10-Hydroxystearic Acid – Identified after Homogenization of Tissue – Is Derived from Bacteria

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Dedicated to Professor Rolf Huisgen on the occasion of his 80th birthday

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10-Hydroxystearic acid seems to be widely distributed in nature: Bacteria generate it by hydroxylation of oleic acid, but it was found also as constituent of plants, in cancer cell cultures and in mammalian tissue homogenates. Investigation of 10-hydroxystearic acid, obtained from mammalian tissue homogenates, revealed its identity with that of bacteria. Thus not 10-hydroxystearic acid is widely distributed in nature but its producers: bacteria. When biological material is processed in aqueous media, lipases are activated, these cleave membrane phospholipids. Thus liberated oleic acid is the substrate for widespread bacteria which are introduced into the media when the work up procedure is done in not sterile surrounding. The bacteria transform then oleic acid to 10*R*-hydroxystearic acid.

Introduction

Homogenization of tissue induces lipid peroxidation of polyunsaturated fatty acids (Wills, 1966; Herold and Spiteller, 1996; Kießling and Spiteller, 1998). In this process linoleic acid is transformed to a mixture of *E,Z*-isomeres of mainly racemic 9-hydroperoxy-10,12-octadecadienoic acids and 13-hydroperoxy-9,11-octadecadienoic acids. In addition small amounts of 11-hydroperoxy-9,12-octadecadienoic acids are generated. Hydroperoxy acids are reduced readily in biological surrounding to corresponding hydroxy acids (Lindstrom and Aust, 1984). Gas chromatographic patterns of hydroxy acids, obtained after hydrogenation and appropriate derivatization, revealed not only the presence of 9-, 11- and 13-hydroxystearic acid, but also of 10- and 12-hydroxystearic acid (Lehmann *et al.*, 1995; Herold and Spiteller, 1996; Kießling and Spiteller, 1998). These compounds are not produced by lipid peroxidation processes and indeed 10-hydroxystearic acid was found as original constituent (not generated by hydrogenation) in tissue homogenates (Herold and Spiteller, 1996). We show in this communication that 10-hydroxystearic acid is generated from oleic acid by water addition in a regio- and enantioselective reaction by bacteria (Wallen *et al.*, 1962; Niehaus and Schröpfer, 1965; Schröpfer, 1966; Yang *et al.*, 1993) introduced from the surrounding.

Abbreviations: GC, gas chromatography, gas chromatogram, gas chromatograph; GC/MS, gas chromatography/mass spectrometry; MSTFA, N-methyl-N-trimethylsilyltrifluoroacetamide; MTPA ester, α-methoxy-α-trifluoromethylphenylacetate-O-ester, Mosher ester; NMR, nuclear magnetic resonance; TRIS, tris(hydroxymethyl)-aminomethane.

Experimental

Nuclear magnetic resonance (NMR) measurement

NMR spectra were recorded on a Bruker DRX 500 spectrometer at 100.3 MHz for ^{13}C, samples in D_6-benzene, chemical shifts in ppm rel. to D_6-benzene (^{13}C: δ 128 ppm).

Gas chromatography/mass spectrometry (GC/MS)

GC/MS measurements were performed on a Hewlett Packard 5890 Series II GC equipped with a fused silica column DB 5 (J & W Scientific, 30 m × 0.25 mm) connected to a Finnigan MAT 95 mass spectrometer. Electron impact mass spectra were recorded at 70 eV. Temperature program: 3 min isotherm at 50 °C, then from 50 °C to 300 °C at a rate of 3 °C/min.

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Gas chromatography (GC)

GC measurements were performed on an United Technologies Packard 438S GC equipped with a fused silica column DB 5 (J & W Scientific, 30 x 0.32 mm). Temperature program: 3 min isotherm at 80 °C, then from 80 °C to 300 °C at a rate of 3 °C/min and finally 15 min isotherm. The temperature of injector and flame ionization detector were kept at 270 °C and 290 °C.

Internal standard

Internal standard for GC/MS was methyl-6-hydroxyheptadecanoate. It was prepared according to Jira (1998), by sodium borohydride reduction of methyl-6-oxoheptadecanoate. 6-oxoheptadecanoic acid was synthesized by reaction of 1-morpholinocyclopentene with lauryl chloride.

Addition of water to oleic acid and 9,10-dideutero-oleic acid in porcine liver homogenates

A porcine liver was removed immediately after slaughtering and transferred in an ice box to the laboratory. 30 minutes after slaughtering the liver was cut (under cooling with ice) in small pieces. A sample (18 g) was homogenized in 100 ml TRIS (tris(hydroxymethyl)aminomethane) buffer (pH 7.4) with a mixer (Ultra Turrax, Janke and Kunkel, Staufen, Germany).

