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# ESR analysis of spin adducts of alkoxyl and lipid-derived radicals with the spin trap Trazon

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**Abstract**

Detection of oxygen-centered radicals was performed using the spin trap 1,3,3-trimethyl-6-azabicyclo[3.2.1]oct-6-ene-*N*-oxide (Trazon), a bicyclic nitrone spin trap that is easily synthesized from the corresponding amine *via* hydrogen peroxide mediated oxidation in the presence of the catalyst, sodium tungstate. Compared to monocyclic spin traps such as 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) or 5-(diethoxyphosphoryl)-5-methyl-1-pyrroline *N*-oxide (DEPMPO), the ESR spectra of Trazon spin adducts provide additional structural information due to long-range hyperfine splitting constants and also due to the fact that different stereoisomers can be distinguished. This is especially helpful for the detection of lipid-derived alkoxyl radicals which can be identified according to their characteristic hyperfine splitting pattern. Due to the relatively high stability of the Trazon spin adducts with lipid alkoxyl radicals, which were formed from peroxidizing linoleic acid, ESR experiments could be performed using a stationary system, whereas a slow-flow system is recommended for DMPO. A series of structurally different alkoxyl radical adducts were synthesized by iron-catalyzed nucleophilic addition of the respective alcohol to the spin trap Trazon and the spectra were analyzed by computer simulation. Both the molecular weight of the alcohol and the position of the alcoholic hydroxyl group were of significant influence on the ESR spectra. Two stereochemically different spin adducts were formed in a ratio typical of the alcohol used, thus allowing structural classification of the alkoxyl radical trapped. 

*Keywords:* Spin traps; ESR; Trazon; Free radicals; Linoleic acid hydroperoxide; Lipid peroxidation

## 1. Introduction

The synthesis of the spin trap Trazon from the respective amine was first described by Sankuratri and Janzen [1]. In contrast to the spin traps DMPO or DEPMPO which are subject to slow degradation even when kept in a freezer, Trazon which crystallizes in white needles, has been reported to have an excellent shelf life [1]. In addition, Trazon is more lipophilic than DMPO and is, therefore, expected to be suitable for the investigation of radicals in lipid membranes, where it can replace the commercially available lipophilic spin trap PBN whose oxygen-centered radical spin adducts are very short-lived ($t_{1/2}$ of PBN/$^{\bullet}$OH: 38 s [2]). Therefore, PBN is mainly used for the detection of carbon-centered radicals. Furthermore, PBN spin adducts often exhibit very similar ESR spectra which renders identification of the trapped radicals rather difficult. An important application of the spin trapping technique is the detection of lipid peroxidation-derived free radicals in their natural environment as was recently shown using DEPMPO-derived spin traps [3,4]. For applications using the commercially available spin trap DMPO a slow-flow system of linoleic acid and lipoxygenase has recently been developed by Dikalov and Mason [5]. Also in their study another model system for the determination of the stereochemical structure of spin adducts of radicals formed during lipid peroxidation was described, consisting of the spin trap DMPO and a series of different alcohols such as 1-penten-3-ol, which bind to the spin trap in an iron-catalyzed nucleophilic addition reaction.

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*Abbreviations:* Trazon, 1,3,3-trimethyl-6-azabicyclo[3.2.1]oct-6-ene-*N*-oxide; EMPO, 5-(ethyoxycarbonyl)-5-methyl-1-pyrroline *N*-oxide; DEPMPO, 5-(diethoxyphosphoryl)-5-methyl-1-pyrroline *N*-oxide; DMPO, 5,5-dimethyl-1-pyrroline *N*-oxide; DTPA, diethylenetriaminepentaacetic acid; ESR, electron spin resonance; LO$^{\bullet}$, lipoxyl radical; $O_2^{\bullet -}$, superoxide anion radical; SOD, superoxide dismutase.

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Aim of the present study was to combine recent advances in the design of more appropriate spin traps [1] for the detection of radicals formed during lipid peroxidation with new developments in the field of structural determination [5]. For this purpose, a series of structurally different Trazon alkoxyl radical adducts were tested with respect to the characteristic differences in their ESR spectra, i.e. the hyperfine splitting constants and the ratio between the two distinguishable diastereomeric forms.

