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9-Hydroxy-10,12-octadecadienoic acid (9-HODE) and 13-hydroxy-9,11-octadecadienoic acid (13-HODE): excellent markers for lipid peroxidation

Peter Spiteller, Gerhard Spiteller *

Lehrstuhl Organische Chemie I, Universität Bayreuth, Universitätsstraße 30, 95440 Bayreuth, Germany

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Abstract

Various conditions for conversion of (9*S*,10*E*,12*Z*)-9-hydroperoxy-10,12-octadecadienoic acid (9*S*-HPODE) and (13*S*,9*Z*,10*E*)-13-hydroperoxy-9,11-octadecadienoic acid (13*S*-HPODE) into the corresponding hydroxy acids, (9*S*,10*E*,12*Z*)-9-hydroxy-10,12-octadecadienoic acid (9*S*-HODE) and (13*S*,9*Z*,10*E*)-13-hydroxy-9,11-octadecadienoic acid (13*S*-HODE), were investigated in vitro. 9*S*-HODE and 13*S*-HODE were subjected to lipid peroxidation under various conditions: oxidation was carried out in air only, and in air/Fe^{2+}/ascorbate, air/H_2O_2/Fe^{2+}, air/Fe^{2+}, and air/Fe^{3+}. In contrast to the corresponding hydroperoxides (9*S*-HPODE and 13*S*-HPODE), 9-HODE and 13-HODE proved to be stable in all these oxidation experiments. Unexpectedly, hydroxy compounds obtained by reduction of hydroperoxides derived from arachidonic acid were not attacked by air/Fe^{2+}/ascorbate or air/Fe^{2+}. Thus, for instance, (15*S*,5*Z*,8*Z*,11*Z*,13*E*)-15-hydroxy-5,8,11,13-eicosatetraenoic acid (15-HETE) remained unchanged in spite of possessing the structural prerequisites for attack by radicals, i.e. a CH_2-group located between two double bonds. Consequently, metal-induced air oxidation reactions of these systems seem to be restricted to hydroperoxides of unsaturated acids (LOOH) and not to corresponding hydroxy compounds (LOH). The reported experiments explain why hydroxy derivatives of unsaturated acids, especially 9-HODE and 13-HODE, are enriched in naturally occurring lipid peroxidation (LPO) processes to a greater extent than any other LPO product and why they are nearly ideal markers for LPO. © 1997 Elsevier Science Ireland Ltd.

Keywords: Lipid peroxidation; Linoleic acid; 9-HODE; 13-HODE; 9-HPODE; 13-HPODE

1. Introduction

The generation of lipid hydroperoxides (LOOH) of unsaturated fatty acids is considered to be involved in the development of numerous

* Corresponding author. Tel.: +49 921 552680; fax: +49 921 552671; e-mail:gerhard.spiteller@uni-bayreuth.de

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chronic diseases, such as atherosclerosis (Morel et al., 1983, Steinbrecher et al., 1984), rheumatoid arthritis (Wingard et al., 1993), diabetes (Nishigaki et al., 1981), multiple sclerosis (Mickel, 1978) and other nervous diseases (Braughler and Hall, 1989).

LOOH is not stable. For example, it is cleaved in the presence of Fe^{2+} in a Fenton-like reaction to alkoxy ($LO^{\bullet}$) radicals (Gardner, 1989):

$$LOOH + Fe^{2+} \rightarrow LO^{\bullet} + Fe^{3+} + OH^{-}$$

Otherwise, Fe^{3+} may produce lipid peroxyl radicals ($LOO^{\bullet}$) (Esterbauer et al., 1989):

