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DOUBLE DIOXYGENATION OF ARACHIDONIC ACID BY SOYBEAN LIPOXYGENASE-1

KINETICS AND REGIO-STEREO SPECIFICITIES OF THE REACTION STEPS

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Summary

The kinetic parameters of the first and second oxygenation of arachidonic acid by soybean lipoxygenase-1 were determined and found to be for the first step at pH 10.0, K_m (arachidonic acid) = 8.5 ± 0.5 μM; k_{cat} = 225 ± 7 s^{-1} and for the second step at pH 8.7, K_m (15-HPETE) = 440 ± 20 μM; k_{cat} = 25 ± 1 s^{-1}.

In the second oxygenation for which 15-L_S-hydroperoxy 5-*cis*, 8-*cis*, 11-*cis*, 13-*trans*-eicosatetraenoic acid is a substrate, two isomeric dihydroperoxy fatty acids are formed. After separation of the corresponding dihydroxy esters by high-performance liquid chromatography, they were identified by mass-spectrometry, ^{1}H- and ^{13}C-NMR spectroscopy as 8-D_S, 15-L_S-dihydroperoxy 5-*cis*, 9-*trans*, 11-*cis*, 13-*trans*-eicosatetraenoic acid and 5-D_S, 15-L_S-dihydroperoxy 6-*trans*, 8-*cis*, 11-*cis*, 13-*trans*-eicosatetraenoic acid. Independent evidence for the absolute configurations was obtained by capillary gas-liquid chromatography of diastereomeric *R*-(—)-2-butyl esters of the acetylated 2-hydroxy carboxylic acids produced by oxidative ozonolysis of the acetylated dihydroxy fatty acids. It is concluded that soybean lipoxygenase-1 produces hydroperoxides with predominantly the *S*-configuration irrespective of the position in the fatty acid which is oxygenated.

Abbreviations: 15-HPETE, 15-L_S-hydroperoxy 5-*cis*, 8-*cis*, 11-*cis*, 13-*trans*-eicosatetraenoic acid; MTPA, α-methoxy-α-trifluoromethyl phenyl acetate. L and D refer to the nomenclature according to the Fischer convention; subscripts S and R refer to the nomenclature according to Cahn, Ingold and Prelog.

Introduction

Soybean lipoxygenase-1 (linoleate:oxygen oxidoreductase, EC 1.13.11.12) is a dioxygenase containing non-heme iron, which catalyses the conversion of polyunsaturated fatty acids containing a 1,4-*cis*, *cis*-pentadiene system into optically active *cis*, *trans*-conjugated hydroperoxydienoic acids. The preference of the enzyme for oxygenation of the n-6 carbon atom as observed for linoleic acid [1,2] is even more pronounced for substances with a larger number of double bonds. Hamberg and Samuelsson [3] showed that aerobic incubation of either all-*cis*-8,11,14-eicosatrienoic acid (bis-homo-γ-linolenic acid) or all-*cis*-5,8,11,14-eicosatetraenoic acid (arachidonic acid) yields the 15-hydroperoxy compound exclusively. Furthermore, these authors showed for bis-homo-γ-linolenic acid that the 15-L_S-hydroperoxide was formed by selective abstraction of the 13-$L_{pro\text{-}S}$ hydrogen atom. Egmond et al. [4] showed that in the formation of 13-L_S-hydroperoxy-linoleic acid by soybean lipoxygenase-1, the 11-$L_{pro\text{-}S}$ hydrogen is selectively abstracted, whereas the 11-$D_{pro\text{-}R}$ hydrogen is abstracted in the formation of 9-D_S-hydroperoxy-linoleic acid by corn germ lipoxygenase. They interpreted these data in terms of an antarafacial reaction. Bild et al. [5] reported that incubation of arachidonic acid at a relatively high concentration of soybean lipoxygenase-1 results in a double dioxygenation with the concomitant formation of a conjugated triene (λ_{max} = 270 nm). They identified the reaction product as 8,15-dihydroperoxy-5,9,11,13-eicosatetraenoic acid.

In the present investigation, this reaction was studied in more detail. The complete analysis of the products was carried out. In particular, attention was paid to the stereospecificity of both oxygenation steps.

Materials and Methods

Soybean lipoxygenase-1 was isolated according to the method of Finnazi-Agrò et al. [6] as modified by Galpin et al. [7]. The enzyme showed a single band on polyacrylamide gel electrophoresis and a specific activity of 245 μmol $O_2 \cdot min^{-1} \cdot mg^{-1}$ (4.08 kat $\cdot kg^{-1}$) as determined polarographically in a Gilson oxygraph equipped with a Clark electrode (1.8 mM linoleic acid in 0.1 M sodium borate, pH 9.0). Arachidonic acid (purity greater than 99%) was obtained from Fluka (Buchs, Switzerland).

Preparation of mono- and dihydroperoxy acids

(a) *15-Hydroperoxy 5-cis, 8-cis, 11-cis, 13-trans-eicosatetraenoic acid.* The monohydroperoxide was prepared at 4°C by incubation of 100 mg arachidonic acid (660 μM) with soybean lipoxygenase-1 (2 nM) in 0.1 M sodium borate buffer (pH 9.0) flushed with oxygen. After the reaction had come to completion (conversion 98%), the hydroperoxide was extracted at pH 3 with diethyl ether and purified by thin-layer chromatography on pre-coated silica gel plates (60 F 254, 0.5 mm) (from Merck) with hexane/diethyl ether/acetic acid (70 : 30 : 1, v/v/v) as eluent. The purified hydroperoxide was dissolved in absolute ethanol and stored at −20°C.

(b) *Mixture of 8,15 and 5,15 dihydroperoxy-eicosatetraenoic acids.* The

dihydroperoxides were prepared by incubation at 4°C of 100 mg arachidonic acid (101 μM) in 0.1 M sodium borate buffer (pH 8.75). The solution was flushed with oxygen and the reaction was started by addition of lipoxygenase-1 to a concentration of 100 nM. The course of the reaction was monitored by recording ultraviolet spectra of aliquots from the reaction mixture. When the absorbance of the triene system (λ_{max} = 270 nm) no longer increased (conversion into dihydroperoxy products, 99%), the reaction was stopped by acidification to pH 3 with 6 M HCl. The reaction products were extracted with diethyl ether and the extract was washed with redistilled water, concentrated and finally dried by azeotropic evaporation with absolute ethanol.

Preparation and purification of mono- and dihydroxy esters

The aforementioned hydroperoxy acids were reduced with a 10-fold molar excess of $NaBH_4$ in methanol. After evaporation of the solvent, the residue was dissolved in redistilled water, acidified to pH 3 and extracted with diethyl ether. The hydroxy acids were subsequently esterified with diazomethane. Methyl-15-hydroxy 5-*cis*, 8-*cis*, 11-*cis*, 13-*trans*-eicosatetraenoate was purified by preparative thin-layer chromatography with hexane/diethyl ether (70 : 30, v/v) as eluent. Methyl-5,15-dihydroxy 6-*trans*, 8-*cis*, 11-*cis*, 13-*trans*-eicosatetraenoate and methyl-8,15-dihydroxy 5-*cis*, 9-*trans*, 11-*cis*, 13-*trans*-eicosatetraenoate were separated by high-performance liquid chromatography on a Perkin-Elmer series 2 chromatograph equipped with a column (0.9 X 25 cm) packed with Partisil-5 (Chrompack, Middelburg, The Netherlands). Elution was performed with a mixture of hexane/chloroform/methanol (90.5 : 8 : 1.5, v/v/v) and detection was carried out with a Pye Unicam LC_3 detector at 254 nm. After evaporation of the solvent, the purified dihydroxy esters were dissolved in absolute methanol and stored at −20°C.

