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# Previously unknown aldehydic lipid peroxidation compounds of arachidonic acid

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**Abstract**

Arachidonic acid was oxidized by iron ascorbate. Samples were withdrawn in time intervals. The aldehydic oxidation products were trapped by preparation of pentafluorbenzyloximes. Their trimethylsilylated derivatives were subjected to analysis by GC/MS. The main aldehydic lipid peroxidation product was found to be the well-known 4-hydroxy-2-nonenal (HNE), but 2-hydroxy heptanal (HH) — a previously unknown lipid peroxidation product of arachidonic acid — was detected to be nearly equally abundant. Malondialdehyde (MDA), glyoxal and 2-hydroxy-4-decenal (HDE) were detected to be produced in up to 100 times lower amounts compared to HNE. The amounts of aldehydes increased steadily with time. In addition, *n*-1-hydroxy-*n*-oxo acids were detected. Similar aldehydes were obtained by iron ascorbate-induced oxidation of hydroxy acids derived by $NaBH_4$-reduction of 13-hydroperoxy-9-*cis*-11-*trans*-octadecadienoic acid. Since this and analogous hydroxy acids (LOHs) are the main biological degradation products of hydroperoxides of unsaturated acids (LOOHs) their further peroxidation seems to be a main source of toxic aldehydes.

*Keywords:* Arachidonic acid; Linoleic acid; Linolenic acid; Lipid peroxidation; 4-Hydroxynonenal; α-Hydroxyaldehydes; *n*-1-Hydroxy-*n*-oxo acids; Hydroxy-oxo-fatty acids; Bishydroperoxides; Hydroxyhydroperoxides; Mechanism of lipid peroxidation

## 1. Introduction

Hydroperoxides of arachidonic acid and other unsaturated acids (PUFAs) are suspected to be involved in the generation of chronic and spontaneous diseases [1–4], due to their ability to be cleaved in the presence of metal ions to radicals, which are further decomposed to aldehydes [5–11] or 4-hydroxynonenal [12]. The latter was especially recognised to be toxic and shown to react

*Abbreviations*: BHT, 2,6-di-*tert*-butyl-4-methyl-phenyl; EDTA-$Na_2$, ethylenediaminetetraacetic acid disodium salt dihydrate; GC, gas chromatography; GC/MS, gas chromatography/mass spectrometry; HDE, 2-hydroxy-4-decanal; HH, 2-hydroxyheptanal; HNE, 4-hydroxy-2-nonenal; LPO, lipid peroxidation; LOOH, hydroperoxy fatty acids derived from unsaturated fatty acids; LOH, hydroxy fatty acids derived by the reduction of the OOH function of a LOOH; MDA, malondialdehyde; MSTFA, *N*-methyl-*N*-trimethylsilyltrifluoroacetamide; PFBA·HCL, pentafluorobenzylhydroxylamine; PFBO,pentafluorobenzyloxime.

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with nucleophiles [13–15] by a Michael-type addition reaction.

We recently detected a new class of hydroxyaldehydes with a hydroxyl group in α-position in biological material [16]. If lipid peroxidation of linoleic [17,18] and linolenic acid [19] was mimicked by oxidation with iron ascorbate, similar α-hydroxyaldehydes were detected.

In this paper, we describe the search for aldehydic compounds derived from arachidonic acid. In contrast to our earlier investigations on the oxidation of linoleic and linolenic acid, we studied the reaction by withdrawing samples at time intervals to get an insight into the kinetics of the oxidation process.

## 2. Experimental

### *2.1. Materials*

*N*-methyl-*N*-trimethylsilyltrifluoracetamide (MSTFA) was obtained from Macherey & Nagel (Dren). All other chemicals were purchased from Fluka (Neu Ulm). Solvents obtained from Merck (Darmstadt) were distilled before use.

1-Hydroxy-2-tridecanone was synthesised according to the method of Meyer [20]. 13-Hydroxy-9,11-octadecadienoic acid was prepared as described previously [21,22]. The fatty acids were stored at −18°C under argon and their purity was determined by GC/MS.

