An Investigation of the Biosynthesis of Carbapenem-Beta-Lactams by

Streptomycetes cattleya

A Thesis submitted to the University of Surrey,
as part of the requirement for the degree of
Master of Philosophy

By

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To Philip
and
my Parents
ABSTRACT

This thesis is divided into five chapters. Chapter one provides a brief outline of the history of β-lactams. New developments in screening methodology and current understanding of the mode of action of β-lactams are presented. A section on the biosynthesis of β-lactams is included.

Our aim in this project was to develop a system for investigation of the biosynthetic pathway leading to Thienamycin, the first representative of a unique class of β-lactams - the carbapenems. It was recognised that due to the intrinsic instability of Thienamycin and non availability of a high yielding strain, that the essential preliminaries of development of a fermentation and convenient assay techniques would form a major part of the effort.

Chapter two describes the fermentation studies carried out with Streptomyces cattleya and includes brief coverage of regulatory controls that may influence antibiotic production in Streptomyces. Possible causes of the significant degree of variability in β-lactam production, observed in S. cattleya fermentation are also presented.

Chapter three covers the development of analytical techniques used in the detection and identification of Thienamycin and co-produced β-lactams. An attempt was made to discriminate between co-produced carbapenems on the basis of their structure-activity relationship. The major β-lactam product of fermentation was identified as Thienamycin.

The experimental section is to be found in chapter four.
Chapter five reviews the results of our studies with *S. cattleya*, the main emphasis of the work being directed to optimising the fermentation and assay systems. Current understanding of the biosynthesis of carbapenems in *Streptomyces* sp. with particular attention to production of Thienamycin by *S. cattleya*, together with recent developments in the field of biosynthesis, such as protoplast and genetic engineering techniques are also discussed.
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### ABBREVIATIONS

<table>
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<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>Ac</td>
<td>acetyl</td>
</tr>
<tr>
<td>7ACA</td>
<td>7 aminocephalosporanic acid</td>
</tr>
<tr>
<td>6APA</td>
<td>6 aminopenicillanic acid</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>c.f.u.</td>
<td>colony forming units</td>
</tr>
<tr>
<td>CoA</td>
<td>coenzyme A</td>
</tr>
<tr>
<td>DHP</td>
<td>Dipeptidase</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethysulphoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>Et</td>
<td>ethyl</td>
</tr>
<tr>
<td>Gram positive</td>
<td>classification of microorganisms, according to uptake of dye stain in a test devised by German Physician H. E. Gram</td>
</tr>
<tr>
<td>Gram negative</td>
<td>to uptake of dye stain in a test devised by German Physician H. E. Gram</td>
</tr>
<tr>
<td>GS</td>
<td>glutamine synthetase</td>
</tr>
<tr>
<td>g</td>
<td>gram</td>
</tr>
<tr>
<td>H.P.L.C.</td>
<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>I.D.</td>
<td>internal diameter</td>
</tr>
<tr>
<td>I.P.N.S.</td>
<td>isopenicillin N synthetase</td>
</tr>
<tr>
<td>L</td>
<td>litre</td>
</tr>
<tr>
<td>LLD-ACV</td>
<td>Arnstein tripeptide</td>
</tr>
<tr>
<td>M</td>
<td>Molar</td>
</tr>
<tr>
<td>m</td>
<td>milli</td>
</tr>
<tr>
<td>μ</td>
<td>micro</td>
</tr>
<tr>
<td>MIC</td>
<td>minimum inhibitory concentration</td>
</tr>
<tr>
<td>M.R.S.A.</td>
<td>methicillin resistant <em>S. aureus</em></td>
</tr>
<tr>
<td>n</td>
<td>nano</td>
</tr>
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</table>

(2)
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>NFT</td>
<td>N-Formimidoyl thienamycin</td>
</tr>
<tr>
<td>OR</td>
<td>origin</td>
</tr>
<tr>
<td>P.B.P.</td>
<td>penicillin binding protein</td>
</tr>
<tr>
<td>Ph</td>
<td>phenyl</td>
</tr>
<tr>
<td>$R_f$</td>
<td>relative mobility</td>
</tr>
<tr>
<td>rpm</td>
<td>revolutions per minute</td>
</tr>
<tr>
<td>RP</td>
<td>reverse phase</td>
</tr>
<tr>
<td>$R_t$</td>
<td>retention time</td>
</tr>
<tr>
<td>SF</td>
<td>solvent front</td>
</tr>
<tr>
<td>TCA</td>
<td>tricarboxylic acid cycle</td>
</tr>
<tr>
<td>TLC</td>
<td>thin layer chromatography</td>
</tr>
<tr>
<td>T</td>
<td>Thienamycin</td>
</tr>
<tr>
<td>TM</td>
<td>trademark</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>&gt;</td>
<td>greater than</td>
</tr>
<tr>
<td>&lt;</td>
<td>less than</td>
</tr>
<tr>
<td>≡</td>
<td>equivalent to</td>
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NOTE

The carbapenem nucleus is numbered as shown below throughout this thesis.
CHAPTER ONE

INTRODUCTION
1.1 INTRODUCTION

Chapter one provides a brief outline of the history of β-lactams. New developments in screening methodology, and current understanding of the mode of action of β-lactams are presented. A section on the biosynthesis of β-lactams includes, recent developments in the field of classical β-lactams, and initial studies for the non-classical groups. Our aims and objectives are set out in a final section.

Naturally occuring β-lactam structures have generally been defined and classified using trivial nomenclature. More recently a system based on a defined parent β-lactam skeleton (or 'nucleus') has been available. Penicillins and cephalosporins being derived from penam and cephem nuclei respectively, while other β-lactams are based on clavam, carbapenem, penem, oxacephem, and monobactam ring systems, see Figure (1.1). Predominantly trivial nomenclature will be used throughout this thesis due to the cumbersome nature of wholly systematic nomenclature for β-Lactam structures. Penicillins, cephalosporins and cephamycins are referred to as classical β-lactams, and the newer groups as nonclassical β-lactams.

Figure (1.2) gives the structures for β-lactam antibiotics referred to in the text, section (1.2). (See pages 8-11).

Figure (1.3) lists structures for the carbapenem class of β-lactam (not exhaustive). (See pages 12-16).
FIGURE (1.1) β-LACTAM SKELETONS

Penam

Cephem

Clavam

Carbapenem

Penem

Oxacephem

Monobactam

Clavem
FIGURE (1.2) \( \beta \)-LACTAM STRUCTURES

(A)  ' Penicillin '  

Penicillin G  Ph.CH\(_2\)–

Penicillin V  Ph.OCH\(_2\)–

Penicillin N  

Ampicillin  Ph.CH–NH\(_2\)–

Methicillin

Carbenicillin  Ph.CH–CO\(_2\)H

Piperacillin  EtN–CO.NH.CH–Ph
Azlocillin

6-Aminopenicillanic acid (6-APA)

(B) 'Cephalosporin'

Cephalosporin C

Cephalothin

Cephalexin

Cefsulodin

Cefuroxime
Cefoperazone

7-Aminocephalosporanic acid (7-ACA)

'Cephemycin'
(7-α-Methoxy cephalosporin)

Cefoxitin

Moxalactam

* Stereochemistry not readily available

(10)
Nocardicin $A^{(4,5)}$

Clavulanic acid $^6$

Monobactam $^7,8$

Sulfazecin $^7,8$

$\beta$-Lactone $^9$

Obafluorin $^9$

References: (1,2,3) and as indicated (11)
FIGURE 1.3  CARBAPENEM STRUCTURES

<table>
<thead>
<tr>
<th>Name</th>
<th>R</th>
<th>R¹</th>
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<tr>
<td>Thienamycin (10,11)</td>
<td>OH</td>
<td>-NH₂</td>
</tr>
<tr>
<td></td>
<td>H-</td>
<td>-NH²</td>
</tr>
<tr>
<td></td>
<td>H₃C</td>
<td>-NHAc</td>
</tr>
<tr>
<td>N-Acetylthienamycin (12,13)</td>
<td>OH</td>
<td>-NH₂</td>
</tr>
<tr>
<td></td>
<td>H-</td>
<td>-NH²</td>
</tr>
<tr>
<td></td>
<td>H₃C</td>
<td>-NHAc</td>
</tr>
<tr>
<td>NS-5 (14,15)</td>
<td>H₃C</td>
<td>-NH₂</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-NHAc</td>
</tr>
<tr>
<td>PS-5 (14,16)</td>
<td>H₃C</td>
<td>-NH₂</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-NHAc</td>
</tr>
<tr>
<td>PS-6 (17)</td>
<td>H₃C</td>
<td>-NH₂</td>
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<td></td>
<td></td>
<td>-NHAc</td>
</tr>
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<td>PS-7 (17)</td>
<td>H₃C</td>
<td>-NH₂</td>
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<tr>
<td></td>
<td></td>
<td>-NHAc</td>
</tr>
<tr>
<td>OA-6129B₂ (18,19)</td>
<td>OH</td>
<td>-NH₂</td>
</tr>
<tr>
<td></td>
<td>H₃C</td>
<td>-NHAc</td>
</tr>
</tbody>
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(12)
Epithienamycin C
MM22381, PS-3A

Epithienamycin D
MM22383, PS-4A
| **Epithienamycin A**  
| MM22380, PS-3B  
|  
| **Epithienamycin B**  
| MM22382, PS-4B  
|  
| **Epithienamycin E**  
| MM13902, PS-E₂  
|  
| **Epithienamycin F**  
| MM17880, PS-E₁  
|  
| **OA6129A**  
|  
| **OA6129B₁**  
|  
| **OA6128C**  
| |
Carpetimycin A
C-19393 H₂

Carpetimycin B
C-19393 S₂

Carpetimycin C

Carpetimycin D
C-19393 E₅

Pluramycin A
SF 2103 A

Pluramycin B

Pluramycin C
Asparenomycin A
PA-31088 IV

Asparenomycin B
PA-39504 x₁

Asparenomycin C
PA-39504 x₂

6643-x (31)

PS-8 (32)

Carbapenam

OA-6129 D (33)

OA 6129 E
1.2 β-LACTAM ANTIBIOTICS - A SHORT HISTORY

1.2.1 The Advent of Penicillin

More than half a century has elapsed since Alexander Fleming discovered penicillin while working at St. Mary's Hospital, London, (34). Three years after his initial discovery Raistrick and co-workers published a study of penicillin which gave details of the solvent extraction of the antibiotic and also noted its instability, (35). Subsequently Howard Florey, Ernst B. Chain, and their colleagues at Oxford University demonstrated the potential of penicillin as a therapeutic agent, (36), Table (1.1). The chemotherapy of bacterial infections was to change drastically as further developments in this field gave rise to a large (and ever increasing) family of antibiotics. It is not possible in this brief review, to give credit to the many great scientists who have made valuable contributions in this area, more comprehensive coverage is available elsewhere, (36,37,38,39).

Penicillin (Pen. G) first became available for clinical use in the early 1940's, (40), initially many common infections yielded to therapy. By 1945 the β-lactam structure proposed for penicillin by Abraham, Baker and Chain, had been confirmed by X-ray crystallographic analysis, (38). Also it had been demonstrated that penicillins with different non-polar side chains could be derived by the addition of the appropriate monosubstituted acetic acid to Penicillium notatum fermentations. Discovery of the relative acid stability of Phenoxymethylpenicillin (Pen. V) in 1954, provided the first penicillin suitable for oral administration, (38).

(17)
TABLE (1.1)

SUMMARY OF SOME OF THE ACHIEVEMENTS OF H. FLOREY, E. B. CHAIN
AND CO-WORKERS, IN THE DEVELOPMENT OF PENICILLIN AS A
THERAPEUTIC AGENT, (36)

1) Cultivation of Penicillin notatum, isolation and
concentration of penicillin by solvent extraction
and freeze drying.

2) Determination of the range of sensitive bacteria,
found that penicillin was active in the presence
of body fluids.

3) Established penicillin as a systematic chemo-
therapeutic agent using animal experiments.

4) Accumulated sufficient penicillin to administer to
first patents in various Oxford hospitals.

5) Made the first important steps in the elucidation
of the chemical structure of penicillin.

6) Discovered an enzyme ( "penicillinase" ) as one
of the main causes of instability of penicillin.
The clinical limitations of the early penicillins had become apparent by the mid 1950's. Antibacterial activity was primarily directed at Gram positive cocci, in addition the incidence of penicillin resistant infections was now significant. Strains of Staphylococci capable of producing an enzyme ('penicillinase') which hydrolysed the β-Lactam ring of penicillin, were frequently isolated. However interesting new developments were to ensure that the penicillin story continued.

1.2.2 Semisynthetic Penicillins and Cephalosporins

1.2.2.1 Semisynthetic Penicillins

An event which was to change the course of β-lactam research, occurred at Beecham Laboratories in 1956. Investigations revealed that the parent substance of penicillin, 6-aminopenicillanic acid (6APA) could be obtained from certain fermentations (an observation that had been made in Japan some years earlier), (38). Later when 6APA became available in bulk quantities, by enzymic or chemical hydrolysis of the natural penicillin, the potential of derivatives prepared by chemical acylation of the nucleus was explored, (41).

During this period attempts were made to solve a number of problems by modification of the penicillin 'nucleus'. Firstly stabilisation of the molecule to staphylococcal penicillinase, secondly expansion of the antibacterial spectrum to include Gram negatives, and thirdly development of acid stable orally absorbable penicillin derivatives, (39).

A vast range of semisynthetic penicillins were now possible. The development of Methicillin, a penicillin stable
to staphylococcal penicillinase, effectively solved the problem of penicillin resistant staphylococcal infections. In 1961 Ampicillin became the first orally administered broad spectrum penicillin. Ampicillin, although susceptible to the staphylococcal penicillinase, was found to be considerably more active than Benzylpenicillin (Pen. G) against many Gram negative organisms.

1.2.2.2 Semisynthetic Cephalosporins

Natural occurrence of a second class of β-lactam, the cephalosporins, was first recognised in 1955. Cephalosporin C was detected as a trace contaminant during isolation and purification of Penicillin N from the fungus Cephalosporium acremonium. (42) Discovery of a chemical method for the removal of the α-aminoadipyl side chain of Cephalosporin C, by workers at Lilly in 1962, and the production of 7-amino cephalosporanic acid (7ACA) in quantity, paved the way for exploration of the potentialities of the cephalosporin ring system, (38).

Production of semisynthetic cephalosporins was accomplished by acylation of the amino group and modification of the C-3 substituent of 7ACA. Cephalosporins generated considerable clinical interest, due to their greater intrinsic resistance to enzymic degradation, and broader spectrum of antimicrobial activity, (43). Cephalothin became available in the early 1960's, as the first semisynthetic cephalosporin. Cephalothin satisfied two goals in β-lactam research, being very resistant to staphylococcal penicillinase, and demonstrating activity against common Gram negative infections, (39).
Cephalexin provided the first semisynthetic cephalosporin suitable for oral administration.

By the late 1960's, changing patterns of infection and the increasing frequency of isolation of opportunistic pathogens, focussed attention on the growing problem of resistance in Gram negative bacteria. An important factor was the ability of many resistant organisms to produce a cell bound β-lactamase, \(^{(38)}\). It was recognised that many β-lactamases with different substrate profiles existed, and while some were chromosomally determined others were mediated by plasmids which could readily be transferred from one organism to another. Additionally the incidence of infections caused by bacterial species, such as Pseudomonas, Serratia and Enterobacter, not amenable to β-lactam antibiotics was now significant.

Research efforts eventually achieved success with the development of new antipseudomonal penicillins and cephalosporins, new types of β-lactamase resistant cephalosporin, and non-classical β-lactam antibiotics.

1.2.3 Expanded Spectrum and Antipseudomonal Penicillins and Cephalosporins

Substitution of the α-amino group of Ampicillin with a carboxyl group, resulted in a penicillin with wide ranging antibacterial activity, that included some strains of Ps. aeruginosa. Carbenicillin (α-carboxypenicillin) was introduced in 1967 as the first parenteral antipseudomonal penicillin. Many similar α-acidic group substitutions were performed, and resulted for example in Cefsulodin in the cephalosporin series.
Modification of the α-amino group of the penicillin or cephalosporin molecule, by acylation with, aliphatic, aromatic or heteroaromatic carboxylic acids gave derivatives with expanded spectrum and especially increased activity against Ps. aeruginosa. This approach led to the development of β-Lactam antibiotics such as Azlocillin, Piperacillin, and Cefoperazone, (39).

1.2.4. New Cephalosporins

Since 1970 new types of cephalosporin have appeared, which show increased stability to a number of β-lactamase, when compared with their predecessors, (39).

The cephamycins (7α-methoxycephalosporins) were discovered as products of Streptomyces, by Nagarajan and co-workers in 1971, (44). The 7α-methoxy substituent was found to confer resistance against β-lactam hydrolysis by β-lactamase. There resulted a number of synthetic cephamycin derivatives, prepared by chemical methoxylation of the β-lactam nucleus. Cefoxitin became clinically available in 1972 and demonstrated good activity against Gram negatives.

In addition a number of cephalosporin derivatives, without the 7α-methoxy substituent demonstrated considerable stability to β-lactamase. A common structural feature was the presence of a 7α-methoxyimino side chain, consequently this group of derivatives became known as the oxime cephalosporins, examples are Cefuroxime, and Cefotaxime.

1.2.5 Non-Classical β-Lactams

1.2.5.1 Chemical Modification of Penicillin and Cephalosporin Nuclei
During the early 1970's Streptomyces species capable of producing β-lactam antibiotics were discovered, (44,45,46). In addition to classical β-lactam antibiotics, naturally occurring compounds with novel β-lactam nuclei were isolated (see section 1.2.5.2). This discovery, together with the clinical success of the semisynthetic cephamycin derivatives may have prompted the re-examination of the concept of nuclear modification that occurred in the late 1970's (47,48, 49,50).

Moxalactam (lately known as Latamoxef) was developed for clinical use in 1978. An oxacephem derivative, chemically transformed from penicillin, with a 7α-methoxy substituent, it was shown to be more active than the cephalosporin analogue and also stable to a wide range of β-lactamase types, (3).

1.2.5.2 Novel β-Lactam Compounds

Further screening of Streptomyces sp., following discovery of the cephamycins in 1971, (44,45,46), has revealed many new and potentially useful compounds, with novel β-lactam structures. Section 1.2.6 deals with the changes in screening procedure that have brought this about. Discoveries include, Clavulanic acid, (51), whose structure is based on the clavam ' nucleus ', Olivanic acids/Epithienamycins, (52,53) together with other β-lactams of the carbapenem class, (16,25) including the highly active β-lactamase resistant carbapenem Thienamycin, (10), and a family of monocyclic β-lactams, the Nocardicins, (4).

A recent development was the discovery of β-lactam compounds produced by bacteria, (54). Independantly workers in Japan and workers at Squibb discovered a new class of
β-lactam antibiotic, termed the monobactams, produced by strains of Gram negative bacteria, (8,55). Sulfazecin, the most commonly found structure was initially isolated from Pseudomonas acidophila, (8). The monocyclic nature of this class of β-lactam changed considerably the definition of structural parameters required for antimicrobial activity.

In addition to the monobactam series, (56,57), bacteria have been shown to produce, a simple carbapenem nucleus, (58), a number of cephalosporins, (59,60), and a group of β-lactam related compounds the β-lactones, (9,61). New β-lactam structures continue to be discovered, (62).

1.2.6. Developments in Screening Techniques for β-Lactam Antibiotics

The abundant discovery of new and novel β-lactam containing molecules in recent years may be attributed to several factors. Significant among these was the renewed enthusiasm on the part of researchers, following discovery of cephamycins as products of Streptomyces, (44,45,46). Also introduction of new isolation techniques, and novel 'mode of action' screens, directed toward the discovery of antibiotic producing organisms have played an important role, (63).

A variety of approaches in screening methology have been adopted, (63,64). β-Lactam antibiotics can be detected in screens which look for inhibitors of peptidoglycan synthesis, (63). The cephamycins were detected using a screen developed to look for agents producing morphological changes in sensitive bacteria, (44). Thienamycin, and subsequently the Epithienamycins, were discovered in the course of screening
soil microorganisms for production of inhibitors of peptidoglycan synthesis in Gram positive and Gram negative bacteria, (10,52,65), the precise details of the method used have not been published.

