papers on methodology

Synthesis of 9-oxononanoyl cholesterol by ozonization[1]

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Abstract A new route for the preparation of 9-oxononanoyl cholesterol (5) and its stable dimethylacetal (4) is described. The core aldehyde 5 is one of the major products formed during lipid peroxidation. The synthesis starts with the ozonization of oleic acid in methanol and further reduction with dimethyl sulfide to yield 9,9-dimethoxy nonanoic acid (2a). The condensation of 2a with cholesterol is achieved with N,N′-dicyclohexylcarbodiimide in dichloromethane to give 4. Further hydrolysis of 4 with the help of an acidic ion exchange resin yields 9-oxononanoyl cholesterol.—Boechzelt, H., B. Karten, P. M. Abuja, W. Sattler, and M. Mittelbach. **Synthesis of 9-oxononanoyl cholesterol by ozonization. *J. Lipid Res.* 1998.** 39: **1503–1507.**

Supplementary key words cholesteryl ester • core aldehyde • ozonolysis • synthesis • lipid peroxidation

During lipid peroxidation (LPO) of lipoproteins a variety of aldehydic compounds is formed. These LPO products, mainly caused by low density lipoprotein (LDL) peroxidation, are thought to promote atherogenesis (1). The determination and biological significance of free short chain aldehydes like alkanals, 2-alkenals, or 4-hydroxyalkenals are well known (2, 3). Most recently also, the biochemistry of secondary LPO products still esterfied to the parent lipid, the compounds termed core-aldehydes, has been thoroughly studied (4, 5). However, very little is known about synthetic routes for the preparation of these compounds. Kamido et al. (6) isolated core aldehydes of cholesteryl esters from copper-catalyzed peroxidation of human plasma lipoproteins. The products were characterized as 2,4-dinitrophenylhydrazones with high performance liquid chromatography (HPLC) and liquid chromatography–mass spectrometry (LC/MS). In a previous paper Kamido et al. (7) described the synthesis of the reference compounds in milligram scale starting from cholesteryl arachidonate and cholesteryl oleate. The esters were split with osmium tetroxide, treated with sodium sulfite, and oxidized again with periodic acid. The aldehydes were further purified by thin-layer chromatography. To our knowledge this is the only preparation described for cholesteryl core aldehydes; it is very laborious for larger scale preparations. Ravandi et al. (8) prepared core aldehydes from unsaturated triacylglycerols, choline and ethanolamine phospholipids by reductive ozonolysis.

Here we present a straightforward two-step method for the synthesis of 9-oxononanoyl cholesterol that is also applicable to the synthesis of other lipid peroxidation core aldehydes. It starts from oleic acid, which is ozonized under reductive conditions leading to 9,9-dimethoxy nonanoic acid which is further condensed with cholesterol.

MATERIALS AND METHODS

Materials

All reagents were of commercial quality; oleic acid, cholesterol and methyl sulfide were purchased from Aldrich Chemical Co., DMAP, DCC, and Amberlyst-15 were from Fluka AG; the 0.3 nm molecular sieve was purchased from Merck KGaA.

Spectroscopic and analytical instruments

^{1}H-NMR spectrum was recorded at 360 MHz on a Bruker AMX-360, TMS was used as internal standard. ^{13}C-NMR spectra were recorded at 90 MHz on a Bruker AMX-360, internal standard DMSO. GC/MS was performed on a Hewlett-Packard MSD equipped with a fused-silica capillary column (0.1 μm DB5-HT, 15 m × 0.25 mm, J&W Scientific Inc.) using He as carrier gas. The column was kept at 100°C and programmed to 200°C at a rate of 10°C/min. A second ramp was programmed from 200°C at 15°C/min to 320°C with an isothermal hold at 320°C for 4 min.

Matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) was performed with a Kratos Compact 2V5.2.0 on DHB matrix. Ozone was generated by using a Fischer instrument, model 503. Purification of **5** was performed by RP/HPLC on a Hewlett-Packard 1100 HPLC. The analytes were separated on a RP 18 column (Ultrasphere ODS, 5 μm material, 25 × 1.0

Abbreviations: DCC, N,N′dicyclohexylcarbodiimide; DHB, 2,5-dihydroxybenzoic acid; DMAP, 4-dimethylaminopyridine; DMSO, dimethylsulfoxide; FA, fatty acid; GC/MS, gas chromatography–mass spectrometry; HPLC, high performance liquid chromatography; LDL, low density lipoprotein; LPO, lipid peroxidation; MALDI/TOF, matrix-assisted laser desorption ionization–time of flight; NMR, nuclear magnetic resonance; RP/HPLC, reversed phase-high performance liquid chromatography; TMS, tetramethylsilane.

[1] This paper is dedicated to the memory of Prof. Hermann Esterbauer, who initiated this project.

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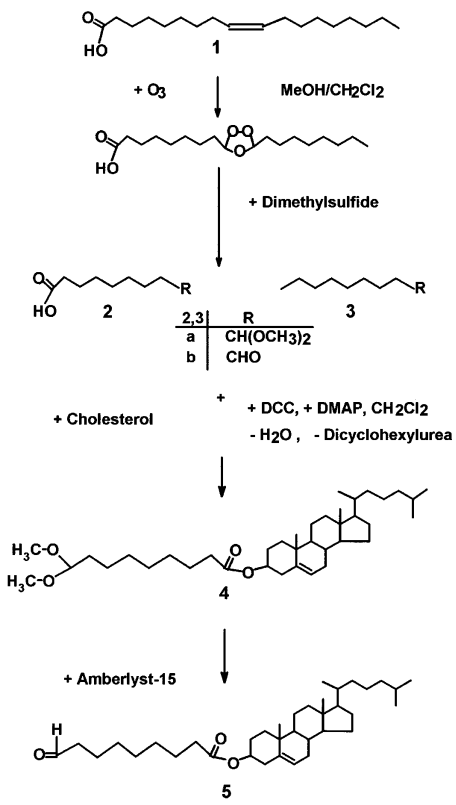

Fig. 1. Main reaction scheme for the synthesis of 9-oxononanoyl-cholesterol.

cm) with acetonitrile/isopropanol at 3 ml/min on the mobile phase at UV detection at 280 nm.

Preparation of products (Fig. 1)

*Synthesis of 9,9-dimethoxy nonanoic acid (**2a**).* A solution of 1.0 g of oleic acid (**1**), 25 ml methanol, and 25 ml dichloromethane was placed into a wash bottle and cooled down to −35°C in an ethanol bath under oxygen flow for 20 min. The mixture was ozonized with an oxygen/ozone mixture with a flow of 50 ml per min containing 5 g/ozone per h. The end of the reaction was indicated by a 5% potassium iodide/water solution turning orange. Afterwards the reaction mixture was flushed for 10 min with nitrogen to remove excess ozone. The wash bottle was then taken from the cooling bath and the contents were stirred well under nitrogen flow with addition of 2 ml dimethylsulfide and slowly brought to room temperature. The reaction mixture was stored well-closed for 24 h at 4°C over 2.0 g molecular sieve (0.3 nm). After filtration, the mixture was washed with 20 ml distilled water to remove DMSO and evaporated at 40°C for 20 min to give 1.4 g of an oily residue. This product mixture consisted of 95% 9,9-dimethoxy nonanoic acid (**2a**) and 5% 9-oxononanoic acid (**2b**) in molar relation to the used oleic acid (GC–MS). The side products were nonane-1, 1-dimethylacetal (**3a**) (98%) and nonanal (**3b**) (2%).

GC/MS fragmentation of 9,9-dimethoxy nonanoic acid: *m/z* (%) = 217 (5) $[M–H^+]$, 75 (100) $[CH(OCH_3)_2]^+$, 71 (25), 155 (22).

