The Synthesis and Biological Evaluation of Novel Nitrogen containing 6 and 7 membered ring Heterocyclic Compounds

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Abstract

Literature reports have previously shown interesting antimicrobial activities of compounds containing the α,β unsaturated carbonyl function and various piperidone and tropanone derivatives have attracted interest. A series of ring substituted N-piperidone dieneones and a novel parent tropan-3-one were synthesized and characterized. Novel compounds along with a previously synthesized library of 1,2-benzothiazines were examined for their antifungal properties. The tropan-3-one and benzothiazines proved to be ineffective inhibitors of *Saccharomyces cerevisiae* growth (<20%). Dieneones of N-piperidone and N-methyl piperidone showed a wide spectrum of activities whereby the free bases are marginally more potent than their respective hydrochloride salts. Based on their potencies against *S. cerevisiae* a subset of dieneones were screened against *Candida albicans* and *Aspergillus niger*. Yeast cells were incubated with the compounds for 24 or 48 hours whereas *A. niger* cells were incubated with the compounds for 16, 24, 48 or 72 hours respectively. Generally the presence of the N-methyl group decreases potency against yeast, whilst the opposite is true in *A. niger*. The compounds are not as effective at inhibiting cell growth against *A. niger* when compared to the yeast. Compound **41n** was the most potent inhibitor giving IC_{90} values of 1.35 x 10^{-3} M (*S. cerevisiae*) and 1.55 x 10^{-3} M (*C. albicans*), whereas compound **39n** was the most potent inhibitor of *A.niger* growth with an MIC value of 3.6 x 10^{-10} M. Selected piperidone dieneones were screened for cytotoxic properties against the A549 cancer cell line using the MTS and LDH assays. Compound **41n** was found to be the most cytotoxic. Potency of benzene ring substitution was para>meta>ortho. Substituents of larger volume and lower electron withdrawing properties produce more potent compounds. The 3'-iodo substituted piperidones (**39n and 41n**) emerged as the most promising agents from the group of compounds in respect to their antifungal and cytotoxic potencies, although the compounds were not as effective as the reference drug miconazole.

***Keywords*: α,β unsaturated carbonyl, piperidone, tropan-3-one, benzothiazine, antifungal, cytotoxicity**

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List of Abbreviations

Synthesis:

NaOH- Sodium hydroxide
KOH- Potassium hydroxide
EtOH- Ethanol
HCl- Hydrochloric acid
MeOH- Methanol
$TMSNEt_2$- Trimethylsilyldiethylamine
$MgBr_2$- Magnesium bromide
$LiClO_4$- Lithium perchlorate
Et_3N- Triethylamine
NaOAc- Sodium acetate
DMF- Dimethylformamide
NaOMe- Sodium methoxide
ABA- Aminobenzoic acid
$ClSO_3H$- Chlorosulfonic acid
H_2N- Amine
NaH- Sodium hydride
$CDCl_3$-D- Deuterated chloroform
Abs- Absolute
RT- Room temperature
Aq- Aqueous
RMM- Relative molecular mass
LC-MS- Liquid chromatography-mass spectrometry
IR- Infrared
UV- Ultraviolet
NMR- Nuclear magnetic resonance
HPLC- High performance liquid chromatography
TLC- Thin layer chromatography

Biological screening:

DMSO- Dimethyl sulfoxide
PDA- Potato dextrose agar
DMEM- Dulbecco's modified eagle medium
FBS- Fetal bovine serum
Pen-strep- Penicillin/Streptomycin
Abs- Absorbance
WT- Wild type

MTS- 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H tetrazolium, inner salt
LDH- Lactate dehydrogenase
MIC- Minimum inhibitory concentration
IC_{10}- Concentration required to inhibit 10% cell growth
IC_{50}- Concentration required to inhibit 50% cell growth
IC_{90}- Concentration required to inhibit 90% cell growth

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"Knowledge without action is insanity, and action without knowledge is vanity."
Imam Abu Hamid al-Ghazali

Chapter 1

Introduction

Chapter 1: Introduction

1.1 Introduction and Rational

The proposed research will study the synthesis and biological activities of novel compounds with six membered heterocyclic rings and 7 membered rings containing nitrogen. Various compounds containing either azo or diaza heterocyclic six or seven membered ring systems will be synthesized and characterized.

The aim is to synthesize a library of various benzene ring substituted tropan-3-one and piperidone dieneones. Along with a synthesized library of 1,2-benzothiazines they will be examined for their antifungal and anticancer properties. The antifungal and anticancer properties of some tropanones (Dimmock *et al,* 2002), piperidones (Pati *et al,* 2009) and 1,2-benzothiazines (Park, Chang, Lee & Kwon, 2002 and Ahmad, Zia-ur-Rehman, Siddiqui, Ullah & Parvez, 2011) reported in previous studies encouraged the present research.

1.2 The Cell Cycle

The cell cycle consists of several phases, through which the immature cell has to undergo in order to divide successfully. For a eukaryotic cell to divide, it requires the replication of its genome (DNA) in the synthesis, or S phase, of the cell cycle and the splitting of that genome in half during the mitotic or M phase. The Gap-1, or G-1, phase is a period in the cell cycle during interphase, before the S phase. For many cells, this phase involves a major period of cell growth including the production or division of new organelles and the duplication of cellular components excluding the chromosomes (Bock, Cardew, & Goode, 2001).

The cells may also enter the resting state otherwise known as the G_0 state where they become inactive and do not replicate or divide. Usually cells enter this phase due to a lack of nutrients or growth factors. In order to divide the cell must re-enter the cycle in the S phase. During the S phase each of the 46 chromosomes undergo duplication in the cell. The main purpose of the S phase is DNA replication where two identical semi-conserved chromosomes are produced. Pre-replication complexes are loaded onto the DNA at replication origins during G1-phase to prevent more than one replication from occurring.

These complexes are subsequently degraded in the S-phase as DNA replication begins (Bock *et al,* 2001).

The Gap-2, or G2-phase, occurs after the S-phase but before the M-phase. During this phase rapid cell growth and protein synthesis occur as the cell prepares itself for mitosis. The G2-phase ends with the onset of the prophase which is the first phase of mitosis, during which the cell's chromatin condenses into chromosomes. The cell is said to be in interphase if it is in any phase of the cell apart from mitosis (Morgan, 2007).

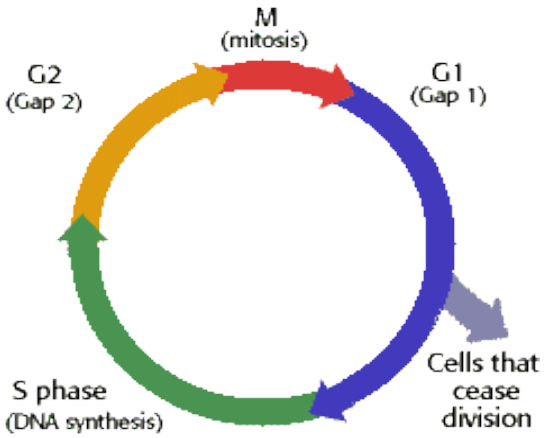


Figure 1- The various phases involved in the cell cycle of a eukaryotic cell (www.biology.arizona.edu (University of Arizona), 2004)

In order to ensure that the cell may divide successfully it is important that the cell cycle is controlled and regulated tightly (Bock *et al*, 2001). Should the normal control and regulation of the cell cycle be lost, it can lead to the development of cancer in higher eukaryotes. Control involves a variety of cytoplasmic proteins including cyclins and cyclin dependant kinases (Cdks) which along with cyclins cause the cell to advance from phases G1 to S, or G2 to M respectively. The Maturation Promoting Factor (MPF) consists of cyclins and Cdks that trigger cell cycle progression by promoting progression to the M phase (Morgan, 2007). The protein p53 plays a fundamental role in DNA repair as it is able to block the cell cycle if DNA is damaged. If the damage is severe this protein can initiate apoptosis (programmed cell death) (Morgan, 2007). Another key protein is p27, which prevents entry into the S phase by inhibiting cyclins A and E (Kaldis, 2006).

1.3 Cancer

This is a disease in which abnormal cells divide in an uncontrolled manner and invade surrounding tissues (Cavallo, De Giovanni, Nanni, Forni & Lollini, 2011). Cancer cells have the ability to spread to different tissues in the body, developing secondary tumours through the blood and lymph systems. This process is known as metastasis (Macdonald, Ford & Casson, 2004). Although many treatments are available for suppressing or eliminating cancers and tumours, the prognosis is still limited in many cancer types. Research to develop novel anti-neoplastic agents is therefore still required.

The role of chemotherapeutic drugs is to slow and hopefully prevent the growth and spread of the cancer. However these drugs are often feared because of their toxicity and many adverse side effects which they may cause the patient (Macdonald *et al*, 2004). Therefore research into producing effective drugs which not only eliminate the cancer but can do so without significantly harming the host is still required.

1.4 A549 cells

Cancer research has advanced over the years due to the accessibility of many cell lines derived from oncological tissues obtained from various cancer patients (Ferreira, Filomenga & Chaves, 2013). The adherent culture strain of the human adenocarcinoma alveolar basal epithelial A549 (ATCC CCL-185) was used in this study. The cell line was introduced in 1972 through explant culture of lung carcinomatous tissue from a Caucasian male 58 years of age. Since then the cell line has been actively used in various anti-cancer studies (Ehrhardt and Kim, 2007).

1.5 Fungi

Fungi are members of large group of eukaryotic organisms including microorganisms such as yeasts and filamentous fungi or moulds of various types (Kavanagh, 2011). Fungal cells are surrounded by a cell wall consisting of chitin, a complex polysaccharide. Some fungi are difficult to notice because of their small structures and their anonymity in soil or on decaying matter. Once the fungi begin to reproduce, either as moulds or mushrooms, they may become visible. Fungi play an important role in nutrient recycling and exchange, as well as decomposition of organic matter (Kavanagh, 2011).

Although fungi are useful in everyday life, certain species of fungi can produce bioactive compounds called mycotoxins. Mycotoxins include certain alkaloids and polyketides which are toxic to animals. Mycotoxins are toxic compounds many of which are produced by *Aspergillus*, *Penicillium* and *Fusarium* species. Under the correct conditions these fungi proliferate and produce harmful mycotoxins (Parker, 2003). Some species have structures containing psychotropic compounds which are consumed recreationally (Kavanagh, 2011). Additionally fungi may have adverse effects on environments such as buildings. Many species are pathogenic to humans and other animals. Fungal diseases also lead to crop losses which can have a detrimental impact on food supplies and human health (Parker, 2003).

Many fungi exist as unicellular and multicellular, in which the fungal cells are unicellular. They are usually oval or round and they reproduce by either budding or by fission (Kavanagh, 2011). Yeasts are classified in the phyla (divisions) Ascomycota or Basidiomycota. Yeasts, particularly those of the *Saccharomyces* genus are of major industrial importance as being used in alcoholic fermentations, bread-making, and in treating various ailments due to brewer's yeast containing a high content of thiamine and other B-complex group vitamins (Kavanagh, 2011). Filamentous fungi such as *Aspergillus niger* are multicellular organisms consisiting of long branched filaments called hyphae which aggregate to form a mass mycelium (Bärlocher, 2008). They have functions in maintenance of ecosystems by breaking down dead organic material, production of commercial enzymes, organic acids and various drugs such as penicillin (El-Enshasya, 2007).

Fungi that cause disease in humans or other organisms are termed pathogenic fungi. Though fungi are eukaryotic organisms many pathogenic fungi are microorganisms (Parker, 2003). *Saccharomyces cerevisiae* is usually not considered a pathogen as diseases caused by this type of yeast are rare however there have been reported incidents (Mermel *et al*, 2009). *Candida* species are the most common human pathogens often causing nasal, subcutaneous and gastrointestinal infections especially in immune compromised patients including AIDS sufferers and transplant patients (Bärlocher, 2008). Other pathogenic C*andida* species include; *C. tropicalis*, *C. parapsilosis* and *C. dubliniensis* which are responsible for various infections (Sardi, Scorzoni, Bernardi, Fusco-Almeida & Mendes-Giannini, 2013). Although most yeast infections are commonly associated with *C. albicans*

exposure to *S. cerevisiae* particularly for immunosuppressed individuals can be fatal. (Mermel *et al*, 2009).

Aspergillus niger is a filamentous fungus which can cause serious lung diseases (aspergillosis) and it is the most common cause of otomycosis (fungal ear infection). We are constantly exposed to airborne *Aspergillus* spores as they are ubiquitous. This exposure is generally harmless and poses no threat to human health; however exposure to large doses of fungal spores can be lethal (Favor, 2004). *Aspergillus* cause fungal diseases in three specific ways. The disease can be caused via localized or systemic infections, through induction of allergenic responses or through the production of mycotoxins. A common pathogenic species is *Aspergillus flavus* which along with being a toxin is also a carcinogen and can contaminate foods such as nuts. Other species include *Aspergillus fumigatus* and *Aspergillus clavatus* which can cause allergy related diseases (Favor, 2004).

Most *Cryptococcal* species are harmless and do not cause disease in humans. The major human and animal pathogen is *Cryptococcus neoformans* which can cause disease of the lungs and secondary infections in HIV and AIDS sufferers (Parker, 2003).

Other pathogenic fungi include: *Histoplasma capsulatum* which can cause histoplasmosis in both humans and animals; *Pneumocystis carinii*, which can cause pneumonia in people with weak immune systems and *Stachybotrys chartarum* which can cause respiratory damage and major headaches (Parker, 2003).

Both *S. cerevisiae* and *C. albicans* have been sequenced to the genome level and have been extensively studied in terms of their cell biology, whereas *A.niger* is the most studied of the filamentous species. These properties mean many useful strains are easily available and therefore they are good models for aiding novel antifungal drug development strategies (Kavanagh, 2011).

1.6 Infection

An infection is caused by the invasion of a host organism's bodily tissues by microorganisms such as bacteria, parasites or fungi. Microorganisms that are naturally found in the body are not considered infectious and they do not usually cause any harm to the host. However

disease-causing microbes such as those previously mentioned can invade bodily tissue, multiply and release toxins which are harmful to the host. The host normally combats infections using their immune system via an innate response such as inflammation followed by an adaptive response. However in many cases the immune response cannot tackle the infection alone so drugs that fight infectious diseases, such as antibiotics or antimicrobials, are used. Many infections can be subclinical and not cause any symptoms, or it may be clinically apparent and cause symptoms. Infections can also be either concentrated in a specific area of the body (localised) for example nails, nose, groin, intestine etc. or they may spread through the lymphatic or blood vessels (disseminated) (Escobar, 1988).

In some cases viral and bacterial infections cause similar symptoms which makes it difficult to determine the cause of the infection. Viral infections are not treated using antibiotics so it is important to distinguish whether it is a viral, bacterial or fungal infection, in order to give the correct drug treatment (Shetty, Tang & Andrews, 2009).

1.7 Antimicrobials and Antimicrobial Resistance

Anti-infective drugs are usually administered prophylactically prior to an infection occurring to prevent people from getting the infection in the first place. They are also administered to patients suffering from an infection to suppress the infection caused by a harmful microorganism attacking and inhabiting tissue in the host's body. There are three types of anti-infective drugs: antiviral, antifungal and antibacterial. Most infectious diseases are caused by bacteria as they are easy to catch and spread. Antibiotics, which are natural or semi-synthetic in origin, are administered to treat bacterial and some fungal infections. They are given either by mouth, injection or applied topically depending on the severity and form of the infection. Intravenous antibiotics may be used to aid the treatment of severe brain infections. Antibiotics function by suppressing bacterial multiplication or by eliminating the bacteria in the host. They may be used in combination to increase efficiency and decrease the risk of resistance (Finberg & Guharoy, 2007).

Antimicrobial drugs are used to selectively inhibit the growth of or kill harmful microorganisms such as protozoans, bacteria or fungi. Since the discovery of antimicrobials such as penicillin and tetracycline the interest in synthesizing novel antimicrobials has increased. Antibiotics are generally derived from natural resources or are semi synthetic

(e.g. most penicillins). Whereas antimicrobials include synthetically produced compounds such as the sulfa drugs (Drlica & Perlin, 2011).

Antibiotics have become an integral part of modern medicine. They either initiate cell death or inhibit growth by targeting essential cell physiology and biochemistry. Today most infections are treated using antibiotics. Antibiotics are also essential in surgery, without which many procedures, such as open heart surgery, cannot be performed (Yount, 2004).

Many antibiotics are available targeting a wide spectrum of bacteria and fungi. The most common classes of antibiotics used in medicine include penicillins, cephalosporins, quinolones, aminoglycosides, macrolides and tetracyclines (Yount, 2004). Synthetic antimicrobials include sulphonamides that are used in treating bacterial infections and the azoles and allylamines which are used in treating fungal infections (Drlica & Perlin, 2011).

Antifungal resistance occurs due to a stable, inheritable modification in a fungal cell against an antifungal agent. Such mutations result in less than normal sensitivity to that antifungal and the effects of the antifungal are decreased. Mutations in genes may cause: changes in the affinity or turnover of the target enzymes; by increasing deactivation of the drug or by exporting the drug from the cell (Projan & Youngman, 2002).

The increasing emergence of drug-resistant strains of fungi is a threat to societal and human health as immunocompromised patients such as those suffering from HIV/AIDS or those undergoing chemotherapy or transplantation are more susceptible to fungal infections. (Norby, Nord & Finch, 2005).

There is a growing concern about the lack of development of new antimicrobial drugs. The European Society of Chemical Microbiology and Infectious Diseases (ESCMID) has noted that development of novel antimicrobial compounds has significantly decreased in the last decade (Norby *et al,* 2005). The number of cases of multidrug resistant fungal infections is increasing (Gulshan & Moye-Rowley, 2007) suggesting an urgent need for new classes of antifungal agent (Oren, Yalcin, Sener & Ucarturk, 2004). There is thus an increasing risk of organisms resistant to most or all existing antimicrobials giving rise to common infections which are untreatable or even lethal (Norby *et al,* 2005). Thus the development of novel

compounds which act as antifungal agents, but differ chemically and target different enzymes and or receptors from existing compounds, is vital.

Various drugs are available that may be used to treat fungal infections. These can be classified on the basis of their mechanism of action. Antifungal agents are available that: impair membrane barrier functions including polyenes such as Amphotericin B (Amp B); inhibit macromolecule synthesis by acting as antimetabolites (flucytosine); interact with microtubules (griseofulvin); inhibit ergosterol synthesis (allylamines and (tri)azole derivatives) or inhibit cell wall (chitin) synthesis (enchinocandins) (Projan & Youngman, 2002).

Azoles are a class of antifungals which act by blocking ergosterol synthesis. Decyl imidazole, the first azole found to inhibit ergosterol production, was the lead compound from which various azoles have been developed. Azole type drugs therefore contain an imidazole or, more recently, a triazole structure. Ergosterol is an essential steroid found in fungi and is required for plasma membrane stability (Lewis, 2011). The pathway of conversion of lanesterol to ergosterol requires specific enzymes called demethylases which are examples of cytochrome P450 enzymes. The enzymes 14 alpha demethylase, 4 alpha demethylase and delta 22, 23 desaturase are blocked by azoles and triazoles. Synthesis of ergosterol is inhibited due to a dative bond formed by a lone pair of electrons on one of the nitrogen atoms of the imidazole with the iron atom in the P450 enzymes (Lewis, 2011). Miconazole is a commonly used example from the class of azole drugs used to treat various fungal infections (Sawyer, Brogden, Pinder, Speight & Avery, 1975). In the present study miconazole nitrate was used as a reference drug. The antifungal properties of all compounds tested were compared against miconazole as an internal control.

In recent years interest in the synthesis of bioactive heterocycles has increased significantly as many compounds with biological activity contain one or more heterocyclic atoms in their structures. Amongst the large family of heterocyclic compounds, those containing sulphur, nitrogen and oxygen attract the most attention from medicinal chemists due to the large range of biological activities observed with these compounds. Nitrogen heterocycles are most commonly used as many classes can be easily synthesized (Projan & Youngman, 2002).

Previous experiments involving the use of tropan-3-one and piperidin-4-one derivatives showed that these compounds had cytotoxic properties against J774 mouse macrophages (Hussain, 2012). One of the aims of this investigation was to explore these compounds in more depth and to introduce different substituent groups in various positions in eneone structures. The potency of these compounds against certain fungal species and a cancer cell line were examined. Also a previously synthesized library of benzothiazine derivatives (Patel, 2012) was screened for their antifungal properties. As previous work involving similar compounds has produced promising results this research will aim to extend those studies in order to determine ways of synthesizing and testing potentially more potent compounds (Patel, 2012 and Hussain, 2012).

Synthesized tropan-3-one along with a library of piperidone dieneones and 1,2-benzothiazines were tested against various fungal species to determine if any of the compounds had antifungal properties. The compounds were compared against miconazole nitrate. Based on their potencies against fungi, a subset of compounds were tested against the epidermal cancer cell line A549 to determine any cytotoxic properties. All compounds were compared to a positive kill control (Triton X-100).

1.8 QSAR

Medicinal chemistry deals with the design and synthesis of new therapeutic chemicals and their development into useful medicines (Thomas, 2011). Medicines are formulated substances used in the treatment of disease, whilst drugs are the active molecules found in the medicines used to treat or prevent disease (Thomas, 2003). Medicinal chemistry involves the isolation of natural compounds or the synthesis of new molecules and investigating how altering the structure of molecules affects their biological activities. The drugs are tested using purified enzymes or receptors, cells, tissues or whole organisms. Such structural changes can lead to an increase, a decrease or no change in the activity of the drug. Such changes may also alter the absorption, bioavailability, distribution, metabolism and excretion of the drug which also alters its overall potency (Patrick, 2001).

Similarly in this investigation modifying the structures of the parent compounds can potentially promote cell death or block cell division. Thus they may prove to be to be useful in developing novel antifungal or anticancer agents.

The results generated by this study can be applied to the development of one or more QSARs to examine whether the hydrophobicity and or the structure of the compounds tested are related to their known bioactivities. If clearly visible relationships are observed, then novel compounds could be developed and synthesized with optimal LogP or, LogD values, sizes volumes or electronic distributions which hopefully will give rise to molecules with higher potencies. To do this the antifungal and anticancer potencies will need to be compared to various physiochemical properties in order to generate QSARS which will allow further molecules with improved potencies to be developed (Thomas, 2003).

1.9 Cell Growth and Dose-response Curves

In biology growth curves are frequently used for quantities such as population size, cell viability or biomass. A graph can be constructed displaying the values of the measured property (i.e. number of viable cells) against the concentration of the novel compound.

The analysis of growth curves is pivotal in cancer research. For patients that are undergoing treatment using anti-cancer drugs, growth curves can be used to determine how efficient the drug is in reducing the cancer (Nadeau, 2012). It is also a vital tool in antifungal studies as it can determine the effects antifungals have on the growth of particular fungal species.

Growth curves were used in this study to analyse the growth of both fungal and cancer cells and for determining their respective exponential phases. The exponential phase is the point at which the cells are proliferating at their maximum rate (Bergman, 2001).

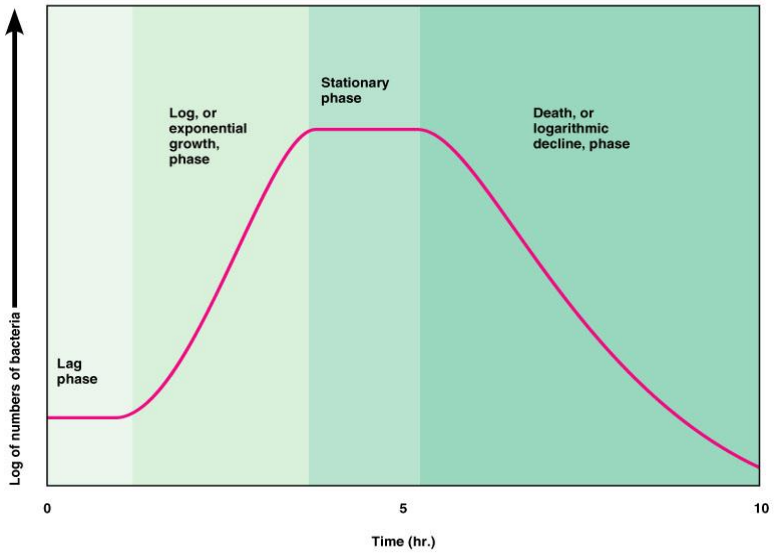


Figure 2- Various phases observed during cell growth (www.biology.arizona.edu (University of Arizona), 2004).

Dose-response curves are used to describe the relationship between the response to drug treatment and the drug concentration (or dose). These are useful in determining safe and hazardous concentrations of the tested drug. The graphical software package GraphPad, Prism 5 is used to construct dose-response curves using a four-parameter logistic equation to construct a suitable sigmoidal curve. A four-parameter logistic nonlinear regression model is commonly used for dose-response curves in both antifungal and anticancer studies (Panetta, 1997).

The equation is as follows;

$$Y= 100 / (1 + 10^{((LogIC50-X) * Hill\ slope)}) \quad \textbf{Equation (1)}$$

Where X is log of drug concentration, Y is the normalized response, 100% down to 0% decreasing as X increases and the Hill slope is the slope factor.

The IC_{50} is defined as the concentration required to induce 50% inhibition of cell growth or equally 50% decrease in cell viability. It measures the efficiency of a compound or agent to inhibit biological or biochemical functions (Rege & Medintz, 2009). IC_{50} measurements specify the concentration of the drug/agent required to successfully inhibit a biochemical or biological process. GraphPad Prism 5 software calculates the IC_{50} values whereas IC_{10} and IC_{90} values (concentration required to inhibit 10% and 90% cell growth or cell viability) were manually obtained from the dose response curves.

1.10 Antifungal Susceptibility Testing using Yeast and Filamentous Fungi

Various methods are available for antifungal susceptibility testing of both yeast and filamentous fungi (Fothergill, 2012). The Clinical Laboratory and Standards Institute (CLSI) and European Committee on Antimicrobial Susceptibility Testing (EUCAST) have approved methods for antifungal susceptibility testing of both yeast and moulds (filamentous fungi). According to the CLSI and EUCAST the liquid broth dilution method is most appropriate for yeasts whereas the disk diffusion assay is more suited to filamentous fungi (Fothergill, 2012, CLSI, 2013 & Kahlmeter *et al*, 2006).

The liquid broth dilution method allows the determination of whether a test compound is fungicidal at a specified concentration. Fungal cells are grown and maintained in broth media which contains all the nutrients required for the cells to grow when incubated at an

appropriate temperature. The drug under examination is incubated with the cells at various concentrations and the inhibition of cell growth by the drug analysed by measuring the optical densities obtained (Fothergill, 2012, CLSI, 2013 & Kahlmeter *et al*, 2006).

Disk diffusion assays use solid media agar plates which are widely used because of their simplicity and cost effectiveness (Sardi *et al*, 2013). Absorbent paper disks containing various amounts of the drugs to be examined are placed onto agar plates which have been inoculated with a specific amount of conidia. The diameter of the zone of inhibition is measured at the end of the incubation period and compared with reference drugs. (Fothergill, 2012, CLSI, 2013).

The effects of the compounds on the inhibition of growth of the yeast species was analysed by determining; IC_{10}, IC_{50} and IC_{90} values. MIC values are generally obtained from susceptibility assays using agar plates or glass tubes (Fothergill, 2012). An earlier study revealed that when three types of yeast species were treated with miconazole the MIC values vary significantly with inoculum size whereas IC_{50} values remain constant (Galgiani & Stevens, 1976). Thus interlaboratory comparisons should be easier if IC_{50} values were measured and quoted.

Assays of microbial susceptibility to drugs via the agar diffusion method were analysed using linear fitting of either the diameter or squared diameter of the inhibition zones against the natural logarithm (ln) of drug concentration at the source;

$$\ln (MIC) = \ln (c) - D^2 / 4dt \qquad \textbf{Equation (2)}$$

Where D= zone of inhibition diameter, d= diffusion coefficient presumed to be independent of concentration and t= time of drug diffusion) (Kronvall, 1982). This reflects a solution of the differential equation describing free diffusion in one dimension which takes into account the dependence of the drug concentration on distance from the source and on time. (Bonev, Hooper & Parisot, 2008). In order to find the most suitable linear fitting, various graphs were plotted of D, D^2, Ď (corrected by subtracting from the diameter of the paper disks) and $Ď^2$ against ln (drug concentration). R^2 values were deduced from each graph and then compared (Microsoft Excel). R^2 values indicate how well data points fit a linear

regression as it represents the variation of the data explained by the fitted line. The closer the R^2 value is to 1 the better the linear regression fit (Colin & Windmeijer, 1997).

1.11 Cytotoxicity Studies

Cell viability was used to determine if cells are living or dead using a total cell sample (Mather & Roberts, 2006). Cell viability assessments have been applied in many areas of research including cancer research. Such methods have been used to determine the viability of cancerous cells, and the rejection of implanted organs. This technique is also useful when testing the effectiveness of insecticides, or to assess the effect of toxins on the environment (Johnson & Spence, 2010).

Cell viability measurements evaluate healthy cells within a sample, which is accomplished by calculating the number of healthy cells by counting them or by measuring an indicator for healthy cells in cell populations (e.g. by adding a specific dye when using a microtitre plate assay). An increase in viability exemplifies cell growth, whereas a decrease is usually due to the toxic effects of the compounds being added or in other cases contaminated culture conditions (Stoddart, 2011).

In comparison to cell viability studies, cell proliferation measures the growth of cell number within a sample. This assessment allows the determination of the actual number or proportion of proliferating cells in cell cultures or tissues, though quiescent non-growing healthy cells are not identified by this analysis (Mather & Roberts, 2006).

Most viability assays focus on the integrity of the cell membrane or some specific metabolic activity of healthy cells. A cell population is incubated with a tetrazolium salt (e.g., MTT, XTT, and WST-1) which is cleaved to a coloured formazan product by metabolically active cells. This procedure is commonly used for measurement of metabolic activity (Cory, Owen, Barltrop, & Cory, 1991). Also analysing the ATP status of the cells can indicate the cellular energy capacity and consequently viability of such cells (Mosmann, 1983). An alternative assay analyses cytoplasmic membrane integrity. Dead cells show a stained cytoplasm while healthy cells are normal and can be directly counted (Mather & Roberts, 2006).

Several cytotoxicity assays have been approved by the National Cancer Institute (NCI) of

which the most commonly used are the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H tetrazolium, inner salt (MTS) and lactate dehydrogenase (LDH) assay (Baharith, Al-Khouli & Raab, 2006).

1.11.1 The MTS Assay

When a cell is damaged it loses the capability to maintain and provide energy for metabolic cell function and growth. Metabolic activity assays work on this principle and usually measure mitochondrial activity. A colorimetric substrate such as MTT, XTT or WST-1 is used and incubated within the cell samples (Davey & Lord, 2003).

The MTS assay is used to determine if compounds have an effect on reducing mitochondrial activity thus indicating the possession of cytotoxic properties. The MTS assay is often defined as a 'one-step' MTT assay as the reagent can be added directly to the cell culture without the need of intermediate steps (Burger & Fiebig, 2004). The test is centred on the ability of viable cells to convert the MTS reagent into a soluble blue/purple formazan dye. Thus viable cells appear blue/purple whereas dead cells are unaffected and remain pale yellow (Burger & Fiebig, 2004). The exact cellular mechanism of MTS reduction into formazan involves reactions with intracellular NADH-oxidoreductases (Berridge, Herst & Tan, 2005). It is believed to occur mainly through the action of mitochondrial succinate dehydrogenase (Lobner, 2000).

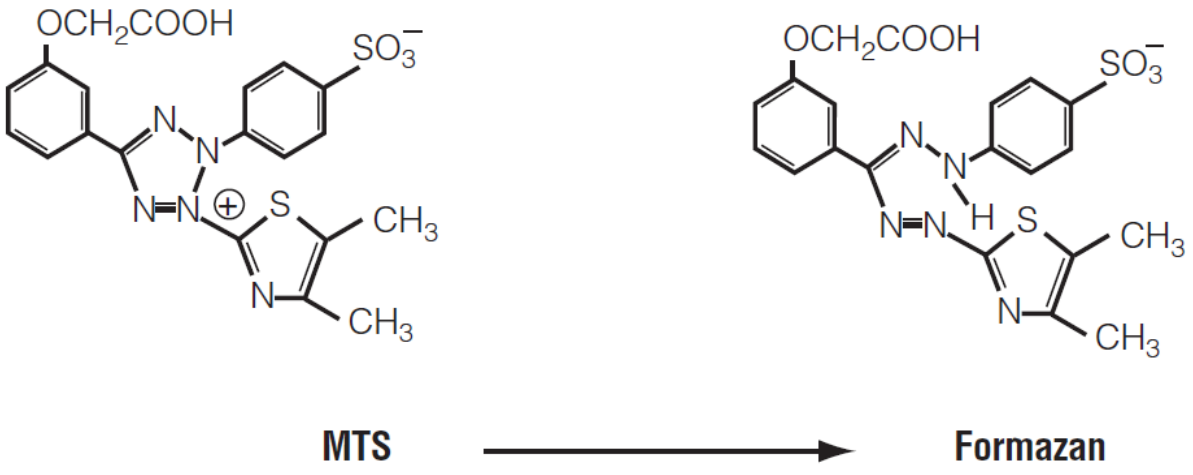


Figure 3- Conversion of MTS into aqueously soluble formazan salt (Promega, 2012)

1.11.2 The LDH Assay

The effect on cell membrane integrity by potentially toxic compounds was measured using the lactate dehydrogenase (LDH) assay. LDH is an oxidoreductase which exists in most organisms and it catalyses the inter-conversion of pyruvate and lactate with an associated inter-conversion of NADH and NAD^+, LDH is used as a catalyst. Pyruvate is used by the cell as a 'dump' for excess reducing power i.e. NADH in the absence of oxygen. This is facilitated by the action of the enzyme LDH (Fotakis & Timbrell, 2006). When a tissue suffers membrane damage, cells release LDH into the bloodstream. Since LDH is a stable enzyme it is widely used for the detection of damaged cells (Fotakis & Timbrell, 2006). The assay measures the release of LDH from cells with damaged membranes by measuring changes in fluorescence of reosurfin which is proportional to the quantity of released LDH (Promega, 2012). An increase in LDH release is shown by an increase in fluorescence which indicates increasing cytotoxicity.

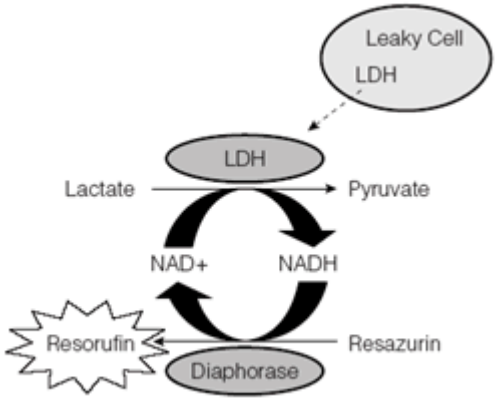


Figure 4- Release of LDH from damaged cells is measured by providing lactate, NAD^+ and resazurin as substrates in the presence of diaphorase. Production of the fluorescent reosurfin product is proportional to the quantity of LDH. (Promega, 2012)

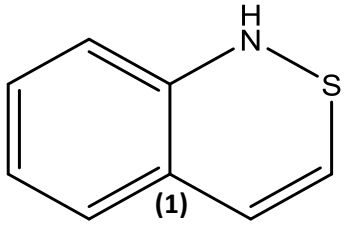
1.11.3 Trypan Blue Assay

The Trypan Blue test is a dye exclusion method which can be used to determine the number of viable and/or non-viable cells within a cell suspension (Kalter, 2010). Trypan Blue is a diazo dye which actively stains the cytoplasm of dead cells. They can consequently be visualized using a microscope. Viable cells with intact cell membranes exclude dyes such as trypan blue; therefore their cytoplasm is not stained by this method. Cells are very selective as to what compounds pass through their membrane. A viable cell will exclude the trypan blue dye whereas non-viable cells will absorb the dye with resultant staining of its

cytoplasm. Under the microscope a living cell will possess a clear cytoplasm, whilst a dead cell will have a blue cytoplasm (Baust & Baust, 2007).

1.12 Benzothiazine

Benzothiazine is a heterocyclic compound composed of a benzene ring conjoined to a 6-membered heterocyclic thiazine (Figure 5). Over the years benzothiazines have been studied in great detail (Niewiadomy, Matysiak & Karpinska, 2011 and Park *et al,* 2002). The preparation of this class of compound along with studies of their pharmacological and biological properties have been investigated in detail (Park, *et al*, 2002) and there is growing interest in this class of compound. (Niewiadomy *et* al, 2011).



(1)

Figure 5- Structure of the 2, 1-benzothiazine nucleus (1)

Several synthetic approaches to produce benzothiaizne and its derivatives have been discovered (Zinnes, Comes & Shavel, 1967). More recently their biological actions such as anticancer or antifungal properties have been studied (Niewiadomy *et al*, 2011).

Ever since Braun and co-workers synthesized the first benzothiazine in 1923, a growing interest into their properties has occurred. Many thousands of compounds for the prospective treatment of diseases have since been synthesized. In the 1950s Abe and co-workers discovered the anti-inflammatory and analgesic action of the benzothiazines by synthesizing various derivatives and noting these compounds exhibited medical, microbiocidal and agrochemical properties (Abe, Yamamoto & Matsui, 1956). Since Abe and co-workers initial discovery, interest into the pharmacological and biological activities of benzothiazines has gradually increased (Park *et al*, 2002).

Depending on the substituents present; benzothiazine derivatives have exhibited a wide range of biological activities ranging from antipsychotic to anti-inflammatory activities (Camoutsis & Catsoulacos, 1992). The medical application of benziothiazine is however

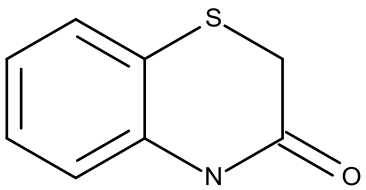
limited as a result of their detrimental side effects such as severe headache and gastrointestinal ulceration (Tumbleson & Schook, 1996). Over the last twenty years extensive research has been conducted to determine ways to limit or prevent these adverse side effects and produce benzothiazines with improved pharmacological properties (Niewiadomy *et al,* 2011).

1.13 Biological Activities of Benzothiazine

1.13.1 Antimicrobial Activities of Benzothizines

3-Phenyl-*N*-(2,2-dimethoxyethyl)-3,4-dihydro-2H-1,4-benzothiazin*e* was tested against *Aspergillus niger* and it demonstrated an antifungal activity 16-fold greater than miconazole (Armenise, Trapani, Stasi & Morlacchi, 1998). The same compound also demonstrated antibacterial activity against strains of *Micrococcus luteus* producing a minimum inhibitory concentration (MIC) of 31 µg/ml (Armenise *et al*, 1998).

Derivatives of 2H-Benzo[1,4]thiazin-3-one **(2)** were administered to bacterial species including *Micrococcus flavus, Proteus vulgaris, Salmonella enteritidis, Staphylococcus aureus, Escherichia coli* and *Bacillus cereus.* The results obtained showed that these derivatives expressed activity against all strains tested (Guarda *et al*, 2001).



(2)

Figure 6- Structure of 2H-Benzo[1,4]thiazin-3-one (2)

2H-1,2-benzothiazine-3-carbohydrazide 1,1-dioxides derivatives were synthesized using a microwave. These compounds displayed anti-microbial activity against fungi and both Gram negative and Gram positive bacteria (Ahmad *et al*, 2011).

1,2-Benzothiazine analogs having tetracycline structures were studied by Zinnes *et al* (1967) which demonstrated antifungal activity against *Aspergillus arogens* and antibacterial activities against *Bacillus megaterium, Escherichia coli* and *Bacillus subtilis.*

The compound groups 7-Chloro-5-trifluoromethyl-4H-1,4-benzothiazines, 7-fluoro-4H-1,4-benzothiazines and 7-trifluoromethyl-4H-1,4-benzothiazines when compared to the reference drugs chloramphenicol and fulvin, showed moderate antimicrobial activity against organisms such as *Bacillus subtilis, Bacillus megaterium, Escherichia coli, Aspergillus arogens* and *Aspergillus awamori* (Rathore, Singh & Kumar, 2006).

4*H*-1,4-benzothiazine derivatives were tested against Gram positive bacteria and *C. albicans* by Armenise *et al* (2006). The compounds 1-(5-chloro-7-fluoro-4H-1,4-benzothiazin-2-yl-ethanone and 1-(5-chloro-7-fluoro-3-methyl-4H-1,4-benzothiazin-2-yl)-ethanone demonstrated antibacterial activity against strains of *Bacillus cereus and Bacillus subtili.* Also *MIC* values of 63 µg/ml and 16 µg/ml were obtained against *Enterococcus faecalis* and *Staphylococcus aureus* respectively. The same compounds also showed antifungal activity (MIC 125 µg/ml) against four strains of *C. albicans* (Armenise *et al*, 2006).

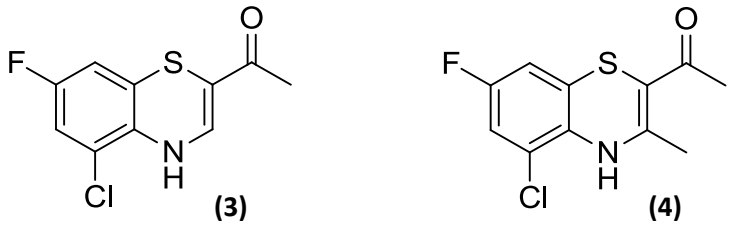


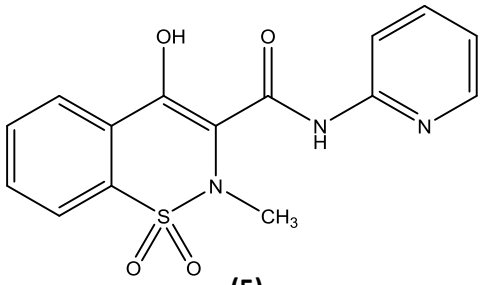
Figure 7: Structures of (1-(5-chloro-7-fluoro-4H-1,4-benzothiazin-2-yl)-ethanone) (3) and (1-(5-chloro-7-fluoro-3-methyl-4H-1,4-benzothiazin-2-yl)-ethanone)(4).

In 2011 Sharma and co-workers reacted substituted 1,4-benzothiazines with either morpholine or N-(2 hydroxyethyl) piperazine to produce Morpholinyl or Piperazinyl benzothiazines. These compounds exhibited activity against both *Bacillus cereus* and *Escherichia coli* (Sharma, Kumar, & Vats, 2011).

1.13.2 Benzothiazines as Anticancer Agents

Peroxicam is a non-steroidal anti-inflammatory drug of the oxicam class which is used as an analgesic (Manjarrez, Perez, Solis & Luna, 1996). It has been shown that the risk of colorectal cancer can be reduced by 40-60% and telomerase activity can be inhibited using non-steroidal anti-inflammatory drugs (NSAIDs) (Brown *et al*, 2000). Recently 1,2 benzothiazine derivatives have been shown to be highly effective non-steroid anti-inflammatory agents (Manjarrez *et al,* 1996). Apart from Piroxicam, Sudoxicam and Isoxicam

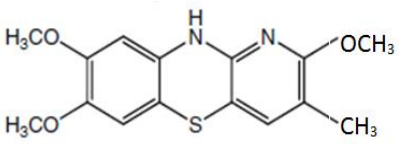
are also examples of drugs used as anti-inflammatory agents (Brown *et al,* 2000). 1,2-Benzothiazine-1,1-dioxides represent a new class of anti-inflammatory agent which includes the drug peroxicam (Manjarrez *et al,* 1996).



(5)

Figure 8- Structure of piroxicam (5)

1,4-Benzothiazine derivatives were synthesized by Abbas and co-workers (2010) and tested against the colon cancer cell line HCT-116, liver carcinoma cell line HPEG2-1 and human breast cell line MCF-7. Results indicated that the compounds 3-Acetyl-7,8-dimethoxy-2-methyl-10H-pyrido-[3,2-b][1,4] benzothiazine, 2-(4-Chlorophenylhydrazono)-6,7-dimethoxy-2H-1,4-benzothiazin-3(4H)-one and 6,7-Dimethoxy-2-(4-nitrophenylhydrazono)-2H-1,4-benzothiazin-3(4H)-one showed activity against all tested cell lines, with the greatest activity being shown against HCT-116 cell line. Some of the 1,4-benzothiazine derivatives also showed antibacterial and antifungal activities with significant activities displayed against *E.coli* and *S. Aureus* (Abbas & Farghaly, 2010).



