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Spin trapping of *C*- and *O*-centered radicals with methyl-, ethyl-, pentyl-, and phenyl-substituted EMPO derivatives

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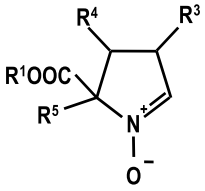
Abstract—In order to develop spin traps with an optimal ratio between hydrophilic and lipophilic properties, low toxicity, and high stability of spin adducts (especially with superoxide radicals), several EMPO-derived spin traps have recently been synthesized forming more stable superoxide adducts ($t_{1/2}$ > 20 min) than DMPO or DEPMPO. In this study, ESR-, ^{1}H-, and ^{13}C-NMR data of several phenyl- or *n*-pentyl-substituted EMPO derivatives are presented with full signal assignment. Methyl groups at position 3 or 4 stabilized the superoxide adducts considerably. Spin adducts from other oxygen- and carbon-centered radicals (e.g., derived from methanol or linoleic acid hydroperoxide) are also described.

1. Introduction

Spin trapping of superoxide radicals using different EMPO derivatives has recently been described by several authors.[1–8] Bulky alkoxycarbonyl substituents[5] or methyl groups in position 3 or 4 of the pyrroline ring[7] considerably stabilized the respective superoxide adducts ($t_{1/2}$ > 20 min) when compared to commonly used spin traps, such as DMPO ($t_{1/2}$ < 1 min),[9] EMPO ($t_{1/2}$ ca. 8 min),[1,2] or DEPMPO ($t_{1/2}$ ca. 14 min).[10,11] In addition to the affects exerted on spin adduct stability, alteration of the 5-alkyl or 5-alkoxycarbonyl substituents also affects the lipophilic properties of the spin trap, thus allowing accumulation of a desired ratio of the spin trap in a particular biological environment, for example, within biological membranes. This can be achieved by replacing either the alkoxycarbonyl[3–5,8] or the 5-methyl group of EMPO by an alkyl or aryl substituent.

The present paper describes synthesis, analytical and spin trapping properties of a series of 5-phenyl- or 5-*n*-pentyl-pyrroline derivatives with increasing lipophilicity, two of which are carrying an additional methyl group at position 3 or 4 of the pyrroline ring (3,5-EMPtPO and 4,5-EMPtPO). This additional substituent exerts a pronounced effect on the stability of the respective superoxide adducts. In continuation of recently published experiments with DMPO,[12] DEPMPO,[10,11] EMPO,[4] and 1,3,3-trimethyl-6-azabicyclo[3.2.1]oct-6-ene-*N*-oxide (TRAZON),[13,14] we also investigated the spin-trapping behavior toward other oxygen- or carbon-centered radicals (spin trap structures, see Fig. 1).



	R_1	R_3	R_4	R_5
EMPO	C_2H_5	H	H	CH_3
EPhPO	C_2H_5	H	H	C_6H_5
PPhPO	C_3H_7	H	H	C_6H_5
EPtPO	C_2H_5	H	H	*n*-C_5H_{11}
PPtPO	C_3H_7	H	H	*n*-C_5H_{11}
3,5-EMPtPO	C_2H_5	CH_3	H	*n*-C_5H_{11}
4,5-EMPtPO	C_2H_5	H	CH_3	*n*-C_5H_{11}
PrMPO	C_3H_7	H	H	CH_3
sBuMPO	*sec*-C_4H_9	H	H	CH_3
tBuMPO	*tert*-C_4H_9	H	H	CH_3
EEPO	C_2H_5	H	H	C_2H_5
PrEPO	C_3H_7	H	H	C_2H_5
iPrEPO	*iso*-C_3H_7	H	H	C_2H_5
BuEPO	*n*-C_4H_9	H	H	C_2H_5
sBuEPO	*sec*-C_4H_9	H	H	C_2H_5
tBuEPO	*tert*-C_4H_9	H	H	C_2H_5

Figure 1. General structure of the spin traps.

Keywords: EPR; Spin trapping; Superoxide; EMPO derivatives.
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2. Results

Incubation of EPhPO in the presence of xanthine/xanthine oxidase at pH 7.4 with a superoxide production rate of about 2 μM/min resulted in the detection of the EPhPO superoxide adduct (Fig. 2a). No EPR spectrum was observed in the presence of SOD (100 U/ml, not shown). Several scans had to be accumulated to detect EPR spectra from EPtPO (Fig. 2b) and PPtPO (not shown), whereas *trans*-4,5-EMPtPO (Fig. 2c) gave a stronger and more persistent EPR signal. The half-life of superoxide adducts was determined incubating 20 mM of the respective spin trap in oxygenated phosphate buffer (pH 7.4) for 10 min in the above-mentioned xanthine/xanthine oxidase system, after which SOD (100 U/mL) was added and the decay of the EPR signal was recorded as a series of consecutive spectra until the superoxide-related lines disappeared. The contribution of secondary lines (mainly hydroxyl adduct) was then subtracted from each individual EPR spectrum before calculating the respective half-life. With all spin traps the resulting intensity decrease of the first two lines was approximated by a first-order exponential decay, which was in good agreement with the experimental data (r^2: EPhPO, PPhPO, *cis*- and *trans*-4,5-EMPtPO (>0.99); PPtPO (0.98), EPtPO (0.95), 3,5-EMPtPO (0.89 (Xa/XOD) or 0.99 (KO_2-system)). In the KO_2 system, SOD (100 U/ml) and catalase (250 U/ml) were added 10 s after starting the incubation in order to decrease possible deleterious effects exerted by the developed peroxide. We also performed a preliminary evaluation of the rate constant of the superoxide spin trapping reaction for EPtPO (k ca. 10–28 $M^{-1} s^{-1}$), PPtPO (k ca. 43–119 $M^{-1} s^{-1}$), 3,5-EMPtPO (k ca. 16–44 $M^{-1} s^{-1}$), 4,5-EMPtPO (k ca. 40-110 $M^{-1} s^{-1}$), and EPhPO (k ca. 27–74 $M^{-1} s^{-1}$). A complete characterization of the rate constants according to Weaver et al.,[15] using two independent methods and a series of different incubations at 100 mM concentration, was, however, not possible due to limited amounts of the isolated diastereomeric forms (3,5- and 4,5-EMPtPO) and the low yields obtained (especially from PPhPO).

