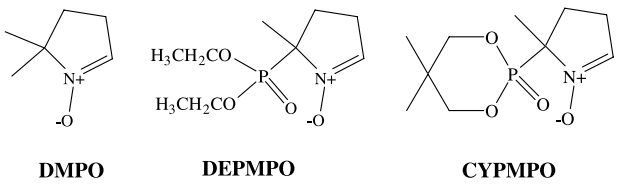

Figure 1. Structures of DMPO, DEPMPO and CYPMPO.

Materials and methods

CYPMPO synthesis and starting chemicals

CYPMPO was synthesized following the reaction scheme shown in Figure 2. The starting cyclic hydrogen phosphite **(1)** was synthesized according to the published procedure [17]. Briefly, ethanol (0.3 mol) and 2,2-dimethyl-1,3-propanediol (0.3 mol) were mixed and stirred while phosphorus trichloride (0.3 mol) was added dropwise. The temperature during the addition was kept below 20°C. The reaction mixture was vacuum-distilled and the product was collected at 142–145°C (2.9 mmHg) and identified as **(1)** using H^1 NMR.

At room temperature, 2-methyl 1-pyrroline **(2)** (17 g, 0.24 mol) was added dropwise under stirring to a dichloromethane solution of the cyclic phosphite **(1)** (36 g, 0.24 mol in 100 ml dichloromethane). The reaction mixture was passed through a silica-packed glass column (2.5 cm diameter, 30 cm long; eluent: chloroform/ethanol = 10/1) and the product **(3)** was separated. The solvent was removed with a vacuum rotary evaporator, yielding crude crystal of **(3)** (24.5 g, 48.2% yield, mp 109–112°C). m-Chloroperbenzoic acid (mCPBA) oxidation was performed as follows: To a dichloromethane solution of **(3)** (100 mmol in 230 ml dichloromethane), mCPBA (100 mmol in 200 ml dichloromethane) was added dropwise on ice. The reactant was washed with a saturated sodium carbonate solution, dried on magnesium sulfate, and subsequently solvent was evaporated off. The product CYPMPO was separated with a silica gel packed glass column (2.5 cm diameter, 30 cm long; eluent: toluene/ethanol = 9/1), yielding 6.3 g of CYPMPO crystal (mp 126°C, yield 49%). CYPMPO was purified with re-crystallization in toluene or ethyl acetate. All chemicals used in the synthesis and free

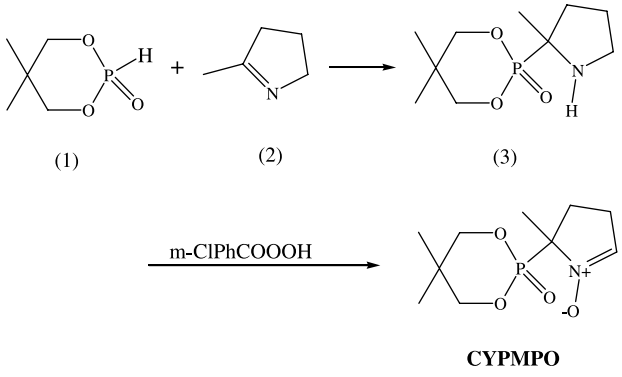

Figure 2. Synthetic reaction scheme of CYPMPO.

radical generation were purchased from Tokyo Chemical Industry Co. Ltd. (Tokyo, Japan) except otherwise specified. Xanthine oxidase (XOD) was purchased from Roche (Indianapolis, USA).

Physico-chemical analysis of CYPMPO

Elemental analysis was preformed by the Elemental Analysis Facility in Kyoto Pharmaceutical University. Octanol/water distribution coefficient was determined using the established spectroscopic method [18]. $^{1}H/^{13}C$ NMR spectra of CYPMPO were recorded in $CDCl_3$ using a Varian Unity Inova 400 MHz NMR spectrometer (Palo Alto, CA, USA). Positive ion mass spectra were obtained in a JEOL JMS-SX 102A QQ mass spectrometer (Akishima, Tokyo, Japan) with the fast-atom bombardment (FAB) method using glycerol as a matrix. IR spectra were obtained with the KBr-disk method by employing a Shimadzu FT-IR spectrometer (Kyoto, Japan).

