Vol. 99, No. 3, 1981 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 15, 1981 Pages 976-986

NOVEL LEUKOTRIENES: PRODUCTS FORMED BY INITIAL OXYGENATION OF ARACHIDONIC ACID AT C-15

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Received February 23,1981

SUMMARY: A preparation of human leukocytes was incubated with arachidonic acid. Two new dihydroxy acids with conjugated triene structures, were isolated and characterized as 8,15-dihydroxy-5,9,11,13-eicosatetraenoic acid (8,15-leukotriene B_4) and 14,15-dihydroxy-5,8,10,12-eicosatetraenoic acid (14,15-leukotriene B_4).

INTRODUCTION

The leukotrienes (LT)[1] constitute a recently discovered group of biologically active compounds derived from arachidonic acid and other polyunsaturated fatty acids (1-4). The first leukotriene described was leukotriene B_4 (LTB_4) (5-7). Later, the slow reacting substance leukotriene C_4 (LTC_4) was characterized (8-10). LTC_4 is metabolized to LTD_4 (11), by elimination of glutamic acid, and further to LTE_4 (12,13), by elimination also

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[1] Abbreviations: LT, leukotriene; LTA_4, leukotriene A_4 (5(S)-trans-5,6-oxido-7,9-trans,11,14-cis-eicosatetraenoic acid); LTB_4, leukotriene B_4 (5(S),12(R)-dihydroxy-6,8,10,14-eicosatetraenoic acid; LTC_4, leukotriene C_4 (5(S)-hydroxy,6(R)-S-glutathionyl-7,9-trans,11,14-cis-eicosatetraenoic acid; LTD_4, leukotriene D_4 (5(S)-hydroxy,6(R)-S-cysteinylglycyl-7,9-trans,11,14-cis-eicosatetraenoic acid; LTE_4, leukotriene E_4 (5(S)-hydroxy,6(R)-S-cysteinyl-7,9-trans,11,14-cis-eicosatetraenoic acid; HPLC, high pressure liquid chromatography; HPETE, hydroperoxyeicosatetraenoic acid; HETE, hydroxyeicosatetraenoic acid.

0006-291X/81/070976-11$01.00/0

of glycine. All the cystein containing derivatives (LTC_4, LTD_4 and LTE_4) have been identified in preparations of slow reacting substance of anaphylaxis (SRS-A), from various sources (4,12).

The formation of leukotrienes from arachidonic acid involves initial oxygenation at C-5 to form a 5-hydroxyperoxy-derivative, which is converted to the unstable epoxide intermediate LTA_4 (5,14). LTA_4 can be hydrolyzed enzymatically to LTB_4 (15), or be converted to LTC_4, by addition of glutahione at C-6 (16).

In this and additional papers (17) we report that leukotriene formation is not restricted to transformations involving oxygenation at C-5 as the initial step. A new group of leukotrienes can also be formed biologically by initial oxygenation at C-15 (Fig. 1).

MATERIALS AND METHODS

Cell preparation and incubations

Human leukocytes were obtained from peripheral blood. The cells were prepared as previously described (18). In large scale incubations the Lymphoprep density gradient centrifugation was omitted. After addition of 75 µM arachidonic acid (Nucheck Corp., Elysian, Minnesota) in ethanol, cells (100 ml of 100 x 10^6 cells/ml) were incubated at 37°C for five minutes. The incubation was stopped by adding 1.5 volumes of methanol. Alternatively the cells were homogenized before incubation. After sonication, the homogenate was centrifuged at 10,000 x g for 30 minutes, and the supernatant incubated with (5,6,8,9,11,12,14,15)-octadeutero-arachidonic acid (preparation, see ref. 19) (15 µM) 37°C for 5 minutes.