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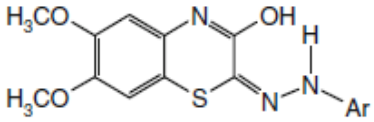

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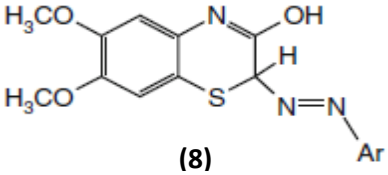


(8)

Figure 9- Structures of 3-Acetyl-7,8-dimethoxy-2-methyl-10H-pyrido [3,2-b][1,4]benzothiazine (6), 2-(4-Chlorophenylhydrazono)-6,7-dimethoxy-2H-1,4-benzothiazin-3(4H)-one (7) and 6,7-Dimethoxy-2-(4-nitro phenylhydrazono)-2H-1,4-benzothiazin-3(4H)-one (8).

Derivatives of 2-aryl-4H-3,1-benzothiazine were synthesized and tested for their anti-proliferative activity by Niewiadomy and co-workers. The compounds were evaluated for their anti-proliferative activity against the human cell lines HCV29T cells (bladder cancer);

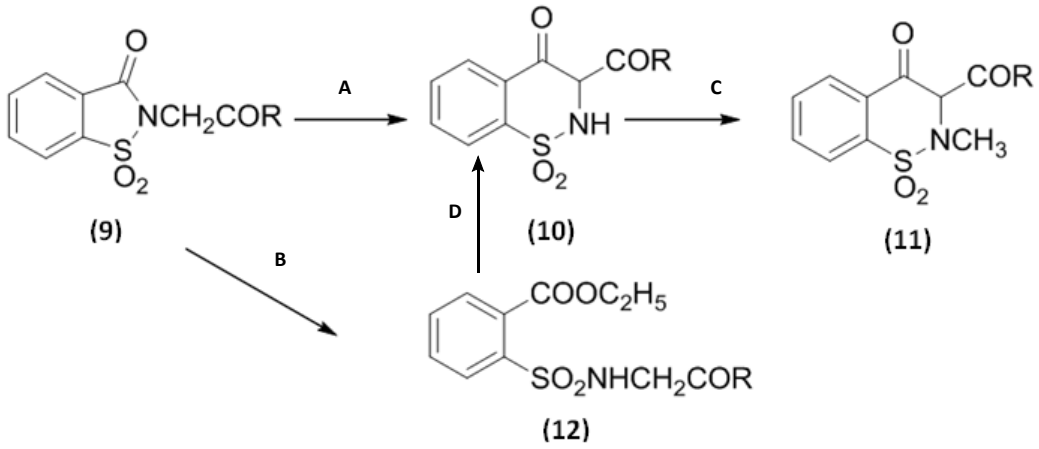
A549 (human non-small cell lung carcinoma); T47D (human breast cancer) and SW707 (human rectal adenocarcinoma) cells. The respective ID_{50} values (the concentration of the compound inhibiting the proliferation rate of the tumour cells by 50% as compared to the untreated control cells) obtained were compared to cisplatin, a drug used in the treatment of bladder cancer. The 2-aryl-4H-3,1-benzothiazine derivatives displayed activity equal to that of cisplatin with some derivatives showing greater activity (Niewiadomy *et al*, 2011).

1.13.3 Other Activities

As well as showing antimicrobial, anticancer and anti-inflammatory activities, benzothiazine derivatives have shown potential as central nervous system (CNS) depressants and anti-depressants, calpain inhibitors, vasodilators, anticonvulsants, antioxidants and urease inhibitors in mammals. Additionally they are used therapeutically as muscle relaxants (Lombardino, Wiseman & McLamore, 1971).

1.14 Synthetic Routes for Benzothiazines

The first methods for the synthesis of benzothiazine compounds were reported by Abe and co-workers (Abe, Yamamoto & Matsui, 1956). The methods to synthesize various benzothiazine derivatives were extensively studied by Zinnes and co-workers (Zinnes *et al*, 1967).



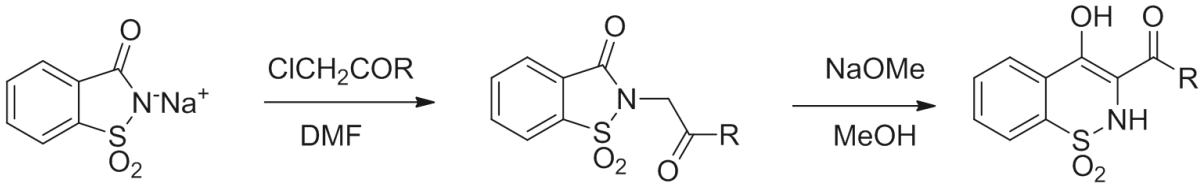
Scheme 1- Synthesis of 1,2-benzothiazine using steps (A-D) by Abe & co-workers (Abe, et al, 1956).

The compound N-Acetonylsaccharin **(9)** together with 2 equivalents of sodium ethoxide gave 80% yield of the 3-acetyl compound **(10) (**step **A).** If one equivalent of sodium ethoxide

was used compound **(12)** was produced **(**step **B).** This was readily converted to compound **(10)** when reacted with 2 equivalents of sodium ethoxide **(**step **D).**

The sodium salt of **(10)** was reacted with methyl iodide in either aqueous or non-aqueous solution to obtain the N-methyl compound **(11) (**step **C).** There is evidence suggesting that a mechanism involving ethanolysis of the carboxamide linkage of **(9)** followed by a Dieckmann ring closure forms 3-acetly-2H-1,2-benzothiazin-4(3H)one 1,1-dioxide **(10)** (Abe *et al*, 1956).

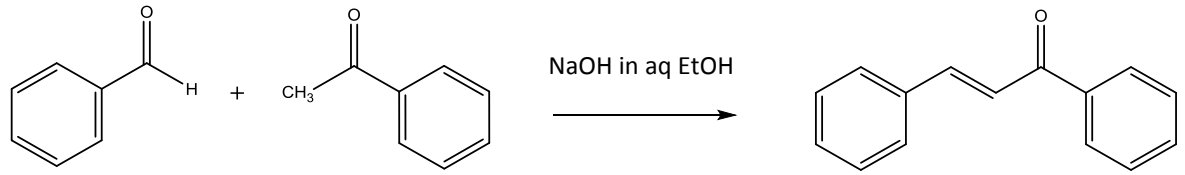
Recently the number of literature references to the 1,2-benzothiazine-1,1-dioxide ring system has increased significantly. Many of these concern the discovery of 3-carboxamides of 2-alkyl-4-hydroxy-2H-1,2-benzothiazine-1,1-dioxides which act as anti-inflammatory agents. A typical example is the drug Piroxicam **(5)**. Although there are a variety of synthetic routes available to obtain 1,2-benzothiazine-1,1-dioxides, the most frequently used in recent years follows the procedure of the Gabriel Colman rearrangement of saccharin derivatives (Scheme 2) (Weeks, Vinick, Breitenbach & Jung, 1983).



(13) **(14)** **(15)**

Scheme 2- Synthesis of 1,2-benzothiazine-1,1-dioxides from saccharin derivatives

Another method for synthesizing 1,2-benzothiazine derivatives is by using chalcones. An aldol condensation between a benzaldehyde **(16)** and an acetophenone **(17)** in the presence of sodium hydroxide as a catalyst produces chalcones **(18)** (Patel, 2012).

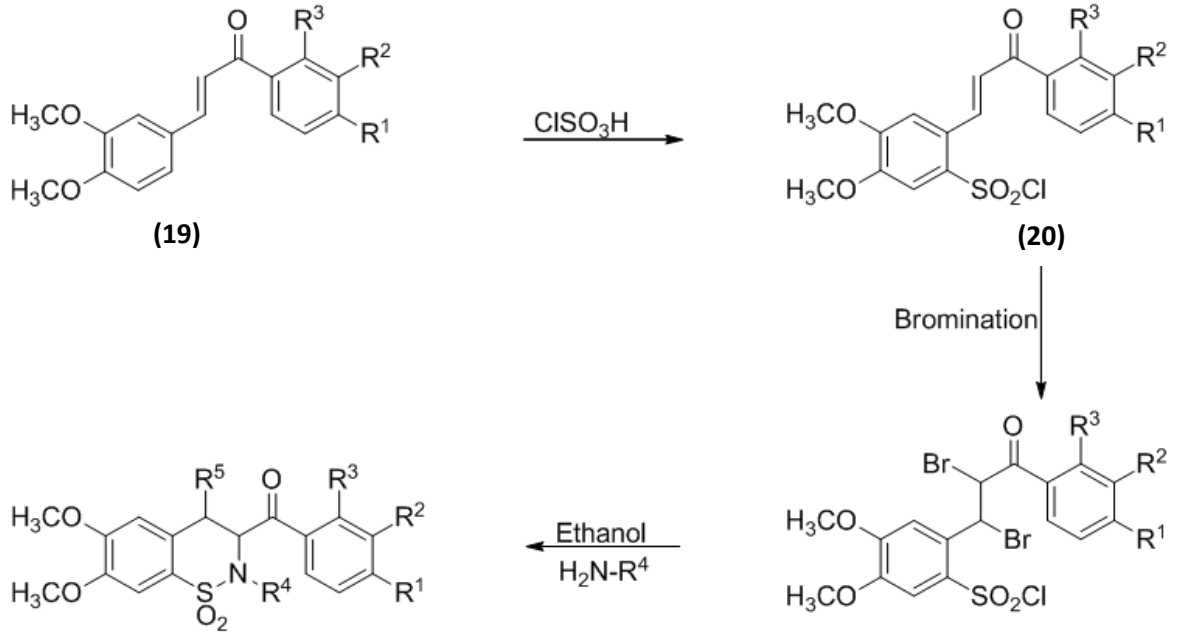


(16) **(17)** **(18)**

Scheme 3- Synthesis of chalcones

Substituted chalcones **(19)** react with chlorosulfonic acid to form sulfonyl chlorides **(20)**, which are then reacted with bromine to form the dibromo compounds **(21).** The dibromo

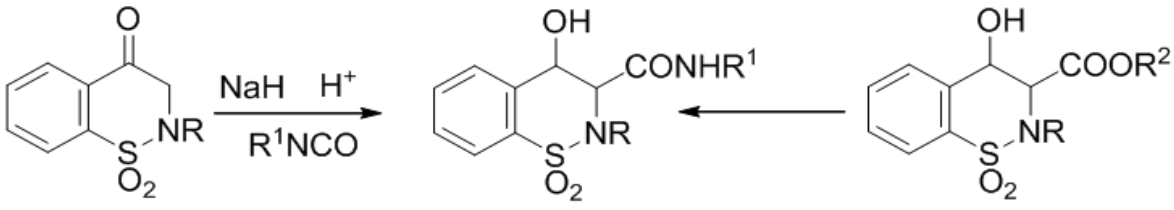
compounds are subsequently treated with various amines to produce a variety of 1,2-benzothiazines **(22)**.



(19) **(20)** **(21)** **(22)**

Scheme 4- A general procedure for the synthesis of 1,2-benzothiazines using chalcones. The 'R' groups are various substituted groups of preference; where R^1, R^2 and R^3 are either H, CH_3, F, Cl or Br; R^4 is either H, CH_3, CH_2CH_3, Ph or $PhCH_2$ and R^5 is Br or $NHCH_3$.

The synthesis of various 3-carbamoyl benzothiazine derivatives **(24)** was described by Lombardino and co-workers (Lombardino, Wiseman & McLamore, 1971). The compounds were produced by the base catalysed reaction of the thiazinone nucleus **(23)** with a variety of aromatic isocyanates. In 1973 Zinnes *et al* used similar reactions to synthesize various other 3-carbamoyl benzothiazine derivatives. A reaction between N-substituted 4-hydroxy-2H-1,2-benzothiazin-3-carboxylic acid methyl ester **(25)** with a suitable amine in refluxing xylene produced a number of carbamoyl derivatives (Zinnes, Lindo, Sircar, Schwartz & Shavel, *1973)*.



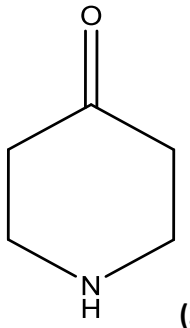
(23) **(24)** **(25)**

Scheme 5- 3-Carboxamides of 2-alkyl -4-hydroxy-2H-1,2-benzothiazine 1,1-dioxide.

1.15 Tropan-3-one and 1-methyl Piperidin-4-one

Tropane alkaloids are extracted from various plants including *Datura stramonium, Datura innoxia, Hyoscyamus niger* and *Atropa belladonna* (Gadzikowska and Grynkiewicz, 2002). Tropane compounds contain a nitrogenous bicyclic ring system (N-substituted-4-azabicyclo [3.2.1] octanes). Many naturally derived tropanes are N-methyl substituted and examples of compounds containing this skeleton include: scopolamine, cocaine and hyoscyamine (Humphery & O'Hagan, 2001).

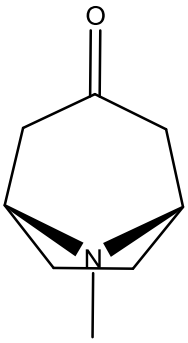
Piperidone comprises of a heterocyclic six-membered ring containing a nitrogen atom as well as a carbonyl group (Schilf, Jameson & Kuroki, 2011). Piperidone, is most commonly found as 1-methyl piperidin-4-one and its N- substituted derivatives are used in; pharmaceutical synthesis including drugs to aid the digestive system and to act as UV stabilizers (Schilf *et al*, 2011).



(26)

Figure 10- The chemical structure of piperidin-4-one (26)

The structures of tropane alkaloids and cocaine were firstly established in 1901 by Richard Martin Willstätter (Willstätter, 1901). The synthethic route for tropane alkaloids such as tropan-3-one was studied and modified by Robinson in 1917 to a three component one-pot double Mannich reaction (Robinson, 1917). As well as tropan-3-one, cocaine is another tropane alkaloid, which also comprises of the tropane core structure. Tropanones are commonly used as intermediates in the production of drugs such as atropine sulphate (Humphrey & O'Hagan, 2001). Tropan-3-one has a piperidone sub structure but it also contains an ethylene bridge as part of a 7-membered ring (Figure 11).



(27)

Figure 11- The chemical structure of tropan-3-one (27)

Various derivatives of tropan-3-one and piperidin-4-one have demonstrated inhibition of cell growth against J774 mouse macrophages (Hussain, 2012). It was intended to extend this study by synthesizing various novel substituted parent tropan-3-one compounds and then along with commercially available piperidin-4-one and N-methyl-piperidin-4-one produce a range of benzylidene derivatives using the aldol condensation with ring substituted benzaldehydes. The interaction is known to occur at positions alpha (α and a') to the carbonyl group on both piperidones and tropan-3-ones. The functional groups C3-C4-O or C5-C4-O on piperidones and C2-C3-O or C4-C3-O on tropan-3-ones exist as enolisable functionalities thus they can undergo keto-enol tautomerisim fairly easily (Schilf, Jameson & Kuroki, 2011). This allows coupling of ring substituted benzaldehydes to occur easily at these points with the formation of benzylidene structures. The piperidone and tropan-3-one libraries would be screened against various fungal species as well as a mammalian cancer cell line to examine their possible antifungal or anticancer properties.

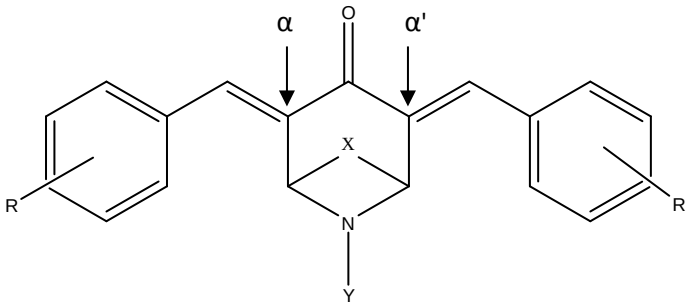


Figure 12- The chemical structure of the intended substituted dibenzylidenes of tropan-3-one and piperidones. Where X= (H, H) for the piperidone and methyl-piperidone derivatives and X= (–CH_2-CH_2-) for the tropan-3-one derivatives. For both tropan-3-ones and piperidones Y=H, whereas it is CH_3 for the methyl-piperidones. Various substituted aromatic aldehydes were reacted at points α and α'. R is various functional groups (e.g. fluorine, chlorine, bromine, methoxy or iodine) and they will be identical for each benzene ring.

Both sets of compounds contain a conjugated α,β unsaturated ketone. These are an important class of carbonyl compounds capable of undergoing the Michael reaction and reacting with nucleophiles such as S^-. The Michael addition involves nucelophilic addition to the β carbon of the α,β-unsaturated carbonyl compound (Otera, 2001). Such α,β-unsaturated compounds can undergo Michael reactions with cellular nucleophiles such as thiolate anions in proteins and cofactors such as glutathione (Pati *et al*, 2009).

Previous studies have revealed that compounds consisting of an α,β -unsaturated ketone undergo the Michael reaction with cellular thiolate anions (Pati *et al*, 2009). Unlike amino and hydroxyl groups thiols are not found in nucleic acids. For the mitochondrial permeability transition pore complex to open it requires alkylation or cross-linking of a critical thiol within the protein permeability transition pore complex (Pati *et al*, 2009). Since some of the compounds used in this investigation contain an α,β-unsaturated ketone they should react easily with thiols via the Michael addition, therefore the mitochondrial permeability transition pore complex cannot open efficiently. This activates the caspase system which leads to the destruction of the mitochondria consequently leading to the initiation of cell death by apoptosis. As a consequence of the interaction of the α,β-unsaturated carbonyl with cellular thiols, compounds containing α,β-unsaturated carbonyls are known to be cytotoxic (Das *et al*, 2007). The interaction is known to occur at the olefinic carbon atoms (shown by C^A and C^B in figure 13) (Das *et al*, 2008, Mutus *et al*, 1989). The nucleophilic attack would be expected to occur at these points due to the electron-withdrawing influences of the carbonyl group (Manavathu, Dimmock, Vashishtha & Chandrasekar, 1999).

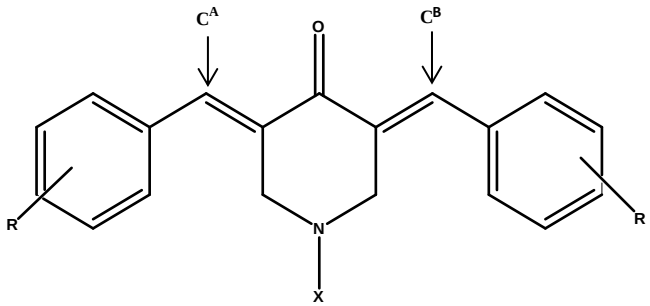


Figure 13- The structure of dibenzylidene methyl piperidin-4-ones (where X= CH_3) and dibenzylidene-1-piperidin-4-ones (where X= H). The various substituents on the benzene rings are represented by R. Olefinic carbon atoms are shown by C^A and C^B

Glutathione is a cellular antioxidant which prevents damage to important cellular components (Kaplowitz, 1981). Glutathione and the side chain of the amino acid cysteine consist of thiolate anions which act as nucleophiles and attack the olefinic carbons on the α,β-unsaturated structure (Das *et al*, 2008, Mutus *et al*, 1989).

Other reports suggest that compounds having an α,β-unsaturated structure block the plasma membrane P-type proton-translocating ATPase (H^+-ATPase) belonging to the P-type ATPase superfamily (Manavathu *et al*, 1999) of various fungal species (Manavathu *et al*, 1999) or block the mitochondrial phosphate transporter (Griffiths, Partis, Sharp & Beechey, 1981)**,** both of which are essential for cell growth (Graham & Stevens, 1999).

Alternatively compounds containing the dieneone structure can have an adverse effect on fungal cell growth by interfering with certain mitochondrial functions such as ROS generation or oxygen consumption by the electron transport chain (Helal *et al*, 2013). An increase in the level of reactive oxygen species (ROS) can lead to an overload of antioxidant defences culminating in cell death (Apel & Hirt, 2004). Oxidative phosphorylation is essential for cell growth and inhibition of this process may lead to an increase in the concentrations of ROS. This would affect the rate of oxygen consumption in the cell and also have a marked effect on the mitochondrial membrane potential (MMP) (Helal *et al*, 2013).

1.16 Biological Activities of Tropan-3-one and Piperidin-4-one

In the past various N-substituted tropanone and piperidone derivatives, as well as similar compounds, all of which contain an α,β-unsaturated carbonyl have exhibited properties of biological and pharmacological interest. They have shown analgesic, anti-depressant, antibacterial, cytotoxic, antihypertensive or fungicidal activities (Das *et al,* 2007).

1.16.1 Tropan-3-ones and Piperdin-4-ones as Anticancer Agents

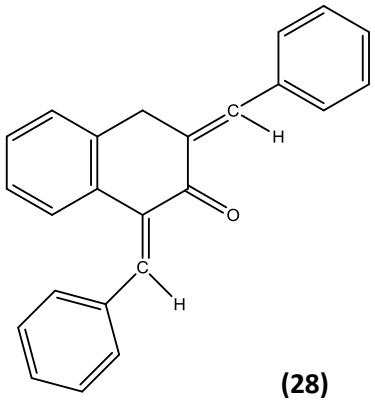
There have been various studies examining the cytotoxic effects of compounds containing the α,β-unsaturated carbonyl on a variety of cell types including cancer cell lines.

Das and co-workers synthesized and analysed the cytotoxic properties of novel 1-[4-(2-alkylaminoethoxy) phenylcarbonyl]-3,5-bis(arylidene)-4-piperidones and associated compounds (Das *et al,* 2007). The compounds were compared with the established

anticancer drugs melphalan and 5-fluorouracil in different bioassays. It was observed that most of the novel compounds exhibited greater potencies than the reference drugs. They found that the compounds had considerably lower IC_{50} values when compared to established anti-cancer drugs, with some compounds showing IC_{50} values nine-fold lower than that of melphalan (a standard anticancer drug often used as an internal standard). The results also indicated that many of the compounds had greater cytotoxic effects on malignant tissue than on normal cells (Das *et al*, 2007). This suggests the series of compounds synthesized are more effective and better cytotoxic agents compared to the recognised anti-cancer drug melphalan.

Pati *et al* (2009) focused their studies on a series of 4-piperidone and tropanone dieneones which are very similar to the compounds proposed for in this investigation. Studies revealed that these groups of compounds also possessed greater cytotoxicity against cancer cell lines when compared to melphalan, a chemotherapeutic drug used in the treatment of multiple myeloma and ovarian cancer (Auyang, 2005). In general the 4-piperidone series of compounds exhibited greater cytotoxic effects when compared to the tropanone series. It was also shown that both sets of compounds, particularly the 4-piperidone series were responsible for increased swelling of rat liver mitochondria. Pati and co-workers found that some of the piperidone dieneones gave greater toxicity towards various malignant cell lines rather than non-cancerous cells (Pati *et al*, 2009).

Dimmock e*t al,* (2002) synthesized and tested a group of 1,3-diarylidene-2-tetralones on human and murine cancer cell lines. The studies revealed that this group of compounds possessed greater cytotoxicity to the cancer cell lines when compared to melphalan. Some compounds exhibited significant toxicity towards leukemic and colon cancer cell lines.



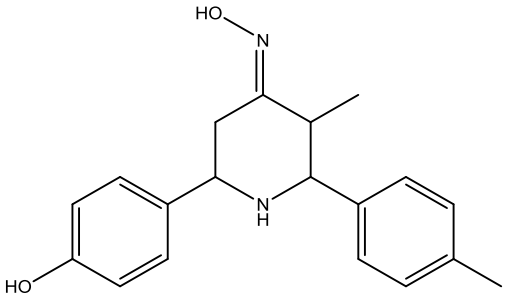
(28)

Figure 14- The chemical structure of 1-((E)-benzylidene)-3-benzylidene-3,4-dihydronaphthalen-2(1H)-one (28) which is from a family of 1,3-diarylidene-2-tetralones

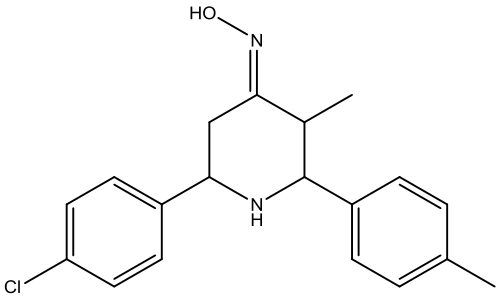
The studies mentioned previously along with numerous other investigations, have all used compounds having structural similarities to the compounds synthesized and tested in this research. These studies have reported that similar compounds to those used in this research, particularly those containing an α,β unsaturated carbonyl show encouraging cytotoxic activities against various cancer cell lines. Therefore it was proposed to synthesize further compounds within the tropanone/piperidone class with varying substituents both on the nitrogen atom and the benzylidene rings.

1.16.2 Antimicrobial Properties of Tropan-3-one and Piperidin-4-one Compounds

Derivatives of 2,6-diaryl-3-methyl-4- piperidone were screened for their antifungal activities by Natesh *et al, (*2003). Of the seven different compounds tested only 2-(4-methylphenyl)-3-methyl-6-(4-hydroxyphenyl)-piperidin-4-oxime **(29)** and 2-(4-methoxyphenyl)-3-methyl-6-(4-chlorophenyl)-piperidin-4-oxime **(30)** exhibited significant antifungal activity against *Aspergillus niger* as they produced the same level of inhibition as the internal standard (ketoconazole). Antifungal activity against *Candida albicans* was observed only with 2-(4-dimethyl-aminophenyl)-3-methyl-6-(4-chlorophenyl)-piperidin-4-oxime which produced the same level of inhibition as ketoconazole (Natesh *et al*, 2003).



(29)

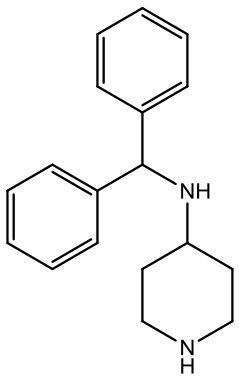


(30)

Figure 15- The chemical structures of 2-(4-methylphenyl)-3-methyl-6-(4-hydroxyphenyl)-piperidin-4-oxime (29) and 2-(4-methylphenyl)-3-methyl-6-(4-chlorophenyl)-piperidin-4-oxime (30)

Senthil and co-workers synthesized and examined the compound 3-methyl, 2-phenyl (3,4-dihydroxy), 6 phenyl, piperidin-4-one for antimicrobial activitiy. Results obtained showed that the compound had no antibacterial activity. However it did exhibit antifungal activity against the yeasts *Candida albicans* and *Candida tropicalis,* and the filamentous fungus *Aspergillus niger* (Senthil, Kumar & Raju, 2011).

Mittal and colleagues (2011) synthesized various substituted N-benzhydrylpiperidin-4-amino derivatives by reductive amination of benzhydrylamine and N-substituted 4-piperidones. These compounds were found to have considerable antibacterial and antifungal activities when compared to ciprofloxacin (standard antibacterial drug) or fluconazole (standard antifungal drug) respectively. All compounds synthesized exhibited better antibacterial activity rather than antifungal activity. Additionally the compounds were more active against Gram positive bacteria rather than Gram negative bacteria (Mittal, Sarode, Shingare, Vidyasagar & Shrivastava, 2011).



(31)

Figure 16- The chemical structure of N-benzhydrylpiperidin-4-amine (31)

Tropan-3-ones can be considered as 2,6 ethylene bridged piperidones **(27).** Tropan-3-one derivatives have also shown promising antifungal and antibacterial activities (Pati *et al,* 2009). Thus these compounds need further study to possibly develop more potent antifungal agents.

1.17 Synthetic Approaches for Tropan-3-one and Piperidin-4-one

Historically tropane derivatives have been synthesized utilizing a variety of routes including: Michael addition of an amine across an unsaturated cycloheptadieneone or oxalyl addition to pyrroles or nitrile oxide cycloaddition (Tufariello, 1979). However the Robinson reaction (Robinson, 1917) as modified in 1954 by Stoll and co-workers (Stoll, Jucker & Lindenmann, 1954) is the most suitable and commonly used route for the synthesis of the tropane skeleton. The Robinson one pot synthesis or a variation of it is still routinely used for the synthesis of tropane and its derivatives (Thuo, 2008).

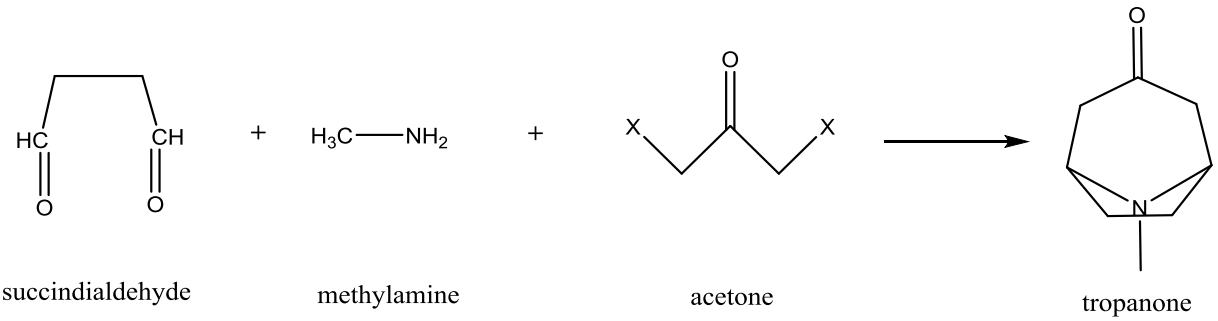


Figure 17- Synthesis of tropanone using Robinson's one-pot synthesis where X= H, CO_2H or CO_2Et (Thuo, 2008)

The Mannich reaction is a very useful organic reaction for the synthesis of carbon-carbon bonds (Azizi, Torkiyan & Saidi, 2006). A number of Mannich reactions have been developed using different catalysts to produce various Mannich products of high enantioselectivities (Humphary & O'Hagan, 2007). The methodology depends on the two component system (also known as indirect). The indirect variants are characterized using electrophiles such as preformed imines, and stable nucleophiles such as enolates, enamines or enol esters. The most favoured method is a direct, one-pot three component strategy as it allows a wide range of structural variations (Azizi *et al*, 2006).

In the synthesis of tropanone Robinson's uses of succindialdehyde, methyl amine, and acetone (or the 1,3-dicarboxylic acid derivative of acetone) are used in a one-pot, three component reaction (Robinson, 1917). The Robinson synthesis is, in effect, a double Mannich reaction (Azizi *et al*, 2006).

Piperidones are usually synthesized by combining an alkyl-1,3-acetonedicarboxylate with benzaldehyde and an amine. This reaction is known as the Petrenko-Kritschenko piperidone synthesis (Li, 2005). However a wide variety of N-substituted piperidones are readily available.

1.17.1 Synthesis of Various Dibenzylidenes using the Aldol Condensation

The aldol condensation involves a nucleophilic addition between an enolate and the carbonyl group of an aldehyde or ketone to produce β-hydroxyketone (aldol) (Noyce & Pryor, 1955). The reaction usually involves the use of a strong base such as NaOH in the presence of a solvent system usually comprised of an alcohol and water. Alternatively the reaction can occur using a strong acid such as HCl in acetic acid. (Leonova, Makarov, Klemenkova & Odinets, 2010). Other methods include the use of Lewis acids such as lithium perchlorate ($LiClO_4$/$TMSNEt_2$) or magnesium bromide ($MgBr_2{\cdot}Et_2O$/MeOH/Et_3N) or alternatively Lewis bases such as Bu_3P or Ph_3P (Leonova *et al*, 2010).

Enolate ions act as nucleophiles with the carbonyl group acting as the electrophile. Dehydration of the aldols by the E1 mechanism subsequently gives rise to conjugated enones by the loss of a water molecule and the formation of an α,β-unsaturated ketone. Again this step is usually base catalysed however an acid may be used instead (Climent, Corma, Fornés, Guil-Lopez & Iborra, 2002).

In the base catalysed reaction the hydroxide ion generates the reactive enolate by removing the weakly acidic α-hydrogen. The carbonyl carbon on the aldehyde is attacked by the nucelophilic enolate carbon atom which produces a tetrahedral intermediate alkoxide. (Climent *et al*, 2002). This is followed by either protonation to yield the β-hydroxy ketone, which may undergo dehydration to form the enone, or an acid-base reaction where the alkoxide deprotonates a water molecule creating the aldol product as well as a hydroxide ion (refer to step 1, figure 15). The dehydration of the aldol product initially involves the

formation of an enolate as a result of the base removing the acidic α-hydrogen (Luo & Falconer, 1999). The C=C double bond is formed from the electrons associated with the negative charge of the enolate thus resulting in the loss of a water molecule and formation of a conjugated α,β enone (refer to Step 2, Figure 18).

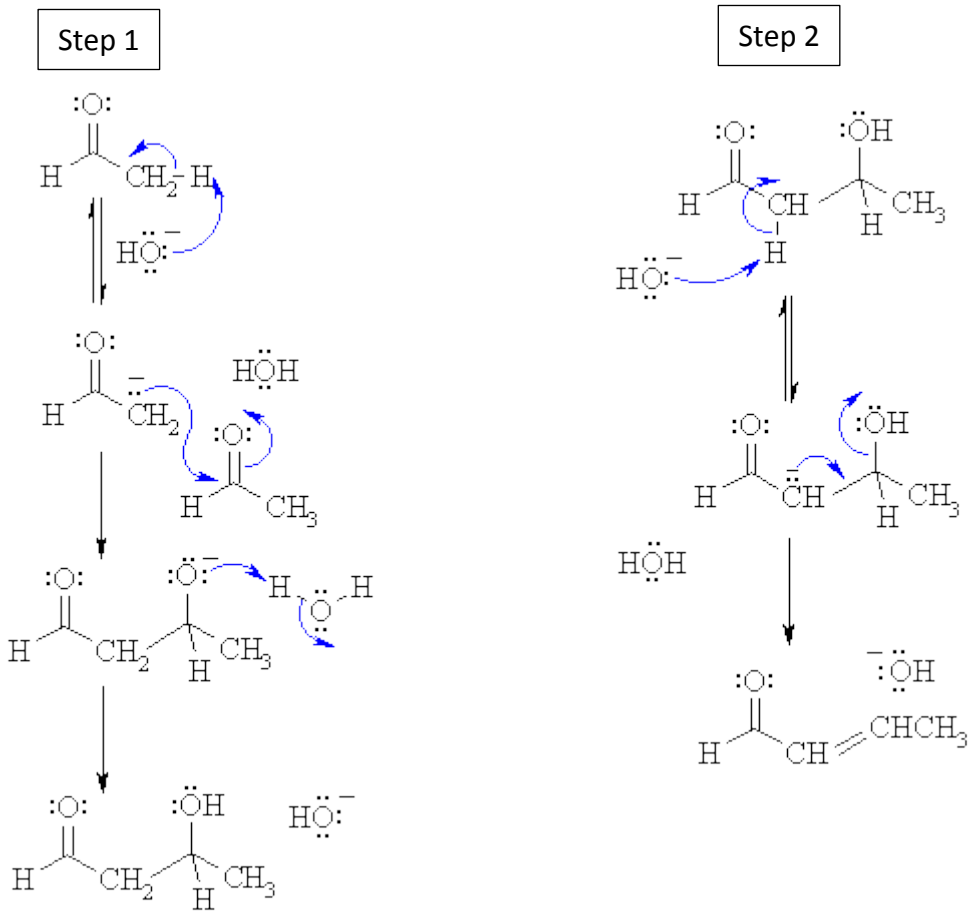


Figure 18- Base catalysed aldol condensation of acetaldehyde (Eagleson, 1993).

In the present study N-substituted piperidones were reacted with various aromatic aldehydes using the aldol condensation. Several synthetic approaches have been deployed for this reaction, which vary in the nature of the solvent and catalytic base used. In most cases the methods used are variations on the condensation of ketones with aldehydes in a basic aqueous alcoholic medium (McElvain & Rorig, 1948).

1.18 Aims and Objectives were to:

- synthesize and characterise various parent tropan-3-one compounds;

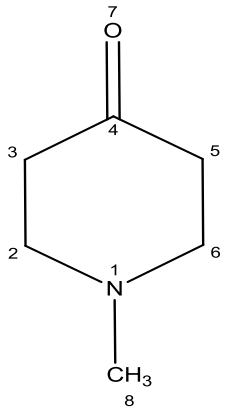
- synthesize and characterise novel benzene ring substituted 3,5-benzylidene derivatives of tropan-3-one and piperidin-4-one;
- characterise compounds using ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectroscopy, mass spectrometry, Fourier transform infrared (FT-IR), melting/boiling point determination and, where required, ultraviolet visible (UV) spectroscopy and retention times from high pressure liquid chromatography (HPLC);
- examine the antifungal activity of the synthesized compounds along with some synthesized 1,2-benzothiazine derivatives by analysing the inhibition of growth of yeasts (*S. cerevisiae and C. albicans*), and filamentous fungi (e.g. *A. niger*) by measuring relevant IC_{10}, IC_{50}, and IC_{90} or MIC values
- test the cytotoxic activity of selected compounds by measuring the corresponding IC_{10}, IC_{50}, and IC_{90} values against a tumour cell line (A549).

Chapter 2

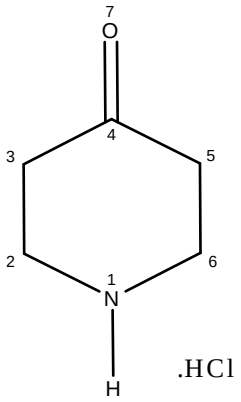
Synthesis and Characterization of Novel Nitrogen Containing 6 and 7-membered Heterocyclic Compounds

2.1.1 Materials:

The coupling of readily available N-methyl-4-piperidone **(32)** or 4-piperidone hydrochloride monohydrate **(33)** with various ring substituted benzaldehydes (Figure 20) at the C-3 and C-5 positions was performed using the aldol condensation to synthesize 3,5-dibenzylidene-piperidin-4-ones along with the N-methyl derivatives. The reaction was utilized using either KOH or 10% NaOH in either aqueous ethanol or aqueous methanol.



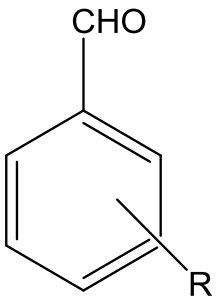
N-methyl-4-piperidone **(32)**



4-piperidone hydrochloride monohydrate **(33)**

Figure 19- Parent piperidin-4-one molecules

Various ring substituted benzaldehydes were coupled with the parent piperidin-4-one compounds to produce a variety of aromatic ring substituted benzylidenes.



(34)

Figure 20- Structure of R-benzaldehyde (34,) where R= various substituents

Table 1- Various R-substituted benzaldehydes used to produce benzylidenes

Compound number	R
34a	**H**
34b	**2-Cl**
34c	**3-Cl**
34d	**4-Cl**
34e	**2-Br**
34f	**3-Br**
34g	**4-Br**
34h	**2-F**
34i	**3-F**
34j	**4-F**
34k	**2-OCH_3**
34l	**4-OCH_3**
34m	**2-I**
34n	**3-I**

In addition to benzylidene derivatives of piperidones, attempts were made to synthesize various N-substituted tropane analogues. Initially the synthesis of various parent tropanone compounds was attempted prior to coupling with various substituted benzaldehydes. The synthesis of: 4-((1R,5S)-3-oxo-8-azabicyclo[3.2.1]octan-8-yl) benzoic acid **(35)**, 2-((1R,5S)-3-oxo-8-azabicyclo[3.2.1]octan-8-yl) benzoic acid **(36)**, 3-((1R,5S)-3-oxo-8-azabicycle[3.2.1] octan-8-yl) benzoic acid **(37)** and ethyl-4-((1R,5S)-3-oxo-8-azabicyclo[3.2.1]octan-8-yl) benzoate **(38)** was attempted using the modified Robinson synthesis (Stoll *et al*, 1954).

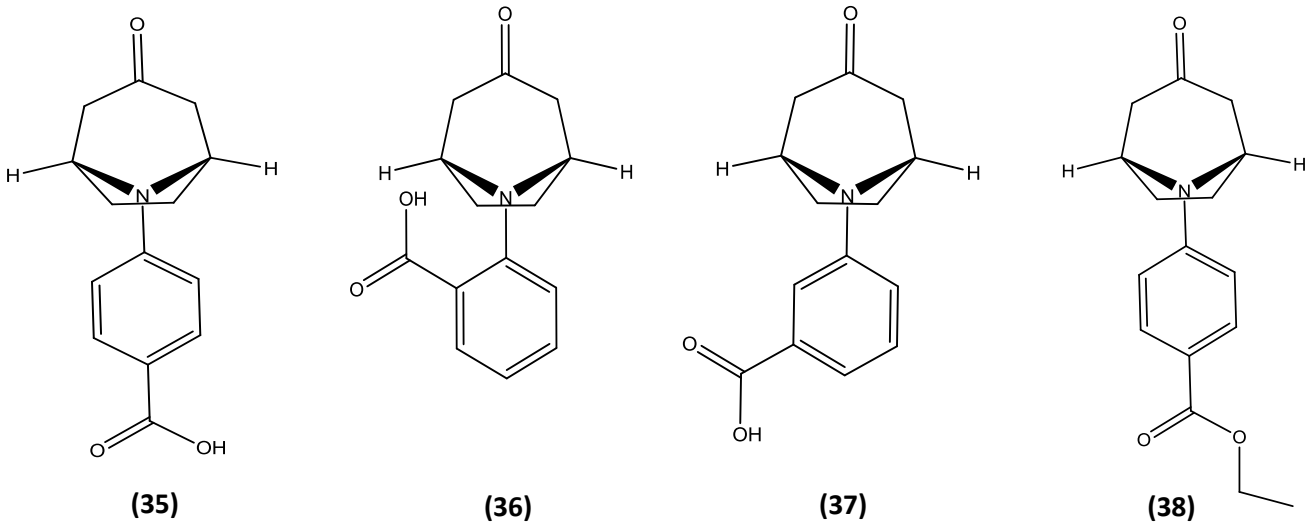


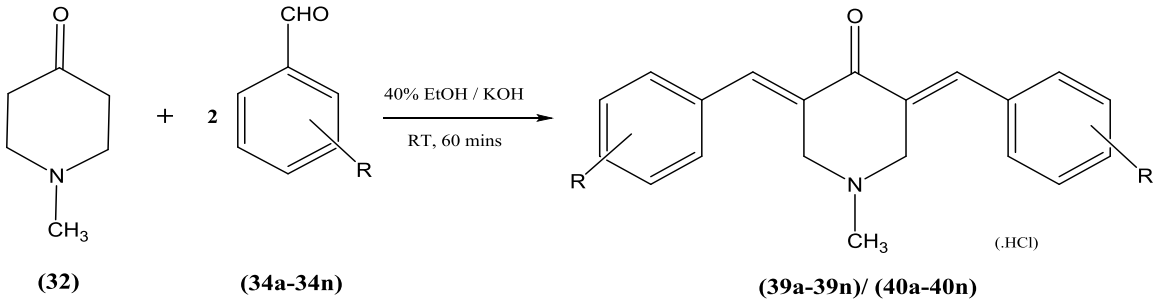
Figure 21- Proposed parent tropan-3-one molecules

All chemicals used were purchased from Sigma Aldrich and used in the form they were delivered without any further purification. The physical properties and chemical structure of all the compounds produced were characterized using various analytical techniques. Melting points were determined using an electrothermal capillary melting point apparatus (Gallenkamp). Thin layer chromatography (TLC) was performed using pre-coated silica gel plates (Macherey-Nagel: DC-fertigfolien polygram sil G/UV254). The compounds were dissolved in an appropriate solvent and a relevant solvent system was used to develop the plates. Visualisation was performed using a mineralight UV lamp. Fourier transform-infrared spectra were obtained using either a FT/IR-Varian Scimitar or Perkin-Elmer Frontier spectrometer with a golden plate attachment and peaks are reported as cm^{-1}. Liquid chromatography-mass spectrometry (LC-MS) was performed using a VG Micromass V15 spectrometer operating at 70 eV (Varian: 210 LC pumps × 2, 1200L Quadrapole MS/MS, 410 autosampler) using a gradient solvent system of A: water/0.1% formic acid and B: acetonitrile/ 0.1% formic acid. LC-MS was used to determine the mass of the compounds as well as molecular fragments. Nuclear magnetic resonance (NMR- ^{1}H, ^{13}C and DEPT) was performed on all compounds using a JEOL ECA600 spectrometer at a frequency of 600 MHz. Chemical shifts were measured in ppm (δ) relative to the solvent used to dissolve the compounds (DMSO-d_6, methanol-d_4 or $CDCl_3$). The following abbreviations are used to describe the signal multiplicities: s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet) and br (broad). The following abbreviations are used to identify the different proton

environments present in the compounds: aliph (aliphatic protons), arom (aromatic protons) and benzyldiene (benzyldiene protons). Signal peaks obtained for the solvents utilised to run the ^{1}H and ^{13}C NMR are not quoted.

