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COMMUNICATION

Stereochemistry of Hydrogen Removal During Oxygenation of Linoleic Acid by Singlet Oxygen and Synthesis of 11(*S*)-Deuterium-Labeled Linoleic Acid

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Abstract Exposure of unsaturated fatty acids to singlet oxygen results in the formation of hydroperoxides. In this process, each double bond in the acyl chain produces two regioisomeric hydroperoxides having an (*E*)-configured double bond. Although such compounds are racemic, the hydrogen removal associated with the oxygenation may, a priori, take place antarafacially, suprafacially or stereorandomly. The present study describes the preparation of [11(*S*)-^{2}H]linoleic acid by two independent methods and the use of this stereospecifically labeled fatty acid to reveal the hidden stereospecificity in singlet oxygenations of polyunsaturated fatty acids. It was found that linoleic acid 9(*R*)- and 13(*S*)-hydroperoxides formed from [11(*S*)-^{2}H] linoleic acid both retained the deuterium label whereas the 9(*S*)- and 13(*R*)-hydroperoxides were essentially devoid of deuterium. It is concluded that polyunsaturated fatty acid hydroperoxides produced in the presence of singlet oxygen in e.g., plant leaves are formed by a reaction involving addition of oxygen and removal of hydrogen taking place with suprafacial stereochemistry. This result confirms and extends previous mechanistic studies of singlet oxygen-dependent oxygenations.

Keywords Singlet oxygen · Oxygenation · Hydroperoxide · Linoleic acid · Oxylipins

Abbreviations

GC–MS	Gas–liquid chromatography–mass spectrometry
CP-HPLC	Chiral phase high performance chromatography
RP-HPLC	Reversed-phase high performance chromatography
SP-HPLC	Straight-phase high performance chromatography
Me_3Si	Trimethylsilyl
HPODE	Hydroperoxyoctadecadienoic acid
HODE	Hydroxyoctadecadienoic acid

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Introduction

Lipoxygenases are non-heme dioxygenases which catalyze the incorporation of molecular oxygen at a 1(*Z*),4(*Z*)-pentadienyl moiety of polyunsaturated fatty acids forming optically active hydroperoxy fatty acids having a pair of (*E*),(*Z*)-configured conjugated double bonds [1–3]. The same type of fatty acid hydroperoxides is formed by autoxidation of polyunsaturated fatty acids by ground state (radical) dioxygen ($^{3}O_2$). In this case oxygenation takes place without control of regiochemistry and the hydroperoxides are racemic. A third possibility of generating hydroperoxides from unsaturated fatty acids is to expose them to singlet oxygen ($^{1}O_2$). This unstable, non-radical form of dioxygen can be generated from ground state oxygen by light irradiation in the presence of a photosensitizer. $^{1}O_2$ is also produced in certain oxygen-evolving reactions such as the oxidation of hydrogen peroxide by hypochlorite and during the thermal decomposition of phosphite ozonides and other oxygenated compounds (see Ref. [4] and references cited therein). In the process, each double bond in the acyl chain will produce two regioisomeric, racemic hydroperoxides.

Mechanistically, the vast majority of lipoxygenase-catalyzed oxygenations proceed with antarafacial stereochemistry, i.e. the initial hydrogen removal and the subsequent attack by $^3O_2$ take place from opposite sides of the plane of the 1,4-pentadiene moiety [2]. Autoxidation, on the other hand, is a stereorandom process where the carbon-centered radical formed following hydrogen removal can be attacked from both sides with equal probability [5]. Mechanistic studies of $^1O_2$ oxygenations of simple model alkenes have indicated a suprafacial process [6, 7], however, it was of interest to extend such studies to a biologically relevant substrate. The present report is concerned with singlet oxygenation of a polyunsaturated fatty acid having the characteristic methylene group-interrupted (*Z*),(*Z*)-diene partial structure. [11(*S*)-^{2}H]Linoleic acid prepared by two different methods was used for this purpose, and the regio- and stereoisomeric hydroperoxides formed during its exposure to $^1O_2$ were analyzed for isotope content using GC–MS.

