TRANSFORMATION OF 15-HYDROPEROXY-5,9,11,13-EICOSATETRAENOIC ACID INTO NOVEL LEUKOTRIENES

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1. Introduction

A new group of biologically active compounds, the leukotrienes, has been described in [1–4]. These derivatives of arachidonic acid and other polyunsaturated fatty acids are formed by initial oxygenation at C-5 forming 5-hydroperoxy acids. The hydroperoxy derivative is further converted to a labile allylic epoxide intermediate leukotriene A_4 (LTA_4) (from arachidonic acid) [5,6]. LTA_4 can be converted to LTB_4 [7,8] by enzymatic hydrolysis [9], and to LTC_4 [10–12], by addition of glutathione [13]. LTC_4 together with the metabolites LTD_4 [14] and LTE_4 [15,16] are the structures responsible for the biological activity referred to as SRS-A in [17]. Here, and in [18], we report that leukotriene formation is not restricted to transformations involving oxygenation at C-5 as the first step. A new group of leukotrienes can also be formed in biological systems by initial oxygenation at C-15.

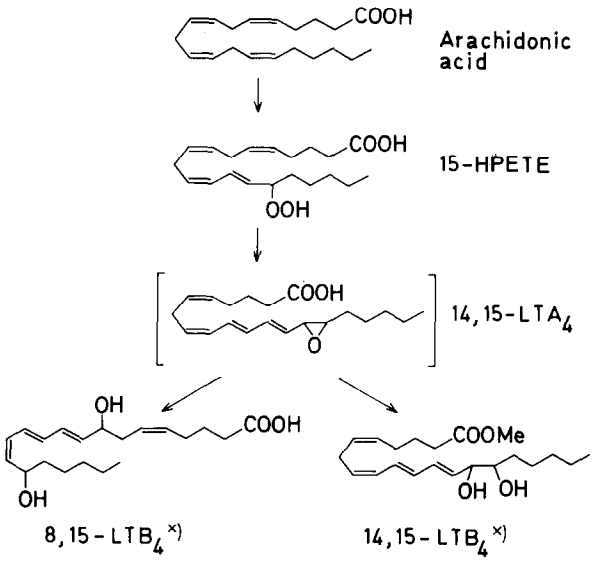

Fig.1. Transformations of arachidonic acid in human leukocytes via initial oxygenation at C-15. Structure in brackets is postulated. Asterisk indicates that the stereochemistry of the conjugated triene has not been determined.

2. Materials and methods

2.1. *Materials*

Dextran T-500 for cell sedimentation was obtained from Pharmacia Fine Chemicals (Uppsala). Arachidonic acid was purchased from Nu-Chek Prep. (Elysian MN) and soybean lipoxygenase (EC 1.13.11.12) type I, from Sigma (St Louis MO). HPLC-equipment was from Waters Assoc. (Milford MA) (pump 6000A, injector U6K) and LDC (Riviera Beach FA) (UV-detector, LDC-III), respectively. HPLC stationary phases were from Macherey-Nagel, (Düren).

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

2.2. *Preparation of human leukocytes*

A concentrate of human leukocytes was obtained from blood component preparation at the Karolinska Hospital. The cells were prepared as in [21]. Briefly, the cell suspension was mixed with an equal volume

of 6% (w/v) Dextran T-500 in 0.9% saline, and the cells were allowed to sediment at 4°C. This separated the majority of the red cells from the white cells. After centrifugation at 400 × *g* 15 min, the pellets were resuspended in a Tris-buffered, ammonium–chloride solution, and incubated at 37°C for 7 min, to induce lysis of remaining red cells. Succeeding centrifugation at 400 × *g* for 15 min, the cells were resuspended in Dulbeccos PBS, 30 × 10^6/ml, for immediate incubation.

2.3. *Preparation of 15-hydroperoxyeicosatetraenoic acid (15-HPETE)*

15-HPETE was produced by incubation of soybean lipoxygenase with arachidonic acid [19]. The product was extracted with diethylether, after acidification (pH 2.5). It was purified by silicic acid column chromatography (CC-4, Mallinkrodt, St Louis MO), activated overnight at 120°C. The sample was applied to the column dissolved in diethylether/hexane (90/10, v/v) and eluted with diethylether/hexane (80/20, v/v). The purified material was stored in methylene chloride at −20°C. The identity and purity of the product was determined by co-chromatography with 15-hydroxyeicosatetraenoic acid on straight phase HPLC, after reduction with triphenylphosphine.

