Isolation and Characterisation of antibacterial agents produced by soil bacterium V₃

Submitted in fulfilment of the requirements for the degree of

MASTER OF SCIENCE (Chemistry)

By

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August 2006

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DECLARATION

I hereby certify that this research is a result of my own investigation which has not already been accepted in substance for any degree and is not being submitted in candidature for any other degree.

Signed

Lindiwe Lucia Khumalo

I hereby certify that this statement is correct

Signed

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August 2006
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Acknowledgements

I would like to thank God Almighty for the strength to overcome all the difficult times that I have been through.

I would also like to thank my supervisor Dr. A. M. Soares for the support, advices and encouragement throughout this project.

My sincere gratitude goes to Dr. M.R Low for the parenthood figure he showed in all the difficult times I had. Thanks also to all my lecturers at Chemistry department for building me up to this level. A special word of thanks is extended to Professor Fanie van Heerden for all the help she has provided as well as contributing to my success.

I would also like to thank Professor O. Q Munro for solving the crystal structure and helping with the crystallographic data. I am grateful to Dr. Mervin Beukes at Genetics department for the assistance with the electrophoresis, and would also like to thank Tommy van der Merwe at University of Witwatersrand for the help with the mass spectroscopy results. Thanks to the National Research Foundation (NRF) for financial support.

I also gratefully acknowledge all the Warren "warriors" including all the postgraduate students, Prof Drewes and technical staff members Raj Somaru and Faizel Shaik for being there for me and providing help in all the times. Thanks to everyone at Chemistry department for making this department a place to be. I am also indebted to Craig Grimmer for all the assistance that he has provided as well as proofreading my work. For the microbiological part of work, I would like to thank all the technical staff at Microbiology department and Vincent Okudoh for proving the soil bacterium.
Finally I would like to thank my family for the love and support that they have shown all the way through. Thanks to my friends for making research enjoyable and for their companionship throughout the studies.
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Meaning</th>
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<tbody>
<tr>
<td>b.p</td>
<td>boiling point</td>
</tr>
<tr>
<td>COSY</td>
<td>correlation spectroscopy</td>
</tr>
<tr>
<td>d</td>
<td>doublet</td>
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<td>dd</td>
<td>doublet of doublets</td>
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<td>ddd</td>
<td>doublet of doublet of doublet</td>
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<tr>
<td>DEPT</td>
<td>distortionless enhancement by polarisation transfer</td>
</tr>
<tr>
<td>FKBP</td>
<td>FK 506-binding protein</td>
</tr>
<tr>
<td>GC</td>
<td>gas chromatography</td>
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<tr>
<td>GC-MS</td>
<td>gas chromatography-mass spectroscopy</td>
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<td>Hz</td>
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<tr>
<td>HMQC</td>
<td>heteronuclear multiple quantum correlation</td>
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<td>HSQC</td>
<td>heteronuclear single quantum correlation</td>
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<tr>
<td>IR</td>
<td>infrared</td>
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<td>m</td>
<td>meta</td>
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<tr>
<td>o</td>
<td>ortho</td>
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<td>m.p</td>
<td>melting point</td>
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<tr>
<td>MS</td>
<td>mass spectrometry</td>
</tr>
<tr>
<td>mTOR</td>
<td>mammalian target of rapamycin</td>
</tr>
<tr>
<td>NP</td>
<td>natural products</td>
</tr>
<tr>
<td>p</td>
<td>para</td>
</tr>
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<td>ppm</td>
<td>parts per million</td>
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<td>singlet</td>
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<td>sp</td>
<td>species</td>
</tr>
<tr>
<td>TLC</td>
<td>thin layer chromatography</td>
</tr>
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<td>UV</td>
<td>Ultraviolet</td>
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ABSTRACT

Actinomycetes are bacteria belonging to the order of Actinomyceteles and are characterised by the formation of branching filaments giving them a fungal appearance. These bacteria are widespread in nature and can be separated into two subgroups: the oxidative forms mostly in soil habitants, and fermentative forms, living in natural cavities of man and animals.

The aim of this project was to isolate and characterise the antibacterial agents produced by a soil bacterium (V₃). This bacterium was isolated from the KwaZulu-Natal midlands soil by Vincent Okudoh. Although not fully characterised, V₃ bacterium belongs to the oxidative subgroup of Actinomycetes. The bacterium V₃ was inoculated into the nutrient broth, and this broth was shaken for 48 hours in a 30°C water bath. The growth production curve was performed so as to determine at what stage was the antibacterial agent produced using this inoculated broth. After determining the stage at which the antibacterial agent was produced, the seed broth was prepared for two days and was used as the starting material for the fermentation of broth for 14 days. Different extraction methods including normal solvent/ solvent extraction, basic, acidic and neutral extraction methods were done to get a faster and easier method for extracting the antibacterial compound. Moreover, it was also important for the method to improve the yield as well as lessening the steps involved in the isolation of antibacterial agent.

After doing these extraction procedures, it was found that the isolated compound was neither a base nor an acid, as it was not able to extract it by basic and acidic methods. It was then assumed that this antibacterial agent behaves like an amino acid. The isoelectric focusing was performed to get the pH at which the antibacterial compound precipitated out of solution. The precipitate was filtered and dried in an oven overnight, and its gas chromatography was recorded. Different instrumental techniques including NMR, IR and GC-MS were carried out to determine the structure of the antibacterial agent. The isolated antibacterial agent was found to be anthranilic acid, and the structure was confirmed by
x-ray crystallography. Gas chromatography was used to compare the isolated antibacterial compound with commercial anthranilic acid and it was found that the two compounds have the same retention time.

Further studies were done to determine if the isolated antibacterial compound was the final product or the intermediate to the formation of the final product that actually inhibits the growth of microorganisms. Different screening tests were carried out to check if the isolated antibacterial agent was able to inhibit the growth of certain microorganisms including *Serratia marsescens*, *Pseudomonas fluoscens*, *Staphylococcus aureus*, *Escherichia coli*, *Streptococcus faecalis* and *Candida utilis*. It was found that anthranilic acid was able to inhibit the growth of all these microorganisms but was most active against *Serratia marsescens* and *Pseudomonas fluoscens*. 
Chapter 1

Natural Products and Drug Discovery

1. Introduction

"Natural products" originate from a wide variety of living organisms and serve different purposes. In addition to those that have essential roles in human life, such as vitamins and nutrients, some are noxious and act as defense mechanisms against predators, while others paralyse prey. Yet others may have no obvious purpose but are metabolic end products that may possess properties from useful to harmful. (For example genistein, widely distributed in plants, which in animals can disrupt endocrine function).

Throughout the ages, humans have depended on nature for their survival, and as a result natural products traditionally have played an important role in drug discovery and were the basis of most early medicines. In this discussion, natural products will be defined and then classified according to chemical classes that are commercially available. They will also be classified according to their medicinal use; finally, with particular attention to antibiotics.

1.1 Definition

According to Strohl, natural products are secondary metabolites produced by organisms in response to external stimuli such as nutritional changes, infection and competition. Examples of widely used natural products include lovastatin (anticholesterol agent) (1), cyclosporin A (2), and tacrolimus FK506 (immunosuppressive agents) (3), paclitaxel (4) which was formerly known as taxol and is an important anticancer drug, and doxorubicin (5), trade name valstar, that is widely used in oncology as an antitumor agent. These natural products are shown in (Figure 1) and are used in biochemical experiments as well as health sectors.
Figure 1: Examples of natural products.

1.2 Classifications

1.2.1 Industrial-Production-Based Classification System

In the pharmaceutical industry drugs that have natural product origin can be divided into three different groups: the unmodified natural products, semisynthetic and natural products-derived
synthetic compounds. If a natural product is produced synthetically for medicinal use, it is still defined as a natural product. Natural products-derived compounds are synthetically derived from a natural product template. In the pharmaceutical industry medicinal agents are subjected to some rigorous standards of efficacy, safety and purity as approved by regulatory agencies such as Food Drug Authority (FDA) in America and Medicines Control Council (MCC) in South Africa.

1.2.1.1 Unmodified Natural Products (NP)

The chemical structures of natural products can be diverse and complex. Nature provided the earliest medicinal agents both as complex mixtures from botanical, marine and microbial preparations and as single drug substances long before synthetic organic chemistry developed to the stage where it could be an important route to new drugs. Natural products continue to be important today as a source of new drugs.

Natural products are compounds isolated directly from natural sources and these are not modified after isolation but they are used as they are obtained from natural sources. Examples include galantamine (6) that can be purchased commercially as Reminyl, an important drug in treating Alzheimer’s disease, as well as mycophenolic acid (7), known as myfortic with immunosuppressive activity, the structures of which are shown in Figure 2. The opium alkaloids are important analgesic drugs, and continue to be manufactured by processing opium exudates and extract. The immunomodulatory cyclosporins originally isolated from the soil fungus *Trichoderma polysporum*, and tacrolimus (FK-506) (3) (Figure 1), a secondary metabolite isolated from *Streptomyces tsukabaensis*, are used to suppress immunological rejection of transplanted organs. These unmodified natural products represent a major breakthrough for organ transplantation.
Natural products have provided the most important successes in the chemotherapy of cancer. Most of the major anticancer drugs are unmodified natural products obtained from plants or microorganisms and include such important drugs as bleomycin, doxorubicin, daunorubicin, vincristine, vinblastine, mitomycin, streptozocin and paclitaxel (Taxol™). Vincristine and vinblastine are complex dimeric indole-indolines obtained from the rose periwinkle (Catharanthus rosea), and are among the most important therapies for the treatment of childhood leukaemia, Hodgkin's disease and metastatic testicular tumours. These unmodified natural products continue to be produced today by mass cultivation and processing of the plant material.

More recently, a complex diterpene, isolated from the bark of the pacific yew tree and named taxol, has been reported to possess significant cytotoxicity to cancer cells. Although it took some fifteen years for the potential of paclitaxel (Figure 1) to be realised, it is now recognized as a breakthrough in the treatment of ovarian and breast cancers and is one of the most exciting new drugs in recent history. Currently, both a semisynthetic derivative with improved water solubility, docetaxel (Taxotere®), and paclitaxel (Taxol®) are approved and used clinically.
1.2.1.2 Synthetic Natural Products

Substances produced by chemical synthesis from basic chemical building blocks are utilized for a variety of purposes and have proliferated over the last half century as synthetic methodology and production technology have developed to highly sophisticated levels. Modern drug research is now predominantly based on substances produced by chemical synthesis, which involve the use of computer-aided drug design, combinatorial libraries and structural optimisation of lead compounds of both natural and synthetic origin to maximize the benefit-risk ratio.

However, the discovery of bioactive natural products, which serve as leads for new drugs, remains an important drug discovery strategy. In the following discussion, two case studies will be conferred, the first one includes the cholesterol lowering agents and the second one involves the antimalarial quinolone drugs. The clinically useful cholesterol-lowering agents known as the "statins" are derived from natural products isolated from a fungus. These drugs inhibit 3-hydroxy-3-methyl glutaryl coenzyme, a reductase (HMG-CoA reductase), and an enzyme critical in the biosynthesis of cholesterol. The first such agent, compactin, was initially reported as an antifungal agent. However, once its mechanism of action was determined, a search for other naturally occurring HMG-CoA reductase inhibitors led to the discovery of lovastatin (1), a metabolite of the fungus Aspergillus terreus that was first introduced to the market in 1989.

Many analogs, both semisynthetic and totally synthetic were later prepared, and from these, several have become important drugs, including simuvastatin (8) (launched 1991), pravastatin (9) (1991) and atorvastatin (10) (1997) (Figure 3). The latter, a chiral, totally synthetic compound, has become the drug of choice in the therapeutic category based on its superior ability to reduce cholesterol at low doses. The development of the statins is a very good example of natural product-based discovery of an important new drug class followed by optimisation of properties yielding improved drugs. It illustrates the interconnection between a natural product and its semisynthetic and total synthetic analogs and the determining role of molecular structure, whether constructed by nature or humans with respect to drug properties.
Figure 3: Structures of synthetic and semisynthetic statin drugs.

With the quinolones the main focus will be on the important antimalarial drugs primaquine, chloroquine and mefloquine, which were all patterned after the alkaloid quinone, the active constituent of the fever tree Cinchona succiruba. When the natural source of quinoline derivatives based on quinine was threatened during the second world war, massive programs to synthesize a multitude of quinoline derivatives based on the quinine prototype ensued, and the aminoquinolines cited above were among some of the successful drugs to emerge from this extensive effort. The effort to design better antimalarial agents led also to the discovery of other important anti-infectives, including a class of synthetic antibacterials that is among the most prescribed in clinical use today, the floroquinolones. Therefore, the quinolone anti-infective agents are purely of synthetic origin and are not modelled after any known natural antibiotic.

The first antimicrobial quinolone was discovered about fifty years ago as an impurity in the chemical manufacture of chloroquine. It demonstrated a small extent activity towards Gram-negative bacteria, not enough to pursue as a therapeutic agent. However, following on this lead, research subsequently resulted in the production of nalidixic acid (11), which was used mainly for urinary tract infections. The sales of these agents were not impressive but this
picture changed with the discovery of norfloxacin (12) and shortly thereafter, ciprofloxacin, ofloxacin and levoflaxin. In fact, ciprofloxacin and levoflaxin are among the top hundred most frequently prescribed drugs worldwide. Pharmaceutical companies are making huge profits from the manufacture of these compounds.

As a result, nalidixic acid (11) was first commercialised and thus is known as the first-generation quinolone. Nalidixic acid was found to be bactericidal in action, and had the advantage of being easily synthesized, and was a convenient orally-active drug. Though nalidixic acid is considered as a good drug, there were limitations to its use, for example, it was found that it had poor pharmacokinetic properties as well as the problem of resistance. The search for other quinolones continued until the first second-generation quinolone norfloxacin (12) was discovered.

Researchers are constantly searching for more accessible and shorter syntheses. Norfloxacin (12) was the breakthrough molecule as its potency and spectrum of activity approximated those of the fermentation-derived antibiotics. Although quinolone antibiotics are of synthetic origin, some were later found in fermented broth.

![Structures of quinolone antibiotics.](image)

The discovery of new quinolone antibiotics is an on-going process and has led to the recent development of the third-generation quinolones. Other fluoroquinolones belong to the group of third-generation quinolones. They are the group of synthetic antimicrobial agents that show
excellent potencies and a broad spectrum of activity against different Gram positive as well as Gram-negative bacteria. These are widely used in the treatment of infectious diseases. The antibacterial activity of fluoroquinolones depends on the bicyclic heteroaromatic pharmacophore as well as the nature of the peripheral substituents and their spatial relationship.7

All fluoroquinolones interfere with DNA transcription, replication and repair and thus leading to bacterial cell death. The mode of action of quinolones is similar to that of aminoglycoside antibiotics in that the bacterial killing is concentration dependent rather than dosage-interval dependent and fluoroquinolones possess post antibiotic action lasting for 1-2 hours.6

As mentioned earlier, quinolones have a synthetic origin, and there are many synthetic methods reported in the literature, for example the Gould-Jacobs reaction, Grohe-Heitzer reaction and the Chu-Mischer synthesis.8 The Gould-Jacobs reaction involves reaction between a substituted aniline and a substituted ethylenemalonate at high temperatures as shown in Scheme 1. The first step is the addition-elimination step; followed by cycloacylation. This is then followed by alkylation step, which is an $S_N2$ mechanism step since the alkyl halide is required. The final step of synthesis involves the ester hydrolysis.

Scheme 1: Basic Gould-Jacobs reaction.
The second basic method is the Grohe-Heitzer reaction shown in Scheme 2. Initially, it involves the acylation of the aromatic ring so that the substituents are present in the acid as the starting material as well as the final product. The first step involves the conversion of a benzoic acid derivative into a benzoylacetic ester. This is followed by the condensation of methylene function with an ortho ester under dehydrating conditions. The enol ether formed is subjected to an addition-elimination reaction with a primary amine, and the resulting product cyclizes by an addition-elimination reaction.

\[
\begin{align*}
\text{COCl} & \quad \text{MgCH(CO}_2\text{Et})_2 \quad ^+ \quad ^X \quad \uparrow \quad \text{H}^+ \\
\text{H}+ \quad \text{CO}_2\text{Et} \quad \text{RNH}^+ \quad \text{CO}_2\text{Et} \quad \text{CI}^O\text{Et} \\
\end{align*}
\]

Scheme 2: Basic Grohe-Heitzer reaction.