2.1.2 Methodology for the synthesis of dibenzylidenes

2.1.2.1 General procedure for the preparation of aromatic ring substituted 3,5-dibenzylidene-1-methyl piperidin-4-ones and their hydrochloride salts by coupling of substituted benzaldehydes with N-methyl-4-piperidone

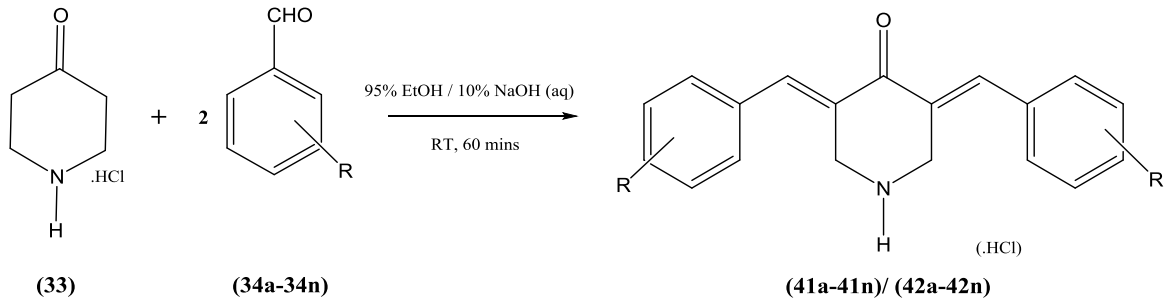


(32) **(34a-34n)** **(39a-39n)/ (40a-40n)**

Figure 22- General protocol for the coupling of substituted benzaldehydes with N-methyl-4-piperidone

N-methyl-4-piperidone (1.13ml, 0.01 moles) and 28ml of 40% ethanol were added to a 100ml round bottomed flask, followed by addition of the relevant benzaldehyde (34a-34n, 0.02 moles (refer to Table 1, Page 37) and powdered potassium hydroxide (1.2g, 0.02 moles). The reaction was stirred at room temperature for 60 minutes during which a yellow solid precipitated. TLC (Silica gel, Macherey-Nagel) was used to monitor the reaction. When the reactants had been consumed the crude solid was filtered, washed with iced methanol (3ml) and dried under nitrogen in a vacuum pistol (50°C, 24 hours). Crude material was recrystallized from either methanol or 95% ethanol (25-30ml, 50°C) over 24 hours. The resultant yellow crystals were filtered, washed with iced methanol (3ml) and dried in a vacuum pistol (50°C). Hydrochloride salts were prepared from crude material by either recrystallization from 30-35ml of 5% hydrochloric acid (50°C) or by dissolving the crude material in 95% ethanol (25-35ml, 50°C) followed by drop-wise addition of 1M hydrochloric acid (7-9ml). The solutions were left on ice for 24 hours to produce the hydrochloride salts. The fine yellow needles produced were filtered, washed with iced methanol (3ml) and dried in a vacuum pistol (50°C). The recrystallized solids, hydrochloride salts, crude materials and the starting materials were compared using silica TLC.

2.1.2.2 General procedure for the preparation of aromatic ring substituted 3,5-dibenzylidene-1-piperidin-4-ones and their hydrochloride salts by coupling of substituted benzaldehydes with 4-piperidone hydrochloride monohydrate



(33) **(34a-34n)** **(41a-41n)/ (42a-42n)**

Figure 23- General protocol for the coupling of substituted benzaldehydes with 4-piperidone hydrochloride monohydrate

4-Piperidone hydrochloride monohydrate (1.54g, 0.01 moles) was dissolved in distilled water (4ml) in a 100ml round bottomed flask followed by the addition of ethanol (95%, 20ml) and the relevant benzaldehyde (34a-34n, 0.02 moles (refer to Table 1, Page 37). The reaction was stirred and sodium hydroxide solution (10%, 20ml, 0.5 moles) was added in small aliquots. After stirring the mixture for 60 minutes a yellow solid had precipitated. The reaction was followed to completion using silica gel TLC (Macherey-Nagel) following which the precipitate was filtered, washed with iced methanol (3ml) and the crude materials dried under nitrogen in a vacuum pistol (50°C, 24 hours). Crude material was recrystallized from 95% ethanol (14-20ml, 50°C) over 24 hours. The resultant yellow crystals were filtered, washed with iced methanol (3ml) and dried in a vacuum pistol (50°C). Further samples from the crude products were recrystallized from dichloromethane (7-20ml), followed by the addition of 95% ethanol (40°C, 3-8ml) and drop-wise addition of concentrated hydrochloric acid (1-1.2ml). The solutions were immersed in ice for 24 hours after which fine cream/yellow needles of the hydrochloride salts were produced. The recrystallized salts were filtered, washed with iced methanol (3ml) and dried in a vacuum pistol (50°C). Silica TLC was used to compare the recrystallized solids, hydrochloride salts, crude material and starting reactants.

Table 2- Various substituent groups present on the benzene rings of the benzylidene products and their associated compound numbers

R	3,5-dibenzylidene-1-methylpiperidin-4-ones	3,5-dibenzylidene-1-methylpiperidin-4-one HCl salts	3,5-dibenzylidene-1-piperidin-4-ones	3,5-dibenzylidene-1-piperidin-4-one HCl salts
H	39a	40a	41a	42a
2-Cl	39b	40b	41b	42b
3-Cl	39c	40c	41c	42c
4-Cl	39d	40d	41d	42d
2-Br	39e	40e	41e	42e
3-Br	39f	40f	41f	42f
4-Br	39g	40g	41g	42g
2-F	39h	40h	41h	42h
3-F	39i	40i	41i	42i
4-F	39j	40j	41j	42j
2-OCH_3	39k	40k	41k	42k
4-OCH_3	39l	40l	41l	42l
2-I	39m	40m	41m	42m
3-I	39n	40n	41n	42n

2.1.3 Methodology for the synthesis of Tropan-3-ones

2.1.3.1 Procedure for the Synthesis of Benzocaine

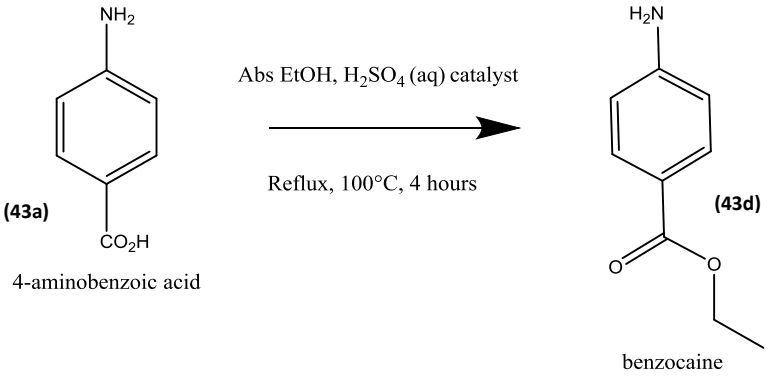


Figure 24- Reaction scheme for the synthesis of benzocaine (43d)

To a 1000ml round bottomed flask containing absolute ethanol (200ml) 4-Aminobenzoic acid (27.4g, 0.2 moles) was added followed by sulphuric acid (1ml) as a catalyst. The reaction was stirred at 100°C for 4 hours under reflux. Silica gel TLC (Macherey-Nagel) was used to follow the reaction to completion. Upon cooling a white solid precipitated, which was filtered and washed with iced methanol (5ml). The crude product was dissolved in absolute ethanol (39ml, 50°C) followed by the addition of distilled water (11ml). The solution was immersed in ice from which fine white crystals formed. The crystals were filtered, washed with iced methanol (5ml) and dried using a vacuum pistol (60°C). Recrystallized and crude materials were compared using silica gel TLC.

2.1.3.2 Attempted Two-step Synthesis of (2, 3 and 4)-((1R,5S)-3-oxo-8-azabicyclo[3.2.1] octan-8-yl) benzoic acid and ethyl-4-((1R,5S)-3-oxo-8-azabicyclo[3.2.1]octan-8-yl) benzoate

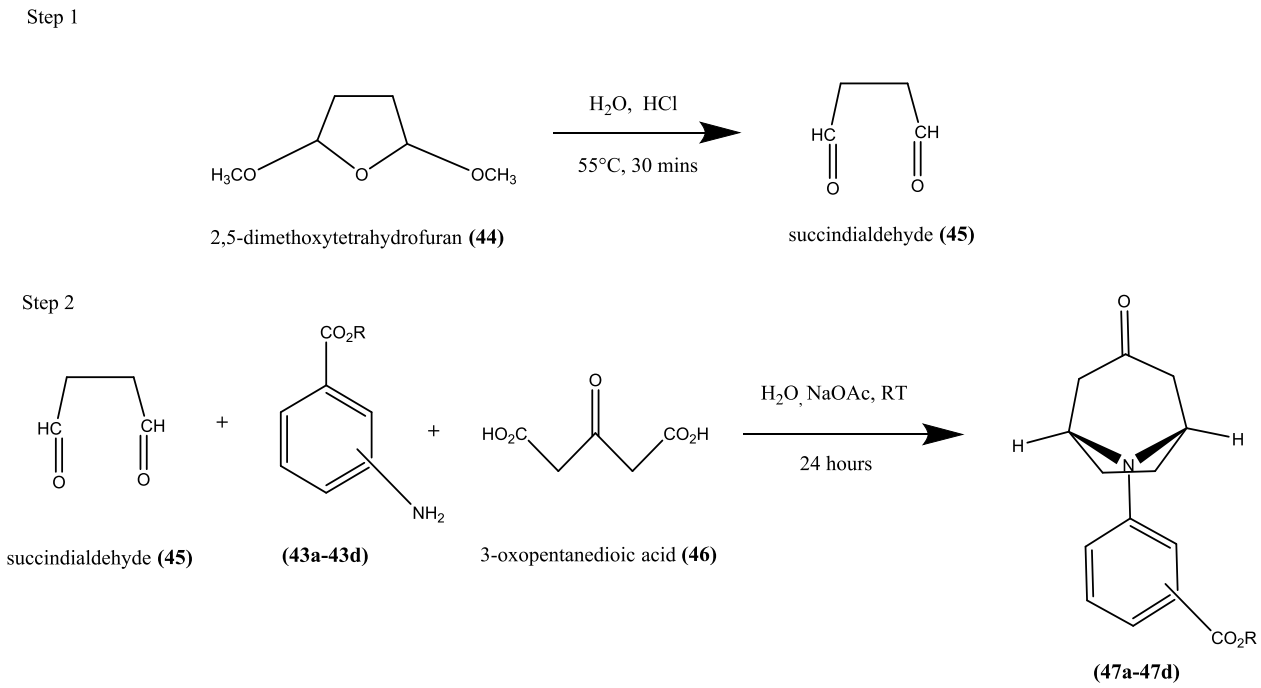


Figure 25- Generalised reaction scheme for the synthesis of (2, 3 or 4)-((1R,5S)-3-oxo-8 azabicyclo [3.2.1] octan-8-yl) benzoic acids and ethyl-4-((1R,5S)-3-oxo-8-azabicyclo[3.2.1]octan-8-yl) benzoate, where 43a: 4-NH_2/R=H, 43b: 2-NH_2/R=H, 43c: 3-NH_2/R=H, 43d: 4-NH_2/R=Et and 47a: 4-CO_2/R=H, 47b: 2-CO_2/R=H, 47c: 3-CO_2/R=H, 47d: 4-CO_2/R=Et.

2.1.3.3 Procedure for The Two-step Synthesis of 4-((1R,5S)-3-oxo-8-azabicyclo[3.2.1]octan-8-yl) benzoic acid (47a)

Succindialdehyde **(45)** was prepared in a 500ml round bottomed flask by mixing 2,5-dimethoxytetrahydrofuran (16.2 ml, 0.120 moles) with deionised water (250ml) along with a few drops of concentrated hydrochloric acid. The mixture was heated for 30 minutes at 55°C (step 1) (Stoll *et al*, 1954).

In a 1 litre conical flask sodium acetate (50g, 0.61 moles) was dissolved in deionised water (300ml) followed by 4-aminobenzoic acid (16.8g, 0.123 moles, **(43a)**), the succindialdehyde mixture from step 1 and acetone 1,3-dicarboxylic acid (3-oxopentanedioic acid) (18g, 0.123 moles). The reaction was stirred overnight at room temperature (22°C) during which a pale yellow solution with a cream/white precipitate formed. The consumption of all reactants was followed by silica TLC (Macherey-Nagel). The solid was filtered, washed with iced methanol (5ml) and dried in a vacuum pistol (70°C). Crude material (0.5g) was recrystallized

from 50ml methanol (68°C) and, following hot filtration, the solution was immersed in ice and left overnight. The recrystallized material was filtered, washed with iced methanol (5ml) and dried in a vacuum pistol (70°C). Remaining crude material (7.45g) was recrystallized from 150ml methanol (68°C).

Post filtration of the solid from the reaction mixture, further solid material formed. The solvent was removed by rotary evaporation (30°C) leaving a solid residue. The solid residue was dissolved in methanol (68°C, 200ml) and the resulting solution was concentrated to half the volume using rotary evaporation (40°C). The solution was then immersed in ice overnight and the resultant crystals were filtered, washed with iced methanol (5ml) and dried (70°C) to constancy. Both recrystallized materials were analysed by silica TLC developed with DCM.

2.1.3.4 Procedure for The Attempted Two-step Synthesis of 2-((1R,5S)-3-oxo-8 azabicyclo [3.2.1]octan-8-yl) benzoic acid (47b)

The synthesis of 2-((1R,5S)-3-oxo-8-azabicyclo[3.2.1]octan-8-yl) benzoic acid was carried out in a similar fashion as described in **2.1.3.3**, replacing 4-aminobenzoic acid **(43a)** with 2-aminobenzoic acid (5.6g, 0.04 moles, **(43b)**). A smaller scale was used in this experiment (step 1: 2,5-dimethoxytetrahydrofuran (5.5ml, 0.04 moles), water (17ml), step 2: sodium acetate- 12.51g, 0.15 moles, water (75ml) acetone 1,3-dicarboxylic acid- 6.0g, 0.04 moles). This reaction was performed under a nitrogen atmosphere to remove any oxygen which could have possibly oxidised the succindialdehyde to succinic acid which might reduce the yield. The water used in the experiment was purged with nitrogen gas to remove any oxygen which could potentially oxidise the aldehyde. The resulting solution was yellow/orange and it was left to stir overnight at room temperature (21°C). A lumpy solid was produced which was dissolved in dichloromethane (150ml). The resulting mixture consisted of two layers. The mixture was washed with brine (50ml) following which the brine was removed and the organic layer was dried using anhydrous magnesium sulphate (2g). The magnesium sulphate was removed by filtration and the solvent removed by rotary evaporation (40°C). Diethyl ether (20ml) was added to the solid residue and the residue was scraped from the side of the flask and separated from the diethyl ether under vacuum. Analysis by silica TLC (Macherey-Nagel) showed that the solid was predominantly 2-aminobenzoic acid and no product had been formed. It is possible that most the product

was lost when washing with brine. As the product formed is possibly zwitterionic, it could have been extracted with the brine.

2.1.3.5 Procedure for The Attempted Two-step Synthesis of 3-((1R,5S)-3-oxo-8 azabicyclo [3.2.1]octan-8-yl) benzoic acid (47c)

The synthesis of 3-((1R,5S)-3-oxo-8-azabicyclo[3.2.1]octan-8-yl) benzoic acid was conducted in a similar fashion as described in **2.1.3.4**, replacing 2-aminobenzoic acid **(43b)** with 3-aminobenzoic acid **(43c)**. The resulting solution was orange/brown and it was stirred overnight at room temperature (21°C). A brown solid was produced which was slightly soluble in methanol, the mixture was left initially on ice and then at room temperature overnight, after which no change was observed.

The experiment was repeated under minimum light conditions to reduce photosensitivity because it is possible that the reaction is sensitive to light, hence the dark colour of the solid obtained from the initial experiment. The crude material obtained was dissolved in 75ml methanol (68°C) and 10ml diethyl ether (20°C) was added. Recrystallization did not occur when the solution was left overnight on ice therefore it was immersed in ice for a further 48 hours, however no solid was formed so it was discarded. Analysis by silica TLC (Macherey-Nagel) showed that the crude material consisted of just starting reactants and no new products had formed.

2.1.3.6 Procedure for The Attempted Two-step Synthesis of ethyl-4-((1R,5S)-3-oxo-8-aza bicycle [3.2.1] octan-8-yl) benzoate (47d)

The synthesis of ethyl-4-((1R,5S)-3-oxo-8-azabicyclo[3.2.1]octan-8-yl) benzoate **(47d)** was conducted in a similar manner as described in **2.1.3.3**, replacing 4-aminobenzoic acid **(43a)** with benzocaine **(43d)**. The quantities of the reactants were as follows; 2,5-dimethoxytetrahydrofuran (8.5ml, 0.06 moles), water (20ml), benzocaine (10.7g, 0.06 moles), sodium acetate (17g, 0.2 moles), water (60ml), acetone 1,3-dicarboxylic acid (9.4g, 0.06 moles). A cream/white precipitate was formed during the course of the reaction. The consumption of the reactants was followed by silica TLC (Macherey-Nagel). Crude material obtained from the reaction was filtered, washed with iced methanol (5ml) and dried using a vacuum pistol (60°C). The crude product was recrystallized from methanol (75ml, 68°C) and incubated overnight in an ice bath. The resultant fine white crystals were filtered, washed

with ice cold methanol (5ml) and dried under vacuum (60°C). Purity of the recrystallized material was checked by comparing it to the crude material and starting reactants using silica gel TLC.

Chapter 2.2: Results- Compound Characterisation

Based on the results obtained by reacting benzaldehyde with piperidone derivatives, various substituted aldehydes were reacted to yield a series of substituted (E) α and α' benzylidene piperidone derivatives. Each derivative was characterized using a variety of physiochemical techniques, the details and values of which are listed below for each compound.

2.2.1 Spectral data for the synthesized aromatic ring substituted 3,5-dibenzylidene-1-methylpiperidin-4-ones and their hydrochloride salts (39a-39n and 40a-40n)

3,5-Dibenzylidene-1-methylpiperidin-4-one (39a)

Chemical Formula**:** $C_{20}H_{19}NO$. M.Wt **:** 289.15. Recrystallized from methanol. Yield: 41%. R_f : 0.4 (Silica TLC; DCM:EtAc, 19:1). M.Pt: 115-118°C (115-150°C, Mcelvain & Rorig, 1948).
IR (golden gate/cm^{-1}): 1679 (C=O), 1587 (C=C), 1621 (aromatic)
^{1}H-NMR (DMSO-d_6): δ 2.49 (br s, 3H, aliph H), δ 4.60 (s, 4H, aliph H), δ 7.49 -7.56 (m, 10H, arom H) and δ 7.87 (br s, 2H, benzyldiene H);
^{13}C-NMR (DMSO-d_6): δ 54.41, 129.51, 130.69, 131.30, 134.11. LC-MS (m/z): 290.4 ($M+H^+$).

3,5-Bis(2-chlorobenzylidene)-1-methylpiperidin-4-one (39b)

Chemical Formula**:** $C_{20}H_{17}Cl_2NO$. M.Wt **:** 358.26. Recrystallized from 95% ethanol. Yield: 74%. R_f : 0.56 (Silica TLC; DCM:EtAc, 2:1). M.Pt: 154-157°C (152-154°C, Mcelvain & Rorig, 1948).
IR (golden gate/cm^{-1}): 1679 (C=O), 1446 (C=C), 1547 (aromatic)
^{1}H-NMR (DMSO-d_6): δ 2.26 (br s, 3H, aliph H), δ 3.60 (s, 4H, aliph H), δ 7.41 -7.44 (m, 8H, arom H) and δ 7.75 (br s, 2H, benzyldiene H);
^{13}C-NMR (DMSO-d_6): δ 56.74, 127.90, 130.30, 131.42, 132.23, 135.46, 186.42 (C=O). LC-MS (m/z): 359.30 ($M+H^+$).

3,5-Bis(3-chlorobenzylidene)-1-methylpiperidin-4-one (39c)

Chemical Formula**:** $C_{20}H_{17}Cl_2NO$. M.Wt **:** 358.26. Recrystallized from 95% ethanol. Yield: 89%. R_f : 0.54 (Silica TLC; DCM:EtAc, 19:1). M.Pt: 156-158°C.
IR (golden gate/cm^{-1}): 1679 (C=O), 1446 (C=C), 1547 (aromatic)
^{1}H-NMR (DMSO-d_6): δ 2.37 (br s, 3H, aliph H), δ 3.72 (s, 4H, aliph H), δ 7.45 -7.49 (m, 8H, arom H) and δ 7.56 (br s, 2H, benzyldiene H);
^{13}C-NMR (DMSO-d_6): δ 56.6, 129.4, 129.5, 130.4, 131.1, 133.8, 186.9 (C=O). LC-MS (m/z): 359.30 ($M+H^+$).

3,5-Bis(4-chlorobenzylidene)-1-methylpiperidin-4-one (39d)

Chemical Formula**:** $C_{20}H_{17}Cl_2NO$. M.Wt **:** 358.26. Recrystallized from 95% ethanol. Yield: 82%. R_f : 0.57 (Silica TLC; DCM:EtAc, 19:1). M.Pt: 156-159°C.

IR (golden gate/cm^{-1}): 1679 (C=O), 1446 (C=C), 1547 (aromatic).
^{1}H-NMR (DMSO-d_6): δ 2.49 (br s, 3H, aliph H), δ 3.71 (s, 4H, aliph H), δ 7.48 -7.52 (m, 8H, arom H) and δ 7.57 (br s, 2H, benzyldiene H);
^{13}C-NMR (DMSO-d_6) δ 56.78, 129.33, 132.74, 133.92, 134.77, 186.88 (C=O). LC-MS (m/z): 359.30 ($M+H^+$).

3,5-Bis(2-bromobenzylidene)-1-methylpiperidin-4-one (39e)

Chemical Formula**:** $C_{20}H_{17}Br_2NO$. M.Wt **:** 447.16. Recrystallized from 95% ethanol. Yield: 76%. R_f : 0.72 (Silica TLC; DCM:EtAc, 19:1). M.Pt: 223-228°C.
IR (golden gate/cm^{-1}): 1672 (C=O), 1586 (C=C), 1618 (aromatic).
^{1}H-NMR (methanol-d_4): δ 2.41 (br s, 3H, aliph H), δ 3.8 (s, 4H, aliph H), δ 7.36 -7.43 (m, 8H, arom H) and δ 7.89 (br s, 2H, benzyldiene H);
^{13}C-NMR (methanol-d_4): δ 57.84, 128.68, 130.21, 130.47, 131.93, 132.66, 134.91, 135.34. LC-MS (m/z): 448.1 ($M+H^+$).

3,5-Bis(3-bromobenzylidene)-1-methylpiperidin-4-one (39f)

Chemical Formula**:** $C_{20}H_{17}Br_2NO$. M.Wt **:** 447.16. Recrystallized from 95% ethanol. Yield: 84%. R_f : 0.74 (Silica TLC; DCM:EtAc, 19:1). M.Pt: 225-230°C.
IR (golden gate/cm^{-1}): 1668 (C=O), 1586 (C=C), 1618 (aromatic).
^{1}H-NMR (methanol-d_4): δ 2.47 (br s, 3H, aliph H), δ 3.81 (s, 4H, aliph H), δ 7.37 -7.43 (m, 8H, arom H) and δ 7.71 (br s, 2H, benzyldiene H);
^{13}C-NMR (methanol-d_4): δ 56.11, 122.32, 128.68, 130.21, 131.93, 132.66, 133.98, 134.90, 137.12. LC-MS (m/z): 448.1 ($M+H^+$).

3,5-Bis(4-bromobenzylidene)-1-methylpiperidin-4-one (39g)

Chemical Formula**:** $C_{20}H_{17}Br_2NO$. M.Wt **:** 447.16. Recrystallized from 95% ethanol. Yield: 75%. R_f : 0.61 (Silica TLC; DCM:EtAc, 19:1). M.Pt: 229-232°C.
IR (golden gate/cm^{-1}): 1668 (C=O), 1587 (C=C), 1619 (aromatic).
^{1}H-NMR (methanol-d_4): δ 2.47 (br s, 3H, aliph H), δ 3.81 (s, 4H, aliph H), δ 7.37 -7.43 (m, 8H, arom H) and δ 7.71 (br s, 2H, benzyldiene H);
^{13}C-NMR (methanol-d_4): δ 56.28, 128.67, 131.86, 132.66, 134.91. LC-MS (m/z): 448.1 ($M+H^+$).

3,5-Bis(2-fluorobenzylidene)-1-methylpiperidin-4-one (39h)

Chemical Formula**:** $C_{20}H_{17}F_2NO$. M.Wt **:** 325.35. Recrystallized from 95% ethanol. Yield: 82%. R_f : 0.67 (Silica TLC; DCM:EtAc, 19:1). M.Pt: 138-140°C.
IR (golden gate/cm^{-1}): 1677 (C=O), 1594 (C=C), 1622 (aromatic).
^{1}H-NMR (methanol-d_4): δ 2.41 (br s, 3H, aliph H), δ 3.73 (s, 4H, aliph H), δ 7.42 -7.45 (m, 8H, arom H) and δ 7.88 (br s, 2H, benzyldiene H);

^{13}C-NMR (methanol-d_4): δ 124.14, 129.23, 130.66, 131.31, 131.37, 134.34, 161.88, 185.70 (C=O). LC-MS (m/z): 326.4 ($M+H^+$).

3,5-Bis(3-fluorobenzylidene)-1-methylpiperidin-4-one (39i)

Chemical Formula**:** $C_{20}H_{17}F_2NO$. M.Wt **:** 325.35. Recrystallized from 95% ethanol. Yield: 71%.
R_f : 0.58 (Silica TLC; DCM:EtAc, 19:1). M.Pt: 135-138°C.
IR (golden gate/cm^{-1}): 1678 (C=O), 1590 (C=C), 1618 (aromatic).
^{1}H-NMR ($CDCl_3$): δ 2.47 (br s, 3H, aliph H), δ 3.72 (s, 4H, aliph H), δ 7.37 -7.38 (m, 8H, arom H) and δ 7.72 (br s, 2H, benzyldiene H);
^{13}C-NMR ($CDCl_3$): δ 46.03, 57.03, 116.03, 116.17, 116.76, 116.90, 126.36, 130.20, 130.26, 134.02, 135.23, 161.91, 186.66 (C=O). LC-MS (m/z): 326.3 ($M+H^+$).

3,5-Bis(4-fluorobenzylidene)-1-methylpiperidin-4-one (39j)

Chemical Formula**:** $C_{20}H_{17}F_2NO$. M.Wt **:** 325.35. Recrystallized from 95% ethanol. Yield: 58%.
R_f : 0.55 (Silica TLC; DCM:EtAc, 19:1). M.Pt: 134-137°C.
IR (golden gate/cm^{-1}): 1674 (C=O), 1586 (C=C), 1608 (aromatic).
^{1}H-NMR ($CDCl_3$): δ 2.47 (br s, 3H, aliph H), δ 3.72 (s, 4H, aliph H), δ 7.37 -7.38 (m, 8H, arom H) and δ 7.72 (br s, 2H, benzyldiene H);
^{13}C-NMR ($CDCl_3$): δ 46.03, 57.04, 116.04, 116.17, 116.76, 116.86, 126.37, 130.21, 130.26, 134.03, 135.23, 161.91, 186.67 (C=O). LC-MS (m/z): 326.3 ($M+H^+$).

3,5-Bis(2-methoxybenzylidene)-1-methylpiperidin-4-one (39k)

Chemical Formula**:** $C_{22}H_{23}NO_3$. M.Wt **:** 349.42. Recrystallized from 95% ethanol. Yield: 72%.
R_f : 0.61 (Silica TLC; DCM:EtAc, 19:1). M.Pt: 176-178°C.
IR (golden gate/cm^{-1}): 1670 (C=O), 1509 (C=C), 1596 (aromatic).
^{1}H-NMR (DMSO-d_6): δ 2.49 (br s, 3H, aliph H), δ 3.79 (s, 4H, aliph H), δ 7.44 -7.46 (m, 8H, arom H) and δ 7.54 (br s, 2H, benzyldiene H);
^{13}C-NMR (DMSO-d_6): δ 55.85, 114.86, 127.79, 132.21, 132.94, 134.78, 160.59. LC-MS (m/z): 350.4 ($M+H^+$).

3,5-Bis(4-methoxybenzylidene)-1-methylpiperidin-4-one (39l)

Chemical Formula**:** $C_{22}H_{23}NO_3$. M.Wt **:** 349.42. Recrystallized from 95% ethanol. Yield: 88%.
R_f : 0.57 (Silica TLC; DCM:EtAc, 10:1). M.Pt: 180-181°C.
IR (golden gate/cm^{-1}): 1670 (C=O), 1504 (C=C), 1596 (aromatic).
^{1}H-NMR (DMSO-d_6): δ 2.49 (br s, 3H, aliph H), δ 3.79 (s, 4H, aliph H), δ 7.44 -7.46 (m, 8H, arom H) and δ 7.54 (br s, 2H, benzyldiene H);
^{1}H-NMR (DMSO-d_6): δ 55.85, 114.86, 127.79, 132.21, 132.94, 134.78, 160.59, 186.68 (C=O). LC-MS (m/z): 350.4 ($M+H^+$).

3,5-Bis(2-iodobenzylidene)-1-methylpiperidin-4-one (39m)

Chemical Formula**:** $C_{20}H_{17}I_2NO$. M.Wt **:** 541.16. Recrystallized from 95% ethanol. Yield: 66%. R_f : 0.36 (Silica TLC; DCM:EtAc, 10:1). M.Pt: 231-234°C.
IR (golden gate/cm^{-1}): 1660 (C=O), 1493 (C=C), 1577 (aromatic).
^{1}H-NMR ($CDCl_3$): δ 2.33 (br s, 3H, aliph H), δ 3.53 (s, 4H, aliph H), δ 7.36 -7.38 (m, 8H, arom H) and δ 7.79 (br s, 2H, benzyldiene H);
^{13}C-NMR ($CDCl_3$): δ 45.49, 56.44, 100.48, 127.85, 129.58, 130.02, 133.55, 139.59, 140.41. LC-MS (m/z): 541.8 ($M+H^+$).

3,5-Bis(3-iodobenzylidene)-1-methylpiperidin-4-one (39n)

Chemical Formula**:** $C_{20}H_{17}I_2NO$. M.Wt **:** 541.16. Recrystallized from 95% ethanol. Yield: 45%. R_f : 0.36 (Silica TLC; DCM:EtAc, 10:1). M.Pt: 232-234°C.
IR (golden gate/cm^{-1}): 1675 (C=O), 1505 (C=C), 1612 (aromatic).
^{1}H-NMR ($CDCl_3$): δ 2.46 (br s, 3H, aliph H), δ 3.75 (s, 4H, aliph H), δ 7.31 -7.34 (m, 8H, arom H) and δ 7.70 (br s, 2H, benzyldiene H);
^{13}C-NMR ($CDCl_3$): δ 94.41, 129.24, 130.21, 137.98, 138.87. LC-MS (m/z): 541.8 ($M+H^+$).

The molecular ion obtained for the hydrochloride salts of the 1-methylpiperidin-4-one dieneones **(40a-40n)** are consistent to that of their free base form **(39a-39n).**

3,5-Dibenzylidene-1-methylpiperidin-4-one Hydrochloride (40a)

Chemical Formula**:** $C_{20}H_{20}ClNO$. M.Wt **:** 325.12. Recrystallized from 5% hydrochloric acid. Yield: 53%. R_f : 0.37 (Silica TLC; DCM: EtAc, 19:1). M.Pt: 243-245°C (243.5-245.5°C, Mcelvain & Rorig, 1948).
IR (golden gate/cm^{-1}): 1679 (C=O), 1587 (C=C), 1621 (aromatic)
^{1}H-NMR (DMSO-d_6): δ 2.49 (br s, 3H, aliph H), δ 4.64 (s, 4H, aliph H), δ 7.5 -7.54 (m, 10H, arom H) and δ 7.87 (br s, 2H, benzyldiene H);
^{13}C-NMR (DMSO-d_6) δ, 47.86, 129.39, 130.27, 131.01, 134.65, 137.94, 184.71 (C=O). LC-MS (m/z): 290.4 ($M+H^+$).

3,5-Bis(2-chlorobenzylidene)-1-methylpiperidin-4-one Hydrochloride (40b)

Chemical Formula**:** $C_{20}H_{18}Cl_3NO$. M.Wt **:** 394.72. Recrystallized from 5% hydrochloric acid. Yield: 96%. R_f : 0.66 (Silica TLC; DCM:EtAc, 2:1). M.Pt: 228-229°C (227-229°C, Mcelvain & Rorig, 1948).
IR (golden gate/cm^{-1}): 1684 (C=O), 1444 (C=C), 1583 (aromatic)
^{1}H-NMR (DMSO-d_6): δ 2.49 (br s, 3H, aliph H), δ 3.32 (s, 4H, aliph H), δ 7.49 -7.53 (m, 8H, arom H) and δ 8.00 (br s, 2H, benzyldiene H);
^{13}C-NMR (DMSO-d_6) δ 54.71, 128.13, 130.51, 131.46, 132.11. 132.21, 134.74. LC-MS (m/z): 359.30 ($M+H^+$).

3,5-Bis(3-chlorobenzylidene)-1-methylpiperidin-4-one Hydrochloride (40c)

Chemical Formula**:** $C_{20}H_{17}Cl_3NO$. M.Wt **:** 394.72. Recrystallized from 5% hydrochloric acid. Yield: 93%. R_f : 0.66 (Silica TLC; DCM:EtAc, 2:1). M.Pt: 228-231°C.
IR (golden gate/cm^{-1}): 1684 (C=O), 1444 (C=C).
^{1}H-NMR (DMSO-d_6): δ 2.05 (br s, 3H, aliph H), δ 2.90 (s, 4H, aliph H), δ 7.53 -7.55 (m, 8H, arom H) and δ 7.63 (br s, 2H, benzyldiene H);
^{13}C-NMR (DMSO-d_6) δ 56.84, 129.64, 130.60, 131.25, 134.17. LC-MS (m/z): 359.30 ($M+H^+$).

3,5-Bis(4-chlorobenzylidene)-1-methylpiperidin-4-one Hydrochloride (40d)

Chemical Formula**:** $C_{20}H_{17}Cl_3NO$. M.Wt **:** 394.72. Recrystallized from 5% hydrochloric acid. Yield: 96%. R_f : 0.46 (Silica TLC; DCM:EtAc, 19:1). M.Pt: 227-228°C.
IR (golden gate/cm^{-1}): 1688 (C=O), 1446 (C=C), 1547 (aromatic)
^{1}H-NMR (DMSO-d_6): δ 2.49 (br s, 3H, aliph H), δ 2.96 (s, 4H, aliph H), δ 7.56 -7.59 (m, 8H, arom H) and δ 7.87 (br s, 2H, benzyldiene H);
^{13}C-NMR (DMSO-d_6) δ 56.61, 129.54, 133.04, 135.52, 138.96. LC-MS (m/z): 360.30 ($M+H^+$).

3,5-Bis(2-bromobenzylidene)-1-methylpiperidin-4-one Hydrochloride (40e)

Chemical Formula**:** $C_{20}H_{18}Br_2ClNO$. M.Wt **:** 483.62. Recrystallized from 5% hydrochloric acid. Yield: 82%. R_f : 0.81 (Silica TLC; DCM:EtAc, 19:1). M.Pt: 275-280°C.
IR (golden gate/cm^{-1}): 1668 (C=O), 1591 (C=C), 1618 (aromatic).
^{1}H-NMR (methanol-d_4): δ 3.29 (br s, 3H, aliph H), δ 3.31 (s, 4H, aliph H), δ 7.44 -7.45 (m, 8H, arom H) and δ 8.02 (br s, 2H, benzyldiene H);
^{13}C-NMR (methanol-d_4) δ 57.75, 122.65, 127.28, 128.78, 130.52, 132.79, 133.01, 135.76, 140.04, 180.59 (C=O). LC-MS (m/z): 448.1 ($M+H^+$).

3,5-Bis(3-bromobenzylidene)-1-methylpiperidin-4-one Hydrochloride (40f)

Chemical Formula**:** $C_{20}H_{18}Br_2ClNO$. M.Wt **:** 483.62. Recrystallized from 95% ethanol/1M hydrochloric acid. Yield: 98%. R_f : 0.70 (Silica TLC; DCM:EtAc, 19:1). M.Pt: 301-308°C.
IR (golden gate/cm^{-1}): 1672 (C=O), 1595 (C=C), 1622 (aromatic).
^{1}H-NMR (methanol-d_4): δ 3.29 (br s, 3H, aliph H), δ 3.31 (s, 4H, aliph H), δ 7.44 -7.45 (m, 8H, arom H) and δ 8.02 (br s, 2H, benzyldiene H);
^{13}C-NMR (methanol-d_4) δ 57.24, 122.64, 128.78, 130.52, 132.79, 132.99, 135.79, 139.93. LC-MS (m/z): 448.1 ($M+H^+$).

3,5-Bis(4-bromobenzylidene)-1-methylpiperidin-4-one Hydrochloride (40g)

Chemical Formula**:** $C_{20}H_{18}Br_2ClNO$. M.Wt **:** 483.62. Recrystallized from 95% ethanol/1M hydrochloric acid. Yield: 84%. R_f : 0.74 (Silica TLC; DCM:EtAc, 19:1). M.Pt: 291-297°C.
IR (golden gate/cm^{-1}): 1666 (C=O), 1583 (C=C), 1615 (aromatic).

^{1}H-NMR (methanol-d_4): δ 3.07 (br s, 3H, aliph H), δ 4.67 (s, 4H, aliph H), δ 7.40 -7.42 (m, 8H, arom H) and δ 8.02 (br s, 2H, benzyldiene H);
^{13}C-NMR (methanol-d_4) δ 57.77, 124.61, 126.59, 132.00, 132.06, 132.60, 140.39, 180.69 (C=O). LC-MS (m/z): 448.1 ($M+H^+$).

3,5-Bis(2-fluorobenzylidene)-1-methylpiperidin-4-one Hydrochloride (40h)

Chemical Formula**:** $C_{20}H_{18}F_2ClNO$. M.Wt **:** 361.81. Recrystallized from 95% ethanol/1M hydrochloric acid. Yield: 98%. R_f : 0.68 (Silica TLC; DCM:EtAc, 19:1). M.Pt: 210-212°C.
IR (golden gate/cm^{-1}): 1677 (C=O), 1595 (C=C), 1623 (aromatic).
^{1}H-NMR (methanol-d_4): δ 3.05 (br s, 3H, aliph H), δ 4.61 (s, 4H, aliph H), δ 7.53 -7.55 (m, 8H, arom H) and δ 8.14 (br s, 2H, benzyldiene H);
^{13}C-NMR (methanol-d_4) δ 56.94, 124.61, 127.83, 130.92, 132.57, 132.63, 134.65, 160.07, 161.73, 180.26 (C=O). LC-MS (m/z): 326.4 ($M+H^+$).

3,5-Bis(3-fluorobenzylidene)-1-methylpiperidin-4-one Hydrochloride (40i)

Chemical Formula**:** $C_{20}H_{18}F_2ClNO$. M.Wt **:** 361.81. Recrystallized from 95% ethanol/1M hydrochloric acid. Yield: 92%. R_f : 0.72 (Silica TLC; DCM:EtAc, 19:1). M.Pt: 210-212°C.
IR (golden gate/cm^{-1}): 1687 (C=O), 1581 (C=C), 1608 (aromatic).
^{1}H-NMR (DMSO-d_6): δ 2.49 (br s, 3H, aliph H), δ 4.63 (s, 4H, aliph H), δ 7.54 -7.57 (m, 8H, arom H) and δ 7.86 (br s, 2H, benzyldiene H);
^{13}C-NMR (DMSO-d_6): δ 57.22, 127.51, 131.52, 136.24, 161.87, 163.49. LC-MS (m/z): 326.2 ($M+H^+$).

3,5-Bis(4-fluorobenzylidene)-1-methylpiperidin-4-one Hydrochloride (40j)

Chemical Formula**:** $C_{20}H_{18}F_2ClNO$. M.Wt **:** 361.81. Recrystallized from 95% ethanol/1M hydrochloric acid. Yield: 95%. R_f : 0.63 (Silica TLC; DCM:EtAc, 19:1). M.Pt: 211-213°C.
IR (golden gate/cm^{-1}): 1678 (C=O), 1588 (C=C), 1613 (aromatic).
^{1}H-NMR (DMSO-d_6): δ 2.49 (br s, 3H, aliph H), δ 4.14 (s, 4H, aliph H), δ 7.55 -7.57 (m, 8H, arom H) and δ 7.85 (br s, 2H, benzyldiene H);
^{13}C-NMR (DMSO-d_6): δ 57.46, 127.49, 131.46, 131.52, 161.87, 163.49. LC-MS (m/z): 326.2 ($M+H^+$).

3,5-Bis(2-methoxybenzylidene)-1-methylpiperidin-4-one Hydrochloride (40k)

Chemical Formula**:** $C_{22}H_{24}ClNO_3$. M.Wt **:** 385.88. Recrystallized from 95% ethanol/1M hydrochloric acid. Yield: 76%. R_f : 0.69 (Silica TLC; DCM:EtAc, 19:1). M.Pt: 216-218°C.
IR (golden gate/cm^{-1}): 1670 (C=O), 1508 (C=C), 1596 (aromatic).
^{1}H-NMR (DMSO-d_6): δ 2.98 (br s, 3H, aliph H), δ 3.34 (s, 4H, aliph H), δ 7.51 -7.53 (m, 8H, arom H) and δ 7.84 (br s, 2H, benzyldiene H);

^{13}C-NMR (DMSO-d_6): δ 55.64, 115.09, 126.67, 133.56, 139.74, 161.45. LC-MS (m/z): 350.4 ($M+H^+$).

3,5-Bis(4-methoxybenzylidene)-1-methylpiperidin-4-one Hydrochloride (40l)

Chemical Formula**:** $C_{22}H_{24}ClNO_3$. M.Wt **:** 385.88. Recrystallized from 95% ethanol/1M hydrochloric acid. Yield: 87%. R_f : 0.62 (Silica TLC; DCM:EtAc, 10:1). M.Pt: 235-237°C.
IR (golden gate/cm^{-1}): 1658 (C=O), 1501 (C=C), 1574 (aromatic).
^{1}H-NMR (DMSO-d_6): δ 2.97 (br s, 3H, aliph H), δ 3.36 (s, 4H, aliph H), δ 7.51 -7.53 (m, 8H, arom H) and δ 7.83 (br s, 2H, benzyldiene H);
^{13}C-NMR (DMSO-d_6): δ 56.12, 115.09, 125.33, 126.68, 133.56, 139.69, 161.42, 181.75 (C=O). LC-MS (m/z): 350.4 ($M+H^+$).

