J. Biochem. **142,** 73–78 (2007)
doi:10.1093/jb/mvm114

Identification of Radicals Formed in the Reaction Mixtures of Rat Liver Microsomes with ADP, Fe^{3+} and NADPH Using HPLC–EPR and HPLC–EPR–MS

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Received March 15, 2007; accepted April 24, 2007;

The reaction of rat liver microsomes with Fe^{3+}, ADP and NADPH was examined using EPR, HPLC–EPR and HPLC–EPR–MS combined use of spin trapping technique. A prominent EPR spectrum ($\alpha^N = 1.58$ mT and $\alpha^H\beta = 0.26$ mT) was observed in the complete reaction mixture. The EPR spectrum was hardly observed for the complete reaction mixture without rat liver microsomes. The radicals appear to be derived from microsomal components. The EPR spectrum was also hardly observed in the absence of Fe^{3+}. Addition of some iron chelators such as EDTA, citrate and ADP resulted in the dramatic change in the EPR intensity. Iron ions seem to be essential for this reaction. For the complete reaction mixture with boiled microsomes, a weak EPR spectrum was observed, suggesting that enzymes participate in the reaction. Five peaks were separated on the HPLC–EPR elution profile of the complete reaction mixture of rat liver microsomes with ADP, Fe^{3+} and NADPH. The retention times of the peaks 1 to 5 were 19.4, 22.5, 27.3, 29.8 and 31.4 min, respectively. To identify the radical adducts, HPLC–EPR–MS analyses were performed for the three prominent peaks. The HPLC–EPR–MS analyses showed that a new radical adduct, 4-POBN/1-hydroxypentyl radical, in addition to 4-POBN/ethyl radical adducts, forms in a reaction mixture of rat liver microsomes with ADP, Fe^{3+} and NADPH.

Key words: ethyl radical, iron ion, lipid peroxidation, 1-hydroxypentyl radical, P450, spin trapping.

Abbreviations: 4-POBN, α-(4-Pyridyl-1-oxide)-*N*-*tert*-butylnitrone.

Extensive EPR spin-trapping studies have shown that free radicals form in the reactions of microsomes with a variety of organic compounds and pharmaceutics, such as ethanol (*1–3*), carbon tetrachloride (*4*), glycerol (*5*), diethylnitrosamine (*6*) and ciprofloxacin (*7*). An EPR spectrum was also obtained when liver microsomes from a malignant hyperthermia susceptible pig were incubated (*8*). After chronic ethanol treatment, superoxide and hydroxyl radicals were also detected in microsomes in the presence of either NADPH or NADH (*1*). Measurements of malondialdehyde showed that lipid peroxidation occurs in the microsomes incubated with iron ion (*9*, *10*), suggesting that iron ion enhances lipid peroxidation in microsomes. Direct evidence for the free radical formation in isolated hepatocytes treated with $FeSO_4$ (or ADP-$FeCl_3$) was also obtained using EPR (*11*).

To identify the radicals formed in the reaction of microsomes, ^{13}C-labelled compounds have been successfully used. It was demonstrated that trichloromethyl ($^{13}CCl_3$) radicals were readily observed in rat liver microsomes metabolizing $^{13}CCl_4$ and in the liver of intact rats given a single dose of $^{13}CCl_4$ (*12*). The generation of 1-hydroxyethyl radicals was verified by using ^{13}C-substituted ethanol (*13*).

Some radicals were identified in the reaction of microsomes incubated with iron ion (*14*) based on HPLC retention times using HPLC–EPR. To our knowledge, the radicals derived from endogenous compounds, however, have not been identified in the reaction of microsomes incubated with iron ion based on mass spectrum data. In this article, a new radical, 1-hydroxypentyl radical is detected and identified, in addition to ethyl radical, in the reaction mixtures of rat liver microsomes with Fe^{3+}, ADP and NADPH using EPR, HPLC–EPR and HPLC–EPR–MS combined use of spin-trapping technique.

