Isolation and Characterization of Two Geometric Allene Oxide Isomers Synthesized from 9S-Hydroperoxylinoleic Acid by Cytochrome P450 CYP74C3

STEREOCHEMICAL ASSIGNMENT OF NATURAL FATTY ACID ALLENE OXIDES*

Received for publication, May 2, 2013, and in revised form, May 22, 2013. Published, JBC Papers in Press, May 24, 2013, DOI 10.1074/jbc.M113.482521

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Background: Allene oxides involved in cyclopentenone biosynthesis are extremely labile and have eluded full stereochemical assignment.

Results: We identify a novel Z-allene oxide that unexpectedly rearranges to a cyclopentenone.

Conclusion: Other natural allene oxides are assigned the E configuration and can be easily distinguished from the Z-allene oxide by NMR.

Significance: Unequivocal determination of the unknown allene oxide configuration is documented and helps elucidate the cyclization chemistry.

Specialized cytochromes P450 or catalase-related hemoproteins transform fatty acid hydroperoxides to allene oxides, highly reactive epoxides leading to cyclopentenones and other products. The stereochemistry of the natural allene oxides is incompletely defined, as are the structural features required for their cyclization. We investigated the transformation of 9S-hydroperoxylinoleic acid with the allene oxide synthase CYP74C3, a reported reaction that unexpectedly produces an allene oxide-derived cyclopentenone. Using biphasic reaction conditions at 0 °C, we isolated the initial products and separated two allene oxide isomers by HPLC at −15 °C. One matched previously described allene oxides in its UV spectrum (λmax 236 nm) and NMR spectrum (defining a 9,10-epoxy-octadec-10,12Z-dienoate). The second was a novel stereoisomer (UV λmax 239 nm) with distinctive NMR chemical shifts. Comparison of NOE interactions of the epoxy proton at C9 in the two allene oxides (and the equivalent NOE experiment in 12,13-epoxy allene oxides) allowed assignment at the isomeric C10 epoxy-ene carbon as Z in the new isomer and the E configuration in all previously characterized allene oxides. The novel 10Z isomer spontaneously formed a cis-cyclopentenone at room temperature in hexane. These results explain the origin of the cyclopentenone, provide insights into the mechanisms of allene oxide cyclization, and define the double bond geometry in naturally occurring allene oxides.

Although allene oxides get their name from the epoxidation of an allene, their origin in biosynthetic pathways involves the enzymatic dehydration of a specific fatty acid hydroperoxide. This reaction was first discovered in the pathways of cyclopentenone biosynthesis in plants and corals (1–4). In the jasmonate pathway in plants (Fig. 1) (5, 6), a lipoxygenase produces the 13S-hydroperoxide of α-linolenic acid, and a peroxide-metabolizing cytochrome P450 (CYP74A, an allene oxide synthase (AOS))2 converts this to the allene oxide, 12,13S-epoxy-octadeca-9Z,11,15Z-trienoic acid. This unstable epoxide is acted upon by the enzyme allene oxide cyclase (AOC) to produce a chiral cyclopentenone, a few steps away from the end product, jasmonic acid. In coral, the lipoxygenase precursor is the 8R-hydroperoxide of arachidonic acid, which is transformed by a catalase-related hemoprotein to the 8,9-epoxy allene oxide, a presumed precursor of the clavulones and related cyclopentenones of corals (7, 8). Recently, an allene oxide formed from 12R-hydroperoxy linolenic acid by a cyanobacterial catalase-related enzyme was described (9).

The allene oxides of these and other pathways have been isolated, stabilized by conversion to the methyl ester and by maintaining at cold temperatures, and HPLC-purified at approximately −15 °C, and the 1H NMR spectra have been acquired at −40 °C (9–11). The NMR analyses confirmed the structures deduced from study of the cyclization and hydrolysis transformations of the allene oxides. Nonetheless, one aspect of the allene oxide stereochemistry remained undefined: the E or Z configuration of the double bond impinging on the epoxide, as illustrated in Fig. 1 for the plant 12,13-epoxy allene oxide of α-linolenic acid. Significantly, the NMR spectra of the allene oxides derived from both the cytochrome P450 AOS and the catalase-related AOS all show a similar pattern of olefinic signals, and with no traces of isomeric allene oxides (9–11). It is likely, therefore, that all the reported allene oxide structures have the same geometry at the epoxy-ene double bond, but it

* This work was supported, in whole or in part, by National Institutes of Health Grant GM-074888 (to A. R. B.), this work was also supported by National Science Foundation (NSF) grant CHE-1212879 (to J. K. C.).

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2 The abbreviations used are: AOS, allene oxide synthase; AOC, allene oxide cyclase; HPODE, hydroperoxyoctadecadienoic acid; HPOTe, hydroperoxyoctadecatrienoic acid; RP-HPLC, reversed-phase high pressure liquid chromatography; SP-HPLC, straight-phase high pressure liquid chromatography.