2.4. *Incubations*

The human leukocyte preparation (30 × 10^6/ml), was pre-warmed to 37°C. 15-HPETE dissolved in ethanol was added to 80 μM final conc. and incubated for 30 min. The amount of ethanol never exceeded 0.1% of the incubation volume. Typically 100 ml cell suspension was used. The incubations were stopped by adding 1.5 vol. methanol.

2.5. *Extraction and purification procedure*

The precipitate obtained upon addition of methanol to the incubation was spun down, and washed once with methanol. The aqueous phase was acidified with hydrochloric acid to pH 2–3, and extracted with diethylether.

After evaporation, the extract was applied to a silicic acid column (CC-4, Mallinkrodt, St Louis MO) 2 g/50 ml incubation vol. The column was eluted with hexane/diethylether (90/10, v/v) followed by ethylacetate. The ethylacete fraction was evaporated and further purified on a semi-preparative reverse phase HPLC-column (Polygosil C18, 10 μm particles, length 500 mm, i.d. 10 mm) that was eluted with methanol/water/acetic acid (70/30/0.01, v/v/v) at 4 ml/min. UV-absorption at 280 nm of the eluate was monitored. Fractions showing a leukotriene-type UV-spectrum were pooled and methylated with diazomethane. Final purification was achieved on a straight phase HPLC-column (Polygosil C60-10, SiO_2, 10 μm particles) eluted at 2 ml/min with hexane/isopropanol/acetic acid (92/8/0.01, v/v/v) UV-recording, 280 nm.

2.3. *UV-spectroscopy*

UV-spectroscopy was performed with a Cary 219 instrument. Spectra were recorded between 320 and 220 nm, with hexane/isopropanol/acetic acid (92/8/0.01, v/v/v) as solvent.

2.4. *Hydrogenation*

Samples were dissolved in 1 ml methanol and cooled to 0°C. PtO_2 (1 mg) was added, and hydrogen bubbled through for 90 s. The catalyst was removed by filtration, on a short column of SiO_2, 100 mesh, eluted with 10 ml methanol.

2.5. *Gas chromatography–mass spectrometry (GC–MS)*

The samples, treated with diazomethane, were converted to trimethylsilylethers using pyridine, hexamethyldizilazane and trimethylchlorosilane, and an LKB-9000 instrument was used for GC–MS analysis. The stationary phase of the GC-column was SE-30 (1%) on Supelcoport. The energy of the ionization beam was 22.5 eV.

3. Results

A preparation of human leukocytes was incubated with 15-hydroperoxyeicosatetraenoic acid. Four compounds were isolated and further characterized. These new metabolites were formed also in incubations of homogenates of leukocytes, with 15-HPETE. Furthermore, when labeled 15-[1-^{14}C]HPETE was incubated, radioactivity was incorporated into the isolated compounds.

3.1. *Compounds I and II*

The material in a peak in the straight phase HPLC, with a retention time of 37 min, was collected. The UV-spectrum of this fraction showed 3 main bands (fig.2A) with maxima at 281 nm, 270 nm and 261 nm. This is in accordance with the occurrence of 3 conjugated double bonds in the isolated material.

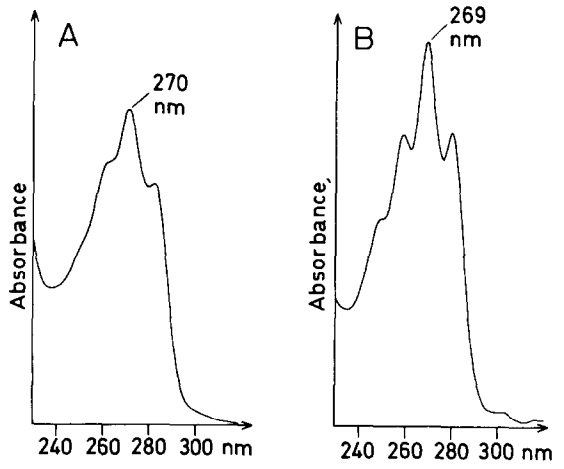

Fig.2. (A) UV-spectrum of compounds I and II; (B) UV-spectrum of compound III.

