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Synthesis and structure of 5,5-diethoxycarbonyl-1-pyrroline *N*-oxide (DECPO). Application to superoxide radical trapping

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Abstract—DECPO, a new analogue of EMPO was synthesized through a two-step synthetic pathway. Its structure and its application to trap superoxide were investigated. The ESR detection of the DECPO–OOH spin adduct is easy even at low concentration of superoxide. In comparison with DEPMPO, the trapping of superoxide with DECPO is faster and the detection of DECPO–OOH can be performed using a very low nitrone concentration (0.5 mM).

5,5-Dimethyl-1-pyrroline *N*-oxide (DMPO) **1** (Fig. 1) has been extensively used to spin trap oxygen-centred radicals such as $O_2^{\cdot-}$ and $HO^{\cdot}$, which play a major role in various oxidative stress processes.[1] The resulting spin adducts are then characterized by ESR. However, at physiological pH, the half-life of the DMPO–superoxide adduct (DMPO–OOH) is close to 60 s and its ESR spectrum is rapidly replaced by the spectrum of DMPO–OH.[2] Moreover, for superoxide the kinetics of trapping by DMPO are slow ($60\ M^{-1}\ s^{-1}$)[2] and thus in many instances the ESR characterization of DMPO–OOH is very tedious. We have shown that replacing one methyl group of DMPO with an electron withdrawing group such as $P(O)(OCH_2CH_3)_2$ or $COOCH_2CH_3$ yielded nitrones, DEPMPO[3] **2**, and EMPO[4] **3**, respectively (Fig. 1), which form superoxide spin adducts with significantly increased half-lives. Moreover, at low superoxide concentration the decay of either DEPMPO–OOH or EMPO–OOH was not accompanied by the formation of significant amounts of the corresponding hydroxyl radical spin adducts. The same trend was observed when a methyl group was replaced with a trifluoromethyl group.[5] However, when the two methyl groups of DMPO were replaced by diethoxyphosphoryl groups, in phosphate buffer, the superoxide spin adduct of the resulting nitrone **4** (Fig. 1) decomposed rapidly to the corresponding hydroxyl spin adduct.[6] In our continuing efforts to understand the electronic and steric effects, which influence the stability of superoxide spin adducts of pyrroline *N*-oxides, we have prepared the 5,5-diethoxycarbonyl-1-pyrroline *N*-oxide (DECPO) **5** and investigated its structure and its use in characterizing superoxide in phosphate buffer at physiological pH. Our preliminary results are presented herein.

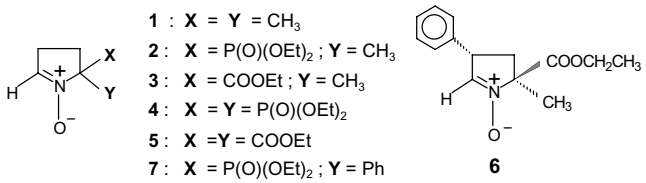

Figure 1. Chemical structure of spin traps.

1. Experimental

The synthesis of a pyrroline *N*-oxide can be achieved by reductive cyclization of the appropriate γ-nitroaldehyde using zinc in the presence of NH_4Cl or CH_3COOH.[7–11]

Keywords: nitrone; spin trapping; DECPO; EMPO; electron spin resonance; superoxide.
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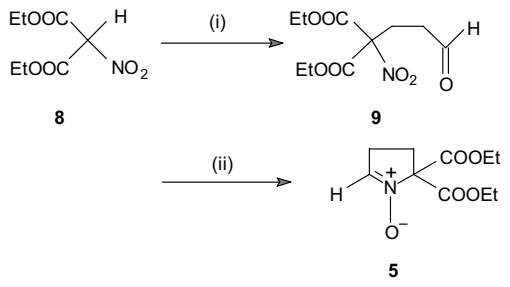

Scheme 1. Synthetic pathway for **5**: (i) acrolein, Et_3N in CH_3CN, 90%; (ii) Zn, H_2O/THF, NH_4Cl, 33%.

