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Novel Fatty Acid Dioxygenases of Human and Plant Pathogenic Fungi

Studies by Gene Deletion and Expression

FREDRIK JERNERÉN



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Abstract

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The dioxygenase-cytochrome P450 fusion proteins (DOX-CYP) comprise a heme-containing enzyme family that shares structural and catalytic properties with mammalian prostaglandin H (PGH) synthases. 7,8-Linoleate diol synthase (7,8-LDS) of *Gaeumannomyces graminis* was first characterized, and DOX-CYP enzymes are of mechanistic and biological interest. The growing number of fungal genome sequences has revealed *DOX-CYP* homologues in medically and economically important species. The aim of this thesis was to identify novel members of the DOX-CYP fusion protein family.

The devastating rice pathogen *Magnaporthe oryzae* contains two *DOX-CYP* genes. The fungus synthesizes 7*S*,8*S*-dihydroxyoctadecadienoic acid (7,8-DiHODE) by dioxygenation of linoleic acid to 8*R*-hydroperoxyoctadecadienoic acid (8*R*-HPODE), and subsequent isomerisation to the diol. 7,8-LDS of *M. oryzae* was identified by gene deletion, but the infection and reproduction processes of the Δ 7,8-LDS strain were not altered. A mutant with constitutive protein kinase A activity profoundly changed the oxygenation profile, possibly due to post-translational modification.

The human pathogens *Aspergillus fumigatus* and *A. clavatus* contain three *DOX-CYP*, designated *psi* producing oxygenase A (*ppoA*), *ppoB*, and *ppoC*, and form three oxylipins: 5*S*,8*R*-DiHODE, 8*R*,11*S*-DiHODE, and 10*R*-hydroxyoctadecadienoic acid. *PpoA* was identified as 5,8-LDS, and *ppoC* as 10*R*-DOX. The 8,11-linoleate hydroperoxide isomerase activity was reduced by two imidazole-containing P450 inhibitors, miconazole and 1-benzylimidazole. *PpoB* could not be linked to the biosynthesis of 8,11-DiHODE for the following reasons: First, the 8,11-hydroperoxide isomerase activity was retained in *A. fumigatus* Δ *ppoB* strains. Second, the P450 domain of the deduced *ppoB* of *A. clavatus* lacks a heme-thiolate cysteine ligand, presumably essential for hydroperoxide isomerase activity.

Linoleate 9*R*-DOX activities of *Aspergillus terreus* and *Lasiodiplodia theobromae* were discovered. 9*R*-HPODE was further converted into unstable allene oxides, as judged by the accumulation of their hydrolysis products, α - and γ -ketols. These allene oxide synthase activities were specific for 9*R*-hydroperoxides. The 9*R*-DOX and AOS were found to have unique characteristics.

In conclusion, novel DOX-CYP enzymes were identified in human and plant pathogenic fungi. These enzymes might be involved in biological processes, and show interesting catalytic similarities to human PGH synthase and thromboxane synthase (CYP5A).

Keywords: aspergilli, dioxygenase, oxygenase, *Magnaporthe oryzae*, *Gaeumannomyces graminis*, *Lasiodiplodia theobromae*, jasmonic acid, linoleate diol synthase, cyclooxygenase, prostaglandin H synthase, cytochrome P450, oxylipin, hydroperoxide isomerase, allene oxide synthase

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List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

- I **Jernerén, F.**, Sesma, A., Franceschetti, M., Hamberg, M., Oliw, E.H. (2010) Gene deletion of 7,8-linoleate diol synthase of the rice blast fungus. Studies on pathogenicity, stereochemistry, and oxygenation mechanisms. *Journal of Biological Chemistry*, 285, 5308-16
- II Garscha, U., **Jernerén, F.**, Chung, D., Keller, N.P., Hamberg, M., Oliw, E.H. (2007) Identification of dioxygenases required for *Aspergillus* development. Studies of products, stereochemistry, and the reaction mechanism. *Journal of Biological Chemistry*, 282, 34707-18
- III Hoffmann, I., **Jernerén, F.**, Garscha, U., Oliw, E.H. (2010) Expression of 5,8-LDS of *Aspergillus fumigatus* and its dioxygenase domain. A comparison with 7,8-LDS, 10-dioxygenase, and cyclooxygenase. *Archives of Biochemistry and Biophysics*. *In press*.
- IV **Jernerén, F.**, Garscha, U., Hoffmann, I., Hamberg, M., Oliw, E.H. (2010) Reaction mechanism of 5,8-linoleate diol synthase, 10*R*-dioxygenase, and 8,11-hydroperoxide isomerase of *Aspergillus clavatus*. *Biochimica et Biophysica Acta*, 1801, 503-7
- V **Jernerén, F.**, Oliw, E.H. The 8,11-linoleate hydroperoxide isomerase activity of *Aspergillus fumigatus* is unaffected by gene deletion of *ppoB* and inhibited by miconazole and benzylimidazole. *Manuscript*.
- VI **Jernerén, F.**, Hoffmann, I., Oliw, E.H. (2010) Linoleate 9*R*-dioxygenase and allene oxide synthase activities of *Aspergillus terreus*. *Archives of Biochemistry and Biophysics*, 495, 67-73
- VII **Jernerén, F.**, Hamberg, M., Oliw, E.H. (2010) Fatty acid dioxygenase and allene oxide synthase activities of *Lasiodiplodia theobromae*. A comparison with *Aspergillus terreus*. *Manuscript*.

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Abbreviations

AA	Arachidonic acid
AOS	Allene oxide synthase
COX	Cyclooxygenase
CP-HPLC	Chiral phase-HPLC
DiHODE	Dihydroxyoctadecadienoic acid
DOX	Dioxygenase
DOX-CYP	Dioxygenase-Cytochrome P450
EET	Epoxyeicosatrienoic acid
ESI	Electrospray ionization
H(P)ETE	Hydro(pero)xyeicosatetraenoic acid
H(P)ODE	Hydro(pero)xyoctadecadienoic acid
H(P)OTrE	Hydro(pero)xyoctadecatrienoic acid
HPLC	High performance liquid chromatography
JA	Jasmonic acid
LA	Linoleic acid
LDS	Linoleate diol synthase
LOX	Lipoxygenase
LT	Leukotriene
MALDI-TOF	Matrix-assisted laser desorption/ionization time-of-flight
NP-HPLC	Normal phase-HPLC
OPDA	Oxophytodienoic acid
P450	Cytochrome P450
PG	Prostaglandin
PGHS	Prostaglandin H synthase
PGI ₂	Prostacyclin
PIOX	Pathogen-inducible oxygenase
PKA	Protein kinase A
Ppo	Psi producing oxygenase
Psi	Precocious sexual inducer
RP-HPLC	Reverse phase-HPLC
RT-PCR	Reverse transcription PCR
sLOX-1	Soybean lipoxygenase-1
TXA ₂	Thromboxane A ₂

Introduction

Oxylipins

Oxylipins are a group of chemical mediators involved in the control of a number of biological functions. In man, such mediators are implicated in inflammation, pain, fever, and in several other physiological processes, e.g., reproduction and thrombocyte aggregation. Drugs that target the biosynthesis of oxylipins include some of the most common and well known drugs on the market, e.g., aspirin, ibuprofen, and diclofenac.

Fatty acids are vital as building blocks of cell membranes and as precursors of lipophilic signaling molecules. Oxylipins are formed by fatty acid dioxygenases (DOX), which are widely distributed among eukaryotes. Fatty acid dioxygenases are metal enzymes and their reaction mechanisms and biological importance have been studied extensively for over 50 years. In addition, oxylipins can be formed by autoxidation, and these reactive oxygen species are also of biological importance.

Mammalian oxylipins

Mammalian oxylipins have been the focus of intensive studies for several decades. The reasons are that many biological processes are regulated by *eicosanoids*. The name is derived from the greek word for twenty, *eikosi*, since these oxylipins are formed from 20 carbon long polyunsaturated fatty acids. The fatty acid substrate can contain three, four, or five double bonds, termed dihomo- γ -linolenic acid, arachidonic acid (AA), and eicosapentaenoic acid, respectively. Of these, AA is by far the most important precursor, see Fig. 1.

Von Euler isolated a vasodilating activity from sheep seminal vesicles, which was termed prostaglandin (PG) [von Euler, 1936]. In the beginning of the 1960s, Bergström *et al.* elucidated the structures of the E and F prostaglandins, which were shown to contain carboxylic acid functional groups and characteristic cyclopentane ring structures [Bergström *et al.*, 1963; Nugteren *et al.*, 1966]. Work by two groups simultaneously revealed the biosynthetic pathway of prostaglandin formation from oxygenation of radiolabelled AA [Bergström *et al.*, 1964; van Dorp *et al.*, 1964].

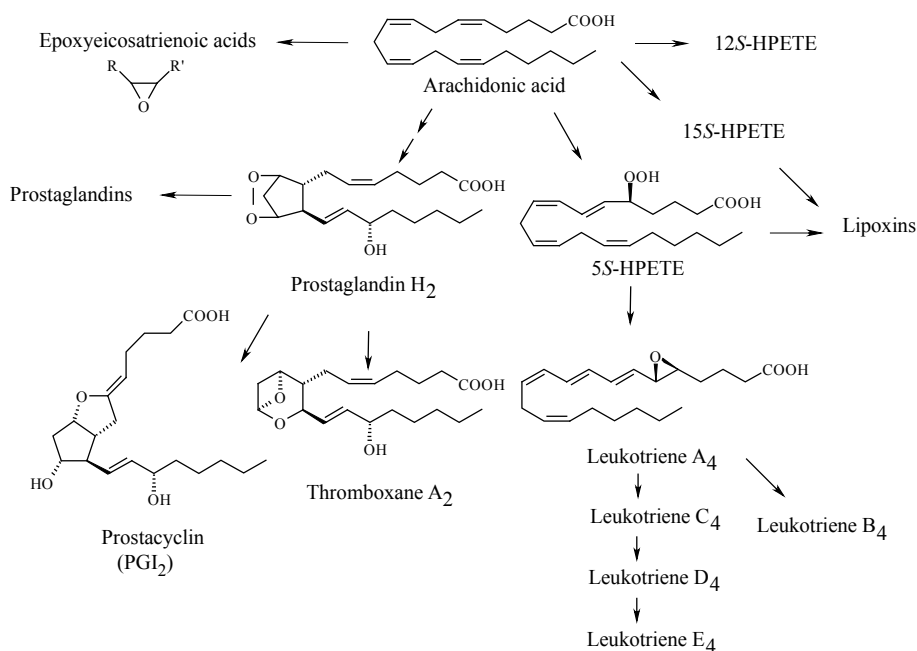


Figure 1. Overview of the AA cascade showing the structures of some key metabolites.

The eicosanoids are subdivided into prostanoids, leukotrienes (LT), and epoxyeicosatrienoic acids (EET). Prostanoids are defined as oxylipins containing a five-membered ring structure as in prostanic acid (Fig. 2), and include the prostaglandins and prostacyclin. These are derived from the key intermediate prostaglandin H_2 (PGH_2), which is also true for thromboxane A_2 (TXA_2 , Fig. 1). Instead of a five-membered ring, TXA_2 includes an oxygen-containing six-membered ring structure [Hamberg *et al.*, 1975]. TXA_2 and the prostaglandins of the E, F, D, and I series, are formed from PGH_2 by specific synthases of which thromboxane and prostacyclin synthase belong to the cytochrome P450 (P450) family.

PGE_2 (Fig. 2) and PGI_2 are both potent vasodilators, and cause hypotension. PGE_2 plays an important role in the inflammatory response and the induction of pain and fever. In thrombocytes, prostaglandin H synthase-1 (PGHS-1)-derived TXA_2 mediates platelet aggregation, which constitutes an important pharmaceutical target in the prevention of myocardial infarction and ischemic stroke with low-dose aspirin.

The leukotrienes, previously collectively known as “slow-reacting substances of anaphylaxis” [Murphy *et al.*, 1979], are formed from AA by the action of 5-lipoxygenase (5-LOX). This group of eicosanoids is defined by their three conjugated double bonds, which is illustrated in their names. The key intermediates in the leukotriene biosynthetic pathway are 5S-hydroperoxyeicosatetraenoic acid (5S-HPETE) and LTA_4 , formed by 5-LOX

in a two-step reaction beginning with the oxygenation at C-5 and the subsequent dehydration into an unstable epoxide (Fig. 1). LTA₄ is further transformed into leukotrienes of the C, D, and E type, collectively known as the cysteinyl-leukotrienes, and to LTB₄. As the cysteinyl-leukotrienes are constrictors of bronchial smooth muscles, leukotriene receptor antagonists (e.g., montelukast and zafirlukast) were developed as remedies for asthma. Another anti-asthmatic drug is zileuton, which is a selective inhibitor of 5-LOX and blocks the synthesis of all leukotrienes. 5-HPETE is also a precursor of lipoxins, which are formed by two sequential LOX reactions.

Other eicosanoids include the EET and 20-hydroxyeicosatetraenoic acid (20-HETE), which are vasoactive substances produced by the action of cytochrome P450 [Oliw, 1994; Spector, 2009].

Plant oxylipins

Plant oxylipins are primarily derived from linoleic acid (LA; 18:2*n*-6) and α -linolenic acid (18:3*n*-3). Key intermediates are 9*S*- and 13*S*-hydroperoxides formed by the action of specific LOX. These hydroperoxides can be further converted into fatty acid hydroxides, epoxy alcohols, divinyl ethers, ketones, and aldehydes [Feussner & Wasternack, 2002; Mosblech *et al.*, 2009]. The most thoroughly studied member of the LOX family in plants is the soybean lipoxygenase-1 (sLOX-1), which forms 13*S*-HPODE, and is used as a model in lipoxygenase research [Brash, 1999].

Plant oxylipins can also be formed by P450 monooxygenases and heme-containing DOX. The latter group consists of α -DOX, which forms 2*R*-hydroperoxy fatty acid metabolites possibly involved in defense responses upon pathogen attack [Sanz *et al.*, 1998; De Leon *et al.*, 2002]. A series of plant P450 enzymes involved in fatty acid metabolism belong to the CYP74 family, and includes hydroperoxide lyase, epoxy alcohol synthase, divinyl ether synthase, and allene oxide synthase (AOS) [Itoh & Howe, 2001; Mosblech *et al.*, 2009].

The fatty acid metabolites of plants which have attracted most interest are unarguably jasmonic acid (JA) and its derivatives. This 12-carbon compound (Fig. 2) was found to mediate hormone-like effects in plants and are involved in defense responses and development [Koo & Howe, 2009]. JA biosynthesis usually originates from α -linolenic acid [Vick & Zimmerman, 1983; Vick & Zimmerman, 1984]. JA is formed via the octadecanoid pathway by the action of a LOX, allene oxide synthase, allene oxide cyclase, and oxophytodienoate reductase [Schaller & Stintzi, 2009]. Overexpression of an allene oxide synthase gene in rice, led to increased levels of jasmonates, and an increased resistance towards infection by *Magnaporthe oryzae* [Mei *et al.*, 2006].