*Synthesis of 9-oxononanoyl cholesterol (**5**).* It is not necessary to purify the product mixture as described above for the synthesis of the 9-oxononanoyl cholesterol. Two hundred mg [~100 mg/0.457 mmol 9,9-dimethoxy nonanonic acid (**2a**)] of the product mixture from above, 5.6 mg (0.046 mmol) DMAP, and 193.3 mg (0.500 mmol) cholesterol were dissolved in a flask with 4 ml of dichloromethane. Also, 94.2 mg (0.457 mmol) of DCC were dissolved in

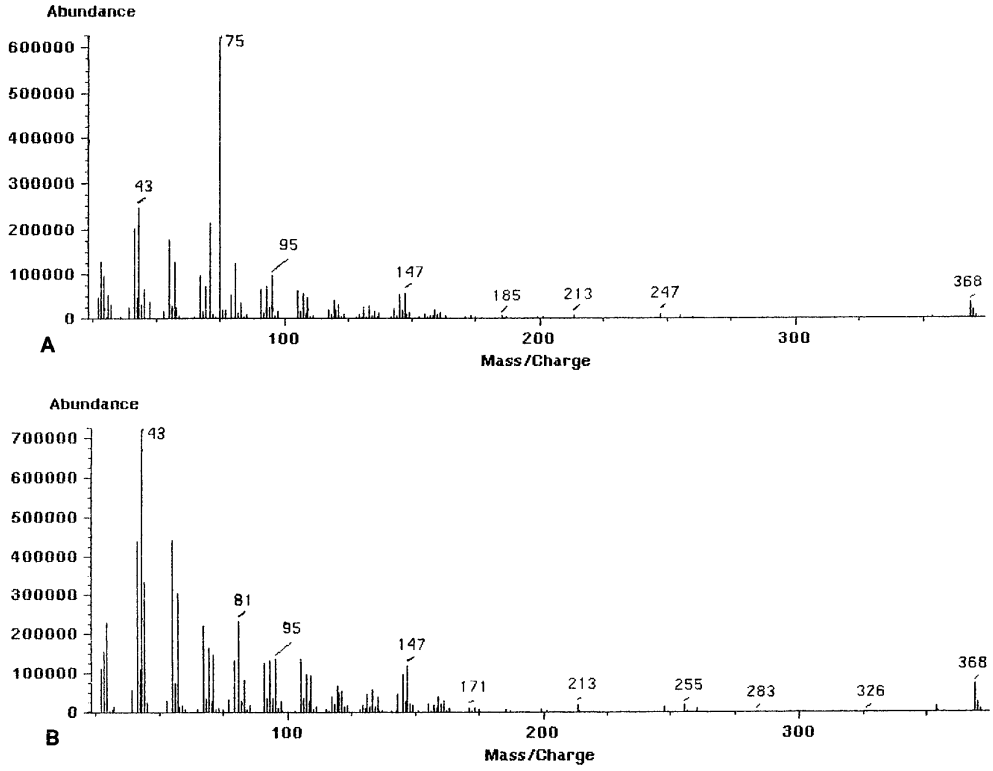

Fig. 2. GC–MS of 9,9-dimethoxynonanoyl cholesterol and 9-oxononanoyl cholesterol were done by direct injection into a HP/MSD without derivatization. GC–MS analysis was performed as described in Materials and Methods. The MS-spectrum was obtained with the quadrupole in EI mode. A: Peak assignment of 9,9-dimethoxynonanoyl cholesterol: *m/z* (%) = 368 (5) $[M–FA]^+$, 75 (100) $[CH(OCH_3)_2]^+$, 43 (41), 95 (26). B: Peak assignment of 9-oxononanoyl cholesterol: *m/z* (%) = 368 (10) $[M–FA]^+$, 43 (100) $[CH_2CHO]^+$, 55 (62), 81 (33), 147 (17), 95 (13).

