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A NMR SHIFT METHOD FOR DETERMINATION OF THE ENANTIOMERIC COMPOSITION OF HYDROPEROXIDES FORMED BY LIPOXYGENASE

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Summary

A method is presented for determination of the enantiomeric composition of hydroxyperoxides formed by enzymic oxygenation of unsaturated fatty acids. After reduction of the hydroperoxy group with $NaBH_4$, and esterification, the positional isomers of the resulting hydroxy compounds are separated by high performance liquid chromatography. The latter are subsequently subjected to a chiral derivatization to form diastereomeric α-methoxy-α-trifluoromethylphenylacetate esters. Determination of the diastereomeric composition by a NMR shift experiment furnishes the enantiomeric composition of the parent hydroperoxides.

The method has been applied to the hydroperoxides formed by incubation of linoleic acid by corn germ or soybean lipoxygenase. Our results indicate that under the conditions used the hydroperoxides are mainly enantiospecifically formed.

Introduction

Lipoxygenase (linoleate : oxygen oxidoreductase EC 1.13.11.12) is a mononuclear non-heme iron dioxygenase, which catalyses the conversion of polyunsaturated fatty acids containing a 1,4-*cis,cis* pentadiene system into conjugated hydroperoxides. Depending on the source of the enzyme and the pH of the incubation medium different isomeric hydroperoxides are formed.

Abbreviations: HPLC, high-performance liquid chromatography; MTPA, α-methoxy-α-trifluoromethylphenylacetate.

Starting from linoleic acid as a substrate, soybean lipoxygenase-1 produces at pH 9 almost exclusively 13-L_S-hydroxyperoxy-9-*cis*, 11-*trans*-octadecadienoic * acid, whereas the corn germ enzyme at pH 6.6 gives rise to mainly 9-D_S-hydroperoxy-10-*trans*,12-*cis*-octadecadienoic acid [1,2]. Other enzymes like soybean lipoxygenase-2 yield mixtures of hydroperoxides. For the understanding of the mechanism of action of these enzymes it is important to know the positional and enantiomeric composition of the products. The molar ratio of the positional isomers has mainly been determined on the basis of mass spectrometry of the hydroxystearates or by TLC of ^{14}C-labelled hydroxy fatty acids. However, the most reliable method seems to be high performance liquid chromatography (HPLC) of the hydroxydienoates or hydroxydienoic acids [3]. This technique has the advantage that it can also be applied on a (-micro)preparative scale.

The determination of the enantiomeric composition of each of the positional isomers is more tedious. In principle the determination of the optical rotation could furnish relevant information in this respect. However, the specific rotation of the unsaturated hydroxy acids is low, therefore giving results with an intrinsic low accuracy. A rather elaborate alternative has been described by Hamberg [4], which makes use of GLC on the methylchloroformate derivatives of the compounds formed after reduction of the hydroperoxy group followed by ozonolysis and esterification.

This paper presents a new and simple NMR method for the determination of the enantiomeric composition based on the lanthanide induced shift of a chiral derivative of the hydroxydienoates.

Materials and Methods

Racemic hydroxydienoates derived from linoleic acid

13-L_S-hydroperoxy-9-*cis*,11-*trans* octadecadienoic acid obtained by preparative incubation of linoleic acid with soybean lipoxygenase-1 at pH 9.0 was converted into the corresponding hydroxy ester by reduction with $NaBH_4$ and subsequent treatment with diazomethane. Oxidation of the hydroxy compound with $Pb(CH_3COO)_4$ in pyridine according to Partch [5] followed by separation on precoated TLC plates (Merck 60 F-154 silicagel 0.5 mm, eluent: hexane/diethyl ether (70 : 30, v/v), afforded the methyl 13-oxo-9-*cis*,11-*trans* octadecadienoate. The structure was confirmed by proton and carbon NMR. Reduction of the oxo compound with $NaBH_4$ in methanol yielded racemic methyl-13-hydroxy-9-*cis*,11-*trans* octadecadienoate. Proton and carbon NMR spectra were identical to those of the optically active compound. A similar sequence of reactions was performed on 9-D_S-hydroperoxy-10-*trans*,12-*cis* octadecadienoic acid obtained by incubation of linoleic acid with corn germ lipoxygenase at pH 6.6 which afforded racemic methyl-9-hydroxy-10-*trans*,12-*cis* octadecadienoate.