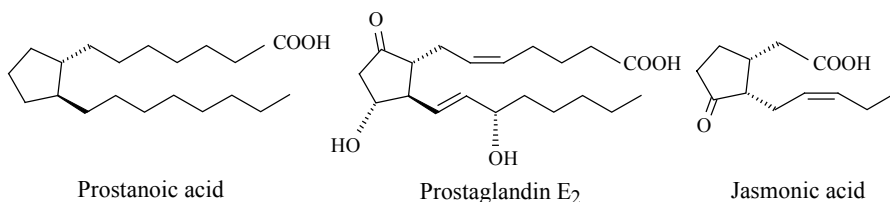


Figure 2. Chemical structures of prostanoic acid, PGE₂, and JA.

Oxygenated fatty acids with 5-membered ring structures can be derived non-enzymatically by autoxidation. The products, called isoprostanes or phytosteranes, are formed in a free radical catalyzed process and their structures resemble prostaglandins, but they are formed without stereospecificity [Imbusch & Mueller, 2000; Thoma *et al.*, 2004]. Phytosteranes accumulate during stress as a result of increasing levels of radical oxygen species [Mueller, 2004].

Fungal oxylipins

In 1986, a fungal oxylipin termed laetisarinic acid was isolated and characterized from the soil basidiomycete *Laetisaria arvalis* [Bowers *et al.*, 1986]. This oxylipin, perhaps more recognizable by its chemical name, 8-hydroxyoctadecadienoic acid (8-HODE), was found to possess antifungal activity against certain phytopathogenic fungi. It was shown that the absolute configuration at C-8 was *R*, and that it was formed by reduction of 8-hydroperoxyoctadecadienoic acid (8*R*-HPODE) to its corresponding alcohol [Brodowsky & Oliw, 1993].

One year after the identification of laetisarinic acid, Champe and co-workers isolated a solvent-extractable activity from certain thermosensitive mutants of *Aspergillus nidulans* [Champe *et al.*, 1987]. This activity was termed *precocious sexual inducer*, psi, because of its ability to increase the production of sexual spores (ascospores), relative to asexual spores (conidia), when added to the fungal culture. Psi was later separated into three components, PsiA α , PsiB α , and PsiC α , which were structurally determined as depicted in Fig. 3, and stem from LA [Champe & el-Zayat, 1989; Mazur *et al.*, 1990; Mazur *et al.*, 1991]. PsiA α is formed from PsiC α (5*S*,8*R*-dihydroxyoctadecadienoic acid, 5,8-DiHODE) by lactonization of the carboxyl group with the C-5 hydroxyl, and is the least potent of the psi factors [Champe & el-Zayat, 1989]. Analogous compounds are formed from oleic acid (18:1*n*-9), named PsiA β , PsiB β , and PsiC β .

As seen in Fig. 3, PsiB α of *A. nidulans* was identified as 8*R*-HODE, which might imply a general function of oxygenated LA as signaling compounds in fungal biology. Since then, 8*R*-HODE was identified in additional fungi. The Take-all fungus, *Gaeumannomyces graminis*, a serious

root pathogen, synthesizes 8*R*-HODE [Brodowsky *et al.*, 1992; Brodowsky & Oliw, 1992], as does the rice pathogen *Magnaporthe oryzae* [Cristea *et al.*, 2003], the sewage fungus *Leptomitius lacteus* [Fox *et al.*, 2000], and *Agaricus bisporus* [Wadman *et al.*, 2005].

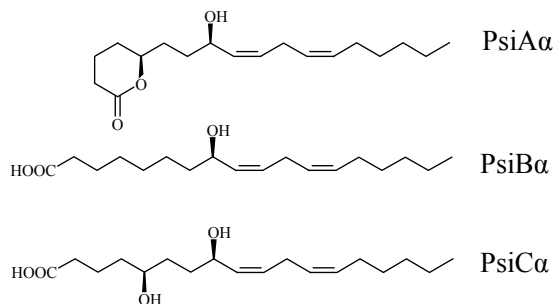


Figure 3. Structures of sporogenic factors of *A. nidulans*.

Along with the biosynthesis of 8*R*-HODE, dihydroxylated metabolites similar to PsiCα (5,8-DiHODE) of *A. nidulans* are formed by these fungi. *G. graminis* and *M. oryzae* produce 7,8-dihydroxyoctadecadienoic acid (7,8-DiHODE), while *L. lacteus* and *A. bisporus* synthesize 8,11-dihydroxyoctadecadienoic acid (8,11-DiHODE). Subsequent work showed that these dihydroxy metabolites are formed from an 8*R*-HPODE intermediate.

Some fungi express lipoxygenases. 9-HODE and 13-HODE were products of a LOX of *Fusarium oxysporum* [Matsuda *et al.*, 1978], and *Saprolegnia parasitica* was shown to produce 15-HPETE from AA [Hamberg *et al.*, 1986; Herman & Hamberg, 1987]. The aforementioned ascomycetous fungus *G. graminis* expresses a LOX with unique characteristics, and is extensively studied. It forms 13*R*-HODE, as opposed to 13*S* formed by plant lipoxygenases, and 11*S*-HODE, an unusual product due to its *bis*-allylic structure [Hamberg *et al.*, 1998]. To date, it is the only LOX shown to contain manganese, instead of iron, as the catalytic metal [Su & Oliw, 1998; Gaffney *et al.*, 2001].

The important plant hormone JA and its derivatives were originally described in filamentous fungi. *N*-jasmonyl-isoleucine was identified 1970 in the pathogen *Giberella fujikuroi* [Cross & Webster, 1970] and JA was described the following year in *Lasiodiplodia theobromae* [Aldridge *et al.*, 1971]. JA derivatives were detected in culture filtrates of *F. oxysporum* [Miersch *et al.*, 1999a], and *Aspergillus niger* was found to be able to hydroxylate jasmonates [Miersch *et al.*, 1999b]. The biological functions of fungal jasmonates are unclear. It was proposed that jasmonates are involved in the pathogenicity of certain fungi towards plants [Thakkar *et al.*, 2004]. The synthesis of JA in *L. theobromae* was found to originate from α-

Beside the four nitrogen ligands of heme, it usually binds two additional axial ligands, sometimes termed the fifth (lower iron) and sixth (upper iron) ligand of heme. The nature of these ligands, of which at least one usually is a functional group of an amino acid of the protein to which it is bound, greatly influence the reactivity of the heme group [Frey & Hegeman, 2007]. The heme-containing DOX can be divided into three groups: the prostaglandin H synthases (PGHS) of vertebrates and corals, the linoleate diol synthases (LDS) and 10R-DOX of filamentous fungi, and the α -DOX of plants. The DOX domains of these subgroups are homologous, and they belong to the same family of heme peroxidases [Sanz *et al.*, 1998; Hörnsten *et al.*, 1999; Garscha & Oliw, 2009].

Prostaglandin H synthases

Identification and purification of the key enzyme in the biosynthesis of the aforementioned prostaglandins, the PGHS, were to a large extent conducted in the 1960 and 1970s using ram seminal vesicles. The formation of PGH₂ was shown to be catalyzed by two activities of a purified fraction, an initial cyclooxygenase (COX) reaction to the intermediate PGG₂ (Fig. 1), followed by a subsequent peroxidase reaction into PGH₂. This compound was further transformed into a series of additional prostaglandins by separate fractions [Miyamoto *et al.*, 1974; Hemler & Lands, 1976; Van der Ouderaa *et al.*, 1977]. The first report of a purified PGHS was published in 1976 by the group of Hayashi [Miyamoto *et al.*, 1976], performed from bull seminal vesicles. Some sources use the name COX as a synonym to PGHS, which is misleading since the name only refers to the first reaction of this bifunctional enzyme. In this thesis, PGHS will be used except when the COX activity of PGHS is discussed.

Catalytic mechanism

The transformation of AA to PGG₂ is a radical-driven chemical process initiated by the stereospecific pro-*S* hydrogen abstraction at C-13 (Fig. 6). This abstraction is catalysed by a tyrosyl radical formed at Tyr385 (numbered according to ovine PGHS-1) [Karthain *et al.*, 1987; Shimokawa *et al.*, 1990; Tsai *et al.*, 1994; Tsai *et al.*, 1995]. The carbon-centered radical ion is delocalized to C-11, where molecular oxygen is incorporated antarafacially to form a peroxy radical. The peroxy radical attacks C-9 and forms an endoperoxide, with a hypothetical carbon-centered radical at C-8. Ring closure is mediated by radical attack at C-12, followed by radical migration and incorporation of a second oxygen molecule to form a peroxy radical at C-15. Finally, PGG₂ is formed by the transfer of hydrogen from Tyr385, which regenerates the tyrosyl radical. PGG₂ is converted by the peroxidase activity of PGHS to its corresponding alcohol, PGH₂, by the reduction of the hydroperoxide at C-15.

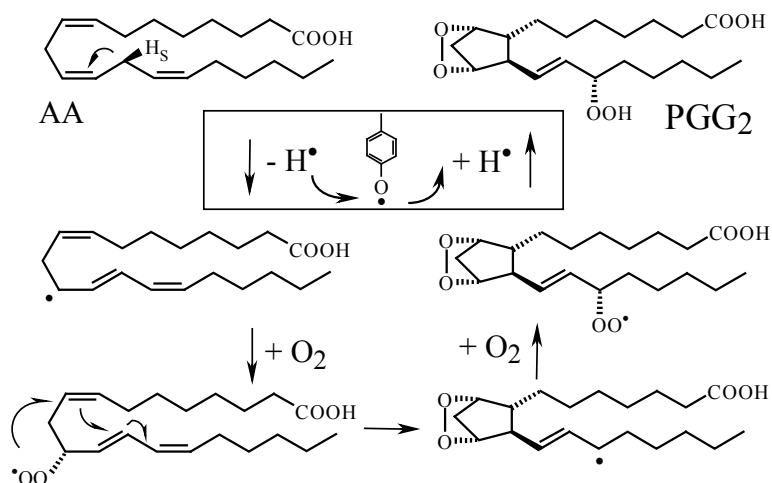


Figure 6. Catalytic mechanism of PGG₂ biosynthesis catalyzed by the COX activity of PGHS, as described by Hamberg and Samuelsson [Hamberg & Samuelsson, 1967]. The stereospecific abstraction of hydrogen by a tyrosyl radical was proposed by Karthein [Karthein *et al.*, 1987].

In addition to Tyr385, several other amino acid residues in PGHS are of importance for its catalytic activity. Arg120 was shown to bind the carboxyl group of AA which aligns Tyr385 with C-13, and is thus involved in the regio- and stereoselectivity of the hydrogen abstraction by PGHS-1 [Malkowski *et al.*, 2000]. Val349, Ser530, and Tyr348 seem to be important for positioning of AA in the active site [Hsi *et al.*, 1995; Schneider *et al.*, 2002]. In PGHS, the heme group is bound by His388, which constitutes the fifth (proximal) heme ligand, while His207 is located at the distal side of the prosthetic group. Both histidines are essential for full enzyme activity [Picot *et al.*, 1994; Landino *et al.*, 1997].

PGHS as a pharmaceutical target

Acetylsalicylic acid (aspirin) is used since 1898 as a remedy for pain, fever, and inflammation. In 1971, Sir John Vane showed that aspirin mediates its effects by inhibition of prostaglandin biosynthesis. The biochemical basis of this effect is the irreversible acetylation of the Ser530 residue of PGHS, which prevents binding of AA in the COX site. Other non-steroidal anti-inflammatory drugs, e.g., ibuprofen, naproxen, and indomethacin, show similar modes of action, but instead block AA entry by competitive inhibition.

In the early 90s, a new isoform of PGHS was discovered [Simmons *et al.*, 1989; Kujubu *et al.*, 1991; Wong & Richards, 1991; Hla & Neilson, 1992]. While PGHS-1 is constitutively expressed in most tissues, PGHS-2 seemed associated with inflammatory responses [O'Banion *et al.*, 1992]. Crystal structures of PGHS-2 were solved and made available. Based on the larger

pocket of the active site of PGHS-2 [Kurumbail *et al.*, 1996; Luong *et al.*, 1996], selective inhibitors of PGHS-2 were developed. The rationale was that these would show sufficient anti-inflammatory, antipyretic, and analgesic effects without the adverse reactions associated with PGHS-1 inhibition (i.e., gastrointestinal bleeding due to the inhibition of PGHS-1-derived TXA₂ in platelets). However, a number of selective PGHS-2 inhibitors (the “coxibs”) were shown to increase the risk of cardiovascular adverse events, possibly due to decreased biosynthesis of the cardioprotective PGI₂, and some of these drugs were withdrawn from the market [Funk & FitzGerald, 2007]. PGHS-2 is not only induced by inflammation, but participates in various other physiological and pathological processes, e.g., renal function, bone metabolism, and cancer [Turini & DuBois, 2002; Wang & Dubois, 2006].

Linoleate diol synthases

A linoleate 8*R*-DOX activity coupled to a hydroperoxide isomerase was originally discovered in the Take-all fungus, *G. graminis* [Brodowsky *et al.*, 1992]. ¹⁸O₂ experiments showed that 7,8-DiHODE was formed from 8*R*-HPODE by intramolecular rearrangement of the hydroperoxide. Subsequent studies increased the knowledge concerning the catalytic mechanism of the responsible enzyme (Fig. 7) [Brodowsky *et al.*, 1994a; Brodowsky *et al.*, 1994b; Hamberg *et al.*, 1994]. 8*R*-HPODE is formed by antarafacial hydrogen abstraction and oxygen insertion at C-8, while the formation of 7,8-DiHODE occurs by suprafacial hydrogen abstraction and oxygen insertion at C-7, with retained configuration of the double bonds.

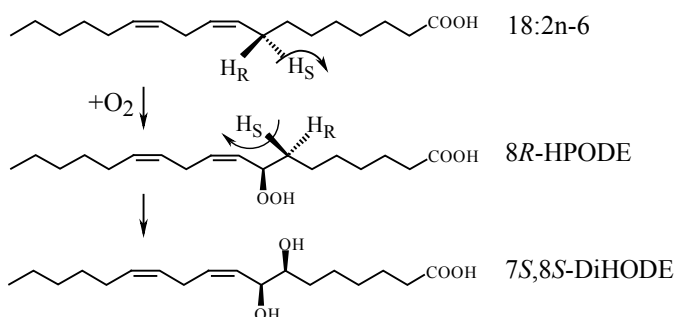


Figure 7. Catalytic reaction mechanism of 7,8-LDS of *G. graminis*. The enzyme is bifunctional, the first step is catalyzed by its DOX activity, while the second reaction is catalyzed by an isomerase activity [Hamberg *et al.*, 1994].

The enzyme responsible for this conversion was purified to homogeneity and was found to contain heme [Su & Oliw, 1996]. Interestingly, EPR studies on the purified enzyme, termed 7,8-linoleate diol synthase (7,8-LDS), suggested a protein radical in coordination with a ferryl metal centre, in analogy with PGHS [Su *et al.*, 1998]. Protein sequence similarities to PGHS

were apparent when the gene of 7,8-LDS was cloned and sequenced in 1999. The deduced amino acid sequence confirmed the presence of a supposedly important catalytic tyrosine residue, as well as proximal and distal histidines [Hörnsten *et al.*, 1999]. Site-directed mutagenesis studies support the importance of the tyrosine residue for DOX activity, which likely acts in analogy with Tyr385 of PGHS [Garscha & Oliw, 2008a; Garscha & Oliw, 2008b].

