Structure Elucidation of Ozonated Olive Oil

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Abstract

Ozonated olive oil was prepared by bubbling ozone-oxygen gas through olive oil until it solidified. ^{1}H- and ^{13}C-NMR spectra of the ozonated oil showed that all C-C double bonds in triacyglycerols were converted to ozonide. The spectra also showed no other structural change and the absence of degraded products of ozonide such as aldehydes and carboxylic acids. HPLC chromatogram of the oil showed three main peaks, two of which were due to ozonides derived from triolein, the major triacylglycerol (45%) in olive oil. These results indicate that main components of the ozonated olive oil are triolein triozonides.

Introduction

Ozonated (ozonized) olive oil is prepared by bubbling ozone-oxygen gas through pure olive oil until it solidifies. In European countries the ozonated olive oil has been applied topically to a variety of cutaneous diseases for disinfecting the lesions and promoting their healing (1). Though little attention has been paid to the therapy with ozonated olive oil in Japan, we have recently demonstrated that the ozonated oil is beneficial to the patients with intractable fistulae or wounds. The ozonated olive oil is supposed to contain ozonides and/or peroxides (2, 3) but no definitive experimental evidences for their structures have appeared in the scientific literatures. For the medical use of the ozonated olive oil, its quality standardization is of great importance. In this paper, we measured spectral data of the ozonated olive oil in order to elucidate the structure of its main component. The stability of the ozonated oil was also investigated by a high-performance liquid chromatography (HPLC).

Experimental

Materials

Olive oil (Japanese Pharmacopoeial drug), triolein and trilinolein were obtained from Wako Pure Chemical Industries Ltd.,(Osaka, Japan). Other authentic standards of triacylglycerols such as LLO, LLP, POL, OPO, OOP and OOS (O: oleic acid, L: linoleic acid, P: palmitic acid, S: stearic acid) were purchased from Sigma Chemical Co.(St.Louis, MO, USA). HPLC-grade 2-propanol and acetonitrile were from Kanto Chemical (Tokyo, Japan). Milli-Q (Nihon

Millipore Kogyo, Yonezawa, Japan) water was used in the preparation of mobile phase. All other reagents used were of analytical grade.

Equipment

^{1}H- and ^{13}C-nuclear magnetic resonance (NMR) spectra were recorded on a JEOL JNM-EX400 spectrometer at 400 and 100 MHz, respectively, in $CDCl_3$ with tetramethylsilane as an internal standard. Infrared (IR) spectra were taken on a JASCO FT-IR-8900 spectrophotometer. Mass spectra and absorption spectra were measured on a JEOL JMS-700TZ and Shimadzu UV-260 spectrophotometer, respectively. HPLC system (Shimadzu, Kyoto, Japan) consisted of an LC-10ADvp pump, a DGU-12A degasser, a Rheodyne Model 7725 sample injector (Rheodyne, Cotati, CA, USA), a CTO-10A column oven, SPD-10AD absorbance detector and C-R5A data processor. For the generation of ozone Nippon Ozone Model OT-31ST-M1 and Model Me-Zone were used.

Preparation of ozonated olive oil

Olive oil (190 g) was warmed in water bath at 30°C. Oxygen gas containing 10 ppm ozone was then bubbled through the olive oil at a rate of 1.0 L/min for 50 hr to give ozonated olive oil as vaselline with the distinctive odor of ozone. The ozonated olive oil was stored in a refrigerator.

HPLC analyses of olive oil and ozonated olive oil

HPLC separation was performed at 40°C with a Develosil ODS UG-3 column (100 × 4.6 mm i.d., 3 μm, Nomura Chemicals, Seto, Japan) as an analytical column which was protected by a guard-pak cartridge column (Develosil ODS UG-5, 10 × 4.0 mm i.d., 5 μm). As mobile phase, a 1:2 mixture of 2-propanol and acetonitrile was used at 1.2 mL/min for olive oil and 84% aqueous acetonitrile at 1.0 mL/min for the ozonated olive oil. The olive oil and authentic triacylglycerols were dissolved in $CHCl_3$ or CH_2Cl_2 (0.036% solution) and the ozonated olive oil in a 1:2 mixture of $CHCl_3$ and acetonitrile (0.63% solution). Twenty microliter of each the solution was injected onto the HPLC column and the eluate was monitored at 210 nm.