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A New Isomer of Fatty Acid Allene Oxides

![Scheme 1](image)

*FIGURE 1. The 11E or 11Z configuration of the allene oxide in the jasmonate pathway. The allene oxide, 12,13-epoxy-octadeca-9Z,11Z,15Z-trienoic acid, is formed by CYP74A (AOS) from 13S-hydroperoxy-linolenic acid and is further converted to the cyclopentenone 12-oxo-phytodienoic acid by AOC and then to jasmonic acid. Note that the Z (cis) and E (trans) nomenclature of the allene oxides is based on the epoxy oxygen having priority over carbon, as highlighted in blue (11E) and red (11Z).*

*E or Z? This is an important question regarding the biosynthetic mechanism(s), as well as for defining the stereochemistry of the natural substrate of the allene oxide cyclase enzyme and for a proper understanding of the “rules” governing the enzymatic or nonenzymatic allene oxide cyclization to cyclopentenones.

The facility for nonenzymatic cyclization of the natural allene oxides has some distinctive characteristics, the most significant elements of which are summarized in Scheme 1. The 18:3ω3 allene oxide of the jasmonate pathway (in addition to being converted to a cyclopentenone by the enzyme AOC) will cyclize spontaneously in physiological buffers (typically ~15% cyclization, 85% hydrolysis to α- and γ-ketols (12)). By contrast, the linoleic acid (C18:2ω6) allene oxide undergoes only hydrolysis (10). By inspection, therefore, the presence of the extra ω3 double bond facilitates cyclization, and study of additional fatty acid analogues supports this deduction (13). Included among these additional allene oxides is the one formed from 9S-hydroperoxylinoleic acid (9S-HPODE) by maize (corn) AOS. Because the hydroperoxy moieties in 9S-HPODE and 13S-HPODE are pseudosymmetrical and superimposable, it is entirely expected that their allene oxide derivatives would have the same symmetry and exhibit similar characteristics to each other. Indeed, neither has a propensity to cyclize (13).

This empirical understanding of allene oxide cyclization was unsettled by the observations of Hamberg (14) that the metabolism of 9S-HPODE in potato microsomes gives rise to an allene oxide-derived cyclopentenone (Scheme 1). Hamberg (14) conducted side-by-side incubations with maize and potato extracts, leaving no doubt that the two plant tissues each form an allene oxide from 9S-HPODE. The maize and potato allene oxides differed slightly in half-life in the microsomes (33 and 44 s, respectively, at 0 °C), their α-ketol hydrolysis products were of different stereochemistry (~70 and 90% 9R-hydroxy, respectively), and although the maize product merely hydrolyzed, as was known, a cyclopentenone was isolated from the potato microsomes (14). Biosynthesis of the cyclopentenone was tied directly to AOS activity when Howe and co-workers (15) showed that a recombinant AOS variant from tomato, designated as CYP74C3, converts 9S-HPODE to ketol hydrolysis products and the same cyclopentenone.

One potential explanation for the unexpected appearance of the cyclopentenone in relation to the potato and tomato AOS activity is that the enzyme itself induces cyclization of the allene oxide, and evidence to support this has been reported (16). Another intriguing possibility is that a different allene oxide double bond isomer is formed by the CYP74C AOS enzymes, and this allene oxide has the propensity to cyclize. Herein we report direct evidence that CYP74C3 transforms 9S-HPODE into two 9,10-epoxy allene oxide isomers, 10E and 10Z, and that the new isomer, 10Z, spontaneously rearranges to a cyclopentenone. A significant practical outcome of these experiments is that, with both isomers in hand, the results permit unambiguous determination of the double bond geometry and thus allow assignment of the complete stereochemistry of the natural allene oxides.

**EXPERIMENTAL PROCEDURES**

*Materials—Fatty acids were purchased from NuChek Prep Inc (Elysian, MN). 9S-HPODE was prepared using the 9-lipoxygenase activity in potato as described (17), and the 13S-hydroperoxides of linoleic (13S-HPODE) and α-linolenic (13S-HPOTrE) acids were prepared using soybean lipoxygenase (Sigma, Type V) (18).*

*AOS Enzyme Preparations—Flaxseed acetone powder was prepared as described (12). The tomato CYP74C3 plasmid was*
a gift of Dr. Gregg Howe (Michigan State University) and was expressed and purified as described (19). The guayule CYP74A2 in plasmid pET28b was a gift of Drs. Zhiquan Pan and Xiaqiang Wang and was expressed and purified by nickel-nitritolactate-acid affinity chromatography by following their methodology (20). The recombinant P450 enzymes were quantified using ε = 100,000 for the main Soret band (393 nm in CYP74A2 and 415 nm in CYP74C3).

