Characterization and biological effects of di-hydroxylated compounds deriving from the lipoxygenation of alpha-linolenic acid.

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Running title: Di-hydroxylated metabolites from ALA

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Abbreviations: ALA, alpha-linolenic acid (octadeca-9,12,15-trienoic acid); BSTFA, *N*,*O*-Bis(trimethylsilyl)-trifluoroacetamide; COX, cyclooxygenase**;** DHA, docosahexaenoic acid; EI, electron impact ionization; EPA, eicosapentaenoic acid; HOTE, hydroxy-octadecatrienoic acid; HpOTE, hydroperoxy-octadecatrienoic acid; LOX, lipoxygenase; LTA_4, leukotriene A_4; LTB_4, leukotriene B_4; $NaBH_4$, sodium borohydride; NICI, negative-ion chemical ionization; NMR, nuclear magnetic resonance; PDX, protectin DX; PMN, polymorphonuclear leukocytes; PtO_2, platinum oxide; PUFA, polyunsaturated fatty acids; RP-HPLC, reverse phase high-performance liquid chromatography; RT, retention time; sLOX, soybean 15-lipoxygenase; TMS, trimethylsilyl; 9,16-di-HOTE, 9,16-dihydroxyoctadecatrienoic acid;

ABSTRACT

We have recently described a di-hydroxylated compound called protectin DX (PDX) which derives from docosahexaenoic acid (DHA) by double lipoxygenation. PDX exhibits anti-aggregatory and anti-inflammatory properties, that are also exhibited by similar molecules, called poxytrins, which possess the same *E,Z,E* conjugated triene geometry, and are synthesized from other polyunsaturated fatty acids with 22 or 20 carbons. Here we present new biological activities of di-hydroxylated metabolites deriving from alpha-linolenic acid (18:3n-3) treated by soybean 15-lipoxygenase (sLOX). We show that 18:3n-3 is converted by sLOX into mainly 13(S)-OH-18:3 after reduction of the hydroperoxide product. But surprisingly, and in contrast to DHA which is metabolized into only one di-hydroxylated compound, 18:3n-3 leads to four di-hydroxylated fatty acid isomers. We report here the complete characterization of these compounds using high field NMR and GC-MS techniques, and some of their biological activities. These compounds are: 9(R),16(S)-dihydroxy-10*E*,12*E*,14*E*-octadecatrienoic, 9(S),16(S)-dihydroxy-10*E*,12*E*,14*E*-octadecatrienoic, 9(S),16(S)-dihydroxy-10*E*,12*Z*,14*E*-octadecatrienoic and 9(R),16(S)-dihydroxy-10*E*,12*Z*,14*E*-octadecatrienoic acids. They can also be synthesized by the human recombinant 15-lipoxygenase (type 2). Their inhibitory effect on blood platelet and anti-inflammatory properties were compared to those already reported for PDX.

Supplementary key words: Octadecatrienoic acid • 15-lipoxygenase • Platelet aggregation • Anti-inflammatory activity

INTRODUCTION

N-3 polyunsaturated fatty acids (PUFA) have been well studied during the last decades since they regulate *in vivo* many physiological functions. It is assumed that long-chain n-3 PUFA have beneficial effects on cardiovascular and coronary artery diseases (1, 2), rheumatoid arthritis (3-5), blood pressure control and heart hypertension (6, 7), and they might prevent multiple sclerosis and Alzheimer's diseases (8-11). More recently, new lipid mediators deriving from eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids have been described. Among them, resolvins are oxygenated molecules that derive from EPA and DHA (12, 13) and are separated into two series: E-series (from EPA) and D-series (from DHA) (14). In addition to the D-series resolvins, protectin D1 (PD1) is produced from DHA *via* a mechanism involving an epoxide intermediate (15), as well as maresin which can be produced by macrophages (16). PD1 has been described as potent anti-inflammatory agents without anti-aggregatory effect (15, 17). In contrast, protectin DX (PDX), an isomer of PD1, which derives from DHA *via* a double lipoxygenation, reveals inhibitory effects on blood platelet aggregation. PDX inhibits platelet cyclooxygenase (COX)-1 and antagonizes the thromboxane A_2-induced aggregation (18). We found that di-hydroxylated compounds called poxytrins, synthesized from C20 and C22 PUFA *via* the soybean 15-lipoxygenase (sLOX) and having the same *E*,*Z*,*E* conjugated triene motif, including PDX, are all anti-aggregatory agents (19). On the other hand, very few data are available concerning the oxygenated metabolism of alpha-linolenic acid (18:3n-3 or ALA). Investigations relating to ALA are particularly relevant since this fatty acid is taken in large amount (1-2 g/day in human adults) as a nutrient and hardly accumulates in plasma, suggesting an active metabolism. Moreover, its conversion into EPA and DHA is relatively limited. A conversion of ALA into its long-chain product DHA was found around 1% in men (20) and 2% in young women (21). Formation of 9,16-dihydroxyoctadecatrienoic acid (9,16-di-HOTE) isomers from ALA by sLOX-2 have already been reported first in 1984 by Vliegenthart *et al*. (22) without establishing the double bond configuration nor the stereochemistry of the asymmetric carbons. Further studies presented by Sok and Kim (23, 24) reported four isomers but again their characterisation was not fully established and especially that of the all trans isomers. In 1991 Grechkin *et al*. (25) showed that potato tuber lipoxygenase (LOX) generated mainly

9(S)-hydroperoxy-octadeca-10*E*,12*Z*,15*Z*-trienoic acid (9(S)-HpOTE) which is different from the sLOX which produces mainly 13(S)-hydroperoxy-9*Z*,11*E*,15*Z*-octadecatrienoic acid (13(S)-HpOTE). Also, two compounds after reduction by $NaBH_4$ were characterized as 9,16-di-HOTE (10*E*,12*Z*,14*E*) except for the stereochemistry of the carbon 16 which remains undetermined and trans isomers were not detected. Moreover, the biological properties of the 9,16-di-HOTE isomers have not been investigated yet. Since ALA is the most abundant n-3 PUFA in human diet, it is relevant to investigate more thoroughly its oxygenated metabolism, especially by 15-LOX that is expressed in blood leukocytes and most endothelial cells, and compare the biological activity of the metabolites to that of PDX, the main 15-LOX product of DHA.

MATERIALS AND METHODS

Materials

ALA, sLOX (E.C. 1.13.11.12, Type 1-B, 131 000 units/mg), platinum oxide (PtO_2), *N,O*-bis(trimethylsilyl)-trifluoroacetamide (BSTFA), COX-1 and COX-2 (E.C. 1.14.99.1) were from Sigma-Aldrich. Human recombinant 15-LOX-2 (0.23 units/mg), prostaglandin D_2-d_4 and prostaglandin E_2-d_4 were from Cayman. 9(S)-hydroxy-10*E*,12*Z*,15*Z*-octadecatrienoic acid (9(S)-HOTE), 9(S)-HpOTE, 13(S)-hydroxy-9*Z*,11*E*,15*Z*-octadecatrienoic acid (13(S)-HOTE) were from Interchim. Organic solvents were from Carlo-Erba. All chemicals used were reagent grade or with the highest quality available.

Biosynthesis of 9,16-dihydroxy-ALA derivatives

ALA was incubated with sLOX (Type 1-B) in sodium-borate buffer. Synthesized hydroperoxides were reduced by sodium borohydride ($NaBH_4$) as previously described (18). After acidification, mono- and di-hydroxylated fatty acids were extracted on a C18 solid-phase cartridge as previously described (18). Commercial 9(S)-HOTE, 9(S)-HpOTE and the home-made racemic 9(±)-HOTE chemically produced by oxygen treatment of ALA and isolated by reverse phase high-performance liquid chromatography (RP-HPLC), were further treated by sLOX in order to generate 9,16-di-HOTEs with defined stereochemistry.

Purification of mono- and di-hydroxylated fatty acids

Mono- and di-hydroxylated fatty acids were analyzed by RP-HPLC on a Waters XBridge C18 column (4.6 × 250 mm, 3.5 µm) by using a linear solvent gradient: solvent A was a mixture of acetonitrile/water acidified to pH 3 (10/90, v/v), and solvent B was acetonitrile. The flow was set at 1 mL/min. Mono- and di-hydroxylated fatty acids were detected with a diode array detector at 235 nm and 270 nm, respectively, and collected separately.

GC-MS analysis of mono- and di-hydroxylated fatty acids

Mono- and di-hydroxylated fatty acids isolated by RP-HPLC were hydrogenated using PtO_2 as a catalyst, and then derivatized into methyl esters and TMS ethers. Samples were then analyzed by GC-MS using the electron impact ionization (EI) mode in order to localize the hydroxyl groups on the fatty chain (18).

Nuclear magnetic resonance (NMR) of 9, 16-di-HOTEs

The NMR experiments were performed on a Bruker-Biospin NMR spectrometer operating at 23.4 T (1000 MHz of Proton Larmor frequency) and equipped with a Cryoprobe. All the spectra were acquired in a $CDCl_3$ solution, working at 298 K and referencing the chemical shifts to tetramethylsilane as an internal standard. Standard sequences were used for 1D, DQF COSY (26-28) and J-resolved (29-31) spectra. The ^1H pulse length was 9.0 µs, 1 s and 5 s were used as recycle delay for J-resolved and COSY spectra. A weak selective decoupling of 270 Hz of power during the acquisition time (900 ms) was used in 1D selective homo-decoupling experiments, to simplify some coupling patterns. 2D DQF COSY was acquired with 8 scans and 2048 × 1024 real points (F2 × F1) with acquisition times of 114 ms and 57 ms for the direct and indirect dimensions. J-resolved 2D was acquired with 16 scans and 8192 × 256 real points, acquiring 458 ms in the direct dimension and 1280 ms in the indirect, J-coupling, dimension. COSY spectra were processed with a 4096 × 4096 point matrix using square cosine and square sine window function for the direct and indirect dimensions. J-resolved 2D spectra were processed with an 8192 × 2048 point matrix with square cosine window function for both the dimensions.

