Synthesis And Evaluation Of Antibacterial Activity Of The Dual-Action Agents: β-Lactamase Inhibitors With Cytotoxic Agents Or β-Lactam Antibiotics

By

Danni Wang

ASTON UNIVERSITY

May 2001

Thesis submitted to Aston University for the degree of
Master of Philosophy

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SUMMARY

The dual-action mechanisms explored in this thesis are:

(1) the conjugates of a β-lactam antibiotic and a cytotoxic agent, aiming at exploitation of synergistic effect in killing bacteria *via* blocking metabolism by the antibiotic and damaging bacterial nucleus by the cytotoxic agent
(2) the conjugates of a β-lactam antibiotic and a β-lactamase inhibitor

The first group of the conjugates consist of a β-lactam antibiotic and a cytotoxic agent: antitumour agents temozolomide, mitozolomide and their derivative ethyl 8-carbamoyl-3,4-dihydro-4-oxo-imidazo[5,1-d]-1,2,3,5-tetrazine-3-acetate, were converted into their corresponded carboxylic acids (**2.5**, **2.6** and **2.9**), then acylated with N-hydroxy-succinimide to give the active esters(**2.7**, **2.8** and **2.10**). Reactions of the active esters (**2.7**, **2.8** and **2.10**) with ampicillin, amoxicillin and cefalexin produced the conjugates of a β-lactam antibiotic with a cytotoxic agent(**2.1a-f**). The second group of the conjugates are the integration of a cytotoxic agent with a cephalosporin *via* its 3-methylene group. Cephalosporines 2-11a-c were synthesised and converted to 3,4-ene precursor (**2.14**), followed by esterification with **2.5** or **2.6** respectively, then deprotection to give the conjugates (**2.2a-f**). Antibacterial activity of the conjugates has been tested against a panel of bacteria including several β-lactamase producing strains. The results of first group conjugates demonstrated little synergistic effect against bacteria, while the second group demonstrated some synergistic effect against bacteria.

β-lactamase inhibitors, tazobactam and sulbactam have been synthesised for preparation of the conjugates of a β-lactam antibiotic with a β-lactamase inhibitor. However, a number of attempts have been made to conjugate amoxicillin and tazobactam without success.

Keywords: *Imipenem, Faropenem, Biapenem, Meropenem, Ritipenem, Panipenem, Pseudomonas aeruginosa.*

To Mum, Dad and my family

Acknowledgements

I would like to thank very much my Supervisor Dr Yongfeng Wang for his expert advice, guidance and constant support.

Thank you to Karen Farrow for her technical support and to Mike Davies for keeping the laboratory up and running.

Thank you to Dr Peter Lambert for the biological test results and for his calming advice and support.

Thank you to all my friends in the Pharmacy Department, especially Linxiang Zhao, Andrew Walsh, Kylie Michelle Loak, Geeta Sood, Julie Simpson, Katy Tims and Nigel for his friendship and company.

A huge thank you to Mum, Dad, my husband Tieqiang and daughter Liz for their continued support over the years and for putting up with me while I wrote this.

Abbreviations

6-APA	6-aminopenicillanic acid
6-BPA	6-bromopenicillanic acid
7-ACA	7-aminocephalosporanic acid
AMP	ampicillin
m-CPBA	m-chloroperbenzoic acid
DCC	dicyclohexylcarbodiimide
DCM	dichloromethane
DMF	*N,N*-dimethylformamide
DMSO	dimethyl sulfoxide
DMSO-d_6	hexadeuterated dimethyl sulfoxide
DNA	deoxyribonucleic acid
FTIR	Fourier transform infrared
HPLC	high pressure liquid chromatography
IC_{50}	half inhibitory concentration
IR	infra red
MDR	multidrug resistant
MIC	minimum inhibitory concentration
MRSA	methicillin-resistant *Staphylococcus aureus*
MS	mass spectroscopy
MSSA	methicillin-sensitive *Staphylococcus aureus*
MTIC	5-(3-methyltriazen-1-yl)imidazole-4-carboxamide
NHS	N-hydroxysuccinimide
NMR	nuclear magnetic resonance
PyBOP	[(benzotriazol- 1-yl)oxy]tripyrrolidophosphonium hexafluorophosphate
Rf	retention factor
RNA	ribonucleic acid
RT	room temperature
TBTU	*O*-Benzotriazol-1yl-N,N,N',N'-tetramethyluronium

	tetrafluroborate
TFA	trifluoroacetic acid
TLC	thin layer chromatography
UV	ultra violet
WHO	world health organisation

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Chapter 1

Introduction

1.1 Infectious diseases

Infectious diseases are the commonest afflictions of humans and are a major source of morbidity and mortality in both developed and developing countries. Table 1.1 shows the estimated morbidity and mortality for the common infectious diseases (Kumar, P and Clark, M 1997).

Table 1.1 League table of infectious diseases world-wide

Disease	Estimated morbidity (No. of cases in thousands per year)	Estimated mortality (No. of deaths in thousands per year)
Diarrhoeal disease	3,000,000 – 5,000,000	10,000
Respiratory infection	?	5,000
Malaria	270,000	1,500
Measles	80,000	1,000
Schistosomiasis	20,000	1,000
Whooping cough	20,000	400
Neonatal tetanus	?	150

The first recognition that fungi could be specifically associated with an animal disease came in 1836 with the work of A. Bassi in Italy on a fungal disease of silkworms. A few years later, J. L. Schönlein showed that certain skin diseases of man were caused by fungal infections (Mann, J *et al* 1996).

In 1876, a German doctor, Robert Koch, discovered that bacteria could act as specific agents of infectious disease in higher animals through the study of anthrax, an infection of domestic animals that is transmissible to man. He proved that the anthrax bacillus was the sole cause of the disease, and demonstrated that its epidemiology was a result of the natural history of the bacterium. Further researches in microscopy and bacteriology led to his discovery of the tubercle bacillus that causes tuberculosis (TB) - a major cause of death in the time of 1882 (Mann, J *et al* 1996).

Such unremitting microbial attacks throughout the infection disease have been the major causes of human death. US Public Health Service statistics for 1910-1920 showed that early in this century tuberculosis killed one in every 1000 US residents. Even today, mainly in developing countries, Mycobacterium tuberculosis remains the leading cause of death attributable to a single infectious agent, killing over three million people worldwide every year (Brickner, S J 1997).

According to an estimate by the World Health Organisation infectious diseases are the world's leading cause of premature death, killing almost 50000 people every day. Tuberculosis (TB) is currently making a dramatic comeback along with cholera, dengue fever, malaria, diphtheria, and bubonic plague as shown in Fig 1.1 (Hook,V 1997).

In addition, in the last two decades at least 30 new infectious diseases have occurred (Clinical, The Pharmaceutical Journal, 1996) such AIDS virus,

Ebola haemorrhagic fever and Legionnaires Disease. Many of these new diseases have no treatment, cure or vaccine (Chemistry In Britain, 1997).

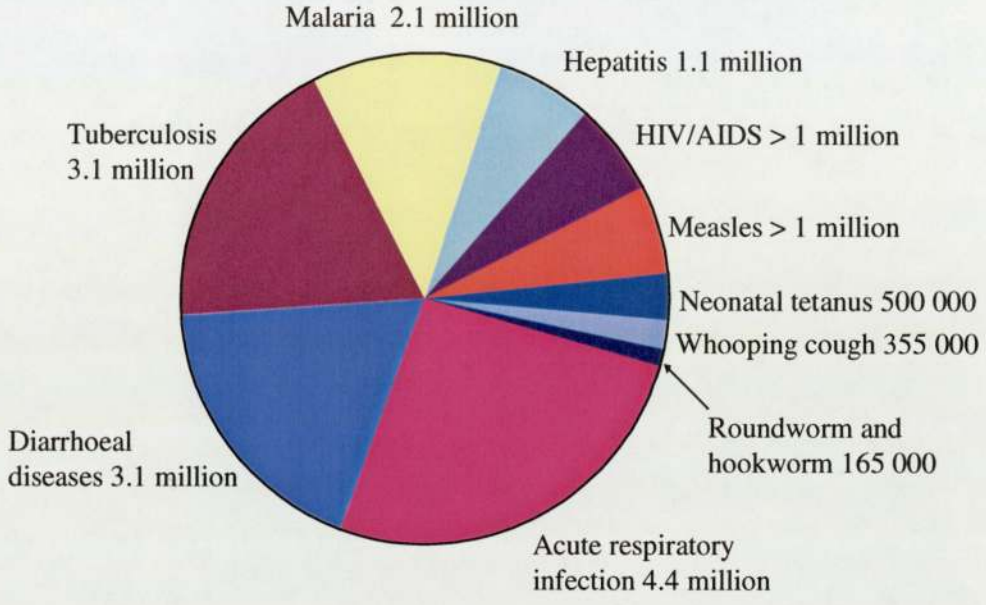


Fig 1.1 The "10 biggest killers" through infection, according to World Health Organisation

1.2 Bacterial Resistance

While old infectious organisms stubbornly come back in resistant forms and new life threatening infections constantly emerge, antibiotics and other life-saving drugs are rapidly losing their efficacy. For example, up to 60 per cent of hospital-acquired infections in the United States were caused by antibiotic-resistant bacteria according to the WHO's 'World health report 1996'.

In fact, antibiotic resistance is not a new phenomenon, but its scale now is far greater than that was predicted in the past. In 1941, just months after penicillin was first used, strains of *Staphylococcus aureus*, which cause a range of infections from boils to pneumonia, were found to be resistant to the drug. At that time, only less than one per cent of all strains of the bacteria was penicillin-resistant, by 1949 this figure had grown to 14%, and today more than 90% of *Staphylococcus aureus* are penicillin resistant (Clinical, The Pharmaceutical Journal 1996).

In the late 1950s and early 1960s multi-drug resistance was reported in *Escherichia coli*, *Salmonella* and *Shigella* in Europe, the US, Japan and Latin America, about a decade after introducing drugs to combat them. By the mid-1970s the problem was widespread: gonorrhoea-causing bacteria had acquired resistance (Hook, V 1997).

Around the globe, methicillin-resistant *Staphylococcus aureus* (MRSA) are very problematic in Japan (where in some hospitals 60% of *S. aureus* isolates

are MRSA), in Spain, France, Italy and the US, each with a greater than 30% incidence (Brickner, S J 1997). In U.K, 40 hospitals per month were affected in 1993 and this had swollen to 80 by 1995.

The problem is so great that some bacteria are resistant to all but one drug, vancomycin. It has become a 'last resort' antibiotic and the only weapon in the antibacterial armoury to retain its efficacy against some multi-resistant strains including methicillin-resistant *S. aureus* and *Enterococci*.

The most alarming shock was the finding of vancomycin-resistant enterococci (VRE) in 1988. Now some of VRE don't respond to any available antibiotics. The enterococci have become the second most frequently encountered hospital acquired pathogen in the US, where the incidence of VRE strains is now about 15% of all clinical enterococcal isolates (Brickner, S J 1997).

Today, antibiotic resistance is becoming prevalent among community acquired infections, such as organisms causing sore throats and ear infections (Hook, V 1997). The situation of antibiotic resistance is further complicated by the varying availability of antibiotics across the globe.

1.3 β-Lactam antibiotics

β-lactam antibiotics are the most varied and widely used antimicrobial agents, accounting for half of all the systemic antimicrobials in use (Livermore, D M

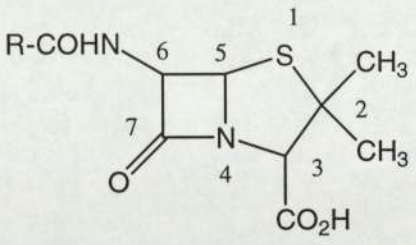
and Williams, J D 1996). β-lactam antibiotics are potent broad spectrum antibacterial agents with low eukaryotic toxicity (Georgopapadakou, N H 1993). The activity of β-lactam antibiotics is influenced by the type of substitutions (R-groups) attached to the basic nucleus (Bryan, L E and Godfrey, A J 1991). Prototypic congeners such as the classic penicillins are limited in activity to certain Gram-positive bacteria such as streptococci and staphylococci. Cephalosporins typically offer an extended spectrum of activity against some Gram-negative bacteria.

The first discovery of the β-lactam antibiotics occurred in 1928 (Coulton, S and Francois, I 1994) when Alexander Fleming observed the antistaphylococcal activity of *Penicillium notatum*. This activity was due to penicillin, the first β-lactam antimicrobial to be discovered (White, L O and Andrews, J M 1999). In 1940, the first time of use of penicillin to treat infection in animals was carried out and shortly, the first clinical use of penicillin in humans was documented (Dax, S L 1997).

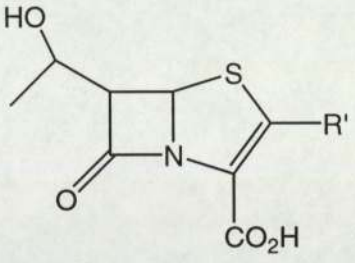
However the chemical structure of penicillin was not correctly characterised as a strained and highly reactive β-lactam ring until 1945. Following this successful example, more screens have been done in a few years of time. By the early 1950s, dozens of penicillins were known. Each congener contains a unique acyl side chain attached to the β-lactam nucleus (Dax, S L 1997).

The family of β--lactam antibiotics have been growing bigger and bigger in fifty years. They encompass an enormous number of semi-synthetic compounds, which can be conveniently divided into three groups: bicyclic penicillins (namely, penams, penems, carbapenems, oxapenams); cephalosporins (namely, cephems, cephamycins, oxacephems, carbacephems) and monocyclic (isolated β-lactam ring) monobactams (Fig 1.2). In addition, a novel tricyclic β-lactam (tribactam), sanfetrinem, has been developed (Di Modugno *et al.*, 1994).

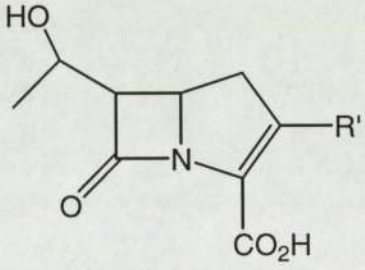
Bicyclic Penicillins



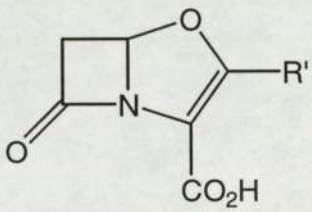
Penam



Penem



Carbapenem



Oxapenem

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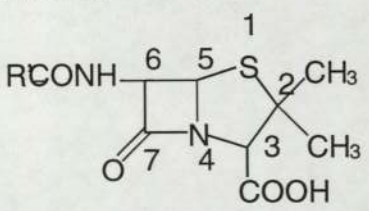
Bicyclic Penicillins



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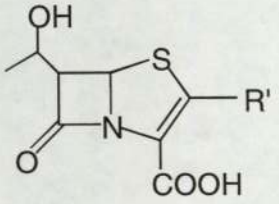
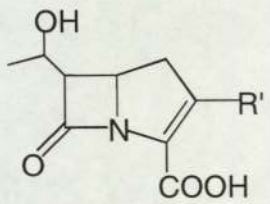
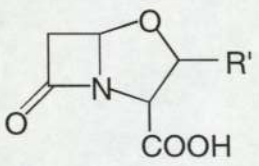


Penem

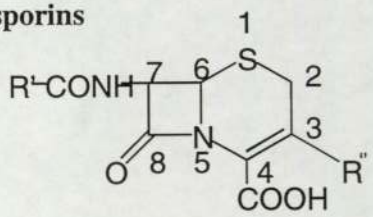


Carbapenem

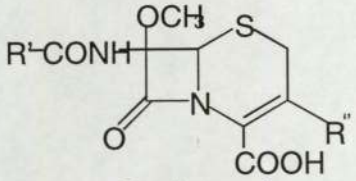


Oxapenam

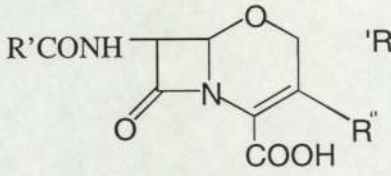
Cephalosporins



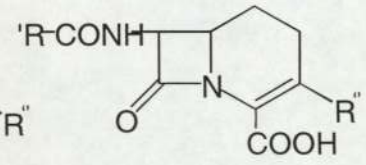
Cephem



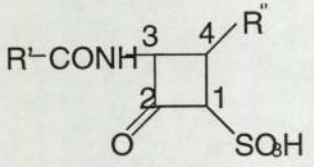
Cephamycin



Oxacephem



Carbacephem



Monobactam

R, R' and R'' = alkyl, aryl and heterocyclitic groups

Fig 1.2 Classes of β-lactam antimicrobial agents

The typically structural feature of the β-lactam family of antibacterials is of a highly reactive β-lactam nucleus which contains a suitably positioned acidic functionality. It is imperative that the lactam carbonyl centre be reactive towards nucleophilic attack by the active site serine residue of the bacterial transpeptidase enzyme. Lactams ensure the strained nature of the amide bond and a four membered β-lactam nucleus remains optimal. The acidic functionality is important for the proper positioning of the β-lactam substrate within the enzyme site so as to mimic the endogenous peptide substrates (i.e. the D-Ala-D-Ala terminus of the MurNAc-pentapeptide). The simplest structural element containing these features is a β-lactam nucleus substituted at the nitrogen atom with an organic acid (Dax, S L 1997) (Fig 1.3).