10 µl (0.35 mmol) oleic acid (Fluka, Neu Ulm, Germany, purity 99%) were emulsified in 2 ml 0.1 M TRIS buffer (pH 7.4) by sonification (Sonorex rapid, Bandelin electronic, Berlin, Germany). 8 ml liver homogenate (28 mg protein/ml) were added to the oleic acid suspension and stirred at 35 °C. After 2, 4, 8, 24 and 48 hours of stirring 1.5 ml aliquot samples were withdrawn. The removed samples were acidified to pH 2 by dropwise addition of 1 N HCl. 15 µg (52.4 nmol) of 6-hydroxyheptadecanoic acid were added as internal standard. Then the homogenate was extracted according to Bligh and Dyer (1959).

The organic solvent was removed (rotatory evaporator). The residue was dissolved in 2 ml methanol:diethyl ether 1:1 (v:v) and treated for 1 min with an etheral solution of diazomethane. Diazomethane was blown off, the remaining methanol was removed by rotatory evaporation. The residue was brought onto a column filled with 5 g silica gel 60 (0.063–0.2 mm, Merck, Darmstadt, Germany) to remove non polar methyl esters of fatty acids and triglycerides by rinsing the column with 150 ml cyclohexane:ethylacetate 98:2 (v:v). In order to obtain the methyl esters of hydroxy fatty acids the column was then eluted with 150 ml ethylacetate.

Solvent was removed as described above. The residue was taken up in dried ethylacetate. Aliquots were removed and trimethylsilylated with N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA, Macherey & Nagel, Düren, Germany). The solution was kept 1 h at 40 °C in a thermomixer (Eppendorf, Hamburg, Germany) to prepare samples for GC/MS measurements.

The same procedure was repeated by adding 9,10-dideutero-oleic acid (Promochem, Wesel, Germany, 98%) to the homogenate instead of oleic acid.

Control experiments

A sample of oleic acid resp. 9,10-dideutero-oleic acid was subjected to workup as described above, but without addition of liver homogenate. 10-Hydroxystearic acid was not detectable.

In a series of other experiments 1.2 g deep frozen porcine liver (liquid nitrogen) was pulverized and added to a solution of 10 µl oleic acid in 10 ml TRIS buffer (0.1 M, pH 7.4). The content of thus formed 10-hydroxystearic acid was compared to equally prepared solutions, which were supplied with different amounts of sodium azide (concentrations: 1 mM, 10 mM and 100 mM) in order to prevent bacterial growth. No effect was observed in the 1 mM solution, but higher concentrations of sodium azide inhibited generation of 10-hydroxystearic acid. In another series of experiments a mixture of penicillin, streptomycin and neomycin was added in different amounts (concentrations: 100 U/ml penicillin to 400 U/ml and 100 µg/ml – 400 µg/ml of streptomycin and neomycin). In all samples generation of 10-hydroxystearic acid was inhibited.

Proof for occurrence of 10-hydroxystearic acid in free form

Aliquots removed after 4 h and 24 h of homogenation time were extracted according to Bligh and Dyer (1959). The extract was divided: One

half was worked up exactly as described above to analyze hydroxy acids, the other half was dissolved in 3 ml tetrahydrofuran and treated for 1.5 h with 10 ml of a 1.4 M methanolic $NaOCH_3$ solution. Then 5 ml of a 2.1 N methanolic HCl solution were added for acidification. The residue obtained after rotatory evaporation was dissolved in 50 ml water. The aqueous solution was extracted 3 times with dichloromethane, the dichloromethane extracts were washed with 50 ml of water each. The combined organic layers were investigated as described above for hydroxystearic acids. Both halfs contained identical amounts of 10-hydroxystearic acid.

Isolation of 10-hydroxystearic acid for NMR measurement

15 g porcine liver – immediately removed after slaughtery – were frozen with liquid nitrogen and pulverized. The powder was added to an emulsion of 300 mg oleic acid in 70 ml of a 0.1 M TRIS buffer solution (pH 7.4). The solution was stirred for 24 h at 35 °C. Fatty acids were then extracted according to Bligh and Dyer (1959). After removal of the organic solvent the residue (2 g) was dissolved in a mixture of methanol:diethyl ether 1:1 (v:v), methyl esters were prepared by addition of CH_2N_2. After removal of solvent the residue was separated on a silica gel column (25 g). Non-polar compounds were eluted from the column with 750 ml cyclohexane:ethylacetate 98:2 (v:v), a fraction containing methyl-10-hydroxystearate was obtained by rinsing the column with 750 ml ethylacetate. The solvent was removed and the residue (250 mg) was separated on 8 thin layer plates (20 cm × 20 cm, RP 18, F_{254S} Merck, Darmstadt, Germany; solvent: methanol p.a.). The fraction containing the methyl-10-hydroxystearate (Rf = 0.49-0.53) was scraped off and extracted with methanol (yield 36 mg).

