its larger conjugated system, to react faster than **8**. Indeed, 2-*trans*,4-*trans*-2,4-decadienal reacts quickly with glutathione to give a large number of products.[20] It is, therefore, understandable that this compound so far escaped detection in blood samples of atherosclerotic patients.

LPO processes are increased by tissue injury, for example, in the course of a myocardial infarction.[21] As a consequence, it is expected that the early oxidation processes described in this paper should also occur in vivo.[22]

Experimental Section

LDL was isolated according to Leiss et al.,[9] or was obtained commercially (Fluka). LDL, obtained from 3–5 mL serum, was dissolved in 1 mL of phosphate buffer (0.1M, pH 7.4) in a GC vial fitted with a stirring bar. 1 mL of a 10 or 100 μM aqueous solution of $CuSO_4$ was added. The vial was closed with a septum, and the mixture stirred at 37 °C. The septum was penetrated by the SPME device and the 100 μm fibre (polydisiloxane, PDMS) half submerged in the solution. After 2 h, 3 h, 4 h, 5 h, 6 h, 10 h, 12 h, 1 d, 2 d, 4 d, 7 d the fibre was removed from the vial and directly introduced into the inlet of the GC/MS instrument (MAT 95 mass spectrometer (Finnigan), EI ion source, 70 eV, HP 5890 series II gas chromatograph, fused silica gel column from J&W Scientific, DB 05, 30 m × 0.32 mm, carrier gas H_2, splitless, injector temperature 280 °C, temperature program: 5 min isothermic at 40 °C, then 3 °C min^{-1} up to 300 °C). The usual flushing of the septum by purge gas prevents detection of compounds with high volatility. In order to avoid this, flushing of the septum was shut down for 30 s when the PDMS fibre was introduced. Use of a special, narrow GC liner tube allowed adjustment of the peak form of the chromatograms and resulted in a considerable improvement of the quality of the mass spectra.

Model oxidations with pure 2,4-decadienal and 4-hydroxy-2-nonenal were carried out in a similar manner as those of LDL samples.

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Oxidation of Linoleic Acid in Low-Density Lipoprotein: An Important Event in Atherogenesis**

Dieter Spiteller and Gerhard Spiteller*

Half of all deaths in Germany are caused by vascular diseases,[1] which are caused in part by deposition of cholesterol-rich plaques in the arteries. Cholesterol, an essential compound in cell membranes, is transfered to the cells by the low-density lipoprotein (LDL). LDL consists of a lipid core composed mainly of cholesterol esters. This core is surrounded by a layer of phospholipids that are wrapped by a protein. Lysyl residues of that protein are recognized by receptors that are arranged in so-called coated pits at the cell surface. The entire coated pit together with receptor-bound LDL molecules is then enclosed by a protein and transported inside the cell where the LDL is separated from the receptor. The liberated LDL is digested: proteins are broken down to amino acids and cholesterol esters and phospholipids are saponified. Depending on the need for cholesterol more or less receptors are generated.[2, 3]

Oxidatively modified LDL[4, 5] is no longer recognized by the LDL receptor but is recognized by scavenger receptors of macrophages. LDL is taken up without limitation by macrophages,[2, 3] which are finally deposited as plaques. LDL is very sensitive to oxidation, which occurs by copper(II) ion catalysis in air[5, 6] or on its own in three to six months when stored at 0 °C.[7] Oxidatively modified LDL (oxLDL) is toxic[4] and the toxic components can be extracted with organic solvents.[5] Thus it was suspected that the toxic compounds might be the products of lipid peroxidation (LPO) of polyunsaturated fatty acids (PUFAs).[4] Some of these products were identified,[8, 9, 10] but most remained unknown until now.