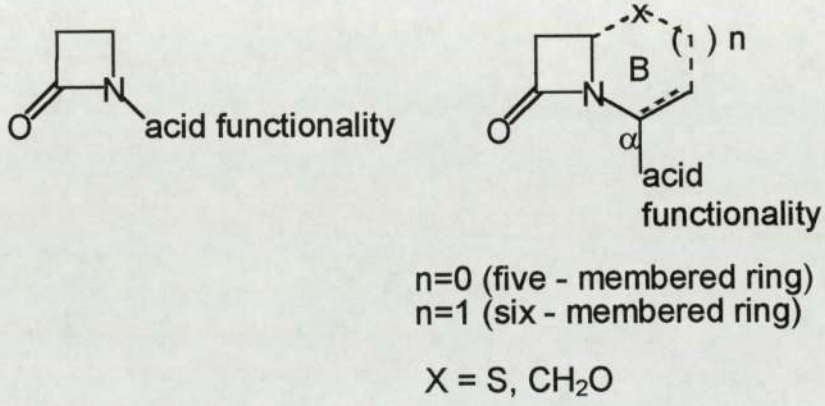


Fig 1.3 Basic β-lactam SAR.

In many cases, the β-lactam ring is fused to either a five or six-membered sulfur-containing heterocyclic ring; the thiazolidine or dihydrothiazine congeners make up the vast penicillin and cephalosporin subfamilies.

However, a bicyclic ring system is not a prerequisite for antibacterial activity, simple monocyclic β-lactams constitute yet another subfamily of the β-lactam antibacterial agents.

In addition to structural variations of the β-lactam nucleus, the substituents that emanate from the lactam core also play a crucial role with regard to antibacterial activity. The first is the acylamino substituent situated alpha to the lactam carbonyl centre, which will be collectively referred to as the 'west-end' substituent. The west-end substituents, being adjacent to the reactive lactam carbonyl centre, play a key role in terms of stability and antibacterial activity of any given β-lactam, and are at least partly responsible for penicillin-binding protein activity. The second type of substitution present in most β-lactam antibacterials are groups extending from the five- or six-membered ring (B in Fig 1.3). The most significant and extensive modifications have been the host of substituents introduced onto the C-3 (C-3') position of the cephalosporin β-lactams, although recent advances have allowed for a number of favourable alterations to penem and carbapenem systems.

β-Lactam antibiotics exert their antibacterial activity by interacting with bacterial enzymes, and in doing so, inhibiting bacterial cell wall synthesis. The growth and division of bacterial cells necessitates production of new cell wall material in which the final step is the cross-linking of specific peptide chains to form a peptidoglycan matrix (Coulton, S and Francois, I 1994).

The major component of the cell wall that confers rigidity is the heteropolymer peptidoglycan (Mann, J *et al* 1996). This substance provides a formidable barrier from the extracellular milieu but it is not the only constituent of the cell wall. Gram negative bacteria also contain a lipopolysaccharide layer and Gram positive organisms utilise other materials to fortify the cell wall. However the peptidoglycan matrix is indeed an integral part of the bacterial cell wall.

Peptidoglycan is a polymer of alternating peptide units and consists of glycan subunits. The glycan subunits consist of alternating N-acetylglucosamine and N-acetylmuramic acid-derived sugars. The peptide chains are ordered and characteristic of the species of bacteria and are attached to the muramic acid glycan.

The peptidoglycan layer forms a covalently closed string-bag-like structure, called sacculus. Thus growth of a bacterium depends on the simultaneous enlargement of its peptidoglycan sacculus. In order to enlarge the peptidoglycan netting the concerted activities of enzyme cleaving meshes and enzymes inserting new sub-units, that is peptidoglycan hydrolases and synthases are needed (Höltje, J V 1997).

Peptidoglycan biosynthesis occurs via a complex series of intracellular reactions that can be divided into three stages:

- The first stage occurs in the cytoplasm and encompasses two distinct processes.
    1. Glucosamine and uridine triphosphate are covalently joined and further modified to afford a uridine diphosphate-N-acetylmuramic acid conjugate (UDP-MurNAc).
    2. A pentapeptide chain is assembled onto this 'anchor' by the action of a host of enzymes that attach individual amino acids onto the growing chain in an orderly and sequential fashion.

- The second stage of peptidoglycan biosynthesis involves a series of membrane-associated transformations in which the cytoplasmic pentapeptide precursor (UDP-MurNAc-pentapeptide) is modified in a way that allows it to traverse the lipophilic membrane.

- The third stage of peptidoglycan biosynthesis is the joining of free peptide strands to one another to rigidify further this bacterial cell wall structure.

β-Lactam antibiotics induce lethal effects on bacteria by inhibiting the transpeptidation process required to complete peptidoglycan biosynthesis. The first step in this process is the binding of the β-lactam antibiotics to certain bacterial proteins that are generally referred to as penicillin binding proteins (PBPs). Penicillin binding proteins are intimately involved in the construction of peptidoglycan; these proteins can provide either transpeptidase or carboxypeptidase activity. Upon PBP binding, the β-lactam

agent is able to adopt a conformation that closely mimics the D-Ala-D-Ala terminus of a peptide-glycan conjugate readied for transpeptidation. The reactive β-lactam moiety is aligned in proximity to the position normally occupied by the scissile D-Ala-D-Ala linkage of the natural substrate. By this association, the β-lactam antibiotics are able to acylate specific serine residues within the active site of transpeptidase enzymes. This renders the enzyme inoperative since the enzyme-(β-lactam) substrate complex is unable to undergo subsequent hydrolysis to regenerate a functional transpeptidase enzyme (Fig 1.4) (Dax, S L 1997)

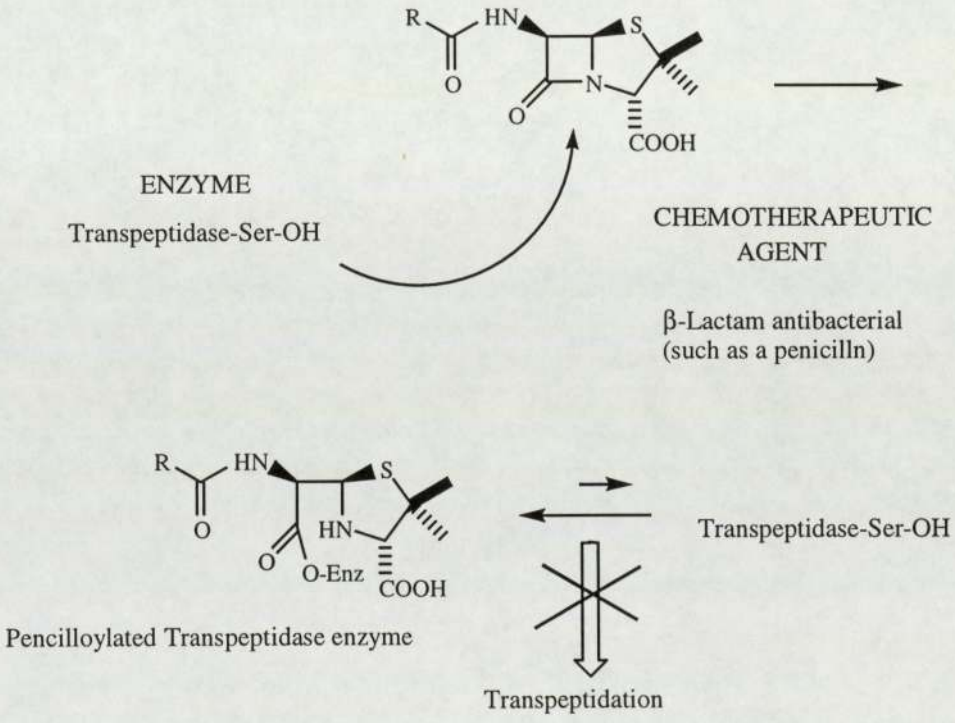


Fig 1.4 Mode of action of β-lactam antibacterial agents.

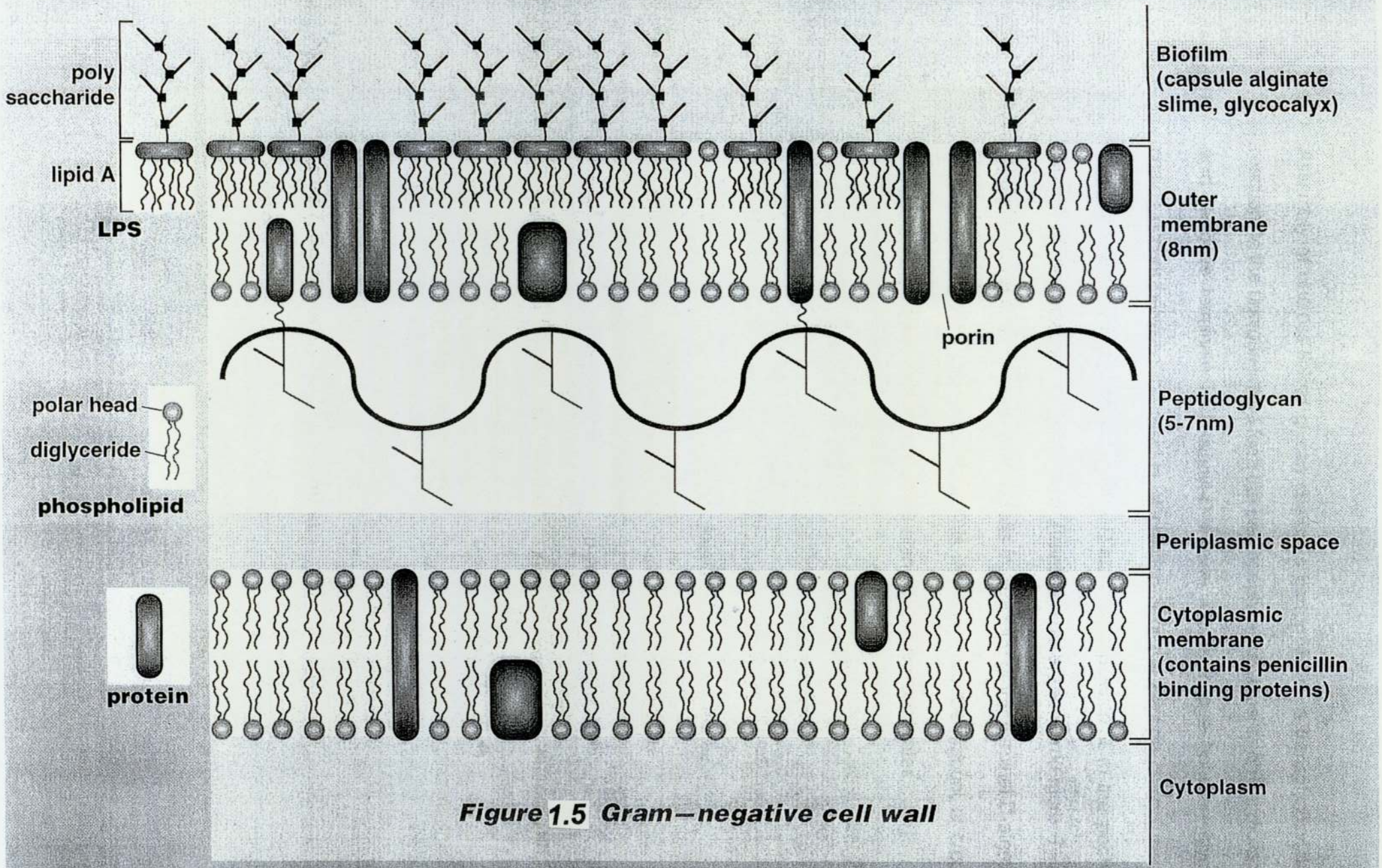


Figure 1.5 Gram—negative cell wall

The Gram-negative bacterial cell wall contains approximately 10% peptidoglycan (murein) (Fig 1.5) which is a meshwork of glycan strands that are cross linked by short peptide bridges (Höltje, J V 1997). The glycan moiety is a chain of alternating β-1,4-glycoside linked units of N-acetyl-D-glucosamine and N-acetyl muramic acid (Bryan, L E and Godfrey, A J 1991). The carboxyl group of muramic acid is substituted with a peptide chain made up of alternating L-and D-amino acids.

The antibacterial activity of β-lactams on Gram-negative bacteria depends essentially on their ability to cross the outer membrane permeability barrier (via water-filled transmembrane pores composed of porin proteins – hydrophilic pathway), their stability to any periplasmically located β-lactamase(s) and their affinity for essential PBPs (Hayes, M V and Ward, J B 1991). Resistance to β-lactams reflects an alteration in any of these three factors (Georgopapadakou, N H 1993).

The β-lactam antibiotics also exert their antibacterial effect by inactivating high molecular weight penicillin-binding proteins (PBPs). All bacteria have such proteins; for example *Staphylococcus aureus* has four PBPs, while *Escherichia coli* has at least seven.

A given bacterial species expresses its own unique set of PBPs reflecting characteristics of the organism, and these proteins are collectively designated based upon molecular size. It is important to realise that PBPs of the same

designation from different species are not related. The role of the larger PBPs has been demonstrated in many bacterial species. In general, PBPs 1a, 1b, and 3 of many species are responsible for a variety of vital cellular operations and most β-lactam antibacterials target one or more of these proteins. Loss of the PBP function has potentially lethal effects on the bacterial cell. In general, the β-lactam antibiotics display a selectivity in undergoing binding to the PBPs that is unique to each individual agent.

In *E. coli*, PBPs are responsible for maintenance of rod shape (PBP 2), cell elongation (PBP 1) and septum formation (PBP 3) (Livermore, D M and Williams, J D 1996). Strains of *P. aeruginosa* exhibit similar functions in bacterial morphogenesis in both organisms (Curtis, N A C *et al*, 1980). The low molecular weight PBPs 4, 5 and 6 are thought to be 'non-essential' since their inhibition has no adverse effect on cell shape. The basis of the bactericidal effect of β-lactams is unclear, the binding of PBPs does not necessarily result in death but indirectly triggers the mechanism(s) responsible for the lethal effect (Odenholt, I S E and Cars, O 1989). Bacterial cells exposed to β-lactams can either lyse, die then lyse, die and not lyse, or survive. The outcome is affected by their autolytic system, growth rate and nutritional state (Bryan, L E and Godfrey, A J 1991). The target PBPs of *E. coli* are present in an excess of the quantity required for normal growth since under in vitro conditions, that is, no lysis was observed when 1a and b were 90% saturated (Gutmann, L et al., 1986). The balance between β-lactam uptake and

clearance determines the fate of the cell rather than either factor alone (Livermore, D M 1988).

In addition, some β-lactam antibiotics can affect the carboxypeptidase function of a PBP (Fig 1.6) although inhibition of transpeptidase activity is usually regarded as the premier event leading to an antibacterial action.

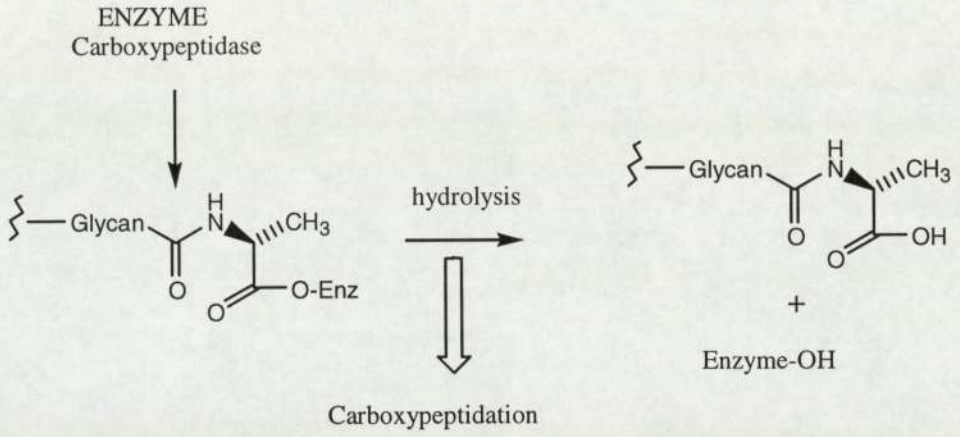


Fig 1.6 Action of carboxypeptidases.

Carboxypeptidase, is analogous to transpeptidase but hydrolyses the D-ala-D-ala termini of pentapeptides, releasing free D-alanine without cross-linking. The role of this enzyme seems to vary in different micro-organisms. It may compete with transpeptidase in order to limit the extent of cross-linkage and prevent excessive rigidity. In some strains, the action of carboxypeptidase upon a pentapeptide may be a prerequisite for the cross-linking reaction involving the amino side-chain of that pentapeptide Fig 1.7 (Williams, R A D and Lambert, P A 1996).

