Synthesis, characterization and biological evaluation of benzo[d]isothiazole ring systems, fused to nitrogen heterocycles

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Abstract

We have developed a simple and efficient synthetic route, starting from easily available starting materials, to the novel ring system benzo[4,5]isothiazolo[2,3-a]pyrazine-6,6-dioxide. A series of chalcones were synthesized which were subsequently reacted with chlorosulphonic acid to generate the chalcone sulphonyl chlorides. The chalcone sulphonyl chlorides were then treated with bromine to generate the dibromo chalcone sulphonyl chlorides. These were subsequently reacted with 1,2-diaminopropane and 2-methyl-1,2-diaminopropane in boiling ethanol resulting in compounds **22-30** and **31-39** respectively, in 12-80% yields. The products were recrystallized using appropriate solvents and were characterized by spectral analysis, melting point, infrared spectroscopy, ^{1}H and ^{13}C NMR and mass spectrometry. In addition to spectral analysis, X-ray crystallography data was also collected for compound **31**, confirming the definitive structure of the synthesized compounds.

The reaction is believed to occur by the domino effect. The less sterically hindered amino group reacts with the sulphonyl chloride group to from the sulphonamide. The sulphonamide group then reacts with the β-carbon by nucleophilic substitution to form the isothiazole ring system and then the second amino group reacts with the α-carbon with elimination of the bromide ion to form the pyrazine ring. X-Ray data confirmed the structure of compound **31** and clearly confirmed that the dimethyl groups were on the carbon atom adjacent to the NH group. The synthesized compounds have been screened for potential antimicrobial properties against *Bacillus subtilis, Escherichia coli, Proteus hauseri* and *Staphylococcus aureus.*

Compound **27** displayed antibacterial activity against *B. subtilis* at a concentration of 416 $\mu g ml^{-1}$. Compounds **23, 24, 25, 26, 34, 35** and **36** all showed activity against *B. subtilis* at a concentration of 6.67 mg ml^{-1}, generally the compounds synthesized displayed activity at the highest concentration and were more active against gram positive bacteria.

A simple, efficient method for the synthesis of the novel benzo[4,5]isothiazolo[2,3-a]pyrazine-6,6-dioxide ring system which should be applicable to generate other fused pyrazine ring systems of biological interest has been developed.

Keywords: benzo[4,5]isothiazolo[2,3-a]pyrazine-6,6-dioxide, dibromochalcone sulphonyl, antibacterial and antifungal

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CONTENTS

Chapter 1

1.1 Introduction

Bacterial infections and diseases have been responsible for the disruption of populations for thousands of years; this includes fatalities of humans, livestock, plants and crops. Noted throughout history, epidemics of certain diseases have successfully wiped out entire communities ranging from hundreds to millions of deaths. Advances in science and technology have provided the medical world with the information required to eradicate many deadly diseases from re-emerging.

Through the development of vaccines and a better understanding hygiene, most developed countries have managed to maintain a strong hold at keeping fatal and highly infectious disease to a bare minimum. From the discovery of penicillin in the beginning of the 20th century there has been an astronomical breakthrough in the variety and quantity of different antibiotics available which gave way to the “golden age” of antibiotics.

Antibacterial resistance has provoked an urgent need for novel antimicrobial compounds. There has been a significant decline in the number of new antibacterial agents being released onto the market, with as few as five new drugs being successfully developed in a four year period (Moellering, 2011).

Research by the Infectious Disease Society of America has stated that there needs to be a minimum of ten new antimicrobial agents developed within the next eight years (Policy, 2010). The interest in antibacterial development has become less intense due to major pharmaceutical companies focusing their R&D into more profitable drugs, such as anticancer and drugs for neurological diseases. According to Theueretzbacher the development of antibacterial agents has slowed considerably, the main research in developing these group of drugs is being carried out in academic institutions (Theuretzbacher, 2009). A possible reason for this could be that there are very few targets available for selective toxic agents to act at. The expected success of High-through put screening and combinatorial libraries did not live up to the expectations *et al*, 2007). The development of antimicrobial resistance has generated a huge demand for successful production of novel pharmaceutically active compounds.

1.2 Antibacterial agents

The success of antibacterial agents is due largely to the fact that they can act selectively against the bacterial cell instead of the mammalian cell. Antibacterial agents have been reported throughout history for many centuries, elements of both ancient Chinese and Ayurvedic are still found in modern medicine (Singh & Barrett, 2006). The discovery of penicillin in 1928 was the beginning of the antibiotic "golden age" and for the next two decades many new antibacterial agent were developed (Saga & Yamaguchi, 2009). In half a century the available antibiotics increased 10 fold with the arrival of sulphonamides, streptomycin, tetracyclines, cephalosporins (Overbye & Barrett, 2005) and quinolone based antibiotics. The number of antibacterial agents available today covered a wide variety of bacterial diseases.

1.3 Antibacterial agents: mechanism of action

The structure, biochemistry and metabolism of a bacterial cell differs greatly to an animal cell, which enables a variety of ways an antibiotic can actively exert a therapeutic effect. The main mechanisms in which antibacterial agents work are commonly categorized into the following groups.

- Disruption of cell wall synthesis these include β-lactam antibiotics, vancomycin and cephalosporins. The bacterial cell wall is encased in layers of peptidoglycan which is a complex matrix consisting of amino sugar chains cross linked via peptide bonds. β-lactam antibiotics work by inhibiting the cross linking of peptidoglycan by inhibition of peptide bond formation, whereas vancomycin binds to the sugar component of peptidoglycan and prevents the synthesis of the glycan backbone (Tenover, 2006).
- Inhibition of protein synthesis these include tetracyclines and aminoglycosides. Bacterial ribosomes are responsible for protein synthesis and comprise of two ribonucleoprotein subunits, 50S and 30S. 50S ribosome inhibitors (macrolides) work by prevention of the initiation phase of mRNA translation whereas the 30S ribsome inhibitors (tetracyclines) work by inhibiting the association of aminoacyl tRNA to the bacterial ribosome (Chopra & Roberts, 2001).

- Metabolic pathway disruption, sulphonamides work by interfering with the synthesis of folate which is essential in the synthesis of bacterial DNA precursors. Bacterium use p-aminobenzoic acid to synthesise folate, using the enzyme dihydropteroate synthetase, sulphonamides competitively inhibit this enzyme and therefore stop bacterial DNA replication (Boufas *et al*, 2014).
- Nucleic acid synthesis inhibitors can be further divided into DNA or RNA synthesis inhibitors. The drug rifampicin inhibits nucleic acid production by binding next to the active site on the RNA polymerase enzyme and blocking the RNA elongation step. Quinolone antibiotics are DNA synthesis inhibitors which inhibit the enzyme topoisomerase, this enzyme is responsible for the winding and unwinding of the double helix that is required for replication. (Aggarwal, Kumar, Dureja, & Khurana, 2011).
- Disruption of the bacterial cell membrane which is achieved by interfering with the integrity of the cell membrane, and results in bacterial cell death. The drug polymyxin disrupts the bacterial cell wall due to the binding of the drug and the lipopolysaccharides (LPS) on the outer membrane. The binding of the drug to the LPS causes both inner and outer membranes to disintegrate (Falagas, Rafailidis & Mattaiou, 2010).

Mode of action	Example of antibacterial agent
Interference with cell wall synthesis	beta-Lactams: penicillins, cephalosporins, carbapenems, monobactams, Glycopeptides: vancomycin, teicoplanin
Protein synthesis inhibition	Bind to 50S ribosomal subunit -macrolides, chloramphenicol, clindamycin, quinupristin-dalfopristin and linezolid
	Bind to 30S ribosomal subunit – aminoglycosides and tetracyclines
Interference with nucleic acid synthesis	Inhibit DNA synthesis: fluoroquinolones
	Inhibit RNA synthesis: rifampin
Inhibition of metabolic pathway	Sulphonamides and folic acid analogues
Disruption of bacterial membrane structure	polymyxins and daptomycin

Table 1: a summary of the different types of antibiotics based on mode of action (Tenover' 2006)

1.4 Antimicrobial resistance

Due to the emergence of superbugs such as methicillin resistant S*taphylococcus aureus* (MRSA), many antibiotics are no longer effective therapeutic agents. Although MRSA and vancomycin resistance appears to be stabilising (Paphitou, 2013) there are new problems occurring. Gonorrhoea is the latest medical condition that has developed a resistance to a wide range of antibiotics with only third generation cephalosporins being effective (Ndowa & Lusti-Narasimhan, 2012).

Drug resistant tuberculosis, multidrug resistant tuberculosis (MDR-TB) and extensively drug resistant tuberculosis (XDR-TB) is becoming increasingly difficult to treat. Drugs such as rifampicin and isoniazid were once freely administered but have recently proved ineffective. Although the global prevalence of TB has seen a decline an estimated nine million new cases

of TB were reported in 2011 (Santos, Pires, Azeredo Bittencourt, Tufik, & Andersen, 2012). With this ancient infectious disease still claiming millions of lives each year there is a desperate need to develop novel and potent anti-TB drugs.

Current trends have indicated a significant rise in the number of gram negative pathogens developing resistance. Over the last decade *E. coli* and *Klebsiella pneumoniae* have displayed that third generation cephalosporins and combination therapies that include aminopenicillins, fluouroquinolones and aminoglycosides are no longer effective (Paphitou, 2013).

The latest research into antimicrobial resistance has stated that multidrug resistant pathogens are spreading at an unprecedented rate. Whilst many gram-positive organisms such as *Staphylococcus aureus* and *Enterococcus species* can be treated with a limited number of drugs whereas gram-negative bacteria have developed resistance to almost all prescribed antibiotics (Theuretzbacher, 2013).

The efficacy of any therapeutic agent becomes limited by the potential risk of resistance. From the late 1920's up until the late 1990's the expansion in the number and variety of antibacterial agents available had increased exponentially however the last decade has seen a decrease in the emergence of novel antibacterial agents. With fewer new antibacterial agents being designed and produced coupled with the increase in antibacterial resistance there has been a significant increase in nosocomial infections and other new fatal medical implications that have reduced the variety of drugs available for prescription. Figure 1 gives a brief outline of the history of antibacterial agents and antibacterial resistance.

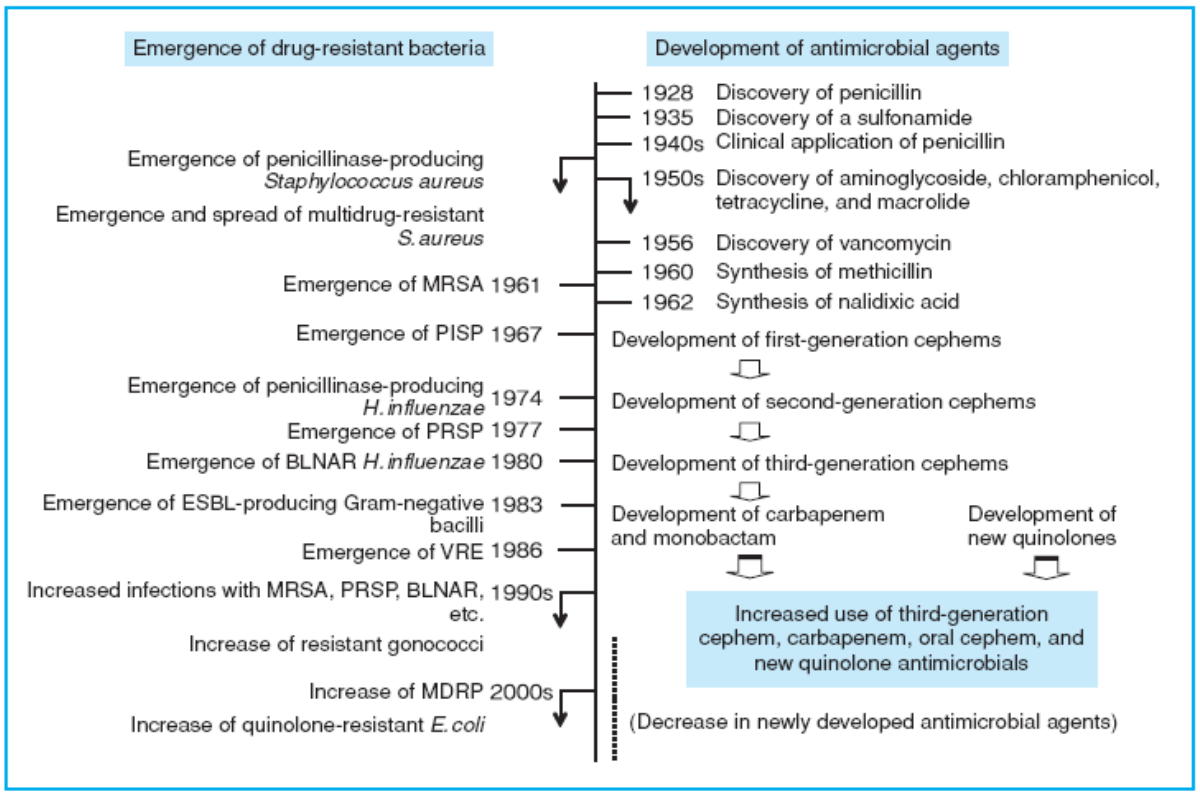


Figure 1: outlines the history of antibacterial agents, and the emergence of antibacterial resistance (Saga & Yamaguchi, 2009).

Although there is much interest in the combination of cephalosporins to a new β-lactamase inhibitor, this presents a challenge. β-Lactamase inhibitors are not effective for all β-lactamase related resistance. It was also stated that one of the only ways to get a stranglehold on emerging resistance is to develop a novel compound or chemical scaffold unrelated to known antimicrobial agents.

1.5 Antimicrobial resistance: Mechanism of action

Antibacterial resistance is thought to be the result of overuse and misuse of antibiotics, (Paphitou, 2013) up until the 1970s most physicians believed that the majority of bacterial infections could be effectively treated by a wide variety of antibacterial agents. Antibiotics were widely prescribed and through this many strains of bacteria have evolved and have become resistant to a broad range of antibiotics. Some bacteria have acquired resistance to more than one line of antibiotics, which has paved way for the emergence of "superbugs". Resistance can either be innate or acquired, innate resistance is naturally occurring in some bacterial species which renders antibiotics ineffective before the pathogen has been introduced to the drug (Ebrahim, 2010). Acquired resistance is mainly a result of genetic mutations, these mutations have enabled bacteria to develop a variety of mechanisms which prevent the efficacy of the antibiotics.

Some bacteria have acquired resistance through genetic mutations; these mutations have enabled bacteria to develop a variety of mechanisms which render antibiotics useless. Some organisms have acquired efflux pumps that have the ability to remove the antibacterial agent before it reaches its target site, other mutations have evolved the bacterial cell wall so that the necessary antibiotic binding sites are present thus preventing the drug from entering the cell (Tenover, 2006). Research into the trends and patterns of antibacterial resistance has been extensively reported over the last three decades. Methicillen resistance *Staphylococcus aureus* (MRSA) has been monitored intensely and it has been reported that the rate of resistance for this bacterium has stabilised (Livermore, 2012). The main mechanism in resistance in MRSA involves target site alteration, through genetic mutations *S. aureus* has acquired penicillin binding proteins and an increase β-lactam antibiotic resistance (Appelbaum, 2007).

Bacteria such *E. coli* and *S. aureus* have become more resistant via the emergence of β-lactamases produced naturally by bacteria which destroy β-lactam antibiotics. Although these enzymes are found in bacteria through genetic mutation they have become more problematic by becoming extended broad spectrum β-lactamases (ESBL's), with over 150 ESBLs being described (Bradford, 2001).

Another mechanism of acquired antimicrobial resistance is horizontal gene transfer (HGT), this is where genetic information coding for resistance for one type of bacteria is laterally transferred to another species of bacteria. This transfer of genetic material is possible through the mobility of some genetic elements such as plasmids, gene cassettes, transposons and bacteriophages (Cruz & Davies 2000). Horizontal gene transfer has several mechanisms and is thought to be one of the main contributing factors of antibiotic resistance. Bacterial HGT mainly occurs through transformation of DNA this is where bacterial cells uptake fragments of naked DNA. Transduction is another mechanism in which genetic material is passed from one cell to another and is done so via a bacteriophage which is a virus that can infect and replicate with a bacterial cell. Plasmids like bacteriophages act as vehicles for HGT, plasmids can pass genetic material via cell to cell contact almost creating a corridor type system where DNA can pass easily from cells (Lindsey, 2014).

This has been observed in *Staphylococcus* species where vancomycin resistance S. aureus have acquired the genes that code for vancomycin resistance from *Enterococci* species. The ability bacteria have to spread antibiotic resistant genes across species has contributed greatly to struggle in combating antimicrobial resistance (Sung & Lindsay, 2007). The actual mechanism of vancomycin resistance in *S. aureus* is yet to be fully elucidated (Srinivasan, Dick & Perl 2002).

Antibiotic class	**Target**	**Mode(s)of resistance**
B-Lactams	Peptidoglycan biosynthesis	Hydrolysis, efflux, altered target
Aminoglycosides	Translation	Phosphorylation, acetylation, nucelotidylation, efflux, altered target
Glycopeptides	Peptidoglycan biosynthesis	Repogramming peptidoglycan biosynthesis
Tetracyclines	Translation	Monooxygenation, efflux, altered target
Macrolides	Translation	Hydrolysis, glycosylation, phosphorylation, efflux, altered target
Lincosamides	Translation	Nucletotidylation, efflux, altered target
Streptogramins	Translation	C-O lyase (type B streptogramins), Acetylation (type A streptogramins), efflux, altered target
Oxazolidinones	Translation	Efflux, altered target
Phenicols	Translation	Acetylation, efflux, altered target
Quinolones	DNA replication	Acetylation, efflux, altered target
Pyrimidines	C_1 metabolism	Efflux, altered target
Sulfonamides	C_1 metabolism	Efflux, altered target
Rifamycins	Transcription	ADP-ribosylation, efflux, altered target
Lipopeptides	Cell membrane	Altered target
Cationic peptides	Cell membrane	Altered target, efflux

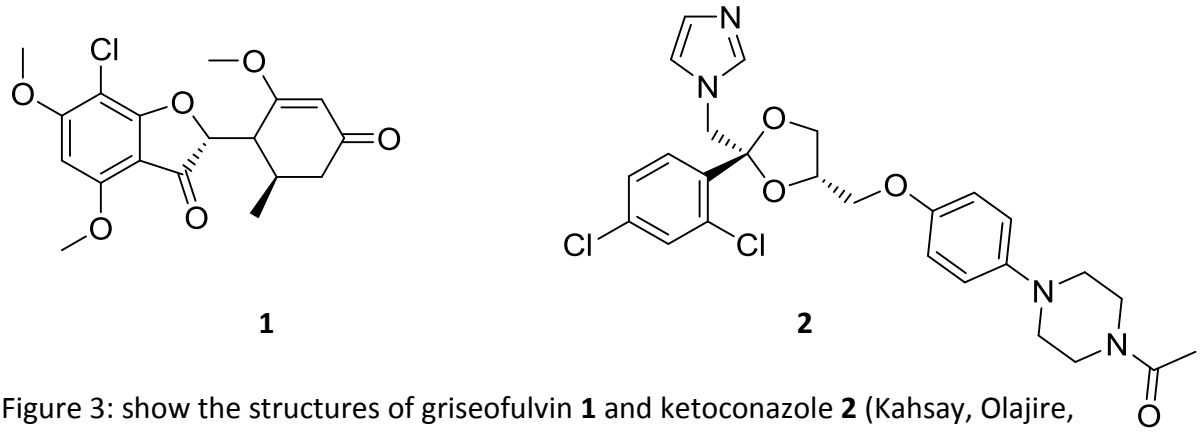
Figure 2: A table to show what mechanisms have been developed by bacteria to combat certain types of antibiotics (Davies & Davies 2010).

1.6 Antifungal agents

Antifungal agents are most easily divided into two categories natural products such as polyenes and echinocandins and synthetic drugs such as the azoles and pyrimidines (Odds, 2003). Fungi are eukaryotic as are humans, so when developing anti-fungal agents it is essential to ensure that the agents to not cause harm to the human host (Thompson, Cadena, & Patterson, 2009), which also this limits the sites of action where antifungal agents can act.

The first antifungal agent in use was griseofulvin **1** in 1939 (Sheehan, Hitchcock, & Carol, 1999) which was the first basic dermal antifungal. This gave way to the azole and polyene antifungal groups. In the 1950s Amphotericin B (Amp B a polyene) isolated from *Streptomyces nodosusand* was discovered and found to be a very effective antifungal in systemic mycosis and is still the gold standard in systemic fungal infections. However Amp B is poorly soluble and is notoriously nephrotoxic (Kleinberg, 2006). To reduce the toxicity of Amp B, lipid based formulations were designed to protect the kidneys. In recent years Amp B has been used in combination with other antifungals to reduce the nephrotoxicity of the drug (Barrett *et al*., 2003). The last 70 years has given rise to numerous novel antifungal agents however azoles are still the most widely used (Odds, 2003). Through understanding of the fungal cell (genome, cell wall and cell membrane) there has been a rapid advance of more effective and less toxic antifungals (Smith & Kauffman, 2012). For the past decade the azole class of antifungals have become the most commonly used and prescribed, however the overuse of this group has lead to increased resistance and poor efficacy (Chai *et al.*, 2011). Ketoconazole **2** is still widely used and is commonly found in many antidandruff shampoos and is used as a starting point for novel azole derivatives (Oh, Yamada, Asami, & Yoshizawa, 2012).

This has sparked a new area of interest into the azole scaffold; the need for novel antifungal agents has become increasingly important with new emerging fungal pathogens coupled with the increase of antifungal resistance.



1 **2**

Figure 3: show the structures of griseofulvin **1** and ketoconazole **2** (Kahsay, Olajire, Schepdael, & Adams, 2013).

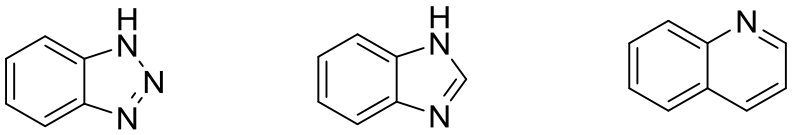
1.7 Antifungals: Mechanism of action

The fundamental difference between fungal cells and mammalian cells is the sterol molecule present in fungi, ergosterol, which is found in the fungal cell membrane. Ergosterol carries out the same functions as cholesterol. Like antibacterial agents, antifungal agents have many mechanisms by which they are effective.

Fungal ergosterol synthesis inhibitors such as azoles inhibits the conversion of lanosterol to ergosterol by targeting the enzyme lanosterol 14α-demethylase (Ahmad, Khan, Manzoor, & Khan, 2010). Squalene epoxidase inhibitors include terbinafin, which inhibit the production of lanosterol, which consequently inhibits the synthesis of ergosterol. Ergosterol distruptors like polyenes bind to ergosterol, creating pores in the cell membrane which causes an efflux of cellular potassium, which ultimately results in cell lysis (Kanafani & Perfect, 2008). Glucan synthesis inhibitors such as the drug class echinocandins inhibit the synthesis of β-glucan which is essential in the fungal cell wall. Chitin synthase inhibitors, chitin plays an important role in maintaining fungal cell wall integrity, inhibition of this glucose derivative will lead to fungal cell impairment and ultimately cell death. Other mechanisms of action include nucleic acid synthesis inhibitors, protein synthesis inhibitors and microtubule synthesis inhibitors.

From the literature available the most recent research into novel antifungal agents is based on developing and synthesizing analogs of known biologically active scaffolds. Compounds

being investigated include benztriazoles **3**, benzimdazoles **4** and quinolones **5** (see figure 3) pyrazines and thiophenes (Kathiravan *et al.*, 2012).



3 **4** **5**

Figure 4: shows chemical structure for benztriazoles **3,** benzimidazoles **4** and quinolone **5**.

1.8 Antifungal resistance

Antifungal resistance is playing a significant role in the fight against invasive fungal infections and is becoming very problematic when treating immunocompromised patients (Kanafani & Perfect, 2008). The understanding and emergence of antifungal resistance has lagged behind antibacterial resistance, this is thought to be due to recent advances in medicines which suppress the immune system (Ghannoum & Rice, 1999). Current antifungals such as azoles, polyenes and echinocandins are no longer as effective. Azole resistance has been categorized into four mechanisms: decreased drug concentrations; target site alteration; up-regulation of target enzyme, and the development of bypass pathways (Kanafani & Perfect, 2008). Similar to bacterial cells, *Candida* fungal species have developed efflux pumps which decrease the amount of antifungal agent that reaches its target site. Resistance to polyenes and echinocandins have been reported but the mechanism in which antifungal resistance has emerged has yet to be elucidated (Kanafani & Perfect, 2008).