Enzyme preparations

Soybean lipoxygenase-1 was isolated according to Finazzi-Agrò et al. [6]. The specific activity of the electrophoretically homogeneous material was 200

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* L and D refer to the nomenclature according to the Fisher convention; subscripts S and R refer to the nomenclature according to Cahn, Ingold and Prelog.

μmol $O_2 \cdot min^{-1} \cdot mg^{-1}$ as determined polarographically on a Gilson oxygraph equipped with a Clark oxygen electrode (1.8 mM NH_4^+ linoleate, pH 9.0). Corn germ lipoxygenase was a partially purified preparation obtained by collecting the ammonium sulfate fraction between 30 and 60% saturation of an extract from acetone-defatted corn germs. The specific activity was 0.7 μmol $O_2 \cdot min^{-1} \cdot mg^{-1}$.

Incubations

Incubations were carried out at 4°C in 0.1 M phosphate buffer (pH 6.6) or in 0.1 M borate buffer (pH 9.0). Linoleic acid was purchased from Lipid Supplies St. Andrews, Scotland, (purity >99%), and added as its ammonium salt to a final concentration of 1.0 mM. During the reaction the solution was flushed with oxygen. Reactions were started by addition of enzyme (0.5 enzyme unit per mg substrate) and the course of the reaction was monitored at 234 nm with a Cary 118 spectrophotometer. After the reaction had come to completion the hydroperoxides were reduced by addition of a 5-fold molar excess of $NaBH_4$. The hydroxydienoic acids were extracted with diethyl ether at pH 3 and esterified with diazomethane. The positional isomers of the hydroxydienoates were separated by HPLC on a Varian 4100 liquid chromatograph equipped with a partisil-5 column (internal diameter 9 mm × 25 cm, Chrompack, Middelburg, The Netherlands) and a Varian CDS 111 electronic integrator. The column was equilibrated with hexane/diethyl ether/ethanol (80 : 20 : 0.08, v/v/v) and eluted with the same solvent mixture. $S(+)$-α-methoxy-α-trifluoromethylphenylacetyl chloride (MTPA-Cl) was prepared from $R(+)$-α-methoxy-α-trifluoromethylphenylacetic acid (MTPA) according to Dale et al. [7] and used as a reagent for the conversion of hydroxydienoates into the corresponding MTPA-esters according to Dale and Mosher [8]. $R(+)$-MTPA was obtained from Aldrich. Pyridine and CCl_4 were of analytical-grade quality, pyridine was dried by distillation from chlorosulfonic acid (1 ml per 100 ml pyridine) and CCl_4 was dried over a 4 Å molecular sieve. These solvents were stored under nitrogen. Proton NMR spectra were recorded on Varian EM 390 (90 MHz) or HA 100 (100 MHz) spectrometers and carbon NMR spectra on a Varian CFT 20 (20 MHz). The spectra were run at room temperature and the samples were dissolved in either CCl_4 (proton spectra) or C^2HCl_3 (carbon spectra) with tetramethylsilane as an internal standard. The shift reagents $Eu(fod)_3$ and $Pr(fod)_3$ were obtained from Merck.

Results and Discussion

For the determination of the enantiomeric composition use is made of the difference in the NMR spectra of diastereomeric MTPA-esters. The 90 MHz proton NMR spectrum of the (+)-MTPA-ester of 13-L_S-hydroxydienoate is given in Fig. 1. Comparison of this spectrum with that of the diastereomeric mixture obtained by reaction of (+)-MTPA-Cl with racemic 13-hydroxydienoate shows significant differences as summarized in Table I. The most pronounced effects are found in the region of the unsaturated protons as shown in the expanded spectra in Fig. 2. The doubling of the triplet of proton 10 indicates a chemical shift increment of this signal for the diastereomers, $\Delta\delta$

