A new class of fatty acid allene oxide formed by the DOX-P450 fusion proteins of human and plant pathogenic fungi, C. immitis and Z. tritici

Ernst H. Oliw,1 Marc Aragó, Yang Chen, and Fredrik Jernerén

Division of Biochemical Pharmacology, Department of Pharmaceutical Biosciences, Uppsala University, SE-751 24 Uppsala, Sweden

Abstract Linoleate dioxygenase-cytochrome P450 (DOX-CYP) fusion enzymes are common in pathogenic fungi. The DOX domains form hydroperoxy metabolites of 18:2n-6, which can be converted by the CYP domains to 1,2- or 1,4-diols, epoxy alcohols, or to allene oxides. We have characterized two novel allene oxide synthases (AOSs), namely, recombinant 8R-DOX-AOS of Coccidioides immitis (causing valley fever) and 8S-DOX-AOS of Zymoseptoria tritici (causing septoria tritici blotch of wheat). The 8S-DOX-AOS oxidized 18:2n-6 sequentially to 8R-hydroperoxy-9Z,12Z-Octadecadienoic acid (8R-HPODE) and to an allene oxide, 8R(9)-epoxy-9,12Z-Octadecadienoic acid, as judged from the accumulation of the α-ketol, 8S-hydroxy-9-oxo-12Z-Octadecenoic acid. The 8S-DOX-AOS of Z. tritici transformed 18:2n-6 sequentially to 8S-HPODE and to an α-ketol, 8R-hydroxy-9-oxo-12Z-Octadecenoic acid, likely formed by hydrolysis of 8S(9)-epoxy-9,12Z-Octadecadienoic acid. The 8S-DOX-AOS oxidized [8R-3H]18:2n-6 to 8S-HPODE with retention of the 3H-label, suggesting suprafacial hydrogen abstraction and oxygenation in contrast to 8R-DOX-AOS. Both enzymes oxidized 18:1n-9 and 18:3n-3 to α-ketols, but the catalysis of the 8R- and 8S-DOX-AOS domains differed. 8R-DOX-AOS transformed 9R-HPODE to epoxy alcohols, but 8S-DOX-AOS converted 9S-HPODE to an α-ketol (9-hydroxy-10-oxo-12Z-Octadecenoic acid) and epoxy alcohols in a ratio of ~1:2. Whereas all fatty acid allene oxides described so far have a conjugated diene impinging on the epoxide, the allene oxides formed by 8-DOX-AOS are unconjugated.—Oliw, E. H., M. Aragó, Y. Chen, and F. Jernerén. A new class of fatty acid allene oxide formed by the DOX-P450 fusion proteins of human and plant pathogenic fungi, C. immitis and Z. tritici. J. Lipid Res. 2016. 57: 1518–1528.

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Eicosanoids in humans and oxylipins in plants and fungi designate oxygenated unsaturated C20 and C18 fatty acids and many of them exert potent biological actions (1–3). Fungal oxylipins can be formed by dioxygenation of C18 fatty acids by two groups of enzymes, lipoygenases (LOXs) and heme-containing dioxygenases (DOXs) (2). The LOXs contain catalytic Fe or Mn, and oxidize unsaturated fatty acids to hydroperoxides by hydrogen abstraction at bisallylic positions (4–8). The heme-containing DOXs belong to the cyclooxygenase (COX) gene family (9). They can oxidize fatty acids at allylic as well as bisallylic positions due to hydrogen abstraction by a tyrosyl radical (10, 11).

The first characterized fungal DOX related to COX was 7,8-linoleate diol synthase (LDS) (12, 13). The 7,8-LDS is a fusion protein with an 8R-DOX domain and a C-terminal cytochrome P450 (CYP) domain with the 7,8-LDS activities (14). This enzyme and the related 5,8- and 8,11-LDSs can be collectively labeled 8R-DOX-LDS for simplicity. There are now five additional characterized groups of enzymes with sequence homology to 8R-DOX-LDS. They usually align with over 60% amino acid sequence identities within each group. The transformation of 18:2n-6 by all eight enzymes is outlined in Fig. 1A. The DOX domains of 8R-, 9R-, 9S-, and 10R-hydroperoxy metabolites of 18:2n-6 and 18:3n-3 (14–17). The C-terminal CYP domains can transform these hydroperoxides by heterolytic cleavage leading to intramolecular hydroxylation at C-7, C-5, or C-11 with formation of 1,2- or 1,4-diols by 8R-DOX-LDS or epoxidation of the n-6 double bond by 10R-DOX-epoxy alcohol synthases (10R-DOX-EASs). The 9R- and 9S-hydroperoxides...
of 18:2\(\text{n-6}\) can also be subject to homolytic cleavage and dehydration to allene oxides by allene oxide synthases (AOSs) (9\(R\) and 9\(S\)-DOX-AOS) (Fig. 1A). AOSs of plants belong to the CYP74 family, but the fungal AOSs form separate CYP families (17, 18). The 8\(R\)-DOX-LDS is often expressed by mycelia in laboratory cultures of many strains, whereas other DOX-CYP may be expressed by certain strains or only in response to specific environmental stimuli (1, 19).

The DOX-CYP enzymes can also be classified from the position of hydrogen abstraction of linoleic acid: C-8 by 8\(R\)- and 10\(R\)-DOX and C-11 by 9-DOX (12, 15, 17). The general theme is antarafacial hydrogen abstraction and oxygen insertion in analogy with COX, but there are two exceptions: 9\(R\)-DOX and 9\(R\)-DOX-AOS (17, 18, 20).  