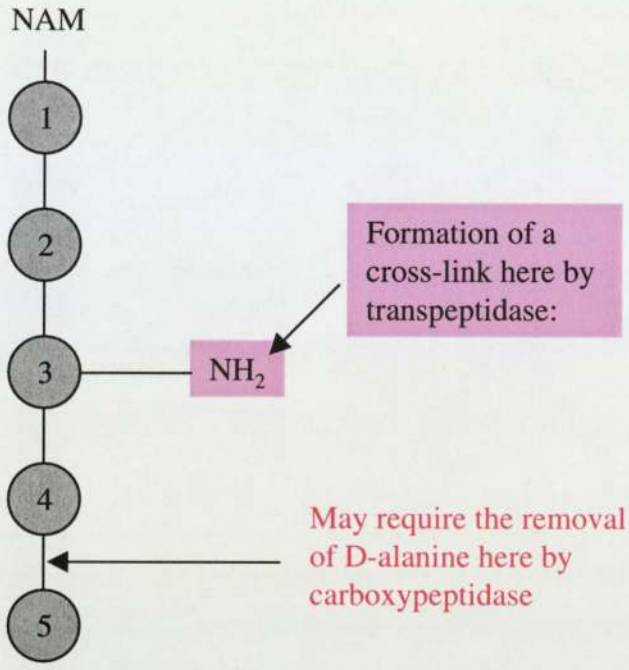
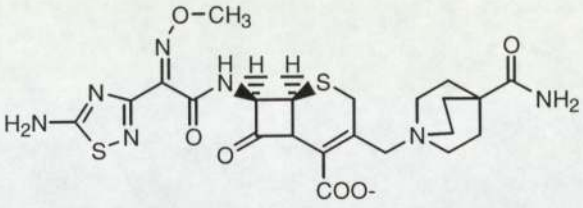
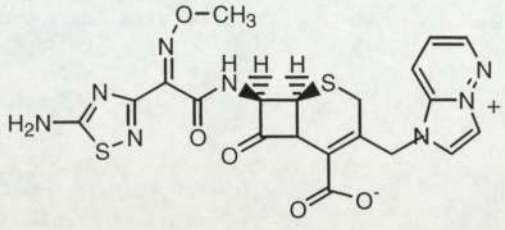


Fig 1.7 Carboxypeptidase action on pentapeptide.

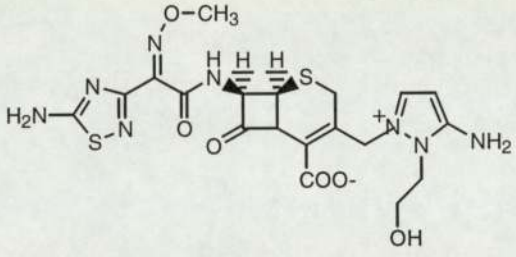
Since the β-lactam antibiotics are able to bind to a number of PBPs with different affinities, and these proteins are performing various functions, it is not surprising that some mechanistic details remain vague. A group of new β-lactam agents have recently entered clinical trials, which oxime-containing west-ends impart an acceptable level of β-lactamase durability to broad spectrum cephalosporins. There remains a need for antipseudomonal cephalosporin agents and several candidates possess a positively charged nitrogen heterocycle as the C-3 substituent, reminiscent of the prototype ceftazidime. Cefclidin, cefozopran, FK-037 and its related congener FK-518, these parenteral agents are truly broad spectrum and possess good stability against many β-lactamases. Compound E-1077 is an interesting variation and



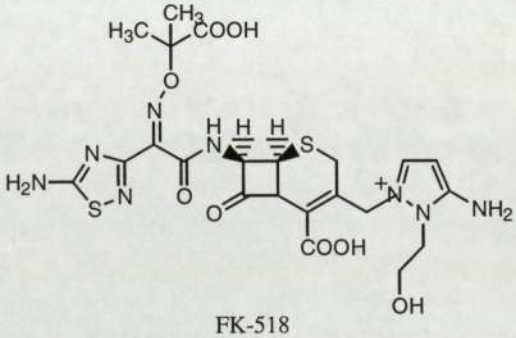
Cefelidin



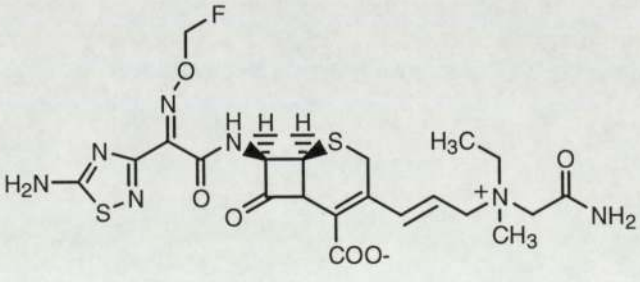
Cefozopran



FK-037



FK-518



E-1077

Fig 1.8 Parenteral cephalosporins under development

demonstrates excellent activity against pseudomonal infections of urinary tract in animal models of urinary tract (Fig 1.8). The incorporation of iron-chelating groups such as catechols and hydroxylated pyridones within the cephalosporin west-end can increase drug penetration into Gram negative bacteria by an iron-uptake mechanism and potentiate antipseudomonal activity. Cefetecol, KP-736 and BOF-12013 are among the most promising of this group. The use of carboxylic ester prodrugs has enhanced oral activity of some cephalosporins that contain small C-3 substituents and an extended spectrum of antibacterial activity: Cefetamet pivoxil, cefdaloxime pentexil, cefcapene pivoxil, cefditoren pivoxil, and cefcanel daloxate(Dax, S L 1997).

In general, these compounds offer a broader spectrum of antibacterial activity than the third generation oral agents and greater potency against many Gram negative pathogens.

1.4 β-lactamases and bacterial resistance to the β-lactam antibiotics

In principle, three basic resistance mechanisms employed by bacteria in response to the action of β-lactam antibiotics are:

1. alteration in target accessibility through decreased permeability,
2. target modification, resulting in a decrease or loss of affinity of the PBPs for the β-lactam antibiotic,
3. the production of β-lactamases (Mascaretti, O A and Boschetti, C E 1995).

In theory, the origins of the first two modes of resistance are encoded by chromosomes and then the possibilities for the development and selection of a mutant in the bacterial population that is insensitive to an antibiotic is virtually endless (Mascaretti, O A and Boschetti, C E 1995).

In practice, β-lactamases are the major enemies of β-lactam antibiotics, as they catalyze the hydrolysis of the β-lactam bond of β-lactam antibiotics, thereby destroying their antibacterial activity (Amyes, S G B *et al* 1996).

The existence of β-lactamases was first noticed as early as 1940, when Abraham and Chain reported that crushed cell of *Escherichia coli* were capable of destroying penicillin (Coulton, S and Francois, I 1994). They concluded that the destruction resulted from the action of an enzyme that they named penicillinase (Dax, S L 1997). By 1944 Kirby had demonstrated that penicillin-resistant strains of *Staphylococcus aureus* caused cleavage of the β-lactam antibiotics to inactive penicillanoic acid, and it was evident that the resistance of *Staphylococci* to penicillin was from β-lactamase activity (Coulton, S and Francois, I 1994). Resistance to the β-lactam antibacterial agents epitomizes the ability of bacteria to mount defences in order to secure survival.

Production of β-lactamases has been reported from Gram-positive and Gram-negative bacteria, actinomycetes, yeasts and blue-green algae (Sykes, R B 1982). In Gram-positive bacteria, the β-lactamase is produced within the

cytoplasm of the cell and is exported through the cell membrane into the surrounding medium. In Gram-negative bacteria, the β-lactamase is again produced in the cytoplasm; however, most of it is exported only as far as the periplasmic space, between the two membranes (Amyes, S G B *et al* 1996). They are probably associated with the cell wall through electrostatic interactions (Mascaretti, O A and Boschetti, C E 1995).

Constitutive β-lactamase production is prevalent among resistant Gram-negative bacteria and results from the regular (and inherent) expression of genetic material that encodes for β-lactamase proteins. In some cases, the enzymes originate from chromosomal genes, although the β-lactamases are more often acquired on plasmids. Constitutive plasmid-mediated β-lactamases account for the majority of β-lactam resistance; the transfer of plasmids is a facile process and widespread among bacterial populations. A number of factors come into play such as the expression of chromosomal β-lactamase genes. The frequency, by which plasmids encode for β-lactamase protein or the copy number for β-lactamase, presents within a transposon. In other words, since β-lactamase production is unaffected by outside forces, the level of β-lactamase production inherent in a given bacteria strain will determine whether resistance is conferred to a particular β-lactam agent (Dax, S L 1997).

However, the mere presence of β-lactamases does not necessarily guarantee resistance. These enzymes must directly encounter the β-lactam agent in order for inhibition of antibacterial activity to occur. For many β-lactams, most of

which are relatively hydrophilic, drug entry into a (Gram-negative) periplasm occurs by passage through porins. Once the β-lactam reaches the periplasm, it can be recognised by a β-lactamase enzyme, undergo binding and be rapidly hydrolysed to the corresponding inactive product (e.g. penicillanoic acid from a penicillin).

As a consequence, the majority of β-lactamase enzyme is not contained within the cell (although most Gram-positive species actually do have a very small intracellular supply of β-lactamase). The supply of β-lactamase is therefore primarily exocellular and subject to external conditions which can alter or destroy the activity of the enzyme. In order for resistance to be conferred, β-lactamase concentrations must reach sufficient levels in the vicinity of the bacterial cell so as to deactivate any β-lactam antibacterial.

Many different β-lactamases are now known, with differing specificities for the various types of β-lactam (Cartwright, S J and Waley, S G 1983). β-Lactamases have been distinguished according to function (specially substrate profile and sensitivity towards inhibitors), physical properties (such as isoelectric point and molecular weight), and genetic location (plasmid or chromosome) (Mascaretti, O A and Boschetti, C E 1995). β-Lactamases have been classified in a number of different ways. β-Lactamases from Gram-positive organisms such as staphylococcal organisms are referred to in general as penicillinases, or penases (Sykes, R B 1982). Gram-negative bacteria,

however, produce a much greater diversity of β-lactamases and therefore require a more complex classification scheme.

Until recently, the most frequently used scheme for the classification of Gram-negative β-lactamases was that of Richmond and Sykes (Coulton, S and Francois, I 1994). In this scheme, five broad enzyme classes were initially identified on the basis of substrate profile and inhibition studies, and each group contained a number of enzyme types.

Class I enzymes, which have been further subdivided Ia to Id, are predominantly active against cephalosporins. They are characteristically produced by strains of *Escherichia coli*, *Enterobacter* species, *Morganella*, *Proteus vulgaris, Pseudomonas*, *Citrobacter*, *Klebsiella* and *Serratia* species. The genetic information of these enzymes is chromosomally mediated and enzyme production may be constitutive or inducible.

Class II enzymes are principally active against penicillins and are chromosomally-mediated. They are found in *proteus mirabilis* and *E. coli.*

Class III enzymes are the plasmid-mediated TEM – type β-lactamases which have approximately equal activity against penicillins and cephalosporins; they are sensitive to inhibition by cloxacillin and resistant to inhibition by *p*-chloromercuribenzoate. They are commonly found in strains of *E. coli* and *Pseudomonas*, as well as *Haemophilus*, *Neisseria*, *Salmonella* and *Shigella.*

The SHV-1 and HMS enzymes also belong to this class of enzyme and together they are the most commonly encountered β-lactamases worldwide.

Class IV enzymes have a similar substrate profile to the enzymes of class III; they are however resistant to inhibition by cloxacillin and sensitive to inhibition by *p*-chloromercuribenzoate. The most important class IV β-lactamases are those produced by strains of *Klebsiella*, and are invariably constitutive and of chromosomal origin.

Class V enzymes have a 'penicillinase' profile, including activity against cloxacillin; they are resistant to inhibition by *p*-chloromercuribenzoate. This class of enzymes includes the oxacillin - hydrolysing enzymes, OXA-1, OXA-2, and OXA-3, and the *Pseudomonas*-specific carbenicillin hydrolysing enzymes, PSE-1, PSE-2, PSE-3, and PSE-4. They are plasmid-mediated enzymes and are found in *E. coli*, *Pseudomonas* and *Serratia* species.

A sixth group (class VI) has subsequently been added into the β-lactamase family produced by *Bacteroides* species (Neu, H C 1985). They hydrolyse cephalosporins better than penicillins and are inhibited by either cloxacillin or carbenicillin.

In all β-lactamases, there is one main active site component. This can either be a serine molecule that provides the catalytic basis for the hydrolysis of penicillins and cephalosporins (classes A, C and D), or a metal ion that

provides the catalytic basis for carbapenem hydrolysis (classes B and E) (Amyes, S G B *et al* 1996).

Most of β-lactamases are extremely efficient at destroying β-lactam agents assuming that the antibacterial is recognised by the enzyme and undergoes binding. Like most PBP_S, the β-lactamases in general are able to accommodate the β-lactam into their reactive pocket through a number of attractive interactions that include hydrogen bonding and lipophilic associations. Typical β-lactam-β-lactamase binding motifs entail complementary charge association of the penicillin carboxylate moiety with specific arginine and lysine residues of the enzyme. A serine or histidine residue in proximity to the β-lactam nitrogen centre serves to stabilise the resultant acyl-enzyme complex. On the other side of the enzyme pocket, an aromatic amino acid residue such as a tryptophan can associate with the lipophilic aromatic group of the β-lactam west-end. These interactions place the reactive β-lactam centre of the drug molecule near the serine residue of the enzyme (Ser-70 in a number of β-lactamase species). This culminates in acylation of the serine with concomitant cleavage of the β-lactam ring (Fig 1.9). The ring-cleaved substrate is released from the enzyme accounting for the catalytic activity of the β-lactamases. Since regeneration of the substrate β-lactam ring is energetically prohibited, antibacterial activity is lost (Dax, S L 1997).

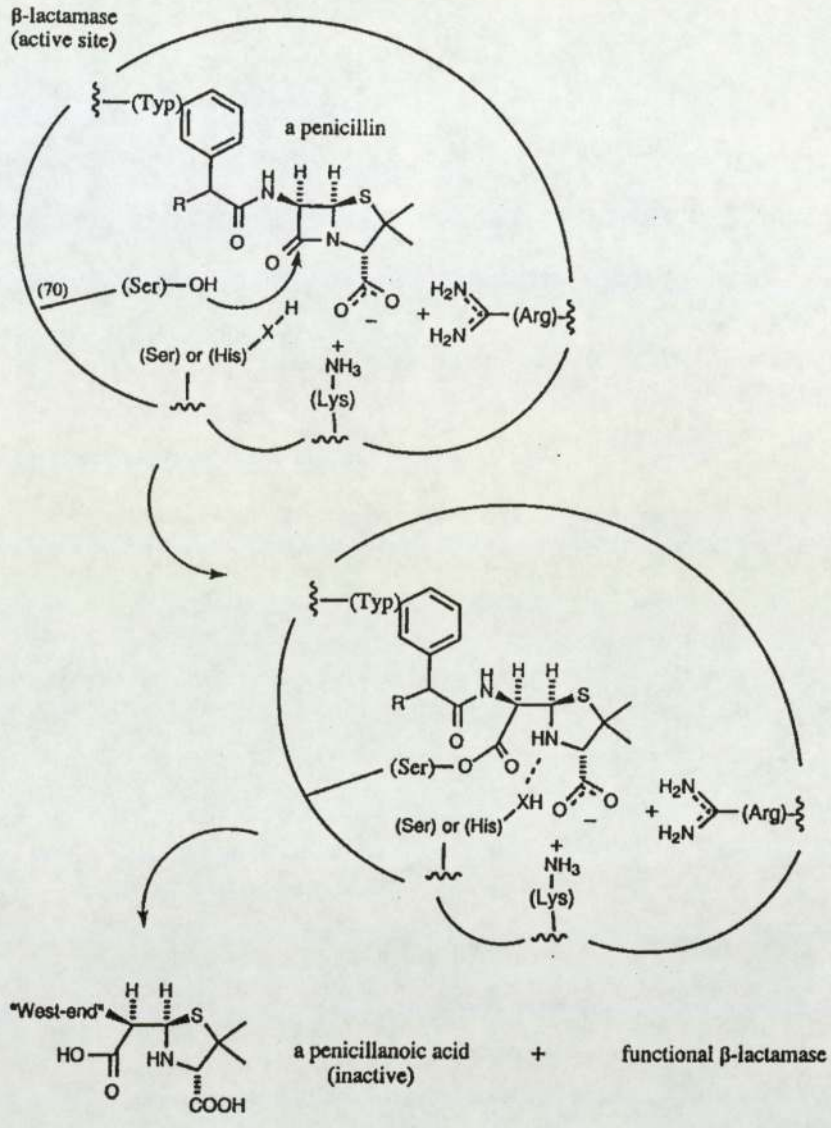


Fig 1.9 Action of β-lactamases

In addition, resistance caused by PBP alteration has recently generated concern for the future of β-lactam antibacterial chemotherapy, though, which may probably remain relatively obscure. Bacteria often acquire resistant PBPs by evolution, but in some alarming cases, resistance is transferred from other strains. Multiple steps/mutations must be achieved before a bacterium can

display β-lactam resistance by altered PBP composition. Gram-positive species such as streptococci and staphylococci, as well as Gram-negative *Haemophilus influenzae, Neisseria gonorrhoeae* and *N. meningitides* have acquired resistance via PBP alteration. The phenomenon of PBP-altered bacterial resistance has now been observed almost worldwide and predictions are that this type of resistance will increase in the near future.