### *2.2. Iron-ascorbate autoxidation of arachidonic acid*

Autoxidation of arachidonic acid (15 mg) was performed in 23 ml of 0.1 M Tris–HCl-buffer (pH 7.4) and 45 ml of 0.15 M aqueous KCl solution containing 3.8 ml of 0.8 mM $Fe^{2+}$ sulphate and 3.8 ml of 20 mM ascorbate (sodium salt) following a modified method of Esterbauer [23].

The solution was incubated in the above oxidation system at room temperature. Oxidation was stopped after 0 min, 10 min, 60 min, 3 h, 12 h, 24 h and 48 h by addition of 80 μl BHT (2% in methanol) and 1.2 ml EDTA-$Na_2$ (1% in bi-distilled water) [24] to investigate the effect of time on the occurrence of aldehydic compounds.

Iron-ascorbate oxidation of 13-hydroxy-9,11-octadecadienoic acid (obtained by $NaBH_4$ reduction of the corresponding hydroperoxide of linoleic acid) was performed similarly as described above for arachidonic acid.

### *2.3. Preparation of PFBO derivatives and MSTFA-adducts of the lipid peroxidation products*

The carbonyl groups of LPO products were transformed to pentafluorobenzyloxime derivatives by derivatisation with PFBA·HCl [17,25]. Quantification of hydroxyaldehydes was carried out by addition of 0.2 mg 1-hydroxy-2-tridecanone. It was used as internal standard. Quantification of MDA and glyoxal was achieved by addition of 0.2 mg PFBO derivative of acetylacetone [26] as internal standard.

The PFBO derivatives were extracted three times with $CHCl_3$. The solvent of organic layers was evaporated and the residue redissolved in ethylacetate. An aliquot (0.2 mg) of PFBO derivatives was added to 10 μl MSTFA for trimethylsilylation. Finally, the mixture was subjected to separation by GC. Identification of compounds was achieved with GC/MS by EI mass spectrometry. Alternatively the aldehydes were investigated by formation of MSTFA adducts as described previously [17].

### *2.4. Gas chromatography/mass spectrometry*

GC was carried out with a Carlo Erba HRGC 5160 Mega series chromatograph equipped with a flame ionisation detector, using a DB-1 fused-silica glass capillary column (30 m × 0.32 mm I.D.), temperature programmed from 80 to 280°C at 3°C/min. The temperature of the injector and detector were kept at 270°C and 290°C, respectively. The carrier gas was hydrogen and the splitting ratio was 1:30. Peak area integration was achieved with a Merck D-2500 integrator.

GC/MS was performed on a Finnigan MAT 312 mass spectrometer connected to a MAT-SS-300 data system. EI mass spectra were recorded at an ionisation energy of 70 eV. A Varian 3700 gas chromatograph with a 30 m × 0.3 mm I.D. DB-1 fused-silica column was used for sample separation. The carrier gas was hydrogen and the temperature programme was the same as used for GC.

## 3. Results

The oxidation of arachidonic acid with iron ascorbate followed the procedure described by Esterbauer [23]. Samples were withdrawn at time intervals; the oxidation reaction of each sample being stopped by addition of BHT and EDTA-$Na_2$ [24].

The aldehydic compounds were transformed into their pentaflourobenzyloxime derivatives [25] which were then reacted with *N*-methyl-*N*-trimethylsilyl-trifluoracetamide (MSTFA). The obtained pentafluorobenzyloxime-trimethylsilyl derivatives (PFBO-TMS) were subjected to separation by GC and identification by MS. Quantification of hydroxyaldehydes was achieved by measuring the ion current of the typical M-197 or M-15 fragments, in comparison to those of the internal standard. In the case of MDA and glyoxal, mass spectrometric ion tracing of the M-197 fragment [26] was used for their quantification.

Hexanal **3**, 2-octenal **5**, 7-oxo-5-heptenoic acid **6** and 5-oxopentanoic acid **9** are already known decomposition products of monohydroperoxy derivatives of arachidonic acid [27,28] (Scheme 1).