Incubation of ALA, 9(S)-HOTE and 9(±)-HOTE with human recombinant 15-LOX-2

ALA, 9(S)-HOTE and 9(±)-HOTE were incubated separately with human recombinant 15-LOX-2 in Tris-HCl buffer as previously described (32, 33). Resulting hydroperoxides were reduced by $NaBH_4$. Lipids were extracted after acidification to pH 3 with acetic acid and di-hydroxylated metabolites were purified as described above.

Preparation of cell suspensions

Platelet suspensions: Venous blood from healthy volunteers who had not taken any medication for at least one week was collected at the local blood bank ("Etablissement Français du Sang") (EFS). Blood was drawn onto anticoagulant and centrifuged at 200 g for 15 min at 20°C to obtain the platelet-rich plasma (PRP) upper phase, and platelets were isolated as described previously (34). Briefly, PRP was acidified to pH 6.4 with 0.15 M citric acid and immediately centrifuged at 900 g for 10 min, and platelet pellets were suspended into Tyrode-HEPES buffer (140 mM NaCl, 2.7 mM KCl, 1.0 mM $MgCl_2$, 12 mM $NaHCO_3$, 0.41 mM NaH_2PO_4, 5 mM HEPES, and 5.5 mM glucose, pH 7.35).

Leukocyte suspensions: Leukocytes were isolated from blood obtained from EFS, as previously described (35). Dextran (2% final concentration) was added to the lower phase obtained after the first blood centrifugation at 200 g. Erythrocytes were then sedimented under gravity for 45 min at 15°C. The erythrocyte-depleted supernatant containing leukocytes was centrifuged at 400 g for 10 min. The resulting leukocyte-rich pellets were re-suspended into saline buffer, and layered over a Ficoll-Paque Plus (density 1.077). After centrifugation, the fraction above the erythrocyte layer was removed and centrifuged, and re-suspended in water for 20 s to lyse the contaminant erythrocytes. Osmotic pressure was immediately restored with NaCl, followed by centrifugation, which provided a white pellet consisting of whole leukocytes that was re-suspended into Tyrode-HEPES buffer. The morphological examination and trypan blue exclusion tests were performed to determine the cell count and purity, and evaluate the viability of neutrophils.

Measurement of platelet aggregation and incubation with human leukocytes

Blood platelet aggregation was determined by the turbidimetric method of Born (36) using a dual aggregometer (ChronoLog, Havertown, PA, USA). To measure changes in the light transmission rate, 400 µL of platelet suspension was heated at 37°C for 1 min with stirring, incubated in the presence or absence of di-hydroxylated compounds (added in 1 µL of ethanol) for 1 more min, and then with collagen as a platelet aggregatory agent for 4 min at 37°C. Concentrations of collagen were adjusted to obtain nearly 75% aggregations for control platelets.

Isolated human leukocytes, suspended in a Tyrode-HEPES buffer containing 2 mM Ca^{2+} were pre-incubated in the presence or absence of 1 µM of different 9,16-di-HOTE isomers for 3 min at 37°C, and triggered with 1 µM ionophore A23187 plus 10 µM arachidonic acid for 10 min. The reaction was stopped by acidifying the media to pH 3 with acetic acid. The oxygenated metabolites were then extracted as previously described (18).

GC-MS measurement of purified cyclooxygenase 1 (COX-1) and cyclooxygenase 2 (COX-2) products

Commercial COX-1 (40 units) and COX-2 (40 units) were pre-incubated for 15 min in a 0.1 M Tris-HCl buffer pH 8.1 with or without 1 µM of different di-hydroxylated metabolites and incubated for 10 min with 10 µM arachidonic acid used as a substrate. Albumin (45 g/L) was added at the end of incubation to facilitate the formation of prostaglandin D_2 (PGD_2) and E_2 (PGE_2) from PGH_2 (37). After acidification to pH 3 with acetic acid, PGE_2 and PGD_2 were extracted by 10 volumes of diethyl ether stabilized by 10% ethanol. Deuterated PGD_2 and E_2 used as internal standards were added for the quantification. Prostaglandins were derivatized into methoxime, pentafluorobenzyl (PFB) esters and TMS and further analyzed by negative-ion chemical ionization (NICI) GC-MS. PGD_2 and E_2 were measured using the SIM mode. Selected ions corresponding to $[M-181]^-$ (loss of the PFB group): m/z: 524 for both derivatized PGD_2 and E_2 and m/z: 528 for their corresponding deuterated internal standards (38, 39) were measured.

Statistical analysis

All results are expressed as means ± standard error of the mean (SEM) or means ± standard deviation (SD). $P < 0.05$ was set as the level of significance as determined by 2-tailed Student's t tests. One-way ANOVA analysis was used to assess differences between groups.

RESULTS

Characterization of metabolites obtained from ALA incubated with sLOX

From ALA incubated with sLOX as described in Materials and Methods, hydroxylated derivatives analyzed by RP-HPLC provided one main compound detected at 50 min with a λ_{max} of 235 nm (Fig. 1A). Its retention time and its UV spectrum were similar to that of commercial 13(S)-HOTE (result not shown).

Regarding di-hydroxylated products (Fig. 1B), and in contrast to DHA which is only converted into one major di-hydroxylated metabolite called PDX, four di-hydroxylated isomers were detected from ALA. They all exhibited characteristic UV spectra with a maximum absorption (λ_{max}) at 270 nm with two shoulder peaks at 260 nm and 280 nm, indicating the presence of a conjugated triene. Moreover, the UV spectra of the two first isomers eluted at 32.1 and 32.6 min are superimposable to that of commercial 12-epi-all trans-leukotriene B_4 (12-epi-all trans LTB_4) with an *E*,*E*,*E* conjugated triene geometry (result not shown), whereas the UV spectra of the two other isomers eluted at 33.2 and 33.5 min are superimposable to that of PDX, suggesting an *E*,*Z*,*E* geometry of these conjugated trienes (Fig. 1B). The spectra of these four isomers differ from those with a Z,*E*,*E* configuration such as LTB_4 and 12-epi-LTB_4 (result not shown).

Localization by GC-MS of hydroxyl groups on the fatty chain

Mono- and di-hydroxylated compounds from ALA mentioned above were hydrogenated and derivatized as methyl esters and TMS ethers as described in "Materials and Methods" and analyzed by GC-MS using the EI mode.

The EI mass spectrum of the hydrogenated mono-hydroxylated ALA derivative (Fig. 2A) shows characteristic ions at m/z: 371 (M-15), (loss of CH_3), 315 (M-71) which corresponds to the loss of CH_2-$(CH_2)_3$-CH_3, 173 (TMS-O-CH-$(CH_2)_4$-CH_3). Such a fragmentation pattern clearly indicates the presence of the hydroxyl group on carbon 13 of the fatty chain. So ALA was mainly converted into 13(S)-hydroperoxy-9*Z*,11*E*,15*Z*-octadecatrienoic acid (13-HpOTE), further reduced by $NaBH_4$ into hydroxylated product for analytical purposes.

All the four hydrogenated di-hydroxylated compounds from ALA exhibited the same mass spectra (Fig. 2B). Their fragmentation pattern shows the following characteristic ions at m/z: 459 (M-15, loss of CH_3), 445 (M-29, loss of CH_2-CH_3), 317 (M-157, loss of CH_2-$(CH_2)_6$-$COOCH_3$), 259 (TMS-O-CH-$(CH_2)_7$-$COOCH_3$), 131 (TMS-O-CH-CH_2-CH_3) and 73 (TMS) which indicates an oxygenation at carbons 9 and 16.

Determination of carbon stereochemistry of di-hydroxylated metabolites synthesized from ALA

Commercial 9(S)-HOTE and racemic 9(±)-HOTE, prepared as described in “Materials and Methods”, were incubated separately with sLOX as described with ALA, and further reduction by $NaBH_4$. 9(S)-HOTE was metabolized into a main compound eluted at 33.4 min (Fig. 3A). This peak was attributed to 9(S),16(S)-di-HOTE because sLOX produces almost exclusively the S enantiomer *via* a double dioxygenation mechanism (40), although soybean LOX generates almost exclusively 13(S)-HpOTE from ALA (40) as it was already shown (Fig. 1A). On the other hand, 9(R),16(S)-di-HOTE peak, eluting at 33.7 min, was also formed (Fig. 3B) when racemic 9(±)-HOTE was incubated with sLOX, compared to 9(S)-HOTE. The two diastereoisomers 9(S),16(S)-di-HOTE and 9(R),16(S)-di-HOTE eluted at 33.4 min and 33.7 min (Fig. 3B), respectively, exhibited the same intensity in agreement with the equivalent amounts of 9(S) and 9(R)-HOTE present in the racemic mixture. The latter chromatogram (Fig. 3B) is similar to that presented in Figure 1B, suggesting that the two peaks (RT 33.2 and 33.5 min, Fig. 1B) observed with ALA as a substrate correspond to the two diastereoisomers 9(S),16(S)-di-HOTE and 9(R),16(S)-di-HOTE, respectively.

