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Tomato CYP74C3 is a Multifunctional Enzyme not only Synthesizing Allene Oxide but also Catalyzing its Hydrolysis and Cyclization

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The mechanism of the recombinant tomato allene oxide synthase (LeAOS3, CYP74C3) was studied. Incubations of linoleic acid (9S)-hydroperoxide with dilute suspensions of LeAOS3 (10–20 s, 0 °C) yield mostly the expected allene oxide (12Z)-9,10-epoxy-10,12-octadecadienoic acid (9,10-EOD), which was detected as its methanol-trapping product. In contrast, the relative yield of 9,10-EOD progressively decreased when the incubations were performed with fourfold, tenfold, or 80-fold larger amounts of LeAOS3, while α-ketol and the cyclopentenone rac-cis-*10-oxo-11-phytoenoic acid (10-oxo-PEA) became the predominant products. Both the α-ketol and 10-oxo-PEA were also produced when LeAOS3 was exposed to preformed 9,10-EOD, which was generated by maize allene oxide synthase (CYP74A). LeAOS3 also converted linoleic acid (13S)-hydroperoxide into the corresponding allene oxide, but with about tenfold lower yield of cyclopentenone. The results indicate that in contrast to the ordinary allene oxide synthases (CYP74A subfamily), LeAOS3 (CYP74C subfamily) is a multifunctional enzyme, catalyzing not only the synthesis, but also the hydrolysis and cyclization of allene oxide.*

Introduction

The lipoxygenase pathway and its products, oxylipins play important physiological roles in aerobic organisms. Allene oxide synthase (AOS, EC 4.2.1.92) is one of the key enzymes of the lipoxygenase pathway in plants and corals.[1–3] The primary products of AOSs are short-lived allene oxides, which are converted in two competing ways: through hydrolysis and cyclization. Cyclization of the allene oxide (9*Z*,11*E*,13*S*,15*Z*)-12,13-epoxy-9,11,15-octadecatrienoic acid (12,13-EOT) occurs both spontaneously and enzymatically.[2,4] The enzyme allene oxide cyclase (AOC, EC 5.3.99.6) controls the stereospecific cyclization of 12,13-EOT into the cyclopentenone (9*S*,13*S*,15*Z*)-12-oxo-10,15-phytodienoic acid (12-oxo-PDA),[1–3] which is a metabolic precursor of the phytohormone 7-*epi*-jasmonic acid.

The majority of plant AOSs that belong to the CYP74A subfamily of cytochromes P450 produce allene oxides,[3] but do not assist in their cyclization. Both spontaneous and AOC-catalyzed cyclization of allene oxides is dependent on the presence of a double bond in the β,γ position to the oxirane ring of allene oxide.[4–6] Thus, allene oxides that are produced from linoleate are hydrolyzed, but not cyclized.[6,7] The recently discovered AOS from the potato stolons and the recombinant tomato AOS (CYP74C subfamily) present an unusual exception.[8,9] These enzymes convert linoleic acid 9-hydroperoxide (9-HPOD) into substantial amounts of the cyclopentenone *cis*-10-oxo-11-phytoenoic acid (10-oxo-PEA) along with α-ketol.[8,9] This phenomenon has not been explained. To address this problem and to obtain new insights into the mechanism of CYP74C subfamily AOSs, we have studied the conversions of 9-HPOD by recombinant tomato AOS (LeAOS3, CYP74C3). We present evidence that LeAOS3 catalyzes not only the synthesis of allene oxide, but also its hydrolysis and cyclization.

Results

Conversion of 9-HPOD by LeAOS3

9-HPOD (100 μg) was incubated with different amounts of LeAOS3 at 0 °C for 10 s, and the products were analyzed as methyl esters/trimethylsilyl (TMS) derivatives by GC–MS (Figure 1). Because the expected primary product of LeAOS3 catalysis is the unstable allene oxide (12*Z*)-9,10-epoxyoctadeca-10,12-dienoic acid (9,10-EOD), the incubation products after a rapid extraction were trapped with methanol. This step efficiently converted 9,10-EOD into the methoxyketone **3a** (see the mass fragmentation of methyl ester **3** in Figure 2B), thus enabling one to monitor the presence of allene oxide.[10]

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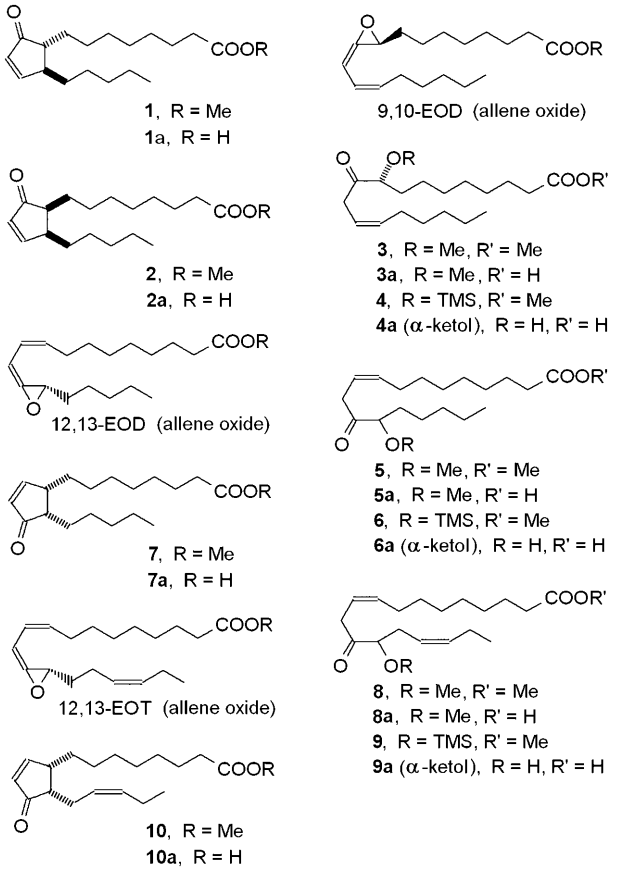