Coccidioides immitis causes valley fever in Western USA with occasionally lethal outcomes (21). Zymoseptoria tritici (teleomorph Mycosphaerella graminicola) causes the most important disease of wheat, septoria tritici blotch (22, 23). Little is known about the DOX-CYP enzymes of these important pathogens. Fungal oxylipins may take part as secondary metabolites in sporulation, the infectious processes, and in biotrophic and necrotic growth (1, 2). It therefore seemed of interest to determine whether C. immitis and Z. tritici might code for known or unique enzymes with homology to 8\(R\)-DOX-LDS and related enzymes. C. immitis and Z. tritici code for three DOX-CYP fusion enzymes each. These tentative proteins can be aligned with characterized DOX-CYP, as shown by the phylogenetic tree in Fig. 1B. The alignment suggests that EGP91582 and EAS36125 are likely related to 8\(R\)-DOX-LDS. EAS34688 is connected to 10\(R\)-DOX-(CYP), but EAS34688 retains the heme-thiolate cysteine in the CYP domain in contrast to 10\(R\)-DOX-(CYP) and might therefore be catalytically related to the nearby 10\(R\)-DOX-EAS subfamily (see Fig. 1B).

The catalytic properties of the three remaining enzymes are more difficult to deduce. EAS28473 and EGP83657 appear to be only distantly related to the known DOX-CYP subfamilies, but they can be aligned with characterized DOX-CYP, as shown by the phylogenetic tree in Fig. 1B. The alignment suggests that EGP91582 and EAS36125 are likely related to 8\(R\)-DOX-LDS. EAS34688 is connected to 10\(R\)-DOX-(CYP), but EAS34688 retains the heme-thiolate cysteine in the CYP domain in contrast to 10\(R\)-DOX-(CYP) and might therefore be catalytically related to the nearby 10\(R\)-DOX-EAS subfamily (see Fig. 1B). The catalytic properties of the three remaining enzymes are more difficult to deduce.

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enzymes, at least one separate subfamily (Fig. 1B). Second, the sequence information deduced from the DOX and CYP domains did not allow any unambiguous conclusions on their dual catalytic activities. Third, C. immitis and Z. tritici are important pathogens, and characterization of novel enzymes might provide important information for future biological studies (1, 2). Work with mycelia of C. immitis has caused fatal infections in laboratory personnel (21), but we assessed the oxidation of fatty acids by mycelia of Z. tritici.

**EXPERIMENTAL PROCEDURES**

**Materials**

Fatty acids were dissolved in ethanol and stored in stock solutions (35–100 mM) at −20°C. The 18:2n-6 (99%), 18:3n-6 (99%), and 18:5n-3 (99%) were from VWR, and 18:1n-9, 20:2n-6, 20:4n-6, and [13C18]18:2n-6 (98%) were from Larodan (Malmö, Sweden). The [115S3H]18:2n-6 (>99% 3H) and [8R6H]18:2n-6 (65% 2H) were prepared by Dr. Hamberg as described (24). The 9S-hydroperoxyoctadecatrienoic acid (HPOTrE) and 9E, 9S, 13R-, and 13S-hydroperoxycytadecanoid acids (HPODEs) were prepared by potato and tomato LOX and by 13E/MnLOX (17). The 8S, 8R-, and 12S-HETE were from Cayman. The [13C6]8R-HPODE was prepared with the 8S-DOX domain of 7,8-LDS (14). The [13C6]8R/HPODE (>95% 13C) was prepared in the same way by incubation under 13C2O2. The labeled 8R/HPODE was purified by reversed-phase (RP)-HPLC. Chemically competent Escherichia coli (NEB5a) were from New England BioLabs. Champion pET101D Directional TOPO kit was from Invitrogen. Restriction enzymes and the gel extraction kit were from Fermentas. Sequencing was performed at Lasergene Genome Center. Restriction enzymes and the gel extraction kit were from Fermentas. Sequencing was performed at Lasergene Genome Center.

**LC-MS analysis**

Recombinant proteins of the crude cell lysate [in 0.05 M Tris-HCl (pH 7.6)/5 mM EDTA/10% glycerol or 0.05 M KHPO4 (pH 7.5)/0.5 M NaCl/0.1 M KCl/1 mM GSH/0.1% (v/v) Tween-20] were incubated with 50–100 μM fatty acids or 30–100 μM hydroperoxides for 40 min on ice; in trapping experiments the hydroperoxides were only incubated for 1 min. The reactions (0.3–0.5 ml) were terminated with 10 ml water and the metabolites were immediately extracted on octadecyl silica (SepPak/C18). The latter was washed with water and retained metabolites were eluted with ethyl acetate (4 ml). After being evaporated to dryness under N2, the residue was dissolved in ethanol (50–100 μl), and 10 μl were subjected to LC-MS/MS analysis.

Nitrogen powder of mycelia of Z. tritici was homogenized in 0.1 M KHPO4 buffer (pH 7.3)/2 mM EDTA/0.04% (v/v) Tween-20. The supernatant, after centrifugation at 16,200 g (10 min, 4°C) was incubated with 100 μM fatty acids for 30 min on ice. The products were extracted as above.