This assignment was also confirmed by the fact that chromatograms of di-hydroxylated isomers from 9(S)-HpOTE and 9(S)-HOTE were almost identical, but different from that with ALA as a substrate (Fig. 4). As a matter of fact, another major *E*,*Z*,*E* product (RT 33.5 min, see also Fig 1B) is likely 9(R),16(S)-di-HOTE. In addition, the same compound appeared when the racemic 9(±)-HOTE was incubated with sLOX (Fig. 3B, RT 33.7 min).

However, we may assume that the two all trans diastereoisomers, synthesized from ALA, as this will be confirmed by NMR below, (RT 32.1 and 32.6 min) (Fig. 1B) were likely to be all trans 9(R),16(S)-di-HOTE and 9(S),16(S)-di-HOTE, respectively, according to the separation of 8(R),15(S)- and 8(S),15(S)-all trans-diHETEs reported in Ref. 41 and 42. Such an elution order has also been established for the separation of 5(S),12(R)- and 5(S),12(S)-all trans LTB_4 and 5(S),12(R)- and 5(S),12(S)-all trans LTB_5 diastereoisomers (43-45).

NMR determination of the conjugated triene double bound geometry

A NMR investigation was performed to determine the *E*/*Z* configuration in the triene moiety. A change in the *E*/*Z* configuration significantly modifies the ^1H-^1H J-couplings among the protons bound to the sp^2 carbons in C=C double bonds, assuming values around ~17 Hz for *E* configuration and around ~10 Hz for *Z* configuration (46). Furthermore, molecules like 9(S),16(S)-dihydroxy-10*E*,12*Z*,14*E*-octadecatrienoic acid (33.2 min, Fig. 1B) and 9(R),16(S)-dihydroxy-10*E*,12*Z*,14*E*-octadecatrienoic acid (33.5 min, Fig. 1B), which only differ for the inversion of the stereochemical configuration at the chiral center of position 9, should show very similar spectra with minor differences in chemical shift and J-couplings, especially in the triene region. On this basis, the inspection of the 1D NMR spectra, and in particular of the triene resonance region (4-7 ppm) allows us to rapidly distinguish among the ensemble of molecules having the same triene configuration (e.g. the *E*,*E*,*E* compounds (32.1 min and 32.6 min, Fig.1B) from the *E*,*Z*,*E* ones (33.2 min and 33.5 min, Fig.1B)), as it can be seen in Fig. 5 and Fig. 6. The resonance assignment has been determined by using DQF COSY, while a large part of the J-couplings were measured through the analysis of 2D J-resolved spectra (data not shown). In such a way, it was possible to determine that the ^1H-^1H ^3J-couplings between protons in position 10-11 and 14-15 is around 15 Hz for all the examined cases, evidencing that the protons are in *trans* position with respect to the double bond. The determination of the ^1H-^1H ^3J-coupling among protons 12 and 13 is complicated by the *quasi*-equivalence of their chemical shifts (strong-coupling condition) and by the presence of additional couplings with the closer protons 11 and 14. To simplify the problem, we observed the resonances of 12 and 13 in the 1D spectra of 9(R),16(S)-dihydroxy-10*E*,12*Z*,14*E*-octadecatrienoic acid (33.5 min, Fig.1B) upon selective decoupling of the protons 11 and 14. This made a significant simplification of the multiplet pattern making evident a $^3J_{12\text{-}13}$ coupling of ~10 Hz typical of *Z* configuration. The same approach could not be done in the case of the *E,E,E* molecules because of the strong overlap among the protons 11,12,13,14 that have a very similar chemical shift. Nevertheless, since the determination of the configuration in the *E,Z,E* molecules is demonstrated above, we can safely assign by exclusion the configuration of the *E,E,E* molecules on the bases of the marked differences among the spectra of *E,E,E* and *E,Z,E*.

Metabolism of ALA by human recombinant 15-LOX-2

This experiment was done to check whether the human recombinant 15-LOX type 2 is also able to synthesize such metabolites. For this purpose, ALA, 9(S)-HOTE and 9(±)-HOTE were separately incubated with this enzyme. We found that human recombinant 15-LOX-2 is able to convert ALA into 13-HOTE as previously described with the soybean 15-LOX (result not shown). However, ALA was less markedly metabolized into di-hydroxylated fatty acids, the main ones being all trans 9,16-di-HOTE isomers. Interestingly enough, the mono-hydroxylated derivatives 9(S)-HOTE and 9(R)-HOTE were well converted into 9(S),16(S)-*E,Z,E*-di-HOTE and 9(R),16(S)-*E,Z,E*-di-HOTE, respectively (result not shown) by the human recombinant LOX.

Inhibitory effect of 9,16-di-HOTEs on COX-1 and anti-aggregatory effects

The inhibition of sheep cyclooxygenase-1(COX-1) by different 9,16-di-HOTE isomers was tested at 1 µM by measuring both PGD_2 and E_2 as described in "Materials and Methods". Results reported in Table 1 show that 1 µM of 9(S),16(S)-dihydroxy-10*E*,12*Z*,14*E*-octadecatrienoic and 9(R),16(S)-dihydroxy-10*E*,12*Z*,14*E*-octadecatrienoic acids decreased significantly the synthesis of both PGD_2 and E_2 by around 28% and 38%, respectively. Such results are in agreement with previous data showing that poxytrins, characterized by a *E,Z,E* triene geometry, are inhibitors of platelet COX-1 (19) whereas the all trans isomers are inactive.

Such results are in agreement with data relating to platelet aggregation induced by collagen. Platelet aggregation was significantly decreased by 64% and 65% in presence of 1 µM 9(S),16(S)-dihydroxy-10*E*,12*Z*,14*E*-octadecatrienoic acid and 9(R),16(S)-dihydroxy-10*E*,12*Z*,14*E*-octadecatrienoic acid, respectively (Fig. 7). In addition, the aggregation started to be reversed from two minutes following the agonist addition. As previously shown (19), all trans isomers had no effect on platelet aggregation.

Anti-inflammatory effect of 9,16-di-HOTEs

After showing that 9,16-dihydroxy-10*E*,12*Z*,14*E*-octadecatrienoic acid isomers possess anti-aggregatory properties as those previously described for poxytrins (19), it was important to know whether such di-hydroxylated compounds issued from ALA may exhibit anti-inflammatory properties. We then investigated the effects of 9,16-di-HOTEs on cyclooxygenase-2 (COX-2) as well as the 5-lipoxygenase pathway.

- Effect of 9,16-di-HOTEs on human recombinant COX-2

The inhibition of human recombinant COX-2 by 1 µM of 9,16-di-HOTE isomers were assessed by measuring PGD_2 and E_2 as described above. Results reported in Table 1 show that 9(S),16(S)-dihydroxy-10*E*,12*Z*,14*E*-octadecatrienoic and 9(R),16(S)-dihydroxy-10*E*,12*Z*,14*E*-octadecatrienoic acids significantly inhibited COX-2 activity by 9% and 21% respectively.

- Effect of 9,16-di-HOTEs on the metabolism of arachidonic acid by the human PMN 5-lipoxygenase

The inhibition of human 5-lipoxygenase pathway by 1 µM of 9,16-di-HOTE isomers was assessed by measuring the following metabolites: 5(S)-HETE, LTB_4 and its two all trans isomers. As reported in Table 2, only 9(R),16(S)-dihydroxy-10*E*,12*Z*,14*E*-octadecatrienoic acid isomer significantly decreased the formation of leukotrienes and 5(S)-HETE by around 10% and 20%, respectively. All other isomers did not inhibit such a production (results not shown). Such data suggest that the inhibition concerns only 5-lipoxygenase but not LTA_4 hydroxylase. Taken together, these data suggest that 9(R),16(S)-dihydroxy-10*E*,12*Z*,14*E*-octadecatrienoic acid isomer may have a potential anti-inflammatory effect.

DISCUSSION

ALA is an essential fatty acid present in vegetable oils, and as such available in human diet. It is the precursor of EPA and DHA, but its conversion into EPA and DHA is rather limiting with around 10% to the former and further 10% to the latter, which means only 1% conversion of ALA to DHA (47, 48). ALA is also rather well β-oxidized (49, 50), that could partially explain its low accumulation in plasma and tissues. In addition, we describe here a pathway which could explain some beneficial effects of ALA in the vascular system at large by reducing both the production of pro-inflammatory eicosanoids PGD_2/E_2 and LTB_4 (51-53). We recently showed that a new di-hydroxylated metabolite from DHA called PDX, which is an isomer of the anti-inflammatory protectin D1, exhibits anti-aggregatory properties, both by inhibiting collagen-induced prostanoids production, including thromboxane A_2, and thromboxane A_2-induced aggregation (19). In the present paper we report the full characterization of the lipoxygenation of ALA, and the anti-inflammatory and anti-aggregatory effects of two LOX end-products. The structure of the four LOX metabolites that we herein describe have already been partially reported (23, 24), but the proposed stereochemistry structures of the 9(S),16(R)-di-HOTE do not fit with our experimental data. On the other hand, their biological properties have not been investigated.