Kinetic measurements

Kinetic measurements were carried out in a 1 cm pathlength cuvette in air-saturated 0.1 M sodium borate or sodium phosphate buffer at 25°C. To measure the first oxygenation step, arachidonic acid was added from a stock solution in 1 M NH_4OH to a cuvette filled with buffer. The reaction was started by addition of enzyme to a concentration of 2 nM and the change in absorbance at 238 nm was recorded on a Cary 118-C spectrophotometer. For the measurement of the second oxygenation, the appropriate amount of a solution of 15-HPETE in ethanol was brought against the wall of an empty cuvette. After evaporation of the ethanol with a stream of air, the buffer was added to the cuvette and the solution was stirred to constant absorbance at 238 nm. This procedure has to be carried out because 15-HPETE is unstable in 1 M NH_4OH, while addition from an ethanolic stock solution has to be avoided because of the strong inhibition of the second oxygenation by ethanol. The reaction was started by addition of enzyme to a concentration of 25 nM and the change in absorbance at 270 nm was recorded.

Analytical methods

Surface tension measurements of solutions of arachidonic acid and 15-HPETE were carried out as described by Verhagen et al. [8]. The number of hy-

droperoxy groups in the hydroperoxy fatty acids was determined according to Koch et al. [9] using the ferrous thiocyanate method. 13-L_S-Hydroperoxy-9-*cis*, 11-*trans*-octadecadienoic acid was used as a reference compound. Molar absorption coefficients of the mono- and dihydroxy-eicosatetraenoates were determined on the basis of a colorimetric method for quantification of the ester function as described by Snyder and Stephens [10] and modified by Renkonen [11]. Methyl stearate was used as a reference compound. Hydrogenation of unsaturated dihydroxy esters was performed by bubbling hydrogen through a methanolic solution, using PtO_2 as a catalyst. The saturated hydroxy esters were trimethylsilylated at room temperature with a mixture of hexamethyl disilazane (0.1 ml) and trimethyl chlorosilane (0.5 ml) in 1 ml pyridine. Gas-liquid chromatography-mass spectrometry was performed on a Finnigan 3100D quadrupole mass spectrometer (electron energy 70 eV) coupled to a 3% SE 30 column. For trimethylsilyl ethers of methyl dihydroxy-eicosatetraenoates, the following temperature programme was used: 195°C for 8 min; 2 K/min to 240°C. *R*(+)-MTPA esters of mono- and dihydroxy-eicosatetraenoates were prepared as described before [2], but using twice the amount of MTPA-Cl for the dihydroxy compounds. ^{1}H-NMR shift experiments were carried out with perdeutero Eu(fod)$_3$ obtained from Merck (Darmstadt, F.R.G.). ^{1}H-NMR spectra were recorded on Varian EM 390 (90 MHz) or Bruker HX 360 (360 MHz) spectrometers at room temperature with C^2HCl_3 as solvent. Chemical shifts are given relative to tetramethylsilane (indirectly to the C^1HCl_3 singlet at 7.262 ppm). Computer simulations of ^{1}H-NMR spectra were carried out on the 620 LOO 16 K computer of a Varian CFT 20 spectrometer with the interactive SIMEQ program developed by C.W.F. Kort and M.J.A. de Bie. ^{13}C-NMR spectra were recorded on a Varian CFT 20 (20 MHz) or a Bruker HX 360 (90.5 MHz) in C^2HCl_3 either at room temperature or at 5°C. Chemical shifts are given relative to tetramethylsilane (indirectly to the C^2HCl_3 signal at 76.89 ppm). Optical rotations were measured at 20°C with a Perkin-Elmer polarimeter (model 241) in a microcuvette of 10 cm pathlength.

Ozonolysis

0.5 mg of each of the unsaturated dihydroxy fatty acid methyl esters were acetylated at 100°C for 30 min with a 1 : 1 mixture of acetic anhydride and pyridine. The acetylated dihydroxy esters were purified by TLC employing hexane/diethyl ether (70 : 30, v/v) as eluent. The purified compounds were dissolved in 1.5 ml chloroform, then oxygen containing approx. 0.5% (v/v) of ozone was bubbled through the solution for 10 min at −15°C. The reaction mixture was treated with a mixture of acetic acid (0.5 ml) and 30% H_2O_2 (0.1 ml) at 50°C for 16 h. The resulting acetylated 2-hydroxy mono- and dicarboxylic acids were esterified with *R*-(−)-2-butanol using HCl as a catalyst. The resulting diastereomeric *R*-(−)-2-butyl esters were reactylated and investigated by capillary GLC on a column (25 m × 0.3 mm) using SP 1000 as a stationary phase (temperature programme, 2 K/min from 110 to 200°C). GC-MS analysis of acetylated *R*-(−)-2-butyl esters of the 2-hydroxy carboxylic acids were carried out on a Kratos MS 80 system equipped with a packed OV 225 column.

Results and Discussion

(I) Kinetics

(a) First oxygenation. 15-Hydroperoxy 5-*cis*, 8-*cis*, 11-*cis*, 13-*trans*-eicosatetraenoic acid can be formed specifically from all-*cis*-5,8,11,14-eicosatetraenoic acid by incubation with soybean lipoxygenase-1 at pH 10.0. Typical reaction conditions: enzyme concentration, 2 nM; arachidonic acid concentration, 50 μM. By high-performance liquid chromatography, no indications were found for the presence of positional isomers. Moreover, the 360 MHz ^{1}H-NMR spectrum contained no indications for products other than 15-HPETE. The exclusive formation of this product is in agreement with the literature [3]. The molar absorption coefficient of the methyl ester of 15-HPETE was found to be $(29.5 \pm 0.5) \cdot 10^3\ M^{-1} \cdot cm^{-1}$ at 238 nm in methanol. The kinetics of the formation of 15-HPETE were studied with substrate concentrations well below the critical micelle concentration of arachidonic acid, which was found to be 180 μM in 0.1 M sodium borate buffer (pH 10.0) at 25°C. Kinetic parameters for this reaction in 0.1 M sodium borate buffer (pH 10.0) were obtained by fitting the initial rates of hydroperoxide formation to the Michaelis-Menten equation