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MATERIALS AND METHODS

Materials—α-(4-Pyridyl-1-oxide)-*N*-*tert*-butylnitrone (4-POBN), a spin-trapping reagent was purchased from Tokyo Kasei Kogyo, Ltd. (Tokyo, Japan). ADP and NADPH were from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). *N,N*-diethylaminoethyl 2,2-diphenylvalerate (SKF-525A) and 1,2-diethylhydrazine dihydrochloride were purchased from Sigma–Aldrich Co. (St Louis, MO, USA). 1-Pentanol was obtained from Kishida Chemical, Ltd. (Osaka, Japan). All other chemicals used were of analytical grade.

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Preparation of Rat Liver Microsomes—Male Sprague–Dawley rats, body weight 250–300 g, were used in the experiments. The rat livers were removed immediately after decapitation. The livers were homogenized in 9 vol of 0.25 M sucrose. The liver homogenate was centrifuged at 16,000 g for 30 min at 4°C. The supernatant fraction was then centrifuged at 120,000 g for 30 min at 4°C. The pallet was suspended in 0.15 M KCl and then centrifuged twice again at 120,000 g. The pallet was suspended in 0.15 M KCl. Protein concentration of the suspension was 5.1 mg/ml. It was kept at –80°C before use.

The Complete Reaction Mixture of Rat Liver Microsomes with ADP, Fe^{3+} and NADPH—The complete reaction mixture contained 0.1 M 4-POBN, 2.6 mg protein/ml rat microsomal suspension, 10 mM ADP, 0.17 mM $FeCl_3$ and 1 mM NADPH in 37 mM phosphate buffer (pH7.4). The reaction was started by adding NADPH and performed for 30 min for the EPR experiment (60 min for the HPLC–EPR and HPLC–EPR–MS) at 37°C.

The Reaction Mixture of 1-Pentanol—The reaction mixture of 1-pentanol contained 0.1 M 4-POBN, 5 µl/ml 1-pentanol, 0.2 mM H_2O_2 and 0.2 mM $FeSO(NH_4)_2SO_4$ in 37 mM phosphate buffer (pH7.4). The reaction was started by adding $FeSO(NH_4)_2SO_4$ and performed for 2 min at 37°C. The reaction mixture was applied to HPLC–EPR.

The Reaction Mixture of Diethylhydrazine—The reaction mixture of diethylhydrazine contained 0.1 M 4-POBN, 8 mg diethylhydrazine dihydrochloride and 0.2 mM $CuCl_2$ in 2 ml of 50 mM carbonate buffer (pH10.0). The reaction was started by adding $CuCl_2$ after bubbling nitrogen gas. It was performed for 120 min under an air atmosphere. The reaction mixture was applied to HPLC–EPR.

EPR Measurements—The EPR spectra were obtained using a model JES-FR30 Free Radical Monitor (JEOL Ltd, Tokyo, Japan). Aqueous samples were aspirated into a Tefron tube centred in a microwave cavity. Operating conditions of the EPR spectrometer were: power, 4 mW; modulation width, 0.1 mT; sweep time, 4 min; sweep width, 10 mT; time constant, 0.3 s. Magnetic fields were calculated by the splitting of MnO ($\Delta H_{3-4} = 8.69$ mT).

HPLC–EPR Chromatography—An HPLC used in the HPLC–EPR consisted of a model 7125 injector (Reodyne, Cotari, CA, USA) and a model 655A-11 pump with a model L-5000 LC controller (Hitachi Ltd, Ibaragi, Japan). A semi-preparative column (300 mm long $\times$ 10 mm i.d.) packed with TSKgel ODS-120T (TOSOH Co., Tokyo, Japan) was used. Flow rate was 2.0 ml/min throughout the experiments. For the HPLC–EPR, two solvents were used: solvent A, 50 mM acetic acid; solvent B, 50 mM acetic acid/acetonitrile (20:80, v/v). A following combination of isocratic and linear gradient was used: 0–40 min, 100–20% A (linear gradient); 40–60 min, 80% B (isocratic). The eluent was introduced into a model JES-FR30 Free Radical Monitor. The EPR spectrometer was connected to the HPLC with a Teflon tube, which passed through the centre of the EPR cavity. The operating conditions of the EPR spectrometer were: power, 4 mW; modulation width, 0.2 mT; time constant, 1 s. The magnetic field was fixed at the third peak in the doublet-triplet EPR spectrum ($\alpha^N = 1.58$ mT and $\alpha^H\beta = 0.26$ mT) of the 4-POBN radical adduct.