## 2. Materials and methods

### 2.1. Chemicals

Cyclopentanol, linoleic acid, 1-pentanol, 3-pentanol, 1-penten-3-ol, superoxide dismutase (SOD) and xanthine oxidase were purchased from Aldrich–Sigma Chemical Co. Petroleum ether (high boiling, 50–70°) was from Fluka. All other chemicals were purchased from Merck.

### 2.2. Trazon

Synthesis and characterization of the spin trap Trazon was according to Sankuratri and Janzen [1] with minor adaptations. 1,3,3-Trimethyl-6-azabicyclo[3.2.1]octane (15.3 g, 100 mmol) was dissolved in a solution of sodium tungstate ($Na_2WO_4 \cdot 2H_2O$, 1.32 g, 4 mmol) in 20 mL water and a 30% solution of hydrogen peroxide (23 mL, 220 mmol) was slowly added under vigorous cooling at −5° under nitrogen. Excess of hydrogen peroxide was destroyed by addition of $NaHSO_3$ and the solution was extracted several times with chloroform after saturation with sodium chloride. The extract was dried with sodium sulfate, the solvent removed and purification of the crude product was performed on silica gel with a gradient consisting of petroleum ether (boiling range 50–70°) and increasing amounts of ethanol. Identification of the product was done by comparison of the data with the literature [1].

### 2.3. Preparation of lipid hydroperoxides

Linoleic acid hydroperoxide was synthesized according to O'Brien [6]. Briefly, linoleic acid was air-oxidized for 72 hr at room temperature in the dark. The oxidation mixture was dissolved in petroleum ether (boiling range 60–90°) and extracted four times with water/methanol (1:3 v/v). The obtained aqueous methanol was then extracted four times with petroleum ether (boiling range 60–90°). The methanolic phase was then evaporated under reduced pressure and the obtained hydroperoxide was dissolved in ethanol and stored in liquid nitrogen. The concentration of hydroperoxide was calculated both iodometrically [7] and spectrophotometrically using an extinction coefficient of $\varepsilon_{233\,nm} = 25{,}250\ M^{-1}\,cm^{-1}$ in ethanol [6].

### 2.4. Instruments

#### 2.4.1. UV–VIS spectrometer

UV–VIS spectra were recorded using the Hitachi model 150-20 and U-3300 UV–VIS spectrophotometers in the double beam mode against a reference sample of the respective solvent. Calculation of the concentrations was done by measuring the absorption maxima in the range between 200 and 300 nm.

#### 2.4.2. IR spectrometer

IR spectra of the synthesized compounds were recorded as film spectra on a ATI Mattson Genesis Series FTIR spectrometer.

#### 2.4.3. ESR measurements

For ESR experiments the Bruker spectrometers ER 200 D-SRC 9/2.7 with the data system ESP1600 and the Bruker ESP300E were used, operating at 9.6 GHz with 100 kHz modulation frequency and equipped with a rectangular $TE_{102}$ or a $TM_{110}$ microwave cavity.

For the formation of the various alkoxyl radical adducts a slightly modified procedure recently published by Dikalov and Mason [5] was used:

Trazon (*ca.* 1–2 M) was incubated for 2–5 min with the respective anhydrous alcohol in the presence of $Fe^{3+}$ (2 mM). The reaction was stopped by 1:50 or 1:100 dilution with 0.3 M phosphate buffer, pH 7.4, containing 20 mM diethylenetriaminepentaacetic acid (DTPA).

## 3. Results

### 3.1. Spin trapping of superoxide radicals

The structure of the spin trap used is shown in Fig. 1. The spatial structure of the spin trap (chiral center) expects the addition of radicals from two different sides. Fig. 2a shows the ESR spectrum of the superoxide adduct of Trazon generated by the xanthine/xanthine oxidase couple in the presence of catalase in phosphate buffer, pH 7.4. A rapid sampler was used since the spectrum disappeared gradually due to the relatively short half-life of the adduct ($t_{1/2} = 3.6$ min). In Fig. 2b the computer simulation of the spectrum is shown.

In order to determine the half-life of the superoxide adduct, Trazon was incubated for 9 min in the presence of

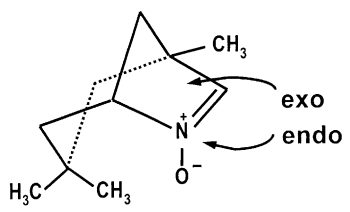



Fig. 1. Structure of the spin trap Trazon.