**Enzyme assays**

Recombinant proteins of the crude cell lysate [in 0.05 M Tris-HCl (pH 7.6)/5 mM EDTA/10% glycerol or 0.05 M KHPO4 (pH 7.5)/0.5 M NaCl/0.1 M KCl/1 mM GSH/0.1% (v/v) Tween-20] were incubated with 50–100 μM fatty acids or 30–100 μM hydroperoxides for 40 min on ice; in trapping experiments the hydroperoxides were only incubated for 1 min. The reactions (0.3–0.5 ml) were terminated with 10 ml water and the metabolites were immediately extracted on octadecyl silica (SepPak/C18).

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methanol (1 mg/ml on ice; 1 h) were routinely used to reduce ketones to alcohols (27). For rapid reduction of the $^{13}$O-labeled α-ketol, we added the 1 min incubation to distilled water (4°C; 1 h) with NaB$_4$H$_4$ (1 M final concentration; 1 h) and reduced foaming by low speed centrifugations. Hydrogenation was performed in ethanol with Pd/C and a gentle stream of H$_2$ for 2 min and the catalyst was then removed by filtration.

**Bioinformatics**

The ClustalW algorithm was used for sequence alignments (Lasergene, DNASTAR, Inc.). The MEGA6 software was used for construction of phylogenetic trees with 200 bootstrap tests of the resulting nodes (28).

**RESULTS**

**Catalytic properties of EAS28473 (8R-DOX-AOS) of C. immitis**

Recombinant EAS28473 oxidized 18:1n-9, 18:2n-6, and 18:3n-3 to 8-hydroperoxides metabolites with MS$^3$ spectra as reported (29, 30). The MS$^2$ spectra of the corresponding nodes (28).

The MS$^2$ spectra of the corresponding alcohols showed the strong and characteristic signal at m/z 157 [OOC-(CH$_2$)$_3$-CHO]. The RP-HPLC-MS/MS analysis of the oxidation of 18:2n-6 is shown in Fig. 2A. Steric analysis by CP-HPLC-MS/MS analysis (Reprosil Chiral-NR) showed that 8-HPODE had the same retention time as authentic $^{13}$C$_{18}$-8HPODE (inset in Fig. 2A).

The 8-hydroperoxides of 18:1n-9, 18:2n-6 and 18:3n-3 were further transformed to α-ketols. The 18:2n-6 was oxidized to 8R-HPODE and an α-ketol, which was identified below as 8-hydroxy-9-oxo-12Z-octadecenoic acid (Fig. 2A). NP-HPLC-MS/MS analysis showed that small amounts of erythro and threo 8(9)-epoxy-10-hydroxy-12Z-octadecoic acids could also be detected (Fig. 2B). The MS$^2$ spectra (m/z 311→full scan) of these epoxy alcohols showed intense signals at m/z 157 [OOC-(CH$_2$)$_3$-CHO] and m/z 187 [OOC-(CH$_2$)$_3$-CHOH-CHO] as described (31).

The structure of the α-ketols formed from 18:2n-6 were confirmed by reduction of the ketone to a hydroxyl group, hydrogenation of the 12Z double bond, comparison with the mass spectra of the $^{13}$C$_{18}$-labeled α-ketol (27), and with the mass spectra of the α-ketol formed from 18:1n-9.

Treatment of 8-hydroxy-9-oxo-12Z-octadecenoic acid with NaB$_4$H$_4$ yielded threo and erythro [9-²H]8,9-dihydroxy-12Z-octadecenoic acid (8,9-DiHODE). Their MS$^3$ spectra (m/z 314→full scan) were identical with strong signals at m/z 157 [OOC-(CH$_2$)$_3$-CHO] and m/z 188 [OOC-(CH$_2$)$_3$-CHOH-CHO] (Fig. 2C).

The 8-hydroxy-9-oxo-octadecanoic acid, which was formed by hydrogenation of 8-hydroxy-9-oxo-12Z-octadecenoic acid, and the α-ketol formed by oxidation of 18:1n-9 by recombinant EAS28473 yielded identical mass spectra (Fig. 2D). The MS$^3$ spectrum (m/z 313→full scan) of 8-hydroxy-9-oxo-octadecanoic acid showed mid-range signals, among other things, at m/z 183 [possibly HC(O)-C(OH)-CH$_2$-CH$_3$], m/z 157 [OOC-(CH$_2$)$_3$-CHO], and m/z 155 (183-28; loss of CO), which supports the proposed fragmentation between C-7 and C-8, and in the upper range at m/z 295 (313-18),

![Fig. 2.](image-url)

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m/z 277 (313-2 × 18), m/z 267 (313-18-28; loss of water and CO), m/z 251 (313-14-18), and m/z 249, 239, and 199. Reduction of 8-hydroxy-9-oxo-octadecanoic acid with NaBH₄ yielded the expected signals at m/z 157 [\text{OCOC}-(CH₂)₄-CHO] and m/z 188 [\text{OCOC}-(CH₂)₆CHOH-C̃CH₂]. The MS² spectrum (m/z 331→full scan) of [¹³C₁₈]8-hydroxy-9-oxo-octadecanoic acid is shown in Fig. 2E. This compound was obtained by hydrogenation of the α-ketol from [¹³C₁₈]18:2n-6. The fragmentation was consistent with the deduced structure, with signals at m/z 165 (157+9) and m/z 194 (183+11) as indicated in the figure.