The most effective and shortest method of synthesizing quinolones is by the Chu-Mischer synthesis (Scheme 3). This method involves the use of a chiral \(\alpha\)-amino alcohol and the starting material is an optically active alanol that will lead to the formation of the chiral product directly.

\[
\begin{align*}
\text{Grohe-Heitzer cycloaracylation} & \quad \text{(Scheme 3: Chu-Mischer synthesis).}^6
\end{align*}
\]

6
1.2.1.3 Semisynthetic Natural Products (Semisynthetic NP)

Semisynthetic natural products have been modified by genetic engineering or chemical synthesis in order to improve their properties. Examples include the numerous antibiotic semisynthetic penicillins, cephalosporin drugs and vitamin derivatives that have improved stability.

The semisynthetic natural products that will be discussed include erythromycin (13) (Figure 5), an important antibacterial agent commercialized as Ketek®. The second semisynthetic natural product is sirolimus (also known as rapamycin, (17)), an immunosuppressant agent whose trade name is Certican (Figure 6) and finally the third examples of semisynthetic natural product deoxyartemisinin (21) an important antimalarial drug (Figure 7).

![Figure 5: Structures of erythromycin and its structural analogues](image-url)

13 Erythromycin: R = H
14 Clarithromycin: R = CH₃
15 Roxithromycin
16 Azithromycin

**Figure 5:** *Structures of erythromycin and its structural analogues.*
Erythromycin (13) and its second-generation derivatives clarithromycin (14), roxithromycin (15) and azithromycin (16) (Figure 5) are the most widely used macrolide antibiotics. Erythromycin derivatives have gained interest for their potential use in the treatment of gastrointestinal disorders and inflammatory diseases and also as precursors for the synthesis of ketolides used in the treatment of emerging drug-resistant bacterial strains. Clarithromycin has been synthesized by methylation of the C6-OH group of erythromycin, whereas roxithromycin has been produced by the insertion of an etheroxime chain at the C9 position. It has been assumed that the higher inhibitory activity of clarithromycin (14), roxithromycin (15) against Gram-negative bacteria, compared with that of erythromycin, is because of their enhanced ability to penetrate the cell envelope of Gram-negative cells. Azithromycin (16) is also derived from erythromycin (13). However, it differs chemically from erythromycin in that a methyl-substituted nitrogen atom is incorporated into the lactone ring. This change has resulted in an improved acid stability and is two to eight times more active against *H. influenzae*.6

Structural modifications have also been made to sirolimus (17) which is also known as rapamycin, an important immunosuppressant and an anticancer therapeutic agent. It has been used widely due to its binding ability to two different proteins, FKBP and mTOR. After structural modification of rapamycin (17) to F1-rapamycin (18), the binding affinity increased 200-fold.9

Figure 6: Structures of rapamycin alongside its structural analogue.12-14
The incidence of malaria has dramatically increased worldwide, because malarial parasites have developed resistance to the commonly-used drugs. This has motivated the discovery of a sesquiterpene endoperoxide, isolated from a Chinese medicinal herb. Following the discovery of artemisinin, different derivatives have been synthesized. The semisynthetic analogues shown in Figure 7 are preferred to the original drug itself because they have higher efficacy. This high activity against resistant Plasmodium falciparum strain is due to their unusual structures. Researchers have agreed that the presence of the endoperoxide group in artemisinin compounds is responsible for its high activity, though the real mechanism of antimalarial activity for these compounds is still not known.

Figure 7: Structures of artemisinin and its structural analogues.

Cheng and co-workers\textsuperscript{11} reported that the poor solubility of artemisinin restricts its employment. As a result, structural modification especially at carbon 12 in artemisinin (19), lead to improved solubility. A series of ether and ester derivatives have been semisynthesized.
However, Chang-Hun and co-workers determined that the deoxyartemisinin (21) and carboxypropyldeoxyartemisinin (22) are more effective against the malarial parasite and this is due to their lipophilicity. Due to this lipophilicity, they also target specific proteins in cancer cells.

It has been reported that the highly reactive endoperoxide in artemisinin is changed to free radicals by the iron in the free heme molecules concentrated in the food vacuoles of malarial parasites and the resulting free radicals lead to cellular destruction.

1.2.1.4: Natural Product-Derived Drugs

However useful they may be, natural products may have several drawbacks. For instance, they may have an impractically short half-life, cause allergic side effects and may be unstable. All these limitations could potentially be overcome either by molecular modifications during the biosynthesis of these drugs, by a chemical synthetic process, or by employing an enzymatic process.

Sheehan and co-workers in 1953 accomplished the total synthesis of penicillins and some other approaches have also been successful. These syntheses are of limited practical value, because of the presence of the lactam ring, which is complex to synthesise, nevertheless, they allow modification of the ring system. On the other hand, the discovery of the parent amine 6-aminopenicillanic acid (6-APA) in fermentation products constituted a major breakthrough in penicillin synthesis. It is formed by acylases that cleave off the side chain of the penicillins, a process that can also be obtained by the selective chemical cleavage of the amide, leaving the lactam intact. After this, 6-APA can be easily acylated by any carboxylic acid and this has yielded thousands of natural product-derived penicillins with improved stability and activity. Table 1 shows some of these antibiotics that are available commercially. Methicillin (24), phenethicilin (26) and oxacillin (27) are lactamase resistant whereas others such as ampicillin (28) are not only orally active but have a broad spectrum of activity. Carbenicillin (25) is particularly active against some microbes such as Pseudomonas and Proteus that are not affected by penicillin.
Table 1: Some commercially available natural product-derived penicillins

<table>
<thead>
<tr>
<th>Name</th>
<th>R-group</th>
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<tbody>
<tr>
<td>24 Methicillin</td>
<td><img src="image" alt="Structure" /></td>
</tr>
<tr>
<td>25 Carbenicillin</td>
<td><img src="image" alt="Structure" /></td>
</tr>
<tr>
<td>26 Phenethicillin</td>
<td><img src="image" alt="Structure" /></td>
</tr>
<tr>
<td>27 Oxacillin</td>
<td><img src="image" alt="Structure" /></td>
</tr>
<tr>
<td>28 Ampicillin</td>
<td><img src="image" alt="Structure" /></td>
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</table>
Other examples of natural product-derived compounds (Figure 8) include elsamicin A (29), commercially known as elsamitrucin, and trichostatin A (30) known commercially as suberoylanilide hydroxamic acid. Elsamicin A (29) is an antitumor antibiotic with a fascinating chemical structure and is a good candidate for pharmaceutical development. The amino substituted glycosyl group of elsamicin A takes part in DNA binding and hence antitumor activity. It inhibits RNA synthesis and causes single-strand scission of DNA via the formation of free radicals. Elsamicin A is also regarded as the most potent inhibitor of topoisomerase II reported so far, whereas trichostatin A is an antifungal antibiotic produced by *Streptomyces platensis*.

### 1.2.2 Biological Activity Based Classification

Natural products can also be classified according to their biological activity. In the following section, antifungals, antivirals, and finally, antibiotics (antibacterials) will be discussed.
1.2.2.1 Antifungal

The incidence of severe, invasive and opportunistic fungal infections in immunocompromised patients is increasing at an alarming rate. The most common fungal pathogens are *Candida* and *Aspergillus* sp.

Antifungal drugs for serious infections are either fungistatic (fluconazole, traconazole, that is, azoles) and vulnerable to resistance or fungicidal (amphotericin B – polyene macrolide) but toxic to the host. A newer option, the enchinocandins are fungicidal and less toxic to the host by virtue of their novel mode of action. Targets for action of antifungal agents are given in Table 2. Cell-wall acting agents are a new class of antifungals with a novel mode of action and are inherently selective and fungicidal in nature. Three classes of such compounds, target respectively, β-1,3 glucan synthase (echinocandins – a derivative of pneumocandin B₆), chitin synthase (nikkomycins) and mannoproteins (pradimicins and benanomicins), were explored for clinical development.
<table>
<thead>
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<th>Target</th>
<th>Specific Target</th>
<th>Antifungal Group</th>
<th>Antifungal agents</th>
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<td>Mannoprotein</td>
<td>Pradmicin</td>
<td>BMS-181184</td>
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<td>β-glucan synthase</td>
<td>Echinocandin</td>
<td>Caspofungin</td>
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Most of the antifungal drugs that are used to date are natural products, but there are also semi-synthetically derived antifungal agents. The natural occurring antifungal agents are the polyenes and griseofulvin, and the semisynthetic ones include the echinocandins. The polyenes are naturally occurring polyketides isolated from various *Streptomyces* species and have broad antifungal activity. The mechanism of action of polyenes involve complexation with ergosterol and thus destabilization of the fungal cell membrane and this results in fungal death. The structures of two polyenes are shown in Figure 9, parmicin A (31) and amphotericin B (32). Amphoteric B is commonly used to treat fungal infections, but has serious side effects, which can be reduced by liposomal preparations.
The echinocandins are a group of naturally occurring lipopeptides produced by various fungal species and display antifungal activity by inhibition of 1,3-β-D-glucan synthesis in the fungal cell wall. Examples of echinocandins are: anidulafungin (33) and aminocandin (structure not available), which are semisynthetic derivatives of echinocandin B (34) and deoxymulundocandin (35), respectively.
1.2.2.2 Antivirals

Viral diseases such as human immunodeficiency virus (HIV), influenza, and yellow fever are known to cause a great health risk in humans, thus a need for the discovery of antiviral drugs has grown considerably.

*Figure 10: Examples of echinochandins.*
Viruses are difficult to target, mainly because they use the metabolic enzymes of their host. One of the most important medical tools for dealing with them is vaccination, that is, using the immune system of the host. However, not all viral infections can be cured in this manner. Some antiviral drugs have been developed to act on the host-cell recognition and insertion phases of viral attack, which involve protein-protein and protein-carbohydrates interactions. Most of today’s successful antiviral agents target nucleic acid metabolism. Most nucleic acid synthesis inhibiting drugs are nucleoside analogues with an altered sugar base or both as shown in Figure 11. Acyclovir (Zovirax) (36) is the best example of a DNA polymerase inhibitor. When it gets incorporated into DNA, it acts as a chain terminator. Azidothymidine (AZT®, Retrovir®, Zidovudine®) (38) is also a chain terminator used against human immunodeficiency virus (HIV).

<table>
<thead>
<tr>
<th>Natural Template</th>
<th>Synthetic Analogue</th>
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<tbody>
<tr>
<td>Guanine nucleotide</td>
<td>Acyclovir</td>
</tr>
<tr>
<td>Thymidine nucleotide</td>
<td>Azidothymidine</td>
</tr>
</tbody>
</table>

**Figure 11**: Examples of Nucleotides used in Natural Products Templates.
Different compounds have been isolated, and synthetic work has been done so as to get drugs that will treat viral diseases, especially HIV. Examples of these compounds shown in Figure 12 include the PA-457 (39), a semi-synthetic derivative of the plant triterpenoid betulinic acid (40). Betulinic acid (40) was found to be a weak inhibitor of HIV replication. Furthermore, calanolide A, which was isolated in a small quantity from the plant source Calophyllum lanigerum, was found to have activity against HIV and also against Mycobacterium tuberculosis, thus patients suffering from tuberculosis as well as HIV can be treated with calanolide A (41).

![Structures of some antiviral drugs](image)

**Figure 12**: Structures of some antiviral drugs
Chapter 2

2. Antibiotics (antibacterials)

2.1. Antibiotics Discovery

The study of antibiotics started in 1929 when Alexander Fleming discovered penicillin from the fungus *Penicillium notatum,* followed by its use as a therapeutic agent around 1940. The discovery of penicillin led to the exploration of nature as a source of biological active agents. As a result, microorganisms were then considered as an important source of structurally different bioactive metabolites and have provided a convenient way for obtaining pharmaceutically useful compounds. Before the introduction of antibiotics in the 1940s and 1950s, patients infected with bacteria in the blood stream had no chance of survival; as a result antibiotics were hailed as miracle drugs because they rapidly cured infections that would otherwise have proven fatal.

2.1.1 Definition

Antibiotics (antibacterials) were traditionally defined as chemical compounds produced by microorganisms of which a small amount has the ability to kill or inhibit the growth of other microorganisms. Recently, antibiotics have been defined as substances synthesised from certain bacteria, fungi and other organisms, that can destroy or inhibit the growth of other microorganisms.

Hammond *et al.* defined antibiotics as substances produced by microorganisms antagonistic to the growth or life of others at high dilution (but excluding organic acids, peroxides and alcohols produced by many microorganisms). According to Hutter *et al.* antibiotics are the products of secondary metabolism with an incidental action in minimal concentration on growth processes. There are five major lines of attack by which the antibiotic can attack the
cell, namely, by inhibiting the cell wall synthesis, inhibiting cell membrane synthesis or permeability, inhibiting nucleic acid replication, inhibiting protein synthesis and interfering with cell metabolism. The need to continue discovering new antibiotics is crucial as microbes mutate very fast and soon become resistant. Natural products have played a crucial role in the development of antibacterial agents. These include the β-lactams (which were introduced in 1941), aminoglycosides (1944), cephalosporins (1945), chloramphenicol (1949), tetracyclines (1950), macrolides (1952), lincosamides (1952), streptogramins (1952), glycopeptides (1956), rifamycins (1957) and lipopeptides (2003). There are a few antibacterials which are synthetically derived, these include sulfonamide (1935), nitroimidazole (1959), quinolone (1962), trimethoprim (1968) and oxazolidinone (2000). Microorganisms have been an important source in discovery of the above-mentioned antibacterial agents. This project has focused on novel antibacterials from soil microorganisms.

2.2 Classification of Antibiotics

There are four classifications of systems of antibiotics:

- The classification according to the modes of action of compounds, that is, whether they are cell-wall or cell-membrane inhibitors.
- They may also be classified based on the antibiotic producing organism, for example, production of penicillin or cephalosporin by the molds *Penicillium notatum* and *Cephalosporium*, respectively.
- Another classification is based on the chemical structure as well as biosynthetic pathway by which antibiotics are formed.
- Lastly, Kahne and co-workers have classified antibiotics into two ways depending on the manner in which they target pathogens. The first way is by the nature of the targets in susceptible bacteria, for example blocking of bacterial cell wall biosynthesis, or protein synthesis. The second way is whether the antibiotic is derived from a natural product (synthetic antibacterial drug).

In our work, however, the classification according to the mode of action as well as classification according to the chemical structure will be followed.
Most antibacterial agents act by inhibiting the synthesis of RNA, DNA or peptidoglycan. The peptidoglycan is a mesh-like carbohydrate polymer that provides mechanical support necessary to prevent the cells from lysing when the osmotic pressure fluctuates. The antibiotics that inhibit the peptidoglycan are derived from natural products. These antibiotics are divided into three classes (Figure 13) depending on their mechanism of action. The first class includes the β-lactams, carbapenems, and cephalosporins. These antibiotics inhibit the enzyme’s active sites that catalyze transpeptidation and thereby preventing glycan cross-linking, for example oxacillin (42). The second class includes moenomycin (44), an important additive to commercially available animal feed; it inhibits the glycan polymerization by binding to the bacterial transglycosylases. The third class includes the ramoplanin, members of lantibiotic family of antibiotics and glycopeptides, for example, vancomycin (43) that binds to substrate and thus inhibits the glycan polymerization of transglycosylases and transpeptidases.
2.2.1 β-Lactam antibiotics

β-Lactam antibiotics are a group of antibiotics having a common characteristic lactam ring. There are a number of antibiotics belonging to this group, including penicillin (sulfur-containing penams), cephalosporin (sulfur-containing cephems), natural and synthetic monocyclic β-lactams, which include carbapenems, oxapenams, carbacephems, and oxacephems (Figure 14).
β-Lactam antibiotics are one of the three largest groups of antibiotics that play an important role in the preservation of human health. The other two groups are the macrolides and the fluoroquinolones, which will be dealt with elsewhere. β-Lactam antibiotics and their derivatives act by inhibiting the bacterial cell wall by binding to the penicillin binding protein which is a protein responsible for the cross linking step in cell wall biosynthesis. Bronson and co-workers reported that the carbapenems are divided into two major categories; these are novel parenteral carbapenems and novel orally-active pro-drugs of carbapenems. The parenteral carbapenems play an important role in improving the pharmacokinetic properties relative to imipenem and meropenem, whereas the orally-active pro-drugs are useful at targeting broader clinical usage since such agents would be used in settings outside the hospital.

β-Lactam antibiotics inhibit the bacterial cell wall and cause the lyses of the cell, and thus result in cell death. The bacterial cell wall has peptidoglycan that is also known as the murein sacculus. The normal biosynthesis of the bacterial cell wall is shown in Scheme 4. The binding of the β-lactam antibiotic leads to the weakening of the cell, and thus death of the
bacterium. **Scheme 5** shows binding of the cephalosporin antibiotic and thus changing the normal structure of the cell.