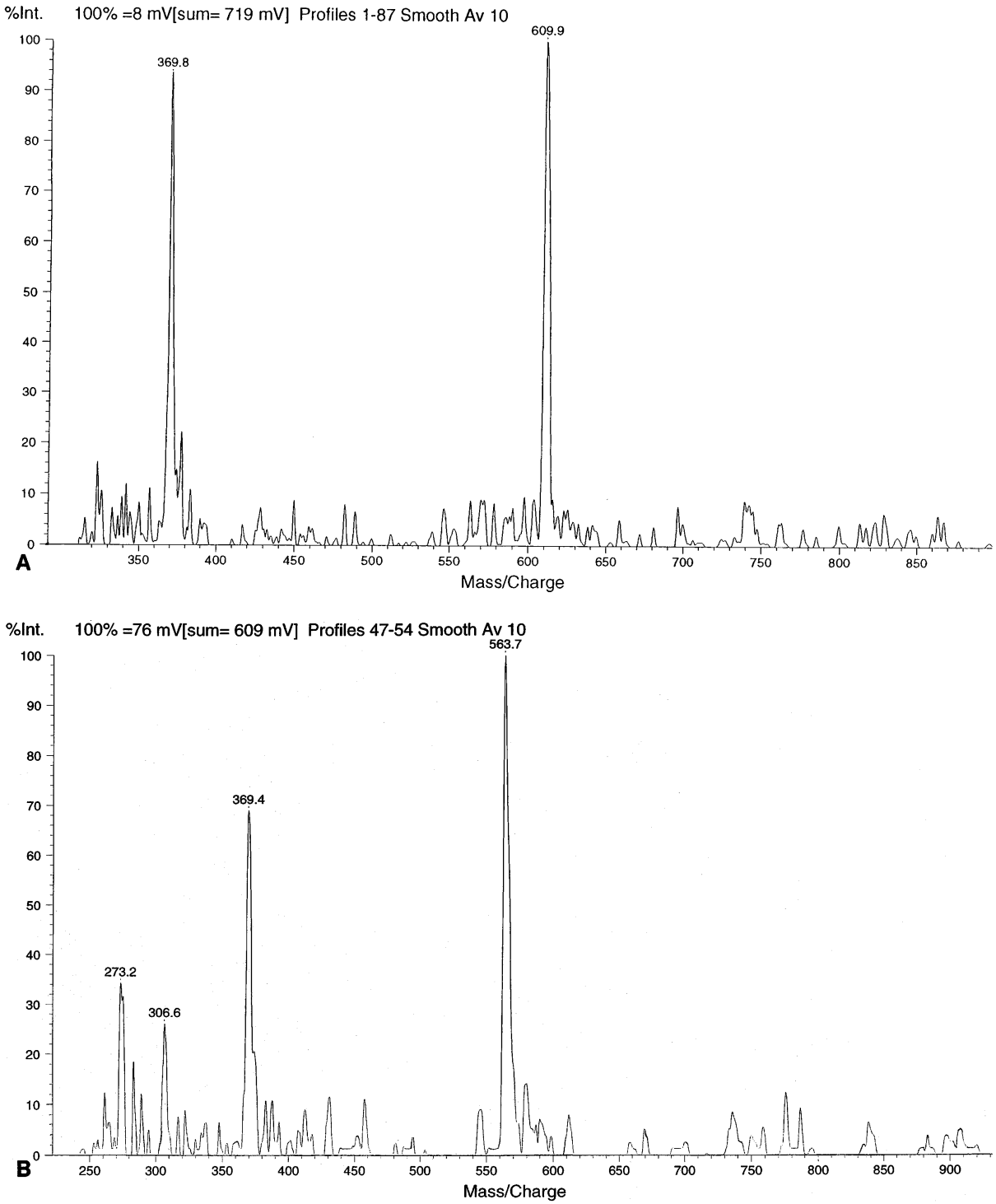

Fig. 3. MALDI/TOF analysis of 9,9-dimethoxynonanoyl cholesterol and 9-oxononanoyl cholesterol. Approximatly 50 ng of 9,9-dimethoxynonanoyl cholesterol or 9-oxononanoyl cholesterol was embedded into a DHB matrix. The beam of a nitrogen-laser was focused on the sample at a diameter of 100 μm. A: The peak at *m/z* 609.8 represents [9,9-dimethoxy cholesterol + Na]$^+$ and 369.8 represents [9,9-dimethoxy cholesterol–FA + H]$^+$. B: The peak at *m/z* 563.7 represents [9-oxononanoyl cholesterol + Na]$^+$ and 369.4 represents [9-oxononanoyl cholesterol–FA + H]$^+$.