Since LPO products occur in LDL in only trace amounts compared to other compounds their identification requires

[*] Prof. Dr. G. Spiteller, Dipl.-Chem. D. Spiteller
Lehrstuhl für Organische Chemie I
Universität Bayreuth
Universitätstrasse 30, 95440 Bayreuth (Germany)
Fax: (+49) 921-552671
E-mail: gerhard.spiteller@uni-bayreuth.de

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highly sensitive and specific methods. Substituted fatty acids and products generated therefrom give, after appropriate derivatization, characteristic electron impact mass spectra (EI-MS). We therefore used this method for the identification of LPO products generated by copper ion induced air oxidation of LDL[5] that are also found after storage of samples of LDL at 0 °C.[7]

Pure PUFAs were first oxidized in the same way as the LDL samples (Cu^{2+} ion catalyzed air oxidation at 37 °C). The lipid fraction of the reaction products was treated with pentafluorobenzylhydroxylamine-hydrochloride to derivatize the carbonyl groups.[9] Free acid groups were methylated by a short treatment with diazomethane. Finally the hydroxyl groups were trimethylsilylated with *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA). The mixture of products thus obtained was analyzed by GC/MS through measurement of the total ion current. The compounds generated turned out to be nearly identical to LPO products that had been identified previously by oxidation of PUFAs with other oxidizing reagents (for example, Fe^{3+}/air),[11, 12] although we recognized an increased tendency for the generation of higher oxidized products (for example, transformation of aldehydes into the corresponding acids). The knowledge of the thus obtained spectra facilitated the identification of analogous products after oxidation of the LDL samples in air. Samples withdrawn at time intervals enabled insight into the reaction sequences. Detection of esterified oxidized PUFAs required an additional hydrolysis step. Figures 1–3 show the time-dependent change in the products in one of the LDL samples.

Figure 1 shows a part of the reconstituted ion-current chromatogram (RIC) of a LDL sample before oxidation. The RIC of the same sample after 6 h oxidation is represented in Figure 2 and after 24 h oxidation in Figure 3. The identified products are listed in Table 1.

The RIC of LDL samples of different donors and commercially available LDL samples are not qualitatively different. In contrast the proportion of acids and of their oxidation products is individually different. They also depend strongly on the kind of diet: High levels of linoleic acid in the diet doubles and triples the amount of oxidation products in LDL 12–16 h after intake of a meal rich in linoleic acid.[13] 9-Hydroxy-10,12-octadecadienoic acid (9-HODE) and 13-hydroxy-9,11-octadecadienoic acid (13-HODE) are already present in trace amounts before oxidation of LDL (Figure 1), which indicates that oxidation of linoleic acid occurs even in healthy persons to a small extent.

Oxidation of LDL does not start immediately as antioxidants present are consumed first.[8] This lag-time is dependent on the amount of copper ions added, but is different for each individual sample. In the present case oxidation products were first recognized after 6 h incubation with $CuSO_4$/air (Figure 2) by an increase of HODE derivatives (peak 33). Although 9-HODE and 13-HODE have been detected after copper ion induced LDL oxidation previously[10] and occur highly enriched in blood samples of patients suffering from atherosclerosis,[14] epoxyhydroxyoctadecenoic acids (EpHODs, peaks 37 and 38), as well as 9- and 13-oxooctadecadienoic acids (KODEs, peaks 35, 36, and 39) have not been described as oxidation products of LDL. Their presence is remarkable,

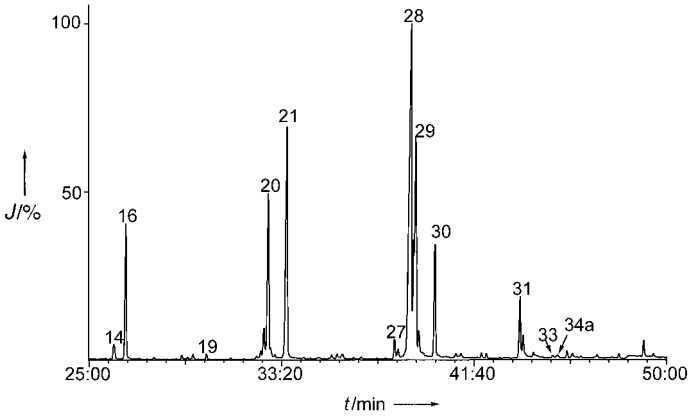

Figure 1. Part of the RIC of a LDL sample before oxidation. The donor was a healthy male aged 67 years (total cholesterol 199 mg dL^{-1}, LDL cholesterol 127 mg dL^{-1}, HDL cholesterol 57 mg dL^{-1}, triglycerides 71 mg dL^{-1}). The numbers above the peaks correspond to the compounds listed in Table 1.

