Identification of Dioxygenases Required for Aspergillus Development

STUDIES OF PRODUCTS, STEROCHEMISTRY, AND THE REACTION MECHANISM

Ulrike Garscha, Fredrik Jerneren, DaWoon Chung, Nancy P. Keller, Mats Hamberg, and Ernst H. Oliw

From the Department of Pharmaceutical Bioscience, Uppsala Biomedical Center, SE-75124 Uppsala, Sweden, the Department of Plant Pathology and the Department of Medical Microbiology and Immunology, University of Wisconsin, Madison, Wisconsin 53706, and the Department of Medical Biochemistry and Biophysics, Karolinska Institutet, SE-171 77 Stockholm, Sweden

Aspergillus sp. contain ppoA, ppoB, and ppoC genes, which code for fatty acid oxygenases with homology to fungal linoleate 7,8-diol synthases (7,8-LDS) and cyclooxygenases. Our objective was to identify these enzymes, as ppo gene replacements show critical developmental aberrancies in sporulation and pathogenicity in the human pathogen Aspergillus fumigatus and the genetic model Aspergillus nidulans. The PpoAs of A. fumigatus and A. nidulans were identified as (8R)-dioxygenases with hydroperoxide isomerase activity, designated 5,8-LDS. 5,8-LDS transformed 18:2n-6 to (8R)-hydroperoxyoctadecadienoic acid ((8R)-HPODE) and (5S,8R)-dihydroxy-9Z,12Z-octadecadienoic acid (5S,8R)-DiHODE. We also detected 8,11-LDS in A. fumigatus and (10R)-dioxygenases in both Aspergilli. The diol synthases oxidized [(8R)−2H]18:2n-6 to (8R)-HPODE with retention of the deuterium label, suggesting antarafacial hydrocarbon abstraction and insertion of molecular oxygen. Experiments with stereospecifically deuterated 18:2n-6 showed that (8R)-HPODE was isomerized by 5,8- and 8,11-LDS to (5S,8R)-DiHODE and to (8R,11S)-dihydroxy-9Z,12Z-octadecadienoic acid, respectively, by suprafacial hydrogen abstraction and oxygen insertion at C-5 and C-11. PpoCs were identified as (10R)-dioxygenases, which catalyzed abstraction of the pro-S hydrogen at C-8 of 18:2n-6, double bond migration, and antafacial insertion of molecular oxygen with formation of (10R)-hydroxy-8E,12Z-hydroperoxyoctadecadienoic acid ((10R)-HPODE). Deletion of ppoA led to prominent reduction of (8R)-H(P)ODE and complete loss of (5S,8R)-DiHODE biosynthesis, whereas biosynthesis of (10R)-HPODE was unaffected. Deletion of ppoC caused biosynthesis of traces of racemic 10-HODE but did not affect the biosynthesis of other oxylipins. We conclude that ppoA of Aspergillus sp. may code for 5,8-LDS with catalytic similarities to 7,8-LDS and ppoC for linoleate (10R)-dioxygenases. Identification of these oxygenases and their products will provide tools for analyzing the biological impact of oxylipin biosynthesis in Aspergilli.

The Aspergilli constitute a family of ascomycete fungi (1, 2). Several species are important human allergens, opportunistic pathogens, and producers of mycotoxins. Their spores are ubiquitous in the environment. Immunocompromised patients are particularly vulnerable to infections by Aspergillus fumigatus, causing farmer’s lung disease and invasive aspergillosis (1, 2). Aspergilli are also plant pathogens and used as industrial microorganisms. Aspergillus nidulans is a model organism for studies of fungal biology (3). The genomes of nine Aspergillus sp. have now been fully or partly sequenced, which highlights their biological importance (2). One set of molecules known to be critical in Aspergillus developmental processes are a series of oxygenated fatty acids originally termed as psi factors.

Champe and co-workers (4, 5) showed in 1989 that A. nidulans oxidized 18:2n-6 and 18:1n-9 to psi factors, e.g. (8R)-HODE, (5S,8R)-DiHODE, and (8R)-HOME, which were identified as inducers of precocious sexual sporulation. Oxidation of polyunsaturated fatty acids to biologically active metabolites was well established in mammals and plants at that time, but this appears to be the first report of hormone-like activities of fungal oxylipins.

Oxidation of 18:2n-6 to (8R)-HODE and DiHODE was not restricted to A. nidulans. (8R)-HODE was originally discovered in Lactisaria arvalis (6, 7). (8R)-HODE is also produced by other fungi, e.g. Gaeumannomyces graminis (the take-all fungus of wheat), Magnaporthe grisea (the rice blast fungus), Leptotium lacteum (the sewage fungus), and Agaricus bisporus (the field mushroom).
Biosynthesis of Oxylinps by Aspergillus sp.

| TABLE 1 | Biological effects of ppo gene loss in Aspergillus sp. |
|---|---|
| **A. nidulans mutants** | Characteristics | Ref. |
| ΔppoA | Increased asexual spore, decreased sexual spore | 23 |
| ΔppoA, B | Increased asexual spore, decreased sexual spore | 23 |
| ΔppoC | Increased sexual spore, decreased asexual spore | 23 |
| ΔppoA, C | Increased sexual spore, decreased asexual spore | 21, 22 |
| RNA interference ppoABC<sup>c</sup> | None detected | 34 |
| | Decreased asexual spore, cell wall aberrancies | Footnote 9 |
| | Enhanced resistance to oxidative stress | Footnote 7 |
| | Germination defects, enhanced virulence attributes<sup>b</sup> | |
| | Enhanced resistance to oxidative stress | 27 |
| | Enhanced virulence<sup>a</sup> | |

**A. fumigatus mutants**

| Characteristics | Ref. |
|---|---|
| ΔppoA | None detected | 34 |
| ΔppoB | None detected | Footnote 9 |
| ΔppoC | Decreased asexual spore, cell wall aberrancies | Footnote 7 |
| | Enhanced resistance to oxidative stress | 27 |
| | Germination defects, enhanced virulence attributes<sup>b</sup> | 27 |
| | Enhanced resistance to oxidative stress | |

<sup>a</sup> Virulence was assessed on host seed (peanut and maize).

<sup>b</sup> Virulence was assessed on a murine model of aspergillosis.

<sup>c</sup> The RNA interference ppoABC<sup>c</sup> strain was created by using RNA interference technology to knock down expression of all three ppo genes simultaneously. However, there is still residual gene expression of all three genes (27).

mushroom) (8–11). *G. graminis* and *M. grisea* also form (7S,8S)-DiHODE and the field mushroom (8R,11S)-DiHODE (8, 10, 11). The mechanism of biosynthesis of (8R)-HODE and (7S,8S)-DiHODE was determined in *G. graminis* (12–14). 18:2n-6 was oxidized to (8R)-HPODE by a heme-containing (8R)-DOX with hydroperoxide isomerase activity, 7,8-LDS. This enzyme abstracts the pro-S hydrogen at C-8 of 18:2n-6 and forms a carbon-centered radical, which reacts with O<sub>2</sub> in an antarafacial way and forms (8R)-HPODE (15). A tyrosyl radical can be detected by EPR in this process (14). (8R)-HPODE is isomerized to (7S,8S)-DiHODE by suprafacial hydroxylation and oxygenation at C-7 (16), catalyzed by a ferryl intermediate (PPIX Fe<sup>4+</sup> = O) (14). Cloning and sequencing of 7,8-LDS revealed that this enzyme was a member of the MPO gene family, which also contains other fatty acid dioxygenases, notably cyclooxygenases and α-DOX (17–19).

