ISOLATION AND CHARACTERIZATION OF ANTIBIOTIC(S) PRODUCED BY BACTERIA FROM KWAZULU-NATAL SOILS

By

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B.Tech. Hons [Futa], M.Sc [Natal]

Submitted in fulfillment of the academic requirements for the degree of

DOCTOR OF PHILOSOPHY

In the

Discipline of Microbiology

School of Biochemistry, Genetics, and Microbiology

Faculty of Science and Agriculture

UNIVERSITY OF KWAZULU-NATAL

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December 2010

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ABSTRACT

This work reports the continued search for new antibiotics in the relatively under investigated region of KwaZulu-Natal, South Africa. A soil bacterium designated strain N8 with antibacterial activity against both Gram-positive and Gram-negative bacteria was isolated from a poultry farm in Pietermaritzburg, South Africa. The organism was one of approximately 2600 strains isolated from various habitats in the KwaZulu-Natal midlands, South Africa during an actinomycete screening programme. The highest number of antimicrobially-active isolates came from a forest soil site whereas the lowest number was present in a riparian soil.

Morphological, physiological and cultural characteristics indicated that strain N8 belonged in the genus *Intrasporangium.* In the literature, members of this actinomycete genus have not been associated previously with antibiotic production. Studies on the influence of different nutritional compounds on antibiotic production showed that the highest antibacterial activities were obtained when glycerol at 1% (w/v) was used as sole carbon source in the presence of mineral trace elements.

Using solvent extraction and various chromatographic techniques, the antibiotic produced by strain N8 was recovered from the fermentation broth. The use of a three-solvent system, petroleum ether: acetone: ethyl acetate enhanced the separation of the antibiotic complex in broth. Bioassay results established that the antibacterial agent was in the ethyl acetate fraction (EAF) and chromatographic methods were used in its purification. The chromatographic methods used were: flash column chromatography (FCC), thin-layer chromatography (TLC), and Harrison research chromatotron (HRC). Further purification was carried out by reverse phase high performance liquid chromatography (HPLC). Most of the inactive, coloured material was removed from the antibiotic extract by FCC, while TLC chromatograms run using a range of the most polar to the least polar solvent systems [SS1 (most polar) – SS5 (least polar)] showed best separation of EAF with SS2. TLC chromatograms using SS2 usually showed 3 bands. Bioautograms of SS2-separated EAF revealed that the antibiotic activity was located in the region with an R_f value of 0.56 – 0.64. The Harrison research chromatotron technique also gave good separation of the EAF sample. Preparative HPLC was used as the final purification step for most of the EAF samples. Although, a number of peaks were observed during isocratic-HPLC (IHPLC) runs, they were not as clearly separated as those obtained with gradient-HPLC (GHPLC).

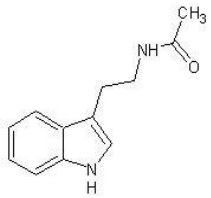
Three major peaks PI, PII and PIII with elution times of 3.56 min, 4.53 min and 23.06 min respectively were revealed under GHPLC runs with decreasing concentrations (100% – 50%) of methanol in water. Methanol concentrations between 50% and 70% in water were considered the optimum GHPLC mobile phases.

Since these chromatographic methods were all time consuming, required large volumes of solvents, and resulted in low yields of the antibiotic, an alternative procedure producing better results was sought. This led to the development of a procedure combining a three-solvent extraction system with a pH precipitation process which efficiently recovered the antibiotic in solid/crystal form. Using this procedure, sufficient quantities of the antibiotic were recovered from the fermentation broth to permit a degree of structural elucidation. Two types of crystals (brown and pink-yellow in colour) were obtained and their chemical natures established by means of ^{1}H- and GCOSY- nuclear magnetic resonance (NMR) and liquid chromatography-mass spectrometry (LC-MS). On further LC-MS analysis, the brown crystals appeared to be a protein and since it did not show inhibitory activity against any of the test organisms, no further studies were carried out on it. The pink-yellow crystals when suspended in a minimal volume of methanol showed inhibitory activity against *S. marcescens* confirming that the antibiotic activity resided therein. The LC-MS spectrum of these crystals showed a prominent/base peak at 304.2724 [mass to charge ratio (m/z) in positive mode]. The elemental composition of this compound suggests a molecular formula close to $C_{16}H_{36}N_2O_3$ with a molar mass of 304.4686 g/mol. No existing name could be assigned to it from the database of known natural compounds. Hence, the possibility that it is a novel antimicrobial compound cannot be excluded.

Characterisation of the antimicrobial substance using GC-MS revealed that it contained at least seven components (A – G). These components were then subjected to mass spectrum analysis and their retention indices compared to computer database listings of known compounds. Components A and B were regarded as representing one compound (possibly isomers) since they have the same molecular weight and formula. Their different retention indices strongly suggest they are indeed isomers. Thus a total of six different compounds were detected in the extract by GC-MS and the molecular formulae assigned to them include: $C_6H_{10}O$ (A and B); $C_6H_{12}O_2$ (C); $C_9H_{14}O$ (D); C_8H_7N (E); $C_{21}H_{44}$ (F); and $C_{12}H_{14}N_2O$ (G). Since only low probability matches were obtained for A – F and as the sample could not be recovered from the analyser, they were not studied further. The closest match (71% probability) with substances listed in the computer database of natural compounds was for compound G ($C_{12}H_{14}N_2O$) which was thus provisionally identified as

N-acetyltryptamine. A structurally related compound known as melatonin is attributed with the ability to inhibit tumour growth *in vivo* and *in vitro*.

Attempts were made to assign a chemical structure to the antibiotic produced by strain N8 using all the data available. The indications are that it is a tryptamine, the chemical structure of which is postulated to be:



In order to monitor the antimicrobial activity of the antibiotic produced by strain N8, bioassays were conducted after all major steps during the isolation and characterization processes. The antimicrobial activity of the pink-yellow crystals was confirmed on the test organisms used during the primary screening phase, namely, *Escherichia coli, Pseudomonas fluorescens, Serratia marcescens, Staphylococcus aureus, Enterococcus faecalis* and *Xanthomonas campestris* pv. *campestris*, and the yeast *Candida utilis*, indicating that the crude substance had maintained its inhibitory activity against Gram-positive and Gram-negative bacteria, and the yeast tested. The study was extended to include investigations into the use of combinations of the GHPLC separated peaks of the antibiotic (PI, PII and PIII) to improve the efficacy of growth inhibition of the test pathogens for possible use in chemotherapy. Data from these studies showed that PI inhibited the growth of *E. coli* and *X. campestris* pv. *campestris* while PII and PIII inhibited the growth of the latter organism and also that of *S. marcescens*. Individually, the peaks showed no growth inhibition on *Pseudomonas fluorescens* but the combination PI+PII+PIII was antimicrobially effective. In all cases, the use of combinations was significantly more effective than the use of any single component alone. For example, the combination of GHPLC PI and PII had a greater growth inhibitory effect (synergic action) against *Serratia marcescens* than did either alone; the inhibition-zone diameter being double (30mm) that caused by the single peaks (15mm) against *S. marcescens*. Likewise mixing PI and PIII resulted in a much improved action against *X. campestris* pv. *campestris*. These findings may meet the current call by many scientists that all infectious diseases should be treated with a combination of two antibiotics with different mechanisms of action in order to counter the serious problem of emerging bacterial resistance.

Since the antibiotic isolated during this study showed activity against both mammalian and plant pathogenic bacteria it is hoped that this work will encourage further investigation in this field in South Africa. The results obtained should impact on the pharmaceutical industry as well as agriculture and will, hopefully, help curb both plant and human infectious diseases in our African communities. This study also confirmed that KwaZulu-Natal soils do harbour rare actinomycetes that produce novel antimicrobial compounds.

PREFACE

The experimental work described in this thesis was carried out in the School of Biochemistry, Genetics & Microbiology; University of KwaZulu-Natal, Pietermaritzburg, South Africa, from January 2004 to December 2009, under the supervision of Professor F.M. Wallis.

These studies represent original work by the author and have not otherwise been submitted in any form for any degree to any tertiary institution. Where use has been made of the work of others it is duly acknowledged in the text.

DECLARATION 1 – PLAGIARISM

I, Vincent Ifeanyi Okudoh declare that

1. The research reported in this thesis, except where otherwise indicated, is my original research.

2. This thesis has not been submitted for any degree or examination at any other university.

3. This thesis does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.

4. This thesis does not contain other persons writing, unless specifically acknowledged as being sourced from other researchers. Where other written sources have been quoted, then:

    a. Their words have been re-written but the general information attributed to them has been referenced
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V. I. Okudoh (Candidate)

Signed……
Prof. F.M. Wallis (Supervisor)

DECLARATION 2 – PUBLICATIONS

DETAILS OF CONTRIBUTIONS TO PUBLICATIONS that form part and /or include research presented in this thesis

Publication 1: Okudoh, V.I and Wallis, F.M. (2007). Antimicrobial activity of rare actinomycetes isolates from natural habitats in KwaZulu-Natal, South Africa. Article published in *South African Journal of Science*, May/June 2007, Vol. **103** No.5/6 pp 216 – 222.

Publication 2: Okudoh, V.I., Laing, M.D. and Wallis, F.M. (?). Improved method for isolation of antibiotics produced by *Intrasporangium* strain N8: Article submitted for publication to the *Canadian Journal of Microbiology*.

Signed: ……
V. I. Okudoh (Candidate)

Signed: ……
Prof. F.M. Wallis (Supervisor)

DEDICATION

This research is dedicated to my whole family: my mother Josephine, my late father Chief Dennis, my wife Irene and my sons Frederick and Michael; including my brothers and sisters; Chudi, Chibuzor, Mbanefo, Nkem and Obianuju for their unconditional and everlasting love.

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ACKNOWLEDGEMENTS

My sincere thanks go to the following individuals and organisations for their time and assistance that led to the successful completion of this thesis:

Professor F. M. Wallis, my supervisor, for expert criticism and superb mentorship. His fatherly guidance and support, right from the time I joined his research team as a Masters student, cannot be quantified in words; Prof. Mark Laing, for his financial support and stimulating suggestions during the developmental stages of the research project; Prof. Fanie van Heerden for productive discussions on chemical aspects of the work.

I thank the following from UKZN for their help, guidance and support: Dr Anna Soares for her expert advice in organic chemistry and extraction procedures; Nicky and Marnie Light in gradient HPLC analysis; Prof. S. Drewes in structural elucidation of the antibiotic compound; Craig Grimmer in NMR analysis; Dr Colin Southway in GCMS analysis; Caryl Janse van Reusberg in LCMS analysis; Marylin Christian and Lindiwe Khumalo in solvent extraction. The staff in microbiology: Diane Fowlds, Igrid Schlosser and Kerry Lindsey for supplying the test organisms; Natalie Jones and Prof Stefan Schmidt for their timely and valuable advice.

Thanks also to my fellow postgraduate students and post-docs: Dr Kwesi Yobo, Habtom, Abraham, Sanisha Naidoo, Kim Govender, Sam Yirenkyi and Daniel Teclu for encouragement and stimulating criticisms. The National Research Foundation (NRF) of South Africa for partial financial support.

LIST OF ABBREVIATIONS

AC	acetone
AF	acetone fraction
ATP	adenosine triphosphate
AZT	azidothymidine
CCD	countercurrent distribution
CI	chemical ionisation
COSY	correlated spectroscopy
d	doublet
DAP	diaminopimelic acid
dd	doublet of doublets
DNA	deoxyribonucleic acid
EAF	ethyl acetate fraction
EI	electron impact
eV	electron volt
ESI-MS	electrospray ionisation mass spectrometry
FCC	flash column chromatography
GC	gas chromatography
GC-MS	gas chromatography mass spectrometry
GCOSY	gradient correlation spectroscopy
GHPLC	gradient high-performance liquid chromatography
HIV	human immunodeficiency virus
^{1}H-NMR	proton nuclear magnetic resonance
HPLC	high-performance liquid chromatography
HRC	Harrison research chromatotron
HR-MS	high-resolution mass spectrometry
IC	ion chromatography
IGHPLC	isocratic high-performance liquid chromatography
IR	infrared
IT	ion trap
J	spin-spin coupling constant in Hz
LC-MS	liquid chromatography mass spectrometry
m/z	mass-to-charge ratio
M^{+}	molecular ion

MALDI-MS	matrix-assisted laser desorption/ionization mass spectrometry
MBC	minimal bactericidal concentration
MeOH	methanol
MF	methanol fraction
MIC	minimum inhibitory concentration
Mp	melting point
MRSA	methicillin resistant *Staphylococcus aureus*
MS	mass spectrometry
NAG	n-acetyl glucosamine
NAM	n-acetyl muramic acid
NMR	nuclear magnetic resonance
NRPS	nonribosomal peptide synthetase
ODS	octadecylsilane
PBPs	penicillin binding proteins
PE	petroleum ether
PKS	polyketide synthetase
ppm	parts per million
PPM	peak-peak mix
QqQ	triple-quadrupole
R_f	retarding factor
RNA	ribonucleic acid
SEM	scanning electron microscope
sp	species
SS	solvent system
TLC	thin-layer chromatography
TOF	time-of-flight
UDP	uridine diphosphate
UTP	uridine triphosphate
UV	ultraviolet
VRSA	vancomycin resistant *Staphylococcus aureus*
WCSP	whole cell sugar pattern

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CHAPTER 1

LITERATURE REVIEW

1.1 INTRODUCTION

"A perfect storm is brewing in infectious diseases" …Scheld, 2003.*

"Infectious diseases physicians are alarmed by the prospect that effective antibiotics may not be available to treat seriously ill patients in the near future. There simply aren't enough new drugs in the pharmaceutical pipeline to keep pace with drug resistant bacterial infections, so-called 'superbugs." … Dalovisio, 2004[#]

Of the thousands of antibiotics discovered over the past ten decades, none has as yet produced a definitive answer to the apparent question of 'microbial resistance'. According to Richard Laing, an expert on medicine policy at the World Health Organization in Geneva, "The relative lack of research on anti-microbials is a matter of concern and there is clearly a potential risk, due to growing antibiotic resistance, that there won't be any effective anti-microbial available in the future" (Hirschler and Ransdell, 2004). The Infectious Diseases Society of America, representing more than 7,500 physicians and scientists, said in a recent report that a "perfect storm" was brewing in infectious diseases (Hirschler and Ransdell, 2004). Microbial resistance to treatment could bring the world back to a pre-antibiotic age (Schmidt, 2004).

Many drug companies in the United States and Europe first made their fortunes from antibiotics discovered through natural products screening, mostly of soil microorganisms. Eventually the collections, or libraries, of organisms assembled in the 1940s and 50s began failing to produce new antibiotics. By the 1970s and 80s, the big pharmaceutical companies started making modifications to the existing basic classes of antibiotics instead of searching for new ones from nature (Erickson, 2003).

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*Scheld, W.M. (2003b). Potential NIAID solutions to the problem posed by emerging and resistant microorganisms. Letter to *Infectious Diseases Society of America*

[#]Dalovisio, J.R. (2004): Bad bugs, No drugs: As antibiotic discovery stagnates, a public health crisis brews. *Infectious Diseases Society of America* report

New versions of antibiotics introduced are described as if they were succeeding generations of a family. Thus, penicillin G and V for example, are called first-generation antibiotics, whereas later examples of the basic penicillin structure have been called second-generation, third-generation, etc. We are currently in the fourth generation of antibiotics about 60 years after their discovery and first use (Walsh and Wright, 2005).

This modification approach has failed because resistance is developed easily to these antibiotics by bacteria which have had billions of years of evolution to cope with harsh environments and can withstand any chemical attack (Projan and Bradford, 2007). This failure is evident in the obvious innovation gap between 1960 and 2000 (Table 1.1). During that period, only three new classes of antibiotics were approved (Walsh and Wright, 2005).

Table 1.1 Classes of antibiotics and their year of approval for clinical use

Classes of antibiotics	Year approved
β- Lactams	1940
Chloramphenicol	1949
Tetracyclines	1949
Aminoglycosides	1950
Macrolides	1952
Quinolones	1962
Streptogramins	1962
Oxazolidinones	2000
Lipopeptides	2003
Glycyclines	2005
Mutilins	2007

Source: Gill (2008)

Over the last decade, it has become clear that the widespread emergence of acquired resistance to antibiotics in bacteria still constitutes a serious threat to public health, hence the need to search for antibiotics with new modes of action (Salyers and Whitt, 2005, Spellberg *et al*., 2008; Fischbach and Walsh, 2009).

The multinational drug companies complain of many problems: the lack of chemical diversity against targets, the difficulty of differentiating new antibiotics without

embarking on huge trials, the financial risks of proving novelty and the habit of reserving truly new antibiotics by hospitals for emergencies (Schmidt, 2004). Of the more than 400 agents described since 1998, only nine new antibiotics have been approved, of which just two, Linezolid (oxazolidinone) and Telithromycin (ketolide) had a novel mechanism of action (Gill, 2008). The 2004 annual reports of major drug companies list a mere five new antibiotics in development. These figures paint a gloomy picture of the fight against infectious diseases (Gill, 2008). Economies of scale usually make it possible for big pharmaceutical companies to produce antibiotics more cheaply for big markets. Thus, the major research inputs on antibiotics relate directly to those diseases occurring within the developed countries and not to those in the developing countries where the need is greatest (Smith, 1986). Although the range of antibiotic therapy is broad, there are still no effective antibiotics for many infecting and disease-producing microorganisms, particularly the diseases predominant in the developing world (Miyo, 2006)*.

As hospitals struggle to deal with superbugs, such as methicillin resistant *Staphylococcus aureus* (MRSA), and with antibiotic resistance in pathogenic organisms in general, the search for new antibiotics has a new sense of urgency. The search for new antibiotics follows certain basic principles (Chopra *et al*., 1997):

- the first is an account of the geographical distribution of the antibiotic-producing microorganisms
- the second is that the producers should be rapidly and properly identified
- the third is concerned with the isolation and purification of the active compounds
- fourthly structural elucidation of the compounds is necessary to demonstrate their novelty
- These requirements can be extended to include investigations into the use of combinations of the compounds to improve the efficacy of growth inhibition of the test pathogens for use in chemotherapy since this is the current trend rather than prescribing single antibiotic treatment for infectious diseases (Balows, 2006).

* Personal communication. Higher education research training attended at the Institute of Development Management, Gaborone, Botswana in September, 2006.

The present investigation has followed these principles in the search for new antibiotics from local sources. The thesis is divided into six chapters: Chapter 1 is a review of the relevant literature; Chapter 2 focuses on the materials and methods used; Chapter 3 covers the isolation and geographical distribution of the antibiotic-producing strains isolated and their subsequent identification. Chapters 4 and 5 describe respectively the purification and structural elucidation of the antibiotic complex isolated; Chapter 6 focuses on the synergistic antimicrobial activities of the compounds produced. Each chapter starts with an introduction and links with the chapter on the materials and methods used in the investigation. To avoid unnecessary repetitions all references are placed together in a general 'References' section at the back of the thesis. The thesis starts with an introduction and ends with an overview. The overview reflects on the achievement (or otherwise) of the original objectives and gives some recommendations for future research.

1.1.1 Aims and objectives

KwaZulu-Natal soils have been reported as a potential source of novel antibiotic producing microorganisms (Baecker and Ryan, 1987; Ntuli, 1994; Okudoh and Wallis, 2007). For example, the organism, *Streptomyces natalensis*, first used in industrial production of Pimaricin, was originally isolated from Natal soils (Baecker and Ryan, 1987).

The main aim of this study was to isolate and identify antibiotic-producing non-streptomycetous actinomycetes from soils and other habitats in the KwaZulu-Natal midlands. The next undertaking was to purify and characterize the most promising of the many strains isolated, viz. *Intrasporangium* stain N8 isolated from a poultry farm in Pietermaritzburg, South Africa.

Other objectives were:

- To optimize production of the antibiotic by manipulation of the fermentation parameters and to test its inhibitory efficacy against selected human and plant pathogens.
- To isolate and purify the biologically active substance(s) using thin layer chromatography (TLC), Harrison research chromatotron and high performance liquid chromatography (HPLC).

- To elucidate, as far as possible, the structure of the biologically active substance(s) using nuclear magnetic resonance (NMR), liquid chromatography/mass spectrometry (LCMS), gas chromatography/mass spectrometry (GCMS) and other physical techniques.

- To investigate the use of combinations of the active substances to improve the efficacy of inhibition of the test pathogens and to compare their synergistic activities with some of the established antibiotics.

It is hoped that the results of this study will impact on the pharmaceutical industry as well as on agriculture and will contribute to the control/curbing of both plant and human infectious diseases in sub-Saharan African communities.

1.2 What are antibiotics?

The term antibiotic was first introduced by Selman Waksman in 1947 to describe, “A chemical substance derived from microorganisms, which has the capacity of inhibiting growth, and even destroying other microorganisms in dilute solutions” (Waksman, 1947). According to Gottlieb and Shaw (1967) antibiotics are “organic substances that are produced by microorganisms and are harmful at low concentrations to growth and metabolic activities of other organisms”. This definition was limited by Lancini and Parenti in 1982 to special inhibitory products of low molecular weight and excluded enzymes, lactic acid, ethanol and other similar substances that prevent growth of some microorganisms.

Debate over the definition of antibiotics has continued. Forsdyke in 2000 included synthetic molecules like AZT in his definition on the basis that they are derivatives of microbial products. According to him an antibiotic is “a chemical of natural or synthetic origin, which at low concentrations inhibits microorganisms of some type within a host organism, while not unacceptably interfering with the life of that organism”. Carlberg (2000) opposed the use of ‘synthetic origin’ in the definition and suggested that AZT and most of the other agents used to treat HIV infections should be referred to as antiviral agents, antiviral drugs or perhaps antimicrobics, because they are synthetic molecules, and the term ‘antibiotic’ should be reserved for such familiar compounds as penicillin and streptomycin.

Most microbiologists are more comfortable with definitions that exclude any synthetic product (Okudoh, 2001) and the present work should be read in that context.

1.3 Microbial sources of antibiotics – General overview

Over 10000 different antibiotics have been isolated from cultures of gram-positive and gram-negative bacteria and of filamentous fungi. However, only about 100 of these have been commercially used to treat human, animal and plant diseases. The reason for this is that only compounds with selective toxicity can be used clinically (Walsh, G. 2003).

Many medically useful antibiotics are produced by members of the genus *Bacillus* e.g. polymyxin and bacitracin produced by *B. polymyxa* and *B. licheniformis* respectively. The classical β-lactam antibiotics, penicillin and cephalosporin, are synthesized by the filamentous fungi *Penicillium* and *Cephalosporium*, but can also be produced by some actinomycetes and other bacteria. However, the actinomycetes, mainly *Streptomyces* species, are responsible for the synthesis of more than 60% of the known antibiotics while a further 15% are made by members of the related actinomycetes: *Micromonospora*, *Actinomadura*, *Actinoplanes*, *Nocardia*, *Streptosporangium*, *Streptoverticillium* and *Thermoactinomyces* (Okudoh, 2001). Thus, almost all commercially used antibiotics are produced by three groups of microorganisms – *Streptomyces*, *Bacillus* and the filamentous fungi (Miyadoh, 1993).

These organisms all have in common that they live in soil and they form some sort of spore or resting structure. It is not known why these microorganisms produce antibiotics, but the answer may be that it affords them some nutritional or spatial advantage in their habitat by antagonizing the competition; or it acts as some sort of hormone or signal molecule associated with sporulation or dormancy or germination (Todar, 2009). Actinomycetes are the producers of most of the clinical important antibiotics (Miyadoh, 1993) and thus will be discussed in more detail.

1.3.1 Antibiotic formation by actinomycetes

Actinomycetes are mainly gram-positive bacteria that form branching filaments or hyphae and asexual spores. They are generally aerobic and have a high percentage of G+C content (about 60 – 70%). They include a very diverse group of organisms such

as streptomycetes, nocardioform and corynebacterium with lots of properties in common (Okudoh, 2001).

Actinomycetes are important from a medical point of view. They constitute a nuisance when they decompose rubber products, grow in aviation fuel and produce odorous substances that pollute water supplies, or grow in sewage treatment plants where they form thick clogging foams. In contrast, they are very useful when they produce clinically important antibiotics. Close to 50% of the known antimicrobial products are produced by actinomycetes (Miyadoh, 1993).

The discovery of new molecules from actinomycetes marked an epoch in antibiotic research and subsequent developments in antibiotic chemotherapy. Since the discovery of streptomycin, a large number of antibiotics, including major therapeutic agents such as aminoglycosides, chloramphenicol, tetracyclines, macrolides and more recently, members of the β-lactam cephamycin group, have been isolated from cultures of *Streptomyces* and *Streptoverticillium* (Atlas of actinomycetes, The society for actinomycetes, Japan 1997).

Streptomyces species have long been recognised as the best antibiotic-producing bacteria and several strains have been rigorously screened by the pharmaceutical industry.Consequently, the chances of isolating a new compound have substantially diminished. More current efforts are concentrated on unusual or difficult to isolate microbes, mostly non-streptomycetous actinomycetes or rare actinomycetes (Bull *et al*., 2000; Lazzarini *et al*., 2000).

1.3.2 Antibiotic formation by rare actinomycetes

As more new antibiotics were discovered, the chances of finding novel antimicrobial leads among conventional actinomycetes dwindled. The focus of industrial screening therefore moved to markers of less exploited genera of rare actinomycetes such as *Actinomadura*, *Actinoplanes*, *Amycolatopsis*, *Dactylosporangium*, *Kibdelosporangium*, *Microbispora*, *Micromonospora*, *Planobispora*, *Streptosporangium* and *Planomonospora* (Lazzarini *et al*., 2000).

The importance of the rare actinomycetes is demonstrated by the fact that many of the successful antibiotics on the market are produced by such actinomycetes; rifamycins are produced by *Amycolatopsis mediterranei*, erythromycin by *Saccharopolyspora erythraea*, teicoplanin by *Actinoplanes teichomyceticus*, vancomycin by *Amycolatopsis orientalis* and gentamicin from *Micromonopsora purpurea* (Lancini and Lorenzetti, 1993).

The richest group of rare actinomycetes is represented by *Actinoplanes* strains which have been extensively isolated by exploiting the mobility of their spores and their chemotactic behaviour to different saline or organic solutions (Palleroni, 1980; Hayakawa *et al*., 1991; Lancini and Lorenzetti, 1993). More than 120 antibiotics have been reported from *Actinoplanes* strains. Amino acid derivatives, such as peptides and depsipeptides are prevalent and among these compounds some are of clinical relevance such as teicoplanin, from *Actinoplanes teichomyceticus* ATCC 31121 (Bardone *et al*., 1978) and ramoplanin from *Actinoplanes* sp. ATCC 33076 (Ciabatti and Cavalleri, 1989).

Another group of rare actinomycetes isolated on a massive scale in recent years are those belonging to the genus *Micromonospora,* which is considered to encompass the second largest group of culturable actinomycetes in soil. Their isolation has been mainly based on the use of antibiotics such as gentamicin and novobiocin as selective agents (Williams and Wellington, 1982) or deleterious agents such as phenol and chlorhexidine gluconate solutions (Hayakawa *et al*., 1991). Important antibiotics produced by *Micromonospora* strains include the aminocyclitols, such as gentamicins; sisomicin; fortimicin; macrolides, such as the mycinamicins and rosamicins and polysaccharide antibiotics such as everninomycin (Lancini and Lorenzetti, 1993).

Among the different genera and families belonging to the order *Actinomycetales*, the genus *Streptomyces* is followed – in terms of the number of strains producing antibiotics - by the family *Micromonosporaceae* (mainly *Micromonospora* and *Actinoplanes*), the family *Pseudonocardiaceae* (mainly Amycolatopsis, *Sacharopolyspora* and *Sacharothrix*) and the family *Thermomonosporaceae* (mainly *Actinomadura*), the family *Nocardiaceae* (*Nocardia* and related genera), the family *Streptosporangiaceae* (mainly *Streptosporangium*) [Lazzarini *et al*., 2000]. The relative distribution of the producing strains among rare actinomycetes is shown in **Figure 1.1**.

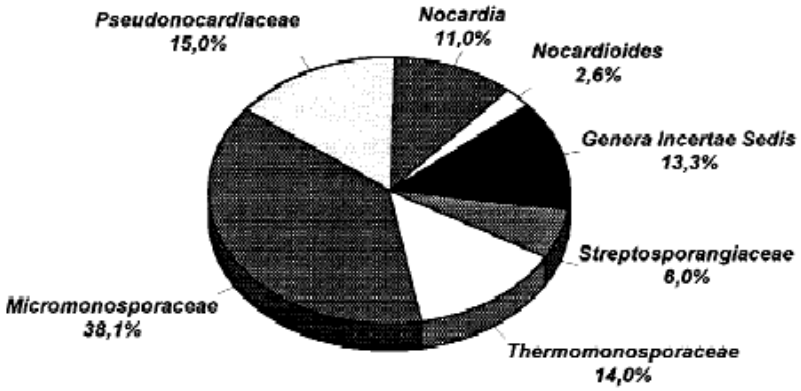


Fig. 1.1 Relative distribution of producing strains among rare actinomycetes: 1,578 producers of bioactive compounds described in the antibiotic literature database (ABL) database are divided into different families and genera according to the recent classification published in the Atlas of Actinomycetes Japan.

The focus organism in the present study, *Intrasporangium,* is also a rare actinomycete, with only one type species *Intrasporangium calvum*. The organism produces branching mycelium which has a definite tendency to fragment, non-motile sporangiospores, and also sporangia intercalary in the mycelial hyphae. *Intrasporangium* is classified as a member of the family *Actinoplanaceae*. To the author's knowledge, no antibiotic formation has been earlier reported for *Intrasporangium* sp.

1.4 Groups of antibiotics – General overview

Various schemes of grouping antibiotics have been proposed, none of which has been adopted (Lancini *et al*., 1995). These include:

- grouping on the basis of the producer organism. However, some organisms may produce several antibiotics, e.g., the production of penicillin-N and cephalosporin C by *Cephalosporium acremonium* (Berdy, 1980).
- grouping according to the route of biosynthesis. This grouping failed because several biosynthetic routes often have much commonality (Okudoh, 2001).
- grouping according to modes of action, i.e., whether they are cell-wall or cell membrane inhibitors, inhibit protein or nucleic acid synthesis, or interfere with the whole system of cellular metabolism. However, several mechanisms of action may operate simultaneously making such classification difficult (Berdy, 1974).

- Currently, those natural and semisynthetic antibiotics that have a common chemical structure are grouped into one “class” and named after the member first discovered or after a principal chemical property (Todar, 2009). The chemical grouping is very useful in practice, as the components of one group usually share many biological properties and also it enables the accommodation of new compounds as they are discovered (Lancini *et al*., 1995). In this work, the groupings according to chemical structure and modes of action will be followed.

1.4.1 Grouping according to chemical structure

Antibiotics are a very heterogeneous group chemically (Lancini and Parenti, 1982). They include substances of molecular weights from 150 to 5000 daltons. The molecules may contain only hydrogen and carbon, or, more commonly, carbon, oxygen, hydrogen and nitrogen. Other molecules also contain sulphur, phosphorus or halogen atoms. Almost all organic structures are represented (aliphatic chains, alicyclic chains, aromatic rings, heterocycles, carbohydrates, polypeptides, etc.). The chemical structures of some commonly used antibiotics are shown in **Figure 1.2** and reveal this diversity in structures. For example, amphotericin B, diflucan, flucytosine and griseofulvin are all antifungal antibiotics but their structures are very different. Likewise, ampicillin, vancomycin, streptomycin, erythromycin, tetracycline, etc., are all antibacterial antibiotics with diverse structures. In fact, the only property that all antibiotics have in common is that they are organic solids, which is obvious from their definition as products of microbial metabolism (Lancini and Parenti, 1982).

There are thirteen main groups of antibiotics based on chemical structure (Berdy, 1974). However, the number of groups may increase to fifteen if the semi-synthetic penicillins and tetracyclines are included (Todar, 2009). A summary of chemical groups of antibiotics and their properties, including their biological source and mode of action, is shown in **Table 1.2.**

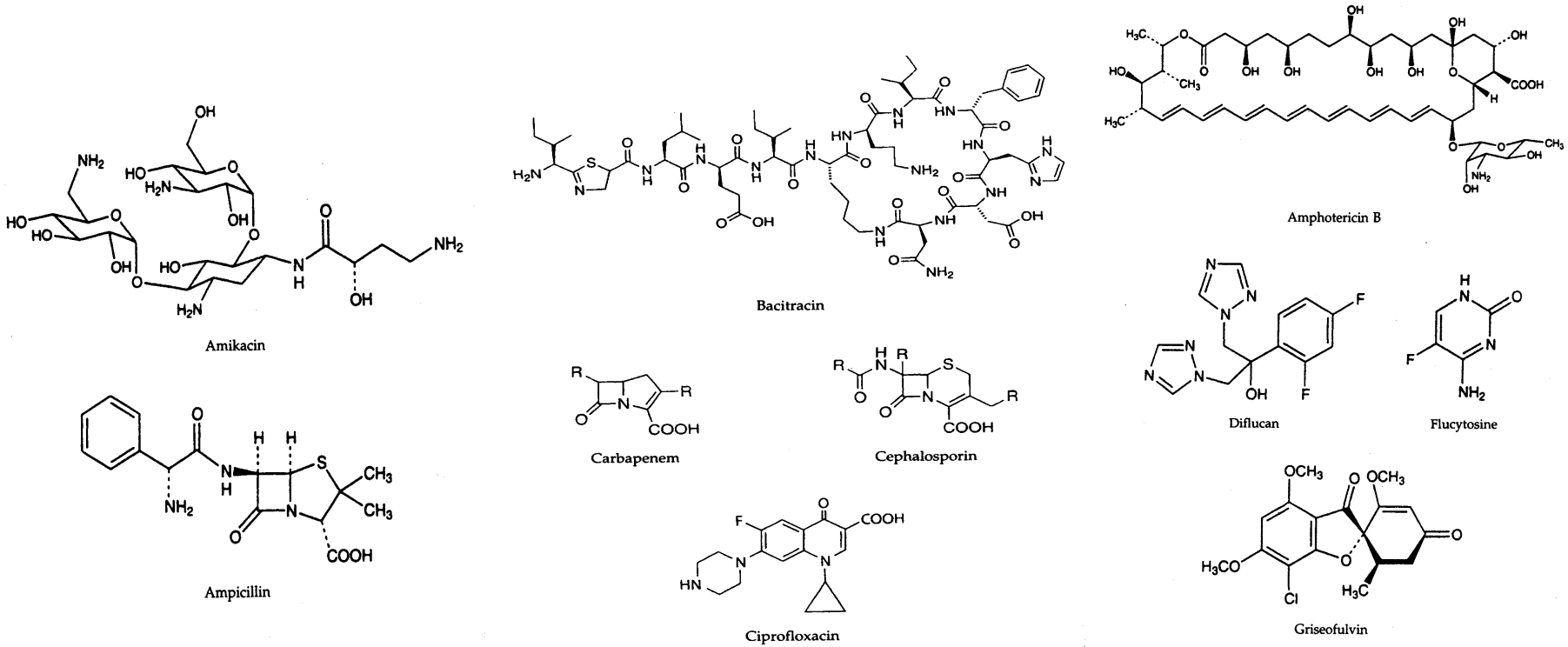
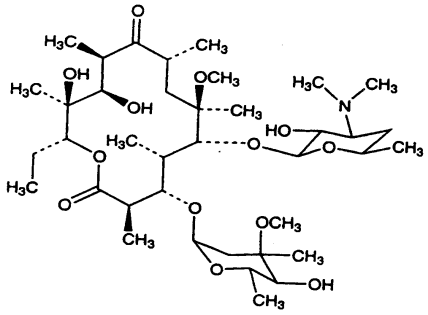
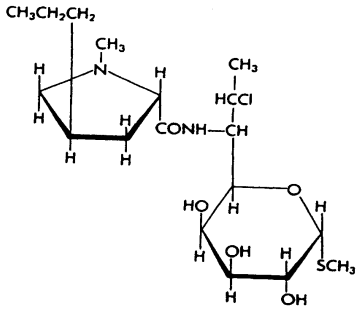


Fig. 1.2 Examples of structures of some commonly used antibiotics. Note the diversity of structures. Source: Salyers and Whitt, (2005).

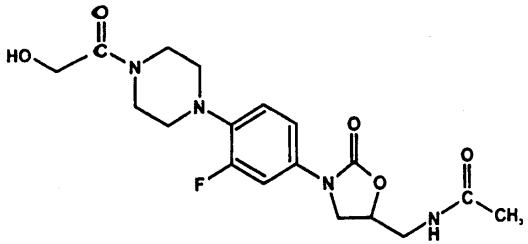
Fig. 1.2 continued



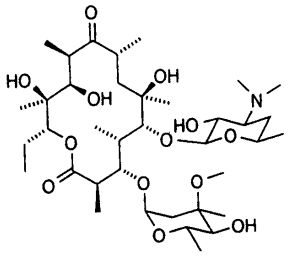
Clarithromycin



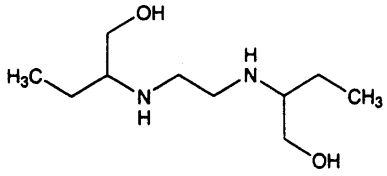
Clindamycin



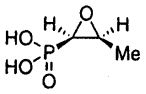
Eperezolid



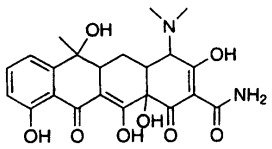
Erythromycin



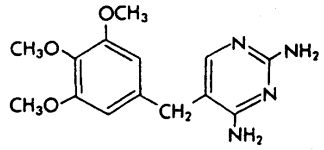
Ethambutol



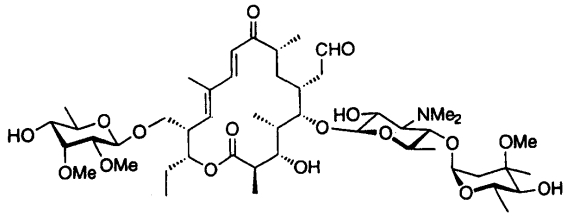
Fosfomycin



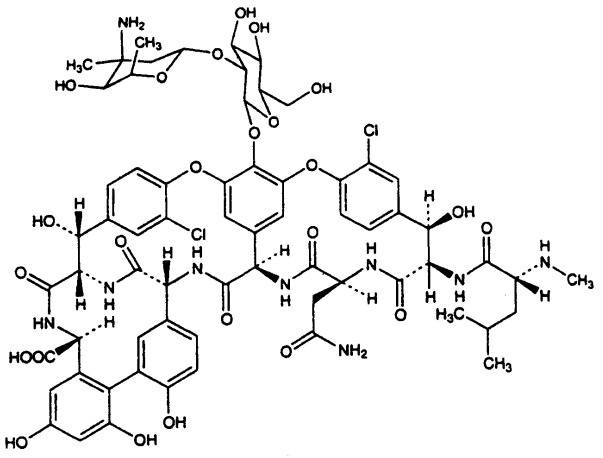
Tetracycline



Trimethoprim

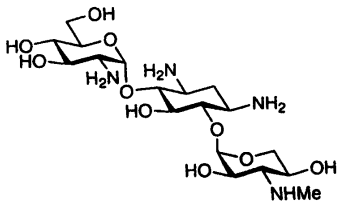


Tylosin

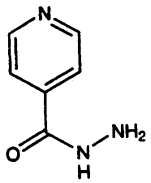


Vancomycin

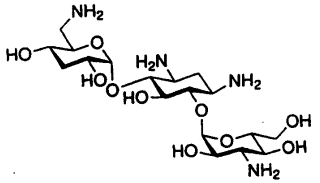
Fig. 1.2 continued



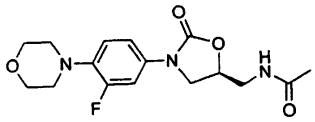
Gentamicin



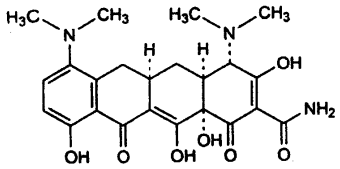
Isoniazid



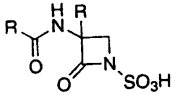
Kanamycin



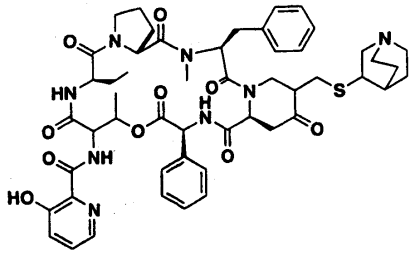
Linezolid



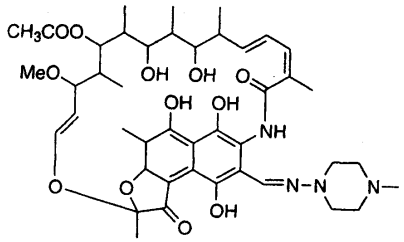
Minocycline



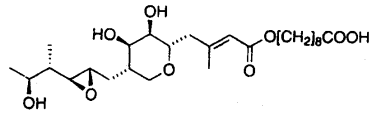
Monobactam



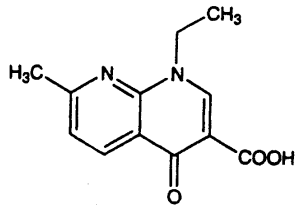
Quinupristin



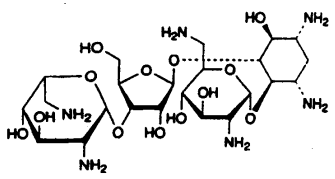
Rifampin



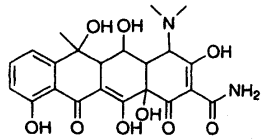
Mupirocin



Nalidixic acid



Neomycin



Oxytetracycline

Table 1.2 Chemical groups of antibiotics and their properties

Chemical group	Examples	Biological source	Spectrum	Principal chemical property	Mode of action
Beta-lactams					
Penicillins	Penicillin G	*Penicillium notatum*	Gram-positive bacteria	β-lactam ring (Lancini *et al*, 1995; Ophardt, 2003)	Inhibits steps in cell wall (peptidoglycan) synthesis and murein assembly
Cephalosporins	Cephalothin	*Cephalosporium* species	"	"	"
Semisynthetic β- lactams					
Aminopenicillins	Ampicillin, Amoxycillin		Gram-positive and Gram-negative bacteria	β-lactam ring (Lancini *et al*, 1995; Ophardt, 2003)	Inhibits steps in cell wall (peptidoglycan) synthesis and murein assembly
Clavulanic acid	Clavamox is clavulanic acid plus Amoxycillin	*Streptomyces clavuligerus*	"	"	Suicide inhibitor of beta-lactamases
Monobactams	Aztreonam	*Chromobacter violaceum*	"	"	Inhibits steps in cell wall (peptidoglycan) synthesis and murein assembly
Carboxypenems	Imipenem	*Streptomyces cattleya*	"	"	"
Peptides					
Polypeptides	Polymyxin	*Bacillus polymyxa*	Gram-negative bacteria	Peptide linked D and L amino acids (Wilson *et al.*, 2004)	Damages cytoplasmic membranes
	Bacitracin	*Bacillus subtilis*	Gram-positive bacteria	"	Inhibits steps in murein (peptidoglycan) biosynthesis and assembly
Glycopeptides	Vancomycin	*Streptomyces orientales*	Gram-positive bacteria, esp. *Staphylococcus aureus*	Peptide linked D and L amino acids with carbohydrate (Wilson *et al.*, 2004, IUPAC, 2009)	Inhibits steps in murein (peptidoglycan) biosynthesis and assembly
Lincomycins	Clindamycin	*Streptomyces lincolnensis*	Gram-positive and Gram-negative bacteria esp. anaerobic *Bacteroides*	6,8-dideoxy-6-aminooctose lincosamine (Spizek and Rezanka, 2004)	Inhibits translation (protein synthesis)

Table 1.2 continued

Chemical group	Examples	Biological source	Spectrum	Principal chemical property	Mode of action
Aminoglycosides	Streptomycin	*Streptomyces griseus*	Gram-positive and Gram-negative bacteria	Amino sugars in a glycosidic linkage (Dale-Skinner and Bonev, 2008)	Inhibit translation (protein synthesis)
	Gentamicin	*Micromonospora* species	Gram-positive and Gram-negative bacteria esp. *Pseudomonas*	"	"
Macrolides	Erythromycin	*Streptomyces erythreus*	Gram-positive bacteria, Gram-negative bacteria not enterics, Neisseria, Legionella, Mycoplasma	Lactone rings linked through glycoside bonds with amino sugars (Todar, 2009)	"
Polyenes	Amphotericin B	*Streptomyces nodosus*	Fungi (*Histoplasma*)	Macrocyclic ring with conjugated double bonds and amino sugars (Hammond and Lambert, 1978)	Inactivates membranes containing sterols
	Nystatin	*Streptomyces noursei*	Fungi (*Candida*)	"	"
Rifamycins	Rifampicin	*Streptomyces mediterranei*	Gram-positive and Gram-negative bacteria, *Mycobacterium tuberculosis*	Naphto and benzoquinone nuclei derivatives (Lancini *et al*., 1995)	Inhibits transcription (eubacterial RNA polymerase)
Tetracyclines	Tetracycline	*Streptomyces* species	Gram-positive and Gram-negative bacteria, *Rickettsias*	Polycyclic naphthacene carboxamide (IUPAC, 2009)	Inhibit translation (protein synthesis)
Semisynthetic Tetracyclines	Doxycycline		Gram-positive and Gram-negative bacteria, *Rickettsias Ehrlichia, Borellia*	derivatives of polycyclic naphthone carboxide ((IUPAC, 2009)	"
Chloramphenicol	Chloramphenicol	*Streptomyces venezuelae*	Gram-positive and Gram-negative bacteria	Nitrobenzene derivative of dichloroacetic acid (Prescott *et al*., 2000)	"

", Same as above; Source: Todar, (2009), original table modified by grouping the semisynthetic β-lactams, the peptides and also by adding the principal chemical properties. The synthetic antibiotics such as quinolones, fluoroquinolones, isoniazid, etc., are omitted from this table.