HPLC–EPR–MS Chromatography—HPLC and EPR conditions were as described in the HPLC–EPR. The operating conditions of the mass spectrometer were: nebulizer, 180°C; aperture 1, 120°C; N_2 controller pressure, 2.0 kg/cm^2 drift voltage, 70 V; multiplier voltage, 1800 V; needle voltage, 3000 V; polarity, positive; resolution, 48.

The mass spectra were obtained by introducing the eluent from the EPR detector into the LC–MS system just before the peaks were eluted. The flow rate was kept at 50 µl/min while the eluent was introducing into the mass spectrometer.

RESULTS

EPR Measurement of the Reaction Mixture of Rat Liver Microsomes with ADP, Fe^{3+} and NADPH—EPR spectrum of the complete reaction mixture of rat liver microsomes with ADP, Fe^{3+} and NADPH was measured (Fig. 1). A prominent EPR spectrum ($\alpha^N = 1.58$ mT and $\alpha^H\beta = 0.26$ mT) was observed in the complete reaction mixture (Fig. 1A). The EPR spectrum was hardly observed for the complete reaction mixture without rat liver microsomes (or Fe^{3+}) (Fig. 1B and D). In the absence of ADP (or NADPH), the intensity of the EPR signal decreased to 50% (or 20%) of the complete reaction mixture (Fig. 1C and E). For the complete reaction mixture with boiled microsomes, weak EPR signals were observed (Fig. 1F). No EPR signal was observed in the presence of P450 inhibitor, SKF-525A (Fig. 1G).

HPLC–EPR and HPLC–EPR–MS Analyses of the Reaction Mixture of Rat Liver Microsomes with ADP, Fe^{3+} and NADPH—The HPLC–EPR analyses were performed for the complete reaction mixture of rat liver microsomes with ADP, Fe^{3+} and NADPH (Fig. 2). Five prominent peaks (peaks 1–5) were separated on the HPLC–EPR elution profile (Fig. 2A). The retention times of the peaks 1–5 are 19.4, 22.5, 27.3, 29.8 and 31.4 min, respectively.

In the absence of ADP (or NADPH), weak peaks were observed (Fig. 2C and E). No peaks were observed in the absence of microsomes (or Fe^{3+}) (Fig. 2B and D).

Time course of the HPLC–EPR peaks was measured for the complete reaction mixture (Fig. 3). Peak heights of the all HPLC–EPR peaks increased with time for 120 min.

To determine the structure of the peak 3, HPLC–EPR–MS analysis was performed. HPLC–EPR–MS analysis of the peak 3 compound gave ions at m/z 282 and m/z 195 (Fig. 4A). The ion m/z 282 corresponds to the protonated molecule of 4-POBN/1-hydroxypentyl radical adduct, $[M+H]^+$. A fragment ion at m/z 195 corresponds to the loss of $(CH_3)_3C(O)N$ from the protonated molecules. HPLC–EPR–MS analysis was performed for the peak 4. HPLC–EPR–MS analysis of the peak 4 compound gave the same ions as peak 3 (Fig. 4B). The peak 4 could be an isomer of the 4-POBN/1-hydroxypentyl radical adducts. HPLC–EPR–MS analysis was performed for the peak 5. HPLC–EPR–MS analysis of the peak 5 compound gave ions at m/z 224 and m/z 137 (Fig. 4C).