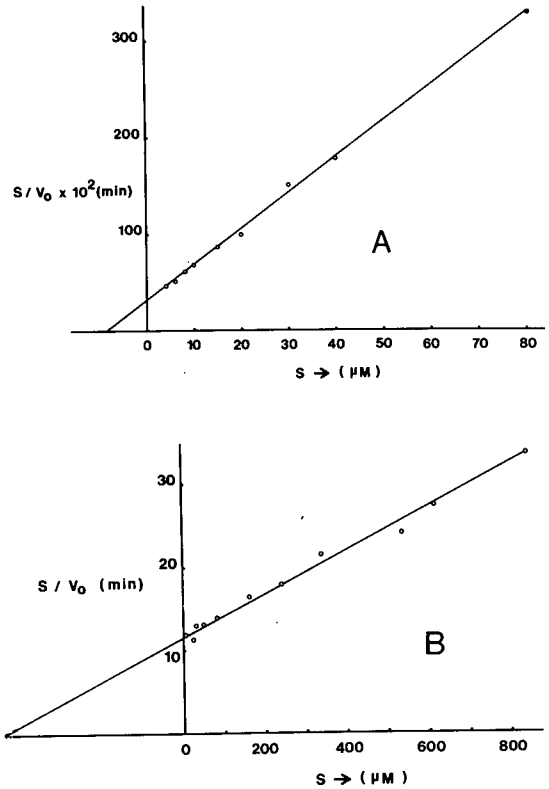

Fig. 1. Hanes plots for the first (A) and the second oxygenation (B) of arachidonic acid. Conditions: (A) substrate, arachidonic acid; [lipoxygenase-1] = 2 nM; [O_2] = 240 μM; temperature = 25°C; 0.1 M sodium borate buffer (pH 10.0). (B) substrate, 15-HPETE; [lipoxygenase-1] = 25 nM; [O_2] = 240 μM; temperature = 25°C; 0.1 M sodium borate buffer (pH 8.7).

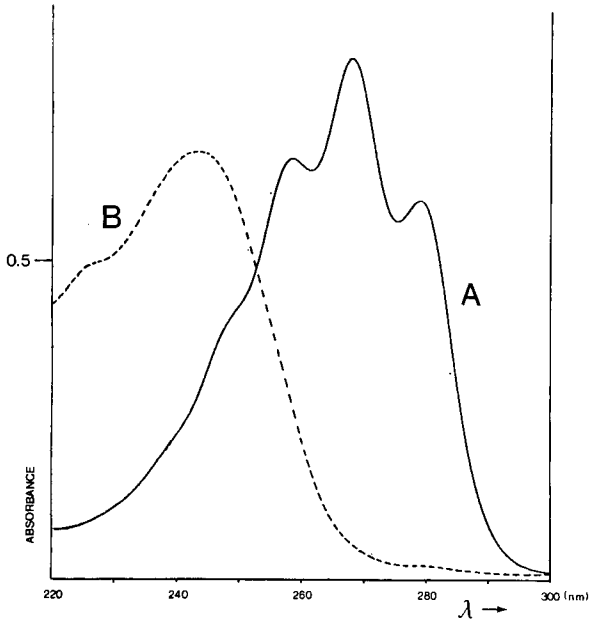

Fig. 2. Ultraviolet spectra of methyl-8,15-dihydroxy 5-*cis*, 9-*trans*, 11-*cis*, 13-*trans*-eicosatetraenoate (A) and methyl -5,15-dihydroxy 6-*trans*, 8-*cis*, 11-*cis*, 13-*trans*-eicosatetraenoate (B). Spectra were recorded in methanol; concentrations: A = 20.8 μM, B = 20.3 μM.

by means of an iterative least squares method. A plot of s/v versus s is shown in Fig. 1A. A K_m of 8.5 μM was found which is higher than the value of 1 μM reported by Cook and Lands [12] at pH 8.5 but considerably lower than the value of 86 μM reported by Bild et al. [13] at pH 9.0.

(b) Second oxygenation. Incubation of arachidonic acid at a relatively high enzyme concentration (typical conditions; enzyme concentration, 25 nM; arachidonic acid concentration, 100 μM) yields two double-dioxygenated products. The presence of two hydroperoxy groups per fatty acid molecule was confirmed by determination with ferrous thiocyanate. One of the products appeared to be identical with the 8,15-dihydroperoxide described before by Bild et al. [5]. The other product which contains two isolated conjugated diene systems (λ_{max} = 243 nm) was identified (vide infra) as 5,15-dihydroperoxy 6-*trans*, 8-*cis*, 11-*cis*, 13-*trans*-eicosatetraenoic acid. The molar ratio of the two isomers (8,15 : 5,15 = 60 : 40) was found to be constant during the course of the reaction as was determined by high-performance liquid chromatography of the corresponding esterified hydroxy compounds. Ultraviolet spectra of the purified compounds are shown in Fig. 2. Molar absorption coefficients of the 8,15- and 5,15-dihydroxy analogues in methanol were found to be $\epsilon_{268.5}$ = $(40 \pm 0.5) \cdot 10^3\ M^{-1} \cdot cm^{-1}$ and $\epsilon_{243} = (33.5 \pm 0.5) \cdot 10^3\ M^{-1} \cdot cm^{-1}$, respectively. For the second oxygenation step, 15-HPETE can be used as a substrate instead of arachidonic acid, leading to the same products in the same molar ratio. The conversion of 15-HPETE can be monitored by measuring the change in absorbance at 270 nm corresponding to the formation of the 8,15-dihydroperoxy compound. Rates of the 15-HPETE conversion can then be calculated

TABLE I

KINETIC PARAMETERS OF THE FIRST AND SECOND OXYGENATION OF ARACHIDONIC ACID BY LIPOXYGENASE-1

K_m and k_{cat} values are expressed ± S.E.

	Substrate	pH	K_m (μM)	k_{cat} (s^{-1})
First step	Arachidonic acid	10.0	8.5 ± 0.5 μM	225 ± 7 s^{-1}
Second step	15-HPETE	8.7	440 ± 20 μM	25 ± 1 s^{-1}

taking into account that the 8,15-dihydroperoxide represents 60% of the reaction products. The pH dependence of the second oxygenation was determined using 15-HPETE as a substrate. A very pronounced optimum was found at pH 8.7 which is approx. 1 pH unit higher than the value reported by Bild et al. [13]. In the first oxygenation, a less pronounced pH optimum was found showing a maximum activity at approx. pH 9—10. The kinetic parameters for the second oxygenation in 0.1 M sodium borate buffer (pH 8.7) were obtained by fitting the initial rates of the 15-HPETE conversion to the Michaelis-Menten equation by means of an iterative least-squares method (Table I). A plot of v/s versus s is given in Fig. 1B. The value of 440 μM for the K_m is much smaller than that of 18 mM reported by Bild et al. [13]. It is remarkable that no deviation is found in the Hanes plot for concentrations up to 850 μM, since surface tension measurements of 15-HPETE in 0.1 M sodium borate buffer showed that premicellar aggregation occurs between 240 and approx. 2000 μM (cf. Ref. 8).

(II) Structural analysis

(a) Products of the first oxygenation. The configurations of the double bonds in 15-HPETE were ascertained by 360 MHz ^{1}H-NMR spectroscopy. The spectrum of the methyl ester, the relevant parameters of which are summarized in Table II, showed a *cis*, *trans*-conjugated diene system next to a carbon bearing a hydroperoxy function in addition to two methylene-interrupted *cis*-double bonds. For the determination of the enantiomeric composition of the 15-HPETE, the hydroperoxy group was reduced with $NaBH_4$ in methanol and the carboxyl group esterified with diazomethane. The NMR parameters of the resulting hydroxy compound (Table II) showed that the configurations of the double bonds remained unaltered. A NMR shift experiment on the corresponding (+)-MTPA ester (for NMR parameters see Table II) yielded a ratio of 15-L_S to 15-D_R of 97 : 3. The same ratio for the 13-hydroperoxide was found in the conversion of linoleic acid with soybean lipoxygenase-1 at pH 9.0 [1,2].