The present review will focus on β-lactams, polypeptides and tetracycline groups of antibiotics as they are among the most clinically successful.

1.4.1.1 The β-Lactams

Penicillins as well as cephalosporins are called beta-lactam antibiotics and are characterized by three fundamental structural requirements: the fused beta-lactam structure, a free carboxyl acid group (shown in red bottom right), and one or more substituted amino acid side chains (shown in black). The lactam structure (**Fig. 1.3**) can also be viewed as the covalent bonding of pieces of two amino acids - cysteine (blue) and valine (red) [Ophardt, 2003].

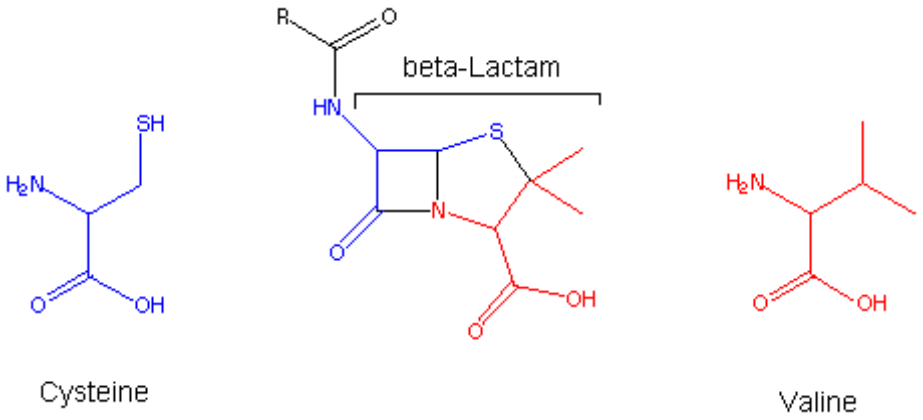


Fig. 1.3 Penicillin showing beta-lactam structure: Lactam (a four-atom ring) fused with a thiazolidine (a five-atom ring). Source: Ophardt (2003).

These antibiotics are the products of two genera of fungi, *Penicillium* and *Cephalosporium*, and are correspondingly represented by the penicillins and cephalosporins (**Fig. 1.4**). Two other classes of beta-lactams are the carbapenems and monobactams. The latter are particularly useful for the treatment of allergic individuals. A person who becomes allergic to penicillin usually becomes allergic to the cephalosporins and the carbapenems as well. Such individuals can still be treated with the monobactams, which are structurally different (**Fig. 1.4**) so as not to induce allergy (Coffman, 2010).

The era of extensive use of antibiotics in medicine began in 1942 when penicillin G was introduced into clinical practice (Lancini *et al*., 1995). However, it has several shortcomings and is effective only against gram-positive bacteria. It may be broken

down in the stomach by gastric acids and is poorly and irregularly absorbed into the blood stream. In addition many disease producing staphylococci are able to produce an enzyme (β-lactamase) capable of inactivating penicillin-G. Various semisynthetic derivatives have been produced which overcome these shortcomings (Ophardt, 2003).

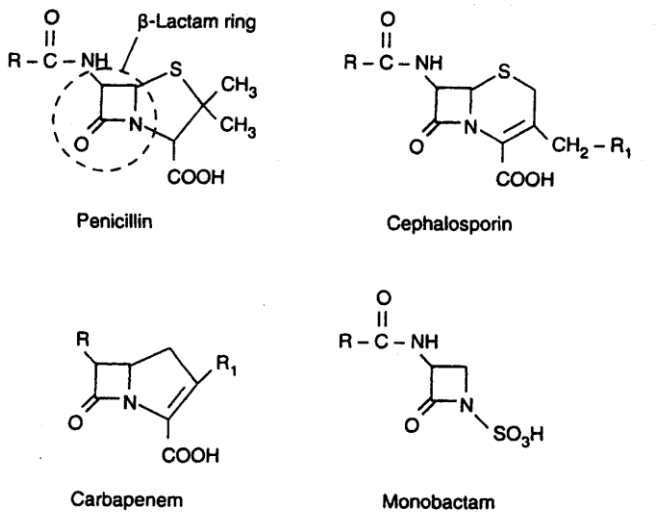


Fig. 1.4 The structure of members of the penicillin family (β-lactams). Clockwise: penicillin, cephalosporin, monobactam, carbapenem**.** They are characterized by a four-member β-lactam ring as shown in the diagram. Penicillin G (Benzylpenicillin) where R = an ethyl phenyl group is the most potent of all penicillin derivatives. Source: Salyers and Whitt, (2005).

The breakthrough that led to the preparation of a thousand semisynthetic penicillins was the isolation of 6-aminopenicillanic acid (6-APA) [Lancini and Parenti, 1982]. Starting with 6-APA many structural analogs of penicillin V (e.g. phenethicillin, propicillin, azidocillin) were synthesised according to the reaction scheme shown in **Figure 1.5**.

Powerful electron-attracting groups attached to the amino acid side chain (position 6) such as in phenethicillin prevent acid attack. A bulky group attached to the amino acid side chain provides steric hindrance which interferes with the lactamase enzyme attachment which would deactivate the penicillins. Finally if the polar character is increased as in ampicillin or carbenicillin, there is a greater activity against Gram-negative bacteria (Ophardt, 2003).

Similar to penicillins, many semisynthetic cephalosporins were prepared through the isolation of 7-aminocephalosporanic acid (7-ACA) [**Fig.1.6B**]. Some of the derivatives prepared include cephalothin, cephaloridine, cephapirin, cephalexin, cefazolin, etc. These were referred to as the first generation cephalosporins (Lancini and Parenti, 1982).

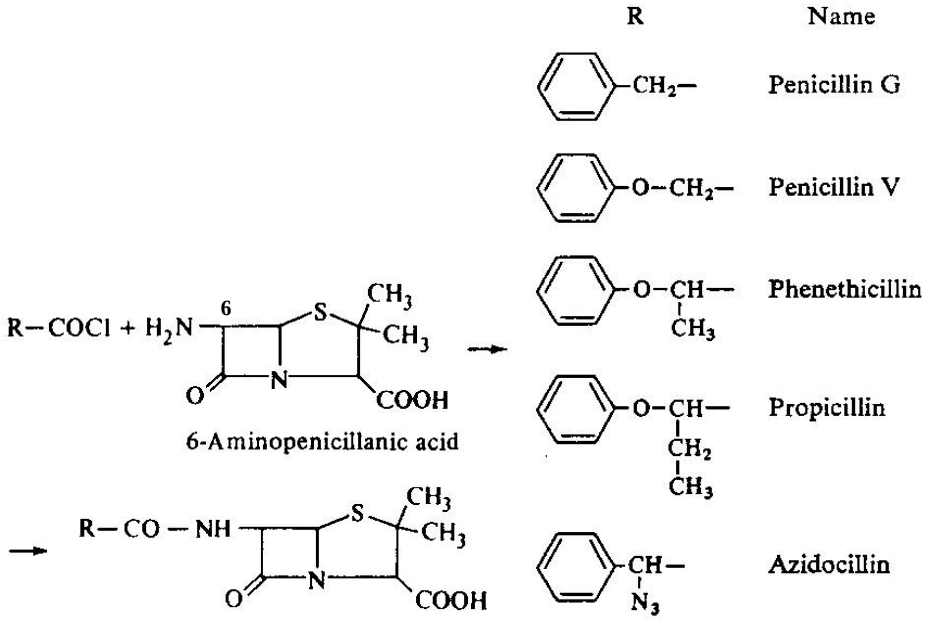


Fig. 1.5 Scheme of synthesis of semisynthetic penicillins from 6-APA and the derivatives of penicillin V. Source: Lancini and Parenti, 1982.

The second- and third-generation cephalosporins were characterised by their enhanced antibacterial activity obtained mainly by the choice of a suitable substituent at the amide chain in position 7 combined with a substituent in position 3 compatible with good pharmacokinetics. The presence of a methoxyl group at position 7 enhanced the stability of the cephalosporins to a broad variety of Gram-negative cephalosporinases, especially those produced by *Bacteroides* species. Cefoxitin was the first compound with a 7-methoxyl group to be developed for clinical use. The cephalosporins which were absorbed orally generally had a simple methoxyl group at the 3 position. Alterations in the side chain at the 3 position of the cephalosporin molecule also enhanced the serum protein binding and renal excretion of the compound (Gorbach *et al*., 2004). Cephalosporins unlike penicillins had the potential of being modified not only at the side chains but also in position 3 of the nucleus, where the acetoxyl group can be easily eliminated or substituted without loss of activity (Gorbach *et al*., 2004).

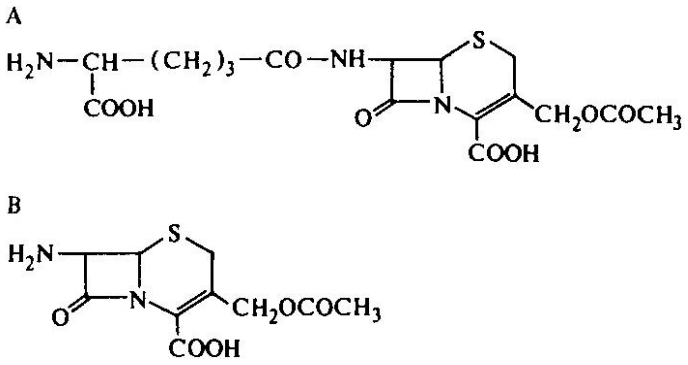


Fig. 1.6 A, Cephalosporin C; **B**, 7-aminocephalosporanic acid (7-ACA): Source: Lancini and Parenti, 1982.

The beta lactam antibiotics are stereochemically related to D-alanyl-D-alanine (**Fig. 1.7**) which is a substrate for the last step in peptidoglycan synthesis, the final cross-linking between peptide side chains. Penicillins bind to and inhibit the carboxypeptidase and transpeptidase enzymes that are required for this step in peptidoglycan biosynthesis. Beta lactam antibiotics are bactericidal and require that cells be actively growing in order to exert their toxicity (Todar, 2009).

Specifically, the cross linking of peptides on the mucosaccharide chains is prevented. If cell walls are improperly made they allow water to flow into the cell causing it to burst. Resemblances between a segment of penicillin structure and the backbone of a peptide chain have been used to explain the mechanism of action of beta-lactam antibiotics (Ophardt, 2003).

Gram-positive bacteria possess a thick cell wall composed of a cellulose-like structural sugar polymer covalently bound to short peptide units in layers. The polysaccharide portion of the peptidoglycan structure is made of repeating units of N-acetylglucosamine linked β-1, 4 to N-acetylmuramic acid (NAG-NAM). The peptide varies, but begins with L-Ala and ends with D-Ala. In the middle is a dibasic amino acid, diaminopimelate (DAP). DAP (orange) provides a linkage to the D-Ala (gray) residue on an adjacent peptide (**Fig. 1.7**). Synthesis of the bacterial cell wall is completed when a cross link between two peptide chains attached to polysaccharide backbones is formed. The cross linking is catalyzed by the enzyme transpeptidase. First the terminal alanine from each peptide is hydrolyzed and secondly one alanine is joined to lysine through an amide bond (Ophardt, 2003).

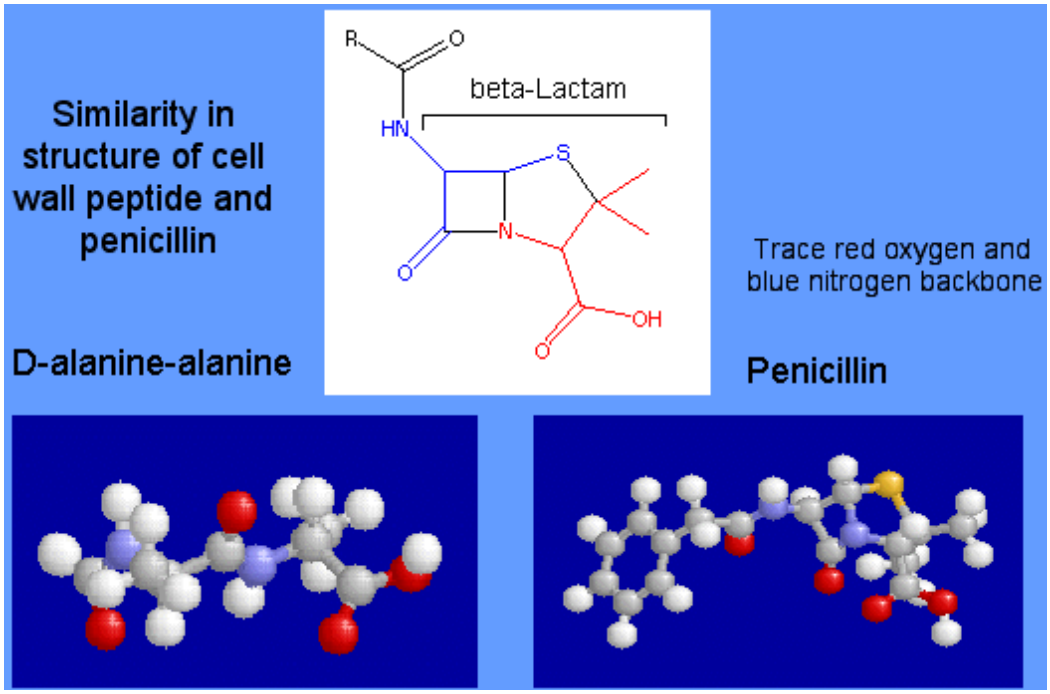


Fig. 1.7 Comparison of structures of D-alanine-D-alanine (left) and beta-lactam antibiotic penicillin (right); Follow the trace of the red oxygen and blue nitrogen atoms. Source: Ophardt, (2003).

Penicillin binds at the active site of the transpeptidase enzyme that cross-links the peptidoglycan strands. It does this by mimicking the D-alanyl-D-alanine residues that would normally bind to this site. Penicillin irreversibly inhibits the enzyme transpeptidase by reacting with a serine residue in the transpeptidase. This reaction is irreversible and so the growth of the bacterial cell wall is inhibited. Since mammalian cells do not have the same type of cell walls, penicillin specifically inhibits only bacterial cell wall synthesis (Ophardt, 2003).

Different beta-lactams differ in their spectrum of activity and their effect on Gram-negative rods, as well as in their toxicity, stability in the human body, rate of clearance from blood, whether they can be taken orally, ability to cross the blood-brain barrier, and susceptibility to bacterial beta-lactamases (Jacoby and Carreras, 1990).

Natural penicillins, such as penicillin G (benzyl penicillin), penicillin V (phenoxymethyl penicillin) and penicillin K (n-Heptyl penicillin) are effective against streptococci, gonococci and staphylococci, except where resistance has developed. They are considered narrow spectrum since they are not effective against Gram-negative rods. Penicillins with enlarged spectra of activity were obtained by substitution of the phenyl group of penicillin G with certain heterocyclic rings. Many derivatives have been

prepared including: ampicillin (D-α-aminobenzylpenicillin), cyclacillin, epicillin, amoxicillin, pivampicillin, etc. Ampicillin with its high level of efficacy inhibited not only the bacteria sensitive to penicillin G, but also most strains of *E. coli*, *Salmonella*, and *Shigella*. However, it was not considered a broad-spectrum antibiotic, as it was inactive against most strains of *Klebsiella*, *Enterobacter*, and *Proteus* and totally inactive against *Pseudomonas* and *S. aureus* (Lancini *et al.,* 1995).

Although nontoxic, penicillins occasionally cause death when administered to persons who are allergic to them. In the U.S. there are 300 - 500 deaths annually due to penicillin allergy. In allergic individuals the beta lactam molecule attaches to a serum protein and initiates an IgE-mediated inflammatory response (Todar, 2009).

Cephalosporins have a low toxicity and a somewhat broader spectrum than natural penicillins. They are often used as penicillin substitutes against Gram-negative bacteria and in surgical prophylaxis. They are subject to degradation by some bacterial beta-lactamases, but they tend to be resistant to beta-lactamases from *S. aureus* (Widmer, 2008).

1.4.1.2 The polypeptides

The term peptide is normally used for compounds that contain a small number of amino acid residues whilst the term polypeptide is used for larger compounds with relative molecular values of 300 or more. Proteins are more complex polypeptides with relative molecular values greater than 2000 (Thomas, 2007). Among the most powerful bactericidal antibiotics are those that possess a peptide structure. Many of them have been isolated, but unfortunately their clinical uses have been limited by their undesirable side reactions, particularly renal toxicity. Examples include: bacitracin, vancomycin, actinomycin, polymyxin B and polymyxin E (colistin) (Wilson *et al*., 2004). Most of them cannot be administered orally due to lack of systemic activity. However, the polymyxins (**Fig. 1.8A**) are very effective against Gram-positive bacteria and therefore useful in severe urethral tract infections (Zuzana *et al*., 2009).

Polypeptide antibiotics variously possess a number of interesting and often unique characteristics; (a) they frequently consist of several structurally similar but chemically distinct entities isolated from a single source; (b) most of them are cyclic with a few

exceptions (e.g., the gramicidins, glycopeptides); (c) they frequently contain D-amino acids not found in higher plants and animals; and (d) many of them contain non-amino acid moieties, such as heterocycles, fatty acids, sugars, etc. Polypeptide antibiotics may be acidic, basic, zwitterionic, or neutral depending on the number of free carbonyl and amino or guanidine groups in their structure (Wilson *et al*., 2004).

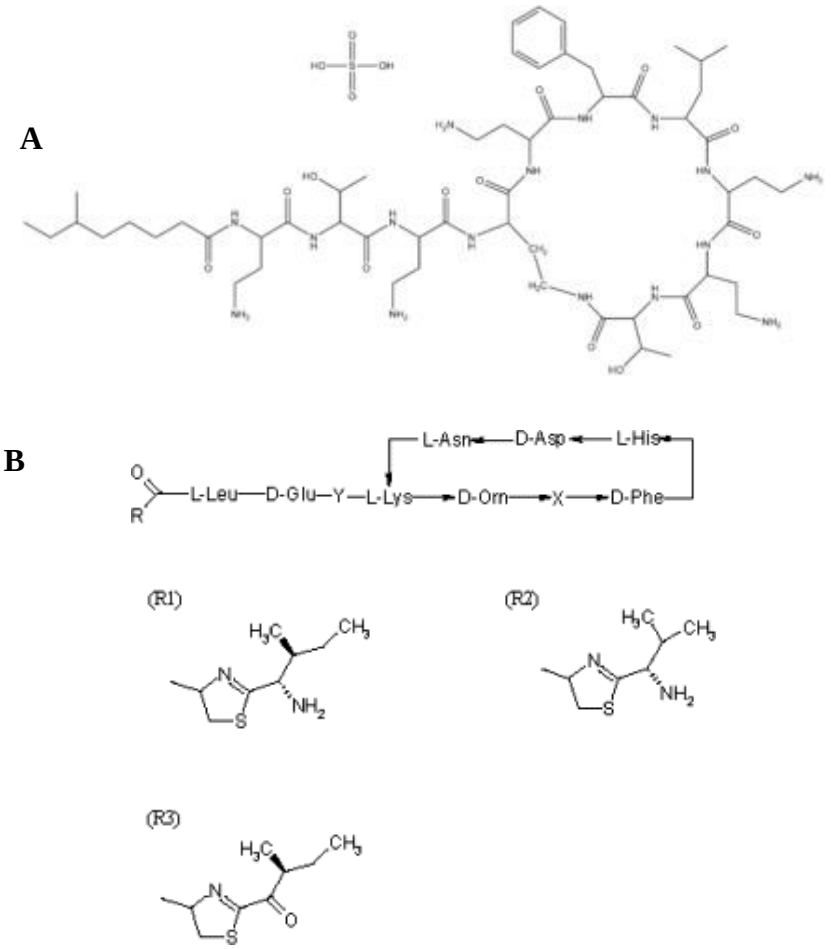


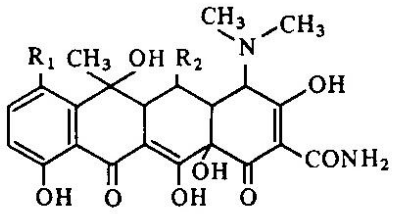
Fig.1.8 Examples of polypeptide antibiotics: **A**, Polymyxin B; **B**, Fundamental structure of bacitracin and the N-terminal thiazoline ring (R=R1, R2 or R3) to give bacitracin A, B, and C respectively. Asn, Asparagine; Asp, Aspartic acid, Glu, Glutamic acid; His, Histidine; Leu, Leucine; Lys, Lysine; Orn, Ornithine, Phe, Phenylalanine; Val, Valine. Source: Wilson *et al*., (2004).

1.4.1.3 The Tetracyclines

The tetracyclines consist of eight related antibiotics which are all natural products of *Streptomyces spp.*, although some can now be produced semisynthetically or synthetically. Tetracycline, chlortetracycline and doxycycline are the best known. The first tetracycline used in therapy, in 1948, was chlortetracycline, produced by

Streptomyces aureofaciens. This was followed two years later by oxytetracycline, isolated from *Streptomyces rimosus*, and in 1952 by tetracycline (**Fig. 1.9**)**.** The latter was originally synthesised by catalytic hydrogenation of chlortetracycline and later by fermentation first by growth of *Streptomyces aureofaciens* in a medium without chlorine and later by fermentation of *Streptomyces* strains that were able to produce it directly. All three of these compounds demonstrated cross-resistance and had very similar spectra of activity (Todar, 2009).

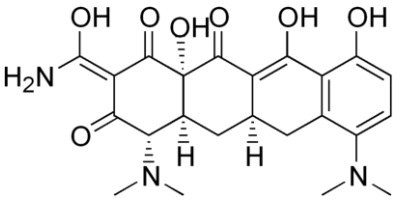
The tetracyclines are broad-spectrum antibiotics with a wide range of activity against both Gram-positive and Gram-negative bacteria. *Pseudomonas aeruginosa* is less sensitive but is generally susceptible to tetracycline concentrations that are obtainable in the bladder (Lancini *et al*., 1995).



			MIC (μg/ml)							
R_1	R_2	Name	*S. aureus*	*S. pneumoniae*	*S. faecalis*	*E. coli*	*K. pneumoniae*	*P. vulgaris*	*S. tiphymurium*	*P. aeruginosa*
H	H	Tetracycline	0.4	0.15	0.7	1.2	0.6	4	2.3	25
Cl	H	Chlortetracycline	0.3	0.1	0.4	1.4	0.3	4.6	1.2	14
H	OH	Oxytetracycline	0.6	0.3	2	1.2	0.6	3.1	1.6	25

Fig. 1.9 Natural tetracyclines showing their broad spectrum activities: Source: Lancini and Parenti (1982).

Comparisons of the activities of the natural tetracyclines and of different derivatives thereof have shown that positions 5, 6, 7 and 9 can have different substituents without any substantial loss of activity. Removal of the hydroxyl group at position 6, with rhodium as catalyst, conferred greater stability to tetracycline but led to the inversion of the configuration of the methyl group and resulted in decreased activity. Substitutions in positions 7 and 9 led to the development of minocycline (**Fig. 1.10**) which is active both in vitro and in vivo against strains of *S. aureus*, *Enterococcus* and *E. coli* that are resistant to tetracycline. However since this achievement most efforts have been without success. Only a few laboratories are still working on new tetracyclines (Gorbach *et al*., 2004).



Minocycline

Fig. 1.10 Structure of the tetracycline derivative minocyline: Source: Gorbach *et al*., (2004).

The tetracyclines have a remarkably low toxicity and minimal side effects when taken by animals. The combination of their broad spectrum and low toxicity has led to their overuse and misuse by the medical community and the wide-spread development of resistance has reduced their effectiveness. Nonetheless, tetracyclines still have some important uses, such as the use of doxycycline in the treatment of Lyme disease (Todar, 2009).

1.4.2 Grouping according to mode of action

Several authors have grouped antibiotics based on the site of action in the microorganism (Hammond and Lambert, 1978; Franklin and Snow, 1981; Lancini and Parenti, 1982; Lancini *et al*, 1995; Todar, 2009). These groupings include:

A. Inhibitors of cell wall synthesis

B. Disruptors of cell membranes

C Inhibitors of protein synthesis

D. Inhibitors of nucleic acid synthesis

A summary of the biological groups of antibiotics and their properties, including their spectra of activity and mode of action, is shown in **Table 1.3**. Refer to **section 1.6** for more detailed discussion on the mechanism of action of these antibiotics.

1.5 Antimicrobial activity of antibiotics

Antibiotics may have a cidal (killing) effect or a static (inhibitory) effect on a range of microbes (Todar, 2009). If a reaction such as cell wall synthesis is blocked irreversibly, the antibiotic is bactericidal, ultimately leading to the death of the organism. The irreversible step in cell wall synthesis is the opening of the existing wall to allow cell growth (Mayberry, 2004)

Table 1.3 **Biological groups of antibiotics and their properties**

Biological group	Examples	Spectrum	Mode of action
1. Inhibitors of cell wall synthesis	Beta-lactams (Penicillins, Cephalosporins, Monobactams, Thienamycins); Beta-lactamase inhibitors (e.g., clavulanic acid)	Gram-positive bacteria and Gram-negative bacteria	β-lactams inhibit steps in cell wall (peptidoglycan) synthesis and murein assembly (Todar, 2009) Clavulanic acid is a suicide inhibitor of beta-lactamases (Reading and Farmer, 1981)
	Cycloserine, Fosfomycin (Phosphonomycin), Vancomycin, Bacitracin, Ristocetin	Gram-positive and Gram-negative bacteria	Inhibit steps in cell wall (peptidoglycan) synthesis and murein assembly (Lancini *et al*., 1995)
2. Disruptors of cell membranes	Lipopeptides (Polymyxin B, Polymyxin E (colistin), circulin);	Gram-negative bacteria,	Damage cytoplasmic membranes (Franklin and Snow, 1981)
	Polyenes (Amphotericin B, Nystatin);	Antitmour, Fungi	Polyenes inactivate membranes containing sterols (Franklin and Snow, 1981)
	Ionophores [linear peptides (gramicidins), cyclic peptides (alamethacin), cyclodepsipeptides (valinomycin, enniantins), macrotetralides, sideromycins]; Tyrocidin		
3. Inhibitors of protein synthesis	**30S ribosome-binding antibiotic** Aminoglycosides (Streptomycin), Amikacin, Gentamycin, Kanamycin, Neomycin, Tobramycin,	Gram-negative bacteria and Mycobacteria	Inhibit translation (protein synthesis) [Todar, 2009]
	Tetracyclines	Gram-positive and Gram-negative bacteria, Protozoa	
	50S ribosome-binding antibiotics Chloramphenicol, Clindamycin, Furadantin, Fusidic acid, Lincomycin, Nitrofuran, Puromycin, Kirromycins, Quinopristin/Dalfopristin, Spectinomycin, Macrolides (Azithromycin, Dirithromycin, Clarithromycin, Erythromycin)	Gram-positive and Gram-negative bacteria, Protozoa, Fungi	Inhibit translation (protein synthesis) [Tritz, 2000]

Table 1.3 continued

Biological group	Examples	Spectrum	Mode of action
4. Inhibitors of Nucleic acid synthesis	The quinolones (nalidixic acid - first generation, norfloxacin, ciprofloxacin - second generation), Showdomycin, Actinomycin D, Adriamycin, Daunomycin, Chromomycin, Azaserine, Alanosine, Hadacidin, Mitomycins, Bleomycins, Ansamycins (rifamycins, streptovaricins, tolipomycins, halomycins), Streptolidigin.	Antitumour agents	Inhibit transcription (eubacterial RNA polymerase) [Tritz, 2000]

If a reaction is blocked reversibly, the antibiotic is bacteriostatic, suppressing the growth of the organism to give the infected individual's immune system a chance to catch up (Mayberry, 2004). Some examples of bactericidal antibiotics in clinical use are shown in **Table 1.4**.

Table 1.4 Bactericidal versus bacteriostatic antibiotics in clinical use

Bactericidal antibiotics	**Bacteriostatic**
All cell wall synthesis inhibitors -Beta-Lactams, bacitracin, fosfomycin, cycloserine, vancomycin, teichoplanin **All DNA synthesis inhibitors** - Nalidixic acid, quinolones, novobiocin **All RNA synthesis inhibitors** - Rifampicin, Actinomycin D **All membrane-active antibiotics** - Polymyxins, gramicidins **Only one of protein synthesis inhibitors** - Aminoglycosides (spectinomycin)	All other antibiotics are bacteriostatic

Source: Mayberry, (2004).

1.5.1 Selective toxicity

Most clinically useful antibiotics exhibit their selective toxicity by specifically blocking one or another type of bacterial macromolecular synthesis (e.g. protein, nucleic acid or cell wall synthesis) -- acting on targets that are not present or accessible in animal/human cells. For example, polyene antibiotics act exclusively on cells which have sterols in their cytoplasmic membrane and thus affect yeasts, fungi and animal cells but have no effect on most bacteria (Franklin and Snow, 1981). Biochemical pathways of microorganisms and man often differ and these points of difference may be exploited in treating infectious diseases, as they can be used to kill disease-causing microorganisms without harming the host (Hammond and Lambert, 1978).

1.5.2 Spectra of activity

Antibiotics are frequently grouped according to their spectra of activity, i.e. according to the class of microorganisms they inhibit. Thus they may be:

A. Antibacterial
B. Antifungal

C. Antiprotozoal
D. Antiviral agents
E. Antitumour agents: justifiably classified also as antibiotics because the compounds were originally isolated on the basis of their antimicrobial activities (Okafor, 1987).

Those antibiotics which are effective against a variety of pathogens are termed broad spectrum antibiotics (Hammond and Lambert, 1978). If effective mainly against Gram-positive or Gram-negative bacteria, they are narrow spectrum. If effective against a single organism or disease, they are referred to as limited spectrum (Todar, 2009).

1.5.3 Minimum inhibitory concentration (MIC) – Its significance

As an index of antibiotic activity, microbiologists and clinicians use the minimum inhibitory concentration (MIC), defined as the lowest antibiotic concentration that will inhibit the growth of a specific organism (Okudoh, 2001). The MIC value is a characteristic of a particular antibiotic for a particular bacterial species under particular test conditions. In addition, within a given species, different strains may, within certain limits, be more or less sensitive to an antibiotic and therefore may have different MIC values (Lancini and Parenti, 1982). The MIC for an antibiotic against a given bacterium will differ according to whether it is determined in liquid or on solid medium, even when the two media are the same except for the agar (Lancini and Parenti, 1982). The MIC of many antibiotics is not affected by variation in the inoculum density in the range commonly used (10^3 – 10^6 bacteria/ ml^{-1}) [Lancini and Parenti, 1982].

In the past, the method for MIC determination used test tubes or small bottles of growth broth containing a test concentration of antibiotics, into which an inoculum of microbes was added (Davidson and Parish, 1989). Currently, the tubes have been replaced by a semi-automated microtitre plate method (Lambert and Pearson, 2000; Rahman *et al*., 2004). To determine the MIC, a micro-titre plate (10 x 10 wells) containing graded doses of antibiotic, are inoculated with the test organism. After suitable incubation, growth will occur in those wells where the antibiotic concentration is below the inhibitory level and the culture will become turbid (cloudy) from the large number of microorganisms present. Growth will not occur when the concentration of the antibiotic is above the inhibitory level and the medium will remain clear (Okudoh,

2001). The MIC is the concentration of antibiotic present in the first “clear” well reading in the downward direction (**Fig.1.11**).

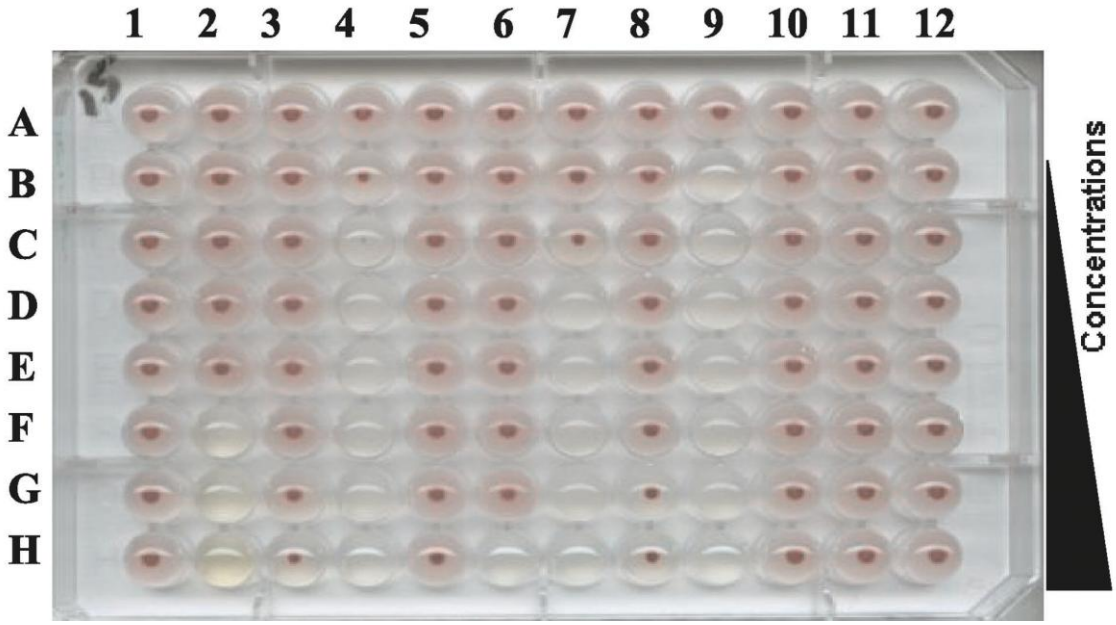


Fig. 1.11 Determination of minimum inhibitory concentration (MIC) on a microtitre plate: Each column contains a decreasing concentration gradient (H to B) of one antibiotic and all wells therein are inoculated with the test organism. Row A does not contain any antibiotic and serves as a growth control. The MIC of each antibiotic can be seen as the first row of each column showing no growth (clear broth). Columns 1 to 12 may contain different antibiotics. Source: Rahman *et al*., (2004).

Where the turbidity of the test compound interferes with the test, indicators such as fluorescin diacetate, resazurin and p-iodotetrazolium can be used (Mann and Markham, 1998; Rahman *et al*., 2004). However, the principal problem encountered is that all MIC techniques currently used are semi-quantitative. Measuring the MIC of a substance by current methods is straightforward, whereas obtaining useful comparative information from the tests can be more difficult (Lambert and Pearson, 2000).

1.5.4 Minimal bactericidal concentration (MBC)

A single antibiotic may be both bacteriostatic and bactericidal, depending on the concentration used (Lancini *et al*., 1995). Sometimes it is useful to determine the minimal bactericidal concentration (MBC). This is done in a similar manner to the MIC except that at the end of the incubation, an aliquot of liquid from each well/ bottle in which no growth can be seen, is diluted to remove the antibiotic and spread on the

surface of Petri plates containing an appropriate nutrient medium. These are incubated for 24h at an appropriate temperature. The MBC is the concentration of the antibiotic in the first clear well from which no colonies could be grown on the Petri plates. In practice, “no colonies” is defined as a 99.9% reduction in colony forming units (CFU) [Lancini and Parenti, 1982].

1.6 Mechanism of action of antibiotics – General overview

The mechanism of action of an antibiotic is the biochemical event by which the growth of a sensitive microorganism is inhibited (Lancini and Lorenzetti, 1993). This is the result of the interference of the antibiotic with a molecule called the target molecule essential for cell growth. Target molecules are normally macromolecules such as DNA, RNA and enzymes, but are occasionally small metabolites such as substrates of enzymatic reactions or membrane components (Lancini and Lorenzetti, 1993).

The mechanism of action of antibiotics is well documented in the literature (Lancini and Parenti, 1982, Nagarajan, 1991, Allen, 2003, Walsh, C. 2003, Salyers and Whitt, 2005**,** Todar, 2009). In this brief review, emphasis is laid on the bacterial cell wall since it is the first point of contact between the cell and all antibiotics and the review is limited to a few common examples of antibiotic classes.

In order to comprehend how an antibiotic inhibits the growth of a bacterial cell, it is important to understand how the life of such an organism is constructed. The life of any organism requires provision of the following: precursors (building blocks), appropriate energy sources; and appropriate enzyme systems and components to carry out the contruction. It also involves the assembly of precursors into polymeric form and arrangement of the polymers into appropriate configurations and structures (Mayberry, 2004).

Most antibiotic mechanisms operate by trying to stop one or two steps in the construction project. This can be achieved by inhibition of the following: production of coenzymes, production of precursors, assembly of enzymatically active structures and transport of structural components to appropriate sites. It also includes the inhibition of production of polymers and their rearrangement, and the disruption of the integrity of pre-existing structures (Mayberry, 2004).

Understanding the mechanism of action of an antibiotic molecule implies the identification of the target molecule(s), the inhibition site and the type of interaction (Lancini and Lorenzetti, 1993). For a large number of antibiotics, the target molecule is known. However, it is easier to identify the metabolic pathway that is blocked than the specific molecule involved. Therefore, we have antibiotics that either inhibits cell wall synthesis, DNA replication and transcription, protein synthesis or cell membrane functions (Lancini and Lorenzetti, 1993).

1.6.1 Inhibition of bacterial cell wall (peptidoglycan) synthesis

Fifty to seventy percent of the Gram-positive bacterial cell wall mass and to a lesser extent (10 – 15%) in Gram-negatives is composed of peptidoglycan **(Fig 1.12)**. Its cross-linked structure provides a tough, fibrous fabric, giving strength and shape to the cell and enabling it to withstand a high internal osmotic pressure (Franklin and Snow, 1981). The bacterial cell wall is the point of inhibition by various classes of antibiotic e.g. penicillins, cephalosporins and vancomycin that affect either peptidoglycan synthesis or assembly of other components of the wall, e.g. teichoic acid (Franklin and Snow, 1981).