The MS³ spectrum (m/z 313→295→full scan) of 8-hydroxy-9-oxo-octadecanoic acid (Fig. 2F) showed signals at m/z 277 (295-18), m/z 267 (295-28; loss of CO), m/z 251 (295-44), and in the lower range m/z 183, 169, and 165. The MS³ spectrum of the [¹³C₁₈]8-hydroxy-9-oxo-octadecanoic acid showed that the corresponding ions in the lower mass range at m/z 194, 180, and 176 (inset in Fig. 2F). We conclude that 18:1n-9 and 18:2n-6 are sequentially oxidized at C-8 to hydroperoxides and dehydrated to unstable allene oxides, which are hydrolyzed to α-ketols with 8-hydroxy-9-oxo configurations.

**MS/MS and MS³ analysis of unsaturated α-ketols formed by 8R-DOX-AOS**

The MS/MS spectra of unsaturated α-ketols, which are formed from 9-hydroperoxy fatty acids, show only weak informative signals, whereas their MS³ spectra are more characteristic (27). We therefore investigated the MS/MS and MS³ spectra of α-ketols derived from 8-hydroperoxy fatty acids.

The MS analyses of 8-hydroxy-9-oxo-12Z-octadecenoic and [¹³C₁₈]8-hydroxy-9-oxo-12Z-octadecenoic acids are summarized in Fig. 3. The MS² spectrum of 8-hydroxy-9-oxo-12Z-octadecenoic acid (m/z 311→full scan) showed important signals at m/z 157 [\text{OCOC}-(CH₂)₄-CHO], m/z 181 [possibly HC(O)C(Õ)=CH-C₈H₂₃], and m/z 187 [\text{OCOC}-(CH₂)₆CHOH-CH₂] as indicated by the inset in Fig. 3A. A characteristic signal of α-ketols was also noted at m/z 265 (Ã; weak loss of CO and water) and m/z 181. The MS² spectrum (m/z 312→full scan) of [¹₁Σ̃H]8-hydroxy-9-oxo-12Z-octadecenoic acid showed that a strong signal at m/z 182 (181+1) (Fig. 3B), which supported the fragmentation. In addition, we used MS³ and MS⁴ spectra to confirm the origin of the signal at m/z 181. The MS³ (m/z 311→181→full scan) and MS⁴ spectra (m/z 311→293→181→full scan) of 8-hydroxy-9-oxo-12Z-octadecenoic acid yielded strong signals at m/z 163 (35%; 181-28; loss of CO). These results were consistent with cleavage between C-7 and C-8 with formation of m/z 181 [possibly HC(O)C(Õ)=CH-C₈H₂₃] (inset in Fig. 3A).

Some signals were likely formed by rearrangement mechanisms, e.g., m/z 199 (Fig. 3A). The MS³ spectrum (m/z 311→199→full scan) yielded m/z 181 (35%; 199-18), m/z 153 (100%; 199-44), and m/z 137 (40%; 155-18) as the major fragment ions. MS³ spectra of α-ketols often contain strong and characteristic signals and can be useful complements to the MS/MS spectra, although the complex MS³ fragmentation is difficult to interpret. The MS³ spectrum of 8-hydroxy-9-oxo-12Z-octadecenoic acid (m/z 311→293→full scan) showed prominent signals at m/z 265 (293-28), m/z 181, 163, 137, and 111. The number of carbon atoms of some fragment ions is marked Cₘ in (E). Important fragments are labeled in a larger font and colored. NL intensity normalized to 100%. The ions at m/z 282 in (D) and (E), which are likely formed by loss of 47 (¹³CO+water) and 29 (¹⁵CO), respectively, are marked *.

Fig. 3. MS/MS and MS³ spectra of the α-ketol (8-hydroxy-9-oxo-12Z-octadecenoic acid) formed from 18:2n-6 and [¹³C₁₈]18:2n-6 by recombinant EAS28473 (8R-DOX-AOS). A: MS/MS spectrum of 8-hydroxy-9-oxo-12Z-octadecenoic acid. B: MS/MS spectrum of the monodeuterated α-ketol, [¹₁Σ̃H]8-hydroxy-9-oxo-12Z-octadecenoic acid. C: MS³ spectrum of 8-hydroxy-9-oxo-12Z-octadecenoic acid. D: MS³ spectrum of [¹³C₁₈]8-hydroxy-9-oxo-12Z-octadecenoic acid. E: MS³ spectrum of [¹³C₁₈]8-hydroxy-9-oxo-12Z-octadecenoic acid. The number of carbon atoms of some fragment ions is marked Cₘ in (E). Important fragments are labeled in a larger font and colored. NL intensity normalized to 100%. The ions at m/z 282 in (D) and (E), which are likely formed by loss of 47 (¹³CO+water) and 29 (¹⁵CO), respectively, are marked *.
and m/z 171 (Fig. 3C), whereas the corresponding spectrum (m/z 311→293→full scan) of [11S-2H]8-hydroxy-9-oxo-12Z-octadecenoic acid yielded many signals increased by one mass unit, notably in the lower mass range at m/z 182 (181+1) and m/z 172 (171+1) (inset in Fig. 3C).