The observation that β-lactam antibiotics could act as β-lactamase inhibitors prompted the screening of microorganisms for naturally occurring inhibitors of this enzyme. Clavulanic acid, (66), and the Olivanic acids, (53,65), together with other β-lactams of the carbapenem class, (67,68), were discovered using this strategy.

β-Lactam supersensitive bacterial mutants have been used very successfully in directed screening procedures. A series of monocyclic β-lactams, the Nocardicins were discovered using a supersensitive mutant of Escherichia coli, (4). Kitano et al reported the production of penicillins and cephalosporins by fungal strains not previously associated with the production of these compounds, using a supersensitive mutant of Ps. aeruginosa, (69). Bacterial mutants have also been used to detect novel β-lactam antibiotics of bacterial origin, (8).

One of the latest developments in this field was the use of β-lactamase induction screens, (64). β-Lactam antibiotics were found to induce β-lactamase production in certain strains of Gram positive and Gram negative bacteria, (64,70). A high degree of specificity was demonstrated for β-lactam containing molecules using this methodology, false positives were obtained with a group of related compounds, the β-lactones, (64).

Introduction of novel strategies for the detection of β-lactam containing molecules has led to the discovery of
new β-lactam antibiotics, novel structural classes, and to the detection of antibiotic production in microorganisms not previously associated with the synthesis of these compounds. Success in the future will depend on the sensitivity and specificity of the screening procedures available. New developments continue to be reported, including the use of isolated enzymes of peptidoglycan synthesis, \(^{(71)}\), and screens to detect the triggering of autolytic enzymes, \(^{(72)}\).

1.2.7 Future Prospects

Further growth in the field of β-lactam antibiotics seems assured, due to their outstanding clinical value, coupled with limitations in the face of changing patterns of infectious disease and bacterial resistance, providing a powerful incentive to continued research.

Developments in screening techniques may yet reveal new β-lactam structures with novel biological properties. The search for new β-lactams with novel activity against specific pathogens is likely to continue, with the trend towards 'custom' antibiotics as opposed to those with wide ranging broad spectrum activity. Future generations of β-lactam antibiotics may include compounds demonstrating high activity against selected organisms (or selected target sites), with the use of synergistic mixtures becoming more significant. Such a strategy may enhance the chance of successfully treating infectious disease. A current example is the co-administration of the β-lactamase inhibitor Clavulanic acid together with the otherwise β-lactamase susceptible penicillin Amoxycillin, \(^{(41)}\). (Augmentin TM).

Although total chemical synthesis of many β-lactam
FIGURE (1.4)

**β-LACTAM CALENDAR: DISCOVERY OF PENICILLIN ONWARDS**

### INFECTIOUS DISEASE

- Many common infections yield to penicillin therapy.
- Penicillin resistant infections clinically significant.
- Infections caused by opportunistic pathogens become significant.

### β-LACTAM ANTIBIOTICS

- Penicillin discovered
- Penicillin clinically available
- β-Lactam structure confirmed
- Cephalosporin C discovered
- 1st 'Cephalosporinase' detected
- 6 APA discovered
- Semi synthetic penicillins
- 7 ACA discovered
- Semi synthetic cephalosporins
- Anti pseudomonal and expanded spectrum penicillins and cephalosporins
- Cephamycin discovered
- Oxime cephalosporins
- Nocardicin discovered
- Thiencamycin discovered
- PS compounds discovered
- Olivanic acids discovered
- Epithienamycins discovered
- Carpetimycins discovered
- Discovery of β-lactam antibiotics produced by bacteria
- Monobactams discovered
- β-Lactones discovered

*(27)*
antibiotics and their nuclear analogues has been achieved, \(^{(73)}\), economic considerations have favoured a semisynthetic approach from natural products. A recent exception to this was the total chemical synthesis of the carbapenem Thienamycin, \(^{(74)}\), ( N-Formimidoyl thienamycin in combination with renal dipeptidase inhibitor, Cilastatin, was marketed in early 1986 under the brand name Primaxin ). The monocyclic nature of the monobactams, has made this class of \(\beta\)-lactam an ideal subject for chemical synthesis, \(^{(75)}\). Azthreonam is an example of a totally synthetic novel structure, with improved properties over naturally occurring compounds, and as such may be an indicator of future trends.

1.3 **MODE OF ACTION OF \(\beta\)-LACTAM ANTIBIOTICS**

Interaction between \(\beta\)-lactam antibiotics and bacteria is a very complex multilevel phenomenon, \(^{(76)}\). Factors such as the penetration of \(\beta\)-lactams into the bacterial cell, and the significance of \(\beta\)-lactamase are not discussed here, \(^{(77)}\).

1.3.1 **Target of \(\beta\)-Lactam Antibiotics**

The \(\beta\)-lactam antibiotics presented a group of ideal chemotherapeutic agents, due to their high selective toxicity for bacteria, which led to speculation as to their mode of action. Important clues were provided by, the localisation of radioactively labelled penicillin in the bacterial cell, and the morphological and biochemical effects of penicillin, \(^{(76,78)}\). It was observed that the sensitivity of bacteria to \(\beta\)-lactams was paralleled by the presence of a rigid cell wall.
Following elucidation of the structure and biosynthesis of the peptidoglycan cell wall, (78), it was demonstrated that one of the terminal steps in cell wall biosynthesis was the penicillin sensitive reaction, (79,80,81). The final cross linking reaction, catalysed by peptidoglycan transpeptidase, was found to be penicillin sensitive, see Figure (1.5). Recent reviews of the structure and biosynthesis of the bacterial cell wall are available, (82,83), Figure (1.6) summarises the events of cell wall biosynthesis.

Tipper and Strominger, (80), proposed that penicillin inactivated peptidoglycan transpeptidase by acting as a structural analogue of the dipeptide terminus of the nascent pentapeptide chain, see Figures (1.5) and (1.6). It was further suggested that β-lactam antibiotics were active site directed acylating agents, (76,80,84), capable of acylating the transpeptidase and thereby inhibiting its activity. Other enzymes concerned with the cross linking step in peptidoglycan biosynthesis, namely D-ala carboxypeptidase and endopeptidase, were also recognised to be penicillin sensitive, (78).

1.3.2 Penicillin Binding Proteins (P.B.P's)

Demonstration of the ability of penicillin to bind covalently to the enzymes that it inhibited, provided a means of detecting the penicillin sensitive enzymes as penicillin binding proteins, (85). The situation, with regard to mode of action of β-lactams, was further complicated when it was shown that bacterial plasma membranes contained groups of proteins that bound penicillin specifically, (86). Existence of multiple P.B.P's suggesting the possibility of
FIGURE (1.5)

DIAGRAMATIC REPRESENTATION OF (A) CROSS-LINKED STRUCTURE OF PEPTIDOGLYCAN AND (B) REACTION INHIBITED BY PENICILLIN (36,87,88)

(A)

M, N-acetylmuramic acid
G, N-acetylglucosamine
vertical lines represent tetrapeptide side chains
horizontal lines represent cross-links

(B)

PEPTIDOGLYCAN BACKBONES

M,G as above
Pentapeptide side chain, L-ALA, L-alanine, D-GLN, D-glutamine (or D-glutamic acid),
X, meso-diaminopimelic acid or L-lysine,
D-ALA, D-alanine

N.B. transpeptidation may be carried out via an additional cross-linking peptide e.g. Pentaglycine. (30)
SUMMARY OF EVENTS OF CELL WALL BIOSYNTHESIS IN BACTERIA

(36, 87, 88)

UDP-N-acetylglucosamine (UDP-N-AGA)

↓

PEP

UDP-N-acetylmuramic acid (UDP-N-AMA)

- activated amino acids
- activated D-alanyl-D-alanine

UDP-N-AMA-Pentapeptide

Linked to membrane bound lipid carrier undecaprenyl phosphate (C_{55})

Undecaprenylpyrophosphate-N-AMA-Pentapeptide

(C_{55}-N-AMA-Pentapeptide)

UDP-N-AGA

UDPG

C_{55}-N-AMA-(\beta 1\rightarrow 4)-N-AGA

Pentapeptide

Addition of short cross-linking peptides to pentapeptide side chain (optional)

Peptidoglycan acceptor

Undecaprenyl pyrophosphate

Peptidoglycan backbone extended by one disaccharide unit

D-alanine

Peptidoglycan with new disaccharide now cross linked

(31)
FIGURE (1.6) Continued

**KEY**

UMP - uridine monophosphate

UDP - uridine diphosphate

PEP - Phosphoenol pyruvate

Pi - inorganic phosphate

Pentapeptide - as Figure (1.5)

- Reaction inhibited by penicillin
multiple targets for penicillin action.

However the role of the P.B.P's in peptidoglycan biosynthesis remains unclear, different subclasses of P.B.P. have been associated with cell elongation, septum formation, control of cell shape and cell division, (76,78,85,89,90). Also experiments with model substrates have revealed that at least some P.B.P.'s can catalyse carboxypeptidase, endopeptidase and transpeptidase reactions, (76). Different \(\beta\)-lactam antibiotics demonstrate selective affinity for different subsets of P.B.P.'s, (78), indicating how their bactericidal effects may be mediated.

1.3.3 Physiological Effect of \(\beta\)-Lactam Antibiotics

The lethal effect of \(\beta\)-lactam antibiotics on bacteria, would appear to be more complicated than the simple inactivation of one or more essential enzymes. Existence of a number of penicillin sensitive proteins (carboxypeptidase, transpeptidase, P.B.P.'s), with distinct physiological roles, and the highly selective affinities demonstrated by different \(\beta\)-lactams, suggest that there may be more than one mechanism for cell inhibition. Thus the single target-unbalanced growth model, (76), in which penicillin inhibits a target enzyme, which then leads to disturbances in peptidoglycan synthesis and ultimately to cell death by uncontrolled lysis, may be an oversimplification of the true situation.

The pathway from inhibited enzyme(s) to inhibited bacterial cell may vary considerably, and is likely to be highly complex depending on the particular \(\beta\)-lactam, the bacterial species, and the environmental conditions, (91).
Contributing factors to this complexity are the number of possible targets for β-lactam antibiotics, and the mechanistically different effects that may result,\(^\text{(76)}\). β-Lactam antibiotics can cause, inhibition of cell wall assembly and therefore growth (the primary response), loss of viability, and cell lysis.

Autolysins (peptidoglycan hydrolase) have an important role to play in the secondary response of cell lysis, as is shown by the phenomenon of 'tolerance',\(^\text{(92,93)}\). A primarily bacteriostatic response to penicillin, being obtained with organisms whose autolytic system was defective, or had been compromised by the use of specific inhibitors of peptidoglycan hydrolase. The bactericidal and lytic effects observed as the secondary response, may well involve factors other than the primary targets of β-lactams, and do not appear to be an automatic consequence of the inhibition of cell wall synthesis,\(^\text{(76,91,93)}\). Recently it has been shown that exposure of cells to β-lactam antibiotics can lead to 'triggering' of the autolytic system,\(^\text{(94)}\), and to a decrease in the degree of O-acetylation of the peptidoglycan polysaccharide moiety,\(^\text{(94)}\), the significance of these observations is not yet clearly understood.

The mechanism of action of β-lactam antibiotics is a complex phenomenon, involving the interplay of a number of different factors, including inhibition of the final cross linking step, and other more subtle disturbances of peptidoglycan synthesis.
1.4 BIOSYNTHESIS OF β-LACTAM ANTIBIOTICS

1.4.1 Classical β-Lactams

Studies of the chemistry of β-lactam containing molecules have been extensive, (1) in addition investigation into the biosynthesis of this group of antibiotics have proved fruitful. Many features of the biosynthesis of β-lactams have been clarified in recent years, however a number of mechanistic steps remain obscure and are currently under investigation, (95). Excellent reviews of this field are available, (43,95,96,97).

A composite picture of penicillin cephalosporin and cephalexin biosynthesis, compiled from data from many microorganisms, is shown in Figure (1.7), (96).

1.4.1.1 Whole Cell Studies

Originally, possible biosynthetic precursors were suggested on the basis of the known chemical structure of penicillins and cephalosporins, (43). Early studies involved addition of potential precursors to the fermentation, and noting the effect on antibiotic production, (41,96). Feeding studies with isotopically labelled cysteine and valine, demonstrated intact incorporation into penicillins and cephalosporins, thus confirming their role as precursors, (95,96). There then followed a search for potential peptide precursors of β-lactam antibiotics. This lead to discovery of the tripeptide δ-(L-α-aminoacidyl)-L-cysteinyl-D-valine, (LLD-ACV), by Arnstein and co-workers, (97,98). LLD-ACV was presumed to be the precursor of penicillins and cephalosporins although direct demonstration of its role was not possible using whole cells due to permeability restrictions.
COMPOSITE PATHWAY OF PENICILLIN, CEPHALOSPORIN AND CEPHAMYCIN BIOSYNTHESIS IN FUNGI AND ACTINOMYCETES (96)

L-α-amino adipate (L-AAA) + L-cysteine (L-CYS) → L-AAA-L-CYS

L-valine (L-VAL)

Phenylacetate

Phenylacetyl-CoA

Isopenicillin N

Benzylpenicillin

Penicillin N

Deacetoxycephalosporin C

O2

Deacetylccephalosporin C

acetate → acetylCoA

Carbamoyl phosphate

Cephalosporin C

O-carbamoyldeacetyl Cephalosporin C

O2

S-adenosylmethionine

7α-methoxy Cephalosporin C

Cephamycin C

7α-methoxy deacetyl Cephalosporin C

acetate

deacetyl Cephalosporin C

Cephamycin B

Cephamycin A

N.B. no single microorganism can carry out all reactions illustrated (36)
imposed by the intact cell envelope, \((43,99)\).

However whole cell studies using isotopic and chiral labelling of precursor have provided many insights into the reactions of \(\beta\)-lactam biosynthesis, \((95)\). For example experiments with valine possessing labelled methyl groups, revealed that the S-C bond of penicillin was formed with retention of configuration at the carbon atom, \((41,96)\). Isotopic labelling of precursors established that \(L\-\alpha\)-amino adipic acid, \(L\)-cysteine and \(L\)-valine were incorporated intact into LLD-ACV tripeptide.

1.4.1.2 Cell Free Systems

Conclusive evidence of a direct role for LLD-ACV in penicillin biosynthesis was provided a decade ago, with the conversion of the tripeptide into isopenicillin N, using a cell free (lysed protoplast) system of *Cephalosporium acremonium*, \((100,101)\). The presumed role of isopenicillin N as a precursor of cephalosporins was also confirmed in this way, \((102)\). Subsequently major advances in understanding of the biosynthetic pathway of penicillins, cephalosporins and cephemycins were made, using various cell free systems, (once the importance of appropriate cofactors was appreciated), together with elegant radiolabelling studies, \((43,95,96,103,104,105)\). Recently it was confirmed that the LLD-ACV tripeptide itself undergoes intact incorporation, although the mechanism of cyclisation remains to be determined, \((106)\).

A more detailed summary of the accepted biosynthetic sequence leading to the classical \(\beta\)-lactams is given elsewhere \((45,95,96,97)\). It should be noted that no single microorganism can carry out all the conversions illustrated, \((96)\). (See Figure (1.7))
1.4.1.3 Studies of the Biosynthetic Enzymes

Although progress has been rapid in determining the biosynthetic pathway of the classical β-lactams, the mechanisms by which the individual reactions occur are not well understood. Currently interest is focussed on transformations at the enzyme level. The biosynthetic enzymes responsible for elaboration of penicillins, cephalosporins and cephemycins have been at least partially elucidated, (43,96,107,108,109).

Baldwin et al (110) have recently succeeded in producing a new type of penicillin by enzymic synthesis, using purified isopenicillin N synthetase (IPNS) and a novel tripeptide precursor. It was observed that the requirement for the valine moiety was not absolute and could be replaced by O-methylallothreonine or isoleucine, leading to the formation of novel penam structures. Baldwin et al indicated that their findings are in agreement with the currently favoured free radical mechanism of cyclisation, involving an enzyme bound monocyclic intermediate, (110).

The molecular genetics of antibiotic biosynthesis are also being approached, (107,111,112,113), with the recent cloning of biosynthetic genes, (114,115), and attempts at sequencing, most notably that for the IPNS gene.

1.4.2 Non Classical β-Lactams

Determination of the biosynthetic pathway leading to novel β-lactam classes (clavam, carbapenem and monobactam compounds), is very much in its infancy. Despite their structural relationship with classical β-lactams, a distinct biosynthetic origin seems likely from the preliminary studies.
FIGURE (1.8)

POSSIBLE BIOSYNTHETIC ORIGIN OF THE CARBON SKELETON, OF THE
β-LACTAMASE INHIBITOR CLAVULANIC ACID (96,117)

\[
\begin{align*}
\text{glycerol} & \quad \text{L-glutamic acid} \\
\xrightarrow[\text{phosphoenol pyruvate]} \quad + & \quad [\text{OR}] \\
\text{oxaloacetate} & \quad \text{L-ornithine} \\
\quad & \quad \text{TCA cycle}
\end{align*}
\]

CLAVULANIC ACID

(39)
so far carried out. Although the LLD-ACV tripeptide does not appear to be involved\(^{(95)}\), the possibility of a common ring closure mechanism (using common enzymes?) cannot be excluded.

1.4.2.1 Clavulanic Acid

Biosynthetic studies using \(^{14}\text{C}\) and \(^{13}\text{C}\) labelled precursors, \(^{(95,96,116)}\), have attempted to identify the metabolic origin of the carbon skeleton of the potent irreversible \(\beta\)-lactamase inhibitor, Clavulanic acid, \(^{(51)}\). It was suggested that carbons 5, 6 and 7 of the \(\beta\)-lactam ring could come from glycerol or a late intermediate of the TCA cycle, \(^{(6)}\), and that carbons 9, 8, 2, 3, and 10 of the oxazolidine ring could be derived from glutamate, \(^{(96)}\), see Figure (1.8). However more recent studies indicate that ornithine may be the precursor of the oxazolidine ring, \(^{(117)}\).

1.4.2.2 The Monobactams

The monobactams are a group of novel monocyclic \(\beta\)-lactams of bacterial origin, \(^{(8,55)}\), Figure (1.2) gives the structure of sulfazecin the most commonly produced monobactam. Studies indicate that serine is the source of the carbon atoms of the \(\beta\)-lactam ring, with methionine the source of the methoxyl substituent, \(^{(7)}\), inorganic sulphate providing the sulphenate group, \(^{(118)}\).

1.4.2.3 The Nocardicins

The nocardicins are a series of novel monocyclic \(\beta\)-lactams produced by \textit{Nocardia uniformis} subspecies tsuyamensis, \(^{(4)}\). A biosynthetic pathway has been postulated on the basis of studies using \(^{14}\text{C}\) labelled amino acids, and the stimulatory
effect of, L-tyrosine, p-hydroxyphenyl pyruvic acid, DL p-hydroxyphenyl glycine, on antibiotic production, \(^{(5,96)}\) see Figure (1.9).

1.4.2.4 The Carbapenems

At the commencement of this project few biosynthetic studies had been carried out for this \(\beta\)-lactam class. It had been reported that glutamate was a precursor for the 5-membered ring of Thienamycin, the \(\beta\)-lactam carbons being supplied by acetate, \(^{(95,119)}\), see Figure (5.1), chapter five. Coverage of the situation currently is given in chapter five.

1.5 Aim of Work

Our aim in this work was to develop a system for investigation of the biosynthetic pathway to Thienamycin and related carbapenems, that would permit biosynthetic-mechanistic studies of the type carried out for penam and cephem \(\beta\)-lactams. The systems we hoped to employ would include, the intact fermentation, resuspended mycelium, and resuspended protoplasts. Extensive use would be made of radiolabelled precursors and autoradiographic techniques.

It was recognised at the outset, due to the intrinsic instability of Thienamycin and the non availability of a high yielding strain, that the essential preliminaries of developing a fermentation, and convenient assay techniques, isolation and purification procedures, would constitute a major part of the effort. In the event production of Thienamycin in adequate yield for isolation was not achieved, and the main emphasis of the work was directed to optimising the fermentation and assay systems.
CHAPTER TWO

FERMENTATION STUDIES AND CONTROL OF SECONDARY BIOSYNTHETIC PATHWAYS IN STREPTOMYCES CATTLEYA
2.1 INTRODUCTION

Chapter two covers the fermentation studies carried out with *Streptomyces cattleya* NRRL 8057, and also includes a brief outline of factors influencing secondary metabolite production, with particular emphasis on the production of β-lactam antibiotics by *Streptomyces*.