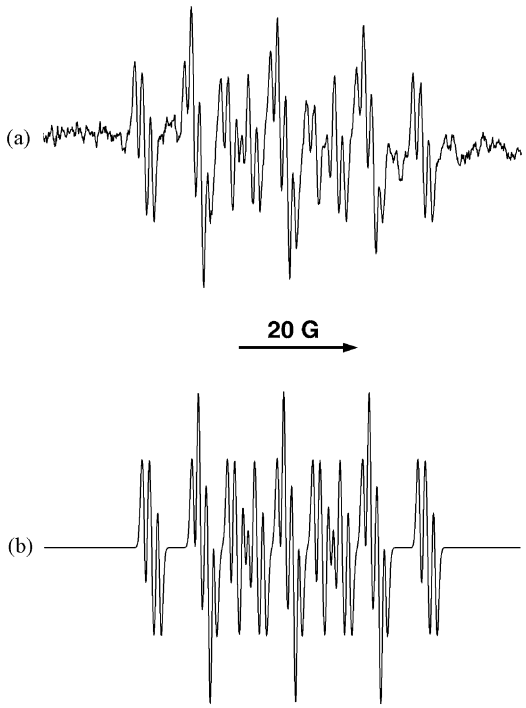



Fig. 2. Formation of the superoxide adducts of the spin trap Trazon using the xanthine/xanthine oxidase system. (a) Trazon (40 mM), catalase (250 U/mL), xanthine (0.4 mM) and xanthine oxidase (50 mU/mL) in phosphate buffer (20 mM, pH 7.4, containing 0.7 mM DTPA ) were mixed together, aspirated into the flat cell using the rapid sampling technique and the ESR spectrum was measured using the following parameters: sweep width, 80 G; modulation amplitude, 0.50 G; microwave power, 20 mW; time constant, 0.16 s; receiver gain, $2 \times 10^5$; scan rate, 28.6 G/min. (b) Computer simulation, based on the hyperfine splitting data listed in Table 1.

xanthine, xanthine oxidase and catalase. The reaction was stopped with SOD (50 U/mL) and the solution was transferred into the ESR spectrometer within 10 s using a rapid sampler. Several consecutive spectra were recorded until the lines disappeared. The decay curve used to calculate the half-life of the Trazon superoxide adduct is a first order exponential fit of the observed intensity of the first triplet. The respective spectroscopic parameters are summarized in Table 1.

### 3.2. Formation of different alkoxyl radical adducts

Formation of Trazon spin adducts with chemically defined primary, secondary, and tertiary alkoxyl substituents, used as model compounds for lipid peroxidation-derived alkoxyl radicals of short and intermediate chain length, was performed by $Fe^{3+}$-catalyzed nucleophilic addition of the respective alcohols to the spin trap according to Dikalov and Mason [5].

In Fig. 3a the ESR spectrum of the methoxyl radical adduct of Trazon is shown. Only one species can be spectroscopically distinguished. The respective computer simulation can be seen in Fig. 3b.

Whereas the respective Trazon/alkoxyl adducts obtained by nucleophilic addition of other short-chain primary alcohols such as ethanol were quite similar, the ESR spectra obtained using bulky substituents such as *tert*-butanol looked quite different (Fig. 4a), indicating stereochemical variations of the structure. The computer simulation shown in Fig. 4b is a 4:1 superposition of two stereoisomers.

Table 1
Comparison of the ESR parameters of various radical adducts of the spin trap Trazon

