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

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AbstractLinoleate 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 transformed 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 8R-DOX-AOS oxidized 18:2n-6 sequentially to 8R-hydroperoxy-9,12Z-octadecadienoic acid (8R-HPODE) and to an allene oxide, 8R(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(epoxy-9,12Z-octadecadienoic acid. The 8S-DOX-AOS oxidized [8R-2H]18:2n-6 to 8S-HPODE with retention of the 2H-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 domains differed. 8R-DOX-AOS transformed 9R-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 8S-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 C_{20} and C_{18} fatty acids and many of them exert potent biological actions (1–3). Fungal oxylipins can be formed by dioxygenation of C_{18} fatty acids by two groups of enzymes, lipoxygenases (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 8RDOX-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 form 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

Abbreviations: AOS, allene oxide synthase; CP, chiral-phase; COX, cyclooxygenase; CYP, cytochrome P450; DiHODE, dihydroxyoctadecadienoic acid; DOX, dioxygenase; EAS, epoxy alcohol synthase; EODE, epoxystadecadienoic acid; HPODE, hydroperoxystadecadienoic acid; HPOTrE, hydroperoxyoctadecatrienoic acid; LDS, linoleate diol synthase; LOX, lipoxygenase; NP, normal-phase; SRS, substrate recognition site; RP, reversed-phase; TPP, triphenylphosphine; α-ketol, C_{18} fatty acids with 8-hydroxy-9-oxo-9-hydroxy-10-oxo structural elements.

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of 18:2n-6 can also be subject to homolytic cleavage and dehydration to allene oxides by allene oxide synthases (AOSs) (9R- and 9S-DOX-AOS) (Fig. 1A). AOSs of plants belong to the CYP74 family, but the fungal AOSs form separate CYP families (17, 18). The 8R-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 8R- and 10R-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: 9R-DOX and 9R-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 8R-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 the CYP domain is homologous to P450 but lacks the critical Cys residue for catalysis. B. Phylogenetic tree of characterized DOX-CYP fusion enzymes except for three orphans each of C. immitis and Z. tritici (marked red). The sequences are (GenBank identification numbers): EDP52540 and APB71131 for 10R-DOX-(CYP); EGU86021 and EQF36272 for 10R-DOX-EAS; for three subfamilies of 8R-DOX-LDS: AGA95448 and EDP50447 for 5,8-LDS, EHA52010 and QGUUS2 for 7,8-LDS, KDN68726 and EQF34869 for 7,8- and 8,11-LDS; EGU88194 and EQF37323 for 9S-DOX-AOS; AGH14485 and EHA29500 for 9R-DOX-AOS; EGU79548 and EQF36675 for 9R-DOX.
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 18:2−6 (99%), 18:3−6 (99%), and 18:5n−3 (99%) were from VWR, and 18:1n−9, 20:2−6, 20:4−6, and [15]18:2−6 (98%) were from Larodan (Malmö, Sweden). The [115S]H18:2−6 (>99% 2H) and [8R]H18:2−6 (65% 2H) were prepared by Dr. Hamberg as described (24). The 95-hydroperoxyoctadecatrienoic acid (HPOTe) and 9E, 9S, 13R, and 13S-hydroperoxyoctadecadienoic acids (HPODEs) were prepared by potato and tomato LOX and by 13R-MnLOX (17). The 8S, 8R, and 12S-HETE were from Cayman. The [3]O2−8R-HPODE was prepared with the 8R-DOX domain of 7,8-LDS (14). The 18O2−8R-HPODE (>95% 18O) was prepared in the same way by incubation under 18O2. The labeled 8R-HPODE was purified by reversed-phase (RP)-HPLC. Chemically competent *Escherichia coli* (NEB5α) were from New England BioLabs. Champion pET101D Directional TOPO kit was from Invitrogen. Restriction enzymes and gel extraction kit were from Fermentas. Sequencing was Directional TOPO kit was from Invitrogen. Restriction enzymes (NEB5α) were from New England BioLabs. Champion pET101D