The material was derivatized into the trimethylsilylethers, as in section 2 and subjected to GC–MS. Two compounds were separated in the gas chromatogram. Their *C*-values were 23.8 and 24.7 (compounds I and II) (SE-30). The mass spectra of the two compounds were practically identical. Fig.3 shows the mass spectrum of the compound with *C*-value 23.8. Ions in the mass spectrum were (*m/e*): 479 (M^+-15), 394 ($Me_3SiO\dot{C}H(CH)_6CH_2(CH)_2(CH_2)_3C(OMe)$-$\dot{O}SiMe_3$, resulting from a rearrangement (see [20]), 321 (M^+-173, loss of $Me_3SiO\dot{C}H(CH_2)_4CH_3$) and 173 ($Me_3Si\dot{O}CH(CH_2)_4CH_3$, base peak).

The ion M^+-15 indicates a tetra-unsaturated dihydroxy acid, with 20 carbons (M = 494). The positions of the hydroxyl groups are at C-14 and C-15, as indicated by the ions at *m/e* 321 and 173. The ion at *m/e* 173 also implies that the molecule is saturated

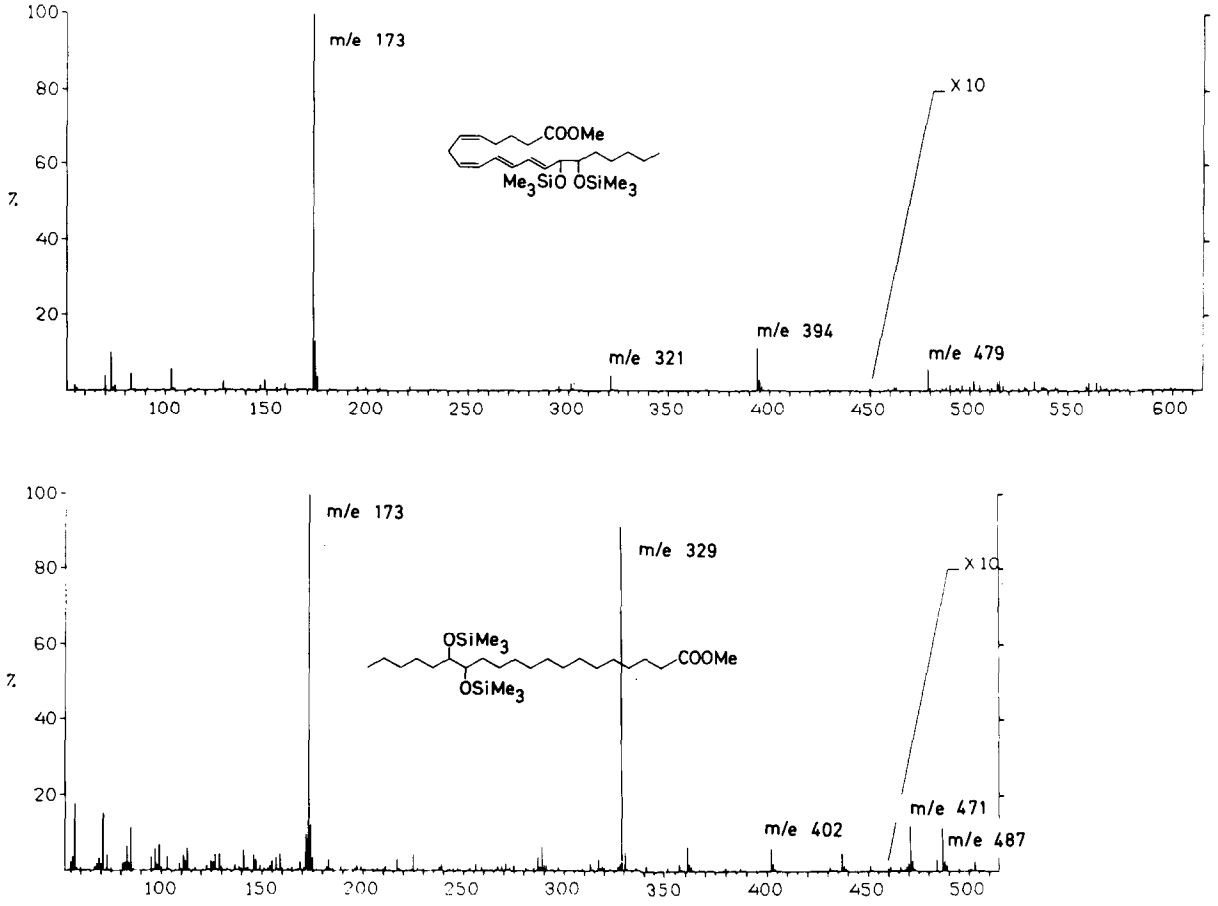

Fig.3. Upper panel – mass spectrum of compound I. Lower panel – mass spectrum of compounds I and II after catalytic hydrogenation (Methyl esters, trimethylsilyl ethers.)