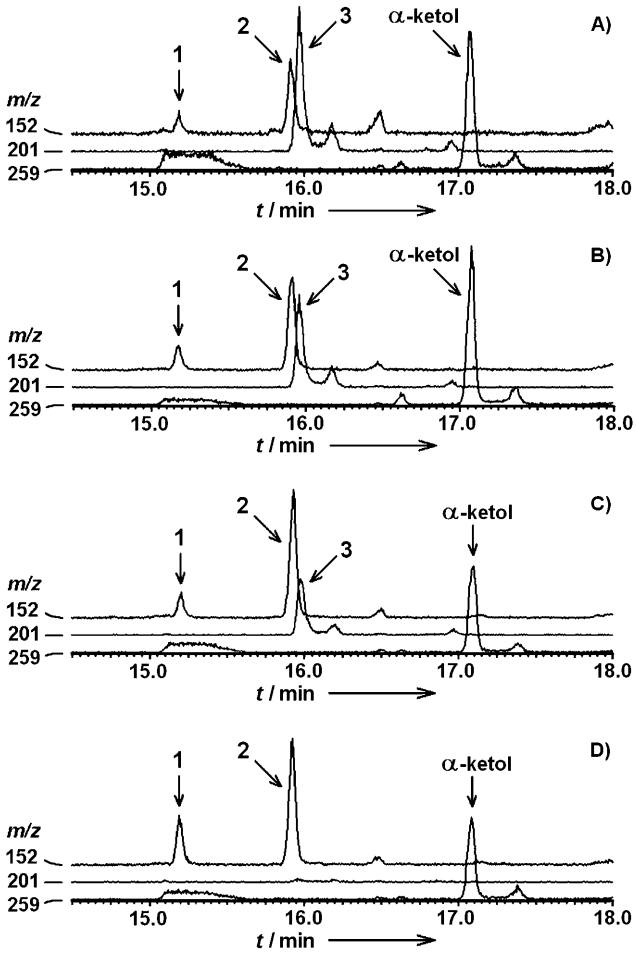


Figure 1. Dependence of 9-HPOD conversion on LeAOS3 concentration. Selected ion GC–MS chromatograms (*m/z* 152, 201 and 259) of methyl esters/TMS derivatives of products formed upon incubations and methanol trapping. 9-HPOD (100 μg) was incubated for 10 s at 0 °C with A) 0.5, B) 2, C) 5, and D) 40 μg, of LeAOS3. See the Experimental Section for more details.

Figure 1 illustrates the analyses data of trapping experiments with different LeAOS3 concentrations, increasing from the top (Figure 1 A) to the bottom (Figure 1 D). As seen in Figure 1 A, the main product detected after incubation of 9-HPOD with a small amount of LeAOS3 (0.5 μg) was trapping product **3 a**, which corresponds to the allene oxide 9,10-EOD. In addition, the α-ketol appeared due to hydrolysis of 9,10-EOD. The mass spectrum of the α-ketol methyl ester trimethylsilyl derivative is presented in Figure 2 C. Along with the trapping product **3 a** and α-ketol, two more products, **1 a** and **2 a** (which were detected as their methyl esters **1** and **2**) were observed (Figure 1 A).

When 9-HPOD was incubated under the same conditions with larger amounts (2, 5 or 40 μg) of LeAOS3, the product patterns changed significantly (Figures 1 B–D, respectively, in comparison to Figure 1 A). The yield of trapping product **3** (corresponding to the remaining allene oxide) progressively decreased. The stepwise disappearance of 9,10-EOD was accompanied by the transient increase of peaks **1** and **2**, as well as the α-ketol peak (Figures 1 B–D in comparison to Figure 1 A). After the incubation with a larger amount of LeAOS3, no trapping product was detected (Figure 1 D). This indicates that all 9-HPOD was quantitatively converted via 9,10-EOD into α-ketol and products **1** and **2** within 20 s. Thus, the observed 9,10-EOD turnover rate in the presence of LeAOS3 is significantly more rapid than one can expect by taking into account the typical rates of spontaneous decomposition of allene oxide (the half life is about 30 s at 0 °C).[8,10]

Compounds **1** and **2** had nearly identical mass spectra, and the mass spectrum of compound **2** and its fragmentation scheme (insert) are presented in Figure 2 A. The spectrum matches well with the previously described data for the methyl ester of 10-oxo-PEA.[8,9,11] Upon mild alkaline treatment, product **2** turned into product **1**; this is in agreement with isomerization of a cyclopentenone **2** (having *cis*-configured side chains) into a *trans*-configured cyclopentenone **1**.[8] On the basis of these experiments and UV spectroscopy data, compounds **1** and **2** were identified as *trans*-10-oxo-PEA and *cis*-10-oxo-PEA, respectively. ^{1}H NMR spectroscopy data (Figure 3 and Supporting Information) were nearly identical to published data for *cis*-10-oxo-PEA,[8] thus confirming the identification of compound **2** as a *cis*-configured 10-oxo-PEA; *cis*-cyclopentenone (**2**) is always the major isomer. It is notable that the cyclopentenone with a *cis* configuration of side chains is an expected product of antarafacial cyclization of allene oxide in accordance with the rules of orbital symmetry conservation.[6,12,13]