(b) Products of the second oxygenation. The presence of two isomeric dihydroperoxy compounds in a reaction mixture obtained after double-dioxygenation of arachidonic acid was shown by GC-MS analysis of the proper derivatives (see Materials and Methods) as well as by ^{1}H- and ^{13}C-NMR spectroscopy. After reduction with $NaBH_4$ and esterification with diazomethane, the reaction mixture could be separated into two components by preparative high-performance liquid chromatography (retention times: 8,15-isomer, 40.0 min; 5,15-isomer,

TABLE II

360 MHz ^{1}H-NMR PARAMETERS OF VARIOUS ARACHIDONIC ACID DERIVATIVES

15-HPETE, methyl-15-hydroperoxy 5-*cis*, 8-*cis*, 11-*cis*, 13-*trans*-eicosatetraenoate; 15-HETE, methyl-15-hydroxy 5-*cis*, 8-*cis*, 11-*cis*, 13-*trans*-eicosatetraenoate; 15-MTPA-ETE, *R*-(+)-MTPA ester of 15-HETE; 5,15-DHETE, methyl-5,15-dihydroxy 6-*trans*, 8-*cis*, 11-*cis*, 13-*trans*-eicosatetraenoate; 8,15-DHETE, methyl-8,15-dihydroxy 5-*cis*, 9-*trans*, 11-*cis*, 13-*trans*-eicosatetraenoate.

Proton	15-HPETE		15-HETE		15-MTPA-ETE		5,15-DHETE		8,15-DHETE		
	δ (ppm)	*J* (Hz)	δ (ppm)	*J* (Hz)	δ (ppm)	*J* (Hz)	δ (ppm)	*J* (Hz)	δ (ppm)	*J* (Hz)	*J* (Hz)
2	2.330		2.316		2.326		2.361		2.314		
3	1.706		1.695		1.709		1.711		1.690		
4	2.111		2.102		2.113		1.596		2.089	$J_{4,5} = 7.5$	$J_{4,6} = -1.1$
5	5.393		5.373		5.375		4.214 *	$J_{5,6} = 7.5$	5.520 *	$J_{5,6} = 11.0$	$J_{5,7} = -1.35$
6	5.393		5.373		5.375		5.726 *	$J_{6,7} = 15$	5.534 *	$J_{6,7} = 7.5$	
7	2.810		2.796		2.798		6.582 *	$J_{7,8} = 11$	2.328		
8	5.393		5.373		5.375		6.018 *	$J_{8,9} = 11$	4.242 *	$J_{8,9} = 6.2$	$J_{8,10} = -0.7$
9	5.393		5.373		5.375		5.423 *	$J_{9,10} = 7.5$	5.750 *	$J_{9,10} = 15.1$	$J_{9,11} = -0.7$
10	2.976	$J_{10,11} = 7.5$	2.948	$J_{10,11} = 7.5$	2.903		3.080	$J_{10,11} = 7.5$	6.702 *	$J_{10,11} = 12.0$	$J_{10,12} = -1.5$
11	5.460	$J_{11,12} = 10.5$	~5.373	$J_{11,12} = 10.5$	~5.514 [c]	$J_{11,12} = 11$	5.440 *	$J_{11,12} = 11$	5.977	$J_{11,12} = 11.0$	$J_{11,13} = -1.5$
12	6.031	$J_{12,13} = 11$	5.988	$J_{12,13} = 11$	5.938	$J_{12,13} = 11$	6.008 *	$J_{12,13} = 11$	5.977	$J_{12,13} = 12.0$	$J_{12,14} = -0.5$
13	6.606	$J_{13,14} = 15.5$	6.510	$J_{13,14} = 15.5$	6.560	$J_{13,14} = 14$	6.565 *	$J_{13,14} = 15$	6.675 *	$J_{13,14} = 14.6$	$J_{13,15} = -0.7$
14	5.611	$J_{14,15} = 7.5$	5.690	$J_{14,15} = 7.0$	~5.514 [c]		5.707 *	$J_{14,15} = 7.5$	5.727 *	$J_{14,15} = 7.1$	
15	4.391		4.160		~5.514 [c]		4.183 *		4.178 *		
16	1.636		1.527		1.604		1.575		1.540		
17	1.297		1.293		1.300		1.309		1.282		
18	1.297		1.293		1.300		1.309		1.282		
19	1.297		1.293		1.300		1.253		1.233		
20	0.883		0.878		0.877		0.891		0.873		
OCH_3 lipid	3.674		3.657		3.666		3.675				
OCH_3 MTPA					3.559						
phenyl					7.486 + 7.392						

* Within these pairs of signals with very similar chemical shifts, the assignment may have to be reversed.

** Coupling constants and chemical shifts of the alkenyl proton resonances are derived from spectrum simulations.

c = complex.