between C-14 and C-20, and the ion at *m/e* 321 that the 4 double bonds are located between C-1 and C-14. The rearrangement product at *m/e* 394 can be expected for a vicinal 14,15-dihydroxy acid, with 4 double bonds between C-1 and C-14 [20]. The exact positions of the double bonds are based on biogenetic considerations (see section 4). It was thus assumed that the double bonds are located at Δ^5, Δ^8, Δ^{10} and Δ^{12}.

When the material in the HPLC-fraction with a retention time of 37 min was hydrogenated, prior to derivatization into trimethylsilyl ethers and GC–MS, a peak with *C*-value 23.6 (SE-30) was detected in the gas chromatogram. The mass spectrum (fig.3) showed ions at (*m/e*): 487 (M^+-15), 471 (M^+-31), 451, 437, 431 (M^+-71, loss of $(CH_2)_4CH_3$), 402 ($Me_3SiO\dot{C}H(CH_2)_{12}$-$C(OMe)\dot{O}SiMe_3$ from a rearrangement (see [20]), 361, 329 (M^+-173, loss of $Me_3SiO\dot{C}H(CH_2)_4CH_3$), 289, and 173 ($Me_3Si\dot{O}CH(CH_2)_4CH_3$, base peak).

This mass spectrum confirms the concept of a C-20 carboxylic acid, with two hydroxyl groups (*M* = 502). The positions of the hydroxyl groups (C-14 and C-15) followed from the ions appearing at *m/e* 329 and 173, respectively. The ion at *m/e* 402, resulting from a rearrangement is in accordance with a 14,15-dihydroxy acid [20].

Based on these data and the biogenetic considerations, compounds I and II are proposed to be two isomers of 14,15-dihydroxy-eicosa-5,8,10,12-tetraenoic acid.

3.2. *Compound III*

The retention time in the straight phase HPLC, of this compound was 40 min. The UV-spectrum (fig.2B) again showed a triplet, with maxima at 280 nm, 269 nm and 259 nm, suggesting a conjugated triene structure also for compound III.

Following conversion into trimethylsilylethers, GC–MS showed a compound with *C*-value 24.8 (SE-30). Ions in the mass spectrum of the compound (fig.4) were at (*m/e*): 494 (M^+), 479 (M^+-15), 463 (M^+-31), 423 (M^+-71, loss of $\cdot(CH_2)_4CH_3$), 404 (M^+-90), loss of trimethylsilanol), 394 (probably $Me_3Si\dot{O}C(OMe)(CH_2)_3(CH)_2CH_2CH(OSiMe_3)(CH)_6^{\cdot}$, from a rearrangement, see [20]), 353 (M^+-141), loss of $\cdot CH_2(CH)_2(CH_2)_3COOMe$), 321 ($M^+$-173, loss of $Me_3SiO\dot{C}H(CH_2)_4CH_3$), 263 ($M^+$-(141 + 90)), 237, 217 (probably $Me_3SiOCH{=}CH{-}CH{=}O\dot{S}iMe_3$, from a rearrangement), 199, 191 (probably $Me_3SiOCH{=}\dot{O}SiMe_3$, from a rearrangement), 173 ($Me_3Si\dot{O}CH(CH_2)_4CH_3$), and 129 (base peak).

The ions at *m/e* 494, 479, 463 and 404 indicate a tetra-unsaturated C_{20} dihydroxy acid. The position of one hydroxyl group (C-15) is determined by the ions appearing at *m/e* 423, 321 and 173. The other hydroxyl group is located at C-8 since there were ions appearing at *m/e* 353 and 263. The positions of the double bonds can be assigned to be at Δ^5, Δ^9, Δ^{11} and Δ^{13} partly by biogenetic considerations (see section 4). This is also supported by the appearance of ions at *m/e* 423 and 173 showing that the molecule is saturated between C-15 and C-20 and ions at *m/e* 353 and 263 indicating that one double bond is present between C-1 and C-8. The conjugated triene (based on UV-absorption) is thus localized between C-8 and C-15.