Extraction and purifications

Ether extraction and silicic acid column chromatography were performed as previously described (5,6,18). Further purification was achieved by reverse phase HPLC. The column (length 500 mm, i.d. 10 mm, Polygosil C 18, 10 µm, Macherey-Nagel, Düren, West Germany) was eluted with methanol/water/acetic acid, 70/30/0.01, 4ml/min. A UV-detector, set at 280 nm, recorded the absorption of the eluate. Fractions showing a leukotriene type UV-spectrum were pooled, treated with diazomethane and injected onto a straight phase HPLC-column (Polygosil 60-10, SiO_2, length 500 mm, i.d. 10 mm) eluted with hexane/isopropanol/acetic acid, 94/6/0,01, v/v/v, at 4 ml/min. The absorbance at 280 nm was recorded. HPLC-pump (6000 A) and injector (U6K) were from Waters Assoc. Inc., Milford, MA. the UV-detector (LDC-III) was from LDC, Riviera Beach, Florida.

UV-spectrometry

Absorption was measured at 320-220 nm in a Cary 219 spectrometer with methanol as solvent.

Gas chromatography - mass spectrometry

For conversion into methyl esters diazomethane in ether was added to the extract dissolved in methanol and samples were left on ice for 5 minutes. The mixture was evaporated under argon and dissolved in 50 µl of acetonitrile. Conversion into trimethylsilyl ethers was performed by addition of 50 µl of bis (trimethylsilyl)-trifluoroacetamide and incubation at 60°C for 30 minutes. Samples were dried down, dissolved in hexane and injected into the Gc-ms apparatus. The instrument (LKB 9000) was equipped with a 1% SE-30 column. The energy of the ionization beam was 22.5 eV.

Catalytic hydrogenation

The catalyst, platinum oxide (1.0 mg) was added to the methyl ester derivatives dissolved in 1.0 ml methanol and hydrogen was bubbled through for 90 seconds. The catalyst was removed by passing the sample through a 200 mg silicic acid (100 mesh) column packed with methanol and eluting with 10 ml of methanol. Pressure was applied. The methanol was evaporated and the samples were subjected to gas chromatography - mass spectrometry as described above.

RESULTS

A preparation of human leukocytes was incubated with arachidonic acid. In addition to the previously described LTB_4, new dihydroxyacids were isolated and characterized.

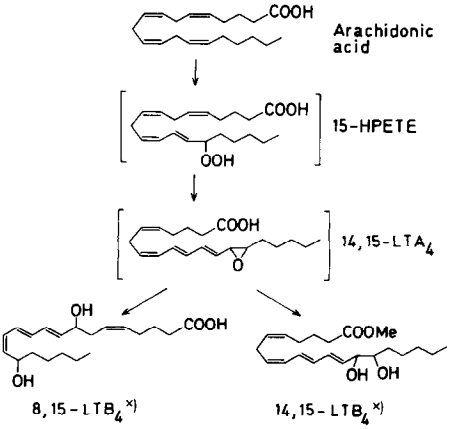

Fig. 1 Metabolic pathways of arachidonic acid in human leukocytes, originating with oxygenation at C-15. Postulated structures are in brackets. Asterisk indicates that the sterochemistry of the double bonds has not been determined.

Identification of two isomers of 14.15-dihydroxy-eicosatetraenoic acid (compounds I and II).

The material in a peak with retention time 25 minutes, was collected from the straight phase HPLC. The ultraviolet spectrum of this material showed three main bands. The maxima, with methanol as solvent, were at 282 nm, 272 nm and 262 nm. This indicates a conjugated triene structure.

Following derivatization (methyl esters, trimethylsilyl ethers) of this material, gas chromatographic analysis separated it into two compounds (I and II) with equivalent chain lengths 23.9 and 24.9, respectively (SE-30). The mass spectra were practically identical for both compounds. Figure 2 shows the mass spectrum of the isomer with C-value 24.9. It was characterized by ions at (m/e): 479(M^+-15), 394($Me_3SiOCH(CH)_6CH_2(CH)_2(CH_2)_3C(OMe)\overset{+}{O}SiMe_3$) resulting from a rearrangement, see ref. 20, 321(M^+-173), loss of $Me_3SiO\dot{C}H(CH_2)_4CH_3$ and 173(M^+-321), loss of $Me_3SiO\dot{C}H(CH)_6CH_2(CH)_2(CH_2)_3COOMe$.