Hydroxyl and superoxide adduct formation and ESR spectra

Hydroxyl radical was produced using two independent methods: (1) short *in situ* illumination (typically 5 s) of UV light [19] from a medium-pressure mercury arc (RUVF-203SF, Radical Research Inc, Hino, Tokyo, Japan) to a dilute aqueous solution (0.3 M, 1%) of hydrogen peroxide in the presence of 5–20 mM CYPMPO, and (2) Fenton reaction system which consists of phosphate buffer (100 mM) solution of 0.05 mM ferrous sulfate, 1 mM hydrogen peroxide, and 5–20 mM CYPMPO. Superoxide radical was spin-trapped *in situ* by illuminating UV to 3 M (10%) aqueous hydrogen peroxide solution for 5 s. Magnetic field scan was initiated immediately after illumination was terminated. In biological systems, superoxide was produced in (1) hypoxanthine (HX)/XOD system, where 250 μM HX, 25 mU/ml of XOD, 0.1 mM DETAPAC, and 5 mM CYPMPO were mixed in phosphate buffer (100 mM); or (2) phorbol myristate acetate (PMA) or opsonized-zymozan (Sigma Chemical Co. St Louis, MO, USA) stimulated bovine neutrophils. Previously published procedures were employed in the isolation and stimulation of neutrophils [20]. In this method, bovine blood was collected strictly following the protocol approved by the institutional animal care and use committee in School of Veterinary Medicine, Hokkaido University.

Spin trapping kinetics

Pseudo first-order decay of the spin adduct was measured by repeatedly recording the ESR signal intensity. Decay constants of CYPMPO superoxide adduct were measured in: (1) UV-illuminated 1% hydrogen peroxide aqueous solution, and 5 s after illumination the decay was recorded; and (2) HX/XOD system and the decay was recorded after the addition of XOD inhibitor allopurinol (0.5 mM) following the method previously published [20]. Although the decay should follow second order kinetics, under the condition that the spin trap concentration or hydronium ion presents in large excess, it can be treated as pseudo first-order, *i.e.* the half-life is not a function of component concentration. Also, decay rates were calculated based on the first-derivative ESR intensity, thus it may differ from the rate calculated from the concentration.

Relative spin trapping rates for superoxide and hydroxyl radicals were determined using a competitive-trapping method, where two spin traps are mixed and ESR signal intensities are compared. However, due to heavy overlap of ESR lines from DEPMPO- and CYPMPO-derived spin adducts, we were unable to directly compare DEPMPO and CYPMPO. Therefore, we obtained relative spin trapping rates with respect to DMPO.

ESR measurements

ESR spectra were recorded at room temperature in a X-band JEOL JES-TE 300 ESR spectrometer (Akishima, Tokyo, Japan) with WIN-RAD operation software (Radical Research Inc.) using disposable ESR flat cells. Typical instrumental settings were as follows: incident-microwave 8 mW, modulation-amplitude 0.1 mT, time-constant 0.1 s, and sweep-rate 5 mT/min. Computer ESR spectrum simulation was carried out using a WIN-RAD software package (Radical Research Inc.)

Results

Chemical and physical analysis of CYPMPO

CYPMPO is slightly hygroscopic colorless crystalline with the melting point of 126. Octanol/water distribution coefficient of CYPMPO is approximately 0.1, indicating it is soluble in water 10 times more than in octanol (DMPO: 0.02–0.09, DEPMPO: 0.06). CYPMPO did not develop ESR-active species when its 50 mM aqueous solution (not buffered) was left at ambient conditions in a closed brown bottle for one month. Table I lists chemical/physical properties of CYPMPO that are relevant to practical spin trapping experiments, including ESR spectroscopic parameters and kinetic constants of CYPMPO hydroxyl and superoxide adducts. Also listed are the properties of DMPO and DEPMPO for the sake of comparison.