Finally, there are certain bacteria containing feature(s) that impart an intrinsic resistance to the β-lactam agents. Upon encountering Gram-negative bacteria, the β-lactam antibacterials need to cross the outer membrane in order to reach the PBP proteins and produce antibacterial effects. Gram-negative bacteria can exclude passage of β-lactam compounds into the periplasmic space thereby eliciting an intrinsic resistance.

1.5 The approaches to overcome bacterial resistance

The problems of bacterial resistance are accentuated due to the fact that the development of antimicrobial agents by the pharmaceutical industry has slowed dramatically in the past decade since most of the easy targets have been completed in the previous half a century. Moreover the expense for development of a new drug is getting greater and greater. However, it is absolutely imperative that new approaches, free of bacterial resistance, to the therapy of infectious diseases should be developed when numbers of effective antibiotics are in crisis.

The major activities of current investigations in overcoming bacterial resistance focus on the following areas.

(1) Development of new chemical entities as novel antibiotics. A great deal of effort is devoted by medicinal chemists is to screen millions of compounds in order to discover new active antimicrobial agents. In many instances, the screens focus on a specific activity against the designated resistant organisms, or look for an activity against targeted metabolic processes specifically. Studies currently on going in this area include continued screens of natural products and of banks of known chemical entities that have known functions other than antimicrobial activity. Possibly the most interesting approach is the use of screens involving synthetic combinatorial libraries. It is thought that these libraries, each made up of tens of thousands of compounds, promise rapid identification of diverse new lead compounds.

(2) Modification of the structures of currently known agents to improve their therapeutical and enzymatic tolerance profiles.

(3) Development of potentiators of known antimicrobials. It is well known that there are efflux pumps with activity against multiple antibiotics (some of which appear similar to the multidrug resistant (MDR) pumps in cancer cells). Several research groups are currently looking for inhibitors of this system that would potentiate the activity of a variety of antimicrobials against multi-resistant strains of *P. aeruginosa* and might have activity against other organisms with MDR-like pumps as well.

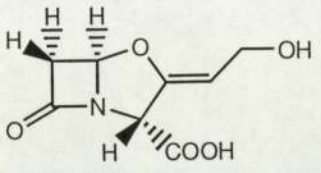
(4) Discovery of new targets in bacterial cellular function. Although a large number of antimicrobial agents exert their mechanism of action *via* the

inhibition of syntheses of bacterial cell walls, still there is a broad variety of metabolic steps in cell wall syntheses for which no effective inhibitors have thus far been developed. Thus, the search for compounds that inhibit unique steps in cell wall syntheses remains an attractive approach. For example, the bacterial translocase transferase reactions, the first membrane associated enzymatic steps in the biosynthesis of the bacterial cell wall, represent attractive targets for the development of additional inhibitors because they are the unique enzymatic systems to bacteria, not found in mammalian cells.

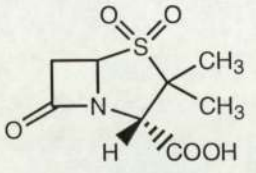
(5) Adherence to certain specific oligosaccharides of host cells is the first step in the process of bacterial infection. It is suggested that an individual oligosaccharide of the host cells acts as a structurally specific receptor for each organism. Therefore design and synthesis of small oligosaccharides that specifically bind to these microbial receptors could result in specific inhibition of bacteria with less chances to induce bacterial resistance.

(6) Development of antisense nucleotides. The idea of developing oligonucleotides that bind to critical segments of DNA or RNA in the bacterial cells and serve as "antisense" molecules has attracted a great deal of attention. This sound excellent approach is under investigation in many laboratories world wide in an attempt to develop new antiviral and antimicrobial agents. However, it has been realised that it is too difficult to implement this approach practically in a short period, largely because of problems, such as non-specific effects from the oligonucleotides, chemical and metabolic instability, lack of binding specificity, and

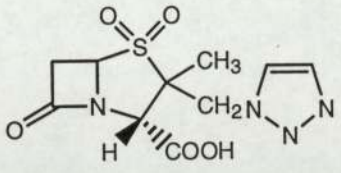
difficulties involved in delivery of intact antisense oligonucleotides to their intracellular site of action. However, if these agents can be successfully produced, they could truly become the "magic bullets", that scientists thought antibiotics would be almost a century ago (Chin, J 1996).



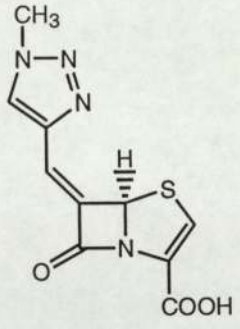
Clavulanic Acid



Sulbactam



Tazobactam



BRL42715

Fig 1.10 Chemical structures of β-lactamase inhibitors.

(7) β-Lactamase inhibitors. The increasing numbers of β-lactamase producing bacteria capable of inactivating β-lactam antibiotics became a major concern in β-lactam therapy, consequently, a great effort has been devoted to the studies of β-lactamase inhibitors in order to overcome this problem. Many unsuccessful attempts to find an inhibitor of β-lactamase were made, as early as the 1950s. It was initially discovered

that some semi-synthetic penicillins, such as oxacillin, could function *in vitro* as β-lactamase inhibitors, however none proved clinically useful. The olivanic acids and clavulanic acid were discovered as part of a large-scale screening of compounds which began in the mid-1960s. In 1981, clavulanic acid became the first β-lactamase inhibitor introduced into clinical practice. Currently used both natural and synthetic β-lactamase inhibitors are β-lactam compounds that lack significant antibacterial activity (McKinnon, P S *et al* 1999). Clavulanic acid is characterised by an oxazolidine ring and the absence of an acylamino side chain in position C6. Sulbactam is a penicillanic sulfone obtained by oxidation of the thiazolidine sulfur of penicillanic acid, whereas tazobactam is a triazolyl-substituted penicillanic sulfone. Finally, BRL 42715 belongs to a novel class of inhibitors, the 6-[substituted methylene] penems (Farmer, T H et al, 1994 (Fig 1.10).

Table 1.2 Intrinsic β-lactamase inhibitory activity of BRL 42715 compared with that of other β-lactamase inhibitors

Organism[a]	I_{50} values (μg/ml) with (+) and without (-) 5 min pre-incubation							
	BRL 42715		Clavulanic acid		Sulbactam		Tazobactam	
	-	+	-	+	-	+	-	+
E.cl P99 (Ia)[b]	0.069	0.002	>50	>50	>50	5.0	>50	0.93
P.v. H (Ic)	0.009	0.003	0.84	0.017	1.8	0.12	0.32	0.006
P.m. C889 (II)	1.4	0.009	3.6	0.021	2.9	0.057	1.0	0.006
E. co. JT4 (TEM-I) (III)	0.044	0.002	0.88	0.055	3.0	1.7	0.12	0.028
K.p. E70 (IV)	0.036	0.001	1.0	0.011	15.7	3.8	0.68	0.047
E.co. (OXA-1) (V)	0.29	0.001	>50	0.71	>50	2.2	>50	1.1
E.co. (PSE-4) (V)	12.5	0.13	2.0	0.022	3.6	0.29	0.42	0.025
S.a. NCTC 11561	3.3	0.016	>50	0.063	>50	1.4	>50	0.27

[a] Enzymes in parentheses were produced particularly by the strains shown;

[b] enzyme classification based upon Richmond-Sykes system

Most β-lactamase inhibitors developed to date are mechanism-based inhibitors or "suicide" inhibitors of β-lactamases. These compounds, initially are recognised as normal substrates by the β-lactamase, but form covalent bonds with various amino acid residues within the enzyme active site, thus leading to irreversible inactivation of the enzymatic activity eventually.

Despite the diversity of the mechanisms reported so far, the scheme depicted in Fig 1.11 is now generally accepted because it accounts for the kinetic data reported for inactivation of various β-lactamases by suicide inhibitors. According to this scheme, the active-site serine hydroxyl group first attacks the β-lactam carbonyl to form acyl-enzyme B. This intermediate can then undergo three competing events. First, the inhibitor is hydrolysed, yielding the intermediate product C in which the β-lactam ring is opened, and then it is spontaneously hydrolysed to give smaller products. Secondly, the inhibitor forms a reversible intermediate (product D) that constitutes a transiently inhibited form of the enzyme. During the course of inhibition, more than 90% of the enzyme can be present in the transiently inhibited form that corresponds to the enamine tautomer of acyl-enzyme B. Finally, interaction of the inhibitor with the enzyme leads to formation of product E, in which the degraded inhibitor is covalently bound to the β-lactamase active site, resulting in irreversible inhibition.

In general, β-lactamase inhibitors have very little antimicrobial activity themselves but rather, typically increase the antibacterial activity approximately 4- to 32-fold to other β-lactams such as amoxycillin, ticarcillin, ampicillin, and piperacillin (see the next section for details). However, β-lactamase inhibitors can paradoxically induce increased production of β-lactamases from the bacteria they encounter. Various studies have shown greater production of β-lactamases when the bacteria have been exposed to β-lactamase inhibitors (McKinnon, P S *et al* 1999).

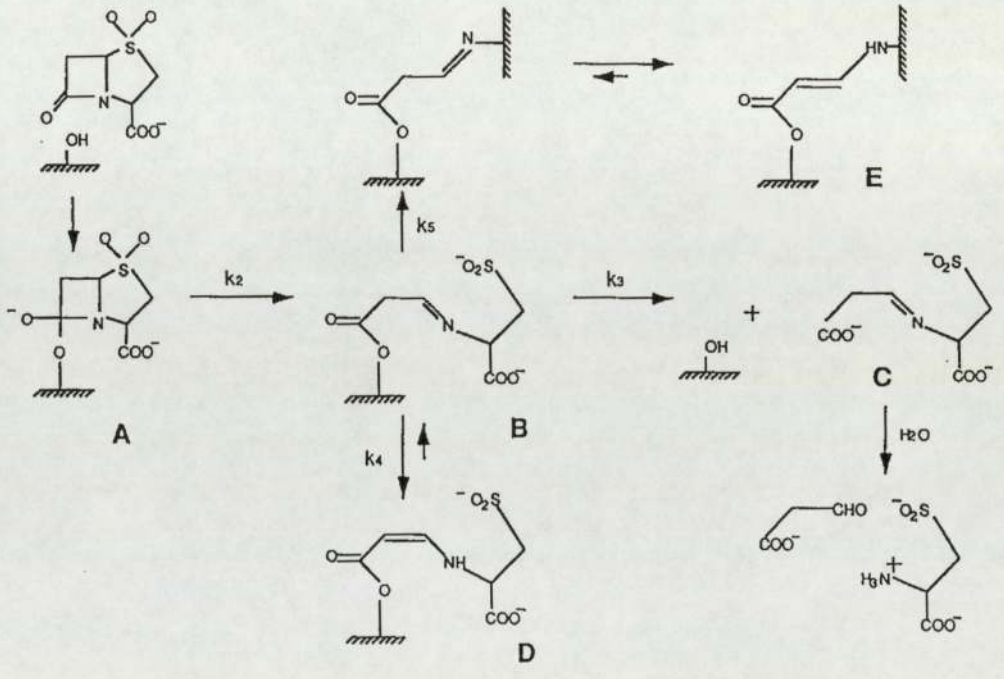


Fig 1.11 General scheme for the mechanism-based inhibitors of class A β-lactamases.

(8) Dual-activity approach. Dual agent approach offered and still is one of most efficient mechanisms in overcoming multidrug-resistance (see the next section for details).

1.6 Mechanism-based Dual-action β-lactam antibacterials

Basically the dual-action therapies are divided into two categories. The first is dual-activity approach. In a simple word, it is a cocktail, which consists of two even three drugs. Each of the drugs possesses an effective action and together they exert a synergistic effect. Currently numbers of cocktails are widely used against cancer, AIDS and bacteria. The second is dual-action mechanism. In this case, two effective drugs are combined *via* a chemical bond to form a new chemical entity, in which two components may exert a continuous or synergistic action *in vivo* either after broken down to two separated compounds or in a single entity.

(1) Dual-activity approach.

A cocktail of a β-lactam antibiotic and a β-lactamase inhibitor is the current widely applied dual-activity approach. Of these compounds are currently in clinical use; e.g. a mixture of clavulanic acid with amoxycillin (SKB, Augmentin) and ticarcillin (Timentin), a mixture of sulbactam with ampicillin (Pfizer, Unasyn) and a mixture of tazobactam with piperacillin (Zosyn, Tazocillin) (Dax, S L 1997). In combination with β-lactamase susceptible antibiotics these β-lactamase inhibitors protect the antibiotic from inactivation by the β-lactamase enzymes, thereby extending the spectrum of activity of the antibiotic.

Table 1.3 Comparative synergistic activities of BRL 42715, tazobactam, clavulanic acid and sulbactam with a range of β-lactamase producing bacteria

Compound	*Concn.*	*MIC (μg/ml)*							
Combination	*of inhibitor (μg/ml)*	*C.f. T1739[a] (I)[e]*	*E. ae. 53[a] (I)*	*E.cl. P99[a] (Ia)*	*E.c. AmpC (high) (Ib)*	*E.c. TEM (high) (III)*	*E.c. OXA-1 (V)*	*K.p. TEM-1 (IV/III)*	*S.a.[b.c]*
Amoxycillin alone		>512	>512	>512	512	>512	>512	>512	>128
Amox + Sulbactam	16	-	-	-	128	>512	32	64	-
Amox +Clav. acid	4	-	-	-	256	32	32	4	2[d]
Amox + Tazobactam	4	512	>512	>512	256	64	64	8	-
Amox + BRL 42715	1	1	2	64	2	4	2	I	0.12[d]
Piperacillin alone		512	>512	>512					
Pip + Tazobactam	4	128	512	512					
Pip + BRL 42715	1	4	16	2					
Cefazolin alone		>512	>512	>512					
Cef + Tazobactam	4	>512	>512	>512					
Cef + BRL 42715	1	4	2	16					
Cefotaxime		64	64	>64					
Cftm + Tazobactam	4	32	>64	64					
Cftm + BRL 42715	1	0.5	2	2					

[a] Cefotaxime resistant; [b]methicillin sensitive strains; [c]*MIC* for 50% of strains tested; [d] inhibitor concentration, 0.2 μg/ml; [e]enzyme classification according to Richmond and Sykes

Resistance to amoxycillin-clavulanate in clinical isolates producing inhibitor-resistant TEM (IRT) enzymes results from point mutations that alter the region of the bla_{TEM} genes encoding penicillinases TEM-1 and TEM-2 (Peggy S Mckinnon 1999).

Hyper-production of wild-type TEM-1 or TEM-2 penicillinases is a frequent mechanism of resistance to β-lactamase inhibitors in clinical isolates (Reguera, J A *et al* 1991). This mechanism causes resistance not only to combinations of amoxycillin and clavulanic acid but also to ampicillin/sulbactam, amoxycillin/tazobactam and piperacillin/tazobactam (Reguera, J A *et al* 1991). At the molecular level, β-lactamase overproduction is generally caused by a point mutation in the nucleotide at position 162 in the promoter region of the bla_{TEM} gene. Overproduction of β-lactamases can also result from an increased number of copies of the plasmid harbouring the *bla* gene in the bacterial cell.

Resistance to the amoxycillin-clavulanate combination has also been described in clinical isolates of *E. coli* with no mutations in the promoter and coding regions of the bla_{TEM} genes, suggesting that other mechanisms can also confer resistance to β-lactamase inhibitors. Mutations in the genes involved in porin synthesis can alter the permeability of the outer membrane. Uptake of β-lactamase inhibitors is lowered. Also, a synergistic effect between β-lactamase overproduction and decreased permeability has been suggested, and the two mechanisms are frequently associated in an individual clinical isolate.

*(2) **Dual-action mechanism.***

In most cases, a known antibacterial combines with a cephalosporin through forming an ester or amide with the 4-carboxylic acid group of the cephalosporin. The first dual-action cephalosporin appeared in the literature

in 1976, when O'Callaghan, Sykes, and Staniforth (O'Callagham, C H et al 1976) described the properties of the cephalosporin (Fig 1.12).