Preparation of Allene Oxides Using Flaxseed Extract (CYP74A1)—Flaxseed acetone powder (100 mg/ml) was stirred for 30 min at 0°C in 0.1 m potassium phosphate buffer, pH 6.5, containing 3 mM Zwittergent 3-14 (Calbiochem) and then centrifuged at top speed in a benchtop microcentrifuge for 5 min at room temperature, and the resulting clear solution was kept at 0°C until use (10). 9

JULY 19, 2013•VOLUME 288•NUMBER 29

A New Isomer of Fatty Acid Allene Oxides

HPLC of Allene Oxide Methyl Esters—The 12,13-epoxy allene oxide methyl esters were purified exactly as described (10) and recovered in 10–25% overall yield from the fatty acid hydroperoxide. The 9,10-epoxy allene oxide methyl esters were purified similarly except that a slightly higher concentration of diethyl ether (3%) was required to keep the retention time near 1 min. In brief, the allene oxide methyl ester was analyzed and purified using an Altex 5 μ silica column (45 × 4.6 mm, originally sold as a guard column), kept at −10 to −20°C in ice/ethanol along with a 2-ml loading loop on the injector to pre-cool the solvent. The column was eluted at a flow rate of 3 ml/min with hexane/diethyl ether (100:1 by volume), with UV detection at 205, 220, 235, and 270 nm using an Agilent 1100 series diode array detector. The allene oxide methyl esters elute at ~1-min retention time. For semipreparative purification runs, up to 0.5–1 mg of crude allene oxide preparation is injected (and 0.25–0.5 mg is collected as pure product).

A New Isomer of Fatty Acid Allene Oxides

The 10E and 10Z isomers of the 9,10-epoxy allene oxide methyl esters were resolved using either a CHIRALCEL OD-H column (solvent hexane/isopropyl alcohol, 100:3 by volume) or a CHIRALPAK IB column (solvent hexane/ethanol, 100:2 by volume), each run at 1 ml/min while submerged in an ice/ethanol bath at approximately −15°C, with UV detection at 205, 220, 235, and 270 nm using an Agilent 1100 series diode array detector. SP-HPLC was conducted using a Thomson Advantage 5 μ silica column (25 × 0.46 cm) and a solvent of hexane/isopropyl alcohol/glacial acetic acid (100:2:0:1) for free acids and omitting the acetic acid for methyl esters, with flow rates of 1 or 2 ml/min.

Analysis of Allene Oxide Methyl Ester Degradation Products—Typically, samples from aqueous incubations were analyzed initially by RP-HPLC using a Waters C18 Symmetry column (25 × 0.46 cm) with a solvent of methanol/water/glacial acetic acid for free fatty acids (80/20/0.01 by volume) and 80/20 methanol/water for methyl esters, with a flow rate of 1 ml/min and UV detection using an Agilent 1100 series diode array detector. SP-HPLC was conducted using a Thomson Advantage 5 μ silica column (25 × 0.46 cm) and a solvent of hexane/isopropyl alcohol/glacial acetic acid (100:2:0:1) for free acids and omitting the acetic acid for methyl esters, with flow rates of 1 or 2 ml/min.

Analysis of Allene Oxide Methyl Ester Degradation Products—Individual allene oxide methyl esters collected from the CHIRALCEL OD-H column in hexane/isopropyl alcohol (100:3) at −15°C were allowed to warm to room temperature and left standing for 1 h in the column solvent. The samples were then evaporated to dryness under nitrogen and analyzed by RP-HPLC using a Waters C18 Symmetry column (25 × 0.46 cm), methanol/water (85:15, by volume) as the running solvent, a flow rate of 1 ml/min, and monitoring of 205-, 220-, 235-, and 270-nm wavelengths using an Agilent 1100 series diode array detector. Subsequent further purification of the products included SP-HPLC using a Thomson Advantage silica column (25 × 0.46 cm) with a solvent system of hexane/isopropyl alcohol (100:2) and flow rates in 1 or 2 ml/min. The enantiomeric composition of the 9S-HPODE-derived cyclopentenone was examined using a CHIRALPAK AD column (25 × 0.46 cm) with a solvent of hexane/methanol (100:2 by volume) and a flow rate of 0.5 ml/min; the enantiomers eluted with close to baseline resolution at retention times of 15.2 and 16.2 min. Alkaline Isomerization of Cyclopentenone—9S-HPODE-derived cyclopentenone was treated with 0.1 m methanolic KOH for 2 h at room temperature, and then aqueous 0.2 m KH2PO4
A New Isomer of Fatty Acid Allene Oxides

was added to quickly neutralize the solution, which was immediately extracted with dichloromethane. The organic phase was washed with water and then taken to dryness under nitrogen, and the isomerized sample was dissolved in a solvent of hexane/isopropyl alcohol (100:2) for SP-HPLC analysis using the above Thomson silica column with a solvent of hexane/isopropyl alcohol (100:2, by volume).