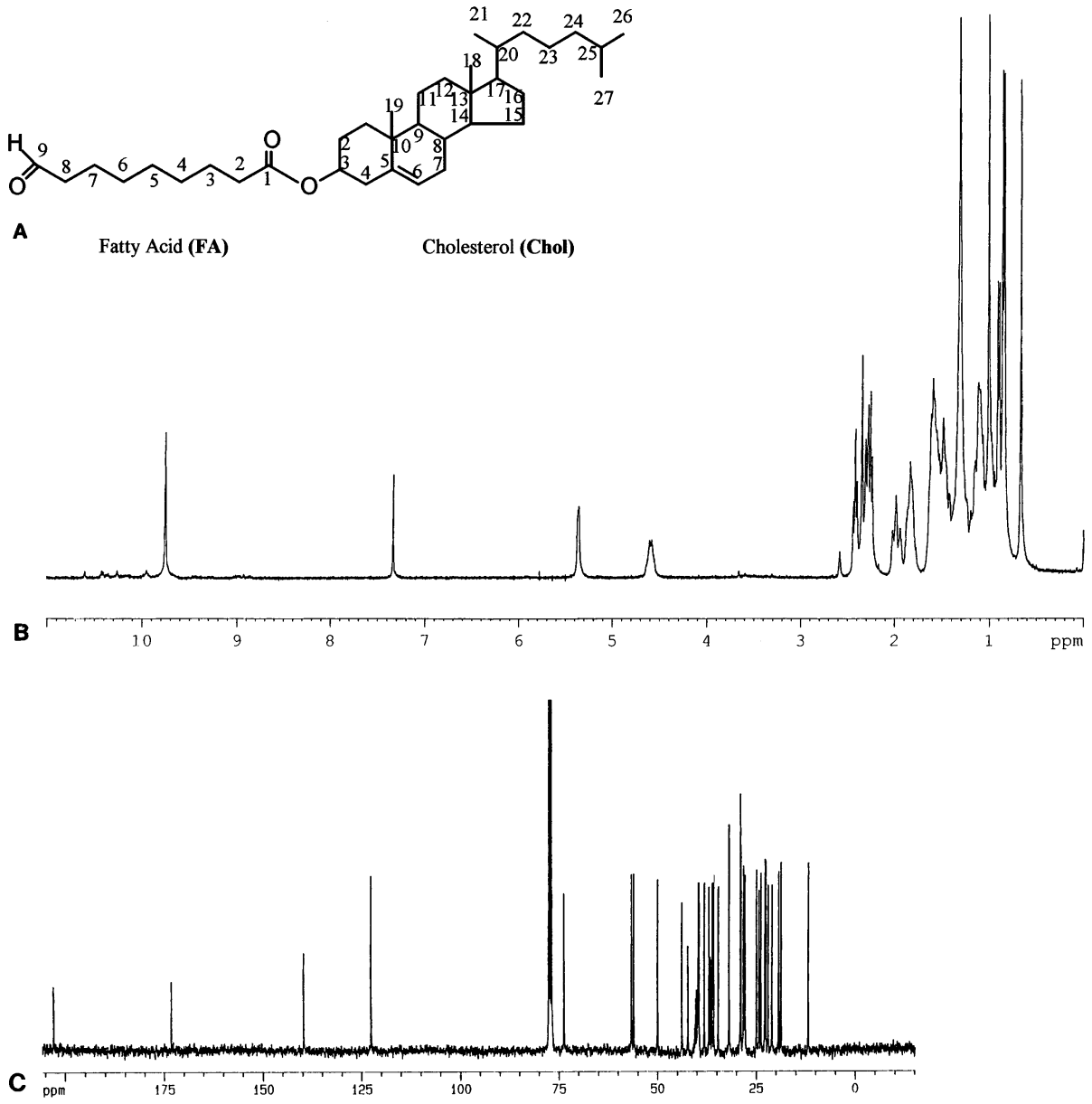

Fig. 4. ^{1}H and ^{13}C-NMR spectra of 9-oxononanoyl cholesterol were recorded as described in Materials and Methods. A: Atom assignment of 9-oxononanoyl cholesterol for ^{1}H and ^{13}C-NMR spectra. B: ^{1}H-NMR spectrum of 9-oxononanoyl cholesterol. Peak assignment: δ = 9.75 (s, 1H, CHO), 5.45 (m, 1H, Chol 6), 4.58 (m, 1H, Chol 3), 0.5–2.6 (57 H, Chol + FA). C: ^{13}C-NMR spectrum of 9-oxononanoyl cholesterol. Peak assignment: δ = 202.82 (FA 9), 173.28 (FA 1), 139.68 (Chol 5), 122.57 (Chol 6), 73.71 (Chol 3), 35.76 (FA 2), 28.93 (FA 7), 28.84 (FA 4,5,6), 28.21 (FA 4,5,6), 27.98 (FA 8), 24.91 (FA 3).

another flask with 1 ml of dichloromethane. Both mixtures were combined and stirred well for 24 h at room temperature. Dicyclohexylurea precipitation indicated the start of the reaction. For purification, the reaction mixture was filtered in the cold and treated with 10 ml dichloromethane, washed with 25 ml 0.5 N HCl, 25 ml saturated $NaHCO_3$ and 25 ml distilled water. Afterwards the organic layer was dried over sodium sulfate and filtered. Yield 50% (146.7 mg (0.250 mmol)) 9,9-dimethoxynonanoyl cholesterol (**4**) in relation to the used cholesterol (GC–MS).

The reaction product (**3a**) still containing some side products was evaporated at 30°C for 30 min and dissolved in 20 ml acetone. To this mixture three drops of water and 20 mg of Amberlyst-15 were added. The mixture was then stirred for 23 h at room temperature to cleave the acetal. Afterwards the mixture was filtered and then evaporated for 20 min at 40°C. Yield 95% (GC-MS) of 9-oxononanoyl cholesterol (**5**).

The product was further purified by column chromatography using Sephadex-LH20 on a 1.5 × 40 cm column with dichloromethane. The pure samples were first dried under nitrogen flow and then under high vacuum. Yield of 98% pure 9-oxonoananoyl cholesterol is 120 mg (0.222 mmol). Final purification was done by RP/HPLC to give a 100% pure product.

RESULTS AND DISCUSSION

Reductive ozonolysis of olefins for the preparation of aldehydes is a well known procedure (9). Due to the