Experimental Procedures

[11(*R*,*S*)-^{2}H]Linoleic Acid

The deuterated linoleic acid was synthesized by acetylene coupling followed by partial hydrogenation of the resulting deuterated octadecadiynoic acid. Briefly, 2-octynal (2.5 g; Sigma-Aldrich, Stockholm, Sweden) was reduced to [1-^{2}H]2-octyn-1-ol using sodium borodeuteride in methanol. The bromide (2.73 g) prepared by refluxing with PBr_3 was coupled to methyl 9-decynoate in the presence of CuI and Cs_2CO_3 [8]. Following purification on a silica gel column, 90% pure methyl [11(*R*,*S*)-^{2}H]9,12-octadecadiynoate (4.4 g; yield from 2-octynal, 75%) was obtained. An aliquot was subjected to partial hydrogenation using P-2 nickel as the catalyst [9]. The resulting deuterated methyl linoleate was purified by RP-HPLC, saponified, and further purified by a second RP-HPLC run to provide >99% pure [11(*R*,*S*)-^{2}H]linoleic acid.

[11(*S*)-^{2}H]Linoleic acid (**5**): Method A

It is well known from previous studies that soybean lipoxygenase-1 stereospecifically abstracts the *pro-S* hydrogen from the ω8 bisallylic methylene group of linoleic acid and other polyunsaturated fatty acids [10], and that substitution of this hydrogen for deuterium is accompanied by a large kinetic isotope effect (k_H/k_D about 40, see [11]). Thus, if [11(*R*,*S*)-^{2}H]linoleic acid is incubated with soybean lipoxygenase, the 11(*R*)-^{2}H-labeled enantiomer will be rapidly consumed whereas the 11(*S*)-^{2}H-labeled acid will largely remain not converted. Method A was based on these facts and involved stirring at 0 °C under oxygen atmosphere of [11(*R*,*S*)-^{2}H]linoleic acid (192 mg) in 300 mL of 0.1 M sodium borate buffer pH 10.4 with soybean lipoxygenase-1 (Sigma-Aldrich type IV, 60 μL (250,000 units) [unit definition: as defined by the manufacturer, 1 U will cause an increase in A_{234} of 0.001/min at pH 9.0 at 25 °C when linoleic acid is the substrate in 3.0 mL volume (1 cm light path)]. After 19 min, additional enzyme was added [20 μL (83,000 U)]. The reaction was followed spectrophotometrically by recording the absorbance at 234 nm and interrupted after 32 min (53% conversion according to UV spectrometry). From the mixture, [11(*S*)-^{2}H]linoleic acid was obtained in >99% pure form following preparative RP-HPLC. The isotope composition was 98.1% deuterated and 1.9% undeuterated molecules. In agreement with the labeling, re-incubation of an aliquot of the [11(*S*)-^{2}H]linoleic acid with soybean lipoxygenase-1 resulted in a very slow conversion and the formation of 13(*S*)-HPODE which was essentially devoid of deuterium.

The above method for enzymatic resolution of [11(*R*,*S*)-^{2}H]linoleic acid has been used in a previous study [11].

