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ω-HYDROXYLATION OF N-ACETYLLEUKOTRIENE E_4 BY RAT LIVER MICROSOMES

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Previous investigations have demonstrated metabolism of leukotriene (LT) C_4 *in vivo* involving transformations of the tripeptide, but not the fatty acid part, yielding N-acetyl LTE_4 as a main biliary metabolite in the rat. In addition, several polar metabolites were detected in the same studies. The present report describes the characterization of a metabolite of N-acetyl LTE_4 formed during incubations with rat liver microsomes. The structure, 5,20-dihydroxy-6-*S*-(2-acetamido-3-thiopropionyl)-7,9-*trans*-11,14-*cis*-eicosatetraenoic acid, of this metabolite showed that it is formed by hydroxylation of the fatty acid part. Preliminary evidence indicates that it is one of several polar metabolites formed *in vivo*.

LTC is metabolized *in vivo* and *in vitro* by alterations of the tripeptide part (1). Successive removal of glutamic acid and glycine and aminoacetylation afforded N-acetyl LTE, originally identified as a fecal metabolite of LTC_4 in the rat (2) and also found endogenously in rat bile after endotoxin induced shock (3). Two kinds of *in vitro* modifications of the fatty acid part of cysteine-containing leukotrienes have been reported viz. conversion of LTC, LTD, and LTE by a 15-lipoxygenase to 15-hydroxy, Δ^{13}-*trans* metabolites (4), and peroxidase mediated oxidation of the sulfur atom in LTC, LTD, and LTE followed by spontaneous degradation to 6-*trans* and 12-*epi*-6-*trans* LTB_4 (5-7). None of these reactions have as yet been demonstrated to occur *in vivo*. In view of extensive formation of polar leukotriene metabolites *in vivo* and *in vitro* (2,3,8-10), it is likely that leukotriene metabolites formed by modifications in the tripeptide part are further metabolized.

Abbreviations: LTB_4, LTC_4, LTD_4, LTE_4, and N-acetyl LTE_4, leukotrienes B_4, C_4, D_4, E_4, and N-acetylleukotriene E_4; RP-HPLC, reverse-phase high performance liquid chromatography; FAB-MS, fast atom bombardment-mass spectrometry; GC-MS, gas liquid chromatography-mass spectrometry; TMS, trimethylsilyl; UV, ultraviolet.

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The present paper describes the characterization of a polar leukotriene metabolite formed during incubations of N-acetyl LTE_4 with rat liver microsomes.

MATERIALS AND METHODS

Chemicals. [5,6,8,9,11,12,14,15-$^3H_8$]LTE_4 and its N-acetyl derivative were prepared as described (2). Synthetic LTE_4 was kindly provided by J. Rokach, Merck-Frosst Canada, Inc.. Glucose-6-phosphate dehydrogenase type XV from Bakers Yeast (EC 1.1.1.49), and NADPH were purchased from Sigma.

Preparation of Subcellular Rat Liver Fractions. Male Sprague Dawley rats (200-250g) were starved for 1-2 days and sacrificed by cervical dislocation. The liver was removed and immediately chilled on ice. It was weighed, minced with scissors and rinsed twice with ice-cold 1.15% aqueous KCl. The mixture was homogenized for 3x15 sec in 2 ml/g tissue of 20 mM Tris-HCl buffer, pH 7.4, containing 0.25 M sucrose, 5 mM EDTA, 1 mM EGTA, and 0.5 mM dithiothreitol, with a Potter-Elvehjelm type homogenizer chilled in an ice-bath. The homogenate was centrifuged at 900xg for 15 min using a Sorvall model RC-2B centrifuge equipped with a SS 34 rotor. The supernatant was removed and centrifuged at 9000xg for 20 min. The resulting supernatant was finally centrifuged at 105,000xg for 75 min in a Beckman model L5-65 ultracentrifuge. The sediments obtained were resuspended in 1 ml/g tissue of 50 mM Tris-HCl (pH 7.4) / 5 mM $MgCl_2$ and immediately used. In some experiments the fractions were resuspended in homogenization buffer and recentrifuged once before use. All steps were performed at 0-4^{0}C.