The results of physico-chemical and spectroscopic analysis are as follows: Elemental analysis ($C_{10}H_{18}HO_4P$ FW. 247.23) *Calcd.* C48.58, H7.34, N5.67. *Found* C48.83, H7.16, N5.50; IR spectra (cm^{-1}): 2956.9, 1573.9, 1473.9, 1465.9, 1249.9,

1060.9, 1020.3, 991.4, 831.3, 792.7; ^{1}H NMR (in $CDCl_3$), δ0.91 (3H, s), 1.29 (3H, s), 1.77(3H, d, $\mathcal{J}$ = 15.0 Hz), 2.20 (1H, dddd, $\mathcal{J}$ = 7.0, 9.5, 13.9, 19.8 Hz), 2.60–2.70 (1H, m), 2.71–2.82 (1H, m), 3.01 (1H, dddd, $\mathcal{J}$ = 4.0, 9.5, 13.9, 16.1 Hz), 3.91–4.01 (2H, m), 4.22 (1H, dd, $\mathcal{J}$ = 3.8, 10.3 Hz), 4.79 (1H, dd, $\mathcal{J}$ = 3.7, 10.4 Hz), 6.94 (1H, q, $\mathcal{J}$ = 2.8 Hz); ^{13}C NMR (in $CDCl_3$), δ 20.4, 20.6, 22.0, 25.7, 30.9, 32.6, 75.3, 77.5, 79.3, 135.0; ^{31}P NMR (in $CDCl_3$): δ 14.5; Mass spectra, FAB$^+$, m/z (%): M$^+$248(100), 149(33), 98(82).

ESR spectra of hydroxyl and superoxide adducts of CYPMPO

Figure 1A and B show the ESR spectra of CYPMPO-trapped product in UV-illuminated hydrogen peroxide solution (1%) and Fenton reaction system, respectively (Figure 3 top, three middle). Both spectra consist of two identical groups of lines which are split by a large hf coupling with phosphorus 31. The spectral pattern is very similar to hydroxyl adduct of DEPMPO, therefore it is rational to assign these ESR spectra to hydroxyl adduct of CYPMPO. The fact that UV-illuminated dilute hydrogen peroxide is a pure hydroxyl radical source supports the assignment [21]. The spectra were reproduced with the computer spectrum simulation (Figure 3 bottom) and hyperfine splitting constants (HFSCs) used are listed in Table I.

ESR spectra of the superoxide adduct of CYPMPO was obtained from well-established superoxide source, *i.e.* in UV-illuminated 10% hydrogen peroxide solution (Figure 4A), HX/XOD system (Figure 4B), and stimulated neutrophils (Figure 5 top and middle). These spectra very similar pattern to DEPMPO superoxide adduct which supports the assignment. The bottom spectrum in Figure 4 is a computer-simulated spectrum for superoxide adduct. HFSCs used in this simulation are listed in Table I.

Kinetic measurement

Half-lives measured with the method described in the experimental section are listed in Table I. The decay curve of the superoxide adduct in HX/XOD system recorded after the addition of allopurinol is shown in Figure 6. Logarithmic plot shown in the left panel of Figure 6 indicates linear curve suggesting that the decay is apparent first-order.

Table I. Chemical and physical properties of the spin trap DMPO, DEPMPO, and CYPMPO.

