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Lipoxygenase Is Irreversibly Inactivated by the Hydroperoxides Formed from the Enynoic Analogues of Linoleic Acid

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ABSTRACT: Triple bond analogues of natural fatty acids irreversibly inactivate lipoxygenase during their enzymatic conversion [Nieuwenhuizen, W. F., *et al.* (1995) *Biochemistry 34*, 10538−10545]. To gain insight into the mechanism of the irreversible inactivation of soybean lipoxygenase-1, we studied the enzymatic conversion of two linoleic acid analogues, 9(*Z*)-octadec-9-en-12-ynoic acid (9-ODEYA) and 12(*Z*)-octadec-12-en-9-ynoic acid (12-ODEYA). During the inactivation process, Fe(III)-lipoxygenase converts 9-ODEYA into three products, *i.e.* 11-oxooctadec-9-en-12-ynoic acid, racemic 9-hydroxy-10-(*E*)-octadec-10-en-12-ynoic acid, and racemic 9-hydroperoxy-10(*E*)-octadec-10-en-12-ynoic acid. Fe(II)-lipoxygenase does not convert the inhibitor and is not inactivated by 9-ODEYA. Fe(III)-lipoxygenase converts 12-ODEYA into 13-hydroperoxy-11(*Z*)-octadec-11-en-9-ynoic acid (34/66 *R/S*), 13-hydroperoxy-11(*E*)-octadec-11-en-9-ynoic acid (36/64 *R/S*), 11-hydroperoxyoctadec-12-en-9-ynoic acid (11-HP-12-ODEYA, enantiomeric composition of 33/67), and 11-oxooctadec-12-en-9-ynoic acid (11-oxo-12-ODEYA) during the inactivation process. Also, Fe(II)-lipoxygenase is inactivated by 12-ODEYA. It converts the inhibitor into the same products as Fe(III)-lipoxygenase does, but two additional products are formed, *viz.* 13-oxo-11(*E*)-octadec-11-en-9-ynoic acid and 13-oxo-11(*Z*)-octadec-11-en-9-ynoic acid. The purified reaction products were tested for their lipoxygenase inhibitory activities. The oxo compounds, formed in the reaction of 9-ODEYA and 12-ODEYA, do not inhibit Fe(II)- or Fe(III)-lipoxygenase. The 9- and 13-hydroperoxide products that are formed from 9-ODEYA and 12-ODEYA, respectively, oxidize Fe(II)-lipoxygenase to its Fe(III) state and are weak lipoxygenase inhibitors. 11-HP-12-ODEYA is, however, the most powerful inhibitor and is able to oxidize Fe(II)-lipoxygenase to Fe(III)-lipoxygenase. 11-HP-12-ODEYA is converted into 11-oxo-12-ODEYA by Fe(III)-lipoxygenase. We propose a mechanism for the latter reaction in which Fe(III)-lipoxygenase abstracts the bisallylic hydrogen H-11 from 11-HP-12-ODEYA, yielding a hydroperoxyl radical which is subsequently cleaved into 11-oxo-ODEYA and a hydroxyl radical which may inactivate the enzyme.

Lipoxygenases (linoleate:oxygen oxidoreductase, EC 1.13.11.12) are non-heme iron-containing dioxygenases that catalyze the conversion of unsaturated fatty acids with one or more 1(*Z*),4(*Z*)-pentadiene systems into *Z,E*-conjugated hydroperoxy fatty acids. Lipoxygenases are widespread among eukaryotes. Mammalian lipoxygenases catalyse the first step in the formation of leukotrienes and lipoxins from arachidonic acid (Yamamoto, 1992). These products play an important role in the immune response and in inflammatory processes. Because of their potential therapeutic applications, there is considerable interest in compounds that inhibit the lipoxygenase reaction (Nuhn *et al.* 1991; Ford-Hutchinson *et al.* 1994). The function of lipoxygenases in plants is only partly understood, but roles have been suggested in plant growth and development, biosynthesis of regulatory molecules, senescence, and response to wounding and pathogens (Siedow, 1991).

Lipoxygenase-1 from soybeans shares many important characteristics with lipoxygenases from other sources. It is available in quantities that allow detailed structural and physical studies [*e.g.* Shibata *et al.* (1988), Van der Heijdt *et al.* (1992), Boyington *et al.* (1993), Minor (1993), Minor *et al.* (1996), Prigge *et al.* (1996), and Gan *et al.* (1996)], and mechanistic studies [*e.g.* De Groot, *et al.* (1973), Verhagen *et al.* (1978), Ludwig *et al.* (1987), and Schilstra *et al.* (1993)], and is therefore widely used as a model for lipoxygenases from other sources.

Triple bond analogues of polyunsaturated fatty acids have been known as inactivators of lipoxygenase since 1965, and their inactivating potential has been investigated by a number of authors (Blain & Shearer, 1965; Downing *et al.* 1970, 1972; Hammarström, 1977; Corey & Munroe, 1982; Corey & Park, 1982; Kühn *et al.* 1984, 1991; Shieh *et al.* 1985). The mechanism by which triple bond analogues of natural substrates inactivate lipoxygenases has not yet been established unambiguously. However, it is generally accepted that the inactivation is caused by an O_2-dependent, suicide-like

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action of the enzyme, since triple bond analogues are converted by lipoxygenase during the inactivating process. We observed that octadec-9,12-diynoic acid (ODYA)[1] was converted into 11-oxooctadec-9,12-diynoic acid (11-oxo-ODYA) by Fe(III)-lipoxygenase and that an unknown short-lived intermediate, formed during the conversion of ODYA, is responsible for the inactivation of lipoxygenase (Nieuwenhuizen *et al.* 1995; Schilstra *et al.* 1996).

To investigate the mechanism of the irreversible inactivation of lipoxygenase by triple bond analogues of linoleic acid, we identified the products that are formed from 9(*Z*)-octadec-9-en-12-ynoic acid (9-ODEYA) and 12(*Z*)-octadec-12-en-9-ynoic acid (12-ODEYA) by soybean lipoxygenase-1.

MATERIALS AND METHODS

Materials. 9(*Z*)-Octadec-9-en-12-ynoic acid (9-ODEYA) and 12(*Z*)-octadec-12-en-9-ynoic acid (12-ODEYA) were synthesized as described (Otsuki *et al.* 1986). On the basis of the ^{13}C-NMR analyses, it was estimated that the 9-ODEYA and 12-ODEYA contained 3−6% of the *E* isomers. Linoleic acid, 9(*Z*),12(*Z*)-octadec-9,12-dienoic acid (99% pure), was from Sigma. 13(*S*)-Hydroperoxy-9(*Z*),11(*E*)-octadec-9,12-dienoic acid (13-HPOD) and 9(*S*)-hydroperoxy-10(*E*),12(*Z*)-octadec-10,12-dienoic acid (9-HPOD) were prepared enzymatically as described (Van Aarle *et al.* 1991; Schilstra *et al.* 1992). Solutions of 13-HPOD (200 mM), 9-HPOD (250 mM), linoleic acid (300 mM), 9-ODEYA (300 mM), and 12-ODEYA (300 mM) were stored in methanol (Merck, gradient grade) under nitrogen at −25 °C until use.

Acetic acid and $NaBH_4$ were from Merck. Pyridine, hexamethyldisilazane, and trimethylchlorosilane were from Fluka. (*S*)-(+)-2-Phenylpropionic acid and oxalyl chloride were from Acros Chimica. Pyridine and toluene were dried by refluxing with CaH_2 for 16 h and then distilled. All reagents were of the purest grade available.