We finally recorded the MS/MS and MS^3 spectra of the [13C18]8-hydroxy-9-oxo-12Z-octadecenoic acid for comparison with these spectra of the unlabeled compound. The MS^2 spectrum (m/z 329→full scan) of [13C18]8-hydroxy-9-oxo-12Z-octadecenoic acid showed signals, among other things, at m/z 165 (see 157+8), m/z 196 (see 187+9), m/z 192 (see 181+11), and weak signals at m/z 208–211 of equal intensities (Fig. 3D). The MS^3 spectrum (m/z 329→311→full scan) of the [13C18]8-hydroxy-9-oxo-12Z-octadecenoic acid showed strong signals, among other things, at m/z 282 (311-29; loss of 13CO), m/z 192, and m/z 180, which likely contained 17, 11, and 9 carbon atoms (Fig. 3E). This confirmed the proposed fragmentation.

The 8R-DOX-AOS differs from 9R-DOX-AOS of Aspergilli by transforming 18:3\textit{n}-3 via 8R-HOPTrE to an allene oxide/\alpha-ketol (Fig. 4A). The structure of the \alpha-ketol was confirmed by LC-MS analysis before and after hydrogenation.

The MS^2 spectrum (m/z 309→full scan) of the \alpha-ketol with two double bonds, 8-hydroxy-9-oxo-12Z,15Z-octadecadienoic acid, showed signals, among other things, at m/z 157, 179, and 197 (see inset in Fig. 4B). The fragmentation ion at m/z 179 was supported by the MS^3 spectrum (m/z 309→291→full scan; data not shown), which yielded m/z 161 (179-18) and m/z 151 (179-28; loss of CO) in analogy with the corresponding ion at m/z 181 in the MS/MS spectrum of 8-hydroxy-9-oxo-12Z-octadecenoic acid discussed above (see Fig. 3A). The signal at m/z 197 might be due to rearrangement. The MS^2 spectrum (m/z 309→291→m/z 197→full scan) yielded m/z 179 (197-18) and m/z 153 (197-44) as the main ions.

The MS^3 spectrum (m/z 309→291→full scan) of the \alpha-ketol showed signals, which were not present in the MS/MS spectrum, e.g., at m/z 193, 171, and 165. The MS^3 spectrum (m/z 309→291→m/z 193→full scan; data not shown) yielded signals at m/z 175 (193-18) and m/z 165 (193-28; loss of CO) (Fig. 4B), suggesting that m/z 193 and 165 could be related.

Finally, the structure of the \alpha-ketol formed from 18:3\textit{n}-3 was confirmed by LC-MS analysis after hydrogenation to 8-hydroxy-9-oxo-octadecanoic acid (see Fig. 2D).

The 18:3\textit{n}-6 appeared to be a poor substrate in analogy with 8R-DOX-LDS. The 20:2\textit{n}-6 was oxidized at C-11, but transformation to \alpha-ketols could not be detected. The product specificity suggested that recombinant EA828473 could be named 8R-DOX-AOS with 18:1\textit{n}-9, 18:2\textit{n}-6, and 18:3\textit{n}-3 as likely natural substrates.

**Oxidation of arachidonic acid by 8R-DOX-AOS of \textit{C. immitis}**

\textit{C. immitis} is a human pathogen and we therefore also assessed the oxidation of 20:4\textit{n}-6 by 8R-DOX-AOS. The metabolites were reduced with TPP and analyzed by NP- and CP-HPLC-MS/MS (supplemental Fig. S1A, B). The three main products were identified as 8-, 10-, and 12-HETE from their MS^2 spectra (m/z 419→full scan), which were as reported previously (32). Steric analysis showed that both 12-HETE and 8-HETE mainly consisted of the S stereoisomers, as judged from reanalysis with added authentic 12S-HETE and 8R-HETE. The minor product, 10-HETE, eluted on CP-HPLC mainly as a single isomer (95%), but the stereo configuration was not further investigated.

**Catalytic properties of recombinant EGP83657 (8S-DOX-AOS) of \textit{Z. tritici}**

EGP83657 oxidized 18:1\textit{n}-9, 18:2\textit{n}-6, and 18:3\textit{n}-3 to 8-hydroperoxy metabolites and to \alpha-ketols in analogy with 8R-DOX-AOS, but with an important difference. Steric analysis of 8-HPODE showed that it mainly consisted of the 8S stereoisomer (Fig. 5A), which apparently was sequentially converted to 8S(9)-epoxy-9,12Z-octadecadienoic acid [8S(9)-EODE] and then hydrolyzed to an \alpha-ketol.

The 8S-HPODE was formed from [8R\textsuperscript{2}H\textsubscript{1}]18:2\textit{n}-6 with retention of the deuterium label (Fig. 5B). These observations
formed by MS/MS analysis, m/z 115 [OOC-(CH2)2-CHO] and m/z 173 [OOC-(CH2)xCHOH-(CH2)x-CHO]. The oxidation of 18:2n-6 to hydroperoxides by nitrogen powder of Z. tritici is shown in Fig. 6A. In addition to 8-HPODE, large amounts of 13-HPODE were also detected. Steric analysis with aid of [13C18]13-HODE showed that 13S-HPODE was the main stereoisomer (>98%; inset in supplemental Fig. S2A). The 18:3n-3 was also oxidized at C-13, but 18:1n-6 was not. The oxidation at C-13 of 18:2n-6 and 18:3n-3 was therefore likely catalyzed by 13S-LOX. We could not detect formation of α-ketols.