**Scheme 4:** *The biosynthesis of bacterial cell wall.*
Scheme 5: Incorporation of cephalosporin onto the bacterial cell.

The discovery of penicillin from a mould contaminant led to the discovery of other β-lactam antibiotics produced by soil organisms, especially the soil bacteria, as shown in Figure 15. Hedge and co-workers\textsuperscript{24} reported a secondary metabolite SCH 42282 (49), with antifungal activity that was isolated from the fermentation broth of a soil Actinomycete identified as a Microtetraspora spp. The search for antimicrobial agents from soil of different places continued and led to the discovery of a new macrocyclic lactam antibiotic, BE-14106 (50) isolated from a soil sample collected in Shiroyama-cho, Mie Prefecture, Japan\textsuperscript{25} This antibiotic (50) was found to be produced by Streptomyces spheroids and has been shown to have both cytotoxic activity against the murine leukaemia cell line and antimicrobial activity.\textsuperscript{10} Moreover, this antibiotic was found to be a 19-membered macrocyclic lactam. It has been noted that different lactam antibiotics have been isolated from different soil environments, as a result Igarashi and co-workers\textsuperscript{26} reported a novel 19-membered macrolactam antibiotic,
cremimycin (51), the antibiotic having been isolated from *Streptomyces species*, which belong to the members of *Actinomyces*. Cremimycin was found to have broad antimicrobial activity against Gram-positive bacteria. Another known antibiotic in this group is a macrocyclic lactam, possessing a β-phenylalanine substituent, which was isolated from cultured broth of an *Actinomyces* strain, and was found to be an antiprotozoal. Biosynthetically, it originates from malonate, methylmalonate and phenylalanine via the polyketide pathway.

![Diagram of Cremimycin](image)

**Figure 15**: Examples of β-lactam antibiotics isolated from soil bacteria.

### 2.2.2 Glycopeptide and Lipoglycopeptide Antibiotics

The two most important glycopeptide antibiotics used clinically, vancomycin (43) and teicoplanin (52) are shown in **Figure 16**. These antibiotics are important in the treatment of bacterial infections that are resistant to other antibiotics like the β-lactam antibiotics.

![Diagram of Vancomycin and Teicoplanin](image)
Gao\textsuperscript{28} reported that vancomycin was isolated from a soil sample collected by the Eli Lilly Company in the mid-1950's. This antibiotic was first used in 1959 and is produced by \textit{Streptomyces orientalis}. Due to the complexity of the structure, structural elucidation of
vancomycin was not easy to achieve. Structural elucidation started back in 1965 and was completed in 1996 when Sheldrick achieved elucidation of the complete structure. In this discussion we will summarise what is known about the structure and the function of the glycopeptide antibiotics, and consider how natural and semisynthetic lipoglycopeptides are developed into new therapeutic agents. Both vancomycin (43) and teicoplanin (52) are similar as they contain a heptapeptide chain, the amino acid sequence of which is not necessarily the same. However, vancomycin (43) is a glycopeptide that has an acylated glycosyl peptide, whereas teicoplanin (52) does not only have a glycosyl group but also a lipid chain.

2.2.2.1 Mode of Action of Vancomycin and Teicoplanin

Kahne and co-workers reported that the mechanism of action of glycopeptide antibiotics, especially vancomycin, involves binding to the peptidoglycan precursors, specifically to the D-Ala-D-Ala terminus. The binding was shown to occur through a set of backbone contacts between the D-Ala-D-Ala dipeptide and the amides that line a cleft formed by the cross-linked heptapeptide of the glycopeptide. Through binding to the D-Ala-D-Ala, the bound glycopeptide acts as an impediment that prevents lipid II and the nascent glycan chain from being processed further. This leads to the inhibition of the transglycosylation and transpeptidation steps of the peptidoglycan synthesis that ends up weakening the peptidoglycan layers leaving the cell susceptible to lyses because of the changes in osmotic pressure of the cell. The mechanism of action of vancomycin is illustrated in Figure 17.
Figure 17: Binding of vancomycin with N-acyl-D-Ala-D-Ala termini, five hydrogen bonds between the underside of the glycopeptide and the acyl-D,D-dipeptide.\textsuperscript{14,29}

Williams and co-workers found that the lipidated glycopeptides are attached to the bacterial cell membrane, whereas nonlipidated glycopeptides are distributed more broadly in the peptidoglycan layers.\textsuperscript{30,31} Due to the lipophilic nature of teicoplanin it has been rationalised that it inserts itself into the membrane, where peptidoglycan precursors terminating in D-Ala-D-Ala are located. This results in the location of these binding partners in close proximity, which leads to the intramolecular interaction between the two, which is more favoured than the intermolecular interaction. Therefore, Williams and co-workers deduced that it is the restriction of motion of intermediates and the intramolecular bond formation that allow teicoplanin (52) to overcome the vancomycin resistance.
2.2.2.2 Biosynthetic Strategies

The total synthesis of the vancomycin (43) and teicoplanin (52) family has been accomplished with the intervention of new chemistry. However, the complexity of the structures of these natural products makes fermentation a more cost-effective and viable route to the bulk product. It has been reported in the literature that the synthesis of most glycopeptide antibiotics, for example vancomycin (43) is tiresome, due to low yields obtained and the cumbersome number of steps involved. These include the assembly of the amino acid components of glycopeptide antibiotics into the heptapeptide backbone by a mechanism known as the multienzyme thiotemplate mechanism. This mechanism involves recognition and activation of each amino acid by a suitable enzyme module; this is then followed by the covalent linking of the enzyme complex to the amino acid through a thioester-pantetheine cofactor unit. A peptide bond is then formed between the two enzyme-bound units. Successive epimerization and further coupling completes the synthesis.

Structural analogues of these compounds have been achieved by semisynthetic methods. A variety of methods have been employed so as to synthesise these compounds and these include the construction of amino acid building blocks, formation of a linear heptapeptide, oxidative coupling processes, glycosidations and reductive alkylation of the amino group of the vancosamine sugar in vancomycin (43) to yield oritavancin (53). Furthermore, feeding experiments in vancomycin-producing bacteria with [1,2-\(^{13}\)C\(_2\)] acetate showed that the m-dihydroxyphenylglycine (AA-7) of vancomycin is formed through the polyketide pathway of four units of acetate. It should be noted that all glycopeptide antibiotics isolated to date are mainly produced by Actinomycetals. Few of these glycopeptides are from the Streptomyces and the majority are from the Actinomyeetes that was originally classified as Nocardia.

Most bacteria have strategies for self-protection and immunity from the action of chemical weapons, which are antibiotics, thus leading to resistance problems. This also happened to glycopeptide antibiotics, as a result some chemical modifications had to be made to vancomycin to give oritavancin (53), which was more effective than vancomycin. Due to the complex structure of vancomycin, synthetic routes were not used and chemical method was preferred to give (53). This method involves a single step reductive alkylation of the amino
group of the vancosamine sugar in vancomycin to give oritavancin (53) as shown in Scheme 6. Malabarba and co-workers\textsuperscript{36} reported three strategies for chemical modification of glycopeptide antibiotics to overcome problems of resistance; these include modifications in the structure of the binding site, synthesis of covalently linked dimers, as well as the alterations of the disaccharide moiety of vancomycin.\textsuperscript{15}
Scheme 6: Reductive alkylation of the amino group of vancosamine sugar in vancomycin to form oritavancin.
2.2.3 Peptide Antibiotics

The search for new antibiotics is a continuous process, because of resistance problems. Zhang and co-workers\textsuperscript{37} reported a new cyclic peptide antibiotic zelkovamycin (54) (Figure 18), which was isolated from the fermentation broth of \textit{Streptomyces sp.} K96-0670. It has been found that zelkovamycin has antibacterial activity against a number of organisms, including the \textit{Xanthomonas oryzae}, \textit{Acholeplasma laidlawii}, \textit{Pyricularia oryzae} and \textit{Staphylococcus aureus}.\textsuperscript{37}

![Figure 18: Structure of zelkovamycin.](image)

Different natural products researchers have discovered different antibiotics from different environments, this includes Schimana and co-workers who discovered three lipopeptide antibiotics from the mycelium extracts of \textit{Streptomyces sp.}\textsuperscript{38} These lipopeptide antibiotics were found to be colourless arylomycin All (55), arylomycin AIII (56), and a yellow arylomycin AIV (57), (Figure 19). These arylomycins have antibacterial activity against Gram-positive bacteria.\textsuperscript{38}
Kimura and co-workers\textsuperscript{39} reported the three liposidomycins, which were produced by \emph{Streptomyces sp.} SN-1061M. These liposidomycins were found to be important in inhibiting the peptidoglycan synthesis and thus are cell wall inhibitors. These liposidomycins A (II) to (IV) 58-60 are shown in Figure 20.

\textbf{Figure 19:} Examples of arylomycins.

\textbf{Figure 20:} Examples of liposidomycins
2.2.4 Aminoglycoside Antibiotics

Magnet et al.\textsuperscript{30} defined aminoglycosides as hydrophilic molecules consisting of a characteristic central aminocyclitol linked to one or more amino sugars by pseudoglycosidic bond(s). According to Vladimir,\textsuperscript{41} the name aminoglycoside refers to the group of antibiotics containing aminosaccharides joined by a glycosidic bond. Aminoglycosides are the most important group of carbohydrate antibiotics used clinically. The first antibiotic isolated in this group was streptomycin (61) which was discovered by Waksman et al. in 1943,\textsuperscript{1,42} and was found to be useful in the treatment of tuberculosis. There are other different types of aminoglycoside antibiotics that were discovered after streptomycin (61), these include kanamycin (62), amikacin (63), gentamicin B (64), tobramycin (65), netilmicin (66).\textsuperscript{17} Streptomycin, which was the first antituberculosis agent to be discovered,\textsuperscript{16} differs from the other aminoglycoside antibiotics in that it has the streptidine ring as the central aminocyclitol, whereas other aminoglycosides have 2-deoxystreptamine as the central aminocyclitol to which glycosidic bonds attach different amino sugars instead of the streptidine ring as shown in Figure 21.

All aminoglycosides are bacteriostatic, and their bacterial killing activity is concentration dependent, that is, the higher their concentration is, the greater the rate at which they kill bacteria.\textsuperscript{43,44} Furthermore, aminoglycosides are polycations and their polarity is in part responsible for the pharmacokinetic properties shared by all members of the group. They all target the small ribosomal subunit by inhibiting the translation process.
2.2.5 Tetracyclines

Tetracycline antibiotics (Figure 22) are all yellow amphoteric substances forming salts with acids or bases or complexes with such metals as aluminium, magnesium, calcium or iron. All tetracyclines are protein synthesis inhibitors. Tetracyclines have found widespread use since their discovery was first reported in 1948. There are six members of tetracycline antibiotics and all these are produced by actinomycetes. These include chlortetracycline (67) (1948), oxytetracycline (68) (1949), tetracycline (69) (1953), and declomycin (70) (1957) (Figure 22). All tetracycline antibiotics contain a hydronaphthacene skeleton as a characteristic structural unit. Tetracyclines are divided into natural and semi-synthetic tetracyclines. The natural tetracyclines include chlortetracycline (67), oxytetracycline (68), and declomycin (70), which are produced by Streptomyces. The semi-synthetic ones are methacycline (1965), doxycycline...
(1967), minocycline (1972) and tigilcycline (1993). Tigilcycline is undergoing phase (III) clinical trials.\textsuperscript{48}

Figure 22: Examples of natural tetracycline antibiotics.

2.2.6 Macrolides Antibiotics

Macrolides antibiotics are a group of closely related compounds having a macrocyclic lactone ring (usually containing 14 or 16 atoms) to which deoxy sugars are attached\textsuperscript{18} as shown in Figure 23. Katz\textsuperscript{49} reported that the first person who originally proposed the definition of macrolides is R.B. Woodward, and he defined the term macrolide as an abbreviation of macrolactone glycoside antibiotics, which is a class of natural products composed of a macrocyclic lactone ring to which one or more deoxysugar residues are attached. Furthermore, Katz and co-workers reported that macrolides are secondary metabolites produced by the actinomycete family of bacteria.\textsuperscript{26} The three known secondary metabolites belonging to the macrolides group were reported by Bertram and co-workers, these include the erythromycin
(13), azithromycin (16) and clarithromycin (14). There are natural as well as semi-synthetically produced macrolides.

The first macrolide antibiotic discovered in 1950 was pikromycin (71). This was then followed by the discovery of erythromycin (13), by Mcquire and co-workers in 1952 from *Streptomyces erythreus*. The other two members of this group are semi-synthetic derivatives of erythromycin. The structures of these antibiotics are shown in **Figure 23**. Macrolide antibiotics were classified based on the chemical viewpoint by Katz and co-workers. These were divided into groups depending on the number of atoms in the macrocyclic ring, and there are 12, 14, 16 and even larger membered macrolide. These are further subdivided depending on the sugar substituent as well as the structure of the lactone ring. From the clinical point of view, macrolides are divided into the first, second and third generation depending on the year they were discovered.

The first generation macrolides are natural products introduced in the 1950s, followed by the semi-synthetic second generation compounds in 1990 and the semi-synthetic third generation molecules in the early 2000s. The present work has concentrated mainly on the macrolides that have been isolated as natural products; some macrolides are the congeners of the parent compound. Congeners are intermediates of the final products found in the late pathway. All macrolides are protein synthesis inhibitors; they bind to ribosome and thus block protein synthesis.

### 2.2.6.1 Fourteen-Membered Macrolides

There are five compound families belonging to this group, these include the erythromycin A (13) and its derivatives pikromycin (71), megalocin A (72), oleandomycin (73), and lankamycin (74) (Figure 23). Each family of compounds has some congeners belonging to it. All 14-membered macrolides have desosamine at C-5, except lankamycin (74), which has the neutral sugar chalcosine at position 5. Pikromycin (71) also differs from other 14-membered macrolides in that the oxygen present at C-3 is in the form of a ketone, thus is known as a ketolide. This ketolide has a higher bacterial potency than erythromycin A.
Figure 23: Examples of 14-membered macrolide antibiotics.
2.2.6.2 Twelve-Membered Macrolides

The two known macrolides that contain twelve-membered rings are methymycin (75) and neomethymycin (76). These two antibiotics both contain a hydroxyl group, but differ in the position of the hydroxyl group. Methymycin (75) has a hydroxyl on C-10 position whereas neomethymycin has a hydroxyl group on C-12 position (Figure 24).

![75 Methymycin](image)

![76 Neomethymycin](image)

*Figure 24: Examples of twelve-membered macrolides.*

2.2.6.3 Sixteen-Membered Macrolides

There are four different groups belonging to the 16-membered macrolide antibiotics, which differ in the structure of the polyketide backbone that forms the macrolactone. These include the tylactone group, platenolide group, mycinamicin, and the chalcomycin-neutramycin groups.

2.2.6.4 Important Uses of Macrolides

Macrolides can be classified into the first, second and third generations depending on their clinical use. The first generation macrolides are those that are isolated as the fermentation products, for example fermented broth, and then used clinically for the treatment of diseases. An example of a first generation macrolide is erythromycin A (13). This antibiotic is very effective but has several disadvantages including a bitter taste and causing stomach cramps. As a result chemical modification was necessary to avoid the use of erythromycin. The
chemical modification of erythromycin is shown in Scheme 7. Other disadvantages of using the first generation macrolides include their short half-life and poor oral bioavailability.

![Scheme 7: Chemical modification of the first-generation macrolide to form the second generation macrolide.](image)

Resistance problems emerged after the development of the second-generation macrolides and as a result some further chemical modification was necessary to form the third generation macrolides. Scheme 8 shows the modification of clarithromycin (14) to form telithromycin (77), which is the third generation macrolide with better activity than clarithromycin (14). There are eight chemical steps involved in the conversion of clarithromycin (14) to telithromycin (77). The first step is an acid hydrolysis of clarithromycin to give the 3-descladinosyl derivative that is then acetylated with acetic anhydride so as to protect the 2'-hydroxyl. Oxidation of the 3-hydroxyl to a ketone is then followed by the introduction of the 11,12-cyclic carbamate. Treatment of the product with methanol results in removal of 2'-acetate group and then the formation of telithromycin (77).
2.2.6.5 Genetic Engineering in the Production of Macrolides.

