Isolation and characterization of natural allene oxides: Unstable intermediates in the metabolism of lipid hydroperoxides

(lipoxygenase/prostaglandin/epoxide)

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ABSTRACT Allene oxides are unstable epoxides that have been implicated as intermediates in the biotransformation of hydroperoxyicosatetraenoic acids and related hydroperoxides to ketols and cyclopentenones. Direct proof of the structure of the putative allene oxide intermediates has been hampered by their extreme instability under the conditions of their biosynthesis ($t\frac{1}{2} \approx$ 15–30 sec at 0°C and pH 7.4). We now report the isolation and structural elucidation of allene oxides prepared from the (13S)-hydroperoxides of linoleic and linolenic acids. The compounds were biosynthesized by using a very active enzyme preparation from flaxseed. After a 5-sec incubation at 0°C, the allene oxide metabolites were extracted and purified as the methyl ester derivatives at −15°C. The structures were established by UV, CD, NMR, and oxygen-18 labeling experiments. 12,13(S)-Oxido-9Z,11-octadecadienoic acid is derived from linoleic acid, and 12,13(S)-oxido-9Z,11,15Z-octadecatrienoic acid is from linolenic acid. Analysis of the breakdown products formed on exposure to water led to identification of hydrolysis and cyclization products previously characterized as enzymic derivatives of the (13S)-hydroperoxides in flaxseed. Our results give direct proof of the structure of the allene oxide intermediates and should facilitate further investigation of the metabolism of this class of epoxide to prostaglandins, clavulones, and other stable end products.

Many key intermediates of lipid hydroperoxide metabolism are unstable chemical structures; as a consequence, their biological activity is short-lived, and their chemical reactivity offers the potential for conversion to a spectrum of active metabolites. The prostaglandin endoperoxides and leukotriene A₄ are two well-known examples. Recently, evidence has emerged of a new derivative of lipid hydroperoxide metabolism, an allene oxide (1–5). This extremely unstable intermediate is considered to be the direct enzymic product of lipid hydroperoxide metabolism in plants (e.g, corn germ and flaxseed) (1, 2) and corals (*Clavularia viridis* and *Plexaura homomalla*) (3–5). Presently, the allene oxides are viewed as intermediates in the formation of more stable end products, notably, α-ketols and cyclopentenones in the plants, and prostaglandins and related icosanoids in the corals (Scheme I).

Formation of allene oxides has precedence in the chemical literature (reviewed in ref. 6). It has proved possible to prepare stable allene oxides and to study their chemistry (7, 8). Heretofore, the more unstable allene oxides have not been isolated, although they are implicated as reactive intermediates in various hydrolysis and cyclization reactions, notably to α-substituted ketones and to cyclopentenones (9–11). We now show that, with appropriate methodology, even extremely unstable derivatives can be prepared. The allene oxides we have isolated have a chemical half-life on the order
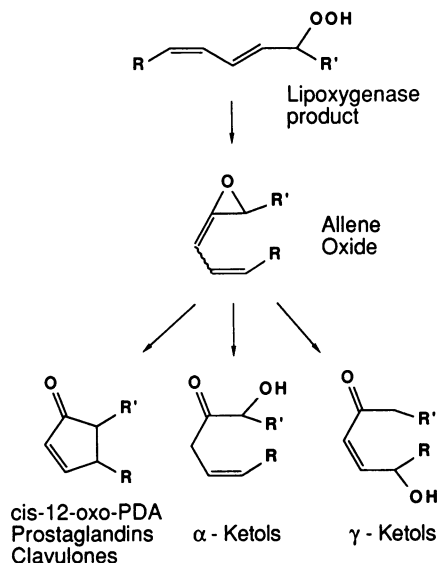
SCHEME: Proposed Lipoxygenase Pathways

of 20 sec at 0°C and pH 7.4. This is substantially less stable than leukotriene A₄ or thromboxane A₂ (12, 13), two of the most unstable of the evanescent family of products derived by oxygenation of polyunsaturated lipids.