This indicates a tetraunsaturated dihydroxy acid with 20 carbons (M = 494). The positions of the hydroxyl groups (C-14 and C-15) were determined by the ions appearing at m/e 173 and m/e 321. The rearrangement ion, m/e 394 is typical of a 14,15-

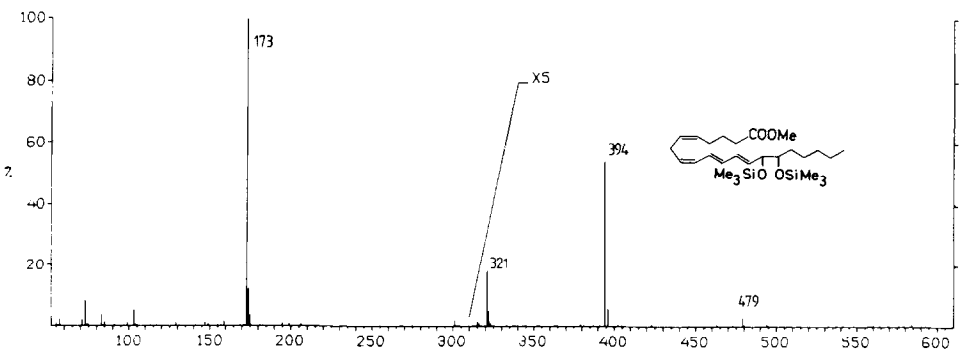

Fig. 2 Mass spectrum of methyl ester trimethylsilyl ether derivative of compound II.

dihydroxy acid (20). The ions m/e 321 and m/e 394 indicated that the four double bonds of the molecule are located between carbons 1 and 14.

Upon catalytic hydrogenation of this material (performed prior to silylation) a single compound, with equivalent chain length 23.5 (SE-30), was detected in the gas chromatogram. The mass spectrum (Fig. 3) now showed ions at (m/e): 487(M^+-15), 471(M^+-31), 402($Me_3SiO\dot{C}H(CH_2)_{12}C(OMe)\overset{+}{O}SiMe_3$), from a rearrangement (see ref. 20), 329(M^+-173, loss of $Me_3SiO\dot{C}H(CH_2)_4CH_3$), and 173($M^+$-329, loss of $Me_3SiO\dot{C}H(CH_2)_{12}COOCH_3$).

This mass spectrum is in full accordance with what would be expected for a saturated C_{20} dihydroxy acid, with hydroxyl groups at C-14 and C-15 (M = 502). The dominating ions m/e 329 and 173 result from cleavage between the carbons bearing the vicinal hydroxyl groups. The shift of the ion bearing the carboxyl group, from 321 in the mass spectrum of the unsaturated compound, to 329 after hydrogenation, aggrees with the positioning of the double bonds between C-1 and C-14. The ion at m/e 402 results from a characteristic rearrangement (20).

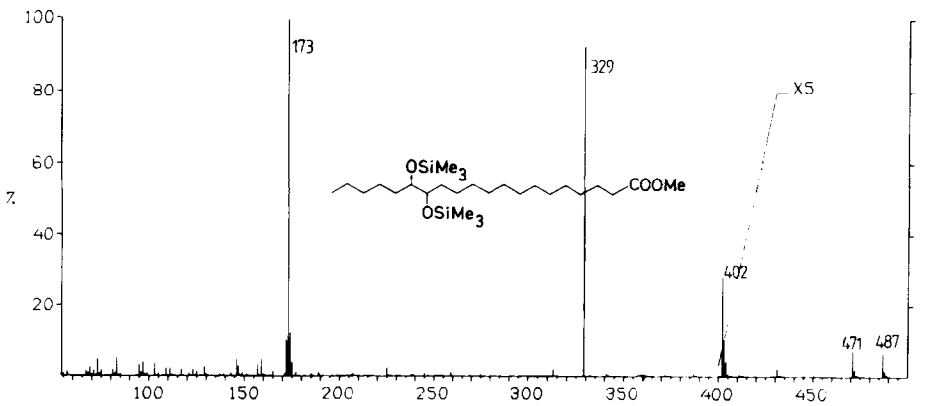

Fig. 3 Mass spectrum of product obtained from catalytic hydrogenation of compounds I and II. Methyl ester trimethylsilyl ether derivative.