| Adduct | (%) | $a_N$ (G) | $a_H$ (G) | $a_H$ (G) | $a_H$ (G) | $a_H$ (G) | $a_H$ (G) |
|---|---|---|---|---|---|---|---|
| $O_2^{\bullet -}$ | – | 14.35 | 9.37 | 8.33 | 1.46 | 1.00 | – |
| $HO^{\bullet}$[a] | – | 15.00 | 9.70 | 9.70 | 1.10 | 1.00 | – |
| $CH_3O^{\bullet}$ | 100 | 14.58 | 9.55 | 8.45 | 1.48 | 1.00 | – |
| $C_2H_5O^{\bullet}$ | 91 | 14.61 | 9.48 | 8.98 | 1.48 | 1.00 | – |
| | 9 | 16.00 | 16.15 | 10.00 | 1.70 | 1.20 | 0.90 |
| 1-$C_3H_7O^{\bullet}$ | 69 | 14.65 | 9.48 | 8.90 | 1.48 | 1.00 | – |
| | 31 | 16.06 | 16.04 | 10.15 | 1.63 | 1.05 | 1.05 |
| 2-$C_3H_7O^{\bullet}$ | 80 | 14.67 | 9.42 | 9.29 | 1.53 | 1.02 | – |
| | 20 | 15.95 | 16.05 | 10.20 | 1.65 | 1.20 | 0.95 |
| *n*-$C_4H_9O^{\bullet}$ | 66 | 14.63 | 9.53 | 8.86 | 1.50 | 1.00 | – |
| | 34 | 15.98 | 16.16 | 10.10 | 1.68 | 1.02 | 1.02 |
| *tert*-$C_4H_9O^{\bullet}$ | 20 | 14.92 | 9.23 | 9.03 | 1.43 | 1.05 | – |
| | 80 | 16.02 | 16.25 | 10.05 | 1.65 | 1.05 | 0.92 |
| *n*-$C_5H_{11}O^{\bullet}$ | 38 | 14.70 | 9.38 | 9.05 | 1.48 | 1.00 | – |
| | 62 | 16.06 | 16.04 | 10.15 | 1.66 | 0.95 | 0.95 |
| 3-$C_5H_{11}O^{\bullet}$ | 63 | 14.67 | 9.35 | 9.29 | 1.53 | 1.02 | – |
| | 37 | 16.10 | 15.95 | 10.10 | 1.65 | 1.03 | 0.97 |
| cyclo-$C_5H_9O^{\bullet}$ | 50 | 14.65 | 9.42 | 9.34 | 1.43 | 1.05 | – |
| | 50 | 16.00 | 16.15 | 10.00 | 1.62 | 0.98 | 0.90 |
| 1-Penten-3-oxyl | 100 | 16.10 | 16.10 | 10.00 | 1.65 | 1.00 | 1.00 |
| *n*-$C_8H_{17}O^{\bullet}$ | 100 | 15.20 | 15.20 | 9.60 | 1.30 | 1.30 | 1.30 |
| $CH_3CH(OH)^{\bullet}$ | 100 | 15.73 | 16.74 | 9.95 | 1.55 | 1.07 | – |
| $LO^{\bullet}$ | 62 | 16.14 | 15.99 | 9.75 | 1.69 | 1.09 | 1.09 |
| | 38 | 15.47 | 16.90 | 10.10 | 1.72 | 1.09 | – |

[a] Sankuratri and Janzen [1].

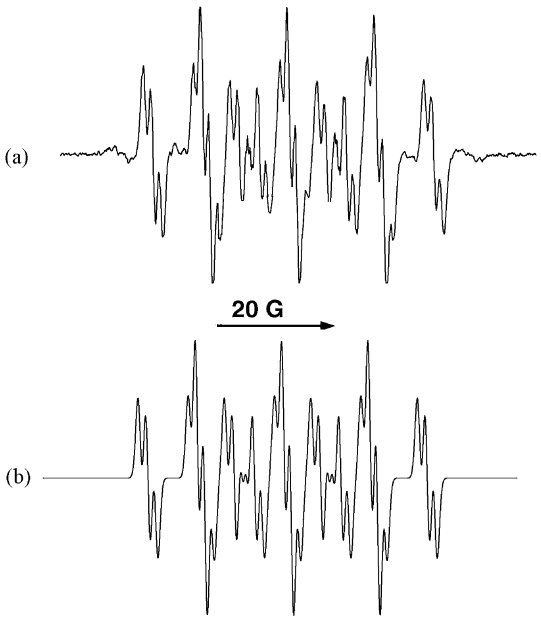



Fig. 3. Formation of the methoxyl radical adduct of Trazon by nucleophilic addition of methanol in the presence of $Fe^{3+}$. (a) Trazon (50 mM) was incubated with methanol in the presence of $FeCl_3$ (2 mM) and the reaction was stopped after 2 min by 1:50 dilution with phosphate buffer (0.3 M, pH 7.4, containing 20 mM DTPA). The spectrum was recorded with the following spectrometer settings: sweep width, 80 G; modulation amplitude, 0.2 G; microwave power, 2 mW; time constant, 0.08 s; receiver gain, $2 \times 10^5$; scan rate, 114.4 G/min. (b) Computer simulation, based on the hyperfine splitting data listed in Table 1.