Our studies reported here are on the formation of allene oxides in flaxseed. It has been known for 10 years that flaxseed can form a cyclopentenone from the (13S)-hydroperoxide of α-linolenic acid (14). The biosynthesis of this product, 12-oxo-[9,13-*cis*]-10,15Z-phytodienoic acid (*cis*-12-oxo-PDA), is known to be closely associated with formation of an α-ketol, 12-oxo-13(R)-hydroxy-9Z,15Z-octadecadienoic acid (15). Directly at issue are the questions whether these compounds are formed via biosynthesis of the allene oxide and which steps are enzymatic. More generally, it is evident that characterization of the natural allene oxides will facilitate investigation of their emerging role as intermediates in the biosynthesis of prostaglandins and of other natural products.

EXPERIMENTAL PROCEDURES

Materials. The sources of chemicals and the preparation of hydroperoxide substrates and of flaxseed acetone powder are described in ref. 2. Oxygen-18 gas (97% ¹⁸O₂) was obtained from Stohler Stable Isotopes (Waltham, MA).

Abbreviations: *cis*-12-oxo-PDA, 12-oxo-[9,13-*cis*]-10,15Z-phytodienoic acid (see Scheme I); RP-HPLC, reversed-phase HPLC; SP-HPLC, straight-phase HPLC; COSY, correlation spectroscopy.
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Incubation and Extraction. Acetone powder of flaxseed was made up as a fresh stock solution [150 mg/ml of 0.1 M potassium phosphate buffer (pH 7.4) containing 3 mM Zwittergent 3-14 (Calbiochem)]. The solution was stirred for 30 min at 0°C, diluted with 2 volumes of phosphate buffer, and centrifuged for 5 min in a Beckman microfuge; the resulting supernatant was used as a source of enzyme. Preparative scale incubations were started by addition of 1 mg of the (13*S*)-hydroperoxide of linoleic or α-linolenic acid (added in 75 μl of ethanol) to 2 ml of ice-cold enzyme preparation, with constant mixing. After about 5 sec, the solution was extracted by addition of 8 ml of ice-cold hexane (or pentane), with mixing for a few more seconds. The organic phase was quickly recovered and reduced in volume to about 2 ml by using a strong stream of nitrogen (with no heating). The sample was then methylated by addition of 20 μl of ethanol and 10 drops of ethereal diazomethane. After a 10-sec reaction on ice, the sample was taken to dryness under nitrogen. The methylated products were immediately redissolved in cold (−15°C) hexane and stored at −70°C. Analytical scale reactions, with monitoring of the hexane extract in the UV, were conducted by using 0.25 ml of enzyme solution, 60 μg of substrate, and 1 ml of hexane for extraction.

HPLC Methods. The methyl ester of the allene oxide intermediate was purified by straight-phase HPLC (SP-HPLC) with an Altex 5-μm Ultrasphere SI column (45 × 4.6 mm), a solvent system of hexane/diethyl ether, 100:1 (vol/vol), and a flow rate of 3 ml/min. The chromatography was performed at *ca.* −15°C. The solvent front was eluted about 15 sec after injection. Retention time of the allene oxide intermediate was about 0.95 min. The eluant was monitored by UV at 235 nm (analytical scale) or 255 nm (preparative runs).

The methyl esters of the *cis*-12-oxo-PDA and the α-ketol were strongly retained under the above conditions. With a higher concentration of diethyl ether (2.5%), they were eluted together at a retention time of ≈12 min (3 ml/min). The compounds were separated and purified by using SP-HPLC and reversed-phase HPLC (RP-HPLC) as described (2).

NMR. Spectra were recorded with a Bruker AM-400 or an IBM/Bruker NR-300 at 400 and 300 MHz, respectively, with perdeuterated hexane ([^{2}H$_{14}$]hexane) as solvent. All spectra were obtained at 233 K. Chemical shifts are reported in relation to tetramethylsilane (δ = 0.0). Parameters for data acquisition were essentially as described (2, 5).