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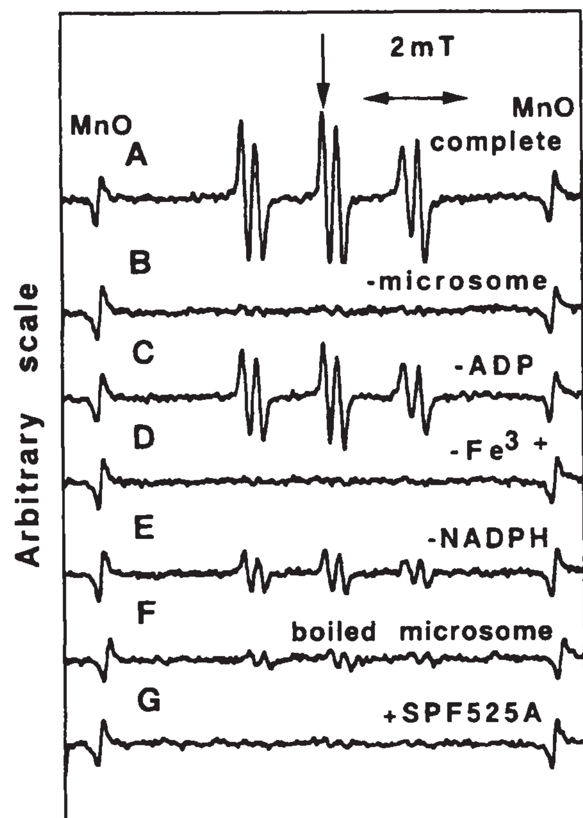

Fig. 1. **EPR spectra of the reaction mixtures of rat liver microsomes with ADP, Fe^{3+} and NADPH.** The reaction and EPR conditions were as described in MATERIALS AND METHODS section. Total volume of the reaction mixtures was 300 μl. (**A**) A complete reaction mixture of rat liver microsomes with ADP, Fe^{3+} and NADPH. (**B**) Same as in A except that microsomes were omitted. (**C**) Same as in A except that ADP was omitted. (**D**) Same as in A except that Fe^{3+} was omitted. (**E**) Same as in A except that NADPH was omitted. (**F**) A complete reaction mixture with boiled microsomes. (**G**) Same as in A except that SKF-525A (10 mM) was added.

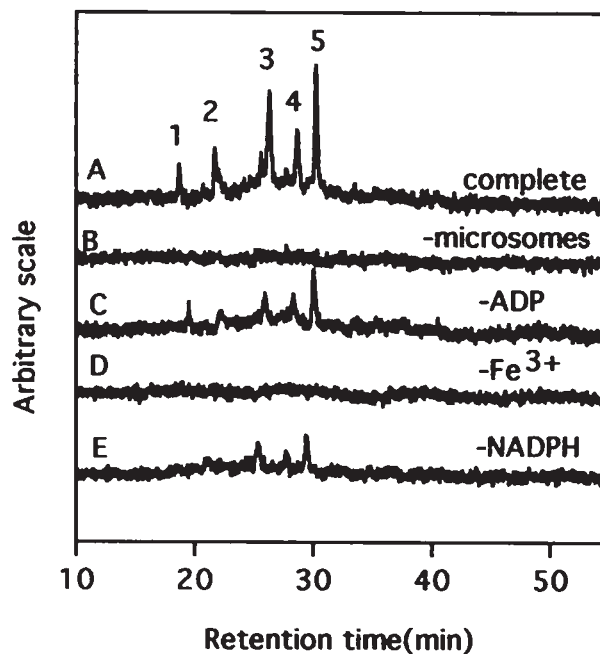

Fig. 2. **HPLC–EPR spectra of the reaction mixtures of rat liver microsomes with ADP, Fe^{3+} and NADPH.** The reaction and HPLC–EPR conditions were as described in MATERIALS AND METHODS section. Total volume of the reaction mixtures was 5 ml. (**A**) A complete reaction mixture of rat liver microsomes with ADP, Fe^{3+} and NADPH. (**B**) Same as in A except that microsomes were omitted. (**C**) Same as in A except that ADP was omitted. (**D**) Same as in A except that Fe^{3+} was omitted. (**E**) Same as in A except that NADPH was omitted.

The ion *m*/*z* 224 corresponds to the protonated molecule of 4-POBN/ethyl radical adduct, $[M+H]^+$. A fragment ion at m/z 137 corresponds to the loss of $(CH_3)_3C(O)N$ from the protonated molecule. HPLC–EPR–MS analyses of the peaks 1 and 2 did not succeed because of the low peak intensities.