2.1.1 PRIMARY AND SECONDARY METABOLISM

Pathways of primary metabolism involve an interconnected series of enzyme mediated reactions which provide, biosynthetic intermediates, and energy, and convert biosynthetic precursors into essential macromolecules. Primary metabolites may be termed 'general' metabolites, in that primary metabolism is essentially identical for all living organisms. In addition certain taxonomic groups are capable of synthesising secondary or 'special' metabolites under specific nutritional conditions (120).

Table (2.1), lists characters used to describe primary and secondary metabolism.

2.2 REGULATION OF SECONDARY METABOLISM

The regulation of secondary metabolism is considered, primarily with respect to β-lactam antibiotic production. although our understanding of the control mechanisms involved in secondary metabolism is limited, regulatory controls similar to those found in primary metabolism may operate.

2.2.1 Physiological Regulation

Physiological control of antibiotic production may be considered at three different levels (121):-
<table>
<thead>
<tr>
<th>Primary metabolism</th>
<th>Secondary metabolism</th>
</tr>
</thead>
<tbody>
<tr>
<td>Essentially identical for all forms of life</td>
<td>May be taxonomically restricted</td>
</tr>
<tr>
<td>Essential for growth of the organism</td>
<td>Not necessary for vegetative growth (may have a survival function in nature)</td>
</tr>
<tr>
<td>Highly specific enzymes involved</td>
<td>Low enzyme specificity, often involves special synthetases</td>
</tr>
<tr>
<td>Metabolites rarely allowed to accumulate</td>
<td>Mixture of compounds of same type may accumulate. Proportion of each component in mixture influenced by environmental and genetic factors</td>
</tr>
<tr>
<td></td>
<td>− Often produced under specific nutritional conditions at low specific growth rates</td>
</tr>
<tr>
<td></td>
<td>− May possess biological activity which is expressed outside the producing organism</td>
</tr>
</tbody>
</table>

(45)
i) Control through environmental factors (sources of carbon, nitrogen, phosphate, oxygen, metal ions).

ii) Control through intermediary metabolism (providing precursors and regulatory signals).

iii) Control exerted at the level of the secondary biosynthetic pathway (feedback regulation formation of autoregulators and autotoxic substances).

A number of control mechanisms may operate at these different levels (9,120,122). Regulatory controls, significant in the production of β-lactam antibiotics by Streptomyces sp., and in particular the production of carbapenems by S. cattleya are outlined here, Table (2.2).

2.2.1.1 Growth Rate Control

The microbial lifestyle places a premium on the capacity for rapid growth. Control mechanisms ensure growth takes priority over less urgent activities such as differentiation and secondary metabolism (123).

As early as 1965 Bu'lock observed biphasic patterns of growth and antibiotic production, which appeared to indicate an incompatibility between unlimited growth rates and secondary metabolism (122). Late expression of the synthesis of antibiotics and other secondary metabolites is well known (124). However, time separation between the growth phase ('trophophase') and the antibiotic production phase ('idiophase') is a function of the nutritional environment, and not the type of molecule (125). In nutritionally deficient media, growth and antibiotic production may occur simultaneously (122).
**TABLE (2.2)**

CONTROL SYSTEMS INVOLVED IN THE REGULATION OF ANTIBIOTIC BIOSYNTHESIS AT THE PHYSIOLOGICAL LEVEL

a) Growth Rate Control  
b) Carbon Metabolite Regulation  
c) Nitrogen Metabolite Regulation  
d) Phosphate Regulation  
e) Metabolic Regulators  
f) Regulation through Mineral Element Requirement  
g) Regulation of Antibiotic Biosynthesis - Branched Pathways
In fermentation growth rate alone may appear to regulate secondary metabolism, antibiotic production occurring at low growth rate, irrespective of the type of nutrient limitation employed to produce the low growth rate. An example of this being the production of Cephamycin C by \textit{S. cattleya} \(^{(126)}\). Alternatively a specific nutrient deficiency may be necessary in addition to the low growth rate, for example production of Thienamycin by \textit{S. cattleya} requires phosphate limited conditions for biosynthesis of this antibiotic to occur \(^{(126)}\). Thus carbon, nitrogen and phosphate regulation may be involved in the growth rate control of antibiotic biosynthesis, \(^{(120)}\).

### 2.2.1.2 Carbon Metabolite Regulation

Rapidly used carbon sources often exert regulatory effects, which can interfere with the biosynthesis of many antibiotics, \(^{(122)}\). Kahan et al indicated that, in \textit{S. cattleya} fermentation, the maximum rate of antibiotic synthesis occurred only after substantial depletion of the major carbohydrate source, glucose \(^{(10)}\). A regulatory mechanism of this type ensures that the most easily metabolised nutrients are used for rapid growth and that antibiotic production is suppressed \(^{(120)}\). Whether cyclic AMP has a role to play in carbon metabolite regulation of antibiotic synthesis remains unclear \(^{(122)}\).

### 2.2.1.3 Nitrogen Metabolite Regulation

Screening of nitrogen sources for inclusion in chemically defined media, used for antibiotic production, frequently results in selection of slowly metabolised amino acids, the use of which probably leads to nitrogen limited growth, \(^{(120)}\).

That nitrogen regulation has a role to play in antibiotic biosynthesis was demonstrated by Aharonowitz and Demain, \(^{(127)}\).
Ammonium was observed to interfere with cephalosporin formation in *Streptomyces clavuligerus*, the 'ammonium effect' appeared to operate at the level of the biosynthesis of the antibiotic synthetases, rather than their inhibition.

Glutamine synthetase is a key enzyme in nitrogen metabolism, nitrogen regulation of antibiotic production may be mediated through the level or activity of this enzyme, \(^{(128)}\), with *S. clavuligerus* high specific cephalexin production was obtained under growth conditions that coincidentally led to high glutamine synthetase activity, \(^{(129)}\). A similar situation being found in the *S. cattleya* fermentation with regard to the antibiotic Thienamycin, \(^{(129)}\).

2.2.1.4 Phosphate Regulation

Phosphate may act as a crucial growth limiting nutrient in many antibiotic fermentations, \(^{(122,130)}\). Phosphate regulation of antibiotic production is widespread, affecting the synthesis of many diverse groups of antibiotics, \(^{(131)}\). The range of environmental concentrations of phosphate tolerated during periods of vegetative growth are often much greater than those which permit synthesis of secondary metabolites, \(^{(131)}\).

It is possible that orthophosphate regulates the level of another intra-cellular effector, that in turn controls the expression of antibiotic biosynthesis, \(^{(132)}\). Evidence suggests that ATP may be involved, a high level of ATP interfering with antibiotic synthesis. Time-course studies have shown a rapid decrease in intracellular ATP just prior to the onset of antibiotic synthesis, \(^{(133)}\).
Thienamycin production in *S. cattleya* (126) and cephalosporin production in *S. clavuligerus*, (133), are thought to be under phosphate regulation. Lilley *et al* observed an increase in respiratory activity in response to phosphate limitation in *S. cattleya* fermentation, (126), it was suggested that a change in glucose metabolism occurred in the phosphate limited condition, that in turn promoted Thienamycin synthesis. Kuenzi, (134) proposed that the negative effect of high levels of phosphate on 3-lactam formation in *C. acremonium*, was due to increased glucose catabolism resulting in carbon source repression. The site of carbon source repression in *C. acremonium* being the ring expansion enzyme, (135).

Recent studies on the individual enzymes of antibiotic biosynthesis in *S. clavuligerus*, indicate that the negative effect of phosphate may be due to enzyme inhibition and/or repression. Interestingly the ring expansion activity was repressed when cells were grown at high phosphate concentrations, (108).

2.2.1.5 Regulation through Mineral Element Requirement

The synthesis of secondary metabolites and structures of cellular differentiation often tolerate a much narrower range of environmental concentrations of specific trace metals, than does vegetative growth of the producer cells. Regulation of secondary metabolism by trace metals and inorganic phosphate has been summarised by E. D. Weinberg, (130).

Biosynthesis of Thienamycin and related carbapenems, demonstrates an unusual requirement for trace amounts of cobalt, (52,53,65). This requirement being partially satisfied by addition of vitamin B12 (cyanocobalamin), to
the fermentation medium during the growth phase, \(^{(136)}\). The precise role(s) of cobalt in Thienamycin biosynthesis remains obscure.

Cobalt may regulate the biosynthesis of the antibiotic synthetases themselves, or may be responsible for the activation of preformed enzymes. Vegetative growth appears unaffected in media not supplemented with cobalt.

In *S. cattleya* fermentation glutamine synthetase activity peaks and then rapidly declines prior to the onset of antibiotic biosynthesis, \(^{(129)}\). Cobalt is known to alter the structural configuration of GS in *Escherichia coli*, \(^{(137)}\), it is possible that cobalt fulfills this role in *S. cattleya* thereby regulating the activity of GS.

Cobalt may be required for formation of vitamin \(B_{12}^{1}\), and may function in enzyme conversions mediated by \(B_{12}^{2}\). The conversion of homocysteine to methionine involves vitamin \(B_{12}^{1}\). Methionine is known to act as a methyl group donor in *S. cattleya*, carbons \(C_8\) and \(C_9\) of the hydroxyethyl side chain of Thienamycin originate from methionine, \(^{(136)}\). Also other independent evidence suggests that cobalt may influence fermentative methylation in *Streptomyces* sp., \(^{(140)}\).

*S. cattleya* produces the non \(\beta\)-lactam antibiotic, cyclopentenedione in fermentation, \(^{(141)}\). The antibiotic is believed to act by sequestration of metal ions \(^{(142)}\), antibacterial activity can be reversed using a mineral salts solution, \(^{(143)}\). If the cyclopentenedione has a role in regulation of antibiotic biosynthesis in *S. cattleya*, this could involve the sequestration of cobalt.
2.2.1.6 Metabolic Regulators

Enzyme induction may be involved in controlling the biosynthesis of antibiotics, \(^{(36,122)}\). Little is known of the metabolic regulatory agents involved, as only a few substances of this type have been studied in the chemically pure state, the stimulatory response may be the result of true enzyme induction or in some cases to increased availability of precursors, \(^{(36)}\). Probably the most well known example is the stimulation of Streptomycin biosynthesis in \textit{Streptomyces griseus} by A-factor, \(^{(144)}\). Processes of differentiation and secondary metabolism may be connected, in that the same regulators may be involved in both activities.

In \textit{S. cattleya} there is evidence that conditions which promote secondary metabolism suppress sporulation, and vice versa, \(^{(145)}\). During the course of this work it was observed that sub-cultures of \textit{S. cattleya} which failed to express the ability to produce Thienamycin in fermentation, frequently sporulated poorly on solid media. Possibly secondary metabolism and sporulation in \textit{S. cattleya} are
under common regulation.

2.2.1.7 Regulation of Antibiotic Biosynthesis in Branched Pathways

Control mechanisms operating in primary metabolism may be partly reflected in secondary metabolism, primary metabolites and intermediates act as precursors for secondary metabolism. In addition to feedback regulation of primary metabolism, they may take part in the regulation of synthesis of, and/or the activity of, enzymes involved in secondary metabolism, \((120,122)\). Secondary metabolism may be regulated by a primary end product of a common branched pathway, the other branch of which leads to the antibiotic. The antibiotic itself may inhibit the activity of the first dedicated enzyme of its biosynthetic pathway, \((146)\).

2.2.2 Genetic Regulation

Streptomyces genetics have been excellently reviewed by Chater and Hopwood, \((147)\), other reviews deal more specifically with the genetics of antibiotic production by Actinomyces \((112,148,149)\).

Genetic regulation of antibiotic biosynthesis is a relatively unexplored field. With the increase in the versatility and scope of genetic analytical techniques \((147)\), and their application to antibiotic producing Actinomyces, hopefully this situation will alter. When considering the control of gene expression, in antibiotic biosynthesis two regulatory models have been proposed, \((36,122)\):

Model 1: A small molecule acts as a co-repressor or inhibitor, repressing the formation of, or inhibiting the action of, the antibiotic
synthetases.

Model 2: An inducer or activator must be synthesised by the producing culture (or added to it!) in order to initiate antibiotic biosynthesis.

Model 1 may fit the experimental data for carbon, nitrogen, or phosphate, regulation. Model 2 could explain the effect of A-factor on Streptomycin production in *S. griseus*, (36).

2.2.2.1 Organisation of Antibiotic Biosynthetic Genes in Streptomyces

Clustering and partial clustering of related metabolic genes is common in the genus Streptomyces, (150). Antibiotic biosynthetic genes, that have been chromosomally mapped, tend to be located in the lower arc of the map, (111). In cases where several genes concerning the biosynthesis of the same antibiotic have been mapped, they are often assembled in clusters, suggesting co-ordinate regulation, (112).

Okanishi proposed that plasmids could also be involved in antibiotic biosynthesis, (151). Evidence that the structural genes could be plasmid borne, was provided by the SCP 1 plasmid of *Streptomyces coelicolor*, (152). SCP 1 carries the biosynthetic genes for production of the antibiotic Methylenomycin A. Plasmids may also be involved in regulation of antibiotic production, or resistance to the antibiotic, rather than carrying the structural genes for biosynthesis, (147).

Umezawa suggested two possible modes of plasmid involvement in the production of secondary metabolites, (153).
Firstly plasmids may be involved in the biosynthesis of products which promote synthesis of secondary metabolites. Secondly plasmids may be directly concerned with the biosynthesis of the enzymes which catalyse the synthesis of whole, or some molecular moieties of secondary metabolites.

2.3 FERMENTATION STUDIES WITH STREPTOMYCES CATTLEYA

2.3.1 Selection of Fermentation Media and Conditions

2.3.1.1 *S. cattleya* Fermentation in Complex Medium

Kahan et al. (65) indicated that a number of complex media may be used for the fermentation of *S. cattleya* and production of Thienamycin. Initially, a range of media used for antibiotic production in Streptomyces, (128,133,145,154, 155), and several of the Kahan media, (65), were tested for growth and antibiotic production. Fermentation was carried out as a batch culture in shake flasks (see Experimental), none of the chemically defined media supported significant growth of *S. cattleya*, antibiotic production was not detected. A complex medium, Kahan medium (E), was selected for use in *S. cattleya* fermentation.

A growth curve for *S. cattleya* when grown on medium (E) is shown in Figure (2.3). Figure (2.2) shows the growth curve for the *S. cattleya* seed inoculum. As expected for batch fermentation in nutritionally rich medium, high titres of antibiotic were achieved only after most of the cellular growth had occurred.

Although standard conditions were imposed, as far as possible, *S. cattleya* demonstrated some variability with
FIGURE (2.2)

A TYPICAL GROWTH CURVE S. CATTLEYA : SEED INOCULUM ($f_1$)

[ 0.25 ml S. cattleya spore suspension (approx. $1 \times 10^8$ C.F.U./ML) inoculated into 25 ml seed medium, in 250 ml conical flask. Flasks incubated $25^\circ C$, 220 rpm for up to 3 days, ($f_1$) then used as inoculum for ($f_2$) fermentation ]
[2.5 ml (f₁) seed inoculum, added to 25 ml Kahan medium (E) in 250 ml conical flask. Flasks incubated at 25°C, 220 rpm, biomass (gram wet cells L⁻¹) and pH recorded. Activity in broth determined using P99 β-lactamase inhibition assay.

\[ \text{Activity in broth determined using P99 } \beta \text{-lactamase inhibition assay.} \]

\[ t₆₅ (f₂) \text{ mycelium from a number of flasks resuspended in defined medium and (f₃) allowed to proceed, (f₃) samples labelled as corresponding time in (f₂) fermentation.} \]

Total activity in broth:

\[ \begin{align*}
(f₂) & \text{ 94 hour, } \quad > 5 \mu g/ml \text{ NFT } \equiv 10 \mu g/ml \text{ NFT} \\
(f₃) & \text{ 118 hour, } \quad \equiv \quad " \quad \equiv 5 \quad "
\end{align*} \]

(M.I.C. broth dilution assay)

T.L.C. analysis of broth samples Figures (3.14), (3.15)

H.P.L.C. analysis of (f₂) 94, (f₃) 118 hour broth Figures (3.17), (3.18) and (3.19)
respect to antibiotic production, antibiotic titre varying considerably from one run to the next. Titres equivalent to, 150 μg/ml N-Formimidoylthienamycin (NFT), as assayed by the Enterobacter cloacae P99 β-lactamase inhibition assay, or equivalent to 10 μg/ml NFT when assayed using Comamonas terrigena or Staphylococcus aureus M.I.C. assays (see Experimental) could be obtained. Fermentation broth with P99 inhibitory activity equivalent to 50 μg/ml N-Formimidoylthienamycin was assayed further to identify the different β-lactam antibiotics co-produced. (chapter 3.)

2.3.1.2 _S. cattleya_ Fermentation in Defined Medium

When fermentation of _S. cattleya_ was carried out in medium (E), a foreshortened stationary phase, followed by a lytic phase was observed in some instances, see figure (2.4). Concommitantly very low or undetectable antibiotic titres were obtained (< 0.156 μg/ml NFT, _S. aureus_ M.I.C. assay).

_S. cattleya_ had previously demonstrated poor growth on chemically defined media, therefore growth was initially carried out on medium (E), the mycelium then being resuspended in a defined medium. The medium used was a modification of that of Aharonowitz and Demain (133), supplemented with cobalt chloride (10 mg/ml). Mycelium was resuspended at different stages during the fermentation, fermentation was then allowed to proceed in the defined system.

In the defined system, the biomass level stabilised after a period of biomass accretion, pH remained stable (between 6.5 - 7.0) for up to five days incubation,
GROWTH CURVE FOR S. CATTLEYA ($f_2$) FERMENTATION ON KAHAN MEDIUM (E), LOSS OF BIOMASS (LYSIS), AND THE EFFECT OF RESUSPENSION IN DEFINED MEDIUM ($f_3$), ON BIOMASS AND FERMENTATION pH

(i) ($f_2$) Kahan Medium (E)

(ii) ($f_3$) Defined Medium

--- antibacterial activity vs S. aureus, determined using M.I.C. broth dilution assay.

($f_1$) and ($f_2$) carried out as described Figures (2.2) and (2.3), mycelium resuspended in defined medium at $t_{47}$ hours ($f_2$) and ($f_3$) allowed to proceed.

($f_3$) samples labelled as corresponding time in parent ($f_2$) fermentation

($f_3$) $t_{125}$ total activity broth $\equiv 12.5 \, \mu g/ml$ NFT

($f_2$) $t_{76}$ " " " $\equiv 0.156 \, \mu g/ml$ NFT
see Figure (2.4). Antibacterial activity equivalent to 12.5 \( \mu g/ml \) NFT ( \textit{S. aureus} M.I.C. assay), was detected in fermentation broth. Antibiotic titres in the defined system were 4 - 80 fold higher than those detected in the parent fermentation. In some instances antibiotic activity could be demonstrated in the defined system, when it was undetectable in the parent fermentation.

The defined system used here for \textit{S. cattleya} fermentation may provide a relatively stable pH that is favourable for antibiotic production, it may allow the maintenance of biomass under nutritionally limited conditions that promote antibiotic biosynthesis. A similar system has recently been reported for \textit{S. cattleya} fermentation, \(^{(136)}\). In addition to obtaining higher antibiotic titres, the defined system permitted use of the hydroxylamine extinguishable absorbance (\( \lambda 299 \text{ nm} \)) of Thienamycin, to follow antibiotic production in fermentation broth and to obtain difference spectra, (chapter 3).

2.3.2 Factors Affecting Antibiotic Productivity

During the course of this work difficulty was experienced in obtaining reproducible antibiotic titres in fermentation. It is appropriate at this point to outline some of the possible causes of this observation, see Table (2.3).

Antibiotic production may be lost (or exhibited) as a result of:

2.3.2.1 Small Alterations in Fermentation Medium or Conditions

Antibiotic titre is often very sensitive to environmental variation, so that even if the underlying genetic determination
FACTORS THAT MAY AFFECT ANTIBIOTIC PRODUCTIVITY IN S. CATTLEYA FERMENTATION

a) Small alterations in fermentation medium or conditions.

b) Method of culture preservation.

c) Genetic factors

1) Mutation
2) Transposable elements
3) Insertions/deletions
4) Plasmid loss
5) Regulation of plasmid copy number.
is relatively simple, any inherent discontinuities may be obscured by environmentally induced variation, \(^{(148)}\).

