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Very stable superoxide radical adducts of 5-ethoxycarbonyl-3,5-dimethyl-pyrroline *N*-oxide (3,5-EDPO) and its derivatives

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Abstract

Oxygen radicals are involved in the onset of many diseases. Adequate spin traps are required for identification and localisation of free radical formation in biological systems. Superoxide spin adducts with half-lives up to 20 min at physiological pH have recently been reported to be formed from derivatives of the spin trap 5-ethoxycarbonyl-5-methyl-1-pyrroline *N*-oxide (EMPO). This is a major improvement over DMPO ($t_{1/2}$ ca. 45 s), and even DEPMPO ($t_{1/2}$ ca. 14 min). In this study, an additional methyl group was introduced into position 3 or 4 of the pyrroline ring which greatly increases the stability of the respective superoxide spin adducts. In addition, the ethoxy group of EMPO was exchanged by either a propoxy- or an *iso*-propoxy group in order to test the influence of increasing lipophilic properties of the investigated spin traps. The structure of all compounds was confirmed by ^{1}H and ^{13}C-NMR with full signal assignment. In comparison with EMPO ($t_{1/2}$ ca. 8 min) or DEPMPO ($t_{1/2}$ ca. 14 min), the superoxide adducts of all novel spin traps were considerably higher ($t_{1/2}$ ca. 12–55 min). In addition, various other spin adducts obtained from oxygen-centered as well as carbon-centered radicals (e.g. derived from methanol or linoleic acid hydroperoxide) were also detected.

Keywords: Spin traps; EPR; EMPO derivatives; Superoxide; Linoleic acid hydroperoxide; Free radicals

1. Introduction

Derivatives of the spin trap EMPO (5-ethoxycarbonyl-5-methyl-1-pyrroline *N*-oxide) have recently been described by several authors to be superior to the parent compound itself [1–3]. Compounds having bulky alkoxycarbonyl substituents formed considerably more stable superoxide adducts ($t_{1/2}$ = 15–25 min [1,3]) as compared to the structurally related spin traps DMPO ($t_{1/2} < 1$ min [4]), EMPO ($t_{1/2}$ ca. 8 min [5–7]), or even DEPMPO ($t_{1/2}$ ca. 14 min [8–10]). Since the observed spin adduct stabilisation was obviously not only due to the electron-withdrawing effect, but also due to steric influences of the alkoxycarbonyl group, it was expected that the incorporation of an additional methyl group at position 3 or 4 of the pyrroline ring of EMPO derivatives might cause increased steric shielding and thus lead to an even higher stability of the superoxide adducts.

Preliminary experiments on trapping of radicals derived from peroxidized linoleic acid using different spin traps, such as DMPO [11], DEPMPO [9,10], EMPO [7] or Trazon [12,13], have recently been reported, although an optimal spin trap for the detection of all types of oxygen- and carbon-centered radicals has not yet been found.

Aim of the present work was the synthesis of a series of 3,5- or 4,5-dimethylated pyrroline derivatives with differ-

Abbreviations: DEPMPO, 5-(diethoxyphosphoryl)-5-methyl-1-pyrroline *N*-oxide; DMPO, 5,5-dimethylpyrroline *N*-oxide; DTPA, diethylenetriaminepentaacetic acid; 3,5-EDPO, 5-(ethoxycarbonyl)-3,5-dimethyl-1-pyrroline *N*-oxide; 4,5-EDPO, 5-(ethoxycarbonyl)-4,5-dimethyl-1-pyrroline *N*-oxide; 3,5-DIPPO, 3,5-dimethyl-5-(iso-propoxycarbonyl)-1-pyrroline *N*-oxide; 4,5-DIPPO, 4,5-dimethyl-5-(iso-propoxycarbonyl)-1-pyrroline *N*-oxide; 3,5-DPPO, 3,5-dimethyl-5-(propoxycarbonyl)-1-pyrroline *N*-oxide; 4,5-DPPO, 4,5-dimethyl-5-(propoxycarbonyl)-1-pyrroline *N*-oxide; EMPO, 5-(ethoxycarbonyl)-5-methyl-1-pyrroline *N*-oxide; EPR, electron paramagnetic resonance; HFS, hyperfine splitting; $LO^{\bullet}$, lipoxyl radical; NMR, nuclear magnetic resonance; $O_2^{\bullet -}$, superoxide anion radical; SOD, superoxide dismutase

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ent lipophilic properties for the detection of superoxide and other radicals in aqueous solution as well as within lipid membranes.

2. Materials and methods

2.1. Chemicals

2-Bromopropionyl bromide, crotonaldehyde, linoleic acid, methacrolein, superoxide dismutase and xanthine oxidase were commercially available from Sigma–Aldrich. Petroleum ether (high boiling, 50–70 °C) was obtained from Fluka, all other chemicals from Merck.

