

# **Organic Synthesis and Fungicidal Activity of Oxylipin-Based Compounds**

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Doctor of Philosophy

ASTON UNIVERSITY

January 2011

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## Thesis summary

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Previous research has shown that the naturally occurring reactive electrophilic species (RES), 12-oxophytodienoic acid (OPDA), not only serves as a precursor for jasmonic acid but is also a potent antifungal compound. However, both the low amount present in plants and the multistep synthesis required to produce this compound on a scale viable for agrochemical use currently limits its practical value. The aim of this research was to generate a range of molecular mimics of OPDA with a minimum number of synthetic steps and screen for antifungal activity. Synthetic 4-octyl-cyclopentenone containing the cyclopentenone ring and an eight carbon alkyl chain was found to show the highest *in vitro* antifungal activity against *C. herbarum* and *B. cinerea* with minimum inhibition concentration (MIC) of 100-200 $\mu$ M. This indicates that structurally simplified 4-octyl-cyclopentenone can be successfully synthesised to mimic the antifungal activity of OPDA against specific fungal strains. Application of 4-octyl-cyclopentenone could act as surfactant by disrupting and disorganising the lipid membrane non-specifically, resulting in the leakage of potassium ions, which was the proposed mode of action of this compound. However, the sensitivity of fungi to this compound is not correlated to the lipid composition of fungal spores. (E)-2-alkenals were also studied for their antimicrobial activity and (E)-2-undecenal was found to have the highest antimicrobial activity against a range of pathogens. The hydrophilic moiety (the  $\alpha,\beta$ -unsaturated carbonyl group), common to both (E)-2-undecenal and 4-octyl-cyclopentenone is essential to their bioactivity, and the hydrophobic moiety plays an important role in their antimicrobial activities. 4-Octyl-cyclopentenone showed no visible toxicity to the test plant, *Arabidopsis thaliana*, suggesting that its high antifungal activity against *Botrytis* and *Cladosporium* could be exploited for commercialisation as a new generation of agrochemical.

**Key words:** fungicides, oxylin, OPDA, RES.

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## Abbreviations

ACP	Acyl carrier protein
AOC	Allene oxide cyclase
AOS	Allene oxide synthase
APCI	Atmospheric pressure chemical ionisation
ATCC	American type culture collection
Avr	Avirulence
CDCl <sub>3</sub>	Deuterated chloroform
COI1	Coronatine Insensitive 1
CrO <sub>3</sub>	Chromium trioxide
CuCN	Copper(I) cyanide
CuI	Copper(I) iodide
DAG	Diacylglycerol
DCM	Dichloromethane
DES	Divinyl ether synthase
DGDG	Digalactosyl diacylglycerol
DMF	Dimethylformamide
DMSO	Dimethylsulfoxide
$\alpha$ -DOX	$\alpha$ -Dioxygenase
EAS	Epoxy alcohol synthase
ESI	Electrospray ionization

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ER	Endoplasmic reticulum
Et <sub>2</sub> O	Diethyl ether
EtOH	Ethanol
EtOAc	Ethyl acetate
FAME	Fatty acid methyl esters
FID	Flame-ionisation detector
GC	Gas Chromatography
GLV	Green leaf volatile
GPL	Glycerophospholipid
GSH	Glutathione
GST	Glutathione-S-transferase
HEPA	High efficiency particulate air
HPL	Hydroperoxide lyase
HPLC	High performance liquid chromatography
HR	Hypersensitive reaction
IsoP	Isoprostane
JA	Jasmonic acid
KAT	L-3-ketoacyl-CoA thiolase
KODE	Ketodiene
KOTE	Ketotriene
$\alpha$ -LeA	$\alpha$ -Linolenic acid
LA	Linoleic acid

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LOX	Lipoxygenase
LRMS	Low resolution mass spectroscopy
LUMO	Lowest unoccupied molecular orbital
MAG	Monoacylglycerol
MeJA	Methyl jasmonic acid
MDA	Malondialdehyde
MFC	Minimum fungicidal concentration
MFP	Multifunctional protein
MGDG	Monogalactosyl diacylglycerol
MIC	Minimum inhibition concentration
MP	Melting point
MS	Mass spectra
MVK	Methyl vinyl ketone
NA	Nutrient agar
NAD	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
NB	Nutrient broth
NCTC	National collection of type cultures
NEFA	Nonesterified fatty acid
NMR	Nuclear magnetic resonance
OPDA (12-oxo-PDA)	12-oxo-10,15c-phytodienoic acids
dnOPDA	Dinor 12-oxo-phytodienoic acid

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OPR	12-oxo-phytodienoate reductase
PCD	Programmed cell death
PDA	Potato dextrose agar
PDB	Potato dextrose broth
PE	Phosphatidylethanolamine
PG	Phosphatidylglycerol
RES	Reactive electrophilic species
PP	Phytostane
PR	Pathogenesis-related
PUFA	Polyunsaturated fatty acid
PXG	Peroxygenase
QSAR	Quantitative structure-activity relationship
ROS	Reactive oxidative species
RT	Room temperature
SA	Salicylic acid
SDA	Sabouraud dextrose agar
SDB	Sabouraud dextrose broth
SE	Sterol esters
SRA	System acquired resistance
TAG	Triacylglycerol
TBAF	Tetrabutylammonium fluoride
TBDPS	tert-Butylchlorodiphenylsilane

TEM	Transmission electron microscopy
TFA	Trienoic fatty acid
THF	Tetrahydrofuran
TMS	Tetramethylsilane
TLC	Thin layer chromatography

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# Chapter1

## Introduction

The development and application of agrochemicals to crops has a history over thousands of years. A growing population is predicted to reach 6 billion by the middle of this century, so there is a huge pressure to increase food production to sustain the global population. Considering the constraints, one of the efficient ways to increase production is to improve the yield of unit area using agrochemicals.

Up to now, most of the chemically synthesised traditional agrochemicals are non-biodegradable, and their residues left in soil and water will have, potentially, severe detrimental effects on environment and biosphere. In addition, some of them are toxic to humans and animals [1]. To address these problems, recent research and development of novel agrochemicals, which are environmental friendly and which have low toxicity towards mammals is being undertaken.

### **1.1 Introduction to fungicides**

Agrochemicals are classified according to their target organisms, e.g. insecticides, herbicides, and fungicides, clearly depict their targets. For example, fungicides are used

extensively in agricultural production to control fungi. In 2004, the market value of fungicides reached over 7 billion dollars, accounting for nearly 25% of the whole agrichemical market share[1]. Since the 1960s, the world fungicide market has been dominated by man-made organic chemicals and most of them pose an environmental risk [2]. In the future, a primary concern for the development of new fungicides will be their effect on the environment and safety application issues. Indeed the concept of an agrochemical could change from killing (-cide) to controlling [1]. Generally speaking, it is extremely expensive and difficult to discover a new compound with antifungal activity. Five approaches of developing fungicides are generally recognised [2]:

- Random screening: This is a traditional method for fungicide development based on wide-scale screening of compounds. The approach works well since it does not depend on knowledge of the modes of action of the compound. However, it requires a high throughput method to efficiently screen the large amount of candidate compounds.
- Combinatorial chemistry: Used widely in pharmaceutical design, in which cheap and low molecular weight compounds are systematically synthesised and tested.
- Analogue synthesis: Based on existing fungicides, some chemical modification can lead to a new fungicide showing enhanced properties.
- Biorational design: This is becoming more increasingly important in novel fungicide discovery, candidates could be synthesised for special mode of action from prior knowledge of related modes of action.
- Speculative approach: Using a simple chemical starting point and an array of

potential substitutions that lie within predetermined physicochemical limits, a family of structural variants can be produced.

To date, most commercial fungicides are chemically, rather than biologically, synthesised. Meanwhile, more and more natural compounds are being discovered to have antifungal activity, which could play an important role in the market of fungicides in future. Using the above approaches, the chemical modification of natural antifungal molecules with enhanced properties can be envisaged to develop a new generation of fungicides. Modifications of some naturally occurring fungicides might mimic natural compound activity, or even improve their activity and increase the efficiency. In short, the approaches mentioned above could be used for the study and exploitation of a new generation of antifungal compounds based on naturally occurring products with fungicidal properties to identify such lead molecules. A range of target compounds with simplified structure from naturally occurring molecules need to be developed to satisfy commercial demand.

## **1.2 Natural fungicides**

Aside from synthesised agrochemicals, fundamental research has revealed that many natural products contribute to plant protection against pathogens. These compounds, including fungicides, insecticides and herbicides, could be derived from bacteria, fungi,

plants and animals. Recently, some of them have been used commercially [3]. They are able to be used directly for industrial manufacture or as a starting point in developing simpler and more efficiently synthesisable compounds, such as pyrrolnitrin, produced by *Pseudomonas pyrocinia*, and strobilurin A, produced by *Strobilurus tenacellus* [4].

In this research, plant-derived antifungal agents were investigated due to their important participation in plant defence against fungal pathogens. Significant progress in our understanding of plant-pathogen interactions has been made in recent years [5]. Although our knowledge about this area is still fragmented, some antifungal agents derived from plants have been discovered, which can be broadly classified into two groups:

1. Direct antifungal substances; they are secreted constitutively by plants or in response to external stresses and have toxicity against fungal strains, inhibiting their growth. These substances include antimicrobial proteins/ peptides [6], oils [7, 8], fatty acids [9, 10], oxylipins [11] and phytoalexins [12]. The expression of some antimicrobial peptides and proteins, like pathogenesis related (PR) proteins, is triggered once plants are attacked by pathogens. Phytoalexins are secondary metabolites of plants and have been well known for their antimicrobial activities for many years. Several chemical types of phytoalexins are also well known, such as isoprenoids and flavonoids [13]. Many oils and fatty acids extracted from different species exhibit the ability of inhibiting fungal growth due to antifungal components

in the complex mixture. A major systemic study of 43 oxylipins revealed their antimicrobial activities towards a range of bacterial and fungal pathogens [11].

2. Indirect antifungal agents; when pathogen invasion is recognised by disease resistance (R) genes binding to specific pathogen-derived avirulence (Avr) proteins and pathogen derived elicitors, a complex signalling network is induced by stimuli [5]. Such stimuli, which normally have no antifungal activity *per se*, initiate plant defence mechanisms by triggering the expression of genes which encode, for example, pathogen related proteins, or enzymes involved in the biosynthesis of phytoalexins, or the enzymes of oxidative stress protection, or regulation of hypersensitive reaction (HR) that lead to rapid host cell death around infected sites. Such events often arrest further microbial invasion. Some of the stimuli contributes to both local inducible host resistance and 'systemic acquired resistance' (SAR) to pathogens. In plants, salicylic acid (SA) and reactive oxidative species (ROS) were well studied as such stimuli of SAR in the 1990s [14, 15]. In the last decade, oxylipins, especially the jasmonate family, such as jasmonic acid (JA) and its precursor 12-oxo-phytodienoic acid (12-oxo-PDA/OPDA), were found to be closely related to plant defence signalling and gene expression during wounding and pathogen attack [16-19]. Non-enzymatically formed oxylipins, phytoprostanes, which are generated from oxidised lipids by oxygen or reactive oxygen species (ROS), were also reported to play a similar role in plants in response to biotic and abiotic stress [20].



Interestingly, oxylipins can play both direct and indirect antifungal roles in plants. In this research, oxylipins are the starting point for developing a new generation of antifungal compounds.

## **1.3 Plant oxylipins**

### **1.3.1 Plant lipids**

Lipids, ubiquitous in all organisms, are fatty acids and their derivatives, and substances related (biosynthetically or functionally) to these compounds [21]. Generally speaking, fatty acids are monocarboxylic acids with a long unbranched chain containing an even number of carbon atoms. The carbon chain length and degree of unsaturation is generally given in a standard nomenclature style, e.g. oleic acid (18:1 $\Delta^9$ ), where ‘18’ represents chain length and the number after the colon is the number of double bond (i.e. ‘1’). The delta ( $\Delta^9$ ) indicates the carbon number at which the double bond occurs counting from the carboxylic end of the fatty acid. In plants the *de novo* synthesis of fatty acids occurs in the plastids, in which long chain fatty acids, such as palmitic acid (16:0), stearic acid (18:0) and oleic acid (18:1), are synthesised from the preformed acetyl-CoA through several cycles of two carbon addition. The reactions involve a series of condensation, reduction, dehydration and further enoyl reduction of acyl thioester on acyl carrier protein (ACP) [22]. The end product of fatty acid synthesis is

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palmitoyl-ACP (16:0-acyl-ACP) and this can be elongated by specific enzymes to stearoyl-ACP (18:0-acyl-ACP). Most of the newly formed 18:0-acyl-ACP (typically 80-85%) are converted to oleoyl-ACP (18:1-acyl-ACP) by  $\Delta^9$  desaturase [23, 24]. These fatty acids are transported to the endoplasmic reticulum (ER) membrane for further elongation and desaturation of chains to form other fatty acids, such as linoleic acid, 18:2 ( $\Delta^{9,12}$ ),  $\alpha$ -linolenic acid, 18:3 ( $\Delta^{9,12,15}$ ) and longer chain fatty acids (C20-C24). Elongation proceeds in a similar way to fatty acid synthesis. For desaturation, after the incorporation of fatty acid acyl groups into glycerophospholipids, such as phosphatidylcholine (PC), desaturases bound to the membrane of ER convert oleate (18:1) to linoleate (18:2) and then to linolenate (18:3) [13, 25]. These fatty acids are mainly esterified onto the glycerol back bone to form glycerolipids in plastids and ER; small amount of fatty acids are consumed to generate minor lipids, such as sphingolipids and sterol esters. Over 1,000 fatty acids are known with different chain lengths, positions, configurations and degrees of unsaturation. However, only around 20 fatty acids occur widely in nature; of these, palmitic, oleic, and linoleic acids make up ~80% of commodity oils and fats [23]. The most commonly occurring fatty acids having an even number of carbon atoms in an unbranched chain are listed in table 1.1.

Table 1.1 Some naturally occurring fatty acid: structure and nomenclature [24].

Carbon skeleton	Systematic name	Common name	Melting point (°C)
12:0	n-Dodecanoic acid	Lauric acid	44.2
14:0	n-Tetradecanoic acid	Myristic acid	53.9
16:0	n-Hexadecanoic acid	Palmitic acid	63.1
16:1( $\Delta^9$ )	cis-9-Hexadecenoic acid	Palmitoleic acid	0.5-1
18:0	n-Octadecanoic acid	Stearic acid	69.6
18:1( $\Delta^9$ )	cis-9-Octadecenoic acid	Oleic acid	13.4
18:2( $\Delta^{9,12}$ )	cis-,cis-9,12-Octadecadienoic acid	Linoleic acid	1-5
18:3( $\Delta^{9,12,15}$ )	cis-,cis-,cis-9,12,15-Octadecatrienoic acid	$\alpha$ -Linolenic acid	-11
18:3( $\Delta^{6,9,12}$ )	cis-,cis-,cis-6,9,12-Octadecatrienoic acid	$\gamma$ -Linolenic acid	-11
20:0	n-Eicosanoic acid	Arachidic acid	76.5
20:4( $\Delta^{5,8,11,14}$ )	cis-,cis-,cis-,cis-5,8,11,14-Icosatetraenoic acid	Arachidonic acid	-49.5
24:0	n-Tetracosanoic acid	Lignoceric acid	86.0

There are three main biological functions of lipids:

1. Non-polar lipids, mainly triacylglycerols (Figure 1.1) assembled in the ER membrane (which consist of a glycerol molecule that is esterified with three fatty acids), are used for energy storage. In plant seeds, triacylglycerols function as a

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carbon store to supply the carbon required for biosynthetic process during seed germination [13].



Figure 1.1 Example of triacylglycerol [24]. A variety of fatty acids could be esterified to one of three carbons of glycerol which are named *sn*-1, *sn*-2, and *sn*-3, with "*sn*" standing for "stereospecific numbering". For example, in this figure at *sn*-1 is stearic acid; linoleic acid and palmitic acid are linked to *sn*-2 and *sn*-3 respectively.

2. Amphiphilic polar lipids can be categorised as glycerophospholipids (GPLs), sphingolipids and sterols (it should be noted that GPLs have been erroneously referred to as 'phospholipids' – this is incorrect as not all phospholipids contain glycerol, Figure 1.2), which are composed of a polar head group (hydrophilic part) and non-polar carbon chains (hydrophobic part). GPLs differ from each other with respect to polar alcoholic head groups linked to phosphate group whose OH group is esterified to the *sn*-3 carbon of glycerol. The polar alcoholic head groups are choline, ethanolamine, serine, glycerol and inositol. In GPLs, the fatty acid at *sn*-1 often has

a saturated chain with 16 or 18 carbon atoms. The longer fatty acids (at least 18 carbon atoms) at *sn*-2 are nearly always unsaturated, with one or more *cis* double bonds (Figure 1.2 a). The backbone structure of sphingolipids contains a so-called sphingo base, and a saturated fatty acid with a long chain (up to 24 carbon atoms) is linked to the amino group of sphingo base with an amide linkage. The acylated sphingosine is referred to as a ceramide. When a sugar or an oligosaccharide is bonded to the hydroxyl group of ceramide, a glycosphingolipid (GSL) forms (Figure 1.2 b). The polar head group of sterol is a single OH group, whereas the sterane skeleton with the side chain serves as the hydrophobic moiety [26]. The hydroxyl group of sterols can be acylated to form sterol ester molecules (Figure 1.2 c). A large variety of sterols (such as cholesterol,  $\beta$ -sitosterol, stigmasterol and ergosterol) constitutes membranes of different species. For example, cholesterol is an important membrane constituent of animals; plants mainly have  $\beta$ -sitosterol and stigmasterol in membrane; and ergosterol is a sterol present in the cell membrane of fungi. Amphiphilic polar lipids are the major membrane forming components which form a bilayer structure (Figure 1.3).

3. It is evident that membranes are key sites of signal perception and transduction. For example, phosphoinositides are the inositol-containing glycerophospholipids of plasma membrane of plant cells and are known to be involved both in transmission of signal across the plasma membrane and in intracellular signalling [27]. Some other lipids derivatives, especially oxylipins which arise from the oxidation of

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unsaturated fatty acids (e.g. jasmonates), regulate gene expression and signal transduction in plants [17].



Figure 1.2 Structure-based classifications of membrane lipids [26]. Plasma membrane lipids consist of glycerophospholipids (GPLs), sphingolipids and sterols.



Figure 1.3 Amphipathic lipid aggregates that form in water [24]. (a) In micelles, there is virtually no water in the hydrophobic interior. (b) Open bilayer. Hydrophobic chains are protected by the hydrophilic heads from interaction with water. (c) Liposome is a closed bilayer, a three-dimensional hollow vesicle enclosing an aqueous cavity.

### 1.3.2 Lipid oxidation

Lipid oxidation is a complex array of important reactions that occur constantly during the life cycle of all aerobic organisms. This process can either be catalysed enzymatically or non-enzymatically, where the later is referred to as autoxidation. On the one hand, lipids can be metabolised by oxidative reactions to maintain health, for example, the fatty acids of triacylglycerol can undergo  $\beta$ -oxidation to supply about 40% calories for animals/plants and sufficient carbon source for the germination of plant seeds. Conversely, excessive oxidation of lipids, especially polar-lipids and polyunsaturated fatty acids in membrane, are potentially harmful to cells due to damage to plasma membrane and toxic derivatives, such as (2E)-4-hydroxyl hexenal and (2E)-4-hydroxyl nonenal formed during oxidation. Interestingly, when plants are stressed by ROS, wounded, or attacked by pathogens, some compounds derived from lipids oxidation, which have a negative effect on plant cells at high levels, were found to

be able to trigger some defence gene expression to protect plants at sub lethal level. During last decade, this family of lipid metabolites, known as oxylipins, has been shown to have an important role in plant defence systems [28, 29].

Oxylipins are a group of compounds derived from the oxidation of unsaturated fatty acids, either through enzymatic or non-enzymatic pathways. In animals, oxylipins including enzymatically formed prostaglandins and non-enzymatically formed neuroprostanes and isoprostanes, have been well studied and their specific roles in the regulation of physiological processes are known to some extent [30, 31].

Plant oxylipins are a diverse class of lipid metabolites that are derived from the oxidation of polyunsaturated fatty acids, linoleic acid (LA, 18:2 n-6),  $\alpha$ -linolenic acid ( $\alpha$ -LeA, 18:3 n-3) or roghanic acid (16:3 n-3). In general, the first step of fatty acid oxidation forms hydroperoxides, either through enzymatic oxidation or non-enzymatic routes, e.g. chemical reactions (Figure 1.4).

So far, research on oxylipins has mainly focused on their important roles in plant defence in response to biotic/abiotic stresses [32], such as wounding, pathogen attack, ROS stresses; and their roles in the regulation of development. In order to elucidate the roles of oxylipins in a given biological context, comprehensive analytical assays are available for the determination of the oxylipin profiles of plant tissue. Methods for oxylipin analysis have been recently summarised in reviews [33, 34].





Figure 1.4 Overview of oxylipin biosynthesis [35]. The formation of oxylipins starts with the conversion of polyunsaturated fatty acids (PUFAs) containing a (1Z, 4Z)-pentadiene system. LOX, lipoxygenase,  $\alpha$ -DOX,  $\alpha$ -dioxygenase, AOS, allene oxide synthase; AOC, allene oxide cyclase; DES, divinyl ether synthase; EAS, epoxy alcohol synthase; HPL, hydroperoxide lyase; PXG, peroxygenase. PUFAs can also be non-enzymatically converted into fatty acid hydroperoxides and hydroxy fatty acids. Non-enzymatic reactions are shaded in dark grey (right), enzymatic reactions in light grey (left).

### 1.3.3 Enzymatic oxidation pathway

The routes of enzymatic oxidation pathways are more known in comparison to non-enzymatic routes, because of their important functions in plants. Several enzymatic synthesis pathways including those initiated by allene oxide synthase (AOS), divinyl ether synthase (DES), hydroperoxide lyase (HPL), peroxygenase (PXG), or epoxy alcohol synthase (EAS) (Figure 1.4 ), have been established [28].

#### 1.3.3.1 Lipoxygenases

In enzymatic oxidation, the first key step in oxylipin metabolism is the formation of regio- and stereo-specific hydroperoxides from free or esterified polyunsaturated fatty acids (PUFAs), which is activated by lipoxygenases (LOXs) or  $\alpha$ -dioxygenase ( $\alpha$ -DOX) [36, 37]. LOXs are non-heme iron containing monomeric proteins (dioxygenases/oxidoreductases) which are very active in catalysing the oxidation reactions of PUFA with a (1Z, 4Z)-pentadiene motif [28]. The functions of LOX have been reviewed elsewhere [38]. When plants suffer external stresses, LOXs gene expression can be up-regulated by several effectors such as wounding, pathogen and even oxylipins, such as jasmonic acid (JA). Plant LOXs can be classified with respect to their positional specificity of fatty acid oxygenation against linolenic acid. With  $\alpha$ -linolenic acid as a substrate molecule in plants, only the hydrogen at C11 could be stereoselectively removed then the oxygen could be added to either the 9 or 13 carbon,

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leading to 9(S)- or 13(S)- hydroperoxy derivatives catalysed by two LOX isomers, 9-LOX and 13-LOX, respectively (Figure 1.5). Different LOX-isomers are located in different subcellular compartments and provide pools of different hydroperoxy PUFAs that undergo oxidation pathways. In this regard, 9-LOXs are exclusively found outside the plastids, whereas 13-LOXs are plastid-localised isoenzymes [39]. LOXs are multifunctional enzymes, which catalyse three different reactions: (i) oxygenation of substrates (dioxygenase reaction), (ii) secondary conversion of hydroperoxy lipids (hydroperoxides reaction) and (iii) formation of epoxy leukotrienes (leukotriene synthase reaction) [40]. The former is the most prevalent in plants.



Figure 1.5 Oxidation catalysed by LOX [41]. Using linolenic acid as starting molecule, the hydrogen at C11 is stereoselectively removed then the oxygen is added to either 9 or 13 carbon, forming 9(S)- or 13(S)- hydroperoxides catalysed by 9-LOX and 13-LOX respectively.

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### 1.3.3.2 Allene oxide synthesis pathway

The 13(S)-hydroperoxy derivatives from linoleic and linolenic acid will be metabolised largely through the pathway catalysed by allene oxide synthase (AOS), an enzyme of the cytochrome hemoproteins of P450 family. This enzyme is responsible for the biosynthesis of jasmonic acid (JA). In *Arabidopsis thaliana*, the level of this enzyme depends on a complex control through wounding, pathogen attack, ethylene (generated from ethephon), SA and feedback regulation through other octadecanoids (like JA and 12-oxo-10,15c-phytodienoic acids (12-oxo-PDA, OPDA)) [42].

#### (a) Jasmonate family and biosynthesis

JA is synthesised through the allene oxide synthesis pathway in higher plants, with OPDA being the key intermediate. When  $\alpha$ -linolenic acid is released from membranes, LOX activity converts it into either 9(S)- or 13(S)-hydroperoxides. 13(S)-hydroperoxide (13(S)-hydroperoxy-9c,11t,15c-octadecatrienoic acid) is then converted to 12,13(S)-epoxy-9c,11t,15c-octadecatrienoic acid by an AOS (Figure 1.6) [42]. This unstable compound readily undergoes further reaction to form  $\alpha$ -ketol through nucleophilic substitution and  $\gamma$ -ketol through electrophilic substitution by ions in the chloroplast, but more importantly is cyclised to OPDA by allene oxide cyclase (AOC) [28]. Four possible enantiomers of OPDA can be synthesised from the two chiral carbon centres (9, 13 C). However only 12-oxo-9(S),13(S)-phytodienoic acid (the

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cis-(+)-enantiomer) exists in most plants and is useful as a precursor of (+)-7-iso-JA which has JA biochemical and physiological properties [43]. These reactions occur in the stroma phase of chloroplasts. The OPDA is transported from the plastids to the peroxisomes as an uncharged free acid and OPDA-CoA esterified from or assisted by the peroxisomal ATP-binding cassette (ABC) transporter COMATOSE (CTS) [44]. In peroxisomes, the cis-(+)-OPDA-specific OPDA-reductase (isozyme 3: OPR3) reduces OPDA to 3-oxo-2-(2-pentenyl)-cyclopentane-1-octanoic acid (OPC-8:0) followed by three cycles of  $\beta$ -oxidation catalysed by acyl-CoA oxidase (ACX), multifunctional proteins (MFP) and 3-ketoacyl-CoA thiolase (KAT) to form (+)-7-iso-JA [45]. Three cycles of  $\beta$ -oxidation are responsible for shortening the carboxylic acid side chain by removing two carbons in each cycle. During the whole pathway, the export of OPDA from chloroplasts to peroxisomes is the rate limiting step [14], resulting in an accumulation of OPDA in the membrane of the chloroplast. Interestingly, some early research reported that OPDA was saturated at the 10, 11-double bond to yield OPC-8:0 in cytoplasm, which was followed by transportation into peroxisomes and  $\beta$ -oxidation [46, 47]. In *Arabidopsis*, most OPDA and dinor 12-oxo-phytodienoic acid (dnOPDA) (derived from rosinic acid (16:3 n-3)) is mainly esterified in mono- and digalactosyl diacylglycerol (MGDG and DGDG). These galactolipids probably serve as a storage compartment for the rapid synthesis of jasmonates following both biotic and abiotic stress [48-50]. Lipids with esterified OPDA and dnOPDA have only been found in *Arabidopsis* to date, and are termed arabidopsides.



Figure 1.6 JA biosynthesis and OPDA metabolism in *Arabidopsis thaliana* [51]. After cis-(+)-OPDA is synthesised from  $\alpha$ -LA in chloroplast, they can be transported from chloroplast to peroxisome for further oxidation to form JA, added to glutathione (GSH) to form OPDA-GSH, or directly trigger expression of genes in nucleus. 13-LOX, 13-lipoxygenase; ACX, acyl-CoA oxidase; AOC, allene oxid cyclase; AOS, allene oxide synthase; CTS/PXA1/PED3, ABC transporter for OPDA or OPDA-CoA import; COI1, F-box protein in JA signal transduction; GST, glutathione-S-transferase; KAT, L-3-ketoacyl-CoA thiolase; MFP, multifunctional protein; OPR, 12-oxo-phytodienoate reductase; PLAI, plastidic acyl hydrolase.

**(b) Biological activity of jasmonates**

In plants, jasmonates are by far the most studied fatty acid derived signalling molecules. They are important plant signal molecules in response to stresses including wounding, insect attack, interaction with pathogens, ROS, and plant development [52]. Jasmonic acid, a member of jasmonate family, is well documented. Its roles include information (signal) transduction to trigger expression of genes related to plant defence [53, 54] and development of plants, such as reproduction in *Arabidopsis* [55]. Its volatile methylated form, methyl jasmonic acid (MeJA), can act in interplant communication [17, 56]. It was also reported that exogenous treatment of MeJA on plants could activate broad-spectrum disease resistance against several fungal pathogens [57, 58]. For the last decade, another member of the jasmonate class of plant hormones, OPDA, has been revealed to be able to regulate gene expression independently or in concert with JA to fine-tune the expression of defence genes [59]. OPDA was proposed to regulate two distinct signal pathways [60, 61], one through COI1 (Coronatine Insensitive 1) similar to JA, and the other COI1-independent pathway via the electrophile effect of its cyclopentenone moiety (such as biological activities mediated through TGA transcription factors by covalently binding to free thiol groups of transcription factors [62]). OPDA and JA have individual signalling activities, however, they share overlapping biological activities [17, 63]. Both of them contribute to plant defence against some species of fungi, like *Botrytis cinerea*, and OPDA has been shown more recently to also play an important role against necrotrophic pathogens [64]. However, in

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some cases, the conversion of OPDA to JA is necessary for the resistance of plants [55]. For example, it is JA, rather than OPDA, that plays an essential role as a signal for wounding-induced defence response in tomatoes [65]. Interestingly, the exogenous treatment of JA could induce the endogenous accumulation of OPDA in some species [66]. Evidently, OPDA and JA appear to operate in concert to contribute to the gene expressions.

Besides its ability to regulate gene expression, OPDA exhibits direct antifungal activity. OPDA (100  $\mu\text{M}$ ) has the highest *in vitro* antifungal activity and chemical stability among 43 oxylipins tested against several plant pathogens, such as *C. herbarum*, *B. cinerea* and *P. parasitica* (Figure 1.7) [11]. In contrast, the JA does not show any direct *in vitro* antifungal activity. The accumulation of OPDA-containing arabidopsides was found in wounded (arabidopside A, B, E, G) and HR (arabidopside E and G) plant tissue, which were induced by two stimuli; wounding [48] and recognition of HR-caused phytopathogenic avirulence peptides [49]. The term arabidopsides has been coined to describe galactolipids (MGDG and DGDG) which contain esterified OPDA and dnOPDA (at *sn*-2 position). In one respect, the arabidopside E and G was reported to have high antifungal activity against *B. cinerea* [49]. However, in the same report, OPDA at concentration of 100  $\mu\text{M}$  was showed to have very limited antifungal activity against *B. cinerea* in comparison with arabidopsides (100  $\mu\text{M}$ ).





Figure 1.7 Antifungal activities of oxylipins [11]. Each test fungal or oomycete strain was exposed to each compound (100  $\mu$ M) in the appropriate liquid medium for 24 h. Inhibition of growth over 24 h was expressed as a percentage relative to the ethanol control. In the diagram, colour intensity is inversely proportional to inhibitory activity of the oxylipin toward the organism. The names of tested organisms are abbreviated as follows: *P. infestans*, *Phytophthora infestans*; *R. spp.*, *Rhizopus species*; *A. brassicicola*, *Alternaria brassicicola*; *F. oxysporum*, *Fusarium oxysporum*; *P. parasitica*, *Phytophthora parasitica nicotianae*; *B. cinerea*, *Botrytis cinerea*; and *C. herbarum*, *Cladosporium herbarum*.

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### 1.3.3.3 Hydroperoxide lyase pathway

Hydroperoxide lyase (HPL), like AOS, belongs to the cytochrome P450 family, and can be classified roughly into two groups, according to their substrate specificity. 13-HPL can cleave the 13-hydroperoxy derivatives of linoleic or linolenic acids to form a group of C6 volatiles (such as (Z)-3-hexenal, (E)-2-hexenal, hexanal, and hexanol [67]) and C12-oxo acids (including traumain and traumatic acid), at the carbon-carbon bond adjacent to the 13-hydroperoxide functionality (Figure 1.8) [28]. In parallel to 13-HPL, 9-HPL will catalyse the cleavage of 9-hydroperoxy isomers of linoleic and linolenic acids, yielding C9 compounds, such as (2E),(6Z)-nonadienal, (Z)-3-nonenal and (E)-2-nonenal. Both C6 and C9 volatiles are referred to as green leaf volatiles (GLVs).

As a group of bio-active compounds, GLVs are formed rapidly when tissues are disrupted and contribute to the plant defence system, especially when they are enhanced by the presence of  $\alpha,\beta$ -unsaturated carbonyl groups [68]. For example, (E)-2-hexenal is a very common GLV in plants and has been well studied for their multifunctional properties correlated with plant defence [69], several features of which are listed below:

1. (E)-2-hexenal was found to be more effective than the corresponding saturated aldehydes and hexanols at inhibiting the growth of fungal spores against some species of fungi, like *Colletorichum acutatum*. This green volatile compound was found to be able to alter the structures of the cell wall and plasma membrane, causing disorganisation and lysis of organelles and, eventually, cell death [70]. Also

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Hamilton-Kemp et al. [71] demonstrated that the  $\alpha,\beta$ -unsaturated aldehyde, (E)-2-hexenal, was considerably more active in the inhibition of hyphal growth of *Botrytis cinerea* than hexenol and hexanal.

2. (E)-2-hexenal exhibits antibacterial activity against several bacterial species, such as *Pseudomonas syringae*, *Xanthomonas campestris* and *Erwinia carotovora* [11].
3. (E)-2-hexenal can trigger the expression of defence-related genes and induce antifungal proteins and phytoalexin accumulation in plants. Therefore, these responses contribute to enhanced resistance against pathogens [72, 73].
4. (E)-2-hexenal accumulates in plants and occurs along with rapidly increased activities of LOX and HPL after wounding [69]. Acetylated C6-aldehydes were reported to be the predominant wound-inducible volatile signal that mediates the indirect defence response by directing tritrophic (plant-herbivore-natural enemy) interactions, whereas the jasmonates are responsible for the activation of the direct plant-defence response [74].

Some recent reports indicate that (E)-2-hexenal antifungal activity was induced by its electrophilic properties,  $\alpha,\beta$ -unsaturated carbonyl groups, that interact with proteins through the reaction with thiol and amine groups of proteins in the cell membrane [75, 76]. Besides (E)-2-hexenal, another 13-HPL derived plant hormone, traumatin (12-oxo-(E)-10-dodecenoic acid), is formed through  $\alpha$ -cleavage of fatty acid hydroperoxides, which was considered to trigger cell division near infected or wound sites [28]. Unsaturated C9-aldehydes, like (E)-2-nonenal, form rapidly after the

disruption of cucumber tissues from the cleavage of fatty acid by 9-HPL from 9-hydroperoxide [77]. Compared with (E)-2-hexenal, this compound is more active towards hindering fungal growth [68, 77]. However, the high concentration required to inhibit growth was also toxic to host cells, leading to cell death and preventing the further spread of pathogens [77].

From the 9-LOX pathway, another important antifungal oxylipin is DES derived divinylethers (colnelenic and colneleic acid), which are formed as fungicides in very high amounts in potato after infection with the oomycete *Phytophthora parasitica* [78]. Therefore, the 9-LOX pathway was reported to perform essential roles in plant defence against microbial pathogens [29, 79], probably resulting from the activity of these antipathogenic substances (nonenal, colnelenic and colneleic acid).

In short, apart from jasmonates, derivatives from other oxylipin bio-synthetic pathways, like HPL and DES pathways, participate and contribute to the plant defence system.



Figure 1.8 Hydroperoxide lyase pathway from 9- and 13- hydroperoxides of linolenic acid [28]. LOX: lipoxygenase; HPL: Hydroperoxide lyase; IF: isomerisation factor. In a similar reaction sequence, linoleic acid yields hexanal as well as (Z)-3- and (E)-2-nonenal.

#### 1.3.4 Non-enzymatic oxidation pathways

ROS, including free radical and oxygen, catalysed non-enzymatic oxidation of PUFAs occur constitutively in membranes of all aerobic organisms, due to the existence of two or three non-conjugated double bonds. Enzymatically formed oxylipins can be classified according to different pathways, such as AOS, DES, HPL, PXG and EAS (Figure 1.4). In contrast, free radicals and oxygen can attack all reactive sites, so it is difficult to give

a classification to non-enzymatically formed oxylipins. Some examples of them are shown in figure 1.4, like cyclopentanoids, (2E)-4-hydroxy alkenals, hydroperoxides, and some ketols, of which the group of cyclopentanoids are of interest because they are structurally related to the established animal and plant defence mediators of the prostaglandin and jasmonate type. In animals, non-enzymatically-formed cyclopentanoids from arachidonic acid (C20:4 $\omega$ 6) are racemic isomers of enzymatically-formed prostaglandins, so they are termed isoprostanes [80]. In plant membrane,  $\alpha$ -linolenic acid (C18:3 $\omega$ 3) is the predominate source for dinor isoprostanes that are termed phytoprostanes (PPs) [81, 82]. Currently, it has been proposed that omega-3 trienoic fatty acids (TFAs) in plants, in particular  $\alpha$ -linolenic acid, serve in the protection of cells by absorbing ROS such that they are oxidised in a free-radical-dependent (non-enzymatic) manner [83]. However, this is debatable, because some products from the oxidation of TFAs could be another source of ROS and harmful to plants.

#### **1.3.4.1 Biosynthesis of phytoprostanes**

As in the case of the enzymatic oxidation pathway, the first step of non-enzymatic oxidation is the formation of hydroperoxides. Enzymatic and non-enzymatic pathways differ from each other with respect to the generation of different hydroperoxides (Table 1.2) [35].

Table 1.2 Two different oxidation pathways generate hydroperoxides.

Enzymatic Oxidation	Non-enzymatic Oxidation
LOXs catalyse forms either S or R hydroperoxides	Free radical reaction forms a racemic mixture of hydroperoxides.
LOXs insert molecular oxygen only at C-9 and C-13 of LA or $\alpha$ -LnA	In case of $\alpha$ -LnA, different positional isomers at C-9, C-10, C-12, C-13, C-15, C-16 could be formed, of which C-10 and C-15 are oxidised by singlet oxygen that is inserted at either end carbon of a double bond [84].

The mechanism of autoxidation of  $\alpha$ -LnA has been reviewed [84]. Briefly, hydrogen abstraction at C-11 and C-14 between two 1,4-diene systems will generate two pentadienyl radicals which are not stable and can undergo oxidation at the end-carbon of each pentadienyl radical leading to four hydroperoxides isomers, conjugated diene 9-, 12-, 13- and 16-hydroperoxides (Figure 1.9). Although 12- and 13- isomers are of lower concentration in comparison to 9- and 16- isomers, the presence of a homoallylic cis double bond in 12- and 13- isomers favours their tendency to undergo 1,3 cyclisation by intramolecular radical addition to the double bond. The newly formed radicals from 12- and 13- peroxy radicals can rapidly react with oxygen to generate the type I phytoprostane G ring (PPG) and the type II PPG, respectively [80]. There are 16 phytoprostane G ring (PP) isomers formed from each starting peroxy radical and 32 PPGs in total from  $\alpha$ -LnA (Figure 1.10). PPGs are very unstable and their non-enzymatic degradation pathway leads to D-, E-, and F- ring compounds, of which

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D- and E- phytoprostanes may dehydrate and isomerise to J-, deoxy-J, A- and B- ring phytoprostanes (Figure 1.11). Plant E<sub>1</sub>- and F<sub>1</sub>- phytoprostane in several species were first identified in the late 1990s [81, 82]. As plant analogues of the animal isoprostanes, the importance and perspective of the discovery of phytoprostanes for future research has been reviewed [85]. Up to now, some sensitive and specific analysis methods have been developed to detect low levels of phytoprostanes in plants, such as GC-MS, HPLC, HPLC-ESI-MS/MS [20, 86]. Among PPs mentioned above, PPD<sub>1</sub> and dPPJ<sub>1</sub> were found to be the most abundant PPs, not only in tomato leaves [20], but also in some other plant species, such as *Arabidopsis* [87].