1.6.1.1 Inhibition of precursor formation

Two carbohydrate precursors (N-acetyl glucosamine and N-acetyl muramic acid) are required for peptidoglycan biosynthesis and are unique to prokaryotic cell walls (Walsh, C. 2003). Several amino acids, especially some of the ‘unnatural’ “D-” configurations, are also required. The process also involves the carrier molecules (UDP, polyprenol) and an energy source ATP (adenosine triphosphate). The enzymes necessary to assemble these precursors into “building blocks” (transglycosylases), form linear glycan polymers which are crosslinked via peptide side chains to form a rigid 3-D peptidoglycan structure (transpeptidases) [Walsh, C. 2003].

The steps in the formation of the carbohydrate precursors are shown in **Figure 1.13**. A typical carbohydrate reaction converts glucose to N-acetyl glucosamine – 1 – phosphate. Uridine triphosphate (UTP) is consumed and uridine diphosphate (UDP) is attached to form UDP – N-acetyl glucosamine (UDP-“G”). Phosphenolpyruvate is added to the 3-oxy position of UDP-“G” via an ether linkage to form UDP – N-acetyl muramic acid (UDP-“M”). This provides a free carboxyl group which serves as an

“anchor” for subsequent additions of amino acids. The reaction is inhibited by fosfomycin, an epoxide analog of phosphenolpyruvate. Both UDP-”G” and UDP-”M” form the basis of the glycan portion of the peptidoglycan wall (Mayberry, 2004).

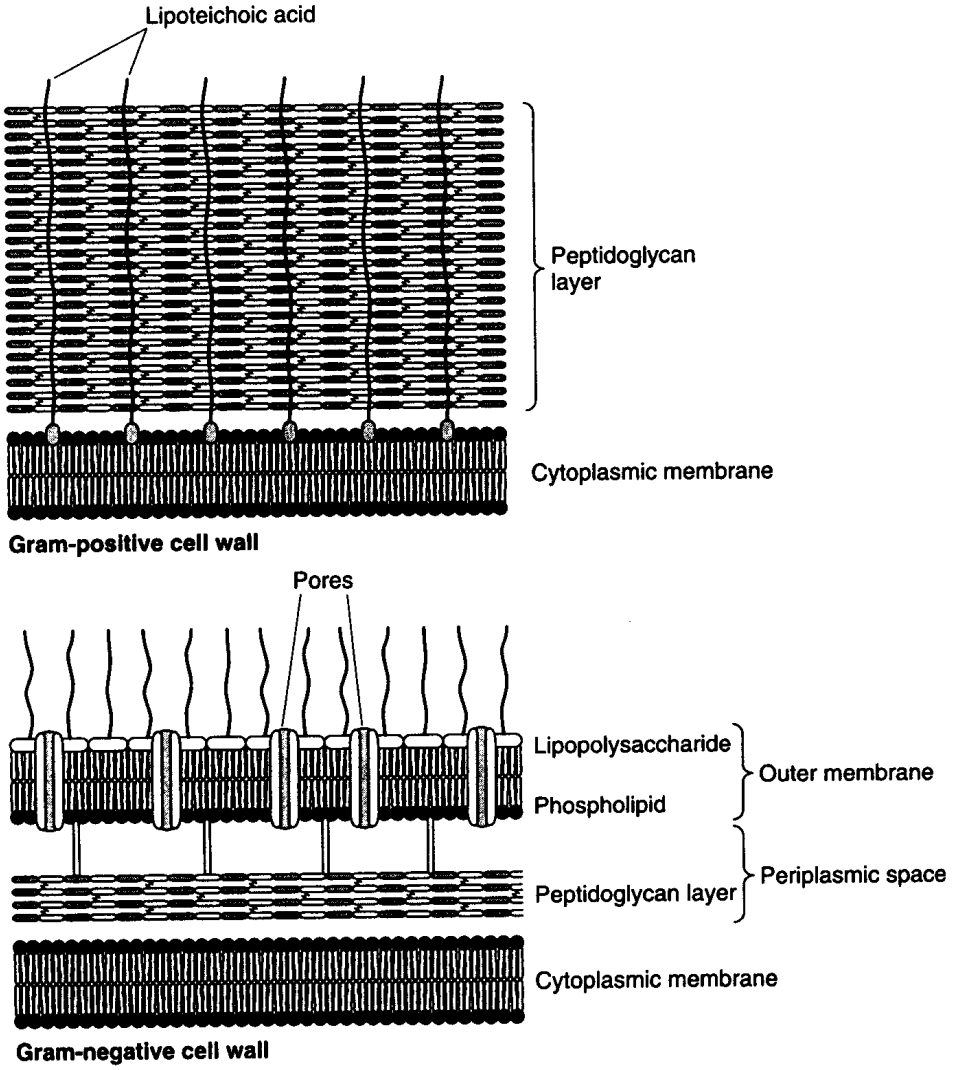


Fig. 1.12 Structure of the bacterial cell wall. Source: Salyers and Whitt, (2005).

The amino acid precursors are mainly glycine, L-alanine, D-glutamic acid, L-lysine and diaminopimelic acid (DAP). The structures of the amino acids found in peptidoglycan (murein) are shown in **Figure 1.14**. Glycine is used without any alteration in its structure as is L-alanine. However, L-alanine is also converted via a racemase to the “unnatural” D-alanine, which is then dimerized to D-alanyl-D-alanine. This D-alanyl-D-alanine dimer is important in the crosslinking of the peptide portion of the peptidoglycan wall. Production of D-alanine and its dimerization are both inhibited by D-cycloserine, a cyclic analog of D-alanine (Lancini *et al*., 1995; Walsh, C. 2003).

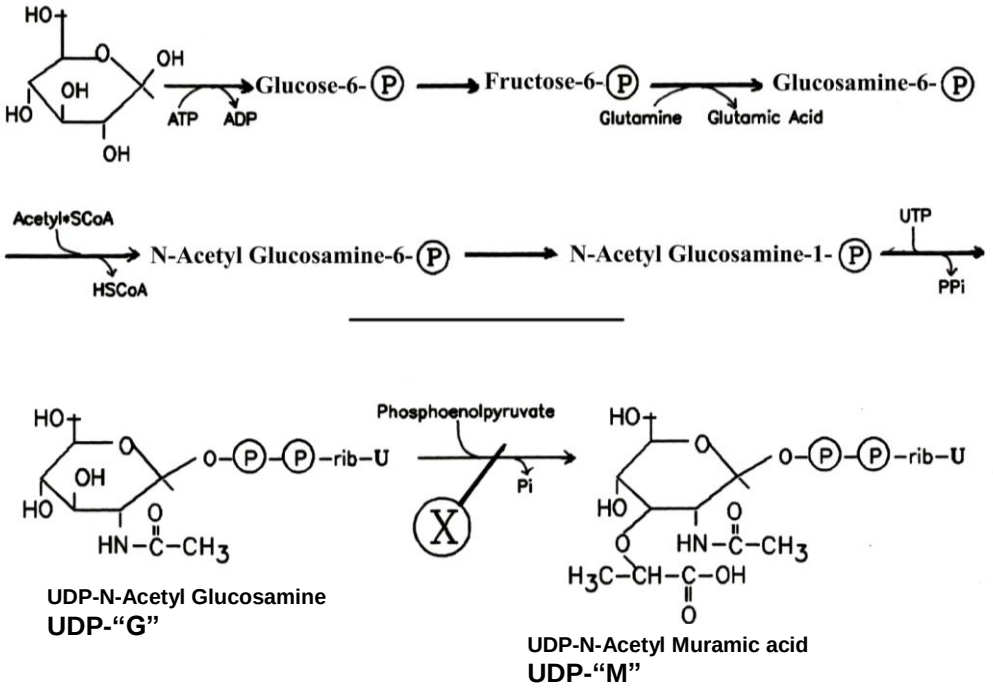


Fig. 1.13 Formation of carbohydrate precursors, UDP-N-acetyl glucosamine and UDP-N-acetyl muramic acid: (X) indicates the site of action of fosfomycin. Source: Mayberry, (2004).

D-glutamic acid is formed by racemization of L-glutamic acid. Currently there is no known inhibitor of this process. L-lysine is found in the walls of some Gram-positive bacteria while diaminopimelic acid (DAP) is found in Gram-negative and acid-fast bacteria (Ghuysen and Hakenbeck, 1994).

1.6.1.2 Inhibition of glycan polymer synthesis

In the assembly of the basic subunit of peptidoglycan, the wall precursors will have to be transported across a lipid membrane. This means that the carrier group on UDP-"M"- pentapeptide must be changed from the water-soluble UDP to a C_{55} polyprenol-P-P, forming lipid I (**Fig. 1.15**). A molecule of N-acetyl glucosamine (derived from UDP-"G") is then linked via a glycosidic bond to lipid I, to form lipid II. Depending on the organism, a number of glycine molecules (activated as tRNA-glycine) are added to the free amine group of lysine (or DAP), forming a "Glycine bridge" of zero to several units (Mayberry, 2004).

The next step in the biosynthesis of peptidoglycan is the extension of the glycan chain (transglycosylation). Lipid II molecules (with or without a glycine bridge) are transported through the cytoplasmic membrane to the site of wall extension. Then

transglycosylases add the carbohydrate dimer to the “growing” end of the glycan polymer, cleaving the polyprenol-P-P, which is transported back into the cell.

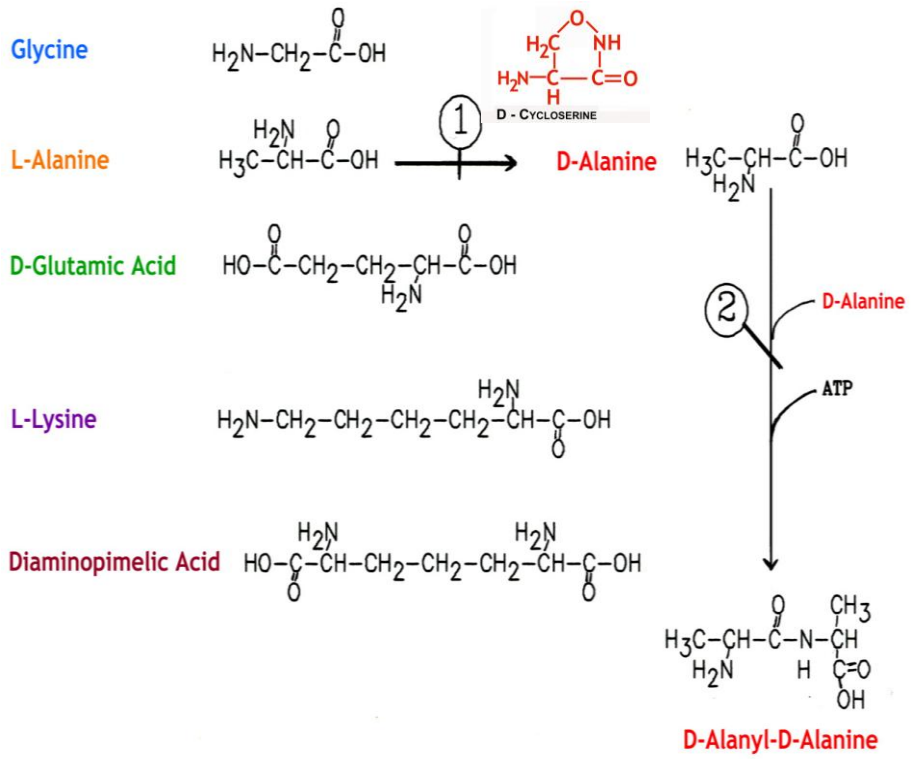


Fig. 1.14 Structures of amino acids found in peptidoglycan (murein). Production of D-alanine and its dimerization are both inhibited [sites (1) and (2)] by D-Cycloserine, a cyclic analog of D-alanine. Source: Mayberry, (2004).

There are no current antibiotics available to inhibit the transglycolases, but the animal feed additive moenomycin is a model compound. The polyprenol-P-P must be dephosphorylated to polyprenol-P to be active in the cycle. This reaction is inhibited by bacitracin and related compounds (Mayberry, 2004).

1.6.1.3 Inhibition of peptide crosslinking

The final step in the biosynthesis of peptidogycan is the crosslinking reaction brought about by transpeptidation **(Fig. 1.16)**. The newly formed glycan chain is a linear polymer and has no dimensional rigidity. Two and three-dimensional stability is then conferred by crosslinking of peptide chains from parallel glycan chains. Transpeptidases (also known as “penicillin-binding proteins”, PBPs) cleave the terminal D-Ala from D-Ala-D-Ala of one pentapeptide and use the energy to form a

peptide bond between the carboxyl group of the subterminal D-Ala and the free amine group of the glycine bridge, L-lysine, or diaminopimelate of a nearby pentapeptide (Mayberry, 2004).

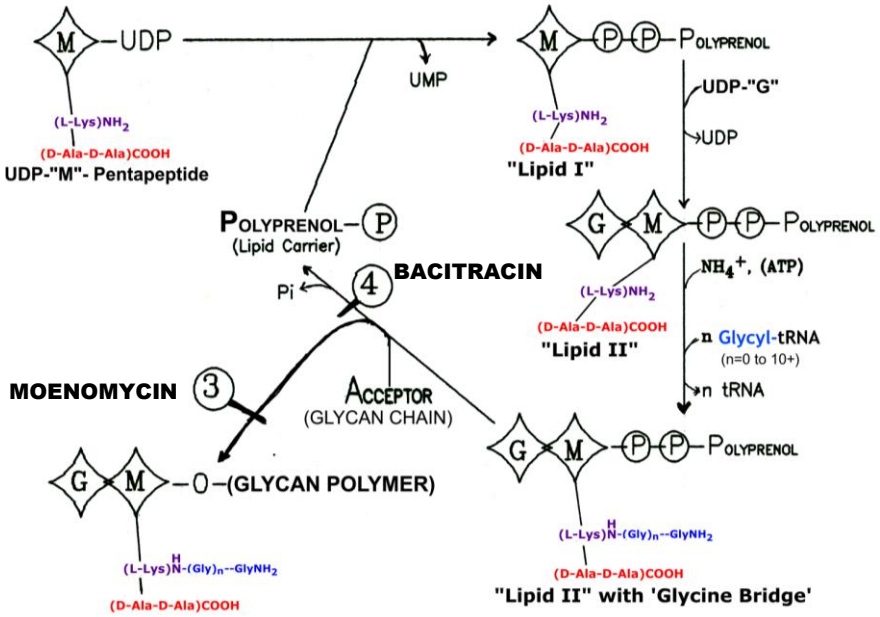


Fig. 1.15 Formation of the basic subunit of peptidoglycan and transglycosylation: (3) and (4) indicate the sites of action of moenomycin and bacitracin respectively. Source: Mayberry, (2004).

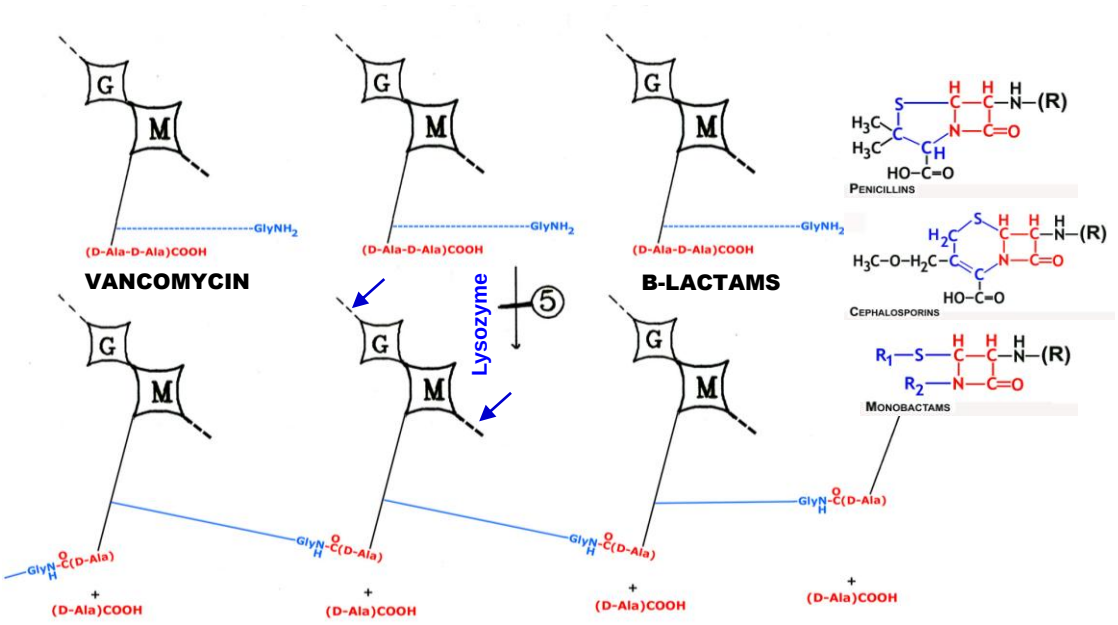
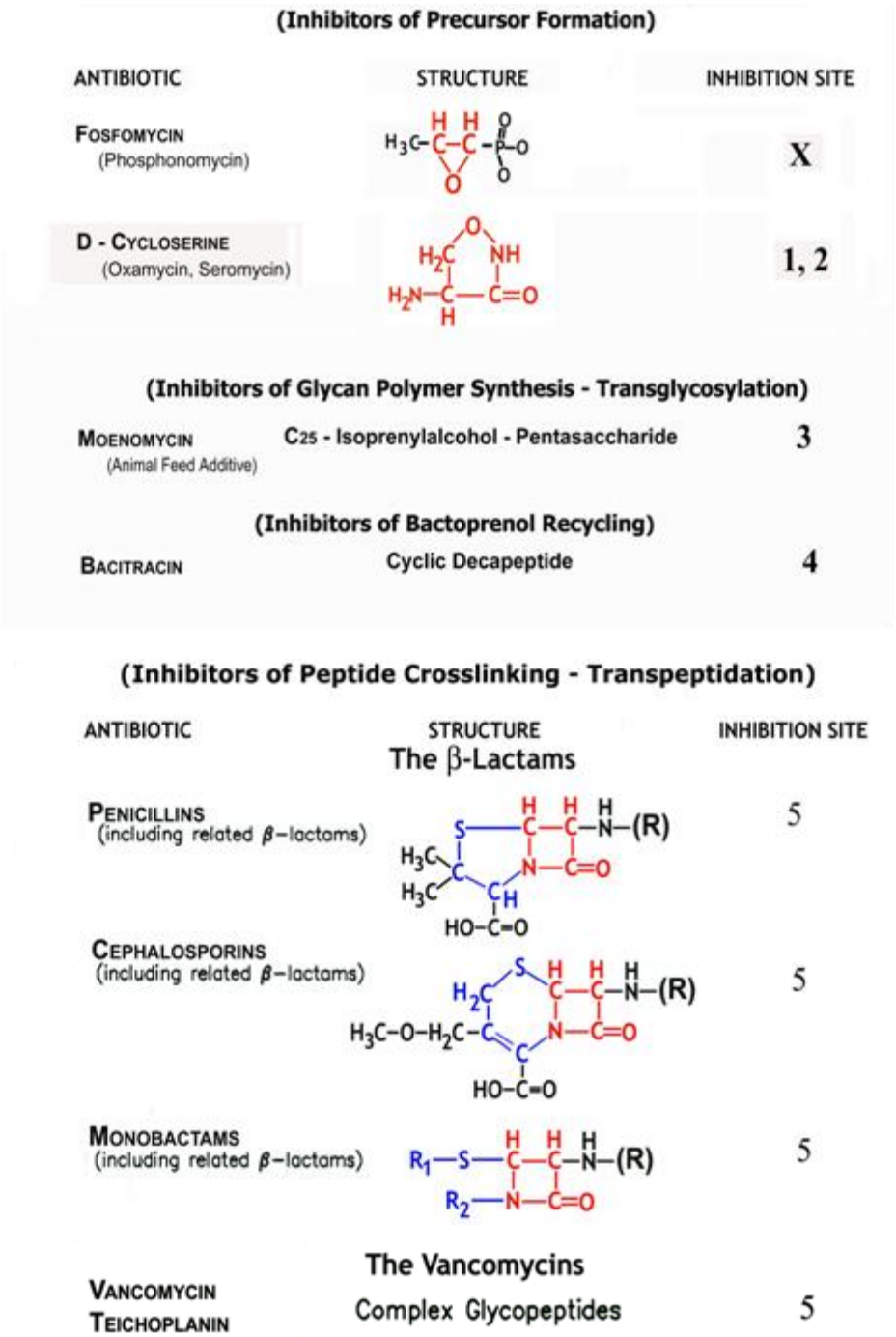


Fig. 1.16 Cross-linking of peptidoglycan (transpeptidation): Vancomycin and teichoplanin bind to D-Ala-D-Ala and prevent proper access by the PBPs; β-Lactam family (penicillins, cephalosporins, monobactams), structural analogs of D-Ala, irreversibly bind to PBPs, thus inactivating them. Source: Mayberry, (2004).

A summary of the target sites in the bacterial cell wall biosynthetic pathway for selected antibiotics is shown in **Table 1.5.**

Table 1.5 Antibiotics that inhibit bacterial cell wall biosynthesis. X and 1– 5 indicate the various sites inhibited during the biosynthetic processes as shown in **Figs. 1.13**, **1.14, 1.15** and **1.16**.



Source: Mayberry, (2004).

1.6.2 Structural disorganization and functional inhibition of bacterial cell membranes – the polypeptide antibiotics

The integrity of the cytoplasmic and, in Gram-negative bacteria, the outer membranes, is vital and compounds that disorganize the membranes rapidly kill the cells. Antibiotics that are active against the bacterial cytoplasmic membrane are the polypeptides [polymyxin B and E (colistin)], valinomycin, amphothericin B, etc. (Hammond and Lambert, 1978). Cell membranes have very similar constituents throughout the phylogenetic ladder from bacteria to mammalian cells. The only important difference is that there are no sterols in bacterial cell membranes, while zymosterol and ergosterol are present in the cell membranes of fungi and plants, and cholesterol in those of mammals. Some antibiotics in this group disorganize the super-molecular structure of the membrane, thus causing loss of cellular substance to the outside, while some act as carriers of specific ions (ionophores) and cause an abnormal accumulation of ions inside the cell (Lancini *et al*, 1995). The majority of inhibitors of cell-membrane functions are non-selective and consequently too toxic to be used systemically (Franklin and Snow, 1981; Axelsen, 2008). This discussion will be limited to the polypeptides since they are the most clinically successful in this group.

Despite thousands of articles about hundreds of the polypeptide antibiotics over several decades, basic questions about their mechanism of action remain unanswered (Axelsen, 2008). Most investigators have concluded that they act solely by permeabilizing the bacterial cell membrane. Although strong evidence for another mechanism of action has not yet emerged, this conclusion may not represent the whole story because polypeptide antibiotics clearly have other significant effects on bacteria such as inhibition of ['4C] aminoacid incorporation into cell walls and the induced accumulation of uridine-nucleotide precursors of the cell wall under conditions in which incorporation of amino acids into protein is unaffected (Stone and Strominger, 1971).

There are numerous polypeptide ‘antibiotics’ from bacterial, fungal, plant and animal (including human) sources which interact with and disrupt the structural and osmotic integrity of the outer and inner membranes of Gram-negative organisms. Some examples are polymyxin B, from *Bacillus polymyxa*, bacitracin from *Bacillus licheniformis* and vancomycin from *Amycolatopsis orientalis (formerly Nocardia orientalis*). Polymyxins bind to membrane phospholipids and thereby interfere with membrane function and are occasionally given for urinary tract infections caused by

Pseudomonas strains that are gentamicin, carbenicillin and tobramycin resistant. They act by competitively replacing Mg^{2+} and Ca^{2+} from negatively charged phosphate groups on membrane lipids. The result is disruption of the cell membrane (**Fig.1.17**) [Tritz, 2000].

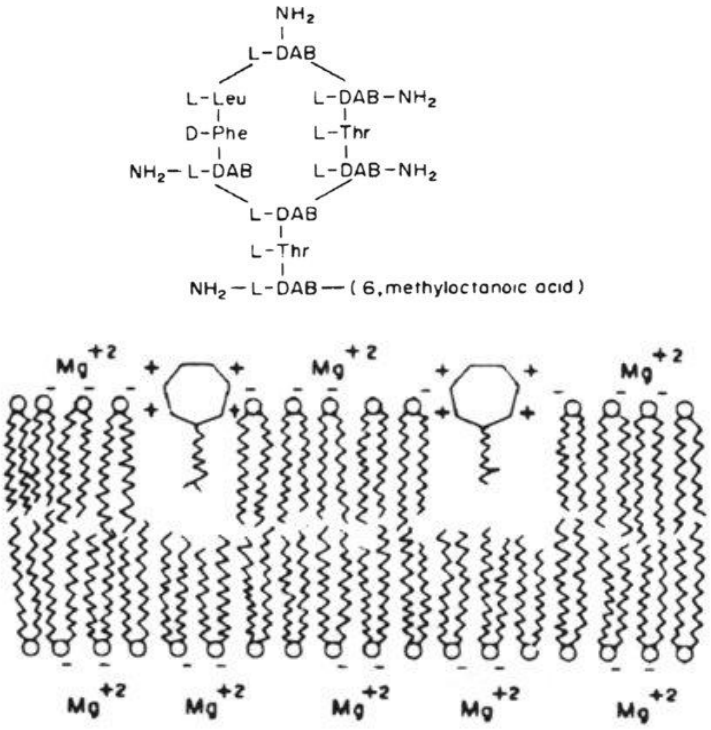


Fig.1.17 Disruption of a cell membrane by a polypeptide, polymyxin B: Source: Tritz, (2000)

Bacitracin (**Fig. 1.8B**) prevents Gram-positive cell wall growth by inhibiting the release of the mucopeptide subunits of peptidoglycan from the lipid carrier molecule that carries the subunit to the outside of the membrane. Teichoic acid synthesis, which requires the same carrier, is also inhibited (Todar, 2009). The principal mode of action of bacitracin is to bind tightly to undecaprenol pyrophosphate (UPP) in the presence of a bound divalent metal cation, sequestering UPP and preventing its interaction with phosphatase (Drablos *et al.*, 1999**).** This in turn prevents the return of undecaprenol monophosphate (UP) lipid carrier to the cycle, thereby inhibiting peptidoglycan and teichoic acid biosynthesis (Toscano & Storm, 1982).

Vancomycin acts by inhibiting proper cell wall synthesis in Gram-positive bacteria. The mechanism inhibited, and various factors related to entering the outer membrane of Gram-negative organisms mean that vancomycin is not active against Gram-negative bacteria. Specifically, vancomycin prevents incorporation of N-acetylmuramic acid

(NAM) - and N-acetylglucosamine (NAG)-peptide subunits from being incorporated into the peptidoglycan matrix; which forms the major structural component of Gram-positive cell walls (Lilly, 1999).

1.6.3 Inhibition of protein synthesis

The inhibitors of protein synthesis mainly interfere with the functions of the 30S or 50S ribosomal subunit (Lancini and Parenti, 1982). Aminoglycosides (e.g. streptomycin, kanamycin, paromomycin) interfere with decoding or translational accuracy of the 30S ribosomal subunit while spectinomycin disrupts translocation between A (aminoacyl), P (peptidyl) and E (exit) sites on ribosomes. Tetracyclines act by blocking proper binding of incoming aminoacyl-tRNA to the A site on the ribosome (Alekshun and Levy, 2007; Todar, 2009). They inhibit protein synthesis on isolated 70S or 80S (eucaryotic) ribosomes, and in both cases, their effect is on the small ribosomal subunit (Todar, 2009). However, most bacteria possess an active transport system for tetracycline that will allow intracellular accumulation of the antibiotic at concentrations 50 times as great as that in the medium. This greatly enhances its antibacterial effectiveness and accounts for its specificity of action, since an effective concentration cannot be accumulated in animal cells. Thus a blood level of tetracycline which is harmless to animal tissues can halt protein synthesis in invading bacteria. Some newly discovered members of the tetracycline family (e.g. chelocardin) have been shown to act by inserting into the bacterial membrane, not by inhibiting protein synthesis (Todar, 2009).

The 50s ribosome-binding antibiotics such as chloramphenicol, block the aminoacyl-tRNA interaction with the peptidyltransferase center (A-site) in the 50S ribosome, inhibiting transpeptidation (‘connection’) [Mayberry, 2004]. Macrolides (e.g., erythromycin, azithromycin, and clarythromycin) block the approach to the peptide exit tunnel, as well as blocking assembly of the 50s ribosome subunit. Ketolides (e.g., telithromycin) are modified macrolides and block the same sites. Linezolid is the first of a new class of synthetic antibiotics and it interacts with the peptidyltransferase center P-site and blocks the first peptide-bond forming step. Synercid is a naturally occuring mixture of compounds that synergistically block peptide translation in the 50s ribosome (Harms *et al*., 2004). Inhibitors of protein synthesis are bacteriostatic if they do not form irreversible bonds with some essential component of the synthetic system. If they do, they are bactericidal (Franklin and Snow, 1981).

1.6.4 Inhibition of nucleic acid synthesis

Antibiotics with this mechanism of action include replication inhibitors and transcription inhibitors such as nalidixic acid and the quinolones (Okudoh, 2001). They act by inhibiting α- (or alpha-) subunit of DNA gyrases and topoisomerases. Novobiocin inhibits the β- (or beta-) subunit of DNA gyrases or topoisomerases. Rifampicin (rifamycin) inhibits RNA synthesis by RNA polymerase (Franklin and Snow, 1981). Most of the inhibitors of genetic material (nucleic acids) are used in cancer chemotherapy rather than as antimicrobial agents (Lancini *et al*, 1995).

1.7 Industrial production of antibiotics

The commercial production of antibiotics has been widespread since the pioneering efforts of Florey and Chain in 1938. Antibiotics are produced industrially by a process of fermentation where the source organism is grown in large fermenters (100 000 – 150 000 litres or more) containing a liquid growth medium. Oxygen concentration, temperature, pH and nutrient levels must be optimal and are closely monitored and adjusted if necessary (Madigan and Martinko, 2005).

As antibiotics are secondary metabolites the microbial population size must be controlled very carefully to ensure that maximum yield is obtained before the cells die. Once the process is complete, the antibiotic must be extracted and purified to a crystalline product. This is simpler to achieve if the antibiotic is soluble in an organic solvent and both the properties of the solvent and of the antibiotic are in equilibrium (Caco *et al*., 2008). The solubility of antibiotics in organic solvents plays a key role in the crystallization process which is a crucial step in the manufacture of many pharmaceuticals as pure products that are recovered easily through filtration (Gracin *et al.,* 2002) Otherwise the antibiotic must first be removed by ion exchange, adsorption or chemical precipitation (Madigan and Martinko, 2005).

1.7.1 Strains used for industrial production

Microorganisms used in industrial fermentations are rarely identical to the wild type. In the antibiotic field, this is because species are often genetically modified to yield the maximum amounts of the substance. Mutation is often used and is encouraged by introducing mutagens such as UV radiation, X-rays or certain chemicals e.g. nitropyrenes, benzopyrene, ethylene oxide, ethionamide, etc. Ongoing selection of the

higher yielding strains over many generations can raise yields 20-fold or more (Madigan and Martinko, 2005).

1.7.2 Antibiotics as secondary metabolites

Products of industrial microorganisms are divided into two: primary and secondary metabolites from primary and secondary metabolism respectively. Primary metabolism is the interrelated series of enzyme-catalysed reactions which provide the cell with energy, synthetic intermediate products and macromolecules such as protein and nucleic acids. Products of primary metabolism are associated with cell growth and their maximum production occurs in the logarithmic phase of growth in a batch culture (Rose, 1979).

In contrast to primary metabolism, secondary metabolism has no apparent function in the organism. The organism continues to exist if secondary metabolism is blocked and would die if primary metabolism were stopped. A summary of the major differences between primary and secondary metabolism is shown in **Table 1.6**. Antibiotics are the most successful products of secondary metabolism and will be the main focus of this review.

1.7.2.1 Trophophase-idiophase relationships in the production of antibiotics

The terms ‘trophophase’ and ‘idiophase’ were introduced by Bu’Lock in 1965 to distinguish two phases of growth of organisms producing secondary metabolites. The trophophase (Greek, tropho = nutrient) is the feeding phase of the growth curve, while the idiophase (Greek, idio = peculiar) is the antibiotic production phase. The trophophase occurs at the logarithmic phase while idiophase occurs at the end of the logarithmic and in the stationary phase of the growth curve. The name ‘idiolites’ has been suggested for secondary metabolites to distinguish them from primary metabolites (Demain, 1974).

Relationship between antibiotic production and sporulation has been reported (Martin and Demain, 1978). An example is in the production of peptide antibiotics by *Bacillus* spp. Both spore formation and antibiotic production were suppressed by glucose; non-spore forming mutants of bacilli also did not produce antibiotics, while reversion to spore formation was accompanied by antibiotic production (Demain, 1974).

Table 1.6 Major **d**ifferencies between primary and secondary metabolism

Primary metabolism	**Secondary metabolism**
Interrelated series of enzyme-catalysed reactions	Specific enzymatic reactions
Same for all species	Species specific
Metabolites not excreted into medium	Metabolites excreted into medium
Metabolites not overproduced	Metabolites often overproduced
Products rarely accumulates in medium	Products accumulates in substantial quantities
Non repression of product synthesis	Frequent feedback inhibition
Product formation parallel with growth	Late formation of products
Products essential for growth and reproduction	Products has no apparent function in the organism
Product completely created from growth substraes	Product initially created from primary metabolites and later from growth substrates
Unrelated products	Family of closely related products
Provide cell with energy	Means of cell protection from adverse environment

Source: Adapted from Madison and Martinko, (2005).

Many roles have been assigned to antibiotics in spore-formers but the most clearly demonstrated has been the essential nature of gramicidin in sporulation of *Bacillus* spp. The absence of the antibiotic leads to partial deficiencies in the formation of enzymes involved in spore formation, resulting in abnormally heat-sensitive spores (Okafor, 1982). The antibiotic, therefore, suppresses the vegetative genes allowing proper development of the spores. It appears, therefore, that the production of secondary metabolites is necessary to regulate some morphological changes in the organism. It could of course be that some external mechanism, nutritional or otherwise, triggers off antibiotic formation as well as the process of spore formation (Okafor, 1982).

1.7.2.2 Control of antibiotic production

Most organisms are able to decide when to manufacture and secrete certain enzymes to enable it to utilize materials in the environment and also to stop the manufacture of certain compounds if they are supplied to it. These sensing mechanisms for the switching on and off of synthetic mechanism enable the organism to avoid the over-production of any particular compound (Okafor, 1982).

The regulatory control of the organism against over-production of secondary metabolites is controlled at three levels: (i) the regulation of enzymes (ii) permeability of the organism and (iii) the genetic level. In this review, the regulation of enzymes as it affects antibiotics control will be discussed.

Enzyme repression

Often the enzymes catalyzing the synthesis of antibiotics are not synthesized if a particular substance is present in the medium. A good example is in penicillin production where the antibiotic is not produced in a glucose-containing medium until after the exhaustion of the glucose, and when the idiophase sets in. Indeed, the 'glucose effect' as it is known is common to a large number of secondary metabolites (Okafor, 1982). It was found that the glucose (substrate) level does not repress the penicillin production but it represses the synthesis of the enzymes necessary for the formation of the antibiotic (Okafor, 1982).

Enzyme induction

Some enzymes are produced by microorganisms only when the substrate on which they act is available in the medium. Examples include enzyme induction by methionine in the synthesis of cephalosporin C by *Cephalosporium ocremonium* and also by tryptophan in ergot alkaloid fermentation (Drew and Demain, 1977).

Feedback inhibition

A major mechanism for the regulation of enzymes involves the phenomenon of feedback inhibition (Madigan and Martinko, 2005). In feedback inhibition, the end product of a biosynthetic pathway inhibits the activity of the first enzyme in this pathway. Thus, as the end product builds up, its further synthesis is inhibited. If the end product is used up, however, synthesis can resume (**Fig. 1.18**).

The above inhibition is made possible by allosteric enzymes which has two combining sites, the active site, where the substrate binds, and the allosteric site, where the inhibitor (effector) binds reversibly. When the effector combines with the allosteric site, the conformation of the enzyme is altered so that the substrate can no longer bind (Madigan and Martinko, 2005).

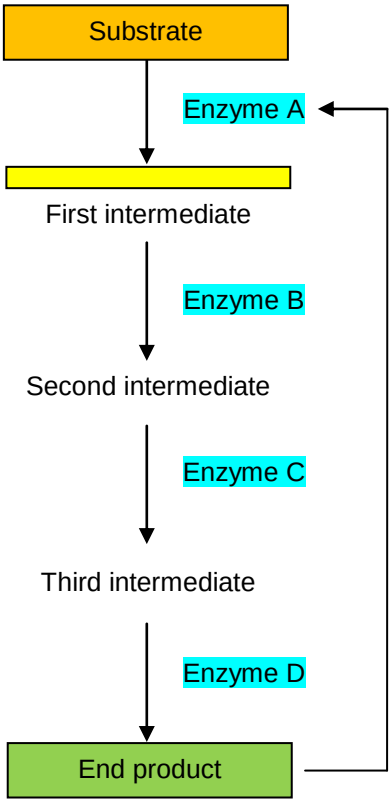


Fig. 1.18 Feedback inhibition: The activity of the first enzyme is controlled by the end product, thus controlling production of end product.

1.7.3 Optimization of antibiotic yield – from laboratory to industry

Most antibiotic production in the laboratory is carried out at the shake flask level and lacks a clear understanding of the production process requirements in industry, offering poor prospects for successful scale-up. There is a need for early data collation on media and physical optima differences between the trophophase (growth phase) and idiophase (production phase) to allow implementation of novel fermentation protocols (Marwick *et. al*. 1999). Cognisance of factors described below is necessary to achieve maximum yield of the antibiotic product.

1.7.3.1 Manipulation of media components

Carbon

The choice of carbon source greatly influences antibiotic production (Spizek and Tichy, 1995). A quickly metabolized substrate such as glucose may often achieve maximum cell growth rates but tends to inhibit the production of many secondary metabolites through catabolite repression. The reason is that intermediate products generated from the rapid catabolism of glucose interfere with the enzymes participating in the secondary metabolism process (Gallo and Katz, 1972). Fast growing cells generally have secondary metabolism switched off until their growth rate slows, via feedback inhibition. This can lead to a fully biphasic fermentation profile, with no production during growth, only during the stationary phase (Liao *et. al.* 1995). Galactose, which is a slowly utilized substrate, can improve antibiotic yields especially in fungi. Nevertheless, the separation of trophophases and idiophases is not well-defined. Production of bacitracin by *Bacillus licheniformis* can be observed during the growth phase, when a slow growth medium is used. Glycerol was found to increase the total yield of manamycin by a *Streptomyces* species when it was fed during the production phase (Kaiser *et al.*, 1994).

Nitrogen

The nitrogen source tends to regulate, secondary metabolism and high nitrogen levels repress idiophase production of many antibiotics (Doull and Vining, 1990; Spizek and Tichy, 1995). However, Zhang *et al.*, (1996) found low ammonium levels to stimulate an antibiotic produced by *Streptomyces griseofuscus*. The use of unstable amino acids as a nitrogen source can inhibit good synthesis of secondary metabolites (Martin and Demain, 1980). The effect may be due to a specific amino acid interacting with the regulation of secondary metabolic pathways. Therefore, it is necessary to optimize the nitrogen type and concentration at different stages of antibiotic production.

Phosphate

Phosphate, although essential for growth, can at below or above optimal concentrations suppress secondary metabolism (Spizek and Tichy, 1995). Interestingly, the level of adenosine triphosphate (ATP) has been observed to decrease significantly before secondary metabolism begins. This was attributed to a rise in phosphatase activity after the cessation of growth (Marwick *et al*., 1999).

Trace elements

The effect of specific metal ions on *Burkholderia glume*, with regard to secondary metabolite production, was investigated by Yamaski *et al.*, (1998), who concluded that certain metal ions such as Zn^{2+}, K^{+} and Fe^{3+} gave optimum yields. If $MgCl_2$ was replaced by $MgSO_4$, no production occurred. Zinc sulphate was found to increase phenazine production by *P. fluorescens* (Slininger and Jackson, 1992). Similarly, KNO_3 or $FeCl_3$ increased phytotoxin production by *P. syringae* (Palmer and Bender, 1993). The above results have interesting implications in fermentation for the control of antibiotic production.

1.7.3.2 Alteration of physical parameters

Temperature

Temperature is a regulatory factor in secondary metabolism of *Streptomyces thermoviolaceus* (James *et al.*, 1991) and mycotoxin production by *Aspergillus species* increased when temperature was reduced from its natural level (Tepsic *et al.*, 1997). A *Lactobacillus sp.* yielded more exopolysaccharide with a temperature shift from 37^{o}C to 25^{o}C at the beginning of the exponential phase (Gamar *et al.*, 1998). Therefore, temperature shifts or cycles may be necessary during fermentations for antibiotic production (Marwick *et al*., 1999).

pH

The pH level of a growth medium has a marked effect on secondary metabolite production, with synthesis falling rapidly on either side of the optimal level. This was the case for violacin production from the marine bacterium *Alteromonas leuteoviolacea*, which ceased at pH 9 after an optimum had been reached at pH 7 (McCarthy *et al.*, 1985). Hays *et al.* (1997) used pH as a stressor to induce methylomycin synthesis by a *Streptomyces* species.

Oxygen

Oxygen transfer is a major limiting factor in antibiotic production. An increase in partial pressure of oxygen was found to induce new metabolite synthesis by *Streptomyces parvulus* (Kaiser *et al.*, 1994). The oxygen transfer from sparged air to the bacterial cell

is partially dependent on medium composition, a viscous medium being harder to oxygenate than a non-viscous one (Švitel and Šturdik, 1995).