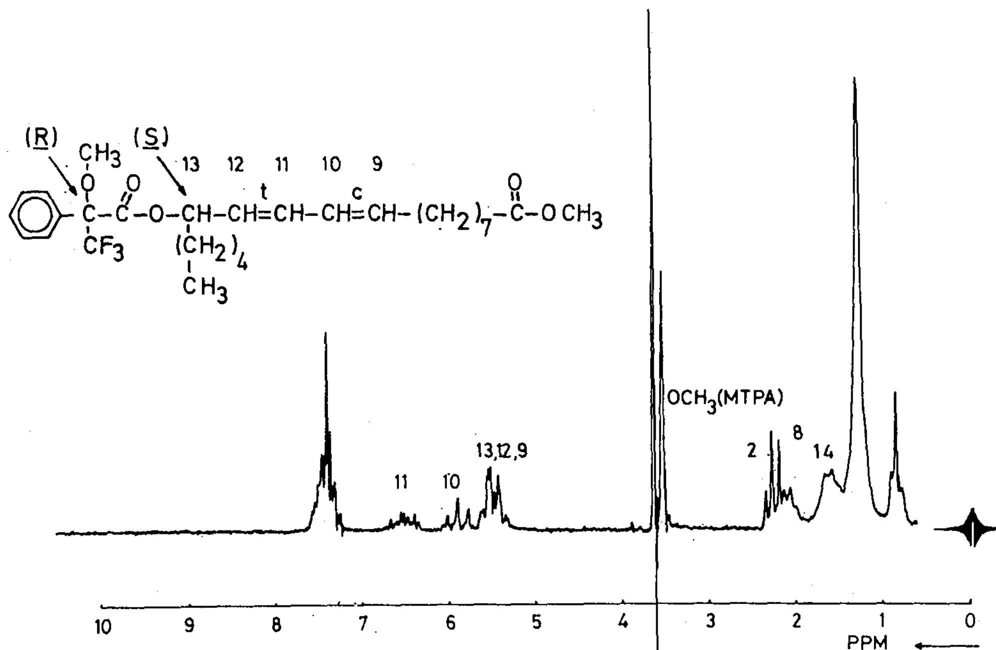


Fig. 1. 90 MHz proton NMR spectrum of the MTPA ester derived from R(+)-MTPA and methyl-13-L$_S$-hydroxy-9-*cis*,11-*trans* octadecadienoate.

of 0.04 ppm. A similar effect is observed on the multiplet of proton 11, where a $\Delta\delta$ value of 0.07 ppm is found. The complex pattern of protons 9, 12 and 13 is also influenced but the complexity of the signal does not allow a rigorous interpretation. In principle, any difference in the spectra of the diastereomers can be used for quantitation provided that integration can be carried out. However, the rather poor separation and relatively low intensity of the signals of the unsaturated protons make these unsuited for an accurate determination of

TABLE I

PROTON NMR PARAMETERS OF DIASTEREOMERIC MTPA ESTERS DERIVED FROM *R*(+)-MTPA

Proton	*S*-hydroxydienoate	*R*-hydroxydienoate
phenyl	7.45 (m)	7.45
11	6.54 (m)	6.61
10	5.95 (t)	5.91
9, 12, 13	5.50 (m)	5.50 *
OCH_3 (lipid)	3.66 (s)	3.66
OCH_3 (MTPA)	3.56 (q)	3.57
2	2.28 (t)	2.28
8	2.14 (q)	2.14
14	1.62 (m)	1.62
3–7, 15–17	1.30 (m)	1.30
18	0.84 (t)	0.87 **

* A downfield shift of at least one of the signals is apparent (cf. Fig. 2).

** Analogous spectra are obtained for MPTA esters derived from 9-hydroxydienoate except for the doubling of the triplet of proton 18, which is absent.