Upon hydrogenation, the *C*-value of compound III shifted to 24.0 (SE-30). The mass spectrum (fig.4) was as follows (*m/e*): 487 (M^+-15), 431 (M^+-71, loss of $\cdot(CH_2)_4CH_3$), 365, 359 (M^+-143, loss of $\cdot(CH_2)_6$-$COOMe$), 341 (M^+-(71 + 90), loss of $\cdot(CH_2)_4CH_3$ + trimethylsilanol), 329 (M^+-173), loss of $Me_3Si\dot{O}CH$-$(CH_2)_4CH_3$), 269 (M^+-(143 + 90)), 245 ($Me_3Si\dot{O}CH$-$(CH_2)_6COOMe$), 216 and 173 that is $Me_3Si\dot{O}CH$-$(CH_2)_4CH_3$ (base peak).

The ions at *m/e* 487 and 431 are consistent with a C_{20} dihydroxy acid. The positions of the hydroxyl groups at C-8 and C-15 were determined by the ions (*m/e*) 359, 269, 245 and by the ions (*m/e*) 431, 341, 173, respectively.

Based on these data, compound III was assigned the structure 8,15-dihydroxyeicosa-5,9,11,13-tetraenoic acid.

3.3. *Compound IV*

The retention time on straight phase HPLC of the methyl ester of compound IV was 36 min. The UV-spectrum was practically the same as for compound III, indicating 3 conjugated double bonds.

The trimethylsilylether derivative of the compound had an equivalent chain length of 22.3 (SE-30). Ions in the mass spectrum were at (*m/e*): 394 (probably $Me_3Si\dot{O}C(OMe)(CH_2)_3(CH)_2CH_2CH(OSiMe_3)(CH)_6^{\cdot}$, from a rearrangement, see [20]), 263 (M^+-(141 + 90) loss of $\cdot CH_2(CH)_2(CH_2)_3COOMe$ plus trimethylsilanol), 243 ($Me_3Si\dot{O}CHCH_2(CH)_2(CH_2)_3COOMe$) and 173 ($Me_3Si\dot{O}CH(CH_2)_4CH_3$, base peak).

The mass spectrum was consistent with a C_{20} tetra-unsaturated dihydroxy acid. The ions at *m/e* 263 and 243 indicated that one hydroxyl group is located at C-8. The ion at *m/e* 173 located the other hydroxyl group to C-15. An ion appearing at *m/e* 394 is in