Lipoxygenase-1 from soybeans (*Glycine max* [L.] Merr. var. Maple Glen, Canada, 1993) was purified as described (Finazzi Agrò *et al.* 1973). Its concentration was estimated from the absorbance at 280 nm ($\epsilon_{280} = 1.6 \times 10^5$ mol^{-1} L cm^{-1}). The enzyme was stored at 4 °C at a concentration of 8 mg/mL in 0.05 M sodium acetate buffer (pH 5.5) containing 20% (w/v) ammonium sulfate. The purified enzyme consists predominantly of Fe(II)-lipoxygenase with traces of Fe(III)-lipoxygenase (Slappendel *et al.* 1981) and is called Fe(II)-lipoxygenase throughout this paper. Fe(III)-lipoxygenase was prepared as described (Nieuwenhuizen *et al.* 1995).

Methods. UV-absorption spectra were recorded with a Hewlett-Packard HP 8452A diode array spectrophotometer.

^{1}H-NMR spectra were recorded with a Bruker AC 300 (300 MHz) spectrometer in $CDCl_3$ at 27 °C. ^{1}H chemical shifts are given in parts per million (δ) relative to internal TMS.

Lipoxygenase activity measurements were carried out using a Hewlett-Packard 8452A diode array spectrophotometer, by measuring the increase of the absorbance at 234 nm. The lag phase and the burst of the lipoxygenase reaction were determined as described (Schilstra *et al.* 1996) using a Hi-Tech SF-51 stopped-flow apparatus equipped with an LS-10 light source and PM-60 photomultiplier.

In order to obtain sufficient amounts of the reaction products, typically, 1 μM lipoxygenase was incubated for 60 min at 25 °C in air-saturated 0.1 M sodium borate buffer (pH 10.0) with 40 μM 9-ODEYA or 12-ODEYA in a total volume of 100 mL. The reaction was terminated by acidifying the mixture to pH 4 with 4 M HCl. Then the reaction products were extracted as described (Nieuwenhuizen *et al.* 1995) and concentrated *in vacuo* using a rotary evaporator. The water bath temperature was kept below 30 °C. Compounds were dissolved in 250 μL of methanol and immediately stored under nitrogen at −25 °C until use.

FAB-MS analyses were carried out on a JEOL JMS SX/SX 102A four-sector mass spectrometer, equipped with a JEOL MS-FAB 10 D FAB gun, as described (Nieuwenhuizen *et al.* 1995).

HPLC analyses were performed using a Hewlett-Packard HP 1090 liquid chromatograph, equipped with an HP 1040A diode array detector and an HP 7994A analytical workstation. HPLC analyses and purification of fatty acids were carried out on Cosmosil 5C18-AR (5 μm, 250 mm × 4.6 mm inside diameter, Nacalai Tesque) or Cosmosil 5C18-AR (5 μm, 250 mm × 10 mm inside diameter, Nacalai Tesque) columns, using methanol/water/acetic acid mixtures (75/25/0.1 or 80/20/0.1, v/v/v) as the eluents at flow rates of 1 or 3 mL/min, respectively. During purification procedures, the collected fractions were kept on melting ice. The fractions were diluted with 5 volumes of water, extracted, concentrated, and stored as described above.

For GC−MS analysis after purification, the products were reduced with $NaBH_4$, hydrogenated, methylated, and silylated as described (Nieuwenhuizen *et al.* 1995).

GC−MS analysis was performed on a Fisons GC 8000 series gas chromatograph, equipped with a DB-1 column (25 μm film thickness, 30 m × 0.31 mm inside diameter, J&W Scientific) using a temperature gradient from 140 to 280 °C (10 min), rising at 4 °C/min. Mass spectra were recorded every 0.2 s with a Fisons Instruments MD 800 MassLab spectrometer under electron impact with an ionization energy of 70 eV.

(*S*)-(+)-2-Phenylpropionic acid chloride was prepared by heating a solution of (*S*)-(+)-2-phenylpropionic acid (50 mg, 0.33 mmol) and oxalyl chloride (0.14 mL, 1.6 mmol) in dry toluene (1 mL) to 50 °C for 3 h. The reaction mixture was concentrated *in vacuo*. The acid chloride was dissolved in 5 mL of dry toluene and stored at −25 °C until use.

To determine the enantiomeric composition of the 9- and 12-ODEYA lipoxygenase products, the lipids were converted into hydroxystearic acid methyl esters as described above and converted into their (*S*)-(+)-2-phenylpropionate diastereoisomer derivatives (Hammarström & Hamberg, 1973). The hydroxystearic acid methyl esters were dissolved in 300 μL of dry pyridine together with a catalytic amount of 4-(dimethylamino)pyridine. To this solution was added 100 μL of 70 mM (*S*)-(+)-2-phenylpropionic acid chloride in dry toluene. After 2 h, 500 μL of methanol was added and the solvents were evaporated under a stream of nitrogen. The residue was dissolved in 400 μL of dichloromethane and

[1] Abbreviations: FAB-MS, fast atom bombardment-mass spectromety; FAB-MS/MS, FAB ionization, collision-induced decompositions (CIDs) in the third field-free region (third FFR); GC−MS, gas chromatography−mass spectrometry; HPLC, high-performance liquid chromatography; 13-HPOD, 13(*S*)-hydroperoxy-9(*Z*),11(*E*)-octadec-9,11-dienoic acid; 9-HPOD, 9(*S*)-hydroperoxy-10(*E*),12(*Z*)-octadec-10,12-dienoic acid; 11-HP-12-ODEYA, 11-hydroperoxyoctadec-12-en-9-ynoic acid; ^{1}H- and ^{13}C-NMR, proton and carbon nuclear magnetic resonance, respectively; 9-ODEYA, 9(*Z*)-octadec-9-en-12-ynoic acid; 12-ODEYA, 12(*Z*)-octadec-12-en-9-ynoic acid; ODYA, octadec-9,12-diynoic acid; 11-oxo-9-ODEYA, 11-oxooctadec-9-en-12-ynoic acid; 11-oxo-12-ODEYA, 11-oxooctadec-12-en-9-ynoic acid; 11-oxo-ODYA, 11-oxooctadec-9,12-diynoic acid; TMS, tetramethylsilane.

washed twice with 400 μL of 1 mM HCl. The organic phase was concentrated under a stream of nitrogen and dissolved in 50 μL of dichloromethane.

Separation of the (*S*)-(+)-2-phenyl propionate diastereoisomer derivatives of the 9-, 11-, and 13-hydroxystearic acid methyl esters was performed on a Fisons GC 8000 series gas chromatograph, equipped with a BPX70 column (25 μm film thickness, 25 m × 0.22 mm inside diameter, SGE) using a temperature gradient from 220 to 280 °C, rising at 3 °C/min. Mass spectra were recorded as described above. The diastereoisomers were identified by coinjection of the diastereoisomers derived from 9(*S*)- or 13(*S*)-HPOD.

Molar absorption coefficients and lipid concentrations were determined with a Varian 3700 gas chromatograph, equipped with a DB-1 column (25 μm film thickness, 30 m × 0.31 mm inside diameter, J&W Scientific) using a temperature gradient from 140 to 280 °C (10 min), rising at 4 °C/min. The concentrations of the $NaBH_4$-reduced, methylated, and silylated compounds were calculated from the response factor of the internal standard, 13(*S*)-*O*-(trimethylsilyl)-9(*Z*),11(*E*)-octadec-9,11-dienoic acid methyl ester. The concentrations were compared with the measured ultraviolet absorbances at 228 and 230 nm.