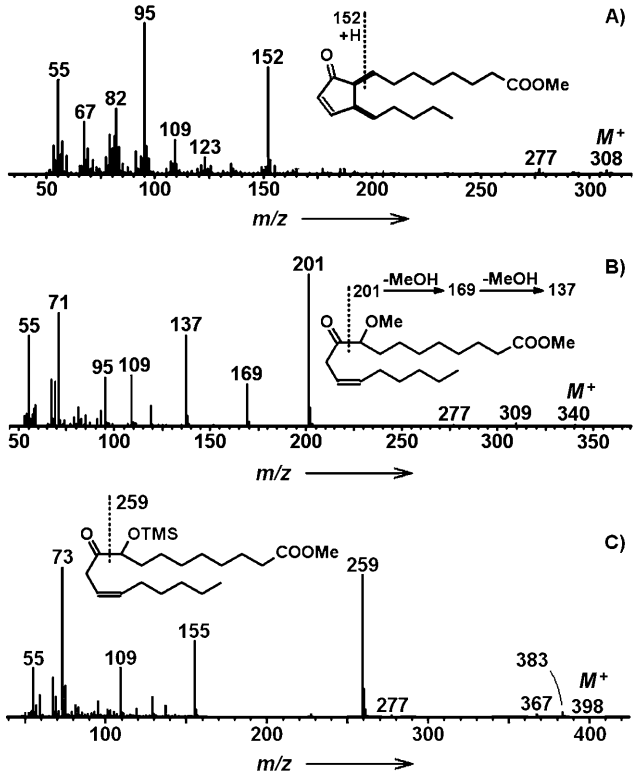


Figure 2. The electron impact mass spectra of compounds A) **2**, B) **3**, C) and **4**.

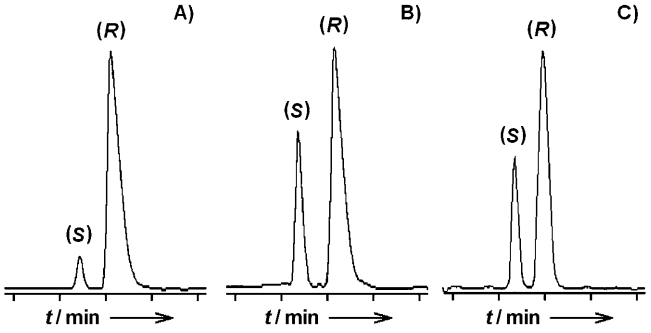
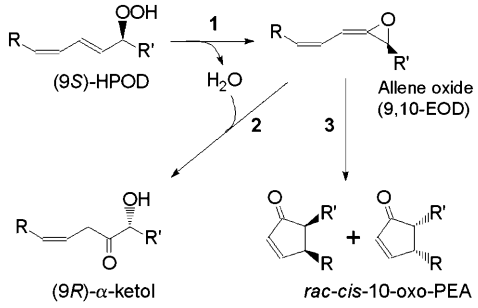


Figure 4. Chiral-phase HPLC analysis of the enantiomeric composition of A) α-ketol **4a** that was synthesized by LeAOS3; B) α-ketol **4a** that was synthesized by maize seed AOS, and C) the methanol-trapping product **3a** that was obtained with LeAOS3. All three products were isolated after incubations of 9-HPOD with the corresponding enzymes, methylated with diazomethane, and purified by reversed-phase and normal-phase HPLC prior to steric HPLC analyses on Chiralcel OB-H column. See the Experimental Section for more details.

Steric analyses of *cis*-10-oxophyto-11-enoic acid (compound 2)

Compound **2**, which was purified by normal-phase HPLC, was subjected to steric analysis on a Chiralcel ODH column (details of analyses are described in the Experimental Section). Unexpectedly, we found that the enantiomers of compound **2** were not separated on this column. Analyses were repeated after the alkaline isomerization of *cis*-disubstituted cyclopentenone **2** into the corresponding *trans* isomer **1**. The results of steric HPLC analyses are presented in Figure 4. The data revealed that the product was fully racemic. Two enantiomers exhibited equal peaks with absorbance maxima at 215 nm (Figure 4). Their identification was confirmed by collection from Chiralcel OD-H column and reanalyses by GC-MS (Scheme 1).

For final approval of steric analysis, a racemic mixture of the 9*R*,13*S* and 9*S*,13*R* enantiomers of 10-oxophytonoic acid was synthesized by starting with commercially available methyl (±)-jasmonate as outlined in Scheme 1. This racemate (a reference standard) and compound **1** (obtained by alkaline isomerization of compound **2**) were reduced with sodium borohydride, and the resulting cyclopentanols (methyl esters) were converted into (−)-menthoxycarbonyl derivatives. Analyses of these derivatives by GC–MS (see the Supporting Information) demonstrated that the synthetic reference and the biologically derived 10-oxophytoenoic acid both provided a pair of fully racemic 10-hydrophytonoates. On



Scheme 1. The proposed scheme of the LeAOS3 (CYP74C3) reaction mechanism. See explanations in the text. R = $(CH_2)_7CO_2Me$; R′ = $(CH_2)_4Me$.