Much research in the past decade has been centred on improving already known antimicrobial agents. The research presented in this thesis is based on taking two known heterocycles and combining them to synthesise a set of novel compounds which display important biological properties.

1.9 Antibiotic susceptibility methods

The definition of antimicrobial resistance is predominantly defined using minimal inhibitory concentration (MIC) breakpoints and a lot of research has been conducted into standardizing susceptibility testing methods to outline how antimicrobial resistance is defined (Ghannoum & Rice, 1999). Antibacterial susceptibility tests were standardized in the late 1970's (Wheat, 2001); only recently have there been standardized and test guidelines for a limited number of fungal species published (Kanafani & Perfect, 2008).

It is important to be able to determine the resistance of a bacterial isolate to a particular antibiotic to gain a better understanding of how resistance has come about. One of the earliest antibacterial susceptibility tests used is the broth dilution method. This method uses 2 fold dilutions of an antibiotic in either tubes or a microtitre plate; to this a standardized inoculated bacterial suspension is added. The lowest concentration of antibiotic that prevents growth is the MIC. The MIC value can then be further investigated by further serial dilution of the MIC (Jorgensen & Ferraro, 2009). Disk diffusion is another method used to determine antimicrobial susceptibility; an appropriate agar plate is inoculated with either bacteria or fungi. Then a standard antibiotic disk or disks will be placed on the agar plate, after incubation the zone of inhibition around the antibiotic disk is measured. If there is a zone of inhibition a further test can be done by taking some of the agar from the zone of inhibition and placed on a sterile plate and left to incubate (Martos *et al*., 2012). If there is no further growth then it can be assumed that the antibiotic used in bactericidal, or if further growth is observed then it could be assumed that the antibiotic is bacteriostatic.

1.10 The relevance of fusing heterocycles

Heterocycles are an enormously diverse group of compounds that are widely distributed in nature, and are found in many pharmaceuticals. Heterocycles are easily manipulated in organic synthetic routes; the compounds can be easily modified to increase or decrease reactivity (Dua, Shrivastava, Sonwane, & Srivastava, 2011). Varying substituent groups (ring activators or deactivators) and their positions around the rings can result in novel libraries of biologically diverse heterocyclic compounds. Heterocycles are used extensively as intermediates in reactions and as building blocks too in synthesis. Incorporation and

substitution of a wide range of functional groups in heterocyclic systems provides an endless number of compounds.

This also leads to synthetic chemists continuously striving for new, more selective synthetic methods to provide a wider range of heterocyclic derivatives; this includes the fusing of different heterocycles and varying functional groups in order to continuously produce novel heterocyclic compounds.

Over the last few decades the study of medicinal chemistry has gone from strength to strength, the discovery and elucidation of the human genome, computational chemistry and the development of a variety of different biological assays have paved the way for research into novel compounds being synthesised and screened for biological importance. With the recent advent of antimicrobial resistance the area of medicinal chemistry has become even more essential in drug discovery.

There is a continuing emergence of new drug resistant strains of a wide variety of bacterial, fungal and viral diseases with gold standard drugs no longer exerting effective activity. Although there is a plethora of medicines and vaccines available to treat and prevent most ailments, supply is no longer meeting demand and the need for novel biologically active compounds is increasing. Over the last decade there has been a reported increase in the rate of resistance in regards to gram negative pathogens (Paphitou, 2013). Methicillen resistance *Staphylococcus aureus* is still a key problem in trying to combat antibacterial resistance (Gould *et al*., 2012).

1.10.1 Benzoisothiazoles

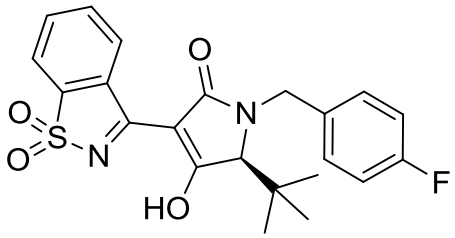
Benzoisothiazoles are heterocycles consisting of a benzene ring fused to an isothiazole ring. The first known benzoisothiazole derivative with known biological importance was saccharin **6**.

6 **7**

Figure 5: shows the chemical structure saccharin **6** and benzo[d]isothiazole **7.**

Saccharin **6** is five hundred times sweeter than sugar and has gained attention over the past century as an alternative to sugar (Sawant, 2011). Benzoisothiazole **8** rings are found in many chemically interesting compounds, these compounds and derivatives have been synthesized and have shown varied biological activity (Yadav & Senthilkumar, 2011). Chaudhary and co-workers (Chaudhary, Sharma, Sharma, & Varshney, 2010), stated that substituted benzoisothiazole compounds have antitumor, anti-allergic, anti-diabetic, anti-inflammatory, anthelmintic and anti-HIV activity (Chaudhary, Sharma, Sharma, & Varshney, 2010).

Benzo[d]isothiazole-1,1-dioxide **8** derivatives have been screened for antimicrobial, antiviral and antiproliferative activity (Vicini *et al*., 2003).



8

Figure 6: shows the structure of a benzo[d]isothiazole-1,1-dioxide derivative **8.**

Benzo[d]isothiazole combined with an arylidene moiety (a methylene derivative of an aryl group) with different substituent such as methyl groups and fluorine atoms, have displayed cytotoxicity against human $CD4^+$ lymphocytes. These compounds inhibited the growth of leukaemia cell lines and also antiproliferative activity against solid tumour-derived cell lines (Vicini *et al.*, 2003). Vicini and co-workers investigated benzoisothiazole hydrazone derivatives as possible antiviral (anti-HIV-1) agents. The investigation focused on the structural requirements that are essential for anti-HIV activity and how modifying the alkene chain length between the benzoisothiazole ring and the hydrazone group would increase or decrease any possible anti-HIV activity. Although many of the compounds did show promising results the mechanism of action remains to be elucidated (Vicini, Incerti, La Colla, & Loddo, 2009). Benzothiazole derivatives have also been found to inhibit human cyclooxygenase-2-enzymes (COX-2) (Paramashivappa, Phani Kumar, Subba Rao, & Srinivasa Rao, 2003).

Recent research in CNS-mediated diseases has given rise to discovery of a family of serotonin (5-HT) receptors, 5-HT_6. In *vivo* studies have shown that inhibition of 5-HT_6 has significant positive impact on cognitive impairment. Sufferers from diseases such as Alzheimer's and schizophrenia could potentially have an effective treatment (Liu *et al*., 2009) within the next decade. In 2012 researchers synthesised a group of benzoisothiazole derivatives that contained a *N,N*-dimethlyformimidamide group of which two of the compounds displayed promising activity as potential 5-HT_6 antagonists (Yoo, Hayat, Rhim, & Park Choo, 2012).

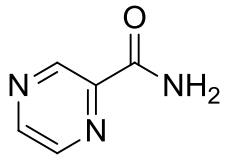
A series of benzo[d]isothiazole-1,1-dioxides were synthesised that inhibited HCV polymerase NS5B. The investigation used structure based design and structure-activity relationships which lead to the addition of a high affinity methyl sulphonamide group (de Vicente *et al.,* 2009). Initially this research group focused on the use of a benzothiazine ring which showed potent inhibition of HCV polymerase. However these compounds had low oral bioavailability and high clearance in rats. The benzothiazines ring was then replaced with a benzo[d]isothiazole-1,1-dioxide (see figure 6) and the new compounds displayed moderate inhibition of the HCV polymerase NS5B (non-structural protein 5 B, this protein is

synthesised by the Hep C virus) (Golub *et al*., 2012). Previous work conducted by this research group showed that the addition of a methylsulphonamide group greatly increased potency however it was ineffective in inhibiting the replication of the virus. A methylene spacer group was introduced between the benzo[d]isothiazole-1,1-dioxide ring and the sulphonamide group, which maintained the potency against the enzyme and increased the replicon potency.

1.10.2 Pyrazine

Pyrazine (paradiazine) is a symmetrical nitrogen containing heterocycle component which is found in a wide variety of drugs, cosmetics and flavour enhancing compounds. Pyrazine derivatives are ubiquitous in medicine and also excellent starting points in the synthesis of complex heterocycles. Pyrazine derivatives have been reported to have a wide range of antimicrobial properties, as well as controlling allergic reactions (Myadaraboina, Alla, Saddanapu, Bommena, & Addlagatta, 2010).

Pyrazinamide **9** has been used to treat tuberculosis (TB) and is still the front line drug, although more recently it has been used in combination with rifampicin and isoniazid. These three drugs have become the gold standard for TB treatment (Mitchison & Fourie, 2010).

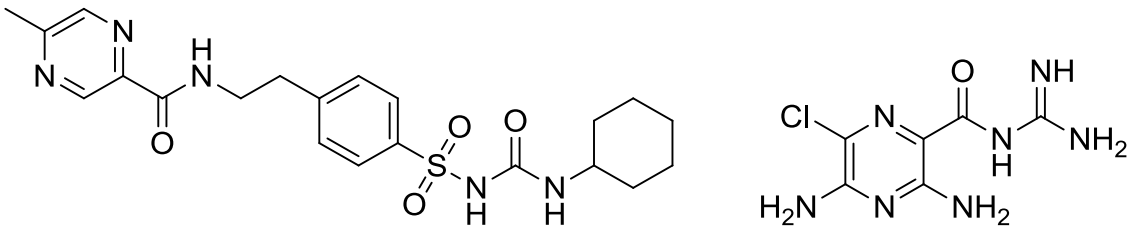


9

Figure 7: shows the structure of pyrazinamide **9**.

The latest research into TB has been coupled with HIV sufferers; TB has become the leading cause of death amongst HIV/AIDS patients. Treating physicians are faced with the dilemma of not knowing which pathogen to treat first as TB increases drug resistance to anti-HIV agents and HIV allows *M.tuberculosis* to become a more effective opportunistic pathogen (Munawwar & Singh, 2012).

Glipizide **10** is an anti-diabetic drug belonging to the sulphonylurea group of drugs. This drug contains a pyrazine ring (Mallikarjun, Ravi, Babu, Kiran, & Kumar, 2009). Amiloride **11** is a potassium sparing diuretic, that has been used in the treatment of congestive heart failure, hypertension and long term use of this drug has been reported to prevent the development of stomach, colon and pancreatic cancer (Tatsuta *et al*., 1997).

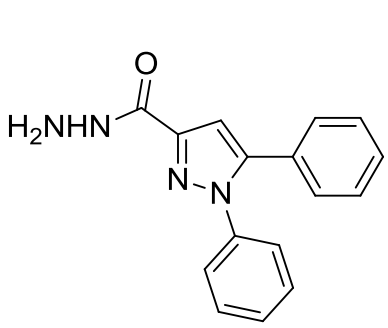


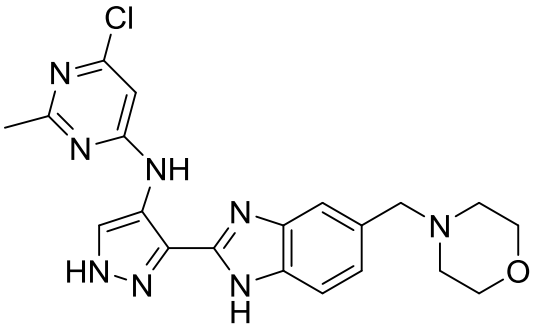
10 **11**

Figure 8: shows the structure of glipizide **10** and amiloride **11.**

Pyrazine derivates play an important role in the food industry; aroma and flavours of many food groups are the result of pyrazine derivatives. Trying to isolate specific derivatives of pyazine is an ongoing investigation by the food industry; by isolating the pyrazines responsible for specific aromas and flavours is key to enhancing the quality of already existing food products on the market (Plutowska & Wardencki, 2007).

Pyrazole is a five membered heterocycle that contains two nitrogen atoms, which is found in a wide variety of biologically active compounds. Figure 8 shows two pyrazole derivatives, compound **12** has shown anti-inflammatory, antipyretic and antinociceptive properties (Malvar *et al.*, 2014) and compound **13** is a pyrazole-benzimidazole derivative that has displayed potent antitumour activity (Zheng *et al*., 2013).



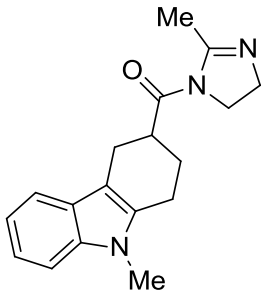


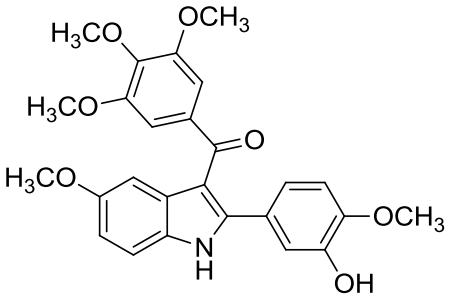
12 **13**

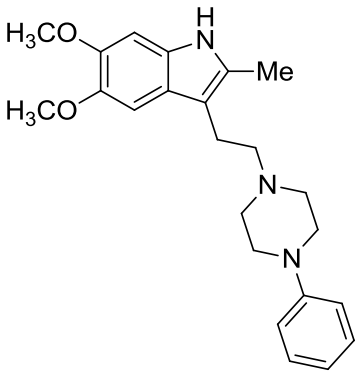
Figure 9: shows structures of compound **12** and **13**

1.10.3 Current research into heterocyclic systems as biologically active compounds.

From the literature available there is a lot of evidence supporting the idea of combining heterocycles to produce novel and biologically important compounds (Kaushik *et al.*, 2013). In a review by Kaushik and co-workers there is an extensive amount of research into the biological importance of indole and indole derivatives. From this review the wide variety of biological importance exerted by indole derivatives are highlighted. Odansetron **14** is a drug given to patients who have undergone chemotherapy to deal with sickness and 2-aryl-3-arylcorbonylindole **15** an anticancer agent which have shown to inhibit tubulin polymerization and oxpertine **16,** which is an antipsychotic (see figure 9).







14 **15** **16**

Figure 10: structure for odansetron **14**, an anticancer 2-aryl-3-arylcorbonylindole **15** and oxpertine **16**.

A review article by Ali and Siddiqui discussed the pharmacological importance of the benzothiazole nucleus. The review article stated that this particular heterocyclic system displayed a range of diverse biological importance such as anti-cancer, antiviral and anticonvulsant properties. In addition the review article also stated that the benzothiazole ring system was a good template for the development of novel biological compounds (Ali & Siddiqui, 2013)

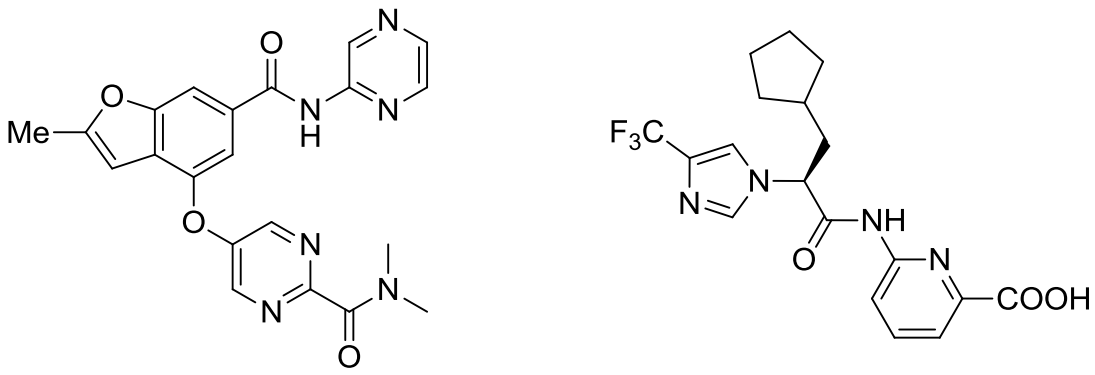
A review article by Rudrapal and De focuses on the biological importance of heterocyclic Schiff bases. Schiff's bases are known for their broad spectrum of biological activity due to the presence of an imine bond. This review includes a vast number of heterocyclic systems including sulphur nitrogen heterocycles, as biologically important compounds (Rudrapal & De, 2013)

Recent research on heterocyclic systems that include sulphur, nitrogen and oxygen has revealed that novel derivatives of bis-benzothiazolyl-pyridines and pyrazine have displayed strong antiproliferative activity. It has been strongly suggested that the synthesized heterocycles work by exerting oxidative stress on the cancer cells (Myadaraboina *et al*., 2010).

In the last three years there has been a lot of interest in aurora kinase inhibitor molecules being synthesised as possible anti-cancer agents. In 2010 a series of novel imidazo[1,2-a]pyrazine aurora kinase inhibitors were synthesized and investigated (Belanger *et al.*, 2010). The aurora kinase family regulate mitosis, over expression of all three mammalian aurora kinases (aurora A, B & C) have been found in human cancer cells; however the implication of these kinases (aurora C) in tumour development has not been ascertained (Katayama & Sen, 2010).

A recent review article on kinase activators as novel antidiabetic agents, focused on designing a set of compounds that would activate glucokinase, which is the enzyme responsible to glucose homeostasis in the body (Matschinsky, 2009). Through structure activity relationships (SAR) the starting compound was a 2-methybenzofuran **17** scaffold from which various structurally diverse analogs were prepared. The review also included the study and discovery of imidazo derivative **18** and related structures see figure 10 (Castro,

2012). Both compounds **17** and **18** are currently undergoing clinical evaluation for type 2 diabetes mellitus patients.



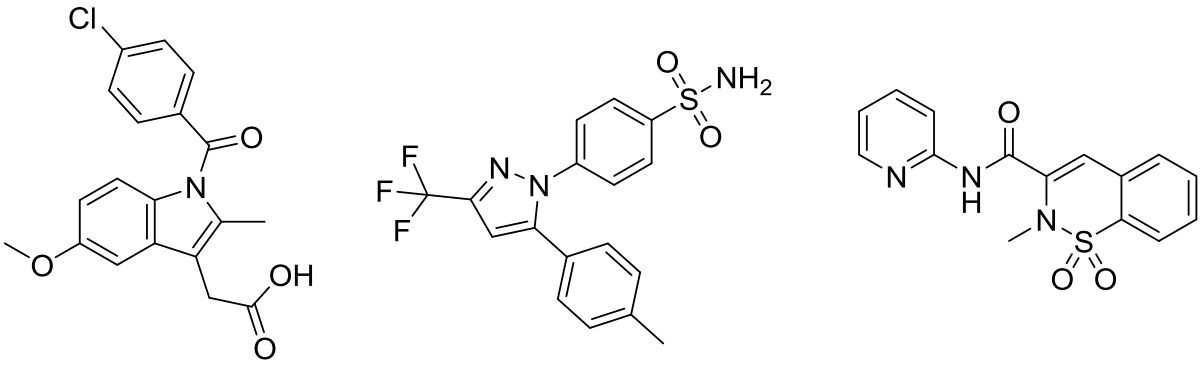
17 **18**

Figure 11: shows a 2-methylbenzofuran derivative **17** and imidazo derivative **18**. that is currently in phase 1 of clinical trials (Castro, 2012).

Chikalia and co-workers synthesized a library of twenty benzimidazole-1,3,4-oxadiazole derivatives and screened all twenty compounds against a variety of bacteria, fungi and have screened them for potential antituberculosis activity. These compounds displayed antibacterial activity at concentrations as low as 4 µgmL and similar results for antituberculosis activity (Patel, Patel, Kumari, Rajani, & Chikhalia, 2012).

Heterocyclic systems are found in many anti-inflammatory agents such as indomethacin **19**, celecoxib **20** and piroxicam **21** (see figure 11). More common anti-inflammatory agents such as ibuprofen are known as non steroidal anti-inflammatory drugs (NSAIDs) which are the most widely used anti-inflammatory agents, with aspirin being marketed in 1860 (Rao, Knaus, Road, & Jolla, 2008) and has remained a gold standard since then. From the development of acetylsalicylic acid (aspirin) there have been a number of breakthroughs in the understanding of inflammatory processes. The elucidation of cylcooxygenase enzymes has helped combat gastrointestinal related side effects caused by older generation NSAIDs. Drugs such as Ibuprofen and diclofenac are the most frequently prescribed antinociceptive and anti-inflammatory agents. Over the last 50 years steroids have been used to treat

chronic inflammatory diseases (DeNatale, Rosenberg, & Gharibo, 2010), however over use of drugs such as corticosteroids have revealed the detrimental effects long term use of steroids. Many drugs will exhibit complication such as resistance or damaging side effects when overused, therefore it is necessary to either limit the amount of drugs we take or continually strive to improve upon the drugs already in use. Constant research is being conducted into the improvement of failing drugs in a broad range of drug classes.

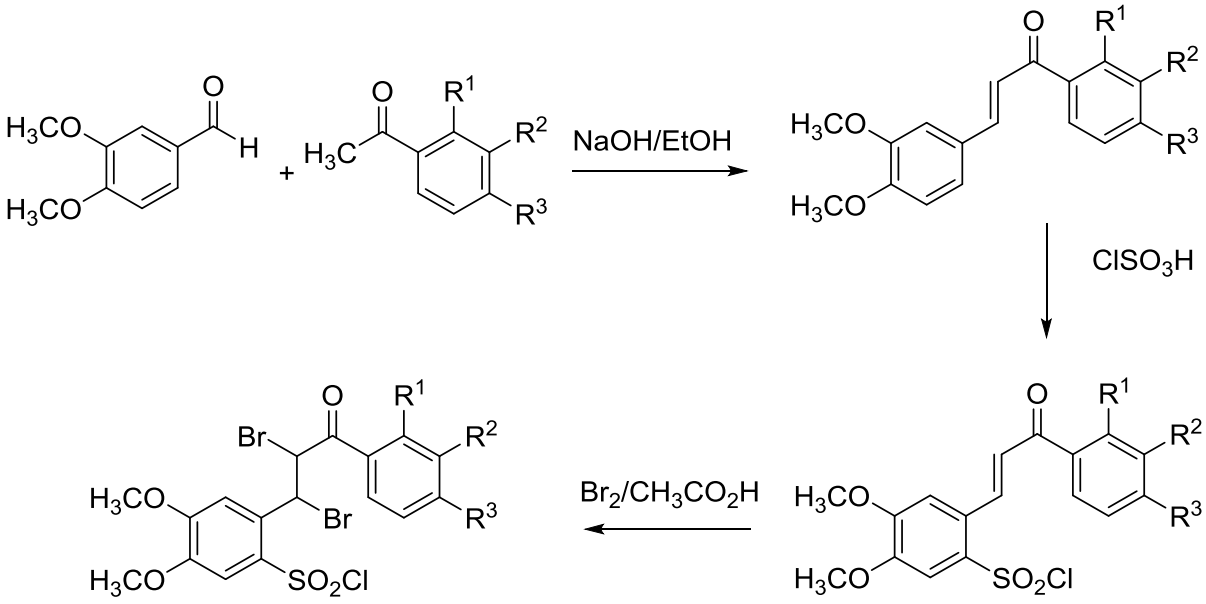


19 **20** **21**

Figure 12: structures of indomethacin **19**, celecoxib **20** and piroxicam **21.**

To our knowledge, the literature shows no information on benzoisothiazoles fused to pyrazine rings as biologically active molecules. There are a small number of articles that report benzoisothiazoles derivatives as being possible drug like molecules with a broad spectrum of biological importance (Yadav & Senthilkumar, 2011).

The research proposed aims to explore the biological properties of benzoisothiazoles fused to pyrazine rings. The aim of the current research was to synthesize benzo[4,5]isothiazolo[2,3-a]pyrazine-6,6-dioxide derivatives. For this, chalcone dibromo sulphonyl chloride were used as a starting materials and ethanol as a solvent, these precursors were synthesized in the laboratory during a previous project (see scheme 1). The resultant product was characterised by using different analytical techniques. The novel library which was successfully synthesized and characterized was then screened for antimicrobial activity.



Scheme 1: Shows the general procedure for synthesis of the precursor used for the synthesis of benzoisothiazole derivatives.

The final sulphonyl chloride precursors were previously synthesized in the lab, from these precursors a series of benzoisothiazole derivates will be synthesised and then screened for any biological activity.

1.10.4 Biological screening

We aimed to screen all the compounds synthesized for a range of different biological activities these include antimicrobial, antifungal, anti-inflammatory, tuberculosis and possibly screen some of the compounds for any activity regarding the CNS. For the antimicrobial screening the compounds will be tested using disk diffusion methods and well diffusion approach which are described in a review by Ncube *et al* (Ncube, Afolayan, & Okoh, 2008).

For bacterial cell viability testing MTT assay will be employed. MTT assay was first described in 1983 by Tim Mosmann, this is a colorimetric technique used in mammalian cells. A yellow tetrazolium salt is reduced to a purple formazan; this method is to provide quantitative data on living or surviving cells. The yellow solution is reduced by mitochondrial dehydrogenase enzymes present in active/living cells; it is common practise to assume that the colour intensity of the purple formazan is directly proportional to the amount of viable cells present. However the mechanism in which the MTT works has been questioned due to the fact that bacteria do not possess mitochondria. It is has been surmised that MTT is mainly reduced by NADH in the cytoplasm and some dehydrogenases associated with the endoplasmic reticulum (Stockert, Blázquez-Castro, Cañete, Horobin, & Villanueva, 2012).