$$LOOH + Fe^{3+} \rightarrow LOO^{\bullet} + Fe^{2+} + H^{+}$$

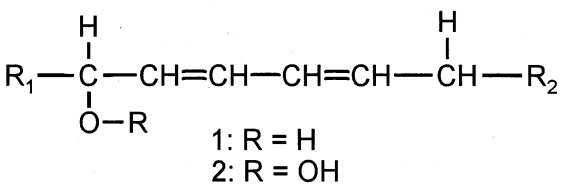
$LO^{\bullet}$ radicals undergo numerous reactions (Marnett and Wilcox, 1995): they abstract hydrogens from other molecules, especially from CH_2-groups activated by two adjacent double bonds to form the corresponding hydroxy acids (LOH) (Frankel, 1982, Esterbauer, 1982); they are decomposed by cleavage of adjacent C–C bonds by generation of aldehydes (Frankel, 1985, Esterbauer et al., 1991); they react with adjacent double bonds by forming epoxy radicals which are transformed to epoxy hydroxy acids (Hamberg, 1973, Gardner and Jursinic, 1981); and they undergo a large number of secondary oxidation reactions. Only a few of the resulting degradation products are known: e.g. malondialdehyde, 4-hydroxynonenal (Esterbauer et al., 1991) and α-hydroxyaldehydes (Loidl-Stahlhofen and Spiteller, 1994, Loidl-Stahlhofen et al., 1994).

Research on the occurrence and physiological activity of lipid peroxidation (LPO) products in mammalians has previously been almost exclusively restricted to those derived from arachidonic acid, in spite of the fact that linoleic acid is much more abundant than arachidonic acid (e.g. the ratio of linoleic acid/arachidonic acid in low-density lipoprotein (LDL) was found to be about 7:1) (Esterbauer et al., 1992) and that $LO^{\bullet}$ radicals seem to be nearly equally able to abstract hydrogen atoms from activated CH_2-groups of linoleic acid as from those of arachidonic acid. Thus, for instance, products obtained by reduction of linoleic acid hydroperoxides—(9-hydroxy-10,12-octadecadienoic acid (9-HODE) and 13-hydroxy-9,11-octadecadienoic acid (13-HODE)—were detected in large amounts in atherosclerotic plaques (Brooks et al., 1970, Harland et al., 1973, Belkner et al., 1991, Kühn et al., 1992) or the arteries and blood of rats after feeding a diet rich in cholesterol (Wang and Powell, 1991). [Note: the expressions 9-HODE and 13-HODE are used if the stereochemistry and E/Z configuration are unknown. This is the case for products generated in non-enzymic LPO processes.]

Recently we detected 9-HODE and 13-HODE in unusually high amounts in heart tissue after myocardial infarction (Dudda et al., 1996b), in LDL of elderly people (Jira and Spiteller, 1996) and in LDL of patients suffering from atherosclerosis or rheumatic arthritis (Jira et al., 1997). Thus, oxidation of linoleic acid seems to be a common process in mammalians after tissue injury, causing cell death.

This pointed to the possibility that hydroxy fatty acids (**1**) such as 9-HODE and 13-HODE are stable to further oxidation. A priori this was not expected since they possess the same conjugated system as the corresponding hydroperoxy fatty acids (**2**), namely 9-hydroperoxy-10,12-octadecadienoic acid (9-HPODE) and 13-hydroperoxy-9,11-octadecadienoic acid (13-HPODE), respectively. These easily undergo further oxidative transformation reactions obviously caused by hydrogen abstraction from C–H bonds activated by the conjugated system (Scheme 1).

In this paper we report on oxidation experiments which demonstrate that 9-HODE and 13-HODE are in fact stable against oxidation under usual LPO conditions. In addition, we demonstrate that (15*S*,5*Z*,8*Z*,11*Z*,13*E*)-15-hydroxy-5,8,11,13-eicosatetraenoic acid (15*S*-HETE) is



Scheme 1. The conjugated systems of HODE (R = H) and HPODE (R = OH) are the same. Hydrogens on carbons adjacent to the conjugated system are expected to be equally prone to abstraction by radicals.

also stable against oxidation, in contrast to its hydroperoxide.