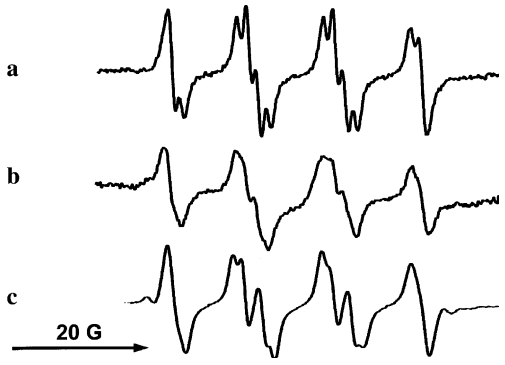


Figure 2. Formation of the superoxide adducts of the spin traps EPhPO, EPtPO, and *trans*-4,5-EMPtPO. (a) EPhPO (40 mM), catalase (250 U/mL), xanthine (0.2 mM), and xanthine oxidase (50 mU/mL) in oxygenated phosphate buffer (20 mM, pH 7.4, containing 0.4 mM DTPA) were incubated and measured using the following EPR parameters: sweep width, 60 G; modulation amplitude, 1.0 G; microwave power, 20 mW; time constant, 0.16 s; receiver gain, 2.5×10^5; scan rate, 42.9 G/min. (b) Same as in (a), except that EPtPO (20 mM) was used. Receiver gain, 4×10^5, 5 scans. (c) Same as in (a), except that *trans*-4,5-EMPtPO (20 mM) was used.

Spin adduct formation with hydroxyl radicals using a Fenton system is shown for EPhPO (Fig. 3a), EPtPO (Fig. 3b), and *trans*-4,5-EMPtPO (Fig. 3c). The EPR spectra of the hydroxyl radical adducts gradually disappeared within about 5 min (Ph-derivatives) to almost 20 min (Pt-derivatives). In the case of the *n*-pentyl derivatives, a significant contribution of secondary products to the spectral intensity was observed, possibly stemming from hydroxyl radical attack at the side chain. The two different types of spin adducts obtained from EPhPO and methanol are shown in Figure 4a (EPhPO/$^{\bullet}OCH_3$, stable for a few minutes, obtained by Fe^{3+}-catalyzed addition of methanol according to Dikalov et al.[12]) and in Figure 4b (EPhPO/$^{\bullet}CH_2OH$, relatively stable, formed using a Fenton system containing 5% methanol as described by Roubaud et al.[16]).

We also investigated the radicals formed in a Fenton-type reaction from peroxidized linoleic acid[18] in the presence of Fe^{2+} under anaerobic conditions,[12] where a mixture of three different radical adducts could be distinguished with EPhPO (Fig. 4c) or PPhPO (not shown). An even more complex mixture of radicals was observed when *cis*-/*trans*-3,5-EMPtPO, *cis*- or *trans*-4,5-EMPtPO (spectra not shown). The spectral parameters of lipid-derived adducts of all investigated spin traps and the comparison with other EMPO derivatives are listed in Table 6.

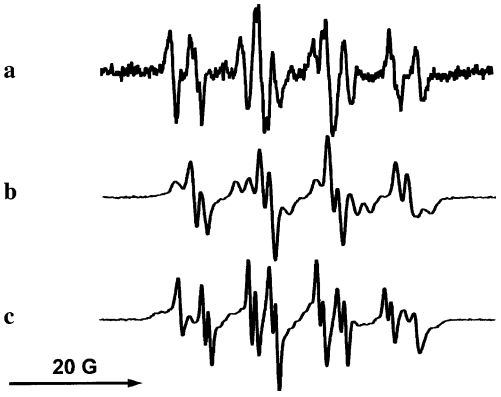


Figure 3. Formation of hydroxyl radical adducts of the spin traps EPhPO, EPtPO, and 4,5-EMPtPO. (a) EPhPO (20 mM) was incubated with a Fenton system containing $FeSO_4$ (1 mM), EDTA (2 mM), and H_2O_2 (0.2%). The reaction was stopped after 10 s by 1:1 dilution with phosphate buffer (300 mM, pH 7.4, containing 20 mM DTPA) and the spectrum was recorded using the following spectrometer settings: sweep width, 80.0 G; modulation amplitude, 0.24 G; microwave power, 20 mW; time constant, 0.08 s; receiver gain, 1×10^4; scan rate, 57.2 G/min. (b) Incubation as in (a), except that EPtPO (20 mM) was used. EPR parameters: sweep width, 80 G; modulation amplitude, 0.1 G; microwave power, 20 mW; time constant, 0.16 s; receiver gain, 2×10^5; scan rate, 28.6 G/min. (c) Incubation and ESR conditions as in (a), except that *trans*-4,5-EMPtPO (20 mM) was used.