Final purification of the fraction containing methyl-10-hydroxystearate was achieved on silica gel plates which were developed with cyclohexane:ethylacetate 3:1 (v:v). The fraction with Rf 0.41–0.52 was scraped off and eluted with ethylacetate. Yield: 25 mg of pure methyl-10-hydroxystearate.

Synthesis of racemic methyl-10-hydroxystearate from methyl-10-oxostearate

250 mg (0.8 mmol) methyl-10-oxostearate (Aldrich, Steinheim, Germany, purity 97%), were added to a solution of 75 mg (2 mmol) $NaBH_4$ (Merck, Darmstadt, Germany, purity 96%), dissolved in 4 ml methanol. This solution was stirred for 20 h at room temperature. After acidification to pH 5 with 1 N HCl 30 ml water were added. Methyl-10-hydroxystearate was extracted 3 times with 40 ml $CHCl_3$. The combined organic layers were washed with 0.88% KCl (w:v) and brought to dryness. Purification of the residue was achieved by thin layer chromatography on 10 home-made silica gel plates (20 cm × 20 cm). The plates were developed as described above. Yield: 120 mg of a racemic mixture of methyl-10-hydroxystearate.

α-Methoxy-α-trifluoromethylphenylacetate-O-ester (MTPA ester) of methyl-10-hydroxyoctadecanoate

The MTPA ester of methyl-10-hydroxystearate was prepared as described by Dale and Mosher (1973) by reacting with α-methoxy-α-trifluoromethylphenylacetyl chloride.

^{13}C NMR data of racemic methyl-10-hydroxyoctadecanoate

14.34, 14.35 (C-18); 23.06 (C-17); 25.12, 25.18, 25.55, 25.61 (C-10, C-14); 25.19 (C-3); 29.33, 29.34, 29.44, 29.48, 29.56, 29.56, 29.58, 29.59, 29.61, 29.63, 29.70, 29.76, 29.80, 29.83 (C-4, C-5, C-6, C-7, C-13, C-14, C-15); 32.20, 32.22 (C-16); 33.79, 33.81, 34.06, 34.09 (C-9, C-11); 34.03, 34.04 (C-2); 50.93 (C-1-OCH_3); 55.38 (C-2′-OCH_3); 77.26 (C-10); 85.02 (q, 27 Hz, C-2′); 124.36 (q, 287 Hz, C-3′); 127.82, 128.49, 129.60 (C-2″, 6″, C-3″,5″, C-4″); 133.22 (C-1″); 166.43 (C-1′); 173.34 (C-1).

^{13}C NMR data of methyl-10R-hydroxyoctadecanoate

14.35 (C-18); 23.06 (C-17); 25.19, 25.55 (C-10, C-14); 25.19 (C-3); 29.33, 29.48, 29.58, 29.59, 29.63, 29.70, 29.80 (C-4, C-5, C-6, C-7, C-13, C-14, C-15); 32.21 (C-16); 33.82, 34.06 (C-9, C-11); 34.03 (C-2); 50.93 (C-1-OCH_3); 55.38 (C-2′-OCH_3); 77.26 (C-10); 85.02 (q, 27 Hz, C-2′); 124.36 (q, 287 Hz, C-

3′); 127.82, 128.52, 129.60 (C-2″,6″, C-3″,5″, C-4″); 133.22 (C-1″); 166.44 (C-1′); 173.34 (C-1).

Results

In a first series of experiments, oleic acid was added to a homogenate of liver and samples were withdrawn in time intervals to recognize the increase in production of 10-hydroxystearic acid with time. The lipid fraction was extracted from the withdrawn samples according to Bligh and Dyer (1959). Free fatty acids were then methylated. Methyl esters of fatty acids are separable from more polar methyl esters of hydroxy fatty acids by chromatography on silica gel. The eluted polar methyl esters of fatty acids were transformed by addition of MSTFA to corresponding trimethylsilylated methyl esters which were subjected to GC/MS. The GC showed besides a peak indicating the presence of trimethylsilylated cholesterol an intense peak, identified by its mass spectrum to be the trimethylsilylderivative of methyl-10-hydroxystearate (Fig. 1).