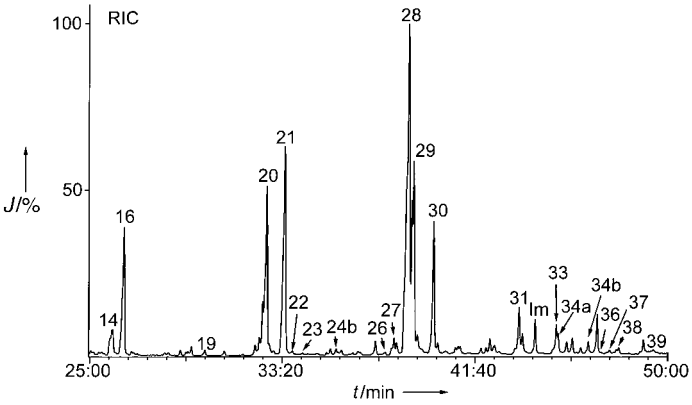

Figure 2. Part of the RIC of the same LDL sample as shown in Figure 1 after 6 h oxidation (5 μM $CuSO_4$, air, 37 °C). Im = impurity.

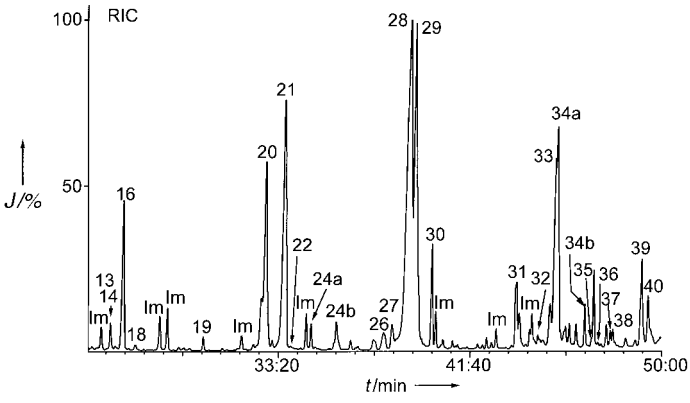

Figure 3. Part of the RIC of the same LDL sample as in Figure 1 after 24 h oxidation. Im = impurity.

since epoxides (by opening of the epoxide ring) and KODEs (in the course of a Michael reaction) react with nucleophiles (especially glutathion) and should develop toxic properties by blocking enzymes containing SH groups.

The product pattern changed considerably within 24 h (Figure 3). The level of acid oxidation products, especially HODEs (peaks 33, 34a, and 34b), increased dramatically, but

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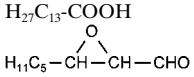
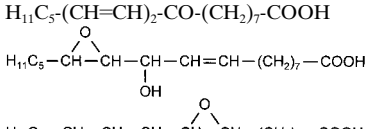
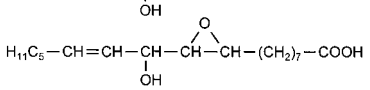
Table 1. Compounds identified before and after oxidation of LDL samples with $CuSO_4$/air and of minimally oxidized LDL samples.[a]

