Dioxygenation of polyunsaturated fatty acids in fungi

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Dioxygenation of polyunsaturated fatty acids in fungi

Dioxygenering van meervoudig onverzadigde vetzuren in schimmels

(met een samenvatting in het Nederlands)

Proefschrift

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Abbreviations

17:0	margaric acid, heptadecanoic acid
18:1	oleic acid, 9Z-octadecenoic acid
18:2	linoleic acid, 9Z,12Z-octadecadienoic acid
18:3	linolenic acid, 9Z,12Z,15Z-octadecatrienoic acid
20:4	arachidonic acid, 5Z,8Z,11Z,14Z-eicosatetraenoic acid
8-H(P)OD	8-hydro(pero)xy octadecadienoic acid
8-HOM	8-hydroxy octadecenoic acid
8-KOD	8-keto octadecadienoic acid
8-K-10-OD	8-keto-10,12-octadecadienoic acid
8-K-9-OD	8-keto-9,12-octadecadienoic acid
9-H(P)OD	9-hydro(pero)xy octadecadienoic acid
13-H(P)OD	13-hydro(pero)xy octadecadienoic acid
10-H(P)OD	10-hydro(pero)xy octadecadienoic acid
12-H(P)OD	12-hydro(pero)xy octadecadienoic acid
10-ODA	10-oxo decenoic acid
8,11-diHOD	8,11-dihydroxy octadecadienoic acid
8,11-diHOT	8,11-dihydroxy octadecatrienoic acid
8,11-FOM	8,11-furan octadecenoic acid
7,8-diHOD	7,8-dihydroxy octadecadienoic acid
5,8-diHOD	5,8-dihydroxy octadecadienoic acid
F-acid	furan fatty acid
PGG_2	9,11-endoperoxy-15*S*-hydroperoxy eicosatetraenoic acid
PGH_2	9,11-endoperoxy-15*S*-hydroxy eicosatetraenoic acid
C_x	carbon chain containing an x number of carbon atoms
C-x	carbon atom number x
FAHD	fatty acid heme dioxygenase
PGS	prostaglandin synthase
LOX	lipoxygenase
α-DOX	α-dioxygenase
LDS	linoleate diol synthase
COX	cyclooxygenase
DRIFT	diffuse reflectance infrared Fourier transform
HSQC	heteronuclear single quantum correlation
TOCSY	total correlation spectroscopy
SPE	solid phase extraction
MC	(–)-menthoxycarbonyl
TMS	trimethylsilyl

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General introduction

Overview of the introduction

In this introduction the possible dioxygenation pathways of polyunsaturated fatty acids in fungi will be described. Its goal is twofold, namely to present the current status of the research in this field, and to put the research described in this thesis in perspective.
The introduction is started by giving a short explanation of the general occurrence of fatty acids and of the metabolism of polyunsaturated fatty acids into so called oxylipins.
One group of enzymes that catalyze this conversion are the dioxygenases and the different types of dioxygenation will be presented briefly. The discussion will then be narrowed down to fungal dioxygenation pathways, giving a short overview of the current status of research in fungi. In the next part, dioxygenation in fungi is further elaborated on. The different types of dioxygenation will be described and the current knowledge of each fungal dioxygenase will be presented. The introduction is finished by summarizing the information about dioxygenation of polyunsaturated fatty acids in fungi and it will be pointed out how the research described in this thesis contributes to the current knowledge.

Polyunsaturated fatty acid metabolism into oxylipins Polyunsaturated fatty acids play a central role in all biological systems. They are usually part of triglycerides and phospholipids in which they fulfill different functions. The fatty acids stored in triglycerides are important sources of energy, while the fatty acids that are esterified in phospholipids are part of the plasma membrane and serve as precursors to signaling molecules generated in response to external events. The conversion of polyunsaturated fatty acids into signaling molecules starts with the hydrolysis of fatty acids from the phospholipid molecules. This hydrolysis is catalyzed by cytosolic phospholipase A_2 and results in the availability of free polyunsaturated fatty acids. The free unsaturated fatty acids can be metabolized into signaling molecules by oxygenation, and the products of this reaction are called oxylipins. The term oxylipin refers to oxygenated compounds synthesized from polyunsaturated fatty acids by mono- or dioxygenase-catalyzed oxygenation. A large part of oxylipins in animals and plants are produced from polyunsaturated fatty acids by the dioxygenase type of oxygenation and it is this type of oxygenation this introduction will focus on.

Catalysis by dioxygenases Dioxygenases add molecular oxygen to polyunsaturated fatty acids in a regio- and stereo-specific manner. The oxylipins formed in animals and plants differ in terms of molecular structure. This is the result of (1) animals and plants containing different polyunsaturated fatty acids and (2) animals and plants having partly different sets of dioxygenases. In animals C_{20} fatty acids (mostly arachidonic acid (20:4)) are converted, while in plants C_{18} fatty acids (mainly linoleic acid (18:2) and linolenic acid (18:3)) are the substrates for dioxygenases. Animals contain prostaglandin synthases (PGSs) and lipoxygenases (LOXs), which catalyze the formation of oxylipins, while plant

Fungal Classification

Fungi are eukaryotic organisms that are non-motile and have a cell wall. Unlike plants they lack chlorophyll, which makes them heterotrophic, *i.e.* they use carbon captured by other organisms for their metabolism. Fungi are thought to be related more closely to animals than to plants. They can grow as single cells (called the yeast form) or as long filamentous strands composed of many cells (referred to as the hyphal form). Hyphal strands often aggregate, forming a mass known as the mycelium. The best known fungi are those that form macroscopic structures like mushrooms, but many other species are also part of the fungal kingdom. The classification of fungi is mainly based on sexual reproductive-structures (see insert fungal reproduction). Currently, five divisions (phyla) are recognized: (1) the chytridiomycota, (2) the zygomycota, (3) the glomeromycota, the (4) ascomycota and (5) the basidiomycota.

The chytridiomycota
The chytridiomycota (1) represent the most ancient group of fungi and occur mostly in aqueous environments. They produce sexual and asexual spores, the zoospores, capable of moving through liquid with flagella. An example of the chytridiomycota is *Batrachochytrium dendrobatidis*, a worldwide occurring pathogen of amphibians.

The zygomycota
The zygomycota (2) are widespread terrestrial saprophytes that reproduce sexually with zygospores and asexually with sporangiospores. *Rhizopus stolonifer* a fungus causing rot of fruits and vegetables is an example of the zygomycota.

The glomeromycota
The glomeromycota (3) are fungi that live in symbiosis with photosynthetic hosts and reproduce asexually through blastospores. An example is *Glomus mossae*, a glomeromycete living in symbiosis with plant roots.

The ascomycota
The mostly land-living ascomycota (4) are the largest division of fungi. They form sexual spores named ascospores in a sac-like structure, and form asexual spores termed conidia. Some ascomycetes grow only as single celled yeasts or as filamentous hypha, others grow in both forms. Examples of ascomycetes are *Aspergillus niger*, used in the industrial production of many substances (*e.g.* citric acid), *Morchella deliciosa*, the morel popular among mushroom pickers worldwide, and *Candida albicans* a fungus that infects immunocompromised hosts.

The basidiomycota
The last division is the basidiomycota (5). These land-living fungi produce sexual basidiospores on club shaped stalks, as well as asexual athrospores spores. Most basidiomycota colonize large areas with their mycelia. *Agaricus bisporus,* the white button mushroom, *Pleurotus ostreatus*, the oyster mushroom, and also *Ustilago maydis*, a pathogen of maize, are examples of the basidiomycota.

For some fungi sexual reproduction has not yet been observed. Since classification used to be based on sexual reproductive structures, these fungi make a separate group, the deuteromycota or 'fungi imperfecti'. Now that classification methods include DNA based comparisons, fungi formerly belonging to the deuteromycota are being assigned to the five divisions. An example of a former deuteromycete is *Aspergillus niger*. This fungus has not yet been shown to reproduce sexually but, based on DNA comparisons, is nonetheless placed in the division of ascomycota.

The oomycota
A distinct group of organisms are the oomycota, the water and slime moulds. They resemble fungi, probably through convergent evolution, but they are positioned in the kingdom protista. An example is *Peronospora sparsa* that causes downy mildew on roses.

Fungal Reproduction

There are two fundamental means by which fungi pass on genes to the next generation, via mitotic (asexual) reproduction or via meiotic (sexual) reproduction. After asexual reproduction the new generation possesses an exact copy of the parental genome. In contrast, mating and recombination of different parental genomes mark sexual reproduction. Sexual reproduction increases genetic variation allowing species to respond to environmental changes, while asexual reproduction avoids the metabolic costs of recombination. Some fungi can reproduce both sexually and asexually, while others are only known to reproduce through one mean.

Sexual reproduction can be homothallic or heterothallic. Homothallic fungi complete their sexual cycle by self-fertilization. As a result, the genome of the next generation may be different from or identical to the parental genome.

An example of a homothallic fungus is *Aspergillus nidulans*. Heterothallic fungi on the other hand, need to cross-fertilize with another individual having complementary mating type to complete the sexual cycle. An example of a heterothallic fungus is *Podospora anserina* used as a model fungus in laboratories. Asexual reproduction occurs through mitosis only. Because of the disadvantages connected with total loss of recombination, it is thought that very few species are truly asexual. For instance, exchange of parental genomes may also occur through the so-called parasexual cycle in which nuclei of different individuals fuse.
An example of a fungus for which only asexual reproduction is observed is *Aspergillus niger*.

It should be noted that this summary necessarily simplifies the possible fungal reproductive strategies.

oxylipins are formed by LOXs and α-dioxygenases (α-DOXs). Plants do not contain PGSs, but α-DOX and PGS are homologous proteins in terms of amino acid sequence. Oxylipins are not stored, but are synthesized as required. Their functions include regulation of inflammatory response and blood pressure in animals, and regulation of defense and growth in plants. Animal and plant dioxygenases have been extensively studied. They have been purified, cloned, sequenced, expressed and their reaction mechanisms, catalytic sites and products have been investigated. Therefore, it seems remarkable that the dioxygenases of the third eukaryotic kingdom, the fungi, are much less well studied.

Dioxygenases in the fungal kingdom The fungal kingdom comprises an enormous group of organisms that can be sub-divided into five different divisions (See insert fungal classification). The few studies on fungal dioxygenases show that most fungi produce oxylipins predominantly from C_{18} fatty acids, but also from C_{20} fatty acids. Some fungi contain LOXs that are comparable to their plant and animal equivalents, while others appear to produce prostaglandins. Some fungi contain linoleate diol synthase (LDS), a dioxygenase that does not occur in animals or plants, but has amino acid homology with PGS and α-DOX.

A few of the fungal dioxygenases have been described in detail, but most studies have either just shown the presence of a catalytic activity or the presence of oxylipins. Also, the biological function of fungal oxylipins is still largely unknown.

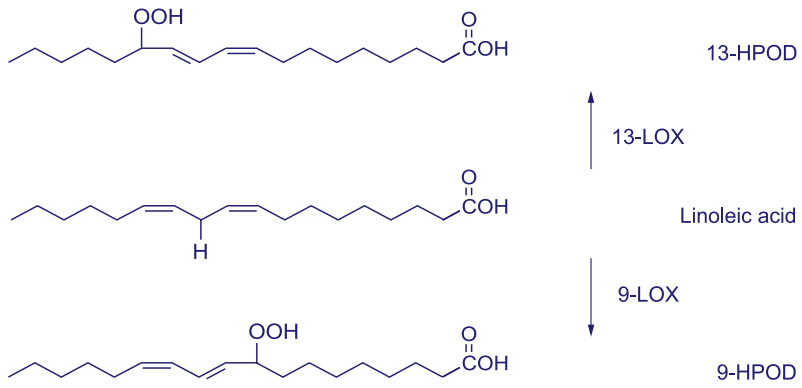
Dioxygenation pathways in fungi

In the following part of the introduction an overview of the current knowledge of dioxygenation pathways of polyunsaturated fatty acids in fungi is given. The inventory is based on the four known dioxygenases (LOX, PGS, LDS and α-DOX) and for each of the different dioxygenases its reaction mechanism, its products, its functions and its occurrence in the five divisions of fungi will be described.

Dioxygenase 1: Lipoxygenase

LOXs are widely distributed in animals, plants and fungi and they generate the precursors to many bioactive compounds. In animals the preferred substrate is usually 20:4, whereas in plants and fungi the most common substrates are 18:2 and 18:3. Animal and plant LOXs have been found as soluble cytosolic enzymes but also in membranes of several cell compartments. Many LOXs have been purified, cloned and expressed and therefore, there is extensive knowledge of this group of dioxygenases. LOXs contain non-heme iron and catalyze the introduction of one oxygen molecule into the fatty acid pentadiene system forming a conjugated hydroperoxy diene (Fig. 1). LOXs are generally named for the position into which they insert the hydroperoxy group. Since animal LOXs metabolize C_{20} fatty acids and plant LOXs metabolize C_{18} fatty acids this nomenclature may cause some confusion. Generally, in animals a 5-LOX, 8-LOX, 12-LOX and a 15-LOX (Funk, 2001) and in plants a 9-LOX and a 13-LOX (Shibata, 1995) occur. The fatty acid hydroperoxides formed can be quickly converted into other oxylipins by enzymatic or non-enzymatic reactions.

Important LOX-derived oxylipins in plants are volatile aldehydes, alcohols and the wound hormones jasmonic acid and traumatin (Noordermeer, 2001). Such wound hormones can induce a wide range of secondary actions. They act as growth promoters and are involved in developmental processes such as seedling growth, pollen formation and flower development (Liavonchanka, 2006). The volatiles have a characteristic green fruity smell and might be a signal for other plants in the surroundings and they are also attractants or repellents of insects.



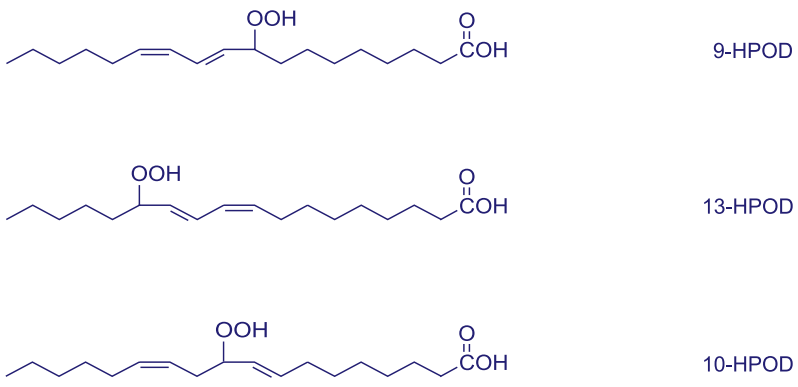
[Figure 1] LOX catalysis

In the first step of LOX catalysis either the *pro S* or *pro R* hydrogen of the bisallylic C-3 of the pentadiene group is abstracted, yielding a radical on the fatty acid substrate. Concomitantly the non-heme iron of the enzyme is reduced from Fe(III) into Fe(II) (Veldink, 1998). Next the pentadiene system rearranges and the radical is then positioned either on C-1 or C-5, depending on the specificity of the LOX (Kuhn, 2000). Molecular oxygen reacts with the radical to form a hydroperoxy radical, which is reduced by Fe(II) into a hydroperoxy anion. The anion subsequently reacts with a proton resulting in a *E,Z* conjugated hydroperoxide (Veldink, 1998). This hydroperoxide has either *S* or *R* configuration, the *S* configuration being the most common (Brash, 1999). Finally, the hydroperoxy fatty acid is released and the enzyme is ready for another round of catalysis.

The biological role of LOX-derived oxylipins in animals is still largely unclear. 5-LOX products are converted into leukotrienes, mediators of anaphylactic and inflammatory disorders. The functions of 8-LOX, 12-LOX and 15-LOX products include modulation of neurotransmission, and influencing cell adhesion, differentiation and maturation (Funk, 2001).

Fungal LOX

In several fungi the presence of LOXs has been demonstrated either directly by showing LOX activity or indirectly via the addition of LOX inhibitors or the demonstration of LOX-derived oxylipins (In Fig. 2 (overleaf) examples of LOX products found in fungi are shown). Since the majority of fungi do not accumulate 20:4, fungal LOXs, similar to plant LOXs, act mainly on C_{18} fatty acids. Most fungi convert C_{18} fatty acids into 9-hydroperoxy or 13-hydroperoxy fatty acids (*i.e.* they contain 9-LOX and 13-LOX, respectively). Interestingly, 10-hydroperoxy and 12-hydroperoxy fatty acids can also be formed as possible



[Figure 2] Examples of fungal LOX products

products of fungal polyunsaturated fatty acid metabolism. The formation of 10-hydroperoxy fatty acids was best studied in *Agaricus bisporus* however, the proposed 10-LOX responsible for its formation has so far not been isolated. Only a few fungal LOXs have been purified and none of the fungal LOXs have been cloned, sequenced or recombinantly expressed so far. As a result there is still limited knowledge of this group of enzymes in fungi. An exception is the Mn-LOX isolated from *Gaeumannomyces graminis*, which has been extensively characterized, and has been cloned and expressed as well.

Fungal 9-LOX and 13-LOX Several studies have described the presence of 9-LOX or 13-LOX activity or the presence of the corresponding oxylipins. Remarkably, fungal LOX activity is frequently accompanied by the formation of 10-hydroperoxy fatty acids and 12-hydroperoxy fatty acids, which in plants, are not known as products of LOX activity. Since the stereochemistry of the LOX products has not been determined and since none of these LOXs have been cloned, sequenced or expressed, it remains unclear whether or not all these oxylipins are indeed produced by a fungal 9-LOX or 13-LOX.

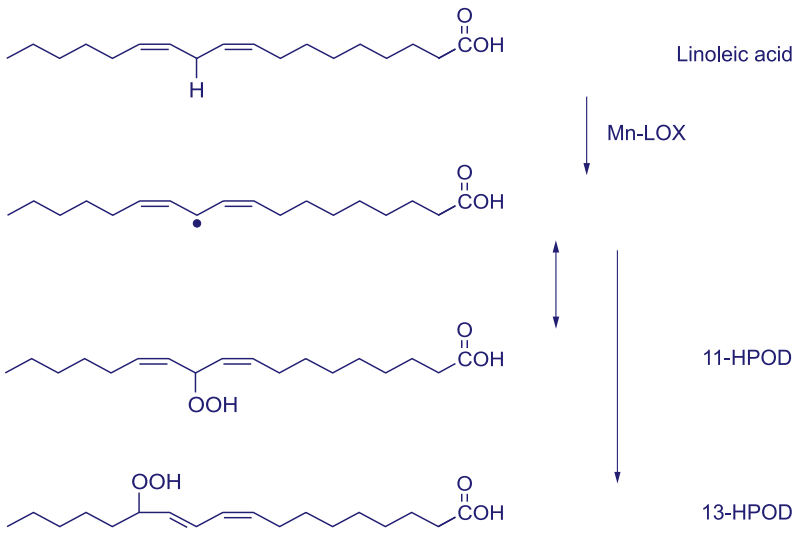
In several members of the division ascomycota, fungal LOX activity has been described. *Fusarium oxysporum*, a pathogen of plants, produced an intracellular LOX, which converts C_{18} fatty acids. Initially, this LOX was demonstrated to form 9-hydroperoxy octadecadienoic acid (9-HPOD) and 13-hydroperoxy octadecadienoic acid (13-HPOD) from 18:2 (Matsuda, 1978; Satoh, 1976), but in a later study, the formation of 10-hydroperoxy octadecadienoic acid

(10-HPOD) and 12-hydroperoxy octadecadienoic acid (12-HPOD) was reported as well (Bisakowski, 1997). A similar LOX activity was found in *Fusarium proliferatum*, a pathogen of orchids that also produced 9-HPOD, 13-HPOD, 10-HPOD and 12-HPOD from 18:2 (Bisakowski, 1995a, 1998). Other LOXs that form the same oxylipins were found in *Morchella esculenta*, the yellow morel (Bisakowski, 2000) and in *Penicillium camemberti* and *Penicillium roqueforti*, fungi used in the production of French cheeses (Perraud, 2000). The latter study showed that 9-HPOD and 13-HPOD occur as a mixture of *R* and *S* stereoisomers, which may either point towards low enantioselectivity (and possibly also low stereospecificity) of the fungal enzymes or the presence of different enzymes forming the different oxylipins (Perraud, 2000, 1999). The possible function of these oxylipins needs further investigation. Other studies on LOX activity in the ascomycota presented the formation of 9-HPOD and 13-HPOD in the mitochondrial fraction of *Saccharomyces cerevisiae* (also known as baker's yeast) (Bisakowski, 1995b, 1997; Shechter, 1983), in *Geotrichum candidum* (an opportunistic human pathogen) (Perraud, 1999), and in *Terfezia claveryi* (a truffle) (Perez-Gilabert, 2005a, 2005b). LOX activity in *Thermomyces lanuginosus* (a soil fungus) produced only 13-HPOD (Li, 2001). Again, the function of these oxylipins has not yet been studied. LOX inhibitors were shown to influence morphology (shift from yeast to hyphal growth) in *Ceratocystis ulmi*, the causal agent of Dutch elm disease (Jensen, 1992). However, the presence of LOX or LOX-derived oxylipins has not yet been demonstrated in *C. ulmi*.

Analysis of LOX activity in the basidiomycetes *Pleurotus ostreatus* (Kuribayashi, 2002) and *Pleurotus pulmonarius* (Assaf, 1995, 1997) showed that these fungi formed 13-HPOD from 18:2. The basidiomycete *Malassezia furfur* also known as *Pityrosporum orbiculare* (the pathogenic agent of pityriasis versicolor) formed 9-HPOD in addition to 13-HPOD (De Luca, 1996). The function of LOX products in the basidiomycota is still unclear.

One study showed LOX activity in the zygomycota. *Mortierella* ssp, fungi common in soil and dung, converted 18:2 and 20:4, but reaction products were not yet identified (Filippovich, 2001).

A number of studies looked into LOX activity in the oomycota. LOX activity of *Saprolegnia parasitica* converted 20:4 into 15*S*-hydroperoxy eicosatetraenoic acid. The purified soluble LOX had also hydroperoxide isomerase activity converting the hydroperoxide into epoxy alcohols (Hamberg, 1986; Herman, 1987). A similar LOX activity associated with vegetative growth was demonstrated in *Saprolegnia ferax* (Herman, 1989a) and *Achlya ambisexualis* (Herman,

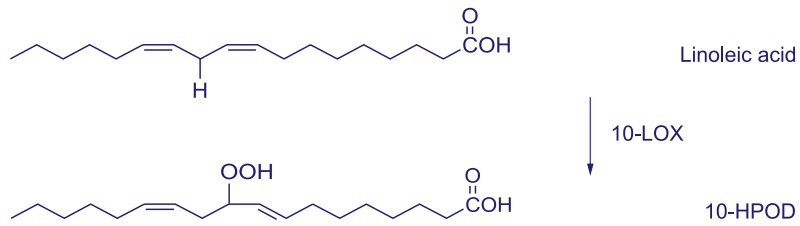


[Figure 3] Mn-LOX catalysis

The first step of Mn-LOX catalysis is the abstraction of *pro S* hydrogen of the bisallylic C-3 of the pentadiene group, yielding a radical on the substrate (Hamberg, 1998). Presumably, the manganese of the enzyme is concomitantly reduced from Mn(III) into Mn(II) (Su, 2000). The fatty acid radical then either reversibly reacts with molecular oxygen to form a 3*S*-hydroperoxy pentadiene derivative via the corresponding peroxy radical, or the radical rearranges to pentadiene C-5 and then irreversibly reacts with molecular oxygen to form the 5*R*-hydroperoxy derivative. The 3*S*-hydroperoxy derivative may subsequently be converted into the 5*R*-hydroperoxy derivative, probably through a reversibly deoxygenated fatty acid radical intermediate (Hamberg, 1998). Mn-LOX differs from other LOX in that it forms a hydroperoxy fatty acid product with *R* configuration (in stead of the most common *S* configuration) and it forms a product with a bisallylic hydroperoxy group (and not a conjugated hydroperoxy diene) (Su, 1998a).

1989b). Indirect evidence for the presence of LOX in the oomycota was found for *Lagenidium giganteum*, a parasite of the mosquito larvae. Addition of LOX inhibitors resulted in downregulation of induction and maturation of oospores that could in part be reversed by the addition of partially purified eicosanoid extracts from *L. giganteum* growth media (Kerwin, 1986).

Fungal Mn-LOX The Mn-LOX from *G. graminis* is the only cloned, sequenced and expressed fungal LOX to date (Cristea, 2005). It differs from all described LOXs in that it is secreted, and contains manganese instead of iron in its catalytic site. Mn-LOX has amino acid homology to animal and plant LOX and also



[Figure 4] 10-LOX catalysis

It should be noted that a 10-LOX has so far not been isolated and characterized. The proposed reaction is derived from indirect experiments that demonstrated either the presence or the conversion of certain oxylipins.

In a study with an *A. bisporus* homogenate incubated with [U-14C] 18:2, labeled 1-octen-3-ol, and labeled 10-ODA, were detected in a ratio of 1:1 (Wurzenberger, 1982). It was proposed that the intermediate in this reaction was 10-HPOD formed from the reaction of 18:2 with an oxygen molecule. Indeed, *A. bisporus* converted 10-HPOD but not 9-HPOD, 12-HPOD or 13-HPOD into 1-octen-3*R*-ol and 10-ODA (Wurzenberger, 1984a). Later it was shown that from a mixture of 10*R/S*-HPOD only the 10*S*-HPOD half was consumed (Wurzenberger, 1984c). The formation of a 10-HPOD from 18:2 has later been confirmed in the basidiomycetes *Lentinus edodes*, *Tricholoma matsutaka* (Akakabe, 2005), *Lentinus decadetes* (Matsui, 2003) and the ascomycetes *Penicillium camemberti* and *Penicillium Roqueforti* (Kermasha, 2002). The first step in 10*S*-HPOD formation is likely the abstraction of hydrogen from the pentadiene system and subsequent isomerisation of the fatty acid radical. Then 10-LOX inserts molecular oxygen forming the corresponding 10*S*-hydroperoxy derivative. This 10-LOX should differ from all known LOXs in that it forms a C-10 hydroperoxy derivative located between the two double bonds (as opposed to a conjugated hydroperoxydiene) Interestingly, a LOX, with non-heme iron, converting 18:2 into 10-HPOD was isolated from the bacterium *Pseudomonas* 42A2 (Busquets, 2004). Later this 10-LOX was cloned and expressed and it was demonstrated that it was homologous to animal and plant LOX, and also contained the conserved His residues that coordinate the iron ligand (Vidal-Mas, 2005) Whether or not this bacterial enzyme resembles the enzyme that forms 10-HPOD in fungi remains to be determined.

contains the highly conserved His residues that, in animal and plant LOX coordinate iron and, as evidenced by site-directed mutagenesis, in Mn-LOX coordinate manganese (Cristea, 2005; Hornsten, 2002b). Substrates for Mn-LOX are C_{18} fatty acids, mostly 18:2 and 18:3, but not 20:4. The enzyme catalyzes the introduction of one oxygen molecule into the fatty acid pentadiene system forming two products, a conjugated *R*-hydroperoxy diene and a structurally remarkable bisallylic *S*-hydroperoxy derivative (Fig. 3) (Su, 1998a). The biological functions of *G. graminis* Mn-LOX and its products are still unknown. Since the enzyme is secreted, highly glycosylated and very stable it was suggested that it might be involved in the infection of wheat roots by *G. graminis* (Su, 1998a).

Fungal 10-LOX The occurrence of a fungal 10-LOX has only been inferred by the demonstration of its products. So far, a 10-LOX has not been isolated. From experiments with *A. bisporus* (formerly known as *Psalliota bispora*), it was proposed that 10-LOX inserts molecular oxygen into C_{18} fatty acids, mainly 18:2 and 18:3 forming their corresponding 10*S*-hydroperoxy derivatives (Wurzenberger, 1984b). For 18:2, the resulting 10*S*-HPOD is then readily converted into a volatile alcohol, 1-octen-3*R*-ol, and an oxo fatty acid, 10-oxo decenoic acid (10-ODA) (Fig. 4) (Grosch, 1985). 1-octen-3-ol is responsible for the characteristic fungal scent of mushrooms. 10-LOX activity and (breakdown) products have been found in many fungi of the divisions basidiomycota and ascomycota.
For example, 10-LOX activity was studied in the basidiomycetes *A. bisporus* (Mau, 1999) and *P. pulmonarius* (Assaf, 1995, 1997) and the ascomycetes *P. camemberti* (Husson, 2004, 2002), and *F. proliferatum* (Husson, 1998). Also, the presence of 1-octen-3-ol was demonstrated in many ascomycetes (Chalier, 1993; Chitarra, 2004; Diaz, 2002; Fischer, 1999; Jacobsen, 1997; Jelen, 2003; Kaminski, 1974, 1980; Nilsson, 1996; Pelusio, 1995; Zeppa, 2004), and basidiomycetes (Assaf, 1995; Beltran-Garcia, 1997; Buchbauer, 1993; Cho In, 2006; Cruz, 1997; Gallois, 1990; Tressl, 1982; Wood, 2001).

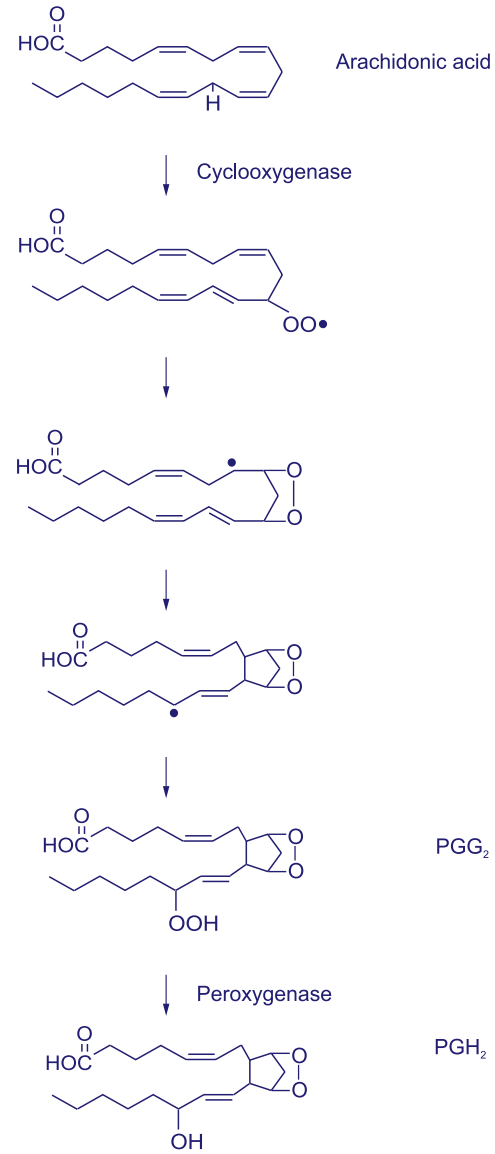
A series of effects have been related to 1-octen-3-ol and 10-ODA, a direct effect of 10-HPOD however, has not yet been found. In *Clitopilus prunulus* 1-octen-3-ol was reported to prevent feasting of banana slugs (Wood, 2001). In *Penicillium paneum* 1-octen-3-ol was a volatile self-inhibitor. Produced by the conidia it inhibited germination at high densities (Chitarra, 2005, 2004). Also several effects have been associated with 10-ODA. In *A. bisporus* 10-ODA was found to stimulate mycelial growth and stile elongation (Champavier, 2000; Mau, 1992) and to increase the number of mushrooms in *Lentinus edodes* (Beelman, 2003). Relatively high concentrations of 10-ODA were shown to inhibit mycelial growth of *Penicillium expansum*, but had a stimulatory effect at relatively low concentrations (Okull, 2003).