Chapter 2

2.1 General methods for synthesis of benzoisothiazole derivatives

All the chemicals were purchased from Sigma Aldrich and used in the reactions without any prior purification. Melting point ranges were determined using a Gallenkamp melting point apparatus. The NMR spectra were recorded using a JeolDextra NMR spectrometer at 600MHz spectrometer with tetramethylsilane as internal standard and solvents as indicated. Chemical shifts were measured in ppm (δ) relative to TMS (0.00 ppm). Coupling constants (J) are reported in Hertz (Hz). The following abbreviations are used to describe the signal multiplicities: s (singlet), d (doublet), t (triplet), q (quartet) and m (multiplet). Infrared spectra were recorded with a Varian spectrophotometer as KBr discs (0.080 g of KBr + 0.0015 g of compound). TLC (thin layer chromatography) was performed using silica gel plates by dissolving the compound in ethyl acetate and using the solvent system cyclohexane:ethyl acetate; 2:1.

2.2 Liquid chromatography Mass Spectroscopy (LC-MS)

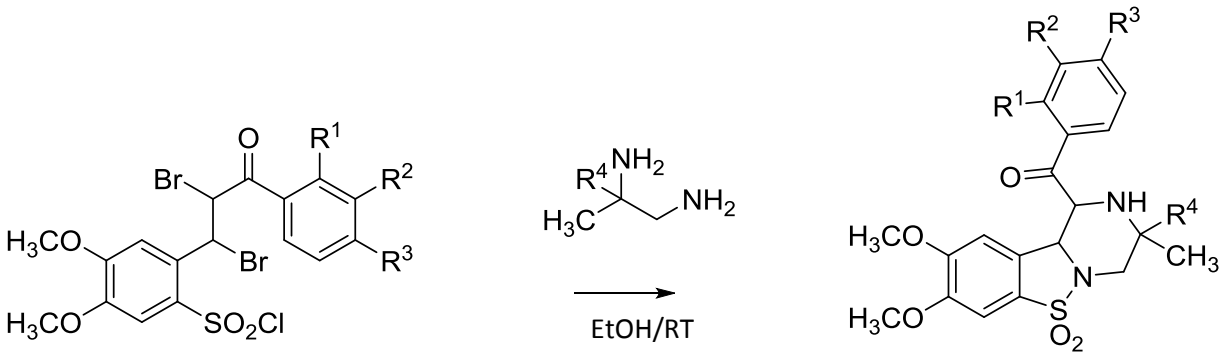
Samples were obtained with a VG Micromass V15 spectrometer operating at 70 eV (Varian: 210 LC pumps × 2, 1200L Quadrapole MS/MS, 410 autosampler) using a gradient solvent system of A: Water/0.1% formic acid and B: acetonitrile/ 0.1% formic acid. Electrospray ionisation was employed.

2.3 X-ray Crystallography

X-Ray analysis has also been used to determine the structure of one of the compounds. The sample of a crystalline compound was diffracted by X-rays. From the diffraction pattern it is possible to predict the spatial arrangement of the atoms in a molecule and therefore characterise the compound. X-Ray analysis was carried out at the University of Nottingham by Dr Lee Martin.

2.4 General method for synthesis of benzo[4,5]-isothiazolo[2,3-a]-pyrazin-1-yl) (phenyl) methanones (22-39)

To a stirred solution of the chalcone dibromo sulphonyl chloride (1g) in ethanol (25 ml) was added 1,2-diaminopropane (2 mole equivalent). The reaction mixture was warmed on a water bath for 10-15 minutes until all the solid had dissolved. The reaction mixture was allowed to cool to room temperature and the resulting solid was filtered and air dried. The product was purified by recrystallization from the appropriate solvent.



Scheme 2: Shows the general procedure for synthesis of the final step in the synthesis of benzoisothiazole fused pyrazine derivatives.

Compound	R^1	R^2	R^3	R^4	% Yield	m.p. (°C)	m/z (M^{+H})
22	H	H	H	H	29	219-220	402.3
23	H	H	CH_3	H	47	180-181	416.3
24	H	H	F	H	30	181-182	420.2
25	H	H	Cl	H	43	204-205	436.2
26	H	H	Br	H	48	204-205	480.4
27	H	Cl	H	H	79	194-195	436.2
28	H	Br	H	H	50	189-190	482.7
29	Cl	H	H	H	46	215-216	436.5
30	Br	H	H	H	75	195-196	480.4
31	H	H	H	CH_3	34	163-164	416.3
32	H	H	CH_3	CH_3	64	205-206	430.3
33	H	H	F	CH_3	12	175-176	434.3
34	H	H	Cl	CH_3	39	180-181	450.2
35	H	H	Br	CH_3	45	195-196	494.1
36	H	Cl	H	CH_3	54	179-180	450.2
37	H	Br	H	CH_3	80	175-176	495.9
38	Cl	H	H	CH_3	64	191-192	452.2
39	Br	H	H	CH_3	29	201-202	498.6

Table 3: shows data for compounds **22-39**.

2.5 Results for Compounds (22-39)

(8,9-Dimethoxy-3-methyl-6,6-dioxido-2,3,4,10b-tetrahydro-1H-benzo[4,5]isothiazolo[2,3-a]pyrazin-1-yl)(Phenyl)methanone (22)

Re-crystallizing solvent used: Ethanol. Yellow crystals were obtained (29% yield); Melting Point: 219-220^{o}C; IR (KBr cm^{-1}): 3337 (NH), 1681 (C=O), 1287 , 1176 (SO_2); ^{1}H NMR ($CDCl_3$): δ 1.8 (d, 3H, CH_3 *J* = 6.42 Hz), δ 1.75 (s, 1H, NH), δ 2.88 (s, 1H, CH), δ 3.45 and δ 3.73 (d, 2H, CH_2 *J* = 12.00 Hz), δ 3.51 (s, 3H, OCH_3), δ 3.91 (s, 3H, OCH_3), δ 4.45 (d, 1H, CH *J* = 10.09 Hz) δ 4.68 (d, 1H, CH *J* = 10.09 Hz), δ 6.43 (s, 1H, CH), δ 7.24 (s, 1H, Ar-H), δ 7.45 (d, 2H, Ar-H *J* = 7.32 Hz), δ 7.60 (s, 1H, Ar-H), δ 7.93 (d, 2H, Ar-H *J* = 8.25); ^{13}C NMR ($CDCl_3$) δ 18.80, 47.20, 48.20, 55.93, 56.31, 58.40, 62.40, 102.72, 107.25, 126.33, 128.98, 129.02, 134.42, 135.42, 150.38, 152.53, 198.21 (C=O); MS (m/z): $(M^{+}H)^{+}$ 402.3.

(8,9-Dimethoxy-3-methyl-6,6-dioxido-2,3,4,10b-tetrahydro-1H-benzo[4,5]isothiazolo[2,3-a]pyrazin-1-yl)(p-tolyl)methanone (23)

Re-crystallizing solvent used: Ethanol. White crystals were obtained (47% yield); Melting Point: 180-181^{o}C; IR (KBr cm^{-1}): 3447 (NH), 1663 (C=O), 1284, 1140 (SO_2): ^{1}H NMR ($CDCl_3$): δ 1.14 (d, 3H, CH_3), δ 1.54 (s, 3H, CH_3), δ 1.73 (s, 1H, NH), δ 2.37 (s, 1H, CH), δ 2.39 and δ 2.43 (s, 2H, CH2), δ 3.44 (s, 3H, OCH3), δ 3.88 (s, 3H, OCH_3), δ 4.45 (d, 1H, CH), δ 4.65 (d, 1H, CH), δ 6.35- 7.78 (m, 6H, Ar-H) ; ^{13}C NMR ($CDCl_3$) δ 21.74, 22.10, 55.87, 56.33, 60.40, 102.71, 107.10, 126.10, 129.22, 129.69, 132.10, 151.30, 152.10, 198.21 (C=O); MS (m/z): $(M^{+}H)^{+}$ 416.3.

(8,9-Dimethoxy-3-methyl-6,6-dioxido-2,3,4,10b-tetrahydro-1H-benzo[4,5]isothiazolo[2,3-a]pyrazin-1-yl)(4-fluorophenyl)methanone (24)

Re-crystallizing solvent used: Ethanol. Yellow crystals were obtained (30% yield); Melting Point: 181-182^{o}C; IR (KBr cm^{-1}): 3297 (NH), 1677 (C=O), 1290, 1144 (SO_2); ^{1}H NMR ($CDCl_3$): δ 1.75 (s, 3H, CH_3), 3.51 (s, 3H, OCH_3), 3.93 (s, 4H, OCH_3, CH), 4.43-4.46 (s, 2H, CH_2), 6.43-7.94 (m, 6H, Ar-H) ; ^{13}C NMR ($CDCl_3$) δ 18.80, 47.20, 48.20, 55.93, 56.31, 58.40, 62.40, 102.72, 107.25, 126.33, 128.98, 129.02, 134.42, 135.42, 150.38, 152.53, 198.21 (C=O); MS (m/z): $(M^{+}H)^{+}$ 420.2.

(8,9-Dimethoxy-3-methyl-6,6-dioxido-2,3,4,10b-tetrahydro-1H-benzo[4,5]isothiazolo[2,3-a]pyrazin-1-yl) (4-chlorophenyl)methanone (25)

Re-crystallizing solvent used: Ethanol. Yellow crystals were obtained (43% yield); Melting Point: 204-205^{o}C; IR (KBr cm^{-1}): 3468 (NH), 1672 (C=O), 1284, 1142 (SO_2); ^{1}H NMR ($CDCl_3$): δ 1.75 (s, 3H, CH_3), 3.51 (s, 3H, 0CH_3), 3.93 (s, 4H, OCH_3, CH), 4.43-4.46 (s, 2H, CH_2), 6.43-7.94 (m, 6H, Ar-H) ; ^{13}C NMR ($CDCl_3$) δ 18.80, 47.20, 48.20, 55.93, 56.31, 58.40, 62.40, 102.72, 107.25, 126.33, 128.98, 129.02, 134.42, 135.42, 150.38, 152.53, 198.21 (C=O); MS (m/z): $(M^{+}H)^{+}$ 436.2

(8,9-Dimethoxy-3-methyl-6,6-dioxido-2,3,4,10b-tetrahydro-1H-benzo[4,5]isothiazolo[2,3-a]pyrazin-1-yl)(4-bromophenyl)methanone (26)

Re-crystallizing solvent used: Ethanol. Yellow crystals were obtained (48% yield); Melting Point: 204-205^{o}C; IR (KBr cm^{-1}): 3458 (NH), 1672 (C=O), 1284, 1141 (SO_2); ^{1}H NMR ($CDCl_3$): δ 1.75 (s, 3H, CH_3), 3.51 (s, 3H, OCH_3), 3.93 (s, 3H, OCH_3), 4.43-4.46 (ss, 2H, CH_2), 6.43-7.94 (m, 6H, Ar-H) ; ^{13}C NMR ($CDCl_3$) δ 18.80, 47.20, 48.20, 55.93, 56.31, 58.40, 62.40, 102.72, 107.25, 126.33, 128.98, 129.02, 134.42, 135.42, 150.38, 152.53, 198.21 (C=O); MS (m/z): $(M^{+}H)^{+}$ 480.4

(8,9-Dimethoxy-3-methyl-6,6-dioxido-2,3,4,10b-tetrahydro-1H-benzo[4,5]isothiazolo[2,3-a]pyrazin-1-yl)(3-chlorophenyl)methanone (27)

Re-crystallizing solvent used: Ethanol. Light yellow crystals were obtained (79% yield); Melting Point: 194-195^{o}C; IR (KBr cm^{-1}): 1691 (C=O), 1270, 1158 (SO_2); ^{13}C NMR ($CDCl_3$) δ 14.78, 41.77, 43.33, 46.23, 56.45, 56.57, 67.33, 105.67, 110.89, 111.89, 111.93, 127.07, 128.91, 130.37, 131.23, 134.19, 135.12, 149.21, 154.67, 188.60 (C=O); M.S (m/z): 436.2 $(M^{+}H)^{+}$

(8,9-Dimethoxy-3-methyl-6,6-dioxido-2,3,4,10b-tetrahydro-1H-benzo[4,5]isothiazolo[2,3-a]pyrazin-1-yl)(3-bromophenyl)methanone (28)

Re-crystallizing solvent used: Ethanol. Yellow crystals were obtained (50% yield); Melting Point: 189-190^{o}C; IR (KBr cm^{-1}): 3455 (NH), 1686 (C=O), 1290, 1140 (SO_2); ^{1}H NMR ($CDCl_3$): δ 1.45 (d, 3H, CH_3 *J* = 7.34 Hz), 3.60-3.61 (d, CH_2, 2H *J* = 14.7 Hz), (s, 3H, OCH_3), 3.93 (s, 3H, OCH_3), 4.33-4.36 (d, H, CH *J* = 10.09 Hz), 4.65-4.66 (d, H, CH *J* = 9.17 Hz), 6.46-8.08 (m, 6H, Ar-H) ; ^{13}C NMR ($CDCl_3$) δ 18.80, 47.27, 48.26, 56.05, 56.34, 58.00, 62.74, 102.80, 106.65, 107.13, 123.35, 126.34, 127.63, 127.71, 130.47, 131.78, 137.12, 150.48, 152.66, 197.04 (C=O); M.S (m/z): $(M^{+}H)^{+}$ 482.7.

(8,9-Dimethoxy-3-methyl-6,6-dioxido-2,3,4,10b-tetrahydro-1H-benzo[4,5]isothiazolo[2,3-a]pyrazin-1-yl)(2-chlorophenyl)methanone (29)

Re-crystallizing solvent used: Ethanol. Yellow crystals were obtained (46% yield); Melting Point: 215-216^{o}C; IR (KBr cm^{-1}): 3388 (NH), 1683 (C=O), 1290, 1190 (SO_2); MS (m/z): $(M^{+}H)^{+}$ 436.5.

(8,9-Dimethoxy-3-methyl-6,6-dioxido-2,3,4,10b-tetrahydro-1H-benzo[4,5]isothiazolo[2,3-a]pyrazin-1-yl)(2-bromophenyl)methanone (30)

Re-crystallizing solvent used: Ethanol. Orange crystals were obtained (75% yield); Melting Point: 195-196^{o}C; IR (KBr cm^{-1}): 3084 (NH), 1682 (C=O), 1286, 1123 (SO_2); ^{1}H NMR ($CDCl_3$): δ 1.21 (s, 3H, CH_3), 1.15 (d, 2H,CH_2), 3.57 (s, 3H, OCH_3), 3.67-3.66 (d, 1H, CH *J* =10.54 Hz), 3.68 (s, 3H, OCH_3), 4.18-4.59 (dd, 2H, CH_2 *J* = 9.17 Hz, *J* = 10.09 Hz), 6.43-8.06 (m, 6H, Ar-H); ^{13}C NMR ($CDCl_3$) δ 18.40, 18.72, 47.16, 48.31, 48.31, 57.97, 58.42, 62.69, 102.81, 107.15, 123.35, 126.35, 127.65, 130.48, 131.75, 137.14, 150.51, 152.66, 196.92 (C=O); MS (m/z): $(M^{+}H)^{+}$ 480.4.

To a stirred solution of chalcone dibromo sulphonyl chloride (1g) in ethanol (25 ml) was added 2-methyl-1,2-diaminopropane (2 mole equivalent). The reaction mixture was warmed on a water bath for 10-15 minutes until all the solid had dissolved. The reaction mixture was allowed to cool to room temperature and the resulting solid was filtered and air dried. The product was purified by recrystallization from the appropriate solvent.

(8,9-Dimethoxy-3,3-dimethyl-6,6-dioxido-2,3,4,10b-tetrahydro-1H-benzo[4,5] isothiazolo[2,3-a]pyrazin-1-yl)(Phenyl)methanone (31)

Re-crystallizing solvent used: Ethanol. White needle shaped crystals were obtained (34% yield); Melting Point: 163-164^{o}C; IR (KBr cm^{-1}): 3420 (NH), 1676 (C=O), 1277, 1141 (SO_2); ^{1}H NMR ($CDCl_3$): δ 1.24 (s, 3H, CH_3), 1.41 (s, 3H,CH_3), 3.46 (s, 3H, OCH_3), 3.64–3.66 (d, 1H, CH *J* = 12.84 Hz), 3.89 (s, 3H, OCH_3), 4.51-4.59 (dd, 2H, CH_2 *J* = 9,17 Hz), 6.37-7.98 (m, 7H, Ar-H); ^{13}C NMR ($CDCl_3$) δ 23.10, 28.25, 49.86, 55.83, 56.34, 57.93, 58.69, 102.73, 106.71, 126.76 129.09, 134.49, 135.59, 150.46, 152.62, 186.45 (C=O); MS (m/z): $(M^{+}H)^{+}$ 416.3

(8,9-Dimethoxy-3,3-dimethyl-6,6-dioxido-2,3,4,10b-tetrahydro-1H-benzo[4,5] isothiazolo[2,3-a]pyrazin-1-yl)(p-tolyl)methanone (32)

Re-crystallizing solvent used: Ethanol. Light yellow crystals were obtained (64% yield); Melting Point: 205-206^{o}C; IR (KBr cm^{-1}): 3312 (NH), 1660 (C=O), 1275, 1177 (SO_2); ^{1}H NMR ($CDCl_3$): δ 1.21 (s, 3H, CH_3), 1.38 (s, 3H,CH_3), 2.39 (s, 3H, CH_3), δ 1.65 (s,1H, NH), δ 3.44 (s, 3H, OCH_3), δ 2.93 and δ 3.63 (d, 2H, CH_2 *J* = 12.84 Hz), 3.88 (s, 3H, OCH_3), 4.45 (s,1H, CH), 4.53 (s, 1H, CH), 6.36-7.86 (m, 6H, Ar-H); ^{13}C NMR ($CDCl_3$) δ 21.73, 23.17, 28.32, 49.90, 55.76, 56.30, 57.84, 58.79, 102.65, 106.74, 126.71, 126.71, 129.18, 129.72, 133.14, 145.65, 150.36, 152.54, 198.06 (C=O); MS (m/z): $(M^{+}H)^{+}$ 430.3

(8,9-Dimethoxy-3,3-dimethyl-6,6-dioxido-2,3,4,10b-tetrahydro-1H-benzo[4,5] isothiazolo[2,3-a]pyrazin-1-yl)(4-fluorophenyl)methanone (33)

Re-crystallizing solvent used: Ethanol. White crystals were obtained (12% yield); Melting Point: 175-176^{o}C; IR (KBr cm^{-1}): 3438 (NH), 1677 (C=O), 1289, 1180 (SO_2); ^{1}H NMR ($CDCl_3$): δ 1.23 (s, 3H, CH_3), 1.41 (s, 3H,CH_3), 3.54 (s,3H, OCH_3), 3.65 (d, 1H, CH *J* = 13.74 Hz), 3.89 (s, 3H, OCH_3), 4.43 (s, 1H, CH), 6.41-8.03 (m, 6H, Ar-H); ^{13}C NMR ($CDCl_3$) δ 22.98, 55.89, 56.36, 58.35, 76.79, 77.00, 77.21, 102.78, 106.65, 116.25, 116.39, 126.81, 131.92, 152.70, 195.67; MS (m/z): $(M^{+}H)^{+}$ 434.3

(8,9-Dimethoxy-3,3-dimethyl-6,6-dioxido-2,3,4,10b-tetrahydro-1H-benzo[4,5] isothiazolo[2,3-a]pyrazin-1-yl)(4-Chlorophenyl)methanone (34)

Re-crystallizing solvent used: Ethanol. Light yellow crystals were obtained (39% yield); Melting Point: 180-181^{o}C; IR (KBr cm^{-1}): 3442 (NH), 1665 (C=O), 1290, 1182 (SO_2); ^{1}H NMR ($CDCl_3$): δ 1.24 (s, 3H, CH_3), 1.41 (s, 3H, CH_3), 2.95 (d, 1H, CH *J* = 12.84 Hz), 3.57 (s, 3H, OCH_3), 3.67 (d, 1H, CH *J* = 13.74 Hz), 3.91 (s, 3H, OCH_3), 4.43 (s, 1H, CH), 4.61 (s, 1H, CH), 6.43-7.95 (m, 6H, Ar-H); ^{13}C NMR ($CDCl_3$) δ23.03, 28.25, 49.87, 55.90, 56.33, 58.21, 58.31, 102.75, 106.62, 126.79, 126.96, 129.37, 130.46, 133.79, 141.07, 150.52, 152.68, 197.31 (C=O); MS (m/z): $(M^{+}H)^{+}$ 450.2

(8,9-Dimethoxy-3,3-dimethyl-6,6-dioxido-2,3,4,10b-tetrahydro-1H-benzo[4,5] isothiazolo[2,3-a]pyrazin-1-yl)(4-bromophenyl)methanone (35)

Re-crystallizing solvent used: Ethanol. Light yellow crystals were obtained (45% yield); Melting Point: 195-196^{o}C; IR (KBr cm^{-1}): 3447 (NH), 1663 (C=O), 1290, 1182 (SO_2); ^{1}H NMR ($CDCl_3$): δ 1.24 (s, 3H, CH_3), 1.41 (s, 3H, CH_3), 2.96 (d, 1H, CH *J* = 13.75 Hz), 3.57 (s, 3H, OCH_3), 3.91 (s, 3H, OCH_3), 3.67 (d, 1H, CH *J* = 13.74 Hz), 4.42 (s, 1H, CH), 4.61 (s, 1H, CH), 6.42-7.86 (m, 6H, Ar-H); ^{13}C NMR ($CDCl_3$) δ 23.03, 28.25, 49.43, 49.86, 55.91, 56.34, 58.20, 58.30, 102.76, 106.62, 126.79, 126.94, 129.88, 130.50, 132.38, 134.16, 150.52, 152.68, 197.53 (C=O) MS (m/z): $(M^{+}H)^{+}$ 494.1

(8,9-Dimethoxy-3,3-dimethyl-6,6-dioxido-2,3,4,10b-tetrahydro-1H-benzo[4,5] isothiazolo[2,3-a]pyrazin-1-yl)(3-chlorophenyl)methanone (36)

Re-crystallizing solvent used: Ethanol. Light yellow crystals were obtained (54% yield); Melting Point: 179-180^{o}C; IR (KBr cm^{-1}): 3449 (NH), 1681 (C=O), 1275, 1190 (SO_2); ^{1}H NMR ($CDCl_3$): δ 1.23 (s, 3H, CH_3), 1.41 (s, 3H,CH_3), 1.55 (s, 1H, CH), 3.57 (s, 3H, OCH_3), 3.65 (s, 1H, CH), 3.89 (s, 3H, CH_3), 4.41 (s, 1H, CH), 4.61 (s,1H,CH), 6.41 (s, 1H, CH), 7.22-7.24 (d, 1H, Ar-H *J* = 5.87 Hz), 7.39-7.42 (t, 1H, Ar-H *J* = 7.79 Hz), 7.56 (d, 1H, Ar-H *J* = 7.34 Hz), 7.82-7.97 (d, 2H, Ar-H *J* = 8.25 Hz), 7.98 (s, 1H, CO); ^{13}C NMR ($CDCl_3$) δ 22.97, 28.18, 49.83, 55.97, 56.36, 58.25, 58.40, 76.79, 77.00, 77.21, 102.80, 106.61, 126.81, 127.22, 128.98, 130.35, 134.28, 137.05, 150.58, 152.76, 198.50 (C=O); MS (m/z): $(M^{+}H)^{+}$ 494.1

(8,9-Dimethoxy-3,3-dimethyl-6,6-dioxido-2,3,4,10b-tetrahydro-1H-benzo[4,5] isothiazolo[2,3-a]pyrazin-1-yl)(3-bromophenyl)methanone (37)

Re-crystallizing solvent used: Ethanol. Light yellow crystals were obtained (80% yield); Melting Point: 175-176^{o}C; IR (KBr cm^{-1}): 3447 (NH), 1681 (C=O), 1265, 1190 (SO_2); ^{1}H NMR ($CDCl_3$): δ 1.24 (s, 3H, CH_3), 1.41 (s, 3H, CH_3), 2.96 (s, 1H, CH_3), 2.92-2.94 (d, 1H, NH), 3.60 (s, 3H, OCH_3), 3.67 (d, 1H, CH), 3.91 (s, 3H, OCH_3), 4.42 (d, 1H, CH *J* = 12.00 Hz), 4.62 (d, 1H, CH), 6.44 (s, 1H CH), 7.34- 7.73 (t, 2H, CH *J* = 7.84 Hz), 7.74-7.75 (d,2H, Ar-H *J* = 9.17 Hz), 7.88-7.89 (d, 2H, Ar-H *J* = 7.43), 8.16 (s, 1H, Ar-H); ^{13}C NMR ($CDCl_3$) δ 22.98, 28.23, 49.86, 55.98, 56.35, 58.25, 58.43, 102.79, 106.62, 123.40, 126.80, 127.64, 130.55, 131.94, 137.15, 137.25, 150.57, 152.76 198.80 (C=O); MS (m/z): $(M^{+}H)^{+}$ 495.9

(8,9-Dimethoxy-3,3-dimethyl-6,6-dioxido-2,3,4,10b-tetrahydro-1H-benzo[4,5] isothiazolo[2,3-a]pyrazin-1-yl)(2-Chlorophenyl)methanone (38)

Re-crystallizing solvent used: Ethanol. White crystals were obtained (34% yield); Melting Point: 195-195.5^{o}C; IR (KBr cm^{-1}): 3448 (NH), 1720 (C=O), 1297, 1179 (SO_2); ^{1}H NMR ($CDCl_3$): δ 1.44 (s, 3H, CH_3), 1.58 (s, 3H, CH_3), 3.22 (d, 1H, CH *J* = 13.75), 3.66 (d, 1H, CH *J* = 14.64 Hz), 3.68 (d, 1H, CH), 3.93 (s, 6H,), 4.62 (d, 1H, CH *J* = 11.00 Hz), 6.76-7.41 (m, 6H, Ar-H); ^{13}C NMR ($CDCl_3$) δ 23.17, 31.25, 42.10, 50.67, 56.46, 58.11, 59.96, 102.81, 104.69, 121.90, 127.46, 129.61, 129.78, 129.92, 143.35, 145.55, 150.68, 153.66, 164.88, 199.80 (C=O); M.S (m/z): $(M^{+}H)^{+}$ 452.2

(8,9-Dimethoxy-3,3-dimethyl-6,6-dioxido-2,3,4,10b-tetrahydro-1H-benzo[4,5] isothiazolo[2,3-a]pyrazin-1-yl)(2-bromophenyl)methanone (39)

Re-crystallizing solvent used: Ethanol. White crystals were obtained (29% yield); Melting Point: 201^{o}C-202^{o}C; IR (KBr cm^{-1}): 3467 (NH), 1715 (C=O), 1297, 1179 (SO_2); ^{1}H NMR ($CDCl_3$): δ 1.44 (s, 3H, CH_3), 1.58 (s, 3H, CH_3), 3.22 (d, 1H, CH *J* = 13.75 Hz), 3.66 (d, 1H, CH *J* = 7.34 Hz), 3.68 (d, 1H, CH *J* = 7.34 Hz), 3.93 (s, 6H), 4.62 (d, 1H, CH *J* = 12.0 Hz), 6.76-7.41 (m, 6H, Ar-H); ^{13}C NMR ($CDCl_3$) δ 23.17, 31.25, 42.10, 50.67, 56.46, 58.11, 59.96, 102.81, 104.69, 121.90, 127.46, 129.61, 129.78, 129.92, 143.35, 145.55, 150.68, 153.66, 164.88, 199.80 (C=O); MS (m/z): $(M^{+}H)^{+}$ 498.6

Chapter 3

3.1 Biological screening methodology

All eighteen compounds were screened against 4 types of bacteria, 3 types of fungi. Before biological testing could be conducted an appropriate solvent needed to be selected.