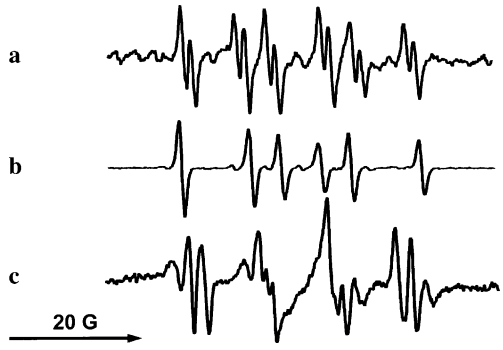
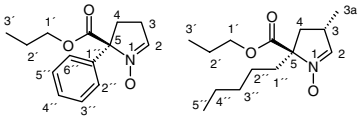


Figure 4. Iron-dependent formation of spin adducts from EPhPO in the presence of methanol or oxidized linoleic acid. (a) After a 10 s incubation of EPhPO (1 M in methanol) with $FeCl_3$ (2 mM), the reaction was stopped by 1:20 dilution with phosphate buffer (0.3 M, pH 7.4, containing 20 mM DTPA), and the spectrum was recorded with the following spectrometer settings: sweep width, 60 G; modulation amplitude, 0.24 G; microwave power, 20 mW; time constant, 0.02 s; receiver gain, 2×10^5; scan rate, 171.7 G/min. (b) EPhPO (20 mM) was incubated with a Fenton system containing $FeSO_4$ (1 mM), EDTA (2 mM), and H_2O_2 (0.2%) in 10% methanol. The reaction was stopped after 10 s by 1:1 dilution with phosphate buffer (300 mM, pH 7.4, containing 20 mM DTPA) and the spectrum was recorded using the following spectrometer settings: sweep width, 80 G; modulation amplitude, 0.1 G; microwave power, 20 mW; time constant, 0.16 s; receiver gain, 5×10^4; scan rate, 28.6 G/min. (c) To a nitrogen-bubbled solution of peroxidized linoleic acid (5 mM) and EPhPO (100 mM) in phosphate buffer (10 mM, pH 7.4, containing 250 units/ml catalase) $FeSO_4$ (0.1 mM) was added and the spectrum was recorded with the following spectrometer settings: sweep width, 80.0 G; modulation amplitude, 1.2 G; microwave power, 20 mW; time constant, 0.16 s; receiver gain, 2.5×10^5; scan rate, 57.2 G/min, 6 scans accumulated.

3. Discussion

Six lipophilic derivatives of the spin trap EMPO with phenyl and *n*-pentyl substituents were synthesized in this study. In contrast to the moderately lipophilic phenyl derivatives, the *n*-pentyl derivatives were considerably more lipophilic in comparison with the parent compound. The structure of the compounds was comprehensively characterized by full NMR assignment (^1H and ^{13}C), ESI Q-TOF MS, and IR spectroscopy (Tables 1–5). Relatively stable superoxide adducts were formed from EPhPO ($t_{1/2}$ = 16.1 min) and the two pentyl compounds having an extra methyl group at either position 3 (3,5-EMPtPO; $t_{1/2}$ = 12.1 min) or 4 (*cis*- and *trans*-4,5-EMPtPO; $t_{1/2}$ ca. 25 min) of the pyrroline ring, slightly more stable than superoxide adducts of DEPMPO derivatives ($t_{1/2}$ = ca. 15 min)[10] but less stable compared to their hydrophilic congeners.[7] This stabilizing effect is most probably due to steric shielding rather than electronic influences as has already been mentioned previously.[5] Determination of the half-lives of the superoxide adducts was done using a first-order exponential decay approximation (Pearson's correlation coefficient $r^2 > 0.98$ with two exceptions). Under the experimental conditions used a second-order decay of the superoxide adducts was not significant. In addition to the half-life of the spin adducts, the rate constant of the spin trapping reaction, the spectral line width, the total number of lines, and additional factors, such as enzyme binding or the solubility (aggregate formation) of the spin trap, reduction by antioxidants or buffer constituents, and degradation by transition metal catalyzed reactions (Fenton type reactions, oxidation by Fe^{3+}, etc.), play also important roles regarding the overall efficiency of the spin trapping reaction. In view of these facts, the half-life values given in Table 4 and the rate constants mentioned in the text are therefore to be interpreted only as preliminary. Further tests will be necessary to test these compounds in different biological systems. Hydroxyl radical adducts were stable for about 15–20 min, except for EPhPO and PPhPO, whose hydroxyl radical adducts were stable only for about 5 min. Sufficiently stable methoxyl radical adducts were obtained only from the spin traps bearing a methyl group in position 3 or 4 of the pyrroline ring, the others decaying within about 3–5 min. Furthermore, in the experiments performed with peroxidized linoleic acid carbon-centered radical adducts were also detected (under anaerobic conditions). Whether originally trapped oxygen-centered radical adducts rearranged rapidly into the respective carbon-centered form or

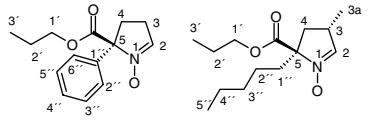
Table 1. ^{13}C NMR data (ppm) of the spin traps