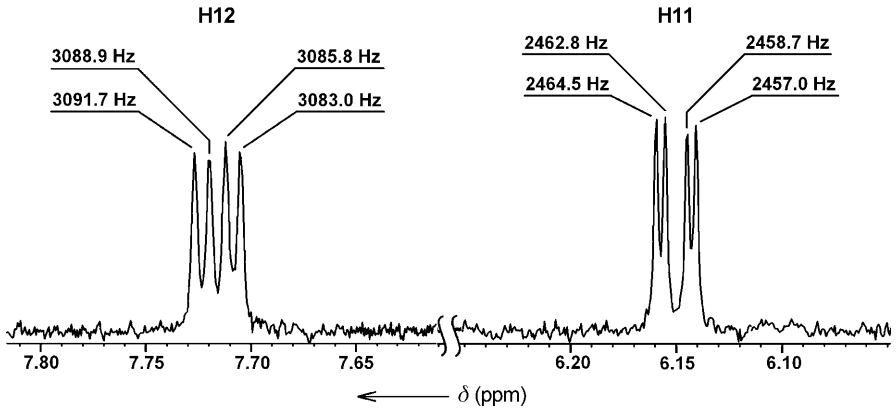


Figure 3. The olefinic region of ^1H NMR spectrum (400 MHz, $CDCl_3$, 296 K) of compound **2**. More detailed data are presented in the Supporting Information.

the basis of this result it was concluded that the 10-oxo-PEA that was generated from 9-HPOD in the presence of LeAOS3 was a racemic mixture of the 9*S*,13*S*- and 9*R*,13*R* enantiomers. This result is consistent with previously published properties of 10-oxo-PEA, which was synthesized by potato CYP74C3.[8]

Steric analyses of α-ketol (4a) and the methanol trapping product 3a

α-Ketol (**4a**) was isolated and purified by normal-phase HPLC after the incubation of LeAOS3 with 9-HPOD. Pure compound **4a** was subjected to steric analysis by chiral-phase HPLC (Figure 5A). The same α-ketol **4a** that was formed by maize AOS (ZmAOS, CYP74A) was used as a reference for steric analyses (Figure 5B). The obtained data (Figure 5A) demonstrate that the α-ketol synthesized by LeAOS3 came predominantly from the 9*R* enantiomer (92%). α-Ketol produced by ZmAOS possessed significantly lower optical purity; the 9*R* and 9*S* enantiomers were present in a ratio of 62:38 (Figure 5B). As found before, potato StAOS3, an enzyme that is closely related to LeAOS3, also produces predominantly (9*R*)-α-ketol.[8] Predominant formation of (9*R*)-α-ketol in the presence of LeAOS3 and StAOS3 indicates that allene oxide 9,10-EOD, the primary product of these enzymes, is hydrolyzed mostly by the S_N2 mechanism. Unlike the hydrolysis, methanolysis of allene oxide is not stereospecific (Figure 5C). The ratio of 9*R* and 9*S* enantiomers of the trapping product **3a** (Figure 5C) and α-ketol **4a**, which were formed by maize AOS (Figure 5B) was nearly identical; this suggests a nearly equal contribution of the S_N2 and S_N1 substitution mechanisms to 9,10-EOD methanolysis and hydrolysis, respectively.

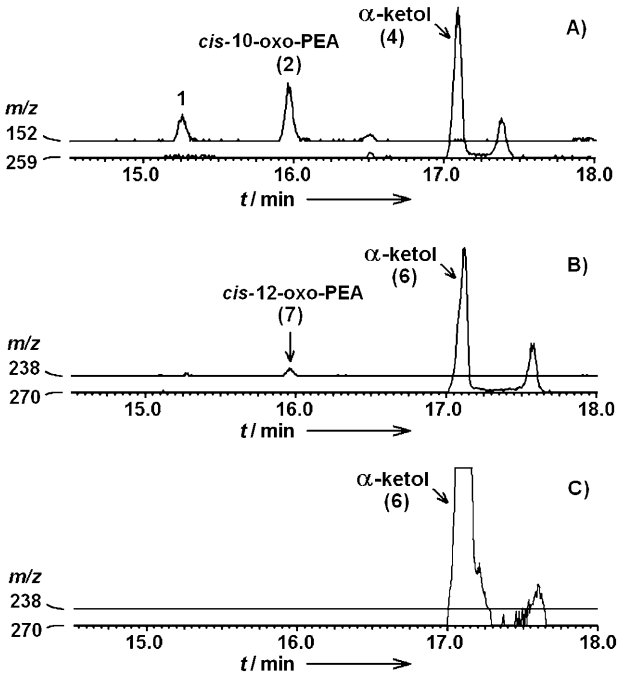


Figure 5. Products of A) 9-HPOD and B) 13-HPOD conversion by LeAOS3, and C) 13-HPOD conversion by flaxseed AOS (CYP74A). Selected ion GC–MS chromatograms of products as their methyl ester TMS derivatives. Hydroperoxides were incubated with LeAOS3 or flaxseed AOS for 20 s at 0 °C, pH 7.0, followed by extraction and derivatization. Intensities of selected ion chromatograms in (C) are increased by fourfold compared to the normalized α-ketol peak. See the Experimental Section for more details.

Conversion of 13-HPOD by LeAOS3

In agreement with the data of Itoh et al.,[9] we found that LeAOS3 was also active towards 13-HPOD (Figure 6). However, the conversion of 13-HPOD afforded predominantly α-ketol (Figure 6B). The relative yield of cyclopentenone (in relation to α-ketol) was about ten times smaller than in the case of 9-HPOD conversion. The main isomer of cyclopentenone (Figure 6B) that was formed from 13-HPOD was identified as *cis*-12-oxo-PEA (data not presented). When 13-HPOD was incubated with flaxseed AOS (CYP74A), no cyclopentenone formation was detectable (Figure 6C).