In the inhibition studies, incubations of lipoxygenase with 9- or 12-ODEYA or their purified reaction products were carried out at 25 °C in 0.1 M sodium borate buffer at pH 10. The total volume of the incubation mixtures was 0.5 mL, and the lipoxygenase concentration was 1 μM unless stated otherwise. Since the lipids were dissolved in methanol, which could affect the lipoxygenase activity, care was taken that the final methanol concentration did not exceed 1% in the incubation mixtures, including the control mixtures. Measurements of the remaining lipoxygenase activity were performed after 4−6 h of incubation, unless stated otherwise. Typically, 10 μL of lipoxygenase solution was added to 1 mL of borate buffer at pH 10, and the reaction was started by adding 10 μL of a 4 mM linoleic acid solution in methanol. Duplicate measurements were performed 30−60 min after the first measurement to ensure that no significant changes in residual activities occurred after the first measurement. All measurements were performed at least in triplicate.

Ab initio quantum mechanical calculations were carried out using the programme GAMESS-UK.[2] All geometries corresponding to the stationary points on the potential energy surface were located by spin-restricted Hartree−Fock (RHF) calculations with use of the 6-31g* basis set. Average gradients of 0.0005 and maximum gradients of 0.0075 were taken as convergence criteria. 2(*Z*)-Hepten-5-yne and 4-hydroperoxy-2(*Z*)-hepten-5-yne were used as model compounds for 12-ODEYA and 11-hydroperoxyoctadec-12-en-9-ynoic acid, respectively.

Atomic structures for 9-ODEYA and 12-ODEYA were constructed and manually fitted to the putative substrate binding site of the three-dimensional structure of soybean lipoxygenase-1 (2sbl; Boyington *et al.* 1993) using the InsightII software package (Biosym/MSI suite) on a Silicon Graphics Indigo computer.

[2] GAMESS-UK is a package of *ab initio* programs written by M. F. Guest, J. H. van Lenthe, J. Kendrick, K. Schoffel, P. Sherwood, and R. J. Harrison with contributions from R. D. Amos, R. J. Buenker, M. Dupuis, N. C. Handy, I. H. Hilier, P. J. Knowles, V. Bonacic-Koutecky, W. von Niessen, V. R. Saunders, and A. J. Stone. The package is derived from the original GAMESS code by M. Dupuis, D. Spangler, and J. Wendoloski in the NRCC Software Catalog Vol. 1 Program No. QG01 GAMESS (1980).

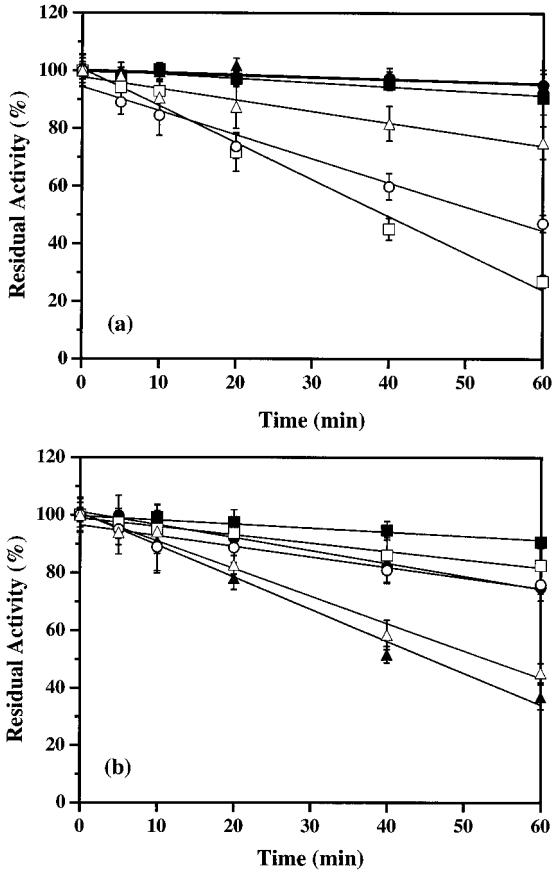


FIGURE 1: Time-dependent inactivation of 1 μM Fe(II)-lipoxygenase (closed symbols) or 1 μM Fe(III)-lipoxygenase (open symbols) in 0.1 M sodium borate buffer at pH 10.0 by (a) 9-ODEYA and (b) 12-ODEYA at concentrations of 10 (squares), 100 (circles), and 1000 μM (triangles) inhibitor. Each point represents the mean value of three to six determinations. The vertical bars depict the SD.

RESULTS

To gain insight into the molecular mechanism of the irreversible lipoxygenase inactivation by triple bond analogues of linoleic acid, we studied the conversion of 9(*Z*)-octadec-9-en-12-ynoic acid (9-ODEYA) and 12(*Z*)-octadec-12-en-9-ynoic acid (12-ODEYA) by soybean lipoxygenase-1.

Inactivation of Fe(II)- and Fe(III)-Lipoxygenase by 9- and 12-ODEYA

In order to investigate the effect of 9-ODEYA and 12-ODEYA on lipoxygenase activities, a 0, 10, 100, or 1000 μM concentration of either inhibitor was added (at $t = 0$) to 1 μM solutions of either Fe(III)-lipoxygenase or Fe(II)-lipoxygenase in 0.1 M sodium borate buffer at pH 10.0. The residual activities were assessed as described in Materials and Methods and are expressed as percentages of the 13-(*S*)-hydroperoxy-9(*Z*),11(*E*)-octadec-9,11-dienoic acid (13-HPOD) formation rate in the control samples. The results are shown in panels a and b of Figure 1.

Over a 1 h incubation period, the residual activity of Fe(II)-lipoxygenase was not affected by 9-ODEYA. The residual activity of Fe(III)-lipoxygenase, however, dropped to 28, 46, and 75% of the control values when incubated with 10, 100, and 1000 μM 9-ODEYA, respectively. The most rapid