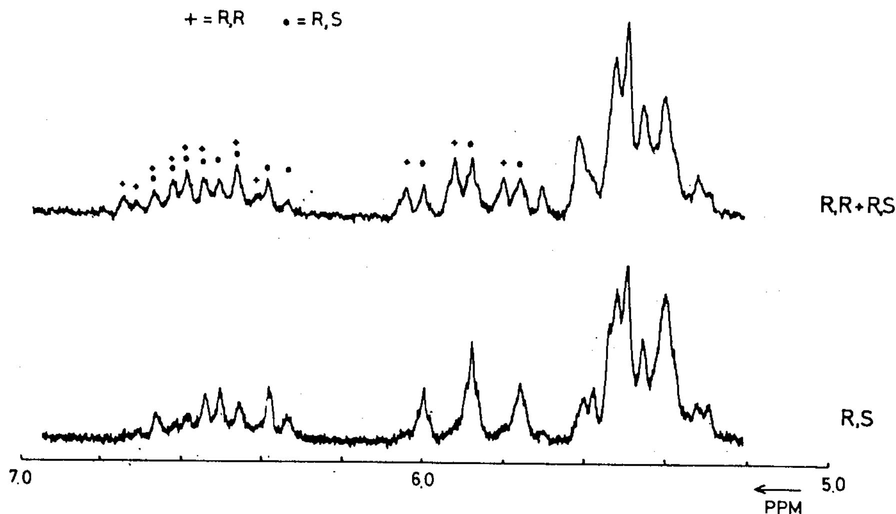
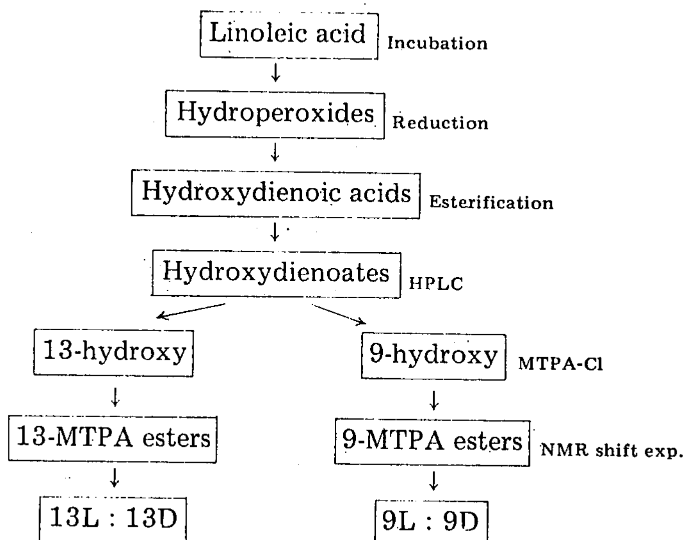


Fig. 2. 90 MHz proton NMR spectra of the MTPA esters derived from R(+)-MTPA and methyl-13-L_S-hydroxy-9-*cis*,11-*trans* octadecadienoate, lower trace, or racemic methyl-13-hydroxy-9-*cis*,11-*trans* octadecadienoate, upper trace.

the enantiomeric composition. The introduction of a non-chiral shift reagent such as $Eu(fod)_3$ or $Pr(fod)_3$ results in a complete separation of the methoxy signals of the MTPA moiety of the diastereomers [8,9]. These signals can adequately be integrated yielding the diastereomeric composition and thereby the enantiomeric purity of the parent hydroperoxides. Typical shift experiments for both $Eu(fod)_3$ and $Pr(fod)_3$ are shown in Fig. 3. Whereas $Eu(fod)_3$ causes a shift to low field, $Pr(fod)_3$ causes a shift to high field. The shift increment of the methoxy signal of the lipid moiety is considerably larger than that of the MTPA moiety. In the case of $Pr(fod)_3$ the lipid methoxy singlet



Scheme I. Summary of the procedure.