3,5-Bis(2-iodobenzylidene)-1-methylpiperidin-4-one Hydrochloride (40m)

Chemical Formula**:** $C_{22}H_{24}ClI_2NO_3$. M.Wt **:** 577.62. Recrystallized from 95% ethanol/1M hydrochloric acid. Yield: 73%. R_f : 0.37 (Silica TLC; DCM:EtAc, 10:1). M.Pt: 272-274°C.
IR (golden gate/cm^{-1}): 1675 (C=O), 1504 (C=C), 1612 (aromatic).
^{1}H-NMR ($CDCl_3$): δ 2.69 (br s, 3H, aliph H), δ 4.08 (s, 4H, aliph H), δ 7.43 -7.46 (m, 8H, arom H) and δ 7.97 (br s, 2H, benzyldiene H);
^{13}C-NMR ($CDCl_3$): δ 52.46, 128.74, 128.74, 129.28, 131.61, 139.89. LC-MS (m/z): 541.8 ($M+H^+$).

3,5-Bis(3-iodobenzylidene)-1-methylpiperidin-4-one Hydrochloride (40n)

Chemical Formula**:** $C_{22}H_{24}ClI_2NO_3$. M.Wt **:** 577.62. Recrystallized from 95% ethanol/1M hydrochloric acid. Yield: 62%. R_f : 0.44 (Silica TLC; DCM:EtAc, 10:1). M.Pt: 271-273°C.
IR (golden gate/cm^{-1}): 1662 (C=O), 1550 (C=C), 1600 (aromatic).
^{1}H-NMR ($CDCl_3$): δ 2.49 (br s, 3H, aliph H), δ 4.08 (s, 4H, aliph H), δ 7.28 -7.46 (m, 8H, arom H) and δ 7.69 (br s, 2H, benzyldiene H);
^{13}C-NMR ($CDCl_3$): δ 53.41, 128.74, 129.28, 131.61, 139.89. LC-MS (m/z): 541.8 ($M+H^+$).

The spectroscopic and spectrometric data for all synthesized methyl piperidone dibenzylidene derivatives was consistent to their assigned structures.

2.2.2 Spectral data for the synthesized aromatic ring substituted 3,5-dibenzylidene-1-piperidin-4-ones and their hydrochloride salts (41a-41n and 42a-42n)

3,5-Dibenzylidenepiperidin-4-one (41a)

Chemical Formula**:** $C_{19}H_{17}O$. M.Wt **:** 275.34. 95% ethanol. Yield: 68%. R_f : 0.38 (Silica TLC; DCM:EtAc, 10:1). M.Pt: 112-115°C.
IR (golden gate/cm^{-1}): 1674 (C=O), 1447 (C=C), 1590 (aromatic), 3249 (N-H).
^{1}H-NMR ($CDCl_3$): δ 1.61 (br s, H, aliph H), δ 4.15 (s, 4H, aliph H), δ 7.37 -7.40 (m, 10H, arom H) and δ 7.79 (br s, 2H, benzyldiene H);
^{13}C-NMR ($CDCl_3$): δ 48.23, 128.65, 129.16, 130.58, 135.09, 136.08, 188.12 (C=O). LC-MS (m/z): 276.3 ($M+H^+$).

3,5-Bis(2-chlorobenzylidene)piperidin-4-one (41b)

Chemical Formula**:** $C_{19}H_{15}Cl_2NO$. M.Wt **:** 344.23. Recrystallized from 95% ethanol. Yield: 58%. R_f : 0.40 (Silica TLC; DCM:EtAc, 10:1). M.Pt: 148-150°C.
IR (golden gate/cm^{-1}): 1669 (C=O), 1396 (C=C), 1590 (aromatic), 3235 (N-H).
^{1}H-NMR ($CDCl_3$): δ 1.57 (br s, H, aliph H), δ 3.98 (s, 4H, aliph H), δ 7.43 -7.44 (m, 8H, arom H) and δ 7.94 (br s, 2H, benzyldiene H);
^{13}C-NMR ($CDCl_3$): δ 48.07, 126.50, 130.03, 130.18, 130.73, 133.78, 187.69 (C=O). LC-MS (m/z): 345.2 ($M+H^+$).

3,5-Bis(3-chlorobenzylidene)piperidin-4-one (41c)

Chemical Formula**:** $C_{19}H_{15}Cl_2NO$. M.Wt **:** 344.23. Recrystallized from 95% ethanol. Yield: 33%. R_f : 0.28 (Silica TLC; DCM:EtAc, 10:1). M.Pt: 153-156°C.
IR (golden gate/cm^{-1}): 1669 (C=O), 1400 (C=C), 1590 (aromatic), 3235 (N-H).
^{1}H-NMR ($CDCl_3$): δ 1.65 (br s, H, aliph H), δ 4.12 (s, 4H, aliph H), δ 7.32 -7.35 (m, 8H, arom H) and δ 7.70 (br s, 2H, benzyldiene H);
^{13}C-NMR ($CDCl_3$): δ 48.09, 128.62, 129.17, 129.91, 130.15, 134.65, 187.56 (C=O). LC-MS (m/z): 345.2 ($M+H^+$).

3,5-Bis(4-chlorobenzylidene)piperidin-4-one (41d)

Chemical Formula**:** $C_{19}H_{15}Cl_2NO$. M.Wt **:** 344.23. Recrystallized from 95% ethanol. Yield: 63%. R_f : 0.51 (Silica TLC; DCM:EtAc, 10:1). M.Pt: 151-153°C.
IR (golden gate/cm^{-1}): 1674 (C=O), 1447 (C=C), 1590 (aromatic), 3245 (N-H).
^{1}H-NMR ($CDCl_3$): δ 1.59 (br s, H, aliph H), δ 3.95 (s, 4H, aliph H), δ 7.16 -7.19 (m, 8H, arom H) and δ 7.84 (br s, 2H, benzyldiene H);
^{13}C-NMR ($CDCl_3$): δ 47.98, 127.12, 130.29, 130.72, 133.24, 135.41, 135.47, 187.62 (C=O). LC-MS (m/z): 345.2 ($M+H^+$).

3,5-Bis(2-bromobenzylidene)piperidin-4-one (41e)

Chemical Formula**:** $C_{19}H_{15}Br_2NO$. M.Wt **:** 433.14. Recrystallized from 95% ethanol. Yield: 58%. R_f : 0.57 (Silica TLC; DCM:EtAc, 10:1). M.Pt: 178-180°C.
IR (golden gate/cm^{-1}): 1669 (C=O), 1396 (C=C), 1586 (aromatic), 3240 (N-H).
^{1}H-NMR ($CDCl_3$): δ 1.59 (br s, H, aliph H), δ 4.10 (s, 4H, aliph H), δ 7.36 -7.38 (m, 8H, arom H) and δ 7.71 (br s, 2H, benzyldiene H);
^{13}C-NMR ($CDCl_3$): δ 48.13, 128.96, 131.76, 134.83, 135.30, 187.64 (C=O). LC-MS (m/z): 434.0 ($M+H^+$).

3,5-Bis(3-bromobenzylidene)piperidin-4-one (41f)

Chemical Formula**:** $C_{19}H_{15}Br_2NO$. M.Wt **:** 433.14. Recrystallized from 95% ethanol. Yield: 55%. R_f : 0.46 (Silica TLC; DCM:EtAc, 10:1). M.Pt: 179-182°C.
IR (golden gate/cm^{-1}): 1674 (C=O), 1396 (C=C), 1586 (aromatic), 3245 (N-H).
^{1}H-NMR ($CDCl_3$): δ 1.67 (br s, H, aliph H), δ 4.12 (s, 4H, aliph H), δ 7.23 -7.25 (m, 8H, arom H) and δ 7.55 (br s, 2H, benzyldiene H);
^{13}C-NMR ($CDCl_3$): δ 48.13, 123.64, 131.96, 131.97, 134.06, 134.89, 135.39, 187.63 (C=O). LC-MS (m/z): 434.0 ($M+H^+$).

3,5-Bis(4-bromobenzylidene)piperidin-4-one (41g)

Chemical Formula**:** $C_{19}H_{15}Br_2NO$. M.Wt **:** 433.14. Recrystallized from 95% ethanol. Yield: 41%. R_f : 0.53 (Silica TLC; DCM:EtAc, 10:1). M.Pt: 185-187°C.
IR (golden gate/cm^{-1}): 1674 (C=O), 1401 (C=C), 1586 (aromatic), 3245 (N-H).
^{1}H-NMR ($CDCl_3$): δ 1.57 (br s, H, aliph H), δ 4.12 (s, 4H, aliph H), δ 7.48 -7.50 (m, 8H, arom H) and δ 7.70 (br s, 2H, benzyldiene H);
^{13}C-NMR ($CDCl_3$): δ 48.07, 129.03, 130.16, 131.94, 132.08, 133.05, 134.56, 135.94, 187.50 (C=O). LC-MS (m/z): 434.1 ($M+H^+$).

3,5-Bis(2-fluorobenzylidene)piperidin-4-one (41h)

Chemical Formula**:** $C_{19}H_{15}F_2NO$. M.Wt **:** 311.33. Recrystallized from 95% ethanol. Yield: 47%. R_f : 0.53 (Silica TLC; DCM:EtAc, 10:1). M.Pt: 126-128°C.
IR (golden gate/cm^{-1}): 1674 (C=O), 1401 (C=C), 1590 (aromatic), 3245 (N-H).
^{1}H-NMR ($CDCl_3$): δ 1.59 (br s, H, aliph H), δ 4.08 (s, 4H, aliph H), δ 7.33 -7.35 (m, 8H, arom H) and δ 7.84 (br s, 2H, benzyldiene H);
^{13}C-NMR ($CDCl_3$): δ 48.27, 115.92, 116.07, 123.19, 123.28, 124.00, 128.80, 131.00, 136.85, 160.14, 161.81, 187.48 (C=O). LC-MS (m/z): 312.3 ($M+H^+$).

3,5-Bis(3-fluorobenzylidene)piperidin-4-one (41i)

Chemical Formula**:** $C_{19}H_{15}F_2NO$. M.Wt **:** 311.33. Recrystallized from 95% ethanol. Yield: 46%. R_f : 0.38 (Silica TLC; DCM:EtAc, 10:1). M.Pt: 127-128°C.
IR (golden gate/cm^{-1}): 1666 (C=O), 1400 (C=C), 1587 (aromatic), 3233 (N-H).

^{1}H-NMR ($CDCl_3$): δ 1.57 (br s, H, aliph H), δ 4.12 (s, 4H, aliph H), δ 7.36 -7.38 (m, 8H, arom H) and δ 7.72 (br s, 2H, benzyldiene H);
^{13}C-NMR ($CDCl_3$): δ 48.02, 115.98, 116.11, 116.79, 116.94, 126.27, 130.14, 134.74, 135.74, 137.22, 161.82, 163.46, 187.59 (C=O). LC-MS (m/z): 312.1 ($M+H^+$).

3,5-Bis(4-fluorobenzylidene)piperidin-4-one (41j)

Chemical Formula**:** $C_{19}H_{15}F_2NO$. M.Wt **:** 311.33. Recrystallized from 95% ethanol. Yield: 52%.
R_f : 0.64 (Silica TLC; DCM:EtAc, 19:1). M.Pt: 126-130°C (127-130°C, Leonova *et al*, 2010).
IR (golden gate/cm^{-1}): 1664 (C=O), 1425 (C=C), 1577 (aromatic), 3312 (N-H).
^{1}H-NMR ($CDCl_3$): δ 1.58 (br s, H, aliph H), δ 4.12 (s, 4H, aliph H), δ 7.36 -7.38 (m, 8H, arom H) and δ 7.72 (br s, 2H, benzyldiene H);
^{13}C-NMR ($CDCl_3$): δ 48.01, 115.98, 116.11, 116.79, 116.94, 126.27, 130.14, 134.74, 135.74, 161.82, 163.46, 187.59 (C=O). LC-MS (m/z): 312.1 ($M+H^+$).

3,5-Bis(2-methoxybenzylidene)piperidin-4-one (41k)

Chemical Formula**:** $C_{21}H_{21}NO_3$. M.Wt **:** 335.40. Recrystallized from 95% ethanol. Yield: 46%.
R_f : 0.57 (Silica TLC; DCM:EtAc, 19:1). M.Pt: 175-177°C.
IR (golden gate/cm^{-1}): 1670 (C=O), 1508 (C=C), 1587 (aromatic), 3247 (N-H).
^{1}H-NMR (DMSO-d_6): δ 2.49 (br s, H, aliph H), δ 3.79 (s, 4H, aliph H), δ 7.42 -7.44 (m, 8H, arom H) and δ 7.52 (br s, 2H, benzyldiene H);
^{13}C-NMR (DMSO-d_6): δ 46.92, 114.75, 128.10, 132.88, 133.91, 134.56, 160.43. LC-MS (m/z): 336.2 ($M+H^+$).

3,5-Bis(4-methoxybenzylidene)piperidin-4-one (41l)

Chemical Formula**:** $C_{21}H_{21}NO_3$. M.Wt **:** 335.40. Recrystallized from 95% ethanol. Yield: 45%.
R_f : 0.51 (Silica TLC; DCM:EtAc, 10:1). M.Pt: 179-180°C.
IR (golden gate/cm^{-1}): 1666 (C=O), 1508 (C=C), 1591 (aromatic), 3242 (N-H).
^{1}H-NMR (DMSO-d_6): δ 2.48 (br s, H, aliph H), δ 3.79 (s, 4H, aliph H), δ 7.42 -7.44 (m, 8H, arom H) and δ 7.52 (br s, 2H, benzyldiene H);
^{13}C-NMR (DMSO-d_6): δ 46.67, 114.75, 132.88, 133.92, 160.43. LC-MS (m/z): 336.2 ($M+H^+$).

3,5-Bis(2-iodobenzylidene)piperidin-4-one (41m)

Chemical Formula**:** $C_{19}H_{15}I_2NO$. M.Wt **:** 527.14. Recrystallized from 95% ethanol. Yield: 35%.
R_f : 0.29 (Silica TLC; DCM:EtAc, 10:1). M.Pt: 222-225°C.
IR (golden gate/cm^{-1}): 1670 (C=O), 1454 (C=C), 1612 (aromatic), 3308 (N-H).
^{1}H-NMR ($CDCl_3$): δ 1.56 (br s, H, aliph H), δ 3.93 (s, 4H, aliph H), δ 7.36 -7.38 (m, 8H, arom H) and δ 7.76 (br s, 2H, benzyldiene H);
^{13}C-NMR ($CDCl_3$): δ 47.86, 100.78, 127.95, 129.98, 130.21, 135.69, 138.89, 139.67. LC-MS (m/z): 528.8 ($M+H^+$).

3,5-Bis(3-iodobenzylidene)piperidin-4-one (41n)

Chemical Formula**:** $C_{19}H_{15}I_2NO$. M.Wt **:** 527.14. Recrystallized from 95% ethanol. Yield: 31%. R_f : 0.40 (Silica TLC; DCM:EtAc, 10:1). M.Pt: 223-225°C.
IR (golden gate/cm^{-1}): 1658 (C=O), 1550 (C=C), 1604 (aromatic), 3308 (N-H).
^{1}H-NMR ($CDCl_3$): δ 1.53 (br s, H, aliph H), δ 4.09 (s, 4H, aliph H), δ 7.65-7.68 (m, 8H, arom H) and δ 7.71 (br s, 2H, benzyldiene H);
^{13}C-NMR ($CDCl_3$): δ 47.95, 94.33, 129.49, 130.17, 134.38, 137.89, 138.92. LC-MS (m/z): 528.9 ($M+H^+$).

The molecular ion obtained for the hydrochloride salts of the piperidin-4-one dieneones **(42a-42n)** are consistent to that of their free base form **(41a-41n).**

3,5-Dibenzylidenepiperidin-4-one Hydrochloride (42a)

Chemical Formula**:** $C_{19}H_{18}ClNO$. M.Wt **:** 311.81. Recrystallized from 95% ethanol/37% hydrochloric acid. Yield: 90%. R_f : 0.51 (Silica TLC; DCM:EtAc, 10:1). M.Pt: 241-243°C.
IR (golden gate/cm^{-1}): 1678 (C=O), 1442 (C=C), 1613 (aromatic), 3245 (N-H).
^{1}H-NMR (DMSO-d_6): δ 2.48 (br s, H, aliph H), δ 4.46 (s, 4H, aliph H), δ 7.50 -7.53 (m, 10H, arom H) and δ 7.87 (br s, 2H, benzyldiene H);
^{13}C-NMR (DMSO-d_6): δ 48.34, 128.57, 129.49, 130.62, 131.08, 134.26, 139.65, 183.04 (C=O). LC-MS (m/z): 276.3 ($M+H^+$).

3,5-Bis(2-chlorobenzylidene)piperidin-4-one Hydrochloride (42b)

Chemical Formula**:** $C_{19}H_{16}Cl_3NO$. M.Wt **:** 380.70. Recrystallized from 95% ethanol/37% hydrochloric acid. Yield: 92%. R_f : 0.51 (Silica TLC; DCM:EtAc, 10:1). M.Pt: 221-223°C.
IR (golden gate/cm^{-1}): 1667 (C=O), 1590 (C=C), 1609 (aromatic), 3245 (N-H).
^{1}H-NMR (DMSO-d_6): δ 2.07 (br s, H, aliph H), δ 4.49 (s, 4H, aliph H), δ 7.55 -7.58 (m, 8H, arom H) and δ 7.84 (br s, 2H, benzyldiene H);
^{13}C-NMR (DMSO-d_6): δ 47.58, 129.49, 130.41, 132.81. LC-MS (m/z): 345.2 ($M+H^+$).

3,5-Bis(3-chlorobenzylidene)piperidin-4-one Hydrochloride (42c)

Chemical Formula**:** $C_{19}H_{16}Cl_3NO$. M.Wt **:** 380.70. Recrystallized from 95% ethanol/37% hydrochloric acid. Yield: 85%. R_f : 0.45 (Silica TLC; DCM:EtAc, 10:1). M.Pt: 220-223°C.
IR (golden gate/cm^{-1}): 1669 (C=O), 1396 (C=C), 1590 (aromatic), 3245 (N-H).
^{1}H-NMR (DMSO-d_6): δ 2.07 (br s, H, aliph H), δ 4.49 (s, 4H, aliph H), δ 7.54 -7.58 (m, 8H, arom H) and δ 7.85 (br s, 2H, benzyldiene H);
^{13}C-NMR (DMSO-d_6): δ 48.41, 128.92, 129.55, 130.45, 132.85, 138.38, 138.54. LC-MS (m/z): 345.2 ($M+H^+$).

3,5-Bis(4-chlorobenzylidene)piperidin-4-one Hydrochloride (42d)

Chemical Formula**:** $C_{19}H_{16}Cl_3NO$. M.Wt **:** 380.70. Recrystallized from 95% ethanol/37% hydrochloric acid. Yield: 95%. R_f : 0.54 (Silica TLC; DCM:EtAc, 10:1). M.Pt: 222-223°C.
IR (golden gate/cm^{-1}): 1674 (C=O), 1447 (C=C), 1609 (aromatic), 3240 (N-H).
^{1}H-NMR (DMSO-d_6): δ 2.48 (br s, H, aliph H), δ 4.45 (s, 4H, aliph H), δ 7.58 -7.59 (m, 8H, arom H) and δ 7.85 (br s, 2H, benzyldiene H);
^{13}C-NMR (DMSO-d_6): δ 46.87, 129.09, 129.54, 132.83, 133.11, 138.40, 182.86 (C=O). LC-MS (m/z): 345.2 ($M+H^+$).

3,5-Bis(2-bromobenzylidene)piperidin-4-one Hydrochloride (42e)

Chemical Formula**:** $C_{19}H_{16}Br_2ClNO$. M.Wt **:** 469.60. Recrystallized from 95% ethanol/37% hydrochloric acid. Yield: 88%. R_f : 0.63 (Silica TLC; DCM:EtAc, 10:1). M.Pt: 252-255°C.
IR (golden gate/cm^{-1}): 1669 (C=O), 1401 (C=C), 1590 (aromatic), 3235 (N-H).
^{1}H-NMR (DMSO-d_6): δ 2.08 (br s, H, aliph H), δ 4.31 (s, 4H, aliph H), δ 7.42 -7.44 (m, 8H, arom H) and δ 7.92 (br s, 2H, benzyldiene H);
^{13}C-NMR (DMSO-d_6): δ 48.34, 125.21, 128.57, 131.39, 132.22, 133.71, 134.03. LC-MS (m/z): 434.0 ($M+H^+$).

3,5-Bis(3-bromobenzylidene)piperidin-4-one Hydrochloride (42f)

Chemical Formula**:** $C_{19}H_{16}Br_2ClNO$. M.Wt **:** 469.60. Recrystallized from 95% ethanol/37% hydrochloric acid. Yield: 97%. R_f : 0.57 (Silica TLC; DCM:EtAc, 10:1). M.Pt: 260-262°C.
IR (golden gate/cm^{-1}): 1678 (C=O), 1442 (C=C), 1608 (aromatic), 3249 (N-H).
^{1}H-NMR (DMSO-d_6): δ 2.07 (br s, H, aliph H), δ 4.41 (s, 4H, aliph H), δ 7.44 -7.48 (m, 8H, arom H) and δ 7.80 (br s, 2H, benzyldiene H);
^{13}C-NMR (DMSO-d_6): δ 44.68, 124.18, 129.61, 132.45, 132.99, 133.49, 138.19. LC-MS (m/z): 434.0 ($M+H^+$).

3,5-Bis(4-bromobenzylidene)piperidin-4-one Hydrochloride (42g)

Chemical Formula**:** $C_{19}H_{16}Br_2ClNO$. M.Wt **:** 469.60. Recrystallized from 95% ethanol/37% hydrochloric acid. Yield: 90%. R_f : 0.63 (Silica TLC; DCM:EtAc, 10:1). M.Pt: 264-269°C.
IR (golden gate/cm^{-1}): 1674 (C=O), 1396 (C=C), 1586 (aromatic), 3249 (N-H).
^{1}H-NMR (DMSO-d_6): δ 2.07 (br s, H, aliph H), δ 4.47 (s, 4H, aliph H), δ 7.46 -7.49 (m, 8H, arom H) and δ 7.83 (br s, 2H, benzyldiene H);
^{13}C-NMR (DMSO-d_6): δ 44.36, 129.86, 131.52, 133.21, 133.27, 138.32. LC-MS (m/z): 434.0 ($M+H^+$).

3,5-Bis(2-fluorobenzylidene)piperidin-4-one Hydrochloride (42h)

Chemical Formula**:** $C_{19}H_{16}F_2ClNO$. M.Wt **:** 347.79. Recrystallized from 95% ethanol/37% hydrochloric acid. Yield: 76%. R_f : 0.70 (Silica TLC; DCM:EtAc, 10:1). M.Pt: 198-201°C.

IR (golden gate/cm^{-1}): 1664 (C=O), 1401 (C=C), 1590 (aromatic), 3318 (N-H).
^{1}H-NMR (DMSO-d_6): δ 2.49 (br s, H, aliph H), δ 4.36 (s, 4H, aliph H), δ 7.55 -7.57 (m, 8H, arom H) and δ 7.89 (br s, 2H, benzyldiene H);
^{13}C-NMR (DMSO-d_6): δ 44.39, 116.52, 116.66, 125.45, 131.53, 132.31, 133.02, 133.08. LC-MS (m/z): 312.3 ($M+H^+$).

3,5-Bis(3-fluorobenzylidene)piperidin-4-one Hydrochloride (42i)

Chemical Formula**:** $C_{19}H_{16}F_2ClNO$. M.Wt **:** 347.79. Recrystallized from 95% ethanol/37% hydrochloric acid. Yield: 55%. R_f : 0.53 (Silica TLC; DCM:EtAc, 19:1). M.Pt: 201-204°C.
IR (golden gate/cm^{-1}): 1672 (C=O), 1487 (C=C), 1606 (aromatic), 3377 (N-H).
^{1}H-NMR (DMSO-d_6): δ 3.32 (br s, H, aliph H), δ 4.49 (s, 4H, aliph H), δ 7.55 -7.58 (m, 8H, arom H) and δ 7.86 (br s, 2H, benzyldiene H);
^{13}C-NMR (DMSO-d_6): δ 45.35, 117.35, 117.45, 117.59, 127.20, 129.54, 131.47, 136.46, 138.43, 161.85, 163.47, 182.89 (C=O). LC-MS (m/z): 312.1 ($M+H^+$).

3,5-Bis(4-fluorobenzylidene)piperidin-4-one Hydrochloride (42j)

Chemical Formula**:** $C_{19}H_{16}F_2ClNO$. M.Wt **:** 347.79. Recrystallized from 95% ethanol/37% hydrochloric acid. Yield: 56%. R_f : 0.69 (Silica TLC; DCM:EtAc, 19:1). M.Pt: 202-204°C.
IR (golden gate/cm^{-1}): 1662 (C=O), 1425 (C=C), 1575 (aromatic), 3316 (N-H).
^{1}H-NMR ($CDCl_3$): δ 1.23 (br s, H, aliph H), δ 1.53 (s, 4H, aliph H), δ 7.24 -7.25 (m, 8H, arom H) and δ 7.51 (br s, 2H, benzyldiene H);
^{13}C-NMR ($CDCl_3$): δ 45.58, 127.94, 129.67, 130.11, 139.68, 140.52. LC-MS (m/z): 312.1 ($M+H^+$).

3,5-Bis(2-methoxybenzylidene)piperidin-4-one Hydrochloride (42k)

Chemical Formula**:** $C_{21}H_{22}ClNO_3$. M.Wt **:** 371.86. Recrystallized from 95% ethanol/37% hydrochloric acid. Yield: 85%. R_f : 0.70 (Silica TLC; DCM:EtAc, 19:1). M.Pt: 215-218°C.
IR (golden gate/cm^{-1}): 1668 (C=O), 1511 (C=C), 1590 (aromatic), 3246 (N-H).
^{1}H-NMR (DMSO-d_6): δ 2.49 (br s, H, aliph H), δ 4.45 (s, 4H, aliph H), δ 7.48 -7.51 (m, 8H, arom H) and δ 7.82 (br s, 2H, benzyldiene H);
^{13}C-NMR (DMSO-d_6): δ 45.48, 115.09, 126.35, 126.85, 133.30, 139.27, 161.32, 182.76 (C=O). LC-MS (m/z): 336.2 ($M+H^+$).

3,5-Bis(4-methoxybenzylidene)piperidin-4-one Hydrochloride (42l)

Chemical Formula**:** $C_{21}H_{22}ClNO_3$. M.Wt **:** 371.86. Recrystallized from 95% ethanol/37% hydrochloric acid. Yield: 92%. R_f : 0.70 (Silica TLC; DCM:EtAc, 10:1). M.Pt: 234-237°C.
IR (golden gate/cm^{-1}): 1666 (C=O), 1508 (C=C), 1596 (aromatic), 3250 (N-H).
^{1}H-NMR (DMSO-d_6): δ 2.49 (br s, H, aliph H), δ 4.45 (s, 4H, aliph H), δ 7.48 -7.50 (m, 8H, arom H) and δ 7.81 (br s, 2H, benzyldiene H);

^{13}C-NMR (DMSO-d_6): δ 45.41, 115.09, 126.85, 133.29, 139.26, 161.32. LC-MS (m/z): 336.2 ($M+H^+$).

3,5-Bis(2-iodobenzylidene)piperidin-4-one Hydrochloride (42m)

Chemical Formula**:** $C_{19}H_{16}ClI_2NO$. M.Wt **:** 563.60. Recrystallized from 95% ethanol/37% hydrochloric acid. Yield: 71%. R_f : 0.38 (Silica TLC; DCM:EtAc, 10:1). M.Pt: 248-251°C.
IR (golden gate/cm^{-1}): 1675 (C=O), 1454 (C=C), 1612 (aromatic), 3312 (N-H).
^{1}H-NMR ($CDCl_3$): δ 1.54 (br s, H, aliph H), δ 3.93 (s, 4H, aliph H), δ 7.34 -7.36 (m, 8H, arom H) and δ 7.76 (br s, 2H, benzyldiene H);
^{13}C-NMR ($CDCl_3$): δ 48.04, 129.59, 130.26, 134.49, 137.98, 139.01. LC-MS (m/z): 528.8 ($M+H^+$).

3,5-Bis(3-iodobenzylidene)piperidin-4-one Hydrochloride (42n)

Chemical Formula**:** $C_{19}H_{16}ClI_2NO$. M.Wt **:** 563.60. Recrystallized from 95% ethanol/37% hydrochloric acid. Yield: 79%. R_f : 0.48 (Silica TLC; DCM:EtAc, 10:1). M.Pt: 251-253°C.
IR (golden gate/cm^{-1}): 1666 (C=O), 1421 (C=C), 1616 (aromatic).
^{1}H-NMR ($CDCl_3$): δ 1.23 (br s, H, aliph H), δ 4.13 (s, 4H, aliph H), δ 7.30 -7.32 (m, 8H, arom H) and δ 7.70 (br s, 2H, benzyldiene H);
^{13}C-NMR ($CDCl_3$): δ 49.98, 93.67, 123.98, 133.06. LC-MS (m/z): 528.8 ($M+H^+$).

The spectroscopic and spectrometric data for all synthesized piperidone dieneone derivatives was consistent to their assigned structures.

2.2.3 Spectral data for the synthesized tropan-3-one (47a, 47d)

4-((1R,5S)-3-oxo-8-azabicyclo[3.2.1]octan-8-yl) benzoic acid (47a)

Chemical Formula**:** $C_{14}H_{15}NO_3$. M.Wt **:** 245.27. Recrystallized from methanol. Yield: 18%. R_f : 0.46 (Silica TLC; DCM). M.Pt: 236-240°C.
IR (KBr disk /cm^{-1}): 1710 (C=O on C-3 of tropanone ring), 1673 (C=0 on C-15 (benzoic acid), 1603 (aromatic).
^{1}H-NMR ($CDCl_3$): δ 1.54 (q, 4H aliphatic H, C6 and C7, predicted: 1.66), δ 2.07 (d, 4H aliphatic H, C2 and C4, predicted: 2.5-2.75), δ 2.37 (m, 4H aliphatic H, C1 and C5, predicted: 3.32), δ 6.6 (m, 4H arom H, C10 and C14, predicted: 7.03), δ 7.69 (m, 4H arom H, C11 and C13, predicted: 7.76). ^{1}H-NMR predictions generated using ChemBioDraw Ultra13 (Perkin Elmer).
^{13}C-NMR ($CDCl_3$): δ 28.62, 39.35, 39.55, 39.76, 39.97, 40.18, 40.39, 40.59, 45.85, 54.07, 113.63, 120.10, 131.87, 148.50, 168.29 (C=O on C-15), 207.14 (C=O on C-3). LC-MS (m/z): 244.1 ($M\text{-}H^+$).

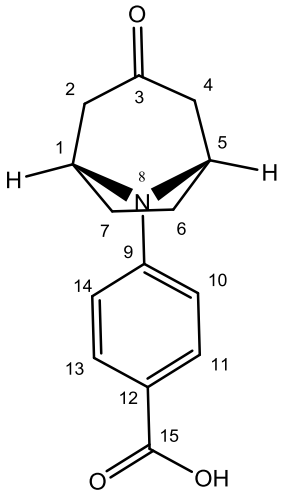


Figure 26- The chemical structure of 4-((1R,5S)-3-oxo-8-azabicyclo[3.2.1]octan-8-yl)benzoic acid (47a) with its relevant carbon and nitrogen numbering.

Benzocaine (43d)

Chemical Formula**:** $C_9H_{11}NO_2$. M.Wt **:** 165.19. Recrystallized from 95% Ethanol/water. Yield: 42%. R_f : 0.48 (Silica TLC; DCM:EtAc, 10:1). M.Pt: 89-91°C (Sigma-Aldrich, 2013).
IR (golden gate/cm^{-1}): 1638 (C=O), 1592 (aromatic), 2984 (C-H), 3343 (N-H).
^{1}H-NMR (DMSO-d_6): δ 1.26 (m, 3H, methyl, C9, predicted: 1.30), δ 4.18 (m, 2H, methylene, C8, predicted: 4.30), δ 5.92 (s, 2H, amine, predicted: 5.48), δ 6.54 (d, 4H, arom, C3 and C5, predicted: 6.47), δ 7.62 (s, 4H, arom, C2 and C6, predicted: 7.63). ^{1}H-NMR predictions generated using ChemBioDraw Ultra13 (Perkin Elmer).

^{13}C-NMR (DMSO-d_6): δ 14.89, 59.99, 113.16, 116.56, 131.55, 153.95, 166.40. LC-MS (m/z): 166.21 ($M+H^+$).

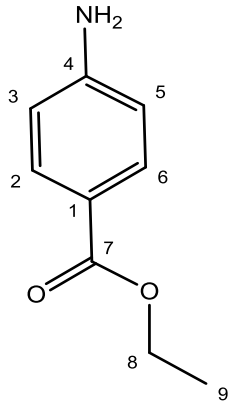


Figure 27- The chemical structure of benzocaine (43d) with its relevant carbon numbering.

ethyl-4-((1R,5S)-3-oxo-8 azabicyclo [3.2.1]octan-8-yl) benzoate (47d)

Chemical Formula**:** $C_{16}H_{19}NO_3$. M.Wt **:** 273.33. Recrystallized from methanol. Yield: 45%. R_f : 0.25 (Silica TLC; Cyclohexane:EtAc, 2:1). M.Pt: 252-255°C.
IR (golden gate/cm^{-1}): 1711 (C=O on C-3 of tropanone ring), 1673 (C=0 on C 15 (benzoate), 1603 (aromatic), 2988 (C-H).
^{1}H-NMR (DMSO-d_6): δ 1.67 (q, 4H aliphatic H, C6 and C7, predicted: 1.66), δ 2.22 (d, 4H aliphatic H, C2 and C4, predicted: 2.5-2.75), δ 3.30 (m, 4H aliphatic H, C1 and C5, predicted: 3.32), δ 6.99 (d, 4H, arom, C10 and C14, predicted: 6.93), δ 7.81 (s, 4H, arom, C11 and C13, predicted: 7.84). ^{1}H-NMR predictions generated using ChemBioDraw Ultra13 (Perkin Elmer).
^{13}C-NMR (DMSO-d_6): δ 60.38, 113.15, 113.90, 117.34, 131.08, 131.64, 148.17, 151.31 (C=O on C-15), 166.37 (C=O on C-3). LC-MS (m/z): 244.1 ($M-H^+$).

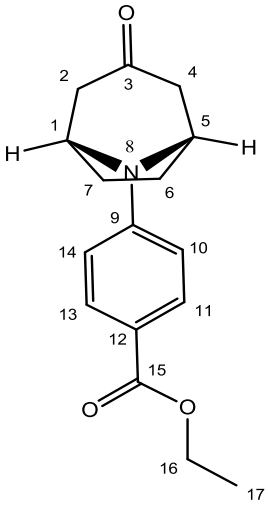


Figure 28- The chemical structure of* ethyl-4-((1R,5S)-3-oxo-8 azabicyclo [3.2.1]octan-8-yl) benzoate (47d) *with its relevant carbon and nitrogen numbering.

Chapter 3

Antifungal and Cytotoxic Screening of Piperidone and Benzothiazine Libraries

Chapter 3.1: Materials and Methods

3.1.1 Materials and Assays:

3.1.1.1 Fungal Strains and Cancer Cell Line

The fungal strains of *Saccharomyces cerevisiae* ATCC 46182, *Candida albicans* NCYC 854 and *Aspergillus niger* ATCC 16888 were obtained from the University of Hertfordshire Fungal Culture collection. The adherent cell line of the adenocarcinoma human alveolar basal epithelial A549 cells ATCC CCL-185 was obtained from the University of Nottingham. Before use the cell line had been re-seeded with media 23 times.

Table 3- Culture strain numbers of University of Hertfordshire collection:

Fungal strain/ Cell line	ATCC/ NCYC number	University's record number
Saccharomyces cerevisiae	ATCC 46182, a wild type strain	Y13a
Candida albicans	NCYC 854	Y100
Aspergillus niger	ATCC 16888, a wild type strain	F8
A549 human carcinoma	ATCC CCL-185	A549

3.1.1.2 Media

- Mueller-Hinton Broth: (Oxoid) was used for seeding the yeasting fungi. It was prepared by dissolving 21g of powder in 1L of distilled water.
- Mueller-Hinton Agar: (Oxoid) was used for seeding and testing against filamentous fungi. It was prepared by dissolving 20g of the powder in 400ml distilled water.

All media were sterilized by autoclaving at 121°C and 15psi pressure for 15-20 minutes.

- The media used to seed the A549 human carcinoma cells was purchased from Sigma-Aldrich and was composed of Dulbecco's Modified Eagle Medium (DMEM, high glucose) supplemented with 10% fetal bovine serum (FBS), 0.1 mM MEM Non-Essential Amino Acids (NEAA), 2 mM L-glutamine, and 1% Pen-Strep.

- The proteolytic enzyme trypsin was used in solution to hydrolyse proteins in the A549 cells so they become detached from the surface of the flask.

3.1.1.3 Stock Solutions of Controls

Miconazole (purchased from Sigma-Aldrich as miconazole nitrate salt) is an azole antifungal which was used as a reference agent. In studies of the inhibition of fungal growth miconazole was used as a positive control. The salt was weighed (2mg) and dissolved in 2.5ml of DMSO (99.98%; Fischer Chemical) giving a concentration of 0.8mg/ml. A 1:10 dilution of this gave a final stock concentration of 0.4µg/ml.

Triton X-100 is a non-ionic surfactant used as a positive (kill) control in cytotoxicity studies. Triton X-100 (50µl) was added to 5ml of DMEM to give final concentration of 5% Triton X-100.

3.1.1.4 Stock Solutions of Compounds (from chapter 2)

Based on the Relative Molecular Mass (RMM) of each of the synthesized dibenzylidenes and the tropan-3-one, a certain amount of material (56.3-200mg) was dissolved in 2ml of DMSO giving a final stock concentration of 2×10^{-1} M. This stock solution was diluted to make further stock solutions ranging from 2×10^{-2} M- 2×10^{-5} M. When treating the fungal cells with test compounds the final concentration of DMSO added was equal or less than 1.5%.

When working with the human carcinoma A549 cell line stock solutions were made fresh and further diluted using the DMEM media to give final concentrations of 2×10^{-3} M- 2×10^{-7} M. Thus ensuring that when treating the cells with test compounds, the final concentration of DMSO was less than or equal to 1%.

All stock solutions were preserved at 4°C and fresh stock solutions were made for each experiment on the day of treatment with the different cell cultures.

3.1.1.5 Stock Solutions of 1,2- Benzothiazine Compounds

The various 1,2-benzothiazine derivatives tested for their antifungal properties against *Saccharomyces cerevisiae* were synthesized by a former research student at the University of Hertfordshire (Patel, 2012).

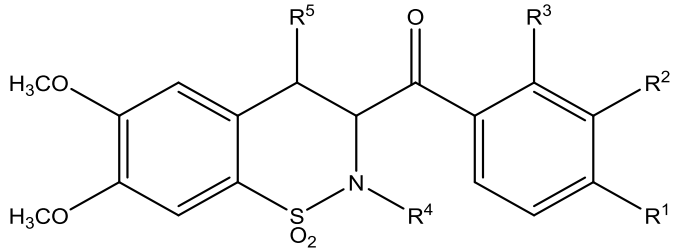


Figure 29- Parent 1,2-benzothiazine molecule with varying R substituents (48a-48as)

Table 4- Various substituent groups present on the 1,2-benzothiazine derivatives.

Compound	R^1	R^2	R^3	R^4	R^5	Molecular Formula
48a	H	H	H	H	Br	$C_{17}H_{16}BrNO_5S$
48b	H	H	H	CH_3	Br	$C_{18}H_{18}BrNO_5S$
48c	H	H	H	CH_2CH_3	Br	$C_{19}H_{20}BrNO_5S$
48d	H	H	H	Ph	Br	$C_{23}H_{20}BrNO_5S$
48e	H	H	H	$PhCH_2$	Br	$C_{24}H_{22}BrNO_5S$
48f	CH_3	H	H	H	Br	$C_{18}H_{18}BrNO_5S$
48g	CH_3	H	H	CH_3	Br	$C_{19}H_{20}BrNO_5S$
48h	CH_3	H	H	CH_2CH_3	Br	$C_{20}H_{22}BrNO_5S$
48i	CH_3	H	H	Ph	Br	$C_{24}H_{22}BrNO_5S$
48j	CH_3	H	H	$PhCH_2$	Br	$C_{25}H_{24}BrNO_5S$
48k	Cl	H	H	H	Br	$C_{17}H_{15}BrClNO_5S$
48l	Cl	H	H	CH_3	Br	$C_{18}H_{17}BrClNO_5S$
48m	Cl	H	H	CH_2CH_3	Br	$C_{19}H_{19}BrClNO_5S$
48n	Cl	H	H	Ph	Br	$C_{23}H_{19}BrClNO_5S$
48o	Cl	H	H	$PhCH_2$	Br	$C_{24}H_{21}BrClNO_5S$
48p	Br	H	H	H	Br	$C_{17}H_{15}Br_2NO_5S$
48q	Br	H	H	CH_3	Br	$C_{18}H_{17}Br_2NO_5S$
48r	Br	H	H	CH_2CH_3	Br	$C_{19}H_{19}Br_2NO_5S$
48s	Br	H	H	Ph	Br	$C_{23}H_{19}Br_2NO_5S$
48t	Br	H	H	$PhCH_2$	Br	$C_{24}H_{21}Br_2NO_5S$
48u	F	H	H	H	Br	$C_{17}H_{15}BrFNO_5S$
48v	F	H	H	CH_3	Br	$C_{18}H_{17}BrFNO_5S$
48x	F	H	H	CH_2CH_3	Br	$C_{19}H_{19}BrFNO_5S$
48y	F	H	H	Ph	Br	$C_{23}H_{19}BrFNO_5S$
48z	F	H	H	$PhCH_2$	Br	$C_{24}H_{21}BrFNO_5S$

Table 4 (continued) various substituent groups present on the 1,2-benzothiazine derivatives.

Compound	R^1	R^2	R^3	R^4	R^5	Molecular Formula
48aa	H	Cl	H	H	Br	$C_{17}H_{15}BrClNO_5S$
48ab	H	Cl	H	CH_3	Br	$C_{18}H_{17}BrClNO_5S$
48ac	H	Cl	H	CH_2CH_3	Br	$C_{19}H_{19}BrClNO_5S$
48ad	H	Cl	H	Ph	Br	$C_{23}H_{19}BrClNO_5S$
48ae	H	Cl	H	$PhCH_2$	Br	$C_{24}H_{21}BrClNO_5S$
48af	H	Br	H	H	Br	$C_{17}H_{15}Br_2NO_5S$
48ag	H	Br	H	CH_3	Br	$C_{18}H_{17}Br_2NO_5S$
48ah	H	Br	H	CH_2CH_3	Br	$C_{19}H_{19}Br_2NO_5S$
48ai	H	Br	H	Ph	Br	$C_{23}H_{19}Br_2NO_5S$
48aj	H	Br	H	$PhCH_2$	Br	$C_{24}H_{21}Br_2NO_5S$
48ak	H	H	Cl	H	Br	$C_{17}H_{15}BrClNO_5S$
48al	H	H	Cl	CH_3	Br	$C_{18}H_{17}BrClNO_5S$
48al	H	H	Cl	CH_2CH_3	Br	$C_{19}H_{19}BrClNO_5S$
48am	H	H	Cl	Ph	Br	$C_{23}H_{19}BrClNO_5S$
48an	H	H	Cl	$PhCH_2$	Br	$C_{24}H_{21}BrClNO_5S$
48ao	H	H	Br	H	Br	$C_{17}H_{15}Br_2NO_5S$
48ap	H	H	Br	CH_3	Br	$C_{18}H_{17}Br_2NO_5S$
48aq	H	H	Br	CH_2CH_3	Br	$C_{19}H_{19}Br_2NO_5S$
48ar	H	H	Br	Ph	Br	$C_{23}H_{19}Br_2NO_5S$
48as	H	H	Br	$PhCH_2$	Br	$C_{24}H_{21}Br_2NO_5S$

As described in section 3.1.1.4 for the piperidone compounds, stock solutions of final concentration 2 x 10^{-1} M were prepared by dissolving an amount of each 1,2-benzothiazine based on their RMM (161-239mg) in 2ml DMSO. This was followed by relevant dilutions to make stock solutions ranging between; 2 x 10^{-2}- 2 x 10^{-5} M, ensuring that the yeast were treated with test compounds in DMSO at DMSO concentration less than or equal to 1.5%. All stock solutions were made on the day of treating the cells and maintained at 4°C.

