The Production Of 3-Hydroxy Fatty Acids By The Yeast *Dipodascopsis uninucleata* And Its Implications

by

PIERRE VENTER

Submitted in fulfillment of the requirements for the degree

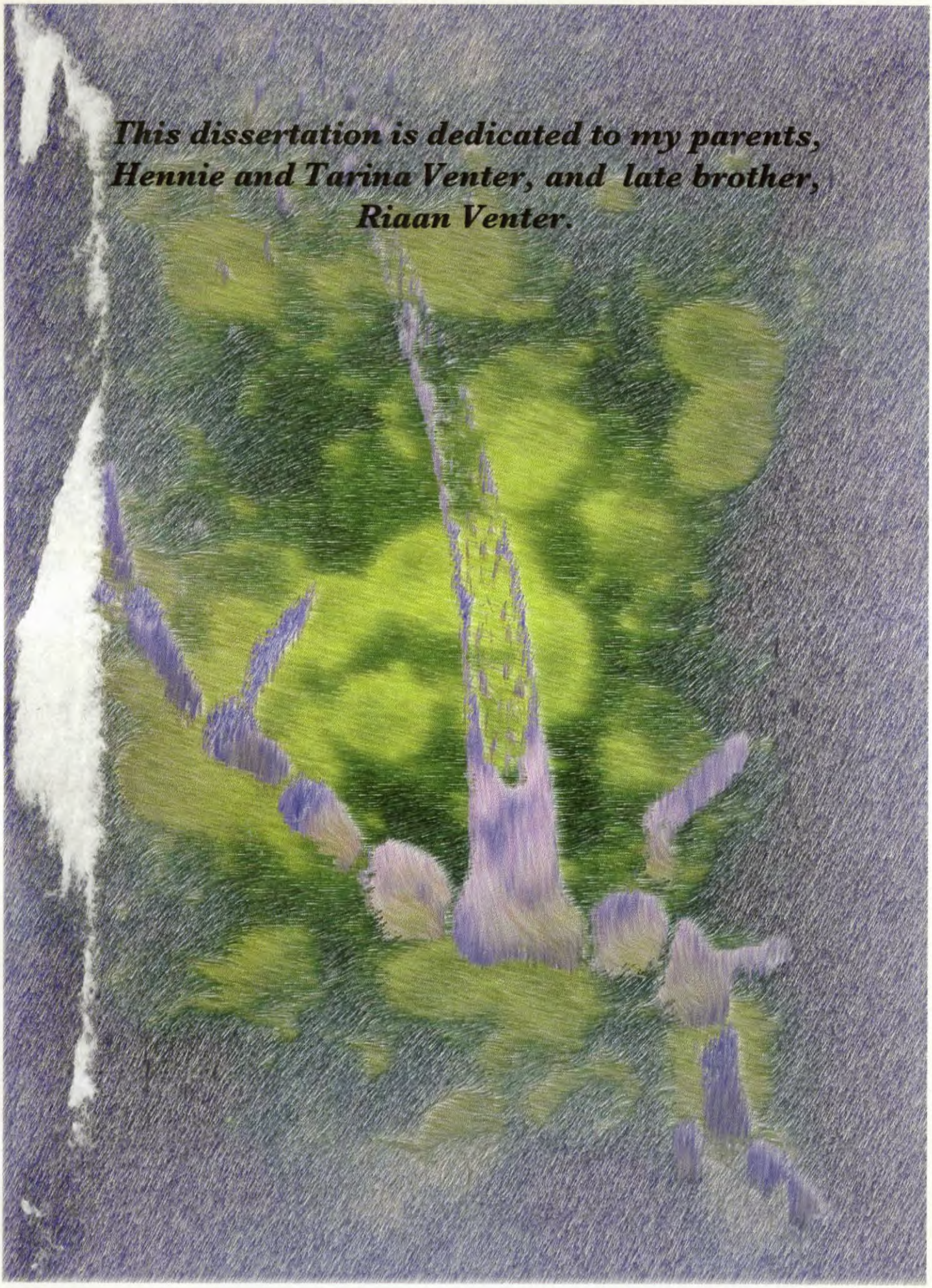
Philosophiae Doctor

In the

Department of Microbiology and Biochemistry, Faculty of Science, University of the Orange Free State, Bloemfontein 9300, Republic of South Africa

Promoter: Prof. J.L.F. Kock
Co-Promoter: Dr. D.J. Coetzee

November 1999


This dissertation is dedicated to my parents, Hennie and Tarina Venter, and late brother, Riaan Venter.

ACKNOWLEDGEMENTS

I wish to express my gratitude and appreciation to the following people for their contribution to the successful completion of this study:

Prof. J.L.F. Kock and **Dr. D.J. Coetzee,** for their creative ideas, stimulating criticism and guidance in planning and executing this study;

Foundation for Research Development, South Africa, as well as the **Volkswagen Foundation**, Germany (1/74643), for financial support;

Dr. P.W.J. van Wyk and **Me D.P. Smith,** for the TEM and SEM information and **Me C.H. Pohl,** for the *Mucor genevensis* IF information that was included in this dissertation;

Co-authors of the different publication, for their contributions, especially **Dr. S. Nigam** and his research group;

Mr. P.J. Botes, for assistance with the GC-MS;

To the rest of my **colleagues in the lab,** especially **Me Elma Pretorius** and **Me Andri van Wyk,** for their support and friendship;

To my **parents,** for their constant support, encouragement and love;

To the **De Wet family,** especially **Tania,** for their friendship, support and interest;

To my **Heavenly Father,** who made this possible and granted me with a glimpse into His spectacular creation.

CONTENTS

CHAPTER 2

Production of 3*R*-hydroxy-polyenoic fatty acids by the yeast *Dipodascopsis uninucleata*

CHAPTER 3

Biological dynamics and distribution of 3-hydroxy fatty acids in the yeast *Dipodascopsis uninucleata* as investigated by immunofluorescence microscopy. Evidence for a putative regulatory role in the sexual reproductive cycle

CHAPTER 4

A novel oxylipin-associated binding phenomenon in yeast flocculation

CHAPTER 5

Aspirin influences Lipid-A composition in the lipopolysaccharide layer of Gram-negative bacteria: implications for therapy of endotoxemia

SUMMARY

CHAPTER 1

Introduction

1. Motivation

In 1991, a novel eicosanoid namely 3-hydroxy-5, 8, 11, 14-eicosatetraenoic acid (3-HETE) was uncovered in the yeast *Dipodascopsis uninucleata* (van Dyk *et al.*, 1991; Kock *et al.*, 1992). Strikingly, the production of this compound was found to be sensitive to low concentrations of non-steroidal anti-inflammatory drugs (NSAIDs) such as aspirin and indomethacin. Furthermore, this compound was found to be associated with the sexual reproductive stage of *D. uninucleata,* which was also NSAID sensitive. These results implicated a possible role of 3-HETE in the sexual reproductive stage of this organism (Botha *et al.*, 1992). Although the sensitivity of 3-HETE towards NSAIDs implies cyclooxygenase activity, this compound contains no cyclopentane ring which is characteristic of the cyclooxygenase formed prostaglandins (Slater and McDonald-Gibson, 1987). In addition, this compound was not formed by a lipoxygenase route due to the lack of conjugated double bonds in the aliphatic chain (Pace-Asciak, 1989). Preliminary investigations also showed that 3-HETE affects signal transduction processes in human neutrophils and tumor cells in multiple ways (Venter *et al.*, 1997). Consequently, the production of larger quantities of this compound for biological testing on mammalian cells became indispensable.

As a result of the above mentioned, it became the purpose of this study to investigate the 3-hydroxy fatty acid metabolism in the yeast *D. uninucleata,* in order to optimize the production of this compound and to study its biological activity in this yeast as well

as mammalian cells. Since 3-hydroxy fatty acids have been implicated in the aggregation of yeast cells in this investigation, the distribution of these fatty acids in well known flocculating strains of the yeast *Saccharomyces cerevisiae* was also investigated. Since NSAIDs affect the outer membrane (i.e. LPS containing 3-hydroxy fatty acids) of Gram-negative bacteria, the influence of these compounds on the 3-hydroxy fatty acid content was also investigated.

2. 3-Hydroxy fatty acids and their importance

2.1 Structure

Hydroxy fatty acids are widely distributed in nature i.e. plants, animals and in some micro-organisms as constituents of various complex lipids or as free carboxylic acids (Finnerty, 1989; Jin *et al.*, 1992; van Dyk *et al.*, 1994). Their basic structure comprises a hydrophilic carboxylate group (polar head) attached to one end of a hydrocarbon chain and a hydroxyl group at the C3 position (counted from the carboxylate group) (Fig. 1) (Finnerty, 1989). This chain can vary considerably in chain length (number of hydrocarbons) and presence of double bonds. Fatty acids containing no double bonds, are referred to as saturated, one double bond - mono unsaturated and more than one - poly-unsaturated. A shorthand designation can be given to fatty acids with the total number of carbon atoms followed by a colon and then the total number of double bonds. These double bonds are usually in the *cis* configuration, but double bonds in the *trans* configuration are known to occur. Two alternative systems for designating the type of unsaturation are used, though in both systems the position(s) of the double bond(s) is indicated immediately after the numeral indicating the number of double bonds. Thus a fatty acid such as 3-hydroxy arachidonic acid (Fig. 2) can be represented as 3-hydroxy-*all cis*-20:4 (5, 8, 11, 14) or 3-hydroxy-20:4 (5*c*, 8*c*, 11*c*, 14*c*). A *trans* isomer can similarly be indicated by the prefix *trans* or suffix *t*. A *Cis* configuration can also be denoted as "*Z*" and *trans* as

"*E*". Another system for locating the double bond(s) is by counting the carbons from the ω – or methyl end of the chain, i.e. for the above-mentioned example - ω6 (Fig. 2) (Ratledge and Wilkinson, 1988).

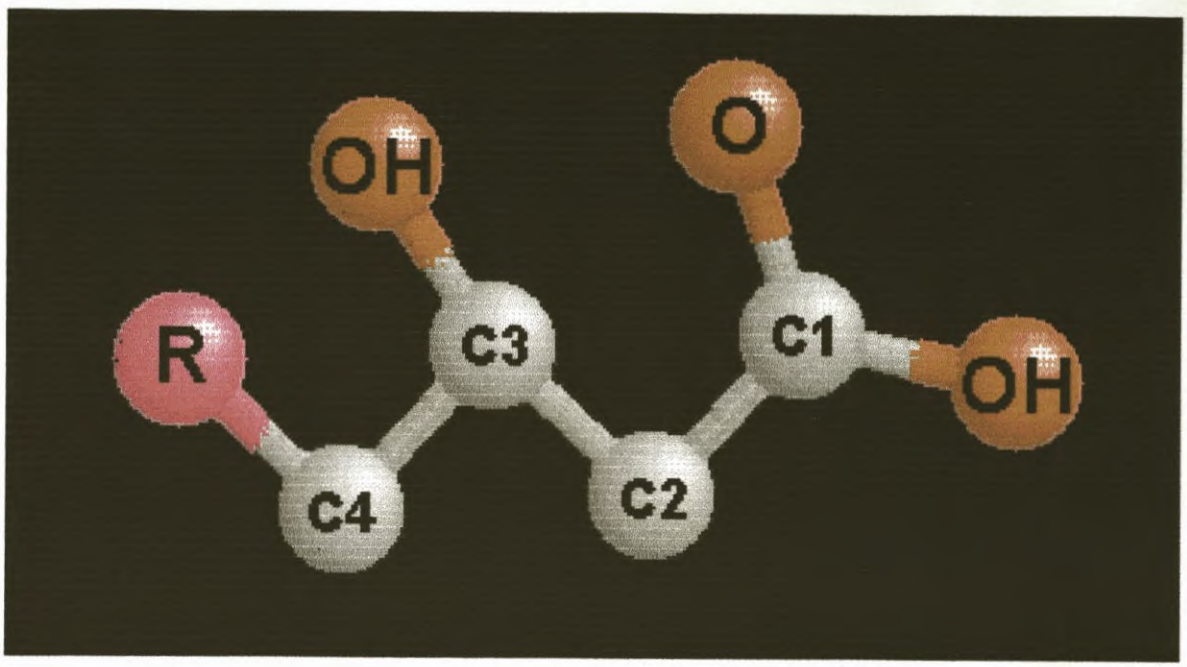


Figure 1. Schematic representation of a 3-hydroxy fatty acid where R can be substituted by a hydrocarbon chain of varying length which may contain double bonds.

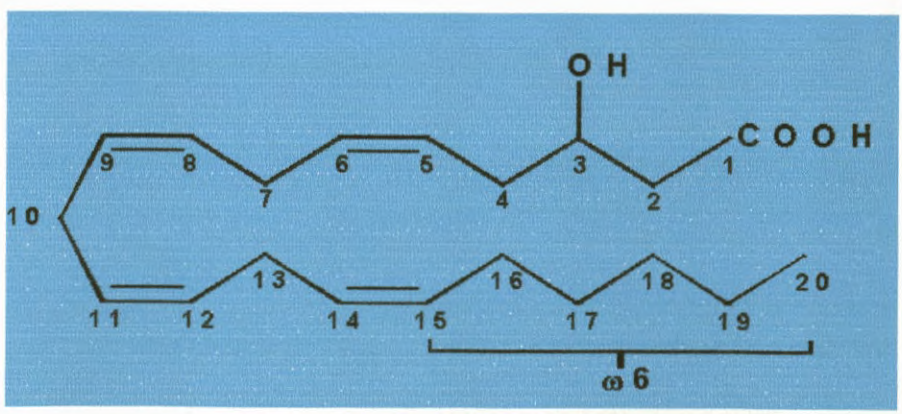


Figure 2. 3-hydroxy-*all cis*- 5, 8, 11, 14 -eicosatetraenoic acid - ω6 series.

3-Hydroxy fatty acids can also be present in two enantiomeric forms (Fig. 3), i.e. 3*R*- and 3*S*-hydroxy fatty acids (March, 1985).

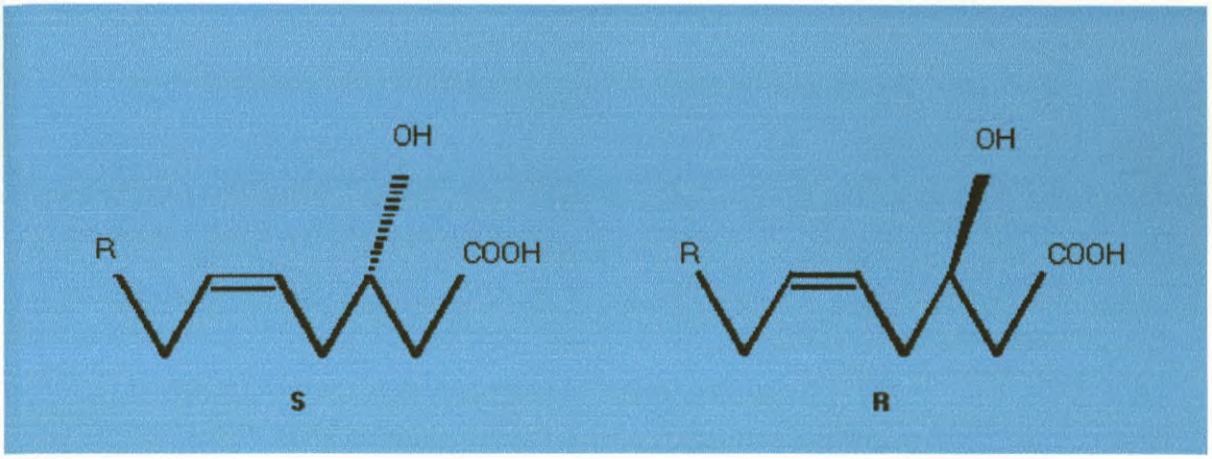


Figure 3. Enantiomers of 3-hydroxy fatty acids.

3-Hydroxy fatty acids also occur in prokaryotic organisms as polymers, which serve as a reserve energy source. Poly-ß-hydroxybutyrate (PHB) consists of fatty acid monomers connected by ester linkages, forming an extended polymer which has plastic-like characteristics (Fig. 4) (Dawes and Senior, 1973).

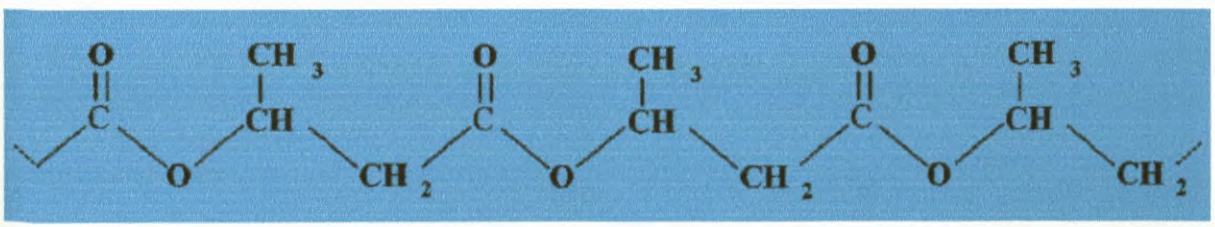


Figure 4. Structure of PHB.

Another well-documented complex structure of 3-hydroxy fatty acids is the glycolipids in the outer leaflet of the membrane of Gram-negative bacteria called Lipid A. Lipid A of *Escherichia coli* for instance, contains D-gluco-configured pyranosidic hexosamine

residues, which are present as β(1→6)-linked homo or heterodimers. The disaccharide carries four 3(*R*)-hydroxy fatty acids, two of which are acylated at their hydroxyl groups by non-hydroxylated fatty acids. In *E. coli,* the 3-hydroxy fatty acids are all 3-hydroxytetradecanoic acid (3-hydroxy 14:0) (Rietchel *et al.,* 1996; Fig. 5).

2.2 Importance in fungi

Eventhough the presence of 3-hydroxy fatty acids in fungi is well documented, (distribution will be discussed in 3.1) very little is known about the biological importance of these fatty acids in fungi. In 1991, van Dyk and co-workers investigated the nature of an aspirin sensitive compound produced during the sexual reproductive phase of the yeast *Dipodascopsis uninucleata* (Fig. 6) when fed with arachidonic acid (AA). After considerable effort they managed to purify this compound and identify this as 3-hydroxy-*all cis*-5, 8, 11, 14-eicosatetraenoic acid (3-HETE) - a novel eicosanoid.

When aspirin was introduced at the start of the life cycle (i.e. from ascospores)(Fig. 6), the sexual stage and production of 3-HETE was most severely inhibited (Botha *et al.*, 1992). Venter and co-workers in 1997 also reported the conversion of linoleic acid to 3(*R*)-hydroxy-*all cis-5*, 8-tetradecadienoic acid by *D. uninucleata.* This phenomenon is intriguing seeing that linoleic acid contributes to about one fourth of the total fatty acid content of this yeast. These 3-hydroxy fatty acids, therefore could exert a regulatory function during the sexual phase of the reproductive cycle of this yeast as has been shown to be the case for other fungal oxylipins (Mazur *et al.*, 1991).

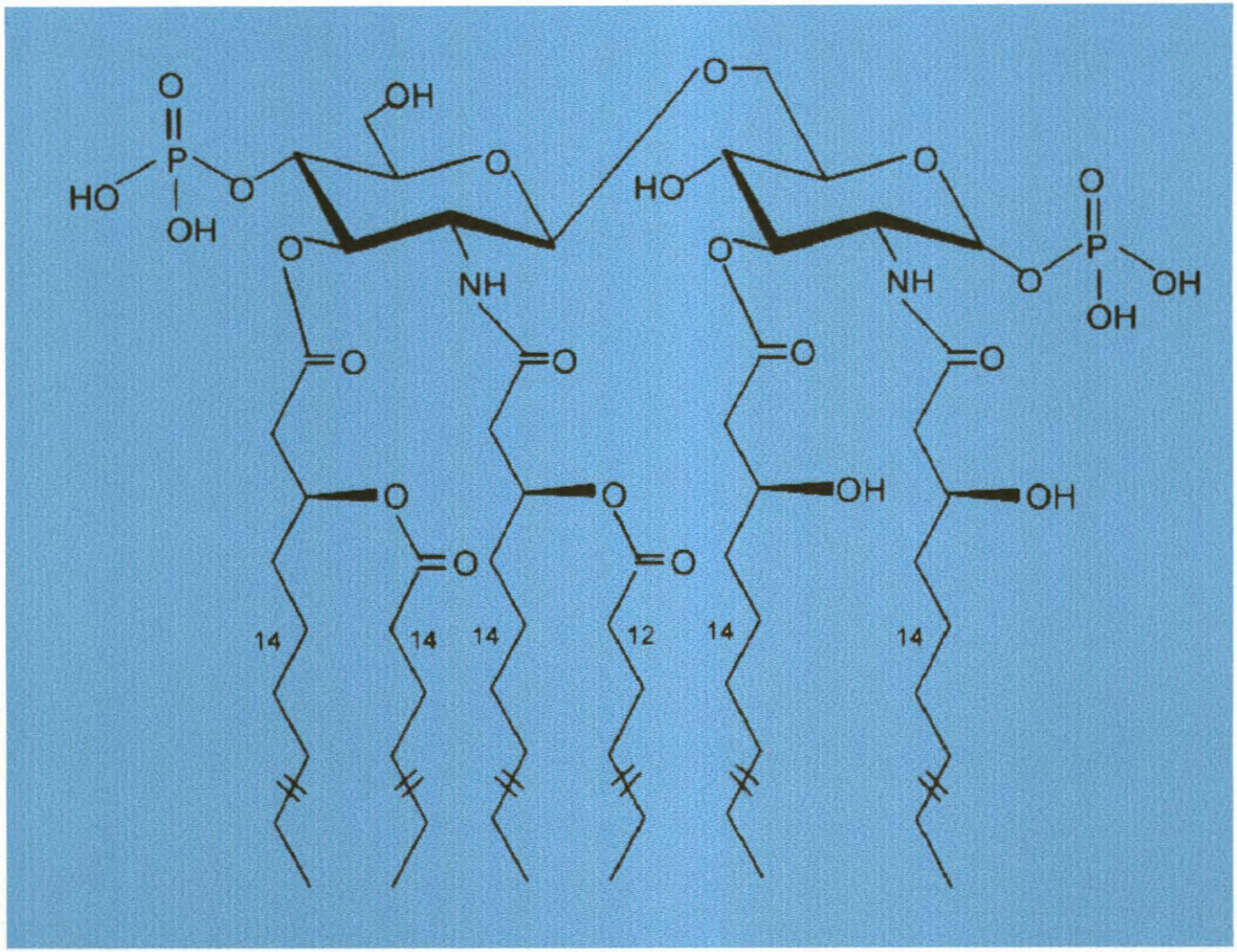


Figure 5. Schematic representation of Lipid A.