As *S. cattleya* fermentation was carried out in shake flasks, fermentation parameters could not be monitored or controlled 'in situ'. However, measures were taken to eliminate any variation in medium composition. Particular attention was paid to cleanliness of glassware, and over autoclaving of media. Medium constituents were obtained from the same batch where possible, freshly prepared batches of media were used throughout.

subsection{2.3.2.2 Method of Culture Preservation}

Variation in culture viability and stability may be due to the method of culture preservation used, and not to inherent genetic instability, \(^{(156)}\). Variations in culture maintenance may affect culture viability, the ability to produce a metabolite or the ability to respond reproducibly to an assay organism, \(^{(156)}\).

When *S. cattleya* was maintained by serial subculture on agar slants, the ability to produce antibiotics and culture viability were rapidly lost in a matter of weeks. Of the other methods used (preservation on sterile soil, lyophilisation) maintenance of *S. cattleya* as a spore suspension in 50% glycerol at minus 20°C, \(^{(150,157)}\), has been the most successful. A viable culture was maintained for a period of six months using this method, however repeated freeze-thaw appeared to affect viability.

subsection{2.3.2.3 Genetic Factors}

i) Mutation, involving straightforward DNA alterations in structural or regulatory genes.
ii) Transposable elements, transposition into a structural
or regulatory gene effecting loss of antibiotic biosynthesis.

iii) Insertions/deletions of genetic material. Baltz and
Stonesifer (158), recently stated that the instability of
antibiotic production in *Streptomyces fradiae* could be
due to a genetic rearrangement associated with the deletion
or amplification of sequences linked to and possibly
encompassing the Tylosin biosynthetic genes.

iv) Plasmid loss, loss of a plasmid carrying structural or
regulatory genes. Plasmid loss, possibly arising through
inefficient segregation or partition of plasmids to
daughter cells at cell division, has been linked with loss
of antibiotic production, (147).

v) Regulation of plasmid copy number. Plasmid copy number
varies considerably between different plasmids, but is
fairly closely controlled for a particular plasmid, (159).
If structural or regulatory genes were encoded on a
plasmid, an increase in the copy number of that plasmid
would increase the cell productivity for that gene product,
and vice versa. Such a system would enable the organism
to preserve the option of antibiotic production, and any
adaptive advantage thereof, as no permanent loss of
 genetic information occurs.

With regard to antibiotic production in *S. cattleya* it
is possible that the situation is obscured by the interplay
of a number of different factors, at the environmental and
the genetic level. The difficulties experienced may have
arisen, not through any genetic instability, but through
insufficient information about the particular environmental
conditions and regulatory controls that promote antibiotic biosynthesis. Expression of antibiotic biosynthesis may occur only under stringent environmental conditions.

2.4 SUMMARY

This chapter outlines some of the regulatory controls that may be relevant to antibiotic production in *S. cattleya*. The experiments included batch fermentation studies with *S. cattleya*, expression of antibiotic production was obtained in complex and in a chemically defined medium. Due to a significant degree of variability in the production of antibiotics by *S. cattleya* some possible causes of this observation are included here.
CHAPTER THREE

IDENTIFICATION OF β-LACTAM ANTIBIOTICS IN S. CATTLEYA

FERMENTATION BROTH
3.1 INTRODUCTION

The carbapenem Thienamycin is produced by *Streptomyces cattleya*, (10,65), N-Acetylthienamycin, N-Acetyl-dehydro-thienamycin, (160,161), Northienamycin, 8-Epithienamycin (162), Penicillin N, Cephamycin C (126), and a cyclopentenedione antibiotic are co-produced in fermentation see Figure (3.1). Kahan et al (10), reported peak Thienamycin levels of 1.4 µg/ml. Thienamycin was purified using mild conditions, at or below 5°C and near neutral pH throughout, with an overall recovery of 1.64%, (10).

Isolation and purification of Thienamycin from *S. cattleya* fermentation broth, was not attempted here, due to its chemical instability and low fermentation productivity, instead use of highly specific and sensitive assays permitted the detection and eventual identification of Thienamycin.

3.2 STRUCTURE–ACTIVITY RELATIONSHIP IN THE CARBAPENEM SERIES

Thienamycin possesses several novel structural features when compared with the classical β-lactams. The five membered ring contains a carbon in place of the more usual sulphur, relative stereochemistry of the 5,6 β-lactam protons involves a trans rather than cis relationship, and Thienamycin incorporates an hydroxyethyl group in place of the customary amide side chain of classical β-lactams. Structural novelty is paralleled by exceptional biological properties. Thienamycin demonstrates very potent and broad spectrum antibacterial activity, is resistant to attack by most bacterial β-lactamases, and is highly active against Pseudomonas sp., (10,163,164,165).
FIGURE (3.1)  ANTIBIOTICS CO-PRODUCED IN S. CATTLEYA FERMENTATION

Thienamycin (10)

N-Acetyltthienamycin (10)

N-Acetyldehydro - thienamycin (162)

8-EPithienamycin (162)

Northienamycin (162)

NB:  Penicillin N, Cephamycin C, Fig. (1.2) and cyclopentenedione, Fig. (2.1) also produced.
Carbapen-3-em-2-carboxylic acid, Figure (3.2), the parent nucleus of Thienamycin and related carbapenems, demonstrates antibacterial activity of the same order of magnitude as the clinically used penicillins and cephalosporins, (166). Addition of the hydroxyethyl side chain at the 6 position further enhances potency of the carbapenem nucleus, (167). The C-6 side chain is also responsible for incorporation of β-lactamase stability and properties of β-lactamase inhibition into the molecule. For maximal antipseudomonal activity substitution of a basic function at the C-3 position is required. The greater effectiveness of Thienamycin (and some analogues) against *Ps. aeruginosa* has been attributed to the Zwitterionic nature of the C-3 cysteaminyl side chain, (21,162), N-acetylated products demonstrating reduced activity, (52).

Antibacterial potency, β-lactamase stability, and β-lactamase inhibitory properties of carbapenems are dependent on the stereochemistry of the 5,6 β-lactam protons, and the C-6 hydroxyethyl side chain. The trans R configuration found in Thienamycin appears to be optimal for both potency and β-lactamase stability, (52). For carbapenems with non sulphated hydroxyethyl side chain, the antibacterial potency conferred by trans R configuration > cis S > trans S, (13,52,162). Trans R configuration gives the highest degree of β-lactamase stability, trans S gives only moderate stability, and cis S low β-lactamase stability. Sulphated derivatives (with a sulphate group esterified onto the C-8 hydroxyl) with 8-S configuration demonstrate the highest degree of β-lactamase inhibition, 8-S non sulphated carbapenems have a similar
i) SQ 27,860 (58)
( Carapen-3-em -2-carboxylic acid )

ii) N-Formimidoyl thienamycin (163)

iii) Cilastatin (11)
Spectrum of $\beta$-lactamase inhibition but are less potent, carbapenems with 8-$R$ configuration are poor $\beta$-lactamase inhibitors, (20,168).

The highly strained nature of the carbapenem nucleus, (169), together with novel structural properties responsible for the exceptional activity of Thienamycin, also contribute to its chemical instability. At high concentration an intermolecular reaction may occur, between the cysteamine residue of one molecule with the azetidinone of a second molecule, thereby inactivating Thienamycin. The problem is circumvented by use of the N-formimidoyl derivative, (11,163) a discovery a great significance in the clinical development of Thienamycin.

Subsequent sections deal with the development of assay techniques, to allow detection of the $\beta$-lactams co-produced in _S. cattleya_ fermentation broth, and to permit their differentiation on the basis of the structure-activity relationship here outlined, see Table (3.1).
TABLE 3.1

ANALYTICAL TECHNIQUES USED IN THE DETECTION AND IDENTIFICATION
OF CARBAPENEMS PRODUCED IN S. CATTLEYA FERMENTATION

1) **β-LACTAMASE INDUCTION**

2) **β-LACTAMASE INHIBITION**

3) **ANTIBACTERIAL ASSAYS**

4) **HYDROXYLAMINE EXTINGUISHABLE ABSORBANCE**
   \[ \lambda 299 \text{ nm} \], AND **DIFFERENTIAL SPECTROPHOTOMETRY**

5) **DEACYLATION OF N-ACETYLATED CARBAPENEMS USING ACYLASE 1**

6) **SUSCEPTIBILITY OF CARBAPENEMS TO RENAL DIPEPTIDASE**

7) **CHROMATOGRAPHY, TLC, H.P.L.C.**
3.3 ANALYTICAL TECHNIQUES

3.3.1 β-Lactamase Induction

β-lactam antibiotics are capable of inducing the synthesis of β-lactamase in both Gram positive and Gram negative organisms, (70). Figure (3.3) illustrates a possible model for the regulation of β-lactamase production. Recently workers at Squibb have published details of a β-lactamase induction assay, used to screen for β-lactams in fermentation broth, (64). A strain of Bacillus licheniformis was employed, which in the absence of an inducer, i.e. β-lactam, produced minimal amounts of β-lactamase. However, in the presence of a β-lactam a large amount of the enzyme was produced and could be detected by the use of a chromogenic cephalosporin substrate, (170).

A β-lactamase induction assay (property of ICI Pharmaceutical PLC) was used for the detection of β-lactam antibiotics in S. cattleya fermentation broth. The assay proved highly specific for β-lactams, and enabled detection down to nanogram levels. Interestingly a false positive was obtained with the dipeptidase inhibitor, Cilastatin, (11), whose structure is shown in Figure (3.2 iii). The induction assay permitted detection of β-lactams, in liquid media (whole fermentation broth, H.P.L.C. fractions) see Figures (3.10), (3.17 - 3.19), and by the use of a TLC-bioautographic approach, see Figures, (3.12), (3.13a).

3.3.2 β-Lactamase Inhibition

Screening of microorganisms for naturally occurring β-lactamase inhibitors lead to the discovery of, Clavulanic
FIGURE (3.3)

MODEL PROPOSED FOR THE REGULATION OF β-LACTAMASE PRODUCTION, (64)

P and O are the promoter and operator sequences for the penicillinase structural gene (Pen P). Pen I is a regulatory gene specifying the penicillinase repressor, Pen A is a regulatory region specifying a penicillinase antirepressor protein. Formation of the repressor/antirepressor complex takes place in the presence of an inducer, thereby inactivating the penicillinase repressor. Subsequently transcription occurs and penicillinase synthesis begins.
acid, (66), and the Olivanic acids, (53). Subsequently other β-lactam antibiotics were shown to act as β-lactamase inhibitors, (67).

β-Lactam antibiotics in the carbapenem series differ in their β-lactamase inhibitory properties, dependant on their stereochemistry. Compounds with 8R configuration, such as Thienamycin and N-Acetyltielenamycin, are reportedly only poor β-lactamase inhibitors, (20). However Thienamycin has been shown to be a relatively potent ' suicide ' inhibitor of the Enterobacter cloacae p99 β-lactamase, (165).

The E. cloacae p99 β-lactamase was prepared, and used in an inhibition assay to detect carbapenems in S. cattleya fermentation broth. Inhibition of the enzyme could be detected in liquid or solid media using the chromogenic substrate Nitrocefin, (171). When hydrolysed by β-lactamase Nitrocefin undergoes a distinct colour change from yellow to red, see Figure (3.4a). Antibiotic standards Cephalosporin C, Cephamycin C, Deacetoxycephalosporin C failed to inhibit P99 at concentration < 100 μg/ml, Penicillin N demonstrated one well of enzyme inhibition at 100 μg/ml but did not inhibit the enzyme at lower concentrations.

A comparison of the P99 inhibitory properties of, N-Formimidoyl thienamycin, MM13902, MM 22383, MM 22382, was made, Figure (3.4b). Structures of the carbapenem standards are given in Figure (3.5 ). MM 13902 and MM 22382 appear to be more potent inhibitors ( approx. 100 fold ) of the P99 β-lactamase, than N-Formimidoyl thienamycin or MM 22383. This observation is in agreement with predicted properties on the basis of their stereochemistry.
FIGURE (3.4)

SHOWING THE E. CLOACAE P99 β-LACTAMASE INHIBITION ASSAY

a) Inhibition of P99 β-lactamase by N-Formimidoyl thienamycin

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<td>D</td>
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<tr>
<td>F</td>
<td>+</td>
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<td>+</td>
<td>+</td>
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<td>G</td>
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<td>+</td>
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<tr>
<td>H</td>
<td>+</td>
<td>-</td>
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<td>-</td>
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<td>-</td>
<td>-</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

NFT (μg/ml)

P99 β-lactamase serially diluted wells A to H, 20 μl sample (antibiotic standard phosphate buffer, H.P.L.C. fraction, fermentation broth) added to wells A to H, row 1, etc., followed by 20 μl Nitrocefin solution, and incubation at 37°C for 15 minutes.

C- row 1 P99 β-lactamase control
C- row 12 Nitrocefin control (enzyme blank)
(+): Nitrocefin hydrolysed by β-lactamase, colour change yellow to red.
(-): Nitrocefin not hydrolysed, no colour change.
FIGURE (3.4)

b) Comparative Inhibitory Properties of Carbapenem Standards

NFT : N-Formimidoyl thienamycin
'O2 : MM 13902
'82 : MM 22382
'83 : MM 22383
C : P99 β-lactamase control
FIGURE (3.5)

STRUCTURAL CONFIGURATION OF CARBAPENEM STANDARDS

(i)

MM13902: sulphated
Olivanic acid, cis 8S

MM22382: hydroxy
Olivanic acid, cis 8S

(ii)

MM22383: hydroxy
Olivanic acid, trans 8S

N-Formimidoyl
-thienamycin
trans 8R

Structure references as given in Figure (1.3)
3.3.3 Assays based on Antibacterial Activity

Antibacterial assays were used to monitor antibiotic production during S. cattleya fermentation. Activity was detected using TLC-bioautography, and broth dilution assays (see Experimental). The test organisms used were Staphylococcus aureus Oxford H, strain VI, Comamonas terigena ATCC 8461, and a strain of Escherichia coli supersensitive to β-lactam antibiotics (obtained from ICI Pharmaceuticals PLC). Differential susceptibilities of the test organisms to β-lactam standards are shown in Table (3.2).

Figures (3.13c), (3.13d) show TLC-bioautographic analysis of the antibiotics in S. cattleya fermentation broth, and their relative activities vs the test organisms. Subsequent Figures (3.14a-c) demonstrate the susceptibility of these broth components to hydroxylamine and Dipeptidase 1 (see later sections). A component (I), Figure (3.13c), with \( R_f = 0.87 \), demonstrated antibacterial activity against E. coli, but not C. terigena or S. aureus, failed to induce β-lactamase, and was not susceptible to hydroxylamine or Dipeptidase 1. It is suggested that this component may have been the cyclopentenedione antibiotic produced by S. cattleya, (141).

Figures (3.17 - 3.19) show H.P.L.C. analysis of S. cattleya fermentation broth, and the detection of antibacterial activity in fractions using the M.I.C. colorimetric assay (broth dilution assay). Attempts were made to assign the antibacterial activity observed in fermentation broth to the presence of a particular β-lactam, on the basis of different intrinsic susceptibility of the test organisms, and the known fermentation products of
### TABLE (3.2)

DIFERENTIAL SUSCEPTIBILITIES OF TEST ORGANISMS TO β-LACTAM ANTIBIOTICS AS DETERMINED USING THE MIC COLORIMETRIC ASSAY

<table>
<thead>
<tr>
<th>M.I.C. (µg/µl) (approx. 10^4 CFU/WELL)</th>
<th>E. COLI SS</th>
<th>S. AUREUS</th>
<th>C. TERIGENA</th>
</tr>
</thead>
<tbody>
<tr>
<td>PENICILLIN N</td>
<td>0.31</td>
<td>1.25-2.50</td>
<td>80.0</td>
</tr>
<tr>
<td>CEPHAMYCIN C</td>
<td>0.31-0.62</td>
<td>80.0</td>
<td>10.0</td>
</tr>
<tr>
<td>CEPHALOSPORIN C</td>
<td>0.15</td>
<td>40.0</td>
<td>40.0</td>
</tr>
<tr>
<td>DEACETOXY-CEPHALOSPORIN C</td>
<td>0.62</td>
<td>&gt;80.0</td>
<td>&gt;80.0</td>
</tr>
<tr>
<td>PS-5</td>
<td>0.62</td>
<td>0.31-0.62</td>
<td>20.0</td>
</tr>
<tr>
<td>N-FORMIMIDOYL THIENAMYCIN</td>
<td>0.078</td>
<td>0.0045</td>
<td>0.15</td>
</tr>
</tbody>
</table>

MIC COLORIMETRIC ASSAY: TEST ORGANISMS E. COLI SS, S. AUREUS and C. TERIGENA
This strategy appears to have been successful in the case of the major \(\beta\)-lactam product, Thienamycin, good correlation was observed between estimates of antibiotic concentration using the different assays. For the minor fermentation products the results were somewhat inconclusive, due to a lack of appropriate \(\beta\)-lactam standards and the very low fermentation yield of these components.

3.3.4 Hydroxylamine Extinguishable Absorbance 299 nm

The highly strained carbapenem nucleus thought to confer exceptional biological activity, may also be responsible for chemical instability of the molecule, rendering it highly susceptible to nucleophilic attack, \(^{(169)}\). Kahan et al observed that a coordinate loss of bioactivity, and absorbance in the 300 nm region of the UV spectrum, occurred when Thienamycin was exposed to unusually low concentrations of hydroxylamine or cysteine, \(^{(10)}\). Carbapenems other than Thienamycin have also been shown to possess a hydroxylamine extinguishable UV chromophore, \(\lambda_{\text{max}} = 297 \sim 300\) nm, \(^{(14,162)}\).

During the course of the work, this property of carbapenems has proved particularly useful. At low concentration (8 \(\mu\)M/ml) hydroxylamine could extinguish the UV chromophore of N-Formimidoyl thienamycin, see Figure (3.6). Concommitant loss of antibacterial activity, \(\beta\)-lactamase inhibitory properties, and the ability to induce \(\beta\)-lactamase formation was observed, Figures (3.14a-c), presumably these activities are dependent on the presence of an intact \(\beta\)-lactam ring. At the concentration used hydroxylamine had no apparent effect on Cephamycin C and the other \(\beta\)-lactam standards,
FIGURE (3.6)
SHOWING THE N-FORMIMIDOYL THIENAMYCIN, HYDROXYLAMINE
EXTINGUISHABLE ABSORBANCE, $\lambda$ 299 nm

[ N-Formimidoyl thienamycin (10 $\mu$g/ml) incubated at room temperature, with hydroxylamine, final concentration 8 $\mu$M/ml, loss of absorbance 299 nm recorded. ]
## Table 3.3

**Effect of Hydroxylamine on β-Lactam Standards**

<table>
<thead>
<tr>
<th>Final Concentration of NH$_2$OH (µM·m$^{-1}$)</th>
<th>4.0</th>
<th>8.0</th>
<th>16.0</th>
<th>32.0</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-Formimidoyl Thienamycin</td>
<td>16.0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>49.0</td>
</tr>
<tr>
<td>Cephamycin C</td>
<td>30.0</td>
<td>30.0</td>
<td>30.0</td>
<td>30.0</td>
<td>30.0</td>
</tr>
<tr>
<td>Cephalosporin C</td>
<td>27.0</td>
<td>27.0</td>
<td>27.0</td>
<td>27.0</td>
<td>27.0</td>
</tr>
<tr>
<td>Deacetoxy Cephalosporin C</td>
<td>17.0</td>
<td>17.0</td>
<td>17.0</td>
<td>17.0</td>
<td>17.0</td>
</tr>
<tr>
<td>Penicillin N</td>
<td>6.0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>28.0</td>
</tr>
</tbody>
</table>

**Diameter of Inhibition Zone (mm):** E. coli TEST ORGANISM, 1 µg STANDARD USED TO PRODUCE THE INHIBITION ZONE, LOADED AS A SINGLE SPOT ON WHATMAN No. 1 PAPER AND OVERLAYED ON E. coli TEST SEEDED AGAR.
FIGURE (3.7)

UV DIFFERENCE SPECTRA

--- SAMPLE FOLLOWING INCUBATION WITH NH₂OH
--- SAMPLE PRIOR TO INCUBATION WITH NH₂OH

(A) N-Formimidoyl
thienamycin
(20 µg/ml)

(B) S. cattleya
fermentation
broth, H.P.L.C.
fraction (II)
See Figure (3.18)

--- TO OBTAIN DIFFERENCE SPECTRA:
Samples scanned in the region 401.5-187 nm, incubated with
hydroxylamine (8µm/ml) at room temp for 25 min. and then
rescanned.
although Penicillin N was susceptible, Table (3.3). Hydroxylamine extinguishable absorbance was used to detect carbapenems in *S. cattleya* fermentation broth, Figures (3.14a-c).