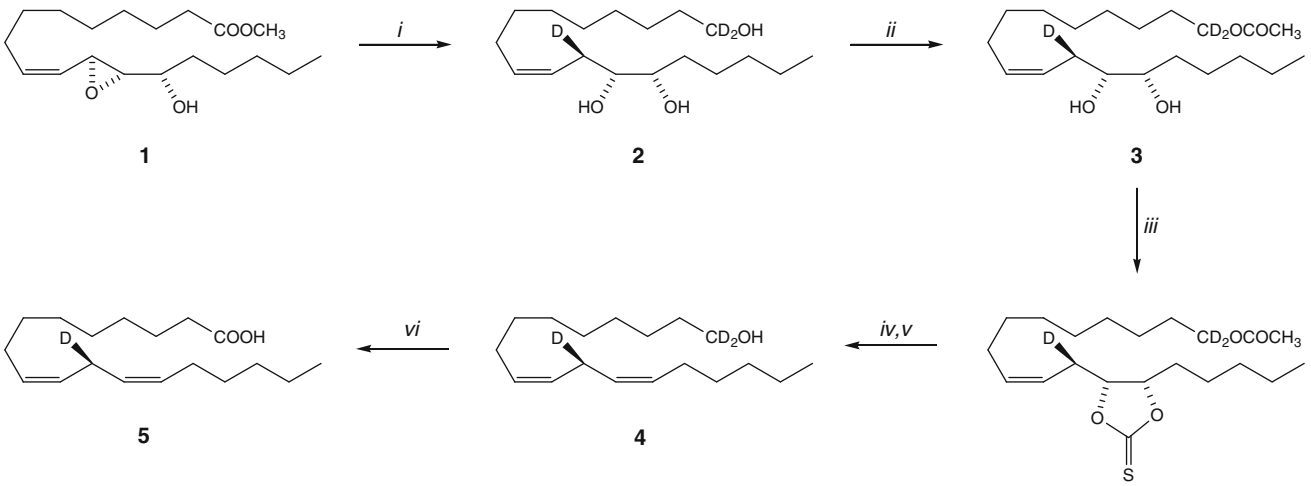
[11(*S*)-^{2}H]Linoleic acid (**5**): Method B

In Method B, a series of stereospecific transformations were used to convert the readily obtainable epoxy alcohol methyl 11(*S*),12(*S*)-epoxy-13(*S*)-hydroxy-9(*Z*)-octadecenoate (**1**) into the desired labeled linoleate (Scheme 1). Thus, **1** (250 mg [12]) in diethyl ether (10 mL) was stirred with lithium aluminium deuteride (155 mg, Sigma-Aldrich) at 23 °C for 5 h affording triol **2** as a white solid [214 mg (92%)]; mass spectrum (Me_3Si derivative) showing *m*/*z* 429 (2%; M^+–Me_3SiOH), 346 (26; M^+–Me_3SiO^+=CH–C_5H_{11}), 275 (100; Me_3SiO^+=CH–CH($OSiMe_3$)–C_5H_{11}), and 173 (Me_3SiO^+=CH–C_5H_{11}). Monoacetylation of **2** using acetyl chloride and 2,4,6-trimethylpyridine [13] afforded diol acetate **3** in quantitative yield; mass spectrum (Me_3Si derivative) showing *m*/*z* 474 (1%; M^+–CH_3), 316 (55; M^+–Me_3SiO^+=CH–C_5H_{11}), 275 (92; Me_3SiO^+=CH–CH($OSiMe_3$)–C_5H_{11}), and 173 (100; Me_3SiO^+=CH–C_5H_{11}). The *erythro*-12,13-diol function of **3** was deoxygenated via the cyclic thionocarbonate using previously described methodology [14]. Following saponification and purification on a silica gel column [1,1,11(*S*)-$^2H_3$]9(*Z*),12(*Z*)-octadecadienol **4** was obtained as a colorless oil [65 mg (32% from epoxy alcohol **1**)]; mass spectrum showing *m*/*z* 269 (7%; M^+), 251 (1; M^+–H_2O), 96 (59), 82 (87), and 68 (100). Oxidation using pyridinium dichromate (273 mg) in dimethylformamide (2.5 mL) containing water (26 mg) and butylated hydroxytoluene antioxidant (2 mg) at 40 °C for 15 h afforded the title compound **5** [23 mg (11% from **1**)]. The

pure compound was obtained following silica gel column chromatography and RP-HPLC. The mass spectrum (methyl ester) showed *m/z* 281 (20%; M^+), 250 (11; M^+–OCH_3), 96 (63), 82 (88), and 68 (100) and an isotopic composition of 2.0% unlabeled and 98.0% monodeuterated molecules. The purity of the sample was in excess of 99% as judged by GLC analysis (Fig. 1). Importantly, this analysis was carried out with the 9(*E*),12(*Z*)- and 9(*Z*),12(*E*)-octadecadienoate isomers as standards and proved that the two double bonds of **5** were both "*Z*".