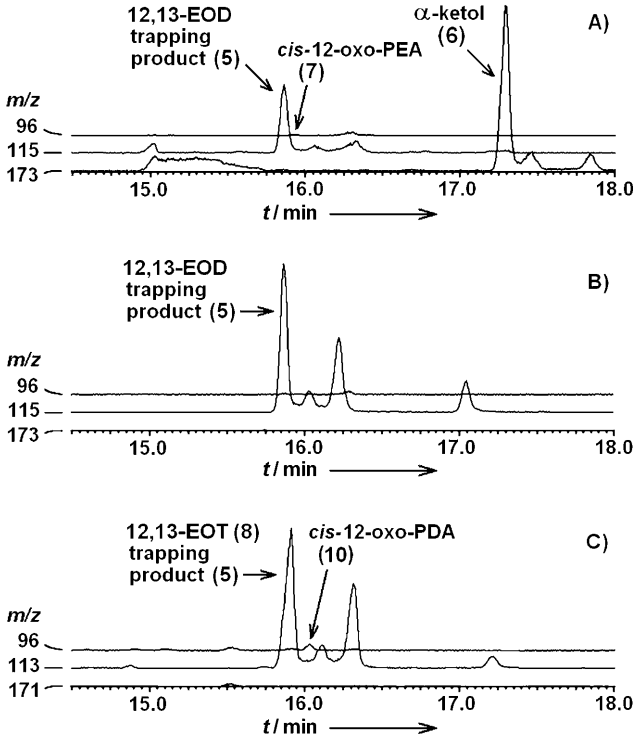


Figure 6. The methanol trapping of incubation products of LeAOS3 and ZmAOS with 9- and 13-hydroperoxides. Selected ion GC–MS chromatograms of product derivatives. The 20 s incubations were performed at 0 °C, followed by rapid extraction, methanol trapping, and derivatization (conversion into methyl esters TMS derivatives). A) products of LeAOS3 incubation with 13-HPOD, B) products of ZmAOS incubation with 13-HPOD, C) products of ZmAOS incubation with 13-HPOT. See the Experimental Section for more details.

Methanol trapping of allene oxides synthesized during the incubations of 13-hydroperoxides with LeAOS3 (CYP74C) and maize AOS (CYP74A)

A series of methanol-trapping experiments were performed for further characterization of LeAOS3 specificity of action and its

comparison to AOSs of the CYP74A subfamily. Incubation of LeAOS3 with 13-HPOD resulted in trapping product patterns (Figure 7A) that were similar to those observed after LeAOS3 incubation with 9-HPOD (Figure 1A). The allene oxide (12,13-EOD) trapping product **5** was detected along with α-ketol **8** (Figure 7A; their mass spectral data are presented in the Supporting Information).

At the same time, 13-HPOD conversion afforded some amount of cyclopentenone **7** (Figure 7A), but significantly less than in the case of 9-HPOD conversion (products **2** and **1**, Figure 1A). When 13-HPOD was incubated for 20 s with ZmAOS, only the methanol-trapping product was detectable; neither the cyclopentenone nor α-ketol were observed. (Figure 7B). This indicates that ZmAOS generated 12,13-EOD, but this allene oxide did not undergo hydrolysis or cyclization within 20 s of incubation.

Because 12,13-EOT is capable of cyclizing spontaneously, it was interesting to reveal how much of its cyclization product is formed under the trapping conditions, but we observed poor substrate conversion when LeAOS3 was incubated with 13-HPOT (result not illustrated). In contrast, ZmAOS rapidly converted 13-HPOT into 12,13-EOT, as seen from its trapping product **8** appearance (Figure 7C). However, even in this case, the extent of allene oxide cyclization into 12-oxo-PDA (**10**) within 20 s of incubation was not substantial (Figure 7C). Thus, the rate of spontaneous cyclization of 12,13-EOT (generated by ZmAOS, Figure 7C) was considerably smaller than the rate of 9,10-EOD cyclization in the presence of LeAOS3 (Figure 1A). These results suggest that unlike CYP74A subfamily AOSs, LeAOS3 catalyzes both cyclization and hydrolysis of 9,10-EOD.

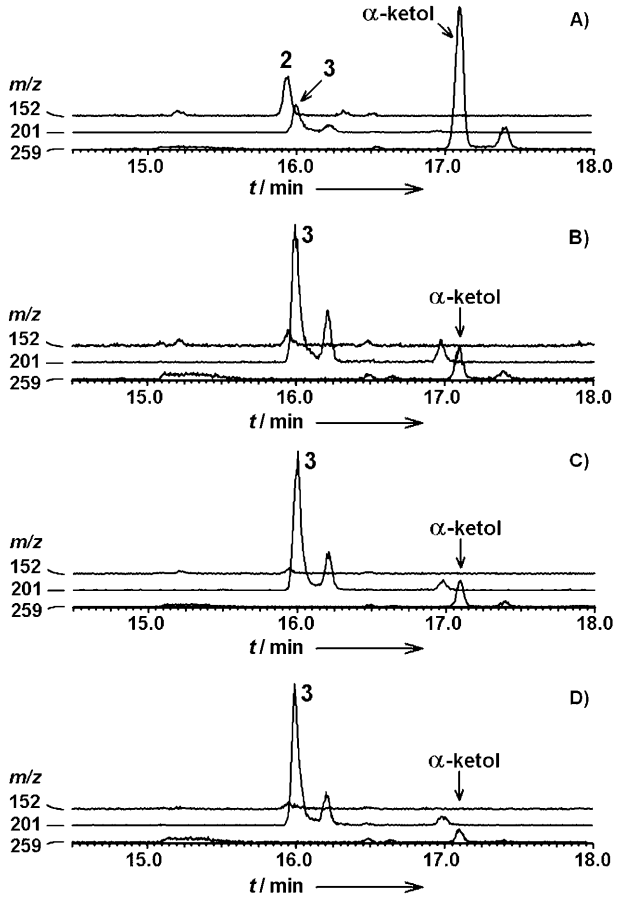


Figure 7. Generation of allene oxide (9,10-EOD) by ZmAOS and its conversion after in situ addition of LeAOS3. Selected ion GC–MS chromatograms (*m/z* 152, 201, and 259) of methyl esters/TMS derivatives of incubations and methanol-trapping products. Experiments A–C included a 10 s preincubation of ZmAOS with 100 μg of 9-HPOD, followed by 10 s incubation after the in situ addition of A) active LeAOS3, B) or boiled LeAOS3, C) or the control protein preparation from *E. coli* cells that harbored the empty expression vector. D) 10 s incubation of ZmAOS with 9-HPOD. See the Experimental Section for more details.