Early experiments have proved that macrolides are produced from acetate, propionate, and butyrate as starter units.\textsuperscript{31} This involves bioconversion of the compounds with five to nine carbons in the chain into the aglycones of macrolides. The aglycones are complex polyketides that are grouped together by consecutive decarboxylative condensations of carboxyl thioesters including malonyl CoA, methylmalonyl CoA; this is done in the same manner as for fatty acid biosynthesis. Each step of the synthesis is programmed to determine the acyl unit incorporated into the growing chain, and the degree to which the resulting β-carbonyl
generated from the condensation is reduced. This process is achieved by the use of polyketide synthase that catalyzes all the steps involved in aglycone assembly. Different enzymes are used for different steps as they perform a variety of functions.

Scheme 9: Genetic engineering in production of macrolides, all modules include ketosynthase (KS), acyltransferase (AT), acyl carrier protein (ACP), ketoreductase (KR), dehydratase (DH), and thioesterase (TE).
2.2.6.6 Mode of action of macrolides

All macrolides are protein synthesis inhibitors and as a result they inhibit translation processes. The mechanism of action of these protein synthesis inhibitors differs in each and every group. Some macrolides bind to the 50S component of bacterial ribosomes and make specific interactions with the 23S RNA. Some macrolides, like the 16-membered macrolides, block the peptide bond formation by binding to the A site and thus block the binding of aminocyl tRNA; these macrolides are peptidyltransferase blockers. The erythromycin and other 14-membered macrolides were found to have no effect on the peptidyltransferase activity.

2.2.7 Streptogramin Antibiotics

Streptogramin antibiotics are natural product antibiotics produced by different members of *Streptomyces*. These antibiotics are divided into two types, type A and type B, which are produced by the same bacterial species in a ratio of 70:30. Type A streptogramins are cyclic polyunsaturated macrolactones consisting of polyketide structures that are cyclized through an internal ester bond between the carboxyl of the C-terminus amino acid and an internal hydroxyl group as shown in Figure 25. Examples of Group A streptogramins are pristinamycin IIₐ (82), madulamycin II (83) and the semisynthetic derivative dalfopristin (85).

The biosynthetic origins of the components of group A, streptogramin pristinamycin IIₐ (82) have been investigated through traditional precursor fermentation experiments and the results are shown in Figure 26. For instance the C-terminal dehydroproline predictably arises from proline.
Figure 25: Examples of type A streptogramin antibiotics.

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<th>R₃</th>
<th>R₄</th>
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Figure 26: Biosynthetic origins of various components of group A streptogramin antibiotics.
Type B streptogramin antibiotics are cyclic hepta- or hexadepsipeptides, for example, pristinamycin IA (86), virginiamycin S2 (87), vernamycin C (88), patrician A (89) and the semisynthetic quinupristin (90) are shown in Figure 27.

![Figure 27: Structures of type B streptogramins.](image)

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</table>

Figure 27: Structures of type B streptogramins.
Streptogramins are protein synthesis inhibitors. They bind to the P site of 50S ribosome and thus block protein synthesis step. These antibiotics share a common mechanism of action with the macrolides, lincosamides, and thiopseudopeptides, but the streptogramins type A and B are different from these antibiotics in that both type A and type B components are separately bacteriostatic but synergistically they exhibit a bactericidal activity.\textsuperscript{28}

2.2.8 Chloramphenicol

Chloramphenicol (91), (Figure 28), was found to be the second interesting antibiotic produced by \textit{Streptomyces}, and it was discovered after streptomycin.\textsuperscript{26} This antibiotic is important because it was found to be the first antibiotic to be a derivative of dichloroacetic acid and nitrobenzene. Furthermore, it is active against a wide range of organisms including bacteria and viruses. Moreover, chloramphenicol attracted attention of most natural product chemists, because like ascorbic acid among the vitamins, it was the first antibiotic to be obtained in quantity by a synthetic process and as a result all commercial supplies of chloramphenicol are synthetic.\textsuperscript{26} Chloramphenicol is a bacteriostatic, broad spectrum antibiotic which inhibits microbial protein synthesis.

\begin{figure}
\centering
\includegraphics[width=0.5\textwidth]{chloramphenicol.png}
\caption{Structure of chloramphenicol antibiotic.}
\end{figure}

2.2.9 Lantibiotics

Lantibiotics are peptide-derived antimicrobial agents made by ribosomes. The name lantibiotics was introduced in 1988 and it is an abbreviation of lanthionine-containing antibiotic peptides.\textsuperscript{51} All natural lantibiotics have meso-stereochemistry and there are three groups of antibiotics belonging to this family. Examples of lantibiotics are shown in Figure 29
These are the nisin (92) group, subtilin (93) group as well as the epidermin (94) groups respectively. Nisin lantibiotic is produced by *Lactococcus lactis* and is widely used as a food preservative. There are two members of this group, the nisin A and Z, which differ from each other by one amino acid, which is Histidine in nisin A and Aspartic acid in nisin Z. The subtilin lantibiotic is produced by *Bacillus subtilis* and is structurally related to nisin; nisin has two more amino acids than subtilin. The third group of lantibiotics is the epidermin group, which consists of 22 amino acids, and was isolated from the *Streptococcus mutans*. All members of the epidermin group have a characteristic Lan ring between positions 3 and 7. Structures of different lantibiotics are shown in **Figure 29**. It should be noted that about 40 lantibiotics are known and these differ from each other not only in structure but in size and mode of action. Some lantibiotics are prepared by chemical synthesis and chemical modification using processes such as the Merrifield synthesis. Protein engineering is the most viable route for preparing lantibiotics since the Merrifield synthesis takes too long due to the complexity of the structure.

![Figure 29: Examples of lantibiotics.](image-url)
2.2.10 Thiopeptide Antibiotics

Figure 30: Examples of thiopeptide antibiotics.
Thiopeptides are naturally-occurring, sulfur-containing, highly modified, macrocyclic peptides.\textsuperscript{52} These antibiotics are grouped as thiazolyl peptides in the Berdy classification system where they are classified according to the chemical structure.\textsuperscript{53} They all have tri- or tetrasubstituted nitrogen heterocycles clustered in a central polyazole domain that is part of macrocyclic ring containing heterocyclic residues like thiazoles, oxazole indoles and dehydroamino acids.\textsuperscript{28} All thiopeptide antibiotics are secondary metabolites produced by \textit{Actinomycetes} especially the \textit{Streptomyces}, and are biological active compounds. They all share a common property, that is, they are growth inhibitors for Gram-positive bacteria and have no activity against Gram-negative bacteria. They are all protein synthesis inhibitors.

### 2.2.11 Antifolates

Antifolate agents are mostly used clinically as antibacterials, antimalarials and anticancer drugs. They are bacteriostatic in nature and thus inhibit bacterial growth but do not actively kill the bacteria. Antifolates interfere with the biosynthetic pathway of tetrahydrofolic acid by inhibiting dihydropteroate synthetase, which is an enzyme responsible for the biosynthesis of folic acid. They also mimic $p$-aminobenzoic acid (PABA), which is essential for the biosynthesis of bacterial cell membrane. These drugs bind in the enzyme active site instead of PABA and have similar electrostatic properties to PABA. Therefore they act as competitive inhibitor of dihydropteroate synthetase. The structures of both antifolate sulfanilamide drug and PABA are shown in Figure 31.

![Figure 31: Structures of PABA (79) and its competitive inhibitor sulfanilamide (80).](image-url)
The administration of sulfanilamide, which is one of the antifolate drugs, causes bacterial to stop growing and dividing. This allows the host time to recognize and overcome the infection. These drugs are not toxic to humans since humans do not have dihydropteroate synthetase and thus do not biosynthesise tetrahydrofolic acid but obtain it from the diet (green vegetables and liver). All living cells need the tetrahydrofolate cofactor for the synthesis of purines, some amino acids and especially thymidine for growth purposes. The mechanism of action of antifolate drugs is shown in Scheme 10.
Scheme 10: *Mechanism for biosynthesis of folic acid and the action of antifolate, sulfonamide and trimethoprim.*
2.2.12 Aims of the study

Throughout the ages, humans have relied on nature for their basic needs including the production of foodstuffs, shelter, clothing, means of transportation, fertilizers, flavors and fragrances, as well as medicines.\textsuperscript{19} Plants have formed the basis of the highly developed traditional medicines that have been in existence for thousands of years. Plant survival depends on certain climatic conditions such as temperature and moisture. Due to their inconsistent growing patterns they cannot be relied upon for the production of medicines. Furthermore, their over-exploitation by illegal chopping has resulted in the reduction of plant growth. For the above reasons soil microorganisms have been chosen for this study since they can be grown anytime, anywhere, and in large quantities. Additionally, the time to reach maturity is greatly reduced.

The unanticipated discovery of penicillin from the fungus \textit{Penicillium notatum} by Fleming in 1929, and its broad therapeutic use in the 1940’s introduced a new era in medicine that is the discovery of antibiotics from microorganisms.\textsuperscript{56} This discovery also resulted in the intensive investigation of nature as a source of bioactive agents. Microorganisms are a productive source of structurally diverse bioactive metabolites and have yielded some of the most important products of the pharmaceutical industry. These include the antibacterial agents such as penicillins, cephalosporins, aminoglycosides, tetracyclines, polyketides, cholesterol lowering agents, and immunosuppressive agents. Many of these useful drugs are produced by \textit{Actinomycete}, which are isolated from soil.

\textit{Actinomycete} are the biggest producers of bacterial secondary metabolites. The objective of this study is to extract and characterize an antibacterial agent produced by soil bacteria obtained from KwaZulu-Natal midlands soil (Pietermaritzburg). The polyene antifungal antibiotic, \textit{natamycin} (\textit{pimaricin}) (\textit{99}) shown in Figure 32 was produced by \textit{Streptomyces natalensis} was isolated from soil near Pietermaritzburg, South Africa. This antibiotic is widely used as a food preservative to control the growth of yeasts and moulds on the surface of cheese and other non-sterile products such as meat and sausages.\textsuperscript{57}
This study has been done as part of the global search for new secondary metabolites with new antibacterial, antifungal, antitumour, as well as herbicidal or pesticidal properties. The primary metabolites involved in secondary metabolite biosynthesis will be identified and also try to obtain as much information about the regulation of their synthesis as possible as this is necessary to further increase the yield of secondary metabolite production.

The first steps in secondary metabolite biosynthesis usually involve a series of very specific enzymatic steps to synthesise a common building block. This compound is then derivatised via non-specific enzymes such as dehydrogenases, transferases, oxidases or other activities, acting in various combinations or sequences, resulting in an enormous diversity of chemical compounds such as end products. The exchange of genes coding for these non-specific enzymes, via natural combination or recombinant DNA techniques, has indeed lead to the formation of new hybrid secondary metabolites (metabolic pathway engineering).\textsuperscript{58}

Mutagenesis and selection of strains overproducing secondary metabolites is necessary to overcome the initially low rate of metabolite synthesis. In the initial strain improvement
procedure, the flux of the specific secondary metabolite biosynthesis pathways themselves may be increased in the production strains. Later, the availability of precursors from primary metabolism will be of influence. At this stage one may expect that increasing the flow towards these building blocks will also increase the productivity of secondary metabolite formation. Incorporation of structural analogues of the building blocks as alternative starter units would be expected to produce structural analogues of the secondary metabolite.
Chapter 3

3. Results and Discussion

3.1 Introduction

Although not fully characterised, bacterium $V_3$ belongs to the oxidative subgroup of the *Actinomycetes*. These are bacteria belonging to the order *Actinomycetales* and are characterised by the formation of branching filaments giving them a fungal appearance as shown in Figure 33. *Actinomycetes* are widespread in nature and can be separated into two subgroups: the oxidative forms mostly in soil habitats, and the fermentative forms, living in natural cavities of man and animals.

![Branching filaments formed by $V_3$.](image)

*Figure 33: Branching filaments formed by $V_3$.*

*Actinomycetes* are undoubtedly the largest producers of bacterial secondary metabolites as shown in Table 3. Secondary metabolites are, in contrast to primary metabolites, not essential
for growth and are synthesized from intermediates, or end-products of primary metabolism via unique biosynthetic pathways. These pathways may have originated from primary pathways within the same organism but gene transfer between different organisms is also an important factor in the evolution of secondary metabolism.⁶⁰
Table 3: Summary of different classes of secondary metabolites originally isolated from soil

<table>
<thead>
<tr>
<th>Class</th>
<th>Common name</th>
<th>Organism</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aminoglycosides</td>
<td>Streptomycin</td>
<td><em>Streptomyces griseus</em></td>
</tr>
<tr>
<td></td>
<td>Hygromycin</td>
<td><em>S. hygroscopicus</em></td>
</tr>
<tr>
<td></td>
<td>Neomycin</td>
<td><em>S. fradiae</em></td>
</tr>
<tr>
<td></td>
<td>Gentamicin</td>
<td>Micromonospora spp.</td>
</tr>
<tr>
<td></td>
<td>Kanamycin</td>
<td><em>S. kanamyceticus</em></td>
</tr>
<tr>
<td></td>
<td>Apramycin</td>
<td><em>S. tenebrarum</em></td>
</tr>
<tr>
<td></td>
<td>Spectinomycin</td>
<td><em>S. spectabilis</em></td>
</tr>
<tr>
<td>Ansamycins</td>
<td>Rifamycin</td>
<td><em>Amycolatopsis mediterranei</em></td>
</tr>
<tr>
<td></td>
<td>Geldanamycin</td>
<td><em>S. hygroscopicus</em></td>
</tr>
<tr>
<td></td>
<td>Herbinycin</td>
<td><em>S. hygroscopicus var. geldamus var. nova</em></td>
</tr>
<tr>
<td></td>
<td>Streptovaricins</td>
<td><em>S. spectabilis</em></td>
</tr>
<tr>
<td>β-lactams</td>
<td>Penicillins</td>
<td><em>S. spp., Nocardia spp.</em></td>
</tr>
<tr>
<td></td>
<td>Cephalosporins</td>
<td><em>S. spp., Nocardia spp.</em></td>
</tr>
<tr>
<td>Macrolides</td>
<td>Oleandomycin</td>
<td><em>S. antibioticus</em></td>
</tr>
<tr>
<td></td>
<td>Erythromycin</td>
<td>Saccharopolyspora erythraea</td>
</tr>
<tr>
<td></td>
<td>Spiramycin</td>
<td><em>Streptomyces ambofaciens</em></td>
</tr>
<tr>
<td></td>
<td>Tylosin</td>
<td><em>S. fradiae</em></td>
</tr>
<tr>
<td>Nucleosides</td>
<td>Sinefungin</td>
<td><em>S. griseolus</em></td>
</tr>
<tr>
<td></td>
<td>Tunicamycin</td>
<td><em>S. griseolus</em></td>
</tr>
<tr>
<td>Peptides</td>
<td>Vancomycin</td>
<td><em>Amycolatopsis orientalis</em></td>
</tr>
<tr>
<td></td>
<td>Actinomycin</td>
<td><em>S. antibioticus</em></td>
</tr>
<tr>
<td></td>
<td>Thioestrepton</td>
<td><em>A. azurea</em></td>
</tr>
<tr>
<td></td>
<td>Avoparcin</td>
<td><em>A. coloradensis</em></td>
</tr>
<tr>
<td></td>
<td>Orientisin A and B</td>
<td><em>A. orientalis</em></td>
</tr>
<tr>
<td></td>
<td>Muracenins</td>
<td><em>A. orientalis</em></td>
</tr>
<tr>
<td></td>
<td>Macrobicyclic peptide</td>
<td><em>A. fastidiosa</em></td>
</tr>
<tr>
<td></td>
<td>Azureomycin A and B</td>
<td><em>A. azurea</em></td>
</tr>
<tr>
<td>Polynes</td>
<td>Amphotericin B</td>
<td><em>S. nodosus</em></td>
</tr>
<tr>
<td></td>
<td>Nystatin</td>
<td><em>S. nouresii</em></td>
</tr>
<tr>
<td></td>
<td>Candidicidin</td>
<td><em>S. griseus</em></td>
</tr>
<tr>
<td>Polyether antibiotics</td>
<td>Monensin</td>
<td><em>S. cinnamomensis</em></td>
</tr>
<tr>
<td></td>
<td>Salinomycin</td>
<td><em>S. albus</em></td>
</tr>
<tr>
<td></td>
<td>Narasin</td>
<td><em>S. aureofaciens</em></td>
</tr>
<tr>
<td>Tetracyclines</td>
<td>(Chlor-) Tetracycline</td>
<td><em>S. aureofaciens</em></td>
</tr>
<tr>
<td></td>
<td>Oxytetracycline</td>
<td><em>S. rimosus</em></td>
</tr>
<tr>
<td></td>
<td>Cetocycline</td>
<td><em>A. sulphurea</em></td>
</tr>
<tr>
<td>Miscellaneous</td>
<td>Chloramphenicol</td>
<td><em>S. venezuelae</em></td>
</tr>
<tr>
<td></td>
<td>Cycloserine</td>
<td><em>S. sp.</em></td>
</tr>
<tr>
<td></td>
<td>Rapamycin</td>
<td><em>S. hygroscopicus</em></td>
</tr>
<tr>
<td></td>
<td>Lincomycin</td>
<td><em>S. lincolnensis</em></td>
</tr>
</tbody>
</table>
The bacterium (V3) used in this work was isolated by Vincent Okudoh from soil in the KwaZulu-Natal midlands. This bacterium was then inoculated onto the nutrient broth as described in the experimental section. Extraction was done using petroleum ether-ethyl acetate (1:1). Chromatography was used to purify the compounds produced by the bacterium.

