

Transformations of 12,13-Epoxy-11-hydroxy-9-octadecenoic Acid and 4,5-Epoxy-N-acetylsphingosine by Incubation with Liver Homogenate and Liver Microsomes

Alexander Möllenberg and Gerhard Spiteller*

Institut für Organische Chemie I, Universität Bayreuth, Universitätsstraße 30, 95440 Bayreuth, Germany. Fax: +0921/552671. E-mail: gerhard.spiteller@uni-bayreuth.de

* Author for correspondence and reprint requests

Z. Naturforsch. **55c**, 981–986 (2000); received June 5/July 7, 2000

12,13-Epoxy-11-hydroxy-9-octadecenoic Acid, 4,5-Epoxy-N-acetylsphingosine, Human Liver Microsomes

Transformation of 12,13-epoxy-11-hydroxy-9-octadecenoic acid and 4,5-epoxy-N-acetylsphingosine by addition of porcine liver homogenate and human liver microsomes, respectively was investigated. Both epoxides were converted to corresponding diols by porcine liver homogenate, but not by human liver microsomes, suggesting location of the hydrolyzing enzymes not in the microsomes, but within the cell wall.

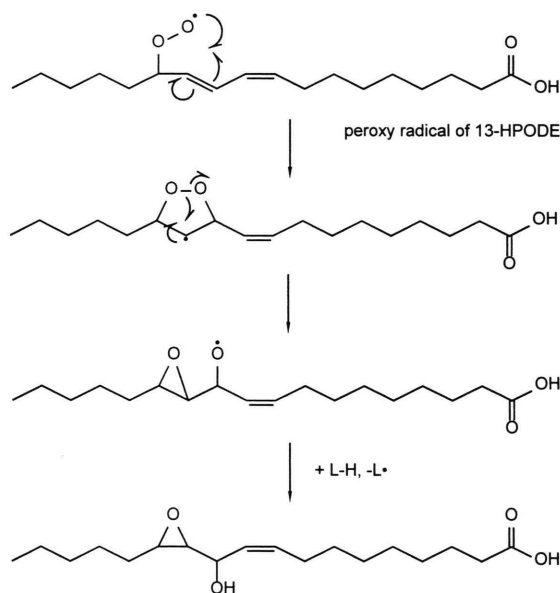
Introduction

Cell injury activates degrading enzymes, for instance lipases – which cleave phospholipids (Parthasarathy *et al.*, 1985) – and lipoxygenases which transform thus liberated polyunsaturated fatty acids (PUFAs) into lipid hydroperoxides (LOOHs, Wills, 1966).

In the course of lipid peroxidation (LPO) processes linoleic acid is transformed to *cis/trans*-isomeric-9-hydroperoxy-10,12-octadecadienoic acids (9-HPODEs) and 13-hydroperoxy-9,11-octadecadienoic acids (13-HPODEs, Dolev *et al.*, 1967). In a biological surrounding HPODEs are readily reduced to corresponding hydroxy compounds (Christophersen, 1968), but also undergo – in presence of iron ions – a cleavage to LO^\bullet radicals (Gardner, 1989). Iron ions are generated by suicidal inhibition of lipoxygenase (Fuchs and Spiteller, 2000). LO^\bullet radicals are further transformed to ep-

oxides (Gardner and Kleiman, 1974; Hamberg and Gotthammar, 1973; Scheme 1).

Epoxides are of high chemical reactivity. They react with all types of nucleophiles, especially glutathione. Thus epoxides generated from arachidonic acid are transformed to the slow reacting factor of anaphylaxis (Denzlinger, 1996).



Scheme 1. Formation of 12,13-epoxy-11-hydroxy-9-octadecenoic acid by decomposition of 13-HPODE.

Abbreviations: GC, gas chromatogram resp. gas chromatography; GC/MS, gas chromatography-mass spectroscopy; HLM, human liver microsomes; HPODE, hydroperoxyoctadecadienoic acid; LOOH, lipid hydroperoxide; LPO, lipid peroxidation; MS, mass spectrometry; MSTFA, *N*-methyl-*N*-trimethylsilyl-trifluoroacetamide; NADPH, nicotinamide-adeninedinucleotide-phosphate; NP-HPLC, normal phase high performance liquid chromatography; PUFA, polyunsaturated fatty acid; RP-HPLC, reversed phase HPLC; TRIS, tris(hydroxymethyl)-aminomethane.