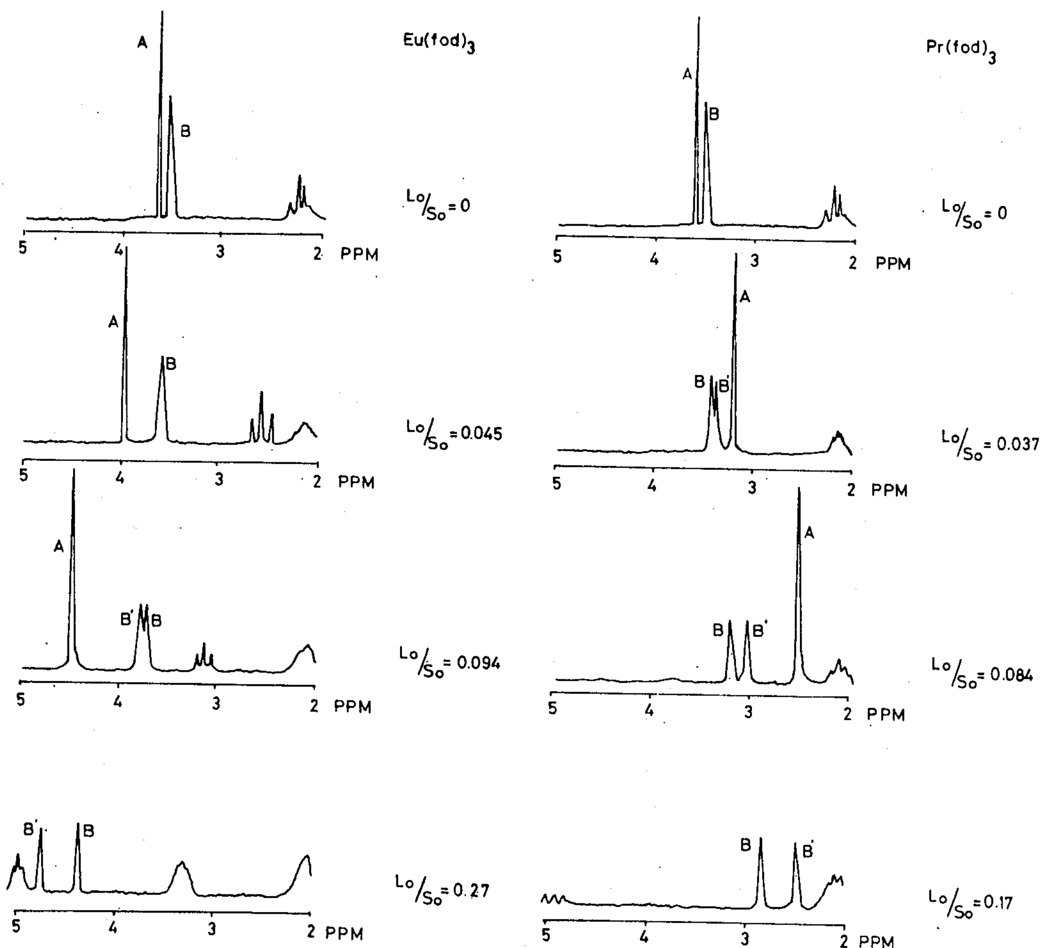


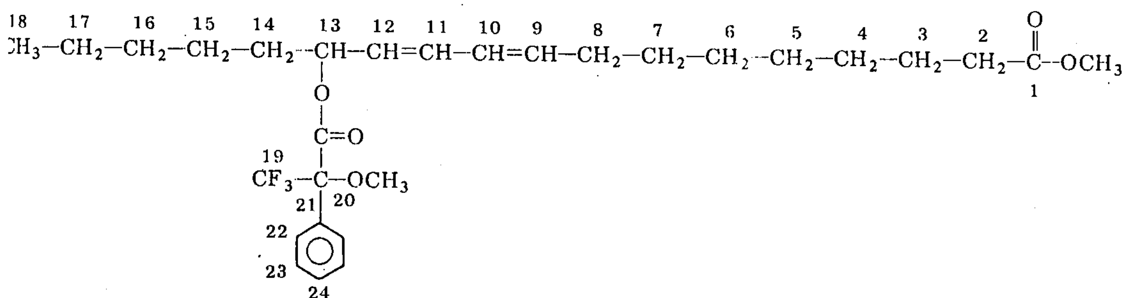
Fig. 3. Lanthanide shift experiments with diastereomeric mixtures of MTPA esters derived from racemic hydroxydienoate Lo = concentration of lanthanide shift reagent, So = concentration of MTPA ester, A = methoxy signal of the lipid, B^1 = methoxy signal of the MTPA moiety of the diastereomer derived from S-hydroxydienoate (i.e. 13-L or 9-D), B = methoxy signal of the MTPA moiety of the diastereomer derived from R-hydroxydienoate.

overtakes the methoxy signal of the MTPA moiety. On addition of increasing amounts of the shift reagent the methoxy signal of the MTPA moiety broadens at first, to split up later in two separate signals representing the different diastereomers. $Pr(fod)_3$ was preferred for the analyses because of a better shift efficiency (a lower shift reagent to substrate ratio). The analysis can be carried out on 10 mg hydroxydienoate using a 90 MHz apparatus in the CW mode. In the FT mode the amount of material can greatly be reduced to less than 1 mg.

In principle proton decoupled carbon NMR would particularly be suited to observe magnetical non-equivalence in diastereomeric compounds since all signals are singlets. A serious disadvantage however, is the intrinsic insensitivity of the method. Table II presents the chemical shift values of the diastereomeric MTPA esters derived from 13-hydroxydienoate. The observed differences in carbon NMR are analogous to those found in proton NMR. For some carbon

TABLE II

CARBON NMR PARAMETERS OF MTPA ESTERS DERIVED FROM (+)-MTPA AND 13-L_S- OR 13-D_R-HYDROXYDIENOATE



Assignments were made by off resonance techniques, lanthanide shift experiments, and by a FT carbon NMR experiment with selective CW irradiation of both carbon satellites in the proton NMR spectrum. The latter experiment was performed on a Varian XL-100 spectrometer.