In order to confirm the structures of the peaks 3, 4 and 5, HPLC–EPR analyses were performed. An HPLC–EPR analysis of the reaction mixture of 1-pentanol, H_2O_2 and Fe^{2+} showed three prominent peaks [1-pentanol(1), (2) and (3)]. The peaks of 1-pentanol (1) and (2) have already identified as two diastereoisomers of 4-POBN/1-hydroxypentyl radical adducts in the previous article (*15*). The peaks of 1-pentanol (1) and (2) showed almost the same retention times as the peaks 3 and 4, respectively. When the 1-pentanol (1) fraction was mixed with the complete reaction mixture, the peak height of the peak 3 increased (Fig. 5C). When the 1-pentanol (2) fraction was mixed with the complete reaction mixture, the peak height of peak 4 increased (Fig. 5E). The result suggests that the

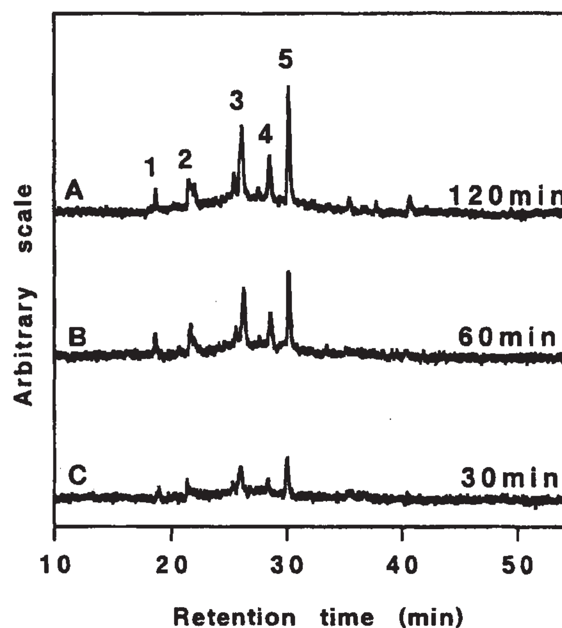

Fig. 3. **Time course of HPLC–EPR analysis of complete reaction mixture.** The reaction and EPR conditions were as described in MATERIALS AND METHODS section. Total volume of the reaction mixtures was 5 ml. (**A**) Reaction time was 120 min. (**B**) Reaction time was 60 min. (**C**) Reaction time was 30 min.

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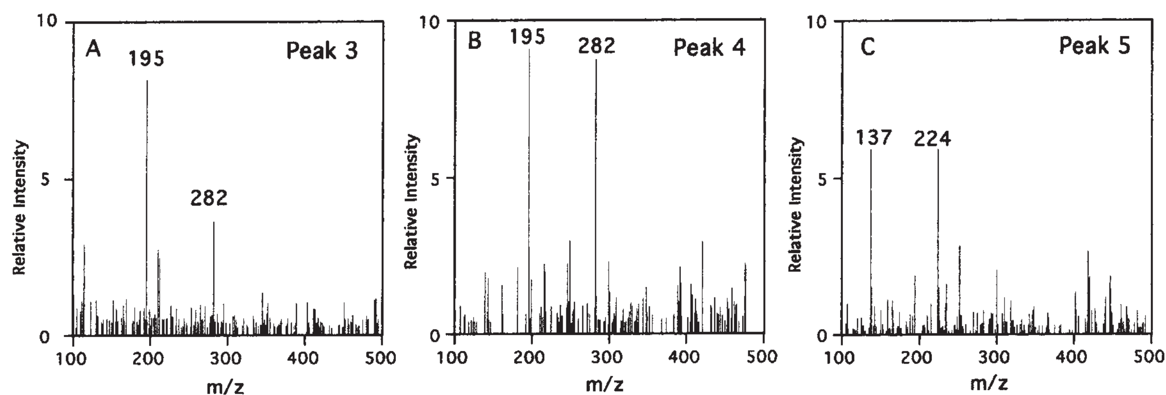

Fig. 4. **HPLC–EPR–MS analysis of the peaks 3–5.** The reaction and HPLC–EPR–MS conditions were as described in MATERIALS AND METHODS section. Total volume of the reaction mixtures was 5 ml. (**A**) A mass spectrum of the peak 3. (**B**) A mass spectrum of the peak 4. (**C**) A mass spectrum of the peak 5.