Differential spectrophotometry was used in conjunction with H.P.L.C. to detect carbapenems in fermentation broth, the UV chromophore was of value when chemically defined growth medium was used. Where complex medium was used the presence of intact β-lactam was demonstrated by β-lactamase induction, β-lactamase inhibition or antibacterial assay. The difference spectrum for the H.P.L.C. fraction thought to contain the Thienamycin component (fraction No. (11), Figure (3.18)), resembled that of the N-Formimidoyl thienamycin standard, Figures (3.7a),(3.7b). When fraction (11) was allowed to react with hydroxylamine, a loss of absorbance in the 300 nm region was observed, with simultaneous increase in the 200 nm region, indicating that this component may indeed be Thienamycin.

3.3.5 Deacylation of N-acetylated Carbapenems

N-acetylated carbapenems are less active antimicrobially, than their counterparts possessing a free C-3 cysteaminyl side chain. Reduction in activity is markedly apparent for *Pseudomonas* sp., (52). The deacetylation of PS-5 to NS-5 by L and D amino acid acylases has been reported, (15), see Figure (3.8).

An attempt was made to design an assay which would allow the deacetylation of N-acetyl carbapenems produced in *S. cattleya* fermentation, and thereby permit their identification. PS-5
was used as a standard to determine the incubation conditions. Carbapenem standard was incubated with Acylase 1 (Sigma), the mixture was separated by T.L.C. and \( \beta \)-lactams detected bioautographically using the \( \beta \)-lactamase induction assay, Figure (3.8), under the conditions used PS-5 has an \( R_f \) value of 0.73, and N-Formimidoyl thienamycin an \( R_f \) of 0.39. In the reaction mixture a new \( \beta \)-lactam product in addition to unchanged PS-5, was detected with \( R_f \) 0.49, presumably the deacetylated analogue NS-5.

A long incubation period was required to ensure detection of product formation. Incubation could not be prolonged indefinitely as the product itself appeared to be unstable, possibly due to the presence of a free amino group on the C-3 side chain, (15). The activity of the acylase was marginally increased by Co\(^{2+}\) enzyme 'activation', (172). To enable detection of the deacetylated product a high concentration of PS-5 (20 \( \mu \)g/ml) was necessary in the incubation mixture. However it was not possible to achieve concentrations, of minor broth components, of this order in fermentation. Following unsuccessful attempts to concentrate the individual minor broth components in H.P.L.C. fractions by freeze-drying this approach to the identification of N-acetylated carbapenems was abandoned.

3.3.6 Susceptibility of Carbapenems to Renal Dipeptidase

Investigators at Merck discovered that Thienamycin and related carbapenems were extensively metabolised in vivo, predominantly in the kidney, (12,173). The renal enzyme, Dipeptidase 1 (Dehydropeptidase) E.C. 3.4.13.11., was
DEACETYLATION OF PS-5 BY ACYLASE 1

[PS-5 (20 µg/ml) incubated with Acylase 1, at 37°C, Co^{2+} 10^{-4} M. T.L.C. performed on Whatman LK 6DF plates with solvent system, ethanol:phosphate buffer (10 mM, pH 7.0), 7:3. β-Lactam zones detected bioautographically using β-lactamase induction assay]

PS-5, PS-5 control (20 µg/ml)
NFT, N-Formimidoyl thienamycin control (10 µg/ml)
1,2,3,4,5,6,PS-5 incubated with Acylase 1, respective incubation period 0, 0.5, 1.0, 1.5, 2.0 and 2.5 Hours.
SF - Solvent front
OR - Origin
found to be responsible for the metabolism of carbapenem antibiotics, which exhibit a structural homology to dehydro-peptides, Figure (3.9). Carbapenems and penems were found to be susceptible to hydrolysis by the Dipeptidase, whereas cephalosporins appeared to be insensitive to attack by the enzyme.

Carbapenem β-lactams vary in their susceptibility to hydrolysis by renal Dipeptidase, compounds with free C-3 cysteaminyl side chain being more stable than their N-acyl analogues, (12). Non basic N-acylated Thienamycin and naturally occurring N-acylated carbapenems were found to degrade 4 to 50 fold faster than Thienamycin when exposed to enzymatic hydrolysis by the Dipeptidase, (12).

Renal Dipeptidase was prepared (see Experimental), and used to detect carbapenems (and penems) in S. cattleya fermentation broth. Action of the enzyme on carbapenems was visualised as loss of, antibacterial activity, β-lactamase inhibitory properties, and ability to induce β-lactamase formation, Figure (3.14a-c). A comparison of the susceptibility of β-lactam standards to Dipeptidase 1 is available in Table (3.4).

S. cattleya co-produces both unacetylated, and N-acetylated carbapenems, (160,161,162), known to vary in their susceptibility to Dipeptidase 1. The β-lactam components in S. cattleya fermentation broth were separated by H.P.L.C., Figure (3.10). Relative susceptibility of the β-lactam fractions to Dipepidase 1 was determined, N-Formimidoyl thienamycin and PS-5 were used as standards, Figure (3.11). β-lactam components in fractions III, IV and V (i.e. 'late off' fractions) and
POSTULATED STRUCTURAL HOMOLOGY BETWEEN THIENAMYCIN AND DEHYDROPEPTIDES

Thienamycin

Dehydropeptide
( for example Glycyldehydro phenylalanine R = NH$_2$ R' = Phenyl )

Arrows indicate site of hydrolysis by Dipeptidase
TABLE (3.4)

SUSCEPTIBILITY OF $\beta$-LACTAM STANDARDS TO DIPEPTIDASE 1

<table>
<thead>
<tr>
<th>ANTIBIOTIC CONCENTRATION (µg/ml)</th>
<th>50 DHP</th>
<th>50 CONTROL</th>
<th>25 DHP</th>
<th>25 CONTROL</th>
<th>10 DHP</th>
<th>10 CONTROL</th>
</tr>
</thead>
<tbody>
<tr>
<td>PENICILLIN N</td>
<td>24</td>
<td>25</td>
<td>18</td>
<td>19</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>CEPHAMYCIN C</td>
<td>28</td>
<td>28</td>
<td>23</td>
<td>23.5</td>
<td>17</td>
<td>16.5</td>
</tr>
<tr>
<td>N-FORMIMIDOYL THIENAMYCIN</td>
<td>33.5</td>
<td>46</td>
<td>29</td>
<td>38</td>
<td>17.5</td>
<td>26</td>
</tr>
<tr>
<td>CEPHALOSPORIN C</td>
<td>27</td>
<td>27</td>
<td>24</td>
<td>24</td>
<td>19.5</td>
<td>19.5</td>
</tr>
<tr>
<td>DEACETOXY CEPHALOSPORIN C</td>
<td>20.5</td>
<td>21</td>
<td>17.5</td>
<td>17.5</td>
<td>13.5</td>
<td>13.5</td>
</tr>
</tbody>
</table>

DIAMETER INHIBITION ZONE / (mm), 20 µl LOADED ONTO WHATMAN ANTIBIOTIC ASSAY DISCS (6.35 mm) AND PLACED ON E. COLI SS SEEDED AGAR
FIGURE (3.10)

H.P.L.C. SEPARATION OF COMPONENTS IN S. CATTLEYA FERMENTATION BROTH, PRIOR TO DETERMINATION OF THEIR SUSCEPTIBILITY TO DIPEPTIDASE 1; FIGURE (3.11)

--- β-Lactamase induction assay

M.I.C. assay vs. S. aureus

H.P.L.C. conditions: column 4 mm ID x 250 mm (Technicol LiChrosorb), 500 μl injection loop, eluant 10 mM phosphate buffer pH 7.0, t₀ to t₂₀ minutes, t₂₀ to t₃₀ 15% methanol gradient added, elution up to t₆₀ at 15% MeOH, flow rate 1.2 ml/min., fractions collected 1 minute intervals

Sample: S. cattleya fermentation broth, Kahan medium (E) 94 hours (f₂), growth curve given Figure (2.3).

β-Lactams located in fractions using β-lactamase induction and MIC broth dilution assays, ( ) on Y axis indicate corresponding well on dilution plate

N.B.: incubation of broth with NH₂OH (conditions as given Figure (3.7) prior to H.P.L.C., reduced all values to zero.
FIGURE (3.11)

RELATIVE SUSCEPTIBILITY OF β-LACTAM COMPONENTS IN S. CATTLEYA FERMENTATION BROTH, TO DIPEPTIDASE 1

I, II, III, IV, V, H.P.L.C. fractions, with respective retention times, 7, 13, 33, 36 and 41 minutes, see Figure (3.10)

(i) N-Formimidoyl thienamycin (ii) PS-5

S. cattleya fermentation broth subjected to H.P.L.C. analysis, Figure (3.10), fractions I-V incubated with Dipeptidase (DHP) at 37°C, 0-30 minutes, remaining activity determined using β-lactamase induction assay (section 4.3.7.3)
THIN LAYER CHROMATOGRAPHY OF β-LACTAM COMPONENTS IN S. CATTLEYA FERMENTATION BROTH, PREVIOUSLY SEPARATED BY H.P.L.C.;

FIGURE (3.12)

T.L.C. performed on Whatman LK 6DF plates, solvent system, ethanol:phosphate buffer (10 mM, pH 7.0) 7:3.
Carbapenem controls, PS-5, N-Formimidoyl thienamycin, (15 μg/ml), 20 μl loaded/track β-Lactam zones detected bioautographically using β-lactamase induction assay

SF - Solvent front, OR - Origin
Zones indicate regions of β-lactamase induction and therefore presence of β-lactam, where 2nd zone observed within induction zone this was due to antibacterial activity of inducing β-lactam vs. B. licheniformis
Relative susceptibility of I, II, III, IV and V, to Dipeptidase 1 determined, Fig. (3.11).
PS-5 standard appear to be significantly more readily hydrolysed by the Dipeptidase than Thienamycin or the 'early off' fractions, I and II. This observation may indicate that the 'late off' fractions contain N-acetylated or N-acylated carbapenems.

3.3.7 Chromatographic Systems

3.3.7.1 Thin Layer Chromatography (T.L.C.)

Thin layer chromatography was used to separate the \( \beta \)-lactams co-produced in \textit{S. cattleya} fermentation broth. \( R_f \) values for the \( \beta \)-lactam standards are given in Table (3.5). \( \beta \)-Lactams were located on the T.L.C. plate using a bioautographic technique with \( \beta \)-lactamase induction, \( \beta \)-lactamase inhibition, or antibacterial assays.

Figures (3.13a-d) show T.L.C. separation and detection of \( \beta \)-lactam components in \textit{S. cattleya} broth, Kahan medium (E) and defined medium (C) (see later H.P.L.C. analysis of identical fermentation broth). Susceptibility of the different components in \textit{S. cattleya} broth (Kahan medium (E), 94 hours (\( f_2 \))), to hydrolysis by hydroxylamine and Dipeptidase 1 are shown in Figures (3.14a-c).

Dilution of fermentation broth allowed resolution of possibly three \( \beta \)-lactam components in the 0.36 - 0.56 \( R_f \) region, and possibly three more \( \beta \)-lactams in the 0.65 - 0.81 \( R_f \) region, detected by \( \beta \)-lactamase induction. H.P.L.C. analysis was therefore carried out to obtain better resolution of these components.

An attempt was made to relate the H.P.L.C. separation of \( \beta \)-lactam components, Figure (3.10), to the situation observed
TABLE (3.5)

THIN LAYER CHROMATOGRAPHY: $R_f$ VALUES FOR $\beta$-LACTAM STANDARDS

### SOLVENT SYSTEM

<table>
<thead>
<tr>
<th>$R_f$ VALUES</th>
<th>ETHANOL: 10mM PO$_4$ 7:3</th>
<th>ACETONITRILE: WATER 7:3</th>
<th>BUTANOL: ACETIC ACID: WATER 4:1:1</th>
</tr>
</thead>
<tbody>
<tr>
<td>PENICILLIN N</td>
<td>0.72</td>
<td>0.36</td>
<td>0.12</td>
</tr>
<tr>
<td>CEPHAMYCIN C</td>
<td>0.68</td>
<td>0.33</td>
<td>0.08</td>
</tr>
<tr>
<td>CEPHALOSPORIN C</td>
<td>0.71</td>
<td>0.34</td>
<td>0.05</td>
</tr>
<tr>
<td>DEACETOXY CEPHALOSPORIN C</td>
<td>0.65</td>
<td>0.32</td>
<td>0.07</td>
</tr>
<tr>
<td>N-FORMIMIDOYL THIENAMYCIN</td>
<td>0.39-0.44</td>
<td>0.21</td>
<td>I.I.T.S.</td>
</tr>
<tr>
<td>THIENAMYCIN</td>
<td>0.39</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>PS-5</td>
<td>0.75</td>
<td>NT</td>
<td>NT</td>
</tr>
</tbody>
</table>

N.T.: NOT TESTED

I.I.T.S.: INACTIVATED IN THIS SYSTEM
FIGURE (3.13)

T.L.C. SEPARATION OF COMPONENTS IN S. CATTLEYA FERMENTATION BROTH

(A) \( \beta \)-Lactamase Induction Assay

(B) P99 \( \beta \)-Lactamase Inhibition Assay

[T.L.C. performed, Whatman LK 6DF plates, solvent system, ethanol:phosphate buffer (10 mM, pH 7.0), 7:3. Broth components detected bioautographically, control NFT 20 \( \mu \)g/ml, 20 \( \mu \)l standard or broth loaded/track]

S. cattleya fermentation broth (growth curve Figure (2.3)), \((f_2)\) indicates Kahan Medium (E), fermentation time given in hours, \((f_3)\) indicates mycelium resuspended in defined medium \((t_{65})\), samples labelled as corresponding time in parent fermentation, \((f_2)\).
(C) T.L.C.-Bioautography vs. E. coli SS

(D) vs. S. aureus vs C. terigena

SF - Solvent front
OR - Origin
T.L.C. performed as Figure (3.13)

*S. cattleya* fermentation broth, \((f_2)\) 94 hours Kahan medium (E), used throughout, control, NFT 20 \(\mu g/ml\)

Susceptibility to Dipeptidase 1: Sample and control incubated with DHP at 37\(^\circ\)C for 30 min.

Susceptibility to hydroxylamine: Sample and control incubated with NH\(_2\)OH (8\(\mu\)m/ml) at room temp. for 25 min.
FIGURE (3.14) continued.

c) T.L.C.–Bioautography vs. E. coli SS

![TLC diagram]

NFT NFT 94 94 NFT NFT 94 94
+ + + + + +
DHP DHP DHP NH$_2$OH NH$_2$OH

(98)
with T.L.C., Figure (3.12). The H.P.L.C. fraction with retention time ($R_t$) 36 minutes revealed three β-lactam components on further T.L.C., with $R_f$ values 0.47, 0.70, 0.77. This may indicate that one of the components with higher $R_f$ was being converted to its deacylated analogue (see section 3.3.5).

Thin layer chromatography permitted separation, and detection, using β-lactamase induction and β-lactamase inhibition assays, of minor components in the N-Formimidoyl thienamycin standard. These minor components appeared to be carbapenems as they were susceptible to hydrolysis by hydroxylamine and Dipeptidase 1. At very high concentrations of the NFT standard, > 100μg/ml, antibacterial activity (vs S. aureus) was detected for these components. Edward O. Stapley (personal communication) suggested that these minor impurities could be minor broth components (or their N-formimidoyl derivatives) produced by S. cattleya, as our NFT standard had been prepared from a fermentation batch of Thienamycin. Later H.P.L.C. analysis of the NFT standard revealed that it also contained some unchanged Thienamycin.

3.3.7.2 High Performance Liquid Chromatography H.P.L.C.

High performance liquid chromatography was used to separate the β-lactams in S. cattleya fermentation as this gave better resolution of components than that obtained with T.L.C. Retention times ($R_t$) for the β-lactam standards are given in Table (3.6). Different retention times were observed for the NFT standard, depending on the column packing material, Figure (3.15) shows retention of NFT with
TABLE (3.6)

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY: RETENTION TIME ($R_t$) FOR $\beta$-LACTAM STANDARDS

<table>
<thead>
<tr>
<th></th>
<th>SPHERISORB 5 ODS 1</th>
<th></th>
<th>TECHNICOL RP$_{18}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RETENTION TIME ($R_t$)</td>
<td></td>
<td>RETENTION TIME ($R_t$)</td>
</tr>
<tr>
<td></td>
<td>(A)</td>
<td>(B)</td>
<td>(A)</td>
</tr>
<tr>
<td>PENICILLIN N</td>
<td>14.0</td>
<td>-</td>
<td>NT</td>
</tr>
<tr>
<td>CEPHAMYCIN C</td>
<td>9.0</td>
<td>8.4</td>
<td>NT</td>
</tr>
<tr>
<td>CEPHALOSPORIN C</td>
<td>35.0</td>
<td>34.5</td>
<td>NT</td>
</tr>
<tr>
<td>DEACETOXY CEPHALOSPORIN C</td>
<td>13.0</td>
<td>12.3</td>
<td>NT</td>
</tr>
<tr>
<td>N-FORMIMIDOYL THIENAMYCIN</td>
<td>38.0</td>
<td>37.5</td>
<td>19.0</td>
</tr>
<tr>
<td>THIENAMYCIN</td>
<td>27.0</td>
<td>26.4</td>
<td>NT</td>
</tr>
<tr>
<td>PS-5</td>
<td>44.0</td>
<td>43.6</td>
<td>38.0</td>
</tr>
</tbody>
</table>

(A): $R_t$ AS DETERMINED BY M.I.C. COLORIMETRIC ASSAY: E.COLISS

(B): $R_t$ AS DETERMINED BY U.V. SPECTROPHOTOMETRY $\lambda$ 260 nm,
($\lambda$ 299 nm FOR CARBAPENEMS)

N.T.: NOT TESTED
FIGURE (3.15)

H.P.L.C. RETENTION TIME FOR N-FORMIMIDOYL THIENAMYCIN WITH
TECHNICOL LiCHROSORB COLUMN PACKING

H.P.L.C. conditions: Column 4 mm I.D. x 250 mm (Technicol
LiChrosorb), 500 µl injection loop, eluant 10 mM phosphate
buffer pH 7.0, \( t_0 \) to \( t_{20} \) minutes, \( t_{20} \) to \( t_{30} \) 15% methanol
gradient added, elution up to \( t_{60} \) at 15% MeOH. Flow rate
1.2 ml/minute, fractions collected 1 minute intervals.

N-Formimidoyl thienamycin: 0.15 µg/ml in 10 mM phosphate
buffer pH 7.0. Retention time \( (R_t) \) for NFT determined
using \( \beta \)-lactamase induction and M.I.C. broth dilution assays.
H.P.L.C. RETENTION TIME FOR THIENAMYCIN AND N-FORMIMIDOYL
THIENAMYCIN, WITH SPHERISORB 5 ODS 1 COLUMN PACKING

(A) M.I.C. Assay
vs. S. aureus

(B) UV 299 nm
Thienamycin

(C) UV 299 nm
N-Formimidoyl thienamycin

H.P.L.C. conditions, as Fig. (3.15), Spherisorb 5 ODS1, standard used at 25 μg/ml. R_t determined using M.I.C. broth dilution assay, and UV absorbance 299 nm.
H.P.L.C. SEPARATION OF COMPONENTS IN S. CATTELYA FERMENTATION BROTH,
( KAHAN MEDIUM (E) )

(A) β-Lactamase Induction and M.I.C. Broth Dilution Assays

Key-
(see Fig. 3.18)

(B) P99 Inhibition Assay

(C) UV Absorbance 299 nm

H.P.L.C. Conditions: as Fig. (3.15), Technicol LiChrosorb Sample: S. cattleya broth, Kahan medium (E) 94 hours ($f_2$), growth curve Fig. (2.3)
H. P. L. C. SEPARATION OF COMPONENTS IN S. CATTELYA FERMENTATION BROTH, (DEFINED MEDIUM)

(A) β-Lactamase Induction and MIC Broth Dilution Assays

(B) P99 Inhibition Assay

(C) UV Absorbance 299 nm

H. P. L. C. Conditions: as Fig. (3.15), Technicol LiChrosorb
Sample: S. cattleya broth, defined medium, 118 hours (f3), growth curve Fig. (2.3)
Technicol LiChrosorb packing. Figure (3.16) shows the retention of NFT and of authentic Thienamycin standard with Spherisorb 5 ODS 1 packing, (NB. NFT standard appears to contain some unchanged Thienamycin). The different retention times observed for the NFT standard, probably reflect a difference in carbon loading of the column packing material.