Following the discovery of 7,8-LDS, new members of this enzyme group were proposed in related fungi. Two homologous genes were identified in *M. oryzae*, which was shown to metabolize 7,8-DiHODE and 8-HODE from LA [Cristea *et al.*, 2003]. In 2005, Keller and co-workers identified three genes present in *Aspergillus fumigatus* and *A. nidulans* based on homology to 7,8-LDS of *G. graminis* [Tsitsigiannis *et al.*, 2004a; Tsitsigiannis *et al.*, 2004b; Tsitsigiannis *et al.*, 2005a], and the authors speculated that these genes could be involved in the biosynthesis of psi compounds in these species. The authors therefore named the genes *psi* producing oxygenase *A* (*ppoA*), *ppoB*, and *ppoC*.

Alpha Dioxygenases

In 1998, cDNA with homology to human PGHS was found to be up-regulated upon defense-response stimulation in tobacco leaves [Sanz *et al.*, 1998]. A homologous gene was found in *Arabidopsis thaliana*. The tobacco enzyme, originally termed *pathogen induced oxygenase* (PIOX), was shown to catalyze the α -dioxygenation of fatty acid substrates to produce 2*R*-hydroperoxy fatty acid metabolites, and was re-named α -DOX [Hamberg *et al.*, 1999].

The reaction of α -DOX with LA is initiated by the stereospecific abstraction of the pro-*R* hydrogen at C-2, followed by suprafacial insertion of molecular oxygen to form 2*R*-HPODE, which disintegrates spontaneously into an aldehyde [Hamberg *et al.*, 2002; Hamberg *et al.*, 2005].

Since the discovery of α -DOX in tobacco and *A. thaliana*, members were identified in other species [Hamberg *et al.*, 2005]. In addition, *A. thaliana* was shown to contain a second gene homologous with PGHS, which was designated α -DOX2 [Hamberg *et al.*, 2002]. When aligned with PGHS-1, all α -DOX sequences show conserved proximal and distal histidines, and a tyrosine residue at similar positions as His-207, His-388, and Tyr-385 of ovine PGHS-1 [Hamberg *et al.*, 2005].

Lipoxygenases

Lipoxygenases are non-heme fatty acid DOX, which metabolize substrates with at least one (1*Z*,4*Z*)-pentadiene. All members contain catalytic iron, with one sole known exception, the manganese LOX of *G. graminis* mentioned earlier [Su & Oliw, 1998; Hörnsten *et al.*, 2002]. The first

described member of the lipoxygenases was discovered in soybeans [André & Hou, 1932]. The sLOX-1 is studied in detail and was crystallized as early as 1947 [Theorell *et al.*, 1947], and is used as a model enzyme in LOX research.

The catalytic iron of sLOX-1 is bound by three histidine residues, along with one asparagine, one C-terminal isoleucine, and one water molecule, which is regarded as the sixth ligand [Boyington *et al.*, 1993; Minor *et al.*, 1996]. The three dimensional crystal structures of several additional lipoxygenases are available, albeit none with a bound fatty acid [Gillmor *et al.*, 1997; Skrzypczak-Jankun *et al.*, 1997; Oldham *et al.*, 2005; Youn *et al.*, 2006; Neau *et al.*, 2009]. The coordination spheres of iron were established in other lipoxygenases. They consist of three histidines as well as the C-terminal isoleucine, while the fifth iron ligand either can be an asparagine (as in sLOX-1) or an additional histidine [Jisaka *et al.*, 2001]. Some LOX contain threonine or valine, instead of isoleucine, as the C-terminal iron ligand [Oliw, 2002].

Little is known about the biological functions of fungal lipoxygenases and their products. Recent reports indicate an involvement in plant-pathogen cross-talk as well as during morphological development [Brodhagen *et al.*, 2008; Horowitz Brown *et al.*, 2008].

Cytochrome P450

P450 is a thoroughly studied heme containing enzyme family. The name cytochrome P450 is due to the fact that the absorption maximum shifts to 450 nm when CO binds heme as the upper axial ligand [Groves, 2005]. The human genome contains 57 functional P450 genes, and the enzymes are of fundamental biological importance due to their roles in the metabolism of endogenous and exogenous substances.

The P450 are monooxygenases, which incorporate single oxygen atoms from molecular oxygen suprafacially into the substrate. Drug metabolizing P450 enzymes are microsomal and membrane-bound, and require NADPH as a reductant provided by P450 reductase. There are exceptions. P450cam from *Pseudomonas putida* is a soluble enzyme and is used as a model in many functional and structural studies [Sono *et al.*, 1996]. A general model of the catalytic cycle of P450 is shown in Fig. 8.

Some P450 do not require NADPH and use endogenous peroxides as substrates, yielding important biological mediators [Vaz & Coon, 1987; Brash, 2009]. PGI₂ and TXA₂ synthase were shown to be P450 enzymes, designated CYP8A and CYP5A, respectively. These enzymes cleave the endoperoxide O-O bond of PGH₂ followed by rearrangement of oxygen, and thus function as peroxide isomerases [Hecker & Ullrich, 1989]. Other related P450 are the allene oxide synthases of plants, which catalyze the dehydration of hydroperoxides formed by lipoxygenases into allene oxides. AOS are

members of the CYP74 family and constitute the second enzymatic step in the biosynthesis of jasmonates. CYP8A, CYP5A, and CYP74 are proposed to convert P450[Fe^{III}] to P450[Fe^{IV}-OH] (compound II) by homolytic cleavage of their hydroperoxide or endoperoxide substrates (Fig. 8) [Brash, 2009].

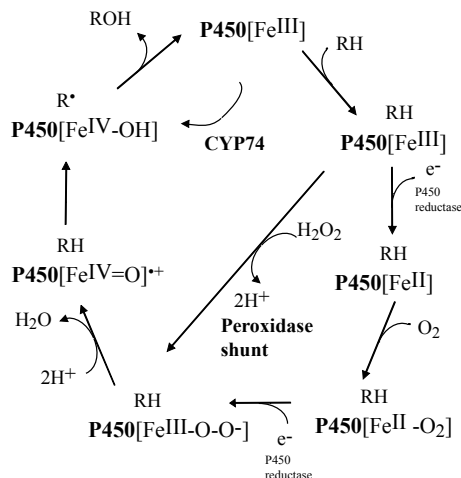


Figure 8. The catalytic cycle of P450 adapted from Sono *et al.* [Sono *et al.*, 1996] The reaction starts with the binding of the substrate converting the catalytic heme iron from its low spin resting state to a high spin state. The first electron is supplied by P450 reductase, reducing iron to a ferrous state. Sequential addition of molecular oxygen and a second electron from P450 reductase yields P450[Fe^{III}-O-O⁻], sometimes called compound 0. Catalyzed dehydration of compound 0 produces compound I, a highly reactive oxidant capable of hydrogen abstraction from the substrate yielding compound II (P450[Fe^{IV}-OH]) and an organic substrate radical. Rebound insertion of the hydroxyl returns the P450 to its resting state and releases the monohydroxylated product. The need for P450 reductase and molecular oxygen can be circumvented by substituting them with H₂O₂ in a pathway called the “Peroxidase shunt”. Another alternative pathway is that of CYP74, CYP8A, and CYP5A (depicted above as the CYP74 pathway), which is discussed in the text as suggested by Brash [Brash, 2009].

Although sequence identities among P450 are generally low, there are a number of characteristic amino acid motifs [Werck-Reichhart & Feyereisen, 2000], two of which are considered to be of larger importance. The first, an E-x-x-R motif, is situated on the proximal side of the heme group, probably stabilizing the protein structure by a salt bridge. The second motif is F-x-x-G-x-R-x-C-x-G, considered to be the most characteristic sequence, which harbours a cysteine residue that serves as the fifth ligand of the heme iron [Werck-Reichhart & Feyereisen, 2000]. Recent studies have revealed that only this heme-thiolate binding cysteine is absolutely conserved among P450 [Rupasinghe *et al.*, 2006].

P450 are ubiquitous in filamentous fungi, implied in a wide variety of processes such as environmental adaptation and secondary metabolism [Park *et al.*, 2008; Cresnar & Petric, 2010]. Some of these are specifically involved in the metabolism of fatty acids. CYP505 was shown to catalyze subterminal ω -1 to ω -3 fatty acid hydroxylations in *F. oxysporum* [Kitazume *et al.*, 2000]. In 2008, sequence alignments of predicted CYP74 homologues of fungi, suggested that P450 domains are present in LDS enzymes [Lee *et al.*, 2008]. Indeed, the 5,8-LDS of *A. nidulans* was later identified as a fusion enzyme between an N-terminal DOX domain, and a C-terminal P450 carrying out the intramolecular rearrangement of 8*R*-HPODE with suprafacial insertion of oxygen at C-5 [Brodhun *et al.*, 2009]. This reaction does not require NADPH and possibly proceed in analogy with the CYP74 enzyme family.

Inhibitors of P450

Inhibitors of P450 are commonly used as drugs in humans. Azole antifungals include several drugs with –conazole suffixes and are used to treat infections by *Candida* and aspergilli. The azoles are subdivided into the imidazoles, which include miconazole and ketoconazole (Fig. 9), and the triazoles, e.g., voriconazole and fluconazole.

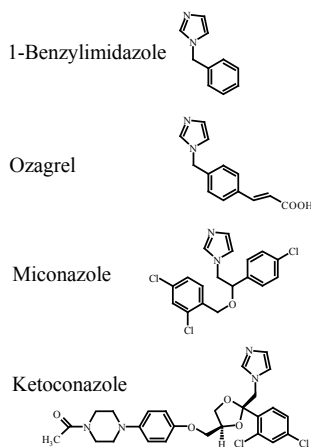


Figure 9. Structures of selected imidazole-containing inhibitors of fungal and human P450.

The azoles act by inhibiting lanosterol 14 α -demethylase (CYP51), presumably by ligation to the heme group which blocks the active site [Gollapudy *et al.*, 2004]. This interferes with the biosynthesis of ergosterol, an essential cell wall component of fungi.

TXA₂ synthase (CYP5A) converts PGH₂ to TXA₂, as mentioned above, and inhibitors of this enzyme are developed as remedies for asthma and cardiovascular events [Arii *et al.*, 2002; Dogne *et al.*, 2002]. Two TXA₂

inhibitors, 1-benzylimidazole and ozagrel, are shown in Fig. 9, and contain the imidazole moiety also encountered in some azole antifungals. None of the P450 inhibitors are specific for only one isoform.

Filamentous ascomycetes

Fungi comprise the third multicellular eukaryotic kingdom, in addition to animals and plants. They have huge impacts on economy and human health, with beneficiary and detrimental effects.

Some fungi are well known human pathogens. Approximately 10% of the food production is lost annually as a result of crop diseases [Strange & Scott, 2005], of which fungal infection is the most common cause [Deacon, 2006]. Fungi are also known as producers of drugs, e.g., penicillin, statins, and griseofulvin, and mycotoxins, which pose another serious threat to food production.

Fungi are valuable as biochemical models. Gene analyses suggest that animals in fact are closer relatives of fungi than of plants [Baldauf & Palmer, 1993]. Fungi, apart from being eukaryotic, are characterized by their typically haploid genomes, their cell wall components (chitin and glucans), and the fact that they produce spores, which can be formed either by sexual or asexual processes [Deacon, 2006]. Some fungi are unicellular (e.g., *Saccaromyces cerevisiae*), while some grow as multicellular filaments, hyphae, which form a network called mycelium. The latter group is collectively termed filamentous fungi.

Fungi (mycota) can be subdivided into four phyla: the zygomycetes (e.g., bread mould), the ascomycetes (e.g., baker's yeast), the basidiomycetes (e.g., mushrooms), and the deuteromycetes (or Fungi Imperfecti). The taxonomy of fungi is based on several characteristics, e.g., cell wall composition, mode of reproduction, genomic arrangement, and spore structure [Deacon, 2006]. The largest of these groups is the ascomycetes, which account for three quarters of all described fungi. This thesis focuses on fatty acid dioxygenases of members of the filamentous ascomycetes, which will be briefly described below.

Aspergilli

Aspergilli is a large group of ascomycetous moulds that primarily reproduce asexually through the formation of a morphological structure called the conidiophore [Bennett, 2009]. These structures give rise to the asexual spores, the conidia. Many members of this phylum are of medical and industrial importance. This is highlighted by the fact that nine aspergilli genomes were sequenced to date [Mabey Gilsenan *et al.*, 2009].

Aspergillus fumigatus

Reflecting its status as the most notorious member of the aspergilli, the genome of *A. fumigatus* AF293 was the first member to be sequenced [Nierman *et al.*, 2005]. In, 2008 the sequence of the clinical isolate A1163 was published [Fedorova *et al.*, 2008].

A. fumigatus is currently the most prevalent fungal pathogen [Denning, 1998] and causes aspergillosis, of which there are several forms. The most serious one, invasive aspergillosis, is a leading cause of death among immunocompromized patients, a group which is steadily increasing [Latzgé, 2001]. The airborne spores of *A. fumigatus* are ubiquitous in our environment, and are usually dealt with by the innate immune system of healthy individuals. In immunocompromized patients, however, the small spores (2-3 μm) can reach the alveoli and breach the lung tissue of the infected patient, leading to mortality rates of up to 90% [Latzgé, 1999]. Aspergillosis is usually treated with amphotericin B and azole antifungals, but none of these show satisfying and reliable results.

A. fumigatus was suspected to lack a sexual stage. However, genetic analyses have revealed genes associated with sexual reproduction [Paoletti *et al.*, 2005; Nielsen & Heitman, 2007; Grosse & Krappmann, 2008], and a sexual cycle was finally identified in 2009 [O'Gorman *et al.*, 2009].

Aspergillus nidulans

The biology of *A. nidulans* is widely studied and it is used as a model organism in classical genetics [Morris *et al.*, 1989; Osmani & Mirabito, 2004; Bennett, 2009]. *A. nidulans* has a well characterized sexual reproductive cycle, in contrast to most other members of the aspergilli family. Many cell- and molecular biology techniques are well established in *A. nidulans* and the genomic sequence of the strain FGSC A4 was published in 2005 [Galagan *et al.*, 2005].

A. nidulans rarely causes aspergillosis, but produces the toxic and carcinogenic precursor of aflatoxin, sterigmatocystine [Yu & Keller, 2005].

Aspergillus clavatus

A. clavatus strain NRRL1 was sequenced in 2008 [Fedorova *et al.*, 2008], and is not known to cause invasive disease. *A. clavatus* produces mycotoxins, which are known to contaminate malted grain, and to cause allergy in man [Fedorova *et al.*, 2008] and neurotoxicity in sheep [Shlosberg *et al.*, 1991] and cattle [Loretto *et al.*, 2003].

Aspergillus terreus

Aspergillus terreus contributes to ~15% of human cases of aspergillosis, but is associated with higher mortality rate compared to *A. fumigatus*, partly

explained by a low susceptibility towards treatment with the antifungal drug amphotericin B [Baddley *et al.*, 2003; Steinbach *et al.*, 2004; Balajee, 2009].

A. terreus is used in the pharmaceutical industry as the biotechnological source of the cholesterol lowering drugs lovastatin and simvastatin [Barrios-Gonzalez & Miranda, 2010]. The unnotated genome sequence of *A. terreus* NIH 2624 was released in 2006 as a part of the Fungal Genome Initiative of the Broad Institute.

In addition to the members described above, there are other important aspergilli. *Aspergillus flavus* and *A. parasiticus* contaminate stored crops with aflatoxins, which are potent liver carcinogens [Payne & Brown, 1998]. *A. flavus* can also cause aspergillosis [Hedayati *et al.*, 2007; Krishnan *et al.*, 2009]. *A. oryzae* and *A. niger* are used in biotechnology, primarily for production of penicillin [Fleissner & Dersch, 2010; Marui *et al.*, 2010].