A steady increase of this GC-peak was observed with time. 24 hours after addition of 10 µl oleic acid to a liver homogenate (corresponding to 224 mg protein) about 0.9 mg 10-hydroxystearic acid were identified.

In order to prove that 10-hydroxystearic acid was generated from oleic acid 9,10-dideutero-oleic acid was added to a liver homogenate in a second experiment.

The resulting 10-hydroxystearic acid was shown after appropriate derivatization by GC/MS to contain two deuterium atoms (Fig. 2). The mass spectrum allowed location of the deuterium atoms since the α-fragment of mass 215 in the unlabeled compound was shifted for 1 mu to *m/z* 216 in the labeled one. The alternative α-cleavage product shifted from *m/z* 273 to *m/z* 275 (Fig. 2). This proves water addition to oleic acid in a regio selective reaction. Unfortunately, enantiomeric saturated hydroxy fatty acids show nearly negligible optical activity (Schröpfer and Bloch, 1965) which considerably complicates determination of the configuration of the 10-hydroxystearic acid. Therefore El-Sharkawy *et al.* (1992) and Yang *et al.* (1993) transformed the hydroxy acid to O-acetylmandelate and deduced the stereochemistry by ^{1}H NMR spectra. For this purpose we used the ^{13}C NMR spectra of the MTPA ester (Dale and Mosher, 1973): The latter was prepared by reacting methyl-10-hydroxystearate with α-methoxy-α-trifluoromethylphenylacetyl chloride. The ^{13}C NMR spectrum of the Mosher ester obtained from methyl-10-hydroxystearate derived from oleic acid after incubation with porcine liver homogenate was compared with that of a racemic methyl-10-hydroxystearate prepared by $NaBH_4$ reduction of methyl-10-oxostearate. The ^{13}C NMR spectrum of the racemic mixture showed in the region between 33.5 to 34.5 ppm six signals, while the NMR spectrum of methyl-10-hydroxystearate derived

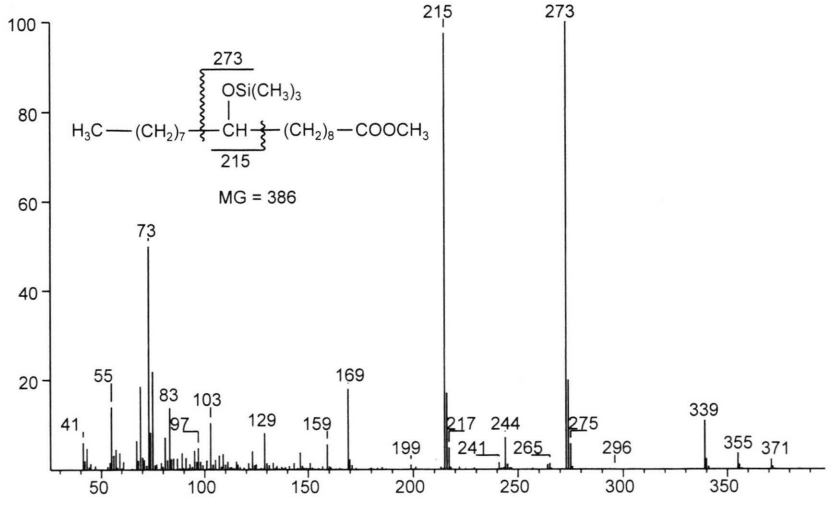

Fig. 1. Mass spectrum of trimethylsilylated methyl-10-hydroxystearate.

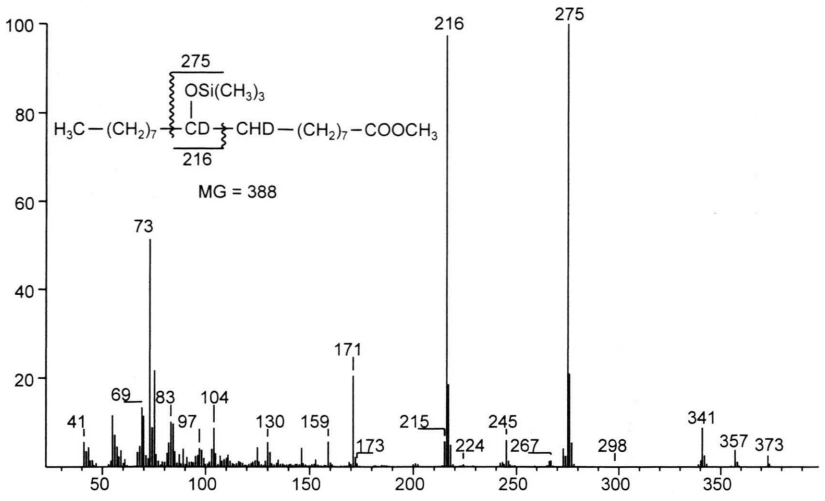

Fig. 2. Mass spectrum of the trimethylsilylated methyl-10-hydroxy-9,10-dideuterostearate.