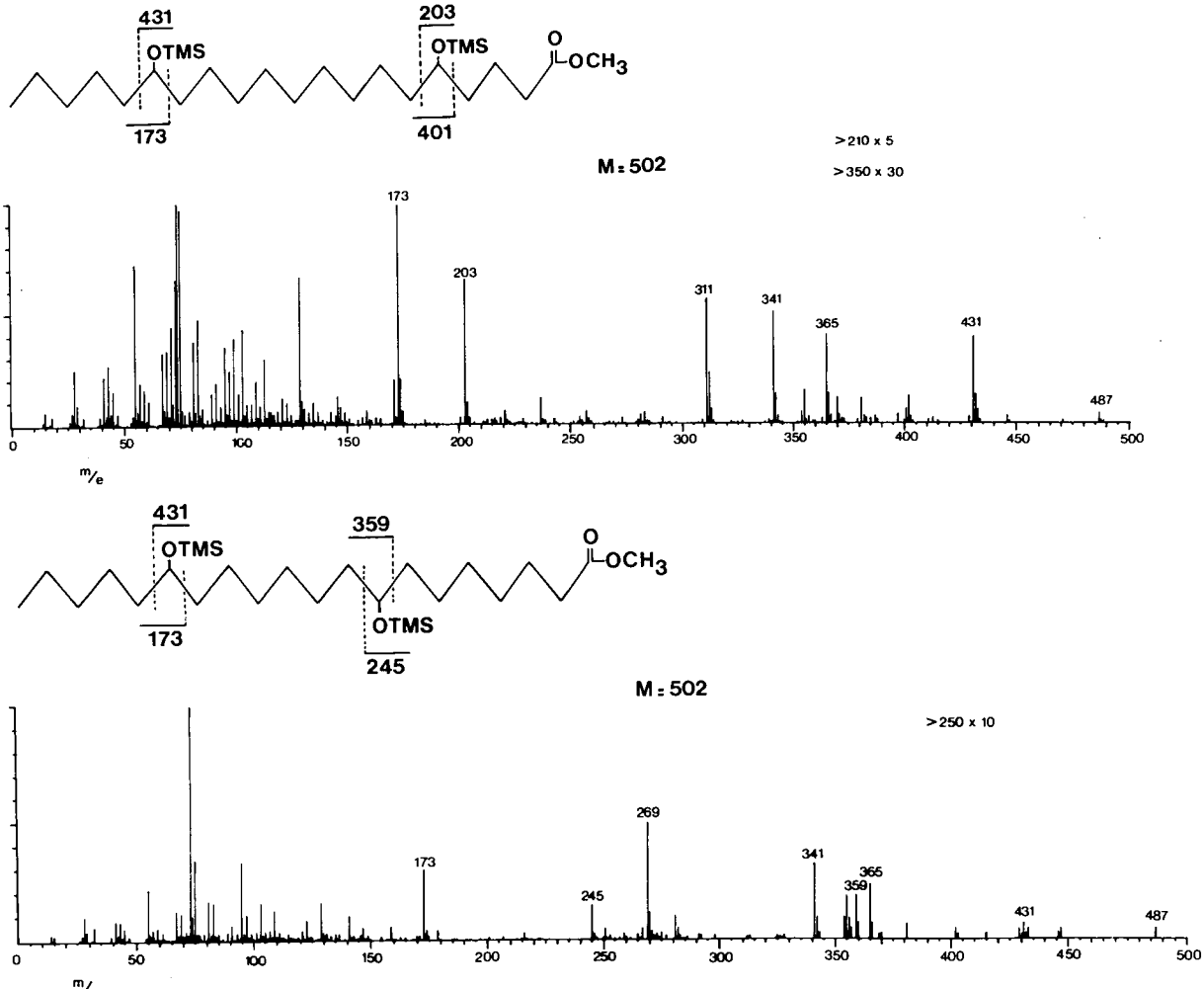

Fig. 3. (a) Mass spectrum of the trimethylsilyl ether of methyl-5,15-dihydroxyeicosanoate. (b) Mass spectrum of the trimethylsilyl ether of methyl-8,15-dihydroxyeicosanaoate.

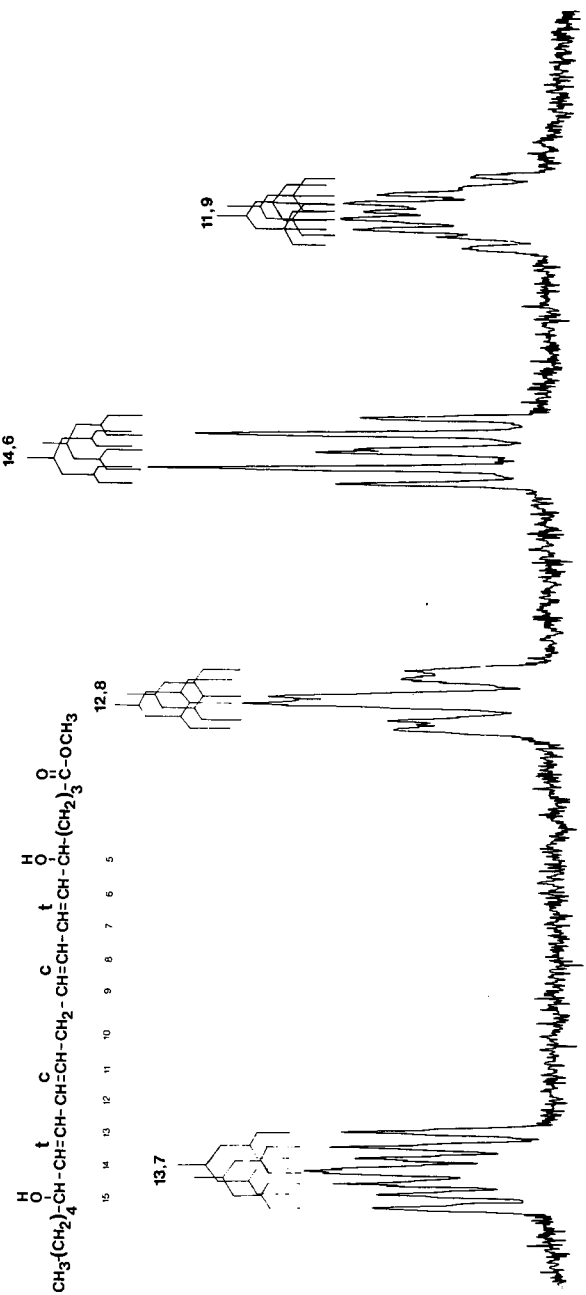

Fig. 4. Expanded 360 MHz ^{1}H-NMR spectrum showing the alkenyl resonances of methyl-5,15-dihydroxy *6-trans*, *8-cis*, 11-*cis*, 13-*trans*-eicosatetraenoate. The spectral parameters are summarized in Table II.

30.4 min; resolution: $R = 2.4$). The mass-spectra of the hydrogenated and trimethylsilylated dihydroxy compounds were shown to be characteristic of C_{20} fatty acids with OTMS groups at positions 5 and 15 (Fig. 3a) or at positions 8 and 15 (Fig. 3b), respectively. Bild et al. [13] did not report the formation of a 5,15 isomer, although the mass spectrum published by these authors is indicative of a similar mixture of products (cf. peak with $m/e = 203$).

The configurations of the double bonds in the unsaturated dihydroxy compounds were determined by 360 MHz ^{1}H-NMR and by 90.5 MHz ^{13}C-NMR. Spectral assignments were made by selective homonuclear (^{1}H-^{1}H) or heteronuclear (^{1}H-^{13}C) double resonance experiments. The 360 MHz ^{1}H-NMR spectrum of the 5,15-dihydroxy methyl ester demonstrates the occurrence of two *cis*, *trans*-conjugated double bond systems which are both located next to a carbon atom bearing a hydroxyl group. This is illustrated by the first-order analysis of the olefinic multiplets given in Fig. 4. The conjugated double-bond systems are separated by a methylene group, as is evident from the relatively low-field resonance position ($\delta = 3.080$ ppm) of the protons of this group.

For the interpretation of the ^{13}C spectrum, the spectral data from the model compounds methyl-13-hydroxy 9-*cis*, 11-*trans*-octadecadienoate and methylstearate [14] were used. Comparison of the ^{13}C chemical shifts of these compounds showed that the presence of a *cis-trans* dienol system is reflected in the

TABLE III

90.5 MHz ^{13}C-NMR PARAMETERS OF UNSATURATED MONO- AND DIHYDROXY COMPOUNDS

5,15-DHETE, methyl-5,15-dihydroxy 6-*trans*, 8-*cis*, 11-*cis*, 13-*trans*-eicosatetraenoate: 8,15-DHETE, methyl-8,15-dihydroxy 5-*cis*, 9-*trans*, 11-*cis*, 13-*trans*-eicosatetraenoate; 13-HOD, methyl-13-hydroxy 9-*cis*, 11-*trans*-octadecadienoate.

Carbon No.	Chemical shift			$\Delta\delta$ **
	5,15-DHETE	8,15-DHETE	13-HOD	
2	33.6	33.2	33.9	
3	20.6	24.5	24.9	
4	36.2	26.5	28.9	
5	72.4 *	131.9	28.9	$\delta = -0.4$
6	136.0 *	125.5	28.9	$\gamma = -0.6$
7	124.8 *	35.1	29.3	$\beta = -0.35$
8	128.1 *	72.5 *	27.5	$\alpha = -2.1$
9	129.5 *	137.3 *	132.5	
10	26.4	125.2	127.8	
11	129.1 *	128.8 *	125.5	
12	127.9 *	128.6 *	135.9	
13	125.2 *	125.2	72.7	
14	136.7 *	136.3 *	37.2	$\alpha' = +7.5$
15	72.0 *	71.7 *	24.8	$\beta' = -4.6$
16	37.1	37.0	31.6	$\gamma' = -0.4$
17	25.0	25.0	22.4	$\delta' = -0.3$
18	31.6	31.6	13.8	
19	22.5	22.5		
20	14.0	14.0		

* Although these pairs of signals with similar chemical shifts have been unambiguously assigned by selective ^{1}H-^{13}C double resonance experiments, the assignments of the signals within such a pair are uncertain.