Salinity

Some bacteria living in a marine environment require salt for growth (Kogure, 1998). However, the effect of salt on antibiotic production has not been extensively researched. Salinity was found by Okami *et al.* (1976) to effect the production of aplasmomycin by a marine *Streptomyces* sp. High salt content may cause problems with bioreactor corrosion, and may also inhibit the dissolution of sparged oxygen into a water-based medium (Garcia and Gordon, 1992).

Pressure

Pressure may act as a stressor to increase antibiotic production via Le Chatelier's principle, if a negative volume change in reactants to products is favoured (Wright *et al*., 1999).

1.7.3.3 Regulatory factors

Induction

Inducing antibiotic production during the growth phase, thereby overriding feedback inhibition can increase antibiotic yields. This has already been achieved in *Streptomyce*s culture, where *virginiae butanolides* (an auto regulator for antibiotic synthesis) was added after 8 hours, resulting in a doubling of virginomycin production (Young *et al.*, 1995). Also, antibiotic production can be induced by antagonizing the bacterial cells into defending themselves against a perceived threat. For example, Fredrickson and Stephanopoulus (1981) stated that microbes competing for a single nutrient would try to eliminate one another via toxic warfare. In fact, a new method has been developed to enhance antibiotic production by marine bacteria, namely the use of terrestrial bacteria as antagonists (Burgess *et al*., 1999).

Induction does not necessarily have to involve microbial products; thus the antibiotic production of a marine *Bacillus* was found to be dependent on the addition of an amino acid analogue, selenomethionine (Imada *et al.*, 1998). Such control of secondary metabolism so as to override normal primary metabolic repression can be very useful in intensified production of antibiotics (Marwick *et al*., 1999).

Inhibition

Auto-inhibition of antibiotic-producing microbes is well documented (Anderson *et al.*, 1974; Lemos *et al.*, 1991). The use of a continuous or semi-continuous fermentation protocol allows extraction of any inhibitory compounds from the medium, ensuring no repression of antibiotic production rates (Marwick *et al*., 1999).

Genetics

Genetic manipulation of antibiotic-producing microbes to increase yield is well established (Matsunaga and Takeyama, 1995; Lloyd *et al.,* 1997; Hosoya *et al*., 1998). Copies of genes encoding for enzymes involved in antibiotic production can be inserted into a cell, via vectors such as plasmids. This process is closely linked with retesting of antibiotic production and effectiveness (Madigan and Martinko, 2005)

1.7.4 Fermentation process development

Optimization of product yield in the laboratory is followed by process scale-up; first to pilot scale of 10 – 100 L and finally to industrial scale of 1000 to 100 000 L, or more, depending upon the specific process. However, during the scale-up process, decreased product yields are often experienced because the conditions in large scale fermenters are not identical to those experienced in the smaller-scale laboratory or pilot plant systems (Waites *et al*, 2001). The essential components of fermentors used in antibiotic production are shown in **Figure 1.19**.

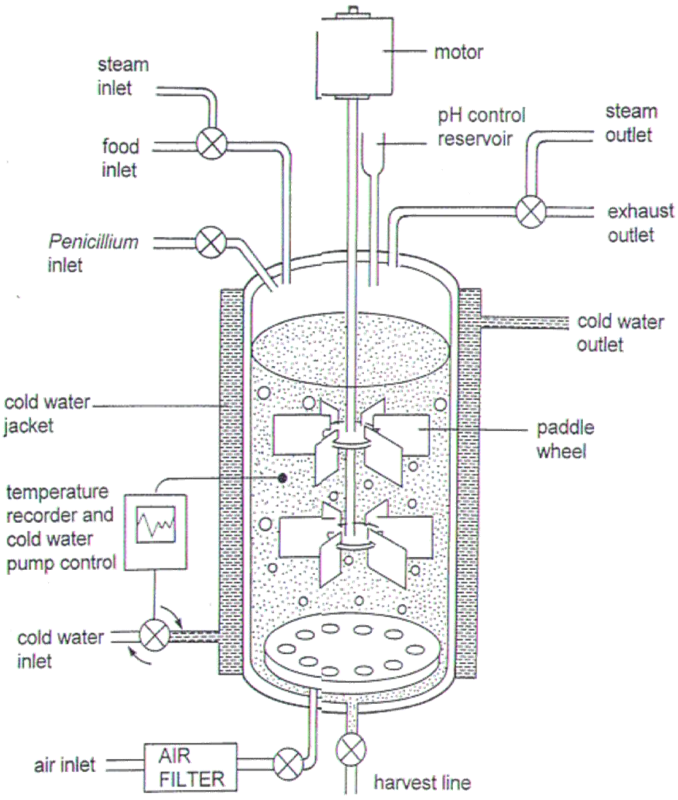


Fig. 1.19 The essential components of a completely stirred tank fermenter used for penicillin production. Source: Cowan, (2009).

Once a microorganism has been selected as a producer organism for a particular antibiotic, media formulation and an examination of operating systems (batch, fed-batch, continuous, etc. **(Fig. 1.20)** are researched along with selection of the most suitable type of fermentation system (stirred tank, airlift, packed bed, solid state, hollow fibre, etc.). Other factors that are considered include fermenter configuration and control of pH, dissolved oxygen, foam and temperature (Cowan, 2009).

A stirred batch fermenter is a closed system where all nutrients are present at the start of the fermentation in a fixed volume, whereas in a fed-batch fermenter fresh medium is introduced throughout the fermentation and the volume of the batch increases with time. Continuous culture is where fresh medium is fed constantly into the vessel and spent medium and cells are removed at the same rate (fixed volume). In all systems, pH, temperature, aeration, etc., are monitored and adjusted (Cowan, 2009).

There are obvious advantages and disadvantages in each of the operating systems. The batch system is the simplest fermenter operation. Sterilisation can be performed in the reactor and all nutrients can be added before inoculation. However, biomass production is limited by carbon/nitrogen (C/N) load and production of toxic waste products. The cells are harvested when biomass levels or product levels start to decline (Cowan, 2009).

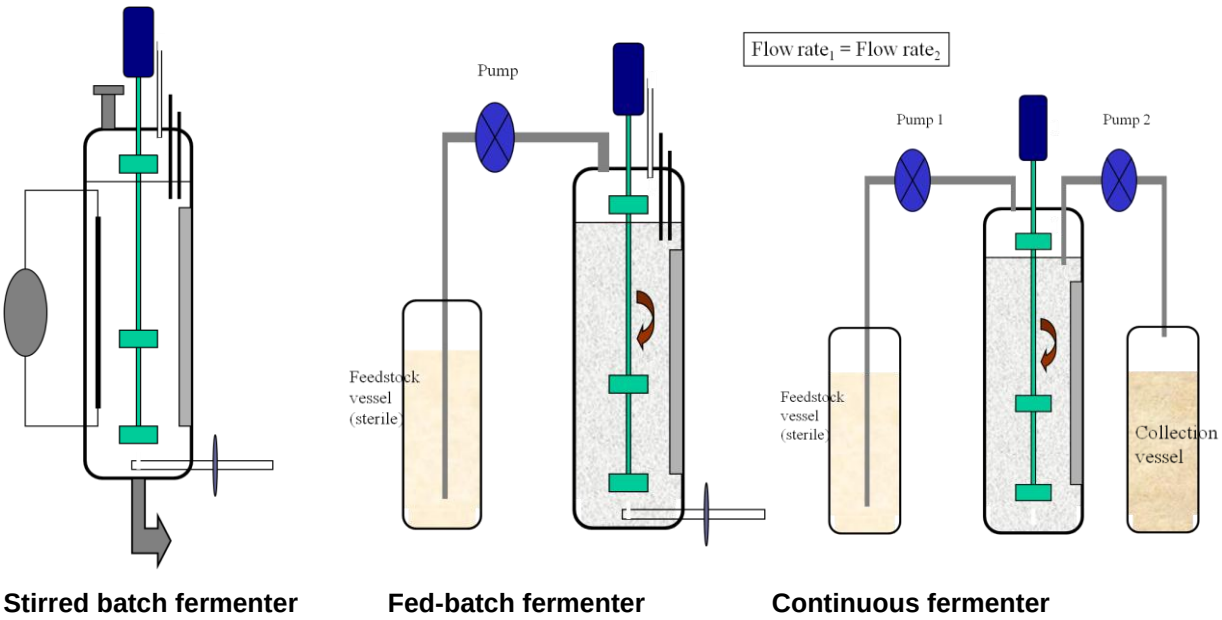


Fig. 1.20 Design of fermentation systems used in antibiotic production. Source; Cowan, (2009).

In the fed-batch system the initial medium concentration is relatively low (no inhibition of culture growth) and the medium constituents (concentrated C and/or N feeds) are added continuously or in increments. This controlled feed results in higher biomass and product yields. However, fermentation is still limited by accumulation of toxic end products (Cowan, 2009).

The continuous fermenter has an input rate that is equal to output rate (total operating volume is constant) and the flow rate can be selected to give steady state growth (growth rate is equal to dilution rate) of the microbial cells. If the dilution rate is greater than, less than or equal to the growth rate the culture either washes out, overgrows or remains stable respectively. The product is harvested from the outflow stream and this system can be operated continuously for weeks or months (Cowan, 2009).

1.8 Bacterial resistance to antibiotics – General overview

Antibiotic resistance is the ability of a microorganism to withstand the effects of antibiotics. This resistance evolves via natural selection acting upon random mutation, but it can also be engineered by applying an evolutionary stress on a population (**Fig. 1.21**). If a bacterium carries several resistance genes, it is called multiresistant or, informally, a superbug. The different resistance genes are linked to each other on segments of DNA able to move efficiently from one bacterial cell to another by a phenomenon known as horizontal gene transfer (HGT) [Clewell, 2008].

Fig. 1.21 Scheme of evolution of resistant strains in a bacterial population, Source: Wikipedia, (2009).

Horizontal gene transfer can occur by three basic mechanisms: transformation (the release of free DNA from one bacterial cell to be actively taken up by another); transduction (transfer of DNA via the use of bacterial viruses [phages] as “vectors”); and conjugation (the movement of DNA from one cell to another via cell-to-cell contact). Conjugation phenomena frequently involve mobile plasmids or conjugative

transposons, which encode their ability to move copies of themselves from one bacterial cell to another and are widespread in the bacterial world (Clewell and Francia, 2004; Lawley *et al*., 2004). Such transferable elements have been the subject of much investigation (Clewell, 1993; Funnell and Phillips, 2004).

Staphylococcus aureus is arguably the most troublesome of all the human pathogens (Kassem *et al*., 2007) and has resisted the effects of almost all clinically used antibiotics (Chopra *et al*., 1997; Weigel, 2003; Tenover and McDonald, 2005) and consequently will merit a brief discussion here.

1.8.1 Methicillin resistant *Staphylococcus aureus* (MRSA)

Staphylococcus aureus is found on mucous membranes and the skin of around a third of the animal population and it is extremely adaptable to antibiotic pressure. It was the first bacterium in which penicillin resistance was found in 1947, just four years after the drug started being mass produced. Methicillin was then the antibiotic of choice, hence the name, methicillin-resistant *Staph aureus* (MRSA). However, methicillin has since then been replaced by oxacillin due to significant kidney toxicity (Kassem *et al*., 2007).

Methicillin-resistant *Staphylococcus aureus* was first detected in Britain in 1961 and is now quite common in hospitals around the world. Half of all *S. aureus* infections in the United States are resistant to penicillin, methicillin, tetracycline and erythromycin. This left vancomycin as the only effective agent available at that time (Barrett, 2005). However, strains with intermediate (4 – 8 µg/ml) levels of resistance, termed GISA (Glycopeptide intermediate *S. aureus*) or VISA (Vancomycin intermediate *S. aureus*), began appearing in the late 1990s (firstly in Japan in 1996). In 2002, the first strain with complete resistance to vancomycin, VRSA (Vancomycin resistant *S. aureus*) appeared in the US (Tenover and McDonald, 2005).

A new class of antibiotics, oxazolidinones, became available in the 1990s to treat MRSA. Linezolid is comparable to vancomycin in effectiveness against MRSA, but resistance to linezolid as well as daptomycin has already been described (Tsiodras *et al*., 2001; Meka and Gold, 2004; Mangili *et al*., 2005).

Community-acquired MRSA (CA-MRSA) has emerged as an epidemic that is responsible for rapidly progressive, fatal diseases including nectrotizing pneumonia, severe sepsis in many hospitals and these infections now appear to be endemic in many urban regions around the world. This scenerio can be likened to the emergence of high-level vancomycin resistance in *Staphylococcus aureus* (Weigel, 2003) and the growing presence of multiple drug-resistant strains of *Mycobacterium tuberculosis* (Wright, 2006).

1.8.2 Mechanism of resistance – General overview

This review focuses mainly on the three major strategies used by bacteria for resistance to most of the important antibiotics in use today. Firstly, they inactivate the antibiotics by producing specific-proteins that chemically modify the antibiotic to a form that no longer interferes with the bacterial function (Salyers and Whitt, 2005). A second strategy is to reduce the intracellular antibiotic concentration by inserting a protein pump (called efflux pumps) into the cytoplasmic membrane. This bacterial protein pump can eject the antibiotic from the cytoplasm as rapidly as it enters the cell. A third resistance strategy is to chemically modify or mutate the target of the antibiotic so that the antibiotic can no longer bind to it. This strategy can be very dangerous to the bacteria because the mutation may disrupt the processes essential for bacterial survival (Salyers and Whitt, 2005).

The strategies used by bacteria to resist the action of β-lactams, polypetides and aminoglycosides will be discussed. The following discussion will also look at three other clinically important antibiotic groups namely, the tetracyclines, macrolides and rifampicin.

The first mechanism of bacterial resistance to penicillin to be described involved the enzyme β-lactamase. This enzyme cleaves the β-lactam ring of penicillin **(Fig. 1.4)**, rendering the antibiotic inactive (Salyers and Whitt, 2005, Hubschwerlen, 2007).

Beta-lactamases seem to have evolved from the proteins that catalyze the cross-linking of peptidoglycan, the proteins that are the target of penicillin. When penicillin binds one of the proteins, the protein mistakes penicillin for the two alanines found at the end of the peptide that normally participate in the cross-linking reaction. The cross-linking

enzyme can start but not complete the hydrolysis of the β-lactam ring. The partially hydrolyzed antibiotic is then trapped in the active site of the enzyme which would normally cease to function. However, the enzyme has evolved to complete the reaction, hence releasing a penicillin molecule with a broken β-lactam ring (Salyers and Whitt, 2005).

To combat bacterial β-lactamases scientists have developed modified forms of penicillin such as carbapenem (**Fig. 1.4**) by surrounding the β-lactam ring with other chemical groups. These new antibiotics are called β-lactamase-resistant β-lactams (BLRBLs). However, β-Lactamases themselves mutate so that the new drugs are no longer effective (Hubschwerlen, 2007).

Another attempt by scientists to deal with β-lactamases was to develop an antibiotic preparation that contains both the antibiotic and an inhibitor of β-lactamase e.g. augmentin which contains a compound (clavulanic acid) that inhibits the bacterial enzyme so that it can no longer destroy amoxicillin, a member of the penicillin family (Salyers and Whitt, 2005).

Vancomycin resistance emerged from a strain of *Staphylococcus aureus* called vancomycin-resistant *S. aureus* (VRSA) that started out being resistant to many antibiotics, including β-lactams. Vancomycin resistance is regarded by many scientists as the last frontier in the battle between bacteria and humans (Dale-Skinner and Bonev, 2008). Vancomycin was originally thought to be the ultimate resistance-proof antibiotic because becoming resistant to it would require bacteria to change their cross-link peptide structure, a change that might be lethal to the bacterium (Salyers and Whitt, 2005). However, resistance to vancomycin actually involves several genes encoding several proteins that comprise a pathway for changing the peptidoglycan cross-linking peptides into a form that no longer binds vancomycin but will still be cross-linked by bacterial enzymes. There is also a gene that encodes an enzyme that degrades the original terminal D-alanine-D-alanine part of the cross-linking peptide (**Fig. 1.22**) [Salyers and Whitt, 2005].

Resistance to polypeptide antibiotics is common, and different polypeptides tend to have different patterns of resistance or susceptibility among bacteria. Resistance to any one polypeptide often varies widely among closely related bacterial species, and

can even vary among different strains of the same species (Axelsen, 2008). In any case, bacteria do not develop secondary resistance when cultivated in sub-inhibitory concentrations of polypeptide antibiotics (e.g. Bacitracin), as is usually the case with antibiotics in other classes (Howard, 2008). This “resistance to acquired resistance” has drawn considerable attention among investigators seeking new antibiotics for use against the growing menace of pathogens with multidrug resistance (Axelsen, 2008).

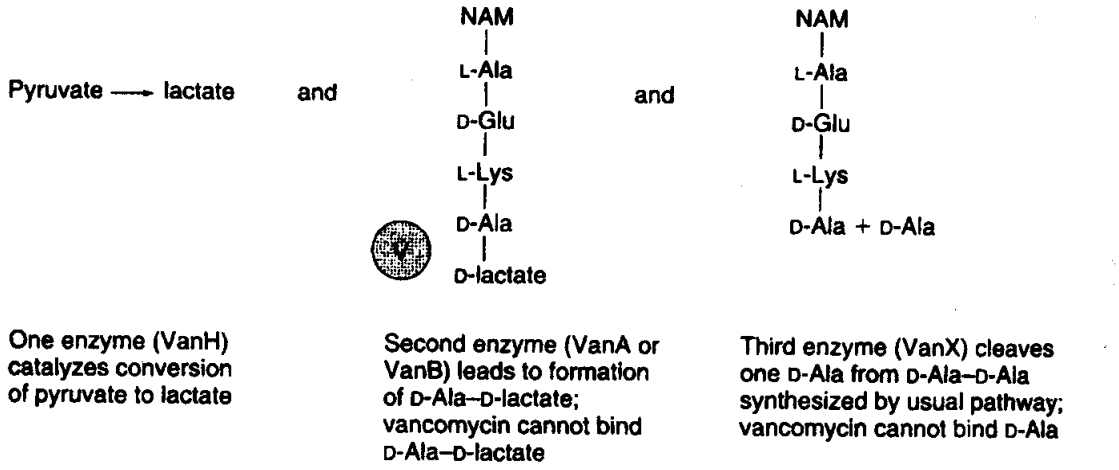


Fig. 1.22 The mechanism of vancomycin resistance. Two enzymes in the resistance pathway replace the D-alanine-D-alanine with D-alanine-D-lactate, which does not bind vancomycin. One enzyme links D-alanine to D-lactate. A second one converts pyruvate, an intermediate in glycolysis, to D-lactate. A third enzyme hydrolyses any normal D-alanine-D-alanine peptides that might form and be bound by vancomycin. The cross-linking enzymes of the cell seem to be able to handle the D-alanine – D-alanine to D-alanine-D-lactate change and thus do not need to be modified. The encircled V represents vancomycin, which is no longer able to bind. (Source: Salyers and Whitt, 2005).

One way bacteria can become resistant to aminoglycosides e.g. streptomycin is to mutate the ribosomal protein that provides the binding site for the antibiotic. The antibiotic will no longer bind to the ribosome and thus will no longer inhibit the growth of the bacteria. The fact that different aminoglycosides bind to different ribosomal proteins means that mutation of one ribosomal protein does not confer resistance to all members of the antibiotic family, yet there are strains of bacteria that are resistant to many aminoglycosides (Davies and Wright, 1997). Many of these multiresistant strains have acquired an enzyme that modifies aminoglycosides by covalently attaching a chemical group (phosphoryl, acetyl, adenyl) to the antibiotic (**Fig. 1.23**). Modification of

the antibiotic prevents it from binding to the ribosome and thus eliminates its ability to stop protein synthesis (Salyers and Whitt, 2005).

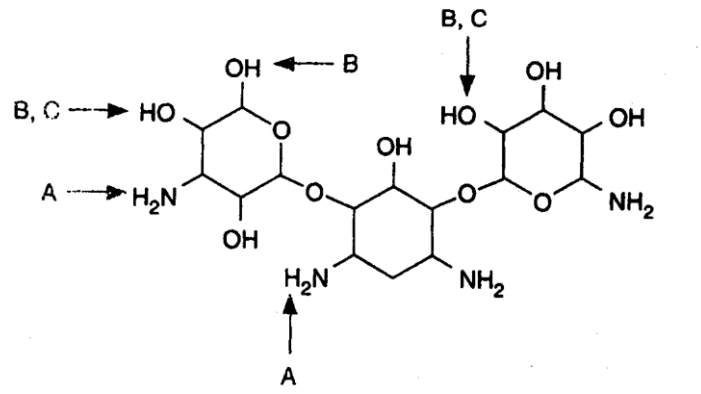


Fig. 1.23 Modification of aminoglycosides by attachment of chemical groups such as acetyl (A), adenyl (B), and phosphoryl (C). Source: Salyers and Whitt, (2005).

Bacteria can also become resistant to aminoglycosides by failing to take them up (Davies and Wright, 1997). Aminoglycosides are charged molecules that do not diffuse readily through the cytoplasmic membrane of a bacterium. Thus, they can enter the bacterial cell only if they are actively transported through the membrane. This type of resistance is not fully understood because scientists still have to identify the proteins that transport aminoglycosides into the bacterial cytoplasm (Davies and Wright, 1997).

In contrast to the aminoglycoside antibiotics, tetracyclines diffuse readily through bacterial membranes, so that cells cannot become resistant to it by failing to take up the antibiotic. However, three mechanisms of resistance to tetracycline have been elucidated (Salyers and Whitt, 2005). The first mechanism to be discovered was called antibiotic efflux in which a protein located in the bacterial cytoplasmic membrane actively pumps tetracycline out of the cell (Levy, 1992; Salyers and Whitt, 2005) [**Fig. 1.24**]. A similar mechanism has also been implicated in resistance of cancer cells to antitumour drugs (Salyers and Whitt, 2005).

A second type of resistance is mediated by a bacterial protein that protects the ribosome (**Fig. 1.25**) by modifying it so that it no longer binds tetracycline but is still able to synthesize proteins (ribosome protection-type tetracycline resistance (RPTS). In this case, tetracycline accumulation within the cell is similar to that in a sensitive cell, but the ribosome is protected (hatching) [**Fig. 1.25**], so that tetracycline no longer binds to it (Salyers and Whitt, 2005).

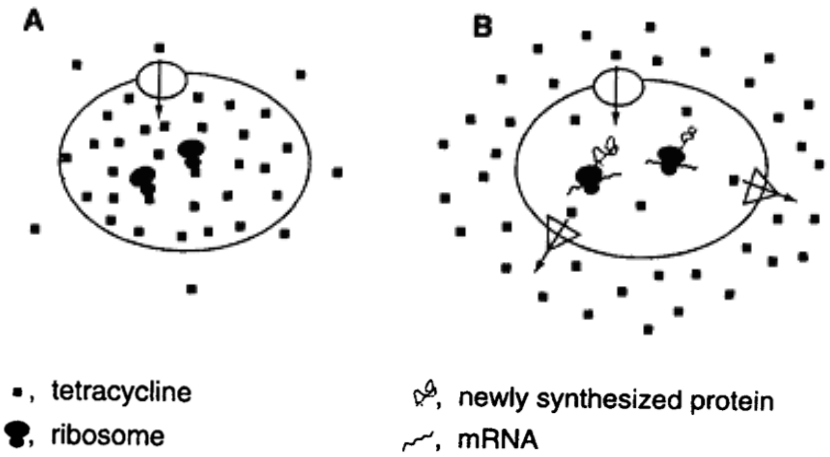


Fig. 1.24 Mechanisms of bacterial resistance to tetracycline: A, Tetracycline (black squares) is taken up by a transporter (open ellipse) causing the intracellular concentration to become higher than the extracellular concentration which allows tetracycline to bind to the ribosomes in sensitive cells thereby stopping protein synthesis. B, cytoplasmic membrane protein (open triangles) pumps tetracycline out of the cell as fast as the transporter takes it up resulting in the intracellular concentration remaining too low for effective binding to ribosomes. Source: Salyers and Whitt, (2005).

A third type of resistance is found only in strains of *Bacteroides* species that normally live in the human intestinal tract. This form of resistance is mediated by an enzyme that chemically modifies tetracycline, rendering it inactive (Salyers and Whitt, 2005).

The macrolides (e.g. clarithromycin and erythromycin) are useful as alternative treatments to patients who are allergic to penicillin (Neu, 1991) and are normally used to treat respiratory and wound infections. More recently clarithromycin has been reported to cure gastric ulcers (Sun *et al*., 2005).

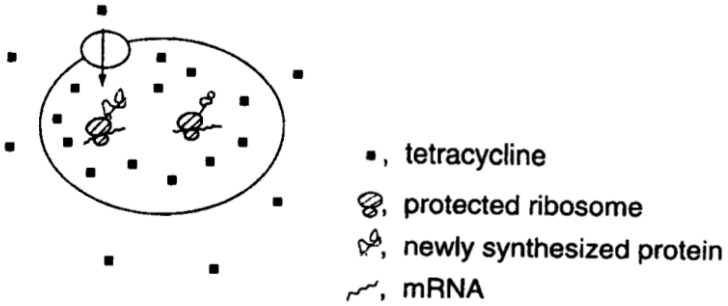


Fig. 1.25 Ribosome protection-type tetracycline resistance. Source: Salyers and Whitt, (2005).

Erythromycin acts by binding to the 50S subunit of the bacterial ribosome and preventing the elongation of bacterial proteins. This type of mechanism is shared by two other classes of antibiotics, lincosamides and streptogramins that do not have the same structure. They also share the same mechanism of resistance. This is because, the 26S rRNA molecule, a large subunit rRNA molecule, forms the centre of the binding site of all three classes of antibiotic. Thus, a bacterial enzyme that methylates a key residue on this rRNA molecule simultaneously confers resistance to all three classes of antibiotics by reducing their binding to the ribosome (Salyers and Whitt, 2005).

Resistance to rifampicin occurs readily from mutations in the β-subunit of RNA polymerase that prevents rifampicin from binding to it. Such mutations occur very readily, which is one reason why rifampicin is used mostly in combination with other tuberculosis (TB) antibiotics (cocktail). Some of the antibiotics included in the cocktail are fluoroquinolones, streptomycin, isoniazid, ethambutol, and pyrazinamide (Salyers and Whitt, 2005). Most TB patients do not comply very well with a drug regimen that forces them to take several antibiotics daily or 2 to 3 times a week for more than 6 months. In the case of HIV patients, the TB treatment takes up to a year or even more. This problem led to the introduction of DOTS (directly observed therapy short course) in the United States. It compels TB patients to come regularly to a clinic or to have health workers visit them in their homes to make sure that the full course is taken. The DOTS programme was highly effective and helped to reduce the risk of *Mycobacterium* resistance to TB drugs in the US (Salyers and Whitt, 2005).

1.8.3 Resistance and chemotherapy

There are many reasons that lead to treatment failure with antibiotics, some of which have been wrongly credited to microbial resistance (Salyers and Whitt, 2005): (1) misdiagnosis of infection; although antibiotics will have no effect if the causative agent of a disease is a virus or a fungus, some physicians still prescribe them; (2) use of an antibiotic that has the wrong pharmacokinetic properties i.e. where an antibiotic fails to get to where it is needed e.g. an antibiotic that does not penetrate the blood-brain barrier will not help a meningitis patient that has bacteria in the cerebrospinal fluid; (3) lack of essential information on the best type of antibiotics to use in treating some disease conditions e.g. the presence of dead tissues in abscesses can inhibit the penetration of some antibiotics; (4) compliance failure e.g. most patients do not finish

the full course of a prescribed antibiotic and this results in treatment failure. In order to work, an antibiotic must interact with the appropriate target, in the appropriate location in the cell, at the appropriate concentration – any change might lead to development of resistance (Salyers and Whitt, 2005; Todar, 2009).

1.9 The future of antibiotic research – General overview

There are few new antibiotics in the pipeline today (Mian and Davis, 2010). The reasons may include starvation at the front of the pipeline due to inadequate sources of suitable compounds to screen coupled with poorly validated discovery methodologies. A successful antibiotic discovery approach in the past, based upon whole cell antibiotic screening of natural products from actinomycetes and fungi, eventually suffered from constipation in the middle of the pipeline due to rediscovery of known compounds, even though low throughput methodology was employed at the front end. The current lack of productivity may be attributed to the poor choice of strategies to address the discovery of new antibiotics (Baltz, 2006). Approaches to accelerate antibiotic discovery from microorganisms as recommended by Baltz (2006) are summarized in **Table 1.7**.

Table 1.7 Approaches to accelerate antibiotic discovery from microorganisms

High-throughput whole cell screening of terrestrial microorganisms
Global soil sampling
Pooling of soils and extraction of spores
Miniaturized fermentations starting with spores
Screening organism resistant to common antibiotics
Improved throughput with automation
Enrichments and selections for uncommon terrestrial and marine microbes
Antibiotics and taxon selective media
Untapped random and exotic soils
Untapped marine sediments
Genome mining
Sequencing multiple common microbes
Sequencing rare and slow growing microbes
Expression of new pathways in robust bacterial hosts
Combinatorial biosynthesis
NRPS pathways
PKS pathways
Glycosylations and other modifications

Source: Baltz (2006)

However, recent applications of high throughput in vitro screening of individual antibacterial targets to identify lead compounds from combinatorial chemical libraries, traditional chemical libraries, and partially purified natural product extracts has not produced any significant clinical candidates. The solution to the current dilemma may be to return to natural product whole cell screening. For this approach to work in the current millennium, the process needs to be miniaturized to increase the throughput by orders of magnitude over traditional screening, and the rediscovery of known antibiotics needs to be minimized by methods that can be readily monitored and improved over time (Baltz, 2006).

It is suggested that an improved understanding of the biological roles of low molecular weight compounds in nature will lead to the discovery of an inexhaustible supply of novel therapeutic agents in the next decade. To support this objective, a functional marriage of biochemistry, genomics, genetics, microbiology, and modern natural product chemistry will be essential (Mian and Davis, 2010).

CHAPTER 2

MATERIALS AND METHODS

2.1 Introduction

All major steps used in the screening programme are shown in **Figure 2.1**.

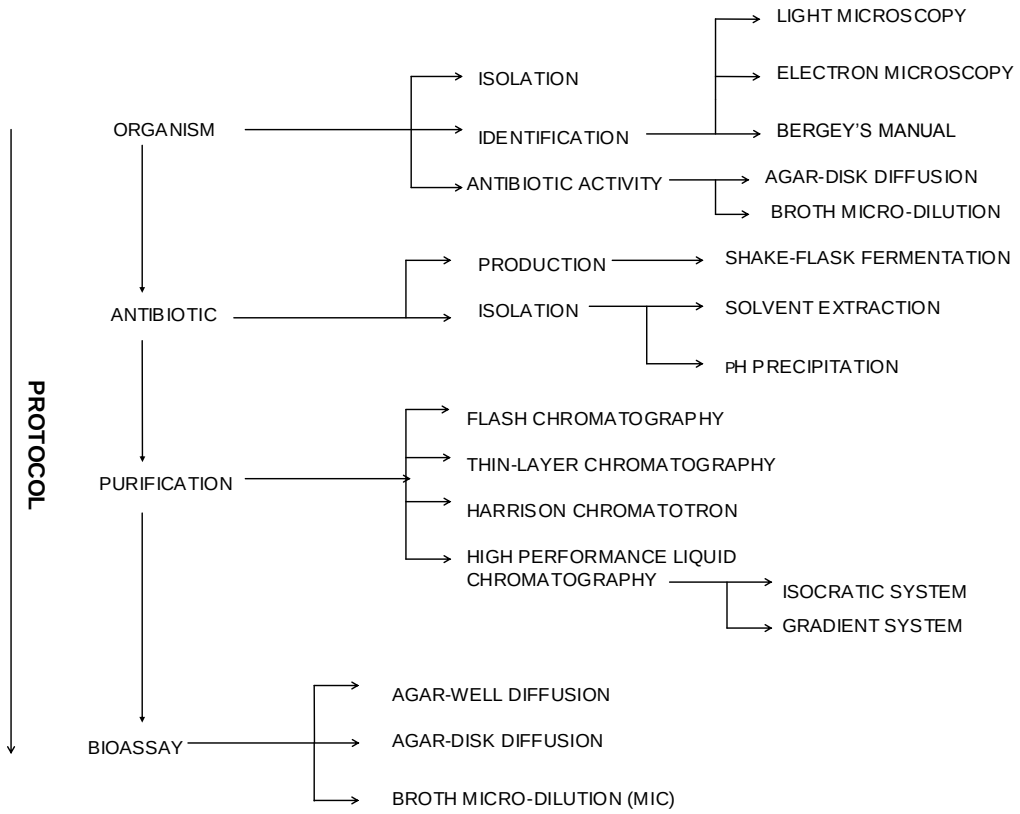


Fig. 2.1 Flowchart indicating sequential steps in the antibiotic recovery, purification and bioassay procedures used.

2.2 Sources and geographical distribution of samples

Soil samples (± 10 g) were collected at depths of 0–5 cm below the surface from various sites in the KwaZulu-Natal Midlands **(Table 3.1, Chapter 3).** The samples were placed in sterile polyethylene bags, closed tightly and stored at 4°C until required.

2.3 Sample preparation

Each sample was prepared, first, by vigorous hand-shaking in an autoclaved (121^{o}C, 15 min) 1-litre beaker. All stones and large debris were subsequently removed aseptically. An approximately 1 g portion of each sample was suspended in 10 ml sterile distilled water from which three dilutions (10^{-2} to 10^{-4}) were prepared with vigorous shaking at each step. Each dilution was divided into four equal aliquots, which were subjected to different treatments before plating on appropriate isolation media. Some samples were heat-pretreated in a waterbath at 70°C for 10 minutes (Cross, 1981) and incorporated together with selective antibiotics into the isolation media. In others, there was no heat-pretreatment but a selective antibiotic was added to the media. The remaining samples had neither heat-pretreatment nor antibiotic incorporation into the media.

2.4 Media

2.4.1 Isolation media

Distilled water was used for all media preparation. The following selective media were used: Czapek's medium (Cross, 1981) supplemented with 25 µg/ml novobiocin and 50 µg/ml cycloheximide; Kosmachev's medium (Kosmachev, 1960); M3 Agar (Rowbotham and Cross, 1977), MGA medium (Nonomura and Ohara, 1971) modified by omitting soil extract and vitamin solution and made more selective by the addition of 10 µg/ml polymyxin B and 12 µg/ml oxytetracycline; Czapek's agar (Higgins and Lechevalier, 1969) modified by addition of 50 µg/ml cycloheximide and 4 µg/ml thiamine-HCl; Winogradsky's nitrite medium (Winogradsky, 1949) and Yeast extract-malt extract agar [International *Streptomyces* Project (ISP) medium 2 (Pridham *et al*., 1957)] modified by addition of gentamicin to the autoclaved and cooled medium to give a final concentration of 50 µg/ml. The cycloheximide was added to inhibit development of invasive fungi. The antibiotics used were chosen on the basis of good results obtained by previous investigators attempting the selective isolation of rare actinomycetes (Williams and Davies, 1965; Hayakawa and Nonomura, 1984; Ntuli, 1994). All plates were incubated at 30°C for 21 days, and each colony was examined directly by light microscopy.

2.4.2 Primary screening media

The media used in primary screening for antibiotic-producing isolates were Iso-sensitest agar (Oxoid) or Mueller-Hinton (MH) agar (Oxoid).

2.4.3 Antibiotic production media

The media used for antibiotic production were Nutrient broth (NB) [meat extract 1 g/l, yeast extract 2 g/l, peptone 5 g/l and sodium chloride 8 g/l, pH 7.1 after sterilization] or Oatmeal broth (ISP medium 3), from which agar had been omitted, (Pridham *et al*., 1956–1957).

2.5 Test organisms

The test organisms, namely, the bacteria *Escherichia coli, Pseudomonas fluorescens, Serratia marcescens, Staphylococcus aureus, Enterococcus faecalis* and *Xanthomonas campestris* pv. *campestris*, and the yeast *Candida utilis* were all from the culture collection in the Department of Microbiology, University of KwaZulu-Natal, and included both human and plant pathogens. The former were selected on the basis of a 1999 survey on microbial infections most commonly encountered at public hospitals in the KwaZulu-Natal Midlands and the latter on the frequency of bacterial infections occurring in the university vegetable gardens.

2.6 Primary screening

Primary screening of isolates for inhibitory action was carried out using a modified cross-streak method. The test organisms were streaked at right angles to the line of growth of the producer isolate inoculated previously and grown for six days at 30°C. The inoculated plates were re-incubated overnight at 30°C and the extent of inhibition of the various test organisms measured in millimeters.

2.7 Secondary screening

Fourteen isolates were selected for secondary screening on the basis of their inhibition of at least two or more of the test organisms. An exception was made in the case of isolates that inhibited only *Pseudomonas* or *Xanthomonas* spp., since members of these genera are resistant to many antimicrobial agents used in the field.

2.8 Purification of isolates and confirmation of activity

All isolates showing antimicrobial activity were purified using the three-way streak method. Purified isolates were streaked separately across the upper third of a plate of either Iso-sensitest agar or Mueller-Hinton agar and the same test organisms cross-streaked to confirm the original screening results (Lancini and Parenti, 1982).

2.9 Bioassay methods

2.9.1 Agar well-diffusion method

Samples of the culture filtrates were assayed for antimicrobial activity using a modification of the agar well-diffusion method of Paik and Glatz, (1995). After aseptically pouring the agar (5 mm deep), plates were incubated overnight at room temperature before a well (7 mm diameter) was cut in the centre of each plate. The plates were dried by further incubation at 37°C for 2 hours to facilitate sample diffusion through the agar. Culture filtrate (50 µl) was transferred to the well with a micropipette and the test organisms streaked radially outwards, starting 1 mm from the edge of the well. The plates were incubated at 30°C for 24 hours and observed for growth inhibition zones. All assays were carried out in triplicate. Plates in which the wells were filled with uninoculated culture medium served as controls.

2.9.2 Agar disk-diffusion method

Some samples were also tested using the agar disk-diffusion method of Eckwall and Schottel, (1997). Sterile commercial paper disks were spotted with 50 µl culture filtrate and allowed to dry before placing on the surface of solid Mueller Hinton medium in Petri dishes freshly seeded with the test organism. The plates were incubated at 30°C for 24 hours before the diameters of growth inhibition zones were measured. All assays were carried out in triplicate. Plates in which the disks were impregnated with either solvents or uninoculated culture medium served as controls.

2.9.3 Broth micro-dilution [Minimum inhibitory concentration (MIC)]

Minimum inhibitory concentration (MIC) was determined using the broth micro-dilution method as recommended by the Swedish reference group for antibiotics (SRGA) and its sub-committee on methodology (Olsson-Liljequist *et al*., 1997) and as per standard

protocol of Hancock's doubling dilution method (Hancock, 1999; Weigand *et al*., 2008) or according to the protocol recommended by the Clinical and Laboratory Standards Institute (CLSI) [formerly National committee for clinical laboratory standards (NCCLS, 1991 and 2004)]. The indicator test organisms were prepared by growing them in Nutrient broth (NB) or Mueller-Hinton (MH) broth to an optical density (OD) of 0.5 at 420 nm, whereafter *X* µl cell suspension was added to 1 ml NB/ MH. The value of *X* was calculated using the equation: *X* µl = 10/OD.

Prior to testing, either 100 µl or 50 µl of the cell suspension was diluted in 10 ml or 5 ml NB/ MH, respectively. Dilutions of the antibiotic-producer culture filtrate (stock solution) were prepared in a micro-titre plate (10× 10 wells). From the stock solution, nine dilutions were made using the growth medium as diluent. A step-wise dilution factor of 2 (1.0, 0.5, 0.25, etc.) was used throughout the dilution procedure. Finally, 100 µl of the prepared suspension of the indicator test organism were added to each of the wells in the micro-titre plate and incubated at 30°C. The plates were examined for growth after 12 h, 24 h and 48 h incubation. The highest dilution showing no visible growth, i.e. a clear medium, was regarded as the MIC.

2.10 Taxonomy

Physiological, morphological, and chemical studies were carried out on eight selected isolates. Selection was based on sizes of inhibition zones greater than 3 mm on at least two or more test organisms, especially *Pseudomonas fluorescens* and *Xanthomonas campestris* pv. *campestris* that are resistant to many commonly used antibiotics. Morphological studies were carried out with a Carl Zeiss Axiophot light microscope and a Hitachi S–570 scanning electron microscope (SEM). The diaminopimelic acid (DAP) isomer in the cell wall was determined by the method of Becker *et al.*, (1965). Carbon source requirements were studied by the method of Nitsch and Kutzner, (1973). The whole cell sugar pattern (WCSP) was obtained by a combination of the methods of Murray and Procter, (1965) and Staneck and Roberts (1974).

2.11 Strain N8

Strain N8 was selected from among 2600 isolates obtained during an actinomycete-screening programme conducted on KwaZulu-Natal soils, South Africa (Okudoh and

Wallis, 2007) and which is described in **Chapter 3**. Stock cultures were kept on Nutrient agar (NA) slants or stored frozen in glycerol at -80^{o}C.

2.11.1 Isolate N8 growth curve

An overnight culture of N8 (10 ml) was seeded into 200 ml of Nutrient Broth (NB). The broth was shaken in a waterbath at 30^{o}C. Aliqouts (5 ml) were removed initially and thereafter at 30 minute intervals and the absorbance measured at 550 nm. This wavelength was used to monitor the increase in cell biomass since NB grown bacterial cultures absorb light optimally at that wavelength (Appendix 3). A portion of each sample was kept in a refrigerator at 4^{o}C for *in vitro* bioassay.