**Expression of recombinant proteins**

The open reading frames of GenBank EAS28473 (Gen; 10 times (30 s sonication, 30 s pause), 4°C). Cell debris was removed by centrifugation and the supernatants were used immediately or stored at −80°C until needed. EAS28473, EGP83657, and EGP87976 were expressed in more than three independent experiments, DNase I (about 0.1 mg/ml). The suspension was frozen and thawed twice and then sonicated (Bioruptor Next Gen; 10 times (30 s sonication, 30 s pause), 4°C). Cell debris was removed by centrifugation and the supernatants were used immediately or stored at −80°C until needed. EAS28473, EGP83657, and EGP87976 were expressed in more than three independent experiments.

We confirmed that cell lysate of untransformed *E. coli* BL21 Star cells does not oxidize 18:2−6 to any of the products discussed below. Analysis of cell lysates of transformed *E. coli* without added 18:2−6 did not form any metabolites of this acid.

**LC-MS analysis**

RP-HPLC with MS/MS analysis was performed with a Surveyor MS pump (ThermoFisher) and an octadecyl silica column (5 μm; 2 × 150 mm; Phenomenex), which was eluted at 0.3–0.4 ml/min with methanol/water/acetic acid, 750/250/0.5. The effluent was used to subject to electrospray ionization in a linear ion trap mass spectrometer (LTQ, ThermoFisher). The heated transfer capillary was set at 315°C, the ion isolation width at 1.5 amu (5 amu for analysis of H2-labeled metabolites and hydroperoxides), the collision energy at 35 (arbitrary scale), and the tube lens at about −110 V. PFG1 was infused for tuning. Samples were injected manually (Rheodyne 7510) or by an autosampler (Surveyor Autosampler Plus, ThermoFisher).

Normal-phase (NP)-HPLC with MS/MS analysis was performed with a silicic acid column (5 μm; 2 × 250 mm; Dr. Maisch) using hexane/isopropanol/acetonic acid, 98:2/0.01 for separation of oxidized fatty acids (0.5 ml/min; Constametric 3200 pump, LDC/MiltonRoy). The effluent was combined with isopropanol/water (3/2; 0.25 ml/min) from a second pump (Surveyor MS pump). The combined effluents were introduced by electrospray ionization into the ion trap mass spectrometer above.

Chiral-phase (CP)-HPLC analysis of 8- and 9HPODE was performed by chromatography on Reprosil Cirial-νR (8 μm; 2 × 250 mm; Dr. Maisch), which was eluted at 0.5 ml/min with hexane/isopropanol/acetonic acid, 98.8/1.2/0.01 (25), and isoforms of 8-HODE were resolved on Ciracel OBH (26). Isomers of HETE and 9- and 13-HODE were resolved on Reprosil Cirial-AM (5 μm; 2 × 250 mm; Dr. Maisch), eluted (0.2 ml/min) with hexane/methanol/acetonic acid, 96/4/0.02 or 95/5/0.02. The effluents of the CP-HPLC columns were mixed with isopropanol/water in a ratio of 2:1 and electro sprayed into the mass spectrometer.

Triphenylphosphine (TPP) in hexane was used to reduce hydroperoxy fatty acids to hydroxy fatty acids. NaBH₄ or Na₂B₄H₆ in
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 18:1\(\,^\text{n-9}\), 18:2\(\,^\text{n-6}\), and 18:3\(\,^\text{n-3}\) to 8-hydroperoxy metabolites with MS\(^3\) spectra as reported (29, 30). The MS\(^2\) spectra of the corresponding \(\alpha\)-ketols, we added the 1 min incubation to distilled water (4°C; 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.

For rapid reduction of the 18O-labeled 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 \(\alpha\)-ketol, we added the 1 min incubation to distilled water (4°C; 1 h) with NaB\(_2\)H\(_4\) (1 M final concentration; 1 h) and reduced foaming by low speed centrifugations. Hydrogenation was performed in ethanol with 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.