### 3.2 Bacterial Growth Production Curve

Bacteria as a group live and grow under a wide range of environmental conditions. Because these microorganisms exert most of their effects through growth, knowledge of the processes involved with microbial growth is essential to the understanding, study and control of fundamental microbial activities. Bacteria can be studied at all stages of growth microscopically, physically, and chemically. The information acquired through such studies can be used to correlate the chemicals formed during growth with the appearance of cellular structures. Secondary metabolites are biosynthesised under sub-optimal conditions near the end of the exponential growth phase or in the stationary phase.

Schlegel\textsuperscript{61} defined growth as the irreversible increase in living substances, which is accompanied by an increase in the size of cells and their division. The term growth as applied to microorganisms usually refers to an increase in the number of microorganisms or mass beyond that present in the original inoculum. A single bacterial cell continually increases in size until it is about double its original dimensions. It then divides and gives rise to two cells approximately the size of the original parent cell. All structural parts of the cell double during this cell growth division cycle.

- The time required for the formation of two new cells from one is called the generation time. During this time, the population of cells doubles. Thus, starting with one bacterium, the increase in population follows a progressive doubling.
- Cell populations can reach levels into the billions, so logarithmic notation that incorporates exponents of 10 is used to handle large numbers.
- Bacteria and other unicellular microorganisms exhibit characteristic growth cycles or patterns, which can be divided into the four distinct growth phases: lag phase;
exponential or logarithmic phase; stationary phase; and death phase. Their general characteristics are as follows:

1. Lag phase: During this phase, cells in a new inoculum adjust to the medium. Increases occur in enzyme production and cell size.

2. Exponential or logarithmic phase: During this phase, cells and cell mass double at a constant rate. Metabolic activities proceed at a constant rate. Environmental conditions, which include pH, temperature, medium properties, etc., influence this phase. Because the doubling of the population (generation time) occurs at regular time intervals, this phase also is referred to as one of balanced growth.

3. Stationary phase: During this phase, toxic products accumulate and/or the availability of nutrients decreases and the number of viable cells reaches a plateau, resulting from some cells dying and others still growing and dividing. There is no net increase or decrease in cell number.

4. Death phase: If toxic substances accumulate and/or cell starvation occurs, the cells of the population enter this phase. The rate of decline becomes exponential with time.

The easiest way to monitor bacterial growth is by using the spectrophotometer. Spectrophotometers are instruments that electronically quantify the kinds and amount of light that are absorbed by molecules in solution. Spectrophotometric measurements such as those described in this exercise are based on the Beer-Bouger Law, which shows the relationship between a solutions concentration of suspended particles and its transmission of light. This relationship provides the basis for the quantitative determinations of the concentration of bacterial cells in a solution by measurement of the light transmitted by the solution.

Normal white light is a mixture of light waves of many different wavelengths (colours) between 380 and 750 nanometers (nm). The eye and brain perceive these waves of different wavelengths as different colours. The growth measurements require that the light entering a solution (incident light) is monochromatic (composed of a single wavelength). A spectrophotometer is equipped with a device to separate white light into its component wavelengths. Absorbance (A), also called optical density (OD), is directly proportional to
concentration (particles suspended) when a solution behaves according to the Beer-Lambert Law (a modification of the Beer-Bouger Law). Absorbance specifically is the logarithm to the base 10 of the reciprocal of the transmittance. In this exercise, absorbance values were used to measure turbidity of the bacterial growth media. On spectrophotometers, the absorbance scale normally is present along with the transmittance scale. The spectrophotometer measures the intensity of light (at various wavelengths) before and after the light has passed through a solution. If the solution contains particles such as bacteria that absorb light, the concentration of the bacteria can be determined spectrophotometrically by measuring the absorption of light.

The procedure for spectrophotometric measurements is relatively simple to perform. In general, it involves placing a solution of a light-absorbing particle into a cuvette (a special, small test tube), selecting the appropriate wavelength, and inserting the cuvette in the light pathway of a spectrophotometer so that the light passes through the cuvette and the solution it contains. Any light transmitted through the cuvette is directed onto a photosensitive device, which converts the radiant energy into electrical energy. A meter in the spectrophotometer then measures the electrical current generated. The absorbance, or percent transmission, is determined by comparing the value produced by the particle-containing solution to that of a blank solution, which does not contain the light-absorbing material. Note that standard plate counts and spectrophotometric measurements should be simultaneously done to follow the bacterial growth cycle and to obtain data with which to plot the corresponding growth curve.

The growth production curve was determined by taking aliquots from the growth media at regular intervals and measuring their absorbance at 550 nm. This is the maximum wavelength at which the bacterial growth medium absorbs light. The results are shown in Figure 34.
The four stages of a bacterial growth curve, namely the lag phase, exponential phase, the stationary phase and the death phase, are all present. As a general rule, the secondary metabolites are formed only when the growth rate of an organism is curtailed. In this study the harvesting of the secondary metabolite was done on the eighth day (near the end of the exponential phase or in the stationary phase).

As shown in chapter two, the formation of antibiotics is directed by organized sets of genes associated with special regulatory mechanisms that control both the timing and the level of gene expression. Their physiological role is not known. Speculations about their functions include a role in overflow metabolism to get rid of excessive amounts of intermediates of primary metabolism. High intracellular concentrations of these intermediates can be toxic to the cell. If export of unmodified metabolites is not possible, their conversion into less toxic, or more easily exported metabolites, may provide a way to prevent build up of toxic intermediates. Another role of secondary metabolites may be in defense mechanisms. Inhibiting other competing cells would leave more nutrients for the survival of the secondary metabolite-producing strain. Indeed many secondary metabolites show antibacterial or other inhibitory activities such as anti-tumor, antifungal, or may function as herbicides. Various groups of secondary metabolites can be distinguished depending on the primary intermediates from which they are derived. This division, however, is not absolute because more than one primary metabolite may be involved in the biosynthesis of a particular secondary metabolite.
Secondary metabolism is an intracellular process; however, few products have been reported to accumulate within the cytoplasm. Therefore, after determining the growth production curve, the next task was to find out using a bioassay whether the bacteriostatic agent was kept intracellular or excreted and hence more abundant in the growth medium. The biomass was separated and the cells or intracellular materials were studied to determine bacteriostatic activity. It was found that only the broth showed bacteriostatic action against *Pseudomonas fluoscens* as shown in Figure 35.

![Figure 35](image)

**Figure 35:** *Results obtained when testing bacteriostatic activity of the broth contents using Pseudomonas fluoscens.*

After determining that the compound with antibacterial action was in the broth, its isolation was then carried out, following the procedure outlined in Figure 36.

### 3.3 Isolation of the Bacteriostatic Agent

The experiment was started by inoculating the nutrient broth with V3 bacterium isolated from the soil. The seed broth was prepared for two days, and this was used as the starting material.
for the fermentation of broth for 14 days. Two solvent systems, which are petroleum ether and ethyl acetate, were used for extracting the antibacterial agent. The petroleum ether aided in removing fatty acids and the ethyl acetate was then used for extracting the remaining organic components, which yielded a yellow solid that inhibited the growth of two bacterial microorganisms, *Serratia marscences* and *Pseudomonas fluoscens*. The other test organisms included the *Staphylococcus auereus*, *Escherichia coli* and the fungus *Candida utilis*. The isolated antibacterial agent was able to inhibit the growth of these microorganisms but their inhibition zones were not as distinct as those for *Serratia marscences* and *Pseudomonas fluoscens*. Chromatographic techniques including the chromatotron were employed so as to purify the isolated antibacterial agent. The whole procedure for the isolation of an antibacterial agent is shown in **Figure 36**.

![Diagram showing different steps involved in extraction of the antibacterial agent.](image)

**Figure 36**: *Diagram showing different steps involved in extraction of the antibacterial agent.*
This procedure involves the separation of compounds with different polarities and since the desired compounds will be soluble in one or another solvent, this technique is meant to 'clear' the solution by removing contaminants. Petroleum ether was used to extract the fermentation broth; it contained no microbial growth inhibitory compounds giving the false impression that isolation of the antibacterial agent would be a simple procedure. Petroleum ether was used as a defatting solvent to remove unwanted waxes and oils from the broth, which would otherwise interfere with further analysis. This also makes further separation easier by removing a large portion of unwanted material. As shown in Figure 36, ethyl acetate was then used to extract the fermentation broth. It contained the bacteriostatic agent. In fact, after the extraction of the broth by ethyl acetate, no antimicrobial activity was detected in the remaining aqueous broth. Attention was focused on the ethyl acetate extract.

3.3.1 Extraction and chromatography

3.3.1.1 Column chromatography

Column chromatography is the most commonly used preparative technique for the isolation of chemical compounds. Initially it was thought essential to develop a good eluting system in order to separate components, therefore, much effort was taken to develop a good eluting system with the ethyl acetate fraction. A combination of methanol: ethyl acetate (1:10) and petroleum ether: ethyl acetate (3:7) was initially chosen as the former separated the polar and the latter the non-polar components and both components are volatile and easily removed from fractions.

3.3.1.2 Preparative Thin-Layer Chromatography (TLC)

Preparative TLC was carried out on the same sample because of its relative low complexity as seen with TLC. Five UV active fractions were scraped off and eluted with acetone and methanol to ensure full recovery of the sample. Only the baseline material showed biological activity. Further investigations revealed that the baseline fraction contained many compounds, which were then separated using a chromatotron to obtain amounts that would be sufficient for
conducting a bioassay and characterisation using spectroscopic techniques like NMR spectroscopy.

Problems encountered with column chromatography were not so much in the procedure involved but in the resulting fractions. Some fractions appeared to be more complex after passing through a column than they had been prior to separation. There was an increase in fractions eluted due to an increase in concentration of the solution applied to the TLC plate. Some Rf values of the fractions differed dramatically from the parent column. It might also be possible that decomposition had taken place and artefacts had formed which contributed to a more complex chromatogram.

3.3.1.3 Chromatotron separation

The ethyl acetate/methanol fractions with antimicrobial activity as seen with the bioassay were subsequently chosen for the separation via a chromatotron. It was decided to remove non-polar components with a solvent such as petroleum ether and another solvent; ethyl acetate was gradually introduced up to 100 %. This method achieved good separation. The third solvent methanol was added gradually to remove non-mobile components. Care was taken that the concentration of methanol did not increase above 50 % as it has tendency to dissolve silica gel, which subsequently interferes with the structural elucidation.

3.3.2 Bioassay Monitoring

There were basically two solvent systems used for the extraction of an antibacterial agent, namely petroleum ether and ethyl acetate. The petroleum ether was used for the extraction of non-polar substances like fatty acids. The bioassay tests were done so as to check if the extracted non-polar substances have any biological activity. The test organisms used were Pseudomonas fluoscens, which is a plant pathogen, and Serratia marcescens, which is a human pathogen. It could be clearly seen that the petroleum ether extract had no biological activity since no inhibition zones were observed around the disk papers (Figure 37). After
removing fatty acids, the ethyl acetate was then used so as to extract any organic polar compounds. The bioassay tests were done so as to check if the ethyl acetate extract had any biological activity. The test organisms used were similar to those used for petroleum ether extract. It was found that the antibacterial agent is a polar organic substance, since clear inhibition zones were observed around the disk papers see Figure 38.

Figure 37: Bioassay results for petroleum ether extract; test organism used was Serratia marcescens

The ethyl acetate extract was found to consist of five different bands when viewed under ultraviolet light. Column chromatography was done so as to separate different bands. The solvent system used was ethyl acetate and petroleum ether (9: 1). Further purification was done using a chromatotron. Each band was tested for biological activity, and there was no biological activity in all these bands. In a thin-layer chromatography plate (TLC), there was a yellow spot observed at the baseline, this spot was extracted with a more polar solvent (methanol) and concentrated on the rotary evaporator. This methanol extract was checked on the TLC plate and it was found that there were four bands observed when viewed under ultraviolet light. These bands were polar because a mixture of two polar solvents (methanol and acetone) in a ratio of (3: 97) was used so as to move these bands from the baseline. It could be stated that there were more than four bands that constituted the lower spot. However, not all of these bands were ultraviolet active because they were not observed under ultraviolet
light of 254 nm, the extra bands were observed when the TLC was developed with anisaldehyde stain. Further purification so as to separate the different components was done using a chromatotron. Each fraction collected was tested for biological activity and only one fraction showed biological activity, the purple spot fraction when viewed under ultraviolet light. After determining that the antibacterial activity was due to the purple spot fraction, further purification was done using preparative thin-layer chromatography. It was found that there were two other fractions that were closer to the purple spot fraction. The R\textsubscript{f} for the purple spot fraction was 0.45 and there was a fraction above this with the R\textsubscript{f} of 0.49 and the fraction below had an R\textsubscript{f} value of 0.41. As a result the chromatotron was run so as to further separate these fractions. The pure fraction crystallized into yellow fluffy shiny crystals. Biological activity for this pure fraction was tested against *Serratia marcescens*, and *Pseudomonas fluorescens* and it was found that the isolated fraction was able to inhibit the growth of these test organisms (Figure 38).

Figure 38: Agar plate showing biological activity of the purple spot fraction, test organism used is *Pseudomonas fluorescens*. 
3.3.3 Structural elucidation of active compound by Nuclear Magnetic Resonance Spectroscopy (NMR)

The application of NMR techniques is growing rapidly in the elucidation of the structure and function of biologically important molecules. In this section the role of NMR in determination of the structure of the active compound will be discussed. Apart from the $^1$H NMR and $^{13}$C NMR, other spectroscopic experiments such as DEPT, COSY, HMBC and HMQC were used for structural determination of the compound.

3.3.3.1 $^1$H NMR Spectrum

The $^1$H NMR of the isolated compound showed that the compound has four protons resonating in the “aromatic” region. Four sets of proton signals observed include the $H_a$ proton signal at 7.94 ppm, a doublet of doublets with coupling constants of 8.1 Hz resulting from ortho coupling with $H_b$ and meta coupling with $H_c$ respectively (Figure 39). The low-field shift of $H_a$ ($\delta = 7.94$) indicated that it must be ortho to a carbonyl-containing functional group. The shift to the lower field is caused by the anisotropic effect of the neighbouring carbonyl group. The $H_c$ proton signal has a chemical shift of 7.32 and is a doublet of doublets of doublets, having a coupling constant of 8.1 Hz, where it ortho couples with $H_d$, and meta couples with $H_c$ with a coupling constant of 7.0 Hz. $H_d$ appeared as a doublet of doublets at 6.70 ppm, with coupling constants of 8.3 Hz and 0.7 Hz from ortho coupling with $H_c$, and meta coupling with $H_b$ respectively. The signal of proton $H_b$ appeared as a doublet of doublets of doublets at 6.66 ppm, with coupling constants of 8.1, 0.7 and 7.0 Hz from ortho coupling with $H_a$, meta coupling with $H_d$ and ortho coupling with $H_c$ respectively.
3.3.3.2 $^{13}$C NMR Spectrum

The $^{13}$C NMR spectrum showed seven carbons, one of which is a carbonyl and the rest are aromatic (Figure 40). In the isolated antibacterial agent there are four methine aromatic carbons at the chemical shifts ($\delta_c$) equal to 116.5, 116.8, 132.1 and 135.1, respectively. Three non-protonated carbons at chemical shift ($\delta_c$) 109.5, 151.1 and 173.2. Of all the non-protonated carbons, C$_3$ with the chemical shift of 109.5 is most upfield. C$_1$ with a chemical shift of 173.2 appears most downfield, and is most likely a carboxylic acid. It could therefore be deduced that X in our compound is a carboxylic group.
3.3.3.3 $^1$H-$^1$H COSY

The COSY (Correlation Spectroscopy) is a 2-Dimensional NMR spectrum that shows correlation between coupled protons. From the upper right to the lower left runs the diagonal, a series of absorptions in which $v_1$ equals $v_2$, these diagonal peaks provide nothing in the way of useful information beyond the simple 1-D $^1$H spectrum. On either side of the diagonal and symmetrical disposed are cross peaks. The structure of isolated compound allows more than one useful entry point. The methine (CH) at the chemical shift of 7.88 has been selected for illustration. If one begins at the diagonal and traces either directly to the right or directly up, the same result is obtained because the spectrum is symmetrical. Two off diagonals or cross peaks are intersected. At right angles to the ones just traced, the chemical shifts of the two-coupled resonances can be obtained. A quick check at the structure of the isolated compound finds $H_a$ adjacent to an ortho proton $H_b$ and also meta proton $H_c$. Likewise $H_b$ at the chemical shift if 6.6 ppm is ortho coupled to $H_c$ and $H_a$ and meta coupled to $H_d$.