3.2 Bacterial Culture and identification

Culture strains of *Bacillus subtilis ATCC 6633*, *Staphylococcus aureus ATCC 6538*, *Escherichia coli ATCC* 11775 *and Proteus hauseri ATCC 13315* were obtained from the Culture collection of University of Hertfordshire. Streaked agar plates were provided, to conduct gram staining and antibacterial screening, inoculums had to be prepared.

- *Bacillus subtilis* - gram-positive bacteria found in soil, has excellent fermentation capacities.(Zweers *et al*., 2008) This bacteria was selected as its related to *Bacillus cereus* which can cause food borne illnesses.

- *Escherichia coli* - rod shaped gram negative, found in lower intestines of warm blooded animals, and recent investigations has shown that the presence of *E. coli* has been strongly linked to both Crohn's disease and inflammatory bowel disease (Barnich, Denizot, & Darfeuille-Michaud, 2013)

- *Proteus hauseri* – rod shaped gram negative bacterium that has been linked with nosocomial disease and urinary tract infections (O'Hara *Et al* 2000).

- *Staphylococcus aureus* – coccal shaped gram positive bacterium. Although *S. aureus* has been extensively reported as a lethal human pathogen it is often ignored that many strains of this bacteria are commensal (Kim, Missiakas & Schneewind, 2014)

Bacteria	ATCC number	University record number
Bacillus subtilis	6633	B30
Escherichia coli	11775	B285
Staphylococcus aureus	6538	B9
Proteus hauseri	13315	B15

Table4: Bacterial cultures and their record numbers

3.3 Inoculum preparation.

Colonies were derived from streaked agar plates were used for gram staining. To prepare inoculums for antibacterial screening the flame loop technique was used and universal bottles containing autoclaved Mueller Hinton (MH) broth were inoculated and the incubated. After 6-8 hours the inoculum was ready to be used in biological testing before placing the inoculum into microtitre plates it was necessary to know the concentration of cells, using McFarland's solution the bacterial culture could be adjusted to a fixed density (see McFarland's solution preparation).

All bacteria were cultured using Mueller Hinton broth and incubated ay 37^{o}C.

Mueller Hinton broth was prepared by adding 21 g of MH broth powder to 1 litre of distilled water; the broth was then autoclaved and kept in an aseptic environment.

3.4 Gram staining technique.

The bacterial cultures provided were further validated by using the gram staining technique. The method used is described by Reed *et al* using a clean microscope slide, a small volume (10-30 µl) of bacterial inoculum was placed on the slide and left to dry. The bacterial smear is then heat fixed by passing it through the blue flame of a Bunsen burner. The slide is the flooded with crystal violet dye mixture. The slide is then rinsed under tap water and stained with Gram's iodine again rinsed with tap water. The counter stain carbonyl fushin is then applied for 10-15 seconds and rinsed with tap water. Once all the water has been removed a

drop of immersion oil is placed on the smear and the slide is then ready to be examination under microscope (Reed, Holmes, Weyers & Jones, 2007).

3.5 Solubility

The library of compounds was tested for solubility in a range of solvents; these solvents include ethanol, methanol, ethyl acetate, dichloromethane, DMSO, acetone and acetonitrile. Of all solvents tested the compounds were only soluble in DMSO and hot ethanol. The compounds were soluble in acetone, however as this solvent is so volatile it was inappropriate to use for biological screening. As the compounds were readily soluble in DMSO this was chosen as the appropriate solvent.

Dimethyl sulphoxide at certain concentrations has been reported as being cytotoxic to a variety of bacteria, fungi and mammalian cells (Da Violante *et al*., 2002). As DMSO was the solvent of choice, it was necessary to ascertain what the maximum concentration or percentage of DMSO that could be implemented in the biological tests.

3.6 Maximum DMSO concentration testing.

Using a microtitre plate 8 different concentrations of DMSO were tested the highest concentration was 10% and the lowest was 3% against all four bacteria. Dilutions were made using eppendorf tubes. A series of dilutions were made using pure 100% DMSO and sterile distilled water see table for dilutions. Each well could hold a maximum volume of 200 µl. From this experiment it was concluded that a maximum of 5% DMSO could be tolerated by the cells. (See results section for graphs)

Amount of inoculum in µl (bacteria + MH broth)	Amount of DMSO (100%) in µl	Percentage DMSO (%)
194	6	3
192	8	4
190	10	5
188	12	6
186	14	7
184	16	8
182	18	9
180	20	10

Table 5: shows the volumes of Muller Hinton (MH) broth and DMSO (100%) to make up the different percentages of DMSO.

3.7 Stock Solution of benzoisothiazole derivatives

20 mg of each benzoisothiazole compound was weighed and dissolved in 1 ml of DMSO (99.98%; Fischer Chemical). The Stock solutions were preserved at - 20°C. The compounds were tested for solubility in a wide range of solvents however the compounds were only readily soluble in DMSO and boiling ethanol.

3.8 Streptomycin (control) Stock:

Streptomycin sulphate salt powder (5g; Sigma Aldrich) was weighed and dissolved in 1ml of sterile distilled water. This was used as a control for antimicrobial testing to compare the activity of synthesized benzoisothiazoles. The concentration of 1 mg ml^{-1} was used for all antibacterial testing.

3.9 MTT dye: (0.2 mg/ml).

MTT (2 mg; 3-4,5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide, a tetrazole) dye (≥97.5%, Sigma Aldrich) was weighed in a fume hood and to this 10ml of sterile distilled water was added under sterile conditions. The solution is stable at 0°C for six months and only stable for four days at 4°C.

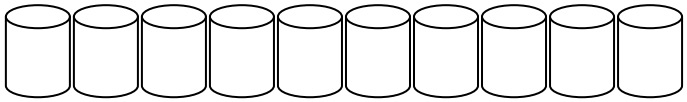
3.10 Preparation of 0.5 McFarland Standard

0.5 McFarland standard was used as a reference to adjust the turbidity of bacterial suspensions, so that the number of bacteria will be within a given range of 1 to 1.5×10^8 CFU/ml, for its further use for susceptibility testing or other procedures that require a standardization of inoculum. A 0.5 McFarland standard was prepared by mixing 0.05ml of 1.175% barium chloride dihydrate ($BaCl_2.2H_2O$) (Sigma Aldrich), with 9.95 ml of 1% sulphuric acid (H_2SO_4) (98%, AR). After preparation, the standards were preserved at 4°C in dark (securely wrapping foil around the falcon). The standard can be measured by the absorbance of the bacterial suspension and the McFarland standard with the help of a spectrophotometer at 590 nm wavelength.

All 18 compounds were soluble in DMSO; however when an aqueous medium was introduced into the solution a precipitate formed. The Muller-Hinton broth used to culture the bacteria was made using distilled water, so it was necessary include DMSO in the broth to increase the solubility of the compounds. So before testing could begin the maximum percentage of DMSO needed to be ascertained. This percentage needed to be high enough to increase solubility if the compounds but cause no harm to the bacteria being tested. This was achieved by using a 96 microtitre plate. The four microorganisms were exposed to different concentrations of DMSO (3-10%). MTT solution was added to test which of the wells contained viable cells, the MTT solution is a colorimetric technique used to show viability of cells through activity in the mitochondria. Data was obtained using a plate reader. From the data obtained the highest that could be applied was 5% of DMSO.

3.10.1 Method for antibacterial screening.

Each well in a 96 microtitre plate can accommodate 200 µl.



Wells 1 2 3 4 5 6 7 8 9 10

Figure 13: represents the microtitre wells.

To well 1, 98 µl of Mueller Hinton (MH) broth was added and then a further 2 µl of pure DMSO was added, this gave well 1 a total of 2% DMSO. To wells 3-8 50 µl of 4% DMSO MH broth was added. To well 1 50 µl of bacterial culture and 50µl of compound was added making the total volume 200 µl. 100 µl was taken from well 1 and diluted down wells 2-8, this gave the wells an approximate DMSO percentage of 4.5%. Well 9 and 10 were the positive control (contained only culture) and negative control (contained only M-H broth). A multichannel pipette was used and all tests were completed in triplicate.

During the incubation period a small amount of condensation is formed so to minimise this, the outer circle of wells were filled with sterile distilled water (SDW). Due to insolubility issues the plates were incubated on an agitator overnight.

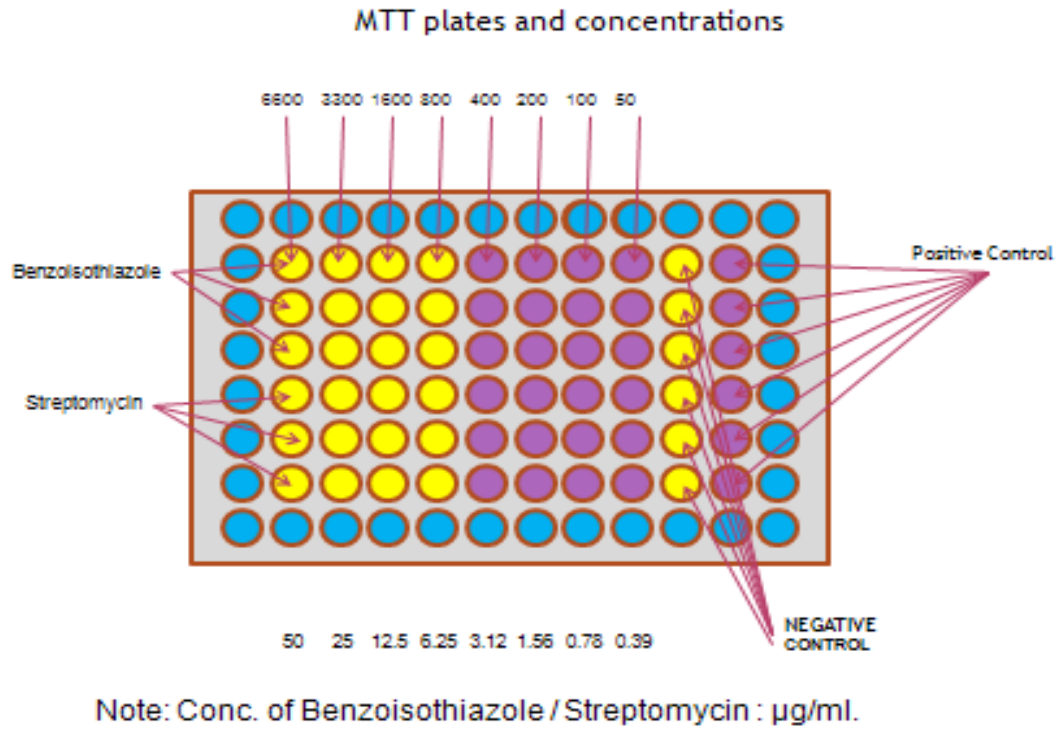


Figure 14: a 96 well microtitre plate the shaded blue circles represent the SDW.

To a microtitre plate (see figure 14) 20 mg of each compound was dissolved in 1ml of DMSO which gave 20 mg ml^{-1} stock concentration. When diluted with Muller-Hinton (50 µl) broth and culture (50 µl) reduced this to a starting concentration of 6.67 mg ml^{-1} and after 8 dilutions a final concentration of 104 µg ml^{-1}. These values were calculated using the following equation.

$$C_1V_1 = C_2V_2$$

C_1 = Starting concentration
V_1 = Starting volume
C_2 = Final concentration
V_2 = Final volume

$$20 \text{ mgml} \times 0.05\text{ml} = ? \times 0.15 \text{ ml}$$

$$\downarrow$$

$$\frac{20 \times 0.05}{0.15} = 6.67 \text{ mg ml}^{-1}$$

Once the culture had been added to the plate, the absorbance was recorded at 590 nm and left to incubate overnight. After the incubation (16-18hrs) period the plates were re-read. To ascertain the activity of the compound MTT assay was used. See MTT preparation.

To gain accurate results for the possible antimicrobial activity of each compound, the compounds must be subjected to the same amount of bacteria culture (ensure that all culture contain approximately the same amount of colony forming units CFU's). To ensure that the cultures were of equal CFU's, McFarland's standard was used; this is a solution of Barium Chloride and sulphuric acid which when mixed forms a barium sulphate precipitate which is of a fixed density that can be used to compare the turbidity of the bacterial culture to provide a numerical value which can be used to determine the amount of bacteria present (see Mcfarland's solution preparation)

After the incubation period 20 µl of MTT solution was added and left to incubate for a further hour at 37^{o}C. After one hour incubation the difference between viable and non-viable cells can easily be seen. Upon the addition of MTT and wells that contain non-viable cells start to developed a mauve to dark purple colour, the solution is left to incubate for an hour to ensure that the assay can work on viable cells, which will remain yellow upon addition of MTT. After the incubation period the plates are read using a spectrophotometer, using the value obtained for the positive control (inoculated MH broth), a graph can be plotted to see at what concentration the cells are no longer viable.

3.10.2 Antifungal screening

Before antifungal screening could begin, the maximum percentage of DMSO had to be determined. Using a similar approach used for antibacterial screening, it was determined that a maximum of 5% DMSO could also be used.

Preliminary experiment.

To determine what concentration of DMSO can be used without affecting the growth of the fungi a preliminary test much like the one used for the antibacterial screening had to be

employed. Using an agar plate that has a lawn of fungi already grown, pre-soaked disks with DMSO concentrations varying from 3-10% (in triplicate) were placed on the plate. The agar plates were incubated for a period of 7-10 days, the results were analysed visibly. Fungal growth was unaffected up to 5%, after this percentage there was a visible decline in the growth of fungi.

3.10.3 Fungi used for testing

- *Aspergillus niger* – This fungus was chosen as some *Aspergillus* species are known to cause Aspergillosis. This fungal infection destroys lung tissue and can be fatal (Bansod & Rai, 2008).

- *Candida krusei* – Candida infections are caused by opportunistic fungi that can result in a wide variety of different fungal infections which can be problematic to treat. Candida fungal infections can be fatal to patients that are immunocompromised (Anaissie, 1992).

- *Mucor* – a filamentous fungi which in rare cases can cause aggressive mucormycosis infections (Badior, Trigo, Eloy & Guimaraes, 2013).

3.10.4 Method fungal sub culturing

An agar plate with a mature strain of each fungus was provided by the University of Hertfordshire. Fresh cultures had to be prepared for antifungal screening. Unlike the bacteria, different fungus requires different media. Potato dextrose agar was used for *Aspergillus niger* and *Mucor*. Malt extract agar was used for *Candida krusei.*

For *C. krusei* the spread plate technique was adopted, spore suspension was prepared and 50µl of the suspension was spread on the appropriate agar plate. This technique provides a thick and evenly distributed lawn of fungi in which any anti-fungal activity could easily be seen. The spore suspension was prepared using sterile tap water, as sterile distilled water would disrupt the fungal cell wall dues to osmotic pressure.

To prepare *A. niger* and *Mucor* plates aseptic PDA Petri dishes were prepared. A cork bore plug (5mm in diameter) of pre-grown fungi was placed in the middle, the fungi was incubated and left to grow until a thick and even lawn of fungal growth was present. To test antifungal activity antibiotic disks were used. Five antibiotic disks were used per plate, 1 disk was inoculated in pure DMSO, 1 disk was inoculated with a control drug, and 3 disks of the same concentration of compound were inoculated. The inoculated disks were placed around the fungi plug and the growth monitored daily.

3.10.5 Results for determination of DMSO percentage

The following graphs show the results of DMSO on the growth of *Bacillus subtilis, Escherichia coli, Proteus hauseri* and*, Staphylococcus aureus.* All tests were carried out in triplicate, the absorbance values for bacterial growth after 16-18 hours without the presence of DMSO was also recorded in triplicate.

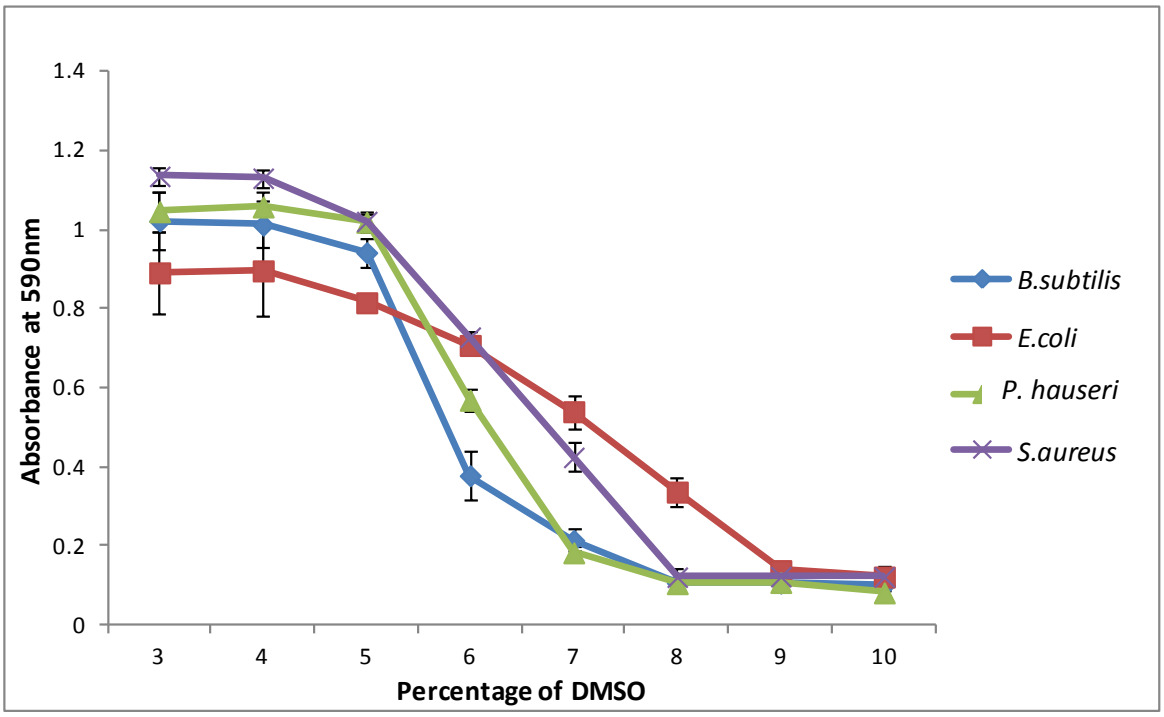


Figure 15: shows the growth of *Bacillus subtilis, Escherichia coli, Proteus hauseri* and, *Staphylococcus aureus* in the presence of DMSO (3-10%) after a 16-18 hour incubation period.

Figure 15 shows after the growth of all four bacteria in the presence of DMSO (3-10%). As the concentration of DMSO was increased, the average absorbance values decreased from an average wavelength of 0.900 at 3% concentration to an average absorbance value of 0.100. Figure 15 shows that for *Bacillus subtilis, Proteus hauseri* and, *Staphylococcus aureus* there is a steep decrease in absorbance after 5% DMSO, which indicates that the DMSO is having an inhibitory effect. The absorbance values for *Escherichia coli* gradually decrease after 5%.

3.10.6 Results for Gram staining.

As expected the gram positive *Bacillus subtilis* and *Staphylococcus aureus* retained the crystal violet dye and had a purple appearance. The gram negative *Escherichia coli* and *Proteus hauseri* were decoloured and retained the counter stain appearing pink in colour.

3.10.7 Minimum inhibitory concentration of reference antibiotic, streptomycin against all four bacteria.

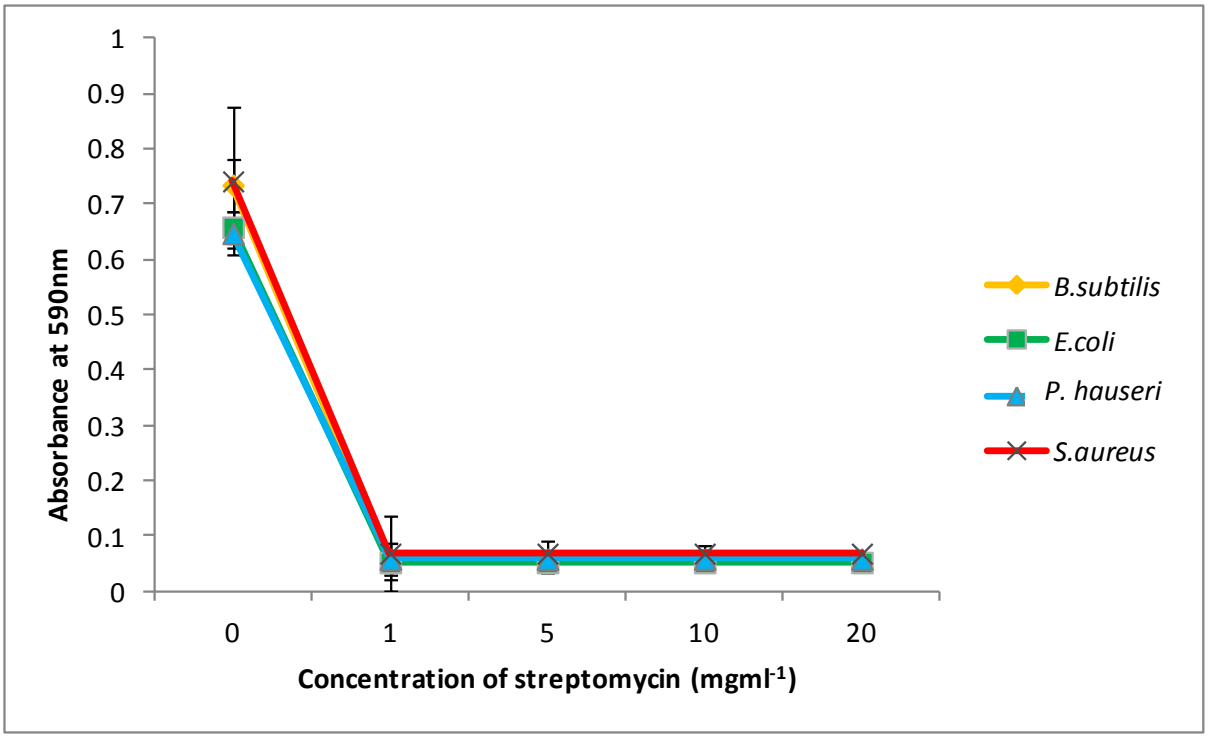


Figure 16: the graph shows the absorbance measured at 590nm after 16-18 hour incubation period for *Bacillus subtilis, Escherichia coli, Proteus hauseri* and*, Staphylococcus aureus* in at different concentrations of streptomycin.