Entry	Origin (point of attack)[b]	Product	Derivative detected[c]	found[d] in LDL[e] 0 h	6 h	24 h	MM-LDL
1	LEA (n-3)	H_5C_2-CHO	PFBO			+	
2	AA (n-6), LA (n-6)	$H_{11}C_5$-CH(OH)-COOH	TMS/OMe			+	
3	AA (n-10), LA (n-10),	$H_{11}C_5$-$(CH{=}CH)_2$-CHO				+	
4	AA, LA	OHC-COOH	PFBO/OMe			+	
5	AA, LA	H_9C_4-CHO	PFBO			+	
6	AA, LA, LEA	OHC-CH_2-COOH	PFBO/OMe			+	
7	LA (n-10), LEA (n-10)	HOOC-$(CH_2)_6$-COOH	OMe/OMe			+	
8	AA (n-6), LA (n-6)	$H_{11}C_5$-CHO	PFBO			+	+
9	LEA (n-3)	H_5C_2-CH(OH)-CHO	TMS/PFBO			+	
10	AA (n-10), LA (n-10)	$H_{11}C_5$-CH(OH)-CH=CH-COOH	TMS/OMe			+	
11		CH_2(OH)-CH(OH)-CHO	TMS/TMS/PFBO			+	
12	LA (n-10), LEA (n-10)	HOOC-$(CH_2)_7$-COOH	OMe/OMe			+	
13	AA (n-6), LA (n-6)	$H_{11}C_5$-CH(OH)-CHO	TMS/PFBO		+	+	
14		$H_{25}C_{13}$-COOH	OMe	+	+	+	+
15	LA (n-6)	$H_{11}C_5$-CO-CH_2OH (artifact, rearrangement product of 13)	TMS/PFBO			+	
16		$H_{27}C_{13}$-COOH	OMe	+	+	+	+
17	AA (n-10), LA (n-10)		PFBO			+	
18	LEA (n-10)	H_5C_2-CH(OH)-CH=CH-CHO	TMS/PFBO			+	
19		$H_{29}C_{14}$-COOH	OMe	+	+	+	+
20		$H_{29}C_{15}$-COOH	OMe	+	+	+	+
21		$H_{31}C_{15}$-COOH	OMe	+	+	+	+
22	AA, LA, LEA	OHC-CHO	PFBO/PFBO			+	+
23	LA (n-10), LEA (n-10)	OHC-$(CH_2)_6$-COOH	PFBO/OMe			+	+
24	LA (n-10), AA (n-10)	$H_{11}C_5$-CH(OH)-CH=CH-CHO	TMS/PFBO		+	+	+
25	LA (n-10), AA (n-10)	$H_{11}C_5$-$(CH{=}CH)_2$-CHO	PFBO		+	+	
26	LA (n-10), LEA (n-10)	OHC-$(CH_2)_7$-COOH	PFBO/OMe		+	+	+
27		H_3C-$(CH_2$-$CH{=}CH)_3$-$(CH_2)_7$-COOH	OMe	+	+	+	+
28		H_3C-$(CH_2)_3$-$(CH_2$-$CH{=}CH)_2$-$(CH_2)_7$-COOH	OMe	+	+	+	+
29		$H_{17}C_8$-CH=CH-$(CH_2)_7$-COOH	OMe	+	+	+	+
30		$H_{35}C_{17}$-COOH	OMe	+	+	+	+
31		H_9C_4-$(CH_2$-$CH{=}CH)_4$-$(CH_2)_3$-COOH	OMe	+	+	+	+
32	OA (n-9)	$H_{17}C_8$-CH=CH-CH(OH)-$(CH_2)_6$-COOH	TMS/OMe			+	+
	OA (n-10)	$H_{15}C_7$-CH=CH-CH(OH)-$(CH_2)_7$-COOH	TMS/OMe			+	+
	OA (n-9)	$H_{17}C_8$-CH(OH)-CH=CH-$(CH_2)_6$-COOH	TMS/OMe			+	+
	OA (n-10)	$H_{15}C_7$-CH(OH)-CH=CH-$(CH_2)_7$-COOH	TMS/OMe			+	+
33	LA (n-6)	$H_{11}C_5$-CH(OH)-$(CH{=}CH)_2$-$(CH_2)_7$-COOH	TMS/OMe	+	++	+++	++
34	LA (n-10)	$H_{11}C_5$-$(CH{=}CH)_2$-CH(OH)-$(CH_2)_7$-COOH	TMS/OMe	+	++	+++	++
35	LA (n-6)	$H_{11}C_5$-CO-$(CH{=}CH)_2$-$(CH_2)_7$-COOH	OMe			+	+
36	LA (n-10)	$H_{11}C_5$-$(CH{=}CH)_2$-CO-$(CH_2)_7$-COOH	OMe			+	+
37	LA (n-6)		TMS/OMe		+	++	++
38	LA (n-10)		TMS/OMe		+	++	++
39	LA (n-6), (n-10)	$H_{11}C_5$-$(CH{=}CH)_2$-C(OH)=CH-$(CH_2)_6$-COOH	ETMS/OMe			+	
		H_9C_4-CH=C(OH)-$(CH{=}CH)_2$-$(CH_2)_7$-COOH	ETMS/OMe				
40	AA (n-10)	$H_{11}C_5$-$(CH{=}CH)_2$-CH(OH)-$(CH_2$-$CH{=}CH)_2$-$(CH_2)_3$-COOH	TMS/OMe			+	+
	AA (n-12)	$H_{11}C_5$-$(CH{=}CH$-$CH_2)_2$-CH(OH)-$(CH{=}CH)_2$-$(CH_2)_3$-COOH	TMS/OMe			+	+
	AA (n-9)	$H_{11}C_5$-CH=CH-CH_2-CH(OH)-$(CH{=}CH)_2$-CH_2-CH=CH-$(CH_2)_3$-COOH	TMS/OMe			+	+
	AA (n-13)	$H_{11}C_5$-CH=CH-CH_2-$(CH{=}CH)_2$-CH(OH)-CH_2-CH=CH-$(CH_2)_3$-COOH	TMS/OMe			+	+
41	LA (n-10)	$H_{11}C_5$-CH = CH-CH(OH)-CH(OH)-CH(OH)-$(CH_2)_7$-COOH	TMS/TMS/ TMS/OMe			+	
42	LA (n-6)	$H_{11}C_5$-CH(OH)-CH(OH)-CH=CH-CH(OH)-$(CH_2)_7$-COOH	TMS/TMS/TMS/OMe			+	
43	LA (n-10)	$H_{11}C_5$-CH(OH)-CH=CH-CH(OH)-CH(OH)-$(CH_2)_7$-COOH	TMS/TMS/TMS/OMe			+	
44	LA (n-10), AA (n-10)	$H_{11}C_5$-CO-CH=CH-CHO	PFBO/PFBO			+	
45	LA (n-6)	OHC-CH=CH-CH(OH)-$(CH_2)_7$-COOH	PFBO/TMS/OMe			+	
46	LA (n-6)	$H_{11}C_5$-CO-$(CH{=}CH)_2$-$(CH_2)_7$-COOH	PFBO/OMe			+	
47	LA (n-10)	$H_{11}C_5$-$(CH{=}CH)_2$-CO-$(CH_2)_7$-COOH	PFBO/OMe			+	