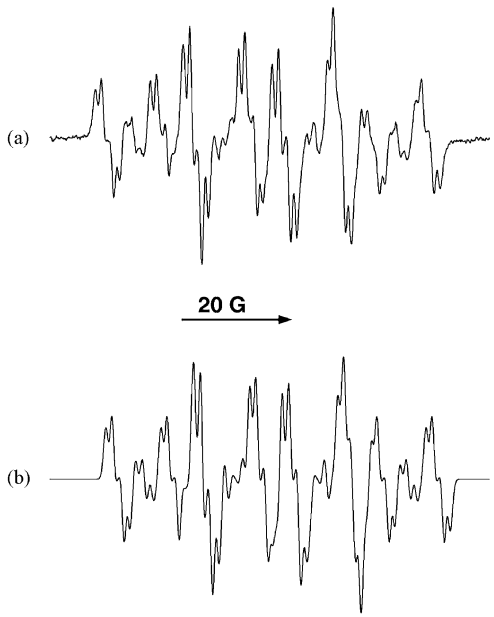



Fig. 4. Formation of the *tert*-butoxyl radical adduct of Trazon by nucleophilic addition of *tert*-butanol in the presence of $Fe^{3+}$. (a) Trazon (50 mM) was incubated with *tert*-butanol in the presence of $FeCl_3$ (2 mM) and the reaction was stopped after 5 min by 1:50 dilution with phosphate buffer (0.3 M, pH 7.4, containing 20 mM DTPA). The spectrum was recorded with the following spectrometer settings: sweep width, 80 G; modulation amplitude, 0.2 G; microwave power, 2 mW; time constant, 0.08 s; receiver gain, $2 \times 10^5$; scan rate, 114.4 G/min. (b) Computer simulation, based on the hyperfine splitting data listed in Table 1.

With some compounds an almost exact 1:1 mixture of the two stereoisomers was obtained, the ESR spectrum of the respective incubation with cyclopentanol and its computer simulation being shown in Fig. 5. The respective ESR parameters are listed in Table 1.

Both the spin trap and the attached alkoxyl residue possess chiral centers, therefore, four diastereomeric forms are to be expected. In the observed ESR spectra only two different forms can be distinguished with a small but observable difference in g-values. The fact that the ratio between the two distinguishable stereoisomers mainly depends on the size of the alkoxyl substituent is consistent with the assumption that the two forms reflect the orientation at the spin trap (exo- and endo-addition) rather than the structure of the alkoxyl residue (*R*- or *S*-form of the trapped radical).

### 3.3. Spin trapping of lipid-derived free radicals

Two different systems have recently been reported for the detection of lipid-derived free radicals. First, a system consisting of peroxidized linoleic acid and the spin trap (dissolved in phosphate buffer, pH 7.4, containing 1% acetonitrile) to which $Fe^{2+}$ (dissolved in water) is added in order to start free radical formation in a Fenton-type reaction. In this way, a series of different DEPMPO derivatives in both aerobic and anaerobic environment has been tested [3,4] using a stationary system in combina-