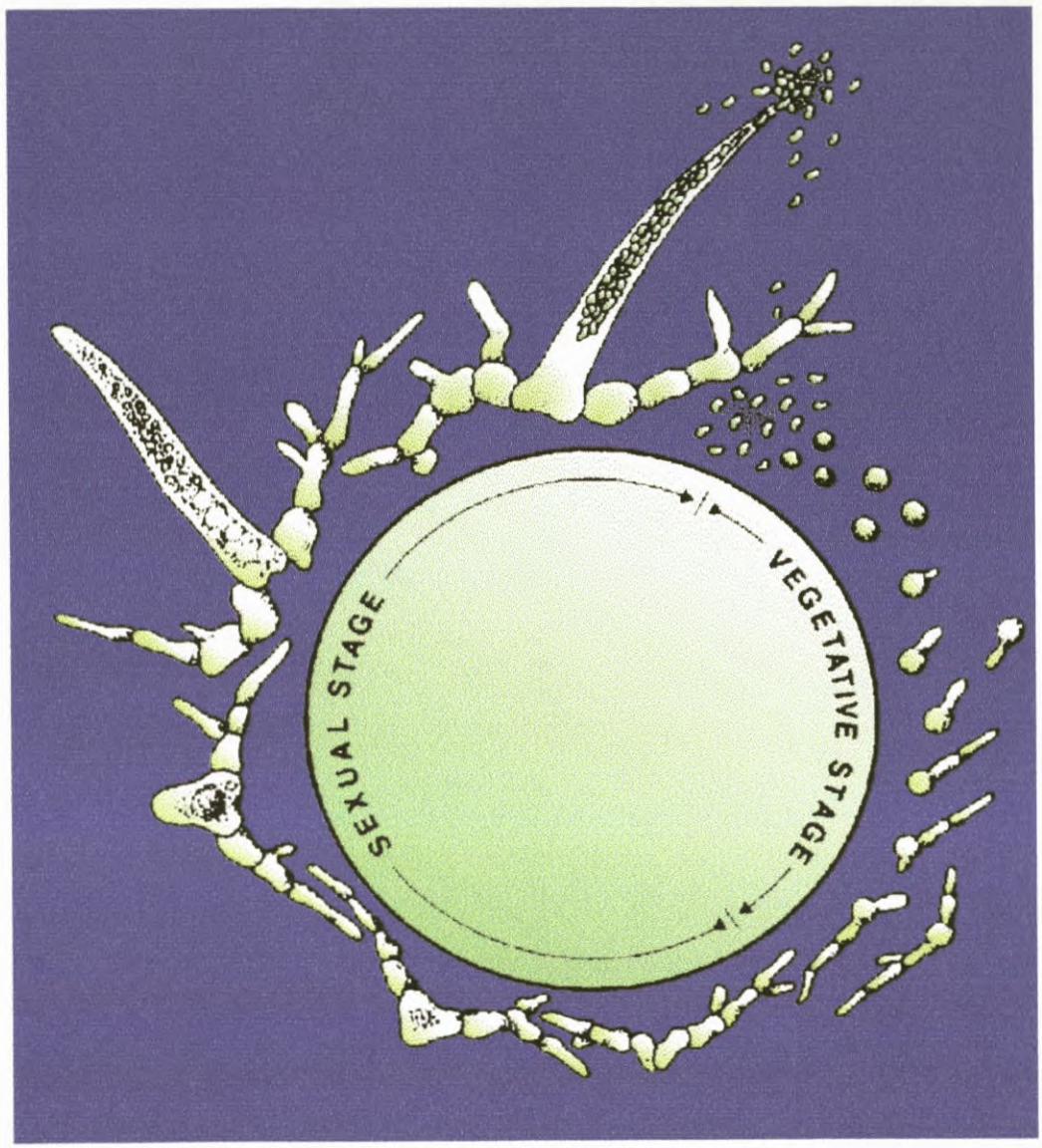


Figure 6. The life cycle of *D. uninucleata*. In short, the life cycle of *D. uninucleata* in liquid medium is characterized by consecutive vegetative and sexual stages. Ascospores swell and after 21 h these spores germinate into hyphae. This is then followed by the sexual reproductive phase where this yeast undergoes plasmogamy, karyogamy, meioses and finally ascosporogenesis (Botha *et al.*, 1992).

2.3 *Importance in bacteria*

A form of 3-hydroxy fatty acids, unique to certain bacteria such as *Rodospirillum* sp. is poly-hydroxybutyrate (PHB)(Sierra and Gibbons, 1962; Dawes and Ribbons, 1964; Lundgren *et al.,* 1965). This polymer is found in special inclusions, which can contribute up to 60% or even more of the cellular dry weight (Fig. 7). Most bacteria do not store triacylglycerols as an energy reserve, it appears likely that this function is served by PHB (Dawes and Senior, 1973).

The most common 3-hydroxy fatty acids are those found in Lipid A, the characteristic lipopolysaccharide of the cell envelope in Gram-negative bacteria (Westphal and Lüderitz, 1954; Burton and Carter, 1964; Nesbitt and Lennarz, 1965; Hancock *et al.,* 1970)(Fig. 8). These compounds are responsible for severe infection, generalised inflammation and pathological disorders in humans, such as sepsis, Disseminated Intravascular Coagulation (DIC)- syndrome and circulatory shock which may, if not urgently treated, lead to death (Parrilo, 1990).

As is the case in *D. uninucleata* (van Dyk *et al.*, 1991), the possibility also exists that 3-hydroxy fatty acids associated with the Lipid A fraction of the lipopolysaccharide (LPS) layer of Gram-negative bacteria are influenced by NSAIDs. Lipid A is considered to be the main contributor to the endotoxic activity of LPS (Holst *et al.,* 1993). It has been found that NSAIDs, which also include salicylates, have a profound influence on the outer membrane permeability of Gram-negative bacteria.

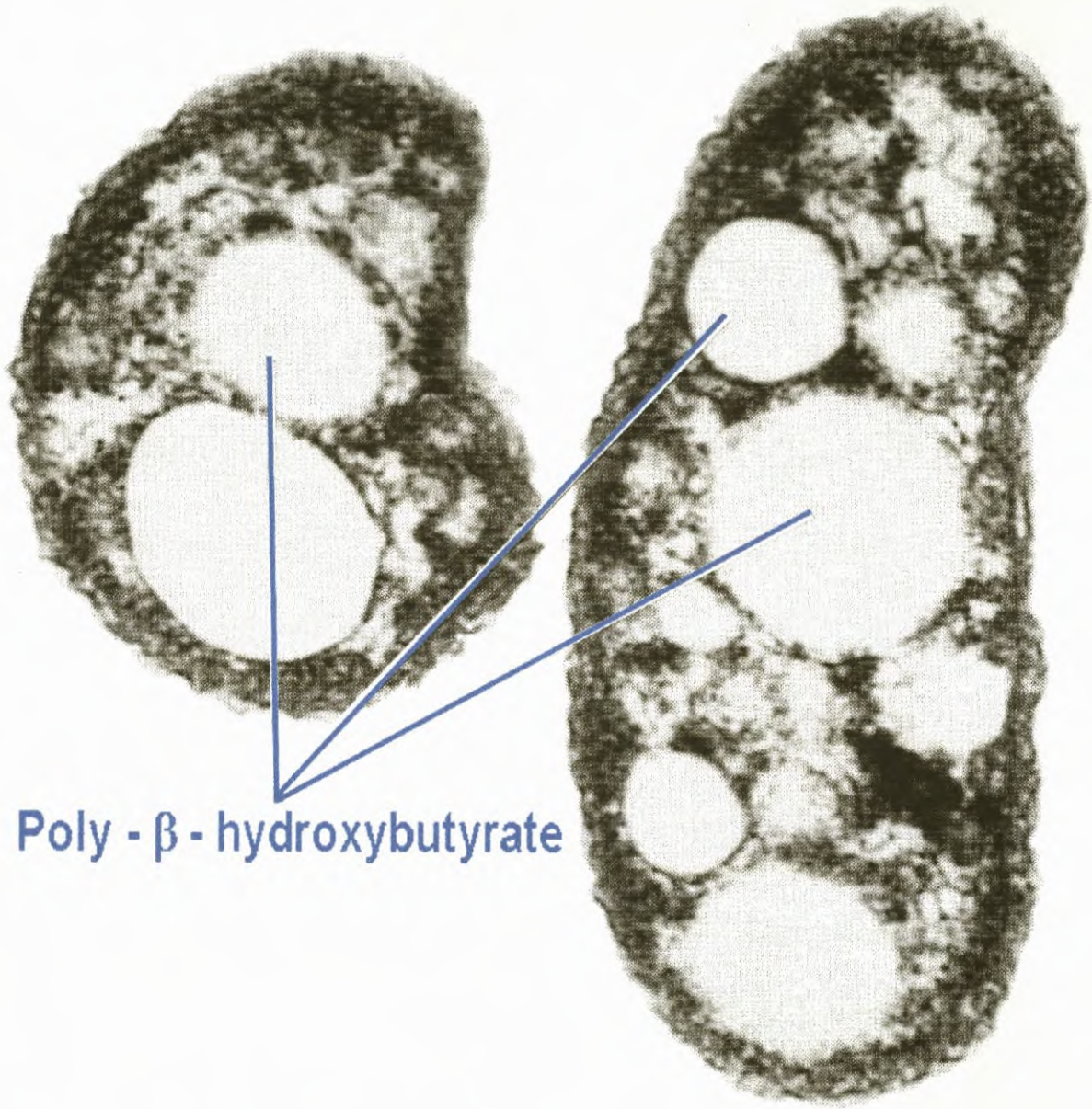


Figure 7. Electron micrograph of a thin section of cells of the phototrophic bacterium *Rhodospirillum* sp., containing granules of PHB (Reproduced from Brock and Madigan, 1991).

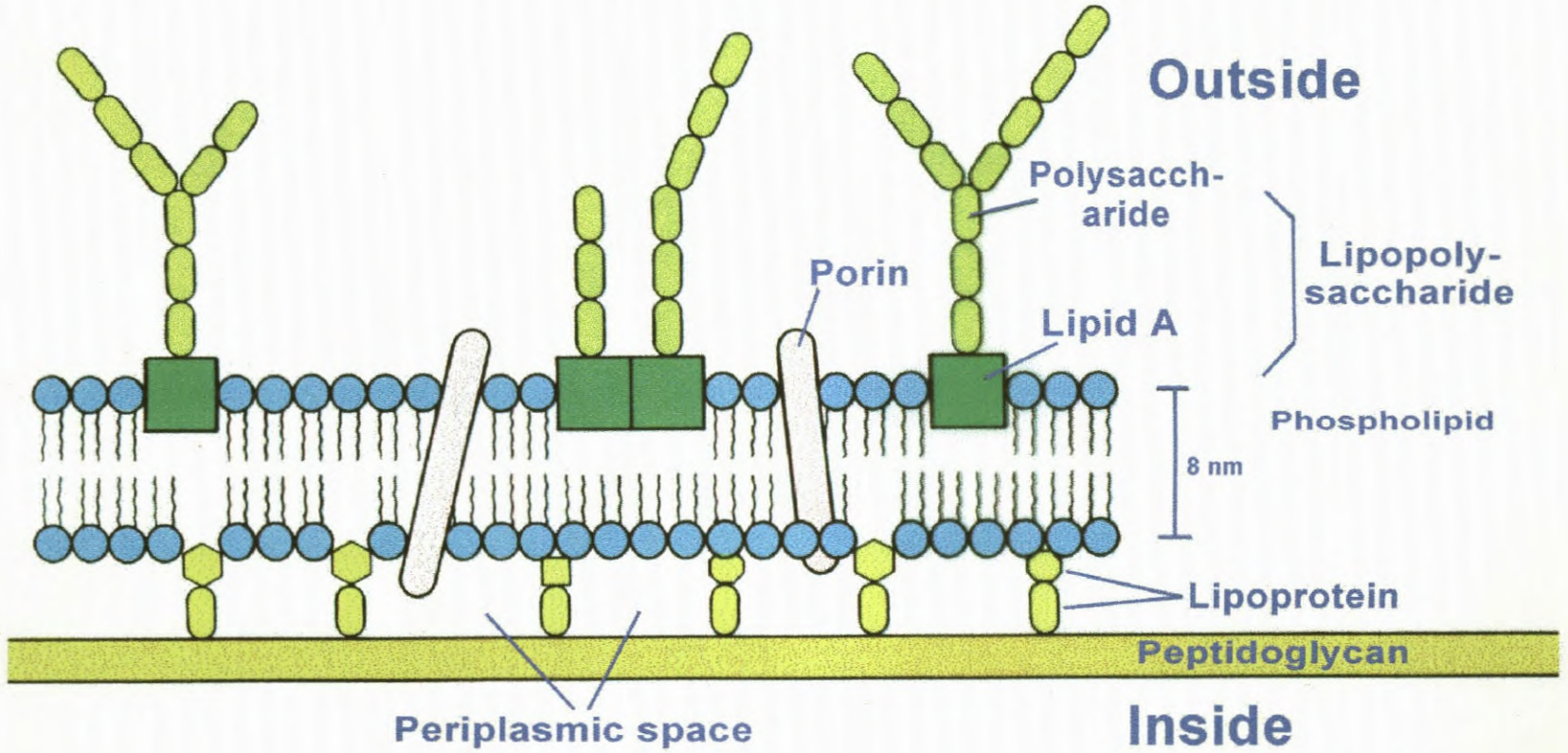


Figure 8. The cell envelope of Gram (-) bacteria containing a lipopolysaccharide layer consisting of polysaccharide and Lipid A. A lipoprotein and peptidoglycan layer is also present (Reproduced from Brock and Madigan, 1991).

These compounds especially affect the porins in the LPS fraction by down-regulating the expression of the outer membrane porin OmpF. The latter serves as a channel for the entry of certain antibiotics and other compounds into the periplasmic space. It is anticipated that, when antibiotics and NSAIDs are administered together, it could improve the therapeutic action of certain antibiotics such as aminoglycosides and other positively charged antibiotics and on the other hand reduce the susceptibility of *E. coli* to a number of beta-lactams, chloramphenicol, nalidixic acid and tetracyclin. This may render certain antibiotic treatments against for instance *E. coli*, ineffective (Aumercier *et al.*, 1990; Burns and Clark, 1992; Rosner and Slonczewski, 1994).

Since research concerning the influence of NSAIDs on the outer membrane of Gram-negative bacteria has mainly concentrated on changes in proteins (porins) associated with the LPS, it is of utmost importance to evaluate the effect of these compounds on the 3-hydroxy fatty acids associated with the Lipid A fraction. This is of special interest since it has previously been demonstrated that NSAIDs inhibit 3-hydroxy fatty acid production in other microorganisms (van Dyk *et al.*, 1991).

2.4 Importance in humans and others

3-hydroxy fatty acids and their products have been found to be important in the diagnosis of several diseases in humans. These fatty acids are important precursors for the production of 3-hydroxydicarboxylic acids, which when present in urine, are important indicators for patients with 3-hydroxyacyl CoA dehydrogenase deficiency, dicarboxylic aciduria and patients who are fasting (Tserng and Jin, 1991). 3-hydroxydicarboxylic acids are excreted in the urine as major metabolites produced via ω-oxidation of 3-hydroxy fatty acids. Interestingly, the latter fatty acids do not exit as pure *L*-enantiomers as expected from ß-oxidation. Instead these compounds were epimerized to a near racemic mixture of *D*- and *L*- isomers with a dominant *D*- isomer (Jin *et al.*, 1992). The metabolism of these compounds will be discussed in 4.3.

It has also been reported that ischemia of the heart (i.e. restriction of vascular flow), is accompanied by the tissue accumulation of long-chain fatty acids and their metabolic derivatives such as 3-hydroxy fatty acids which may be detrimental to proper myocardial function. This happens through micelle formation, which may be incorporated into the membranes thereby interfering with their physiological function and causing membrane instability and disruption. During ischemia, oxygen delivery to the heart tissue is drastically lowered, which is accompanied by a significant decrease of mitochondrial function. This results in the accumulation of long-chain and 3-hydroxy fatty acids (Glatz *et al.*, 1993).

There are also examples where fatty acid derived compounds such as Leukotrien B_4 (LTB_4) is transformed to the 3-hydroxy derivative. LTB_4 is a biologically active product of the arachidonic acid metabolism and are produced by neutrophils, mast cells and others involved in the immune response of humans. Incubation of LTB_4 with a human-derived hepatoma cell line (Hep G_2 cells) resulted in the production of both 3(*R*)-hydroxy-LTB_4 and 3(*S*)-hydroxy-LTB_4 (Wheelan and Murphy, 1995).

Interestingly, 3-hydroxy fatty acid (as part of a secretory lipid) production has also been reported to be induced during the mating season of mallard ducks (Hiremath *et al.*, 1992). It has been found that the monoester wax of the female gland is completely replaced with diesters, composing of 3-hydroxy C8, C10, and C12 fatty acids esterified with C16 and C18 fatty alcohols which constitute the female pheromones. It has also been reported that estradiol, or a combination of estradiol and thyroxine, results in the proliferation of peroxisomes and the production of diesters of 3-hydroxy fatty acids in the uropygial gland of male and female mallard ducks. Here the production of 3-hydroxy fatty acids occurs by the action of fatty acyl-CoA oxidase (Hiremath *et al.*, 1992).

3. Distribution of 3-hydroxy fatty acids

3.1 Fungi

Documentation of the presence of 3-hydroxy fatty acids in fungi started as early as 1967 when Stodola *et al.* reported the presence of 3(*D*)-hydroxy-hexadecanoic (16:0) and -octadecanoic acid (18:0) as part of the extracellular glycolipids of strains representing the yeast *Rhodotorula graminis* and *Rh. glutinis*. The year 1968 was marked by a discovery by Vesonder *et al.* who reported the formation of large quantities extracellular 3(*D*)-hydroxy-16:0 by *Saccharomycopsis malanga*. Further reports on the formation of extracellular 3(*D*)-hydroxy-16:0 by this yeast followed in 1974 by Kurtzman *et al.* 3-hydroxy hexanoic (6:0) and -octanoic acid (8:0) were also reported to be present in the glycolipids of the smut fungi, *Ustilago nuda* and *U. zeae* (Lösel, 1988).

Not only 3-hydroxylated saturated fatty acids were found to be produced by fungi, but also a 3-hydroxy unsaturated fatty acid. A discovery was made in 1991 by van Dyk and co-workers who reported the biotransformation of exogenously fed AA to 3-HETE by the yeast *D. uninucleata*. In an elaborate study, Kock *et al.*, 1997 have managed to construct a process by which approx. 4% of the AA fed to the yeast are transformed to 3-HETE. The biotransformation of AA to 3-HETE was also found to occur in *Babjevia anomala* and *D. tothii* (Kock *et al.*, 1991; van Dyk *et al.*, 1991; Coetzee *et al.*, 1992).

3.2 Bacteria

The presence of 3-hydroxy fatty acids in bacteria was reported much earlier than is the case for fungi. In 1926, Lemoigne isolated a compound from *Bacillus megaterium*, which he considered to be the hydrolysis product of a polymer of β-

hydroxy butyrate. This compound was named poly-3-hydroxy-butyric acid (PHB), a storage product of this bacterium. Many reports followed during the sixties and seventies outlining a variety of bacteria producing PHB (Dawes and Senior, 1973; Table 1).

Table 1. Occurrence of PHB in microorganisms.

Genera		
Actinomycetes	*Azotobacter*	*Bacillus*
Beijerinckia	*Chlorogloea*	*Chromatium*
Chromobacterium	*Derxia*	*Ferrobacillus*
Hydrogenomonas	*Lampropaedia*	*Micrococcus*
Nocardia	*Pseudomonas*	*Rhizobium*
Rhodopseudomonas	*Rhodospirillum*	*Sphaerotilus*
Spirillum	*Streptomyces*	*Zoogloea*

(Dawes and Senior, 1973).

PHB, which is a member of the poly-hydroxyalkanoic acid (PHA) series of polyesters, is a biodegradable and biocompatible microbial thermoplastic produced on industrial scale. One example of a company doing this is ZENECA (formerly ICI), which uses *Alcaligenes eutrophus* for PHB production (Holmes, 1985; Byrom, 1987). A vast array of 3-hydroxy fatty acids are known that constitutes these polymers, a few are shown in Fig. 9 (Steinbüchel *et al.,* 1994).

In Gram-negative bacteria, the varieties of 3-hydroxy fatty acids are also not limited. They occur as part of the complex structure referred to as Lipid A (structure shown in

2.1). The chain lengths of these fatty acids vary however from bacterium to bacterium. A few are shown in Table 2. For more information the reader is referred to Ratledge and Wilkinson (1988). All these 3-hydroxy fatty acids found in prokaryotic organisms are mainly in the *R*-configuration.

Table 2. 3-hydroxy fatty acids found in Lipid A of Gram (-) bacteria.

Bacterium	Fatty acid
Escherichia coli	3-OH-14:0
Salmonella sp.	3-OH-14:0
Klebsiella pneumoniae	3-OH-14:0
Pseudomonas alcaligenes	3-OH-12:0
Xanthomonas sp.	3-OH-13:0
Agrobacterium tumefaciens	3-OH-16:0
Bacteroides gingivalis	3-OH-17:0

(Galanos *et al.*, 1977; Wilkinson, 1977; Masshimo *et al.*, 1985).

3.3 ***Mammalians*** (see 2.4)

Table 3 summarizes the distribution of 3-hydroxy fatty acids in mammalians.

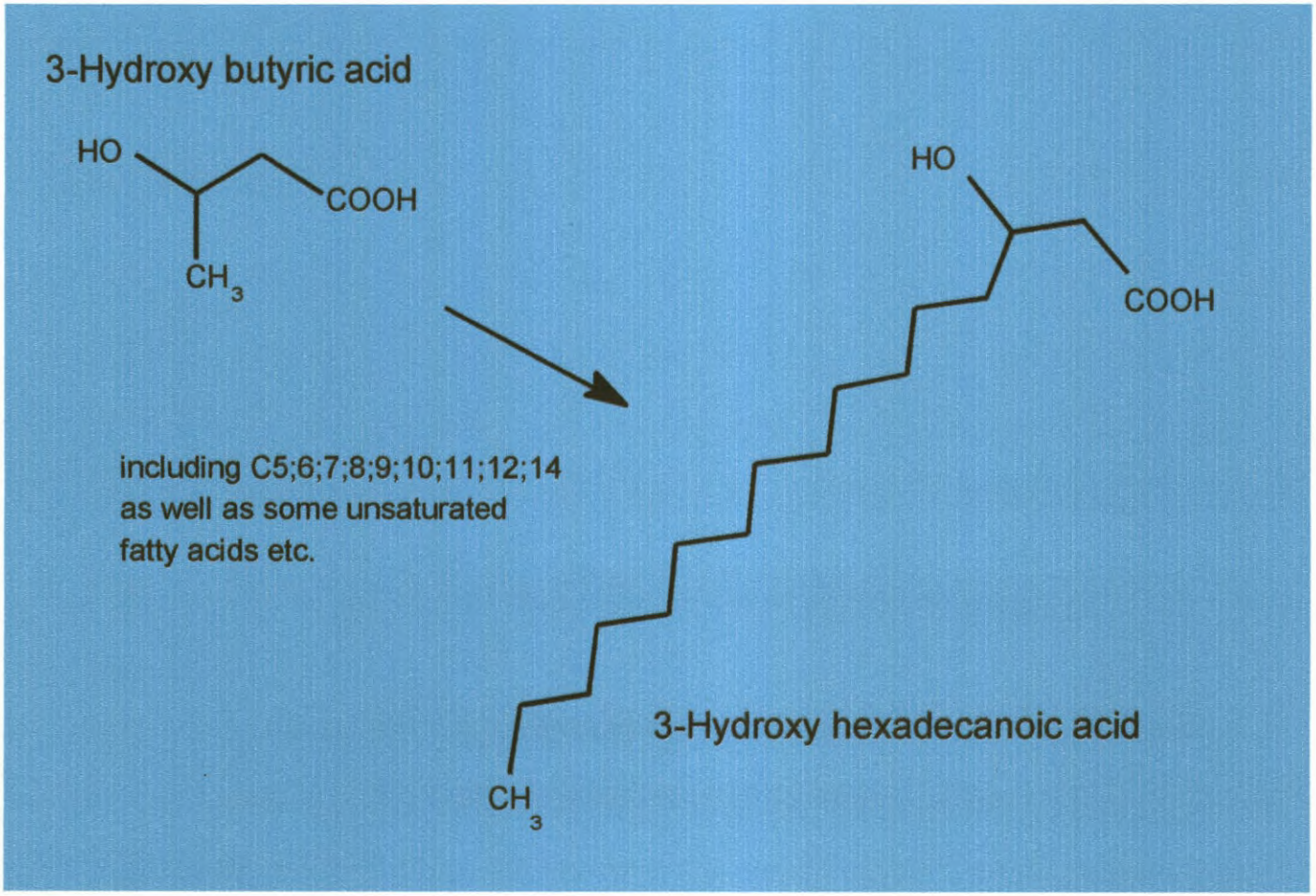


Figure 9. Some 3-hydroxy fatty acids ranging from C4 to C16 that are used in the production of bacterial polymers (the reader is referred to Steinbüchel *et al.*, (1994) for more structural information).

Table 3. Presence of 3-hydroxy fatty acids in mammalian cells and the Mallard duck.