As shown in figure 16 all the bacteria were inhibited at a concentration of 1 mg ml^{-1}, this concentration was used in all antibacterial screening.

3.10.8 Results for antibacterial screening.

All 18 compounds were screened for antibacterial activity. Compound **27** exerted the most noticeable antibacterial effect. Some compounds displayed some activity, at the concentration of 6.67 mg ml^{-1}; further testing would be needed to investigate whether this concentration is viable.

The compounds were screened at 8 different concentrations the highest concentration used was 6.67 mg ml^{-1} and the lowest was 52 µg ml^{-1}.

Compound concentrations used mg ml^{-1}
6.67
3.3
1.6
0.833
0.416
0.208
0.104
0.052

Table 6: shows the different compound concentrations used.

3.10.9 Cytotoxicity studies of compounds 22-39 against *Bacillus subtilis Escherichia coli*, *Proteus hauseri* and, *Staphylococcus aureus.*

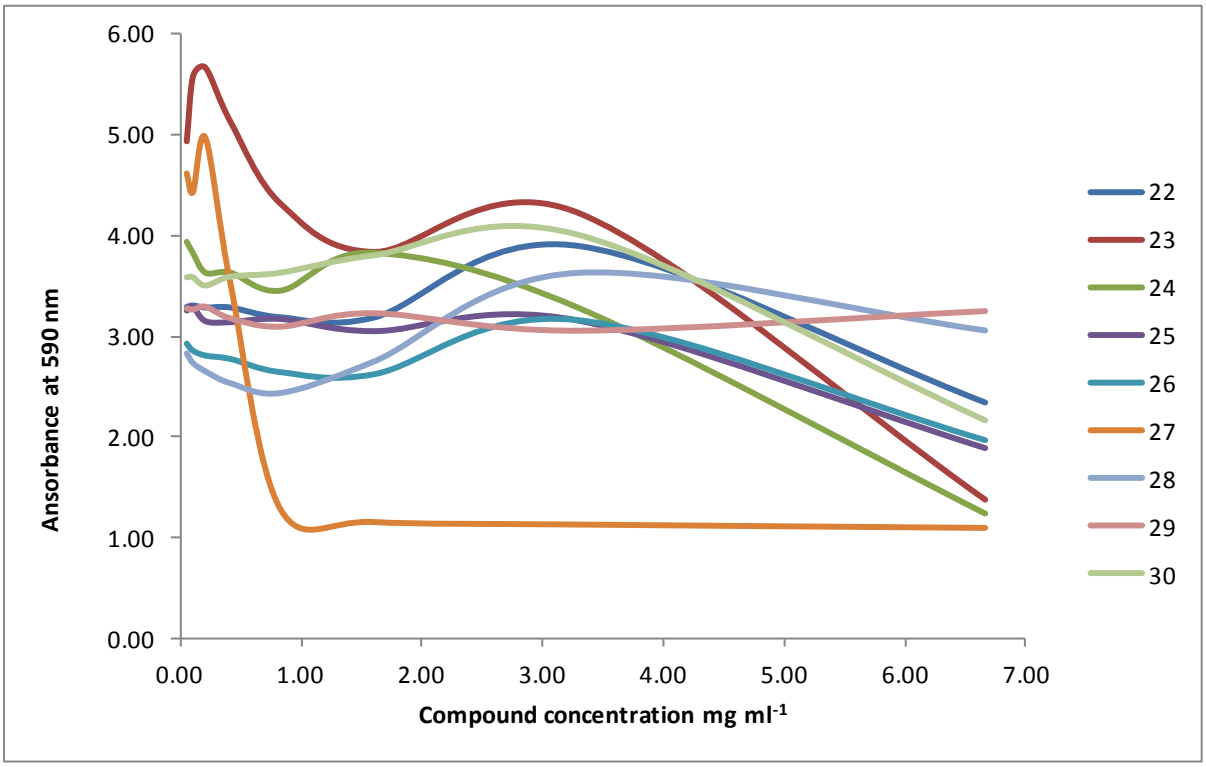


Figure 17: shows the absorbance of *B. subtilis* measured at 590nm with compounds **22-30** after 16-18hrs incubation and the addition of MTT.

From figure 17 it can be seen that the growth of *B. subtilis* is inhibited by Compound **27** at concentration of 0.833 mg ml^{-1} and continued to decrease Streptomycin was the reference drug at a concentration of 1 mg ml^{-1}. Compounds **22, 23, 24, 25** and **30** displayed inhibition at the highest concentration (6.67 mg ml^{-1}) only.

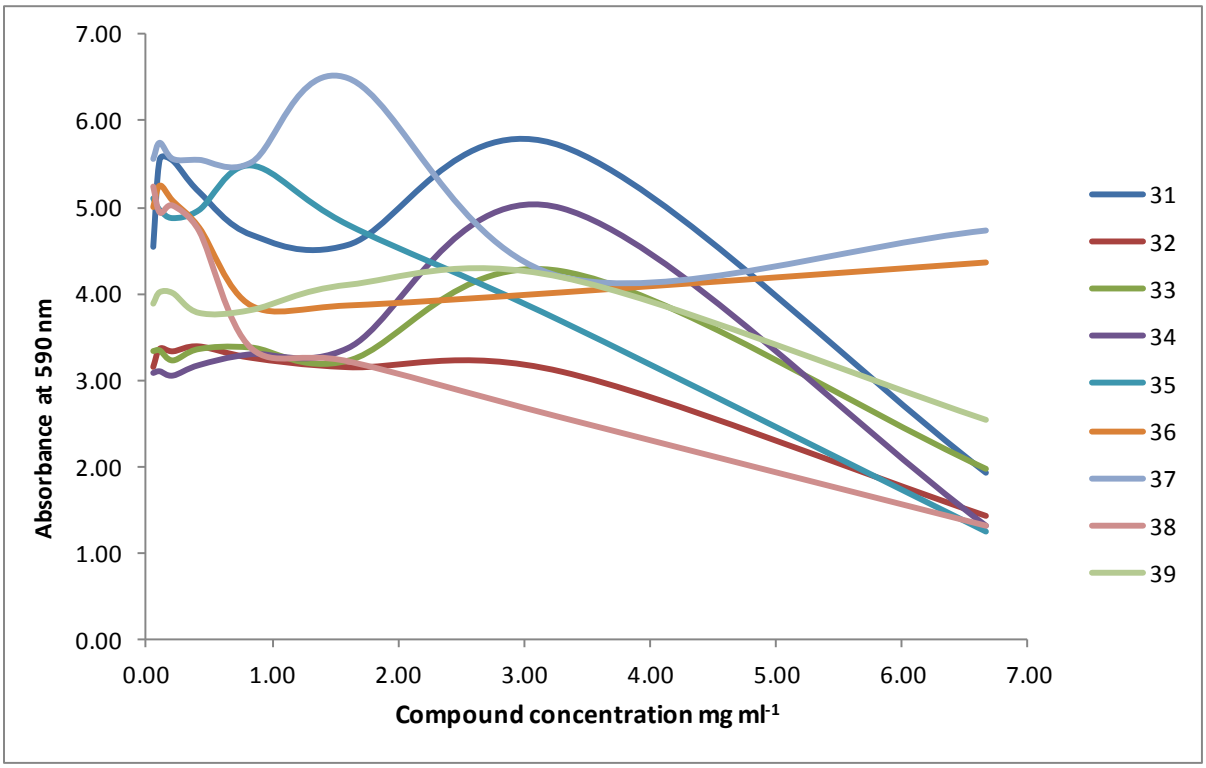


Figure 18: shows the absorbance of *B. subtilis* measured at 590 nm with compounds **31-39** after 16-18hrs incubation and the addition of MTT.

From figure 18 it can be seen that the growth of *B. subtilis* is inhibited by compounds **31, 32, 33, 34, 35, 38** and **39** at the highest concentration of 6.67 mg ml^{-1}. Compounds **36** and **37** show no inhibition. Streptomycin was the reference drug at a concentration of 1 mg ml^{-1}.

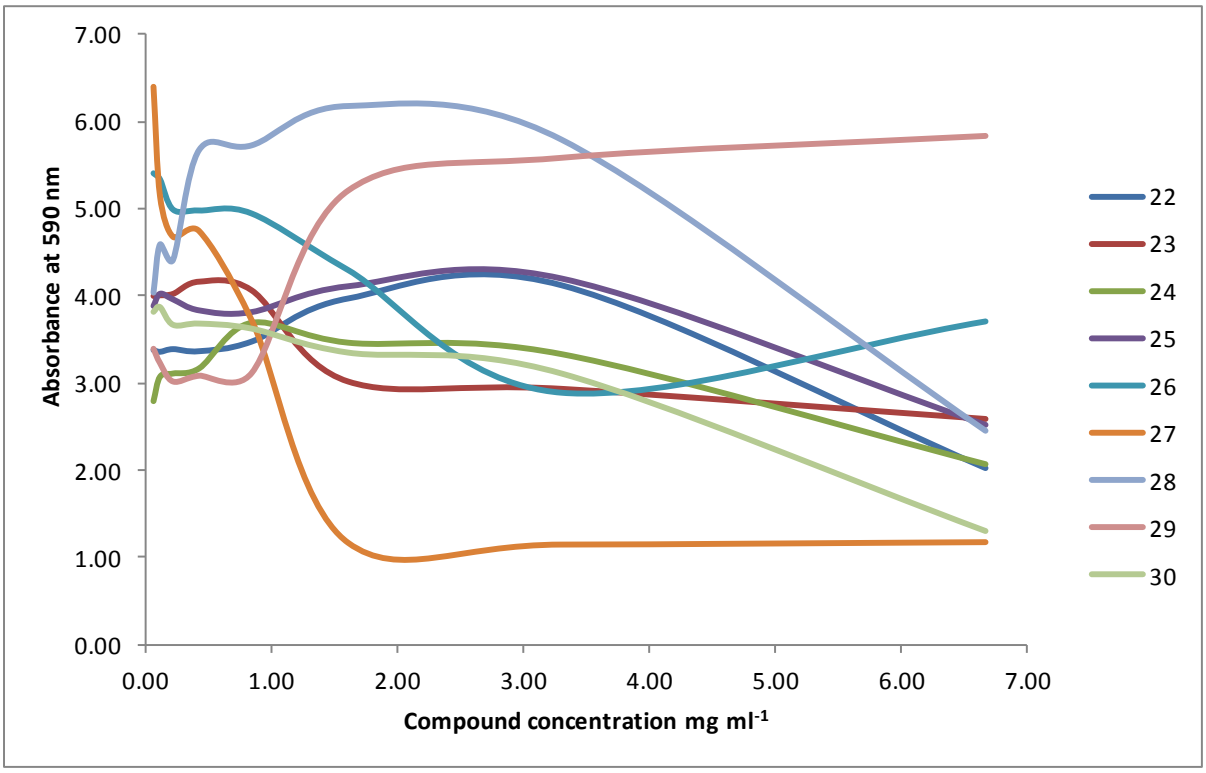


Figure 19: shows the absorbance of *E. coli* measured at 590 nm with compounds **22-30** after 16-18hrs incubation and the addition of MTT.

From figure 19 it can be seen that the growth of *E. coli* is inhibited by Compound **27** from the highest concentration of 6.67 mg ml^{-1} down to the concentration of 1.6 mg ml^{-1}. From the graph it can be seen that concentrations below 1.6 mg ml^{-1} have a steep decrease in inhibitory effect on the growth of *E. coli*. Streptomycin was the reference drug at a concentration of 1 mg ml^{-1}. Compounds **22, 23, 24, 25, 27** and **29** displayed inhibition at the highest concentration.

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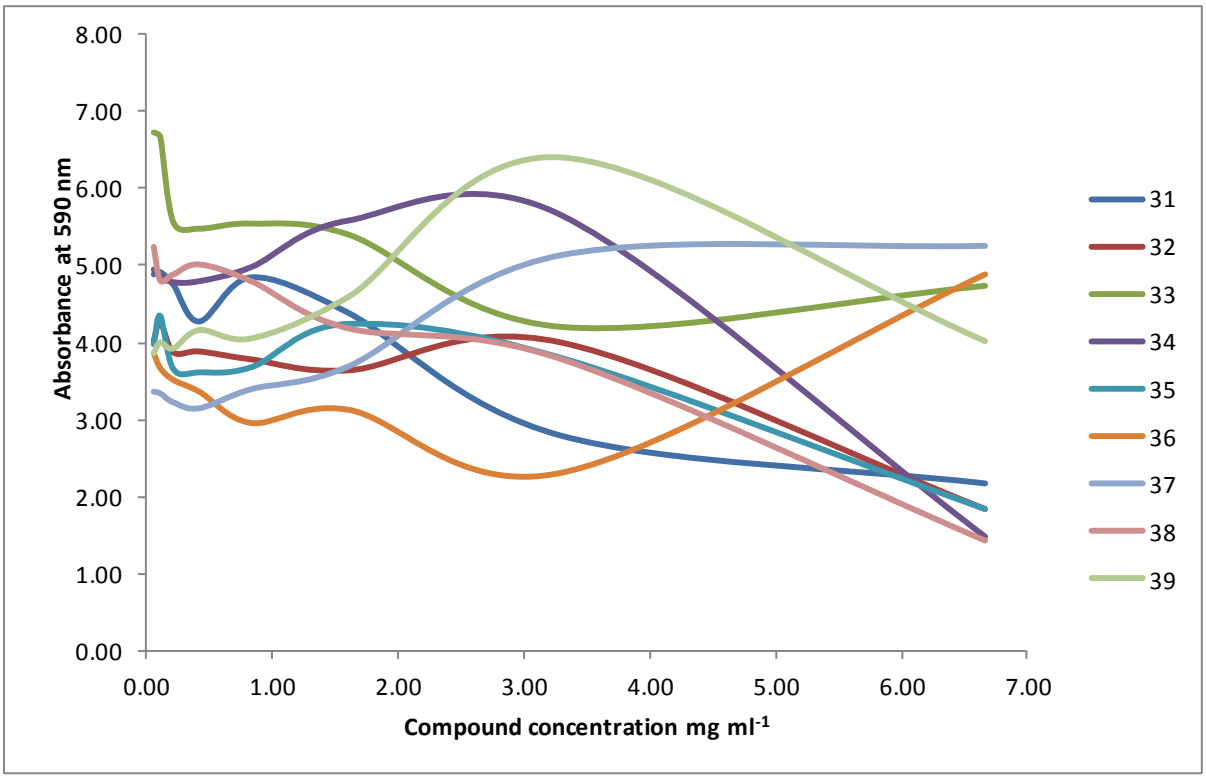


Figure 20: shows the absorbance of *E. coli* measured at 590nm with compounds **31-39** after 16-18hrs incubation and the addition of MTT.

From figure 20 it can be seen that the growth of *E. coli* is inhibited by compounds **31, 32, 33, 35** and **38** from the highest concentration of 6.67 mg ml^{-1}. For compounds **31, 32, 33** and **35** there is a steep increase in absorbance values which indicates that after the highest concentration the compounds no longer inhibit the growth of *E. coli.* Streptomycin was the reference drug at a concentration of 1 mg ml^{-1}.

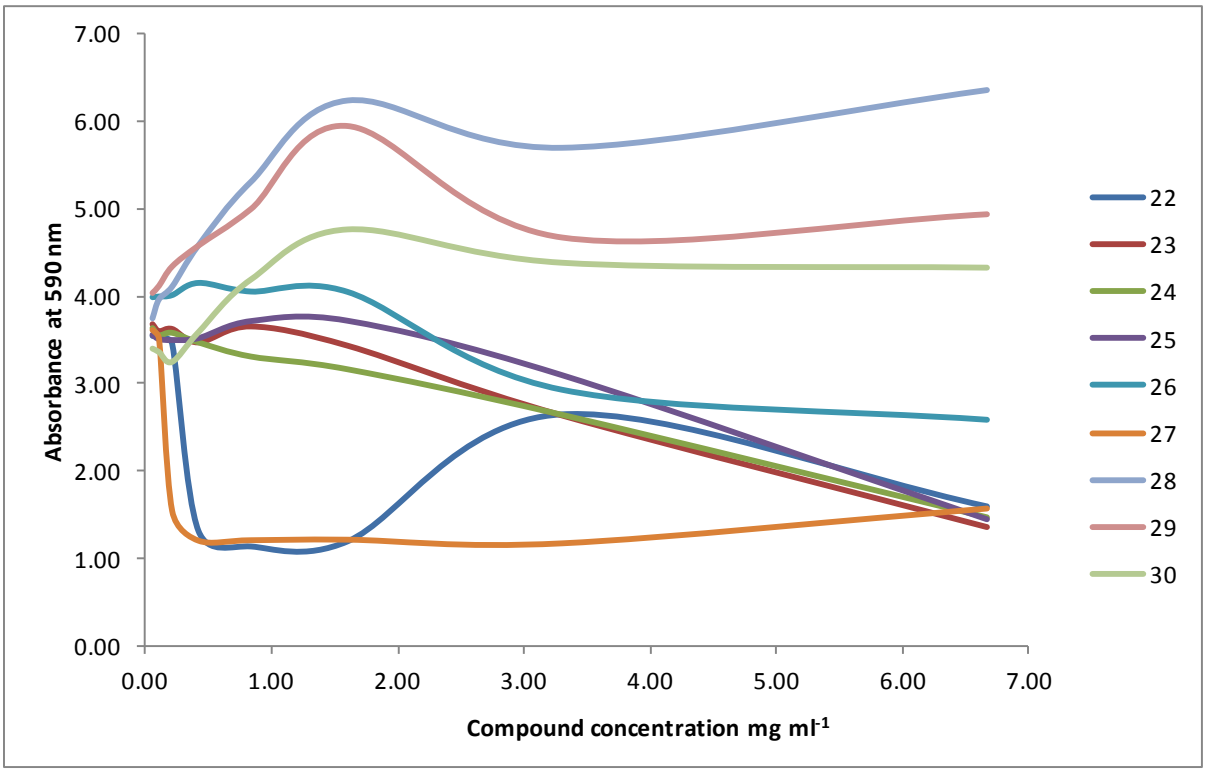


Figure 21: shows the absorbance of *P. hauseri* measured at 590nm with compounds **22-30** after 16-18hrs incubation and the addition of MTT.

From figure 21 it can be seen that the growth of *P. hauseri* is inhibited by Compound **27** from the highest concentration of 6.67 mg ml^{-1} down to the concentration of 0.833 mg ml^{-1}. From the graph it can be seen that concentrations below 0.833 mg ml^{-1} have a steep decrease in inhibitory effect on the growth of *P. hauseri.* As the concentration falls below 0.208 mg ml^{-1} the absorption plateaus this indicates that Compound **27** has no effect on the growth of *P. hauseri*. Streptomycin was the reference drug at a concentration of 1 mg ml^{-1}. Compounds **22, 23, 24** and **25** are having an inhibitory effect on the growth of the bacteria at the highest concentration.

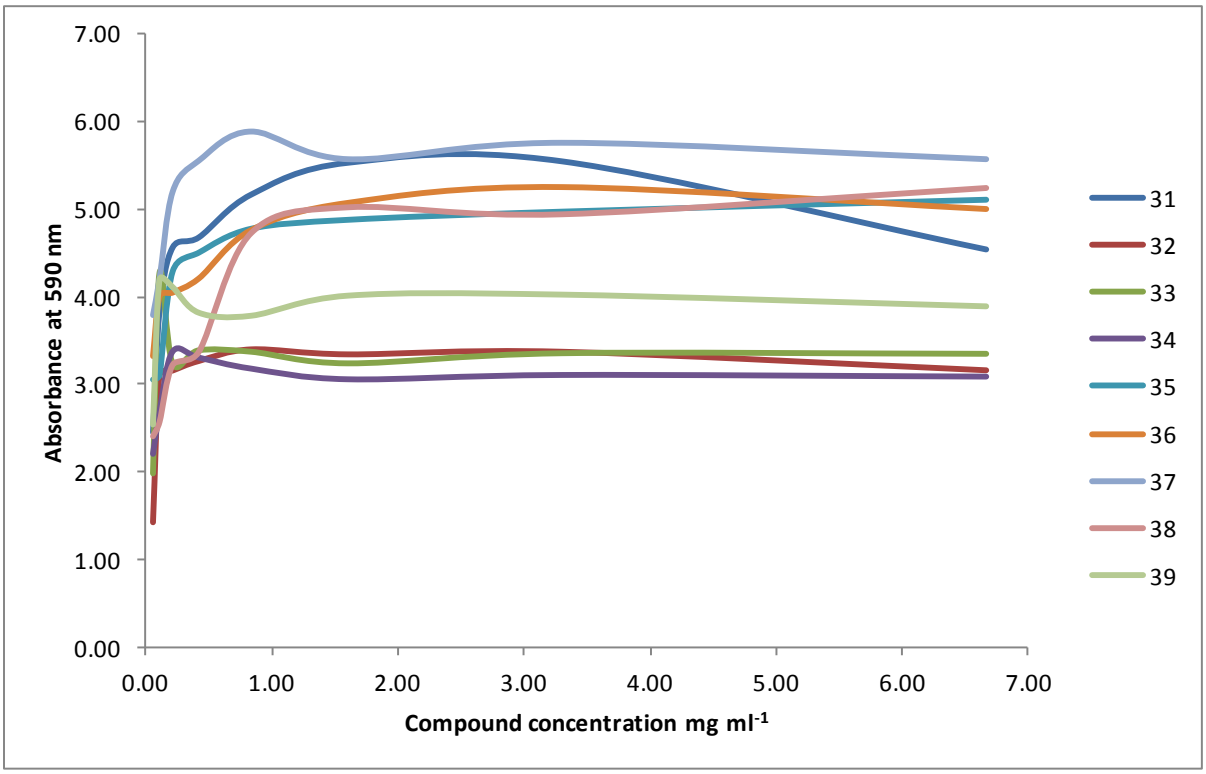


Figure 22: shows the absorbance of *P. hauseri* measured at 590nm with compounds **31-39** after 16-18hrs incubation and the addition of MTT.

