Vol. 118, No. 3, 1984 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 14, 1984 Pages 943-949

TRIHYDROXYTETRAENES: A NOVEL SERIES OF COMPOUNDS FORMED FROM ARACHIDONIC ACID IN HUMAN LEUKOCYTES

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Received January 6, 1984

Summary: Addition of 15L-hydroperoxy-5,8,11,13-eicosatetraenoic acid (15-HPETE) to human leukocytes led to the formation of a novel series of compounds containing four conjugated double bonds. The yield of tetraenes was increased approx. 100-fold when ionophore A23187 (5 μM) was added simultaneously with 15-HPETE. The structure of the major tetraene was established by physical methods as well as by chemical degradation and found to be 5,6,15L-trihydroxy-7,9,11,13-eicosatetraenoic acid.

When stimulated, a large number of cells liberate arachidonic acid from its esterified forms (for review, see ref. 1). Non-esterified arachidonic acid may be oxygenated by action of either fatty acid cyclooxygenase or lipoxygenases. The lipoxygenases of mammalian tissue, i.e. the 5-, 12- and 15-lipoxygenases (2-4), transform arachidonic acid into a number of biologically active derivatives. For example, arachidonic acid 5-lipoxygenase catalyzes the first step in the formation of leukotrienes (for review, see ref. 5). Leukotrienes, particularly leukotrienes B_4, C_4 and D_4, are believed to play a mediator role in eg. immediate hypersensitivity reactions and in inflammation (5).

In view of the potential biological importance of lipoxygenase products we have studied the interaction of two major lipoxygenase pathways and examined the products formed. In the present paper we describe a new series of oxygenated derivatives of arachidonic acid containing a conjugated tetraene structure as a characteristic feature and report the structure of the major compound.

The abbreviations used are: 15-HPETE, 15L-hydroperoxy-5,8,11,13-eicosatetraenoic acid; 5,15-DHETE, 5,15-dihydroxy-6,8,11,13-eicosatetraenoic acid; 8,15-DHETE, 8,15-dihydroxy-5,9,11,13-eicosatetraenoic acid; 14,15-DHETE, 14,15-dihydroxy-5,8,10,12-eicosatetraenoic acid; TLC, thin layer chromatography; RP-HPLC, reversed phase high performance liquid chromatography; Me_3Si, trimethylsilyl.

0006-291X/84 $1.50

MATERIALS AND METHODS

Arachidonic acid was purchased from Nu-Chek-Prep., Elysian, Minn. 15-HPETE[1] was obtained by incubation of arachidonic acid with soybean lipoxygenase (6). Reference compounds for oxidative ozonolysis were prepared as previously described (2,7).

Cell Preparation and Incubation Conditions

Human leukocytes from peripheral blood were prepared as in (8). Neutrophils accounted for more than 90% of the leukocytes present as determined by Giemsa stain and light microscopy. Cells were washed and suspended in a buffered salt solution (138 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 1.0 mM $MgCl_2$ and 0.6 mM $CaCl_2$; pH 7.45) at 100×10^6 cells/ml. Leukocytes (100-500 ml suspension) were preincubated at 37° for 5 min with slow stirring. Subsequently, either 15-HPETE (100 µM) or 15-HPETE (100 µM) plus A23187 (5 µM) were added in ethanol (final concentration, lower than 0.1%, v/v) and the mixture was stirred for 30-40 min at 37°. Incubations were stopped by addition of 2 volumes of methanol.

Extraction and Purification

Material obtained by extraction with diethyl ether was subjected to silicic acid chromatography (2,4). The ethyl acetate eluate was taken to dryness and the residue mixed with approx. 25 nCi of each [1-^{14}C]11,12,15-trihydroxy-5,8,13-eicosatrienoic acid and [1-^{14}C]11,14,15-trihydroxy-5,8,12-eicosatrienoic acid (cf. ref. 9). The mixture was esterified by treatment with diazomethane and subjected to thin layer radiochromatography (solvent system, ethyl acetate - 2,2,4-trimethylpentane (5:1, v/v)). Methyl esters of trihydroxytetraenes appeared in a zone (R_F = 0.56-0.77) just above the zone containing the labeled trihydroxyeicosatrienoates. Material in the trihydroxytetraene zone was further purified by RP-HPLC (column, 500 x 10 mm Polygosil-C_{18}; solvent, methanol - water (7:3, v/v); flow rate, 3 ml/min).

Methods for Structure Determination

Catalytic hydrogenation and oxidative ozonolysis were carried out as described (10). Gas-liquid chromatographic analysis of products obtained on oxidative ozonolysis was carried out with an F&M Biomedical gaschromatograph model 402. The stationary phase was 5% QF-1 and helium was used as carrier gas. Mass spectrometry was performed with an LKB 9000S instrument equipped with a column of 1% OV-1 on Chromosorb W. The energy of the electron beam was set at 22.5 eV and the trap current was 60 µA. n-Butylboronate derivatives were prepared by treatment with 2 mg of n-butylboronic acid in 0.2 ml of acetone for 30 min at 37°.