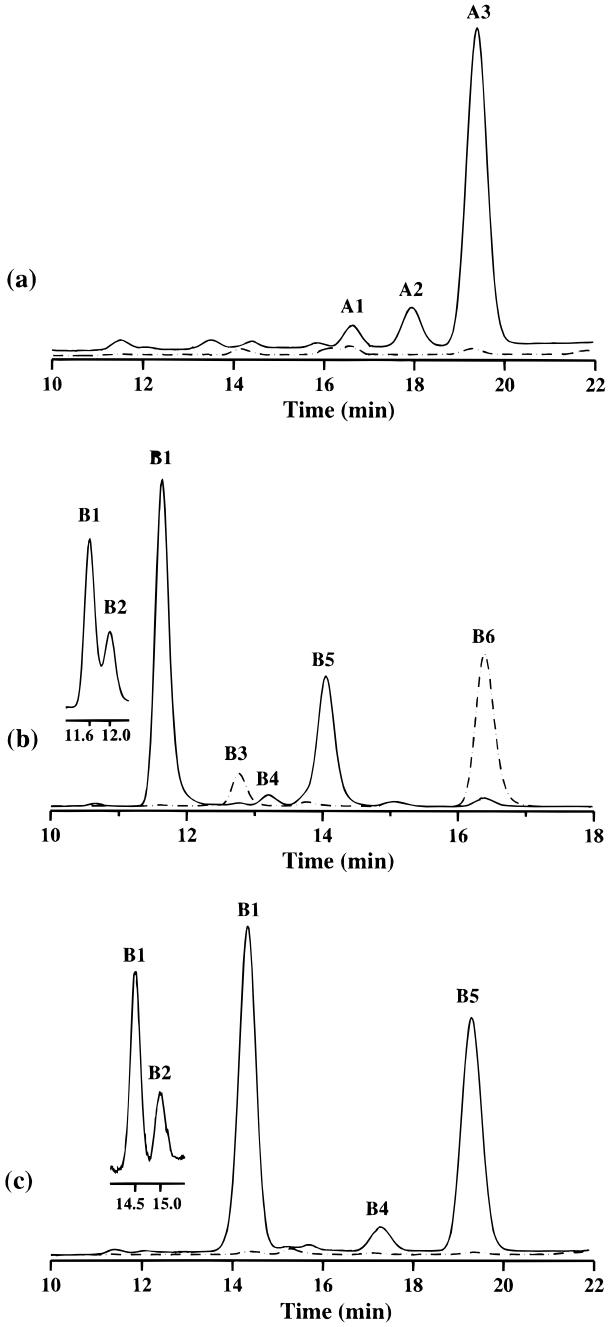


FIGURE 2: (a) Reversed phase HPLC chromatogram (75/25/0.1 methanol/water/acetic acid, 1 mL/min, 5 μm C_{18}, 250 mm × 4.6 mm inside diameter) of the products formed in the reaction of 9-ODEYA with Fe(III)-lipoxygenase. (b) Reversed phase HPLC chromatogram (80/20/0.1 methanol/water/acetic acid, 3 mL/min, 5 μm C_{18}, 250 mm × 10 mm inside diameter) of the products formed in the reaction of 12-ODEYA with Fe(II)-lipoxygenase. (c) Reversed phase HPLC chromatogram (75/25/0.1 methanol/water/acetic acid, 1 mL/min, 5 μm C_{18}, 250 mm × 4.6 mm inside diameter) of the products formed in the reaction of 12-ODEYA with Fe(III)-lipoxygenase. The solid lines represent the traces at 228 nm; the dashed lines represent the traces at 270 nm. The insets in panels b and c depict magnification of the trace recorded at 205 nm around **B1**.

decrease in residual activity was observed at low inhibitor concentrations (see Discussion). From this experiment, it can be concluded that 9-ODEYA inactivates Fe(III)-lipoxygenase, but not Fe(II)-lipoxygenase.

Both Fe(II)- and Fe(III)-lipoxygenase are inhibited by 12-ODEYA. The rates at which the residual activities of the two lipoxygenase preparations decreased did not differ significantly. The residual activity dropped to approximately

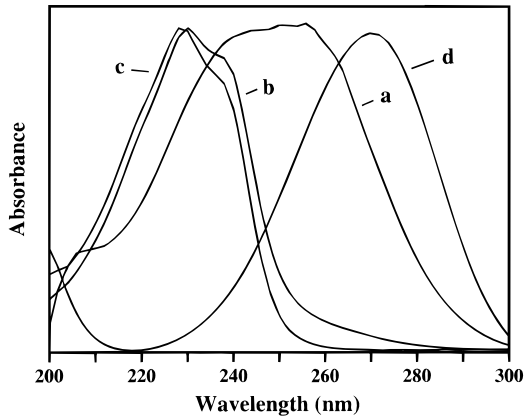


FIGURE 3: UV-absorption spectra, recorded in methanol, of the purified reaction products formed in the reactions of 9-ODEYA and 12-ODEYA with lipoxygenase. The intensities of the spectra were arbitrarily matched to facilitate comparison. (a) Spectrum of **B4**. The spectrum of **A1** is identical with that of **B4**. (b) Spectrum of **A2**. The spectra of **A3** and **B5** are identical with that of **A2**. (c) Spectrum of **B1**. (d) Spectrum of **B3**. The spectrum of **B6** is identical with that of **B3**.

87, 74, and 40% of the control values for both Fe(II)- and Fe(III)-lipoxygenase when incubated with 10, 100, and 1000 μM 12-ODEYA, respectively. In contrast to the results with 9-ODEYA, the residual activity decreased faster with increasing 12-ODEYA concentrations (see Discussion).

HPLC and UV Analysis of Reaction Products

Fe(III)- but not Fe(II)-lipoxygenase converted 9-ODEYA. In Figure 2a, the HPLC chromatogram of the products (**A1**−**A3**) formed in the reaction of 1 μM Fe(III)-lipoxygenase with 40 μM 9-ODEYA for 60 min in borate buffer at pH 10.0 is shown. The average peak area ratio of **A3** and **A2** was 7/1.

12-ODEYA was converted by both Fe(II)- and Fe(III)-lipoxygenase. In panels b and c of Figure 2, the HPLC chromatograms of the products formed in the reactions of 1 μM Fe(II)-lipoxygenase or 1 μM Fe(III)-lipoxygenase with 40 μM 12-ODEYA for 60 min in borate buffer at pH 10.0 are shown, respectively. When 12-ODEYA is converted starting with Fe(II)-lipoxygenase, six products (**B1**−**B6**) are formed. The average peak area ratios of **B1** and **B5** and of **B6** and **B3** were 2.1/1 and 4.5/1, respectively. When the reaction with 12-ODEYA is started with Fe(III)-lipoxygenase, only the compounds **B1**, **B2**, **B4**, and **B5** are formed, and not **B3** and **B6**. In this case, the average peak area ratio of **B1** and **B5** was 1.4/1.

The UV spectra of the lipoxygenase products of 9- and 12-ODEYA are shown in Figure 3. Compounds **A1** and **B4** have identical UV spectra with an absorption maximum at 256 nm (spectrum a); compounds **A2**, **A3**, and **B5** have identical UV spectra with an absorption maximum at 230 nm (spectrum b), and compound **B1** has a UV spectrum with an absorption maximum at 228 nm (spectrum c). Compounds **B3** and **B6** have identical UV spectra with an absorption maximum at 270 nm (spectrum d). Compound **B2** has no chromophore and was detected at 205 nm (see the insets in panels b and c of Figure 2).

Upon reduction with $NaBH_4$, compounds **A1** and **B4** lost their chromophores. After reduction with $NaBH_4$, **A3** coeluted with **A2** on HPLC. The UV spectra of the $NaBH_4$-reduced **B3** and **B6** are identical to those of **B1** and **B5**, respectively, and they coelute on HPLC with $NaBH_4$-reduced

B1 and **B5**, respectively. This indicates that **B3** is the oxo derivative of **B1** and **B6** is the oxo derivative of **B5**. After reduction with $NaBH_4$, compounds **A3**, **B1**, **B2**, and **B5** had shorter retention times on HPLC, but the retention time of **A2** did not change, indicating that **A3**, **B1**, **B2**, and **B5** are hydroperoxides and **A2** is a hydroxide.

Mass Spectrometric Analysis of the Reaction Products

The reaction products were converted into their hydroxystearic acid methyl ester derivatives, silylated (see Materials and Methods), and then analyzed with GC−MS (EI ionization; data not shown). The mass spectra of compounds **A1**, **B2**, and **B4** show major ions at *m/z* 201 and 287 which were assigned to $[CH_3(CH_2)_6 - (OTMS)CH]^+$ and $[CH(OTMS)(CH_2)_9COOCH_3]^+$, respectively, indicating the presence of a hydroxyl group at C-11. The mass spectra of compounds **A2** and **A3** show major ions at *m/z* 229 and 259, which were assigned to $[CH_3(CH_2)_8 - (OTMS)CH]^+$ and $[CH(OTMS)(CH_2)_7COOCH_3]^+$, respectively, indicating the presence of a hydroxyl group at C-9. The mass spectra of compounds **B1**, **B3**, **B5**, and **B6** show ions at *m/z* 173 and 315, assigned to $[CH_3(CH_2)_4 - (OTMS)CH]^+$ and $[CH(OTMS)(CH_2)_{11}COOCH_3]^+$, respectively, indicating the presence of a hydroxyl group at C-13.

^{1}H-NMR Analysis of the Reaction Products

In order to establish the geometry of the double bonds, the $NaBH_4$-reduced reaction products were analyzed with ^{1}H-NMR spectroscopy. The ^{1}H-NMR data are listed in Table 1, and the structures of the products are shown in Figure 4.

A2 and A3. The $^3J_{10,11}$ of 15.82 Hz in the spectrum of **A3** indicates that the conjugated double bond has the *E* geometry. **A2** and **A3** have identical UV spectra, and the silylated hydroxystearic acid methyl ester derivatives of **A2** and **A3** have identical mass spectra. Furthermore, **A3** has a longer retention time on reversed phase HPLC than **A2** but coelutes with **A2** after reduction with $NaBH_4$. **A2** is therefore identified as 9-hydroxy-10(*E*)-octadec-10-en-12-ynoic acid and **A3** as 9-hydroperoxy-10(*E*)-octadec-10-en-12-ynoic acid.