Fatty acid	Distribution
3-Hydroxydicarboxylic acid (dominant *R*)	Urine (human)
3-Hydroxy long chain fatty acids	Ischemia of the heart (human)
3-Hydroxy LTB_4 (*R/S*)	Neutrophils, mast cells, hepatoma cell line (human)
3-Hydroxy fatty acid monoester wax (C8, C10, C12, esterified with C16, C18)	Female pheromones (Mallard ducks)

(Tserng and Jin, 1991; Hiremath *et al.*, 1992; Glatz *et al.*, 1993; Wheelan and Murphy, 1995).

4. 3-Hydroxy fatty acid metabolism

4.1 Fungi

The metabolic pathway for the production of 3-hydroxy fatty acids in fungi is β-oxidation (Finnerty, 1989). This catabolic pathway can be distinguished by two phases (Fig. 10). First a long chain fatty acid suitable for this catabolism enters the cell from the environment. This is achieved mainly by diffusion of the compound into the cell through the cytoplasmic membrane. Secondly, the formation of a fatty acyl CoA is catalysed by acyl CoA synthetase. Following this, is dehydrogenation by removal of a pair of hydrogen atoms from the α - and β carbon atoms of the fatty acyl

CoA to yield a Δ^2- unsaturated acyl CoA (Δ^2-refers to the double bond positioned directly after C2 counted from the carboxylate group), which is then enzymatically hydrated to form a racemic mixture (equivalent amounts of *D* and *L*) of β-hydroxy-acyl-CoA (3-hydroxy fatty acids). If it happens that a *D-cis*-3-unsaturated fatty acid enters the system, an isomerase enzyme converts the Δ^3- to a Δ^2-unsaturated fatty acyl CoA. In fungal β-oxidation only the *L*-enantiomer of the 3-hydroxyacyl CoA are further dehydrogenated via an NAD^+ / NADH system to yield a β-ketoacyl CoA, which in turn then undergoes enzymatic cleavage to yield an acetyl CoA as well as a fatty acid with two less carbon atoms than the original fatty acid. The *D*-enantiomer undergoes epimerization to yield an *L*-enantiomer, which then proceed through the latter degradation system. ß-oxidation has been implicated to occur mainly in peroxysomes in yeasts (Sheridan and Ratledge, 1996).

4.2 Bacteria

In bacteria the production of 3-hydroxy fatty acids differ somewhat from the proposed metabolism in fungi. Even between different genera of bacteria small differences may occur. Commonly accepted however is the metabolism as proposed by Moskowitz and Merrick in 1969 for the production of PHB by *Rhodospirillum rubrum*. This is the metabolic pathway that will be discussed in this section. For more information the reader is referred to a review by Dawes and Senior (1973).

The anabolic pathway in *R. rubrum* appears to be the opposite of β-oxidation in fungi (Fig. 11), where two acetyl CoAs are enzymatically fused and hydrogenated to yield a 3(*L*)-hydroxybutyryl CoA. Epimerization from the *L*- to the *D*-enantiomer follows via a Δ^2 - unsaturated butyryl CoA. Polymerisation of the *D*-enantiomers occurs with the help of a PHB granule-bound hydroxy butyryl-CoA polymerase. Polymers of the β(*D*)-hydroxybutyric acid (the final storage product) can be catabolised again to acetyl CoA (necessary for energy production) via the opposite route - similar to β-oxidation.

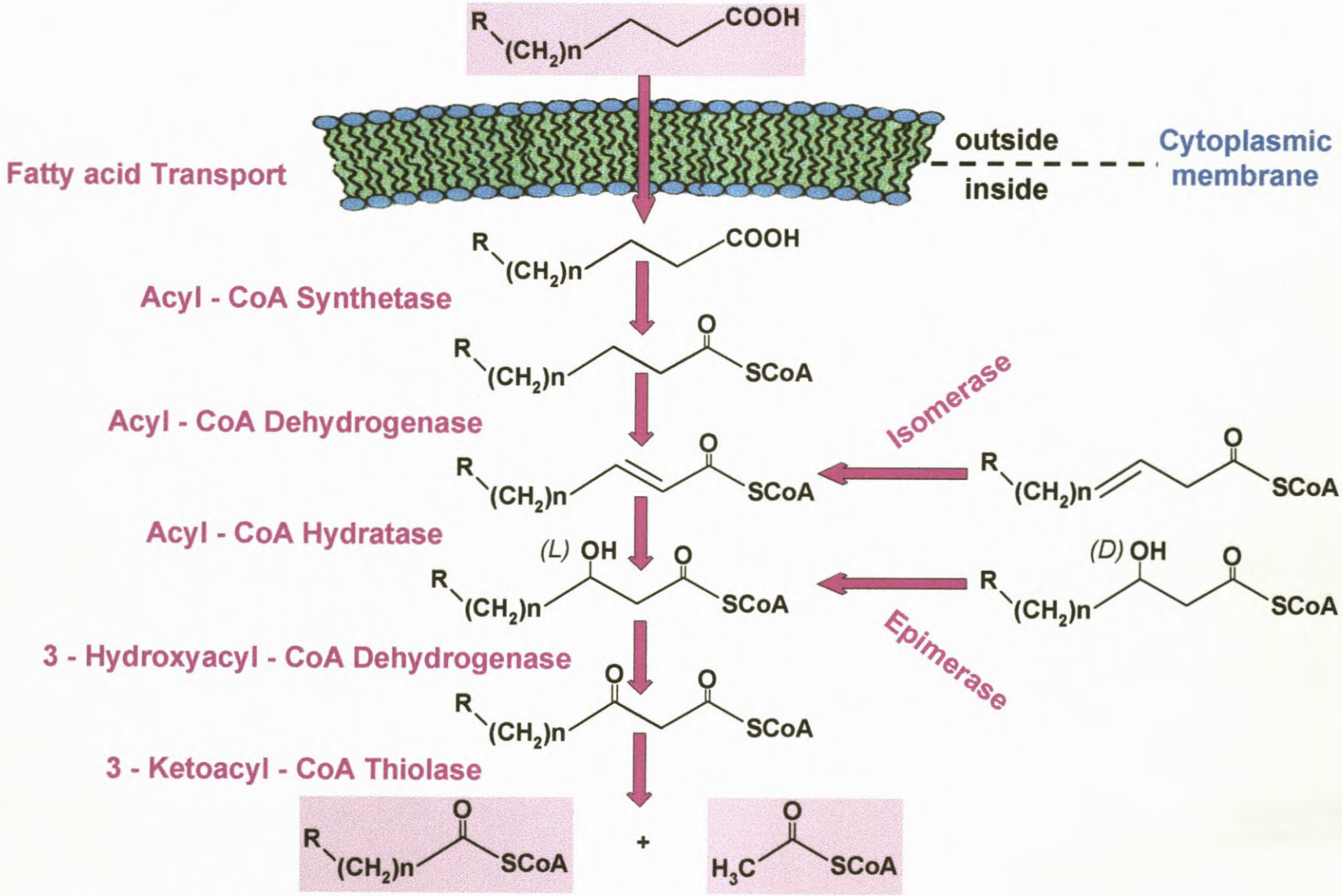


Figure 10. β-oxidation of fatty acids (Reproduced from Finnerty, 1989).

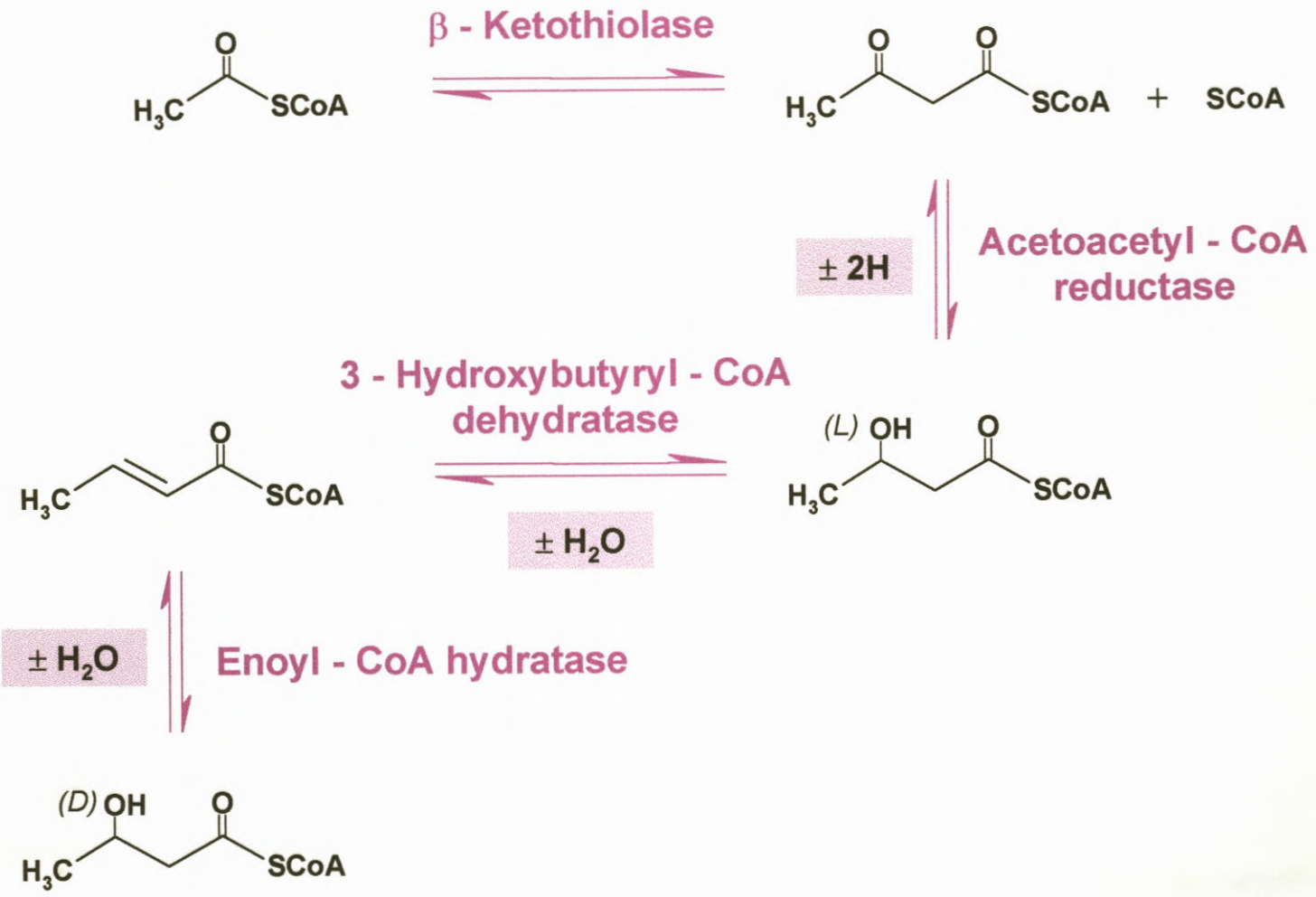


Figure 11. Metabolic pathway for the production of PHB (Reproduced from Dawes and Senior, 1973).

4.3 Mammalians

Stanley and Tubbs (1975), proposed the "leaky hosepipe" model, which emphasized that fatty acid catabolism occurs via two pools in mammalian cells. The major pool is the fast turnover pool of the main pathway of β-oxidation (same as in 4.1 - Fig. 10) and the smaller slower pool i.e. the leakage of metabolites from the main β-oxidation pathway. Riudor *et al.* (1986) reported 3-hydroxydicarboxylic acids to be major urinary metabolites produced from the ω - oxidation of 3-hydroxy fatty acids. Later in 1992, Jin *et al.*, did metabolic studies on rat liver homogenates to investigate the latter phenomenon. The results obtained pointed to an incomplete oxidation of fatty acids, which was consistent to the "leaky hosepipe" model. One contradictory aspect was however that the 3-hydroxy metabolites released were epimerised to a near racemic mixture, in stead of a pure *L*-enantiomer as would be expected from β-oxidation. Consequently a pathway for the racemization of 3-hydroxy fatty acids and the production of *cis*-3 and *trans*-3 fatty acids was proposed (Fig. 12).

Contrary to the conventional concept of net epimerization from 3(*D*)-hydroxy- to 3(*L*)-hydroxy fatty acids (necessary for conventional β-oxidation, see 4.1), Jin *et al.*, (1992) also reported a net conversion from 3 (*L*)-hydroxyacyl CoA to 3(*D*)-hydroxyacyl CoA in the metabolism of saturated fatty acids. It is therefore not surprising that the 3-hydroxy-dicarboxylic acids - constituents of mammalian urine - are break-down products of the latter, which leaked out of the β-oxidation cycle as mentioned previously.

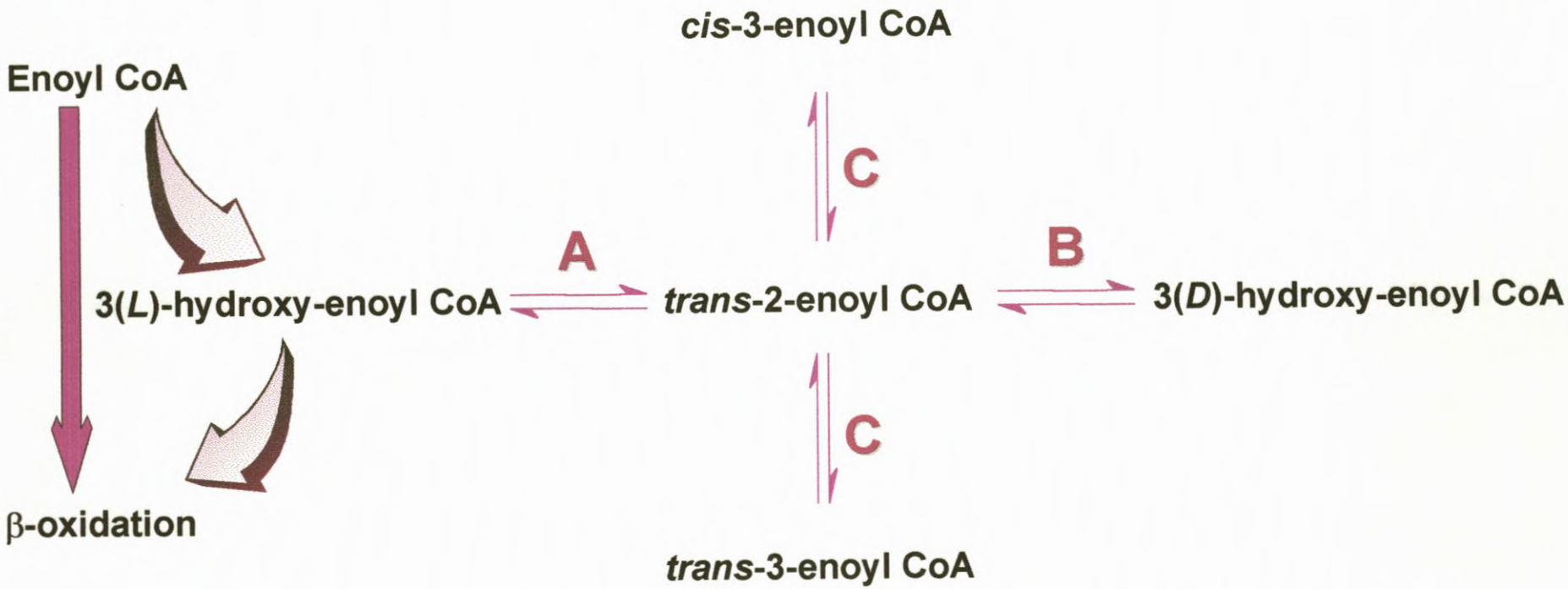


Figure 12. The postulated pathway for the racemization of 3-hydroxy fatty acids and the production of *cis*-3- and *trans*-3 fatty acids. 3(*L*)-hydroxy-enoyl CoA is enzymatically transformed (A → crotonase) to *trans*-2-enoyl-CoA which in turn is converted to 3(*D*)-hydroxy-enoyl CoA (B → enoyl CoA-hydratase). This reaction is reversible and yields a racemic mixture of *D/L*. The intermediate in the latter reaction, *trans*-2-enoyl CoA, is also converted to *cis*-3-enoyl CoA and trans-3-enoyl CoA (C → enoyl CoA isomerase) (Reproduced from Jin *et al.*, 1992).

5. Purpose of research

With this as background the following investigations have been launched:

1. To investigate the production of 3-hydroxy fatty acids by the yeast *D. uninucleata*. In this part of the study the production of different 3-hydroxy polyenoic fatty acids from different precursors will be highlighted as well as the possible metabolic pathways **(Chapter 2)**.

2. To locate the production of 3-HETE during the life cycle of *D. uninucleata*. Here, immunofluorescence microscopy will be applied to identify where this substance is produced. The possible biotechnological implications will also be discussed **(Chapter 3)**.

3. To determine if 3-hydroxy fatty acids also occur in a flocculant strain of the biotechnological important yeast *Saccharomyces cerevisiae*. Here, immunofluorescence microscopy will be applied to determine if and where this substance occurs **(Chapter 4)**.

4. To determine if 3(*R*)-hydroxy fatty acids associated with the LPS layer of Gram-negative bacteria i.e. *Escherichia coli*, is also affected by NSAIDs, as is the case in *D. uninucleata* **(Chapter 5)**.

6. References

Aumercier, M., Murray, D.M. and Rosner, J.L. (1990). *Antimicrob Agents Chemother* **34(5),** 786-791.

Botha, A., Kock, J.L.F., Coetzee, D.J., Van Dyk, M.S., Van der Berg, L. and Botes, P.J. (1992). *System Appl Microbiol* **15,** 148-154.

Brock, T.D. and Madigan, M.T. (1991). In: *Biology of Microorganisms,* **Sixth Edition,** pp. 58-74. Edited by T.D. Brock and M.T. Madigan. Prentice-Hall Inc., USA.

Burns, J.L. and Clark, D.K. (1992). *Antimicrob Agents Chemother* **36(10),** 2280-2285.

Burton, A.J. and Carter, H.E. (1964). *Biochem* **3,** 411-418.

Byrom, D. (1987). *TIBTECH* **5,** 246-250.

Coetzee, D.J., Kock, J.L.F., Botha, A., Van Dyk, M.S., Smit, E.J., Botes, P.J. and Augutyn, O.P.H. (1992). *System Appl Microbiol* **15,** 311-318.

Dawes, E.A. and Ribbons, D.W. (1964). *Bacteriol Rev* **28,** 126-149.

Dawes, E.A. and Senior, P.J. (1973). *Adv Microbial Physiol* **10**, 135-266.

Finnerty, W.R. (1989). In: *Microbial Lipids* **Vol 2**, pp. 525-566. Edited by C. Ratledge and S.G. Wilkinson. California Academic Press Limited, San Diego, USA.

Galanos, C., Lüderitz, O., Rietschel, E.T. and Westphal, O. (1977). *Int Rev Biochem* **14,** 239-335.

Glatz, J.F.C., Vork, M.M. and Van der Vusse, G.J. (1993). *Mol Cell Biochem* **123,** 167-173.

Hancock, I.C., Humphreys, G.O. and Meadow, P.M. (1970). *Biochim Biophys Acta* **202,** 389-391.

Hiremath, L.S., Kessler, P.M., Sasaki, G.C. and Kolattukudy, P.E. (1992). *Eur J Biochem* **203,** 449-457.

Holmes, P.A. (1985). *Phys Technol* **16,** 32-36.

Holst, O., Muller-Loennies, S., Lindner, B. and Brade, H. (1993). *Eur J Biochem* **214,** 695-701.

Jin, S.J., Hoppel, C.L. and Tserng, K.Y. (1992). *J Biol Chem* **267,** 119-125.

Kock, J.L.F., Coetzee, D.J., Van Dyk, M.S., Truscott, M., Botha, A. and Augustyn, O.P.H. (1992). *Ant v Leeuwenhoek* **62,** 251-259.

Kock, J.L.F., Coetzee, D.J., Van Dyk, M.S., Truscott, M., Cloete, F.C., Van Wyk, V. and Augustyn, O.P.H. (1991). *S Afr J Sci* **87,** 73-76.

Kock, J.L.F., Jansen van Vuuren, D., Botha, A., Van Dyk, M.S., Coetzee, D.J., Botes, P.J., Shaw, N., Friend, J., Ratledge, C., Roberts, A.D., and Nigam, S. (1997). *System Appl Microbiol* **20**, 39-49.

Kurtzman, C.P., Vesonder, R.F. and Smiley, M.J. (1974). *Mycologia* **66,** 582-587.

Lemoigne, M. (1926). *Soc Biol* **94,** 1291-1292.

Lösel, D.M. (1988). In: *Microbial Lipids* **Vol 1**, pp. 699-806. Edited by C. Ratledge and S.G. Wilkinson. California Academic Press Limited, San Diego, USA.

Lundgren, D.G., Alper, R., Schnaitman, C. and Marchessault, R.H. (1965). *J Bacteriol* **89,** 245-251.

March, J. (1985). In: *Advanced organic chemistry,* **third edition,** pp. 82-109. Edited by J. March. John Wiley and Sons, NY, USA.

Masshimo, J., Yoshida. M., Ikeuchi. K., Hata, S., Arata, S., Kasai, N., Okuda, K. and Takazoe, I. (1985). *Microbial Immunol* **29,** 395-403.

Mazur, P., Nakanishi, K., El-Zayat, A.E. and Champe, S.P. (1991). *J Chem Soc Chem Commun* **20,** 1486-1487.

Moskowitz, G.J. and Merrick, J.M. (1969). *Biochem* **8,** 2748-2755.

Nesbitt, J.A. and Lennarz, W.J. (1965). *J Bacteriol* **89,** 1020-1025.

Pace-Asciak, C.R. (1989). In: *Advances in prostaglandin, thromboxane and leukotriene research* **Vol. 18**. Raven Press, New York, USA.

Parrilo, J.E. (1990). *Ann Intern Med* **113,** 227-229.

Ratledge, C. and Wilkinson, S.G. (1988). In: *Microbial lipids* **Vol. 1**, pp. 23-54. Edited by C. Ratledge and S.G. Wilkinson. California Academic Press Limited, San Diego, USA.

Rietchel, E.T., Brade, H. and Holst, O. (1996). In : *Current Topics in Microbiology Immunology* **Vol. 216**, pp. 39-81. Edited by E.T. Rietschel and H. Wagner. Springer-Verlag, Heidelberg, Germany.

Riudor, E., Ribes, A., Boronat, M., Sabado, C., Dominquez, C. and Ballabriga, A. (1986). *J Inherited Metab Dis* **9,** Suppl. 2, 297-299.

Rosner, J.L. and Slonczewski, J.L. (1994). *J Bacteriol* **176(20),** 6262-6269.

Sheridan, R. and Ratledge, C. (1996). *Microbiol* **142,** 3171-3180.

Sierra, G. and Gibbons, N.E. (1962). *Can J Microbiol* **8,** 249-253.

Slater, T.F. and McDonald-Gibson, R.G. (1987). In: *Prostaglandins and related substances,* pp. 1-5. Edited by C. Benedetto, R.G. Mcdonald-Gibson, S. Nigam and T.F. Slater. Academic Press, London, UK.

Stanley, K.K. and Tubbs, P.K. (1975). *Biochem J* **150,** 77-88.

Steinbüchel, A., Valentin, H.E. and Schönebaum, A. (1994). *J Environ Polym Degrad* **2,** 67-74.

Stodola, F.H., Deinema, M.H. and Spencer, J.F.T. (1967). *Bact Rev* **31**, 194-213.

Tserng, K.Y. and Jin, S.J. (1991). *J Biol Chem* **266,** 11614-11620.

Van Dyk, M.S., Kock, J.L.F. and Botha, A. (1994). *World J Microbiol Biotech* **10**, 495-504.

Van Dyk, M.S., Kock, J.L.F., Coetzee, D.J., Augustyn, O.P.H. and Nigam, S. (1991). *FEBS Lett* **283(2)**, 195-198.

Venter, P., Kock, J.L.F., Sravan Kumar, G., Botha, A., Coetzee, D.J., Botes, P.J., Bhatt, R.K., Falck, J.R., Schewe, T. and Nigam, S. (1997). *Lipids* **32,** 1277-1283.

Vesonder, R.F., Wickerham, L.J. and Rohwedder, W.K. (1968). *Can J Chem* **46,** 2628-2629.