NMR Analyses—NMR experiments were acquired using an 11.7-Tesla Oxford magnet equipped with a Bruker DRX console operating at 500.13 MHz. Spectra were acquired in 3- or 5-mm NMR tubes using a Bruker 5-mm BBFO NMR probe. For allene oxide samples, chemical shifts were referenced internally to the CH₃ triplet in hexane-d₁₄, measured at 0.84 ppm in relation to TMS, which also served as the 2H lock solvent. A probe temperature of 233 K was used for samples of allene oxide methyl esters. For one-dimensional ¹H NMR, typical experimental conditions included 32,000 data points, 13-ppm sweep width, a recycle delay of 1.5 s, and 16–32 scans depending on sample concentration. Selective one-dimensional NOE spectra were recorded using the double pulse field gradient spin echo technique (22, 23). Experimental parameters for this experiment were similar to those for the standard one-dimensional ¹H NMR experiment with the addition of 600 ms, which was used for the NOE mixing time, and 512–1024 scans per acquisition depending on sample concentration. Selective NOE experiments on a sample of the allene oxide methyl ester product of 13S-HPOTrE were run at 243 K using a Bruker 600 MHz instrument equipped with a Bruker TCI cryoprobe.

Computation NMR Methods—The proton NMR spectra of E- and Z-allene oxide models (bearing the truncated methyl side chains) were calculated by the Spartan’08 program (Wavefunction, Inc., Irvine, CA) with the Hartree-Fock method at 3-21G basis sets. For computation of interatomic distances, the E and Z allene oxide molecules were built in Moe 2012.10 (Chemical Computing Group, Montreal, Canada), with methyl termination on either side at the 7- and 15-positions. Unrestricted energy minimization in a gas phase was conducted using the MMFF94 force field implemented in Moe. The lowest energy conformers for the E and Z isomers were retrieved, and distances between the epoxide proton (H9 in the 9,10-epoxy allene oxides) and H11 (doublet) or H12 (triplet) were measured in the respective isomers, providing the interproton distances of each pair. These distances were then used in the interpretation of the observed NOE measurements.

Other Spectroscopic Methods—In addition to the on-line diode array detection of HPLC, UV spectra were recorded using a PerkinElmer Life Sciences Lambda-35 scanning instrument. GC-MS was conducted using the methyl ester trimethylsilyl derivatives or the further methoxime derivatives of the products. The analyses were carried out in the positive ion electron impact mode (70 eV) using a Thermo Finnigan DSQ mass spectrometer. Typically the initial oven temperature was set for 150 °C, held for 1 min, and then increased to 300 °C at 20 °C/min increment and held at 300 °C for 3 min.

RESULTS

Preparation of Allene Oxides—We employed biphasic reaction conditions at ice-cold temperature in which 9S-HPODE in pentane or hexane is vigorously mixed with a concentrated solution of CYP74 at pH 6.5. At this pH, the 9S-HPODE partitions into the aqueous phase where it is metabolized to the allene oxide; the allene oxide is less polar than the hydroperoxide and is instantly back-extracted into the hexane phase and thus protected from hydrolysis. The allene oxide is then further stabilized by conversion to the methyl ester by a 60-s treatment of the hexane solution at 0 °C with diazomethane. The allene oxide methyl ester can be chromatographed on HPLC in hexane-based solvents at −10 °C or below (10).

Isolation of Allene Oxides from Reaction of 9S-HPODE with CYP74C3—Our initial HPLC analyses of the CYP74C3/9S-HPODE products used a short silica column (4.5 cm × 0.46 cm) run under the conditions we used previously for purification of allene oxide methyl esters (9–11). This system resolved a single peak of product having the characteristic UV spectrum of a fatty acid allene oxide (cf. Refs. 1, 9, and 10). Notwithstanding the appearance of a single chromatographic peak, the results of ¹H NMR analysis of the CYP74C3/9S-HPODE reaction clearly displayed the existence of what we termed “old” and “new” allene oxide isomers (Fig. 2A). As there are only three protons on the two double bonds of a C18:2 allene oxide, the olefinic region has just three signals: a triplet (t) and a doublet of triplets (dt) representing the protons on the cis double bond and a doublet (d) for the single proton on the epoxy-ene double bond. For comparison, Fig. 2A is aligned with spectra recorded on the CYP74A allene oxides formed from 13S-HPODE (Fig. 2B) and 13S-HPOTrE (Fig. 2C), the latter two representing the well described, “old” allene oxide isomer (10). Based on this alignment, it is obvious that the more abundant set of signals in Fig. 2A represents the “new” allene oxide, whereas the minor set matches the chemical shifts of the isomers isolated before (cf. Ref. 10). In the “old” and “new” isomers, the coupling constants for the double bond signals are indistinguishable, providing no clues to the undetermined stereochemistry at the epoxy-ene double bond.

