Oxylipins in *Cryptococcus neoformans* and related yeasts

by

Olihile Moses Sebolai

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Department of Microbial, Biochemical and Food Biotechnology
Faculty of Natural and Agricultural Sciences
University of the Free State
Bloemfontein
South Africa

Promoter: Prof. J.L.F. Kock
Co-promoters: Prof. P.W.J. van Wyk
Dr. C.H. Pohl

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This thesis is lovingly dedicated to my parents, **Rre le Mme Sebolai.** Thank you for everything.

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NB: This thesis consists of different chapters, each in publication format according to the style requested by the journal of submission. As a result repetition of some information could not be avoided.

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Chapter 1

Literature review

Parts of this study have been published in FEMS Yeast Research (2007), **7:** 1207-1217.

1.1 Motivation

Cryptococcus neoformans is an important human pathogen and a significant cause of worldwide morbidity and mortality especially in immunocompromised persons, mainly due to the prevalence of HIV/AIDS (Levitz, 1991; Powderly, 1993). *Cryptococcus* infections are acquired through the respiratory canal and mainly cause asymptomatic pulmonary infections in immunocompetent persons. However, in immunocompromised persons, this yeast disseminates through the central nervous system and often presents as meningioencephalitis (Bose *et al.,* 2003).

Treatment of cryptococcal infections with conventional drugs such as amphotericin B and fluconazole remains difficult, especially in developing countries (UNAIDS, 1998). This is largely due to the high cost of these drugs and their subsequent unavailability. In addition, there has recently been a marked increase in antifungal resistance by pathogenic yeasts, including *Cryptococcus neoformans* (Pina-Vaz *et al.,* 2000). This is mainly attributed to the widespread use of antifungals as curative agents. Therefore, the challenge is to find or develop alternative effective and low cost drugs to combat yeast growth and/or infection.

It is suggested in literature that the non steroidal anti-inflammatory drug (NSAID), acetylsalicylic acid (aspirin, ASA), which is mainly prescribed for the treatment and prevention of inflammatory responses, has potential as an antifungal agent against yeasts (Kock *et al.,* 2003; Coccoli *et al.,* 2005; Leeuw *et al.,* 2007). The ASA

action/response can be attributed to its effect on whole cells, mitochondria as well as the inflammatory prostaglandin (PG) cascade in mammalian cells. In mitochondria, ASA was shown to influence/inhibit aerobic respiration and the β-oxidation pathway, which facilitates amongst others, the production of 3-hydroxy (OH) oxylipins (Kock *et al.,* 2007; Leeuw *et al.,* 2007).

3-OH oxylipins are widely distributed in nature (Kock *et al.,* 2003; Noverr *et al.,* 2003), where they have also been implicated in the pathogenesis of certain microorganisms. Interestingly, during the pathogenic hyphal phase of *Candida albicans*, this pathogen produces ASA-sensitive 3-OH oxylipins. These oxylipins facilitate yeast colonization and elicit pro-inflammatory responses in infected host tissue (Deva *et al.,* 2001; Ciccoli *et al.,* 2005). Furthermore, ASA was shown to inhibit yeast-to-hyphal transition as well as biofilm formation by this pathogenic yeast (Deva *et al.,* 2001; Alem & Douglas, 2004). This research suggests new NSAID-sensitive targets for the control of yeast infections.

With this as background, this thesis aims to address the following questions:

[1] Can *Cryptococcus neoformans* produce 3-OH oxylipins? (see Chapter 2)

[2] Is the production of these oxylipins as well as growth, sensitive to ASA? (see Chapter 3)

[3] Is the same true for other pathogenic yeasts related to *Cryptococcus neoformans*? (see Chapter 4)

1.2 Background: *Cryptococcus*

Kützing first used the generic name *Cryptococcus* in 1833. Then in 1901, Vuillemin proposed that *Cryptococcus* should be reserved to accommodate pathogenic yeasts (Fell & Statzell-Tallman, 1998). *Cryptococccus* species are ubiquitous basidiomycetous yeast, characterized by encapsulated cell walls and are non fermentative (Fell & Statzell-Tallman, 1998). This yeast genus includes one of the important human pathogens, namely *Cryptococococus neoformans.* This yeast comprises of four distinct serotypes (i.e. A, B, C and D) according to the immunologic properties of the capsular antigens (Fraser *et al.,* 2003). Strains of serotype A are assigned to *Cryptococcus neoformans* var. *grubii* and those of serotype D to *Cryptococcus neoformans* var. *neoformans. Cryptococcus neoformans* var. *gattii* is limited to strains of serotypes B and C. This genus has 34 species currently recognized (with *Cryptococcus neoformans* designated as type species) according to the latest yeast monograph by Kurtzman & Fell (1998).

Species currently accepted (Fell & Statzell-Tallman, 1998)

1. *Cryptococcus aerius* (Saito) Nannizzi (1927)*
2. *Cryptococcus albidosimilis* Vishniac & Kurtzman (1992)*
3. *Cryptococcus albidus* (Saito) C.E. Skinner (1947)*
4. *Cryptococcus amylolentus* (van der Walt, D.B. Scott & van der Klift) Golubev (1981)*
5. *Cryptococcus antarcticus* Vishniac & Kurtzman (1992)*

6. *Cryptococcus aquaticus* (Jones & Slooff) Rodrigues de Miranda & Weijman (1988)*
7. *Cryptococcus ater* (Castellani ex Cooke) Phaff & Fell (Lodder 1970)*
8. *Cryptococcus bhutanensis* S. Goto & Sugiyama (1970)*
9. *Cryptococcus consortionis* Vishniac (1985)*
10. *Cryptococcus curvatus* (Diddens & Lodder) Golubev (1981)*
11. *Cryptococcus dimennae* Fell & Phaff (1967)*
12. *Cryptococcus feraegula* Saëz & Rodrigues de Miranda (1988)*
13. *Cryptococcus flavus* (Saito) Phaff & Fell (1970)*
14. *Cryptococcus friedmannii* Vishniac (1985)*
15. *Cryptococcus fuscescens* Golubev (1984)*
16. *Cryptococcus gastricus* Reiersöl & di Menna (1958)*
17. *Cryptococcus gilvescens* Chernov & Bab'eva (1988)*
18. *Cryptococcus heveanensis* (Groenewege) Baptist & Kurtzman (1976)*
19. *Cryptococcus huempii* (Ramírez & González) Roeijmans, van Eijk & Yarrow (1989)*
20. *Cryptococcus humicolus* (Daszewska) Golubev (1981)*
21. *Cryptococcus hungaricus* (Zsolt) Phaff & Fell (1970)*
22. *Cryptococcus kuetzingii* Fell & Phaff (1967)*
23. *Cryptococcus laurentii* (Kufferath) C.E. Skinner (1950)*
24. *Cryptococcus luteolus* (Saito) C.E. Skinner (1950)*
25. *Cryptococcus macerans* (Frederiksen) Phaff & Fell (1970)*
26. *Cryptococcus magnus* (Lodder & Kreger-van Rij) Baptist & Kurtzman (1976)*
27. *Cryptococcus marinus* (van Uden & Zobell) Golubev (1981)*

28. *Cryptococcus neoformans* (Sanfelice) Vuillemin (1901)*
29. *Cryptococcus podzolicus* (Bab'eva & Reshetova) Golubev (1981)*
30. *Cryptococcus skinneri* Phaff & do Carmo-Sousa (1962)*
31. *Cryptococcus terreus* di Menna (1954)*
32. *Cryptococcus uniguttulatus* (Zach) Phaff & Fell (1970)*
33. *Cryptococcus vishniacii* Vishniac & Hempfling (1979)*
34. *Cryptococcus yarrowii* A. Fonseca & van Uden (1991)*

*References included in Fell & Statzell-Tallman (1998).

Pathogenicity and antifungals used

Cryptococcus neoformans is an opportunistic yeast pathogen frequently isolated from bird droppings, soil as well as trees and can cause infections in both immunocompetant and immunocompromised persons (Buchanan & Murphy, 1998). Inhalation of the yeast cells results in the subsequent infection of the central nervous system. The associated symptoms include respiratory and neurological effects, such as coughing and headaches. This yeast pathogen has a number of virulence factors, of these the capsule [comprised of glucuronoxylomannan (GXM), which is the major polysaccharide], is the most important. The capsule can inhibit phagocytosis and influence cytokine production - functions crucial for mounting an efficient immune response (Buchanan & Murphy, 1998). Other mammalian pathogenic *Cryptococcus* species include *Cryptococcus albidus*, *C. ater*, *C. curvatus, C. feraegula*, *C. gastricus*, *C. laurentii*, *C. macerans* and *C. uniguttulatus* (Fell & Statzell-Tallman, 1998).

Life-threatening cryptococcal infections have steadily increased over the decades largely due to the increasing number of HIV-infected persons (Buchanan & Murphy, 1998). Although cryptococcosis is relatively easy to diagnose, its treatment is often impossible in developing countries (UNAIDS, 1998). With no prospect of treatment, patients' life expectancy is probably less than a month. Amphotericin B has been the drug of choice for many years in combating fungal infections (Ghannoum & Rice, 1999). However, due to its severe side effects in humans, i.e. nephrotoxicity, new generation drugs (allylamines and azoles) were introduced in the late 1980s and early 1990s. However, the widespread use of these new generation drugs has led to fungal resistance. Nonetheless, amphotericin B is still the most effective antifungal drug available but its clinical use is limited by (1) its narrow therapeutic index, and (2) its demonstrated low safety profile (Ghannoum & Rice, 1999). For more information on *Cryptococcus* see reviews by Buchanan & Murphy (1998), Ghaummon & Rice (1999) and Boekhout (2006).

1.3 Oxylipins: new targets for antifungals

Over the years, studies of oxylipin metabolism and function suggest that their metabolism may serve as targets for antifungal action (Kock & Coetzee, 1990; Noverr *et al.,* 2003; Erb-Downward & Huffnagle, 2006). This led to compounds such as NSAIDs to be considered as new antifungals (Kock & Coetzee, 1990; Kock *et al.,* 2003, 2007; Leeuw *et al.,* 2007). These studies yielded promising results in the control of yeast

growth, including that of pathogenic yeasts. In this section research implicating NSAIDs, such as ASA, as new antifungals, is discussed.

1.3.1 Acetylsalicylic acid-sensitive oxylipins: discovery and structure

The word “oxylipin” describes a group of oxidized fatty acids, some characterized by the presence of one or more hydroxyl groups (Bhatt *et al.,* 1998). An initiative to assess if a cheap biological source, i.e. yeasts, could produce ASA-sensitive oxylipins such as PGs, led to the discovery of ASA-sensitive 3-hydroxy fatty acids (3-OH oxylipins) in the early 1990s (Kock *et al.,* 1991; Van Dyk *et al.,* 1991, 1993). Prostaglandins mediate several responses in mammalian cells, amongst others labour induction and the inhibition of blood platelet aggregation (Samuelsson *et al.,* 1983; Needleman *et al.,* 1986), and are produced chemically at high cost for medical applications (Dixon, 1991). Using radio thin-layer chromatography and radio immuno assay, an ASA-sensitive metabolite was uncovered when the yeast *Dipodascopsis uninucleata* (used as a model organism), was fed arachidonic acid (AA), a precursor for PGs (Kock *et al.,* 1991, 1992). The chemical structure of this metabolite was later elucidated as 3-OH 5Z, 8Z, 11Z, 14Z eicosatetraenoic acid (3-HETE, a 20:4 fatty acid with an OH group at C-3) following ^{1}H two-dimensional correlation spectroscopy nuclear magnetic resonance, gas chromatography – mass spectrometry (electron impact and fast atom bombardment) as well as infrared spectrometry analysis (Fig. 1). The production pathway of this metabolite was regarded as a target site for ASA action.

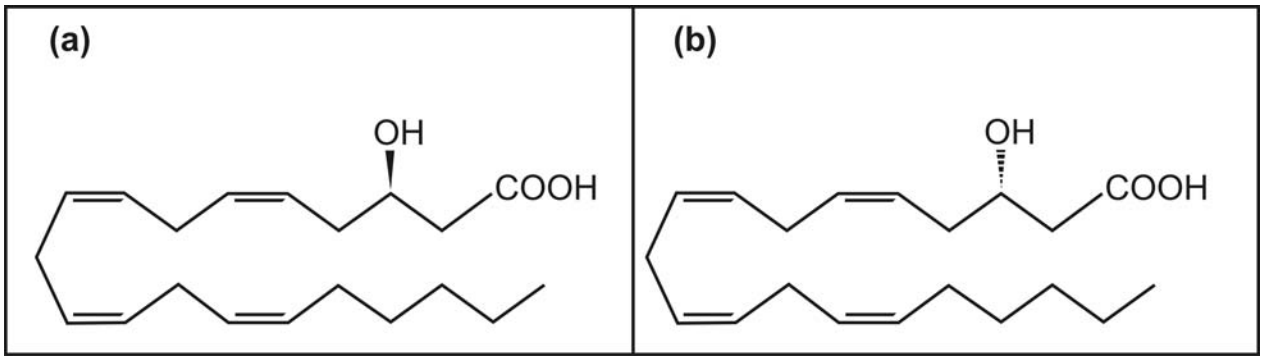

Fig. 1: The chemical structures of typical 3-hydroxy oxylipins. (a) *R*-3-hydroxy-5,8,11,14-eicosatetraenoic acid; (b) *S*-3-hydroxy-5,8,11,14-eicosatetraenoic acid.

During these bioprospecting studies, the first PGs were also uncovered in yeasts (Kock *et al.,* 1991; Noverr *et al.,* 2003). Prostaglandins were later found in other yeasts, such as *Cryptococcus neoformans* and *Candida albicans*, where they probably play a role as virulence factors (Noverr *et al.,* 2001, 2002). Here, indirect immunological techniques (which are prone to cross-reactions) were used to analyze these PGs. Recently, more direct evidence of PGs in yeasts was provided using sophisticated mass spectrometry techniques. It was shown that the unrelated pathogenic yeasts, *Cryptococcus neoformans* and *Candida albicans* produce PGE_2, which may act as ASA-sensitive virulence factors during infection (Erb-Downward & Huffnagle, 2007; Erb-Downward & Noverr, 2007).

1.3.2 Distribution: 3-OH oxylipins in yeasts

The presence of 3-OH oxylipins in yeasts is well documented (Kock *et al.,* 1998, 2003, 2004, 2006; Noverr *et al.,* 2003; Leeuw *et al.,* 2007). Using antibodies raised against chemically synthesized 3-OH oxylipins (Bhatt *et al.,* 1998; Groza *et al.,* 2002, 2004), the distribution of these compounds was visually mapped using immunofluorescence microscopy (Fig. 2) (Kock *et al.,* 1998). These antibodies were found to be specific against 3-OH oxylipins in general, i.e. 3-OH oxylipins of different chain lengths and level of desaturation (Kock *et al.,* 1998). Consequently, 3-OH oxylipins where found to be mainly associated with yeast sexual stages (asci), in particular, coating cell wall surfaces of ascospores (Fig. 2) (Kock *et al.,* 1998). So far, in addition to antibody mapping, chemical analysis by gas chromatography-mass spectrometry in most cases confirmed the presence of 3-OH oxylipins.

Further studies reported these oxylipins to be associated mainly with the ascospores of *D. uninucleata* (Fig. 2) (Kock *et al*., 1998); *Dipodascus* (Van Heerden *et al*., 2005, 2007); many lipomycetaceous species i.e. *Lipomyces doorenjongii, L. kockii, L. kononenkoae, L. starkeyi, L. yamadae, L. yarrowii, Smithiozyma japonica* and *Zygozyma oligophaga* (Smith *et al*., 2000b); *Saturnispora saitoi* (Bareetseng *et al*., 2006); *Saccharomycopsis* (Sebolai *et al*., 2001, 2004, 2005); *Eremothecium* (Bareetseng *et al*., 2004; Kock *et al*., 2004; Leeuw *et al*., 2006, 2007) and *Ascoidea* (Figs. 3 and 4) (Ncango *et al*., 2006). Although 3-OH oxylipins were reported in lipomycetaceous yeasts, no 3-HETE could be detected when these yeasts were fed with AA (Kock *et al*., 1992). Furthermore, in contrast to *Dipodascopsis uninucleata*, 3-OH oxylipins accumulate mainly on the ascus tip of the closely related *Dipodascopsis tothii*, as observed by immunofluorescence microscopy (Smith *et al*., 2000a).

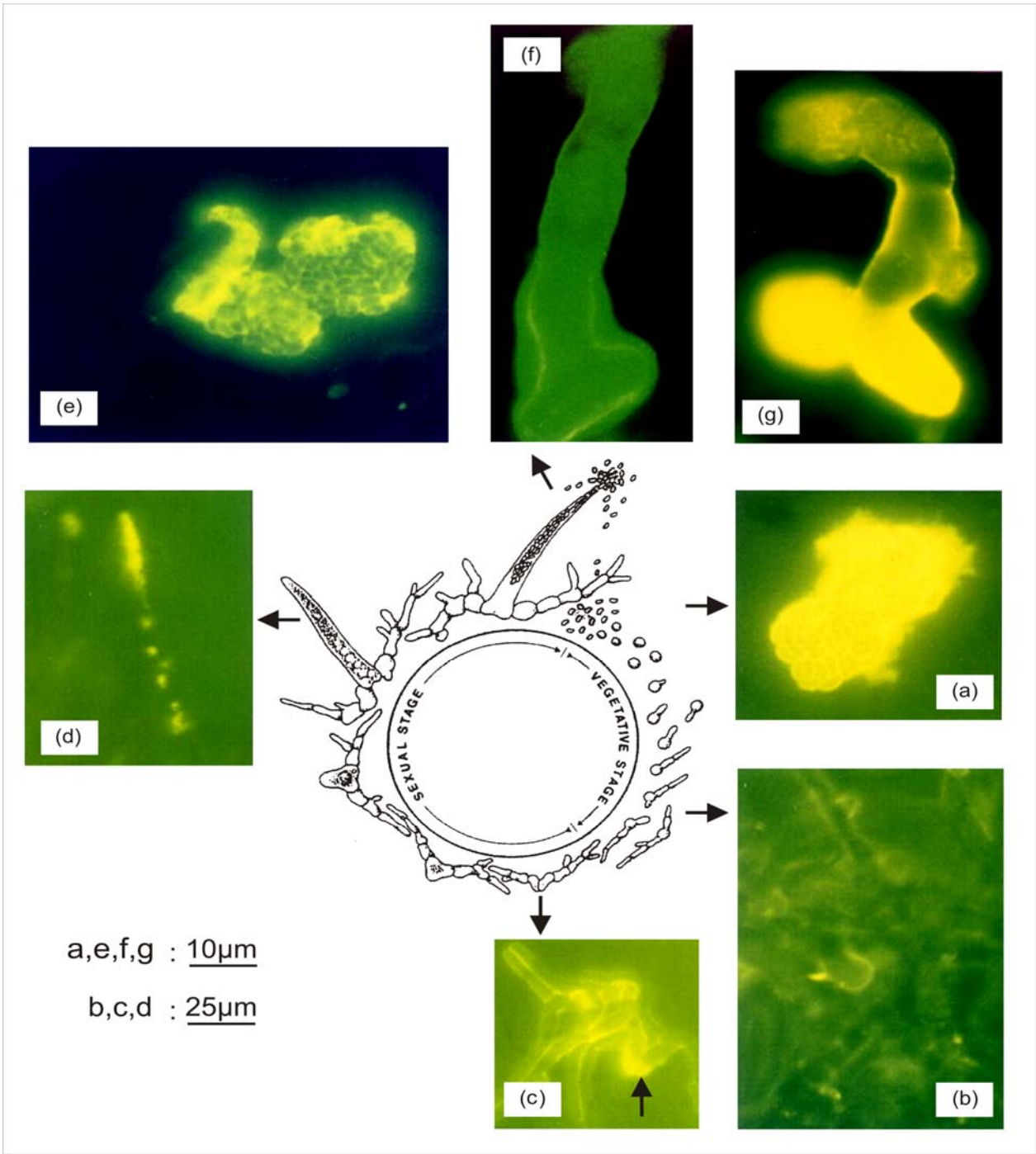

Fig. 2: The life cycle of *Dipodascopsis uninucleata* and distribution of 3-HETE visualized through immunofluorescence mapping. (a) Liberated ascospores showing high affinity for oxylipin antibody. (b) Hyphae with low oxylipin antibody affinity. (c) Gametangiogamy with tip of adhering gametes showing high affinity for oxylipin antibody. (d) Young ascus with ascospores demonstrating high affinity for oxylipin antibody. (e), Liberated

fluorescing ascospores from ascus. (f) Empty ascus protoplast: still with characteristic morphology. (g) Deformed mature ascus protoplast containing fluorescing ascospores mainly at base. (a, b) Asexual vegetative stage. (c, d, e, f, g) Sexual stage. Reprinted by permission of Federation of the European Biochemical Societies from Kock *et al*. (1998©).