B1 and B5. The $^3J_{11,12}$ of 11.61 Hz in the spectrum of **B1** indicates that the conjugated double bond in **B1** has the *Z* geometry, and the $^3J_{11,12}$ of 15.88 Hz in the spectrum of **B5** indicates that the double bond in **B5** has the *E* geometry. **B1** and **B5** are therefore identified as 13-hydroperoxy-11(*Z*)-octadec-11-en-9-ynoic acid and 13-hydroperoxy-11(*E*)-octadec-11-en-9-ynoic acid, respectively.

B3 and B6. The $^3J_{11,12}$ of 11.61 Hz in the spectrum of **B3** indicates that the conjugated double bond in **B3** has the *Z* geometry, and the $^3J_{11,12}$ of 15.95 Hz in the spectrum of **B6** indicates that the conjugated double bond in **B6** has the *E* geometry. Compound **B3** is therefore identified as 13-oxo-11(*Z*)-octadec-11-en-9-ynoic acid and **B6** as 13-oxo-11(*E*)-octadec-11-en-9-ynoic acid.

B2. From the ^{1}H-NMR spectrum of **B2**, the geometry of the double bond could not be deduced. Because the exact chemical shifts of H-12 and H-13 cannot be derived from the spectrum, attempts to determine the geometry of the double bond *via* a simulation of the spectrum failed.

A1 and B4. There was insufficient material available for accurate ^{1}H-NMR analyses of **A1** and **B4**.

Table 1: 300 MHz ^{1}H-NMR Data of Compounds **A3**, **B1**−**B3**, **B5**, and **B6**[a]

protons	**A3**	**B1**	**B2**	**B3**	**B5**	**B6**
H-2	2.35 (t, $J_{2,3}$ = 7.50)	2.36 (t, $J_{2,3}$ = 7.35)	2.36 (t, $J_{2,3}$ = 7.37)	2.36 (t, $J_{2,3}$ = 7.46)	2.36 (t, $J_{2,3}$ = 7.48)	2.36 (t, $J_{2,3}$ = 7.41)
H-7	1.57 (m)	1.48 (m)	1.45 (m)	1.45 (m)	1.47 (m)	1.50 (m)
H-8	2.33 (m)	2.33 (dt, $J_{7,8}$ = 7.57; $J_{8,11}$ = 2.23)	2.21 (dt, $J_{7,8}$ = 6.87; $J_{8,11}$ = 1.95)	2.44 (dt, $J_{7,8}$ = 7.05; $J_{8,11}$ = 2.39)	2.30 (dt, $J_{7,8}$ = 7.01; $J_{8,11}$ = 1.98)	2.38 (dt, $J_{7,8}$ = 6.79; $J_{8,11}$ = 2.09)
H-9	4.12 (q, $J_{8,9}$ = 7.01)	–	–	–	–	–
H-10	6.04 (dd, $J_{9,10}$ = 6.46)	–	–	–	–	–
H-11	5.68 (dd, $J_{10,11}$ = 15.82)	5.53 (dd, $J_{11,12}$ = 11.61)	5.14 (td, $J_{11,12}$ = 7.16)	6.10 (td, $J_{11,12}$ = 11.61)	5.67 (dd, $J_{11,12}$ = 15.88)	6.64 (td, $J_{11,12}$ = 15.95)
H-12	–	5.81 (dd, $J_{12,13}$ = 6.50)	5.54 (m, H-12 + H-13)	6.20 (d)	6.04 (dd, $J_{12,13}$ = 6.47)	6.44 (d)
H-13	–	4.65 (q, $J_{13,14}$ = 6.90)	–	–	4.12 (q, $J_{13,14}$ = 6.84)	–
H-14	2.29 (dt, $J_{11,14}$ = 2.06; $J_{14,15}$ = 7.09)	2.35 (m)	2.12 (m)	2.76 (t, $J_{14,15}$ = 7.46)	2.35 (m)	2.51 (t, $J_{14,15}$ = 7.42)
H-15	1.53 (m)	1.54 (m)	1.55 (m)	1.64 (m)	1.55 (m)	1.64 (m)
H-3, -4, -5, -6, -16, -17	1.28−1.49 (m)	1.29−1.45 (m)	1.22−1.43 (m)	1.21−1.39 (m)	1.22−1.44 (m)	1.18−1.45 (m)
H-18	0.91 (t, $J_{17,18}$ = 6.88)	0.90 (t, $J_{17,18}$ = 6.83)	0.89 (t, $J_{17,18}$ = 6.83)	0.90 (t, $J_{17,18}$ = 6.87)	0.89 (t, $J_{17,18}$ = 6.87)	0.89 (t, $J_{17,18}$ = 6.79)

[a] Chemical shifts (δ) are given in parts per million at 27 °C and were measured in $CDCl_3$ relative to internal TMS. Coupling constants (^3J, in parentheses) are given in hertz. t = triplet. m = multiplet. d = doublet. dd = double doublet. td = triple doublet. q = quartet. dt = double triplet.

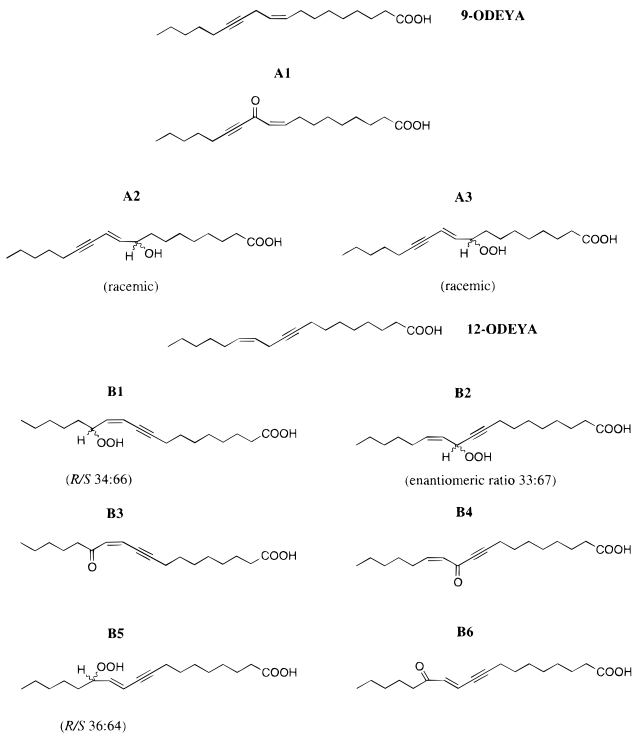


FIGURE 4: Structures of 9-ODEYA, 12-ODEYA, and their lipoxygenase products. The structures of **A1**, **B2**, and **B4** are shown as the *Z* isomers, although the geometries of the double bonds were not established.

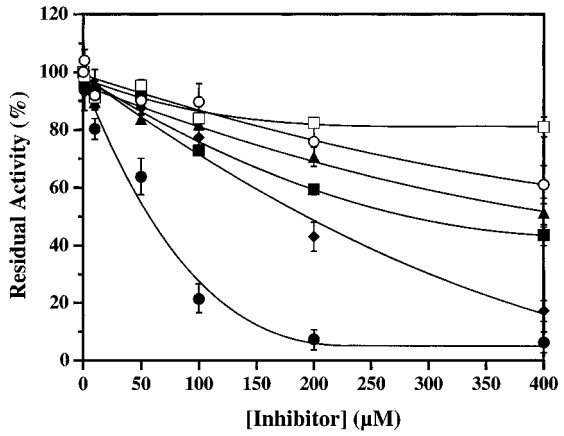


FIGURE 5: Influence of increasing concentrations of compounds **B1** (■), **B2** (●), **B5** (▲), **A3** (♦), 13-HPOD (□), and 9-HPOD (○) on the residual activity of 1 μM lipoxygenase in 0.1 M sodium borate buffer at pH 10.0 and 25 °C. The residual activities were measured 4−6 h after addition of the lipids. Duplicate measurements were performed 30−60 min after the first measurement to ensure that no significant changes in residual activities occurred after the first measurement. Each point represents the mean value of three to six determinations. The vertical bars depict the SD.