Chromatographic Separation of Two Allene Oxide Isomers—With the recognition that there are two allene oxide isomers formed by CYP74C3 from 9S-HPODE, we set about attempting to separate the two chromatographically. Even at extended retention times, the silica column gave a single peak. Trials with a silver-loaded silica column run at −15 °C gave peaks too broad for practical chromatographic resolution. We turned, therefore, to chiral-phase columns, which have proved useful in other circumstances for the separation of stereoisomers that are not enantiomers (17, 24). Two different chiral phases (see “Experimental Procedures”), with the columns run at −15 to −20 °C, successfully resolved the “old” and “new” allene oxide isomers, illustrated in Fig. 3A using the CHIRALCEL OD-H column. The two isomers display similar smooth conjugated diene UV spectra, with the “new” isomer displaying a slightly higher λmax, Fig. 3B.

Preparation of Allene Oxides for NOE Experiments—Given the need for high sample concentrations for NOE measurements, requiring at least hundreds of micrograms of these highly unstable allene oxides in the NMR tube, we had to make some compromises. We used the mixture of isomers produced by CYP74C3 for the NOE experiments because we could not
recover sufficient individual isomers from the chiral column separation. (In fact, having the two isomers in the same tube has the advantage of providing side-by-side comparison of NOE effects on the one sample.) Also, to obtain the best quality data on the “old” isomer, we made separate preparations of the allene oxides derived from \textit{13S}-HPODE and \textit{13S}-HPOTrE rather than using the \textit{9S}-HPODE-derived allene oxide (the minor component of the CYP74C3 mixture). In the NMR, signals from the epoxy-ene region of the spectra of \textit{9,10-} and \textit{12,13}-allene oxides from \textit{9S-} and \textit{13S}-HPODE are pseudosymmetrical and essentially indistinguishable. This allowed us to use the CYP74A2 enzyme from guayule for production of the allene oxide from its preferred substrate \textit{13S}-HPODE (21) and flaxseed AOS (CYP74A1) for preparation of the allene oxide from \textit{13S}-HPOTrE.

NOE Experiments to Assign the Epoxy-ene Configuration in Two Allene Oxide Isomers—We acquired NOESY spectra on the CYP74C3-derived mixture of “old” and “new” allene oxide isomers, but the results were indecisive because of insufficient concentration of sample. More conclusive were selective NOE experiments targeting the key epoxy-ene protons, particularly the epoxide proton located at 3.43 ppm. Firstly, consider the results from the “old” isomer, using the allene oxide from \textit{13S}-HPODE. Irradiation at 3.43 ppm (H13) gives clear NOE signals to the doublet (H11, 5.62 ppm) and triplet (H10, 5.83 ppm) protons, with the triplet signal slightly stronger (Fig. 4A). Using the same \textit{13S}-HPODE-derived allene oxide and selective NOE approach, Grechkin and colleagues (25) reported seeing the NOE between the epoxy proton H13 and the triplet at H10, with no NOE observed to the doublet at H11; taking support from computer modeling of the interatomic distances involved, they assigned the 11E configuration to this isomer. Although this is in agreement with our new data, we consider that only direct comparison of the two isomers permits the definitive assignment.

Thus, when this selective NOE experiment is repeated using the mixture of \textit{9,10-}epoxy allene oxides (CYP74C3 products), it is apparent that the two isomers give different results (Fig. 4B). Although the minor isomer (the “old” isomer equivalent to the CYP74A product from \textit{13S}-HPODE) gives similar responses to the triplet and doublet as just described (and marked with an O for “old” in Fig. 4B), the more abundant “new” isomer gives a
studied the allene oxide configuration using NOE effects. Irradiation of the H13 epoxide proton at 3.4 ppm showed the NOEs to the triplet and doublet protons in the conjugated diene (and to the adjacent 15,16-double bond) (Fig. 4C), confirming assignment of this allene oxide as the “old” isomer, 11E. The full NOE spectra (0–7 ppm) on a complete set of experiments irradiating this allene oxide at H9, H10, H11, and H13 are given in Fig. 5. With 2.4 mg of purified allene oxide methyl ester at a concentration of 14.5 mg/ml in the NMR solvent, the results represent a definitive data set, illustrating the extensive NOEs around the epoxy-ene system, including the reciprocal NOEs back to the epoxy proton H13 upon irradiation of the triplet (H10) and doublet (H11) of the conjugated diene.