Westphal, O. and Lüderitz, O. (1954). *Angew Chem* **66,** 407-417.

Wheelan, P. and Murphy, R.C. (1995). *Arch Biochem Biophys* **321(2),** 381-389.

Wilkinson, S.G. (1977). In: *Surface Carbohydrates of the Procaryotic Cell,* pp. 97-175. Edited by I.W. Sutherland. Academic Press, London, UK.

CHAPTER 2

Production of 3*R*-Hydroxy-polyenoic Fatty Acids by the Yeast *Dipodascopsis uninucleata*

This work has been published in *Lipids* **32**, 1277-1283 (1997).

Abstract

Various fatty acids were fed to the yeast *Dipodascopsis uninucleata* UOFS Y128, and the extracted samples were analysed for the accumulation of 3-hydroxy metabolites with the help of electron impact gas chromatography - mass spectrometry (GC-MS). Fatty acids containing a *5Z, 8Z* - diene system (*5Z, 8Z, 11Z* - eicosatrienoic, *5Z, 8Z, 11Z, 14Z* – eicosatetraenoic and *5Z, 8Z, 11Z, 14Z, 17Z* - eicosapentaenoic acids) yielded the corresponding 3-hydroxy – all *Z* - eicosapolyenoic acids. Moreover, linoleic acid (*9Z, 12Z* - octadecadienoic acid) and *11Z, 14Z, 17Z* - eicosatrienoic acid were converted to the 3-hydroxylated metabolites of shorter chain length, e.g., 3-hydroxy - *5Z, 8Z* - tetradecadienoic acid and 3-hydroxy - *5Z, 8Z, 11Z* - tetradecatrienoic acid, respectively. In contrast, no accumulation of a 3-hydroxy metabolite was observed with oleic acid (*9Z* - octadecaenoic acid), linolelaidic acid (*9Z, 12E* - octadecadienoic acid), γ-linolenic acid (*6Z, 9Z, 12Z* - octadecatrienoic acid) and eicosanoic acid as substrate. These findings pinpoint that the 3-hydroxylation of a fatty acid in *Dipodascopsis uninucleata* requires a *5Z, 8Z* - diene system either directly or following initial incomplete β-oxidation. Following analysis of the enantiomer composition, the arachidonic acid (AA) metabolite was identified as 3*R*-hydroxy - *5Z,*

8Z, 11Z, 14Z - eicosatetraenoic acid (3*R* - HETE), which rules out normal β-oxidation as a biosynthetic route to this new class of oxylipins.

1. Introduction

Long-chain hydroxy fatty acids and other oxygenated fatty acid derivatives (oxylipins) are widely distributed in both the animal and plant kingdom and exert a plethora of biological actions (Nomura and Ogata, 1976; Gardner, 1991; van Dyk *et al.*, 1994). During the past ten years, plenty of knowledge has accumulated regarding the synthesis of oxylipins by fungi and related organisms. A large number of unsaturated hydroxy fatty acids which are formed by either lipoxygenase, dioxygenase or cytochrome P - 450 - mediated pathways have been found in various fungal species and oomycetes (Mantle *et al.*, 1969; Schechter and Grossman, 1983; Hamberg *et al.*, 1986; Hamberg *et al.*, 1987; Mazur *et al.*, 1991; Brodowsky *et al.*, 1992; Brodowsky and Oliw, 1992; Su *et al.*, 1995). These compounds carry one or more hydroxy groups at carbon atoms 5, 7, 8, 9, 12, 13, 15 or 17 of the fatty acid molecule. While the majority of fungal oxylipins are formed from oleic or linoleic acid, there are only a few examples for AA - derived oxylipins (eicosanoids) in fungi. The 15*S* - hydroxyeicosanoids have been identified in *Saprolegnia* and *Achlya* species (Hamberg *et al.*, 1986; Hamberg *et al.*, 1987). In earlier communications, we have reported on the formation of 3-hydroxy - *5Z, 8Z, 11Z, 14Z* - eicosatetraenoic acid (3-HETE) by the yeast *Dipodascopsis uninucleata* fed with AA (van Dyk *et al.*, 1991; Kock *et al.*, 1997). In the present study, we demonstrate that 3-hydroxy fatty acids are produced by *D. uninucleata* from other polyenoic fatty acids, provided they contain a *5Z, 8Z* - diene system either directly or upon preceding β-oxidation. Moreover, the stereochemical identification of the 3-hydroxy group (3*R*) rules out a normal β-oxidation as putative biosynthetic pathway for these oxylipins.

2. Experimental procedures

The compound 3-HETE was isolated and purified as described elsewhere (van Dyk *et al.*, 1991). The methylation and trimethylsilylation were performed according to the instructions supplied with the kits from Aldrich (Deisenhofen, Germany) and Merck Chemicals (Darmstadt, Germany), respectively. Fatty acids 18:1 (*9Z*) and 18:2 (*9Z, 12E*) were obtained from Sigma (Deisenhofen, Germany), all other fatty acids were supplied by Cayman (Ann Arbor, MI).

2.1 Synthesis of 3R- and 3S-HETE

The synthetic strategy for 3*R*- and 3*S*-HETE involved a convergent approach coupling chiral aldehyde with Wittig salt; these were derived from 2-deoxy-D-ribose and AA, respectively (Bhatt *et al.*, 1998). Briefly, lead tetraacetate oxidation of readily available methyl 5,6-dihydroxyeicosatetraenoate (Bhatt *et al.*, 1994) and $NaBH_4$ reduction of the resultant aldehyde gave an alcohol. Alcohol to bromide interconversion under standard conditions followed by displacement with triphenylphosphine in acetonitrile generated a phosphonium salt. The 3*R*-*t*-butyldiphenylsilyloxy *d*-valerolactone (Saiah *et al.*, 1992) prepared from 2-deoxy-D-ribose as described (Chauhan *et al.*, 1994) was converted to an ester *via* saponification and treatment with ethereal diazomethane, the oxidation of which with pyridinium chlorochromate yielded chiral aldehyde. The condensation of chiral aldehyde with the ylide of phosphonium salt gave, after fluoride-mediated deprotection and high-performance liquid chromatography (HPLC) purification, methyl 3*R*-hydroxy-5, 8, 11, 14-eicosatetraenoate. Mitsunobu inversion of it using chloroacetic acid, saponification of the resultant ester, and diazomethane esterification yielded methyl 3*S*-hydroxy-5, 8, 11, 14-eicosatetraenoate. The corresponding free acids were prepared by saponification of the respective esters.

2.2 *Cultivation of the yeast Dipodascopsis uninucleata*

Dipodascopsis uninucleata UOFS Y128 was grown to stationary phase (sexual stage) after which 12.5 mg/l of the fatty acid (Table 1) were added. Following 6 h of incubation, the cells were harvested. The growth and harvesting procedure were as described earlier (van Dyk *et al.*, 1991).

2.3 *Extraction of fatty acid metabolites from the yeast*

The cells obtained in each experiment were mixed with absolute ethanol to a final concentration of 80% ethanol. The suspension was kept at 5 °C for 18 h and then filtered. The filtrate was adjusted to 15% aqueous ethanol. The yeast sample was acidified to pH 3.0 with formic acid and chromatographed on a preconditioned Sep-Pak C_{18} cartridge (Millipore, Bradford, MA) as described (Nigam, 1987). The AA metabolites were finally eluted with 5 ml of freshly distilled ethyl acetate. The eluate was evaporated under a stream of nitrogen, and the fatty acid metabolites were separated from other hydrophobic compounds by applying their triethylamine salts on Sep-Pak silica gel cartridges and eluting them with 15% ethanol. Following evaporation of ethanol under a stream of nitrogen, the samples were adjusted to pH 3.0 and extracted with ethyl acetate as described above. Each sample was again chromatographed on a preconditioned Sep-Pak C_{18} cartridge as described before and finally dissolved in chloroform.

Table 1. Accumulation of 3-hydroxy metabolites from selected fatty acids fed to *Dipodascopsis uninucleata*.

Fatty acids fed	Accumulation of oxylipins *	Identified structure
18:1 (9Z)	-	
18:2 (9Z, 12Z)	+	3-OH-14:2 (5Z, 8Z)
18:2 (9Z, 12E)	-	
18:3 (6Z, 9Z, 12Z)	-	
20:0	-	
20:3 (5Z, 8Z, 11Z)	+	3-OH-20:3 (5Z, 8Z, 11Z)
20:3 (11Z, 14Z, 17Z)	+	3-OH-14:3 (5Z, 8Z, 11Z)
20:4 (5Z, 8Z, 11Z, 14Z)	+	3-OH-20:4 (5Z, 8Z, 11Z, 14Z)
20:5 (5Z, 8Z, 11Z, 14Z, 17Z)	+	3-OH-20:5 (5Z, 8Z, 11Z, 14Z, 17Z)

* as judged from the detection of a fraction co-migrating with 3-HETE in thin-layer chromatography

2.4 Thin Layer Chromatography (TLC)

Chloroform fractions obtained from yeasts fed with polyenoic fatty acids in the presence or absence of 1 mM acetyl-salicylic acid were chromatographed together with the 3-HETE standard prepared in our own laboratory on silica gel plates (Merck) as described (van Dyk *et al.*, 1991). The compounds were visualised by placing TLC plates in an iodine tank.

2.5 *Electron Impact Mass Spectra*

Electron impact mass spectra of 3-HETE and other 3-hydroxy fatty acids were recorded on a Hewlett-Packard 5890 gas chromatograph (Palo Alto, CA) equipped with a Hewlett-Packard 5972 MSD at 1447 EM Volts. An HP-1 fused-silica capillary column (30m X 0.25 mm i.d.) was used for separation with helium as carrier gas. Other operating conditions were: ion source 170 °C and electron impact energy 70 eV.

Prior to analysis, the methyl-trimethylsilyl (ME-TMSi) derivatives of the samples were prepared as described (Barrow and Taylor, 1987) and reconstituted in 100 µl chloroform/hexane (1:4) before injection. Samples were injected by split ratio of 1:50 at 230 °C and the column temperature programmed from 140–300 °C at 5 °C per min.

2.6 *Analysis of enantiomeric composition of 3-HETE*

Enantiomeric analysis of 3-HETE from *D. uninucleata* was performed on an Apex Chiral AU 50 HPLC column (4.6 X 250 mm, Jones chromatography, Hengoed, Glamorgan, United Kingdom) with a variable wavelength ultraviolet - detector (Pharmacia, Freiburg, Germany) using 1% isopropanol in n-hexane as isocratic solvent system. The flow rate was 1.0 ml/min. The ultraviolet absorption peaks were monitored at 208 nm.

3. Results

A number of fatty acids were fed to *D. uninucleata*. After 6 h the fatty acids which were not incorporated in cellular lipids, were extracted by ethanol and separated as described in the Experimental procedures section. It is evident from Table 1 that a selected number of fatty acids produced various compounds which comigrated on TLC

with authentic 3-HETE. These compounds were isolated, converted to their ME-TMSi derivatives, and their structures were established by gas chromatography / electron impact mass spectrometry (Figs. 1-4). The characteristic fragments of the compounds are listed in Table 2.

Table 2. Characteristic mass fragments of the 3-hydroxy derivatives.

Metabolite	Fragments	Figure
3-OH-20:3 (5Z, 8Z, 11Z)	175; 408 [M^+]; 393 [M^+-15 (CH_3)]	1
3-OH-20:4 (5Z, 8Z, 11Z, 14Z)	175; 406 [M^+]; 391 [M^+-15 (CH_3)]	2A
3-OH-20:5 (5Z, 8Z, 11Z, 14Z, 17Z)	175; 404 [M^+]; 389 [M^+-15 (CH_3)]	2B
3-OH-14:2 (5Z, 8Z)	175; 326 [M^+]; 311 [M^+-15 (CH_3)]	3
3-OH-14:3 (5Z, 8Z, 11Z)	175; 309 [M^+-15 (CH_3)]	4

All compounds exhibited a base peak of *m/z* 175 [$CH_3O(CO)$-CH_2-CHO-TMSi] which is indicative of a hydroxylation at C3 position. A 3-hydroxy derivative accumulated when *5Z, 8Z, 11Z* - eicosatrienoic, *5Z, 8Z, 11Z, 14Z* - eicosatetraenoic (AA), *5Z, 8Z, 11Z, 14Z, 17Z* - eicosapentaenoic, *11Z, 14Z, 17Z* - eicosatrienoic, or *9Z, 12Z* - octadecadienoic (linoleic) acids were fed to the yeast. From the structural identification of the corresponding derivatives, it was evident that the first three fatty acids were 3-hydroxylated without alteration of the chain length, whereas the other two were shortened by 6 and 4 carbon atoms, respectively. In contrast, 3-hydroxy derivatives were not observed upon feeding of *9Z* - octadecaenoic (oleic), *9Z, 12E* - octadecadienoic (linolelaidic), *6Z, 9Z, 12Z* - octadecatrienoic (γ-linolenic), or eicosanoic acids, although these fatty acids were shown to be metabolised to a large extent by the yeast, most probably *via* β-oxidation. Feeding experiments with C_{22} polyenoic fatty acids failed since the latter turned out to be toxic to *D. uninucleata*.

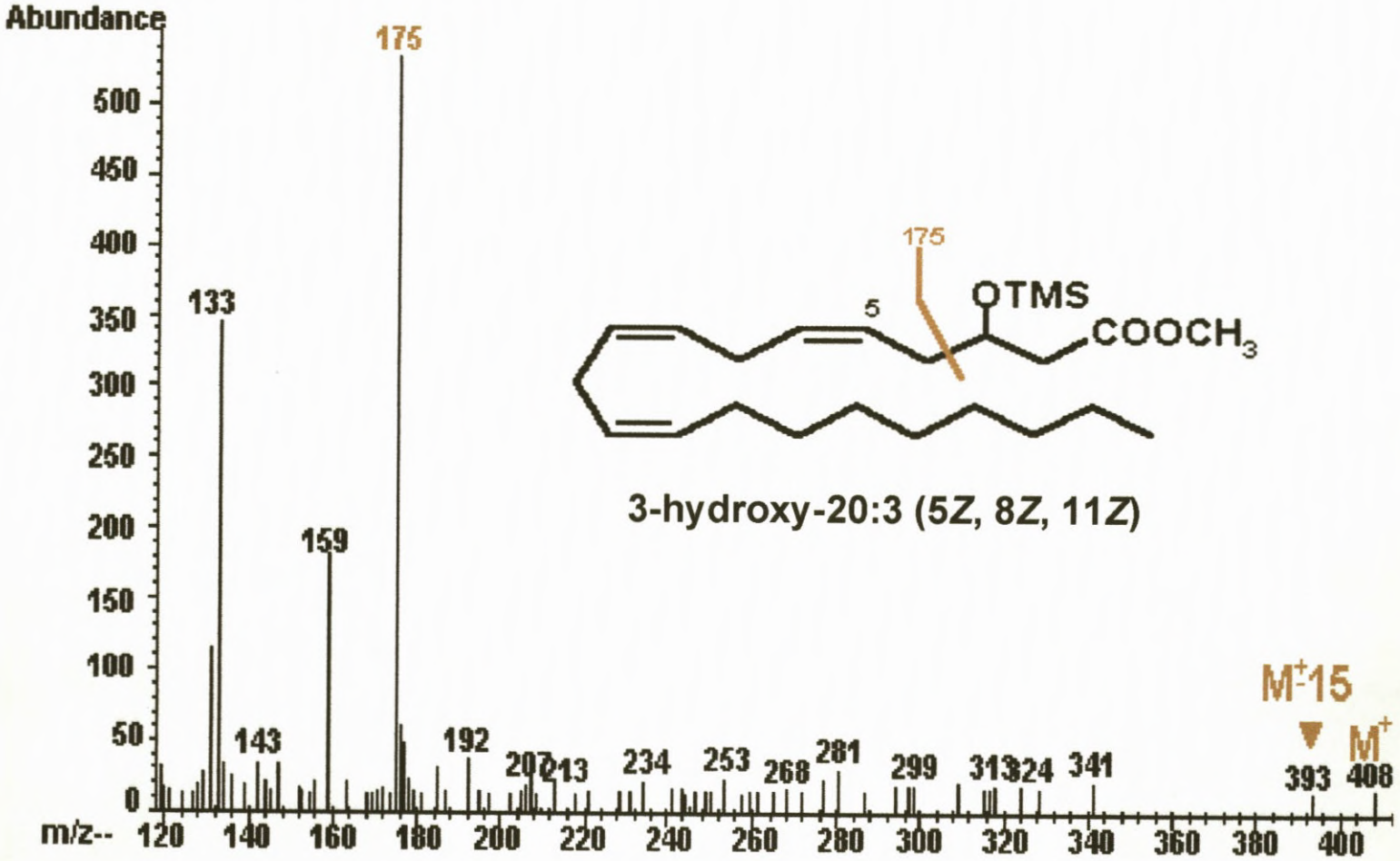


Figure 1. Electron impact-mass spectra of methyl-trimethylsilylated 3-hydroxy 20:3 (5Z, 8Z, 11Z).

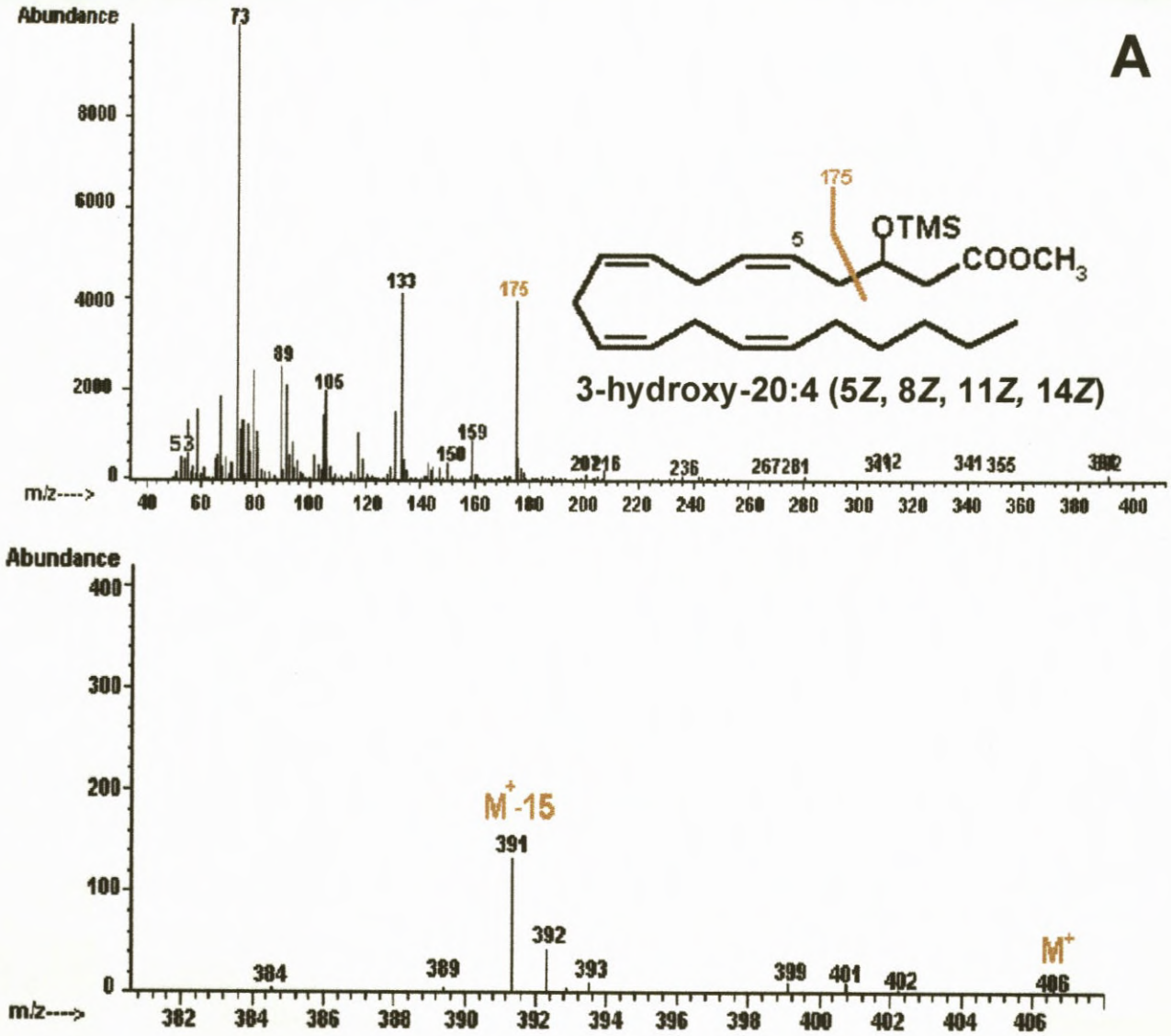


Figure 2 A. Electron impact-mass spectra of methyl-trimethylsilylated 3-hydroxy-5*Z*, 8*Z*, 11*Z*, 14*Z*-eicosatetraenoic acid (3-HETE). Lower panel show expanded spectra of *m/z* 380 to 408. OTMS, *O*-Trimethylsilyl.

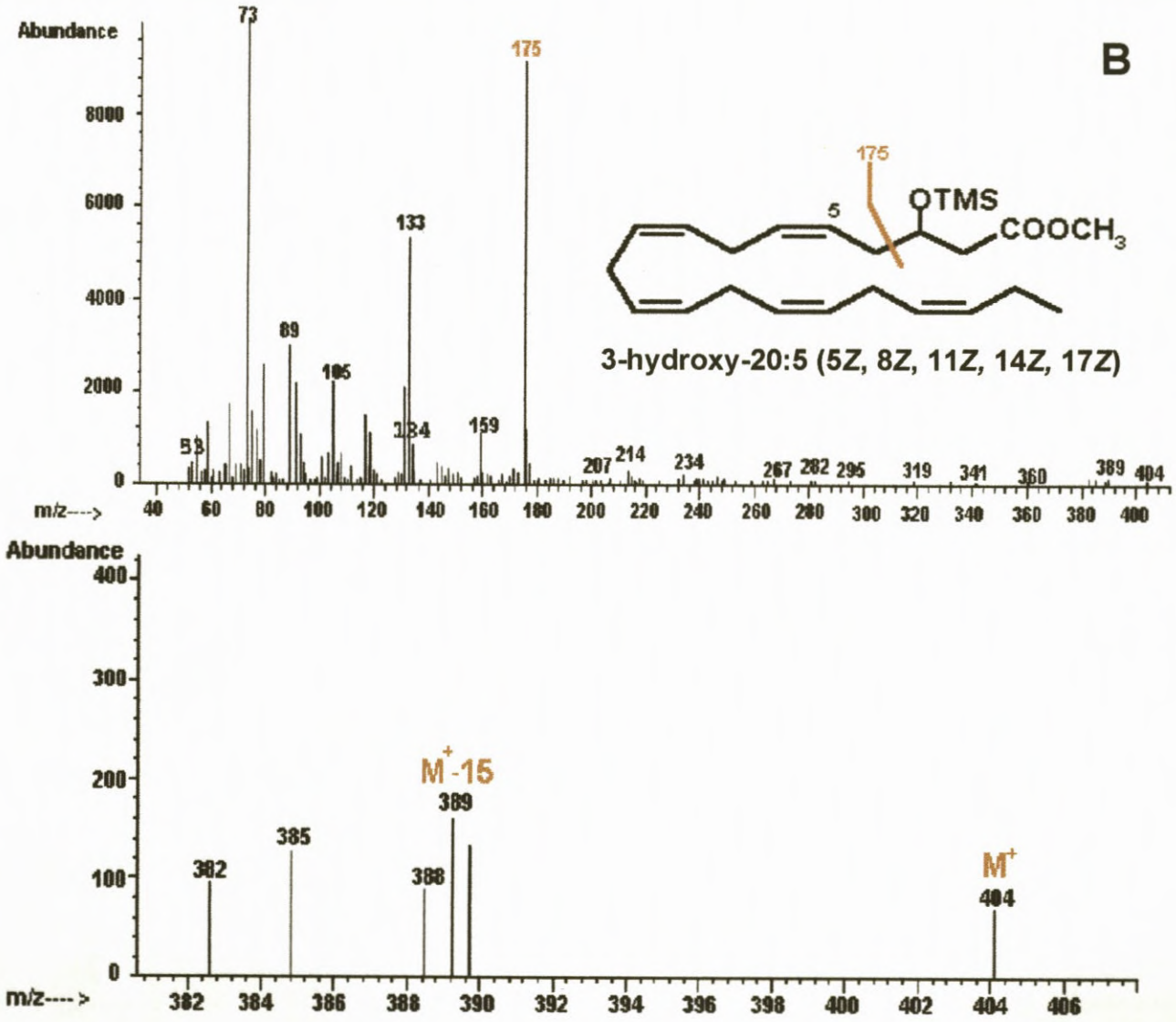


Figure 2 B. Electron impact-mass spectra of methyl-trimethylsilylated 3-hydroxy-5*Z*, 8*Z*, 11*Z*, 14*Z*, *17Z*-eicosapentaenoic acid (3-HEPE). Lower panels show expanded spectra of *m/z* 380 to 408. OTMS, *O*-Trimethylsilyl.