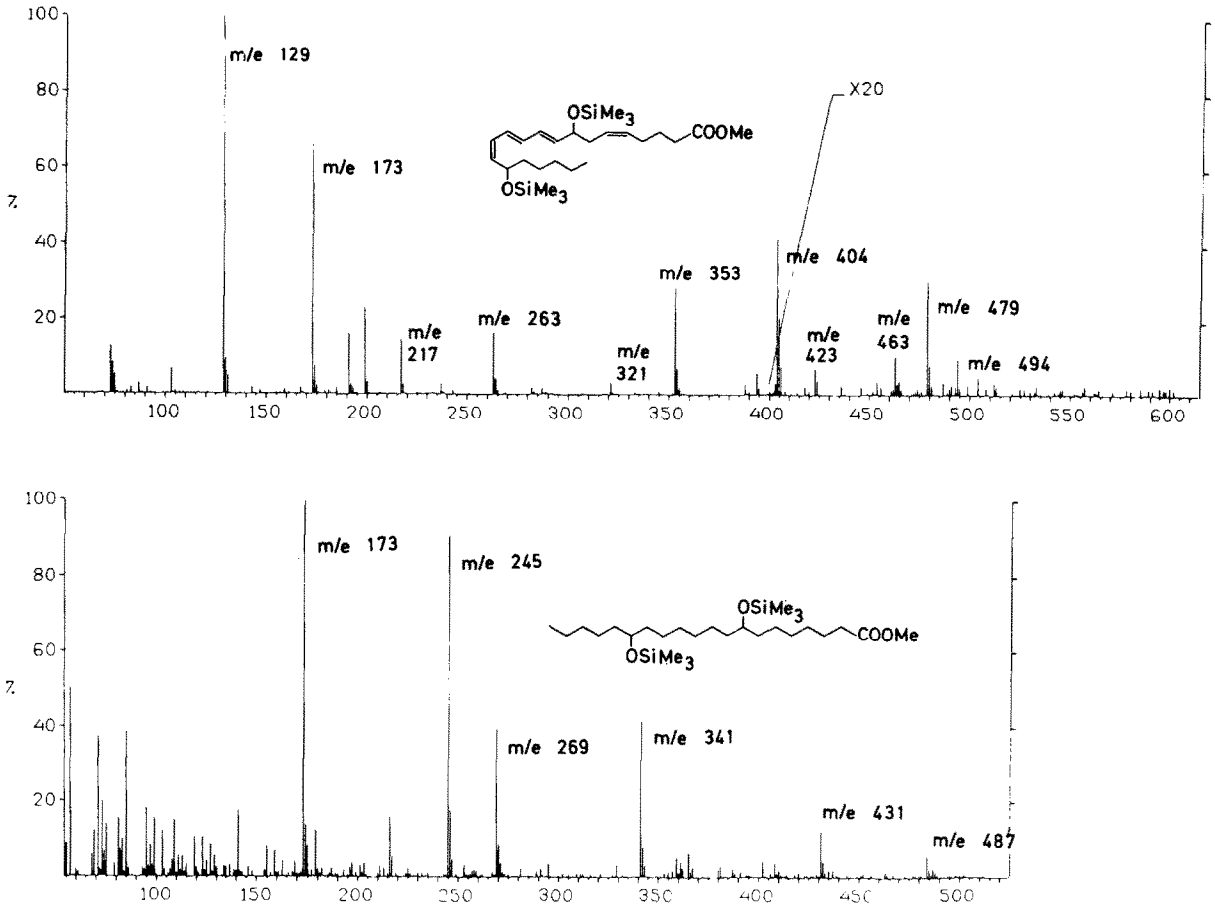

Fig.4. Upper panel – mass spectrum of compound III. Lower panel – mass spectrum of compound III after catalytic hydrogenation. (Methyl esters, trimethylsilylethers.)

agreement with a rearrangement [20] expected for a 8,15-dihydroxy acid.

The double bonds in compound IV are proposed to be at Δ^5, Δ^9, Δ^{11} and Δ^{13} (cf. compound III).

Furthermore, when compound IV was hydrogenated, a product with a *C*-value of 24.0 (SE-30) was obtained. The mass spectrum was practically identical to that of hydrogenated compound III, described above.

Based on these data, compound IV was assigned to be an isomer of 8,15-dihydroxy-eicosa-5,9,11,13-tetraenoic acid.

4. Discussion

In [21] it was shown that leukocytes generate 15-hydroxy-5,8,11,13-eicosatetraenoic acid in addition to products oxygenated at C-5. It therefore seemed of interest to study the biosynthesis of products formed by initial oxygenation at C-15. Here, 15-hydroperoxy-5,8,11,13-eicosatetraenoic acid (15-HPETE) was incubated with a preparation of human leukocytes. The covalent structures of 4 metabolites are described. These are 2 isomers of 14,15-dihydroxy-5,8,10,12-eicosatetraenoic acid and 2 isomers of 8,15-dihydroxy-5,9,11,13-eicosatetraenoic acid.

The assignment of structures was partly based on chromatographic properties in reverse phase and straight phase HPLC and in gas chromatography of both the parent compounds and their hydrogenated derivatives. Furthermore, ultraviolet absorbance and mass spectrometry provided detailed information about location of double bonds and alcohol groups.

The proposed structures are also supported by the biogenetic relationship with 15-HPETE and the analogy with corresponding transformations previously described for 5-hydroperoxy-6,8,10,14-eicosatetraenoic acid. It should be stressed that the stereochemistry of the alcohol groups at C-8 and C-14 have not been determined whereas the stereochemistry at C-15 is assumed to be retained. Also, the configurations of the double bonds have not been determined.

The new metabolites of arachidonic acid are proposed to be formed via an allylic epoxide, viz, 14,15-oxido-5,8,10,12-eicosatetraenoic acid. This epoxide is suggested to be protonated and converted to the 14,15-dihydroxy or 8,15-dihydroxy derivatives by addition of hydroxyl ion at C-14 and C-8, respectively. This mechanism leads to retention of a conjugated triene structure in the dihydroxylated derivatives.

In view of the structural and biogenetic relationship with the leukotrienes formed via oxygenation at C-5 it is proposed to retain the leukotriene nomenclature for this group of compounds and to indicate the positions of the functional groups. The products described are thus 8,15-LTB_4 (two isomers) and 14,15-LTB_4 (two isomers) and the proposed precursor is 14,15-LTA_4. Corresponding compounds have also been found as metabolites of arachidonic acid [18].

Additional work is in progress to elucidate the stereochemistry and possible biological roles of these new leukotrienes.

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