From figure 22 it can be seen that the growth of *P. hauseri* is inhibited by compounds **31, 32, 33, 34, 35** and **38** at the highest concentration of 6.67 mg ml^{-1}. Compound 31 displayed inhibition of growth down to a concentration of 3.3 mg ml^{-1} but as the concentration decreases the inhibition of growth stops. Streptomycin was the reference drug at a concentration of 1 mg ml^{-1}

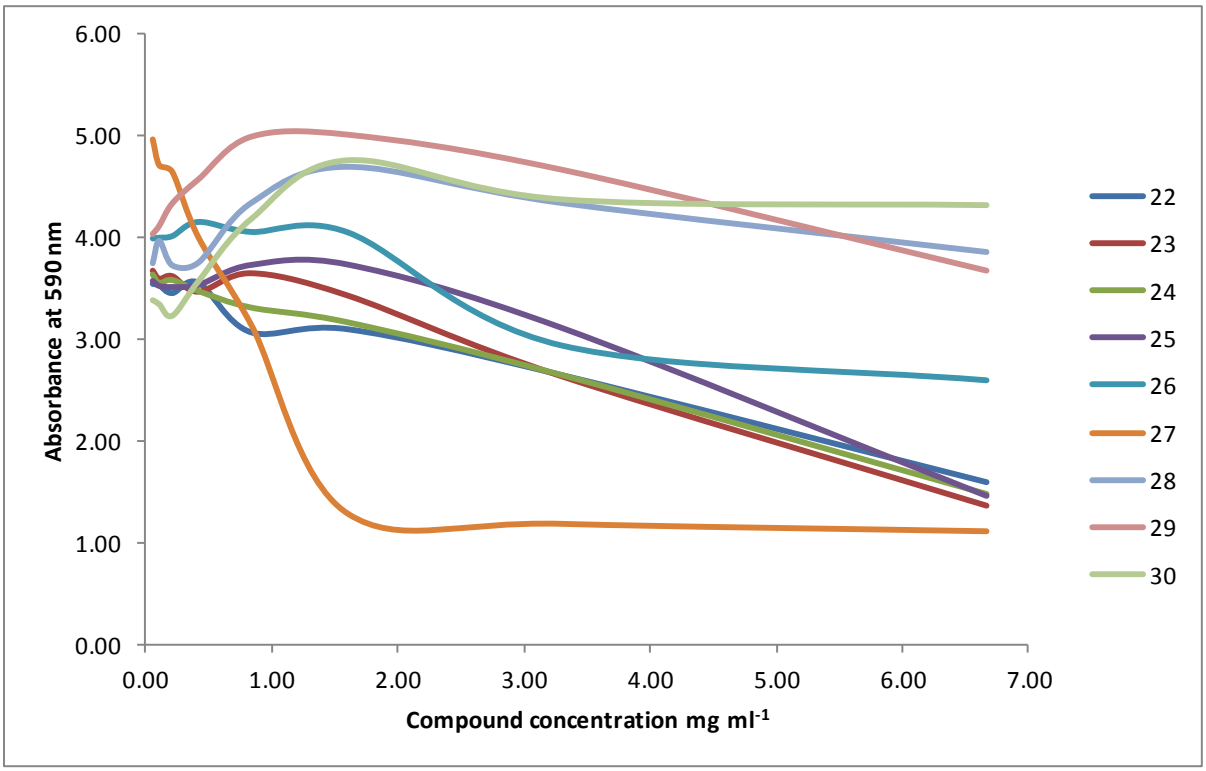


Figure 23: shows the absorbance of *S. aureus* measured at 590nm with compounds **22-30** after 16-18hrs incubation and the addition of MTT.

From figure 23 it can be seen that the growth of *S. aureus* is inhibited by Compound **27** from the highest concentration of 6.67 mg ml^{-1} down to the concentration of 0.833 mg ml^{-1}. From the graph it can be seen that concentrations below 0.833 mg ml^{-1} have a steep decrease in inhibitory effect on the growth of *S. aureus*. As the concentration fall below 0.208 mg ml^{-1} the absorption plateaus, this indicates that Compound **27** has no effect on the growth of *S. aureus.* Compounds **22, 23, 25, 26** and **30** displayed some inhibition at the highest concentration. Streptomycin was the reference drug at a concentration of 1 mg ml^{-1}

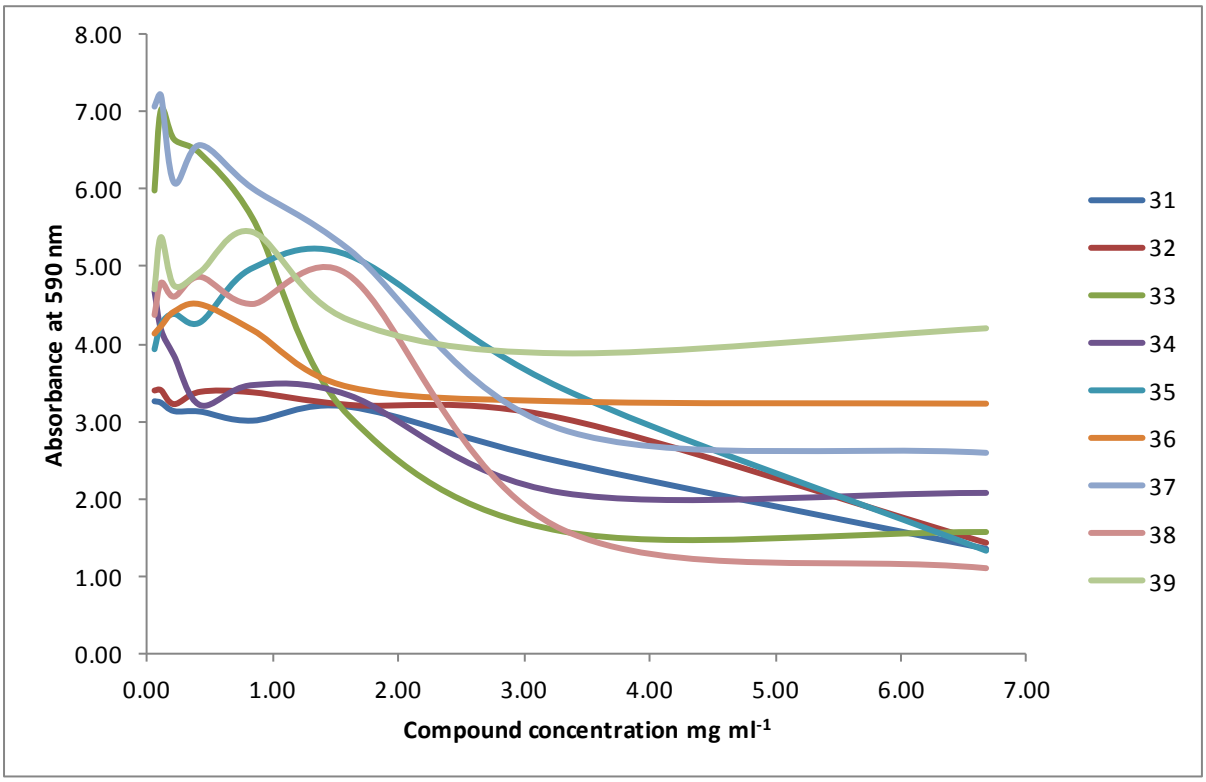


Figure 24: shows the absorbance of *S. aureus* measured at 590nm with compounds **31-39** after 16-18hrs incubation and the addition of MTT.

From figure 24 it can be seen that the growth of *S. aureus* is inhibited by compounds **31, 32, 33, 37** and **38** at the highest concentration of 6.67 mg ml^{-1}. Streptomycin was the reference drug at a concentration of 1 mg ml^{-1}

3.11 Results for antifungal screening

Two different methods were applied to determine if any of the 18 compounds had any antifungal properties, after the compounds were added and the agar plates were left to incubate no inhibition of growth was observed which indicated that the benzoisothiazoles had no significant antifungal properties.

Chapter 4

4.1 Discussion

The problems that have arisen due to the emergence of antimicrobial resistance have resulted in the desperate need for novel classes of antimicrobial agents. The varieties of diseases and bacterial infections that have now become resistance to many gold standard drugs have reached a potentially lethal stage. Antibiotic resistance has been the focal point of concern for the past decade however less attention has been paid to the development of antifungal resistance. A review article of current antifungal agents, published by a research group in India has outlined the interest of novel heterocyclic compounds being synthesized and screened for antifungal activity (Kathiravan *et al*., 2012). The biological properties of heterocycles that include nitrogen, oxygen and sulphur have been reported extensively over the last century although there have been no reports on benzoisothiazoles fused pyrazine rings. This has prompted interest into the synthesis and biological screening of the novel class of benzoisothiazoles derivatives.

According to the literature the synthesis and spectral characterization of benzo[4,5] isothiazolo[2,3-a]pyrazine-6,6-dioxide derivatives has not been reported previously. The present work reports the synthesis and spectral characterisation of the novel ring system benzo [4,5]isothiazolo[2,3-a]pyrazine-6,6-dioxide.

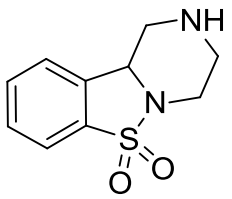
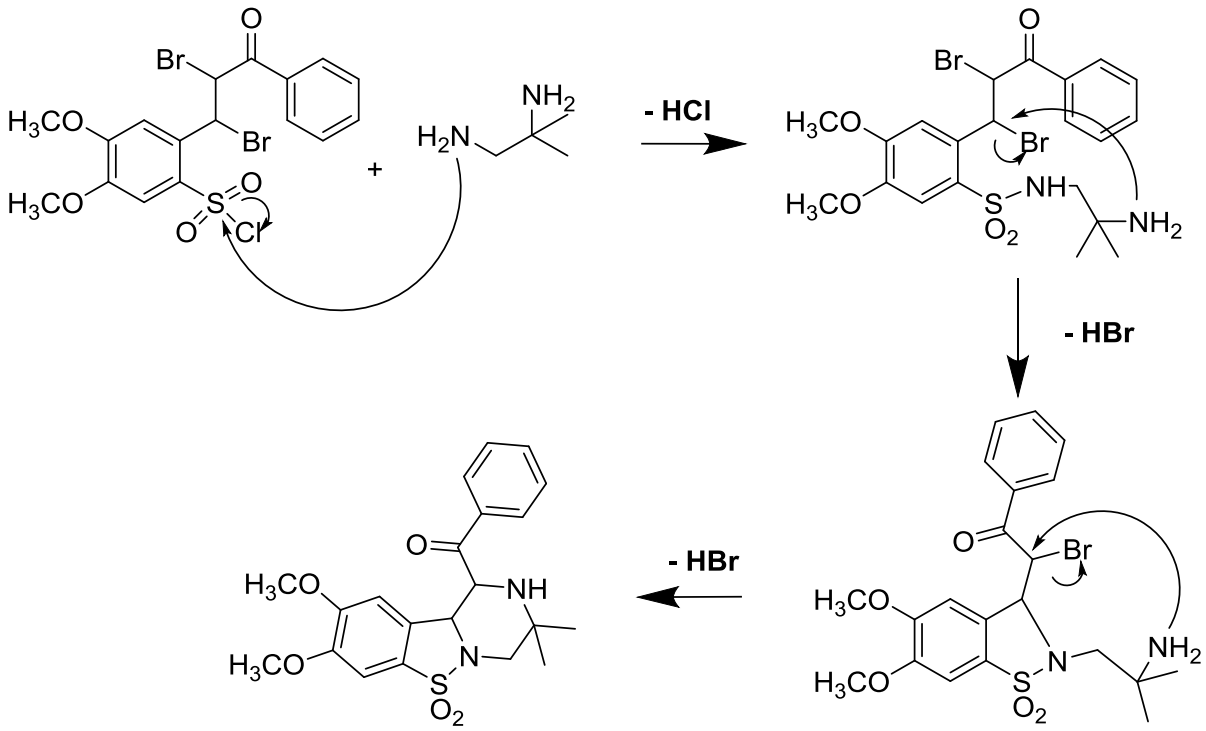


Figure 25: Structure of 1,3,4,10b-tetrahydro-2*H*-benzo[4,5]isothiazolo[2,3-a]pyrazine-6,6-dioxide**.**

The compounds were synthesized by reacting a series of dibromo chalcones sulphonyl chlorides with 1,2-diaminopropane and 2-methyl-1, 2 diamino-propane respectively. The yield of compounds, ranged from 12-80% with compound **33** obtaining a yield of 12% and compound **37** with a yield of 80%.



Scheme 3: shows the full proposed mechanism for the formation of the benzoisothiazole derivative.

All compounds were recrystallized and spectral analysis conducted on the purified products. Thin layer chromatography confirmed purity of compounds; proton and carbon NMR spectra confirmed the structures. For all the compounds the infrared spectra indicated the presence of the carbonyl group in the region of 1681 cm^{-1} and 1660 cm^{-1}. Similarly, presence of the suphonyl group was confirmed by absorption in the region 1130-1170 and 1330-1350 cm^{-1}. The expected peak for (-NH) group was observed in the region of 3310-3320 cm^{-1}.

The definitive structures of the compound were confirmed by the X-ray analysis of compound **31**. The x-ray structure clearly confirmed that the two methyl groups were on the carbon atom next to the NH group (see figure 29). All the compounds were reacted under the same reaction conditions. Using NMR data combined with the X-ray data the structures for all 18 compounds were correctly elucidated.

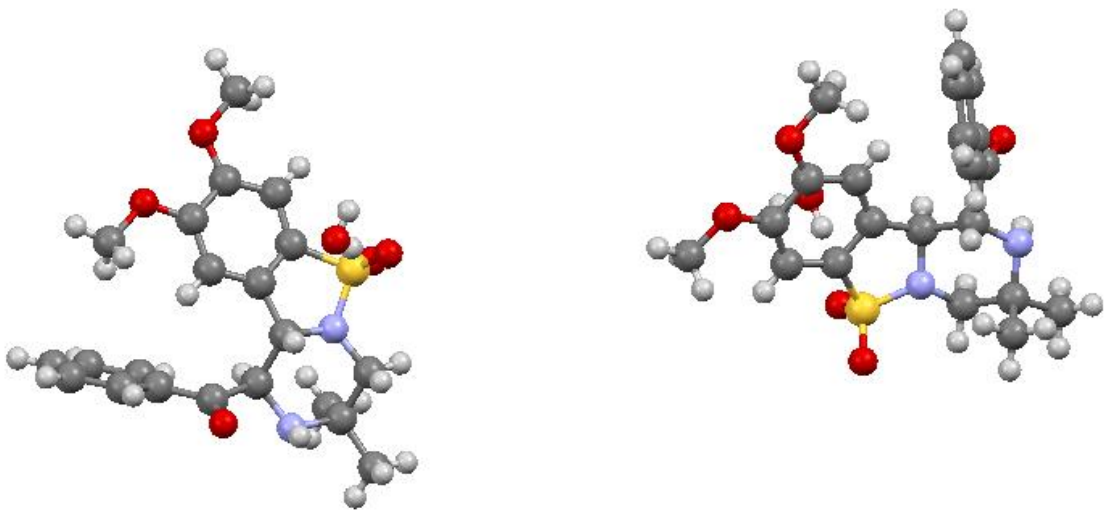


Figure 26: X-Ray structure of compound **31.**

Table 9: Crystal data and structure refinement for compound **31.**

Identification code	Compound **31**	
Empirical formula	$C_{21}H_{24}N_2O_5S$	
Formula weight	416.5	
Temperature	171(2) K	
Wavelength	0.71073 Å	
Crystal system	Monoclinic	
Space group	P2(1)	
Unit cell dimensions	a = 5.8645(9) Å	α= 90°.
	b = 19.946(3) Å	β= 97.459(3) °.
	c = 8.8732(14) Å	γ = 90°.
Volume	1029.1(3) $Å^3$	
Z	2	
Density (calculated)	1.402 Mg/m^3	
Absorption coefficient	0.199 mm^{-1}	
F(000)	460	
Crystal size	0.30 x 0.19 x 0.08 mm^3	
Theta range for data collection	2.04 to 28.30°.	
Index ranges	-7<=h<=7, -26<=k<=26, -11<=l<=11	
Reflections collected	11737	
Independent reflections	4948 [R(int) = 0.0394]	
Completeness to theta = 28.30°	98.6 %	

Absorption correction	None
Refinement method	Full-matrix least-squares on F^2
Data / restraints / parameters	4948 / 3 / 283
Goodness-of-fit on F^2	0.894
Final R indices [I>2sigma(I)]	R1 = 0.0419, wR2 = 0.0978
R indices (all data)	R1 = 0.0523, wR2 = 0.1053
Absolute structure parameter	0.04(7)
Largest diff. peak and hole	0.278 and -0.298 e.$Å^{-3}$

4.2 Biological screening

All eighteen benzoisothiazoles compounds were screened against four bacteria *Bacillus subtilis, Escherichia coli, Proteus hauseri* and *Staphylococcus aureus.* The compounds were also screened against three fungi *Aspergillus niger, Candida krusei and Mucor*. Due to solubility problems, the precise concentration of each compound interacting with the bacterial cells could not be ascertained. Activity was displayed by some compounds and this was analysed using MTT assays however no firm statistical data could be obtained. Using the MTT reduction assay, rapid and reproducible results were obtained for antibacterial screening. From the literature used many novel compounds that were screened for biological activity were done so without a particular mechanism of action in mind.

4.3 Antibacterial screening

Antibacterial screening was conducted using the following bacteria *Bacillus subtilis*, *Escherichia coli*, *Staphylococcus aureus* and *Proteus hauseri.* 20 mg of the compound was dissolved in 1 ml of pure DMSO to prepare stock solutions of 20 mg ml^{-1}. Initial screening started with 6.67 mg ml^{-1} and was diluted down to 0.1 mg ml^{-1} (104 µg ml^{-1}) in a 96 well plate. Due to solubility issues the stock solutions could not be diluted with distilled water, therefore the compound solution was diluted down in concentration using the culture inoculated broth. Initial tests showed that a maximum concentration of 5% DMSO could be used and had no cytotoxic effect on the four types of bacteria. To improve solubility of the compounds the 96 well plates were incubated on a rotary shaker. The rotary shaker improved the solubility of the compounds it was noticed that there was less precipitate present after the incubation period; however this was only noticed at concentrations below 3 mg ml^{-1}

The concentration of the compounds was tested in a range of 6.67 mg ml^{-1} down to 104 mg ml^{-1}. Dilutions were carried out so that each well had 50 µl of medium with a particular concentration of compound. An equal volume of fixed bacterial culture was added to the wells and incubated at 37^{o}C for 16-18 hours. The inoculums was adjusted to 0.5 McFarland turbidity standard (Ncube, Afolayan, & Okoh, 2008). Streptomycin was used as a reference drug throughout all the antibacterial testing.

Using MTT assay as an activity detection method is less conventional than the more commonly used disk diffusion assay. As the MTT assay was used as a preliminary investigation to ensure a more accurate set of results a method of using disk diffusion should be used to determine a narrower range of concentration at which the compound is active.

Compound **27** displayed promising antibacterial activity against all four bacteria at concentrations of 6.67 mg ml^{-1} down to 104 mg ml^{-1}. Compound **27** exerted the most activity against *B. subtilis.* Inhibition was observed from a concentration of 0.416 mg ml^{-1}, from the MTT assay it can be seen that compound was as effective as the control drug streptomycin at the concentration of 0.4 mg ml^{-1}.

Compound **27** inhibited the growth of both *E. coli* and *S. aureus* at a concentration of 1.6 mg ml^{-1}. As the concentration of Compound **27** decreased from 1.6 mg ml^{-1} down to the lowest concentration of 0.052 mg ml^{-1} the inhibitory effect of the compound decreased rapidly. Compound **27** had the least antibacterial activity against *P. Hauseri*; inhibition was displayed at the highest concentration of Compound **27** this indicates that the compounds are less potent against gram-negative bacteria. Streptomycin is bactericidal, which indicates that the benzoisothiazoles derivates that had antibacterial activity in higher concentration had bactericidal activity. As the concentration and inhibition decreased the compounds appear to be bacteriostatic.

From the antibacterial screening it was observed that Compound **27** was the most active against gram positive bacteria. This indicates that a chlorine atom in the *meta* position on the benzene ring is structurally significant. Compound **27** had one methyl group present on the fused pyrazine ring. There was no visible trend or similarity in results that correlated to the number of methyl groups on the pyrazine ring. There was no visible correlation between activity and halogen present; however there is much evidence in the literature to support the potency enhancing effects of halogen substituents. A research group in India synthesised a novel library of benzimidazole-oxadiazole derivatives with aim of developing newer and more potent antimicrobial agents. The research conducted stated that benzothiazole templates are important lead compounds in the design of pharmacologically active heterocycles (Patel, Patel, Kumari, Rajani, & Chikhalia, 2012) from the biological

screening it was concluded that the novel compounds bearing a benzothiazole ring were active against all the pathogenic strains tested and found that the benzothiazole rings that were attached to an electron withdrawing halogen had the highest potency (Patel *et al*., 2012). The position of the chlorine atom is structurally significant. When comparing Compound **27** to established fused heterocyclic antibacterial agents it can be noted that this compound has structural similarities to a variety of antibacterial agents such as the sulphone group which is common in some sulphonamide antibiotics. Another structural s similarity is the presence of a five membered ring which contains electronegative atoms which is found in carbapenem antibiotics, although the mechanism of action for the synthesized compounds would need to be validated before any strong correlation between structures could be ascertained.

Heterocyclic systems containing nitrogen, sulphur and oxygen atoms are essential building blocks in chemistry and fundamental to life. From what is available in the literature antimicrobial agents more often than not contain at least one or more of these three elements. The addition of halogens are frequently employed to increase antimicrobial activity, however with the exception of compound **27,** there was no significant difference in activity when varying the substituent group.

Research conducted by Rani and co-workers has shown that nitrogen and sulphur bicyclic thiadiazoles derivatives containing the electron withdrawing group fluorine displayed excellent antimicrobial properties against *E. coli, S. aureus, A. niger* and *C .albicans.* This research found a trend in activity with fluorine being the most active substituent followed by chlorine, methyl then methoxy groups. Antimicrobial activity was observed at concentrations of 6.25-100 µg ml^{-1}. Compounds that contained a methoxy group had significantly less activity against the bacteria and fungi tested (Rani, Ramachandran and Kabilan, 2010). This could possibly indicate that the presence of a methoxy group reduces the antimicrobial activity of a compound.

Azo dyes have recently attracted interest as potential antimicrobial agents. A library of novel heterocyclic disazo dyes were synthesised and screened for antimicrobial activity. Although no antibacterial activity was observed, two novel azo dye compounds displayed potent antifungal activity with a chlorine substituent group with antifungal activity more potent than that of the control drug fluconazole (Karci, Şener, Yamaç, Demirçal, 2009).

Fluoroquinolones (FQs) are still noted for their antibacterial effectiveness, this is due to the drugs ability to inhibit two essential bacterial enzymes. Research and development into this group of drugs is continually being conducted as FQs are a broad spectrum antibiotic. A review article by Bush & Pucci has discussed a novel group of DNA topoisomerase inhibitors all containing a fluoroquinolone moiety with excellent antibacterial activity against *Staphylococcus aureus* (Bush & Pucci, 2012). An interesting area of research to pursue would be to introduce a quinolone structure onto the benzoisothiazole derivatives, this could lead to a more potent set of compounds.

The library of compounds synthesized were found to be more active on gram positive bacteria. This suggests that the mechanism of action could be cell wall synthesis inhibition; although for this to be validated further investigation would be required. Calculated log P values of 1.51-1.73 were obtained which indicates the compounds were lipophilic enough to penetrate the cell wall of gram positive bacteria, which is lipophilic in nature. For further research into the antimicrobial properties of this group of benzoisothiazoles derivatives, it is necessary for more work to be conducted on improving the solubility of these compounds.

4.4 Antifungal screening

Like the antibacterial screening, initial testing for DMSO fungal toxicity needed to be investigated. A range of different DMSO concentrations were tested, a maximum of 5% DMSO was tolerated by the fungi. The 3 fungi screened were *Aspergillus niger, Candida krusei* and *Mucor.* 70 µl of the compound mixture was absorbed onto antibiotic disks the disks were then placed on an agar plate that had an evenly distributed lawn of fungal growth. The agar plates were left to further grow to see if the compounds had any

fungicidal or fungiostatic activity. The growth activity of the fungi was monitored at 24hr intervals.

Although the compounds synthesized contained an azole moiety there was no antifungal activity exhibited by any of the compounds. This could be due to the presence of a SO_2 group in the benzoisothiazole ring. The majority of the azole based antifungals contain halogens but no sulphur atoms; this indicates that the presence of a SO_2 decreases antifungal activity.

In a study conducted by Koparir and co-workers a novel library of 1,2,4-triazole derivatives were synthesized and screened for antibacterial activity against *E. coli, S. aureus, K. pneumoniae* and *P. aeruginosa* and antifungal activity against two strains of *Aspergillus*. The compounds that bore a hydroxyl group on the phenyl ring attached to the triazole ring displayed potent antibacterial activity at a concentration of 6.25µgml^{-1} against *E. coli* and *S. aureus* and similar results for *Aspergillus flavus* (Koparir, Orek, Koparir & Sarac, 2012). From this it can be deduced that triazole rings fused to phenol rings are structurally significant, in comparison to the compounds synthesized in the current research a possible way to increase the antifungal activity of these compounds would be to incorporate a triazole ring.

A series of novel benzimidazole derivatives were synthesized by Desai and co-workers in 2013. These compounds have displayed excellent antifungal properties, one compound in particular showed a fungal MIC that was lower than the standard antifungal drugs chloramphenicol and ketoconazole that were used in the tests (Desai, Shihory & Kotadiya, 2013). A significant finding of this research was that compounds that contained fluoro or nitro electron withdrawing groups displayed the most potent activity.

Compounds **22-39** all contained two methoxy groups which are electron donating groups (EDGs), from the literature available there is a significant amount of evidence showing that the presence of an electron donating group limits the potential antimicrobial activity of a compound. To improve both potential activity and solubility of the compounds it would be beneficial to substitute the two methoxy groups, to groups that have stronger electron withdrawing capabilities.

Chapter 5

5.1 Conclusion

In summary, the study conducted in the work has designed a new synthetic route to benzoisothiazole derivatives, from readily available starting materials. The compounds synthesized showed potential biological activity against gram positive bacteria *B. subtilis* and *P. hauseri.* Due to insolubility of the compounds in DMSO future work would involve the incorporation of hydroxyl groups or an added ring structure. Another possible line of investigation could be to synthesize the amine salt of the benzoisothiazoles.