	DMPO⋆	DEPMPO†	CYPMPO
Appearance at ambient conditions	Colorless solid (not weighable)	Viscous oil	White powder (weighable)
Melting point	35°C	< RT(n.d.)	126°C
Water solubility	Good	Good	Good
Octanol/water distribution coefficient	0.02–0.09	0.06	0.11
Hygroscopic property	Unknown	High	Low
Free radical impurities in neat form	Rapid development	Slow development	Slow development
Stability of aqueous solution at RT	Spontaneous free radical formation	Unknown	No formation of free radicals
Purify with re-crystallization?	No	No	Yes
Recommended store temperature	−80°C	−80°C	4°C
Commercial availability	Yes†	Yes‡	–¶
OH adduct			
Diastereomer	No	Yes	Yes
HFSC(A_N, A_H, A_P)/mT	1.49, 1.49, –	1.40, 1.30, 4.74	1.37, 1.37, 4.88 1.35, 1.23, 4.70
Half life (pH 7.4)	>60 min	>60 min	>60 min
Trapping rate	$>10^9\,M^{-1}\,s^{-1}$	$>10^9\,M^{-1}\,s^{-1}$	$>10^9\,M^{-1}\,s^{-1}$
O_2^- adduct			
Diastereomer	No	Yes	Yes
HFSC(A_N, A_H, A_P)/mT	1.42, 1.15, 0.13($A\gamma_H$)	1.34, 1.19, 5.25 1.32, 1.05, 4.85	1.34, 1.19, 5.15 1.35, 1.07, 4.95
Half life	1 min§	15 min§ 68 min‖,#	15 min§ 51 min‖
Trapping rate ratio (Actual rate)	1($16\,M^{-1}\,s^{-1}$)	1.5($24\,M^{-1}\,s^{-1}$)	3($48\,M^{-1}\,s^{-1}$)

⋆ Information gathered from MSDS and reference [5]. † From Aldrich Chemical Co. (Milwaukee, WI, USA), Sigma Chemical Co. (St Louis, MO, USA), and Dojin Chemicals (Kumamoto, Japan). Other commercially available DMPO-type spin trap includes BMPO [11], which is available from Northwest Life Science Specialties (Vancouver, WA, USA). ‡ From Alexis Biochemicals (San Diego, CA, USA). ¶ Commercialization considered by Radical Research Inc. § Calculated from spontaneous decay curve of ESR signal intensity of CYPMPO superoxide adduct produced with *in situ* illumination of UV light to 1% hydrogen peroxide aqueous solution. ‖ Calculated from the decay curve of ESR signal intensity of CYPMPO superoxide adduct produced in hypo xanthine/XOD system. The decay was recorded after the addition of XOD inhibitor allopurinol (Figure 6). # From Ref. [20].

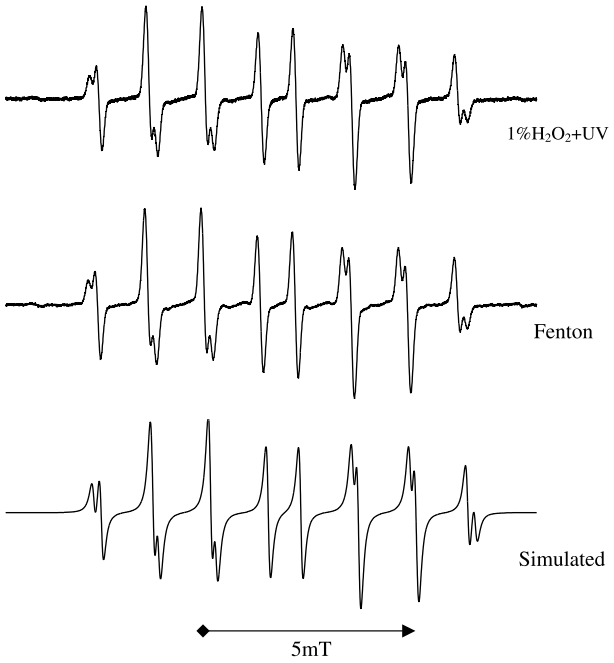

Figure 3. ESR spectrum of hydroxyl adduct of CYPMPO. OH adduct produced in: UV-illuminated hydrogen peroxide (1% 0.3 M) (top); Fenton system, 1 mM H2O2 plus 0.05 mM ferrous sulfate (middle); and computer-simulated spectrum for OH adduct of CYPMPO (HFSCs used for computer-simulation are listed in Table I) (bottom).