The locations of the four double bonds of these compounds were partially based on biogenetic considerations (see discussion). It is assumed that the compounds are derived from a 14,15-epoxy-acid, and that the Δ^5 double bond of arachidonic acid is retained isolated (not in conjugation) during the transformations. The double bonds forming the conjugated triene should thus be located at Δ^8, Δ^{10} and Δ^{12}.

These data indicate that compounds I and II are two isomers of 14,15-dihydroxy-5,8,10,12-eicosatetraenoic acid.

Identification of two isomers of 8,15-dihydroxy-eicosatetraenoic acid (compounds III and IV).

Compounds III and IV could be separated on straight phase HPLC. Compound III (more polar) had a retention time of 32 min, while the less polar compound IV appeared at 23 min.

Both isomers showed practically identical UV-spectra, with three absorption maxima at 280 nm, 269 nm and 259 nm, with methanol as solvent.

Compound III, after conversion into the methyl ester trimethylsilyl ether, showed an equivalent chain length of 24.8 (SE-30), when analyzed by Gc-ms. Characteristic ions in the mass spectrum of this compound were (m/e) (Fig. 4): 494(M^+), 479(M^+-15), 463(M^+-31), 423(M^+-71, loss of $\cdot(CH_2)_4CH_3$), 404(M^+-90, loss of trimethylsilanol), 353(M^+-141, loss of $\cdot CH_2(CH)_2(CH_2)_3$COOMe), 263(M^+-(141+90)), 243(M^+-251, loss of $\cdot(CH)_6CH(OSiMe_3)(CH_2)_4CH_3$), 217(probably $Me_3SiO-CH=CH-CH=\overset{+}{O}SiMe_3$, from a rearrangement), 199, 191(probably $Me_3SiO-CH=\overset{+}{O}-SiMe_3$ from a rearrangement), 173(M^+-321), loss of $\cdot(CH)_6CH(OSiMe_3)CH_2(CH)_2(CH_2)_3$COOMe and 129 (base peak).

The molecular ion and the ions M^+-15, M^+-31 and M^+-90 are consistent with a C_{20} tetraunsaturated fatty acid with two hyd-

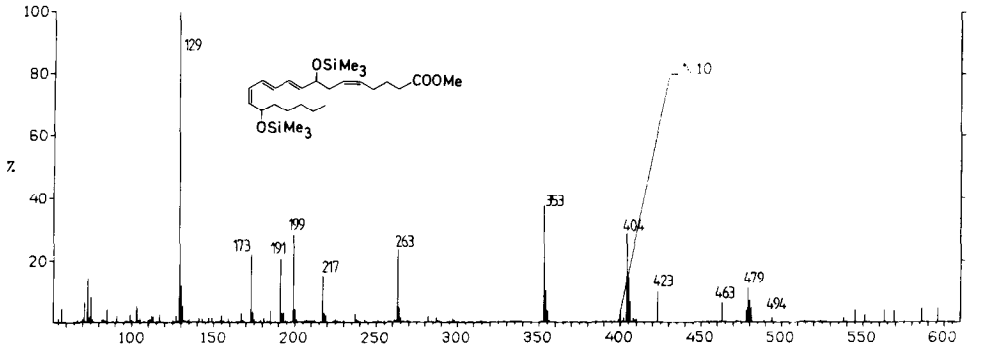

Fig. 4 Mass spectrum of methyl ester trimethylsilyl ether derivative of compound III.

roxyl groups (M = 494). The position of the hydroxyl group at C-15 is determined by the ions m/e 173 and 423. The other hydroxyl group should be located at C-8 because of the ions at m/e 253, 263 but also by the appearance of a small ion at m/e 243.