Figure 1.9 Mechanism of linolenic acid autoxidation [84].





Figure 1.10 Non-enzymatic phytoprostane biosynthesis from  $\alpha$ -linolenic acid [86].



Figure 1.11 The phytyloprostanol pathway in plants [20]. G<sub>1</sub>-phytyloprostanols rapidly decompose in aqueous environment. Non-enzymatic or enzymatic reduction of both peroxy groups yields chemically stable F<sub>1</sub>-phytyloprostanols. The rearrangement of the endoperoxide group and reduction of the side chain hydroperoxy group of G<sub>1</sub>-phytyloprostanols yields D<sub>1</sub>- and E<sub>1</sub>-phytyloprostanols which may dehydrate/isomerise to J<sub>1</sub>- and deoxy-J<sub>1</sub>-phytyloprostanols or A<sub>1</sub>- and B<sub>1</sub>-phytyloprostanols, respectively.

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### 1.3.4.2 Bioactivity of phytoprostanes

It was found that the levels of PP in fresh plant tissues are much higher than isoprostane (IsoP) in mammalian tissues, and the levels increase dramatically when plants are dried and stored after harvest [88]. Recent research shows that singlet oxygen generated during photosynthesis in chloroplast may accelerate radical driven formation of lipid hydroperoxides which are the precursors of PPs [89]. It is also known that the production of PPs in plants could be induced in several ways, such as reactive oxygen species (ROS) *in vivo* [82, 90], wounding [82] and pathogen infection [90].

Two distinct groups of PPs are classified by chemical structure difference. The first group comprises reactive electrophiles with a cyclopentenone ring moiety, which have thiol and amine reactivity. More research was focused on the biological activities of these PPs including A-, B-, and J- ring PPs (due to their important roles in inducing several plant-protection mechanisms), such as gene triggering for plant defence and detoxification [91], induction of phytoalexins [90] and recently they were found to have anti-inflammatory activities similar to prostaglandin A<sub>1</sub> (PGA<sub>1</sub>) and 15-Deoxy- $\Delta^{12,14}$ -prostaglandin J<sub>2</sub> (dPGJ<sub>2</sub>) in human cells [92]. These cyclopentenone PPs belong to a large family of compounds with  $\alpha,\beta$ -unsaturated carbonyl structure, which are termed reactive electrophilic species (RES). The second group of PPs includes D-, E- and F- ring phytoprostanes. These compounds have been shown to have lower bioactivity than PPs with cyclopentenone rings. Like malondialdehyde (MDA),

the F-ring of PPs can also be used as an oxidation marker, not only because of their stability but their direct generation from the G-ring of PPs [82]. Some reports also showed that E<sub>1</sub>-phytoprostane could possibly induce secondary metabolism (scopoletin levels in tobacco cell) [90]. However, the conversion of PPE to the active PPA (RES) could not be ruled out.

Most studies have used a mixture of various PP isomers because of the difficulty to purifying individual isomers. Only a few reports indicated the effect of different regioisomers with interesting results [90, 93]. Some organic synthetic approaches to prepare PPs have been published [86, 94], which provide good standards and biological controls for further phytoprostane research on the structure activity relationship. Although the interest and discovery of PPs were enlightened by analogy to the mammalian pathway, our knowledge about phytoprostanes and their functional importance is limited compare to the analogous (iso- and neuro- prostanes in humans). More effort is needed to elucidate their full spectrum of biological activities.

#### **1.4 Fatty acid derived reactive electrophilic species (RES)**

As discussed above, oxylipins are composed of various fatty acid derivatives, of which those compounds with an  $\alpha,\beta$ -unsaturated carbonyl group, like (E)-2-hexenal, OPDA and cyclopentenone PPs, have attracted the most interest due to their bioactivities. In

general, RES are classified as compounds having chemical reactivity with nucleophilic groups, such as thiol- (SH-) and amine- (H<sub>2</sub>N-) groups. In plants, compounds containing  $\alpha,\beta$ -unsaturated carbonyl groups or other types of RES derived from fatty acids are termed 'oxylipin RES' [95, 96].

#### **1.4.1 Oxylipin reactive electrophilic species**

Fatty acid derived  $\alpha,\beta$ -unsaturated epoxides,  $\alpha,\beta$ -unsaturated aldehydes, cyclopentenone products and other electrophilic hydroperoxides or ketones are classified as oxylipin RES [96]. These representative oxylipin RES could be generated, enzymatically or non-enzymatically, from lipid oxidation of PUFAs (Table 1.3 and Figure 1.4) [97].

Both enzymatically and non-enzymatically derived oxylipin RES are constantly generated *in vivo* even in healthy plant tissues, however, both pathways could be activated by a variety of biotic and abiotic stress conditions leading to accumulation of RES oxylipins [52]. These oxylipin RES are derived from different synthetic origins and play different roles in plants. However, they have two common properties: electrophilicity and lipophilicity, which endow them with important biological activities.

Table 1.3 RES oxylipins in plants and their bio-synthetic pathway [97].

	Synthetic Pathway	RES	Example
Enzymatic Pathway	Jasmonate pathway	Jasmonates	OPDA, dinor-OPDA, arabidopside
	Reduction of enzymatically formed LOOH	Hydroxyl and oxo-fatty acids	KOTE, KODE, Traumatin
	Fragmentation of hydroperoxides through enzymatic HPL pathway	Aldehydes and other small fragments	Hexenals, Nonenals
Non-enzymatic Pathway	PPs pathway	Cyclopentenone phytoprostanes	J <sub>1</sub> -, A <sub>1</sub> - and B <sub>1</sub> - PPs
	Reduction of nonenzymatically formed LOOH	Hydroxyl and oxo-fatty acids	KOTE, KODE
	Fragmentation of formed LOOH under free radical catalysis	Aldehydes and other small fragments	Hexenals, Nonenals, MDA, 4-hydroxyl-(2E)-nonenal

### 1.4.2 Electrophilicity and lipophilicity of RES

Oxylipin RES normally consist of an electrophilic part (e.g. an  $\alpha,\beta$ -unsaturated carbonyl moiety) and saturated or unsaturated carbon chains together with other functional groups, like carboxyl or hydroxyl moieties.

As for electrophilic  $\alpha,\beta$ -unsaturated carbonyl groups, it should be noticed that hard electrophiles having small atomic radii, high electronegativity (high  $E_{\text{LUMO}}$ ) and low polarisability, tend to react with hard nucleophiles (e.g. amino groups of lysine and DNA) (Figure 1.12). Soft electrophiles, on the other hand, having larger radii, high polarisability, containing unshared pairs of electrons and low electronegativity (low  $E_{\text{LUMO}}$ ) prefer soft nucleophiles (such as, thiol group of cysteine or glutathione) [97, 98]. Theoretically, the  $\alpha,\beta$ -unsaturated carbonyl group in oxylipins maybe (or are) closed as soft electrophilic species which tend to react with soft nucleophiles, like cysteine residues in GSH [99, 100]. Similar to RES in animals, it is hypothesised that bioactive plant RES could act as Michael acceptors, reacting not only with GSH, but also directly with other enzymes and transcription factors [51]. Different RES have different reactivities with nucleophiles as a consequence of their different electrophilicities. Notably, the smallest RES molecule, acrolein, has much higher reactivity with thiol and amine groups in comparison with other  $\alpha,\beta$ -unsaturated aldehydes, explaining its high cytotoxicity [100] and bioactivity [61]. In addition, conjugates of the RES with protein could be used as markers of oxidative stress in tissues.



Figure 1.12 Product formations after reaction with thiol and primary amine nucleophiles [100]. (a) Michael addition, product in the reaction between glutathione or thiol and an  $\alpha,\beta$ -unsaturated aldehyde; (b) Michael addition, product in the reaction between primary amine (e.g. lysine) and  $\alpha,\beta$ -unsaturated aldehyde; (c) Schiff base, product in the reaction between primary amine and  $\alpha,\beta$ -unsaturated aldehyde.

Besides electrophilicity, lipophilicity(or hydrophobicity) is another important parameter of oxylipin RES. Lipophilicity refers to the ability of a chemical compound to dissolve in lipophilic substances, such as oils, fats, lipids and non-polar solvents, and obey the axiom that *like dissolves like*. The partition coefficient,  $\log P$ , is often used as a measurement of lipophilicity, which is defined to be the logarithm of the ratio of the concentrations of an unionised compound in the two phases of a mixture of two immiscible solvents (normally a water and octanol solvent system) at equilibrium [101].



$$\log P_{\text{oct/wat}} = \log \left( \frac{[\text{solute}]_{\text{octanol}}}{[\text{solute}]_{\text{water}}^{\text{deionised}}} \right)$$

Log  $P$  is one of the major determinants of estimating the behaviour of drugs in organisms; therefore it is likely to be an important factor for the design of drugs and agrochemicals. In plant biochemistry, log  $P$  has a major importance in all biological activities as it represents the ability of molecules to reach the active sites [102]. With respect to RES, log  $P$  could affect the biological activity and cytotoxicity in two ways. The first is the ability of the compound to permeate through a membrane since plasma membranes are composed typically of a bilayer structure, in the middle of which are non-polar tails of fatty acids. Secondly, lipophilicity is an important physicochemical parameter referring to the affinity for the hydrophobic binding sites of proteins. The different affinities with the proteins will also influence the subsequent reaction rates with the substrates.

### **1.4.3 Biological activities of oxylipin RES in plant defence**

In mammals, lipid derived RES, such as prostaglandins and isoprostanes, and their biological activities have been well studied [30]. By comparison, research on plant RES is somewhat lagging behind. Recently, a growing number of biological roles of oxylipin RES in plants have been unveiled and some of them are related to the plant defence system.

Low levels of RES oxylipins are ubiquitously in all higher organisms and are naturally present, even in unstressed cells [91]. Studies showed that in general, the amount of RES increases rapidly following both biotic and abiotic stresses leading to a transient over accumulation [35]. In order to keep RES to low levels in healthy organisms, the expressions of genes related to their detoxification are increased. Some genes encoding detoxification proteins (such as glutathione-S-transferases (GST)) and other plant defence related gene expressions have been reported to be inducible by various kinds of RES with  $\alpha,\beta$ -unsaturated carbonyls, such as PPs [62, 90], OPDA [18, 52, 63, 103], MDA [104], hexenal [72, 105], KODE and KOTE [106]. Interestingly, RES B<sub>1</sub>-PP, that can not react with thiol groups, was also reported to trigger plant defence and detoxification [93]. RES oxylipins with similar structures could induce a common set of defence genes. For example, two cyclopentenone rings containing compounds, PPA<sub>1</sub> and OPDA share similar biological activities (reviewed elsewhere [107]), although phytoprostanes may also induce a unique set of responses. In addition, exogenous RES can influence endogenous RES pools. For example, in *Arabidopsis* the level of OPDA can be increased by the treatment with acrolein, which may be attributed to the action of lipase on esterified OPDA [61]. Enzymatic and non-enzymatic lipid peroxidation is implicated in the hypersensitive response (HR), during which, some of the oxylipins serve as signals necessary for defence gene activation whereas others contribute to pathogen killing or participate in the execution of programmed cell death (PCD) associated with this resistance [17, 79, 96]. A range of RES oxylipins, such as derivatives of 9, 13-LOX pathways, jasmonates and arabidopsides were found in

*Arabidopsis* during HR [17, 78, 96, 108, 109]. These enzymatically-formed oxylipins were found at an early stage, whereas non-enzymatically oxidised lipids were at low levels [109]. ROS mediated non-enzymatic peroxidation appeared as a late stage [79, 110] and both of them contribute to HR. This reveals the defence-related functions of enzymatically and non-enzymatically-formed oxylipins RES during biotic and abiotic stress.

Phytoalexins are disease-inducible low molecular mass secondary metabolites, which are commonly toxic to fungi, bacteria, plants and animal cells. Therefore, most of phytoalexins are absent from unchallenged, healthy tissue. When invaded by pathogens, plants will endeavour to protect themselves by a rapid accumulation of phytoalexin at the site of invasion. The production of phytoalexins often accompanies the hypersensitive response and is elicited by a variety of stimuli [111], of which RES compounds are important. Phytoalexins accumulate in plant cells in response to treatment with some oxylipin RES. For example, camalexin accumulates in cells after pre-treatment with cyclopentenone phytoprostanes [90, 93] and (E)-2-hexenal [73]. The accumulation of scopoletin was found in tobacco cells in response to OPDA and cyclopentenone phytoprostanes [94]. In short, RES induced accumulation of phytoalexins contributes to plant defence against some pathogens through their general toxicity.

Some oxylipins also show direct antimicrobial activities. 43 oxylipins were screened against several fungal and bacterial strains [11]. Oxylipin RES, like OPDA, KOTE, hexenals, have high antimicrobial activities. OPDA was considered as the best antifungal candidate and was also more stable in comparison to other effective compounds. (E)-2-hexenal had the best antibacterial activity. Interestingly, JA does not show any ability to inhibit fungal growth and (E)-2-nonenal that is reported to have antifungal activities [77] is not as active as (E)-2-hexenal against bacterial strains. The simple RES,  $\alpha,\beta$ -unsaturated aldehydes, were revealed to have antifungal activities against *Saccharomyces cerevisiae* and the length of the carbon chain plays an important role in balancing the hydrophilic  $\alpha,\beta$ -unsaturated carbonyl and hydrophobic carbon chain [68]. During HR, the accumulation of arabidopside E along with keto and hydroxyl fatty acids and jasmonates, display direct antipathogenic effects against secondary infectious agents. Considering that PPs, particularly cyclopentenone containing PPs, appear in the late stage of HR and some of them have similar chemical structure with OPDA, it is possible that these compounds also have antimicrobial activity.

#### **1.4.4 Phytotoxicity of RES**

Oxylipin RES play important roles in plant defence. However, this group of compounds can also cause severe damage to host cells due to their reactive chemical properties. They can affect plant cells in at least two ways. Firstly, the  $\alpha,\beta$ -unsaturated carbonyl can

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be reduced by enzymes such as NADPH-oxidoreductases and oxo-phytodienoic acid reductase, and cause indirect damage by the depletion of pools of reductants (NADPH and NADH). Secondly, RES can damage cells directly by modifying cell substances (proteins) due to their chemical reactivity with nucleophiles [95]. The photosystem II fluorescence of *Arabidopsis* was measured to quantify the damaging effects of lipid derivatives, especially oxylipin RES and was in the order: acrolein > methyl vinyl ketone (MVK) > 2E,6Z-nonadienal > (E)-2-nonenal > (E)-2-hexenal > OPDA=13-KOTE > 13-KODE > traumatin [61]. This investigation shows a correlation of activities with molecular weight and the increased degree of unsaturation. The general cytotoxicity of  $\alpha,\beta$ -unsaturated RES largely depends on electrophilic reactivity and lipophilicity. For example, the hepatocyte toxicity of  $\alpha,\beta$ -unsaturated aldehydes generally increases with increasing electrophilic reactivity with thiol and amine groups and often increases with lipophilicity value higher than 1.8 [100]. Although the phytotoxicity of RES was believed to have a similar relationship with their chemical reactivity, more work is necessary to clarify the mechanism. Summarising, oxylipin RES have powerful biological activity at relatively low levels, but are highly phytotoxic. Therefore, it is important to control the accumulation of oxylipin RES to a sub-lethal in plant cells.

In short, oxylipin RES play an important role in plants, and to some extent, are more active than their non-electrophilic counterparts, such as (E)-2-hexenal vs hexanal, OPDA vs JA and A-, B-, J- ring PPs vs E-, D-, F- ring PPs. Enzymatically or

non-enzymatically formed oxylipins RES accumulate in response to biotic and abiotic stresses, however, the detailed mechanism of RES recognition and signalling remains to be identified.

## **1.5 Molecular design based on 12-oxo-PDA**

So far, oxylipin RES contribute to the plant defence system through several mechanisms in response to environmental stresses. They are very promising candidates for the development of a new generation of agrochemicals, of which OPDA with a cyclopentenone ring is one of the most interesting compounds. This is not only because of its biological activity [63, 103], but also it has the most effective *in vitro* antifungal activity and stability among 43 oxylipins tested against fungal strains [11]. The cyclopentenone moiety is considered to confer the bioactivity of OPDA due to its electrophilicity as discussed above.

In this project, 12-oxo-PDA (OPDA) was considered as a natural lead molecule for the development of new agrochemicals. OPDA can be found in almost all higher plants. The biosynthetic pathway takes place in chloroplasts where most OPDA exists as free and esterified lipids, but only at low levels. OPDA is only present in plant tissue at nanogram levels [112] and the degradation could easily happen during extraction. Hence, it is difficult to extract and purify a large amount of this compound directly from

plant tissues. OPDA can be successfully produced in the laboratory through organic synthesis [113], however, the published process is complicated and consists of many steps. Therefore, at the start of this research, neither natural nor organic synthesis was reviewed as viable for large-scale commercial production of the compound for antifungal applications. Consequently, a new approach needed to be developed to enable the production of a new generation of agrochemicals based on the modification of OPDA.

The basic idea and fundamental principle underlying our approaches is to modify the chemical structure of OPDA, keeping the functional cyclopentenone ring and simplifying the substituents whilst preserving the biological and antifungal activities of the molecule. Undoubtedly, the electrophilic cyclopentenone ring is the key structural motif that needed to be retained in the design and development of new compounds. The substituent on C9 of OPDA is a hydrophobic carbon chain terminated with a carboxyl group, which is known to be related to the membrane permeability of OPDA [44]. The hydrophobic carbon chain provides the lipophilicity which is balanced by the hydrophilic moiety of OPDA. The terminal carboxyl group forms an ester with glycerol and plays an important role in  $\beta$ -oxidation during metabolism. In addition, the formation of OPDA-CoA esters makes it easier to traverse the membrane of peroxisomes [44]. Similar to OPDA, another 16-carbon cyclopentenoic acid present at low levels in plants, dinor 12-oxo-phytodienoic acid (dnOPDA), is derived from plastid 16:3 fatty acids rather than by  $\beta$ -oxidation of the 18-carbon OPDA [114]. With respect to different chain

length, dnOPDA is 2C shorter than OPDA. However, both free and esterified dnOPDA were reported to have similar behaviour to OPDA [59] in plants but their antifungal activities have not been evaluated. The other substitute on C13 of OPDA is the pentenyl side-chain and very little is known about its role. With a similar structure to OPDA, the 15,16-dihydro-12-oxo-phytodienoic acid (DH-OPDA), which was proposed to be generated from linoleic acid (18:2 n-6), has a saturated pentyl substituent on C13 and is involved in a defence signalling pathway parallel to OPDA but somewhat lower in terms of overall activity [115]. Another function of the pentenyl side-chain on C13 could be that the presence of the double bond (C15=C16) favours the cyclisation similar to the function of the homoallylic cis double bond involved in both enzymatic and non-enzymatic 1,3-cyclisation during synthesis [84, 116]. The delocalisation of this double bond assists in epoxide opening and undergoes subsequent cyclisation catalysed by the enzyme AOC [117]. So far, the side chain on C9 is known to be important in the activities of OPDA and closely associated with its bio-activity. The pentenyl substituent on C13 seems to enable the formation of OPDA. However, limited information is known on how it contributes to the biological properties of OPDA.

According to two common properties of RES, electrophilicity and lipophilicity, the preferred modification is to synthesise a compound lacking a substituent on the C13 of OPDA, i.e. compounds composed of just the cyclopentenone ring along with hydrophobic carbon chains of varying lengths. Altering the length of the hydrophobic chain will mainly affect the lipophilic properties of the molecule. Appropriate



lipophilicity is required for antifungal performance of the compound and in this project it is determined by the length of the hydrophobic chain. Experimentally, the log *P* value of most of commercial agrochemicals is lower than 5 and was suggested to be controlled between 3 and 4.5 to achieve high permeability to cells [1]. The calculated log *P* value of the lead molecule, OPDA is around 4.6. A low lipophilicity is helpful for solubility of the compound in tissue and transportation of the compound to reach infected sites in plants. Another reason for maintaining low lipophilicity is the increase in toxicity with increasing lipophilicity [100, 118]. Furthermore, the shorter the chain, the shorter the half-life of the compound, this could translate to the compound having less adverse effects on the environment. Bearing in mind the need to control two common properties of the designed compound (electrophilicity and lipophilicity), a targeted organic synthesis will be more efficient and economical than random screening.

The synthetic design centred therefore on producing compounds with a cyclopentenone ring along with a hydrophobic carbon chain substituent on the C4 of the cyclopentenone ring with varying lengths to control the log *P* value to less than 5. Some designed target compounds to be synthesised for evaluation of antifungal activity are given in Figure 1.13 and their log *P* values were calculated by Molinspiration log *P* calculator.

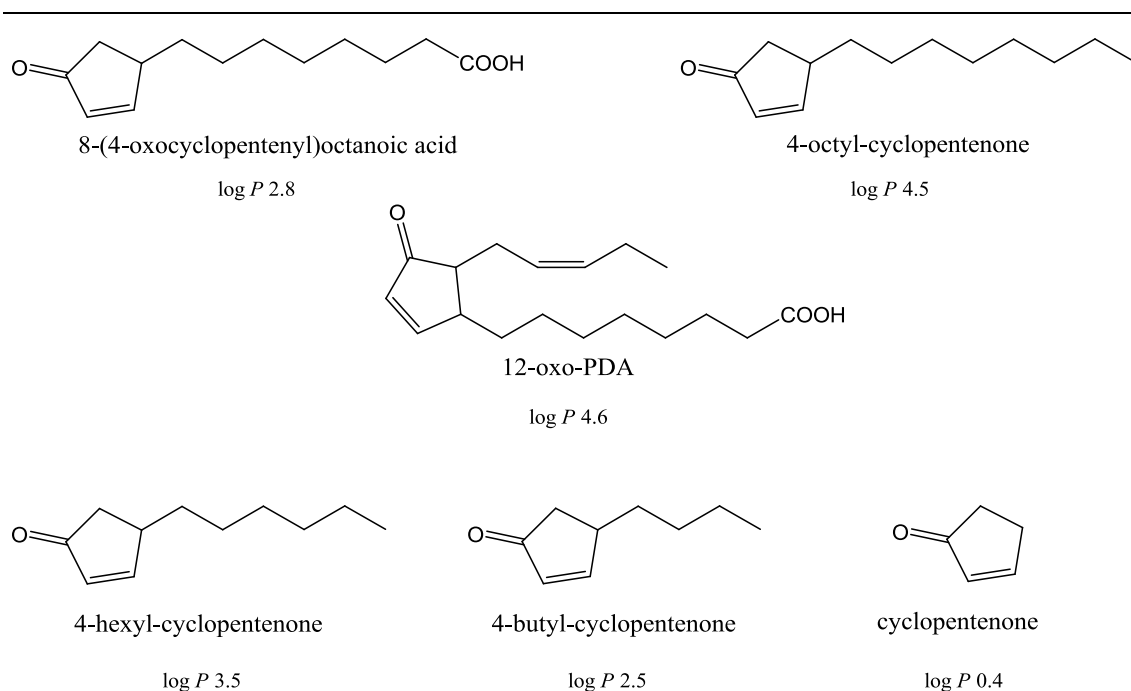


Figure 1.13 Designed target compounds. The value of log *P* was calculated by Molinspiration log *P* calculator.

## 1.6 Aims

The aim of this project was to synthesise compounds with a cyclopentenone ring and different length alkyl chains on the C4 of the cyclopentenone ring. These compounds have a similar chemical structure to OPDA and may thus mimic its natural antifungal properties. These designed compounds needed to be highly effective on specific targeted fungal pathogens rather than possessing a general toxicity towards many organisms, which would thus minimise their side effects on users. Like many naturally occurring compounds, the ready biodegradation of these synthetic compounds will make them environmentally friendly by reducing persistent application residue. In

addition, the synthetic pathway for the production of these compounds should have as few steps as possible, which favours large scale manufacture and commercial application. The main work focuses on the following research:

- Synthesis of OPDA analogues
- Screening of synthesised OPDA analogue for antifungal activity
- Investigation into the mode of action of active compounds
- Antimicrobial activity of (E)-2-alkenals

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# Chapter 2

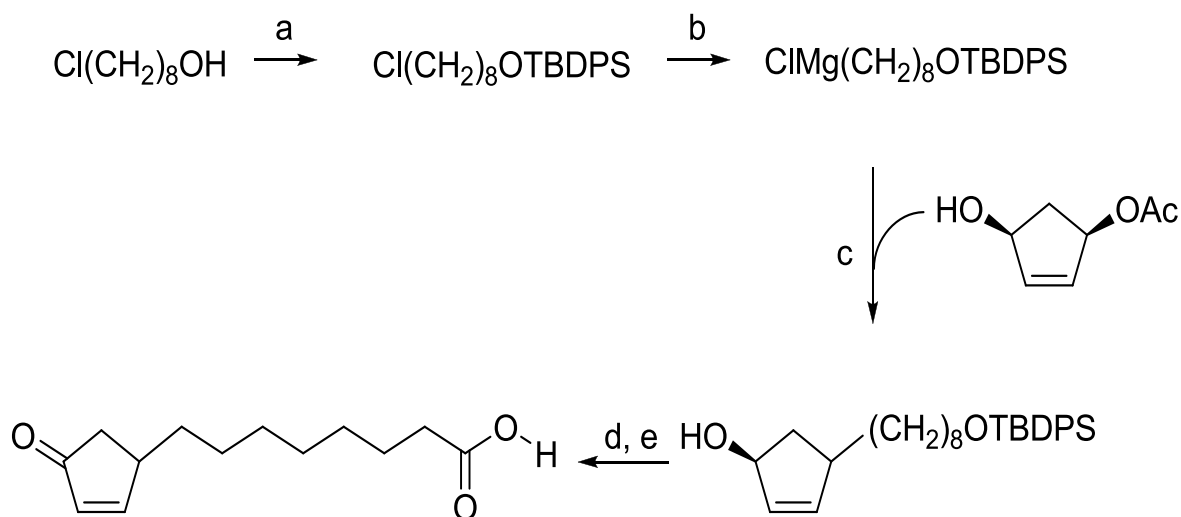
## Organic Synthesis and Characterisation of Target Compounds

### 2.1 Introduction

The Nazarov and Pauson-Khand reactions provide two common methods to synthesise cyclopentenone rings [94, 119, 120], however, neither of them have been used to synthesise OPDA. Instead, other methods have been developed to synthesise OPDA and three synthetic pathways were published by Grieco [121], Ernst [122] and Kobayashi [113, 123]. In this project, the synthetic pathway published by Kobayashi was simplified and used for the preparation of the target compounds.

The most recent method of synthesising OPDA [113] starts with (1R,3S)-(+)-cis-4-cyclopentene-1,3-diol-1-acetate and requires several further steps to complete. As mentioned in the introduction, the function of the pentenyl side group on the cyclopentenone ring is not clear, thus the hydrophobic long carbon chain on the cyclopentenone ring can be installed first of all. The synthesis can then be generally simplified to less than five steps to produce the target compounds in Figure 1.13. Copper-catalysed installation of an alkyl chain onto the cyclopentene ring published by Kobayashi [124], provides a simple method for the addition of varying lengths of

carbon chains to the cyclopentenone ring which, in turn, should change the balance between the hydrophilic and hydrophobic portions of the molecule. Target compounds were synthesised as given below (Scheme 2.1).



Scheme 2.1 Pathway to synthesise compound simplified from OPDA. Reagents and conditions: (a) imidazole (1.5 equiv), TBDPS-Cl DMF, RT; (b) Mg, 1,2-dichloroethane; (c) CuI, THF, -18 °C; (d) TBAF, 4Å molecular sieves, 55 °C; (e) CrO<sub>3</sub>/H<sub>2</sub>SO<sub>4</sub>, acetone.

### 2.1.1 Grignard reagent

The important step in the copper-catalysed addition of the alkyl chain to the cyclopentenone ring is the preparation of the corresponding Grignard reagent. The Grignard reaction is widely used to form carbon-carbon bonds and some carbon-heteroatom bonds. Preparation of a Grignard reagent is achieved via reaction between an alkyl or aryl halide and magnesium. This is a relatively simple reaction, however reaction conditions are critical. Moisture inhibits the reaction by quenching the reagent

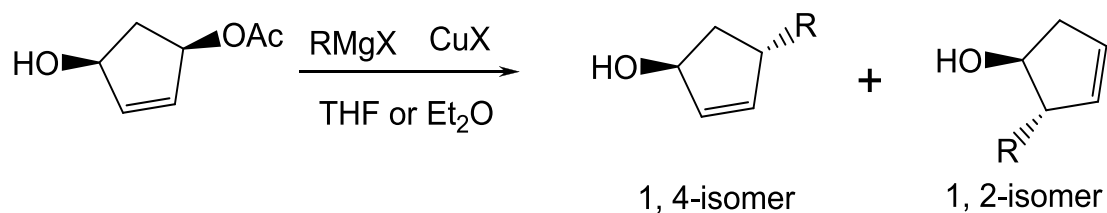
to the corresponding hydrocarbon, so the synthesis should be conducted using dry solvents and under an inert atmosphere. Other factors can also influence the preparation of Grignard reagent [125]:

1. Quantity of the magnesium. Usually, a 5-10% stoichiometric excess, relative to the alkyl halide of fresh magnesium turnings are used.
2. Activators and inhibitors. In order to increase the activity of the magnesium, iodine and ethyl bromide are often used as activators. The concentration of the activator and the reaction temperature will affect the efficiency of the activation process.
3. Effect of halide purity and of the rate of its addition. High purity of the halide and slow addition are required for carrying out a high yielding reaction. In addition, vigorous stirring is necessary to eliminate the local overheating that can occur during this exothermic reaction.
4. Effect of the nature of the halogen and of the alkyl group. Given the same organic moiety, iodides react more readily with magnesium than bromides do, which in turn are more active than chlorides. This order of reactivity is entirely consistent with any nucleophilic substitution reaction employing these leaving groups.

### **2.1.2 Installation of alkyl group**

The synthetic pathway for the larger compounds starts with (1R, 3S)-(+)-cis-4-cyclopentene-1,3-diol-1-acetate, with the key step being the copper-catalysed addition of the alkyl chain onto the unsaturated five-member ring

(scheme 2.2).



Scheme 2.2 Installation of alkyl group [124]. RMgX (X=Cl, Br), CuX (X=CN, I), R: alkyl chain

This reaction generates trans 1,4- or 1,2- isomers (Scheme 2.2). The ratio of these two regioisomeric products and their yields of formation depends on the composition of RMgX (X= Cl, Br)/CuX (X= CN, I) and the choice of solvent (Et<sub>2</sub>O or THF) [124]. Typically, the 1,4-isomer predominates and is the major product. This is fortunate as this regioisomer is the precursor of the target compounds. The composition of RMgX/CuX and the choice of solvent were found to be the most important factors for increasing the yield of the 1,4-isomer. According to the previous report [124], several combinations of reagent/solvent were tested, and the two of which gave the optimum yield and highest ratio of 1,4-isomer: 1,2-isomer are shown in Table 2.1.

Since there was very little difference between the CuCN and CuI catalysed reaction, CuI was used to catalyse the reaction since it is far less toxic than CuCN. Compared with Et<sub>2</sub>O, THF is more polar and tends to determine the regioselectivity, preferentially forming the 1,4-isomer in this reaction. In addition, it was found that a bromide Grignard reagent will influence the regioselectivity of the installation and increase the

Table 2.1 Synthetic conditions of the reactions with high yield of 1,4-isomer [124].

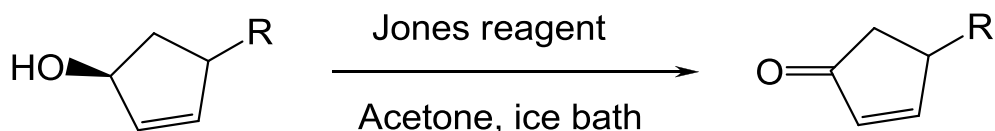


yield of 1,2-isomer [124], thus chloride Grignard reagent is needed to achieve high yield of 1,4-isomer. This is an efficient method to add an alkyl chain onto the cyclo-ring. In this project, a  $\text{RMgCl/CuI}$  (10 mol %) in THF reaction system was used to add an alkyl substituent to the cyclo-ring and achieve high yield of 1,4-isomer followed by the oxidation of hydroxyl group to produce the cyclopentenone ring.

### 2.1.3 Hydroxyl group oxidation

Jones reagent ( $\text{CrO}_3/\text{H}_2\text{SO}_4$ ) is an ideal oxidation reagent for the oxidation of primary and secondary hydroxyl groups to carboxylic acids and ketones, respectively, without influencing the double bond in cyclopentenone ring (Scheme 2.3). Jones reagent is prepared by dissolving  $\text{CrO}_3$  into concentrated  $\text{H}_2\text{SO}_4$  and diluting this mixture with distilled water.





Scheme 2.3 Hydroxyl group oxidation.

## 2.2 Materials

All reactions involving moisture-sensitive and/or air-sensitive reagents were conducted under a nitrogen atmosphere, in oven dried (120°C) glassware. Tetrahydrofuran (THF) was dried by refluxing over sodium/benzophenone and was distilled immediately prior to use. Distilled water was used throughout. All other reagents were used as received from chemical suppliers Sigma-Aldrich Chemical Co. Acros, Fluka and Fisher. Thin layer chromatography (TLC) and column chromatography were carried out using Merck Kieselgel 60 TLC plates and Merck Kieselgel 60 silica gel, respectively.

Infra red (IR) spectra were recorded on a PerkinElmer FT-10 FT-IR spectrometer. Nuclear Magnetic Resonance (NMR) spectra,  $^1\text{H}$  NMR (300 MHz) and  $^{13}\text{C}$  NMR (75.58 MHz), were recorded on a Bruker AC300 spectrometer. Deuterated chloroform ( $\text{CDCl}_3$ ) was used as the solvent (7.24 for  $^1\text{H}$  and 77.0 for  $^{13}\text{C}$  respectively) and chemical shift values are reported relative to TMS.  $^{13}\text{C}$  NMR spectra were recorded using the PENDANT program [126]. Mass spectra (MS) were recorded by Karen Farrow (LHS, Aston University, UK). A Sartorius ME36S microbalance was used to

measure the weight of synthesis products. The chemicals used in organic synthesis and their supplier are listed in Table 2.2.

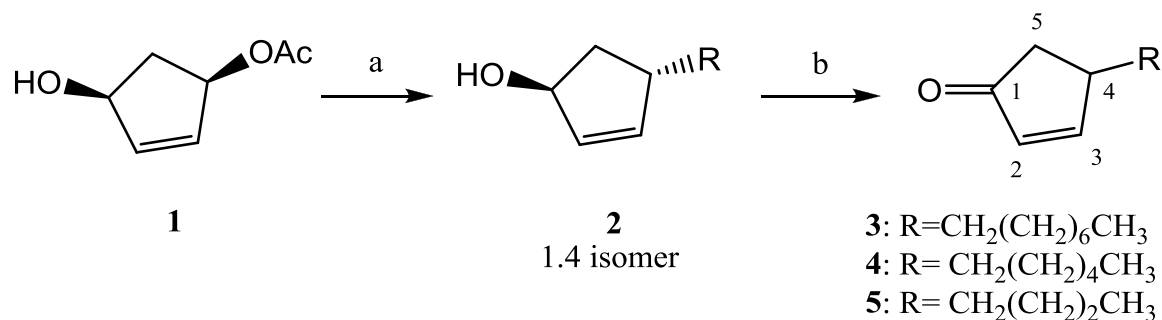
Table 2.2 Chemicals used in the organic synthesis.

Chemicals	Manufacture
(1R,3S)-(+)-cis-4-cyclopentene-1,3-diol 1-acetate $\geq 99\%$	Sigma-Aldrich
8-Chloro-1-octanol 98%	Sigma-Aldrich
Butylmagnesium chloride 2.0M in THF	Sigma-Aldrich
Hexylmagnesium chloride 2.0M in THF	Sigma-Aldrich
Octylmagnesium chloride 2.0M in THF	Sigma-Aldrich
tert-Butyl(chloro)diphenylsilane 98%	Sigma-Aldrich
Ammonium chloride 99.5%	Sigma-Aldrich
Ammonium Hydroxide solution 28%	Sigma-Aldrich
Tetrabutylammonium fluoride solution 1.0 M in tetrahydrofuran	Sigma-Aldrich
Silica Gel 200-400mesh 60Å for column chromatography	Sigma-Aldrich
Celite 521	Acros
Chromium (VI) trioxide 99+%	Acros
Copper (I) iodide 98%	Acros
1, 2-Dichloroethane puriss over MS ( $H_2O \leq 0.005\%$ )	Fluka

### 2.3 Synthetic procedure of target compounds

A simplified version of Kobayashi's synthesis of OPDA [113] was employed to produce the target compounds **3**, **4**, **5**, and **8** (Scheme 2.4 and 2.5). FT-IR,  $^1H$  NMR,  $^{13}C$  NMR spectra and APCI-MS of target compounds can be found in the Appendices.

### 2.3.1 Synthesis of compounds 3-5



Scheme 2.4 Synthesis of compounds **3-5**. Reaction conditions: (a) CuI, THF, -18 °C, (b) CrO<sub>3</sub>/H<sub>2</sub>SO<sub>4</sub>, acetone.

The synthetic method was modified from Kobayashi's method [124]. A two necked round bottomed flask was charged with CuI (14.0 mg, 0.071 mmol), suspended in dry THF (1.8 mL), the resultant slurry stirred magnetically and cooled to -18 °C (NaCl/ice bath: 1:3). A solution of RMgCl (octylmagnesium chloride in THF 1.5 mL, 1.39 M) was then added dropwise whilst maintaining this temperature. Stirring was then continued for a further 20 minutes at -18 °C, after which a solution of (1R,3S)-(+)-*cis*-4-cyclopentene-1,3-diol 1-acetate **1** (100.3 mg) in THF (0.2 mL) was added dropwise. The reaction was then stirred at -18°C for 5 hours before being quenched by the addition of a saturated aqueous solution of NH<sub>4</sub>Cl (5 mL) and an aqueous solution of NH<sub>4</sub>OH (28%, 5 mL) with vigorous stirring. The crude product was extracted with Et<sub>2</sub>O (3 x 25 mL). And the combined organic extracts were then dried by standing over anhydrous MgSO<sub>4</sub>, collected by filtration and concentrated under reduced pressure to furnish the crude product as a colourless oil. The crude product was purified by column chromatography (Hexane/EtOAc: 75/25) to give the pure and major 1,

4-isomer **2** (132.1 mg, 0.674 mmol) (TLC Hexane/EtOAc: 75/25,  $R_f$  = 0.40). This product was used in subsequent oxidation reactions directly without further analysis.

The pure 1, 4-isomer **2** (132.1 mg 0.674 mmol) was dissolved in freshly distilled acetone (1.7 mL) and the resultant solution cooled to 0 °C. Freshly prepared Jones reagent was then added dropwise until the colour of the reagent persisted and the resultant orange solution were stirred for a further 30 minutes at 0 °C. After this time, the reaction was quenched by the addition of 2-propanol, and the resultant mixture filtered through a pad of silica gel using Et<sub>2</sub>O as the eluent. The filtrate was washed with brine (3 x 25 mL) before being dried by standing over anhydrous MgSO<sub>4</sub>, collected by filtration and concentrated under reduced pressure to furnish the crude products **3-5** as yellow oils. The crude products were purified by column chromatography (Hexane/EtOAc: 50/50) to give the pure compounds **3-5**.