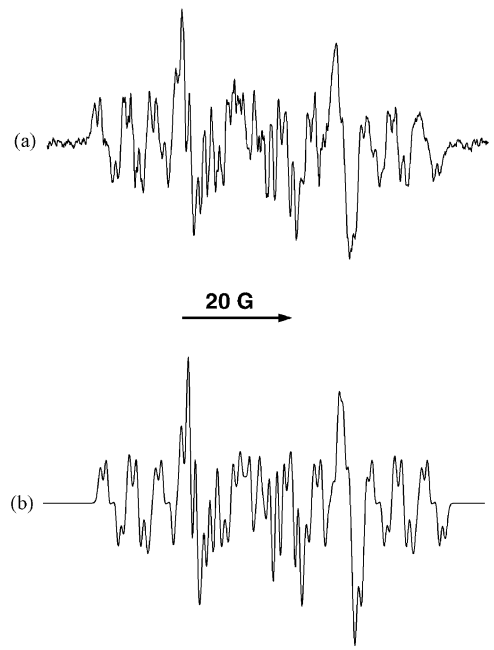



Fig. 5. Formation of the cyclopentoxyl radical adduct of Trazon by nucleophilic addition of cyclopentanol in the presence of $Fe^{3+}$. (a) Trazon (50 mM) was incubated with cyclopentanol in the presence of $FeCl_3$ (2 mM) and the reaction was stopped after 2 min by 1:50 dilution with phosphate buffer (0.3 M, pH 7.4, containing 20 mM DTPA). The spectrum was recorded with the following spectrometer settings: sweep width, 80 G; modulation amplitude, 0.2 G; microwave power, 5 mW; time constant, 0.02 s; receiver gain, $2 \times 10^5$; scan rate, 114.4 G/min; 5 scans. (b) Computer simulation, based on the hyperfine splitting data listed in Table 1.

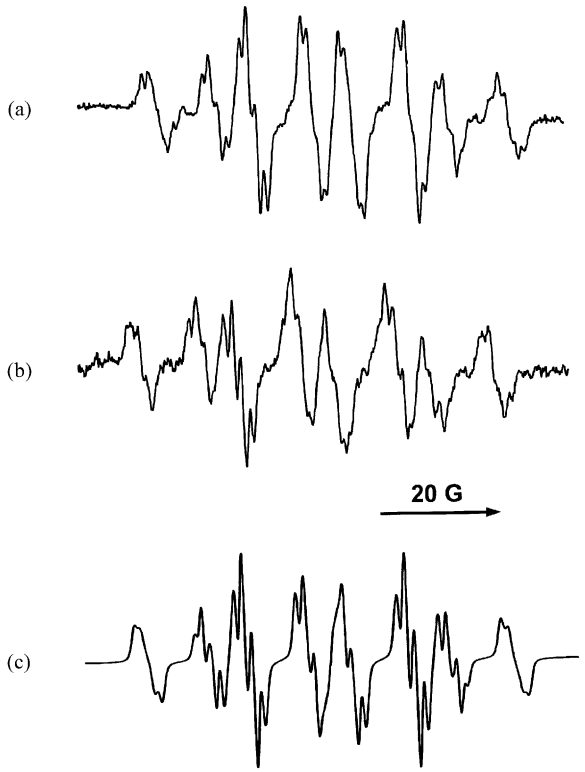



Fig. 6. Detection of Trazon spin adducts with linoleic acid-derived alkoxyl radicals. (a) A nitrogen-bubbled solution of peroxidized linoleic acid (1 mM) and Trazon (70 mM) in phosphate buffer (20 mM, pH 7.4, containing 1% acetonitrile) were mixed with a nitrogen-bubbled solution of $FeSO_4$ (0.25 mM) in water using a rapid sampler. The spectrum was recorded with the following spectrometer settings: sweep width, 80 G; modulation amplitude, 0.7 G; microwave power, 20 mW; time constant, 0.16 s; receiver gain, $1 \times 10^5$; scan rate, 28.6 G/min. (b) Linoleic acid (1 mM) and SOD (5000 U/mL) were mixed with lipoxidase (1 mg/mL; 126,500 U/mL) and 80 mM of the spin trap Trazon in phosphate buffer (100 mM, pH 8.5, 1 mM DTPA, containing 1% ethanol) at a total flow rate of 1.67 mL/min. The spectrum was recorded with the following spectrometer settings: sweep width, 80 G; modulation amplitude, 1.0 G; microwave power, 40 mW; time constant, 0.08 s; receiver gain, $2 \times 10^5$; scan rate, 114.4 G/min. (c) Computer simulation, based on the hyperfine splitting data listed in Table 1.

tion with the rapid sampling technique. The second system, consisting of linoleic acid, lipoxidase (dissolved in phosphate buffer, pH 8.5, 1 mM DTPA, 1% ethanol) has recently been described by Dikalov and Mason [5] for a slow-flow system with the spin trap DMPO.

The ESR spectra obtained in the Fenton-type reaction are shown in Fig. 6a. Here, a nitrogen-bubbled solution of peroxidized linoleic acid and Trazon in phosphate buffer was rapidly mixed with a nitrogen-bubbled solution of $FeSO_4$ in water. In contrast to the results obtained earlier with the spin traps DMPO or DEPMPO, the Trazon adduct was stable for almost half an hour (not shown).