On the other hand, if another equally useful entry point corresponding to $H_c$ at $\delta_{H} 7.2$ is selected, again beginning at the diagonal, this time it can be found that $H_c$ is correlated with three distinct resonances. It first correlates to $H_b$ and $H_d$ that are ortho to it. In addition, $H_c$ is correlated to $H_a$ doublet of doublets to the left of the diagonal. These correlations of course make perfect sense with the structure, and hence connectivities can be made. The COSY spectrum for the isolated antibacterial agent showed four signals, this agreed well with the proton spectrum. The information obtained from COSY spectrum helped in determining which proton is correlated to which one. From this spectrum it was then observed that $H_d$ is correlating with $H_b$, and also true with correlating with $H_b$ and $H_c$ that are correlating with each other and also with both $H_a$ and $H_d$. (Figure 41).
The HSQC and HMQC spectra were then used to show the single-range coupling and multiple-range coupling of protons and carbon respectively. The NMR spectra for the single-range and multiple range couplings are shown in Figures 42 and 43 respectively.

3.3.3.4 $^1$H- $^{13}$C HMQC and HSQC

The $^1$H- $^{13}$C experiment correlates $^{13}$C nuclei with directly attached (that is coupled) protons; these are one-bond ($^1J_{CH}$) couplings. The frequency domains of $F_1 (v_1)$ and $F_2 (v_2)$ are of different nuclei, and so there is no apparent diagonal or symmetry. Historically, the HMQC (Heteronuclear Multiple Quantum Correlation) experiment was preceded by the HETCOR (HETeronuclear CORrelation) experiment. Although experimentally there are many differences, the essential difference is that, while the HETCOR experiment is carbon detected, the HMQC experiment is proton detected$^{67,68}$ Since there are great differences between proton and carbon in their relative abundances and sensitivities, the HMQC is greatly preferred and the outcome of HSQC and HMQC experiments on the isolated antibacterial agent will be discussed below. In the HMQC spectrum, the proton scale is on the $F_2$ axis along the y-direction and the carbon-13 scale in on the $F_1$ axis along the x-direction. Immediately obvious is the fact that there is no diagonal or symmetry. This is true because as mentioned above $F_1$ and $F_2$ represent different nuclei. Since the HSQC experiment provides the information about
correlation of protons with directly bonded carbons, so it could be seen from Figure 42, that proton H_a is bonded to C_7, H_c bonded to C_5, H_d bonded to C_4 and H_b bonded to C_6, respectively.

Figure 42: HSQC spectrum for antibacterial agent.

The same is true with the HMQC experiment that gives information about the correlation of protons with neighbouring carbons. From the results in Figure 43; one could observe the coupling between H_a and C_1, H_a and C_3 as well as H_a and C_5. There is also a coupling between H_c and C_7, and C_3 respectively. The carbon-hydrogen coupling is also observed between H_b and C_2, and H_b and C_4. The last observed carbon-hydrogen coupling is between H_d and C_2 and C_5 respectively. All these couplings are three bond couplings and are stronger than the one and two bond coupling. Since the compound is aromatic, one could also observe the four-bond coupling though this is a rare case which occurs in aromatic systems.
Interpretation of this spectrum is straightforward. One begins at any carbon atom and mentally draws a line horizontally until a cross peak is encountered. Another line is mentally drawn perpendicular to the first to find the proton or protons with which it correlates. There are only three cases possible for each carbon atom. If a line drawn encounters no cross peaks, then the carbon has no attached hydrogens. If the drawn line encounters only one cross peak, then the carbon may have 1, 2 or 3 protons attached. If two protons are attached, they are either chemical shift equivalent or they overlap. If the line encounters two peaks, there is a special case of diastereotropic protons attached to methylene group. Much of this information is
confirmed by the DEPT spectrum (Figure 44), which shows the presence of \(-\text{CH}_3\), \(-\text{CH}_2\) and \(-\text{CH}\) groups. In the case the compound, only the presence of \(-\text{CH}\) groups is shown by the DEPT spectrum. Therefore, the HMQC spectrum should, whenever possible, be considered along with the DEPT spectrum.

![DEPT Spectrum](image)

**Figure 44**: DEPT Spectrum for the isolated antibacterial agent.

### 3.3.3.5 Infra Red Spectroscopy (IR)

The infrared spectroscopy was used to identify the functional groups present in the structure. It was deduced that our compound displays a strong broad \(-\text{NH}_2\) stretching absorptions in the 3474 and 3374 cm\(^{-1}\) region, due to the symmetrical and unsymmetrical \(-\text{NH}_2\) stretching. The very strong and broad \(\text{OH}\) stretching absorption that extends from 2500 to 3300 cm\(^{-1}\) shows the presence of the carboxylic acid functionality (Figure 45)
This further suggests that the compound exists predominantly as a hydrogen bonded dimer. The carbonyl stretching frequency for this dimer is found at the wavenumber of 1672 cm$^{-1}$. The bands that appear between 2000 and 1667 cm$^{-1}$ are due to the aromatic overtones and combinations. These weak absorptions can be used to assign the ring substitution pattern, whether mono-, di- or tri- substitution pattern and as shown in Figure 46 positional isomers can also be distinguished. Usually, if there is a carbonyl group present, the frequency due to that carbonyl can disturb the overtone pattern. Fortunately, the carbonyl stretching frequency appears slightly lower than this region and had no effect on the pattern.
Figure 46: Different regions for different substitutions on the aromatic ring

When looking at the infrared spectrum of the compound, it could be noted that its overtone region is similar to that of ortho-substitution Figures 45 and 46. By using all of the above information, the antibacterial agent was found to be anthranilic acid.

3.3.3.6 Mass Spectrometry

There are three basic functions that the mass spectrometer performs. It firstly subjects the molecules to the bombardment by a stream of high-energy electrons, whereby the molecule is converted to ions by high-energy electrons. These ions then get accelerated towards the electric field. Different fragments are then detected by the mass spectrometer. From the mass spectrometer results one could observe six different fragments with high intensities.
Taking into account the NMR and IR spectroscopic data, a molecular formula of $C_7H_7NO_2$ could be elucidated for the molecule and it is clear that anthranilic acid can account for all the data.

On the mass spectrum (Figure 47), the base peak appears at m/z 137. It has an odd mass due to the presence of one nitrogen atom in the molecule. In the Ms spectrum a second peak appears at m/z 119 that corresponds to the loss of water from the anthranilic acid structure as shown in (Scheme 11). The third fragment with m/z of 92 corresponds to the decarboxylation of anthranilic acid as shown in Scheme 12.

![Figure 47: Low resolution mass spectrum for anthranilic acid.](image)

Scheme 11: Fragmentation of anthranilic acid by loss of water.
Scheme 12: Fragmentation of anthranilic acid by loss of carboxyl group.

In addition to the low-resolution mass spectrum of anthranilic acid, the high-resolution mass spectrum was obtained and is shown in Figure 48. The calculated mass of anthranilic acid (C₇H₇O₂N) was 137.04768 and experimentally determined to be 137.04609.

Figure 48: High-resolution mass spectrum for anthranilic acid.

3.3.3.7 X-ray studies of anthranilic acid

The structure of anthranilic acid was confirmed by x-ray crystallography and was found to be in the orthorhombic space group Pbca. The dimensions of the unit cell were found to be \( a = 7.106(2) \text{ Å}, \ b = 15.835(4) \text{ Å}, \ c = 11.641(3) \text{ Å}, \ \alpha = 90^\circ, \ \beta = 90^\circ, \ \gamma = 90^\circ \) Volume 1309.8(6) Å³ The crystallographic structure of anthranilic acid is shown in Figure 49.
Figure 49: ORTEP drawing of anthranilic acid with thermal ellipsoid plot (50% probability surfaces)

Further crystal structure data for anthranilic acid is contained in the appendix. One of the features of the crystal structure is that there are eight molecules in the symmetric unit as shown in Figure 50.

Figure 50: Unit cell contents viewed approximately down the a-axis.
Figure 51: Hydrogen bonding between molecules of anthranilic acid in the lattice.

There are several hydrogen bonds and \( \pi - \pi \) stacking interactions between the molecules in the crystal structure as shown in Figure 51 and 52. The hydrogen bonds observed are:

- Discrete carboxylic acid dimers are evident. Both carboxylates are bound symmetrically to each other with a hydrogen bonding of 1.641 Å.
- Weak intramolecular N-H—O interactions with a distance of 1.944 Å.
- The dimers interact through weaker non-stereospecific intermolecular N-H—O interaction with the distance of 2.260 Å.

Brown, using X-rays\(^7\) and later using neutron diffraction,\(^7\) showed the novel coexistence of neutral molecules and zwitterions in the same crystal of anthranilic acid as shown in Figure 53. Contrary to these findings, in this project no evidence of the formation of zwitterions was found. The centre-to-centre phenyl ring stacking interaction has a distance of 3.278 Å as shown in Figure 52. The anthranilic acid dimers lay in parallel sheets that are all fairly planar. It is therefore concluded that in the solid state, these hydrogen bonds and \( \pi - \pi \) stacking stabilise anthranilic acid. However, it is suspected that x-ray crystallography of anthranilic acid isolated by precipitation would have zwitterions. Some attempts to grow a suitable crystal to run x-ray crystallography from the precipitated anthranilic acid was not successful; therefore, the speculation presented in the preceding sentence could not be tested.
Figure 52: The anthranilic acid dimers lay in parallel sheets that are all fairly planar.

Figure 53: Evidence of the formation of zwitterions from studies by Brown.\textsuperscript{71}

3.3.4 Acid-Base extraction

A common and important use of extraction is the separation of acidic, basic and neutral organic compounds. An organic acid, \( \text{RCO}_2\text{H} \), or a base, such as \( \text{RNH}_2 \), is usually much more soluble in organic solvents than in water. However, the salts of these compounds, for example \( \text{RCO}_2\text{Na}^+ \) or \( \text{RNH}_3^+\text{Cl}^- \), have much higher solubilities in water, since they are ionic substances. To separate an acid, for example, the broth was basified with sodium hydroxide and extracted with ethyl acetate to remove basic and neutral entities. Following the isolation procedure outlined in Figure 54, the acid was converted to the salt (Equation 1). After
separation of the broth and ethyl acetate, the acid was recovered by reacidification of the aqueous solution (Equation 2).

\[
\begin{align*}
RCO_2H + NaOH & \rightarrow RCO_2Na^+ + H_2O \quad (1) \\
RCO_2Na^+ + HCl & \rightarrow RCO_2H + Na^+Cl^- \quad (2)
\end{align*}
\]

The acidic component was then extracted with ethyl acetate and this sample was analysed by gas chromatography. By the same principle, the basic component was separated from acidic and neutral compounds by acidifying the broth with aqueous acid, (in this experiment 2 N HCl (Equation 2) was used). After removal of acidic and neutral entities with ethyl acetate, the solution was basified by adding NaOH (equation 4), and then extracted with ethyl acetate.

\[
\begin{align*}
RNH_2 + HCl & \rightarrow RNH_3^+Cl^- \quad (3) \\
RNH_3Cl + NaOH & \rightarrow RNH_2 + H_2O + NaCl \quad (4)
\end{align*}
\]

The ethyl acetate extract was also analysed by gas chromatography. The third procedure followed was that the broth was simply extracted with ethyl acetate. All three extracts were analysed by gas chromatograph as shown in Figure 55.
Figure 54: General outline for the separation of acidic, basic, and neutral components of a mixture.\textsuperscript{22}
When looking at the chromatogram in Figure 55, it is clear that the peak corresponding to the anthranilic acid is only observed in the neutral extract. This means that anthranilic acid is neither an acid nor a base because if it was an acid it would have been present in the acid extract and same is true for basic extract. This agrees well with the low yield that was obtained when conducting solvent to solvent extraction of the fermentation broth, and isolating anthranilic acid was not easy. As a result, further experiments were conducted to find the best method of increasing the yield of anthranilic acid and shortening the procedure involved in the isolation of anthranilic acid.

Figure 55: Gas chromatogram of anthranilic acid (commercially available), acidic, basic and neutral components.

3.3.5 Electrophoresis

O’Farell and Klose first introduced two-dimensional electrophoresis in 1975.\textsuperscript{73,74} This is a powerful and widely used technique for the analysis of complex protein mixtures extracted from cells, tissues or other biological samples. This method separates proteins according to two independent properties in two discrete steps, namely the first-dimension step, isoelectric focusing (IEF), which separates proteins according to their isoelectric point (pI) and the second-dimension step, SDS-polyacrylamide gel electrophoresis (SDS-PAGE), which separates proteins according to their molecular masses. In the work carried out in this study, the isolation of the bacteriostatic agent (anthranilic acid) had been achieved by solvent-solvent
extraction. The yield of this procedure was very low and the product required many chromatographic separations to determine the pure compound. It was therefore necessary to optimise the isolation procedure by making sure that there were fewer isolation steps and the yield was improved.

Isoelectric focusing (IEF) can be described as electrophoresis in a pH gradient set up between a cathode and anode with the cathode at a higher pH than the anode, and is one of the most discerning separating methods based upon the gross physical properties such as charge or size. Proteins are amphoteric molecules; they carry either positive, negative or zero net charge, depending on the pH of the solvent. The net charge of a protein is the sum of all the negative and positive charges of its amino acid side chains including the amino and carboxyl-termini. The isoelectric point (pI) is the specific pH at which the net charge of protein is zero. Proteins are positively charged at pH values below their pI and negatively charged at pH above their pI. When the electrical potential is applied across the gradient, the pH gradient is established by the carrier ampholytes, and the protein species migrate and focus at their isoelectric points. In this manner a mixture of proteins is separated, as each focuses at different pI as shown in Figure 56.

Figure 56: Separation of an amino acid mixture by electrophoresis, neutral molecules do not migrate, protonated molecules migrate toward the negative electrode and deprotonated molecules toward the positive electrode.

In this work 2-D electrophoresis has been employed, and the anthranilic acid is separated in the first dimension according to its isoelectric point and in the second dimension, according to
its molecular mass by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).\textsuperscript{77} In the present case the second dimension of anthranilic acid was not determined, since the interest was only in its pH and not in its molecular mass. As result the isoelectric point of anthranilic acid was found to be 8.25 (see Table 4).

Since anthranilic acid has both carboxyl and the amino functional groups, it was assumed that it would behave as a neutral amino acid. The amino acids that have both acidic and basic groups in the same molecule undergo an intramolecular acid-base reaction and exist primarily in the form of a dipolar ion or zwitterions.\textsuperscript{78} In acid solution (low pH) amino acids become protonated and thus exist as cations, and in basic solution (high pH), they exist as anions. The intermediate step where an amino acid is balanced by the anionic and a cationic form is called the isoelectric point, and that is where it exists as dipolar ions.

It has been reported from the literature that all neutral amino acids have isoelectric points in the pH range of 5.0-6.5 and not exactly 7.0 as one might expect.\textsuperscript{5} This is caused by the fact that carboxyl groups are stronger acids in aqueous solution than amino groups and as a result they tend to dominate. For example, amino acids with acidic side chains (for example aspartic acid) as shown in Table 4 have isoelectric points at lower or more acidic pH to suppress the dissociation of the extra carboxylate group in the same side, while amino acids with basic side chain (for example histidine) also shown in Table 4 have isoelectric points at higher or more basic pH to restrain protonation of the extra amino group.
<table>
<thead>
<tr>
<th>Amino acids</th>
<th>Isoelectric point</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartic acid</td>
<td>3.0</td>
<td>HOOC—C—CH$_2$COOH</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>5.5</td>
<td>NH$_2$</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>5.7</td>
<td>NH$_2$</td>
</tr>
<tr>
<td>Histidine</td>
<td>7.6</td>
<td>NH$_2$</td>
</tr>
<tr>
<td>Anthranilic acid</td>
<td>8.25</td>
<td>COOH</td>
</tr>
</tbody>
</table>

Anthranilic acid as a neutral molecule was expected to behave like neutral aromatic amino acids, such as phenylalanine and tyrosine with the isoelectric points of 5.5 and 5.7 respectively. However, the isoelectric point found experimentally for anthranilic acid is 8.25. It is proposed that anthranilic acid has this unusually high isoelectric point because its protonated form is very stable, since the cationic charge on the nitrogen atom can be stabilised mesomerically (Scheme 13).
3.3.6 Isolation of anthranilic acid by precipitation

Having determined that the anthranilic acid displayed neither acidic nor basic properties, it was then believed that the anthranilic acid behaved like a neutral amino acid. The electrophoresis for the anthranilic acid was then done so as to determine the pH at which it precipitates from solution. The pH at which anthranilic acid precipitated was found to be 8.25. The nutrient broth was then adjusted to the pH of 8.25, and a small amount of precipitate was formed. This was filtered; dried in an oven and dissolved in ethyl acetate and the gas chromatography results are shown in Figure 57. The gas chromatogram of the precipitate and that of the commercially available anthranilic acid match with each other, and both have a retention time of 12.3 minutes.