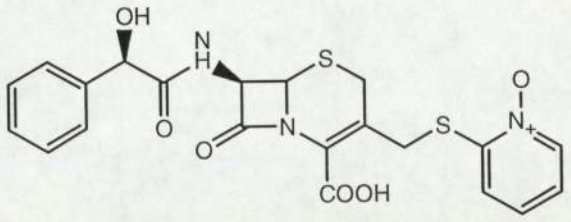
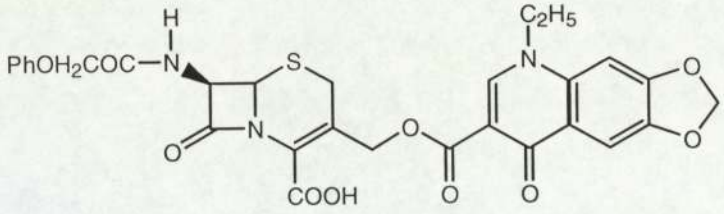
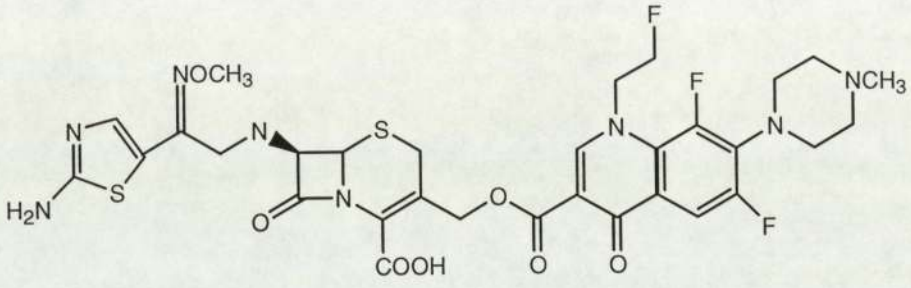


Fig 1.12 Cephalosporin MCO

This provides a fascinating rationale for drug design, and has led to the syntheses of antibacterials with interesting and unusual properties (Keith, D D *et al* 1994) as shown in Fig 1.13, which are examples of this class of dual agents. They are molecular hybrids of cephalosporins and quinolone antibacterials constructed in a way that allows both components to exert their individual antibacterial properties (Fig 1.14).



Ro 23-5068



Ro 23-9424

Fig 1.13 Dual action β-lactams

The antibacterial mechanism of this group of dual action compounds is that when a β-lactamase is deactivating the cephalosporin by opening of the β-lactam ring the second antibacterial agent is released on site to exert its antibacterial activity. The dual-action mechanism of action has been demonstrated: upon PBP binding, the β-lactam centre undergoes acylation of bacterial transpeptidases which result in subsequent disruption of peptidoglycan biosynthesis. As a consequence of β-lactam ring cleavage, free quinolone is released at the cellular level that allows for inhibition of DNA gyrase activity (Dax, S L 1997). Carbapenem-and penem-based dual-action agents have also been reported (Keith, D D *et al* 1994).

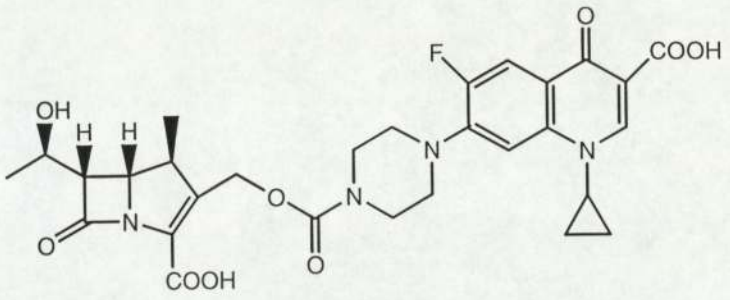


Fig 1.14 Dual action β-lactams

Many other variations on this theme are being pursued; some include the linking group between the quinolone and the β-lactam (ester, carbamate, amine, ammonium, etc.).

Dual-action β-lactam antibacterials characteristically display a composite spectrum of antibacterial activity indicating contributions from both agents. This can be advantageous since β-lactam (cephalosporin) activity can typically bestow potent activity against Gram-positive pathogens such as streptococci and staphylococci while quinolone potency is often primarily manifested against Gram-negative species including *Pseudomonas aeruginosa*. In addition, anaerobic organisms can be covered if a penem or carbapenem is incorporated into a dual-action agent. Finally, some of the undesirable properties of each component can be notably reduced by combining the two antibacterial agents as a dual-action hybrid (Dax, S L 1997).

A cell wall of peptidoglycan surrounds most bacteria, providing rigid support and protection against environmental changes in osmotic pressure. This structure is unique in nature. The biochemistry of cell-wall synthesis is highly specific, and provides selective targets for useful chemotherapeutic agents (Keith D D *et al* 1994). One lethal target is the final cross-linking step, through which rigidity is introduced into the peptidoglycan. β-Lactams inhibit this step by acylating active-site serine residues of the transpeptidases responsible for carrying out the cross-linking. Similarly, acylation of active-site serine residues occurs as a key step in the mechanism by which most β-lactamases inactivate these antibiotics. In either case, when a cephalosporin containing a potential leaving group, X, at the 3'-position reacts with a bacterial enzyme, that group is eliminated (Fig 1.15).

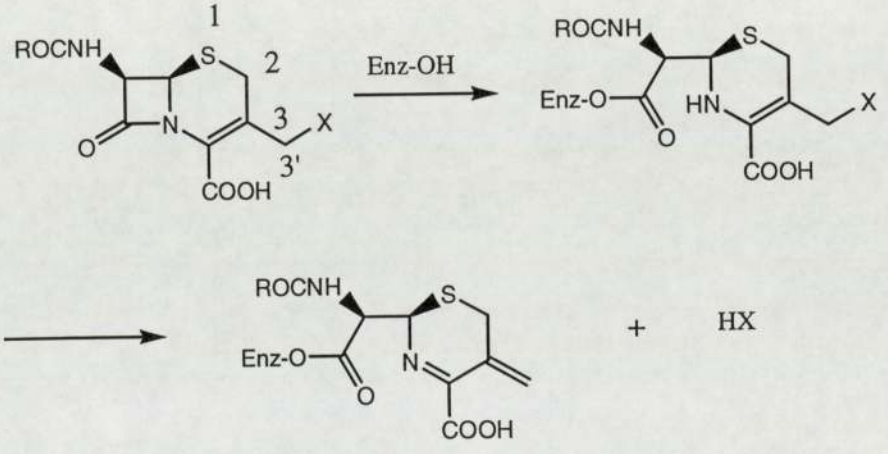


Fig 1.15 Dual-action mechanism.

It has been reported that opening of the β-lactam ring correlates with elimination of the leaving group, although the reaction is probably not

concerted. Quantitative release of such 3'-substituents as acetate, azide, and pyridine can be brought about by treatment with β-lactamases, and this reaction appears to be quite general. If the leaving group possesses intrinsic antibacterial activity, then the cephalosporin should exhibit a dual mode of action. In addition to providing its own β-lactam activity, the cephalosporin should also act as a targeted prodrug for the second antibacterial agent, delivering it close to its site of action (Keith, D D *et al* 1994).

1.7 Aim and Objectives

The aim of the project is to study syntheses and antibacterial activities of a group of dual agents designed to be resistant to β-lactamases in line with recent achievements of dual-activity approaches in combating super-bugs (Chin, J 1996).

The dual agents designed for this study fall into two categories. The first is a combination of β-lactam antibiotic. The cytotoxic agents used here are temozolomide and mitozolomide discovered in Aston University. The cytotoxic agents combine on α-amino group of α-aminophenylacetamido of amoxicillin and cefalexin. Locations of cytotoxic agents are similar to the stereo-blocking group in piperacillin. Therefore they can protect the β-lactam ring of the antibiotic on one hand, and they can be cytotoxic lethal drugs to the bacteria as they are on the surface of the bacteria even after the antibiotic is deactivated by the β-lactamases.

The second is a combination of the β-lactam antibiotics and β-lactamase inhibitors. We chose ampicillin, amoxycillin and cephalexin to combine with sulbactam and tazobactam because presently they are the most popular agents used in clinic. Both sulbactam and tazobactam have been successfully used in the design of prodrugs or the cocktails. However, bacterial resistance against them is increasing. A variety of ways could be applied to constitute dual agents. We foresee that loading a β-lactamase inhibitor on the amino groups of 6-(aminophenylacetyl) and 6-[amino(4-hydroxyphenyl)acetyl] in ampicillin and amoxycillin will produce novel dual agents, which integrate the merits of both semi-synthetic N-acyl ampicillin or amoxycillin antibiotics, such as piperacillin (Qingxiang, Y and Wang, Y F 1987) and the cocktail dual agents to achieve two goals:

1. To introduce a stereo-block protecting the β-lactam ring from an attack of β-lactamase, which has been proved to be a very successful strategy in the past (Evans, M A *et al* 1978)
2. To introduce a bodyguard protecting the β-lactam ring against an attack of β-lactamases.

The attempted model compound is shown in the Fig 1.16. At present, the research indicated the carboxylic acid groups in sulbactam and tazobactam are not the key element required by the receptor binding. Therefore they are the right β-lactamase inhibitors for our dual agent studies. The same idea can be applied to a wide range of β-lactam antibiotics and β-lactamase inhibitors, for example clavulanic acid, sulbactam, tazobactam, BRL42715.

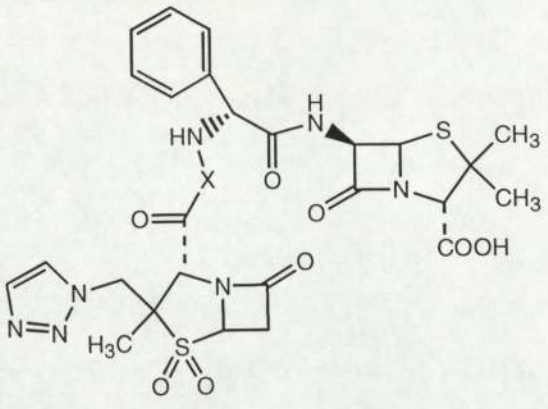


Fig 1.16 Designed model conjugate

Chapter 2

Synthesis Of Conjugates Of Cytotoxic Agents Temozolomide And Mitozolomide With β-Lactams

2.1 Introduction

β-Lactamases are the most serious threats posed by bacteria to β-lactam antibiotic chemotherapy. Almost every member of the β-lactam antibiotics has met resistance in clinical use.

Numbers of approaches have been developed to overcome β-lactamases, including cocktails made of antibiotics with β-lactamase inhibitors, as well as conjugates of antibiotics with β-lactamase inhibitors. However, ability of bacteria to adapt themselves to new environments through mutation or increase of self-protecting armours has limited application of these approaches dramatically. The limitation of these approaches, either cocktails or conjugates, are that they only act on bacterial metabolic processes and bacterial self-protection enzymes (β-lactamases); as a result, mutations of bacterial DNA are induced. It seems feasible to us that if a dedicated conjugate is made of a β-lactam antibiotic and a cytotoxic agent, the cytotoxic agent can exert cytotoxic effect to kill bacteria before or after the β-lactam antibiotic is disassembled by β-lactamases. In this strategy, the β-lactam antibiotic acts as

an antibacterial and also as a targeting auxiliary to carry and direct the cytotoxic agents to surfaces of bacteria.

Two types of the conjugates were designed in this study reflecting possible synergetic effect and synthetic feasibility of a conjugate of β-lactam antibiotic and a cytotoxic agent. The cytotoxic agents, mitozolomide and temozolomide, were chosen in this study because they are discovered in Aston and readily available.

In the type one of conjugates (Fig 2.1), we envisaged that if the cytotoxic agents link on the α-amino group of 6- or 7- α-aminobenzyl of ampicillin and cefalexin, the group of cytotoxic agents can effect stereo hindrance similar to the piperazine group in piperacillin on the one hand; it can exert cytotoxic activity before or after the β-lactam antibiotics are de-activated on the other hand.

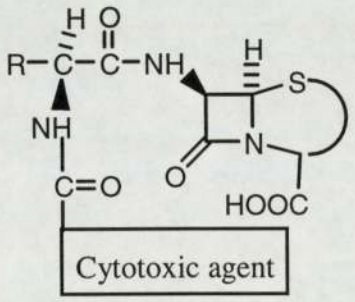
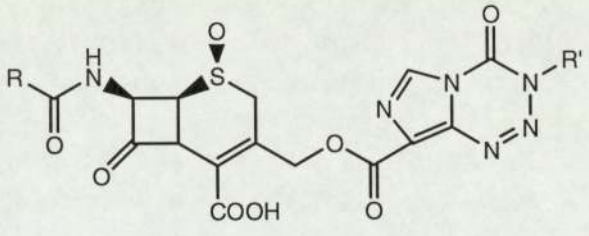


Fig 2.1 Conjugates 2.1

In the type two of conjugates (Fig 2.2), the cytotoxic agents were located on the 3-methylene group of cephalosporin. According to the established mechanism, when the carbonyl group in β-lactam is attacked by a nucleophile, such as a β-lactamase, a cascade reaction is initiated Fig. 1.15. As a result, 3-methylene group is converted to exo C=C to expel the group on it. Therefore, when a cephalosporin β-lactam is de-activated by β-lactamases, the cytotoxic agent will be released on surface of bacteria and can easily exert cytotoxic effect on bacteria.



2.2a	R=Bzl;	R'=CH_2CH_2Cl
2.2b	R=2-Thiophene;	R'=CH_2CH_2Cl
2.2c	R=$PhCH_2O$;	R'=CH_2CH_2Cl
2.2d	R=Bzl;	R'=CH_3
2.2e	R=2-Thiophene;	R'=CH_3
2.2f	R=$PhCH_2O$;	R'=CH_3

Fig 2.2 Type two conjugates (Conjugate 2.2a – 2.2f)

2.2 Cytotoxic agents

Mitozolomide

Mitozolomide, (NSC 353451) (Stevens *et al.*, 1984) or 8-carbamoyl-3-(2-chloroethyl)-imidazo-[5,1-d]-1,2,3,5-tetrazin-4(3H)-one (Fig 2.3), is a cytotoxic agent with anticancer activity. It has shown striking activity against rodent tumours (Erba, E *et al* 1986).

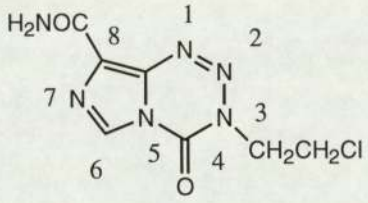


Fig 2.3 Mitozolomide

The antitumour effect of mitozolomide is thought to be caused by chloroethylation and cross-linking of DNA, as occurs with the chloroethylnitrosoureas (CNUs). The overall shared mechanism of action of mitozolomide with CNUs is indicated by their cross-resistance in cell lines and in *vivo* tumours, including those demonstrated to exhibit relatively high levels of the DNA alkyltransferase (ATase) (Dive, C *et al* 1989).

Its wide spectrum of activity, similarity to chloroethylnitrosoureas in the formation of DNA-interstrand cross-links and reported cross-resistance with nitrosoureas, are consistent with a mechanism of action involving the formation of a chloroethyltriazene (MCTIC).

Like the chloroethylnitrosoureas, mitozolomide does not produce DNA-ISC in cells that are able to remove the crosslinkable monoadducts bound to O^6 of guanine (i.e. cells with MER+ phenotype) (Erba E *et al* 1986). On the other hand, in contrast to the most commonly used chloroethylnitrosoureas (e,g. BCNU), mitozolomide exhibited only modest inhibition of glutathione reductase, gammaglutamyl transpeptidase and chymotrypsin. Since these enzymes are sensitive to inhibition by carbamoylating agents, it was concluded that such species were not formed under physiological conditions. DNA damage by mitozolomide is thought to be mediated by the chloroethylating metabonate MCTIC (Dive, C *et al* 1989).

Temozolomide

Temozolomide (8-Carbamoyl-3-methyl [5,1-d]-1, 2, 3, 5-tetrazin-4(3H)-one) is an imidazotetrazine derivative of the agent dacarbazine (Fig 2.4). It undergoes chemical conversion to species 5-(3-methyl-1-triazeno)-imidazole-4-carboxamide (MTIC) at physiological pH (Hvizdos, K M and Goa, K L 1999). MTIC methylates the 6 position of guanine, although this lesion may be repaired by the enzyme O-6-alkylguanine-DNA alkyltransferase (Britten, C D *et al* 1999).

