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## PpoC from *Aspergillus nidulans* is a Fusion Protein with only one active Heme

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### SYNOPSIS

In *Aspergillus nidulans* psi factor producing oxygenases (Ppos) are required for the production of so-called psi factors (precocious sexual inducer) - compounds that control balance between the sexual and asexual life cycle of the fungus. The genome of *A. nidulans* harbours three different *ppo* genes: *ppoA*, *ppoB* and *ppoC*. For all three enzymes two different heme containing domains are predicted: a fatty acid heme peroxidase/dioxygenase domain for the N-terminal region and a P450 heme thiolate domain for the C-terminal region. While PpoA was shown to use both heme domains for its bifunctional catalytic activity (linoleic acid 8-dioxygenation and 8-hydroperoxide isomerisation), we found that PpoC apparently only harbours a functional heme peroxidase/dioxygenase domain. Consequently, we observed that PpoC catalyzes mainly the dioxygenation of linoleic acid (18:2<sup>Δ9Z,12Z</sup>), yielding 10-hydroperoxy linoleic acid (10-HPODE). No isomerase activity was detected. Additionally, 10-HPODE was converted at lower rates into 10-keto linoleic acid (10-KODE) and 10-hydroxy linoleic acid (10-HODE). In parallel, decomposition of 10-HPODE into 10-oxo decenoic acid (10-ODA) and volatile C<sub>8</sub> alcohols that are inter alia responsible for the characteristic mushroom flavour. Beside these principle differences we also found that PpoA and PpoC can convert 8-HPODE and 10-HPODE to the respective epoxy alcohols: 12,13-Epoxy-8-hydroxy octadecenoic acid and 12,13-epoxy-10-hydroxy octadecenoic acid. By using site directed mutagenesis we could demonstrate that both enzymes share a similar mechanism for the oxidation of 18:2<sup>Δ9Z,12Z</sup>: They both use a conserved tyrosine for catalysis and the directed oxygenation at the C-8 and C-10 is most likely controlled by conserved valine/leucine residues in the dioxygenase domain.

Short Title: PpoC is a Fusion Protein with one active Heme

Key Words: Fatty acid dioxygenase, oxylipin formation, psi factor forming oxygenase.

### INTRODUCTION

In *Aspergillus nidulans* (teleomorph *Emericella nidulans*) oxidized unsaturated fatty acids, so-called oxylipins, derived from endogenous fatty acids, have been described to regulate the developmental life cycle of the fungus [1-7]. In *A. nidulans* these oxylipins are collectively called psi factors (precocious sexual inducer). Psi factors are a mixture of oxidized 18:1<sup>Δ9Z</sup>, 18:2<sup>Δ9Z,12Z</sup> and 18:3<sup>Δ9Z,12Z,15Z</sup>, where x:y<sup>Δz</sup> is a fatty acid containing x carbons and y double bonds in position z counting from the carboxyl group [8] and are termed psiβ, psiα and psiγ, respectively. The number and the position of the hydroxyl groups on the fatty acid backbone leads to further classification of psi factors: psiB (OH at C-8, e.g. (8R)-HODE<sup>2</sup>), psiA (OH at C-5 and C-8, e.g. (5S,8R)-DiHODE) and psiC (OH at C-8 and δ-lactone ring) [1, 2].

The genes encoding enzymes that are involved in the production of psi factors have been first identified by Keller and co-workers and were termed psi factor producing oxygenases (Ppos). The genome of *A. nidulans* harbours three different genes possibly encoding Ppos: *ppoA*, *ppoB* and *ppoC* [9-11]. The same group could show that deletion of these genes had a significant effect (i) on the developmental ratio between asexual conidiospores and sexual ascospores, (ii) on the production of psi factors and (iii) on the production of secondary metabolites, the mycotoxins [9, 11-13]. Ppo enzymes show a significant sequence identity to mammalian prostaglandin endoperoxide H synthase (25-29 % identity) and fungal 7,8-linoleate diol synthase (40-45 % identity) [10]. In contrast to 7,8-linoleate diol synthase a search for conserved domains using the National Centre for Biotechnology Information (NCBI) database predicted for Ppo enzymes the presence of two different heme domains: a heme

peroxidase/dioxygenase domain for the N-terminal region and a P450 heme thiolate for the C-terminal region. Indeed, we could show on the basis of sequence alignments and spectroscopic analysis that PpoA consists of those different heme domains [19]. Furthermore, we demonstrated that both domains are essential for the two different catalytic activities of PpoA: In the N-terminal peroxidase/dioxygenase domain of PpoA the dioxygenase reaction takes place and 18:2<sup>A9Z,12Z</sup> is oxidized to (8*R*)-HPODE. This intermediate product is then isomerized by the P450 heme thiolate domain of PpoA to 5,8-DiHODE as is also shown in Fig.1 [19]. Based on these results PpoA was assigned as CYP6001A1, as first member of a new P450 subfamily.

Recently Garscha *et al.* reported that homogenates of *A. nidulans* mutants that are deficient in PpoC failed to form (10*R*)-HPODE and (10*R*)-HODE [20]. From that Garscha *et al.* concluded that PpoC is a (10*R*)-dioxygenase. Recombinant expression of *A. fumigatus* PpoC in insect cells confirmed this hypothesis [21]. Moreover mutational studies of 7,8-linoleate diol synthase and PpoC implicated a general mechanism for the directed oxygenation of 18:2<sup>A9Z,12Z</sup> at the C-8 and C-10 in fungal dioxygenases. It was proposed that conserved leucine/valine residues in the dioxygenation site of both dioxygenases determine the oxygenation site [21]. In the same study the formation of low amounts of the epoxy alcohol 12,13-epoxy-(10*R*)-hydroxy octadecenoic acid was observed. It was suggested that in analogy to the isomerisation activity of the bifunctional 7,8-linoleate diol synthase that transforms 8-HPODE to (7*S*,8*S*)-DiHODE, PpoC is also a bifunctional enzyme with epoxy alcohol synthase activity that converts 10-HPODE into 12,13-epoxy-(10*R*)-hydroxy octadecenoic acid [21].

In the present study we aimed to compare the biochemical properties of the two Ppo-enzymes PpoA and PpoC from *A. nidulans* sharing a sequence identity of 44 % to elucidate the base for differences in the produced compounds. Therefore, we expressed PpoC in the same expression system as reported for PpoA [19], purified PpoC and analyzed the biochemical properties of this enzyme.

## EXPERIMENTAL

### Materials

Chemicals were obtained from Sigma-Aldrich (Munich, Germany) and Carl Roth & Co. (Karlsruhe, Germany). Agarose was from Biozym Scientific GmbH (Hess. Oldendorf, Germany). All fatty acids were purchased from Sigma-Aldrich (Munich, Germany) or Cayman (Ann Arbor, USA). Acetonitrile was from Fisher Scientific UK Limited (Leicestershire LE11 5RG, UK). Restriction enzymes were provided by MBI Fermentas (St. Leon-Roth, Germany).

### Cloning and Expression of recombinant PpoC in *E. coli*

*A. nidulans ppoC* (GenBank<sup>TM</sup> accession no. AY613780.1) was amplified from fungal cDNA of sexual and vegetative stages using gene specific primers containing *NdeI* and *NotI* recognition sites (forward primer: 5'-CCATATGTTGCGAAGATTTTCTACCTTCAG-, reverse primer: 5'-AGCGGCCGCTCAAGCAGAATCAATCTGCTTCTTAG-) via the Phusion<sup>TM</sup> Hot Start High Fidelity DNA Polymerase System (Finnzymes, Espoo, Finland). PCR amplification was performed under the following conditions: 98 °C for 3 min followed by 30 cycles at 98 °C for 30 sec, at 53 °C for 30 sec and at 72 °C for 3 min and terminated by 5 min at 72 °C. The resulting fragment was cloned into pJET2.1/blunt (Fermentas, St. Leon Rot, Germany), yielding the plasmid pJET-PpoC. For expression in *E. coli ppoC* was cloned into the pET24 expression vector (Novagen) by *NdeI* and *NotI*, yielding the plasmid pET24-PpoC and transformed together with the plasmid pGro7 (encoding the Chaperone groES-groEL) (Takara Bio Inc.) into *E. coli* Bl21\* cells (Invitrogen life technologies, Karlsruhe, Germany).

### Site directed mutagenesis

*In vitro* mutagenesis was carried out using Phusion<sup>TM</sup> Hot Start High Fidelity DNA Polymerase System (Finnzymes, Espoo, Finland) and using the protocol from the QuickChange® mutagenesis kit (Stratagene, La Jolla, CA, USA). The following primers were used: PpoA\_V328L sense (5'-CAAATATTATCCTAAAAGATTATUCUTUAUCGTACGATTTTGAATATAAAC -3') and antisense (5'-GTTTATATTCAAAATCGTACGTAGATAATCTTTTAGGATAATATTG -3'); PpoA\_V328A sense (5'-

GTACGCAAATATTATCCTTAAUGUGATTATGCACGAACGATTTTGAATATAAAA-3') and  
 antisense primer (5'-  
 GTTTATATTCAAATCGTTCGTGCATAATCCTTAAGGATAATATTTGCGTAC  
 -3')  
 PpoC\_Y427F sense (5'-  
 GAAACCAATGTTCTGTGCGAATTTAACCTGGCCTTTCGGTGGCACTCTGCTATCAGTG-3')  
 and antisense (5'-  
 CACTGATAGCAGAGTGCCACCGAAAGGCCAGGTTAAATTCGACAGAACATTGGTTTC-3')  
 PpoC\_G1039C sense (5'- GGCATCGGTTCTCAAATCTGCCTAGGCAAGGACGCCACACTG-3')  
 and antisense (5'-CAGTGTGGCGTCCTTGCCCTAGGCAGATTTGAGAACCGATGCC-3')  
 PpoC\_L385V sense (5'-  
 CATCACCTGTACGATTACGTACGCACGATCGTTAACCTGAACCGAGTGAACAG-3') and  
 antisense (5'-  
 CTGTTCACTCGGTTTCAGGTTAACGATCGTGCGTACGTAATCGTACAGGGTGATG-3')  
 PpoC\_L385A sense (5'-  
 CATCACCTGTACGATTACGCACGCACGATCGTTAACCTGAACCGAGTGAACAG-3') and  
 anti sense (5'-  
 CTGTTCACTCGGTTTCAGGTTAACGATCGTGCGTACGTAATCGTACAGGGTGATG-3')

### Expression of recombinant Ppo-enzymes in *E. coli*

*E. coli* BL21\* cells containing pET24-PpoC and the plasmid pGro7 (encoding the Chaperone groES-groEL) (Takara Bio Inc. Otsu, Japan) were cultivated in 2xYT broth containing 50 µg/mL chloramphenicol, 25 µg/mL kanamycin, 25 µg/mL arabinose to induce the expression of the chaperone and 0.02 mM δ-aminolaevulinic acid. Cells were grown to an OD<sub>600</sub> of 0.6-0.8. Induction of the expression of the recombinant protein was performed by adding isopropyl-beta-D-thiogalactopyranoside to a final concentration of 0.1 mM and cultures were cultivated for 3 d shaking at 16° C. Cells were harvested by centrifugation at 4,000 x g and 6° C for 10 min. The pellet was shock frozen in liquid nitrogen and stored at -20° C.