Magnaporthaceae

The fungal family Magnaporthaceae was suggested in 1994 based on the similarities between *Gaeumannomyces* and *Magnaporthe* species [Cannon, 1994]. This family includes several root and leaf infecting fungi, and comprises a significant threat to production of staple foods (e.g., rice and wheat) worldwide.

Gaeumannomyces graminis

G. graminis is a well known plant pathogen, also known as the “Take-all fungus” of wheat. This soil-borne fungus can cause disease of several other cereals, e.g., rice, maize, barley, and oat [Besi *et al.*, 2009]. There is no effective fungicide available for disease control, and the disease is distributed over large areas of the world [Cook, 2003].

The infection originates from the roots of the plants. It causes the root to rot, and disrupts the water flow, which leads to premature death. No stable method of transfection of *G. graminis* is available, which makes studies on the impact of certain genes on the infection process difficult.

Magnaporthe oryzae

M. oryzae is a heterothallic haploid ascomycetous filamentous fungus, and is one of the best studied pathogenic fungi. It is used extensively as a model organism to study interactions between plant hosts and fungal pathogens [Ebbole, 2007]. *M. oryzae* is a world-wide threat to rice production as it causes the rice blast disease. The annual production loss was calculated to be in the range of 10-30% [Talbot, 2003], equivalent to loss of rice sufficient to feed over 60 million people [Dean *et al.*, 2005]. Rice blast can be devastating, since rice is the staple crop of many poor areas of the world [Strange & Scott, 2005].

M. oryzae was previously included in the species *M. grisea*. *M. grisea* is capable of infecting a large number of grass species, and some of these strains are non-pathogenic towards rice. In 2002, a phylogenetic study of a number of genes from different strains of *M. grisea* suggested that the rice infecting strains form a separate species, which was named *M. oryzae*. These names are sometimes incorrectly used interchangeably in the literature. In this study, only rice infecting strains were used, and will be referred to as *M. oryzae*.

M. oryzae was the first plant-pathogenic fungus that was sequenced [Dean *et al.*, 2005]. Its haploid genome consists of approximately 40 mega base pairs in seven chromosomes with just over 11000 genes (5th annotation). A method for genetic transformation of *M. oryzae* became available in 1987 [Parsons *et al.*, 1987]. Since then, more sophisticated and effective transformation systems were developed [Sweigard *et al.*, 1998; Khang *et al.*, 2005; Betts *et al.*, 2007], with the first report using *Agrobacterium tumefaciens* published in 2001 [Rho *et al.*, 2001].

Pathogenicity and infectious cycle

Several excellent reviews cover the biology of rice infection of *M. oryzae* [Talbot, 1995; Talbot, 2003; Wang *et al.*, 2005; Wilson & Talbot, 2009]. Only a brief summary will be given here. The infectious cycle starts with the attachment of the asexual conidia to the leaf surface of the rice host. Germination occurs almost immediately, and cuticle components trigger the formation of an infectious structure called the appressorium [Gilbert *et al.*, 1996], which penetrates the leaf surface by generating an enormous pressure of about 80 kPa. The turgor pressure was shown to be dependent on mobilization of glycerol, and lipid and fatty acid metabolism [Wang *et al.*, 2007]. After breaching the cuticle, the fungus rapidly spreads within the rice plant and destroys it. *M. oryzae* was shown to be able to infect rice by penetrating the roots, in analogy with the infection of wheat by the Take-all fungus, *G. graminis* [Sesma & Osbourn, 2004].

Lasiodiplodia theobromae

Lasiodiplodia theobromae (synonym *Botryodiplodia theobromae*) is a pathogenic fungus which mainly infects plants and trees in tropic and subtropic regions. It is also known to cause keratitis and other diseases in humans, although this occurs infrequently [Rebell & Forster, 1976; Thomas, 2003; Woo *et al.*, 2008; Kindo *et al.*, 2010]. *L. theobromae* is recognized by the perfume industry as a source of JA. Studies showed that the capacity to synthesize JA is highly strain specific [Aldridge *et al.*, 1971; Eng *et al.*, 1998].

Aims

7,8-LDS of *G. graminis* was discovered, purified to homogeneity, and cloned in the 1990s. 7,8-LDS was the first member of a novel group of enzymes to be biochemically characterized. In recent years, genomic information from several related ascomycetous fungi were made available. Homology-based genomic analyses and biochemical data suggested that new members of the linoleate diol synthase family could be present in important filamentous fungi.

The general aims of this study were to identify homologues to 7,8-LDS, investigate new members of this enzyme family, and study their catalytic mechanisms.

Specific aims of this study were to:

- Delete the putative *7,8-LDS* gene of *M. oryzae* and to study the mutant strain during infection and sporulation.
- Elucidate the catalytic properties of 7,8-LDS of *M. oryzae*.
- Investigate the impact of the cAMP signaling pathway on the oxylipin profile of *M. oryzae*.
- Study the formation of oxylipins in *A. fumigatus* and *A. nidulans*, and identify the *ppo* genes using knock-out strains.
- Develop expression systems for 5,8-LDS of *A. fumigatus* and compare its DOX domain with 7,8-LDS of *G. graminis*.
- Characterize the 8,11-linoleate hydroperoxide isomerase activities of *A. fumigatus* and *A. clavatus*.
- Study the fatty acid oxygenation of *A. terreus* and *L. theobromae*, and characterize novel oxylipins.

Comments on Methodology

Filamentous fungi

All fungal strains used in this thesis are listed in Table 1. Information concerning the handling and cultivation of these fungi can be found in the respective papers.

Agrobacterium tumefaciens-mediated transformation

As a natural plant pathogen, the gram-negative soil bacterium *Agrobacterium tumefaciens* infects plant roots and cause crown-gall disease, recognized by tumor-like outgrowths. The pathogenicity of *A. tumefaciens* is due to its ability to transfer DNA from a large tumor-inducing (Ti) plasmid into the host plant [Michielse *et al.*, 2005]. A specific region of the Ti-plasmid, called T-DNA, is integrated randomly by non-homologous recombination into the host genome where it expresses enzymes causing the plant disease [Michielse *et al.*, 2005]. The T-DNA is defined by its border sequences. It is possible to delete all genes within the T-DNA and replace them with other genetic material for integration into the host genome. This principle has formed the basis of transformation of plant cells with *A. tumefaciens* for several decades.

Lately, *Agrobacterium tumefaciens*-mediated transformation (ATMT) protocols were developed for fungi and yeasts. *Saccharomyces cerevisiae* was successfully transformed in 1995 [Bundock *et al.*, 1995], followed by transformation of a number of filamentous fungi in 1998 [de Groot *et al.*, 1998]. The first report of transformation of *M. oryzae* was published three years later [Rho *et al.*, 2001], and this transformation system is used extensively ever since. Transformation of *A. fumigatus* is generally performed using protoplasts, and only a single report in English used ATMT [Sugui *et al.*, 2005].

It is possible to achieve targeted deletion of genes by ATMT using transformation constructs containing large homologous regions flanking a selection marker. One potential drawback of this method is the low number of targeted transformants due to the competing random integration of the construct. This problem can partially be circumvented by using strains of fungi with deletions of proteins involved in the non-homologous end-joining

machinery [van Attikum *et al.*, 2001]. Such strains of *A. tumefaciens* were developed, and deletion of the Ku80 protein led to significantly increased numbers of transformants generated by targeted recombination without affecting the pathogenicity of the fungus [da Silva Ferreira *et al.*, 2006].

In this study, a so-called binary system was used. This system utilizes two plasmids among which the components of the Ti-plasmid are divided. One considerably smaller plasmid carries the T-DNA along with genes allowing convenient manipulation in *E. coli*, as well as high stability in *A. tumefaciens*. CAMBIA (Canberra, Australia) distributes a variety of binary vectors for *Agrobacterium* transformations. Plasmids used in this thesis were constructed in pCAMBIA0380 with a cassette conferring hygromycin B resistance. Constructs were introduced in *A. tumefaciens* AGL-1, which harbours the attenuated Ti-plasmid pTiBo542. The latter supplies the proteins required for the transformation machinery [Lazo *et al.*, 1991]. A schematic overview of the ATMT protocol used for transformation of *M. oryzae* and *A. tumefaciens* is given in Fig. 10.

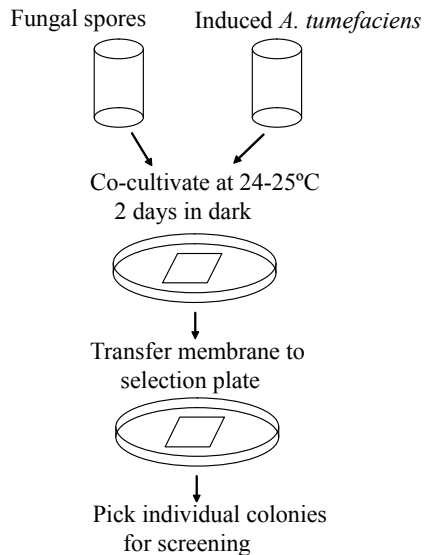


Figure 10. ATMT of *M. oryzae* and *A. fumigatus*. In this study, fungal spores were used for transformation. Prior to co-cultivation, *A. tumefaciens* was induced with acetosyringone, a phenolic compound which is an inducer of the virulence genes required for T-DNA transfer. Fungal spores and *A. tumefaciens* cells were mixed and the suspension was spread on nitrocellulose or nylon membranes placed on agar plates containing a co-cultivation medium with acetosyringone. After 2 days of incubation in dark, the membranes were transferred to selection plates containing hygromycin B. These plates were incubated in suitable growth conditions for the different fungi until transformants could be picked and grown for further analysis by PCR and/or Southern blot hybridization.

Table 1. *Strains used in this thesis*

Strain	Comment	Paper	Reference
<i>M. oryzae</i>			
Guy11	Wild type	I	
Guy11 Δ 7,8- <i>LDS</i>	Δ 7,8- <i>LDS</i>	I	This study
Δ <i>mac1</i>	Δ Adenylate cyclase	I	[Choi & Dean, 1997]
Δ <i>mac1 sum1-99</i>	Constitutive PKA	I	[Adachi & Hamer, 1998]
Δ <i>cpka</i>	Δ Catalytic subunit of PKA	I	[Xu <i>et al.</i> , 1997]
PMK1	Δ Pathogenicity MAPK 1 ^a	I	[Xu & Hamer, 1996]
MST12	Δ MAPK substrate and transcription factor	I	[Park <i>et al.</i> , 2002]
<i>A. fumigatus</i>			
AF293	Wild type, sequenced	II	[Xue <i>et al.</i> , 2004]
Fresen.	Wild type	II	[de Boer <i>et al.</i> , 2005]
TDWC1.13	Δ <i>ppoA</i>	II	[Dagenais <i>et al.</i> , 2008]
TDWC2.4	Δ <i>ppoB</i>	II	[Dagenais <i>et al.</i> , 2008]
TDWC4.17	Δ <i>ppoC</i>	II	[Dagenais <i>et al.</i> , 2008]
TDWC10.5	Wild type	II	[Dagenais <i>et al.</i> , 2008]
J272	Wild type	III, V	CBS Fungal Biodiversity Centre
J272 Δ <i>ppoB</i>	DOX deletion	V	This study
H237	Wild type, clinical isolate	V	[Zhao <i>et al.</i> , 2006]
AF: Δ ku80	Impaired NHEJ ^b	V	[da Silva Ferreira <i>et al.</i> , 2006]
AF: Δ ku80 Δ <i>ppoB</i>	DOX-CYP deletion	V	This study
A1163	CEA17 PyrG ⁺ , sequenced	V	[Fedorova <i>et al.</i> , 2008]
<i>A. nidulans</i>			
RDIT9.32	Wild type	II	[Tsitsigiannis <i>et al.</i> , 2004b]
RDIT12.9	Δ <i>ppoA</i>	II	[Tsitsigiannis <i>et al.</i> , 2004b]
RDIT59.1	Δ <i>ppoB</i>	II	[Tsitsigiannis <i>et al.</i> , 2005b]
RDIT28.12	Δ <i>ppoC</i>	II	[Tsitsigiannis <i>et al.</i> , 2004a]
<i>A. clavatus</i>			
CBS 514.65	Wild type	IV	CBS Fungal Biodiversity Centre
<i>A. terreus</i>			
IBT1948	Wild type	VI	IBT culture collection
<i>G. graminis</i>			
CBS 870.73	<i>G.g. var. avanae</i>	III	CBS Fungal Biodiversity Centre
<i>L. theobromae</i>			
CBS 122127	Wild type	VII	CBS Fungal Biodiversity Centre
CBS 117454	Wild type	VII	CBS Fungal Biodiversity Centre

^aMAPK, mitogen-activated protein kinase ^bNHEJ, Non-homologous end-joining

Phenotype assays

The pathogenicity of *M. oryzae* strains towards rice (*Oryza sativa*) were assayed for its two different modes of infection. The main route of rice blast infection is by leaf penetration. The pathogenicity assay of leaf infection is a well known method, using two-week old plants for spray-inoculation of a spore-suspension. After five days the numbers of lesions (dark brown spots) on the leaves were counted.

In 2004, Sesma and Osbourn showed that *M. oryzae* can infect rice through the roots, in analogy with *G. graminis* infection of wheat [Sesma & Osbourn, 2004]. This assay was used to investigate root infection by *M. oryzae* mutants. In short, vermiculite (a brown hydrous silicate mineral) was used to bury mycelial plugs of *M. oryzae* beneath rice seeds in 50 ml Flacon tubes. These tubes were sealed to prevent loss of water, and grown for 15 days before the roots of the seedlings were assayed for lesions.

Appressorium is the fungal structure, which like a syringe penetrates the cuticle of the rice leaf. The formation of appressoria can be assayed on plastic microscope coverslips with hydrophobic surfaces, as this can imitate the interaction between the waxy cuticle and the spore, and stimulate appressorium formation. Germination and appressorium formation was analyzed microscopically and compared to wild type *M. oryzae*.

Mycelial growth was assessed as the radial growth of the fungal strains on agar plates. Depending on the fungus (*M. oryzae* or *A. fumigatus*), different growth periods, temperatures, and media were used (generally complete medium for *M. oryzae* and *Aspergillus* minimal medium for *A. fumigatus*) in these assays. The radial distance was measured and spores were harvested using deionized water and counted in a haemocytometer (Bürker chamber).

Recombinant systems

Cloning

RNA was prepared from nitrogen powder of mycelia using protocols based on the selective precipitation of RNA with LiCl. RNA quality was assessed by microchip chromatography (Experion, Bio-Rad), and genomic DNA was removed with DNaseI prior to reverse transcription. Superscript III First-Strand Synthesis System (Invitrogen) was used for RT-PCR. Superscript III is a variant of the Moloney murine leukemia virus (M-MLV) reverse transcriptases with reduced RNaseH activity, which minimizes RNA degradation and increases the sizes of the cDNA. In addition, M-MLV does not possess DNA endonuclease activity, which makes this reverse transcriptase suitable for amplification of full length cDNA. Generally, poly(dT)-primers are suitable for generation of cDNA towards the 3' end of

the target gene, since they bind to the poly(A)-tail of mRNA, whereas the 5' ends often were efficiently amplified using random hexamers.