0939–5075/2000/1100–0981 \$ 06.00 © 2000 Verlag der Zeitschrift für Naturforschung, Tübingen · www.znaturforsch.com · D



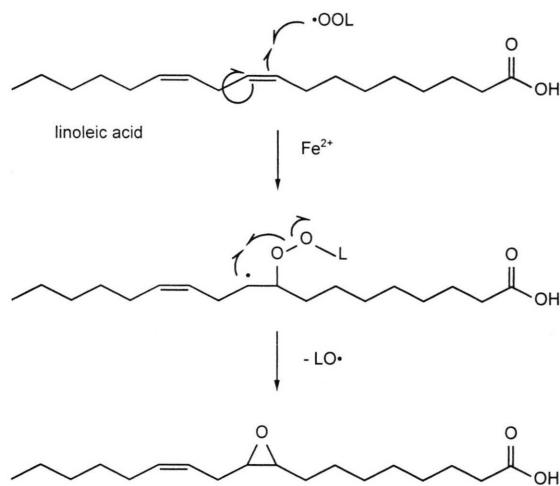
Dieses Werk wurde im Jahr 2013 vom Verlag Zeitschrift für Naturforschung in Zusammenarbeit mit der Max-Planck-Gesellschaft zur Förderung der Wissenschaften e.V. digitalisiert und unter folgender Lizenz veröffentlicht: Creative Commons Namensnennung-Keine Bearbeitung 3.0 Deutschland Lizenz.

Zum 01.01.2015 ist eine Anpassung der Lizenzbedingungen (Entfall der Creative Commons Lizenzbedingung „Keine Bearbeitung“) beabsichtigt, um eine Nachnutzung auch im Rahmen zukünftiger wissenschaftlicher Nutzungsformen zu ermöglichen.

This work has been digitalized and published in 2013 by Verlag Zeitschrift für Naturforschung in cooperation with the Max Planck Society for the Advancement of Science under a Creative Commons Attribution-NoDerivs 3.0 Germany License.

On 01.01.2015 it is planned to change the License Conditions (the removal of the Creative Commons License condition “no derivative works”). This is to allow reuse in the area of future scientific usage.

Epoxides are generated not only by intramolecular reactions by decomposition of hydroperoxides derived from arachidonic acid and linoleic acid but also by intermolecular reaction by action of double bonds with peroxy radicals (Spiteller, 1998).



Scheme 2. Formation of 12,13-epoxy-9-octadecenoic acid by epoxidation of linoleic acid.

For instance, peroxy radicals transform linoleic acid and linolenic acid to a variety of epoxides, known as leukotoxines (Ozawa, 1986). They also react with cholesterol to generate cholesterol epoxide, detected in atherosclerotic plaques (Smith *et al.*, 1980; Watabe *et al.*, 1982). Many epoxides are toxic. As a consequence epoxides should be removed as fast as possible from biological systems.

In order to investigate the fate of epoxides in biological systems we offered 12,13-epoxy-11-hydroxy-9-octadecenoic acid, a product formed by conversion of HPODEs, and 4,5-epoxy-*N*-acetyl-sphingosine, an artificial epoxidation product of sphingolipids, to a porcine liver homogenate and human microsomes: Homogenization activates the same enzymes (Wills, 1966; Herold and Spiteller, 1996) which are stimulated by cell injury, for instance in diseases connected with inflammation. The results of these investigations are reported in this paper.

Results

Preparation of liver homogenate

Porcine livers were removed immediately after slaughter, and transferred in an ice box to the laboratory where they were homogenized instantly. Homogenates were incubated with the epoxides.

Preparation of 12,13-epoxy-11-hydroxy-9-octadecenoic acid

13-Hydroperoxy-9,11-octadecadienoic acid was prepared by action of soybean lipoxygenase on linoleic acid (Hamberg and Samuelsson, 1967). The raw 13-hydroperoxy-9,11-octadecenoic acid was purified by twofold normal phase NP-HPLC (Galasch, 1998).