Results and discussion

Olive oil is a mixture of triacylglycerols (triglycerides) and contains unsatsurated fatty acid residue such as oleic acid and linoleic acid, the contents of which are known to be 75-85% and 6-10%, respectively (2). In our experiments for preparation of ozonated olive oil, about 160 mg of ozone was absorbed per 1.0 g of olive oil which seemed to be enough to react with all C-C double bonds in the unsaturated fatty acid residues.

Figure 1 shows ^{13}C-NMR spectra of the olive oil and the ozonated olive oil. In comparison with ^{13}C-NMR spectra of authentic triolein and trilinolein, large signals at 130 ppm was assigned to the olefinic carbon of oleic acid whereas small signals at 128 ppm were due to those of linoleic acid. Ozonation of the olive oil resulted in the complete disappearance of the olefinic carbon signals with the appearance of new signals around 105 ppm. In accordance with this change, two sets of triplet proton signal appeared at 5.14 and 5.18 ppm, respectively, with the complete disappearance of olefinic proton signals around 5.35 ppm in ^{1}H-NMR spectrum of the ozonated olive oil. A Criegee ozonide of methyl oleate was reported to show

the ring carbon signals at about 104 ppm and two triplet signals at 5.13 and 5.18 ppm for the ozonide ring protons of the cis and trans isomers, respectively (4). In addition, the ozonated olive oil showed no absorption above 210 nm in its UV spectrum but showed the absorption at 1110 cm^{-1} in its IR spectrum, which was assignable to O-C-O bond of the ozonide ring (4). These spectral data clearly indicated that all C-C double bonds of unsaturated fatty acid residues in the olive oil were converted to a Criegee ozonide. Since ^{1}H- and ^{13}C-NMR spectra of the ozonated olive oil showed no other structural change in triacylglycerol and the absence of the decomposed product such as aldehydes and carboxylic acids. Therefore, it is concluded that the ozonated olive oil is a mixture of triacylglycerol ozonides.