2. Materials and methods

2.1. Materials

N-Methyl-*N*-trimethylsilyltrifluoracetamide (MSTFA) was obtained from Macherey and Nagel (Düren, Germany). Soybean lipoxygenase (5.3 U), $Fe_2SO_4 \cdot 7H_2O$, linoleic acid, arachidonic acid and sodium ascorbate were purchased from Fluka (Neu Ulm, Germany). HCl and $FeCl_3 \cdot 6H_2O$ were obtained from Merck (Darmstadt). Solvents were distilled before use.

Gas chromatography (GC) was carried out with a Carlo Erba HRGC 5160 Mega Series gas chromatograph equipped with a flame ionization detector using a DB-1 fused silica gel capillary column (30 m × 0.32 mm i.d.) (J&W Scientific, Germany), covered with a 0.1 μm layer of liquid phase. The temperature of the detector was kept at 290°C, the injector temperature was 280°C. Injector volume was 0.2–0.7 μl of a 1% (m/v) solution. Temperature programme: 3 min isothermal at 80°C, 3°C/min from 80°C to 280°C, then 15 min isothermal at 280°C. The carrier gas was hydrogen and the split ratio was 1:30. Peak integration and data recording were done with a Merck Hitachi Chromatointegrator D-2500.

Gas chromatography/mass spectrometry (GC/MS) was performed on a Finnigan MAT 95 double focusing mass spectrometer with inverse Nier-Johnson geometry, equipped with an EI ion source operated at 70 eV. A Hewlett Packard 5890 Series II gas chromatograph with a fused silica DB-05 glass capillary column (30 m × 0.25 mm i.d., covered with a 0.1 μm layer of liquid phase; J&W Scientific, Germany) was used for sample separation. The injector temperature was kept at 280°C; injection volumes were 0.6–1.5 μl of a 1–2% (m/v) solution. Temperature programme: 3 min isothermal at 50°C, increased within 2 min to 100°C, then 3°C/min until 300°C, finally 10 min isothermal at 300°C.

Kovats indices were determined by co-injection of a 0.2 μl sample of a standard mixture of saturated straight chain alkanes (C_{10}–C_{36}) (Kovats, 1958).

2.2. Thin-layer chromatography

Preparative thin-layer chromatography was carried out on glass plates 20 cm × 20 cm covered with a 0.75 mm layer of silica gel 60 PF254. The plates were prechromatographed with methanol; the 2 cm zone at the upper border containing impurities was then discarded. Before use the plates were activated for 2 h at 120°C.

2.3. High-performance liquid chromatography (HPLC)

2.3.1. Analytical normal phase HPLC (NP-HPLC)

The HPLC system comprised a Pumpsystem Beckman System Gold with programmable solvent module 126 and a Beckman System Gold diode array detector 168, with a LKB Bromma 2210 channel recorder. The column was a Bischoff Nucleosil 3 μm (25 cm × 4.6 mm i.d.) column, with a Bischoff Spherisorb 5 μm Si/NP (2 cm × 4 mm) precolumn.

2.3.2. Preparative NP-HPLC

This was carried out with a Beckman System Gold with programmable solvent module 125, programmable detector module 166, a Kipp & Zonen BD 41 recorder, a Bischoff Ultrasep FS 100 (6 μm) column (25 cm × 20 mm i.d.) and a Bischoff precolumn Ultrasep (2 cm × 20 mm i.d.).

2.4. Synthesis of (13*S*,9*Z*,11*E*)-13-hydroperoxy-9,11-octadecadienoic acid (13*S*-HPODE)

Synthesis of 13*S*-HPODE was carried out in a slightly modified procedure as described by Hamberg and Samuelsson (1967). Briefly, 1 g of linoleic acid (3.57 mmol) was suspended in 600 ml of 0.1 M borate buffer (pH 9.0) and emulsified by ultrasonic vibration. After removal of the ultrasonic bath the emulsion was vigorously magnetically stirred at 20°C, and 100 mg soybean lipoxygenase (5.3 U) were added. A few minutes later the solution clarified. After 1 h oxidation