This approach was applied to synthesize DECPO. 1,4-Addition to acrolein[12,13] of the carbanion derived from diethyl nitromalonate **8** (Scheme 1, (i)) afforded diethyl 5-oxo-2-nitropentan-2-yl-dicarboxylate **9**. DECPO **5** was obtained after reduction of the nitro function to the hydroxylamine and subsequent in situ cyclization (Scheme 1, (ii)).[14]

2. X-ray structure of DECPO

The X-ray structure of DECPO[15] is shown in Figure 2. Some of its geometric features together with those of $EMPPO_t$[16] **6** and DEPPPO[17] **7** (Fig. 1) are collected in Table 1. For these three nitrones the pyrroline *N*-oxide ring adopts a ^4E conformation, the envelope amplitude being rather large for **7** and **5**. As a consequence of the large envelope amplitude on C4, one ethoxycarbonyl group of **5** is equatorial and one face of **5** (face A, Fig. 2) should be approached easily by the attacking radical. The presence of two electron withdrawing ethoxycarbonyl groups in **5** favors the dipolar canonical electronic

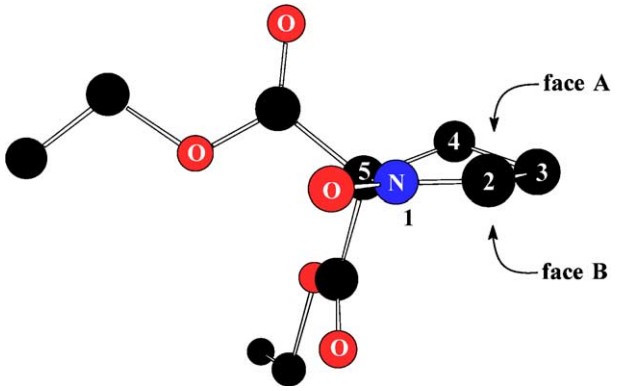

Figure 2. X-ray structure of DECPO **5**.

Table 1. Selected geometric features determined by X-ray for the nitrones **5**, **6**, and **7**

Nitrones	N–O (Å)	C=N (Å)	C_2NC_5 (°)	$C_2NC_5C_4$ (°)
6	1.285	1.272	113	−7.8
5	1.283	1.294	111.6	−17.3
7	1.300	1.303	111	−17.8

structure ($^-$O–N–C$^+$) and leads to a significant lengthening of the ON=C bond (1.272 Å for **6** and 1.294 Å for **5**) (Table 1).

3. ESR studies

When superoxide is produced via the hypoxanthin (HX; 0.2 mM)—xanthin oxidase (XOD; 0.05 units per mL) system in the presence of 25 mM of DECPO in oxygenated phosphate buffer (0.1 M, pH 7.4), we observed an ESR signal corresponding to superimposition of the signals of DECPO–OOH (♦) and DECPO–OH (•) spin adducts (Fig. 3a). The presence of a high concentration of superoxide dismutase (SOD) (1250 units per mL) in the incubation mixture abolished the signal (data not shown) proving that it was due to the trapping of superoxide and that the signal of DECPO–OH resulted from the chemical fate of DECPO–OOH. To investigate the effect of the superoxide concentration on the for-