Dioxygenase 2: Prostaglandin synthase

PGSs occur mostly in animals in which they generate prostaglandins from polyunsaturated fatty acids with a C_{20} carbon chain, mainly 20:4. PGSs are often referred to as cyclooxygenases (COXs). PGSs are integral membrane proteins bound to the luminal surface of the ER and to the contiguous outer membrane of the nuclear envelope. There are two PGS isoforms; PGS-1 (or COX-1) is the constitutive enzyme and PGS-2 (or COX-2) the inducible enzyme (Smith, 1996).

[Figure 5] PGS Catalysis

PGS catalysis can be conceived as a two-step sequence of a cyclooxygenase and a peroxidase reaction, which take place at distinct but structurally and functionally interconnected sites. In the first step of prostaglandin biosynthesis, a tyrosyl radical abstracts the bisallylic *pro* 13*S* hydrogen from 20:4 yielding a radical on the substrate. After this initial hydrogen removal, the double bonds of the C-11 - C-15 pentadiene rearrange and the radical is then positioned on C-11. Next, molecular oxygen is added antarafacially to C-11 and the resultant 11*R*-peroxy radical is cyclized onto the double bond between C-8 and C-9. The radical formed at C-8 next cyclizes onto the C-12 - C-15 conjugated diene to generate a bicyclic endoperoxide. This last cyclization also generates an allylic radical on C-13 that rearranges to C-15 and reacts with molecular oxygen to provide the 15*S*-hydroperoxy radical. This radical then recovers the hydrogen atom from the Tyr residue thus yielding 9,11-endoperoxy-15*S*-hydroperoxy eicosatetraenoic acid, PGG_2, and regenerating the tyrosyl radical for a next round of catalysis (McGinley, 2003; Smith, 1996). In the subsequent peroxidase reaction, the 15*S*-hydroperoxy group of PGG_2 is coordinated to the heme-iron and the proton of the terminal oxygen atom is donated. Next there is a transfer of a proton to the other oxygen atom that results in an acid-base catalyzed heterolytic cleavage of the oxygen-oxygen bond yielding 9,11-endoperoxy-15*S*-hydroxy eicosatetraenoic acid, PGH_2 (Seibold, 2004).



Many animal PGSs have been purified, cloned and expressed, which has resulted in detailed knowledge of the catalysis and function of these enzymes. PGS contains heme-iron and catalyzes the introduction of two oxygen molecules into 20:4. First, it converts 20:4 into 9,11-endoperoxy-15*S*-hydroperoxy eicosatetraenoic acid (PGG_2) in the cyclooxygenase reaction, and then it forms 9,11-endoperoxy-15*S*-hydroxy eicosatetraenoic acid (PGH_2) in the peroxidase reaction (Fig. 5).

PGH_2 is unstable and is the precursor to several bioactive prostanoids, formed through conversion by various cell-specific enzymes. These prostanoids play a role in numerous basic physiological processes. Well-studied effects include contraction of vascular smooth muscle (thereby regulating blood pressure), maintenance of cardiovascular homeostasis and stimulation of uterine contraction (Cha, 2006; Funk, 2001).

Fungal PGS

Indirect evidence has been presented for the occurrence PGSs in fungi but despite these indications, a fungal PGS has not yet been isolated. PGS activity has either been inferred by the addition of PGS inhibitors or by the demonstration of prostaglandins. It should be noted that most studies did not use a direct method (*e.g.* GC/MS) to demonstrate prostaglandins but relied on indirect detection with antibodies. This may have led to a misinterpretation of the results. A function has not yet been related to the fungal prostaglandins.

Direct evidence for fungal PGS The most direct evidence for the occurrence of fungal prostaglandins comes from two studies on oxylipins from the basidiomycete *Cryptococcus neoformans* a human pathogen. One study showed indirectly by using antibodies that *C. neoformans* produced a series of different prostaglandins. This study also showed that prostaglandin production was repressed by PGS inhibitors (Noverr, 2001). In the second study, the presence of prostaglandins in *C. neoformans* was confirmed directly by LC/MS-MS. However, this study also showed that prostaglandin production was not affected by the addition of PGS inhibitors. The authors suggested that the decrease in the first study was probably the result of reduced cell viability and not the result of PGS inhibition (Erb-Downward, 2007). Since the genome of *C. neoformans* lacks a PGS homolog; the authors hypothesized that the prostaglandins should be produced by another enzyme. This enzyme remains to be isolated.

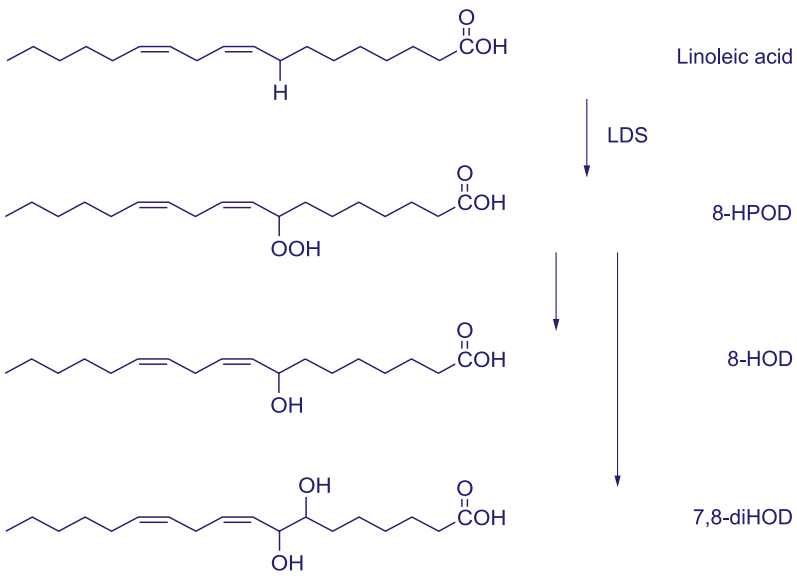
Additional direct evidence for fungal prostaglandins comes from studies on species of the family *Saccharomycetales*. The occurrence of prostaglandins was demonstrated by using GC/MS and their formation was shown to be repressed by the addition of PGS inhibitors (Botha, 1993, 1992; Kock, 1991). However, the enzyme involved in the production of these PGS has still to be identified. The *Saccharomycetales* prostaglandins appeared to be connected to growth and development, as PGS inhibitors affected these processes (Botha, 1993, 1992; Kock, 1991).

Indirect evidence for fungal PGS Other evidence for the occurrence of fungal prostaglandins is derived from indirect studies in a number of ascomycota. In the study on *C. neoformans* oxylipins, the presence of prostaglandins in the ascomycetes *Candida albicans* was demonstrated as well (using antibodies and PGS inhibitors) (Noverr, 2001). Prostaglandins appeared to be connected to *C. albicans* morphology as addition of exogenous prostaglandins induced hyphal growth over yeast growth (Noverr, 2004). Further indirect evidence comes from studies on *A. fumigates* and *A. nidulans* in which the occurrence of prostaglandins was also demonstrated by using antibodies (Tsitsigiannis, 2005a). In *Trichoderma viride*, *Fusarium sambucinium* and *Trichothecium rosum* evidence for the presence of fungal prostaglandins was derived from the observation that addition of PGS inhibitors decreased growth of these fungi (Krystofova, 1994; Lamacka, 1998).

PGS inhibitors also reduced the growth of the oomycetes *Achlya Carolina*, *Achlya ambisexualis* and *S. parasitica* in a dose-related manner. Exogenous addition of prostaglandins partially overcame this inhibition, suggesting a role for prostaglandin-like compounds in oomycete development (Herman, 1985). In a single study on prostaglandins in the zygomycota, PGS occurrence in *Mortierella* ssp and *Cunninghamella* spp was demonstrated by using antibodies (Lamacka, 1998).

Dioxygenase 3: Linoleate diol synthase

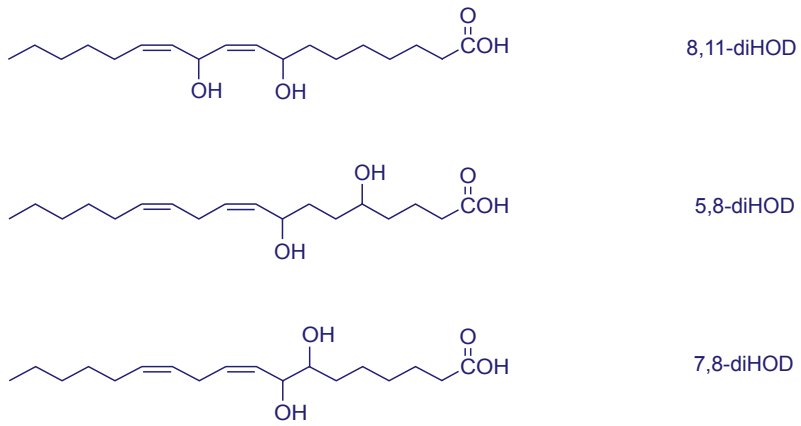
LDSs are, as yet, exclusively found in fungi and they convert polyunsaturated fatty acids with a C_{18} carbon chain, mostly 18:2 (Brodowsky, 1992). An LDS has been purified, characterized, cloned and sequenced from the ascomycete *G. graminis* the pathogen that causes 'take all' root disease of wheat. LDS has amino acid homology to PGS and also contains the highly conserved residues (*i.c.* the proximal and distal His, and the Tyr residues involved in the formation of the tyrosyl radical) necessary for PGS catalysis. LDS, like PGS, contains heme-iron and catalyzes two reactions. The first reaction is the insertion of molecular oxygen in 18:2 forming 8*R*-hydroperoxy octadecadienoic acid (8*R*-HPOD) that is either reduced into 8*R*-hydroxy octadecadienoic acid (8*R*-HOD) or, in the second reaction, isomerized into 7*S*,8*S*-dihydroxy octadecadienoic acid (7*S*,8*S*-diHOD) (Fig. 6, overleaf) (Brodowsky, 1992; Su, 1996). Initially it was thought that these two activities belonged to separate enzymes, namely a cytosolic dioxygenase and a microsomal hydroperoxy isomerase, but later it became clear that LDS is actually a bifunctional enzyme (Su, 1995).



[Figure 6] LDS catalysis

Similar to PGS, the first step of LDS catalysis is the abstraction of the *pro* 8*S* hydrogen of 18:2, probably by a tyrosyl radical (Hornsten, 2002a). Molecular oxygen is then inserted antarafacially to the fatty acid radical producing an 8*R*-peroxy radical. It is assumed that the peroxy radical subsequently recovers the hydrogen from the Tyr residue yielding 8*R*-hydroperoxy octadecadienoic acid and regenerating the tyrosyl radical for a next round of catalysis. 8*R*-hydroperoxy octadecadienoic acid is then most likely reduced into its corresponding hydroxy derivative by donation of the terminal oxygen atom under the formation of another tyrosyl radical. 8*R*-Hydroxy octadecadienoic acid is then either released, or further converted by LDS. In this second step, the *pro S* hydrogen from C-7 is abstracted and the donated oxygen atom is added suprafacially so that eventually 7*S*,8*S*-dihydroxy octadecadienoic acid is formed (Hamberg, 1994; Oliw, 1999). LDS differs from PGS in that it abstracts an allylic hydrogen (and not a bisallylic hydrogen), introduces molecular oxygen at the same carbon atom of hydrogen abstraction (in contrast to hydrogen abstraction and oxygen insertion at different carbon atoms) and it retains double bond configuration (instead of allowing rearrangement to *E*,*Z* conjugation) (Hamberg, 1994; Oliw, 1999; Su, 1998b).

The formation of 8-HOD and 7,8-diHOD from 18:2 has also been demonstrated in the ascomycete *Magnaporthe grisea*, the causal agent of rice blast disease. The genome of *M. grisea* contains a gene with close homology to the LDS gene of *G. graminis*, which also encodes the catalytically important residues (Cristea, 2003). However, it has not yet been demonstrated that this gene is indeed connected to the formation of 8-HOD and 7,8-diHOD in *M. grisea*. The function of 8-HOD and 7,8-diHOD in *G. graminis* and *M. grisea* is unknown.



[Figure 7] Examples of fungal LDS products

Putative fungal LDS

Other fungi form 8-HOD as well and in some cases, also oxylipins with a similar structure to 7,8-diHOD. In Fig. 7 examples of LDS-derived products found in fungi are shown. Their formation might be catalyzed by LDSs with novel specificities. However, the occurrence of such LDSs remains to be confirmed since none of the responsible enzymes have been isolated or characterized.

Putative fungal 5,8-LDS *A. nidulans* secretes a set of oxylipins, called psi factors (for "precocious sexual inducer"). They were characterized as 8*R*-HOD (psiBα), 5*S*,8*R*-dihydroxy octadecadienoic acid (5*S*,8*R*-diHOD) (psiCα) and lactonized 5*S*,8*R*-diHOD (psiAα). Their corresponding 18:1 derivatives (psiBβ, psiAβ, psiCβ, respectively) were present as well (Champe, 1989, 1987; Mazur, 1990).
The enzymes involved in their formation have not yet been isolated; however, the genome of *A. nidulans* contains three genes, *ppoA*, *ppoB* and *ppoC* (for "putative psi-producing oxygenase") with homology to *G. graminis* LDS and PGS. The putative proteins all contain the in LDS and PGS conserved distal and proximal heme-ligands and the Tyr residue involved in the formation of a tyrosyl radical (Tsitsigiannis, 2004a, 2005b, 2004b). Deletion of the *ppoA* gene resulted in *A. nidulans* strains deficient in the production of 8-HOD (psiBα) (Tsitsigiannis, 2004b). The deletion of *ppoB* or *ppoC* resulted in strains, reduced in 8-HOM (psiBβ) formation (Tsitsigiannis, 2004a, 2005b). A relation between these genes and the formation of 5,8-dihydroxy and lactonized 5,8-dihydroxy fatty acids on the other hand, was not found.

There are indications that *A. nidulans* oxylipins are involved in the balance between sexual and asexual reproduction. Deletion of *ppoA* (connected to 8-HOD production) increased the ratio of asexual to sexual spores 4-fold, but deletion of *ppoB* (linked to 8-HOM formation) increased this ratio by 8-fold (Tsitsigiannis, 2005b, 2004b). On the other hand, deletion of *ppoC* (also related to 8-HOM formation) resulted in a 3-fold decrease of the ratio of asexual spores to sexual spores (Tsitsigiannis, 2004a). The observation that the disruption of *ppoB* and *ppoC* lead to a different phenotype suggests that other factors besides 8-HOM also influence sporulation in *A. nidulans* (Tsitsigiannis, 2005a). In a study with purified psi factors, the *in vitro* addition of 8-HOD and 5,8-diHOD decreased the ratio asexual spores to sexual spores; lactonized 5,8-diHOD however, increased this ratio (Champe, 1989).

Putative fungal 8,11-LDS A crude extract of *A. bisporus* formed 8*R*-HOD and 8*R*,11*S*-dihydroxy octadecadienoic acid (8*R*,11*S*-diHOD) from 18:2. It was proposed that the formation of 8,11-diHOD occurred either through an 8,11-endoperoxy, an 8-peroxo free radical or an 8-hydroperoxy intermediate. In the latter case the reaction is probably catalyzed by an LDS however, with a novel specificity. The formation of 8-HOD was also demonstrated in the basidiomycetes *Piptoporus betulinus*, *Tricholoma fulvum*, *Tricholoma flavovirens*, *L. edodes* and *P. ostreatus* however, only *T. fulvum* and *P. ostreatus* formed both 8-HOD and 8,11-diHOD (Wadman, 2005). The function of these oxylipins has yet to be demonstrated.

In the oomycete *Leptomitus lacteus* a portion of 8,11-diHOD was among the many dioxygenase and monooxygenase products present (Fox, 2000). It is not yet clear whether or not the formation of 8,11-diHOD in *L. lacteus* is similar to its formation in *A. bisporus*.

Fungal LDS miscellaneous In the basidiomycete *Laetisaria arvalis*, a soil fungus, the presence of 8*R*-HOD was described, but the enzyme catalyzing its formation was not characterized (Bowers, 1986). Later, it was found that 8-HOD was derived from 8-HPOD, produced by a dioxygenase with similar properties as LDS from *G. graminis*. However, *L. arvalis* did not form dihydroxy fatty acids (Brodowsky, 1993). 8-HOD had antifungal properties against certain fungal pathogens but the mechanism through which 8-HOD achieved this antifungal effect has not yet been studied (Bowers, 1986).

In the basidiomycete *Ustilago maydis* a protein abundant in the sexual spores was isolated and named Ssp1 (for "spore specific protein"). Ssp1 had homology with *G. graminis* LDS and PGS but the nature and function of the oxylipins formed by this protein, remains as yet unclear (Huber, 2002).

Dioxygenase 4: α-dioxygenase (α-DOX)

α-DOX, sometimes called pathogen inducible oxygenase (PIOX) or plant α-dioxygenase (PADOX) has only been found in higher plants so far, and uses as substrates C_{16} to C_{20} fatty acids possessing 0 - 4 double bonds (Hamberg, 1999; Sanz, 1998). α-DOX has homology to PGS and LDS and also contains the amino acid residues important in PGS catalysis (Liu, 2004; Sanz, 1998). α-DOX contains heme-iron and catalyzes the addition of molecular oxygen to the α-carbon atom (C-2) of fatty acids (Hamberg, 1999). The formed 2*R*-hydroperoxy fatty acids are chemically unstable and undergo spontaneous decarboxylation to yield the corresponding chain-shortened aldehydes. Possibly, since α-DOX is pathogen inducible, these oxylipins act as signalcompounds important for the plant defense mechanisms or have a direct toxic effect (Hamberg, 1999). Furthermore, the accumulation of α-DOX transcripts is enhanced in leaves subjected to artificial senescence, and it has been shown that α-DOX plays a role in plant development (Hamberg, 2005).

Fungal α-DOX

Currently, there is no evidence for α-DOX activity or α-DOX derived oxylipins in any of the five divisions of fungi. It is interesting to note that a set of structurally similar oxylipins was found in the *Saccharomycetales* (Bareetseng, 2004; Deva, 2000; Pohl, 1998; Sebolai, 2004; Smith, 2003). In these fungi, 3-hydroxy fatty acids of different chain lengths occurred in close association with the surfaces of sexual spores (van Dyk, 1991), however, they are not produced by a dioxygenase (Akpinar, 1997).

Outline of this thesis

Dioxygenation is a general part of polyunsaturated fatty acid metabolism in fungi. The occurrence of oxylipins has been demonstrated among the five divisions of the fungal kingdom and there is evidence for the presence of three types dioxygenases (*i.e.* PGS, LOX and LDS). Many fungal oxylipins differ from their animal and plant counterparts with respect to their molecular structures. Their formation is likely catalyzed by similar enzymes with different specificities from their plant and animal homologs, or by a novel group of dioxygenases altogether. Despite such remarkable differences, surprisingly little research is available for fungal dioxygenation. The current knowledge of fungal dioxygenases with respect to reaction mechanism, structure and function of its products is still very limited. Most studies have either shown the occurrence of oxylipins or the occurrence of an activity. More complete studies in fungi connecting dioxygenase genes, proteins, products and functions are currently not available. In depth analysis of dioxygenation pathways of polyunsaturated fatty acids in fungi will lead to a better understanding of the complex fungal life cycle, and will add to the knowledge of its animal and plant equivalents.

The research described in this thesis contributes to the current knowledge of fungal polyunsaturated fatty acid metabolism in different ways. In **Chapter 2** a set of novel dioxygenase metabolites formed by *A. bisporus*, are described. These metabolites could be potential products of an LDS with unknown specificity or they could be produced by a new sort of dioxygenase. To relate oxylipins to a possible function in growth and development, in **Chapter 3** their occurrence was studied in different developmental stages of *S. commune*, a model fungus closely related to *A. bisporus*. In **Chapter 4** the cloning of a putative dioxygenase gene from *S. commune* is described. The encoded protein has close amino acid homology to LDS and PGS and has homologs in many members of the fungal kingdom. *A. niger* has three of these homologs, and the result of their disruption an over-expression is described in **Chapter 5**.

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2

Conversion of linoleic acid into novel oxylipins by the mushroom *Agaricus bisporus*

Abstract

Oxylipins are associated with important processes of the fungal life cycle, such as spore formation. Here, we studied the formation of fatty acid metabolites in *Agaricus bisporus*. Incubation of a crude extract of lamellae with linoleic acid (18:2) led to the extensive formation of two oxylipins. They were identified as 8*R*-hydroxy-9*Z*,12*Z*-octadecadienoic acid (8*R*-HOD) and 8*R*,11*S*-dihydroxy-9*Z*,12*Z*-octadecadienoic acid (8*R*,11*S*-diHOD) by using RP-HPLC, GC/MS, IR, GC/MS analysis of diastereomeric derivatives, ^{1}H NMR and ^{13}C NMR spectroscopy. Both compounds have not been reported before in *A. bisporus*. Oleic (18:1), α-linolenic (α-18:3), and γ-linolenic (γ-18:3) acid were converted into their 8-hydroxy derivatives as well, and α-18:3 was further metabolized into its 8,11-diol derivative.

Reactions with [U-^{13}C] 18:2 demonstrated that the compounds 8-HOD and 8,11-diHOD were formed from exogenously supplied 18:2. When [U-^{13}C] 8-HOD was supplied, it was not converted into 8,11-diHOD, indicating that it is not an intermediate in the formation of 8,11-diHOD. When *A. bisporus* crude extract was incubated under an atmosphere of $^{16}O_2$/ $^{18}O_2$ the two hydroxyl groups of 8,11-diHOD contained either two ^{18}O atoms or two ^{16}O atoms. Species that contained one of each isotope could not be detected. It is proposed that the formation of the 8,11-dihydroxy compounds either occurs through an 8,11-endoperoxy, a 8-peroxo free radical or an 8-hydroperoxy intermediate. In the latter case, the reaction should be catalyzed by a dioxygenase with novel specificity.

Introduction

Metabolism of polyunsaturated fatty acids is a central feature in all biological systems, including animals, plants and fungi. An important class of enzymes involved in polyunsaturated fatty acid metabolism are the dioxygenases, which include lipoxygenases (LOXs), prostaglandin synthases (PGS), and linoleate diol synthases (LDSs) (Hornsten, 1999). LOXs are non-heme-iron-containing proteins that catalyze the regio- and stereospecific insertion of molecular oxygen in polyunsaturated fatty acids with one or more 1*Z*,4*Z*-pentadiene systems thus forming 1*S*-hydroperoxy-2*E*,4*Z*-pentadiene derivatives. Secondary metabolites of these hydroperoxides have a wide variety of functions. PGSs are ferric-heme proteins that exist in vertebrates and presumably in corals (Varvas, 1999). They catalyze two sequential reactions: (a) the double dioxygenation and cyclisation of arachidonic acid by forming an endoperoxide function and (b) the reduction of the remaining hydroperoxide (Funk, 2001). The reaction products are involved in a variety of biological functions. LDSs contain heme-iron and have two related enzyme activities namely (a) the dioxygenation of the C-8 of linoleic acid (9*Z*,12*Z*-octadecadieonoic acid, 18:2) and (b) the isomerisation of the hydroperoxide group into 7*S*,8*S*-dihydroxy octadecadienoic acid (7*S*,8*S*-diHOD). This 7,8-LDS has been reported only in fungi and its amino acid sequence is homologous to PGSs (Brodowsky, 1992a; Hornsten, 1999).

Despite the enormous number (over 1.5 million) of known species in the fungal kingdom, fungal polyunsaturated fatty acid metabolism has received relatively little attention. In several species LOXs have been reported that have activities similar to the plant and mammalian enzymes (Bisakowski, 2000; Filippovich, 2001; Kuribayashi, 2002; Li, 2001; Perraud, 1999). However, some of the dioxygenating enzymes and intermediates differ remarkably from the fatty acid metabolites present in plants and mammals. For instance, the LOX

proposed to catalyze the formation of 1-octen-3*R*-ol, the typical mushroom flavor has an unusual specificity. It should convert the pentadiene system into a 4*S*-hydroperoxy-1*Z*,5*E* pentadiene derivative, but the mechanism of this reaction is still unclear (Matsui, 2003; Wurzenberger, 1984). Also, a non-heme-manganese LOX has been reported that converted the pentadiene system into a 3*S*-hydroperoxy-1*Z*,4*Z* pentadiene derivative (Hamberg, 1998).

A growing number of oxygenated fatty acids, and their secondary metabolites are being identified as products of fungal fatty acid metabolism. Also, fungal dioxygenases seem to possess novel specificities compared to their plant and mammalian counterparts. As part of a study on fungal polyunsaturated fatty acid metabolism, we have carried out an investigation into the capacity of *A. bisporus* to transform polyunsaturated long chain fatty acids.

Materials and methods

Materials

All chemicals used were commercially obtained and of analytical grade. Oleic acid (9Z-octadecenoic acid, 18:1, 99% pure), 18:2 (99% pure), α-linolenic acid (9Z,12Z,15Z-octadecatrienoic acid, α-18:3, 99% pure), γ-linolenic acid (6Z,9Z,12Z-octadecatrienoic acid, γ-18:3, 99% pure), and Z-vaccenic acid (11Z-octadecenoic acid, 99% pure) were obtained from Sigma (St. Louis, MO). [U-^{13}C] 18:2 (99% pure) was obtained from Isotec (Matheson Trigas, Irving, TX). Solutions of 30 mM fatty acid were stored in methanol under N_2 at -20˚C until use. 2*S*- and 2*R*,*S*-hydroxynonanoic acids as well as 2*S*- and 2*R*,*S*-hydroxy-1,9-nonanedioic acids were purchased from Larodan Fine Chemicals (Malmö, Sweden). Cysteine, glutathione and glutathione peroxidase were purchased from Acros (Fairlawn, NJ) and Sigma. $^{18}O_2$ (500 mL) was 99.51% pure.

Mushrooms (*A. bisporus*, *Lentinus edodes* and *Pleurotus ostreatus*) were purchased from a local supermarket and stored at 4˚C until use. Mushrooms (*Piptoporus betulinus*, *Tricholoma fulvum* and *Tricholoma flavovirens equesne*) were picked in a local forest and identified on guidance of a fungi handbook.

Preparation of crude extracts of lamellae

Lamellae were separated from the cap and homogenized directly with 1 mL buffer (50 mM sodium phosphate pH 6.5)/g lamellae in a commercial Waring blender and centrifuged at 4˚C, 2500 × g for 20 min. The supernatant (crude extract) was filtered through cheesecloth and used immediately or after boiling in a waterbath for 10 min as a control for enzyme activity.

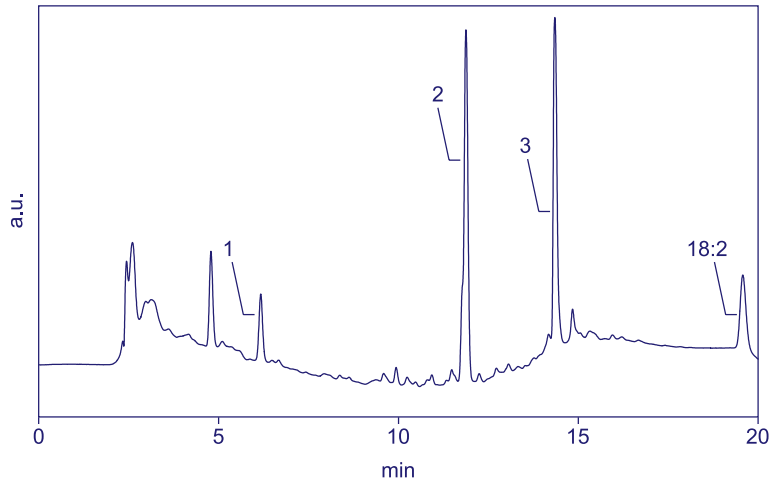
Extraction and purification of fatty acid products

Typically, 4 mL phosphate buffer (50mM, pH 6.5) was mixed with 1 mL crude extract, rigorously stirred and incubated with 120 µM substrate for 30-45 min at room temperature under a continuous flow of O_2. In some cases, cysteine (250 µM) or glutathione (1mM) and glutathione peroxidase (2 or 8 U/mL) were added at the start of the incubation. For the large-scale production of 18:2 metabolites, reactions were carried out with 100 mL crude extract and 400 mL phosphate buffer. Incubations under an atmosphere of $^{16}O_2/^{18}O_2$ were carried out after repeated evacuation and purging the reaction vessel that contained the dissolved substrate with nitrogen. The experimental atmosphere had an $^{18}O_2$ to $^{16}O_2$ ratio of either 6:1 (v/v) or 3:2 (v/v). Fatty acids and reaction products were recovered directly by solid phase extraction (SPE, Oasis HLB 200 mg; Waters, Milford, MA). The eluate was concentrated under N_2, and analyzed by RP-HPLC. Analysis by GC/MS of the reaction products as TMS ethers of methyl ester derivatives was performed as described previously (van Zadelhoff, 1998). The fatty acid methylation reagent was diazomethane. For GC/MS analysis, samples were analyzed before and after hydrogenation. Endogenously present oxylipins were extracted from frozen mushrooms according to the method of Bligh and Dyer using dichloromethane instead of chloroform (Bligh, 1959).

Characterisation of fatty acid products

Infrared spectral analysis was carried out by diffuse reflectance infrared Fourier transform (DRIFT) spectroscopy. Samples were prepared by slowly evaporating a few droplets of the concentrated eluate at 40˚C onto powdered KBr in a DRIFT cup. Similarly, solutions of methyl 18:1 and methyl 18:2 in methanol were used for reference purposes. Blank KBr exposed to a few droplets of pure methanol, was used as background. After evaporation, the cups were transferred to a DRIFT accessory (Spectra-tech) in a Perkin-Elmer-2000 FTIR spectrometer. Spectra were recorded by accumulating 25 scans at an optical resolution of 4 cm^{-1}. All spectra were converted into Kubelka-Munk units prior to interpretation.

^{1}H NMR spectra were recorded at 300K with a Bruker AMX 500 (500 MHz) spectrometer; δ_H values are given in ppm relative to the signal for internal TMS (δ_H = 0, $CDCl_3$). 2D ^{1}H-^{1}H TOCSY (mixing times 7 ms or 100 ms) and ^{1}H-^{13}C HSQC spectra were recorded at 300 K with a Bruker AMX 500 (500 MHz and 126 MHz).