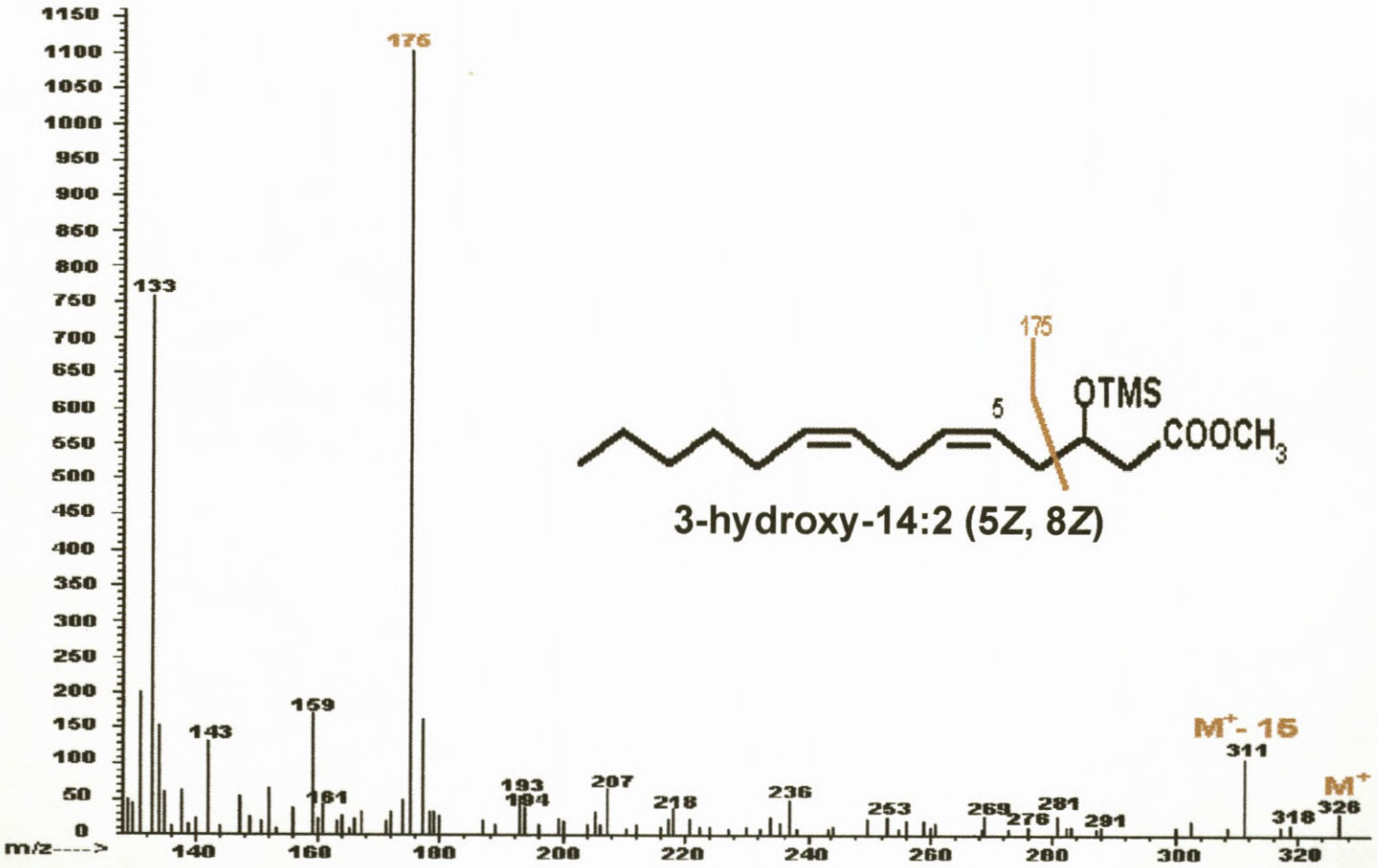


Figure 3. Electron impact-mass spectra of methyl-trimethylsilylated 3-hydroxy 14:2 (5Z, 8Z).

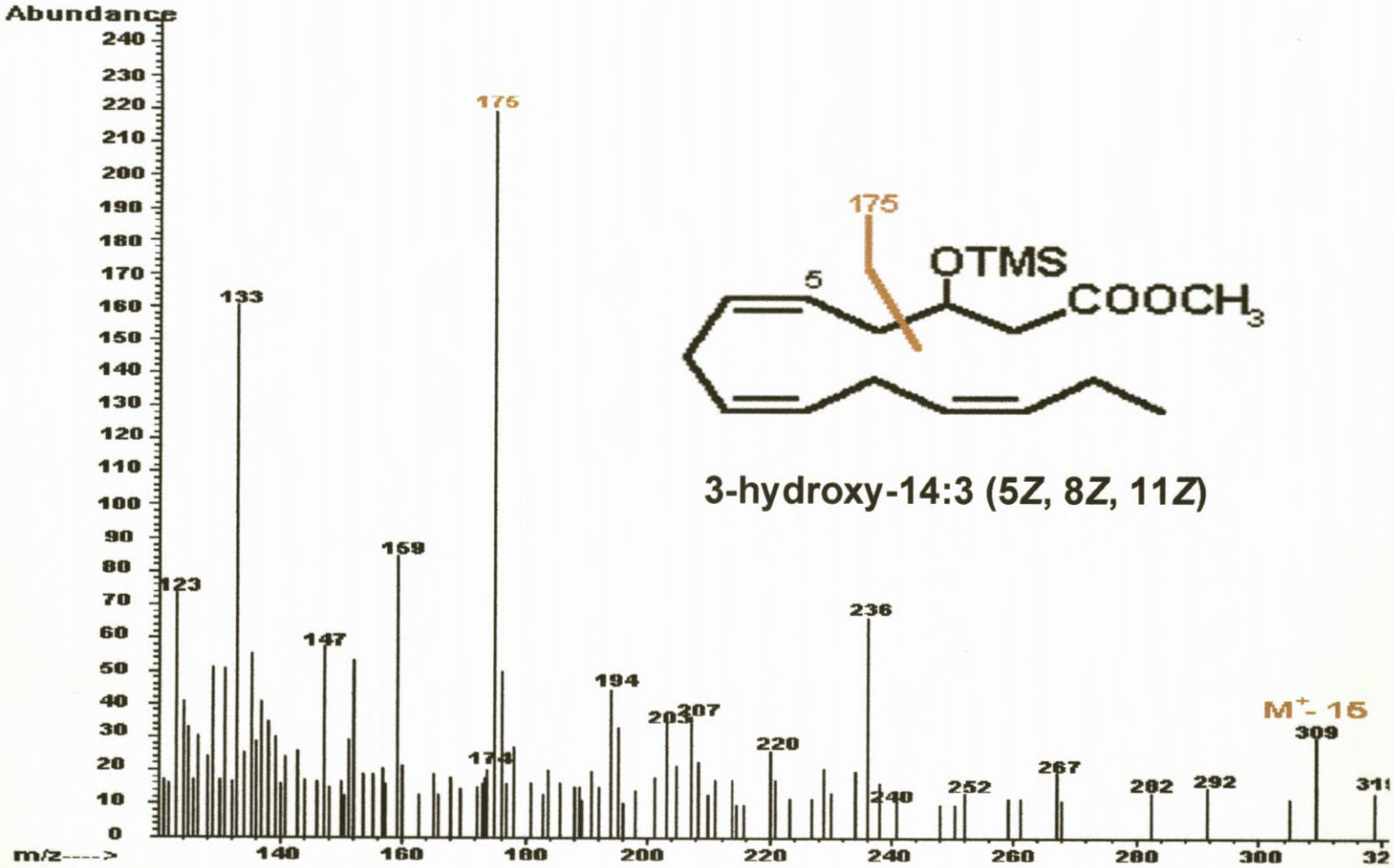


Figure 4. Electron impact-mass spectra of methyl-trimethylsilylated 3-hydroxy 14:3 (5Z, 8Z,11Z).

The accumulation of the 3-hydroxy polyenoic fatty acids was generally suppressed by 1 mM acetylsalicylic acid.

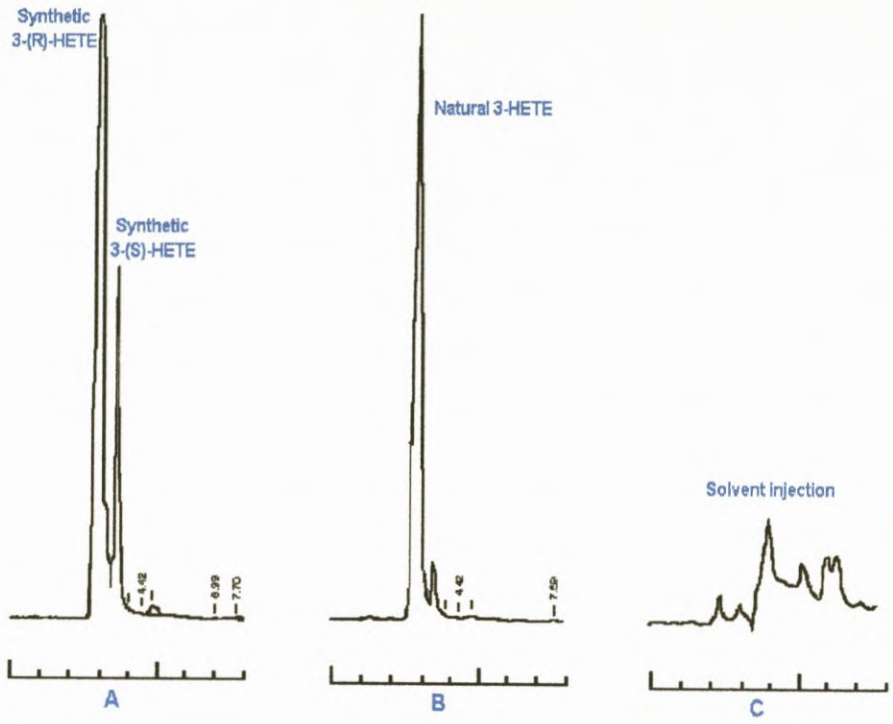


Figure 5. Chiral phase HPLC analysis of 3-HETE.

For the product from AA, 3-HETE, an analysis of the enantiomeric composition was performed. Figure 5A shows the resolution of the enantiomers when injected at a ratio of 4:1 (40μg 3*R*-HETE and 10μg 3*S*-HETE). The retention volumes were 3.0 and 3.8 ml solvent, respectively. Figure 5B clearly shows that the natural 3-HETE from *D. uninucleata* consist of 95% 3*R*-HETE. Interfering peaks were not observed in the HPLC chromatogram at the above retention volumes (Fig. 5C). [In independent experiments these data were confirmed by Dr. M. Hamberg, Karolinska Institute, Stockholm, Sweden, who analysed a sample of 3-HETE from *D. uninucleata* supplied by Dr. J. Friend University of Hull, United Kingdom (*personal communication*)].

4. Discussion

In this report we have demonstrated that *D. uninucleata* produced a 3-hydroxy derivative not only from AA but also from a variety of other polyenoic fatty acids, including linoleic acid which contributes to about one-fourth of the total fatty acids of this yeast (Kock *et al.,* 1997). It is interesting to note that the 3-HETE exhibited an enantiomeric ratio 95:5 (*R/S*). On the basis of structural similarities between 3*R*-HETE and other 3-hydroxy fatty acids, we postulate that the other 3-hydroxy fatty acids should also bear the *R*-configuration. The observation that a number of other fatty acids, among them saturated fatty acids, oleic acid and γ-linolenic acid, were not converted to detectable amounts of 3-hydroxy derivatives, despite the fact that they were metabolised, suggest some specific structural requirements for the 3*R*-hydroxylation. From the comparison of the structures of the hydroxylation - susceptible and nonsusceptible fatty acids, and of those of the hydroxy derivatives, it follows that the presence of a *5Z, 8Z* - diene system in the fatty acid molecule is a prerequisite for the 3*R*-hydroxylation by *D. uninucleata.* Such a diene arrangement can also be accomplished by a preceding partial breakdown of the fatty acid *via* β-oxidation, which explains the formation of 3-hydroxylated C_{14} polyenoic fatty acids from linoleic and *11Z, 14Z, 17Z* - eicosatrienoic acid.

The *5Z, 8Z* - diene system in the fatty acid does not take part in the aforementioned biotransformation reaction, nor does it seem to be capable of activating the methylene

group at the C3 position at which the hydroxylation occurs. Therefore, the *5Z, 8Z* - diene system might fulfill a signal function for the recognition of the substrate by the corresponding enzyme in *D. uninucleata via* binding at the active site.

At first sight, a 3-hydroxy fatty acid may be principally formed *via* incomplete β-oxidation. In that case, however, the 3*S*-enantiomer (corresponding to 3-[*L*] according to the Fischer nomenclature) would have been found as the main metabolite. Since we clearly demonstrated that *D. uninucleata* produced nearly exclusively 3*R*-HETE (3-[*D*]-HETE) from AA, such an assumption has to be rejected. The different stereospecificities of the biosynthetic pathway in *D. uninucleata* and β-oxidation further imply that the 3*R*-hydroxy fatty acids cannot be degraded *via* normal β-oxidation, which might be the reason for the accumulation of these oxylipins upon feeding of the corresponding precursor fatty acids to the yeast.

The capability of producing 3*R*-hydroxy fatty acids is apparently not restricted to *D. uninucleata* alone. The 3*R*-hydroxypalmitic acid and related saturated compounds have been reported in other yeasts such as *Rhodotorula graminis, Rh. glutinis* (Stodola *et al.,* 1967), and *Saccharomycopsis malanga* (Vesonder *et al.,* 1968). Moreover, medium-chain 3-hydroxy fatty acids have been shown to be present in the glycolipids of the smut fungi *Ustilago zeae* and *U. nuda* (Lösel, 1988). Finally, saturated 3*R*-hydroxy fatty acids with 10, 12 and 14 carbons are encountered in Lipid A, the component of the lipopolysaccharides of Gram-negative bacteria, that is essential for the endotoxin activity (Holst *et al.,* 1993). The biosynthetic system in *D.*

uninucleata appears to differ, however, from those of other fungi and of bacteria in its marked preference for polyunsaturated fatty acids possessing a *5Z, 8Z* - diene system.

The biosynthetic pathway to the 3*R*-hydroxy polyenoic fatty acids remains to be elucidated. Lipoxygenase- or dioxygenase - mediated reactions can be excluded, since they would require a neighbouring double-bond system (at C2 and/or C4). The same is true for an involvement of prostaglandin endoperoxide synthase, eventhough the synthesis of the 3*R*-hydroxy fatty acids was found to be inhibited by acetylsalicylic acid (van Dyk *et al.*, 1991). It should be stressed that the action of acetylsalicylic acid on enzymes is fairly unspecific. Moreover, acetylsalicylate and salicylate act in higher plants as hormones inducing certain hypersensitivity reactions by changing the gene expression program (Finnerty, 1989; Slusarenko, 1996). Thus, two metabolic routes to 3*R*-hydroxy fatty acids are conceivable: (1) a reaction sequence analogous to β-oxidation, however, implicating a 2-enoyl-CoA hydratase with opposite steric specificity; and (2) a direct monooxygenase reaction at C3, e.g., by a cytochrome P-450 type enzyme.

The conversion of linoleic acid to 3*R*-hydroxy - *5Z, 8Z* - tetradecadienoic acid needs special attention in view of the fact that the precursor fatty acid contributes to about one-fourth of the total fatty acid content of *D. uninucleata* (Kock *et al.*, 1997). Therefore, this oxylipin could exert a regulatory function during the sexual phase of the reproductive cycle of this yeast as has been shown to be the case for other fungal oxylipins (Mazur *et al.*, 1991).

Since *D. uninucleata* does not synthesize adequate amounts of AA, the conversion of the latter to 3*R*-HETE does not appear to be of major importance for the reproduction biology of this yeast. This reaction may, however, play a pivotal role in the cell-cell interaction with AA - producing organisms. In separate investigations we found that 3*R*-HETE affects signal transduction processes in human neutrophils and tumour cells in multiple ways (Nigam, S., Venter, P., Grierman, M., Schewe, T., Kock, J.L.F., Botha, A., Kumar, G.S. and Franke, J., Submitted for publication). Thus, this novel eicosanoid may be capable of modifying inflammatory processes and mitogenesis. In view of these observations, it would be of vital interest to investigate whether 3*R*-HETE and related compounds can also be produced by those fungal strains that are pathogenic to humans.

5. References

Barrow, S.E. and Taylor, G.W. (1987). In: *Prostaglandins and Related Compounds*, pp. 99-141. Edited by C. Benedetto, R.G. McDonald-Gibson, S. Nigam and T.F. Slater. IRL Press, Oxford, United Kingdom.

Bhatt, R.K., Chauhan, K., Wheelan, P., Murphy, R.C. and Falck, J.R. (1994). *J Am Chem Soc* **166**, 5050-5056.

Bhatt, R.K., Falck, J.R. and Nigam, S. (1998). *Tetrahedron Lett* **39**, 249-252.

Brodowsky, I.B. and Oliw, E.H. (1992). *Biochim Biophys Acta* **1124**, 59-65.

Brodowsky, I.B., Hamberg, M. and Oliw, E.H. (1992). *J Biol Chem* **267**, 14738-14745.

Chauhan, K., Bhatt, R.K., Falck, J.R. and Capdevilla, J. (1994). *Tetrahedron Lett* **35**, 1825-1828.

Finnerty, W.R. (1989). In: *Microbial Lipids* **Vol 2**, pp. 525-566. Edited by C. Ratledge and S.G. Wilkinson. California Academic Press Limited, San Diego.

Gardner, H.W. (1991). *Biochim Biophys Acta* **1024**, 221-239.

Hamberg, M., Herman, C.A. and Herman, R.P. (1986). *Biochim Biophys Acta* **877**, 447-457.

Hamberg, M., Herman, C.A. and Herman, R.P. (1987). *Biochim Biophys Acta* **879**, 410-418.

Holst, O., Muller-Loennies, S., Lindner, B. and Brade, H. (1993). *Eur J Biochem* **214,** 695-701.

Kock, J.L.F., Jansen van Vuuren, D., Botha, A., Van Dyk, M.S., Coetzee, D.J., Botes, P.J., Shaw, N., Friend, J., Ratledge, C., Roberts, A.D. and Nigam, S. (1997). *System Appl Microbiol* **20**, 39-49.

Lösel, D.M. (1988). In : *Microbial Lipids* **Vol 1**, pp. 699-806. Edited by C. Ratledge and S.G. Wilkinson. California Academic Press Limited, San Diego.

Mantle, P.G., Morris, L.J. and Hall, S.W. (1969). *Trans Brit Mycol Soc* **53**, 441-447.

Mazur, P., Nakanishi, K., El-Zayat, A.E. and Champe, S.P. (1991). *J Chem Soc Chem Commun* **20**, 1486-1487.

Nigam, S. (1987). In: *Prostaglandins and Related Compounds,* pp. 55-73. Edited by C. Benedetto, R.G. McDonald-Gibson, S. Nigam and T.F. Slater. IRL Press, Oxford, United Kingdom.

Nomura, T. and Ogata, O. (1976). *Biochim Biophys Acta* **431**, 127-131.

Saiah, M., Bessodes, M. and Antonakis, K. (1992). *Tetrahedron Lett* **33**, 4317-4320.

Schechter, G. and Grossman, S. (1983). *Int J Biochem* **15**, 1295-1304.

Slusarenko, A.J. (1996). In: Lipoxygenase and lipoxygenase pathway Enzymes, pp. 176-197. Edited by G.J. Piazza. AOCS press Champaign.

Stodola, F.H., Deinema, M.H. and Spencer, J.F.T. (1967). *Bact Rev* **31**, 194-213.

Su, C., Brodowsky, I.B. and Oliw, E.H. (1995). *Lipids* **30**, 43-50.

Van Dyk, M.S., Kock, J.L.F. and Botha, A. (1994). *World J Microbiol Biotech* **10**, 495-504.

Van Dyk, M.S., Kock, J.L.F., Coetzee, D.J., Augustyn, O.P.H. and Nigam, S. (1991). *FEBS Lett* **283(2)**, 195-198.

Vesonder, R.F., Wickerham, L.J. and Rohwedder, W.K. (1968). *Can J Chem* **46**, 2628-2629.

CHAPTER 3

Biological dynamics and distribution of 3-hydroxy fatty acids in the yeast *Dipodascopsis uninucleata* as investigated by immunofluorescence microscopy. Evidence for a putative regulatory role in the sexual reproductive cycle

This work has been published in *FEBS Letters* **427**, 345-348 (1998).

Abstract

Dipodascopsis uninucleata has been recently shown to produce 3-hydroxy polyenoic fatty acids from several exogenous polyenoic fatty acids. In order to examine whether endogenous 3-hydroxy fatty acids (3-hydroxy-FA) may be implicated in the developmental biology of this yeast, we mapped by immunofluorescence microscopy their occurrence in fixed cells with or without cell walls using an antibody raised against 3*R*-hydroxy-5*Z*, 8*Z*, 11*Z*, 14*Z*-eicosatetraenoic acid (3*R*-HETE), the biotransformation product from arachidonic acid (AA). This antibody turned out to cross-react with other 3-hydroxy-FA. 3-Hydroxy-FA were detected *in situ* in gametangia, asci, as well as between released ascospores, and proved to be associated with the sexual reproductive stage of the life cycle of the yeast. Acetylsalicylic acid (1 mM), which is known to suppress the formation of 3-hydroxy-FA from exogenous polyenoic fatty acids, inhibited the occurrence of immunoreactive material as well as the sexual phase of the life cycle suggesting a prominent regulatory role of 3-hydroxy-FA for the latter.

1. Introduction

Long chain fatty acids are widely distributed in plants and animals. These oxylipins exert a myriad of biological actions (Nomura and Ogata, 1976; Schweizer, 1989; Gardner, 1991; van Dyk *et al.,* 1994). A number of fungal species, particularly representatives of the Endomycetaceae, Lipomycetaceae and Dipodascaceae has been reported to produce unsaturated fatty acids, e.g. linoleic and α-linolenic acid (Botha and Kock, 1993), which form the basis of oxylipins. Screening of members of the Lipomycetaceae in our laboratory revealed that *Dipodascopsis uninucleata* is capable of transforming the exogenously fed AA to 3-HETE (van Dyk *et al.,* 1991; Kock *et al.,* 1997) and other polyenoic fatty acids to respective 3-hydroxy-FA through a sequence of reactions analogous to β-oxidation, however stereochemically inverse, or through a direct monooxygenase reaction (Venter *et al.,* 1997). The prerequisite for the formation of 3-hydroxy-FA from the corresponding polyenoic fatty acids turned out to be the presence of a 5*Z*, 8*Z*-diene system either directly or upon preceding partial β-oxidation (Venter *et al.,* 1997).