Expression in insect cells

Cell lines derived from the fall armyworm *Spodoptera frugiperda*, Sf9/Sf21, can be utilized for expression of recombinant proteins. Baculovirus based transfection systems were successfully used for the expression of PGHS-1 [Shimokawa & Smith, 1992], and a number of P450, e.g., human CYP3A4 [Lee *et al.*, 1995]. Insect cells offer a eukaryotic expression system capable of efficient protein folding and most post-translational modifications. Baculovirus systems are based on the homologous recombination of the expression construct into the genome of the insect cells. Although the techniques were refined in recent years, this method is still laborious. An alternative to the baculovirus system is the transient plasmid-driven expression offered by the InsectSelect system [Invitrogen, 2006], which was used in this thesis.

The open reading frames of the genes of interest were inserted into the pIZ/V5-His vector. The plasmid constructs were introduced in Sf21 cells by liposome based transfection. The expression in pIZ/V5-His was driven by the OpIE2 promoter from *Orgyia pseudotsugata* multicapsid polyhedrosis virus. If the native stop codons were interrupted, the expression vectors fused the expressed proteins with the V5 epitope and a 6xHis tag to facilitate detection and purification. Recombinant proteins were harvested two days post-transfection. This expression system is not suitable for large scale expressions due to its transient nature, low yield, and the high cost of liposomes, but it is ideal for rapid analysis and screening of generated protein mutants.

In this study, several expression constructs were developed in pIZ/V5-His. 5,8-LDS of *A. fumigatus* and 7,8-LDS of *G. graminis*, along with their generated mutants and partial constructs, were expressed in Sf21 cells. Constructs of ppoB of *A. fumigatus*, with and without the native stop codon, were also expressed using this system, essentially as described [Garscha & Oliw, 2008b].

Expression in *E. coli*

E. coli expression systems are widely used for production of recombinant proteins. Major advantages are the possibilities of high scale production and low cost. *E. coli* is well characterized and extensively used, which render it popular in non-specialized laboratories. While *E. coli* can be regarded as a first-choice system, it has some major drawbacks. First and foremost, it is a prokaryotic system, and lacks the machineries for post-translational modifications and proper protein folding of eukaryotes. As a result, many

eukaryotic proteins will not be properly processed when expressed in *E. coli*. Another limiting factor is the formation of inclusion bodies during high-level expression of foreign proteins. These insoluble aggregates usually contain inactive proteins. This constitutes a common problem with bacterial expression systems. In some cases, the problem with inclusion bodies can be overcome by refolding and solubilisation of the aggregates, or avoided by certain biotechnological techniques [Sahdev *et al.*, 2008]. Despite its limitations, a number of fungal fatty acid oxygenases were recently successfully expressed using *E. coli* hosts, namely 10R-DOX and 5,8-LDS of *A. nidulans* [Brodhun *et al.*, 2009; Brodhun *et al.*, 2010].

Open reading frames were amplified by PCR, followed by directional cloning in-frame with C-terminal V5 epitope and 6xHis tag of pET/101D-TOPO. Expressions were driven by the IPTG inducible bacteriophage T7 promoter. *E. coli* BL21 Star (DE3) cells were used which express T7 RNA polymerase for efficient transcription. Some experiments utilized the Rosetta 2 (DE3) (Novagen) bacterial host strain. These cells are BL21 derivatives that supply tRNA for codons rarely used by *E. coli*, thereby potentially increasing the expression of foreign eukaryotic proteins. In combination with titration of expression with IPTG, this host strain can decrease misfolding and inclusion body formation.

Several expression constructs were developed for *E. coli*. Full-length and partial constructs of 5,8-LDS of *A. fumigatus* and 7,8-LDS of *G. graminis* were expressed, as well as key mutants suitable for investigations of enzyme kinetics. *E. coli* expression was also developed for ppoB of *A. fumigatus*.

Analysis of protein expression

Protein over-expression in *E. coli* was usually monitored by SDS-PAGE and Coomassie G-250 based staining (Page Blue, Fermentas). However, SDS-PAGE only offers identification based on molecular size of the expected protein product. In addition, expression levels in insect cells, and occasionally in *E. coli*, were not high enough to allow detection by Coomassie staining. For these reasons, protein expressions were also confirmed by Western blot hybridization. After transfer and immobilization of proteins on nitrocellulose membranes, recombinant proteins carrying a V5 epitope (Gly-Lys-Pro-Ile-Pro-Asn-Pro-Leu-Leu-Gly-Leu-Asp-Ser-Thr) or a 6xHis tag could be detected using specific primary antibodies. Horseradish peroxidase labelled secondary antibodies were used in conjunction with the ECL Advance system (GE Healthcare) for detection of chemoluminescence.

Mass spectrometry was occasionally used for identification of expressed proteins. After excision of a protein band from a polyacrylamide gel, the protein was digested with trypsin as described [Hellman *et al.*, 1995]. The tryptic peptides were subsequently subjected to Matrix Assisted Laser

Desorption Ionization (MALDI) coupled to a Time-of-Flight (TOF) mass spectrometer for identification of the parent protein.

Enzyme activity assays

Enzyme assays were performed in four different ways. Whole mycelium from fungal cultures, homogenate of mycelium ground up in liquid nitrogen, or recombinant enzymes from either *E. coli* or insect cell expressions, were incubated and the products were analyzed by HPLC-MS/MS.

Crude, large-scale assays were performed using intact mycelium as described (*paper II*). In short, fungal mycelium was incubated in 5 volumes (w/v) of buffer typically using 0.5-1 mg/ml LA (1.8-3.6 mM) at room temperature for 4-6 h. The incubation buffer was filtered and extracted by conventional liquid-liquid extraction for subsequent analysis by HPLC-MS/MS.

Cell free preparations from either homogenates of nitrogen powder of fungal mycelium (*paper I*), or recombinant proteins expressed in insect cells [Garscha & Oliw, 2009] or *E. coli* (*paper III*), were used. They were assayed for enzyme activity by incubating 0.3-0.5 ml with exogenous fatty acids. After 30-40 minutes of incubation at 4°C, the reactions were stopped with 4 volumes of ethanol.

Metabolites were extracted using solid phase extraction on C₁₈ silica cartridges (SepPak/C₁₈). Solid phase extraction has some advantages over liquid-liquid extraction. First and foremost, the procedure is more convenient and faster, making this method more amendable when dealing with multiple samples. The method also consumes less organic solvent and the recovery of the analytes is generally more complete [Snyder *et al.*, 1997]. After elution with ethyl acetate, the volume is easily reduced, and the sample can be reconstituted for further analysis using HPLC-MS/MS.

HPLC-MS/MS analysis

Analyses of extracted metabolites were mainly performed using HPLC coupled to tandem mass spectrometry. This method combines the separation by structural features (e.g., hydrophobicity and chirality) by the HPLC system, with the separation by their mass-to-charge ratio (m/z) by the mass spectrometer. Some experiments also utilized a photodiode array detector before introduction of the effluent into the mass spectrometer.

Three modes of HPLC were used. In most routine analyses reverse phase HPLC (RP-HPLC) with C₁₈-silica columns was utilized. Separations of diastereoisomers of dihydroxylated metabolites were performed on normal phase HPLC (NP-HPLC) using silica columns. These columns were eluted with hexane/isopropyl alcohol/acetic acid. To enable proper ionization

during electrospray ionization (ESI), the effluent was combined in a T-junction with a mixture of isopropyl alcohol/water. Chiral phase HPLC (CP-HPLC), with columns listed in Table 2, was used to separate enantiomers. The choice of column when analysing a metabolite was mainly empirical, since the theory of chiral selection is not well elaborated.

Table 2. *CP-HPLC columns used for separations of enantiomers of LA metabolites.*

Column	Chiral selector	Enantiomers separated
Reprosil Chiral-NR	Aromatic (undisclosed)	10-HODE, 13-HODE
Reprosil Chiral-AM	Amylose tris-3,5-dimethylphenyl-carbamate	8-HODE, 9-HODE, α -ketol
Chiralcel OB-H	Cellulose tribenzoate	8-HODE

For some qualitative analyses the lack of baseline separation of the chromatographic system could be compensated for by the possibility of monitoring representative fragment ions formed during MS/MS. Matrix effects are potential problems in HPLC-MS/MS. These are caused by the co-elution of components not seen in the chromatogram, which can cause suppression or enhancement of the detection of an analyte [Van Eeckhaut *et al.*, 2009]. This problem was partially solved by analyzing particularly important samples on both NP-HPLC and RP-HPLC.

The mass spectrometer used in this study was a linear quadrupole ion trap (LTQ, Thermo Finnigan) with an ESI interface. Since the analytes in this thesis carries carboxylic acid functional groups, mass spectrometry was carried out in the negative ion mode. Negative mode ESI requires deprotonation of the fatty acid analytes. This might seem incompatible with the use of acid in the HPLC mobile phase, but it was shown that acidity and pK_a values are inconclusive parameters in negative mode ESI [Cech & Enke, 2001; Henriksen *et al.*, 2005]. As a consequence, acidic analytes can still ionize efficiently using mobile phases with pH below the pK_a of the analytes.

In MS/MS experiments, the collision energy was set at 35-50% of 5V (thus using 1.75-2.5V). The isolation width was set at 1.5 for most analytes, but was increased to 5 in analyses of hydroperoxide metabolites [Garscha *et al.*, 2008], and to 6 for deuterium labelled fatty acid products.

Bioinformatic tools

Protein and nucleotide sequence data were generally obtained from GenBank (www.ncbi.nlm.nih.gov). Predictions of phosphorylation sites were performed using the NetAspGene 1.0 server developed at the Technical University of Denmark [Blom *et al.*, 2004]. Database searches using

structural annotations of predicted proteins were performed using the SUPERFAMILY database version 1.75 [Gough *et al.*, 2001].

Alignments and phylogenetic trees were constructed using the MEGA 4 program [Tamura *et al.*, 2007]. The alignments were performed using the Clustal W algorithm [Thompson *et al.*, 1994], which is widely used for alignments of protein sequences. Phylogenetic trees were constructed using the neighbour-joining method [Saitou & Nei, 1987] followed by bootstrap tests of the resulting nodes. The distances within the trees are based on the expected number of substitutions per amino acid site. Each node in a tree has a bootstrap value, which could be perceived as the reliability of a constructed node when the sequences are provoked with random amino acid substitutions. The bootstrap values lies between 0-100, where nodes with values above 95 can be considered as reliable [Tamura *et al.*, 2007].

Sequencing was performed by the Uppsala Genome Center (Rudbeck Laboratory, Uppsala, Sweden) using AB BigDye[®] Terminator v.3.1 Sequencing Kits and separation with capillary electrophoresis on ABI3730XL automatic sequencer (Applied Biosystems). The results were analyzed using the Lasergene suite software (DNAS[®]Star).

Comments on nomenclature

The nomenclature in the field of fatty acid oxygenases can be confusing. As a principle, trivial names are avoided in this text unless they are generally accepted within the scientific community. Examples of the latter are many members of the arachidonic acid cascade, i.e., prostaglandins, leukotrienes, and their associated enzymes.

Abbreviations of chemical names of oxylipins are commonly used in the literature, e.g., 15-HPETE for 15-hydroperoxyeicosatetraenoic acid. Oxygenated metabolites of LA will be abbreviated and denoted by the same principle. Hence, 8-hydroxyoctadecadienoic acid will be designated 8-HODE, and not PsiB α or laetiseric acid, as these names do not provide structural information.

Enzymes will be named by their activities, when these are known. The enzyme responsible for the conversion of LA to 10*R*-HPODE will therefore be referred to as 10*R*-DOX, and not by its previous name, ppoC, since this can be confusing and misleading. Putative proteins are referred to by their GenBank accession numbers. If an enzymatic activity is conclusively linked to a specific gene, this will take on the name of the enzyme. Again, the gene encoding 10*R*-DOX should be referred to as 10*R*-DOX, and not *ppoC*, but both are often used to avoid confusion.

This thesis discusses a group of enzymes which oxygenate LA, of which 7,8-LDS of *G. graminis* is the prototype. The deduced protein sequences consist of N-terminal DOX domains with homology to animal heme

peroxidases, and C-terminal P450 domains, with or without hydroperoxide isomerase activities. This gene/protein family is referred to as dioxygenase-P450 fusion proteins (DOX-CYP).

Results

Linoleate diol synthases of *M. oryzae* (paper I)

Mycelium of *M. oryzae* was previously reported to produce 7,8-DiHODE [Cristea *et al.*, 2003], but its stereochemistry and mechanism of formation was not elucidated. CP-HPLC and NP-HPLC using appropriate standards showed that the absolute configurations at C-7 and C-8 were *S*. 7*S*,8*S*-DiHODE was formed from 8*R*-HPODE with retained configuration at C-8. The change from *R* to *S* at C-8 is merely due to the Cahn-Ingold nomenclature. Studies using stereospecifically deuterated LA showed that 7*S*,8*S*-DiHODE was formed in analogy with *G. graminis* (Fig. 7), i.e., by abstraction of the pro-*S* hydrogen and antarafacial insertion of molecular oxygen at C-8, followed by pro-*S* hydrogen abstraction and suprafacial oxygen insertion at C-7. While 7,8-DiHODE was the main product, detailed studies of oxygenated metabolites showed formation of 10-HODE, 8*R*,11*S*-DiHODE, 6*S*,8*R*-DiHODE, and traces of 5,8-DiHODE.

Identification of 7,8-LDS (paper I)

The genome of *M. oryzae* contains two genes with homology to 7,8-LDS of *G. graminis*, *MGG_13239* and *MGG_10859*, with sequence identities of 63% and 41%, respectively. A gene deletion construct of *MGG_13239* was developed, and transformation of *M. oryzae* Guy11 resulted in homologous integration and targeted disruption of the gene, as judged by PCR analysis and Southern blot hybridization. This strain, which completely lacked 7,8-LDS activity and only showed traces of residual 8-DOX activity (~1% compared to wild-type), was termed Δ 7,8-LDS. The phenotype of Δ 7,8-LDS was not altered compared to wild-type Guy11 in the parameters investigated. These included leaf and root infection of rice, appressoria formation, spore amount and morphology, and vegetative growth.

DOX and isomerase activities of sum1-99 (paper I)

Several mutants of *M. oryzae* with altered infection capacities towards rice were analyzed with regard to their abilities to oxygenate LA. One mutant, Δ *mac1 sum1-99*, showed a dramatic increase in the biosynthesis of 5*S*,8*R*-DiHODE and 10*R*-HODE. Later analyses showed that these metabolites are

the main products of $\Delta mac1\ sum1-99$ under certain conditions, (Jernerén, unpublished data, Fig. 11).

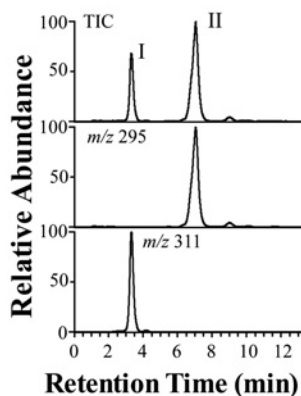


Figure 11. Chromatogram of metabolites formed from LA by *M. oryzae* $\Delta mac1\ sum1-99$ grown for four days at room temperature. MS² spectrum of peak I corresponds to 5,8-DiHODE. Peak II contains a mixture of 10-HODE and 8-HODE, the former being the main metabolite. Only traces of 7,8-DiHODE could be detected in this experiment.