Other Spectroscopic Methods. GC/MS was carried out with a Nermag (Houston) R10-10C mass spectrometer coupled to a Varian Vista 6000 gas chromatograph; mass spectra were recorded under electron impact (70 eV). A Beckman DU-7 scanning spectrophotometer was used for recording UV spectra. A Jasco (Easton, MD) J-500A recording spectropolarimeter was used to obtain CD measurements; samples were scanned from 300 to 200 nm in a 0.8-ml sample holder with 1-cm path length in hexane at 5°C.

Measurement of the Absolute Configuration of the α-Ketol. All derivatizations and HPLC procedures were essentially identical to an earlier method (5). The α-ketol is converted to hydrogenated 12,13-diols, and the absolute configuration of the *threo* diol is determined by HPLC separation of the benzyl esters of the (13*R*)- and (13*S*)-12,13-diol enantiomers, chromatographed as the (−)-menthoxycarbonyl diastereomers. A chiral (13*S*)-12,13-diol standard was prepared from (13*S*)-hydroxy C18.3ω6 C$_{18}$ fatty acid by controlled autoxidation (5); and a racemic 12,13-diol standard, by epoxidation of linoleic acid (5).

RESULTS

Isolation of C18.3 Allene Oxide. We developed the method using the (13*S*)-hydroperoxide of α-linolenic acid as substrate. An acetone powder extract of flaxseed metabolized up

to 500 μg of this compound per ml within a few seconds at 0°C. Rapid extraction with 4 volumes of ice-cold hexane and monitoring of the hexane phase by UV spectroscopy revealed the appearance of a new chromophore with a λ_{max} of 236 nm. This metabolite was unstable, and decomposed to non-UV-absorbing material with a half-life of a few minutes at room temperature. The unstable intermediate was not detected in incubations allowed to proceed for 60 sec or longer prior to extraction. It seemed likely that this unstable intermediate was the C18.3 analog of the linoleate allene oxide previously detected by Hamberg in corn germ (1).

Our finding that the intermediate could be recovered by extraction with hexane was the first step in the development of an isolation and purification scheme. We used the hexane extract/UV assay to investigate the yield of product with changes in the conditions of incubation and extraction. We eventually settled on using a short incubation time, no pH changes, and rapid conversion to the methyl ester derivative. The methyl ester has a half-life of several hours in ice-cold hexane and is stable at −70°C.

The first attempts at chromatographic purification of the intermediate indicated that interaction with the polar stationary phase almost completely destroyed the compound. However, the use of a short column operated at −15°C allowed isolation of the intermediate in good yield. In this way we were able to prove that the radiolabeled hydroperoxide substrate is converted to the radiolabeled intermediate, Fig. 1. The yield from substrate to pure product was 20–25%.
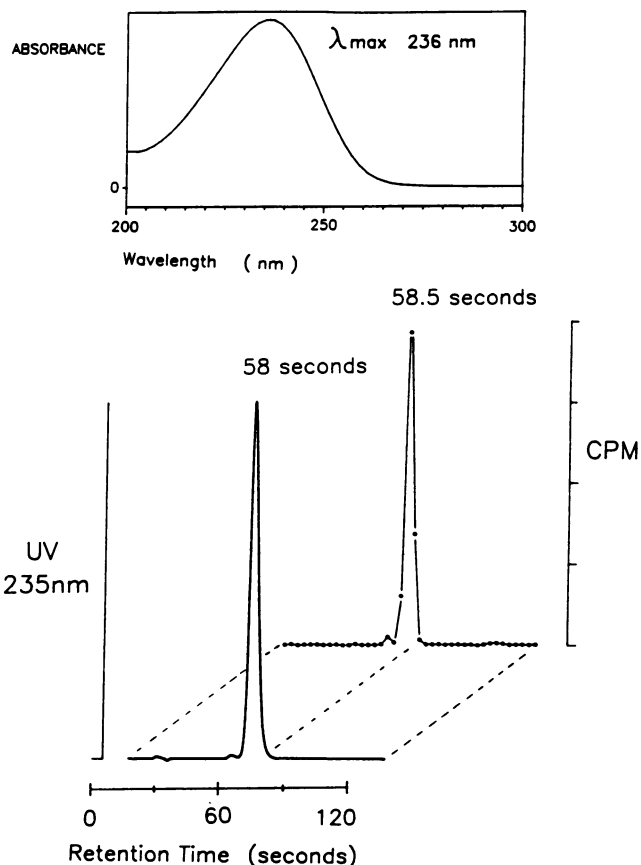