**4-octyl-cyclopentenone (3)** was obtained as a yellow oil (47.3 mg, 0.24 mmol, yield 35.6%). (TLC Hexane/EtOAc: 50/50  $R_f$  = 0.75). IR (neat):  $\nu_{\max}/\text{cm}^{-1}$  3052, 1716, 1669, 1178. <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>):  $\delta$  0.97 (t, 3, <sup>3</sup>J = 6.9 Hz, CH<sub>3</sub>), 1.28-1.80 (m, 14, CH<sub>2</sub>), 2.07 (dd, 1, <sup>2</sup>J<sub>5a,5b</sub> = 18.9 Hz, <sup>3</sup>J<sub>5a,4</sub> = 2.1 Hz, 5-H<sub>a</sub>), 2.61 (dd, 1, <sup>2</sup>J<sub>5b,5a</sub> = 18.9 Hz, <sup>3</sup>J<sub>5b,4</sub> = 6.3 Hz, 5-H<sub>b</sub>), 2.95-3.08 (m, 1, 4-H), 6.22 (dd, 1, <sup>3</sup>J<sub>3,2</sub> = 5.7 Hz, J = 2.1 Hz, 3-H), 7.71 (dd, 1, <sup>3</sup>J<sub>2,3</sub> = 5.7 Hz, J = 2.4 Hz, 2-H). <sup>13</sup>C NMR (75.58 MHz, CDCl<sub>3</sub>, PENDANT):  $\delta$  14.15 (CH<sub>3</sub>), 22.70 (CH<sub>2</sub>), 27.67 (CH<sub>2</sub>), 29.28 (CH<sub>2</sub>), 29.50 (CH<sub>2</sub>), 29.64 (CH<sub>2</sub>), 31.89 (CH<sub>2</sub>), 34.80 (CH<sub>2</sub>), 41.11 (C-5), 41.56 (C-4), 133.59 (C-3), 168.83 (C-2), 210.20 (C-1).

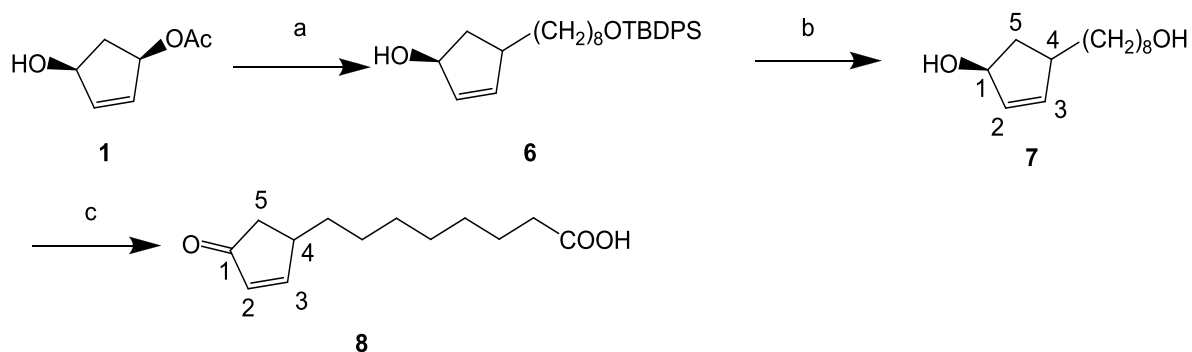
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LRMS (APCI+):  $m/z$  195 ( $[M+H]^+$ ); calculated for  $C_{13}H_{22}O$ : 194.32.

**4-hexyl-cyclopentenone (4)** was obtained as a yellow oil (11.0 mg, 0.066 mmol, yield 25.8%). (TLC Hexane/EtOAc: 50/50  $R_f$  = 0.75). IR (neat):  $\nu_{max}/cm^{-1}$  3046, 1716, 1669, 1181.  $^1H$  NMR (300 MHz,  $CDCl_3$ ):  $\delta$  0.95 (t, 3,  $^3J = 6.9$  Hz,  $CH_3$ ), 1.25-1.72 (m, 10,  $CH_2$ ), 2.05 (dd, 1,  $^2J_{5a,5b} = 18.8$  Hz,  $^3J_{5a,4} = 2.0$  Hz, 5- $H_a$ ), 2.59 (dd, 1,  $^2J_{5b,5a} = 19.0$  Hz,  $^3J_{5b,4} = 6.4$  Hz, 5- $H_b$ ), 2.93-3.06 (m, 1, 4- $H$ ), 6.22 (dd, 1,  $^3J_{3,2} = 5.6$  Hz,  $J = 2.0$  Hz, 3- $H$ ), 7.71 (dd, 1,  $^3J_{2,3} = 5.7$  Hz,  $J = 2.4$  Hz, 2- $H$ ).  $^{13}C$  NMR (75.58 MHz,  $CDCl_3$ , PENDANT):  $\delta$  14.10 ( $CH_3$ ), 22.63 ( $CH_2$ ), 27.63 ( $CH_2$ ), 29.30 ( $CH_2$ ), 31.73 ( $CH_2$ ), 34.79 ( $CH_2$ ), 41.11 (C-5), 41.55 (C-4), 133.60 (C-3), 168.79 (C-2), 210.13 (C-1). LRMS (APCI+):  $m/z$  167 ( $[M+H]^+$ ); calculated for  $C_{11}H_{18}O$ : 166.26.

**4-butyl-cyclopentenone (5)** was obtained as a yellow oil (9.2 mg, 0.067 mmol, yield 25.7%). (TLC Hexane/EtOAc: 50/50  $R_f$  = 0.75). IR (neat):  $\nu_{max}/cm^{-1}$  3049, 1716, 1669, 1183.  $^1H$  NMR (300 MHz,  $CDCl_3$ ):  $\delta$  0.99 (t, 3,  $^3J = 6.9$  Hz,  $CH_3$ ), 1.26-1.74 (m, 6,  $CH_2$ ), 2.14 (dd, 1,  $^2J_{5a,5b} = 18.8$  Hz,  $^3J_{5a,4} = 2.1$  Hz, 5- $H_a$ ), 2.61 (dd, 1,  $^2J_{5b,5a} = 18.9$  Hz,  $^3J_{5b,4} = 6.3$  Hz, 5- $H_b$ ), 2.90-3.08 (m, 1, 4- $H$ ), 6.22 (dd, 1,  $^3J_{3,2} = 5.6$  Hz,  $J = 2.0$  Hz, 3- $H$ ), 7.72 (dd, 1,  $^3J_{2,3} = 5.6$  Hz,  $J = 2.4$  Hz, 2- $H$ ).  $^{13}C$  NMR (75.58 MHz,  $CDCl_3$ , PENDANT):  $\delta$  14.01 ( $CH_3$ ), 22.72 ( $CH_2$ ), 29.84 ( $CH_2$ ), 34.48 ( $CH_2$ ), 41.11 (C-5), 41.53 (C-4), 133.61 (C-3), 168.85 (C-2), 210.42 (C-1). LRMS (APCI+):  $m/z$  139 ( $[M+H]^+$ ); calculated for  $C_9H_{14}O$ : 138.21.

### 2.3.2 Synthesis of 8-(4-oxocyclopentenyl)octanoic acid (**8**)



Scheme 2.5 Synthesis of 8-(4-oxocyclopentenyl)octanoic acid (**8**). Reaction conditions: (a) CuI, ClMg(CH<sub>2</sub>)<sub>8</sub>OTBDPS, THF, -18 °C; (b) TBAF, 4 Å molecular sieves, 55 °C; (c) CrO<sub>3</sub>/H<sub>2</sub>SO<sub>4</sub>, acetone.

#### Grignard preparation of ClMg(CH<sub>2</sub>)<sub>8</sub>OTBDPS (Scheme 2.1)

A one-necked flask (25 ml) was charged with 8-chloro-1-octanol (1.0 g, 6.0 mmol), TBDPSCl (2.0 g, 7.2 mmol) and DMF (10 ml) in ice bath with stirring. Imidazole (1.0 g, 15.0 mmol) was added slowly and stirred until complete dissolution in DMF was achieved. The reaction was carried out at room temperature (RT) overnight. The reaction was quenched by the addition of water and extracted with DCM (25 ml x 3) then the combined extracts were washed with HCl solution (1 M) (25 ml x 3). After being dried with MgSO<sub>4</sub>, the mixture was concentrated to a transparent oil. The crude oil was purified by column chromatography (Hexane/EtOAc: 90/10) to give the pure oil (Cl(CH<sub>2</sub>)<sub>8</sub>OTBDPS, 2.27 g, 5.64 mmol, yield: 94%) (TLC Hexane/EtOAc: 90/10, R<sub>f</sub> = 0.86). IR (neat):  $\nu_{\max}/\text{cm}^{-1}$  3071.3, 2931.7, 2857.5, 1428.0, 1111.8, 701.8. <sup>1</sup>HNMR (300 MHz, CDCl<sub>3</sub>):  $\delta$  1.13 (s, 9, CH<sub>3</sub>), 1.31-1.54 (m, 8, CH<sub>2</sub>), 1.63 (m, 2, CH<sub>2</sub>), 1.83 (m, 2, CH<sub>2</sub>), 3.61 (t, 2, <sup>3</sup>J<sub>HCCH</sub> = 6.7 Hz, CH<sub>2</sub>), 3.73 (t, 2, <sup>3</sup>J<sub>HCCH</sub> = 6.7 Hz, CH<sub>2</sub>), 7.41-7.54 (m, 6,

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Ph-*H*), 7.70-7.79 (m, 4, Ph-*H*).  $^{13}\text{C}$ NMR (75.58 MHz,  $\text{CDCl}_3$ , PENDANT):  $\delta$  22.34 (Si-C), 25.71 ( $\text{CH}_2$ ), 26.60 ( $\text{CH}_2$ ), 26.92 ( $\text{CH}_3$ ), 28.88 ( $\text{CH}_2$ ), 29.22 ( $\text{CH}_2$ ), 32.55 ( $\text{CH}_2$ ), 32.67 ( $\text{CH}_2$ ), 45.23 ( $\text{CH}_2$ ), 63.97 ( $\text{CH}_2$ ), 127.61 (Ph-C), 129.54 (Ph-C), 133.97 (Ph-C), 135.63 (Ph-C). HRMS (TOF MS ES<sup>+</sup>):  $m/z$  403.221 ( $[\text{M}+\text{H}]^+$ ); calculated for  $\text{C}_{24}\text{H}_{35}\text{OClSi}$ : 402.214.

A two necked round bottom flask (25 ml) was charged with fresh magnesium turnings (54.1 mg) and dry THF (0.5 ml) which just covered the turnings. Several drops of  $\text{Cl}(\text{CH}_2)_2\text{Cl}$  (0.06 ml) were added, then the mixture was heated up to 50 °C for a few minutes without stirring. When the heat removed, effervescence was observed from the surface of the turnings and solution became grey and turbidity.  $\text{TBDPSO}(\text{CH}_2)_8\text{Cl}$  (0.45 g) in THF (1.5 ml) was added dropwise slowly with vigorous stirring. Then heat the reaction up to refluxing temperature and leave it overnight. Grignard reagent ( $\text{ClMg}(\text{CH}_2)_8\text{OTBDPS}$ ) prepared was used in next step without any purification and characterisation.

#### **4-(8-hydroxyoctyl)cyclopentenol (7)**

The synthetic pathway was simplified from Kobayashi's method [123]. Grignard reagent,  $\text{ClMg}(\text{CH}_2)_8\text{OTBDPS}$ , (2.0 mL, 0.53 M in THF, 1.06 mmol) was added slowly to a slurry of CuI (10.5 mg, 0.054 mmol) in THF (1.0 mL) at -18 °C. The reaction mixture was then stirred for a further 20 minutes at -18 °C, after which time a solution of (1R, 3S)-(+)-*cis*-4-cyclopentene-1,3-diol-1-acetate **1** (50 mg, 0.35 mmol) in THF

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(0.2 mL) was added dropwise. The resultant reaction mixture was then stirred at  $-18\text{ }^{\circ}\text{C}$  for 5 hours before being quenched by the addition, with vigorous stirring, of a saturated aqueous solution of  $\text{NH}_4\text{Cl}$  (5 mL) and an aqueous solution of  $\text{NH}_4\text{OH}$  (28%, 5 mL). The crude product was extracted with EtOAc (3 x 10 mL). The combined organic extracts were dried by standing over anhydrous  $\text{MgSO}_4$ , collected by filtration and concentrated under reduced pressure to furnish the crude product as an oil. After that, the crude product was purified by column chromatography (Hexane/EtOAc: 75/25) to afford the major 1, 4-isomer **6** (142.5 mg, 0.316 mmol) (TLC Hexane/EtOAc: 75/25,  $R_f = 0.35$ ). Finally, the purified product was used directly in a subsequent deprotection step without being subjected to any analysis.

A mixture of **6** (107.9 mg, 0.24 mmol), tetrabutylammonium fluoride (TBAF) (1.20 mL, 1.0 M in THF, 1.20 mmol), 4Å molecular sieves (89 mg) and THF (2.50 mL) was stirred and heated to  $55\text{ }^{\circ}\text{C}$  for one hour before being cooled to  $0\text{ }^{\circ}\text{C}$  and diluted by the addition of EtOAc (2.5 mL) and an aqueous solution of saturated  $\text{NH}_4\text{Cl}$  (2.5 mL). The resultant mixture was filtered through a pad of silica gel using EtOAc as the eluent. After filtration, the phases were separated and the aqueous phase was extracted with EtOAc (3 x 25 mL). The combined organic extracts were dried by standing over anhydrous  $\text{MgSO}_4$ , collected by filtration and concentrated under reduced pressure to furnish the crude product as a yellow oil. The crude product was purified by column chromatography (DCM/Acetone: 75/25) to give 4-(8-hydroxyoctyl)cyclopentenol **7** (34.7 mg, 0.158 mmol, yield: 68.3%). (TLC DCM/Acetone: 75/25,  $R_f = 0.40$ ). IR (neat):



$\nu_{\max}$  / $\text{cm}^{-1}$  3418, 3034, 1465, 1022.  $^1\text{H NMR}$  (300 MHz,  $\text{CDCl}_3$ ):  $\delta$  1.20-1.51 (m, 14,  $\text{CH}_2$ ), 1.59-1.61 (m, 2,  $\text{OH}$ ), 1.80 (ddd, 1,  $^2J_{5a,5b}=14.0$  Hz,  $^3J_{5a,1}=7.1$  Hz,  $^3J_{5a,4}=5.2$  Hz, 5- $H_a$ ), 1.92 (ddd, 1,  $^2J_{5b,5a}=14.1$  Hz,  $^3J_{5b,4}=7.5$  Hz,  $^3J_{5b,1}=2.9$  Hz, 5- $H_b$ ), 2.82-2.90 (m, 1, 4- $H$ ), 3.71 (dd, 2,  $^2J=12.0$  Hz,  $^3J=6.6$  Hz,  $\text{CH}_2\text{CH}_2\text{OH}$ ), 4.83-4.91 (m, 1, 1- $H$ ), 5.83 (dt, 1,  $^3J_{3,2}=5.4$  Hz,  $J=2.2$  Hz, 3- $H$ ), 5.96 (ddd, 1,  $^3J_{2,3}=5.6$  Hz,  $J=2.0$  Hz,  $J=0.8$  Hz, 2- $H$ ).  $^{13}\text{CNMR}$  (75.58 MHz,  $\text{CDCl}_3$ , PENDANT):  $\delta$  25.76 ( $\text{CH}_2$ ), 27.95 ( $\text{CH}_2$ ), 29.43 ( $\text{CH}_2$ ), 29.59 ( $\text{CH}_2$ ), 29.72 ( $\text{CH}_2$ ), 32.83 ( $\text{CH}_2$ ), 35.92 ( $\text{CH}_2$ ), 40.73 ( $\text{CH}_2$ ), 44.09 (C-4), 63.13 ( $\text{CH}_2\text{-OH}$ ), 77.20 (C-1), 132.30 (C-3), 140.40 (C-2).

**8-(4-oxocyclopentenyl)octanoic acid (8)** 4-(8-hydroxyoctyl)cyclopentenol **7** (34.7 mg 0.158 mmol) was dissolved in freshly distilled acetone (1.0 mL) and the resultant solution cooled 0 °C. Freshly prepared Jones reagent was then added dropwise until the colour of the reagent persisted and the resultant orange solution was stirred for a further 30 minutes at 0 °C. After this time, the reaction was quenched by the addition of 2-propanol (5 mL), and the resultant mixture filtered through a pad of silica gel using  $\text{Et}_2\text{O}$  as the eluent. The filtrate was washed with brine (3 x 25 mL) before being dried by standing over anhydrous  $\text{MgSO}_4$ , collected by filtration and concentrated under reduced pressure to furnish the crude product as a white solid. The crude product was purified by column chromatography (DCM/Acetone: 75/25) to give pure 8-(4-oxocyclopentenyl)octanoic acid **8** as a white crystalline solid (8.7 mg, 0.039 mmol, yield: 24.3%) (TLC DCM/Acetone: 75/25,  $R_f=0.70$ ). Melting Point (MP): 83-85 °C. IR (KBr disc):  $\nu_{\max}$  / $\text{cm}^{-1}$  3430, 3050, 1734, 1698, 1670, 1172.  $^1\text{H NMR}$  (300 MHz,  $\text{CDCl}_3$ ):

$\delta$  1.33-1.68 (m, 12,  $\text{CH}_2$ ), 2.07 (dd, 1,  $^2J_{5a,5b} = 18.9$  Hz,  $^3J_{5a,4} = 2.1$  Hz, 5- $H_a$ ), 2.43 (t, 2,  $J = 7.5$  Hz,  $\text{CH}_2\text{CH}_2\text{COOH}$ ), 2.61 (dd, 1,  $^2J_{5b,5a} = 18.6$  Hz,  $^3J_{5b,4} = 6.3$  Hz, 5- $H_b$ ), 2.95-3.05 (m, 1, 4- $H$ ), 6.22 (dd, 1,  $^3J_{3,2} = 5.7$  Hz,  $J = 1.8$  Hz, 3- $H$ ), 7.75 (dd, 1,  $^3J_{2,3} = 5.4$  Hz,  $J = 2.4$  Hz, 2- $H$ ), 9.00-11.0 (br, 1,  $\text{COOH}$ ).  $^{13}\text{C}$  NMR (75.58 MHz,  $\text{CDCl}_3$ , PENDANT):  $\delta$  24.64 ( $\text{CH}_2$ ), 27.56 ( $\text{CH}_2$ ), 28.97 ( $\text{CH}_2$ ), 29.11 ( $\text{CH}_2$ ), 29.39 ( $\text{CH}_2$ ), 34.02 ( $\text{CH}_2$ ), 34.70 ( $\text{CH}_2$ ), 41.07 (C-5), 41.53 (C-4), 133.62 (C-3), 168.89 (C-2), 179.68 ( $\text{COOH}$ ), 210.41 (C-1). LRMS (APCI NEG):  $m/z$  223 ( $[\text{M}-\text{H}^+]$ ); calculated for  $\text{C}_{13}\text{H}_{20}\text{O}_3$ : 224.30.

## 2.4 Discussion

In the synthesis of 8-(4-oxocyclopentenyl)octanoic acid, tert-butylchlorodiphenylsilane (TBDPS) was used to protect the hydroxyl group because of its stability both under acidic and basic conditions [127]. The preparation of the Grignard reagent,  $\text{ClMg}(\text{CH}_2)_8\text{OTBDPS}$ , was proved to be the most difficult step to accomplish in the whole pathway (Scheme 2.1), since the less active catalyst,  $\text{Cl}(\text{CH}_2)_2\text{Cl}$ , recommended by Kobayashi was used. The purpose of using this catalyst is to control the regioselectivity during the installation step to achieve a high yield of the desired 1,4-isomer [113]. Given the same organic moiety, chlorides have lower reactivity with magnesium than bromides and iodides. In addition, the bulky end hydroxyl protecting group, TBDPS, made the reaction hard to start, so it was time consuming to establish the optimum concentration of catalyst and temperature to initiate the Grignard reaction.

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The key point in the initiation of the Grignard formation reaction is to achieve high concentration of  $\text{Cl}(\text{CH}_2)_2\text{Cl}$  by adding this catalyst into the mixture of magnesium turnings in only a very small volume of dry THF. The freshly prepared Grignard reagent was used immediately for the chain installation.

Side chain installation is the key step of the whole pathway and the reaction conditions should be strictly controlled.  $\text{CuI}$  is used as the catalyst rather than  $\text{CuCN}$  which is much more toxic. Mechanistically the  $\sigma$ -allylcopper intermediates was produced by coordination of the copper reagent to the olefin moiety present within the starting molecule, (1R,3S)-(+)-cis-4-cyclopentene-1,3-diol 1-acetate, followed by the oxidative, nucleophilic substitution of the Grignard reagent at C1 position of the ring [124].

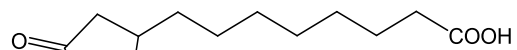
Jones reagent ( $\text{CrO}_3/\text{H}_2\text{SO}_4$ ) was used for oxidation in this project. The yield of this step is lower than expected; only 20-30%. This finding might be caused by the moisture of improperly prepared acetone. Distilled acetone should be dried using molecular sieves before use as the solvent for the Jones reagent. In future work, distilled acetone needs to be dried to overcome this problem.

The 8-(4-oxocyclopentenyl)octanoic acid synthesised in this project is a new molecule that has not been published before, however, 4-octyl-cyclopentenone has been synthesised as an intermediate which is subsequently converted to other target compounds through different synthetic pathways [128, 129]. The biological properties

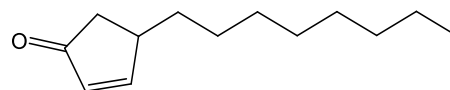
of this cyclopentanone intermediate (4-octyl-cyclopentenone) are not disclosed. Compared with the two synthetic routes published, the synthetic pathway in this project is much shorter and simpler. Interestingly, the same final target compound and an intermediate (4-octyl-cyclopentenone) were synthesised using different synthetic routes in these two published works [128, 129]. The final target compound is alkylidene cyclopentanone prostaglandin, TEI 9826, which is known to have anti-tumour activity. With respect to a similar synthetic method, it is possible to add different chains onto the ring of 4-octyl-cyclopentenone to generate prostaglandins and phytoprostanes-like compounds, which may be of interest in other applications. Therefore, 4-octyl-cyclopentenone could also be used as a multifunctional intermediate for other interesting biological compounds and potential antimicrobial agents.

## **2.5 Conclusion**

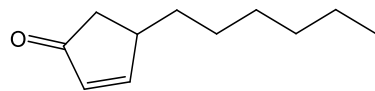
The successful synthesis of target compounds listed below (Figure 2.1) was achieved by successfully modifying of Kobayashi's method [124]. All of the target compounds were characterised by IR, NMR and MS (see Appendix). The structural similarity of these molecules to OPDA is evident and they were tested for antifungal activity in order to assess their efficacy in comparison with the lead compound, OPDA.



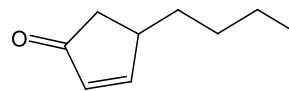
8-(4-oxocyclopentenyl)octanoic acid (8)



4-octyl-cyclopentenone (3)



4-hexylcyclopentenone (4)



4-butylcyclopentenone (5)

Figure 2.1 Synthetic compounds mimic of OPDA.

# Chapter 3

## Antifungal Activity Assays

### 3.1 Introduction

#### 3.1.1 Fungal pathogens

Fungi are now recognised as one of five kingdoms of life and they differ from plants and animals with respect to unique biological features. So far, more than 70,000 species of fungi have been described and about 700 new species are discovered each year [111]. Some of them we are familiar with in daily life, such as mushrooms, *Penicillium*, yeasts and so on. The fungi studied by mycologists include three groups of fungus-like organisms, the straminipila, the slime moulds and the true fungi. Formal classification and nomenclature of fungi are set out in Figure 3.1.

Humans have cultivated and harvested fungi for millennia as food source and for the use in fermentation processes. For example, mushrooms are an important food; yeasts are required for fermentation of wine and bread making; fungal derived antibiotics are essential against some diseases. However, many fungi are harmful to human society, especially serious fungal pathogens of cultivated plants. For example, a million people died of starvation in Ireland when the late blight fungus, *Phytophthora infestans*,



Figure 3.1 Flowchart summarising classification of fungus-like organisms [130].

infected potatoes in the 1840s [131]. Some fungi are necrotrophic pathogens, like *Botrytis* spp [132], which first kill plant cells by producing toxins, then feed upon the host plants by producing enzymes to degrade the constituents. In comparison with necrotrophs, in biotrophic associations, the plant tissues are invaded but remain alive. Necrotrophic parasites survive and are easily cultured in the absence of their hosts, whereas most biotrophic pathogens can only live together with their hosts.

In order to control fungal growth and minimise damage to crops, a variety of fungicides have been developed and applied for thousands of years. Most research on the interaction of fungi and plants has been motivated by crop loss caused by parasitic or pathogenic fungi through two distinct infection sites, aerial surfaces and root surfaces.

The first step in the development of fungicides is targeting the pathogens, since for each crop, there are more than one pathogen causing disease. Fungicide pathogens which become targets for development are driven by their economic impact, such as disease severity, value of economic loss and commercial risk. The grapevine fungicide market, for example, with an estimated a million dollar market in Europe, is largely targeted at *Botrytis cinerea* infections.

In this research, four major plant fungal pathogens of phylum Ascomycota were chosen to test the antifungal activity of the synthesised compounds, they were: *F. oxysporum*, *Fusarium oxysporum*; *A. brassicicola*, *Alternaria brassicicola*; *C. herbarum*, *Cladosporium herbarum* and *B. cinerea*, *Botrytis cinerea*.

#### *Fusarium oxysporum*

*Fusarium oxysporum* is a fungus that causes *Fusarium* wilt disease in more than a hundred species of plants. *F. oxysporum* has been characterised as causing the following symptoms: vascular wilt, yellows, corm rot, root rot, and damping-off. The most important of these is vascular wilt. Losses often exceed 30,000 tons of canning tomatoes, or 10% to 35% of the crop in the USA [133]. In solid media culture, such as potato dextrose agar (PDA), the aerial mycelium of *F. oxysporum* first appears white and then may change to a variety of colours - ranging from violet to dark purple - according to the strain (Figure 3.2).



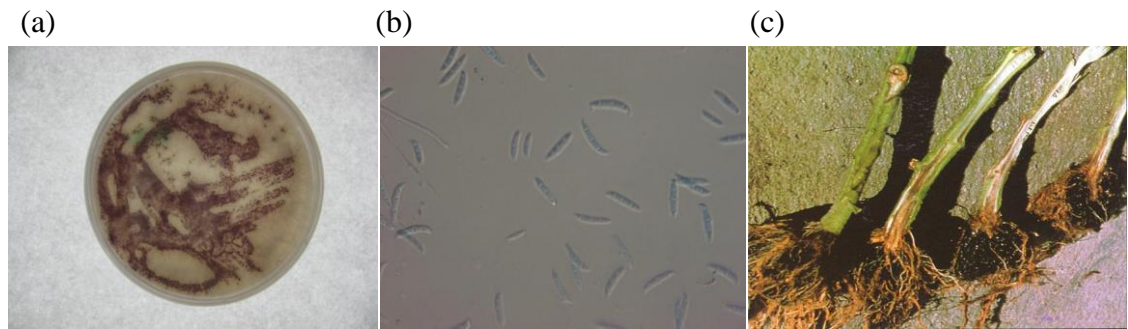


Figure 3.2 *Fusarium oxysporum*. (a) The culture of *F. oxysporum* on PDA, (b) Spores and (c) Rot root of plants infected by *F. oxysporum*.

### *Alternaria brassicicola*

*Alternaria brassicicola* is a ubiquitous necrotrophic fungal pathogen. *A. brassicicola* causes black spot disease (also called dark leaf spot) on virtually every important cultivated *Brassica* species including broccoli, cabbage, canola, and mustard. It is of worldwide economic importance resulting occasionally in 20-50% yield reductions in crops [133]. When cultured in PDA at 25 °C, *A. brassicicola* forms dark green to black colonies with an abundance of spores (Figure 3.3).

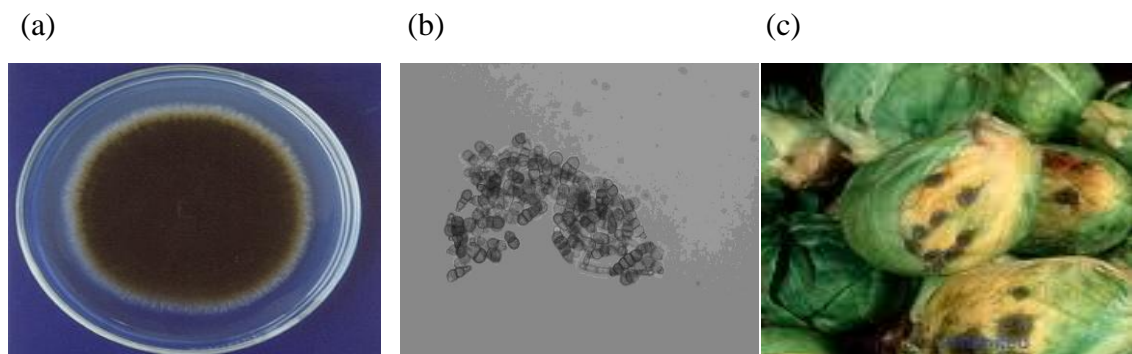


Figure 3.3 *Alternaria brassicicola*. (a) The culture of *A. brassicicola* on PDA, (b) Spores and (c) Leaf Spot (*A. brassicicola*) on harvested Brussel Sprouts (*Brassica oleracea*).

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*Cladosporium herbarum*

*Cladosporium herbarum* is the most frequently encountered mold in the air. It causes leaf mold and is one of the most common colonisers of dying and dead plants and also occurs in various soil types [133]. Also, this mold is frequently found in uncleaned refrigerators, foodstuffs, on moist window frames, in houses with poor ventilation, with straw roofs, situated in low, damp areas. *Cladosporium herbarum* is cultured on PDA and the colour of the colony ranges from dark green to black (Figure 3.4).

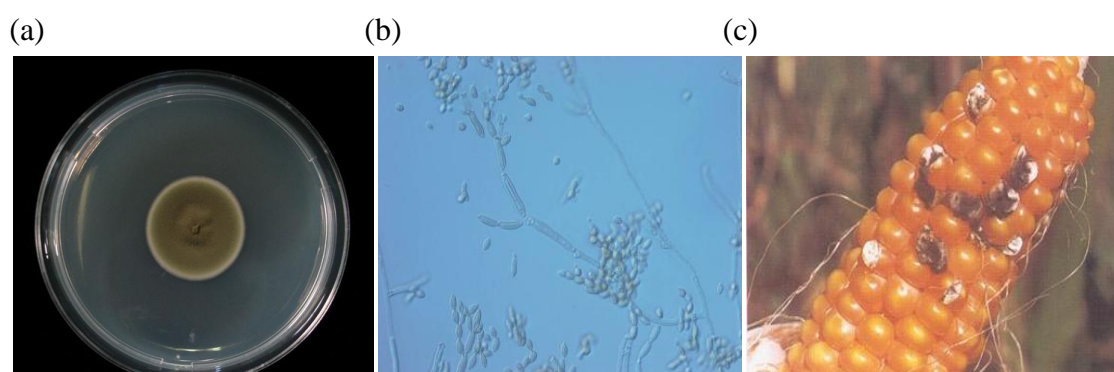


Figure 3.4 *Cladosporium herbarum*. (a) The culture of *C. herbarum* on PDA, (b) Spores and (c) Sweet corn infected by *C. herbarum*.

*Botrytis cinerea*

*Botrytis cinerea*, the cause of grey mould disease, is an important commercial target for fungicide, as it is the major necrotrophic fungal pathogen for vine, vegetables and top fruits world-wide [134]. Juicy fruits favour the growth of this pathogen in which it spread much quicker than on plant leaves, hence *B. cinerea* causes huge damage to fruit crops and is responsible for much post harvest spoilage. Apart from traditional azoles

and dicarboximides fungicides, many new fungicides have been developed to eliminate the crop loss caused by this pathogen, such as pyrimethanil and mepanipyrim [132]. The west European fungicide market of *B. cinerea* was estimated to be worth about 50 million dollars in 2004 [2]. In culture on PDA, as on host plant, the colonies are grey in colour (Figure 3.5).

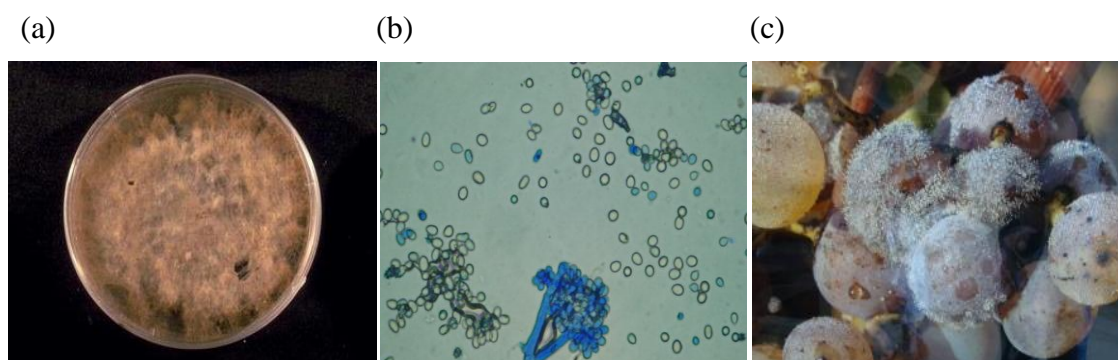


Figure 3.5 *Botrytis cinerea*. (a) The culture of *B. cinerea* on PDA, (b) Spores and (c) bunch rot *B. cinerea* of grapes.

### 3.1.2 Laboratory facilities and safety

Some micro-organisms are hazardous to humans, for example, spores of *Botrytis*, *Cladosporium*, *Aspergillus*, and *Rhizopus*, give rise to allergic (hypersensitive) reactions [135]. Although the fungi tested in this project have low pathogenicity to humans, safety precautions should still be followed carefully. In the laboratory, cultural procedures were carried out in a designated culture room which was maintained clean and key facilities were sterilised with 70% EtOH. A class II bio-safety cabinet was used to reduce contamination of both culture and operator. In such a cabinet, the air pressure inside was higher than outside, and the circulating air was sterilised by a high efficiency

particulate air (HEPA) filter which removes over 99.7% of airborne particles of 0.3  $\mu\text{m}$  or greater in diameter. All apparatus and culture media were sterilised to eliminate any transmissible agents (such as fungus, bacterial, virus and so on) by steam autoclave (121  $^{\circ}\text{C}$ , 20 min). After experimentation, bio-hazard waste was sterilised by autoclave before disposal.

### **3.2 *In vitro* antifungal activity assays**

*In vitro* antifungal assay was established in a 96-well plate in order to assess the effect of compounds on different fungal species in a controlled environment.

#### **3.2.1 Material and equipment**

##### **3.2.1.1 Fungal strains**

Four fungal strains tested were from the fungal stocks used by Prost I et al. [11] including *C. herbarum*, *Cladosporium herbarum* (Pers.:Fr.); *B. cinerea*, *Botrytis cinerea* (MUCL30158); *F. oxysporum*, *Fusarium oxysporum* (unspecified) and *A. brassicicola*, *Alternaria brassicicola* (MUCL20297). Another isolate of *Botrytis cinerea*, *B. cinerea* (GLUK-1), was supplied by Dr. Katherine J. Denby (Warwick HRI) (Table 3.1).

Table 3.1 Fungal strains tested.

Fungal strain	Plant disease	Growth condition
<i>C. herbarum</i>	Leaf mould, Indoor and outdoor mould	PDA, 25 °C, in darkness.
<i>B. cinerea</i> (MUCL30158)	Major pathogen for vine, Grey mould, Root and fruit rotting,	PDA, 25 °C, 12 h light, 12 h dark.
<i>B. cinerea</i> (GLUK-1)	Major pathogen for vine, Grey mould, Root and fruit rotting,	Sterilised apricot half, 25 °C, in darkness.
<i>F. oxysporum</i>	Breakdown of xylem, Leaf wilting, Head blight (wheat),	PDA, 25 °C, in darkness.
<i>A. brassicicola</i>	<i>Brassica</i> dark leaf spot on most <i>Brassica</i> species,	PDA, 25 °C, in darkness.

### 3.2.1.2 Antifungal activity of synthesised compounds

Four synthetic compounds (Figure 2.1) and two compounds, (E)-2-nonenal and a cyclopentenone ring, purchased from Sigma-Aldrich were tested.

### 3.2.1.3 Chemicals

Distilled water was used to prepare aqueous media for all experiments. Other solvents used were of high purity (High performance liquid chromatography (HPLC) grade) for all routine experiments.

### **3.2.1.4 Apparatus**

A Max Mono Monocular biological Microscope (Fisher) was used for spore counting; Omega sterilising system (Prestige Medical Autoclave) for sterilisation; Versatile Environmental test Chamber MRL-350H (SANYO) for fungal culture; Microplate reader Model 680 (Bio-rad) for monitoring turbidity. All the solvents, glassware and pipette tips used for fungal culture were sterilised by autoclaving.

## **3.2.2 Methods**

### **3.2.2.1 Fungal culture**

The method used for fungal culture was modified from that used for the evaluation of oxylipins [11, 135]. Briefly, *C. herbarum*, *F. oxysporum* and *A. brassicicola* were cultured on autoclaved potato dextrose agar (PDA 39g/L, Sigma-Aldrich). These samples were sealed with parafilm (Fisher) and stored in an incubator at 25 °C. *F. oxysporum* and *A. brassicicola* were sub-cultured every week. *C. herbarum* was sub-cultured every two weeks.

*B. cinerea* (MUCL30158) was grown on PDA (24 g/L, Sigma-Aldrich) for 3 to 4 weeks with a photoperiod of 12 hours at 25 °C in a growth chamber (SANYO, Versatile Environmental Test Chamber, Fluorescent light: 40W×3(FL40SSW/37)). *B. cinerea*

(GLUK-1) was grown on half apricots purchased from Tesco, and these samples were sealed with micro-pore tape (Boots) and cultured for 2 to 3 weeks in darkness at 25 °C in an incubator.

### **3.2.2.2 Harvest of fungal spores**

Spore numbers were counted in suspension using a hemocytometer (Bright-Line, Sigma-Aldrich) (Figure 3.6).

#### *F. oxysporum* and *A. brassicicola*

Petri dishes were flushed with sterilised potato dextrose broth (PDB, 48 g/L, Sigma-Aldrich) solution and newly cultured fungal spores were removed by pipette. The spore suspension was filter through a 50 µm autoclaved Cell Micro Sieves (VWR) and adjusted to 100,000 to 120,000 spores/mL by dilution with PDB.

#### *C. herbarum* and *B. cinerea* (MUCL30158 & GLUK-1)

For *C. herbarum* spore production, petri dishes were flooded with distilled water. Spores were scraped off into distilled water using a glass pipette which had been heat treated to form a right angle configuration. The spores were filtered through an autoclaved Cell Micro Sieves (VWR) with micropore size of 50 µm and the

concentration was adjusted to 40,000 spores/mL with distilled water. A similar method was used to collect *B. cinerea* (MUCL30158 & GLUK-1) spore suspension. Once *B. cinerea* mycelium was suspended in water, spores were gently scraped into distilled water. Spore concentration was adjusted to 100,000 spores/mL after filtration through Cell Micro Sieves (VWR) with micropore size of 50  $\mu\text{m}$ .

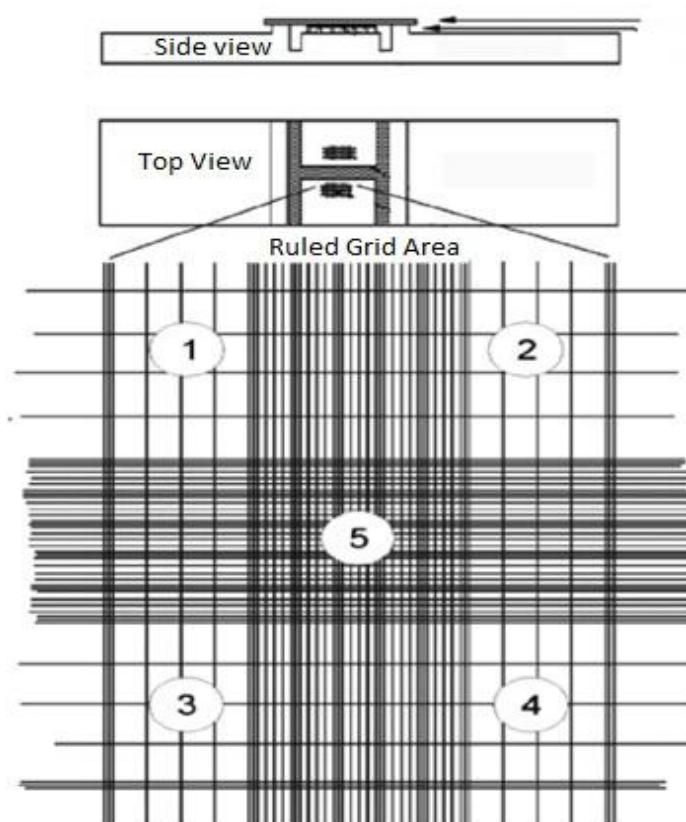


Figure 3.6 Hemocytometer (Bright-Line, Sigma-Aldrich). The volume of each squares marked with 1 to 5 = 1 mm (length)  $\times$  1 mm (width)  $\times$  0.1 mm (depth) =  $1 \times 10^{-4}$  mL. The concentration of cell (cells/mL) = (Total cells counted in five squares)  $\div$  5  $\times 10^4$ .