2-Hydroxyheptanal **1**, 2-hydroxy-4-decenal **4**, 5-hydroxy-6-oxohexanoic acid **7** and 5-hydroxy-8-oxo-6-octenoic acid **8** were previously unknown to be produced by oxidation of arachidonic acid. They are derived by decomposition of bishydroperoxy or hydroxyhydroperoxy acids.

The results of the investigation of the increase in aldehydes with time are represented in the form of diagrams: The first one (Fig. 1) relates to hydroxyaldehydes. This group of aldehydes consists of 4-hydroxy-2-nonenal (HNE) **2**, 2-hydroxyheptanal (HH) **1**, and 2-hydroxy-4-decenal (HDE) **4**.

The second group of aldehydes (Fig. 2), which are generated in amounts of a magnitude 10–100 times less than HNE or HH, are dialdehydes: glyoxal and malondialdehyde (MDA). Thus, MDA is produced in this oxidation reaction in remarkable low yield.

Due to lack of a standard compound (the synthesis of hydroxyaldehydic acids is rather difficult), we were not able to quantify the hydroxyaldehydic acids **7** and **8** exactly, but **7** seems to be present in amounts equal to HH **1**, and **8** to HNE **2**.

The iron ascorbate-induced oxidation of 13-hydroxy-9-*cis*-11-*trans*-octadecadienoic acid resulted in the production of the aldehydic compounds, HH, HNE and MDA, which were obtained also by the analogous oxidation of the corresponding hydroperoxy acid.