**Spontaneous Cyclization of the “New” Allene Oxide**—As we mentioned earlier, the initial impetus for our experiments derived from the observations that a 9S-HPODE-derived allene oxide has a facility to cyclize in buffer to a cyclopentenone (14, 15), a property not associated with the linoleate allene oxides described before (1, 10, 13). To follow up on this with the purified allene oxides, we utilized a chiral column to collect “old” and “new” allene oxide methyl esters (Fig. 3A), which allowed us to stand in column solvent (hexane/isopropyl alcohol, 100:3) for 60 min at room temperature, and then we analyzed the products by RP-HPLC. Two major peaks are detected at 8.5 and 11 min from the “new” 10Z allene oxide methyl ester (Fig. 6A), whereas the 10E (“old”) isomer shows a dominant 8.5-min peak and only a trace of the second (Fig. 6B). The UV spectrum of the first product resembles that of an allene oxide-derived γ-ketol (of which it is a structural analog), exhibiting a broad conjugated enone absorbance, λmax 229 nm in RP-HPLC solvent. The second peak, formed from the 10Z allene oxide, exhibits a conjugated enone profile very similar to that of cis-12-oxo-phytodienoic acid (12); the λmax is shifted to 222 nm in the wetary RP-HPLC solvent when compared with the 9S-HPODE-derived cyclopentenone from potato extracts (220 nm in ethanol (14)).

The first product was identified by additional HPLC analyses and by NMR including COSY, heteronuclear single quantum correlation, and heteronuclear multiple bond correlation analyses as two diastereomers of an oxygenated isopropyl alcohol adduct, 9-O-isopropyl-10-oxo-13RS-hydroperoxy-octadec-11E-enolate (Table 1). The adduct appears to be formed by isopropyl alcohol attack on the allene oxide, forming an α-ketol ether (9-O-isopropyl-10-oxo), which is autoxidized to the 13-hydroperoxide. The 13-hydroperoxide diastereomers appear as a single peak on RP-HPLC in MeOH/water solvent (Fig. 6), but resolve using CH₃CN/water, and also on SP-HPLC either as the 13-hydroperoxide or after triphenylphosphine reduction to the 13-hydroxy (data not shown). In our experience, allene oxide-derived α-ketols are not prone to extensive oxygenation under mild conditions (1 h at room temperature in HPLC solvent), suggesting that the oxygenation occurs during the course of transformation from the allene oxide. Facile abstraction of the OH hydrogen of the enol form of the ketone at C10 may account for the efficient peroxidation under short term exposure to dissolved oxygen in the hexane/isopropyl alcohol solvent. Oxygenated α-ketols (except O-H rather than O-isopropyl) are prominent in some plant extracts (26–28). The
synthesis is attributed to lipoxygenase-catalyzed oxygenation of allene oxide-derived α-ketols (a reaction shown to occur in vitro (27)), but perhaps this efficient nonenzymatic oxygenation during hydrolysis of the allene oxide could contribute to their observed abundance in plant tissues.

The second product, formed only from the 10Z allene oxide, was examined by GC-MS in the electron impact mode after hydrogenation and conversion to the methoxime derivative. Two peaks representing the syn- and anti-methoxime isomers gave diagnostic ions at m/z 339 (M⁺, ~2% relative abundance), 308 (M-31, 4%), 268 (M-71, 20%), 183 (M-[C₁-C₈], ~40%), and 112, (base peak), compatible with the expected spectrum of the 9-HPODE-derived cyclopentenone. The structure was confirmed by NMR as the cis-cyclopentenone 10-oxo-11-phytoenoate methyl ester (data not shown), identical to the previously characterized product of potato and CYP74C3 (14–16).

Treatment of the cis-cyclopentenone with 0.1 M KOH in methanol for 2 h at room temperature induced isomerization to the trans isomer, resolved at a slightly shorter retention time by SP-HPLC, 4.7 min for trans, 5.0 min for cis (using hexane/isopropyl alcohol 100:2 as solvent, 1 ml/min). Chiral analysis of the cis-cyclopentenone methyl ester using a CHIRALPAK AD column resolved the enantiomers. In two independent analyses, cyclopentenone prepared by allowing the 10Z allene oxide methyl ester to degrade in SP-HPLC column solvent gave peak areas of 61:39 and 53:47, indicating some retention of chirality in the cyclization. By contrast, the product recovered from aqueous incubation was racemic, as has been reported before for this 9S-HPODE-derived cyclopentenone (14, 16).