2.12 Antibiotic production and recovery

All steps used in the recovery process are shown in **Figure 2.2.**

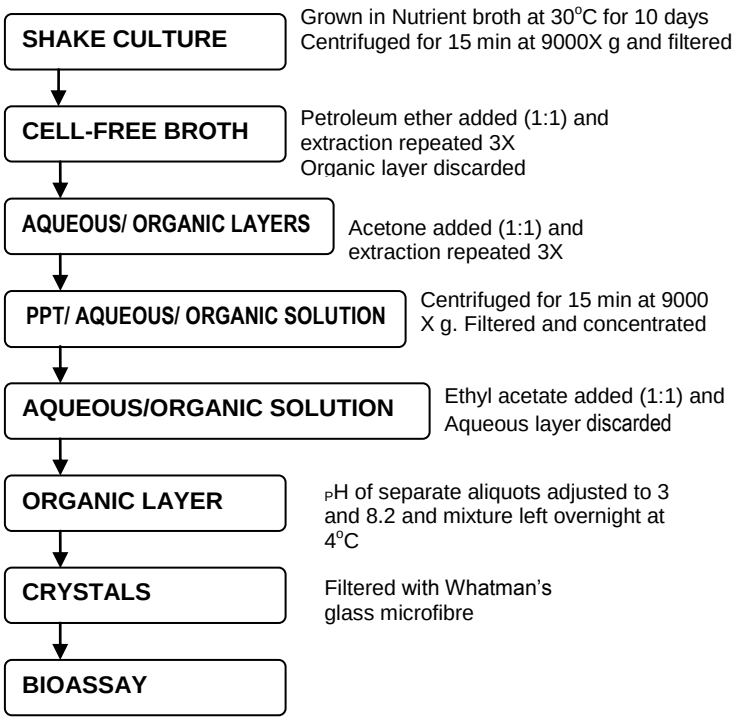


Fig. 2.2 Protocol used for extraction of the antibiotic produced by strain N8

2.12.1 Shake flask fermentation

Growth from a mature slant culture of strain N8 was inoculated aseptically into 250-ml Erlenmeyer flasks each containing 100 ml of production media as described in **Section**

2.4.3 and incubated in a New Brunswick rotary shaker at 30°C and 250 rpm for 2 days. The seed culture (10 ml) was transferred into 200 ml of the same medium in a 500-ml flask and incubated at 30°C for 14 days under agitation at 250 rpm. After incubation, the cultures were centrifuged in a Heraeus Labofuge 200 at 9500×*g* for 15 minutes. Aliquots of the supernatant (5 ml) were filter-sterilized (Cameo 25 AS acetate membrane) into sterile screw-capped glass vials. The filtration procedure was repeated to ensure that supernatant (culture filtrate) used for the antimicrobial activity bioassay was cell-free. Altogether ten litres of culture were produced in 50 flasks.

2.12.2 Solvent extraction

A three-solvent system comprising petroleum ether, acetone and ethyl acetate, was used for the recovery of the antibiotic from the broth. The non-polar solvent, petroleum ether, was used to remove lipids from the broth. Acetone precipitation helped to remove most solutes from the broth and ethyl acetate was used for extracting the remaining polar-organic substances. A ratio of 1:1 for solvent and broth was used and the extraction procedure was repeated three times. The first solvent, petroleum ether (PE), was added to the cell-free broth, the mixture shaken thoroughly and left overnight to settle. Separation of the organic and aqueous layers was achieved by means of a separating funnel. The PE extract was tested for antimicrobial activity before being discarded. The second solvent, acetone, was added and the resultant precipitate removed by centrifugation at 9000xg for 15 minutes. The precipitate too was tested for antimicrobial activity by redissolving in methanol (10ml) and following the agar disk-diffusion method of Eckwall and Schottel, (1997) described in **Section 2.9.2**. The remaining solution was concentrated 10 fold with a BUCHI rotary evaporator set at 30^{o}C. Finally, the solution was extracted with an equal volume of ethyl acetate (1:1). The aqueous layer was discarded after establishing through bioassay that the antimicrobial agent was in the ethyl acetate extract.

2.12.3 pH precipitation

Separate aliquots from the cell-free broth were adjusted to pH 3 and 8.2 with 2N HCL and 2N NaOH respectively, and then concentrated *in vacuo,* with a BUCHI Rotavapor EL 130 at 30^{o}C or left overnight in a refrigerator set at 4^{o}C to crystallize. The crystals were removed through filtration with Whatman's glass microfiber and dried overnight at

ambient temperature. Two bioassay methods, agar well- and agar disk-diffusion, were used to monitor the antimicrobial activity of the extracts. The above procedure led to an efficient recovery of the antimicrobial compound.

2.13 Purification by chromatography

Various chromatographic techniques were tried to purify the antibacterial agent present in the ethyl acetate extract.

2.13.1 Flash column chromatography (FCC)

Initially, flash column chromatography (FCC) was used to separate the compound(s) with antibiotic activity. Freeze-dried samples were loaded onto a column packed with silica gel (Merck 60) as solid support and eluted with various solvents, namely: methanol, ethyl acetate, acetone, petroleum ether and n-hexane. Each fraction was evaporated to dryness using a BUCHI Rotavapor EL 130 at 30^{o}C and then resuspended in 1ml methanol before testing for biological activity.

2.13.2 Thin layer chromatography (TLC)

Thin layer chromatography (TLC) was carried out on each extract using coated Merck Kieselgel 60 F_{254} plastic sheets. Various solvent systems (SS1 – SS5), ranging from strongly polar to non-polar, were used in attempts to optimize the eluting process. These were: Methanol: Ethyl acetate (80:20) – SS1; Ethyl acetate: Methanol (85:15) – SS2; Ethyl acetate: Petroleum ether (80:20) – SS3; Dichloromethane (100) – SS4; and n-Hexane: Ethyl acetate (20:80) – SS5.

Thin layer chromatography plates were visualized, first by checking for fluorescence under ultraviolet (UV) light at 254 nm, or after staining with anisaldehyde, and drying with a heat gun. To recover any biologically-active compound(s) from the TLC plates, all visible bands were excised from the TLC chromatogram, the matrix (silica gel) packed into a Pasteur pipette and eluted through glass wool using ethyl acetate. The samples were evaporated to dryness in a BUCHI Rotavapor EL 130 at 30^{o}C and resuspended in 1 ml methanol before testing for biological activity.

2.13.3 Harrison research chromatotron (HRC)

The biologically-active extract was further purified by the Harrison research chromatotron technique. A column plug was used to remove all impurities in the sample before loading into the chromatotron. As before, petroleum ether was first added to remove non-polar compounds in the sample. Ethyl acetate was then gradually introduced up to 100%. The third solvent, methanol, was added to remove non-mobile components. Care was taken not to increase the concentration of methanol beyond 50% because of its tendency to dissolve the silica gel on chromatotron plates. In attempts to increase the concentration of the antibiotic, 30 fractions collected at 1min intervals were pooled into six groups (A – F) consisting of five fractions each. The samples were evaporated to dryness in a BUCHI Rotavapor EL 130 at 30^{o}C and resuspended in 1 ml methanol before testing for biological activity.

2.13.4 High performance liquid chromatography (HPLC)

High performance liquid chromatography (HPLC) was carried out under both isocratic and gradient conditions using the procedures reported by Schreiber *et al*. (1988) and Joshi (2002). The HPLC procedure was repeated to provide as much material as possible for structural elucidation and synergistic activity testing.

2.13.4.1 Isocratic high performance liquid chromatography (IHPLC)

The isocratic HPLC analysis was conducted on active extracts using a Data Apex Clarity chromatography station. Fifty microlitres of extract were injected into an Alltech C-18 column (3.9mm X 300 mm) followed by isocratic elution with constant concentrations of methanol in water throughout the separation run. Five separations were carried out on decreasing concentration of methanol in water (100%, 80%, 70%, 60% and 50% respectively). Elution was monitored at 280 nm. Column output was manually collected, and each aliquot was subsequently concentrated in a BUCHI Rotavapor EL 130 at 30^{o}C. The concentrates were dissolved in minimal methanol before bioassay. Pure methanol was used as the control during the bioassay. However, the purity of the peaks was doubtful at this stage due to manual collection of the samples.

2.13.4.2 Gradient high performance liquid chromatography (GHPLC)

N8 extract was subjected to a gradient high pressure liquid chromatography (GHPLC) analysis to improve separation of the peaks thereby achieving higher purity of the compounds through separation with a Thermo Separation Products automated fraction collector. Various separations were carried out at decreasing concentrations (80%, 70%, 60%, and 50%) of methanol in water. Separations were performed on active preparations from the N8 extract using a Varian Analytical Instruments (CA, USA) HPLC station equipped with a Prostar 240 pump and a Spectra System variable wavelength UV detector model 3000HR. A Hypersil 5 octadecylsilane (ODS) analytical column (25 cm x 10 mm) purchased from HPLC Technology Ltd, Cheshire, UK was used. Absorbance was monitored at 280nm on a C-18 reverse-phase column.

The samples were eluted at a flow rate of 0.7 ml. min^{-1} over a total time of 45 min. Peaks were collected with an automated fraction collector and assayed for growth inhibitory activity against the test organisms.

2.14 Structural elucidation methods

2.14.1 Gas chromatography-Mass spectroscopy (GC-MS)

Mass spectra were recorded on a ThermoFinnigan Trace GC coupled to a PolarisQ mass spectrometer. The column used was 30m x 0.25mm internal diameter with a 0.25µm stationary phase film containing 5% phenyl (equivalent) / 95% methyl polysilphenylene / siloxane. The mobile phase was helium gas. Low and high resolution mass spectra were measured using a Kratos MS 80 RF double focusing magnetic sector instrument at 70 eV. Spectra were obtained using electron impact (EI) and chemical ionization (CI) modes.

2.14.2 Liquid chromatography-Mass spectroscopy (LC-MS)

The sample was diluted to a concentration of 2-5 ng/µl (sample dependant) in HPLC grade methanol and introduced by direct infusion into a Mass spectrometer (Waters® Micromass® LCT PremierTM, Time-of-Flight (TOF)) by means of a syringe pump. The conditions were as follows: capillary voltage: 2500 kV; cone voltage: 20 kV; desolvation temperature: 180°C; desolvation gas flow rate: 500 L/hr. The sample was ionised using electrospray ionisation in negative (ESI−) and positive (ESI+) modes.

2.14.3 ^{1}H- and GCOSY- Nuclear magnetic resonance (NMR) spectroscopy

NMR spectra were recorded on a Bruker Avance III spectrometer fitted with a 5mm BBOZ probe using deuterated methanol as solvent and residual protonated methanol as the internal standard. The chemical shifts were recorded in parts per million (ppm, δ) and the coupling constants at 500 mHertz (MHz). Details of the experimental conditions were as follows: Operating temperature 30 °C; Proton spectra recorded at 64K (F2), AQ 3.17 seconds, SW 20.66 ppm, D1 2.5 seconds, NS 256; GCOSY spectra recorded at 2048 x 128 (F2 x F1), SW 20.66 ppm, D1, 2.5 seconds, NS 32.

CHAPTER 3

ISOLATION AND SCREENING FOR ANTIMICROBIAL ACTIVITY OF RARE ACTINOMYCETES FROM NATURAL HABITATS IN KWAZULU–NATAL*

3.1 Introduction

For the last five decades, antibiotics have revolutionized medicine by providing cures for life-threatening infections (Cohen, 2000). Lately, however, there has been a growing concern about the emergence and management of antibiotic-resistant bacteria (Jack, 1996; Chopra *et al*., 1997). Most of the commonly used antibiotics in our hospitals have become ineffective due to these resistant bacteria. The need to discover and develop more-effective antibiotics with unique modes of action is greater than ever (Kong *et al*., 1998).

KwaZulu-Natal province of South Africa is a potentially rich field for the discovery of new antibiotic-producing microorganisms (Baecker and Ryan, 1987). For example, the organism, *Streptomyces natalensis*, an actinomycete first used in the industrial production of pimaricin, was originally isolated from KwaZulu-Natal soil (Wang *et al*., 2006). However, interest waned in this area as the number of secondary metabolites identified as 'novel' decreased. Unsurprisingly, researchers had concentrated on the compounds produced by members of the *Streptomyces* genus and thus the probability of finding novel compounds became increasingly remote.

Interest then switched to the rarer actinomycetes as potential sources of novel bioactive metabolites (Lynda *et al*., 2002; Cook and Meyers, 2003; Boudjella *et al.*, 2006) and methods designed to isolate (Pisano *et al*., 1986; Hayakawa and Nonomura, 1989; Labeda, 1990; Kizuka *et al*., 1997) and identify (Staneck and Roberts, 1974; Harvey *et al*., 2001; Lynda *et al*., 2002) a wide variety of such actinomycetes have been developed.

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* Parts of this chapter have been published in the *South African Journal of Science*, **103**, 216 – 222. May/June 2007 – No. 5/6

Modern isolation methods involve five steps: choice of a substrate (Pisano *et al*., 1987; Kitouni *et al*., 2005;), composition of the isolation medium (Houang *et al*., 1983; Hayakawa and Nonomura, 1984), pre-treatment and incubation conditions (Pisano *et al*., 1986), colony selection (Williams and Davies, 1965; Nolan and Cross, 1988; Hotta and Okami, 1996) and, finally, purification (Paik and Glatz, 1995; Hotta and Okami, 1996). Of these factors, composition of the isolation medium, pre-treatment and incubation conditions are the most important, since they determine which organism will develop on the isolation plates (Nolan and Cross, 1988).

Actinomycetes are mainly aerobic soil inhabitants and are widespread in nature (Lancini and Lorenzetti, 1993). The terms ‘rare actinomycetes’ or ‘rare actinos’ refers to those genera less easily detected than *Streptomyces*. No clear-cut habitat preference can be established for the different genera of rare actinomycetes; however, some, such as *Micromonospora* or *Actinoplanes*, are more abundant in decaying plant material or muddy soils on freshwater shores. Thermophilic species are often found in natural warm habitats, such as compost or hay mounds (Lancini and Lorenzetti, 1993).

The aim of the present study is to continue the search for new antibiotic producers in KwaZulu-Natal by targeting rare actinomycetes using improved isolation methods. Members of the genus *Streptomyces* were excluded from the search, because they have been extensively studied in the past (Okami and Hotta, 1988; Miyadoh, 1993; Ntuli, 1994).

3.2 Materials and methods

3.2.1 Bacterial isolation

Soil samples were collected at depths of 0–5 cm below the surface from various sites in the KwaZulu-Natal Midlands (**Table 3.1**)**.** The storage and preparation of the samples is described in **Sections 2.2** and **2.3**. All the media used and procedures involved for selective isolation of rare actinomycetes are described in **Section 2.4**.

3.2.2 Primary screening

Screening of isolates for inhibitory action was carried out using a modified cross-streak method as described in **Section 2.6**. Purification of the isolates showing antimicrobial activity was carried out using the three-way streak method as described in **Section 2.8**.

3.2.3 Secondary screening

The production of antibiotics by the organisms was previously discussed in **Section 2.12**. Samples of the culture filtrates were assayed for antimicrobial activity using a modification of the agar well-diffusion method of Paik and Glatz, (1995) which was described in **Section 2.9.1**. Some samples were also tested using the agar disk-diffusion method of Eckwall and Schottel, (1997) described in **Section 2.9.2**.

Minimum inhibitory concentration (MIC) was determined using the broth micro-dilution method as recommended by the Swedish reference group for antibiotics (SRGA) [Olsson-Liljequist *et al*., 1997] and its sub-committee on methodology and as per standard protocol of Hancock's doubling dilution method (Hancock, 1999) as described in **Section 2.9.3**.

3.2.4 Taxonomy

Physiological, morphological, and chemical studies on eight selected isolates were carried out as described in **Section 2.10**.

3.3 Results

The number of antibiotic-producing isolates varied with the sample source and the isolation pretreatment applied. Eighty of the approximately 2600 isolates screened, showed some antimicrobial activity. The highest number of active isolates came from the Forest site, while the lowest number came from the Riverine soil sample. Calculation of the number of antimicrobially active isolates in the total number of isolates screened from the various samples showed that the number of culturable antibiotic-producing microorganisms constituted only about 3% of the total overall microbial population of the samples studied (**Table 3.1**).

The average inhibition zone size was sometimes different for the secondary screening stage, but the spectrum of activity of the respective isolates remained unchanged from those recorded in the primary screening phase. Some of the isolates lost a substantial part of their activity on extended incubation. Isolated colonies, which grew within an inhibition zone, were recorded as resistant variants of the strain (**Table 3.2** and **Fig. 3.1A**). Isolate N8 inhibited all the test organisms in the primary screen on both NA (**Fig. 3.1A**) and ISTA plates (not shown). This broad-spectrum inhibition was verified during secondary screening on MH medium (**Fig. 3.1B**), with uninoculated MH medium in the wells serving as a control (**Fig. 3.1C**). Surprisingly, this isolate showed no visible activity against the test organisms during secondary screening using the agar-well diffusion method. However, the disk-diffusion method, with *Serratia marcescens* as the test organism, gave positive results (**Fig. 3.1D**). In some cases, discoloration of *S. marcescens* was also observed.

Isolate N2 showed highest activity against *P. fluorescens* with an MIC of 0.0039 µg/ml, whereas the MIC for *X. campestris* pv. *campestris* was 0.25 µg/ml (**Table 3.3**). Isolate N8 showed good antimicrobial activity against all the test organisms used especially the *Pseudomonas* and *Xanthomonas* species, with MICs of 0.0625 µg/ ml and 0.0025 µg/ ml, respectively (**Table 3.3**). Isolates N33 and N35 were also strongly inhibitory, with values of 0.002 µg/ml and 0.0039 µg/ml against *P. fluorescens* and *X. campestris* pv. *campestris*, respectively. The MIC results for all the selected antimicrobially active isolates against the test organisms are shown in **Table 3.3**. Isolate N16 inhibited four of the seven test organisms namely *S. aureus* (0.125 µg/ml), *E. coli* (0.0039 µg/ml), *P. fluorescens* (0.0039 µg/ml) and *X. campestris* pv. *campestris* while isolate N19 inhibited three of the seven test organisms including *P. fluorescens* (0.125 µg/ml), *C. utilis* (0.25 µg/ml) and *X. campestris* pv. *campestris.* Isolate N12 and N30 inhibited only *P. fluorescens*. The results for N16 were better against *P. fluorescens* and *X. campestris* pv. *campestris* than those of N8 whereas the MIC result for isolate N2 against *P. fluorescens* (0.0039 µg/ml) was better than that against *X. campestris* pv. *campestris* (0.25 µg/ml).

The physiological, morphological, and chemical characteristics of all isolates showing antimicrobial action are presented in **Table 3.4**. Isolate N8, which had the broadest spectrum of activity, formed colonies with no aerial mycelium on NA. When young, the organism produced branched substrate mycelium, but on maturing, dark brown to grey

round to oval dome-like bodies or vesicles were formed (**Fig. 3.2A**). The smooth-surfaced spores were produced in dense clusters contained in a thick sheath of extracellular material or inside the vesicles (**Fig. 3.2B**). In broth culture, the hyphae merged together to form synnemata, but when dried, extensive branching fragments were observed. The isolate could not utilize raffinose and rhamnose as carbon sources and no diffusible pigments were formed in either solid or liquid media. The cells were non-acid fast and catalase-positive. Optimum growth occurred between 27°C and 30°C.

High-performance amino-acid analysis (Beckman 6300 analyser) showed that N8 contained substantial amounts of L- or *meso*-DAP. None of the diagnostic sugars for specific actinomycetes was present in the whole cell hydrolysate. Environmental scanning electron microscopy revealed that N8 spores are enclosed in a thick slimy layer which, when disrupted, exposes the spore mass. Scanning electron microscopy (SEM) revealed the smooth-surfaced, oval to rod-shaped spores.

A characteristic morphological feature of isolate N35 was the coryneform-like angular arrangement of bacillary elements as revealed by SEM (**Fig. 3.3A**) with some of the rod-like elements measuring up to 4 µm in diameter (**Fig. 3.3B**).

The spectra of activity of the isolates showed that N2, N8 and N16 were active against both Gram-positive and Gram-negative bacteria (**Fig. 3.4**). Isolate N8 produced a broad-spectrum antibiotic(s) active against both bacteria and fungi (yeasts), whereas N35 was inactive against Gram-positive bacteria.

Table 3.1 Percentage of the total number^ of isolates active against the test organisms* on primary screening

Sample source	No. Isolates per pre-treatment		Total no. isolates screened	No. Isolates active	%
Chicken manure [CM]	H +A	38	354	12	3.39
	H + NA	46			
	NH +A	94			
	NH + NA	166			
Chicken litter [CL]	H +A	34	423	18	4.26
	H + NA	61			
	NH + A	123			
	NH +NA	205			
Cow manure [COW]	H + A	43	313	11	3.51
	H + NA	55			
	NH + A	58			
	NH +NA	157			
Compost soil [CS]	NH +A	45	121	2	1.66
	NH + NA	76			
	NH + A	56			
Uncultivated farm soil [US]	H + A	23	182	4	2.19
	NH + NA	103			
River bank soil [RB]	NH + A	13	94	0	0
	NH + NA	45			
	H + A	36			

Sample source	No. Isolates per pre-treatment		Total no. isolates screened	No. Isolates active	%
SAPPI† forest soil [SF]	NH + A	97			
	NH + NA	167			
	H + A	66			
	H + NA	105	435	23	5.28
Creosote-contaminated soil (CCS)	NH + NA	5	5	1	20.00
Sweet waters (dry soil) [SW]	NH + A	71			
	NH + NA	128			
	H + A	61	260	2	0.77
Illovo sugarcane field [CSF]	NH + A	101			
	NH + NA	169			
	H + A	56			
	H + NA	60	386	7	1.88
	Grand Total		**2573**	**80**	**3.11**

**Escherichia coli, Pseudomonas fluorescens, Serratia marcescens, Staphylococcus aureus, Enterococcus faecalis, Xanthomonas campestris* pv. *campestris* and *Candida utilis;* NH = non – heated; H = Heated; A, antibiotics added; NA, no antibiotics added.

^ Number based on the average colony count of the 10^{-2} to 10^{-4} dilutions of the sample.

†South African Paper and Pulp Industries.

Table 3.2 Average inhibition zone sizes (mm) of selected isolates* against test organisms^ on primary screening

Isolate*	Source	Test organisms^						
		Sa	Ec	Pf	Sm	Ef	Cu	Xc
N2	CL	13	10	20	21	–	20	15
N3	CM	25	20	25	–	–	nt	–
N4a	SW	15	–	30	20	–	nt	–
N4b	SW	–	30	15	–	–	–	–
N8	CM	20	25	20^{Rs}	23	22	27	24
N12	CSF (NH+A)	3	7	8	–	–	–	5
N15	CL	20	21	–	2	–	30	--
N16	CL	25	5	14	–	–	Rs	–
N19	SF (NH+A)	3	4	10	–	–	–	5
N25	CL	2	30	–	–	–	30	–

Isolate*	Source	Test organisms^						
		Sa	Ec	Pf	Sm	Ef	Cu	Xc
N30	CSF (H)	5	–	20	–	–	–	–
N33	FS (NH)	–	3	10	–	–	–	–
N35	SF (NH)	–	3	–	–	–	–	10
N48	CS	18	30	–	–Dc	–	21	19

^Sa, *Staphylococcus aureus*; Ec, *Escherichia coli*; Pf, *Pseudomonas fluorescens*; Sm, *Serratia marcescens*; Ef, *Enterococcus faecalis*; Cu, *Candida utilis*; Xc, *Xanthomonas campestris* pv. *campestris*; Rs, Resistant variant; CL, Chicken litter; CM, Chicken manure; CS, Compost soil; SW, Sweet waters (dry soil); nt = not tested; -Dc, discoloration; -, no inhibition; H, heated; NH, non-heated; A, antibiotics added; SF, SAPPI forest; CSF, sugarcane field; *selected for secondary screening.

Table 3.3 Minimum inhibitory concentrations (µg/ml) of the selected antimicrobially active isolates against the test organisms

Isolate	Test organisms						
	Sa	Ec	Pf	Sm	Ef	Cu	Xc
N2	0.0078	0.0156	0.0039	uns*	uns	nt	0.25
N8	0.0078	0.0039	0.0625	0.125	0.25	0.002	0.0025
N12	nt	nt	0.25	uns*	uns	uns	nt
N16	0.125	0.0039	0.0039	uns	uns	uns	0.002
N19	nt	nt	0.125	uns	uns	0.25	0.25
N30	nt	uns	0.0625	uns	uns	uns	uns
N33	uns	nt	0.002	uns	uns	uns	uns
N35	uns	0.031	uns	uns	uns	uns	0.0039

*uns, unsusceptible; nt, not tested; Sa, *Staphylococcus aureus*; Ec, *Escherichia coli*; Pf, *Pseudomonas fluorescens*; Sm, *Serratia marcescens*; Ef, *Enterococcus faecalis*; Cu, *Candida utilis*; Xc, *Xanthomonas campestris* pv. *campestris.*

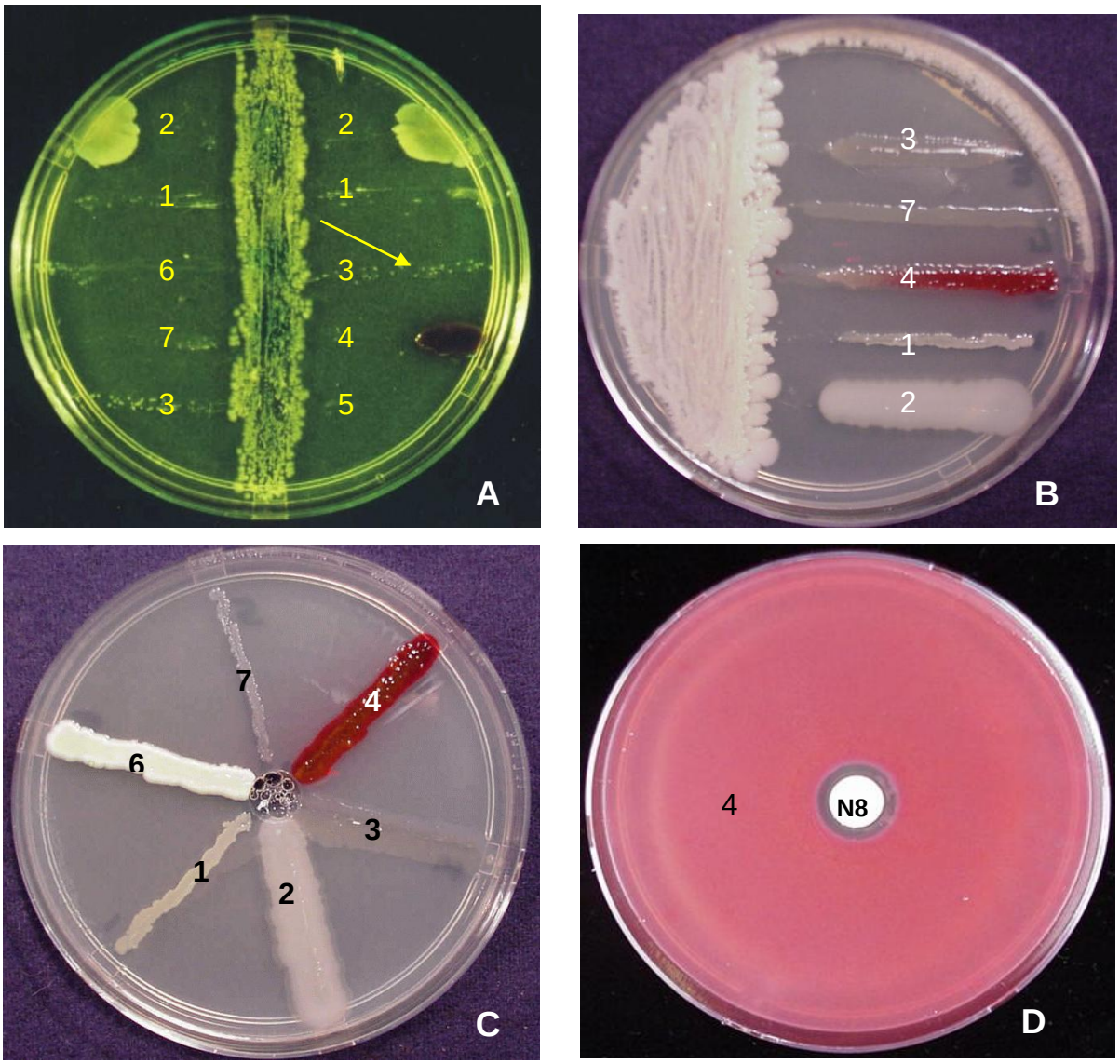

Fig. 3.1 Inhibition of test organisms by isolate N8: **A**, inhibition of test organisms on primary screening (arrow shows resistant variants); **B**, confirmation of inhibition on MH agar; **C**, control showing uninhibited growth of test organisms on MH agar; **D**, inhibition of *Serratia marcescens* by N8 purified extract on secondary screening. Test organisms include: *Staphylococcus aureus* (1), *Escherichia coli* (2), *Pseudomonas fluorescens* (3), *Serratia marcescens* (4), *Enterococcus faecalis* (5), *Candida utilis* (6) and *Xanthomonas campestris* pv. *campestris* (7).

Table 3.4 Physiological, morphological and chemical characteristics of selected isolates antimicrobially active against the test organisms

	Isolates							
Characteristic	N2	N8	N12	N16	N19	N30	N33	N35
Physiological								
Gram reaction	+	+	Variable	+	+	Variable	nt	+
Catalase	+	+	–	+	+	–	–	+
Oxidase	–	–	+	–	+	+	+	+
Acid-fastness	–	–	–	–	–[a]	–	–	–
Spore production	+	+	+[b]	+	–	–	–	+
V-formation	–	–	–	–	+	+	–	–
Motility	–	–	–	–	–	–	–	–
Morphological								
Aerial mycelium color (surface)	Pale white orange	Pale white orange	Milky	Milky	NA	NA	NA	NA
Substrate mycelium color (underneath)	*YBR	*YBR	–	*YBR	Extensive	–	–	–

	Isolates							
Characteristic	N2	N8	N12	N16	N19	N30	N33	N35
Cells/spores shape	Oval spores	Oval spores	Rod shaped cells	Oval spores	Rod shaped cells [c]	Drumsticks/ Hammer	Rods	Rod shaped cells
Spore surfaces	Smooth	Smooth	–	Smooth	–	–	–	–
Pellicle formation	+	+	–	+	nt	nt	–	nt
Chemical								
Diaminopimelic acid (DAP) isomer	Meso	L - or Meso	Meso	L	L - or Meso	None	L - or Meso	None
Diagnostic sugar	NC	NC	Ara+Gal	Ara +Gal	Ara+Gal	Gal	Ara +Gal	Gal
Cell wall type	I, III	I, III	IV	I	VI sensu	VI	NA	VI
WCSP type	C	C	A	A sensu	A	NA	A	NA
Glycine	+	++	nt	+	+++	nt	++	nt
Melanin pigment	nt	+	nt	+	nt	–	–	–

+ = Positive; – = negative; nt = not tested; NA = Not applicable; WCSP = Whole-cell sugar pattern; Ara = Arabinose; Gal = Galactose; NC = No Characteristic sugar; *YBR=Yellow-Brown-Red; [a] binds and retains fuschin; [b] endospores; [c] appear as doublets with tapered ends.

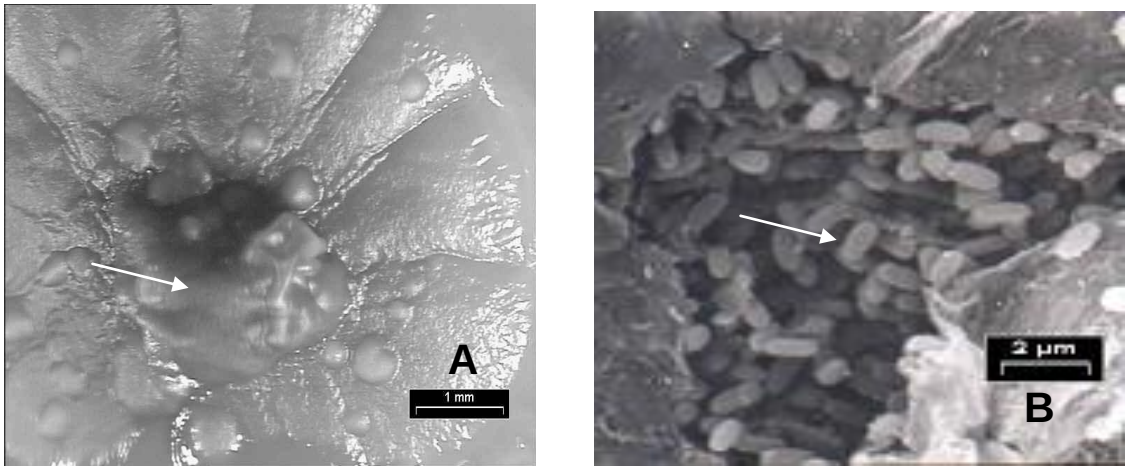

Fig. 3.2 Growth of N8 culture on Nutrient agar: A = mature mycelium (arrow shows dome like body or spore vesicles); B = Scanning electron micrograph of mycelium (arrow shows oval shaped spores exposed inside a broken vesicle).

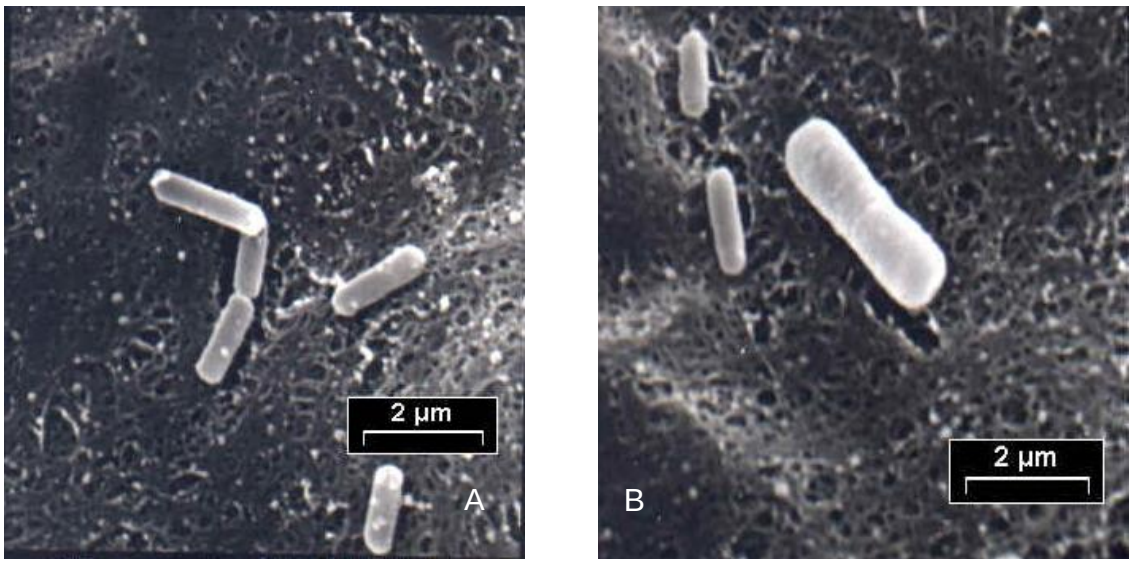

Fig. 3.3 Scanning electron micrograph of isolate N35: A = Angular arrangement of bacillary elements; B = Enlarged cell (up to 4 µm).

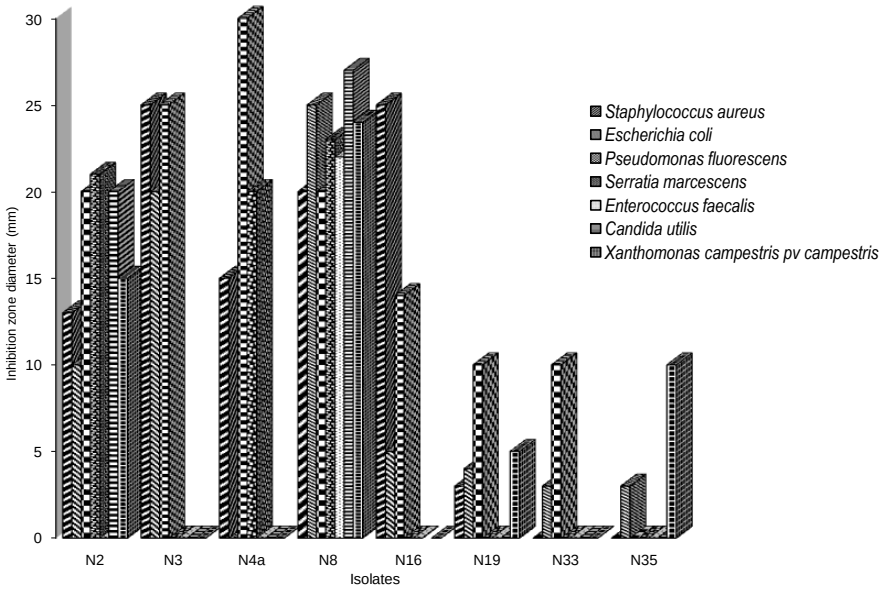

Fig. 3.4 Comparison of antimicrobial spectra of the active isolates selected for secondary screening

3.4 Discussion

Enumeration of the exact number of colonies isolated in this investigation is not possible, since many of the organisms that were obviously non-actinomycetes were disregarded. However, a rough estimate of the scale of the isolation programme may be obtained by considering the number of plates used. For each of 10 samples investigated, three dilutions (10^{-2} to 10^{-4}) were prepared and divided into four equal aliquots and each aliquot was subjected to a different treatment before plating on triplicate plates of at least seven appropriate isolation media. There were four treatment conditions as shown in **Table 3.1**. Thus, a total of 10 × 3 × 4 × 3 × 7 = 2520 isolation plates were used in this investigation.

Heat pretreatment of the samples was beneficial as it eliminated most of the unwanted Gram-negative bacteria that produce mucoid, spreading colonies on soil dilution plates (Williams and Wellington, 1982), thereby facilitating the isolation of actinomycetous organisms. The addition of antibacterial antibiotics such as gentamicin, streptomycin and novobiocin to the isolation media also enhanced the selection of members of the

actinomycetales. The combined treatment of heat and antibiotic incorporation into the media eliminated most of the unwanted fungi, yeasts and eubacteria and consequently reduced the number of colonies developing on the plates.

The disadvantage of adding antibiotic(s) to the isolation media is that potent antibiotic producers, sensitive to the antibiotic added, might be inhibited, and hence remain undetected. On the other hand, the antibiotic(s) makes the medium more selective by limiting the types of organisms developing on the plates. Different members of the actinomycetales differ significantly in their sensitivity toward antibacterial antibiotics. Streptomycetes fortunately belong to the antibiotic-sensitive group (Williams and Davies, 1965; Jawetz *et al*., 1984), making it easier to target the rarer actinomycetales.

Some of the isolation media used, namely Winogradsky's nitrite medium (Winogradsky, 1949), M3 medium (Rowbotham and Cross, 1977), and Kosmachev's medium (Kosmachev, 1960), were found to be selective for actinomycetes without heat pre-treatment and/or antibiotic incorporation, although a few colonies of other bacteria did occasionally develop on the plates. Thus, the selectivity of some of the so-called selective media used in this study is questionable.

Although the media used in this investigation were not compared directly, some differences were nonetheless evident. Modified Czapek's agar appeared to enhance pigment-producing strains of actinomycetes, most probably the Nocardioform group. MGA supported growth of mainly filamentous organisms. Of all the media used, Kosmachev's medium appeared to be the most selective, as most of the plates contained virtually pure cultures of non-streptomycetous actinomycetes. This medium has been reported to be selective for *Excellospora* and related genera (Kosmachev, 1960).

Screening results indicate that forest sites, with their usually high organic content and acidic nature, may offer a good hunting ground for antibiotic-producing microorganisms, especially in KwaZulu-Natal. Waksman and his co-workers in 1942 reported the presence of antibiotic-producing microorganisms in acidic forest soils. The soil samples from the riverine site were waterlogged and hence devoid of oxygen, thus making them unfavourable for growth of aerobic antibiotic-producing actinomycetes.

Our negative results with this soil may also reflect an inability of the media used to support growth and/or antibiotic production by the riparian microbiota.

Many of the other samples screened, such as compost soil, cow and chicken manure, and sugar-cane field soil, were rich in organic matter and contained a large variety of actinomycetes. As could be expected, the dry, uncultivated soil sample also contained many actinomycete isolates, since it has been reported that most arid soils usually have an alkaline pH and contain a number of the rare actinomycetes (Kizuka *et al.*, 1997). These authors reported on the distribution of actinomycetes in South African soils in 1997. Apart from this report, current quantitative data on the geographical and ecological distribution of actinomycetes in South Africa is scarce in the scientific literature.

Results of the present studies confirm the contention that it is erroneous to compare one antibiotic agent against another on the basis of inhibition zone size, since the size and position of the growth inhibition zone are a consequence of many dynamic systems, including diffusion rate, proceeding simultaneously (Linton, 1983). The observed discoloration of *Serratia marcescens* was not the result of metabolic interference, as incubation at a lower temperature restored the characteristic red colour. The resistance of *Enterococcus faecalis* to most of the isolates tested, with the exception of isolate N8, may have been a medium affect or the strain of *Enterococcus faecalis* used may produce an enzyme, which inactivated any antibiotic produced or due to an efflux pump that expelled the antibiotic (Salyers and Whitt, 2005).

The loss of some activity during secondary screening using the agar well-diffusion method could be ascribed to procedural effect, including preparation of the culture filtrate, media composition, and incubation conditions. Another explanation may be that in the primary screens actively growing cells (with ongoing antibiotic production) were used, whereas cell-free filtrates were used in the secondary screening protocol and a large amount of antibiotic or antibiotic activity may have been lost. Furthermore, losses might occur if the active compounds are labile.

The MIC values shown in **Table 3.3** serve as an index of the relative antimicrobial activity and the antimicrobial spectra of the isolates. After a lot of thought, isolate N8 was selected for further detailed description because it exhibited activity against all the

test organisms used in this investigation. It also showed larger diameter inhibition zones than another active isolate, N2. Furthermore, isolate N2 did not inhibit a resistant strain of *Enterococcus faecalis* and the inhibitory activity it showed against *Serratia marcescens* during primary screening was not confirmed during secondary screening whereas with N8 it was.