FIG. 1. Purification of the C18.3 allene oxide by SP-HPLC. Conditions: column, Altex Ultrasphere silica 4.5 × 0.46 cm; solvent, 1% diethyl ether in hexane; flow rate, 3 ml/min; temperature, −15°C. Fractions were collected every 3 sec for liquid scintillation counting. (*Lower*) UV (235 nm) chromatogram in the foreground and the corresponding radiolabeled profile behind. (*Upper*) UV spectrum of the allene oxide.

In addition, we also isolated the intermediate derived from the (13S)-hydroperoxide of linoleic acid. This C18.2 intermediate showed similar properties to the α-linolenate analog and was purified by the same protocol.

UV and CD Spectra. The UV chromophore has a smooth symmetrical appearance more typical of an enone than of a conjugated diene. However, like a typical conjugated alkene, there was no noticeable shift in λ_{max} on changing from hexane to the more polar acetonitrile. The CD spectra revealed Cotton effects, with a positive band at 240–250 nm and a stronger negative extremum at 215–220 nm.

^{1}H-NMR of the Allene Oxides. Interpretation of the NMR data is aided by the knowledge that the intermediates are chiral (CD) and that there is retention of oxygen from the hydroperoxide substrate (see below). The spectrum and 2-dimensional correlation spectroscopy (COSY) of the α-linolenate derivative are shown in Fig. 2, and the assignments for both allene oxides are given below. There are several aspects of these data that support the allene oxide structure. Of particular note are (i) the splitting patterns of the olefinic protons, particularly the carbon-11 proton H11 (a doublet coupled only to H10), (ii) the absence of a proton on carbon-12, (iii) the similarity of the chemical shift of the proton geminal to the epoxide (H13, 3.42 ppm) to the value reported for chemically synthesized allene oxides [3.25 ppm (16, 17)], (iv) the fact that H13 is a triplet, coupled only to the two protons on carbon-14, and (v) the fact that the two H14

protons are nonequivalent, thus confirming assignment of the chiral center at carbon-13. The assignments are fully supported by the COSY spectrum (Fig. 2) and also by selective decoupling experiments. The coupling constants across the 9,10 and 15,16 double bonds indicate that both are cis ($J_{9,10}$ = 10.5 Hz, $J_{15,16}$ = 10.8 Hz).