![Scheme 13: Mesomeric effect in anthranilic acid](image)

**Figure 57:** Gas chromatogram for anthranilic acid.
3.4 Fate of anthranilic acid

From the work done it is not clear whether the anthranilic acid isolated was the end product, biodegradation product or a precursor leading to some other secondary metabolites. The literature has many examples of the three scenarios and each case will be considered below.

3.4.1 Anthranilic acid as a precursor

It has been reported in the literature that anthranilic acid has been used as a precursor in the synthesis of different compounds including anthranilohydrazide, 3,4-dihydro-4-oxo-1; 2,3-benzotriazine, triazine-β-naphthol adduct and n-phenylanthranilic acid. These compounds showed antibacterial activity but none of the compounds showed any noticeable antifungal effect. For instance, Hurley and co-workers accounted for the feeding of antitumor antibiotics produced by various Actinomycetes, which are anthramycin, tomaymycin, and sibiromycin with anthranilic acid, 3-hydroxy-4-methylanthranilic acid and tryptophan. The results are shown in Table 5. From these results, it could be clearly seen that anthranilic acid was efficiently incorporated into tomaymycin, but this was almost certainly via tryptophan, since the incorporation into tomaymycin was reduced by greater than 80% when cold tryptophan was added in a parallel feeding experiment. Anthranilic acid and 3-hydroxy-4-methylanlanthranilic acid were not successfully taken up by the anthramycin and as a result their precursor role was not properly measured. But anthranilic acid was effectively taken up by sibiromycin. However, 3-hydroxy-4-methylanlanthranilic acid was incorporated into sibiromycin.
Table 5: Incorporation of labelled substrates into anthramycin, tomaymycin and sibiromycin\textsuperscript{22}

<table>
<thead>
<tr>
<th>Compound fed</th>
<th>Percentage incorporation into:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Anthramycin</td>
</tr>
<tr>
<td>[COOH-\textsuperscript{14}C]anthranilic acid</td>
<td>(&lt;0.1)^a</td>
</tr>
<tr>
<td>[COOH-\textsuperscript{14}C]anthranilic acid + DL-tryptophan</td>
<td>NE</td>
</tr>
<tr>
<td>3-hydroxy-4-methyl-2[-14C]anthranilic acid</td>
<td>(&lt;0.1)^a</td>
</tr>
<tr>
<td>DL-[7a-14C] tryptophan</td>
<td>18.0</td>
</tr>
</tbody>
</table>

\( ^a \) Radiolabelled substrates failed to be taken up significantly by the organism.
\( ^b \) Parallel feeding experiments in which a 100-ml culture of S.achromogenes was divided into halves prior to the addition of [COOH-\textsuperscript{14}C]anthranilic acid to each flask and DL-tryptophan to one of the two flasks. NE, not examined.

3.4.1.1 Shikimic Acid Pathway

Shikimic acid is an organic acid, which was first isolated from the fruits of \textit{Illicium} species, where it was present in large quantities (up to 20 \%)\textsuperscript{81}. The name shikimate was derived from Japanese name for the tree \textit{Illicium religiosum}, shikimi-no-ki (anise tree). About 60 years later it was revealed that shikimate was an intermediate in the aromatic amino acids biosynthesis in plants and microorganisms, since animals cannot carry out \textit{de novo} synthesis of aromatic amino acids.\textsuperscript{82} \textbf{Scheme 13} shows the shikimic acid pathways. There are seven enzymes of the shikimate pathway that catalyse sequential conversion of erythro-4-phosphate and phosphoenol pyruvate to chorismic, which is used as a substrate for other pathways that culminate in the production of folates, ubiquinones, naphthoquinones and the aromatic amino acids tryptophan, phenylalanine and tyrosine as shown in \textbf{Scheme 14} and \textbf{Scheme 15}.\textsuperscript{83} 4-Aminobenzoate and 4-hydroxybenzoate, the precursors of tetrahydrofolate and benzo-and
naphthoquinones, respectively, are directly biosynthesised from chorismate. Menaquinone, 2,3-dihydroxybenzoate and salicylate biosyntheses proceed via isochorismate. 

Scheme 13: The shikimate pathway.
Scheme 14: Chorismate and beyond primary metabolism pathways.
An interesting group of secondary metabolites containing aromatic rings is derived from intermediates of aromatic amino acid biosynthesis or the aromatic acids themselves (Scheme 15). Many of these compounds are clinically important, especially vancomycin and teicoplanin. These glycopeptide antibiotics are active against methicillin resistant *Staphylococcus aureus* (MRSA).\textsuperscript{86-87}
**Scheme 15: Aromatic amino acid biosynthesis**
3.4.1.2 Alkaloids biosynthesised from Anthranilic acid

All of the antibiotics featured in Scheme 15 are alkaloids. Alkaloids belong to an important class of natural products, which are nitrogenous compounds occurring in plants, toads and animals including mammalian and fungi. Most of them are optically active, and nearly all of them are of basic nature.

Figure 59: Diagram showing anthranilic acid-derived antibiotics
Among the commonly known naturally occurring alkaloids, benzodiazepines form a class of biologically active compounds from which widely prescribed psychoactive drugs have been developed. These compounds also have potential as highly selective anti-infective agents. To date, about thirteen members of well-known antibiotics are produced by various *Streptomyces* species. These include anthramycin (101), and tomatoycin (102), abbeymycin (103), chicamycin (A) (104), DC-81 (105), mazethramycin (106), sibanomicin (DC-102) (107), sibiromycin (108) and prothracarcarcin (109) (Figure 59). Hurley has studied the biosynthesis of a number of these compounds; they all have anthranilic acid as a precursor. The anthranilic acid nucleus has been highlighted.

3.4.1.3 Biosynthesis of Enediyne Antitumor Antibiotics

![Figure 60: Structure of an enediyne antibiotic.](image)

Enediyne antibiotics (Figure 60) are potent antitumor agents. Although natural enediyynes have seen limited use as clinical drugs mainly because of substantial toxicity, various polymer-based delivery systems or enediyne-antibody conjugates have shown clinical promise and success in anticancer chemotherapy. A great challenge will be to develop ways to make
new enediynes for mechanistic and clinical studies. A closer look at the enediynes reveals an anthranilic acid nucleus as shown in Figure 60. Anthranilic acid is used as a precursor; the structural analogues of enediyne could be obtained by feeding *Streptomyces globisporus* with analogues of anthranilic acid.

### 3.4.1.4 Biosynthesis of 2-heptyl-3-hydroxy-4-quinolone from anthranilic acid

Through $^{14}$C labelling studies, Pesci et al. found that anthranilate is a precursor of the production of 2-heptyl-3-hydroxy-4-quinolone (112) (Figure 61); it is referred to as the *Pseudomonas quinolone signal* (PQS), which is responsible for *Pseudomonas aeruginosa* virulence.

![Beta-ketodecanoic acid](image)

**Figure 61:** Biosynthesis of 2-heptyl-3-hydroxy-4-quinolone from anthranilic acid.

However, methyl anthranilate was found to inhibit the production of PQS, and could be used in the development of therapeutic agents that will reduce the effectiveness of *Pseudomonas aeruginosa*.

### 3.4.2 Anthranilic acid as a metabolic degradation product

It has been reported in the literature that anthranilic acid could be formed as a product of catabolism, for instance some strains of *Bradyrhizobium japonicum* have the ability to catabolize indole-3-acetic acid (plant hormone) to anthranilic acid (Scheme 16).
Furthermore, anthranilic acid can be obtained as the metabolic end product of the kynurenine pathway of tryptophan (Scheme 17). The other end product of this pathway is the neurotoxin quinolic acid, which is responsible for the neuronal cell death in a number of important neurological diseases. As a result this makes kynureninase a possible therapeutic target for diseases such as Huntington’s, Alzheimer’s and AIDS related dementia. This is an enzyme-catalysed reaction, and the enzyme involved in catalysis is kynureninase.

3.4.3 Anthranilic acid as a secondary metabolite

Anthranilic acid also known as vitamin L₁ can exist as a secondary metabolite; which is required for lactation in human females. Bovine liver and also Aloe vera products are rich sources of vitamin L₁. Anthranilic acid is the ortho-isomer of para-aminobenzoic acid.
(PABA), which is widely used as the active inhibitor of the bacteriostasis produced by the sulfonylamide drugs. It is also used as the growth factor for several species of bacteria including *Neurospora crassa*.\(^9\) PABA is an essential metabolite for bacteria that is involved in the biosynthesis of folic acid that is necessary for the growth of bacteria and is therefore widely distributed in nature. The sulfonamides have chemical structures similar to that of PABA, and may act as bacteriostatic agents by competing for the bacterial enzyme concerned with the metabolism of PABA.\(^98\) Unlike anthranilic acid, PABA has been isolated from yeast by Rubbo and Blanchard.\(^99,100\) Anthranilic acid, however, is an aromatic amine which occurs physiologically as a metabolite of the amino acid tryptophan and may be further broken down into carbon dioxide, water and ammonia.\(^101\)

### 3.5 Conclusion

The aim of this project was to isolate and characterise the antibacterial agent produced by *Actenomyces* bacterium V3. The extraction and characterisation as well as isolation was successfully achieved. Much was learnt during this process about the behaviour of microorganisms and the use of aseptic techniques. Different extraction procedures were successfully accomplished. A crystal structure was obtained to conclusively prove the structure of the isolated antibacterial agent as anthranilic acid, also known as ortho-aminobenzoic acid.

The solvent-to-solvent extraction procedure was first used for the extraction of the antibacterial agent. Due to the low yield obtained, lengthy steps involved and large volumes of solvent used in the solvent-to-solvent extraction, the method was improved by the use of an acid-base extraction process. At this time the extraction of the antibacterial agent was not successful. It was therefore deduced that the isolated compound is neither a base nor an acid. The electrophoresis experiment was carried out so as to determine the pH of the isolated compound. It was then assumed that this compound behave like an amino acid. The pI at which the anthranilic acid precipitate out of the solution was found to be 8.25, and the compound precipitated out of solution.
Gas chromatography of this precipitate was compared to that of the commercially available anthranilic acid, and it was found that both these compounds eluted at the same time. It was therefore concluded that the faster and effective method for isolating the antibacterial agent produced by soil bacterium V₃ is by precipitation.
4. Experimental

4.1 Materials and Methods

4.1.1 General Experimental Procedures

Flash column chromatography was carried out using Merck silica gel 60 as a solid support in accordance with literature procedure. The crude material was either preadsorbed onto the column support or applied as a concentrated solution on top of the column. The columns were then eluted with various ratios of ethyl acetate (b.p 40-60 °C) in petroleum ether.

Thin-layer chromatography (TLC) was performed on precoated Merck Kieselgel 60 F$_{254}$ plastic sheets. TLC plates were used for monitoring separations in both column chromatography and chromatotron and also as a separation technique. Visualisation of the plates was achieved using the following techniques:

- Fluorescence on exposure to ultraviolet light (254 nm).
- Developing by dipping them in an anisaldehyde stain. The anisaldehyde stain was prepared whilst cooling the reagents in nitrogen by adding 5 ml of glacial acetic acid, 17 ml of concentrated sulfuric acid, 13 ml p-anisaldehyde in 465 ml of absolute ethanol. The dipped TLC plate was heated with a heat gun.
- Developing in an aqueous cerium sulphate (0.2%), ammonium molybdate (5%) and sulphuric acid (5%) solution followed by heating with a heat gun.
- Developing in an aqueous potassium permanganate (0.5%) and sodium hydrogen carbonate (2.5%) solution and drying.

Further purification of compounds was achieved by using the Harrison research chromatotron or preparative TLC plates. Preparative TLC plates were prepared on baked glass plates (12 cm x 20 cm x 20 cm) coated with silica gel. The Silica gel (Merck Kieselgel 60 F$_{254}$) (200 g) was homogeneously suspended in distilled water (500 ml). This paste was rapidly spread on cleansed grease-free glass plates with a spreader. These plates
were kept in a draft free place for 48 hours at room temperature and subsequently activated for 48 hours at 120 °C.

NMR spectra were recorded on a Varian Unity Inova 500 spectrometer at 25°C using deuterated chloroform as solvent and residual protonated solvent as the internal standard. The chemical shifts are reported in parts per million (ppm, δ) and the coupling constants in Hertz (Hz).

Infrared spectra were obtained on a Perkin Elmer one spectrometer as films on KBR discs.

The mass spectra were recorded on a ThermoFinnigan Polaris / QCQ Plus instrument coupled to a Varian CP-3800 gas chromatograph. Low and high resolution mass spectra were measured on a Kratos MS 80 RF double-focussing magnetic sector instrument at 70 eV. Spectra were obtained using the electron impact mode (EI) and chemical ionisation mode (CI).

Melting points were determined using a Kofler hot-stage apparatus by placing a few crystals on a dish and is uncorrected.

X-ray diffraction studies were performed on an Oxford Diffraction Xcalibur 2 CCD 4-circle Diffractometer equipped with an Oxford Instruments Cryojet.

Autoclaving was accomplished by using a Vertical type model HL-341; Aseptic techniques were all performed on a Julian ward laminar bench. The bacterial growth curve was determined on a Milton Roy Spectronic spectrophotometer at the wavelength of 550 nm. Centrifuging was achieved on a 8 tube continuous flow centrifuge (Szent-Gyrgyi Blum, KBS-3, Sorvall type SS-3).

Electrophoresis was done on a Pharmacia Biotech instrument using a 3-10 pl, 110 mm strip from Amersham Scientific, and an immobiline dry strip reswelling tray from Pharmacia Biotech.
4.2 Microbiological Materials and Methods

4.2.1. Microorganisms

The organism (V3) used was obtained from Vincent Okudoh (PhD student from the department of Microbiology and Plant Pathology) at University of KwaZulu-Natal Pietermaritzburg campus. This microbe was reported to have antibiotic properties by Mr Okudoh in his MSc thesis. The test organisms used to evaluate the antibiotic properties for V3 are Staphylococcus aureus, Escherichia coli, Streptococcus faecalis, Serratia marcescens and Pseudomonas fluorescens.

4.2.2 Storage and growth on agar

The organism (V3) used was preserved as a frozen culture. For maintenance on agar slants, the bacterium was grown on a medium consisting of beef extract (0.2 g); acid hydrolysate of casein (1.7 g), starch (0.15 g), in 100 ml of water and the final pH for this medium was adjusted to 7.3. The stock cultures were maintained in two ways:

- Mature slopes were stored in the refrigerator at 4 °C and working slopes sub-cultured from them as required.
- The mycelium from mature slope were taken up in a sterile solution of 20 % w/v glycerol in water and stored in 1.5 ml sterile vials at -196 °C.
- The defrosted spore (mentioned above) suspensions were transferred to each slope of agar slants when necessary.

4.2.3 Mueller-Hinton Agar

This is a general-purpose medium suitable for the growth of a wide variety of microorganisms. It is composed of beef extract (2.0 g/L); acid hydrolysate of casein (17 g/L), starch (1.5 g/L),
agar (17 g/L) and the final pH for this medium was adjusted to 7.3 +/- 0.1 at 25°C. Mueller-Hinton agar was prepared by dissolving 38 g of medium in one litre of distilled water. This was heated with frequent agitation for one minute to completely dissolve the medium. Autoclaving at 121°C for 15 minutes was performed, and the medium was cooled to about 40°C and then poured into petri dishes. Poured agar was allowed to cool and solidify before use.

4.2.4 Nutrient Broth

The nutrient broth is composed of meat extract (1.0 g/L), yeast (2.0 g/L), peptone (5.0 g/L) and sodium chloride (8.0 g/L). Preparation procedure involved dissolving 16 g of nutrient broth in 1000 ml of distilled water, followed by heating with frequent agitation to completely dissolve the medium and autoclaving at 121°C for 15 minutes. The medium was cooled and the V3 bacterium was inoculated into this medium. This medium served as food for the bacteria. This was then incubated for fourteen days on a rotary shaker at 30 °C and 150 rpm.