High-Performance Liquid Chromatography (HPLC)

Purification of **5** and its methyl ester was performed by reversed-phase (RP) HPLC using a column of Nucleosil C_{18} 100-7 (250 × 10 mm; Macherey–Nagel, Düren, Germany) and solvent systems of acetonitrile–water (85:15, by vol) (**5** methyl ester) or acetonitrile–water–acetic acid (75:25:0.005, by vol) (**5**) at a flow rate of 4 mL/min. Regioisomeric hydroxyoctadecadienoic acids were separated by straight-phase (SP) HPLC using a column of Nucleosil 50-7 (250 × 10 mm, Macherey–Nagel, Düren,



Scheme 1 Synthesis of [11(*S*)-^{2}H]linoleic acid **5** from epoxy alcohol **1**. (*i*) $LiALD_4$, diethyl ether; (*ii*) CH_3COCl, 2,4,6-trimethylpyridine; (*iii*) thiophosgene, 4-dimethylaminopyridine; (*iv*) 1,3-dimethyl-2-phenyl-1,3,2-diazaphospholidine; (*v*) NaOH, aq. ethanol; (*vi*) pyridinium dichromate, dimethylformamide, water

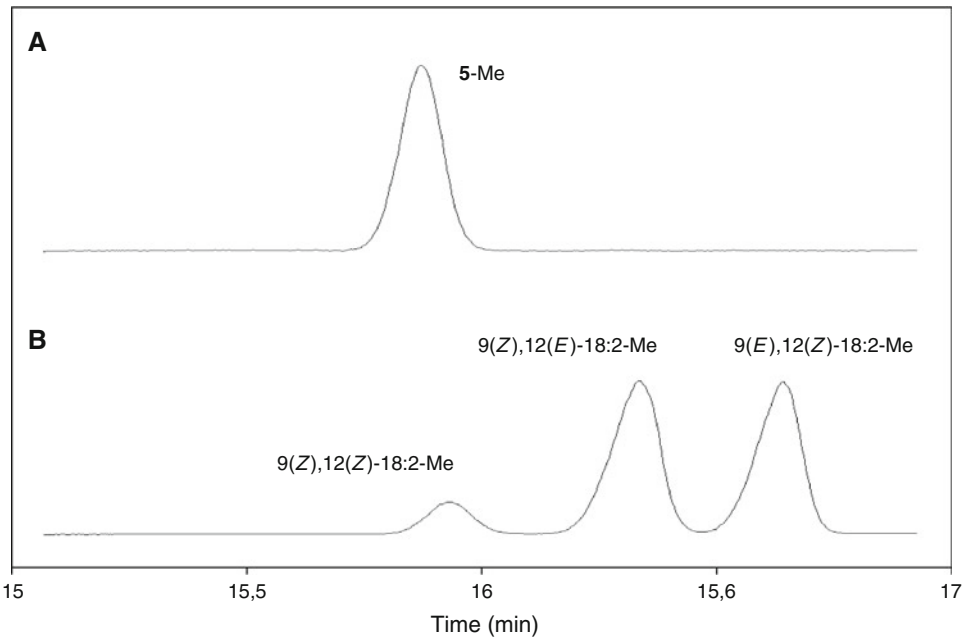


Fig. 1 GLC analysis of the methyl ester of **5** (**a**). The methyl esters of 9(*Z*),12(*Z*)-, 9(*Z*),12(*E*)- and 9(*E*),12(*Z*)-octadecadienoates were used as references (**b**). A methyl silicone capillary column (25 m, 0.33 μm film thickness) was used with helium as the carrier gas. Column temperature, 180 °C