### Cell lysis and protein purification

Harvested cells of 500 mL culture expressing recombinant PpoC were resuspended in 50 ml 50 mM Tris/HCl, pH 8.0. After addition of lysozyme to a final concentration of 0.1 mg/mL and phenylmethylsulfonylfluorid to a final concentration of 0.2 mM, cells were incubated for 30 min on ice. Cells were sonified and this extract was centrifuged at 54,000 x g and 4° C for 20 min to clear debris, yielding crude bacterial extract. The supernatant obtained from cell lysis was loaded on a Source 30Q resin (25 ml, XK 16/20 column; GE Healthcare, Munich, Germany) at a flow rate of 1 mL/min using an ÄKTA-Prime-System (GE Healthcare, Munich, Germany). The column was washed with 50 ml of 50 mM Tris/HCl (pH 8.0) and protein was eluted with a linear gradient of 0-0.3 M NaCl in 50 mM Tris/HCl (pH 8.0) within 30 min at a flow rate of 2 ml/min. Fractions of 2 ml were collected. Fractions with highest activity (as judged by Clark-type O<sub>2</sub>-electrode) and highest purity (as judged by SDS-PAGE) were combined and concentrated to a final volume of 2 ml using Vivaspin 20 (100.000 MWCO) (Sartorius Stedim Biotech GmbH, Goettingen, Germany). For gel filtration the concentrated protein extract was loaded on a Superdex S200 26/60 pg column (GE Healthcare, Munich, Germany) equilibrated with 50 mM Tris/HCl (pH 8.0) containing 1 M NaCl. Elution was performed at a flow rate of 2 ml/min. Fractions containing enzyme activity were combined and loaded on a desalting 26/10 column (GE Healthcare, Munich, Germany) equilibrated with 50 mM Tris/HCl (pH 8.0). The desalted protein solution was loaded on a MonoQ 10/10-column (GE Healthcare, Munich, Germany). After loading and washing with 50 mM Tris/HCl (pH 8.0) the protein was eluted at 3 ml/min with a linear gradient to 50 mM Tris/HCl (pH 8.0) containing 1 M NaCl within 30 min. Recombinant PpoA and related mutants (PpoA\_V328A & PpoA\_V328L) were purified as described [19].

### UV/Vis-Spectroscopy

UV/vis-spectroscopy was performed in 50 mM HEPES, pH 7.4, at room temperature using a dualbeam Varian Cary 100 Bio UV/vis Spectrophotometer (Varian Inc., Palo Alto, CA, USA)



### Activity assays

250 µg of fatty acid (18:1<sup>Δ9Z</sup>, 18:2<sup>Δ9Z,12Z</sup>, 18:3<sup>Δ9Z,12Z,15Z</sup>) were dissolved in 1 mL or 800 µL of 50 mM Tris/HCl, pH 8.0. The reaction was started by the addition of either 15 µg purified PpoC or 200 µL PpoC bacterial crude extract and proceeded for 30 min at room temperature. Incubations were stopped by adding 2 mL diethylether to extract fatty acids and their derivatives. A second extraction was performed additionally with 2 ml diethylether and 100 µl glacial acid. After evaporating under nitrogen stream the sample was dissolved in 30 µl HPLC-eluent containing water, acetonitrile and glacial acid (50/50/0.1).

Incubations of 18:2<sup>Δ9Z,12Z</sup> and <sup>18</sup>O<sub>2</sub> (Campro Scientific, Berlin, Germany) with PpoC were performed in 50 mM Tris/HCl (pH 8.0). 900 µl buffer were degassed and supplied with <sup>18</sup>O<sub>2</sub> on ice until the system was saturated. 100 µl PpoC bacterial crude extract and 250 µg 18:2<sup>Δ9Z,12Z</sup> were added and incubated for 30 min at room temperature. The reaction system was further enriched with <sup>18</sup>O<sub>2</sub> during incubation. The reaction was terminated by addition of diethyl ether and reaction products were extracted as described above.

Incubations of 18:2<sup>Δ9Z,12Z</sup> with purified PpoA, PpoA\_V328A and PpoA\_V328L were performed in 50 mM HEPES (pH 7.4) as described [19]. Incubations with 8-HPODE and 10-HPODE were either performed in 200 µl of PpoC crude bacterial extract (in 50 mM Tris/HCl pH 8.0) or with 200 µl 50 µM HEPES (pH 7.4) containing 1-5 µM PpoA.

### RP-HPLC/MS analysis

The HPLC assembly was a Surveyor HPLC system (Thermo Finnigan, Austin, Texas, USA) with an EC250/2 100-5 C18 column (2.1×250 mm, 5 µm particle size; Macherey-Nagel, Düren, Germany). The solvent system for the following gradient was A: acetonitrile/water/acetic acid (40/60/0.1 [v/v/v]) and B: acetonitrile/acetic acid (100/0.1 [v/v]). The gradient elution profile was as follows: first a flow rate of 0.2 ml/min: 0-10 min, 80 % A and 20 % B; 10-30 min from 80 % A and 20 % B to 100 % B; 30-35 min flow rate increase to 0.3 ml/min; 35-40 min 100 % B; 40-44.5 min from 100 % B to 80 % A and 20 % B; 44.5-45 min 80 % A and 20 % B and flow rate decreased to 0.2 ml/min. The mass spectrometer was an LCQ ion trap mass spectrometer (Thermo Finnigan, Austin, Texas, USA) with electrospray ionisation and monitoring of negative ions. The capillary temperature was 300 °C and the capillary voltage was 27 kV. For tandem MS analysis, the collision energy was 1 V.

### Analysis of volatile compounds

Volatile compounds were measured from the reaction of 10 ml crude bacterial extract incubated with 2.5 mg of fatty acid (18:1<sup>Δ9Z</sup>, 18:2<sup>Δ9Z,12Z</sup>, 18:3<sup>Δ9Z,12Z,15Z</sup>) by solid phase micro-extraction. Incubation of the reaction mixture was performed in a vial, sealed with a teflon septum and a polydimethylsiloxane/divinylbenzene coated fibre (Supelco, Germany) was inserted. After 30 min the reaction products were extracted as described [23]. The analysis of the hydroxy fatty acids was carried out using an Agilent (Waldbronn, Germany) 5973 Network mass selective detector connected to an Agilent (Waldbronn, Germany) 6890 gas chromatograph equipped with a capillary DB-23 column (30 m x 250 µm; 0.25 µm coating thickness; J&W Scientific, Agilent (Waldbronn, Germany)). Helium was used as carrier gas (1 ml/min). The injection temperature was 250 °C, the temperature gradient was 40 °C for 2 min, 150 – 200 °C at 15 °C/min, and 200 °C for 3 min. Electron energy of 70 eV, an ion source temperature of 230 °C, and a temperature of 260 °C for the transfer line was used.

### Lipid derived radical trapping by nitroxyl radicals and peroxidase assay

Lipid derived radicals generated during the incubation of 18:2<sup>Δ9Z,12Z</sup> with PpoC were trapped and analyzed as nitroxyl radicals as previously described [19, 24]. We used the peroxidase assay according to Kulmacz [25] to demonstrate peroxidase activity of PpoC as previously described [19].

### Determination of kinetic parameters

Kinetic experiments were performed in 50 mM Tris/HCl (pH 7.9) at room temperature with a Clark-type O<sub>2</sub>-electrode (Rank Brothers, Bottisham, Cambridge, UK). Initial rates of oxygen consumption were measured for different substrate concentrations in buffer that was pre-equilibrated against air at 1 atm. The reaction was started by the addition of 200 µl PpoC bacterial crude extract to 800 µl of rapidly stirring buffer containing a defined amount of substrate. The following substrates were used:

Sodium salts of 18:1<sup>A9Z</sup>, 18:2<sup>A9Z,12Z</sup>, and 18:3<sup>A9Z,12Z,15Z</sup>. Kinetic parameters were determined by fitting seven data points to Michaelis-Menten equations by using the software Origin 6.0.

### Determination of the pH-optimum

The pH-optimum was determined by measuring initial rates of oxygen consumption at a defined pH-value using a Clark-type O<sub>2</sub>-electrode (Rank Brothers, Bottisham, Cambridge, UK): 200 µL PpoC bacterial crude extract were added to 800 µL of buffer containing 100 µM of the sodium salt of 18:2<sup>A9Z,12Z</sup>. The following buffers were used for the different pH-ranges: 0.2 M acetate buffer (pH 4.5-5.5), 0.2 M phosphate buffer (pH 5.5-8.0), 0.2 M borate buffer (pH 8.0-10.5), respectively.

## RESULTS

### PpoC is a fusion protein of the CYP6001 family

BLAST search analysis of PpoC predicts the presence of two domains in its sequence: Within the N-terminal region of the protein a heme peroxidase/dioxygenase domain is predicted whereas a P450 heme thiolate domain is proposed for the C-terminal part. Therefore, PpoC from *A. nidulans* was assigned as CYP6001C1 by Dr. D. R. Nelson of the P450 Nomenclature Committee (Department of Molecular Sciences, University of Tennessee). A similar domain architecture has also been described recently for PpoA, another member of the Ppo-family of *A. nidulans*, for which functionality of both domains has been proven [19]. Sequence alignments of the predicted N-terminal heme peroxidase/dioxygenase domain of PpoC with those of *A. fumigatus* PpoC (Fig. 2A, AfPpoC), *A. nidulans* PpoA (Fig. 2A, AnPpoA), *G. graminis* 7,8-linoleate diol synthase (Fig. 2A, GgLDS), sheep prostaglandin endoperoxide H synthase-1 (Fig. 2A, sheep PGHS1) and sheep prostaglandin endoperoxide H synthase-2 (Fig. 2A, sheep PGHS2) showed that residues reported to be essential for the catalytic dioxygenase activity are conserved in all six sequences. However, sequence alignments of the predicted C-terminal P450 domain of PpoC with those of known P450 heme thiolate enzymes, the recently described *A. nidulans* PpoA [19] and *A. fumigatus* PpoC [21], showed that the crucial cysteine, which is known as the 5<sup>th</sup> heme iron ligand, is neither conserved in PpoC from *A. nidulans* nor from *A. fumigatus*. Instead of a cysteine a glycine and a phenylalanine, respectively, are found at the particular position of the PpoC sequences (Fig. 2B). This may indicate that the P450 domain is not functional and does not show catalytic activity (*vide infra*).