Regarding the positions of the four double bonds, the ions at m/e 173 and 423 imply that the molecule is saturated from C-15 to C-20. The ions at m/e 353 and 263 indicate one double bond between C-1 and C-8. The positions of the three conjugated double bonds (according to the UV-spectrum) are therefore Δ^9, Δ^{11} and Δ^{13}. This is in agreement with the proposed biogenetic mechanism (see Discussion).

The compound corresponding to the more polar 8,15-dihydroxy acid (compound III), obtained from an incubation of leukocyte 10,000 x g supernatant with 5,6,8,9,11,12,14,15-octadeutero-arachidonic acid (see ref. 19), had an equivalent chain length of 24.2. Ions in the mass spectrum were now shifted to (m/e): 487(M^+-15), 471(M^+-31), 411(M^+-90), 359(M^+-143), 269(M^+-(143+90)), 219, 201, 192, 174(M^+-328) and 132 (base peak).

These shifts in the m/e-values are in accordance with the structures assigned to the various ions.

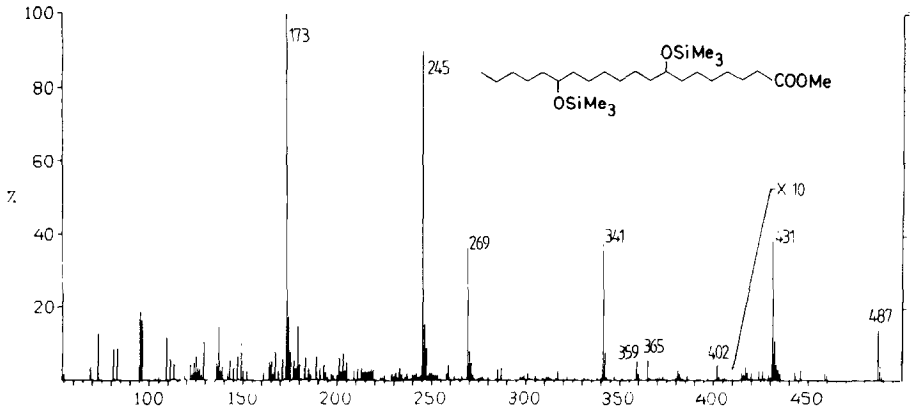

Fig. 5 Mass spectrum of product obtained from catalytic hydrogenation of compound III. Methyl ester trimethylsilyl ether derivative.

After catalytic hydrogenation of compound III, obtained from nondeuterated arachidonic acid, gas chromatographic analysis showed a compound with equivalent chain length 24.0 (SE-30). The mass spectrum of the hydrogenation product was (m/e) (Fig.5): 431(M^+-71, loss of $\cdot(CH_2)_4CH_3$), 402(probably $Me_3\overset{+}{S}iOC(OMe)(CH_2)_6CH(OSiMe_3)(CH_2)_6\cdot$, from a rearrangement, see ref. 20), 265, 359(M^+-143, loss of $\cdot(CH_2)_6COOMe$), 341(M^+-(71+90), loss of $\cdot(CH_2)_4CH_3$+trimethylsilanol), 269(M^+-(143+90)), 245($Me_3\overset{+}{S}iOCH(CH_2)_6COOMe$), and 173($Me_3\overset{+}{S}iOCH(CH_2)_4CH_3$).

The assignments of the hydroxyl groups, at C-8 and C-15 are validated by the ions at m/e 359, 269, 245 and at m/e 431, 341 and 173, respectively. The rearrangement ion m/e 402 is also in agreement with an 8,15-dihydroxy acid.

The less polar isomer of the 8,15-dihydroxy acid, with retention time 23 minutes in the straight phase HPLC (compound IV), showed a UV-spectrum practically identical to that of compound III.

Gc-ms showed an equivalent chain length of 22.3 for compound IV (SE-30), methyl ester, trimethylsilylether.