Our data show that ALA is mainly converted into its 13-oxygenated derivative 13-HOTE by 15/ω6-lipoxygenase. However, formation of a small amount of the 9-oxygenated product 9-HOTE could also be observed. In contrast to DHA, which is converted into only one main di-hydroxylated compound (PDX) *via* a double lipoxygenation, four di-hydroxylated compounds were observed from ALA following incubation with 15-lipoxygenase, characterized as 9,16-di-HOTEs. The stereochemistry of these di-hydroxylated compounds was assessed by incubating 9(S)-HOTE and racemic 9(±)-HOTE separately with sLOX (see the above results). The UV spectra of the two main di-hydroxylated products are superimposed to that of PDX, which possesses a *E,Z,E* conjugated triene geometry. The diastereoisomers are likely produced *via* a dioxygenation mechanism as previously described for PDX (18) and these two compounds are likely to have an *E,Z,E* conjugated triene geometry. Such a geometry was further confirmed by high field NMR which allowed to determine precisely the ^1H-^1H J-couplings among the protons bound to the sp^2 carbons in C=C double bonds. The geometry of the conjugated trienes of two additional but less abundant (around half the amount of the *E,Z,E*-9(S),16(S)-di-HOTE and 9(R),16(S)-di-HOTE) di-hydroxylated products was supposed to be *E,E,E* since their UV spectra were superimposed to that of all trans LTB_4, which has such an *E,E,E* conjugated triene geometry. This all trans geometry was also confirmed by NMR to determine the ^1H-^1H J-couplings among the protons bound to the sp^2 carbons in C=C double bonds. Based on data from the literature, we may suggest that the stereochemistry of these two minor products (Fig. 1B) is 9(R),16(S)- and 9(S),16(S)-all trans-di-HOTEs, in order of increasing RT, respectively. Such a configuration is also in agreement with the 5(S),12(R)-

and 5(S),12(S)-all trans LTB_4 and 5(S),12(R)- and 5(S),12(S)-all trans LTB_5 diastereoisomers already described (43-45). We conclude that the four di-hydroxylated compounds issued from ALA treated by sLOX are 9(R),16(S)-dihydroxy-10*E*,12*E*,14*E*-octadecatrienoic, 9(S),16(S)-dihydroxy-10*E*,12*E*,14*E*-octadecatrienoic, 9(S),16(S)-dihydroxy-10*E*,12*Z*,14*E*-octadecatrienoic, 9(R),16(S)-dihydroxy-10*E*,12*Z*,14*E*-octadecatrienoic acids, in order of increasing RT, respectively. Such data suggest that there are two different mechanisms explaining the formation of these 9,16-di-HOTE isomers. The *E*,*Z*,*E* compounds 9(S),16(S)-di-HOTE and 9(R),16(S)-di-HOTE would result from a dioxygenation mechanism as previously shown for PDX (18), whereas the all trans 9,16-di-HOTE isomers might derive from unknown intermediates.

We also investigated the metabolism of ALA incubated with human recombinant 15-LOX-2. Interestingly, 9(S)-HOTE or 9(R)-HOTE were both well converted into the corresponding 9,16-di-HOTEs whereas ALA was less efficiently metabolized into those di-hydroxylated fatty acids than by sLOX. Such a result might be biologically relevant since 9(S)-HOTE can be produced through cyclooxygenases, and 9(R)-HOTE can be produced *via* aspirinated COX-2, both in an aborted cyclooxygenation process (54).

The present study also shows some biological effects of the *E*,*Z*,*E* di-hydroxylated 9,16-di-HOTE isomers. We observed that 9(S),16(S)-dihydroxy-10*E*,12*Z*,14*E*-octadecatrienoic and 9(R),16(S)-dihydroxy-10*E*,12*Z*,14*E*-octadecatrienoic acids decreased significantly prostaglandin synthesized by recombinant COX-1. Moreover, these *E*,*Z*,*E* isomers inhibit platelet aggregation triggered by collagen both by decreasing the extent of aggregation and making it partially reversible (Fig. 7). However, these *E*,*Z*,*E* di-hydroxylated ALA metabolites appear to be less potent inhibitors than PDX under the same conditions, although the anti-aggregatory effects observed is in agreement with our previous data showing that poxytrins, characterized by an *E*,*Z*,*E* conjugated triene, are inhibitors of COX-1 (19).

COX-2 is an inducible enzyme becoming abundant at sites of inflammation, and is up-regulated in various carcinomas, having a central role in tumorigenesis (55). The inhibition of COX-2 is a major mode of action to reduce inflammation (56). In our study, we found that 9(S),16(S)-dihydroxy-10*E*,12*Z*,14*E*-octadecatrienoic and 9(R),16(S)-dihydroxy-10*E*,12*Z*,14*E*-octadecatrienoic acids exhibit an inhibitory effect on the human recombinant COX-2.

Moreover, 9(R),16(S)-dihydroxy-10*E*,12*Z*,14*E*-octadecatrienoic acid also decreased significantly the formation of LTB_4 and 5(S)-HETE from arachidonic acid incubated with human PMN, indicating the inhibition of 5-lipoxygenase.

It is interesting to note that in all the tests evaluated, the *E,Z,E* 9(R),16(S) isomer was a more potent inhibitor than the *E,Z,E* 9(S),16(S) one. This reinforces the interest of the 15-lipoxygenation of 9(R)-HOTE produced by aspirinated COX-2 (54) to synergize with aspirin in atherothrombogenesis.

In conclusion, we observe from the present study that four di-hydroxylated compounds are produced from ALA treated by sLOX. They are 9(R),16(S)-dihydroxy-10*E*,12*E*,14*E*-octadecatrienoic, 9(S),16(S)-dihydroxy-10*E*,12*E*,14*E*-octadecatrienoic, 9(S),16(S)-dihydroxy-10*E*,12*Z*,14*E*-octadeca trienoic, and 9(R),16(S)-dihydroxy-10*E*,12*Z*,14*E*-octadecatrienoic acids. Interestingly, 9,16-dihydroxy-10*E*,12*Z*,14*E*-octadecatrienoic acid isomers exhibit anti-aggregatory properties as other poxytrins (19). In addition, 9(R),16(S)-dihydroxy-10*E*,12*Z*,14*E*-octadecatrienoic acid, which can be produced from 9(R)-HOTE by aspirinated COX-2, inhibits both cyclooxygenases and the 5-lipoxygenase pathway and appears to be a potential anti-inflammatory compound *in vitro.*

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Figure legends:

Figure 1. Typical RP-HPLC chromatogram of ALA metabolites. ALA was incubated with sLOX, and further treated by $NaBH_4$ to reduce hydroperoxides into hydroxylated derivatives. The lipid extract was then analyzed by RP-HPLC. Mono-hydroxylated fatty acids (A) and di-hydroxylated fatty acids (B) were detected at λ = 235 nm and 270 nm, respectively. On the rightern part of chromatograms, the UV spectra acquired on the top of chromatographic peaks are reported.

Figure 2. Electron impact mass spectrum of the Me-TMS derivative of the hydrogenated HOTE (A) and hydrogenated di-HOTE (B) synthesized from ALA by sLOX followed by reduction of hydroperoxides into hydroxylated derivatives by $NaBH_4$ treatment.

Figure 3. RP-HPLC profile of 9(S),16(S)-di-HOTE synthesized from 9(S)-HOTE by sLOX (A); RP-HPLC profile of 9(S),16(S)-di-HOTE and 9(R),16(S)-di-HOTE synthesized from 9(±)-HOTE by sLOX (B). The intermediate hydroperoxide products were reduced by $NaBH_4$ prior to analysis.

Figure 4. RP-HPLC profile of 9,16-di-HOTEs synthesized by sLOX from ALA (A); from 9(S)-HpOTE (B); and from 9(S)-HOTE (C). The intermediate hydroperoxide products were reduced by $NaBH_4$ prior to analysis.

Figure 5. 1D spectra and J-couplings for 9(S),16(S)-dihydroxy-10*E*,12*E*,14*E*-octadecatrienoic acid (A) and 9(R),16(S)-dihydroxy-10*E*,12*E*,14*E*-octadecatrienoic acid (B). Resonance assignment and J-couplings were determined from the analysis of a DQF COSY, and 2D J-resolved spectra (data not shown). Spectra were acquired with 8 scans and 2 s of recycle delay, the acquisition time was 1.8 s. Spectra were processed with exponential line broadending of 0.3 Hz as window function.

Figure 6. 1D spectra and J-couplings for 9(S),16(S)-dihydroxy-10*E*,12*Z*,14*E*-octadecatrienoic acid (A) and 9(R),16(S)-dihydroxy-10*E*,12*Z*,14*E*-octadecatrienoic acid (B). Resonance assignments and J-couplings were determined from the analysis of a DQF COSY, and 2D J-resolved spectra (data not shown). Spectra were acquired with 8 scans and 2 s of recycle

delay, the acquisition time was 1.8 s. Spectra were processed with exponential line broadending of 0.3 Hz as window function.

Figure 7. Effect of *E,Z,E*-9,16-di-HOTEs on platelet aggregation triggered by collagen. Platelet suspension (400 µL) was pre-incubated for 1 min at 37°C and triggered by ~0.05 ng/µL collagen in the presence or absence of 1 µM 9,16-di-HOTEs. Platelet aggregation was monitored for 4 min. The aggregation profiles shown are representative of 3 independent experiments.