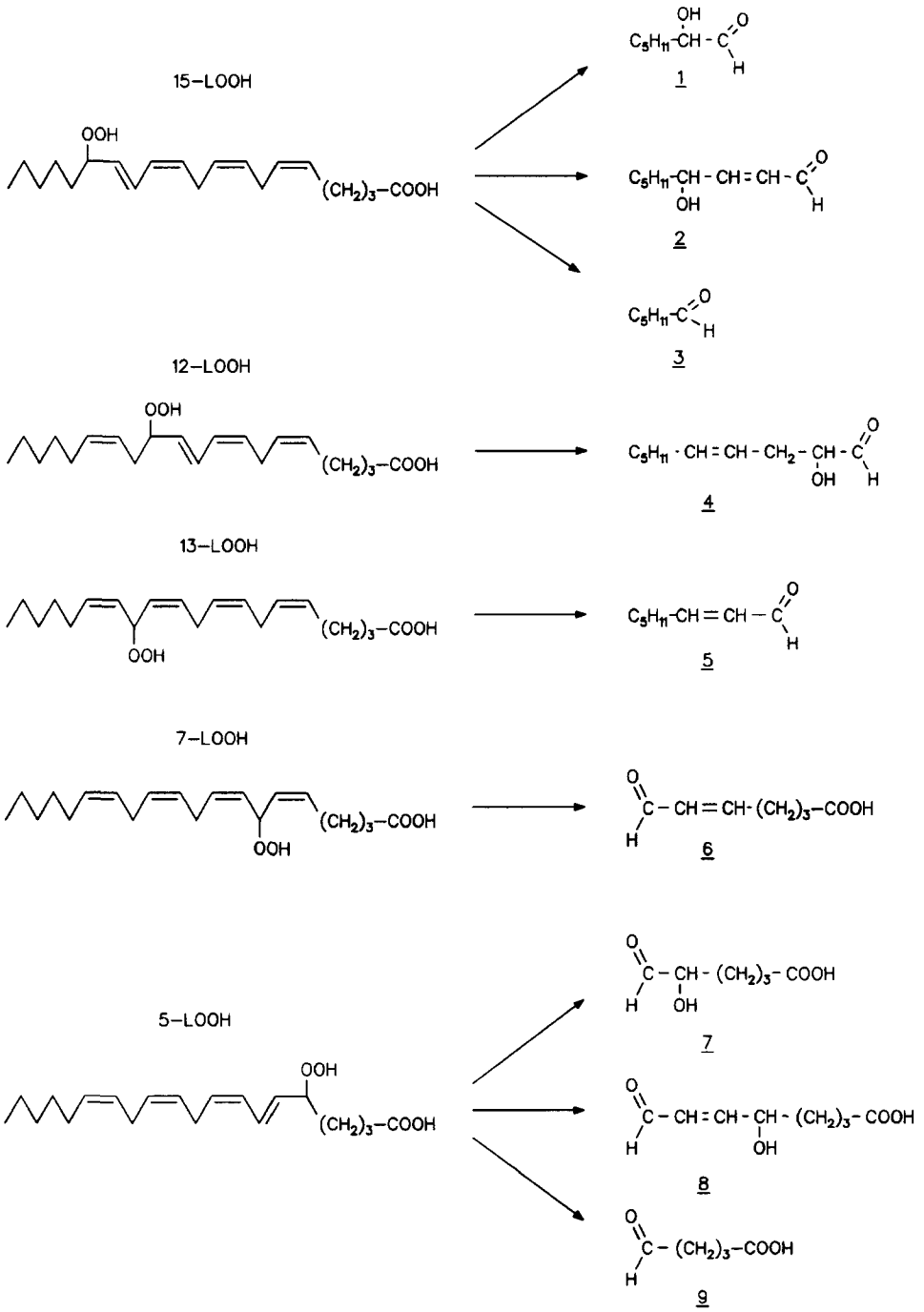
## 4. Discussion

Arachidonic acid contains three equivalent double allylically activated $CH_2$ groups in positions 7, 10 and 13. In an autocatalytic reaction, but not in an enzymatic one, each of the hydrogens can be removed with about the same probability. Thus, six isomeric hydroperoxides with a hydroperoxy group in position 5, 8, 9, 11, 12 or 15 are expected to be produced. As shown by Yamagata et al. [6], in fact those products with a hydroperoxy function at an inner position (8, 9, 11, 12), suffer much easier degradation than those with the hydroperoxy group at an outer position (5 and 15), resulting in the prevalence of 5 and 15 monohydroperoxy compounds.

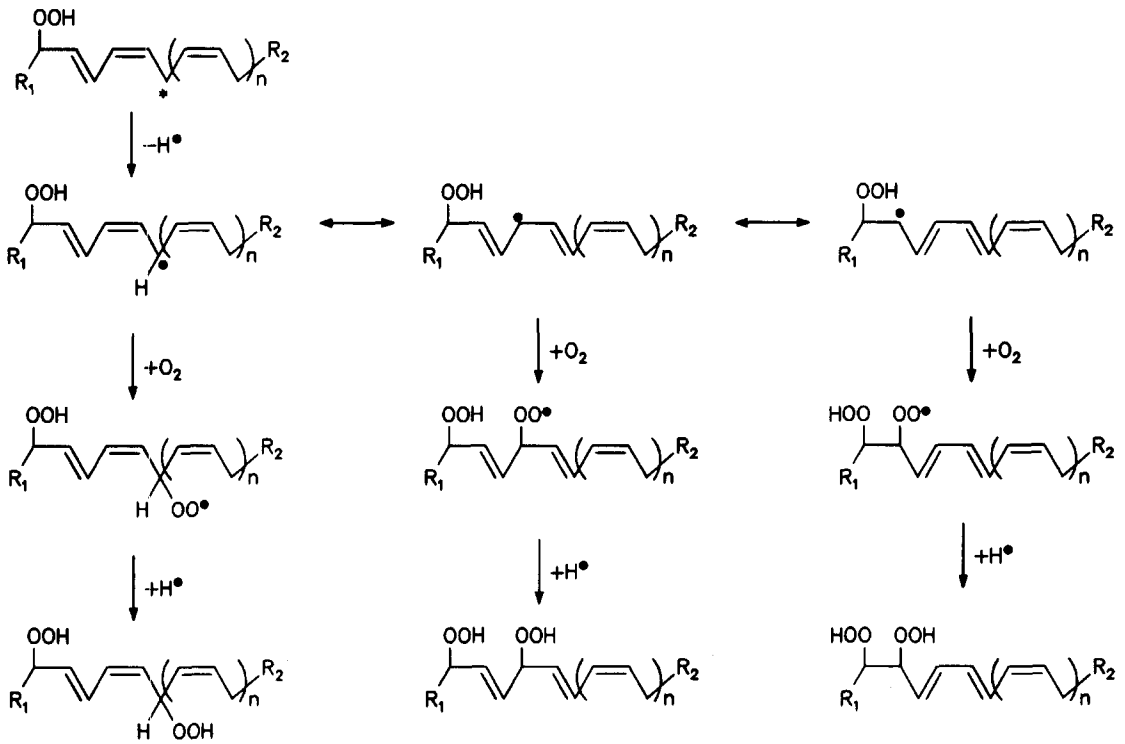
As a consequence, decomposition products of the latter hydroperoxy acids are also dominant after oxidation of arachidonic acid with iron ascorbate.