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

Treatment of 8-hydroxy-9-oxo-12Zoctadecenoic acid with NaBH\(_4\) yielded threo and erythro \([9-\text{H}]8,9\text{-diHODE}\). Their MS\(^2\) spectra (m/z 314→full scan) were identical with strong signals at m/z 157 \([\text{OOC-(CH\(_3\))}_2\text{CHO}]\) and m/z 188 \([\text{OOC-(CH\(_3\))_2\text{CHOH-CHO}]}\) (Fig. 2C).

The 8-hydroxy-9-oxo-octadeceanoic acid, which was formed by hydrogenation of 8-hydroxy-9-oxo-12Zoctadecenoic acid, and the \(\alpha\)-ketol formed by oxidation of 18:1\(\,^\text{n-9}\) by recombinant EAS28473 yielded identical mass spectra (Fig. 2D). The MS\(^2\) spectrum (m/z 314→full scan) of 8-hydroxy-9-oxo-octadeceanoic acid showed mid-range signals, among other things, at m/z 183 [possibly H\(_2\text{O})\text{C(O')=CH-CH}_2\text{H}_2]\), m/z 157 \([\text{OOC-(CH\(_3\))}_2\text{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),
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-octadecenoic acid with NaBH₄ yielded the expected signals at m/z 157 \([\text{OOC-(CH}_2\text{)}_2\text{C=CH-C(OH)}_2\text{CHO}]\) and m/z 188 \([\text{OOC-(CH}_2\text{)}_2\text{C=CH-C(OH)}_2\text{CHO}]\). The MS\(^3\) spectrum (m/z 331–full scan) of \([^{13}\text{C}_{18}]\)8-hydroxy-9-oxo-octadecenoic acid is shown in Fig. 2E. This compound was obtained by hydrogenation of the \(\alpha\)-ketol formed from \([^{13}\text{C}_{18}]\)8-hydroxy-9-oxo-12Z-octadecenoic and \([^{13}\text{C}_{18}]\)8-hydroxy-9-oxo-12Z-octadecenoic acids are summarized in Fig. 3. The MS\(^2\) spectrum of 8-hydroxy-9-oxo-12Z-octadecenoic acid (m/z 311→full scan) showed important signals at m/z 157 \([\text{OOC-(CH}_2\text{)}_2\text{C=CH-C(OH)}_2\text{CHO}]\), m/z 181 (possibly HC(O)-C(O=)=CH-C\(_6\)H\(_4\)\(_3\)), and m/z 187 \([\text{OOC-(CH}_2\text{)}_2\text{C=CH-C(OH)}_2\text{CHO}]\) as indicated by the inset in Fig. 3A. A characteristic signal of \(\alpha\)-ketols was also noted at m/z 265 (A\(^{-}\)-6; likely loss of CO and water) and m/z 181. The MS\(^2\) spectrum (m/z 312→full scan) of \([^{11}\text{H}]\)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 MS\(^3\) and MS\(^4\) spectra to confirm the origin of the signal at m/z 181. The MS\(^3\) (m/z 311→181→full scan) and MS\(^4\) spectrum (m/z 311→293→191→full scan) of 8-hydroxy-9-oxo-12Z-octadecenoic acid yielded strong signals at m/z 163 (35%; 181-18) and m/z 153 (base peak, 100%; 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(O=)=CH-C\(_6\)H\(_4\)\(_3\)) (inset in Fig. 3A).

Some signals were likely formed by rearrangement mechanisms, e.g., m/z 199 (Fig. 3A). The MS\(^3\) 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.

MS\(^3\) spectra of \(\alpha\)-ketols often contain strong and characteristic signals and can be useful complements to the MS/MS spectra, although the complex MS\(^3\) fragmentation is difficult to interpret. The MS\(^3\) 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,