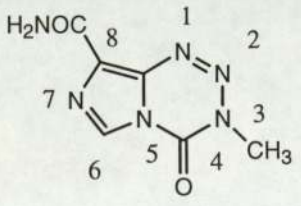


Fig 2.4 Temozolomide

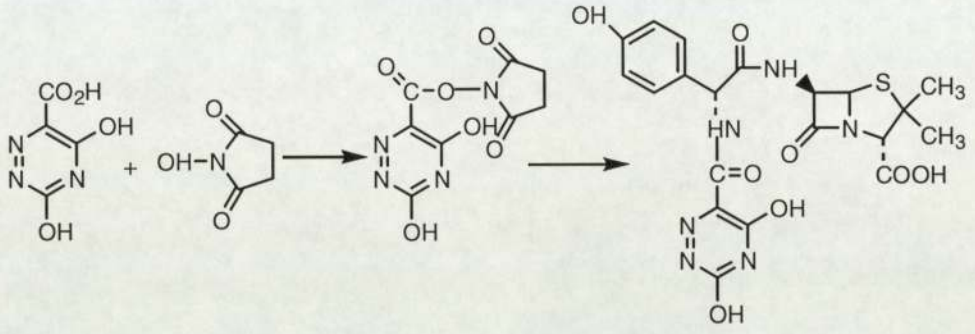
Temozolomide is a robust antitumour drug of significant benefit to brain tumour patients, and has been approved in Europe and may be available in the USA soon as an anti-brain tumour drug (Wang, Y F 1996). Detailed NMR and crystallographic and molecular modelling studies have confirmed that temozolomide is an effective molecular device (MW 194 Da) for targeting an electrophilic and fugitive methyldiazonium fragment to the major groove of guanine-rich sequences in DNA. Clinical activity correlates with methyl group transfer to a guanine O6-position of DNA since tumour types which can repair this lesion are less sensitive to the drug (Wang, Y F *et al* 1994).

Another major reason for choosing mitozolomide and temozolomide as the cytotoxic agents for our conjugates is that they are small molecules and find no difficulty to pass through cell membranes. Therefore, when they are linked on a β-lactam antibiotic or after they are disconnected with the β-lactam antibiotic, they could access bacterial genetic materials through diffusion once they are on the surface of bacteria.

2.3 Synthesis of the type one of conjugates 2.1: the cytotoxic agents link on the α-amino group of 6- or 7-α-aminobenzyl of ampicillin, amoxicillin and Cephalexin

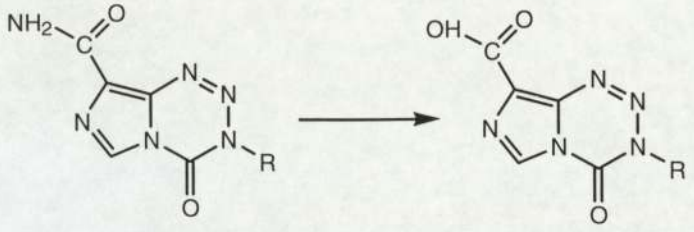
In 1970s when the advanced antibacterial activity of N-acyl ampicillin or amoxicillin type of antibiotics, such as, piperacillin and BL-P1908 were discovered, the synthetic methods for acylation of α-amino group of α-aminobenzyl and α-amino(p-hydroxybenzyl) groups in ampicillin and amoxicillin were explored. Activation of carbonyl groups is essential for a successful acylation especially when a selection is required between two or

among more active groups such as amino or hydroxyl. The active ester method is one of the most successful methods for completion of this task. N-hydroxy succinimide active ester approach is the ideal for synthesis of our designed conjugates because they are easy to prepare and have been successfully applied in the acylation of ampicillin and amoxicillin (**Scheme 2.1**).



Scheme 2.1

In order to prepare the conjugate through acylation of α-amino group of α-aminobenzyl and α-amino (p-hydroxybenzyl) groups in ampicillin, amoxicillin and cephalexin, 8-carboxamide group of temozolomide and mitozolomide has to be converted to carboxylic acid. It is well known that the imidazotetrazinone nucleus is sensitive to basic media where tetrazinone ring opens to give N^3-alkyl imidazotriazine, the active form of the drug. In contrast, the nucleus is very stable to acidic media, including both organic and inorganic acids. Conversion of 8-carboxamide in temozolomide and mitozolomide to 8-carboxylic acid was accomplished



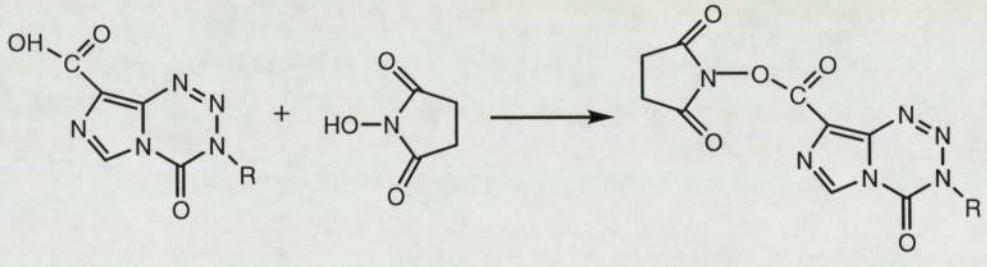
2.3 R=CH_2CH_2Cl **2.5** R=CH_2CH_2Cl

2.4 R=CH_3 **2.6** R=CH_3

Scheme 2.2

with sodium nitrite in concentrated sulfuric acid. The reaction was initiated by careful addition of 3.5 equivalent sodium nitrite to a suspension of temozolomide and mitozolomide in sulfuric acid at 0 °C, followed by continuous stirring at room temperature for 6 hours to give the acid **2.5** and **2.6** (Scheme 2.2) as white precipitate (**Scheme 2.2**).

N-hydroxy succinimide active esters of temozolomide and mitozolomide were prepared in the following manner (**Scheme 2.3**).



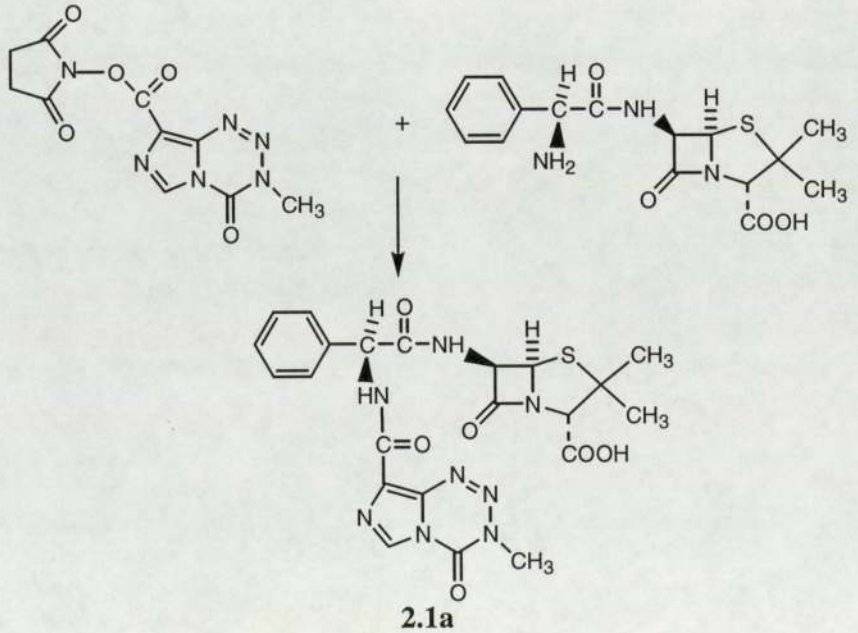
2.7 R=CH_2CH_2Cl **2.8** R=CH_3

Scheme 2.3

In presence of dehydrating agent DCC, a dry DMF solution of equal mole of temozolomide or mitozolomide acid and N-hydroxysuccinimide was stirred at room temperature for 8 hours. After dicyclohexylurea precipitate was

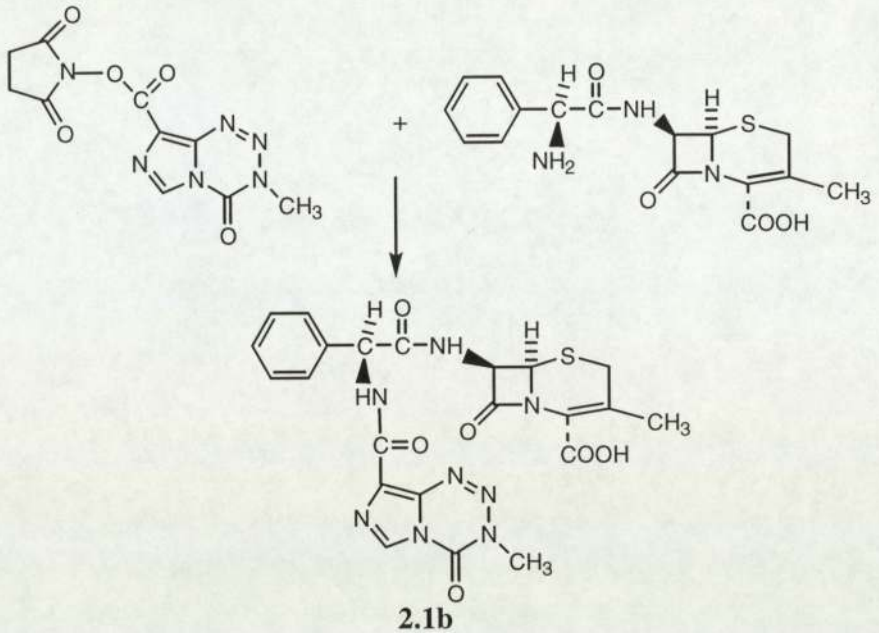
removed by filtration, the solution was concentrated under high *vacuum* to give white solid temozolomide or mitozolomide active ester in a moderate yield.

Acylation of α-amino group of α-aminobenzyl and α-amino(p-hydroxybenzyl) groups in ampicillin, amoxicillin and cephalexin were carried out smoothly. First the mixture of equal mole of the β-lactam antibiotic and the active ester in dry DMF was cooled and exposed to triethylamine. The reaction normally finished in one day at ambient temperature. After work-up with water and an organic solvent extraction to remove both water soluble and organic soluble impurity, the conjugates 2.1a (Scheme 2.4) of the β-lactam antibiotics and temzolomide were obtained (**Scheme 2.4**).



2.1a

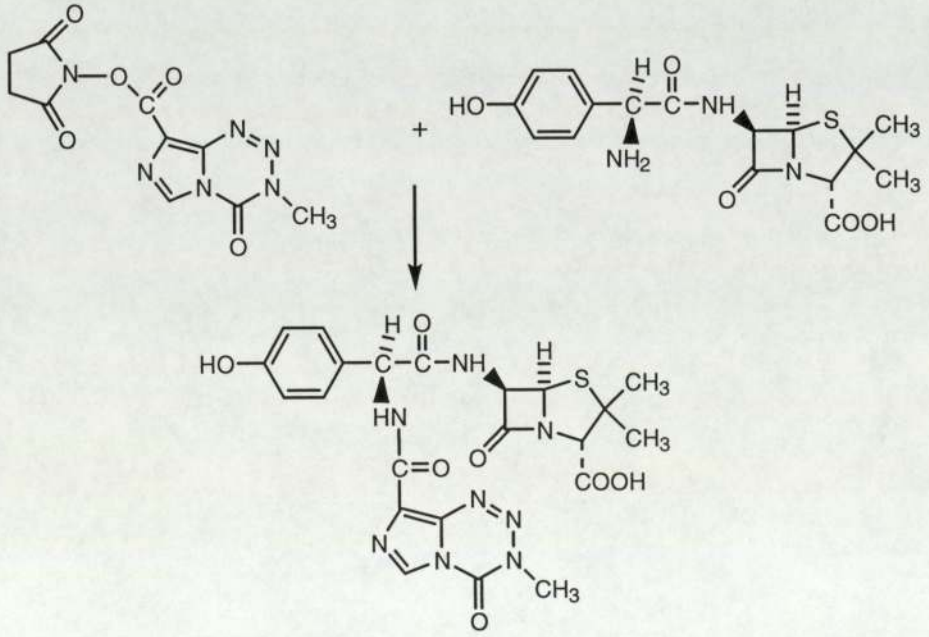
Scheme 2.4



2.1b

Scheme 2.5

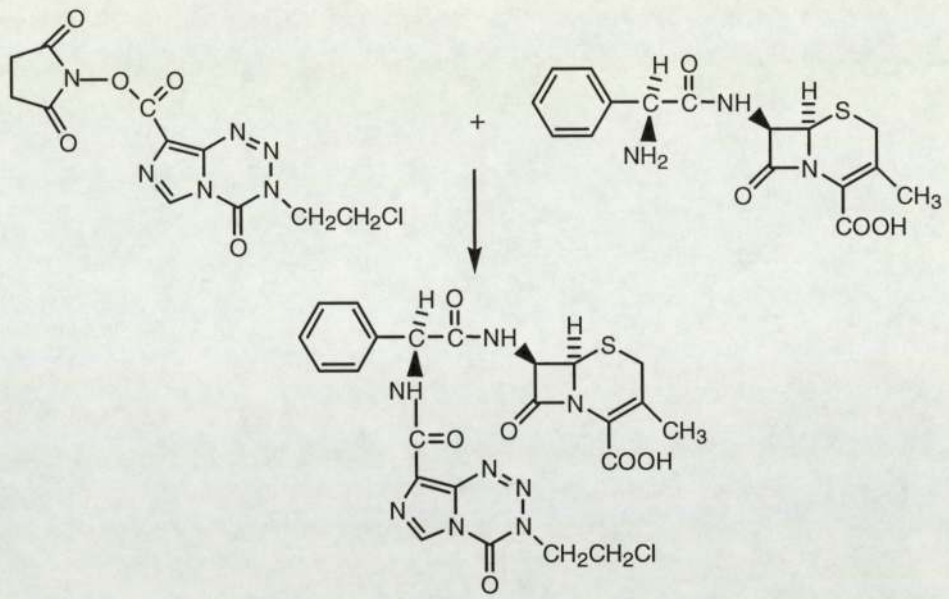
In the same manner acylation of cephalexin was successful (**Scheme 2.5**) to give the conjugate 2.1b. The selective acylation of amino group of amoxicillin was accomplished in the same approach to give the conjugate 2.1c - **Scheme 2.6**



2.1c

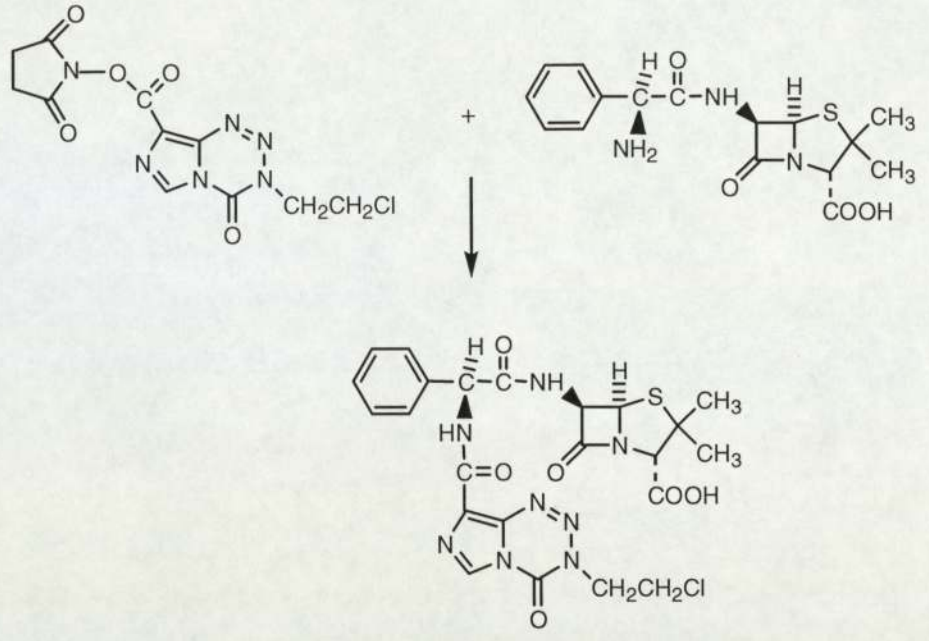
Scheme 2.6

The conjugates containing mitozolomide were also synthesised in the same approach (**Scheme 2.7, 2.8** and **2.9**)