Conversion of Fe(II)- into Fe(III)-Lipoxygenase by the Hydroperoxide Products

To 100 nM Fe(II)-lipoxygenase solutions in borate buffer at pH 10.0 was added 500 nM, 13-HPOD, **A3**, **B1**, **B2**, or **B5**. At these hydroperoxide concentrations, no significant lipoxygenase inactivation is observed (see below). The concentration of hydroperoxides was determined using molar absorption coefficients ϵ_{234} of 2.5×10^4 mol^{-1} L cm^{-1} for 13-HPOD and $\epsilon_{228}/\epsilon_{230}$ of $(1.5 \pm 0.2) \times 10^4$ mol^{-1} L cm^{-1} for **A3**, **B1**, and **B5**. The latter value is close to that reported for 11(*E*)-octadec-11,17-dien-9-ynoic acid methyl ester ($\epsilon_{229} = 1.6 \times 10^4$ mol^{-1} L cm^{-1}; Hopkins *et al.* 1968). The concentration of **B2** was determined as described in Materials and Methods.

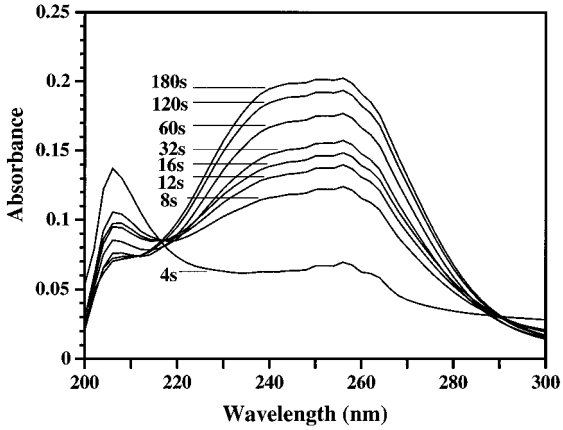


FIGURE 6: Spectra, corrected for the protein absorbance, of the reaction between 500 nM lipoxygenase and 20 μM **B2** in 0.1 M sodium borate buffer at pH 10.0.

After 10 min, the mixtures were stopped-flow mixed in a 1/1 ratio with a solution of 100 μM linoleic acid. The redox state of lipoxygenase at the start of the reaction is apparent from the shape of the curve during the pre-steady-state phase (Schilstra *et al.* 1996). All tested hydroperoxides were able to oxidize Fe(II)-lipoxygenase to the Fe(III)-lipoxygenase. The initial lag phase of 0.67 ± 0.03 s changed into a burst (Schilstra *et al.* 1994) of 1.18 ± 0.04 s in the samples containing 13-HPOD, **B1**, **B2**, and **B5** and into a burst of 0.42 ± 0.10 s in the sample containing **A3**.

Inactivation of Lipoxygenase by the Purified Lipid Hydroperoxides

The lipid hydroperoxides **A3**, **B1**, **B2**, **B5**, 9-HPOD, and 13-HPOD were added to Fe(II)-lipoxygenase (see Figure 5). The time course of the inactivation processes was not determined, and the residual activities were determined after several hours to ensure that the inactivation reactions were complete. The residual activities with increasing hydroperoxide concentrations show that 9- and 13-HPOD have only a minor effect on the enzymatic activity, the 13-hydroperoxides **B1** and **B5** reduced the enzymatic activity slightly, whereas the 9-hydroperoxide **A3** is a more potent inhibitor. The strongest inhibition, however, was observed with the 11-hydroperoxide **B2**, reducing the residual activity to less than 10% at 200 μM concentrations. The oxo compounds **A1**, **B3**, **B4**, and **B6** did not affect the enzymatic activity of either Fe(II)- or Fe(III)-lipoxygenase.

*Conversion of **B2** during Lipoxygenase Inhibition*

After the inactivation of lipoxygenase with **A3**, **B1**, or **B5,** no UV spectral changes or reaction products were detected with UV/VIS or HPLC analyses. However, when 20 μM **B2** was added to a 500 nM Fe(II)-lipoxygenase solution in borate buffer at pH 10.0, the formation of a compound with a UV spectrum identical to that of **B4** was observed (Figure 6). When **B2** was added to a 500 nM solution of heat-denatured lipoxygenase, no spectral changes were observed, indicating that the conversion of **B2** is an enzymatic process. The irreversible inactivation of lipoxygenase by **B2** is not a suicide inactivation because more than one **B2** molecule per lipoxygenase molecule is converted.

The disodium salt of the **B2**-derived product was analyzed without further derivatization with FAB-MS/MS, using

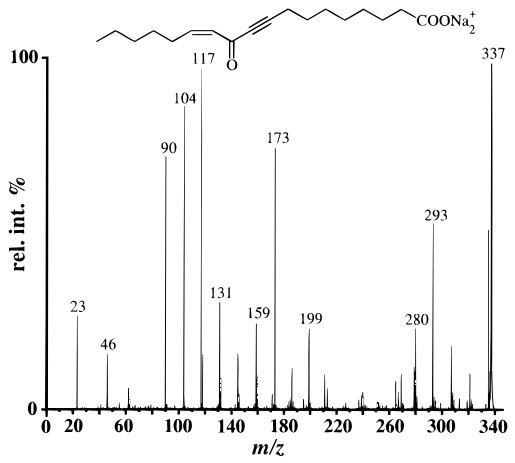


FIGURE 7: FAB-MS/MS spectrum of the disodium salt of the compound formed in the reaction of lipoxygenase with **B2**.

collision induced decomposition (CID) in the third field-free region (third FFR) of the mass spectrometer. In the high-mass region of the FAB-MS/MS spectrum (Figure 7), the sodium-cationized molecular ion $[M + 2Na - H]^+$ at *m/z* 337 is observed, indicating that the compound has a molecular mass of 292. The ions at *m/z* 293 and 280 are assigned to fragments $[M - C_3H_8]^+$ and $[M - C_4H_9]^{\bullet+}$, respectively. The spectrum also shows ions at *m/z* 90, 104, 117, and 173, corresponding to $[CO_2Na_2]^{\bullet+}$, $[C_2H_2O_2Na_2]^{\bullet+}$, $[C_3H_3O_2Na_2]^+$, and $[C_7H_{11}O_2Na_2]^+$, respectively, proving that the seven methylene groups of the molecules adjoining the carboxyl group have not changed. The mass spectrum is identical to that of **B4** (not shown), and it can be concluded that 11-hydroperoxyoctadec-12-en-9-ynoic acid (**B2**) is converted into 11-oxo-octadec-12-en-9-ynoic acid (**B4**) during the irreversible inactivation of Fe(III)-lipoxygenase.