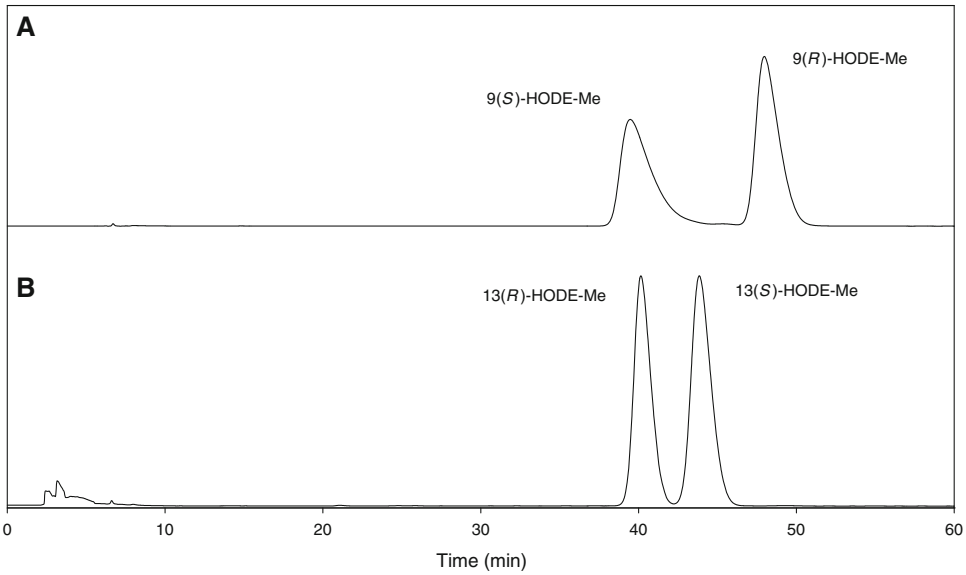


Fig. 2 Separation by CP-HPLC of the methyl esters of 9-HODE (**a**) and 13-HODE (**b**) obtained by singlet oxygenation of **5**. A Chiralcel OB-H column (250 × 4.6 mm) eluted with 2-propanol/hexane (1.5:98.5, v/v) at 0.5 mL/min was used. Detection by UV (234 nm)

Germany) and a solvent system of 2-propanol-hexane-acetic acid (2:98:0.01, by vol) at a flow rate of 4 mL/min. The column effluent was passed through serially connected detectors for measurement of UV absorbance (210 or 234 nm) and refractive index. Methodology for separation of enantiomers of the methyl esters of 9- and 13-HODE by chiral phase (CP) HPLC has been described in detail [15, 16].

Oxygenation Procedure and Isolation of Products

[11(*S*)-^{2}H]Linoleic acid (4.7 mg) was dissolved in methanol (6 mL) containing methylene blue (3 mg). The solution was stirred at 5–8 °C under continuous bubbling of O_2 and irradiated by a 250 W halogen lamp giving a light intensity of approximately 30,000 lx. After 100 min, the solution was cooled on ice and treated with $NaBH_4$ (15 mg). Water was added, and the material extracted with diethyl ether was subjected to SP-HPLC (refractive index detection). Four peaks were observed due to 13-HODE (29%; 42.7 mL effluent), 12-HODE (21%; 51.3 mL), 10-HODE (21%; 57.0 mL), and 9-HODE (29%; 73.1 mL). The 9- and 13-hydroxyoctadecadienoates were methyl-esterified and individually resolved into enantiomers using CP-HPLC (Fig. 2). Derivatization of the methyl esters into Me_3Si derivatives [16] was performed prior to analysis by GC–MS.

GC–MS

A Hewlett–Packard model 5970B mass selective detector connected to a Hewlett–Packard model 5890 gas chromatograph was used. For determination of the isotope content of methyl ester–Me_3Si derivatives, the instrument was operated in the selected ion monitoring mode using the following ions: *m/z* 382.3/383.3 (M^+), 311.2/312.2 (Me_3SiO^+=CH–CH=CH–CH=CH–$(CH_2)_7$–$COOCH_3$), and 225.1/226.1 (Me_3SiO^+=CH–CH=CH-CH=CH–$(CH_2)_4$–CH_3).

Results and Discussion

Shown in Table 1 are results of GC–MS analyses of 9- and 13-hydroxyoctadecadienoates isolated following exposure of [11(*S*)-^{2}H]linoleic acid to singlet oxygen. It is clear that most of the deuterium label was retained in the 9(*R*)-and 13(*S*)-hydroxy derivatives, and that the 9(*S*)- and 13(*R*)-hydroxy compounds had lost most of the label. Thus, the first-mentioned pair was formed by stereospecific elimination of the 11(*R*)-hydrogen, whereas the 11(*S*)-deuterium was lost in the formation of the latter pair. This means that the stereochemical relationship between oxygen addition and hydrogen abstraction is suprafacial in the formation of all four stereoisomers, a finding which confirms and extends previous studies carried out using simple synthetic