double bond in cholesterol, direct ozonization of cholesteryl oleate to form **5** could not be performed. Therefore we decided to start with oleic acid (**1**), which can be ozonized under reductive conditions to form 9-oxononanoic acid (**2b**) (10). Surprisingly, we found that the ozonization in a mixture of dichloromethane and methanol and further reduction with dimethyl sulfide leads to very pure 9,9-dimethoxy nonanoic acid (**2a**) and only small amounts of 9-oxononanoic acid (**2b**). As the protected derivative of **2b**, 9,9-dimethoxy nonanoic acid (**2a**) is an ideal compound for the condensation with cholesterol, the condensation was performed with DMAP and DCC (11). 9,9-Dimethoxynonanoyl cholesterol (**4**) was obtained in 50% yield. This stable derivative can easily be hydrolyzed under mild conditions using the ion exchange resin Amberlyst-15 (12). The final product was further purified by column chromatography using Sephadex-LH20. The overall yield of the reaction for the synthesis of 9-oxononanoyl cholesterol (**5**) was 44%. The core aldehyde **5** as well as the dimethyl acetal **4** were characterized by ^{1}H- and ^{13}C-NMR spectroscopy as well as by gas chromatography with MS-detection and by MALDI-TOF spectroscopy. All data were consistent with the structures of 9-oxononanoyl cholesterol (**5**) and 9,9-dimethoxynonanoyl cholesterol (**4**), respectively. The results are shown in **Fig. 2, Fig. 3,** and **Fig. 4**.

The suitability of the above method for the preparation of other core aldehydes is currently under investigation.

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REFERENCES

1. Esterbauer, H., M. Dieber-Rotheneder, G. Waeg, G. Striegl, and G. Jürgens. 1990. Biochemical, structural and functional properties of oxidized low-density lipoprotein *Chem. Res. Toxicol*. **3:** 77.
2. Curzio, M., H. Esterbauer, C. Di Mauro, G. Cecchini, and M. U. Dianzani. 1986. Chemotactic activity of the lipid peroxidation product 4-hydroxynonenal and homologous hydroxyalkenals. *Biol. Chem. Hoppe-Seyler.* **367:** 321–329.
3. Waeg, G., G. Dimsity, and H. Esterbauer. 1996. Monoclonal antibodies for detection of 4-hydroxynonenal modified proteins. *Free Radical Res.* **25:** 149–160.
4. Smith, L. L. 1996. Review of progress in sterol oxidations: 1987–1995. *Lipids.* **31:** 453–487.
5. Hoppe, G., A. Ravandi, D. Herrera, A. Kuksis, and H. F. Hoff. 1997. Oxidation products of cholesteryl linoleate are resistant to hydrolysis in macrophages, form complexes with proteins, and are present in human atherosclerotic lesions. *J. Lipid Res.* **38:** 1347–1360.
6. Kamido, H., A. Kuksis, L. Marai, and J. J. Myher. 1995. Lipid ester-bound aldehydes among copper-catalyzed peroxidation products of human plasma lipoproteins. *J. Lipid Res.* **36:** 1876–1887.
7. Kamido, H., A. Kuksis, L. Marai, J. J. Myher, and H. Pang. 1992. Preparation, chromatography and mass spectrometry of cholesteryl ester and glycerolipid-bound aldehydes. *Lipids.* **27:** 645–650.
8. Ravandi, A., A. Kuksis, J. J. Myher, and L. Marai. 1995. Determination of lipid ester ozonides and core aldehydes by high-performance liquid chromatography with on-line mass spectromentry. *J. Biochem. Biophys. Methods.* **30:** 271–285.
9. Mittelbach, M. 1993. Ozonides and ozonolysis of lipids. *In* CRC Handbook of Chromatography. Analysis of Lipids. J. Sherma, K. D. Mukherjee and N. Weber, editors. CRC Press, Boca Raton, FL. 163–172.
10. Ackman, R. G., M. E. Retson, L. R. Gallay, and F. A. Vandenheuvel. 1961. Ozonolysis of unsaturated fatty acids. *Can. J. Chem.* **39:** 1956–1963.
11. Ziegler, F. E., and G. D. Berger. 1979. A mild method for the esterification of fatty acids. *Synth. Commun*. **9:** 539–543.
12. Coppola, G. M. 1984. Amberlyst-15, a superior acid catalyst for the cleavage of acetals. *Synthesis.* 1021–1023.