[a] The compounds are arranged according to increasing elution time from the GC column; the entry number corresponds to the peaks in Figures 1–3. The origin of identified compounds, their structural formulas, and the type of derivative are given in a separate column. [b] AA = arachidonic acid, LA = linoleic acid, LEA = linolenic acid, OA = oleic acid. The point of attack is described by the number of the carbon atom in the starting material by counting from the carbon (n) at the alkyl end of the chain. [c] ETMS = enol trimethylsilyl ester, OMe = methyl ester, PFBO = pentafluorobenzyloxime, TMS = trimethylsilyl ether. [d] The grading of + to + ++ represents the relative proportion of product formed. [e] The duration of the oxidation (Cu^{2+}/O_2) is given.

also an increased generation of aldehydes was observed, for example, of 4-hydroxy-2-nonenal[15] (4-HNE; peaks 24a and 24b two isomers) or of 2-hydroxyheptanal (peak 13).

In contrast to the typical linoleic acid oxidation products (HODEs, KODEs, EpHODs) oxidation products of arachidonic acid, for example, hydroxyeicosatetraenoic acids (peak 40, several isomers) were present only in low amounts. This result is somewhat surprising because it was previously assumed[3] that LPO preferentially affects arachidonic acid. A nearly equal tendency for linoleic and arachidonic acid to be oxidized is also indicated by the nearly equal decrease of the peaks corresponding to these acids (peak 28 and 31) in the oxidized samples).