### Cloning and functional expression of PpoC

In order to analyze the enzymatic reaction catalyzed by PpoC from *A. nidulans*, its cDNA was cloned from vegetative and sexual stages of *A. nidulans* by using specific primers. Sequencing of the resulting plasmid confirmed the ORF with 3933 bp and 1117 amino acids, respectively, showing one amino acid residue exchange (S215P) according to the published genome sequence [26]. Functional expression of PpoC was performed in *E. coli* BL21\* cells and optimal cultivation conditions (16 °C, 3 d) were determined. Expression of PpoC could be enhanced by co-expression of Chaperone groES-groEL and the addition of 0.2 mM δ-aminolaevulinic acid before induction (heme biosynthesis). Protein expression was verified by SDS-PAGE (Fig. 3A).

### Purification of PpoC

In order to purify PpoC a protocol was used that based on the previously described protocol for PpoA purification [19]. Extraction and solubilization of PpoC using crude extract from bacteria was performed with 50 mM Tris/HCl, pH 8.0. The addition of detergents and NaCl to the extraction buffer did not improve protein extraction. For the initial purification step a Source 30Q resin was chosen to isolate and concentrate PpoC from the crude cell extract. Fractions showing the highest activity were combined and further purified using size exclusion chromatography. The native molecular mass of PpoC using gel-filtration chromatography was 480 kDa. This was 4-fold larger than that determined by SDS-PAGE. Therefore, the native protein is a homo tetramer as it has previously been demonstrated for 7,8-linoleate diol synthase [18] and PpoA [19]. With the final chromatography step using a MonoQ column most of the remaining contaminants were removed. After each purification step the protein fractions showing the highest activity were combined and subjected to SDS-PAGE (Fig. 3A). A protein band at approximately 120 kDa became subsequently more prominent. Using this protocol we were able to purify approx. 0.5-2 mg of protein from 500 ml of cell culture. The activity

of the pure protein was estimated to be  $6.5 \mu\text{mol } [\text{O}_2]/(\text{min}\cdot\text{mg})$  using the Clark-type  $\text{O}_2$ -electrode. However, we found that the activity of the purified protein decreased significantly within a few hours. The addition of neither 10 % glycerol nor 5 mM EDTA to the buffer improved PpoC stability.

### Spectral analysis and prosthetic groups

The UV/vis absorption spectrum of purified PpoC is shown in Fig. 3B. The highest absorption maxima were at 280 nm (probably due to aromatic amino acid side chains, not shown) and 403 nm ( $\gamma$ , Soret band) indicating the presence of heme. Additionally two weaker maxima at approximately 530 nm and 630 nm could be detected and are indicated by arrows. However, the heme absorption spectrum appeared to be weak – a fact that might indicate that heme was partially lost during the purification procedure.

### Identification of products formed by recombinant PpoC

As we found purified PpoC to be highly unstable under our assay conditions, we decided to investigate most of the biochemical properties of PpoC by using bacterial crude extract of PpoC expressing *E. coli* cells and cells expressing the empty vector as controls.

Bacterial crude extract of PpoC expressing cells was incubated with  $18:2^{\Delta 9Z,12Z}$  for 30 min on a shaker. After extraction of fatty acids and their derivatives we analyzed product formation with RP-HPLC/MS<sup>2</sup>. We detected the formation of 10-HPODE ( $m/z$  311, RT 22.1 min), 8/10-HODE ( $m/z$  295, RT 19.5 min/20.9 min), 10-KODE ( $m/z$  293, RT 22.6 min) and 10-ODA ( $m/z$  183, RT 3.8 min) (Fig. 4A-D). Additionally we found the formation of 12,13-epoxy-10-hydroxy octadecenoic acid ( $m/z$  311, RT 9.9 min; Fig. 4A). The corresponding MS/MS spectra of 12,13-epoxy-10-hydroxy octadecenoic acid, 8-HODE, 10-HODE, 10-KODE and 10-ODA are shown in Supplemental Fig. 1A-E and are in agreement with previously reported spectra [20, 21]. Bacterial crude extract of empty vector controls did not metabolize  $18:2^{\Delta 9Z,12Z}$  to any of these products. Comparing the relative amounts of different products formed during the incubation, we found that 10-H(P)ODE appeared to be the main products that are formed from  $18:2^{\Delta 9Z,12Z}$  by PpoC (Fig 4E), while 12,13-epoxy-10-hydroxy octadecenoic acid and 10-ODA accumulated to lesser extents.

We next examined which products are formed by PpoC from other fatty acid substrates. We were particularly interested in products formed from fatty acids that occur endogenously in *A. nidulans*, such as other unsaturated C18-fatty acids. As supposed,  $18:1^{\Delta 9Z}$  and  $18:3^{\Delta 9Z,12Z,15Z}$  were mainly converted to their corresponding 10-hydro(pero)xy and 8-hydro(pero)xy derivatives (Tab. 1). Interestingly,  $18:1^{\Delta 9Z}$  was predominantly oxidized at the C-8, yielding 8-hydro(pero)xy oleic acid (8-H(P)OME), while  $18:3^{\Delta 9Z,12Z,15Z}$  was mainly oxidized at the C-10, yielding 10-hydro(pero)xy linolenic acid (10-H(P)OTrE).

Additionally, we observed that during incubations of  $18:3^{\Delta 9Z,12Z,15Z}$  with PpoC a polar product was formed that eluted at 3.8 min from RP-HPLC. The corresponding tandem MS spectrum ( $m/z$  199) of this compound is shown in Supplemental Fig. 1F and shows the following signals  $m/z$  181 (M-H<sub>2</sub>O), 155 (M-44, probably loss of CO<sub>2</sub>), 139 (M-60) and 137(M-62). Due to similarity to previously reported MS/MS spectra of 10-ODA [20] this product was tentatively assigned as (2E)-decendioic acid.

### Identification of volatile products

10-ODA, formed during the incubation of PpoC and  $18:2^{\Delta 9Z,12Z}$ , has been described to be a cleavage product of 10-HPODE [27]. It is known that this compound is formed from  $\beta$ -scission of the hydroperoxide – a reaction which is catalyzed by metals [27] and by still unidentified fungal lyases [28]. As additional cleavage products volatile octenol-isomers have also been reported [28-30]. To elucidate if similar volatile compounds are also formed during the reaction of PpoC with  $18:1^{\Delta 9Z}$ ,  $18:2^{\Delta 9Z,12Z}$  and  $18:3^{\Delta 9Z,12Z,15Z}$ , we analyzed the gas phase above the reaction mixture using solid phase micro extraction and GC/MS analysis. Using the NIST library and spectra reported earlier [31, 32], we were able to identify most of these compounds.

We found that during the reaction of PpoC and  $18:1^{\Delta 9Z}$  two volatile compounds were formed that could be identified as 1-octanol and 2-undecenal, respectively (Fig. 5A and Tab. 1). The latter compound has been described as an odorant formed from 8-HPOME [33]. Volatile compounds that were formed during the reaction of PpoC and  $18:2^{\Delta 9Z,12Z}$  were identified as 1-octen-3-ol, 2-octen-1-ol,



3-octenone and 2-octenal, respectively (Fig. 5B and Tab 1). Furthermore, we also detected the formation of two volatile products during incubations of PpoC with 18:3<sup>A9Z,12Z,15Z</sup> (Fig. 5C). These products could not be reliably identified by using the NIST library. However, incubation with [<sup>13</sup>C<sub>18</sub>]-18:3<sup>A9Z,12Z,15Z</sup> indicated that these products are octadiene-isomers (data not shown).

As these volatile compounds were also detected during incubation of 10-hydroperoxy derivatives with empty vector controls, we suggest that these cleavage reactions were mainly caused by non-enzymatic reactions or may be at least catalyzed independently from PpoC. Similar observations have been reported before [20].

### Peroxidase activity and pH-Optimum

In order to determine if the high amounts of 10-HODE (Fig. 4E) can be formed by PpoC within an enzymatic peroxidase reduction, we performed a peroxidase assay using TMPD as a co-substrate. When purified PpoC was incubated with 100 μM of TMPD and 100 μM of 18:2<sup>A9Z,12Z</sup>, we were able to follow the characteristic increase at 611 nm for several minutes. A typical progress curve is shown in Supplemental Fig. 2 and is indicative for the peroxidase activity of PpoC.

We analyzed the pH-optimum by measuring the initial rates of oxygen consumption for the reaction of PpoC crude extract with 100 μM 18:2<sup>A9Z,12Z</sup>. The initial rate of oxygen consumption reaches a maximum between pH 7.4 and 8.0. pH values >8.0 led to an rapid decrease in enzymatic activity.

### Kinetic properties

Kinetic parameters were determined by measuring the initial rates of oxygen consumption for the reaction with different substrate concentrations using again the Clark-type O<sub>2</sub>-electrode. The reaction was performed with PpoC crude extract; thus we were able to determine kinetic parameters that are independent of the enzyme concentration such as K<sub>M</sub>. We used sodium salts of 18:1<sup>A9Z</sup>, 18:2<sup>A9Z,12Z</sup> and 18:3<sup>A9Z,12Z,15Z</sup> as substrates and measured the initial rate of oxygen consumption for the different concentration in triplicates. The reaction was performed at pH 7.9 (pH-optimum) and started by the addition of substrate to the oxygen-saturated stirring solution. The K<sub>M</sub> values are summarized in Tab. 2 and suggest that 18:2<sup>A9Z,12Z</sup> may be the preferred substrate.