EGP91582 aligns with 8R-DOX-LDS, and this protein is a strong candidate for the observed 5,8-LDS activities. The oxidation at C-13 of 18:2n-6 and 18:3n-3 was likely due to the only LOX of Z. tritici (GenBank identification number: EGP90986), which belongs to the family of fungal iron LOX (Fig. 6B). The 13S-LOX of Z. tritici thus has the same are consistent with suprafacial hydrogen abstraction and oxygen insertion, whereas 8R-DOX catalyzes antarafacial hydrogen abstraction and oxygenation as discussed above (33).

The 20:2n-6 was oxidized at C-11, but an α-ketol could not be detected. The 18:3n-6 was a poor substrate. We conclude that EGP83657 can be described as 8S-DOX-AOS with 18:1n-9, 18:2n-6, and 18:3n-3 as likely natural substrates.

Catalytic properties of recombinant EGP87976 (9R-DOX-AOS) of Z. tritici

Recombinant EGP87976 oxidized 18:2n-6 and transformed 9R-HPODE to two polar products, as judged by RP-HPLC-MS analysis (peaks I and II in supplemental Fig. S2A). The MS/MS and MS3 spectra were as reported for the α- and α-ketols, respectively, of 9HPODE-derived allene oxides (27). The 18:2n-6 was also oxidized to 9-HPODE, and steric analysis by CP-HPLC (Reprosil Chiral-AM) and MS2 analysis showed that 9-HPODE consisted of the 9S and 9R stereoisomers in a ratio of ~1:3 (supplemental Fig. S2B). This underestimates the relative formation of the 9R stereoisomer as 9R-HPODE was further transformed to α- and γ-ketols as major products.

The 18:3n-3 was a poor substrate, and 9S-HPODE was transformed mainly to epoxy alcohols, but a 9-HPODE-derived α-ketol was also detected by its characteristic MS3 spectrum (27). Recombinant EGP87976 thus belongs to the group of 9R-DOX-AOS, which is found in Aspergilli (Fig. 1B).

Oxidation of fatty acids by mycelia of Z. tritici

Nitrogen powder of mycelia of Z. tritici oxidized 18:1n-9, 18:2n-6, and 18:3n-3 to hydroperoxides at C-8 and to variable amounts of 5,8-diols. Steric analysis (Chiralcel OBH) showed that 8HPODE stereoisomer was formed (>95%), and the 5,8-diols were identified by two characteristic fragments formed by MS/MS analysis, m/z 115 [OOC-(CH2)2-CHO] and m/z 173 [OOC-(CH2)xCHOH-(CH2)x-CHO]. The oxidation of 18:2n-6 to hydroperoxides by nitrogen powder of Z. tritici is shown in Fig. 6A. In addition to 8-HPODE, large amounts of 13-HPODE were also detected. Steric analysis with aid of [13C18]13-HODE showed that 13S-HPODE was the main stereoisomer (>98%; inset in supplemental Fig. S2A). The 18:3n-3 was also oxidized at C-13, but 18:1n-6 was not. The oxidation at C-13 of 18:2n-6 and 18:3n-3 was therefore likely catalyzed by 13S-LOX. We could not detect formation of α-ketols.

EGP91582 aligns with 8RDOX-LDS, and this protein is a strong candidate for the observed 5,8-LDS activities. The oxidation at C-13 of 18:2n-6 and 18:3n-3 was likely due to the only LOX of Z. tritici (GenBank identification number: EGP90986), which belongs to the family of fungal iron LOX (Fig. 6B). The 13S-LOX of Z. tritici thus has the same
catalytic activity as reported for the LOX of *Fusarium oxysporum* and *Pleurotus ostreatus* (8, 34).

A summary of the oxidation of 18:2n-6 by mycelia and by 8S- and 9R-DOX-AOS is shown in Fig. 6C. The DOX-AOS activities could not be detected in mycelia and may only be expressed in response to environmental stimuli in analogy with other secondary metabolites.

**Non-enzymatic hydrolysis of allene oxides**

It seems likely that the α-ketols, which are derived from oxidation of 18:2n-6, are formed from nonenzymatic hydrolysis of allene oxides, 8R(9)- and 8S(9)-epoxy-10,12Z-octadecadienoic acids [8R(9)- and 8S(9)-EODE] (18). Hydrolysis of allene oxides to α-ketols occurs mainly with inversion of configuration of the hydroxyl group compared with the configuration of the precursor hydroperoxide (17, 35, 36). The two α-ketols, which are formed by hydrolysis of 8R(9)- and 8S(9)-EODE, are therefore expected to consist mainly of the 8S and 8R-hydroxy-9-oxo-12Z-octadecenoic acids, respectively.

CP-HPLC-MS/MS analysis showed that 8R(9)-EODE and [13C18]8S(9)-EODE were hydrolyzed to α-ketols with different retention times (Fig. 7A). The stereoisomer with the shortest retention time was formed by hydrolysis of 8R(9)-EODE and thus tentatively identified as the α-ketol with 8S configuration. “S” before R” is also the elution order of the major α-ketols formed by hydrolysis of 9R(10)- and 9S(10)-EODE, respectively (17).

