



# Biotransformation of [U-<sup>13</sup>C]linoleic acid suggests two independent ketonic- and aldehydic cycles within C8-oxylipin biosynthesis in *Cyclocybe aegerita* (V. Brig.) Vizzini

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## Abstract

Although the typical aroma contributing compounds in fungi of the phylum Basidiomycota are known for decades, their biosynthetic pathways are still unclear. Amongst these volatiles, C8-compounds are probably the most important ones as they function, in addition to their specific perception of fungal odour, as oxylipins. Previous studies focused on C8-oxylipin production either in fruiting bodies or mycelia. However, comparisons of the C8-oxylipin biosynthesis at different developmental stages are scarce, and the biosynthesis in basidiospores was completely neglected. In this study, we addressed this gap and were able to show that the biosynthesis of C8-oxylipins differs strongly between different developmental stages. The comparison of mycelium, primordia, young fruiting bodies, mature fruiting bodies, post sporulation fruiting bodies and basidiospores revealed that the occurrence of the two main C8-oxylipins octan-3-one and oct-1-en-3-ol distinguished in different stages. Whereas oct-1-en-3-ol levels peaked in the mycelium and decreased with ongoing maturation, octan-3-one levels increased during maturation. Furthermore, oct-2-en-1-ol, octan-1-ol, oct-2-enal, octan-3-ol, oct-1-en-3-one and octanal contributed to the C8-oxylipins but with drastically lower levels. Biotransformations with [U-<sup>13</sup>C]linoleic acid revealed that early developmental stages produced various [U-<sup>13</sup>C]oxylipins, whereas matured developmental stages like post sporulation fruiting bodies and basidiospores produced predominantly [U-<sup>13</sup>C]octan-3-one. Based on the distribution of certain C8-oxylipins and biotransformations with putative precursors at different developmental stages, two distinct biosynthetic cycles were deduced with oct-2-enal (aldehydic-cycle) and oct-1-en-3-one (ketonic-cycle) as precursors.

**Keywords** Lipoxygenase · 1-octen-3-ol · 3-octanone · Mushroom aroma · Linoleic acid

## Introduction

Volatile compounds like oct-2-en-1-ol (**1**), octan-1-ol (**2**), oct-2-enal (**3**), octanal (**4**), octan-3-ol (**5**), oct-1-en-3-ol (**6**), oct-1-en-3-one (**7**) and octan-3-one (**8**) are known as the main C8-oxylipins from which octan-3-ol (**5**), oct-1-en-3-ol (**6**), oct-1-en-3-one (**7**) and octan-3-one (**8**) are the predominant C8-oxylipins contributing to the characteristic

mushroom aroma (Kleofas et al., 2014; Malheiro et al., 2013; Matsui et al., 2003; Tressl et al., 1982). Although these oxylipins are known for several decades, their biosynthesis is scarcely understood. It is widely accepted that these oxylipins are lipoxygenase-derived metabolites, which play an important role, e.g. in sporulation, sexual and asexual life cycle or act as infochemicals (Brodhun & Feussner 2011; Holighaus et al., 2014). The proposed steps of the C8-oxylipin pathway in mushrooms are the oxygenation of polyunsaturated fatty acids (PUFA) by lipoxygenases (LOX) with a subsequent cleavage by hydroperoxide lyases (HPL) (Combet et al., 2006). In higher fungi of the phyla Basidiomycota and Ascomycota, the C18-PUFA (9Z,12Z)-Octadeca-9,12-dienoic acid (subsequently stated with the trivial name linoleic acid) is the predominant fatty acid (Brodhun and Feussner, 2011). This indicates that the oxygenation of linoleic acid by LOX is the initial step in oxylipin biosynthesis. LOX targets a (1Z,4Z)-pentadiene motif in linoleic acid

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and produces either 13-hydroperoxy-9,11-octadecadienoic acid (13-HPOD) or 9-hydroperoxy-10,12-octadecadienoic acid (9-HPOD), via radical rearrangements and the insertion of molecular oxygen. Until now, only three LOX-type enzymes from Basidiomycota have been characterised that mainly produce 13-HPOD (Karrer & Rühl 2019; Kuribayashi et al., 2002; Plagemann et al., 2013). Although homogenates of *Aspergillus* species converted linoleic acid into 8-hydroperoxy-9,12-octadecadienoic acid (8-HPOD) and 5*S*,8*R*-dihydroxy octadecadienoic acid (5,8-diHODE) as major products, 10*R*-hydroperoxy-8,12-octadecadienoic acid (10*R*-HPOD) and 10*R*-hydroxy-8,12-octadecadienoic acid (10*R*-HODE) have been detected as minor products. The characterisation of the precocious sexual inducer (psi) factor producing genes (*ppoA*, *ppoB* and *ppoC*) revealed that *ppoC* from *A. nidulans* encodes for a 10*R*-dioxygenase (10-DOX), which mainly produces 10-HPOD (Brodhun et al., 2010). Nonetheless, LOX/DOX that oxygenate linoleic acid to 10-HPOD in Basidiomycota yet have to be found. Several studies showed that homogenates of fruiting bodies from Basidiomycota produce a variety of C8-oxylipins and are able to convert linoleic acid (Assaf et al., 1995; Husson et al., 2001; Wurzenberger & Grosch 1982). Remarkably, the most detected oxylipins in fruiting bodies include high amounts of compounds 5–8 (Cruz et al., 1997; Li et al., 2016; Mau et al., 1997). The C8-oxylipins 1–4 are represented more unregularly with notably lower amounts in fruiting bodies from which either oct-2-en-1-ol (1) and octan-1-ol (2) or oct-2-enal (3) and octanal (4) are predominant (Cho et al. 2006, Li et al., 2016; Matsui et al., 2003; Mau et al., 1997).

Furthermore, the comparison of the volatile composition at different developmental stages or parts focused mainly on oct-1-en-3-ol (6) or, in case several C8-oxylipins have been analysed, was limited to fruiting bodies. However, when different developmental stages of fruiting bodies from fungi of the phylum Basidiomycota were observed, maturation was accompanied with vigorous variations in the occurrence of mainly compounds 5–8 while compounds 1–4 seem to undergo minor variations (Cho et al. 2006, Li et al., 2016; Matsui et al., 2003; Mau et al., 1997). Unfortunately, all studies lack a comparison of the C8-oxylipin composition at different stages of the fungal life cycle. So far, differences in the occurrence of C8-oxylipins were only shown for fruiting bodies, where different maturity stages of the basidiocarp were investigated. An exception is the recently published work on the volatilome of *Cyclocybe aegerita* (V. Brig.) Vizzini, where different stages during fructification have been analysed. Nevertheless, C8-oxylipin biosynthesis in basidiospores was completely neglected, and only oct-1-en-3-ol (6) and octan-3-one (8) have been detected (Orban et al., 2020). In this study, this gap is addressed, and the C8-oxylipin composition as well

as the C8-oxylipin-biosynthesis via biotransformations with [ $U$ - $^{13}C$ ]labeled linoleic acid to C8-oxylipins in mycelium, primordia, immature fruiting bodies, mature fruiting bodies, basidiospores and fully mature fruiting bodies after sporulation within the mushroom *C. aegerita*, used as a model fungus (Frings et al., 2020), is investigated. Additionally, the fatty acid composition between different morphological stages is compared.

