Stereochemical Studies of Epoxides Formed by Lipoxygenase-Catalyzed Co-oxidation of Retinol, β-Ionone, and 4-Hydroxy-β-ionone

Doris Waldmann and Peter Schreier*

Lehrstuhl für Lebensmittelchemie, Universität Würzburg, Am Hubland, 97074 Würzburg, Germany

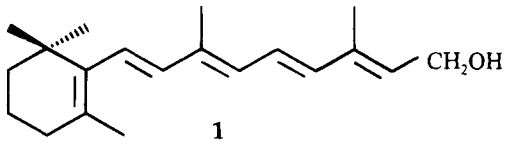
High-pressure liquid chromatographic (HPLC) analysis of the products formed during co-oxidation of retinol (**1**), β-ionone (**2**), and 4-hydroxy-β-ionone (**3**) by purified soybean lipoxygenase isoenzymes (LOX-1/LOX-2) revealed 5,6-epoxides as major transformation products; with **1**, also the 5,8-epoxy derivative was found. The epoxides were characterized by comparison of their chromatographic and spectroscopic data with those of synthesized reference compounds. Using chiral phases, multidimensional gas chromatography and HPLC analyses showed the occurrence of racemic mixtures of the epoxides. The assignment of their stereochemistry and chromatographic order of elution was performed by circular dichroism spectroscopy. The observed lack of selectivity of product formation during co-oxidation supports the hypothesis of a free peroxyl radical mechanism.

Keywords: *Chiral analysis; co-oxidation; 4-hydroxy-β-ionone; β-ionone; lipoxygenase; retinol*

INTRODUCTION

Lipoxygenase (linoleate:oxygen oxidoreductase, EC 1.13.11.12) (LOX) catalyzes the regioselective and enantioselective dioxygenation of (*Z,Z*)-1,4-pentadienoic fatty acids (Gardner, 1991; Crooke and Wong, 1991). A rich source for the enzyme is soybean seed, in which at least four isoenzymes are present (Axelrod et al., 1981). The capacity of LOX to co-oxidize plant pigments, such as carotenoids and chlorophyll in the presence of linoleic acid, has long been known (Weber et al., 1973). The isoenzymes of LOX differ in their ability to co-oxidize polyene structures. LOX-1 is considered to be more effective in co-oxidation under anaerobic conditions, whereas co-oxidation with LOX-2 has been observed under aerobic conditions (Klein et al., 1984).

Despite the abundant information on lipoxygenase-catalyzed co-oxidation (Cohen et al., 1985; Klein et al., 1985; Abbas et al., 1988; Macias et al., 1991), the mechanism of the reaction remains undefined to date. The reasons have to be looked for in the insufficient structural and stereochemical information of co-oxidized substrates. Recently, the stereochemistry of the co-oxidation of all-*trans*-retinoic acid catalyzed by LOX-2 and -3 has been described for the first time (Matsui et al., 1994). In this paper, the structures and the stereochemistry of products formed during LOX-1/LOX-2-catalyzed co-oxidation of retinol (**1**), β-ionone (**2**), and 4-hydroxy-β-ionone (**3**) are reported.



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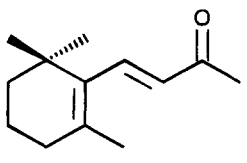

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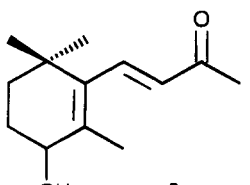

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EXPERIMENTAL PROCEDURES

Chemicals. All commercial chemicals used were of analytical grade quality; solvents were redistilled before use. Retinol (**1**) and β-ionone (**2**) were from Fluka, Neu-Ulm. LOX-1 (116 units/mg at pH 9.0) and LOX-2 (70 units/mg at pH 6.5) were purified from soybean seed according to the method of Weyd (1993). 13-Hydroperoxylinoleic acid was prepared using LOX-1 (Matsui et al., 1989) and purified by flash chromatography on silica gel (ethyl acetate + hexane, 9/1 v/v) to remove linoleic acid.