3.1.1.6 Antimicrobial Disks

Blank disks (Oxoid) made from paper with a diameter of 6mm were used for antifungal diffusion susceptibility testing against *A.niger*. All disks used were sterilized by autoclaving at 121°C and 15psi pressure for 5 hours. After inoculation with the various compounds the disks were stored at -20°C prior to placing on agar plates.

3.1.1.7 MTS Assay

The MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt) assay kit was purchased from Promega. A one solution reagent containing the MTS (317µg/ml) and an electron coupling reagent (phenazine ethosulfate; PES) was stored in the dark at -20°C. Before use it was thawed in a water bath at 37°C.

3.1.1.8 LDH Assay

The lactate dehydrogenase (LDH) assay kit (Promega) included; a substrate mix, assay buffer and stop solution, all of which were stored at -20°C. The CytoTox-ONE solution was prepared by thawing the substrate mix and assay buffer in an incubator (22°C), followed by the addition of 11ml of the assay buffer to the substrate mix. The stop solution was thawed by incubating at 22°C before use.

3.1.1.9 Absorbance and Fluorescent Measurement

A UV spectrophotometer (UNICAM; helios α) was used to measure the absorbance (600nm) in inhibition of yeast cell growth. The absorbance values from the MTS assay were measured using an ELISA plate reader (Labsystems; Multiskan Ascent). Fluorescent measurements were obtained from the LDH assay using a fluorescence spectrometer (PerkinElmer LS55 with a 96-well plate reader attachment).

3.1.2 Methods

3.1.2.1 Inhibition of growth of *S. cerevisiae* and *C. albicans*

3-((1R,5S)-3-oxo-8azabicyclo[3.2.1]octan-8-yl) benzoic acid along with benzylidene derivatives of piperidone and 4-piperidone and a library of 1,2-benzothiazines were used for susceptibility testing against actively growing yeast species; *S. cerevisiae* ATCC 46182 and *C. albicans* NCYC 854. Initially the cell growth of both fungi was analysed for each experiment to determine the mid-exponential (log) phase, defined as the point at which the cells are proliferating at their maximum rate.

Using standard aseptic techniques a few colonies of the yeast were inoculated in a 250ml conical flask containing Mueller-Hinton broth (80ml). The flasks were incubated at 32°C and shaken at 100 revs/min overnight. The flasks were mixed well and cell counts (Neubauer haemocytometer) and an absorbance reading at 600nm (UNICAM; helios α) were obtained from 1ml of the cell suspension, thus giving an approximation of the number of cells present in the flasks after 24 hours incubation. After mixing the flasks thoroughly 1ml (9.13×10^8 cells/ml) of cell suspension was re-suspended in a fresh 250ml conical flask containing 80ml Mueller-Hinton broth. The flasks were incubated at 32°C and shaken at 100 revs/min. Cell counts and absorbance readings at 600nm were noted hourly between 1-8 hours and then after 24 hours incubation. Cell growth curves were constructed plotting the absorbance at 600nm against time. From the growth curves it was concluded that the mid-exponential phase for both the yeasting fungi was between 5-7 hours of incubation (refer to Figure 30, Page 75).

The susceptibility test was performed using a liquid broth dilution method (Fothergill, 2012, CLSI, 2013). A set of 5ml glass bijous were acid washed and dried before adding 2ml of Mueller-Hinton broth followed by autoclaving (121°C, 15psi, 5 hours). Relevant stock solutions of miconazole nitrate, synthesized benzylidene piperidones, methyl-piperidones, 3-((1R,5S)-3-oxo-8 azabicyclo[3.2.1]octan-8-yl) benzoic acid and 1,2-benzothiazines were made using DMSO. Various concentrations of the compounds and controls were added to the 2ml media in each bijou. DMSO (1.5%) was also used as a control to verify that DMSO had no effect on the growth of the cell line as all compounds including the control were dissolved in DMSO.

S. cerevisiae cell suspension (1ml) taken from an overnight growth was re-suspended in a 250ml conical flask containing 80ml Mueller-Hinton broth. The flask was incubated for 5 hours (32°C, 100 revs/min) to get the cells in the mid-exponential phase, after which 1ml of cell suspension was removed and absorbance at 600nm recorded to estimate the number of cells present per ml. A broth dilution method was performed and after a 1:300 dilution, 100µl of the diluted cell suspension was added to each bijou (final number of cells per bijou: 4.76×10^4 cells/ml). All tests were conducted in duplicate. Bijous were incubated at 32°C and shaken at 100 revs/min with absorbance readings (600nm) being recorded after 24 and 48 hours incubation. The most potent compounds from the first screen were re-tested using a fresh growth of *S. cerevisiae.*

The most potent compounds were also used for susceptibility testing against *C. albicans.* The method was as described previously for the susceptibility testing on *S. cerevisiae.* The experiment was repeated twice in duplicate using a fresh growth of cell culture. The final number of cells per bijou was between 5.3×10^4- 5.4×10^4 cells/ml.
Results were expressed as percent inhibition of cell growth as compared to the 1.5% DMSO treated control using the following formula:

$$100 - (Abs_D / Abs_C \times 100) \quad \textbf{Equation (3)}$$

Where Abs_D = absorbance (600nm) of cells + drug and Abs_C = absorbance (600nm) of cells + control (1.5% DMSO).

3.1.2.2 Studies on the inhibition of growth of *A. niger*

The inhibitory effects of the compounds on the growth of *A. niger* ATCC 16888 was analysed using a disk diffusion assay (Fothergill, 2012, CLSI, 2013). A few mycelial fragments of *A. niger* were aseptically inoculated onto two sterile Potato Dextrose Agar (PDA) plates and grown for 7 days at 35°C. Stock inoculum suspensions of conidia were prepared from the 7-day old cultures grown by adding sterile distilled water (2.5ml) to each plate. The plates were shaken on a plate shaker (Heidolph, Titramax 100) at 150rpm/min for 20 minutes. The conidial suspension from each plate (1ml) was added to two separate sterile 20ml universals. Absorbance at 530nm for each sample was read before the samples were simultaneously mixed and then split again.

The samples were adjusted spectrophotometrically on the day of treatment with compounds to optical densities ranged between 0.09-0.11 using a 1:10 dilution with sterile distilled water. (Espinel-Ingroff *et al*, 2007). The final concentrations of the stock inoculum suspensions ranged from 8.6 x 10^5 to 2.4 x 10^6 CFU/ml, as demonstrated by quantitative colony counts on Sabouraud dextrose agar. The entire surface of each agar plate was inoculated simultaneously in three directions with 100µl of the stock inoculum using a sterile glass spreader. The inoculated agar was allowed to dry for 20 minutes. Sterile paper disks were loaded with various quantities of the most potent compounds (as defined by the inhibition studies on yeasts), miconazole nitrate (0.6µg) or DMSO (1.5%). The disks were allowed to dry for 60 minutes before being applied to the inoculated agar using sterile forceps. The plates were covered with lids and incubated in ambient air at 35°C within 10 minutes of disk application to the inoculated agar. Each experiment was conducted in duplicate and the experiment repeated using a fresh growth of *A. niger* ATCC 16888.

In the disk diffusion assay zone of inhibition diameters were measured to the nearest whole millimetre at the point where there was a measurable reduction of growth after 16, 24 and 72 hours of incubation. Corrected diameters were acquired by subtracting the diameter of the paper disks (6mm) from the inhibition zone.

3.1.2.3 Cytotoxic Evaluation of Compounds Against Human Carcinoma A549 Cell Line

3.1.2.3.1 A549 Cell Harvest and Growth Analysis

The A549 (ATCC CCL-185) human lung carcinoma cell line obtained from the University of Nottingham was used to analyse the cytotoxicity of the most potent compounds as defined by inhibition studies on yeasts. Selected compounds were tested against A549 cells for their effect on mitochondrial activity (MTS) and membrane integrity (LDH). Due to time limitations only four compounds were analysed for their cytotoxic properties. Cells were harvested in sterile T75 flasks consisting of Dulbecco's Modified Eagle Medium (DMEM, high glucose) supplemented with 10% fetal bovine serum (FBS), 0.1 mM MEM Non- essential amino acids (NEAA), 2 mM L-glutamine, and 1% Pen-Strep and kept in conditions of 37°C, 5% CO_2. Cells were passaged into fresh sterile T75 flasks every fortnight containing fresh media warmed to 37°C. PBS was used to remove any excess media and/or dead cells. Trypsin (0.5%), a serine protease was used to cleave proteins bonding the cultured cells to

the flask. Trypsin was removed by centrifuging the cell suspension at 250g, 18-25°C, 5 mins (Thermo scientific; Heraeus Labofuge 400R).

The growth of A549 cells was studied to determine the mid-exponential phase. A549 cells were seeded into a 96-well plate at a concentration of 1 x 10^4 cells/100µl/well. The plate was incubated at 37°C, 5% CO_2 and cell counts were obtained in triplicate from six wells daily for a total of 9 days. Cells were counted using a Neubauer haemocytometer with dilution and scaling factors applied to determine the number of cells per ml. A dye exclusion assay (Trypan blue) was used when performing cell counts to discount any dead cells. Trypan Blue actively stains the cytoplasm of deceased cells so they can be easily viewed using a light microscope (Baust & Baust, 2007). The mid exponential phase of the cell line was found to be after 3 days of cultivation (see Figure 52, Page 143).

For testing purposes; 96-well plates were seeded with the cells at a concentration of 1 x 10^4 cells/100µl/well. Plates were incubated for three days at 37°C, 5% CO_2. The compounds tested were initially dissolved in DMSO and then diluted using DMEM such that the final concentration of DMSO was 1%. The cells were then treated with a positive (kill) control (5% Triton X-100), negative control (1% DMSO) and varying concentrations of each compound tested (2 x 10^{-3}- 2 x 10^{-7} M). Each control and concentration of compound tested was examined in sextuplicate. The experiment was repeated using a fresh growth of A549 cells.

3.1.2.3.2 Cytotoxicity Studies of Test Compounds Using The MTS Assay

The MTS assay was used to determine if the compounds have an effect on reducing mitochondrial activity thus possessing cytotoxic properties. The MTS assay is often defined as a 'one-step' MTT assay as the reagent can be added directly to the cell culture without the need of intermediate steps. (Burger & Fiebig, 2004). The test is centred on the ability of viable cells to convert the MTS reagent into a soluble blue/purple formazan dye. Thus viable cells appear blue/purple whereas dead cells are unaffected and remain pale yellow. (Burger & Fiebig, 2004). The exact cellular mechanism of MTS reduction into formazan involves reactions with intracellular NADH-oxidoreductases (Berridge *et al,* 2005). It is believed to occur mainly through the action of mitochondrial succinate dehydrogenase (Lobner, 2000).

After the cells were treated with the test compounds for 48 hours 20µl of the MTS reagent (317µg/ml) was added to each well of the 96-well plates. Plates were covered in aluminium foil to avoid interaction with light and were briefly shaken on a plate shaker (Heidolph, Titramax 100) for 30 seconds. All plates were incubated for 3 hours at 37°C, 5% CO_2. The absorbance was determined using a multi-well plate reader (Labsystems; Multiskan Ascent) at 490nm. Results were expressed as percent cytotoxicity as compared to the controls (1% DMSO, 5% Triton X-100). The following formula was utilised to determine percent cytotoxicity:

$$100 - (100 \times (Abs_{D} - Abs_{PC}) / (Abs_{NC} - Abs_{PC})) \quad \textbf{Equation (4)}$$

Where Abs_{D}= absorbance (600nm) of cells + drug, Abs_{PC}= absorbance (600nm) of cells + positive control (5% Triton X-100) and Abs_{NC}= absorbance (600nm) of cells + negative control (1.5% DMSO).

3.1.2.3.3 Cytotoxicity Studies of Test Compounds Using The LDH assay

The effect on cell membrane integrity by potentially toxic compounds was measured using the lactate dehydrogenase (LDH) assay. LDH is an oxidoreductase which exists in most organisms and it catalyses the inter-conversion of pyruvate and lactate with an associated inter-conversion of NADH and NAD^{+}, LDH is used as a catalyst. Pyruvate is used by the cell as a 'dump' of excess reducing power i.e. NADH in the absence of oxygen. This is facilitated by the action of the enzyme LDH (Fotakis & Timbrell, 2006). When a tissue suffers membrane damage, cells release LDH into the bloodstream. Since LDH is a stable enzyme it is widely used for detection of damaged cells (Fotakis & Timbrell, 2006). The enzymatic activity of LDH is temperature sensitive, thus it is recommended to equilibrate the temperature of the assay plates with the CytoTox-ONE reagent at 22 °C. The reagent consists of resazurin which is converted to resorufin when LDH is released into the culture medium. Production of the fluorescent reosurfin product is proportional to the quantity of LDH (Promega, 2012). An increase in LDH release is shown by an increase in fluorescence which indicates increasing cytotoxicity. Both the resazurin and the resorufin product formed during the assay are light-sensitive. Therefore this experiment was carried out under minimum light conditions and black 96-well plates were used which were stored in dark conditions until the fluorescence was read to avoid discrepancies.

Plates containing cells incubated with test compounds for 48 hours were initially incubated in ambient air at 22°C for 30 minutes. The CytoTox-ONE reagent was made by mixing the assay buffer (11ml) with the lyophilised substrate mix post incubation (22 °C). The CytoTox-ONE reagent (100µl) was added to each well of the plates. The plates were covered in aluminium foil and shaken briefly on the plate reader (Heidolph, Titramax 100) for 20 seconds before incubation at 22°C for 10 minutes. Post incubation 50µl of 'stop' solution was added to each well. The 'stop' solution is used to rapidly stop the continued generation of fluorescent product thus allowing the plate to be read without variability in the time of incubation. All plates were shaken for 10 seconds and the fluorescence was measured using an excitation wavelength of 560nm and an emission wavelength of 590nm (PerkinElmer LS55 Fluorescence spectrophotometer). Results were expressed as percent cytotoxicity as compared to the controls (1% DMSO, 5% Triton X-100). The following formula was applied to determine percent cytotoxicity:

$$100 \times (F_{D}-F_{NC}) / (F_{PC}-F_{NC}) \qquad \textbf{Equation (5)}$$

Where F_{D}= fluorescence (E_x 560nm, E_m 590nm) of cells + drug, F_{PC}= fluorescence (E_x 560nm, E_m 590nm) of cells + positive control (5% Triton X-100), F_{NC}= fluorescence (E_x 560nm, E_m 590nm) of cells + negative control (1.5% DMSO), E_x= excitation wavelength and E_m= emission wavelength.

3.2.1 Inhibition of Growth of *Saccharomyces cerevisiae* and *Candida albicans* by Novel Compounds

3.2.1.1 Growth Curves for *S. cerevisiae* and *C. albicans*

The growth of the yeasts *S. cerevisiae* and *C. albicans* was analysed in order to determine the mid exponential phase. The exponential phase is defined as the point at which the cells are proliferating at their maximum rate. Absorbance readings at 600nm and cell counts were determined hourly between 1-8 hours and after 24 hours incubation (37°C, 100 revs/min). Cell growth curves were constructed of absorbance at 600nm against time.

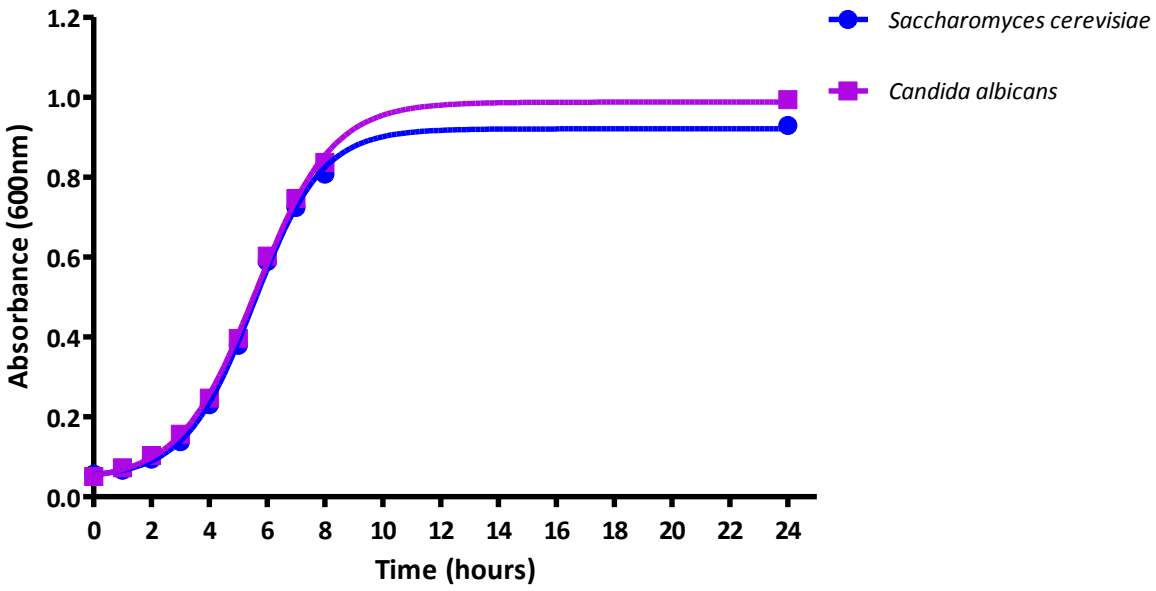
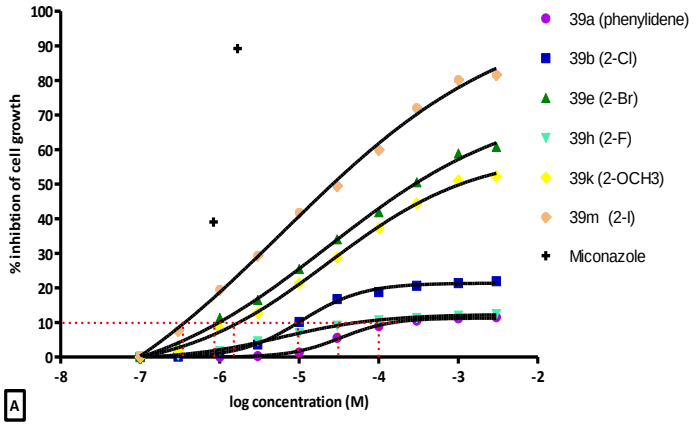


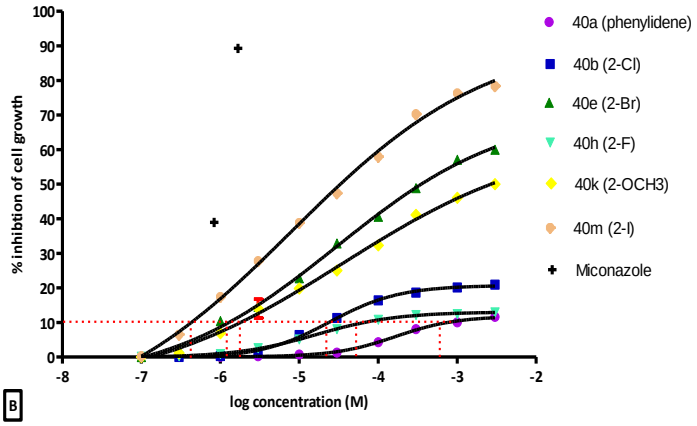
Figure 30- Growth curve showing the absorbance at 600 nm for S. cerevisiae and C. albicans taken at hourly intervals during growth (37°C).

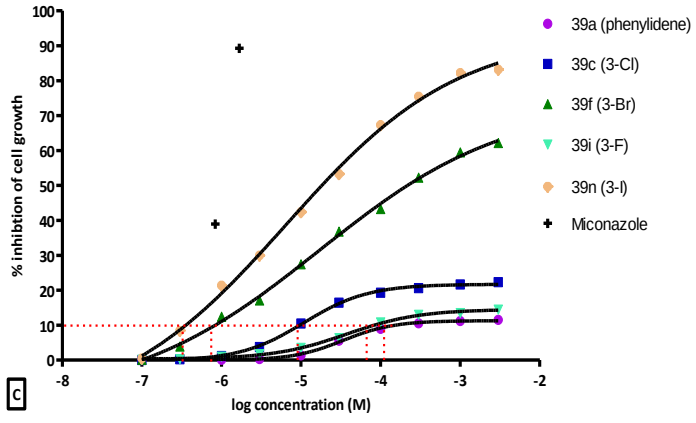
As shown in figure 30, both the fungal species were in lag phase until 4-5 hours of growth. The exponential (or log) phase is seen between 5-7 hours. After 7 hours the cells enter the stationary phase followed by the deceleration phase after 24 hours incubation. When determining the inhibitory potencies of the novel compounds against the growth of these fungi, the cells were initially incubated at 37°C for 5 hours (to obtain an absorbance reading between 0.3-0.4) to ensure they were in exponential phase and actively metabolizing.

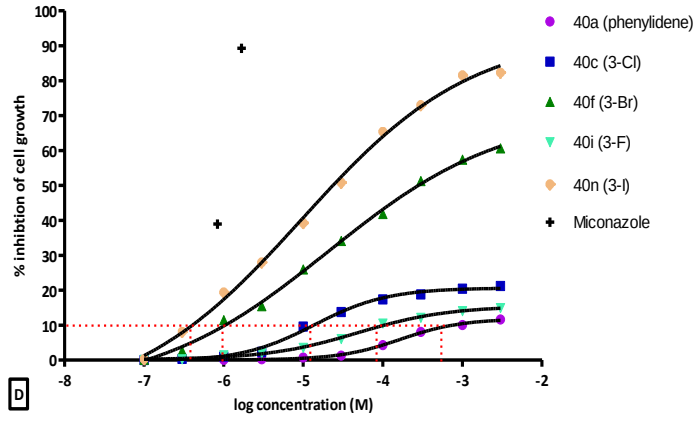
3.2.1.2 Inhibition of Growth of *S. cerevisiae* After 24 hours Treatment With Ring Substituted 3,5-dibenzylidene-1-methyl Piperidin-4-ones (39a-39n) and Their Hydrochloride Salts (40a-40n)

The ability of the novel compounds to inhibit cell growth in *S. cerevisiae* ATCC 46182 was analysed using a liquid broth dilution method. Bijous initially containing 4.76 x 10^4 cells/ml in 2ml Mueller-Hinton broth media were treated with various concentrations of the compounds, along with controls and the antifungal drug miconazole as a reference. Bijous were incubated for 24 hours (37°C, 100 revs/min). Post incubation absorbance (600nm) was obtained from a 1ml sample from each bijou. All tests were conducted in duplicate. As the compounds and controls were dissolved in DMSO (1.5%), an initial test to determine the effect of DMSO at a final concentration of 1.5% on cell growth was conducted. DMSO at a concentration of 1.5% had no effect on cell growth with results being expressed as percent inhibition (see Equation **3**, Page 70) as compared to the untreated controls. Ring substituted dieneones were compared against dieneones without any ring substituents.









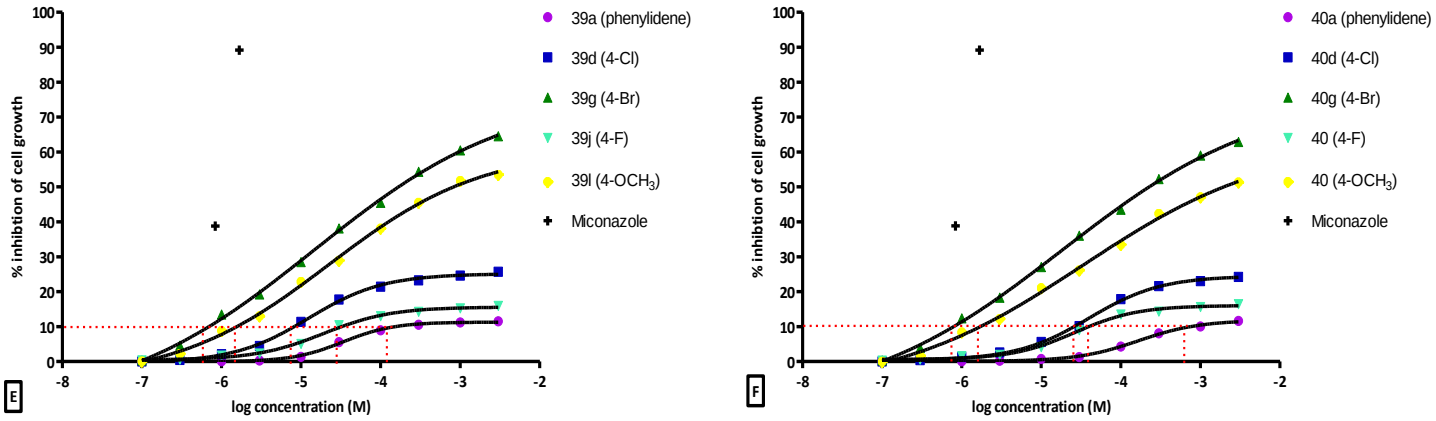
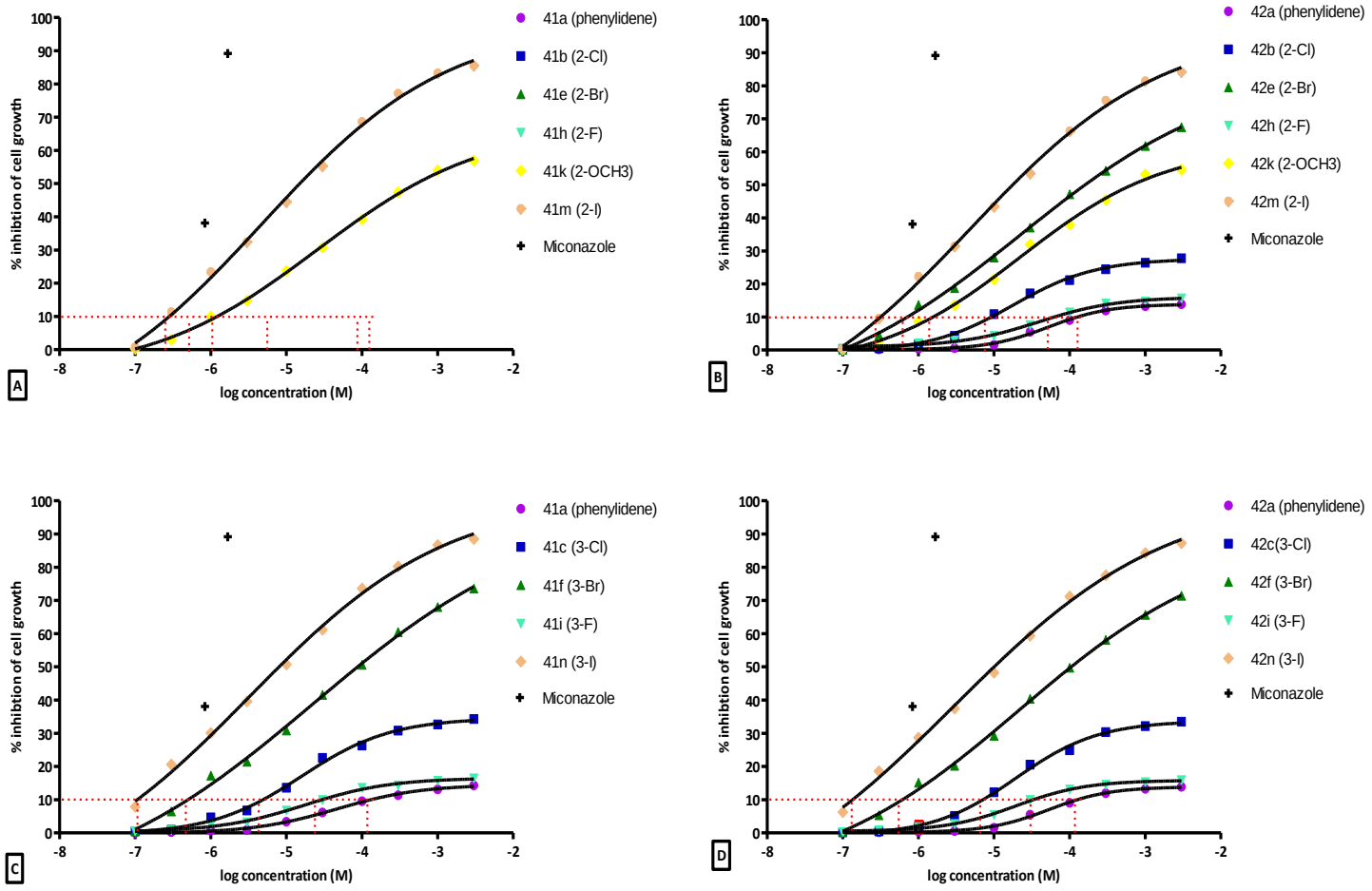


Figure 31- Dose response curves of the free base and hydrochloride salts of ortho-substituted (A, B), meta-substituted (C, D) and para-substituted (E, F) 3,5-dibenzylidene-1-methyl piperidin-4-ones; showing the effect of increasing concentration on the % inhibition of cell growth of S. cerevisiae after a 24 hour treatment. The data is presented as percentages of the control (1.5% DMSO). The reference drug miconazole at a concentration of 0.835 x 10^{-6} M gave an average absorbance (600nm) of 0.609 (39% inhibition) and 0.107 (89% inhibition) at 1.67 x 10^{-6} M. The concentrations of compound resulting in 10% inhibition (IC_{10}) were determined from the dose-response curve. IC_{50} values were determined using the graphical software package GraphPad, Prism 5.

Figure 31 shows that increasing the concentration of each of the compounds causes inhibition of growth of the yeast. However there is a point where increasing concentration has no increased effect on inhibition (as seen by plateau in the dose-response curves) which varies from compound to compound. The highest concentration used (3 x 10^{-3} M) of each compound gave a significantly higher percentage inhibition. However it can be seen that the Iodo-substituted 3,5-dibenzylidene-1-methyl piperidin-4-ones (**39m and 39n**) and their hydrochloride salts (**40m and 40n**) gave the highest level of inhibition, whereas compounds without ring substituents gave the lowest level of inhibition (**39a, 40a**). There was very little variability between the free base compounds and their hydrochloride salts, with the free base compounds generally giving higher levels of inhibition. However, the major factor was the 'R' substituent where iodo substituted compounds were the most potent growth inhibitors and un-substituted compounds produced the lowest level of inhibition. Another factor affecting the level of inhibition was the position of the substituent where para substituted compounds were the most potent and ortho substituted compounds were the least potent. Compound **39n** was the most potent giving 83% inhibition at a concentration of 3 x 10^{-3} M, whereas miconazole gave 89% inhibition at 1.67 x 10^{-6} M.

3.2.1.3 Inhibition of Growth of *S. cerevisiae* After 24 hours Treatment with Ring Substituted 3,5-dibenzylidene-1-piperidin-4-ones (41a-41n) and Their Hydrochloride Salts (42a-42n)

Ring substituted 3,5-dibenzylidene-1-piperidin-4-ones (**41a-41n**) and their hydrochloride salts (**42a-42n**) were examined for their inhibitory effect on the cell growth of *S. cerevisiae* ATCC 46182 as described in **3.2.1.2.**



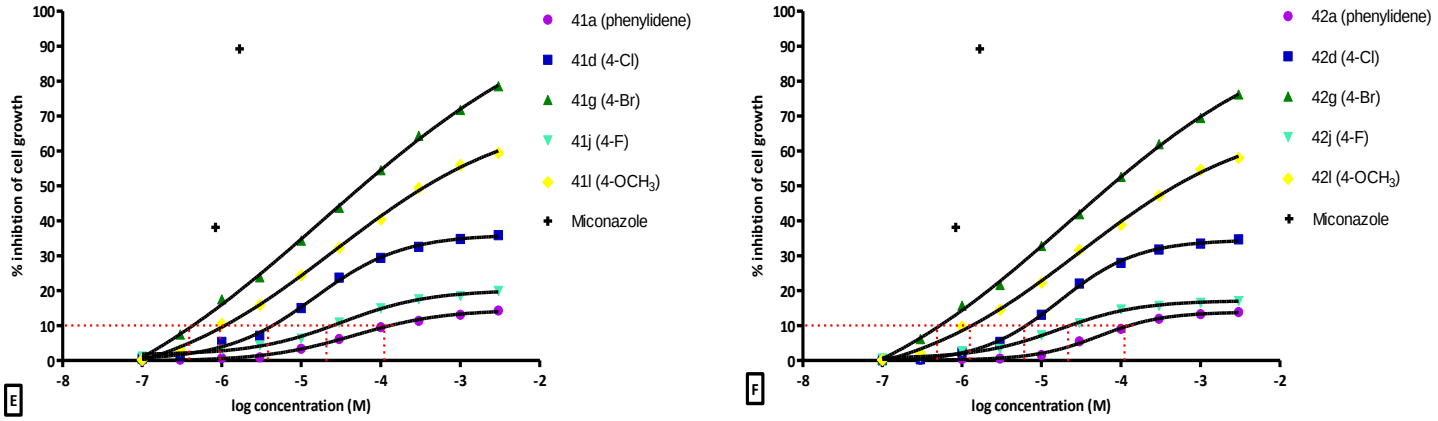


Figure 32- Dose response curves of the free base and hydrochloride salts of ortho-substituted (A, B), meta-substituted (C, D) and para-substituted (E, F) 3,5-dibenzylidene-1- piperidin-4-ones; showing the effect of increasing concentration on the % inhibition of cell growth of S. cerevisiae after a 24 hour treatment. The data is presented as percentages of the control (1.5% DMSO). The reference drug miconazole at a concentration of 0.835 x 10^{-6} M gave an average absorbance (600nm) of 0.618 (38% inhibition) and 0.108 (89% inhibition) at 1.67 x 10^{-6} M. The concentrations of compound resulting in 10% inhibition (IC_{10}) were determined from the dose-response curve. IC_{50} values were determined using the graphical software package GraphPad, Prism 5.

The results obtained resemble the data obtained in **3.2.1.3**; increasing concentrations of each compound increased the inhibition of cell growth with DMSO (1.5%) having no effect on growth. These compounds however show higher degrees of growth inhibition in comparison to the ring substituted 3,5-dibenzylidene-1-methyl piperidin-4-ones and their respective hydrochloride salts. There is not a significant difference between the results obtained for the free base and the hydrochloride salts of the ring substituted 3,5-dibenzylidene-1-piperidin-4-ones. In both cases the iodo-substituted compounds produced the highest level of inhibition and un-substituted compounds were the least potent growth inhibitors. The position of the substituent conveyed an effect on inhibition, where para-substituted substituents gave rise to the most potent inhibitors and ortho-substituted substituents produced the least potent compounds. Compound **41n** was the most potent giving 89% inhibition at a concentration of 3 x 10^{-3} M, whereas miconazole gave the same level of inhibition at 1.67 x 10^{-6} M.

3.2.1.4 Inhibition of Growth of *S. cerevisiae* After 48 hours Treatment with Ring Substituted 3,5-dibenzylidene-1-methyl piperidin-4-ones (39a-39n) and Their Hydrochloride Salts (40a-40n)

The experiment as described in **3.2.1.2** was repeated except that cells were treated with the compounds for 48 rather than 24 hours. Results of inhibition studies were repeated using the most potent compounds. The data was combined and percent inhibition calculated.

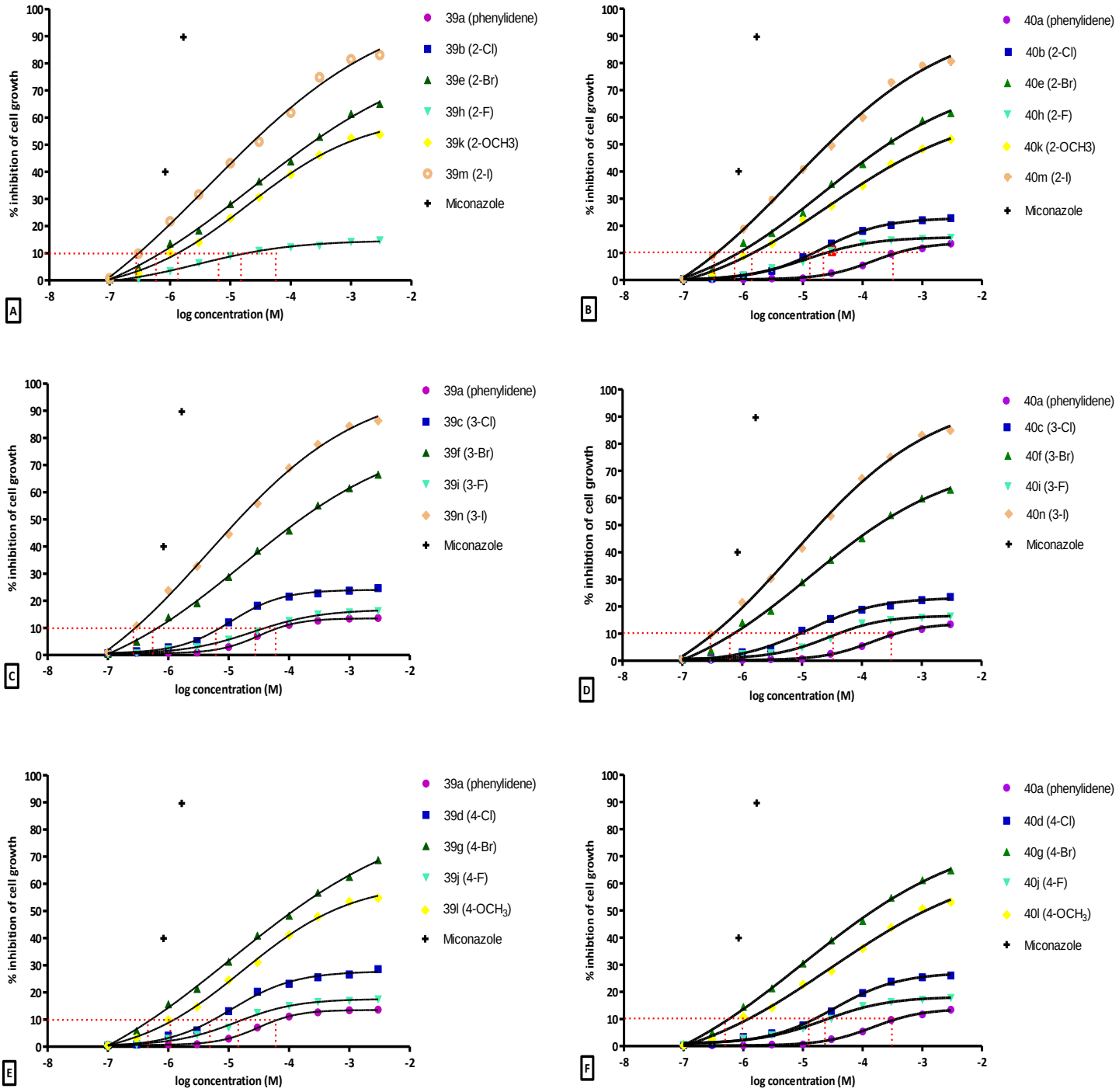
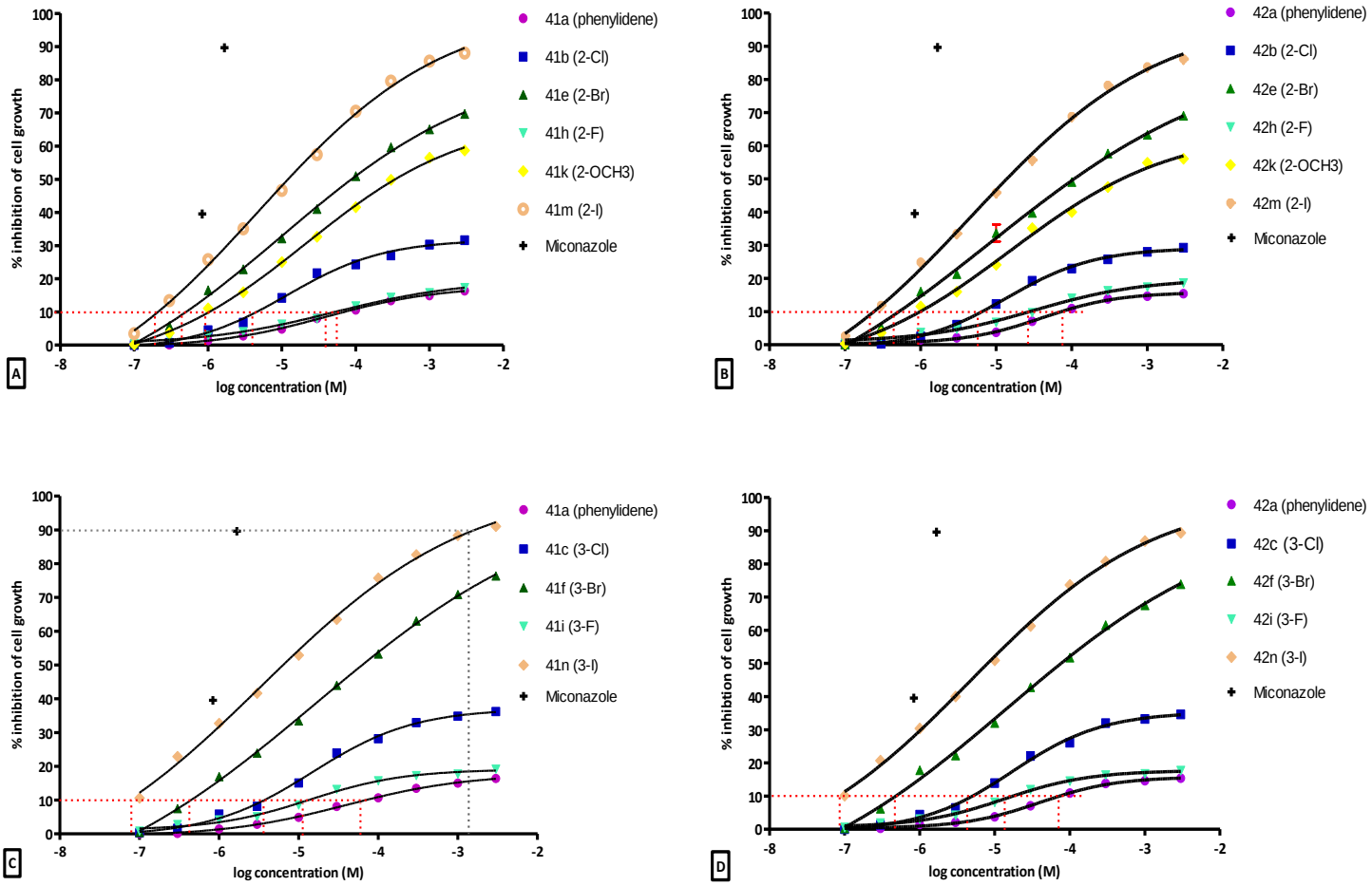


Figure 33- Dose response curves of the free base and hydrochloride salts of ortho-substituted (A, B), meta-substituted (C, D) and para-substituted (E, F) 3,5-dibenzylidene-1-methyl piperidin-4-one; showing the effect of increasing concentration on the % inhibition of cell growth of S. cerevisiae after a 48 hour treatment. The data is presented as percentages of the control (1.5% DMSO). The reference drug miconazole at a concentration of 0.835 x 10^{-6} M gave an average absorbance (600nm) of 0.603 (39% inhibition) and 0.104 (90% inhibition) at 1.67 x 10^{-6} M. The concentrations of compound resulting in 10% inhibition (IC_{10}) were determined from the dose-response curve. IC_{50} values were determined using the graphical software package GraphPad, Prism 5.

Incubating the cells with the compounds for a longer period resulted in an increase in growth inhibition, whereby increasing the incubation period by a further 24 hours has resulted in further increase in inhibition of growth. This is the case for both the free base and hydrochloride salts of the ring substituted 3,5-dibenzylidene-1-methyl piperidin-4-ones. Compound **39n** was the most potent giving 86% inhibition at a concentration of 3 x 10^{-3} M, whereas miconazole gave 90% inhibition at 1.67 x 10^{-6} M.

3.2.1.5 Inhibition of Growth of *S. cerevisiae* After 48 hours Treatment With Ring Substituted 3,5-dibenzylidene-1-piperidin-4-ones (41a-41n) and Their Hydrochloride Salts (42a-42n)

3,5-dibenzylidene-1-piperidin-4-ones and their hydrochloride salts were re-tested for their inhibitory potencies against *S. cerevisiae* as described in **3.2.1.2**, however the cells were treated with the compounds for 48 hours rather than 24.