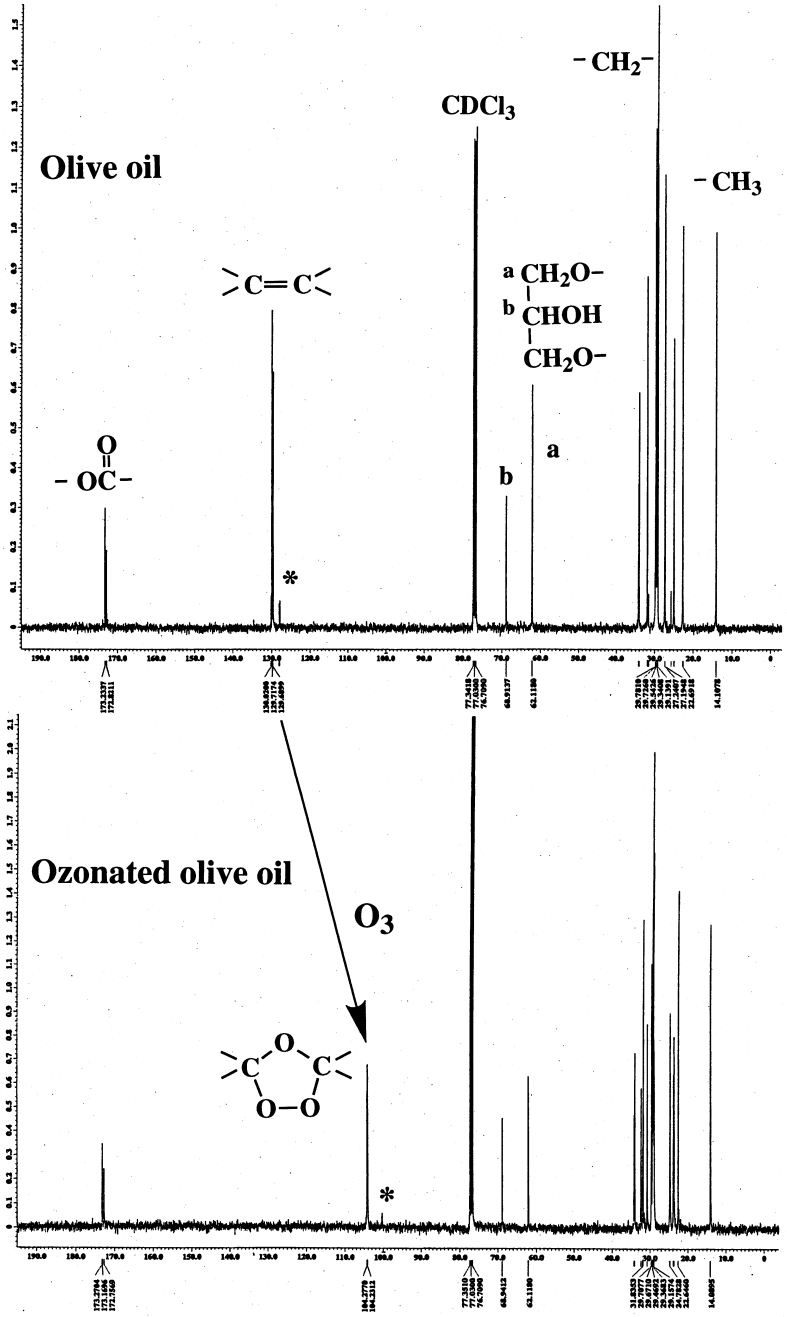

Figure 1. ^{13}C-NMR spectra of the olive oil and the ozonated olive oil.
* Signals due to linoleic acid

Figure 2 shows HPLC chromatograms of olive oil and the ozonated olive oil. With the mobile phase of a 1:2 mixture of 2-propanol and acetonitrile, triolein (OOO) appeared at 23.2 min (peak 5) and comprised about 45% of total triacylglycerols in the olive oil.

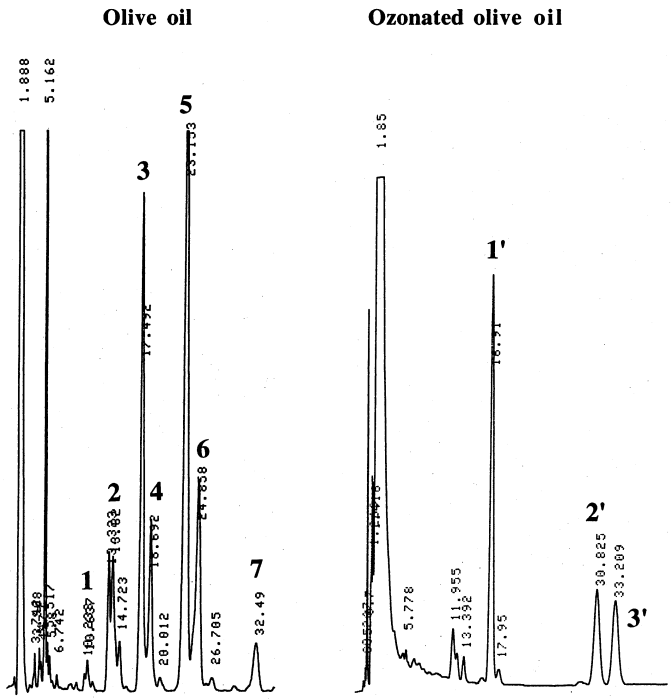

Figure 2. HPLC chromatograms of the olive oil and the ozonated olive oil Mobile phase were a 1:2 mixture of 2-propanol and acetonitrile at 1.2 mL/min for olive oil and 84% aqueous acetonitrile at 1.0 mL/min for the ozonated olive oil.

Compared with the retention times of authentic triacylglycerols, other peaks of 1, 2, 4, 6 and 7 were tentatively assigned to trilinolein (LLL), LLP, POL, OPO (or OOP) and OOS, respectively. However, the sum of these triacylglycerols was less than 20%. Though peak 3 was not assigned because of lack of authentic standard, it was assumed to be due to LOO or OLO from its retention time.

Upon ozonation of the olive oil, all the peaks due to triacylglycerols disappeared whereas the three main peaks were detected in the HPLC chromatogram with a more polar mobile phase of 84% aqueous acetonitrile. The peak 2' and 3' were also seen in the HPLC chromatogram of triolein triozonides prepared by ozonation of triolein. Therefore, it can be concluded that the main components (about 45%) in the ozonated olive oil is triolein triozonide (Fig. 3). Because both cis- and trans isomers of ozonide are present in the ozonated olive oil as described above, there is the possibility that the ozonated olive oil contains six isomers of triolein triozonide. Thus, the peak 2' and 3' are probably due to the isomeric mixture of triolein triozonide. The same study was also conducted on the ozonated safflower oil to give similar results.

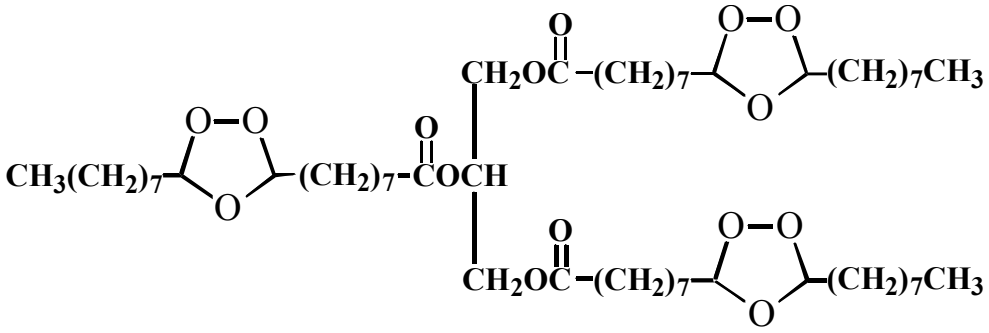

Figure 3 The structure of triolein triozonide

The ozonated olive oil has been presumed to be stable because its bactericidal effect was reported to decrease only to a low extent when stored for 8 years (5). The stability of the ozonated olive oil was also investigated by iodometric titration (2). However, little is known on the stabilities of triacylglycerol ozonides. Therefore, we examined their stabilities by using the HPLC method described above. The thermostabilities of triolein triozonides (peak 2' and 3' in Fig.2) in the ozonated olive oil were found to be relatively high since the extent of their decomposition were less than 5% upon heating at 50°C for 30 hr in cyclohexane. However, the contents of the triozonides decreased to 95-87% when the melting and freezing of the ozonated olive oil was repeated 6 times in a open tube. In a sealed tube, on the other hand, the triozonides were stable at room temperature for 3 months and in a refrigerator for at least 2 years.

References

1. Viebahn, R. "*The Use of Ozone in Medicine*", 2 nd revised edition (English), Heidelberg, Karl F. Haug Publishers (1994).
2. Cronheim, G. "Organic Ozonides as Chemotherapeutic Agents. I. Chemical Studies", J. Am. Pharm. Assoc., 36: 274-278 (1947).
3. Rainbauer, H., Washüttl, J., Steiner, I., Kroyer, G., Winker, N., Streichsbier, F. "Chemische Untersuchungen an Ozonisiertem Olivenöl", Fette. Seifen. Anstrichmittel., 84 (5): 188-191 (1982).
4. Wu, M., Church, D. F., Mahier, T. J., Barker, S. A., Pryor, W. A. "Separation and Spectral Data of the Six Isomeric Ozonides from Methyl Oleate", Lipids, 27 (2): 129-135 (1992).
5. Streichsbier, F., Rainbauer, H., Washüttl, J., Kroyer, G., Steiner, I., Winker, N., "Mikrobiologische Untersuchungen an Ozonisiertem Olivennöl", Fette. Seifen. Anstrichmittel., 84 (8): 304-308 (1982).