The H.P.L.C. system used here was a modification of that of Wilson et al.,(162), who indicated that Thienamycin was less well retained than NFT. An observation that can be explained by the more polar nature of Thienamycin, when compared with its N-formimidoyl derivative. Thus prior to obtaining the Thienamycin standard from Merck, the Thienamycin component in S. cattleya broth was tentatively assigned as a β-lactam with retention time less than that of the NFT standard.

Separation by H.P.L.C., of the β-lactam components in S. cattleya fermentation broth ( Kahan medium (E) ) is shown in Figure (3.17). Figure (3.18) shows the separation of β-lactam components when the defined medium was used, and illustrates the value of the UV chromophore of Thienamycin, (λ 299 nm ). This UV absorption of carbapenems could not be applied to complex fermentation broth, for example medium (E), due to the high UV background.

The major β-lactam component in S. cattleya broth had a retention time (R_t) of 11/12 minutes, notably less than that of the NFT standard ( R_t 19 minutes ), with Technicol LiChrosorb packing. In addition several other β-lactam components were detected, a number having considerably longer retention time than NFT, suggesting they were of a less polar nature, and supporting the view that these components could
H.P.L.C. SEPARATION OF COMPONENTS IN S. CATTLEYA FERMENTATION BROTH, (KAHAN MEDIUM (E)), WITH SPHERISORB 5 ODS 1 COLUMN PACKING

(A) M.I.C. Broth Dilution Assay
(Activity vs. E. coli SS
omitted for clarity.
Fractions 29, 36 act.
≡ 1/16
dilution factor)

(B) P99 β-Lactamase Inhibition Assay

H.P.L.C. Conditions: as Figure (3.15), Spherisorb 5 ODS 1

Sample: S. cattleya Fermentation broth Kahan medium (E),
94 hours (f₂), growth curve Figure (2.3).
Broth used here identical to that in Figure (3.17) - note effect of column packing on (Rₜ) of major β-lactam component
be N-acylated carbapenems. Figure (3.19) demonstrates the effect of hydroxylamine on the β-lactam components in fermentation broth. Relative susceptibility of the different β-lactams to hydrolysis by Dipeptidase 1 is discussed in section (3.3.6).

H.P.L.C. separation of β-lactams in *S. cattleya* fermentation broth, was also performed using Spherisorb 5 ODS 1 packing. Identical broth was used, so a direct comparison can be made between Figure (3.17) and (3.19). With Spherisorb packing the retention time ($R_t$) of the major β-lactam component was 29 minutes, ($R_t$) for NFT was 38 minutes and 27 minutes for the authentic Thienamycin standard.

3.4 IDENTIFICATION OF β-LACTAMS IN S. CATTLEYA FERMENTATION BROTH

3.4.1 Thienamycin

On the following criteria Thienamycin was identified in *S. cattleya* broth as a component that:-

i) Demonstrated the ability to induce β-lactamase formation in *B. licheniformis*

ii) Demonstrated the ability to inhibit P99 β-lactamase.

iii) Demonstrated antibacterial activity vs *S. aureus*, *C. terigena*, and *E. coli*

iv) Demonstrated susceptibility to hydroxylaine at low concentration, and gave the desired difference spectrum

v) Demonstrated susceptibility to renal Dipeptidase 1, of the same order as the N-Formimidoyl thienamycin standard.

iv) Demonstrated a close correlation with expected $R_f$ value on T.L.C.
vii) Demonstrated a close correlation with expected $R_t$ on H.P.L.C.

3.4.2 Minor Broth Components

In addition to the major fermentation product Thienamycin, several minor broth components were detected, Figures (3.17, 3.18, 3.19), which acted as inducers for $\beta$-lactamase formation in \textit{B. licheniformis}. These components were initially detected by their ability to inhibit the P99 $\beta$-lactamase, which appeared to be superior to that of the NFT standard. Susceptibility of these components to hydroxylamine and Dipeptidase 1 suggested they were carbapenems. In some cases they were hydrolysed more readily by the Dipeptidase, than the NFT standard.

The minor broth components may be carbapenems of the $N$-acetylated or $N$-acylated variety (their mobilities on T.L.C. and H.P.L.C. suggest they are of a less polar nature than NFT), with varying stereochemistry, which would account for their $\beta$-lactamase inhibitory properties.

3.5 SUMMARY

This chapter covers a number of analytical techniques, used in the detection and identification of Thienamycin and co-produced $\beta$-lactam antibiotics, in \textit{S. cattleya} fermentation broth.

Assays were designed to discriminate between co-produced carbapenems and $\beta$-lactams on the basis of their structure-activity relationships.

The major $\beta$-lactam fermentation product was identified as Thienamycin.
CHAPTER FOUR

EXPERIMENTAL
4.1 CULTURE MAINTENANCE

Streptomyces cattleya NRRL 8057 was obtained, as freeze dried ampoules, from ICI Pharmaceuticals PLC. The organism was incubated on Malt-Yeast agar (g/L, malt extract, 10.0, yeast extract, 4.0, glucose, 4.0, bacteriological agar (Oxoid No. 1), 20.0), at 25°C for a period of 14 days. Spore suspensions were prepared by pipetting a solution of 50% glycerol : 50% distilled water, onto the surface of the agar, spores were evenly dispersed by gentle pipetting.

Spore counts were carried out as follows; the suspension was serially diluted in 0.85% saline, over the range 10^{-2} - 10^{-10}, 0.1 ml of each dilution was spread on the surface of an agar plate, all operations being carried out in duplicate. Colony counts were performed after incubation of plates at 25°C for 3 days. The spore suspension diluted to 1 x 10^8 c.f.u/ml, was stored in aliquots at -20°C.

4.2 BATCH FERMENTATION OF S. CATTLEYA

4.2.1 Preparation of Seed Inoculum, (f₁)

The spore suspension was allowed to come to room temperature, 0.25 ml was inoculated into 25 ml of seed medium in a 250 ml conical flask. Flasks were incubated in a Gallenkamp Cooled Orbital Incubator, at 25°C, 220 r.p.m. for up to 3 days. Figure (2.2) shows a typical growth curve for the seed inoculum stage.

4.2.2 Batch Fermentation in Complex Medium, (f₂)

2.5 ml of the seed culture (f₁), was used to inoculate 25 ml of medium (E), (10), in a 250 ml conical flask. Flasks
were incubated, at 25°C, 220 r.p.m., as for the seed culture. Biomass (as g wet cell/litre) and fermentation pH were recorded. Fermentation broth was analysed for biological activity (see section (4.3)). Figure (2.3) shows a typical growth curve.

4.2.3 Batch Fermentation in 'Defined' Medium, (f₃)

*S. cattleya* was grown in medium (E) as indicated, after exponential growth had ceased (approx. 30 hours (f₂)) and at subsequent stages during the fermentation, the mycelium was washed and resuspended in medium (C),(¹³³). The culture was spun in a bench centrifuge for 5 minutes, the supernatant removed and an equal volume of medium (C) added, mycelium was gently resuspended using a glass rod and centrifuged a second time. The supernatant was replaced by an equal volume of fresh medium (C). Resuspended mycelium from all flasks was pooled to ensure uniformity, and finally returned to the incubator (25 ml resuspended culture/250 ml flask), all procedures were carried out aseptically.

Fermentation was allowed to proceed, biomass and pH were recorded, broth samples were stored at -70°C prior to analysis for biological activity (as were samples from (f₂) fermentation). Figure (2.4) shows a typical growth curve.

4.3 ANALYTICAL TECHNIQUES

4.3.1 Chromatographic Systems

4.3.1.1 Thin Layer Chromatography

Thin layer chromatography (T.L.C.) was carried out using Whatman linear K silica T.L.C. plates, LK 6 DF 20 x 20 cm, 250μ layer thickness. Solvent systems used were, acetonitrile: water 7:3, ethanol:phosphate buffer (10 millimolar, pH 7.0),
The ethanol:phosphate buffer system was used routinely. T.L.C. plates were run to completion in the solvent system and dried thoroughly. Beta lactam zones were located bioautographically using, the $\beta$-lactamase induction assay, the P99 inhibition assay, or antibacterial assays. N-Formimidoyl thienamycin was used as a marker.

$R_f$ values for $\beta$-lactam standards in the different solvent systems are given in Table (3.5). T.L.C. was used to separate the $\beta$-lactam components in S. cattleya fermentation broth, see Figures (3.13), (3.14).

4.3.1.2 High Performance Liquid Chromatography

The procedure used was a modification of that of Wilson et al. (162).

A Spectra-Physics Solvent Delivery System II model SP8 700 was used. Column effluent was monitored at 299 nm (carbapenems) or 260 nm (other $\beta$-lactams), using a Pye Unicam LC-UV variable wavelength detector. Column dimensions were 4 mm I.D. x 250 mm (pre-column 4 mm I.D. x 40 mm) with packing material, Technicol LiChrosorb RP$_{18}$ (10$\mu$ pore size), or Spherisorb 5 ODS 1 (5$\mu$ pore size). Chromatography was performed at the ambient temperature (approx. 22°C), a 500 microlitre injection loop was used. The column was eluted (at flow rate 1.2 ml/minute) with 10 millimolar phosphate buffer, pH 7.0, $t_{z0}$ to $t_{20}$ minutes, between $t_{20}$ - $t_{30}$ minutes a 15% methanol gradient was added, and elution allowed to proceed up to $t_{60}$ minutes. Fractions were collected at one minute intervals and assayed for activity.
Retention times ($R_t$) for the $\beta$-lactam standards are given in Table (3.6). H.P.L.C. was used to resolve the $\beta$-lactam components in *S. cattleya* fermentation broth, Figures (3.17), (3.18), (3.19). Prior to H.P.L.C. fermentation samples were centrifuged in a Gallenkamp Minifuge for 5 minutes at the high speed setting, or Millipore filtered (type HA, 0.45µm pore size) to remove particulate matter. Following H.P.L.C. collected fractions could be stored at 4°C for 48 hours with no apparent loss of activity.

Wilson et al.,(162), indicated that column performance deteriorated significantly with age. Therefore column efficiency was always checked prior to use, and the column head repacked as necessary.

4.3.2 Antibacterial Assays

4.3.2.1 Broth Dilution : M.I.C. Colorimetric Assay

i) Preparation of Test Organism Cultures

Broth cultures of the test organisms were prepared by overnight incubation at 37°C. *E. coli* and *C. terigena* were grown in Nutrient Broth (Oxoid CM 1), *S. aureus* was grown in Brain Heart Infusion Broth (Gibco).

A viable cell count was performed by serial dilution of the overnight cultures, in 0.85% saline, over the concentration range $10^{-3} - 10^{-8}$. 0.1 ml of each dilution in the series was spread on the surface of a nutrient agar plate and incubated overnight at 37°C. All operations were carried out in duplicate. Following overnight incubation of plates, colony forming units per millilitre (c.f.u./ml) of the original cultures were determined.
Prior to use in the M.I.C. assay, overnight cultures of the test organisms were diluted to $10^{-3}$ in broth, incubated at 37°C for one hour and used immediately.

ii) M.I.C. Colorimetric Assay

80 μl of Nutrient Broth or Brain Heart Infusion Broth (Phenol red indicator 0.02%) was added to each of the 96 wells of a microtitre plate (purchased from Flow Laboratories LTD). 80 μl of the test sample added to well (A) and 2-fold serially diluted through wells (A) to (H), twelve samples tested per plate. 20 μl of a culture of the test organism was then added to each well of the plate (approx. $10^4$ c.f.u./well). The microtitre plate was sealed using acetate strip (Flow Laboratories), and incubated overnight at 37°C. In wells in which growth had occurred a colour change from red to yellow was observed, where growth was inhibited by antibiotic no colour change occurred, see Figure (4.1).

iii) Determination of M.I.C. of β-lactam Standards vs. Test Organisms

Solutions of β-lactam antibiotic standards, Cephamycin C, Penicillin N, Cephalosporin C, N-Formimidoyl thienamycin PS-5, were prepared in 10mM phosphate buffer, at a concentration of 160 μg/ml. Standard solutions were filter sterilised using Millipore filters (type HA, 0.45 μ). Solutions were 2-fold serially diluted in sterile 10 mM phosphate buffer, over the concentration range 100 - 0.313 μg/ml. Test solutions were used to determine the M.I.C.'s for the β-lactam standards vs. the test organisms, Table (3.2).
FIGURE (4.1)

THE M.I.C. COLORIMETRIC ASSAY

Samples (H.P.L.C. fraction, antibiotic standard, fermentation broth or phosphate buffer) serially diluted in broth (+ 0.02% phenol red indicator), 20 μl overnight culture of test organism added (10^4 c.f.u./well), plate read following overnight incubation, 37°C

(+) denotes growth of test organism (S. aureus, C. terigena E. coli SS) phenol red indicator undergoes colour change red to yellow.

(-) absence of growth of test organism.

C-rows 9,10 - negative growth control i.e. no inoculation of test organism

C-rows 11,12 - positive growth control i.e. inoculated with test organism, phosphate buffer used for dilutions.

AB- antibiotic standard, e.g. NFT M.I.C. vs S. aureus = 0.0045 μg/ml

SAMPLE (4) demonstrates antibacterial activity, growth of test organism inhibited wells A to F.
4.3.2.2 T.L.C. - Bioautography

Samples were separated using thin layer chromatography, section 4.3.1.1. The T.L.C. plates were then dried and placed in contact with agar seeded with one of the test organisms. Large bioassay plates (20 x 20 cm) were used, approximately $10^8$ C.f.u./100 ml, Diagnostic Sensitivity Test agar (Oxoid CM 261) gave just confluent growth. After 30 minutes at room temperature the T.L.C. plates were removed, the bioassay plates were incubated overnight at 37°C.

Bioautographic detection limit for β-lactam standards, determined by spotting solutions of the standards on Whatman No. 1 paper allowing the papers to dry and overlaying them on seeded agar as indicated, are shown in Table (4.1).

4.3.3 β-Lactamase Inhibition Assay

4.3.3.1 Preparation of Nitrocefin Reagent

Fifty milligrams of Nitrocefin was dissolved in 5 ml D.M.S.O., and made up to 120 ml with 50 mM phosphate buffer, as required.

4.3.3.2 Preparation of Nitrocefin Impregnated Paper

Whatman No. 1 paper was cut to size, and wetted completely in Nitrocefin solution (as above). Papers were shaken free of excess solution, air dried in a fume cupboard, and stored at -20°C. (100 mls of the solution was sufficient for approximately 20 papers, 9 x 20 cm).

4.3.3.3 Preparation of Enterobacter cloacae P 99 β-Lactamase

A strain of _E. cloacae_ P 99 was obtained from I.C.I. Pharmaceuticals, the organism was maintained on Nutrient agar slopes at 4°C.
### TABLE (4.1)

**BIOAUTOGRAPHIC DETECTION LIMIT FOR β-LACTAM STANDARDS**

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>E. COLI SS</th>
<th>S. AUREUS</th>
<th>C. TERIGENA</th>
</tr>
</thead>
<tbody>
<tr>
<td>PENICILLIN N</td>
<td>0.1</td>
<td>0.5</td>
<td>10.0</td>
</tr>
<tr>
<td>CEPHAMYCIN C</td>
<td>0.1</td>
<td>15.0</td>
<td>2.0</td>
</tr>
<tr>
<td>CEPHALOSPORIN C</td>
<td>0.05</td>
<td>2.0</td>
<td>5.0</td>
</tr>
<tr>
<td>DEACETOXY CEPHALOSPORIN C</td>
<td>0.1</td>
<td>15.0-20.0</td>
<td>10.0</td>
</tr>
<tr>
<td>N-FORMIMIDOYL THIENAMYCIN</td>
<td>0.02</td>
<td>0.002</td>
<td>0.05</td>
</tr>
</tbody>
</table>

**MICROGRAMS (µg) ANTIBIOTIC REQUIRED TO PRODUCE AN INHIBITION ZONE**
E. cloacae P 99′ was used to inoculate 50 ml of Brain Heart Infusion broth (Gibco) in a 250 ml conical flask. The culture was incubated overnight, 37°C, 180 cycles/minute, in a Gallenkamp Orbital Incubator. A further 10 flasks were inoculated with 1 ml of the overnight culture, and shaken at 37°C, 180 cycles/min., for 18 hours, (174). Cells were harvested by centrifugation at 5500g for 15 min. (Beckman J2-21, J 14 rotor 4 x 250 ml). The cells were washed in 100 ml 0.1 M phosphate buffer and centrifuged at 5500g for 15 min. (J 17 rotor 14 x 50 ml). Following resuspension in 50 ml 0.1 M phosphate buffer, cells were subjected to ultrasonic disruption to release the cell bound β-lactamase. A Sonicator™ Cell Disrupter (Heat Systems Ultrasonics Inc) Model W375 was used. Sonication was allowed to proceed for 4 minutes (pulse 50% of cycle), with ice/water cooling. Cell debris was removed by centrifugation at 17,000g for 45 minutes, (J 17 rotor, 14 x 50 ml). The crude enzyme preparation was dialysed against 10 mM phosphate buffer overnight, and stored at -20°C. Enzyme activity was assayed using the chromogenic substrate Nitrocefin.

4.3.3.4 P 99 β-Lactamase Inhibition Assay

i) Broth Dilution Assay

Assays were performed in microtitre plates, 8 x 12 wells (Flow Laboratories). Eighty microlitres of 10 mM phosphate buffer was added to each well. Eighty microlitres of the standardised enzyme preparation was added to wells 1 - 12 in row (A), the enzyme was serially diluted down rows (A) to (H). Twenty microlitres of standard antibiotic solution or test broth was added to wells (A) to (H) in line 1 of the plate, and so on for each sample (12 samples tested/plate).
Twenty microlitres of the Nitrocefin solution was then added to each well, and the plate incubated at 37°C for 15 min.

N-Formimidoyl thienamycin was used as the antibiotic standard (see Figure (3.4)), thus P99 inhibitory activity in fermentation broth could be estimated.

ii) T.L.C. - Bioautography

Samples were separated by T.L.C. (section 4.3.1.1), the T.L.C. plates was dried and placed in contact with agar containing the P99 β-lactamase. After 30 minutes at room temperature, the T.L.C. plate was removed, Nitrocefin impregnated paper was placed on the surface of the agar. Following incubation at 37°C for 15 minutes, β-lactamase inhibitors were visualised as yellow zones on a red background. Detection limit for N-Formimidoyl thienamycin was of the order 0.05 µg.

4.3.3.5 Inhibition of P99 β-Lactamase by β-Lactam Standards

Solutions of the β-lactam standards Cephemycin C, Penicillin N, Cephalosporin C, Deacetoxycephalosporin C, N-Formimidoyl thienamycin, MM 13902, MM 22383 and MM 2238 were prepared in 10 mM phosphate buffer. The β-lactam standards were tested for P99 β-lactamase inhibitory properties using the broth dilution assay.

4.3.4 β-Lactamase Induction Assay

A β-lactamase induction assay (property of I.C.I. Pharmaceuticals PLC.) was used to detect β-lactam antibiotics in S. cattleya fermentation broth.

4.3.4.1 Broth Dilution Assay

Samples were serially diluted with an overnight culture
of the test organism, there followed a period of incubation to allow for $\beta$-lactamase induction. The enzyme was detected by addition of the Nitrocefin reagent and further incubation. A standard solution of Ampicillin (0.02 μg/ml) was used as a control, see Figure (4.2).