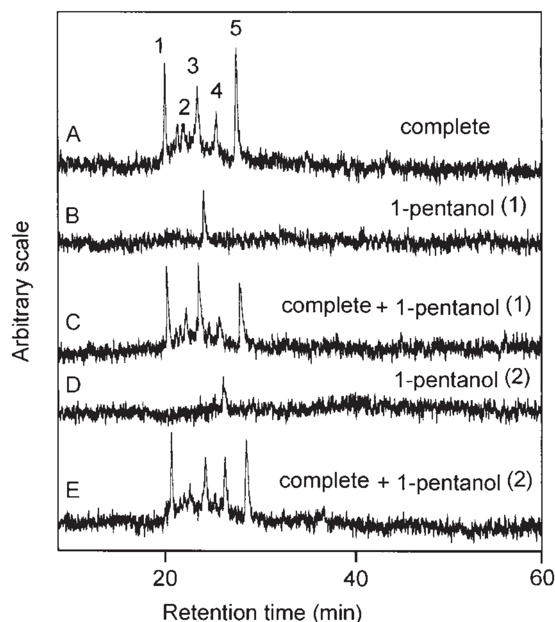

Fig. 5. **Identification of the peaks 3–5.** The reaction and HPLC–EPR–MS conditions were as described in MATERIALS AND METHODS section. (**A**) An HPLC–EPR analysis of the complete reaction mixture (2 ml). (**B**) An HPLC–EPR analysis of 1-pentanol (1) (0.4 ml). (**C**) An HPLC–EPR analysis of a mixture of the complete reaction mixture (1 ml) and 1-pentanol (1) (0.2 ml). (**D**) An HPLC–EPR analysis of 1-pentanol (2) (0.4 ml). (**E**) An HPLC–EPR analysis of a mixture of the complete reaction mixture (1 ml) and 1-pentanol (2) (0.2 ml).

peak 3 and peak 4 are isomers of 4-POBN/1-hydroxypentyl radical adduct.

An HPLC–EPR analysis of the reaction mixture of diethylhydrazine gave a peak with a similar retention time of peak 5. When the peak fraction of diethylhydrazine was mixed with the complete reaction mixture, the peak height of peak 5 increased (data not shown). The result shows that the peak 5 is 4-POBN/ethyl radical adduct.

Effect of Some Compounds on the Radical Formation—Effect of some iron chelators such as EDTA, citrate and ADP on the radical formation was examined (Table 1). The addition of EDTA (or citrate, or ADP) resulted in dramatic change in the EPR intensity (EDTA, 42 ± 6% of control; citrate, 0% of control; ADP, 187 ± 16% of control).

Table 1. **Effect of some compounds on the radical formation.**

Compounds	% Control
+ EDTA (10 mM)	42 ± 6[a]
+ Citrate (10 mM)	0[a]
+ ADP (10 mM)	187 ± 16[a]
+ Azide (10 mM)	92 ± 8
+ Cyanide (1 mM)	92 ± 0
+ Cyanide (10 mM)	58 ± 4

Results are means ± SD of three experiments. [a]Control is complete reaction mixture without ADP. ESR measurement and reaction were performed as described in MATERIALS AND METHODS section.

Effect of azide and cyanide ions was studied. On addition of 10-mM azide ion, no effect was observed. On the other hand, the EPR peak height decreased to 58% of the control when 10-mM cyanide ion was added to the reaction mixture.

DISCUSSION

In this study, the reaction of rat liver microsomes with Fe^{3+}, ADP and NADPH was examined using EPR, HPLC–EPR and HPLC–EPR–MS combined use of spin trapping technique. A prominent EPR spectrum ($\alpha^N = 1.58$ mT and $\alpha^H\beta = 0.26$ mT) was observed in the complete reaction mixture. The EPR spectrum was hardly observed for the complete reaction mixture without rat liver microsomes. The radical could be derived from microsomal components. The EPR spectrum was also hardly observed in the absence of Fe^{3+}. Addition of iron chelator EDTA (or citrate, or ADP) resulted in dramatic change in the EPR intensity. Iron ions seem to be essential for this reaction. For the complete reaction mixture with boiled microsomes, a weak EPR spectrum was observed, suggesting that enzymes participate in the reaction.