While in arachidonic acid the $CH_2$ groups in position 7, 10 and 13 are double allylically activated equally, one $CH_2$ group in the hydroperoxy acids is activated by a conjugated diene system and an allylic system as well (Scheme 2). As a consequence, hydrogen abstraction is more prone from this exceptional activated $CH_2$ group than from any other CH group. Thus, mesomeric radicals are produced which can react again with oxygen. Therefore, a great number of bishydroperoxides is produced as exemplified in Scheme 2.

By the well-known decomposition of monohydroperoxides to aldehydes in the presence of iron ions, LO· radicals are generated [19,29–31]. If these remove an activated hydrogen from monohydroperoxides, LOHs are produced. These contain the same diene-allylically activated $CH_2$ groups as the corresponding LOOHs and, there-




Scheme 1. Identified degradation products of hydroperoxy derivatives of arachidonic acid.




$R_1$, $R_2$ = alkyl or acidic end of LOOH, respectively

n = 0 : linoleic acid

n = 1 : linolenic acid

n = 2 : arachidonic acid

Scheme 2. Generation of bishydroperoxy PUFAs.

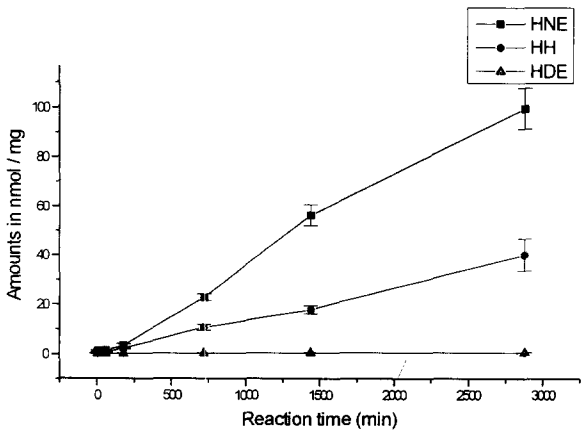



Fig. 1. Formation of hydroxyaldehydes with time during the autoxidation of arachidonic acid.