Incubations. N-acetyl[$^3H_8$]LTE_4 (1-9 µM; 0-20 Ci/mol) was dissolved by sonication in ethanol/water 1:3 (v/v; 3% of final volume) and added to the microsomal suspension. The reaction was started by adding an NADPH-regenerating system (NADPH 2 mM, glucose-6-phosphate 12.5 mM and glucose-6-phosphate dehydrogenase 2.2 U/ml). The incubation was performed at 37^{0}C for 5-60 min with rotation of the incubation flask and under normal atmosphere or under 93% O_2 plus 7% CO_2. The reaction was terminated by adding four volumes of ice-cold methanol. The mixture was left on ice for 2 hours and then centrifuged at 1000xg for 20 min at 4^{0}C. The pellet was washed twice with 5-10 ml of methanol. Supernatants were pooled, evaporated to near dryness under reduced pressure, dissolved in 1-2 ml of 30% aqueous methanol and purified by RP-HPLC. Using this protocol the recovery was 95-99%.

HPLC. Samples were purified by RP-HPLC on columns of C_{18} Nucleosil (250 x 4.5 mm) eluted at a flow rate of 1 ml/min with methanol/water/acetic acid/ phosphoric acid 55:45:0.07:0.03 (v/v/v/v, adjusted to pH 5.4 with NH_4OH; solvent 1) for 30 min and then with methanol/water/acetic acid/ phosphoric acid 72:28:0.07:0.03 (v/v/v/v; solvent 2) for 30 min. Compound I (see Results) was rechromatographed on a C_{18} Nucleosil column eluted with methanol/ water/ acetic acid 55:45:0.1 (v/v/v; solvent 3).

Analytical methods. UV spectra were recorded on a Hewlett-Packard model 8450A spectrophotometer. Reductive desulfurizations and analyses of diethyl ether-extractable products by GC-MS were carried out on a VG 7070E double focusing mass spectrometer connected to a DANI 3800 gas chromatograph (VG Analytical, Manchester, UK). A fused silica column (25 m x 0.31 mm i.d., 0.17 µM dimethyl silicone film, Hewlett Packard, USA) was used at a temperature of 250^{0}C. Injections were performed using an all-glass falling needle system. FAB-MS was performed as described (2).

RESULTS

Metabolism of N-acetyl LTE_4 by liver microsomes. When N-acetyl LTE_4 was incubated with rat liver microsomes in the presence of NADPH for 30 min at 37^0C, three products were formed (Fig.1). The major one (compound I) had a retention time of 0.20 relative to N-acetyl LTE_4 when using solvent 1. The conversion of N-acetyl LTE_4 into compound I required NADPH and was enhanced by ca 60% when the incubation was performed under 93% O_2 plus 7% CO_2 instead of normal atmosphere (Fig.2). The reaction was almost completed after 10 min although 80-90% of the added N-acetyl LTE_4 was unaltered. Readdition of NADPH did not significantly increase the yield of products whereas readdition of microsomes enhanced product formation by ca 50%. The highest yield of compound I, 209 pmol/g wet weight of tissue, was obtained using 105,000xg sediment from liver homogenates; 900xg and 9000xg sediments gave 98 and 94 pmol/g wet weight of tissue, respectively. No product was formed when the 105,000xg supernatant was used.

Structure of compound I. The ultraviolet absorption spectrum of compound I (λ_{max} at 280 nm and shoulders at 270 and 292 nm) was identical to the UV

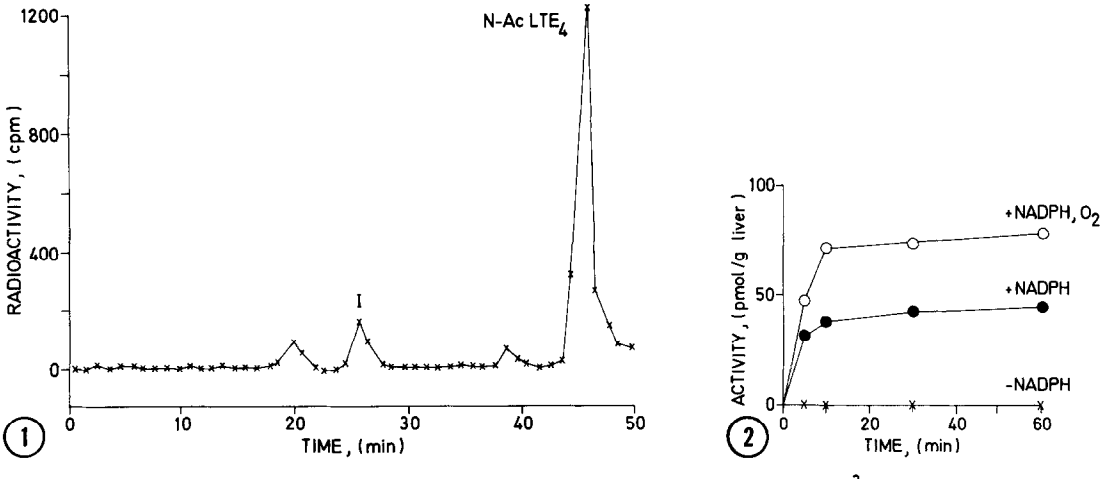

Fig.1. RP-HPLC of products obtained by incubating N-acetyl[$^3H_8$]LTE_4 with a 105,000xg sediment from rat liver. Conditions: Elution was isocratic with solvent 1 from 0-30 min and solvent 2 from 30-50 min.

Fig.2. Time-course for the formation of compound I from N-acetyl LTE_4 in absence of NADPH (X), in presence of NADPH, under normal atmosphere (●), and in presence of NADPH, under 93% O_2 plus 7% CO_2 (O). Conditions: see Materials and Methods.

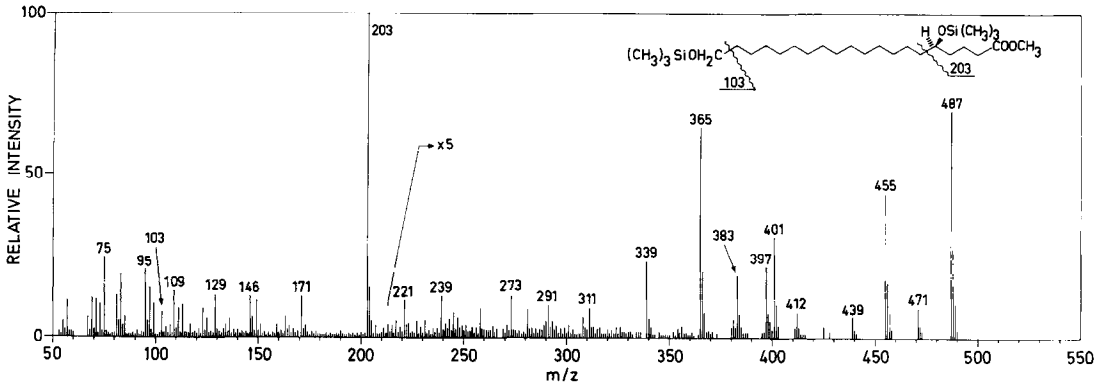

Fig.3. Positive ion EI mass spectrum of the TMS ether, methylester derivative of compound I.

spectrum of N-acetyl LTE_4 (2), suggesting that the conjugated triene at $\Delta^{7,9,11}$ and the allylic thioether substituent at C-6 had been retained. Reductive desulfurization and GC-MS analysis showed an equivalent chain length on GC, for the methylester, TMS ether derivative of the product obtained, of 25.3. This value is 3.6 C longer than the equivalent chain length of the methylester, TMS ether derivative of 5-hydroxyeicosanoic acid (11). This difference is similar to the difference in retention time observed between 5,12,20-trihydroxy- and 5,12-dihydroxy-eicosanoic acid (methylester, TMS ether derivatives; 3.3 C; ref. 12). The mass spectrum of the desulfurized, hydrogenated compound I (methylester, OTMS derivative; Fig.3) showed significant ions at m/z 487 (M-15, loss of $\cdot CH_3$), 471 (M-31, loss of $\cdot OCH_3$), and 455 (M-47, loss of $CH_3OH + \cdot CH_3$). These ions are typical fragment ions of trimethylsilyloxy fatty acid methylesters and indicated that the molecular weight of the compound was 502. The presence of TMS ether groups in the molecule was obvious from ions at m/z 412 (M-90, loss of $\cdot(CH_3)_3SiOH$), 397 (M-15-90), 365 (M-47-90), 129 ($((CH_3)_3Si^+OCH_2CH{=}CH_2$), 75 ($((CH_3)_2Si^+{=}O$), and 73 ($((CH_3)_3Si^+$). The isotope intensity ratios at m/z 487/488 and at m/z 455/456 were 100:39 and 100:38, respectively, suggesting that each of these ions contained two silicone atoms (theoretical values: 100:39.8 and 100:38.6, respectively). The ions at m/z 203 (base peak, $(CH_3)_3SiO^+{=}CH(CH_2)_3COOCH_3$), 171 (203-32), and 113 (probably 203-90) demonstrated that one trimethylsilyloxy group was located at

C-5. Ions at m/z 401 (M-101, loss of $\bullet(CH_2)_3COOCH_3$), 311 (M-101-90), and 221 (M-101-2x90) indicated that the second trimethylsilyloxy group was located somewhere between C-6 and C-20. A TMS ether group at C-20 would give rise to ions at m/z 103 ($CH_2=O^+Si(CH_3)_3$, due to α-cleavage, ref. 13,14) and at m/z 146 ($CH_2=C^+(OCH_3)OSi(CH_3)_3$, due to McLafferty rearrangement of the ω-TMS ether group and the carboxyl group, ref. 14). Both of these ions were present in the mass spectrum shown in Fig.3. Computer generated mass chromatograms confirmed that the ion intensities at m/z 103 and m/z 146 had maxima at the same retention time as the pure compound. The UV spectrum of compound I excluded that the second hydroxyl group was located between carbon atoms 5 and 15 (c.f. 11). Carbon atoms 6, 12, and 15 were also excluded by the absence of mass spectral ions at m/z 299 and 276 (15), 389 and 215 (16), and 431 and 173 (17). Finally, positions C-16, C-17, C-18, or C-19 would give rise to significant ions at m/z 159, 145, 131, and 117, respectively, due to α-cleavage. Of these ions only one of low intensity at m/z 159 was present in the mass spectrum in Fig.3. This ion is most likely explained by a rearrangement of the methylester, TMS ether derivatized ω-hydroxy fatty acid ($CH_2=CH-C(OSi(CH_3)_3)=O^+CH_3$, ref. 14). Therefore, the mass spectrum in Fig.3 is only compatible with the structure 5,20-dihydroxyeicosanoic acid of the underivatized compound.

Negative ion FAB-MS of compound I (Fig.4) showed ions of significant intensities at m/z 496, 518, and 534. These were interpreted as representing $[M-H]^-$, $[M-2H+Na]^-$, and $[M-2H+K]^-$, respectively (2,18), suggesting that the molecular weight of compound I was 497. This is consistent with the introduction of an oxygen atom in the substrate, N-acetyl LTE_4 (mol. wt. 481). The

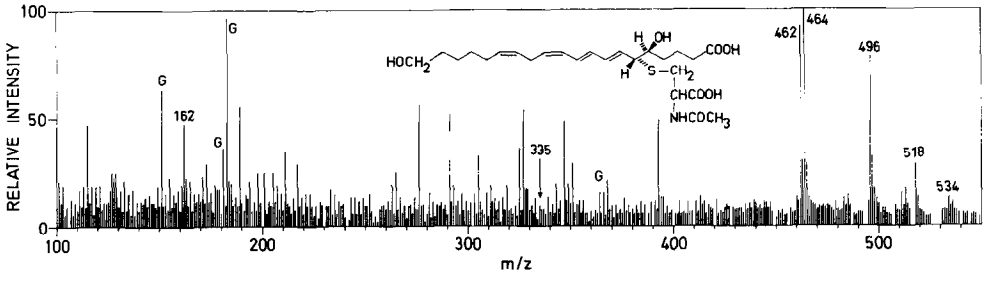

Fig.4. Negative ion FAB mass spectrum of compound I. Ions designated G are due to the glycerol matrix. Conditions: see ref. 2.

FAB-MS data thus agreed with the GC-MS identification of 5,20-dihydroxy acid and further indicated that compound I had retained the N-acetylcysteine substituent and the four double bonds of N-acetyl LTE_4. A major ion was seen at m/z 162 $[CH_3CONHCH(CH_2S\bullet)COOH]^-$ and small ions were observed at m/z 335 $[M-162]^-$, 349 [M-130-18, loss of $CH_3CONHCH(CH_2)COOH$ plus $H_2O]^-$, and 367 $[M-130]^-$. The former two ions are most likely formed by cleavage of the C-S bond between C-6 of the fatty acid and sulfur of the N-acetylcysteine group and the latter two by cleavage of the C-S bond between the sulfur atom and C-3 of the N-acetylcysteine group (cf. 2,18), supporting the presence of an N-acetylcysteine moiety in the molecule. The ion at m/z 367 may also be derived from the glycerol matrix (according to: $91+(92)_n$), but the absence of an analogous ion at m/z 275 makes this less likely. Some fragments in the mass spectrum shown in Fig.2 viz. at m/z 462, 347, 327, and 115 were due to impurities eluting shortly before compound I on RP-HPLC. Ions at m/z 464, 393, 305, 291, 277, and 189 are probably also derived from impurities. The FAB mass spectrum of compound I is consistent with the structure 5,20-dihydroxy-6-*S*-(2-acetamido-3-thiopropionyl)-eicosatetraenoic acid. It is likely that the double bonds have retained their original position and geometry and that the stereochemistry at C-5 and C-6 is also unchanged.

Based on the results described above it is concluded that the chemical structure of compound I is 5(S),20-dihydroxy-6(R)-*S*-(2-acetamido-3-thiopropionyl)-7,9-*trans*-11,14-*cis*-eicosatetraenoic acid (20-hydroxy N-acetyl LTE_4).

DISCUSSION

The present report describes the identification of a novel leukotriene metabolite, 20-hydroxy N-acetyl LTE_4, which was formed when N-acetyl LTE_4 was incubated with rat liver microsomes in the presence of NADPH and oxygen (Fig.5). The characterization of this metabolite may provide a metabolic pathway for the conversion of cysteine-containing leukotrienes into polar, hitherto unidentified, metabolites which are formed from LTC under *in vivo* and *in vitro* conditions. GC-MS data of the product obtained after reductive

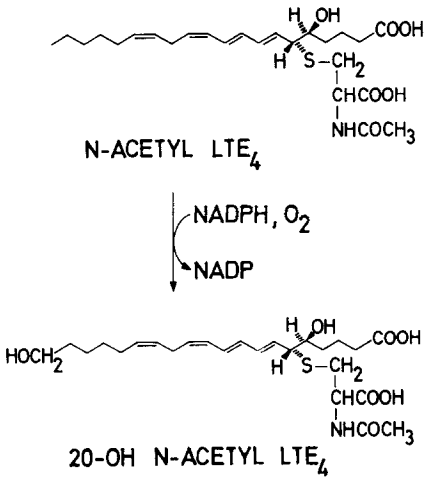

Fig.5. Conversion of N-acetyl LTE_4 to 20-hydroxy N-acetyl LTE_4 by rat liver microsomes.

desulfurization in conjunction with UV spectroscopy and FAB-MS of intact, underivatized compound I indicated that a hydroxyl group had been introduced at C-20 in N-acetyl LTE_4. The formation of this metabolite required NADPH and the rate of the reaction increased with increasing oxygen concentration. It was also enhanced by starvation of the rats, suggesting that a microsomal liver cytochrome P_{450} catalyzed the hydroxylation reaction (cf. 19). The N-acetyl LTE_4 hydroxylase activity was present predominantly in the microsomal fraction. The enzyme was quite labile: EDTA, EGTA, and dithiothreitol protected against inactivation during the preparation of microsomes. The enzymatic activity was also destroyed within 10 min at 37^0C during incubations with N-acetyl LTE_4. ω1-Oxidation of fatty acids (19) and prostaglandins (20) occur in kidney, liver, and lung microsomes. Human polymorphonuclear leukocytes transform LTB_4, 5(S),12(S)-dihydroxy eicosatetraenoic acid (12,21), and 12-HETE (22) to ω1-hydroxy products. Enzymatic ω-hydroxylation of cysteine-containing leukotrienes has, however, not been previously demonstrated. The enzyme catalyzing formation of 20-hydroxy N-acetyl LTE_4 seems to be different from the LTB_4 ω1-hydroxylase because a 60-fold excess of LTB_4 did not inhibit the ω1-hydroxylation of N-acetyl LTE_4 by rat liver microsomes. Preliminary evidence indicates that 20-hydroxy N-acetyl LTE_4 is formed *in vivo* in rats given [$^3H_8$]LTC_4 i.v.. The importance of the ω1-

hydroxylase reaction for the formation of other polar LTC_4 metabolites *in vivo* are currently being investigated in our laboratory.

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REFERENCES

1. Hammarström,S., Örning,L., and Bernström,K., (1985) Mol. Cell. Biochem. 69, 7-16.
2. Örning,L., Norin,E., Gustafsson,B., and Hammarström,S., (1986) J. Biol. Chem. 261, 766-771.
3. Hagman,W., Denzlinger,C., Rapp,S., Weckbecker,G., and Keppler,D., (1986) Prostaglandins 31, 239-251.
4. Örning,L., and Hammarström,S. (1983) FEBS Lett. 153, 253-256.
5. Goetzl,E.J. (1982) Biochem. Biophys. Res. Commun. 106, 270-275.
6. Hendersson,W., and Klebanoff,S.. (1983) Biochem. Biophys. Res. Commun. 110, 266-272.
7. Lee,C., Lewis,R., Tauber,A., Mehrotra,M., Corey,E.J., and Austen,K.F. (1983) J. Biol. Chem. 258, 15004-15010.
8. Örning,L., Kaijser,L., and Hammarström,S., (1985) Biochem. Biophys. Res. Commun. 130, 214-220.
9. Appelgren,L.-E., and Hammarström,S., (1982) J. Biol. Chem. 257, 531-535.
10. Ormstad,K., Uehara,N., Orrenius,S., Örning,L., and Hammartröm,S., (1982) Biochem. Biophys. Res. Commun. 104, 1434-1440.
11. Murphy,R., Hammarström,S., and Samuelsson,B., (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 4275-4279.
12. Lindgren,J-Å., Hansson,G., and Samuelsson,B., (1981) FEBS Lett. 128, 329-335.
13. Oliw,E., Fahlstadius,P., and Hamberg,M., (1986) J. Biol. Chem. 261, 9216-9221.
14. Eglinton,G., Hunneman,D., and McCormick,A. (1968) Org. Mass. Spec. 1, 593-611.
15. Borgeat,P., and Samuelsson,B., (1979) J. Biol. Chem. 254, 7865-7869.
16. Borgeat,P., and Samuelsson,B., (1978) J. Biol. Chem. 254, 2643-2646.
17. Maas,R., Turk,J., Oates,J., and Brash,A., (1982) J. Biol. Chem. 257, 7056-7067.
18. Murphy,R., Mathews,R., Rokach,J., and Fenselau,C., (1982) Prostaglandins 23, 201-206.
19. Ellin,Å., and Orrenius,S. (1975) Mol. Cell. Biochem. 8, 69-79.
20. Oliw,E., Granström,E., and Änggård,E. (1983) In Prostaglandins and related substances (C.Pace-Asciak and E.Granström, eds.) pp. 1-44 Elsevier, Amsterdam.
21. Hansson,G., Lindgren,J.-Å., Dahlen,S.-E., Hedqvist,P., and Samuelsson,B. (1981) FEBS Lett 130, 107-112.
22. Wong,P., Westlund,P., Hamberg,M., Granström,E., Chao,P., and Samuelsson,B. (1984) J. Biol. Chem. 259, 2683-2686.