### Investigating epoxy alcohol synthase activity of Ppo-enzymes

We detected the formation of 12,13-epoxy-10-hydroxy octadecenoic acid (approx. 3 % of all products formed) when PpoC was incubated with 18:2<sup>A9Z,12Z</sup>. It has been suggested that this product is formed from 10-HPODE by *A. fumigatus* PpoC [21]. To clarify the enzymatic formation of epoxy alcohols by PpoC, we incubated 10-HPODE with PpoC harbouring bacterial crude extract. Clearly, formation of 12,13-epoxy-10-hydroxy octadecenoic acid could be detected. Next, we also incubated 8-HPODE with PpoC and detected the formation of the corresponding 12,13-epoxy-8-hydroxy octadecenoic acid. The individual RP-HPLC/MS chromatograms of both experiments are shown in Fig. 6. The corresponding MS<sup>2</sup> spectra of both epoxy alcohol derivatives, which are in agreement with those published before [21] are shown in supplemental Fig. 3A & B. It should be noted, however, that we also detected small amounts of both epoxy alcohols when using cell lysates of empty vector controls (Fig 6A&B). As the detected amounts in control assays were much lower compared to incubations with PpoC, we conclude that the major part of the detected epoxy alcohols are formed by an enzymatic reaction as previously proposed in [21], but rather by the peroxidase/dioxygenase domain of Ppo enzymes, than by the isomerase domain.

To investigate the origin of both oxygen atoms in the epoxy alcohol derivatives, we incubated 18:2<sup>A9Z,12Z</sup> with bacterial crude lysate under an atmosphere enriched in <sup>18</sup>O<sub>2</sub>. The MS spectrum of the epoxy alcohol product is shown in Fig. 7 and displays two major signals: *m/z* 311 corresponding to ([<sup>16</sup>O<sub>2</sub>]-12,13-epoxy-10-hydroxy octadecenoic acid) and *m/z* 315 ([<sup>18</sup>O<sub>2</sub>]-12,13-epoxy-10-hydroxy octadecenoic acid). As no abundant signal with *m/z* 313 could be detected, we conclude that the epoxy and the hydroxyl group contain either two atoms of <sup>16</sup>O or two atoms of <sup>18</sup>O. This indicates that both oxygen atoms of the epoxy alcohol group are derived from the same substrate molecule – the fatty acid hydroperoxide.

We next examined a potential epoxy alcohol synthase activity of the related PpoA enzyme (purified to homogeneity). Therefore, we incubated 10-HPODE with different concentrations of PpoA. In Fig. 8 the relative amounts of 12,13-epoxy-10-hydroxy octadecenoic acid, 10-ODA and 10-HPODE are



plotted against PpoA concentrations. We did observe a linear correlation between the PpoA concentrations deployed and product formation or substrate consumption, respectively. Thus, we suggest PpoA to exhibit epoxy alcohol synthase activity with 10-HPODE as a substrate, paralleling the results obtained with PpoC (see above). As reported previously 8-HPODE is predominantly converted to 5,8-DiHODE by the 8-hydroperoxide isomerase P450 domain of PpoA [19]. The tandem MS spectrum of this product is shown in Supplemental Fig. 3C. Additionally, we detected traces of 12,13-epoxy-8-hydroxy octadecenoic acid formed by PpoA from 8-HPODE.

#### Site directed mutagenesis of determinants that control site of dioxygenation

Val-385 is a conserved residue in the peroxidase/dioxygenase domain of PpoC, PpoA (Val-328), 7,8-linoleate diol synthase (Val-330) and prostaglandin endoperoxide H synthase (Val-349 in sheep PGHS-1). In the latter, Val-349 is known to be important for substrate positioning and the dioxygenase reaction [34]. To compare the oxygenation mechanisms of *A. nidulans* PpoA and PpoC, we mutated the corresponding amino acids in both enzymes: Leu-385 of PpoC was substituted by either valine or alanine, while Val-328 of PpoA was exchanged either by alanine or leucine. Crude bacterial extract from wild type or mutated PpoC and purified wild type or mutated PpoA was incubated with 18:2<sup>A9Z,12Z</sup>. The relative amounts of hydroxyl products formed were determined by RP-HPLC/MS and the results are visualized as column diagrams (Fig. 9). For PpoC (Fig. 9A) we observed that substitution of Leu-385 with the smaller residue valine enhanced the formation of 8-HODE by 2-3-fold compared to the wild type enzyme. Reducing the size to the smaller residue like alanine enhanced the formation of 8-HODE by even 3-4 fold as judged by RP-HPLC/MS analysis.

We previously reported that we were unable to detect any 10-H(P)ODE formed during incubations of 18:2<sup>A9Z,12Z</sup> and PpoA by RP-HPLC. Using an improved RP-HPLC/MS approach we now observed the formation of 10-H(P)ODE and especially found that approx. 30 % of the formed hydroxides are 10-HODE (Fig. 9B). The substitution of Val-328 of PpoA by alanine reduced the formation of 8-HODE and increased the production of 10-HODE, whereas substitution with leucine only led to a modest increase in 8-HODE formation compared to the wild type enzyme (Fig. 9B).

#### Site directed mutagenesis of the catalytic tyrosine

As shown in Fig. 1A Tyr-427 of *A. nidulans* PpoC is conserved in the enzymes analyzed. Recent mutagenesis studies indicated that this tyrosine residue is important for catalytic activity [19, 34-36] presumably by generating a tyrosyl radical [37, 38]. Substitution of PpoC Tyr-427 by Phe (Y427F) led to an inactive enzyme that showed no detectable dioxygenase activity when incubated with 18:2<sup>A9Z,12Z</sup>. The expression of the Y427F was confirmed by SDS-PAGE analysis (Supplemental Fig. 4).

#### Site directed mutagenesis of the predicted P450 heme thiolate domain

As shown in Fig. 2B we found that the predicted P450 domain of PpoC does not contain the conserved cysteine residue that is known to be the 5<sup>th</sup> heme iron ligand in P450 enzymes [22]. Substitution of this cysteine (Cys-1006) in PpoA by alanine led to an enzyme that showed dioxygenase, but no detectable isomerase activity presumably through distortion of the P450 heme moiety [19]. As PpoC contains a glycine residue instead of a cysteine at this particular position, we hypothesized that substitution of Gly-1039 by cysteine may reconstitute the heme binding moiety and thereby lead to an enzyme with isomerase activity as it is known for PpoA. However, we did not detect formation of any DiHODE derivative when PpoC\_G1039C was incubated with 18:2<sup>A9Z,12Z</sup>. Moreover, the enzyme showed the same catalytic activity as the wild type enzyme. Moreover, we also incubated the enzyme with 8-HPODE Upon the incubation of PpoC\_G1039C with 8-HPODE no DiHODE was detectable.

#### Radical spin trapping adducts

In order to investigate the formation of carbon centred fatty acid radicals during the reaction of PpoC and 18:2<sup>A9Z,12Z</sup> we used the method of Koshiishi *et al.* to scavenge the hypothetical radical with the radical scavenger CmΔP. Recently, we adapted this approach for the PpoA/linoleate system and found the formation of two radical spin trapping adducts which we tentatively assigned as the trapped C-8 and C-10 radical adducts [19] - a finding that now goes along with our observation that PpoA is also able to form 10-H(P)ODE (Fig. 9B). To test the existence of radical intermediates in the PpoC/linoleate system we performed the trapping reaction with CmΔP using PpoC and 18:2<sup>A9Z,12Z</sup>. The linoleate allyl radical CmΔP adducts of *m/z* 463 were identified using RP-HPLC/MS. Two distinct

peaks could be detected; a major peak eluting at 28.8 min and a smaller one at 30.8 min [19]. We suggest from this result, that the major peak corresponds to the trapped C-8 radical adduct while the smaller peak may correspond to the C-10 radical adduct, in analogy to the PpoA/linoleate system. The MS spectra of both trapping adducts of the PpoC/linoleate system showed the characteristic fragment with  $m/z$  185; this fragment arose from the reduced form of Cm $\Delta$ P through heterolysis of the C-O-N bond [40]. We also detected trapping adducts with  $m/z$  479 that may correspond to nitroxyl radical-lipid epoxy allyl adducts [40].

## DISCUSSION

The present study aimed to characterize *A. nidulans* PpoC, a putative fatty acid dioxygenase, and to compare it with *A. nidulans* PpoA - another member of the *A. nidulans* Ppo-family. The Ppo enzyme family in *A. nidulans* consists of three dioxygenase genes, PpoA, PpoB and PpoC. Genetic evidence indicated Ppo products to be involved in the regulation of the developmental life cycle of *A. nidulans* [13]. Interestingly, NCBI conserved domain search predicted that all three protein sequences seem to be fusion proteins because of the presence of two conserved domains: a heme peroxidase/dioxygenase domain in the N-terminal region of the enzymes and a cytochrome P450 region in the C-terminal part. Recently, we showed that the bifunctional activity of PpoA is indeed related to the presence of both domains: within the peroxidase/dioxygenase domain of PpoA the (8*R*)-dioxygenase reaction takes place while in the P450 domain the (8*R*)-hydroperoxide isomerisation is accomplished [19]. Although PpoC is predicted to be a fusion protein as well having a similar C-terminal P450 domain, we found on the basis of sequence alignments, that the crucial cysteine residue is not conserved in its sequence (Fig. 2B). In P450 enzymes the corresponding residue acts as the 5<sup>th</sup> heme iron ligand and mutation of this cysteine leads to an inactive enzyme [41]. Therefore, it appeared doubtful that this domain is functional in PpoC and has catalytic activity. To analyze the influence of this changed domain structure we isolated PpoC cDNA, expressed the enzyme in *E. coli* and investigated its biochemical properties.

Our results indicate that both fusion proteins, PpoA and PpoC, have many properties in common, but there are also essential differences that are most likely caused by the differences in their C-terminal P450 domain. PpoA and PpoC resemble 7,8-linoleate diol synthase tetrameric heme proteins that catalyze the oxidation of 18:2 <sup>$\Delta^{9Z,12Z}$</sup> . While PpoA predominantly oxidizes 18:2 <sup>$\Delta^{9Z,12Z}$</sup>  at the C-8, yielding 8-HPODE, PpoC mainly performs oxygenation at the C-10 forming 10-HPODE. These dioxygenase reactions are both catalysed within the peroxidase/dioxygenase domain of the fusion proteins (Fig. 9). Sequence alignments showed that PpoA and PpoC contain the conserved tyrosine within their heme peroxidase/dioxygenase domain (Tyr-374 in PpoA and Tyr-427 in PpoC) that is also found in 7,8-linoleate diol synthase (Tyr-376), sheep prostaglandin endoperoxide H synthase-1 (Tyr-385) and sheep prostaglandin endoperoxide H synthase-2 (Tyr-370). Previous studies showed that dioxygenase activity was reduced or even abolished in these enzymes when this tyrosine residue was mutated [19, 34, 35]. As also reported previously for PpoA, we now provide evidence that substitution of this tyrosine by phenylalanine also abolished dioxygenase activity of PpoC. Thus, we conclude that PpoC also uses a tyrosyl radical for the abstraction of the hydrogen from the C-8 of 18:2 <sup>$\Delta^{9Z,12Z}$</sup>  and forms a carbon centred radical at this position. The unpaired electron is delocalized and can migrate from the C-8 to the C-10 by shifting the C-9-C-10 double bond to C-8-C-9. The formation of carbon centred substrate radicals at the C-8 and C-10 was also supported by experiments in which we trapped these radicals using the radical scavenger Cm $\Delta$ P and as reported before for other enzymes [19, 40].