[Figure 1] RP-HPLC chromatogram (λ = 200 nm) of the reaction of *A. bisporus* crude extract of lamellae and 18:2

Indicated are peak 1 (10-ODA), peak 2 (8,11-diHOD) and peak 3 (8-HOD), the major fatty acid metabolites. RP-HPLC analysis and purification of the fatty acid products was carried out on a Cosmosil 5C18-AR (5 µm; 250 × 4.6 mm i.d.; Nacalai Tesque, Kyoto, Japan) reversed-phase column using a gradient system (solvent A: methanol/water/acetic acid (50:50:0.01, v/v/v); solvent B: methanol/water/acetic acid (95:5:0.01, v/v/v)) with the following gradient program: 100% solvent A for 1 min, followed by a linear increase of solvent B up to 100% within 10 min and finally an isocratic post-run at 100% solvent B for 10 min. The flow-rate was 1 mL/min.

Stereoconfiguration of the reaction products from 18:2 was determined by formation of methyl MC derivatives followed by oxidative ozonolysis and methyl esterification (Hamberg, 1971). Methyl 8,11-diHOD (approx. 1 mg) was dissolved in ethyl acetate (3 mL) and stirred for 20 min under hydrogen gas, in the presence of palladium-on-calcium carbonate (5 mg) (Hamberg, 1998). An aliquot of the partially hydrogenated material was derivatized with trimethylchlorosilane (trimethylchlorosilane/hexamethyldisilazane/pyridine 1:1:5, v/v/v) and the resulting TMS ethers of methyl ester derivatives were analyzed by using GC/MS. GC was performed with a Hewlett-Packard model 5890 gas chromatograph equipped with a methylsilicone capillary column (length, 25 m; film thickness, 0.33 µm; Agilent Technologies, Wilmington, DE). Helium was used as the carrier gas, at a flow rate of 25 cm/s. For analysis of methyl MC derivatives the start temperature was either 190˚C and increased by 2˚C/min (analysis of methyl MC 2-hydroxy nonanoates) or 210˚C and increased by 3˚C/min (analysis of dimethyl MC 2-hydroxy-1,9-nonanedioates). Under the conditions used, the MC derivative of methyl 2*S*-hydroxy nonanoate eluted with a shorter retention time (11.80 min) compared to the corresponding derivative of the methyl 2*R*-hydroxy compound (11.94; separation factor, 1.01). In the same way, the MC derivative of dimethyl 2*S*-hydroxy-1,9-nonanedioate eluted with a shorter retention time (11.56 min) than the MC derivative of the dimethyl 2*R*-hydroxy compound (11.67 min; separation factor, 1.01). GC/MS of methyl MC derivatives was carried out with a Hewlett-Packard model 5970B mass selective detector connected to a Hewlett-Packard model 5890 gas chromatograph equipped with a 5% phenylmethylsiloxane capillary column (12 m; film thickness, 0.33 µm; Agilent Technologies, Deerfield, IL). The start temperature was 120˚C and was increased by 10˚C/min.

Results

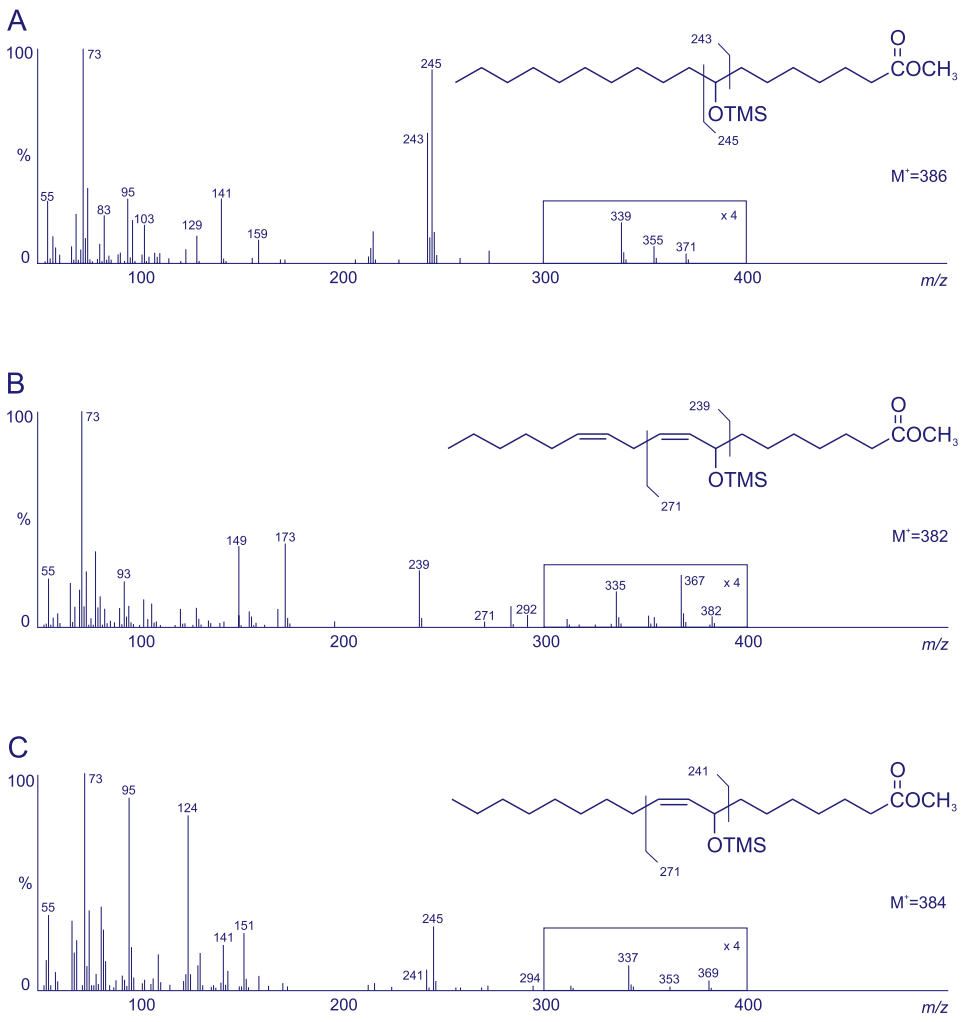
RP-HPLC analysis

A crude extract of lamellae of *A. bisporus* was incubated with 18:2 and the reaction mixture was extracted with SPE and analyzed by RP-HPLC. A typical HPLC chromatogram is shown in Fig. 1. Incubation with 18:2 resulted in the appearance of two large peaks in the HPLC chromatogram and a smaller one. Peak 2 (Fig. 1) had a retention time of 11.9 min, and peak 3 (Fig. 1) of 14.5 min. Both had absorbance at 200 nm. Peak 1 (Fig. 1), at 6.1 min retention time contained a compound with λ_{max} at 220 nm, characteristic of a conjugated oxo-ene in methanol. Reference compounds of dihydroxy fatty acid reaction products had a retention time of 11-12 min, whereas the monohydroxy fatty acid references eluted between 13-15 min. Minor amounts of regioisomeric dihydroxy and monohydroxy fatty acids were also separated in the subsequent GC/MS analysis of the RP-HPLC peaks. Several had a λ_{max} at 234 nm characteristic of a conjugated diene dissolved in methanol.

GC/MS analysis

Dihydroxy and monohydroxy fatty acid reaction products were fractionated on HPLC and after derivatization further investigated with GC/MS. Structures of oxygenated fatty acids were deduced from the spectra of the TMS ethers of methyl esters of hydroxy and dihydroxy compounds. In some cases reaction products were also hydrogenated.

[continued on p. 50]



[Figure 2] Mass spectrum and fragmentation patterns of (A) TMS ether of fully hydrogenated methyl 8-HOD, (B) TMS ether of methyl 8-HOD and (C) TMS ether of methyl 8-HOM

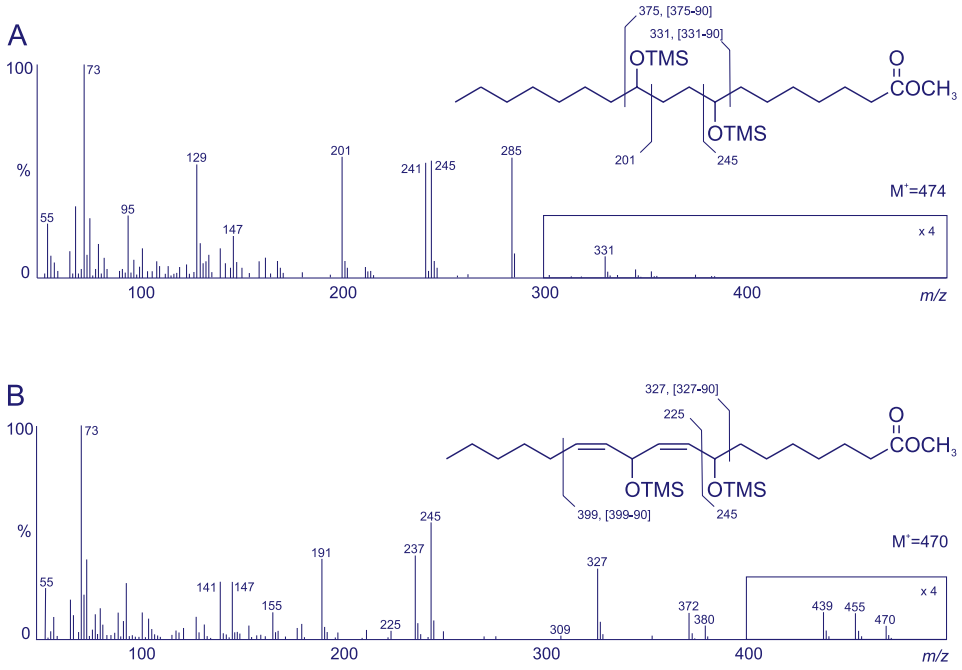
GC/MS analysis was performed on a Carlo Erba GC 8060 equipped with a Fisons MD800 MassLab spectrometer equiped with a column (30 m AT-1 × 0.25 mm × 0.25 µm; Alltech) The column temperature was held at 140°C for 2 min and then allowed to rise from 140°C to 280°C at 6°C/min and then held at this temperature for 2 min. Mass spectrometry was performed in positive ion electron impact mode over a mass range of 50-500 Da and electron ionisation at 70 eV.

	Hydrogenated		Non-hydrogenated		
Mass identity	**8-HOD** ***m/z***	**8,11-diHOD** ***m/z***	**8-HOD** ***m/z***	**8,11-diHOD** ***m/z***	**8-HOM** ***m/z***
M^+	386 (<1)	-	382 (<1)	470 (<1)	384 (<1)
M^+-CH_3	371 (<1)	-	367 (1)	455 (1)	369 (1)
M^+-CH_3O	355 (1)	-	-	439 (1)	353 (<1)
M^+-CH_3OO	339 (3)	-	335 (<1)	-	337 (2)
M^+-TMSOH	-	384 (<1)	292 (7)	380 (8)	294 (3)
F1	245 (44)[a]	375 (<1)[c]	271 (4)[f]	399 (<1)[h]	245 (30)[a]
F1-TMSOH	-	285 (57)	-	309 (3)	-
F2	243 (28)[b]	331 (3)[d]	239 (27)[g]	327 (35)[i]	241 (10)[j]
F2-TMSOH	-	241 (54)	-	237 (42)	151 (27)
F3	-	245 (55)[a]	-	245 (58)[a]	-
F3-TMSOH	-	-	-	-	-
F4	-	201 (58)[e]	-	-	-
F4-TMSOH	-	-	-	-	-
TMS^+	73 (100)	73 (100)	73 (100)	73 (100)	73 (100)

[Table 1] Characteristic mass fragments of (hydrogenated) TMS ethers of methyl esters of *A. bisporus* metabolites of 18:2 (mass fragment; relative abundance)

GC/MS analyses were performed under similar conditions as described in the legend to Fig. 2.

[a]Cleavage between C-8 and C-9; $TMSO^+$=CH-$(CH_2)_6$-$COOCH_3$
[b]Cleavage between C-7 and C-8; $TMSO^+$=CH-$(CH_2)_9$-CH_3
[c]Cleavage between C-11 and C-12; ($TMSO^+$=CH)-$(CH_2)_2$-(TMSO-CH)-$(CH_2)_6$-$COOCH_3$
[d]Cleavage between C-7 and C-8; $TMSO^+$=CH-$(CH_2)_2$-(TMSO-CH)-$(CH_2)_6$-CH_3
[e]Cleavage between C-10 and C-11; $TMSO^+$=CH-$(CH_2)_6$-CH_3
[f]Cleavage between C-10 and C-11; $^+$CH=CH-(TMSO-CH)-$(CH_2)_6$-$COOCH_3$
[g]Cleavage between C-7 and C-8; $TMSO^+$=CH-CH=CH-CH_2-CH=CH-$(CH_2)_4$-CH_3
[h]Cleavage between C-13 and C-14; $^+$CH=CH-(TMSO-CH)-CH=CH-(TMSO-CH)-$(CH_2)_6$-$COOCH_3$
[i]Cleavage between C-7 and C-8; $TMSO^+$=CH-CH=CH-(TMSO-CH)-CH=CH-$(CH_2)_4$-CH_3
[j]Cleavage between C-7 and C-8; possibly $TMSO^+$=CH-$(CH_2)_3$-CH=CH-$(CH_2)_4$-CH_3.



[Figure 3] Mass spectrum and fragmentation patterns of (A) TMS ether of fully hydrogenated methyl 8,11-diHOD, (B) TMS ether of methyl 8,11-diHOD

GC/MS analyses were performed under similar conditions as described in the legend to Fig. 2.

[continued from p. 47]

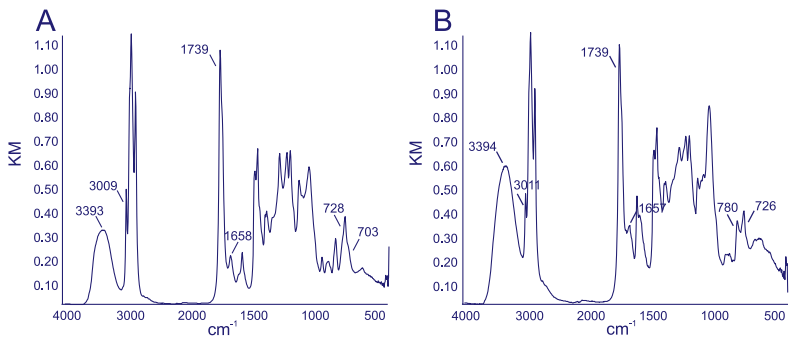
Analysis of monohydroxy fatty acids In the GC chromatogram of the hydrogenated monohydroxy fatty acids as TMS ethers of methyl ester derivatives, one prominent peak was present. The mass spectrum identified it as TMS ether of methyl of 8-hydroxy octadecanoate in view of the prominent fragment at *m/z* 245, produced by cleavage between C-8 and C-9 towards the carboxylic headgroup (Table 1 and Fig. 2A). GC/MS analysis of the corresponding non-hydrogenated monohydroxy fatty acids as TMS ethers of methyl ester derivatives, revealed that the TMS ether of methyl 8-hydroxy octadecanoate was derived from TMS ether of methyl 8-hydroxy-9,12-octadecadienoate (Table 1 and Fig. 2B, pp. 48-49). Thus, the monohydroxy fatty acid, peak 3 (Fig. 1) was identified as 8-hydroxy octadecadienoic acid (8-HOD) (Brodowsky, 1992b). Furthermore, minor amounts (5-10% as estimated from peak intensity on GC/MS) of other monohydroxides, namely TMS ether of methyl 10-hydroxy octadecadienoate,

TMS ether of methyl 9-hydroxy octadecadienoate and TMS ether of methyl 13-hydroxy octadecadienoate (data not shown), were observed in the GC chromatogram as a portion of RP-HPLC peak 3. Interestingly, another monohydroxy compound *viz.* 8-hydroxy octadecenoic acid (8-HOM) was identified by GC/MS of its TMS ether of methyl ester derivative (Table 1 and Fig. 2C). Originally the compound contained two double bonds (9*Z* and 12*Z*). One double bond (9*Z*) appeared to be hydrogenated by an *A. bisporus* homogenate. Probably, the remaining double bond still was 12*Z*.

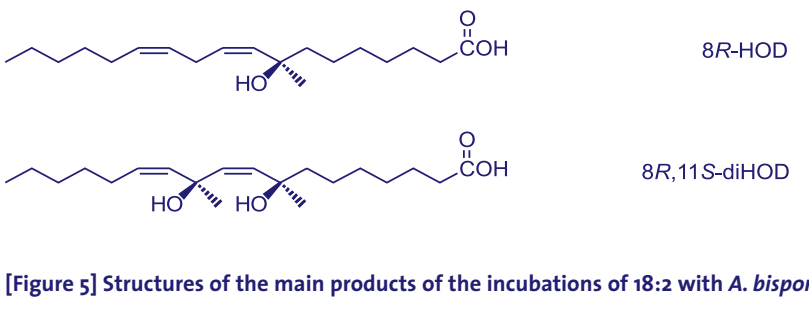
Analysis of dihydroxy fatty acids Hydrogenated dihydroxy fatty acids as TMS ethers of methyl ester derivatives were separated by using GC and one dominant peak was present in the chromatogram. The mass spectrum showed four ion peaks stemming from cleavages around the two oxygenated C atoms and thereby indicating the presence of a TMS ether of methyl 8,11-dihydroxy octadecanoate (Table 1 and Fig. 3A) Comparison of spectra from the hydrogenated and non-hydrogenated samples, showed that TMS ether of methyl 8,11-dihydroxy octadecanoate was derived from TMS ether of methyl 8,11-dihydroxy-9,12-octadecadienoate. This was evidenced by the molecular ion at *m/z* 470 and by the characteristic fragments resulting from cleavage around the double bonds and the oxygenated C atoms (Table 1 and Fig. 3B). Thus, the major dihydroxy fatty acid product in peak 2 (Fig. 1) proved to be 8,11-dihydroxy octadecadienoic acid (8,11-diHOD). In addition, small amounts (5-10% as estimated from peak intensity on GC/MS) of 8,9-dihydroxy octadecadienoic acid and 8,13-dihydroxy octadecadienoic acid were formed (data not shown). Furthermore, peak 1 (Fig. 1) was characterized by using GC/MS as its methyl ester. Its mass spectrum was compared to reported data and the compound was identified as 10-oxo decenoic acid (10-ODA) (Mau, 1993; Wurzenberger, 1982).

IR spectroscopy

The *Z* configuration of the double bonds in methyl 8-HOD as well as in methyl 8,11-diHOD are proved by DRIFT spectroscopy analysis. Both compounds exhibit absorption bands characteristic of the *Z* configuration, *i.e.*, a =C-H stretching vibration around 3005 cm^{-1}, a C=C stretching at 1655 cm^{-1} and a =C-H out-of-plane bending vibration at about 690 cm^{-1} (Fig. 4A and 4B, overleaf). In agreement with this assignment, bands typical of an *E* double bond geometry at 3025, 1675 and 965 cm^{-1} are absent in the spectra of both products.



[Figure 4] IR spectrum of (A) methyl 8-HOD and (B) methyl 8,11-diHOD



[Figure 5] Structures of the main products of the incubations of 18:2 with *A. bisporus*

Steric analysis

Stereoconfiguration of the hydroxyl groups in 8-HOD and in 8,11-diHOD were analyzed as the methyl MC derivatives of the fatty acids.

Steric analysis of methyl 8-HOD Methyl 8-HOD (approx. 0.5 mg) was derivatized with (–)-menthoxycarbonyl chloride and the MC derivative was isolated by thin layer chromatography. The material obtained was subjected to oxidative ozonolysis and the methyl-esterified product analyzed by GC. A main peak was observed, which coeluted with the MC derivative of dimethyl 2*R*-hydroxy-1,9-nonanedioate, whereas, less than 3% of the 2*S*-derivative was present. Accordingly, C-8 of the degraded oxylipin had *R* configuration (Fig. 5).

Steric analysis of methyl 8,11-diHOD Methyl 8,11-diHOD was partially hydrogenated and analyzed as its TMS ether derivative. Four main peaks were observed, designated A-D. Peak A (12.55 min; 49%) corresponded to TMS ether of the starting methyl 8,11-diHOD, whereas peak D (13.06 min, 11%) was due to the fully hydrogenated derivative, *i.e.* TMS ether of methyl 8,11-dihydroxy octadecanoate. Peak B (12.68 min; 20%) showed in the mass spectrum prominent ions at *m/z* 373 [M^+-$(CH_2)_6CH_3$], 329 [M^+-$(CH_2)_6COOCH_3$], 283 [373-TMSOH], and 239 [329-TMSOH] and originated from the TMS ether derivative of methyl 8,11-dihydroxy-9-octadecenoate. Peak C (12.80 min; 20%) showed ions at *m/z* 457 [M^+-CH_3], 245 [$TMSO^+$=CH-$(CH_2)_6COOCH_3$], and 199 [$TMSO^+$=CH-CH=CH-$(CH_2)_4CH_3$; base peak] and represented the TMS ether derivative of methyl 8,11-dihydroxy-12-octadecenoate. The above mentioned mixture of free fatty acids was converted into MC derivatives, fractionated by TLC, and subjected to oxidative ozonolysis. The product was analyzed by GC as its methyl ester derivative. Peaks were observed coeluting with the MC derivative of methyl 2*S*-hydroxy nonanoate (less than 3% of the 2*R*-isomer) and with the MC derivative of dimethyl 2*R*-hydroxy-1,9-nonanedioate (less than 3% of the 2*S*-isomer), respectively. The former component was formed by cleavage of the double bond of methyl 8,11-dihydroxy-9-octadecenoate, and the latter fragment from methyl 8,11-dihydroxy-9-octadecenoate and also from unhydrogenated methyl 8,11-diHOD. It was concluded that C-11 of the degraded oxylipin had *S* configuration and C-8 had *R* configuration (Fig. 5). Thus, the two main metabolites from the reaction of 18:2 with an *A. bisporus* crude extract of lamellae were identified as 8*R*-HOD and 8*R*,11*S*-diHOD, respectively.

^{1}H NMR and ^{1}H-^{13}C HSQC Spectra

The identification of methyl 8-HOD and methyl 8,11-diHOD was further confirmed by recording ^{1}H NMR, ^{1}H-^{1}H TOCSY and ^{1}H-^{13}C HSQC spectra (Tables 2 and 3, overleaf).

Occurrence of 8-HOD and 8,11-diHOD in other fungi

The question arises whether the formation of these compounds is specific to *A. bisporus*. Therefore, we randomly tested several other mushroom species. In all species, one or both of these metabolites were formed as well: upon 18:2 incubation, *P. betulinus*, *T. fulvum*, *T. flavovirens equesne*, *L. edodes* and *P. Ostreatus* formed 8-HOD as identified by RP-HPLC and GC/MS. *T. fulvum* and *L. edodes* formed both 8-HOD and 8,11-diHOD as evidenced by RP-HPLC and GC/MS.

$CDCl_3$ (δ)		Coupling constant ^3J (Hz)	Protons	Assignment
0.90	t	(H-18,H-17) = 6.9	3 H	C-18**H**$_3$ [a]
1.36	m		14 H	C-4**H**$_2$C-5**H**$_2$C-6**H**$_2$C-7**H**$_2$ and C-15**H**$_2$C-16**H**$_2$C-17**H**$_2$
1.61	m		2 H	C-3**H**$_2$
2.07	dt	(H-14,H-13) = 7.2 (H-14,H-15) = 7.2	2 H	C-14**H**$_2$
2.31	t	(H-2,H-3) = 7.6	2 H	C-2**H**$_2$
2.85	m		2 H	C-11**H**$_2$
3.67	s		3 H	OC**H**$_3$
4.46	dt	(H-8,H-9) = 8.3 (H-8,H-7) = 6.4	1 H	C-8**H**
5.40	m		4 H	C-9**H**C-10**H**C-11**H**$_2$C-12**H**C-13**H**

[Table 2] Relevant resonances in the ^1H NMR spectrum of methyl 8-HOD

Spectrum recorded at 500 MHz, in $CDCl_3$, and assigned by using 2D ^1H-^1H TOCSY and ^1H-^{13}C HSQC (500 and 126 MHz, $CDCl_3$). ^{13}C resonances: δ = 133.0 (C-9), δ = 131.2 (C-13), δ = 130.7 (C-10), δ = 127.3 (C-12), δ = 67.8 (C-8), δ = 51.6 (OCH_3), δ = 37.5 (C-7), δ = 34.2 (C-2), δ = 31.7 (C-16), δ = 29.3 (C-4, C-5, C-15), δ = 27.3 (C14), δ = 26.1 (C-11), δ = 25.2 (C-6), δ = 25.0 (C-3), δ = 22.1 (C-17), δ = 14.1 (C-18).

[a]Protons involved are printed in **boldface**.

$CDCl_3$ (δ)		Coupling constant ^3J (Hz)	Protons	Assignment
0.90	t	(H-18,H-17)= 6.6	3 H	C-18**H**$_3$ [a]
1.37	m		14 H	C-4**H**$_2$C-5**H**$_2$C-6**H**$_2$C-7**H**$_2$ and C-15**H**$_2$C-16**H**$_2$C-17**H**$_2$
1.61	m		2 H	C-3**H**$_2$
2.07	m		2 H	C-14**H**$_2$
2.31	t	(H-2,H-3) = 7.5	2 H	C-2**H**$_2$
3.67	s		3 H	OC**H**$_3$
4.46	dt	(H-8,H-9) = 6.8 (H-8,H-7) = 6.4	1 H	C-8**H**
5.31	dd	(H-11,H-12) = 7.8 (H-11,H-10) = 7.8	2 H	C-11**H**
5.53	m		4 H	C-9**H**C-10**H**C-11**H**$_2$C-12**H**C-13**H**

[Table 3] Relevant resonances in the ^1H NMR spectrum of methyl 8,11-diHOD

Spectrum recorded under the same conditions as described at Table 2.
^{13}C resonances: δ = 134.9 (C-9), δ = 133.1 (C-13), δ = 130.7 (C-10, C-12), δ = 68.0 (C-8), δ = 63.9 (C-11), δ = 51.6 (OCH_3), δ = 36.9 (C-7), δ = 34.0 (C-2), δ = 31.6 (C-16), δ = 29.3 (C-4, C-5, C-15), δ = 27.5 (C-14), δ = 25.2 (C-6), δ = 24.6 (C-3), δ = 22.3 (C-17), δ = 14.2 (C-18).

[a]Protons involved are printed in **boldface**.

Enzyme characteristics

Boiling the crude extract prior to incubation results in a complete loss of activity. In the corresponding RP-HPLC chromatogram peak 1, peak 2 and peak 3 disappeared completely (data not shown). Since the energetically unfavorable *Z* configuration is retained and the product is chiral, it is likely that 8-HOD and 8,11-diHOD are formed by an enzymatic reaction. Their syntheses were found to occur in the eluate after filtration through a 0.22 µm filter. Therefore, it is expected that 8-HOD and 8,11-diHOD are formed by a cytosolic enzymatic reaction (Song, 1991).

Minute amounts (about 1 a.u. on RP-HPLC) of endogenous 8-HOD and 8,11-diHOD were present in Bligh and Dyer extracts of mushrooms. Directly after blending and centrifugation only minor amounts (around 10 a.u. on RP-HPLC) of the metabolites were present. In a reaction without added substrate, 8-HOD and 8,11-diHOD were also formed (around 60 a.u. on RP-HPLC), but to a lesser extent than in a reaction with added substrate (about 100 a.u. on RP-HPLC) and arising from the presence of free endogenous 18:2. Incubation with [U-^{13}C] 18:2 showed that products represent a mixture of converted 18:2 from endogenous and exogenous sources. The conversion of exogenously supplied 18:2 was about 60% of the total conversion, as judged by the ratio of ^{13}C labeled fragments to unlabeled fragments by using GC/MS.

Incubation with [U-^{13}C] 18:2 was performed to study the connection between 18:2 and its metabolites. Analysis by using GC/MS showed that incubation with [U-^{13}C] 18:2 resulted in the formation of labeled 8-HOD, labeled 8,11-diHOD and labeled 8-HOM. Also unlabeled 8-HOD, 8,11-diHOD and 8-HOM were formed. Incubation with labeled [U-^{13}C] 8-HOD resulted in the formation

of labeled and unlabeled 8-HOM, however, only unlabeled 8,11-diHOD could be detected. This indicates that it is most unlikely that 8-HOD is an intermediate in the formation of 8,11-diHOD. To evaluate whether 8,11-diHOD may be formed through a hydroperoxy or endoperoxy intermediate, experiments were carried out in the presence of a reducing agent (250 µM cysteine or 1 mM glutathione and glutathione peroxidase 2 or 8 U/mL). In such a reducing environment hydroperoxides and endoperoxides are reduced into hydroxides, but the amount and the ratio 8-HOD to 8,11-diHOD remained unchanged. Also during time-based experiments (t = 0, 2, 4, 6, 8, 10, 15, 20, 25 and 30 min) no change in the ratio 8-HOD to 8,11-diHOD was observed. When 8,11-diHOD was isolated and characterized after incubation under an atmosphere of $^{16}O_2/^{18}O_2$, the hydroxyl groups of 8,11-diHOD contained either two atoms of ^{16}O or two atoms of ^{18}O. Species containing one of each isotope could not be detected. This finding indicated that the two hydroxyl groups were derived from the same molecule of gaseous oxygen. Therefore, the intermediate in the formation of 8,11-diHOD is possibly an 8,11-endoperoxy, an 8-peroxo free radical or an 8-hydroperoxy intermediate. However, experiments in the presence of a reducing agent failed so far to confirm the presence of such intermediates.

Substrate requirements for the 8- and 8,11-hydroxylating activity were studied by adding various substrates to the homogenate and analyzing the reaction products by using RP-HPLC and GC/MS. 18:1, α-18:3, and to a limited extent γ-18:3, were converted into 8-hydroxylated derivatives. However, *Z*-vaccenic acid was not metabolized. Only α-18:3 was also converted into 8,11-dihydroxy octadecatrienoic acid (8,11-diHOT). Apparently, a 9*Z* double bond, with no other double bond towards the carboxylic headgroup, is necessary for the 8- and 8,11-hydroxylating activity.

Discussion

The main finding of the present study is the formation of novel oxygenated products from 18:2 when incubated with *A. bisporus* crude extract of lamellae. The major reaction products were two oxylipins, identified as 8*R*-HOD and 8*R*,11*S*-diHOD. Analysis by using IR showed that all double bonds had retained the *Z* configuration. Steric analysis showed that the hydroxyl group at the C-8 position in 8-HOD and 8,11-diHOD had *R* configuration and that the hydroxyl group at C-11 in 8,11-diHOD had *S* configuration. These identifications were further confirmed by ^1H NMR and ^1H-^{13}C HSQC spectra. Repeating the reaction with [U-^{13}C] 18:2 demonstrated that these compounds were formed from exogenously supplied 18:2. A crude extract supplied with [U-^{13}C] 8-HOD did not convert it into 8,11-diHOD. This suggests that 8-HOD as such is not an intermediate in the formation of 8,11-diHOD. Incubating an *A. bisporus* crude extract under an atmosphere of $^{16}O_2/^{18}O_2$ showed that the two hydroxyl groups of 8,11-diHOD contained either two ^{18}O atoms or two ^{16}O atoms. It is proposed that this reaction occurs through 8,11-endoperoxy, an 8-peroxo free radical or 8-hydroperoxy intermediates, involving a novel dioxygenase. Probably the conversion of the intermediate is too fast to be trapped by a reducing agent.