** Shift increments relative to the corresponding ^{13}C-resonances of methylstearate (see text).

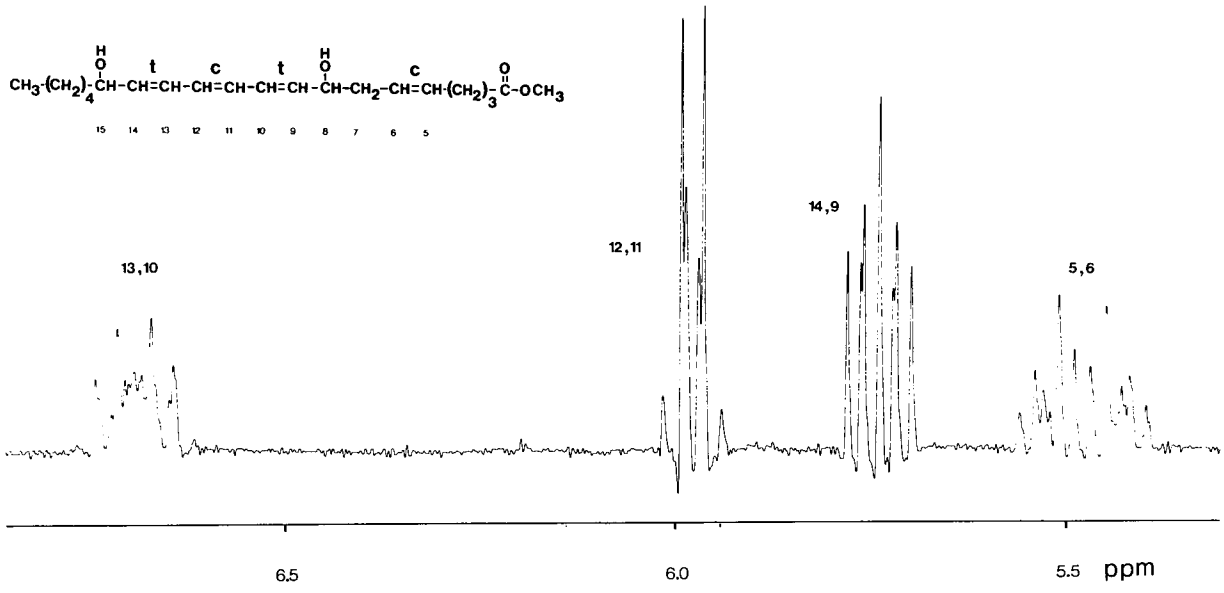

Fig. 5. Expanded 360 MHz ^{1}H-NMR spectrum showing the alkenyl resonances of methyl-8,15-dihydroxy 5-*cis*, 9-*trans*, 11-*cis*, 13-*trans*-eicosatetraenoate. Resolution enhancement was achieved by Lorentzian to Gaussian transformation according to Ernst [24]. The spectral parameters are summarized in Table II.

chemical shifts of the neighbouring carbon atoms. The observed chemical shift increments obtained in this way (Table III) are applicable to recognize this structural element in other compounds. In the 5,15 compound, two of such systems separated by a methylene group occur for the following reasons. For the calculated chemical shifts of C_2—C_4 and C_{16}—C_{19} in the 5,15-dihydroxy compound an excellent agreement (within 0.1 ppm) with the experimental values (Table III) was found. Bus et al. [15] have shown that for skipped methylene carbon atoms the application of additivity relations yields systematically too low chemical shift values. For C_{10}, a similar result was obtained, since the calculated chemical shift was 0.9 ppm lower than the experimental shift. The chemical shifts of the alkenyl atoms $C_{14,6}$, $C_{13,7}$ and $C_{12,8}$ agree well with those of the corresponding atoms C_{12}, C_{11} and C_{10} in the unsaturated model compound. The chemical shifts of $C_{11,9}$ differed from that of C_9 in the unsaturated model compound due to the presence of a second diene system on the other side of the methylene group. This effect is similar to that reported in the literature [14]. On the basis of the foregoing, the 5,15 compound can now be described as methyl-5,15-dihydroxy 6-*trans*, 8-*cis*, 11-*cis*, 13-*trans*-eicosatetraenoate.

Determination of the configurations of the double bonds from the 360 MHz ^{1}H-NMR spectrum of the 8,15-dihydroxy methyl ester is more difficult because higher-order effects preclude the direct deduction of the relevant coupling constants from the ^{1}H-NMR spectrum. Therefore, these coupling constants were determined by spectrum-simulation of the region between 5 and 7 ppm shown in Fig. 5. The NMR parameters summarized in Table II are consistent with a *trans*, *cis*, *trans* configuration of the conjugated triene moiety having on both sides a carbon atom bearing a hydroxyl group. The configuration of the isolated double bond is *cis*. For the interpretation of the ^{13}C spectrum (Table III), the shift increments obtained from the model compounds discussed before, in combination with the α-effect of an isolated double bond ($\Delta\delta$ = —2.45 ppm) [15], were used. The values calculated for C_4 and C_7 agree with the experimental shifts within 0.4 ppm and prove the *cis* geometry of the isolated double bond. For C_{16} to C_{20}, the experimental shifts were found to be the same as in the 5,15 isomer showing that these parts of the molecules are identical. The symmetry of the triene system is expressed by the occurrence of three pairs of signals. The chemical shifts of $C_{14,9}$, $C_{13,10}$ and $C_{12,11}$ agree well with the corresponding shifts of C_{12}, C_{11} and C_{10}, respectively, in the unsaturated model compound. Therefore, the configuration of the triene system is *trans*, *cis*, *trans* and the structure of the 8,15 compound is methyl-8,15-dihydroxy 5-*cis*, 9-*trans*, 11-*cis*, 13-*trans*-eicosatetraenoate.