RESULTS

Human leukocytes and platelets transform 15-HPETE into several dihydroxy acids (8,11). In order to probe possible interactions between the 5- and 15-lipoxygenase pathways we have prepared 15-HPETE and examined the products formed upon incubation with human leukocytes. Analysis by RP-HPLC of material isolated by silicic acid chromatography demonstrated the presence of 5,15-DHETE, 8,15-DHETE and 14,15-DHETE, i.e. dihydroxy-eicosatetraenoates recognized in previous work (8,11). In addition, a new series of more polar compounds having strong ultraviolet absorption at 301 nm was observed. In order to obtain the new com-

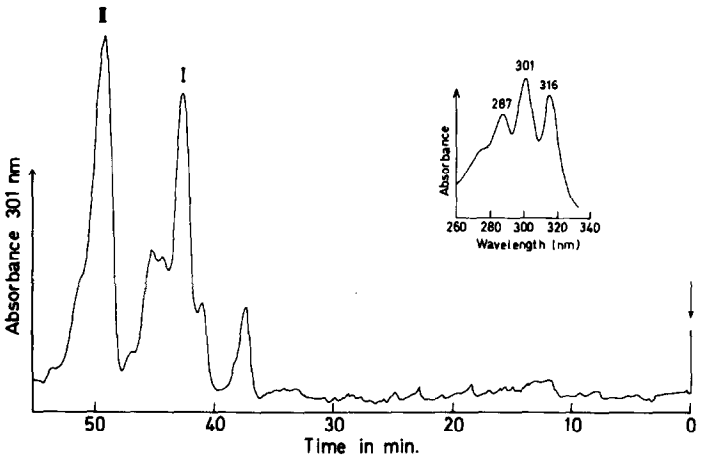

Fig. 1. RP-HPLC chromatogram of esterified material obtained following incubation of 15-HPETE plus ionophore A23187 with a suspension of human leukocytes.
Column, 500x10 mm Polygosil-C_{18}; solvent, methanol-water (7:3, v/v); flow rate, 3 ml/min. The ultraviolet detector was set at 301 nm.
Insert: Ultraviolet spectrum of Compound II. Solvent, methanol.

pounds in pure form it was necessary to include a TLC-purification step of the esterified material. When the TLC purification was omitted the new compounds were obtained heavily contaminated with 11,12,15-trihydroxy-5,8,13-eicosatrienoate and 11,14,15-trihydroxy-5,8,12-eicosatrienoate. These compounds which are well known degradation products of 15-HPETE are formed by non-enzymatic reactions (9).

Isolation of Compound II. Human leukocytes were incubated with 15-HPETE plus ionophore A23187 as described above. Material obtained following TLC was subjected to RP-HPLC. As seen in Fig. 1 several peaks of material showing strong absorption at 301 nm appeared. Material present in the major peak (Compound II) was rechromatographed and subjected to structural analysis.

Structure of Compound II. The ultraviolet spectrum of Compound II showed three main bands of intense absorption with λ_{max}^{MeOH} = 287, 301 and 316 nm (Fig. 1, insert), indicating the presence of a conjugated tetraene structure (cf. ref. 12).

An aliquot of Compound II was converted into the Me_3Si derivative and subjected to gas-liquid chromatographic - mass-spectrometric analysis. The C-value was 24.1 and the mass spectrum showed ions of high intensity at m/e 379 (M-203; loss of $\cdot CH(OSiMe_3)-(CH_2)_3-COOCH_3$), 289 (379-90; elimination of Me_3SiOH), 203 ($Me_3SiO^+=CH-(CH_2)_3-COOCH_3$), 173 ($Me_3SiO^+=CH-(CH_2)_4-CH_3$) and 171 (203-32; elimination of CH_3OH). Ions of low intensity were observed at