As a preliminary experiment, MTT assay was employed, additional work to validate the antimicrobial properties of these compounds would include disk diffusion assays and determination of minimal inhibitory concentration (MIC) however the solubility issues would need to be resolved first.

5.2 Future work

The first avenue of work would be to increase the solubility of the 18 compounds, a possible route for this would be to convert the two methoxy groups on the 1,2-dimentoxybenzene ring to hydroxyl, in a method described by Alonso *et al* two methoxy groups were demethylated to produce two hydroxyl groups. Methylated resveratrol was refluxed at $0^{o}C$ for 5 hours in a mixture of Boron tribromide (BBr_3) and chloroform; this gave a yield of 70% for the demethylated final product (Alonso, Riente & Yus 2009).

If all the compounds are successfully demethylated and solubilities enhanced then possible re-screening for activity could be an option, and could potentially lead to more potent set of compounds which could be further modified to produce a larger library of compounds. From this, a more in-depth investigation of biological activity could be achieved, with possible statistical analysis being employed.

Other routes to pursue would be introducing another ring to the core structure or possibly making an amine salt.

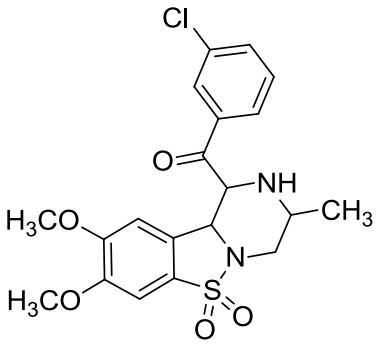


Figure 27: (8,9-Dimethoxy-3-methyl-6,6-dioxido-2,3,4,10b-tetrahydro-1Hbenzo[4,5] isothiazolo[2,3-a]pyrazin-1-yl)(3-chlorophenyl)methanone **27**.

Compound **27** see Figure 27, displayed promising activity against *B. subtilis* and some activity against the other 3 bacteria. Compound 27 could either be further tested for any possible biological importance such as anti-tuberculosis and possibly anti-inflammatory activity solubility permitting. If Compound **27** was structurally modified and solubility increased then it could possibly be reassessed via *in silico* methods. To fully explore the idea of a QSAR model it would be ideal to have between 20-30 analogues of compounds **27** which is another area of work that could be investigated.

Structure activity relationships (SARs) provide a correlation between chemical structure and biological activity whilst being reinforced with statistical analysis. To further enhance the antimicrobial properties of this class of compounds it would be useful to develop a SAR model by collecting data on the 3D structure of known antimicrobial agents and their biological activity (McKinney, Richard, Waller, Newman & Gerbericks, 2000).

From the available literature, benzoisothiazoles are an essential starting point for pharmaceutically active compounds. Benzoisothiazoles as single units do not appear to show much biological importance, however when fused to other heterocyclic ring systems, these derivatives display a broad spectrum of activity. The aim of this project is to fuse benzoisothiazoles to quinoxalines, benzodiazepines and diazocines to produce a library of novel benzoisothiazole derivatives that hopefully display promising biological properties.

Using the literature data and synthetic routes available we plan to fuse the benzoisothiazole ring to known pharmaceutically active heterocycles i.e. quinoxalines, benzodiazepines and diazocines.

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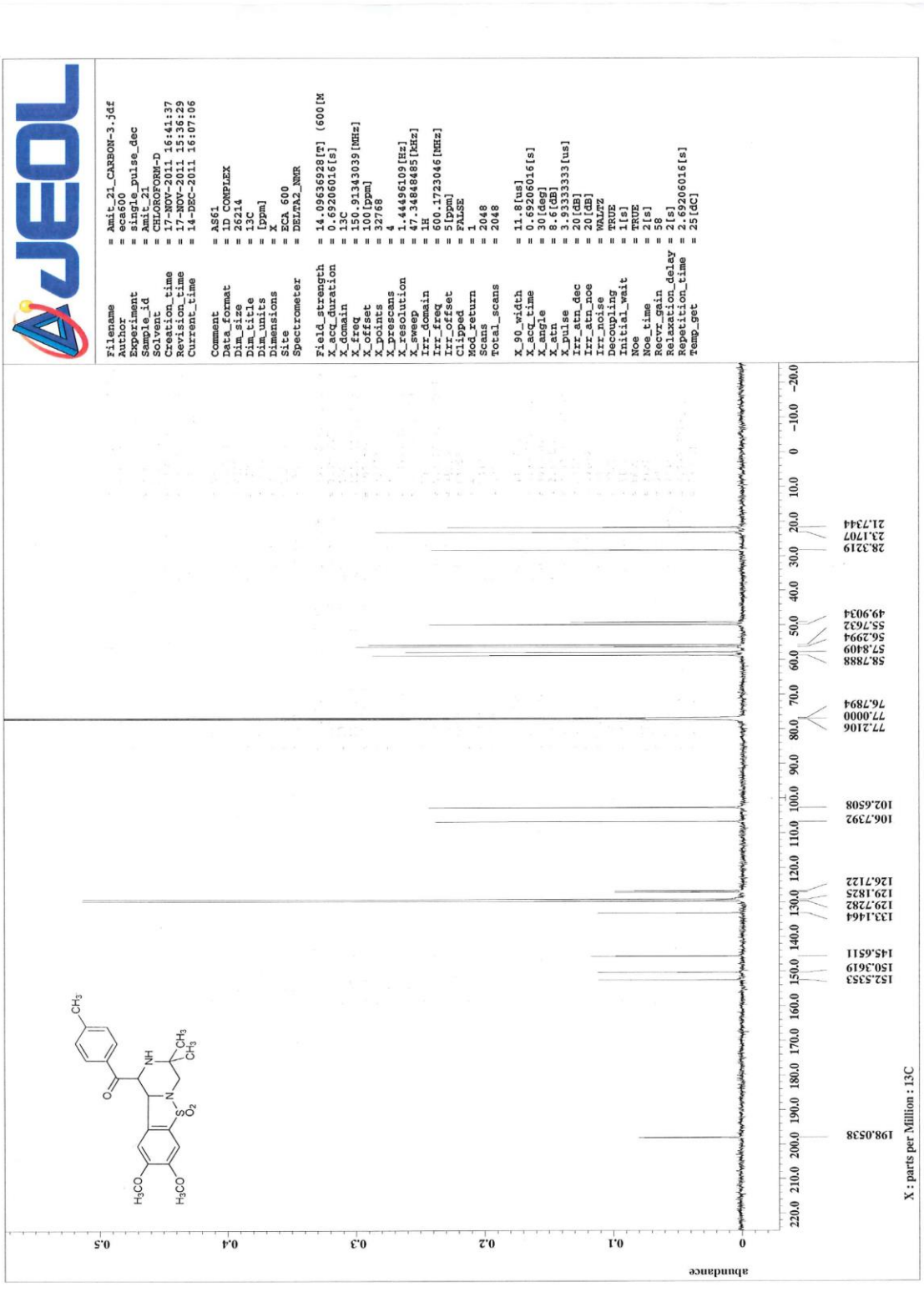
Appendix

Appendix I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Filename = Amit_21_CARBON-3.jdf
Author = eca600
Experiment = single_pulse_dec
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Solvent = CHLOROFORM-D
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Revision_time = 17-NOV-2011 15:36:29
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Comment = AS61
Data_format = 1D COMPLEX
Dim_size = 26214
Dim_title = 13C
Dim_units = [ppm]
Dimensions = X
Site = ECA 600
Spectrometer = DELTA2_NMR
Field_strength = 14.09636928[T] (600[M
X_acq_duration = 0.69206016[s]
X_domain = 13C
X_freq = 150.91343039[MHz]
X_offset = 100[ppm]
X_points = 32768
X_prescans = 4
X_resolution = 1.44496109[Hz]
X_sweep = 47.34848485[kHz]
Irr_domain = 1H
Irr_freq = 600.1723046[MHz]
Irr_offset = 5[ppm]
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Scans = 2048
Total_scans = 2048
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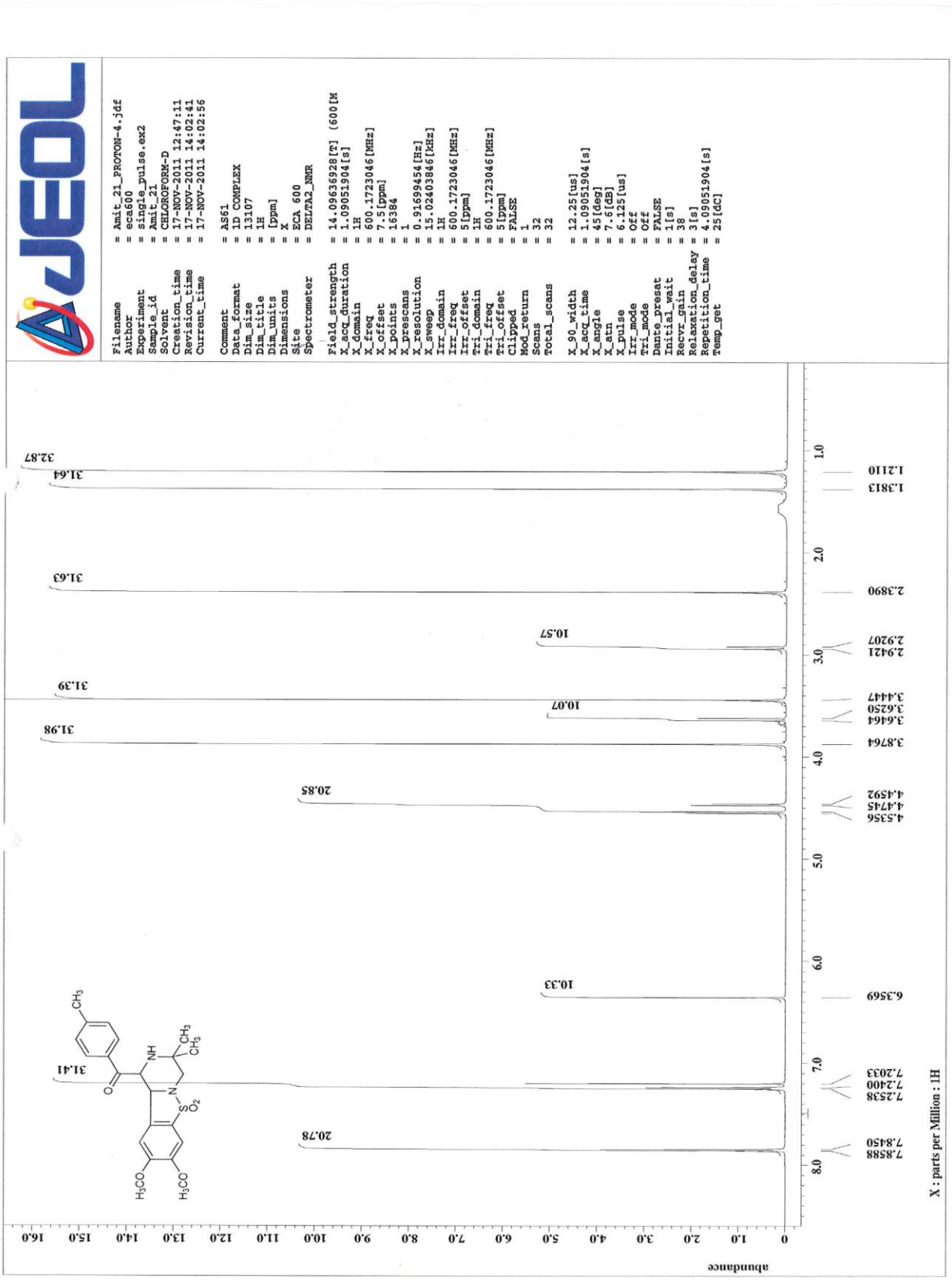
X_acq_time = 0.69206016[s]
X_angle = 30[deg]
X_atn = 8.6[dB]
X_pulse = 3.93333333[us]
Irr_atn_dec = 20[dB]
Irr_atn_noe = 20[dB]
Irr_noise = WALTZ
Decoupling = TRUE
Initial_wait = 1[s]
Noe = TRUE
Noe_time = 2[s]
Recvr_gain = 58
Relaxation_delay = 2[s]
Repetition_time = 2.69206016[s]
Temp_get = 25[dC]
abundance
X : parts per Million : 13C
198.0538
152.5353
150.3619
145.6511
133.1464
129.7282
129.1825
126.7122
106.7392
102.6508
77.2106
77.0000
76.7894
58.7888
57.8409
56.2994
55.7632
49.9034
28.3219
23.1707
21.7344
CH3
NH
CH3
CH3
O
N
SO2
H3CO
H3CO

Appendix II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X_offset = 7.5[ppm]
X_points = 16384
X_prescans = 1
X_resolution = 0.91699454[Hz]
X_sweep = 15.02403846[KHz]
Irr_domain = 1H
Irr_freq = 600.1723046[MHz]
Irr_offset = 5[ppm]
Tri_domain = 1H
Tri_freq = 600.1723046[MHz]
Tri_offset = 5[ppm]
Clipped = FALSE
Mod_return = 1
Scans = 32
Total_scans = 32
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X_acq_time = 1.09051904[s]
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X_atn = 7.6[dB]
X_pulse = 6.125[us]
Irr_mode = Off
Tri_mode = Off
Dante_presat = FALSE
Initial_wait = 1[s]
Recvr_gain = 38
Relaxation_delay = 3[s]
Repetition_time = 4.09051904[s]
Temp_get = 25[dC]
32.87
31.64
31.63
31.39
31.98
31.41
20.85
20.78
10.57
10.07
10.33
1.2110
1.3813
2.3890
2.9207
2.9421
3.4447
3.6250
3.6464
3.8764
4.4592
4.4745
4.5356
6.3569
7.2033
7.2400
7.2538
7.8450
7.8588
abundance
X : parts per Million : 1H

Appendix III

Table i. Atomic coordinates (x 10^4) and equivalent isotropic displacement parameters ($Å^2$x 10^3) for compound **31.**

U(eq) is defined as one third of the trace of the orthogonalized U^{ij} tensor.

	x	y	z	U(eq)
S(1)	260(1)	4525(1)	4207(1)	19(1)
N(1)	-1286(3)	4472(1)	5593(2)	24(1)
N(2)	-3581(4)	4271(1)	8097(2)	22(1)
O(1)	-871(3)	4889(1)	2912(2)	30(1)
O(2)	2508(3)	4773(1)	4770(2)	29(1)
O(3)	2077(3)	2198(1)	1904(2)	29(1)
O(4)	-1094(3)	1656(1)	3240(2)	29(1)
O(5)	-6807(3)	3350(1)	7124(2)	28(1)
C(1)	124(4)	3663(1)	3872(3)	20(1)
C(2)	1398(4)	3307(1)	2911(3)	22(1)
C(3)	958(4)	2624(1)	2747(3)	23(1)
C(4)	-782(4)	2322(1)	3495(3)	22(1)
C(5)	-2039(4)	2700(1)	4406(3)	22(1)
C(6)	-1569(4)	3378(1)	4618(3)	18(1)
C(7)	-2752(4)	3871(1)	5551(3)	20(1)
C(8)	-2006(4)	5066(1)	6367(3)	22(1)
C(9)	-2094(4)	4875(1)	8034(3)	22(1)
C(10)	-2915(4)	3686(1)	7241(3)	20(1)
C(11)	-4816(4)	3161(1)	7311(3)	20(1)
C(12)	-4258(4)	2439(1)	7605(3)	21(1)
C(13)	-2147(5)	2215(1)	8322(3)	30(1)
C(14)	-1770(6)	1538(2)	8607(4)	39(1)
C(15)	-3484(5)	1077(1)	8153(3)	37(1)
C(16)	-5602(5)	1293(1)	7438(3)	32(1)
C(17)	-5998(5)	1963(1)	7170(3)	26(1)
C(18)	3689(5)	2486(1)	1012(3)	30(1)
C(19)	-2774(5)	1339(1)	4041(3)	32(1)
C(20)	297(4)	4725(1)	8852(3)	31(1)
C(21)	-3180(5)	5447(1)	8839(3)	29(1)
O(1W)	-4746(4)	4118(1)	1223(3)	51(1)

Table ii.Bond lengths [Å] and angles [°] for **31.**

S(1)-O(2)	1.4353(18)
S(1)-O(1)	1.4449(18)
S(1)-N(1)	1.6232(19)
S(1)-C(1)	1.747(2)
N(1)-C(8)	1.460(3)
N(1)-C(7)	1.473(3)
N(2)-C(10)	1.472(3)
N(2)-C(9)	1.492(3)
N(2)-H(1N2)	0.75(3)
O(3)-C(3)	1.357(3)
O(3)-C(18)	1.430(3)
O(4)-C(4)	1.356(3)
O(4)-C(19)	1.434(3)
O(5)-C(11)	1.217(3)
C(1)-C(6)	1.385(3)
C(1)-C(2)	1.398(3)
C(2)-C(3)	1.391(3)
C(2)-H(2)	0.9500
C(3)-C(4)	1.421(3)
C(4)-C(5)	1.386(3)
C(5)-C(6)	1.388(3)
C(5)-H(5)	0.9500
C(6)-C(7)	1.511(3)
C(7)-C(10)	1.559(3)
C(7)-H(7)	1.0000
C(8)-C(9)	1.536(3)
C(8)-H(8A)	0.9900
C(8)-H(8B)	0.9900
C(9)-C(20)	1.523(3)
C(9)-C(21)	1.528(3)
C(10)-C(11)	1.536(3)
C(10)-H(10)	1.0000
C(11)-C(12)	1.493(3)
C(12)-C(13)	1.390(4)
C(12)-C(17)	1.410(3)
C(13)-C(14)	1.386(4)
C(13)-H(13)	0.9500
C(14)-C(15)	1.384(4)
C(14)-H(14)	0.9500
C(15)-C(16)	1.388(4)
C(15)-H(15)	0.9500
C(16)-C(17)	1.372(4)
C(16)-H(16)	0.9500
C(17)-H(17)	0.9500
C(18)-H(18A)	0.9800

C(18)-H(18B)	0.9800
C(18)-H(18C)	0.9800
C(19)-H(19A)	0.9800
C(19)-H(19B)	0.9800
C(19)-H(19C)	0.9800
C(20)-H(20A)	0.9800
C(20)-H(20B)	0.9800
C(20)-H(20C)	0.9800
C(21)-H(21A)	0.9800
C(21)-H(21B)	0.9800
C(21)-H(21C)	0.9800
O(1W)-H(1W)	0.83(3)
O(1W)-H(2W)	0.88(3)

O(2)-S(1)-O(1)	114.24(11)
O(2)-S(1)-N(1)	109.70(11)
O(1)-S(1)-N(1)	113.03(11)
O(2)-S(1)-C(1)	114.69(11)
O(1)-S(1)-C(1)	110.66(11)
N(1)-S(1)-C(1)	92.67(11)
C(8)-N(1)-C(7)	118.01(18)
C(8)-N(1)-S(1)	121.80(16)
C(7)-N(1)-S(1)	114.58(15)
C(10)-N(2)-C(9)	115.20(18)
C(10)-N(2)-H(1N2)	110(2)
C(9)-N(2)-H(1N2)	106(2)
C(3)-O(3)-C(18)	117.08(19)
C(4)-O(4)-C(19)	115.83(19)
C(6)-C(1)-C(2)	123.9(2)
C(6)-C(1)-S(1)	110.02(17)
C(2)-C(1)-S(1)	125.98(18)
C(3)-C(2)-C(1)	117.0(2)
C(3)-C(2)-H(2)	121.5
C(1)-C(2)-H(2)	121.5
O(3)-C(3)-C(2)	125.1(2)
O(3)-C(3)-C(4)	114.8(2)
C(2)-C(3)-C(4)	120.1(2)
O(4)-C(4)-C(5)	124.1(2)
O(4)-C(4)-C(3)	115.3(2)
C(5)-C(4)-C(3)	120.6(2)
C(4)-C(5)-C(6)	119.9(2)
C(4)-C(5)-H(5)	120.1
C(6)-C(5)-H(5)	120.1
C(1)-C(6)-C(5)	118.5(2)
C(1)-C(6)-C(7)	113.9(2)
C(5)-C(6)-C(7)	127.6(2)
N(1)-C(7)-C(6)	103.60(18)

N(1)-C(7)-C(10)	106.06(18)
C(6)-C(7)-C(10)	117.59(19)
N(1)-C(7)-H(7)	109.7
C(6)-C(7)-H(7)	109.7
C(10)-C(7)-H(7)	109.7
N(1)-C(8)-C(9)	107.41(18)
N(1)-C(8)-H(8A)	110.2
C(9)-C(8)-H(8A)	110.2
N(1)-C(8)-H(8B)	110.2
C(9)-C(8)-H(8B)	110.2
H(8A)-C(8)-H(8B)	108.5
N(2)-C(9)-C(20)	109.3(2)
N(2)-C(9)-C(21)	107.77(19)
C(20)-C(9)-C(21)	109.8(2)
N(2)-C(9)-C(8)	109.22(19)
C(20)-C(9)-C(8)	111.3(2)
C(21)-C(9)-C(8)	109.4(2)
N(2)-C(10)-C(11)	106.05(18)
N(2)-C(10)-C(7)	111.24(18)
C(11)-C(10)-C(7)	109.62(19)
N(2)-C(10)-H(10)	110.0
C(11)-C(10)-H(10)	110.0
C(7)-C(10)-H(10)	110.0
O(5)-C(11)-C(12)	120.4(2)
O(5)-C(11)-C(10)	118.2(2)
C(12)-C(11)-C(10)	121.4(2)
C(13)-C(12)-C(17)	118.6(2)
C(13)-C(12)-C(11)	123.5(2)
C(17)-C(12)-C(11)	117.9(2)
C(14)-C(13)-C(12)	120.6(3)
C(14)-C(13)-H(13)	119.7
C(12)-C(13)-H(13)	119.7
C(15)-C(14)-C(13)	120.1(3)
C(15)-C(14)-H(14)	119.9
C(13)-C(14)-H(14)	119.9
C(14)-C(15)-C(16)	120.0(3)
C(14)-C(15)-H(15)	120.0
C(16)-C(15)-H(15)	120.0
C(17)-C(16)-C(15)	120.2(3)
C(17)-C(16)-H(16)	119.9
C(15)-C(16)-H(16)	119.9
C(16)-C(17)-C(12)	120.6(3)
C(16)-C(17)-H(17)	119.7
C(12)-C(17)-H(17)	119.7
O(3)-C(18)-H(18A)	109.5
O(3)-C(18)-H(18B)	109.5
H(18A)-C(18)-H(18B)	109.5

O(3)-C(18)-H(18C)	109.5
H(18A)-C(18)-H(18C)	109.5
H(18B)-C(18)-H(18C)	109.5
O(4)-C(19)-H(19A)	109.5
O(4)-C(19)-H(19B)	109.5
H(19A)-C(19)-H(19B)	109.5
O(4)-C(19)-H(19C)	109.5
H(19A)-C(19)-H(19C)	109.5
H(19B)-C(19)-H(19C)	109.5
C(9)-C(20)-H(20A)	109.5
C(9)-C(20)-H(20B)	109.5
H(20A)-C(20)-H(20B)	109.5
C(9)-C(20)-H(20C)	109.5
H(20A)-C(20)-H(20C)	109.5
H(20B)-C(20)-H(20C)	109.5
C(9)-C(21)-H(21A)	109.5
C(9)-C(21)-H(21B)	109.5
H(21A)-C(21)-H(21B)	109.5
C(9)-C(21)-H(21C)	109.5
H(21A)-C(21)-H(21C)	109.5
H(21B)-C(21)-H(21C)	109.5
H(1W)-O(1W)-H(2W)	97(4)

Symmetry transformations used to generate equivalent atoms:

Table iii. Anisotropic displacement parameters ($Å^2 x\ 10^3$) for compound **31.** The anisotropic

displacement factor exponent takes the form: $-2\pi^2[\ h^2\ a^{*2}U^{11} + 2\ h\ k\ a^*\ b^*\ U^{12}\ ]$

	U^{11}	**U^{22}**	**U^{33}**	**U^{23}**	**U^{13}**	**U^{12}**
S(1)	20(1)	16(1)	22(1)	0(1)	6(1)	0(1)
N(1)	31(1)	18(1)	27(1)	-5(1)	16(1)	-5(1)
N(2)	21(1)	22(1)	24(1)	-3(1)	7(1)	0(1)