Co-oxidation Experiments. *Stock Solutions of Linoleic Acid (a) and Cosubstrate (**1**; **2**; **3**) (b).* (a) Ten milligrams of linoleic acid and 10 mL of Tween 80 were dissolved in 1 mL of distilled water; the pH was adjusted to 11.0 with NaOH. (b) A solution of 10 mg of cosubstrate and 100 mL of Tween 80 in 20 mL of dichloromethane was carefully evaporated under vacuum to dryness (rotavapor) and the residue redissolved in 1 mL of Na-EDTA solution (0.25%).

Incubation Conditions. Using a and b, the standard reaction mixture contained 100 mM cosubstrate and 670 mM linoleic acid in 0.1 M buffer solution (LOX-1, sodium borate pH 9.0; LOX-2, potassium phosphate, pH 6.5). The reaction was started by the addition of enzyme (0.1 unit–3.0 kilounits) and proceeded with stirring at 25 °C under air. After 30 min, the mixture was extracted with ethyl acetate, the solvent was dried and carefully concentrated under vacuum (rotavapor) to approximately 0.5 mL, and the concentrated extract was redissolved in 1 mL of methanol (or 1 mL of methyl *tert*-butyl ether) for subsequent HPLC, MDGC, and CD analyses.

To determine the rate of cosubstrate conversion during LOX-1/LOX-2 co-oxidation, both the decrease of cosubstrate and the increase of product formation were simultaneously checked by HPLC.

High-Performance Liquid Chromatography (HPLC). HPLC analyses were performed with (a) achiral phases and (b) chiral phases using a Knauer HPLC pump 64 and a Hewlett-Packard 1040A diode array detector interfaced with a HP 9153C computer system. (a) (i) Spherisorb ODS 2 columns (250 mm × 4.6 mm i.d. and 250 mm × 16 mm i.d.; 5 mm; Knauer, Berlin) were employed at flow rates of 0.9 and 4.5 mL/min, respectively, using methanol + 70 mM aqueous ammonium acetate solution (pH 6.5) (86/14 v/v) for analytical and preparative separation of the co-oxidation products of retinol (**4**; **5**). (ii) Eurospher Si 100 columns (250 mm × 4.6 mm i.d. and 250 mm × 16 mm i.d.; 5 mm; Knauer) were employed at flow rates of 1 and 5 mL/min, respectively, using methyl *tert*-butyl ether + pentane (9/1 v/v) for analytical and preparative separation of the co-oxidation products from β-ionone (**6**) and 4-hydroxy-β-ionone (**7**). (iii) The separation

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of the diastereomeric 5*R*,8*S* (**5a**) and 5*R*,8*R* (**5b**) as well as 5*S*,8*S* (**5c**) and 5*S*,8*R* (**5d**) epoxyretinol isomers was achieved on a Spherisorb S5-CN column (250 mm × 4.6 mm i.d.; 5 mm; Knauer) using A/B (98/2 v/v) (A = hexane + 0.1% *N*-ethyldiisopropylamine; B = dichloromethane + 0.25% methanol) at a flow rate of 1.5 mL/min. The same type of column was employed for the separation of 4*S*- (**7a**) and 4*R*-hydroxy-5,6-epoxy-β-ionone (**7b**) diastereomers using pentane + methyl *tert*-butyl ether (7/3 v/v) at 0.9 mL/min. (b) (i) Separation of 4*S*- (**6a**) and 4*R*-hydroxy-β-ionone (**6b**) enantiomers was achieved on a Chiralcel OB-H column (250 mm × 4 mm i.d.; Daicel, Gross-Gerau) using hexane + 1-propanol (9/1 v/v) at a flow rate of 0.6 mL/min. (ii) The enantiomeric 5,6-epoxy derivatives of retinol (**4a/4b**) and β-ionone (**6a/6b**) as well as the two diastereomeric pairs **5a/5b** and **5c/5d** of 5,8-epoxyretinol were separated on a Ceramospher Chiral RU-1 column (250 mm × 4 mm i.d., 5 mm; Shisheido, Japan) using 0.7 mL/min of methanol.