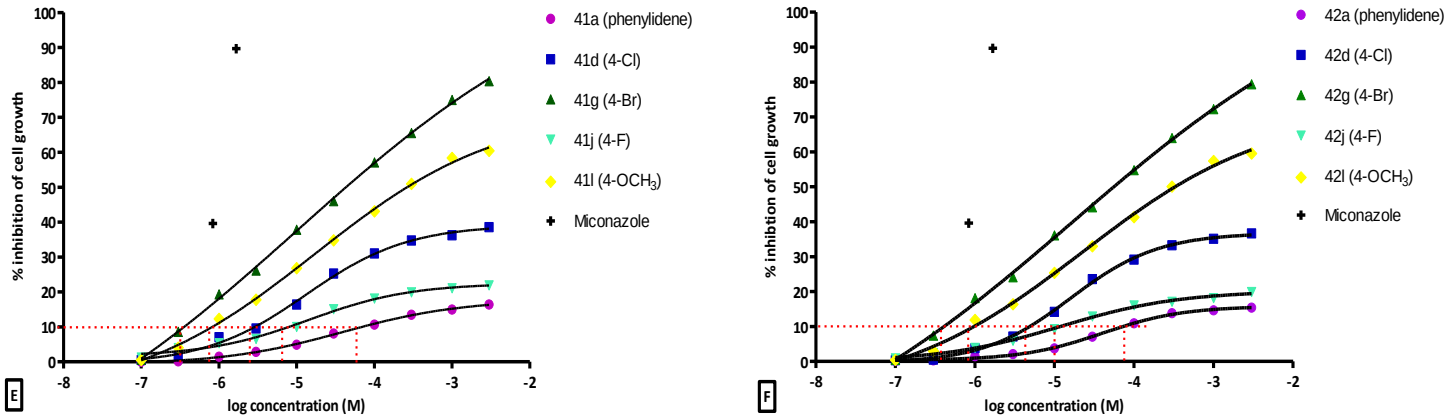


Figure 34- Dose response curves of the free base and hydrochloride salts of ortho-substituted (A, B), meta-substituted (C, D) and para-substituted (E, F) 3,5-dibenzylidene-1- piperidin-4-ones; showing the effect of increasing concentration on the % inhibition of cell growth of S. cerevisiae after a 48 hour treatment. The data is presented as percentages of the control (1.5% DMSO). The reference drug miconazole at a concentration of 0.835 x 10^{-6} M gave an average absorbance (600nm) of 0.606 (40% inhibition) and 0.103 (90% inhibition) at 1.67 x 10^{-6} M. The concentrations of compound resulting in 10% and 90% inhibition (IC_{10}, IC_{90}) were determined from the dose-response curve. IC_{50} values were determined using the graphical software package GraphPad, Prism 5.

In comparison to the 24 hour treatment (**3.2.1.3**), 48 hours incubation with compounds resulted in a higher degree of cell growth inhibition. Both the free base and the hydrochloride salts of the ring substituted 3,5-dibenzylidene-1- piperidin-4-ones showed an increase in cell growth inhibition when incubated for a further 24 hours. Compound **41n** was the most potent giving 91% inhibition at a concentration of 3 x 10^{-3} M, whereas miconazole gave 90% inhibition at 1.67 x 10^{-6} M.

3.2.1.6 Inhibition of Growth of *S. cerevisiae* After 24 and 48 hours Treatment With Substituted 1,2-Benzothiazines (48a-48as)

The 1,2-benzothiazine derivatives (see section **3.1.1.5)** which have previously shown activity against various bacterial species (Patel, 2012) were tested in an identical manner as described in **3.2.1.2**. Cells were treated with the compounds for 24 and 48 hours to determine if incubation time had an effect on cell growth inhibition. Of the 45 compounds tested; only 4 compounds displayed significant inhibition (10% or above inhibition) after 24 or 48 hours incubation with the *S. cerevisiae* cells.

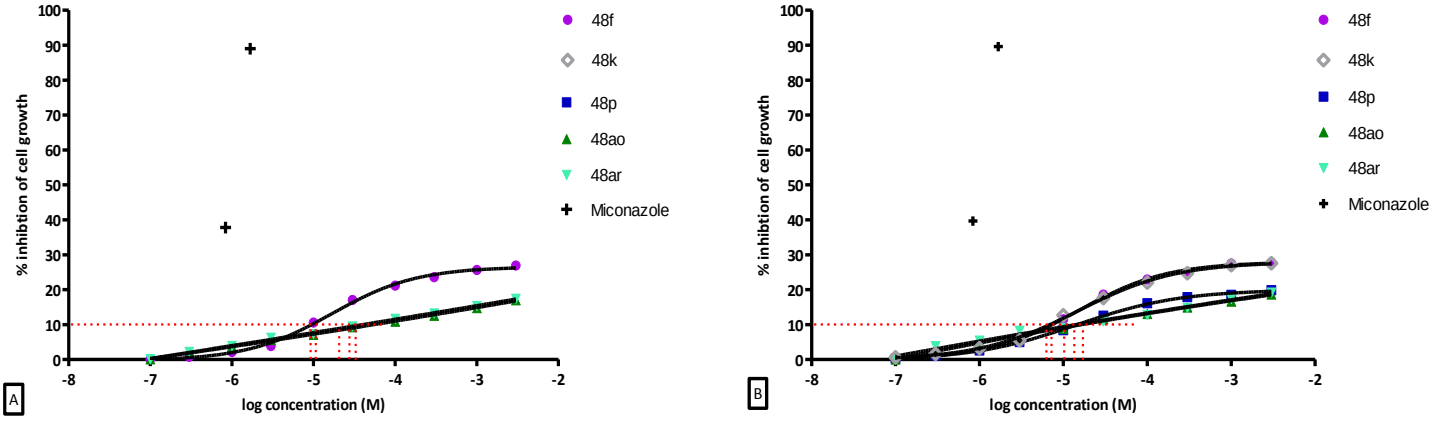


Figure 35- Dose response curves of the various substituted 1,2-benzothiazines; showing the effect of increasing concentration on the % inhibition of cell growth of S. cerevisiae after a 24 (A) and 48 (B) hour treatment. The data is presented as percentages of the control (1.5% DMSO).The reference drug miconazole at a concentration of 0.835 x 10^{-6} M gave an average absorbance (600nm) of 0.614 (38% inhibition) and 0.107 (89% inhibition) at 1.67 x 10^{-6} M. The concentrations of compound resulting in 10% inhibition (IC_{10}) were determined from the dose-response curve. Dose response curves were generated using a graphical software package GraphPad, Prism 5.

The benzothiazine derivatives were not as effective as the piperidone derivatives at inhibiting the growth of *S. cerevisiae*. Only 4 out of the 45 compounds tested exhibited inhibition of 10% and above. Although increasing the concentration increased the level of inhibition, only the compounds (4-bromo-6,7-dimethoxy-1,1-dioxido-3,4-dihydro-2H-benzo[e][1,2]thiazin-3-yl)(p-tolyl) methanone (**48f**) and (4-bromo-6,7-dimethoxy-1,1-dioxido-3,4-dihydro-2H-benzo[e][1,2]thiazin-3-yl)(4 chlorophenyl) methanone (**48k**) gave significant levels of inhibition (approximately 20%). Increasing the incubation time had little effect on the degree of inhibition, with little variability between the data obtained for 24 and 48 hours treatment. Miconazole was a far more potent inhibitor providing 89% inhibition at a concentration of 1.67 x 10^{-6} M.

3.2.1.7 Inhibition of Growth of *S. cerevisiae* After 24 and 48 hours Treatment With 4-((1R,5S)-3-oxo-8-azabicyclo[3.2.1]octan-8-yl) Benzoic acid (47a)

Tropanones belong to the same family of compounds as piperidones, with the difference being the presence of an ethylene bridge. They are also known to possess antifungal properties (Humphery & O'Hagan, 2001). The synthesized 4-((1R,5S)-3-oxo-8-azabicyclo[3.2.1]octan-8-yl) benzoic acid was tested against *S. cerevisiae* for its potency to inhibit cell growth as described in section **3.2.1.2** over 24 and 48 hours incubation. Results indicated that the compound had a minute effect on growth inhibition as it produced less than 10% inhibition. Thus a dose response curve could not be constructed.

3.2.1.8 Inhibition of Growth of *C. albicans* After 48 hours Treatment With A Subset of Ring Substituted 3,5-dibenzylidene-1-methyl piperidin-4-ones

The most potent ring substituted 3,5-dibenzylidene-1-methyl piperidin-4-ones indicated by the results obtained from the screening against *S. cerevisiae* were tested as described in section **3.2.1.2** except that *S. cerevisiae* was replaced with *C. albicans* NCYC 854. Candida cells were treated with the compounds for 48 hours as testing against *S. cerevisiae* had revealed that 48 hour incubations resulted in higher levels of inhibition compared to the 24 hour treatment. All tests were conducted in duplicate and the experiment repeated using a fresh cell growth. The hydrochloride salts were not tested because although the differences were small, the free base compounds generally gave higher levels of inhibition when testing against *S. cerevisiae.* Initial tests confirmed DMSO (1.5%) had no effect on *C. albicans* cell growth.

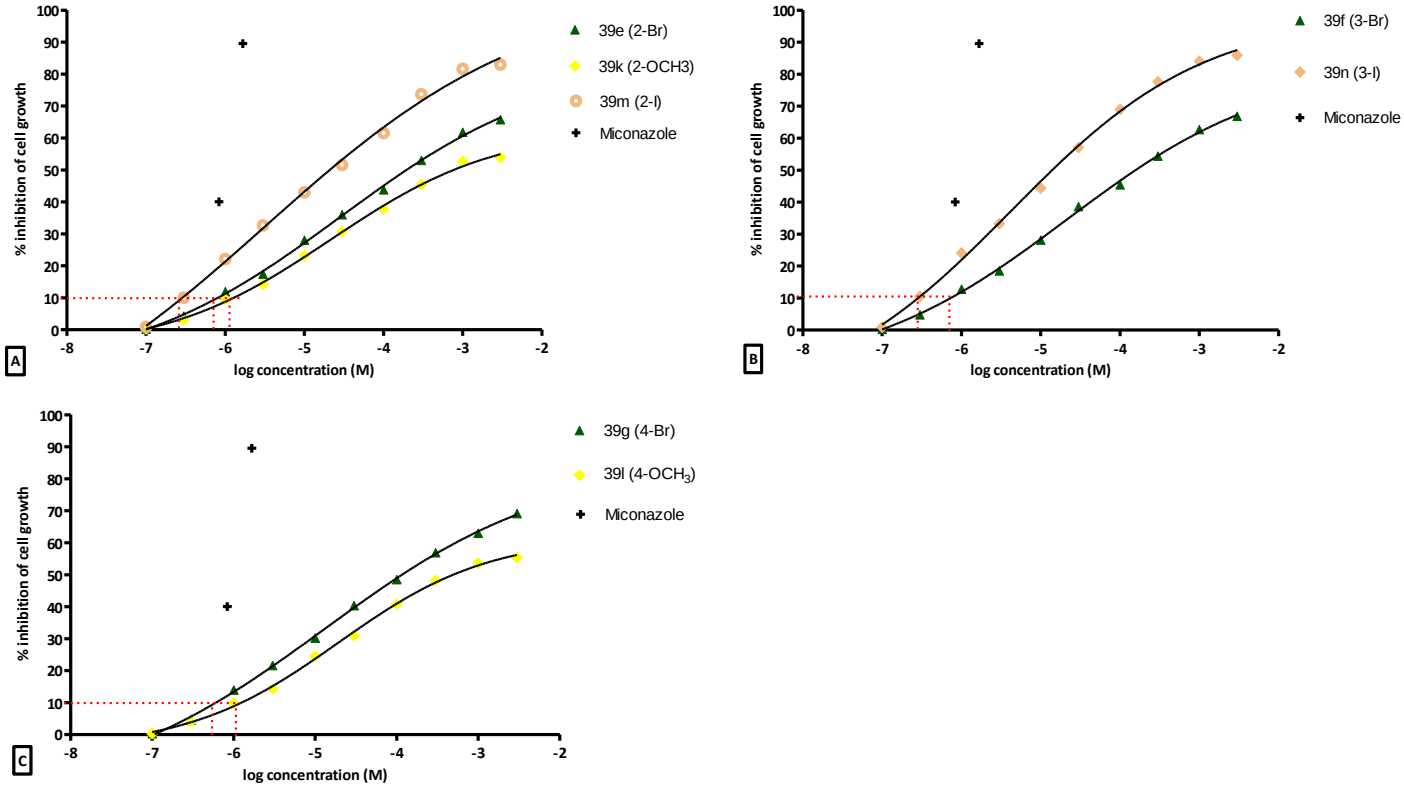


Figure 36- Dose response curves of the free base ortho-substituted (A), meta-substituted (B) and para-substituted (C) 3,5-dibenzylidene-1-methyl piperidin-4-ones; showing the effect of increasing concentration on the % inhibition of cell growth of C. albicans after a 48 hour treatment. The data is presented as percentages of the control (1.5% DMSO). The reference drug miconazole at a concentration of 0.835 x 10^{-6} M gave an average absorbance (600nm) of 0.603 (40% inhibition) and 0.105 (90% inhibition) at 1.67 x 10^{-6} M. The concentrations of compound resulting in 10% inhibition (IC_{10}) were determined from the dose-response curve. IC_{50} values were determined using the graphical software package GraphPad, Prism 5.

The data obtained correlated with that of testing against the *S. cerevisiae* species (**3.2.1.4**). Increasing concentration of each compound resulted in an increase in inhibition of *C. albicans* cell growth. The iodo-substituted compounds gave rise to the most potent inhibitors and para-substituted substituents produced the most potent inhibitors.

3.2.1.9 Inhibition of Growth of *C. albicans* After 48 hours Treatment with Selected Ring Substituted 3,5-dibenzylidene-1-piperidin-4-ones

A selection of ring substituted 3,5-dibenzylidene-1-piperidin-4-ones which gave the highest levels of inhibition as exhibited by screening against *S. cerevisiae* (**3.2.1.5**) after 48 hours incubation were screened against the *C. albicans* species in the same manner. The hydrochloride salts were not tested as the free base compounds produced higher levels of *S. cerevisiae* growth inhibition.

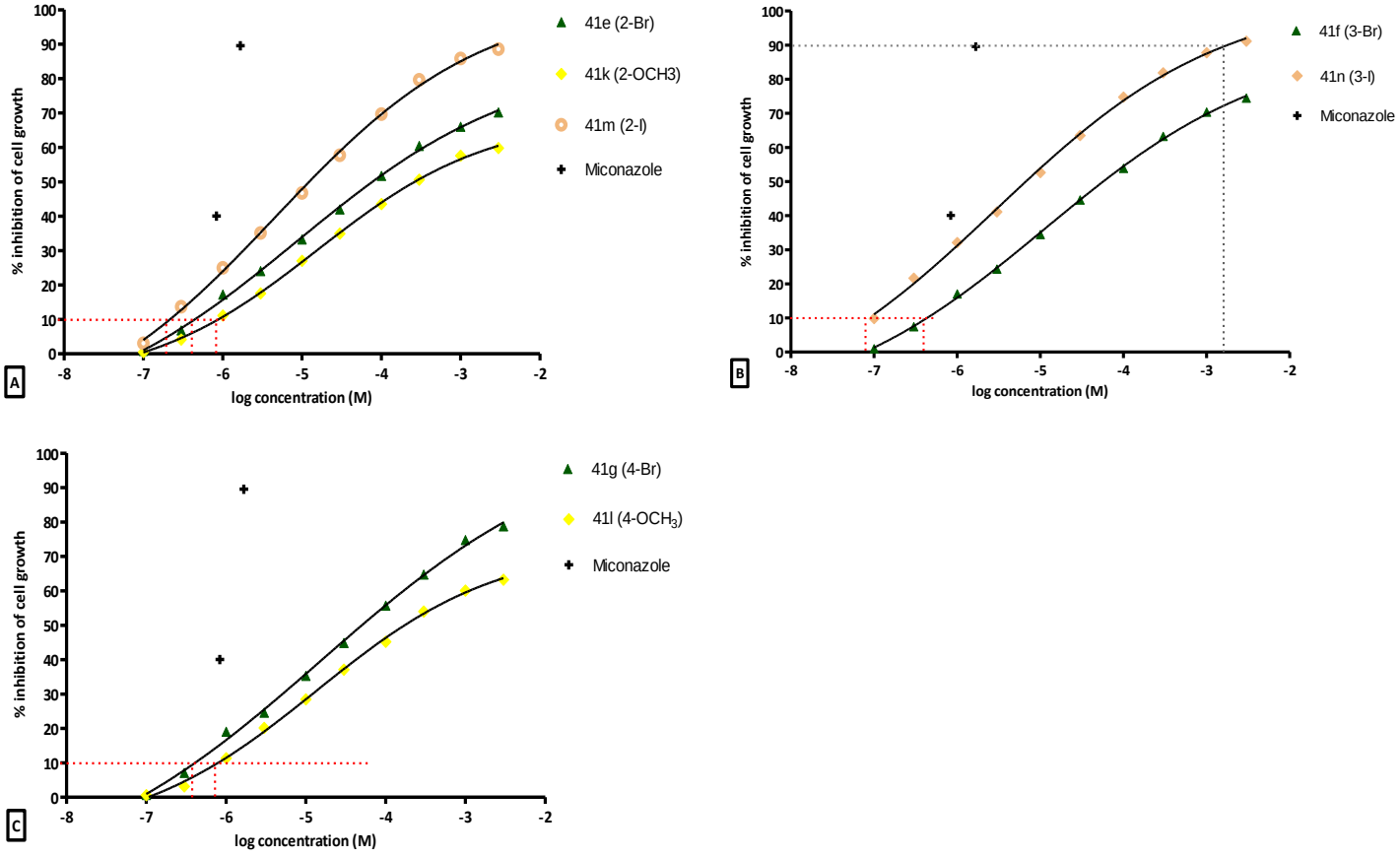


Figure 37- Dose response curves of the free base ortho-substituted (A), meta-substituted (B) and para-substituted (C) 3,5-dibenzylidene-1-piperidin-4-ones; showing the effect of increasing concentration on the % inhibition of cell growth of C. albicans after a 48 hour treatment. The data is presented as percentages of the control (1.5% DMSO). The reference drug miconazole at a concentration of 0.835 x 10^{-6} M gave an average absorbance (600nm) of 0.603 (40% inhibition) and 0.105 (90% inhibition) at 1.67 x 10^{-6} M. The concentrations of compound resulting in 10% and 90% inhibition (IC_{10}, IC_{90}) were determined from the dose-response curve. IC_{50} values were determined using the graphical software package GraphPad, Prism 5.

The compounds had a similar inhibitory effect on the growth of *C. albicans* as compared to *S. cerevisiae* (section **3.2.1.5, Figure 34**). In both cases increasing the concentration of the compound gave an increase in inhibition. Para substituted compounds were more potent than ortho or meta substituted compounds. The iodo-substituted compounds gave rise to the most effective inhibitor, with the 3'-iodo substituted 3,5-dibenzylidene-1-piperidin-4-one giving the highest level of inhibition (91%) at a concentration of 3×10^{-3} M. Whereas miconazole nitrate at a concentration of 1.67×10^{-6} inhibited 90% of cell growth.

Table 5- The relevant IC_{10}, IC_{50} and IC_{90} values of each compound tested for growth inhibition against various fungal species over 24 and 48 hours.

Compound type	Compound no.	Fungal species	*S. cerevisiae* ATCC 46182						*C. albicans* NCYC 854		
		% inhibition	IC_{10} (x 10^{-6} M)		IC_{50} (x 10^{-6} M)		IC_{90} (x 10^{-3} M)		IC_{10} (x 10^{-6} M)	IC_{50} (x 10^{-6} M)	IC_{90} (x 10^{-3} M)
		Time of incubation (hours)	24	48	24	48	24	48	48	48	48
Ring substituted 3,5-dibenzylidene-1-methyl piperidin-4-ones	39a		129	59	N/R	N/R	N/R	N/R	N/R	N/R	N/R
	39b		9.12	6.3	N/R	N/R	N/R	N/R	N/R	N/R	N/R
	39c		8.71	5.9	N/R	N/R	N/R	N/R	N/R	N/R	N/R
	39d		7.24	4.8	N/R	N/R	N/R	N/R	N/R	N/R	N/R
	39e		0.87	0.59	23.12	19.7	N/R	N/R	0.63	21.5	N/R
	39f		0.79	0.56	17.97	16.85	N/R	N/R	0.59	17.3	N/R
	39g		0.58	0.46	14.57	10.93	N/R	N/R	0.51	13.1	N/R
	39h		32.4	23.9	N/R	N/R	N/R	N/R	N/R	N/R	N/R
	39i		32.3	19.5	N/R	N/R	N/R	N/R	N/R	N/R	N/R
	39j		28.2	14.1	N/R	N/R	N/R	N/R	N/R	N/R	N/R
	39k		16.2	1.35	22.89	22.5	N/R	N/R	1.15	28.8	N/R
	39l		14.5	0.91	22.46	11.7	N/R	N/R	1.02	19.3	N/R
	39m		0.33	0.28	6.52	4.44	N/R	N/R	0.26	3.18	N/R
	39n		0.32	0.26	6.19	4.27	N/R	N/R	0.26	2.67	N/R
HCl salts of ring substituted of 3,5-dibenzylidene-1-methyl piperidin-4-ones	40a		603	316	N/R	N/R	N/R	N/R	N/R	N/R	N/R
	40b		25.1	12.6	N/R	N/R	N/R	N/R	N/R	N/R	N/R
	40c		22.4	10.5	N/R	N/R	N/R	N/R	N/R	N/R	N/R
	40d		12.3	7.6	N/R	N/R	N/R	N/R	N/R	N/R	N/R
	40e		1.15	0.68	29.9	22.9	N/R	N/R	N/R	N/R	N/R
	40f		0.96	0.62	20.6	20.14	N/R	N/R	N/R	N/R	N/R
	40g		0.72	0.5	18.4	13.7	N/R	N/R	N/R	N/R	N/R
	40h		64.6	30.2	N/R	N/R	N/R	N/R	N/R	N/R	N/R
	40i		52.5	25.1	N/R	N/R	N/R	N/R	N/R	N/R	N/R
	40j		38	22.9	N/R	N/R	N/R	N/R	N/R	N/R	N/R
	40k		1.74	1.29	35.4	27.7	N/R	N/R	N/R	N/R	N/R
	40l		1.55	0.98	33.2	29.2	N/R	N/R	N/R	N/R	N/R
	40m		0.42	0.32	9.5	7.38	N/R	N/R	N/R	N/R	N/R
	40n		0.38	0.29	8.03	6.87	N/R	N/R	N/R	N/R	N/R

N/R: not reached, particular level of inhibition not reached by tested compound.

Table 5 (continued) - The relevant IC_{10}, IC_{50} and IC_{90} values of each compound tested for growth inhibition against various fungal species over 24 and 48 hours.

Compound type	Compound no.	Fungal species	*S. cerevisiae* ATCC 46182						*C. albicans* NCYC 854		
		% inhibition	IC_{10} (x 10^{-6} M)		IC_{50} (x 10^{-6} M)		IC_{90} (x 10^{-3} M)		IC_{10} (x 10^{-6} M)	IC_{50} (x 10^{-6} M)	IC_{90} (x 10^{-3} M)
		Time of incubation (hours)	24	48	24	48	24	48	48	48	48
Ring substituted 3,5-dibenzylidene-1-piperidin -4-ones	41a		123	56.2	N/R	N/R	N/R	N/R	N/R	N/R	N/R
	41b		5.62	3.89	N/R	N/R	N/R	N/R	N/R	N/R	N/R
	41c		4.17	3.47	N/R	N/R	N/R	N/R	N/R	N/R	N/R
	41d		3.63	2.45	N/R	N/R	N/R	N/R	N/R	N/R	N/R
	41e		0.5	0.44	17.03	7.96	N/R	N/R	0.44	8.54	N/R
	41f		0.45	0.42	15.89	7.89	N/R	N/R	0.38	7.98	N/R
	41g		0.39	0.33	13.2	7.62	N/R	N/R	0.35	7.53	N/R
	41h		67.6	30.2	N/R	N/R	N/R	N/R	N/R	N/R	N/R
	41i		23.9	11.2	N/R	N/R	N/R	N/R	N/R	N/R	N/R
	41j		20.9	6.61	N/R	N/R	N/R	N/R	N/R	N/R	N/R
	41k		1.02	0.91	20.37	11.8	N/R	N/R	0.79	15.49	N/R
	41l		0.93	0.76	19.77	10.9	N/R	N/R	0.69	12.88	N/R
	41m		0.26	0.19	4.71	4.15	N/R	N/R	0.2	2.9	N/R
	41n		0.17	0.11	3.22	3.39	N/R	1.35	0.083	2.34	1.55
HCl salts of ring substituted 3,5-dibenzylidene- 1-piperidin-4-ones	42a		126	74.1	N/R	N/R	N/R	N/R	N/R	N/R	N/R
	42b		7.24	5.37	N/R	N/R	N/R	N/R	N/R	N/R	N/R
	42c		5.37	3.8	N/R	N/R	N/R	N/R	N/R	N/R	N/R
	42d		4.57	3.4	N/R	N/R	N/R	N/R	N/R	N/R	N/R
	42e		0.63	0.49	22.72	8.49	N/R	N/R	N/R	N/R	N/R
	42f		0.51	0.46	21.7	11.9	N/R	N/R	N/R	N/R	N/R
	42g		0.47	0.37	16.8	7.79	N/R	N/R	N/R	N/R	N/R
	42h		38	33.1	N/R	N/R	N/R	N/R	N/R	N/R	N/R
	42i		30.2	13.5	N/R	N/R	N/R	N/R	N/R	N/R	N/R
	42j		21.4	9.77	N/R	N/R	N/R	N/R	N/R	N/R	N/R
	42k		1.35	0.96	22.3	15.25	N/R	N/R	N/R	N/R	N/R
	42l		1.15	0.81	22.1	10.3	N/R	N/R	N/R	N/R	N/R
	42m		0.26	0.22	4.83	4.35	N/R	N/R	N/R	N/R	N/R
	42n		0.21	0.18	4.62	3.93	N/R	N/R	N/R	N/R	N/R
Substituted 1,2-benzothiazines	48f		7.94	6.03	N/R	N/R	N/R	N/R	N/R	N/R	N/R
	48k		8.71	6.92	N/R	N/R	N/R	N/R	N/R	N/R	N/R
	48p		18.62	9.55	N/R	N/R	N/R	N/R	N/R	N/R	N/R
	48ao		26.3	12.3	N/R	N/R	N/R	N/R	N/R	N/R	N/R
	48ar		31.6	16.59	N/R	N/R	N/R	N/R	N/R	N/R	N/R

The dose response curves and IC_{50} values were generated using the software package GraphPad, Prism 5. The concentrations required to inhibit 10% or 90% cell growth (IC_{10} and IC_{90}) were obtained manually from the dose response curves. The free base compounds produced slightly lower IC_{10} and IC_{50} values when compared to their respective hydrochloride salts. Results indicated that 48 hours treatment with the compounds gave lower IC_{10} and IC_{50} values rather than 24 hours. The most potent compound was **41n** (3'-iodo 3,5-dibenzylidene-1-piperidin-4-one) with the lowest IC_{10} and IC_{50} values against both species of yeast respectively. It is also the only compound to give greater than 90% inhibition of cell growth for both species giving IC_{90} values of 1.35×10^{-3} M against *S. cerevisiae* and 1.55×10^{-3} M against *C. albicans* after 48 hours incubation, whereas miconazole nitrate, the reference antifungal, at a concentration of 1.67×10^{-6} M gave 89.5% inhibition of cell growth against both species with the same incubation period. Compounds with no ring substituents (**39a, 40a, 41a and 42a**) produced the highest IC_{10} and IC_{50} values thus the lowest level of growth inhibition. Para substitution gave lower IC_{10} and IC_{50} values when compared to meta or ortho substituted compounds. The free base 3,5-dibenzylidene-1-piperidin-4-ones (**41a-41n**) produced the lowest corresponding IC_{10} and IC_{50} values against both *S. cerevisiae* and *C. albicans.* A selected subset of compounds as defined by their potencies against *S. cerevisiae* were tested against *C. albicans* producing similar results suggesting that the compounds had an equal effect on both types of species.

The benzene ring substituted piperidones gave lower IC_{10} and IC_{50} values when compared to the 1,2-benzothiazine derivatives or the tropan-3-one. Of the 45 1,2-benzothiazine compounds tested; only four compounds displayed inhibition of greater than 10% after 24 or 48 hours incubation with *S. cerevisiae.* Compound **47a** (4-((1R,5S)-3-oxo-8-azabicyclo[3.2.1]octan-8-yl) benzoic acid) was an ineffective inhibitor of cell growth as it produced inhibition of below 10%.

3.2.2 Inhibition of Growth of *Aspergillus niger*

3.2.2.1 Inhibition of growth of *A. niger* After 48 and 72 hours Treatment With A Subset of Ring Substituted 3,5-dibenzylidene-1-methyl piperidin-4-ones

A subset of compounds selected by their ability to inhibit growth of yeasts to levels greater than 50% were examined for their effect on the inhibition of growth of the filamentous

fungus *A. niger* ATCC 16888 using the disk diffusion assay (Fothergill, 2012, CLSI, 2013). PDA plates were inoculated with 8.6 x 10^5 to 2.4 x 10^6 cfu/ml which were obtained from 7-day old cultures and adjusted to optical densities (530nm) between 0.09-0.11 by dilution with sterile deionised water. Plates were loaded with pre-soaked paper disks containing various amounts of the test compounds, miconazole (0.6µg) and DMSO (1.5%). The plates were incubated in ambient air at 35°C within 10 minutes of disk application and zone of inhibition diameters were measured after 16, 24 and 72 hours of incubation. All tests were carried out in duplicates and the experiment repeated using a fresh growth of *A. niger*. Preliminary tests revealed that DMSO had no effect on the cell growth of *A. niger.* Of the seven selected 3,5-dibenzylidene-1-methyl piperidin-4-ones tested only three showed meaningful growth inhibition after 72 hours. None of the compounds tested inhibited cell growth after 16 or 24 hours incubation. Zone of inhibition diameters were determined by measuring the radius of the zone of inhibition (r) and the width/diameter (D). The following equation was then applied to determine an average diameter: 2r+D /2.

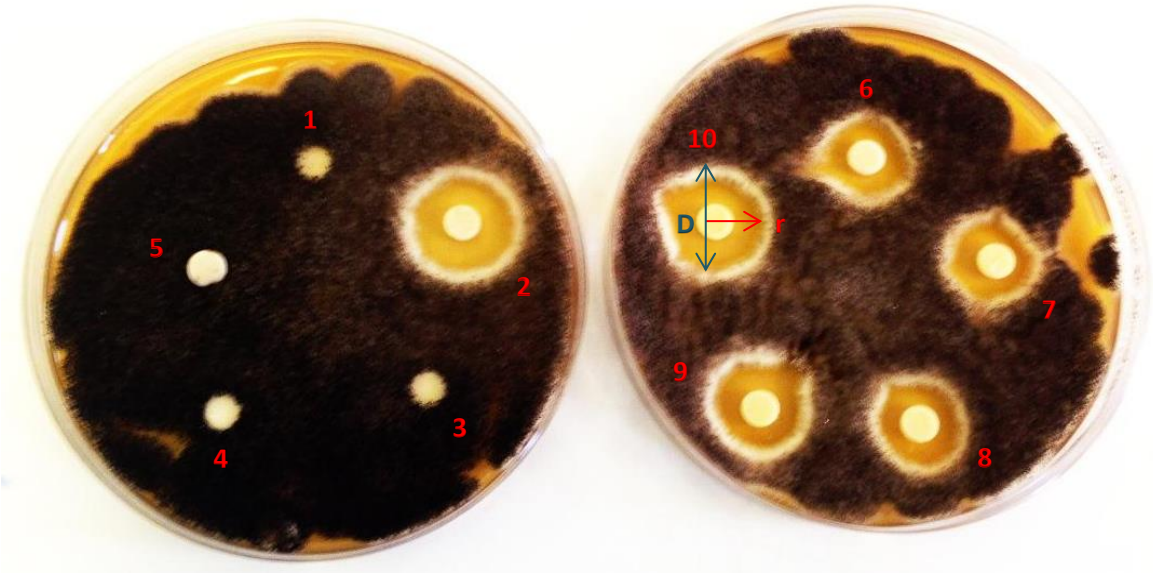


Figure 38- Disk diffusion assay showing the zone of inhibition diameters against A. niger ATCC 16888 for 1.5% DMSO (1), 10µl of 1.253µM (12.53 nmoles) miconazole (2) and various amounts (10µl of 1 x10^{-6} M to 3 x10^{-3} M) of 3,5-Bis(3-bromobenzylidene)-1-methylpiperidin-4-one (4f) (3) to (10) following 72 h incubation. Zone of inhibition diameters were determined by measuring 'D' and 'r' of each relevant inhibition zone. Corrected diameters were determined by subtracting the diameter of the paper disks (6mm) from the diameter of the zone of inhibition.

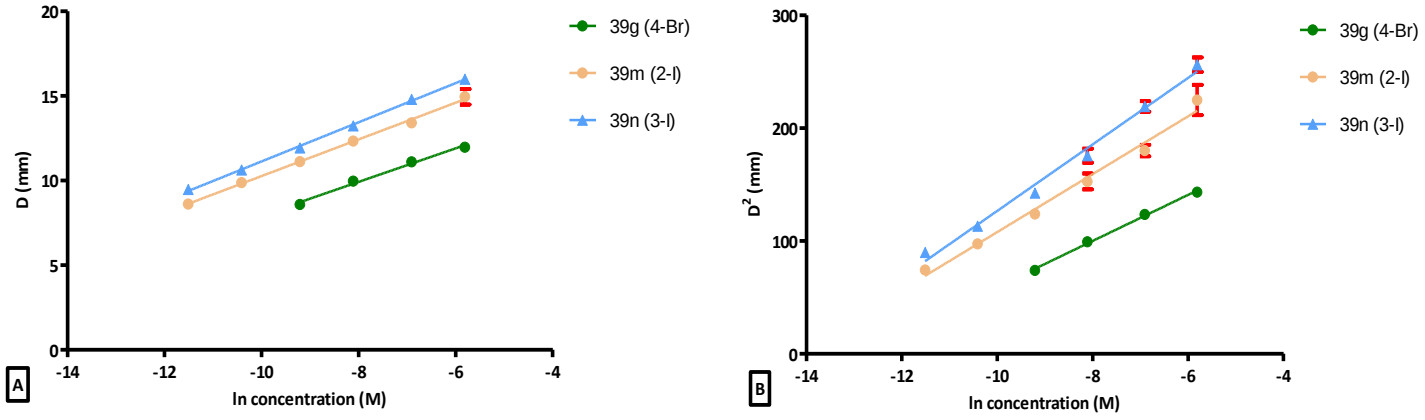
Assays of microbial susceptibility to drugs by the agar diffusion method are analysed using linear fitting of the diameter or squared diameter of the inhibition zones against the natural

logarithm (ln) of drug concentration at the source. (Bonev, Hooper, & Parisot, 2008).This reflects a solution of the differential equation describing free diffusion in one-dimension (refer to Equation **2**, Page 13). In order to find the most suitable linear fitting various graphs were plotted of D, D^2, Ď (corrected by subtracting from the diameter of the paper disks) and $Ď^2$ against ln of the drug concentrations. The R^2 values were deduced from each graph and then compared (Microsoft Excel). R^2 values indicate how well data points fit in a linear regression as it represents the variation of the data explained by the fitted line. The closer the R^2 value is to 1 the better the linear regression fit.

Table 6- R^2 values for ring substituted 3,5-dibenzylidene-1-methyl piperidin-4-ones from linear regression analysis using quadratic or linear dependence of zone size (mm) against ln (c)

		R^2 value							
Compound no.	Compound type	Ring substituted 3,5-dibenzylidene-1-methyl piperidin-4-ones							
	Incubation time (hours)	48				72			
	Parameter	D	D^2	D - Ď	$(D - Ď)^2$	D	D^2	D - Ď	$(D - Ď)^2$
39g		0.990	0.997	0.990	1.000	0.986	0.980	0.986	0.966
39m		0.998	0.988	0.998	0.965	0.989	0.961	0.989	0.913
39n		0.999	0.990	0.999	0.974	0.980	0.954	0.980	0.918

From the data in table 6 it was evident that plots of D and D^2 against ln (concentration) gave better fitted linear regressions as their corresponding R^2 values were closer to 1. Therefore plots of D and D^2 against ln (concentration) were generated using the software package GraphPad, Prism 5 following which the minimum concentration required to induce cell growth inhibition (MIC) was deduced for each of the compound tested. MIC values were deduced from the x-intercept of each graph.



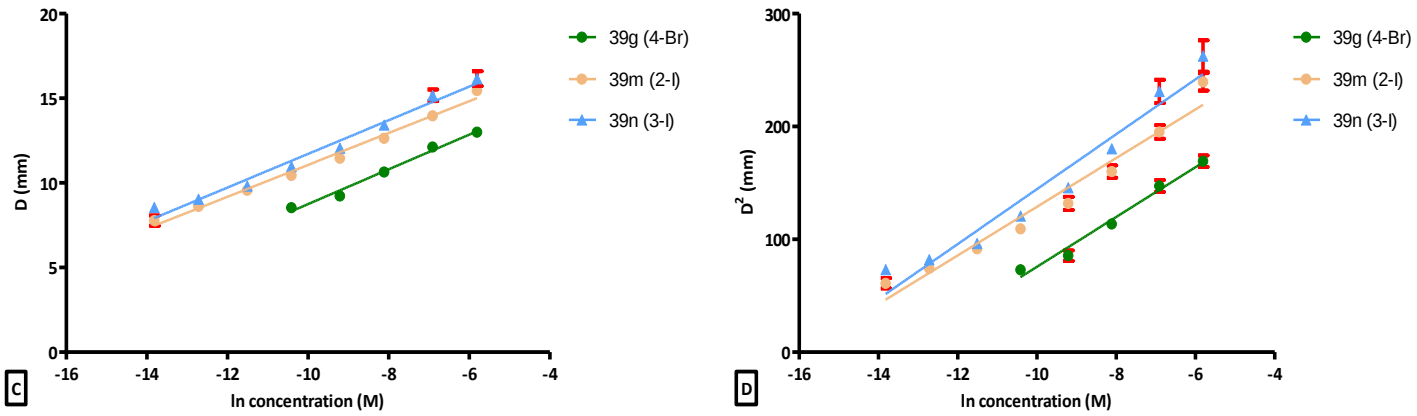


Figure 39- Linear regression fits of selected 3,5-dibenzylidene-1-methyl piperidin-4-ones against A. niger showing the effect on the zone of inhibition diameter (D) as amount of compound loaded is increased. Inhibition zone diameters D (mm) and their squared values (D^2) were plotted against ln (conc) in linear fits. The effects on D and D^2 after 48 hours treatment with the test compounds is shown by graphs A and B, whereas graphs C and D show the effect of 72 hours treatment. The reference drug miconazole nitrate (10µl of 1.253µM (12.53 nmoles) gave an average zone of inhibition diameter of 17mm after 48 hours incubation and 18mm after 72 hours incubation. All graphs were constructed using the graphical software package GraphPad, Prism 5 and MIC values were determined by each respective intercept from the linear fits.

None of the test compounds produced zone of inhibition at 16 and 24 hours of incubation. From the 7 selected compounds only 3 compounds produced measurable zone of inhibition diameters after 48 and 72 hours incubation. Increasing the concentration of these compounds increased the zone of inhibition diameters. Increasing the incubation period from 48 to 72 hours also showed an improvement in cell growth inhibition. Compound **39g** (3,5-bis(4-bromobenzylidene)-1-methylpiperidin-4-one) was the least potent giving lower diameters at each given concentration, whereas compound **39n** (3,5-bis(3-iodobenzylidene)-1-methylpiperidin-4-one) was the most potent growth inhibitor. Graph D shows that the iodo-substituted compounds (**39m** and **39n**) do not give linear regression of best fit when plotting D^2 against ln (concentration) after a 72 hour treatment suggesting that hydrophobicity is a key factor affecting their potency to inhibit cell growth. (Bonev *et al*, 2008).

3.2.2.2 Inhibition of Growth of *A. niger* After 48 and 72 hours Treatment With A Subset of Ring Substituted 3,5-dibenzylidene-1-piperidin-4-ones

On the basis of the highest potency as distinguished by susceptibility testing against yeast a selection of ring substituted 3,5-dibenzylidene-1-piperidin-4-ones were examined for their potency to inhibit growth of *A. niger* ATCC 16888 in the same manner described in **3.2.2.1**.

Of the seven compounds selected for screening only three compounds produced zone of inhibition diameters after 48 hours incubation and four compounds showed activity after 72 hours.

Table 7- R^2 values for ring substituted 3,5-dibenzylidene-1-piperidin-4-ones from linear regression analysis using quadratic or linear dependence of zone size (mm) against ln (c)

		R^2 value							
Compound no.	Compound type	Ring substituted 3,5-dibenzylidene-1-piperidin-4-ones							
	Incubation time (hours)	48				72			
	Parameter	D	D^2	D - Ď	$(D - Ď)^2$	D	D^2	D - Ď	$(D - Ď)^2$
41g		0.996	0.997	0.996	0.992	0.995	0.992	0.995	0.977
41l		N/A	N/A	N/A	N/A	0.989	0.978	0.989	0.956
41m		0.993	0.988	0.993	0.976	0.978	0.949	0.978	0.913
41n		0.995	0.986	0.995	0.971	0.983	0.962	0.983	0.936

The R^2 values for plots of D and D^2 against ln (concentration) were closer to 1 thus giving better linear fits. Plots of D and D^2 against ln (concentration) were constructed and MIC values deduced from the x-intercepts respectively.

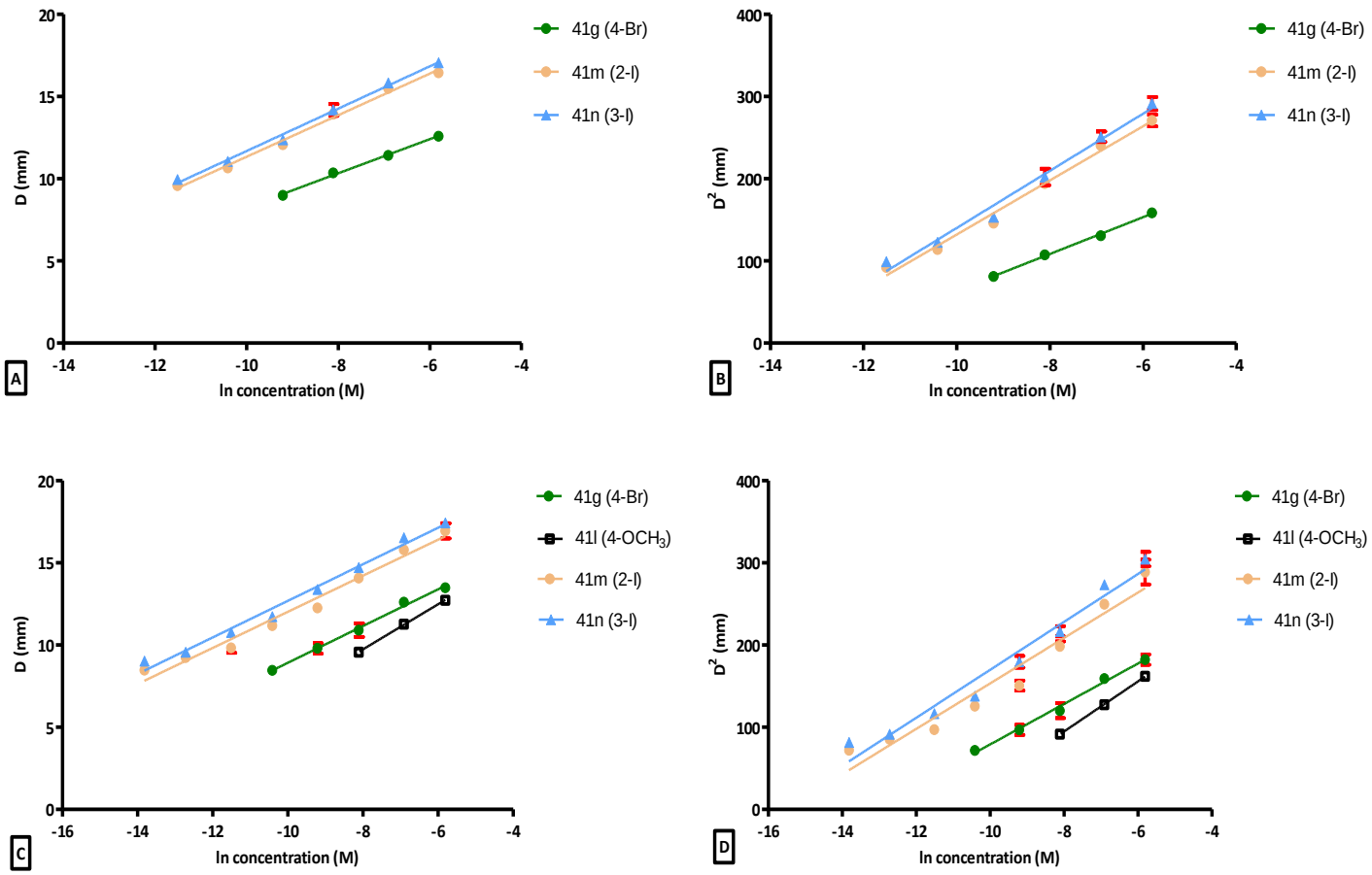


Figure 40- Linear regression fits of selected 3,5-dibenzylidene-1-piperidin-4-ones against A. niger showing the effect on the zone of inhibition diameter (D) as amount of compound loaded is increased. Inhibition zone diameters D (mm) and their squared values (D^2) were plotted against ln (conc) in linear fits. The effects on D and D^2 after 48 hours treatment with the test compounds is shown by graphs A and B, whereas graphs C and D show the effect of 72 hours treatment. The reference drug miconazole nitrate (10µl of 1.253µM (12.53 nmoles) gave an average zone of inhibition diameter of 17mm after 48 hours incubation and 18mm after 72 hours incubation. All graphs were constructed using the graphical software package GraphPad, Prism 5 and MIC values were determined by each respective intercept from the linear fits.