The biological importance of psi factors in the sporulation process of *A. nidulans* was further extended by Keller and co-workers (20–23). The genomes of *A. nidulans* and *A. fumigatus* were published in 2005 (24, 25). Keller and co-workers found with the aid of the 7,8-LDS sequence that both genomes contained three genes (*ppoA*, *ppoB*, and *ppoC*), which coded for putative fatty acid oxygenases of the MPO family with about 40% amino acid identity with 7,8-LDS (22). The exon-intron borders and the amino acid sequences of the gene transcripts could be deduced from sequence homology to 7,8-LDS, including homology to the presumed distal and proximal heme ligands of 7,8-LDS and the critical Tyr residue for catalysis. The deduced sequence of PpoA of *A. nidulans* was confirmed by cDNA analysis (23). Keller and co-workers (20–23) reported that deletion of these genes affected the ratio of asexual spores (conidia) to sexual spores (ascospores), the biosynthesis of (8R)-HODE, and mycotoxin production in *A. nidulans*. In addition to the impact on the sporulation process, deletion of these genes also led to alterations in virulence on host seed (20). Deletion of *ppoA* reduced formation of (8R)-HODE and increased the ratio of conidia to ascospores, whereas forced expression of *ppoA* had the opposite effect (23). Most recently, deletion of *ppoB* also increased conidia formation, whereas deletion of *ppoC* decreased conidia formation (20, 21). These results were recently extended to *A. fumigatus*. An initial study demonstrated that down-regulation of all three *A. fumigatus* ppo genes by RNA interference technology produced a hypervirulent strain (27). Further work showed that deletion of *ppoC* yielded a pleiotrophic phenotype with formation of aberrant conidia and increased virulence in a mouse model of aspergillosis. The biological effects of *ppo* gene loss in *A. nidulans* and *A. fumigatus* are summarized in Table 1.

Studies of recombinant 7,8-LDS suggested that (5S,8R)-DiHODE could be formed by an enzyme of *A. nidulans* with a closely related oxygenation mechanism (26). 7,8-LDS expressed in insect cells had similar properties as the native enzyme. 7,8-LDS expressed in *Pichia pastoris* oxygenated 18:2n-6 to (8R)-HPODE and (5,8S)-DiHODE and transformed exogenous (8R)-HPODE to (5,8R)-DiHODE (26). Mycelia and cell-free preparations of *A. nidulans* were found to oxidize 18:2n-6 to (8R)-HPODE and (5,8R)-DiHODE and transformed (8R)-HPODE to (5,8R)-DiHODE. In addition, (10R)-HODE was formed as a major metabolite under certain conditions (26, 29).

Fungi have been known to produce 10-HODE with R or S absolute configuration. The shiitake mushroom, *Lentinula edodes*, and the field mushroom form (10S)-HPODE, which can be transformed to an aroma compound, 1-octen-3-ol, or reduced to (10S)-HODE (30, 31). (10R)-HODE is formed by *Epichloë typhina* (32), and this stereoisomer also predominates in *G. graminis* and *A. nidulans* (26, 29, 33). The corresponding (10R)-hydroperoxide has not been identified, and little is known about the mechanism of biosynthesis of (10R)-HODE.

The first aim of the present study was to examine the biosynthesis of oxylipins from 18:2n-6 by the human pathogen *A. fumigatus*. We next extended these studies to *A. nidulans*, as this has traditionally been used as a model organism (4). We found that both species transformed 18:2n-6 to (8R)-HPODE/(8R)-HODE, (5S,8R)-DiHODE, and (10R)-HPODE/(10R)-...
Biosynthesis of Oxylipins by Aspergillus sp.

**TABLE 2**

Strains of Aspergillus sp. used in this study

| Strains of *A. fumigatus* | Genotype | Ref. |
|---------------------------|----------|-----|
| Fres.                     | Wild type | 34  |
| AF293                     | Wild type | 41  |
| TDWC1.13<sup>a</sup>      | Δ*ppoA; A. parasiticus (A.p) pyrG; pyrG1* | Footnote 7 |
| TDWC1.4<sup>b</sup>       | Δ*ppoB; A. parasiticus (A.p) pyrG; pyrG1* | Footnote 7 |
| TDWC1.7<sup>c</sup>       | Δ*ppoC::N::argB; argB1; pyrG1; ppoC::pyrG* | Footnote 7 |
| TDWC10.5<sup>d</sup>      | Wild type | 7   |

*Δ*ppoC::N::argB; argB1; *A. fumigatus* ppoA, ppoB, and ppoC genes were identified as described previously (27).<sup>a</sup>
*Δ*ppoC knock out was prepared using the *A. nidulans* argB cassette and the *pyrG1* and *argB1* double auxotrophic strain AF293.6.7<sup>b</sup>
TDWC10.5 was obtained by ectopic complementation with 5 kb of *ppoC* (containing 1 kb of its promoter<sup>d</sup>). All mutants were characterized by PCR and Southern hybridization.<sup>c</sup>

**Strains of *A. nidulans***

| Strains of *A. nidulans* | Genotype | Ref. |
|--------------------------|----------|-----|
| RDTI9.32                 | veA, wild type | 23  |
| RDTI12.9                 | *methG1; Δ*ppoA::*methG6; veA* | 23  |
| RDTI50.1                 | pyrA4; Δ*ppoB; pyroA6; veA | 22  |
| RDTI58.12                | Δ*ppoC::trpC; veA; trpC801 | 21  |

**EXPERIMENTAL PROCEDURES**

**Materials**

18:1n-9 (99%), 18:2n-6 (99%), 18:3n-3 (99%), 18:3n-6 (99%), and imidazole were from Merck. 18:2n-6 (94–96%) was from Carl Roth (Karlsruhe, Germany). [9,10,12,13-<sup>2</sup>H<sub>4</sub>]<sub>18:2n-6</sub> (99%), 16:3n-3 (99%), 17:3n-3 (99%), 19:3n-3 (99%), 20:2n-6 (99%), 10-KODE (99%), and 10-ODA (99%) were obtained from Larodon (Malmö, Sweden). 16:1n-7 (99%), malt extract, and TNM were from Sigma. [(11S)-<sup>2</sup>H]<sub>18:2n-6</sub> (>95%) and [(11R)-<sup>2</sup>H]<sub>18:2n-6</sub> were prepared as described (16), whereas [(8R)-<sup>2</sup>H]<sub>18:2n-6</sub> and [(5S)-<sup>2</sup>H]<sub>18:2n-6</sub> were synthesized as described in the Supplemental Material. The strains of *Aspergillus* sp. are summarized in Table 2 (cf. Refs. 20–23). *A. fumigatus* Fres. will be referred to as *A. fumigatus*, and this strain was a kind gift of Dr. Levenfors (MASE Laboratories, Sveriges Lantbruksuniversitet, Uppsala, Sweden (34)). Spores of *A. fumigatus* were isolated from mycelia on potato dextrose agar, harvested in 0.5% Bactopeptone (5 × 10<sup>7</sup> spores/ml), and kept at 4 °C. Solvents were HPLC grade from Merck and J. T. Baker Inc. Cartridges with C<sub>18</sub> silica and silica (SepPak/C<sub>18</sub> and SepPak, respectively) were from Waters. BW4AC was a kind gift from Wellcome Research Laboratories (Beckenham, UK), and stock solutions (10 mM) were made in ethanol. Zileuton was from Abbott. Paracetamol (acetaminophen) was obtained locally. (8R,11S)-DiHODE was obtained as described (11, 29).

**Fungal Preparations**

**Fungal Growth**—*A. fumigatus* and *A. nidulans* were grown in liquid media (1.5% malt extract) from spores or mycelia on agar in a rotary shaker (150 rpm) at 37 or 22 °C (dark or in laboratory light) for 3–10 days. Mycelia were harvested by filtration, washed with saline, and then used directly or blotted dry and ground to a fine powder in liquid nitrogen. *A. fumigatus* and *A. nidulans* were also grown in 9-cm plastic Petri dishes either in the dark or 50 cm under a fluorescent lamp (30 watts, Tr-lite fluorescent, Duro-test, Fairfield, NJ, or without light-dark cycles) for a few days at 22 or 37 °C. Colonies were picked by forceps, blotted dry, and incubated with 18:2n-6.

**Nitrogen Powder of *A. fumigatus* and *A. nidulans***—*A. nidulans* was grown in liquid culture for 3 days at 37 °C (150 rpm), and *A. fumigatus* was grown for 24 h at 37 °C and then at room temperature (150 rpm) for 48 h. *Mycelia* (10–20 g) were harvested by filtration, washed with saline, and ground with liquid nitrogen to a fine powder, which was stored at −80 °C. The nitrogen powder was homogenized (glass-Teflon, 10 passes; 4 °C) in 10 volumes (w/v) of 0.1 M KHPO<sub>4</sub> buffer (pH 7.3), 2 M EDTA, 0.04% Tween 20, centrifuged at 13,000 × g (10 min, 4 °C), and used immediately for enzyme assay.

**Enzyme Assays**

**Incubation with Mycelia**—Mycelia (0.5–20 g) were incubated with 5 volumes (w/v) of 0.1 M NaBO<sub>3</sub> buffer (pH 8.0 or 8.2) containing 18:2n-6 (0.5–1 mg/ml) for 5–6 h at 22 °C with shaking. The pH of the incubation buffer was typically above pH 7.3 at the end. The mycelia were separated from the incubation medium by filtration. Medium from large scale incubations was extracted with ethyl acetate and from small scale incubations on SepPak/C<sub>18</sub>. The ethyl acetate extract was dried (Na<sub>2</sub>SO<sub>4</sub>) and evaporated to dryness, and the products were purified by preparative TLC (ethyl acetate/hexane/acetic acid, 60:40:0.01) or by silicic acid chromatography. The latter was performed on a Sep/Pak cartridge or a short column with silica (Silicar CC-4, Mallinkrodt), which were eluted stepwise with increasing concentrations of diethyl ether (7, 25, and 50%) in hexane and finally with ethanol; 8- and 10-HPODE were eluted with 25%
ether. The major metabolites were identified by LC-MS and by gas chromatography-MS analysis.

**Incorporation with Subcellular Fractions**—An aliquot (0.5 ml) of nitrogen powder supernatant was incubated with 30–100 μM of 18:2n-6, 30–50 μM [13H]18:2n-6 or 15 μM (8R)-HPODE for 30–45 min on ice. The reaction was terminated with 0.5 ml of methanol, and the products were extracted on a cartridge of C18 silica (SepPak/C18, Waters) as described (29). In some experiments, 50 pmol of (13R)-[3H]HODE was added as an internal standard, and TPP (10 μg) was added to reduce hydroperoxides to alcohols. The formation of (8R)-HODE and other oxylipins was quantified with help of the internal standard, (13R)-[3H] HODE, as follows: 8-HODE, MS/MS analysis (m/z 295 → full scan) with monitoring of m/z 157; (13R)-[3H] HODE, MS/MS analysis (m/z 299 → full scan) with monitoring of m/z 198; (5S,8R)-DiHODE, MS/MS analysis (m/z 173 → full scan); (8R,11S)-DiHODE, MS/MS analysis (m/z 213 → full scan). Standard curves were prepared with 50 pmol of (13R)-[3H]HODE and variable amounts of (8R)-HODE from a stock solution. The concentration of (8R)-HODE was determined by conversion to (8R,13R)-DiHODE by oxidation with manganese lipoxygenase (UV analysis) as described (29). Effects of drugs were assessed in duplicates with 100 μM 18:2n-6 as substrate.

**LC-MS/MS Analysis**

An ion trap mass spectrometer (LTQ, Thermo Fisher Scientific) was used with electrospray ionization and monitoring of negative ions. The pump for HPLC was from Thermo Fisher Scientific (Surveyor MS). The HPLC columns contained octadecyl silica (5-μm, 150 × 2 mm) and were usually eluted with methanol/water/acetic acid, 75:25:0.01 or 80:20:0.01, at 0.2–0.3 ml/min. The capillary temperature was 325 °C, and prostaglandin F1α was used for tuning. For analysis of deuterium labeling of products formed from [13H]18:2n-6, we used MS/MS analysis of m/z 295 and 311 (isolation width 6 atomic mass units) and studied with the zoom scan rate function over 10 min. The main product at 22 °C and the latter at 37 °C (cf. Ref. 29). At 22 °C, (10R)-HODE was formed as a major metabolite (Fig. 1C); this difference between 22 and 37 °C remained after incubation of mycelia for 10 days with a relative increase in (10R)-HODE formation (cf. Fig. 1B). Steric analysis by CP-HPLC-MS/MS showed that 10-HODE, isolated from mycelia grown at 22 °C consisted of ~98% of the R stereoisomer (Fig. 1D, cf. Ref. 29). *A. fumigatus* formed (8R)-HODE with ~95% stereoselectivity (29).

The relative amounts of (8R,11S)- and (5S,8R)-DiHODE also appeared to be changed by temperature. The former was the main product at 22 °C and the latter at 37 °C (cf. Fig. 1, A and C).

**Results**

**Oxylipin Biosynthesis by A. fumigatus**

Mycelia of *A. fumigatus*, which had been growing in liquid culture at 37 °C in the dark, were incubated with 18:2n-6 at room temperature, and the products were analyzed by RP-HPLC-MS/MS. The profiles of oxylipins formed are shown in Fig. 1A. The products were identified by LC-MS/MS analysis, and the stereoisomers were analyzed by CP-HPLC-MS/MS and NP-HPLC-MS (cf. Refs. 29 and 35) and as discussed below. The two major dihydroxy fatty acids were (8R,11S)-DiHODE and (5S,8R)-DiHODE, and they eluted in this order. (8R,13)-DiHODEs were also noticed in some experiments. The latter eluted before 8,11-DiHODE on RP-HPLC and was likely formed from (8R,11S)-DiHODE by during the isolation procedure (29). MS/MS analysis showed that the main hydroxy fatty acid was (8R)-HODE, but ~10% (10R)-HODE was also detected (29), as judged from the reconstructed ion chromatograms of m/z 183 (10-HODE; OOC-CH2=CH=CH-CHO) and m/z 157 (8-HODE; OOC-CH2=CH-CHO) during MS/MS analysis.

We next examined whether growth conditions (fluorescent light/darkness, growth temperature, growth length) changed the oxylipin profile. The results are summarized in Fig. 1B. Temperature (22 versus 37 °C) had the greatest effect and increased the relative formation of (10R)-HODE in comparison with (8R)-HODE, whereas fluorescent light yielded inconsistent results (Fig. 1B). At 22 °C, (10R)-HODE was formed as a major metabolite (Fig. 1C); this difference between 22 and 37 °C remained after incubation of mycelia for 10 days with a relative increase in (10R)-HODE formation (cf. Fig. 1B). Steric analysis by CP-HPLC-MS/MS showed that 10-HODE, isolated from mycelia grown at 22 °C consisted of ~98% of the R stereoisomer (Fig. 1D, cf. Ref. 29). *A. fumigatus* formed (8R)-HODE with ~95% stereoselectivity (29).

**Isolation and Identification of (8R)-HPODE and (10R)-HPODE**

(8R)-HPODE was obtained in milligram amounts in some experiments by incubation of mycelia of *A. fumigatus* with 18:2n-6, as illustrated by LC-MS/MS analysis in Fig. 2A. The MS/MS spectrum (m/z 311 → m/z 293 → full scan) of 8-HPODE was as reported (35). (8R)-HPODE was then purified by silicic acid chromatography. The purity was assessed after reduction with TPP.
Hydroperoxy fatty acids will be partly dehydrated to the corresponding keto fatty acids in the heated transfer line of the mass spectrometer (35, 36). We found that cell-free preparations (nitrogen powder) of mycelia of A. fumigatus transformed 18:2n-6 to 10-ODA, 10-KODE, and to 10-HPODE, as shown in Fig. 2B. 10-HPODE was identified by the signal at m/z 311 in full scan, which was associated with signals at m/z 183 (carboxylate anion of 10-ODA) and m/z 293 (carboxylate anion of 10-KODE). Incubation with [9,10,12,13-2H4]18:2n-6 yielded consistent MS/MS results. This suggested that (10R)-HPODE was transformed to 10-KODE and to 10-ODA in the heated transfer system of the mass spectrometer. In addition, (10R)-HPODE will also converted to 10-ODA and to 10-KODE during the incubation, whereas (8R)-HPODE was partly transformed to 8-KODE (cf. Fig. 2B). These transformations are catalyzed by metals and metalloproteins (37).

We compared the transformation of (10R)-HPODE to 10-ODA by subcellular fractions of A. fumigatus with a heat-inactivated control without detecting significant hydroperoxide lyase activity. The MS/MS spectra of 10-KODE and 10-ODA are shown in Fig. 3, A and B. The MS/MS spectrum of 10-KODE (m/z 293 → full scan) showed signals, inter alia, at m/z 275 (A−18), m/z 249 (A−44), m/z 233, and in the lower mass range at m/z 199, m/z 155, m/z 153, m/z 139, and m/z 137. Authentic 10-KODE yielded an identical MS/MS spectrum. The corresponding MS/MS spectrum of 10-[9,12,13-2H3]KODE, obtained by incubation with [9,10,12,13-2H4]18:2n-6, showed signals, inter alia, at m/z 277 and m/z 278, m/z 252, m/z 201, and in the lower mass range at m/z 155 and 156, and at m/z 138 and 139.

The MS/MS spectrum of 10-ODA (m/z 183 → full scan) showed signals at m/z 165 (A−18), m/z 155 (A−28, unidentified, possibly loss of CO), and m/z 139 (A−44), and the spectrum of authentic 10-ODA was identical. The corresponding MS/MS spectrum of 10-ODA, derived from an incubation with [9,10,12,13-2H4]18:2n-6, showed major signals at m/z 167 and 166, m/z 157 and 156, m/z 140 and 141. This spectrum suggested that 10-[9,10-2H2]ODA was formed.

The mycelia were harvested and incubated with 18:2n-6 for 6 h at 22 °C. Top trace, total ion current (TIC); middle trace, MS/MS analysis (m/z 295 → full scan) of HODE; bottom trace, MS/MS analysis (m/z 311 → full scan) of DIHODE. The RP-HPLC column was eluted with methanol/water/acetic acid, 750:250:0.1. The major oxylipins are marked except 8,11-DiHODE, which eluted after 4.5 min. B, relative formation of (8R)-HODE and (10R)-HODE by mycelia in liquid culture, which were grown for 3 days at 37 °C (in dark or fluorescent light) and for 3 and 10 days at 22 °C (in dark or light). The traces show the reconstructed ion chromatograms (MS/MS analysis; m/z 295 → full scan) of m/z 157 (8-HODE) and m/z 183 (10-HODE), normalized to the largest of the two peaks. The RP-HPLC column was eluted with methanol/water/acetic acid, 800:200:0.1. The area under the two peaks were measured by integration (XCalibur software) and used as relative measures of biosynthesis of 10- and 8-HODE. MA%, measured area of m/z 183 in percent of total area under m/z 157 and m/z 183. D, dark; L, light. C, reconstructed ion chromatograms of oxylipins formed by A. fumigatus grown in liquid media at 37 °C. Top trace, total ion current (TIC); middle trace, MS/MS analysis (m/z 295 → full scan) of 10-HODE; bottom trace, MS/MS analysis (m/z 311 → full scan) of DIHODE. D, CP-HPLC analysis of mycelia grown at 22 °C. Integration of the signal at m/z 183 suggested that 98% of R stereoisomer of 10-HODE was formed. The Reprosil Chiral-NR column was eluted with 3% isopropyl alcohol in hexane (0.25 ml/min).

**FIGURE 1.** Identification by LC-MS/MS analysis of major oxylipins formed from 18:2n-6 by mycelia of A. fumigatus. A, reconstructed ion chromatograms of oxylipins formed by A. fumigatus grown in liquid culture at 37 °C (dark).
Mechanism of Biosynthesis of DiHODE and HPODE

Cell-free preparations of A. fumigatus transformed (8R)-HPODE to (5S,8R)-DiHODE and (8R,11S)-DiHODE, but (8R)-HPODE did not increase the biosynthesis of (10R)-HODE (Fig. 4). We conclude that (8R)-HPODE is transformed into the two diols by hydroperoxide isomerases, whereas (10R)-HODE is apparently formed directly from 18:2n-6.

The reaction mechanism of the (8R)- and (10R)-DOX and the hydroperoxide isomerases were studied with stereospecifically deuterated 18:2n-6 as substrates for oxygenases/hydroperoxide isomerases of cell-free preparations of A. fumigatus. The results are summarized in Table 3.

A. fumigatus metabolized [(8R)-2H]18:2n-6 to (8R)-HODE with retention of the pro-R hydrogen at C-8, and this deuterium atom was also retained in (5S,8R)-DiHODE, (8R,11S)-DiHODE, and (10R)-HODE (Fig. 3C). Formation of some fragments is suggested by the inset of the MS/MS analysis of (8R)- and (10R)-HODE (m/z 293 → full scan) showed that the major peaks of 8-KODE and 10-KODE co-eluted with (8R)-HPODE and (10R)-HPODE, respectively. Pre-formed 10-KODE and 8-KODE eluted before and after the two hydroperoxides.

These results suggest that biosynthesis of (8R)-HPODE occurs by abstraction of the pro-S hydrogen at C-8 and ant-
arafacial insertion of $O_2$, whereas biosynthesis of (10$R$)-HPODE occurs by abstraction of the hydrogen at C-8, double bond migration to C-8 and C-9, and antarafacial dioxygenation at C-10 of the planar structure of C-8 to C-10. The hydroperoxidase activities, leading to hydroxylation reactions at C-5 and C-11, apparently occur by abstraction of the pro-S hydrogens at C-5 and C-11 of (8$R$)-HPODE and suprafacial insertion of oxygen.

**Effects of Inhibitors on Oxylipin Biosynthesis**

TNM causes nitration of tyrosine residues and inhibits 7,8-LDS, possibly by blocking formation of the tyrosyl radical (14), whereas BW4AC is a redox inhibitor of lipoxygenases and 7,8-LDS, possibly by blocking formation of the tyrosyl radical (14), whereas BW4AC is a redox inhibitor of lipoxygenases and 7,8-LDS, possibly by blocking formation of the tyrosyl radical (14).

Oxygenation of C$_{16}$-C$_{20}$ Fatty Acids—The transformation of unsaturated C$_{16}$-C$_{20}$ fatty acids by the fatty acid oxygenases of *A. fumigatus* is summarized in Table 4. (10$R$)-DOX apparently oxidized 16:1$\alpha$-8, 18:1$\alpha$-8, 18:2$\alpha$-6, and 18:3$\alpha$-6. Several fatty acids were oxidized at their C-8 carbon and the corresponding hydroperoxide was apparently transformed to 5,8- and 8,11-diols. This seemed to require a saturated carbon chain from the carboxyl group to the first double bond of 7 or 9 carbons.

The LC-MS/MS analysis showed that absence of double bonds in the $\omega$-6 position changed the fragmentation pattern. MS/MS analysis of 10-HODE showed a characteristic fragment at m/z 183 ($^{18}OOC(CH_2)_6-CH=CH-CHO$), whereas 10-HOME showed a characteristic intense signal at m/z 155 ($^{18}OOC(CH_2)_{16}-CH=CH_2$).

**Effects of Deletion of ppoA and ppoC on Oxylipin Biosynthesis by *A. fumigatus* AF293**

*A. fumigatus* AF293 formed (8$R$)-H(P)ODE, (5S,8$R$)-DiHODE, and (10$R$)-H(P)ODE as major metabolites. We could not detect biosynthesis of 8,11-DiHODE with certainty, but we cannot exclude that traces of this metabolite could be formed. Deletion of *ppoA* resulted in complete loss of biosynthesis of (5S,8$R$)-DiHODE, as judged from RP-HPLC analysis, and deletion of *ppoC* led to diminished biosynthesis of (10$R$)-HODE (Table 5). Deletion of *ppoB* had little effect on oxylipin formation and transformed 18:2$\alpha$-6 in the same way as *A. fumigatus* AF293.

**Oxygenation of Fatty Acids by *A. nidulans***

In agreement with the original report by Champe and co-workers (4, 5), mycelia of *A. nidulans* grown at 37 °C transformed 18:2$\alpha$-6 to (8$R$)-HODE and (5S,8$R$)-DiHODE as major products. In addition, we also noticed biosynthesis of (8$R$)-HPODE and (10$R$)-HODE (29). Biosynthesis of (10$R$)-HODE was confirmed in cell-free preparations of *A. nidulans*, as described above for *A.

---

**TABLE 3**

Isotopic composition of stereospecifically deuterated linoleic acids and their oxygenated products formed by *A. fumigatus*

| Substrate                  | Monodeuterated molecules$^a$ | Product                  | Monodeuterated molecules | Retention of deuteron |
|----------------------------|------------------------------|--------------------------|--------------------------|-----------------------|
| [(5S)-$^2$H]18:2$\alpha$-6 | 24                           | 55,8$R$-DiHODE           | 0.6                      | 89                    |
| [(8R)-$^2$H]18:2$\alpha$-6 | 64                           | 8$R$-HODE                | 61                       | 95                    |
| [(11S)-$^2$H]18:2$\alpha$-6| 95                           | 8$R$,11S-DiHODE          | 1.0                      | <1                    |
| [(11R)-$^2$H]18:2$\alpha$-6| 25                           | 8$R$,11S-DiHODE          | 0.6                      | <1                    |

$^a$ The isotopic compositions of the linoleic acids were determined by selected monitoring of the ions m/z 294 and 295 (M+, methyl linoleates; gas chromatography-MS with electron impact ionization). LC-MS/MS analysis in the full scan mode yielded slightly lower values. The deuterium content of 18:2$\alpha$-6 in the incubations was reduced slightly due to the endogenous 18:2$\alpha$-6 present in the incubations.

$^b$ The isotopic compositions of the oxygenated products were determined by HPLC-MS/MS analysis of the following ions: (8$R$)-HODE, m/z 157 and 158; (10$R$)-HODE, m/z 183 and 184; (5S,8$R$)-DiHODE, m/z 173 and 174; (8$R$,11S)-DiHODE, m/z 213 and 214.

---

**Figures**

**FIGURE 4.** Transformation of (8$R$)-HPODE by *A. fumigatus*. The relative abundance of (8$R$,11S)-DiHODE, (5S,8$R$)-DiHODE, and (10$R$)-HODE was estimated with the aid of the internal standard in incubations with (open box) or without (8$R$)-HPODE (black box). Mean ± S.D. of three experiments.

**FIGURE 5.** Effects of TNM and BW4AC on the (8$R$)- and (10$R$)-DOX activities of *A. fumigatus*. The relative abundance of the integrated signal intensities at m/z 183 (10-HODE, black bars) and m/z 157 (8-HODE, white bars) were set to 100% in control incubations of nitrogen powder of *A. fumigatus*, and the drug effects are given in percent of these values (mean ± S.D.).
**Biosynthesis of Oxylipins by Aspergillus sp.**

### TABLE 4

Oxygenation of unsaturated fatty acids by dioxygenases and hydroperoxide isomerases of *A. fumigatus*

The products were identified by MS/MS analysis of the carboxylate anions (A⁻ → full scan) and by detection of characteristic fragments.

| Fatty acid | Products related to oxygenation by | 10R-DOX | 8R-DOX | 5,8-LDS | 8,11-LDS |
|------------|----------------------------------|---------|---------|---------|---------|
| 16:1n-7    | 10-HHME                          | 8-HHME  | 5,8-DHHME | 8,11-DHHME |
| 18:1n-9    | 10-HOME                          | 8-HOME  | 5,8-DHOME | 8,11-DHOME |
| 18:2n-6    | 10-HODE                          | 8-HODE  | 5,8-DHODE | 8,11-DHODE |
| 18:3n-3    | 10-HOTrE                         | 8-HOTrE | 5,8-DHOTrE | 8,11-DHOTrE |
| 19:3n-3    | 11-HNTrE                         | 9-HNTrE | ND      | ND      |
| 20:2n-6    | 10-HEDE                          | 7,10-DHEDE | 10,13-DHEDE |

* 8,11-DHHME and 8,11-DHOME fragmented during LC-MS/MS analysis fragmented between C-10 and C-11 with formation of a characteristic signal at m/z 185.
* OOC-(CH2)5-CH(OH)-CH2, 16:3n-3, 17:3n-3, and 18:3n-6 were not oxidized in appreciable amounts.
* DDHTrE indicates dihydroxyoctadecatrienoic acid, HHME indicates hydroxyhexadecenoic acid, and HNTrE indicates hydroxynonadecatrienoic acid.
* ND indicates not detected.

### TABLE 5

Relative abundance of characteristic ions during LC-MS/MS analysis of 5,8R-DiHODE, 8R-HODE, and 10R-HODE formed by *A. fumigatus* AF293 and its two mutants ΔppoA and ΔppoC.

| Strains | Deleted presumed oxygenase (common name) | Relative abundance | 5,8-DiHODE m/z 173 | 8-HODE m/z 157 | 10-HODE m/z 183 |
|---------|------------------------------------------|-------------------|-------------------|----------------|----------------|
| TDWC10.5 | None (wild type)                         | %                 | %                 | %              |               |
| TDWC11.3 | Exp. 1                                   | EAL98912 (ΔppoA)  | <0.01b            | 14%           | 86            |
| TDWC16.7 | Exp. 1                                   | EAL92371 (ΔppoC)  | 14                | 85             | 1              |

* The strains were grown at 37 °C for 3 days before incubation with 18:2n-6. The strains are described in Table 2. TDWC10.5 is TDWC4.17 with reconstitution of the ppoC gene.
* 5,8-DiHODE was undetectable.
* Steric analysis showed moderate access of the 8R stereoisomer (~65% R and ~35% S).

### TABLE 6

Relative abundance of characteristic ions of 5,8-DiHODE, 8-HODE, and 10-HODE formed by *A. nidulans* and its two mutants ΔppoA and ΔppoC.

| Strains | Deleted oxygenase | Relative abundance | 5,8-DiHODE m/z 173 | 8-HODE m/z 157 | 10-HODE m/z 183 |
|---------|-------------------|--------------------|-------------------|----------------|----------------|
| Wild type Exp. 1 | 15 | 75 | 10 | | |
| Wild type Exp. 2 | 17 | 56 | 27 | | |
| Wild type Exp. 3 | 11 | 65 | 24 | | |
| ΔppoA Exp. 1 | AAR88626 | <0.1 | 9a | 91 | |
| ΔppoA Exp. 2 | <0.1 | 6 | 94 | | |
| ΔppoA Exp. 3 | <0.1 | 11 | 89 | | |
| ΔppoC Exp. 1 | AAT36614 | 34 | 63 | 3b | |
| ΔppoC Exp. 2 | 14 | 83 | 3 | | |
| ΔppoC Exp. 3 | 32 | 63 | 9 | | |

* The strains of *A. nidulans* were grown at 37 °C for 3 days before incubation with 18:2n-6 in experiment 1 and after a few additional days at 22 °C in experiments 2 and 3.
* CP-HPLC analysis showed that 8-HODE formed by the ΔppoA strain contained moderate access of the R stereoisomer (~65% R, 35% S).
* CP-HPLC analysis showed that 10-HODE formed by ΔppoC was racemic (cf. Fig. 7). The strains are described in Table 2.

**Wild Type and ΔppoB**—These two strains appeared to oxidize 18:2n-6 to the same spectrum of metabolites, e.g. (8R)-H(P)ODE, (10R)-H(P)ODE, and (5,5R)-DiHODE, although the relative amounts differed. NP-HPLC of the hydroxy fatty acids formed by reduction with TPP did not reveal any qualitative difference in formation of hydroxy fatty acids between the wild type and ΔppoB. Biosynthesis of (8R,11S)-DiHODE could not be detected.

**ΔppoA**—This strain had completely lost the capacity to form (5S,8R)-DiHODE, and the biosynthesis of (8R)-HODE was strongly reduced (Table 6). The main metabolite of 18:2n-6 was (10R)-HODE. The (10R)-DOX activity appeared to increase when the mycelia were grown at room temperature compared with 37 °C (Table 6). Steric analysis showed that 8-HODE contained some excess of the 8R stereoisomer (65%), suggesting enzymatic biosynthesis, possibly related to (10R)-DOX activity. Incubation of nitrogen powder of ΔppoA with 18:2n-6 led to identification of 10-ODA, 10-KODE, and 10-HPODE (Fig. 6), and products formed from [9,10,12,13-2H4]18:2n-6 yielded consistent results. Steric analysis showed that 10-HODE consisted mainly of the 10R stereoisomer (Fig. 7A). We conclude that ppoA may code for 5,8-LDS.

**ΔppoC**—This strain had reduced the capacity to form (10R)-HODE (Table 6), and steric analysis of the small amounts of 10-HODE formed by this mutant showed that it was racemic (Fig. 7B). We conclude that ppoC codes for linoleate (10R)-DOX.

**DISCUSSION**

We have studied three previously uncharacterized fatty acid oxygenases of the human pathogen *A. fumigatus* and extended the results to the model organism, *A. nidulans*. (10R)-DOX and 5,8-LDS were found in both species and 8,11-LDS in *A. fumiga-
We have studied their reaction mechanisms by aid of stereospecifically deuterated 18:2n-6 and conclusively identified the genes of 5,8-LDS and (10R)-DOX by gene targeting of *ppoA* and *ppoC*. The genes are homologous and belong to the MPO family along with 7,8-LDS, cyclooxygenases, and α-DOX. Our results suggest that diol synthases may have a common reaction mechanism.

The three *Aspergillus* oxygenases catalyze abstraction of the pro-5 hydrogen at C-8 of 18:2n-6. Antarafacial insertion of O₂ occurred either at C-8 with formation of (8R)-HPODE by the two diol synthases or, after double bond migration, at C-10 with formation of (10R)-HPODE by (10R)-DOX (Fig. 8). This steric course is in agreement with the antarafacial relationship between hydrogen abstraction and oxygen insertion found for most lipoxygenases and for cyclooxygenases-1 and -2 (39). A few dioxygenase reactions deviate from this rule. Manganese lipoxygenase catalyzes suprafacial formation of linoleic acid 11- and 13-hydroperoxides (40), and the major α-DOX of tobacco and *Arabidopsis* produces α-hydroperoxides with retention of absolute configuration at C-2 (19). Furthermore, (10S)-DOX from the field mushroom (*A. bisporus*) catalyzes formation of (10S)-HPODE in a reaction that involves suprafacial stereochemistry. In the next step (Fig. 8), (8R)-HPODE was transformed to (5S,8R)-DiHODE or to (8R,11S)-DiHODE. Studies with stereospecifically deuterated 18:2n-6 at positions 5 and 11 suggested that these diols were formed by suprafacial hydrogen abstraction and oxygenation; cytochromes P450s catalyze aliphatic hydroxylation by the same mechanism (39). The results are also summarized in Table 3. The oxygenation mechanism of 5,8- and 8,11-LDS is similar to 7,8-LDS of *G. graminis* (14).

(10R)-HPODE partly decomposed to 10-ODA and 10-KODE during incubation (37), and it was also reduced to (10R)-HODE (Figs. 3B and 6). We could not detect transformation to diols. 10-ODA and 10-KODE were also formed during the LC-MS/MS analysis of (10R)-HPODE, presumably in the heated capillary. The transformation of (10R)-HPODE to 10-ODA appeared to be mainly nonenzymatic, as it was noted to a similar extent in heat-inactivated controls.

The genes of 5,8-LDS and (10R)-DOX were identified by gene targeting. Deletion of *ppoA* of both *Aspergilli* resulted in complete loss of biosynthesis of (5S,8R)-DiHODE and to biosynthesis of only small amounts of (8R)-H(P)ODE, whereas (10R)-HPODE now was formed as the major metabolite. Steric analysis showed that 8-HODE from these *ppoA* mutants contained moderate excess of the R stereoisomer. Deletion of *ppoC* resulted in almost complete loss of biosynthesis of (10R)-H(P)ODE, and the small amounts of 10-HODE formed was a racemic mixture of R and S stereoisomers. The changes in product formation in the *ppoA* and *ppoC* mutants (Tables 4 and 5) may not only be due to loss of the particular gene in question but also to a release of feedback inhibition observed by previous transcript analysis of *ppo* expression in *ppo* mutants (8, 10). We conclude that *ppoA* codes for 5,8-LDS and *ppoC* for (10R)-DOX in both species. Table 7 summarizes these findings. The two 5,8-LDS enzymes can be aligned with 78% and the two (10R)-DOX enzymes with 66% amino acid identity. Whether 8-HODE can be formed by (10R)-DOX as a minor product or by other fungal enzymes awaits further studies.

Interestingly, the biosynthesis of (10R)-HPODE was augmented in both species grown at 22 °C compared with 37 °C. Deletion of *ppoC* in *A. fumigatus* augments the survival of this mutant in a mouse model of invasive aspergillosis. Whether biosynthesis of (10R)-HPODE will affect the virulence of *A. fumigatus* may merit further investigation, as little is known about its virulence factors. In contrast, loss of *ppoC* had no effect on virulence in the *A. nidulans*/seed interaction. How-

---

* M. Hamberg and E. Oliw, unpublished observations.
ever, a double mutant deleted in both ppoC and ppoA showed a decrease in virulence on peanut seed (20). These results suggest possible different roles of oxylipins dependent on the host/fungal pathosystem.

Both A. nidulans and A. fumigatus contain a third gene (ppoB, see Table 2 and Table 7) that might code for fatty acid oxygenases. Alignment suggests that these genes may code for proteins with less than 40% amino acid identity and may not be closely related. Loss of ppoB had no discernible effect on virulence in A. fumigatus but had in A. nidulans a large effect on increasing virulence on seed. Disruption of ppoB did not influence oxylipin biosynthesis in this study. Recent mRNA analysis shows A. nidulans ppoB to be a pathogenesis-induced gene, which may explain why changes in oxylipin profile were not observed in this strain in the present study. A. fumigatus AF293 may only produce traces of (8R,11S)-DiHODE, whereas A. fumigatus Fres. formed this metabolite as one of the major products. In analogy, Aspergillus clavatus contains three genes with homology to the three oxygenase genes of A. fumigatus, and this fungus also expresses 5,8-LDS, 8,11-LDS, and (10R)-DOX activity. It is possible that the PpoB of A. fumigatus forms 8,11-LDS, a topic of further studies.

The MPO family contains fatty acid dioxygenases with hydrogen abstraction by a tyrosyl radical as a common feature. The homology of oxygenases of A. nidulans and A. fumigatus includes the distal and proximal heme His ligands and the catalytically important Tyr residue of cyclooxygenases. In agreement with this oxygenation mechanism, TNM (30–100 μM) inhibited the (8R)- and (10R)-DOX activities of A. fumigatus, possibly by interfering with the oxygenation mechanism by nitration of Tyr residues.

FIGURE 8. Overview over the reaction mechanism of 5,8-LDS, 8,11-LDS, and (10R)-DOX. In the first step marked (i), the pro-5 hydrogen at C-8 of 18:2n-6 is abstracted with formation of a carbon-centered radical by all three enzymes, 5,8-LDS, 8,11-LDS, and (10R)-DOX. Oxygen is then inserted at C-8 in an antarafacial way with formation of (8R)-HPODE by the (8R)-DOX activities of 8,11-LDS and 5,8-LDS. (8R)-HPODE is isomerized to (8R,11S)-DiHODE by 8,11-LDS, to (5S,8R)-DiHODE by 5,8-LDS, or reduced to (8R)-HODE, as indicated in the top right column. Alternatively, the 9–10 double bond migrates to position 8–9, and oxygen is inserted in the carbon-centered radical at C-10 in an antarafacial way in relation to the C-8 to C-10 structural element with formation of (10R)-HPODE by (10R)-DOX. (10R)-HPODE can be reduced to (10R)-HODE and decomposed to 10-KODE and 10-ODA by metal complexes (37), as indicated in the bottom right column.

D. Tsitsigiannis and N. P. Keller, submitted for publication.

F. Jernérén, U. Garscha, M. Hamberg, and E. Oliw, unpublished data.
TABLE 7
Overview of genes of A. nidulans and A. fumigatus with homology to 7,8-LDS

| Organism            | Identified genes and oxygenases by gene targeting | LOCUS       | Enzyme          | Common name (NCBI) |
|---------------------|---------------------------------------------------|-------------|-----------------|-------------------|
| A. nidulans         |                                                  | AAR88626    | 5,8-LDS         | PpoA*             |
| A. fumigatus        |                                                  | EAL89712    | 5,8-LDS         | PpoA              |
| A. nidulans         |                                                  | AAT36614    | 10R-DOX         | PpoC              |
| A. fumigatus        |                                                  | EAL92371    | 10R-DOX         | PpoC              |
| Presumed fatty acid oxygenases |                                  | AAX35769    | Unknown         | PpoB*             |
| A. nidulans         |                                                  | EAL84400    | 8,11-LDS        | None              |

* 5,8-LDS oxygenates 18:2n-6 to psi factors.
* The biological function of [10R]-DOX and its products are unknown.
* The biological effects of deletion of this presumed oxygenase gene have been investigated (27).
* Tentatively identified by exclusion, as the genome of A. fumigatus contains only three genes with homology to 7,8-LDS: 5,8-LDS, (10R)-DOX, and 8,11-LDS activities were detected in A. fumigatus Fres, and the two former were identified by gene targeting of A. fumigatus AF293.

What are the structural differences between (8R)-DOX and (10R)-DOX? As discussed above, a Tyr radical formed by both groups of enzymes likely abstracts the pro-S hydrogen at C-8 of 18:2n-6, but oxygen then reacts either at C-8 or at C-10 of 18:2n-6. It is known from lipoxygenase biochemistry that mutation of a single amino acid can change the position of oxygenation (26). It is therefore of interest to determine the conserved differences in the primary sequences of 5,8-LDS and (10R)-DOX and to compare them with homologous positions in cyclooxygenases. Replacement of Ser-530 of cyclooxygenase-1 with threonine or acetylation of the corresponding Ser residue of cyclooxygenase-2 with aspirin shifted the position of oxygenation (26). It is therefore of interest to determine the conserved differences in the primary sequences of 5,8-LDS and (10R)-DOX and to compare them with homologous positions in cyclooxygenases. Replacement of Ser-530 of cyclooxygenase-1 with threonine or acetylation of the corresponding Ser residue of cyclooxygenase-2 with aspirin shifted the position of oxygenation (26).

In summary, we have identified novel oxygenases and oxylipins in two Aspergillus sp. Our results indicate that dioxygenases and (10R)-DOX have fundamental catalytic and structural properties in common. The genes of these dioxygenases and (10R)-DOX of A. nidulans and A. fumigatus have been deleted, and the resulting phenotypes can now be interpreted in the light of our report.

Acknowledgments—We thank Drs. V. Laszlo and G. Csaba (Semmelweis University, Hungary) for the generous gift of Tetrahymena pyriformis.

REFERENCES
1. Wilson, D. M., Mukhtar, W., and Juric, Z. (2002) Adv. Exp. Med. Biol. 504, 3–17
2. Jones, M. G. (2007) Microbiology 153, 1–6
3. Doonan, J. H. (1992) J. Cell Sci. 103, 599–611
4. Champe, S. P. and el-Zayat, A. A. (1989) J. Bacteriol. 171, 3982–3988
5. Mazur, P., Meyers, H. V., Nakanishi, K., El-Zayat, A. A. E., and Champe, S. P. (1990) Tetrahedron Lett. 31, 3837–3840
6. Bowers, W. S., Hoc, H. C., Evans, P. H., and Kayatama, M. (1986) Science 232, 105–106
7. Brodovsky, I. D., and Oliw, E. H. (1993) Biochim. Biophys. Acta 1168, 68–72
8. Brodovsky, I. D., and Oliw, E. H. (1992) Biochim. Biophys. Acta 1124, 59–65
9. Fox, S. R., Akpinar, A., Prabhune, A. A., Friend, J., and Ratledge, C. (2000) Lipids 35, 23–30
10. Cristea, M., Osbourn, A. E., and Oliw, E. H. (2003) Lipids 38, 1275–1280
11. Wadman, M. W., van Zadelhoff, G., Hamberg, M., Visser, T., Veldink, G. A., and Vliegenthart, J. F. (2005) Lipids 40, 1163–1170
12. Brodovsky, I. D., Hamberg, M., and Oliw, E. H. (1992) J. Biol. Chem. 267, 14738–14745
13. Su, C., and Oliw, E. H. (1996) J. Biol. Chem. 271, 14112–14118
14. Su, C., Sahlin, M., and Oliw, E. H. (1998) J. Biol. Chem. 273, 20744–20751
15. Hamberg, M., Gerwick, W. H., and Asén, P. A. (1992) Lipids 27, 487–493
16. Hamberg, M., Zhang, J. Y., Brodovsky, I. D., and Oliw, E. H. (1994) Arch. Biochem. Biophys. 309, 77–80
17. Hörsten, L., Su, C., Osbourn, A. E., Garosi, P., Hellman, U., Wernstedt, C., and Oliw, E. H. (1999) J. Biol. Chem. 274, 28219–28224
18. Daujas, H., and Toh, H. (2000) J. Mol. Evol. 51, 433–445
19. Hamberg, M., Ponce de León, I., Rodriguez, M. J., and Castresana, C. (2005) Biochem. Biophys. Res. Commun. 338, 169–174
20. Tsitsigiannis, D. I., and Keller, N. P. (2006) Mol. Microbiol. 59, 882–892
21. Tsitsigiannis, D. I., Kowieski, T. M., Zarnowski, R., and Keller, N. P. (2004) Eukaryot. Cell 3, 1398–1411
22. Tsitsigiannis, D. I., Kowieski, T. M., Zarnowski, R., and Keller, N. P. (2005) Microbiology 151, 1809–1821
23. Tsitsigiannis, D. I., Zarnowski, R., and Keller, N. P. (2004) J. Biol. Chem. 279, 11344–11353
24. Galagan, J. E., Calvo, S. E., Cuomo, C., Ma, L. J., Wortman, J. R., Batzoglou, S., Lee, S. I., Basturkmen, M., Spevak, C. C., Clutterbuck, J., Kapitonov, V., Jurka, J., Scassacchio, C., Farman, M., Butler, J., Purcell, S., Harris, S., Braus, G. H., Draht, O., Busch, S., D’Enfert, C., Bouchier, C., Goldman, G. H., Bell-Pedersen, D., Griffiths-Jones, S., Doonan, J. H., Yu, J., Vienken, K., Pain, A., Freitag, M., Selker, E. U., Archer, D. B., Penalva, M. A., Oakley, B. R., Momany, M., Tanaka, T., Kumagai, T., Asai, K., Machida, M., Nierman, W. C., Denning, D. W., Caddick, M., Hynes, M., Paolletti, M., Fischer, R., Miller, B., Dyer, P., Sachs, M. S., Osmani, S. A., and Birren, B. (2005) Nature 438, 1105–1115
25. Nierman, W. C., Pain, A., Anderson, M. I., Wortman, J. R., Kim, H. S., Arroyo, J., Berriman, M., Abe, K., Archer, D. B., Bermejo, C., Bennett, J., Bowyer, P., Chen, D., Collins, M., Coulson, R., Davies, R., Dyer, P. S., Farman, M., Fedorova, N., Fedorova, N., Feldhlyum, T. V., Fischer, R., Fosker, N., Fraser, A., Garcia, J. L., Garcia, M. J., Gohl, A., Goldman, G. H., Gomi, K., Griffiths-Jones, S., Gwilliam, R., Haas, B., Haas, H., Harris, D., Horiuichi, H., Huang, J., Humphray, S., Jimenez, J., Keller, N., Khouri, H., Kitamoto, K., Kobayashi, T., Konzack, S., Kulkarni, R., Kumagai, T., Lafon, A., Latge, J. P., Li, W., Lord, A., Lu, C., Majoros, H. W., May, G. S., Miller, B. L., Mohamoud, Y., Molina, M., Monod, M., Mouyna, I., Mulligan, S., Murphy, L., O’Neill, S., Paulsen, I., Penalva, M. A., Pertea, M., Price, C., Pritchard, B. L., Quail, M. A., Rabinowitsch, E., Rawlins, N., Rajandream, M. A., Reichard, U., Renaud, H., Robson, G. D., Rodriguez de Cordoba, S., Rodriguez-Pena, J. M., Ronning, C. M., Rutter, S., Salzberg, S. L., Sanchez, M., Sanchez-Ferrero, J. C., Saunders, D., Seeger, K., Squares, R., Squares, S., Takeuchi, M., Tekaija, F., Turner, G., Vazquez of Aldana, C. R., Weidman, J., White, O., Woodward, J., Yu, J. H., Fraser, C., Galagan, J. E., Asai, K., Machida, M., Hall, N., Barrett, B., and Denning, D. W. (2005) Nature 438, 1151–1156

Synthesis of Oxylipins by Aspergillus sp.
Biosynthesis of Oxylipins by Aspergillus sp.

26. Coffa, G., Schneider, C., and Brash, A. R. (2005) Biochem. Biophys. Res. Commun. 338, 87–92
27. Tsitsigiannis, D. I., Bok, J. W., Andes, D., Nielsen, K. F., Frisvad, J. C., and Keller, N. P. (2005) Infect. Immun. 73, 4548–4559
28. Thuresson, E. D., Lakkides, K. M., Rieke, C. J., Sun, Y., Wingerd, B. A., Micielli, R., Mulichak, A. M., Malkowski, M. G., Garavito, R. M., and Smith, W. L. (2001) J. Biol. Chem. 276, 10347–10357
29. Garscha, U., and Oliw, E. H. (2007) Anal. Biochem. 367, 238–246
30. Wurzenberger, M., and Grosch, W. (1984) Biochim. Biophys. Acta 794, 25–30
31. Akakabe, Y., Matsui, K., and Kajiwara, T. (2005) Biosci. Biotechnol. Biochem. 69, 1539–1544
32. Koshino, H., Togiya, S., Yoshihara, T., and Sakamura, S. (1987) Tetrahedron Lett. 28, 73–76
33. Brodowsky, I. D., Zhang, L. Y., Oliw, E. H., and Hamberg, M. (1994) Ann. N. Y. Acad. Sci. 744, 314–316
34. de Boer, H. J., Kool, A., Broberg, A., Mziray, W. R., Hedberg, I., and Levenfors, J. J. (2005) J. Ethnopharmacol. 96, 461–469
35. Oliw, E. H., Su, C., Skogström, T., and Benthin, G. (1998) Lipids 33, 843–852
36. Murphy, R. C., Barkley, R. M., Zemski Berry, K., Hankin, J., Harrison, K., Johnson, C., Kran, J., McAnoy, A., Uhlnson, C., and Zarini, S. (2005) Anal. Biochem. 346, 1–42
37. Labeque, R., and Marnett, L. J. (1987) J. Am. Chem. Soc. 109, 2828–2829
38. Brodowsky, I. D., Hamberg, M., and Oliw, E. H. (1994) Eur. J. Pharmacol. 254, 43–47
39. Schneider, C., Pratt, D. A., Porter, N. A., and Brash, A. R. (2007) Chem. Biol. 14, 473–488
40. Hamberg, M., Su, C., and Oliw, E. (1998) J. Biol. Chem. 273, 13080–13088
41. Xue, T., Nguyen, C. K., Romans, A., Kontoyiannis, D. P., and May, G. S. (2004) Arch. Microbiol. 182, 346–353