was stopped by adding 2 N HCl until the solution became slightly cloudy. The solution was extracted three times with $CHCl_3$. The $CHCl_3$ layers were dried by addition of Na_2SO_4 and filtered. The solvent was then removed in vacuum at 20°C. The residue was dissolved in 5 ml of hexane/isopropanol solution (10:1, v/v) and slowly passed through a silica gel cartridge applying a slight excess of pressure. The cartridge was rinsed with another 5 ml of solvent. After solvent evaporation, 0.96 mg (86% yield) of raw 13*S*-HPODE was obtained as a colourless oil.

2.5. Synthesis of (9S,10E,12Z)-9-hydroperoxy-10,12-octadecanoic acid (9S-HPODE)

Synthesis of 9*S*-HPODE was carried out as described by Galliard (Matthew et al., 1977). Briefly 1.00 g of linoleic acid was suspended in 300 ml of 0.1 M phosphate buffer (pH 5.6) and 1 ml of Tween 20. Then 1 kg of fresh tomatoes, homogenized in 500 ml of phosphate buffer (pH 5.6) using a Waring blender, were added. The solution was vigorously magnetically stirred in air for 5 h. The reaction was terminated by adjusting the pH to 2–3 by addition of 2 N H_2SO_4. The aqueous solution was extracted three times each with 100 ml of diethyl ether. Separation of the layers required centrifugation (1500 rpm, 10 min). The ethereal solution was dried with Na_2SO_4. After filtration the solvent was removed in vacuum at 20°C. Yield was 0.91 g of yellow oil which still contained about two-thirds of linoleic acid.

2.6. Synthesis of (15S,5Z,8Z,11Z,13E)-15-hydroperoxy-5,8,11,13-eicosatetraenoic acid (15S-HPETE)

This acid was prepared in a similar way as described for synthesis of 13*S*-HPODE following the descriptions of Van Os et al. (1981) and Funk et al. (1976). Instead of 1 g of linoleic acid, 100 mg of arachidonic acid were emulsified with 60 ml of borate buffer pH 9.0 and only 4 mg lipoxygenase were added by stirring. Completion of the reaction was checked after stirring for 1 h. A sample was withdrawn, reduced with $P(OCH_3)_3$ (see below) and trimethylsilylated and analysed by GC; if arachidonic acid methylate was still detected, another 4 mg of lipoxygenase were added and the reaction mixture was stirred for another hour. Usually the reaction was complete after 2 h.

Preparation of (9*S*,10*E*,12*Z*)-9-hydroxy-10,12-octadecadienoic acid (9*S*-HODE and (13*S*,9*Z*,11*E*)-13-hydroxy,9,11-octadecadienoic acid (13*S*-HODE) was done by reduction of 13-HPODE and 9-HPODE, respectively, with $SnCl_2 \cdot 2H_2O$ in a modified procedure of Hamberg (1971). First, 0.50 g (1.60 mmol) of 13*S*-HPODE (obtained by soybean lipoxygenase oxidation) or 9*S*-HPODE (obtained by tomato lipoxygenase) was dissolved in a mixture of 100 ml of $CHCl_3$, 200 ml of methanol and 80 ml of H_2O. The pH of the solution was adjusted to 3 by addition of 2 N HCl. Immediately afterwards 395 mg (1.75 mmol) of $SnCl_2 \cdot 2H_2O$ were added. The solution was stirred for 1 h at room temperature. Separation into an aqueous and an organic layer was achieved by addition of 100 ml each of H_2O and $CHCl_3$. After centrifugation the chloroform layer was separated. The aqueous layer was extracted another time with 100 ml $CHCl_3$. The combined chloroform layers were dried with Na_2SO_4, filtered and then the solvent was removed at 20°C in vacuum.