	^2C	^3C	$^{3a}CH_3$	^4C	$^{4a}CH_3$	^5C	COO	$^{1'}C$	$^{2'}C$	$^{3'}C$	$^{1''}C$	$^{2''}C$	$^{3''}C$	$^{4''}C$	$^{5''}C$	$^{6''}C$
EMPO[c]	134.9	25.4	—	31.9	—	78.5	169.3	61.7	13.4	—	20.3	—	—	—	—	
EPhPO	135.6	26.0	—	34.4	—	85.6	168.7	62.8	13.9	—	135.7	128.3[a]	128.5[b]	127.0	128.5[b]	128.3[a]
PPhPO	135.9	26.1	—	34.3	—	85.6	168.8	68.2	21.7	10.2	135.6	127.0	128.4	126.2	128.4	127.0
EPtPO	135.7	26.2	—	28.5	—	82.0	169.9	67.0	13.8[a]	—	32.6	22.2[b]	31.7	22.4[b]	13.9[a]	—
PPtPO	135.0	26.1	—	28.5	—	82.0	170.0	67.5	21.8	10.2	32.6	22.4[a]	31.7	22.5[a]	13.9	—
c3,5EMPtPO	140.1	33.7	19.0	37.2	—	82.7	170.2	62.0	13.8	—	32.8	22.3	31.5	22.5	13.8	—
t3,5EMPtPO	139.6	33.1	18.4	36.7	—	82.5	169.8	62.0	13.8	—	32.4	22.3	31.5	22.5	13.8	—
c4,5EMPtPO	136.4	34.0	—	35.0	15.4	85.1	168.4	61.7	13.9[a]	—	30.7	22.3	31.8	22.4	14.0[a]	—
t4,5EMPtPO	134.6	35.0	—	37.2	14.6	84.5	170.0	62.1	14.0[a]	—	29.6	22.4	32.1	23.7	14.1[a]	—

[a] Tentative assignment (reversed order also possible).
[b] Tentative assignment (reversed order also possible).
[c] Data from Stolze et al.[5]

Table 2. ^{1}H NMR data [ppm] of the spin traps



	^{2}CH	^{3}CH$_x$	^{3a}CH$_3$	^{4}CH$_x$	^{4a}CH$_3$	$^{1'}$CH$_2$	$^{2'}$CH$_2$	$^{3'}$CH$_3$	$^{1''}$CH$_x$	$^{2''}$CH$_x$	$^{3''}$CH$_x$	$^{4''}$CH$_3$	$^{5''}$CH$_x$	$^{6''}$CH
EMPO[a]	6.97t	2.75m	—	2.16m 2.60m	—	4.26m	1.31t	—	1.72s	—	—	—	—	—
EPhPO	7.13t	2.64–2.84m	—	2.65m 3.06–3.16m	—	4.34q	1.30t	—	—	7.34–7.43m	7.46–7.51m	7.34–7.43m	7.46–7.51m	7.34–7.43m
PPhPO	7.16t	2.64–2.82m	—	2.65m 3.04–3.16m	—	4.22t	1.68m	0.90t	—	7.32–7.42m	7.44–7.52m	7.32–7.42m	7.44–7.52m	7.32–7.42m
EPtPO	7.00t 2.74m	2.63m 2.50m	—	2.26m	—	4.26m	1.29t	—	2.04m 2.16m	1.24–1.38m	1.24–1.38m	1.24–1.38m	0.89t	—
PPtPO	6.96t	2.63m 2.74m	—	2.26m 2.50m	—	4.17m	1.70m	0.95t	2.04m 2.16m	1.24–1.40m	1.24–1.40m	1.24–1.40m	0.89t	—
c3,5EMPtPO	6.99d	3.08m	1.24d	2.08ddc 2.49ddt	—	4.25m	1.29t	—	1.95m 2.20m	1.29m	1.29m	1.29m	0.90t	—
t3,5EMPtPO	6.99d	3.15m	1.19d	1.80ddt 2.66ddc	—	4.25m	1.29t	—	1.95m 2.20m	1.29m	1.29m	1.29m	0.89t	—
c4,5EMPtPO	7.05t	2.38m 2.74m	—	2.70m	1.16d	4.27m	1.30t	—	1.92m 2.25m	1.30–1.41m	1.30–1.41m	1.30–1.41m	0.87t	—
t4,5EMPtPO	6.98t	2.26m 2.83m	—	2.96m	1.16d	4.27m	1.30t	—	1.99m 2.10m	1.30–1.41m	1.30–1.41m	1.30–1.41m	0.87t	—

Abbreviations: s, singlet; d, doublet; t, triplet; m, multiplet; ddc, doublet of doublets (*cis* to ester); ddt, doublet of doublets (*trans* to ester).

[a] Data from Stolze et al.[5]

Table 3. ESI Q-TOF MS analysis of the spin traps

	Acquired $[MH]^+$	Calcd $[MH]^+$	Error [ppm]	mDa	Acquired $[MNa]^+$	Calcd $[MNa]^+$	Error [ppm]	mDa
Sample 1 EPhPO	234.1201	234.1125	32.62	7.64	256.1016	256.0950	25.92	6.64
Sample 2 PPhPO	248.1323	248.1281	16.87	4.19	270.1157	270.1106	18.84	5.09
Sample 3 EPtPO	228.1673	228.1594	34.57	7.89	250.1516	250.1419	38.73	9.69
Sample 4 PPtPO	242.1872	242.1751	50.11	12.14	264.1636	264.1576	22.86	6.04
Sample 5 3,5-EMPtPO	242.1808	242.1751	23.69	5.74	264.1636	264.1576	22.86	6.04
Sample 6 *cis*-4,5-EMPtPO *trans*-4,5-EMPtPO	242.1872	242.1751	50.11	12.14	264.1702	264.1576	47.84	12.64