Determination of the absolute configuration at the chiral centers by NMR

The absolute configurations of the methyldihydroxytetraenoates were determined by a ^{1}H-NMR shift method [2] on the di-(+)-MTPA esters as described before. NMR shift experiments were carried out with perdeutero-Eu(fod)$_3$ at 360 MHz in C^2HCl_3. The broad singlet representing the methoxy signal of both MTPA moieties in the 8,15 di-MTPA ester splits into four signals upon addition of a sufficient amount of shift reagent (Fig. 6). The relative intensities of the signals demonstrate that both hydroperoxy groups have been

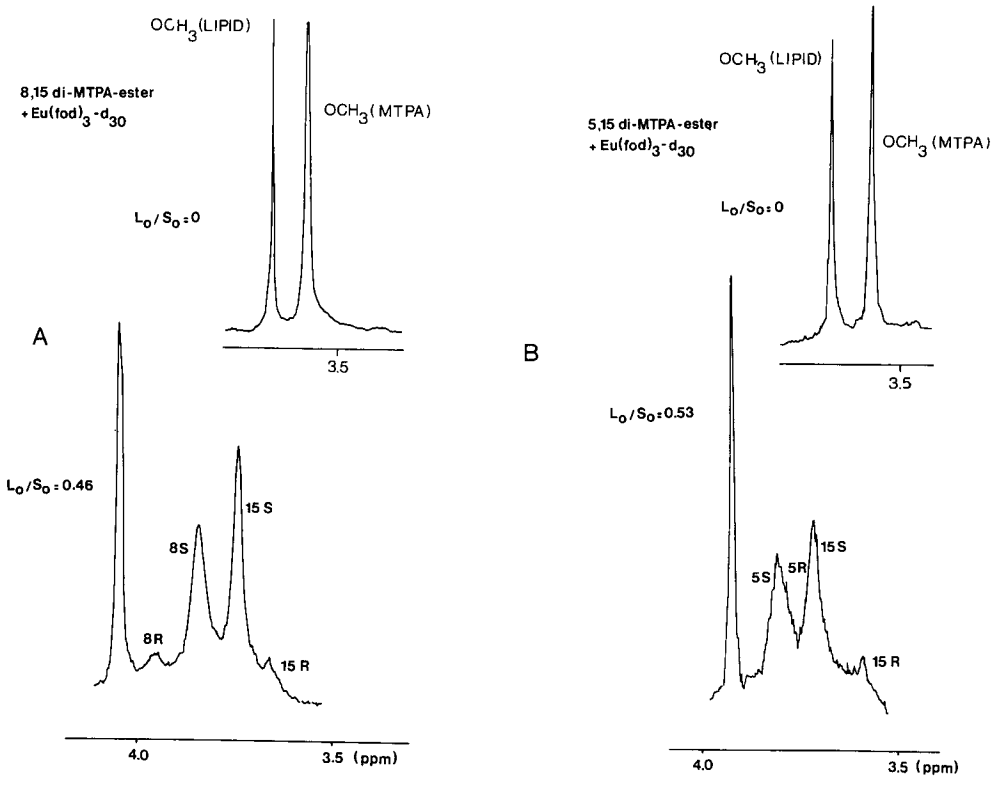

Fig. 6. Lanthanide-induced shift experiments on the di-(+)-MTPA esters of the 8,15 (A) and 5,15 (B) unsaturated dihydroxy esters. L_O = concentration of shift reagent; S_O = concentration of di-MTPA ester.

introduced almost enantiospecifically. Since 15-HPETE is a precursor of the dihydroperoxy compounds, it is assumed that the configuration at C_{15} remains unaltered. Comparison of the shifted methoxy signals with the methoxy signal of the (+)-MTPA ester of methyl-15-hydroxy eicosatetraenoate in a similar shift experiment enabled the assignment of the methoxy signals at highest field to the 15-MTPA moiety. For the determination of the absolute configuration at C_8, comparison with methyl-13-L$_S$-hydroxy 9-*cis*, 11-*trans*, 15-*cis*-octadecatrienoate [16] obtained by incubation of α-linolenic acid with soybean lipoxygenase-1 at pH 9.0, is appropriate. A ^{1}H-NMR shift experiment on the (+)-MTPA ester showed that the methoxy signal with the smaller induced shift is associated with the diastereomer derived from the *S* fatty acid. Therefore, the configuration at C_8 of the 8,15-dihydroxy compound is predominantly *S*, which corresponds to the D configuration in the Fischer convention.

A ^{1}H-NMR shift experiment on the di-(+)-MTPA ester of the 5,15-dihydroxy compound showed that the original MTPA methoxy peak gives rise to three signals (Fig. 6). The two signals at higher field which differ greatly in intensity were again assigned to the 15-MTPA moiety. The occurrence of only one signal at lower field means that either the chiral center at C_5 is enantiopure or that a signal belonging to C_5 overlaps with a signal of C_{15}. The ratio of the signals belonging to the 15-MTPA moiety is very similar to that found in a shift

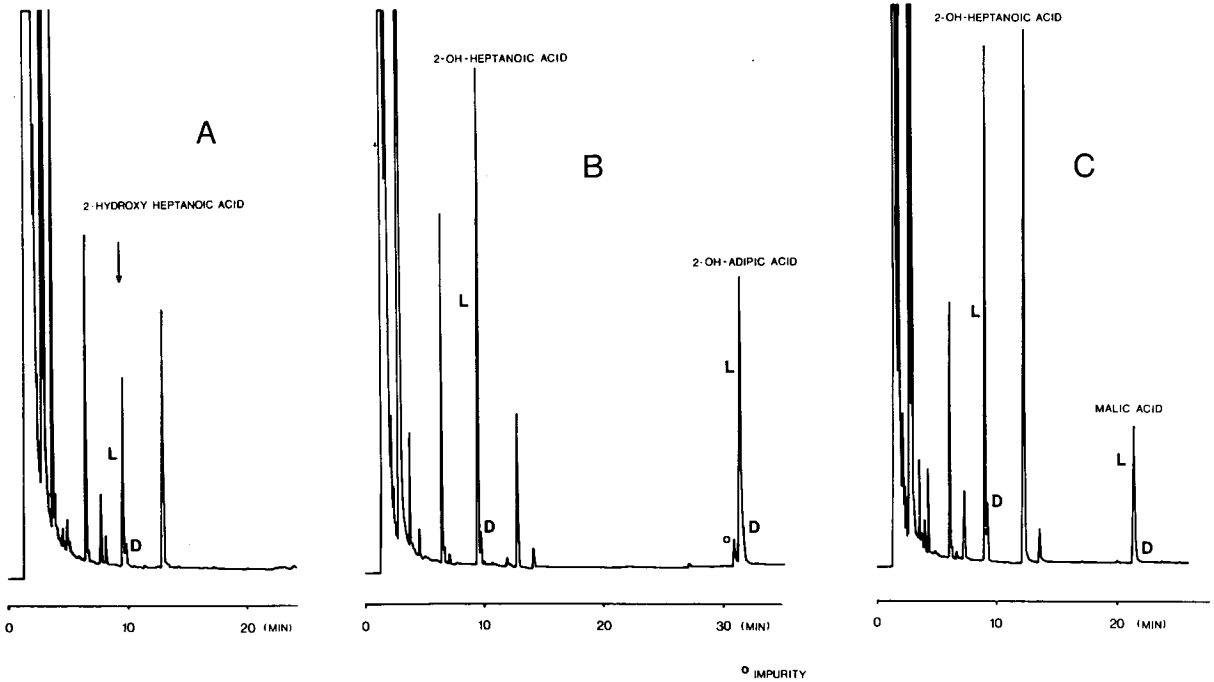

Fig. 7. Capillary gas chromatograms of acetylated *R*-2-butyl esters of 2-hydroxy-carboxylic acids and dicarboxylic acids obtained after oxidative ozonolysis of acetylated methyl-15-hydroxy 5,8,11,13-eicosatetraenoate (A), methyl-5,15-dihydroxy 6,8,11,13-eicosatetraenoate (B) and methyl-8,15-dihydroxy 5,9,11,13-eicosatetraenoate (C). The signals designated D are at least in part derived from contamination of the commercially available *R*-butanol with 6% of the *S*-enantiomer [18].

experiment on the MTPA ester of the 15-hydroxy compound, illustrating that the contribution of the overlapping signal from C_5 could only be small. Therefore, both hydroperoxy groups are formed stereospecifically. Both MTPA moieties are part of similar structural elements, leading to the assumption that the largest induced shift corresponds to the *S* configuration for the C_5 chiral center as well [2]. Therefore, the configuration at C_5 is predominantly *S*, corresponding to the D configuration according to the Fischer convention.