4.2.4.1 Seed Cultures

A culture medium consisting of meat extract (1.0 g), yeast (2.0 g), peptone (5.0 g) and sodium chloride 8.0 g in 1000 ml distilled water was prepared. The medium (100 ml) in 250 ml conical flask was inoculated and incubated for 2 days on a rotary shaker at 30°C and 150 rpm.

4.2.4.2 Production of Cultures

For the production of cultures, a medium consisting of meat extract (1.0 g), yeast (2.0 g), peptone (5.0 g) and sodium chloride (8.0 g) in 1000 ml distilled water was prepared. The medium (100 ml) in 250 ml conical flasks was inoculated with the seed culture (7.5 ml) and incubated for 14 days at 30°C and 150 rpm.
4.3 Interpolation of \((V_3)\) growth curve

\(V_3\) culture was inoculated into the nutrient broth growth medium (100 ml). This broth was then shaken in a 30°C water bath for 48 hours. Aliquots (5.0 ml) of the broth were taken every 30 minutes and the absorbance was measured at 550 nm using the spectrometer. The remaining sample was stored in a refrigerator to stop further growth. This residual sample was going to be used for further bioassay analysis. The results of this study are shown in Table 6 and are represented graphically in Figure 34.
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4.4  Isolation of an antibacterial agent, anthranilic acid, using solvent extraction and chromatographic techniques.

The bacterial extract was prepared by inoculating 12 L of nutrient broth with V3 Sample, which was seeded for 48 hours. The inoculated broth was shaken for 336 hours at 37°C. Fatty acids were removed using a non-polar solvent, petroleum ether, and ethyl acetate was used for extracting other organic compounds. A ratio of 1:1 for solvent and nutrient broth was used and this was washed three times and the concentrated extracts were tested for antibacterial activity. The whole procedure used for extraction of a bacteriostatic agent is illustrated in Chapter 3, Figure 36.

Inoculated nutrient broth (250 ml) was extracted with petroleum ether (5 x 100 ml) to remove fatty acids. Thereafter, ethyl acetate (5 x 100 ml) was used to extract the organic material from the broth. The organic layer was then dried with anhydrous MgSO4, filtered and evaporated in a rotary evaporator, which resulted in a yellow liquid. Preparative thin-layer chromatography, using multiple elutions with 10 % petroleum ether in ethyl acetate afforded four separate bands. Elution of these bands plus the material on the baseline yielded five fractions. The material on the baseline was extracted with methanol. The fractions were tested for biological activity.

When spotted on the TLC plate, the baseline fraction showed three compounds that were separated with the chromatroton. Further purification of the active fraction was achieved by using preparative chromatography using 5 % methanol in ethyl acetate, and this resulted in the isolation of a yellow solid anthranilic acid, which showed activity against *Serratia marcescens* and *Pseudomonas fluorescens* (0.028 g/L, 0.30 %), mp: (140-145°C, lit104 144-148°C); δH (500 MHz, CDCl3): 7.94 (dd, 1H, 8.1 Hz, 1.7 Hz, Hα) 7.32 (ddd, 1H, 8.1 Hz, 0.7 Hz, 7.0 Hz, Hc), 6.70 (dd, 1H, 8.3 Hz, 0.7 Hz, Hd), 6.66 (ddd, 1H, 8.1 Hz, 0.7 Hz, 7.0 Hz, Hb); δc (500 MHz, CDCl3): 109.5 (C2), 116.5 (C4), 116.8 (C6), 132.1 (C7), 135.1 (C5), 151.1 (C3), and 173.2 (C1); m/z (EI): 137 ([M]+, 100 %), 119 (C7H5NO, loss H2O, 63), 92 (C5H6N, loss CO2 and H+, 81), 65 (38), 52 (12), 39 (24), HRMS: [M]+ 137.04768 C7H7NO2 requires 137.04609; νmax (cm⁻¹): 3400 (s, NH2), 3300 (s, OH), 1660 (s, CO)
Crystal structure determination of anthranilic acid. A yellow-needle shaped crystal was grown by slow evaporation of a saturated ethyl acetate. The general data collected and used for structural determination is described in Tables 6, 7 and 8. All the crystallographic data was analysed and solved by Professor O.Q. Munro of this department. The following information was obtained from the crystallographic data, C7H7NO2, fw =137.14, a = 7.106(2) Å, b = 15.835(4) Å, c = 11.641(3) Å, β= 90°, V=1309.8(6) Å³, monoclinic, Z =8, Dc= 1.391 Mg/m³, μ =0.103 Mg/m³, T = 100(2) K, R1 = 0.0669, wR2 = 0.1311

4.5 Purification of bacteriostatic agent using thin-layer chromatography (TLC)

Different ratios of solvent systems were used to find the best solvent system to use for preparative TLC separation technique. These were 30:70, 50:50 and 70:30 ethyl acetate to petroleum ether, and it was found that the one that gave clear separation was 90:10 ethyl acetate to petroleum ether. On the TLC plate, four fractions were observed. Another fraction was at the baseline. All the other fractions were tested for biological activity. The one at baseline was washed with methanol and concentrated by the rotary evaporator. When spotted on the TLC plate, it showed three compounds that were separated by chromatotron using 5% methanol in ethyl acetate. Further purification was achieved by using preparative chromatography.

4.6 Electrophoresis

To perform an isoelectric focusing (IEF) analysis, anthranilic acid was applied to a polyacrylamide gel and current was applied. The anthranilic acid was focused on an Immobilon 3-10 pH ready strip (Amersham Biosciences) overnight, for 16 hrs and 200 µl of a 0.08021 M was loaded onto the gel. The electrophoresis was done by Dr Mervyn Beukes and the results are shown in Figure 62. The anthranilic acid migrated according to charge density until it reached that part of the gel with a pH corresponding to its pl. At this point, net charge was zero and migration stopped. Most of the compound was focused at a pl of approximately 8.25
4.7 Isolation of bacteriostatic agent by precipitation

The bacterial extract was prepared by inoculating nutrient broth (100 ml) with V3 Sample, which was seeded for 48 h. The inoculated broth was shaken for 336 h at 37 °C. The pH of the resulting broth was adjusted to 8.3 by adding NaOH. The product precipitated out of solution and was filtered and dried to give white crystals of anthranilic acid (1.2 g. 1.2%); mp: (140-145 °C, lit104 144-148); δH (500 MHz, CDCl3): 7.94 (dd, 1H, 8.1 Hz, 1.7 Hz, Hα) 7.32 (ddd, 1H, 8.1 Hz, 0.7 Hz, 7.0 Hz, Hc), 6.70 (dd, 1H, 8.3 Hz, 0.7 Hz, Hd), 6.66 (ddd, 1H, 8.1Hz, 0.7Hz, 7.0Hz, Hb).

4.8 Antibiotic screening test

The agar diffusion method was used. In this method the agar was poured into a petri dish seeded with a test microorganism used for antibiotic screening. Paper discs were dipped into a extract of test compound. After drying, these discs were placed onto the seeded agar and the compound was allowed to diffuse. For instance, the petroleum ether extract was concentrated on paper discs and allowed to diffuse into the agar containing the microorganism for 14 days.
and the results are shown in Figure 63. No inhibition zones were observed; therefore the petroleum ether extract contained none of the antimicrobial active agents. On the other hand when the ethyl acetate extract was concentrated on the paper discs and placed on the agar containing the microorganism, after 14 days as shown in Figure 64, an inhibition zone was observed.

Figure 63: Paper discs saturated with petroleum ether extract on Pseudomonas fluorescens.

Figure 64: Paper discs saturated with ethyl acetate extract on Serratia marcescens.
Chapter 5

5. References

7 Dizman, B., Elasri, M. O., Mathias, L. J., Biomacromolecules, 2004, 6, 514.


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* Described in experimental section


APPENDICES
Table 7: Crystal data and structure refinement for anthranilic acid.

Empirical formula  C₇H₇N O₂
Formula weight  137.14
Temperature  100(2) K
Wavelength  0.71073 Å
Crystal system  Orthorhombic
Space group  P b c a
Unit cell dimensions  a = 7.106(2) Å  Δ = 90°
  b = 15.835(4) Å  Δ = 90°
  c = 11.641(3) Å  Δ = 90°
Volume  1309.8(6) Å³
Z  8
Density (calculated)  1.391 Mg/m³
Absorption coefficient  0.103 mm⁻¹
F(000)  576
Crystal size  0.40 x 0.20 x 0.08 mm³
Theta range for data collection  4.23 to 25.04°
Index ranges  -8 <= h <= 7, -18 <= k <= 18, -13 <= l <= 13
Reflections collected  7566
Independent reflections  1154 [R (int) = 0.1064]
Completeness to theta = 25.00°  99.2 %
Absorption correction  Empirical (DIFABS)
Max. and min. transmission  0.8363 and 0.4890
Refinement method  Full-matrix least-squares on F²
Data / restraints / parameters  1154 / 0 / 103
Goodness-of-fit on F²  1.208
Final R indices [I>2sigma(I)] R1 = 0.0669, wR2 = 0.1311
R indices (all data) R1 = 0.0904, wR2 = 0.1400

Largest diff. peak and hole 0.201 and -0.202 e Å⁻³
Table 8: Atomic coordinates ($x \times 10^4$) and equivalent isotropic displacement parameters ($A^2 x 10^3$) for anthranilic acid. $U(eq)$ is defined as one third of the trace of the orthogonalized $U_{ij}$ tensor.

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Table 9: Bond lengths [Å] and angles [°] for anthranilic acid.

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</tr>
<tr>
<td>O(1)-C(7)-C(1)</td>
<td>123.8(2)</td>
<td></td>
</tr>
<tr>
<td>O(2)-C(7)-C(1)</td>
<td>115.3(2)</td>
<td></td>
</tr>
<tr>
<td>C(6)-N(1)-H(1N)</td>
<td>117(2)</td>
<td></td>
</tr>
<tr>
<td>C(6)-N(1)-H(2N)</td>
<td>113.6(19)</td>
<td></td>
</tr>
<tr>
<td>H(1N)-N(1)-H(2N)</td>
<td>124(3)</td>
<td></td>
</tr>
<tr>
<td>C(7)-O(2)-H(1O)</td>
<td>105(2)</td>
<td></td>
</tr>
</tbody>
</table>

Symmetry transformations used to generate equivalent atoms:
Table 10: Anisotropic displacement parameters ($A^2 \times 10^3$) for anthranilic acid. The anisotropic displacement factor exponent takes the form: $-2\pi^2 \left[ h^2 a^* U^{11} + \ldots + 2 h k a^* b^* U^{12} \right]$

<table>
<thead>
<tr>
<th></th>
<th>$U^{11}$</th>
<th>$U^{22}$</th>
<th>$U^{33}$</th>
<th>$U^{23}$</th>
<th>$U^{13}$</th>
<th>$U^{12}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>C(1)</td>
<td>25(1)</td>
<td>14(1)</td>
<td>23(2)</td>
<td>-2(1)</td>
<td>1(1)</td>
<td>4(1)</td>
</tr>
<tr>
<td>C(2)</td>
<td>30(2)</td>
<td>20(2)</td>
<td>24(2)</td>
<td>0(1)</td>
<td>0(1)</td>
<td>5(1)</td>
</tr>
<tr>
<td>C(3)</td>
<td>34(2)</td>
<td>26(2)</td>
<td>29(2)</td>
<td>1(1)</td>
<td>9(2)</td>
<td>2(1)</td>
</tr>
<tr>
<td>C(4)</td>
<td>29(2)</td>
<td>16(1)</td>
<td>38(2)</td>
<td>-3(1)</td>
<td>5(1)</td>
<td>-2(1)</td>
</tr>
<tr>
<td>C(5)</td>
<td>26(2)</td>
<td>21(2)</td>
<td>33(2)</td>
<td>-6(1)</td>
<td>-2(1)</td>
<td>3(1)</td>
</tr>
<tr>
<td>C(6)</td>
<td>29(2)</td>
<td>14(1)</td>
<td>23(2)</td>
<td>-3(1)</td>
<td>0(1)</td>
<td>5(1)</td>
</tr>
<tr>
<td>C(7)</td>
<td>26(1)</td>
<td>17(1)</td>
<td>18(1)</td>
<td>-3(1)</td>
<td>-1(1)</td>
<td>6(1)</td>
</tr>
<tr>
<td>N(1)</td>
<td>30(1)</td>
<td>32(1)</td>
<td>21(1)</td>
<td>-2(1)</td>
<td>-3(1)</td>
<td>-1(1)</td>
</tr>
<tr>
<td>O(1)</td>
<td>27(1)</td>
<td>26(1)</td>
<td>20(1)</td>
<td>1(1)</td>
<td>1(1)</td>
<td>-2(1)</td>
</tr>
<tr>
<td>O(2)</td>
<td>28(1)</td>
<td>32(1)</td>
<td>22(1)</td>
<td>0(1)</td>
<td>-2(1)</td>
<td>-4(1)</td>
</tr>
</tbody>
</table>
Table 11: Hydrogen coordinates ($x \times 10^4$) and isotropic displacement parameters ($A^2 \times 10^3$) for anthranilic acid.

<table>
<thead>
<tr>
<th>Atom</th>
<th>x</th>
<th>y</th>
<th>z</th>
<th>U(eq)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H(2)</td>
<td>4027</td>
<td>6288</td>
<td>-1870</td>
<td>30</td>
</tr>
<tr>
<td>H(3)</td>
<td>6791</td>
<td>7056</td>
<td>-2034</td>
<td>36</td>
</tr>
<tr>
<td>H(4)</td>
<td>8738</td>
<td>7226</td>
<td>-419</td>
<td>33</td>
</tr>
<tr>
<td>H(5)</td>
<td>7886</td>
<td>6661</td>
<td>1335</td>
<td>32</td>
</tr>
<tr>
<td>H(1N)</td>
<td>5840(50)</td>
<td>5880(20)</td>
<td>2480(30)</td>
<td>52(10)</td>
</tr>
<tr>
<td>H(10)</td>
<td>260(50)</td>
<td>5210(20)</td>
<td>-860(30)</td>
<td>63(12)</td>
</tr>
<tr>
<td>H(2N)</td>
<td>3860(50)</td>
<td>5410(20)</td>
<td>1890(30)</td>
<td>43(9)</td>
</tr>
</tbody>
</table>
Table 12: Torsion angles [$^\circ$] for anthranilic acid.

<table>
<thead>
<tr>
<th>Bond Sequence</th>
<th>Torsion Angle [$^\circ$]</th>
</tr>
</thead>
<tbody>
<tr>
<td>C(6)-C(1)-C(2)-C(3)</td>
<td>-1.2(4)</td>
</tr>
<tr>
<td>C(7)-C(1)-C(2)-C(3)</td>
<td>176.9(2)</td>
</tr>
<tr>
<td>C(1)-C(2)-C(3)-C(4)</td>
<td>1.5(4)</td>
</tr>
<tr>
<td>C(2)-C(3)-C(4)-C(5)</td>
<td>-0.7(4)</td>
</tr>
<tr>
<td>C(3)-C(4)-C(5)-C(6)</td>
<td>-0.4(4)</td>
</tr>
<tr>
<td>C(4)-C(5)-C(6)-N(1)</td>
<td>-177.6(3)</td>
</tr>
<tr>
<td>C(4)-C(5)-C(6)-C(1)</td>
<td>0.7(4)</td>
</tr>
<tr>
<td>C(2)-C(1)-C(6)-N(1)</td>
<td>178.3(3)</td>
</tr>
<tr>
<td>C(7)-C(1)-C(6)-N(1)</td>
<td>0.2(4)</td>
</tr>
<tr>
<td>C(2)-C(1)-C(6)-C(5)</td>
<td>0.1(4)</td>
</tr>
<tr>
<td>C(7)-C(1)-C(6)-C(5)</td>
<td>-178.0(2)</td>
</tr>
<tr>
<td>C(2)-C(1)-C(7)-O(1)</td>
<td>-177.3(3)</td>
</tr>
<tr>
<td>C(6)-C(1)-C(7)-O(1)</td>
<td>0.8(4)</td>
</tr>
<tr>
<td>C(2)-C(1)-C(7)-O(2)</td>
<td>1.7(3)</td>
</tr>
<tr>
<td>C(6)-C(1)-C(7)-O(2)</td>
<td>179.8(2)</td>
</tr>
</tbody>
</table>

Symmetry transformations used to generate equivalent atoms: