FORMATION OF NOVEL HYDROXYLATED EICOSATETRAENOIC ACIDS IN PREPARATIONS OF HUMAN POLYMORPHONUCLEAR LEUKOCYTES

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1. Introduction

Recent studies of arachidonic acid metabolism in polymorphonuclear leukocytes have led to the discovery of the biologically active leukotrienes [1]. The synthesis of these compounds is initiated by oxygenation of arachidonic acid at C-5 forming 5-hydroperoxy-eicosatetraenoic acid [2], which is further converted to the unstable 5,6-epoxy-7,9,11,14-eicosatetraenoic acid (leukotriene A_4, LTA_4) [3,4]. The epoxide is hydrolysed either enzymatically to 5*S*,12*R*-dihydroxy-6-*cis*,8-*trans*,10-*trans*,14-*cis*-eicosatetraenoic acid (LTB_4) [5–7] or non-enzymatically to additional isomeric 5,12- and 5,6-dihydroxy eicosatetraenoic acids [8]. LTA_4 is also converted by addition of glutathione into LTC_4 [9,10]. This compound and two metabolites, LTD_4 and LTE_4, are responsible for the biological activity of most preparations of slow-reacting substance of anaphylaxis (SRS-A) [11,12]. We have reported that a new group of leukotrienes can also be formed by initial oxygenation at C-15 [13,14].

Here, we describe the formation of 5*S*,12*S*-dihydroxy-6,8,10,14-eicosatetraenoic acid and 5*S*,12*S*,20-trihydroxy-6,8,10,14-eicosatetraenoic acid in preparations of human leukocytes. These novel metabolites are not formed via the leukotriene pathway. Instead arachidonic acid is transformed by a double oxygenation to the dihydroxy acid and further ω oxidized to the trihydroxy acid.

2. Materials and methods

2.1. *Materials*

Arachidonic acid was obtained from Nu-Check Prep. (Elysian MN) ionophore A23187 from Calbiochem-Behring (La Jolla CA), Dextran T-500 from Pharmacia Fine Chemicals (Uppsala) and Silicar CC-4 from Mallinkrodt (St Louis MO). Ampulles of $^{18}O_2$ (100 ml) were purchased from Miles Labs. (Elkhurt IN).

2.2. *Cell preparation and incubations*

Preparation of leukocyte suspensions from partially purified concentrates of human leukocytes (from the Karolinska Hospital, Stockholm) was performed as in [15]. The cells (mainly neutrophil granulocytes contaminated with platelets) were suspended in phosphate buffered saline (pH 7.4) (containing 0.87 mM $CaCl_2$) to give final conc. $30–100 \times 10^6$ white cells/ml. Cell suspensions (200 ml) were preincubated at 37°C for 10 min. After addition of ionophore A23187 and arachidonic acid (final conc. 5 and 150 μM, respectively) the cells were incubated for 10 min and the incubations terminated by addition of 3 vol. ethanol.

2.3. *Labeling experiments with $^{18}O_2$*

Phosphate-buffered saline (90 ml) was introduced into a 3-necked round flask and the flask was evacuated and flushed with argon prior to introduction of $^{18}O_2$. Finally, the system was brought to atmospheric pressure with argon and 6×10^9 leukocytes in 10 ml phosphate-buffered saline were injected into the flask. Thereafter the cells were incubated with ionophore A23187 and arachidonic acid as above.

2.4. *Purification*

After filtration of precipitated material the filtrate was evaporated to remove the ethanol. The remaining water phase was extracted at pH 3 with diethyl ether

and the extract was purified on a silicic acid (Silicar CC-4) column eluted with diethyl ether/hexane, 10/90 (v/v) and 40/60 (v/v) and methanol/ethyl acetate, 5/95 (v/v). The last fraction was subjected to reverse phase high performance liquid chromatography (RP-HPLC) using a Polygosil 60-10 C_{18} column (500 X 10 mm, Macherey Nagel, Düren) eluted with methanol/water/acetic acid, 70/30/0.01 (v/v/v) at 4 ml/min. Eluted material was monitored with a UV-detector at 270 nm. The compounds were converted to methyl esters by treatment with diazomethane prior to straight phase (SP-)HPLC on a Nucleosil 50-5 column (250 X 4.6 mm, Macherey Nagel) using hexane/isopropyl alcohol/acetic acid 95/5/0.01 (v/v/v) (dihydroxy acids), or 90/10/0.01 (v/v/v) (trihydroxy acids) at a flow rate of 1 ml/min.

2.5. *Steric analysis of alcohol groups*

(−)-Menthoxycarbonyl derivatives, prepared as in [16], were purified on a RP-HPLC column (Nucleosil 5 C_{18}; 250 X 4.6 mm, Macherey Nagel) eluted with methanol/water, 97.5/2.5 (v/v) at 1 ml/min, dissolved in chloroform and subjected to oxidative ozonolysis [16]. The short chain menthoxycarbonyl derivatives were treated with diazomethane prior to gas-chromatographic analysis using a 3% OV-210 column (on Chromosorb W,HP).

2.6. *Gas chromatography–mass spectrometry (GC/MS)*

Combined GC/MS was carried out on a LKB 9000 S instrument with a 1% SE-30 (on Chromosorb W,HP) column at 235°C (ionizing voltage 22.5 eV). Mass spectra were recorded with a computer system as in [17]. Methyl esters of the compounds were transformed into trimethylsilyl (Me_3Si) ethers by treatment with hexamethyldisilazane and trimethylchlorosilane in pyridine. [$^2H_{18}$]Me_3Si derivatives were prepared similarly using [$^2H_9$]hexamethyldisilazane and [$^2H_9$]trimethylchlorosilane. Hydrogenated compounds were prepared using platinum oxide as catalyst [5].

3. Results

Leukocyte suspensions were incubated with ionophore A23187 and arachidonic acid. The RP-HPLC chromatogram of the extracted and purified products was similar to those obtained with human peripheral blood and rabbit peritoneal polymorphonuclear leukocytes (PMNL) [6,8]. The main dihydroxylated product formed in these systems had been described as LTB_4. Eluted material corresponding to this peak and to a more polar peak (retention time 12 min) were collected separately and converted to methyl esters.

Further purification of the less polar material on SP-HPLC resulted in the appearance of two separate main peaks (fig.1). Ultraviolet spectrometry of the major compound (A) showed absorption maximum at 268 nm with shoulders at 258 and 278 nm indicating that the compound contained a conjugated triene structure. GC/MS of the trimethylsilyl ether methyl ester of compound A showed a peak with *C*-value 22.4 (1% SE-30). The mass spectrum (fig.2) had prominent ions at *m/e* 494 (M), 479 (M-15), 463 (M-31), 404 (M-90), 393 (M-101, loss of $^{\cdot}CH–(CH_2)_2–COOCH_3$), 383 (M-111, loss of $^{\cdot}CH_2–CH{=}CH–(CH_2)_4–CH_3$), 354 (probably $^{\cdot}CH{=}CH–(CH{=}CH)_2–C(OSiMe_3)–(CH_2)_3–C(OCH_3){=}O^{+}SiMe_3$ from a rearrangement), 293 (M-(111 + 90)), 279, 213 ($Me_3SiO^{+}{=}CH–CH_2–CH{=}CH–(CH_2)_4–CH_3$), 203 (base peak; $Me_3SiO^{+}{=}CH–(CH_2)_3–COOCH_3$ and

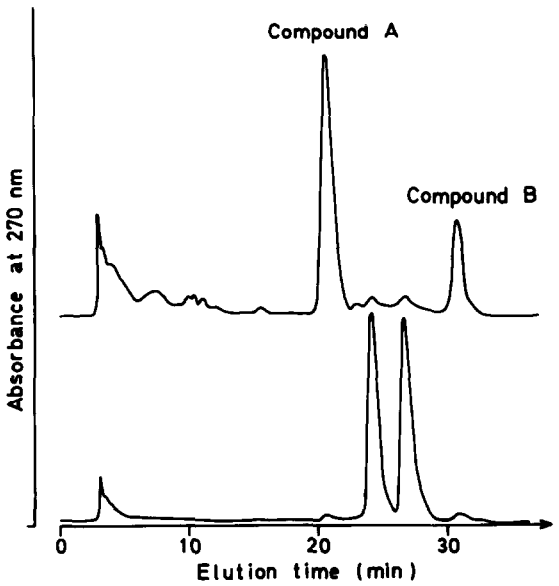

Fig.1. The upper curve shows a SP-HPLC chromatogram of the methyl esters of the products corresponding to the main peak from RP-HPLC. The lower curve shows a SP-HPLC chromatogram of the methyl esters of the two epimers (at C-12) of 5,12-dihydroxy-6-*trans*,8-*trans*,10-*trans*,14-*cis*-eicosatetraenoic acid.

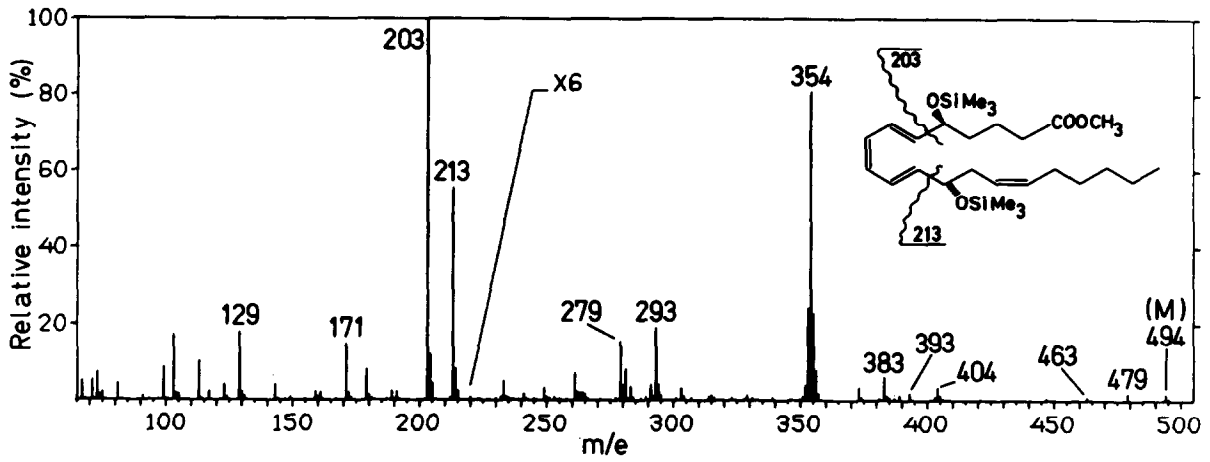

Fig.2. Mass spectrum of the Me_3Si derivative of the methyl ester of compound A.

M-(111 + 90 + 90)), 171 and 129. This suggests a tetraunsaturated C-20 fatty acid structure with hydroxyl groups at C-5 and C-12. The structures of the ions above were supported by the mass spectrum of the [$^2H_{18}$]trimethylsilyl ether methyl ester of compound A. The mass spectrum of the Me_3Si derivative of the methyl ester of hydrogenated compound A (*C*-value 24.0) was in complete agreement with the reported mass spectrum of the same derivative of hydrogenated LTB_4 [5]. The results strongly indicated that compound A was a new isomer of 5,12-dihydroxy-6,8,10,14-eicosatetraenoic acid. The UV- and GC/MS-data confirmed that the minor compound (B) was LTB_4 since the results obtained correspond to those described for that compound [5].

Methyl esters of the polar products were further purified on SP-HPLC. The UV-spectrum of a main compound (C) was identical to that of compound A. GC/MS of the Me_3Si derivative of the methyl ester of compound C gave a peak with *C*-value 25.4. The mass spectrum (fig.3) contained ions of high intenstity at *m/e* 582 (M), 567 (M-15), 551 (M-31), 492 (M-90), 481 (M-101, see above), 402 (M-(2 X 90)), 391 (M-(90 + 101)), 383 (M-199, loss of $^{\cdot}CH_2$–CH=CH–$(CH_2)_4$–CH_2OSiMe_3), 354 (see above), 301 ($(Me_3SiO^+$=CH–CH_2–CH=CH–$(CH_2)_4$–CH_2OSiMe_3), 203 (base peak; see above), 171 and 129. This indicates a tetraunsaturated C-20 structure with three hydroxyl groups, located at C-5, C-12 and the third at a position beyond C-13. The relative high intensity

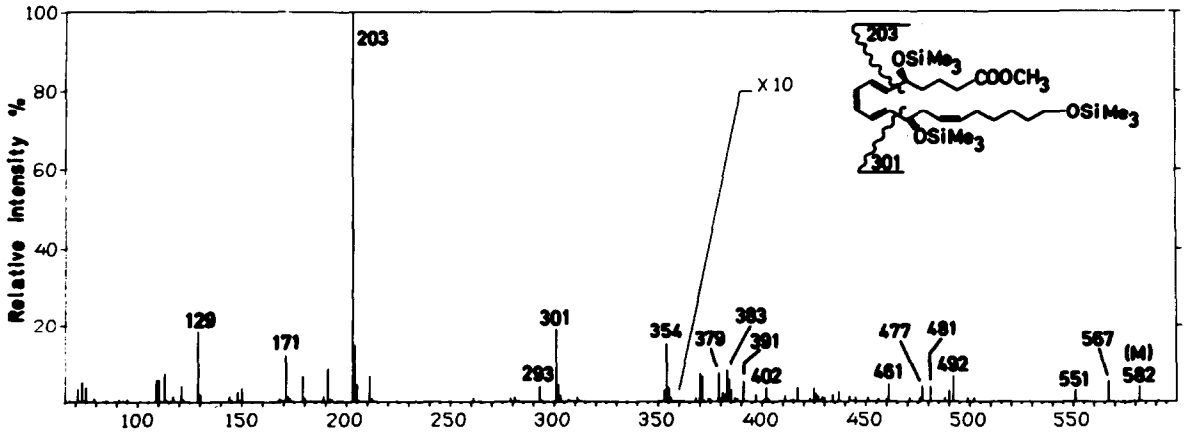

Fig.3. Mass spectrum of the Me_3Si derivative of the methyl ester of compound C.

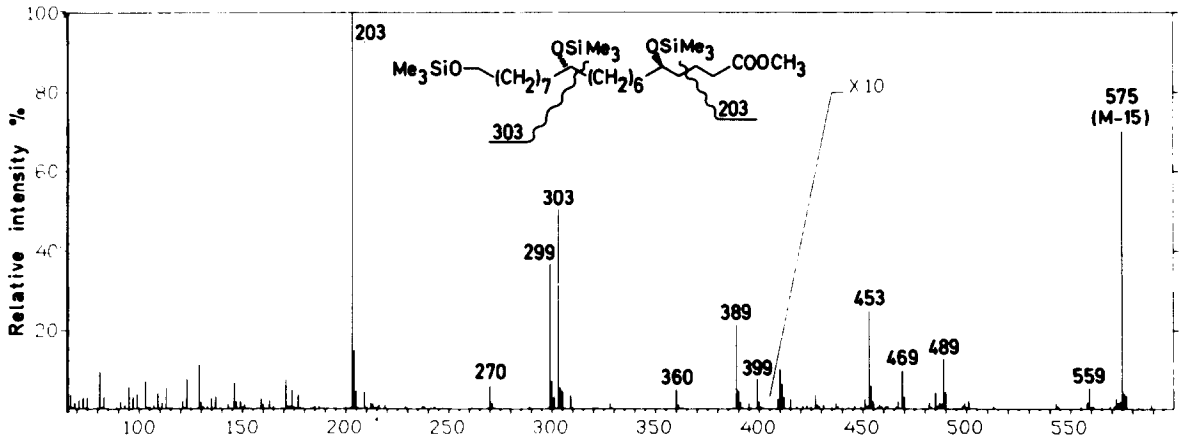

Fig.4. Mass spectrum of the Me_3Si derivative of the methyl ester of hydrogenated compound C.

of ions at *m/e* 203, 301 (i.e., 213 + 88) and 354 indicate that compound C is structurally related to compound A (c.f., fig.2). Evidence for an ωl hydroxylation was obtained from the equivalent chain length (*C*-25.4) which was increased with 3.0 *C* [18,19] as compared to the *C*-value of compound A (*C*-22.4). Thus, the indicated structure of 5,12,20-trihydroxy-6,8,10,14-eicosatetraenoic acid. In agreement with the proposed structure the mass spectrum (fig.4) of the Me_3Si derivative the methyl ester of hydrogenated compound C (*C*-value 27.3) had prominent ions at *m/e* 575 (M-15), 559 (M-31), 489 (M-101), 469 (M-(90 + 31)), 453, 399 (M-(101 + 90)), 389 (M-201, loss of $^{\cdot}CH_2-(CH_2)_6-CH_2OSiMe_3$), 303 ($Me_3SiO^+=CH-(CH_2)_7-CH_2OSiMe_3$), 299 (M-(201 + 90)), 203 (base peak; see above), 129 and 103 ($^{\cdot}CH_2=O^+SiMe_3$). Mass chromatogram of specific fragments generated by the computer [20], showed that the ion at *m/e* 103 (i.e., indicating an ωl hydroxyl group) appeared in a peak which cochromatographed with the total ion current of the pure compound.

As shown in fig.5 the mass spectrum of the trimethylsilyl ether methyl ester of hydrogenated compound A formed in $^{18}O_2$-labeling experiments contained several ions that were shifted 4 units to higher *m/e* values (i.e., *m/e* 491, 475, 405 and 393) while other ions (i.e., *m/e* 313, 301, 217 and 205) were shifted 2 units to higher *m/e* values (cf., fig.3). These

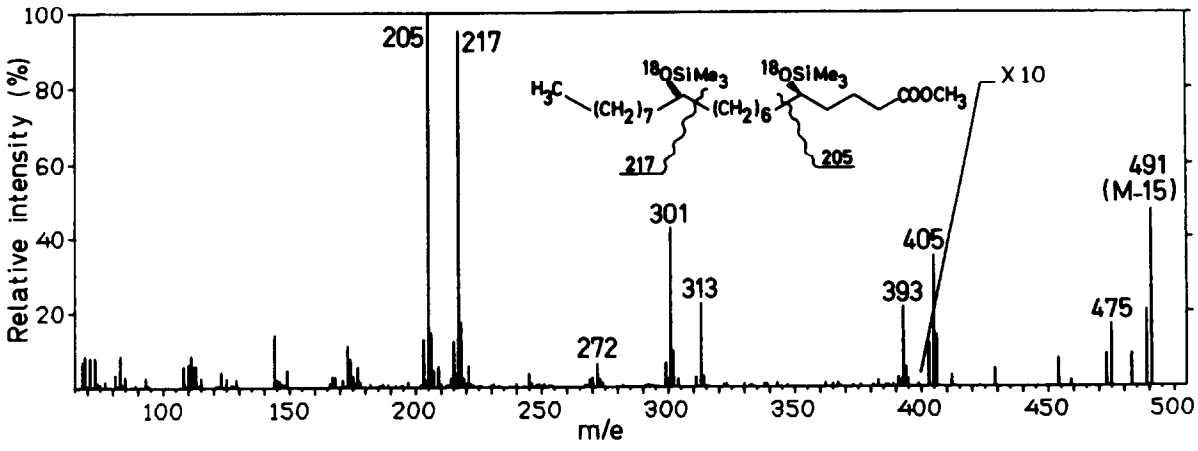

Fig.5. Mass spectrum of the Me_3Si derivative of the methyl ester of hydrogenated compound A formed in labeling experiments with $^{18}O_2$.

data show that compound A carried 2 atoms of ^{18}O, which were located at C-5 and C-12. The results demonstrate that the hydroxyl groups at C-5 and C-12 in compound A were both derived from molecular oxygen. The mass spectrum of the trimethylsilyl ether methyl ester of hydrogenated compound C from the $^{18}O_2$-labeling experiments contained ions that were shifted to higher *m/e* values 6 units (i.e., *m/e* 581, 565, 495), 4 units (i.e., *m/e* 403, 393, 307) or 2 units (i.e., *m/e* 301, 205 and 105). Thus, compound C carried 3 atoms of ^{18}O, showing that the hydroxyl groups of this compound were all derived from molecular oxygen.

The (−)menthoxycarbonyl derivatives of the methyl esters of compounds A and C were subjected to oxidative ozonolysis and the fragments were treated with diazomethane prior to gas chromatographic analysis. The products from both compounds gave peaks corresponding to (−)-menthoxycarbonyl dimethyl *S*-malate and (−)-menthoxycarbonyl dimethyl 2-*S*-hydroxy adipate. These results demonstrate that the structures of compounds A and C are 5*S*,12*S*-dihydroxy-6,8,10,14-eicosatetraenoic acid and 5*S*,12*S*,20-trihydroxy-6,8,10,14-eicosatetraenoic acid, respectively.

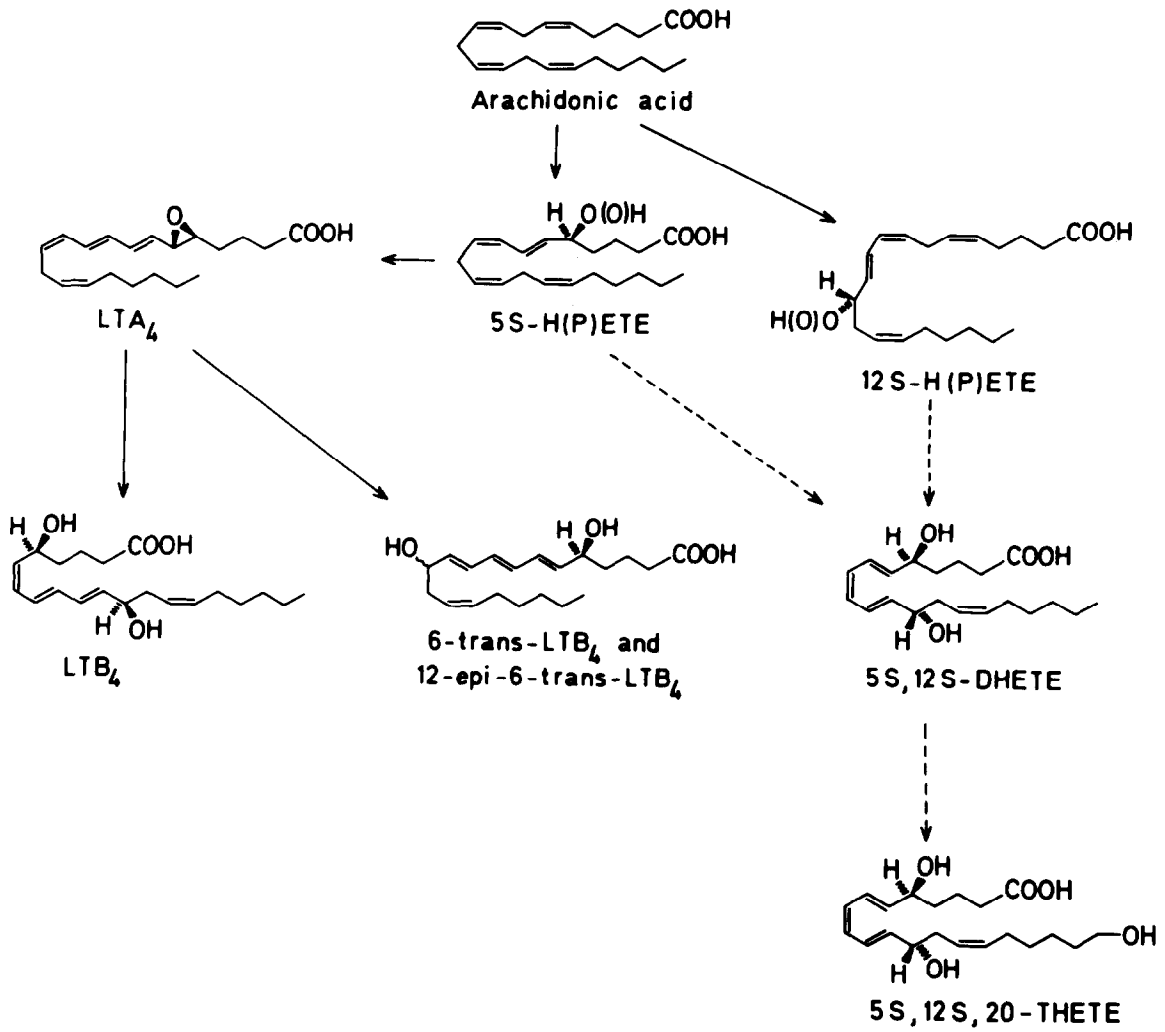

Fig.6. Scheme of transformation of arachidonic acid to 5*S*,12*S*-dihydroxy-6,8,10,14-eicosatetraenoic acid and 5*S*,12*S*-20-trihydroxy-6,8,10,14-eicosatetraenoic acid by preparations of human polymorphonuclear leukocytes. Dashed lines indicate proposed reactions.

4. Discussion

Here we describe the transformation of arachidonic acid into a new leukotriene-like dihydroxy acid and a related trihydroxy acid in a preparation of human polymorphonuclear leukocytes. The structure of the novel dihydroxy acid is 5*S*,12*S*-dihydroxy-6,8,10,14-eicosatetraenoic acid. In addition, an ω1-hydroxylated metabolite, 5*S*,12*S*,20-trihydroxy-6,8,10,14-eicosatetraenoic acid has been identified. Elucidation of the structures was based on chromatographic properties, UV-absorbance and GC/MS-analysis of various derivatives, including hydrogenated compounds.

Oxidative ozonolysis of the (−)-menthoxycarbonyl derivatives of compounds A and C supported the proposed locations of the 4 double bonds and made it possible to determine the absolute configuration of the alcohol groups at C-5 and C-12. The geometry of the double bonds has not yet been demonstrated. However, the chromatographic, UV-absorbance and GC/MS data distinguish the new dihydroxy acid from the two C-12-epimers of 5,12-dihydroxy-6,8,10,14-eicosatetraenoic acid [8] which both have an all-*trans* configuration of the triene. Therefore, the conjugated triene of the compound must contain at least one *cis* double bond since an all-*trans* geometry would make the new metabolite identical to 12-epi-6-*trans*-LTB_4. Because of mass spectrometric similarities it is likely that compound C is formed from compound A via an ω-oxidation and therefore has the same geometry of the double bonds. Labeling experiments with $^{18}O_2$ indicate that the compounds are formed by double and triple oxygenation of arachidonic acid. Incorporation of molecular oxygen at C-5 and C-12 of arachidonic acid in lipoxygenase catalyzed reactions should lead to isomerization of the double bonds *cis*-Δ^5 and *cis*-Δ^{11} to *trans*-Δ^6 and *trans*-Δ^{10}, respectively. Since the double bonds at Δ^8 and Δ^{14} are not involved in these transformations they have probably retained their original *cis* configuration. Therefore, the geometry of the double bonds in both compounds is most likely 6-*trans*,8-*cis*,10-*trans*,14-*cis*.

Since the compounds are not formed from the unstable leukotriene epoxide (LTA_4) they are not regarded as leukotrienes. Instead, the trivial names 5*S*,12*S*-DHETE and 5*S*,12*S*,20-THETE are introduced. It has not been established whether the formation of the compounds is initiated by oxygenation at C-5 or C-12 (see fig.6). Leukocyte preparations are usually contaminated by platelets. Since the lipoxygenase of these cells produce 12-*S*-HETE [21], it is possible that the hydroxylation of C-12 occurs in the platelets while the hydroxylation at C-5 is carried out by the leukocytes. Control experiments with washed human platelets showed that 5*S*,12*S*-DHETE or 5*S*,12*S*,20-THETE could not be produced by platelets alone (not shown). Further investigations are required to determine the cellular origin of the new metabolites.

Additional studies in progress indicate that 5*S*,12*S*-DHETE and 5*S*,12*S*,20-THETE are biologically active. These results will be reported separately.

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