### 3.2.2.3 Antifungal tests

The method used for antifungal test was modified from the one used for the evaluation of oxylipins [11]. *F. oxysporum* and *A. brassicicola* were grown in potato dextrose



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broth (PDB, 48g/L, Sigma-Aldrich) in an incubator at a speed of 250 rpm at 25 °C. *C. herbarum* and *B. cinerea* were grown in clarified V8 juice broth (5% V8 juice (100%, Campbell Foods, Belgium) in water, pH was adjusted to 5.0 (Hydrus 300 pH meter, Fisher) by the addition of NaOH and centrifuged three times (10,000 g, 5 min, Eppendorf Centrifuge 5804)) in an incubator at 25 °C. Cultures were started with 2,000 spores/well (*C. herbarum*) or 5,000 spores/well (*B. cinerea*, *F. oxysporum* and *A. brassicicola*). Microorganisms were grown in sterile, flat-bottom, 96-well flat-bottom microplates (NUNC Sigma-Aldrich), sealed with parafilm, in a final volume of 100 µL. Test compounds were dissolved in ethanol and the ethanol concentration was kept to a maximum of 1% final concentration in each well. Controls were treated with 1% ethanol. Fungal growth was detected by the absorbance of the microcultures at 595 nm with Bio-rad plate reader Model 680, after 24 to 72 h of incubation in the presence or absence of compounds. For *C. herbarum* and *B. cinerea*, compounds were added after 16 h of growth and  $A_{595}$  measured at 16 h and 24 to 72 h period. The amount of light transmitted through the culture suspension was compared with that through a blank, measuring the turbidity of each well according to the Lambert-Beer Law:

$$A = -\log\left(\frac{I}{I_0}\right) = \epsilon lc$$

A: absorbance (also called optical density (OD))

$I_0$ : amount of incident light

I: amount of transmitted light

$\epsilon$ : extinction coefficient

l: path length across the suspension

c: concentration

For homogenous spore suspensions, turbidity equates with spore germination and mycelium growth and largely follows the Lambert-Beer Law [135]. However, the germination and growth of some fungal species (e.g. *Rhizopus*) form non-uniform suspensions which clearly can lead to wide discrepancies in values. Fungal strains tested here can form relatively homogenous growing suspensions and provide reliable results.

#### **3.2.2.4 Spore germination assay**

This method was modified from the one published by Prost I [11]. Spore germination was assessed in the presence of the test compounds in a 96-well plate. Compounds were added to each well to a final concentration of 100  $\mu$ M (all compounds) together with the spore suspension. For *B. cinerea* (GLUK-1) and *C. herbarum*, spores were suspended in clarified 5% V8 juice broth, whereas for *F. oxysporum* and *A. brassicicola*, potato dextrose broth was used instead. The plate was incubated for a few hours (16 h for *C. herbarum*, 10 h for *B. cinerea* (GLUK-1), 8 h for *F. oxysporum*, *A. brassicicola*), before the observation was carried out under the microscope (Max Mono Monocular biological Microscope, magnification: 100 $\times$ ) and 50 spores were assessed for germination status. Spores with visible germ tubes were counted as being germinated and results were expressed as a percentage of germination.

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### 3.2.3 Results

#### 3.2.3.1 Antifungal tests

*A. brassicicola*, *F. oxysporum*, *C. herbarum*, *B. cinerea* (MUCL30158) and *B. cinerea* (GLUK-1) were exposed to each of the test compounds and their growth was measured over a 72 h period (Figure 3.7). None of the test compounds (100  $\mu$ M) had significant antifungal activity against *F. oxysporum*. Only 4-octyl-cyclopentenone showed limited inhibition (60% inhibition,  $P < 0.01$ ) to fungal growth after 24 h. However, fungal growth recovered and overcame inhibition after 72 h (Figure 3.7 a). Similar to *F. oxysporum*, none of the test compounds at 100  $\mu$ M showed good antifungal activities against *A. brassicicola* and appeared to have significant antifungal activity in comparison to the control after 72 h. 4-Octyl-cyclopentenone, 4-hexyl-cyclopentenone and (E)-2-nonenal showed limited inhibition (50% inhibition,  $P < 0.01$ ) on fungal growth within 24 h and these two target microorganisms overcame inhibition after 72 h (Figure 3.7 b). In contrast to *A. brassicicola* and *F. oxysporum*, *C. herbarum* were more sensitive to test compounds, especially to 4-octyl-cyclopentenone. The absorbance value of the treatment of 4-octyl-cyclopentenone (100  $\mu$ M) after 24 h culture was nearly zero and visually the suspension was clear without any fungal growth observable to the naked eye, whereas fungal growth could be seen in the other treatments at the same concentration after 24 h culture. Fungal growth with the treatment of 4-octyl-cyclopentenone (100  $\mu$ M) recovered gradually but slower than the other

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treatments. After 72 h culture, its inhibition decreased to nearly 50% ( $P < 0.01$ ). Therefore, this compound (at 100  $\mu\text{M}$ ) was just able to control fungal growth rather than kill it. None of the other test compounds showed as good antifungal activity against *C. herbarum* as 4-octyl-cyclopentenone. (E)-2-nonenal showed very limited inhibition against this fungal strain (Figure 3.7 c).

The inhibition of *B. cinerea* (MUCL30158) was similar to that of *C. herbarum*. Mycelium growth following exposure to 4-octyl-cyclopentenone (100  $\mu\text{M}$ ) was inhibited by nearly 100% ( $P < 0.01$ ) after 24 h. Fungal growth recovered by about 50% ( $P < 0.01$ ) after 48 h culture. No other test compounds showed significant antifungal activity in comparison to the control after 48 h (Figure 3.7 d). As to the other fungal isolate of *Botrytis*, *B. cinerea* (GLUK-1), the antifungal activities of treatments at higher concentration of 200  $\mu\text{M}$  were assessed. The antifungal activity of 4-octyl-cyclopentenone at 200  $\mu\text{M}$  against *B. cinerea* (GLUK-1) is similar to that of *B. cinerea* (MUCL30158) treated with 4-octyl-cyclopentenone at 100  $\mu\text{M}$ . None of the other compounds tested at the concentration of 200  $\mu\text{M}$  inhibited fungal growth (Figure 3.7 e). The interesting finding is that fungal growth recovery occurred after 48 h in wells of these two *B. cinerea* isolates, and it was found that among eight wells treated with 4-octyl-cyclopentenone, fungal growth was only found in three to four wells and meanwhile the other wells were completely clear. This accounted for the wide varieties in the absorbance/optical density (OD) values of 4-octyl-cyclopentenone treatments of these two *B. cinerea* isolates had large variations (Figure 3.7 d and e) after

72 h culture.

In short, previous work reported that (E)-2-nonenal (100  $\mu$ M) has no effect on all tested fungal strains [11], which was confirmed in this work. The cyclopentenone ring on its own did not appear to have any antifungal activity. 4-Hexyl-cyclopentenone and 4-butyl-cyclopentenone having six and four carbon alkyl chains, respectively, are much less effective than 4-octyl-cyclopentenone (containing an eight carbon alkyl chain) on fungi, which shows the importance of the length of alkyl chain on antifungal activity of compounds. Surprisingly, 8-(4-oxocyclopentenyl)octanoic acid is very similar to the lead molecule, OPDA, yet little antifungal activity was observed. Overall, 4-octyl-cyclopentenone showed the best antifungal activities among these test compounds. It has similar antifungal activities to its natural analogue, OPDA, which is also more active against *C. herbarum* and *B. cinerea* (MUCL30158) than *F. oxysporum* and *A. brassicicola* [11]. Further tests were therefore focused on 4-octyl-cyclopentenone using different concentrations of the compounds.

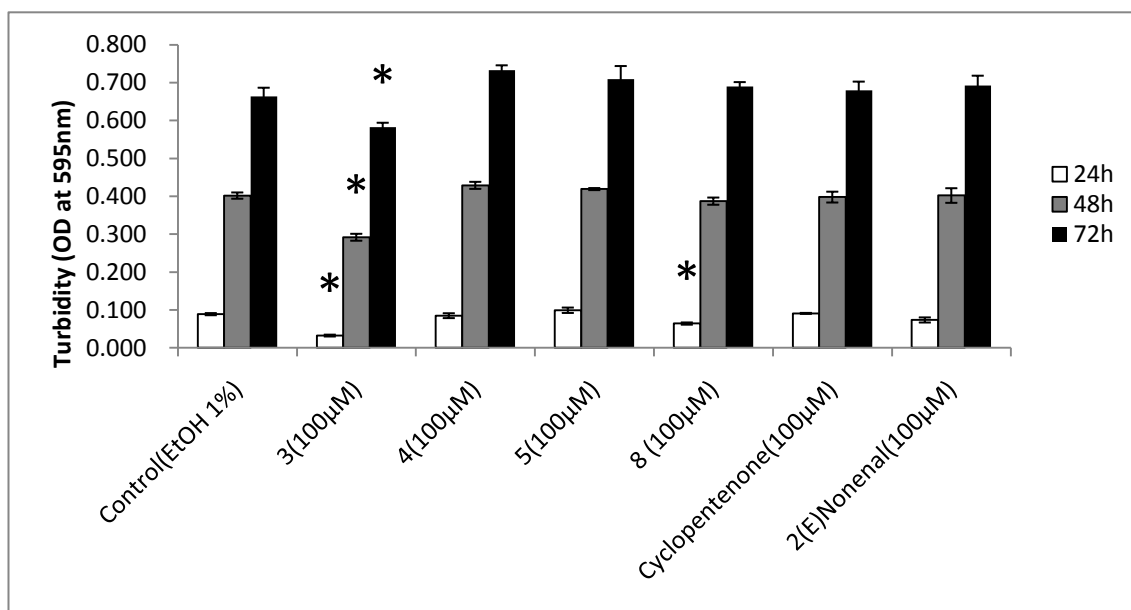
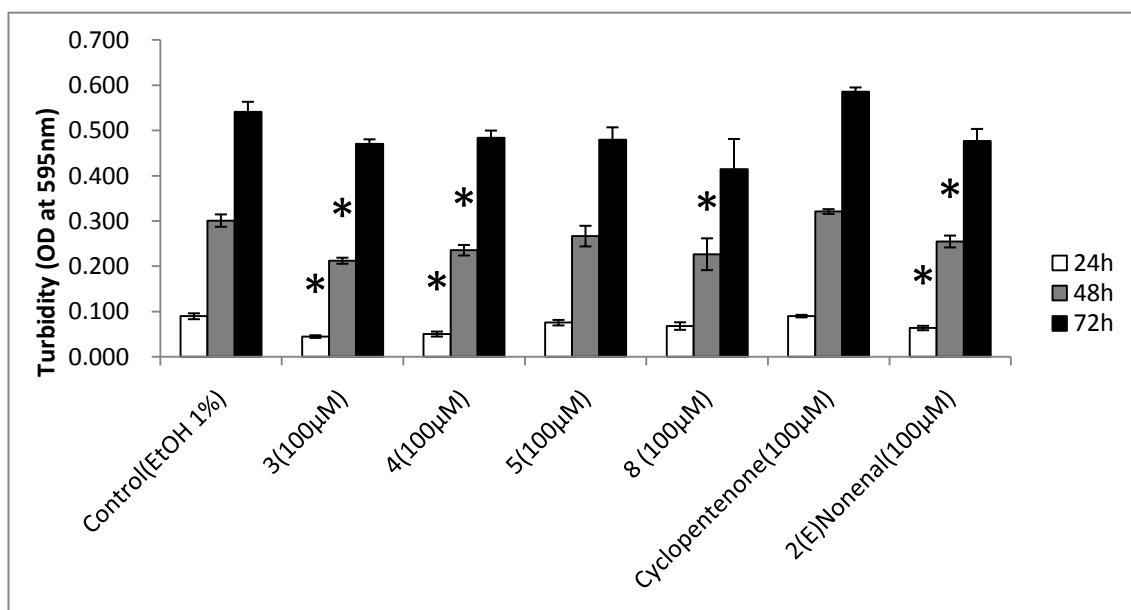
(a) Effect of test compounds on the growth of *F. oxysporum*.(b) Effect of test compounds on the growth of *A. brassicicola*.

Figure 3.7 Results of *in vitro* antifungal assay. Fungal strains were exposed to a series of test compounds in appropriate liquid culture medium, starting from the time of spore addition to wells (*F. oxysporum* and *A. brassicicola*) or 16 h later (*C. herbarum* and *B. cinerea*). Results show means  $\pm$ SD (n=6). Asterisk indicates a significant difference at  $P < 0.01$  (Dunnett's test) against control. 4-octyl-cyclopentenone (**3**), 4-hexyl-cyclopentenone(**4**), 4-butyl-cyclopentenone(**5**), 8-(4-oxocyclopentenyl)octanoic acid (**8**).

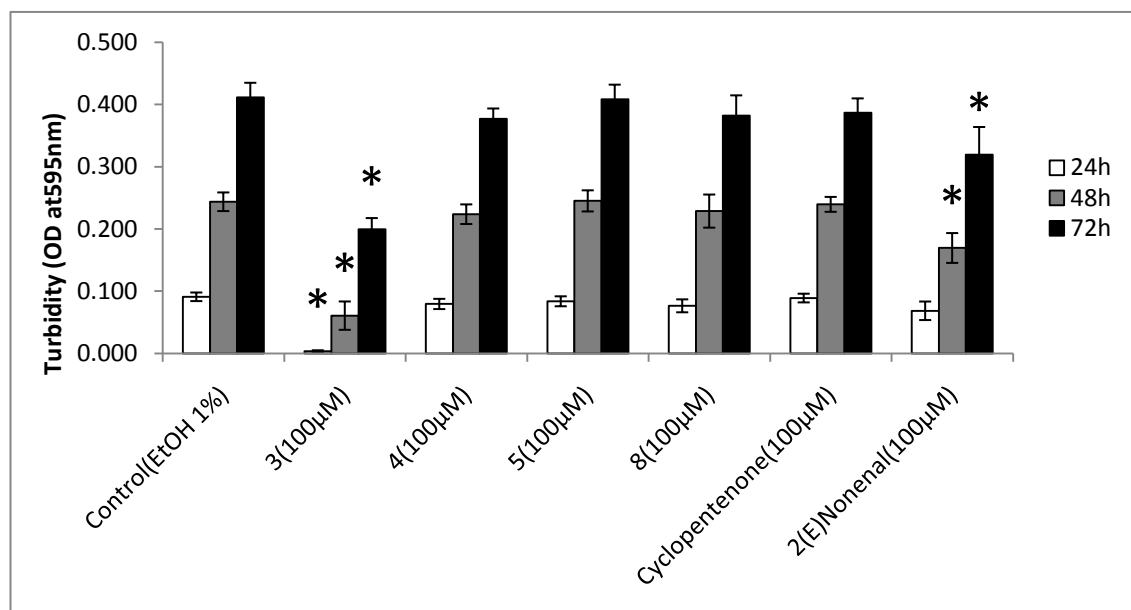
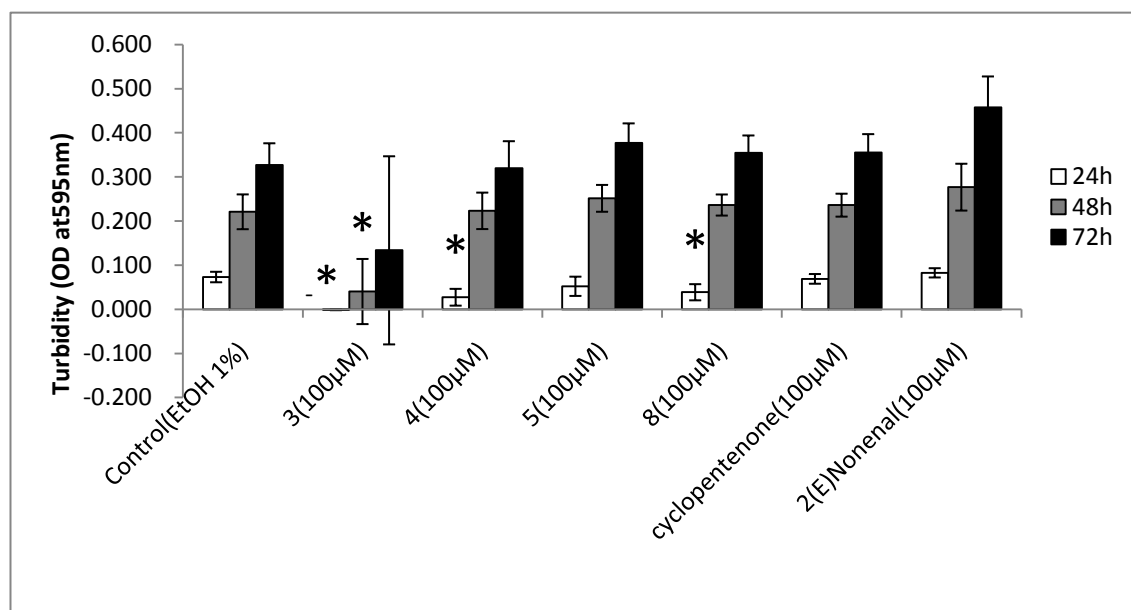
(c) Effect of test compounds on the growth of *C. herbarum*.(d) Effect of test compounds on the growth of *B. cinerea* (MUCL30158).

Figure 3.7 Results of *in vitro* antifungal assay. Fungal strains were exposed to a series of test compounds in appropriate liquid culture medium, starting from the time of spore addition to wells (*F. oxysporum* and *A. brassicicola*) or 16 h later (*C. herbarum* and *B. cinerea*). Results show means  $\pm$ SD (n=6). Asterisk indicates a significant difference at  $P < 0.01$  (Dunnett's test) against control. 4-octyl-cyclopentenone (**3**), 4-hexyl-cyclopentenone(**4**), 4-butyl-cyclopentenone(**5**), 8-(4-oxocyclopentenyl)octanoic acid (**8**).

(e) Effect of test compounds on the growth of *B. cinerea* (GLUK-1).

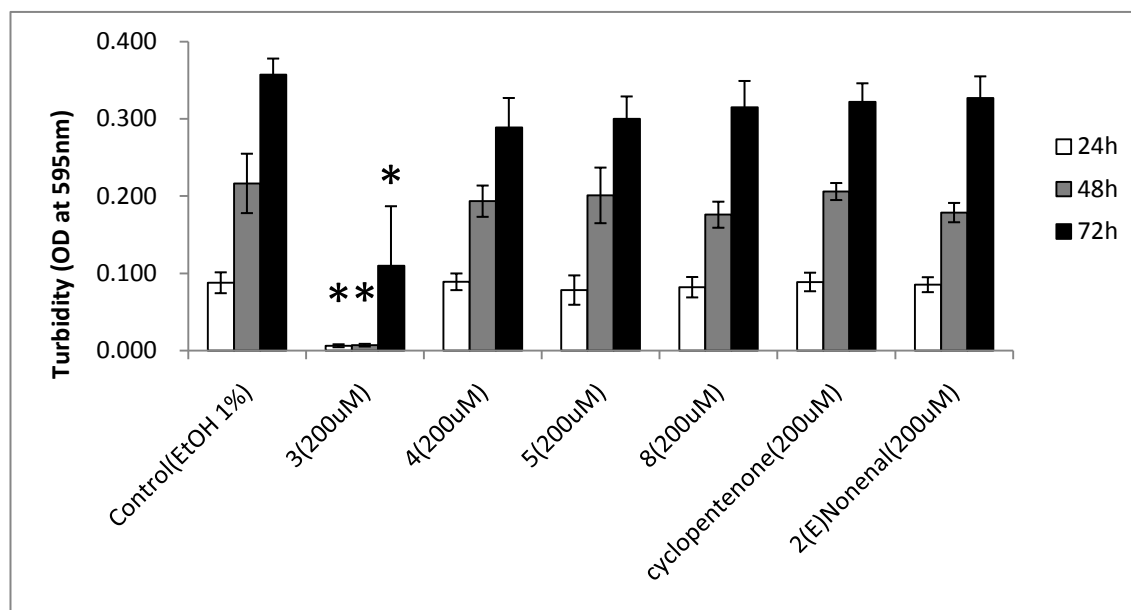


Figure 3.7 Results of *in vitro* antifungal assay. Fungal strains were exposed to a series of test compounds in appropriate liquid culture medium, starting from the time of spore addition to wells (*F. oxysporum* and *A. brassicicola*) or 16 h later (*C. herbarum* and *B. cinerea*). Results show means  $\pm$ SD (n=6). Asterisk indicates a significant difference at  $P < 0.01$  (Dunnett's test) against control. 4-octyl-cyclopentenone (**3**), 4-hexyl-cyclopentenone(**4**), 4-butyl-cyclopentenone(**5**), 8-(4-oxocyclopentenyl)octanoic acid (**8**).

### 3.2.3.2 Antifungal activities of 4-octyl-cyclopentenone

4-Octyl-cyclopentenone at various concentrations (400  $\mu$ M, 200  $\mu$ M and 100  $\mu$ M) was tested against fungal strains (Figure 3.8). *F. oxysporum* was not very sensitive to 4-octyl-cyclopentenone at the lowest concentration, 100  $\mu$ M. However, at higher concentrations significant inhibition ( $P < 0.01$ ) occurred. At 200  $\mu$ M, the inhibition of mycelium growth decreased from 70% ( $P < 0.01$ ) after 24 h to nearly 40% after 72 h, reaching 50% growth inhibition ( $P < 0.01$ ) after 48 h (Figure 3.8 a). 4-Octyl-cyclopentenone at 100  $\mu$ M showed limited antifungal activity against *A.*



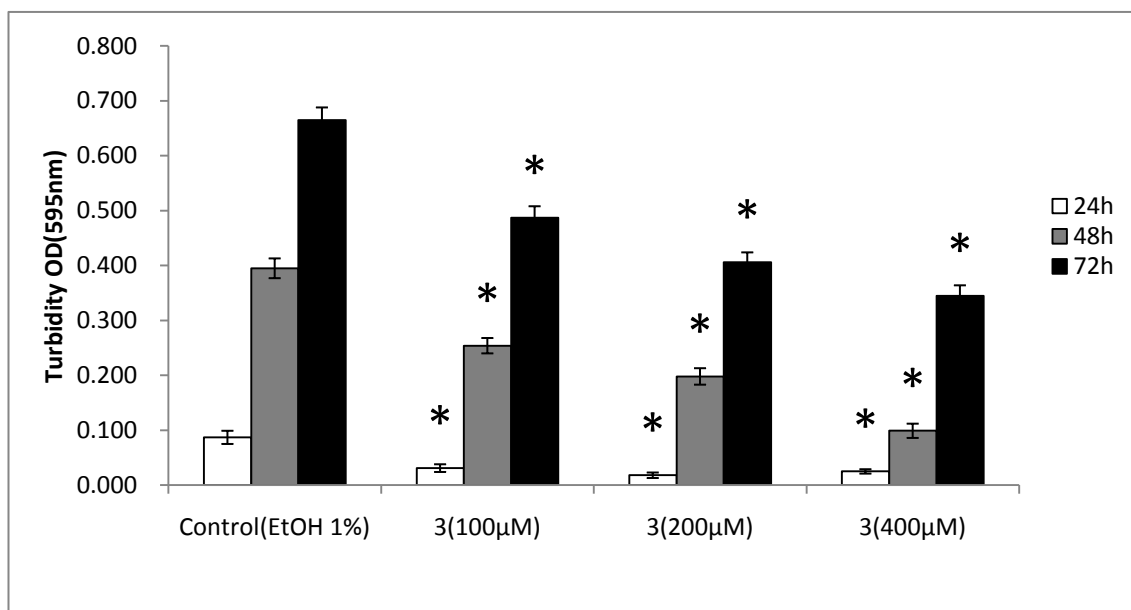
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*brassicicola*, although this activity increased with increase in concentration. 4-Octyl-cyclopentenone only inhibits hyphal growth rather than kill it, and growth recovery was found after 48 h. At concentrations of 200  $\mu\text{M}$  and 400  $\mu\text{M}$ , 4-octyl-cyclopentenone showed increased antifungal activity against *A. brassicicola* than *F. oxysporum*, and the inhibitions of 200  $\mu\text{M}$  and 400  $\mu\text{M}$  were approximately 50% ( $P<0.01$ ) and 65% ( $P<0.01$ ) respectively after 72 h culture (Figure 3.8 b). *C. herbarum*, 4-octyl-cyclopentenone at 400  $\mu\text{M}$ , 200  $\mu\text{M}$  and 100  $\mu\text{M}$  had nearly 100% inhibition ( $P<0.01$ ) of fungal growth after 24 h and no mycelium growth was evident within a week in 400  $\mu\text{M}$  and 200  $\mu\text{M}$  treated samples (Figure 3.8 c). For concentrations lower than 100  $\mu\text{M}$ , very limited growth inhibition was found and growth recovered very quickly in two day culture (data not shown). Similar to *C. herbarum*, the fungal growth of *B. cinerea* (MUCL30158) was totally inhibited at 200  $\mu\text{M}$  and 400  $\mu\text{M}$  and no mycelium growth was detected even after one week culture. For 100  $\mu\text{M}$  application, inhibition was nearly 100% ( $P<0.01$ ) after 24 h and recovered a little after 48 h (Figure 3.8 d). The fungal growth of *B. cinerea* (GLUK-1) was inhibited by this compound within 48 h, however the recovery of fungal growth was found in 72 h culture. As long as the concentration increased to 400  $\mu\text{M}$ , 4-octyl-cyclopentenone inhibited the growth of this isolate within 72 h, and no recovery was observed (Figure 3.8 e).

In short, antifungal activity against all fungi increases with an increase in concentration of 4-octyl-cyclopentenone. For the two less sensitive fungi, *F. oxysporum* and *A. brassicicola*, their spores can survive and grow when exposed to

4-octyl-cyclopentenone at high concentration of 400  $\mu\text{M}$  after 24 h, i.e. the minimum inhibitory concentration (MIC) must be over 400  $\mu\text{M}$ . For *C. herbarum* and *B. cinerea* (MUCL30158), no visual fungal growth was found in the treatment of 4-octyl-cyclopentenone at 100  $\mu\text{M}$  after 24 h, e.g. MIC is 100  $\mu\text{M}$ , and the fungal growth recovered slowly after 48 h. When the concentration of 4-octyl-cyclopentenone increased to 200  $\mu\text{M}$ , no recovery of fungal growth of these two fungi was detected within 72 h and even after a week (data not shown), e.g. the minimum fungicidal concentration (MFC) of this compound against *C. herbarum* and *B. cinerea* (MUCL30158) is between 100 and 200  $\mu\text{M}$ . The other isolate of *B. cinerea* (GLUK-1) (from infected pepper in Scotland) is less susceptible to 4-octyl-cyclopentenone than *B. cinerea* (MUCL30158) isolated in mainland Europe (source not given). 4-Octyl-cyclopentenone at 200  $\mu\text{M}$  can only inhibit spore germination and partially inhibit spore germination/mycelial growth within 24 h. The fungicidal effect against *B. cinerea* (GLUK-1) can be achieved by doubling the concentration to 400  $\mu\text{M}$ . Hence, the MIC of 4-octyl-cyclopentenone against *B. cinerea* (GLUK-1) is 200  $\mu\text{M}$  and corresponding MFC is 400  $\mu\text{M}$ . In summary, although the antifungal activity increases with concentration, 4-octyl-cyclopentenone is effective against *C. herbarum* and *B. cinerea* at concentrations lower than 400  $\mu\text{M}$ .

(a) Effect of 4-octyl-cyclopentenone on the growth of *F. oxysporum*.



(b) Effect of 4-octyl-cyclopentenone on the growth of *A. brassicicola*.

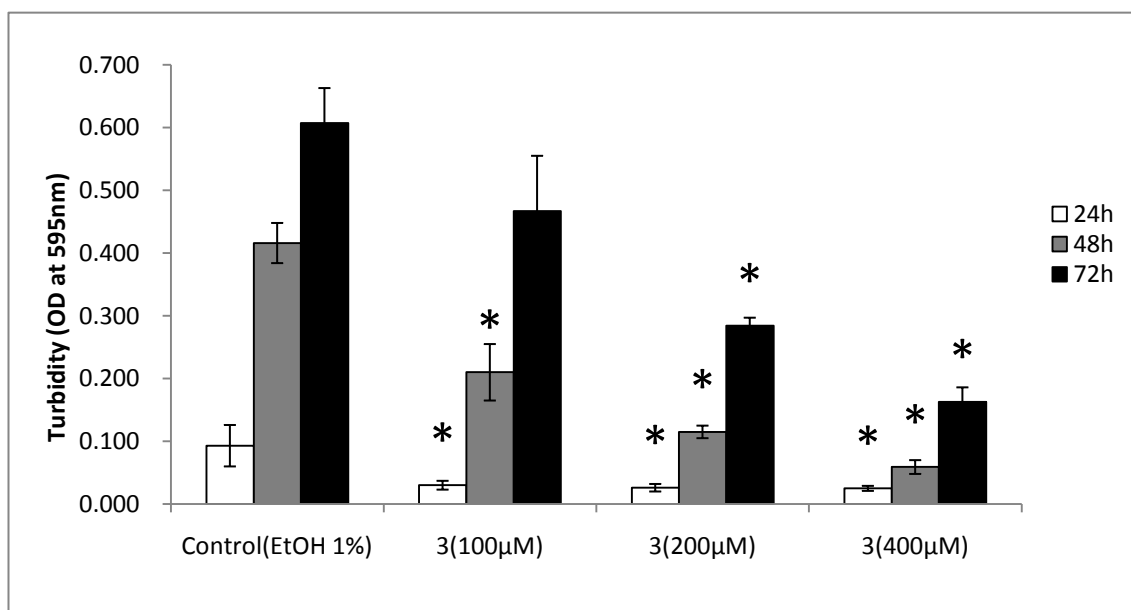
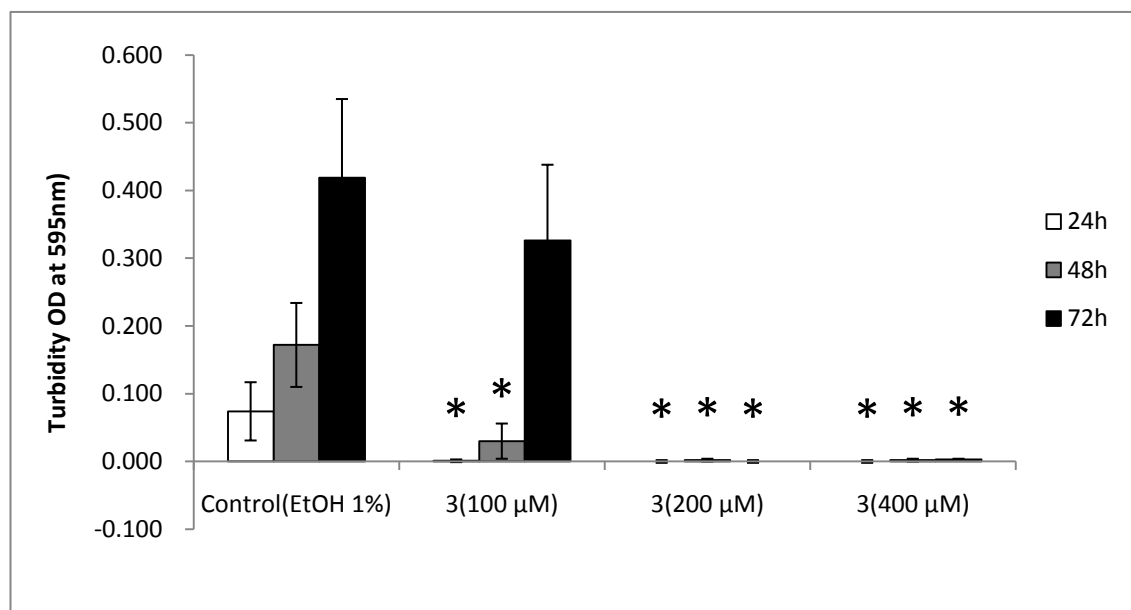


Figure 3.8 *In vitro* antifungal assay of 4-octyl-cyclopentenone (**3**). Fungal strains were exposed to 4-octyl-cyclopentenone (**3**) of different concentrations of 100, 200 and 400  $\mu\text{M}$ , starting from the time of spore addition to wells (*F. oxysporum* and *A. brassicicola*) or 16 h later (*C. herbarum* and *B. cinerea*). Results show means  $\pm$ SD (n=6). Asterisk indicates a significant difference at  $P < 0.01$  (Dunnett's test) against control.

(c) Effect of 4-octyl-cyclopentenone on the growth of *C. herbarum*.



(d) Effect of 4-octyl-cyclopentenone on the growth of *B. cinerea* (MUCL30158).

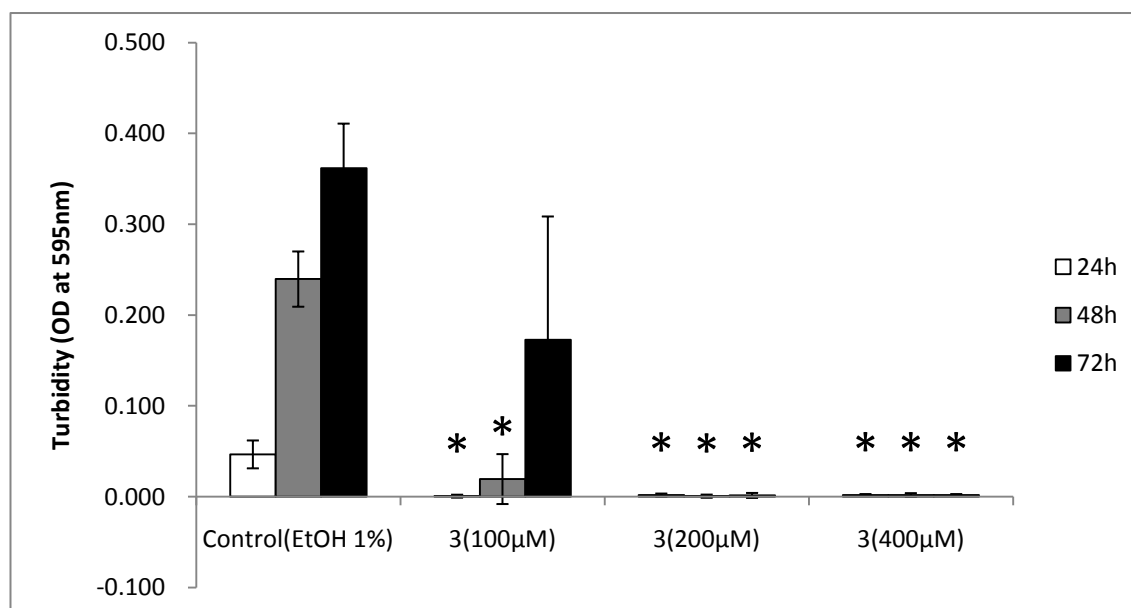


Figure 3.8 *In vitro* antifungal assay of 4-octyl-cyclopentenone (**3**). Fungal strains were exposed to 4-octyl-cyclopentenone (**3**) of different concentrations of 100, 200 and 400  $\mu\text{M}$ , starting from the time of spore addition to wells (*F. oxysporum* and *A. brassicicola*) or 16 h later (*C. herbarum* and *B. cinerea*). Results show means  $\pm$ SD (n=6). Asterisk indicates a significant difference at  $P < 0.01$  (Dunnett's test) against control.

(e) Effect of 4-octyl-cyclopentenone on the growth of *B. cinerea* (GLUK-1)

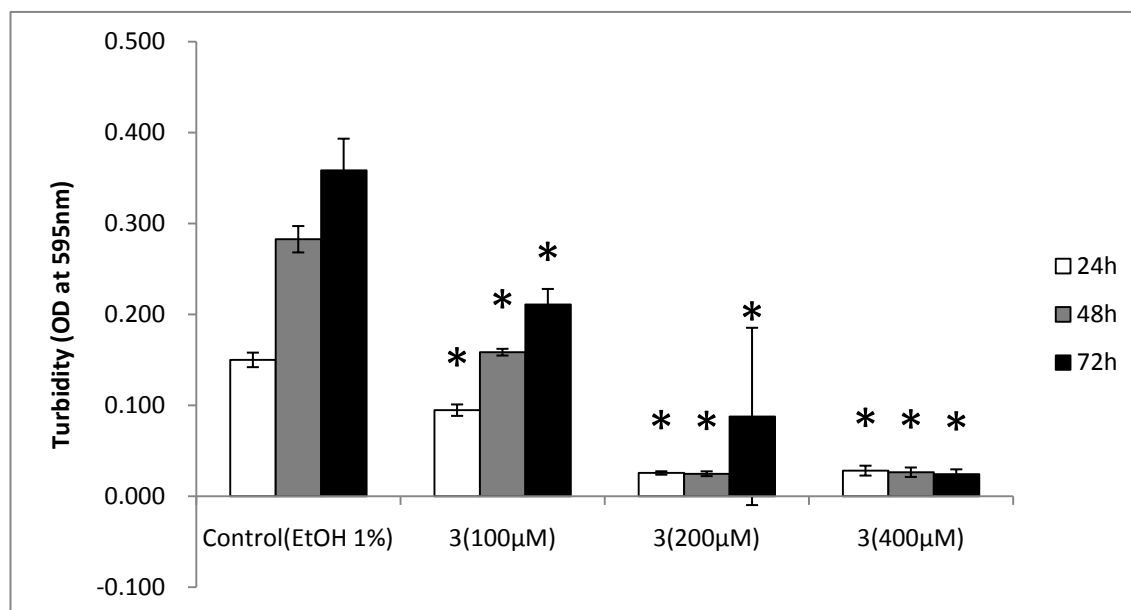


Figure 3.8 *In vitro* antifungal assay of 4-octyl-cyclopentenone (**3**). Fungal strains were exposed to 4-octyl-cyclopentenone (**3**) of different concentrations of 100, 200 and 400 µM, starting from the time of spore addition to wells (*F. oxysporum* and *A. brassicicola*) or 16 h later (*C. herbarum* and *B. cinerea*). Results show means  $\pm$ SD (n=6). Asterisk indicates a significant difference at  $P < 0.01$  (Dunnett's test) against control.

### 3.2.3.3 Effect of compounds on fungal spore germination

Spore germination is an important developmental stage in the life cycle of fungi, therefore it is worthy to show the performance of compounds on spore germination after short time exposure. Spores were incubated for a few hours in the presence of selected compounds. For each fungus, the incubation time for germination assessment was optimised for high number of germinated spores in the controls for better visualisation under the microscope. The percentage of germinated spores of each treatment was calculated and compared with the percentage of germination in control treated with 1% EtOH. The results are shown in figure 3.9. In the controls of *C. herbarum* and *B.*

*cinerea*, nearly all spores germinated after 16 hours and 10 hours cultured, however, only 65% of spores of *F. oxysporum* germinated after 8 h culture. 4-Octyl-cyclopentenone is again the most active compound tested, which has about 80% inhibition of the spore germination of test species at the concentration of 100  $\mu$ M and 100% inhibition at 200  $\mu$ M. Other test compounds showed very limited inhibition of fungal spore germination. The effect of compounds on spore germination of *A. bassicicola* was not able to be evaluated by this method, because the clustering of spores made it difficult to count them under the microscope. However, it was still evident that 4-octyl-cyclopentenone at test concentrations showed good inhibition of spore germination of *A. bassicicola* after 7-8 h culture (data not shown).