The loss of some activity during secondary screening using the agar well-diffusion method could be ascribed to procedural effect, including preparation of the culture filtrate, media composition, and incubation conditions. Another explanation may be that in the primary screens actively growing cells (with ongoing antibiotic production) were used, whereas cell-free filtrates were used in the secondary screening protocol and a large amount of antibiotic or antibiotic activity may have been lost. Furthermore, losses might occur if the active compounds are labile.

Comparisons with descriptions of actinomycetales genera in *Bergey's Manual of Systematic Bacteriology* (Lechevalier, 1989) showed N8 to closely resemble members of the *Nocardioides* group. However, unlike N8, *Nocardioides* produce aerial mycelium that ultimately fragments. Hence, the isolate is unlikely to belong in this group. The absence of diagnostic sugars in whole-cell hydrolysates (WCSP C) of isolate N8, the presence of L-DAP (wall chemotype I), the formation of substrate mycelium with round to oval spore-like bodies and vesicles, together with other cultural and physiological properties listed in **Table 3.4**, placed N8 in the genus *Intrasporangium*.

Similar approaches to those described above were used to identify tentatively isolates N2, N12, N16, N19 and N35 as *Thermomonospora* sp., *Saccharopolyspora* sp., *Nocardia* sp., *Corynebacterium* sp. and *Promicromonospora* sp., respectively.

3.5 Conclusions

These preliminary findings confirm that KwaZulu-Natal soils harbour antibiotic-producing microorganisms, as shown by previous investigators (Baecker and Ryan, 1987; Ntuli, 1994; Kizuka *et al*., 1997; Wang *et al*., 2006), and that it could be worthwhile undertaking a more comprehensive search for rare forms of antibiotic-producing organisms in this province of South Africa.

Of all the isolates assayed in the present investigation, N8, tentatively identified as an *Intrasporangium* species, appears to have the greatest potential. The results obtained from screening and MIC determinations showed it to be active against all the test organisms used, especially the *Pseudomonas* and *Xanthomonas* species, with MICs of 0.0625 µg/ ml and 0.0025 µg/ ml, respectively. However, specificity of action by antibiotics is often favoured by medical science (Jawetz *et al*., 1984). It may be that the substance(s) produced by isolate N8 is/are toxic to many additional microorganisms. The chemical nature of the antimicrobial compound(s) produced by N8 is reported in **Chapter 5**. The indications are that the major antimicrobial substance is a tryptamine.

CHAPTER 4
ISOLATION AND PURIFICATION OF THE ANTIBIOTIC COMPOUND(S) PRODUCED BY *INTRASPORANGIUM* STRAIN N8*

4.1 Introduction

Solvent extraction is widely used to extract antibiotics (Alves *et al*., 2002; Kumar *et al*., 2005). However, most antibiotic compounds exist in two main forms, as a salt or as a basic compound which are respectively soluble and insoluble in hydrophilic solvents. Therefore, a careful choice of solvent system provides a more efficient and faster extraction of the antimicrobial compounds from complex mixtures (Oka *et al*., 1998).

Historically the first separations of organic mixtures were accomplished by countercurrent distribution (CCD) followed soon after by partition chromatography on paper or on columns of cellulose or Sephadex. The CCD procedure offers a high resolving power when the proper solvent system is used and becomes a powerful tool to separate the various components of antibiotic complexes (Oka *et al*., 1998).

More recent developments in antibiotic purification include the use of thin-layer (TLC) and high-pressure liquid chromatography [HPLC] (Giuliano *et al*., 2002; Joshi, 2002). One of the shortcomings of chromatography is that standards are required in order to identify compounds in the sample and, even then, one cannot be absolutely certain of the identification, especially if a novel substance is involved. In contrast, spectroscopic techniques can give much information about the structure and identity of unknown substances (Giuliano *et al*., 2002).

In this chapter the isolation and purification of an antibacterial agent produced by *Intrasporangium* strain N8 obtained from a barnyard soil in the KwaZulu-Natal midlands, South Africa is described. The study is part of the current worldwide search for new antibiotic compounds as multiply resistant organisms continue to be a threat to global public health (Cohen, 2000).

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*Parts of this chapter have been submitted for publication to the *Canadian Journal of Microbiology*.

4.2 Materials and methods

All major steps used in the purification of the antibacterial agent are shown in **Figure 2.1.**

4.2.1 Organism and primary activity

The source of strain N8 was described in **Section 2.11**. Stock cultures were kept on Nutrient agar slants or stored frozen in glycerol at -80^{o}C. The organism was provisionally identified as an *Intrasporagium* species according to Bergey's Manual of Systematic Bacteriology (Williams *et al*., 1989) as was described in **Section 2.10**. Antimicrobial activity was determined *in vitro* by agar disk diffusion or micro-dilution methods as described in **Section 2.9**.

4.2.2 Strain N8 growth curve

The growth curve of strain N8 was established in conjunction with bioassays to determine the best time to harvest the antibiotic produced by the organism. The procedure used is described in **Section 2.11.1**. The results of the study are shown in **Appendix 3** and graphically represented in **Figure 4.1**.

4.2.3 Antibiotic production and recovery

The fermentation and recovery procedures are described in **Section 2.12**. Ten litres of culture was produced which was centrifuged at 9000xg for 15 minutes and the supernatant filtered through Cameo 25 AS acetate membrane filters prior to analysis. A flowchart indicating all steps used in the recovery process is shown in **Figure 2.2 (Chapter 2)**.

4.2.4 Purification by chromatography

Various chromatographic techniques were tried during attempts to purify the antibacterial agent present in the ethyl acetate extract as described in **Section 2.13**.

4.3 Results

4.3.1 Organisms and primary activity

Isolate N8 showed good antimicrobial activity against all the test organisms used during the primary and secondary screening phases, especially *Pseudomonas fluorescens* and *Xanthomonas campestris* pv. *campestris*, with average inhibition zone diameters of 26 mm and 28 mm, respectively. Its spectrum of activity included both Gram-negative and Gram-positive bacteria and the test yeast, *Candida utilis* **(Table 4.1).**

Table 4.1 Spectrum of activity produced by *Intrasporangium* strain N8 against test organisms during confirmatory primary* screening

Test Organism	Extent of inhibition	Average inhibition zone diameter (mm)
Staphylococcus aureus	+	10.0
Escherichia coli	+++	25.0
Enterococcus faecalis	++	20.0
Serratia marcescens	++	15.0
Pseudomonas fluorescens	++++	26.0
Candida utilis	+++	21.0
Xanthomonas campestris pv. *campestris*	++++	28.0

* Screening on Mueller-Hinton (MH) agar (Oxoid), + = positive inhibition (3–10 mm); ++ = 11–20 mm; +++ = 21–25 mm; ++++ = 26–30 mm

4.3.2 Antibiotic production and recovery

The stationary phase of growth commenced after about 58 h of incubation (**Fig. 4.1**). Antibiotic production commenced after 58 h and continued until maximum yield at 168 h incubation at 30^{o}C on the neutral (pH 7.0) media NB and/or ISP medium 3.

Addition of petroleum ether, a non-polar solvent, successfully removed much of the lipids from the broth, while subsequent addition of ethyl acetate helped in extracting the remaining organic material from the broth. Acetone precipitation eliminated most solutes from the broth. Some pink-yellow needle-like crystals formed on the sides of the flask containing the concentrated ethyl acetate extract. The crystals showed inhibitory activity against *Serratia marcescens* (**Fig. 4.2A**).

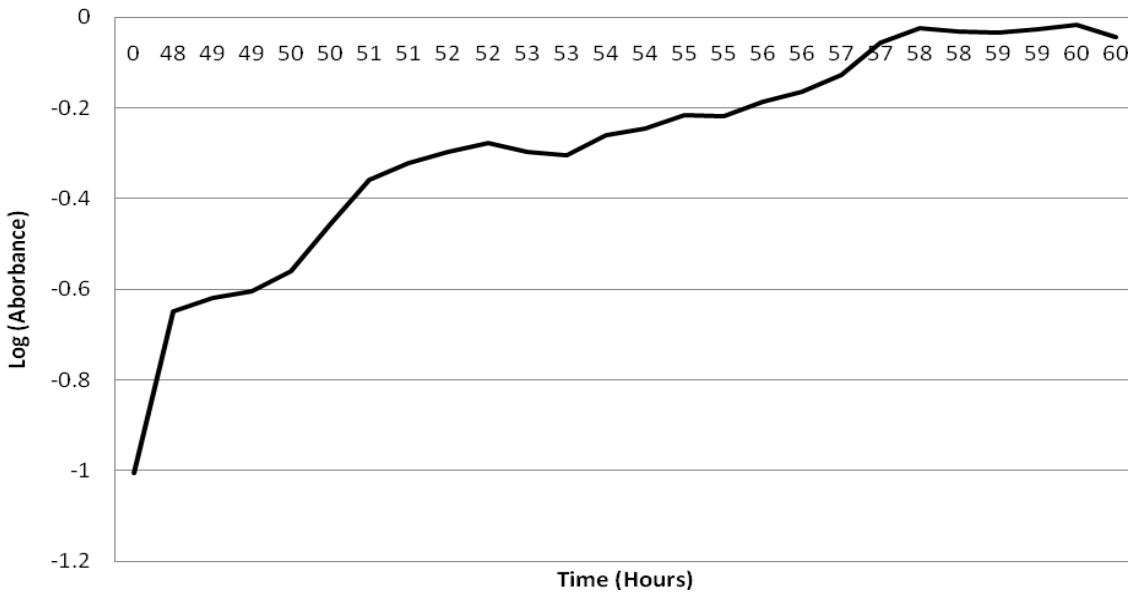


Fig. 4.1 Growth curve of strain N8 at 30^{O}C in Nutrient broth.

Thin-layer chromatography of the ethyl acetate fraction revealed three separate bands when viewed under UV light at 254 nm. The eluate from the upper band inhibited the growth of *Serratia marcescens* (**Fig. 4.2B**) and also *E. coli* and *X. campestris* pv. *campestris* (results not shown). The middle and lower bands did not inhibit the growth of any of the test organisms.

Bioassay results of solvent extracts showed clear inhibition zones around paper disks dipped in the ethyl acetate extract (EH) [**Fig. 4.2C**]**.** No zone of inhibition against the test organism, *E. coli,* was observed for the petroleum ether extract (PE). A very large inhibition zone (30 mm) was also observed for the alcohol extract (AH), although portions of the zone were cloudy compared to that of the ethyl acetate extract (EH) [21 mm] which was clear. A slight inhibition was observed for the alcohol used as control (AHC) [**Fig. 4.2D**] and was consequently dropped as an extraction solvent. The other solvents, petroleum ether (PEC), acetone (ACC), and ethyl acetate (EAC) used as control (**Fig. 4.2D**) did not show inhibition against the test organism.

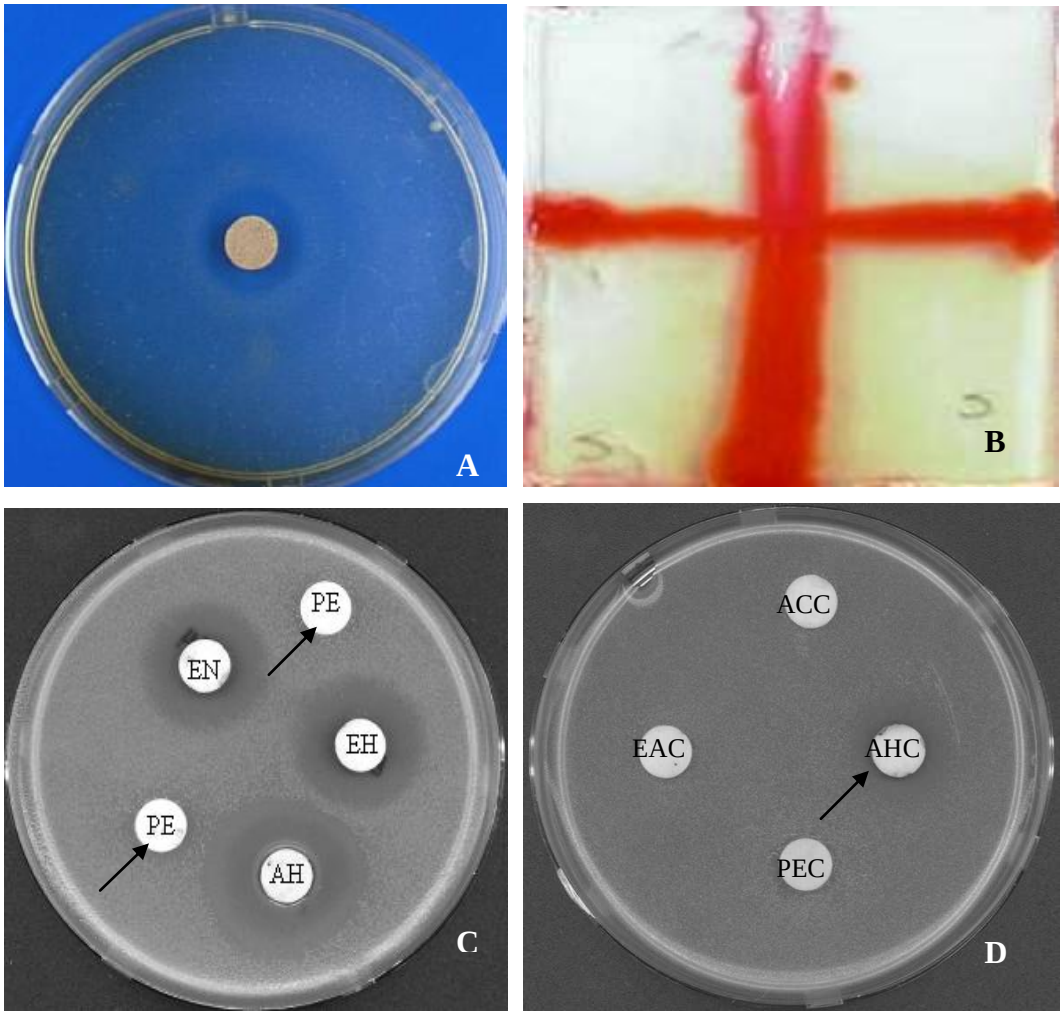


Fig. 4.2 Antimicrobial activity of *Intrasporangium* strain N8. A = Inhibition of *Serratia marcescens* by pink-yellow needle-like crystals of the antibiotic produced by strain N8; B = Inhibition of *Serratia marcescens* by the uppermost band on TLC chromatograms following separation of strain N8 culture fluid extract, EAF with SS2; C = Inhibition of *E. coli* by paper disks soaked in solvent extracts (arrow shows non-inhibition of *E. coli* by petroleum ether extract (PE); D = Non-inhibition of *E. coli* by paper disks soaked in each of the solvents petroleum ether (PEC), acetone (ACC), and ethyl acetate (EAC) used as controls [arrow shows slight inhibition by alcohol control (AHC)]; EN = Ethyl acetate: NaCl (NaCl was added to allow for complete micelle removal and later discarded with the aqueous layer); EH = Ethyl acetate: H_2O; AH =Alcohol: H_2O; PE = petroleum ether.

4.3.3 Purification by chromatography

Flash column chromatography (FCC) removed most of the inactive, colored material from the antimicrobially-active extract. However, after passing through the column, some extracts appeared to be more complex than was originally thought judging from the number of bands observed on TLC chromatograms (results not shown). The components of the solvent extracts were clearly separated on TLC chromatograms run

with the most polar to the least polar solvent systems (SS1 – SS5). The solvent system SS1 eluted four bands from the ethyl acetate fraction (EAF) [**Fig. 4.3A**] whereas both the methanol fraction (MF) [**Fig. 4.3B**] and acetone fraction (AF) [**Fig. 4.3C**] contained only three bands. The solvent system SS2 eluted four bands each from MF and EAF but only two from AF. The solvent system SS3 eluted five bands from MF while SS5 eluted three bands from EAF. There were no bands observed from any of the fractions run with solvent system SS4 while no separation was observed for the n-Hexane fraction used as a control (**Fig. 4.3D**). In summary, good separation from most of the fractions was achieved with SS2. The average R_f values of all the eluted bands are shown in **Appendix 1**. Bioassay of the chromatogram from ethyl acetate extract separated with the SS2 system revealed antibiotic activity in the region that had an R_f value of 0.56 – 0.64.

The Harrison research chromatotron technique gave good separation of the antimicrobially-active sample. In attempts to increase the concentration of the antibiotic 30 fractions collected at 1min intervals were pooled into six groups (A – F) as described in **Section 2.13.3** before being tested for biological activity. Two groups (A and E) of the pooled fractions showed biological activity against *Serratia marcescens* (results not shown).

High performance liquid chromatography (HPLC) analysis results revealed peaks [**Fig. 4.4A**] that eluted within 5 minutes under isocratic conditions. When the methanol concentration of the mobile phase was decreased during the analysis, an increase in retention time was observed [**Fig. 4.4B – D**]. Optimal separation following isocratic HPLC was observed at 50% methanol concentration [**Fig. 4.4E**]. Although, a number of peaks were observed during isocratic HPLC runs, they were not as clearly separated as when run under gradient conditions. Three major peaks (PI, PII and PIII) [**Fig. 4.5**] were revealed when run under decreasing concentrations of methanol in water. Methanol concentrations between 50% and 70% in water were considered the optimum gradient HPLC mobile phases (**Fig. 4.5B – C)**. A methanol concentration of 100% in water was not suitable (**Fig. 4.5A**). On further purification, the peak at 5 minutes (PII) was collected as pure (**Fig. 4.5D**) since it inhibited the growth of most test organisms including *S. aureus*, *E. coli*, and *S. marcescens* as well as *M. luteus* and *X. campestris* pv*. campestris* (**Table 6.1**, **Chapter 6**). PI and PIII were also collected as pure (results not shown).

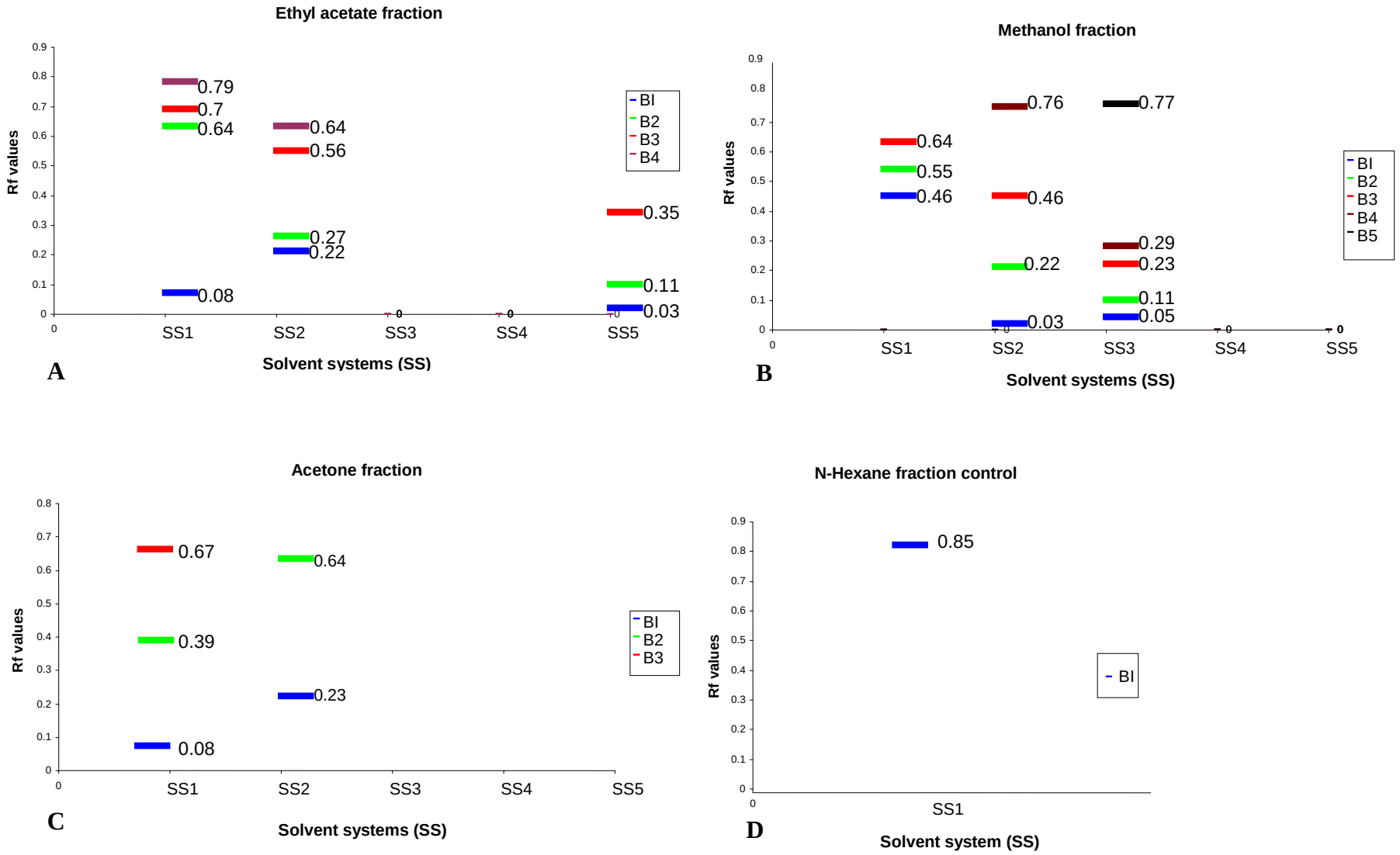


Fig. 4.3 Separation of solvent extracts by thin layer chromatography: **A**, Ethyl acetate fraction; **B**, Methanol fraction; **C**, Acetone fraction; **D**, n-Hexane control; B1, B2, B3, B4, and B5 indicate band numbers 1, 2, 3, 4 and 5 respectively eluted by each solvent system (SS). SS1, Methanol: ethyl acetate (80:20); SS2, Ethyl acetate: methanol (85:15); SS3, Ethyl acetate: diethyl ether (80:20); SS4, Dichloromethane (100%) did not separate any of the extracts; SS5, n-Hexane: ethyl acetate (20:80). Various band positions on the chromatograms are shown by their R_f values.

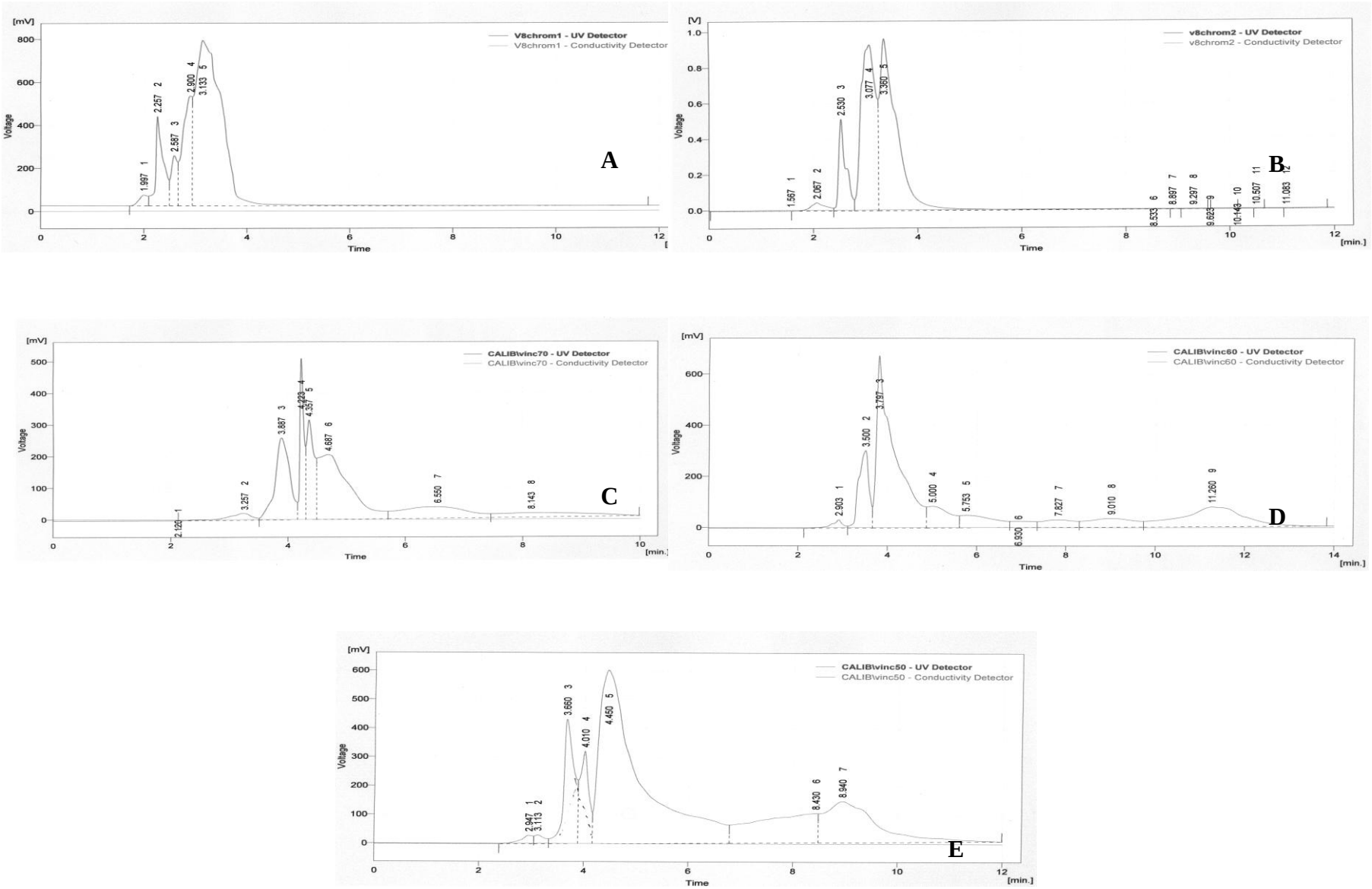


Fig. 4.4 High performance liquid chromatograms following isocratic separation of *Intrasporangium* strain N8 culture fluid extract. Fifty microlitres of extract were injected into an Alltech C-18 column (3.9mm X 300 mm) followed by isocratic elution with constant concentrations of methanol in water throughout the separation run. A – E = Separations achieved on decreasing concentration of methanol in water (100%, 80%, 70%, 60% and 50% respectively). Elution was monitored at 280 nm. Voltage on the vertical axis equals absorbance units.

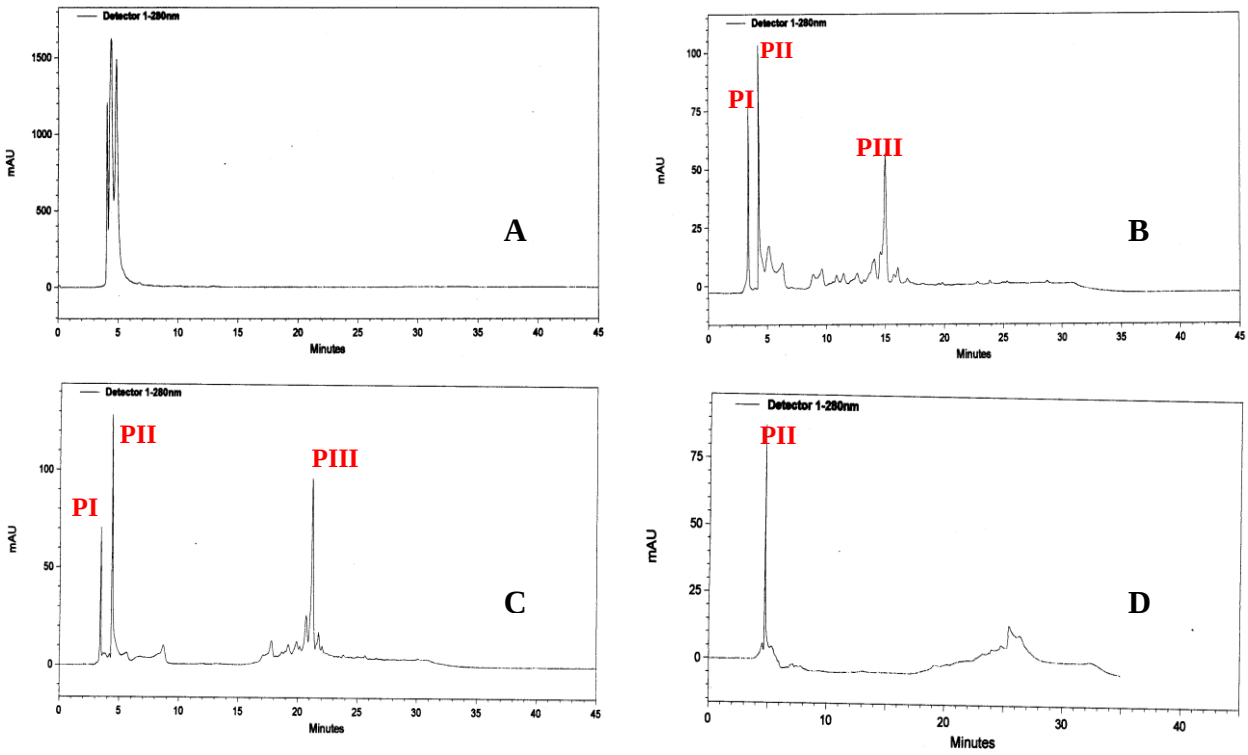


Fig. 4.5 High performance liquid chromatograms following gradient separation of culture fluid extract of *Intrasporangium* N8. Fifty micro litres of extract were injected into a Hypersil 5 ODS analytical column (25 cm X 10 mm) followed by gradient elution in decreasing concentrations of methanol in water. Elution was monitored at 280 nm. The unit on the vertical axis signifies milliabsorbance units (mAU). A-D= Separations achieved at various concentrations of methanol in water [**A**, 80% methanol; **B**, 70% methanol; **C**, 60% methanol with three major peaks (PI – PIII) observed; **D**, 50% methanol following targeting and further purification of PII since it inhibited the growth of most organisms tested including *S. aureus*, *E. coli*, and *S. marcescens* as well as *M. luteus* and *X. campestris* pv. *campestris* (note that each of the peaks was targeted and collected as pure prior to efficacy testing).

4.4 Discussion

The zone of inhibition observed during the primary and secondary screening phases is due to antibiotic diffusing into the medium from the paper disks impregnated with N8 culture filtrate. Concentration of the inhibitory compound(s) decreased with distance from the disk, creating a concentration gradient. At a certain distance from the disk the concentration was minimal for any inhibition to occur, hence, an activity zone was delineated. Strain N8 was most effective against *X. campestris* pv. *campestris* with an

inhibition zone diameter of 28 mm (**Table 4.1**)**.** This result will be interesting to plant pathologists as the organism is notoriously difficult to contain in the field.

By monitoring antibiotic production every 12 hours during the culture period, it was possible to establish the time of maximum antibiotic production. Most secondary metabolites, including antibiotics, are produced at a very late stage during growth of an organism (Zahner, 1979). *Intrasporangium* strain N8 should be grown for 7 to 10 days in simple medium before harvesting the broth for antibiotic separation. In this study, harvesting of the secondary metabolite was done on the tenth day. This ensured the stationary phase had been reached which is imperative since most antibiotics start being produced at the onset of this phase (Eckwall and Schottel, 1997).

Glycerol was found to limit the multiplication of N8 cells. However, to what extent glycerol enhanced or decreased antibiotic yield by N8 was not evident from the bioassay results. This substance has been used by some authors (Hadder *et al*., 2007; Jamil *et al*., 2007) as a stress factor to increase antibiotic yield.

The recovery and purification of the antibiotic produced by strain N8 posed a number of problems including the separation of compounds with different polarities. To this end, substances in the broth were targeted and extracted with both polar and non-polar solvents. Acetone precipitation helped to eliminate solutes thereby aiding extraction of the active compound(s) by removing a large proportion of the inactive substances.

The successful extraction of the antibacterial agent with polar solvents only indicated that it was a polar organic molecule and not a lipid. This is in keeping with the findings of other writers that the majority of antibiotic compounds are polar (Lancini and Lorenzetti, 1993). The polar nature of the active substance was confirmed when the petroleum ether extract failed to show any biological activity against the test organisms (**Fig. 4.2C**)**.** The successful extraction of the antimicrobial agent from the broth with ethyl acetate led to the sole use of ethyl acetate soluble extracts (EAF) in all subsequent chromatographic analyses.

Column chromatography is the most commonly used preparative technique for the isolation of chemical compounds; therefore considerable effort was expended in developing a good eluting system for the EAF. The active substance was soluble in

methanol, alcohol, acetone and ethyl acetate but not in hexane or petroleum ether. The anomaly of the extracts from column chromatography having more bands than the original sample could be attributed to possible decomposition of the original compound and formation of artifacts which resulted in a more complex chromatogram being obtained (Zapata and Garrido, 1991).

TLC separation of the EAF repeatedly produced clearly isolated bands and the results obtained with all the solvent systems used were reproducible. However, the procedure is very laborious and time-consuming. The most important parameters viz; the stationary phase, vapour phase, suitable solvents, mode of development as well as mobile phase optimisation and transfer are selected by trial and error (Nyiredy, 2000). In this investigation, the strongly polar solvent system SS2 which comprised ethyl acetate: methanol (85:15) was found to be the most suitable eluent. The advantage of SS2 is that both components are volatile and are thus easily removed from the sample.

The advantages of using a Harrison research chromatotron for separation are that they are relatively cheaper and quicker to run than TLC since less solvent is consumed and the components are visible as they elute (Yrjonen, 2004). In addition, the eluted fractions are easily pooled together for subsequent bioassay analysis. However, preparation of the plates is time consuming and tedious. The procedure served as the penultimate step in the purification protocol.

Preparative HPLC was used as the final purification step for most of the EAF samples. The main difference between isocratic and gradient HPLC conditions is in the concentration of the mobile phase (Purcell *et al*., 1999). In the latter case, mobile phase concentration is important when trying to separate a mixture of two compounds since the separation improves with varying concentration of the solvents (Purcell *et al*., 1999; Guzzetta, 2001). With gradient HPLC a suitable mobile phase combination makes possible the targeting and collection of particular compounds of interest often not possible with isocratic runs (Stoll *et al*., 2006). The noticeable increases in retention time as methanol concentration in the mobile phase decreased during HPLC analysis may be due to molecules adhering more strongly to the C-18 column and hence being eluted more slowly by the weaker mobile phase.

4.5 Conclusions

The successful chemical extraction, purification and subsequent bioassay results confirmed that *Intrasporangium* strain N8 produces organic compound(s) with antibiotic properties and that this/these is/are readily soluble in acetone, ethyl acetate and methanol, but insoluble in petroleum ether and hexane thereby indicating a polar nature.

Since the chromatographic methods used initially were time consuming, and produced low yields of the compound(s) of interest, an alternative procedure producing better results was sought. This led to the development of a three-solvent system and pH precipitation techniques for efficient recovery of the antimicrobially-active compound in solid form. The procedures for this are detailed in **section 2.12** (**Chapter 2**).

CHAPTER 5

STRUCTURAL ELUCIDATION OF THE ANTIBIOTIC COMPOUND(S) PRODUCED BY *INTRASPORANGIUM* STRAIN N8

5.1 Introduction

Antibiotics are mainly polar compounds containing acidic or basic functional groups (e.g., carboxylic acids, phenols, and amines) that may be subject to direct and indirect breakdown during fermentation and/or isolation/purification. The result can be a complex mixture of intermediates and transformation products. Once released into the broth, antibiotics are subjected to processes (e.g., dilution, biodegradation, etc.) that contribute to their elimination from the broth. Hydrolytic processes are of great importance in determining the fate of antibiotic compounds (Petrović and Barceló, 2007).

Antibiotic identification is complicated and a cumbersome process, requiring the application of advanced instrumental methods (e.g., liquid chromatography (LC) with mass spectrometry (MS) [LC-MS], LC with ultraviolet or fluorescence (UV/FL) detection, nuclear magnetic resonance (NMR), matrix-assisted laser desorption/ionisation (MALDI)-MS, gas chromatography (GC) with mass spectrometry (GC-MS), ion chromatography (IC) or infrared (IR) spectroscopy. Of these, LC-MS has gained popularity and has become one of the preferred techniques for analysing antibiotics (Petrović *et al*., 2005). Two common strategies are applied, depending on the LC-MS instrumentation used. One relies on accurate measurement of molecular mass, and, subsequently, the determination of the empirical formula using orthogonal acceleration time-of-flight (oaTOF) instruments; whereas the other involves elucidation on the basis of structural information gained in tandem MS experiments that can be accomplished either by coupling mass analyzers in series (e.g., triple-quadrupole (QqQ)) or by using a single ion-trap (IT) analyzer (Petrović *et al*., 2005).

The sensitivity and selectivity of modern LC-MS instruments have made this technique a powerful tool and as mentioned above, a method of choice for identifying antibiotic products and elucidating their structures. In many LC-MS applications, the MS

component is the most relevant factor in the elucidation process, especially when no databases are available. Another significant problem when dealing with novel compounds is the lack of authentic standards that can be used to confirm the structure, because, although LC-MS and other accurate measurement systems (e.g., LC-Qq-TOF) can be used for compound identification, authentic standards are still needed for the final, definitive confirmation, as well as for quantification (Petrović *et al*., 2005). There are three basic functions that a mass spectrometer performs. Firstly it subjects the molecules to bombardment by a stream of high energy electrons, whereby the molecule is converted to its ionic form. Secondly, these ions then get accelerated in an electric field. Finally the different ionized fragments are detected (Hites, 1997).

In addition to LC-MS, GC-MS is also a useful tool for structural elucidation of antibiotic compounds. It is the single most important tool for the identification and quantification of volatile and semi volatile organic compounds in complex mixtures (Santos and Galceran, 2002). As such, it is very useful for the determination of molecular weights and (sometimes) the elemental compositions of unknown organic compounds in complex mixtures (Santos and Galceran, 2002). However, the major disadvantage of GC-MS is that the sample must be volatile, since its components will be separated in the gas phase and only compounds with vapour pressures exceeding about 10^{-10} torr can be analysed. This means that usually biological samples need to be derivatised (Hites, 1997). Also, determining positional substitutions on aromatic rings is often difficult. Certain isomeric compounds cannot be distinguished by mass spectrometry (for example, naphthalene versus azulene), but they can often be separated chromatographically (Hites, 1997).

To ensure qualitative identification of an organic compound using GC-MS, several criteria should be met. Firstly, the mass spectra of the unknown compound and of the authentic compound must agree over the entire mass range of the spectra. It is particularly important to compare the patterns within narrow mass ranges; these patterns should agree almost exactly. In this case, the spectrum of the authentic compound might come from a library of reference spectra or from the actual compound itself. In the latter case, the compound could be purchased or synthesized. Secondly, the GC retention times of the unknown compound and of the authentic compound must agree within about ±1 to 2 sec. It is often convenient to co-inject the unknown mixture and the authentic compound. The GC peak in question should increase in size by the

correct factor. Thirdly, a compound cannot be considered fully identified in a mixture unless two other questions are addressed: is the identification plausible? Why is it present in a given sample? If identification is implausible or if there is no reason for a compound to be present in a sample, the identification could be wrong or the compound could be a contaminant (Hites, 1997). However, as mentioned previously, a complicating factor might be the lack of authentic standards.

Another powerful tool used by many researchers to elucidate the structure and function of many biologically important molecules is NMR. In this investigation, correlation spectroscopy (COSY) and proton nuclear magnetic resonance (^{1}H NMR) experiments were used for structural determination of the antimicrobial compound. COSY produces a 2-dimensional NMR spectrum that shows correlation between coupled protons in a molecule (Jacobsen, 2007). On the spectrum is a diagonal with cross peaks on either side that are symmetrically disposed. Two cross peaks can interact at right angles where the chemical shifts of two-coupled resonances can be obtained. The gradient correlation spectroscopy (GCOSY) spectrum indicates which of the lines in the proton spectrum are connected. The advantage of GCOSY is that it filters out noise and also is faster than normal COSY (Bendiak *et al*., 2002).

The ^{1}H NMR spectrum gives the coupling constants or the splitting in the peaks. A single peak is normally referred to as a singlet. It is called a 'doublet' when the peak is split into two and a 'doublet of doublet' when two corresponding peaks have the same splitting. A 'multiplet' refers to three or more splits within two corresponding peaks. The difference between two peaks at one position and two peaks at another position which is the same is known as the coupling constant (Hornback, 2005). It is measured in Hertz (Hz). A line list showing the coupling constants for the antimicrobial compound produced by N8 is shown in **Appendix 2.** In this chapter attempts to elucidate the structure of the secondary metabolite with antibiotic activity produced by strain N8 is described.

5.2 Materials and Methods

5.2.1 Liquid chromatography-Mass spectroscopy (LC-MS)

LC-MS analysis of the crystals was carried out as described in **Section 2.14.2.** A three-solvent system together with pH adjustment was used for the recovery of the crystals

from the broth as described in **Section 2.12**.

5.2.2 ^{1}H- and GCOSY- Nuclear magnetic resonance (NMR) spectroscopy

NMR spectra were recorded on a Bruker Avance III spectrometer at 30^{o}C using deuterated methanol as solvent and residual protonated methanol as the internal standard as described in **Section 2.14.3**. Gradient correlation spectroscopy (GCOSY) spectra were recorded on the same instrument. The sample was dissolved in d-methanol using residual protonated methanol as chemical shift reference as described in **Section 2.14.3**.

5.2.3 Gas chromatography-Mass spectroscopy (GC-MS)

Characterization of the antimicrobial substance was carried out by GC-MS analysis as described in **Section 2.14.1**. The sample was evaporated to dryness in a BUCHI Rotavapor EL 130 at 30^{o}C and resuspended in a minimal volume of acetone before assay.