3-OH oxylipins are also associated with cell wall surfaces of aggregating/flocculating yeast vegetative cells of *Saccharomycopsis malanga* and *Saccharomyces cerevisiae* (Kock *et al.,* 2000; Sebolai *et al.,* 2001; Strauss *et al.,* 2005; Speers *et al.,* 2006). This was revealed during transmission electron microscopy (including immunogold labelling) as well as immunofluorescence microscopy studies. Furthermore, these compounds are also associated with surfaces of pathogenic hyphal stages of *Candida albicans* (Deva *et al.,* 2000, 2001, 2003).

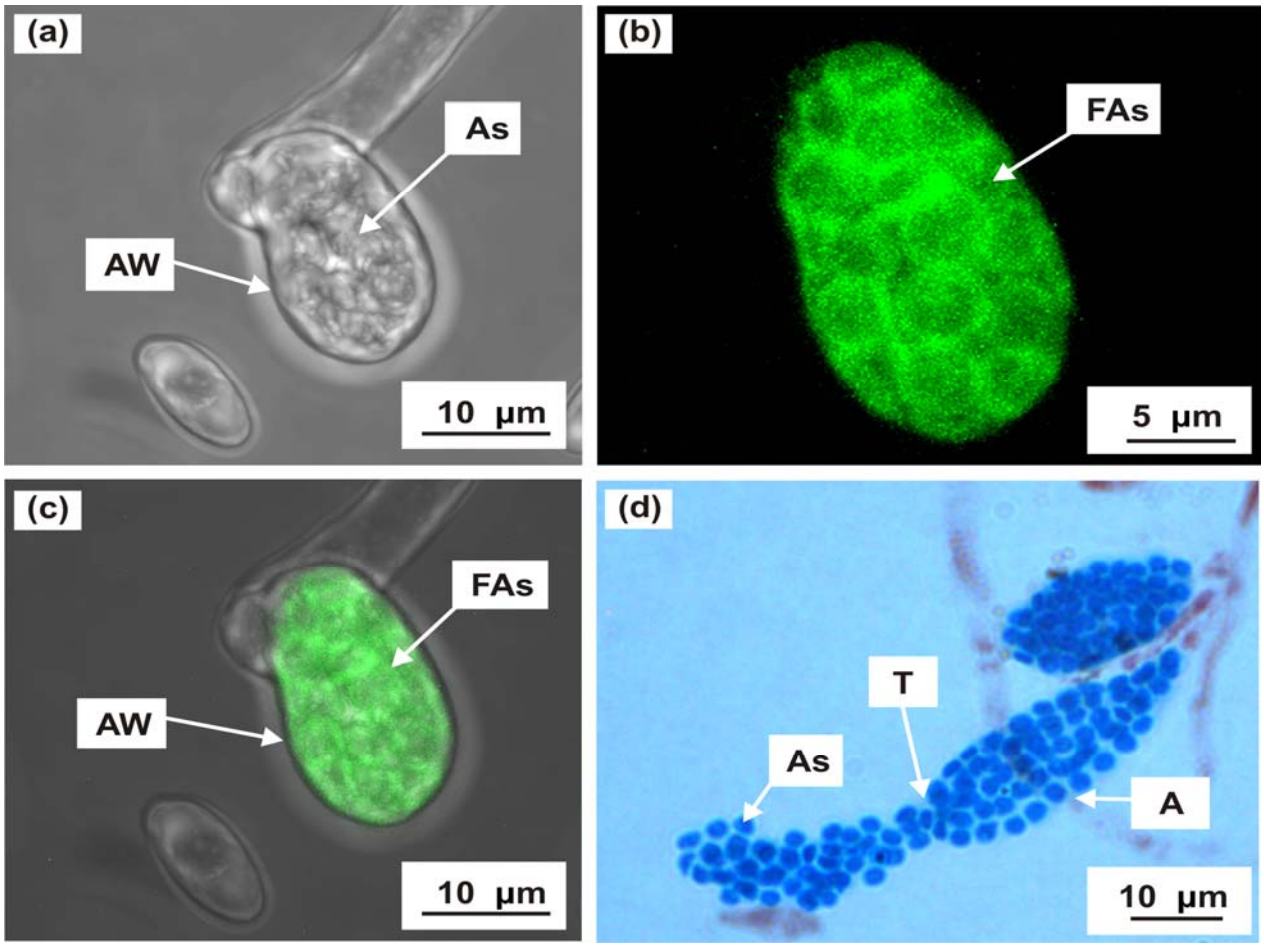

Fig. 3: Light micrograph (a), immunofluorescence-only micrograph showing in more detail selectively fluorescing brims surrounding hat-shaped ascospores in circles (b, compare Fig. 4a), light combined with immunofluorescence micrograph (c), and light micrograph of stained ascospores (d) of *Ascoidea corymbosa*. A, ascus; As, ascospore; AW, ascus wall; FAs, fluorescing ascospores; T, ascus tip. Taken with permission from Ncango *et al*. (2006).

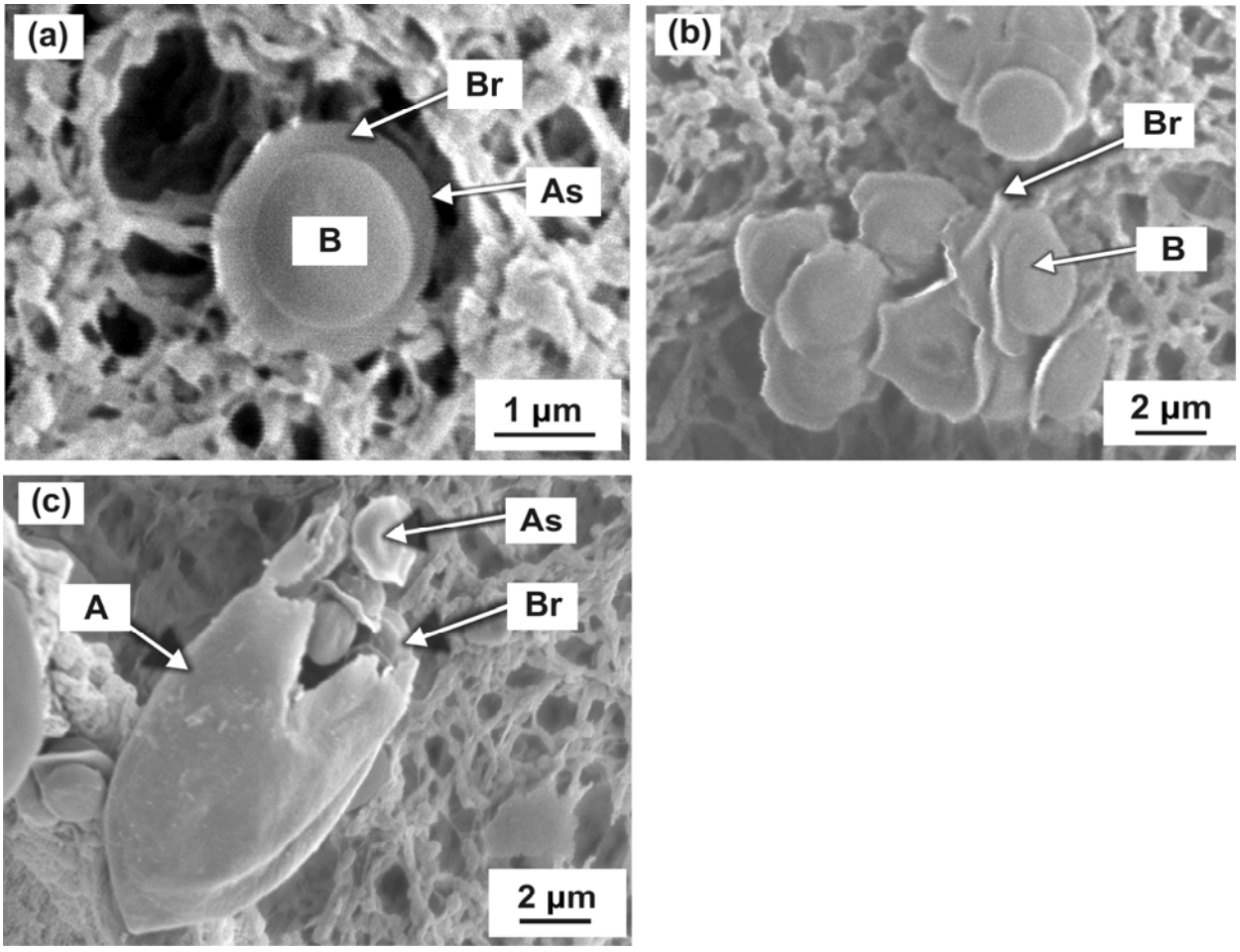

Fig. 4: Scanning electron micrographs of individually released ascospore (a) and aggregated released ascospores (b) in *Ascoidea corymbosa*. The release of ascospores from the ascus opening (tip) is shown in (c). A, ascus; As, ascospore; B, bowl; Br, brim. Taken with permission from Ncango *et al*. (2006).

1.3.3 Functions: 3-OH oxylipins

The first evidence concerning the biological function of 3-OH oxylipins was presented in the 1990s. It was reported that 3-HETE affects signal transduction processes in human neutrophils and tumour cells in multiple ways (Nigam *et al*., 1999) and acts as a strong chemotactic agent, the potency of which is comparable with those

of leukotriene B_4 or fMet-Leu-Phe. The cell signaling cascade triggered by 3-HETE appears to imply G-protein-dependent processes. A novel 3-OH oxylipin, 3,18-dihydroxy-5,8,11,14-eicosatetraenoic acid, was identified in *Candida albicans*, a pathogen in vulvovaginal candidiasis (Deva *et al*., 2000, 2001, 2003). These researchers concluded that the administration of ASA should be beneficial in the treatment of this disease in two ways: (1) by inhibiting 3-OH oxylipin formation - mainly associated with the hyphal phase; and (2) by inhibiting PGE_2 formation in the infected host tissue.

Recently, Ciccoli *et al.,* (2005) uncovered a novel mode of infection of the yeast pathogen *Candida albicans*. They found that this yeast converts AA, released from infected or inflamed host cells, to a 3-HETE-like compound. This oxylipin then acts as substrate for the host cyclooxygenase-2 (COX-2), leading to the production of the potent pro-inflammatory 3-hydroxy prostaglandin E_2 (3-OH-PGE_2). They uncovered a cascade of novel bioactive 3-OH PGs, produced from 3-HETE via mammalian COX-2 (Fig. 5).

When infected, mammalian cells usually release AA for transformation via ASA-sensitive COX-1 and COX-2 to pro-inflammatory eicosanoids such as PGs, thromboxanes and prostacyclin. These compounds are potent regulators of the host immune responses, and play a role in numerous basic host cell physiological processes. Ciccoli *et al.* (2005) have shown that 3-HETE is also an appropriate substrate for COX-2, being almost as effective as AA, and produces novel 3-OH eicosanoids, including 3-hydroxyprostaglandin B_2, 3-hydroxyprostaglandin D_2, 3-hydroxyprostaglandin E_2 and 3-hydroxyprostaglandin $F_{2\alpha}$.

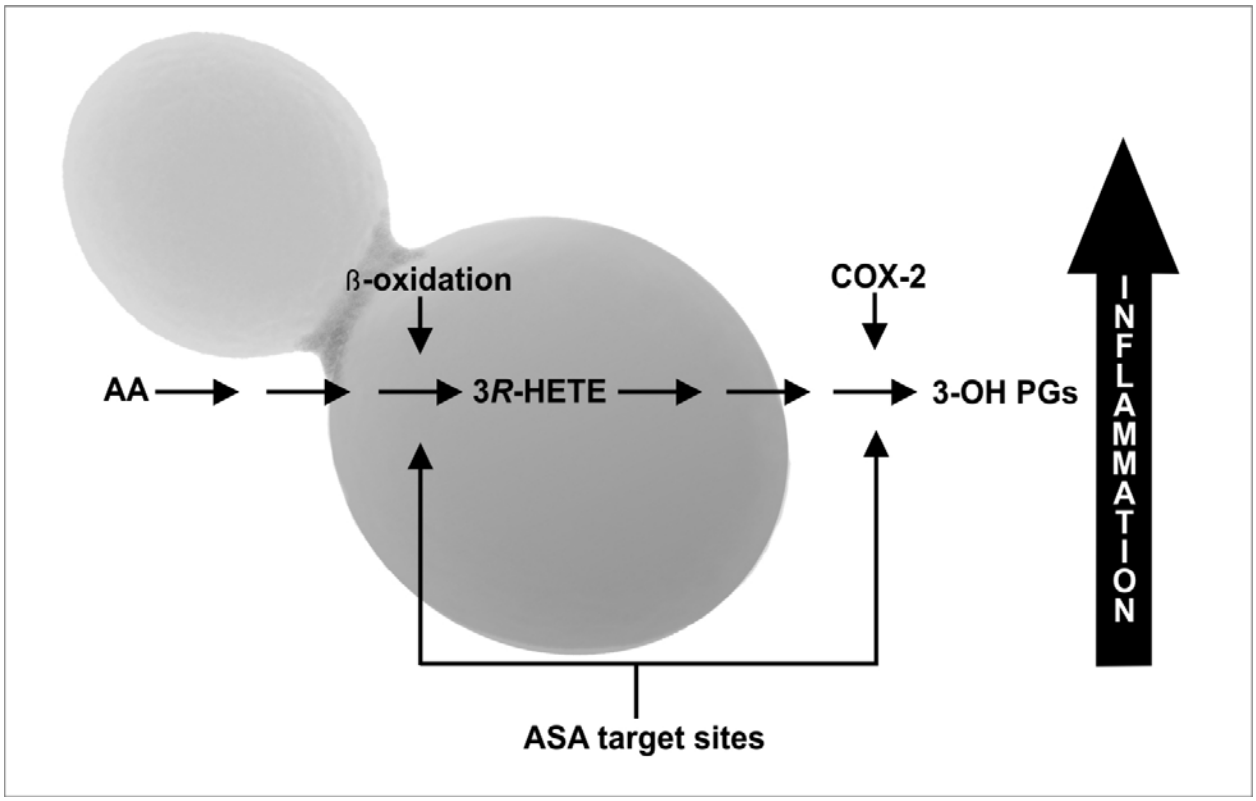

Fig. 5: A diagram showing the formation of potent inflammatory 3-hydroxy prostaglandins in host cells from 3-HETE produced via incomplete β-oxidation from host-released AA by the yeast *Candida albicans*. ASA = acetylsalicylic acid, COX-2 = cyclooxygenase–2. Taken with permission from Kock *et al*. (2005).

These authors showed that 3-OH eicosanoids have strong biological activities similar to and in some cases even more potent than those of the normally produced eicosanoids.

As yeast growth, formation of virulent hyphal stages as well as 3-HETE and COX-2-produced 3-OH PGs are inhibited by low concentrations of ASA, this research suggests new targets for the control of yeast infection. Research concerning the applicability of ASA and other NSAIDs as antifungals in order to control yeast infection should now be addressed – an idea first proposed in 1990 (Kock & Coetzee, 1990).

Interestingly, studies on flocculating *Saccharomyces cerevisiae* showed that the strains studied were incapable, under the conditions tested, of producing 3-HETE (*ab initio* or from exogenously fed AA) that is necessary for the synthesis of inflammatory COX-2-produced 3-OH PGs in mammalian cells. These results thus affirmed the Generally Regarded as Safe (GRAS) status of biotechnologically important *Saccharomyces cerevisiae* strains, as no known inflammatory eicosanoids or COX-2 precursors were detected (Strauss *et al.,* 2005).

3-OH oxylipins are not only strong pro-inflammatory lipid mediators (Nigam *et al*., 1999; Ciccoli *et al*., 2005), but also show potent antifungal activity against some moulds and yeasts (Sjogren *et al*., 2003). The literature shows that 3-OH 10:0, 3-OH 11:0, 3-OH 12:0 and 3-OH 14:0 have antifungal activity with minimum inhibitory concentrations between 10 and 100 μg mL^{-1} against some species of *Aspergillus*, *Penicillium, Kluyveromyces, Pichia* and *Rhodotorula*. It will be of interest to determine whether yeasts produce specific 3-OH oxylipins for their own protection against other fungi. Furthermore, 3-OH oxylipins are also found in Gram-negative bacteria as a crucial part of the inflammatory disease-causing lipopolysaccharide endotoxin component (Rietschel *et al*., 1994; Annane *et al*., 2005). Here, lipopolysaccharide plays an important role in the development of inflammation, which may eventually lead to septic shock, the most severe complication of sepsis and a deadly disease worldwide.

1.3.4 Oxylipins, mitochondria and acetylsalicylic acid inhibition

3-OH oxylipins are not only found in yeasts. According to the literature, these compounds may also be produced in mitochondria by β-oxidation in mammalian cells (Szponar *et al*., 2003). So far, indirect evidence suggests that 3-OH oxylipins are probably produced in a similar way, especially in the sexual cells of various yeasts. This is based on the link found between yeast oxylipin production and mitochondria, both of which are inhibited by a known mammalian mitochondrial inhibitor, ASA (Glasgow *et al*., 1999). This is contrary to the general belief that β-oxidation occurs only in peroxisomes of yeast (Hiltunen *et al*., 2003, 2005). In these elegant biochemical studies, mainly *Saccharomyces cerevisiae* was analysed without reference to the sexual cell types of the large diversity of non related yeasts.

In a groundbreaking study, Botha *et al*., (1992) analysed the life cycles of the non fermenting yeasts *Dipodascopsis tothii* and *Dipodascopsis uninucleata*, as well as the inhibitory effect of the NSAIDs ASA and indomethacin. When the yeasts were grown in synchronous culture, the life cycles of both yeasts were characterized by similar consecutive asexual and sexual reproductive stages (Fig. 2). In the presence of different concentrations of ASA (i.e. 0.1, 0.2, 0.5 and 1.0 mM), dose-dependent inhibition of the asexual vegetative stage was observed in both yeasts, although 0.1 and 0.2 mM ASA did not inhibit this stage in *Dipodascopsis uninucleata*. The sexual stages were found to be more sensitive to these NSAIDs, and spore liberation was completely inhibited by a concentration of ASA as low as 0.1 mM in *Dipodascopsis tothii*. Similar results were

obtained with indomethacin, although at much lower concentrations. Later studies reported some liberation of spores by *Dipodascopsis uninucleata* after 40 h of growth in the presence of 0.1 mM ASA, which also indicates dose-dependent inhibition of ascospore release, although at much lower concentrations than those needed to inhibit asexual vegetative cells (Kock *et al*., 1999). Consequently, these results suggest that both ASA and indomethacin inhibit both asexual and sexual stages in yeast, although the sexual stage proved to be much more sensitive.

It was also shown that *Dipodascopsis uninucleata* produces 3-OH oxylipins that are inhibited by ASA in a dose-dependent manner. This hinted at the possibility that these oxylipins are mainly produced during the sexual cycle (Van Dyk *et al.,* 1991, 1993). This was proven with immunofluorescence microscopy, which showed that 3-OH oxylipins accumulate in sexual cells (asci, including gametangia), whereas only limited amounts are associated with the filamentous vegetative stage (Fig. 2) (Kock *et al*., 1998).

According to the literature, 3-OH oxylipins in *Dipodascopsis uninucleata* may be produced by β-oxidation (Ciccoli *et al*., 2005). It was found that *Dipodascopsis uninucleata*, during its sexual stage, is capable of synthesizing the oxylipins 3-OH 5Z,8Z-tetradecadienoic acid from exogenously fed linoleic acid (9Z,12Z-octadecadienoic acid) and 3-OH 5Z,8Z,11Z-tetradecatrienoic acid from exogenously fed 11Z,14Z,17Z-eicosatrienoic acid after, probably, several cycles of β-oxidation (Venter *et al*., 1997).

Evidence supporting a link between oxylipins and yeast mitochondria was presented by Strauss *et al.,* (2007). Mitochondrial function is generally accepted as being important for expression of flocculation in yeasts. This has been demonstrated by the use of drugs such as antimycin A and ethionine (Nishihara *et al*., 1976; Egilsson *et al*., 1979; Iung *et al*., 1999), which inhibit mitochondrial function, cells that carry deletions in mitochondrial genes (Hinrichs *et al*., 1988), and petite (respiratory-deficient) mutants (Holmberg & Kielland-Brandt, 1978; Ernandes *et al*., 1993). In a recent study by Strauss *et al.,* (2007), a link between mitochondrial activity, oxylipin production and flocculation was demonstrated in a flocculating strain of *Saccharomyces cerevisiae*. Here, strongly flocculating cells showed both increased mitochondrial activity and oxylipin production as compared to weakly flocculating cells. Also, in the presence of ASA, flocculation, mitochondrial activity and oxylipin production declined sharply. This suggests that ASA, also a mitochondrial inhibitor in mammalian cells (Somasundaram *et al*., 1997; Glasgow *et al*., 1999), inhibits mitochondrial function in yeasts, resulting in the decrease of flocculation and probably oxylipin levels as well. Whether flocculation decrease is due to general mitochondrial or only oxylipin inhibition is not clear.

When long-chain fatty acids such as AA were exogenously fed to asexual vegetative cells of *Saccharomyces cerevisiae*, no hydroxylation to 3-HETE or shorter-chain oxylipins could be detected, as was evident in sexual cells of *Dipodascopsis uninucleata*. Only a short-chain 3-OH 8:0, produced *ab initio* in the presence or absence of AA, could be identified (Strauss *et al*., 2005). Is it possible that this oxylipin is produced via the fatty acid synthesis type II (FAS II) route in mitochondria of vegetative

cells (Hiltunen *et al*., 2005)? Will such a route be followed also in sexual cells of this yeast?

Strikingly, a recent study further strengthens the link between oxylipins and mitochondria. Here, concomitant increases in mitochondrial activity as well as 3-OH oxylipins in sexual cells of non related fermentative and non fermentative yeasts were reported (Ncango *et al*., 2007). These were found in the yeasts *Ascoidea africana*, *Asc. corymbosa*, *Asc. rubescens*, *Dipodascopsis uninucleata* and *Pichia anomala*.

Also, when ASA was added to *Ascoidea*, the sexual stage proved to be most susceptible to inhibition (Ncango *et al*., unpublished data), similar to what was found for *Dipodascopsis* (Botha *et al*., 1992; Kock *et al*., 1998), *Dipodascus* (Van Heerden *et al*., 2007) and *Eremothecium* (Leeuw *et al*., 2007). This is to be expected, as ASA is known to inhibit β-oxidation in mammalian mitochondria and therefore also 3-OH oxylipin synthesis (Glasgow *et al*., 1999). This is ascribed to ASA metabolites having structural similarities to the acyl-portions of the substrate and product of the 3-hydroxyacyl-CoA dehydrogenase activity of the β-oxidation pathway. In addition to the above, ASA may also inhibit mitochondrial activity by uncoupling mitochondrial oxidative phosphorylation and/or inhibiting electron transport (Somasundaram *et al.,* 1997; Norman *et al.,* 2004). It is therefore not surprising that the yeast sexual cycle, which has previously been found to be dependent on mitochondrial activity (Marmiroli *et al*., 1983; Codon *et al*., 1995), is more susceptible to ASA than are asexual vegetative cells (Kock *et al*., 2003). This is particularly true for *Ascoidea, Dipodascopsis*, *Dipodascus* and *Eremothecium*, in which high mitochondrial activity is presumably necessary to produce sufficient energy through

aerobic respiration to sustain high production and assembly throughput during the formation of numerous ascospores within a single enlarged sexual cell.

As expected, ASA addition and oxygen deprivation yielded similar results in inhibiting the sexual cycle of *Dipodascopsis*. When this yeast was grown under anoxic conditions, the sexual cycle was completely inhibited, whereas limited asexual growth was still observed. This further emphasizes the importance of aerobic respiration in sexual cell development of this yeast (Botha *et al*., 1993). Any disruption in mitochondrial activity by low concentrations of ASA or oxygen will surely negatively affect the proper development of the many sexual spores per sexual cell rather than the relatively less productive vegetative cells during sexual reproduction.

The clear link between oxylipin production, mitochondria and ASA sensitivity reported in various non related yeasts and different cell types calls for further biochemical studies to determine whether β-oxidation in yeast may occur in cell inclusions other than peroxisomes. The effect of ASA on peroxisomal β-oxidation and possible oxylipin production via FAS II should also be further researched.

1.3.5 Hypothesis

As mitochondrial dependence seems to be linked to ASA sensitivity in yeasts, it can be concluded that yeast with a mitochondrion-dependent strict aerobic metabolism

will be more sensitive to this NSAID than those that can also produce energy through an alternative anaerobic glycolytic fermentative pathway in which mitochondria are not involved. This has been suggested recently (Leeuw *et al*., 2007) by growth experiments with several yeasts with both energy generation options, such as the ASA-sensitive non fermentative *Ascoidea africana*, *Asc. corymbosa*, *Asc. rubescens*, *Eremothecium ashbyi*, *E. coryli* (weakly fermentative), *E. cymbalariae*, *E. gossypii*, *E. sinecaudum*, *Cryptococcus neoformans*, *Dipodascus albidus*, *Dipodascopsis uninucleata*, *Rhodotorula glutinis* and *Lipomyces starkeyi*, and the more resistant fermenting yeasts *Candida magnoliae*, *Ca. tropicalis*, *Kluyveromyces marxianus*, *Pichia anomala*, *Saccharomyces cerevisiae*, *Schizosaccharomyces octosporus*, *Sc. pombe*, *Zygosaccharomyces baillii*.

This review prompts the following holistic hypothesis (Fig. 6): (1) the asexual vegetative reproductive phase of strictly aerobic yeasts are more sensitive to ASA than are yeasts with an additional fermentative pathway; (2) the sexual reproductive phase of yeasts is more sensitive to ASA than is the asexual vegetative growth phase; (3) flocculation in fermentative yeasts is partially inhibited by ASA; (4) these phenomena are probably attributable to mitochondrial inhibition by ASA, which in turn may be linked to the inhibition of products such as 3-OH oxylipins - not necessarily indicating oxylipin function; and (5) mitochondrial respiration and β-oxidation are more pronounced during the sexual phase of yeasts than in their asexual vegetative phase. The general validity of this hypothesis in the fungal domain should now be assessed.

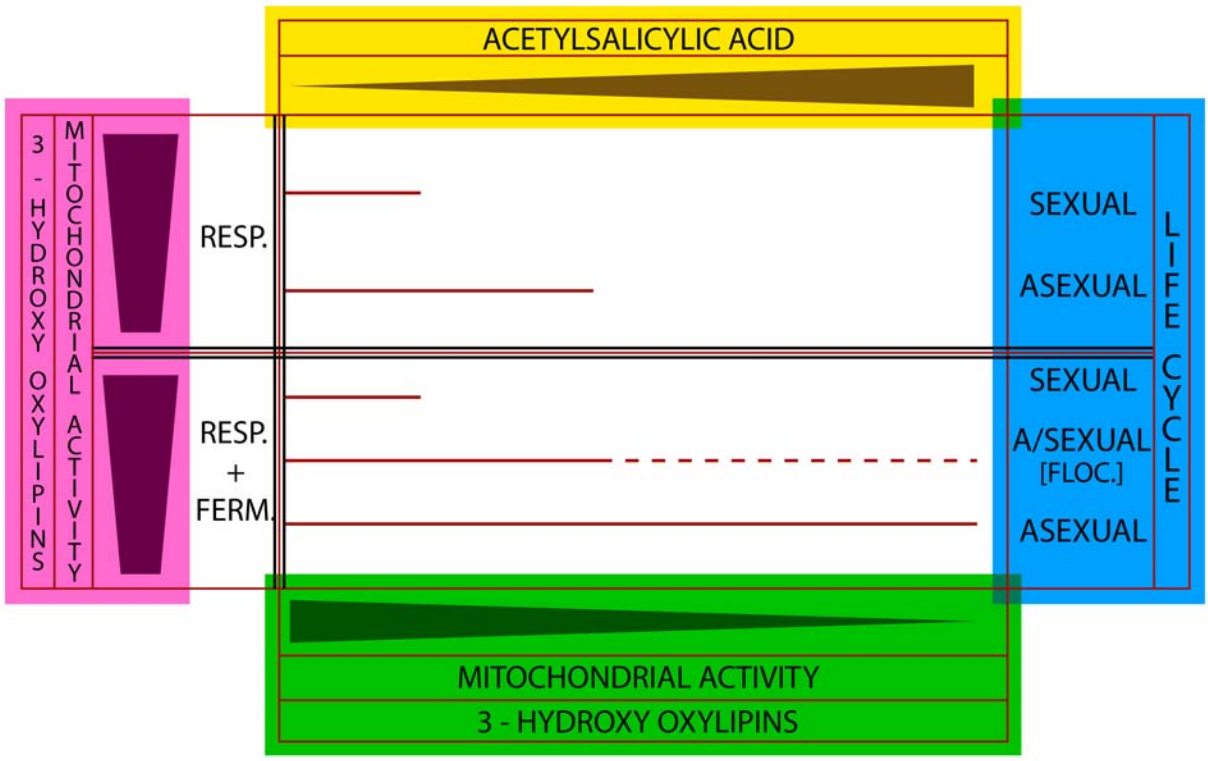

Fig. 6: A visual representation of a hypothesis suggesting a possible link between 3-OH oxylipin production, mitochondrial activity, and ASA sensitivity. *x*-axis, top: increase in ASA concentration from left to right. *x*-axis, bottom: decrease in mitochondrial activity and 3-OH oxylipin levels from left to right. *y*-axis, left: decrease in mitochondrial activity and 3-OH oxylipin levels from sexual reproductive to asexual growth phases in both strict aerobic yeasts (RESP.) and yeasts with both aerobic and fermentative pathways (RESP. + FERM.). *y*-axis, right: different phases of yeast life cycles i.e. sexual, asexual as well as asexual/sexual flocculation (FLOC.). Middle block: response surface showing the relative sensitivities of different yeast phases towards increasing levels of ASA.

When interpreting the literature, it is important to realize that ASA may have additional effects. As well as inhibiting mitochondrial β-oxidation (Glasgow *et al*., 1999) and uncoupling mitochondrial oxidative phosphorylation and/or inhibiting electron transport (Somasundaram *et al.,* 1997; Norman *et al.,* 2004), ASA may also cause side effects in mitochondria as well as whole cells. For instance, ASA may induce apoptosis in many cell types by caspase activation through mitochondrial cytochrome *c* release

(Pique *et al*., 2000). In the cell, ASA may cause acetylation of COX-1, resulting in the cessation of the production of physiologically important PGs (Cena *et al*., 2003). Research on ASA also suggests that this NSAID has beneficial antioxidant properties by reducing O_2^- production through lowering of NADPH oxidase activity (Wu *et al*., 2002).

1.4 Purpose of study

Considering the preceding discussion, this study addresses the following:

[1] 3-OH oxylipin production by *Cryptococcus neoformans* var. *neoformans* UOFS Y-1378 (Chapter 2).

[2] The influence of ASA on oxylipins and growth of this yeast pathogen (Chapter 3).

[3] Distribution of 3-OH oxylipins and ASA sensitivity in other *Cryptococcus* species (Chapter 4).

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Chapter 2

3-Hydroxy fatty acids found in capsules of *Cryptococcus neoformans*

This study has been published in Canadian Journal of Microbiology (2007), **53:** 809-812.

2.1 Abstract

Using immunofluorescence confocal laser scanning microscopy, immunogold transmission electron microscopy and gas chromatography - mass spectrometry, we demonstrate the presence of 3-hydroxy fatty acids in *Cryptococcus neoformans*. Our results suggest that these oxylipins accumulate in capsules where they are released as hydrophobic droplets through tubular protuberances into the surrounding medium.

Key words: Capsule, *Cryptococcus neoformans*, 3-Hydroxy fatty acid, Protuberances.

2.2 Introduction

Cryptococcus infection remains a significant cause of worldwide morbidity and mortality, especially in immunosuppressed AIDS subjects (Levitz 1991; Powderly 1993). A major virulence factor of this pathogenic yeast is the capsule, which consists of polysaccharides (Pirofski 2006; Yauch et al. 2006). This structure contributes to virulence by inhibiting phagocytosis and shedding (Yauch et al. 2006).

In 1991, we reported the presence of acetylsalicylic acid (also known as ASA and aspirin) sensitive 3-hydroxy (3-OH) fatty acids (oxylipins) i.e., 3(*R*)–hydroxyeicosatetraenoic acid (3*R*-HETE), in the yeast *Dipodascopsis uninucleata* (Van Dyk et al. 1991). These oxylipins were later found in various yeasts (Kock et al. 1999, 2000, 2003; Deva et al. 2001; Noverr et al. 2003; Strauss et al. 2005; Van Heerden et al. 2005; Leeuw et al. 2007), while subsequent studies further exposed the production pathway of these compounds as new target sites for developing novel antifungals, especially in nonfermentative yeasts (Leeuw et al. 2007). 3*R*-OH fatty acids are found in Gram-negative bacteria as a crucial part of the inflammatory-disease-causing lipopolysaccharide endotoxin component (Rietschel et al. 1994), while on their own, they modulate several human neutrophil functions (Nigam et al. 1999), act as precursors to inflammatory 3-OH prostaglandins (Ciccoli et al. 2005), and serve as antifungals (Sjogren et al. 2003). Consequently, in this study, we focus on the production of 3-OH fatty acids in the pathogenic, nonfermentative, and acetylsalicylic acid-sensitive (Leeuw et al. 2007) yeast *Cryptococcus neoformans*.

2.3 Materials and methods

Strain used and cultivation

Cryptococcus neoformans var. *neoformans* UOFS Y-1378, isolated from human bone lesions and held at the University of the Free State, Bloemfontein, South Africa, was cultivated in 500 mL conical flasks, each containing 100 mL defined YNB (Difco Laboratories, Detroit, Michigan, USA) broth supplemented with 4 % glucose (Saarchem, Wadeville, South Africa), at 25 ^{O}C on a rotary shaker (160 r/min) for 42 h, after which the

cells were used for transmission electron microscopy (TEM) and 3-OH fatty acid analyses. In addition, cells grown on YM agar plates (25 ^{O}C for 2 days) were used for immunofluorescence microscopy. All experiments were performed at least in triplicate.

Immunofluorescence microscopy studies

Previously described antibodies (Kock et al. 1998) raised against chemically synthesized 3*R*-HETE (Bhatt et al. 1998; Groza et al. 2002) were used together with immunofluorescence confocal laser scanning microscopy (CLSM) to map 3-OH fatty acids in *C. neoformans* (Leeuw et al. 2007). In short, 30 µL of primary antibody was added to the cells and incubated to allow sufficient binding to the oxylipins. After adequate washing, fluorescein isothiocyanate-conjugated secondary antibodies (Sigma, St. Louis, Missouri, USA) were added and the sample was further incubated before analysis with a Nikon TE 2000 CLSM (Tokyo, Japan) (Kock et al. 1998).

Ultrastructural studies

Transmission electron microscopy (TEM) was performed as described previously (Kock et al. 2000). In short, yeast sections were de-osmified and reacted with above primary antibody and then reacted with a gold probe (Sigma). Following adequate washing, sections were stained using uranyl acetate (Merck, Darmstadt, Germany) and lead citrate (Merck), and then viewed using a Phillips EM 100 TEM (Eindhoeven, the Netherlands).

Oxylipin analysis studies

Gas chromatograph–mass spectrometry analysis (GC-MS) was performed on derivatised extracts containing 3-OH fatty acids as described previously (Strauss et al. 2005). These oxylipins were first extracted from cultures at low pH using ethyl acetate (Saarchem) and then methylated and silylated using diazomethane (Aldrich, Steinheim, Germany) and bis-(trimethylsilyl) trifluoroacetamide (BSTFA, Merck). GC-MS was subsequently performed on the resulting derivatives using a Finnigan Trace Ultra gas chromatograph (Thermo Electron Corp., San José, California, USA) equipped with a Finnigan Trace DSQ MS (Thermo Electron Corp.) and HP-5-60 m fused silica capillary column (0.23 mm inside diameter and 0.1 μm coating thickness).

2.4 Results and discussion

Using well tested and extensively applied antibodies (Kock et al. 1999, 2000, 2003; Deva et al. 2001; Noverr et al. 2003; Strauss et al. 2005; Van Heerden et al. 2005; Leeuw et al. 2007) prepared against chemically synthesized (Bhatt et al. 1998; Groza et al. 2002) 3-OH fatty acids (epitope, carbons 1-3), we mapped the presence of these oxylipins in the capsule of a selected strain of *C. neoformans*. Confocal laser scanning microscopy on cells treated with 3-OH fatty acid primary antibody and fluorescein

isothiocyanate-conjugated secondary antibody show fluorescing protuberances (fluorescing micrograph) attached to the capsules (superimposed light micrograph) of this yeast (Fig. 1). A detailed fluorescing micrograph shows protuberances originating from a fluorescing base (site of oxylipin accumulation) situated in the thin capsule and ending in a fluorescing globule-like structure (Fig. 2a). These protuberances with base are clearly shown with TEM as osmiophilic structures (Fig. 2b). Since the globule-like terminal structures could not be observed by TEM they are probably not contained in cellular material and are therefore destroyed during preparation. Immunogold TEM clearly shows the accumulation of gold particles representing 3-OH fatty acids in the capsule, especially at the base of the protuberance, as well as inside this tubular structure (Figs. 2c and 2d). GC-MS on derivatised culture extracts (cells and supernatant) further confirmed the association of these oxylipins with external capsular material and supernatant. Only a single 3-OH fatty acid, i.e., 3-OH 9:1 (as free fatty acid), with a major fragment ion at 175, and which depicted a hydroxyl group at carbon 3 and an M^+ -15 ion at 242.7, could be observed (Fig. 3) (Van Dyk et al. 1991). This is similar to the 3-OH oxylipin found in *Saccharomycopsis javanensis* and *Saccharomycopsis vini* (Sebolai et al. 2005). No bacterial contamination that could contribute to these results was observed using light and electron microscopy or during repeated purification (streaking out) of the yeast culture. We conclude that the globular ends of the protuberances are hydrophobic droplets containing 3-OH fatty acids that are released through tubular stalks (each about 30 nm x 400 nm) into the surrounding medium. This data is in accordance with the findings of Rodrigues et al. (2007), who found a novel release mechanism for the major virulence factor of *C. neoformans*, whereby polysaccharide-packaged lipid vesicles crosses the cell wall and the capsule

into the surrounding environment. In our study, oxylipins and their transport are reported for the first time in this yeast.

These results prompt further research into the production and biological activity of this group of oxylipins, as well as their chemical association with capsular polysaccharides in different strains of *C. neoformans*. 3-OH fatty acids are not only strong pro-inflammatory lipid mediators, but also show potent antifungal activity against some moulds and yeasts (Sjogren et al. 2003). Besides assisting with yeast sexual spore release (Kock et al. 2003) and flocculation (Strauss et al. 2005), these oxylipins may also function as protective agents against fungal competition in the environment. The antifungal activity of the commonly used non steroidal anti-inflammatory drugs, such as aspirin, and their effect on cryptococcal infection *in vivo*, need further attention, especially since *in vitro* studies show growth inhibition of this yeast at just 2 mmol/L aspirin (Leeuw et al. 2007). It has been suggested that 3-OH fatty acids in yeasts are produced via incomplete β-oxidation in mitochondria (Ciccoli et al. 2005), an organelle that probably originated through endosymbiosis from lipopolysaccharide-containing Gram-negative bacteria millions of years ago (Gray et al. 2001).

In conclusion, we have identified 3-OH fatty acids in the capsule of *C. neoformans* that expand the known spectrum of biologically active compounds associated with this main virulence factor.

2.5 Acknowledgements

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2.7 Figures

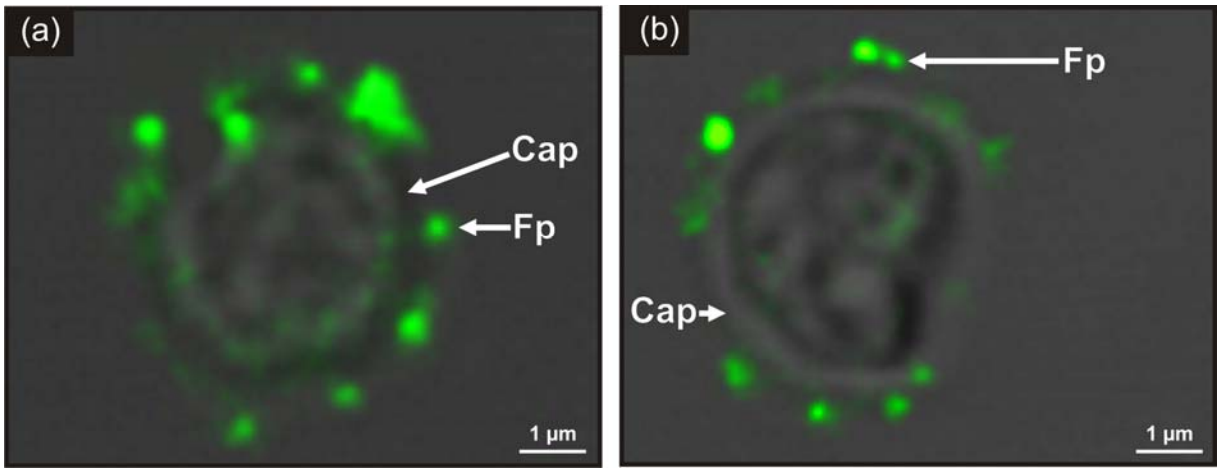

Fig. 1: Confocal laser scanning microscopy with light micrograph superimposed onto corresponding fluorescing micrograph of *Cryptococcus neoformans*. Fluorescing protuberances (Fp) indicate the presence of 3-hydroxy fatty acids attached to capsules (Cap) of *Cryptococcus neoformans* (a, b).

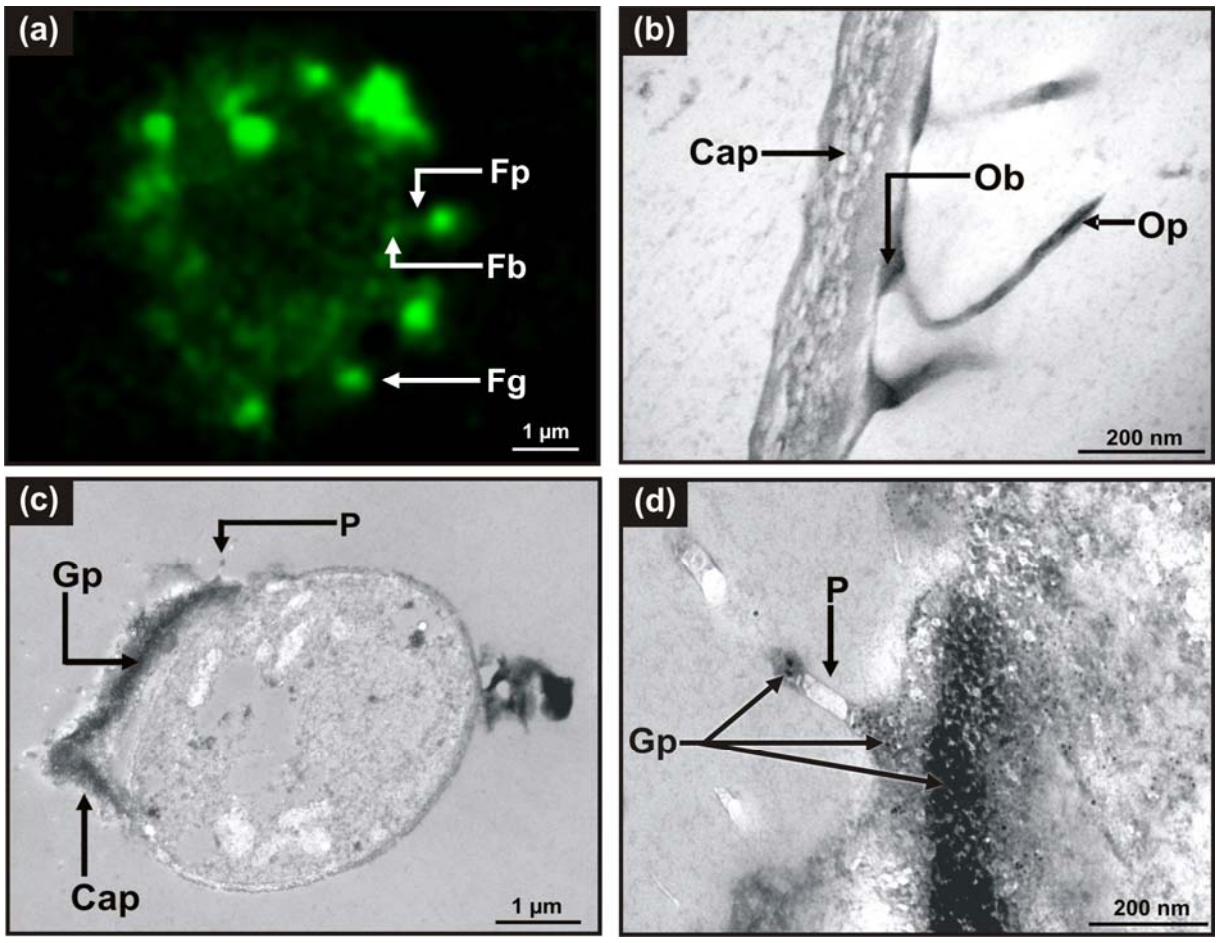

Fig. 2: Mapping 3-hydroxy fatty acids on capsular material of *Cryptococcus neoformans*. A confocal laser scanning micrograph of a yeast sample treated with antibodies directed against 3-hydroxy fatty acids and coupled to a fluorescing secondary antibody. Here, fluorescing protuberances (Fp) are imbedded in a fluorescing base (Fb), while the opposite tip forms a fluorescing globule-like (Fg) structure (a). Transmission electron micrograph showing an osmiophilic base (Ob) and osmiophilic protuberance (Op) attached to a thin capsule (b). Transmission electron micrograph of immuno labeled section showing concentration of 3-hydroxy fatty acids as gold particles (Gp, 10 nm in diameter) in shedding capsule (Cap). A capsule protuberance (P) is visible (c). Larger magnification of labeled capsule showing protuberance (P) and gold particle (Gp) distribution (d).

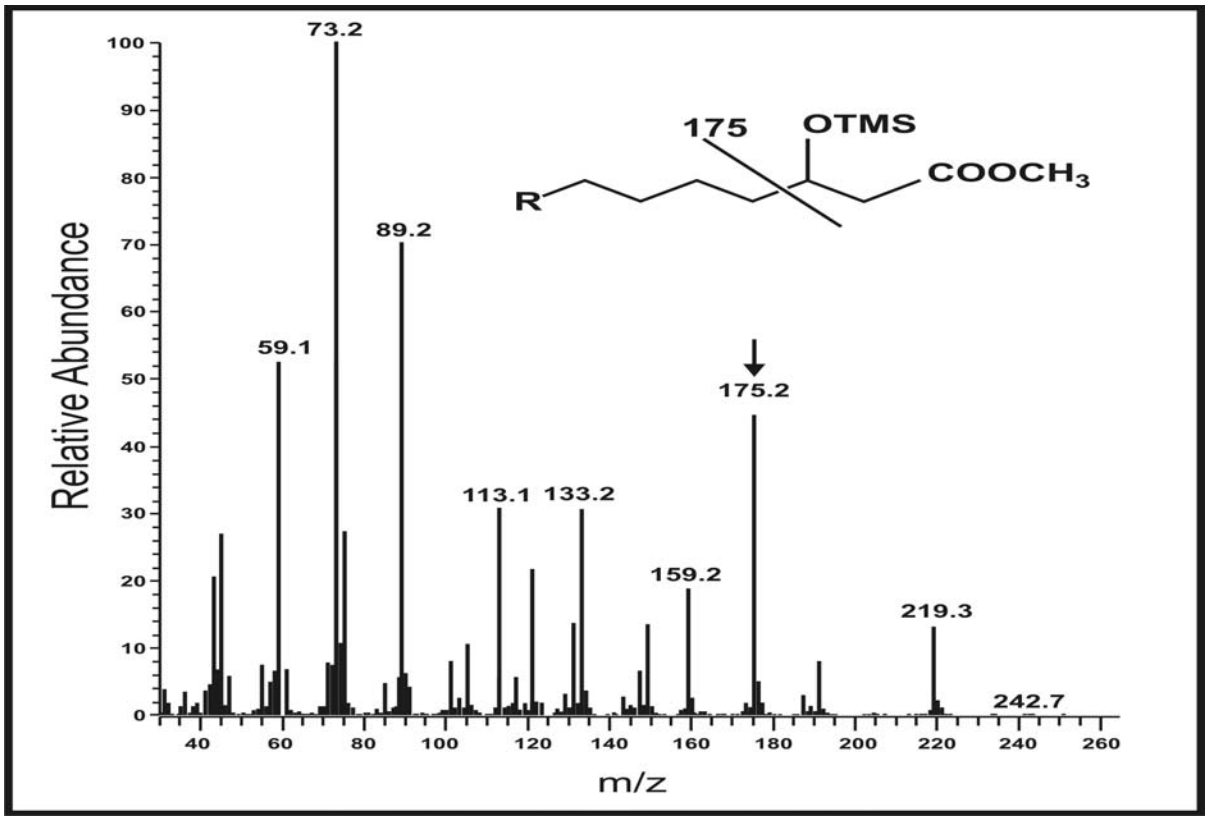

Fig. 3: A mass spectrum of a 3-hydroxy fatty acid obtained from derivatised culture extracts of *Cryptococcus neoformans* after 42 h of growth. The peak at 175 is one of the major peaks indicating the presence of a hydroxyl group at C-3. This 3-hydroxy 9:1 fatty acid eluted after 16.3 min. *m/z*, mass-to-charge ratio.

Chapter 3

The influence of acetylsalicylic acid on oxylipin migration in *Cryptococcus neoformans* var. *neoformans* UOFS Y-1378

This study has been accepted for publication in Canadian Journal of Microbiology (2007).

3.1 Abstract

In this paper we report the influence of acetylsalicylic acid on oxylipin migration in *Cryptococcus neoformans* var*. neoformans* UOFS Y-1378, previously isolated from human bone lesion. Transmission electron microscopy suggests that osmiophilic material originates in mitochondria and is deposited inside the yeast cell wall, from where it is excreted into the environment, along capsule protuberances or through capsule detachment. Previous studies using immunogold labeling, indicate that these osmiophilic layers contain 3-hydroxy oxylipins. In the present study, the addition of acetylsalicylic acid (an inhibitor of mitochondrial function) in increasing amounts to the cells, abrogated the migration of the osmiophilic material as well as capsule detachment from cell walls and hence oxylipin excretion. Consequently, we hypothesize that 3-hydroxy oxylipins are produced in mitochondria, probably via incomplete β-oxidation or fatty acid synthesis, from where it is deposited inside the cell wall and excreted through tubular protuberances attached to the surrounding capsules and/or through detachment of these oxylipin containing capsules.

Key words: Acetylsalicylic acid, Capsules, *Cryptococcus neoformans*, 3-Hydroxy oxylipins, Mitochondria.

3.2 Introduction

3-Hydroxy fatty acids (3-OH oxylipins) are widely distributed in the yeast domain (amongst pathogens and non-pathogens) where they are mainly associated with sexual structures (asci and ascospores) and sometimes vegetative cells (Kock et al. 2003; Sebolai et al. 2004). These oxylipins are implicated in a number of biological functions such as cell aggregation/flocculation and ascospore release (Sebolai et al. 2001, 2007; Kock et al. 2003; Strauss et al. 2005). These fatty acids, characterized by a hydroxyl group on the β-carbon, are probably produced by incomplete β-oxidation and/or fatty acid synthesis of type II (FAS II) in mitochondria (Venter et al. 1997; Bhatt et al. 1998; Ciccoli et al. 2005; Hiltunen et al. 2005).

In 1991, Van Dyk and co-workers reported an acetylsalicylic acid (aspirin, ASA) sensitive 3-OH oxylipin associated with the sexual cycle of *Dipodascopsis uninucleata* (Van Dyk et al. 1991, 1993; Kock et al. 1998). The sexual stage of this yeast, which is presumably dependent on active mitochondria (Marmiroli et al. 1983; Codon et al. 1995) for sufficient energy production, was shown to be more sensitive to ASA than the asexual stage (Botha et al. 1992; Kock et al. 1999). In addition, Leeuw et al. (2007) reported that yeasts, dependent on mitochondrial aerobic respiration for growth, are

more sensitive to ASA than those that can switch to anaerobic glycolytic fermentation for energy production.

In mammalian cells, ASA is reported to inhibit mitochondrial β-oxidation of medium and long chain fatty acids leading to subsequent inhibition of 3-OH oxylipin production (Glasgow et al. 1999). This is a result of structural similarities between the acetylsalicylic acid metabolite, salicylate, and acyl-portions of the substrate and product of the 3-hydroxyacyl-CoA dehydrogenase activity of the β-oxidation pathway (Glasgow et al. 1999). In addition to the antagonistic effect of salicylate on mitochondrial β-oxidation, ASA can also uncouple oxidative phosphorylation and/or inhibit mitochondrial electron transport (Somasundaram et al. 1997; Norman et al. 2004). Therefore, it is also possible that ASA can inhibit 3-OH oxylipin production via β-oxidation or FAS II through this inactivation route.

Previously, we reported that *Cryptococcus neoformans* (a nonfermentative, encapsulated basidiomycetous yeast) produces 3-OH oxylipins that are associated with capsules and presumably excreted through tubular protuberances (Sebolai et al. 2007). The possibility that ASA can also inhibit 3-OH oxylipin production in this pathogen as reported for other yeasts (Kock et al. 2003), will be addressed in this study.

3.3 Materials and methods

Strain used

Cryptococcus neoformans var*. neoformans* UOFS Y-1378 (isolated from a human bone lesion), held at the University of the Free State, Bloemfontein, South Africa, was maintained on complex media, yeast-malt (YM) agar slants. *Cryptococcus neoformans* var*. neoformans* UOFS Y-1378 was used for growth, ultrastructural as well as oxylipin studies. All experiments were done at least in triplicate.

Growth studies: Influence of ASA on growth and capsule detachment

A 250 mL conical flask containing 50 mL of standardized chemically defined media i.e. YNB-glucose broth [yeast nitrogen base 6.7 g/L (Difco Laboratories, Detroit, Michigan, USA), glucose 40 g/L (Saarchem, Wadeville, South Africa), pH 5.4], was inoculated with a loopful of yeast cells from two day old YM slants. This culture was allowed to grow to stationary phase until optimum capsule detachment was observed (at 25 ^{o}C on a rotary shaker at 160 r/min for 20 h) before appropriate volumes were transferred to three 500 mL conical flasks containing 100 mL of the same standardized medium in order to obtain an absorbency reading of approximately 10 Klett units. All cultures were incubated as before. After 18 h of cultivation, ASA [(Aldrich, Steinheim, Germany), dissolved in absolute ethanol (Merck, Darmstadt, Germany) at room temperature (80 g/L stock solution)] was added to each flask to yield final concentrations of 0 mmol/L (control), 1 mmol/L and 5 mmol/L respectively. Following this, cultivation was continued for another 24 h. During the 42 h cultivation period, Klett readings were recorded at 6 h time intervals using a photoelectric colorimeter (Klett-Summerson, Klett MFG. Co., Inc, New York, USA) in order to establish yeast growth under the influence of different ASA concentrations. At the end of the cultivation period, the percentage of

capsules detaching from cell walls was determined microscopically for each ASA concentration. This was done by counting cells with visually loosened capsules (Figs. 3c and 3d) and cells with intact capsules (Figs. 3a and 3b). In a separate experiment, the percentage capsule detachment in untreated cells (control) was also determined over the course of the 42 h growth period at 6 h time intervals.

Ultrastructural studies: Influence of ASA on migration of osmiophilic layers and mitochondrial ultrastructure

Material for transmission electron microscopy (42 h old cultures at 0 mmol/L, 1 mmol/L and 5 mmol/L ASA) was prepared according to van Wyk and Wingfield (1991). In short, material was chemically fixed using sodium phosphate-buffered 3 % glutardialdehyde (Merck) and similarly buffered osmium tetroxide (Merck). Sections were made with a LKB III ultramicrotome (Stockholm, Sweden) and viewed with a Phillips EM 100 transmission electron microscope (Eindhoven, the Netherlands).

Material for scanning electron microscopy, i.e. ASA untreated 42 h old cultures was chemically fixed as mentioned above. Next, the material was dehydrated in a graded ethanol (Merck) series, and critical-point-dried (Biorad Microscience Division, Watford, England), mounted on stubs and sputter-coated with gold (confer electron conductivity) using SEM coating system (Biorad Microscience Division). Preparations were examined using a Shimadzu Superscan SSX 550 scanning electron microscope (Tokyo, Japan).

Oxylipin studies: Influence of ASA on 3-hydroxy oxylipin production

3-Hydroxy oxylipins were extracted directly from the 42 h cultures (0 mmol/L, 1 mmol/L and 5 mM mmol/L) using two volumes (200 mL) of ethyl acetate (Saarchem) at pH 4 after formic acid was added to the medium (Sebolai et al. 2004). The organic phase was evaporated under N_2 (AFROX, Bloemfontein, South Africa), followed by derivitization using diazomethane (Aldrich) and bis-(trimethylsilyl) trifluoroacetamide (BSTFA, Merck). Samples were reconstituted in 200 μL chloroform:hexane (4:1, v/v) (Merck) before 1 μL was injected into a gas chromatograph mass spectrometer at an inlet temperature of 230 ^{o}C and a split ratio of 1:50. A Finnigan Trace Ultra Gas Chromatograph (Thermo Electron Corp., San José, California, USA) equipped with a Finnigan Trace DSQ MS (Thermo Electron Corp.) and an HP-5-60 m fused silica capillary column (0.1 μm coating thickness), was used to analyze the methylated trimethylsilylated samples.

3.4 Results and discussion

Influence of ASA on growth and capsule detachment

The growth patterns of *Cryptococcus neoformans* var. *neoformans* UOFS Y-1378, were similar in all cases for the first 18 h of growth i.e. in the absence of ASA. After 18 h (i.e. when ASA was added to some flasks), growth of the control experiment (0 mmol/L ASA) still increased to some extent while cell density of cultures containing 1 mmol/L and 5 mmol/L ASA decreased in a dose dependent manner (Fig. 1). This phenomenon may be ascribed to cell lysis as observed microscopically and is in accordance with

results reported for another non steroidal anti-inflammatory drug (NSAID), ibuprofen, which was shown to have fungistatic and fungicidal effects against actively growing *Candida albicans* cells (Pina-Vaz et al. 2000).

Results show that untreated cells nearing stationary growth phase (after 30 h) are more likely to detach their capsules from cell walls (Figs. 1, 3c and 3d) compared to actively growing cells (Figs. 3a and 3b). At the start of cultivation (0 h), a higher incidence of capsule detachment (39.2 %) was observed compared to actively growing cells after 18 h of cultivation. This is explained by the fact that cells used for inoculation (50 mL culture in 250 mL flask), were already in late stationary growth phase (similar to 42 h grown cells in 100 mL media in 500 mL flask). After 18 h, when these cells neared stationary growth phase, capsule detachment again increased sharply.

When ASA was added to cultures after 18 h of growth i.e. at 1 mmol/L and 5 mmol/L respectively, a drastic decrease in capsule detachment occurred after 42 h of growth (Fig. 2). However, no significant difference in capsule detachment could be observed between 42 h old cultures with 1 mmol/L and 5 mmol/L ASA respectively. Here, the percentage capsule detachment for both ASA concentrations was similar to that obtained for untreated cells after 18 h of growth (Figs. 1 and 2). We conclude that capsule detachment was inhibited by ASA at a concentration as low as 1 mmol/L.

Migration of osmiophilic layers

Results obtained using transmission electron microscopy (Figs. 3a, 3b and 3c) implicate a migration route for osmiophilic material, i.e. from (i) the mitochondria (suggested production site, Fig. 3a) (Ciccoli et al. 2005), (ii) to deposition of this material

on the inside of cell walls (Figs. 3a and 3b), and (iii) eventual migration through the cell wall to the outside of capsules that become detached (Fig. 3c). Results suggest that cells during steps (i) and (ii) are actively growing with capsules still firmly attached to cell walls, while cells with detached capsules (step iii) are older with less active growth (Fig. 1). No peroxisomes could be detected in any of the cells analysed. A 3-dimensional view of detaching capsules is illustrated in Fig. 3d.

Strikingly, a similar link between mitochondria and osmiophilic material was observed, although not discussed, in one of the transmission electron micrographs published by Garciá-Rivera et al. (2004) for *Cryptococcus neoformans*. Since osmiophilic layers in previous yeast studies (Kock et al. 2000), which include *Cryptococcus neoformans* (Sebolai et al. 2007) contained 3-OH oxylipins, we suggest the same in this study.

Influence of ASA on migration of osmiophilic layers and oxylipin production

Interestingly, in the presence of 5 mmol/L ASA (Fig. 3e), the migration of osmiophilic layers was abrogated. Furthermore, mitochondrial ultrastructure became altered (i.e. cristae and matrix were not visible) while capsule detachment could not be observed (Fig. 3e). 3-Hydroxy oxylipin production was also inhibited by ASA in a dose-dependent manner (Fig. 4). Here, oxylipins were detected in trace amounts when cells were treated with 1 mmol/L ASA and could not be detected when treated with 5 mmol/L ASA. The mass spectrum of this metabolite was characterized by a pronounced peak of *m/z* = 175 [$CH_3O(CO).CH_2.CHO.TMSi$], which is characteristic of hydroxylation of fatty acids at the β-carbon (Van Dyk et al. 1991) (Fig. 5). Since an M^+ - 15 of *m/z* = 242.7 was

obtained, the structure of this oxylipin is probably 3-OH 9:1 with M^+ = 258. This was also reported previously by Sebolai et al. (2007). However, nuclear magnetic resonance studies and others should be conducted to confirm the complete chemical structure of this oxylipin.

We conclude that 3-OH oxylipins are produced in mitochondria after which they are deposited inside cell walls from where they migrate across the cell wall through capsule associated protuberances eventually to be released as droplets (vesicles) into the surrounding medium (Fig. 6). This production and migration phenomenon is inhibited by ASA.

Recently, Rodrigues et al. (2007) reported a novel release mechanism for the major polysaccharide virulence factor of *Cryptococcus neoformans*. They found that polysaccharide-packaged lipid vesicles cross the cell wall and the capsule into the surrounding environment. It will be of interest to determine if 3-OH oxylipins are also present in these lipid vesicles and if so, what role they play in pathogenesis. The effect of ASA on *Cryptococcus* pathogenicity also needs urgent investigation. Strikingly, literature reports that 3-OH oxylipins are not only strong pro-inflammatory lipid mediators (Ciccoli et al. 2005), but also show potent inhibitory activity against other eukaryotes (Sjogren et al. 2003).

According to Ciccoli et al. (2005), 3-OH oxylipins may be produced through incomplete β-oxidation in yeast. Is this phenomenon conserved for all yeasts especially since a fatty acid synthesis (FAS) type II pathway has also been reported in yeast

mitochondria (Hiltunen et al. 2005)? In bacteria, this route is used to produce 3-OH oxylipins of medical importance i.e. 3-OH mycolic acid, which is a virulence factor in *Mycobacterium tuberculosis* (Takayama et al. 2005).

3.5 Acknowledgements

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3.6 References

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3.7 Figures

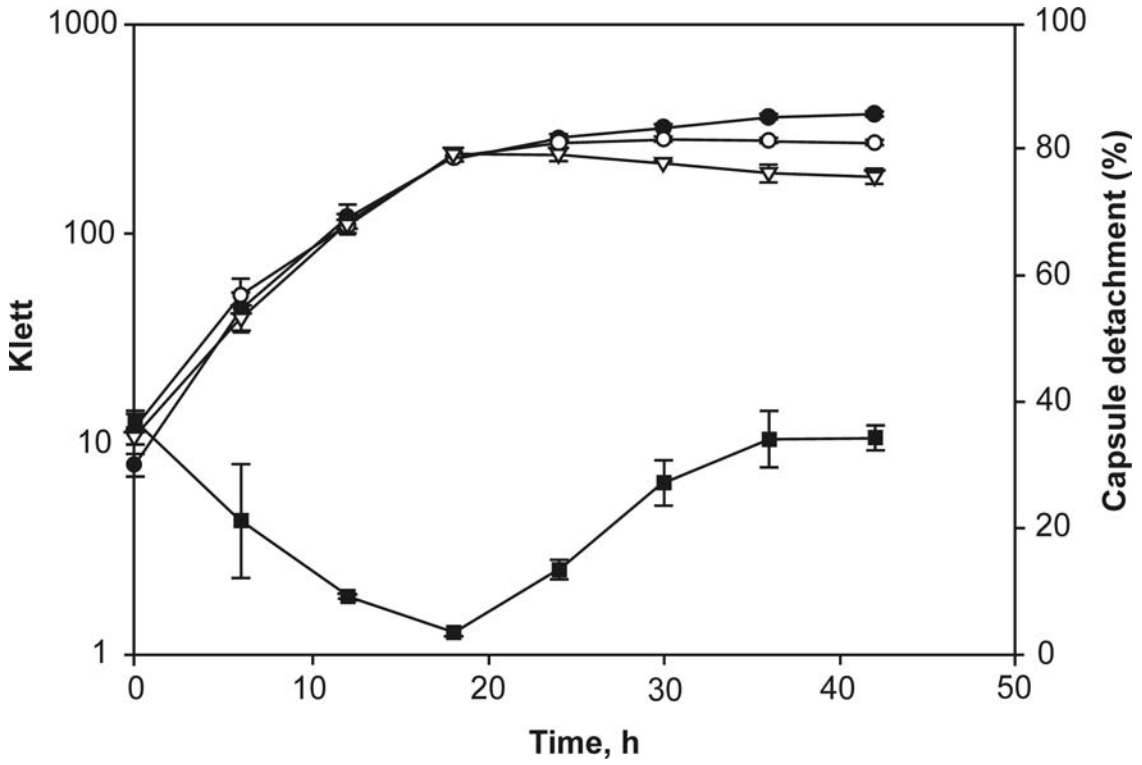

Fig. 1: The growth patterns of *Cryptococcus neoformans* var. *neoformans* UOFS Y-1378 when cultivated for the first 18 h in the absence of acetylsalicylic acid (ASA) and then under different concentrations of ASA [0 mmol/L (-●-), 1 mmol/L (-○-), and 5 mmol/L (-∇-)]. In addition, the percentage capsule detachment was mapped over 42 h growth period for ASA untreated cells (-■-).

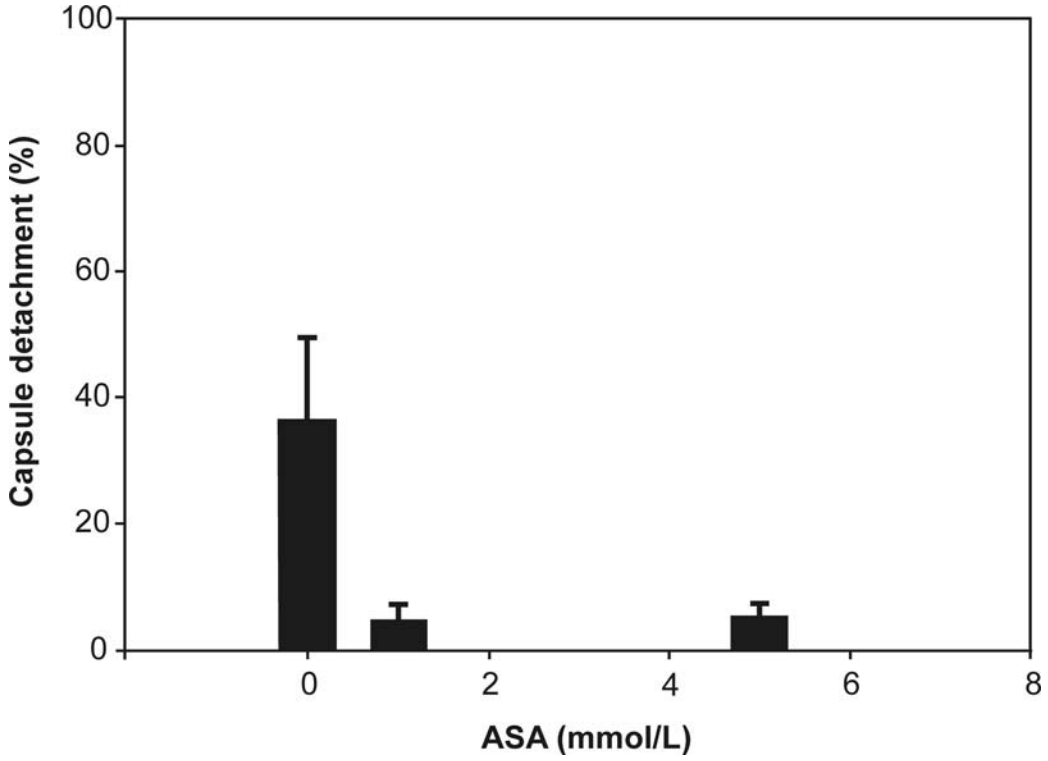

Fig. 2: The influence of different concentrations of acetylsalicylic acid (ASA) on capsule detachment after 42 h of cultivation.

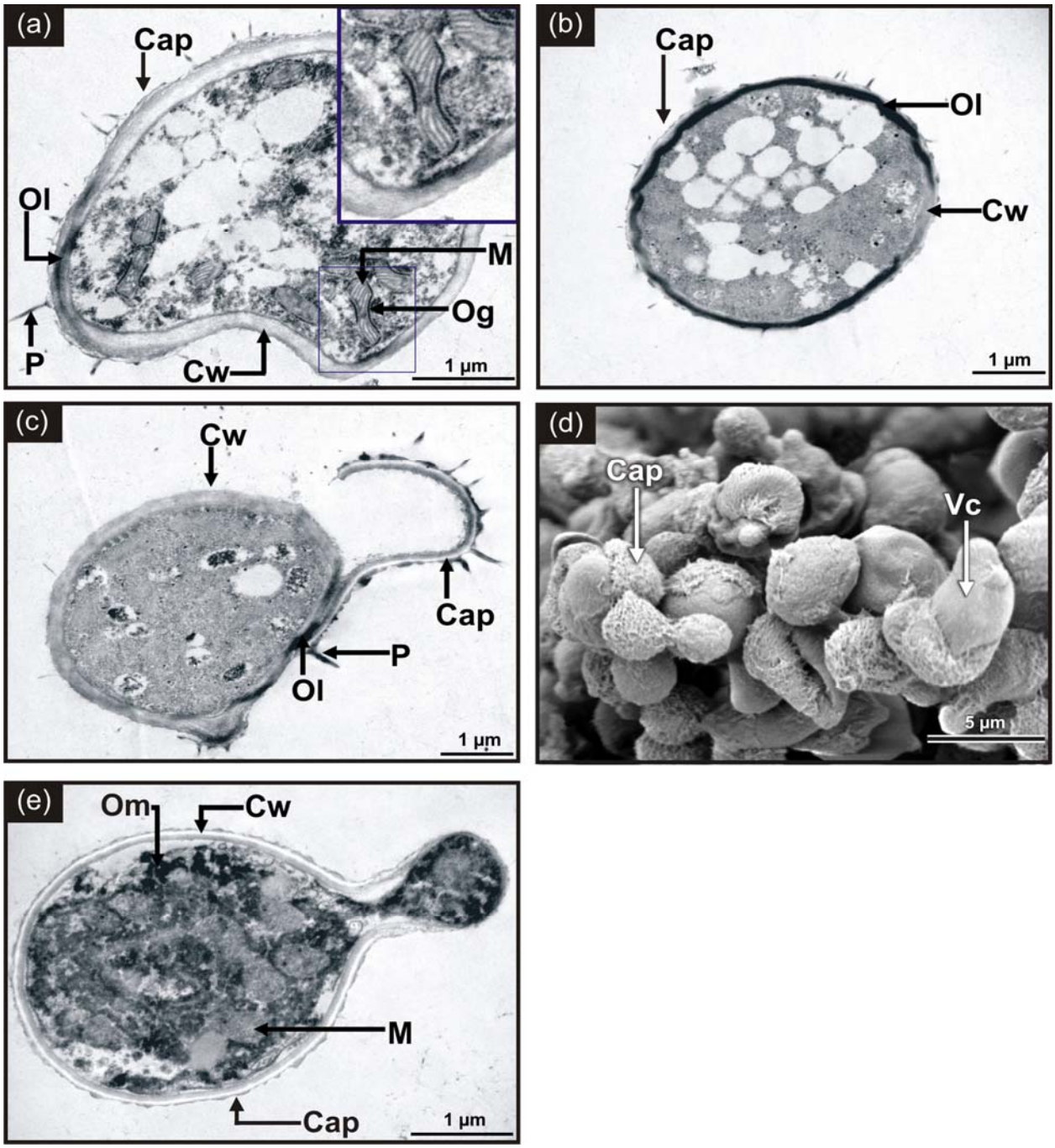

Fig. 3: Transmission electron micrographs depicting a possible migration route of osmiophilic material (containing 3-OH oxylipins) from (i) mitochondria (a) (ii) to depositing of this material on the inside of the cell wall (b) and (iii) eventual excretion through the cell wall to the outside of capsule that becomes detached (c). Scanning electron micrograph depicting capsule detachment (d). In the presence of 5 mmol/L

acetylsalicylic acid (ASA) a mitochondrial ultrastructural change is observed while capsule detachment as well as osmiophilic material migration is inhibited (e). Cap = capsule, Cw = cell wall, M = mitochondrion, Og = osmiophilic globules, Ol = osmiophilic layer, Om = osmiophilic material, P = protuberance. Vc = vegetative cell.

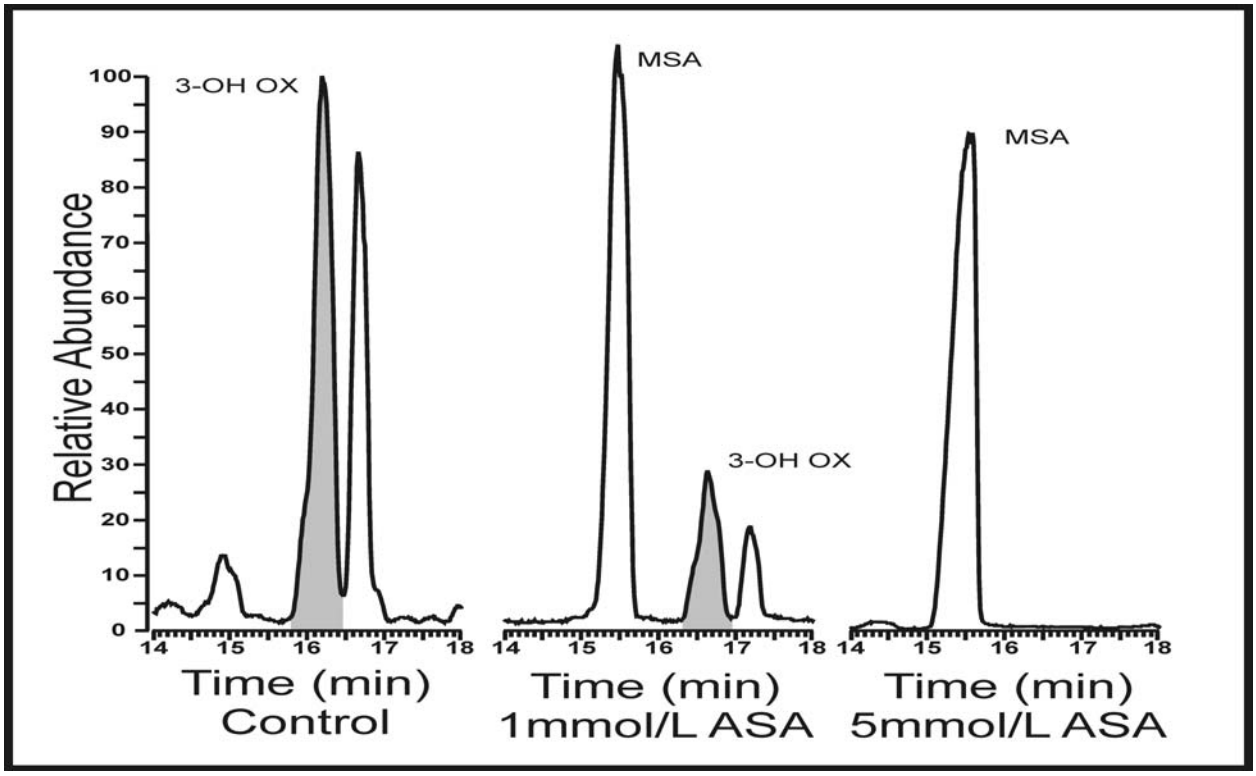

Fig. 4: Partial ion chromatograms of the methylated trimethylsilylated samples obtained from cells treated with 0 mmol/L (control), 1 mmol/L and 5 mmol/L acetylsalicylic acid (ASA) respectively. MSA = Methylsalicylate, 3-OH OX = 3-Hydroxy oxylipin (filled peaks).

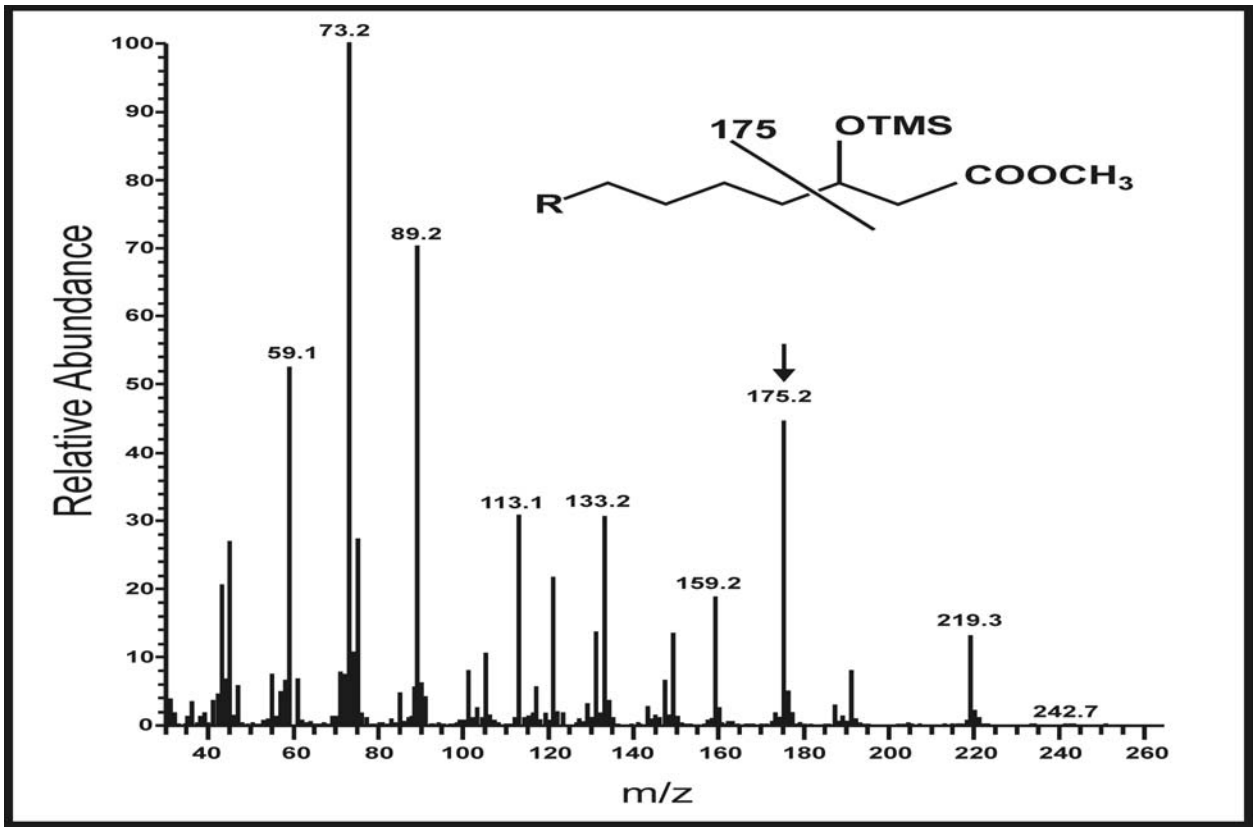

Fig. 5: Electron impact mass spectrum of the methylated trimethylsilylated 3-OH oxylipin obtained from cultivated cells (0 mmol/L and 1 mmol/L ASA). The *m/z* = 175 was derived from the derivatised first three carbon positions as counted from the carboxylic group. *m/z*, mass-to-charge ratio. (Reproduced from Sebolai et al. 2007).

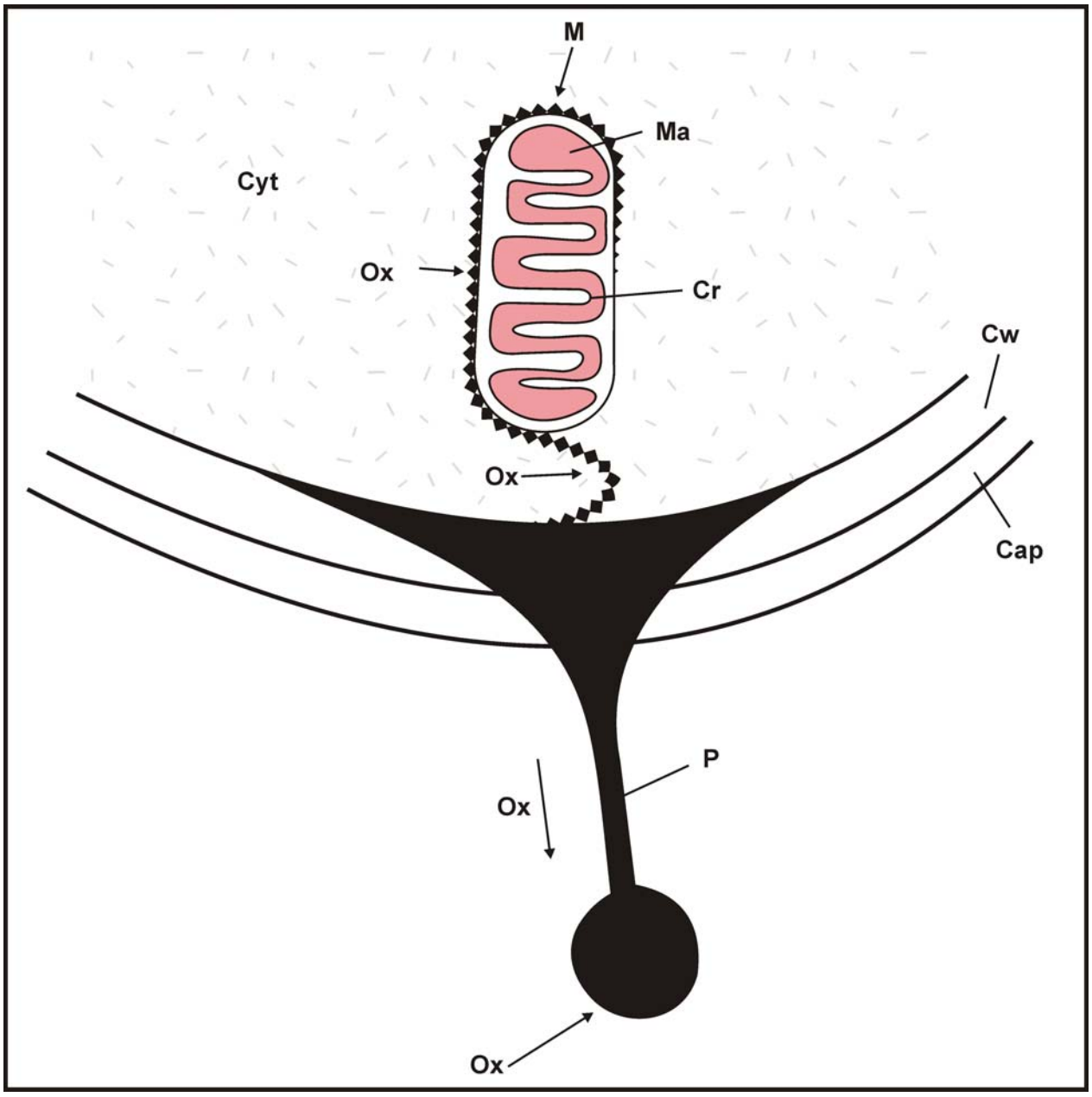

Fig. 6: Hypothesis depicting the migration of 3-OH oxylipins from mitochondria through cell walls into the surrounding environment. Cap = capsule, Cr = Cristae, Cw = cell wall, Cyt = cytoplasm, M = mitochondrion, Ma = matrix, Ox = oxylipin, P = protuberance.

Chapter 4

Distribution of 3-hydroxy oxylipins and acetylsalicylic acid sensitivity in *Cryptococcus* species

This study has been accepted for publication in Canadian Journal of Microbiology (2007).

4.1 Abstract

Using a well-tested antibody specific for 3-hydroxy oxylipins, we mapped the presence of these oxylipins in selected *Cryptococcus* species. Immunofluorescence microscopy studies revealed that these compounds are deposited on cell wall surfaces, appendages and collarettes. *In vitro* studies revealed that growth of *Cryptococcus* species, was inhibited by acetylsalicylic acid (a known inhibitor of mitochondrial function - including production of 3-hydroxy oxylipins), from a concentration as low as 1 mmol/L. The results suggest that acetylsalicylic acid is effective in controlling the growth of tested pathogens by probably targeting their mitochondria. This study further expands the known function of this anti-inflammatory drug as antifungal agent.

Key words: Acetylsalicylic acid, *Cryptococcus*, 3-Hydroxy oxylipins, Mitochondria.

4.2 Introduction

The yeast genus *Cryptococcus* includes important pathogens, such as *Cryptococcus neoformans* (Fell and Statzell-Tallman 1998). This basidiomycetous genus is non-fermentative, therefore requires functional mitochondria to support growth and other energy-driven cell processes (Fell and Statzell-Tallman 1998; Litter 2004). In yeasts, mitochondria are important in facilitating amongst others (1) completion of sexual life cycles (include sporulation and ascospore release), (2) flocculation and, (3) production of 3-hydroxy oxylipins through incomplete β-oxidation or fatty acid synthesis type II (FAS II) (Botha et al. 1992; Kock et al. 1999, 2007; Ciccoli et al. 2005; Hiltunen et al. 2005; Strauss et al. 2005, 2007). These functions, mediated by mitochondria, have been reported to be inhibited by acetylsalicylic acid (ASA) in a dose-dependent manner (Kock et al. 2003, 2007). Consequently, ASA may find application in controlling (1) flocculation processes, (2) sexual life cycles and, (3) production of 3-hydroxy oxylipins.

3-Hydroxy oxylipins are widely distributed in the yeast domain. Here, these compounds are mainly associated with surfaces of aggregating ascospores and vegetative cells, and also facilitate the release of ascospores from asci (Kock et al. 2003). In addition, these compounds are produced by pathogenic microorganisms where they act as virulence factors, and hence influencing the hosts' immune response

(Rietschel et al. 1994; Deva et al. 2000, 2001; Ciccoli et al. 2005; Takayama et al. 2005).

Recently, Sebolai et al. (2007*a*) discovered that a strain of *Cryptococcus neoformans* produces a 3-hydroxy oxylipin (3-OH 9:1), which is associated with the capsule (major virulence factor of this pathogen) and protuberances (associated cell wall structures). Furthermore, growth of this strain was inhibited in a dose-dependent manner by increased amounts of ASA (Leeuw et al. 2007; Sebolai et al. 2007*b*). Consequently, this study aims to further test the antifungal activity of ASA against other strains of *Cryptococcus neoformans* and related yeasts, some of which have been reported to be pathogenic. In addition, the localization of 3-hydroxy oxylipins will be mapped with immuno-confocal laser scanning microscopy (CLSM) while their chemical structures are assessed using gas chromatography-mass spectrometry (GC-MS).

4.3 Materials and methods

Strains used and cultivation

Yeast strains used in this study are listed in Table 1, and are held at the University of the Free State, Bloemfontein, South Africa. Cultures for immuno-confocal laser scanning microscopy (CLSM) and growth inhibition studies were cultivated on yeast-malt (YM) extract agar slants for 2 days at room temperature. Cultures used for oxylipin and ultrastructural studies were cultivated in 500 mL conical flasks, containing

100 mL of chemically defined yeast nitrogen base (YNB; 6.7 g/L) (Difco Laboratories, Detroit, Michigan, USA) broth supplemented with 4 % glucose (Saarchem, Wadeville, South Africa). These experiments were conducted at 30 ^{o}C while shaking (160 r/min) on a rotary shaker for 42 h. All experiments were performed at least in triplicate.

Oxylipin studies

Immunofluorescence: Polyclonal antibodies raised specifically against 3-hydroxy oxylipins (Bhatt et al. 1998; Groza et al. 2002) were employed during immunofluorescence confocal laser scanning microscopy (CLSM), to map the distribution of oxylipins in the selected yeast strains (*Cryptococcus neoformans* var. *neoformans* UOFS Y-1376, *Cryptococcus neoformans* var. *neoformans* UOFS Y-1380, *Cryptococcus neoformans* var. *grubii* UOFS Y-2783, *Cryptococcus neoformans* var. *grubii* UOFS Y-2784, *Cryptococcus neoformans* var. *grubii* UOFS Y-2785, *Cryptococcus neoformans* var. *grubii* UOFS Y-2786, *Cryptococcus albidus* UOFS Y-2127, *Cryptococcus ater* UOFS Y-2585, *Cryptococcus curvatus* UOFS Y-0812^{T}, *Cryptococcus gastricus* UOFS Y-0477, *Cryptococcus humicolus* UOFS Y-1345, *Cryptococcus laurentii* UOFS Y-1349). Briefly, cells from 2 day old YM agar slants were reacted with 30 µL of the primary antibody (incubated in the dark for 1 h). Subsequently, cells were reacted with 30 µL of the fluorescein isothiocyanate-coupled secondary antibody (Sigma, St. Louis, Missouri, USA). Following adequate washing, microscopic slides were prepared and viewed with a Nikon TE 2000 CLSM (Tokyo, Japan) (Kock et al. 1998). Appropriate controls were also included to ensure correct interpretation (Kock et al. 1998).

Chemical analysis: Two volumes (200 mL each) of ethyl acetate (Saarchem) were used to extract 3-hydroxy oxylipins directly from 42 h old cultures of selected yeast strains (*Cryptococcus albidus* UOFS Y-2127, *Cryptococcus ater* UOFS Y-2585, *Cryptococcus curvatus* UOFS Y-0812^T, *Cryptococcus gastricus* UOFS Y-0477, *Cryptococcus humicolus* UOFS Y-1345, *Cryptococcus laurentii* UOFS Y-1349) at acidic pH (Sebolai et al. 2004). The pH was decreased using 3 % formic acid (Saarchem). The organic phase was subsequently evaporated under a stream of nitrogen (AFROX, Bloemfontein, South Africa), followed by derivitization using diazomethane (Aldrich, Steinheim, Germany) and bis-(trimethylsilyl) trifluoroacetamide (BSTFA, Merck, Darmstadt, Germany). Finally, the methylated trimethylsilylated samples were analyzed using a Finnigan Trace Ultra gas chromatograph (Thermo Electron Corp., San Josè, California, USA) equipped with a Finnigan Trace DSQ MS (Thermo Electron Corp.) and HP-5-60 m fused silica capillary column (0.1 µm coating thickness) (Sebolai et al. 2004).

Electron microscopy

Fixation of ultrastructural material: Material for electron microscopy (42 h old cultures of *Cryptococcus gastricus* UOFS Y-0477 and *Cryptococcus ater* UOFS Y-2585) was chemically fixed using sodium phosphate-buffered 3 % glutardialdehyde (Merck) and similarly buffered osmium tetroxide (Merck) (van Wyk and Wingfield 1991).

Scanning electron microscopy: *Cryptococcus ater* UOFS Y-2585 was dehydrated in a graded ethanol (Merck) series for scanning electron microscopy (SEM). Next, the material was critical-point-dried (Biorad Microscience Division, Watford, England), mounted, and coated with gold using a SEM coating system (Biorad Microscience

Division) (van Wyk and Wingfield 1991). Preparations were examined using a Shimadzu Superscan SSX 550 scanning electron microscope (Tokyo, Japan).

Transmission electron microscopy: *Cryptococcus gastricus* UOFS Y-0477 was dehydrated in a graded acetone (Merck) series for transmission electron microscopy (TEM). Next, the material was embedded in epoxy resin (Spurr 1969) before thin-sections were made with a LKB III ultramicrotome (Stockholm, Sweden), and viewed with a Phillips EM 100 transmission electron microscope (Eindhoeven, the Netherlands) (van Wyk and Wingfield 1991).

Growth inhibition studies

Yeast strains used for this study are listed in Table 2. The effect of ASA (Aldrich; 80 g/L stock solution in absolute ethanol (Merck)) on yeast growth was assessed in test tubes containing 5 mL of YNB (6.7 g/L; Difco Laboratories) broth supplemented with 2 % glucose (Saarchem) (Petrou and Shanson 2000). The tubes were incubated aerobically with 2 day old cultures on YM agar, while agitating on a Rollordrum (30 ^{o}C for 4 days) according to the assimilation tests in liquid medium protocol (Yarrow 1998). All yeasts were subjected to the following ASA concentration gradient: 0 mmol/L, 1 mmol/L, 2 mmol/L, 3 mmol/L, 4 mmol/L and 5 mmol/L ASA. Ethanol (ETOH) control (equivalent to the ethanol volume used to reconstitute 5 mmol/L ASA) as well as a negative control (i.e. no ASA, ethanol or inoculum) were included. Growth was determined visually as described by Yarrow (1998).

4.4 Results and discussion

Oxylipin mapping

Fluorescence depicting the presence of 3-OH oxylipins was observed on cell wall surfaces as protuberances in *Cryptococcus neoformans* var. *neoformans* UOFS Y-1376 and *Cryptococcus neoformans* var. *grubii* UOFS Y-2784 (Fig. 1). Similar observations were also noted for *Cryptococcus neoformans* var. *neoformans* UOFS Y-1380, *Cryptococcus neoformans* var. *grubii* UOFS Y-2783, *Cryptococcus neoformans* var. *grubii* UOFS Y-2785 as well as *Cryptococcus neoformans* var. *grubii* UOFS Y-2786 (data not shown). This 3-OH oxylipin distribution pattern is in accordance with that previously reported for *Cryptococcus neoformans* var. *neoformans* UOFS Y-1378 (Sebolai et al. 2007*a*, *b*). In the latter yeast, protuberances were proposed to facilitate oxylipin release into the surrounding medium. Are these protuberances and function conserved characteristics for *Cryptococcus neoformans*? In addition, 3-OH oxylipins were found to be associated with cell walls of *Cryptococcus albidus* UOFS Y-2127, *Cryptococcus curvatus* UOFS Y-0812^{T}, *Cryptococcus humicolus* UOFS Y-1345 as well as *Cryptococcus laurentii* UOFS Y-1349, although, no appendages could be observed (data not shown).

Strikingly, in *Cryptococcus gastricus* UOFS Y-0477, fluorescence indicates accumulation of 3-OH oxylipins in the vicinity of the collarette (i.e. at site of enteroblastic

bud formation) (Figs. 2a and 2b). At this position, a selective accumulation of osmiophilic material is also observed (Fig. 2c). Previous studies show that osmiophilic material in yeasts also contains 3-OH oxylipins (Kock et al. 2000; Sebolai et al. 2007*a*). Is it possible that 3-OH oxylipins are involved during bud formation and/or mother-daughter cell separation?

Results obtained for *Cryptococcus ater* UOFS Y-2585 are shown in Fig. 3. Here, oxylipins are mainly associated with the cell wall surfaces as well as extended thread-like appendages (Fig. 3a). These appendages extended to reach adjacent cells, possibly to effect cell aggregation. These appendages are shown in more detail in Fig. 3b using SEM.

Oxylipin characterization

Analytical studies using gas chromatography-mass spectrometry, confirmed the presence of 3-OH oxylipins in the tested yeast strains *Cryptococcus albidus* UOFS Y-2127, *Cryptococcus ater* UOFS Y-2585, *Cryptococcus curvatus* UOFS Y-0812^{T}, *Cryptococcus gastricus* UOFS Y-0477, *Cryptococcus humicolus* UOFS Y-1345 and *Cryptococcus laurentii* UOFS Y-1349. The mass spectra of the analyzed methylated trimethylsilylated samples were all characterized by a mass fragment of *m/z* = 175, which is indicative of hydroxylation of fatty acids at the C-3 position, when counting from the carboxylic group (Figs. 4a – 4f) (Van Dyk et al. 1991). However, the total structures of these 3-OH oxylipins could not be determined since it was not possible to identify their corresponding mother ions.

Growth inhibition studies

Literature suggests that yeasts that are dependent only on mitochondrial-aerobic respiration for growth, are more sensitive to ASA compared to yeasts that possess both energy production pathways i.e. aerobic respiration and fermentation (Leeuw et al. 2007). Our results further corroborate this hypothesis (Table 2). In this study, all non-fermenting *Cryptococcus* species were much more sensitive to ASA compared to the fermentative yeast, *Saccharomyces cerevisiae* (Table 2). Already at an ASA concentration of 2 mmol/L, a decrease in growth of most *Cryptococcus* species (exception: *Cryptococcus humicolus* UOFS Y-1345 and *Cryptococcus terreus* UOFS Y-1136^{T}) was evident. From 3 mmol/L ASA, the growth of all *Cryptococcus* species was significantly inhibited. Literature suggests that the ASA response may be due to inhibition of mitochondrial function, which includes inhibition of oxidative phosphorylation and respiratory electron transport chain – functions important for energy generation (Somasundaram et al. 1997; Norman et al. 2004). This data suggests that ASA can be used as an antifungal agent to combat growth of these pathogenic yeasts.

In conclusion, the data presented show that 3-OH oxylipins are widely distributed in the yeast genus *Cryptococcus*. In the examined representatives, these compounds were mainly associated with structures such as protuberances, collarettes, appendages as well as cell wall surfaces. Is it possible that these 3-OH oxylipins may be virulence factors contributing to *Cryptococcus* pathogenicity? According to the endosymbiotic theory, mitochondria once freely existed as Gram negative bacteria (Gray et al. 2001). Is

it possible that Gram negative bacteria, that function as mitochondria, are still capable of causing infections indirectly through 3-OH oxylipin release in *Cryptococcus* (Sebolai et al. 2007*a*, *b*)? It has been found that 3-OH oxylipins are an important part of the released lipopolysaccharide (LPS) layer (endotoxin) responsible for inflammatory responses upon infection by Gram negative bacteria (Rietschel et al. 1994).

Furthermore, growth inhibition studies support the suggested link between mitochondrial dependence and ASA sensitivity in yeasts (Kock et al. 2007; Leeuw et al. 2007; Strauss et al. 2007). Is it possible that ASA, a commonly used non steroidal anti-inflammatory drug (NSAID), can also be used to combat *Cryptococcus* infections? However, care should be taken when proceeding to *in vivo* tests since ASA administered to humans over extended periods of time and at high dosages can be toxic (Wolfe et al. 1999).

4.5 Acknowledgements

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4.7 Tables

Table 1: Yeasts used in this study.

Yeasts tested	Strain no.	Source
Cryptococcus aerius	UOFS Y-1340^T	air, Japan
Crypt. albidus	UOFS Y-0223	unknown
Crypt. albidus	UOFS Y-2127	soil, South Africa
Crypt. ater	UOFS Y-2585	unknown
Crypt. bhutanensis	UOFS Y-0476^T	soil, Laya, Bhutan
Crypt. bhutanensis	UOFS Y-1341	rumen of musk ox
Crypt. curvatus	UOFS Y-0812^T	sputum of TB patient, Netherlands
Crypt. curvatus	UOFS Y-1342	uterus of cow, United Kingdom
Crypt. fuscescens	UOFS Y-2591	ex saline takyr soil, Russia
Crypt. gastricus	UOFS Y-0477	soil, New Zealand
Crypt. gastricus	UOFS Y-1344	rumen of musk ox
Crypt. humicolus	UOFS Y-1345	mushrooms
Crypt. hungaricus	UOFS Y-1346	flower of *Lewesia pygmaea*, United States
Crypt. laurentii	UOFS Y-1348	palm wine, Congo
Crypt. laurentii	UOFS Y-1349	ex tumour, Netherlands
Crypt. luteolus	UOFS Y-1351	ex acidic sludge, Netherlands
Crypt. macerans	UOFS Y-1352^T	dew-retted flax straw, Denmark

Crypt. neoformans var. *grubii*	UOFS Y-2783	AIDS patient, South Africa
Crypt. neoformans var. *grubii*	UOFS Y-2784	AIDS patient, South Africa

Table 1: Yeasts used in this study (continued).

Yeasts tested	**Strain no.**	**Source**
Crypt. neoformans var. *grubii*	UOFS Y-2785	AIDS patient, South Africa
Crypt. neoformans var. *grubii*	UOFS Y-2786	AIDS patient, South Africa
Crypt. neoformans var. *neo.*	UOFS Y-0474	ex fermenting fruit juice
Crypt. neoformans var. *neo.*	UOFS Y-1376	brain, Netherlands
Crypt. neoformans var. *neo.*	UOFS Y-1380	ex man, United States
Crypt. terreus	UOFS Y-0479	soil, Belgium
Crypt. terreus	UOFS Y-1136^{T}	garden soil, New Zealand
Saccharomyces cerevisiae	UOFS Y-1529	unknown

Crypt. = *Cryptococcus* (*Filobasidiella*); *neo. = neoformans;* T= type strain; UOFS = University of the Free State; var. = variety. Y = yeast.

Table 2: Influence of acetylsalicylic acid on growth of *Cryptococcus neoformans* and related yeasts (after 4 days of cultivation).

Yeast	Cult. No.	Growth ASA concentration range (mmol/L)								Ferment
		0	1	2	3	4	5	ETOH	-*	
Crypt. aerius	UOFS Y-1340^T	+++	++	+	+	+	+	+++	-	n
Cypt. albidus	UOFS Y-0223	+++	++	+	+	+	+	+++	-	n
Cypt. albidus	UOFS Y-2127	+++	+	+	+	+	+	+++	-	n
Cypt. bhutanensis	UOFS Y-0476^T	+++	+++	++	++	+	+	+++	-	n
Cypt. bhutanensis	UOFS Y-1341	+++	+	+	+	+	+	+++	-	n
Crypt. curvatus	UOFS Y-0812^T	+++	++	+	+	+	+	+++	-	n
Crypt. curvatus	UOFS Y-1342	+++	++	++	+	+	+	+++	-	n
Crypt. fuscescens	UOFS Y-2591	+++	++	+	+	+	+	+++	-	n
Crypt. gastricus	UOFS Y-0477	+++	++	++	+	+	+	+++	-	n
Crypt. gastricus	UOFS Y-1344	+++	++	++	+	+	+	+++	-	n
Crypt. humicolus	UOFS Y-1345	+++	+++	+++	+	+	+	+++	-	n
Crypt. hungaricus	UOFS Y-1346	+++	++	+	+	+	+	+++	-	n
Crypt. laurentii	UOFS Y-1348	+++	+++	++	+	+	+	+++	-	n
Crypt. laurentii	UOFS Y-1349	+++	+	+	+	+	+	+++	-	n
Crypt. luteolus	UOFS Y-1351	+++	+++	++	+	+	+	+++	-	n
Crypt. macerans	UOFS Y-1352^T	+++	+++	++	++	+	+	+++	-	n
Cypt. neo. var. *grubii*	UOFS Y-2783	+++	+++	++	++	+	+	+++	-	n
Cypt. neo. var. *grubii*	UOFS Y-2784	+++	++	++	+	+	+	+++	-	n
Cypt. neo. var. *grubii*	UOFS Y-2785	+++	++	++	+	+	+	+++	-	n
Cypt. neo. var. *grubii*	UOFS Y-2786	+++	++	++	+	+	+	+++	-	n
Cypt. neo. var. *neo.*	UOFS Y-0474	+++	++	++	++	+	+	+++	-	n
Cypt. neo. var. *neo.*	UOFS Y-1376	+++	++	++	+	+	+	+++	-	n
Cypt. neo. var. *neo.*	UOFS Y-1380	+++	+++	++	+	+	+	+++	-	n
Crypt. terreus	UOFS Y-0479	+++	++	++	++	+	+	+++	-	n
Crypt. terreus	UOFS Y-1136^T	+++	+++	+++	+	+	+	+++	-	n
Sacch. cerevisiae	UOFS Y-1529	+++	+++	+++	+++	+++	+++	+++	-	p

-* = negative control; 0-5 = ASA concentration in mmol/L; ETOH = ethanol control; n = fermentation negative; p = fermentation positive; +++: good growth; ++: growth; + weak or no growth. *Crypt.* = *Cryptococcus (Filobasidiella)*; *neo*. = *neoformans*; *Sacch.* = *Saccharomyces*; t = type strain; var. = variety.

4.8 Figures

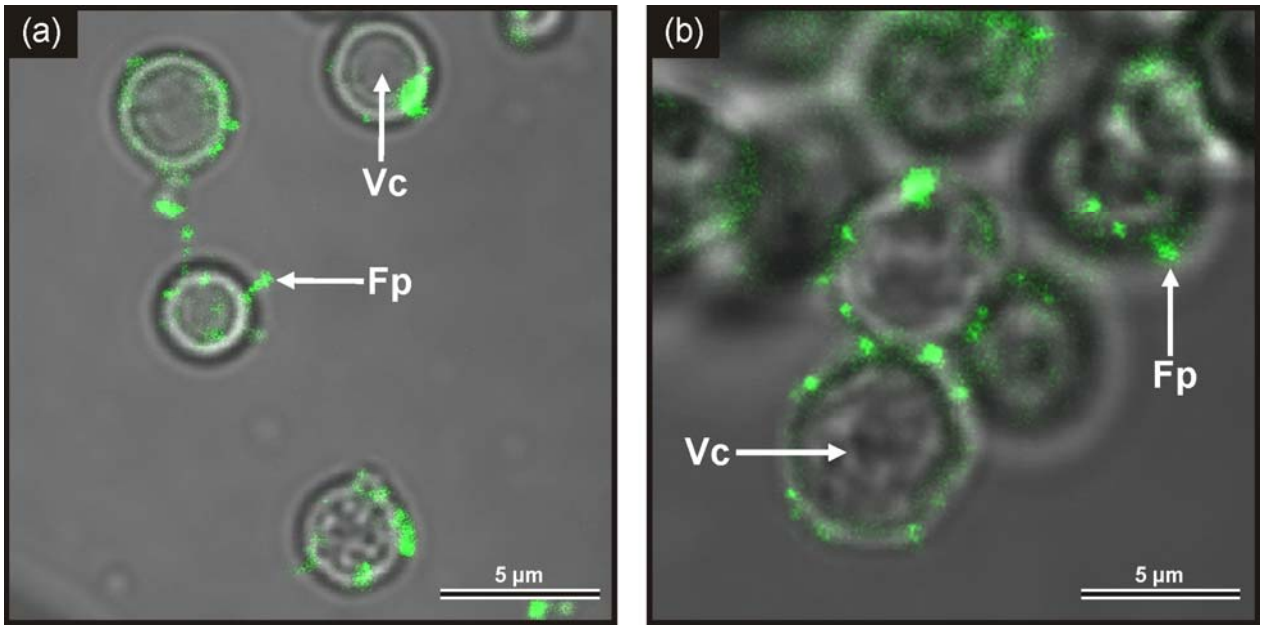

Fig. 1: Light micrographs with super-imposed immunofluorescence showing the association of fluorescence (depicts 3-OH oxylipins) with cell wall surfaces including protuberances in *Cryptococcus neoformans* var*. neoformans* UOFS Y-1376 (a), and *Cryptococcus neoformans* var. *grubii* UOFS Y-2784 (b). Fp = fluorescing protuberance; Vc = vegetative cell.

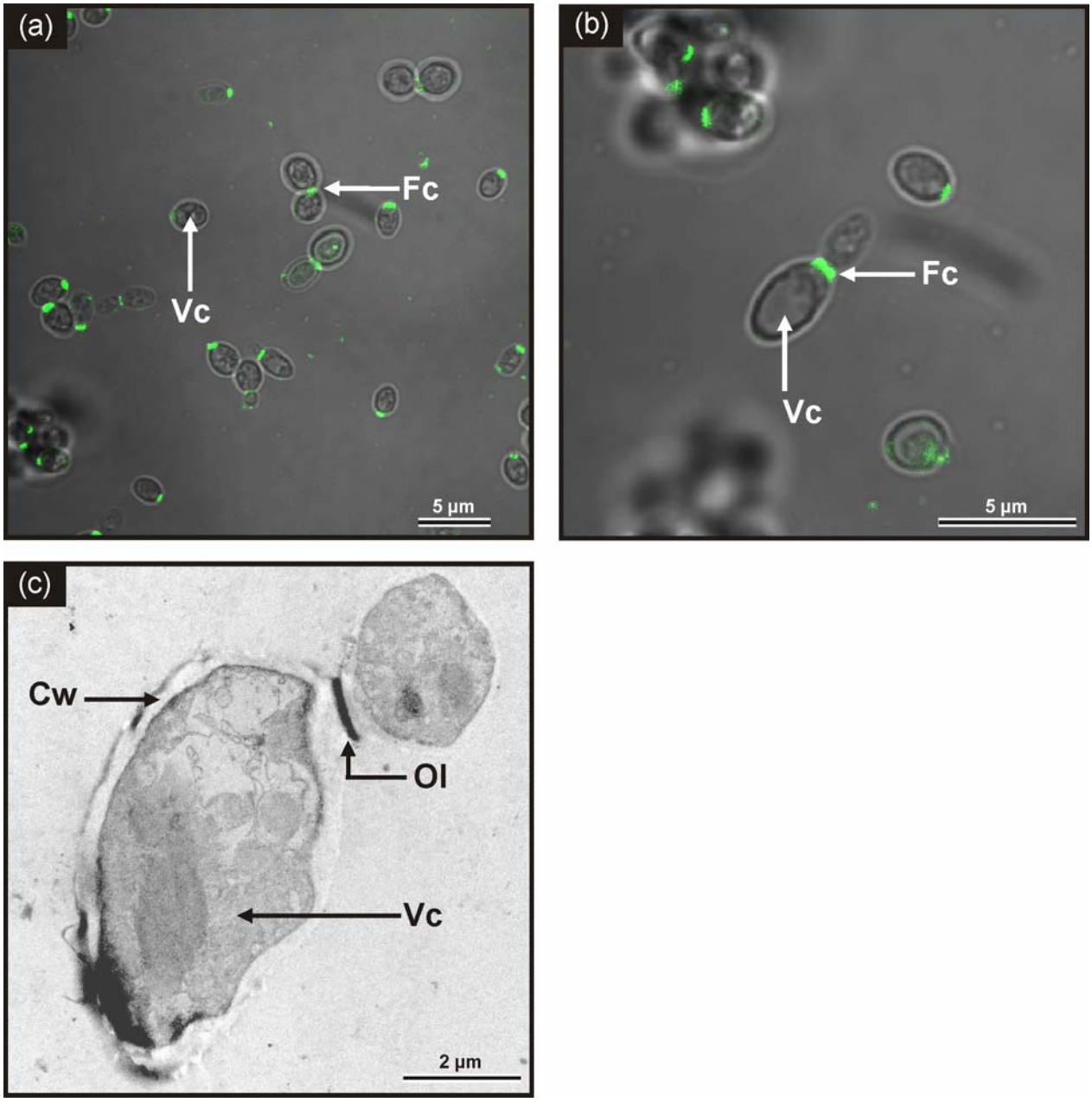

Fig. 2: Light micrographs with super-imposed immunofluorescence (a, b), showing the deposition of fluorescing material (depicts 3-OH oxylipins) at the budding sites (collarettes) in *Cryptococcus gastricus* UOFS Y-0477, the corresponding TEM micrograph also shows the deposition of osmiophilc material at the budding site (c). Cw = cell wall; Fc = fluorescing collarette; Ol = osmiophilic layer; Vc = vegetative cell.

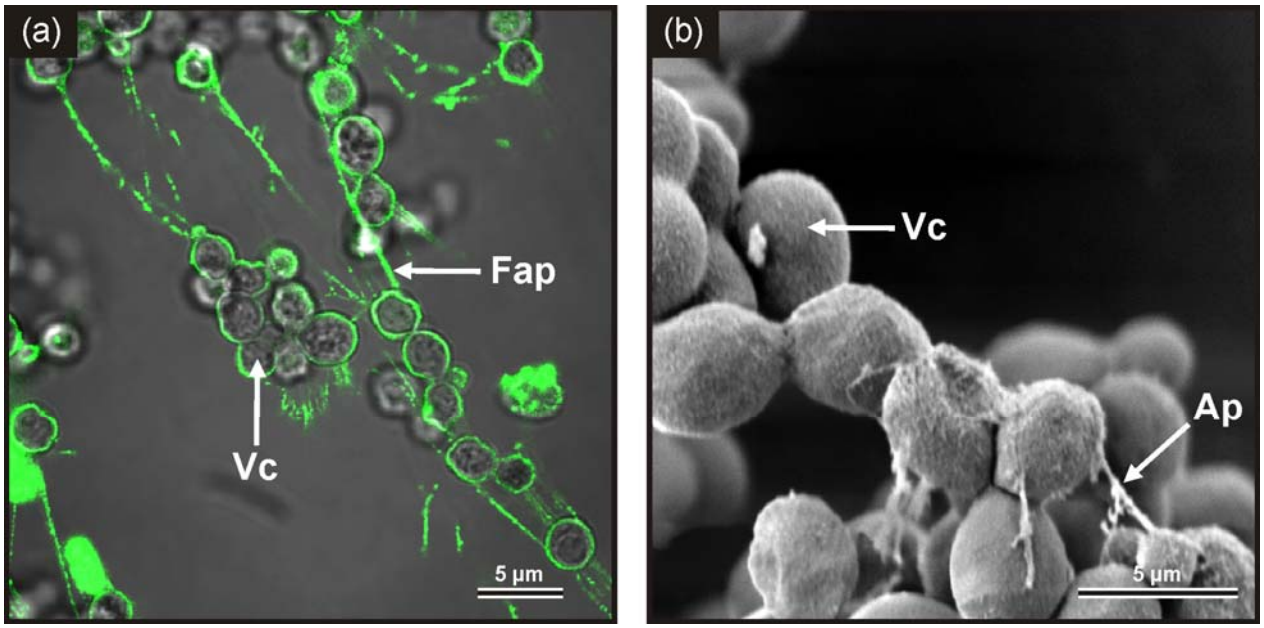

Fig. 3: Light micrograph with super-imposed immunofluorescence (a), showing fluorescing material (depicts 3-OH oxylipins) associated with cell wall surfaces and appendages in *Cryptococcus ater* UOFS Y-2585, the corresponding SEM micrograph reveals detail of cell wall-associated appendages (b). Ap = appendage; Fap = fluorescing appendage; Vc = vegetative cell.

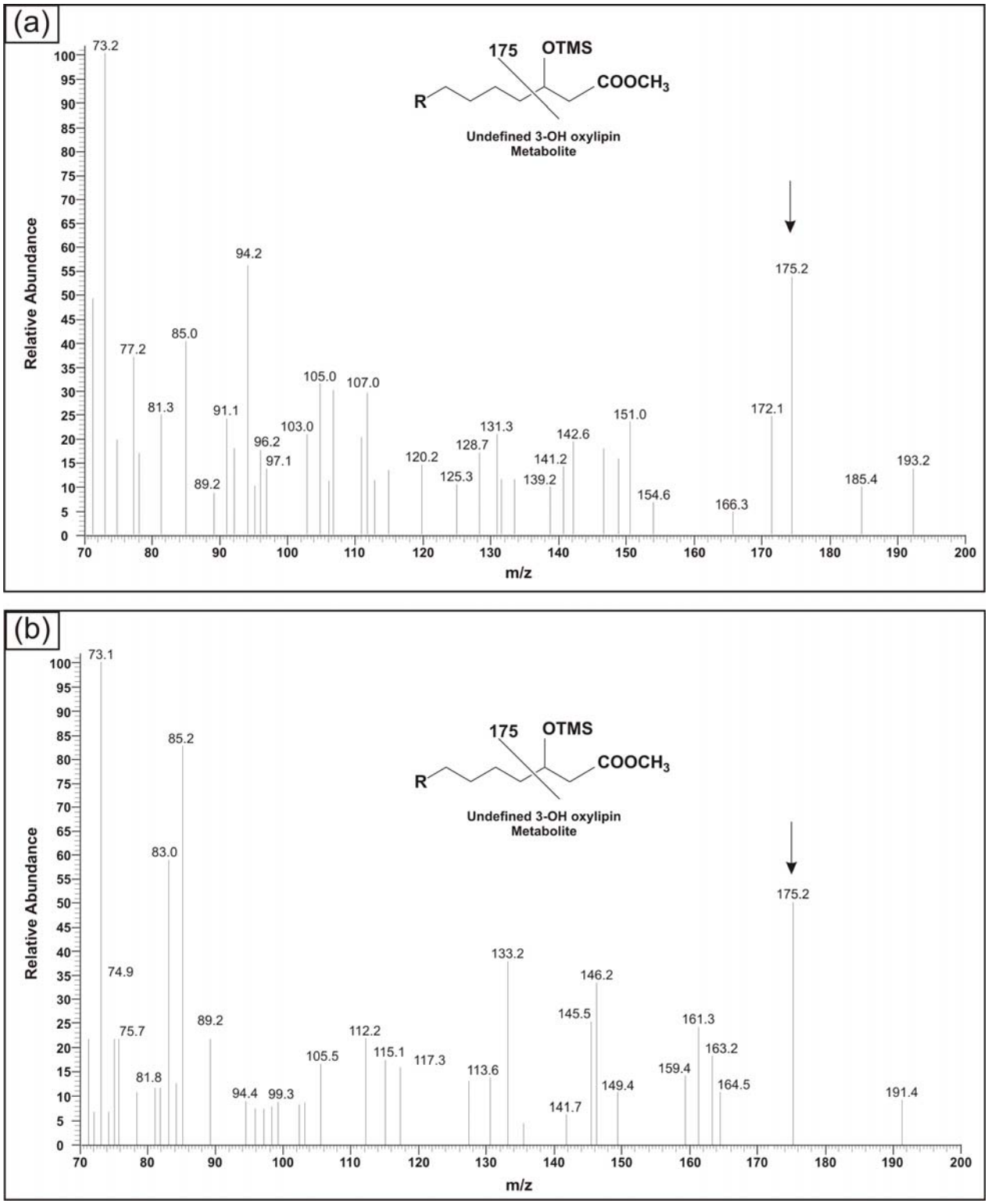

Fig. 4 (a, b): Mass spectra of 3-hydroxy oxylipin metabolites obtained from derivatised culture extracts of *Cryptococcus albidus* UOFS Y-2127 (a), *Cryptococcus ater* UOFS Y-2585 (b). The mass fragment at *m/z* = 175, indicates the presence of a hydroxyl group at C-3 position, when counting from the carboxylic group. *m/z*, Mass-to-charge ratio.

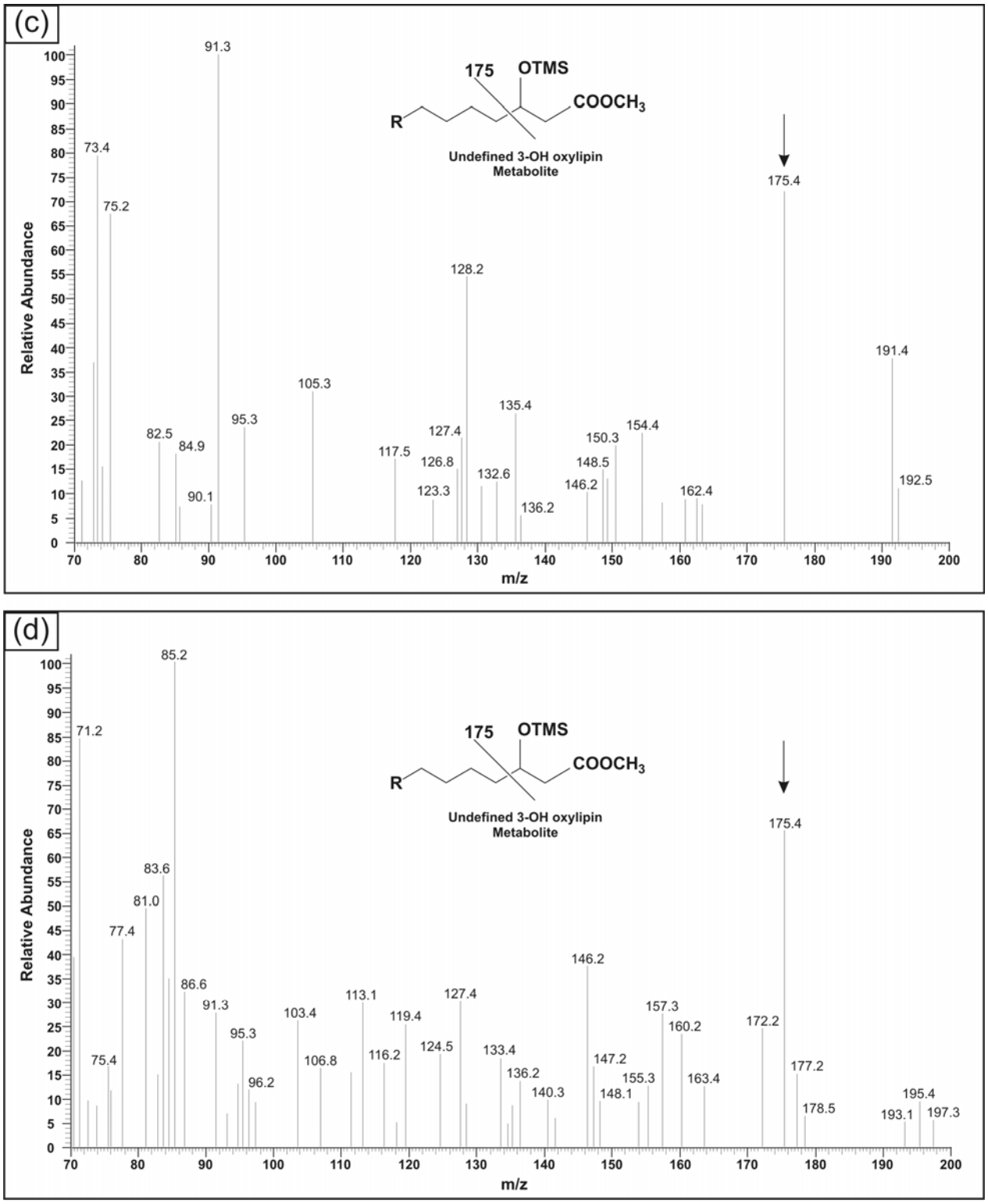

Fig. 4 (c, d): Mass spectra of 3-hydroxy oxylipin metabolites obtained from derivatised culture extracts of *Cryptococcus curvatus* UOFS Y-0812^T (c), *Cryptococcus gastricus* UOFS Y-0477 (d). The mass fragment at *m/z* = 175, indicates the presence of a hydroxyl group at C-3 position, when counting from the carboxylic group. *m/z*, Mass-to-charge ratio; T = type strain.

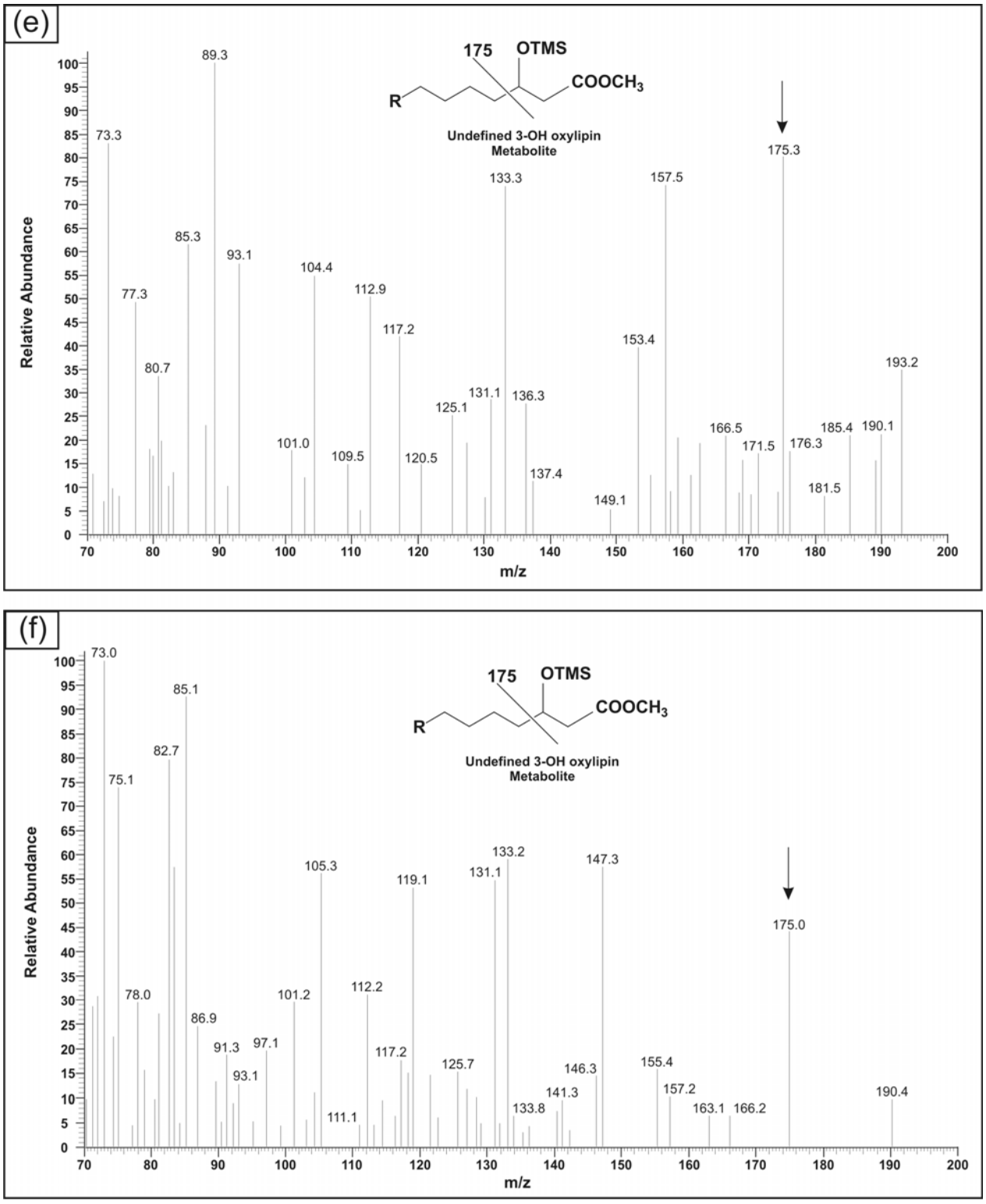

Fig. 4 (e, f): Mass spectra of 3-hydroxy oxylipin metabolites obtained from derivatised culture extracts of *Cryptococcus humicolus* UOFS Y-1345 (e), *Cryptococcus laurentii* UOFS Y-1349 (f). The mass fragment at *m/z* = 175, indicates the presence of a hydroxyl group at C-3 position, when counting from the carboxylic group. *m/z*, Mass-to-charge ratio.

Summary

Literature shows that *Cryptococcus neoformans* is an important human pathogen responsible for many deaths worldwide. To compound this, treatment of cryptococcal infections has over the years been difficult. This is largely due to the widespread use of antifungals, leading to the emergence of drug resistant strains. The capsule (with glucuronoxylomannan as major polysaccharide) is the principal virulence factor of this pathogen, and can influence the hosts' immune response. Moreover, recent studies have identified novel bioactive compounds, which can also contribute to the virulence of pathogens such as *Cryptococcus neoformans* and *Candida albicans*. These include compounds such as oxylipins (oxidized fatty acids), which have been reported to modulate the hosts' immune response during infections. This exposes new targets for antifungal action.

In this study, the 3-hydroxy fatty acid, 3-OH 9:1, has been discovered in *Cryptococcus neoformans* var. *neoformans* UOFS Y-1378 using gas chromatography-mass spectrometry. Immunofluorescence confocal laser scanning microscopy and immunogold transmission electron microscopy revealed that this 3-OH oxylipin accumulates in capsules, where it is released as hydrophobic droplets through protuberances (each about 30 nm x 400 nm) into the extracellular environment. This discovery further expands our knowledge of the known spectrum of biologically active compounds associated with this main virulence factor of *Cryptococcus neoformans*.

3-OH 9:1 is produced in yeast mitochondria probably through β-oxidation or fatty acid synthesis pathway type II (FAS II). Evidence supporting this statement, was provided after mapping the migration of 3-OH oxylipin-containing osmiophilic material

during ultrastructural studies. Here, osmiophilic material was shown to originate in mitochondria and is deposited inside the yeast cell wall, from where it is released into the surrounding medium, along capsule protuberances or through capsule detachment. When acetylsalicylic acid (ASA, an inhibitor of mitochondrial function – including 3-OH oxylipin production) was added, the migration of the osmiophilic material as well as capsule detachment from cell walls and hence oxylipin release was abrogated. This data is in accordance with literature, where a novel release mechanism for the major virulence factor of *Cryptococcus neoformans* is reported. Here, virulent polysaccharide packaged lipid vesicles are reported to cross the cell wall and the capsule into the surrounding environment. This Ph.D. study implicates the lipid vesicles to contain 3-OH oxylipins.

It was also demonstrated that 3-OH oxylipins are widely distributed in other members of the pathogenic yeast genus *Cryptococcus*, following immunofluorescence confocal laser scanning microscopy (using antibodies directed towards 3-OH oxylipins) and gas chromatography-mass spectrometry. In the examined strains these compounds were mainly associated with cell wall surfaces, protuberances, appendages and collarettes. According to literature, yeasts that are dependent only on mitochondrial-aerobic respiration for growth, are more sensitive to ASA compared to yeasts that possess both energy production pathways i.e. aerobic respiration and fermentation. In this study, *in vitro* data corroborate this hypothesis. Here, the growth of all non-fermenting *Cryptococcus* species was much more sensitive to ASA compared to the fermentative yeast, *Saccharomyces cerevisiae* (which could tolerate as much as 5 mM ASA). Already at an ASA concentration of 2 mM, a decrease in growth of most

Cryptococcus species was evident, and at 3 mM ASA, the growth of all *Cryptococcus* species was significantly inhibited. The observed ASA effect may be due to inhibition of mitochondrial function, which includes inhibition of oxidative phosphorylation and respiratory electron transport chain – functions important for energy generation. These data suggest that ASA can be used as an antimitochondrial antifungal agent to combat growth of these pathogenic yeasts. This discovery should now be further researched *in vivo* taking into account the toxicity of ASA and other non steroidal anti-inflammatory drugs.

Key words: Acetylsalicylic acid; Antifungal agent; Capsule; *Cryptococcus*; Electron microscopy; Growth inhibition; 3-Hydroxy oxylipins; Immunofluorescence confocal laser scanning microscopy; Mitochondria; Yeast.

Opsomming

Volgens literatuur is *Cryptococcus neoformans* ‘n belangrike menslike patogeen, verantwoordelik vir ‘n groot aantal sterftes wêreldwyd. Om dit te vererger, was behandeling van cryptococcale infeksies oor die jare moeilik. Dit is grootliks as gevolg van die algemene gebruik van antifungale middels, wat gelei het tot die ontstaan van weerstandbiedende stamme. Die kapsule (met glukuronoxylomannaan as hoof polisakkaried) is die belangrikste virulensiefaktor van dié patogeen, en kan die gasheer se immuunrespons beïnvloed. Verder het onlangse studies nuwe bioaktiewe verbindings, wat ook mag bydra tot die virulensie van patogene soos *Cryptococcus neoformans* en *Candida albicans*, geïdentifiseer. Dit sluit verbindings soos oksielipiene (geöksideerde vetsure), wat gerapporteer is om die gasheer se immuunrespons te moduleer gedurende infeksies, in. Dit lê nuwe teikens vir antifungale aksies bloot.

In hierdie studie is m.b.v. gaschromatografie-massaspektrometrie die 3-hidroksievetsuur, 3-OH 9:1, in *Cryptococcus neoformans* var. *neoformans* UOFS Y-1378 ontdek. Met behulp van immunofluoresensie konfokale laser skandeermikroskopie en immunogoudtransmissie-elektronmikroskopie is aangetoon dat hierdie 3-OH oksielipien in kapsules versamel, vanwaar dit as hidrofobiese druppels deur uitsteeksels (elk ongeveer 30 nm x 400 nm) in die ekstrasellulêre omgewing vrygestel word. Hierdie ontdekking verbreed ons kennis verder oor die bekende spektrum van biologies-aktiewe verbindings wat met die hoof virulensiefaktore van *Cryptococcus neoformans* geässosieer is.

3-OH 9:1 word in gismitochondria geproduseer, waarskynlik deur β-oksidasie of ‘n tipe II vetsuursinteseweg (FAS II). Getuienis ter stawing van hierdie stelling is verkry

nadat die migrasie van 3-OH oksielipienbevattende osmiofilliese material gevolg is gedurende ultrastrukturele studies. Dit is aangetoon dat osmiofilliese materiaal in die mitochondria onstaan en binne die gisselwand neergelê word, van waar dit in die omliggende omgewing vrygestel word d.m.v. kapsulêre uitsteeksels of d.m.v. vrystelling van die kapsule self. As asetielsalisielsuur (ASA, ‘n inhibeerder van mitochondriale funksie – insluitend 3-OH oksielipienproduksie) bygevoeg word, word die migrasie van die osmiofilliese material sowel as vrystelling van die kapsule van die selwand en dus oksielipienvrystelling, opgehef. Hierdie data is in ooreenstemming met literatuur, waar ‘n nuwe vrystellingsmeganisme vir die hoof virulensiefaktor van *Cryptococcus neoformans* gerapporteer word. Hier word gerapporteer dat virulente polisakkariede, verpak in lipiedvesikels, die selwand en kapsule oorsteek na die omliggende omgewing. Hierdie Ph.D. studie impliseer dat die lipiedvesikels 3-OH oksielipiene bevat.

Met behulp van immunofluoresensie konfokale laser skandeermikroskopie (met teenliggaampies teen 3-OH oksielipiene) en gaschromatografie-massaspektrometrie is dit ook gedemonstreer dat 3-OH oksielipiene wyd verspreid is in ander lede van die gisgenus *Cryptococcus*. In die bestudeerde stamme is hierdie verbindings hoofsaaklik geässosieer met selwandoppervlakke, uitsteeksels, aanhangsels en kragies. Volgens literatuur is giste wat slegs afhanklik is van mitochondriale aerobiese respirasie vir groei meer sensitief vir ASA in vergelyking met giste wat beide aerobiese respirasie en fermentasie kan uitvoer om energie te verkry. In hierdie studie ondersteun *in vitro* data hierdie hipotese. Hier is gevind dat die groei van alle nie-fermenterende *Cryptococcus* spesies baie meer sensitief was vir ASA in vergelyking met die fermenterende gis, *Saccharomyces cerevisiae* (wat soveel as 5 mM ASA kon hanteer). ‘n Afname in die

groei van meeste *Cryptococcus* spesies was reeds duidelik teen ‘n ASA konsentrasie van 2 mM, en teen 3 mM ASA is die groei van alle *Cryptococcus* spesies beduidend geïnhibeer. Die waargenome effek van ASA mag wees a.g.v. die inhibisie van mitochondriale funksie, insluitend oksidatiewe fosforilering en respiratoriese elektrontransport – belangrike energiegenererende funksies. Hierdie data impliseer dat ASA as antimitochondriale antifungale middel gebruik kan word om die groei van hierdie patogene giste te beheer. Hierdie ontdekking behoort nou verder *in vivo* ondersoek te word, met inaggenome die toksisiteit van ASA en ander nie-steroïed anti-inflammatoriese middels.

Sleutel woorde: Antifungale middel; Asetielsalisielsuur; *Cryptococcus*; Elektron mikroskopie; Giste; Groei inhibisie; 3-Hidroksie oksielipiene; Immunofluoresensie konfokale laser skandering mikroskopie; Kapsule; Mitochondriale.

Appendix

Synthesis of 3-hydroxy oxylipins (performed by Prof. S. Nigam in Germany)

The 3-hydroxy oxylipins, 3*R*- and *S*-hydroxy 5,8,11,14-eicosatetraenoic acid [3(*R/S*)-HETE] were specifically synthesized for antibody production (Kock *et al.,* 1998). 3(*R/S*)-HETE were chemically synthesized using a convergent approach of coupling chiral aldehyde with a Wittig salt (Bhatt *et al.,* 1998; Groza *et al.,* 2002). These were derived from 2-deoxy-D-ribose and arachidonic acid (AA) respectively.

Preparation of antibodies (performed by Prof. S. Nigam and Prof. J.L.F. Kock)

Antibodies against the synthesized 3(*R/S*)-HETE were raised in rabbits using the following protocol (Kock *et al.,* 1998): the carboxyl group of 3*R*-HETE was conjugated to bovine serum albumin amino group via a N-succinimidyl ester bond. An initial injection of 1 mg (of conjugated protein) was emulsified in an equal volume of Freunds' complete adjuvant, and in incomplete adjuvant for later injections. This protein emulsion was injected subcutaneously on the back of a female New Zealand white rabbit every second week, for three months. Following this treatment, blood from the rabbits' carotid artery was collected, left for two hours at room temperature and centrifuged for 20 min (1200 *g* at 4^{o}C). The sera were affinity purified by Biogenes in Berlin, Germany (Kock *et al.,* 1998).

Characterization of antibodies (performed by Prof. S. Nigam and Prof. J.L.F. Kock)

The raised antibodies against 3*R*-HETE were characterized by determining their titer, sensitivity and specificity. Since radio-labeled [^{14}C]-3-HETE is not commercially available, a biological tracer in small quantities was prepared (using the yeast *Dipodascopsis uninucleata*), through the biotransformation of [^{14}C]- arachidonic acid (AA) to [^{14}C]-3-HETE. Next, the radio-labeled [^{14}C]-3-HETE was purified using radio-high performance liquid chromatography. The antibody titer was shown to give a binding of approximately 30 % radio-labeled 3-HETE at a dilution of 1:100 in the absence of unlabeled 3-HETE. Minimum detectable amounts of 3-HETE (i.e. sensitivity) was 30 pmol as determined by 10 % displacement of radioactivity by unlabeled 3-HETE from the zero point (maximum binding of labeled 3-HETE). The specificity of the antibody was analyzed, using various structurally related compounds to determine the possible cross-reactions with the antibody. The antibody showed cross-reactions of < 0.5 % with 5-, 12-, or 15-HETE, while significant cross reactions occurred with other 3*R*-hydroxy (OH) fatty acids of different chain lengths and degrees of desaturation (Kock *et al.,* 1998). No immunoreactivity was observed against free fatty acids during immunofluorescence microscopy in this study. Therefore, immunoreactivity solely indicates the presence of 3-OH fatty acids.

Immunofluorescence confocal laser scanning microscopy (performed by candidate: O.M. Sebolai)

This was performed as described by Kock *et al.,* (1998). A loopful of cells were reacted with 30 µL of the primary antibody and incubated in the dark for 1 h to allow sufficient binding to the oxylipins. Subsequently, cells were reacted with the secondary antibody (coupled to fluorescein isothiocyanate**)** (30 µL, 1 h in the dark) (Sigma, St. Louis, USA) to allow sufficient binding to the primary antibody. In order to maintain cell structure integrity, antibody, fluorescence and wash treatments, were carried-out in 2 mL plastic tubes. Following adequate washing, cells were mounted in 1,4-diazabicyclo[2.2.2]octane (DABCO) (Aldrich Chemical Company, USA). DABCO is a free radical scavenger which helps sustain the fluorescence. Fluorescing material was photographed using a Nikon TE 2000 CLSM (Tokyo, Japan). Appropriate controls were also included to ensure correct interpretation (Kock *et al.,* 1998).

3-Hydroxy oxylipin extraction and analysis (performed by candidate: O.M. Sebolai; Mr. P. Botes assisted with setting-up of gas chromatograph - mass spectrometer)

This was performed as described by Sebolai *et al.,* (2004). The pH of cultivation media was decreased to less than 4 using 3 % formic acid (Saarchem, Wadeville, South Africa). Next, 200 mL of ethyl acetate (Saarchem, Wadeville, South Africa) was used to extract 3-hydroxy oxylipins directly from cultivation flasks. The organic phase was subsequently evaporated under a stream of nitrogen (AFROX, Bloemfontein, South

Africa). The extracts were methylated using diazomethane (Aldrich, Steinheim, Germany) and silylated using bis-(trimethylsilyl) trifluoroacetamide (BSTFA, Merck, Darmstadt, Germany). Finally, 1 μL (of derivatised sample) was injected into a gas chromatograph - mass spectrometer at an inlet temperature of 230 ^{o}C and a split ratio of 1:50. A Finnigan Trace Ultra Gas Chromatograph (Thermo Electron Corp., San José, California, USA) equipped with a Finnigan Trace DSQ MS (Thermo Electron Corp.) and an HP-5-60 m fused silica capillary column (0.1 μm coating thickness), were used to analyze the methylated trimethylsilylated samples (Sebolai *et al.,* 2004).

Electron microscopy (performed by candidate: O.M. Sebolai)

Fixation of material

Material for electron microscopy was chemically fixed using sodium phosphate-buffered 3 % glutardialdehyde (Merck, Darmstadt, Germany) for 3 h and similarly buffered osmium tetroxide (Merck, Darmstadt, Germany) for 1 h (van Wyk & Wingfield, 1991).

Scanning electron microscopy

Following washing with the same buffer, the material was dehydrated in a graded ethanol (Merck, Darmstadt, Germany) series (50 %, 70 %, 95 %, 2X 100 %) for scanning electron microscopy (with each step lasting 30 min). Next, the material was critical-point-dried (Biorad Microscience Division, Watford, England), mounted on stubs, and coated

with gold (confer electron conductivity) using a SEM coating system (Biorad Microscience Division, Watford, England) (van Wyk & Wingfield, 1991). Preparations were examined using a Shimadzu Superscan SSX 550 scanning electron microscope (Tokyo, Japan).

Transmission electron microscopy

Following washing (using same buffer), the material for transmission electron microscopy was dehydrated in a graded acetone (Merck, Darmstadt, Germany) series (50 %, 70 %, 95 %, 2X 100 %). Each dehydration step lasted for 30 min. The material was embedded in epoxy resin (Spurr, 1969) and left to polymerise in an oven (70 ^{o}C for 8 h) before sections were made with a LKB III ultramicrotome (Stockholm, Sweden). Next, sections were stained with uranyl acetate for 10 min and lead citrate for 5 min. Finally, sections were viewed with a Phillips EM 100 transmission electron microscope (Eindhoeven, the Netherlands) (van Wyk & Wingfield, 1991).

Immuno-gold labeling transmission electron microscopy (performed by candidate: O.M. Sebolai)

This was performed as described by Kock *et al.,* (2000). Sections collected on grids (studied during transmission electron microscopy) were de-osmified using sodium metaperiodate (Merck, Darmstadt, Germany). Sodium metaperiodate assists in enhancing immuno-gold labeling yield. Next, the sections were treated with 30 µL of the

primary antibody (specific for 3-hydroxy fatty acids [Kock *et al.,* 1998]) for 1 h in the dark and finally with 30 µL of the gold-probe (Sigma, St. Louis, USA) for 1 h in the dark. Following adequate washing with phosphate buffer solution (Oxoid, Hampshire, England), sections were stained using uranyl acetate (Merck, Darmstadt, Germany) and lead citrate (Merck, Darmstadt, Germany). Finally, stained sections were viewed with a Phillips EM 100 TEM (Eindhoeven, the Netherlands) (Kock *et al.,* 2000).

Growth inhibition studies (performed by candidate: O.M. Sebolai)

The effect of ASA [(Aldrich, Steinheim, Germany); 80 g L^{-1} stock solution in absolute ethanol (Merck, Darmstadt, Germany)] on yeast growth was assessed in test tubes containing 5 mL of YNB (6.7 g L^{-1}; Difco Laboratories, Detroit, Michigan, USA) broth supplemented with 2 % glucose (Saarchem, Wadeville, South Africa) (Petrou & Shanson, 2000). The tubes were incubated aerobically with 2 day old cultures on YM agar while agitating on a Rollordrum (30 ^{o}C for 4 days) according to the assimilation tests in liquid medium protocol (Yarrow, 1998). All yeasts were subjected to the following ASA concentration gradient: 0 mM, 1 mM, 2 mM, 3 mM, 4 mM and 5 mM ASA. Ethanol (ETOH) control (equivalent to the ethanol volume used to reconstitute 5 mM ASA) as well as a negative control (i.e. no ASA, ethanol or inoculum) were included. Growth was determined visually using a white card containing black lines as described by Yarrow (1998). Here, +++ indicates good growth (no black lines visible), ++ indicates growth (black lines just visible) and + indicates weak or no growth (black lines similar to that of inoculated tubes at the start of growth).

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