As suggested earlier, the intracellular production of 3-hydroxy-FA may be implicated in the sexual reproductive stage of *D. uninucleata* (Kock *et al.,* 1997). The life cycle of this yeast is characterized by alternating asexual and sexual reproductive stages (Biggs, 1937; Zsolt, 1963; Botha and Kock, 1993). During the asexual stage, i.e. first 32 h, if grown in synchronous culture from ascospores (eventually present in attached clusters) the latter separate themselves, swell and germinate to produce well-separated hyphae. This process is followed by the sexual stage (32 to 45 h) which is characterized by the formation of conjugated gametangia, karyogamy, meioses and ascus formation. Each ascus contains hundreds of ascospores formed through the process of ascosporogenesis and are liberated in clusters within 45 h. In as much as the cell cycle has been proved to be sensitive to acetylsalicylic acid which at the same time also inhibits the formation of 3-HETE from AA, it was tempting to speculate

that the formation and action of certain 3-hydroxy-FA may be involved in ascosporogenesis. If this assumption holds true, 3-hydroxy-FA must be detectable *in situ* at the corresponding developmental stages of the yeast.

In the present study we investigated the *in situ* occurrence and localization of 3-hydroxy-FA in *D. uninucleata* during the course of the vegetative and sexual reproductive stages of the cell cycle with the help of immunofluorescence microscopy. For this purpose we used an antibody raised against 3-HETE in rabbits, that turned out to cross-react also with other 3-hydroxy-FA. Despite the apparent impediments to effective immunofluorescence such as encasement of the *D. uninucleata* cells in a tough and impervious cell wall, the method described here has permitted the effective visualization of intracellular structures in yeast cells.

2. Experimental procedures

2.1 Strain used

D. uninucleata UOFS-Y128 was used throughout this study and is held at The University of the Orange Free State, Bloemfontein, South Africa.

2.2 Cultivation and harvesting of cells

D. uninucleata was cultivated for 72 h in three 250 ml conical flasks each containing 50 ml synthetic medium (4 g/l glucose; 6.7 g/l Yeast Nitrogen Base from Difco Laboratories, USA). The cultures were incubated on a rotary shaker (160 r.p.m., throw = 50 mm) at 30 °C. To one of the flasks, 1.0 mg AA in ethanol (Sigma, Germany) was added after 72 h followed by further cultivation for 3 h. To another

flask, acetylsalicylic acid was added to a final concentration of 1 mM prior to cultivation (Botha and Kock, 1993) followed by the addition of AA as described previously. No extra additions were made to the third flask. This experiment was performed at least in duplicate.

2.3 *Detection of 3-HETE and other 3-hydroxy-FA by immunofluorescence microscopy*

2.3.1 *Preparation of antibody*

3*R*-HETE was synthesized by us as described previously (Bhatt *et al.*, 1998). The antibodies against 3*R*-HETE were raised in rabbits as follows: the carboxyl group of 3*R*-HETE was conjugated to amino groups of bovine serum albumin (BSA) by the *N*-succinimidyl ester method (Hosoda *et al.*, 1981). The conjugate (1 mg protein) was emulsified in an equal volume of Freund's complete adjuvant (for the first injection) or incomplete adjuvant (for later injections). The emulsion was injected subcutaneously into several sites on the back of a female New Zealand white rabbit every second week for a total of 7 times for about 3 months. Finally the whole blood was collected from the carotid artery, left at room temperature for 2 h and centrifuged at 1200 x *g* for 20 min at 4 °C. The sera were affinity purified by Biogenes, Berlin.

2.3.2 *Characterization of antibody*

Characterization of the antibody was accomplished by the determination of its titer, sensitivity and specificity. Since the [^{14}C]-3-HETE is not commercially available, a little amount of the tracer was prepared by the transformation of [^{14}C]-AA (sp. act. 52 mCi/mmol) by *D. uninucleata* to [^{14}C]-3-HETE and purified with the help of radio-HPLC. The titer of the antibody gave a binding of approximately 30% labeled 3-HETE at a dilution of 1:100 in the absence of unlabeled 3-HETE. The sensitivity

(minimum detectable amount) of 3*R*-HETE 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 expressed in terms of cross-reactions with structurally related compounds. Cross-reactivity of the antibody with 5-, 12- or 15-HETE was < 0.5%. While investigating the distribution of 3-HETE during the life cycle of *D. uninucleata* we observed substantial cross-reactivity of the antibody against other 3-hydroxy-FA produced in the yeast. Therefore, we checked the immunoreactivity of several commercially available hydroxylated and free fatty acids against our antibody with the help of immunofluorescence microscopy. The cross-reactivities expressed in terms of fluorescence intensities (maximum, +++; medium, ++; minimum, +; none, -) were for the FA as follows: 3*R*-HETE, +++; 3*S*-HETE, ++; 3*R*-hydroxy-5*Z*, 8*Z*-tetradecadienoic acid, +++; 3-hydroxy-palmitic acid, ++; 3-hydroxy-myristic acid, ++; 3- hydroxy-lauric acid, +; 3- hydroxy-butyric acid, +; AA, -; linoleic acid, -; palmitoleic acid, -; oleic acid, -; stearic acid, -; palmitic acid, -; myristic acid, -; lauric acid, -. Hence in our study the immunoreactivity indicates solely the presence of 3-hydroxy-FA.

2.3.3 Immunofluorescence microscopy

Fixation and immunofluorescence of yeast cells were performed as described (Kilmartin and Adams, 1984; Harlow and Lane, 1988). Briefly, yeast cells were centrifuged at 900 r.p.m. for 5 min onto glass microscope slides using a Cytospin (Shandon, Germany) and fixed in acetone at room temperature. The slides were then treated with the antibody against 3-HETE (30 µl; 1:10 dilution v/v) and left for 30 min. After washing with BSA-PBS, the affinity purified FITC anti-rabbit IgG (Sigma, Germany) was added (30 µl; 1:10 dilution v/v). Following adequate washing, the slides were photographed using Kodak Gold Ultra 400 ASA film on an Olympus BX40 standard microscope equipped for epifluorescence with a 500 W high-pressure mercury lamp. The stained cells for immunofluorescence detection were compared with appropriate controls including preimmune sera from the rabbit.

The detection of 3-HETE and other 3-hydroxy-FA in protoplasts was accomplished by fixing the yeast cells (100 µl) from each culture in paraformaldehyde for 90 min at room temperature followed by stripping of the cell walls with β-glucuronidase and lyticase (Sigma, Germany) as described (Kilmartin and Adams, 1984). Protoplasts were then attached to ChemMate Capillary Gap Plus slides (DAKO biotek, USA) and fixed by methanol for 6 min and acetone for 30 s at –20 °C. The treatment with antibodies and detection was performed as described above.

3. Results and discussion

In earlier communications we described a sizeable formation of 3-HETE in *D. uninucleata* when fed with AA which is not present in this yeast (Kock *et al.*, 1997). Since this 3-HETE is now available in sufficient amounts, we raised an antibody against 3*R*-HETE in a rabbit and checked it for its specificity. Fortunately, the antibody exhibited substantial cross-reactivity against authentic 3-hydroxy-tetradecanoic acid. Owing to this observation it is reasonable to presume that it has also cross-reacted with those 3-hydroxy-FA which are derived from endogenously occurring polyenoic FA in *D. uninucleata*, in particular 3*R*-hydroxy-5*Z*, 8*Z*-tetradecadienoic acid that has been shown to be formed from linoleic acid (Venter *et al.*, 1997). The endogenous distribution of these 3-hydroxy-FA should be in relation to the composition of free polyenoic fatty acids in *D. uninucleata* in the order linoleic acid >> oleic acid ≈ α-linolenic acid (Kock *et al.*, 1997). However, when 5*Z*, 8*Z*, 11*Z* – eicosatrienoic, 5*Z*, 8*Z*, 11*Z*, 14*Z*-eicosatetraenoic (AA), 5*Z*, 8*Z*, 11*Z*, 14*Z*, 17*Z*-eicosapentaenoic (EPA), 11*Z*, 14*Z*, 17*Z*-eicosatrienoic or 9*Z*, 12*Z*-octadecadienoic (linoleic) acids were exogenously fed, *D. uninucleata* transformed the first 3 FA to their 3*R*-hydroxylated form without alteration of the chain length, whereas the last 2 FA were shortened by 6 and 4 carbon atoms to 3*R*-hydroxy-5*Z*, 8*Z*, 11*Z* –

tetradecatrienoic and 3*R*-hydroxy-5*Z*, 8*Z*–tetradecadienoic acids, respectively (Venter *et al.*, 1997).

By using this antibody we mapped by immunofluorescence microscopy the occurrence and localization of 3-hydroxy-FA in cells representing different stages of the life cycle of *D. uninucleata*. For these experiments we used fixed cells with cell walls from AA-fed non-synchronous cultures of the yeast (Fig. 1A-E). No significant fluorescence could be visualized in the vegetative hyphae (Fig. 1B). Marked fluorescence was however observed at the stage of initiation of the sexual phase, i.e. during gametangiogamy (Fig. 1C). Immunoreactive compounds were also visualized in immature asci (Fig. 1D) and were eventually released together with the ascospores (Fig. 1E, A). With protoplasts of the cells positive immunoreaction was also observed only in asci before (Fig. 1G) and after ascospore release (Fig. 1F) as well as between ascospores. This immunoreaction was however abrogated when *D. uninucleata* was grown in the presence of 1 mM acetylsalicylic acid (data not shown).

Identical results were obtained with cells grown in the absence of AA with the exception that the fluorescence intensities in the micrographs were diminished (data not shown). It must be, however, emphasized that this observation must not reflect a lower concentration of 3-hydroxy-FA under these conditions, but rather a relative low immunoaffinity of the antibody against the 3-hydroxy-FA formed from endogenous polyenoic fatty acids.

Our data show clearly that 3-hydroxy-FA are formed in *D. uninucleata* during the sexual phase of the reproductive cycle but not during the vegetative phase. The changing patterns of 3-hydroxy-FA localization during the yeast cell cycle provide some clues as to regulatory function of this family of oxylipins, which is further, supported by the specific suppression of the sexual phase by acetylsalicylic acid. A crucial role in reproduction biology of fungi has also been proposed for some other oxylipins, so-called psi (precocious sexual inducers) factors. They include 5*Z*, 8*R*-dihydroxy-octadeca-9*Z*, 12*Z*-dienoic acid (5, 8-DiHODE), 5*Z*, 8*R*-dihydroxy-octadeca-

9*Z*-(mono)enoic acid (5, 8-DiHOME), 8*R*-hydroxy-octadeca-9*Z*, 12*Z*-dienoic acid (8-HODE), 8*R*-hydroxy-octadeca-9*Z*-(mono)enoic acid (8-HOME), etc. (Champe *et al.*, 1987; Champe and El-Zayat, 1989; Mazur *et al.*, 1990; Mazur *et al.*, 1991). Since the yeast contains linoleic acid in abundance, its corresponding conversion product 3*R*-hydroxy-5*Z*, 8*Z*-tetradecadienoic acid was predominantly present when the yeast was grown in the absence of AA. In the presence of AA, however, 3-HETE may more or less substitute this compound. Hence, we conjecture that the 3*R*-hydroxy-5*Z*, 8*Z*-tetradecadienoic acid is the natural growth regulator in *D. uninucleata*. This assumption is firmly supported by the maximal cross-reactivity of the antibody with the 3*R*-hydroxy-5*Z*, 8*Z*-tetradecadienoic acid (see 2.3). The 3-hydroxy-FA are presumably formed during gametangiogamy and ascosporogenesis. Their absence in other stages may indicate a relatively high turnover rate. The prospective biological functions in the sexual reproductive phase of the life cycle of *D. uninucleata* remain to be elucidated in future studies. The availability of an effective immunofluorescence procedure presented here should greatly facilitate the study of this genetically tractable yeast.

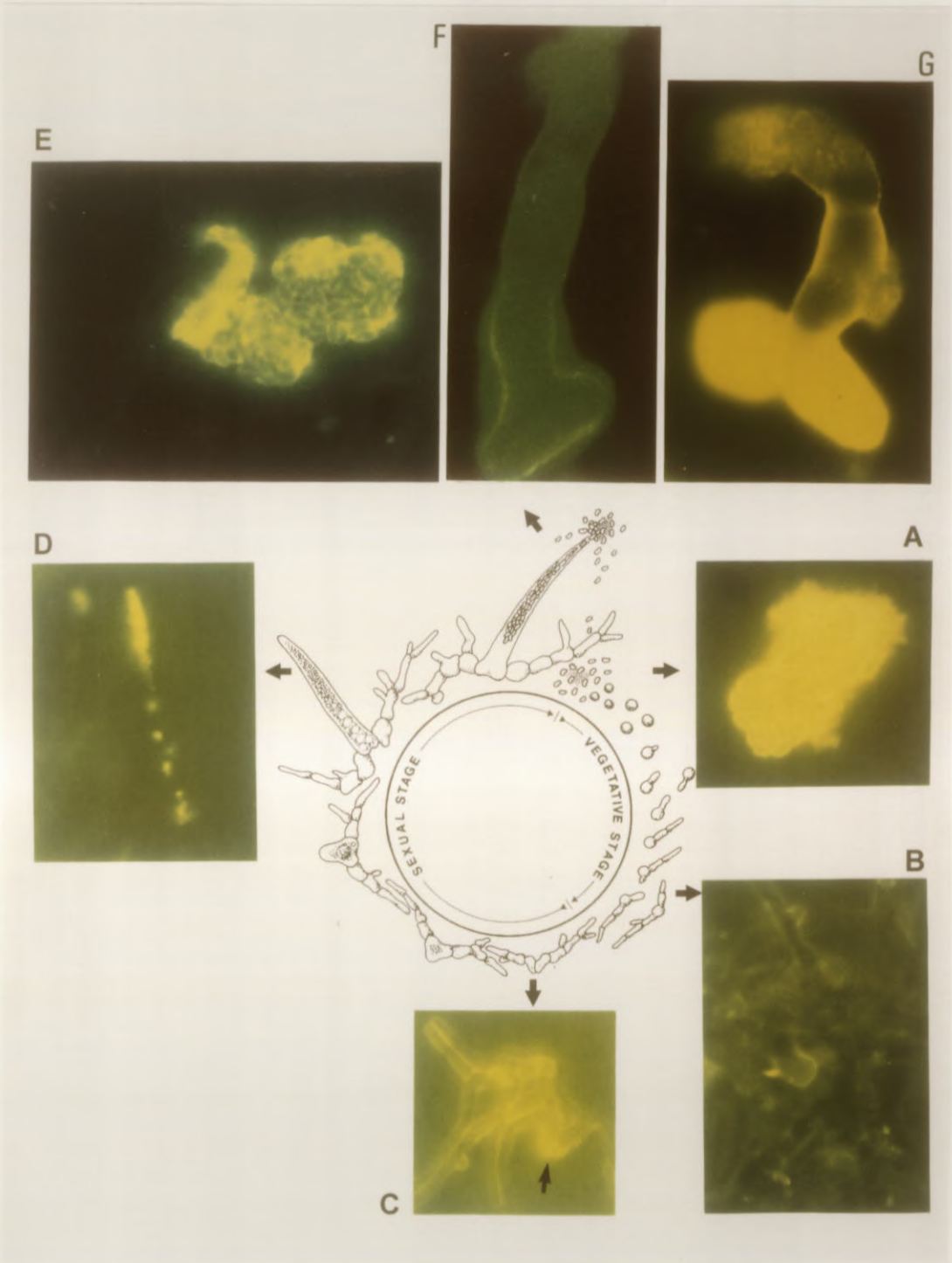


Figure 1. The life cycle of *Dipodascopsis uninucleata* and distribution of 3-HETE visualized through immunofluorescence mapping. A: liberated ascospores; B: hyphae with cell wall; C: gametangiogamy; D: young ascus with cell wall; E: liberated ascospores from ascus with cell wall; F: empty ascus protoplast: still with characteristic morphology i.e. base (bottom) and shaft; G: deformed mature ascus protoplast containing ascospores mainly at base. (A, E, F, G: 10 mm on photo = 10 μm cell size; B, C, D: 10 mm on photo = 25 μm cell size).

4. References

Bhatt, R.K., Falck, J.R. and Nigam, S. (1998). *Tetrahedron Lett* **39**, 249-252.

Biggs, R. (1937). *Mycologia* **29**, 34-44.

Botha, A. and Kock, J.L.F. (1993). *Antonie van Leewenhoek* **63**, 111-123.

Champe, S.P. and El-Zayat, A.E. (1989). *J Bacteriol* **17**, 3982-3988.

Champe, S.P., Rao, P. and Chang, A. (1987). *J Gen Microbiol* **133**, 1383-1387.

Gardner, H.W. (1991). *Biochim Biophys Acta* **1024**, 221-239.

Harlow, E. and Lane, D. (1988). In : *Antibodies: A laboratory Manual* **Chapter 10**. Edited by E. Harlow and D. Lane. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Hosoda, H., Kawamura, N. and Nambara, T. (1981). *Chem Pharm Bull* **29**, 1969-1974.

Kilmartin, J.V. and Adams, A.E.M. (1984). *J Cell Biol* **98**, 922-933.

Kock, J.L.F., Jansen van Vuuren, D., Botha, A., Van Dyk, M.S., Coetzée, D.J., Botes, P.J., Shaw, N., Friend, J., Ratledge, C., Roberts, A.D. and Nigam, S. (1997). *System Appl Microbiol* **20**, 39-49.

Mazur, P., Meyers, H.V., Nakanishi, K., El-Zayat, A.E. and Champe, S.P. (1990). *Tetrahedron Lett* **31**, 3837-3840.

Mazur, P., Nakanishi, K., El-Zayat, A.E. and Champe, S.P. (1991). *J Chem Soc Chem Commun* **20,** 1486-1487.

Nomura, T. and Ogata, O. (1976). *Biochim Biophys Acta* **431**, 127-131.

Schweizer, E. (1989). In : *Microbial Lipids* **Vol 2**, pp. 3-50. Edited by C. Ratledge and S.G. Wilkinson. California Academic Press Limited, San Diego.

Van Dyk, M.S., Kock, J.L.F. and Botha, A. (1994). *World J Microbiol Biotech* **10**, 495-504.

Van Dyk, M.S., Kock, J.L.F., Coetzee, D.J., Augustyn, O.P.H. and Nigam, S. (1991). *FEBS Lett* **283(2)**, 195-198.

Venter, P., Kock, J.L.F., Sravan Kumar, G., Botha, A., Coetzee, D.J., Botes, P.J., Bhatt, R.K., Falck, J.R., Schewe, T. and Nigam, S. (1997). *Lipids* **32,** 1277-1283.

Zsolt, J. (1963). *Acta Bot Acad Sci Hung* **9**, 217-226.

CHAPTER 4

A novel oxylipin-associated binding phenomenon in yeast flocculation

This work has been submitted for publication in *Antonie van Leeuwenhoek*.

Abstract

Research on the distribution of oxylipins (3-hydroxy fatty acids) in flocculant strains of the yeast *Saccharomyces cerevisiae* led to the uncovering of a novel binding phenomenon observed during lectin-mediated aggregation. We found that oxylipins such as 3-hydroxy 8:0 and 3-hydroxy 10:0 are produced during the growth cycle of the flocculating yeast *Saccharomyces cerevisiae* ATCC 26602. Using oxylipin specific antibodies in immunofluorescence microscopic studies, we uncovered that these compounds are synthesized continuously from an early stage of growth, are associated with the cell wall and are present between flocculating cells. Similar results were obtained with the NewFlo phenotype flocculent brewing yeast strain *Saccharomyces cerevisiae* NCYC 1364. This implicated the involvement of oxylipins in cell aggregation. Further investigations using scanning and transmission electronmicroscopy, indicated that changes in the depositing of osmiophilic layers in the yeast followed the same pattern as the immunofluorescence results. The presence of oxylipins in the osmiophilic layer was finally demonstrated using immunogold labeling. We found that intracellular oxylipin-containing osmiophilic layers migrate through yeast cell walls in a "ghostlike" fashion without visually affecting the cell wall structure or the layers. This migration resulted in the binding of

these layers to cell walls of adjacent cells. Our results also implicate a similar mechanism in other distantly related fungi.

1. Introduction

Yeast flocculation is of prime importance to the brewing industry in order to effectively separate yeasts and thereby producing a desired product (Calleja, 1987). In brewing yeasts, lectins are synthesized continuously from an early stage of growth and are rapidly inserted into the cell wall where they remain inactive before being activated at flocculation onset (Stratford, 1993). This activation mechanism is however still unknown.

It is interesting to note that, Straver *et al.,* (1993) reported that cell surface hydrophobicity is a major determinant for yeast cells to become flocculant. This is in accordance with the results obtained in this study. Here, we report on a novel lipid (oxylipin)-associated binding phenomenon observed during the flocculation of yeasts.

2. Experimental procedures

2.1 Strains used

Saccharomyces cerevisiae ATCC 26602, a known flocculant, (Shieh and Chen, 1986) and NCYC 1364, a NewFlo phenotype (Stratford, 1993), were used in this study and are held at the American Type Culture Collection (ATCC), Rockville, Maryland, USA and the National Collection for Yeast Cultures (NCYC), Norwich, UK, respectively. In

addition, *Mucor genevensis* MUFS 038, held at the University of the Orange Free State, Bloemfontein, South Africa, was investigated.

2.2 Cultivation and oxylipin analysis

Saccharomyces cerevisiae ATCC 26602 was cultivated as described elsewhere (*Growth experiment*) until flocculation occurred. Oxylipins were extracted by using 2x volume (200 ml) of ethyl acetate followed by evaporation according to the method of Nigam (1987). Extracts were derivatised and subjected to gas chromatography-mass spectrometry analysis (Venter *et al.*, 1997). This procedure was repeated on YM (yeast-malt) broth alone without yeast growth.

2.3 Growth experiments

Saccharomyces cerevisiae ATCC 26602 was inoculated from an agar slant into 500 ml conical flasks containing 200 ml YM broth (Difco). The culture was incubated at 30 ^{0}C while shaking (160 rpm) for 20 h. Growth was measured at regular intervals by measuring changes in optical density using a Klett Summerson colorimeter (red filter). After 11 h of growth, non-flocculant cultures were boiled (Stratford, 1993) (heat experiment) and the flocculation rate determined by measuring the optical density over time. This was compared to an 11 h untreated culture (heat control). After 20 h of growth under normal cultivation procedures, significant flocculation occurred and was measured as before (normal). All experiments were performed at least twice and similar trends were observed. *S. cerevisiae* NCYC 1364 (a NewFlo phenotype flocculant) was cultivated and analysed in a similar way. Material from the heat control and heat experiment as well as normal flocculating cells were sampled for immediate immunofluorescence and electronmicroscopy analysis.

Cultivation procedures for *Mucor genevensis MUFS 038* were followed as described by Pohl *et al.*, (1998) without the addition of fatty acids. Material was subjected to immediate immunofluorescence analysis.

2.4 Immunofluorescence microscopy

Synthesis of 3-hydroxy fatty acid: These oxylipins were first synthesized for antibody preparation. The synthetic strategy for the production of 3*R*- and 3*S*-hydroxy fatty acids (i.e. 3-hydroxy- 5, 8, 11, 14- eicosatetraenoic acid or 3-HETE), involved a convergent approach coupling chiral aldehyde with Wittig salt: these were derived from 2-deoxy-D-ribose and arachidonic acid (AA), respectively (Bhatt *et al.*, 1998).

Preparation and characterization of antibody: This was done as described by Kock *et al.*, (1998). Briefly, antibodies against chemically synthesized 3-HETE were raised in rabbits and then characterized by determining its titer, sensitivity and specificity.

Microscopy: Immunofluorescence of fungal cells was performed as described (Kock *et al.*, 1998). In order to maintain aggregated cell floc structure and *Mucor* sporangia, antibody, fluorescence and wash treatments were performed in 2 ml plastic tubes. Following adequate washing, the slides with fluorescing material were photographed using Kodak Gold Ultra 200 ASA film on a Zeiss Axioskop microscope equipped for epifluorescence with a 50 W high-pressure mercury lamp. The stained cells were compared with appropriate controls as described (Kock *et al.*, 1998).

2.5 Electron microscopy

Material for electron microscopy was chemically fixed (gluteraldehyde and osmiumtetroxide) (Van Wyk and Wingfield, 1991). Electronmicrographs were taken with a Jeol 6400 WINSEM (Japan) and a Philips CM 100 (The Netherlands) TEM.