Table1. Effect of 9,16-di-HOTEs on cyclooxygenase-1 and 2

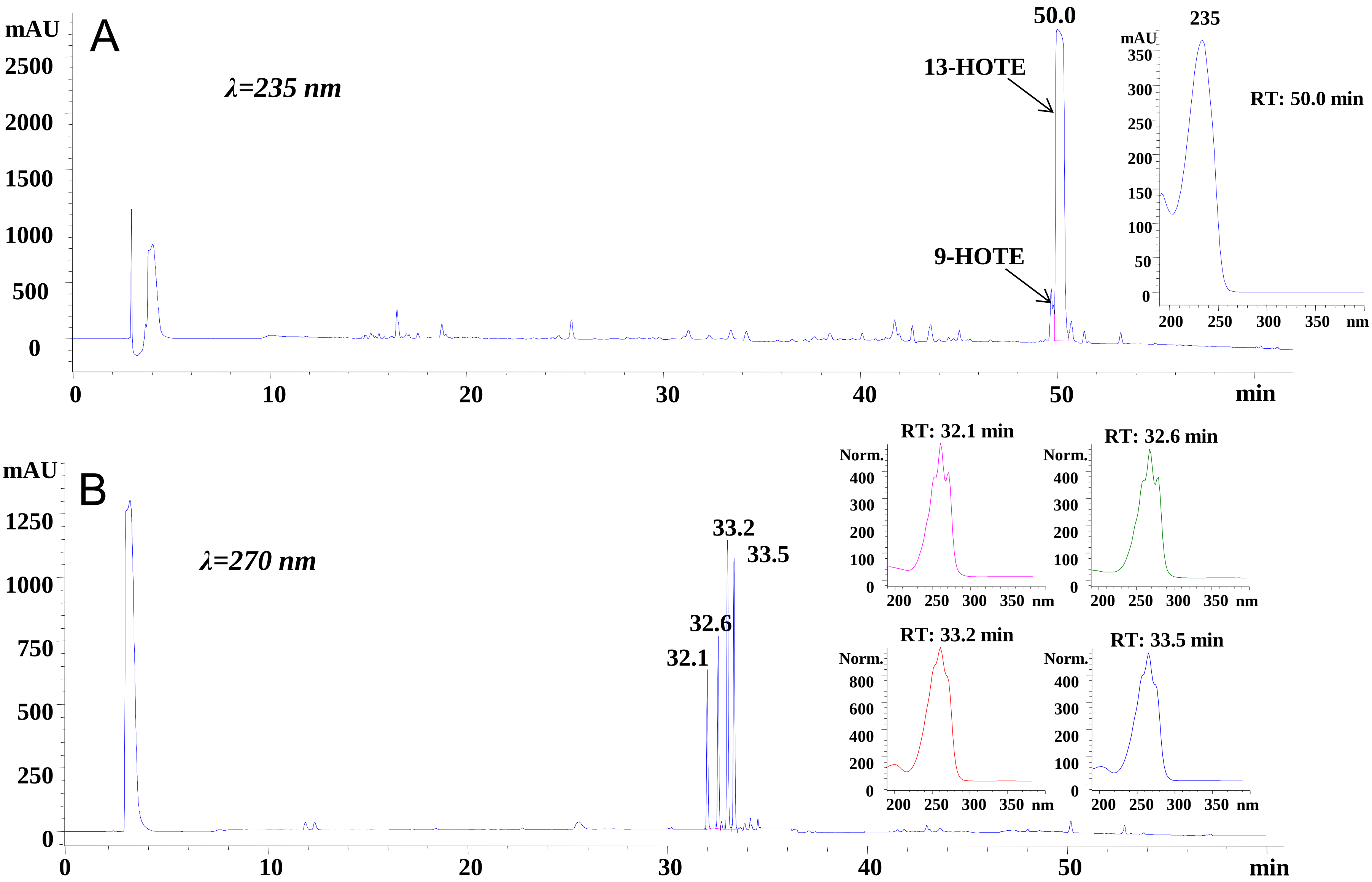
	Control	9(S),16(S)-di-HOTE *E*,*Z*,*E*	9(R),16(S)-di-HOTE *E*,*Z*,*E*
COX-1			
(PGD_2+ PGE_2)	134.9 ± 15.8	96.8 ± 14.0**	83.2 ± 9.0**
COX-2			
(PGD_2+ PGE_2)	136.1 ± 11.0	124.5 ± 8.8*	107.4 ± 7.2**

Purified COX-1 and COX-2 were incubated with or without 1 µM of different 9,16-di-HOTE isomers and with 10 µM arachidonic acid. PGD_2 and E_2 were measured by GC-MS. Results expressed in ng of PGD_2 and PGE_2 represent the mean ± SEM of 4 determinations. *P < 0.05, **P < 0.01 versus control. Only the inhibitory effects of *E*,*Z*,*E* isomers are shown.

Table2. Effect of 9,16-di-HOTEs on human leukocyte 5-lipoxygenase

	Control	9(R),16(S)-di-HOTE *E*,*Z*,*E*
LTB_4s	9.57 ± 0.66	8.78 ± 0.78*
5-HETE	0.89 ± 0.03	0.71 ± 0.05*
5-LOX products	10.46 ± 0.68	9.49 ± 0.81*

Isolated human leukocytes were incubated in the presence or absence of 1 µM of different 9,16-di-HOTE isomers at 37 °C and triggered with 1 µM of ionophore A23187 and 10 µM of arachidonic acid. Oxygenated metabolites were then extracted and quantified by RP-HPLC. Results expressed in nmol of total LTB_4s (LTB_4 + LTB_4 isomers) and 5-HETE represent the mean ± SEM of 5 determinations, *$P < 0.05$, versus control. Only the inhibitory effect of 9(R),16(S)-di-HOTE is shown..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13-HOTE
9-HOTE
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235
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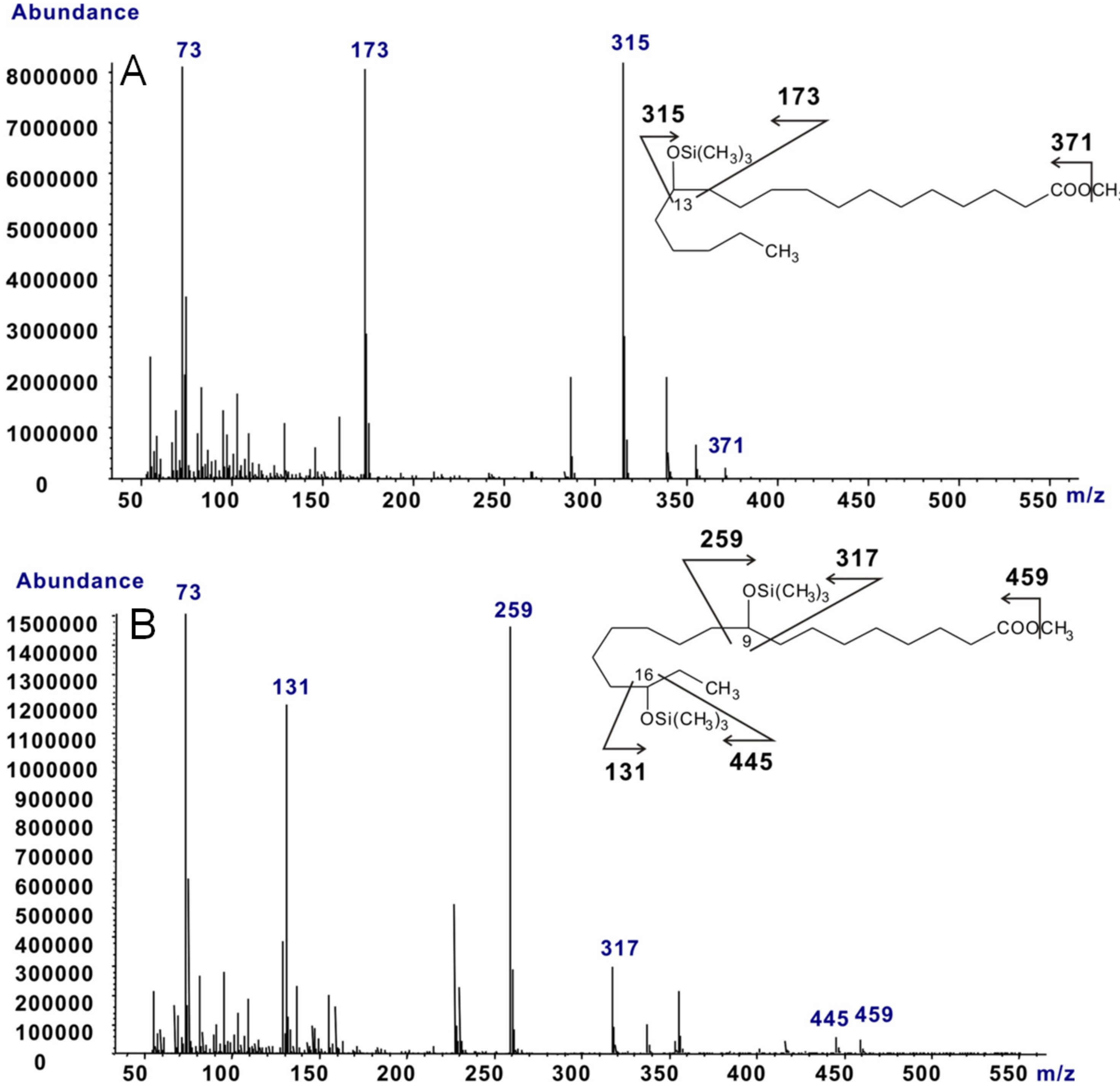