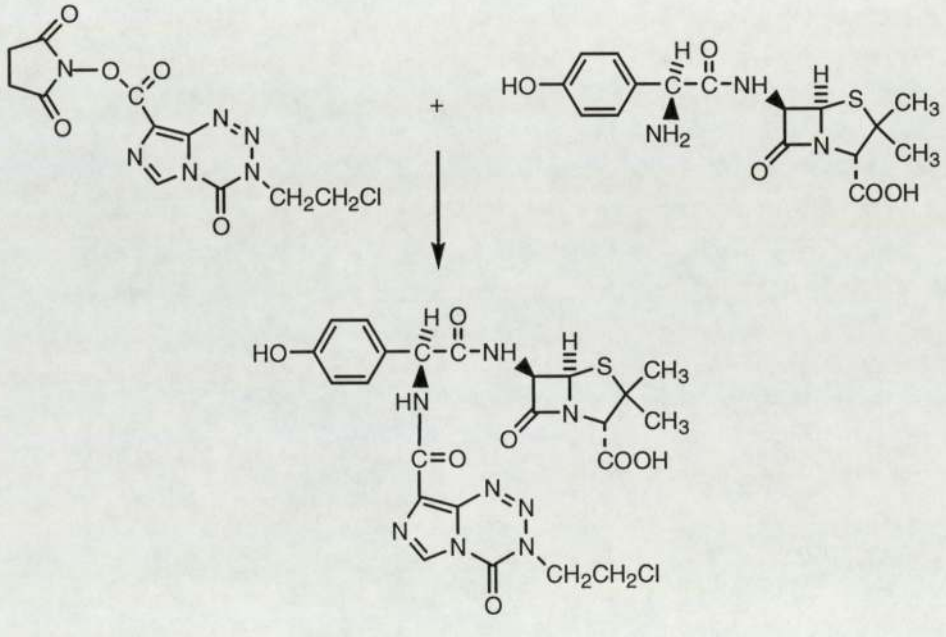
2.1d

Scheme 2.7



2.1e

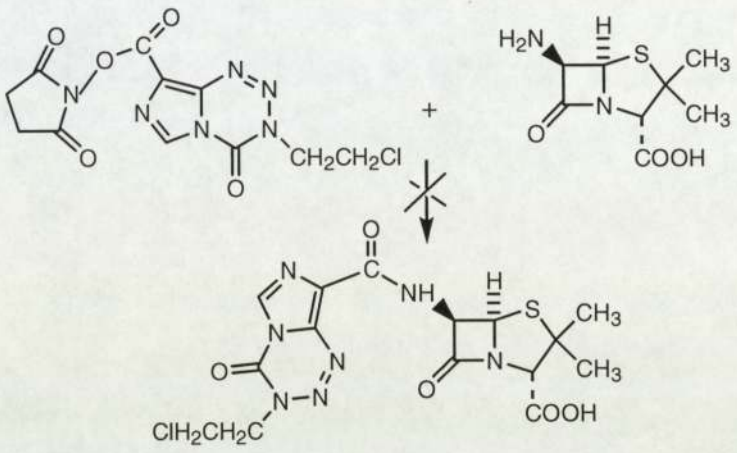
Scheme 2.8



2.1f

Scheme 2.9

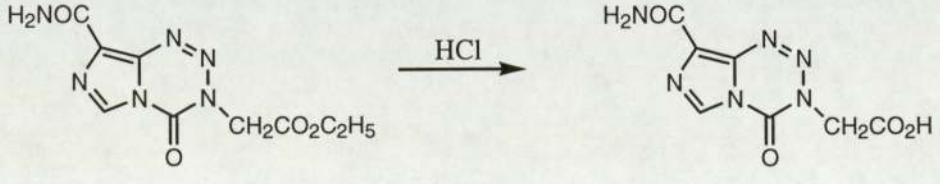
The acylation of 6-APA with mitozolomide active ester was intended to achieve **2.1g** (**scheme 2.10**) but it was not successful.



2.1g

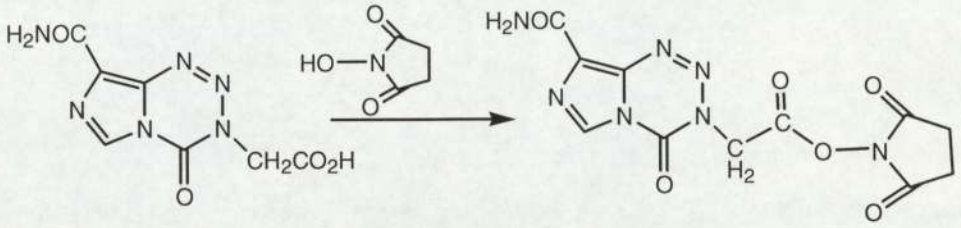
Scheme 2.10

A group of 3-acylate 4-oxo-imidazo[5,1-d]-1,2,3,5-tetrazine conjugates were also synthesised as described in **Schemes 2.13, 2.14, 2.15, 2.16** and **2.17**. First, ethyl 8-carbamoyl-3,4-dihydro-4-oxo-imidazo[5,1-d]-1,2,3,5-tetrazine-3-ylacetate was hydrolysed under acidic condition to 8-carbamoyl-3, 4-dihydro-4-oxo-imidazo [5,1-d] -1,2,3,5- tetrazine-3-ylacetic acid **2.9**, **Scheme 2.11**, followed by preparation of the active ester **2.10** in the same manner as described above **Scheme 2.12**.



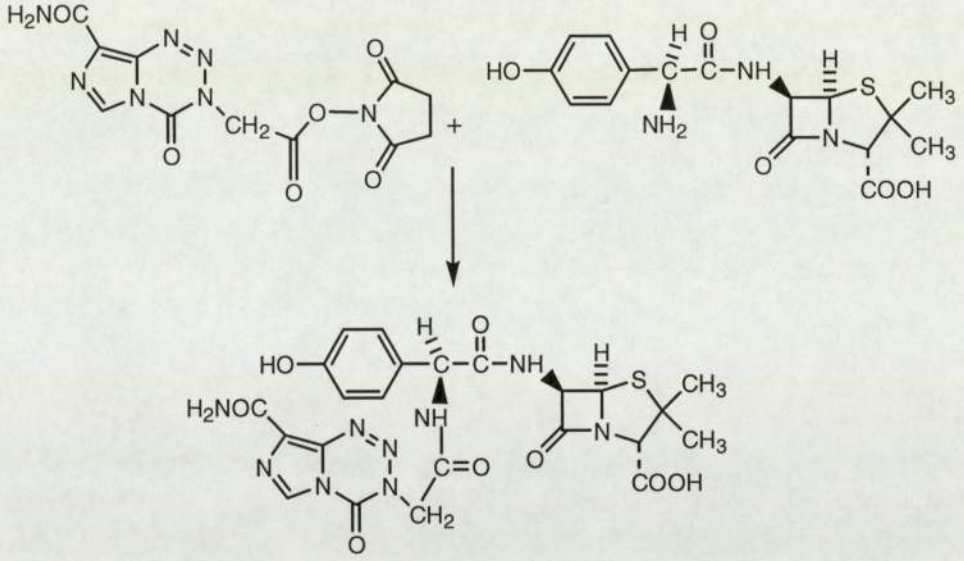
2.9

Scheme 2.11



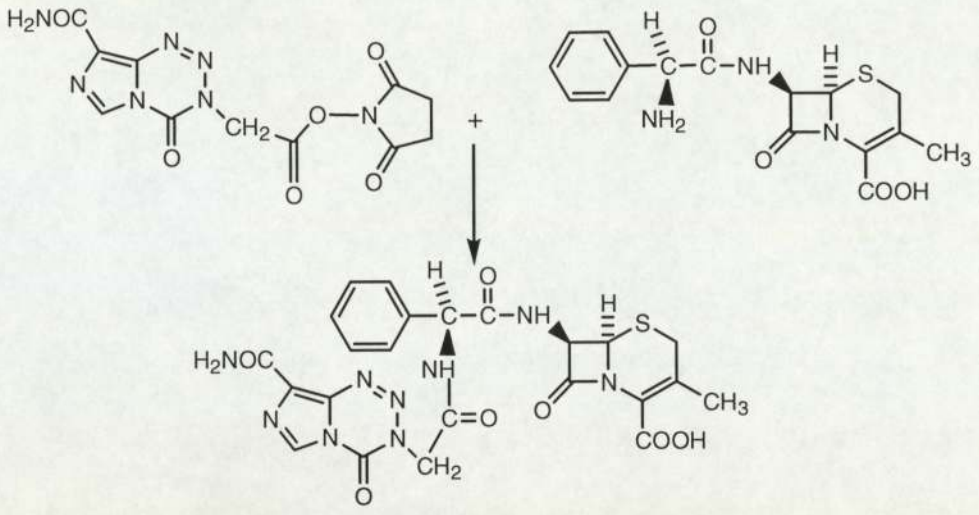
2.10

Scheme 2.12



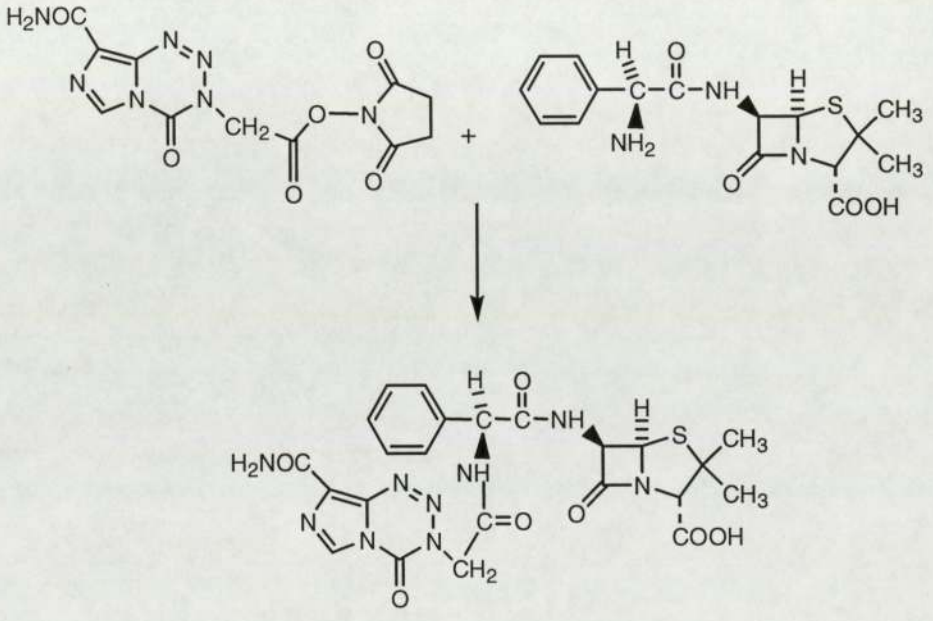
2.1h

Scheme 2.13



2.1i

Scheme 2.14



2.1j

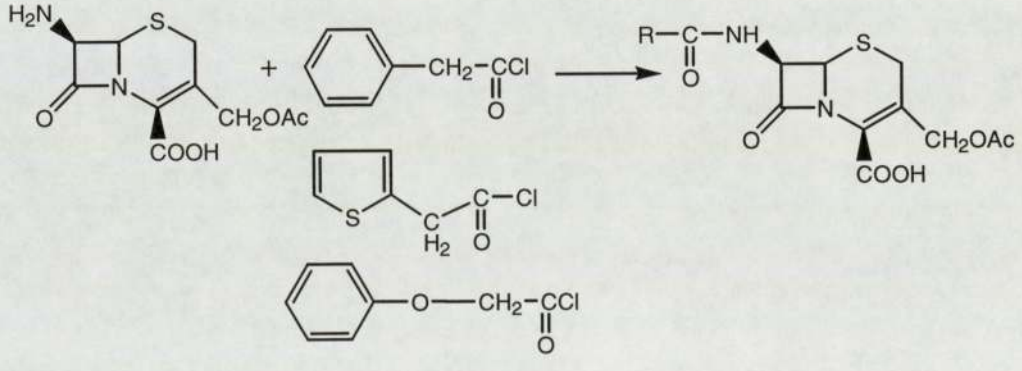
Scheme 2.15

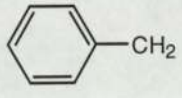
Attempts to convert the conjugates **2.1a-j** into their corresponding water soluble sodium salts with both organic and inorganic reagents resulted in

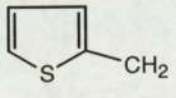
decomposition of the conjugates. This might indicated that the carbonyl group in the triazinone is vulnerable to a weak base, such as an organic carboxylic salt.

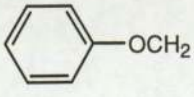
2.4 Synthesis of the type two of conjugates 2.2: the cytotoxic agents were located on 3-methylene group of cephalosporin

Cephalosporins, **2.11a, b** and **c**, were chosen as the cephem in this study since it is economically easy to access. A reaction of 7-ACA (7β-aminocephalosporanic acid) with arylacetyl chloride in the presence of TEA in DMF at –40 °C gave high yield of **2.11a, b** and **c** as a single pure product after a routine partition of reaction mixture between an organic and an aqueous phase (**Scheme 2.16**).



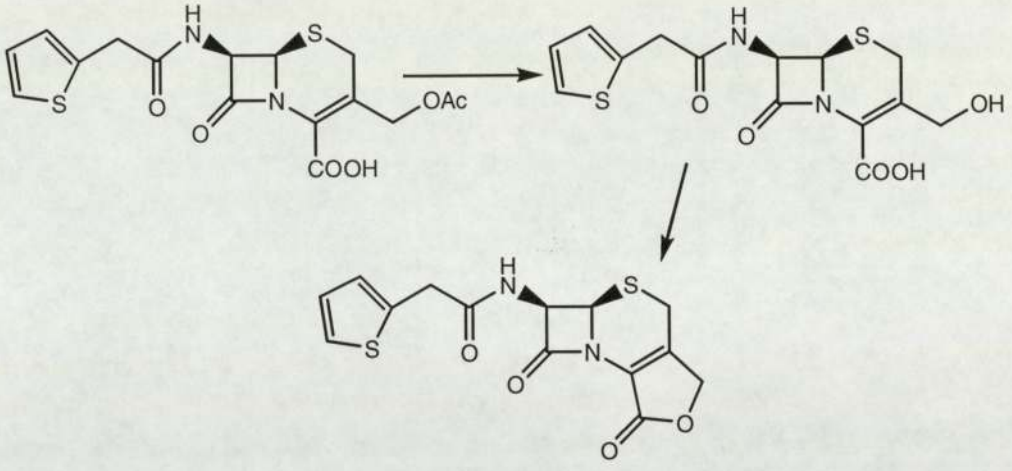
R =  **2.11 a**

 **2.11 b**

 **2.11 c**

Scheme 2.16

Hydrolysis of 3-acetoxymethyl of **2.11a, b and c** into 3-hydroxylmethyl was recorded in literature and foreseen as a conventional reaction. However when the reaction was performed accordingly, the only visible product was 2,3-lactone **2.12** (**Scheme 2.17**). This outcome was attributed to a driving force of plane geometry of 3-hydroxylmethyl and 2-carboxylic acid in dilute HCl for the intramolecular esterification.

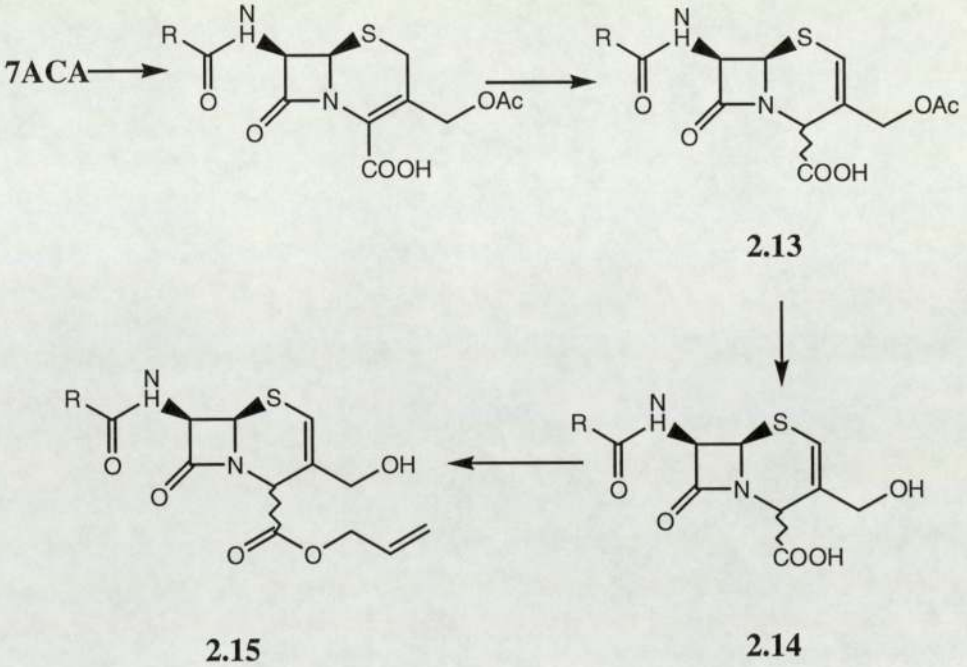


2.12

Scheme 2.17

It was predicated that 3,4-ene isomer **2.14**, in which the plane geometry was destroyed, will stay in a free acid and alcohol form. The isomerization of double bond in **2.11a, b and c** was promoted by dry pyridine and acetic anhydride to furnish a pyridine salt first. After neutralisation with 2M HCl, **2.13** was obtained as a white powder. The hydrolysis of **2.13** with 1M NaOH gave **2.14.** Protection of 3-carboxylic acid of **2.14** was accomplished with allylbromide in DMF to give cephem **2.15** which was ready for a reaction with the imidazotetrazinone **(Scheme 2.18)**.

The reaction between **2.15** and mitozolomide acid **2.3** was proved to need a strong catalyst. First we tried the mild reagents DCC and TBTU; no reaction was observed. Finally the reaction was completed by applying PyBOP and diisopropylethylamine in dichloromethane at 0 °C to afford **2.16** in 42% yield after flash column purification. As 3,4-cephem in **2.16** is not an active substrate for β-lactamases, an isomerization has to be carried out to shift the double bond to 2,3-position.