^{1}H-NMR (300 MHz in [^{2}H$_{14}$]hexane) of the C18.3 allene oxide methyl ester gave (ppm): δ 0.95 (t, H18abc), 1.18–1.43 (m, H4–6, 6 protons), 1.28–1.43 (m, H7ab), 1.525 (m, H3ab), 2.03 (p, H17ab), 2.11 (q, H8ab, $J_{8a,9}$ = 7.4 Hz), 2.19 (t, H2), 2.29–2.42 ($\approx$p, H14a, $J_{14a,15}$ = 8.1 Hz, $J_{14a,14b}$ = 14.4 Hz), 2.46–2.59 ($\approx$p, H14b), 3.42 (t, H13), 5.13 (dt, H9, $J_{9,10}$ = 10.5 Hz), 5.33 ($\approx$q, H15, $J_{15,16}$ = 10.8 Hz), 5.51 ($\approx$q, H16), 5.67 (d, H11), 5.86 (t, H10, $J_{10,11}$ = 11.7 Hz). For the linoleate analog: the H18 protons were under the hexane peak (0.9 ?), 1.23–1.53 (m, H4–7, H15, H16 and H17, 14 protons), 1.51–1.68 (m, H3ab), 1.53–1.83 (m, H14ab), 2.11 (q, H8ab, $J_{7,8}$ = 6.6 Hz), 2.18 (t, H2ab), 3.45 (t, H13), 5.11 (dt, H9, $J_{9,10}$ = 10.5 Hz), 5.65 (d, H11), 5.86 (t, H10, $J_{10,11}$ = 11.6 Hz).

Taken together, the UV, CD, oxygen-18 labeling (see below), and the NMR data are completely in accord with the proposed structures of the linolenate and linoleate allene oxides. Thus, the derivative of α-linolenic acid is identified as the allene oxide 12,13(S)-oxido-9Z,11,15Z-octadecatrienoate, and the linoleate analog is identified as 12,13(S)-oxido-9Z,11-octadecadienoate.

Hydrolysis and Cyclization of the Allene Oxides. The methyl ester derivatives were evaporated from cold hexane, instantly dissolved in cold ethanol, and mixed with water (pH 7.4 phosphate buffer). The products were subsequently analyzed by HPLC with on-line acquisition of UV spectra. From the α-linolenate analog, we detected a number of polar products and also two prominent derivatives corresponding in retention time and UV characteristics to the methyl esters of cis-12-oxo-PDA and the α-ketol, 12-oxo-13-hydroxy-9,15-octadecadienoate. The identification was confirmed by GC/MS. An α-ketol was also formed from the linoleate allene oxide, but there was only a trace of the corresponding cyclopentenone ($\approx$1% abundance).

Retention of ^{18}O from the Hydroperoxy Substrate in the Allene Oxide Product. Biosynthesis of cis-12-oxo-PDA and the corresponding α-ketol is known to proceed with retention of one of the oxygens of the hydroperoxy substrate (18, 19). We used this property to investigate the retention of hydroperoxy oxygen through the key sequence (i) biosynthesis and chromatographic purification of the allene oxide, and (ii) nonenzymic conversion to cis-12-oxo-PDA and the α-ketol. Allene oxide was prepared from (13S)-[^{18}O$_2$]hydroperoxylinolenic acid. The purified methyl ester was treated with water, and the cis-12-oxo-PDA and α-ketol were recovered by RP-HPLC. The two compounds were then reduced with sodium borohydride, converted to the trimethylsilyl ether derivatives, and analyzed by GC/MS.

The hydroxycyclopentane reduction product of cis-12-oxo-PDA showed complete retention of one of the original oxygen-18 labels from the hydroperoxy substrate. The shift of 2 atomic mass units is evident in the high-mass ions and in the base peak of the product from the ^{18}O experiment (Fig. 3). These results clearly prove retention of one ^{18}O atom in the cis-12-oxo-PDA molecule, and, it naturally follows, in the allene oxide parent.