Table 4. Half-life of the superoxide adducts and *n*-octanol/buffer partition coefficients of the spin traps

Compound	Apparent $t_{1/2}$ (min)	Partition coefficient *n*-octanol/phosphate buffer (100 mM, pH 7.0)
EMPO[a]	8.6[a]	0.15[a]
EPhPO	16.1	1.79
PPhPO	7.6	5.03
EPtPO	2.2	15.28
PPtPO	10.2	35.16
cis/*trans*-3,5-EMPtPO[b]	12.1	44.75
cis-4,5-EMPtPO	25.1	32.48
trans-4,5-EMPtPO	25.3	33.08
i-PrMPO[a]	18.8[a]	0.20[a]
s-BuMPO[a]	26.3[a]	1.06[a]
t-BuMPO[a]	15.7[a]	0.80[a]
EEPO[a]	18.0[a]	0.25[a]
PrEPO[a]	20.0[a]	0.78[a]
i-PrEPO[a]	23.3[a]	0.66[a]
BuEPO[a]	18.0[a]	2.88[a]
s-BuEPO[a]	14.1[a]	1.89[a]
t-BuEPO[a]	18.2[a]	1.53[a]

[a] Data from Stolze et al.[5]

whether the originally formed alkoxyl radicals underwent rapid β-scission (rate constant ca. $10^6\ M^{-1}\ s^{-1}$)[17] prior to radical trapping cannot be decided. A variety of secondary reactions has been reported leading to carbon-centered radicals according to different pathways[19] involving intermediates such as LOO$^\bullet$ or LO$^\bullet$.[20–25] (see Table 6).

4. Conclusion

In conclusion, EPhPO and 4,5-EMPtPO can be recommended for trapping of superoxide radicals, since the half-life of their superoxide adducts is higher than 15 min. EPtPO, on the other hand, forms a rather unstable superoxide adduct and can, therefore, not be recommended.

5. Experimental

5.1. Chemicals

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

5.2. Syntheses

Synthesis and characterization of the compounds were performed as reported previously,[4,5] in analogy to the synthesis of EMPO and its derivatives[1,2] with minor adaptations as given below.

5.2.1. α-Bromophenylacetyl chloride. α-Bromophenylacetic acid (25 g, 100 mmol) was refluxed for 2 h in 40 ml of thionyl chloride. The evolving gas (sulfur dioxide and hydrogen chloride) was absorbed into 10% sodium carbonate solution. Twenty milliliters of excess thionyl chloride was recovered by distillation for reuse, the remaining part was removed after addition of heptane (50 ml). After removal of heptane in a rotary evaporator, the crude product was used without further purification.

5.2.2. Alkyl α-bromophenylacetate. α-Bromophenylacetyl chloride (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 aq $NaHCO_3$ (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.

5.2.3. Propyl 2-bromoheptanoate. Ethyl 2-bromoheptanoate (25 ml, 128 mmol) was refluxed for 3 h in 100 ml 1-propanol containing 3 ml concentrated hydrochloric acid as a catalyst. The solvent was removed in a rotary evaporator, petroleum ether was added, and traces of remaining acid were removed after addition of a small amount of CaO. After 1 h, the solution was filtered and the petroleum ether was distilled off. If necessary, the product was purified by distillation.

5.2.4. Synthesis of the alkyl 2-nitroheptanoates and alkyl α-nitrophenylacetates. The respective alkyl 2-bromoheptanoate or α-bromophenylacetate (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 *N*,*N*-dimethylformamide (120 ml) at room temperature. The solution was stirred overnight, poured into ice water (240 ml), and extracted four times with ethyl acetate (100 ml). The combined extracts were treated twice with 100 ml of a $NaHCO_3/Na_2CO_3$ solution and dried over Na_2SO_4. After removal of the solids by filtration, the solvent was evaporated in vacuo.

Table 5. IR data (cm^{-1}) of the spin traps

Intensities: strong (**1741**), medium (**1464**), weak (950)																							
EMPO[a]	**2985**	**2940**	2874	**1741**	**1582**	**1464**	**1446**	**1377**	—	1341	**1288**	—	**1236**	**1182**	—	**1107**	—	**1024**	950	926	**862**	796				
EPhPO	**2978**	2904	**2851**	**1737**	**1572**	1497	**1462**	**1447**	1389	1366	1341	**1305**	**1252**	**1218**	**1155**	**1096**	**1051**	**1030**	**1012**	921	**857**	799	**758**	**699**	**675**	**643**
PPhPO	**2965**	2877	2849	**1741**	**1574**	1497		**1449**	1387	1345	1308		**1252**	**1218**	1158	1096	1054	1031	1000	950	901	797	758	**699**	676	644
EPtPO	**2957**	**2930**	**2859**	**1742**	**1583**	**1465**		1368	1343			**1229**	**1180**	1125	1114	1096	**1063**	1037		933	861	771	726	694	62	
PPtPO	**2959**	**2918**	**2871**	**1741**	**1580**	**1463**		1379	1345			**1229**	**1178**	1123			1064	1038	966	945	908	792	732	693	63	
3,5EMPtPO	**2958**	**2932**	**2872**	**1741**	**1578**	**1464**	**1458**	**1368**		**1300**	**1270**	**1228**	**1214**	**1140**	**1110**	1095	1085	**1028**	964		860	768	727	705	**636**	
c4,5EMPtPO	**2959**	**2931**	**2872**	**1741**	**1577**	**1465**	**1458**	**1382**	**1368**	**1324**	**1260**	**1237**		**1176**	**1139**	**1119**	**1096**	**1030**	957	931	861	804	774	728	**689**	
t4,5EMPtPO	**2957**	**2935**	**2872**	**1741**	**1583**	**1465**	**1458**	**1382**	**1369**	1348	**1251**	**1229**	**1192**	**1175**	**1138**	**1119**	**1096**	1066	**1024**	951	930	860	805	772	745	**692**

[a] Data from Stolze et al.[5]

The obtained colorless or pale yellow products were used further without purification.