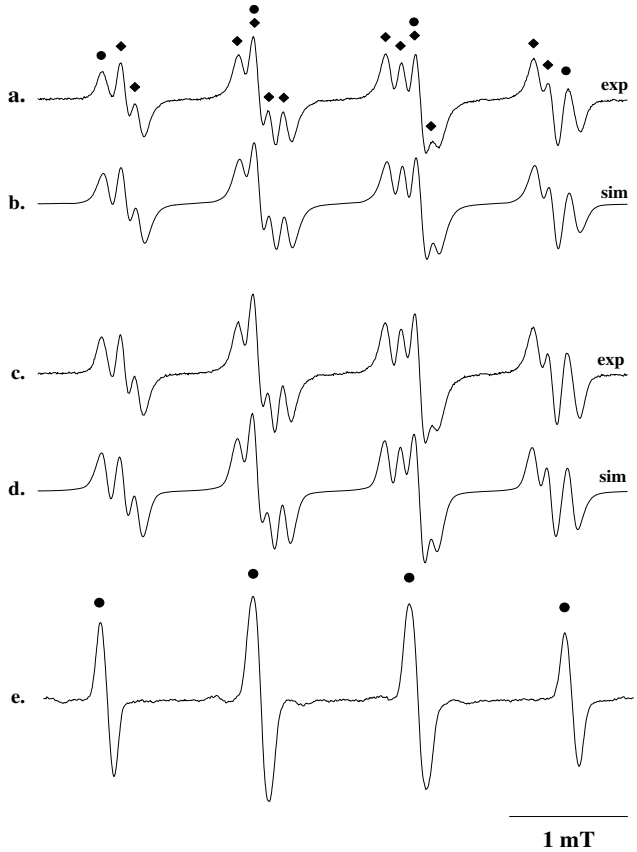

Figure 3. ESR spectra of DECPO–superoxide and DECPO–hydroxyl spin adducts. (a) ESR signal obtained upon incubating for 60 s an oxygenated-phosphate buffer (0.1 M, pH 7.4) solution containing HX (0.2 mM), DTPA (0.5 mM), and XOD (0.05 units per mL) in the presence of DECPO (25 mM); (b) computer simulation of experimental spectrum (a); (c) as (a) but in the presence of XOD (0.1 units per mL); (d) computer simulation of experimental spectrum (c); (e) authentic DECPO–OH spin adduct signal obtained after adding $FeSO_4$ (0.5 mM) to a solution containing DECPO (5 mM) and H_2O_2 (1 mM) in deoxygenated phosphate buffer (0.1 M, pH 7.4).

mation of DECPO–OH, we used different xanthin oxidase concentrations.

The intensity of the DECPO–OH signal increased when either the time of incubation (data not shown) or the xanthin oxidase concentration was increased (16% for 0.01, 23% for 0.05, and 35% for 0.1 units per mL). This result indicates that the formation of DECPO–OH resulted from the reaction of superoxide with DECPO–OOH. The superoxide reaction with nitroxide spin adducts and stable nitroxides has already been mentioned[18–20] and the same influence of the superoxide concentration was observed during its trapping with DMPO, EMPO, and DEPMPO.[21] An authentic DECPO–OH signal was generated using a Fenton system [H_2O_2 (2 mM), $FeSO_4$ (0.5 mM)] in deoxygenated phosphate buffer in the presence of DECPO (5 mM) (Fig. 3e). The 1/2/2/1 four-line signal ($a^N = 1.33$ and $a^{H\beta} = 1.275$ mT) (•) obtained is very similar to that of the DMPO–OH spin adduct.

In order to avoid the continuous formation of superoxide in our incubation mixtures, we decided to use KO_2 (0.1 M) in DMSO in the presence of 1 equiv of crown ether (18C6). When 60 μL of KO_2 (20 mM final concentration) solution in DMSO was added to 240 μL of a deoxygenated phosphate buffer solution containing DECPO (2.5 mM final concentration), an intense 12-line signal was observed (Fig. 4). In the presence of SOD (2500 units per mL) in the incubation mixture the signal was dramatically reduced proving that it was the result of the trapping of superoxide with DECPO. The 12 line ESR signal of DEPCO–OOH is very similar to those of

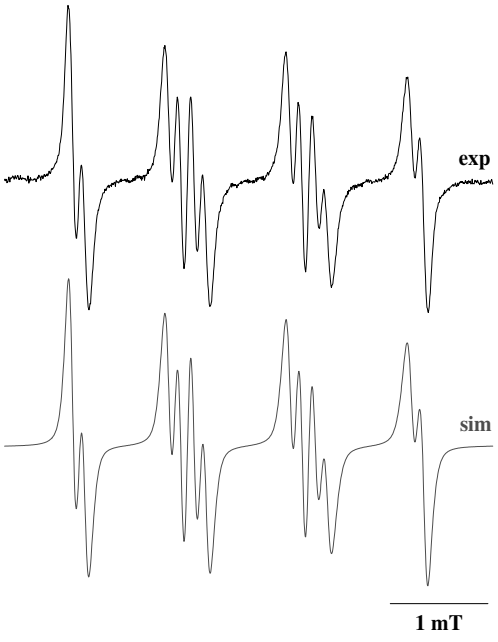

Figure 4. ESR spectrum of DECPO–superoxide spin adduct at physiological pH. ESR signal observed upon incubating for 60 s the reaction mixture obtained after adding a DMSO solution of KO_2 (20 mM final concentration) containing 1 equiv of crown ether to a deoxygenated-phosphate buffer (0.1 M, pH 7.4) solution containing DECPO (2.5 mM final concentration).

DMPO–OOH and EMPO–OOH, it was satisfactorily simulated assuming a slow exchange between two DECPO–OOH conformers **A** and **B** (**A** (8%): $a^N = 1.24$ and $a^{H\beta} = 1.135$ mT and **B** (92%) $a^N = 1.24$, $a^{H\beta} = 0.98$ and $a^{H\gamma} = 0.10$ mT) (Fig. 4). No satisfactory simulation could be obtained assuming a significant contribution of the DECPO–OH signal to the observed spectrum.