The residue obtained after reduction of 9-HPODE contained large amounts of linoleic acid. This was removed by thin-layer chromatography: 0.47 g (1.59 mmol) of raw 9*S*-HODE was dissolved in 2.5 ml of a mixture of cyclohexane, ethyl acetate and acetic acid 596:400:4 (v/v) and applied to five thin-layer plates. The plates were developed with the solvent mixture given above. Immediately after drying, identification of 9*S*-HODE was achieved by irradiation of the plate with UV light of 254 nm. 9*S*-HODE showed fluorescence in a band with R_F between 0.33 and 0.45. This band was scraped off. The silica gel was eluted three times each with 20 ml of dried ethyl acetate. After filtration the solvent was removed at 30°C using a rotor vap. Yield: 0.11 g of 9*S*-HODE (25%).

Separation from isomeric byproducts of 9-HODE and 13-HODE was accomplished by purification with HPLC in a modified procedure reported by Wu et al. (1995): 100 mg of 9*S*-

HODE, prepurified by thin-layer chromatography, or 13*S*-HODE obtained after reduction of 13*S*-HPODE, were dissolved in a mixture of 500 μl of hexane/isopropanol 95:5 (v/v). This mixture was subjected to separation on a silica gel column (25 cm × 10 mm i.d.) with spherical beads (6 μm) using a mixture of hexane/isopropanol/acetic acid (99:1:0.1, v/v). Detection was achieved by measuring the UV absorbance at 234 nm (diene system):

13*S*-HODE: elution between 10.8 and 12.0 min.

(13*S*,9*E*,11*E*)-HODE: elution between 17.6 and 18.6 min.

9*S*-HODE: elution between 22.6 and 24.2 min.

(9*S*,10*E*,12*E*)-HODE: elution between 25.8 and 27.4 min.

9*S*-HODE as its TMS methylate: RI 2309; MS (70 eV), *m*/*z* (%): 382 (17, M^+), 311 (15), 259 (2), 225 (100), 155 (44), 143 (23), 130 (35), 73 (82).

13*S*-HODE as its TMS methylate: RI 2297; MS (70 eV), *m*/*z* (%): 382 (25, M^+), 311 (100), 225 (40) 155 (17), 143 (23), 130 (50), 73 (80).

15*S*-HETE was obtained by reduction of 15*S*-HPETE: 20 mg HPETE were dissolved in 0.5 ml $CHCl_3$ (top shaped vial), then 50 μl of $P(OCH_3)_3$ were added. The solution was shaken for 15 min at room temperature. The solvent and excess of reagent were blown off with nitrogen. The residue was dissolved in 200 μl of ethyl acetate; 30 μl of this solution (containing 3 mg of HETE pure enough for use in oxidation experiments) were transferred to a 250 ml round bottom flask for further reaction.

2.7. Autoxidation experiments with 9S-HODE and 13S-HODE

First, 15 mg (51 μmol) 9*S*-HODE and 13*S*-HODE were emulsified in 22 ml of phosphate buffer (pH 7.4) and 45 ml of 0.2 M KCl solution were added. Autoxidation was started by addition of: (1) 15 mg (85 μmol) of sodium ascorbate and 0.6 ml (1.0 mM) of $FeSO_4$ solution; (2) 15 mg (53 μmol) of $FeSO_4 \cdot 7H_2O$; (3) 15 mg (53 μmol) of $FeSO_4 \cdot 7H_2O$ and 100 μl (50 μmol) of 3% H_2O_2 solution; (4) 15 mg (53 μmol) of $FeCl_3 \cdot 6H_2O$. Aliquots of 1 ml were removed after 0, 1, 12 and 24 h, 2 and 5 days, and 2 weeks. Oxidation was stopped by the addition of 10 ml of $P(OMe)_3$. After addition of 0.5 ml of $CHCl_3$, the solution was shaken at room temperature for 15 min and then extracted three times with 0.5 ml of $CHCl_3$. The $CHCl_3$ layer was separated and dried with Na_2SO_4. Then the solvent was removed under a flow of nitrogen at room temperature. The residue was subjected to methylation and trimethylsilylation and investigated by GC and GC/MS as described below.