## Materials and methods

### Chemicals

All chemicals were commercially obtained and used without further treatment. [ $U$ - $^{13}C$ ] linoleic acid (99%  $^{13}C$ , 97% pure) was purchased from Sigma-Aldrich (St. Louis, USA).

### Fungal cultivation and harvesting

For mycelium propagation, 1.5% malt-extract-agar was used. High petri-dishes with 1.5% malt-extract were inoculated with an *C. aegerita* AAE-3 (Herzog et al., 2016) mycelium-overgrown circular agar slice (diameter 0.5 cm) and incubated at 24 °C in darkness until it reached the edge of the petri-dishes. The obtained mycelium was gently removed from the agar-plate and used subsequently for volatile measurements and biotransformations without storage or any other treatment. For fruiting, the overgrown petri-dishes were further cultivated at 24 °C with a 12 h dark/night shift at 95% relative air humidity (Rumed-Rubarth Apparate GmbH, Laatzen, Germany) until the desired developmental stage was obtained. Primordia, immature fruiting bodies with closed caps and post sporulation fruiting bodies were harvested with a scalpel, sliced and immediately used for further experiments. Basidiospores of *C. aegerita* were harvested by cultivation of substrate blocks with immature fruiting bodies (provided by druid-Austernpilze, Ottrau, Germany) at 24 °C in a 12 h dark/night shift at 85% relative air humidity in a climate chamber until sporulation. Dropped basidiospores were gently isolated from the fruiting bodies without damaging them and used for further measurements. Images of the different developmental stages were taken with a Samsung 32 MP camera. Microscopic images were taken using an Olympus microscope BX43 (Olympus Europa SE & CO. KG, Hamburg, Germany) with 20× and 100× magnification equipped with a SC50 (5.0 MP) camera.

### Biotransformations and gas chromatography

For biotransformation, one of the following substrates [ $U$ - $^{13}C$ ]linoleic acid, (*E*)-oct-2-en-1-ol (1), octan-1-ol (2), (*E*)-oct-2-enal (3), octanal (4), octan-3-ol (5), oct-1-en-3-ol

(6), oct-1-en-3-one (7) or octan-3-one (8) with a final concentration of 50  $\mu\text{M}$  were incubated with either mycelium, primordia, immature fruiting bodies, mature fruiting bodies, post sporulation fruiting bodies or basidiospores in 50 mM phosphate buffer (pH 7.5) in a headspace-vial at room temperature. All experiments have been conducted in triplicates. For volatile composition measurements, the fungal parts were soaked in 50 mM phosphate buffer and immediately used for GC-analysis. The resulting products were extracted for 30 min at 30 °C by a solid phase microextraction (SPME) fibre (polydimethylsiloxane/divinylbenzene). After extraction, the volatiles were desorbed in the GC inlet at 250 °C. Analysis was carried out on a Agilent Technologies 7890A GC-system (Santa Clara, USA), equipped with a VFWax column (30 m  $\times$  0.25 mm  $\times$  0.25  $\mu\text{m}$  film thickness, Santa Clara, USA) operated in splitless or 1/50 split mode under the following parameters: carrier gas, He with a constant flow of 1.2 mL  $\cdot$  min<sup>-1</sup>. Oven temperature was at 40 °C (3 min), 5 °C  $\cdot$  min<sup>-1</sup> to 240 °C and hold for 7 min. The mass spectrometer operated in electron impact mode with an electron energy of 70 eV and scanned in a range of m/z 33–300.

### C8-oxylin identification and quantification

C8-oxylin were identified by using authentic standards. Furthermore, the identities were confirmed by their characteristic fragmentation pattern based on standards and the National Institute of Standards and Technology (NIST) Chemistry WebBook. All oxylin were measured in the headspace of commercial vials. To avoid contaminations and minimise known discriminations of headspace compositions by internal standards or other supplemented compounds, no internal standard was added.

### Fatty acid analysis

Fatty acid extraction was carried out via homogenation of the samples with N<sub>2</sub>(liq), followed by the addition of 3 mL *n*-hexane. The mixture was incubated for 20 min at room temperature. Cell debris were removed by centrifugation for 10 min at 4,063 g. Fatty acid containing organic phase was removed from the aqueous phase and concentrated to ~1 mL via a rotary evaporator. Next, 4 mL 0.5 M methanolic NaOH-solution was added to the concentrated extract, followed by incubation for 10 min at 80 °C. For methylation, 3.5 mL 20% methanolic boron trifluoride was added to the extract and incubated for another 5 min at 80 °C. After the mixture was cooled down, 5 mL saturated NaCl-solution was added. The organic phase was separated, dried over anhydrous Na<sub>2</sub>SO<sub>4</sub> and analysed via GC–MS using a VFWax column (30 m  $\times$  0.25 mm  $\times$  0.25  $\mu\text{m}$  film thickness, Santa Clara, USA) operating in splitless mode under the following parameters: carrier gas, He with a constant flow of 1.2 mL  $\cdot$

min<sup>-1</sup>. Oven temperature was at 40 °C (3 min), 3 °C  $\cdot$  min<sup>-1</sup> to 240 °C and hold for 12 min. The mass spectrometer operated in electron impact mode with an electron energy of 70 eV and scanned in a range of m/z 33–300. Fatty acids were identified by their characteristic fragmentation pattern based on authentic standards and the National Institute of Standards and Technology (NIST) Chemistry WebBook.