O(1)	40(1)	23(1)	26(1)	4(1)	4(1)	3(1)
O(2)	20(1)	23(1)	43(1)	-5(1)	4(1)	-3(1)
O(3)	36(1)	21(1)	35(1)	-5(1)	20(1)	0(1)
O(4)	37(1)	17(1)	36(1)	-4(1)	17(1)	-3(1)
O(5)	18(1)	24(1)	41(1)	2(1)	6(1)	0(1)
C(1)	22(1)	15(1)	23(1)	-2(1)	3(1)	-1(1)
C(2)	24(1)	20(1)	22(1)	-1(1)	8(1)	-2(1)
C(3)	26(1)	22(1)	20(1)	-2(1)	7(1)	2(1)
C(4)	28(1)	17(1)	22(1)	-1(1)	3(1)	0(1)
C(5)	22(1)	20(1)	24(1)	-2(1)	7(1)	-4(1)
C(6)	20(1)	18(1)	17(1)	0(1)	4(1)	2(1)
C(7)	21(1)	17(1)	24(1)	-2(1)	6(1)	-1(1)
C(8)	24(1)	17(1)	26(1)	-5(1)	8(1)	-1(1)
C(9)	21(1)	21(1)	26(1)	-4(1)	9(1)	-2(1)
C(10)	20(1)	19(1)	21(1)	-2(1)	6(1)	-1(1)
C(11)	21(1)	21(1)	20(1)	0(1)	5(1)	-2(1)
C(12)	24(1)	22(1)	20(1)	0(1)	8(1)	-2(1)
C(13)	26(1)	27(1)	35(1)	7(1)	3(1)	-1(1)
C(14)	41(2)	32(2)	45(2)	11(1)	6(1)	8(1)
C(15)	48(2)	22(1)	43(2)	9(1)	18(1)	5(1)
C(16)	40(2)	25(1)	34(1)	-1(1)	13(1)	-7(1)
C(17)	27(1)	25(1)	28(1)	0(1)	8(1)	-3(1)
C(18)	32(1)	33(1)	28(1)	-3(1)	15(1)	0(1)
C(19)	35(2)	19(1)	45(2)	0(1)	16(1)	-4(1)
C(20)	22(1)	35(1)	34(1)	-5(1)	3(1)	-4(1)
C(21)	35(1)	24(1)	31(1)	-7(1)	12(1)	-1(1)
O(1W)	51(1)	72(2)	30(1)	8(1)	6(1)	-29(1)

Table iv. Hydrogen coordinates (x 10^4) and isotropic displacement parameters (Å^{2}x 10^3) for compound **31.**

	x	y	z	U(eq)
H(2)	2514	3522	2392	26
H(5)	-3220	2496	4884	26
H(7)	-4319	3977	5018	24
H(8A)	-3541	5216	5888	26
H(8B)	-898	5436	6302	26
H(10)	-1409	3505	7729	24
H(13)	-954	2528	8620	35
H(14)	-331	1390	9114	47
H(15)	-3211	612	8330	44
H(16)	-6781	976	7133	39
H(17)	-7456	2108	6688	32
H(18A)	4392	2130	466	45
H(18B)	4888	2723	1679	45
H(18C)	2892	2802	279	45
H(19A)	-2869	861	3782	48
H(19B)	-4277	1550	3751	48
H(19C)	-2325	1390	5138	48
H(20A)	187	4606	9912	46
H(20B)	1274	5122	8821	46
H(20C)	969	4350	8349	46
H(21A)	-4708	5547	8300	44
H(21B)	-2207	5847	8845	44
H(21C)	-3319	5315	9887	44
H(1W)	-4250(80)	4180(30)	400(40)	95(17)
H(2W)	-3700(60)	4360(20)	1790(40)	73(13)
H(1N2)	-4770(50)	4381(15)	7780(30)	24(8)

Table v. Torsion angles [°] for compound **31.**

O(2)-S(1)-N(1)-C(8)	67.0(2)
O(1)-S(1)-N(1)-C(8)	-61.7(2)
C(1)-S(1)-N(1)-C(8)	-175.51(19)
O(2)-S(1)-N(1)-C(7)	-139.92(17)
O(1)-S(1)-N(1)-C(7)	91.33(18)
C(1)-S(1)-N(1)-C(7)	-22.48(18)
O(2)-S(1)-C(1)-C(6)	127.07(17)
O(1)-S(1)-C(1)-C(6)	-101.91(18)
N(1)-S(1)-C(1)-C(6)	13.94(18)
O(2)-S(1)-C(1)-C(2)	-57.2(2)
O(1)-S(1)-C(1)-C(2)	73.8(2)
N(1)-S(1)-C(1)-C(2)	-170.3(2)
C(6)-C(1)-C(2)-C(3)	-2.0(4)
S(1)-C(1)-C(2)-C(3)	-177.14(19)
C(18)-O(3)-C(3)-C(2)	-5.8(4)
C(18)-O(3)-C(3)-C(4)	174.1(2)
C(1)-C(2)-C(3)-O(3)	-177.8(2)
C(1)-C(2)-C(3)-C(4)	2.3(3)
C(19)-O(4)-C(4)-C(5)	-2.9(3)
C(19)-O(4)-C(4)-C(3)	177.3(2)
O(3)-C(3)-C(4)-O(4)	-0.9(3)
C(2)-C(3)-C(4)-O(4)	179.0(2)
O(3)-C(3)-C(4)-C(5)	179.3(2)
C(2)-C(3)-C(4)-C(5)	-0.7(4)
O(4)-C(4)-C(5)-C(6)	179.0(2)
C(3)-C(4)-C(5)-C(6)	-1.3(4)
C(2)-C(1)-C(6)-C(5)	0.0(4)
S(1)-C(1)-C(6)-C(5)	175.83(19)
C(2)-C(1)-C(6)-C(7)	-178.1(2)
S(1)-C(1)-C(6)-C(7)	-2.3(2)
C(4)-C(5)-C(6)-C(1)	1.7(3)
C(4)-C(5)-C(6)-C(7)	179.5(2)
C(8)-N(1)-C(7)-C(6)	177.43(18)
S(1)-N(1)-C(7)-C(6)	23.3(2)
C(8)-N(1)-C(7)-C(10)	-58.1(3)
S(1)-N(1)-C(7)-C(10)	147.74(15)
C(1)-C(6)-C(7)-N(1)	-12.1(3)
C(5)-C(6)-C(7)-N(1)	170.0(2)
C(1)-C(6)-C(7)-C(10)	-128.7(2)
C(5)-C(6)-C(7)-C(10)	53.4(3)
C(7)-N(1)-C(8)-C(9)	60.8(3)
S(1)-N(1)-C(8)-C(9)	-147.06(17)
C(10)-N(2)-C(9)-C(20)	-66.5(3)
C(10)-N(2)-C(9)-C(21)	174.2(2)
C(10)-N(2)-C(9)-C(8)	55.5(3)

N(1)-C(8)-C(9)-N(2)	-53.9(2)
N(1)-C(8)-C(9)-C(20)	66.9(2)
N(1)-C(8)-C(9)-C(21)	-171.64(19)
C(9)-N(2)-C(10)-C(11)	-173.64(19)
C(9)-N(2)-C(10)-C(7)	-54.5(3)
N(1)-C(7)-C(10)-N(2)	50.8(2)
C(6)-C(7)-C(10)-N(2)	166.10(19)
N(1)-C(7)-C(10)-C(11)	167.82(18)
C(6)-C(7)-C(10)-C(11)	

Appendix IV

Table of absorbance readings at 590nm for *Bacillus subtilis* after adding MTT

[mg ml-1]	Compounds																		
	22	**23**	**24**	**25**	**26**	**27**	**28**	**29**	**30**	**31**	**32**	**33**	**34**	**35**	**36**	**37**	**38**	**39**	**Ctrl**
0.052	1.191	1.597	1.37	1.181	1.073	1.529	1.038	1.189	1.279	1.514	1.15	1.207	1.126	1.629	1.611	1.717	1.658	1.357	0.05
0.104	1.195	1.72	1.341	1.192	1.049	1.488	1.003	1.185	1.279	1.715	1.215	1.209	1.132	1.602	1.66	1.75	1.599	1.391	0.05
0.208	1.189	1.735	1.289	1.148	1.032	1.602	0.972	1.194	1.256	1.712	1.206	1.174	1.116	1.583	1.625	1.717	1.615	1.388	0.05
0.416	1.189	1.635	1.289	1.145	1.020	1.255	0.926	1.156	1.281	1.6448	1.223	1.214	1.155	1.602	1.562	1.715	1.555	1.329	0.05
0.833	1.159	1.462	1.239	1.155	0.972	0.237	0.887	1.128	1.292	1.544	1.183	1.219	1.194	1.701	1.348	1.71	1.216	1.338	0.05
1.6	1.158	1.345	1.342	1.116	0.964	0.139	1.008	1.173	1.338	1.519	1.149	1.173	1.215	1.567	1.649	1.872	1.171	1.412	0.05
3.3	1.359	1.441	1.188	1.150	1.149	0.117	1.289	1.114	1.39	1.742	1.132	1.451	1.606	1.301	1.388	1.436	0.945	1.433	0.05
6.67	0.852	0.318	0.218	0.635	0.680	0.087	1.116	1.180	0.775	0.660	0.351	0.683	0.278	0.214	1.472	1.556	0.279	0.929	0.05

[mg ml-1] = Concentration of compound in mg ml-1

Ctrl = Control is the reference drug streptomycin

Table of absorbance readings at 590 nm in natural log for *Bacillus subtilis* after adding MTT

	22	23	24	25	26	27	28	29	30
0.052	3.29036993	4.93819559	3.9353507	3.2576302	2.92413877	4.61356095	2.82356423	3.28379577	3.593044884
0.104	3.30355777	5.58452846	3.82286446	3.29366195	2.8547949	4.4282302	2.72644892	3.27068682	3.593044884
0.208	3.28379577	5.6689278	3.62915558	3.15188284	2.80667357	4.9629484	2.64322563	3.30025586	3.511347967
0.416	3.28379577	5.129458	3.62915558	3.14244136	2.77319476	3.50783837	2.52439139	3.17719903	3.600238164
0.833	3.18674494	4.31458007	3.45215958	3.17402342	2.64322563	1.26744112	2.42783521	3.08947137	3.640059399
1.6	3.18355979	3.83818654	3.82668924	3.05261927	2.62216418	1.1491241	2.7401153	3.23167313	3.811413051
3.3	3.89229906	4.22491862	3.28051361	3.15819291	3.1550363	1.12411943	3.62915558	3.04652014	4.014850053
6.67	2.34433083	1.37437626	1.24358707	1.88702214	1.97387773	1.09089668	3.05261927	3.2543742	2.170592127

	31	32	33	34	35	36	37	38	39
0.052	4.54487398	3.15819291	3.34343927	3.08329861	5.09877339	5.00781654	5.56779998	5.24880273	3.884522237
0.104	5.55667551	3.37029406	3.35013284	3.10185401	4.9629484	5.25931084	5.75460268	4.94808187	4.018866911
0.208	5.54003047	3.34009751	3.23490642	3.05261927	4.86954255	5.07841904	5.56779998	5.02788792	4.006828377
0.416	5.11920933	3.39736455	3.36692545	3.17402342	4.9629484	4.76834841	5.55667551	4.73508653	3.777264234
0.833	4.683286	3.26415198	3.38380224	3.30025586	5.47942408	3.84971839	5.52896148	3.37366604	3.811413051
1.6	4.56765526	3.1550363	3.23167313	3.37029406	4.79224986	3.85357003	6.50128598	3.22521624	4.104155512
3.3	5.70874951	3.10185401	4.26737976	4.98283995	3.6729678	4.00682838	4.20384675	2.57281338	4.191254112
6.67	1.93479233	1.42048733	1.97980826	1.3204862	1.23862265	4.35794232	4.73982398	1.32180734	2.531975935

Table of absorbance readings at 590nm for *Escherichia coli* after adding MTT

[mg ml-1]	Compounds																		
	22	**23**	**24**	**25**	**26**	**27**	**28**	**29**	**30**	**31**	**32**	**33**	**34**	**35**	**36**	**37**	**38**	**39**	**Ctrl**
0.052	1.218	1.385	1.026	1.356	1.687	1.855	1.395	1.221	1.341	1.587	1.393	1.908	1.599	1.382	1.353	1.212	1.658	1.352	0.050
0.104	1.212	1.387	1.122	1.391	1.674	1.636	1.524	1.171	1.355	1.593	1.452	1.899	1.581	1.47	1.301	1.205	1.571	1.388	0.050
0.208	1.221	1.391	1.134	1.375	1.607	1.541	1.485	1.103	1.301	1.559	1.355	1.717	1.565	1.298	1.258	1.17	1.587	1.368	0.050
0.416	1.214	1.425	1.153	1.343	1.605	1.555	1.737	1.125	1.305	1.453	1.358	1.702	1.568	1.286	1.213	1.146	1.613	1.427	0.050
0.833	1.246	1.401	1.306	1.338	1.598	1.312	1.746	1.135	1.289	1.579	1.331	1.714	1.606	1.305	1.084	1.222	1.568	1.401	0.050
1.6	1.378	1.106	1.242	1.412	1.458	0.148	1.822	1.651	1.21	1.479	1.293	1.688	1.72	1.446	1.141	1.304	1.432	1.525	0.050
3.3	1.415	1.078	1.203	1.433	1.063	0.130	1.757	1.721	1.135	1.025	1.387	1.436	1.732	1.336	0.837	1.634	1.331	1.857	0.050
6.67	0.711	0.954	0.725	0.929	1.311	0.154	0.894	1.765	0.261	0.774	0.613	1.556	0.388	0.614	1.588	1.657	0.354	1.392	0.050

[mg ml-1] = Concentration of compound in mg ml-1

Ctrl = Control is the reference drug streptomycin

Table of absorbance readings at 590 nm in natural log for *Escherichia coli* after adding MTT

	22	23	24	25	26	27	28	29	30
0.052	3.38042013	3.9948259	2.78988395	3.88063966	5.40324663	6.39169825	4.03497457	3.39057662	3.822864459
0.104	3.36019833	4.00282355	3.07099005	4.01886691	5.33345902	5.13459002	4.59055072	3.22521624	3.876760957
0.208	3.39057662	4.01886691	3.10806392	3.95507672	4.98782528	4.66925719	4.41496541	3.01319205	3.6729678
0.416	3.36692545	4.15785784	3.16768171	3.83051784	4.9778596	4.73508653	5.680277	3.08021685	3.687689094
0.833	3.47640947	4.0592572	3.69137863	3.81141305	4.94313626	3.71359348	5.73163024	3.11117354	3.629155585
1.6	3.96695977	3.0222452	3.46253161	4.10415551	4.29735622	1.1595129	6.18421452	5.21218941	3.353484653
3.3	4.11648647	2.93879608	3.33009223	4.19125411	2.8950431	1.13882838	5.79502621	5.59011579	3.111173543
6.67	2.03602627	2.59607321	2.0647311	2.53197594	3.70988174	1.16649089	2.44488968	5.84157236	1.298227665

	31	32	33	34	35	36	37	38	39
0.052	4.88905973	4.02691269	6.73959612	4.94808187	3.98285939	3.86901518	3.36019833	5.24880273	3.865148102
0.104	4.91848226	4.27164928	6.67921189	4.8598132	4.34923514	3.6729678	3.33675908	4.81145725	4.006828377
0.208	4.7540648	3.87676096	5.56779998	4.78267494	3.66196541	3.51837769	3.22199264	4.88905973	3.92748786
0.416	4.27592306	3.8884087	5.48490624	4.7970445	3.61828443	3.36356021	3.14558537	5.0178422	4.16618188
0.833	4.85010328	3.78482632	5.55112161	4.98283995	3.68768909	2.95648186	3.39396889	4.7970445	4.059257195
1.6	4.38855493	3.64370128	5.40865258	5.58452846	4.24609612	3.1298967	3.68400325	4.18706495	4.595143569
3.3	2.78709546	4.00282355	4.20384675	5.65194651	3.80379784	2.30942829	5.1243311	3.78482632	6.404494439
6.67	2.16842262	1.84596098	4.73982398	1.47402978	1.84780787	4.89395123	5.24355656	1.42475519	4.022887788

Table of absorbance readings at 590nm for *Staphylococcus aureus* after adding MTT

[mgml^{-}]	Compounds																		
	22	**23**	**24**	**25**	**26**	**27**	**28**	**29**	**30**	**31**	**32**	**33**	**34**	**35**	**36**	**37**	**38**	**39**	**Ctrl**
0.052	1.266	1.303	1.292	1.272	1.385	1.602	1.32	1.397	1.221	1.182	1.224	1.79	1.543	1.367	1.418	1.957	1.477	1.547	0.050
0.104	1.262	1.281	1.269	1.258	1.387	1.549	1.377	1.417	1.209	1.176	1.225	1.951	1.431	1.445	1.441	1.977	1.568	1.681	0.050
0.208	1.24	1.288	1.277	1.255	1.391	1.535	1.314	1.469	1.175	1.142	1.171	1.895	1.346	1.477	1.485	1.807	1.530	1.557	0.050
0.416	1.269	1.245	1.246	1.261	1.425	1.385	1.322	1.522	1.275	1.14	1.219	1.867	1.164	1.452	1.506	1.884	1.584	1.594	0.050
0.833	1.121	1.296	1.198	1.315	1.401	1.144	1.467	1.609	1.432	1.102	1.217	1.728	1.242	1.604	1.429	1.795	1.509	1.693	0.050
1.6	1.131	1.234	1.153	1.316	1.400	0.246	1.546	1.613	1.561	1.16	1.168	1.121	1.203	1.636	1.234	1.654	1.588	1.459	0.050
3.3	0.973	0.971	0.973	1.133	1.078	0.164	1.467	1.542	1.477	0.905	1.112	0.453	0.725	1.228	1.178	1.063	0.466	1.354	0.050
6.67	0.463	0.314	0.386	0.374	0.954	0.100	1.349	1.304	1.464	0.303	0.363	0.448	0.725	0.278	1.169	0.954	0.089	1.434	0.050

[mg ml-1] = Concentration of compound in mg ml-1

Ctrl = Control is the reference drug streptomycin

Table of absorbance readings at 590 nm in natural log for *Staphylococcus aureus* after adding MTT

	22	23	24	25	26	27	28	29	30
0.052	3.5466376	3.68032109	3.6400594	3.56798139	3.9948259	4.9629484	3.74342138	4.0430526	3.390576616
0.104	3.53247939	3.60023816	3.55729349	3.51837769	4.00282355	4.70676107	3.96299479	4.12472768	3.350132844
0.208	3.45561346	3.62552824	3.58586598	3.50783837	4.01886691	4.64132553	3.7210281	4.34488808	3.238142944
0.416	3.55729349	3.4729348	3.47640947	3.52894867	4.15785784	3.9948259	3.75091571	4.5813788	3.57870141
0.833	3.06792059	3.6546488	3.31348332	3.72475099	4.0592572	3.13930049	4.33620699	4.99781092	4.187064953
1.6	3.0987537	3.43494186	3.16768171	3.7284776	4.05519997	1.27889957	4.69266194	5.0178422	4.763582447
3.3	2.64587018	2.64058372	2.64587018	3.10495741	2.93879608	1.17821432	4.33620699	4.67392879	4.379786592
6.67	1.58883334	1.36888974	1.47108467	1.45353715	2.59607321	1.10517092	3.85357003	3.68400325	4.32321786

	31	32	33	34	35	36	37	38	39
0.052	3.26088946	3.40076362	5.98945247	4.67860505	3.92356233	4.12885447	7.078061	4.37978659	4.697356952
0.104	3.24138271	3.40416608	7.03571978	4.18287998	4.24185214	4.22491862	7.22104732	4.7970445	5.370924211
0.208	3.13302816	3.22521624	6.6525484	3.84202665	4.37978659	4.41496541	6.09214356	4.61817682	4.744566175
0.416	3.12676837	3.38380224	6.46886068	3.20271856	4.27164928	4.50866004	6.57977138	4.87441453	4.923403206
0.833	3.01018037	3.3770414	5.62938387	3.46253161	4.97288423	4.17452258	6.01947472	4.52220633	5.435763559
1.6	3.18993328	3.21555509	3.06792059	3.33009223	5.13459002	3.43494186	5.22784946	4.89395123	4.301655721
3.3	2.47193192	3.04043318	1.57302419	2.0647311	3.41439392	3.24787196	2.8950431	1.593607	3.872886133
6.67	1.35391446	1.43763586	1.5651787	2.0647311	1.3204862	3.21877226	2.59607321	1.09308066	4.195447463

Table of absorbance readings in 590 nm for *Proteus hauseri* after adding MTT

[mg ml-1]	Compounds																		
	22	**23**	**24**	**25**	**26**	**27**	**28**	**29**	**30**	**31**	**32**	**33**	**34**	**35**	**36**	**37**	**38**	**39**	**Ctrl**
6.67	0.463	0.314	0.386	0.374	0.954	0.455	1.851	1.597	1.464	1.514	1.15	1.207	1.126	1.629	1.611	1.717	1.658	1.357	0.050
3.3	0.973	0.971	0.973	1.133	1.078	0.163	1.741	1.542	1.477	1.715	1.215	1.209	1.132	1.602	1.660	1.750	1.599	1.391	0.050
1.6	0.172	1.234	1.153	1.316	1.400	0.198	1.832	1.782	1.561	1.712	1.206	1.174	1.116	1.583	1.625	1.717	1.615	1.388	0.050
0.833	0.121	1.296	1.198	1.315	1.401	0.193	1.67	1.609	1.432	1.644	1.223	1.214	1.155	1.562	1.562	1.772	1.555	1.329	0.050
0.416	0.269	1.245	1.246	1.261	1.425	0.189	1.522	1.522	1.275	1.544	1.183	1.219	1.194	1.503	1.438	1.714	1.216	1.338	0.050
0.208	1.240	1.288	1.277	1.255	1.391	0.437	1.413	1.469	1.175	1.519	1.149	1.173	1.215	1.454	1.399	1.648	1.171	1.412	0.050
0.104	1.262	1.281	1.269	1.258	1.387	1.259	1.377	1.417	1.209	1.345	1.132	1.451	0.987	1.131	1.388	1.436	0.945	1.433	0.050
0.052	1.266	1.303	1.292	1.272	1.385	1.287	1.320	1.397	1.221	0.894	0.351	0.683	0.789	1.114	1.201	1.333	0.879	0.929	0.050

[mg ml-1] = Concentration of compound in mg ml-1

Ctrl = Control is the reference drug streptomycin

Table of absorbance readings at 590 nm in natural log for *Proteus hauseri* after adding MTT

	22	23	24	25	26	27	28	29	30
6.67	1.58883334	1.36888974	1.47108467	1.45353715	2.59607321	1.57617338	6.36618252	4.93819559	4.32321786
3.3	2.64587018	2.64058372	2.64587018	3.10495741	2.93879608	1.17703669	5.70304362	4.67392879	4.379786592
1.6	1.18767783	3.43494186	3.16768171	3.7284776	4.05519997	1.21896239	6.24636691	5.941728	4.763582447
0.833	1.12862491	3.6546488	3.31348332	3.72475099	4.0592572	1.21288279	5.3121678	4.99781092	4.187064953
0.416	1.30865514	3.4729348	3.47640947	3.52894867	4.15785784	1.20804095	4.5813788	4.5813788	3.57870141
0.208	3.45561346	3.62552824	3.58586598	3.50783837	4.01886691	1.54805608	4.10826172	4.34488808	3.238142944
0.104	3.53247939	3.60023816	3.55729349	3.51837769	4.00282355	3.52189783	3.96299479	4.12472768	3.350132844
0.052	3.5466376	3.68032109	3.6400594	3.56798139	3.9948259	3.62190453	3.74342138	4.0430526	3.390576616

	31	32	33	34	35	36	37	38	39
6.67	4.54487398	3.15819291	3.343439274	3.083298606	5.098773395	5.007816541	5.567799984	5.248802734	3.884522237
3.3	5.556675512	3.370294064	3.350132844	3.101854009	4.962948402	5.259310844	5.754602676	4.948081868	4.018866911
1.6	5.540030466	3.340097506	3.234906419	3.052619273	4.869542548	5.078419037	5.567799984	5.027887923	4.006828377
0.833	5.175831487	3.397364554	3.366925455	3.174023418	4.768348412	4.768348412	5.882606818	4.735086525	3.777264234
0.416	4.683285999	3.264151985	3.383802239	3.300255864	4.495154325	4.212262861	5.551121614	3.373666044	3.811413051
0.208	4.567655255	3.155036295	3.23167313	3.370294064	4.280201124	4.051146794	5.196576275	3.225216243	4.104155512
0.104	3.838186541	3.101854009	4.267379762	2.683172867	3.098753705	4.006828377	4.203846754	2.572813379	4.191254112
0.052	2.444889677	1.420487326	1.979808257	2.201194131	3.046520135	3.3234387	3.792403549	2.408490012	2.531975935