The *sum1-99* mutant has a spontaneous compensatory mutation in the regulatory subunit of protein kinase A (PKA), which renders it constitutively active. Mutants with impaired function of PKA were not altered in their oxygenation profiles. The emergence of new metabolites could be explained by an up-regulation of the second 7,8-LDS homologue, *MGG_10859*. However, real-time PCR analysis failed to show an increased expression of this transcript compared to control strains with insignificant 5,8-DiHODE and 10-HODE formation. These results indicate that the regulation of fatty acid oxygenation by PKA of *M. oryzae* must be explained by other mechanisms.

Dioxygenases of *A. fumigatus* and *A. nidulans* (paper II)

Three main metabolites were detected in *A. nidulans* and *A. fumigatus* AF293 when incubated with LA: 10-H(P)ODE, 8-H(P)ODE, and 5,8-DiHODE. The absolute configurations of the monohydroxy compounds were analysed by CP-HPLC, and were found to be *R* in both cases (Fig. 12). Diastereoisomers of 5,8-DiHODE formed from 8*R*-HPODE were resolved by NP-HPLC and the metabolite could be identified as 5*S*,8*R*-DiHODE. In addition, *A. fumigatus* Fresen. was found to synthesize 8,11-DiHODE. This metabolite was formed from 8*R*-HPODE and was identified as 8*R*,11*S*-DiHODE.

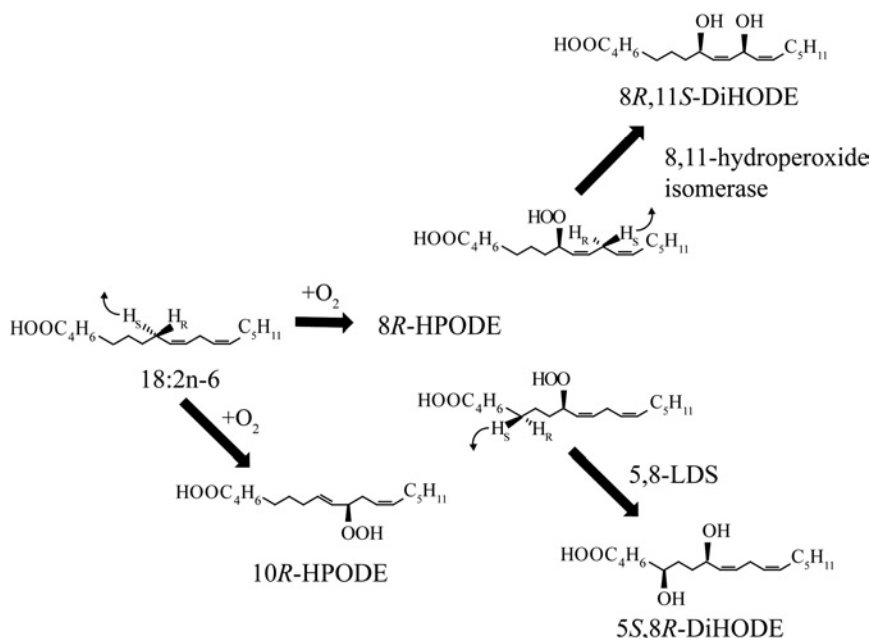


Figure 12. Catalytic reaction mechanisms of fatty acid DOX of *A. nidulans* and *A. fumigatus*. Note that 8,11-DiHODE only is formed by *A. fumigatus* strain Fresen.

These aspergilli contain three genes with homology to 7,8-LDS of *G. graminis*, designated *ppoA*, *ppoB*, and *ppoC* [Tsitsigiannis *et al.*, 2004a; Tsitsigiannis *et al.*, 2004b; Tsitsigiannis *et al.*, 2005a]. By studying knock-out strains of *A. nidulans* and *A. fumigatus*, it was evident that *ppoA* coded for a 5,8-LDS, while *ppoC* coded for a 10R-DOX. Since the gene deletions were created in a genetic background, which lacked capacity to form 8,11-DiHODE, it was not possible to conclusively link this metabolite to any particular gene. Based on these results in isolation, it seemed reasonable to assume that *ppoB* encodes 8,11-LDS or an 8,11-linoleate hydroperoxide isomerase.

The mechanisms of the reactions were determined with stereospecifically deuterated LA. By comparing the deuterium contents of the substrates with those of the different metabolites, it was possible to determine the stereochemistry of the hydrogen abstractions and oxygen insertions. As seen in Fig. 12, all reactions were initiated by the stereospecific abstraction of the pro-*S* hydrogen at C-8 to form a carbon centred radical which formed 8R-HPODE after antarafacial insertion of molecular oxygen, or 10R-HPODE after double-bond migration and antarafacial oxygen insertion. 8R-HPODE could be further converted into either 5S,8R-DiHODE or 8R,11S-DiHODE by pro-*S* hydrogen abstraction and suprafacial oxygen insertion at C-5 and C-11, respectively.

Recombinant 5,8-LDS (*paper III*)

5,8-LDS (ppoA, XP_75150) was cloned by RT-PCR using RNA generated from *A. fumigatus* J272. The deduced amino acid sequence differed at two positions (Cys33, Gly261) compared with the predicted protein derived from the genome sequence of *A. fumigatus* AF293. These differences did not significantly alter the catalytic activity of the enzyme since recombinant protein from *E. coli* and insect cells readily transformed LA to 8-HPODE and 5,8-DiHODE.

Members of the LDS protein family were suggested to be fusion proteins of heme-containing DOX domains with catalytic and structural homology with PGHS, and C-terminal cytochrome P450 domains [Lee *et al.*, 2008; Brodhun *et al.*, 2009]. 5,8-LDS of *A. fumigatus* was investigated, and the proposed hydrogen abstracting tyrosine of the DOX domain, and the heme-binding cysteine of the P450 domain, were replaced by site-directed mutagenesis. The results showed that replacement of the tyrosine residue with phenylalanine completely abolished the 8-DOX activity, while its capacity to carry out the subsequent isomerization reaction to 5,8-DiHODE was retained. Conversely, replacement of the cysteine residue with serine, abolished the isomerase activity, while the DOX activity remained unchanged.

Furthermore, it was of interest to examine if the two domains could be expressed separately in active forms. An N-terminal partial construct expressing amino acids 1-674 (of 1079) was shown to be catalytically active, producing a prominent amount of 8*R*-H(P)ODE after incubation with LA. Analogous experiments were carried out with 7,8-LDS of *G. graminis*. The recombinant enzyme showed 7,8-LDS activity when expressed in *E. coli* and insect cells. A partial construct containing the first 673 amino acids (of 1165) retained 8*R*-DOX activity, but lacked isomerase activity.

DOX and hydroperoxide isomerase activities of *A. clavatus* (*paper IV*)

A phylogenetic tree was constructed using 17 sequences of predicted proteins of the fatty acid DOX/P450 family. These sequences were collected from *A. clavatus* and four additional aspergilli species with known fatty acid oxygenation profiles. The predicted proteins of *A. clavatus* include three DOX-CYP members. Members of the 10*R*-DOX (ppoC) subgroup contain inactive P450 domains, and lack the conserved cysteine residues acting as heme-thiolate ligands. In contrast to *A. nidulans* and *A. fumigatus*, only one of the three DOX-CYP sequences of *A. clavatus* seemed capable of providing a thiolate-heme cysteine ligand. This protein belongs to the 5,8-

LDS (ppoA) cluster in the phylogenetic tree, and likely encodes an enzyme with this activity.

The fatty acid oxygenation profile was therefore assessed in *A. clavatus*. As expected, it produced significant amounts of 10*R*-HODE and 5*S*,8*R*-DiHODE. More surprisingly, it also readily produced 8*R*,11*S*-DiHODE. It seemed reasonable to assume that the third gene encodes an 8,11-LDS. Partial sequencing of the putative 8,11-LDS gene confirmed a serine, instead of a cysteine, at the crucial position of the corresponding enzyme. This was highly unexpected, given the ability of this fungus to produce two dihydroxy metabolites from LA, and raised the question concerning the origin of 8,11-DiHODE in this fungal species.

Gene deletion and expression of ppoB (*paper V*)

10*R*-DOX (ppoC) and 5,8-LDS (ppoA) of *A. fumigatus* were identified (*paper II*), cloned, and expressed ([Garscha & Oliw, 2009], *paper III*). The third putative DOX/P450 enzyme member, ppoB, was linked with 8,11-LDS activity only by exclusion. RNA was extracted from *A. fumigatus* J272, which readily converts LA to 8,11-DiHODE. Full length cDNA was considerably more difficult to obtain, compared with 10*R*-DOX and 5,8-LDS, and genomic DNA was utilized in the cloning process. The full-length cDNA differed at two positions in its deduced amino acid sequence compared to ppoB of strain AF293 (which does not produce 8,11-DiHODE) and at three positions compared to A1163 (an 8,11-DiHODE producing strain). None of these substitutions were unique for J272.

Recombinant ppoB was produced in insect cells and *E. coli* as judged by SDS-PAGE, MALDI-TOF, and Western blot analysis. In *E. coli*, the protein was mainly expressed as inclusion bodies, but could still be detected to some extent in soluble fractions. Neither expression system produced recombinant enzymes with detectable activity after incubations with LA, purified 8*R*-HPODE, or any other fatty acid substrate tested.

As experiments using recombinant ppoB failed to show catalytic activity, the connection between ppoB and 8,11-DiHODE biosynthesis was addressed by gene deletion. Two separate deletion constructs were developed. One targeted the proposed DOX domain of ppoB, while the other targeted both the DOX and the isomerase domain. Knock-out strains obtained with these deletion constructs were successfully developed from strains of *A. fumigatus* with high capacities to form 8,11-DiHODE. Contrary to what was expected, the deletions had no effects whatsoever on the production of 8,11-DiHODE.

The 8,11-linoleate hydroperoxide isomerase activity of *A. fumigatus* was present in cytosolic fractions. Considerable inhibition was obtained when fungal homogenate was incubated with LA in the presence of certain inhibitors of fungal and human P450, i.e., 1-benzylimidazole, ozagrel,

ketoconazole, and miconazole (Fig. 13). The common feature of these drugs is the imidazole moiety (Fig. 9).

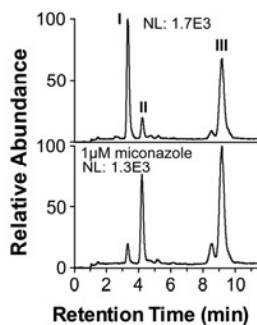


Figure 13. The chromatograms show metabolites formed from homogenate of *A. fumigatus* Δ KU80 after incubation with 2 μ M 8*R*-HPODE without (*top*), and with (*bottom*) supplementation of 1 μ M miconazole. Peak I was identified as 8,11-DiHODE, peak II as 5,8-DiHODE, and peak III contains the internal standard used in the experiment (13-HODE).

9*R*-DOX and AOS activities of *A. terreus* (paper VI)

The genome of *A. terreus* contains five genes of the DOX-CYP gene family. A phylogenetic tree was constructed using the deduced protein sequences, which showed that two of the proteins cluster with 10*R*-DOX and 5,8-LDS of other aspergilli. The remaining sequences (ATEG_03171, ATEG_00985, and ATEG_02036) clustered in three separate groups with members of unknown functions. Therefore, the ability of *A. terreus* to form novel oxygenated metabolites from fatty acids was examined.

In addition to the expected monohydroxy metabolites, 10*R*-HODE and 8*R*-HODE, *A. terreus* synthesized an enzymatic product from LA, 9-HODE, which had never before been described in aspergilli as an enzymatic product. 9-H(P)ODE can be formed by autoxidation, but this pathway was excluded by comparing the rate of formation with other autoxidation products, and by steric analyses. Separation of the stereoisomers on CP-HPLC, and comparison with 9*S*-HODE formed by potato stolons, showed that 9*R*-HODE was formed with high stereoselectivity. 9*R*-HODE was formed from [11*S*-²H]-LA with retention of the deuterium label, suggesting hydrogen abstraction at C-11 followed by double bond migration and suprafacial oxygen insertion at C-9 [Jernerén *et al.*, 2010].

Polar metabolites of LA were also produced by *A. terreus*. 5,8-DiHODE was readily formed by nitrogen powder of this fungus. More surprisingly, two novel metabolites were detected as major products, and were identified as 9-hydroxy-10-oxo-12*Z*-octadecenoic acid and 13-hydroxy-10-oxo-11*E*-octadecenoic acid. Analogous metabolites are formed from 9*S*-HPODE in

certain plants as non-enzymatic products of an unstable allene oxide intermediate. The presence of these compounds, known as α - and γ -ketols, suggested that *A. terreus* expresses an AOS. The genome of the fungus contains 125 putative P450 enzymes, none of these show obvious similarities with the AOS (CYP74) of *A. thaliana*.

The AOS activity of *A. terreus*, as judged by the accumulation of the α - and γ -ketol, was shown to be specific to 9*R*-HPODE, while the 9*S* stereoisomer is converted by AOS of plants. Experiments showed that the AOS activity was located in microsomal fractions of *A. terreus*, while the 9*R*-DOX activity was found to be soluble. Since the genome of *A. terreus* lacks LOX, it seemed possible that the 9*R*-DOX activity is encoded by a member of the DOX-CYP fusion protein family. The genome contains three candidates as discussed above.

9*R*-DOX and AOS activities of *L. theobromae* (paper VII)

Allene oxide synthases are known for their involvement in the enzymatic conversion of 13*S*-HPOTrE to jasmonates in plants. As mentioned in the introduction, JA is known as a product formed by the tree pathogenic filamentous fungi *L. theobromae*. A biosynthetic pathway of JA formation from α -linolenic acid was recently conclusively identified, but the exact mechanism was not elucidated [Tsukada *et al.*, 2010].

The oxygenation of polyunsaturated fatty acids by *L. theobromae* was analyzed. JA could only be detected in small amounts by GC-MS, and its mechanism of formation could not be established. 9*R*-hydroperoxides were readily formed from linoleic and α -linolenic acid. These were subsequently transformed into their corresponding allene oxides as judged by the accumulation their non-enzymatic degradation products: the α - and γ -ketols. In analogy with its formation in *A. terreus*, 9*R*-HPODE was formed from LA by abstraction of the pro-*R* hydrogen at C-11, followed by double bond migration and insertion of molecular oxygen suprafacially at C-9. In both fungi, α -linolenic acid was transformed to 9*R*-HPOTrE. But only the AOS activity of *L. theobromae* converted this hydroperoxide into its corresponding allene oxide.

Interestingly, γ -linolenic acid (18:3*n*-6) was also a substrate for the 9-DOX activity, which resulted in the formation of 9-HPOTrE(*n*-6). This is of mechanistic interest due to the fact that characterized members of the DOX-CYP family (i.e. 10*R*-DOX, 7,8-LDS, and 5,8-LDS) usually do not accept this fatty acid as a substrate, while LOX do. The genome of *L. theobromae* had not been sequenced. Therefore, an *in silico* investigation of the genetic origin of this 9*R*-DOX activity was not possible.