The purified 13-HPODE was decomposed by treatment with Fe^{2+} ions (Gardner and Kleiman, 1981; Spiteller and Spiteller, 1998) to a great number of products, which were separated by NP-HPLC. Fractions were collected, trimethylsilylated with MSTFA and subjected to GC/MS. The fraction thus identified to contain 12,13-epoxy-11-hydroxy-9-octadecenoic acid was collected and repurified by RP-HPLC.

Incubation of 12,13-epoxy-11-hydroxy-9-octadecenoic acid with porcine liver homogenate

Fresh porcine liver homogenate was then incubated with one diastereomer of the obtained purified 12,13-epoxy-11-hydroxy-9-octadecenoic acid. Lipids were extracted according to Bligh and Dyer (1959). The lipid fraction was trimethylsilylated and investigated by GC/MS. The GC indicated the presence of two main products, which according to their mass spectra represent a mixture of two diastereomeric trimethylsilylated 11,12,13-trihydroxy-9-octadecenoic acids in a ratio of about 13:10. This is expected by autocatalytic hydrolytic opening of the epoxide-ring of 12,13-epoxy-11-hydroxy-9-octadecenoic acid.

Incubation of porcine liver homogenates with 4,5-epoxy-*N*-acetyl-sphingosine

4,5-epoxy-*N*-acetyl-sphingosine was prepared as recently described (Möllenberg and Spiteller, 2000) by reacting *N*-acetyl-sphingosine with 1,1-dimethyldioxirane (Adam *et al.*, 1991). After incubation of 4,5-epoxy-*N*-acetyl-sphingosine with por-

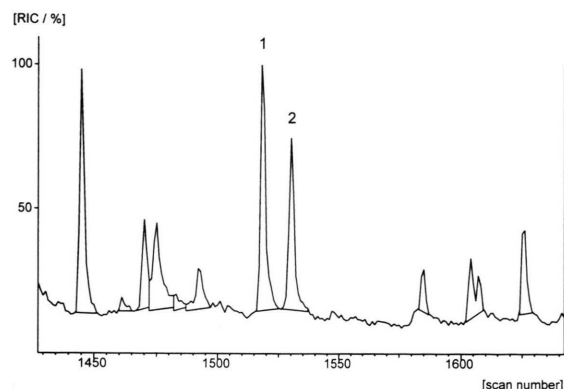
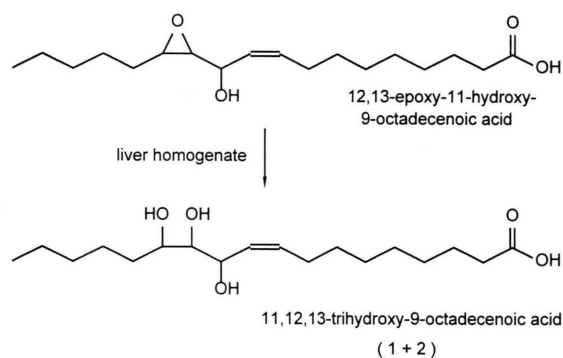


Fig. 1. Part of the GC after incubation of 12,13-epoxy-11-hydroxy-9-octadecenoic acid with liver homogenate (**1**, **2**: diastereomers of 11,12,13-trihydroxy-9-octadecenoic acid) (RIC = recalculated ion current).



Scheme 3. Ring opening of 12,13-epoxy-11-hydroxy-9-octadecenoic acid in liver homogenate to diastereomeric 11,12,13-trihydroxy-9-octadecenoic acid (**1** + **2**).

cine liver homogenate for 24 h the lipids were extracted, trimethylsilylated and investigated by GC/MS. The GC (Fig. 2) indicated besides unchanged 4,5-epoxy-*N*-acetyl sphingosine the presence of 4-*N*-acetyl amino-3-hydroxy-2-(1-hydroxytetradecyl)-1-oxa-cyclopentane (Möllenberg, 2000) and 4,5-dihydroxy-*N*-acetyl sphingosine (Polito *et al.*, 1969) (see *et al.*, Scheme 1). Peak 3 was tentatively identified by its mass spectrum to be 4,5-epoxy-3-*O*-acetyl sphingosine, formed by transfer of the acetyl group from the nitrogen to the adjacent hydroxyl group. The molecular mass of the compound (identical with the mass of the isomeric 4,5-epoxy-*N*-acetyl sphingosine) was derived from the M-15 peak 486 to be 501. An intense peak at *m/z* 204 confirmed the presence of a

HOCH₂-CH=NHTMS⁺ fragment. Loss of 59 from the molecular mass indicates the presence of -OCOCH₃ in α -position of the carbon atom attached to the nitrogen.