Multidimensional Gas Chromatography (MDGC). A Siemens Sichromat 2 double-oven gas chromatograph with split injection (250 °C, 1:20) and FIDs on oven 1 and 2 (250 °C each) was used. Preseparation was achieved in oven 1 on a J&W fused silica DB-Wax capillary column (30 m × 0.25 mm i.d.; film thickness 0.25 μm). The temperature was programmed from 60 to 240 °C at 10 °C/min. A "live" switching device (Schomburg et al., 1984) in oven 1 was used to perform effluent cut (12.1−12.4 min) onto an octakis(2,3-di-*O*-methyl-6-*O*-pentyl)-γ-cyclodextrin column (30 m × 0.25 mm i.d.; film thickness 0.3 μm) in oven 2. Temperature program: 15 min isothermal at 60 °C, then increased from 60 to 200 °C at 2 °C/min. Helium was used as carrier gas at 1.2 mL/min in oven 1 and at 1.8 mL/min in oven 2. The flow rates for the detector gases were each 30 mL/min of hydrogen and 300 mL/min of air. Results of analyses were verified by comparison of MDGC data of synthesized reference compounds.

Synthesis of Reference Compounds. Synthesis of 4-hydroxy-β-ionone (**3**) was performed by epoxidation of α-ionone and subsequent cleavage of the epoxide to the hydroxylated β-ionone (Rosenberger, 1982). The 5,6-epoxy derivatives of retinol (**4**), β-ionone (**6**), and 4-hydroxy-β-ionone (**7**) as well as 5,8-epoxyretinol (**5**) were synthesized as described (Jungalwala and Cama, 1965). The identities of **3**−**7** were checked by NMR spectroscopy (Acemoglu and Eschenmoser, 1981; Acemoglu and Eugster, 1984; Englert, 1985).

Nuclear Magnetic Resonance (NMR). ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AC 200 instrument using $CDCl_3$ or CD_3OD as solvent and Me_4Si as reference standard.

Circular Dichroism (CD). CD spectra were recorded in methanol (20 °C) using an ISA Jobin Yvon Dichrograph CD6 instrument.

RESULTS

Purified soybean lipoxygenase type 1 (LOX-1) and type 2 (LOX-2) (Weyd, 1993) were used. In the presence of linoleic acid, both isoenzymes effectively co-oxidized the cosubstrates under study, i.e. retinol (**1**), β-ionone (**2**), and 4-hydroxy-β-ionone (**3**), under aerobic conditions. Both isoenzymes formed the same products; only quantitative differences were found. The molar amounts (related to the substrate linoleic acid) of co-oxidation products were determined to be 25 and 40 μM for LOX-1 and LOX-2, respectively. When the active enzyme was replaced by a heat-denaturated preparation (10 min at 100 °C) or when 13-hydroperoxylinoleic acid was used instead of linoleic acid, no reaction was observed.

The products formed by LOX-1/LOX-2-catalyzed co-oxidation from **1**−**3** were analyzed by HPLC and CD spectroscopy. Structural elucidation was performed by comparison of chromatographic and spectroscopic data with those of synthesized reference substances. Stereochemical product analysis was carried out by CD spectroscopy as well as multidimensional gas chromatography and HPLC using chiral phases. In the following, the results obtained are represented in relation to the cosubstrate used.

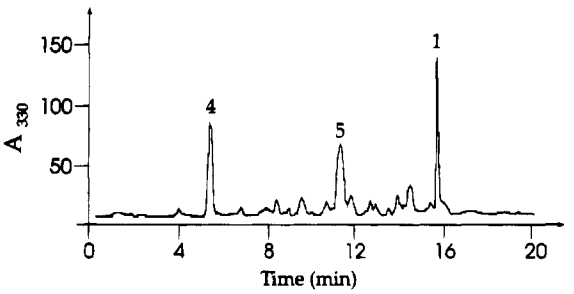

Figure 1. HPLC separation of the products formed by LOX-1/LOX-2 co-oxidation of retinol (**1**) on Spherisorb ODS 2.