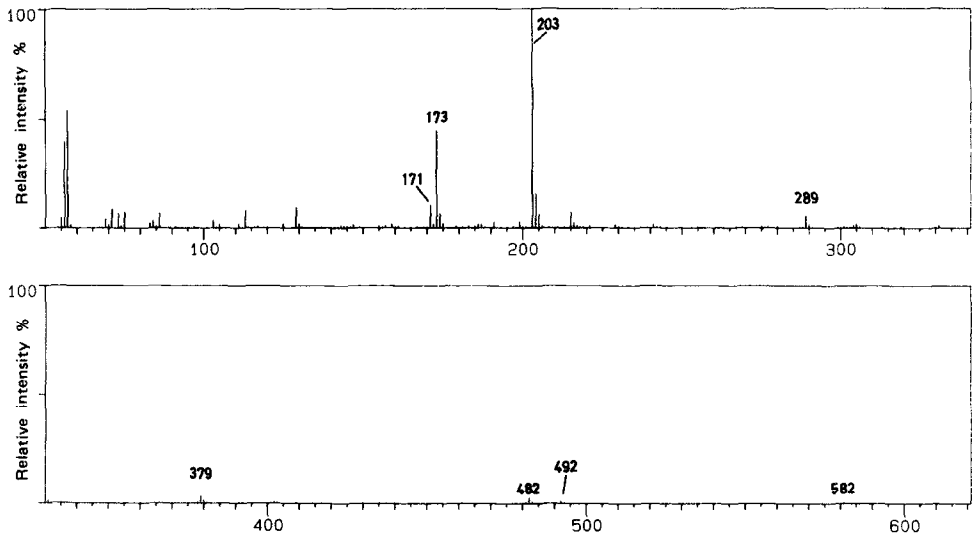

Fig. 2. Mass spectrum of the Me_3Si derivative of Compound II.

inter alia m/e 582 (M), 492 (M-90; elimination of Me_3SiOH) and 482 (M-100; rearrangement followed by elimination of $O{=}HC{-}(CH_2)_4{-}CH_3$)(Fig. 2).

We next examined the product formed upon catalytic hydrogenation of Compound II. The major peak of the gas-chromatogram (C-25.3) was due to the saturated derivative of Compound II (Me_3Si derivative). Ions of high intensity were observed at m/e 575 (M-15; loss of $\cdot CH_3$), 519 (M-71; loss of $\cdot(CH_2)_4{-}CH_3$), 490 (M-100; rearrangement followed by loss of $O{=}HC{-}(CH_2)_4{-}CH_3$), 297 (M-(203+90); loss of $\cdot CH(OSiMe_3){-}(CH_2)_3{-}COOCH_3$ plus Me_3SiOH), 276 (M-314; rearrangement followed by loss of $O{=}HC{-}(CH_2)_8{-}CH(OSiMe_3){-}(CH_2)_4{-}CH_3$), 203 (base peak; $Me_3SiO^+{=}CH{-}(CH_2)_3{-}COOCH_3$) and 173 ($Me_3SiO^+{=}CH{-}(CH_2)_4{-}CH_3$). A minor peak (C-23.4) also appeared in the gas-chromatogram of hydrogenated Compound II. The mass spectrum showed prominent ions at m/e 487 (M-15; loss of $\cdot CH_3$), 471 (M-31; loss of $\cdot OCH_3$), 299 (M-203; loss of $\cdot CH(OSiMe_3){-}(CH_2)_3{-}COOCH_3$), 276 (M-226; rearrangement followed by loss of $O{=}HC{-}(CH_2)_{13}{-}CH_3$) and 203 (base peak; $Me_3SiO^+{=}CH{-}(CH_2)_3{-}COOCH_3$). Material present in the minor peak was assigned the structure methyl 5,6-dihydroxy-eicosanoate (Me_3Si derivative) based on the C-value and the mass spectrum.

Taken together, the mass spectrometry data suggested that Compound II was a methyl eicosatetraenoate carrying hydroxyl groups at C-5, C-6 and C-15. The location of the hydroxyl groups was supported by the high intensity of the ion at m/e 203 (base peak

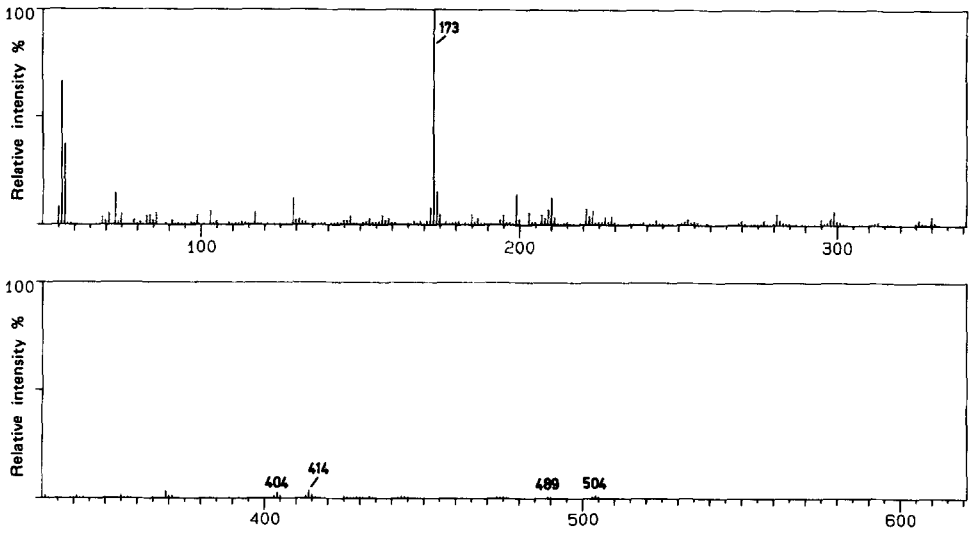

Fig. 3. Mass spectrum of the n-butylboronate-Me_3Si derivative of Compound II.