The data so far led us to ask what the amino acid determinants are that direct the oxygenation at C-8 and C-10, respectively. Recently, Garscha *et al.* reported the recombinant expression of a PpoC from *A. fumigatus* that catalyzes a similar reaction as PpoC [21]. By substituting Leu-384 and Val-388 with larger and smaller amino acids they could show that these residues act as determinants that direct oxygenation either at C-8 or C-10 of 18:2 <sup>$\Delta^{9Z,12Z}$</sup>  [21]. As similar substitutions of the homologue residue in *G. graminis* 7,8-linoleate diol synthase (Val-330) showed a related effect, they developed a general model for the oxygenation by fungal 8-dioxygenases and 10-dioxygenases, respectively. This model proposed that Leu-384 shields the C-8 of 18:2 <sup>$\Delta^{9Z,12Z}$</sup>  in the active site of (10*R*)-dioxygenase or PpoC [21]. To gain further insights into the oxygenation mechanisms, we also mutated the related Val-328 (in PpoA) and Leu-385 (in PpoC) residues in the Ppo-enzymes from *A. nidulans*. This experiment enabled for the first time the direct comparison of two dioxygenases present in the same species and

with high amino acid sequence similarity but different product specificities. For the substitution of PpoC Leu-385 by alanine and valine we found similar effects as reported [21] that may also indicate that Leu-385 shields the C-8 of 18:2<sup>A9Z,12Z</sup>. However, the substitution of Val-328 in PpoA by leucine and alanine conversely indicated that this residue might shield C-10 rather than C-8. This effect may be explained by a different positioning or even by a different orientation of the fatty acid in the active site of both enzymes, leading to the conclusion that our results in contrast to those of Garscha *et al.* [21] indicate that there either is no general model for oxygenation by these fungal dioxygenases or the role of the mutated amino acids is as yet unclear. Clearly, a crystal structure is needed to further address this question. A further explanation may lay in the fact that the isomerase activity of recombinant 7,8-linoleate diol synthase is very weak and, therefore, 8-H(P)ODE can accumulate. This accumulation might influence the equilibrium in a different way compared to PpoA where almost all 8-HPODE is immediately converted to 5,8-DiHODE.

We further investigated the downstream products of the dioxygenase reaction of PpoC. 10-HPODE that was formed during incubations of PpoC and 18:2<sup>A9Z,12Z</sup> was mainly converted into 10-HODE, which was formed by an enzymatic peroxidase reaction of PpoC within the C-terminal heme peroxidase/dioxygenase domain. Another product of this domain was 10-KODE. However, 10-HPODE appeared to be unstable on its own and 10-ODA and volatile compounds like 2-octen-1-ol, 2-octenal, 3-octanone and 1-octen-3-ol were formed most likely by chemical fragmentation from this PpoC product. The latter compound has also been reported to be formed from 18:2<sup>A9Z,12Z</sup> by homogenates of *Lentinus decedetes* [42], *Psalliotia bispora* [28-30] and *Tricholoma matsutake* [43]. Although the reaction has been discovered 25 years ago, the enzyme involved in formation of these compounds known as mushroom flavour was never identified from neither of these sources. Interestingly, at first an independent metabolic reaction leading to formation of the same volatiles 2-octen-1-ol and 1-octen-3-ol was described for the moss *Physcomitrella patens* [32]. Here a bifunctional lipoxygenase uses the C-20 fatty acid arachidonic acid and the C-8 alcohols are formed via the (12*S*)-hydroperoxide of this fatty acid [44].

As a further product 12,13-epoxy-10-hydroxy octadecenoic acid was formed during the reaction of PpoC and 18:2<sup>A9Z,12Z</sup>. This product has also been reported to be formed by PpoC from *A. fumigatus* [21]. It was suggested that this enzyme has epoxy alcohol synthase activity and converts specifically 10-HPODE to 12,13-epoxy-10-hydroxy octadecenoic acid in analogy to the isomerisation reaction that is performed with 8-HPODE by 7,8-linoleate diol synthase [21]. Indeed, by incubating purified 10-HPODE with PpoC bacterial crude extract we confirmed the enzymatic conversion of this substrate to 12,13-epoxy-10-hydroxy octadecenoic acid. Thus, we showed for the first time that 10-HPODE is in fact the precursor of 12,13-epoxy-10-hydroxy octadecenoic acid. Additionally, we found that PpoC can also convert 8-HPODE into 12,13-epoxy-8-hydroxy octadecenoic acid but not into 5,8-DiHODE. This finding underlines that a functional P450 domain, as in case of PpoA, is essential for an isomerisation reaction but not for epoxy alcohol synthase activity.

As 7,8-linoleate diol synthase displays its bifunctionality by conversion of 18:2<sup>A9Z,12Z</sup> to 8-HPODE followed by the isomerisation yielding 7,8-DiHODE, we next investigated if purified PpoA also harboured a similar epoxy alcohol synthase side activity besides the described dioxygenase and isomerase activity. Incubations of purified PpoA and 8-HPODE led mainly to the formation of 5,8-DiHODE by the 8-hydroperoxide isomerase P450 domain as reported. As we also detected low amounts (< 1 % of total reaction products) of 12,13-epoxy-8-hydroxy octadecenoic acid during these incubations we concluded that like PpoC also PpoA harbours a epoxy alcohol synthase side activity. This was further confirmed by incubations of PpoA with 10-HPODE (Fig. 8). In contrast to 8-HPODE this substrate was not converted into a dihydroxy fatty acid but rather transformed into 12,13-epoxy-10-hydroxy octadecenoic acid. The finding that the amount of 12,13-epoxy-10-hydroxy octadecenoic acid showed a linear dependency from concentrations of the PpoA used in the experiment additionally indicate an enzymatic reaction. However, as we also found production of epoxy alcohols in negative controls in minor amounts, we suggest that epoxy alcohol derivatives can also be formed from 10-HPODE and 8-HPODE under the used assay conditions.

As the isomerase activity of the bifunctional PpoA is located in the P450 domain and PpoC seems to contain an inactive P450 domain, due to the lack of a highly conserved cysteine residue, we tried to obtain a bifunctional PpoC by mutation analysis. To reconstitute the P450 heme binding moiety and to obtain functional 8-hydroperoxide isomerase domain we substituted of Gly-1039 with cysteine and incubated the enzyme with 18:2<sup>A9Z,12Z</sup> and 8-HPODE. No functional 8-hydroperoxide isomerase

activity was retrieved. This might indicate that several more residues in the PpoC sequence are important for correct binding of heme and a correct folding of the P450 domain. However, for conclusive identification of further essential amino acids constituting a functional P450 motif a crystal structure is required. A domain switching approach may also be suitable to address this question in the future, but the lack of information about the crystal structure of the enzyme is hindering further investigations in that direction as well.

Taken together, our results show that PpoA and PpoC are fusion proteins that employ similar mechanisms to catalyze the formation of 8- and 10-hydroperoxy fatty acids. In this context the leucine/valine residues that have been reported recently for *A. fumigatus* PpoC and *G. graminis* 7,8-linoleate diol synthase seem also to determine the position of oxygenation of 18:2<sup>Δ<sup>9</sup>,12Z</sup>, i.e. C-8 and C-10, of dioxygenases in *A. nidulans*, but a general mechanism how these amino acid residues influence the site of oxygenation remains unclear. PpoC and PpoA both have an epoxy alcohol synthase side activity and can convert 8-HPODE and 10-HPODE into the corresponding epoxy alcohols. However, this is employed by the peroxidase/dioxygenase domain of these enzymes. This is further supported by the fact that PpoA converts 8-HPODE mainly into 5,8-DiHODE, while PpoC cannot perform a similar reaction, probably due to a degenerated P450 heme thiolate domain.

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### ABBREVIATIONS FOOTNOTES

The abbreviations used are: CmΔP, 3-carbamoyl-2,2,5,5-tetramethyl-3-pyrroline-N-oxyl; DiHODE, dihydroxy octadecadienoic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethane-sulfonic acid; H(P)OME, hydro(pero)xy octadecenoic acid; H(P)ODE, hydro(pero)xy octadecadienoic acid; H(P)OTrE, hydro(pero)xy linolenic acid; 10-KODE, (8*E*,12*Z*)-10-oxo-8,12-octadecadienoic acid; ODA, 10-oxo-8-decenoic acid; Ppo, psi producing oxygenase; *ppo*, gene coding for Ppo; Psi, precocious sexual inducer; RP, reversed phase; SP, straight phase; TMPD, N,N,N',N'-tetramethyl-p-phenylenediamine; Tris, 2-Amino-2-(hydroxymethyl)propane-1,3-diol.

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## FIGURE-LEGENDS

## FIGURES

**Figure 1: Psi factor formation in *A. nidulans* and *A. fumigatus*.** Ppo enzymes abstract a hydrogen atom from the C-8 of the backbone of unsaturated C-18 fatty acids and form a carbon centred radical at this position - a reaction that is presumably accomplished via a tyrosyl radical mechanism within the N-terminal fatty acid heme peroxidase/dioxygenase domain. In case of enzymes belonging to PpoA subfamily molecular oxygen attacks the C-8 centred radical and forms (8*R*)-HPODE. This intermediate product is either used as a substrate for peroxidase reaction, yielding 8-HODE, or it serves as a substrate for the 8-hydroperoxide isomerase reaction, yielding mainly 5,8-DiHODE. The isomerase reaction is believed to be accomplished by the C-terminal P450 heme thiolate domain of PpoA [19]. Thirdly the fatty acid hydroperoxide is reduced to 8-HODE or transformed to small amounts of 12,13-epoxy-8-hydroxy octadecenoic acid, respectively. In case of PpoC enzymes the carbon centred radical migrates to C-10 and molecular oxygen is inserted at this position, yielding 10-HPODE. This product appears to be unstable and either oxidizes to 10-KODE or decomposes to 10-ODA and volatile compounds as 1-octen-3-ol. Furthermore, 10-HPODE can be reduced to 10-HODE or transformed to small amounts of 12,13-epoxy-10-hydroxy octadecenoic acid, respectively.