In order to exclude the possibility of bacterial action within the liver tissue and possibly artificial generation of hydrolysis products of the added epoxides, the incubation of 4,5-epoxy-*N*-acetyl sphingosine with liver homogenate was also carried out with addition of antibiotics (neomycin, penicillin, streptomycin). The addition of antibiotics showed no influence on the result, proving that bacterial action was not involved in the generation of the obtained products.

Incubation of 12,13-epoxy-11-hydroxy-9-octadecenoic acid and of 4,5-epoxy-N-acetyl sphingosine with human liver microsomes

In contrast to the reactions described above incubation of 12,13-epoxy-11-hydroxy-9-octadecenoic acid with human liver microsomes applying similar conditions as described for porcine liver (except for the reaction time of 3 h) the starting material was recovered nearly unchanged. Also incubation of 4,5-epoxy-*N*-acetyl sphingosine with human liver microsomes resulted mainly in recovery of starting material, but to a small extent 4-*N*-acetyl amino-3-hydroxy-2-(1-hydroxytetradecyl)-1-oxa-cyclopentane was formed. The diol resulting

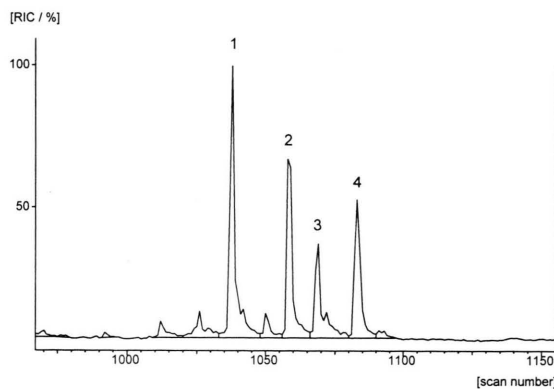
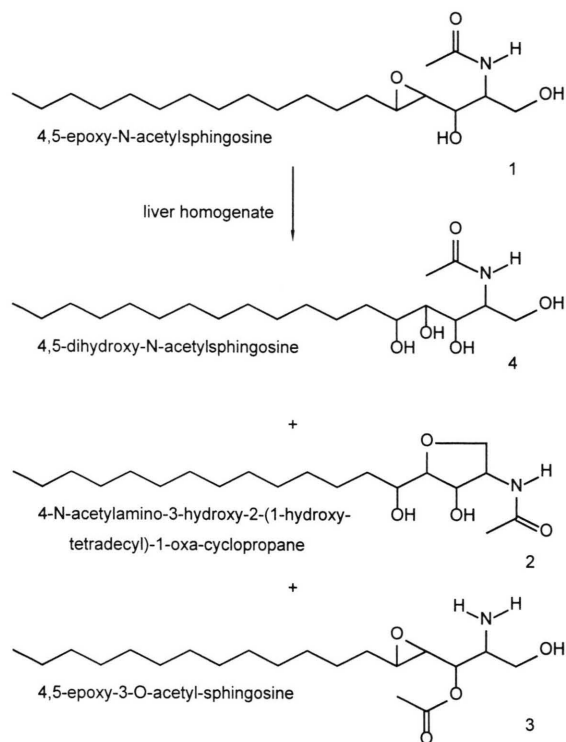


Fig. 2. Part of the GC after incubation of 4,5-epoxy-*N*-acetyl sphingosine (**1**) with liver homogenate (**2**: 4-*N*-acetyl amino-3-hydroxy-2-(1-hydroxytetradecyl)-1-oxa-cyclopentane, **3**: 4,5-epoxy-3-*O*-acetyl sphingosine, **4**: 4,5-dihydroxy-*N*-acetyl sphingosine) (RIC = recalculated ion current).



Scheme 4. In liver homogenate, 4,5-epoxy-N-acetylsphingosine (**1**) is converted to 4,5-dihydroxy-N-acetylsphingosine (**4**), 4-N-acetylamino-3-hydroxy-2-(1-hydroxy-tetradecyl)-1-oxa-cyclopentane (**2**) and 4,5-epoxy-3-O-acetylsphingosine (**3**).

from ring opening of the epoxide was observed only in traces.