Autoxidation of 15*S*-HETE was performed in a similar manner, except that instead of 15 mg only 3 mg were used; the amounts of the other reagents were equally reduced.

2.8. Esterification with diazomethane

The reduced aliquots (0.1–0.2 mg sample), dissolved in 10 μl of ethyl acetate, were transformed to a top shaped vial and 200 μl of ethereal diazomethane solution were added; after 10 min, excess diazomethane and solvent was blown off under a flow of nitrogen.

2.9. Trimethylsilylation

MSTFA (10 μl) was added to the methylated sample and shaken in a thermomixer for 60 min at 40°C.

2.10. Compound identification

A 0.2–1.5 μl volume of the MSTFA solution obtained after methylation and trimethylsilylation was subjected to GC and GC/MS. Mass spectra were recorded from all GC peaks.

3. Results

Since hydroperoxides in the presence of traces of Fe^{2+} readily undergo further oxidation to products which might also be generated from the corresponding hydroxy acids (see Scheme 1), perfect sample purification was obligatory to study oxidation of the latter.

13*S*-HPODE was prepared using soybean lipoxygenase which develops its highest activity at

pH 9.0 (Hamberg and Samuelsson, 1967). Linoleic acid is of low solubility in borate buffer. Therefore an emulsion of linoleic acid was prepared applying vigorous stirring under ultrasonification. After addition of soybean lipoxygenase the solution clarified; thus obviously linoleic acid was bound to lipoxygenase.

It turned out to be unnecessary to blow pure oxygen through the solution, as recommended earlier (Hamberg, 1983, Dolev et al., 1967), as this did not increase the yield of 13*S*-HPODE. The oxidation was terminated by addition of 2 N HCl. HCl addition must be done carefully until slight clouds become visible (pH 4.5). If more acid is added, 13*S*-HPODE may decompose. In addition, the voluminous precipitated enzyme is then transferred by following extraction with $CHCl_3$ into the organic layer. Excess enzyme was removed by cartrige filtration.

The synthesis of 15*S*-HETE was performed analogous to that of 13*S*-HPODE starting with arachidonic acid and soybean lipoxygenase. The product was only obtained in satisfactory yield if the amount of soybean lipoxygenase was kept low. The progress of the reaction was controlled by sample withdrawal and product analysis. If after 2 h of reaction time starting material was still present, a second portion of soybean lipoxygenase was added and after another 2 h a second control was performed. When the reaction was done with the same or higher amounts of enzyme compared to arachidonic acid, 14,15-epoxy-13-hydroxy-5,8,11-eicosatrienoic acid was obtained as the main product, indicated by MS.

9*S*-HPODE was prepared by a tomato lipoxygenase in borate buffer at pH 5.6 obtained by homogenation of tomatoes. Tomato homogenate is acidic, therefore the pH must be adjusted during the reaction by careful addition of 1 N NaOH if necessary. Unfortunately, only a limited amount of linoleic acid was converted to 9*S*-HPODE.

Hydroperoxides readily undergo isomerization (Hamberg and Samuelsson, 1967, Chan et al., 1979, Frankel, 1982), even when stored in solution at 0°C. Decomposition is provoked in the presence of traces of acid which is necessary to exclude peak broadening during HPLC separation (Wu et al., 1995). We therefore decided to reduce the raw hydroperoxides without further purification, since the hydroxy derivatives produced are stable to isomerization.

Either $NaBH_4$ (Hamberg and Samuelsson, 1965, Graveland, 1970), $SnCl_2$ (Hamberg, 1971), $P(Ph)_3$ (Porter et al., 1980, Neff et al., 1981), $P(OCH_3)_3$ or $P(CH_3)_3$ (Shulman, 1977) is recommended for reduction of hydroperoxides to the corresponding alcohols: $NaBH_4$ reduces not only hydroperoxides, but also attacks oxo compounds. Since hydroxyoxo acids are generated in the course of further oxidation of LOOH (Loidl-Stahlhofen et al., 1994), $NaBH_4$ was not considered as a reducing agent.