Discussion

In this paper, we describe the synthesis and characterization of a new DEPMPO-type spin trap, CYPMPO. The trapping rate for hydroxyl and superoxide radicals and the stability of the spin adducts were very similar to DEPMPO. Furthermore, like spin adducts of DEPMPO almost all spin adducts of CYPMPO possess two asymmetric (chiral) carbons, which results in the presence of diastereomer. Thus, ESR spectrum consists of the overlap of two radical species with very similar HFSCs. As we noted in the previous papers there have been considerable confusion in the description of diastereomer of spin adduct origin [22]. It should be emphasized that diastereomer is neither conformational isomer nor cis-trans isomer. Diastereomers have different chemical and physical properties including ESR parameters, and are essentially isolable.

The ability of CYPMPO in trapping superoxide was as effective as DEPMPO both in chemical and biological superoxide generating systems. In PMA-stimulated bovine neutrophils, previous report indicated that unlike human or mouse neutrophils DMPO was unable to trap superoxide radical and only hydroxyl radical adduct was detected, however, DEPMPO did trap superoxide [20]. We show in this report that the ESR spectra of CYPMPO adduct in

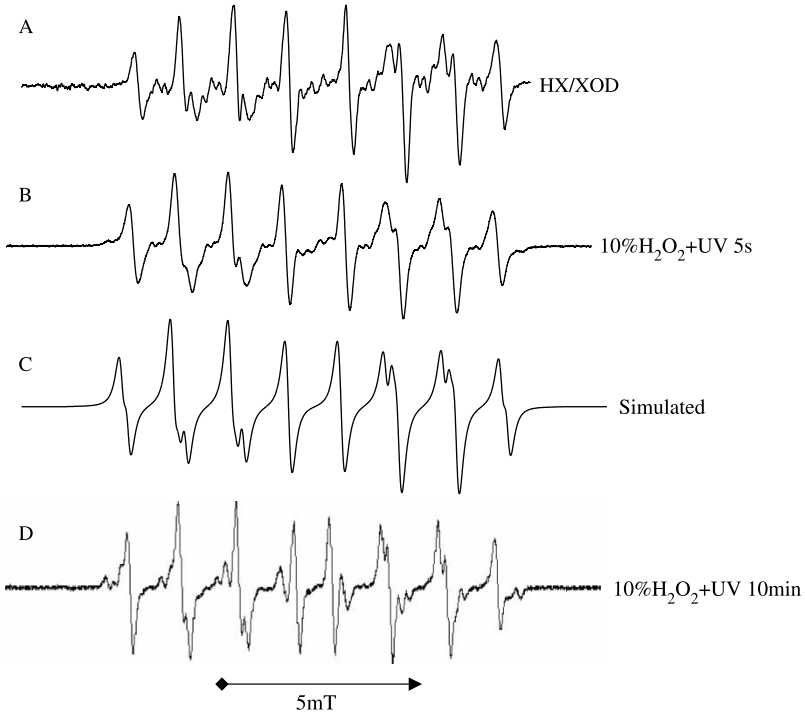

Figure 4. ESR spectrum of CYPMPO superoxide adduct. Superoxide adduct produced in: (A) HX/XOD; (B) UV-illuminated hydrogen peroxide (10%, 1 M); (C) Computer-simulated spectrum for superoxide adduct (HFSCs used for computer-simulation are listed in Table I); and (D) Both hydroxyl and superoxide adducts were produced after 10 min UV illumination to 10% hydrogen peroxide. Well separated two pairs of lines at the center are assigned to superoxide adduct (wider pair, weaker intensity) and hydroxyl adduct (narrower pair, stronger intensity), respectively.

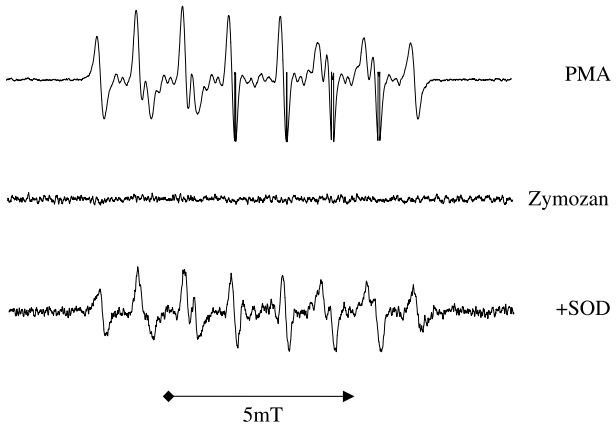

Figure 5. ESR spectrum recorded in stimulated bovine neutrophils in Hanks balanced salt solution: PMA-stimulated (top); PMA-stimulated in the presence of SOD (middle); and Opsonized zymozan stimulated (bottom).