Discussion

Degradation of lipid hydroperoxides generated in the course of LPO is a multistep process not yet fully understood which is observed in all diseases combined with cell degradation (Spiteller, 1996). LPO involves mainly linoleic acid (Spiteller, 2000). Products obtained by opening of an epoxidizing are rather common in nature: unsaturated compounds, epoxidation products thereof and corresponding diols were detected in plants, for instance sterols (Meyer and Spiteller, 1997) or terpene derivatives (Ziegler, 1992). Primary LPO products of linoleic acid are 9- and 13-HPODE. In order to elucidate the further fate of these compounds pure 13-HPODE, generated enzymically

by soybean lipoxygenase (Hamberg and Samuelsson, 1967), was subjected to artificial decomposition by air oxidation induced by Fe^{2+} ions (Gardner and Kleiman, 1981; Spiteller and Spiteller, 1998). Important products of this treatment, also detected after artificial oxidation of low density lipoproteins (Spiteller, 2000) are epoxyhydroxy octadecenoic acids (Gardner and Kleiman, 1981).

To elucidate the fate of epoxides in the body we isolated 12,13-epoxy-11-hydroxy-9-octadecenoic acid from the product mixture obtained by treatment of 13-HPODE with iron ions and incubated it with porcine liver homogenate. This resulted in opening of the epoxide ring and formation of two diastereomeric 11,12,13-trihydroxy-9-octadecenoic acids.

4,5-dihydroxylated derivatives of sphingosine have been reported to occur in biological tissue (Prostenik *et al.*, 1982). Therefore 4,5-epoxy-N-acetylsphingosine was prepared and subjected to treatment with liver homogenate as well. It reacted also by ring opening.

Microsomes represent a concentrated extract of liver enzymes. Thus we also expected ring opening of epoxides by addition of epoxides to microsomes. In spite of addition of cofactors (NADPH, Mg ions) only traces of dihydroxy derivatives were observed. These results suggest – if not a special cofactor is involved – that transformation of epoxides to dihydroxyderivatives is not achieved in microsomes, possibly indicating that the necessary enzymes for transformation of epoxides to their corresponding diols are not located in the microsomes but in the cell membranes.

Experimental

Materials

N-Methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) was obtained from Machery and Nagel (Düren, Germany). Solvents were distilled before use, water was distilled twice. Sphingosine was purchased from BIOMOL (Hamburg, Germany), reagents of analytical grade were bought from FLUKA AG (Buchs, Switzerland). Fresh porcine liver tissue was obtained from Dr. Brunnbauer, (slaughterhouse Bayreuth). Human liver microsomes were obtained from GENTEST Corporation (Woburn, USA).

Preparation of 12,13-epoxy-11-hydroxy-9-octadecenoic acid by conversion of 13-HPODE

13-Hydroperoxyoctadecadienoic acid (13-HPODE) was synthesized according to Hamberg and Samuelsson (1967). Raw 13-HPODE was purified twice by NP-HPLC (detection at 212 and 234 nm) on a Bischoff Ultrasep column: Isocratic separation 76 % A (A: n-hexane/AcOH 100/0.1, v/v) and 24 % B (B: n-hexane/isopropanol/AcOH 95/5/0.1, v/v/v) at 20 ml/min. Retention time: 17.0 min.

13-HPODE (181 mg, 580 μ mol) was emulsified in 0.1 M phosphate buffer (266 ml, pH 7.4) and 543 ml 0.2 M KCl (KCl solution prevents the 13-HPODE to form micelles) by sonification. Oxidation was started by addition of 1 mM FeSO₄ (36 ml) and vitamin C (176 mg, 1 mmol), which keeps the iron ions in the oxidation state +2. The reaction mixture was stirred for 24 h at 37 °C (Spiteller and Spiteller, 1998). Purification of 12,13-epoxy-11-hydroxy-9-octadecenoic acid was performed by NP-HPLC (detection at 212 nm) on the same column and with the same solvent mixture as described above for 13-HPODE. Retention time: 30.5 min. Yield: 12 mg. A further purification step was performed by RP-HPLC (Bischoff Spherisorb ODS II). Isocratic separation 35 % A (A: methanol/water/AcOH 95/5/0.1, v/v/v) and 65 % B (B: methanol/water/AcOH 5:95:0.1, v/v/v) at 1 ml/min. Retention time: 3.7 min. Yield: 11 mg.