DISCUSSION

Identification of Two Geometrical Allene Oxide Isomers—Until now, all the reported allene oxide structures for which an NMR spectrum is available corresponded to one and the same configuration of the epoxy-ene double bond, herein referred to as the “old” isomer, and now assigned as the E configuration.
A New Isomer of Fatty Acid Allene Oxides

How Are Two Allene Oxide Isomers Formed by CYP74C3?—CYP74C3 may catalyze nonselective synthesis of 10E and 10Z allene oxides or E-to-Z conversion. Although the latter has not been demonstrated directly, some observations of Grechkin et al. (16) are relevant to the question. They studied allene oxide synthesis by recombinant CYP74C3 (prepared using the identical cDNA plasmid as employed in our studies), and while inferring that only one allene oxide is formed, they had concluded that CYP74C3 promoted stereospecific hydrolysis and racemic cyclization of the allene oxide. The higher the concentration of CYP74C3 included in the biosynthesis (beyond the racemic cyclization of the allene oxide. The higher the concentration of CYP74C3 included in the biosynthesis (beyond the racemic cyclization of the allene oxide. The higher the concentration of CYP74C3 included in the biosynthesis (beyond the racemic cyclization of the allene oxide. The higher the concentration of CYP74C3 included in the biosynthesis (beyond the racemic cyclization of the allene oxide. The higher the concentration of CYP74C3 included in the biosynthesis (beyond the racemic cyclization of the allene oxide. The higher the concentration of CYP74C3 included in the biosynthesis (beyond the racemic cyclization of the allene oxide. The higher the concentration of CYP74C3 included in the biosynthesis (beyond the racemic cyclization of the allene oxide. The higher the concentration of CYP74C3 included in the biosynthesis (beyond the racemic cyclization of the allene oxide. The higher the concentration of CYP74C3 included in the biosynthesis (beyond the racemic cyclization of the allene oxide. The higher the concentration of CYP74C3 included in the biosynthesis (beyond the racemic cyclization of the allene oxide. The higher the concentration of CYP74C3 included in the biosynthesis (beyond the racemic cyclization of the allene oxide. The higher the concentration of CYP74C3 included in the biosynthesis (beyond the racemic cyclization of the allene oxide. The higher the concentration of CYP74C3 included in the biosynthesis (beyond the racemic cyclization of the allene oxide. The higher the concentration of CYP74C3 included in the biosynthesis (beyond the racemic cyclization of the allene oxide. The higher the concentration of CYP74C3 included in the biosynthesis (beyond the racemic cyclization of the allene oxide. The higher the concentration of CYP74C3 included in the biosynthesis (beyond the racemic cyclization of the allene oxide. The higher the concentration of CYP74C3 included in the biosynthesis (beyond the racemic cyclization of the allene oxide. The higher the concentration of CYP74C3 included in the biosynthesis (beyond the racemic cyclization of the allene oxide. The higher the concentra

Based on the very similar appearance of the double bond protons in the spectra, this generality applies to the allene oxides formed by cytochrome P450 CYP74A (10), by the catalase-related AOS in coral (substrate 8R-hydroperoxyicosatetraenoic acid) (11, 29), and by the catalase-related AOS of the cyanobacterium Acaryochloris marina (substrate 12R-hydroperoxy linolenic acid) (9) (Fig. 7). Remarkably, there is no trace of the Z isomer in these reported spectra, as clearly illustrated for the CYP74A allene oxides in Figs. 2, B and C and 4, A and C. Characterization of the “new” (Z) isomer formed as the prominent product by CYP74C3 is indeed novel.

Two Allene Oxide Isomers Are Formed by CYP74C3—It is striking that CYP74C3 forms both the 10Z and the 10E allene oxides from 9S-HPODE. We cannot be definite concerning the relative proportions of the two because of issues of relative stability during the preparation and purification. Nonetheless, the NMR spectra of the mixture recovered from SP-HPLC showed a 2–4-fold excess of the “new” Z isomer, whereas the chiral column separation (which involves a long retention on the column) tended to show similar sized peaks of the two allene oxide isomers and left us with the impression that the “new” isomer is the more unstable during sample preparation and on the column. This contrasts with what might be inferred from the findings of Hamberg (14) under aqueous conditions, in which the measured stability of 9S-HPODE-derived allene oxide(s) in maize and potato extracts at 0 °C and pH 7.4 indicated a slightly shorter half-life of the maize (10E) allene oxide, 33 s in maize microsomes versus 44 s in potato.

TABLE 1

NMR chemical shifts and correlations for the oxidized isopropyl alcohol adduct

The spectra were recorded on a Bruker AV-II 600 MHz spectrometer equipped with a cryoprobe (600.13 MHz, CD3CN). HMBC, heteronuclear multiple bond correlation; HSQC, heteronuclear single quantum correlation.