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259
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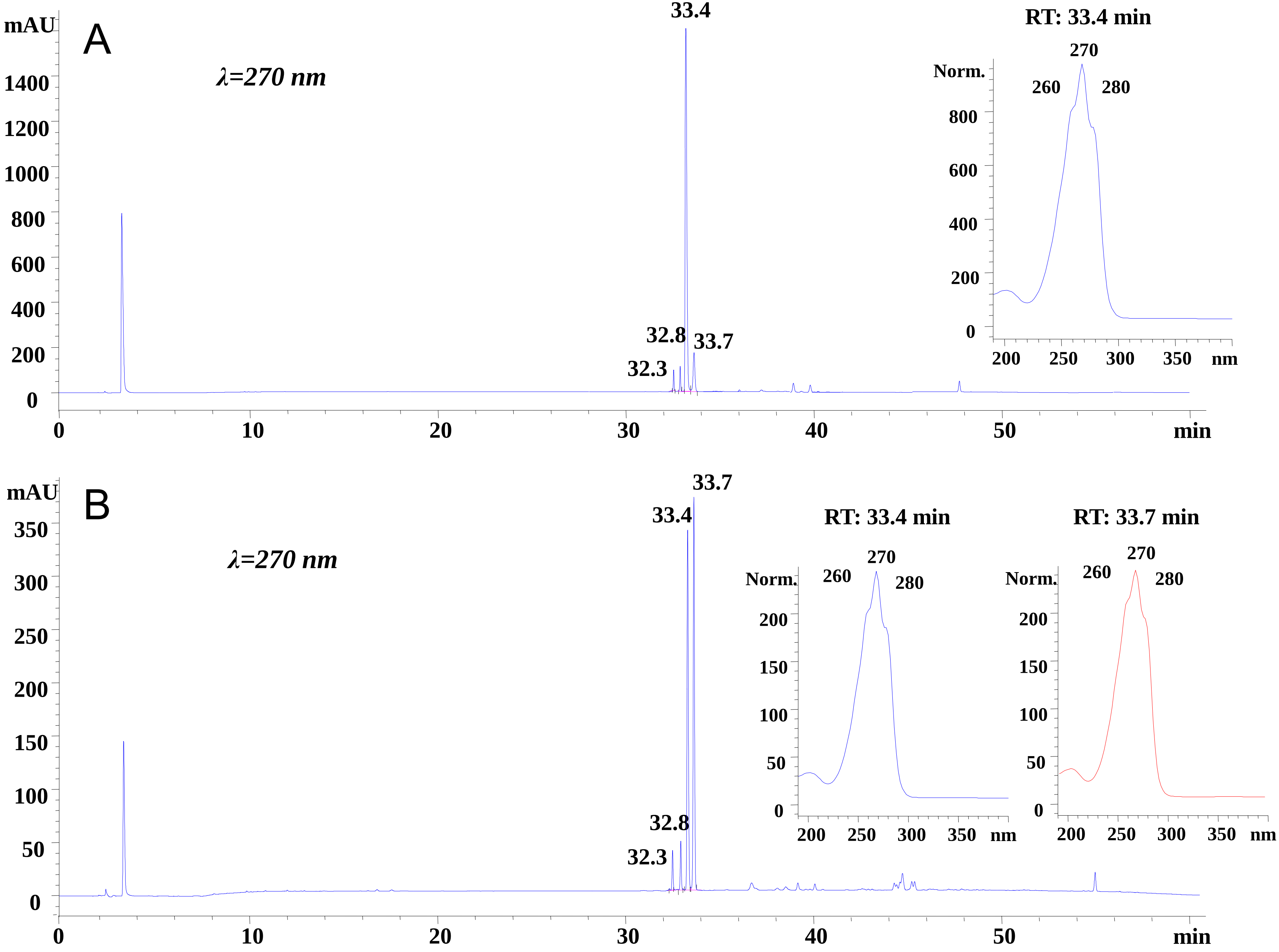

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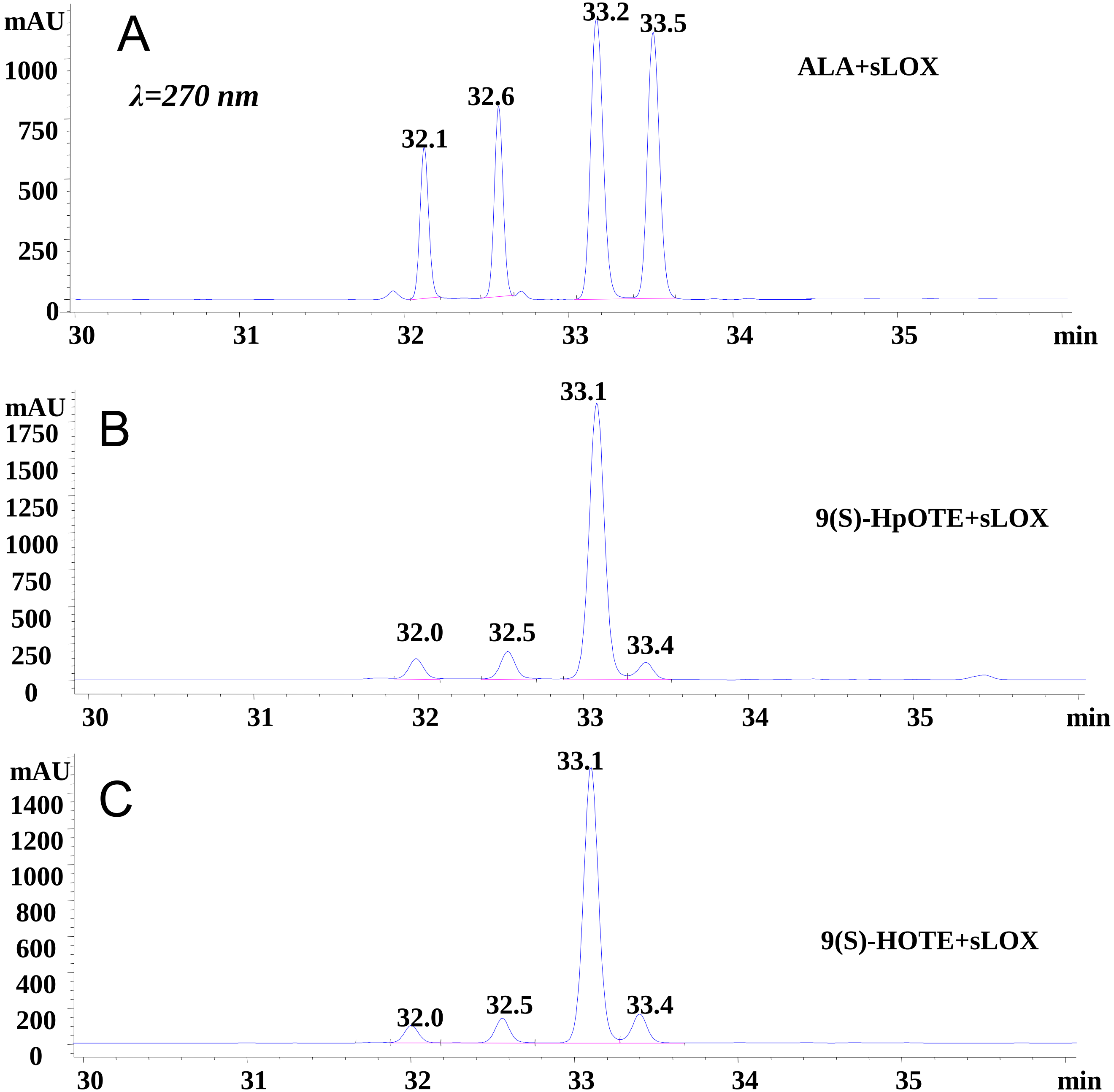
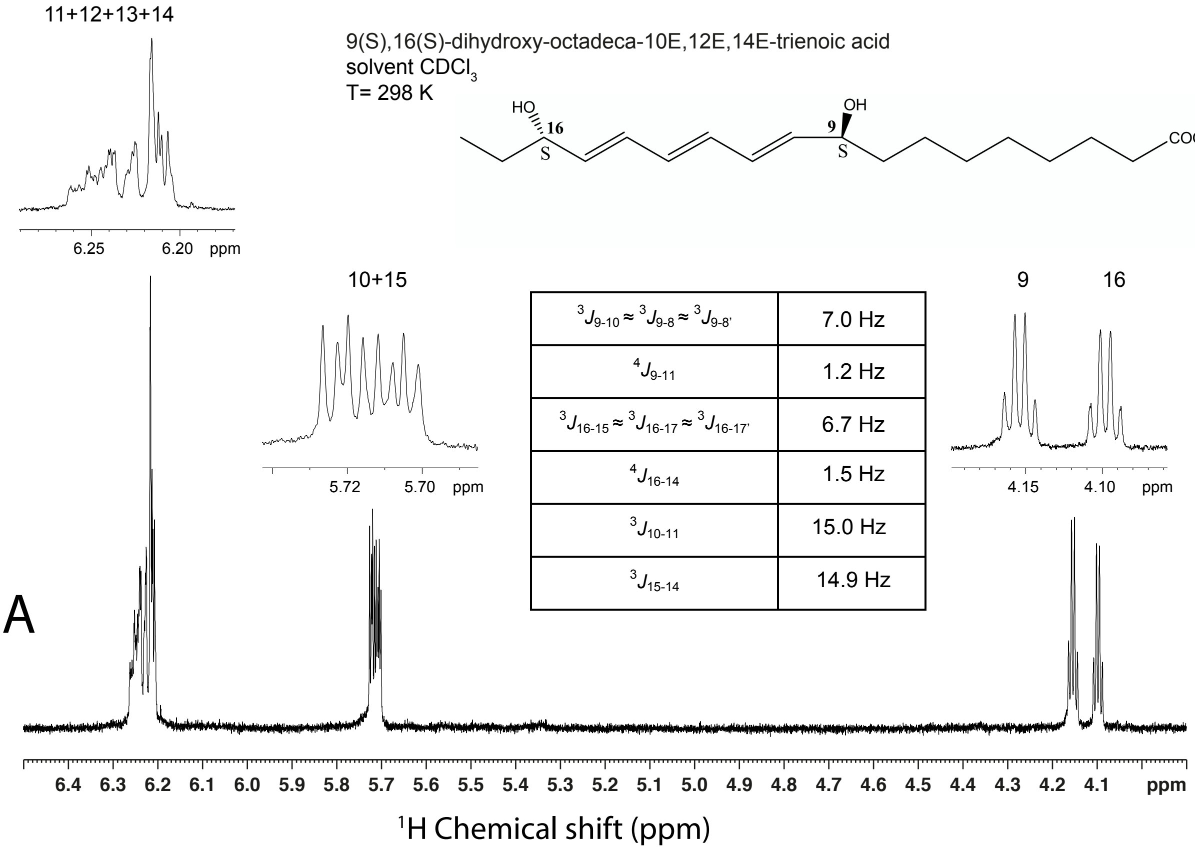
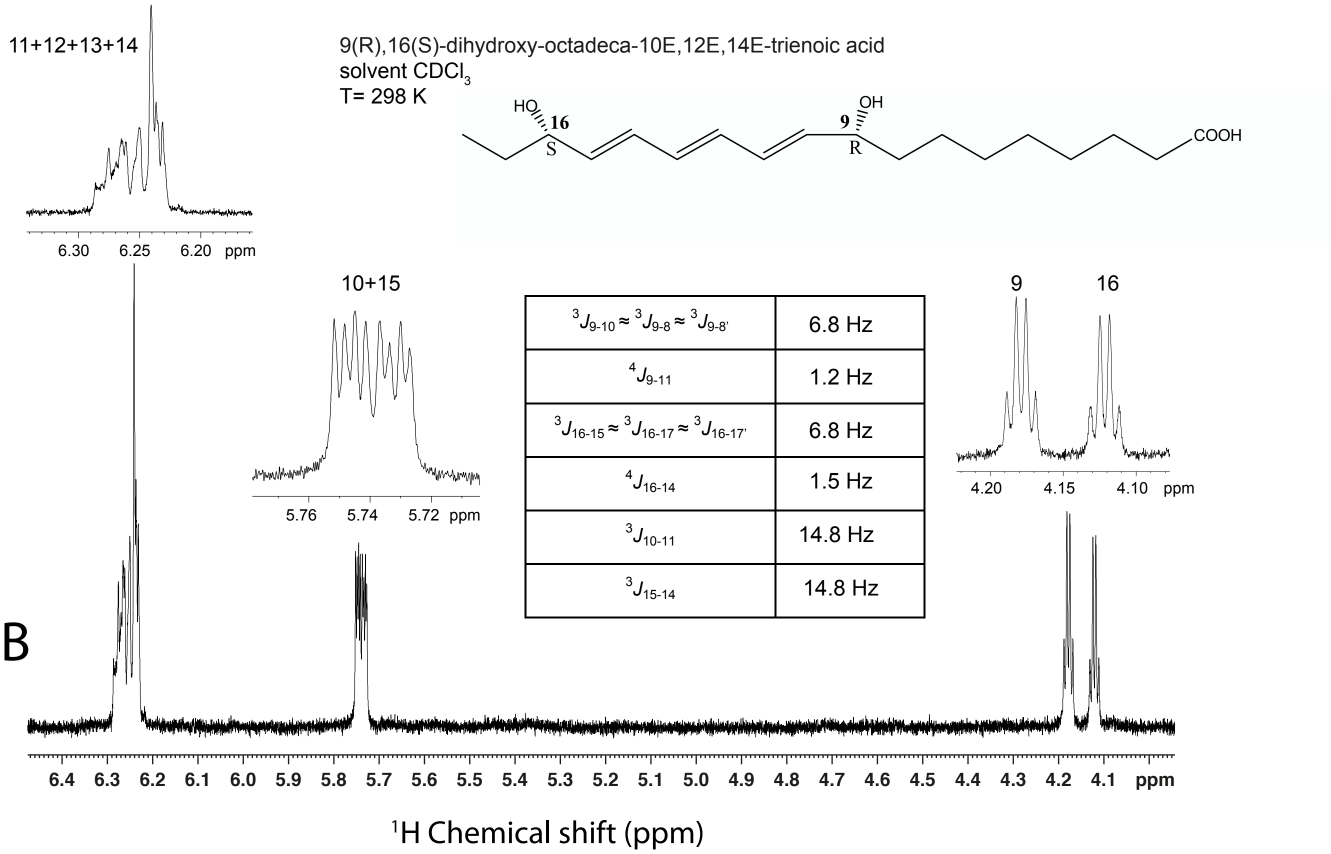
260
280
800
600
400
200
0
200
250
300
350
nm
B
mAU
λ=270 nm
350
300
250
200
150
100
50
0
33.7
33.4
32.8
32.3
0
10
20
30
40
50
min
RT: 33.4 min
Norm.
270
260
280
200
150
100
50
0
200
250
300
350
nm
RT: 33.7 min
Norm.
270
260
280
200
150
100
50
0
200
250
300
350
nm