Reduction with $SnCl_2$ should be carried out in aqueous acidic media at pH 3–4 to ensure solution of $SnCl_2$. In contrast to previous reported reductions (Hamberg, 1971), where $SnCl_2$ in an ethanolic emulsion was applied, we used a homogeneous solution for this purpose. Hydroperoxy fatty acids are almost insoluble in water, therefore the reduction was done in a mixture of chloroform, methanol and water (Bligh and Dyer, 1959). In this single phase solution both $SnCl_2$ and fatty acid are well soluble. The reaction was terminated after a few minutes. Separation of reaction products was achieved by adding chloroform and water, forming two layers. This reduction proceeded nearly completely and without any E/Z isomerization. Therefore this method was used for reduction of sample amounts in milligram scale.

$P(Ph)_3$ turned out not to be suitable for reduction of fatty acid hydroperoxides, since $P(Ph)_3$ and $P(Ph_3)O$ (produced as oxidation product after reduction of LOOH) are soluble in organic solvents and therefore $P(Ph_3)O$ is extracted with organic solvents. The GC retention times of $P(Ph_3)O$ are nearly identical with those of the main oxidation products of 9-HPODE and 13-HPODE, causing severe peak overlapping.

In contrast, $P(OCH_3)_3$ was volatile enough for removal in the course of solvent evaporation. The reduction was achieved at pH 7.4, avoiding the danger of acid-induced decomposition of LOOH, the reaction was complete within minutes, but still some E/Z isomerization was observed. Nevertheless, this method was always applied for reduction

in miniscale. Reduction with $P(CH_3)_3$ was also checked, but proved less sufficient than that with $P(OCH_3)_3$ due to the high volatility of the reagent.

Samples of 9*S*-HODE had to be purified by thin-layer chromatography to remove the large amounts of linoleic acid by use of a solvent mixture consisting of cyclohexane, ethyl acetate and traces of acetic acid. Purification of all hydroxy derivatives was achieved with the same solvent mixture (Wu et al., 1995).

The air oxidation experiments with 9*S*-HODE and 13*S*-HODE were carried out by addition of amounts of iron ions greatly surpassing the Fe^{2+} concentration in biological samples. Consequently, we deduce that the much smaller iron ion amounts in biological material are not able to induce oxidation of 9*S*-HODE and 13*S*-HODE. In first experiments, 9*S*-HODE and 13*S*-HODE as well as 15*S*-HETE were treated in air at pH 7.4 and room temperature with Fe^{2+}/ascorbate, thus using typical oxidation conditions applied also for model oxidation of unsaturated acids (Thiele and Huff, 1964, Yamagata et al., 1983, Esterbauer et al., 1986). Even if the reaction temperature was increased to 45–50°C, and the reaction time was extended to 2 weeks, oxidation products were only observed in traces by GC/MS, and 95% of 9*S*-HODE and 13*S*-HODE were recovered unchanged. Under similar oxidation conditions 15*S*-HETE also remained unattacked.

Since preliminary experiments with 9*S*-HPODE and 13*S*-HPODE revealed that addition of equimolar amounts of Fe^{2+} induces oxidation reactions much faster than Fe^{2+}/ascorbate, 9*S*-HODE, 13*S*-HODE and 15*S*-HETE were also subjected to oxidation in air with Fe^{2+} alone. Also in these cases oxidation was not observed.

H_2O_2 and Fe^{2+} produce $^{\bullet}OH$ radicals in the Fenton reaction. Therefore as well as Fe^{2+} we also added H_2O_2 to samples of 9*S*-HODE and 13*S*-HODE. Even after 2 weeks' reaction time, GC revealed only the presence of unchanged starting material. Also, autoxidation experiments of 13*S*-HODE with air using Fe^{3+} as catalyst showed that no reaction occurred: the starting material was recovered unchanged. Addition of Fe^{3+} caused also no oxidation. Similarly, the oxidation experiments with 9*S*-HODE and with 15*S*-HETE were negative, only the starting material was recovered.