| Proton No. | 1H (ppm) | Multiplicity | Coupling constant (Hz) | HMQC 13C (ppm) | HSQC 13C (ppm) | HMBC 13C (ppm) |
|------------|----------|--------------|------------------------|----------------|----------------|----------------|
| 2          | 2.26     | t            | J2,3 = 7.5             | 1.53           | 33.5           | 173.3, 28.7, 24.7 |
| 3          | 1.53     | m            | 2.26                   | 24.7           |                 | 173.3, 28.7 |
| 4          | 1.27     | m            | 1.53                   | 22.3, 24.7     | 28.7, 31.3     |                |
| 5          | 1.53     | m            | 3.90                   | 21.3           | 210.1          | 71.2, 32.3, 24.7 |
| 6          | 3.90     | t            | J8,9 = 6.7             | 1.53           | 82.0           | 71.2, 32.3, 24.7 |
| 11         | 6.62*    | d            | J11,12 = 16.0          | 6.79           | 125.8          | 210.1, 84.3    |
| 12         | 6.79/6.80b | dd           | J12,13 = 16.0, J12,14 = 6.7 | 6.62, 4.45   | 145.6          | 201.6          |
| 13         | 4.45     | d            | J13,14 = 6.7           | 6.79, 1.53     | 84.3           | 125.8, 32.0    |
| 14         | 1.53     | m            | 1.27                   | 32.0           |                |                |
| 15-17      | 1.27     | m            | 1.53                   | 22.3, 24.7     | 31.3           |                |
| 18         | 0.87     | t            | J17,18 = 7.2           | 1.27           | 33.1           | 32.3, 22.3     |
|<CH3>      | 3.49*    | m            | 1.10                   | 71.2           |                |                |
|<CH2>      | 1.10     | d            | J2 = 6.1               | 3.49           | 20.8/22.2      | 71.2           |
|OCH3        | 3.59     | s            |                        | 50.7           | 173.3          |                |
|OOG         | 9.55*    | s            |                        |                |                |                |

a The chemical shift was determined in the HMBC experiment.
b Two overlapping signals from two diastereomers were observed.
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All reported allene oxides (ref. 9, 10, 11):-
13S-HPOTEs and 13S-HPODE (CYP74A)
9S-HPODE (using maize AOS)
8R-HPETE (P. homomalla cAOS)
12R-HPOTEs (A. marina cAOS)

FIGURE 7. Summary of natural allene oxide structures at the epoxy-ene carbon. cAOS, catalase-related AOS; HPETE, hydroperoxyeicosatetraenoic acid.

FIGURE 8. Computed NOE distances in E and Z allene oxides. A and B, three-dimensional representation of the relationship of the epoxide proton of the E and Z allene oxides to the doublet (d) and triplet (t) on the conjugated diene, with computed distances shown.

Located out-of-plane, and it nearly bisects the diene framework (Fig. 8, A and B). Thus, the stereochemical assignment on the basis of NOE measurements of only one isomer was deemed to be fraught with uncertainties. The availability of the “new” isomer (10Z) allowed comparative NOE studies, which proved to be more compelling. Although the “old” isomer (10E) gives triplet-to-doublet NOE signal intensities of ∼2:1, the “new” isomer shows a very strong NOE to the doublet and a particularly weak NOE to the triplet (Fig. 4B). Calculations (using truncated methyl side chains) indicate a significantly shorter difference in distance from the epoxy proton to the two nuclei of interest in the “old” isomer (E, 3.49 versus 4.10, difference 0.61 Å) when compared with the “new” (Z, 3.58 versus 4.59, difference 1.01 Å) (Fig. 8, A and B). Taken together, these NOE studies on both allene oxide isomers provided unequivocal determination of their double bond geometry. With the geometry thus established, the pattern of chemical shifts of the olefin protons, common to all natural allene oxides with the exception of the “new” Z isomer, can now be taken as a reliable diagnostic criterion of the E or Z configuration.

Cyclization of the Natural Allene Oxides—The conclusion that the jasmonate pathway AOC substrate has the E configuration on the epoxy-ene double bond is satisfying and in excellent agreement with cyclization involving backside attack at the epoxide (cf. Fig. 1). However, for the two allene oxides from 9S-HPODE, the results seem counterintuitive; the 10E isomer does not spontaneously cyclize (similar to the 13-HPODE-derived allene oxide in the absence of a homoallylic double bond), whereas the “new” 10Z isomer, apparently in the “wrong” (or unfavorable) configuration, is the one that spontaneously forms a cyclopentenone. Z-Allene oxides cannot possibly cyclize via a concerted mechanism, but after stepwise pathway is available (i.e., ring opening of the epoxide followed by a conrotatory ring closure of the resulting oxyallyl intermediate). In comparison, both concerted and stepwise cyclizations can be operative for the corresponding E-allene oxides, and an ab initio study indicated that both pathways have comparable activation energies (30). The aforementioned difference in cyclization between the 10E and 10Z allene oxides can be rationalized by an attractive postulate that the initial ring-opening step is the rate-determining step and has the lower activation barrier for the 10Z isomer. Full elucidation on cyclization mechanisms must await additional studies.

Acknowledgments—We thank Dr. Gregg A. Howe for the CYP74C plasmid and Drs. Xiaoliang Wang and Zhiquiang Pan for supplying the CYP74A2 plasmid. We thank Dr. M. Wade Calcutt for the LC-MS analysis. Darika Kongrit contributed in the early phases of this study.

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A New Isomer of Fatty Acid Allene Oxides

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