The ESR spectrum obtained using the slow-flow linoleic acid/lipoxidase system reported by Dikalov and Mason [5] is shown in Fig. 6b, where linoleic acid and SOD were mixed 1:1 with soybean lipoxidase and Trazon at a total flow rate of 1.67 mL/min. The ESR spectrum is practically identical to the one obtained in the Fenton system shown above (Fig. 6a), consisting of at least two different isomers of alkoxyl radical adducts. A tentative computer simulation is shown in Fig. 6c, assuming two different stereoisomers of equal g-value. The respective data are summarized in Table 1.

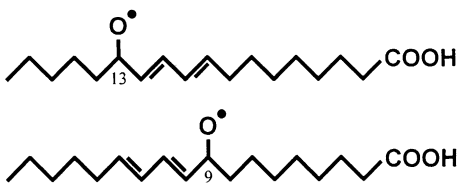
We also checked the influence of the added solvent. Whereas no effect was observed in the presence of acetonitrile, the addition of ethanol resulted in the appearence of additional lines from the Trazon/α-hydroxyethyl radical adduct in the Fenton system (ESR spectrum not shown, hyperfine splitting, see Table 1). In the lipoxygenase system the addition of 1% ethanol did not have any effect.

## 4. Discussion

The spin trap Trazon forms especially stable alkoxyl radical adducts which can easily be detected in a stationary system since they are more stable than those previously reported using DMPO [5] and more selective for oxygen-centered radicals in lipid phases than PBN. The ESR spectra of Trazon adducts with primary alkoxyl radicals of short chain length (methoxyl and ethoxyl) are characteristically different from those observed with alkoxyl radicals of longer chain length (such as the *n*-octyloxy substituent) or with a bulky tertiary structure (such as the *tert*-butoxy residue), most probably reflecting the formation of two different stereoisomers. Alkoxy substituents of intermediate size such as the cyclopentoxy residue are forming two different stereoisomers at an almost equal ratio.

The two possible diastereomers formed from Trazon and a non-chiral alcohol such as methanol or *tert*-butanol result from the orientation with respect to the spin trap (exo- vs. endo-addition [1]) (see Fig. 1).

On the other hand, the two different species observed in the spectrum of the spin adducts of Trazon with lipid alkoxyl radicals merely reflect the different possible isomers of the trapped radical itself since the lipid alkoxyl residue is so large, that it can only bind from the sterically more favorable side (most probably exo-addition). The two most likely positions of the oxygen on the peroxidized linoleic acid are positions 9 and 13, leading to at least two different structural isomers of the alkoxyl radical.




Each of these two structural isomers exists in racemic form giving rise to two pairs of diastereomers after addition to the chiral spin trap Trazon which at high resolution

should be spectroscopically distinguishable. A complete analysis of the ESR spectra obtained is, therefore, impossible unless the oxidation products can be separated into their pure optical forms.

On the other hand, the Trazon superoxide radical adduct ($t_{1/2} = 3.6$ min), is only slightly more stable than the respective DMPO adduct ($t_{1/2} = 45$ s, [8]), whereas the superoxide adducts of 5-(ethyoxycarbonyl)-5-methyl-1-pyrroline *N*-oxide (EMPO) ($t_{1/2} = ca.$ 8 min [9]) and DEPMPO ($t_{1/2} = ca.$ 15 min [3,10]) are considerably more stable.

Spin adducts of Trazon with alkylperoxyl radicals, formed during lipid peroxidation as a result of oxygen addition to transiently formed carbon-centered lipid radicals, were not detected. In this respect secondary reactions of lipidperoxyl radicals with other reactants have to be taken into account [11–16], especially the decay into molecular oxygen and secondary lipoxyl radicals, which in turn are rapidly converted into carbon-centered lipid radicals *via* several pathways [17].

In summary, the spin trap Trazon can be recommended for the detection of lipid-derived alkoxyl radicals especially with respect to the trapping of alkoxyl radicals formed during lipoxidase-catalyzed peroxidation of lipids, where it is both better suitable than the spin traps DMPO or PBN and provides additional stereochemical information.

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