### Data processing

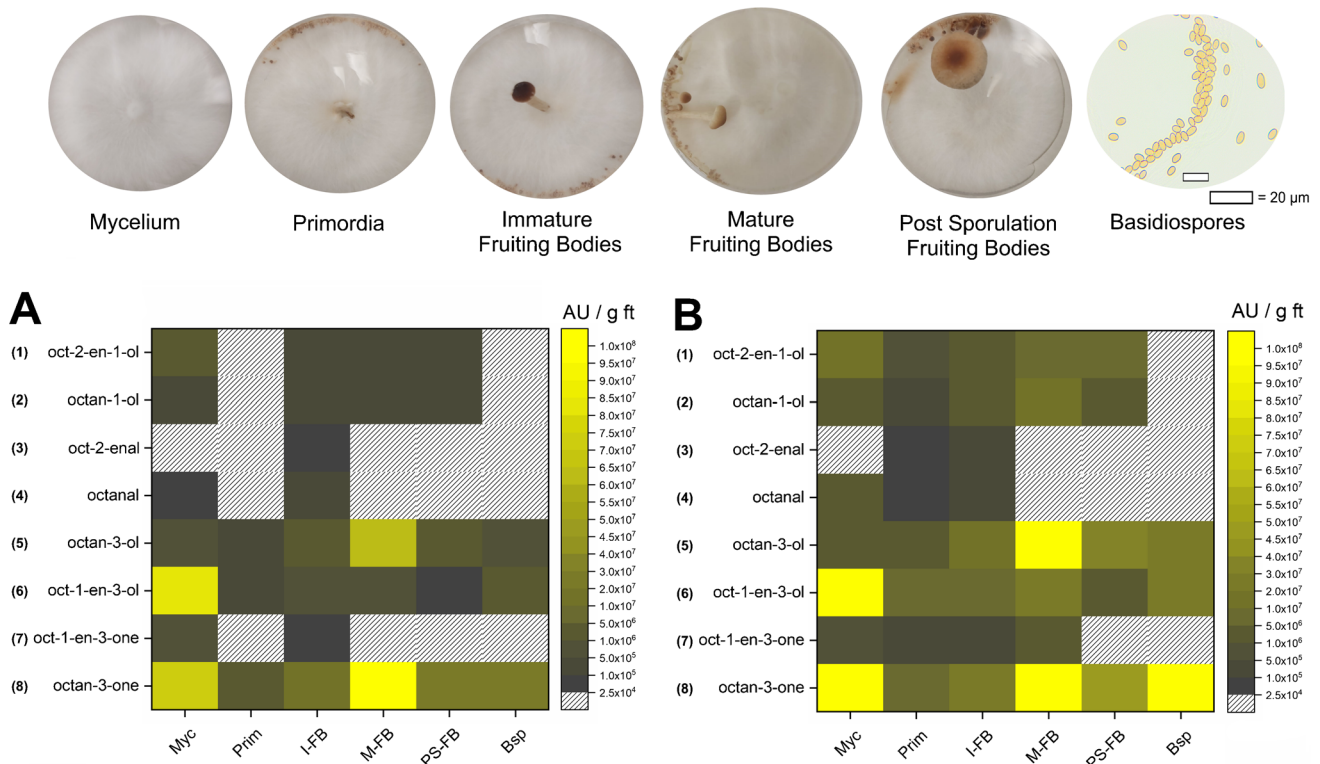
The peak areas of the identified C8-oxylin were used to determine the relative amount of the respective substance. Each relative peak area of a detected C8-oxylin at a certain developmental stage was added up and considered 100%, meaning that the peak area of a certain C8-oxylin correlates to the added up peak areas. This approach enables an appropriate overview of the C8-oxylin composition. Additionally, means of the peak area of a certain C8-oxylin were expressed as AU/g of fresh weight tissue to display the occurrence of each C8-oxylin at different developmental stages to distinguish between the relative composition of C8-oxylin and the actual levels. The processed data were used to generate the heatmaps using OriginPro (OriginLab Corporation, Northampton, MA, USA).

## Results

### Endogenous C8-oxylin composition and biotransformation of [U-<sup>13</sup>C]linoleic acid

The composition of endogenous C8-oxylin at different developmental stages were analysed by means of SPME-GC/MS peak areas. To verify which developmental stage produced the highest amount of a certain C8-oxylin, the obtained peak areas were expressed as arbitrary units per gram of fresh weight tissue (AU/g ft) (Fig. 1). Measuring the volatilome at different developmental stages only allows detection of accumulated C8-oxylin prior to harvesting. Since we were also interested in the overall enzymatic activity towards linoleic acid conversion at a certain developmental stage, additional biotransformation experiments with [U-<sup>13</sup>C]linoleic acid were carried out to provide informations on enzymatically produced [U-<sup>13</sup>C]C8-oxylin and conceivable induction of the endogenous biosynthesis. In total, eight C8-oxylin were identified, which eluted as a mixture of endogenous and [U-<sup>13</sup>C]-labeled compounds (Fig. 2).

The highest AU/g ft of oct-2-en-1-ol (1), octan-1-ol (2), oct-1-en-3-ol (6), oct-1-en-3-one (7) and octan-3-one (8) was detected in mycelial samples followed by a striking decrease at the primordial stage. With ongoing maturation, the levels of most C8-oxylin recovered in immature fruiting bodies



**Fig. 1** Various developmental stages of *C. aegerita* that were used for SPME-GC/MS measurements and biotransformations with [ $^{13}\text{C}$ ]linoleic acid. Endogenous C8-oxylipin occurrence is shown as arbitrary units per gram of fresh weight (AU/g ft) (**A**). C8-oxylipin occurrence after biotransformation of [ $^{13}\text{C}$ ]linoleic acid is shown as arbitrary

units per gram of fresh weight (AU/g ft) (**B**). Myc=mycelium, Prim=Primordia, I-FB=immature fruiting bodies, M-FB=mature fruiting bodies, PS-FB=post sporulation fruiting bodies, Bsp=basidiospores (Fig. 1). Dashed boxes = not detected/low peak area

and decreased afterwards. An exception was octan-3-one (**8**), which steadily increased or remained at high levels after the primordial stage (Fig. 1A). C8-aldehydes were only detected at two stages being most present in immature fruiting bodies (Fig. 1A). Octan-3-ol (**5**) was detected throughout all developmental stages, starting with low levels in the mycelium and primordia, followed by an increased extent in immature and mature fruiting, and decreased thereafter. Addition of [ $^{13}\text{C}$ ]linoleic acid resulted in a pattern comparable to non-treated fungal tissue for oct-2-en-1-ol (**1**), oct-1-en-3-ol (**6**), oct-1-en-3-one (**7**) and octan-3-one (**8**) (Fig. 1B). Interestingly, primordia were able to produce low levels of compounds **1–4** and oct-1-en-3-one (**7**) from [ $^{13}\text{C}$ ]linoleic acid, although no endogenous production was detected (Fig. 1A). Surprisingly, mature and post sporulation fruiting bodies produced slightly more octan-1-ol (**2**) than mycelium after incubation, which is opposing to the naturally occurring levels (Fig. 1A, B). Somewhat comparable results were obtained for octanal (**4**) and octan-3-ol (**5**) production of non-treated and incubated fungal tissue. Although highest endogenous octanal levels were detected in immature fruiting bodies, after incubation with [ $^{13}\text{C}$ ]linoleic acid mycelium produced higher levels of octanal than