The α-ketol isolated in the same experiment was found to contain only 20% of one of the original ^{18}O labels. As expected, the ^{18}O was located on the carbon-12 hydroxyl of the two diols (NaBH$_4$ product from the α-ketol). The loss of oxygen-18 is undoubtedly due to exchange of the carbon-12 oxygen during extraction and chromatographic work-up of the α-ketol prior to the NaBH$_4$ reduction. This could be avoided, as in earlier studies of ^{18}O retention in the α-ketol
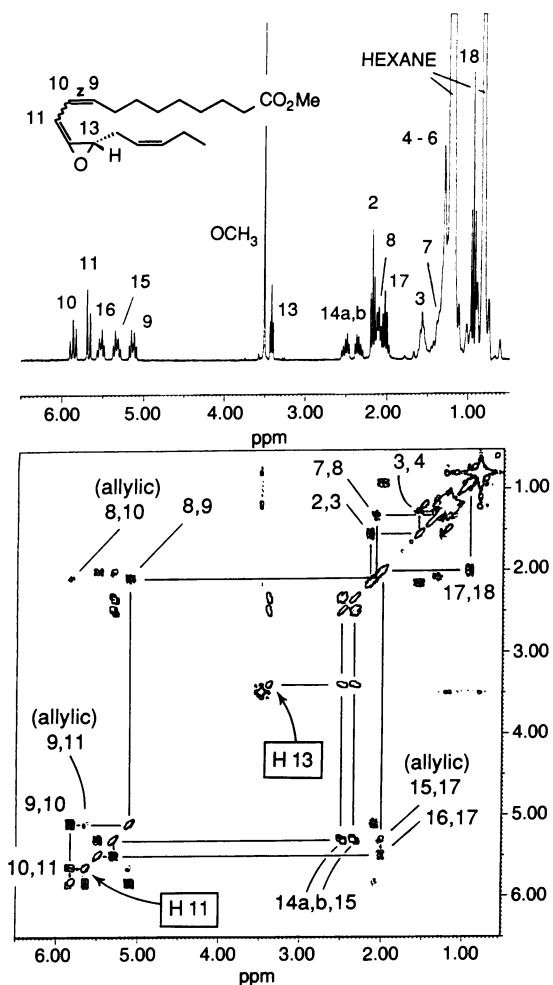

FIG. 2. ^{1}H-NMR (*Upper*) and 2-dimensional COSY (*Lower*) spectra of the C18.3 allene oxide. The data were recorded on about 1.5 μmol of the purified compound [12,13(S)-oxido-9Z,11,15Z-octadecatrienoate methyl ester] in [^{2}H$_{14}$]hexane at −35°C.
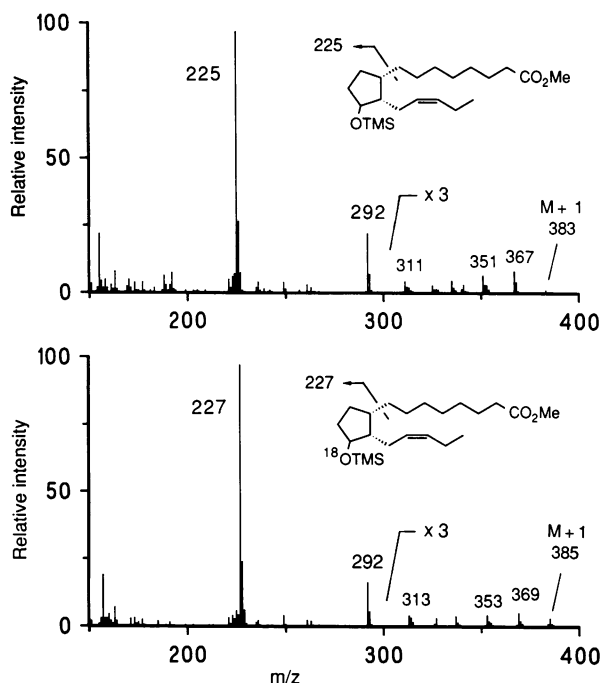
FIG. 3. Evidence for hydroperoxide-derived oxygen in the allene oxide and its retention in the cyclization to *cis*-12-oxo-PDA. (*Upper*) Mass spectrum of unlabeled hydroxycyclopentane. (*Lower*) Mass spectrum is that of [^{18}O]hydroxycyclopentane prepared by the following sequence: (*i*) enzymic conversion of (13S)-[^{18}O$_2$]hydroperoxylinolenic acid to allene oxide, (*ii*) chromatographic isolation of allene oxide methyl ester, (*iii*) reaction of pure allene oxide with water, (*iv*) isolation of *cis*-12-oxo-PDA, (*v*) NaBH$_4$ reduction, and (*vi*) GC/MS of the trimethylsilyl ether (OTMS) derivative. There is complete retention of one ^{18}O in formation of the allene oxide and its cyclization product.