2.2. Syntheses

Synthesis and characterization of the compounds were performed as reported earlier [1,7], in analogy to the synthesis of EMPO and its derivatives [5,6] with minor adaptations as given below.

2.2.1. Alkyl 2-bromopropionate

2-Bromopropionyl bromide (70 mmol) was slowly added to a solution of the respective alcohol (100 mmol) and pyridine (70 mmol) in $CHCl_3$ at 0 °C (ice bath). After stirring for 1 h, the reaction mixture was successively washed with water (50 mL), sulfuric acid (10%, 50 mL) and concentrated aqueous Na_2CO_3 (50 mL), and dried over Na_2SO_4 overnight. Solvent and excess alcohol were removed under reduced pressure. The crude, nearly colorless product was used without further purification.

2.2.2. Alkyl 2-nitropropionate

The respective alkyl 2-bromopropionate (60 mmol) was added under stirring to a solution of sodium nitrite (7.2 g, 104 mmol) and phloroglucinol dihydrate (8.5 g, 52 mmol) in dry dimethylformamide (120 mL) at room temperature. The solution was stirred overnight, poured into ice water (240 mL), and extracted four times with ethyl acetate/petroleum ether (v/v = 4:1, 100 mL). The combined extracts were treated twice with 100 mL of saturated Na_2CO_3 solution and dried over Na_2SO_4. After removal of the solids by filtration, the solvent was evaporated in vacuo. The obtained colorless or pale yellow products were used further without purification.

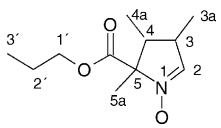
2.2.3. Alkyl 2,3-dimethyl-4-formyl-2-nitro-butanoate and alkyl 2,4-dimethyl-4-formyl-2-nitro-butanoate

23 mmol of the respective alkyl 2-nitropropionate was dissolved in a mixture of acetonitrile (10 g, 244 mmol) and triethylamine (0.2 g, 2 mmol). Crotonaldehyde (2.5 g, 38 mmol: for the 4-methyl derivatives) or methacrolein (2.5 g, 38 mmol: for the 3-methyl derivatives) was slowly added at 0 °C. The solution was stirred at room temperature overnight and then poured into a solution of ice-cold HCl (5 mL concentrated HCl in 150 mL water). The solution was extracted three times with CH_2Cl_2 and dried over anhydrous Na_2SO_4. After filtration the mixture was distilled under reduced pressure, and the purity of the obtained product was assessed by thin layer chromatography and IR spectroscopy. If necessary, the product was repurified by column chromatography.

2.2.4. Synthesis of the N-oxides

Synthesis of the nitrones was performed according to the procedure described previously for the synthesis of EMPO derivatives [1,7]. To a solution of the respective alkyl dimethyl-4-formyl-2-nitrobutanoate (25 mmol) in H_2O/CH_3OH (v/v = 3:2, ca. 250 mL) an aqueous NH_4Cl solution (1.87 g in 8 mL water) was added. The mixture was carefully kept at room temperature while 8.5 g (130 mmol)

Table 1
^{13}C NMR data (ppm) of the spin traps



	$^{2}C{=}N$	^{3}C	^{3a}C	^{4}C	^{4a}C	^{5}C	COO	$^{1'}C$	$^{2'}C$	$^{3'}C$	$^{5a}CH_3$
EMPO*	134.9	25.4	–	31.9	–	78.5	169.3	61.7	13.4	–	20.3
c-3,5EDPO	138.8	33.3	18.6	40.1	–	79.6	170.1	62.2	13.9	–	21.3
t-3,5EDPO	139.8	33.3	18.6	41.5	–	79.6	169.8	62.2	13.9	–	21.7
c-4,5EDPO	135.6	33.8	–	40.7	19.9	82.0	168.1	61.9	14.0	–	14.7
t-4,5EDPO	133.8	34.4	–	36.8	15.3	82.0	170.0	62.0	13.9	–	14.7
t-3,5DPPO	139.7	33.2	18.5	41.5	–	79.6	169.8	67.5	21.68	10.1	21.66
c-4,5DPPO	135.9	33.9	–	40.8	19.9	82.2	168.2	67.5	21.7	10.3	14.7
t-4,5DPPO	134.1	34.5	–	36.9	15.4	82.1	170.0	67.6	21.7	10.2	14.7
c-3,5DIPPO	139.5	33.3	18.5	40.1	–	79.6	169.5	69.9	21.2	–	21.3
t-3,5DIPPO	139.8	33.2	18.5	41.5	–	79.6	169.2	69.8	21.3 21.4	–	21.7
c-4,5DIPPO	135.7	34.0	–	41.0	20.0	82.1	168.3	70.1	21.7	–	14.8
t-4,5DIPPO	134.2	34.6	–	37.0	15.5	82.1	169.5	70.0	21.7	–	14.8