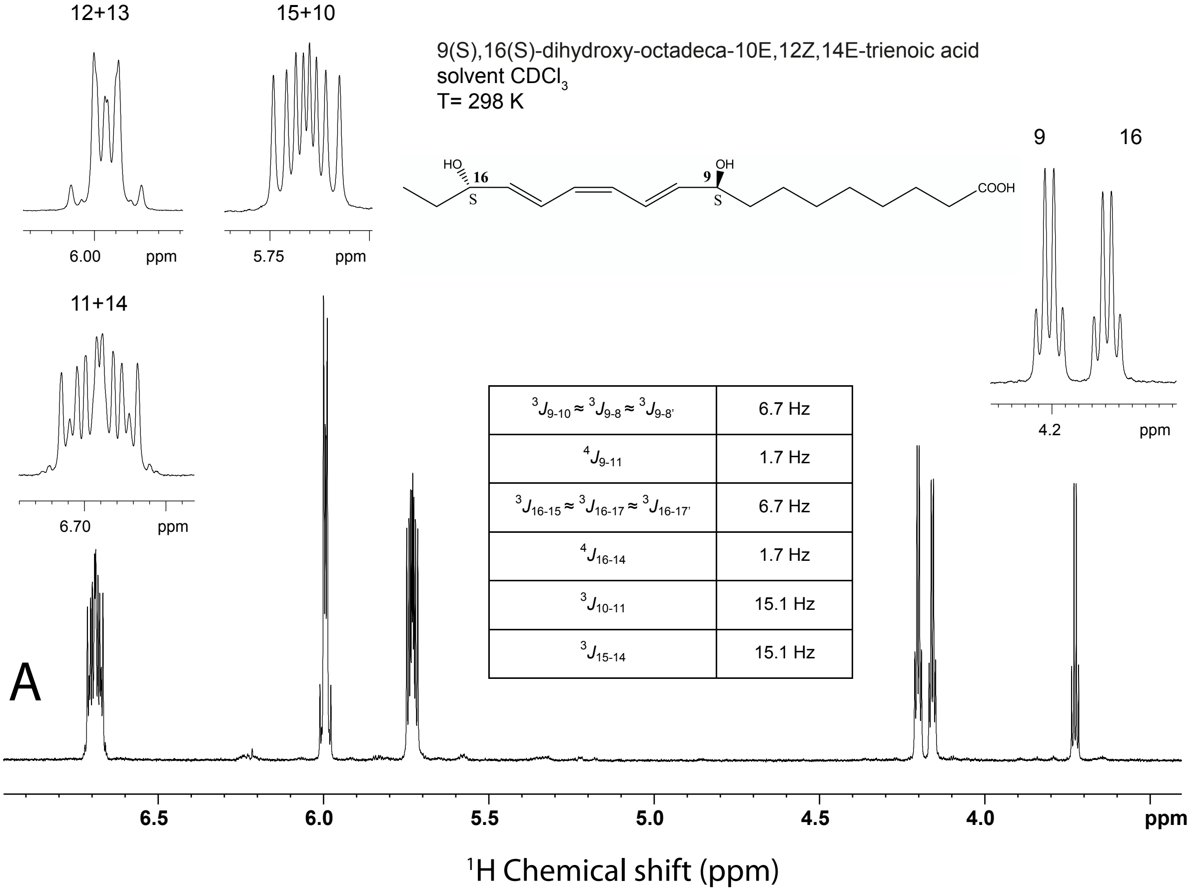
A
λ=270 nm
ALA+sLOX
mAU
1000
750
500
250
0
32.1
32.6
33.2
33.5
30
31
32
33
34
35
min
B
9(S)-HpOTE+sLOX
mAU
1750
1500
1250
1000
750
500
250
0
32.0
32.5
33.1
33.4
30
31
32
33
34
35
min
C
9(S)-HOTE+sLOX
mAU
1400
1200
1000
800
600
400
200
0
32.0
32.5
33.1
33.4
30
31
32
33
34
35
min