5.2.5. Alkyl 4-formyl-2-nitro-2-*n*-pentyl-butanoate (alkyl 4-formyl-2-nitro-2-phenylbutanoate). Alkyl 2-nitroheptanoate (23 mmol) or alkyl α-nitrophenylacetate (for EPhPO and PPhPO) was dissolved in a mixture of acetonitrile (10 g, 244 mmol) and triethylamine (0.2 g, 2 mmol). Acrolein (2 g, 38 mmol) was slowly added at 0 °C. The solution was kept at 10 °C for 1.5 h and then poured into a solution of ice-cold HCl (5 ml of concentrated HCl in 150 ml water). The solution was extracted three times with CH_2Cl_2 and dried over Na_2SO_4. After filtration, the mixture was distilled under reduced pressure, and the purity of the remaining product was assessed by thin layer chromatography and IR spectroscopy.

5.2.6. Ethyl 4-formyl-2-nitro-2-*n*-pentylpentanoate. Same conditions as indicated above for the synthesis of alkyl 4-formyl-2-nitro-2-*n*-pentyl-butanoate, except that methacrolein (2.5 g, 36 mmol) was used instead of acrolein. After slow addition of methacrolein at 0 °C, the solution had to be stirred for several days at room temperature.

5.2.7. Ethyl 4-formyl-3-methyl-2-nitro-2-*n*-pentylbutanoate. Same conditions as indicated above for the synthesis of alkyl 4-formyl-2-nitro-2-*n*-pentyl-butanoate, except that crotonaldehyde (2.5 g, 36 mmol) was used instead of acrolein. After slow addition of crotonaldehyde at 0 °C, the solution had to be stirred for several days at room temperature.

5.2.8. Synthesis of the *N*-oxides. Synthesis of the nitrones was performed according to the procedure described recently for the synthesis of EMPO derivatives.[4,5] To a concentrated solution of 25 mmol of the respective alkyl 4-formyl-2-nitro-2-*n*-pentylbutanoate (or alkyl 4-formyl-2-nitro-2-phenylbutanoate for EPhPO and PPhPO) in H_2O/CH_3OH (v/v = 6:4), an aqueous solution of ammonium chloride (1.87 g in 8 ml of water) was added. While zinc dust (8.5 g, 130 mmol) was slowly added within 30 min, the mixture was carefully kept at room temperature. The mixture was stirred for 4.5 h at room temperature, the white precipitate and the remaining zinc powder were removed by filtration, and the residue was washed five times with methanol (30 ml). The liquid phase was concentrated to about 10 ml and extracted four times with 60 ml CH_2Cl_2. The organic phase was dried with Na_2SO_4, filtered, and concentrated. Careful purification by column chromatography on silica gel with a petroleum ether/ethanol gradient allowed the separation from the majority of side products and provided the product as a dark yellow oil or yellow needles (yield ca. 20% for the pentyl derivatives, ca. 5% for the phenyl derivatives). Additional purification was done immediately before the EPR experiments on a 1 ml solid phase extraction column using a Chromabond C-18 100 mg column obtained from Macherey-Nagel (Düren, Germany). The purity of the obtained products was assessed by TLC and UV spectroscopy. Final identification of the purified products was performed by ^1H NMR, ^{13}C NMR, ESI Q-TOF MS, and IR spectroscopy (see Tables 1–5).

Table 6. Comparison of the EPR parameters of radical adducts of different EPhPO, EPtPO, EMPO and EEPO derivatives