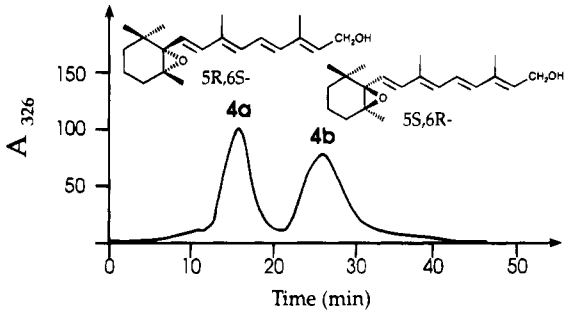

Figure 2. HPLC separation of 5,6-epoxyretinol enantiomers on Ceramospher Chiral RU-1: **4a**, 5*R*,6*S* enantiomer; **4b**, 5*S*,6*R* enantiomer.

Retinol (1). LOX-1/LOX-2-catalyzed co-oxidation of **1** yielded two major products (Figure 1), which were identified as 5,6-epoxyretinol (**4**) and 5,8-epoxyretinol (**5**). Product **4** was successfully separated into its enantiomers (**4a/4b**). Similar to our previous findings obtained with *all-trans*-retinoic acid (Matsui et al., 1994), a racemic mixture was detected. CD spectroscopy allowed the assignment of the configuration of the enantiomers; the 5*R*,6*S* enantiomer (**4a**) eluted before the 5*S*,6*R* enantiomer (**4b**) on Ceramospher Chiral RU-1 (Figure 2).

After the isolation of 5,8-epoxyretinol (**5**) by preparative HPLC, the two diastereomeric pairs **5a/5b** and **5c/5d** were separated on the chiral phase Ceramospher Chiral RU-1, before subsequent separation of the diastereomers on Spherisorb S5-CN was carried out. The HPLC runs are outlined in Figure 3a,b. The assignment of the stereochemistry was achieved by comparison of HPLC retention times and CD data with those of synthesized reference compounds. As shown in Figure 4, purified 5*R*,6*S*- (**4a**) and 5*S*,6*R*-epoxyretinol (**4b**) were converted separately into the 5,8-epoxides **5a**−**5d** (Jungalwala and Cama, 1965). During this acid-catalyzed rearrangement, the configuration at C5 does not change; thus, the resulting diastereomeric 5,8-epoxides only differ in the configuration at C8 (Eschenmoser and Märki-Fischer, 1984). These diastereomers were nicely separated on Spherisorb S5-CN (Figure 3b), and CD spectra were recorded from the four isomers (Figure 3c). Comparison of the CD spectra of the enantiomers with data previously reported for structurally related compounds (Uebelhart and Eugster, 1988) revealed for **5a** the 5*R*,8*S*, for **5b** the 5*R*,8*R*, for **5c** the 5*S*,8*S*, and for **5d** the 5*S*,8*R* configuration. As shown in Figure 3b, again racemic mixtures of **5a/5b** and **5c/5d** were obtained.