from oleic acid incubated with a liver homogenate had peaks only at 33.83, 34.02 and 34.06 ppm, thus proving its enantiomeric character.

10*R*-Hydroxystearic acid was then prepared by incubation of oleic acid with bakers yeast (we observed that "bakers yeast" is not always able to generate 10*R*-hydroxystearic acid by addition of oleic acid (El-Sharkawy *et al.*, 1992), presumably its microorganism content is variable). 10*R*-Hydroxystearic acid was transformed into its MTPA ester (Dale and Mosher, 1973). Its ^{13}C NMR spectrum proved identical with the ^{13}C NMR spectrum of the MTPA ester derived from 10-hydroxystearic acid obtained by addition of oleic acid to a porcine liver homogenate. Thus presuming that 10-hydroxystearic acid obtained from yeast is *R* configurated, 10-hydroxystearic acid detected in tissue homogenates is also *R* configurated.

Apparently, the enzymes which perform this reaction require free oleic acid, because we were unable to detect increased amounts of 10-hydroxystearic acid after tissue saponification compared to tissue samples not saponified.

Since 10-hydroxystearic acid detected in mammalian tissue homogenate is *R* configurated likewise as 10-hydroxystearic acid isolated from bacteria (Niehaus and Schröpfer, 1965) we considered the possibility, that the compound might be produced by action of bacteria during work-up of tissue in a non-sterile surrounding. Therefore immediately after homogenation of liver tissue, either sodium azide resp. antibiotics, preventing bacterial growth, were added in different concentrations. Generation of 10-hydroxystearic acid and bacterial growth was observed only in the sample with the lowest content of sodium azide. In another experiment a liver homogenate was heated to 100 °C to destroy the enzymes. In spite of that after addition of oleic acid and after stirring in air for 24 h 10-hydroxystearic acid was identified. We conclude that 10*R*-hydroxystearic acid is generated by bacteria, which seem to be present everywhere.

Discussion

10*R*-hydroxystearic acid is a well known water addition product to oleic acid generated by bacteria (Wallen *et al.*, 1962), especially pseudomonades (Niehaus and Schröpfer, 1965; Wallen *et al.*, 1962). Schröpfer has investigated this reaction in detail and determined the stereochemistry of the product (Schröpfer and Bloch, 1965).

10-Hydroxystearic acid was detected also in lipid extracts of cell cultures derived from Lewis lung carcinoma in vitro line C 108 (Cavalli *et al.*, 1991). This observation led to the speculation that 10-hydroxystearic acid is involved in cell growth (Cavalli *et al.*, 1991). 10-Hydroxystearic acid was also detected in dead bodies under water (Tomita, 1984) and after processing of plants in cuticula membranes of *Rosmarinus officinalis L* (Brieskorn and Kabelitz, 1971).

All these experiments were not done in sterile surrounding. Considering our results it seems very

probably that 10-hydroxystearic acid has been also generated in the above described cases by bacteria.

Bacteria can use only free oleic acid as substrate. Free oleic acid is present in healthy tissue only in traces, if at all, but it is abundant in conjugates:

In the course of cell degradation processes of plants (Galliard, 1975) and mammalians (Cheeseman, 1993) lipases are activated. Action of lipases is induced by all work up procedures of tissue in aqueous surrounding. A very severe type of cell damage is homogenization. Homogenization of tissue is usually done in aqueous solution. Due to the action of lipases oleic acid, the substrate for bacteria, is produced by homogenization. If the homogenized mixtures are not worked up instantly the apparently everywhere existing bacteria start working and produce 10*R*-hydroxystearic acid.

As a consequence all procedures connected with homogenization of plant or mammalian tissue should be carried out in sterile surrounding or should be done in organic solvents (this does not perfectly exclude activation of enzyme activity since tissue contains always considerable amounts of water). The obtained homogenates should be processed as fast as possible, in order to avoid too excessive action of bacteria.

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