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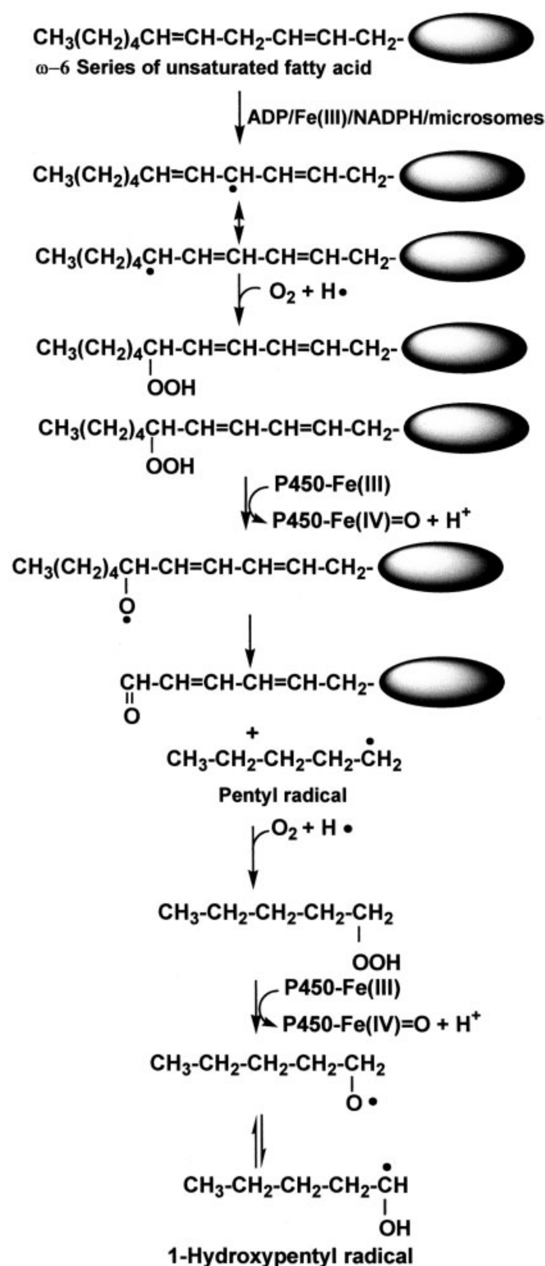
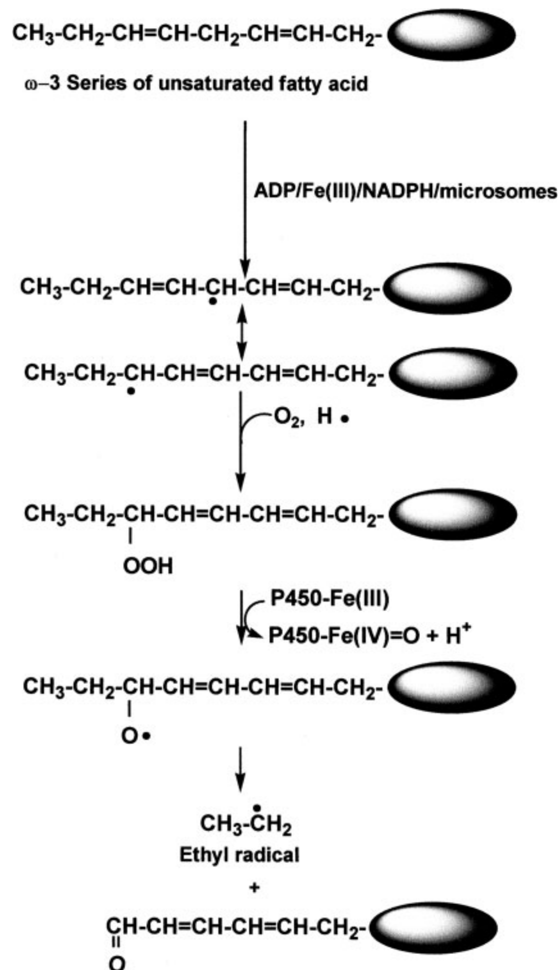