4.3.4.2 T.L.C. - Bioautography

Samples were separated by T.L.C., the T.L.C. plate was dried and placed in contact with agar seeded with test organism, for 30 minutes at room temperature. After removal of the T.L.C. plate, the agar plate was incubated to allow for enzyme induction. $\beta$-Lactamase was detected (as a red zone on yellow background), by placing Nitrocefin impregnated paper on the agar surface and further incubation. See Figure (3.13).

4.3.5 Hydroxylamine Extinguishable Absorbance 299 nm

4.3.5.1 Preparation of Hydroxylamine Reagent

The hydroxylamine reagent was prepared just prior to use by equimolar addition of potassium hydroxide to hydroxylamine hydrochloride.

4.3.5.2 N-Formimidoyl thienamycin, Hydroxylamine Extinguishable Absorbance, 299 nm

A solution of NFT at concentration 10μg/ml in 10 mM phosphate buffer was prepared, and incubated with hydroxylamine, (final concentration in reaction mixture 8 μM/ml), at room temperature for 25 minutes. Loss of absorbance at 299 nm was recorded, see Figure (3.6).

4.3.5.3 Effect of Hydroxylamine on $\beta$-Lactam Standards

Solutions of the $\beta$-lactam standards, Cephamycin C,
**FIGURE (4.2)**

**β-LACTAMASE INDUCTION ASSAY**

Samples (H.P.L.C. fraction, antibiotic standard fermentation broth, or phosphate buffer) serially diluted in *B. licheniformis* broth culture, following incubation at 37°C, 20 μl Nitrocefin solution added to each well, to detect presence of induced β-lactamase.

(+) denotes presence of induced β-lactamase, Nitrocefin hydrolysed, colour change yellow → red.

(-) no β-lactamase induction, in absence of β-lactamase
Nitrocefin remains yellow.

Samples (9-12) Ampicillin control (0.02 μg/ml), wells 9-12 A, (-) due to antibacterial activity of Ampicillin vs *B. licheniformis*

Sample (4) demonstrates β-lactamase induction, wells A-G, and is the major β-lactam containing fraction.
Penicillin N, N-Formimidoyl thienamycin, Cephalosporin C, Deacetoxy cephalosporin C were prepared at a concentration of 1 mg/ml (1 μl = 1 μg standard).

Antibiotic standards were incubated with hydroxylamine (final concentration in reaction mixture 32 - 4 μM/ml), for 25 minutes at room temperature. 1 μl of the reaction mixture was spotted onto Whatman No. 1 paper, after drying the papers were overlayed on seeded agar plates for 30 minutes at room temperature. The plates were incubated at 37°C overnight, *E. coli* was used as the test organism, (100 ml D.S.T. agar seeded with 0.1 ml of an overnight culture), see Table (3.3).

4.3.5.4 Differential Spectrophotometry

Difference spectra were obtained using a Pye-Unicam SP8-100 Ultraviolet Spectrophotometer, in the scanning mode. Reacted and unreacted samples were scanned in the region 401.5 ~ 187 nm. Bandwidth was set at 2 nm, with a wavelength speed of 1 nm/cm. See Figure (3.7)

4.3.6 Deacylation of PS-5

Acylase I (N-acylamino acid amido hydrolase E.C. 3.5.1.14) was purchased from Sigma Chemical Company. One unit of activity hydrolysed 1 μM of N-acetylmethionine per hour, pH 7.0, 25°C.

The enzyme was prepared over the concentration range 2 - 32 mg/ml in 10mM phosphate buffer pH 7.0 (1 mg solids contained 1805 units of enzyme activity). PS-5 was used at a concentration of 20 μg/ml. Ninety microlitres of the antibiotic solution was incubated with 10 μl of enzyme solution for up to 4 hours at 37°C, samples were taken at half hourly
intervals. Samples were loaded on Whatman LK 6DF chromatography plates, 20 μl loaded/track. T.L.C. plates were run in the solvent system, ethanol:phosphate buffer, 10 mM pH 7.0, 7:3, for 3 hours and air dried. Deacylation of PS-5 was detected using the β-lactamase induction assay. Acylase 1 was 'activated' by Co²⁺ over the range 1 x 10⁻² - 1 x 10⁻⁵ M, for optimal deacylation of PS-5, the acylase was used at a concentration of 32 mg/ml, Co²⁺ 1 x 10⁻⁴ M, and incubation at 37°C for 2 hours, see Figure (3.8).

4.3.7 Susceptibility of Carbapenems to Renal Dipeptidase

4.3.7.1 Preparation of Dipeptidase from Pig Kidney

The method used was a modification of that of B. J. Campbell (175) and is summarised in Figure (4.3).

Dipeptidase activity was increased by addition of ZnSO₄·7H₂O to the enzyme preparation, to a final concentration of 3 mM. 'Activation' was allowed to proceed overnight at 4°C, and the enzyme used within 3 days. Addition of Zn²⁺ permitted a reduction of the incubation period from 3 hours to 30 minutes. Enzyme activity was diluted to obtain a standard preparation, which under the incubation conditions used (see below) gave a reduction of 8 to zero wells in the β-lactamase induction assay, with the NFT standard.

4.3.7.2 Effect of Renal Dipeptidase on β-Lactam Standards

Solutions of the β-lactam standards Cephamycin C, Penicillin N, N-Formimdoyl thienamycin, Cephalosporin C, Deacetoxy cephalosporin C, in 10 mM phosphate buffer were made at concentrations of 50, 25, and 10 μg/ml. One hundred and sixty microlitres of the β-lactam solution was incubated with forty microlitres of Dipeptidase or phosphate buffer for
SUMMARY OF PREPARATION OF DIPEPTIDASE FROM PIG KIDNEY,

Fresh pig kidney, 2 Kg, (skin, fat and medulla removed), homogenised with 2 litres of iced water

pellet discarded ← centrifuged, 1300g, 20 min.

pellet discarded ← centrifuged, 1300g, 20 min.

supernatant adjusted to pH 5, 1 M HCl

pellet discarded ← supernatant adjusted to 20% saturation (NH₄)₂SO₄, left 1 hour, 0°C.

pellet retained ← supernatant adjusted to 30% saturation (NH₄)₂SO₄, 1 hour, 0°C.

pellet retained ← supernatant adjusted to 60% saturation (NH₄)₂SO₄

pellet retained ← centrifuged, 1300g, 20 min.

pellets and supernatant assayed for activity vs glycyldehydrophenyl alanine by following loss of absorbance, 275 nm.

Dipeptidase activity located in 40% pellet, crude Dipeptidase was stored in aliquots at -70°C.
30 minutes at 37°C. Twenty microlitres of incubation mixture was loaded on Whatman Antibiotic Assay discs, (6.35 mm diameter). Assay discs were dried and placed on seeded agar (E. coliSS, 0.1 ml overnight culture to 100 ml D.S.T. agar). After a 30 minute period at room temperature (prediffusion) seeded plates were incubated overnight at 37°C, see Table (3.4). N-Formimidoyl thienamycin was found to be appreciably more sensitive to hydrolysis by Dipeptidase than other β-lactam standards, under these conditions.

4.3.7.3 Relative Susceptibility of β-Lactams Co-produced in S. cattleya Fermentation Broth, to Renal Dipeptidase (DHP)

A sample of S. cattleya fermentation broth, medium (E) 94 hours (f2), was separated using H.P.L.C., fractions 1-60 were collected and assayed for β-lactamase induction activity and antibacterial activity vs. S. aureus, Figure (3.10). Peak fractions were labelled I to V with corresponding retention times 5/7, 13, 33, 36 and 41 minutes.

Susceptibility of fractions I to V to the Dipeptidase was determined using the β-lactamase induction assay. N-Formimidoyl thienamycin and PS-5 were used as standards. Sixty four microlitres of the sample (H.P.L.C. fraction or antibiotic standard) was incubated with 16 μl DHP, a control was included in which DHP was replaced by 10 mM phosphate buffer. Incubation was carried out at 37°C, 0 - 30 minutes, in microtitre plates, the induction assay was then carried out as previously described. Reduction in the ability to induce β-lactamase formation (expressed as % t = 0) was plotted against incubation time for fractions I to V and for NFT and PS-5, see Figure (3.11).
CHAPTER FIVE

DISCUSSION
5.1 INTRODUCTION

Chapter five reviews the results of our studies with S. cattleya, and presents an outline of current understanding of the biosynthesis of carbapenems in Streptomyces sp., with particular attention to the production of Thienamycin by S. cattleya. Recent developments in the field of biosynthesis, such as the use of genetic engineering and protoplast fusion techniques, are also discussed.

Thienamycin is of considerable importance as a chemotherapeutic agent, in spite of its superior properties Thienamycin (or the N-formimidoyl derivative NFT) may be subject to problems associated with the clinical use of β-lactam antibiotics. NFT was found to be capable of inducing β-lactamase formation in clinical isolates, which could then interfere with activity of the antibiotic. Clinical strains of Bacteroides fragilis, and Pseudomonas maltophilia, have been shown to produce β-lactamase which can hydrolyse NFT. Preincubation of Methicillin resistant Staphylococcus aureus (M.R.S.A.), clinical isolates with sub-inhibitory concentrations of NFT resulted in markedly increased M.I.C.'s for both Methicillin and NFT, a phenomenon thought to be due to induction of an altered penicillin binding protein, P.B.P.2', rather than β-lactamase induction.

Emergence of resistant organisms emphasises the continuing need for new and novel β-lactam antibiotics, together with novel strategies for administration of already known β-lactams. Synthetic schemes for modification of the carbapenem nucleus,
have been designed and may provide altered carbapenem structures of clinical significance.\(^{(181,182)}\) Increased knowledge of the biosynthetic pathway and its control, may lead to discovery of new carbapenems via 'directed biosynthesis'.

5.2 **PRODUCTION OF THIENAMYCIN AND RELATED CARBAPENEMS BY**

**S. CATTLEYA**

Chapters two and three present the results of studies on the production of Thienamycin and related carbapenems by *S. cattleya*. Fermentation was carried out in both complex and defined medium. The major β-lactam product, of fermentation, was identified as Thienamycin. Although progress of the work was impeded by problems of culture maintenance, and a significant degree of variability in growth of the organism and in antibiotic production, it may be possible to draw some general conclusions.

A batch fermentation system, as used here, presents a poorly defined system for investigation of the parameters controlling antibiotic formation. The organism is constantly adapting to a new environment, thus a steady state may never be achieved. With continuous culture a steady state could be maintained, and individual variables studied more rationally. Distinction between growth rate effects and effects due to specific substrates,\(^{(126)}\), may then have been possible. Use of washed cells may have permitted description of certain features at the enzyme level,\(^{(183)}\). A particular pattern of primary metabolic enzymes (e.g. enzymes of nitrogen assimilation) may be conducive to antibiotic production. Manipulation of growth conditions could allow imposition of this desirable pattern of enzymes in the cell.
Several analytical techniques for the identification of carbapenems in *S. cattleya* fermentation broth have been evaluated. The major β-lactam product behaved in a manner consistent with it being Thienamycin. Other minor β-lactam products were also detected, but have not been satisfactorily identified. Their behaviour in a number of assays suggested they were carbapenem in nature, (see chapter three).

Further identification of these minor broth components will necessitate their isolation and purification, with subsequent chemical analysis, an approach that would require large scale fermentation, due to the low fermentation productivity and chemical instability of these components, (160,161,162).

Although in this instance isolation and purification of fermentation products was not achieved, feeding studies using radiolabelled precursors and application of autoradiographic techniques, may have yielded interesting results without the need for purification. However time limitations prevented our work in this area from proceeding beyond a very preliminary stage.

5.3 BIOSYNTHESIS OF CARBAPENEMS

Present knowledge of the biosynthetic pathway leading to the carbapenem class of β-lactams is very sparse and fragmentary. Limited studies involving administration of isotopically labelled likely precursors, and the use of blocked mutants, have allowed speculation as to the nature of the biosynthetic sequence, (30). An overview of currently available information is given here. Figure (1.3) shows the chemical structures of carbapenems, (See page 12).
5.3.1 Studies using Isotopically Labelled Precursors

5.3.1.1 Origin of the Five-Membered Ring of Thienamycin

Preliminary studies suggested that glutamic acid was the precursor of the five-membered ring of Thienamycin, and that the glutamate molecule was incorporated intact,\(^{(119)}\), see Figure (5.1). An observation that allowed comparison with the origin of the five-membered ring of Clavulanic acid, also thought to be derived from glutamate,\(^{(96)}\). Recent studies however suggest that ornithine is in fact the precursor of Clavulanic acid,\(^{(117)}\), see Figure (1.8).

5.3.1.2 Origin of \(\text{C}_6\) and \(\text{C}_7\) \(\beta\)-Lactam Carbons

Investigations by workers at Merck indicated that acetate was efficiently incorporated into Thienamycin by \(S.\text{cattleya}\),\(^{(119)}\). Feeding experiments with \((1,2-^{13}\text{C})\) and \((2-^{14}\text{C})\) acetate demonstrated incorporation of intact acetate units predominantly in \(\text{C}_6-\text{C}_7\)\(^{(136)}\). An acetate unit was also incorporated to a lesser extent at \(\text{C}_4-\text{C}_5\), which could be explained as \((1,2-^{13}\text{C})\) labelled acetate yields \((4,5-^{13}\text{C})\) glutamate via the tricarboxylic acid cycle (TCA), which in turn gives Thienamycin enriched for \((^{13}\text{C})\) at \(\text{C}_4-\text{C}_5\), see Figure (5.2).

5.3.1.3 Origin of the \(\text{C}_6\) Hydroxyethyl Side Chain

The possibility that the hydroxyethyl side chain of Thienamycin could be formed by two single carbon transfers has been investigated,\(^{(136)}\). \(\text{L- (methyl }^{14}\text{C})\) methionine was very efficiently utilised in biosynthesis of the antibiotic by \(S.\text{cattleya}\). Experiments with \(\text{L- (methyl }^{13}\text{C})\) methionine revealed a high degree of incorporation of \((^{13}\text{C})\) at \(\text{C}_8\) and
PRELIMINARY STUDIES OF THIENAMYCIN BIOSYNTHESIS USING RADIOLABELLED PRECURSORS, ORIGIN OF THE CARBON SKELETON, (119)

I) Incorporation of deuterium from L-(2,4,4,2\text{H}_3) glutamic acid

\[
\text{L-(2,4,4,2\text{H}_3) glutamic acid} \xrightarrow{S. cattleya} \text{(4,2\text{H}_1) Thienamycin}
\]

II) Incorporation of $^{13}\text{C}$ from (\gamma-carboxyl $^{13}\text{C}$) glutamic acid

\[
\text{L-(5,$^{13}\text{C}$) glutamic acid} + \text{\textbullet CO}_2 \xrightarrow{S. cattleya} \text{(5,$^{13}\text{C}$) Thienamycin}
\]

(131)
INCORPORATION OF $^{1,2\,^{13}}C$ ACETATE INTO THIENAMYCIN, (119)

![Chemical diagram](image)

$(6,7^{13}C)$Thienamycin
(predominantly)

$(4,5^{13}C)$Thienamycin

(132)
C(9), with no apparent incorporation at any other site. The hydroxymethyl side chain of Northienamycin was also found to have methionine as its precursor. $^{14}$C) formate was very poorly incorporated into the C(6) side chain probably due to the high level of formate dehydrogenase in S. cattleya (136). Formation of an ethyl group from two methyl groups has already been documented with respect to the C(24) ethyl group of certain plant sterols, (139).

5.3.1.4. Origin of the C(3) Side Chain

Experiments were carried out with $^{14}$C) or $^{35}$S) cystine, and $^{35}$S) pantethine to determine the likely precursor of the C(3) side chain of Thienamycin, (S. cattleya possesses enzymes capable of reducing a number of disulphide compounds to their sulphydryl form ), (136).

L-(U-$^{14}$C) and $^{35}$S) cystine were very efficiently incorporated into the Thienamycin molecule, radioactivity being located in the C(3) side chain. Molar specific incorporations in excess of 70% of the theoretical maximum were observed. In contrast $^{35}$S) pantethine was very poorly incorporated, (136). Williamson et al favour a direct role for cysteine in the formation of the cysteaminyl side chain of Thienamycin, (136).

Studies with $^{3}$H) & $^{35}$S) cystine demonstrated that the cysteaminyl side chain was not unsaturated during its synthesis. The Thienamycin synthesised was found to have the same $^{3}$H/$^{35}$S ratio as the starting material, ruling out the possibility of a dehydrothienamycin species as a direct precursor of Thienamycin. Cysteine appeared to be decarboxylated after addition to the antibiotic nucleus as cysteamine itself was poorly incorporated into Thienamycin, (136).
5.3.2 Studies using Blocked Mutants

5.3.2.1 The OA-6129 Carbapenems

Following discovery of the OA-6129 group of carbapenems, (184), and their structure elucidation, (18), OA-6129 carbapenems were distinguishable from other β-lactams by possession of a C(3) pantothienyl side chain, see Figure (1.3). Speculation ensued as to their biosynthetic relationship with other carbapenem groups.

A mutant of Streptomyces fulvoviridis A933 17M9, was isolated, and found to produce, OA 6129A, OA 6129B\(_1\), OA6129B\(_2\), and OA 6129C, instead of PS-5, Epithienamycins A and C, and MM 17880 respectively, (19), see Figure (1.2), demonstrating that carbapenems with C(3) pantothienyl side chain may act as direct biosynthetic precursors for at least some carbapenems.

Subsequent studies, (185,186,187), revealed that the mutant lacked depantothenylating activity found in the parent strain and designated as A933 acylase. Similar enzyme activities have been found in other Streptomyces sp that produce carbapenems, including S. cattleya, (185). Figure (5.3).

5.3.2.2 S. cattleya Blocked Mutants

Rosi et al isolated two S. cattleya mutants that were altered in carbapenem biosynthesis. One produced an N-Acetylthienamycin, and the other a deshydroxy analogue of Thienamycin corresponding to NS-5, (14), see Figure (1.3).

Williamson et al, (136), concluded that the addition of the C(8) hydroxyl group is one of the terminal steps in the biosynthetic pathway. Whether the oxygen originates from O\(_2\) or H\(_2\)O has yet to be determined. The dehydroxy analogue
(A) DEPANTOOTHENYLATION OF OA 6129 CARBAPENEMS BY A933 ACYLASE (185)

\[
\begin{align*}
\text{OA 6129} & \quad \rightarrow \\
& \quad \text{A 933} \\
& \quad \text{OA 6129} \quad \text{NH} \\
& \quad \text{H3C} \quad \text{CH3} \\
& \quad \text{NCO} \quad \text{1} \\
& \quad \text{OH} \\
& \quad \text{Pantothenic Acid}
\end{align*}
\]

(B) ACYL EXCHANGE BETWEEN OA 6129 CARBAPENEMS AND ACYL CoA'S BY A933 ACYLASE, (185)

\[
\begin{align*}
\text{OA 6129} & \quad \rightarrow \\
& \quad \text{Acyl CoA} \\
& \quad \text{A933} \\
& \quad \text{OA 6129} \quad \text{NH} \\
& \quad \text{H3C} \quad \text{CH3} \\
& \quad \text{NCO} \quad \text{1} \\
& \quad \text{OH} \\
& \quad \text{Pantothenate} -\text{Acyl} + \text{CoA-SH}
\end{align*}
\]

( \text{R} = -\text{CH}(\text{CH}_3)\text{OH} \)
( \text{R} = -\text{CH}(\text{CH}_3)\text{OSO}_3\text{H} \text{ or } -\text{CH}_2\text{CH}_3 )
of Rosi et al may in fact be the biosynthetic precursor of Thienamycin itself. Addition of a hydroxyl group to the prochiral centre of C(8) may account for the small amount of 8-Epithienamycin produced by *S. cattleya*, if the reaction involved is not absolutely stereospecific.

5.3.2.3 *Streptomyces griseus* Blocked Mutants

Nozaki et al isolated three types of blocked mutant from *S. griseus* subsp. *cryophilus*, which appeared to be impaired in specific steps of carbapenem biosynthesis, (24). A biosynthetic pathway for the terminal stages of formation of 5,6-cis carbapenems was proposed, see Figures (5.4) and (5.5).