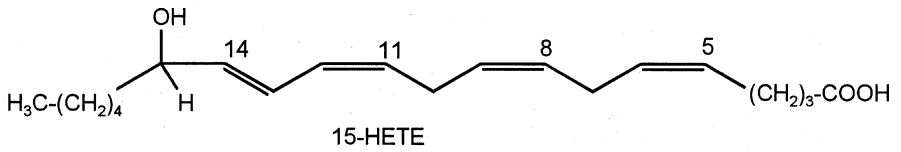
In contrast, 9*S*-HODE and 13*S*-HODE are not stable in an organic solvent. If a solution of 13*S*-HODE in ethyl acetate was stored for 4 months at room temperature in daylight and air, nearly all the 13*S*-HODE disappeared.

4. Discussion

Hydroperoxides of linoleic acid (LOOH) and the corresponding hydroxy acids (LOH) possess an identical conjugated diene system (see Scheme 1). It was therefore assumed that LOOH and LOH are equally prone to oxidation (Spiteller, 1996, Dudda et al., 1996a). This assumption was checked by subjecting 9-HODE and 13-HODE to air oxidation in the presence of catalytic amounts of Fe^{2+} and ascorbate. The latter is a common reagent to reduce Fe^{3+} generated in the course of the reaction (Thiele and Huff, 1964, Wills, 1965). 9-HODE and 13-HODE proved to be stable in this system. They proved stable also even after prolonged reaction time (14 days): only traces of oxidation products were detected. Similar results were obtained by oxidation with equimolar amounts of Fe^{2+}/O_2, while HPODEs are oxidized nearly instantly with this reagent. Even addition of H_2O_2 to a solution of 13*S*-HODE and Fe^{2+} ($Fe^{2+} + H_2O_2$ generates $^{\bullet}OH$ radicals) was not successful: 95% of starting material was recovered unchanged. Also after addition of Fe^{3+} only the starting material was re-isolated.

This high stability of 9*S*-HODE and 13*S*-HODE seems to indicate that either the hydrogens in positions 9 and 13, respectively (see Scheme 1), are not equally activated as in the corresponding hydroperoxides (since LOOH readily undergo decomposition) or that the oxidizing agent is peroxyl radicals.

Hydroxy acids derived by reduction of corresponding hydroperoxides of arachidonic acid, e.g. (15*S*,5*Z*,8*Z*,11*Z*,13*E*) - 15 - hydroxy - 5,8,11,13 - eicosatetraenoic acid (15*S*-HETE), possess activated CH_2-groups in positions 7 and 10. We assumed therefore that these CH_2-groups (Scheme 2) would suffer hydrogen abstraction as easily as



15-HETE

Scheme 2. CH_2-groups in positions 7 and 10 are activated by two adjacent double bonds. In spite of this, 15*S*-HETE is not attacked by LO$^{\cdot}$, in contrast to 15*S*-HPETE.

the activated CH_2-groups in arachidonic acid or linoleic acid. Unexpectedly this assumption proved not to be true. When we subjected 15*S*-HETE to oxidation with air/Fe^{2+}/ascorbate or Fe^{2+} even after 2 days nearly no loss of starting material was recognized. Thus we are forced to conlcude that further degradation of lipid peroxidation products requires the presence of a hydroperoxy group or a peroxyl radical.

The high stability of 9*S*-HODE and 13*S*-HODE to further oxidation explains why increased amounts of these compounds compared to other LPO products are detected in biological samples after oxidative stress. Because linoleic acid usually occurs in biological samples in much higher amounts than arachidonic acid and due to the high stability of 9*S*- and 13*S*-HODE, these hydroxy acids are well suited to estimate oxidative stress exerted in human LDL samples and tissue.

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