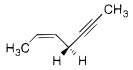
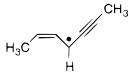
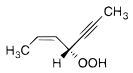
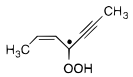
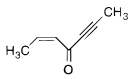
Enantiomeric Composition of the Hydro(pero)xide Products

Compounds **A2**, **A3**, **B1**, **B2**, and **B5** were reduced with $NaBH_4$, methylated, and hydrogenated as described (Nieuwenhuizen *et al.* 1995). The hydroxystearic acid methyl esters were converted into diastereoisomers with (*S*)(+)-2-phenylpropionic acid chloride (Hammarström & Hamberg, 1973). The assignment of the diastereoisomers was made by coinjection of the diastereoisomers derived from 13(*S*)- or 9(*S*)-HPOD. Compounds **A2** and **A3** are formed as racemic mixtures. Interestingly, compounds **B1** and **B5** have *R/S* ratios of 34/66 and 36/64, respectively. The *R/S* ratio of **B2** could not be established because a chiral 11-hydroxystearic acid standard was not available. However, an enantiomeric ratio of 33/67 was observed.

Quantum Mechanical Study of the Reactions of Bisallylic Hydroperoxides

Because **B2** is converted into **B4** during the irreversible inactivation of lipoxygenase, the putative intermediates of the reaction mechanism were evaluated using *ab initio* quantum mechanical calculations in which 2(*Z*)-hepten-5-yne and 4-hydroperoxy-2(*Z*)-hepten-5-yne were used as model compounds for 12-ODEYA and **B2**, respectively. The structures of the model compounds and the calculated total energies are shown in Table 2. To compare the different reaction steps, the energies relative to the energy of 2(*Z*)-hepten-5-yne or 4-hydroperoxy-2(*Z*)-hepten-5-yne were calculated. The energies of the free radicals were corrected for the missing hydrogen, hydroxyl radical, or water. Hence, the number of atoms is the same in every reaction step, and energy differences are only caused by the breaking of chemical bonds.

Table 2: Total Energies Calculated with the Program GAMESS-UK Using Spin-Restricted Hartree−Fock (RHF) Calculations with the 6−31g* Basis Set[a]

	compounds	relative energy (kJ/mol)[b]	total energy (kJ/mol)
a		0	-711040.9
b		+296.5	-709436.3
c		0	-1103823.7
d		+303.9	-1102211.7
e		-77.5	-904532.2
f	•H	-	-1308.1
g	•OH	-	-197905.9
h	H_2O	-	-199566.2

[a] Compound *a* is a model for 12-ODEYA, compound *b* for the 12-ODEYA radical, compound *c* for **B2**, compound *d* for the 11-hydroperoxyl-12-ODEYA radical, and compound *e* for **B4**. [b] The energies relative to 2(*Z*)-hepten-5-yne (*a*) or 4-hydroperoxy-2(*Z*)-hepten-5-yne (*c*) are corrected for the missing hydrogen (*f*) or hydroxyl radical (*g*).

Because **B2** consists of both enantiomers of the bisallylic hydroperoxide (enantiomeric composition of 33/67, see above), and Fe(III)-lipoxygenase is capable of abstracting the bisallylic *pro-S* hydrogen of an unsaturated fatty acid (Egmond *et al.* 1972), the first step in the formation of **B4** (Table 2, *e*) is most likely the abstraction of the H-11 (see also Figure 8). The H-11 abstraction from **B2** (*c*) yields radical *d* and costs 303.9 kJ/mol ($d + f - c$), which is on the same order of magnitude as the abstraction of the first bisallylic hydrogen H-11 from 12-ODEYA (*a*) that costs +296.5 kJ/mol ($b + f - a$) and yields a 12-ODEYA radical (*b*). Radical *d* is then cleaved into a hydroxyl radical (*g*) and **B4** (*e*), yielding −77.5 kJ/mol ($e + g + f - c$). If the abstracted hydrogen and the hydroxyl radical react and form water, the total dehydration reaction yields −274.7 kJ/mol ($e + h - c$; see Discussion).

DISCUSSION

In previous studies, we showed that, during the irreversible inactivation of lipoxygenase by the linoleic acid analogue octadec-9,12-diynoic acid (ODYA), the inhibitor is converted into 11-oxooctadec-9,12-diynoic acid (11-oxo-ODYA) but that 11-oxo-ODYA *per se* does not inactivate the enzyme (Nieuwenhuizen *et al.* 1995). The irreversible inactivation of lipoxygenase is caused by a short-lived intermediate, possibly 11-hydroperoxyoctadec-9,12-diynoic acid (11-HP-ODYA; Schilstra *et al.* 1996). To further investigate the inactivation mechanism, we tested the linoleic acid analogues 9(*Z*)-octadec-9-en-12-ynoic acid (9-ODEYA) and 12(*Z*)-

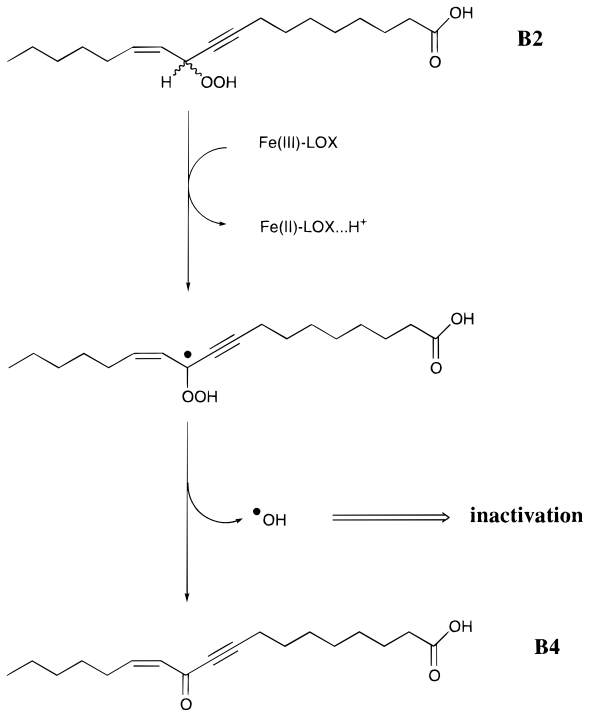


FIGURE 8: Proposed mechanism for the formation of 11-oxooctadec-12-en-9-ynoic acid (**B4**) from 11-hydroperoxyoctadec-12-en-9-ynoic acid (**B2**) during the irreversible inactivation of Fe(III)-lipoxygenase [Fe(III)-LOX].

octadec-12-en-9-ynoic acid (12-ODEYA) as substrates and inhibitors for soybean lipoxygenase-1.

When the conversion of 12-ODEYA is started with Fe(II)-lipoxygenase, the products 13-hydroperoxy-11(*Z*)-octadec-10-en-12-ynoic acid (34/66 *R/S*, **B1**), 11-hydroperoxyoctadec-12-en-9-ynoic acid (enantiomeric composition of 33/67, **B2**), 13-oxo-11(*Z*)-octadec-11-en-12-ynoic acid (**B3**), 11-oxooctadec-12-en-9-ynoic acid (**B4**), 13-hydroperoxy-11(*E*)-octadec-11-en-12-ynoic acid (36/64 *R/S*, **B5**), and 13-oxo-11(*E*)-octadec-11-en-12-ynoic acid (**B6**) are formed (see Figure 4). Fe(III)-lipoxygenase also converts 12-ODEYA into **B1**, **B2**, **B4**, and **B5**, but no **B3** or **B6** is formed. Interestingly, Fe(II)-lipoxygenase does not convert 9-ODEYA, whereas Fe(III)-lipoxygenase converts 9-ODEYA into 11-oxooctadec-9-en-12-ynoic acid (**A1**), racemic 9-hydroxy 10-en-12-ynoic acid (**A2**), and racemic 9-hydroperoxy 10-en-12-ynoic acid (**A3**) (see Figure 4).