The formation of 8-HOD has previously been observed in several instances *viz.* the ascomycete *Gaeumannomyces graminis*, a pathogen of agricultural crop (Brodowsky, 1992a, 1992b); in the oomycete *Leptomitus lacteus*, a sewage fungus (Fox, 2000); in the basidiomycete *Laetisaria arvalis*, a soil fungus (Brodowsky, 1993); in the ascomycete *Magnaportha grisea*, which causes rice blast disease (Cristea, 2003), and in the ascomycete *Aspergillus nidulans*, a seed infesting fungus (Mazur, 1990). Upon incubation with 18:2, 8-HPOD, 8-HOD and 7,8-diHOD were the major reaction products formed by *G. graminis* and *M. grisea*. This activity was assigned to a 7,8-LDS that dioxygenates 18:2 into 8-HPOD and either isomerizes this into 7,8-diHOD or reduces it to 8-HOD (Brodowsky, 1994). This LDS differs from LOX in that it contains heme-iron and abstracts the monoallylic hydrogen from the C-8 of 18:2. Molecular oxygen is then inserted antarafacially at C-8 with no change in the position of the double bonds (Hamberg, 1994). This process is energetically less favorable than the abstraction of allylic hydrogen at C-11 (Su, 1996). LOX abstracts a hydrogen atom from the allylic C-11 of 18:2 and inserts molecular oxygen at C-9 or C-13 with formation of *E*/*Z* conjugated double bonds. Unlike LOXs, the 7,8-LDS has two secondary activities, *i.e.* isomerisation or reduction of the hydroperoxide formed in the first step. *A. nidulans* produced 8-HOD, 5,8-dihydroxy octadecadienoic acid (5,8-diHOD) and the lactone of 5,8-diHOD from 18:2 (Mazur, 1990; Tsitsigiannis, 2004). The mechanism of this reaction has not yet been described. In a *L. lacteus* homogenate, it was demonstrated that a portion of 8,11-diHOD was among the many dioxygenase and monooxygenase products present (Fox, 2000).

It is possible that in analogy to 7,8-diHOD in *G. graminis* and *M. grisea*, 8,11-diHOD in *A. bisporus* is formed by 8,11-LDS that forms and isomerizes a 8-hydroperoxy intermediate. Another possibility is that in analogy with PGSs, an 8,11-endoperoxy intermediate is formed that in a second step is reduced into two hydroxyl groups. However, the occurrence of such intermediates has yet to be demonstrated.

Interestingly, in other studies on mushroom fatty acid metabolism, 10-hydroxy octadecadienoic acid (10-HOD) was found to be the most prominent metabolite (Matsui, 2003). In these studies 10-HOD was derived from 10-HPOD, the proposed intermediate in the formation of 1-octen-3-ol, and 10-ODA (Wurzenberger, 1982, 1984). In our experiments we detected only minor amounts of 10-HOD and 10-ODA. This indicates that this pathway is active, but much less than the pathway forming 8,11-diHOD. It remains subject of further studies, whether or not there is a connection between the formation of 8-HOD and 8,11-diHOD, and 1-octen-3-ol and 10-ODA.

In *A. nidulans* 8-HOD acts as a sporulation hormone (Calvo, 1999; Champe, 1989; Tsitsigiannis, 2004); 8-HOD (in some papers termed psiBα), and 5,8-diHOD (also known as psiCα) stimulated ascospore (sexual) and inhibited conidia (asexual) development. The lactone of 5,8-diHOD (or psiAα) had the opposite effect. In *L. arvalis,* 8-HOD (or laetisaric acid) was reported to possess antifungal properties by inducing rapid hyphal lysis of soil plant pathogens, including *Rhizoctonia solani*, *Phythium ultimum*, *Fusarium oxysporum* and *Mucor globusus* (Bowers, 1986). The functions of 8-HOD and 7,8-diHOD in *G. graminis* and *M. grisea* and 8-HOD and 8,11-diHOD in *A. bisporus* have still to be established. It is interesting to note that when added to human white blood cells *in vitro*, 8-HOD attenuated leukotriene B_4 formation (Vasange-Tuominen, 1994).

The results presented here extend previous studies performed on the formation of oxylipins in fungi in general and *A. bisporus* in particular. This is the first time that extensive formation of 8-HOD and 8,11-diHOD is reported and that a complete structural elucidation including stereoconfiguration is described. We demonstrated the formation of these compounds in several different mushroom families, indicating these kinds of oxylipins may be general to fungal species. To facilitate characterization of this novel dioxygenating activity, more detailed experiments are required, most importantly the isolation, purification and cloning of the enzyme.

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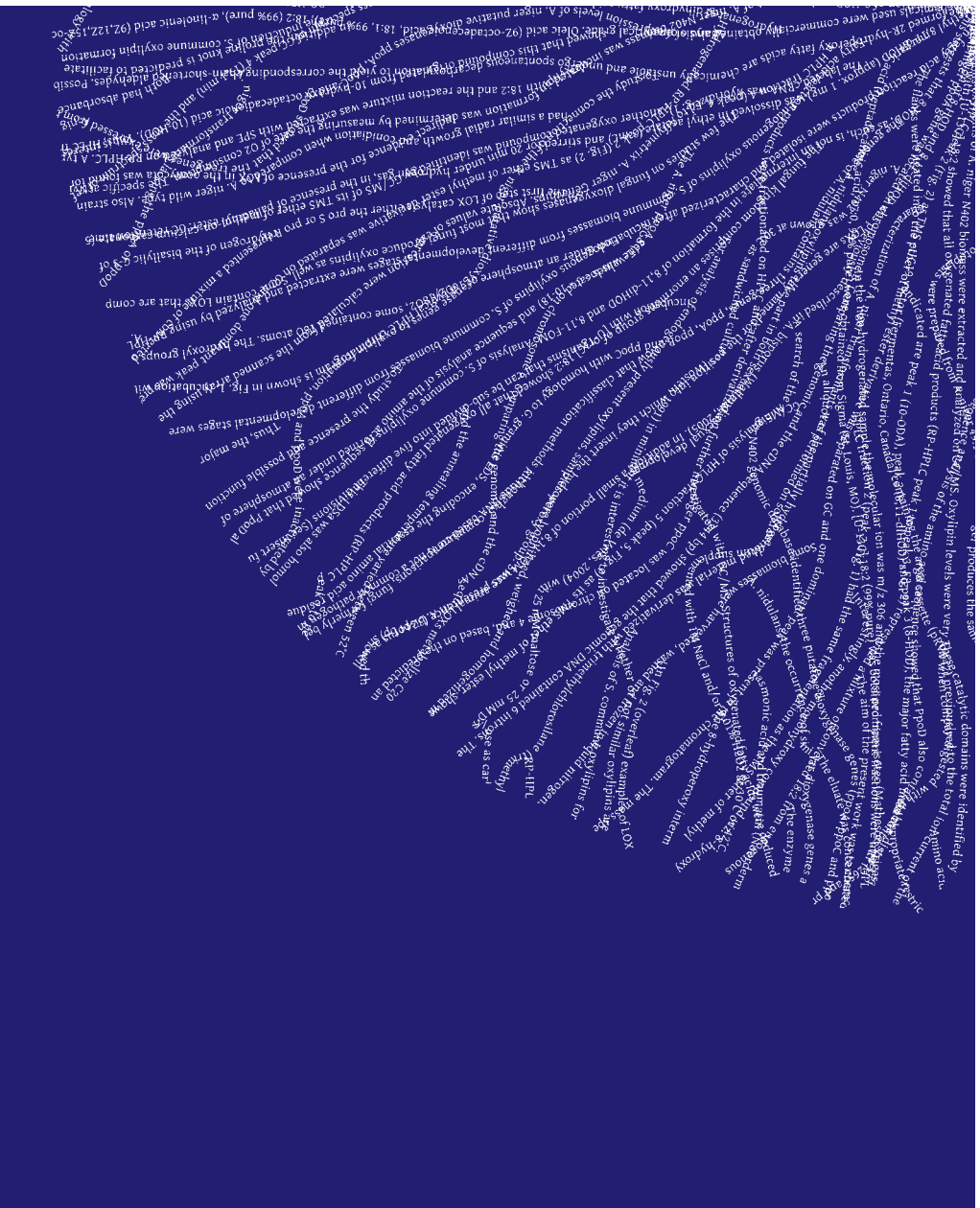


Analysis of oxylipin formation in *Schizophyllum commune*

Abstract

Several studies have demonstrated the formation of oxylipins in fungi however, few of these oxylipins have been connected to a biological function. The developmental stages of *Schizophyllum commune* can easily be separated, which makes it possible to study the presence and possible functions of oxylipins during the *S. commune* life cycle. The main oxylipins of *S. commune* were characterized as 8-hydroxy octadecadienoic acid (8-HOD), 8,11-dihydroxy octadecadienoic acid (8,11-diHOD) and 8,11-furan octadecenoic acid (8,11-FOM) using GC/MS analysis, ^{1}H NMR and ^{1}H-^{13}C HSQC spectra. The furan fatty acid 8,11-FOM, was a novel compound formed through the reaction of linoleic acid (18:2) with molecular oxygen. Oxylipin profile analysis showed that *S. commune* vegetative mycelium and fruiting bodies oxylipin profiles were similar. Also, the oxylipin profiles of *S. commune* mycelia grown in either 18:2 supplemented or non-supplemented medium were comparable. Potentially, *S. commune* oxylipins play a constant role during its entire life cycle and it is likely that, in analogy with plants and animals, oxylipins are important signaling compounds in fungi.

Introduction

Oxylipins are formed in the enzymatic reaction of polyunsaturated fatty acids with molecular oxygen. Their molecular structures differ depending on the fatty acid substrate and the enzyme specificity. Oxylipins are formed in plants, animals and fungi and have a wide variety of biological functions. For example, plant oxylipins are involved as signal molecules in defense against pathogens and also have direct antimicrobial properties (Noordermeer, 2001). Animal oxylipins, the prostaglandins, thromboxanes and leukotrienes, take part in inflammation and cell homeostasis (Cha, 2006; Funk, 2001). The enzymes that form the plant oxylipins include the lipoxygenases (LOXs) and α-dioxygenases (α-DOXs), while the animal oxylipins are produced by LOXs and prostaglandin synthases (PGSs). The function and formation of plant and animal oxylipins are fairly well understood; however, knowledge of function and formation of fungal oxylipins is much more limited.

Several studies on fungi have demonstrated the formation of oxylipins and showed that such oxylipins differ markedly from their plant and animal counterparts in terms of molecular structure. Examples of fungal oxylipins are 8-hydroxy octadecadienoic acid (8-HOD) and 8,11-dihydroxy octadecadienoic acid (8,11-diHOD) from *Agaricus bisporus* (Wadman, 2005), the psi (precocious sporulation inducer) factors 8-HOD and 5,8-dihydroxy octadecadienoic acid (5,8-diHOD) from *Aspergillus nidulans* (Mazur, 1990; Tsitsigiannis, 2004) and 8-HOD and 7,8-dihydroxy octadecadienoic acid (7,8-diHOD) from *Gaeumannomyces graminis* (Brodowsky, 1992). These oxylipins have a retained *Z* configuration of their double bonds and have unique positions of inserted molecular oxygen compared to their plant and animal counterparts. Not surprisingly, the enzymes that catalyze the formation of these compounds are different from their plant and animal counterparts as well. An example is the linoleate diol

synthase (LDS) from *G. graminis* that forms 8-HOD and 7,8-diHOD. This enzyme differs from the LOXs, α-DOXs and PGSs in that it abstracts the allylic hydrogen, retains the original *Z* configuration of the double bonds and has two secondary activities, *i.e.*, isomerisation or reduction of the hydroperoxide formed in the first step. (Brodowsky, 1992; Hamberg, 1994; Hornsten, 1999; Su, 1996, 1998). The enzyme that catalyzes the formation of 8-HOD and 8,11-diHOD in *A. bisporus* may resemble the LDS from *G. graminis* however, an 8-hydroperoxy intermediate has still to be demonstrated (Wadman, 2005). The mechanism of 8-HOD and 5,8-diHOD formation in *A. nidulans*, has not yet been described.

Few studies have connected fungal oxylipins to a biological function. It seems likely that, in analogy to plant and animal oxylipins, they fulfill roles as signal molecules. It has been shown that fungal oxylipins influence events such as spore development and change of morphogenesis. The psi factors 8-HOD and 5,8-diHOD stimulate sexual sporulation and inhibit asexual sporulation in *A. nidulans*, whereas lactonized 5,8-diHOD has the opposite effect (Tsitsigiannis, 2005b). In *Aspergillus fumigatus*, the causal agent of invasive aspergilloses, oxylipins are connected to pathogenicity as they can modulate fungal development and host immune responses (Tsitsigiannis, 2005a). In *Candida albicans*, an opportunistic human pathogen, oxylipins stimulate yeast to hypha transition enabling the pathogen to colonize under different environmental conditions (Noverr, 2004). The functions of 8-HOD and 7,8-diHOD in *G. graminis* and 8-HOD and 8,11-diHOD in *A. bisporus* have yet to be established.

The aim of the present work was to characterize the oxylipins formed by *Schizophyllum commune*. *S. commune*, the split gill fungus, is closely related to *A. bisporus* and is used as a model organism for research on the basidiomycota. Its developmental stages can easily be separated, which makes it possible to study the presence and possible function of oxylipins during the *S. commune* life cycle.

Materials and methods

Materials

All chemicals used were commercially obtained and of analytical grade. Linoleic acid (9Z,12Z-octadecadienoic acid, 18:2, 99% pure), arachidonic acid (5Z,8Z,11Z,14Z-eicosatetraenoic acid, 20:4, 99% pure) and margaric acid (heptadecanoic acid, 17:0, 99% pure) were obtained from Sigma (St. Louis, MO). [U-^{13}C] 18:2 (99% pure) was acquired through Isotec (Matheson Trigas, Irving, TX). Solutions of 30 mM fatty acid were stored in methanol under N_2 at -20˚C. $^{18}O_2$ (500 mL) was 99.51% pure. Bovine serum albumin (BSA) was from New England Biolabs (Ipswich, MA).

Strains, media and culture conditions

Strains of *S. commune* monokaryon 4-40 (*MAT*A41 *MAT*B41, CBS 341.81) and dikaryon resulting from the mating between 4-40 and 4-39 (*MAT*A43 *MAT*B43, CBS 340.81) were conserved at -80˚C. Mycelia were maintained on agar/inoculum medium at 25˚C. The inoculum medium contained $MgSO_4 \cdot 7H_2O$ (0.5 g/L), L-Asn·H_2O (1.5 g/l), thiamine dichloride (0.12 mg/L), $FeCl_3 \cdot 6H_2O$ (5 mg/L), HBO_3 (60 mg/L), $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (40 mg/L), $CuSO_4 \cdot 5H_2O$ (0.2 g/L), $ZnSO_4 \cdot 7H_2O$ (2 g/L), $MnSO_4 \cdot 4H_2O$ (0.1 g/L), $CoCl_2 \cdot 6H_2O$ (0.4 g/L), $Ca(NO_3)_2 \cdot 4H_2O$ (1.2 g/L), KH_2PO_4 (0.46 g/L), K_2HPO4 (1 g/L). Ten days old *S. commune* mycelium agar discs (3 cm radius) were homogenized in 100 mL inoculum medium in a Monell cup for 60 s. Homogenates were transferred to a 500 mL flask and were incubated (200 rpm, 30˚C, four days) on an platform shaker (Innova 2300, New Brunswick Scientific, Edison NJ). Homogenates used for studies on induction of oxylipin formation were supplemented with 18:2 (0.1-1% v/v) before or during incubation. Fresh fruiting bodies of *S. commune* were picked in a local forest and were divided over three stages (stage IV, stage IV-V and stage V (Leonard, 1968)).

Preparation of crude extracts

S. commune mycelia were harvested by centrifugation (4000 × g, 10 min, 4˚C). Supernatants were discarded, pellets were washed with cold phosphate buffer (50 mM sodium phosphate, pH 6.5) and centrifuged under the same conditions. Biomasses were resuspended in phosphate buffer (1:2 w/v) and lysed with a French Pressure Cell (2 × 1000 psi, Amico, piston diameter 2.5 cm). *S. commune* fruiting bodies were homogenized directly with 1 mL/g phosphate buffer in a commercial Waring blender. Lysates were centrifuged (10000 × g, 20 min, 4°C) and the supernatant, referred to as the crude extract, was filtered over cheesecloth and used directly. Some biomasses were harvested, washed and frozen in liquid nitrogen. Part of the frozen material was lyophilized and weighed for biomass determination, and part was extracted with methanol/water (4:1 v/v) for GC/MS analysis of endogenous oxylipins. The internal standard was 17:0.

Enzymatic reactions

Typically, 4 mL phosphate buffer was mixed with 10 mL crude extract, rigorously stirred and incubated with 120 µM substrate for 30-45 min at room temperature under a continuous flow of O_2. For the large-scale production of 18:2 metabolites, reactions were carried out with 100 mL crude extract and 400 mL

phosphate buffer. Incubations under an atmosphere of $^{16}O_2/^{18}O_2$ were carried out after repeated exhaustion and purging with N_2 of the reaction vessel that contained the dissolved substrate. The experimental atmosphere had an $^{18}O_2$ to $^{16}O_2$ ratio of 3:1 (v/v).

Extraction, purification and characterization of oxylipins

Fatty acids and reaction products were recovered directly by solid phase extraction (SPE, Oasis HLB 200 mg, Waters, Milford, MS). The eluate was concentrated under N_2, analyzed by RP-HPLC and converted into methyl esters with diazomethane and into TMS ethers for GC/MS analysis as described previously (van Zadelhoff, 1998). For GC/MS analysis, samples were analyzed before and after hydrogenation. In some cases reaction products were reduced with sodium borodeuteride to distinguish between hydroxyl and oxo groups. ^1H NMR spectra of fatty acid methyl esters were recorded at 300K with a Bruker AMX 500 (500 MHz) spectrometer; δ_H values are given in ppm relative to the signal for internal TMS (δ_H = 0, $CDCl_3$). 2D ^1H-^1H TOCSY (mixing times 7 ms or 100 ms) and ^1H-^{13}C HSQC spectra were recorded at 300 K with a Bruker AMX 500 (500 MHz and 126 MHz).

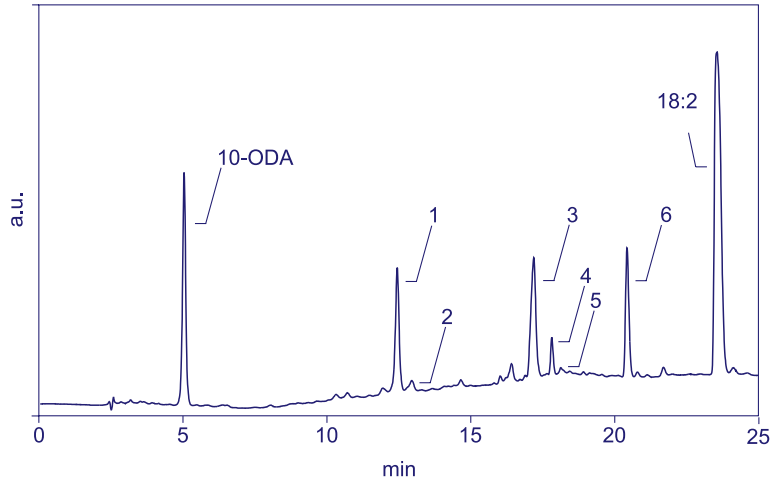
Enzymatic activity assay

Oxylipin formation was determined by measuring the rate of O_2 consumption with a DW-1 Clarke type electrode (Hansatech Instruments, Norfolk, UK). The reaction mixture consisted of 0.5 mL phosphate buffer with 600 µM 18:2 and 0.5 mL crude extract. Specific activity (µmol O_2/min/mg) was calculated by dividing the activity (µmol O_2/min/mL) by the protein concentration (mg/mL). Protein concentration of the crude extract was measured according to the Bradford assay using BSA as a standard for calibration (Bradford, 1976).

Results

RP-HPLC and GC/MS analysis of *S. commune* oxylipins

A crude extract of four day old *S. commune* 4-40 mycelium was incubated with 18:2 and the reaction mixture was extracted with SPE and analyzed by RP-HPLC. A typical HPLC chromatogram is shown in Fig. 1. Incubation with 18:2 resulted in the appearance of three large peaks in the HPLC chromatogram and three smaller ones. Reference compounds of dihydroxy fatty acids had a retention time of 9-11 min, whereas the monohydroxy fatty acid references eluted between 15-18 min. The chemical nature of HPLC fraction 6 (peak 6, Fig. 1) was

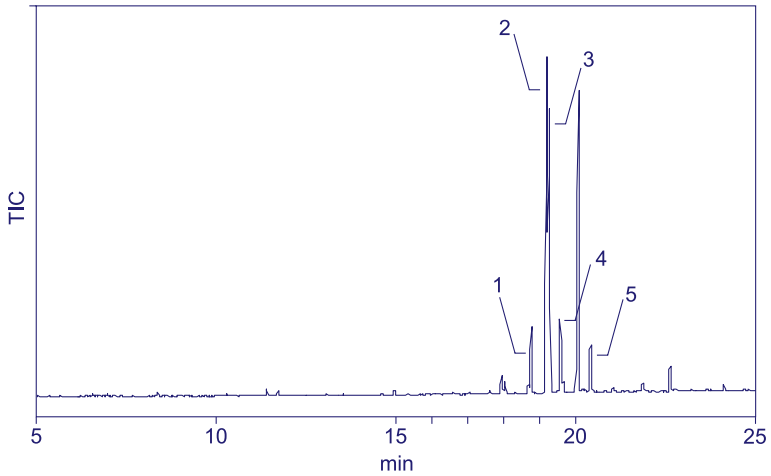


[Figure 1] RP-HPLC chromatogram (λ = 200 nm) of the reaction of *S. commune* with 18:2

Indicated are peak 1 (9.9 min; 8,11-diHOD), peak 2 (10.2 min, λ_{max} 234 nm; 8,13-diHOD), peak 3 (16.2 min, λ_{max} 230 nm; 8-HOD, 8-K-9-OD, 8-K-10-OD, 10-HOD, and 13-HOD, peak 4 (17.0 min, λ_{max} 226 nm; 8-KOD), peak 5 (17.2 min, λ_{max} 280 nm; 8-KOM) and peak 6 (20.2 min, λ_{max} 270 nm; 8,11-FOM), all metabolites from 18:2. HPLC analysis and purification of the fatty acid products was carried out on a Cosmosil 5C18-AR (5 µm; 250 × 4.6 mm i.d.; Nacalai Tesque, Kyoto, Japan) reversed-phase column using a gradient system (solvent A: methanol/water/acetic acid (50:50:0.01, v/v/v); solvent B: methanol/water/acetic acid (95:5:0.01, v/v/v)) with the following gradient program: 100% solvent A for 1 min, followed by a linear increase of solvent B up to 100% within 10 min and finally an isocratic post-run at 100% solvent B for 10 min. The flow-rate was 1 mL/min.

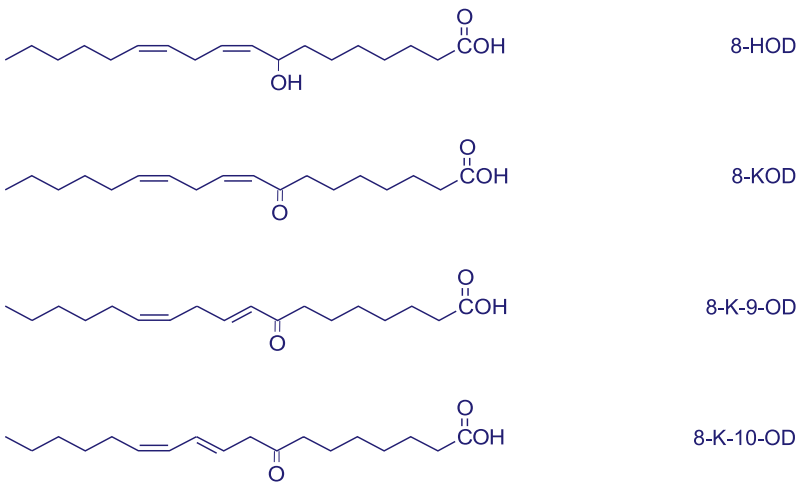
analyzed by using GC/MS. Fatty acid reaction products were fractionated on HPLC and after derivatization further investigated with GC/MS. Structures of oxygenated fatty acids were deduced from the spectra of the TMS ethers of methyl esters. 10-ODA was characterized by comparing its mass spectrum with reported data (Mau, 1993; Wurzenberger, 1982).

Analysis of dihydroxy fatty acids (HPLC fractions 1 and 2) Hydrogenated dihydroxy fatty acids as TMS ethers of methyl esters from HPLC fraction 1 (peak 1, Fig. 1) were separated on GC and one dominant peak was present in the chromatogram. The mass spectrum was identical to the mass spectrum of the TMS ether of methyl 8,11-dihydroxy octadecanoate. Comparing the GC retention time and the mass spectrum and of the non-hydrogenated sample to the GC retention time and mass spectrum of TMS ether of methyl 8,11-dihydroxy-



[Figure 2] GC chromatogram of HPLC fraction 3 (peak 3, Fig. 1) as TMF ethers of methyl esters

Indicated are peak 1 (19.4 min; 8-HOD), peak 2 (19.8 min; 10-HOD), peak 3 (20.0 min; 8K-9-OD), peak 4 (20.1 min; 13-HOD) and peak 5 (20.6 min; 8K-10-OD). GC/MS analysis was performed on a Carlo Erba GC 8060 equipped with a Fisons MD800 MassLab spectrometer equipped with a column (30 m AT-1 × 0.25 mm × 0.25 µm, Alltech, Deerfield, IL) The column temperature was held at 140°C for 2 min and then allowed to rise from 140°C to 280°C at 6°C/min and then held at this temperature for 2 min. Mass spectrometry was performed in positive ion electron impact mode over a mass range of 50-500 Da and electron ionisation at 70 eV.



[Figure 3] Structures of 8-HOD and its isomers 8-KOD, 8-K-9-OD and 8-K-10-OD produced in the reaction of 18:2 with *S. commune*

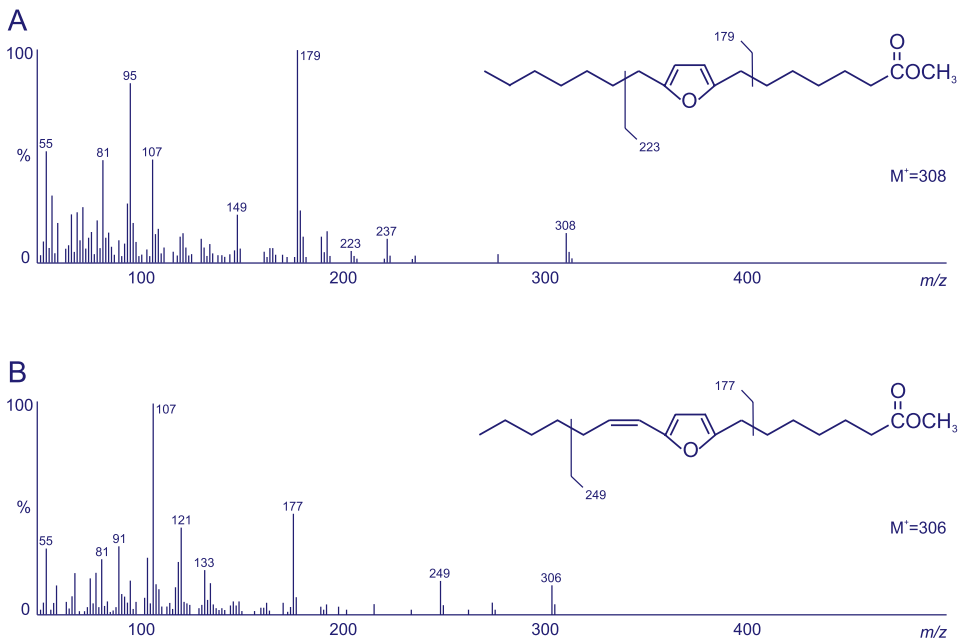
9,12-octadecadienoate showed that the major fatty acid product in HPLC fraction 1 was 8,11-dihydroxy octadecadienoic acid (8,11-diHOD) (Wadman, 2005).

HPLC fraction 2 (peak 2, Fig. 1) had the same fragmentation as the TMS ether of methyl 8,11-diHOD. However, after hydrogenation, the material proved to be derived from 8,13-dihydroxy octadecadienoic acid, in view of the fragments *m/z* 245 (cleavage between C-8 and C-9 towards the carboxylic headgroup) and *m/z* 173 (cleavage between C-12 and C-13 towards the aliphatic carbon chain). In addition, small amounts (1-5% as estimated from peak intensity on GC/MS) of 9,10-dihydroxy octadecenoic acid and 12,13-dihydroxy octadecenoic and (partially) hydrogenated dihydroxy fatty acid derivatives were present in HPLC fraction 2 (data not shown).

Analysis of monohydroxy fatty acids (HPLC fractions 3, 4 and 5) The GC chromatogram of the hydrogenated monohydroxy fatty acids of HPLC fraction 3 (peak 3, Fig. 1) as TMS ethers of methyl esters showed that this fraction was a mixture of the TMS ethers of methyl 8-hydroxy octadecanoate, methyl 10-hydroxy octadecanoate and methyl 13-hydroxy octadecanoate. In the analysis of the corresponding non-hydrogenated monohydroxy fatty acids as TMS ethers of methyl esters, five peaks were visible in the GC chromatogram (Fig. 2).

GC fraction 1 (peak 1, Fig. 2) and GC fraction 3 (peak 3, Fig. 2) had the same mass spectrum. GC fraction 1 was TMS ether of methyl 8-hydroxy-9,12-octadecadienoate in view of the retention time and mass spectrum (Fig. 3) (Wadman, 2005). Likely, GC fraction 3 was a *Z/E* isomer of GC fraction 1, one of the double bonds now having *E* configuration, presumably the original 9*Z* double bond. Reduction of this compound with sodium borodeuteride revealed that the hydroxyl group at C-8 originated from an oxo group. This identified GC fraction 3 to be derived from 8-keto-9,12-octadecadienoic acid (8-K-9-OD) (Fig. 3).

GC fraction 5 (peak 5, Fig. 2) had an intense fragment of *m/z* 245 indicating cleavage between C-8 and C-9 towards the carboxylic head group. The molecular ion of *m/z* 382 specified that GC fraction 5 was a C18 fatty acid with two double bonds and one oxygenated carbon atom. Under the used conditions, fragmentation occurs around double bonds and oxygenated carbon atoms, but not when these are next to each other in the carbon chain. Therefore, the fragment of *m/z* 245 indicated that the double bond originally between C-9 and C-10 had probably moved to C-10 and C-11, now having *E* configuration.



[Figure 4] Mass spectrum and fragmentation of (A) fully hydrogenated methyl 8,11-FOM, (B) methyl 8,11-FOM and (C) methyl [U-^{13}C] 8,11-FOM

GC/MS analysis was performed as indicated in the legend to Fig. 2.