Hydrolysis of allene oxides in an excess of methanol will form methoxy derivatives. We incubated 8R-DOX-AOS for 1 min with excess 8R-HPODE, added 30 vol of methanol and let the hydrolysis proceed for 1 h. To facilitate the analysis, we reduced the α-ketols with NaBH4. Analysis showed the presence of the expected products, 8,9-DHOME and 8-methoxy-9-hydroxy-12Z-octadecenoic acid, in a ratio of ~9:1 (Fig. 7B). The MS/MS spectrum (m/z 327→full scan) of the latter is shown in Fig. 7C. Signals were noted at m/z 309 (327-18), m/z 295 (327-32; loss of methanol), m/z 277 (295-18), and m/z 201 [OOC-(CH2)6CHOH-CHO] and in the lower mass range at m/z 186, 171, and 141. The MS2 spectrum (m/z 327→201→full scan) showed signals at m/z 186 (100%; 201-15, loss of -CH3), and m/z 169 (10%; 201-32, loss of methanol), whereas MS2 spectrum (m/z 327→295→full scan) yielded intense signals at m/z 277 (100%; 295-18), m/z 171 (35%), and m/z 141 (20%). These spectra were consistent with the proposed structure. The trapping experiment indicates a short half time of the unconjugated allene oxide, 9R(10)-EODE, as about 90% was hydrolyzed by water after 1 min of incubation (Fig. 7B).

We next analyzed the transformation of [18O2]8R-HPODE by 8R-DOX-AOS to determine the incorporation of 18O into the α-ketol (8-hydroxy-9-oxo-12Z-octadecenoic acid). The ketone at C-9 of the α-ketol can be exchanged with water and we therefore reduced the products formed after 1 min with NaBH4. LC-MS analysis of erythro and threo 8,9-DHOME in the full scan mode showed incorporation of one molecule of 18O (Fig. 7D) and the MS2 spectrum (m/z 315→full scan) showed a signal at m/z 189, which demonstrated the 18O-label at C-9 (Fig. 7E).

**Transformation of hydroperoxides by 8R- and 8S-DOX-AOS**

The transformation of other hydroperoxides by 8R- and 8S-DOX-AOS may indicate their relation to other AOS of fungi. We therefore assessed whether the 8R-AOS of 8R-DOX-AOS (EA82473) and 8S-AOS of 8S-DOX-AOS (EGP83657) could transform 9- and 13-HPDE.

The 8R-DOX-AOS efficiently transformed 9H-HPDE to an epoxy alcohol as a major product, but an α-ketol
could not be detected. The epoxy alcohol was tentatively identified as three 9R(10R)-epoxy-11-hydroxy-12Z-octadecenoic acid, as judged from NP- and RP-HPLC-MS/MS analysis (m/z 311→full scan) (31) along with small amounts of the erthro isomer (supplemental Fig. S3A). The 9S-, 13S-, and 13R-HPODE were only converted to small fractions (5–10%) of epoxy alcohols, as judged by RP-HPLC-MS/MS analysis.

The 8S-DOX-AOS (EGP83657) converted only a fraction of 8R-HPODE to 8-hydroxy-9-oxo-12Z-octadecenoic acid (5–10%). In contrast, about 50% of 9SHPODE was transformed to an α-ketol, 9-hydroxy-10-oxo-12Z-octadecenoic acid, and to erthro and three 9S(10S)-epoxy-11-hydroxy-12Z-octadecenoic acids (supplemental Fig. S3B) [see (31)]. The relative amounts of the α-ketol and the epoxy alcohols were ~1:2. The 9SHPOTre was transformed in the same way. The α-ketols were identified by their characteristic MS² and MS³ spectra (16, 27). The 9R-HPODE and 13S- and 13R-HPODE were not transformed by 8S-DOX-AOS, as only small amounts of epoxy alcohols were detected (<10%).

The transformation of 9SHPODE by the 8S-AOS activities to an α-ketol, which originates from 9S(10)-EODE, suggests that the 8S-AOS may have evolved from 9S-AOS of 9S-DOX-AOS.

**DISCUSSION**

Our main goal was to investigate the catalytic properties of three putative DOX-CYP fusion enzymes of *C. immitis* (EAS28473) and *Z. tritici* (EGP83657 and EGP87976). We report that two of the recombinant enzymes form novel alkene oxides and they are named 8S and 8S-DOX-AOS, respectively, whereas the third enzyme belongs to the 9R-DOX-AOS subfamily. The sequential oxidation of 18:1n-9 by 8R- and 8S-DOX-AOS to allene oxides and hydrolysis to α-ketols are outlined in Fig. 8. We detected 5,8-LDS and 13S-LOX activities of mycelia of *Z. tritici* (Fig. 6C), which could be attributed to expression of EGP91582 and EGP99086, respectively.

Previously described alkene oxides are formed from cis-trans conjugated hydroperoxy fatty acids, e.g., from 9S- and 13S-HPOTre by plant CYP74, 8R-hydroperoxyeicosatetraenoic acid by the AOS-LOX of the coral, *Plexaura homomalla*, and from 9R- and 9R-HPOTre by fungal 9-DOX-AOS (37–39). The alkene oxides can be transformed enzymatically or nonenzymatically to cyclopentenone fatty acids, e.g., analogs of jasmonic acid and prostaglandin A₂, respectively (37–39). The process leading to a cyclopentenone from the 10Z-isomer of the 9SHPODE-derived alkene oxide was recently studied in detail (40, 41). This alkene oxide undergoes homolytic cleavage with formation of a ketone at C-10, a radical at C-9, and an allyl-like radical at C-11 to C-13. This oxoallylidrational may then form a cyclopentenone. In contrast, 8-HPOTre-derived alkene oxides lack conjugated double bonds, but they are nevertheless rapidly hydrolyzed by methanol or water (Fig. 7). The oxidation of 18:1n-9 to allene oxides by 8S-DOX-AOS is unprecedented as this fatty acid is a poor substrate of LOXs and fungal 9R- and 9S-DOX-AOSs (17, 42).