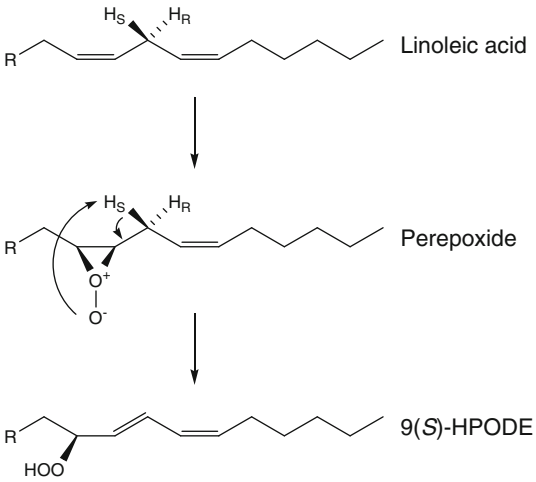
Table 1 Deuterium-content of 9- and 13-hydroxyoctadecadienoates formed by $^1O_2$ oxygenation of [11(*S*)-^{2}H]linoleic acid (**5**)

Hydroxyoctadecadienoate[a]	Monodeuterated molecules (%)	
	Exp 1[b]	Exp 2[c]
9(*R*)-HODE	97.1	96.9
9(*S*)-HODE	3.5	2.3
13(*R*)-HODE	3.4	2.5
13(*S*)-HODE	97.2	97.1

[a] The deuterium label was fully retained in the 10- and 12-hydroxyoctadecadienoates

[b] **5** prepared from [11(*R,S*)-^{2}H]linoleic acid was used

[c] **5** prepared from epoxy alcohol **1** was used



Scheme 2 Possible mechanism in the formation of 9(*S*)-HPODE from linoleic acid in the presence of singlet oxygen

alkenes [6, 7]. Although the detailed mechanism of singlet oxygenation is still being discussed [17–19], it appears that the most plausible one consists of initial addition of $^1O_2$ to the C=C bond to form a doubly charged perepoxide, in which the geometrical configuration of the two carbon atoms is retained (e.g., a (*Z*) double bond gives rise to a *cis*-configured epoxide group) [7]. As illustrated for the linoleic acid → 9(*S*)-HPODE conversion (Scheme 2), suprafacial abstraction of a proton from the allylic methylene by the negatively charged perepoxide oxygen, opening of the epoxide ring and creation of an (*E*)-configured double bond completes formation of the hydroperoxide.

Fatty acid peroxidation in biological systems can take place by lipoxygenase-catalyzed oxygenation, autoxidation, or oxygenation by singlet oxygen, and such oxygenations frequently take place in parallel. This has been best studied in plant leaves, which possess lipoxygenase activity, can initiate $^3O_2$-dependent autoxidation, and produce $^1O_2$ during photosynthesis [20, 21]. Whereas the first- and second-mentioned oxygenations take place antarafacially and stereorandomly, respectively, the present report shows that oxygenation of polyunsaturated fatty acids by singlet oxygen proceeds suprafacially. The distribution of regio- and stereoisomeric hydroperoxides formed by the three oxygenation pathways differ, and determination of specific hydroxide isomers by GC–MS or HPLC has been used to assess the relative importance of the different modes of fatty acid peroxidation in leaves [20]. Conceivably, a further aid in such studies could be provided by isotope analysis of the various hydroxide isomers if a stereospecifically deuterated fatty acid precursor is included as a probe.

Finally, singlet oxygenation has been reported to be accompanied by a weak intramolecular deuterium isotope effect (k_H/k_D 1.4 or less) [7]. An isotope effect would lead to a preponderance of the 10- and 12-hydroxyoctadecadienoates at the expense of the 9- and 13-hydroxy isomers, however, this was not observed using SP-HPLC (refractive index-based detection) or during analysis of the reaction product by GC–MS. Also, as seen in Fig. 2, the ratio of the enantiomers of 9- and 13-hydroxyoctadecadienoates observed on CP-HPLC was close to 1.

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