2.6 Immunogold labeling

The pre-treated ultra thin sections for electron microscopy were labeled as described by Stirling (1990). All steps were performed in a humidified chamber. Firstly, sections on nickel grids were floated on a 100 µl drop of a saturated aqueous solution of sodium metaperiodate (room temperature, 1 h) for de-osmification. The grids were then transferred to a 100 µl drop of 1 % BSA in PBS for 10 minutes to block background labeling. After blocking, the grids were transferred to a 20 µl drop of a 1:10 primary antibody dilution (Kock *et al.*, 1998) at 4 °C overnight. Following adequate washing in PBS, the sections were blocked again for 5 minutes and transferred to a 1:10 dilution of the gold probe at room temperature for 1 h. Subsequent washings in PBS and deionised water were followed by staining of the sections with uranyl acetate and lead citrate. Electron micrographs were taken with a Philips CM 100 (The Netherlands) TEM.

3. Results and discussion

We found that oxylipins such as 3-hydroxy 8:0 and 3-hydroxy 10:0 are produced over the growth cycle of the flocculating yeast *Saccharomyces cerevisiae* ATCC 26602 when grown in complex medium (Table 1). This group of novel oxylipins was previously isolated through ethanol extraction followed by purification using C18 and silica gel columns. The extracts were finally derivatised and analysed by gas chromatography mass-spectrometry (Venter *et al.*, 1997).

In order to determine the distribution of these lipids in this yeast, we used specific 3-hydroxy-oxylipin antibodies in an immunofluorescence microscopic investigation. Antibodies were raised in rabbits against chemically synthesized 3*R*-hydroxy- 5Z, 8Z, 11Z, 14Z -eicosatetraenoic acid (3-HETE). Testing for specificity showed that

antibodies react with 3-hydroxy fatty acids in general but not with other oxylipins or free fatty acids (Kock *et al.,* 1998). We found that these oxylipins are synthesized continuously from an early stage of growth and are associated with the cell wall. When flocculation was induced under natural conditions or by heat treatment (Stratford, 1993) (Fig. 1A, B), the oxylipins differentiated into localized fluorescing protuberances (Fig. 1D) on the cell wall and were also present between the flocculating cells. This implicated the involvement of these compounds in cell aggregation. In contrast, no fluorescence was detected on the daughter (developing) cells (Fig. 1C) and the budscars formed when daughter cells became detached from the mother cells (Kurtzman and Fell, 1998) (Fig. 1C). Similar results were obtained with the NewFlo phenotype flocculent brewing yeast strain *Saccharomyces cerevisiae* NCYC 1364 when grown under similar conditions (results not shown).

Table 1. Oxylipins found in *Saccharomyces cerevisiae* ATCC 26602.

Metabolite	Fragments	Media
3-OH 8:0	175; 246 [M^+]; 231 [M^+-15(CH_3)]	A
3-OH 10:0	175; 259 [M^+-15(CH_3)]	A
3-OH 12:1	175; 285 [M^+-15(CH_3)]	B

A - yeasts cultivated in complex medium i.e. yeast malt (YM) broth; B - complex medium i.e. yeast malt (YM) broth analysed without yeast growth.

These results prompted us to further investigate the distribution of 3-hydroxy fatty acids in *Saccharomyces cerevisiae ATCC 26602.* For this purpose, transmission electronmicroscopy (TEM) was performed on osmium tetroxide fixed yeast samples. Osmium tetroxide is known to stain fatty acids electron dense during fixation for

easier detection (DeRobertis and DeRobertis, 1980). To our surprise, changes in the depositing of osmiophilic layers in the yeast followed the same pattern as the immunofluorescence results (Fig. 2). No osmiophilic layers could be detected in young developing cells (Fig. 2C). In the more developed growing cells, the osmiophilic layers started to appear and were closely associated and overlapped to a limited extent with the cell walls - probably enough to cause fluorescence across the cell surface (Fig. 2D, E). At the start of flocculation, these layers became detached (layers move to the inside of cells) while protuberances were formed which crossed the cell wall (Fig. 2F) to "reach" for other adjacent cells in order to attach to their cell walls, probably through lectin-sugar interbridging (Fig. 2G). This could explain the fluorescing protuberances and the decrease in fluorescence on other parts of the flocculating yeast (Fig. 1D). Interestingly, the osmiophilic layers crossed the cell walls without visually disturbing the cell wall or layer structure. Similar protubing knobs could be observed on the cell surfaces of some mature flocculating cells when viewed with scanning electron microscopy (SEM) (Fig. 2B), while the cell walls of non-flocculating growing yeasts were smooth (Fig. 2A). The possibility of these knobs to be similar to the fluorescing protuberances is under investigation. In order to further verify the nature of the osmiophilic layer, the *in situ* localization of 3-hydroxy oxylipins in *Saccharomyces cerevisiae* ATCC 26602 were mapped using immunogold labeling. The gold particles were mainly concentrated on the osmiophilic layer (Fig. 3A), as well as the corresponding protubing osmiophilic layer (Fig. 3B). Since the primary antibody is specific for 3-hydroxy fatty acids, it can be concluded that the osmiophilic layer contains these compounds. These results thus coincide with that obtained by immunofluorescence microscopy.

It is subsequently hypothesized that oxylipins may play an important role in the maintenance of the integrity and the "ghostlike" movement of these osmiophilic layers through the cell wall pores (Robinow and Johnson, 1991), probably by means of entropic based hydrophobic forces (Rudolph, 1994). These layers may also contain the activated lectins necessary for binding to the cell walls of adjacent cells. These

results support the hypothesis that the cell surface hydrophobicity is a major determinant for yeast cell flocculation (Straver *et al.*, 1993).

The oxylipin 3-hydroxy 14:2 has been found in a distantly related fungus, i.e. *Mucor genevensis,* by using gas chromatographic mass-spectrometry analysis as described before (Pohl *et al.*, 1998). We have demonstrated, using immunofluorescence microscopy, that this oxylipin is associated with cell surfaces of aggregating reproductive structures of this fungus (Alexopoulos and Mims, 1979) (Fig. 4). Oxylipins, and presumably also the accompanying "ghosting" phenomenon, may be widely distributed in fungi and other organisms. Its association with cell aggregation is of interest and should be further investigated in these and other eukaryotic and bacterial taxa. The influence of different oxylipins on yeast flocculation is under investigation.

Figs. 1-4 and their legends are given on the following pages 74-81.

LEGENDS TO FIGURE 1

Changes in oxylipin formation over the growth cycle of *Saccharomyces cerevisiae* ATCC 26602. A, Growth curve with flocculation onset after 20 h. B, Flocculation curves of normally flocculating cells (♦), heat induced flocculation (■) and corresponding cells not heated (▲). C, Oxylipin specific immunofluorescing micrographs of yeast cells in exponential growth showing fluorescing mature cells (FMC), non-fluorescing buds or young cells (NFB) and non-fluorescing budscars (NBS). D, Flocculating yeast cells after 20 h of growth. Oxylipin specific fluorescing protuberances are observed on cell surfaces (FP). Scale bar, 5μm in (C) and (D).

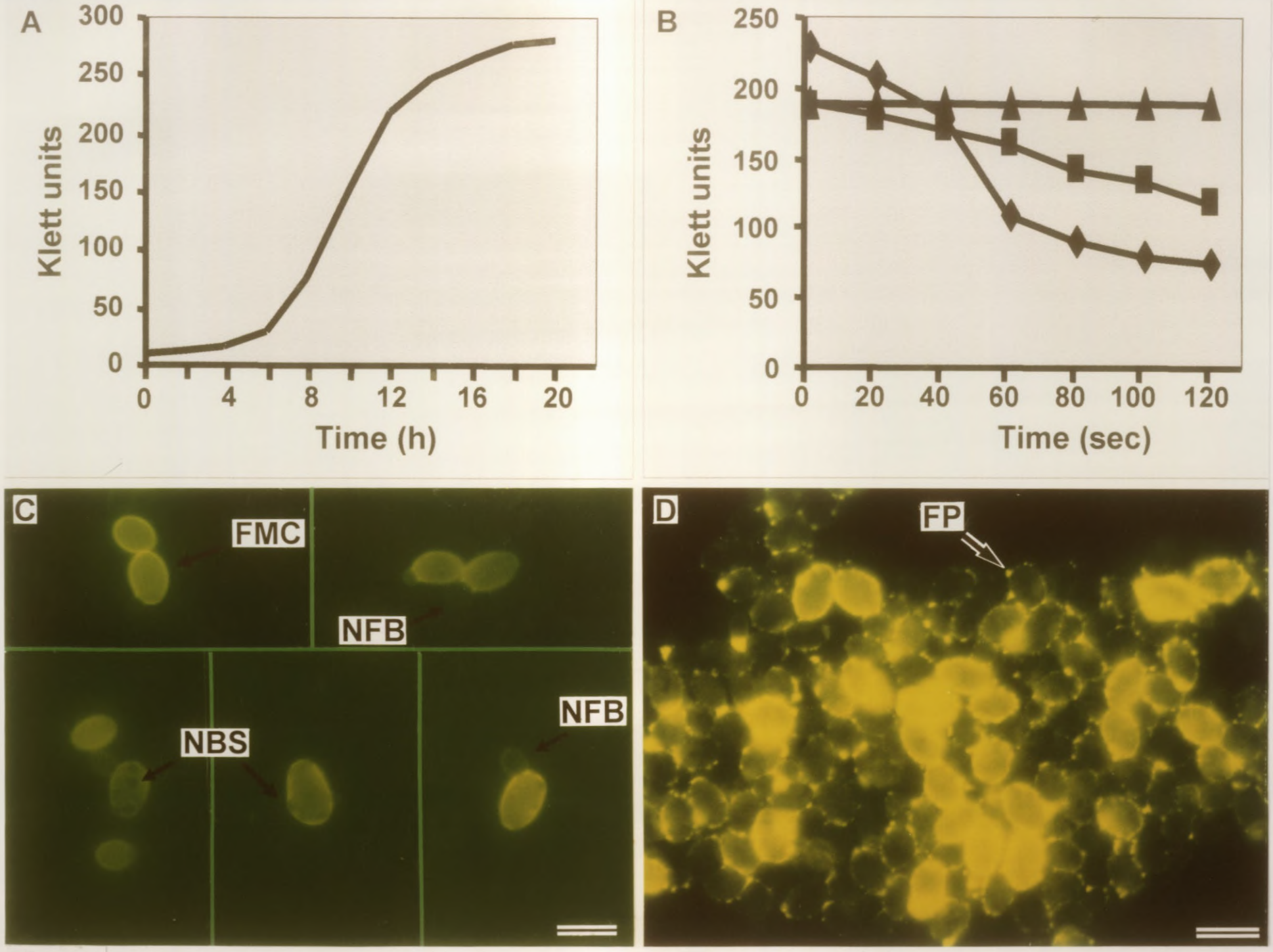

A
Klett units
300
250
200
150
100
50
0
0
4
8
12
16
20
Time (h)
B
Klett units
250
200
150
100
50
0
0
20
40
60
80
100
120
Time (sec)
C
FMC
NFB
NBS
NFB
D
FP

Figure. 1

LEGENDS TO FIGURE 2

Changes in cell wall protuberances as well as osmiophilic layer formation over the growth cycle of *Saccharomyces cerevisiae* ATCC 26602. A, A SEM of actively growing, non-flocculating cells. B, SEM photomicrographs showing protuberances (PR) on surface of mature yeast cells. C, TEM micrograph of immature young yeast cells indicating lack of osmiophilic layer and characteristic double layered cell walls (DCW). D, TEM micrograph indicating osmiophilic layer (OL) with budscar (BS). E, TEM micrograph of cell wall with closely associated overlapping osmiophilic layer. F, TEM micrograph indicating protubing osmiophilic layer (POL). G, TEM micrograph showing adhesion (AD) of POL to cell wall of neighboring cell. Scale bar, 5μm in (A) and (B); 2 μm in (F); 1.5 μm in (D); 1 μm in (E) and (G); 0.5 μm in (C).

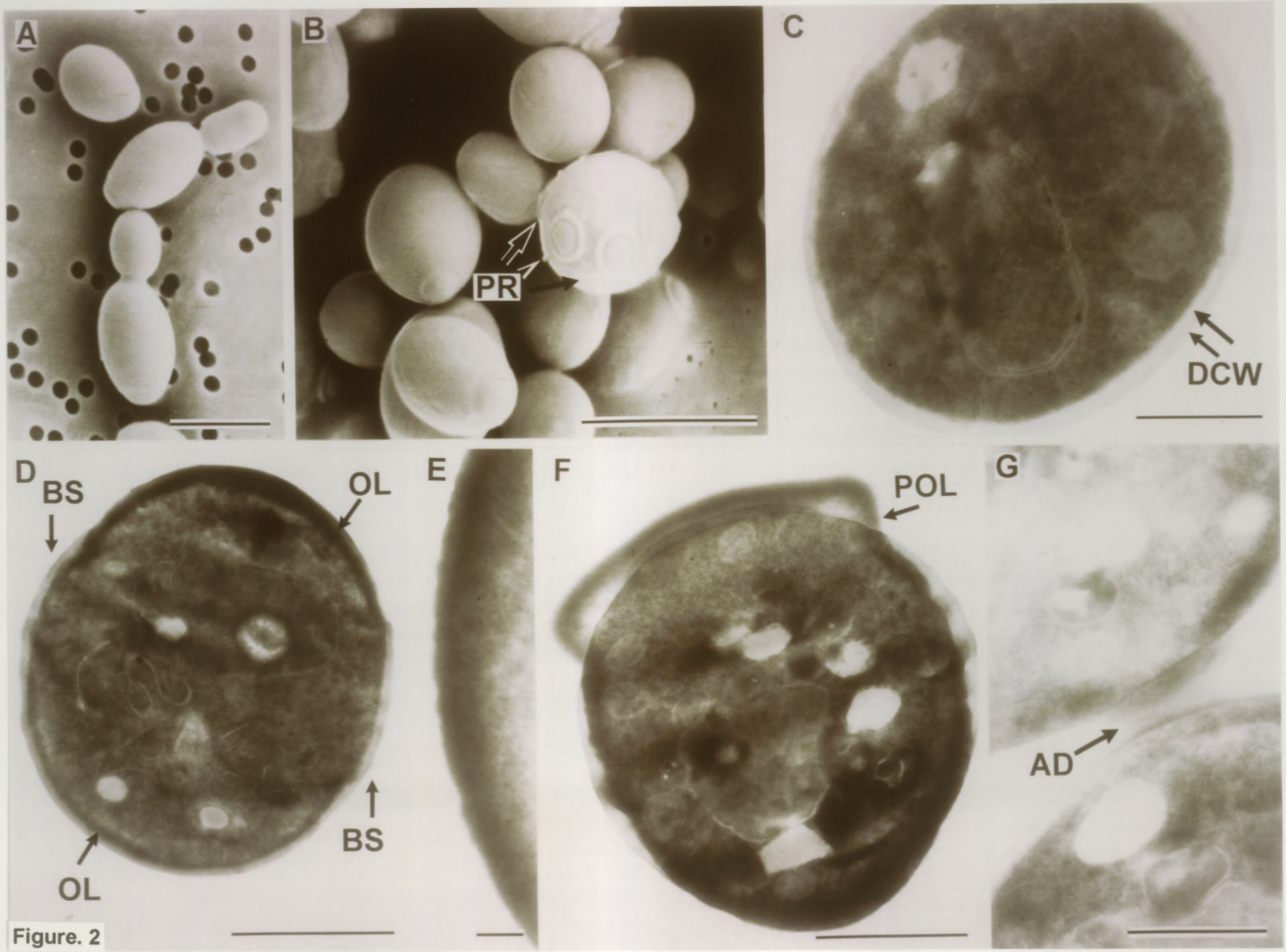


Figure. 2

LEGENDS TO FIGURE 3

Immunogold localization of 3-hydroxy fatty acids in *Saccharomyces cerevisiae* ATCC 26602. A, TEM micrograph indicating the osmiophilic layer (OL), clearly gold-labeled (GL) for 3-hydroxy fatty acids. B, TEM micrograph indicating protubing osmiophilic layer (POL), with similar labeling. Scale bar, 0.5μm in (A) and 0.1μm in (B).

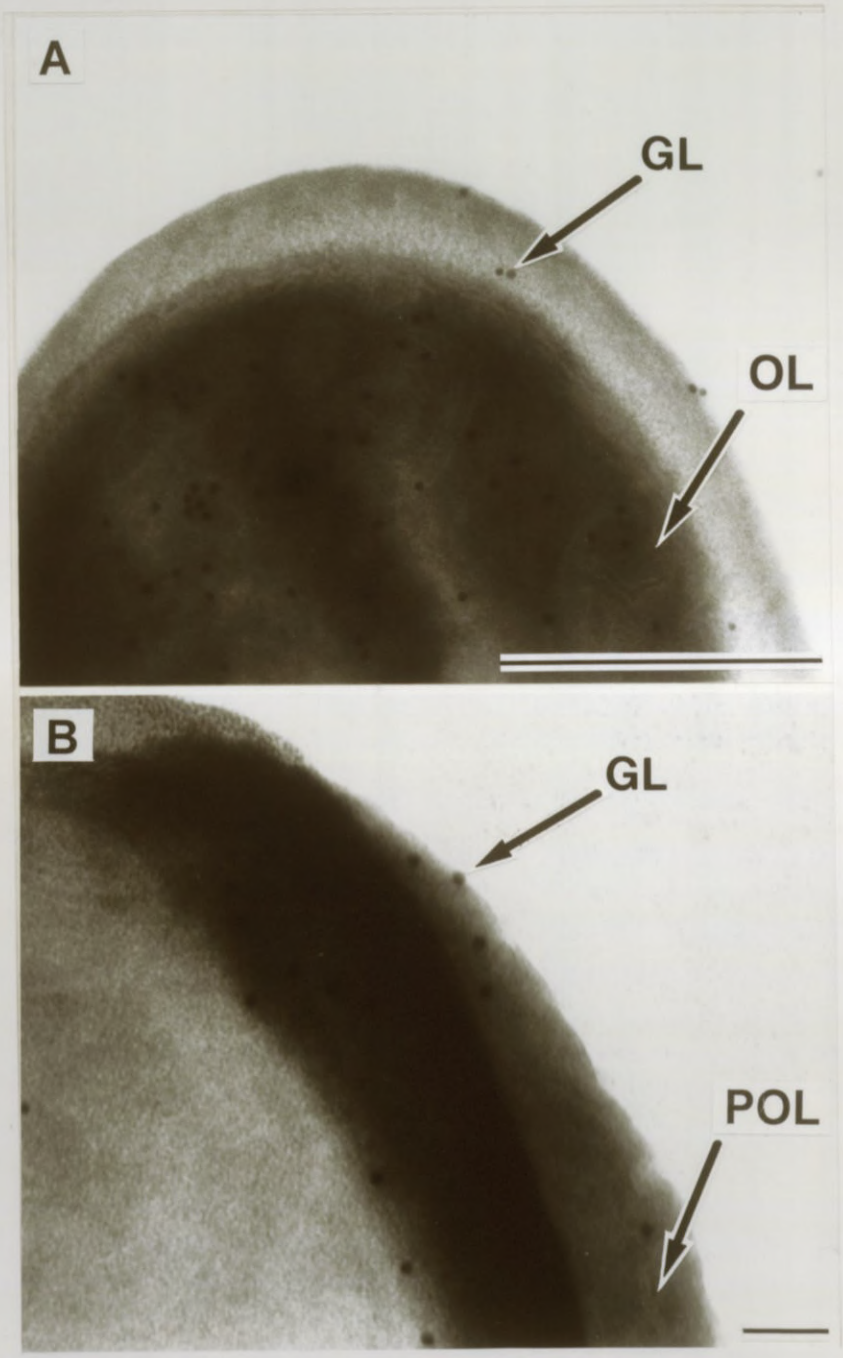


Figure. 3

LEGENDS TO FIGURE 4

Immunofluorescing micrographs of the zygomycetous fungus *Mucor genevensis* MUFS 038 showing fluorescing sporangium (SP), collumella (COL) and sporangiospores (S). Scale bar, 5 μm.

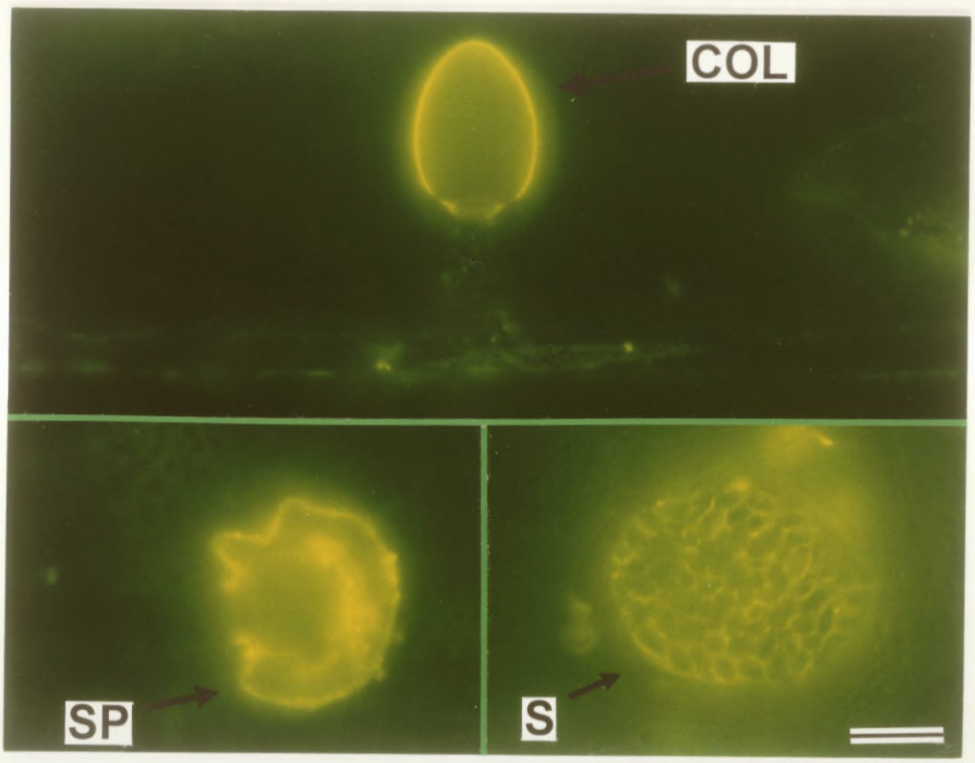


Figure. 4

4. References

Alexopoulos, C.J. and Mims, C.W. (1979). In: *Introductory Mycology,* **3rd Ed.**, pp. 191-228. Edited by C.J. Alexopoulos and C.W. Mims. John Wiley & Sons, New York, USA.

Bhatt, R. K., Falck, J. R. and Nigam, S. (1998). *Tetrahedron Lett* **39**, 249-252.

Calleja, C.B. (1987). In: *The Yeasts* **Vol 2**, pp. 165-238. Edited by A. H. Rose and J.S. Harrison. Academic Press Inc. Ltd., London, UK.

DeRobertis, E.D.P. and DeRobertis, E.M.F. (1980). In: *Cell and molecular Biology*, pp. 673-674. Edited by E.D.P. DeRobertis and E.M.F. DeRobertis. Saunders College, Philadelphia, USA.

Kock, J.L.F., Venter, P., Linke, D., Schewe, T. and Nigam, S. (1998). *FEBS Lett* **427**, 345-348.

Kurtzman, C.P. and Fell, J.W. (1998). In: *The Yeasts,* pp. 33-44. Edited by C.P. Kurtzman, J.W. Fell. Elsevier Science Publishers, Amsterdam, The Netherlands.

Nigam, S. (1987). In: *Prostaglandins and Related Compounds,* pp. 55-73. Edited by C. Benedetto, R.G. McDonald-Gibson, S. Nigam and T.F. Slater. IRL Press, Oxford, United Kingdom.

Pohl, C.H., Botha, A., Kock, J.L.F., Coetzee, D.J., Botes, P.J., Schewe, T. and Nigam, S. (1998). *Biochem Biophys Res Com* **253**, 703-706.

Robinow, C.F. and Johnson, B.F. (1991). In *The Yeasts,* **Vol 4**, pp. 18-25. Edited by A. H. Rose and J.S. Harrison. Academic Press Inc. Ltd., London, UK.