Radical	HFS (G)	EMPO[a]		EPhPO		PPhPO			EPtPO		PPtPO		c/t-3,5EMPtPO			*cis*-4,5EMPtPO				*trans*-4,5EMPtPO		
˙OOH		(43%)	(57%)	(60%)	(40%)	(61%)	(39%)		(55%)	(45%)	(62%)	(38%)	(35%)	(35%)	(30%)	(74%)	(15%)	(11%)		(67%)	(33%)	
	a^N	13.28	13.28	13.00	13.05	12.98	13.04		13.35	13.35	13.20	13.18	13.40	12.75	13.40	13.24	13.18	13.18		13.48	13.55	
	a^H	9.67	11.89	11.65	9.00	11.51	8.82		9.55	11.98	11.61	9.00	15.03	6.80	8.10	11.60	9.81	7.83		10.99	8.00	
	a^H	—	—	—	—	—	—		—	—	0.50	—	—	—	—	—	—	—		—	0.66	
˙OH		(50%)	(50%)	(50%)	(50%)	(40%)	(34%)	(26%)	(52%)	(48%)	(52%)	(48%)	(67%)	(33%)		(26%)	(4%)[b]	(25%)	(24%)	(21%)[c]	(13%)	(11%)	(76%)[d]
	a^N	14.00	14.00	13.43	13.43	13.66	13.37	13.66	13.85	14.00	14.00	13.85	13.75	14.30		14.30	14.00	13.85	13.82	13.80	13.85	13.87	
	a^H	15.00	12.58	12.70	14.70	12.93	15.15	11.38	12.16	15.97	15.87	12.16	9.30	19.25		19.60	16.37	10.85	12.40	9.20	8.40	10.88	
	a^H	0.90	—	0.75	0.75	—	—	—	0.75	—	—	0.80	—	—		—	—	—	—	—	—	0.40	
		(100%)		(100%)		(100%)			(100%)		(75%)	(25%)	(38%)	(31%)	(31%)		(51%)	(49%)			(100%)		
˙H	a^N	15.52		15.12		15.06			15.34		15.33	15.13	15.43	15.34	15.03		15.24	15.34			15.42		
	a^H	22.21		21.38		21.37			22.91.		22.96	25.14	26.08	25.08	26.28		24.46	26.08			26.02		
	a^H	20.82		20.48		20.47			19.87		19.89	17.37	16.15	17.21	16.68		18.22	17.40			17.45		
	a^H	—		—		—			—		—	—	0.60	0.55	0.50		1.15	—			—		
˙CH_3		(100%)		(100%)		(100%)			(52%)	(48%)	(51%)	(49%)	(68%)	(16%)	(16%)		(51%)	(49%)			(89%)	(11%)	
	a^N	15.42		14.90		14.87			15.08	15.15	15.05	15.14	15.00	15.00	15.25		15.15	15.20			15.20	15.00	
	a^H	22.30		21.63		21.51			23.80	21.22	23.60	21.14	15.75	18.50	26.65		19.26	17.43			17.43	19.30	
	a^H	—		—		—			—	—	—	—	—	—	—		0.60	—			0.45	0.70	
˙OCH_3		(50%)	(50%)	(53%)	(47%)	(56%)	(44%)		(62%)	(38%)	(57%)	(43%)	(50%)	(35%)	(15%)		(42%)	(40%)	(18%)		(50%)	(42%)	(8%)
	a^N	13.74	13.74	13.30	13.30	13.27	13.27		13.48	13.48	13.49	13.49	13.40	13.75	13.85		13.38	13.40	13.72		13.60	13.60	13.88
	a^H	10.87	7.81	9.80	7.11	9.78	7.17		7.70	10.49	10.52	7.73	5.80	14.75	17.25		8.75	5.64	16.04		5.38	9.02	17.92
	a^H	0.55	0.50	0.60	0.50	—	—		—	—	—	—	—	—	—		—	—	1.86		—	—	—
˙CH_2OH		(100%)		(100%)		(100%)			(100%)		(100%)		(53%)	(47%)			(100%)				(100%)		
	a^N	14.95		14.54		14.57			14.78		14.76		14.86	14.66			14.70				14.94		
	a^H	21.25		20.85		20.62			21.40		21.08		24.69	16.68			18.85				18.18		
˙C (LOOH)		(62%)	(38%)	(58%)	(26%)	(16%)	(45%)	(40%)	(15%)	(50%)	(26%)	(24%)	(56%)	(26%)	(18%)	(58%)	(22%)	(20%)		(67%)	(21%)	(12%)		(33%)	(29%)	(15%)	(14%)[e]
	a^N	13.45	13.45	13.40	14.10	14.95	14.90	13.67	14.18	15.00	13.85	14.00	15.30	13.85	14.00	15.20	14.25	13.65		14.80	13.70	14.00		15.00	14.80	15.00	14.65
	a^H	11.45	8.55	13.50	17.40	21.60	21.70	12.68	17.25	23.00	12.16	15.97	22.90	12.16	15.87	24.90	19.25	8.80		19.55	10.80	17.90		21.00	17.25	15.00	19.50
	a^H	—	—	1.00	—	0.76	0.76	0.80	—	1.40	0.75	—	1.50	0.80	—	—	—	—		—	—	—		—	—	—	—
	a^H			0.75																						

Radical		*i*-PrMPO		*s*-BuMPO		*t*-BuMPO		EEPO		PrEPO		*i*-PrEPO		BuEPO		*s*-BuEPO		*t*-BuEPO	
˙OOH		(57%)	(43%)[a]	(56%)	(44%)[a]	(56%)	(44%)[a]	(63%)	(37%)[a]	(61%)	(39%)[a]	(62%)	(38%)[a]	(67%)	(33%)[a]	(62%)	(38%)[a]	(65%)	(35%)[a]
	a^N	13.30	13.30	13.03	13.30	13.38	13.38	13.15	13.15	13.23	13.23	13.21	13.21	13.21	13.21	13.22	13.22	13.27	13.27
	a^H	11.88	9.66	11.82	9.54	11.95	9.70	11.40	8.75	11.52	8.80	11.60	9.03	11.55	8.92	11.55	8.90	11.65	9.07
	a^H	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