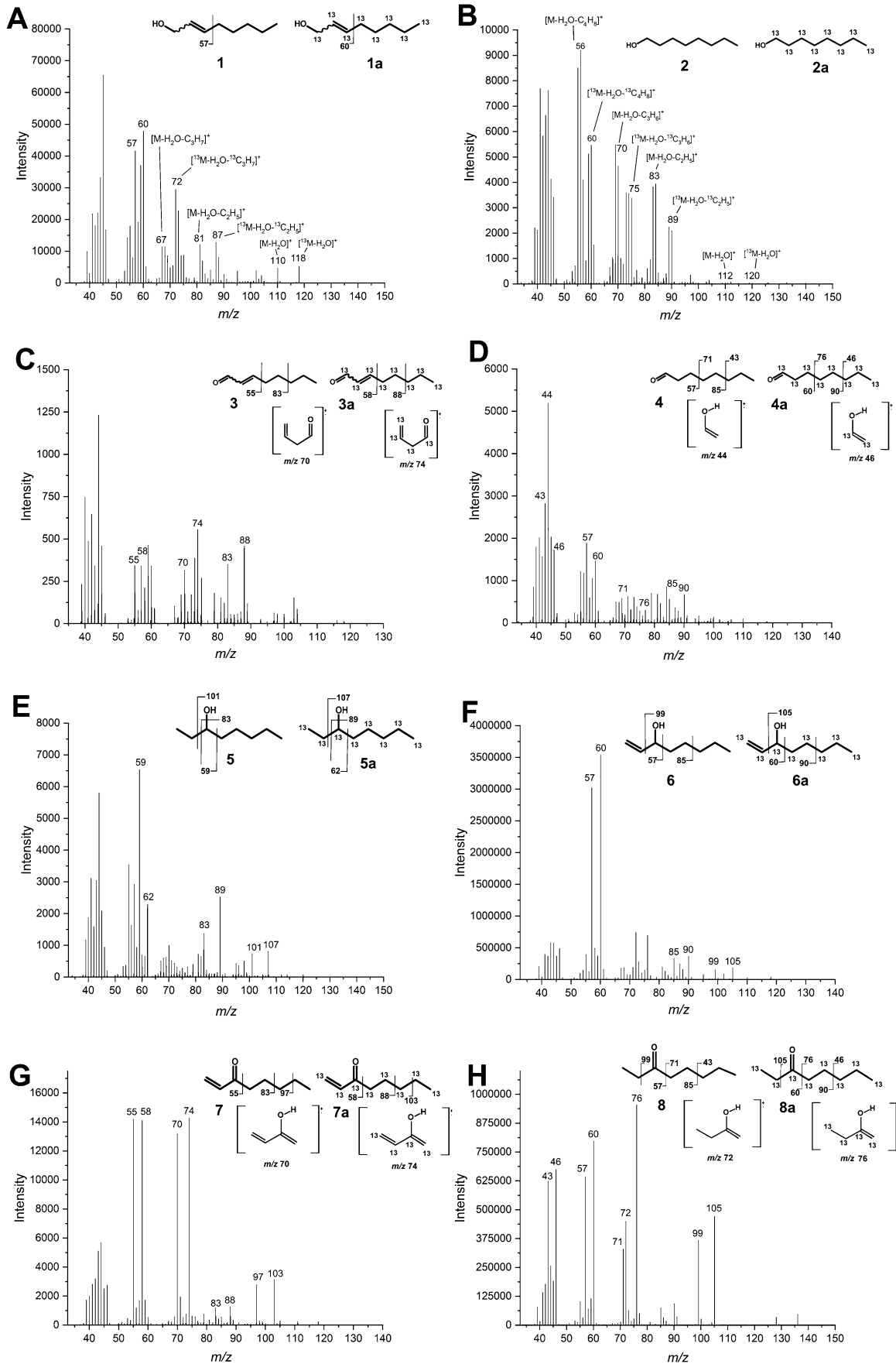
immature fruiting bodies (Fig. 1A, B). Levels of octan-3-ol (**5**) increased with ongoing maturity when [ $^{13}\text{C}$ ]linoleic acid was added to the fungal tissue. This is also opposing the endogenous occurring levels (Fig. 1A, B).

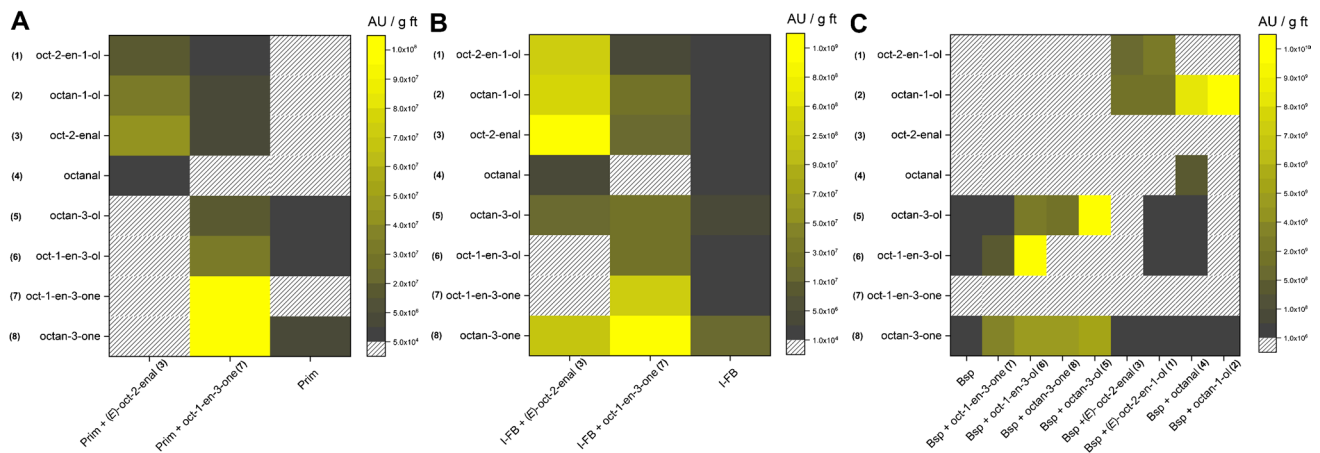
### Effects of putative precursors on oxylipin composition

Based on an emerged pattern of mutual occurring C8-oxylipins, especially between primordia and immature fruiting bodies, the hypothesis that certain C8-oxylipins derive from putative short chain precursors, oct-2-enal (**3**) or oct-1-en-3-one (**7**), has been evaluated via additional biotransformation experiments (Fig. 3).

Primordia showed a completely different C8-oxylipin pattern when oct-2-enal (**3**) or oct-1-en-3-one (**7**) was added.