In our experimental conditions, the rate constant for the spontaneous disproportionation of superoxide is close to $2.4 \times 10^5\ M^{-1}\ s^{-1}$ at pH 7.4[22] and the half-life of superoxide is very short (2.1×10^{-4} s for 20 mM of KO_2 concentration). Thus, when KO_2 is reacted in phosphate buffer with a pyrroline *N*-oxide the decay of the resulting superoxide spin adduct is not mediated by superoxide, as is observed when a continuous flux of superoxide is generated by the hypoxanthin/xanthin oxidase system.

Using KO_2 as a source of superoxide, we evaluated the persistency of the DECPO–OOH adduct at pH 7.4. The decay of the DECPO–OOH signal was monitored by following the evolution of the low field peak. The monitoring was started 150 s after the addition of KO_2 (5 mM) to the phosphate buffer solution of DECPO (10 mM). The half-life (305 ± 13 s)[†] was obtained assuming a first-order decay and was not dependent on the presence of either SOD (1250 units per mL) or catalase (630 units per mL) in the incubation mixtures. The same experiment was performed with DEPMPO, which remains the most effective nitrone to detect superoxide. We found for the DEPMPO–OOH spin adduct a half-life of 1090 s, a value slightly greater than the values previously reported.[23,24] However, although the DEPMPO–OOH spin adduct was found to be more persistent than DECPO–OOH, we observed that under the same experimental conditions the concentration of the latter was larger (close to 8 times according to the signal intensities). The larger concentration of DECPO–OOH could be accounted for by a faster kinetic of trapping. Villamena[24] has shown that the kinetic of superoxide trapping on pyrroline *N*-oxides increases when the positive charge on the carbon of the nitronyl function (C2, Fig. 2) increases. We have calculated at the B3LYP/6-31G(d) level the net atomic charges (Mulliken) on C2 for DMPO (+0.052), EMPO (+0.061), DECPO (+0.069), and DEPMPO (+0.074). According to these calculations the faster kinetics of superoxide trapping observed for DECPO cannot be explained by electronic factors. Tanigushi has shown that the trapping of free radicals with DMPO is very sensitive to steric factors.[25] As we discussed above, in the case of DECPO one face of the nitrone (face A, Fig. 2) is easily available to the attacking free radical and that could explain the faster kinetics of radical trapping.

Owing to the reactivity of superoxide with nitroxide spin adducts, the concentration of superoxide is a key factor to be considered during the analysis of a superoxide spin trapping experiment. Due to the fast kinetics of

[†] Value corresponding to the mean average of three measurements.

superoxide trapping, DEPCO can be used at very low concentrations (as low as 0.5 mM) and it appears to be an interesting tool for characterizing superoxide in a biological medium. Detailed kinetic analysis of our experiments is in progress and will be published in a forthcoming paper.

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13. To a cooled (10 °C) mixture of diethyl nitromalonate **8** (15.8 g, 77 mmol) and acrolein (4.35 g, 77.8 mmol) in CH_3CN (50 mL) were slowly added 20 drops of triethylamine. The mixture was then stirred for 2 h at 20 °C and then concentrated under reduced pressure to give **9** as an orange oil (20 g, 99%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.77 (s, 1H); 4.36 (q, 4H, $J = 9$ Hz); 2.73–2.87 (m, 4H); 1.33 (t, 6H, $J = 9$ Hz); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 198.7, 162.3, 96.3, 63.9, 38.6, 26.1, 13.6.
14. To **9** (4 g, 15.3 mmol) in THF (160 mL) at 0 °C was added a solution of NH_4Cl (5 g, 93.45 mmol) in water (25 mL). The resulting mixture was stirred at 0 °C and then Zn dust (3.12 g, 47.7 mmol) was added portionwise over 2 h. The mixture was then stirred for 2 h at 0 °C. The mixture was saturated with NaCl and Et_2O (50 mL) was added. The mixture was filtered off and water was separated. The organic phase was washed with brine (20 mL), dried over Na_2SO_4 and concentrated under reduced pressure. The residue was purified by column chromatography (silica, CH_2Cl_2/EtOH 96:4) to give pure DECPO as a white powder, which was crystallized from CH_2Cl_2 (1.15 g, 33%) (mp 75 °C). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.01–7.03 (m, 1H); 4.37 (q, 4H, $J = 9$ Hz); 2.70–2.90 (m, 4H); 1.34 (t, 6H, $J = 9$ Hz); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 165.4, 135.8, 84.8, 63.2, 29.9, 26.2, 13.9.
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