(18), by *in situ* reduction of the α-ketol prior to isolation and chromatography.

Absolute Configuration of the *cis*-12-oxo-PDA and α-Ketol. The *cis*-12-oxo-PDA formed from the purified C18.3 allene oxide showed no Cotton effects in the CD, indicating that the product is racemic. This is precisely the result obtained on *cis*-12-oxo-PDA recovered from enzyme incubations (2).

We developed a method for measurement of the absolute configuration of the α-ketol derivatives based on the approach we used before for an α-ketol formed by *P. homomalla* (5). The results revealed that α-ketol derived by allene oxide hydrolysis has a similar enantiomeric excess of (13R)-hydroxy as the product formed in enzyme incubations. Both have undergone partial racemization with inversion of configuration at carbon-13 (Fig. 4 and Table 1).

DISCUSSION

We describe the structural elucidation of allene oxides derived from the (13S)-hydroperoxides of α-linolenic and linoleic acids. The firm structural assignment is based on (*i*) the UV chromophore of the isolated pure products, (*ii*) the retention of ^{18}O from (13S)-[^{18}O$_2$]hydroperoxide substrate in the structure, (*iii*) the chirality of the allene oxides as indicated by the CD analysis and the fact that the α-ketol hydrolysis products retain partial chirality, and (*iv*) the proton NMR analyses. The configuration of the allene oxide at carbon-11 is the one aspect of the structure that is not yet resolved. A *cis* arrangement of the carbons (11E) is required for cyclization, but whether the isolated allene oxide exists in this configuration is yet to be established. Certainly, the samples are *not* a mixture of the 11E and 11Z isomers; the NMR data
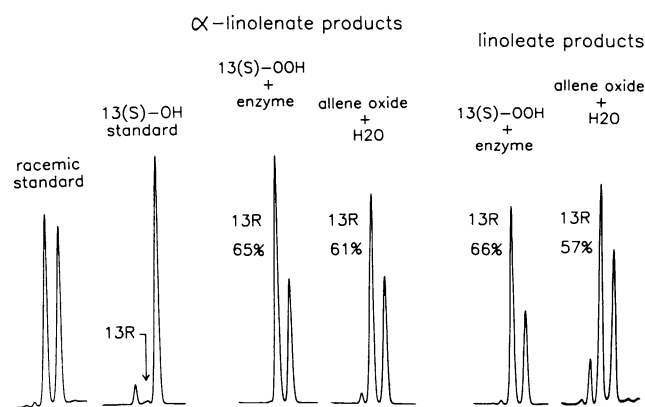

FIG. 4. Absolute configuration of the 13-hydroxyl of the α-ketol. The partial chromatograms show resolution of (13R)- and (13S)-hydroxy derivatives of the α-ketols. The compounds were analyzed as the benzyl esters of (13R)- and (13S)-*threo*-12,13-diols, derivatized as the menthoxycarbonyl diastereomers and separated by RP-HPLC with UV detection at 210 nm. (*Left*) Standards: authentic racemic (13R)/(13S)-hydroxy and authentic (13S)-hydroxy standards. (*Center*) α-Linolenate products: A comparison of α-ketol recovered from incubation of the (13S)-hydroperoxide of α-linolenic acid with an acetone powder of flaxseed with α-ketol formed by hydrolysis of purified allene oxide. (*Right*) Linoleate products: Corresponding analyses of the C18.2 derivatives.

indicate the presence of a single isomer, and the SP-HPLC chromatogram shows only one peak of allene oxide.