Carbon No.	Chemical shift		Increment
	13-L_S	13-D_R	
1	173.9	173.9	
2	33.8	33.8	
3	24.7	24.7	
4	28.8	28.8	
5	28.8	28.8	
6	27.5	27.5	
7	28.8	28.8	
8	29.2	29.2	
9	127.1	127.1	
10	128.9	129.1	+0.19
11	129.0	129.3	+0.29
12	134.1	134.4	+0.25
13	77.3	77.3	
14	34.1	34.1	−0.05
15	24.5	24.2	−0.28
16	31.2	31.2	−0.08
17	22.2	22.2	
18	13.7	13.7	
19	165.5	165.5	
20	84.4 (q) $^2J_{CCF}$ = 30 Hz	84.2	−0.15
21	132.2	132.4	+0.20
22	127.2	127.2	
23	128.0	128.0	
24	129.2	129.2	
OCH_3 (lipid)	51.1	51.1	
OCH_3 (MTPA)	55.2	55.2	
CF_3	123.2 (q) $^1J_{CF}$ = 290 Hz	123.2	

nuclei the separation between the signals of the diastereomers is sufficient to allow determination of the ratio without a shift experiment.

The mixtures of hydroperoxides formed by various lipoxygenases under different conditions have been analysed according to Scheme I.

The results in terms of regio- and stereospecificity of the enzymic conversion of linoleic acid are summarized in Table III.

TABLE III

ANALYSES OF HYDROPEROXYOCTADECADIENOIC ACIDS FORMED FROM LINOLEIC ACID ON INCUBATION WITH SOYBEAN-1 OR CORN GERM LIPOXYGENASE

Conditions *	% Conversion **	9 : 13	9-D_S : 9-L_R	13-L_S : 13-D_R
Soybean-1 (pH 9.0)	98	2.5 : 97.5 (±0.5) ***	61 : 39 (±1)	97 : 3 (±1)
Soybean-1 (pH 6.6)	92	23 : 77 (±2)	92 : 8 (±1)	94 : 6 (±1)
Corn germ (pH 6.6)	63	86 : 14 (±1)	94 : 6 (±1)	79 : 21 (±2)

* Three incubations were performed in each case, mean values are given.
** As calculated from absorbance at 234 nm.
*** Figures between brackets indicate the accuracy.

The proportion of the 9-isomer formed by soybean lipoxygenase-1 increases considerably when the pH of the incubation medium is lowered from 9.0 to 6.6 (cf. Axelrod [2]). The enantiomeric purity of the 13-isomer is hardly diminished, whereas the enantiomeric purity of the produced 9-isomer is dramatically increased by this change in pH. The corn germ preparation produces predominantly the 9-D_S-hydroperoxide but in view of the possible presence of hydroperoxide isomerase in the preparation [10], it should be kept in mind that the results refer to that part of the hydroperoxides which is not converted by the isomerase. Analyses of the reaction mixtures show that none of the produced positional isomers is racemic. This observation can be explained by the assumption that there is only a small contribution from a nonenantiospecific process. It has to be noted that this process does not necessarily lead to the formation of equal amounts of the racemic 9- and 13-isomer [11].

These results differ from those reported by Hamberg [4], who suggested that only one isomer is enzymically formed and that the three remaining isomers are the result of autoxidation.

According to Egmond et al. [12,13] an antarafacial relation exists between hydrogen abstraction and oxygen insertion, i.e. the formation of the 9-D_S-hydroperoxide from linoleic acid is coupled to the abstraction of the $11\text{-D}_{pro\text{-}R}$ hydrogen and formation of the 13-L_S-hydroperoxide is coupled to abstraction of the $11\text{-L}_{pro\text{-}S}$ hydrogen. Comparison of the results of the present investigation with those of Egmond et al. [13] demonstrates that the main course of the reaction proceeds via this mechanism. For some of the chiral compounds formed in minor quantities this relation does not seem to hold, but the reason for this deviation is as yet unclear. An interesting finding is the ability of soybean lipoxygenase-1 at low pH to abstract either the $\text{L}_{pro\text{-}S}$ or the $\text{D}_{pro\text{-}R}$ hydrogen. This feature can be interpreted in terms of a head-tail reversion of the substrate fatty acid in the active site of the enzyme [14].

Conclusion

The present investigation describes a new method for the determination of the enantiomeric composition of hydroperoxides based on a simple derivatization procedure followed by a lanthanide shift NMR experiment. It can be carried out on small amounts of material and seems to be generally applicable to unsaturated hydroxy acids as ricinoleic acid or derivatives of linolenic acid.

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