Oleic acid oxidation products (peak 32) are detectable in small amount after oxidation for 24 h.[10] This observation demonstrates that oxidative attack is also possible only at monoallylically activated methylene groups in LDL.

In contrast to previous views[3] the preferential presence of linoleic acid oxidation products can be understood if one considers that radicals, which induce LPO processes, only recognize the -CH=CH-CH_2-CH=CH- group. They therefore attack free PUFAs as well as their esters with comparable probability. LDL consists predominantly of cholesterol linoleate,[16] while cholesterol arachidonate is present in much less amounts. Furthermore, LPO products of arachidonic acid degrade faster than those of linoleic acid. Thus the observed prevalence of linoleic acid oxidation products is explained.

HODEs, the main products of LPO of LDL, are physiologically highly reactive compounds: In combination with other LPO products, for example, 2,4-decadienal, HODEs effect the release of interleukin-1β from macrophages,[17] they activate the peroxisome-proliferator-activated receptor protein (PPAR-γ),[18] which plays a role in cell differentiation,[19] they induce inflammation,[20] and cause swelling of mitochondria.[21] Toxic properties are ascribed in particular to 4-HNE, which undergoes, like all α,β-unsaturated aldehydes, Michael reactions.[15, 22, 23] Even more toxic is 2,4-decadienal[24] because of its higher conjugation and hence higher reactivity. Since the same structural element is present in KODEs, the latter should have analogous toxicity, but this had not been investigated.

Copper-ion induced air oxidation is an unphysiological method. A more physiologically similar mild oxidation of LDL occurs just by storing LDL samples at 0 °C.[7] This minimally modified LDL (MM-LDL) is still recognized by the LDL cell receptor, but not by the scavenger receptor.[7] We isolated such a MM-LDL from a serum sample stored for six months at 0 °C. The sample was investigated in exactly the same way as that oxidized with Cu^{2+}/air. The chromatogram was similar to that obtained from a LDL sample oxidized for 10 h with Cu^{2+}/air. Since this MM-LDL is still recognized by the LDL receptor[7] it should be transferred into the cell and digested there (see above). Consequently HODEs, KODEs, EpHODs, 4-HNE, and 2,4-decadienal should be introduced into the cell. This sequence might explain why fatty streaks, which are the first signs of a beginning atherosclerosis, develop beneath a structurally intact endothel.[25] In contrast it was suggested previously that LDL would be oxidized during atherosclerosis by a prolonged stay at the surface of endothelial cells, which would result in potential oxidation by an unknown manner.[26]

The increase in HODEs occurs years before the first signs of atherosclerotic changes become evident in the arteries,[27] and thus also before oxidized LDL is recognized by the scavenger receptor of macrophages. In model experiments we detected that lysine residues of the LDL protein, which are responsible for receptor binding, are much less susceptible to oxidation than PUFAs. Consequently our investigations suggest that atherosclerosis starts with an increased oxidation of the PUFAs in cholesterol esters and phospholipids in LDL. The increase of the cholesterol level in atherosclerotic patients is therefore only a side effect of the much more important oxidation of the fatty acid part. The increase in the oxidation products exceeds that of cholesterol by more than one order of magnitude.[16]

Experimental Section

LDL was purchased (Fluka AG) or prepared according to Leiss[28] by fractional precipitation of serum from healthy donors immediately after withdrawal of blood. MM-LDL was obtained by storage of serum samples at 0 °C for 3–6 months.