·OH		(72%)	(28%)[a]	(77%)	(23%)[a]	(81%)	(19%)[a]	(55%)	(45%)[a]	(59%)	(41%)[a]	(61%)	(39%)[a]	(65%)	(35%)[a]	(80%)	(20%)[a]	(62%)	(38%)[a]
	a^N	14.10	14.25	14.06	14.22	14.13	14.20	13.86	14.00	13.84	13.98	13.87	14.00	13.80	13.98	13.95	13.80	13.90	14.05
	a^H	12.80	15.35	12.60	15.35	12.71	15.47	12.05	15.90	12.01	15.90	12.01	15.94	11.97	15.95	16.20	11.90	12.16	16.10
	a^H	0.65/0.45	0.5	0.72	0.45	0.70	0.65	0.78	0.62	0.80	0.58	0.78	0.63	0.78	0.65	—	0.72	0.80	0.60
		(100%)		(100%)		(100%)		(100%)		(100%)		(100%)		(100%)		(100%)		(100%)	
·H	a^N	15.52		15.49		15.59		15.32		15.32		15.33		15.25		15.32		15.40	
	a^H	22.25		22.27		22.42		22.99		22.97		23.00		23.06		22.99		23.04	
	a^H	20.80		20.79		20.84		19.87		19.85		19.90		19.87		19.85		20.00	
$\cdot CH_3$		(100%)		(100%)		(100%)		(53%)	(47%)	(59%)	(41%)	(50%)	(50%)	(51%)	(49%)	(60%)	(40%)	(59%)	(41%)
	a^N	15.35		15.32		15.44		15.14	15.14	15.14	15.08	15.14	15.09	15.10	15.09	15.13	15.07	15.21	15.11
	a^H	22.14		22.03		21.95		23.99	21.25	21.20	24.17	21.36	24.18	21.19	24.13	21.10	24.03	21.29	24.25
$\cdot OCH_3$		(100%)		(100%)		(100%)		(100%)		(100%)		(100%)		(100%)		(100%)		(100%)	
	a^N	13.76		13.62		13.72		13.45		13.41		13.42		13.41		13.42		13.47	
	a^H	9.31		9.12		9.25		9.00		8.98		8.92		8.88		8.86		8.91	
	a^H	1.28		1.43		1.40		1.45		1.45		1.47		1.47		1.47		1.47	
$\cdot CH_2OH$		(100%)		(100%)		(100%)		(100%)[f]		(100%)[f]		(100%)[f]		(100%)[f]		(100%)[f]		(100%)[f]	
	a^N	14.95		14.94		15.01		14.76		14.74		14.77		14.74		14.78		14.80	
	a^H	21.15		20.97		20.98		21.70		21.50		21.60		21.52		21.35		21.31	
	a^H	—		—		—		0.87		0.87		0.90		0.90		0.92		0.88	
·C (LOOH)		(77%)	(23%)	(71%)	(29%)	(67%)	(33%)	(56%)	(44%)	(54%)	(46%)	(64%)	(36%)	(65%)	(35%)	(61%)	(39%)	(73%)	(27%)
	a^N	15.22	14.96	15.12	14.96	15.12	14.96	14.99	15.05	15.03	15.03	15.03	15.13	14.47	14.80	15.00	15.05	15.10	15.22
	a^H	22.33	20.52	22.33	20.62	22.34	20.64	21.18	23.82	20.94	23.49	21.29	24.30	24.42	21.20	21.00	24.02	21.18	24.36
	a^H	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

[a] Data from Stolze et al.[4]
[b] Approximate values calculated from difference spectra taken 3 min after the first scan.
[c] Approximate values calculated from difference spectra taken 30 min after the first scan.
[d] Secondary radicals from side chain attack (unresolved mixture).
[e] Rest mainly ·OH adduct.
[f] Broad lines due to unresolved HFS.

5.2.9. Preparation of lipid hydroperoxides. Linoleic acid hydroperoxide was synthesized according to O'Brien.[18] Briefly, linoleic acid was air-oxidized for 72 h in the dark at room temperature. The oxidation mixture was dissolved in petroleum ether (boiling range 60–90 °C) and extracted four times with water/methanol (v/v = 1:3). The obtained methanolic phase was counter-extracted four times with petroleum ether (boiling range 60–90 °C) and evaporated under reduced pressure. The obtained hydroperoxide was dissolved in ethanol and stored in liquid nitrogen. The concentration of hydroperoxide was determined by UV spectroscopy based on an extinction coefficient of $\varepsilon_{233\ nm} = 25250\ M^{-1}\ cm^{-1}$ in ethanol.[18]

5.3. Instruments

UV–vis spectra were recorded on Hitachi 150-20 and U-3300 spectrophotometers in double-beam mode against a blank of the respective solvent. Determination of the concentrations was done measuring the absorption maxima in the range between 200 and 350 nm. IR spectra were recorded as film on an ATI Mattson Genesis Series FTIR spectrometer (see also Table 5).

For EPR experiments, Bruker spectrometers (ESP300E and EMX) were used, operating at 9.7 GHz with 100 kHz modulation frequency, equipped with a rectangular TE_{102} or a TM_{110} microwave cavity.

NMR spectra were recorded on a Bruker Avance at 300.13 MHz for ^{1}H and 75.47 MHz for ^{13}C. $CDCl_3$ was used as the solvent throughout, TMS (tetramethylsilane) as the internal standard. ^{13}C peaks were assigned by means of APT (attached proton test), HMQC (^{1}H-detected heteronuclear multiple-quantum coherence), and HMBC (heteronuclear multiple bond connectivity) spectra. All chemical shift data are given in ppm units.

Mass spectra were obtained as follows: samples were diluted in the ratio 1:10.000 in 70% methanol containing 0.1% formic acid and injected offline to ESI Q-TOF MS on a Waters Micromass Q-TOF Ultima Global at a flow rate of 5 μl/min. To acquire appropriate spectra for every sample, capillary voltage was adjusted between 1.2 and 3.0 kV. The mass spectrometer had been previously tuned with [Glu1]-fibrinopeptide B to give the highest possible sensitivity and a resolution of 10.000 (FWHM). Mass tuning of the TOF analyzer was done in the tandem MS mode using again [Glu1]-fibrinopeptide B. Data analysis was performed with MassLynx 4.0 SP4 Software (Waters Micromass).

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