Fe(II)-lipoxygenase preparations contain traces of catalytically active Fe(III)-lipoxygenase (Slappendel *et al.* 1981; Nieuwenhuizen *et al.* 1995) that produce lipid hydroperoxides which initially are used to convert Fe(II)-lipoxygenase into the Fe(III) form. Conversion of Fe(II)- to Fe(III)-lipoxygenase should lead to the conversion of **B1** and **B5** into the oxo compounds **B3** and **B6**. The absence of **B3** and **B6** in the reaction of Fe(III)-lipoxygenase with 12-ODEYA suggests that the dioxygenation reaction goes through a complete catalytic cycle and that no 12-ODEYA radicals or peroxyl radicals are released from the enzyme because this would leave the enzyme in the Fe(II) state. Both **B1** and **B5** can oxidize Fe(II)-lipoxygenase to catalytically active Fe(III)-lipoxygenase. However, the ratios of **B1** and **B5** of 2.1/1 and 1.4/1 formed in the reaction of Fe(II)- and Fe(III)-lipoxygenase, respectively, indicate that predominantly **B5** is used for the formation of Fe(III)-lipoxygenase. The hydroperoxides **B1** and **B5** do not efficiently oxidize Fe(II)-lipoxygenase to the Fe(III) state because more **B3** and **B6** are formed than is expected on the basis of a 1/1 reaction of hydroperoxide and lipoxygenase. It is conceivable that 9-ODEYA is not converted by Fe(II)-lipoxygenase if the C-9 hydroperoxide **A3** is less active than the C-13 hydroperoxides **B1** and **B5** in oxidizing Fe(II)- to Fe(III)-lipoxygenase. The latter is indicated by the shorter burst phase that is observed after the addition of **A3** to Fe(II)-lipoxygenase than that with the same **B1** or **B5** concentrations. This implies that in the presence of 9-ODEYA too little **A3** is formed to oxidize Fe(II)-lipoxygenase to the catalytically active Fe(III) state to start the reaction.

Because the oxo compound **B4** is formed in the reaction of Fe(II)- and Fe(III)-lipoxygenase with 12-ODEYA, whereas the oxo compounds **B3** and **B6** are formed only by Fe(II)-lipoxygenase, it is proposed that **B2** is converted into **B4** by Fe(III)-lipoxygenase. Fe(III)-lipoxygenase can abstract bisallylic hydrogen atoms, and the enzymatic abstraction of the H-11 from **B2** and the concomitant formation of **B4** and a hydroxyl radical is conceivable (Figure 8), although a mechanism involving a peroxyl radical cannot be excluded on the basis of the present data.

It is not clear if both enantiomers of **B2** are converted, but it is most likely that the bisallylic *pro-S* H-11 is abstracted (Egmond *et al.* 1972) since the required energy (303.9 kJ/mol, see Results) is comparable with the abstraction of the *pro-S* H-11 (296.5 kJ/mol) from 12-ODEYA. The abstraction of the bisallylic H-11 by Fe(III)-lipoxygenase yields a bisallylic hydroperoxide radical. The subsequent cleavage of the O−O bond is energetically favorable (−77.5 kJ/mol), and a hydroxyl radical and **B4** are formed. The hydroxyl radical is an extremely reactive species that may inactivate the enzyme, possibly by the oxidation of the iron histidine ligands, resulting in the release of iron from the enzyme (Höhne *et al.* 1991). The competition between the inactivation of the enzyme and the formation of water from the hydroxyl radical and the abstracted hydrogen atom may explain why more than one **B2** molecule per lipoxygenase molecule is converted, and no true suicide inhibition is observed. The inactivation mechanism is in line with the observed inactivation of Fe(III)-lipoxygenase by a short-lived intermediate formed during the conversion of ODYA into 11-oxo-ODYA (Nieuwenhuizen *et al.* 1995; Schilstra *et al.* 1996).

A1 is formed during the irreversible inactivation of Fe(III)-lipoxygenase by 9-ODEYA. Although no 11-hydroperoxyoctadec-9-en-12-ynoic acid was isolated, the presence of the corresponding 11-oxo compound suggests that an 11-hydroperoxide has been formed, and an inactivation mechanism identical to that presented above is conceivable. Because **A3** is also a lipoxygenase inhibitor, the effects of both compounds will be additive in the irreversible inactivation of lipoxygenase by 9-ODEYA.

The rate of decrease in the residual activity of lipoxygenase decreases with increasing 9-ODEYA concentrations, whereas the residual activity of lipoxygenase decreases faster with increasing 12-ODEYA concentrations (see Figure 1). This indicates that the conversion of 9-ODEYA is slow and small amounts of the inactivating species compete with the other substrates for interaction with lipoxygenase, leading to slower inactivation at high 9-ODEYA concentrations (Schilstra *et al.* 1996). The faster inactivation with increasing 12-ODEYA concentrations corresponds to the faster production of the inactivating species.

Insight into the interactions of a substrate with lipoxygenase can be obtained through molecular modeling, starting from the soybean lipoxygenase-1 crystal structure. A recent study (Prigge *et al.* 1996) suggests that the positional specificity of soybean lipoxygenase-1 is determined by residues Thr556 and Phe557 in the (putative) substrate binding site (Boyington *et al.* 1993). More bulky amino acids at these positions would not permit the C-18 methyl of the substrate to bind as deeply, placing the ω-6 carbon atom at a position unfavorable for the dioxygenation reaction. The identity of the substrate binding site is, however, uncertain, and an alternative substrate binding site, where the C-18 methyl of the substrate binds in a cavity that terminates near Leu541, has been suggested (Minor *et al.* 1996). Comparison of human 15-lipoxygenase with soybean lipoxygenase-1 in a recent mutagenesis and molecular modeling study on human 15-lipoxygenase suggests that the substrate may bind in an orientation where the carboxylic group is located near Lys260, which is close to Leu541, with the C-18 methyl of the substrate near Phe557 (Gan *et al.* 1996). Although it is impossible at present to define a specific binding mode for the substrate on the basis of the available structural data, the presence of the pentadiene moiety of the fatty acid in the vicinity of the iron opposite His690 is obvious from the current models.

If 12-ODEYA is bound in the putative substrate binding site (Boyington *et al.* 1993) in a conformation wherein C-9−C-13 are essentially coplanar, and its C-9 triple bond and C-12 double bond make an angle of approximately 90° during the dioxygenation at C13, **B1** will be formed. In this orientation, the C-12 double bond is in the vicinity of the iron opposite His690 and the C-18 methyl near Phe557. The $(CH_2)_7COOH$ lies in the cavity lined by Gln495, Val750, Gln697, and Leu754, with the carboxyl group near Ser747/Thr709. Interestingly, the rigid C-12 triple bond in 9-ODEYA makes a bent conformation similar to that of 12-ODEYA impossible, and hence, the formation of a *Z* conjugated product is unlikely. However, if 12-ODEYA or 9-ODEYA is bound in a more extended conformation wherein their triple bonds and double bonds make an angle of approximately 168° and C-9−C-13 are essentially coplanar during the dioxygenation reaction at C-13 or C-9, **B5**, **A2**, or **A3** will be formed. In this orientation, the C-18 methyl groups of 12-ODEYA and 9-ODEYA lie in the cavity terminating near Leu541. The C-12 double bond of 12-ODEYA and the C-12 triple bond of 9-ODEYA are in the vicinity of the iron opposite His690, and their $(CH_2)_7COOH$ groups lie in the same cavity described above with their carboxyl groups near Arg707. Dioxygenation of 12-ODEYA at C-11 will yield **B2** in both conformations.

This study has shown that the irreversible inactivation of soybean lipoxygenase by 9- and 12-ODEYA is caused by the hydroperoxide products derived from these linoleic acid analogues. 11-Hydroperoxyoctadec-12-en-9-ynoic acid (**B2**) is an especially strong lipoxygenase inhibitor, which is converted into 11-oxooctadec-12-en-9-ynoic acid (**B4**) during the inactivation. We propose that during the conversion of **B2** to **B4** a hydroxyl radical is formed which may inactivate the enzyme.

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