The purified allene oxides are rapidly hydrolyzed when mixed with water, giving an α-ketol as the major product. Analysis of the absolute configuration of the 13-hydroxyl of the α-ketol(s) gave results that were similar (but not identical) for α-ketol prepared from pure allene oxide and that recovered from enzyme incubations. The slight difference in chirality measured in these experiments may reflect differences in the conditions of hydrolysis of the allene oxide methyl ester (measured with the purified compound) compared with the free acid studied in the enzyme incubations. Although the slight quantitative differences appear to be real, the results are still very similar [61% vs. 65% (13R)-hydroxy] and almost certainly indicate that, even in the enzyme milieu, hydrolysis of the allene oxide is a nonenzymatic event.

The purified allene oxides are also subject to cyclization when mixed with water. The C18.3 allene oxide forms the cyclopentenone *cis*-12-oxo-PDA in about 20–25% of the yield of the α-ketol. The cyclic product is racemic, the same result obtained previously on a product recovered from enzyme incubations (2). Significantly, the C18.2 allene oxide formed α-ketol but only a trace of the cyclopentenone. This is in accord with the findings of Vick and Zimmerman (20), who only detected cyclization in the presence of the Δ^{15} double

Table 1. Steric analysis of the 13-hydroxyl group of the α-ketol products

	% 13R configuration in preparations	
Sample	Allene oxide hydrolysis	Enzyme incubation
C18.2 α-ketol	57.2*	66.5, 66.4* 65.5,
C18.3 α-ketol	60.7, 60.9*	65.3*
Racemic standard[†]	50.1, 49.8, 49.4, 50.3	
13S standard	≈1	

*The α-ketol was reduced *in situ* with NaBH$_4$ prior to isolation. Values without the asterisk were separate analyses on purified α-ketol.
[†]Separate derivatizations/injections of the same racemic standard.

bond. Our findings prove that this is not a substrate requirement of a "cyclase" enzyme but rather a property of the chemistry of the allene oxide intermediate. Notably, the NMR spectra of the C18.2 and C18.3 allene oxides exhibit almost identical chemical shifts and coupling constants of the protons attached to carbons 9 through 13. This strongly implies that both compounds have the same configuration in the epoxyene moiety, and hence that there must be another explanation for the propensity of the C18.3 allene oxide to cyclize. Interestingly, we found that the C18.3 allene oxide is less stable than the C18.2 analog; we measured a half-life at 0°C and pH 7.4 of about 16 sec for the C18.3 allene oxide and about 27 sec for the C18.2 analog (unpublished data). Although a faster rate of cyclization of the C18.3 allene oxide cannot fully account for the observed decrease in half-life, it might be inferred that the Δ^{15} double bond promotes destabilization of the allene oxide such that cyclopentenone formation more efficiently competes with the on-going hydrolysis.

The conditions we used to isolate the natural allene oxides should be applicable to related biochemical intermediates, to chemically synthesized analogs, and to other natural products (e.g., thromboxane A_2). The key to preparation of these unstable compounds is to achieve a rate of synthesis that significantly exceeds the rate of degradation. We were able to attain this condition, using a very active enzyme preparation of flaxseed. Once the compound is prepared and converted to the methyl ester, the isolation is fairly straightforward; the important provisos are that the temperature is kept below 0°C and that there is only brief exposure to polar materials such as the stationary phase of the SP-HPLC.

A key point of interest in this work is the potential role of the allene oxides as reactive intermediates in the formation of ketols, cyclopentanones (prostaglandins, clavulones) and possibly other metabolites of polyunsaturated lipids. We have shown that the allene oxide derivative of linolenic acid decomposes to form an α-ketol and the cyclopentenone *cis*-12-oxo-PDA when exposed to water. This finding constitutes direct proof that these well known metabolites arise from an allene oxide intermediate. Isolation and characterization of other natural allene oxides will help delineate their role as intermediates in the biochemical pathways of polyunsaturated lipid metabolism.

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