***β*-Ionone (2).** In Figure 5 the HPLC separation of the products formed from **2** by LOX-1/LOX-2 co-oxida-

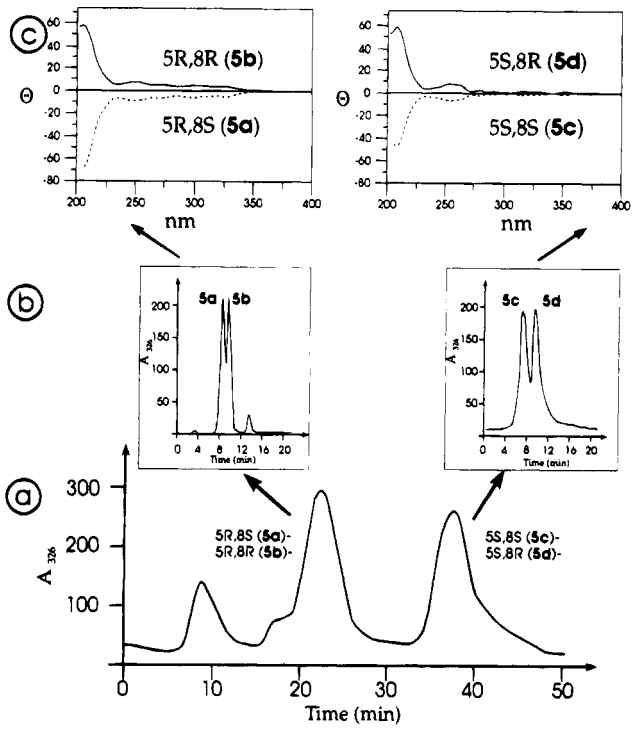

Figure 3. (a) HPLC separation of isolated 5,8-epoxyretinol on Ceramospher Chiral RU-1. (b) HPLC separation of the four 5,8-epoxyretinol isomers on Spherisorb S5-CN. (c) CD spectra of 5*R*,8*S*- (**5a**), 5*R*,8*R*- (**5b**), 5*S*,8*S*- (**5c**), and 5*S*,8*R*-epoxyretinol (**5d**).

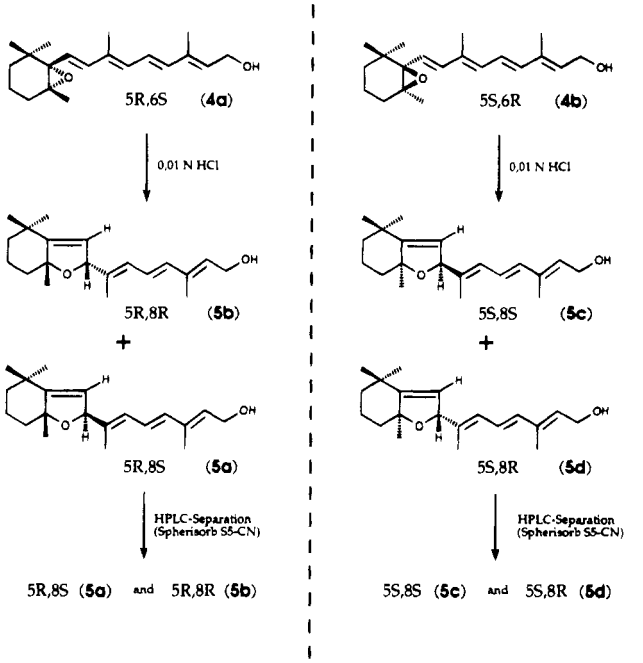

Figure 4. Synthesis of the four 5,8-epoxyretinol isomers (**5a**–**5d**) from pure 5,6-epoxyretinol isomers **4a** and **4b**.

tion is represented. As major transformation product, 5,6-epoxy-β-ionone (**6**) was identified. The epoxide **6**, isolated by preparative HPLC, could be separated into its enantiomers **6a/6b** by chirospecific MDGC; also with **2** as cosubstrate a racemic product was formed. Successful separation of enantiomers was also achieved using HPLC on chiral phase (Figure 6). Comparison of the CD spectra of the separated isomers with previously published data (Eschenmoser et al., 1981) allowed the assignment of the stereochemistry and the determination of the order of elution on the chiral phases. In both

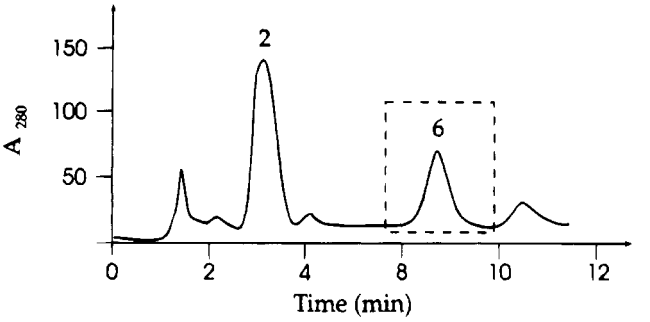

Figure 5. HPLC separation of the products formed by LOX-1/LOX-2 co-oxidation of β-ionone (**2**) on Eurospher Si 100.