Scheme 1. **Possible reaction paths for the formation of 1-hydroxypentyl radical.**

HPLC–EPR and HPLC–EPR–MS analyses showed that 4-POBN/1-hydroxypentyl radical, a new radical adduct, and 4-POBN/ethyl radical adducts form in a reaction mixture of rat liver microsomes with ADP, Fe^{3+} and NADPH. Possible reaction paths for the formation of the radicals (1-hydroxypentyl and ethyl radicals) are shown as follows (Schemes 1 and 2).

Reaction of ADP/Fe(III)/NADPH/microsomes with unsaturated fatty acid LH such as linoleic acid, arachidonic acid (Scheme 1) and eicosapentaenoic acid (Scheme 2) results in allyl radical $L^{\bullet}$ (Equation 1).

$$LH \overset{(ADP/Fe(III)/NADPH/microsomes)}{\rightarrow} L^{\bullet} \quad (1)$$



Scheme 2. **Possible reaction paths for the formation of ethyl radical.**

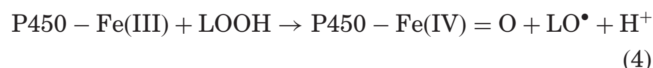
Peroxyl radical $LOO^{\bullet}$ possibly forms through the reaction of the allyl radical $L^{\bullet}$ with molecular oxygen O_2 (Equation 2).

$$L^{\bullet} + O_2 \rightarrow LOO^{\bullet} \quad (2)$$

The peroxyl radicals $LOO^{\bullet}$ turn to hydroperoxides LOOH by removing $H^{\bullet}$ from surrounding molecules (Equation 3).

$$LOO^{\bullet} + H^{\bullet} \rightarrow LOOH \quad (3)$$

Since SPF-525A completely inhibited the formation of the radical formation, cytochrome-P450 participates in the reaction. Rota *et al*. (*16*) reported that cytochrome-P450-catalysed homolytic scission of hydroperoxides LOOH. This reaction yields radical intermediates $LO^{\bullet}$ (Equation 4) (*17*).



$$P450 - Fe(III) + LOOH \rightarrow P450 - Fe(IV) = O + LO^{\bullet} + H^{+} \quad (4)$$

β-Scission of the $LO^{\bullet}$ possibly forms the ethyl radical (Scheme 2) [or pentyl radical (Scheme 1)] (Equation 5). Thus, oxidation of ω3 series of unsaturated fatty acids may form the ethyl radical (Scheme 2). The pentyl

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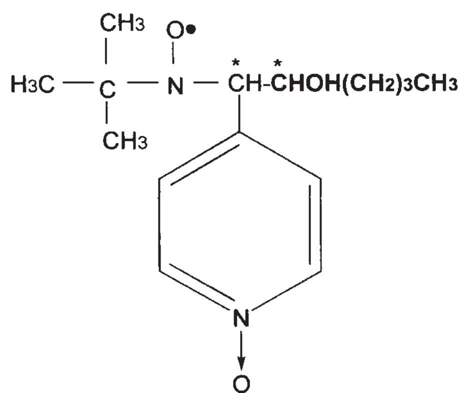

Fig. 6. **Chemical structure of 4-POBN/1-hydroxypentyl radical adduct.** Asymmetric carbon atoms are indicated by * marks.

radical could be derived from ω6 series of unsaturated fatty acids (Scheme 1) (*18*).

$$\mathrm{LO}^{\bullet} \rightarrow \text{Ethyl radical (Pentyl radical)} \quad (5)$$

Reaction of the pentyl radical with molecular oxygen O_2 may form 1-hydroxypentyl radical through the reaction paths described in Scheme 1.

Two HPLC–EPR peaks were observed for 4-POBN/1-hydroxypentyl radical adducts (peaks 3 and 4, Fig. 2). Because two asymmetric carbon atoms exist in the radical adduct (Fig. 6), peaks 3 and 4 appear to be the two diasteremers of the 4-POBN/1-hydroxypentyl radical adduct.

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