Reduction with sodium borodeuteride showed that the hydroxyl group on C-8 was derived from an oxo group. This indicated that the compound in GC fraction 5 was originally 8-keto-10,12-octadecadienoic acid (8-K-10-OD) (Fig. 3).

Comparing the mass spectrum of GC fraction 4 (peak 4, Fig. 2) with reference compounds showed that TMS ether of methyl 13-hydroxy octadecanoate was derived from 13-hydroxy octadecadienoic acid (13-HOD). The mass spectrum of the non-hydrogenated TMS ether of methyl 10-hydroxy octadecanoate, GC fraction 2 (peak 2, Fig. 2), showed that this compound originated from 10-hydroxy octadecadienoic acid (10-HOD). Thus, HPLC fraction 3 (peak 3, Fig. 1) was composed of 8-HOD, its isomers, 8-K-9-OD and 8-K-10-OD, 10-HOD, and 13-HOD, in estimated ratios of 6:2:5:5:1, respectively, as judged from peak intensities on GC/MS.

In HPLC fraction 4 (peak 4, Fig. 1), another oxygenated compound was identified by GC/MS of its TMS ether of methyl ester. GC retention time and fragmentation of this compound were identical to those of 8-HOD. However, reduction with sodium borodeuteride showed that this compound originally had an oxo function. This identified RP-HPLC fraction 4 as 8-keto octadecadienoic acid (8-KOD) (Fig. 3).

GC/MS analysis of HPLC fraction 5 (peak 5, Fig. 1) as its TMS ether of methyl ester, showed that this compound had same fragmentation as the TMS ether of methyl ester of 8-hydroxy octadecenoic acid (8-HOM). Reduction with sodium borodeuteride revealed that the hydroxyl function originated from an oxo function, identifying the compound as 8-keto octadecenoic acid (8-KOM).

Analysis of HPLC fraction 6 Analysis of HPLC fraction 6 (peak 6, Fig. 1) on GC/MS as its hydrogenated methyl ester showed that the molecular ion was *m/z* 308 (Fig. 4A and Table 1, overleaf). Also one prominent fragment was present at *m/z* 179 and a smaller fragment at *m/z* 223. In the non-hydrogenated sample the molecular ion was *m/z* 306 and the most prominent fractions were *m/z* 177 and *m/z* 249 (Fig. 4B and Table 1). The (hydrogenated) compound could not be converted into the TMS ether indicating that no free hydroxyl group was present. The mass spectrum of the methyl ester of HPLC fraction 6 isolated after the reaction under an atmosphere of $^{18}O_2/^{16}O_2$ (*vide infra*) showed that the compound was labeled with one atom of ^{18}O (Table 1). The mass spectrum of the methyl ester of HPLC fraction 6 isolated after the reaction with [U-^{13}C] 18:2 (*vide infra*) showed that this compound was a C_{18} fatty acid derivative (Fig. 4C and Table 1). The ion at *m/z* 177 represented a C_{12} fragment, containing one oxygen atom resulting from the cleavage between C-6 and C-7 towards the aliphatic carbon chain and the ion at *m/z* 249 was a C_{14} fragment also with one oxygen atom resulting from the cleavage between C-14 and C-15 towards the carboxylic head group. HPLC fraction 6 was identified as a 18:2 derived metabolite containing a furan moiety with its oxygen group between C-8 and C-11. This compound is referred to as 8,11-furan octadecenoic acid (8,11-FOM).

Mass identity	8,11-FOM (m/z)	hydrogenated (m/z)	[U-^{13}C] labeled (m/z)	^{18}O labeled (m/z)
M^+	306 (17)	308 (13)	324 (27)	308 (40)
M^+-CH_3	291 (<1)	293 (<1)	309 (<1)	293 (3)
M^+-CH_3O	275 (5)	277 (3)	293 (12)	277 (12)
M^+-CH_3OO	259 (<1)	261 (<1)	277 (4)	261 (<1)
F1	249 (15)[a]	223 (10)[c]	263 (23)[a]	251 (26)[a]
F2	177 (47)[b]	179 (100)[d]	189 (59)[b]	179 (64)[b]

[Table 1] Characteristic mass fragments of either hydrogenated, ^{18}O labeled, [U-^{13}C] labeled 8,11-FOM

GC/MS analysis was performed under similar conditions as described in the legend to Fig. 2.

[a]Cleavage between C-14 and C-15; $^+CH_2$-CH=CH-(C1=CC=CO1)-$(CH_2)_6$-$COOCH_3$

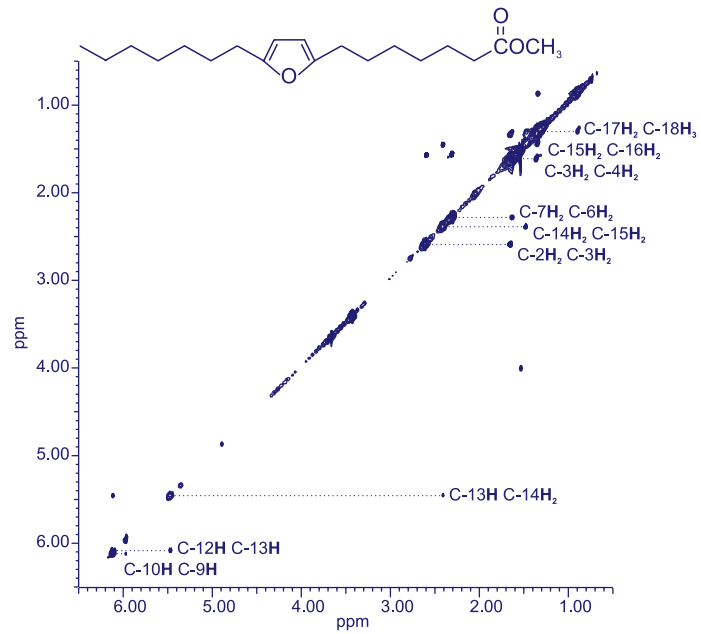
[b]Cleavage between C-6 and C-7; CH_3-$(CH_2)_6$-(C1=CC=CO1)-CH_2^+

[c]Cleavage between C-12 and C-13; $^+CH_2$-(C1=CC=CO1)-$(CH_2)_6$-$COOCH_3$

[d]Cleavage between C-6 and C-7; CH_3-$(CH_2)_4$-CH=CH-(C1=CC=CO1)-CH_2^+

The identity of methyl 8,11-FOM was further confirmed by ^{1}H NMR, ^{1}H-^{1}H TOCSY and ^{1}H-^{13}C HSQC spectra (Fig. 5, Tables 2 and 3 (p. 76)). In the spectrum of the ^{1}H-^{1}H TOCSY with mixing time 7 ms, couplings between protons on adjacent carbon atoms were observed (Fig. 5). As C-8 and C-11 do not contain hydrogen atoms, there were no cross peaks in the TOCSY spectrum between the protons on C-8 and C-9, the protons on C-8 and C-7, the protons on C-11 and C-12, and the protons on C-11 and C-10. In the ^{1}H-^{13}C HSQC spectrum of 8,11-FOM, correlation between carbon atoms and their protons is measured. Since C-8 and C-11 did not contain protons, correlation peaks were absent.

Therefore, the oxylipins formed from 18:2 by *S. commune* were 8,11-diHOD, 8,13-diHOD, 8-HOD, its isomers 8-KOD, 8K-9-OD and 8K-10-OD, 10-HOD, 13-HOD, 8-KOM, 8,11-FOM and 10-ODA.



[Figure 5] ^{1}H-^{1}H TOCSY spectrum (mixing time 7 ms) of the methyl 8,11-FOM

Indicated are the couplings between protons on adjacent carbon atoms. Couplings between C-4H_2 and C-5H_2 and between C-16H_2 and C-17H_2 could not be discriminated from the diagonal. Since C-8 and C-11 do not contain hydrogen atoms, cross peaks between C-8 and protons on C-9 and C-7, and cross peaks and C-11 and protons on C-12, and C-10 were absent.

Presence of oxylipins during the *S. commune* life cycle

Endogenous oxylipins of *S. commune* biomasses from different developmental stages were extracted and analyzed by using RP-HPLC and GC/MS. *S. commune* vegetative monokaryon 4-40 and dikaryon 4-40 × 4-39 had the same oxylipin profiles. Also the oxylipin profiles of fruiting bodies divided over three groups (stage IV, stage IV-V and stage V (Leonard, 1968)), were identical to those of the *S. commune* mycelia. In all cases the ratios of the different oxylipins and the oxylipin content were comparable.

Characteristics of oxylipin formation

Incubation with [U-^{13}C] 18:2 showed that all oxygenated fatty acids (HPLC peak 1 to peak 6, Fig. 1) represented a mixture of converted 18:2 from endogenous and exogenous sources. The conversion of exogenous 18:2 was about

$CDCl_3$ (δ)		Coupling constant ^3J (Hz)	Protons	Assignment
0.90	t	(H-18,H-17) = 6.9	3 H	C-18**H**$_3$ [a]
1.31	m		10 H	C-4**H**$_2$C-5**H**$_2$ and C-15**H**$_2$C-16**H**$_2$C-17**H**$_2$
1.63	m		4 H	C-3**H**$_2$ and C-6**H**$_2$
2.30	t	(H-7,H-6) = 7.3	2 H	C-7**H**$_2$
2.41	dt	(H-14, H-13) = 7.5 (H-14, H-15) = 7.3	2 H	C-14**H**$_2$
2.61	t	(H-2, H-3) = 7.7	2 H	C-2**H**$_2$
3.66	s		3 H	OC**H**$_3$
5.47	dt	(H-13,H-12) = 11.6 (H-13,H-14) = 7.3	1 H	C-13**H**
5.97	d	(H-9, H-10) = 2.8	1 H	C-9**H**
6.11	m		2 H	C-10**H** and C-12**H**

[Table 2] Relevant resonances in the ^{1}H NMR spectra of methyl ester of 8,11-FOM

Spectra recorded at 500 MHz, in $CDCl_3$, and assigned with 2D ^{1}H-^{1}H TOCSY and ^{1}H-^{13}C HSQC (500 and 126 MHz, $CDCl_3$). [a]Protons involved are printed in **boldface**.

$CDCl_3$ (δ)	Assignment	$CDCl_3$ (δ)	Assignment
131.0	C-13	29.7	C-4
118.3	C-12	29.0	C-5
109.8	C-10	28.2	C-2
106.9	C-9	26.7	C-6
51.8	OCH_3	25.1	C-3
34.5	C-7	22.7	C-17
32.0	C-16	14.1	C-18
29.8	C-15, C-14		

[Table 3] Correlations in the and ^{1}H-^{13}C HSQC spectra of the methyl ester of 8,11-FOM

Spectra were recorded under the same conditions as described in the legend to table 2.

50% of the total conversion, as judged by the ratio of ^{13}C labeled fragments to unlabeled fragments on GC/MS. Incubation with labeled [U-^{13}C] 8-HOD resulted in the formation of labeled 8,13-diHOD (as well as partially hydrogenated forms, data not shown), labeled 8-HOD, labeled 8K-9-OD, labeled 8K-10-OD and labeled 8-KOD. Minute amounts of labeled 8-KOM, and 8-HOM were present. 8,11-diHOD and 8,11-FOM were unlabeled after a reaction with [U-^{13}C] 8-HOD. When fatty acid reaction products were isolated and characterized after incubation under an atmosphere of $^{16}O_2$/$^{18}O_2$, some contained ^{18}O atoms. The hydroxyl groups of 8,11-diHOD contained either two atoms of ^{16}O or two atoms of ^{18}O. Species containing one of each isotope, which was the case for 8,13-diHOD, could not be detected. The hydroxyl groups of 8-HOD, 10-HOD, 13-HOD, and the oxygen group of 8,11-FOM were labeled as well. *S. commune* did not convert 20:4 into the corresponding oxylipins.

Induction of oxylipin formation in *S. commune*

The induction of *S. commune* oxylipin formation in medium supplemented with 18:2 (0.1% v/v daily or 1% v/v at day 1) was monitored in terms of changes in dry weight biomass and enzymatic activity. Growth was compared to growth in non-supplemented cultures. *S. commune* cultures were inoculated, harvested after 0, 1, 2, 3, 4, 5, and 7 days, dried and weighed. At day 3, non-supplemented cultures had reached maximum biomass (1.3 g). Cultures supplemented with 1% 18:2 at day 1 also reached maximum biomass (1.3 g) at day 3, but growth was slowed down during the first day, most likely due to fatty acid hydroperoxides formed by auto-oxidation of 18:2. Biomasses of cultures supplemented with 0.1% 18:2 daily increased steadily until reaching maximum biomass (1.5 g) at day 7. There was no significant difference in biomass between supplemented and non-supplemented cultures.

Oxylipin formation was determined by measuring the rate of O_2 consumption. The specific activities of non-supplemented and 18:2 supplemented cultures were both around 0.3 U/mg. Clearly, oxylipin formation of *S. commune* could not be induced by culturing the fungus in a 18:2 rich medium.

Discussion

This study describes the characterization of oxylipins formed from 18:2 by *S. commune*. The main reaction products were identified as 8-HOD, 8,11-diHOD and 8,11-FOM using GC/MS, ^{1}H NMR and ^{1}H-^{13}C HSQC spectra. Analysis of oxylipins from the reaction with [U-^{13}C] 18:2 showed that these were derived from a mixture of exogenously added and endogenously present 18:2. A crude extract supplied with [U-^{13}C] 8-HOD did not convert it into 8,11-diHOD and 8,11-FOM. This suggests that 8-HOD, as such, is not an intermediate in the formation of 8,11-diHOD and 8,11-FOM. Analysis of *S. commune* oxylipins formed under an atmosphere of $^{16}O_2/^{18}O_2$ showed that the 8,11-FOM molecules contained an ^{18}O atom indicating that 8,11-FOM is formed in the reaction of 18:2 with molecular oxygen. The reaction with [U-^{13}C] 8-HOD demonstrated that 8-HOD is converted into 8-K-9-OD, 8-K-10-OD, 8-KOD and 8-KOM. The formation of these compounds is most likely catalyzed by secondary dehydrogenating and reducing enzymes (Wanner, 1998).

The formation of 8-HOD and 8,11-diHOD was previously described in *A. bisporus* (Wadman, 2005). In addition, a small portion of 8,11-FOM was present in *A. bisporus* as well (M.W. Wadman, unpublished results). Likely, 8-HOD, 8,11-diHOD and 8,11-FOM in *S. commune* are formed by a similar dioxygenase as is present in *A. bisporus*. This enzyme could resemble the LDS from *G. graminis*. LDS abstracts an allylic hydrogen from C-8 and inserts oxygen at the same atom without rearrangement of the neighboring double bond. The 8-hydroperoxy intermediate is then either reduced into 8-HOD or isomerized into 7,8-diHOD (Su, 1998). It is unclear whether or the not the formation of 8-HOD and 8,11-diHOD occurs through an 8-hydroperoxy intermediate. Another possibility is that, in analogy with PGS, an 8,11-endoperoxy intermediate is formed that in a second step is reduced into two hydroxyl groups. The occurrence of such intermediates has yet to be demonstrated.

So called furan fatty acids (F-acids) are common constituents of plants, but their structures and properties differ from fungal 8,11-FOM. Plant F-acids are tri or tetra substituted furan derivatives that have a propyl or pentyl side chain in one of the α positions, a saturated carboxylic acid on the other α position, and one or two methylated β positions. The basic skeleton of F-acids is derived from a fatty acid, the oxygen is derived from molecular origin and the methyl groups originate from methionine. Their mode of formation however, is still unclear. F-acids are potent antioxidants as they are scavengers of peroxyl radicals forming dioxoenes. As a result, F-acids are completely degraded during homogenization of tissues in aqueous solutions (Batna, 1994; Spiteller, 2005). In contrast 8,11-FOM was isolated from a aqueous *S. commune* homogenate. 8,11-FOM differs from the F-acids in that it is an unmethylated furan derivative with a heptene side chain in one of the α positions. The mode of 8,11-FOM formation remains to be elucidated.

It was reported that 1-octen-3-ol levels vary between different developmental stages of *A. bisporus* (Cruz, 1997). To study the presence and possible function of oxylipins during the *S. commune* life cycle, oxylipin profiles at different developmental stages were analyzed. *S. commune* vegetative monokaryon and dikaryon mycelium and fruiting bodies (stage IV to V) had the same oxylipin profiles.

Other studies reported changed oxylipin formation under certain conditions. In *Pleurotus pulmonarius* and *Fusarium proliferatum* lipoxygenase activity was enhanced by supplementing the medium with 18:2 (Belinky, 1994; Husson, 2001). *Penicillium camemberti* and *A. bisporus* formed increased levels of 1-octen-3-ol, a metabolite from 10-HPOD, when the medium was supplemented with 18:2 (Husson, 2004, 2002; Mau, 1999). Analysis of *S. commune* oxylipin formation in mycelia grown in 18:2 supplemented and non-supplemented medium, showed that oxylipin formation was not induced by addition of 18:2. Apparently, *S. commune* oxylipin formation was unaltered under all used conditions and potentially, oxylipins play a constant role in the *S. commune* life cycle.
Like LDS from *G. graminis*, *S. commune* dioxygenase could not form oxylipins from 20:4 (Brodowsky, 1992) but the conversion of 20:4 has been demonstrated in *C. albicans* (Noverr, 2004). *C. albicans* takes up exogenous 20:4 from the host and converts it into a prostaglandin that stimulates yeast to hypha transition (Deva, 2000; Noverr, 2001). This transition enables the fungus to colonize different tissues of the host. Although *S. commune* is not a common pathogen in humans, it might cause several clinical symptoms in immuno-compromised patients. Manifestations included maxillary sinus infection (Kern, 1986), fungus ball of the lung (Sigler, 1995), brain abscess (Rihs, 1996) and bronchogenous cyst (Bulajic, 2006). Since *S. commune* does not convert 20:4, an infection with this fungus should pass off in another way than a *C. albicans* infection.
In summary, oxylipin formation appears to be a general phenomenon in the fungal kingdom. It is likely that, in analogy with plants and animals, oxylipins are important signaling compounds during the entire fungal life cycle. Interestingly, the structures of fungal oxylipins are considerably different from their plant and animal counterparts. Cloning and expression of the involved enzymes will likely show novel modes of dioxygenation.

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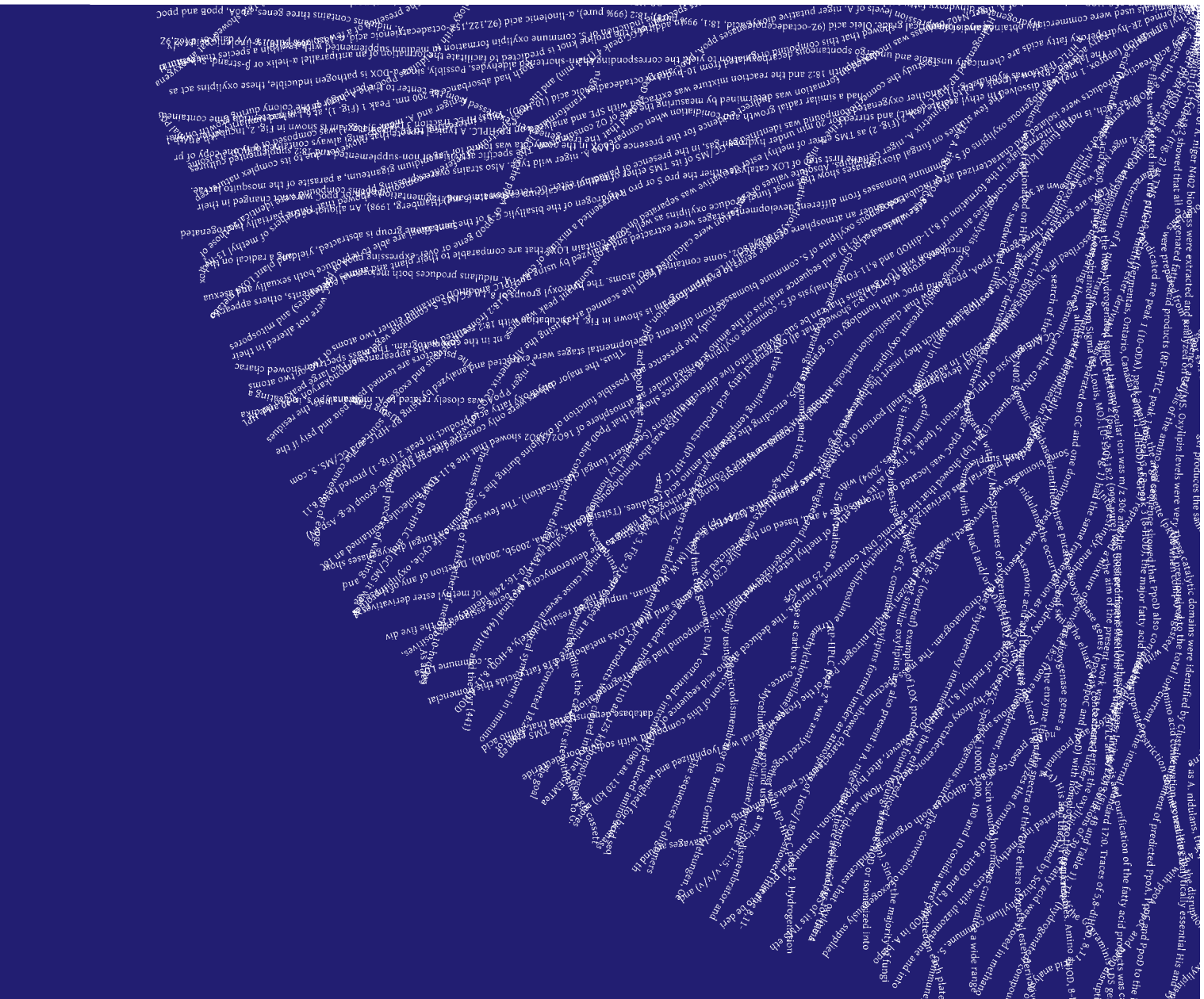


Identification and phylogenetic analysis of a *Schizophyllum commune* fatty acid heme dioxygenase

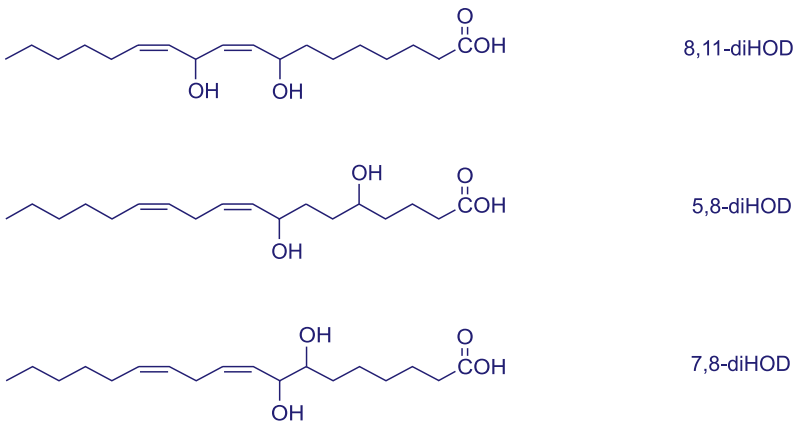
Abstract

S. commune produces 8-hydroxy octadecadienoic acid (8-HOD) and 8,11-dihydroxy octadecadienoic acid (8,11-diHOD) and it seems likely that these oxylipins are formed by a dioxygenase (Chapter 3, this thesis). In this study, a dioxygenase gene from *S. commune* was cloned and sequenced. The encoded protein, IdsA contains the essential amino acids (*i.c.* distal and proximal His and Tyr residues) for catalysis, and belongs to the fatty acid heme dioxygenase family (FAHD). The online available fungal genomes were searched for putative FAHDs and a phylogenetic tree was constructed. This tree showed that the fungal FAHD family has diverged into six different gene sub-groups (FAHD-1, FAHD-2, FAHD-3, FAHD-4, FAHD-5 and FAHD-6). Since complete studies connecting fungal FAHD genes, putative proteins, oxylipins and functions are currently not available, it remains unclear if proteins belonging to the same FAHD gene sub-group also form similar oxylipins or have similar functions.

Introduction

Free polyunsaturated fatty acids can be metabolized into signaling molecules, called oxylipins. The term oxylipin refers to oxygenated compounds synthesized from fatty acids by a mono- or dioxygenase-catalyzed oxygenation. In the formation of oxylipins in animals and plants different sets of dioxygenases are involved, and the availability of substrates in animals and plants may vary. Animals contain lipoxygenase (LOX) and prostaglandin synthase (PGS), while plant oxylipins are formed by LOX and α-dioxygenase (α-DOX). Plant α-DOX and animal PGS are homologous proteins in terms of amino acid sequence and form distinct sub-families of the fatty acid heme dioxygenase (FAHD) family (Hornsten, 1999; Sanz, 1998). The few studies on fungal dioxygenases show that most fungi produce oxylipins as well. Some contain LOXs that are comparable to their plant and animal equivalents, others appear to possess enzymes that produce prostaglandins. Some fungi contain a dioxygenase, namely linoleate diol synthase (LDS) that does not occur in animals or plants, that forms a structurally unusual set of oxylipins.

To date, only the LDS from *Gaeumannomyces graminis*, the pathogen that causes 'take all' root disease of wheat, has been studied in detail. The enzyme was purified and characterized, the corresponding gene was cloned and sequenced, and it was shown that LDS has amino acid homology with PGS and α-DOX (Hornsten, 1999; Su, 1996, 1998). It also contains the highly conserved amino acid residues (*i.c.* distal and proximal His, and Tyr involved in the formation of the tyrosyl radical) essential for PGS catalysis (Hornsten, 1999). LDS, like PGS, contains heme-iron and catalyzes two reactions. The first reaction is the insertion of molecular oxygen into linoleic acid (9Z,12Z-octadecadienoic acid, 18:2) forming 8-hydroperoxy octadecadienoic acid (8-HPOD) that is either reduced into 8-hydroxy octadecadienoic acid (8-HOD) or, in the second reac-



[Figure 1] Examples of fungal oxylipins

tion, isomerized into 7,8-dihydroxy octadecadienoic acid (7,8-diHOD) (Fig. 1) (Brodowsky, 1992; Su, 1998). *Magnaporthe grisea*, the causal agent of rice blast disease, also converts 18:2 into 8-HOD and 7,8-diHOD. The genome of *M. grisea* contains a gene with close homology to the *G. graminis* LDS gene (Cristea, 2003), and it is likely that the encoded protein is responsible for the conversion of 18:2.

The production of 8-HOD and oxylipins structurally related to 7,8-diHOD has been reported in several other fungi (Champe, 1989; Mazur, 1990; Wadman, 2005) (Fig. 1). Their formation might be catalyzed by LDSs with novel specificities; however, these LDSs remain to be confirmed since none of the responsible enzymes have been isolated or characterized.

For example, *Aspergillus nidulans*, the model system for the ascomycota, forms a number of oxylipins: 8-HOD, 5,8-dihydroxy octadecadienoic acid (5,8-diHOD) (Fig. 1) and lactonized 5,8-diHOD (Champe, 1989; Mazur, 1990). The genome of *A. nidulans* contains three genes, *ppoA*, *ppoB* and *ppoC* with homology to *G. graminis* LDS, encoding the essential amino acid residues. (Tsitsigiannis, 2004a, 2005b, 2004b). Deletion of any of these genes resulted in *A. nidulans* strains deficient in the production of 8-hydroxy oxylipins, but a relation to 5,8-dihydroxy and lactonized 5,8-dihydroxy oxylipins was not found (Tsitsigiannis, 2004a, 2005b, 2004b). There are indications that *A. nidulans* oxylipins are involved in the balance between sexual and asexual reproduction and therefore, they are referred to as psi factors (for "precocious sexual inducer") (Tsitsigiannis, 2004a, 2005b, 2004b).

The basidiomycete *Agaricus bisporus*, the white button mushroom, forms 8-HOD and 8,11-dihydroxy octadecadienoic acid (8,11-diHOD) (Fig. 1) from 18:2. It was demonstrated that this formation is catalyzed by a dioxygenase, however, the gene encoding this dioxygenase has not yet been identified (Wadman, 2005). *Schizophyllum commune*, the split gill fungus, is closely related to *A. bisporus* and is used as a model organism for the basidiomycota. *S. commune* also produces 8-HOD and 8,11-diHOD and it seems likely that these oxylipins are formed by a similar dioxygenase as is present in *A. bisporus* (Chapter 3, this thesis). The aim of the present investigation was to clone and sequence the *S. commune* dioxygenase gene involved in the formation of 8,11-diHOD and to compare it to *G. graminis* LDS and to other fungal proteins that belong to the FAHD family.

Materials and methods

Nucleic acid manipulations

The amino acid sequence of *G. graminis* LDS (Hornsten, 1999) was used to perform a BLASTp search of the *S. commune* partial genomic database (Microbiology, Utrecht University, the Netherlands). Five oligomers (s5d10896, 354 bp; s1k10628, 192 bp; s1p02824, 132 bp; s5do7627, 279 bp; s1k02847, 370 bp) were identified that had homology to the LDS gene. Primers to amplify these oligomers were designed at the 5' and 3' flanking regions (Table 1). *S. commune* genomic DNA was used as the PCR template. PCRs were carried out with AccuTaq LA™ DNA polymerase according to the manufacturer's protocol (Sigma, St. Louis, MO) and the annealing temperature varied between 52˚C and 60˚C. Amplified PCR products were cloned into the pGEMTeasy vector (Promega, Madison, WI) and used to transform competent *Escherichia coli* DH5α. Positive clones containing amplified oligomers (s5d10896dw/s5d10896up, ± 450 bp and s5d10896dw/s1k10628up, ± 1000 bp) were analyzed by restriction mapping and sequence analysis and sequence comparisons to the NCBI genetic database using the tBLASTn algorithm (www.ncbi.nlm.nih.gov).

Screening of *S. commune* λZAP libraries

Total RNA of *S. commune* monokaryon 4-40 (*MAT*A43 *MAT*B43, CBS 340.81), and dikaryon resulting from the cross of 4-40 with 4-39 (*MAT*A41 *MAT*B41, CBS 341.81), grown on either glucose or xylose/arabinose supplemented medium, was isolated and a λZAP coding DNA library was constructed (de Vries, Alves and Wösten, unpublished results). Amplified oligomers were digested with *Eco*RI, randomly labeled with [α-^{32}P]dCTP and used as the probe for λZAP

	Sequence 5' → 3'
Genomic oligomers	
s5d10896-dw	CTGCGACTGCTCTTGGTTG
s1k10628-dw	GGAGCAGCTGGACGCTTG
s1p02847-dw	CATGACCTCGATGACACGG
s5d07627-dw	CGCGTGCTCGCAAAGTGC
s5d10896-up	GATGTTCACGTCGTCGTGG
s1k10628-up	GGGTCAAGCGTCCAGCTG
s1p02847-up	GCTTCCAGGACTGGAATCC
s5d07627-up	CGCGCAGGAAGAGTCTCG
s1k02847-up	CGATGCGGATACGAAGACG
Site-directed mutagenesis	
*lds*A-SDM-*Bam*HI-dw	GATCAATGAGAGGGGCTCCTGGCAGCAAGACC
*lds*A-SDM-*Bam*HI-up	GGTCTTGCTGCCAGGAGCCCCTCTCATTGATC
Expression constructs	
*lds*A-EXPR-*Bam*HI-dw	GGATCCTTTATCAGGAGGTTGTC
*lds*A-EXPR-*Not*I-up	GCGGCCGCAATATCGTACTGGATCGGATCATCATC

[Table 1] Primers used in this study

library screening and Northern analysis. Plaque lifts were performed following the manufacturer's instructions (Stratagene, La Jolla, CA). Hybridization with radioactive probes was done as described previously (de Vries, 2002), except that washing of the filters was done at 65˚C. Positive plaques were isolated and screened again under the same conditions until single plaques could be obtained. pCMV-Script phagemids were isolated from positive plaques by *in vivo* excision as described in the manufacturer's protocol (Stratagene) and analyzed by restriction mapping and sequence comparisons to the NCBI genetic database.