Mass spectrum: 441(1), 385(4), 356(5), 343(100), 341(22), 326(6), 277(4), 253(4), 217(4), 173(8), 155(24), 147(29), 129(35), 75(50), 73(86) (Hamberg and Gotthammar, 1973)

Synthesis of 4,5-epoxy-N-acetyl sphingosine

Synthesis of 4,5-epoxy-N-acetyl sphingosine was carried out as recently described (Möllenberg and Spiteller, 2000).

Incubation of epoxides with liver homogenate

Fresh porcine liver tissue (10.6 g) was cut in small pieces, 40 mM TRIS/HCl buffer (100 ml,

pH 7.4) was added. The mixture was homogenized in a mixer (Waring Blendor) for 2 min. In order to decrease viscosity, a 5 ml portion of the homogenate was diluted with 3 ml buffer. 1.8 mg of 12,13-epoxy-11-hydroxy-9-octadecenoic acid deposited in a flask were added to the liver homogenate and the mixture was stirred at 37 °C for 24 h. The lipids were extracted according to Bligh and Dyer (1959), converted to the TMS-derivatives and subjected to GC/MS.

In the same manner N-acetyl sphingosine (1.8 mg in 1 ml buffer) was incubated and processed.

Incubation of epoxides with human liver microsomes (HLM)

Commercially available HLM contain 10 mg total protein in 500 μ l 250 mM sucrose. The system is recommended to be used with 1.3 mM NADPH and 3.3 mM MgCl₂ (Gentest Corporation, 1997). A solution containing 10 ml TRIS/HCl (40 mM, pH 7.4) MgCl₂ · 6 H₂O (6.7 mg, 3.3 mM) and NADPH (10.8 mg, 1.3 mM) was prepared. This solution was divided into five portions and to each 2 ml 100 μ l of the thawed HLM solution were added. 12,13-epoxy-11-hydroxy-9-octadecenoic acid (1.4 mg, 4.5 μ M) was added to one portion of HLM and after addition of 2 ml H₂O (to decrease the viscosity of the solution) the reaction mixture was stirred at 37 °C for 3 h. N-acetyl sphingosine (1.4 mg, 4 μ M) was added to the next portion of HLM and treated as described for 12,13-epoxy-11-hydroxy-9-octadecenoic acid. The lipids were extracted according to Bligh and Dyer (1959), converted to the TMS-derivatives and subjected to GC/MS.

Acknowledgements

We thank Deutsche Forschungsgemeinschaft and Fischer-Stiftung for financial support. We are obliged to M. Gläeßner for running the mass spectra and W. Kern for purification of solvents. We are also indebted to Dr. Brunnbauer (slaughterhouse Bayreuth) for providing porcine liver samples.