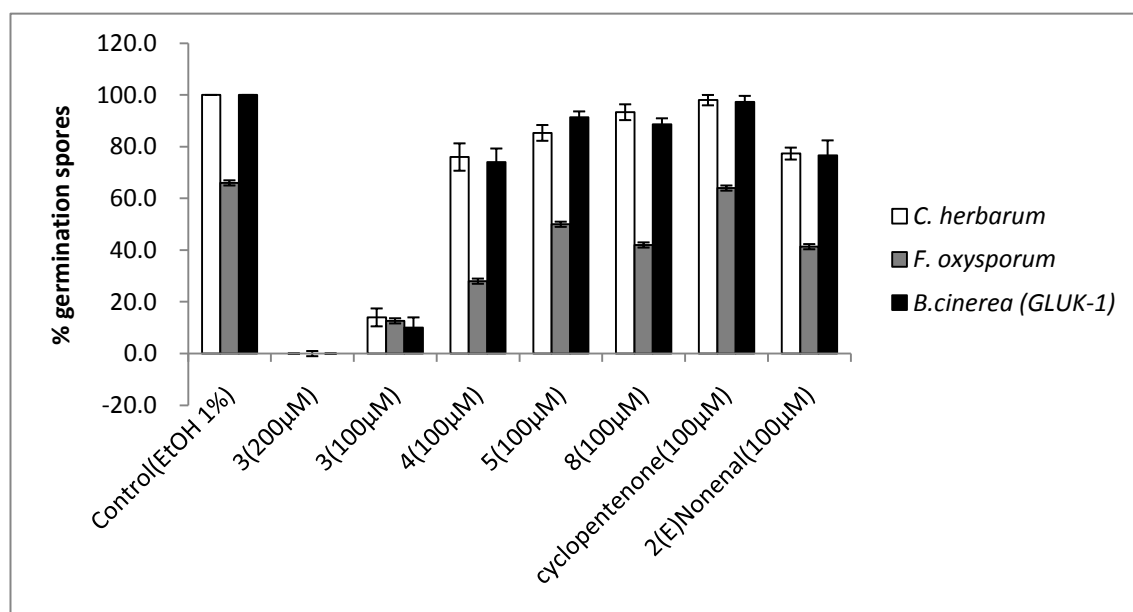


Figure 3.9 Effect of compounds on spore germination. Freshly prepared spores of *C. herbarum* (20,000 spores/mL), *F. oxysporum* (50,000 spores/mL) and *B. cinerea* (50,000 spores/mL) were exposed to each compounds and assessed for germination status after 16 h (*C. herbarum*) or 8 h (*F. oxysporum* and *B. cinerea* (GLUK-1)). Each bar represents mean of three independent experiments  $\pm$ SD. 4-octyl-cyclopentenone (**3**), 4-hexyl-cyclopentenone(**4**), 4-butyl-cyclopentenone(**5**), 8-(4-oxocyclopentenyl)octanoic acid (**8**).

### 3.2.4 Discussion

Apart from cyclopentenone and 8-(4-oxocyclopentenyl)octanoic acid, synthetic RES compounds belong to non-ionised surfactants, with polar heads and non-polar tails, which contribute to their different  $\log P$  values. Surfactants are known as an important group of materials having active properties, of which antimicrobial activity is an important application. Lipophilicity is a key property and plays an important role in modulating their antimicrobial activities.

4-Octyl-cyclopentenone, 4-hexyl-cyclopentenone and 4-butyl-cyclopentenone have the same hydrophilic head, the cyclopentenone ring, and differ in the length of alkyl chain. This compound is the most effective antifungal agent and significantly better than the other two compounds against the tested fungal species. Their cyclopentenone ring will provide similar electrophilicity and corresponding biological activities. The different lengths of the hydrophobic chain that have influence on the lipophilicity ( $\log P$ ) of compounds seem to affect the antifungal activity of compounds. In this project,  $\log P$  value was calculated by Molinspiration  $\log P$  (milog  $P$ ) calculator (Table 3.2).

The antifungal activities of some other non-ionised surfactants against several fungal species have been well studied [9, 68, 136-143]. Their conclusion was that the antifungal activity of non-ionised surfactants primarily depends on the properties of the biological hydrophilic head of test compounds, however, the length of the alkyl group

Table 3.2 Lipophilicity ( $\log P$ ) of compounds tested for *in vitro* antifungal activity. The value of  $\log P$  was calculated by Molinspiration  $\log P$  calculator.

Chemicals	$\log P$ value
OPDA	4.6
4-octyl-cyclopentenone ( <b>3</b> )	4.5
4-hexyl-cyclopentenone ( <b>4</b> )	3.5
4-butyl-cyclopentenone ( <b>5</b> )	2.5
8-(4-oxocyclopentenyl)octanoic acid ( <b>8</b> )	2.8
Cyclopentenone	0.4

also plays an important role. This alkyl chain is considered to balance the hydrophobic and hydrophilic moieties to achieve an optimal lipophilicity which favours compounds to reach the active sites. Given the same hydrophilic part, the antifungal effectiveness of test compounds, to some extent, depends on their lipophilicity, i.e. the balance between hydrophobic and hydrophilic parts.

4-Octyl-cyclopentenone with an eight carbon aliphatic chain has the highest antifungal activity of the compounds tested in this project. From Table 3.2, it can be seen that the  $\log P$  value of this compound is 4.5. 4-Hexyl-cyclopentenone and 4-butyl-cyclopentenone are much less effective on inhibiting fungal growth and their  $\log P$  values are 3.5 and 2.5, respectively. Interestingly, 4-octyl-cyclopentenone and OPDA have very close values of  $\log P$ , 4.5 and 4.6, respectively. In addition, OPDA was elucidated to have very similar antifungal activities against several fungal strains to 4-octyl-cyclopentenone. Both have more effective antifungal activity against *C. herbarum* and *B. cinerea* (MUCL30158) than *F. oxysporum* and *A. brassicicola*. As



RES, their similar electrophilicity and lipophilicity might endow them comparable performances, such as solubility in tissue matrices, affinity to amphiphilic substances (lipids and proteins) and membrane interaction. However, OPDA and other oxylipins tested were reported to be less effective than those commercial fungicides (such as benomyl and metalaxyl) against the same target organisms [11]. The synthetic analogous of OPDA, 4-octyl-cyclopentenone, behave similar antifungal activity to OPDA, therefore further modification will be needed to enhance its performance which would compete with those commercial products.

(E)-2-nonenal is an effective compound among plant volatile compounds derived from HPL pathway displaying higher antifungal activity than other common GLVs, like (E)-2-hexenal and (Z)-3-nonenal [144]. (E)-2-nonenal was reported to have antifungal activity against *A. brassicicola*, *F. oxysporum* and *B. cinerea* [71, 77]. It showed inhibition of spore germination of *F. oxysporum* and *B. cinerea* at concentration lower than 100  $\mu\text{M}$ . However, here we observed that (E)-2-nonenal (100  $\mu\text{M}$ ) showed little effect on spore germination and fungal growth of test fungal species. The different results may be caused by using different pathogen isolates and experimental systems in our laboratory.

8-(4-Oxocyclopentenyl)octanoic acid which contains a carboxyl group on the end of the alkyl chain like OPDA was not as effective as 4-octyl-cyclopentenone against these fungal species. One possibility is that the loss of antifungal activity could be due to the

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lack of a pentenyl chain on C13 of OPDA leading to a much lower log  $P$  value (2.8) than that of OPDA (log  $P = 4.6$ ) and 4-octyl-cyclopentenone (log  $P = 4.5$ ). Such lower lipophilicity will affect the membrane affinity and permeability of compounds to fungal pathogens. Another reason for the inactivity of 8-(4-oxocyclopentenyl)octanoic acid could be that the presence of two polar heads (cyclopentenone ring and carboxyl end group) on 8-(4-oxocyclopentenyl)octanoic acid could interact with each other forming an intramolecular ring when the eight carbon chain bends. Such a change will form a two-ring structure of 8-(4-oxocyclopentenyl)octanoic acid so that it is hard to penetrate the plasma membrane. In the case of natural occurring cis- (+) OPDA, the presence of side pentenyl substitution will block the bending of the longer chain due to steric hindrance and its antifungal activity was preserved or even enhanced. Pure OPDA and 4-octyl-cyclopentenone have the physical appearance of oil, whereas 8-(4-oxocyclopentenyl)octanoic acid with carboxyl end forms crystal when concentrated. This observation reflects the influence of the pentenyl side chain of OPDA on its physical property. Besides the two possibilities mentioned, the biodegradation of 8-(4-oxocyclopentenyl)octanoic acid might cause the suppression of its bio-activity. Although it is known that OPDA is very stable during *in vitro* antifungal assays [11], the degradation of 8-(4-oxocyclopentenyl)octanoic acid by  $\beta$ -oxidation cannot be excluded due to the presence of the carboxyl group. A carboxyl group is needed for the esterification to CoA which is subsequently necessary for  $\beta$ -oxidation [25, 65]. The  $\beta$ -oxidation would shorten the alkyl chain, and in this way, the antifungal activity of 8-(4-oxocyclopentenyl)octanoic acid could be degraded. From another view,

the oxidation of compounds will accelerate the speed of biodegradation, which would decrease the adverse effect of compounds on the environment. If 8-(4-oxocyclopentenyl)octanoic acid was sprayed onto plants to induce host defence response, this compound could also be a potential indirect antifungal candidate. These hypotheses need to be evaluated in the future and more research undertaken to clarify the detailed biological activities of 8-(4-oxocyclopentenyl)octanoic acid.

For *B. cinerea*, 4-octyl-cyclopentenone shows a different performance against different isolates. *B. cinerea* (MUCL30158) is more sensitive to this compound than *B. cinerea* (GLUK-1). The large variation of results after 48 h culture was found for *Botrytis* (MUCL30158) treated with 4-octyl-cyclopentenone at 100  $\mu$ M and *B. cinerea* (GLUK-1) treated with the same compound at 200  $\mu$ M. As the antifungal action mode of 4-octyl-cyclopentenone is still unknown, it is difficult to explain this interesting finding. One explanation could be that 4-octyl-cyclopentenone inhibited most of the fungal cells and the recovery of fungal growth only started from the cells which survived from the treatment. As discussed above, the antifungal activity of 4-octyl-cyclopentenone at concentration of 100  $\mu$ M against *B. cinerea* (MUCL30158) is comparable to that of 4-octyl-cyclopentenone at 200  $\mu$ M against *B. cinerea* (GLUK-1). This observation indicated that although different antifungal sensitivities were found for different isolates, they might share similar antifungal modes of action. In this project, *B. cinerea* (MUCL30158) was used for *in vivo* test and since *B. cinerea* (GLUK-1) provides a larger yield of spore than *B. cinerea* (MUCL30158), this makes it more convenient for

the study of modes of action due to the requirement of large amount of spores used in these types of assays.

### **3.2.5 Conclusion**

In conclusion, 4-octyl-cyclopentenone is the most active compound against the fungal species tested and has similar activities to OPDA. The simplified 4-octyl-cyclopentenone, therefore, can mimic some biological activities of OPDA. Our data shows that as a RES, the series of synthesised compounds has a range of antifungal activity even though they have the same electrophilic cyclopentenone ring. The different lengths of alkyl chains therefore play an important role in balancing hydrophobic and hydrophilic moieties (i.e. lipophilicity, measured as  $\log P$ ) which is likely to improve the membrane permeability of the compound to reach the active sites of target pathogens.

### **3.3 *In vivo* antifungal assay of 4-octyl-cyclopentenone**

From the *in vitro* tests, 4-octyl-cyclopentenone was established as the strongest antifungal compound against the test organisms. However, the controlled environment of the *in vitro* test differs from the condition in whole tissues (*in vivo* test). It is therefore necessary to determine whether *in vitro* results would translate into the desired

efficacy *in vivo*. To this end, a series of *in vivo* experiments was conducted to evaluate 4-octyl-cyclopentenone's antifungal activity.

### **3.3.1 Materials**

Test fungal strains were mentioned in 3.2.1. Seeds of two plant species, *Arabidopsis thaliana* (Col-5) and *Brassica oleracea* were supplied by Professor Eric Holub from Warwick HRI. The *in vivo* test on leaves of *Arabidopsis thaliana* was carried out in the laboratory of Dr. Katherine J. Denby (Warwick HRI), following their standard procedure given below.

### **3.3.2 Methods**

#### **3.3.2.1 Growing of the test plants**

*Arabidopsis thaliana* and *Brassica oleracea* are popular as model organisms in plant biology and genetics. Their wild-type and mutants are widely used in many laboratories to study plant defence and interface between pathogens and host plants [49, 63, 145-147].

**(a) Growing of *Arabidopsis thaliana* (Col-5)**

The basic method used for growing *Arabidopsis thaliana* in this project was modified from the classic method published by the *Arabidopsis* Biological Resource Centre [148]. Seeds were placed on the surface of premixed soil (4 scoops of potting soil, 2 scoops of sand and 2 scoops of vermiculite) in cell planting trays. Trays with seeds were placed at 3-4 °C for at least 2-4 days to break dormancy and improve germination rate. The trays were then placed in a culture chamber (SANYO, Versatile Environmental Test Chamber) under 100-150  $\mu\text{mol}/\text{m}^2\text{s}^{-1}$  photosynthetic photon flux density light with a 12 hour photoperiod at 24 °C. Plants were regularly watered and the tray was covered with plastic wrap which provided a high humidity level for successful seed germination.

**(b) Growing of *Brassica oleracea***

Seeds were grown on the surface of premixed soil (6 scoops of potting soil, 1 scoop of sand and 1 scoop of vermiculite) under 100-150  $\mu\text{mol}/\text{m}^2\text{s}^{-1}$  photosynthetic photon flux density light with a 16 hour photoperiod in SANYO culture chamber at 24 °C. One week old plants were used for phytotoxicity testing.

**3.3.2.2 Phytotoxicity test of 4-octyl-cyclopentenone on *Brassica oleracea***

One week old *Brassica oleracea* plants were used and 20  $\mu\text{L}$  of 4-octyl-cyclopentenone

at different concentrations (100  $\mu\text{M}$ , 200  $\mu\text{M}$  and 400  $\mu\text{M}$ ) were applied to the surface of the leaves virtually covering the small leaves at this early growing stage. 20  $\mu\text{L}$  of 1% EtOH treatment was used as a control. The plants were placed back in SANYO culture chamber under the same growth conditions (100-150  $\mu\text{mol}/\text{m}^2\text{s}^{-1}$  photosynthetic photon flux density light with a 16 hour photoperiod at 24  $^{\circ}\text{C}$ ). Visual evidence of disease and difference between control and 4-octyl-cyclopentenone treatments were recorded every day and photos were taken 2 weeks later.

### **3.3.2.3 *In vivo* antifungal test on leaves of *Arabidopsis thaliana***

Experiments to test how 4-octyl-cyclopentenone can inhibit the growth of *B. cinerea* (MUCL30158) on leaves of plants followed the method published by Denby (Warwick HRI) [146, 149]. Leaves were harvested from five-week old *Arabidopsis thaliana* and placed into plastic humidity trays containing 1.5% phytoagar at a depth of 1 cm. Leaves were inoculated with 10  $\mu\text{L}$  of suspension of 16 hour pre-cultured germinated spores in 5% V8 juice (10,000 spores/mL) treated with 4-octyl-cyclopentenone to achieve a final concentration of 200  $\mu\text{M}$ . 10  $\mu\text{L}$  of germinated spore suspension treated with 1% EtOH as a control. Total leaf area and diameter of the developing lesion were measured after 48 and 72 h using IMAGEJ and high resolution digital pictures of the leaves. A 1 cm scale was included in each image, which enabled calibration of the measurements.

### 3.3.2.4 *In vivo* antifungal test on apricot fruits

The *in vivo* antifungal activity of 4-octyl-cyclopentenone was assessed by culturing *B. cinerea* (MUCL30158) on apricot halves. This test was evaluated to determine whether the compound was effective *in vivo* using a susceptible host substrate. Apricots were purchased from Tesco and cleaned by washing surface three times with autoclaved distilled water, then placed in a petri-dish. The fruit surface was punctured to form 3-4 holes with a depth of 4 mm using a glass rod of 3 mm diameter. The 16 h pre-cultured germinated spores (50,000 spores/mL) suspension was mixed with 4-octyl-cyclopentenone to achieve a final concentration of 200  $\mu$ M and 10-20  $\mu$ L of the suspension was injected into the holes. The petri-dish was sealed with parafilm and cultured in an incubator. After 48 h, the growth of the pathogen on the fruit was recorded photographically and compared to 1% EtOH control treated sample.

### 3.3.3 Results

#### 3.3.3.1 Phytotoxicity test of 4-octyl-cyclopentenone

Phytotoxic means harmful or lethal to plants and phytotoxic effects can range from slight burning or browning of the leaves to death of the plants. The phytotoxic effect of 4-octyl-cyclopentenone on *Brassica* leaves was monitored visually and recorded photographically. The results in Figure 3.10 showed that there were no damage lesions,



burning, browning or death of leaves. No abnormal growth was observed in the following three weeks with regard to the number and radii of leaves. In short, there was no visual difference between control and all the treatments, even at highest concentration of 400  $\mu\text{M}$ . Although this visualisation approach cannot measure the overall phytotoxic effects, the results indicate that no obvious damage had occurred to the plant leaves after compound application.

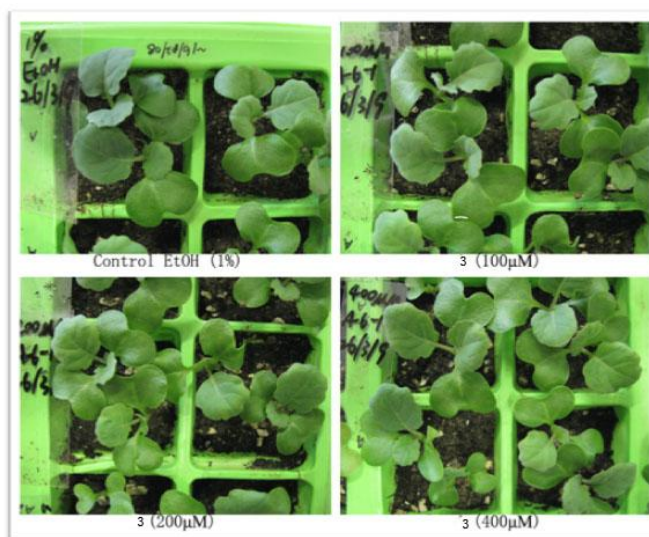
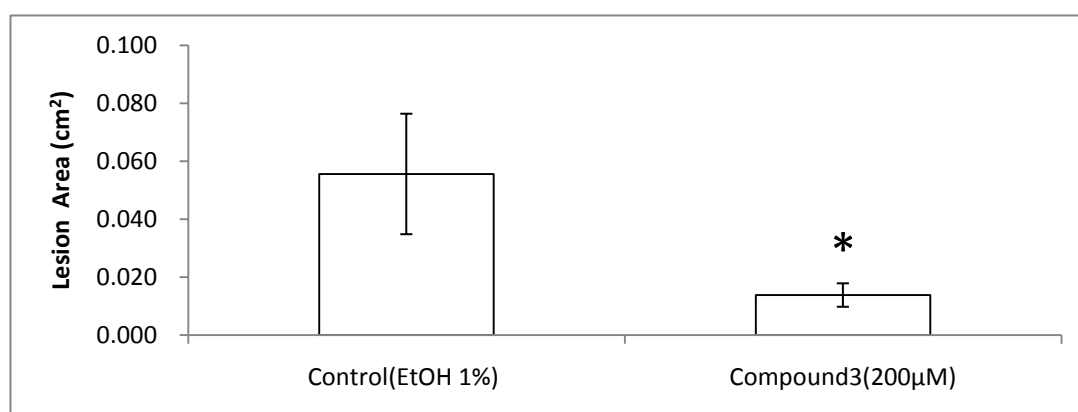


Figure 3.10 Phytotoxicity assay of 4-octyl-cyclopentenone on *Brassica oleracea*. 4-octyl-cyclopentenone (**3**) with concentration up to 400  $\mu\text{M}$  has no visual effects on the growth of *Brassica oleracea* even after two weeks culture.

### 3.3.3.2 *In vivo* antifungal test on leaves of *Arabidopsis thaliana*

Although *B. cinerea* is a main pathogen of fruits, it can be pathogenic on *Arabidopsis thaliana* resulting in an interaction which is amenable to biological analysis [149]. 4-Octyl-cyclopentenone at 200  $\mu\text{M}$  showed significant antifungal activity against *B. cinerea* (MUCL30158) on leaves of *Arabidopsis thaliana* in 72 h culture and the

damage lesion area of the treatment is approximately 70-75% less than the control (Figure 3.11). By contrast, *in vitro*, complete growth inhibition of this fungal strain was observed in the treatment of this compound at 200  $\mu\text{M}$ . However, given that conditions on a leaf surface are quite different from a controlled sterile environment, the inhibition achieved *in vivo* was still quite significant.



(a) Control (1% EtOH)



(b) Treatment of 4-Octyl-cyclopentenone (**3**) (200  $\mu\text{M}$ )

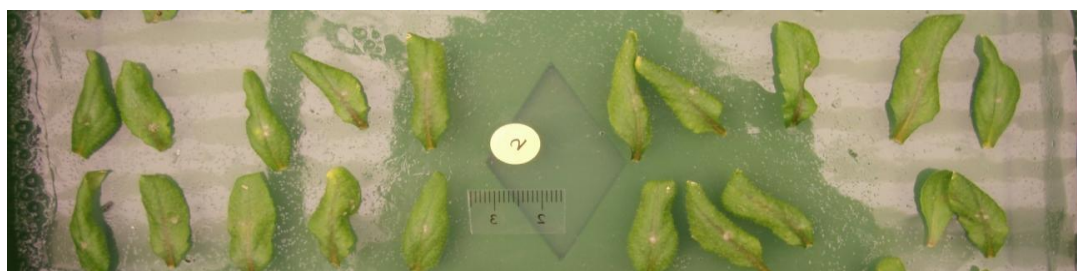


Figure 3.11 *In vivo* antifungal test on leaves of *Arabidopsis thaliana*. 4-Octyl-cyclopentenone (**3**) (200  $\mu\text{M}$ ) was added to suspension of *B. cinerea* (MUCL30158) after 16 h. The significant difference of damage lesion between control (a) and treatment (b) indicates the inhibition of fungal growth after 72 h. Asterisk indicates a significant difference at  $P < 0.01$  (Dunnett's test) against control. Error bars indicate  $\pm\text{SD}$  ( $n=13$ ).

### 3.3.3.3 Antifungal test on apricot fruits

Apricot halves were used to test the antifungal activity of 4-octyl-cyclopentenone (200  $\mu\text{M}$ ) against *B. cinerea* (MUCL30158). After 48 h, fungal growth was found around all three inoculation sites for control treatments, in comparison, only one out of three inoculation sites was infected with *B. cinerea* growth for 4-octyl-cyclopentenone treatment and the radii of the infected site was smaller than that of the control (Figure 3.12). However, unlike the *in vitro* tests, 4-octyl-cyclopentenone at 200  $\mu\text{M}$  could not inhibit growth of *Botrytis* completely and slow recovery of fungal growth was observed on all inocula sites after 72 h culture. Summarising, complete inhibition of fungal growth of *B. cinerea* (MUCL30158) was achieved by *in vitro* treatment of 4-octyl-cyclopentenone (200  $\mu\text{M}$ ), however, *in vivo* fungal growth recovered and mycelium growth initiated after 48 h delay.

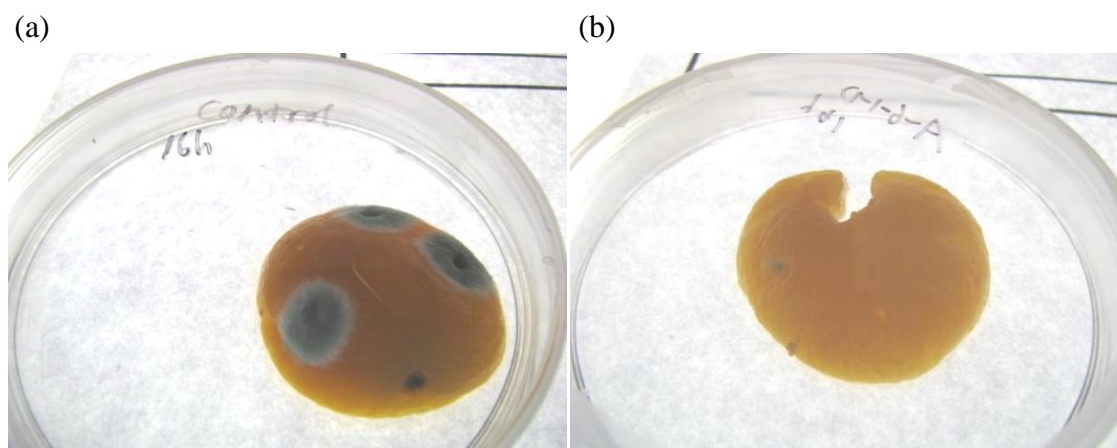


Figure 3.12 *In vivo* antifungal test on apricots. *B. cinerea* (MUCL30158) was inoculated to apricot halves in the absence (b) and presence (a) of 4-octyl-cyclopentenone and incubated for 48 h. Fungal growth was found around all three inoculated sites without 4-octyl-cyclopentenone (a) and only a little growth was found in one inoculated site incubated with 4-octyl-cyclopentenone (b).

### 3.3.4 Discussion

Most commercial agrochemicals are known to be toxic to host plants if not applied according to their instructions; therefore any commercial agrochemical should be tested for its phytotoxicity before being introduced to the market. RES oxylipins with  $\alpha,\beta$ -unsaturated carbonyl group have both positive and negative effects on the host plant cells. On the one hand they have important biological activities during the life span of plants, on the other hand, this group of compounds has phytotoxicity to host plants and certain cytotoxicity to mammal cells at high levels [61, 100]. The application of 4-octyl-cyclopentenone up to 400  $\mu\text{M}$  on one-week old leaves of *Brassica* shows no visual adverse effect on plants. In comparison, OPDA was reported to cause harmful effects to plant tissue at higher concentration than 200  $\mu\text{M}$  [115]. When this compound accumulates in cells, it can be detoxified by GSH reductants as shown for phytoprostanes [93]. Although it is still unknown whether 4-octyl-cyclopentenone was taken up by the plant or not, it is predicted that the similar electrophilicity and lipophilicity between this compound and OPDA might endow them similar biological and physical properties. In addition, the exogenous application of  $\alpha,\beta$ -unsaturated RES on plants could induce the accumulation of endogenous RES oxylipins [61] and phytoalexin [90], which were reported to be related to HR response in tobacco leaves [150]. So far, the exogenous application of RES, 4-octyl-cyclopentenone, was not found to induce any HR response or other visual symptoms of disease on *Brassica*. Nevertheless, more experiments need to be performed to evaluate the overall

phytotoxicity related to this compound.

As to the antifungal test of 4-octyl-cyclopentenone on the system of *Arabidopsis* and *B. cinerea*, this compound at concentration of 200  $\mu\text{M}$  does not show 100% inhibition of fungal growth. The direct antifungal activity of 4-octyl-cyclopentenone has been already established *in vitro*. Whilst the compound was effective *in vitro*, complete inhibition of fungal growth was not achieved *in vivo* and some fungal growth recovery was observed. The antifungal activity of 4-octyl-cyclopentenone against *B. cinerea* during *in vivo* testing is attributed to at least two factors:

1. Complex environment, including light, air flow and contaminations that will affect both on plants and pathogens.
2. The changes of chemical and physical properties of the applied compound, including unwanted concentration reduction due to degradation (chemical degradation and biological degradation by the host tissue).

Therefore, these common factors will contribute to the difference between *in vitro* and *in vivo* results.

Apricot fruits are natural hosts for *B. cinerea*, whereas *Arabidopsis* and *B. cinerea* system is mainly used as a model in the laboratory [149]. For the test on apricots, the environment that the compound is exposed to is likely to consist of an array of degradation enzymes that may inactivate it. In addition, the diffusion of the compound into fruit tissue will reduce local concentration. 4-Octyl-cyclopentenone at 200  $\mu\text{M}$  was

effective against *B. cinerea* cultured in apricots within 48 h, however, some fungal growth was evident after 72 h incubation. It is therefore necessary to do more chemical structural modifications of compound in order to improve the performance of these compounds.

### 3.3.5 Conclusion

RES oxylipins are known to have an adverse effect on host plants and the accumulation of hydroperoxides and OPDA was found to have a harmful effect on plants [61]. 4-Octyl-cyclopentenone (with similar antifungal activity to OPDA) so far shows no visual phytotoxicity to *B. oleracea* at 400  $\mu$ M. Complex conditions *in vivo* reduce the antifungal efficacy of 4-octyl-cyclopentenone observed *in vitro* by about 25%. Improved antifungal activity may be achieved through further modification of its chemical structure.

### 3.4 Summary

4-octyl-cyclopentenone was found to be the most effective antifungal compound among six tested compounds *in vitro*. This compound showed the ability of inhibiting spore germination of all tested fungal strains and growth of two fungal strains, *C. herbarum* and *B. cinerea*, at low concentration. Fungal strain inhibition was also achieved against

*B. cinerea* on a leaf surface of *Arabidopsis* and on apricot fruits.

# Chapter 4

## Study on the mode of action of 4-octyl-cyclopentenone

### 4.1 Introduction

4-octyl-cyclopentenone has good *in vitro* antifungal activity against two fungi, *C. herbarum* and *B. cinerea*, however, the knowledge about the mode of action is still lacking, which is important for further modification to improve its fungicidal activity. In order to better understand the observations on the antifungal activity of 4-octyl-cyclopentenone, we have analysed electrolyte leakage and lipid composition of the spores of both sensitive and non-sensitive fungi to determine whether the difference in spore lipids composition related to the sensitivity of the applied compound.

#### 4.1.1 Mode of action of fungicides

Fungicides have been used for thousands of years, but research into their modes of action is relatively recent (last 40 years). Some modes of actions are well known and used to direct the research and development of fungicides [1, 2, 151, 152], which will be more effective to pathogens, less harmful to the environment and lower chance of resistance risk. Some modes of action for fungicides are listed in table 4.1:



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Table 4.1 The various modes of action of different fungicides [2].



For the past decade, many research efforts have been focused on the concept of antifungal agents as so called ‘plant activators’, and their modes of action related to host plant resistance, such as systemic acquired resistance (SAR). As mentioned in Chapter 1, this group of compounds could have direct antifungal activity against several fungal species or act indirectly on host plants by triggering host resistance. The benefit of the application of these compounds is to minimise the adverse effect on the environment.

4-Octyl-cyclopentenone modified from the naturally occurring antifungal agent, OPDA, was shown to have good antifungal activity (see Chapter 3). Two candidate modes of action could contribute to its antifungal activity.

1. Membrane disruption and disorganisation: As a non-ionised surfactant with optimum lipophilicity, it would cause the non-specific disruption of cell membrane integrity. In this way, the fluidity of cell membranes would be disturbed, resulting in increased permeability, modification of proteins, nutrient leakage and subsequent cell death. In addition, the reactivity of this RES with nucleophiles of protein should be taken account as well.
2. Induced host resistance (induced gene expression): As discussed in the introduction, the biological activities of RES, such as OPDA, green leaf volatiles (GLVs) and phytoprostanes, are reported to act through complex defence gene expression and induction of phytoalexins in host plants. The synthetic RES, 4-octyl-cyclopentenone, was predicted to be able to mimic these self-defence activities of plant RES due to the bioactivity of the  $\alpha,\beta$ -unsaturated carbonyl group of cyclopentenone ring, which would reacts with nucleophiles, such as –SH containing proteins.

In this chapter, the research focuses on the effect of 4-octyl-cyclopentenone on membrane disruption and disorganisation.

#### **4.1.2 Plasma membrane structure of cells**

In general, the main function of the plasma membrane is to separate the interior of a cell from the external environment and maintain the transportation and exchange of substances. The plasma membrane is mainly composed of several groups of molecules, such as polar lipids, sterols and membrane proteins (intrinsic and extrinsic), which are essential for the unique structure and biological functions of the membrane (Figure 4.1). Glycerophospholipids are the major lipids of most biological membranes supplemented with sphingolipids and sterols. The basic bilayer structure formed by amphiphilic membrane lipids and their lateral diffusion contribute to membrane fluidity which plays an important role in maintaining membrane structure and regulating basic biological functions. The fluidity of the membrane largely depends on the nature of the concentration of sterols and lipophilicity of lipids. It is commonly assumed that more saturated fatty acids esterified to membrane lipids or adding more sterols makes membrane less fluid. However, the influence of adding sterols on fluidity is still debatable. For example the introduction of cholesterol into the phosphatidylcholine model membrane has no effect on the speed of movement of lipids [25]. Membrane proteins interact with lipids through amphiphilic parts or are anchored to the membrane by covalent lipid modification and are essential for cell function, such as receptors, transporters and signalling molecules.



Figure 4.1 Fluid mosaic model for general cell membranes and their composition [24]. Polar lipids bilayer of membrane forms a fluid structure in which membrane proteins are held by hydrophobic interaction with non-polar chains and free to move laterally in the plane of bilayer.

#### **4.1.3 Electrolyte leakage**

One of the important fungicidal modes of action is disruption of cell membrane caused by dodine (guanidine) or polyenes [2]. The exact structural and functional modification of the cell membrane caused by these compounds is not fully understood. However, the cellular membrane disfunction due to the exposure of these compounds is well expressed in increased fluidity and leakage of ions (e.g. potassium ions), which can be readily measured by the efflux of electrolytes. Hence, the estimation of membrane disruption by measuring cellular electrolyte leakage from affected spores into an aqueous medium is finding a growing use as a measure of cell membrane stability.

Polyene antibiotics are a group of important antifungal antibiotics, including nystatin, amphotericin B, and pimaricin. These drugs interact with sterols in cell membranes (ergosterol in fungal cells; cholesterol in human cells) to form channels through the membrane, causing the cells to become leaky. In this project, nystatin was used to alter the fluidity and permeability of the membrane by causing leakage of potassium ions and other essential substances from cells [153, 154]. In order to evaluate potential cell membrane disruption by 4-octyl-cyclopentenone, we have compared the effect of this compound with nystatin on the efflux of potassium ions from spores.

#### **4.1.4 Lipid extraction and analysis**

Several efficient methods exist for the total extraction of lipids from tissues. Bligh & Dyer and Folch methods using methanol and chloroform-methanol solvent system are very efficient for total lipid extraction [155, 156]. Other solvents and methods have also been used to extract lipids, such as hexane, iso-propanol and solid phase. In order to increase the efficiency of extraction, tissue disruption, such as mechanical homogeniser [9], ultrasonic sound [157] and microwaves [158], have all been used.

Gas chromatography (GC) and high-performance liquid chromatography (HPLC) are two common methods used for analysis of lipids, especially their fatty acid components. In this project, the lipid composition was determined and quantified by GC. Briefly, after converted to their corresponding methyl ester derivatives, volatile fatty acid methyl

esters (FAME) were transported through the column by the flow of an inert, gaseous mobile phase. The column itself contains a liquid stationary phase which separate the FAMEs according to their different affinities for the two phases. The outlet gas from column is ionised and detected by either a flame ionisation detector (FID) or mass spectrometry (MS).

## **4.2 Materials**

All the solvents used in the experiment are HPLC grade unless stated otherwise. Four fungal strains, *C. herbarum*, *F. oxysporum*, *A. brassicicol* and *B. cinerea* (GLUK-1) were used for the study of the modes of action. A large volume of spore suspension at high concentration needed to be prepared in each case.

## **4.3 Methods**

The methods of fungal culture and spore harvest were described in 3.2. The freshly prepared spore suspension was diluted with growth medium and used for the adsorption and leakage tests. For lipid extraction, spores were collected by centrifuge (4,000 *g*, 5 min, Eppendorf Centrifuge 5424) and the resultant pellet was dried in a speed vacuum system (SPD 1010 SpeedVac system, Thermosavant) for 10 min and stored at -80 °C, at

which temperature they could be stored until required without significant loss of activity.

#### **4.3.1 Adsorption of compounds to spores**

The method used for measuring the adsorption of 4-octyl-cyclopentenone and 8-(4-oxocyclopentenyl)octanoic acid to spores was modified from the published method [159]. Briefly, spores of four fungal strains (*F. oxysporum*, *A. brassicicola*, *C. herbarum* and *B. cinerea* (GLUK-1)) were harvested and washed three times with HPLC grade water. After 10 min pre-incubation at room temperature, 4-octyl-cyclopentenone and 8-(4-oxocyclopentenyl)octanoic acid (100  $\mu$ M and 200  $\mu$ M) were mixed with and without fungal spores (concentration of  $10^7$  spores/mL) at room temperature. The suspension was vortexed for 5 s, and the absorbance of the supernatants (obtained by centrifugation at 4,000 g for 5 min) was measured at 227 nm (JENWAY 6505 uv/vis Spectrophotometer).

#### **4.3.2 Leakage test of potassium ions from spores**

Fungal spores were incubated with 4-octyl-cyclopentenone and the leakage of potassium ions was monitored. Nystatin is a polyene antifungal drug which is well studied for its antifungal activity caused by bonding with sterols and the leakage of ions from cells [153, 160]. In this experiment, leakage of potassium ions was monitored by

measuring the potassium concentration in the medium with a potassium selective electrode (Corning Electrode, Corning Ltd.) and the potential derived from the potassium-sensitive electrode was recorded on a mV/pH meter (Corning 240 pH meter). A range of increasing concentrations of KCl solutions was measured to give a standard curve of  $K^+$  before each test. The precultured fungal spores were harvested and washed three times with HPLC grade water and then resuspended in HPLC water to give  $2 \times 10^6$  spores/mL. After one min pre-incubation at room temperature, 4-octyl-cyclopentenone and nystatin were added and the conductivity of suspension was measured every 5-10 min with continuous stirring using a magnetic stirring bar. Spore suspensions without compounds were used as controls.

### **4.3.3 Lipid extraction and analysis**

Two methods were evaluated for lipid extraction from whole cells:

1. Method one was modified from Bligh & Dyer method [161]. The fungal spores in potassium phosphate buffer (1 mL, pH 7.4) were homogenised using an ultrasonic probe (Ultrasonic Processor UP 200S) for 30 min. The suspension was acidified with 0.15 M acetic acid (1mL) followed by the addition of chloroform/methanol (1:2 v/v, 7.5 mL) and this extraction system was kept stirring with a magnetic stirrer for 2 hours at 0 °C. After which, HPLC grade water (2.25 mL) and chloroform (2.25 mL) were added to form phase separation assisted by low-speed centrifugation and the lower chloroform phase containing



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the lipids was removed with a glass pipette and dried under N<sub>2</sub> on a heating block (Dri-Block DB-3A & Sample Concentrator). Samples were resuspended in a small volume of chloroform and stored at -80 °C prior to analysis.

2. Method two was modified by Kates [162] from the method of Bligh & Dyer and used for extraction of lipids from *B. cinerea* by some groups [163]. Hot iso-propanol (IPA) is used in order to inactivate lipid degradative enzymes. Dried spores were transferred to test tubes and extracted with hot IPA (70 °C, 3 mL). After vortexing, the mixture was left to extract for 15 min in a heating block at 70 °C before centrifuging at 1,000 g for 10 min. The supernatant was decanted and the pellet was washed twice more with hot IPA (2×3 mL) and then with IPA/chloroform (3 mL, 1 : 1, v/v). The supernatants were combined, dried under N<sub>2</sub> at 50 °C, resuspended in chloroform-methanol (1 : 1, v/v) and stored at -80 °C.

Lipids were separated and purified by thin-layer chromatography (TLC) on precoated silica gel plates (Merck, TLC silica gel 60) using a non-polar solvent system of iso-hexane/diethyl ether/acetic acid (70:30:1 by vol.). Lipids were identified by lightly staining with iodine vapour. Each stained lipid band on the TLC plate was scraped off into a test tube and fatty acid methylation was performed by the addition of concentrated sulfuric acid (2.5% v/v) in anhydrous methanol (2 mL) [161]. The fatty acid methyl esters (FAME) were extracted by iso-hexane and dried under N<sub>2</sub> and either analysed by GC or stored at -80 °C prior to analysis.