Northern analysis of *S. commune*

Total RNA was isolated from mycelium of *S. commune* monokaryon 4-40 and dikaryon 4-40 × 4-39 that were grown for 4 days at 25˚C either in the light or in the dark and from 3-day-old mushrooms that were picked from an 8-day-old colony dikaryon. Samples were ground in liquid nitrogen and extracted using Trizol reagent (Invitrogen, Carlsbad, CA). RNA electrophoresis and hybridization with radioactive probes was done as described previously (de Vries, 2002), except that washing of the filters was done at 65˚C.

Site-directed mutagenesis and recombinant expression of *S. commune ldsA* gene

The coding sequence of the *S. commune ldsA* was amplified by PCR with primers that introduced an in frame *Bam*HI restriction site and a *Not*I restriction site at the 5' flank and 3' flank, respectively (Table 1). As a template pCMV-Script containing the *ldsA* gene (isolated from positive plaques) was used. The PCR fragment was ligated into pGEMTeasy generating pGEMTeasy-*ldsA* and the sequence was verified. The internal *Bam*HI site at position 2815 of the *ldsA* gene in pGEMTeasy-*ldsA* was removed by Quickchange mutagenesis (Stratagene), using the primers described in Table 1, generating pGEMTeasy-*ldsA*-dB. The full coding sequence of pGEMTeasy-*ldsA*-dB was confirmed by sequencing. Next, the *Bam*HI/*Not*I fragment of pGEMTeasy-*ldsA*-dB was ligated into a pTT3 expression vector encoding an N-terminal hexa-His tag (Durocher, 2002), generating pTT3-*ldsA*. HEK293E cells were transiently transfected with pTT3-*ldsA* using PEI. 120 h post transfection, cells were harvested by centrifugation and lysed by sonication in PBS. The cell-free supernatant (1.5 mL) was used for Western blot analysis and assayed for dioxygenating activity.

Dioxygenating activity assay

Typically, 4 mL phosphate buffer containing 10 µM hematin and 1mM phenol was mixed with 1 mL cell-free supernatant and stirred (250 rpm) at 4°C for 15 min (Adapted from (Miller, 1994; Shimokawa, 1992)). Next, 120 µM 18:2 was added and the mixture was incubated for 30-45 min at room temperature under a continuous flow of O_2. Fatty acids and reaction products were recovered directly by solid phase extraction (SPE, Oasis HLB 200 mg, Waters, Milford, MA). The eluate was concentrated under N_2, converted into methyl esters with diazomethane and into TMS ethers for GC/MS analysis as described previously (van Zadelhoff, 1998).

Phylogenetic analysis

To compare the *S. commune ldsA* gene to the *G. graminis* LDS gene and to study the conservation of FAHD proteins across the fungal kingdom, the online available fungal genetic databases were searched for LDS homologs (See website references). The amino acid sequences were aligned and a phylogenetic tree was created using the ClustalW program (www.ebi.ac.uk/clustalw).

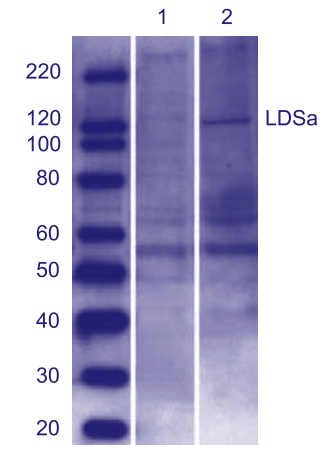
Results and Discussion

Identification and analysis of *S. commune* ldsA gene

In a search with the amino acid sequence of *G. graminis* LDS through the *S. commune* partial genomic database five oligomers (s5d10896, 354 bp; s1k10628, 192 bp; s1p02824, 132 bp; s5do7627, 279 bp; s1k02847, 370 bp) were identified, which had homology to the LDS gene. PCR with primers designed on the 5' and 3' flanking regions yielded two fragments (s5d10896dw/s5d10896up, ± 450 bp and s5d10896dw/s1k10628up, ± 1000 bp) that were used as a probe to search a *S. commune* λZAP library. The pCMV-Script phagemid isolated from a positive plaque was sequenced, and comparison to the NCBI genetic database showed that this phagemid contained the coding sequence for a protein, LDSa (1078 aa, 120 kD), with strong homology to *G. graminis* LDS (*E*-value = 4×10^{-123}; 37% identity, 52% positives, 7% gaps). This LDSa was also homologous to PGS (*E*-value = 2×10^{-16}; 24% identity, 39% positives, 17% gaps) and contained the essential distal His (211) and proximal His (388) and the Tyr (385) residues. Conserved domain search of *S. commune* LDSa using the Pfam database (www.sanger.ac.uk/Software/Pfam) indicated that residues 223 - 248 encoded a domain comparable to animal heme peroxidases and that residues 620 - 1052 were similar to cytochrome P450 monooxygenase. *S. commune* LDSa also contained the proline knot motif that targets proteins to oil bodies in plants (Abell, 1997; Tsitsigiannis, 2004b). Finally, LDSa contained 5 protein kinase C phosphorylation sites. The sequences of oligomers s1p02824 and s1k02847 were not present in the *ldsA* sequence, suggesting the presence of another LDS homolog (*ldsB*) in *S. commune*.

Recombinant expression of *S. commune* LDSa

S. commune LDSa had high homology to *G. graminis* LDS suggesting that this dioxygenase also converts fatty acids. Recombinant *S. commune* LDSa was transiently expressed in HEK293E cells and the expression was confirmed by using Westernblot analysis (Fig. 2). Previously, it was reported that the activity



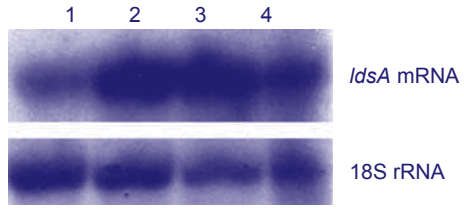
[Figure 2] *S. commune* LDSa (120 kD) produced by HEK239E cells

Lane 1: lysate of mock transfected HEK239 cells
Lane 2: lysate of HEK293E cells transfected with pTT3-*ldsA*.

of recombinant PGS dependents on heme incorporation into the apo-enzyme (George, 1996; Miller, 1994; Shimokawa, 1992). Since *S. commune* LDSa has amino acid homology to PGS and also contains the essential proximal and distal His, heme was added to ensure holo-enzyme formation. However, in spite of this exogenous addition of heme, recombinant *S. commune* LDSa produced by HEK239E cells could not convert 18:2. It can be suggested to try another expression system, for example *Pichia pastoris*, or overexpression in *S. commune* to provide evidence for the connection LDSa to polyunsaturated fatty acid metabolism.

Northern analysis of *S. commune* ldsA

Total RNA was isolated from 4-day-old colonies of *S. commune* monokaryon 4-40 grown in the dark and dikaryon 4-40 × 4-39 grown in either the dark or the light. The monokaryon abundantly formed aerial hyphae, but no fruiting structures. The dikaryon formed stage I aggregates in the dark and stage II primordia in the light (the first two stages of fruiting (Leonard, 1968)). In addition, RNA was isolated from 3-day-old mushrooms (stage IV-V) harvested from 8-day-old mycelium of dikaryon. The strongest expression of *ldsA* was observed in stage II aggregates (Fig. 3, overleaf). Expression of *ldsA* was lowest in the monokaryon. Despite the low levels of mRNA, *S. commune* fruiting bodies, vegetative mycelium of monokaryon 4-40 and dikaryon 4-40 × 4-39 all converted 18:2 into 8-HOD and 8,11-diHOD (Chapter 3, this thesis).



[Figure 3] Northern analysis of *S. commune ldsA*

Accumulation of mRNA of *ldsA*.
Lane 1: monokaryon (4-40);
Lane 2: dikaryon stage I aggregates (4-40 × 4-39);
Lane 3: dikaryon stage II primordia (4-40 × 4-39);
Lane 4: dikaryon stage IV-V fruiting bodies (4-40 × 4-39).
Methylene blue staining of 18S rRNA served as loading control.

		FAHD						
Basidiomycetes	**Class**	**1**	**2**	**3**	**4**	**5**	**6**	**Tot**
Coprinus cinereus	Homobasidiomycetes						2	2
Schizophyllum commune	Homobasidiomycetes						1	?
Laccaria bicolor	Homobasidiomycetes						2	2
Phanerochaete chrysosporium	Homobasidiomycetes						3	3
Sporobolomyces roseus	Microbotryomycetes						2	2
Ustilago maydis	Ustilaginomycetidae						1	1

[Table 2] FAHD in the basidiomycota*

**Puccinia graminis* and *Cryptococcus neoformans* serotype A/B did not contain LDS homologs.*

Phylogenetic analysis

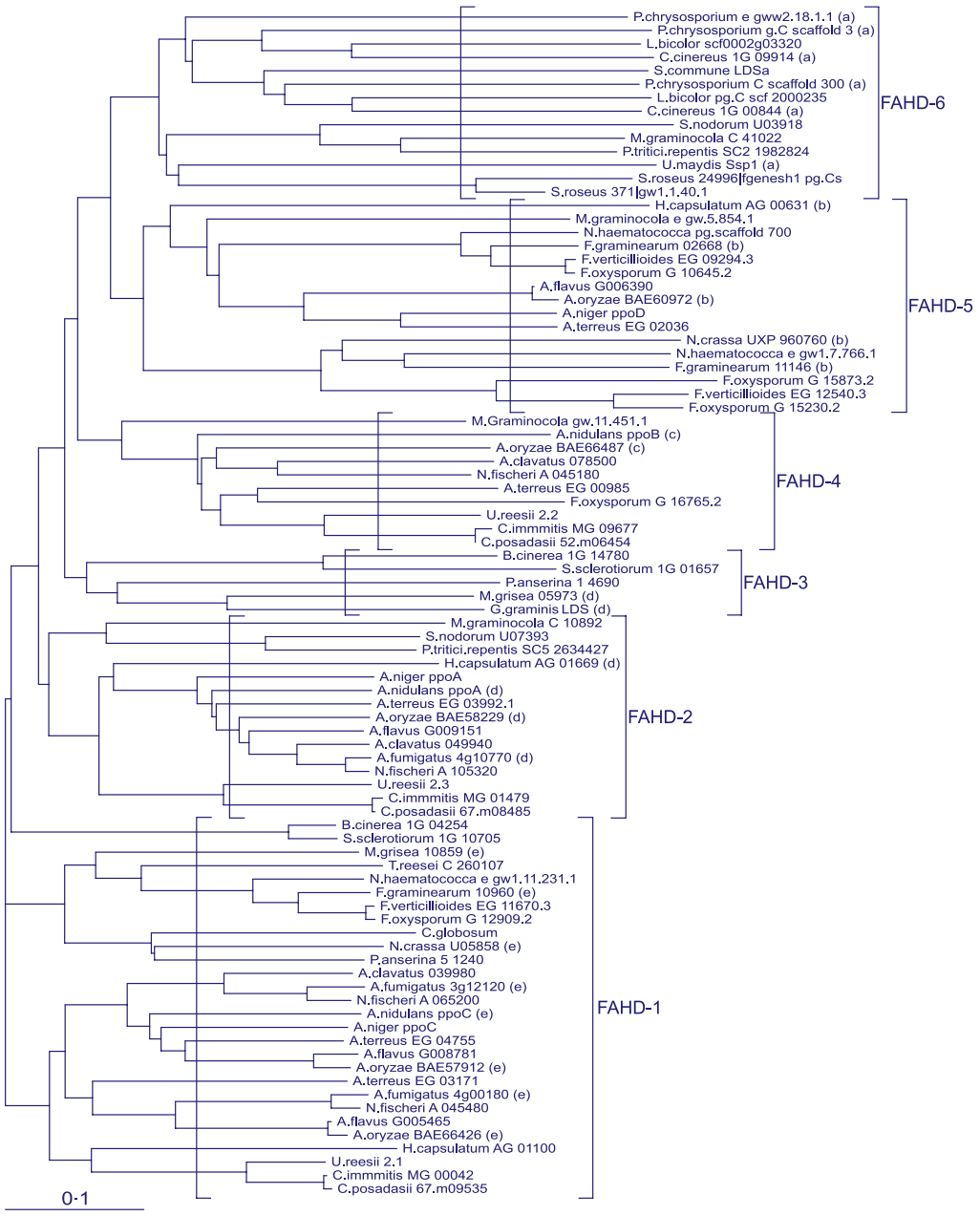
To compare putative *S. commune* LDSa to *G. graminis* LDS and to study the conservation of FAHDs in the ascomycota and basidiomycota, the online available fungal genetic databases were searched for LDS homologs. Most fungi did contain one or more (predicted) LDS homologs. However, some of the ascomycetes and two of the basidiomycetes did not contain LDS homologs (Tables 2 and 3). Putative proteins were aligned using the ClustalW program and a phylogenetic tree was created (Fig. 4, p. xx) (Chenna, 2003). The distance of proteins belonging to the same gene sub-group resembled the taxonomic distance of the different fungi.

		FAHD						
Ascomycetes	**Class**	**1**	**2**	**3**	**4**	**5**	**6**	**Tot**
Mycosphaerella graminicola	Dothideomycetes		1		1	1	1	4
Stagonospora nodorum[a]	Dothideomycetes		1				1	2
Pyrenophora tritici repentis	Dothideomycetes		1				1	2
Aspergillis fumigatus	Eurotiomycetes	2	1					3
Aspergillus nidulans[b]	Eurotiomycetes	1	1		1			3
Aspergillus niger	Eurotiomycetes	1	1			1		3
Aspergillus flavus	Eurotiomycetes	2	1			1		4
Aspergillus terreus	Eurotiomycetes	2	1		1	1		5
Aspergillus oryzae	Eurotiomycetes	2	1		1	1		5
Aspergillus clavatus	Eurotiomycetes	1	1		1			3
Neosartorya fischeri	Eurotiomycetes	2	1		1			4
Histoplasma capsulatum[c]	Eurotiomycetes	1	1			1		3
Coccidioides immitis (RS and H538.4)	Eurotiomycetes	1	1		1			3
Coccidioides posadasii	Eurotiomycetes	1	1		1			3
Uncinocarpus reesii	Eurotiomycetes	1	1		1			3
Sclerotinia sclerotiorum	Letiomycetes	1		1				2
Botrytis cinerea[d]	Letiomycetes	1		1				2
Trichoderma reesei[e]	Sordariomycetes	1						1
Fusarium graminearum[f]	Sordariomycetes	1				2		3
Fusarium verticillioides[g]	Sordariomycetes	1				2		3
Fusarium oxysporum	Sordariomycetes	1			1	3		5
Nectria haematococca	Sordariomycetes	1				2		3
Magnaporthe grisea	Sordariomycetes	1	1					2
Gaeumannomyces graminis	Sordariomycetes		1					?
Chaetomium globosum	Sordariomycetes	1						1
Podospora anserina	Sordariomycetes	1	1					2
Neurospora crassa	Sordariomycetes	1				1		2

[Table 3] FAHD in the ascomycota*

[a]*Phaeosphaeria nodorum*, [b]*Emericella nidulans*, [c]*Ajellomyces capsulatus*, [d]*Botryotinia fuckeliana*, [e]*Hypocrea jecorina*, [f]*Gibberella zeae*, [g]*Gibberella moniliformis*

**Pneumocystis carinii*, *Yarrowia lipolytica*, *Candida albicans*, *C. dubleinsis*, *C. glabrata*, *C. guilliermondii*, *C. lusitaniae*, *C. parapiplosis*, *C. tropicalis*, *Ashbya gossypii*, *Debaryomyces hansenii*, *Kluyveromyces lactis*, *Lodderomyces elongisporus*, *Pichia stipitis*, *Saccharomyces cerevisiae*, *S. cerevisiae* (RM11-1a), *Schizosaccharomyces japonicus*, and *S. pombe* did not contain LDS homologs.



[Figure 4] Phylogenetic tree of fungal FAHD gene sub-groups

Sequence names are indicated according to the annotation performed by the different databases.
The scale bar represents 0·1 amino acid substitutions per site.

[a]previously assigned to basidio *ppo* group [b]previously assigned to *ppoD* group
[c]previously assigned to *ppoB* group [d]previously assigned to *ppoA* group [e]previously assigned to *ppoC* group

The phylogenetic tree showed that the fungal FAHD family has diverged into six different gene sub-groups (FAHD-1, FAHD-2, FAHD-3, FAHD-4, FAHD-5 and FAHD-6) (Fig. 4). In contrast, a previous study on the fungal FAHD family showed that it was divided into five sub-groups (basidio *ppo*, *ppoA*, *ppoB*, *ppoC* and *ppoD*) (Tsitsigiannis, 2005b). At the time of this earlier study, only the genomes of 4 basidiomycetes and 12 ascomycetes were available; to date, the genomes of 8 basidiomycetes and 45 ascomycetes are accessible allowing better separation between gene sub-groups.

The former *ppoA* sub-group was now divided into two groups, FAHD-2 and FAHD-3 (Fig. 4 and Table 3). Proteins in FAHD-2 occurred in the dothiomycetes and eurothiomycetes, but not in the lethiomycetes and sordariomycetes (Table 3). For proteins belonging to the FAHD-3 sub-group it was the other way around. These occurred in the lethiomycetes and some of the sordariomycetes, but not in the dothiomycetes and eurothiomycetes (Table 3). It is interesting to note that fungi always contained only one copy of proteins from the FAHD-2 or FAHD-3 sub-group and that organisms never had both kinds of proteins. This may either suggest that FAHD-2 and FAHD-3 are complementary or that the presence of FAHD-2 or FAHD-3 is reflected in differences in physiology. Similarly, fungi containing FADH-1 (former *ppoC*) type proteins, did not have FAHD-6 (previously named basidio *ppo* sub-group) proteins.

In contrast to the previous reported study (Tsitsigiannis, 2005b), FAHD-6 did not only contain homologs from basidiomycetes. FAHD-6 type proteins were also found in three ascomycetes, suggesting that FADH-6 is not exclusive to the basidiomycota. On the other hand, all LDS homologs found in basidiomycetes, belonged to FAHD-6 (Table 2).

Proteins from FAHD-4 (formerly *ppoB*) and FAHD-5 (formerly *ppoD*) gene type were scattered trough the pezizomycotina (Table 3).

Amino acid conservation around the catalytically essential His and Tyr residues was investigated by partial alignment of the six FAHD gene sub-groups (Table 4 and Table 5, overleaf). In all gene sub-groups the proximal and distal His and Tyr residues were conserved. Some amino acids were only conserved in five of the FAHD gene sub-groups (*e.g.* Glu (E) in FADH-6 at position minus 5 from the conserved Tyr residue (Table 5)), others were only conserved in one FAHD sub-group (*e.g.* Asp (N) in FAHD-4 at position plus 5 from the proximal His (table 5)). Blasting the amino acid consensus around the distal

[continued on p. 100]

FAHD

1	F/L	X	X	W/I	A	S/T	L/I	I/V	I	H*	D	X	F	X	T
	F(13), L(11)	F(13), W(9), Y(2)	Y(11), D(7), T(3), V(1), A(1), G(1)	W(22), I(2)	A(24)	S(18), T(6)	L(13), I(11)	I(22), V(2)	I(24)	H(24)	D(14)	I(11), I(2), C(2), V(1)	F(23)	Q(14), W(7), R(2)	T(24)
2	L	F	Y	L/I	A	S/A	X	I	I	H*	D	X	F	X	T
	L(13)	F(13)	Y(13)	L(8), I(5)	A(13)	S(10), A(3)	I(9), L(3), V(1)	I(13)	I(13)	H(13)	D(13)	L(9), I(3), C(2)	F(13)	R(6), Q(7), E(1)	T(13)
3	L	F	Y	L	A	T	I	I	X	H*	D	X	F	X	T
	L(4), M(1)	F(5)	Y(5)	L(4), F(1)	A(5)	T(5)	I(4), L(1)	I(5)	T(2), I(3)	H(5)	D(5)	I(2), L(2), C(1)	F(5)	Q(3), L(2)	T(5)
4	L	F	X	F/L	A	T	X	I	I	H*	D	X	F	X	T
	L(7), A(1)	F(7), I(1)	H(4), Y(3), S(1)	F(5), L(3)	A(8)	T(7), I(1)	I(4), V(2), L(1)	I(8)	I(8)	H(8)	D(7), G(1)	I(3), L(3), D(1), V(1)	F(7), M(1)	R(5), H(2), Q(1)	T(7), F(1)
5	L	F/I	Y	H	A	X	I	I	I	H*	D	I	F	R	T
	L(15)	F(9), I(5)	Y(15)	H(15)	A(15)	T(9), A(4), S(2)	I(15)	I(15)	I(15)	H(15)	D(15)	I(13), V(1), L(1)	F(15)	R(14), C(1)	T(13), S(2)
6	X	F	X	F	A	X	X	X	X	H*	X	X	F	X	T
	F(6), M(4), L(3), T(1)	F(14)	A(8), S(4), G(1), N(1)	F(14)	A(14)	T(5), A(3), D(3), N(2), S(1)	L(7), I(3), C(2), T(1), H(1)	V(9), I(3), A(1), R(1)	I(9), T(3), H(2)	H(13), S(1)	S(7), R(2), T(2), E(2), D(1)	C(4), V(4), I(4), S(1), L(1)	F(13), G(1)	R(5), N(4), Q(2), F(1), S(1), K(1)	T(13), P(1)
C[a]	**L**	**F**	**Y**	**X**	**A**	**X**	**X**	**I**	**I**	**H***	**D**	**X**	**F**	**X**	**T**

[Table 4] Analysis of amino acid conservation around the distal His

Dark blue indicates amino acids that are conserved in only one FAHD gene sub-group, light blue indicates amino acids that are conserved in (most) FAHD gene sub-groups.
[a]consensus *distal His

FAHD

1	X	E	F	N	L	X	Y	R	W	H*	S	X	X	S/G	X
	V(16), A(10), F(1)	E(27)	F(27)	N(27)	L(27)	A(17), C(8), V(2)	Y(27)	R(27)	W(27)	H(27)	S(27)	C(14), A(8), T(5)	I(23), L(3), T(1)	S(21), G(6)	A(10), Q(6), E(6), R(4), N(1)
2	A	E	F	N	L	V	Y	R	W	H*	S	X	X	S	X
	A(15)	E(15)	F(15)	N(15)	I(14), V(1)	V(14), I(1)	Y(15)	R(15)	W(15)	H(15)	S(13), A(2)	C(12), T(2), A(1)	I(8), V(4), L(3)	S(15)	Q(6), D(5), E(2), K(1)
3	X	E	F	N	L	X	Y	R	W	H*	X	X	X	S	X
	V(3), A(2)	E(5)	F(5)	N(5)	L(5)	I(4), L(1)	Y(5)	R(5)	W(5)	H(5)	S(3), C(2)	A(2), G(2), T(1)	I(3), M(1), V(1)	S(5)	Q(4), E(1)
4	X	E	F	N	X	I	Y	R	W	H*	A/P	A/T	X	S	N
	A(7), V(1), M(1)	E(9)	F(9)	N(9)	M(5), V(3), L(1)	I(9)	Y(9)	R(9)	W(7), F(2)	H(9)	A(6), P(3)	A(7), T(2)	I(5), V(3), T(1)	S(8), N(1)	N(9)
5	X	E	F	N	L	L	Y	R	F	H*	S/C	X	I	S	X
	V(12), A(2), T(1), S(1)	E(16)	F(16)	N(16)	L(16)	L(15), M(1)	Y(15)	R(15)	F(15)	H(15)	S(10), C(6)	A(6), C(5), V(3), I(2)	I(15), T(1)	S(15)	R(6), K(4), Q(2), L(2), M(2)
6	X	X	F	X	X	L	Y	R	W	H*	A	X	X	X	X
	V(6), A(3), I(2), S(1), T(1)	E(12), Q(1), G(1)	F(14)	N(9), A(3), D(1), S(1)	L(6), C(4), V(3), I(1)	L(12), M(2)	Y(14)	R(11), H(3)	W(14)	H(14)	A(12), T(3)	T(7), A(4), C(2), V(1)	L(6), T(5), V(2), I(1)	S(11), G(2), A(1)	A(5), E(3), R(2), L(1), V(1), Q(1)
C[a]	**X**	**E**	**F**	**N**	**L**	**X**	**Y**	**R**	**W**	**H***	**X**	**X**	**X**	**S**	**X**

[Table 5] Analysis of amino acid conservation around the proximal His and Tyr residues

Dark blue indicates amino acids that are conserved in only one FAHD gene sub-group, light blue indicates amino acids that are conserved in (most) FAHD gene sub-groups.
[a]consensus *proximal His

[continued from p. 97]

and proximal His (LFYXAXXIIHDXFXT, Table 4 and EFNLXYRWHXXXS, Table 5, respectively) against the NCBI genomic database showed that these consensus sequences are unique to fungal FAHDs.

Regrettably, complete studies connecting fungal FAHD genes, putative proteins, oxylipins and functions are currently not available. Therefore, it remains unclear if proteins that are part of the same gene sub-group also form similar oxylipins or have similar functions. Experimental studies on the capacity of different fungi to oxygenate polyunsaturated fatty acids will show how the different gene sub-groups are connected.

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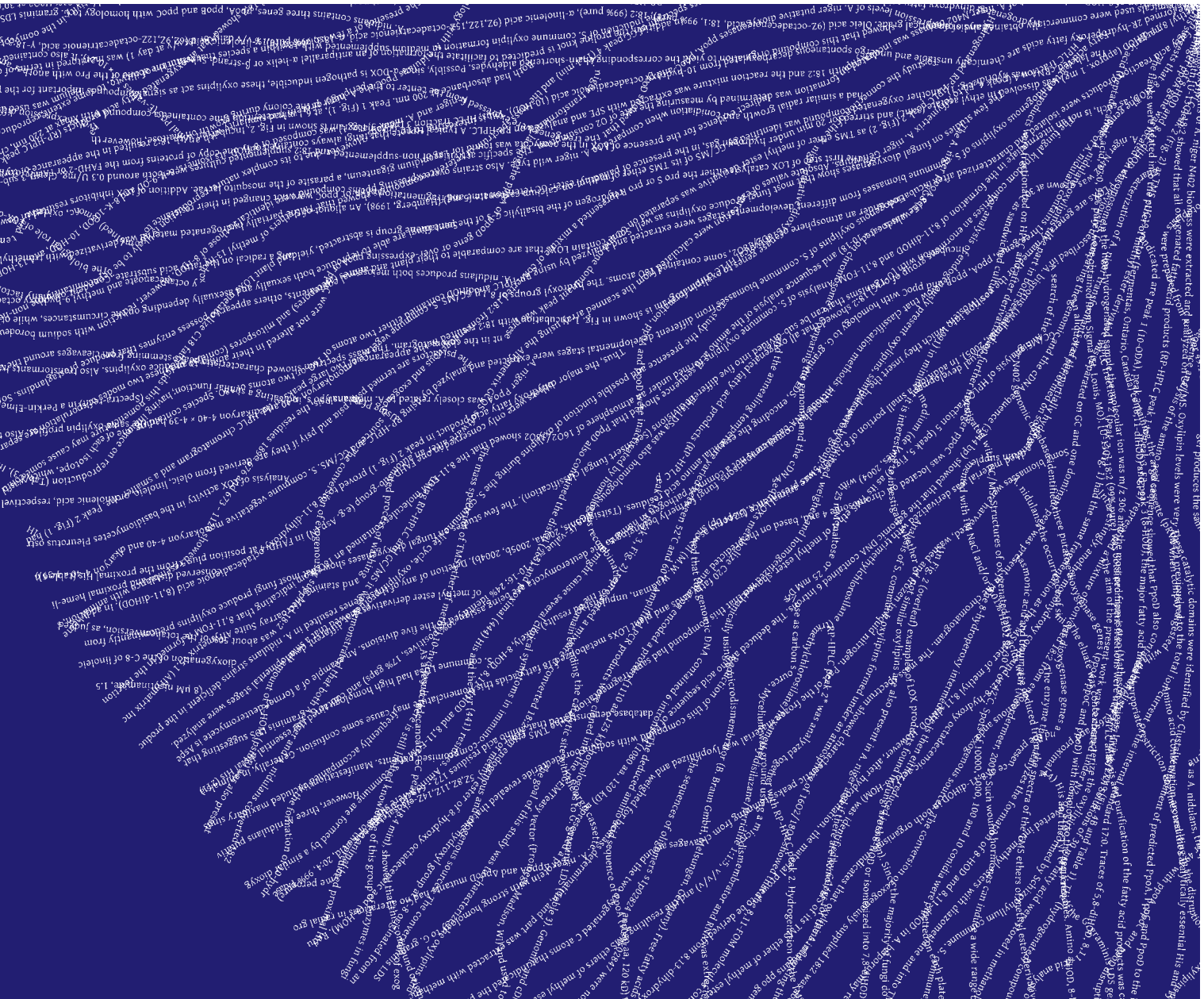


Comparison of oxylipin production and putative dioxygenase genes in *Aspergillus niger* and *Aspergillus nidulans*

Abstract

Exogenous and endogenous factors influence fungal reproduction and the oxylipins produced by *Aspergillus nidulans* are examples of such factors (Tsitsigiannis, 2005). Three *A. nidulans* putative dioxygenase genes, *ppoA*, *ppoB* and *ppoC* have been related to oxylipin formation. Transformants lacking the *ppoA*, *ppoB* or *ppoC* gene were deficient in their oxylipin production and had a modified balance between sexual and asexual sporulation (Tsitsigiannis, 2004a, 2005, 2004b). *A. niger* is closely related to *A. nidulans*, but it only produces asexual spores.