The 8R- and 8S-DOX-AOS can be aligned with 51% amino acid identity, but the four amino acids in the 8-DOX domains from the catalytic Tyr to the proximal His ligand are not identical. The consensus sequence TyrArgPheHis of all 9S- and 9R-DOX domains is conserved in 8R-DOX-AOS, but it is replaced in 8S-DOX-AOS with the consensus sequence TyrArgTrpHis of 8R-DOX-LDS, 10R-DOX-(CYP), and 10R-DOX-EAS. 8S-DOX-AOS is, nevertheless, the first described enzyme with a catalytic 8S-DOX domain.

The substrate recognition sites (SRSs) 4 (or I-helices) of the 8R- and 8S-AOS domains differ. The hexamer SRS 4 sequence ValAlaThrGlnAlaGln of 8S-AOS aligns except for one or two positions (in bold type) with the SRS 4 sequence ValAlaAsnGlnAlaGly of 5,8-LDS [see (18, 43)]. The CYP domain of 8S-DOX-AOS is catalytically related to 9S-DOX-AOS, as it transformed 9S-HPOTre to an α-ketol, but this relation is not evident from SRS 4 or other sequence alignments of 8S- and 9S-AOS domains.

The general rule for oxygenation of unsaturated fatty acids by COXs, LOXs, and DOX-CYP fusion enzymes is antarafacial hydrogen abstraction and oxygen insertion. Exceptions to this rule are, for example, fungal LOXs with catalytic manganese, 9R-DOX, and 9R-DOX-AOS (6, 17, 20). The 9R- and 9S-DOX-AOSs both abstract the proR hydrogen at C-11 of 18:2n-6, but oxygen is inserted from opposite directions. An outline of the sequential biosynthesis of 8S-HPOTre and 8S(9)-EODE from 18:2n-6 by 8S-DOX-AOS and the nonenzymatic hydrolysis of 8S(9)-EODE to an α-ketol is shown in Fig. 9A. Due to the Cahn-Ingold-Prelog rule, the proR hydrogen at C-11 points in the same direction as the proS hydrogen at C-8 of 18:2n-6, which is abstracted

![Fig. 8](image-url) Overview of the sequential oxidation of oleic acid to 8-hydroperoxyoctadecenoic acid (HPOME) and to 8S-DOX-AOS. The two alkene oxides, 8R(9)- and 8S(9)-epoxy-9-octadecamonoenoic acids (EOMEs), are mainly hydrolyzed to α-ketols by inversion of configuration at C-8 (solid arrows).
by both 8R- and 8S-DOX-AOS (Fig. 9B). The 8S-DOX-AOS thus catalyzes suprafacial hydrogen abstraction and oxygenation in analogy with 9R-DOX-AOS.

Interestingly, 9R-DOX-AOS of Aspergillus niger can be transformed to 9S-DOX-AOS by replacement of two amino acids with those conserved in these two positions of 9S-DOX-AOS, Gly616leu and Phe627Leu (17). This shifts the direction of oxygenation, but it does not change the hydrogen abstraction (see Fig. 9B). Gly616 and Phe627 were not conserved in the corresponding positions of either 8R-DOX-AOS or 8S-DOX-AOS, which both contain Leu residues in these positions.

What is the biological function of 8S-DOX-AOS? C. immitis and its closely related species, Coccioidoides posadasii, cause common and potentially serious human infections, which are endemic in California and Arizona (21). It was therefore of interest to determine whether 8R-DOX-AOS could metabolize arachidonic acid, as eicosanoids have potent actions in inflammation and its resolution (44). Arachidonic acid was transformed to 8S, 10-, and 12-hydroperoxyeicosatetraenoic acid. None of these metabolites are known to have specific biological effects (44), but it would be of interest to determine whether 8R-DOX-AOS is expressed during infection of human cells and metabolizes arachidonic acid of the host.

Z. tritici is spread worldwide, and septoria tritici blotch is often considered to be the most devastating disease of wheat (22, 23). Plants transform 13SHPOTe sequentially to allene oxides and to jasmonates, which act as growth and defense hormones (37, 38). The fungal AOS domains can only be aligned with plant AOSs (CYP74) and related P450 with a low degree of amino acid identity (18). Fungal AOSs and CYP74 have likely evolved independently. The parallel evolution of 8R-, 8S-, 9R-, and 9S-DOX-AOSs in fungi and CYP74 linked to LOX in plants suggest that the allene oxides may be of biological importance. This is well-established in the pathway to jasmonic acid with potent actions in plants (37, 38). Lasiodiplodia theobromae and a few other fungal pathogens overproduce jasmonic acid to induce pathological plant growth (45). Fungal jasmonates may be formed by the biochemical pathway in plants, but details are lacking (8, 46). The fungal repertoire of oxylipins likely participates in the struggle between the pathogen and its host, as well as in reproduction and development (1).

In summary, we have characterized fatty acid oxygenases of Z. tritici and report biosynthesis of two novel allene oxides formed by 8R- and 8S-DOX-AOS of C. immitis and Z. tritici. Their natural substrates are likely unsaturated C18 fatty acids, and the biological function of these secondary metabolites should be evaluated in the context of related allene oxides formed by plants.

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