**Fig. 3.** MS/MS and MS\(^3\) spectra of the \(\alpha\)-ketol (8-hydroxy-9-oxo-12Z-octadecenoic acid) formed from 18:2\(^{\text{n-6}}\) and \([^{13}\text{C}_{18}]\)18:2\(^{\text{n-6}}\) 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 \(\alpha\)-ketol, \([^{11}\text{H}]\)8-hydroxy-9-oxo-12Z-octadecenoic acid. C: MS\(^3\) spectrum of the monodeuterated \(\alpha\)-ketol, \([^{11}\text{H}]\)8-hydroxy-9-oxo-12Z-octadecenoic acid. D: MS\(^3\) spectrum of \([^{13}\text{C}_{18}]\)8-hydroxy-9-oxo-12Z-octadecenoic acid. E: MS\(^3\) spectrum of \([^{13}\text{C}_{18}]\)8-hydroxy-9-oxo-12Z-octadecenoic acid. The number of carbon atoms of some fragment ions is marked C\(_{\text{subscript}}\) 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 (\(^{13}\text{CO}+\text{water}\)) and 29 (\(^{15}\text{CO}\)), respectively, are marked *.
and m/z 171 (Fig. 3C), whereas the corresponding spectrum (m/z 311→293→full scan) of [11S,18S]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 MS3 spectra of the [13C18]8-hydroxy-9-oxo-12Z-octadecenoic acid for comparison with these spectra of the unlabeled compound. The MS2 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 MS3 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 Aspergillus by transforming 18:3n-3 via 8R-HPOTrE to an allene oxide/α-ketol (Fig. 4A). The structure of the α-ketol was confirmed by LC-MS analysis before and after hydrogenation.

The MS2 spectrum (m/z 309→full scan) of the α-ketol with two double bonds, 8-hydroxy-9-oxo-12Z,15Z-octa decadienoic 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 MS3 spectrum (m/z 309→291→179→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 MS3 spectrum (m/z 309→291→197→full scan) yielded m/z 179 (197-18) and m/z 153 (197-44) as the main ions.

The MS3 spectrum (m/z 309→291→full scan) of the α-ketol showed signals, which were not present in the MS/MS spectrum, e.g., at m/z 193, 171, and 165. The MS4 spectrum (m/z 309→291→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 α-ketol formed from 18:3n-3 was confirmed by LC-MS analysis after hydrogenation to 8-hydroxy-9-oxo-octadecenoic acid (see Fig. 2D).

The 18:3n-6 appeared to be a poor substrate in analogy with 8R-DOX-LDS. The 20:2n-6 was oxidized at C-11, but transformation to α-ketols could not be detected. The product specificity suggested that recombinant EAS28473 could be named 8R-DOX-AOS with 18:1n-9, 18:2n-6, and 18:3n-3 as likely natural substrates.

**Oxidation of arachidonic acid by 8R-DOX-AOS of C. immitis**

*C. immitis* is a human pathogen and we therefore also assessed the oxidation of 20:4n-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 MS2 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 stereo isomer, 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 Z. tritici**

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

The 8SHPODE was formed from [8R-2H]18:2n-6 with retention of the deuterium label (Fig. 5B). These observations...
are consistent with suprafacial hydrogen abstraction and oxygen insertion, whereas RDOX 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 γ- and α-ketols, respectively, of 9H-PODE-derived alkenes (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 9H-PODE 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 9H-PODE was further transformed to α- and γ-ketols as major products.

The 18:3n-3 was a poor substrate, and 9S-PODE was transformed mainly to epoxy alcohols, but a 9H-PODE-derived α-ketol was also detected by its characteristic MS3 spectrum (27). Recombinant EGP87976 oxidizes 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 8H-HPODE 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)]-CHO] and m/z 173 [OOC-(CH2)3CHOH-(CH2)2-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-HPDE were also detected. Steric analysis with aid of [13C18]13-HOPE showed that 13S-HPDE 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

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**Fig. 5.** Steric analysis of 8-HPODE formed by EGP83657 (8S-DOX-AOS) and the retention of deuterium during oxidation of [8D3H]18:2n-6 to 8HPODE. A: The 8-HPODE formed by EGP83657 eluted before [13C18]8HPODE on the CP-HPLC column and it was thus identified as the 8S stereoisomer. B: MS/MS spectrum (m/z 293–297→full scan) of 8-HOPE formed from [8D3H]18:2n-6 (65% 2H) (33). The even numbered fragments contain the 2H label, and the signal at m/z 158 is likely due to the fragment ion OOC-(CH2)3-C2HO. The inset shows the labeling of hydrogens at C-8 of 18:2n-6.

**Fig. 6.** The 13S-LOX and 5,8-LDS activities of mycelia and an overview of fatty acid DOXs of Z. tritici. A: RP-HPLC-MS/MS analysis of hydroperoxides after reduction to alcohols. The inset shows that 13S-LOX was the main product (top) as judged from the separation of 13C18-labeled 13R- and 13S-LOX (bottom). B: Phylogenetic tree of five iron LOXs. The GenBank identification numbers are (from top to bottom): EGP90986, EF177850, EAL84806, EKX38530, and BAH9788. The phylogenetic tree was constructed with MEGA6. The percent sequence identity with EGP90986 is indicated. C: Overview of the oxidation of 18:2n-6 by mycelia (left) and by two recombinant DOX-AOSs (right) of Z. tritici.
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-DiHOME 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)₆-CHOCH₃-CHO] 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 MS³ 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-DiHOME 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 hydroperoxydes 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 8S-AOS of 8R-DOX-AOS (EAS28473) and 8S-AOS of 8S-DOX-AOS (EP83657) could transform 9- and 13-HPDE.

The 8S-DOX-AOS efficiently transformed 9R-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-12-Z-octadecenoic acid, as judged from NP- and RP-HPLC-MS/MS analysis (m/z 311→full scan) (31) along with small amounts of the erythro 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-12-Z-octadecenoic acid (5–10%). In contrast, about 50% of 9S-HPODE was transformed to an α-ketol, 9-hydroxy-10-oxo-12-Z-octadecenoic acid, and to erythro 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 9S-HPOTrE 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 9S-HPODE 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 allene oxides and they are named 8S- and 9S-AOS domains. The sequential oxidation of 18:1 to 8S-DOX-AOS can be aligned with 51% amino acid identity, but the four amino acids in the 8S-AOS domains from the catalytic tyrosine of the proximal His heme ligand are not identical. The consensus sequence TyrArgPheHis of all 9S- and 9R-DOX-AOS 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 8R-DOX-AOS aligns except for one or two positions (in bold type) with the SRS 4 sequence ValAlaAsnGln(Ala/Gly)Gln of 8S-AOS [see (18, 43)]. The CYP domain of 8S-DOX-AOS is catalytically related to 9S-DOX-AOS, as it transformed 9S-HPODE 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 antiperiplanar 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-HPODE 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. Overview of the sequential oxidation of oleic acid to 8-hydroperoxystearic acid (HPOME) and to allene oxides by 8R- and 8S-DOX-AOS. The two allene 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).](http://www.jlr.org/content/suppl/2016/06/09/jlr.M668961.DC1.html)
Fig. 9. Illustration of the sequential biosynthesis of allene oxides from 18:2n-6 by 8S-DOX-EAS (EGP83657) and the suprafacial and antarafacial oxidation mechanisms of 8- and 9-DOX-AOS. A: Overview of the sequential biosynthesis of allene oxides by 8S-DOX-AOS and important amino acid residues for catalysis. B: The 8S and 8R-DOX-AOSs abstract the proS hydrogen at C-8, whereas 9S and 9R-DOX-AOSs abstract the proR hydrogen at C-11. The figure illustrates that these proS and proR hydrogens have the same absolute configuration relative to the 9,12Z-pentadiene structure. The red and blue arrows indicate the direction of oxygen insertion at C-8 and C-9, respectively, in relation to the pentadiene structure. The S and R assignments are due to the Cahn-Ingold-Prelog nomenclature rule.

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, Gly616Ile and Phe626Leu (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 8S-DOX-AOS or 8R-DOX-AOS, which both contain Leu residues in these positions.

What is the biological function of 8-DOX-AOS? *C. immitis* and its closely related species, *Coccidioides posadasi*, 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 12S-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 caused by rust fungi and the species *Lasiodiplodia theobromae* and *Lasiodiplodia theobromae*. The fungal AOS domains 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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