Generation of allene oxide by maize AOS (CYP74A) and its further conversion after the in situ addition of LeAOS3

The observed dependence of the allene oxide (9,10-EOD) lifetime on the LeAOS3 concentration (Figure 1) suggests that LeAOS3 controls not only the formation of allene oxide, but its hydrolysis and cyclization as well. Additional experiments were performed to substantiate this assumption. These experiments included the generation of 9,10-EOD by a 10 s incubation of 9-HPOD with ZmAOS (CYP74A) followed by the in situ addition of LeAOS3 and incubation for additional 10 s. Incubations were terminated by extraction, methanol trapping and derivatization, as described above. Allene oxide (detected as the methanol trapping product **3**) was the predominant product that was formed by ZmAOS in 10 s (Figure 8D), whereas 9-HPOD conversion was nearly complete. Continued incubation after LeAOS3 addition resulted in the conversion of allene oxide into 10-oxo-PEA, mostly the *cis* (**2**) one and α-ketol (Figure 8A). To confirm the dependence of 9,10-EOD hydrolysis and cyclization on LeAOS3, two control experiments were performed: one with boiled LeAOS3 and another with a protein preparation from *E. coli* cells that harbored the empty expression vector pET23-b. In both of these controls allene oxide (detected as trapping product **3**) remained unrecovered (Figure 8B and C, respectively). These results demonstrate that both hydrolysis and cyclization of 9,10-EOD depend on the presence of native LeAOS3.

Discussion

The discovery of the CYP74C subfamily AOSs revealed a new important facet of the plant lipoxygenase pathway.[8,9] The intriguing capability of CYP74C subfamily AOSs to produce 10-oxo-PEA distinguishes them from the majority of AOSs (CYP74A), which are unable to produce cyclopentenones from linoleic acid hydroperoxides.[8,9] This capability has not yet been explained.

As mentioned in the Introduction, the majority of AOSs (CYP74A subfamily) have a single function: synthesis of unstable allene oxide, which is further converted (hydrolyzed or cyclized) spontaneously.[1–3] Half lives of allene oxides (27–44 s at 0 °C, pH 7.0) in the presence of CYP74A type AOSs do not depend on the AOS concentration.[3,10]

In full agreement with literature data,[8,9] the results of trapping experiments demonstrate that a short-lived allene oxide is a primary product produced and liberated by LeAOS3. Thus,

the synthesis of allene oxides is the common function of AOSs of both CYP74A and CYP74C subfamilies. At the same time, the fate of allene oxide in the presence of LeAOS3 is dramatically different in a few respects. First, allene oxide half life is strongly dependent on the LeAOS3 concentration. The larger the LeAOS3 concentration, the shorter is the lifetime of 9,10-EOD. Second, 9,10-EOD is transiently converted into 10-oxo-PEA and α-ketol. Third, the α-ketol is formed enantiospecifically (9*R*). Finally, when the LeAOS3 concentration achieves a saturated value, 9-HPOD conversion via 9,10-EOD into 10-oxo-PEA and α-ketol is complete within 20 s at 0 °C, pH 7.0.

The obtained results enable us to propose a mechanistic scheme of LeAOS3 action (Scheme 1). The data demonstrate that LeAOS3 is a multifunctional enzyme that catalyzes three conversions. The first is the synthesis of allene oxide 9,10-EOD (Scheme 1, reaction 1), which is the primary product that is synthesized and liberated by LeAOS3. Then LeAOS3 recaptures 9,10-EOD to catalyze two subsequent competing conversions, namely the hydrolysis and cyclization (Scheme 3, reactions 2 and 3, respectively). The hydrolysis, but not the cyclization, occurs stereospecifically. The final products of LeAOS3 are (9*R*)-α-ketol **4a** and the racemic *cis*-10-oxo-PEA **2a** (Scheme 3).

LeAOS3, as well as other CYP74C subfamily enzymes, is active towards both 9- and 13-hydroperoxides (except 13-HPOT). We found that LeAOS3 efficiently converts both 9-HPOD and 13-HPOD into the corresponding allene oxides and α-ketols. On the other hand, the cyclase activity of LeAOS3 possesses regiospecificity. 12,13-EOD is significantly less efficient as a substrate for cyclopentenone production by LeAOS3 than 9,10-EOD.

The earlier-described enzyme AOC also catalyzes allene oxide cyclization.[4,14–17] However, AOC is clearly distinct from LeAOS3 in several respects: 1) unlike LeAOS3, AOC is not a P450 protein; 2) AOC does not possess AOS activity; 3) AOC specifically utilizes only 12,13-EOT, but not 9,10-EOT, or allene oxides formed from linoleic acid; 4) unlike LeAOS3, AOC acts stereospecifically.