The mass spectrum was characterized by ions at (m/e): 494(M^+), 479(M^+-15), 463(M^+-31), 394(probably $Me_3Si\overset{+}{O}C(OMe)(CH_2)_3(CH)_2CH_2CH(OSiMe_3)(CH)_6^{\cdot}$, from a rearrangement, see ref. 20), 263(M^+-(141+90)), 243($Me_3Si\overset{+}{O}CHCH_2(CH)_2(CH_2)_3COOMe$), 211, 173($M^+$-321, loss of $^{\cdot}(CH)_6CH(OSiMe_3)CH_2(CH)_2(CH_2)_3COOMe$) base peak.

M^+, M^+-15 and M^+-31 are again in accordance with a tetra-unsaturated C_{20} fatty acid with two hydroxyl groups. The ions appearing at m/e 263, 243 (second to the base peak in intensity) indicate that the hydroxyl group is located at C-8 and the occurrence of a hydroxyl group at C-15 is supported by the ion at m/e 173. The locations of the double bonds are indicated to be at Δ^5, Δ^9, Δ^{11} and Δ^{13}, based on the same reasons as for compound III.

When deuterated arachidonic acid was incubated with 10,000 x g supernatant, the compound corresponding to the less polar 8,15-dihydroxy acid (compound IV) showed an equivalent chain length of 22.1. Ions in the mass spectrum were now at (m/e): 269(M^+-(143+90)), 246($Me_3Si\overset{+}{O}$-$CDCH_2(CD)_2(CH_2)_2COOMe$) and 174 ($Me_3SiO\overset{+}{C}D(CH_2)_4CH_3$), in accordance with the expected shifts.

In summary, two isomers of 8,15-dihydroxy-5,9,11,13-eicosatetraenoic acid, and two isomers of 14,15-dihydroxy-5,8,10,12-eicosatetraenoic acid, were isolated from incubation of human leukocyte preparations with arachidonic acid. The 14,15-dihydroxy-acids were most abundant.

DISCUSSION

This paper describes the formation of new dihydroxy acids in a human leukocyte preparation incubated with arachidonic acid. The isolated and characterized compounds are 14,15-dihydroxy-5,8,10,12-eicosatetraenoic acid (2 isomers), and 8,15-dihydroxy-5,9,11,13-eicosatetraenoic acid (2 isomers).

The structure elucidation was based on chromatographic properties, ultraviolet spectroscopy and Gc-ms of the parent compounds, and hydrogenated derivatives (methyl esters, trimethylsilylethers). The proposed structures are also in agreement with previously established biogenetic relationships.

These compounds all contain a conjugated triene structure. They are proposed to be formed *via* the allylic epoxide 14,15-oxido-5,8,10,12-eicosatetraenoic, which in turn is visualized to be formed by dehydration of 15-hydroperoxyeicosatetraenoic acid (15-HPETE). In a separate study it has been shown that 15-HPETE is a precursor of these new compounds (17). Also, the existence of a 15-lipoxygenase pathway in human leukocytes was demonstrated earlier by the isolation of 15-HETE from these cells (18).

The proposed reaction scheme is analogous to that previously described for the biosynthsis of 5,12-leukotriene B_4 and 5,6-leukotriene B_4, *via* 5-HPETE and the epoxide 5,6-leukotriene A_4. Accordingly, the leukotriene nomenclature is also used for the new metabolites, which are named 14,15-leukotriene B_4 and 8,15-leukotriene B_4, respectively.

The stereochemistry of the compounds described has not been studied. The stereochemical features indicated in Fig. 1 are based on the assumption that the stereochemistry of leukotrienes formed *via* the 5- and 15-lipoxygenase catalyzed pathways are analogous.

Additional studies are in progress to elucidate the stereochemistry and possible biological functions of these new arachidonic acid metabolites.

ACKNOWLEDGEMENTS

We wish to thank Ms Ulla Andersson and Ms Inger Tollman-Hansson for excellent technical assistance. This study was supported by a grant from the Swedish Medical Research Council (Project 03X-217).

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