**Fig. 2** Mass spectra of the co-eluted endogenous and [ $^{13}\text{C}$ ]labeled C8-oxylipins. Characteristic fragmentations pattern of **A** oct-2-en-1-ol (**1**), [ $^{13}\text{C}$ ]oct-2-en-1-ol (**1a**). **B** octan-1-ol (**2**), [ $^{13}\text{C}$ ]octan-1-ol (**2a**). **C** oct-2-enal (**3**), [ $^{13}\text{C}$ ]oct-2-enal (**3a**). **D** octanal (**4**), [ $^{13}\text{C}$ ]octanal (**4a**). **E** octan-3-ol (**5**), [ $^{13}\text{C}$ ]octan-3-ol (**5a**). **F** oct-1-en-3-ol (**6**), [ $^{13}\text{C}$ ]oct-1-en-3-ol (**6a**). **G** oct-1-en-3-one (**7**), [ $^{13}\text{C}$ ]oct-1-en-3-one (**7a**). **H** octan-3-one (**8**), [ $^{13}\text{C}$ ]octan-3-one (**8a**)





**Fig. 3** Influence of different C8-oxylipins on their composition at different developmental stages. Means of SPME-GC/MS peak areas were expressed as arbitrary units per gram of fresh weight tissue

(AU/g ft). **A** Prim = primordia, **B** I-FB = immature fruiting bodies, **C** Bsp = basidiospores. Dashed boxes = not detected or low peak area ( $< 5 \cdot 10^4$  AU/g ft)

Addition of oct-1-en-3-one (7) led to strikingly increased levels of octan-3-ol (5), oct-1-en-3-ol (6) and octan-3-one (8), while addition of oct-2-enal (3) led to a drastic increase of oct-2-en-1-ol (1), octan-1-ol (2) and octanal (4), compared to the endogenously produced C8-oxylipin composition (Fig. 1, Fig. 3A). A similar pattern was observed in immature fruiting bodies with a strong increase of octan-3-ol (5), oct-1-en-3-ol (6) and octan-3-one (8) when oct-1-en-3-one (7) was added. However, an increase of oct-2-en-1-ol (1) and octan-1-ol (2) with oct-2-enal (3) as a substrate was detected (Fig. 3B). Due to the small number of endogenously produced C8-oxylipins, basidiospores showed the lowest endogenous background. Therefore, they were used for a more detailed investigation of the C8-oxylipin pathway using various substrates for additional biotransformation experiments. In basidiospores, a complete conversion of oct-1-en-3-one (7) to octan-3-one (8), oct-1-en-3-ol (6) and octan-3-ol (5) was observed (Fig. 3C), whereas oct-1-en-3-ol (6) was converted to octan-3-ol (5) and octan-3-one (8) but in drastically lower levels. A weak reduction of octan-3-one (8) resulted in a slight increase of octan-3-ol (5), while the reversed oxidation of octan-3-ol (5) to octan-3-one (8) was also detected (Fig. 3C). Just like oct-1-en-3-one (7), oct-2-enal (3) was completely transformed resulting in high octan-1-ol (2) and oct-2-en-1-ol (1) levels. Furthermore, basidiospores reduced octanal (4) to octan-1-ol (2) and the addition of oct-2-en-1-ol (1) led to increased octan-1-ol (2) levels, whereas octan-1-ol (2) was not converted (Fig. 3C).

### Free fatty acid composition at different developmental stages

Due to the fact that fatty acids are the precursors of C8-oxylipins, the free fatty acid composition between mycelium, immature fruiting bodies, mature fruiting bodies and

basidiospores was investigated. The analysed fatty acids were grouped into saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) of which palmitic acid (hexadecanoic acid, 16:0) was the most prominent SFA and linoleic acid ((9Z,12Z)-Octadeca-9,12-dienoic acid, 18:2 (9Z, 12Z)) the most prominent PUFA. Regarding MUFA, oleic acid ((9Z)-Octadeca-9-enoic acid, 18:1 (9Z)) and *cis*-vaccenic acid ((11Z)-Octadec-11-enoic acid, 18:1 (11Z)) were similarly distinctive (Table 1).

In the mycelium and fruiting body samples, the fatty acid composition was alike (Table 1). However, the ratio of SFA and MUFA increased by ~15% with ongoing maturation. Simultaneously, the linoleic acid ratio decreased with

**Table 1** Fatty acid composition in % at various developmental stages. SFA saturated fatty acids, MUFA monounsaturated fatty acids, PUFA polyunsaturated fatty acids. Myc mycelium, I-FB immature fruiting bodies, M-FB mature fruiting bodies, Bsp basidiospores

Fatty acid	Myc / %	I-FB / %	M-FB / %	Bsp / %
14:0	0.4 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.6 ± 0.0
15:0	0.3 ± 0.1	1.2 ± 0.1	2.8 ± 0.1	0.4 ± 0.0
16:0	19.0 ± 0.7	20.0 ± 0.3	23.4 ± 0.2	34.8 ± 1.0
17:0	-	0.2 ± 0.1	0.2 ± 0.0	-
18:0	1.1 ± 0.1	2.4 ± 0.2	0.9 ± 0.1	2.5 ± 0.1
SFA*	21%	24%	27%	38%
16:1 (11Z)	-	0.3 ± 0.1	0.4 ± 0.0	-
18:1 (9Z)	1.4 ± 0.0	3.4 ± 0.3	3.5 ± 0.1	18.7 ± 0.6
18:1 (11Z)	1.3 ± 0.0	4.2 ± 0.3	7.2 ± 0.2	1.6 ± 0.0
MUFA*	3%	8%	11%	20%
18:2 (9Z, 12Z)	76.5 ± 0.6	68.2 ± 0.6	61.6 ± 0.2	41.4 ± 0.4
20:2 (11Z, 14Z)	-	0.1 ± 0.1	-	-
PUFA*	77%	68%	62%	41%

\* = summated and rounded values of the corresponding fatty acids. Significance can be calculated from the data above

maturation. In basidiospores, the fatty acid composition was drastically different compared to the other analysed compartments. SFA, especially palmitic acid rose to ~35% while linoleic acid accounted for only ~41%. Furthermore, oleic acid soared to ~19% in basidiospores (Table 1).

## Discussion

### C8-oxylipin composition

Unlike prior studies in which oct-1-en-3-ol (**6**) was the predominant volatile in basidiomycetous mycelium or fruiting body homogenates (Akakabe et al., 2005, Cho et al. 2006, Cruz et al., 1997, Li et al., 2016, Matsui et al., 2003, Tressl et al., 1982), the most abundant C8-oxylipins in *C. aegerita* throughout all developmental stages was octan-3-one (**8**). This discrepancy is mainly caused due to the used extraction and homogenation method, which is often overlooked. This becomes clear in studies of Combet et al., (2009) and Rapior et al., (1998), who compared either homogenised, sliced and whole samples of fruiting bodies of *A. bisporus* or different extraction methods of fruiting bodies of *C. aegerita*, respectively. Disruption of fungal tissue could lead to the release of membrane bound fatty acids, interfering with endogenous formation and, thus, complicate the understanding of C8-oxylipin biosynthesis. Moreover, cellular compartments could also be harmed and, thus, might trigger the formation and release of C8-oxylipins (Combet et al., 2009). While homogenised samples and liquid extraction led to higher production of oct-1-en-3-ol (**6**), headspace measurements of whole or sliced samples showed higher octan-3-one (**8**) levels (Combet et al., 2009). Previous studies focused on disruption and liquid extraction methods and consequently found oct-1-en-3-ol (**6**) as the main C8-oxylipin in different fungal species. Nonetheless, the variety of different C8-oxylipins (up to eight) detected in these studies is widely in accordance with our findings (Akakabe et al., 2005, Cho et al. 2006, Cruz et al., 1997, Li et al., 2016, Matsui et al., 2003, Tressl et al., 1982).