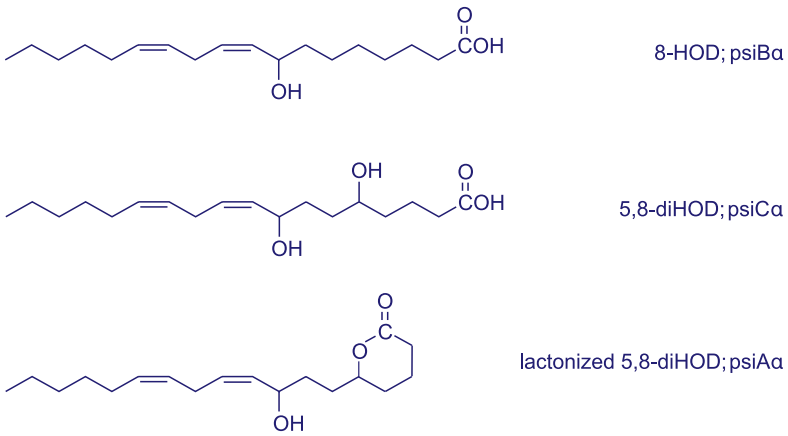
This study showed that both *A. niger* and *A. nidulans* formed 8,11-dihydroxy octadecadienoic acid (8,11-diHOD), 5,8-diHOD, lactonized 5,8-diHOD, 8-HOD, 10-hydroxy octadecadienoic acid (10-HOD), small amounts of 8-hydroxy octadecenoic acid (8-HOM; psiBβ), 9-hydroxy octadecadienoic acid (9-HOD) and 13-hydroxy octadecadienoic acid (13-HOD). Analysis of the *A. niger* genome revealed that this fungus contains three putative dioxygenase genes, *ppoA*, *ppoC* and *ppoD* with homology to the *ppo* genes from *A. nidulans*. *A. niger* transformants lacking the *ppoA* or *ppoD* gene or over-expressing *ppoA* or *ppoC* however, were unaltered in their production oxylipins and in their sporulation. *A. niger* and *A. nidulans* produce the same oxylipins and also have similar dioxygenase genes. It is likely that oxylipins not only influence reproduction but have a more general role in the fungal life cycle.

Introduction

The fungal kingdom comprises a large group of organisms (estimated to consist of over 1.5 million species) with only 5% identified thus far. They can grow as single cells (the yeast form) or as long filamentous structures composed of many cells (the hyphal form). Fungal species can survive in virtually all biotopes on earth, as they have been identified in water, in soil, on plants and on animals.

Part of their success comes from the ability to use different reproductive strategies, which provide increased flexibility for diverse environmental requirements. Fungal species can make sexual cells (meiospores) and/or asexual cells (mitospores) produced in distinct reproductive structures. Sexual reproduction results from fusion of two nuclei followed by meiosis. The genomes of the meiospores produced during this process are different from the parental genome due to genetic recombination. Recombination allows the repair of naturally occurring mutations and results in new genotypes and phenotypes that allow for natural selection (Taylor, 1999). On the other hand, asexual reproduction provides the ability to disperse numerous genetically identical mitospores, without the metabolic costs of sexual reproduction (Taylor, 1999). Some fungi are able to reproduce both sexually and asexually depending on the circumstances, while others display only one mode of reproduction. However, this summary necessarily simplifies the possible fungal reproductive strategies.

Several exogenous and endogenous factors that influence fungal reproduction have been identified. Oxylipins produced by *Aspergillus nidulans* are an example of such factors (Tsitsigiannis, 2005). *A. nidulans* produces both meiospores (ascospores) and mitospores (conidia) and these two modes of sporulation are separated in time with asexual preceding sexual development.



[Figure 1] *A. nidulans* psi factors are hydroxylated fatty acids

A. nidulans oxylipins, collectively named psi factors, influence the timing and balance of sexual and asexual development. Analysis of the psi factors showed that they are a mixture of hydroxylated fatty acids (Fig. 1) (Mazur, 1990). The psi factors are termed psiβ, psiα and psiγ if they are derived from oleic, linoleic, or linolenic acid, respectively. The position and number of hydroxylations further identifies the psi compounds as psiA (lactonized 5,8-dihydroxy fatty acid derivatives), psiB (8-hydroxy fatty acid derivatives) and psiC (5,8-dihydroxy fatty acid derivatives). Feeding studies with purified psi factors from *A. nidulans* showed that 8-hydroxy octadecadienoic acid (8-HOD; psiBα) and 5,8-dihydroxy octadecadienoic acid (5,8-diHOD; psiCα) stimulated ascospore and inhibited conidial development, whereas lactonized 5,8-diHOD (psiAα) had the opposite effect (Champe, 1989, 1987).

The proteins that synthesize these oxylipins have not yet been identified. However, three *A. nidulans* putative dioxygenase genes with homology to mammalian prostaglandin synthase (PGS), *ppoA*, *ppoB* and *ppoC* have been related to their formation. Deletion of *A. nidulans ppoA* reduced the level of 8-HOD and increased the ratio of asexual to sexual spore development (Tsitsigiannis, 2004b). Elimination of *ppoC* from the genome led to the reduced formation of 8-hydroxy octadecenoic acid (8-HOM; psiBβ) and significantly reduced the ratio of asexual to sexual spores (Tsitsigiannis, 2004a). The *ppoB* deleted mutants were also reduced in the formation of 8-HOM however, unlike the *ppoC* deleted mutants

they had an increased ratio of asexual to sexual spores (Tsitsigiannis, 2005). Interestingly, a connection of the three *ppo* genes to the formation of 5,8-dihydroxy and lactonized 5,8-dihydroxy fatty acid derivatives was not found.

The genus *Aspergillus* comprises a diverse group of species with members that reproduce both sexually as well as asexually and members that only use one type of reproduction. *Aspergillus niger* is closely related to *A. nidulans*, but it only reproduces through asexual spores. The goal of this study was to characterize the oxylipins and putative dioxygenase genes in *A. niger* and to compare them to their equivalents in *A. nidulans*. Since *A. nidulans* oxylipins take part in both sexual and asexual development it is interesting to investigate whether or not similar oxylipins are also present in *A. niger* and if there are homologs for the *A. nidulans ppo* genes in the *A. niger* genome. Their presence could point towards alternative functions for *Aspergillus* oxylipins or indicate a latent potential for sexual reproduction in *A. niger*.

Materials and methods

Materials

All chemicals used were commercially obtained and of analytical grade. Linoleic acid (9Z,12Z-octadecadienoic acid, 18:2, 99% pure), arachidonic acid, 5Z,8Z,11Z,14Z-eicosatetraenoic acid, 20:4, 99% pure) and margaric acid (heptadecanoic acid, 17:0, 99% pure) were obtained from Sigma (St. Louis, MO). [U-^{13}C] 18:2 (99% pure) was obtained from Isotec (Matheson Trigas, Irving, TX). Solutions of 30 mM fatty acid were stored in methanol under N_2 at -20˚C until use.

Strains, media and culture conditions

Aspergillus strains used are listed in Table 1 (overleaf). Cultures were grown in minimal medium containing trace elements and 1% glucose as carbon source, unless otherwise indicated in the text (de Vries, 2004). Appropriate supplements (8 µM nicotinamide, 1.5 mM leucine, 5 mM uridine) were added to the media to complement auxotrophic mutations. *Aspergillus* cultures were inoculated with 10^6 spores/ml and grown at 30˚C on a rotary shaker (Inova 2300; New Brunswick Scientific, Edison, NJ) at 250 rpm. For growth on solid media 1.5% of agar was added.

Strain	Genotype
A. niger N402	*cspA*1
A. niger 49.1	*nicA*1, *leuA*1, *pyrA*6, *ΔargB*:: *A. niger argB*
A. niger ΔppoA	*nicA*1, *leuA*1, *pyrA*6, *ΔargB*:: *ppoA* disruption construct
A. niger ΔppoD	*nicA*1, *leuA*1, *pyrA*6, *ΔargB*:: *ppoD* disruption construct
A. niger over-expressing *ppoA*	*nicA*1, *leuA*1, *pyrA*6:: pGW635, *ppoA* functional construct
A. niger over-expressing *ppoC*	*nicA*1, *leuA*1, *pyrA*6:: pGW635, *ppoC* functional construct
A. nidulans WG096	*pabaA*1, *yA*2

[Table 1] *Aspergillus* strains used in this study

Oxylipin characterization and analysis of enzymatic capacity

Strains were grown in 25 ml of liquid medium in Petri dishes under stationary conditions at 30˚C. Alternatively, strains were grown in 50 ml of liquid medium at 30˚C in a rotary shaker at 250 rpm. Mycelial mats were collected after 72 h, dried between paper and frozen in liquid nitrogen.

For analysis of endogenously present oxylipins, samples were lyophilized, weighed and homogenized mechanically using a microdismembrator (B. Braun GmbH, Melsungen, Germany). Free fatty acids and their derivatives were extracted with 80% methanol 1:10 (w/v), centrifuged at 4˚C, 2500 × g for 20 min and recovered by solid phase extraction (SPE, Oasis HLB 200 mg; Waters, Milford, MA) 17:0 was used as an internal standard.

The enzymatic capacity to oxygenate fatty acids of *Aspergillus* strains was examined as follows. Samples were homogenized, extracted with phosphate buffer (50 mM sodium phosphate pH 6.5, 5:1 w/v) and centrifuged at 4˚C, 2500 × g for 20 min. The supernatant (crude extract) was filtered through cheesecloth and used immediately. Typically, 4 mL phosphate buffer was mixed with 1 mL crude extract, rigorously stirred and incubated with 120 µM substrate for 30-45 min at room temperature under a continuous flow of O_2. Fatty acids and reaction products were recovered directly by SPE.

SPE eluates were concentrated under N_2, and analyzed by RP-HPLC.

Analysis by GC/MS of the fatty acid products as TMS ethers of methyl ester derivatives was performed as described previously (van Zadelhoff, 1998). The fatty acid methylation reagent was diazomethane. For GC/MS analysis, samples were analyzed before and after hydrogenation. Oxylipins were identified by mass spectrum on the basis of their fragmentation patterns.

Nucleic acid manipulations

The amino acid sequence of *Gaeumannomyces graminis* LDS (Hornsten, 1999) was used to perform a BLASTp search of the *A. niger* N402 (Bos, 1988) genomic database (DSM food specialties, Delft, The Netherlands). Three putative dioxygenase genes (*ppoA*, *ppoC* and *ppoD*) were identified that predicted proteins with high similarity to LDS. These genes were aligned to the *ppo* genes from *A. nidulans* and to the LDS from *G. graminis* and a phylogenetic tree was created using the ClustalW program (www.ebi.ac.uk/clustalw). Pairwise scores between amino acids were represented as the number of identities and positives.

Primers to amplify fragments for functional and disruption constructs were based upon the *A. niger* N402 genome sequence. These primers introduced restriction sites at either site of the amplified fragment during a PCR reaction (Table 2, overleaf). *A. niger* genomic DNA was isolated using previously described techniques and used as the PCR template (de Graaff, 1988). PCRs were carried out with AccuTaq LA™ DNA polymerase according to the manufacturer's protocol (Sigma) and the annealing temperature varied between 52˚C and 60˚C. Amplified PCR products were cloned into the pGEMTeasy vector (Promega, Madison, WI) and used to transform competent *Escherichia coli* DH5α. Positive clones containing the fragments for functional or disruption constructs were analyzed by restriction mapping and sequence comparisons to the NCBI genetic database using the tBLASTn algorithm (www.ncbi.nlm.nih.gov).

Creation of disruption constructs

Primers for fragments for disruption constructs were designed at the 5' and 3' flanking regions of predicted catalytic domains of PpoA, PpoC and PpoD. These catalytic domains were identified by ClustalW alignment of predicted PpoA, PpoC and PpoD to the LDS from *G. graminis* of which the catalytic domain has been identified (Hornsten, 1999).

Disruption constructs for *ppoA*, *ppoC* and *ppoD*, including the *argB* marker gene, were created as follows (Lenouvel, 2002). First the 5' and 3' flanking regions were amplified by PCR introducing the indicated restriction sites

	Sequence 5'→ 3'
Functional constructs	
ppoA-dw	GAGGTGGGTCTTGTTTG
ppoA-up	GACAAACAGGGAGTTGC
ppoC-dw	CCCTATTCTTCCGACGG
ppoC-up	GACAGCAGTCTAGAGGC
Disruption constructs	
*ppoA3'-Nsi*I-dw	ATGCATGGTGGCAAACCAAGCC
*ppoA3'-Kpn*I-up	GGTACCGGTGAGGAGCACTACTTG
*ppoA5'-Hind*III-dw	AAGCTTATTTGTAGAGTCGAGG
*ppoA5'-Sph*I-up	GCATGCCATGCTTACCGTGAATG
*ppoD5'-Kpn*I-dw	GGTACCTTCCAGCTGGCATTGGTG
*ppoD5'-BamH*I-up	GGATCCGTGCAGGGCCTTGAGCC
*ppoD3'-Sph*I-dw	GCATGCTGAAGCGCAACGTCTAAC
*ppoD3'-Hind*III-up	AAGCTTCAGCCCGTAGTTCTG

[Table 2] primers used in this study

(Table 2). The amplified products were digested from pGEMTeasy, separated on 0.8% agarose gel and isolated. The flanks were ligated into the pUC19 vector (Fermentas, Ontario, Canada) containing the *argB* cassette (pRV542) previously digested with the appropriate restriction enzymes resulting in the disruption constructs for *ppoA*, *ppoC* and *ppoD*. Disruption constructs were linearized by digestion with *Kpn*I/*Hind*III and used for *A. niger* transformations.

Creation of functional constructs

Primers for functional fragments were designed approximately 80 bp outside of the coding region. Functional fragments of *ppoA*, *ppoC* and *ppoD* in pGEM-Teasy were used directly for *A. niger* transformations.

A. niger transformations

Protoplasts were prepared from *A. niger* 49.1 as described and transformed using polyethylene glycol (Kusters-van Someren, 1991). Transformation of *A. niger* 49.1 with *ppoA*, *ppoC* and *ppoD* disruption constructs created transformants to ArginineB prototrophy with the catalytic domain of the corresponding gene product deleted. Co-transformation of *A. niger* 49.1 with *ppoA*, *ppoC* and *ppoD* functional constructs and pGW635 (containing *A. niger pyrA*) created transformants having multiple gene copies. Transformants were purified by repeated streaking of conidia. Gene replacement and ectopic integration of the *argB* marker gene and the presence of multiple gene copies were checked by PCR and Southern analysis using internal fragments as probes.

Probe construction and Southern analysis

Functional constructs of *ppoA*, *ppoC* and *ppoD* were digested with *Eco*RV, *Pst*I and *Sph*I, respectively, yielding internal probes for the catalytic domain encoding region. Fragments were separated on an 0.8% agarose gel, isolated and randomly labeled with [α-^{32}P]dCTP. This resulted in 1082 and 1146 bp fragments for *ppoA*, 905 and 749 bp fragments for *ppoC* and a 1241 bp fragment for *ppoD*.

Chromosomal DNA of *A. niger* transformants was digested with the appropriate restriction enzymes. Hybridization with radioactive probes was done as described, except that washing of the filters was done at 65˚C (de Vries, 2002). Positive transformants were selected and used for further characterization.

Phenotypic characterization of *A. niger* transformants

Characterization of *A. niger* transformants was performed on solid medium supplemented with 1M NaCl and/or 0.01% H_2O_2 at 30˚C or 42˚C. Spots of 10000, 1000, 100 and 10 conidia were pipetted on each plate and incubated. Strains *A. niger* 49.1 and *A. niger* N402 were used as wild type.

A. niger microarray analysis

A. niger N402 was grown at 30˚C as sandwiched cultures (Wösten, 1991) in minimal medium (de Vries, 2004) with 25 mM maltose or 25 mM D-xylose as carbon source. Mycelium was ground using a microdismembrator and RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the instructions of the manufacturer. RNA was purified using Nucleospin RNA clean up (Macherey-Nagel GmbH, Düren, Germany). Concentration of RNA was measured at λ_{260}. Quality of the RNA was analyzed on an Agilent 2100 BioAnalyzer

using the RNA6000 labchip kit (Agilent Technologies, Palo Alto, CA). For microarray analysis, biotin-labeled antisense cRNA was generated by labeling 20 or 2 µg of total RNA with the BioArray High Yield RNA transcription labeling kit (ENZO) or the Affymetrix Eukaryotic One-Cycle Target Labeling and Control Reagent package, respectively. The quality of the cRNA was checked using the Agilent 2100 bioanalyzer. The labeled cRNA was hybridized to Affymetrix *A. niger* Genechips. Absolute values of expression were calculated from the scanned array using the Affymetrix GCOS software after an automated process of washing and staining. Microarray Suite Affymetrix v5.1 (Affymatrix Inc., Santa Clara, CA), Spotfire DecisionSite (Spotfire Inc, Somerville, MA), GeneData Expressionist Analyst V Pro 2.0.18 (GeneData, Basel, Switzerland) and the R statistical environment (www.r-cran.org) were used for data analyses.

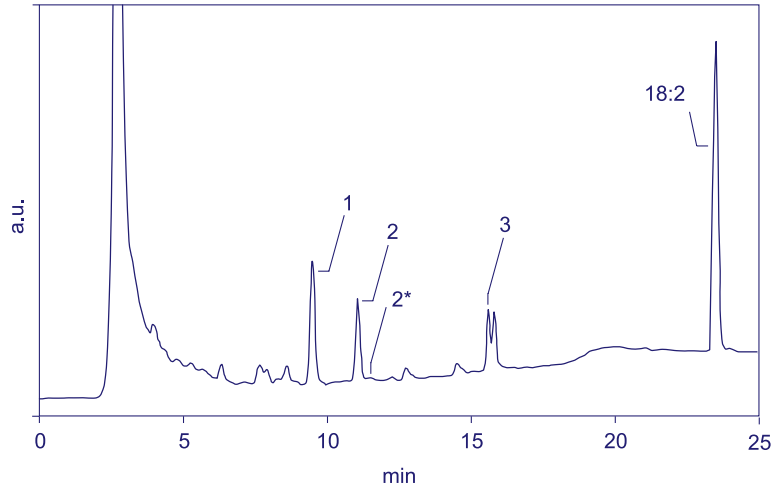
Results

RP-HPLC and GC/MS analysis

A crude extract of *A. niger* N402 biomass was incubated with 18:2 and the reaction mixture was extracted with SPE and analyzed on RP-HPLC. A typical HPLC chromatogram is shown in Fig. 2. Incubation with 18:2 resulted in the appearance of three large peaks in the HPLC chromatogram and a smaller one. Reference compounds of dihydroxy fatty acids had a retention time of 9-11 min, whereas monohydroxy fatty acid references eluted between 15-18 min. Similar results were obtained for *A. niger* 49.1, *A. niger ΔppoA*, *A. niger ΔppoD*, *A. niger* over-expressing *ppoA*, *A. niger* over-expressing *ppoC* and *A. nidulans* WG096 (data not shown).

Fatty acid reaction products were fractionated on HPLC and after derivatization further investigated with GC/MS. Structures of oxygenated fatty acids were deduced from the spectra of the TMS ethers of methyl ester derivatives.

Analysis of dihydroxy fatty acids (RP-HPLC peak 1, peak 2 and peak 2*) Hydrogenated dihydroxy fatty acids as TMS ethers of methyl ester derivatives from RP-HPLC peak 1 (Fig. 2) were separated on GC and one dominant peak was present in the chromatogram. The mass spectrum was similar to the mass spectrum of the TMS ether of methyl 8,11-dihydroxy octadecanoate (Wadman, 2005). The GC retention time and mass spectrum of the non-hydrogenated sample and the GC retention time and mass spectrum of TMS ether of methyl 8,11-dihydroxy-9,12-octadecadienoate showed that the major fatty acid product in RP-HPLC peak 1 (Fig. 2) was 8,11-dihydroxy octadecadienoic acid (8,11-diHOD) (Wadman, 2005).



[Figure 2] RP-HPLC chromatogram (λ = 200 nm) of the reaction of a crude extract of *A. niger* wild type biomass with 18:2

Indicated are peak 1 (9.2 min; 8,11-diHOD), peak 2 (10,8 min; 5,8-diHOD), peak 2* (10.9 min, λ_{max} 218 nm; lactonized 5,8-diHOD), and peak 3 (15.1 min; 8-HOD), the major fatty acid metabolites. RP-HPLC analysis and purification of the fatty acid products were carried out on a Cosmosil 5C18-AR (5 µm; 250 × 4.6 mm i.d.; Nacalai Tesque, Kyoto, Japan) reversed-phase column using a gradient system (solvent A: methanol/water/acetic acid (50:50:0.01, v/v/v); solvent B: methanol/water/acetic acid (95:5:0.01, v/v/v)) with the following gradient program: 45% solvent A for 1 min, followed by a linear increase of solvent B up to 100% within 10 min and finally an isocratic post-run at 100% solvent B for 10 min. The flow-rate was 1 mL/min.

Hydrogenated RP-HPLC peak 2 (Fig. 2) as TMS ether of methyl ester derivative was separated on GC and one dominant peak was present in the chromatogram. The mass spectrum showed characteristic peaks stemming from cleavages around the two oxygenated C atoms that indicated the presence of TMS ether of methyl 5,8-dihydroxy octadecanoate. Comparison of the mass spectrum from hydrogenated and non-hydrogenated samples showed that the TMS ether of methyl 5,8-dihydroxy octadecanoate was derived from the TMS ether of methyl 5,8-dihydroxy-9,12-octadecadienoate. This was evidenced by the molecular ion at *m/z* 470 and by the characteristic fragments resulting from cleavage around the double bonds and oxygenated C atoms (Calvo, 2001). Thus RP-HPLC peak 2 (Fig. 2) proved to be 5,8-diHOD (psiCα).

RP-HPLC peak 2* was analyzed together with RP-HPLC peak 2. Hydrogenation of the TMS ether derivative showed peaks stemming from cleavage around an oxygenated C-atom. The molecular ion at *m/z* 370 evidenced that

this compound was TMS ether of lactonized 5,8-dihydroxy octadecanoate. Comparing the hydrogenated sample with the non-hydrogenated sample showed that TMS ether of lactonized 5,8-dihydroxy octadecanoate probably originated from lactonized 5,8-diHOD (psiAα).

Analysis of monohydroxy fatty acids (RP-HPLC peak 3) In the GC chromatogram of the hydrogenated monohydroxy fatty acids of RP-HPLC peak 3 (Fig. 2) as TMS ethers of methyl ester derivatives, one prominent peak was present. The mass spectrum identified it as a mixture of the TMS ethers of methyl 8-hydroxy octadecanoate, methyl 10-hydroxy octadecanoate and a small amount of methyl 9-hydroxy octadecanoate. Also, a small peak of methyl 13-hydroxy octadecanoate was present in the GC chromatogram. In the GC/MS analysis of the corresponding non-hydrogenated monohydroxy fatty acids as TMS ethers of methyl ester derivatives, three peaks were visible in the GC chromatogram. Reference compounds indicated that GC peak 1 (18.3 min) was TMS ether of methyl 8-hydroxy octadecadienoate in view of the fragmentation pattern and retention time of the non-hydrogenated sample (Wadman, 2005). The mass spectrum of TMS ether of methyl 10-hydroxy octadecanoate, GC peak 2 (18.4 min), showed that this compound originated from 10-hydroxy octadecadienoic acid (10-HOD). The mass spectrum of GC peak 4 (19.1 min) and the mass spectrum of reference compounds showed that TMS ethers of methyl 13-hydroxy octadecanoate and methyl 9-hydroxy octadecanoate were derived from 13-hydroxy octadecadienoic acid (13-HOD) and 9-hydroxy octadecadienoic acid (9-HOD). Thus, RP-HPLC peak 3 (Fig. 2) was composed of 8-HOD (20), 10-HOD (18), 13-HOD (1) and 9-HOD (1). ((x) are relative amounts estimated from peak intensities on GC/MS)

GC/MS analysis of monohydroxy fatty acids eluting after RP-HPLC peak 3 (Fig. 2) as TMS ethers of methyl ester derivatives showed that a small amount of 8-HOM (psiBβ) was also present in the *Aspergillus* strains (Table 1, data not shown).

Characteristics of oxylipin formation

Incubation with [U-^{13}C] 18:2 showed that all oxygenated fatty acid products (RP-HPLC peak 1 to peak 3, Fig. 2) represented a mixture of converted 18:2 from endogenous and exogenous sources. The conversion of exogenously supplied 18:2 was about 50% of the total conversion, as judged by the ratio of [U-^{13}C] labeled fragments to unlabeled fragments on GC/MS. In a reaction with 20:4 was shown that 20:4 it was not converted by an *A. niger* N402 crude extract.

Endogenous oxylipins

Endogenous oxylipins of *A. niger* N402 biomass were extracted and analyzed on GC/MS. Oxylipin levels were very low when compared to the total ion-current of the internal standard 17:0. Traces of 5,8-diHOD, 8,11-diHOD, 8-HOD, 10-HOD, 13-HOD and 8-HOM were detected, however oxylipin levels were generally just above background. Similar results were obtained for *A. niger* 49.1, *A. niger ΔppoA*, *A. niger ΔppoD*, *A. niger* over-expressing *ppoA*, *A. niger* over-expressing *ppoC* and *A. nidulans* WG096.

Identification of three putative *A. niger* dioxygenase genes, ppoA, ppoC and ppoD

A search of the *A. niger* N402 genomic database identified three putative dioxygenase genes (*ppoA*, *ppoC* and *ppoD*) with homology to the sequence of the *G. graminis* LDS gene. These genes encoded proteins with heme peroxidase and cytochrome P450 domains.

A. niger ppoA The *A. niger ppoA* gene was located on chromosome 6. Comparing the genomic and the cDNA sequence (3244 bp) showed that the genomic DNA contained 6 introns. The deduced amino acid sequence of PpoA (1080 aa, 120 kD) represented a protein with strong homology to *G. graminis* LDS suggesting that *A. niger* PpoA also generates oxylipins (Table 3, overleaf). *A. niger* PpoA was closely related to *A. nidulans* Ppo's, indicating a similar function; having influence on reproduction (Table 3). In analogy with *G. graminis* LDS and *A. nidulans* PpoA, *A. niger* PpoA showed homology to animal PGS (*E*-values > 7×10^{-21}). *A. niger* PpoA also contained the distal (202) and proximal (377) His and the Tyr (374) residues, essential for catalytic activity of PGS. A conserved domain search of *A. niger* PpoA using the Pfam database (www.sanger.ac.uk/Software/Pfam) indicated that residues 128 - 608 encoded a domain comparable to animal heme peroxidases and that residues 935 - 991 were similar to cytochrome P450 monooxygenase. Putative *A. niger* PpoA also contained the proline knot motif that targets proteins to oil bodies in plants (Abell, 1997; Tsitsigiannis, 2004b). Finally PpoA contained 17 protein kinase C phosphorylation sites.

A. niger ppoC *A. niger ppoC* was located on chromosome 4 and, based on the predicted annotation encoded a protein (1110 aa, 125 kD) homologous to *G. graminis* LDS (Table 3). Genomic and cDNA (3333 bp) analysis showed that *ppoC* contained 12 introns. *A. niger* PpoC showed strong homology with *A. nidulans*

Protein	Protein	E- value	Identities %	Positives %	Gaps %
A. niger PpoA	*A. nidulans* PpoA	0	69	81	7
	A. nidulans PpoC	0	37	56	10
	A. nidulans PpoB	1×10^{-68}	43	53	21
	G. graminis LDS	0	45	60	8
A. niger PpoC	*A. nidulans* PpoC	0	60	75	10
	A. nidulans PpoA	0	47	64	10
	A. nidulans PpoB	8×10^{-86}	39	51	20
	G. graminis LDS	3×10^{-174}	41	58	10
A. niger PpoD	*A. nidulans* PpoA	5×10^{-177}	38	55	11
	A. nidulans PpoC	8×10^{-161}	31	46	12
	A. nidulans PpoB	5×10^{-70}	41	52	19
	G. graminis LDS	1×10^{-143}	38	55	2
A. niger PpoA	*A. niger* PpoC		47		
A. niger PpoA	*A. niger* PpoD		38		
A. niger PpoC	*A. niger* PpoD		37		

[Table 3] Comparison of predicted *A. niger* putative dioxygenases PpoA, PpoC and PpoD

PpoC (Table 3). In analogy with *A. nidulans* PpoC the *A. niger* protein had similarity with animal PGS (*E*-values > 3×10^{-24}). It also contained the distal (246) and proximal (424) His and the Tyr (420) residues. Analysis of putative *A. niger*


```
                  ●  ○
GNQVSAEFNLVYRWHACISQRDEKWT 390  PpoA A. niger
GNQVSAEFNVVYRWHACISKRDEKWT 390  PpoA A. nidulans
GNQCSVEFNLAYRWHSAISANDEKWT 436  PpoC A. niger
GNQCSVEFNLAYRWHSAISANDEKWT 443  PpoC A. nidulans
GNQVSAEFNLLYRFHSVISRRDEQWT 455  PpoD A. niger
GNQVSAEFNMIYRWHATTSNEDEKWL 333  PpoB A. nidulans
*** *.**    **:*:   * .* *:*
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[Figure 3] Amino acid alignment of the predicted proximal His domain in *A. niger* PpoA, PpoC and PpoD to *A. nidulans* PpoA, PpoB and PpoC

Identical amino acids are marked with asterisks; similar amino acids are marked with colons. The proximal His and the Tyr residue important for catalysis in PGS are marked with ○ and ● , respectively. Deviating amino acids in *A. niger* PpoD are indicated with boxes.

PpoC with the Pfam database demonstrated that amino acid residues 172 - 648 were similar to animal heme peroxidase and that residues 692 - 1087 encoded a cytochrome P450 domain. PpoC also contained the proline knot motif (Abell, 1997; Tsitsigiannis, 2004b) and had 18 protein kinase C phosphorylation sites.

A. niger ppoD *A. niger ppoD* was located on chromosome 3 and encoded a protein (1164 aa, 131 kD) with near identity to *G. graminis* LDS (Table 3). Comparative sequence analysis of the genomic DNA and cDNA (3495 bp) indicated that *ppoD* contained 11 introns. Comparing the sequence of *A. niger* PpoD with PpoA, PpoB and PpoC from *A. nidulans* showed that *A. niger* PpoD had strongest similarity to *A. nidulans* PpoA and PpoC and not to *A. nidulans* PpoB (Table 3). PpoD shared similarity with animal PGS (*E*-values > 3×10^{-18}) and sequence analysis of the amino acid sequence showed that PpoD also contained the distal (265) and proximal (444) His and the Tyr (441) residues. Amino acid analysis of the predicted proximal His domain revealed that PpoD differed from the other Ppo's having a Phe instead of a Trp between the proximal His and Tyr residues and that a Lys, conserved in the other Ppo's, was replaced by a Gln residue (Fig. 3). Also, in contrast to the other Ppo's, *A. niger* PpoD did not contain the proline knot motif, because the third Pro residue was replaced by an Arg residue (Fig. 4). Using Pfam analysis, the residues 189 - 676 and 673 - 1120 showed resemblance with animal heme peroxidase and cytochrome P450 domains, respectively. Finally *A. niger* PpoD contained 16 protein kinase C phosphorylation sites.