Determination of the absolute configuration by gas-liquid chromatography

To obtain independent evidence for the configuration at C_5 and C_8 in the dihydroxy compounds, the enantiomeric composition of the 2-hydroxy acids obtained by oxidative ozonolysis [17] of the acetylated dihydroxy methyl esters was determined. To this purpose their *O*-acetylated *R*-2-butyl esters were analysed by capillary GLC [18]. From the 8,15-dihydroxy compound, inter alia 2-hydroxyheptanoic acid (C_{14}—C_{20}) and malic acid (2-hydroxysuccinic acid) (C_6—C_9) are obtained, whereas the 5,15 isomer gives rise to 2-hydroxyheptanoic acid (C_{14}—C_{20}) and 2-hydroxyadipic acid (C_1—C_6). The chromatograms given in Fig. 7 clearly demonstrate the large preponderance of one of the enantiomers over the other for all 2-hydroxy acids. By cochromatography of proper reference compounds, it was proved that the peaks with the highest intensity belong to 2-hydroxy acids having the L_S configuration. In consequence, all chiral centers in the authentic C_{20} acids have mainly the *S* configuration. Translation of the observed chirality in terms of the Fischer convention [19] affords the following structures for the parent compounds: 8-D_S, 15-L_S-dihydroperoxy 5-*cis*, 9-*trans*, 11-*cis*, 13-*trans*-eicosatetraenoic acid and 5-D_S, 15-L_S-dihydroperoxy 6-*trans*, 8-*cis*, 11-*cis*, 13-*trans*-eicosatetraenoic acid. These results corroborate the conclusions from the NMR investigation.

Optical rotations

The optical rotations of both unsaturated dihydroxy esters were determined and found to be: $[\alpha]_{546}^{20} = +21.8°$ ($c = 1.072$, methanol) for the 8,15 isomer and $[\alpha]_{546}^{20} = +25.7°$ ($c = 0.698$, methanol) for the 5,15 isomer. The signs of these rotations are in line with the positive optical rotations of methyl-13-L_S-hydroxy octadecadienoate and of methyl-9-D_S-hydroxy octadecadienoate. It is interesting to note that one of the dihydroxy acids obtained in this study bears a great structural resemblance to the 5-D_S, 12-D_R-dihydroxy 6,8,10,14-eicosatetraenoic acid obtained as a by-product in the incubation of arachidonic acid with polymorphonuclear leukocytes [20,21]. It seems that also in the animal system, the formation of the dihydroxy compounds proceeds as a biphasic process wherein the introduction of the hydroxyl at position 12 is a relatively slow step. However, from a mechanistic point of view the plant and animal systems are significantly different because in the plant system both hydroxyls are derived from hydroperoxy groups, whereas in the animal system only for the hydroxy group at C_5 can a hydroperoxide be conceived as a precursor [22].

Concluding remarks

Both the first and second step of the double dioxygenation of arachidonic acid by soybean lipoxygenase-1 were found to proceed highly stereospecifi-

cally. However, the regio specificity of the second step (8,15 : 5,15 = 60 : 40) is considerably lower than that of the first step (15-HPETE > 99%). A similar specificity pattern has been observed for the oxygenation of linoleic acid with soybean lipoxygenase-1 at low pH [2]. From the data presented in this study, as well as from literature [1—3,16,23], it can be concluded that stereospecific oxygenation by lipoxygenase-1 produces almost exclusively the *S* enantiomer, irrespective of the position of the fatty acid carbon atom which is oxygenated.

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References

1 Hamberg, M. (1971) Anal. Biochem. 43, 515—526
2 Van Os, C.P.A., Vente, M. and Vliegenthart, J.F.G. (1979) Biochim. Biophys. Acta 574, 103—111
3 Hamberg, M. and Samuelsson, B. (1967) J. Biol. Chem. 242, 5329—5335
4 Egmond, M.R., Vliegenthart, J.F.G. and Boldingh, J. (1972) Biochem. Biophys. Res. Commun. 48, 1055—1060
5 Bild, G.S., Ramadoss, C.S., Lim, S. and Axelrod, B. (1977) Biochem. Biophys. Res. Commun. 74, 949—954
6 Finnazi-Agrò, A., Avigliano, L., Veldink, G.A., Vliegenthart, J.F.G. and Boldingh, J. (1973) Biochim. Biophys. Acta 326, 462—470
7 Galpin, J.R., Tielens, L.G.M., Veldink, G.A., Vliegenthart, J.F.G. and Boldingh, J. (1976) FEBS Lett. 69, 179—182
8 Verhagen, J., Vliegenthart, J.F.G. and Boldingh, J. (1978) Chem. Phys. Lipids 22, 255—259
9 Koch, R.B., Stern, B. and Ferrari, C.G. (1958) Arch. Biochem. Biophys. 78, 165—179
10 Snyder, F. and Stephens, N. (1959) Biochim. Biophys. Acta 34, 224—245
11 Renkonen, O. (1961) Biochim. Biophys. Acta 54, 361—362
12 Cook, H.W. and Lands, W.E.M. (1975) Can. J. Biochem. 53, 1220—1231
13 Bild, G.S., Ramadoss, C.S. and Axelrod, B. (1977) Arch. Biochem. Biophys. 181, 36—41
14 Tulloch, A.P. and Bergter, L. (1979) Lipids 14, 996—1002
15 Bus, J., Sies, I. and Lie Ken Jie, M.S.F. (1976) Chem. Phys. Lipids 17, 501—518
16 Vick, B.A. and Zimmerman, D.C. (1979) Plant Physiol. 63, 490—494
17 Bergström, S., Ryhage, R., Samuelsson, B. and Sjövall, J. (1963) J. Biol. Chem. 238, 3555—3564
18 Van Os, C.P.A., Rijke-Schilder, G.P.M., Kamerling, J.P., Gerwig, G.J. and Vliegenthart, J.F.G. (1980) Biochim. Biophys. Acta 620, 328—333 → 320 — 331
19 IUPAC Commission on the nomenclature of organic chemsitry (1976) Pure Appl. Chem. 45, 13—30
20 Borgeat, P., Hamberg, M. and Samuelsson, B. (1976) J. Biol. Chem. 251, 7816—7820
21 Borgeat, P. and Samulesson, B. (1979) J. Biol. Chem. 254, 2643—2646
22 Borgeat, P. and Samuelsson, B. (1979) Proc. Nat. Acad. Sci. U.S.A. 76, 3213—3217
23 Egmond, M.R., Veldink, G.A., Vliegenthart, J.F.G. and Boldingh, J. (1975) Biochim. Biophys. Acta 409, 399—401
24 Ernst, R.R. (1966) in Advances in Magnetic Resonance (Waugh, J.S., ed.), Vol. 2, pp. 1—135, Academic Press, New York