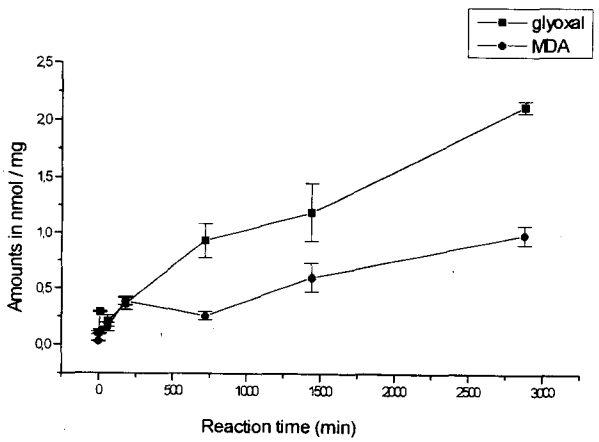
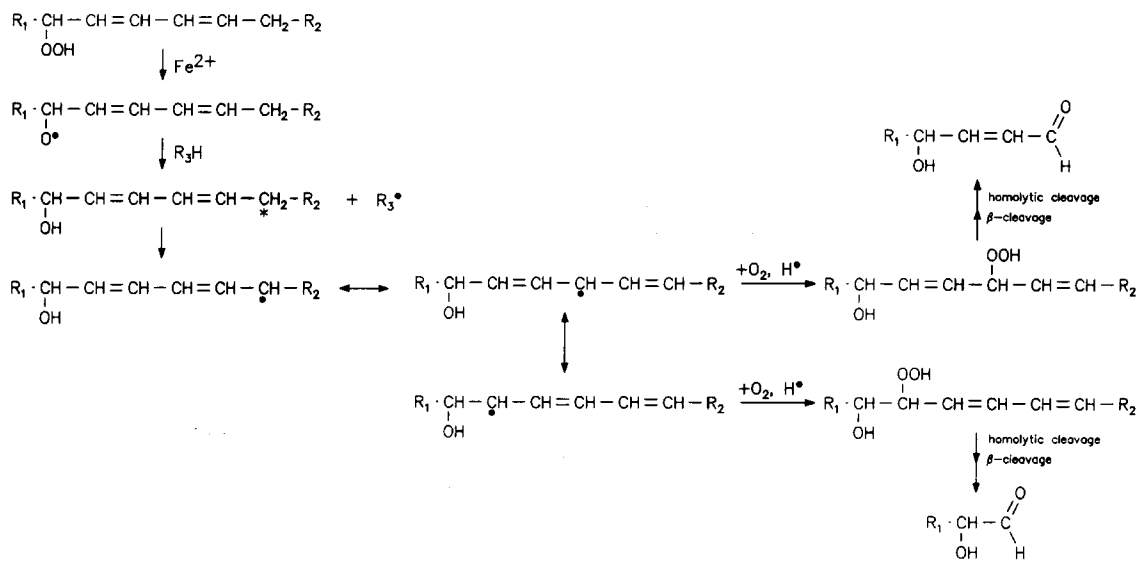



Fig. 2. Formation of glyoxal and malondialdehyde with time during the autoxidation of arachidonic acid.




Scheme 3. Production of LOHs and their further oxidation and decomposition to hydroxyaldehydes.

fore, they are also subjected to a second oxidation reaction, producing hydroxyaldehydes (Scheme 3).

Thus, the iron ascorbate-mimicked oxidation of 13-hydroxy-9-*cis*-11-*trans*-octadecadienoic acid produced the same hydroxyaldehydes as found by oxidation of the precursor hydroperoxy acids except glyoxal (the generation of glyoxal requires the presence of a 1,2 bishydroperoxide; Scheme 2).

In nature, monohydroperoxides are very easily reduced enzymatically to corresponding LOHs [32]. Therefore, we assume that most of the hydroxyaldehydes found after oxidation of natural occurring PUFAs are generated in the way outlined above.

Withdrawing of samples at time intervals demonstrates that the amount of aldehydes obtained depends on the time which had elapsed after the start of oxidation. Thus, quantification also requires considering the time of samples withdrawal.

Aldehydes containing more than one double bond were not detected. This probably reflects their high tendency to undergo further oxidation as already stated [33,34]. Therefore, unsaturated aldehydic products derived by decomposition of the monohydroperoxides of arachidonic acid with the functional group at an inner position (8, 9, 11, 12) were not detected or only in tiny amounts.

All *n*-6 acids produce 4-HNE and 2-hydroxyheptanal. Therefore, it is not possible to distinguish in a biological sample if these compounds are produced from arachidonic or linoleic acid. But this distinction becomes possible if the content of 5-hydroxy-6-oxohexanoic acid **7**, 5-hydroxy-8-oxo-6-octenoic acid **8** and 5-oxopentanoic acid **9** (derived from arachidonic acid) and 9-hydroxy-10-oxodecanoic acid, 9-hydroxy-12-oxo-10-dodecenoic acid and 9-oxononanoic acid (derived from linoleic acid) is measured. These acids are specific for arachidonic or linoleic acid oxidation, respectively.

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