Rudolph, A.S. (1994).*J Cell Biochem* **56**, 183-187.

Shieh, W-J. and Chen, L-F. (1986). *Appl Microbiol Biotechnol* **25**, 232-237.

Stirling, J.W. (1990). *J Histochem Cytochem* **38**, 145-157.

Stratford, M. (1993). *Yeast* **9**, 85-94.

Straver, M.H., VD Aar, P.C., Smit, G. and Kijne, J.W. (1993). *Yeast* **9**, 527-532.

Van Wyk, P.W.J. and Wingfield, M.J. (1991). *Mycologia* **83**, 698-707.

Venter, P., Kock, J.L.F., Sravan Kumar, G., Botha, A., Coetzee, D.J., Botes, P.J., Bhatt, R.K., Falck, J.R., Schewe, T. and Nigam, S. (1997). *Lipids* **32**, 1277-1283.

CHAPTER 5

ASPIRIN INFLUENCES LIPID-A COMPOSITION IN THE LIPOPOLYSACCHARIDE LAYER OF GRAM-NEGATIVE BACTERIA: IMPLICATIONS FOR THERAPY OF ENDOTOXEMIA

This work has been submitted for publication in *SAJS*

Abstract

Endotoxins from Gram-negative bacteria are macromolecular amphiphilic lipopolysaccharides (LPS), located exclusively in the outer leaflet of the outer bacterial membrane. The Lipid A component of the LPS, which expresses as free Lipid A all the toxic *in vivo* activities of LPS, represents the endotoxic principle of LPS and is specifically responsible for severe infections, generalized inflammation and pathophysiological disorders in humans. Cellular responses to LPS include the generation and release of mediators like interleukin-1, interleukin-6 and tumour necrosis factor alpha which in turn cause hypotention, ischemia, vascular injury etc. Treatment of endotoxemia includes generally removal of the source of infection, hemodynamic and respiratory support and therapy with antimicrobial agents. On the basis of our previous observations that aspirin inhibited the biosynthesis of 3-hydroxylated arachidonic acid produced by the yeast *Dipodascopsis uninucleata* we

report in the present study that aspirin at moderate concentrations influence the biosynthesis of 3-hydroxylated myristic acid in Lipid A of Gram-negative bacteria. Our results therefore suggest an important role for aspirin as a therapeutic agent in the treatment of LPS mediated diseases.

1. Introduction

Endotoxins from Gram-negative bacteria are macromolecular amphiphilic lipopolysaccharides (LPS), located exclusively in the outer bacterial membrane (Raetz, 1990; Rietchel *et al.,* 1996). These compounds are responsible for severe infections, generalized inflammation and pathophysiological disorders in humans, such as sepsis, Disseminated Intravascular Coagulation (DIC) - syndrome and circulatory shock, which may, if not urgently treated, lead to death (Parrilo, 1990). Moreover, LPS stimulate host cells to generate mediators like interleukin-1, interleukin-6 and tumour necrosis factor alpha (Wright *et al.,* 1990), which in turn initiate the transmembrane signaling leading to cell activation.

For several bacterial strains the detailed chemical structures have been established, thus describing the 3 main regions: (1) the inside lipid part, which serves as an anchor for LPS in the outer membrane and constitutes the toxophore group – Lipid A, (2) the middle core oligosaccharide, predominantly presented at the cell surface and (3) the outer O-specific chain, which is attached to the core oligosaccharide via the Gal $\alpha(1\rightarrow4)$GlcII linkage. In most enterobacterial strains, the Lipid A component has been

shown to exert endotoxic activities, whereby the number, position and nature of fatty acids are important for the expression of biological activity (Holst *et al.*, 1993). The Lipid A in *Escherichia coli*, for instance, contains (*D*)-gluco-configurated pyranosidic hexosamine residues, which are present as β(1→6) - linked homo - or heterodimer (Rietchel *et al.*, 1996). The disaccharide carries four 3(*R*)-hydroxy fatty acids, two of which are acylated at their hydroxyl group by non-hydroxylated fatty acids. In *E. coli* the 3-hydroxy fatty acids are all 3-hydroxytetradecanoic acid. Strikingly, 3-hydroxylated fatty acids in Lipid A shows structural similarity with the 3-hydroxy arachidonic acid, so called 3-HETE, which was isolated from *Dipodascopsis uninucleata* and characterized by us previously (Van Dyk *et al.*, 1991). Since the synthesis of 3-HETE in yeast culture could be inhibited by aspirin (van Dyk *et al.*, 1991), it was of interest to investigate if aspirin could also in general inhibit the Lipid A biosynthesis in Gram-negative bacteria. In the present study, we report that aspirin at physiological concentration inhibits the synthesis of Lipid A and thus of LPS in *E. coli*.

2. Experimental procedures

2.1 Cultivation of E. coli

Lyophilized bacterial culture of *E. coli* UOFS Y2 (generously provided by Dr. Karl-Heinz Riedel from the UNESCO-MIRCEN bacterial culture collection at the University of the Orange Free State) was grown in pure culture in 500 ml conical flasks containing 100 ml medium. The medium contained in g/l: TSB, 30; $MgSO_4$, 0.25; K_2HPO_4, 10.

Experimental flasks contained 1 mM acetylsalicylic acid (aspirin - Sigma) diluted in a small volume of ethanol and the control flasks contained the same amount of ethanol without aspirin. Three day old cultures grown on TSB-agar plates at 37 °C were inoculated into liquid medium and cells incubated for 16h at 37 °C until late logarithmic growth phase. Experiments were repeated 7 times.

2.2 Extraction

Cells were harvested by centrifugation at 10 000 g for 15 min and washed thoroughly with 0.02 M Tris (pH 8). Extractions were performed according to the method proposed by Nichols (1994). Briefly, a small amount of cells (0.3 g) were transferred to Scott tubes (20 ml - 120 mm X 12 mm) and suspended by the addition of 0.5 ml distilled H_2O. In addition, 2 ml of $CHCl_3$-methanol (1:2 vol/vol) were added to each sample, and the samples were vortexed. After standing for 2 h, 0.75 ml of $CHCl_3$ and 0.75 ml of 0.5 M K_2HPO_4 - 2 M KCl were added to each sample. Samples were vortexed, and the lower $CHCl_3$ phase (lipid phase) was removed and dried under N_2 gas. The aqueous phase, expected to contain LPS, was retained, and the hydroxy fatty acid content was then analysed as described below.

Alkaline extraction was performed on samples by the addition of 4 N KOH (0.5 ml, 100 °C, 90 min) and the hydrolysate acidified with concentrated HCl. The acidified samples were then extracted with $CHCl_3$ (1 ml, three times) and dried under N_2 gas. Lauric acid (12:0, internal standard; Sigma) was added to each sample of the bacterial extract.

2.3 Lipid analysis by gas chromatography - mass spectrometry (GC-MS)

This was performed according to the method proposed by Kock *et al.,* (1997). Dried samples were methylated using diazomethane and then silylated using O-bis (trimethylsilyl) trifluoroacetamide with kits from Aldrich (Deisenhofen, Germany). The samples were dissolved in 200 µl $CHCl_3$/hexane (1:4) for GC-MS analysis.

GC-MS analysis was carried out on a Hewlett Packard 5890 gas chromatograph equipped with a Hewlett Packard 5972 MSD. GC conditions: Column; HP-1 30 m fused silica capillary, 0.25 mm i.d. 0.25 µm coating. Carriergas, He at 154 psi head pressure. Temperature Program: Injector 230 °C, Initial column temperature 140 °C, Final column temperature 300 °C, Ramp rate 5 °C min^{-1}, Final time 20 min. Injection; Samples were dissolved in 200 µl $CHCl_3$/hexane (1:4) and 0.5 µl injected with a 50:1 split. Mass Spectrometer Parameters: EMV 1447, AMU Gain 619, Scan 50 to 650 AMU, GC-MS interface temperature 280 °C.

3. Results and discussion

The multiplication, death as well as lysis of bacteria set LPS free from the cell surface. LPS were collected from bacterial cell cultures and the fatty acids in Lipid A extracted as described above (Nichols, 1994). After preparing derivatives, the fatty acids were

determined by GC-MS. For quantitative evaluation, an internal standard, lauric acid (12:0), was added to the extracts.

Fig. 1 shows the mass spectrum of 3-hydroxy myristic acid, a component of Lipid A from *E. coli* with M-15 (315) as a prominent peak. The amount as quantitated from 7 repetitions was 831.4 ± 10 ng/g wet cells. Upon addition of 1 mM aspirin to cultures of *E. coli,* an average decrease of 63.7% ± 19.6% in the synthesis of 3-hydroxy myristic acid was observed (Fig. 1B).

This effect of aspirin is surprising, since no evidence has yet been found for the presence of cyclooxygenases in either *Dipodascopsis uninucleata* or in *E. coli* (Kock *et al.*, 1997). Inasmuch as salicylic acid also inhibits the formation of 3-hydroxy fatty acids (van Dyk *et al.,* 1991), it can be conjectured that the level of salicylic acid might be essential for the inhibition of Lipid A synthesis. Salicylic acid has also been shown to be essential for resistance of many immunized plants against a broad spectrum of pathogenic viruses and fungi (Slusarenko, 1996). We believe that the hydroxylation of fatty acids is a consequence of incomplete (leaky) β-oxidation, which involves free radical reaction (Venter *et al.,* 1997). The salicylic acid and aspirin thus function as radical scavengers and consequently as potent inhibitors of 3-hydroxylation of fatty acids. For the growing and multiplying bacteria, the minimal LPS structure consist of one 2-keto-3-deoxyoctonoic acid (Kdo)-residue linked to Lipid A (Holst *et al.,* 1993). How far the biosynthesis of Kdo and its incorporation into LPS are affected by aspirin is presently under investigation.

In conclusion, we have demonstrated that aspirin can almost completely inhibit the synthesis of Lipid A, which shares the common structural element in all endotoxins from Gram-negative bacteria. Aspirin may therefore represent the most suitable drug for the therapy of patients suffering from life-threatening endotoxemia. However, we have yet to transfer our *in vitro* observations in bacterial cell culture to animal models and also to humans, for which trails are under consideration. In addition, the effect of aspirin on 3-hydroxy fatty acid production in the LPS of other bacterial strains of *E. coli* and other Gram-negative bacterial species, will be investigated.

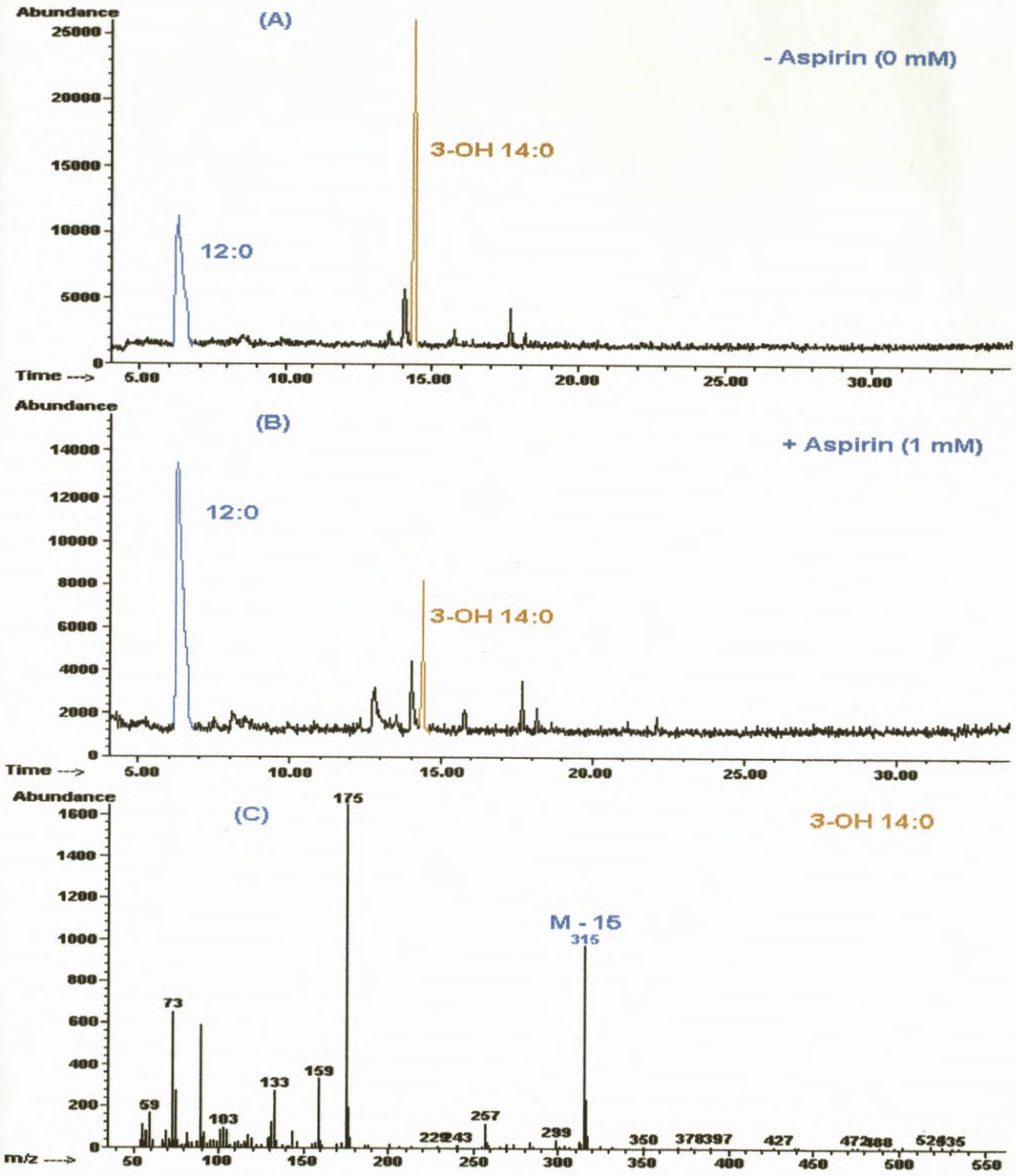


Figure 1. The inhibitory effect of aspirin on the synthesis of Lipid A as demonstrated by the production of 3-hydroxy myristic acid. Lauric acid was used as an internal standard. GC-MS chromatogram represents one single recording out of 3 similar experiments. (A) Generation of 3-hydroxy myristic acid in absence of aspirin; (B) Generation of 3-hydroxy myristic acid in the presence of 1 mM aspirin; (C) Mass spectrum of the 3-hydroxy myristic acid with M-15 as the prominent peak.

4. References

Holst, O., Muller-Loennies, S., Lindner, B. and Brade, H. (1993). *Eur J Biochem* **214,** 695-701.

Kock, J.L.F., Jansen van Vuuren, D., Botha, A., Van Dyk, M.S., Coetzee, D.J., Botes, P.J., Shaw, N., Friend, J., Ratledge, C., Roberts, A.D. and Nigam, S. (1997). *System Appl Microbiol* **20**, 39-49.

Nichols, F.C. (1994). *Infect Immun* **62(9),** 3753-3760.

Parrilo, J.E. (1990). *Ann Intern Med* **133**, 227-228.

Raetz, C. (1990). *Ann Rev Biochem* **59**, 129-170.

Rietchel, E.T., Brade, H. and Holst, O. (1996). In : *Current Topics in Microbiology Immunology* **Vol. 216**, pp. 39-81. Edited by E.T. Rietschel and H. Wagner. Springer-Verlag, Heidelberg, Germany.

Slusarenko, A.J. (1996). In: Lipoxygenase and lipoxygenase pathway Enzymes, pp. 176-197. Edited by G.J. Piazza. AOCS press Champaign.

Van Dyk, M.S., Kock, J.L.F., Coetzee, D.J., Augustyn, O.P.H. and Nigam, S. (1991). *FEBS Lett* **283(2)**, 195-198.

Venter, P., Kock, J.L.F., Sravan Kumar, G., Botha, A., Coetzee, D.J., Botes, P.J., Bhatt, R.K., Falck, J.R., Schewe, T. and Nigam, S. (1997). *Lipids* **32,** 1277-1283.

Wright, S.D., Ramos, R.A., Tobias, P.S., Ulevitch, R.J. and Mathison, J.C. (1990). *Science* **249**, 1431-1433.

Summary

In 1991, a novel eicosanoid namely 3-hydroxy-5, 8, 11, 14-eicosatetraenoic acid (3-HETE) was uncovered in the yeast *Dipodascopsis uninucleata* by van Dyk and co-workers. Strikingly, the production of this compound was found to be sensitive to low concentrations of aspirin and indomethacin. With this as background, a study was conducted that unveiled the possible biochemical pathway used by this yeast for the production of 3-HETE. Here, various fatty acids were fed to *D. uninucleata*, and the extracted samples analysed for the accumulation of 3-hydroxy metabolites with the help of electron impact gas chromatography - mass spectrometry. It was found that 3-hydroxylation of fatty acids in *D. uninucleata* requires a *5Z, 8Z* - diene system either directly or following initial incomplete β-oxidation. Following analysis of the enantiomer composition, the arachidonic acid metabolite was identified as 3*R*-hydroxy - *5Z, 8Z, 11Z, 14Z* - eicosatetraenoic acid (3*R* - HETE), which rules out normal β-oxidation as a biosynthetic route. Consequently, studies on the biological dynamics and distribution of 3-hydroxy oxylipins in *D. uninucleata* followed. The occurrence of oxylipins was mapped by immunofluorescence microscopy (IF) in fixed cells, with or without cell walls, using an antibody raised against 3*R*-HETE. This antibody turned out to cross-react with other 3-hydroxy oxylipins. These compounds were detected *in situ* in gametangia, asci, as well as between released aggregating ascospores. Aspirin (1mM), which is

known to suppress the formation of 3-hydroxy oxylipins from exogenous polyenoic fatty acids, inhibited the occurrence of immunoreactive material as well as cell aggregation, suggesting a prominent regulatory role of 3-hydroxy oxylipins for the latter. Since these oxylipins are associated with the aggregation of sexual cells in *D. uninucleata*, the next step was to screen for the presence of these compounds in aggregating cells of other yeasts such as the biotechnological important *Saccharomyces cerevisiae*. It was found that oxylipins such as 3-hydroxy-8:0 and 3-hydroxy-10:0 are produced over the growth cycle of the flocculating yeast *Saccharomyces cerevisiae* ATCC 26602. Using oxylipin specific antibodies in IF studies, it was demonstrated that these compounds are synthesized continuously from an early stage of growth and are associated with the cell wall and are present between flocculating cells. Similar results were obtained with a NewFlo phenotype flocculent brewing yeast strain. This implicated the involvement of oxylipins in cell aggregation. Further investigations using scanning- and transmission electronmicroscopy, indicated that changes in the depositing of lipid rich osmiophilic layers in the yeast followed the same pattern as the IF results. Immunogold studies verified the presence of oxylipins in these osmiophilic layers. It was uncovered that the oxylipin containing osmiophilic layers play an important role in cell aggregation. Surprisingly, further investigations implicated the presence of aspirin sensitive 3-hydroxy oxylipins in the LPS layer of the Gram-negative bacterium *Escherichia coli*. In this study it was found that aspirin, at moderate concentrations, influences the biosynthesis of the endotoxic 3-hydroxylated

myristic acid in the Lipid A of Gram-negative bacteria. This discovery therefore suggests an important role for aspirin as a therapeutic agent in the treatment of LPS mediated diseases.

Opsomming

'n Nuwe eikosanoïed genaamd 3-hidroksie - 5, 8, 11, 14 - eikosatetraenoësuur (3-HETE) is in 1991 deur Van Dyk en medewerkers in die gis *Dipodascopsis uninucleata* ontdek. Dit was opvallend dat die produksie van hierdie komponent sensitief was vir lae konsentrasies aspirien en indometasien. Met hierdie inligting as agtergrond is 'n studie uitgevoer wat 'n moontlike biochemiese weg uitwys vir die produksie van 3-HETE deur hierdie gis. In hierdie studie is verskeie vetsure vir *D. uninucleata* gevoer en die geëkstraheerde monsters geanaliseer vir die teenwoordigheid van 3-hidroksie-metaboliete m.b.v. elektron impak gaschromatografie - massaspektrometrie. Daar is bevind dat 3-hidroksielering van vetsure in *D. uninucleata*, 'n 5*c*, 8*c* - diëen sisteem benodig en deur direkte of opvolgende onvolledige β-oksidasie plaasvind. Analises aangaande die enantiomeer-samestelling het gevolg en die aragidoonsuur-metaboliet is geïdentifiseer as 3*R*-hidroksie – 5*c*, 8*c*, 11*c*, 14*c* - eikosatetraenoësuur (3*R*-HETE) wat normale β-oksidasie as 'n biosintetiese weg onwaarskynlik maak. Gevolglik is studies rakende die biologiese dinamika en distribusie van 3-hidroksie-oksilipiene in *D. uninucleata* gedoen. Immunofluoresensie mikroskopie (IF) is gebruik om die posisie van oksilipiene in gefikseerde selle, met en sonder selwande, vas te stel deur gebruik te maak van 'n teenliggaampie wat opgewek is teen 3*R*-HETE. Gevolglik is ook vasgestel dat hierdie teenliggaampie met ander 3-hidroksie-

oksilipiene kruisreageer. Hierdie verbindings is waargeneem *in situ* in gametangia, askusse, asook tussen vrygestelde aggregerende askospore. Aspirien (1mM), wat daarvoor bekend is dat dit die produksie van 3-hidroksie-oksilipiene vanaf eksogeniese poliënoïese vetsure onderdruk, het ook die teenwoordigheid van immunoreaktiewe materiaal en selaggregasie onderdruk wat daarop wys dat 3-hidroksie-oksilipiene 'n prominente regulerende rol by laasgenoemde speel. Siende hierdie oksilipiene geassosieer is met die aggregerende seksuele selle van *D. uninucleata*, was die volgende stap om te bepaal of hierdie verbindings ook teenwoordig is in aggregerende selle van ander giste soos die biotegnologies-belangrike *Saccharomyces cerevisiae.* Dit is bevind dat oksilipiene soos 3-hidroksie-8:0 en 3-hidroksie-10:0 geproduseer word tydens die groeistadia van die flokkulerende gis *Saccharomyces cerevisiae* ATCC 26602. Deur gebruik te maak van die oksilipien-spesifieke teenliggaampies in IF studies, is gedemonstreer dat hierdie verbindings voortdurend gesintetiseer word vanaf die vroeë stadia van groei en later geassosieer is met die selwand. Die laasgenoemde is ook teenwoordig tussen flokkulerende selle. Soortgelyke resultate is verkry met 'n "NewFlo" fenotiepe flokkulerende brouersgis-stam. Dit impliseer ook die betrokkenheid van oksilipiene by selaggregasie. Verdere ondersoeke is ook uitgevoer met die skandeer- en transmissie elektronmikroskoop. Dit het uitgewys dat veranderinge in die neerslag van lipiedryke osmiofiliese lae in die gis dieselfde patroon as die IF resultate volg. Immunogoud-studies het die teenwoordigheid van oksilipiene in hierdie osmiofiliese lae bevestig. Dit is dus

duidelik dat die osmiofiliese lae 'n belangrike rol by selaggregasie speel. Verdere navorsing het, bo-verwagting, die teenwoordigheid van aspirien-sensitiewe 3-hidroksie-oksilipiene in die LPS laag van die Gram-negatiewe bacterium *Escherichia coli* uitgewys. Dit is bevind dat aspirien, teen matige konsentrasies, die biosintese van die endotoksiese 3-hidroksie-miristiensuur in die Lipied A van Gram-negatiewe bakteriëe beïnvloed. Die laasgenoemde bevinding is van uiterste belang, veral in die behandeling van LPS-bemiddelde siekte toestande. Die bevinding wys daarop dat aspirien 'n belangrike rol kan speel as 'n geneesmiddel in bogenoemde siekte toestande.

Key words: Arachidonic acid; *Dipodascopsis uninucleata*; Flocculation; Gram negative bacteria; 3-HETE; 3-Hydroxy fatty acids; Immunofluorescence; NSAIDs; ß-oxidation; *Saccharomyces cerevisiae*.