LeAOS3 and the related potato AOS (StAOS3, CYP74C) control a new route from linoleic acid to cyclopentenones. LeAOS3 is specifically expressed in tomato roots.[9] StAOS3 has recently been cloned and the specific expression of StAOS3 gene was observed in the underground organs of potato, including the sprouting eyes of tubers and roots.[18] The closely related gene CYP74C9 is specifically expressed in aging *Petunia inflata* petals.[19] Although the corresponding *Petunia* CYP74C9 protein has not yet been characterized biochemically, its phylogeny enables one to attribute it to the AOSs of CYP74C subfamily. Taken together these data indicate that the AOSs of CYP74C subfamily might play specific physiological roles in some plant tissues.[8,9,18,19] Recently we detected a similar enzyme that uses 13-HPOD and affords a high yield of *cis*-12-oxo-10-phytoenoic acid in sunflower roots.[21]

Experimental Section

Materials: Linoleic and α-linolenic acids, as well as soybean lipoxygenase type V were purchased from Sigma. Silylating reagents were purchased from Fluka (Buchs, Switzerland). 9-HPOD was prepared by incubation of linoleic acid with tomato fruit lipoxygenase at 0 °C, pH 6.0, under continuous oxygen bubbling. 13-HPOD and 13-HPOT were obtained by incubations of linoleic and α-linolenic acids, respectively, with soybean lipoxygenase type V as described before.[6] All hydroperoxides were purified by normal-phase HPLC.

Expression of recombinant LeAOS3 (CYP743): The vector for expression of LeAOS3 was a generous gift from Dr. Gregg A. Howe. Expression and enzyme preparation was performed as described before.[9]

Purification of recombinant LeAOS3: The recombinant LeAOS3 was purified by immobilized metal affinity chromatography IMAC). All the purification procedures were performed at 4 °C. The crude *E. coli* lysate (1 mL) was added to TALON CellThru cobalt-based IMAC resin (0.5 mL, Clontech, Mountain View, CA, USA) that had been pre-equilibrated with buffer A (50 mM sodium phosphate, 0.3 M NaCl, 1.56 mM Emulphogene, pH 7.0). Then the suspension was gently agitated for 15 min. The resin was applied to a column (1×10 cm) and washed with buffer A (4×10 mL). LeAOS3 was eluted with buffer A (0.5 mL) that contained 150 mM imidazole. The eluate was diluted tenfold with 50 mM sodium phosphate buffer (pH 7.0). Imidazole was removed by ultrafiltration by using Amicon Ultra 30 kDa molecular mass cutoff centrifugal filter devices (Millipore). The relative purity of recombinant LeAOS3 was estimated by SDS-polyacrylamide gel electrophoresis and staining of gels with Coomassie brilliant blue R-250. Protein concentration was estimated as described previously.[9]

Incubations of recombinant LeOAS3 with 9-HPOD: The reaction was initiated by the injection of a solution of (9*S*)-HPOD (100 μg) in EtOH (10 μL) into suspensions of LeAOS3 (0.5, 2, 5, or 40 μg of purified protein) in 100 mM phosphate buffer (1 mL), pH 7.0, at 0 °C. The mixture was vigorously vortexed for 10–20 s and rapidly extracted with cold hexane (1.5 mL). The hexane solution was cooled to about −20 °C, concentrated in vacuo by about twofold, and treated with ethereal diazomethane at −20 °C for 3 min. The solvent was evaporated in vacuo and ice-cold MeOH (3 mL) was added to the dry residue. After 30 min at 23 °C, solvent was evaporated and the dry residue was treated with a trimethylsilylating mixture as described before.[7] Control experiments were performed in the same way with protein preparation from *E. coli* cells that had been transformed with an empty pET23-b vector that carried no LeAOS3 cDNA.

Preparation of maize and flax allene oxide synthases: Preparation of ZmAOS and flax AOS, as well as fatty acid hydroperoxide incubations with these enzymes were performed as described before.[11]

Synthesis of allene oxide by maize allene oxide synthase (CYP74A) and its further conversion after in situ addition of LeAOS3: For preparation of ZmAOS (CYP74A) maize seed acetone powder (6 g) was extracted with phosphate buffer (20 mL), pH 7.0 at 0 °C for 40 min followed by centrifugation at 15000 *g* for 5 min. The supernatant (1 mL) was incubated with 9-HPOD (100 μg) for 10 s at 0 °C under the vigorous vortexing. Then these incubations were continued for 10 s longer after the addition of either a) active LeAOS3 (20 μg), b) the same amount of boiled LeAOS3, c) the equivalent amount of protein preparation from *E. coli* cells that harbored the empty expression vector pET23-b. In separate control incubations (d) 10 s incubation of 9-HPOD with ZmAOS was terminated directly with no additions. All incubations were terminated by extraction, MeOH trapping and derivatization, as described above.

Micropreparative incubations: LeAOS3 preparation (20 μg of protein) was incubated with 9-HPOD (200 μg) at 23 °C and pH 7.0 for 5 min and the products were extracted with hexane and methylated with diazomethane. 12-Oxo-PDA was prepared as described previously.[6]

Chromatographic analysis of products: Products were separated as methyl esters by RP-HPLC on Macherey–Nagel Nucleosil 5 ODS column (250×4.6 mm) that was eluted with MeOH/H_2O (linear gradient from 76:24 to 96:4, *v*/*v*) at a flow rate of 0.4 mL min^{-1}. Products were collected and re-chromatographed by NP-HPLC on two serially connected Separon SIX columns (150×3.2 mm; 5 μm) eluted with hexane/iPrOH (98.2:1.8, *v*/*v*), flow rate 0.4 mL min^{-1}. Enantiomers of purified *cis*-10-oxo-PEA acid methyl ester were separated on Chiralcel OD-H column (250×0.46 mm, 5 μm) with hexane/iPrOH 98:2 (*v*/*v*), flow rate 0.4 mL min^{-1}. Alternatively, *cis*-10-oxo-PEA methyl ester (**2**) was reduced with $NaBH_4$ and the resulting cyclopentanols were converted to the (−)-menthoxycarbonyl derivatives as described previously.[8] These derivatives were separated by GC–MS.