```
AFPNHFKGDSVYAHFPLVIPS  627   PpoA A. niger
AFPNYFKPDSIYAHYPMTIPS  672   PpoC A. niger
ALPGWFPYNSLHATQPMFTRK  696   PpoD A. niger
ATPXXX----XFS--PXXXPA  Proline knot (X = V, I, L, F in plants)
* *          .:   *
```

[Figure 4] Amino acid alignment of the predicted proline knot motif in *A. niger* PpoA, PpoC and PpoD to the proline knot motif in plants (Abell, 1997; Murphy, 2001)

Identical amino acids are marked with asterisks; similar amino acids are marked with colons. The conserved Pro residues are indicated with boxes. The third Pro residue in *A. niger* PpoD is replaced with an Arg residue.

Phenotypic characterization of *A. niger* transformants

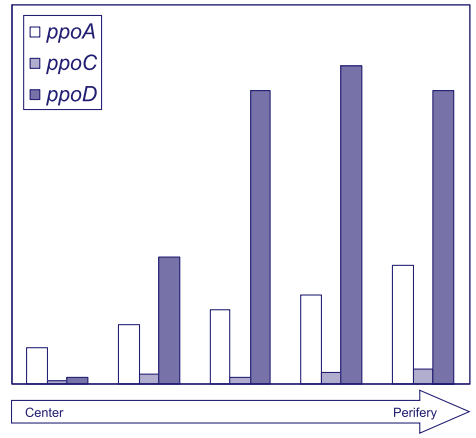
To study the connection of the *A. niger* putative dioxygenase genes to oxylipin formation, *ppoA* and *ppoD* were inactivated by homologous recombination of the domain encoding the catalytic site with the *argB* cassette. *A. niger* Δ*ppoA* and Δ*ppoD* mutants had no alterations in radial growth nor were they altered in conidiation. Also their reactions on (combined) osmotic, oxidative and temperature stress did not differ from the reaction of the wild type.

The effect of over-expressing putative dioxygenase genes in *A. niger* was examined as well. *A. niger* strains over-expressing *ppoA* or *ppoC* had a similar radial growth and conidiation when compared to *A. niger* wild type. Also strains over-expressing *ppoA* or *ppoC* were not changed in their resistance to (combined) osmotic, oxidative and temperature stress. It might be possible that the additional gene copies do not result in elevated expresssion due to a tight regulatory system. This will require further study.

A. niger microarray analysis

Analysis of expression levels of *A. niger* putative dioxygenases *ppoA*, *ppoC* and *ppoD* showed that the tree genes were expressed from the center to the periphery of the colony during growth on maltose, however the level of expression differed (Fig. 5). The genes *ppoA* and *ppoD* were expressed mainly in the periphery, while levels of *ppoC* expression were constant throughout the colony and low in comparison to the expression of *ppoA* and *ppoD*. Similar results were obtained during growth on D-xylose (data not shown).

[Figure 5] Microarray analysis of expression levels of *A. niger* putative dioxygenase genes *ppoA*, *ppoC* and *ppoD* on maltose



Heterologous expression of *A. niger* putative dioxygenase genes

A. niger ppoA, *ppoC* and *ppoD* were cloned into vector pQE30 (Invitrogen) and vector pTT3 (Durocher, 2002) for expression in *E. coli* M15 and HEK293E cells, respectively. Both bacterial and mammalian cells failed to produce recombinant PpoA, PpoC and PpoD (results not shown).

Discussion

In this study the characterization of *A. niger* and *A. nidulans* oxylipins and the identification of three putative dioxygenase genes, *ppoA*, *ppoC* and *ppoD* in *A. niger* is described.

Using RP-HPLC and GC/MS it was demonstrated that both *A. niger* and *A. nidulans* converted 18:2 mainly into 8,11-diHOD, 5,8-diHOD, lactonized 5,8-diHOD, 8-HOD and 10-HOD. Small amounts of 8-HOM, 9-HOD and 13-HOD were also formed. The reaction with [U-^{13}C] 18:2 showed that these compounds were produced from a mixture of exogenously added and endogenously present 18:2.

The formation of 10-HOD, 9-HOD and 13-HOD, but not the formation of 8,11-diHOD, 5,8-diHOD, lactonized 5,8-diHOD and 8-HOD, was previously described for *A. niger* (Hall, 2004). It seems likely that in this previous study, as a result of extensive sample preparation (*i.e.* multiple lyophilization steps, homogenisation, defatting with acetone and diethylether, DNA removal, and ammonium sulfate precipitation), the enzymatic capacity to form the latter oxylipins was partly lost. In contrast, biomasses in the present study were only homogenized.

The presence of 5,8-diHOD, lactonized 5,8-diHOD, 8-HOD and 8-HOM but not the presence of 10-HOD, 9-HOD, 13-HOD and 8,11-diHOD was reported earlier in *A. nidulans* (Champe, 1989; Mazur, 1990, 1991) A later study confirmed the presence of 8-HOD and 5,8-diHOD in *A. nidulans*, but failed to demonstrate the presence of lactonized 5,8-diHOD (Calvo, 2001). Another previous study in *A. nidulans* demonstrated the presence of 8-HOD and 8-HOM as well, but did not show the occurrence of 5,8-diHOD and lactonized 5,8-diHOD (Tsitsigiannis, 2004a, 2005, 2004b). This may be explained by the characterization of only the endogenous oxylipins. In contrast, the current research characterizes both the presence of endogenous oxylipins as well as the enzymatic capacity to form oxylipins. Although the endogenous level of 5,8-diHOD was very low and endogenous lactonized 5,8-diHOD was not detected, *A. nidulans* and *A. niger* had nonetheless the capacity to form it. The presence of 8,11-diHOD has never been demonstrated before in fungi belonging to the phylum ascomycota. The extensive formation of 8,11-diHOD was reported for the first time in the phyllum basidiomycota (Wadman, 2005). Interestingly, the current study shows the presence of 8,11-diHOD in both *A. niger* and *A. nidulans* indicating that the formation of this compound is a more general feature of the fungal kingdom.

Analysis of the *A. niger* genome revealed that this fungus contains three putative dioxygenase genes, *ppoA*, *ppoC* and *ppoD* with homology to the *ppo* genes from *A. nidulans*. The *A. nidulans ppoB* gene was not present in *A. niger*, instead *A. niger* contained a *ppoD* gene. In contrast to the other Ppo's, *A. niger* PpoD did not contain the proline knot motif (Fig. 2). This motif targets plant proteins to oil bodies and it has been demonstrated that fungi target such proteins to oil bodies as well (Ting, 1997). In addition, the proline knot is predicted to facilitate the formation of an antiparallel α-helix or β-strand. Substitution of one of the Pro with another amino acid residue, will likely result in significant changes in the protein structure (Abell, 1997). Therefore, *A. niger* PpoD might differ considerably from the other Ppo's.

A. niger transformants lacking the *ppoA* or *ppoD* gene or over-expressing *ppoA* or *ppoC* were not altered in their ability to produce oxylipins. Also transformants had no alterations in radial growth, conidiation and reaction to stress. It seems likely that *ppoA*, *ppoC* and *ppoD* code for proteins able to produce similar oxylipins. Their expression may vary, depending on temporal, developmental and environmental conditions. Expression analysis of *A. niger ppoA*, *ppoC* and *ppoD* shows that these genes are expressed in different ratios depending on the fungus' developmental stage (Fig. 5).

In contrast to the results reported in this study, *A. nidulans* transformants lacking the *ppoA*, *ppoB* or *ppoC* gene were deficient in oxylipin production and had a modified balance between sexual and asexual sporulation. Deletion of *ppoA*, *ppoB* or *ppoC* reduced the level of 8-HOD, 8-HOM and again 8-HOM, respectively (Tsitsigiannis, 2004a, 2005, 2004b). It should be noted that these studies did not detect 5,8-diHOD, lactonized 5,8-diHOD and 8,11-diHOD in *A. nidulans*. Deletion of *ppoA* or *ppoB* from the *A. nidulans* genome increased the ratio of asexual to sexual spore development (Tsitsigiannis, 2005, 2004b). Elimination of *ppoC* on the other hand, significantly reduced the ratio of asexual to sexual spores (Tsitsigiannis, 2004a).

Since *A. niger* produces the same oxylipins and has similar putative dioxygenase genes as *A. nidulans*, the question arises whether oxylipins possess additional functions, or whether the asexual fungus *A. niger* has a latent potential for sexual reproduction. Possibly, the presence of *ppoD* instead of *ppoB* in *A. niger* is related to the reproductive differences between *A. niger* and *A. nidulans*. However, due to its complex nature, reproduction is likely to be dependent on many factors. Strictly asexual species are considered an evolutionary endpoint, and therefore truly asexual species are thought to be extremely rare (Taylor, 1999). The fungus *Aspergillus fumigatus* for example, has long been considered an asexual organism, yet recent findings suggest that it may have a latent potential for sexual reproduction (Dyer, 2005). The presence of a few isolates within a species that retain the ability to, to some extent, reproduce sexually may be enough to overcome the disadvantages connected to asexuality (Hurst, 1996). Therefore, failure to demonstrated asexuality may be due to the use of inappropriate strains or wrong environmental conditions (Taylor, 1999).

The demonstration of 8-HOD, 5,8-diHOD and lactonized 5,8-diHOD in both *A. niger* and *A. nidulans*, the occurrence of similar dioxygenase genes and the presence of 8,11-diHOD in both organisms, indicates that oxylipins influencing the balance between asexual and sexual reproduction have a more general role in the fungal life cycle.

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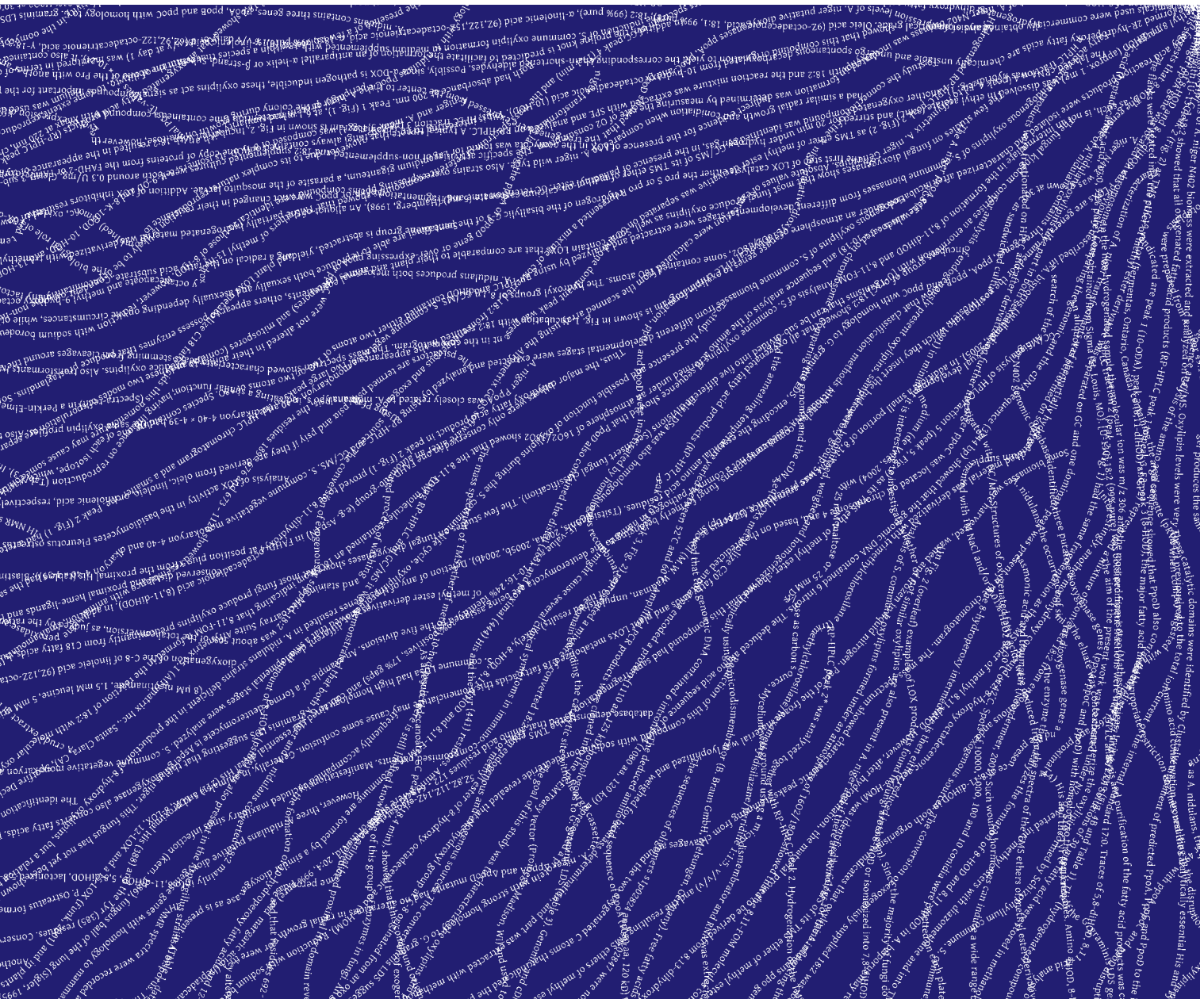

Summary

Polyunsaturated fatty acids play a central role in all biological systems. They are constituents of the plasma membrane and serve as precursors to signaling molecules generated in response to external events. The conversion of polyunsaturated fatty acids into signaling molecules starts by the hydrolysis of fatty acids from phospholipid molecules and the free fatty acids are subsequently oxygenated. The products of this reaction are called oxylipins. Many of the oxylipins in animals, plants and fungi are produced by the dioxygenase-type of oxygenation and this type of oxygenation is the focus of this thesis.

Dioxygenases add molecular oxygen to polyunsaturated fatty acids, in a regio- and stereospecific manner. The oxylipins formed in the three eukaryotic kingdoms (animals, plants and fungi) differ in terms of molecular structure, because these organisms have partly different sets of dioxygenases. Animals use prostaglandin synthases (PGSs) and lipoxygenases (LOXs), which catalyze the formation of oxylipins, while plant oxylipins are formed by LOXs and α-dioxygenases (α-DOXs). Plants do not contain PGSs, but α-DOX and PGS are homologous proteins in terms of amino acid sequence.

The functions of oxylipins include regulation of inflammation and blood pressure in animals, and regulation of defense and growth in plants. Animal and plant dioxygenases have been extensively studied. The dioxygenases of the third eukaryotic kingdom, the fungi, have received comparatively little attention.

The few studies on fungal dioxygenases show that most fungi produce oxylipins. Some fungi contain LOXs that are comparable to their plant and animal equivalents, while others appear to produce prostaglandins. Some fungi contain linoleate diol synthase (LDS), a dioxygenase that is not found in animals or plants, but has amino acid homology with PGS and α-DOX. A few of the fungal dioxygenases have been described in detail, but most studies have either just shown the presence of a catalytic activity or the presence of oxylipins. Many fungal oxylipins differ from their animal and plant counterparts with respect to their molecular structures. Their formation is likely catalyzed either by similar enzymes with specificities different from their plant and animal homologs, or by a novel group of dioxygenases. Again, despite such remarkable differences, surprisingly little research is available on fungal dioxygenation. In depth analysis of dioxygenation pathways of polyunsaturated fatty acids in fungi will lead to a better understanding of the complex fungal life cycle, and will add to the knowledge on their animal and plant equivalents.

In **Chapter 2** the characterization is described of two previously unknown oxylipins in the mushroom *Agaricus bisporus*. They were identified as 8-hydroxy octadecadienoic acid (8-HOD) and 8,11-dihydroxy octadecadienoic acid (8,11-diHOD). It is proposed that the formation of the 8,11-diHOD occurs through either an 8,11-endoperoxy, an 8-peroxo free radical or an 8-hydroperoxy intermediate. These oxylipins are potential products of an LDS with unknown specificity or they could be produced by a new sort of dioxygenase.

To relate fungal oxylipins to a possible function in growth and development, in **Chapter 3** their occurrence was studied in different developmental stages of *Schizophyllum commune*, a model fungus closely related to *A. bisporus*. The main oxylipins of *S. commune* were characterized as 8-HOD, 8,11-diHOD and 8,11-furan-octadecenoic acid (8,11-FOM). 8,11-FOM, is a novel compound formed through the reaction of linoleic acid with molecular oxygen. Oxylipin analysis of *S. commune* vegetative mycelium and fruiting bodies showed that their oxylipin profiles are similar. Potentially, oxylipins play a constant role during the *S. commune* life cycle and it is likely that, in analogy with plants and animals, oxylipins serve as signaling molecules in fungi.

In **Chapter 4** the cloning of a putative dioxygenase gene from *S. commune* is described. The encoded protein contains the essential amino acids (*i.e.* distal and proximal His and Tyr residues) necessary for LDS and PGS catalysis, and it belongs to the fatty acid heme dioxygenase (FAHD) family. The online available fungal genomes were searched for putative FAHDs and a phylogenetic tree was constructed, which showed that the fungal FAHD family has diverged into six different gene sub-groups. Since complete studies connecting fungal FAHD genes, proteins, oxylipins and functions are currently not available, it remains unclear if proteins belonging to the same FAHD gene sub-group also form similar oxylipins or have similar functions.

Analysis of the *A. niger* genome revealed that this fungus contains three putative dioxygenase genes, *ppoA*, *ppoC* and *ppoD* and the result of their disruption an over-expression is described in **Chapter 5**. In *A. nidulans* putative dioxygenase genes are related to oxylipin formation and to the balance between sexual and asexual sporulation. In contrast, *A. niger* transformants lacking the *ppoA* or *ppoD* gene or over-expressing *ppoA* or *ppoC*, are not altered in their ability to produce oxylipins or sporulation. *A. niger* is closely related to *A. nidulans*, but it reproduces through asexual spores only. Since *A. niger* and *A. nidulans* produce the same oxylipins and also have similar dioxygenase genes, it is likely that oxylipins not only influence the balance between asexual and sexual reproduction, but that they play a more general role in the fungal life cycle.

Samenvatting

Meervoudig onverzadigde vetzuren spelen een centrale rol in alle eukaryoten (dieren, planten en schimmels). Ze zijn onderdeel van het plasmamembraan en fungeren daarnaast als uitgangsstof voor verschillende signaalmoleculen die onder andere geproduceerd worden als reactie op gebeurtenissen van buiten af. De omzetting van meervoudig onverzadigde vetzuren naar signaalmoleculen begint met de hydrolyse van vetzuren uit (fosfo-)lipiden. Deze vrije vetzuren worden vervolgens door enzymen geoxygeneerd. De producten die zo ontstaan worden oxylipines genoemd. Veel oxylipines uit de drie eukaryotische rijken worden gemaakt door dioxygenases. Oxygenering door dioxygenases is het onderwerp van dit proefschrift.

Het inbouwen van zuurstof in meervoudig onverzadigde vetzuren door dioxygenases gebeurt op een specifieke plaats in het molecuul met een specifieke stereo-configuratie. Doordat eukaryoten deels verschillende sets dioxygenases hebben, verschilt de structuur van de oxylipines die ze maken.
In dieren worden oxylipines gevormd door prostaglandine synthases (PGSs) en lipoxygenases (LOXs), terwijl planten LOXs en α-dioxygenases (α-DOXs) gebruiken. PGS komt in planten niet voor, maar de aminozuursequenties van α-DOXs zijn homoloog aan die van PGSs.

In dieren zijn oxylipines betrokken bij ontstekingsreacties en bij bloeddrukregulatie, in planten beïnvloeden ze de groei en de afweer. Er is veel bekend over dioxygenering in dieren en planten, over de dioxygenases in schimmels is relatief weinig bekend. Het is bekend dat de meeste schimmels oxylipines maken. Sommige schimmels hebben LOX equivalenten die vergelijkbaar zijn met de LOXs in dieren en planten. Andere schimmels maken juist prostaglandines. In schimmels is ook een dioxygenase gevonden die niet bekend is uit dieren en planten, namelijk linoleaat diol synthase (LDS). LDS heeft aminozuurhomologie met PGSs en α-DOXs.

Er zijn relatief weinig gedetailleerde onderzoeken over schimmel dioxygenases beschikbaar. De meeste onderzoeken laten of alleen een activiteit of alleen de aanwezigheid van oxylipines zien. Wel is bekend dat de structuur van veel schimmeloxylipines verschilt van die van hun tegenhangers uit het dieren- en plantenrijk. Het is waarschijnlijk dat deze oxylipines gemaakt worden door enzymen die lijken op de enzymen uit dieren en planten maar ze hebben wel een andere specificiteit. Dit roept eens te meer de vraag op waarom er maar zo weinig bekend is over dioxygenering in schimmels. Een uitgebreidere analyse van de verschillende manieren van dioxygenering in schimmels zal immers leiden tot een beter begrip van hun equivalenten in dieren en in planten; het zal ook de kennis van het leven van schimmels vergroten.

In **hoofdstuk 2** wordt de karakterisering van twee nog onbekende oxylipines in *Agaricus bisporus* (de witte champignon) beschreven. Ze werden geïdentificeerd als 8-hydroxy octadecadieenzuur (8-HOD) en 8,11-dihydroxy octadecadieenzuur (8,11-diHOD). Waarschijnlijk wordt 8,11-diHOD gevormd via een 8,11-endoperoxide, een 8-peroxo vrij radicaal, of een 8-hydroperoxide intermediair. De vorming wordt mogelijk gekatalyseerd door een LDS met een nog onbekende specificiteit of door een geheel nieuwe soort dioxygenase.

Om schimmeloxylipines te relateren aan een mogelijke functie in groei en ontwikkeling, werd hun aanwezigheid in verschillende ontwikkelingsstadia van *Schizophyllum commune* bestudeerd en beschreven in **hoofdstuk 3**. *S. commune* (het waaiertje) wordt door onderzoekers gebruikt als een modelschimmel; het is nauw verwant aan *A. bisporus*. De oxylipines die door *S. commune* worden geproduceerd zijn vooral 8-HOD, 8,11-diHOD en 8,11-furan octadeceenzuur (8,11-FOM). 8,11-FOM wordt gevormd uit de reactie van linolzuur met zuurstof; het is een nog onbekende soort oxylipine. Vegetatief mycelium en vruchtlichamen van *S. commune* bleken hetzelfde oxylipineprofiel te hebben. Het is waarschijnlijk dat oxylipines een rol spelen in alle levensstadia van *S. commune*. Mogelijk worden ze, evenals in dieren en planten, gebruikt als signaalmoleculen.

In **hoofdstuk 4** wordt de klonering van een mogelijk dioxygenase gen uit *S. commune* beschreven. Het gecodeerde eiwit bevat de essentiële aminozuren (*i.c.* de distale en proximale His residuen en het Tyr residu) die nodig zijn voor katalyse van LDS en PGS. Het eiwit dat gecodeerd wordt door dit gen is onderdeel van de vetzuur heem dioxygenase (FAHD) familie. Er werd een fylogenetische boom gemaakt met alle FAHDs, gevonden in de in online databases beschikbare schimmelgenomen. Het blijkt dat de FAHD familie in schimmels uit zes gen-groepen bestaat. Omdat er geen gegevens beschikbaar zijn die FAHD genen, eiwitten, oxylipines en functies met elkaar verbinden, is het voorlopig nog onduidelijk of eiwitten uit dezelfde gen-groep ook dezelfde oxylipines maken, en of deze oxylipines verwante functies hebben.

In het genoom van *Aspergillus niger* werden drie mogelijke dioxygenase genen gevonden, namelijk *ppoA*, *ppoC* en *ppoD*. Het resultaat van hun disruptie en over-expressie staat beschreven in **hoofdstuk 5**. Gelijksoortige genen zijn bekend in *Aspergillus nidulans*; ze zijn in die schimmel betrokken bij oxylipinevorming en bij de balans tussen geslachtelijke en ongeslachtelijke voortplanting. Echter, *A. niger* stammen waarin *ppoA* of *ppoD* waren uitgeschakeld of waarin *ppoA* of *ppoC* tot overexpressie werden gebracht, bleken onveranderd in hun vermogen oxylipines te maken; ook hun voortplanting was ongewijzigd. *A. niger* is nauw verwant aan *A. nidulans* maar kan zich alleen ongeslachtelijk voortplanten. Omdat twee verschillende schimmels als *A. niger* en *A. nidulans* vergelijkbare dioxygenasegenen hebben en omdat ze hetzelfde oxylipineprofiel hebben, is het waarschijnlijk dat oxylipines niet alleen de voortplanting beïnvloeden, maar dat ze, net als prostaglandines in dieren, een meer algemene rol spelen in het leven van schimmels.

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eyefordetail.nl zette de kroon op het werk. Robert, met zelf een doctoraat in de fysica ben je met afstand de meest geschikte grafisch ontwerper die ik me kon wensen. Je ontwerpbureau is terecht een groot succes!

Resume

Mayken Wadman was born on October 11th, 1978 in Waalre, the Netherlands. She graduated from secondary school in 1997 (Van Maerlant Lyceum, Eindhoven). From 1997 to 2002 she studied chemistry at Utrecht University. Her graduation project, on the relation between phospholipid synthesis and schizophrenia, was carried out at the Children's Hospital Oakland Research Institute in Oakland (CA, USA) under the supervision of Dr F.A. Kuypers and Dr J.A.F. op den Kamp. After graduating, she worked as a PhD student at the section of Bioorganic Chemistry of the Bijvoet Center for Biomolecular Research at Utrecht University under the supervision of prof. Dr G.A. Veldink en prof. Dr J.F.G. Vliegenthart. The results of her research on the metabolism of polyunsaturated fatty acids in fungi are described in this thesis. At present she holds a position as a postdoctoral researcher with prof. Dr H.A.B. Wösten in the Department of Microbiology at Utrecht University.

Curriculum Vitae

Mayken Wadman werd geboren op 11 oktober 1978 te Waalre. Na het behalen van het atheneum diploma aan het Van Maerlant Lyceum te Eindhoven in 1997, werd in hetzelfde jaar begonnen met de studie scheikunde aan de Universiteit Utrecht. Het afstudeeronderzoek werd verricht bij het Childrens Hospital Oakland Research Institute te Oakland (CA, USA) onder begeleiding van dr. F.A. Kuypers en dr. J.A.F. op den Kamp. Het onderzoek richtte zich op de relatie tussen fosfolipiden synthese en schizofrenie. Na het behalen van het doctoraalexamen werkte zij van juni 2002 tot september 2006 onder begeleiding van prof. dr. G.A. Veldink en prof. dr. J.F.G. Vliegenthart als assistent in opleiding bij de sectie Bioorganische Chemie van het Bijvoet Centrum voor Biomoleculair Onderzoek van de Universiteit Utrecht. Het onderzoek, naar het metabolisme van meervoudig onverzadigde vetzuren in schimmels, staat beschreven in dit proefschrift. Vanaf november 2006 werkt zij als postdoc in de leerstoelgroep Microbiologie van de Universiteit Utrecht bij prof. dr. H.A.B. Wösten.

Vereenvoudigde samenvatting

In dit proefschrift wordt beschreven hoe een groep eiwitten, de dioxygenases, meervoudig onverzadigde vetzuren kan omzetten naar signaalmoleculen. Dit is onderzocht in schimmels. Wat zijn meervoudig onverzadigde vetzuren? Waarom is de omzetting van deze vetzuren belangrijk en wat voor soort signaalstoffen worden er dan gevormd? Wat zijn dioxygenases en waarom is dit onderzocht in schimmels? De motivering voor dit onderzoek en het antwoord op bovenstaande vragen wordt hieronder op vereenvoudigde wijze samengevat.

Vetzuren zijn overal in de natuur aanwezig. Zij vormen bestanddelen van oliën en vetten en dus van plant en dier. Uiteraard komen ze voor in alle producten waarin oliën en/of vetten verwerkt zijn, zoals salami, kaas, koekjes en melk. Er zijn verzadigde en onverzadigde vetzuren; zoals bekend uit de vele advertenties die consumptie van vooral de tweede soort áánprijzen. 'Onverzadigd' betekent in chemische zin dat het vetzuur niet helemaal gevuld is met waterstofatomen: het vetzuur heeft een dubbele koolstofbinding. Meervoudig onverzadigde vetzuren hebben meerdere van deze dubbele bindingen. Voorbeelden van meervoudig onverzadigde vetzuren zijn linolzuur en arachidonzuur (bekender als omega-6 vetzuren) en linoleenzuur (een omega-3 vetzuur). Meervoudig onverzadigde vetzuren worden in plant en dier onder andere benut als brandstof, maar ook als grondstof voor signaalstoffen die een belangrijke rol vervullen als boodschappers tijdens verschillende levensprocessen. Signaalstoffen gevormd in de reactie van meervoudig onverzadigde vetzuren met zuurstof, heten oxylipines. Voorbeelden van oxylipines zijn de prostaglandines, stoffen die onder andere betrokken zijn bij pijngewaarwording. Andere voorbeelden van oxylipines zijn de geuren van pas gemaaid gras en van vers gesneden komkommer.

Deze geurende oxylipines bieden de plant bescherming tegen ongedierte en zijn een waarschuwingssignaal voor andere planten in de omgeving.

De vorming van oxylipines loopt via eiwitten. Omdat de eiwitten die oxylipines vormen met zuurstof uit de lucht (O_2) werken, worden zij dioxygenases genoemd. Er zijn verschillende dioxygenases die elk hun eigen soort oxylipines maken en die op hun beurt weer allemaal een eigen functie hebben. Omdat oxylipines als signaalstof op veel verschillende processen invloed hebben (bijvoorbeeld als pijn- of waarschuwingssignaal), is kennis over omzetting van meervoudig onverzadigde vetzuren door dioxygenases belangrijk. Er is dan ook veel onderzoek naar dioxygenases gedaan, zowel in dieren als in planten. Naast dieren en planten is er echter nog een grote groep andere organismen: de schimmels. Schimmels komen veel voor: van paddenstoelen in het bos en schimmelende boterhammen in de vuilnisbak tot in infecties zoals kalknagels bij de mens; ze zijn de makers van penicilline geproduceerd in de farmaceutische industrie en zijn te koop als vleesvervangers in de supermarkt. Omdat oxylipines zo belangrijk zijn voor dieren en planten is de verwachting dat zij ook in schimmels een centrale rol hebben. Aangezien er betrekkelijk weinig onderzoek aan de vorming van oxylipines in schimmels is verricht, is het een uitdaging hier een bijdrage aan te leveren.

De belangrijkste uitkomst van ons onderzoek is dat schimmels bijzondere, tot nog toe onbekende soorten oxylipines maken, en dat deze - evenals in dieren en in planten - een centrale rol spelen in het leven van die schimmels. Dit is onderzocht door oxylipines uit verschillende schimmels te analyseren, hun vóórkomen op verschillende leeftijden te bestuderen of hun productie stil te leggen. Wat de rol van schimmeloxylipines precies is en door wat voor soort dioxygenases zij gemaakt worden daar kunnen we voorlopig alleen nog maar naar gissen en moet door nader onderzoek worden opgehelderd.

Polyunsaturated fatty acids play a central role in all biological systems. They are the constituents of oil and fat and therefore of plants and animals. In nature, fatty acids are used as fuel, but also as precursors to signaling compounds. Oxylipins are examples of such signaling compounds. They are formed by enzymes called dioxygenases. Animal and plant dioxygenases have been extensively studied. The dioxygenases of the third eukaryotic kingdom, the fungi, have received comparatively little attention. In depth analysis of dioxygenation pathways in fungi – the subject of this thesis – will lead to a better understanding of the complex fungal life cycle, and will add to the knowledge on their animal and plant equivalents.