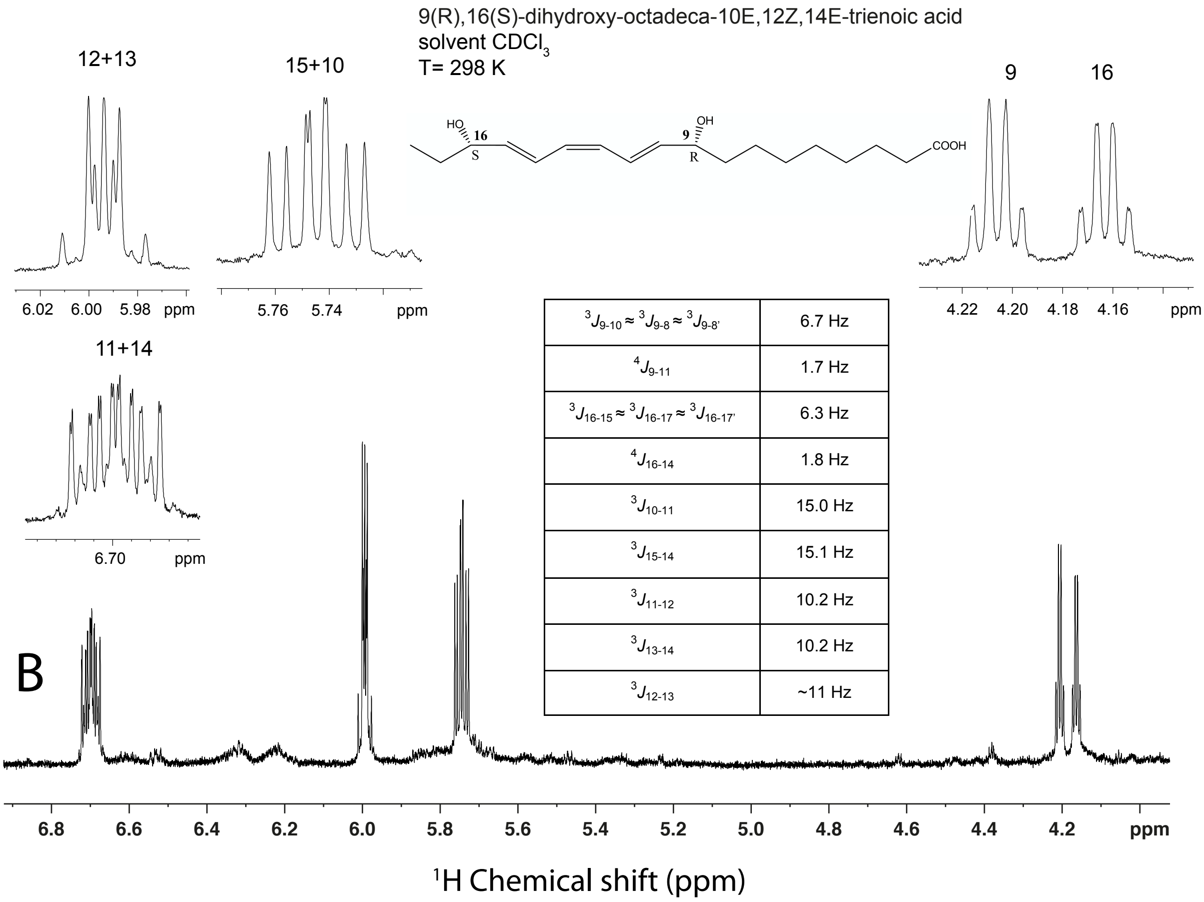
$^3J_{9-10} \approx {}^3J_{9-8} \approx {}^3J_{9-8'}$	7.0 Hz
$^4J_{9-11}$	1.2 Hz
$^3J_{16-15} \approx {}^3J_{16-17} \approx {}^3J_{16-17'}$	6.7 Hz
$^4J_{16-14}$	1.5 Hz
$^3J_{10-11}$	15.0 Hz
$^3J_{15-14}$	14.9 Hz



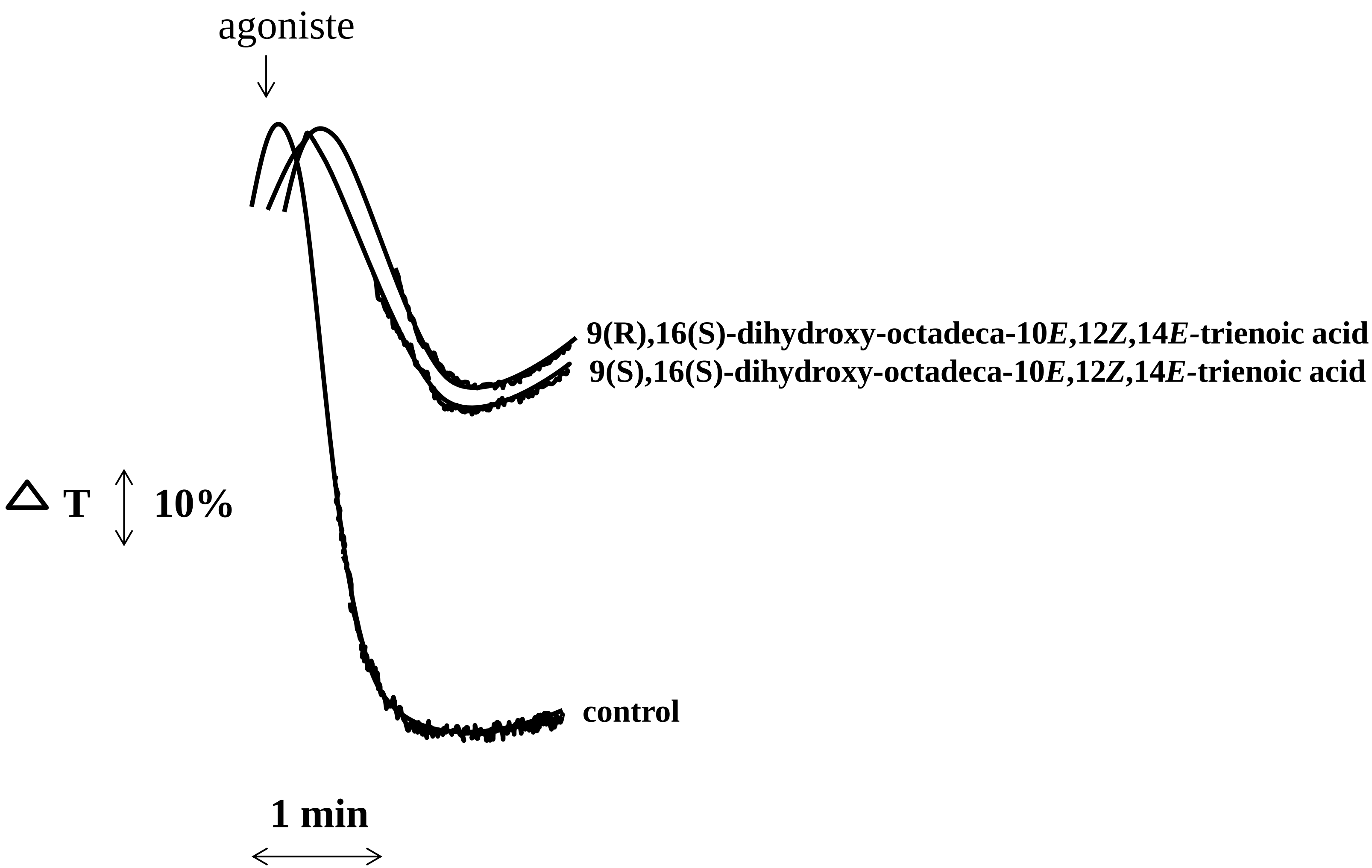
$^3J_{9-10} \approx {}^3J_{9-8} \approx {}^3J_{9-8'}$	6.8 Hz
$^4J_{9-11}$	1.2 Hz
$^3J_{16-15} \approx {}^3J_{16-17} \approx {}^3J_{16-17'}$	6.8 Hz
$^4J_{16-14}$	1.5 Hz
$^3J_{10-11}$	14.8 Hz
$^3J_{15-14}$	14.8 Hz



$^3J_{9\text{-}10} \approx {}^3J_{9\text{-}8} \approx {}^3J_{9\text{-}8'}$	6.7 Hz
$^4J_{9\text{-}11}$	1.7 Hz
$^3J_{16\text{-}15} \approx {}^3J_{16\text{-}17} \approx {}^3J_{16\text{-}17'}$	6.7 Hz
$^4J_{16\text{-}14}$	1.7 Hz
$^3J_{10\text{-}11}$	15.1 Hz
$^3J_{15\text{-}14}$	15.1 Hz



$^3J_{9\text{-}10} \approx {}^3J_{9\text{-}8} \approx {}^3J_{9\text{-}8'}$	6.7 Hz
$^4J_{9\text{-}11}$	1.7 Hz
$^3J_{16\text{-}15} \approx {}^3J_{16\text{-}17} \approx {}^3J_{16\text{-}17'}$	6.3 Hz
$^4J_{16\text{-}14}$	1.8 Hz
$^3J_{10\text{-}11}$	15.0 Hz
$^3J_{15\text{-}14}$	15.1 Hz
$^3J_{11\text{-}12}$	10.2 Hz
$^3J_{13\text{-}14}$	10.2 Hz
$^3J_{12\text{-}13}$	~11 Hz



agoniste
9(R),16(S)-dihydroxy-octadeca-10E,12Z,14E-trienoic acid
9(S),16(S)-dihydroxy-octadeca-10E,12Z,14E-trienoic acid
ΔT 10%
control
1 min