Individual FAME were quantified by a GC (PERKIN ELMER Auto System XL Gas Chromatograph) coupled to a flame-ionisation detector (FID), using a DB-23 capillary column (Agilent 30 m, 0.25 mm, J&W Scientific) with helium as carrier gas. The oven temperature program used was as follows: 100 °C, 20 °C/min to 200 °C, held for 5 min, 10 °C/min to 240 °C, and finally held for 5 min. FID temperatures were 230 °C. FAMES were identified by comparing retention times with standard FAMES and quantified using an internal standard of methyl-heptadecanoate (17:0).

## **4.4 Results**

### **4.4.1 Adsorption of compounds to spores**

Ultraviolet spectroscopy is a useful method of spectroscopic analysis to detect organic molecules containing conjugated systems, such as dienes,  $\alpha,\beta$ -unsaturated carbonyl compounds and aromatics [164]. The solution of 4-octyl-cyclopentenone and 8-(4-oxocyclopentenyl)octanoic acid containing cyclopentenone rings were scanned from 200 nm to 300 nm and the absorbance peak was found near 227 nm. According to the Beer-Lambert law, the absorbance of compounds is proportional to the concentration. Fungal spores were mixed with compounds and incubated for 1 min based on the published method [159]. Centrifuge was then used to obtain the supernatant from the cell suspension. The residual of 4-octyl-cyclopentenone after

treatments of *B. cinerea*, *C. herbarum* and *A. brassicicola* was approximately 45%; with respect to *F. oxysporum*, it was about 70%. In case of 8-(4-oxocyclopentenyl)octanoic acid only 2% or less was adsorbed to all species after treatments. This results shows that 4-octyl-cyclopentenone has much higher affinity to spores than 8-(4-oxocyclopentenyl)octanoic acid that shows very little adsorption to cells.

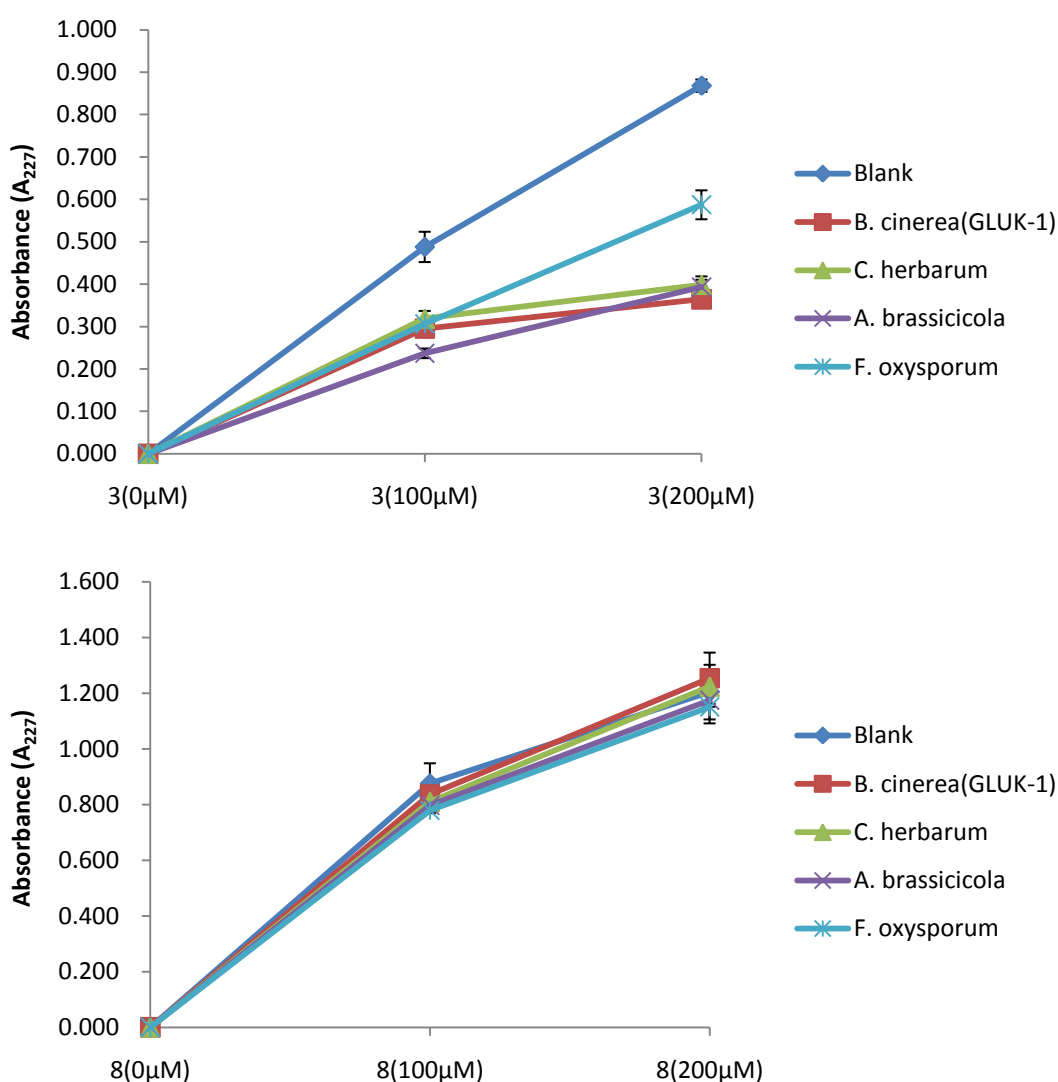


Figure 4.2 Adsorption of 4-octyl-cyclopentenone (**3**) and 8-(4-oxocyclopentenyl)octanoic acid (**8**) to fungal spores. Compounds at two concentrations of 100  $\mu\text{M}$  and 200  $\mu\text{M}$  were mixed with different spore suspensions (Blank, *B. cinerea*, *C. herbarum*, *A. brassicicola* and *F. oxysporum* with  $10^7$  spores/mL) for 1 min. Absorbance at 227 nm was measured in the supernatant obtained. Values are means  $\pm$  SD (n=3).

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#### 4.4.2 Leakage of potassium ions from spores

The leakage of  $K^+$  caused by the addition of 4-octyl-cyclopentenone (200  $\mu$ M, 39  $\mu$ g/mL) was measured and compared with nystatin (50  $\mu$ g/mL). Spores were incubated in the presence of compounds for 1 hour and the efflux of  $K^+$  was recorded every 5 min. Nystatin induced efflux of  $K^+$  to the highest level for all the fungal spores within 20 min. 4-Octyl-cyclopentenone caused leakage of  $K^+$  in the incubation of two fungal strains, *B. cinerea* and *C. herbarum*, however, the leakages were only about 35% and 25%, respectively, of that caused by nystatin. For the other two fungal strains, *A. brassicicola* and *F. oxysporum*, the leakage caused by 4-octyl-cyclopentenone was almost undetectable (Figure 4.3). Although the leakage caused by 4-octyl-cyclopentenone was lower than nystatin. This result is still correlated to the results of the *in vitro* antifungal test, i.e. the more sensitive the fungal strains (*B. cinerea* and *C. herbarum*), the more leakage of  $K^+$  caused by 4-octyl-cyclopentenone. Summarising, leakages of  $K^+$  were only detected for two fungal strains sensitive to 4-octyl-cyclopentenone, however the level was lower in comparison to the commercial polyene drug, nystatin.

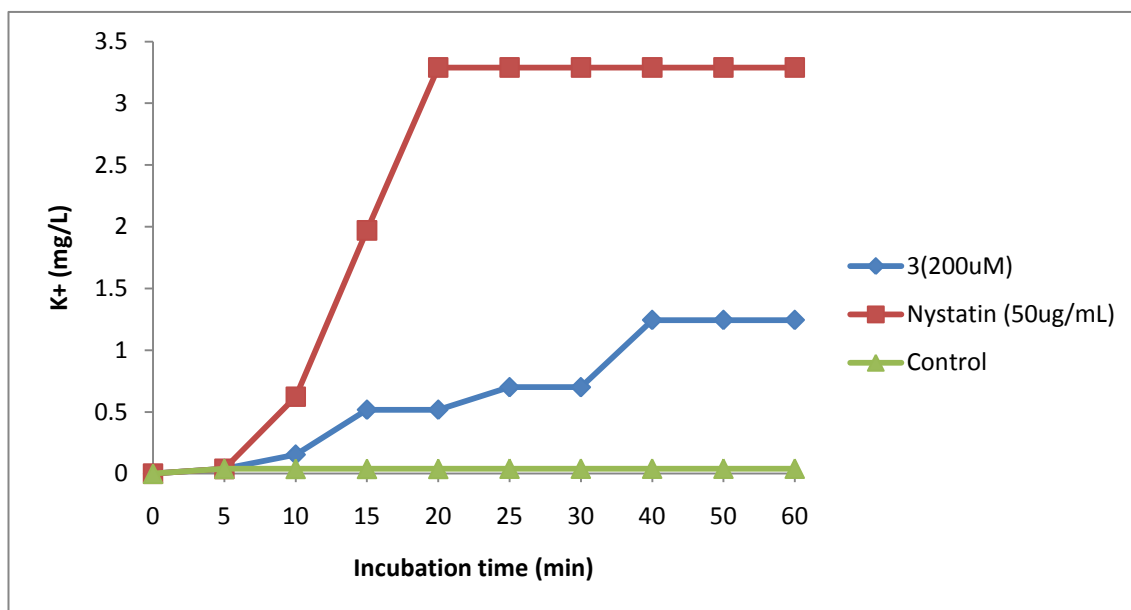
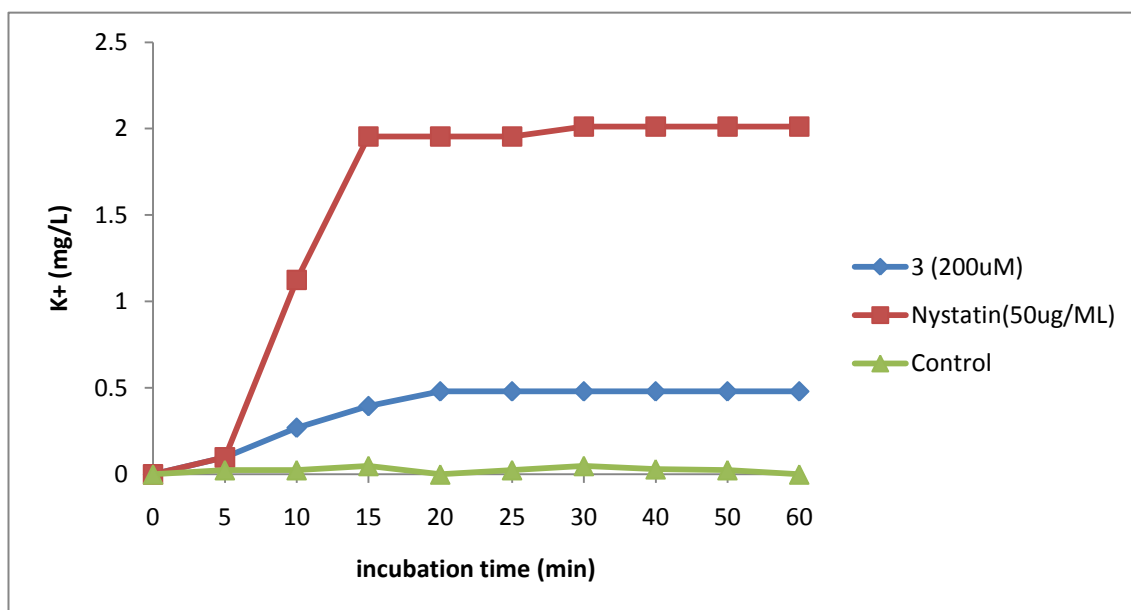
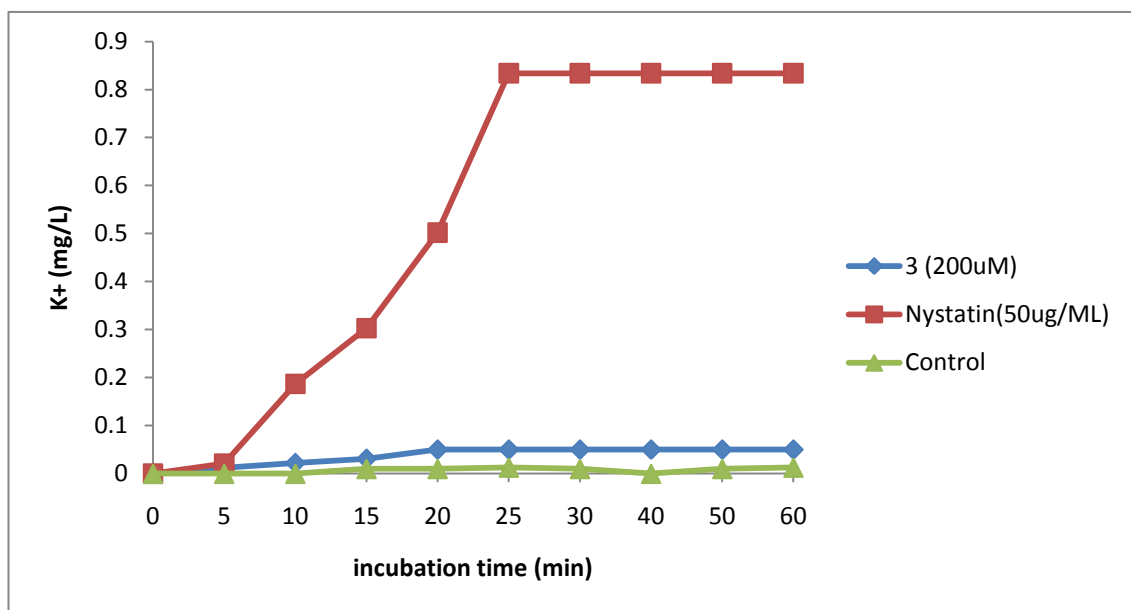
(a) Leakage test of potassium ions from spores of *B. cinerea* (GLUK-1)(b) Leakage test of potassium ions from spores of *C. herbarum*.

Figure 4.3 Leakage test of potassium ions. 4-octyl-cyclopentenone (**3**) at 200  $\mu\text{M}$  and nystatin at 50  $\mu\text{g/mL}$  were added to spore suspensions (a) *B. cinerea* (GLUK-1), (b) *C. herbarum*, (c) *F. oxysporum* and (d) *A. brassicicola* with concentration of  $2 \times 10^6$  spores/mL). Spore suspension in the absence of compounds was used as a control.  $\text{K}^+$  efflux into the medium was measured with a  $\text{K}^+$  selective electrode.

(c) Leakage test of potassium ions from spores of *F. oxysporum*.



(d) Leakage test of potassium ions from spores of *A. brassicicola*.

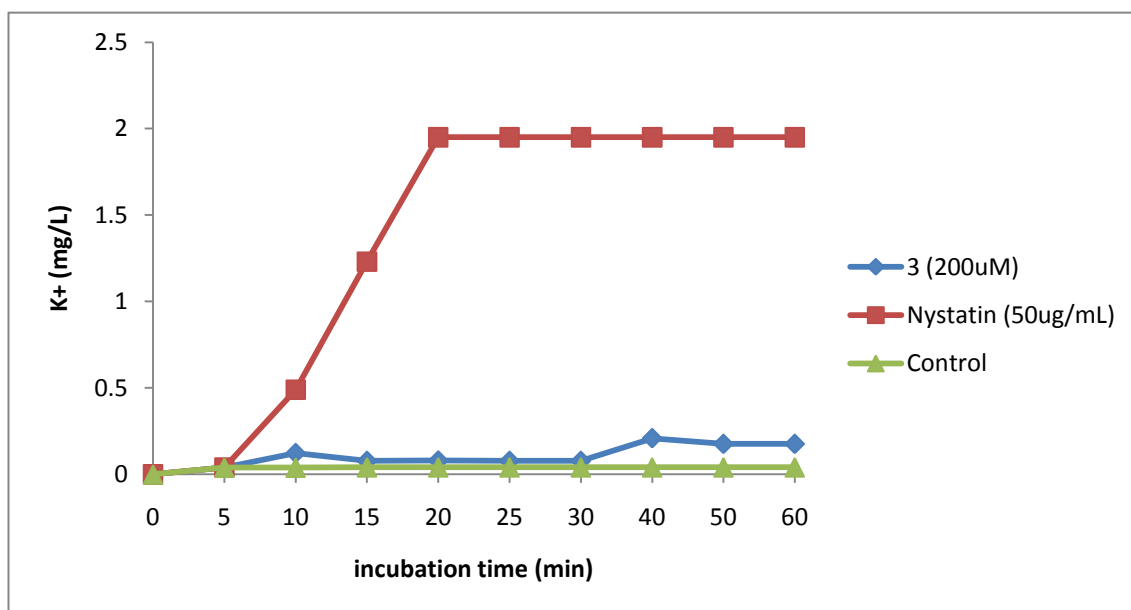


Figure 4.3 Leakage test of potassium ions. 4-octyl-cyclopentenone (**3**) at 200  $\mu\text{M}$  and nystatin at 50  $\mu\text{g}/\text{mL}$  were added to spore suspensions (a) *B. cinerea* (GLUK-1), (b) *C. herbarum*, (c) *F. oxysporum* and (d) *A. brassicicola* with concentration of  $2 \times 10^6$  spores/mL). Spore suspension in the absence of compounds was used as a control.  $\text{K}^+$  efflux into the medium was measured with a  $\text{K}^+$  selective electrode.

#### 4.4.3 Lipid composition of fungal spores

Two lipid extraction methods, Bligh & Dyer and hot IPA extraction, were used for lipid extraction and analysis. Hot IPA is considered to be a better extraction solvent than chloroform/methanol (Bligh & Dyer method) and used for lipid extraction, due to its reasonable yield (Table 4.2) and inactivation of lipid degradative enzymes (Table 4.3).

Table 4.2 Comparison of the yield of lipid extractions using different methods.

Methods of Extraction	Yield (Total lipid/Dry weight %)			
	<i>C. herbarum</i>	<i>F. oxysporum</i>	<i>B. cinerea</i>	<i>A. brassicicola</i>
Bligh & Dyer	0.54	0.52	0.10	0.61
Hot IPA	0.37	8.15	0.25	0.27

Table 4.3 Comparison of the composition of NEFA from extractions using different methods for fungal strains.

Methods of Extraction	Composition of NEFA (wt%)			
	<i>A. Herbarum</i>	<i>F. oxysporum</i>	<i>B. cinerea</i>	<i>A. brassicicola</i>
Bligh & Dyer	31.5	33.5	22.4	8.7
Hot IPA	4.0	2.9	7.83	4.7

The fatty acids extracted from spores are mainly composed of palmitate (16:0), stearate (18:0), oleate (18:1 n-9), linoleate (18:2 n-6) and  $\alpha$ -linolenate (18:3 n-3). The major fatty acid composition of four fungi is shown in table 4.4. Linoleic acid (18:2 n-6) was the major fatty acid in three species, *A. brassicicola*, *C. herbarum* and *B. cinerea*. Two fungi, *F. oxysporum* and *C. herbarum*, are rich in oleic acid (18:1 n-9). The amount of

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linolenic acid (18:3 n-3) was higher in *A. brassicicola* and *B. cinerea* (GLUK-1) than the other two fungal strains.

Lipid extracts were separated by TLC using a non-polar solvent system of iso-hexane/diethyl ether/acetic acid (70:30:1 by vol.) to achieve the separated lipid classes including polar lipids, monoacylglycerols (MAGs), diacylglycerols (DAGs), non-esterified (free) fatty acids (NEFAs), triacylglycerols (TAGs) and sterol esters (SE). Lipid class distribution of the four fungi examined is shown in Table 4.5, Figure 4.4 and Figure 4.5. Lipid composition of fungal strains was found to be different for each fungal strain. *C. herbarum* and *B. cinerea* (GLUK-1) had higher levels of polar lipids than non-polar lipids. In contrast, there were lower polar lipids in the other two fungal strains in comparison with non-polar lipids (Table 4.5). Non-polar lipids of all species were composed mainly of TAG (over 70% of non-polar lipids, except for *B. cinerea* in which TAG constitutes 54%), primarily used as lipid storage for subsequent germination and growth of the spores. The other non-polar lipid classes (MAG, DAG, NEFA and SE) are relatively minor components. Low amounts of NEFA indicate that little hydrolysis of endogenous lipids occurred during the extraction.

Linoleic acids (18:2, n-6) was the main polyunsaturated fatty acid of both polar and non-polar lipids, while linolenic acid (18:3, n-3) was only detected in *A. brassicicola* and *B. cinerea* and at much lower levels than linoleic acids (18:2, n-6). Oleic acid (18:1, n-9) was detected in non-polar lipids with high levels of TAG in *F. oxysporum*, while a



low level of oleic acid in the lipids was detected in *B. cinerea*. Palmitic acid (16:0) is the dominant saturated fatty acids accumulated in both polar and non-polar lipids of all fungal strains, which was higher than the other saturated fatty acid, stearic acid (18:0).

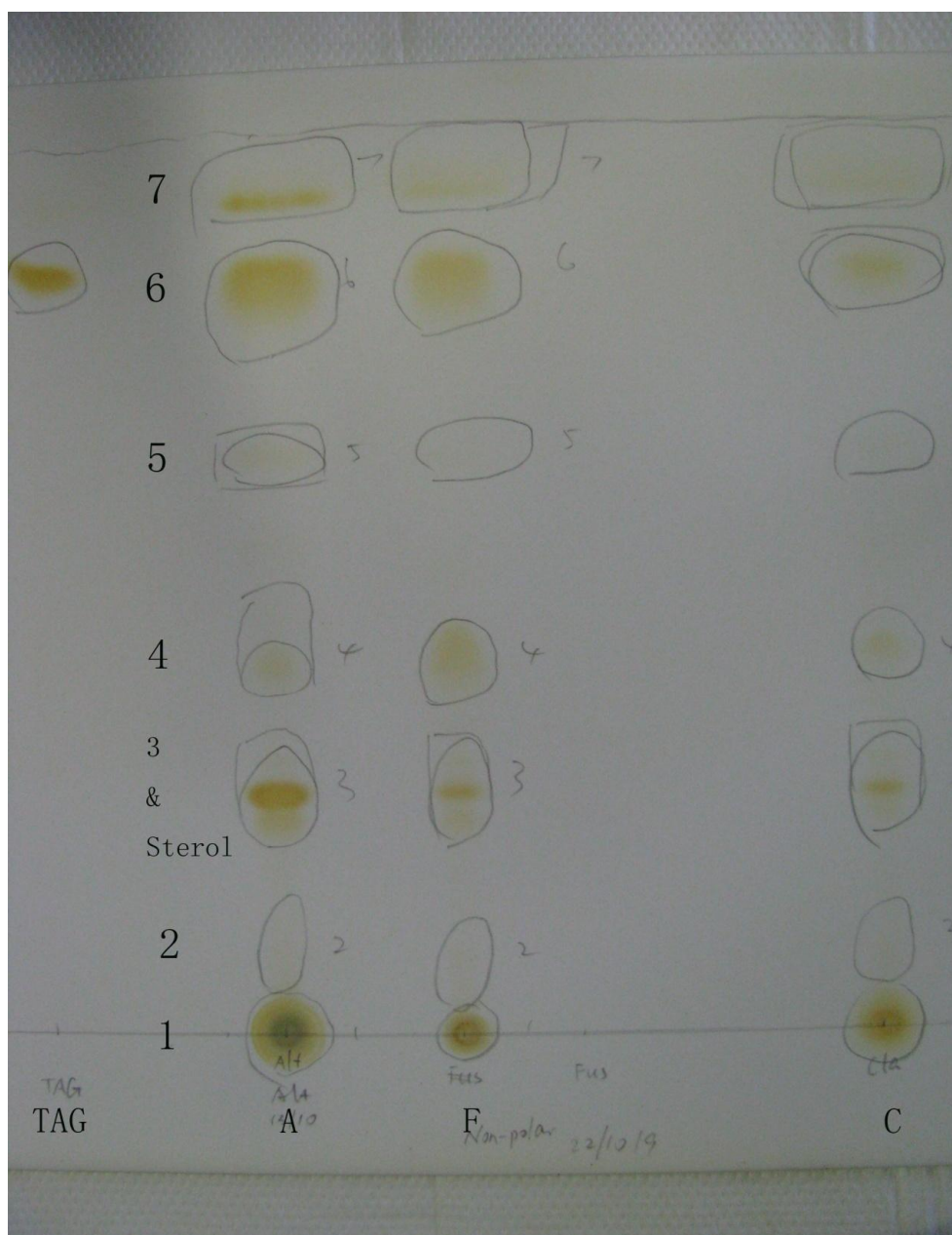


Figure 4.4 Lipid classes of three fungal strains. Lipid classes of three fungal strains (A: *A. brassicicola*; F: *F. oxysporum*; C: *C. herbarum*) were separated by non-polar TLC plate and stained in iodine vapour. Class 1, Polar lipids; 2, MAG; 3, DAG (Sterols were shown in the middle of the band of DAG); 4, NEFA; 5, Unknown band; 6, TAG (TAG standard was used.); 7, Sterol esters.

Table 4.4 The major fatty acid composition of four fungal strains.

Fatty Acid	Lipid Composition (wt. %)			
	<i>A. brassicicola</i>	<i>F. oxysporum</i>	<i>C. herbarum</i>	<i>B. cinerea</i> (GLUK-1)
Palmitate(16:0)	21.6±1.3	27.6±3.0	12.8±0.5	27.0±0.8
Stearate(18:0)	9.9±1.7	11.3±2.5	5.2±0.4	12.5±1.0
Oleate(18:1 n-9)	15.2±2.1	29.1±2.7	32.1±0.5	4.3±0.2
Linoleate(18:2 n-6)	48.3±0.6	29.6±3.2	49.5±0.5	48.1±1.0
α-linolenate(18:3 n-3)	5.0±0.4	2.6±0.5	0.3±0.1	8.1±1.2

Results show means ±SD for three separate experiments.

Table 4.5 Lipid class distribution of four fungal strains.

	Lipids (fatty acids wt. %)			
	<i>A. brassicicola</i>	<i>F. oxysporum</i>	<i>C. herbarum</i>	<i>B. cinerea</i> (GLUK-1)
Polar lipids	34.6±3.1	11.9±0.8	62.2±2.6	60.7±0.8
Monoacylglycerols	1.0±0.7	0.2±0.3	0.4±0.1	2.7±0.2
Diacylglycerols	4.7±0.6	1.1±0.3	5.5±0.1	5.7±0.3
Nonesterified fatty acids	5.2±0.8	2.9±1.5	4.0±0.1	6.6±2.4
Triacylglycerols	51.9±4.6	82.9±1.2	25.8±2.3	21.4±1.2
Sterol esters	2.6±0.6	1.1±0.2	2.2±0.4	3.0±0.1
Non-polar lipids	65.4±3.1	88.1±0.8	37.8±2.6	39.3±0.8

Total lipid extracts were separated TLC by the non-polar solvent system and the separated class were quantified by GC of their derived fatty acids. Results show means ±SD for three separate experiments.

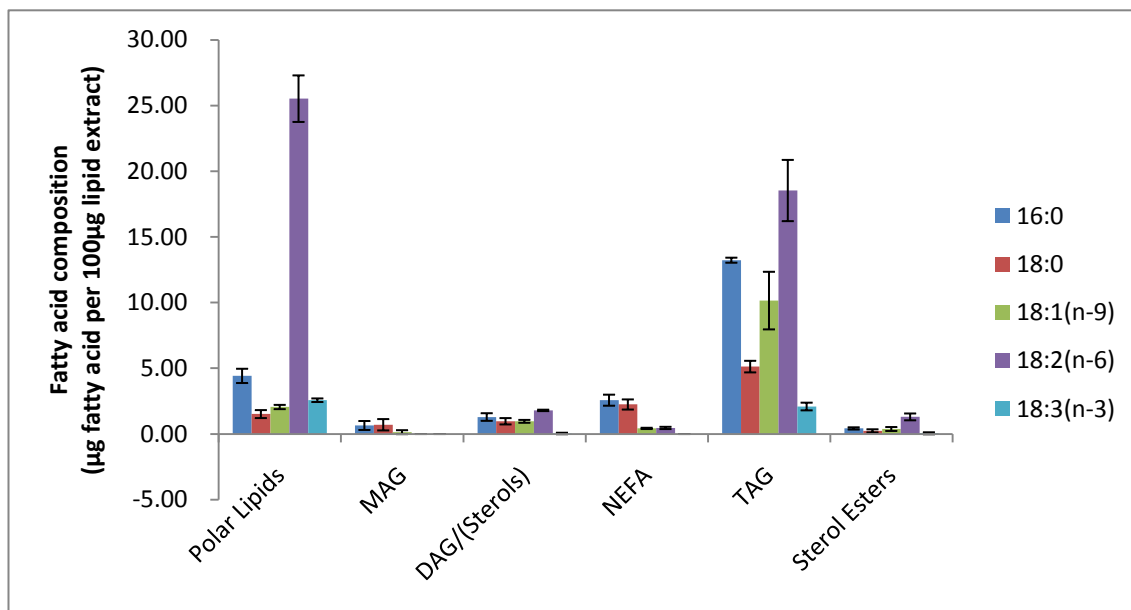
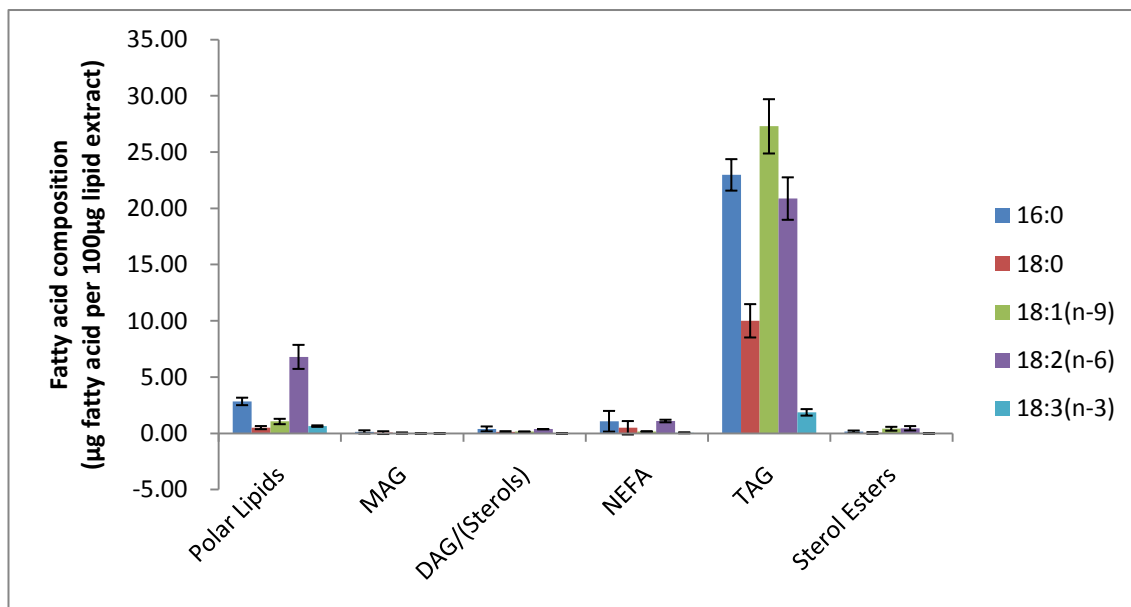
(a) Fatty acid composition of lipid classes of *A. brassicicola*.(b) Fatty acid composition of lipid classes of *F. oxysporum*.

Figure 4.5 Fatty acid composition of lipid classes. Fatty acid abbreviations: 16:0, palmitate; 18:0, stearate; 18:1 (n-9), oleate; 18:2 (n-6), linoleate; 18:3 (n-3),  $\alpha$ -linolenate. Lipid abbreviations: DAG, diacylglycerol; MAG, monoacylglycerol; NEFA, nonesterified fatty acids; TAG, triacylglycerol. Results as means  $\pm$ SD for three separate experiments.

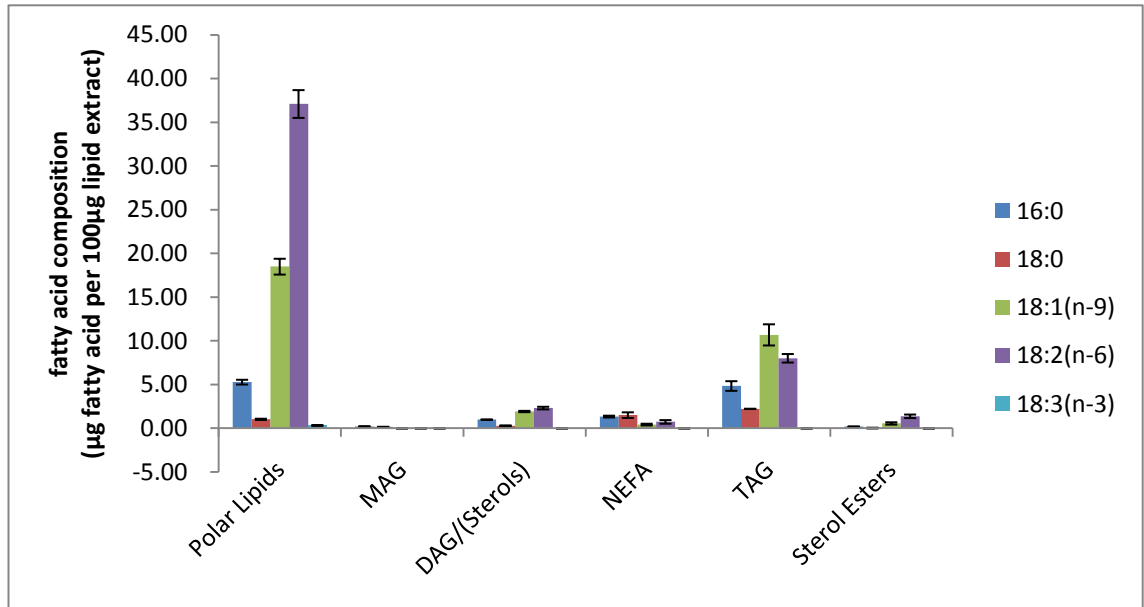
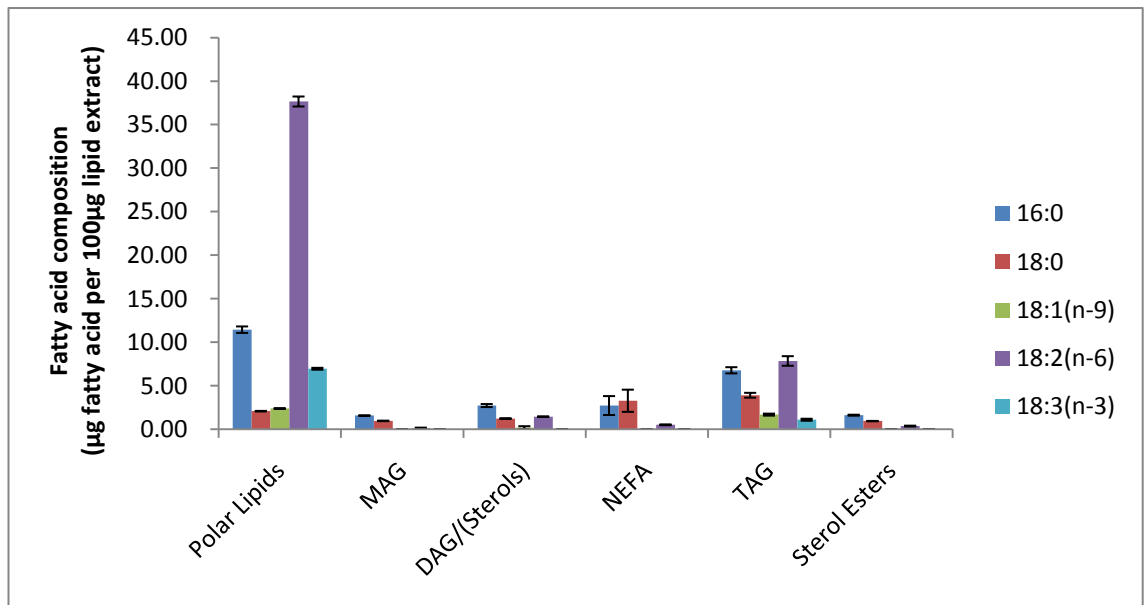
(c) Fatty acid composition of lipid classes of *C. herbarum*.(d) Fatty acid composition of lipid classes of *B. cinerea* (GLUK-1).

Figure 4.5 Fatty acid composition of lipid classes. Fatty acid abbreviations: 16:0, palmitate; 18:0, stearate; 18:1 (n-9), oleate; 18:2 (n-6), linoleate; 18:3 (n-3),  $\alpha$ -linolenate. Lipid abbreviations: DAG, diacylglycerol; MAG, monoacylglycerol; NEFA, nonesterified fatty acids; TAG, triacylglycerol. Results as means  $\pm$ SD for three separate experiments.

The plasma membrane of spores is rich in polar lipids and its fluidity largely depends on the degree of unsaturation of hydrophobic membrane lipids and the concentration of sterols [25]. The degree of unsaturation ( $\Delta/\text{mol}$ ) is described as:

$$\Delta/\text{mol} = [1 \times (\% 18:1) + 2 \times (\% 18:2) + 3 \times (\% 18:3)] / 100$$

The polar lipids of *A. brassicicola* and *B. cinerea* are rich in linoleic acids (18:2, n-6) which results in a higher  $\Delta/\text{mol}$  value than for the other two species (Table 4.6). For *C. herbarum*, the degree of unsaturation is lowered by the high level of oleic acid (18:1 n-9) in polar lipids. *F. oxysporum* had the lowest UNSAT/SAT ratio and degree of unsaturation. Since *B. cinerea* and *C. herbarum* are the species which are more sensitive to 4-octyl-cyclopentenone, the data obtained from the lipid analysis of the species reveals no obvious correlation between lipid composition and antifungal activity.

Table 4.6 The values of UNSAT/SAT and degree of unsaturation ( $\Delta/\text{mol}$ ) of four fungal strains: *A. brassicicola*, *F. oxysporum*, *C. herbarum* and *B. cinerea* (GLUK-1).

Fungal strains	UNSAT/SAT (mol/mol)	$\Delta/\text{mol}$
<i>A. brassicicola</i>	4.8 $\pm$ 0.4	1.7 $\pm$ 0.3
<i>F. oxysporum</i>	2.4 $\pm$ 0.6	1.4 $\pm$ 0.1
<i>C. herbarum</i>	8.2 $\pm$ 0.3	1.5 $\pm$ 0.1
<i>B. cinerea</i> (GLUK-1)	3.2 $\pm$ 0.1	1.6 $\pm$ 0.1

Results show means  $\pm$ SD for three separate experiments.

## 4.5 Discussion

The process by which test compounds go through cell wall is usually neglected in this research. The fungal cell wall (consisting largely of chitin and other polysaccharides) is usually known as a semi-permeable layer which permits the passage of small molecule and proteins. In addition, there is no correlation between the antifungal activity of oxylipins and different cell wall structures of fungal species (true fungi have chitin and oomycete have cellulose) [11]. For these reasons, we have focussed our discussion of the mode of action of these synthetic compounds on their effects on cell membrane components. The adsorption test of compounds to spores revealed that 4-octyl-cyclopentenone had good affinity to fungal membranes for all fungal species. As a non-ionic surfactant, polar and non-polar moieties of 4-octyl-cyclopentenone are balanced to achieve the optimal lipophilicity resulting in a favourable membrane interaction. Antifungal activity of other non-ionised surfactants [138] suggested that they interact with the plasma membrane and disrupt its functions. The adsorption of 4-octyl-cyclopentenone is a pre-requisite for further investigation into its mode of action. This finding could explain, at least in part, the inhibition of spore germination of fungal species that were exposed to 4-octyl-cyclopentenone. In contrast, 8-(4-oxocyclopentenyl)octanoic acid shows very poor affinity to cells and has low antifungal activity (as discussed in Chapter 3). The results of this experiment indicate that its inactivity could be caused by its low affinity to fungal spores.