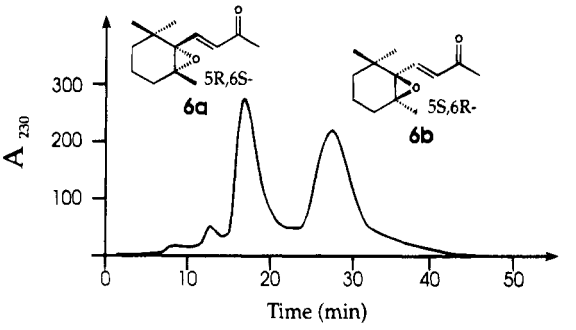

Figure 6. HPLC separation of 5,6-epoxy-β-ionone (**6**) on Ceramospher Chiral RU-1.

MDGC and HPLC analyses the 5*R*,6*S* enantiomer (**6a**) was found to be the first eluting isomer.

4-Hydroxy-β-ionone (3). Compound **3** was used to introduce a chiral center in the cosubstrate, expecting a potential stereospecific preference of the co-oxidation reaction. First of all, **3** was separated into the pure 4*S* (**3a**) and 4*R* enantiomers (**3b**) using HPLC on Chiralcel OB-H. LOX-1/LOX-2-catalyzed co-oxidation of **3a** resulted in the epoxidation of the 5,6-double bond. As shown in Figure 7a, the two diastereomeric 4*S*-hydroxy-5,6-epoxy-β-ionones (**7a/7b**) were separated by HPLC on Spherisorb S5-CN. Their absolute configuration was assigned by CD spectroscopy using previously published data (Eschenmoser et al., 1981; Acemoglu and Eschenmoser, 1981) (cf. Figure 7b). Subsequent co-oxidation of **3b** led to identical results, i.e., also with **7a/7b** a racemic mixture of the epoxidized product was found. The order of elution of the 4*R*-hydroxy-5,6-epoxy-β-ionone diastereomers corresponded to that of the 4*S* isomers.

DISCUSSION

The postulated preference of LOX-2 for the aerobic co-oxidation (Klein et al., 1984) could not be confirmed by our experiments, in which both LOX-1 and LOX-2 were found to be effective catalysts. The co-oxidative effect of LOX-1 under aerobic conditions has been stressed previously (Weyd, 1993).

According to the results of our earlier (Matsui et al., 1994) and present studies, isoprenoids differing in the length and functionalization of the polyene chain as well as the substitution of the cyclohexenyl ring exhibited the same behavior. During their LOX-1/LOX-2-catalyzed co-oxidation, the 5,6-double bond was epoxidized nonselectively; for **1**, a rearrangement of the 5,6-epoxide to the 5,8-epoxide occurred. Epoxidation of polyenes catalyzed by hematin (Labeque and Marnett, 1988) and rat liver microsomes (Samokyszyn and Marnett, 1990) has been reported to proceed by a peroxyl radical