- Adam W., Bialas J. and Hadjiarapoglou L. (1991), A convenient preparation of acetone solutions of dimethyldioxirane. *Chem. Ber.* **124**, 2377..
- Bligh E. G. and Dyer W. J. (1959), A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **37**, 911–917.
- Christophersen B. O. (1968), Formation of monohydroxy-polyenic fatty acids from lipid peroxides by a glutathione peroxidase. *Biochim. Biophys. Acta* **164**, 35–44.
- Denzlinger C. (1996), Biology and pathophysiology of leukotrienes. *Crit. Rev. Oncol. Haematol.* **23**, 167–223.
- Dolev A., Rohwedder W. K. and Dutton H. J. (1967), Mechanism of lipoxidase reaction. I. Specificity of hydroperoxidation of linoleic acid. *Lipids* **2**, 28–33.
- Fuchs C. and Spiteller G. (2000), Iron release from the active site of lipoxygenase. *Z. Naturforsch.* **55c**, 643–648.
- Gallasch B. (1998), Untersuchungen pflanzlicher Fettsäureoxidationsprodukte mittels GC/FID/MSD-Kopplung. Dissertation, Universität Bayreuth.
- Gardner H. W. and Kleiman R. (1974), Homolytic decomposition of linoleic acid hydroperoxide. Identification of fatty acid products. *Lipids* **9**, 696–706.
- Gardner H. W. and Kleiman R. (1981), Degradation of linoleic acid hydroperoxides by a cysteine ferric chloride catalyst as a model for similar biochemical reactions. II. Specificity in formation of fatty acid epoxides. *Biochim. Biophys. Acta* **665**, 113–122.
- Gardner H. W. (1989), Oxygen radical chemistry of polyunsaturated fatty acids. *Free Radic. Biol. Med.* **7**, 65–68.
- Gentest Corporation (1997), Human Liver Microsomes, Supplementary description of the product. Woburn, USA.
- Hamberg M. and Samuelsson B. (1967), Mechanism of the biosynthesis of prostaglandins E1 and F1 α . *J. Biol. Chem.* **242**, 5336–5343.
- Hamberg M. and Gotthammar B. (1973), New reaction of unsaturated fatty acid hydroperoxides. Formation of 11-hydroxy-12, 13-epoxy-9-octadecenoic acid from 13-hydroperoxy-9,11-octadecadienoic acid. *Lipids* **8**, 737–744.
- Herold M. and Spiteller G. (1996), Enzymic production of hydroperoxides of unsaturated fatty acids by injury of mammalian cells. *Chem. Phys. Lipids* **79**, 113–121..
- Möllenberg A and Spiteller G. (2000), Investigations on Ceramide-Epoxides. Submitted to *Chem. Phys. Lipids*.
- Meyer W. and Spiteller G. (1997), Oxidized phytosterols increase by ageing in photoautotrophic cell cultures of *Chenopodium rubrum*. *Phytochemistry* **45**, 297–302.
- Ozawa T., Hayakawa M. T., Tadanobu S., Satoru S., Kiyoshi I., Masaru T. and Fumio T. (1986), Biosynthesis of leukotoxin, 9,10-epoxy-12-octadecenoate, by leukocytes in lung lavages of rat after exposure to hyperoxia. *Biochem. Biophys. Res. Commun.* **134**, 1071–1078.
- Parthasarathy S., Steinbrecher U. P., Barnett J., Witztum J. L. and Steinberg D. (1985), Essential role of phospholipase A2 activity in endothelial cell-induced modification of low density lipoprotein. *Proc. Natl. Acad. Sci. U. S. A.* **82**, 3000–3004.
- Polito A. J., Naworal J. and Sweeley C. C. (1969), Determination of the structures of sphingolipid bases by combined gas chromatography-mass spectrometry. *Biochemistry* **8**, 1811–1815.
- Prostenik M., Cosovic C., Gospocic Lj., Jandric Z. and Ondrusek V. (1982), Studies in the sphingolipids series. XXXVII. Long-chain bases in cerebroside of central nervous tissue. *Rad Jugosl. Akad. Znan. Umjet., kem [407]* **3**, 5–12.
- Smith L. L., Teng J. I., Lin Y. Y. and Seitz P. K. (1980), Lipid Peroxides Biol. Med., Proc. Int. Conf. 1980, (K. Yagi, ed.). Academic Press, New York p. 89.
- Spiteller D. (2000), Oxidation of linoleic acid in low-density lipoprotein: an important event in atherogenesis. *Angewandte Chemie, Int. Ed.* **39**, 585–589.
- Spiteller G. and Spiteller P. (1998), Strong dependence of the lipid peroxidation product spectrum whether Fe²⁺/O₂ or Fe³⁺/O₂ is used as oxidant. *Biochim. Biophys. Acta* **1392**, 23–40.
- Spiteller G. (1996), Enzymic lipid peroxidation – a consequence of cell injury? *Free Rad. Biol. Med.* **21**, 1003–1009.
- Spiteller G. (1998), Linoleic acid peroxidation – the dominant lipid peroxidation process in low density lipoprotein – and its relationship to chronic diseases. *Chem. Phys. Lipids* **95**, 105–116.
- Watabe T., Isobe M., Tsubaki A. and Ozawa N. (1982), Epoxidation of cholesterol by hepatic microsomal lipid hydroperoxides. *Biochem. Biophys. Res. Commun.* **108**, 724.
- Wills E. D. (1966), Mechanisms of lipid peroxide formation in animal tissues. *Biochem. J* **99**, 667–676.
- Ziegler H. (1992), Analytik der Cumarine des Zitronenöls (*Citrus limon*, Burm. f.). Dissertation, University of Bayreuth.