**Figure 2: Partial alignment of the predicted N-terminal heme peroxidase/dioxygenase domain and C-terminal P450 domain from PpoC with sequences from different fatty acid heme dioxygenases and P450 enzymes.** A. Partial amino acid alignment of the predicted heme peroxidase/dioxygenase domain from *A. nidulans* PpoC (XP\_662632; AnPpoC) with *A. fumigatus* PpoC (ABV21633; AfPpoC), *A. nidulans* PpoA (XP\_659571; AnPpoA), *G. graminis* 7,8-linoleate diol synthase (Q9UUS2.1; GgLDS), sheep prostaglandin endoperoxide H synthase-1 (P05979.2) and sheep prostaglandin endoperoxide H synthase-2 (P79208.1). The catalytic tyrosine as well as the proximal and distal histidines, which are conserved in the six sequences and have been identified before [35, 45] are shaded black. Additionally, leucine/valine residues that are responsible for direction of oxygenation in *A. fumigatus* PpoC and *G. graminis* 7,8-linoleate diol synthase are shaded black. B. Partial amino acid alignment of the predicted P450 heme thiolate domain from *A. nidulans* PpoC with known P450 enzymes [CYP450\_119 (1F4T:B), CYP450\_cin (1T2B:B), CYP450\_130 (2UUQ:A), CYP450\_nor (1JFB:A), CYP4503A4 (1TQN:A), CYP4502C9 (1R9O:A)] and predicted CYP450 domains from different Ppo-enzymes [*A. fumigatus* PpoC (ABV21633), *A. nidulans* PpoA (XP\_659571) and *A. fumigatus* PpoA (XP\_751750)]. Amino acids matching the P450 consensus sequence according to the PROSITE database are shaded grey. Additionally the cysteine responsible for the coordination of heme iron is shaded black. The sequences were aligned using the ClustalW algorithm.

**Figure 3: SDS-PAGE at different stages of purification (A) and UV/vis absorption of purified PpoC (B).** A. Aliquots after each purification step were analysed by 8 % SDS-PAGE. Lane 1, crude cell extract; lane 2, after Source 30Q; lane 3, after Sephadex S200 (60/20), lane 4, after MonoQ (10/10). B. A spectrum of the native enzyme in 50 mM HEPES (pH 7.4) is shown. The UV/vis spectrum of the purified enzyme showed absorption maxima at 403 nm ( $\gamma$ , Soret) indicating the presence of heme. Additionally two weaker maxima at approximately 530 nm and 630 nm are indicated by arrows.

**Figure 4: RP-HPLC/MS analysis of metabolites formed from 18:2<sup>A9Z,12Z</sup> by recombinant PpoC.** RP-HPLC/MS analysis after 30 min incubation of 18:2<sup>A9Z,12Z</sup> with PpoC in 50 mM Tris buffer (pH 8.0) and extractive isolation. RP-HPLC/MS analysis of (A) 10-HPODE and 12,13-epoxy-10-hydroxy octadecenoic acid ( $m/z$  311; full scan), (B) 8-HODE and 10-HODE ( $m/z$  295; full scan), (C) 10-KODE ( $m/z$  293; full scan) and (D) 10-ODA ( $m/z$  183; full scan). As reported previously [20], 10-HPODE decomposed in the mass spectrometer into 10-ODA which co-elutes with 10-HPODE at 20.9 min, while pre-formed 10-ODA elutes at 3.8. E. Relative amounts of products formed during the incubation. Mean values and standard deviations were calculated from three different experiments.



**Figure 5: GC/MS-analysis of volatile compounds of PpoC and different fatty acid substrates.** Volatile compounds formed during the incubation of PpoC with (A) 18:1<sup>Δ9Z</sup>, (B) 18:2<sup>Δ9Z,12Z</sup> and (C) 18:3<sup>Δ9Z,12Z,15Z</sup>. The products were identified with SPME, GC/MS and by using the NIST library and spectra reported earlier [31, 32]. Incubations of PpoC with 18:1<sup>Δ9Z</sup> led to the production of 1-octanol and 2-undecenal, while during incubations with 18:2<sup>Δ9Z,12Z</sup> predominantly 1-octen-3-ol, 2-octen-1-ol, 2-octenal and 3-octenon were formed. 18:3<sup>Δ9Z,12Z,15Z</sup> was converted by PpoC into two volatile products which could not be identified using the NIST library. By experiments with [<sup>13</sup>C<sub>18</sub>]-labelled 18:3<sup>Δ9Z,12Z,15Z</sup> these products were tentatively assigned as octadiene isomers.

**Figure 6: Conversion of 8-HPODE and 10-HPODE to epoxy alcohols by PpoC.** Shown are the results from RP-HPLC/MS analysis after incubation of 10-HPODE (A & C) and 8-HPODE (B & D) with empty vector controls (A & B) and recombinant PpoC bacterial crude extract (C & D) after 40 min incubation at room temperature.

**Figure 7: Mass spectrometric analysis of 12,13-epoxy-10-hydroxy octadecenoic acid formed from 18:2<sup>Δ9Z,12Z</sup> by PpoC under <sup>18</sup>O<sub>2</sub>.** RP-HPLC/MS analysis of the 12,13-epoxy-10-hydroxy octadecenoic acid obtained after incubation of 18:2<sup>Δ9Z,12Z</sup> with recombinant PpoC bacterial crude extract under enriched <sup>18</sup>O<sub>2</sub> atmosphere.

**Figure 8: Conversion of 10-HPODE to 12,13-epoxy-10-hydroxy octadecenoic acid and 10-ODA as a function of PpoA concentration.** The reaction system contained 500 μM 10-HPODE and various concentration of purified PpoA in 50 μM HEPES (pH 7.4). The reaction was terminated after 40 min.

**Figure 9: Relative amounts of 8-HODE formed by PpoA\_WT, PpoC\_WT and their corresponding mutants.** A. Relative amounts of 8-HODE formed by recombinant PpoC\_WT, PpoC\_L385A and PpoC\_L385V bacterial crude extract in 50mM Tris/HCl (pH 8.0). B. Relative amounts of 8-HODE formed by purified PpoA\_WT, PpoA\_V328A and PpoC\_V328L in 50 mM HEPES (pH 7.4). Mean values and standard deviations were calculated from three different experiments.

## TABLES

TABLE 1:

**Oxygenation products of *A. nidulans* PpoC.**

Products were identified using RP-HPLC/MS<sup>2</sup> analysis and by detection of characteristic signals (8-hydroperoxide derivative with  $m/z$  157 (cleavage between C-8 and C-9) and 10-hydroperoxide derivative with  $m/z$  183 (cleavage between C-10 and C-11, as also shown in Supplemental Figure 2). Volatile products were extracted by solid phase micro extraction and identified by GC/MS analysis using the NIST database (Figure 6).

| Substrate                   | Hydroperoxide products | Hydroperoxide derived products                                  | Volatile products  |
|-----------------------------|------------------------|---|--|
| 18:1 <sup>Δ9Z</sup>         | 8-HPOME<br>10-HPOME    | 8-HOME<br>10-HOME   | 1-octanol,<br>2-undecenal                                  |
| 18:2 <sup>Δ9Z,12Z</sup>     | 8-HPODE<br>10-HPODE    | 8-HODE,<br>10-HODE,<br>12,13-Ep-10-HOME,<br>10-ODA,<br>10-KODE, | 1-octen-3-ol,<br>2-octen-1-ol,<br>2-octenal,<br>3-octanone |
| 18:3 <sup>Δ9Z,12Z,15Z</sup> | 8-HPOTE<br>10-HPOTE    | 8-HOTrE,<br>10-HOTrE,<br>2-decenedioic acid                     | octadiene-isomers  |

TABLE 2:

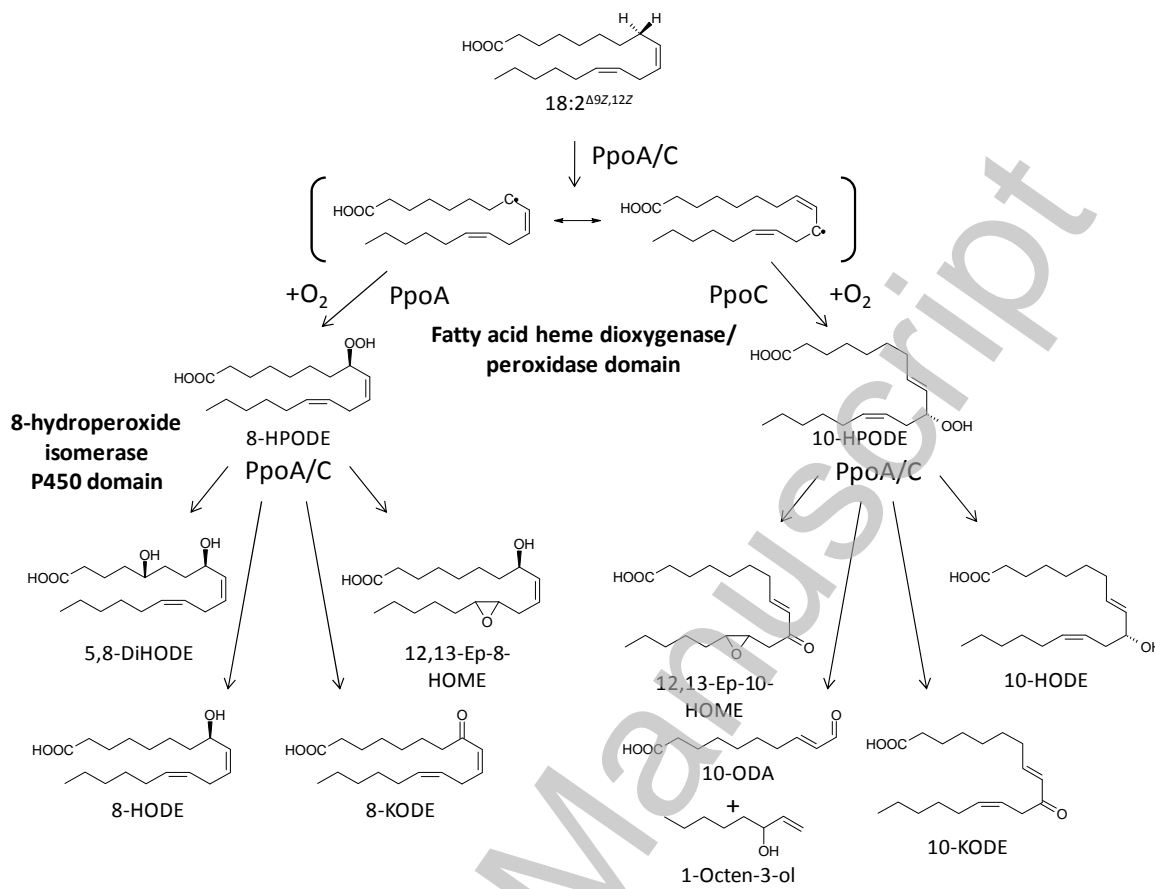
**Kinetic parameters for different substrates.**