Having established that 4-octyl-cyclopentenone can be adsorbed to the spores, the next question to address is by what mode of action 4-octyl-cyclopentenone impedes fungal membrane activity. As a non-ionic surfactant, 4-octyl-cyclopentenone is first likely to interact with the membrane and alter its fluidity leading to leakage of essential internal substances. In this way, the membrane will be disrupted and spore growth will be inhibited by electrolyte leakage. This is one of the established mechanisms of cell disruption, which are shared by many antifungal substances such as other non-ionic surfactants [159] (including (E)-2-alkenals [70]), polyenes [153] and plant defensins [160]. In order to prove this, the efflux of potassium ions from spores was measured in presence of 4-octyl-cyclopentenone. Nystatin was used as a reference in this experiment as it has previously been shown to have broad antifungal activity. The notable leakage of potassium ions from cells exposed to 4-octyl-cyclopentenone was only found in suspensions of *C. herbarum* and *B. cinerea* whose fungal growths were inhibited by 4-octyl-cyclopentenone during *in vitro* tests. However, the leakage caused by 4-octyl-cyclopentenone was lower than nystatin, which has greater antifungal activity. A very low level leakage of  $K^+$  was detected for the other two fungal strains, which are less sensitive to 4-octyl-cyclopentenone on fungal growth. The leakage of  $K^+$  caused by the disruption of the fungal membrane correlated with the results of *in vitro* antifungal test (see Chapter 3). It is still unknown whether 4-octyl-cyclopentenone interacts with lipids or sterols or lipid proteins; also other potential modes of action could not be ruled out, therefore, it will be important in the future study to identify the active sites.

Lipids were extracted from organisms and analysed to explore the relationship between antifungal mechanisms and lipid composition. Apart from the differences between fungal species, there are many important factors involved in determining the influence of the environment on fungal lipids, such as temperature, pH, carbon source, inorganic nutrients and aeration (reviewed in [23]). Therefore, the growth conditions of each fungus were controlled to minimise the difference between batches. Two lipid extraction methods, Bligh & Dyer and hot IPA extraction, were compared for their efficiency of lipid extraction. The classic Bligh & Dyer method using chloroform/methanol is the most widely used and ultrasonic homogeniser was used to aid cellular disruption to increase the yield. The yields of these two methods are listed in Table 4.2. Hot IPA was found to be more efficient for lipid extraction from *F. oxysporum* and *B. cinerea*, whereas Bligh & Dyer extraction had higher yield for *A. brassicicola* and *C. herbarum*. Therefore, hot IPA also shows good effectiveness for lipid extraction. The reason for choosing hot IPA for lipid analysis here is not only because of the yield of extraction, but also to achieve a low level of NEFA (Table 4.3). Using Bligh & Dyer extraction assisted by ultrasonic sound, a significant high level of NEFA was detected from extracts of fungal spores, which indicates that fatty acids were readily hydrolysed from TAG or polar lipids by endogenous enzymes. In addition, the high energy and force generated from the ultrasonic homogeniser could also increase the level of NEFA through hydrolysis. In contrast, hot IPA was used as a solvent to inactivate enzymes of degradation of lipids to obtain the lowest NEFA level. In future, further modifications could be done to increase yield and eliminate lipid degradation



during Bligh & Dyer extraction, such as using mechanical homogenisers at low temperature and inactivating degrading enzymes (for example, inhibiting phospholipase by using mepacrine [165]).

From the results of lipids analysis, there is little correlation between sensitivities of fungal strains to 4-octyl-cyclopentenone and lipid/ fatty acid composition of the spores. For polar lipids, *A. brassicicola* and *C. herbarum* have higher values of UNSAT/SAT ratios than *F. oxysporum* and *B. cinerea*. In contrast, *A. brassicicola* and *B. cinerea* have higher degrees of unsaturation ( $\Delta$ /mol) than *F. oxysporum* and *C. herbarum*. Major findings are as follows:

1. Sensitive fungal strains, *C. herbarum* and *B. cinerea*, have a higher proportion of polar lipids.
2. *F. oxysporum* has the lowest proportion of polar lipids and also contains the lowest content of linoleic acid (18:2 n-6) compared with that of polar lipids of other species resulting in the lowest UNSAT/SAT ratio and  $\Delta$ /mol of all four species examined. Additionally, the lowest adsorption of 4-octyl-cyclopentenone to *F. oxysporum* and the lowest leakage of  $K^+$  caused by either nystatin or 4-octyl-cyclopentenone were found as well.
3. No correlation was found between the sensitivity of *A. brassicicola* to test compounds and their lipid composition.

Up to now, it is known that 4-octyl-cyclopentenone is able to disrupt cell membranes of

selective species and causes leakage, although the exact mechanism requires further investigation to elucidate the process. The data presented here suggested similar ion leakage and membrane disruption caused by the application of the compound. However, no clear relationship was evidenced showing the lipid composition and the degree of unsaturation in relation to the sensitivity toward 4-octyl-cyclopentenone.

Besides the degree of unsaturation, two remaining important factors are the concentration of ergosterols and modification of proteins, which could be potential active sites for test compounds. Ergosterols in the membrane, influence membrane fluidity lead to the leakage of  $K^+$ . Nystatin (and some other compounds) target and bind to sterols to change fluidity were reported [166]. It is therefore necessary to analyse the concentration of ergosterols in the membrane. On non-polar TLC plates of lipids, strong sterol bands were observed near DAG in all samples (Figure 4.4). The details of how compound interacts with membrane protein and which protein is targeted are still unknown. More research needs to be done to unveil the influence of these two factors on the interaction between test compounds and pathogens. The utilisation of other techniques could be helpful to reveal further information of the mode of action of 4-octyl-cyclopentenone. Transmission electron microscopy (TEM) is capable of imaging at a significantly higher resolution than light microscopes and is used by many research groups to show the structural changes and cell disruption of fungi exposed to antifungal chemicals, in order to identify active sites [167]. Another approach is to use radio-labelled compounds to highlight potential candidate target sites [76].

## **4.6 Conclusion**

4-octyl-cyclopentenone was readily adsorbed to the spores of all fungal species tested. However, fungal spore sensitivity to the test compound showed that high electrolyte leakage might account for their antifungal mode of action. In this project, no correlation was found between the lipid composition of spores and their sensitivity to test compounds. In short, the fungicidal mode of action could be the direct electrolyte leakage and membrane disruption caused by permeation of compound and binding to membrane active sites.

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# Chapter 5

## Antimicrobial Activities of (E)-2-Alkenals

### 5.1 Introduction

As a reactive electrophilic species (RES), 4-octyl-cyclopentenone was simplified to mimic the biological activity of OPDA. Could it be simplified even further and still have antifungal activity? As mentioned above, the  $\alpha,\beta$ -unsaturated carbonyl group is the important motif which endows the compounds with biological activities. How essential is the cyclopentenone ring or could just an  $\alpha,\beta$ -unsaturated carbonyl group with alkyl chain exhibits the same properties? For comparative purposes, we have used aliphatic (E)-2-alkenals, in order to assess, indirectly, the relative importance of the cyclopentenone ring. (E)-2-alkenals are a group of RES containing electrophilic  $\alpha,\beta$ -unsaturated carbonyl groups and hydrophobic alkyl chains. They have been well studied due to their common availability. This group of compounds is reported to have antimicrobial activities and potential cytotoxicities [100, 168]. In this chapter, their antifungal activities against plant pathogens (*A. brassicicola*, *F. oxysporum*, *C. herbarum*, *B. cinerea* (MUCL30158 and GLUK-1)) were tested and compared with 4-octyl-cyclopentenone. In addition, another two bacteria (*Escherichia coli*, *Staphylococcus aureus*) and one human fungal pathogen (*Candida albicans*) were tested

to evaluate the antifungal activity of (E)-2-alkenals. Based on previous work [68, 169], the antimicrobial activity of medium-chain (E)-2-alkenals proved to be the most efficient in a range from C9 to C13. Here we evaluate (C9-C13) (E)-2-alkenals and compare them directly with the synthetic compound, 4-octyl-cyclopentenone.

## 5.2 Materials

Medium-chain (C9-C13) (E)-2-alkenals were purchased from Sigma-Aldrich. HPLC grade solvents were used in experiment unless stated otherwise; five plant pathogenic fungal strains (*A. brassicicola*, *F. oxysporum*, *C. herbarum*, *B. cinerea* (MUCL30158 and GLUK-1)) and equipment used for antifungal test were used as reported in Chapter 3.

Antimicrobial minimum inhibitory concentration (MIC) assays of three new microorganisms (*Escherichia coli*, *Staphylococcus aureus* and *Candida albicans*) were supervised by Professor Peter Lambert (Life & Health Science, Aston University). The organisms listed in table 5.1 were supplied by Professor Lambert.

Table 5.1 Classification and culture method of human pathogens.

Name	Classification	Growth condition
<i>Escherichia coli</i> (NCTC 10418)	Gram negative bacteria	Nutrient agar (NA) at 37 °C
<i>Staphylococcus aureus</i> (NCTC 6571)	Gram positive bacteria	Nutrient agar (NA) at 37 °C
<i>Candida albicans</i> (ATCC 76615)	Fungus	Sabouraud dextrose agar (SDA) at 30 °C

NCTC: National Collection of Type Cultures; ATCC: American Type Culture Collection

### 5.3 Methods

#### 5.3.1 Antifungal tests against plant fungal strains

The methods used for these assays were the same as described in Chapter 3. Briefly, fungal spores (for *A. brassicicola* and *F. oxysporum*, spore suspensions with concentration of 5,000 spores/well in PDB were prepared) or precultured germinated spores (*C. herbarum* 2,000 spores/well and *B. cinerea* (MUCL30158 & GLUK-1), 5,000 spores/well in 5% V8 juice) for 16 h, were incubated in the presence of test compounds in 96 well microplate, sealed with parafilm, in a final volume of 100  $\mu$ L. The fungal growth/turbidity was measured by a Bio-rad microplate reader at 595 nm after 24, 48 and 72 hours.

### 5.3.2 Agar diffusion tests

A modified agar diffusion test [170] was also set up to reveal any inhibition of growth by the test compounds, which were placed on the agar. When the compound inhibits the growth of microbes, it is seen as a clear zone on the agar gel. An inoculation loop was used to transfer microbes from agar (*E. coli*, *S. aureus* on nutrient agar (NA) and *C. albicans* on sabouraud dextrose agar (SDA), Oxoid) plates and suspended into broth (*E. coli*, *S. aureus* in nutrient broth (NB) and *C. albicans* in sabouraud dextrose broth (SDB), Oxoid) forming uniform solutions. Cells in broth solutions were swabbed uniformly across culture plates. Compounds were dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich) and the concentration was diluted to 10 mg/mL. DMSO has little effect on pathogen growth. 20  $\mu$ L of compounds solution was added to the application sites where a round piece of agar was removed by using the end of a sterilised glass pipette (Figure 5.2). Petri-dishes were sealed with parafilm and incubated at 37 °C for bacteria and at 30 °C for fungi. The effects of several compounds on the inhibition of growth are shown photographically after 24 h.

### 5.3.3 Antimicrobial MIC assays

The method used for antimicrobial assays is that modified from Kubo [68, 169]. All antimicrobial susceptibility tests in this study were performed under a standard condition using fresh inoculums from agar culture (*E. coli*, *S. aureus* on NA and *C.*

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*albicans* on SDA), with final inoculum size of  $10^5$  cells/mL in the broth (*E. coli*, *S. aureus* in NB and *C. albicans* in SDB, Oxoid). Test compounds were dissolved in DMSO and a wide range of concentrations were prepared for the tests against the pathogens in order to calculate the minimum inhibitory concentration (MIC). A serial 2-fold dilution of test compounds was prepared in DMSO [170], and 30  $\mu$ L of the compound solution was added to 3 mL of inoculated suspension with a final concentration of  $10^5$  cells/mL. The assay tubes were incubated at 37 °C for bacteria and at 30 °C for fungi without shaking for 24 h. MIC values were recorded as the lowest concentration of compound that demonstrated no visible growth.

## 5.4 Results

### 5.4.1 Antifungal activity against plant pathogens

The effect of medium-chain (C9-C13) (E)-2-alkenals on the growth of four plant pathogens is given in Figure 5.1. There was no significant difference between control and treatments of (E)-2-alkenals (100  $\mu$ M) against *A. brassicicola* and *C. herbarum*. 4-Octyl-cyclopentenone showed much better antifungal effectiveness than (E)-2-alkenals against these two fungi (Figure 5.1 a and c). For *F. oxysporum*, (E)-2-dodecenal at 100  $\mu$ M was the most effective one against this fungal strain, which was slight better than 4-octyl-cyclopentenone. The inhibition was nearly 90% ( $P < 0.01$ )

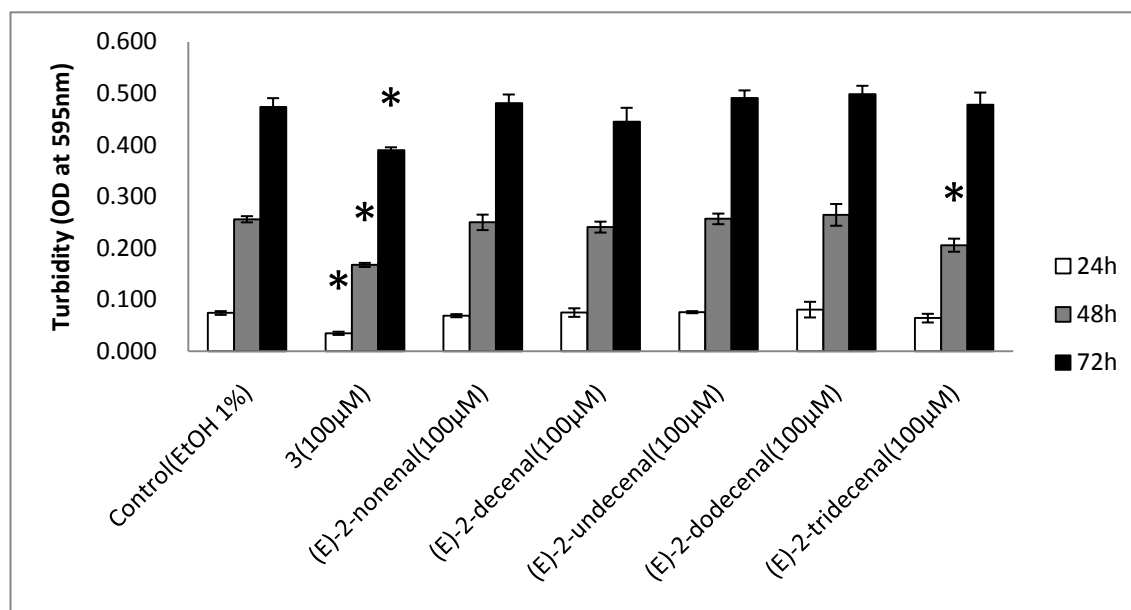


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after 24 h and fungal growth recovered after 48 h (Figure 5.1 b). (E)-2-dodecenal and (E)-2-undecenal at 100  $\mu$ M were effective on fungal growth of *B. cinerea* (MUCL30158) and the inhibition was over 90% ( $P < 0.01$ ) after 24 h. However, fungal growth in wells treated with (E)-2-alkenals recovered much quicker than those treated with 4-octyl-cyclopentenone (Figure 5.1 d). For *B. cinerea* (GLUK-1), only (E)-2-dodecenal had limited antifungal activity (60% inhibition after 24 h,  $P < 0.01$ ) at 200  $\mu$ M (Figure 5.1 e).

In conclusion, (E)-2-alkenals showed limited growth inhibition against *F. oxysporum* and *B. cinerea* and had little effect on the growth of *A. brassicicola* and *C. herbarum*. (E)-2-undecenal and (E)-2-dodecenal at concentration of 100  $\mu$ M inhibited growth of *F. oxysporum* and *B. cinerea* (MUCL30158) for the first 24 h, however, growth recovered very quickly after 48 h culture for all fungal pathogens. 4-Octyl-cyclopentenone exhibited higher antifungal activity than (E)-2-alkenals tested against *C. herbarum* and both isolates of *B. cinerea*, especially its effect on recovery of fungal growth after 48 h culture. Only (E)-2-dodecenal showed comparable antifungal activity with 4-octyl-cyclopentenone against *F. oxysporum*. Its inhibition achieved about 25% against *F. oxysporum* after 72 h culture which was slightly more efficacious than 4-octyl-cyclopentenone. In summary, 4-octyl-cyclopentenone was still the most effective antifungal compound against plant pathogenic fungi and was better than medium-chain (C9-C13) (E)-2-alkenals.

(a) Effect of (E)-2-alkenals on the growth of *A. brassicicola*.



(b) Effect of (E)-2-alkenals on the growth of *F. oxysporum*.

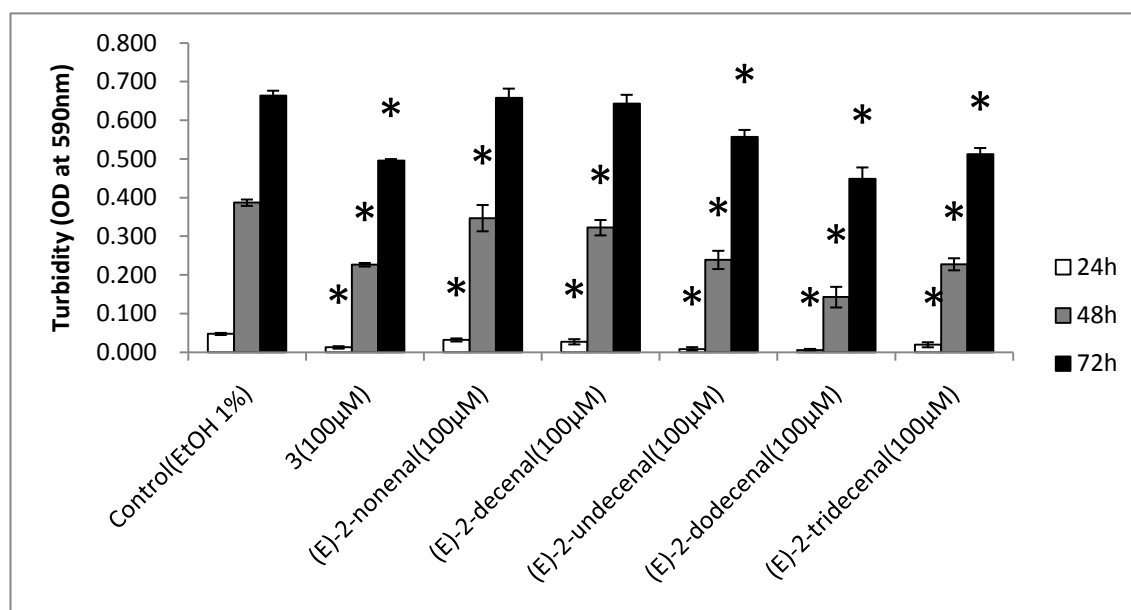
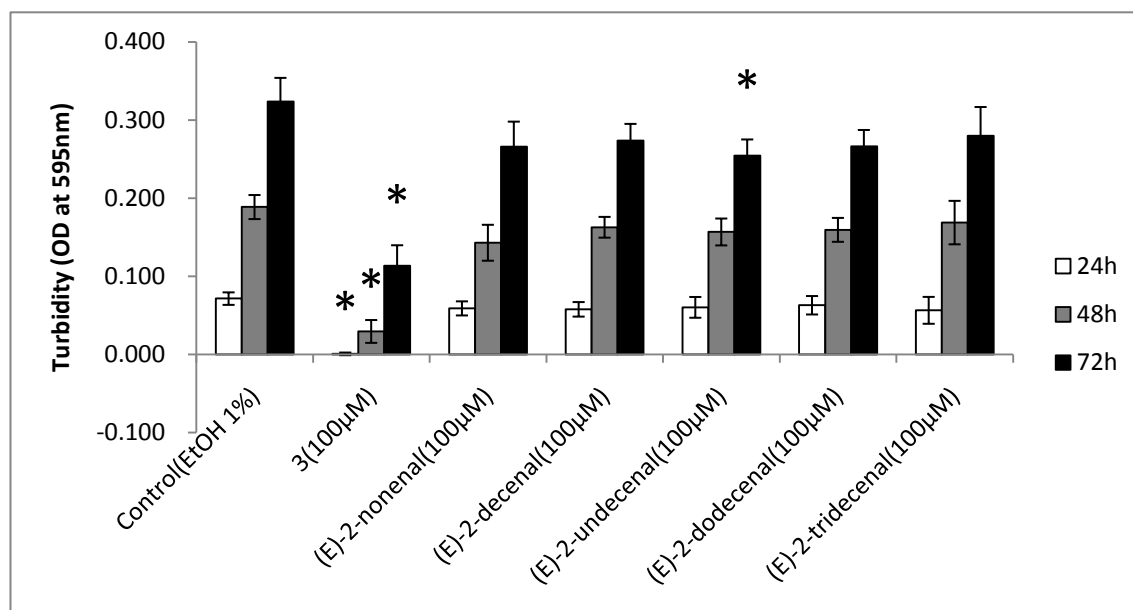


Figure 5.1 Antifungal activity of (E)-2-alkenals against plant pathogens. Fungal strains were exposed to a series of (E)-2-alkenals and 4-octyl-cyclopentenone (**3**) in appropriate liquid culture medium, starting from the time of spore addition to wells (*F. oxysporum* and *A. brassicicola*) or 16 h later (*C. herbarum* and *B. cinerea*). Results show means  $\pm$ SD (n=6). Asterisk indicates a significant difference at  $P < 0.01$  (Dunnett's test) against control.

(c) Effect of (E)-2-alkenals on the growth of *C. herbarum*.



(d) Effect of (E)-2-alkenals on the growth of *B. cinerea* (MUCL30158).

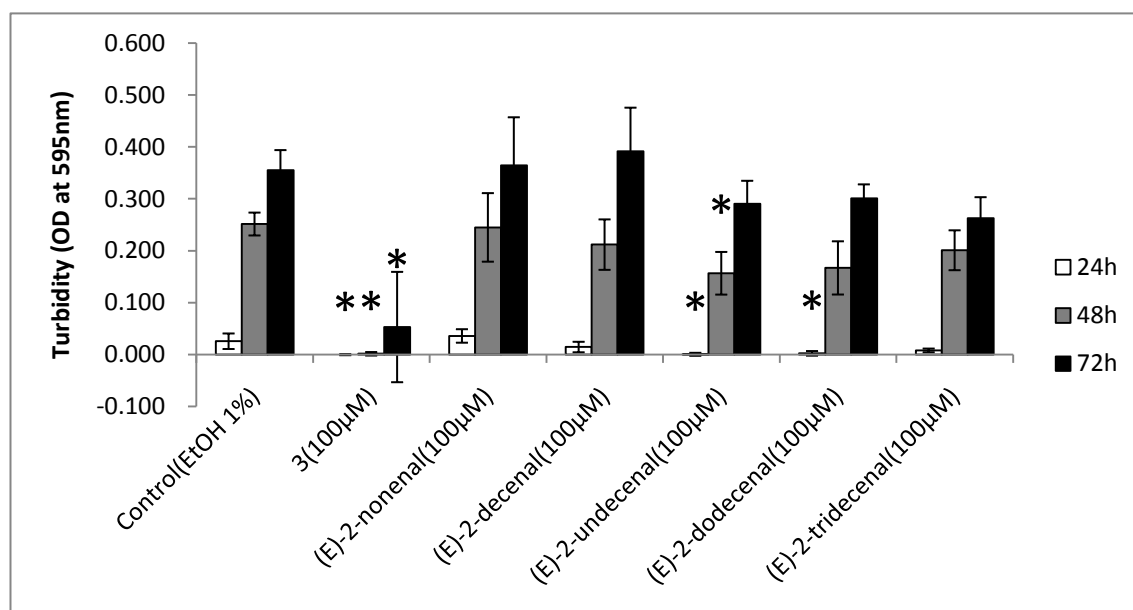


Figure 5.1. Antifungal activity of (E)-2-alkenals against plant pathogens. Fungal strains were exposed to a series of (E)-2-alkenals and 4-octyl-cyclopentenone (**3**) in appropriate liquid culture medium, starting from the time of spore addition to wells (*F. oxysporum* and *A. brassicicola*) or 16 h later (*C. herbarum* and *B. cinerea*). Results show means  $\pm$ SD (n=6). Asterisk indicates a significant difference at  $P < 0.01$  (Dunnett's test) against control.

(e) Effect of (E)-2-alkenals on the growth of *B. cinerea* (GLUK-1).

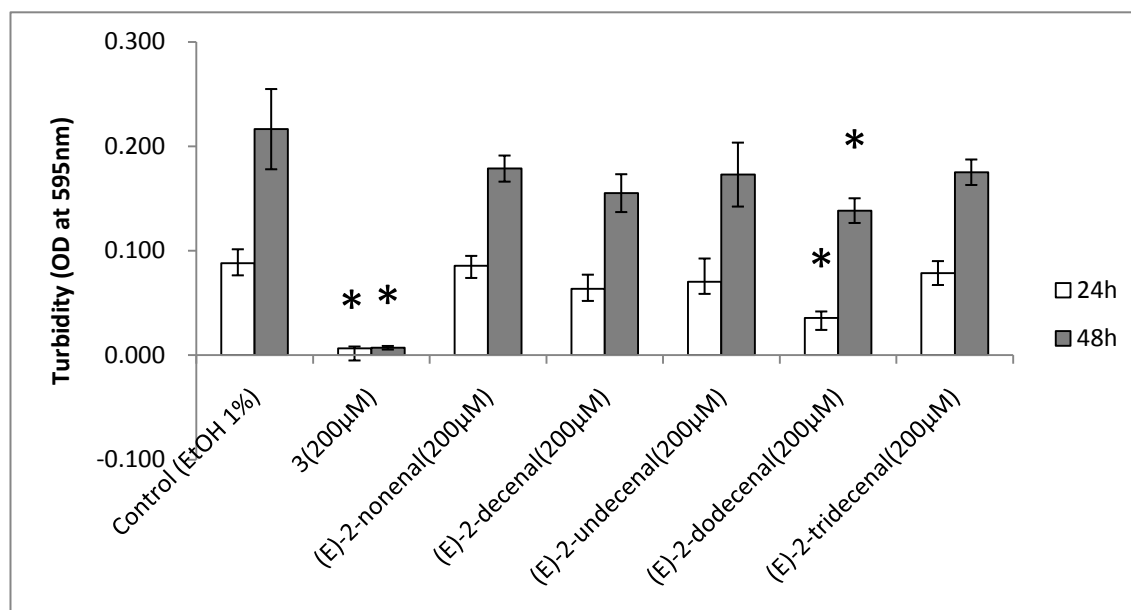


Figure 5.1 Antifungal activity of (E)-2-alkenals against plant pathogens. Fungal strains were exposed to a series of (E)-2-alkenals and 4-octyl-cyclopentenone (**3**) in appropriate liquid culture medium, starting from the time of spore addition to wells (*F. oxysporum* and *A. brassicicola*) or 16 h later (*C. herbarum* and *B. cinerea*). Results show means  $\pm$ SD (n=6). Asterisk indicates a significant difference at  $P < 0.01$  (Dunnett's test) against control.

#### 5.4.2 Antimicrobial activity against human pathogens

In the agar diffusion test, the compound diffuses into the agar. The concentration of the compound will be highest around the application sites, and will decrease as the distance from the sites increases. If the compound is effective against organisms at a certain concentration, no colonies will grow where the concentration in the agar is greater than or equal to the effective concentration. This is the zone of inhibition. Thus, the size of the zone of inhibition is a measure of the compound's effectiveness: the larger the clear area around application sites, the more effective the compound. The results of agar diffusion tests show that the three human pathogens respond differently to (2E)-alkenals

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(Figure 5.2). (E)-2-nonenal, (E)-2-decenal, and (E)-2-undecenal were found to be effective on *C. albicans*. (E)-2-undecenal and (E)-2-dodecenal showed antibacterial activity against *S. aureus*, but not against *E. coli*. From the agar diffusion test, *S. aureus* was more sensitive to (E)-2-alkenals than *E. coli*.

Although agar diffusion tests can be used to predict the antimicrobial activity of compounds, the antimicrobial MIC assay is a more accurate mean to measure the inhibition of fungal growth. The MIC values against *C. albicans*, *S. aureus* and *E. coli* are listed in Table 5.2. As expected, their antimicrobial activity against these two pathogens correlated with the hydrophobic alkyl chain length. Inhibition activity determination on various chain lengths indicates that an optimum chain length is reached after which there is a dramatic decrease of activity with the further increase of chain length. This is known as the ‘cut-off’ phenomenon. (E)-2-undecenal and (E)-2-decenal were found to be very effective against *C. albicans* with MIC of 16  $\mu\text{g/mL}$ , whereas (E)-2-tridecenal showed little activity up to 128  $\mu\text{g/mL}$ . As for *S. aureus*, (E)-2-dodecenal and (E)-2-undecenal had the strongest antibacterial activity with MIC less than 4  $\mu\text{g/mL}$ . The antifungal effect of (E)-2-tridecenal against this organism is lower with a MIC of 16  $\mu\text{g/mL}$ . In conclusion, (E)-2-undecenal was found to have the best antimicrobial activity against tested pathogens among (E)-2-alkenals. 4-Octyl-cyclopentenone with a MIC of 19  $\mu\text{g/mL}$  against *C. albicans* has similar antifungal activity to (E)-2-undecenal and (E)-2-decenal, whereas this compound was ineffective against *S. aureus*. As for *E. coli*, MIC values of all test compounds were

higher than 128  $\mu\text{g/mL}$ , which shows very little sensitivity of this bacteria to (E)-2-alkenals. Thus, these non-ionic surfactants have specific antimicrobial activity against certain species.

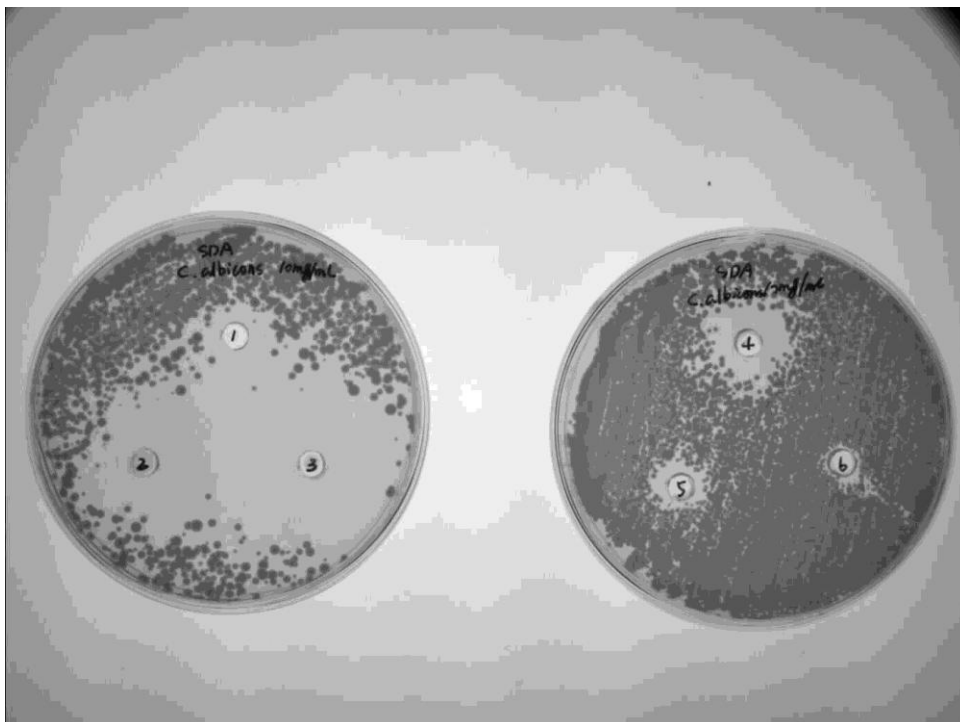
Table 5.2 Antimicrobial (MIC) activity (micrograms per milliliter) of (E)-2-alkenals against *C. albicans*, *S. aureus* and *E. coli*.

Chemicals	log <i>P</i> value	MIC*		
		<i>C. albicans</i>	<i>S. aureus</i>	<i>E. coli</i>
(E)-2-hexenal	2.3	128	128	>128
(E)-2-nonenal	3.8	64	64	>128
(E)-2-decenal	4.3	16	16	>128
(E)-2-undecenal	4.8	16	<4	>128
(E)-2-dodecenal	5.4	32	<4	>128
(E)-2-tridecenal	5.9	>128	16	>128
4-octyl-cyclopentenone	4.5	19	>76	>76

Log *P* values were calculated by Molinspiration log *P* calculator.

\*MIC values of compounds were monitored after incubated for 24 h and no visual fungal growth in treatments.

(a) Effect of (E)-2-alkenals on *C. albicans*.



(b) Effect of (E)-2-alkenals on *E. coli* (b right) and *S. aureus* (b left).

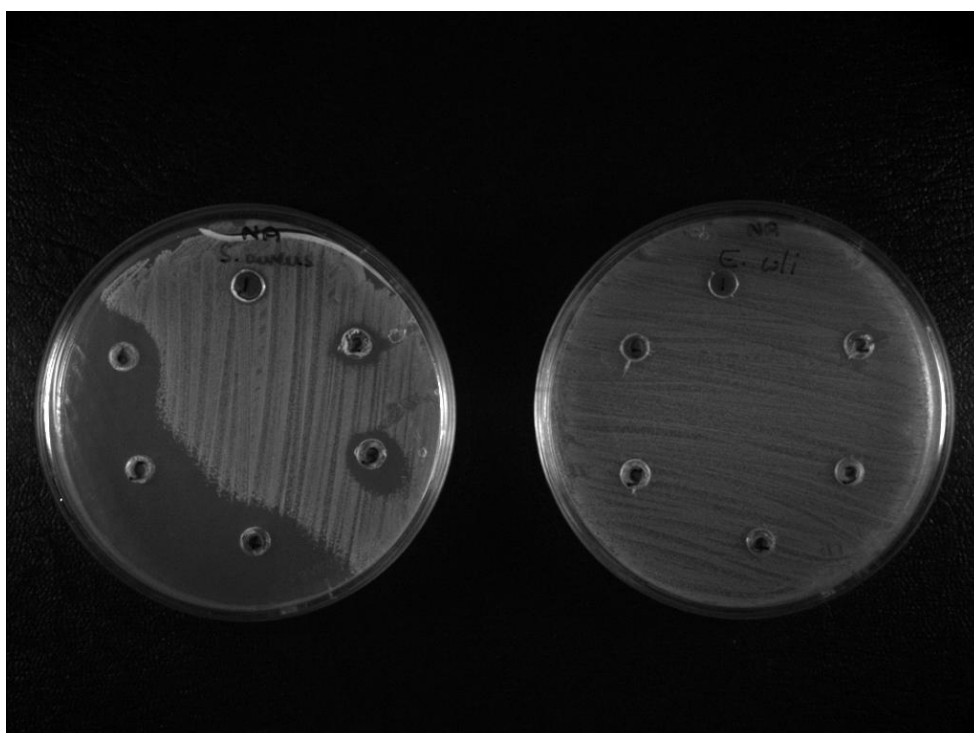


Figure 5.2 Effect of (E)-2-alkenals on *C. albicans* (a), *E. coli* (b right) and *S. aureus* (b left) on SDA. A series of (E)-2-alkenals with concentration of 10 mg/mL: (E)-2-hexenal (1), (E)-2-nonenal (2), (E)-2-decenal (3), (E)-2-undecenal (4), (E)-2-dodecenal (5) and (E)-2-tridecenal (6) were added onto agar. Photos of plates were taken after 24 h.

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## 5.5 Discussion

The tests performed on a series of (E)-2-alkenals revealed that the chain length of the alkyl group was a primary determinant of antifungal activity (Table 5.2). (E)-2-undecenal has the best antimicrobial activity against *C. albicans* and *S. aureus*. Our findings agree with the results of previous work done by other groups [68, 169]. In Kubo's studies on the structure antimicrobial activity-structure relationship with a homologous series of (E)-2-alkenals, the activity of medium chain (C9-C13) (E)-2-alkenals against, *Saccharomyces cerevisiae* [68], and *Salmonella choleraesuis* [169], was evident. They revealed that (E)-2-undecenal was the best antifungal agent against *Saccharomyces cerevisiae* and (E)-2-dodecenal was the most effective against *Salmonella choleraesuis*. In addition, the activity "cut-off" phenomena occurred in both cases when the chain length reached C13. Their observations regarding the properties of (E)-2-alkenals concur with our results against different microbes (Table 5.2), therefore, it is plausible that they share similar mechanisms and interactions with sensitive pathogens. It is plausible that the hydrophilic head group (the  $\alpha,\beta$ -unsaturated carbonyl group) is essential. This head binds like a 'hook' to active sites, such as nucleophilic groups of membrane protein and the hydrophilic portions of cytoplasmic membrane surface or sterols. The hydrophobic moiety, the alkyl chain, is able to enter the membrane lipid bilayers through hydrophobic effect. In this way, these aliphatic (E)-2-alkenals can disrupt and disorganise the membrane. The "cut-off" phenomena could be explained by the inappropriate lipophilicity of compounds, or the



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bond-breaking concept between  $\alpha,\beta$ -unsaturated carbonyl group and specific membrane protein domains [68]. Apart from Kubo's group, another study of antibacterial activity of  $\alpha,\beta$ -unsaturated aldehydes shows that (E)-2-alkenals cause the release of carboxylfluorescein (which is trapped in liposomes of phosphatidylcholine (PC)), and the effectiveness order correlates well with the chain length and the presence of the  $\alpha,\beta$ -unsaturated carbonyl group [171]. Therefore, apart from the hydrophilic head group, the lipophilicity plays an important role in determining activity [172]. Notably, two effective compounds against *C. albicans*, including (E)-2-undecenal and 4-octyl-cyclopentenone, have log *P* value around 4.7, however they have different antimicrobial activities against *S. aureus*. In short, the activity of the hydrophilic moiety is important to the antimicrobial activity of (E)-2-alkenals through balancing the hydrophobic and the hydrophilic moieties.

In this project, it was also found that none of the (E)-2-alkenals tested show any activity against *E. coli* in the agar diffusion test. These antimicrobial agents, therefore, seem to have specific activity to different organisms due to the biological structure of these organisms, such as membrane structure (the gram-negative bacterium, *E. coli*, has outer and inner membrane, whereas, gram-positive bacteria, only have one layer of membrane). Potentially, the outer membrane barrier could cause the different susceptibility of bacteria to test compounds. However, the detailed mechanism and clarification of membrane structures of different species need to be further investigated. The different response towards the  $\alpha,\beta$ -unsaturated alkenals could also reflect the

diversity of glycerophospholipids composition, which would be particularly important in the cell membrane. Unlike animals, plants and fungi, bacteria generally do not contain polyunsaturated fatty acids and characteristically contain odd-numbered and branched fatty acids. Glycerophospholipids are usually the major constituents of bacterial membranes. The glycerophospholipid composition of bacterial membranes is reported [23, 173] and summarised in Table 5.3. Phosphatidylethanolamine (PE), phosphatidylglycerol (PG) and diphosphatidylglycerol (DPG) are the major glycerophospholipids present in bacteria although the lipid compositions in species differ. For example, the major membrane lipid of gram-negative bacteria, *E. coli*, is PE, whereas there is very little PE detected in gram-positive *Staphylococcus* spp. (where PG is the main component). This difference between membrane lipid compositions could result in the different susceptibility of species to a series of alkenals. Possibly, the presence of large amount of PE could consume (E)-2-alkenals through the reaction of the  $\alpha,\beta$ -unsaturated carbonyl group with the amine group on the polar head group of PE. In this case, most of the (E)-2-alkenals would be anchored to the surface of the membrane rich in PE, therefore they could not permeate the membrane or interact with other active sites. More studies should be undertaken on the reactivity of (E)-2-alkenals with the positive charged amine group of PE. Other possible active sites for test compounds could be proteins. The diversity of cell membrane structure and the existence of specific proteins of different pathogen species make it difficult to explain the specificity of the compounds tested.

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Table 5.3 Major phospholipid components of bacteria [23].