bovine neutrophils were very similar to DEPMPO, suggesting that the superoxide trapping ability of CYPMPO is as good as DEPMPO. Unlike DMPO, we did not see the decomposition of the superoxide adduct into hydroxyl adduct (data not shown), therefore the small hydroxyl adduct signal in Figure 2 is not artifact. The kinetic measurement of CYPMPO superoxide adduct in HX/XOD system at pH 7.4 indicated that pseudo first-order decay half-life is 15 ± 1 min. This half-life is the same as

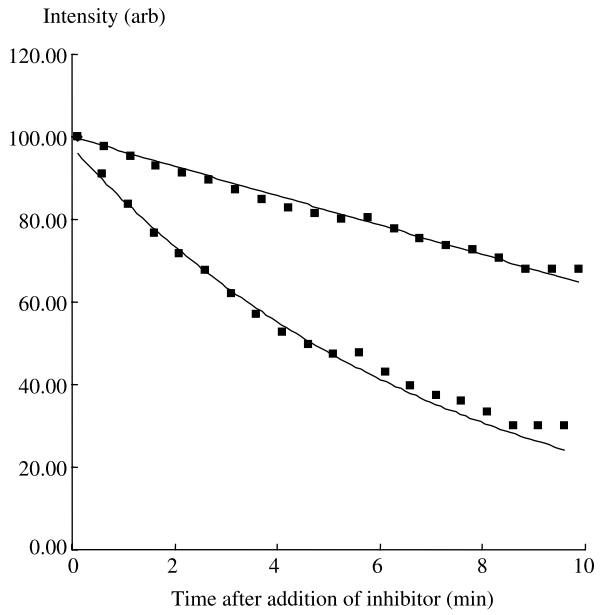

Figure 6. Decay of the ESR signal of CYPMPO superoxide adduct produced in the HX/XOD system after the addition of XOD inhibitor, allopurinol. ESR signal intensities (arbitrary unit) are plotted as a function of time (min) after the addition of allopurinol. Logarithmic plot (top line) of the decay is linear (no scale is shown). The half-life of CYPMPO superoxide adduct was calculated to be 50.6 min (see, Table I for the comparison with DEPMPO).

DEPMPO superoxide adduct, suggesting that electronic influence to the adduct stability by the diethoxy phosphoryl and alicycloxy phosphoryl group is similar.

Both CYPMPO and DEPMPO have excellent spin trapping capabilities toward superoxide and hydroxyl radicals, in addition, some ESR lines in the both adducts are clearly separated from each other and readily identifiable. However, practically speaking, we found that the handling of CYPMPO is considerably less demanding than DEPMPO and DMPO. Moreover, commercial DEPMPO was reported to contain hydroxyl amines as contaminants, which interfered with spin trapping reaction [23]. In contrast, CYPMPO is less hydrophilic and can be readily purified with re-crystallization from organic solvent such as toluene or ethyl acetate. Our test showed that CYPMPO aqueous solution (50 mM) did not develop ESR-active species for more than one month at room temperature (data not shown), suggesting that stock solutions of CYPMPO may be storable for a long period. Whereas, in the case of DMPO, in addition to poor commercial availability of pure (free-radical impurity free) spin trap, one must prepare fresh spin trap solution immediately before spin trapping experiments. We believe that CYPMPO will become practically very useful spin trapping agent.

Acknowledgements

We thank Masashi Shinmei, Radical Research Inc., Hino, Tokyo, Japan for his enthusiastic support and encouragement in developing CYPMPO. We also thank Shunji Terada and Kayoko Oda at Kyoto Pharmaceutical University for their elemental analysis (ST) and mass spectrometer operation (KO).

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