Kinetic analysis was performed with a Clark-type O<sub>2</sub>-electrode. Initial time dependent rates of oxygen consumption were measured with different substrate concentrations and K<sub>M</sub>-values were determined by fitting seven data points to the Michaelis-Menten-equation using the software Origin 6.0. To prevent mycel formation the sodium salts of each fatty acid was used as a substrate for the kinetic analysis. Mean values were calculated from three different experiments.

| Substrate                   | K <sub>M</sub> -value [μM] |
|-----------------------------|----------------------------|
| 18:1 <sup>Δ9Z</sup>         | 13.1 ± 1.7                 |
| 18:2 <sup>Δ9Z,12Z</sup>     | 3.6 ± 0.97                 |
| 18:3 <sup>Δ9Z,12Z,15Z</sup> | 7.9 ± 1.6                  |

**FIGURES**

**Figure 1**



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Figure 3

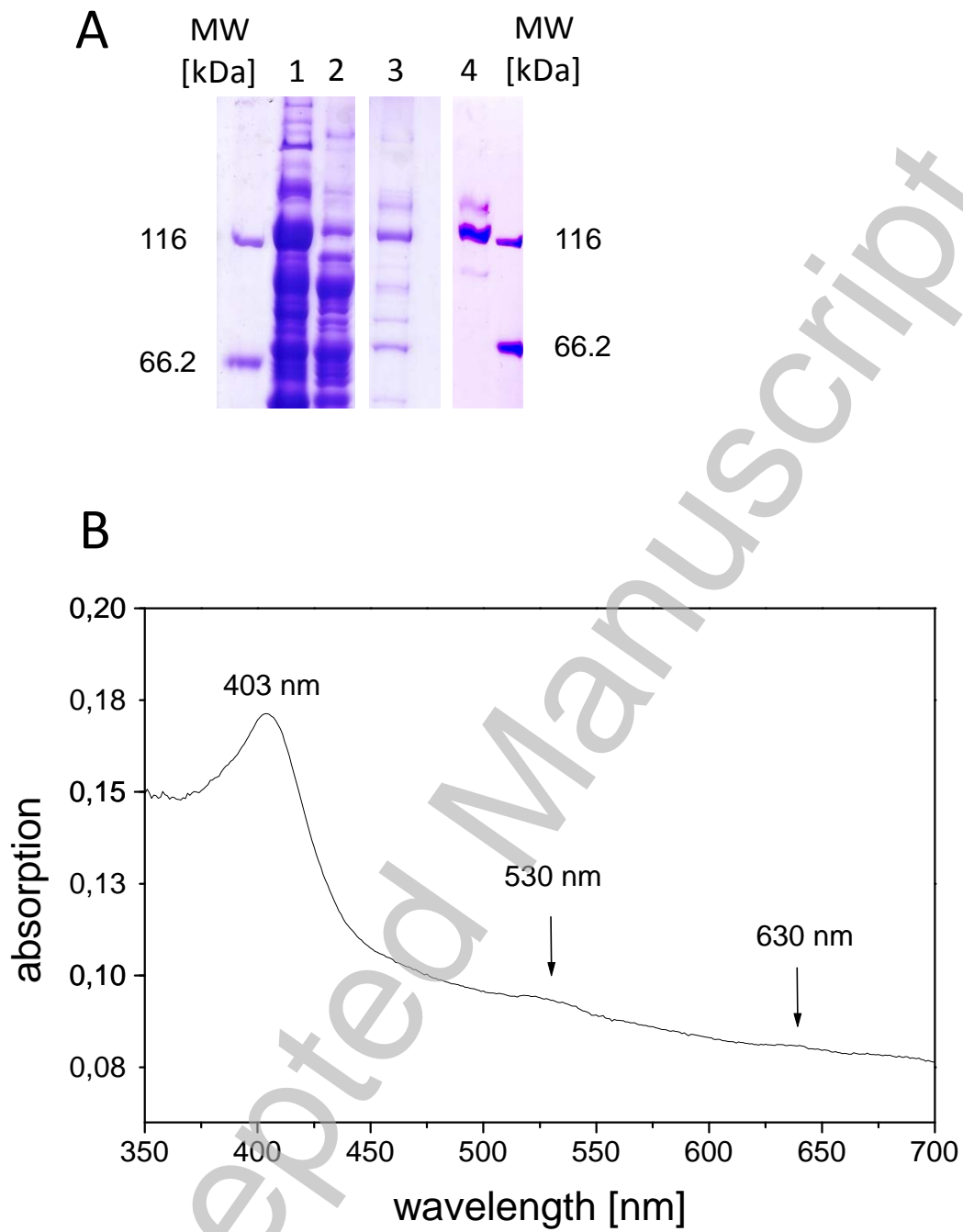


Figure 4

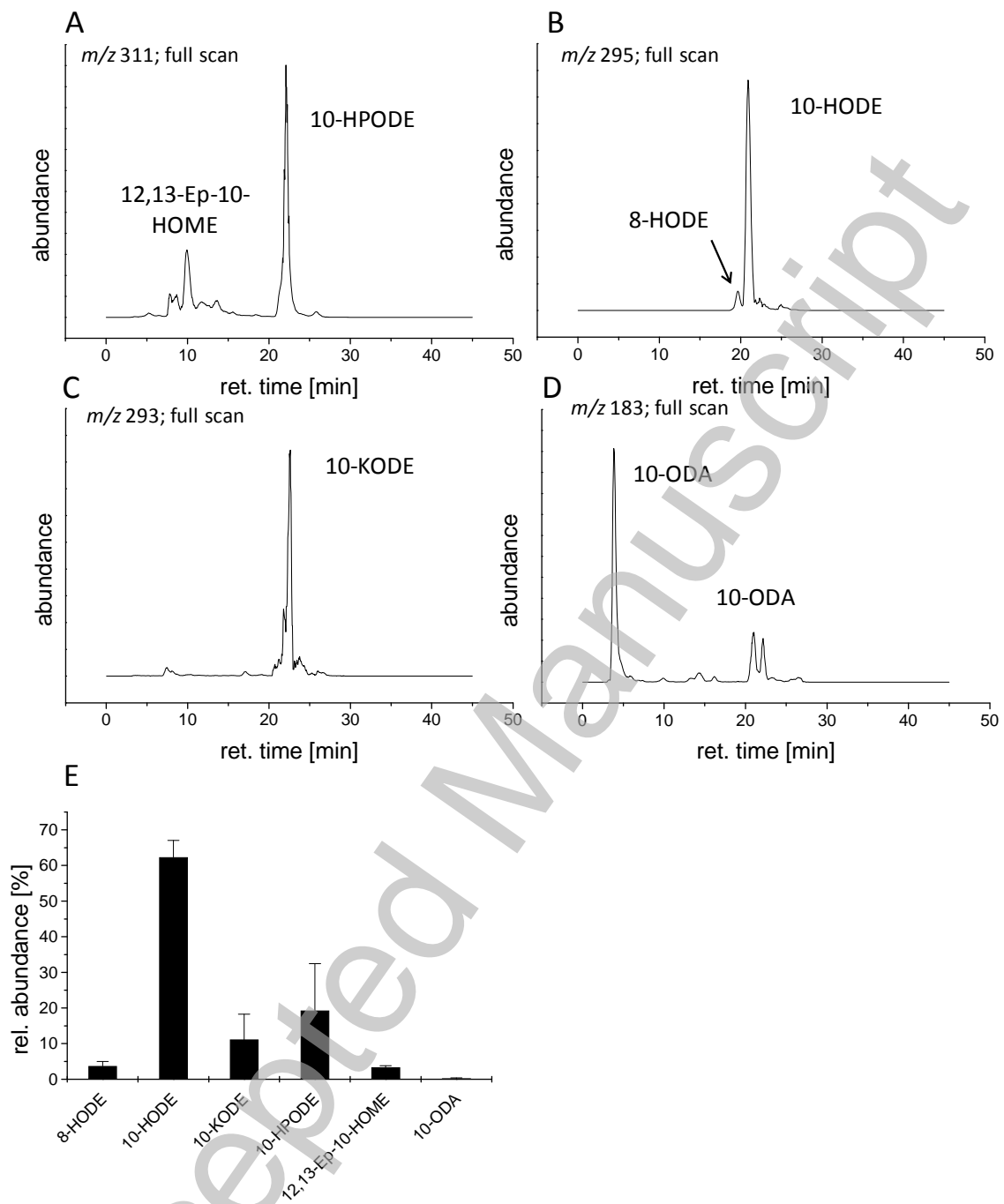
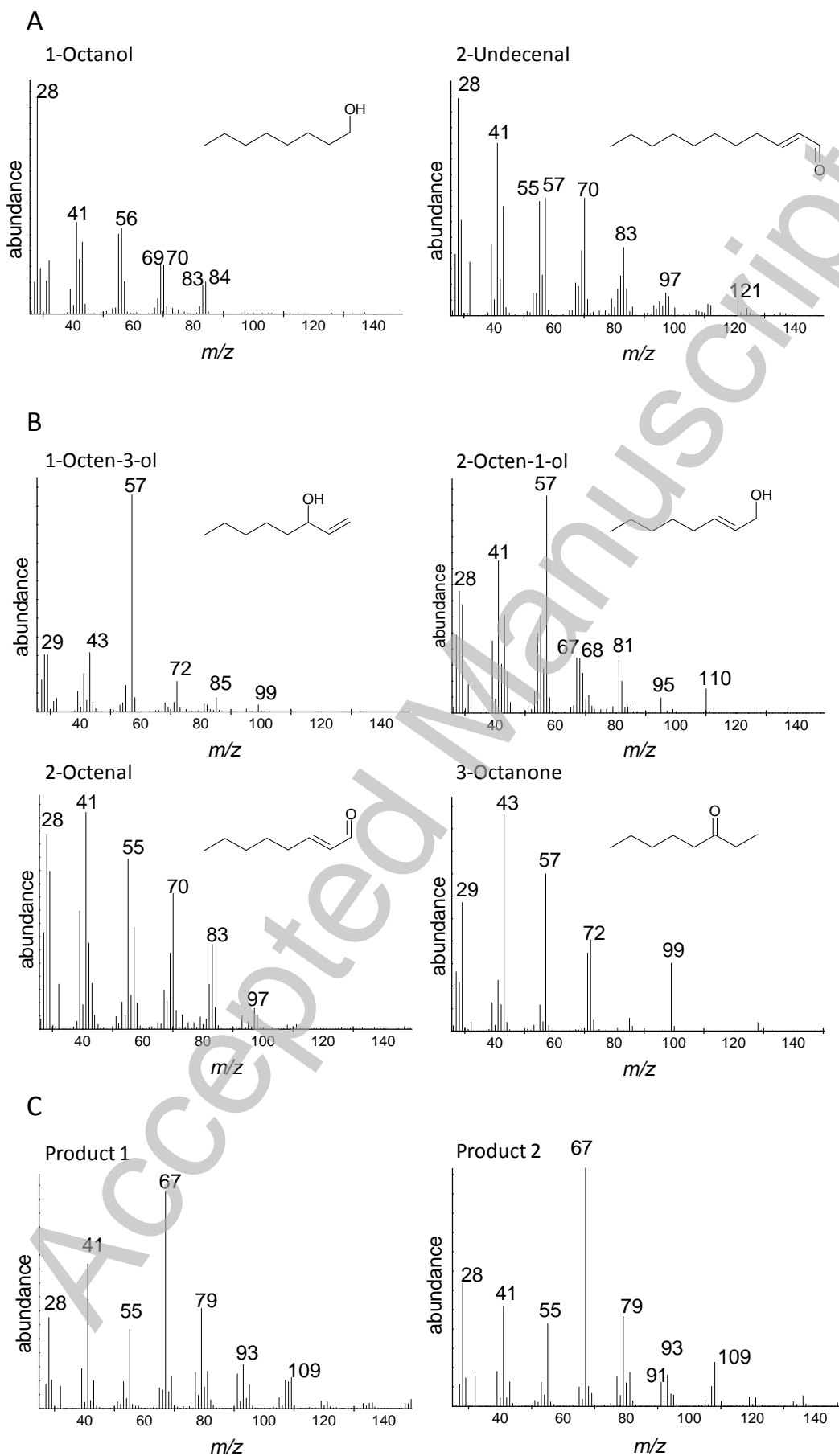


Figure 5



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Figure 6

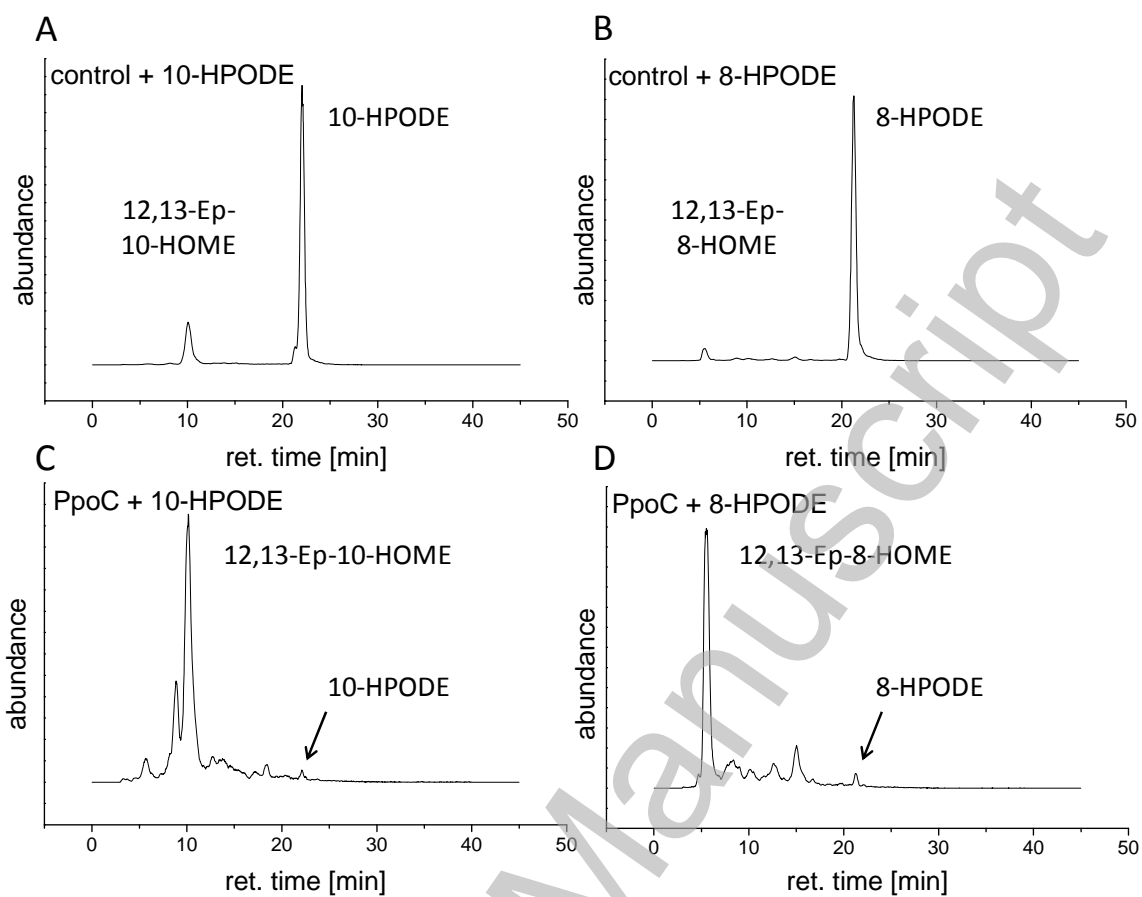


Figure 7

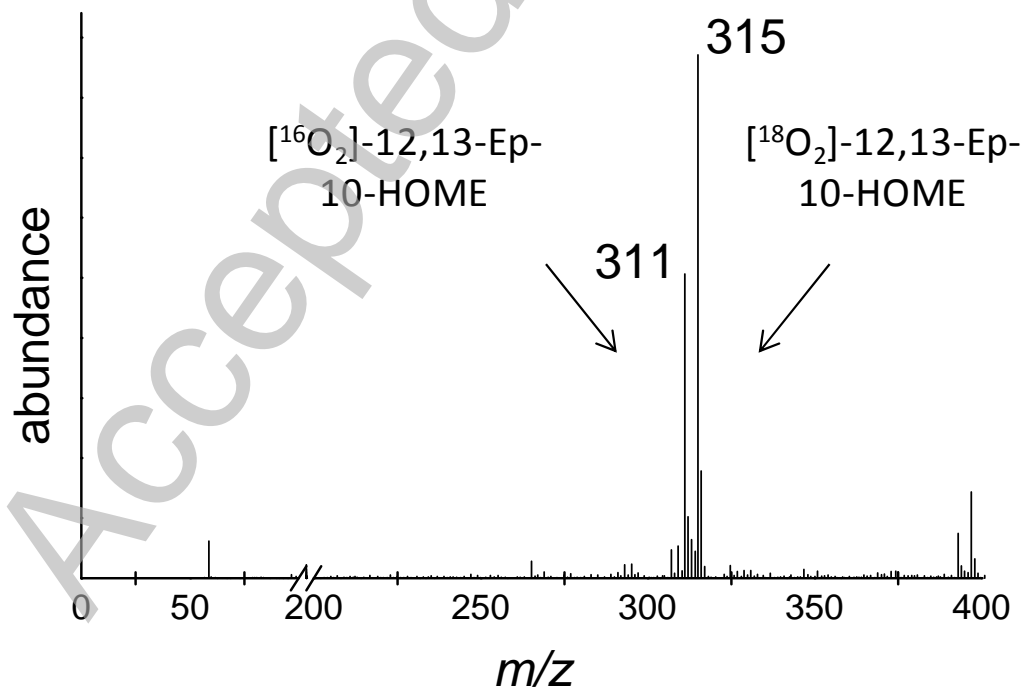




Figure 8

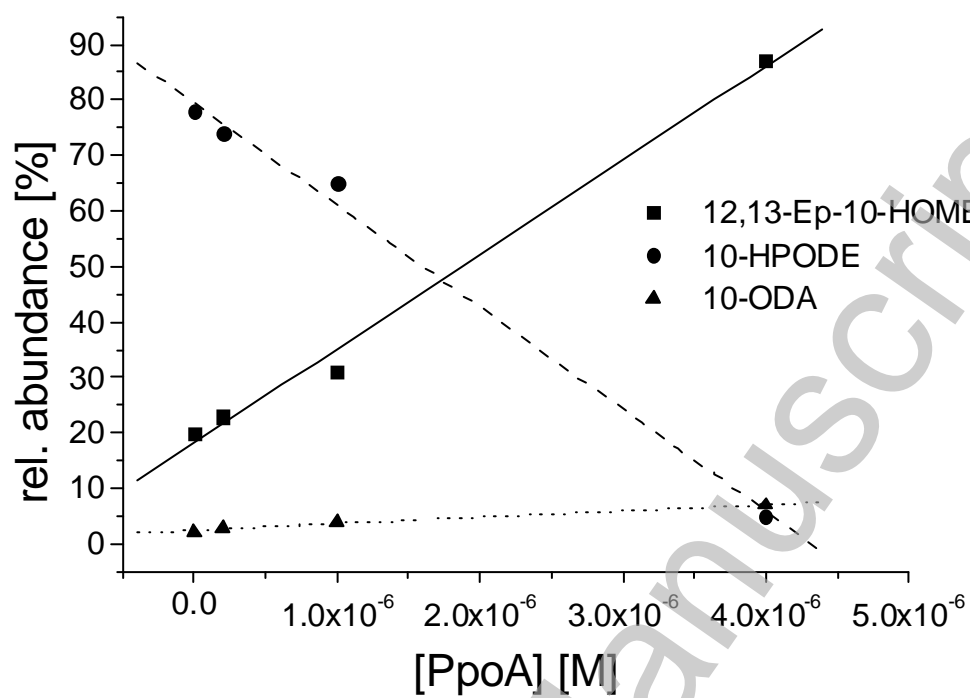


Figure 9