Synthesis of 10-oxophytonoic acid: The synthetic scheme that was used was similar to that previously described for the 12-oxophytonoic acid synthesis.[22] A racemic mixture of the 9*R*,13*S* and 9*S*,13*R* enantiomers of 10-oxophytonoic acid was synthesized by starting with commercially available methyl (±)-jasmonate as outlined in Scheme 1. The sequence involved $NaBH_4$ reduction of methyl (±)-jasmonate (1 g) into diastereomeric cyclopentanols, cleavage of the Δ^9 double bond by oxidative ozonolysis, and anodic coupling with *tert*-butyl hydrogen suberate. Selective removal of the methyl ester protecting group by mild alkaline hydrolysis followed by anodic coupling with pentanoic acid, removal of the *tert*-butyl protecting group by treatment with trifluoroacetic acid in CH_2Cl_2, and oxidation of the ring alcohol group by using Dess–Martin periodinane afforded the desired 10-oxophytonoic acid in a yield of 20%.

Steric analysis of 10-oxophytoenoic and 10-oxophytonoic acids: 10-Oxo-PEA (90 μg) was obtained by incubation of 9-HPOD with LeAOS3 was epimerized into the side-chain *trans* compound by treatment with 0.1 M NaOH in 90% aq MeOH at 23 °C for 30 min and subsequently methyl esterified. The resulting material, as well as the methyl ester of the above-mentioned synthetic 10-oxophytonoic acid, were separately reduced with $NaBH_4$ into diastereomeric methyl 10-hydroxyphytonoates. Analysis of the corresponding (−)-menthoxycarbonyl derivatives was performed by GC–MS by using a capillary column of 5% phenylmethylsiloxane (12 m, 0.33 μm film thickness, carrier gas, helium) as described before.[22]

Steric analyses of α-ketols and the methanol trapping product: Samples of α-ketols **4a** that were obtained after incubations of 9-HPOD with LeAOS3 and ZmAOS were esterified with diazomethane and purified by reversed-phase and normal-phase HPLC as described above. Pure α-ketol methyl ester samples were subjected to steric analyses on Chiralcel OB-H column (250×0.46 mm, 5 μm), solvent hexane/iPrOH, 94:6 (*v*/*v*), flow rate 0.5 mL min^{-1}.

The methanol-trapping product **3a** was prepared by incubation of LeAOS3 (5 μg of protein) with [1-^{14}C]9-HPOD (37 Bq, 20.7 KBq μmol^{-1}) in phosphate buffer (15 mL), pH 7.0 at 0 °C for 20 s. The reaction was stopped by the addition of ice-cold MeOH (200 mL) under the vigorous stirring and allowed to stand for 20 min at 0 °C. Then most of the MeOH was evacuated in vacuo, and the rest was extracted with hexane/ethyl acetate 1:1 (*v*/*v*). The extracted products were methylated with diazomethane and separated by the RP-HPLC as described above. The methanol-trapping product methyl ester **3** was finally purified by NP-HPLC generally as described above, but under elution by solvent mixture hexane/iPrOH 99:1 (*v*/*v*), flow rate 0.4 mL min^{-1}. The steric analysis of compound **3** was performed by using a Chiralcel OB-H column (250×0.46 mm, 5 μm), hexane/iPrOH 94:6 (by volume), and a flow rate of 0.5 mL min^{-1}.

Spectral studies: The UV spectra of compounds that were purified by HPLC were recorded on-line by using an SPD-M20 A diode array detector (Shimadzu). Alternatively, the UV spectra were recorded off-line with a Perkin–Elmer Lambda 25 spectrophotometer. Products were analyzed as methyl esters or methyl esters/TMS derivatives by GC–MS as described before.[20] GC–MS analyses were performed by using a Shimadzu QP5050A mass spectrometer that was connected to Shimadzu GC-17A gas chromatograph that was equipped with an MDN-5S (5% phenyl 95% methylpolysiloxane) fused capillary column (length, 30 m; ID 0.25 mm; film thickness, 0.25 μm). Helium at a flow rate of 30 cm/s was used as the carrier gas. Injections were made in the split-mode by using an initial column temperature of 120 °C. The temperature was raised at 10 °C min^{-1} until 240 °C. Full-scan or selected-ion monitoring (SIM) analyses were both performed by using the electron impact ionization (70 eV). The ^{1}H NMR and 2D-COSY spectra were recorded with a Bruker Avance 400 instrument (400 MHz, $CDCl_3$, 296 K). The homonuclear ^{1}H double resonance experiments were performed with the same instrument.

Abbreviations

AOS, allene oxide synthase; LeAOS, tomato allene oxide synthase; ZmAOS, maize allene oxide synthase; AOC, allene oxide cyclase; 9-HPOD, (9*S*,10*E*,12*Z*)-9-hydroperoxyoctadeca-10,12-dienoic acid; 13-HPOD, (9*Z*,11*E*,13*S*)-13-hydroperoxyoctadeca-9,11-dienoic acid; 9,10-EOD, (12*Z*)-9,10-epoxyoctadeca-10,12-dienoic acid; 12,13-EOD, (9*Z*)-12,13-epoxyoctadeca-9,11-dienoic acid; 12,13-EOT, (9*Z*,11*E*,13*S*,15*Z*)-12,13-epoxyoctadeca-9,11,15-trienoic acid; 10-oxo-PEA, 10-oxo-11-phytoenoic acid; 12-oxo-PDA, (15*Z*)-12-oxophyto-10,15-dienoic acid; 12-oxo-PEA, 12-oxo-10-phytoenoic acid; TMS, trimethylsilyl; IMAC, immobilized metal affinity chromatography.

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