5.3 Results

The antimicrobial agent was successfully extracted from the fermentation medium as mentioned in **Chapter 4 (section 4.4**). Adjusting the pH of the EAF to either 3 or 8.2, resulted in the formation of two types of crystals; brown and pink-yellow in colour respectively. On further analysis using LC-MS (**Fig 5.1**), the brown crystals appeared to be a protein and since it did not show inhibitory activity against any of the test organisms, no further studies were carried out on it. The pink-yellow crystals when suspended in a minimal volume of methanol showed inhibitory activity against *S. marcescens* (discussed in Chapter 4: **Fig. 4.2A**).

The MS spectrum (**Fig. 5.2**) of the pink-yellow crystals shows a prominent/base peak at 304.2724 (mass to charge ratio (m/z) in positive mode. Analysis of this peak indicated that it could be $C_{16}H_{36}N_2O_3$. There was no matching compound in the database of natural products [National Institute of Standards and Technology (NIST), www.nist.gov].

The ^{1}H-NMR spectrum (**Fig. 5.3**) of the pink-yellow crystals showed signals at 1.6, 1.7,

4.3, 4.4, 5.6, 6.7, 7.0, 7.4, 7.6, and 7.7 ppm. There is evidence of one or two aromatic protons (possibly part of a benzene ring) at positions 7 – 7.5 ppm. In addition, there is clear evidence of approximately six CH_2 groups, about the same number of CH_3 groups and possibly five CHOH, CHNHCO and CHR (R=alkyl) groups.

The gradient correlation spectroscopy (GCOSY) spectrum of the pink-yellow crystals (**Fig. 5.4**) revealed a connection between the protons at 1.6 and 4.4 ppm or a sharing of the same neighbouring carbons. Similarly, the protons at 1.7 and 4.3, 5.6 and 7.4, 6.7 and 7.0, 7.6 and 7.7 ppm are also connected.

Gas chromatography-mass spectroscopic (GC-MS) data analysis of the EAF indicated that the substance showing antibiotic activity comprised at least seven components labeled A – G in **Figure 5.5**. These components separated by gas chromatography were then subjected to mass spectrum analysis and their retention indices compared to computer database listings of known compounds [National Institute of Standards and Technology (NIST), www.nist.gov]. Although A and B have different retention indices, their molecular weight and formula are the same and therefore they were regarded as representing a single compound. Thus a total of six different compounds were detected by GC and their molecular formulae elucidated as: $C_6H_{10}O$ (A and B); $C_6H_{12}O_2$ (C); $C_9H_{14}O$ (D); C_8H_7N (E); $C_{21}H_{44}$ (F); and $C_{12}H_{14}N_2O$ (G). The closest match from the computer database of known compounds [National Institute of Standards and Technology (NIST), www.nist.gov] was found for $C_{12}H_{14}N_2O$ (G) with 71% probability **(Table 5.1)** and identified as N-[2-(1H-indol-3-yl) ethylacetamide (**Fig. 5.6A; Fig. 5.6B**). The putative identities of A – G are also shown in **Table 5.1.** Since low probability matches were obtained for A – E and as the sample could not be recovered from the analyser, they were not studied further.

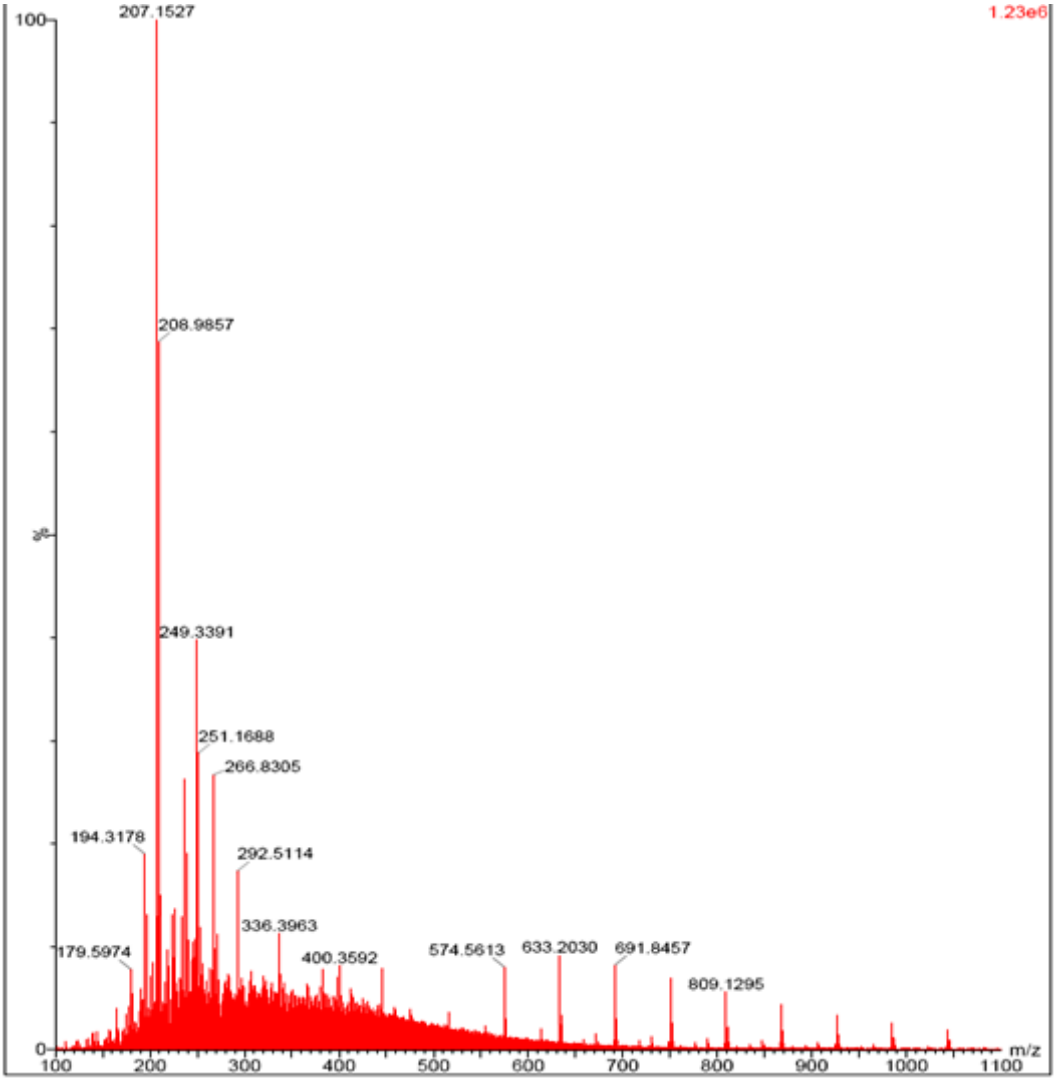


Fig. 5.1 Mass spectrum of the brown-coloured crystals produced from ethyl-acetate extract of the culture fluid of strain N8.

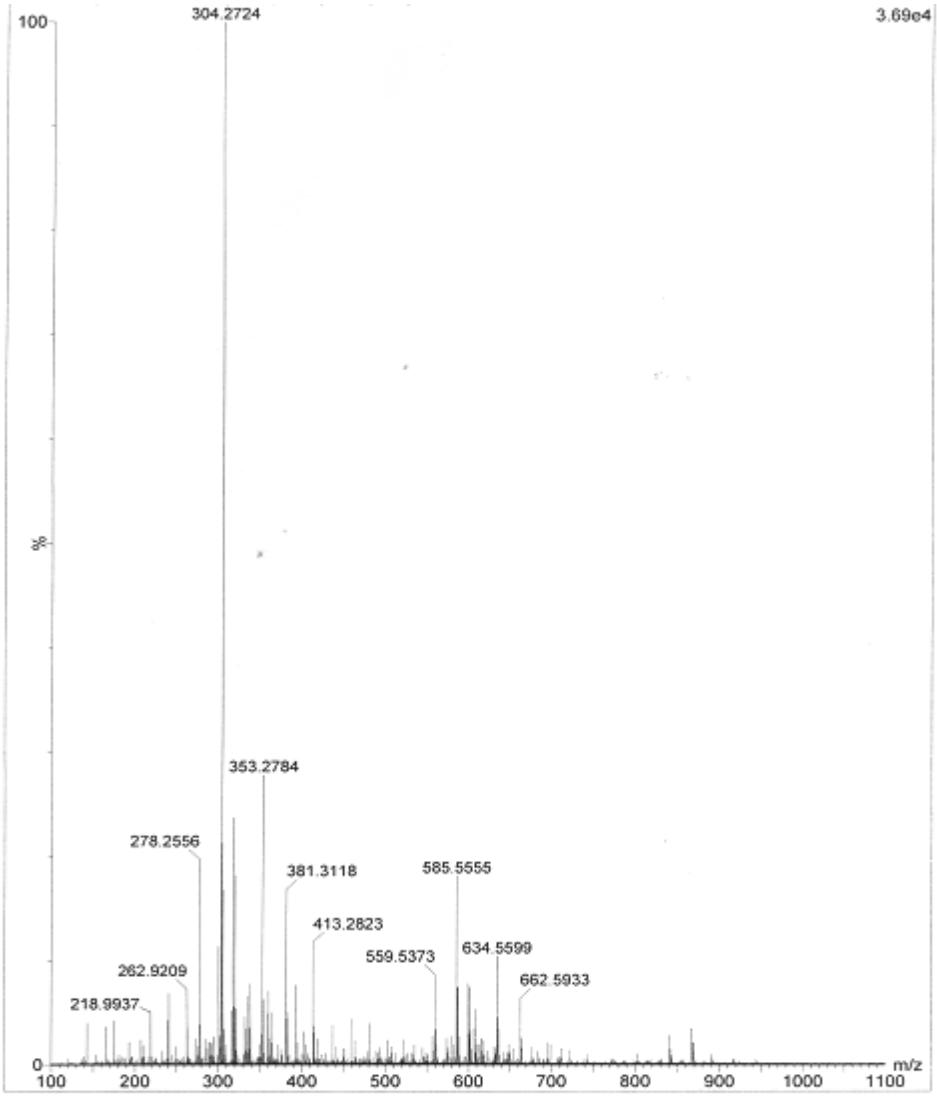


Fig. 5.2 Mass spectrum of the needle-like pink-yellow crystals produced from the ethyl-acetate extract of the culture fluid of strain N8.

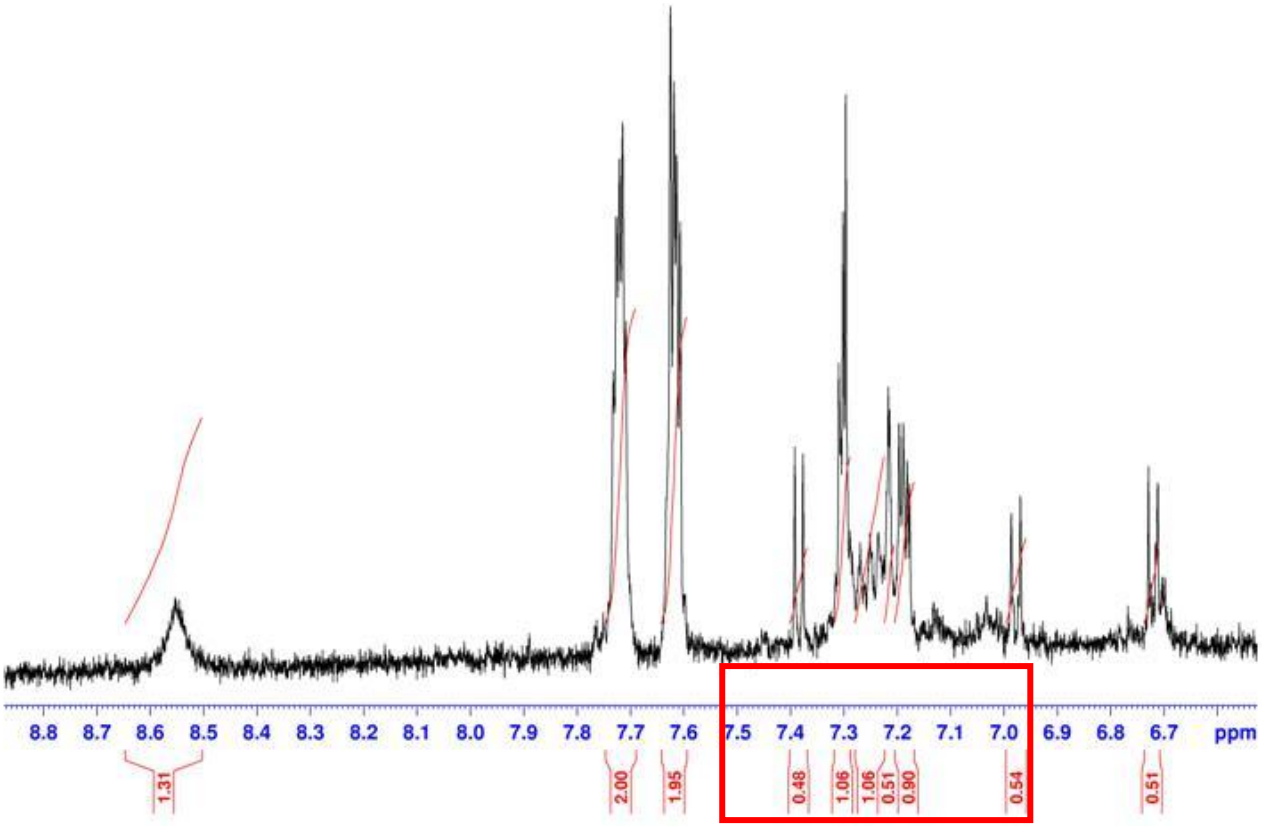


Fig. 5.3 Proton nuclear magnetic resonance (^{1}H-NMR) analysis of the antimicrobial compound produced by strain N8. Note the portion of the spectrum showing evidence of one or two aromatic protons

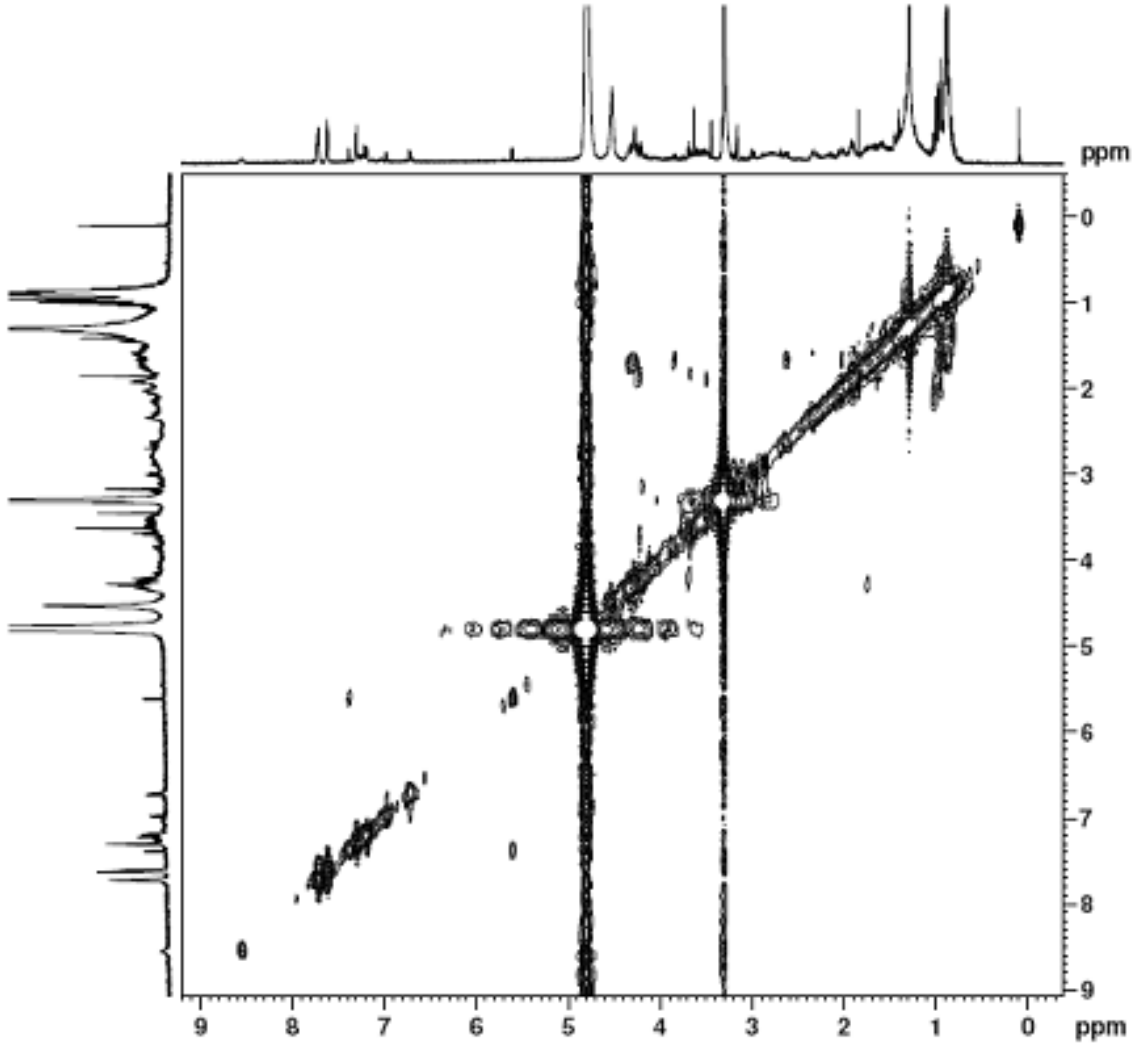


Fig. 5.4 Gradient correlation spectroscopy spectrum of the antimicrobial compound produced by strain N8

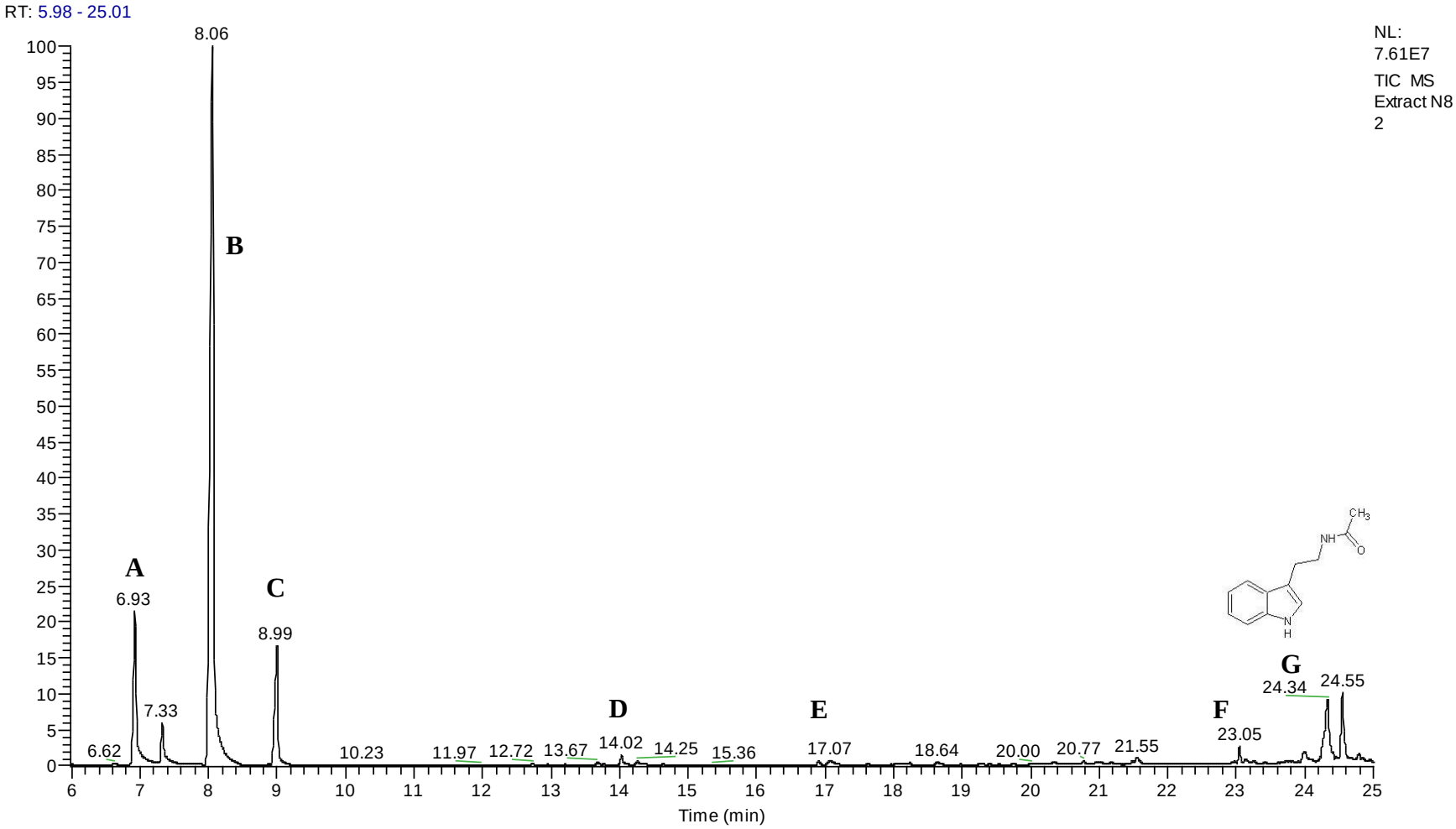


Fig. 5.5 Gas chromatogram of the antimicrobial substance in the ethyl acetate extract (EAF) after extraction of the culture fluid of strain N8. Components A – G were subjected to mass spectroscopy analysis. Note the occurrence of a possible tryptamine structure (G) and is essential for antimicrobial activity (Korkmaz *et al*., 2008; Raoudha *et al*., 2009).

Table 5.1 Compounds identified* by mass spectroscopic data analysis following gas chromatography separation of the antimicrobial substance produced by strain N8.

Components of antibiotic molecule	Retention time (min)	Mol. weight	Mol. formula	Index name of compound	Prob. (%)
A	6.93	98	$C_6H_{10}O$	• 3-hexen-2-one	37
				• 4 methyl-4-penten-2-one	11
B	8.06	98	$C_6H_{10}O$	• 3-hexen-2-one	20
				• 4 methyl-3-penten-2-one	39
C	8.99	116	$C_6H_{12}O_2$	• 4-hydroxy-4-methyl-2-pentanone	57
D	14.02	138	$C_9H_{14}O$	• 2,6-dimethyl-2,5, Heptadien-4-one	57
E	17.07	117	C_8H_7N	• Indole	18
				• m-aminophenylacetylene	16
				• Indolizine	12
F	23.05	296	$C_{21}H_{44}$	• 10-methyl elcosane (alkane hydrocarbon)	6
G	24.30	202	$C_{12}H_{14}N_2O$	• N-[2-(1H-indol-3-yl) ethyl acetamide	71
				• 2-[z]-(3,4-dimethyl 2H-pyrrol-2-yledene) methyl-3,4-dimethyl[1H] pyrrol	43

* Identification based on the built-in computer database of known compounds [National Institute of Standards and Technology (NIST), www.nist.gov].

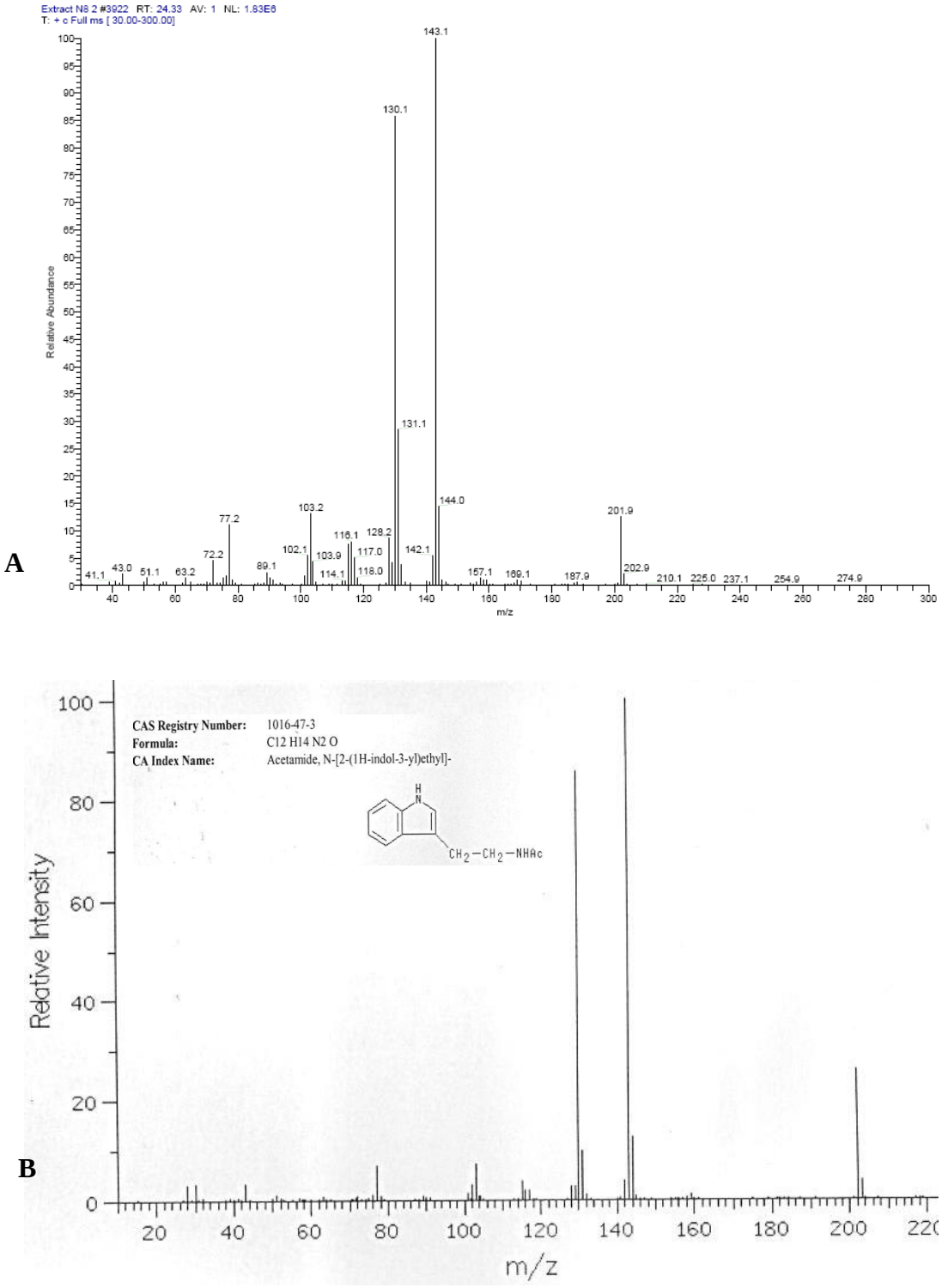
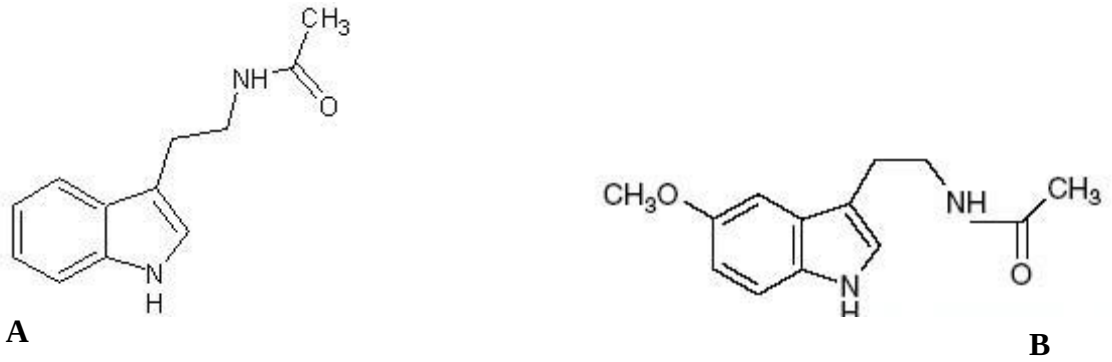


Fig. 5.6 Structural identity of component G: **A**, Full mass spectrum of component G; **B**, Full mass spectrum of N-[2-(1H-indol-3-yl) ethylacetamide (=N-acetyltryptamine).



N-[2-(1H-indol-3-yl) ethylacetamide (=N-acetyltryptamine) **Melatonin**

Fig. 5.7 The tentative structure of the antimicrobial compound produced by strain N8. **A**, Structure of N-acetyltryptamine; **B**, Melatonin

5.4 Discussion

The LC-MS spectrum of the pink-yellow crystals showed evidence of a base/prominent peak at 304.2724 m/z in positive mode (**Fig. 5.2**). The base peak in a mass spectrum corresponds to the separated ion beam which has the greatest intensity. The elemental composition of the compound that yielded this base peak suggests a molecular formula closely similar to $C_{16}H_{36}N_2O_3$ with a molar mass of 304.4686 g/mol. However, the compound could be assigned a name from the databases of natural compounds [National Institute of Standards and Technology (NIST), www.nist.gov].

On further analysis of the pink yellow crystals MS data it became evident that the metabolite is not pure since bands appear at a range of masses above 304. The ^{1}H- and GCOSY- nuclear magnetic resonance (NMR) spectroscopy (**Fig. 5.3** and **Fig. 5.4** respectively) profiles of the crystals appear to have some properties of tryptamine in them but mixed with another compound making it impossible to assign a single structure based on these profiles.

However, when the GC-MS profile of the antimicrobial substance produced by strain N8 was compared to MS profiles from the databases of natural compounds, a better fit was obtained with 71% probability of being accurate. GC-MS is the single most important tool for the identification and quantification of organic compounds in complex mixtures (Santos and Galceran, 2002). GC-MS analysis of strain N8 metabolite revealed seven prominent components (A – G). It appears that components A and B

represent two similar molecular compounds (possibly isomers) since they have the same molecular weight and formula. Their different retention indices strongly suggest that they are indeed isomers. In a GC-MS, the lighter molecules elute first followed by heavier molecules (Krupp and Donard, 2005). It is evident from the GC-MS results that G is the heaviest molecule since it eluted last from the column. It has a molecular formula of $C_{12}H_{14}N_2O$ with a structure that closely resembles an indolethylacetamide (=N-acetyltryptamine) [**Fig. 5.7A**]. The 71% probability (**Table 5.1**) refers to how closely the structure of the compound in question matches the index value from a chemical structure library or a computer database of known compounds [National Institute of Standards and Technology (NIST), www.nist.gov].

The structure of component G (N-acetyltryptamine) was compared to structures of commonly used antibiotics (**Refer to Fig. 1.2, Chapter 1**) in an attempt to tentatively suggest its mode of action. N-acetyltryptamine has been reported to be produced by both *Thermoactinomyces* strain TA66-2 (Korkmaz *et al*., 2008) and *Streptomyces* species strain TN58 (Raoudha *et al*., 2009). Thiazole was also reported to be produced simultaneously with N-acetyl tryptamine by the above mentioned authors. Tryptamine is a monoamine alkaloid found in plants, fungi, and animals. It is based around the indole ring structure, and is chemically related to the amino acid tryptophan, from which its name is derived. Tryptamine is found in trace amounts in the brains of mammals and is believed to play a role as a neuromodulator or neurotransmitter. Tryptamine is also the backbone for a group of compounds known collectively as tryptamines. This group includes many biologically active compounds, including neurotransmitters and psychedelic drugs (Jones, (1982).

A closely related structure is known as melatonin (**Fig. 5.7B**) which has been attributed with the ability to inhibit tumour growth *in vivo* and *in vitro*. Recent studies have shown that melatonin can profoundly retard tumour progression induced by chemical carcinogens and can also modulate enzymes involved in xenobiotic metabolism and detoxification (Tan *et al.*, 1993). An example of a group of enzymes involved in xenobiotic metabolism is hepatic microsomal cytochrome P450. These enzymes that metabolize xenobiotics are very important for the pharmaceutical industry, because they are responsible for the breakdown of medications. Melatonin is also known for its radical scavenger activity, which had been related to its ability to protect neuronal cells from different kinds of oxidative stress (Mor *et al*., 2003). Melatonin, both alone and in

combination with other agents, has been studied for anticarcinogenic or carcinostatic activity against a variety of human cancers (Lissoni *et al*., 1987).

The antibiotic produced by strain N8 appears to be a mixture of closely related compounds the main component of which is shown in **Figure 5.6A** with a peak at 143.1 m/z ($M + Na^+$). A feature of the antibiotic is the occurrence of a tryptamine structure and is essential for antimicrobial activity (Korkmaz *et al*., 2008; Raoudha *et al*., 2009). It is postulated that the antibiotic produced by strain N8 shares the same mechanism of action with tryptamine.

5.5 Conclusion

The chemical nature of the antibiotic produced by *Intrasporangium* strain N8 was partly revealed by the results of the GC-MS analysis. However, the true chemical identity of the antibiotic could not be confirmed by comparison with any known compounds in the database of natural products available. More work involving additional chemical analytical techniques is required to conclusively prove its novelty. This however, is beyond the scope of the present investigation which is essentially microbiological. The indications are that the antibiotic is a tryptamine, the tentative structure of which is shown in **Figure 5.7.** To the authors knowledge no known antibiotic produced by *Intrasporangium* have been earlier reported.

CHAPTER 6

ANTIMICROBIAL EFFICACY OF COMPOUND(S) PRODUCED BY *INTRASPORANGIUM* STRAIN N8

6.1 Introduction

The use of a single antibiotic in the initial treatment of infectious diseases has continued for many, many years. This strategy has too often resulted in strains showing induced resistance to the antibiotic. As new antibiotics are discovered, the microorganisms keep inventing new ways of becoming resistant to them. The development of resistance easily outpaces the appearance of new antibiotics (Fox, 2007).

There are many reasons for using a combination of two or three antibiotics with different mechanisms of action in the initial treatment of infectious diseases. (1) At normal mutation rates the chances are infinitesimally small that two or three resistance mutations will occur spontaneously against two or three antibiotics with different mechanisms of action; (2) It has been shown that “the effect of combined antibiotics in serum attainable levels is bactericidal as opposed to the ineffectiveness of the maximum dosage of each antibiotic when given singly” (Balows, 2006); (3) A combination of the most pharmaco-dynamically effective members of an antibiotic class, reduces the duration and overall costs of a disease treatment, due to fewer relapses and follow-up therapy for patients contending with an antibiotic resistance problem (Scheld, 2003a).

A typical example of combination therapy is the use of Rifater antibiotic. Rifater is a triple combination antibiotic of isoniazid, rifampicin and pyrazinamide. Use of a triple combination antibiotic to treat tuberculosis became necessary because isoniazid which was used for many years to treat TB (mostly in Southeast Asia) eventually became ineffective since TB strains became resistant to it. Rifater was formulated in an attempt to try to prevent TB strains from becoming resistant to rifampicin and/or pyrazinamide. Ethambutol was also added to the anti-TB regimen (Hibbard, 2007).

Unfortunately, since only one antibiotic was frequently added to the treatment regimen

at a time and combination therapy was not used; a few strains of *Mycobacterium tuberculosis* eventually became resistant to all TB therapies. In the Rifater clinical studies, the combination of the three antibiotics mentioned above did not result in any more adverse contraindications in patients than when each antibiotic was given separately. If each antibiotic has a different mechanism of action, it seems logical that the adverse effects due to each antibiotic would be independent of one another (Hibbard, 2007).

The aim of this part of the study was to optimize the efficacy of the antibiotic produced by *Intrasporangium* strain N8. The six compounds identified during GC-MS analysis were not recoverable due to the nature of the equipment used so it was not possible to conduct further tests on each of them individually. However, three major compounds (peaks) were successfully collected and purified by GHPLC analysis for this part of the study. The compounds (peaks) viz. PI, PII and PIII were tested against major plant and human pathogens both singly and in various combinations to see whether they are biologically more effective when combined than when used separately. The results were compared with some standard antibiotics.

6.2 Materials and Methods

6.2.1 Test media

All media used were prepared in distilled deionized (DI) water. Mueller-Hinton (MH) medium (Beef extract, 2g; Casein hydrolysate, 17g; Starch, 1,5g; Agar, 17g; per litre (pH 7.4) was used for susceptibility testing.

6.2.2 Target microbes

The test organisms were all from the culture collection in the Discipline of Microbiology, University of KwaZulu-Natal as described in **Section 2.5.** *Micrococcus luteus* (ML), a human pathogen and two unidentified endogenous bacterial pathogens of *Eucomis autumnalis autumnalis* (Eaa) and *Veltheimia bracteata* (Vyb) were added to the list. The fungal pathogen of potato, *Rhizoctonia solani (Rhz)* was also tested. The unidentified organisms were supplied by UKZN's Plant Pathology Department. *Eucomis autumnalis autumnalis* commonly known as 'pineapple flower' is used medicinally in South Africa for the treatment of low backache and *Veltheimia bracteata*, known as

'Yellow flame' is touted to have a purgative effect.

6.2.3 Recovery and purification of the antimicrobial agent

All major steps used in the recovery procedure are shown in **Figure 2.2** and described in **Section 2.12**. The compound(s) was purified using GHPLC as described in **Section 2.13.4**. Corresponding individual peaks were pooled from several HPLC runs of the sample and concentrated before bioassay.

6.2.4 Bioassay

The peaks used for susceptibility tests were collected from the GHPLC analysis. The *in vitro* antimicrobial activities were determined by agar disk-diffusion as described in **Section 2.9.2**. All samples were suspended in a minimal volume of methanol which was also used as the control. Some standard antibiotics, namely, Penicillin G (10 i.u.), Bacitracin (10 i.u.) and Tetracycline (10 mcg), supplied by Oxoid, and an unidentified locally produced antimicrobial agent (V3)* were used in order to compare the results with those obtained with the antibiotic compounds produced by N8. The standard antibiotics used are secondary metabolites of three major antibiotic-producing groups of microorganisms, viz. moulds, eubacteria and actinomycetes. The minimum inhibitory concentration was determined by broth micro-dilution methods according to the protocol recommended by the Clinical and Laboratory Standards Institute (CLSI) [formerly National committee for clinical laboratory standards (NCCLS)] as described in **Section 2.9.3**. The MIC of the mixture (PPM) was compared to that of the individual components, PI, PII and PIII. Synergy in this study referred to instances where the average diameter of the inhibition zone was greater, or the MIC was less, for the combined peaks than was the average inhibition zone diameters, or MICs, of the individual peaks when tested separately. Contrary to the above instance where the MIC for the combined peaks was more than was the single peaks, it was referred to as antagonistic. A flowchart showing the steps used to test the efficacy of the GHPLC separated peaks obtained from the antibiotic produced by *Intrasporangium* strain N8 is shown in **Figure 6.1**.

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* Supplied by UKZN Plant Pathology department

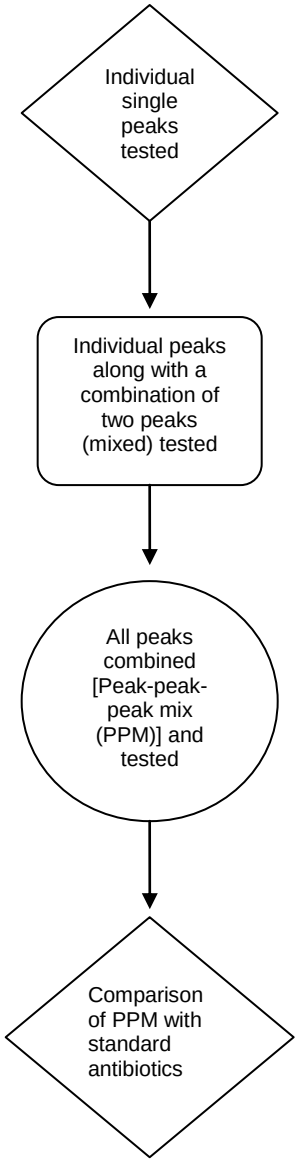

Fig. 6.1 Flowchart showing steps used to test the efficacy of the gradient high performance liquid chromatography separated peaks obtained from the antibiotic produced by *Intrasporangium* strain N8.

6.3 Results

During primary screening, *Intrasporangium* strain N8 inhibited all the test organisms used on both NA (**Fig. 3.1A**) and ISTA plates (**Fig. 3.1B**) as described **in Chapter 3**. After extraction of the fermentation broth of the organism with various solvents (**section 2.12.2**), all the extracts, with the exception of petroleum ether extract (**Fig. 4.2C**) showed good antimicrobial activity against the test organism as described **in Chapter 4.** The ethyl acetate extract (EAF) had the best activity [**Fig. 4.2C**] and was selected for

further analysis. Purification of the EAF with GHPLC resulted in three peaks (PI, PII and PIII) [**Fig. 4.5C]** collected with an automated fraction collector (**section 2.13.4**).

Results from growth-inhibition efficacy testing of the individual peaks showed that they induced different responses in the different test organisms (**Fig. 6.2**; **Table 6.1**). For example, PII (targeted and collected as pure during GHPLC analysis) showed clear inhibitory activity against *E. coli* (**Fig. 6.2C**) with average inhibition zone diameter of 12mm and an MIC of 0.031µg/ml (**Table 6.1**). It also showed growth inhibitory activity against *S. marcescens* (15 mm) [**Fig. 6.2A**], *X. campestris pv. campestris* (12 mm) [**Fig. 6.2B**] and *S. aureus* (15 mm) [**Table 6.1**]. Likewise, PI inhibited the growth of *E. coli* (11 mm), *X. campestris pv. campestris* (11 mm) but showed no activity on *S. marcescens* **(Table 6.1)**. The presence of resistant cells was observed when PIII was tested against *S. marcescens* (**Fig. 6.2A)**. The average inhibition zone diameters together with the MIC results of the individual peaks on each of the test organisms are shown in **Table 6.1**.