in the spectra) and by the formation of methyl 5,6-dihydroxy-eicosanoate as a hydrogenolysis product during catalytic hydrogenation of Compound II. In order to exclude alternative structures and to prove the presence of a vicinal diol structure in Compound II, an aliquot was treated with n-butylboronic acid in acetone followed by treatment with hexamethyldisilazane and trimethylchlorosilane in pyridine. This resulted in the formation of an n-butylboronate-Me_3Si derivative of Compound II (C-25.3). The mass spectrum showed a prominent ion at m/e 173 (Me_3SiO^+=CH-$(CH_2)_4$-CH_3) demonstrating that the non-vicinal hydroxyl group was located at C-15 (Fig. 3). Ions were also observed at m/e 504 (M), 489 (M-15), 414 (M-90), 404 (M-100) and 199 (possibly [CH=CH-CH($OSiMe_3$)-$(CH_2)_4$-CH_3]$^+$).

In order to assign the stereochemistry at C-15 the (-)menthoxycarbonyl derivative of Compound II (cf. ref. 10) was subjected to oxidative ozonolysis. Gas-liquid chromatographic analysis of the ozonolysis product showed the presence of the (-)menthoxycarbonyl derivative of 2L-hydroxyheptanoic acid, thus demonstrating that the configuration of the hydroxyl group at C-15 of Compound II was "L" and that one of the four double bonds of Compound II was located at Δ^{13}. The latter finding together with the ultraviolet spectrometry data which showed that the four double bonds of Compound II were conjugated (see above) demonstrated that the locations of the four double bonds were at Δ^7, Δ^9, Δ^{11}, Δ^{13}.

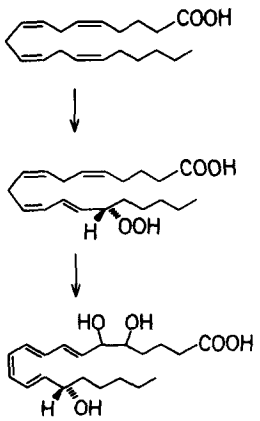

Fig. 4. Scheme of formation of 5,6,15L-trihydroxy-7,9,11,13-eicosatetraenoic acid. The geometrical configurations of the double bonds are tentative.

On basis of these results the parent acid of Compound II was assigned the structure 5,6,15L-trihydroxy-7,9,11,13-eicosatetraenoic acid.

DISCUSSION

In the present paper we describe the structure of a new compound isolated from human leukocytes, i.e. 5,6,15L-trihydroxy-7,9,11,13-eicosatetraenoic acid. The compound has four conjugated double bonds and three alcohol groups. It appears to be the major product of a new series of compounds which contain four conjugated double bonds as a distinguishing characteristic of the group (Fig. 4).

Samples of the new compound were isolated from the diethyl ether extract of incubations of human leukocytes exposed to 15-HPETE in the presence or absence of the ionophore A23187. In order to remove contaminating material (i.e. 11,12,15-trihydroxy- and 11,14,15-trihydroxy-eicosatrienoic acids) thin layer chromatography was included as an essential step in the purification scheme. The presence and positions of three hydroxyl groups as well as the presence of four conjugated double bonds were established by gas-liquid chromatographic - mass-spectrometric analysis of several derivatives. Analysis of the fragments formed upon oxidative ozonolysis of the (-)menthoxycarbonyl derivative of Compound II showed that the configuration of the hydroxyl group at C-15 was retained ("L" or 15(S)) and that one of the four double bonds was located at Δ^{13}. The latter finding taken together with the ultraviolet data showed that the

locations of the double bonds were at Δ^7, Δ^9, Δ^{11} and Δ^{13}. However, in the present study we have not examined the geometrical configurations of the four conjugated double bonds. Nevertheless it is likely that the double bond at Δ^{13} retained the trans configuration present in 15-HPETE.

The biological effects of this new derivative of arachidonic acid will be reported later.

ACKNOWLEDGMENTS

We wish to thank Mrs. G. Hamberg and Mrs. A. Öhrström for excellent technical assistance. This work was supported by the Swedish Medical Research Council (proj. no. 03X-00217 (B.S.) and 03X-05170 (M.H.)).

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