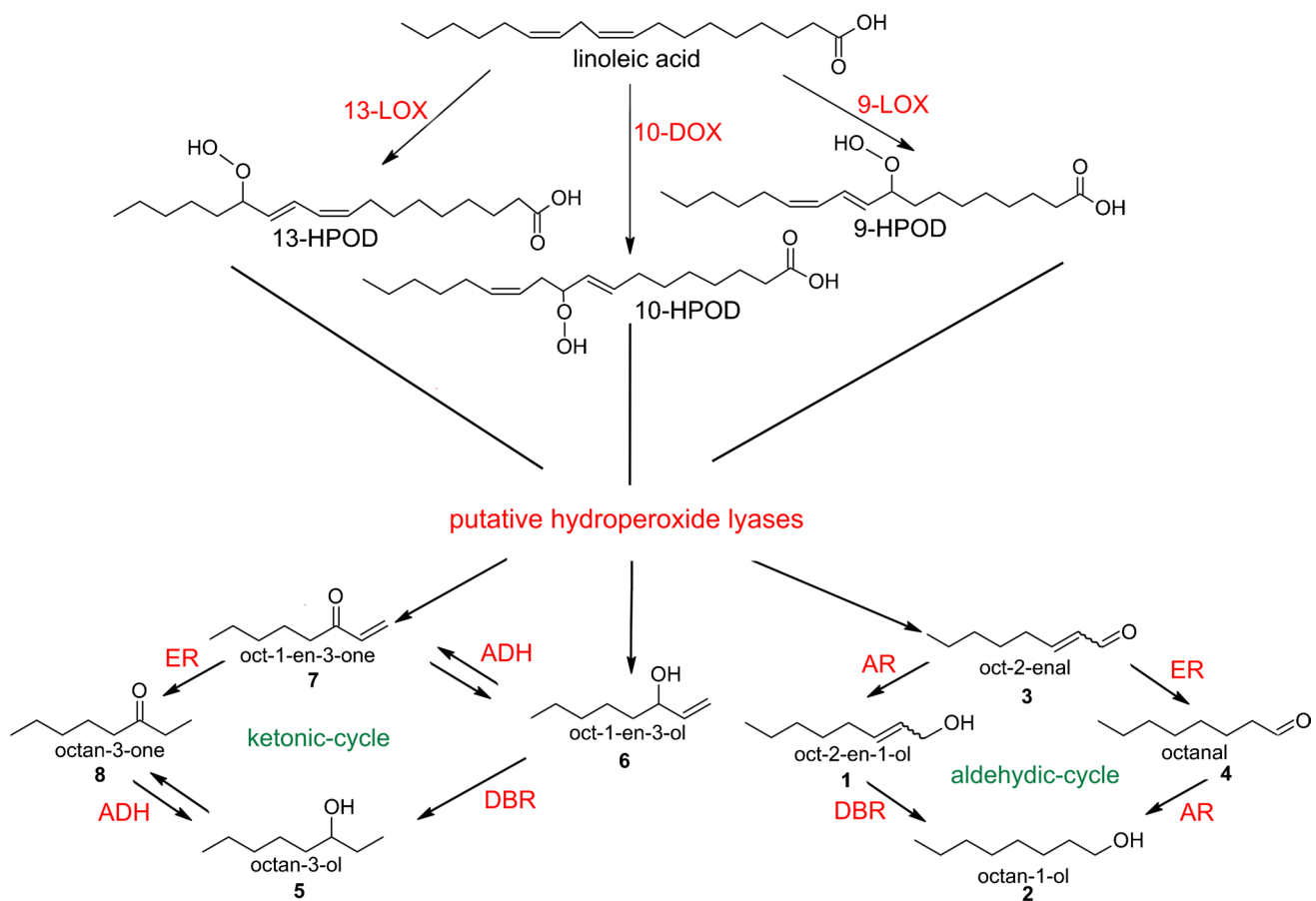
Although octan-3-one is the predominant oxylipin in this study and not oct-1-en-3-ol (**6**), both were detected throughout all developmental stages emphasising their biological importance. Electroantennographic experiments showed that the fungivorous beetle *Bolitophagus reticulatus* is able to differentiate between the most common C8-oxylipins to assess host quality (Holighaus et al., 2014). Here, oct-1-en-3-ol (**6**) acts as repellent and octan-3-one (**8**) as attractant. Contradictory, the three wood-living generalist beetles *Malthodes fuscus*, *Anaspis marginicollis*, *Anaspis rufilabris* and the moth *Epinotia tedella* were attracted to oct-1-en-3-ol (**6**), whereas the

generalist predator on fungus-insects *Lordithon lunulatus* distinguished between oct-1-en-3-ol (**6**) and octan-3-one (**8**) and was attracted by a mixture of both C8-oxylipins (Fäldt et al. 1998). Besides interspecies communication, C8-oxylipins might also operate as intra-species signals as shown in *Penicillium paneum* where oct-1-en-3-ol (**6**) functions as a self-inhibitor signal in spore germination (Schulz-Bohm et al., 2017). These studies reveal that C8-oxylipins have different functions and, consequently, their occurrence depends on the developmental stage of the fungus. To investigate the enzymatic potential of each developmental stage towards the production of different C8-oxylipins, [U-<sup>13</sup>C]linoleic acid has been added to different fungal tissues. The resulting [U-<sup>13</sup>C]C8-oxylipins co-eluted with the endogenous C8-oxylipins and, therefore, the detected mass spectra were a mixture of non-labeled  $\alpha$ -cleavage fragmentations, McLafferty ions and their corresponding fragmentations with a <sup>13</sup>C-mass shift (Fig. 2A–H).

### Influence of linoleic acid on C8-oxylipin composition

In general, major differences in the production of [U-<sup>13</sup>C] C8-oxylipins were detected that indicates varying enzymatic activities at certain developmental stages (Fig. 1B). Especially the developmental process of fruiting bodies shows drastic morphology dependent C8-oxylipin production (Fig. 1). This is highlighted by the cluster-like occurrence of certain C8-oxylipins, noticeable particularly for compounds **5–8** in late developmental stages (Fig. 1B) and compounds **1–4** in early developmental stages (Fig. 1B). Since post sporulation fruiting bodies and basidiospores showed low oct-1-en-3-one (**7**) but increasing octan-3-one (**8**) production, accompanied with fairly consistent levels of octan-3-ol (**5**) and oct-1-en-3-ol (**6**), we propose the existence of a biocatalytic cycle with oct-1-en-3-one (**7**) as precursor defined as ketonic-cycle (oct-1-en-3-one-cycle) (Fig. 1, Fig. 4). In contrast, compounds **1–4** were predominantly detected in early developmental stages with decreasing contribution in more mature developmental stages. Therefore, we propose a second biocatalytic cycle with oct-2-enal (**3**) as precursor that is defined as aldehydic-cycle (oct-2-enal-cycle) (Fig. 1, Fig. 4).

In primordia, only three C8-oxylipins (octan-3-ol (**5**), oct-1-en-3-ol (**6**) and octan-3-one (**8**)) occurred endogenously, although primordia were able to produce all eight C8-oxylipins when [U-<sup>13</sup>C]linoleic acid was added (Fig. 1A, B). Interestingly, with the addition of [U-<sup>13</sup>C]linoleic acid to primordia, all C8-oxylipins were detected as non-labeled and [U-<sup>13</sup>C]compounds that indicates the induction of endogenous C8-oxylipin biosynthesis. In contrast, all eight C8-oxylipins emerged endogenously in immature fruiting bodies. Therefore, an upregulation of aldehydic- and



**Fig. 4** Hypothetical C8-oxylipin pathway in Basidiomycota. The oxygenation of linoleic acid by lipoxygenases/dioxygenases (LOX, DOX) is considered as the initial step. Subsequently, the hydroperoxy-fatty acids (HPOD) are cleaved by putative hydroperoxide lyases (HPL) through an unknown mechanism which could lead to two distinct

biocatalytic cycles with oct-1-en-3-one (7) and oct-2-enal (3) as precursors. Further modifications via ene-reductases (ER), double bond reductases (DBR), aldo-keto reductases (AR) and alcohol dehydrogenases (ADH) could lead to the variety of known C8-oxylipins

ketonic-cycle related genes in the transition from primordia to immature fruiting bodies seems conceivable. By comparing the AU/g ft of each C8-oxylipin in these two developmental stages, it is clearly noticeable that immature fruiting bodies produced more C8-oxylipins of both cycles compared to primordia, which is consistent with a conceivable upregulation of the aldehydic- and ketonic-cycle between these two stages (Fig. 3A, B). Furthermore with ongoing maturity, a downregulation of the aldehydic and an upregulation of the ketonic-cycle seems reasonable (Fig. 1). This is elucidated by the comparison of the C8-oxylipin composition and biosynthesis in immature fruiting bodies, mature fruiting bodies, post sporulation fruiting bodies and basidiospores, where the total number of different C8-oxylipins declined from eight in immature fruiting bodies, to five in mature and post sporulation fruiting bodies, and three in basidiospores (Fig. 1). The vanishing C8-oxylipins with maturation are all related to the aldehydic-cycle, while the ketonic-cycle related C8-oxylipins remain in high amounts. This

strengthens the hypothesis of two distinct biosynthetic pathways. Similar observations were made by Li et al., (2016), who showed that egg-shaped, bell-shaped and mature fruiting bodies of *Tricholoma matsutake* produced predominantly octan-3-one (8) and oct-1-en-3-ol (6). With ongoing maturation of *T. matsutake* increasing levels of octan-3-one and decreasing oct-1-en-3-ol (6) levels have been detected. On the other hand, octan-1-ol (2), octanal (4) and oct-2-en-1-ol (1) were either not detected or in low levels that decreased with ongoing maturity. This is in accordance with our hypothesis that a ketonic cycle (e.g. octan-3-one (8)) is getting more prominent during maturation, whereas the aldehydic-cycle (e.g. octan-1-ol (2)) is decreasing concurrently. Furthermore in *T. matsutake*, oct-2-enal was present in higher proportions in immature fruiting stages (egg- and bell-shaped fruiting bodies) followed by a strong decrease with maturation, which underlines the downregulation of the aldehydic-cycle in mature developed stages and is fairly in accordance with our hypothesis. Mau et al., (1997)



compared the octan-3-ol (5), oct-1-en-3-ol (6), octan-1-ol (2) and oct-2-en-1-ol (1) levels in liquid extracts from aging fruiting bodies of *V. volvacea*. Amounts of ketonic-cycle related C8-oxylipins, particularly oct-1-en-3-ol (6) increased with maturity. This indicates a higher activity of the ketonic-cycle in more developed fruiting bodies. In contrast, aldehydic-cycle related C8-oxylipins were detected overall in lower amounts, which did not undergo any significant change in their composition. Moreover, Cho et al. (2006) analysed different maturity grades of fruiting bodies from *T. matsutake* that showed an increase in the overall level of ketonic- and aldehydic-cycle related C8-oxylipins. Yet, higher relative peak areas of the ketonic-cycle related C8-oxylipins were identified in all developmental stages conceivably induced by a higher biocatalytic activity of the ketonic-cycle. Nevertheless, it has to be mentioned that liquid extracts from *T. matsutake* and not the headspace was analysed. Studies by Kleofas et al., (2015) and Tressl et al., (1982) demonstrated that liquid extracts of fruiting bodies of *Calocybe gambosa* and *Agaricus campestris* contained strikingly more ketonic-cycle related C8-oxylipins compared to the aldehydic-cycle, which supports our hypothesis of an increased respectively high activity of the ketonic-cycle in mature developmental stages.

It is noteworthy that the total amount and number of C8-oxylipins decline with maturation (Fig. 1). Although fatty acids are involved in many biological functions, the differences in the fatty acid composition, especially the steady decline of linoleic acid contribution at more mature developmental stages and the decrease of C8-oxylipins is noticeable (Table 1). Furthermore, we were able to demonstrate that the fatty acid composition of basidiospores consists of significantly higher ratios of SFA and MUFA, which could be explained by their differing role in morphological and physiological stages (Table 1).

### Biotransformations of precursors from the ketonic- and aldehydic-cycle

As already mentioned, an upregulation of the aldehydic- and ketonic-cycle in the transition from primordia to immature fruiting bodies is hypothesised. Therefore, these two developmental stages were chosen for biotransformation experiments with the precursors oct-1-en-3-one (7) and oct-2-enal (3). As presumed, a specific and strong cluster-like increase of C8-oxylipins from the ketonic-cycle was detected in immature fruiting bodies when oct-1-en-3-one (7) was used as a substrate. Additionally, oct-1-en-3-one (7) also seemed to induce endogenous C8-oxylipin production explained by the slight increase of octan-1-ol (2), oct-2-en-1-ol (1) and oct-2-enal (3) (Fig. 3B). An opposing effect was observed when oct-2-enal (3) was added to immature fruiting bodies. The drastic cluster-like increase of aldehydic-cycle related

C8-oxylipins was accompanied with a minimal increase of octan-3-ol (5) and octan-3-one (8), while oct-1-en-3-ol (6) and oct-1-en-3-one (7) decreased (Fig. 3B). A comparable impact was observed for primordia. Addition of oct-1-en-3-one (7) led to a strong increase of ketonic-cycle related C8-oxylipins and a slight increase of aldehydic-cycle related C8-oxylipins 1–3 (Fig. 3A). In contrast, adding oct-2-enal to primordia, aldehydic-cycle related C8-oxylipins raised vigorously with a distinct reduction of ketonic-cycle related C8-oxylipins (Fig. 3A). Based on these observations, oct-1-en-3-one (7) and its derived compounds could have an inducing effect on the ketonic- and aldehydic-cycle. An opposing inhibitory effect on the ketonic-cycle seems to be caused by oct-2-enal-derived compounds.

### Biotransformations in Basidiospores

As related to primordia and immature fruiting bodies, addition of oct-1-en-3-one (7) to basidiospores led to a strong increase of the ketonic-cycle related oxylipins octan-3-one (8), oct-1-en-3-ol (6) and octan-3-ol (5). Furthermore, addition oct-1-en-3-ol (6) to basidiospores resulted in a slight increase of octan-3-one (8) and octan-3-ol (5) suggesting an oxidation of the alcohol with a following reduction of the double-bond leading to octan-3-one (8). The oxidation of octan-3-ol (5) was also observed when added to basidiospores, indicating an alcohol dehydrogenase (ADH) equilibrium system between octan-3-ol (5), oct-1-en-3-ol (6) and the corresponding ketones. A comparable equilibrium system was not observed for the aldehydic-cycle in basidiospores. Adding oct-2-en-1-ol (1) or octan-1-ol (2) to basidiospores did not lead to an oxidation to the aldehydes octanal (4) or oct-2-enal (3). However, oct-2-enal (3) and octanal (4) were completely or strongly reduced to oct-2-en-1-ol (1) and/or octan-1-ol (2) (Fig. 3C). The addition of oct-2-enal (3) and octan-1-ol (2), likewise in primordia and immature fruiting bodies (Fig. 3A, B), seems to inhibit the ketonic-cycle, which is reflected by the decrease of oct-1-en-3-ol (6) and octan-3-ol (5) levels (Fig. 3C).

### Independent ketonic- and aldehydic cycle

The aldehydic- and ketonic-cycle seems to consist mainly of ene-reductases (ER), keto-/aldehyde-reductases (KR, AR) and alcohol dehydrogenases (ADH), which all play an important role after the oxidation of linoleic acid and the subsequent, putative cleavage of 13-, 10-, 9-HPOD to shortened C8-oxylipins (Fig. 4). A recently published study by Orban et al., (2021) investigating the transcriptome of different fruiting stages in *C. aegerita* supports our hypothesis of distinct pathways within the C8-oxylipin biosynthesis. By combining transcriptome and volatilome data, different sets of putative LOX, DOX, ER and ADH genes potentially

involved in oct-1-en-3-ol (**6**) and octan-3-one (**8**) biosynthesis could be identified. Darriet et al., (2002) were able to show the reduction of oct-1-en-3-one (**7**) and oct-1,5-dien-3-one in *S. cerevisiae* cells. Chen and Wu (1983) pointed out a reduction of oct-1-en-3-one (**7**) to oct-1-en-3-ol (**6**) and octan-3-one (**8**) in *A. bisporus*. Furthermore, Wanner and Tressl (1998) were able to identify two enone-reductases from *S. cerevisiae* with a reduction activity towards oct-1-en-3-one (**7**). The hypothesis of ERs reducing C8-oxylipin related alkenals and alkenones are reinforced by our recently characterised ene-reductase CaeEnR1 from *C. aegerita*. On the one hand, we were able to show that oct-1-en-3-one (**7**) and oct-2-enal (**3**) were reduced to octan-3-one and octanal. On the other hand, a reduction of oct-1-en-3-ol (**6**) or oct-2-en-1-ol (**1**) was not observed, suggesting a different set of enzymes involved in the transformation of C8-oxylipin related alcohols (Fig. 4) (Karrer et al., 2021a, b). Nevertheless, the role of the putative intermediates 13-, 10- and 9-HPOD has to be clarified in further experiments. Yet, only a coherence of 10-HPOD and oct-1-en-3-ol (**6**) was indicated by Wurzenberger and Grosch (1984b), who showed that a crude extract of *A. bisporus* was able to cleave 10-HPOD to oct-1-en-3-ol (**6**), while 9-, 12- and 13-HPOD were not converted to any C8-oxylipin. Furthermore, Assaf et al., (1995) detected an accumulation of 13-HPOD, accompanied with an increase of oct-1-en-3-ol (**6**) after adding linoleic acid to a homogenate of *Pleurotus pulmonarius*. Nevertheless, the addition of 13-HPOD as a precursor did not lead to an increase of oct-1-en-3-ol (**6**). Although, Joh et al. (2012) proposed a cleavage of 13-HPOD to oct-1-en-3-one (**7**) with subsequent reductions or a direct cleavage of 10-HPOD to oct-1-en-3-ol (**6**) in *P. ostreatus*. This would imply two biosynthetic pathways including the involvement of a DOX producing 10-HPOD and a LOX converting linoleic acid to 13-HPOD. However, no coherence between 9-HPOD and certain C8-oxylipins exists, although it is well known that fungi of the phylum Basidiomycota harbour various LOX in their genomes. Additionally, it was shown that gene expression levels highly depend on the developmental stages (Orban et al., 2021; Tasaki et al., 2019). Few of the known LOX genes from *P. ostreatus*, *P. sapidus*, *P. dryinus*, *P. sajor-caju* and *C. aegerita* have been cloned, heterologously expressed and tested for their specific activity towards linoleic acid (Karrer & Rühl 2019; Karrer et al., 2021a, b; Kuribayashi et al., 2002; Leonhardt et al., 2013; Plagemann et al., 2013). However, among these LOX, only three were comprehensively characterised which predominantly produce 13-HPOD and rarely minor amounts of 9-HPOD from linoleic acid (Karrer & Rühl 2019; Kuribayashi et al., 2002; Plagemann et al., 2013), whereas 10-HPOD has not been detected as a LOX product. Only DOX from Ascomycota are known to produce 10-HPOD from linoleic acid (Brodhun et al., 2010). Taken together previous findings and results

from our biotransformation experiments, the existence of two independent ketonic- and aldehydic-cycles, branching after HPOD-cleavage, seems reasonable (Fig. 4).

## Conclusion

Although further research in C8-oxylipin biosynthesis is required, we were able to show that two morphology dependent and distinct biocatalytic cycles seem reasonable by combining metabolomic analysis. The aldehydic-cycle appears to be more active in early developmental stages, whereas the ketonic-cycle is active throughout all developmental stages, peaking at the stage of sporulation. The existence of distinct pathways was underlined with biotransformation experiments using the putative precursor of the aldehydic-cycle oct-2-enal (**3**) and the precursor of the ketonic-cycle oct-1-en-3-one (**7**). With the addition of each precursor, a drastic increase of the corresponding aldehydic- or ketonic-cycle related C8-oxylipins was detected, which supports our hypothesis. Nonetheless, genes encoding for putative LOX, DOX as well as the modifying enzymes of shortened oxylipins have to be the focus of further studies.

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**Data availability** All datasets are presented in the main manuscript and in the supporting file.

**Code availability** Not applicable.

## Declarations

**Ethics approval** Not applicable.

**Conflict of interest** The authors declare no competing interests.

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