In an attempt to check for synergy, the individual peaks were tested along with combinations of two peaks (e.g. PI +PII mix) on different test organisms. The results showed that the individual activities of the peaks were minimal compared to when they were combined. For example, when PI and PII were combined, the diameter of the inhibition zone on plates inoculated with *S. marcescens* (**Fig. 6.2A**) was double (30 mm) that produced by PII alone (15 mm). Likewise, a combination of PI and PIII on *X. campestris* pv*. campestris* (12 mm) showed inhibitory activity as compared to the little or no inhibition observed when PIII alone was tested (**Fig. 6.2B)**. The mixture of PII and PIII did not inhibit the growth of most test organisms. The spectra of activities produced by individual and combined peaks against Gram-postive and Gram-negative test organisms are graphically represented in **Figure 6.3A** and **6.3B** respectively.

Table 6.1 Antimicrobial activities of individual and combined peak(s) produced by *Intrasporangium* strain N8 against test organisms compared to standard antibiotics

Test organism*	Average Inhibition zone diameter (mm)									MIC (µg/ml)					
	PI	PII	PIII	PI+ PII	PI+ PIII	PII+ PIII	PPM	V3	Pen (10 i.u)	Bac (10 i.u)	Tet (10 µg)	PI	PII	PIII	PPM
Gram-positive organisms															
Staphylococcus aureus	18	15	12	23	nt	uns	38	uns	28	uns	16	0.25	0.13	>1.0	0.0078
Enterococcus faecalis	uns	uns	uns	06	05	uns	22	uns	14	>1.0	>1.0	uns	uns	uns	0.25
Micrococcus luteus	15	10	13	22	25	19	35	uns	25	uns	uns	0.25	0.50	nt	0.02
Gram-negative organisms															
Escherichia coli	11	12	uns	uns	uns	nt	25	uns	05	uns	16	0.50	0.031	uns	0.039
Pseudomonas fluorescens	uns	uns	uns	uns	20	rs	30	uns	uns	uns	uns	uns	uns	uns	0.063
Serratia marcescens	uns	15	15^{rs}	30	nt	12	28	uns	uns	uns	0.13	uns	0.25	uns	0.125
Xanthomonas campestris pv. *campestris*	11	12	uns	22	12	nt	25	uns	uns	uns	uns	0.03	0.06	uns	0.002
Yeast															
Candida utilis	uns	uns	14	uns	uns	uns	27	uns	uns	uns	uns	uns	uns	0.25	0.002
Plant pathogens															
Eucomis autumnalis autumnalis pathogen	12	13	17	11	nt	12	30	uns	30	uns	uns	0.13	0.13	nt	0.06
Veltheimia bracteata pathogen	uns	uns	uns	uns	uns	uns	uns	30	uns	uns	uns	uns	uns	uns	uns
Fungi^															
Rhizoctonia solani	uns	uns	uns	uns	uns	uns	uns	uns	uns	uns	uns	uns	uns	uns	uns

*****Cultured on MH agar for disk diffusion or in MH broth for MIC determination; ^ cultured on PDA; uns, unsusceptible; nt, not tested; rs, resistant strains; Pen, penicillin; Bac, bacitracin; Tet, Tetracycline.

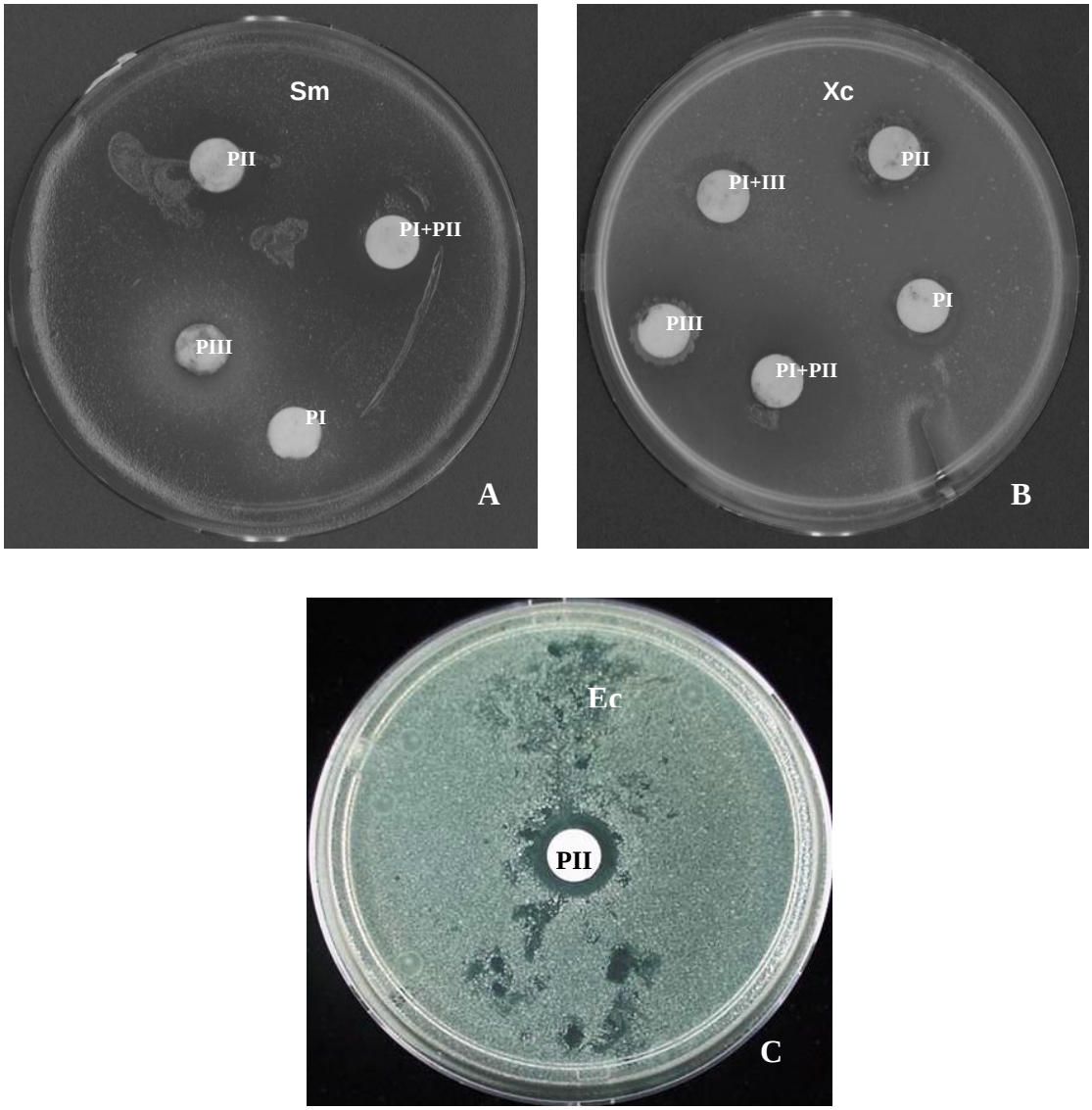

Fig. 6.2 Growth inhibition of test organisms by individual and combined components of the antibiotic produced by strain N8. **A**, Inhibition of *Serratia marcescens* (Sm) [note the larger inhibition zone produced by the PI+PII mixture (30mm) than that produced by PII (15mm) alone and note also the presence of cells resistant to PIII]; **B**, Stronger inhibition of *Xanthomonas campestris* pv. *campestris* (Xc) by the combined action of PI+PII and also PI+PIII compared to the inhibition caused by each component separately; **C**, Inhibition of *Escherichia coli* (Ec) by PII: in this case combining components did not improve growth inhibitory action.

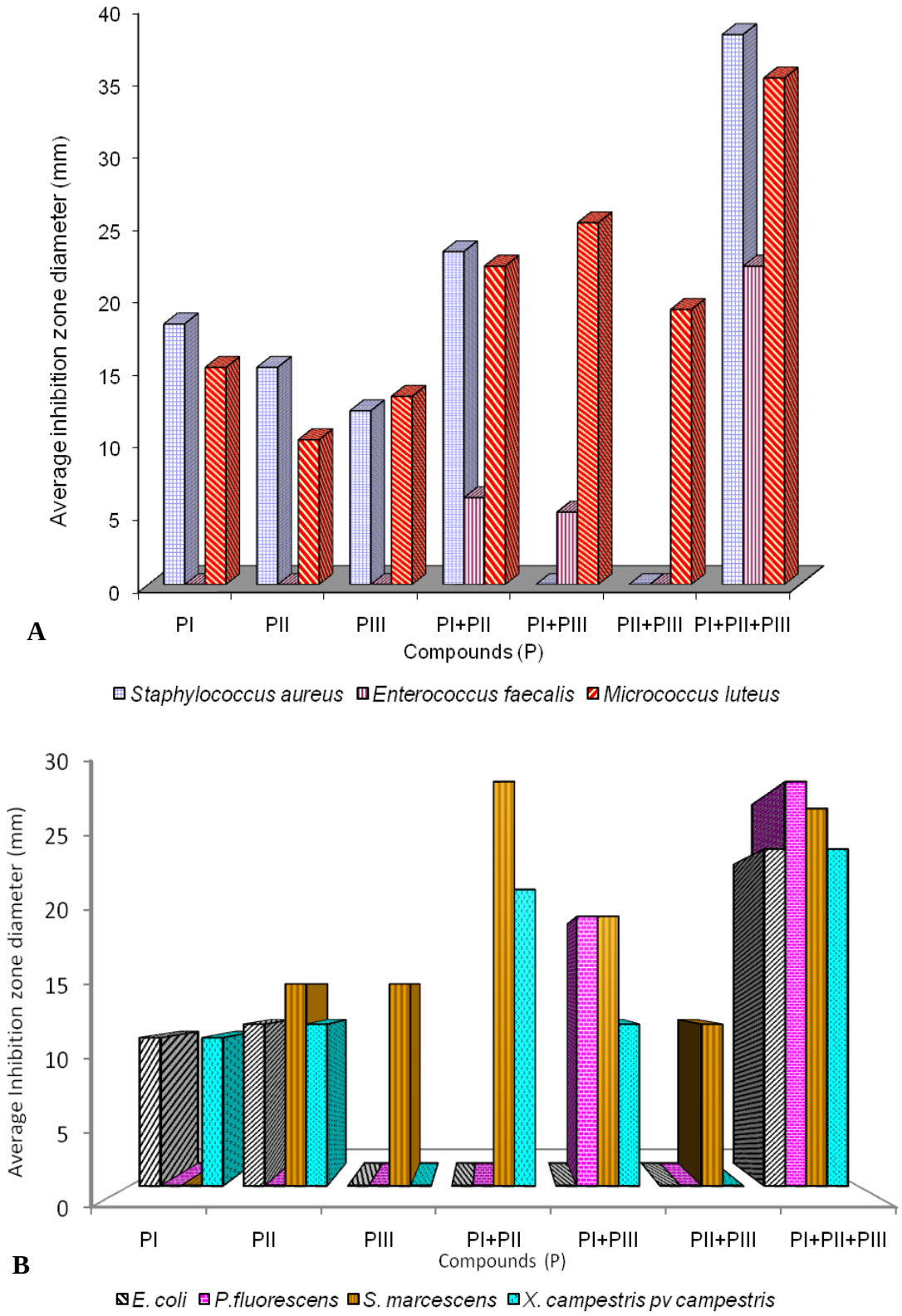

Fig. 6.3 Spectra of antibacterial activities of individual and combined peaks obtained from the antibiotic produced by *Intrasporangium* strain N8 against the test organisms: **A**, Gram-positive test organisms; **B**, Gram-negative test organisms.

As a sequel to the above analysis, all the peaks (PI +PII +PIII) were combined to check for improved efficacy against the test organisms. The peak-peak-peak mixture (PPM) showed strong growth inhibition against most of the test organisms (**Fig. 6.4**). For example, it strongly inhibited the growth of *S. marcescens* (**Fig. 6.4A)**, *S. aureus* (**Fig. 6.4B)**, *Pseudomonas fluorescens* (**Fig. 6.4C)**, and *Micrococcus luteus* (**Fig. 6.4D)** but failed to inhibit the growth of the fungal pathogen of potato, *Rhizoctonia solani* (**Fig. 6.4E)**. The diameters of inhibition zones observed were large [ranging from 28 mm for *S. marcescens* to 38 mm for *S. aureus* (**Fig. 6.4**)] compared to those produced by the individual components in the foregoing experiment.

Graphical analysis of the peaks bioassay results showed a reduction in the MIC values required to inhibit the test organisms when the peaks were combined as compared to when used individually (**Fig. 6.5**). This synergistic effect was evident from the plot of the combined peak MICs which lies below the plot of the average and single peak MIC values (**Fig. 6.5**). For example, the inhibitory effectiveness of PI (**Fig. 6.5A**) was less than the combined peak effect for most of the test organisms except for *X. campestris* pv*. campestris* where both showed good inhibitory effect with MIC,s of 0.03µg/ml and 0.002µg/ml respectively. Likewise, the inhibitory effects produced by a combination of the peaks (PI+PII+PIII) were by far better than the effect produced by PIII alone (**Fig. 6.5C**) on most of the test organisms. In some instances, the combination produced an antagonistic effect with a higher MIC value than that obtained for a single peak. For example, the MIC for *E. coli* (0.039 µg/ml) using PII (**Fig. 6.5B**) was less than that obtained for the combined peaks with an MIC of 0.031µg/ml). A graphical analysis of **s**ingle and synergic inhibition of plant and human pathogens by peak(s) produced by *Intrasporangium* strain N8 is shown in **Figure 6.5**.

The inhibitory potential of PPM was then compared to standard antibiotics on MH agar medium. The inhibition of the pathogen of *Eucomis autumnalis autumnalis* (Eaa) by PPM is similar to that shown by commercial penicillin G (**Fig. 6.6A)**. The unidentified antibiotic, V3, did not inhibit the growth of any of the test organisms except the yellow flame pathogen (Vyb) [**Fig. 6.6B**]. Both PPM and penicillin G showed no activity against strain Vyb.

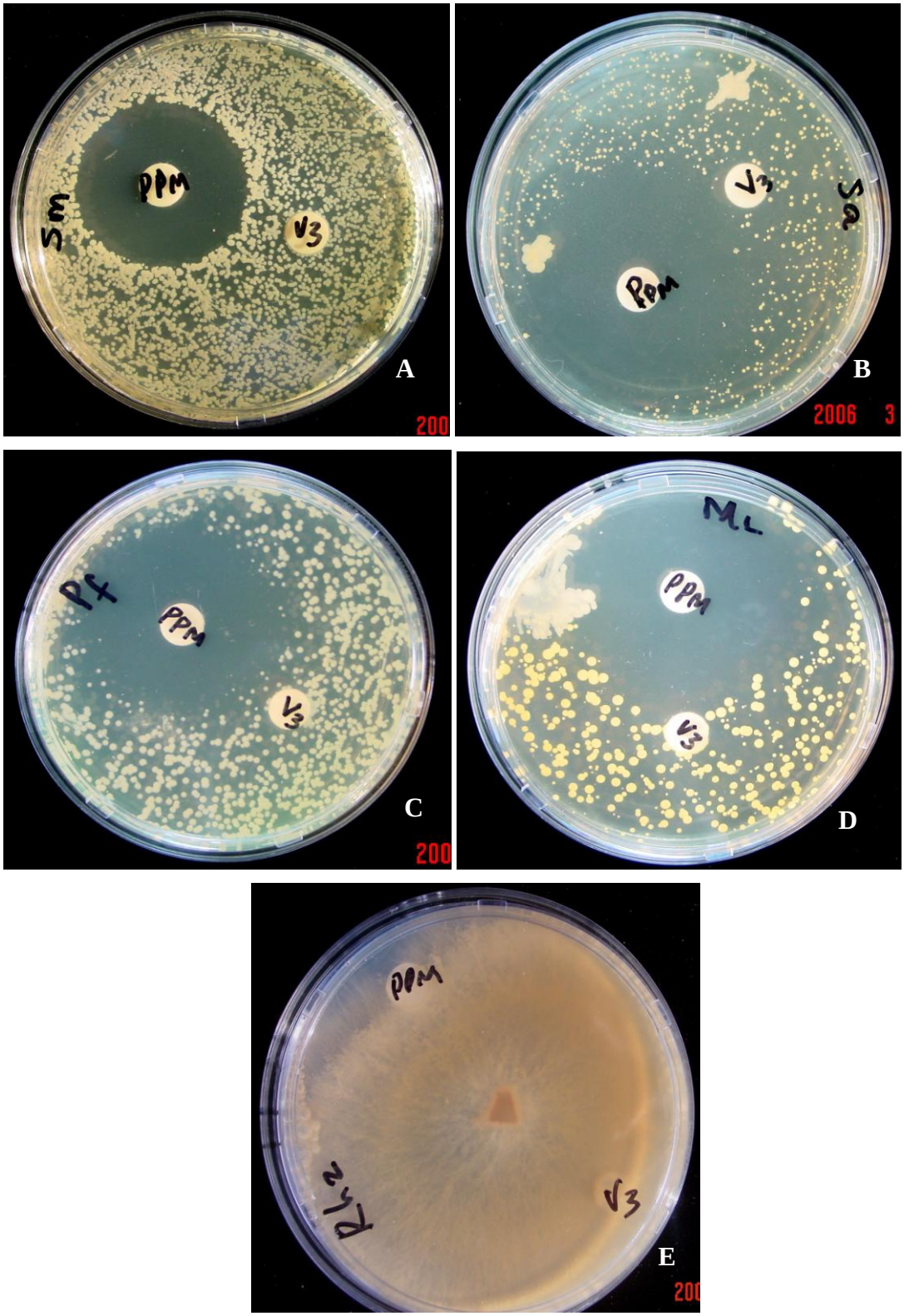

Fig. 6.4 Efficacy of PPM (PI+PII+PIII Mix): A - E = Synergic inhibition of plant and human pathogens by PPM. Test organisms include: **A**, *Serratia marcescens* (Sm); **B**, *Staphylococcus aureus* (Sa); **C**, *Pseudomonas fluorescens* (Pf); **D**, *Micrococcus luteus* (ML); **E**, *Rhizoctonia sp.* (Rhz) a fungal pathogen of potato; V3, Unidentified inhibitory substance supplied by UKZN Plant Pathology Dept.

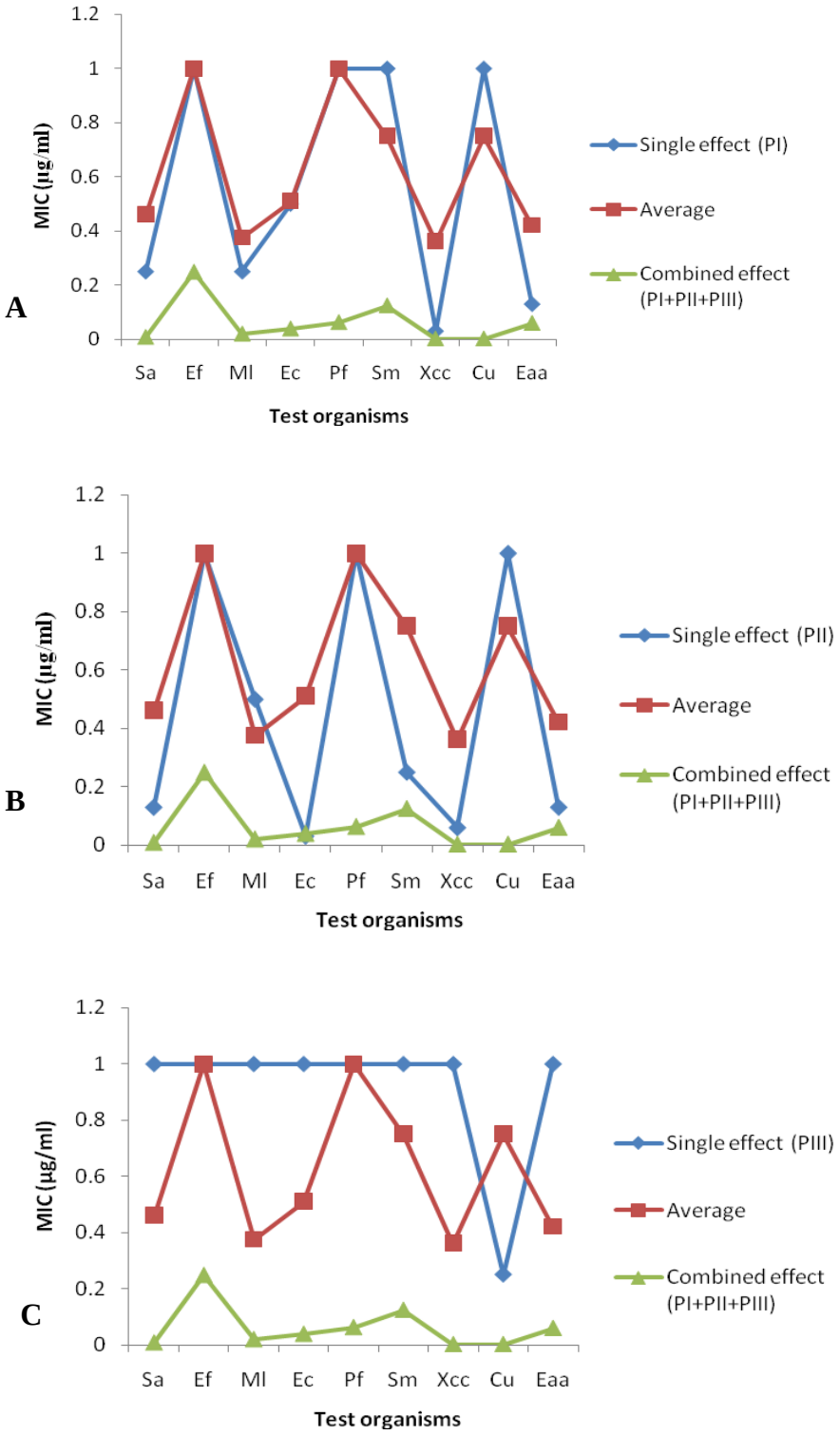

Fig. 6.5 Graphs showing single and synergic inhibition of plant and human pathogens by peak(s) produced by *Intrasporangium* strain N8: **A**, Peak 1 (PI); **B**, Peak II (PII); **C**, Peak III (PIII). Sa, *Staphylococcus aureus*; Ef, *Enterococcus faecalis*; Ml, *Micrococcus luteus*; Ec, *Escherichia coli*; Pf, *Pseudomonas fluorescens*; Sm, *Serratia marcescens*; Xcc, *Xanthomonas campestris* pv. *campestris*; Cu, *Candida utilis*; Eaa, *Eucomis autumnalis autumnalis*

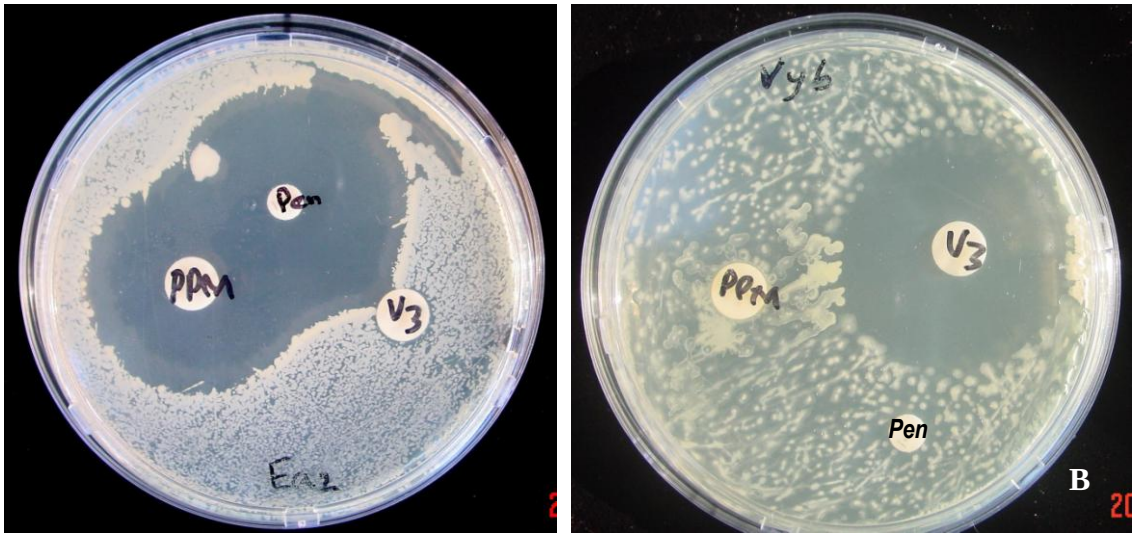

Fig. 6.6 Growth inhibitory efficacy of PPM (PI+PII+PIII Mix) compared to that of penicillin (Pen) and an unidentified antimicrobial substance (V3) supplied by UKZN Plant Pathology Department against **A**, an unidentified bacterial pathogen of *Eucomis autumnalis autumnalis* (Eaa), and **B**, an unidentified pathogen of *Veltheimia bracteata* (Vyb); Pen, Penicillin G. Note similar activities of PPM and penicillin.

MIC determinations conducted in MH broth showed that in general the mixture (PPM) had a higher inhibitory activity than the individual components (**Table 6.1).** For example, the MIC for PPM against *Staphylococcus aureus* was 0.0078µg/ml whereas the MICs for the individual components ranged from 0.13µg/ml to >1.00µg/ml. PPM generally showed a strong inhibitory activity against the Gram-positive test bacteria, the exception being *Enterococcus faecalis* which was only weakly inhibited (MIC = 0.25µg/ml). A strong inhibitory activity was also observed against the Gram-negative test bacteria *Pseudomonas fluorescens* (0.063µg/ml), *Serratia marcescens* (0.125µg/ml) and *Xanthomonas campestris* pv. *campestris* (0.0025µg/ml). The two unidentified plant pathogens were less sensitive to the individual components (PI, PII and PIII) than to the combination (PI+PII+PIII). Of note is the inhibitory spectrum of PIII which showed minimal activity against all the bacteria tested but was active against the yeast, *Candida utilis* (0.25µg/ml). None of the individual components showed any activity against *Pseudomonas fluorescens*. In the case of the fungal plant pathogen *Rhizoctonia solani*, neither the components nor the mixture showed any inhibitory action. A summary of the antimicrobial activities of the antibiotic produced by *Intrasporangium* strain N8 is given in **Table 6.1**.

6.4 Discussion

Testing for synergy often requires the use of pure compounds. To obtain such compounds usually involves many purification steps which can be a very challenging task, particularly if the sophisticated instrumentation required for the task is not available. To achieve 100% purity may not be possible, and hence less pure compounds may be used initially.

However, a brief mention of the efforts expended to obtain a pure compound for the synergy tests will give some idea of the scope of the present study. The antimicrobial agent of interest was extracted with ethyl acetate and alcohol but was not extractible with petroleum ether. This suggested that it was possibly a polar organic compound. Having established that the substance was extractible with a polar solvent, the next step involved purification since it could be a mixture of several compounds.

To this end, thin layer chromatography (TLC) was employed and revealed the presence of three bands on the chromatogram. Two identical aluminum-backed silica TLC plates overlaid with a thin layer of MH agar were sprayed separately with the test organisms *E. coli* and *S. marcescens* and observed for inhibition. These organisms were chosen because they showed susceptibility during primary screening. The bioautograms obtained were not conclusive on these plates but subsequently much better results were obtained when plastic-based silica TLC plates were used. Much effort was expended in standardizing the bioautogram procedures which were problematic since the equipment for spraying the test organisms onto the plates was difficult to quantitate as was standardisation and optimisation of the thickness of the agar overlay. Diffusion and concentration of the antibiotic compound(s) were major factors in assessing the practicality of the bioautogram technique. For example, substances separated on the chromatogram could be bound to the silica plate depending on their polarity thereby hindering the diffusion of the antibiotic into the agar growth medium.

Therefore extraction was carried out by scraping the silica off duplicate plates at positions corresponding to the bands on the initial chromatogram and eluting with ethyl acetate as described in **Section 2.13.2, Chapter 2**. At this stage, further biological activity testing was carried out using either broth micro-dilution or agar-disk diffusion methods during which large volumes of solvents and many silica plates were

consumed. The numbers of bands eluted per solvent system are shown in **Appendix 1**. Based on the bioassay results, attention was focused on the ethyl acetate extract which was further purified by HPLC. Corresponding peaks were pooled from several runs to obtain sufficient amounts of the compounds for bioassay.

The compounds used for synergy tests were separated by gradient HPLC to a high level of purity. Initially, individual fractions eluting at 1 min intervals were assayed on the test organisms. Unfortunately, the results of these bioassays were not conclusive, possibly as a result of dilution of the antibiotic compound. Corresponding fractions from several repeat runs were then pooled to increase the concentration of the putative active substances before bioassay.

The larger diameters of the inhibition zones obtained following mixing of the compounds (PPM) indicated a stronger inhibition of the test organisms, suggesting that they were more susceptible to the combination than to the individual substances. The reason may be that with the mixtures the test organisms were hit simultaneously at different sensitive targets in the cell thus enhancing the inhibitory action of the compounds. Alternatively, uptake and transport of the active compound within the cells is enhanced by the presence of the other substances. Two or more different targets inside the cell may be adversely affected and this may mean that more than one mechanism of action is involved (Yeaman and Yount, 2003; Balows, 2006).

Both PPM and penicillin G inhibited the growth of the bacterial pathogen of *Eucomis autumnalis autumnalis* (Eaa) but failed to inhibit the growth of the pathogen o*f Veltheimia bracteata* (Vyb) under standardized conditions suggesting they had similar modes of action. The target site of these two antibiotics could be the same which may or may not, be essential to the growth of the two test organisms respectively. The major substance in PPM indicated a structure related to the peptide class of antibiotics while penicillin G is a β-lactam antibiotic. Both classes, however, have similar modes of action in that they inhibit steps in peptidoglycan biosynthesis and murein assembly (Franklin and Snow, 1981; Todar, 2009).

The MIC results shown in **Table 6.1** serve as an index of the antimicrobial spectra of the components of the antibiotic produced by *Intrasporangium* strain N8. Of note is the difference in susceptibility of the test organisms to the different components (peaks)

when determined by the broth micro-dilution or agar-diffusion techniques. The reason is that the MIC determinations were done in a liquid medium without agar. Agar with its SO_3^- groups can absorb antibiotics, thus changing the conditions for diffusibility of the antibiotic, of dissolved oxygen, and of nutrients (Lancini *et al*., 1995).

It is clear from the MIC results that strain N8 produces a broad-spectrum antibiotic with inhibitory activity against prokaryotes including both Gram-positive and Gram-negative bacteria and against the eukaryotic yeast *Candida utilis*. The antifungal activity of the antibiotic could not be confirmed conclusively as it failed to show significant inhibitory activity against the fungal pathogen, *Rhizoctonia solani*. A further test on additional fungal pathogens is required to confirm its fungicidal capabilities.

To date, information on the genus *Intrasporangium* is sparse, and to the author's knowledge, the production of antibiotics by species of *Intrasporangium* has not been reported before.

6.5 Conclusion

The synergistic inhibition of *S. marcescens* by a combination of PI and PII, together with the results obtained with different combinations of P1, PII and PIII on various test organisms, especially *Pseudomonas fluorescens* and *Xanthomonas campestris* pv. *campestris*, showed that it was preferable to use combinations of the isolated compounds rather than any of the individual components singly for treatment of these serious agents of infectious plant diseases. Single antibiotic administration has been used for many years in the initial treatment of infectious diseases; a strategy that too often resulted in inducing resistance to the antibiotic in some strains. According to Fox (2007), development of resistance easily outpaces the appearance of new antimicrobial drugs on the market and, therefore, it is time to change our strategy for treatment of infectious diseases by using either appropriate combinations of the available antibiotics or novel compounds to counteract this life-threatening problem.

CHAPTER 7

CONCLUSIONS

Most of the objectives set out at the beginning of this study have to some extent been successfully met.

Initially, there was a strong belief that the microbiota inhabiting South African soils has the potential to produce new antibiotics judging from the successes achieved by Baecker and Ryan in 1987 and Wang *et al.* in 2006. Both reported that novel antibiotic-producing soil-inhabiting, actinomycetes had previously been discovered in Natal habitats, viz. pimaricin and platencin respectively.

It seemed reasonable to suppose that other genera and species producing new antimicrobial substances could be discovered. The literature showed that much work has been done by many scientists on actinomycetes, especially *Streptomyces* species, so the present investigation was designed to exclude *Streptomyces spp*. and target instead rare actinomycetes, also known as rare actinos. Thus a search for non-streptomycetous, antibiotic-producing actinomycetes was undertaken in KwaZulu-Natal soils from areas never investigated before. The media used by earlier scientists like Waksman (Waksman *et al.,* 1942) and Lechevalier (Lechevalier, 1989) to isolate actinomycetes were modified, based on current knowledge. These adaptations made the media more selective for the target organisms. The geographical distribution and antimicrobial activity of the organisms isolated is discussed in **Chapter 3**.

Ten isolates showing antimicrobial activity against two or more of the seven test organisms used were studied further. The test organisms were selected on the basis of a 1999 survey of the most common animal and plant bacterial pathogens in KwaZulu-Natal hospitals and the University vegetable gardens respectively. All ten of the producer organisms were tentatively identified to genus level.

Of these ten organisms, one isolate stood out from the rest, viz. N8. It showed activity against all seven test organisms especially *Pseudomonas fluorescens* and *Xanthomonas campestris* pv. *campestris* which are notoriously difficult to contain in the

field. The results of this aspect of the investigation have been published in a peer reviewed journal.

Because of these useful attributes further studies on N8 were undertaken. The first step was to identify the organism using standard bacteriological methods. The organism was identified as an *Intrasporangium* species and given the appellation *Intrasporangium* strain N8.

Next, an attempt was made to isolate and purify the antibiotic produced by N8. These processes were successfully carried out using solvent-to-solvent extraction procedures to separate the antibiotic from the culture broth followed by various chromatographic techniques. As these procedures were time consuming, required large volumes of solvents, and resulted in low antibiotic yields, they were replaced with a process entailing a combination of a three-solvent system and pH precipitation.

Using this procedure, large enough quantities of the antibiotic were recovered from the fermentation broth to permit a degree of structural elucidation.

The chemical nature of the antibiotic was successfully established by means of ^{1}H- and nuclear magnetic resonance (NMR), liquid chromatography – mass spectrometry (LCMS) and gas chromatography – mass spectrometry (GCMS). The empirical formula was determined to be $C_{16}H_{36}N_2O_3$. At this stage, it became clear that a full structural elucidation of the compound(s) showing antimicrobial activity was a project on its own and thus beyond the scope of this thesis which is largely microbiological.

For microbiologists, the chemical structure of antimicrobial compounds is of interest since it may indicate a possible mode of action of the substance. However, their biological activity and efficacy as growth inhibitors is more important. In chapter six, the inhibitory potential of the components of the antibiotic produced by strain N8 was examined. Data from the bioassay results demonstrated that the use of combinations of the components producing the individual peaks on the trace were significantly more effective than any of the single components when used alone, thereby providing evidence of synergistic action. For example, the diameters of the inhibition-zones were doubled when a combination of the components PI and PII was used against both *S. marcescens* and *Pseudomonas fluorescens*.

Genetic engineering is also recommended to improve the yield of the antibiotic produced by N8. The toxicity of the antibiotic produced by N8 should also be studied to establish its clinical significance. An extension of this study can be carried out on the use of N8 in agriculture by testing its efficacy in the field on a broader range of fungal and bacterial plant pathogens. This is important since the current trend is towards biocontrol using natural substances rather than synthetic chemicals to treat plant diseases.

In conclusion, the initial objective of targeting rare actinomycetes was successfully met, leading to the isolation of *Intrasporangium* strain N8 and characterization of the antibiotic produced by the organism which has not been reported previously. However, further investigations mainly of a chemical nature and outside the scope of this thesis should be carried out to determine if the antibiotic produced by N8 is indeed novel.

Hopefully, the results of this study will encourage renewed efforts in the search for novel antibiotics in South African soils. Owing to its spectrum of activity the antibiotic produced by N8 could, if found suitable for commercialization, impact on the pharmaceutical and plant protection industries for the treatment of both human/animal or plant infectious diseases, especially in sub-Saharan Africa.

The following recommendations are made through the experiences gained during this investigation:

- Obtaining unknown antibiotic compounds in pure form is laborious and time-consuming. Careful consideration regarding the order in which chemical analyses are done is therefore essential. Investigators interested in the structure of the compound under study should begin with chemical analyses that do not consume the purified substance, i.e. NMR, LC-MS, before proceeding to bioassays and GC-MS analysis. This will eliminate the problem of running out of purified sample early in the investigation and will also save the time and energy expended on repeated fermentations and isolation/purification procedures.

- Improvement of strain N8 by genetic engineering is recommended to produce new strains with increased antibiotic production capacities.

- The toxicity of the antibiotic produced by N8 should also be investigated to establish its clinical significance.

- The usefulness of N8 in agriculture should be assessed by testing its efficacy in the field on a broader range of fungal and bacterial plant pathogens.

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APPENDICES

Appendix 1 Average R_f values* of bands on TLC chromatograms from solvent extracts of strain N8

Extract source	No. of bands per solvent system (SS)^		Total no. of bands	R_f values
Methanol [MF]			12	
	SS1	3		0.45; 0.55; 0.64
	SS2	4		0.03; 0.22; 0.46; 0.76
	SS3	5		0.05; 0.11; 0.23; 0.29; 0.77
Acetone [AF]			5	
	SS1	3		0.08; 0.39; 0.67
	SS2	2		0.23; 0.64
Ethyl acetate			11	
[EAF]	SS1	4		0.08; 0.64; 0.70; 0.79
	SS2	4		0.22; 0.27; 0.56; 0.64
	SS5	3		0.03; 0.11; 0.35
N-hexane			1	
(NHF)	SS1	1		0.85
	Grand Total		**29**	

*average values from triplicate runs of samples on TLC chromatograms (R_f values calculated from distance traveled by spot divided by distance traveled by solvent). ^SS1= methanol: ethyl acetate (80:20); SS2= ethyl acetate: methanol (85:15); SS3= ethyl acetate: diethyl ether (80:20); SS4= dichloromethane (100%); SS5= n-hexane: ethyl acetate (20:80).

Appendix 2 Line list for ^{1}H-NMR of antimicrobial compound produced by strain N8

Peak	v(F1) [ppm]	v(F1) [Hz]	Intensity [abs]
1	8.5530	4276.5856	617424.00
2	7.7256	3862.8773	2615280.00
3	7.7195	3859.8272	2920848.00
4	7.7140	3857.0772	3120000.00
5	7.6242	3812.1763	3657600.00
6	7.6176	3808.8762	3332864.00
7	7.6130	3806.5762	2944880.00
8	7.6064	3803.2761	2488896.00
9	7.3921	3696.1240	1255584.00
10	7.3768	3688.4738	1382528.00
11	7.3094	3654.7731	1858288.00
12	7.3017	3650.9231	2650672.00
13	7.2964	3648.2730	3192128.00
14	7.2166	3608.3722	1734144.00
15	7.1962	3598.1720	1542768.00
16	7.1888	3594.4719	1545216.00
17	7.1818	3590.9719	1349392.00
18	6.9861	3493.1199	1039728.00
19	6.9693	3484.7197	1160976.00
20	6.7288	3364.4673	1184800.00
21	6.7116	3355.8672	1231872.00
22	5.6147	2807.4062	1323712.00
23	5.5995	2799.8060	1285824.00
24	4.5292	2264.6453	6354864.00
25	4.3232	2161.6433	1867344.00
26	4.2870	2143.5429	2715088.00
27	4.2736	2136.8428	3208640.00
28	4.2605	2130.2926	1831264.00
29	4.2344	2117.2424	1531040.00
30	4.2249	2112.4923	1740384.00
31	4.2154	2107.7422	2002848.00
32	4.1996	2099.8420	1584736.00
33	3.6939	1846.9870	1527536.00
34	3.6844	1842.2369	1976000.00
35	3.6304	1815.2363	3397632.00
36	3.0124	1506.2301	1220016.00
37	3.0027	1501.3800	1368480.00
38	2.9849	1492.4799	1182240.00
39	2.9753	1487.6798	1098288.00
40	2.3337	1166.8734	1350240.00
41	2.0258	1012.9203	1451424.00
42	1.9202	960.1192	2120080.00
43	1.8989	949.4690	1890480.00
44	1.8502	925.1185	4549408.00
45	1.8478	923.9185	4507072.00
46	1.4085	704.2641	4245680.00
47	1.2917	645.8629	26360880.00
48	1.0021	501.0600	5540256.00
49	0.9933	496.6599	4279920.00
50	0.9890	494.5099	6602080.00
51	0.9724	486.2097	6010336.00
52	0.9645	482.2597	6204576.00
53	0.9593	479.6596	6823328.00
54	0.9513	475.6595	7959616.00
55	0.9149	457.4592	6672464.00
56	0.9004	450.2090	12109472.00
57	0.8893	444.6589	14884432.00
58	0.8793	439.6588	12780336.00
59	0.8751	437.5588	12485024.00
60	0.8610	430.5086	6911680.00
61	0.8556	427.8086	6954768.00

Appendix 3: The absorbance values of strain N8 during growth in Nutrient broth (λ = 550 nm)

Time (hours)	Absorbance	Log (Absorbance)
0.0	0.099	-1.004
48.0	0.225	-0.648
48.5	0.241	-0.618
49.0	0.249	-0.604
49.5	0.276	-0.559
50.0	0.350	-0.456
50.5	0.438	-0.359
51.0	0.477	-0.322
51.5	0.505	-0.297
52.0	0.528	-0.277
52.5	0.503	-0.298
53.0	0.495	-0.305
53.5	0.549	-0.260
54.0	0.567	-0.246
54.5	0.609	-0.215
55.0	0.606	-0.218
55.5	0.652	-0.186
56.0	0.685	-0.164
56.5	0.746	-0.127
57.0	0.879	-0.056
57.5	0.944	-0.025
58.0	0.932	-0.031
58.5	0.925	-0.034
59.0	0941	-0.026
59.5	0.964	-0.016
60.0	0.904	-0.044