Discussion

Following the characterization of 7,8-LDS of *G. graminis*, several putative fatty acid oxygenases of related fungi were identified. Some of these are proposed to be involved in virulence and reproduction [Tsitsigiannis *et al.*, 2004a; Tsitsigiannis *et al.*, 2004b; Tsitsigiannis *et al.*, 2005a]. One of the products of 7,8-LDS, 8*R*-HODE, was originally described as a fungicidal agent [Bowers *et al.*, 1986], and was later shown to affect reproduction processes in aspergilli [Champe *et al.*, 1987; Mazur *et al.*, 1991; Calvo *et al.*, 1999]. These results suggest that 7,8-LDS is a member of a larger group of enzymes in filamentous fungi involved in fundamental biological processes.

The overall aim of the present study was to expand the knowledge of this proposed enzyme group by identifying new members and characterize their activities. In order to do this, biochemical and analytical techniques were used, along with molecular biology methods such as gene deletion using *A. tumefaciens*, and protein expression.

Common catalytic mechanism of the DOX-CYP family

7,8-LDS of *G. graminis* is a bifunctional enzyme [Hamberg *et al.*, 1994]. Molecular oxygen is incorporated into LA antarafacially after hydrogen abstraction at C-8, and a tyrosyl radical is formed during the catalysis [Su *et al.*, 1998]. The 8*R*-hydroperoxide produced in this reaction can either be non-enzymatically reduced to its corresponding alcohol, 8*R*-HODE, or isomerized into 7*S*,8*S*-DiHODE by suprafacial hydrogen abstraction and oxygen insertion at C-7. The psi compounds were identified and described in *A. nidulans* [Champe *et al.*, 1987; Mazur *et al.*, 1990]. The reaction mechanisms of their biosynthesis were identified in the current study, along with several analogous activities in a number of related ascomycetous filamentous fungi (Table 3 and 4). Common for the formation of 8-HPODE and 10-HPODE is the pro-*S* hydrogen abstraction of LA to produce a carbon-centered radical at C-8. This abstraction is presumably catalyzed by a tyrosyl radical in the YRWH motif found in all members of the DOX-CYP enzyme family characterized to date. Molecular oxygen is inserted antarafacially at C-8 or C-10 (following double bond migration), yielding hydroperoxide products. The enzymatic synthesis of 9-HPODE in *A. terreus* and *L. theobromae* occurs by a slightly different mechanism, and will be discussed in a separate section below.

Table 3. Major hydroperoxy metabolites of LA formed by studied fungi.

Fungus	8-HPODE	10-HPODE	9-HPODE
<i>A. fumigatus</i>	+++	+++	-
<i>A. nidulans</i>	+++	+++	-
<i>A. clavatus</i>	+++	+++	-
<i>A. terreus</i>	+++	+++	+++
<i>M. oryzae</i>	+++	+	-
<i>M. oryzae</i> sum1-99	+++	+++	-
<i>L. theobromae</i>	++	++	+++

The hydroperoxides listed in Table 3, are commonly reduced to their respective alcohols, 8-HODE, 10-HODE, and 9-HODE. However, all fungi studied are able to convert 8R-HPODE into at least one dihydroxylated compound (Table 4). These are formed by hydrogen abstraction and suprafacial oxygen insertion at carbon 5, 11, and 7, respectively. The suprafacial oxygen insertions of the isomerase reactions are typical of P450.

Table 4. Major dihydroxy metabolites of LA formed by studied fungi.

Fungus	5,8-DiHODE	8,11-DiHODE	7,8-DiHODE
<i>A. fumigatus</i>	+++	+++	-
<i>A. fumigatus</i> AF293	+++	-	-
<i>A. nidulans</i>	+++	-	-
<i>A. clavatus</i>	+++	+++	-
<i>A. terreus</i>	+++	-	-
<i>M. oryzae</i>	+	+	+++
<i>M. oryzae</i> sum1-99	+++	+	+
<i>L. theobromae</i>	+++	-	-

The substrate specificities of the DOX-CYP follow a certain pattern. γ -Linolenic acid is not accepted as a substrate by the DOX activities, which therefore seem to require a saturated carbon chain from the carboxyl group to the first double bond at C-9 of 18-carbon unsaturated fatty acids. This is in contrast to the LOX enzymes.

In addition to the experiments conducted in this thesis, the oxylipin profile of an additional member of the aspergilli was investigated by Wadman *et al.* *A. niger* was shown to produce 8,11-DiHODE, 5,8-DiHODE, and 10-HODE as main metabolites when incubated with LA [Wadman *et al.*, 2009]. This oxygenation profile closely resembles that of *A. clavatus*.

Identification of DOX-CYP fusion enzymes

7,8-LDS was cloned and sequenced in the late 90s [Hörnsten *et al.*, 1999]. Homologous genes (*ppoA*, *ppoB*, *ppoC*) were identified in *A. nidulans* and *A. fumigatus* [Tsitsigiannis *et al.*, 2005a]. Two of these genes were conclusively identified in the present study, and the catalytic mechanisms of

their gene products were elucidated. A summary of all identified enzymes is given in Table 5.

The rice pathogen *M. oryzae* contains two genes of the *DOX-CYP* family. One of these, *MGG_13239*, shows a considerable degree of sequence identity to 7,8-LDS of *G. graminis*. In *paper I*, *MGG_13239* was deleted in order to identify the encoded activity and to study its biological significance. As predicted, gene deletion of *MGG_13239* completely abolished the capacity to form 7,8-DiHODE, and reduced the 8R-HODE biosynthesis to trace amounts. It was concluded that *MGG_13239* encodes a 7,8-LDS with similar catalytic functions as its homologue in *G. graminis* (*paper I*).

Table 5. *DOX-CYP* enzymes identified in this thesis by gene deletion studies, in some cases confirmed by protein expression experiments

Fungus	Enzyme	Gene deletion	Expression
<i>M. oryzae</i>	7,8-LDS	<i>Paper I</i>	-
<i>A. fumigatus</i>	10R-DOX	<i>Paper II</i>	[Garscha & Oliw, 2009]
	5,8-LDS	<i>Paper II</i>	<i>Paper III</i>
<i>A. nidulans</i>	10R-DOX	<i>Paper II</i>	[Brodhun <i>et al.</i> , 2010]
	5,8-LDS	<i>Paper II</i>	[Brodhun <i>et al.</i> , 2009]
<i>G. graminis</i>	7,8-LDS	-	<i>Paper III</i> ^a

^a7,8-LDS of *G. graminis* was originally identified by protein purification [Su *et al.*, 1998] and was expressed earlier [Garscha & Oliw, 2008a; Garscha & Oliw, 2008b]. The reference in the table refers to the first recombinant system with full LDS activity.

Studies using gene deletion mutants of aspergilli showed that the capacities to form 10R-HODE and 5S,8R-DiHODE were abolished in Δ *ppoC* and Δ *ppoA* strains, respectively. The encoded enzymes were therefore named 10R-DOX and 5,8-LDS (*paper II*). The enzymatic activities of these gene products were verified by protein expression studies (Table 5), and catalytically important amino acid residues were determined. Interestingly, Brodhun *et al.* showed that the 5,8-LDS of *A. nidulans* is a fusion enzyme between an N-terminal DOX domain, and a C-terminal P450 isomerase domain [Brodhun *et al.*, 2009]. Sequence analyses suggest that this is a common feature of the LDS and 10R-DOX family, here designated DOX-CYP. Experiments using partial constructs of 7,8-LDS of *G. graminis* and 5,8-LDS of *A. fumigatus* showed that the DOX domains remained catalytically active when expressed independently (*paper III*). Furthermore, site-directed mutagenesis of the conserved cysteine residues of the proposed P450 domains to serine, abolished the isomerase activities of the enzymes (*paper III*). The same result was observed when this cysteine residue of 5,8-LDS of *A. nidulans* was replaced with alanine [Brodhun *et al.*, 2009]. These results highlight the importance of cysteine as the fifth heme ligand of P450.

Biological significance of fungal oxylipins

As mentioned, Champe *et al.* showed that the psi factors regulate reproduction in *A. nidulans*. PsiBa (8-HODE) and PsiCa (5,8-DiHODE) promote sexual reproduction and inhibit conidiation [Champe & el-Zayat, 1989]. Prior to the identification of the psi factors, it was known that LA itself possesses sporogenic activity in several fungi [Katayama & Marumo, 1978; Nukina *et al.*, 1981]. This was confirmed in more recent studies [Goodrich-Tanrikulu *et al.*, 1998; Calvo *et al.*, 1999]. Calvo *et al.* developed *A. nidulans* strains with deletions in the Δ -12 desaturase (Δ odeA), responsible for converting oleic acid to LA. These strains are devoid of 8-HODE and 5,8-DiHODE and show increased sexual and decreased asexual sporulation [Calvo *et al.*, 2001], in contrast to what was expected from previous reports.

Keller *et al.* have studied the biological impacts of deletions of *ppo* genes extensively. A deletion of *ppoA* (5,8-LDS) of *A. nidulans* increased the ratio between asexually and sexually derived spores [Tsitsigiannis *et al.*, 2004b], while deletion of *ppoC* (10R-DOX) produced the opposite effect [Tsitsigiannis *et al.*, 2004a]. Deletion of *ppoB*, with unknown biochemical functions, produced similar effects as Δ *ppoA*, i.e., an increased ratio between conidia and ascospores [Tsitsigiannis *et al.*, 2005b]. There are indications that the *ppo* gene products are involved in oxylipin-mediated cross-talk between *A. nidulans* and peanut seeds [Brodhagen *et al.*, 2008]. The interactions between *A. flavus* and its hosts were shown to be partly regulated by fatty acids and oxylipins, which also affect aflatoxin synthesis [Burow *et al.*, 1997; Wilson *et al.*, 2001; Brown *et al.*, 2009; Gallo *et al.*, 2010].

A sexual reproduction cycle in *A. fumigatus* was only recently discovered [O'Gorman *et al.*, 2009]. Whether this process is influenced by oxylipins, in analogy with *A. nidulans*, is unknown. The fact that cleistothecia (the structure in which the sexually derived spores are produced) develop six months after mating of certain strains with opposite mating types, makes such studies of sporegenic effects in *A. fumigatus* complicated. All three *ppo* genes of *A. fumigatus* AF293 were deleted and the capacities of the deletion strains to form conidia were assessed. Deletion of *ppoC* increased the number of conidia, while Δ *ppoA* and Δ *ppoB* showed no alterations compared to wild-type [Dagenais *et al.*, 2008]. The deletion of *ppoB* in an 8,11-DiHODE producing strain of *A. fumigatus* (*paper V*) were in concordance with this report.

Very little is known regarding the biological impact of oxylipins on the biology and pathogenicity of *M. oryzae*. Certain oxylipins present on the rice leaf surface were shown to induce appressorium formation during the infectious process of *M. oryzae*, e.g., *cis*-9,10-epoxy-18-hydroxyoctadecadienoic acid [Gilbert *et al.*, 1996]. 9- and 13-H(P)ODE

inhibit growth of *M. oryzae* and levels of these oxylipins are elevated in transgenic strains of rice with increased resistance towards the pathogen [Yara *et al.*, 2007]. As stated in the introduction of this thesis, jasmonates are important in defense responses of plants, and are induced in rice upon infection with *M. oryzae* [Mei *et al.*, 2006].

A strain of *M. oryzae* was developed with deletion of the *7,8-LDS* gene (*paper I*). The pathogenicity towards rice was assessed using two different models. These experiments showed no alterations of phenotypes during infection, and appressoria developed as normal. Furthermore, the strain was also unchanged in regard to colony morphology, sporulation, and growth. This was in concordance with the gene deletion of a *7,8-LDS* homologue in *Ustilago maydis*, hypothesized to be involved in the mobilization of lipids, which did not alter its phenotype [Huber *et al.*, 2002]. A comparative genome analysis showed that members of the *DOX-CYP* gene cluster are conserved in, and are specific to, filamentous fungi [Soanes *et al.*, 2008]. The authors of this report speculated that these findings may indicate housekeeping functions of the enzymes.

Characteristics of DOX-CYP sequences

To date, members of the DOX-CYP enzyme family were identified in *G. graminis* [Su *et al.*, 1998; Hörnsten *et al.*, 1999], *M. oryzae* (*paper I*), *A. fumigatus* (*paper II*), and *A. nidulans* (*paper II*). Additionally, information on the oxylipin profiles of *A. niger* [Wadman *et al.*, 2009], *A. clavatus* (*paper IV*), and *A. terreus* (*paper VI*) are published. Genomic information from these fungi is available.

Figure 14 shows a phylogenetic tree constructed from an alignment of all identified and putative DOX-CYP of these fungi. All included aspergilli show 10R-DOX and 5,8-LDS activities, and the identified and putative members of these enzyme groups form two distinct clusters in the phylogenetic tree. It is evident from this comparison that ppoB proteins do not form a homogenous group. This was highlighted by Wadman *et al.* who named the third putative DOX-CYP of *A. niger* ppoD, since it is not closely related to ppoB of either *A. fumigatus* or *A. nidulans* [Wadman *et al.*, 2009].

A partial alignment of the identified and putative enzymes included in the phylogenetic tree shows amino acid residues of the P450 domains around the presumed heme-thiolate cysteine ligand (Fig. 15). Strikingly, all members of the 10R-DOX cluster lack the essential cysteine, explaining their absent isomerase activities [Garscha & Oliw, 2009; Brodhun *et al.*, 2010].

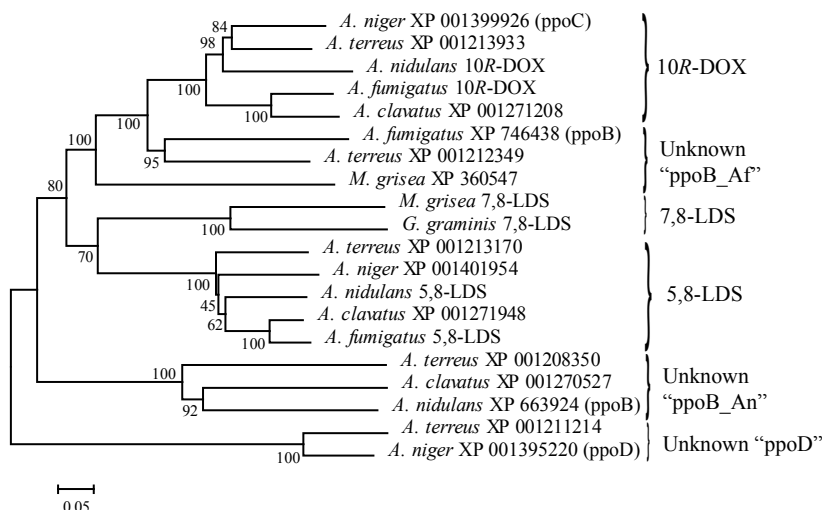


Figure 14. Phylogenetic tree showing members of the DOX-CYP protein family of fungi studied in this thesis and *A. niger* [Wadman *et al.*, 2009]. Proteins with unknown function are denoted by their hypothetical protein number (GeneBank). Two clusters with characterized members appear, the 10R-DOX and the 5,8-LDS clusters. PpoB_Af, ppoB_An, and ppoD designate three protein clusters with unknown functions.

<i>A. niger</i> ppoC	1012	EVRLDRPLDSY	IHYGLG	PQSG	CLG
<i>A. terreus</i> XP 001213933	1014	EVRLDRPLDSY	IQFGLG	PHAG	CLG
<i>A. nidulans</i> 10R-DOX	1019	EVRLDRPDES	LYNYGIG	SQIG	CLG
<i>A. fumigatus</i> 10R-DOX	1035	EVRLDRPMNSY	IINPTLGP	HCF	FLS
<i>A. clavatus</i> XP 001271208	1031	EVRLDRPLDAY	IINHSLGP	HGF	FLS
<i>A. fumigatus</i> ppoB	1027	EVDLNRPMESY	IHYGVG	PHTC	CLG
<i>A. terreus</i> XP 001212349	1036	EVRLDRPMSAY	LHYGEG	PHM	CLG
<i>M. grisea</i> XP 360547	1022	VVNPRRP	PAKKY	IHYGVG	PHACL
<i>M. grisea</i> 7,8-LDS	1069	KVRLDRDLDSY	TFFFLG	LP	HRCAG
<i>G. graminis</i> 7,8-LDS	1060	EVRLDRPLESY	VHFGLG	LP	HRCAG
<i>A. terreus</i> XP 001213170	977	KVRLDRDMDLY	VHFGSG	PHK	CLG
<i>A. niger</i> XP 001401954	986	KVKLDRDMSLY	AHFGFG	PHC	CLG
<i>A. nidulans</i> 5,8-LDS	986	KVKLDRDMNLY	AHFGFG	PHK	CLG
<i>A. clavatus</i> XP 001271948	986	KVKLDRDMNLY	AHFGFG	PHC	CLG
<i>A. fumigatus</i> 5,8-LDS	986	KVKLDRDMNLY	AHFGFG	PHC	CLG
<i>A. terreus</i> XP 001208350	958	SIRLDRPDDLY	IHHGWG	PHS	CLG
<i>A. clavatus</i> XP 001270527	968	KIRLDRPADVY	IHRGWEP	HAS	SLG
<i>A. nidulans</i> ppoB	928	EIRLDRPLQLY	IHHGWG	THA	CLG
<i>A. terreus</i> XP 001211214	1053	KFRLDRPASAY	IQWCGY	GAHE	CLG
<i>A. niger</i> ppoD	1055	TFKLRPSNAY	IHFYGY	GAHE	CLG
P450 consensus			FxxGxRxCxG		

Figure 15. Partial alignment of DOX-CYP proteins of aspergilli. The sequences are shown in the same order as in the phylogenetic tree (Fig. 14). The alignment shows the area around the cysteine residue (marked) required for heme-binding and isomerase activity. This residue is evidently absent in the 10R-DOX cluster. The apparently conserved tyrosine present in DOX-CYP protein sequences is also highlighted. The P450 consensus sequence of the heme binding region, according to [Werck-Reichhart & Feyereisen, 2000], is shown at the bottom for comparison.

All sequences of the DOX-CYP family of these fungi and additional aspergilli (e.g., *A. oryzae* and *A. flavus*) show conserved tyrosine residues ten amino acids prior to the cysteines (Fig. 15). This tyrosine is not a part of any of the described consensus sequences of P450, and might be a hallmark of fungal DOX-CYP sequences.

8,11-Linoleate hydroperoxide isomerase activities of aspergilli

In *paper II*, we were unable to identify the function of *ppoB* since Δ *ppoB* showed the same oxylipin profile as its corresponding wild-type strain (AF293). However, we hypothesized that *ppoB* encodes an 8,11-LDS since *A. fumigatus* Fresen. forms 8,11-DiHODE from LA.

There are now data contradicting this assumption. As mentioned previously, *ppoB* proteins do not form a distinct group in phylogenetic comparisons (Fig. 14). In fact, the *ppoB* proteins of *A. fumigatus* and *A. nidulans* are apparently not close relatives, which makes this nomenclature confusing. Two additional members of the aspergilli family were shown to synthesize 8*R*,11*S*-DiHODE, *A. clavatus* and *A. niger*. Their respective “third genes” (XP 001270527 and XP 001395220, Fig. 14), are not closely related to *ppoB* of *A. fumigatus*, as expected if these encode 8,11-LDS enzymes.

When the deduced amino acid sequence is examined, it is evident that XP 001270527 of *A. clavatus* lacks the essential cysteine residue discussed above, which is replaced with serine (Fig. 15). This likely renders the P450 domain of this putative DOX-CYP enzyme inactive, since it presumably would be unable to support heme. The serine residue of XP 001270527 was confirmed by partial sequencing of its corresponding gene (*paper IV*). As mentioned, the cysteine residue is absolutely conserved among P450 [Rupasinghe *et al.*, 2006], and replacement of cysteine with serine by site-directed mutagenesis resulted in negligible activities of CYP2B4 [Vatsis *et al.*, 2002], 5,8-LDS of *A. fumigatus*, and 7,8-LDS of *G. graminis* (*paper III*). Therefore, it seems feasible that 8,11-DiHODE of *A. clavatus* is formed by other mechanisms.

The origin of the 8,11-linoleate hydroperoxide isomerase activity of *A. fumigatus* is obscure. The subcellular localization of this activity was assessed by differential centrifugation, and resided in the soluble fraction (100,000 x g). The activity was selectively inhibited by a number of fungal and human P450 inhibitors containing imidazole moieties. This suggests an involvement of a soluble P450, possibly expressed as a fusion enzyme, consistent with the DOX-CYP family.

The connection between *ppoB* and 8,11-DiHODE biosynthesis of *A. fumigatus* was studied in more detail. Full length cDNA was cloned and expressed in insect cells and *E. coli*, but the recombinant proteins failed to show enzymatic activity (*paper V*). The failure of expression systems to produce active enzymes must not, however, be regarded as conclusive proof

that a link between a gene and an enzymatic activity does not exist. Targeted deletion of the DOX and DOX-CYP domains of *ppoB* resulted in fungal strains with retained capacities to form 8,11-DiHODE (*paper V*). These data suggest that *ppoB* of *A. fumigatus* does not encode 8,11-LDS or 8,11-linoleate hydroperoxide isomerase.

Impact of PKA on hydroperoxide isomerase activities and 10R-DOX

Several studies highlight the importance of cAMP and protein kinase mediated signaling on the pathogenic process of *M. oryzae*. Gene deletion of the catalytic subunit of the cAMP dependent PKA, *cpkA*, resulted in the inability of forming functional penetrative structures, and the mutant strain was non-pathogenic [Mitchell & Dean, 1995; Xu *et al.*, 1997]. A deletion mutant of the adenylate cyclase gene *MAC1* failed to form appressoria unless exogenous cAMP was added to the fungal culture [Choi & Dean, 1997]. Interestingly, a spontaneous mutation suppressing the *MAC* deletion phenotype (*sum*) restored this ability, and partially its pathogenicity [Adachi & Hamer, 1998]. The mutant strain in question, $\Delta mac1 sum1-99$, carries a mutation in the regulatory subunit of PKA, which renders the kinase activity independent of cAMP. Lipid degradation involved in the mobilization of the glycerol-dependent turgor generation of appressoria, seemed to be very rapid in this mutant [Thines *et al.*, 2000].

PKA was previously postulated to be involved in oxylipin signaling in aspergilli, based on indirect evidence. Double ($\Delta ppoA\Delta ppoC$) and triple ($\Delta ppoA\Delta ppoB\Delta ppoC$) mutants of oxylipin-related genes of *A. nidulans* produced the opposite phenotype as a mutant in which the G_{α} subunit of a G-protein coupled receptor, known to mediate transcriptional effects *via* PKA, was constitutively active [Tsitsigiannis & Keller, 2006]. The biological effects of oxylipins were therefore attributed to PKA-dependent transcriptional regulation. In this thesis, $\Delta mac1 sum1-99$ with a constitutively active PKA substantially influenced the oxylipin profile (*paper I*) (Fig. 11). This is intriguing, as it is the first observation suggesting an additional mode of regulation of fungal oxylipin biosynthesis. PKA is well known to mediate many of its effects *via* downstream transcriptional regulation, but the effect on the oxylipin profile of *M. oryzae* was not associated with an up-regulation of the second *DOX-CYP*. Even so, this would not sufficiently explain the effects of the mutation, since the number of oxylipin metabolites exceeds the number of members of the *DOX-CYP* protein family of this fungus.

There are other possible explanations of the effect of $\Delta mac1 sum1-99$. One obvious possibility would be the presence of additional enzymes capable of catalyzing these fatty acid oxygenase and fatty acid hydroperoxide isomerase reactions. The isomerase reactions of LDS are catalyzed by their P450 domains. The genome of *M. oryzae* contains 133 members of the cytochrome P450 gene family. Therefore, the 5S,8R-

DiHODE metabolite of $\Delta mac1\ sum1-99$ might be formed from 8*R*-HPODE by a separate P450. This seems unlikely, since most fungal P450 are microsomal unless they are fusion enzymes. The 5,8-hydroperoxide isomerase activity was found to reside in the soluble fraction after ultracentrifugation of fungal homogenate. Several post-translational modifications of P450 are described [Aguiar *et al.*, 2005]. PKA mediated phosphorylation was shown to influence enzyme activity, but this is so far only shown to occur as an on-off switch [Oesch-Bartlomowicz *et al.*, 2001; Oesch-Bartlomowicz & Oesch, 2003]. The amino acid sequence of the 7,8-LDS in *M. oryzae* contains several potential sites for PKA phosphorylation, and altered isomerase regioselectivity by PKA is an intriguing possible explanation for the sudden change in oxylipin profile of $\Delta mac1\ sum1-99$. An analogous mechanism could potentially help to explain the origin of the unidentified 8,11-linoleate hydroperoxide isomerase activity of aspergilli.

9*R*-DOX and AOS activities of ascomycetous fungi

Allene oxides are formed in plants from α -linolenic acid by the action of lipoxygenases followed by AOS (CYP74). The allene oxide formed in plants from 13*S*-HPOTrE is either non-enzymatically hydrolyzed into α - and γ -ketols, or enzymatically converted into jasmonates by allene oxide cyclase, oxophytodienoate reductase, and three cycles of β -oxidations [Schaller & Stintzi, 2009]. An allene oxide can also be formed from 9*S*-HPOTrE by specific isoforms of AOS of plants. This allene oxide is not converted into jasmonates, but is non-enzymatically converted into its corresponding α - and γ -ketols by hydrolysis, or to 10-OPDA by cyclization [Hamberg, 2000].

In *paper VI*, the fatty acid oxygenation of *A. terreus* was investigated, as this fungus harbors five genes of the *DOX-CYP* family (Fig. 14). *A. terreus* was found to synthesize an allene oxide from 9*R*-HPODE, as judged by the accumulation of associated α - and γ -ketols. α -Linolenic acid was also accepted as a substrate of the 9*R*-DOX activity, while oleic acid was not. This indicates that a *bis*-allylic structure is essential for hydrogen abstraction to take place, which is typical of lipoxygenases.

Lipoxygenases catalyze the stereospecific dioxygenation of polyunsaturated fatty acids containing at least one (1*Z*,4*Z*)-pentadiene structure. The reaction mechanism of sLOX-1 serves as a model, and starts with the abstraction of the *bis*-allylic pro-*S* hydrogen at C-11 of LA, followed by rearrangement of the carbon-centered radical, and antarafacial insertion of molecular oxygen forming 9- and 13-hydroperoxides with *cis-trans* conjugated dienes [Oliw, 2002].

The AOS activity of *A. terreus* is specific for 9*R*-HPODE, as neither 9*S*-HPODE, 9*R*-HPOTrE, 13*R/S*-HPODE, or 13*R*-HPOTrE was converted into ketols. In this respect, the AOS activity of *L. theobromae* differs, as α -linolenic acid was converted to an α -ketol, 9-hydroxy-10-oxo-12*Z*,15*Z*-octadecadienoic acid, *via* 9*R*-HPOTrE (*paper VII*). The preference for 9*R*-

hydroperoxides of fungal AOS is in sharp contrast to plant 9-AOS, which are specific for hydroperoxides of *S*-configuration.

The 9*R*-DOX activities of *A. terreus* and *L. theobromae* are of mechanistic interest. The oxygenation involves suprafacial insertion of molecular oxygen, which is uncommon for fatty acid DOX. All DOX-CYP fusion enzymes and LOX catalyze antarafacial insertions of oxygen, with the sole exception of MnLOX of *G. graminis* [Hamberg *et al.*, 1998]. As stated, the substrate specificities of 9*R*-DOX resemble that of lipoxygenases. Genomic information from *L. theobromae* is not available, but the genome of *A. terreus* is devoid of apparent LOX genes (Table 6). This raises the question regarding the origin of the 9*R*-DOX activity.

Table 6. Number of proteins of four superfamilies of five different fungi listed in the Superfamily server, release 1.73 [Gough *et al.*, 2001]

Fungus	P450	Catalse	Lipoxygenase	Heme-dependent peroxidase
<i>A. terreus</i>	125	4	-	9
<i>A. fumigatus</i> AF293	77	4	2	6
<i>A. fumigatus</i> A1163	79	4	2	6
<i>A. nidulans</i>	96	5	-	6
<i>M. oryzae</i>	133	2	1	11

The AOS activity of *Plexaura homomalla* is fused with an 8*R*-lipoxygenase, providing its hydroperoxide substrate [Brash *et al.*, 1987; Boutaud & Brash, 1999]. However, the 9*R*-DOX and AOS activities of *A. terreus* were separated by ultracentrifugation. The DOX activity resides in the soluble fraction, while the AOS is microsomal. Despite the mechanistic differences, it is possible that the 9*R*-DOX activity is encoded by a member of the *DOX-CYP* gene family. The similarities between the reaction mechanisms of the 9*R*-DOX activities of *L. theobromae* and *A. terreus* suggest a common enzymatic pathway in these ascomycetous fungi, a topic of future studies.

JA was only formed in small amounts by two commercial strains of *L. theobromae*, insufficient for detailed studies. The capacity of *L. theobromae* to form JA is highly strain specific and research is limited by restrictions due to its pathogenicity. Recently, JA synthesis in *L. theobromae* was shown to originate from α -linolenic acid, in analogy with the plant octadecanoid pathway [Tsukada *et al.*, 2010]. There was, however, an interesting difference in the reduction of the cyclopentenone double bond of the intermediate 12-OPDA. This caused the authors to speculate that the biosynthetic pathway of fungi may have evolved independently. The mechanisms of the enzymes involved need to be further investigated. Of course, such studies require strains with high JA biosynthesis. This thesis demonstrates that *L. theobromae* and *A. terreus* can form hydroperoxides and allene oxides, but an allene oxide cyclase has not yet been described.

Conclusions

- 7,8-LDS was conclusively identified in *M. oryzae* by gene deletion, but this enzyme is not essential for rice infection or sporulation.
- The reaction mechanisms of 7,8-LDS of *M. oryzae* and *G. graminis* are virtually identical.
- Oxygenation of LA by *M. oryzae* shifts to new metabolites in a mutant with increased PKA activity.
- ppoA and ppoC of *A. nidulans* and *A. fumigatus* were identified as 5,8-LDS and 10R-DOX, respectively.
- 5,8-LDS of *A. fumigatus* was successfully expressed. The 8R-DOX domain of 5,8-LDS can be expressed independently of its P450 domain.
- *A. clavatus* has 8,11-linoleate hydroperoxide isomerase activity despite the lack of a heme-thiolate cysteine ligand in the protein sequence of ppoB.
- Gene deletion and expression showed that the 8,11-linoleate hydroperoxide isomerase activity is unrelated to ppoB of *A. fumigatus*.
- Novel linoleate 9R-DOX and AOS activities were discovered in *A. terreus* and *L. theobromae*.

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