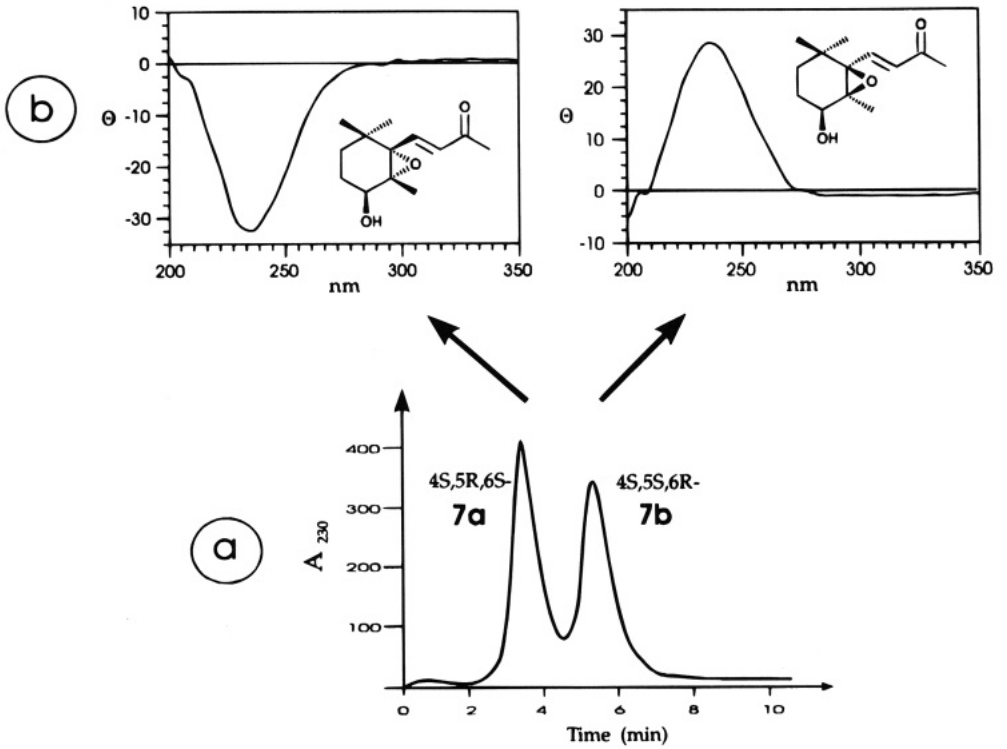

Figure 7. (a) HPLC separation of 4S-hydroxy-5,6-epoxy-β-ionone diastereomers (**7a/7b**) on Spherisorb S5-CN. (b) CD spectra of **7a** and **7b**.

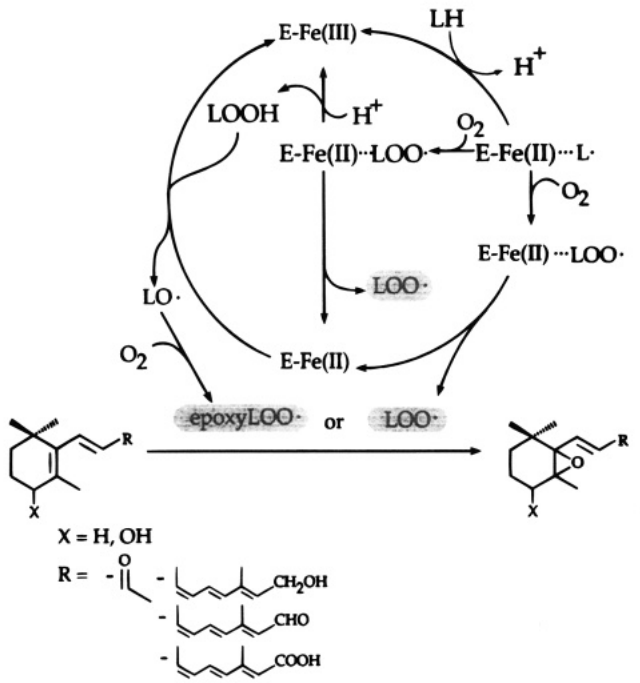

Figure 8. Postulated mechanism of LOX-1/LOX-2-catalyzed aerobic co-oxidation: L, fatty acid; E, lipoxygenase.

mechanism. It is quite conceivable that a peroxyl radical formed from fatty acid is also operative in the LOX-1/LOX-2 catalyzed epoxidation of **1**–**3** and analogous isoprenoids (Matsui et al., 1994). In Figure 8, the structures of potential peroxyl radicals are indicated. These free radicals may be responsible for the nonselective co-oxidation of the cosubstrates. Released peroxyl radicals may attack randomly the favored double bond in the cosubstrate structure, yielding racemic epoxides. Together with our previous findings (Matsui et al., 1994) the present stereochemical studies clearly demonstrate that the enzyme is not directly involved in the formation of co-oxidation products.

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