LDL oxidation: LDL derived from serum (40 mL) was dissolved in phosphate buffer (PBS; 20 mL, 0.1M, pH 7.4), and an aqueous $CuSO_4$ solution (10 μM, 20 mL) added. The solution was stirred at 37 °C. Samples (10 mL) were withdrawn after 0, 6, 12, and 24 h, and extracted according to the procedure of Bligh and Dyer.[29] A 0.05 mM methanolic pentafluorobenzylhydroxylamine hydrochloride solution (50 μL) was added. After 10 min the solvent was removed on a rotatory evaporator at room temperature. The residue was dissolved in 0.1M PBS (10 mL, pH 7.4) by ultrasonification, and cholesterolesterase (Fluka AG, 1 mg) was added. The solution was stirred for 1 h at 37 °C. Hydroperoxides were destroyed by addition of $P(OCH_3)_3$ (20 μL)[30] (the enzyme is deactivated if $P(OCH_3)_3$ is added before enzymatic hydrolysis). The solution was brought to pH 2 after hydrolysis and the saponified lipids were extracted according to Bligh and Dyer.[29] After removal of $CHCl_3$, the residue was dissolved in MeOH/H_2O (85/15, 5 mL) by ultrasonification and filtered over a previously equilibrated RP-18 cartridge. The methanol was removed, conc. HCl added (pH 2), and the products were extracted with diethyl ether. The diethyl ether was removed by a stream of nitrogen. The residue was treated for 2 min with 5% solution of diazomethane in diethyl ether (0.5 mL), and the excess CH_2N_2 and solvent were removed under a stream of nitrogen (Caution: if HCl is not removed completely, hydroxy functions are partly transferred to methyl ethers by the subsequent treatment with diazomethane). The residue was treated with MSTFA (10 μL; 60 min, 40 °C). The samples prepared were then subjected to GC/MS analysis.

The oxidation of linoleic acid and arachidonic acid was performed as described recently,[31] except that a 5 μM solution of $CuSO_4$ was used instead of Fe ions.

GC/MS: Finnigan MAT 95, 70 eV, ICIS data system, connected to a HP 5890 Series II gas chromatograph, fused silica gel column coated with DB-05 (J&W Scientific Instruments) i.d. 0.25 mm, 30 m, H_2, injector 280 °C, temperature program: 3 min isothermal at 50 °C, 4 °C min^{-1} to 100 °C, 3 °C min^{-1} to 300 °C, 10 min isothermal at 300 °C, injecton volume 0.6–3 μL.

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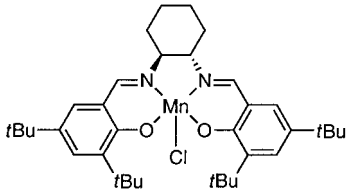
Radical Intermediates in the Jacobsen – Katsuki Epoxidation**

Luigi Cavallo and Heiko Jacobsen*

The generation of optically active epoxides by oxygen-transfer reactions represents one of the most elegant techniques used in the formation of carbon – oxygen bonds in asymmetric synthesis.[1] The catalytic protocol developed by Jacobsen and co-workers,[2] which involves the use of the Mn^{III} – salen complex **1** and derivatives thereof, currently stands as the most powerful method in this field; it has been



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proven effective for virtually every class of unfunctionalized, conjugated olefins,[3] and industrial production of the catalyst on a ton scale has become possible.[4] Although the importance of steric bulk in the 3,3′-position of the salen ligand and the profound influence of electronic effects due to the substituents in 5,5′-position were recognized early on[5] and rationalized in model calculations,[6] the mechanism of the Mn^{III} – salen catalyzed epoxidation is still a matter of controversy. The *cis* – *trans* isomerization observed in the transformation of conjugated alkenes gave rise to several proposed mechanisms. Starting with a Mn^{V}-oxo-salen complex as the catalytically active species—experimental evidence has recently been provided for this[7]—the reaction might proceed either by direct substrate attack at the oxo ligand in a concerted or sequential fashion involving radical intermediates, or by substrate attack at both the metal and oxo centers to generate an oxametallacyclic intermediate (Scheme 1, pathways **Ic**, **Is**, and **II**, respectively). New experimental results seem to provide evidence for substrate attack at the oxo ligand only,[8] as well as for the existence of manganaoxetanes;[9] the controversial mechanistic debate has recently been summarized in a highlight.[10]

[*] Dr. H. Jacobsen
Anorganisch-Chemisches Institut
Unversität Zürich
Winterthurerstrasse 190, 8057 Zürich (Switzerland)
Fax: (+41) 1-635-6802
E-mail: jacobsen@aci.unizh.ch
Dr. L. Cavallo
Dipartimento di Chimica
Università degli Studi di Napoli "Federico II"
Via Mezzocannone 4, 80134 Napoli (Italy)

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