The cysteaminyl side chain of Thienamycin does not appear to be unsaturated during its synthesis, (136). A dehydrothienamycin species as a precursor of Thienamycin therefore seems extremely unlikely. N-Acetylthienamycin and N-Acetyl dehydrothienamycin are possibly produced by *S. cattleya* as shunt metabolites, by a divergent branch in the biosynthetic pathway. A pathway similar to that of *S. griseus* may operate in the dehydrogenation of N-Acetylthienamycin to give N-Acetyldehydrothienamycin.

5.3.3 Studies using Metabolic Inhibitors

5.3.3.1 Inhibition of Thienamycin and Cephamycin C Synthesis in *S. cattleya*

The biosynthetic relationship between Thienamycin, and Cephamycin C, was probed by Williamson et al, by manipulation of the growth conditions, and the use of selective metabolic inhibitors, (136).

Thienamycin synthesis alone could be substantially reduced by elimination of Co^{2+} from the growth medium.

(136)
SHOWING A PROPOSED BIOSYNTHETIC PATHWAY FOR 5,6 CIS CARBAPENEMS

Broken arrows indicate biosynthetic steps blocked in mutant types I, II and III

EPI-T EPITHIENAMYCIN
**SULPHONYLOXY COMPOUND**

<table>
<thead>
<tr>
<th>Compound</th>
<th>R</th>
<th>R¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-19393 S₂</td>
<td><img src="image" alt="Structure" /></td>
<td><img src="image" alt="Structure" /></td>
</tr>
<tr>
<td>MM 4550</td>
<td><img src="image" alt="Structure" /></td>
<td><img src="image" alt="Structure" /></td>
</tr>
<tr>
<td>MM 13902</td>
<td><img src="image" alt="Structure" /></td>
<td><img src="image" alt="Structure" /></td>
</tr>
<tr>
<td>MM 17880</td>
<td><img src="image" alt="Structure" /></td>
<td><img src="image" alt="Structure" /></td>
</tr>
</tbody>
</table>

**HYDROXY COMPOUND**

<table>
<thead>
<tr>
<th>Compound</th>
<th>R</th>
<th>R¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-19393 H₂</td>
<td><img src="image" alt="Structure" /></td>
<td><img src="image" alt="Structure" /></td>
</tr>
<tr>
<td>C-19393 E₅</td>
<td><img src="image" alt="Structure" /></td>
<td><img src="image" alt="Structure" /></td>
</tr>
<tr>
<td>Epithienamycin B</td>
<td><img src="image" alt="Structure" /></td>
<td><img src="image" alt="Structure" /></td>
</tr>
<tr>
<td>Epithienamycin A</td>
<td><img src="image" alt="Structure" /></td>
<td><img src="image" alt="Structure" /></td>
</tr>
</tbody>
</table>
Thiaisoleucine (a sulphur analogue of isoleucine) was found to inhibit Thienamycin but not Cephamycin C synthesis, by an unknown mechanism. Isoleucine may provide acetate units for Thienamycin biosynthesis, and may explain the stimulatory effect of branched chain amino acids on synthesis, (30). Conversely an analogue of lysine, aminohydroxy caproic acid, was found to inhibit Cephamycin C synthesis but not Thienamycin synthesis, possibly by blocking conversion of lysine to α-aminoacidipic acid, (188).

Thus it would appear that Thienamycin and Cephamycin C are formed by independent biosynthetic pathways, although they share the common precursors cysteine and methionine. Conceivably the biosynthetic pathways could also involve common enzymes.

5.3.3.2 Reverse Transsulphuration in S. cattleya

Reverse transsulphuration describes the reactions by which the sulphur atom of homocysteine and the carbon chain of serine are combined to form cysteine, see Figure (5.6). Clearly cystathionine γ lyase is a key enzyme in the pathway. Williamson et al suggest that reverse transsulphuration is a general feature of sulphur metabolism in Actinomycetes, (189).

Studies have shown that cystathionine, methionine, and cysteine are all efficiently utilised by S. cattleya for antibiotic synthesis, (189). A mechanism based inhibitor of cystathionine γ lyase, propargylglycine, was found to inhibit the synthesis of both Thienamycin and Cephamycin C in S. cattleya, (189). Inhibition was reversed by providing the cells with another source of cysteine, such as cystine,
FIGURE (5.6) SHOWING THE REACTIONS OF REVERSE TRANSSULPHURATION (198)

METHIONINE

\[ \rightarrow S \text{AM} \]

\[ \rightarrow \text{SAHC} \]

HOMOCYSTEINE

\[ \rightarrow \text{NH}_3 \]

\[ \rightarrow \text{Pyruvate} \]

CYSTATHIONINE

\[ \rightarrow \alpha\text{-KETO butyrate} \]

\[ \rightarrow \text{Cysteine} \]

\[ \rightarrow \text{Serine} \]

\[ \rightarrow \text{SO}_4^{2-} \]
which would suggest that reverse transsulphuration is a major pathway used to provide cysteine in \textit{S. cattleya}, \cite{189}.

The ability of methionine to reverse the effects of propargylglycine inhibition, may be explained if the addition of methionine to the culture spares the small endogenous pool of cysteine for antibiotic synthesis, \cite{189}. Assuming cysteine sulphur can be converted to methionine sulphur via direct transsulphuration in \textit{S. cattleya}. Evidence suggests that direct transsulphuration does occur in other \textit{Streptomyces} sp., \cite{190}.

5.3.4 \textbf{Hypothetical Scheme for Biosynthesis of Thienamycin}

A hypothetical scheme for the biosynthesis of Thienamycin, and other carbapenems has been proposed by Williamson \textit{et al}, \cite{136}, based on their own studies, and data currently available in the literature, see Figure (5.7). The difficulty arises in reconciling information often of a fragmentary or contradictory nature, into a single biosynthetic pathway, given the diversity of carbapenem structures. The scheme proposed by Williamson \textit{et al} involves cyclisation of the five member and four member rings to form the carbapenem nucleus, with subsequent addition of \(C_3\) and \(C_6\) substituents.

In carbapenem biosynthesis we may be dealing with a multiply branched pathway, involving a common biosynthetic stem, a complex array of possible terminal pathways may exist. The type and proportion of different carbapenems produced could be determined by such factors as :

1) \textit{Streptomyces} species ( or \textit{Bacterial} species ).
2) Growth conditions, for example the amount of free sulphate
THIENAMYCIN AND OTHER CARBAPENEMS, (136)

\[ \text{CoA-C-CH}_3 + \gamma\text{-glutamylphosphate} \]

\[ \xrightarrow{\text{Pi}} \]

\[ \xrightarrow{\text{H}_2\text{O}} \]

\[ \text{HSCoA} \]

\[ \text{SAM} \]

\[ \text{SAHC} \]

\[ \text{Carbapenem Carboxylic acid} \]

\[ \text{HSCoA} \]

\[ \text{Pantethiene} \]

\[ \text{OA-6129 Series} \]

\[ \text{Northienamycin} \]

\[ \text{Asparenomycins} \]

\[ \text{PS-Series} \]

\[ \text{SAM} \]

\[ \text{SAHC} \]

\[ (2,3) \text{ oxidation} \]

\[ \text{H}_2\text{O} \]

\[ (6,8) \text{ oxidation} \]

\[ (6,8) \text{ reduction} \]

\[ \text{Thienamycin (in S. cattleya ?)} \]
FIGURE (5.7) Continued

\[
\begin{align*}
\text{CH}_3\text{-CH}_2 \quad \text{(2,3) oxidation} \\
\text{CH}_3\text{-CH}_2 \quad \text{hydroxylation C}_8\text{(S)} \\
\text{OR} \quad \text{hydroxylation C}_8\text{(R)} \\
\text{2,3 Dihydrothienamycin} \\
\text{THIENAMYCIN} \\
x = \text{pantothenate}
\end{align*}
\]
in the case of sulphated/nonsulphated carbapenems.

3) Enzymes produced, for example acylase activity, depantothenylating activity.

4) Amount and type of acyl-CoA's available for acyl exchange.

The carbapenem nucleus gives wide scope for chemical modification, (181,182), it is hardly surprising that this also appears to be the case for microbial modification. As indicated by Williamson, (30), areas that require further investigation are, the origin of the C(8) hydroxyl group of carbapenems, and whether the isotope labelling studies used in determining the origin of the carbon skeleton of Thienamycin hold true for other carbapenem producers. An important investigative tool is likely to be the use of advanced intermediates as precursors in cell free systems/extracts.

5.4 THE ROLE OF PANTETHIENE IN CARBAPENEM BIOSYNTHESIS

Discovery of the OA 6129 carbapenems, (184), led to speculation about the involvement to pantethiene in the biosynthesis of carbapenems. Several possible roles for pantethiene have been proposed, (187). Firstly the pantethiene could act as a precursor for the C(3) pantothienyl side chain. Secondly Coenzyme A (with a pantothienyl moiety as acyl carrier), could be involved in cell growth, and also in the derivation of the C(3) acetylaminooethylthio side chain of some carbapenems, by an acyl exchange reaction. And finally that a thiotemplate system of peptide antibiotics, (191), might operate in the biosynthesis of carbapenems.
That structures possessing a C(3) pantothienyl side chain, could act as intermediates in biosynthesis of certain carbapenems, was demonstrated in *Streptomyces fluvoviridis*, (19). Whether this applies to carbapenem biosynthesis generally, is not yet clear. In *S. fulvoviridis* a specific acylase catalyses the depantothenylation of OA 6129 carbapenems, (185). The enzyme also catalyses acyl exchange, of the C(3) side chain of OA 6129 carbapenems, with acyl CoA's, see Figure (5.3). Similar enzyme activities have been determined in other carbapenem producing *Streptomyces*, including *S. cattleya*, (185).

To date, C(3) pantothienyl intermediates have not been detected in *S. cattleya* fermentations. Incorporation of (35S) labelled cysteine, and (35S) pantethiene, ( at a level 74 times lower than that of (35S) cysteine ) into the C(3) side chain of Thienamycin have been reported, (136). As *S. cattleya* is able to utilise exogenous pantethiene, (136) it would appear that the preferred precursor of the C(3) side chain in *S. cattleya* is in fact cysteine. Radiolabelled pantothenate was not taken up into the mycelium of *S. fulvoviridis*, however radiolabelled β-alanine was specifically incorporated into the β-alanyl portion of the C(13) pantothienyl side chain of OA 6129 carbapenems, (187).

The thiotemplate system described by Lipmann, (191), involves a pantethiene moiety, within a multienzyme unit which functions as a carrier arm for peptide elongation. Such a system could operate in carbapenem biosynthesis, subsequent cyclisation of the peptide giving rise to the carbapenem nucleus. Although judging from the structure of the OA 6129 carbapenems, one molecule of pantethiene
appears to be consumed in producing one molecule of the antibiotic, \(^{186}\). Incorporation of an intact glutamate unit into the five-membered ring of Thienamycin by \textit{S. cattleya}, \(^{119}\) would seem to be incompatible with thiotemplate peptide synthesis, and subsequent cyclisation of Thienamycin.

The role of pantethiene in carbapenem biosynthesis, and the origin of the \(C_{(3)}\) side chain of carbapenems are open to question. A number of potential precursors may exist, that are preferentially used by different \textit{Streptomyces} sp. depending on the metabolic pool, to provide the \(C_{(3)}\) side chain. This in itself points away from thiotemplate synthesis, in which pantethiene would play a very central role in biosynthesis.

5.5 **THE ROLE OF COBALT IN CARBAPENEM BIOSYNTHESIS**

The role that cobalt fulfills in carbapenem biosynthesis is not yet understood. A number of possibilities are presented and discussed in greater detail in chapter two, including, regulation of biosynthesis, or activity of, the antibiotic synthetases, formation and correct functioning of vitamin \(B_{12}\), and regulation of glutamine synthetase activity. To date the studies carried out consist largely of adding \(Co^{2+}\) or vitamin \(B_{12}\) to the fermentation and noting the effect on antibiotic production.

Trace metals can greatly influence secondary metabolism, and often have an important role to play, \(^{130}\). At present the effect of manganese on patulin production in \textit{Penicillium urticae} is probably the most well characterised system, for the involvement of a metal ion, in this case in fungal secondary metabolism, \(^{192}\). These studies present a
detailed investigation of the essential role of manganese, and indicate a growing awareness of the significance of metal ions in antibiotic biosynthesis.

An insight into the site and mode of action of metal ions in secondary metabolism will influence our ability to manipulate organisms and their biosynthetic pathways to enhance the formation of desirable products. It may be possible to remove a 'key' metal ion from its functional site using highly selective chelating agents, demonstrating very strong affinity for single metal. Conceivably a natural product could fulfill this requirement, presently available chelating agents may not be sufficiently specific for individual trace metals.

The cyclopentenedione antibiotic (a cobalt chelator), produced by S. cattleya, could be involved in the control of secondary metabolism in this organism. For example the cyclopentenedione may sequester available cobalt, and thereby prevent activation of preformed enzymes until after the growth phase, thus delaying the onset of antibiotic biosynthesis. However, the precise role(s) of cobalt (and the cyclopentenedione) remain to be determined.

5.6 FUTURE PROSPECTS

The biosynthetic pathway leading to penicillin, cephalosporin and cephamycin β-lactams is reasonably well established, (see chapter one), and at least partially elucidated at the enzyme level, (96,105,109,193,194). Attention is now being given to the mechanism by which individual enzyme reactions occur, (110). Biosynthetic pathways for recently discovered β-lactam classes are
currently under investigation. It is recognised that a number of regulatory controls may influence secondary metabolism, (120,122). Largely, the actual regulatory mechanisms remain obscure, as does the underlying genetic determination of secondary metabolism. Knowledge of the gene-enzyme relationships involved in antibiotic synthesis is extremely scanty, (148).

Factors contributing to the lack of progress, in understanding of some aspects of secondary metabolism and antibiotic biosynthesis, may have included:

1) The success of industrial strain development programs to furnish mutants with increased production ability, using the traditional random mutation and screening approach.

2) The considerable empirical knowhow, that existed, of the nutritional and environmental factors affecting antibiotic production, (195).

Thereby obviating the need for comprehensive understanding of antibiotic biosynthetic pathways and their control. However there are indications that developments in fundamental biochemistry and genetics will provide a sound basis for research into the genetics and molecular biology of antibiotic biosynthesis. Further knowledge will be necessary to fully exploit some of the new techniques available. Genetic engineering, and protoplast fusion techniques are likely to be of considerable importance to the future of antibiotic research.

Conditions for the formation and reversion of Streptomyces protoplasts were originally developed by Okanishi et al, (196). More recently the technique has
been applied to antibiotic producing Streptomyces, (197, 198, 199). Protoplast fusion techniques permit intra and inter species recombination, to improve antibiotic yields, (190), or provide novel antibiotics, (200).

Protoplasting and regeneration may result in increased antibiotic yield, (201), or in plasmid ' curing '. Protoplast techniques have an extensive role to play in the genetic analysis of antibiotic biosynthesis, (112), see Table (5.1).

As cloning vectors are increasingly available for Streptomyces sp., recombinant DNA techniques should prove very useful in the genetic analysis of antibiotic biosynthesis, (107, 114). Recently the S. clavuligerus genes, for Clavulanic acid biosynthesis have been cloned, (115).

Ultimately genetic manipulation of the biosynthetic pathways, of antibiotic producing organisms should be achievable. For example, increases in antibiotic yield by the use of multicopy genes, or transfer of the ability to make an antibiotic from an organism with less desirable characteristics, to one more amenable to economic industrial fermentation.

Increased knowledge of biosynthetic pathways, and the individual enzyme reactions involved, together with an understanding of the regulatory mechanisms controlling antibiotic biosynthesis, will allow a more direct approach to the design/manipulation of producing organisms. Bottlenecks in the biosynthetic pathway could be removed by deregulation of the rate limiting step, (122). Advanced intermediates could be designed in the form of modified substrates i.e., structural analogues, which could lead to formation of novel products, (110, 202, 203, 204). Current immobilised
### TABLE (5.1)

THE ROLE OF PROTOPLAST TECHNIQUES IN GENETIC ANALYSIS, (112)

1) Generalised recombination through protoplast fusion.
2) Plasmid "Curing" by protoplasting and regeneration.
3) Transformation/transfection of protoplasts by plasmid or 'phage' DNA.
4) Gene cloning on plasmid or 'phage' vectors.
5) Transformation of protoplasts by chromosomal DNA entrapped in Liposomes.
6) Artificial feeding of metabolic precursors using Liposomes.

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(150)
enzyme methology, (205,206,207) and protein engineering techniques, (11) may have a significant role to play, in that use of substrate analogues or altered enzymes could give rise to novel β-lactam structures, (110). The rational/semirational design of antibiotic producing strains, with increased antibiotic yields, novel antibiotic products, or other desirable properties may be feasible. In conclusion we are moving into an exciting period in antibiotic research in which a detailed understanding of biosynthesis and its regulation is no longer of purely academic interest.
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APPENDIX A

Media Recipes

1) Seed (inoculum) Medium

<table>
<thead>
<tr>
<th>Component</th>
<th>%</th>
<th>g/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycerol</td>
<td>3.0</td>
<td>30.0</td>
</tr>
<tr>
<td>Peptone (Oxoid L37)</td>
<td>2.0</td>
<td>20.0</td>
</tr>
<tr>
<td>CaCO₃</td>
<td>0.1</td>
<td>1.0</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>0.024</td>
<td>0.24</td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
<td>0.02</td>
<td>0.20</td>
</tr>
<tr>
<td>M.E.C.</td>
<td>0.01</td>
<td>1 ml</td>
</tr>
</tbody>
</table>

**Minor Element Concentrate (M.E.C.)**

<table>
<thead>
<tr>
<th>Component</th>
<th>%</th>
<th>g/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ferrous sulphate·7H₂O</td>
<td>0.1</td>
<td>1.0</td>
</tr>
<tr>
<td>Cupric sulphate·5H₂O</td>
<td>0.015</td>
<td>0.15</td>
</tr>
<tr>
<td>Zinc sulphate·7H₂O</td>
<td>0.1</td>
<td>1.0</td>
</tr>
<tr>
<td>Manganese sulphate·4H₂O</td>
<td>0.01</td>
<td>0.1</td>
</tr>
<tr>
<td>Potassium molybdate</td>
<td>0.01</td>
<td>0.1</td>
</tr>
</tbody>
</table>

Acidify to obtain a clear solution, add to media (v/v)%

2) Medium (E), Kahan et al (10)

<table>
<thead>
<tr>
<th>Component</th>
<th>g/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>25.0</td>
</tr>
<tr>
<td>Corn steep liquor</td>
<td>15.0</td>
</tr>
<tr>
<td>Peptone (Oxoid L37)</td>
<td>10.0</td>
</tr>
<tr>
<td>Cotton seed medium</td>
<td>5.0</td>
</tr>
<tr>
<td>CoCl₂·2H₂O</td>
<td>10mg</td>
</tr>
</tbody>
</table>

Adjusted to pH 7.3 with NaOH

<table>
<thead>
<tr>
<th>Component</th>
<th>g/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaCO₃</td>
<td>3.0</td>
</tr>
</tbody>
</table>

Corn steep liquor (C.P.C. Corn Products Ltd) and cotton seed media (Milou-Pro) were obtained from ICI Pharmaceuticals PLC.
Corn steep liquor stored at 4°C.

3) **Medium (C)**, Aharonowitz and Demain, (133)

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycerol</td>
<td>10.0  g/l</td>
</tr>
<tr>
<td>L-asparagine</td>
<td>2.0 g/l</td>
</tr>
<tr>
<td>MgSO$_4$ (anhydrous)</td>
<td>0.6 g/l</td>
</tr>
<tr>
<td>K$_2$HPO$_4$</td>
<td>1.0 mM</td>
</tr>
<tr>
<td>Morpholinepropane sulphonic acid (MOPS)</td>
<td>20.93 (100 mM)</td>
</tr>
<tr>
<td>CoCl$_2$·2H$_2$O</td>
<td>10mg</td>
</tr>
</tbody>
</table>

Trace salts solution 1 ml

Adjusted to pH 6.8 with NaOH

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>FeSO$_4$·7H$_2$O</td>
<td>1.0 g/l</td>
</tr>
<tr>
<td>MnCl$_2$·4H$_2$O</td>
<td>1.0 g/l</td>
</tr>
<tr>
<td>CaCl$_2$·2H$_2$O</td>
<td>1.0 g/l</td>
</tr>
</tbody>
</table>

Media autoclaved 20 minutes 121°C and used within 3 days.