Some natural (E)-2-alkenal products, such as (E)-2-hexenal and (E)-2-nonenal, were reported to play important roles in plant defence through direct antimicrobial activity [11] and indirect signalling to trigger the expression of plant defence genes [56]. In this test, these two (E)-2-alkenals with low lipophilicity showed very limited antimicrobial activity, which disagrees with the antibacterial activity of (E)-2-hexenal against bacteria *Pseudomonas syringae*, *Xanthomonas campestris* and *Erwinia carotovora* [11]. More surprisingly, in the same paper, (E)-2-nonenal was reported to be much less effective than (E)-2-hexenal against bacteria. Our finding is that (E)-2-nonenal is more active than (E)-2-hexenal against *S. aureus* and *C. albicans*. Again, it indicates that the

antimicrobial activity of (E)-2-alkenals is species dependent.

It has been reported that (E)-2-alkenals have better antimicrobial activity than their corresponding alkanals [68] and alkanols [137], which indicates that different properties of the head groups will influence the performance of the molecule. Both 4-octyl-cyclopentenone and (E)-2-alkenals have  $\alpha,\beta$ -unsaturated carbonyl groups, and the only difference between them is the five-membered carbon ring, which causes the difference in antimicrobial activity between them. Cyclopentenone is a relatively weak electrophile (higher  $E_{LUMO}$  than that of (E)-2-alkenals) [168] and has a lower reactivity with soft nucleophiles than acyclic  $\alpha,\beta$ -unsaturated aldehydes. However, no correlation could be made between the reactivity and the antimicrobial activity of the test compounds. The weaker electrophile, 4-octyl-cyclopentenone, is more active against four plant pathogenic fungi; some stronger electrophiles, (E)-2-alkenals, are more effective to *S. aureus*; and both of them have no effect on *E. coli*. Therefore, antimicrobial activity of this series of compounds is not only related to reactivity of  $\alpha,\beta$ -unsaturated carbonyl groups with nucleophiles in cells, but also to other processes. Nevertheless, it is still important to identify the target sites for these compounds.

As mentioned above, aliphatic (E)-2-alkenals can inhibit growth of some microorganisms by disrupting and disorganising the membrane. In addition, it is also assumed that similar to (E)-2-hexenal and (E)-2-nonenal, other (E)-2-alkenals are able to play a role in plant signalling because of the presence of the  $\alpha,\beta$ -unsaturated carbonyl

group which is considered to contribute to their biological activities, as mentioned in Chapter 1. The details are still not known, and more research is needed to treat plants with these compounds and check the gene expression. Widescale gene expression patterns using genomic techniques, such as DNA microarrays, are required to unveil the effects of these agents on the regulation of gene expressions.

## **5.6 Conclusion**

In studies on the structure-antifungal activity relationship with a homologous series of acyclic (E)-2-alkenals, the antimicrobial activity of (E)-2-alkenals against pathogens is largely due to their non-ionic surface-active properties. The hydrophilic  $\alpha,\beta$ -unsaturated carbonyl head group could bind to active sites and the maximum activity can be obtained when the hydrophilic and hydrophobic moieties becomes the most appropriately balanced. The antimicrobial effects of (E)-2-alkenals likely result from several processes, such as:

1. Direct perturbation of the plasma membrane lipids, resulting in increased membrane permeability and in leakage of intracellular substances.
2. Enhanced permeability of the membrane caused by the alternation of other membrane functions, e.g. binding to specific proteins which act as ion gates/receptors or signal transduction mediators.
3. Penetration into the interior of the cell and interact with other intracellular sites.

# Chapter 6

## Summary and Future Work

### 6.1 Summary

Fungi have been competing with man for crops since early cultivation and fungicides have been utilised since then. The big challenge of traditional fungicides in the 21<sup>st</sup> century is their environmental risk and high toxicity. In order to cope with the adverse effects of traditional fungicides and improve their efficacy against target pathogens, more studies have been devoted to developing a new generation of fungicides for the future which is environmental friendly.

After millions of years of evolution, mature plant defence systems have been developed to protect themselves from external stresses. Molecular signalling and antimicrobial substances are generated by plants in response to the attack of pathogens. These include oxylipins which are a large family of lipid derivatives formed from either enzymatic or non-enzymatic oxidation pathways, which are of interest, not only because of their important role in defence signalling, but also their direct antimicrobial activities against many pathogenic species. The precursor of JA, OPDA, is one of the active RES oxylipins containing an  $\alpha,\beta$ -unsaturated carbonyl group. This bio-active compound was reported not only to be able to regulate gene expression related to defence but also has

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the best antifungal activity and stability among 43 oxylipins [11]. OPDA is therefore a promising naturally occurring antifungal agent. In this project, a series of synthetic OPDA analogues were designed and synthesised from the installation of alkyl chains onto the cyclopentenone ring. In this way, all of the synthetic compounds are composed of a hydrophilic cyclopentenone ring containing an  $\alpha,\beta$ -unsaturated carbonyl group and a hydrophobic alkyl chain with different carbon chain lengths.

The antifungal activities of the synthetic compounds were tested against several fungal species which cause severe damage to agricultural production. 4-Octyl-cyclopentenone containing a cyclopentenone ring and an eight carbon alkyl chain was found to show the best *in vitro* antifungal activity against *C. herbarum* and *B. cinerea* with a MIC of 100-200 $\mu$ M. However, this compound, like OPDA, was less effective against *A. brassicicola* and *F. oxysporum*. The results indicate that the structurally simplified 4-octyl-cyclopentenone was successfully synthesised and could mimic the antifungal activity of OPDA against specific fungal strains due to their close electrophilicity and lipophilicity. *In vivo* assays showed that this compound does not induce any visible damage to *Brassica*. Studies *in vivo* using apricots were promising although recovery was noted on some inoculation sites, so it appears from the preliminary studies that the almost total inhibition of growth *in vitro* may be more challenging to achieve *in vivo*.

The exploitation of the mode of action of 4-octyl-cyclopentenone indicates that its interaction with the fungal plasma membrane causes the leakage of internal substances,

such as potassium ions, leading to the inhibition of fungal growth. However, the lipids composition has no relationship with 4-octyl-cyclopentenone uptake and leakage of ions. Straight chain (E)-2-alkenals were also evaluated as antimicrobial compounds. These molecules have the same  $\alpha,\beta$ -unsaturated carbonyl group as 4-octyl-cyclopentenone. Straight chain alkenals are able to interact with the active sites on the membrane surface and disrupt the cell membrane. This mode of action also reflects the importance of two common properties of RES oxylipins: electrophilicity and lipophilicity.

In short, the synthetic 4-octyl-cyclopentenone is found to have similar antifungal activity to the naturally occurring RES, OPDA, but has a simpler chemical structure which makes it easier to synthesise in a large scale for commercial exploitation.

## **6.2 Future work**

This project has gone some way to generating a useful antifungal agent with promise as a commercial product. Although 4-octyl-cyclopentenone could mimic the antifungal activity of OPDA, there are still several remaining problems awaiting resolution. They are outlined below:

1. Organic synthesis. In chapter 2, a two step synthetic pathway was used to produce 4-octyl-cyclopentenone. The challenge is to improve the yield and minimise the cost of the starting material which is very expensive from the chemical supplier. In



addition, large scale manufacture of the Grignard reagent comes with safety risks and would have to be manufactured in accordance with stringent health and safety regulations. Two other methods have been used to synthesise 4-octyl-cyclopentenone [128, 129], however, both of them proved to be more complicated than the current method.

2. Antifungal activity. Fungi are highly mutagenic and the possibility of adapting to new agrochemicals is inevitable. Therefore, it is not enough that the test compounds only can simulate the properties of naturally occurring molecules, but that they can elicit even stronger responses. As for *in vitro* antifungal activity against *C. albicans*, 4-octyl-cyclopentenone is more effective than some synthetic surfactant antifungal agents [174, 175], but still much lower than most commercial fungicides against a broad spectrum of fungal pathogens. More efforts are therefore required to improve the *in vitro* antifungal performance. In addition, more tests still need to be done to indicate the antifungal activity of 4-octyl-cyclopentenone against a broad spectrum of microbes.
3. Mode of action. Although, it is assumed that 4-octyl-cyclopentenone and (E)-2-alkenals share similar antifungal mode of action and the leakage of potassium ions was detected, the actual active site is still unknown. Understanding of the active site is essential for improving antifungal activity and dealing with the fungal resistance. It has been shown that the analysis of the lipid composition of fungal species does not appear to have any correlation to their sensitivity to 4-octyl-cyclopentenone. The remaining two major components of the fungal

membrane are sterols and membrane proteins, both of which could be the possible target sites interacting with (E)-2-alkenals.

4. Biological activity. Aside from the direct antifungal activity, the other important role of OPDA is to contribute to the plant defence system through regulating defence gene expression. Many RES oxylipins, such as (E)-2-hexenal, PPA and PPB, were reported to be able to trigger gene expression in response to stresses. It would not be surprising that RES 4-octyl-cyclopentenone has such ability; however, this requires direct testing.

This project is just the beginning of work on this promising group of compounds modified from natural antifungal RES oxylipins. In order to cope with these problems mentioned above, several lines of work should be carried out to improve and develop the product for commercial applications:

1. Modification of the compound. The idea is to combine the bioactive part, i.e. the cyclopentenone ring and an improved hydrophobic part to enhance antifungal activity. The easiest change to make is the installation of longer chains (C9, C10, C11, C12, C13...) onto the ring and hopefully the “cut-off” phenomena could be found as for (E)-2-alkenals. Notably, the longer chain could increase phytotoxicity and therefore further tests would be required. Other groups, such as double bonds, hydroxyl, amine and aromatic substituents could be added to the alkyl chain. Substituents could also be added to the cyclopentenone ring, however, it was noted that the derivatives with different substituents on the ring will affect the

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electrophilicity of the  $\alpha,\beta$ -unsaturated carbonyl group, leading to different reactivity with nucleophilic proteins [176]. For 8-(4-oxocyclopentenyl)octanoic acid, oxygen could be inserted to the alkyl chain to form a 3-oxo-carboxyl end group which is resistant toward  $\beta$ -oxidation and has the potential for displaying enhanced biological activity in situations where activity is limited by metabolic degradation [177]. 4-Octyl-cyclopentenone could be used as a multifunctional intermediate that offers fast access to the cyclopentenone prostaglandin analogues with powerful biological activities. Therefore, the synthesis of these cyclopentenone compounds is also a useful step for more possibilities of synthetic pathways to prostaglandins and phytoprostane analogues. All of these derivatives could have enhanced antifungal activity. Again, more attention should be given to the potential increase in toxicity with the increasing number of double bonds and hydroxyl groups. Some techniques commonly used in drug development could be used to design new fungicides, such as quantitative structure- activity relationship (QSAR) models and computer modelling. They could predict the potential antimicrobial activity and toxicity of newly designed molecules [168, 178] prior to screening.

2. Regulation of gene expression. The genes related to pathogen attack and detoxification could be assessed by DNA microarray technology. In addition, the accumulation of other secondary metabolites such as phytoalexins, could also be monitored after the treatment.
3. Determination of the mode of action. The plasma membrane of fungi is rich in sterols, e.g. ergosterol, which adjusts the fluidity of the membrane. Several

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fungicides were reported to affect this important membrane component by inhibiting its synthesis or binding onto its surface, leading to the disruption and disorganisation of the membrane. For 4-octyl-cyclopentenone, ergosterol could be an important active site; therefore the analysis of the sterol composition of the target organism is necessary. As to the influence on membrane protein, it is more complicated than the analysis of the sterol. TEM could be used to identify the subcellular active site with fluorescence or molecules targeted by other means, such as radio-labelled compounds/proteins. Two-dimensional gel electrophoresis could be used to identify potential target proteins. Recently, OPDA has become commercially available from Cayman Chemical and can be used to explore the details of its mode of action.

4. Successful small scale *in vitro* examples need to be scaled up to field trials to show that these compounds are efficient in a natural environment. Nevertheless, the antifungal agent should be applied to real crops to evaluate their performance. Field trials are the definitive approach to assess antifungal activity of promising compounds.
5. Applications in the future. Recently, some new anti-cancer properties of jasmonates were published [52]. 4-Octyl-cyclopentenone and other cyclopentenone prostaglandin analogues could share bio-activities of prostaglandins and OPDA. Therefore, the application of these compounds could be used in a variety of commercial applications. Some possible aspects of application of this family of compounds are listed in Table 6.1.

Table 6.1 Potential applications in the future.

Applications	Examples
Agriculture/Horticulture industry	Crop protection Seed treatment Protection of fruit postharvest Ornamental plant protection Forest protection
Cosmetic industry	Antifungal activity in solid fats Antifungal activity in liquid oils
Food industry	Preservatives Additives
Environmental Antifungal Sector	Additives to deodorising sprays
Human healthcare	Human antifungal drugs Human antifungal creams Anti-cancer agents
Veterinary industry	Veterinary antifungal agents

In short, 4-octyl-cyclopentenone and other synthetic compounds derived from the lead molecule, OPDA, are a group of promising antimicrobial agents. In order to develop efficient products suitable for commercial use, more efforts and investment are needed to continue the related research. This project has revealed the potential application of one such analogue of OPDA as an antifungal agent. More research into the wide application of this interesting group of molecules is wanted to satisfy societal demands for safer, less toxic but efficient chemicals that benefit people's lives.

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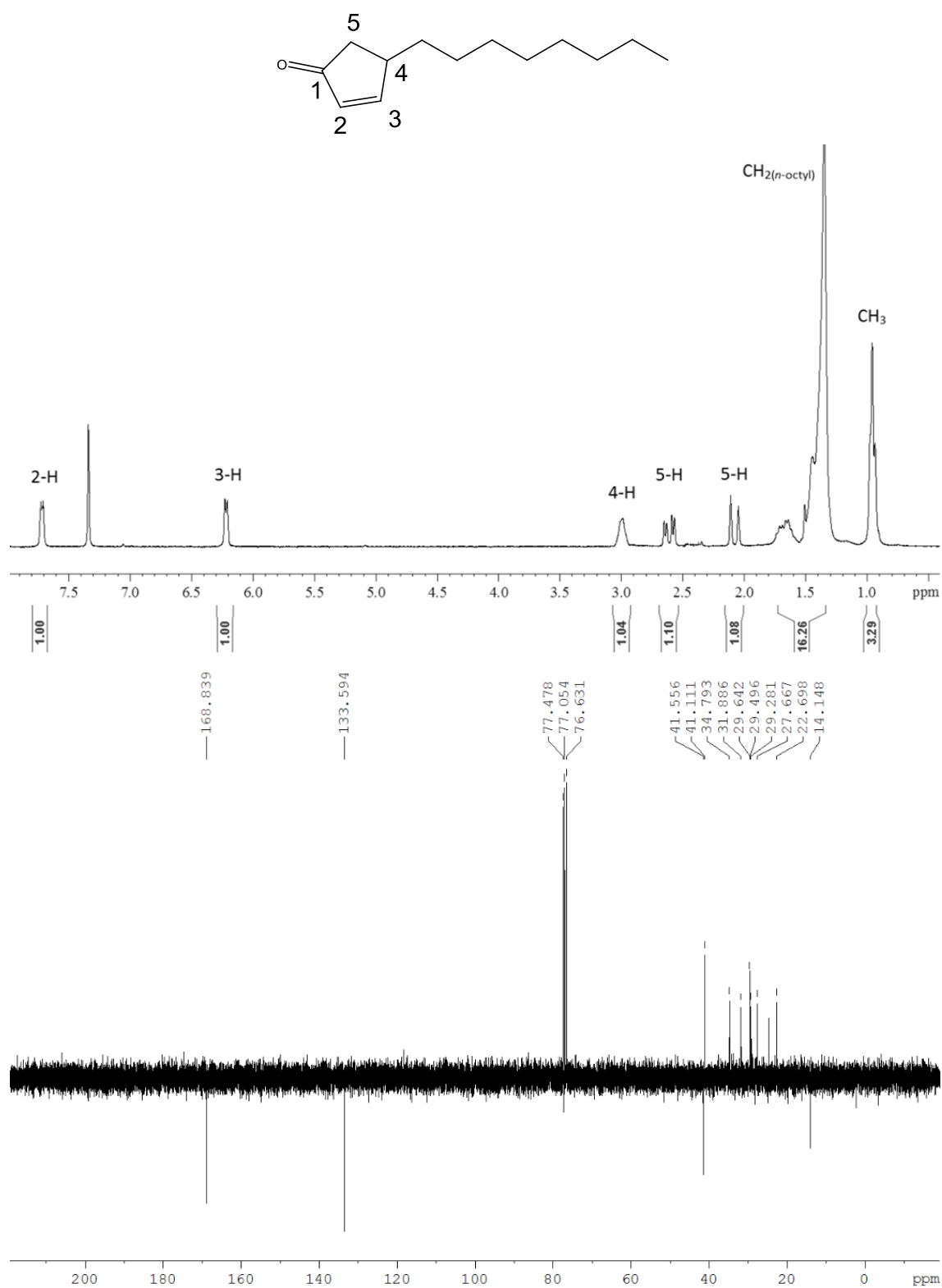


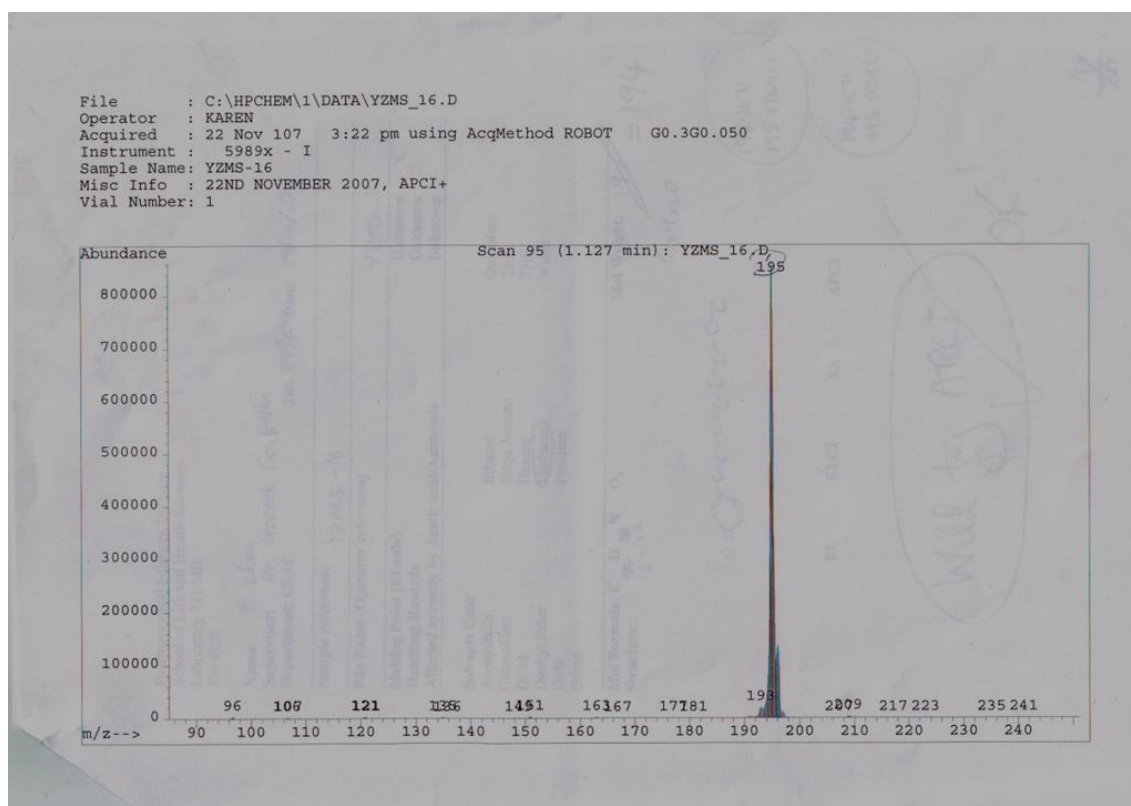
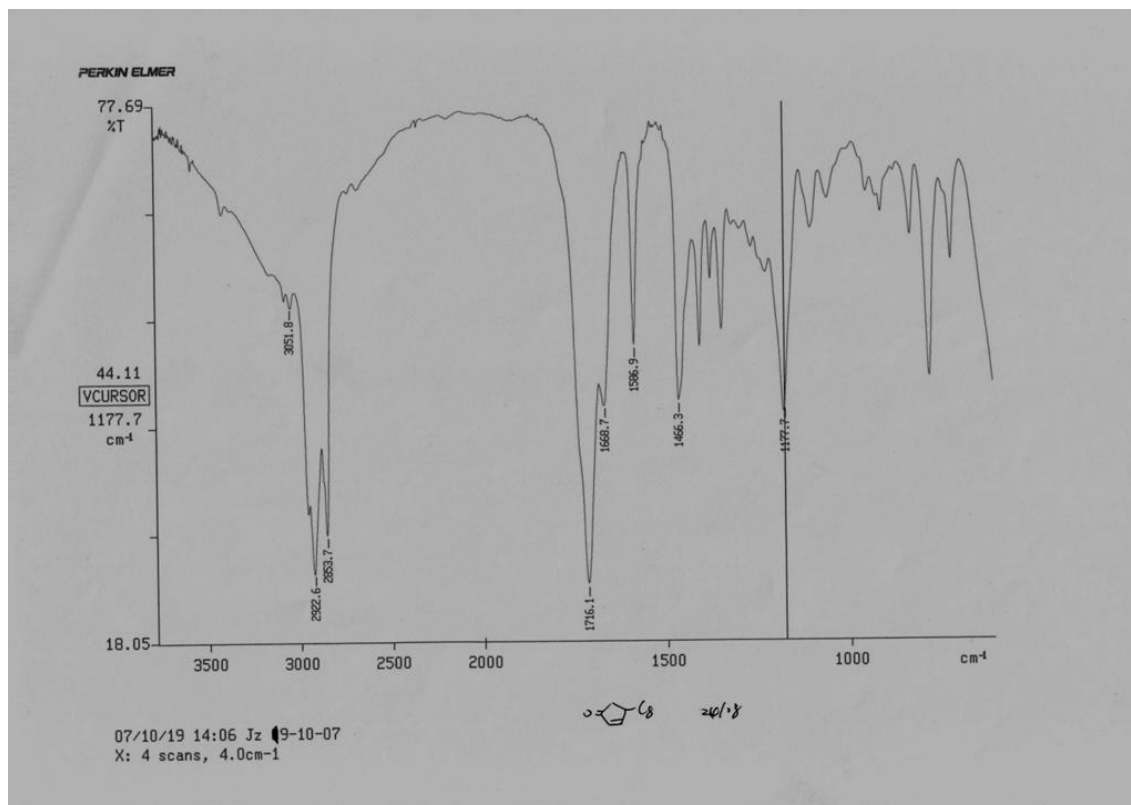
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# Appendices

## Appendix 1

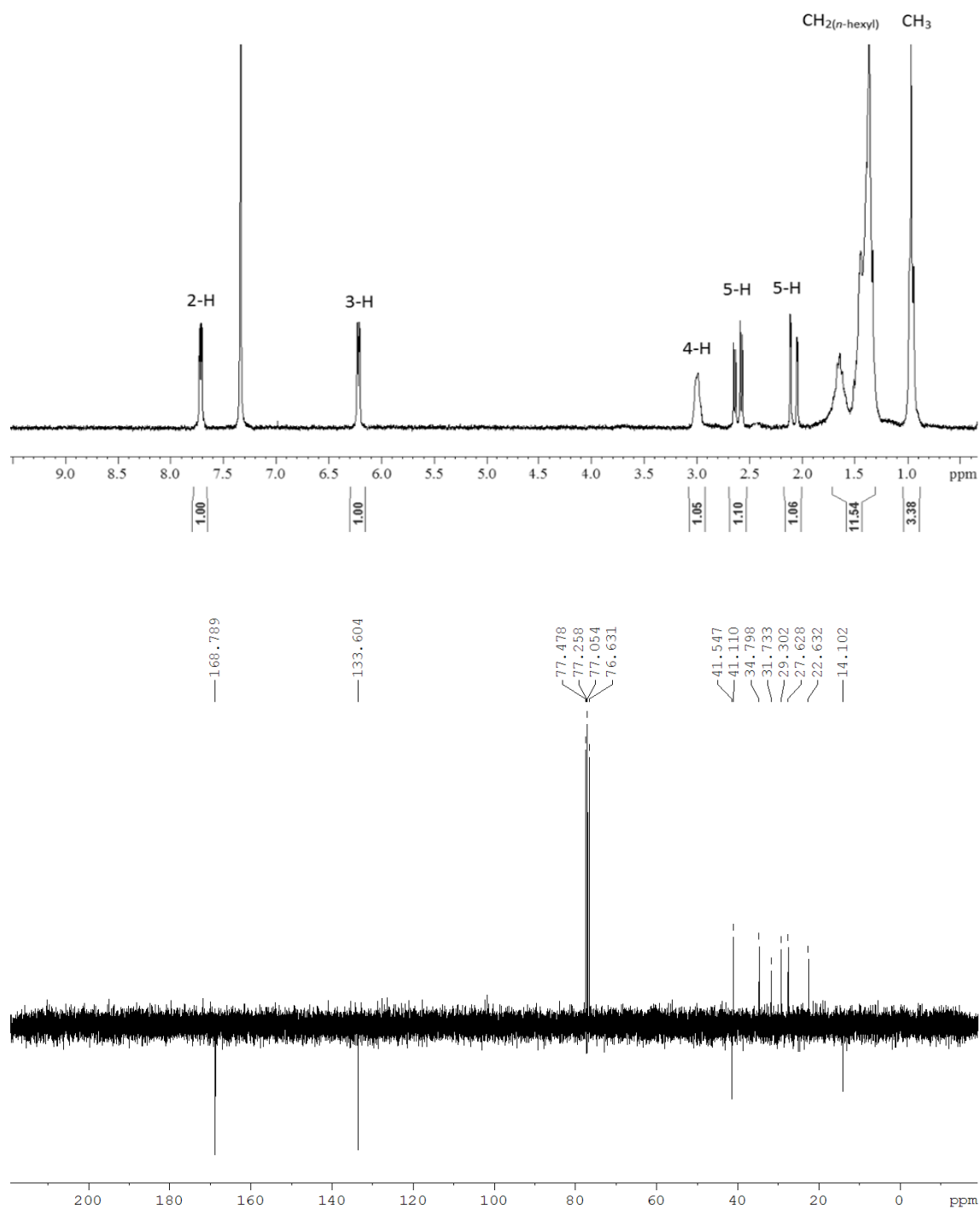
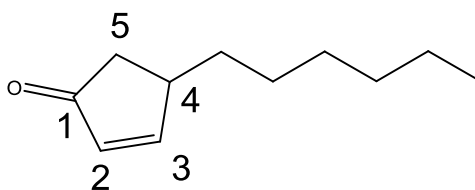
Spectra of  $^1\text{H}$  NMR,  $^{13}\text{C}$  NMR, FT-IR and APCI-MS of **4-octyl-cyclopentenone (3)**

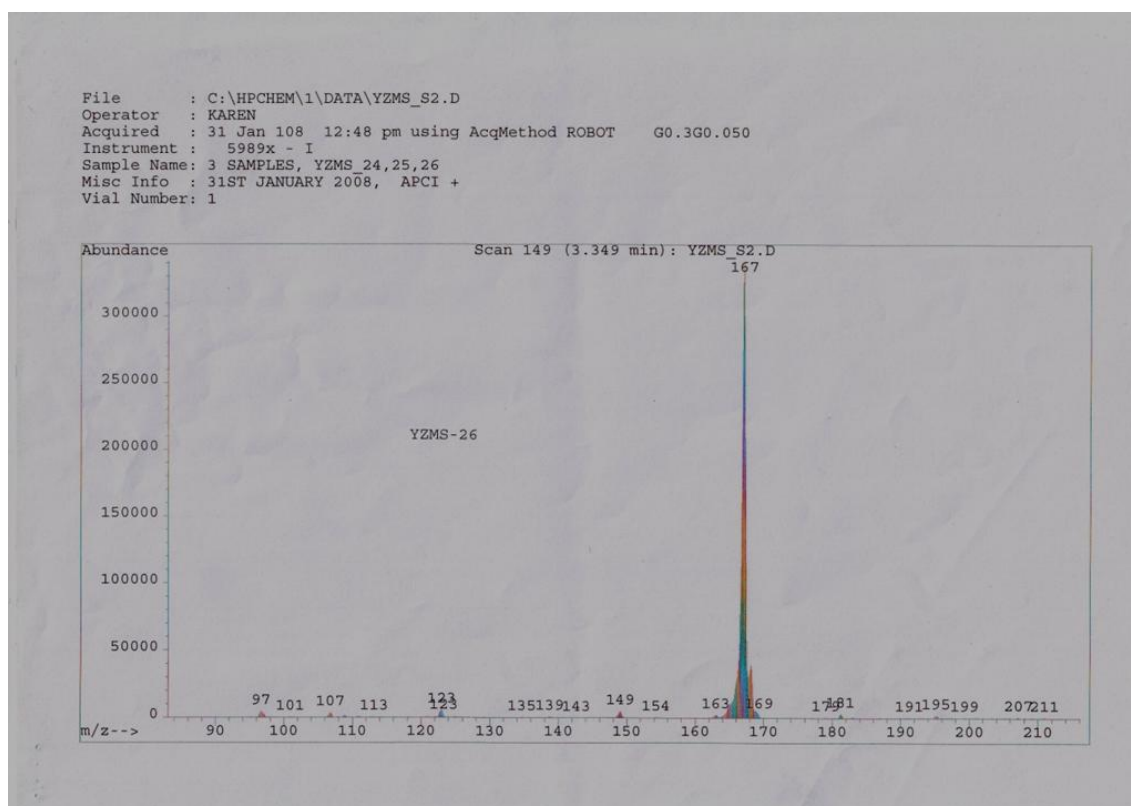
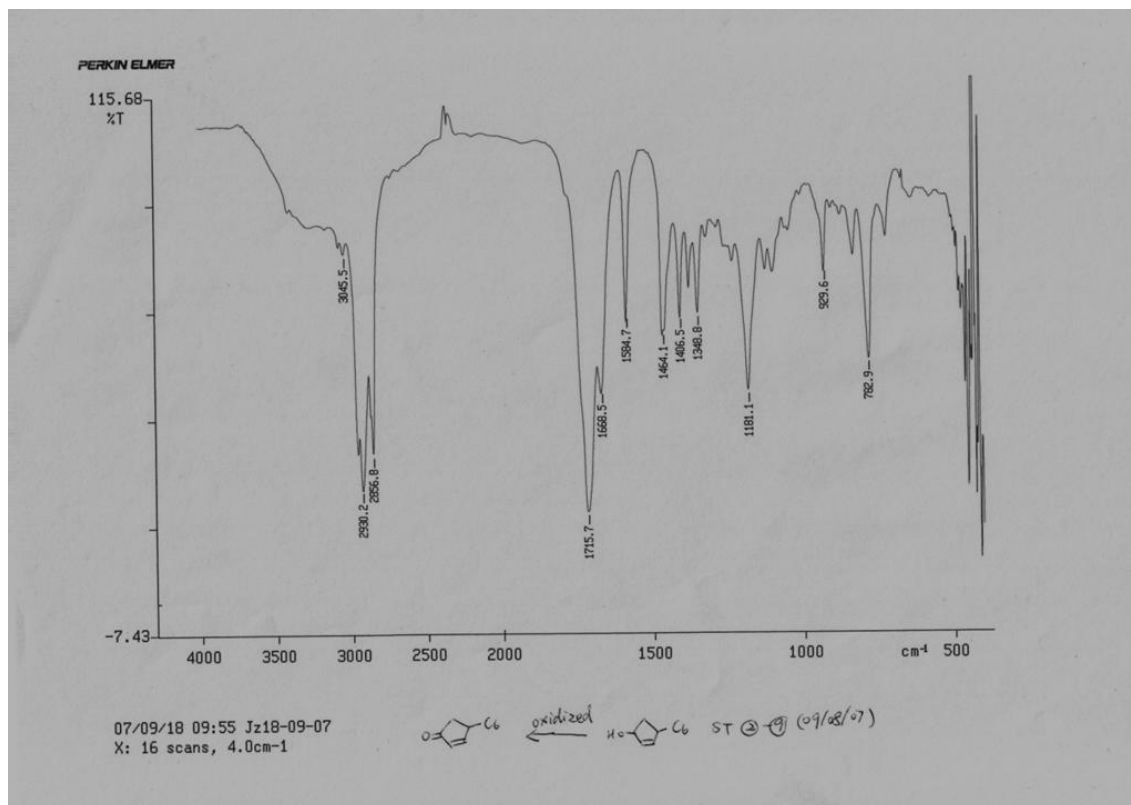




## Appendix 2

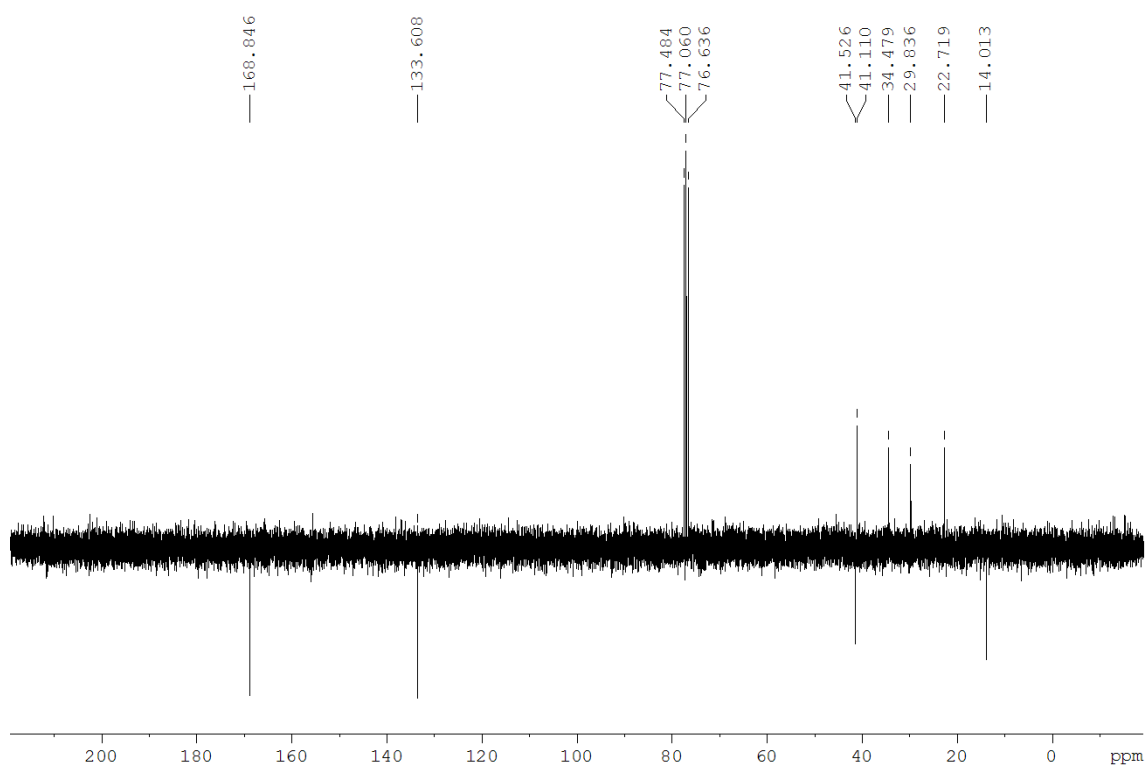
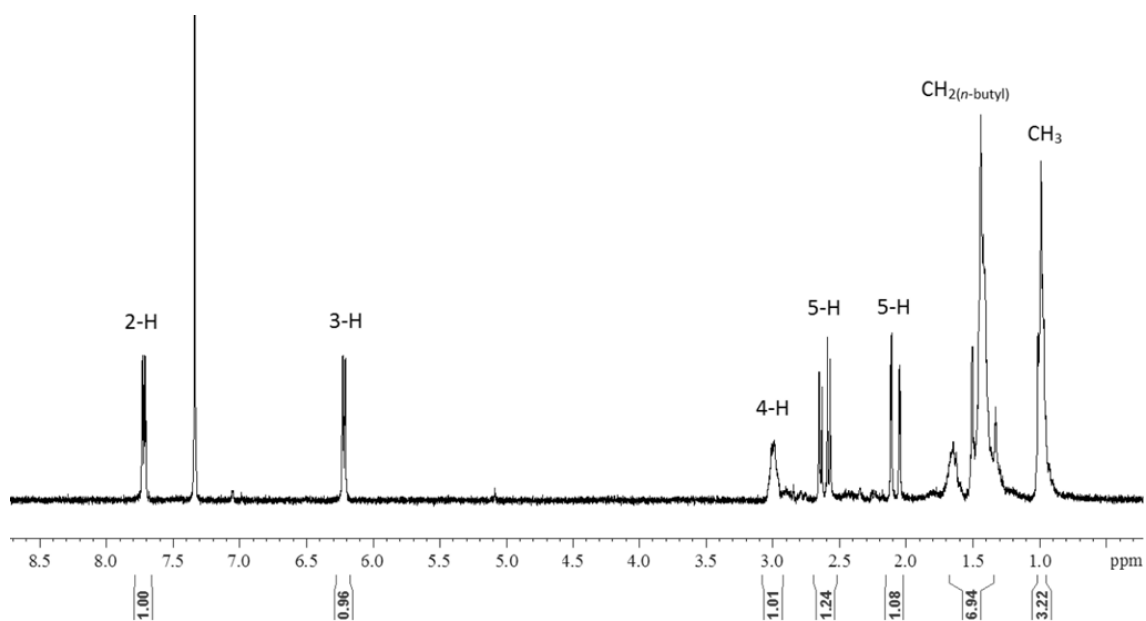
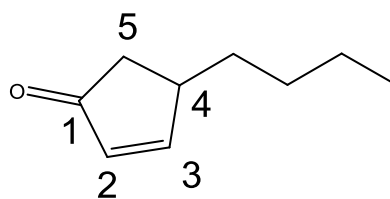
Spectra of  $^1\text{H}$  NMR,  $^{13}\text{C}$  NMR, FT-IR and APCI-MS of **4-hexyl-cyclopentenone (4)**



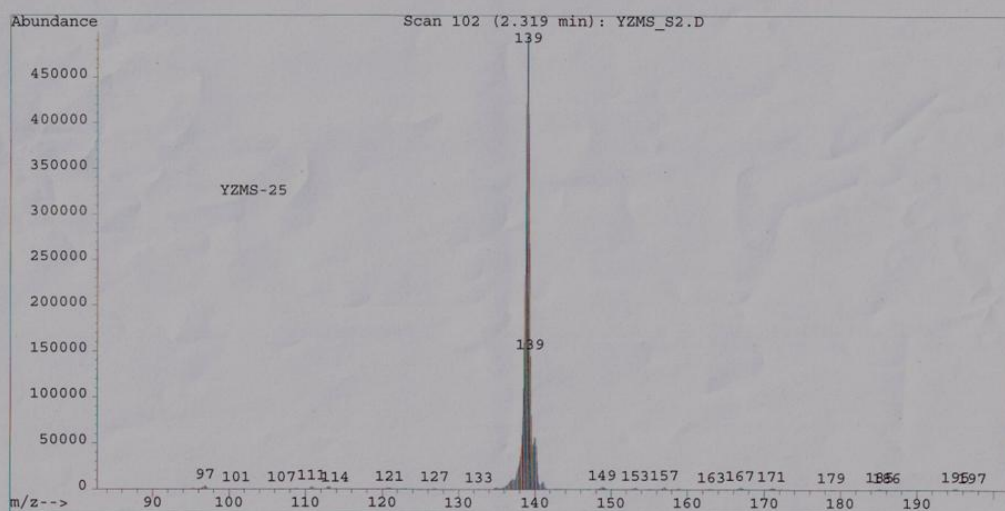


## Appendix 3

Spectra of  $^1\text{H}$  NMR,  $^{13}\text{C}$  NMR and APCI-MS of **4-butyl-cyclopentenone (5)**



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Acquired : 31 Jan 108 12:48 pm using AcqMethod ROBOT G0.3G0.050  
Instrument : 5989x - I  
Sample Name: 3 SAMPLES, YZMS\_24,25,26  
Misc Info : 31ST JANUARY 2008, APCI +  
Vial Number: 1





## Appendix 4

Spectra of  $^1\text{H}$  NMR,  $^{13}\text{C}$  NMR, FT-IR and APCI-MS of  
**8-(4-oxocyclopentenyl)octanoic acid (8)**