Equally to the ring substituted 3,5-dibenzylidene-1-methyl piperidin-4-ones (figure 39), the 3,5-dibenzylidene-1- piperidin-4-ones (figure 40) showed no zone of inhibition after 16 and 24 hours incubation. From the 7 test compounds screened only 4 compounds showed activity after 48 and 72 hours incubation. Increasing the concentration of the compounds resulted in an increase in inhibition of cell growth as shown by an increase in zone of inhibition diameters. The screened compounds were more effective after 72 hours incubation with *A. niger* than 48 hours. Of the compounds tested the 4'-methoxy substituted derivative (**41l**) was the least potent inhibitor whereas the 3'-iodo substituted compound (**41n**) was the most potent. As shown in graph D (D^2 against ln (concentration)

the iodo substituted compounds (**41m and 41n**) do not give linear regression of best fit after 72 hours of treatment proposing that hydrophobicity of these compounds affects the potency to inhibit cell growth (Bonev *et al,* 2008).

Table 8- Susceptibility of *A. niger* to a subset of 3,5-dibenzylidene-1-methyl piperidin-4-ones and 3,5-dibenzylidene-1-piperidin-4-ones. MIC (M) and R^2 values from linear regression analysis using quadratic $((D - \check{D})^2)$ or linear dependence of zone diameter (mm) on ln (concentration)

Compound	Compound Nº	Incubation time (hours)	48		72	
		Parameter	D/ln (C)	D^2/ln (C)	D/ln (C)	D^2/ln (C)
Ring substituted 3,5-dibenzylidene-1-methyl piperidin-4-ones	39g	MIC (x 10^{-8} M)	1.51	247	0.91	147
		R^2	0.9897	0.9971	0.9857	0.9803
	39m	MIC (x 10^{-8} M)	0.35	67.4	0.036	11.6
		R^2	0.9976	0.9881	0.9894	0.961
	39n	MIC (x 10^{-8} M)	0.29	61.6	0.035	11.9
		R^2	0.9989	0.99	0.9802	0.9543
Ring substituted 3,5-dibenzylidene-1-piperidin-4-ones	41g	MIC (x 10^{-8} M)	1.62	263	1.55	183
		R^2	0.9956	0.9973	0.995	0.9915
	41l	MIC (x 10^{-8} M)	N/A	N/A	28.2	1490
		R^2	N/A	N/A	0.9889	0.9779
	41m	MIC (x 10^{-8} M)	0.58	82.3	0.079	17.8
		R^2	0.9932	0.988	0.9776	0.9494
	41n	MIC (x 10^{-8} M)	0.52	80.7	0.05	13.5
		R^2	0.9949	0.9855	0.9831	0.9624

The MIC for each of the test compounds decreased significantly as the time of incubation was increased from 48 to 72 hours. In general the iodo- substituted 3,5-dibenzylidene-1-methyl piperidin-4-ones and 3,5-dibenzylidene-1-piperidin-4-ones were the most potent as displayed by their lower MIC values compared to the other compounds. However meta substitution provided a more potent inhibitor. Compound **39n** was the most potent compound giving the lowest MIC values after 72 hours incubation from both plots (3.6 x 10^{-10} M (D/ln (c)) and 11.6 x 10^{-8} M (D^2/ln (C)). Compound **41l** was the least potent compound with the highest MIC values after 72 hours incubation as determined from each plot (2.82 x 10^{-7} M (D/ln (C)) and 1.49 x 10^{-5} M (D^2/ln (C)). The quadratic model was accurate for compounds **39g** and **41g** whereas linear fits are more accurate in all other cases.

3.2.3 Cytotoxic Evaluation of Selected Compounds Against human carcinoma A549 cell line

3.2.3.1 Cytotoxic Studies of Selected 3,5-dibenzylidene-1-piperidin-4-ones using The MTS Assay

The cytotoxic properties of four selected 3,5-dibenzylidene-1-piperidin-4-ones (as exemplified by their higher potencies against yeasts or filamentous fungi) against mammalian A549 ATCC CCL-185 cells were analysed using the MTS assay. The MTS assay was utilised to determine the effect on the mitochondrial activity of the cells by the test compounds. Sterile polystyrene 96-well plates were seeded with cells (1 x 10^4 cells/100µl/well) and incubated for three days at 37°C, at an atmosphere of 5% CO_2 to ensure the cells were in mid exponential phase for testing purposes. Post incubation the cells (3.7 x 10^4 cells/100µl/well) were treated with various concentrations of the test compounds as well as a positive (kill) control (5% Triton X-100) and a negative control (1% DMSO) and re-incubated for a further 48 hours. To each well of the 96-well plates 20µl of the MTS reagent (317µg/ml) was added, following which the plates were incubated for 3 hours. Absorbance readings at 490nm were obtained (Labsystems; Multiskan Ascent) and results were expressed as percent cytotoxicity as compared to the controls (1% DMSO, 5% Triton X-100) (see Equation **4**, Page 73). Preliminary results showed DMSO (1%) had no cytotoxic effect on the cells.

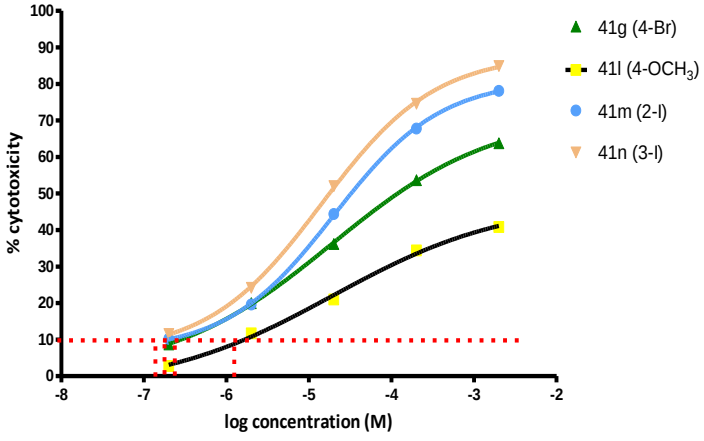


Figure 41- Dose response curves of the free base ring substituted 3,5-dibenzylidene-1-piperidin-4-ones; showing the effect of increasing concentration on the % cytotoxicity of A549 ATCC CCL-185 after 48 hours of treatment. The data is presented as percentages of the controls (1% DMSO and 5% Triton X-100). The positive control (5% Triton X-100) gave an average absorbance (490nm) of 0.2 after 48 hours incubation. The concentrations of compound giving 10% cytotoxicity (IC_{10}) were determined manually from the dose-response curve. IC_{50} values were determined using the graphical software package GraphPad, Prism 5.

Increasing the concentration of the test compounds consequently increased the percent cytotoxicity against the A549 cells suggesting that each compound is reducing the mitochondrial activity of these cells. However it was evident that the iodo substituted 3,5-dibenzylidene-1-piperidin-4-ones were the most effective compounds as shown by their increased cytotoxicity. 3,5-bis(3-iodobenzylidene) piperidin-4-one (**41n**) was the most effective compound as it had the highest percent cytotoxicity compared to the other screened compounds whereas compound **41l** (3,5-bis(4-methoxybenzylidene) piperidin-4-one was the least potent.

3.2.3.2 Cytotoxic studies of Selected 3,5-dibenzylidene-1-piperidin-4-ones using The LDH Assay

The cytotoxic properties of the selected compounds in respect to their effect on the membrane integrity of A549 ATCC CCL-185 cells was analysed using the Lactate Dehydrogenase (LDH) assay. The assay measures changes in fluorescence where an increase indicates higher levels of LDH thus indicating a greater loss of membrane integrity. The cells were seeded and treated with the same test compounds in a similar fashion as described in **3.2.3.2.** Post 48 hour treatment CytoTox-ONE reagent (100µl) was added to each well of the plates and the plates were incubated at 22°C for 10 minutes, after which 50µl of 'stop' solution was added to each well. Plates were shaken for 10 seconds and the fluorescence was measured using an excitation (E_x) wavelength of 560nm and an emission (E_m) wavelength of 590nm (PerkinElmer LS55 Fluorescence spectrophotometer fitted with a 96-well plate reader attachment). Results were expressed as percent cytotoxicity as compared to the controls (1% DMSO, 5% Triton X-100) (see Equation **5**, Page 74).

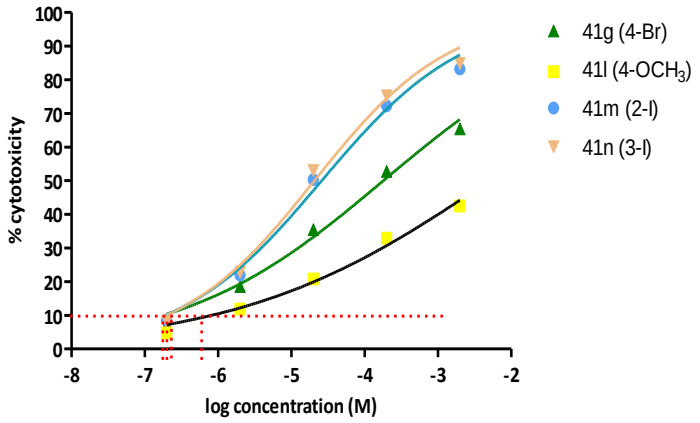


Figure 42- Dose response curves of the free base ring substituted 3,5-dibenzylidene-1-piperidin-4-ones; showing the effect of increasing concentration on the % cytotoxicity of A549 ATCC CCL-185 cells after a 48 hour treatment. The data is presented as percentages of the controls (1% DMSO and 5% Triton X-100). The positive control (5% Triton X-100) gave an average Fluorescence (E_x: 560nm, E_m: 590nm) of 869 after 48 hours incubation. The concentrations of compound resulting in 10% cytotoxicity (IC_{10}) were determined manually from the dose-response curve. IC_{50} values were determined using the graphical software package GraphPad, Prism 5.

Increasing the concentration of test compounds resulted in an increase in damage to the membrane integrity thus an increase in cytotoxicity. The data obtained bears a resemblance to the results in figure 41 (section **3.2.3.1**) with compound **41n** being the most potent cytotoxic compound thus having the most effective damaging effect on membrane integrity and compound **41l** the least cytotoxic compound.

Table 9- The relevant IC_{10}, IC_{50} and IC_{90} values of selected 3,5-dibenzylidene-1-piperidin-4-ones compounds tested for cytotoxicity studies against A549 ATCC CCL-185 cells after 48 hours treatment using the MTS and LDH assays.

Compound Number	Assay type	MTS			LDH		
	% cytotoxicity	IC_{10} (x 10^{-7} M)	IC_{50} (x 10^{-4} M)	IC_{90}	IC_{10} (x 10^{-7} M)	IC_{50} (x 10^{-4} M)	IC_{90}
41g		2.60	1.05	N/R	2.19	1.8	N/R
41l		12.3	54	N/R	5.62	50.6	N/R
41m		1.95	0.30	N/R	1.95	0.26	N/R
41n		1.41	0.17	N/R	1.62	0.21	N/R

N/R: not reached, particular level of inhibition not reached by tested compound.

The IC_{50} values obtained for each of the tested compounds between the two assays were fairly similar**.** In both cases compound **41n** (3,5-bis(3-iodobenzylidene) piperidin-4-one) provided the lowest IC_{50} values (MTS: 0.17 x10^{-4} M, LDH: 0.21 x10^{-4} M) implying it is the

most cytotoxic compound thus having the most potent effect on membrane integrity and mitochondrial activity. With IC_{50} values of 5.4 x 10^{-3} M (MTS) and 5.06 x 10^{-3} M (LDH) 3,5-bis(4-methoxybenzylidene) piperidin-4-one (**41l**) is the least cytotoxic compound of those tested. Of the two iodo-substituted compounds tested meta substitution gave rise to a more cytotoxic compound.

Chapter 4

Discussion

Chapter 4.1: Synthetic Procedures

The need for novel antifungal and anticancer agents has significantly increased over recent years due to an rise in cancer cases and increased resistance of pathogenic fungi to clinically used antifungal agents, with only a few novel antifungal and anticancer agents being introduced in the last decade (Norby *et al*, 2005). The European Society of Chemical Microbiology and Infectious Diseases (ESCMID) has noted that production of novel compounds has decreased considerably over recent years (Norby *et al,* 2005). Resistance occurs predominantly as a result of altered genetics (Projan & Youngman, 2002). The number of cases of multidrug resistant fungal infections is increasing (Gulshan & Moye-Rowley, 2007) suggesting an urgent need for new classes of antifungal agents (Oren, Yalcin, Sener & Ucarturk, 2004). The emergence of drug-resistant strains of fungi is a significant threat to human and animal health as immunocompromised patients such as those suffering from HIV/AIDS, undergoing chemotherapy or organ transplantation are more susceptible to fungal infections (Norby *et al,* 2005). The antifungal and anticancer properties of some novel tropan-3-ones (Dimmock *et al,* 2002), piperidones (Pati *et al,* 2009) and libraries of 1,2-benzothiazines (Park *et al,* 2002, Ahmad *et al*, 2011) have encouraged research on the synthesis of novel tropan-3-ones, piperidones and 1,2-benzothiazines, with subsequent analysis of their antifungal and anticancer properties.

4.1.1 Synthesis of Ring Substituted 3,5-dibenzylidene-1-methyl piperidin-4-ones, 3,5-dibenzylidene-1- piperidin-4-ones and Their Hydrochloride Salts.

In the present study an aldol condensation of N-methyl-4-piperidone (**32**) or 4-piperidone hydrochloride monohydrate (**33**) with various aromatic aldehydes (refer to Table 1, Page 37) was performed. The aldol condensation involves a nucleophilic addition between an enolate and the carbonyl group of an aldehyde or ketone to produce β-hydroxyketone (aldols) (Noyce & Pryor, 1955). The reaction requires the use of a strong base such as NaOH in the presence of a solvent system usually comprised of ethanol and water or alternatively the reaction can occur using a strong acid such as a Lewis acid (Leonova *et al*, 2010*)*. Dehydration of the aldols by the E1 mechanism then gives rise to a conjugated α,β-

unsaturated enone with the loss of a water molecule. Again this step is often base catalysed however an acid may be used instead (Climent *et al*, 2002)

Several synthetic approaches have been deployed for this reaction, which vary in the nature of the solvent and catalyst used. In most cases the methods used are variations on the condensation of ketones with aldehydes in a basic aqueous alcoholic medium (McElvain & Rorig, 1948).

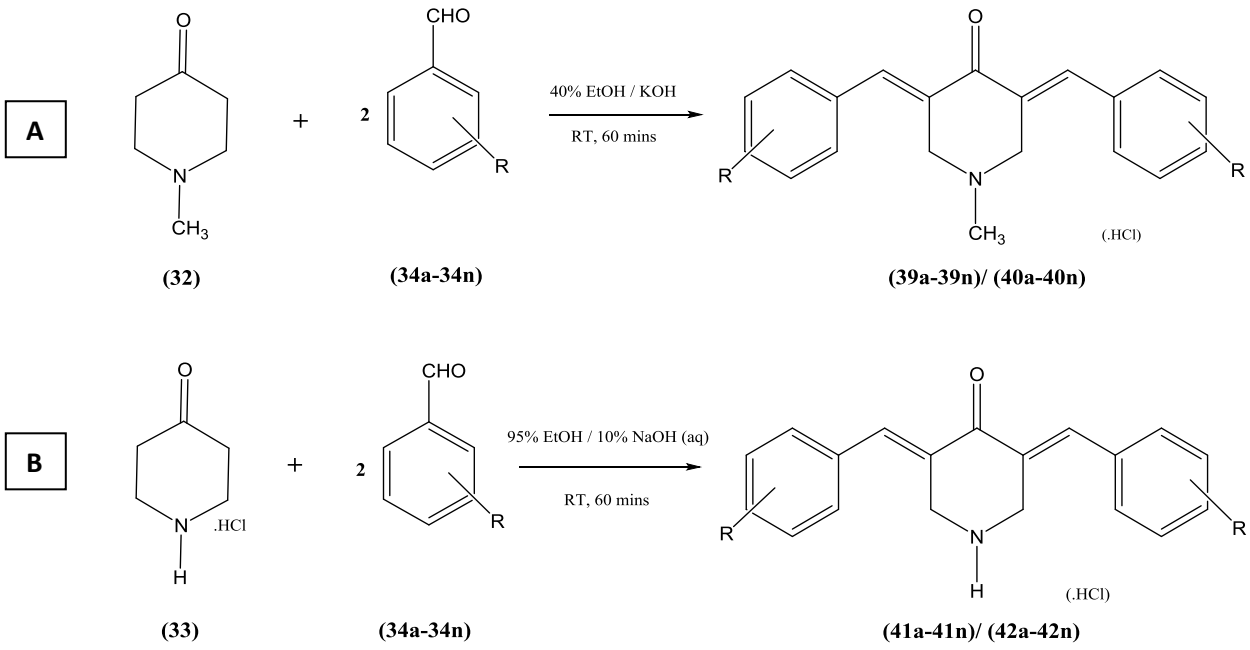


Figure 43- General protocol for the coupling of substituted benzaldehydes with N-methyl-4-piperidone (A) or 4-piperidone hydrochloride monohydrate (B)

An effective method for the coupling of various substituted aromatic aldehydes at the C-3 and C-5 position of either the N-methyl-4-piperidone or the 4-piperidone hydrochloride monohydrate was used (McElvain & Rorig, 1948). The groups alpha (α) to the carbonyl group in each parent compound are enolisable meaning they can undergo keto-enol tautomerism (Otera, 2001), thus an aldol condensation at these points results in formation of C=C double bond from the coupling of R-substituted benzaldehydes. Compounds were purified by recrystallization using various solvents. All compounds synthesized showed only one spot on silica TLC and where characterized by ^{1}H and ^{13}C NMR, IR and mass spectrometry. Generally the condensation occurred in good yield with yields ranging from 41-89% achieved for the ring substituted 3,5-dibenzylidene-1-methyl piperidin-4-ones (**39a-39n**) and 53-98% for their respective hydrochloride salts (5a-5n). Yields of 31-68% were

obtained for the ring substituted 3,5-dibenzylidene-1-piperidin-4-ones (**39a-39n**) and 55-97% for their corresponding hydrochloride salts. For all compounds synthesized the range of the melting points was within 3°C.

The structures of the resultant condensation products were established using ^{1}H and ^{13}C NMR, IR spectroscopy and mass spectrometry. The IR spectra of all compounds show a peak between 1658-1688 cm^{-1} corresponding to an unsaturated carbonyl (C=O) group. The ketonic C=O peak of N-methylpiperidin-4-one is observed at about 1721.2 cm^{-1} however due to the conjugation with the α,β-unsaturated bond the peaks are shifted to a lower wavenumber. The presence of the carbonyl peak is further confirmed by chemical shifts (between δ 180-188 ppm) in the ^{13}C NMR spectra for compounds (**39b**, **39c**, **39d**, **39h**, **39i**, **39j**, **39k**, **40a**, **40e**, **40g**, **40h**, **40l**, **41a**, **41b**, **41c**, **41d**, **41e**, **41f**, **41g**, **41h**, **41i**, **41j**, **42a**, **42d**, **42i** and **42k**). The carbonyl peak is not observed for the other compounds in their ^{13}C NMR spectra which suggests low levels of keto-enol tautomerism. This can also be explained by shifts in the ^{13}C NMR caused by the fusion of the benzaldehydes on respective α carbons which shifts the carbonyl peak to a lower ppm as addressed by a previous study which studied the chemical shift correlations for changes in structure for progestrones, androst-4-enedione and testosterones (Reich, Jautelat, Messe, Weigert, & Roberts, 1966). However it is not fully understood why the carbonyl peaks are seen in some compounds and not all. IR peaks between 1396-1595 cm^{-1} for all compounds are indicative of presence of a (C=C) double bond whereas peaks between 1547-1623 cm^{-1} suggest presence of the aromatic C-C single bond. The ring substituted 3,5-dibenzylidene-1-piperidin-4-ones (**41a-41n**) and their hydrochloride salts (**42a-42n**) display peaks between 3233-3377 cm^{-1} which indicates the existence of the N-H group.

Analysis by UV spectroscopy on various free bases of 3,5-dibenzylidene-1-methyl piperidin-4-ones produced UV doublets on the spectra in regions between 220-241nm and 316-357nm indicating the presence of an unsaturated carbonyl (Obili, 2014).

For all synthesized compounds the theoretical molecular mass was predicted using the software package ChemBioDraw Ultra13 (Perkin Elmer). The molecular ion for each compound obtained from mass spectrometry was equivalent to the predicted molecular mass (+/- Hydrogen atom).

The ^{1}H NMR of the 3,5-dibenzylidene-1-methyl piperidin-4-ones (**39a-39n**) and their hydrochloride salts (**40a-40n**) showed the presence of aliphatic protons on the CH_3 group with broad singlet peaks appearing at δ 2.05-3.29. A peak between δ 1.57-2.49 confirms the presence of an amino proton on the N-H group associated with the 3,5-dibenzylidene-1-piperidin-4-ones (**41a-41n**) and their hydrochloride salts (**42a-42n**).

In the ^{1}H NMR spectra of all compounds, the signals for protons of the substituted aromatic rings appear as multiplets at δ 7.16-7.68, which is characteristic of *E* isomers (Das *et al,* 2008). Singlet peaks are observed at δ 2.96-4.67 for the aliphatic protons on the piperidone ring and benzyldiene protons are distinguished by singlet peaks between δ 7.54-8.14.

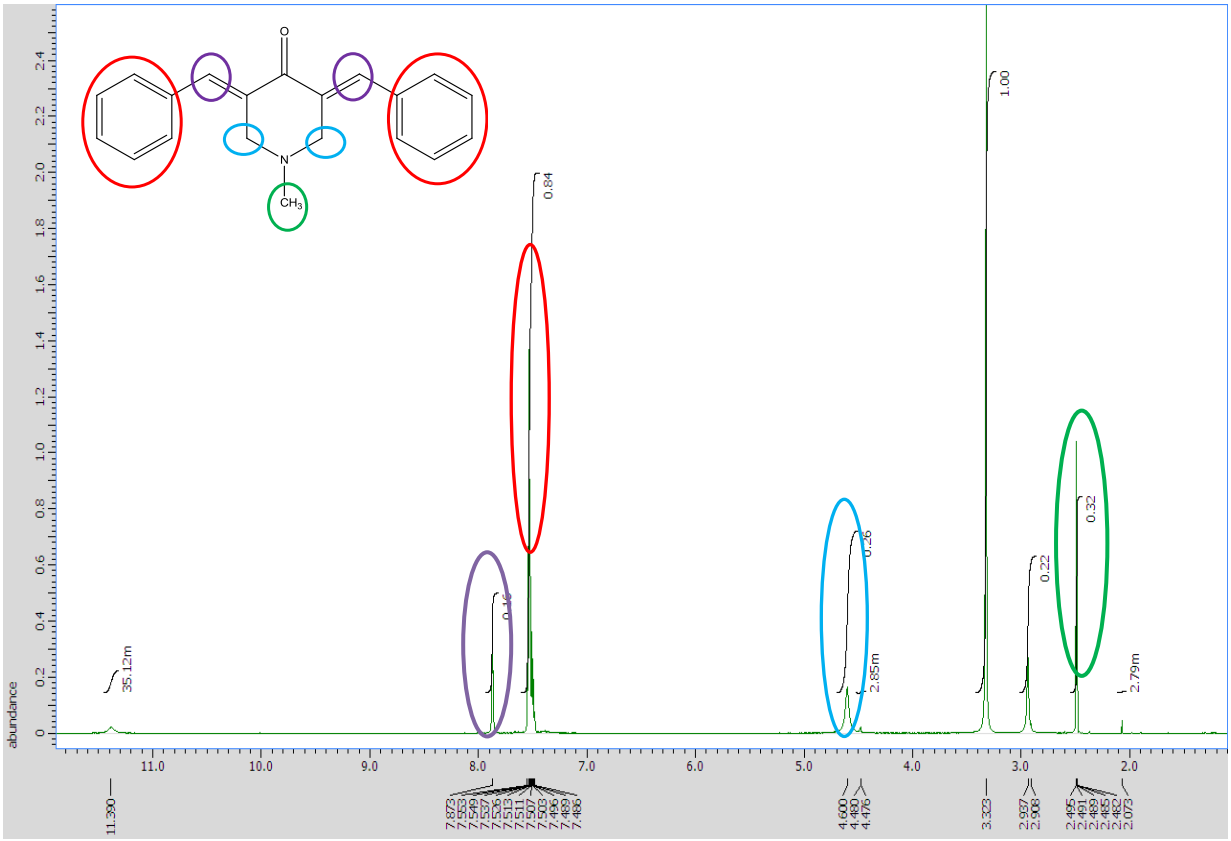


Figure 44- The ^{1}H NMR spectra of un-substituted 3,5-dibenzylidene-1-methyl piperidin-4-one (39a) where the relevant peaks on the spectra have been assigned to the different proton environments found on the compound. ^{1}H-NMR (DMSO-d_6): δ 2.49 (br s, 3H, aliph H), δ 4.60 (s, 4H, aliph H), δ 7.49 -7.56 (m, 10H, arom H) and δ 7.87 (br s, 2H, benzyldiene H)

High-performance liquid chromatography analysis on various free base 3,5-dibenzylidene-1-methyl piperidin-4-ones (**39a-39n**) has shown that these compounds degrade in aqueous

methanol to about five unidentified molecules following incubation at 4°C for 5-7 days (Obili, 2014).

4.1.2 Attempted Synthesis of (2, 3 and 4)-((1R,5S)-3-oxo-8-azabicyclo[3.2.1]octan-8-yl) benzoic acid (47a, 47b, 47c) and ethyl-4-((1R,5S)-3-oxo-8 azabicyclo [3.2.1]octan-8-yl) benzoate (47d)

A variety of synthetic approaches have been identified for synthesizing tropane derivatives including Michael addition of an amine to substituted cycloheptadieone, oxalyl addition to pyrroles or nitrile oxide cycloaddition (Tufariello, 1979). However the modified Robinson reaction (Stoll *et al*, 1954) is the most suitable and commonly used route for the synthesis of various tropane derivatives (Thuo, 2008).

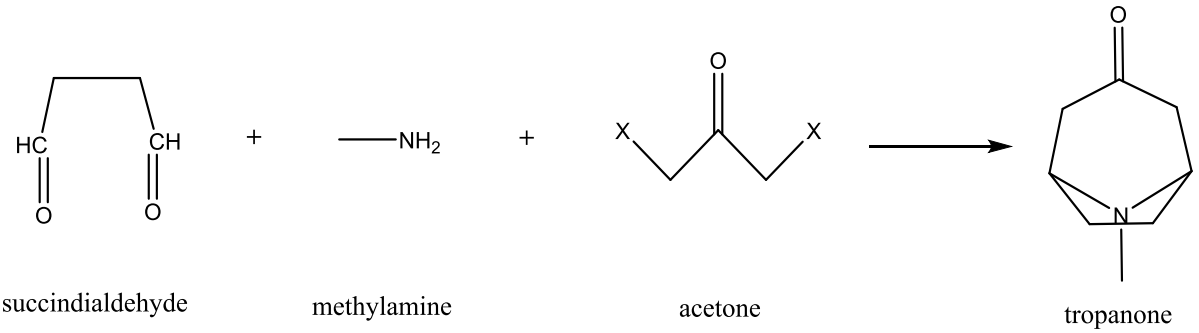


Figure 45- Synthesis of tropanone using Robinson's one-pot synthesis where x= H, CO_2H or CO_2Et (Thuo, 2008)

A two-step procedure was used to synthesize various tropane-3-one derivatives. Step one involved the synthesis of succindialdehyde from 2,5-dimethoxytetrahydrofuran (H_2O, HCl, 55°C) (Stoll *et al*, 1954). The second step involved reacting the succindialdehyde with various substituted aminobenzoic acids or their ethyl ester and acetone 1,3-dicarboxylic acid (3-oxopentanedioic acid) in the presence of aqueous NaOAc to yield the corresponding products. Step two of the reaction is the Robinson one pot synthesis (1917) which is widely used for the synthesis of tropane and its various derivatives (Thuo, 2008).

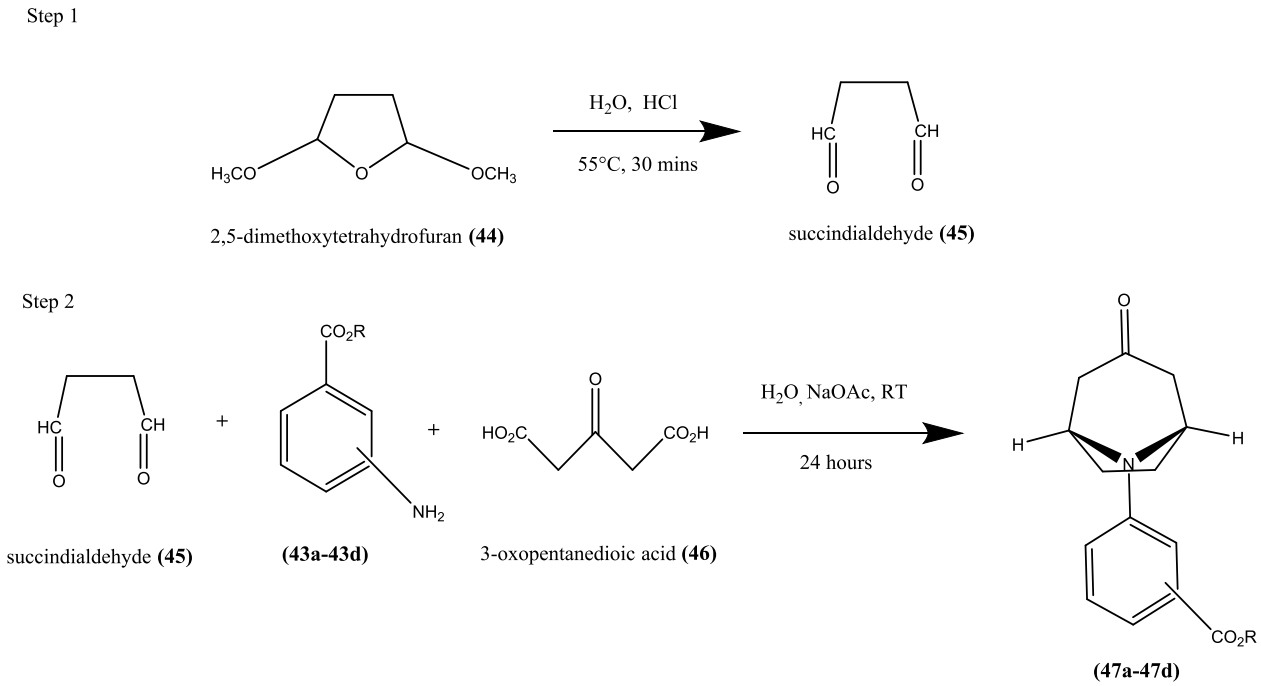


Figure 46- Generalised reaction scheme for the synthesis of (2, 3 or 4)-((1R,5S)-3-oxo-8 azabicyclo [3.2.1] octan-8-yl) benzoic acids and ethyl-4-((1R,5S)-3-oxo-8-azabicyclo[3.2.1]octan-8-yl) benzoate, where 43a: 4-NH_2/R=H, 43b: 2-NH_2/R=H, 43c: 3-NH_2/R=H, 43d: 4-NH_2/R=Et and 47a: 4-CO_2/R=H, 47b: 2-CO_2/R=H, 47c: 3-CO_2/R=H, 47d: 4-CO_2/R=Et.

Compound **47a** produced a yield of only 18% with the range of the melting point within 4°C. The compound was purified by recrystallizing from methanol and only one spot was observed on silica TLC. ^{1}H and ^{13}C NMR, IR spectroscopy and mass spectrometry were employed to characterize the compound and determine its structure. The molecular weight of the compound was confirmed using mass spectrometry with the molecular ion being 244.1 ($M\text{-}H^{+}$) and the theoretical molecular mass of the compound 245. IR spectra showed the presence of the C=O group on C-3 of the tropanone ring at 1710 cm^{-1} and the C=O (as part of the benzoic acid) group on C-15 at 1673 cm^{-1}. The presence of these carbonyl peaks was further confirmed by the ^{13}C NMR spectra. The carbonyl group at C-3 is predicted to be at δ 207.3 by ChemBioDraw Ultra13 (Perkin Elmer) and at approximately 200.4 using literature tables (Silverstein & Webster, 2005) and the peak was observed at δ 207.14. The C=O group at C-15 is predicted at δ 169.3 by ChemBioDraw Ultra13 and at approximately δ 168 using literature tables and it was observed at δ 168.29. The difference in the peaks is possibly due to a long range inductive effect caused by the oxygen molecules in the benzoic acid which may cause the peaks to shift up/downfield (Silverstein & Webster, 2005). A sharp peak at 1603 cm^{-1} in the IR spectrum confirms presence of the aromatic C-C single bond.

The ^{1}H NMR analysis of the compound shows the presence of aliphatic protons on; C-6 and C-7 at δ 1.54 (predicted δ 1.66), C-2 and C-4 at δ 2.07 (predicted between δ 2.5-2.75) and C-1 and C-5 at δ 2.37 (predicted 3.32). The aromatic protons on C-10 and C-14 are observed at δ 6.6 (predicted δ 7.03) and at δ 7.69 for C-11 and C-13 (predicted δ 7.76).

Succindialdehyde is a very reactive aldehyde which can oxidise with ease (Mondal & Mukherjee, 2003) forming succinic acid or undergo disproportionation to produce 4-hydroxybutyric acid. If one or both of these occurred before the completion of the reaction it would explain the low yield obtained.

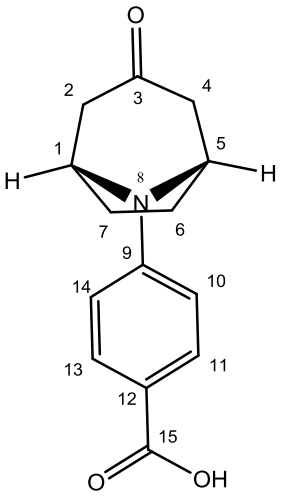


Figure 47- Chemical structure of 4-((1R,5S)-3-oxo-8-azabicyclo[3.2.1]octan-8-yl) benzoic acid (47a) with its relevant carbon and nitrogen numbering.

The synthesis of ethyl-4-((1R,5S)-3-oxo-8 azabicyclo [3.2.1]octan-8-yl) benzoate **(47d)** initially required the synthesis of benzocaine (43d). 4-Aminobenzoic acid in the presence of absolute ethanol was stirred under reflux (100°C, 4 hours) yielding the product. The reaction scheme is shown below.

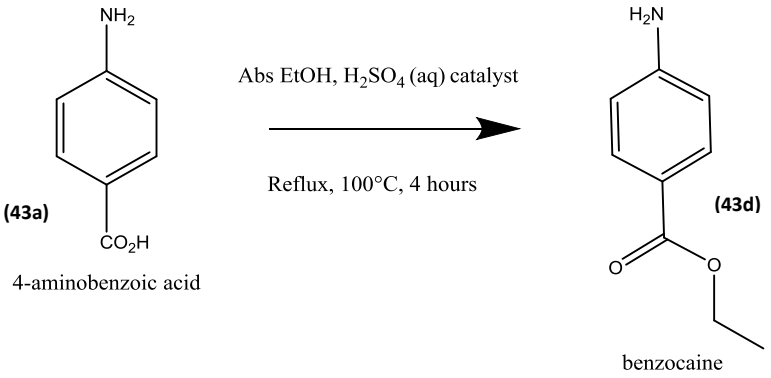


Figure 48- reaction scheme for the synthesis of benzocaine (43d)

Compound **43d** was purified by recrystallization from ethanol/water with only one spot being observed on silica TLC. The yield of the product was 42% and a melting point range of 89-91°C (literature range: 88-90°C (Sigma-Aldrich, 2013) was obtained. The carbonyl group on C-7 was detected at 1638 cm^{-1} on the IR spectrum and further confirmed at δ 166.40 on the ^{13}C NMR spectra (predicted: δ 164.6). IR spectroscopy suggests the existence of the aromatic C-C bond at 1592 cm^{-1}, a peak at 2984 cm^{-1} provides evidence of C-H bonds and indication of the N-H group at 3343 cm^{-1}. The ^{1}H NMR analysis of the compound distinguished the presence of the methyl protons on C-9 at δ 1.26 (predicted: δ 1.30), the methylene protons on C-8 at δ 4.18 (predicted δ 4.30) and the protons associated with the amino group at δ 5.92 (predicted: 5.48). The aromatic protons on C-3 and C-5 where evident at δ 6.54 (predicted: 6.47), whereas those on C-2 and C-6 were shown at δ 7.62 (predicted: δ 7.63). Analysis by mass spectrometry revealed the molecular ion to be 166.21 ($M\text{-}H^{+}$) with the theoretical molecular mass of the compound being 165.19.

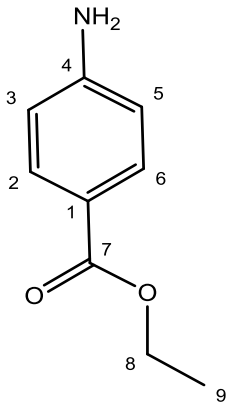


Figure 49- Chemical structure of benzocaine (43d) with its relevant carbon numbering.

The resultant benzocaine **(43d)** was used for the attempted synthesis of ethyl-4-((1R,5S)-3-oxo-8 azabicyclo [3.2.1]octan-8-yl)benzoate **(47d)** (refer to Figure 50). The product had a yield of 45% with a melting point range within 3°C. The product was recrystallized from methanol and analysis using silica TLC gave one spot for the product. The IR spectrum exhibited the presence of the C=O group on C-3 of the tropanone ring at 1711 cm^{-1} and the C=O group on C-15 at 1673 cm^{-1}. Further peaks at 1603 cm^{-1} and 2988 cm^{-1} were consistent with aromatic C-C bonds and C-H bonding respectively. The C=O peak for C-15 (predicted at δ 165.9 by ChemBioDraw Ultra13 and at δ 170.5 using literature tables was observed at δ 166.37 on the ^{13}C NMR. However the carbonyl peak for C-3 (predicted at δ 207.3 by ChemBioDraw Ultra13 and at 200.4 using literature tables) had been shifted to a lower ppm and was shown at δ 151.63. The shift is possibly due to a long range inductive effect caused by the oxygen molecules in the ester (Reich *et al,* 1966).

Analysis by ^{1}H NMR analysis indicate the presence of the aliphatic protons on; C-6 and C-7 at δ 1.67 (predicted δ 1.66), C-2 and C-4 at δ 2.22 (predicted between δ 2.5-2.75) and C-1 and C-5 at δ 3.30 (predicted 3.32). The aromatic protons on C10 and C14 are detected at δ 6.99 (predicted δ 6.93) and at δ 7.81 for C-11 and C-13 (predicted δ 7.84). The methyl protons on C-17 and the methylene protons on C-16 are not observed. As these are very distinctive peaks it suggests that there is no ester group present on C-15. Furthermore the ^{1}H NMR resembles that of compound **47a** and the molecular ion of the compound was also the same (244.1 ($M-H^{+}$) with the theoretical molecular mass predicted to be 273.33. Therefore it seems that the compound is structurally identical to **47a** suggesting that during or before

the reaction the ester group on the benzocaine reactant has been hydrolysed to form its benzoic acid derivative (PABA).

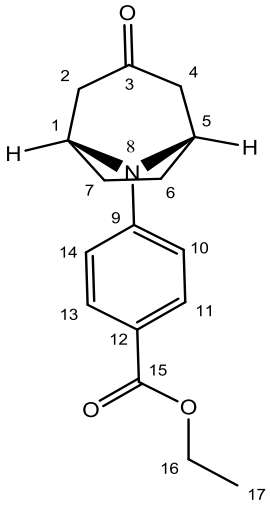


Figure 50- The chemical structure of ethyl-4-((1R,5S)-3-oxo-8 azabicyclo [3.2.1]octan-8-yl) benzoate (47d) with its relevant carbon and nitrogen numbering.

The attempted synthesis of 2-((1R,5S)-3-oxo-8-azabicyclo[3.2.1]octan-8-yl)benzoic acid **(47b)** was unsuccessful as analysis by TLC on the obtained material revealed the solid was predominantly 2-aminobenzoic acid and no product had been formed. Although the reaction was repeated under a nitrogen atmosphere to remove any oxygen which could have possibly oxidised the succindialdehyde to succinic acid the same outcome occurred. Addition of methanol resulted in the formation of an emulsion. Brine was added in an attempt to break down the emulsion but this proved ineffective.

Several problems were encountered in the attempted synthesis of 3-((1R,5S)-3-oxo-8-azabicyclo[3.2.1]octan-8-yl) benzoic acid **(47c)**. Initially the brown solid formed did not recrystallize when dissolved in methanol. Analysis by silica TLC showed the material consisted of starting material with no new products being formed. The experiment was repeated under minimum light conditions to reduce photosensitivity and an excess of succindialdehyde and acetone 1,3-dicarboxylic acid was used. However no product was formed as defined by silica TLC. The synthesis of both (2 and 3)-((1R,5S)-3-oxo-8 azabicyclo [3.2.1]octan-8-yl) benzoic acids were unsuccessful and the reasons for no products being formed are not understood.

Chapter 4.2: Antifungal Testing

Fungi belong to a large group of eukaryotic organisms including microorganism such as yeasts, filamentous fungi and *moulds of various types.* Fungi play important roles in nutrient cycling and exchange, as well as decomposition of organic matter (Kavanagh, 2011).

Although fungi have various industrial and medical benefits they are also the cause of many infectious diseases in both plants and animals. Additionally there is an increase in resistance of pathogenic fungi to clinical and agricultural antifungals. Fungi that cause disease in humans or other organisms are termed pathogenic fungi. *Candida* species are the largest group of human pathogens which often cause nasal, subcutaneous or GI infections particularly in immunocompromised patients such as AIDS sufferers or transplant patients. *Saccharomyces cerevisiae* is usually not considered a pathogen as diseases caused by this type of yeast are rare however there have been reported incidents (Mermel *et al*, 2009). Most yeast infections are associated with *C. albicans*. Exposure to *S*. *cerevisiae* particularly for immunosuppressed individuals can be fatal. *Aspergillus niger*, a filamentous fungus can cause serious lung disease (aspergillosis) and is the most common cause of otomycosis (fungal ear infection). As genomes of the yeast species *S*. *cerevisiae* and *C. albicans* have been sequenced and are easily cultured, and *A.niger* is the most abundant of the pathogenic filamentous species, it makes them good models for aiding diagnostic and novel antifungal drug development strategies (Kavanagh, 2011). The Clinical Laboratory and Standards Institute (CLSI) and European Committee on Antimicrobial Susceptibility Testing (EUCAST) have approved methods for antifungal susceptibility testing of both yeast and moulds (filamentous fungi). According to the CLSI and EUCAST the liquid broth dilution method is most appropriate for yeasts whereas the disk diffusion assay is more suited to filamentous fungi (Fothergill, 2012, CLSI, 2013 & Kahlmeter *et al*, 2006)**.**

Azoles are a class of antifungals which work by blocking ergosterol synthesis. N-decyl imidazole was the first azole found to inhibit ergosterol production, and it was the lead compound from which various azoles have been developed. Azole type drugs therefore contain an imidazole structure. Ergosterol is an essential steroid found in fungi and is required for plasma membrane stability (Lewis, 2011). Miconazole is a commonly used

example of azole drugs used to treat various fungal infections (Sawyer *et al,* 1975). In the present study miconazole was used as a reference drug against which all the synthesized compounds were compared.

When analysing the effects of the various piperidone dieneones, tropan-3-ones and 1,2 benzothiazines on the inhibition of growth of yeast species; IC_{10}, IC_{50} and IC_{90} (concentrations required to inhibit 10%, 50% or 90% of cell growth) values were determined rather than the minimum inhibitory concentration (MIC). An earlier study revealed that when three yeast species were treated with miconazole the MIC values vary significantly with inoculum size whereas IC_{50} values remain constant (Galgiani & Stevens, 1976). Thus inter-laboratory comparisons are easier.

Because the disk diffusion assay was used for examining the potential growth inhibitory potencies of the compounds against *A. niger* IC_{50} values could not be determined hence MIC values were obtained instead.

Compounds with similar chemical and physical properties to those used in this study i.e. containing an α,β-unsaturated ketone, have previously shown activity against various fungal species (Dimmock *et al*, 1974, Natesh *et al*, 2003, Pati *et al*, 2009, Senthil *et al*, 2011 & Mittal *et al*, 2011).

Various substituted compounds consisting of a piperidone or tropanone ring have shown to possess antifungal properties (Das *et al*, 2007). It has been suggested that the presence of an α,β-unsaturated ketone particularly in dieneone structures is linked with reducing the level of reduced glutathione in the cells by reaction with glutathione thiolate anions (Michael addition) leading to inhibition of cell growth or death (Pati *et al*, 2009). Other reports propose that structures having α,β–unsaturated carbonyls are responsible for blocking proton export by having an effect on the plasma membrane ATPase (Manavathu *et al*, 1999) or by blocking the mitochondrial phosphate transporter (Griffiths *et al*, 1981), both of which are essential for cell growth (Graham & Stevens, 1999).

Various compounds consisting of a parent benzothiazine structure have shown antifungal activity (Niewiadomy *et al,* 2011). It is believed that presence of sulphur, nitrogen or oxygen in the heterocyclic structure results in antifungal properties (Projan & Youngman, 2002).

4.2.1 Antifungal Analysis of Some 3,5-dibenzylidene-1-methyl piperidin-4-ones, 3,5-dibenzylidene-1-piperidin-4-ones, Their Hydrochloride Salts, Tropan-3-ones and 1,2-Benzothiazines Against The yeasts *S. cerevisiae* and *C. albicans*

Growth of the yeast species *S. cerevisiae* and *C. albicans* was studied to determine the mid exponential phase of growth. The exponential (or log) phase for both species was seen after 5-7 hours.

All test compounds as well as the reference drug (miconazole) were dissolved in DMSO thus initial tests were carried out to determine the effect of DMSO on cell growth. DMSO had no effect on the growth of either *S. cerevisiae* or *C. albicans* at a final concentration of 1.5%.

Although 1,2-benzothiazines have shown antibacterial properties (Patel & Bassin, 2012), their ability to inhibit the growth of *S. cerevisiae* was poor. All 45 derivatives (**48a-48as**) were tested, of which only four compounds exhibited inhibition at or above 10% after either 24 or 48 hours incubation (refer to Figure 35, Page 83). Increasing the incubation time from 24 to 48 hours had little effect on the degree of inhibition, with little variability in the data obtained.

Tropanones contain the same carbon skeleton as piperidones, with the addition of an 1,5 ethylene bridge. They are also known to possess antifungal properties (Humphery & O'Hagan, 2001). However the data obtained with **47a** suggested that the compound had little effect on the cell growth as it gave less than 10% inhibition. The compound consists of a parent tropan-3-one structure with no additional groups (i.e. ring substituted dieneones) attached on the carbons α to the carbonyl group therefore it does not contain a highly conjugated system unlike the piperidone dieneones. Also there is no α,β-unsaturated or styryl ketone like structure embedded into the compound. The lack of the α,β-unsaturated carbonyl could lead to their low potencies. Previous studies suggest that the presence of α,β-unsaturated carbonyls contributes to their antifungal potencies (Dimmock and Taylor, 1974).

Data obtained showed that incubating the *S. cerevisiae* cells with various piperidone derivatives for 48 hours produced greater levels of growth inhibition compared to 24 hours incubation. The free bases of 3,5-dibenzylidene-1-methyl piperidin-4-ones (**39a-39n**) and

3,5-dibenzylidene-1-piperidin-4-ones (**41a-41n**) were slightly more effective cell growth inhibitors when compared to their respective hydrochloride salts. In general the presence of the methyl group on the heterocyclic nitrogen (compounds **39a-40n**) results in lower potencies whereas presence of a hydrogen atom results in more potent compounds (**41a-42n**). The un-substituted compounds were the least effective, whereas the iodo-substituted compounds gave the greatest level of inhibition with the 3'-iodo substituted 3,5-dibenzylidene-1-piperidin-4-one producing the highest degree of inhibition (91%) at a concentration of 3×10^{-3} M. Para substitution produced the most effective compound with higher levels of inhibition obtained when compared to ortho or meta substituted compounds. However none of the compounds were found to be as potent as the reference drug (miconazole nitrate) which produced 89% inhibition at concentration of 1.67×10^{-6} M.

Further antifungal testing was carried out using only the free bases of the piperidone derivatives due to time constraints and their increased potencies in comparison to the tropan-3-ones and 1,2-benzothiazines. Based on their potency against *S. cerevisiae* a subset of compounds were screened against *C. albicans.* The selected compounds were incubated with the cells for 48 hours and their ability to inhibit cell growth determined. Results showed these compounds had similar potencies against *C. albicans* when compared to *S. cerevisiae.* This was further confirmed by the determination of the respective IC_{10}, IC_{50} and IC_{90} values for each compound tested against both fungal species (refer to Table 5, Page 87). Both species of yeast gave lower IC_{10} and IC_{50} values for para substituted compounds when compared to meta or ortho substituted analogues (para>meta>ortho). Compounds with no aromatic substituents had the highest IC_{10} and IC_{50} values (refer to Table 5, Page 87) hence they were the least potent. It appears that increasing the volume of the substituent group results in an increase in their potency with the iodo-substituted compounds being the most potent growth inhibitors. This can be further explained by the difference in electronic properties of the substituents on the benzene rings where the antifungal potency of the piperidone dieneones decreased with an increase of electron withdrawing properties of the R substituent. This is the opposite to that found in recent anticancer studies using novel N-phosphorylalkyl substituted E,E-3,5-bis (arylidene) piperid-4-ones and N-phosphorylated E,E-3,5-bis (thienylidene) piperid-4-ones which suggest that increasing the electron

withdrawing properties of the R substituents increases potency. (Makarov *et al*, 2009, Makarov *et al*, 2010).

The most potent compound was **41n** (3'-iodo 3,5-dibenzylidene-1-methyl piperidin-4-one) having the lowest IC_{10} and IC_{50} values against both species of yeast respectively. It is also the only compound to give greater than 90% inhibition of cell growth for both species giving IC_{90} values of 1.35×10^{-3} M against *S. cerevisiae* and 1.55×10^{-3} M against *C. albicans* after 48 hours incubation. However the reference drug (miconazole nitrate) under the same conditions, was more potent against both species producing 89-90% inhibition at a lower concentration (1.67×10^{-6} M).

The synthesized piperidones all contain a double α,β-unsaturated ketone structure (dieneone) and it has been proposed by various studies that interactions with thiolate anions are believed to occur at the olefinic carbon atoms (as shown by C^A and C^B in Figure 51) (Das *et al*, 2008, Mutus *et al*, 1989). The nucleophilic attack would be expected to occur at these points due to the electron-withdrawing influences of the carbonyl group (Manavathu *et al*, 1999). α,β-Unsaturated compounds can undergo Michael reactions with cellular nucleophiles such as thiolate anions found in proteins and cofactors such as glutathione with little or no affinity for hydroxyl and amino groups in nucleic acids (Pati *et al*, 2009). Thiols are distinctive as unlike amino and hydroxyl groups they are not found in nucleic acids. For the mitochondrial permeability transition pore complex to open it requires alkylation or cross-linking of a critical thiol on the protein permeability transition pore complex (Das *et al,* 2007). Since some of the compounds used in this investigation contain an α,β-unsaturated ketone they should react easily with thiolate anions via the Michael addition. Thus the mitochondrial permeability transition pore complex cannot open efficiently which activates the caspase system resulting in the destruction of the mitochondria, consequently leading to the initiation of cell death by apoptosis (Das *et al*, 2007).

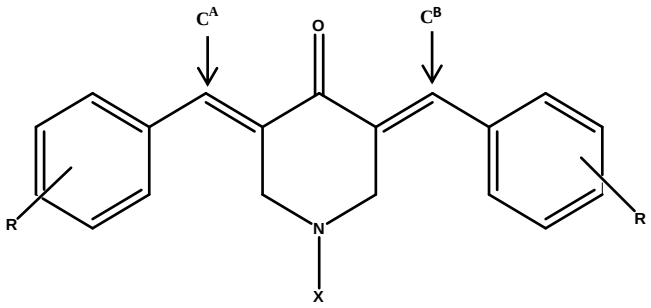


Figure 51- The structure of synthesized ring substituted 3,5-dibenzylidene-1-methyl piperidin-4-ones (where x= CH_3) or 3,5-dibenzylidene-1-piperidin-4-ones (where x= H). The various substituents on the benzene rings are represented by R (see Table 1, Page 37). Olefinic carbon atoms are shown by C^A and C^B

Glutathione is a cellular antioxidant which prevents damage to important cellular components (Kaplowitz, 1981). Glutathione and the side chain of the essential amino acid cysteine contain thiolate anions which act as nucleophiles and attack the electrophilic olefinic carbons on the α,β-unsaturated structure (Das *et al*, 2008, Mutus *et al*, 1989).

Alternatively compounds containing a dieneone structure can have an adverse effect on fungal cell growth by interference with certain mitochondrial functions such as ROS generation or oxygen consumption by the electron transport chain (Helal *et al*, 2013). An increase in the level of reactive oxygen species (ROS) can lead to an overload of antioxidant defences culminating in cell death (Apel & Hirt, 2004). Oxidative phosphorylation is essential for cell growth and inhibition of this process may lead to an increase in the concentrations of ROS. This could affect the rate of oxygen consumption in the cell and also have a marked effect on the mitochondrial membrane potential (MMP) (Helal *et al*, 2013).

Other reports suggest that compounds containing the α,β-unsaturated structure block the P-type proton-translocating ATPase (H^+-ATPase) of various fungal species (Manavathu *et al*, 1999) or they block the mitochondrial phosphate transporter (Griffiths *et al*, 1981), both of which are essential for cell growth (Graham & Stevens, 1999). The H^+-ATPase is a plasma-membrane located ATP-driven proton pump belonging to the P-type ATPase superfamily (Manavathu *et al*, 1999).

4.2.2 Analysis of Antifungal Activity By a Subset of Ring Substituted 3,5-dibenzylidene-1-methylpiperidin-4-ones and 3,5-dibenzylidene-1-piperidin-4-ones Against the Filamentous Fungus *A. niger*

The piperidone dieneones which produced inhibition of yeast growth at levels greater than 50% were screened for their capacity to inhibit the growth of the filamentous fungus *A. niger*. The standard method for susceptibility testing using filamentous (mould) fungi was applied (Fothergill, 2012, CLSI, 2013). Initial tests revealed that DMSO up to a concentration of 1.5% had no effect on the cell growth of *A.niger.* None of the compounds tested showed inhibition of cell growth after either 16 or 24 hours incubation. Results showed that the compounds were not as effective at inhibiting growth of *A.niger* as only 7 of the 14 tested compounds showed activity. Filamentous fungi are multicellular organisms containing long branched filaments called hyphae which aggregate to form a mycelial mass thus making it more difficult for compounds to cross both the chitin based cell wall and the plasma membrane (Bärlocher, 2008). Therefore adverse effects by the piperidone dieneones on filamentous fungi would be expected to be lower in contrast to yeast which would be more vulnerable to these compounds.

Increasing the incubation period from 48 to 72 hours enhanced inhibition of fungal growth. Compound **39g** (3,5-bis(4-bromobenzylidene)-1-methylpiperidin-4-one) was the least potent giving smaller zone of inhibition diameters at each given concentration when compared to the other dieneones. As with the yeasts tested, compound **39n** (3,5-bis(3-iodobenzylidene)-1-methylpiperidin-4-one) was the most potent inhibitor of growth. Again as also demonstrated by the yeast studies the data obtained showed that para-substitution produced the most effective compound. Increasing the volume of the aromatic substituent and decreasing its electronegativity (electron withdrawing properties) also improved potency.

Assays of microbial susceptibility to drugs via the agar diffusion method were analysed using linear fitting of either the diameter or squared diameter of the inhibition zones against the natural logarithm (ln) of drug concentration at the source;

$$\ln (MIC) = \ln (c) - D^2 / 4dt \qquad \textbf{Equation (2)}$$

Where D= zone of inhibition diameter, d= diffusion coefficient presumed to be independent of concentration and t= time of drug diffusion) (Kronvall, 1982). This reflects a solution of the differential equation describing free diffusion in one dimension which takes into account the dependence of the drug concentration on distance from the source and on time. (Bonev, Hooper & Parisot, 2008). In order to find the most suitable linear fitting, various graphs were plotted of D, D^2, Ď (corrected by subtracting the diameter of the paper disks from D) and $Ď^2$ against ln (drug concentration). The R^2 values were deduced from each graph and then compared (Microsoft Excel). R^2 values indicate how well data points fit in a linear regression as it represents the variation of the data explained by the fitted line. The closer the R^2 value is to 1 the better the linear regression fit (Colin & Windmeijer, 1997).

As seen in Tables 6 and 7 (Pages 91 & 93 respectively) plots of D or D^2 against ln (concentration) gave better fitted linear regressions as their corresponding R^2 values were closer to 1 as compared to plots for (D - Ď) and $(D - Ď)^2$ against log (concentration). MIC values were attained from the x intercept of each graph. MIC values from Table 8 (Page 95) suggest that increasing the incubation time from 48 hours to 72 hours increased the level of inhibition. Due to the multicellular nature of *A. niger* it is likely that the piperidone dieneones took longer to have an effect on these cells. Generally the iodo substituted dieneones were the most potent as shown by their lower MIC values compared to the other compounds, though meta substitution provided a more potent inhibitor. Compound **39n** was the most potent with MIC values after 72 hours incubation from both plots (3.6×10^{-10} M (D/ln (c)) and 11.6×10^{-8} M (D^2/ln (C)). Compound **41l** was the least potent compound with the highest MIC values after 72 hours incubation from each plot (2.82×10^{-7} M (D/ln (C)) and 1.49×10^{-5} M (D^2/ln (C)). The quadratic model was accurate for compounds **39g** and **41g** whereas linear fits are more accurate in all other cases. The reference drug miconazole nitrate was a far more effective inhibitor of *A. niger* as it produced average zone of inhibition diameters of 17mm after 48 hours incubation and 18mm after 72 hours incubation at a concentration of 1.253µM (10µl applied, 12.53 nmoles).

When using the disk diffusion assay the concentration of compound loaded is not a true concentration, it is an apparent concentration based on the amount of drug loaded on the disks which diffuses into the agar (Bonev *et al*, 2008)**.**

The hydrophobicity of the piperidone dieneones also has a part in level of inhibition as well as other factors including thickness of the medium gel (Bonev *et al*, 2008).

There is approximately a 150-300 fold difference between the MIC values obtained from plots of D/ln (C) and D^2/ln (C); however it can be assumed that because D/ln (C) give R^2 values closer to 1, the MIC values obtained from these plots are more accurate (Bonev *et al*, 2008).

It is evident from the data obtained that the compounds possess antifungal properties as they have an inhibitory effect on the growth of both yeast and filamentous fungi. In general it appears that the presence of hydrogen on the heterocyclic nitrogen of the piperidone ring gives compounds with lower potencies than compounds having a methyl group in the same position. The compounds however are not as effective against filamentous fungi as they are against yeasts. This is probably due to the multicellular structure of filamentous fungi. Further experiments are needed to confirm this theory as there is substantial difference between $IC_{10,}$ IC_{50} and IC_{90} values obtained with yeasts and the MIC values acquired from the filamentous fungus. Using the disk diffusion assay for yeasts as well as filamentous fungi could be useful for making accurate comparisons.

As mentioned previously there are many means by which the piperidone dieneones may affect the growth of these fungi. This requires further exploration. The present study has revealed that the position of the substituent on the benzene rings, its volume as well as its electron withdrawing properties play a vital role in the overall potency. Although not as potent as the marketed antifungal drug miconazole; there is indication that further study on the structures of the dieneones can give predictions of a more potent agent.

Chapter 4.3: Anticancer Testing

Cancer is a disease in which abnormal cells divide in an uncontrolled manner ultimately invading surrounding tissues. (Macdonald *et al*, 2004). Although many treatments are available for suppressing or eliminating cancers and tumours, the prognosis in many types of cancer is limited. Research to develop novel anti-neoplastic agents is therefore required (Macdonald *et al*, 2004). Cancer research has advanced over the years due to the availability of many cancer cell lines derived from various cancer patients (Ferreira, 2013). The adherent culture strain of the human adenocarcinoma alveolar basal epithelial A549 ATCC CCL-185 was used in this study. Several cytotoxicity assays have been approved by the National Cancer Institute (NCI) of which the most commonly used are the lactate dehydrogenase (LDH) and the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy methoxy- phenyl)-2-(4-sulfophenyl)-2H tetrazolium, inner salt (MTS) assays (Baharith et al, 2006).

Due to time restrictions four selected 3,5-dibenzylidene-1-piperidin-4-ones (as exemplified by their potencies against both yeasts and filamentous fungi when compared to other dieneones) were chosen for the testing of their cytotoxic properties using the MTS and LDH assays.

The MTS assay was utilised to determine if the compounds had an effect on reducing mitochondrial dehydrogenase activity thus indicating they possessed cytotoxic properties (Burger & Fiebig, 2004). The effect on cell membrane integrity of the compounds was analysed using the LDH assay (Fotakis & Timbrell, 2006).

Although both assays are approved by the NCI they are not very accurate indicators of cytotoxicity as results can be affected in various ways (Berridge *et al*, 2005). As the MTS assay measures optical density and the LDH assay measures fluorescence there is always an issue of light interference which can have an effect on the accuracy of the collected data (Lobner, 2000). Also, due to the small volumes of reagents used, precautions are required to reduce errors which in many cases can be difficult.

4.3.1 Cytotoxic Evaluation of Test Compounds using The MTS and LDH assays

Initial tests were carried out to study the effect of DMSO on the cells. Results showed that DMSO up to a concentration of 1% was not cytotoxic to A549 cells. The cytotoxicity analysis revealed that the iodo-substituted compounds were the most cytotoxic with the methoxy-substituted compound being the least potent (see Figure 41 (Page 96) and Figure 42 (Page 98). Compound **41n** (3,5-bis(3-iodobenzylidene) piperidin-4-one) had the greatest effect on membrane integrity and mitochondrial activity as indicated by having the lowest IC_{50} values; 1.7 $x10^{-5}$ M (MTT) and LDH: 2.1 $x10^{-5}$ M (LDH) in comparison to the other compounds (Table 9, Page 98). Compound (**41l**) (3,5-bis(4-methoxybenzylidene) piperidin-4-one) was the least cytotoxic with IC_{50} values of 5.4 x 10^{-3} M (MTS) and 5.06 x 10^{-3} M (LDH) (Table 9, Page 98). Of the two iodo-substituted compounds tested meta substitution gave the most cytotoxic compound.

The cytotoxic analysis of the piperidone dieneones on the mammalian cancer cell line A549 reveals that these compounds are having an adverse effect on both mitochondrial activity and membrane integrity. It provides further evidence that the position of the R substituent on the benzene ring is important in determining the compounds potency. Additionally increasing the volume of the molecular group and decreasing its electron withdrawing properties increased potency.

As a result of time restrictions a subset of piperidone dieneones were tested against A549 cells based on their increased potencies against fungal cells when compared to other dieneones. However a recent study using N-phosphorylalkyl substituted E,E-3,5-bis (arylidene) piperid-4-ones, which also contain an α,β-unsaturated ketone structure revealed that increasing the electron withdrawing properties of the R substituents increases potency. Using a nitro group in the para-position of the benzene ring produced the most cytotoxic compound out of an analogue of fourteen ring substituted piperidones against various cancer cell lines including A549 cells (Makarov *et al*, 2009). Evidence from this study suggests that the piperidone dieneones containing substituents of high electronegativity (i.e. F, Cl) should be examined for their cytotoxic properties.

It is expected that these compounds have similar effects on both fungal and mammalian cells. Both cell types are eukaryotic with similar structures and contain many of the same

enzymes (Brown, 2010). As previously mentioned for the antifungal analysis there are many ways at which these compounds may have their cytotoxic effects on cell membrane and mitochondrial activity. Most commonly these compounds are said to be involved in alkylation of thiols present on important cellular components such as glutathione. (Dimmock *et al,* 2002). However as reported by Manavathu *et al*, (1999) compounds of similar nature i.e containing an α,β-unsaturated ketone are responsible for blocking the plasma membrane proton-translocating ATPase (H^+-ATPase) of various cells which is essential for cell growth. Alternatively other reports suggest that compounds of this kind interfere with mitochondrial functions such as ROS generation, oxygen consumption by the electron transport chain and phosphate transport (Helal *et al*, 2013 & Griffiths, *et al*, 1981).

Chapter 5

Further Work and Conclusion

Chapter 5.1: Further Work

Due to limited time available it was not possible to synthesize various dibenzylidene derivatives of the synthesized tropan-3-one. Tropan-3-ones are from the same family of compounds as piperidones and they have also shown antifungal and cytotoxic properties (Pati *et al*, 2009). By synthesizing various dibenzylidene derivatives of tropan-3-one using the aldol condensation it could be determined whether the presence of an ethylene bridge has an effect on the potency.

With all synthesized compounds the definitive structures could have been further confirmed using X-ray crystallography. This is a fairly accurate tool used to determine the atomic and molecular structure of a solid compound. It uses a beam of x-ray which atoms diffract at different angles and intensities. Measurement of these properties can enable construction of a three-dimensional image from which the position of atoms, electron densities and chemical bonds can be distinguished (Sayre, 2002).

Although the piperidone derivatives were not as effective as the reference drug miconazole, they show potential for further improvement which can lead to increased potencies. The introduction of hydrophobic or bioactive groups on the heterocyclic nitrogen in the piperidone derivatives can increase both the potency and also the bioavailability by assisting approach of the cytotoxin to a specific binding site (Makarov *et al*, 2010).

The current study revealed that para substitution produced the most effective compound and increasing the volume and decreasing the electron withdrawing properties of the R substituent increased potency. Whereas this is contradicted by recent anticancer studies using novel N-phophorylalkyl substituted E,E-3,5-bis (arylidene)piperid-4-ones and N-phosphorylated E,E-3,5-bis (thienylidene) piperid-4-ones which suggest that increasing the electron withdrawing properties of the R substituents increases potency (Makarov *et al*, 2009, Makarov *et al*, 2010). Based on these findings further novel structures can be synthesized with stronger electron withdrawing R substituents such as acids or nitro groups or effectively addition of more than one group on the relevant benzene rings. The data collected in this study in conjunction with the theoretical logP values which distinguish the

hydrophobic properties of the compounds can be used to study structure-activity relationships. From this a QSAR analysis can be generated and the structure of a theoretically more potent compound. The same can be applied to the benzothiazine compounds.

Additional hydrophobic groups can also be added to the carbonyl group on the piperidone ring to increase potency and bioavailability. The use of substituted pyridine rings rather than benzene can be beneficial as it would increase solubility thus bioavailability as suggested by Leonova and co-workers (2010). Alternatively thienyl rings can be added to the parent piperidone compounds which may also increase potency (Makarov *et al*, 2010).

All compounds were tested for their antifungal properties against *S*. *cerevisiae,* from which the most potent were selected for screening against *C. albicans* and *A.niger.* Four compounds were analysed against the A549 cancer cells due to restriction of time. In order to further validate the findings all compounds could be screened against additional cell lines as well as those used in this study.

Though it was found that certain compounds inhibit fungal cell growth and reduce cell viability of a particular cancer cell line, the manner in which they do so is not fully understood. Various biological techniques can be used to further study this field which would be of great interest as it can give ideas on how these compounds function as potential antifungal or anticancer agents. Most compounds of similar nature are believed to play a role in alkylation of critical cellular thiols such as glutathione. Therefore the effect of these compounds on glutathione and cysteine levels can be analysed using a inactivation assay as proposed by Manavathu and co-workers (1999). The assay is a good tool to determine the inactivation of the antifungal activity of the piperidone dieneones by various thiol reagents such as cysteine or glutathione. Manavathu and co-workers (1999) showed that incubation of various Mannich bases of a series of cyclic conjugated stryl ketones with various concentrations of glutathione and cysteine eliminated their antifungal activity in a concentration-dependant fashion.

Alternatively the proton pumping activities of the cells after incubation with the compounds can be analysed by monitoring glucose-induced acidification of the external medium by measuring differences in pH (Manavathu *et al*, 1999). This would provide evidence whether

the compounds play a part in blocking the plasma membrane proton-translocating ATPase (H^+-ATPase). This can be verified by isolating the plasma membrane and to assay the ATPase activity with and without presence of the compounds.

The effect of the compounds on mitochondrial function such as ROS generation can be analysed using a sulforhodamine B assay which measures fluorescent intensities that are proportional to the level of ROS generated (Helal *et al*, 2013).

Alternative assays which could have been applied to cytotoxicity studies include various NCI approved assays such as the 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) assay which is proposed to give an improved dynamic range and higher sensitivity (Berridge *et al,* 2005).

An improved approach to quantify the number of viable and non-viable cells is the use of a flow cytometer. Flow cytometry uses a computer software programme to quantify small particles such as cells (Ormerod, 2000). It can simultaneously examine chemical and physical characteristics of many thousands of particles. The technique can also determine if cell death is occurring by means of apoptosis or necrosis and it can also study cell viability and the cell cycle of the cell line being examined. Although this technique is more time consuming it reduces human error and can produce more precise data.

Further work exploring other pharmacological properties of these compounds can be conducted such as anti-inflammatory and analgesic action. Several piperid-4-one containing analogues of curcumin exhibited inhibition on lipopolysaccharide (LPS) induced IL-6 release in RAW2647 macrophage cell showing cytokine-inhibitory activity (Wu *et al*, 2013). This study suggests that compounds containing an α,β unsaturated carbonyl possess anti-inflammatory properties thus the anti-inflammatory potencies of the piperidone dieneones should be investigated.

Chapter 5.2: Conclusion

In summary a library of 3,5-dibenzylidene-1-methyl piperidin-4-ones, 3,5-dibenzylidene-1-piperidin-4-ones, and their respective hydrochloride salts was synthesized. A parent tropan-3-one structure was also synthesized which will be used as a precursor to develop a library of tropanone dieneones. The synthesized compounds, along with a previously synthesized library of 1,2-benzothiazines were examined for their antifungal properties against various fungal cell lines and anticancer properties against a cancer cell line. Results obtained suggest that the tropan-3-one and benzothiazines are ineffective as antifungal agents, whereas the synthesized piperidone dieneones show a wide spectrum of activity. The free base piperidone dieneones are slightly more potent than their corresponding hydrochloride salts. In general the presence of a hydrogen atom on the heterocyclic nitrogen rather than a methyl group increases activity against yeast while the opposite is true in filamentous fungi. Antifungal testing against yeast cells showed that 48 hours incubation leads to a higher percentage of growth inhibition rather than 24 hours, with 72 hours of incubation proving most effective when tested against the filamentous fungi. The compounds are not as effective at inhibiting cell growth against filamentous fungi when compared to yeast. This is probably due to the multicellular structure of filamentous fungi.

The aromatic 'R' substituent on the benzene rings of piperidone dieneones plays a role in potency whereby para substitution produces the most potent compound followed by meta and ortho substitution. The volume and electron withdrawing properties of the R substituent is also vital, where R substituents of larger volume and lower electron withdrawing properties produce more potent compounds. The 3'-iodo substituted piperidones (**39n and 41n**) emerged as the most promising agents from the group of compounds in respect to their antifungal and cytotoxic potencies, though the compounds were not as effective as the reference drug miconazole. This study has revealed the potential of these substituted piperidones in particular the iodo substituted to be used as the starting point for further development to improve potency.

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Appendix

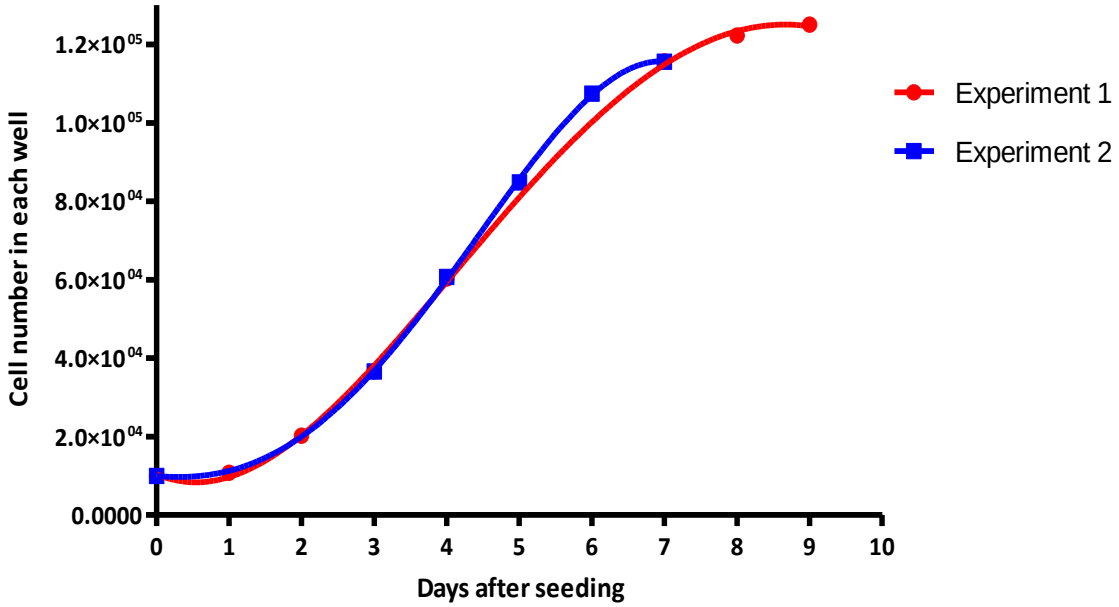

Figure 52- Growth curve showing cell counts of A549 cells taken at daily intervals. Initially A549 cells were seeded into a 96-well plate at a concentration of 1 x 10^4 cells/100µl/well at day 0. The plate was incubated at 37°C, 5% CO_2 and cell counts were obtained in triplicate from six wells daily for a total of 9 days.

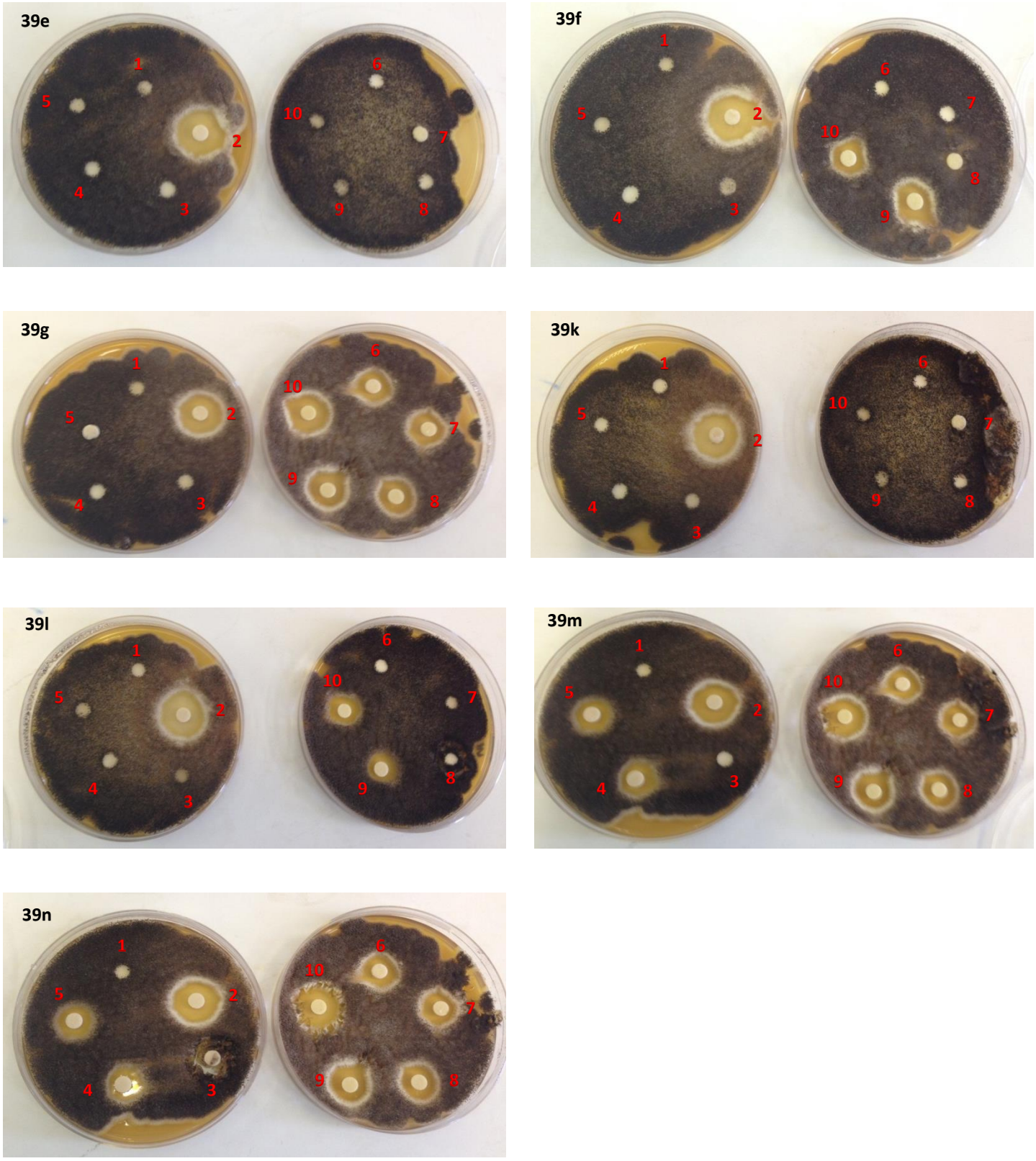

Figure 53- Disk diffusion assay showing the zone of inhibition diameters against Aspergillus niger ATCC 16888 for 1.5% DMSO (1), 10µl of 1.253µM (12.53 nmoles) miconazole (2) and various amounts (10µl of 1 x10^{-6} M to 3 x10^{-3} M) of selected ring substituted 1-methylpiperidin-4-one dieneones (3) to (10) following 72 h incubation. Zone of inhibition diameters were determined by measuring 'D' and 'r' of each relevant inhibition zone. Corrected diameters were determined by subtracting the diameter of the paper disks (6mm) from the diameter of the zone of inhibition.

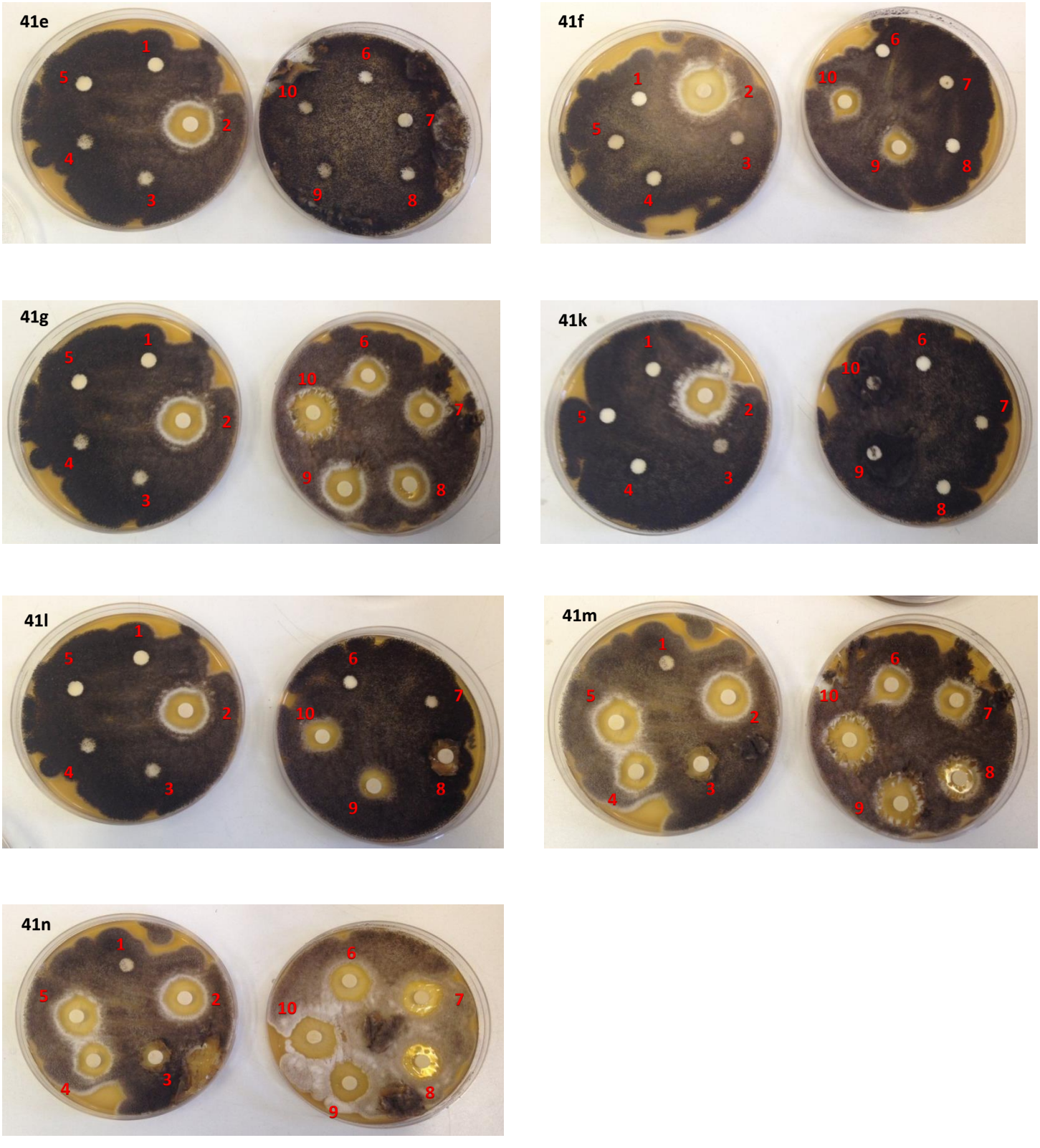

Figure 54- Disk diffusion assay showing the zone of inhibition diameters against Aspergillus niger ATCC 16888 for 1.5% DMSO (1), 10µl of 1.253µM (12.53 nmoles) miconazole (2) and various amounts (10µl of 1 x10^{-6} M to 3 x10^{-3} M) of selected ring substituted piperidin-4-one dieneones (3) to (10) following 72 h incubation. Zone of inhibition diameters were determined by measuring 'D' and 'r' of each relevant inhibition zone. Corrected diameters were determined by subtracting the diameter of the paper disks (6mm) from the diameter of the zone of inhibition.

Table 10- The relevant compound numbers and codes for ring substituted 1-methylpiperidin-4-one dieneones as displayed in the thesis, logbook and spectral data

Compound type	Compound no. (thesis)	Compound code (logbook and spectral data)
Ring substituted 3,5-dibenzylidene- 1-methyl piperidin-4-ones	39a	Pip 1a
	39b	Pip 3a
	39c	Pip 4a
	39d	Pip 5a
	39e	Pip 6a
	39f	Pip 7a
	39g	Pip 8a
	39h	Pip 9a
	39i	Pip 20a
	39j	Pip 21a
	39k	Pip 26a
	39l	Pip 27a
	39m	Pip 28a
	39n	Pip 29a
HCl salts of ring substituted of 3,5-dibenzylidene-1-methyl piperidin-4-ones	40a	Pip 1b
	40b	Pip 3b
	40c	Pip 4b
	40d	Pip 5b
	40e	Pip 6b
	40f	Pip 7b
	40g	Pip 8b
	40h	Pip 9b
	40i	Pip 20b
	40j	Pip 21b
	40k	Pip 26b
	40l	Pip 27b
	40m	Pip 28b
	40n	Pip 29b

Table 11- The relevant compound numbers and codes for ring substituted piperidin-4-one dieneones as displayed in the thesis, logbook and spectral data

Compound type	Compound no. (thesis)	Compound code (logbook and spectral data)
Ring substituted 3,5-dibenzylidene-1-piperidin -4-ones	41a	Pip 10a
	41b	Pip 11a
	41c	Pip 12a
	41d	Pip 13a
	41e	Pip 14a
	41f	Pip 15a
	41g	Pip 16a
	41h	Pip 17a
	41i	Pip 18a
	41j	Pip 19a
	41k	Pip 24a
	41l	Pip 25a
	41m	Pip 30a
	41n	Pip 31a
HCl salts of ring substituted 3,5-dibenzylidene- 1-piperidin-4-ones	42a	Pip 10b
	42b	Pip 11b
	42c	Pip 12b
	42d	Pip 13b
	42e	Pip 14b
	42f	Pip 15b
	42g	Pip 16b
	42h	Pip 17b
	42i	Pip 18b
	42j	Pip 19b
	42k	Pip 24b
	42l	Pip 25b
	42m	Pip 30b
	42n	Pip 31b

Table 12- The relevant compound numbers and codes for ring substituted 1,2-benzothiazine derivatives as displayed in the thesis and logbook

Compound no. (thesis)	Compound code (logbook and spectral data)
48a	BT1
48b	BT2
48c	BT3
48d	BT4
48e	BT5
48f	BT6
48g	BT7
48h	BT8
48i	BT9
48j	BT10
48k	BT11
48l	BT12
48m	BT13
48n	BT14
48o	BT15
48p	BT16
48q	BT17
48r	BT18
48s	BT19
48t	BT20
48u	BT21
48v	BT22
48x	BT23
48y	BT24
48z	BT25
48aa	BT26
48ab	BT27
48ac	BT28
48ad	BT29
48ae	BT30
48af	BT31
48ag	BT32
48ah	BT33
48ai	BT34

Table 12 (continued) - The relevant compound numbers and codes for ring substituted 1,2-benzothiazine derivatives as displayed in the thesis and logbook

Compound no. (thesis)	Compound code (logbook and spectral data)
48aj	BT35
48ak	BT36
48al	BT37
48al	BT38
48am	BT39
48an	BT40
48ao	BT41
48ap	BT42
48aq	BT43
48ar	BT44
48as	BT45