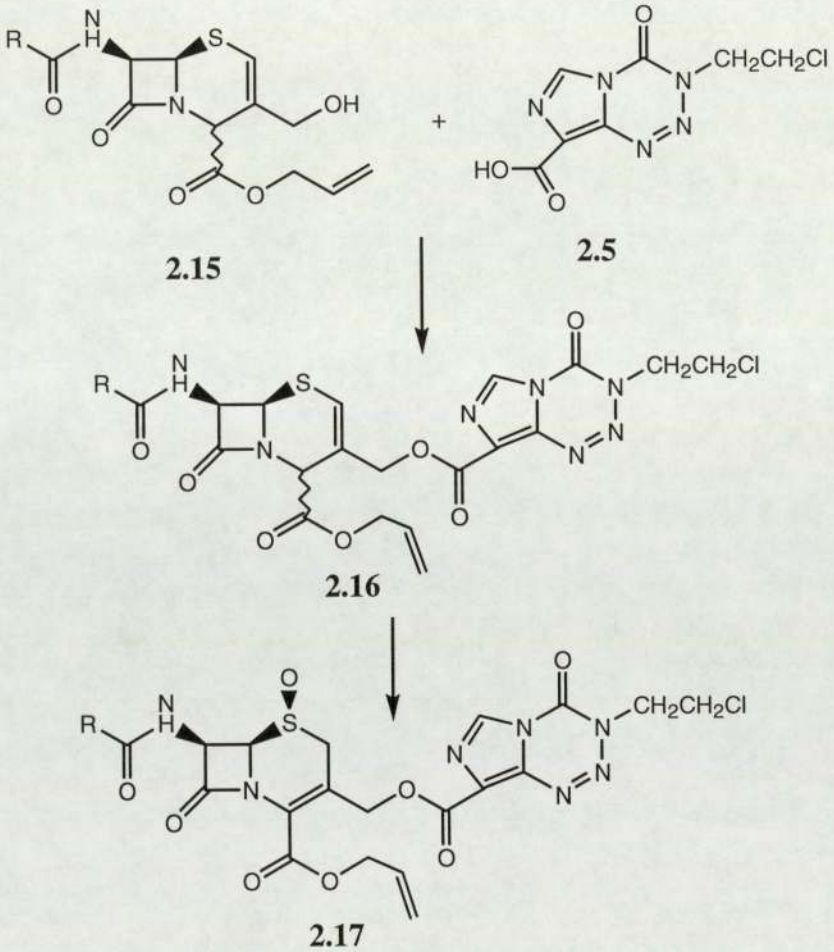


Scheme 2. 18

It was found in present study that from **2.17** to the final target **2.2a, b and c** has to go isomerization first followed by deprotection. If deprotection first, the isomerisation will give a messy mixture. The isomerization was achieved with *m*-CPBA in dichloromethane and the deprotection was accomplished with lead

triphenylphosphine and tetrakis(triphenylphosphine)-palladium to give the targets **2.2a, b and c.**

The conjugates **2.2d, e** and **f** were synthesised in the same manner.



2.15 **2.5**

2.16

2.17

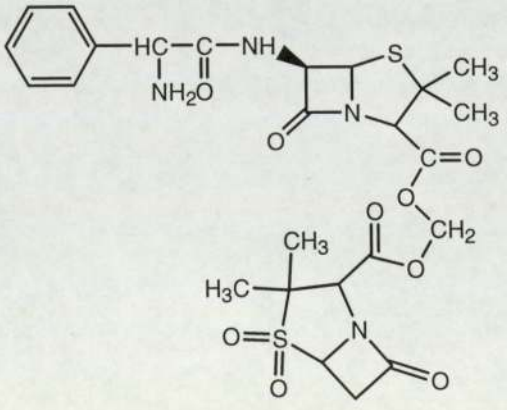
Scheme 2.19

Chapter 3

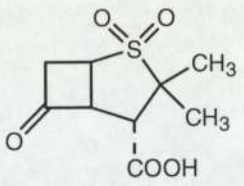
Attempts of Synthesis of Conjugates of Sulbactam And Tazobactam With Ampicillin And Amoxicillin Via An Amide Linker

3.1 Introduction

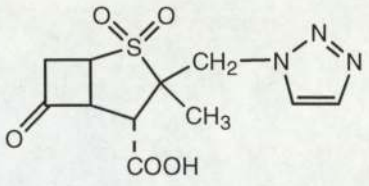
Sultamicillin **3.1** is a successful prodrug and a dual action agent of sulbactam **3.2** and ampicillin in a conjugate form of a β-lactam antibiotic and a β-lactamase inhibitor. After sulbactam, another deamino-dioxide β-lactam tazobactam **3.3** was discovered with more potent activity against β-lactamases and a very low toxicity (Brown, A G 1981). In a combination cocktail with piperacillin, tazobactam displayed a synergistic effect, superior to that of sulbactam (Serranndell, M N and Castanner, J 1986; Gutmann, L et al 1986; Ishida, N et al 1985).



3.1 Sultamicillin



3.2 Sulbactam



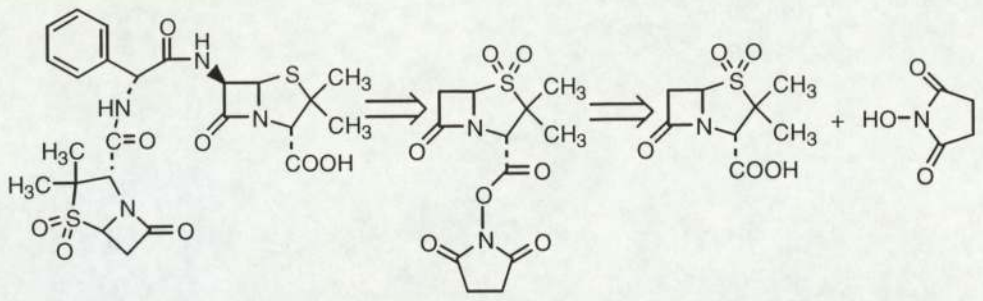
3.3 Tazobactam

Fig 3.1 Sultamicillin, Sulbactam and Tazobactam

As mentioned above, a group of semisynthetic N-acyl ampicillin or amoxicillin derivatives that emerged during the late 1970s, such as piperacillin 2, have been proved to be a very successful generation of β-lactam antibiotics and are widely used clinically (Kuck, N A and Redin, G S 1978).

The carboxylic acid group in sulbactam and tazobactam is able to undergo free rotation (Toomer, C A et al 1991) and there is no evidence suggesting the carboxylic acid group is a key element required for the receptor binding.

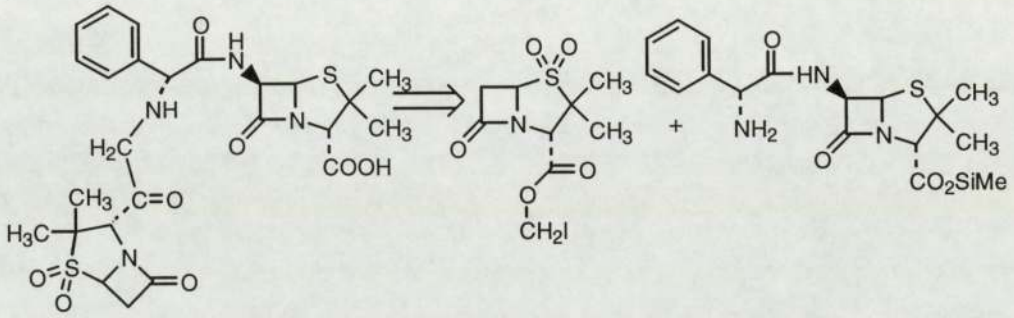
Therefore, sulbactam or tazobactam is a perfect acyl component for acylation of the α-amino group of ampicillin or amoxicillin to produce an acyl ampicillin or amoxicillin antibiotics with a β-lactamase inhibitor as a structural block covering the bond at which the enzyme is aiming. If the stereo-chemistry of sulbactam, tazobactam and β-lactams allows this direct link to happen, the dual agent **3.4** should be produced (**Scheme 3.1**).



3.4

Scheme 3.1

In the synthesis of sultamicillin, the iodomethyl ester of sulbactam is used in the reaction with carboxylate anion of ampicillin, where the amine group of ampicillin is protected to avoid the alkylation by the iodomethyl ester. We envisaged that if the carboxylic acid group of ampicillin is protected leaving the amine group free for alkylation, the dual agent **3.5** could be formed (**Scheme 3.2**).



3.5

Scheme 3.2

Synthetic methodologies used in Chapter 2, synthesis of conjugates of cytotoxic agents temozolomide and mitozolomide with β-lactams, could be applied in synthesis of this group of conjugates.

It needs to start with synthesis of sulbactam **3.2** and tazobactam **3.3** as starting materials, since no commercial products are available. The carboxylic group in sulbactam and tazobactam undergoes activation to form the active esters, then acylation with ampicillin and amoxicillin to give the conjugates.

Once the target compounds have been obtained, structural study with X-ray, if one was lucky to get good quality mono-crystals, and computer molecular mechanics packages will be carried out to determine their preferred conformation and structure activity relationship in combination with evacuation of their activity against bacteria and activity resistant β-lactamases.

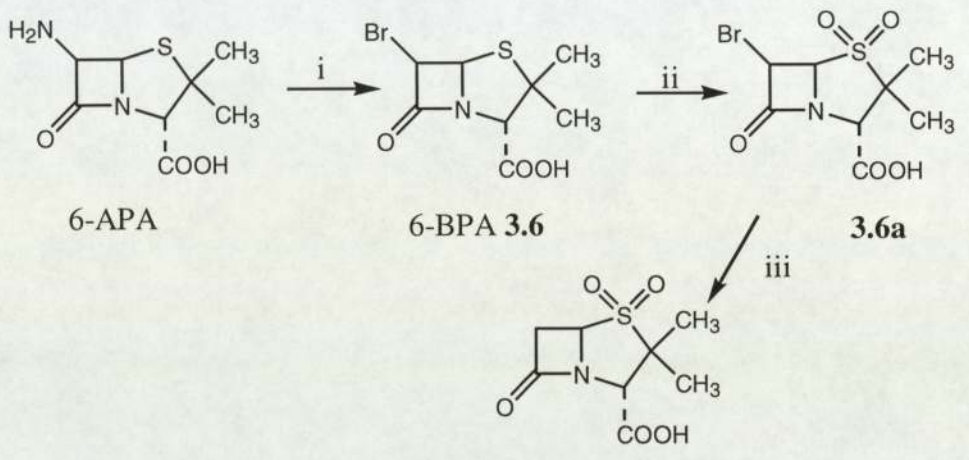
3.2 Synthesis of Sulbactam

In the literature (Micetich, R G et al 1986), sulbactam was synthesized from 6-APA through 6,6-dibromopenicillanic acid followed by oxidation and debromination *via* a catalytic hydrogenation. This synthesis has certain drawbacks such as low yield and high cost.

In our laboratory, we have established an efficient method to perform deamination of 6-aminopenicillanic acid (6-APA) the resulting in 6-bromopenicillinanic acid (6-BPA), and also the method for debromination by using cheap Zn dust.

6-APA was treated with sodium nitrite in the presence of potassium bromide under acidic condition to give 6-BPA in 87% yield. The resulting 6-BPA was applied directly in the next step of oxidation with potassium permanganate to

yield 1,1-dioxide 6-BPA. The debromination was furnished with Zn dust in an acidic medium to give sulbactam **3.2** in a reasonable yield (**Scheme 3.3**)



3.2

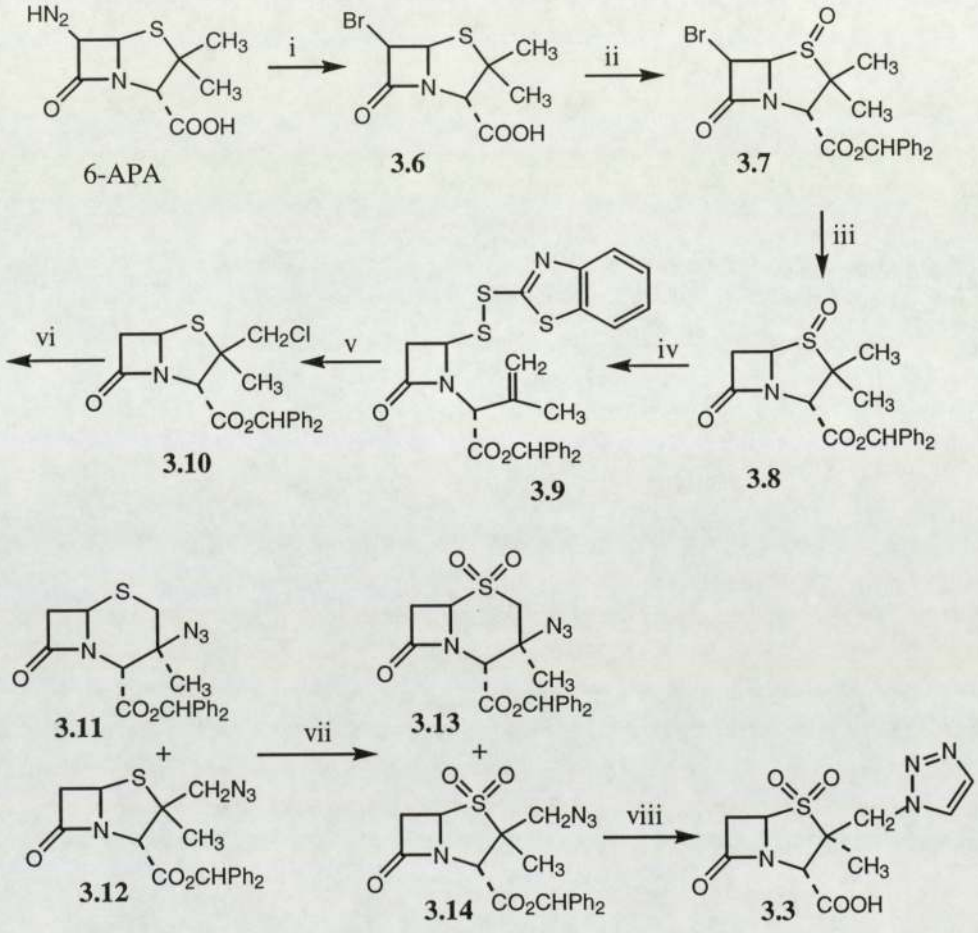
i. a 2.5N H_2SO_4/KBr, C_2H_5OH, 6-10 °C; b $NaNO_2/H_2O$, 6-8 °C. **ii**. $KMnO_4$, H_3PO_4, H_2O. **iii**. Zn dust, CH_3CO_2H.

Scheme 3.3

3.3 Synthesis of Tazobactam

Tazobactam was synthesized by application of modified literature methods (**Scheme 3.4**) (Setti, E L et al 1995) 6-APA was converted to 6-BPA by diazotization with sodium nitrite in the presence of potassium bromide. Oxidation, protection and debromination of 6-BPA did not happen as efficiently as described in the literature. However, after column purification the benzhydryl 6,6-dihydropencillanate-1-oxide **3.8** was in good quality for the next step ring opening reaction. The benzhydryl 6,6-dihydropencillanate-1-oxide was heated with 2-mercaptobenzothiazole in toluene yielding a good

yield of azetidine disulfide **3.9**, which reacted with copper (II) chloride in DCM at 0 °C to furnish a high yield of 2-chloromethylpenicillin **3.10**. The substitution of chloro in 2-chloromethylpenicillin by azide yields a mixture of products, 2 β-azidomethylpenicillin **3.12** and 3 β-azido-3α-methylcepham **3.11** in a ratio

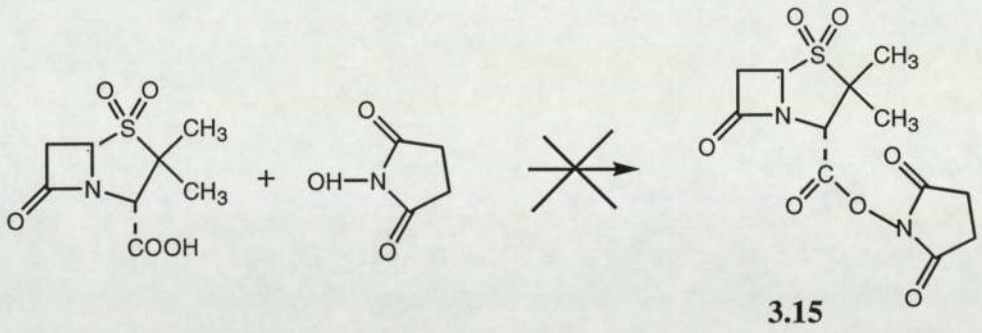


i. a 2.5N H_2SO_4/KBr, C_2H_5OH, 6-10 °C; b $NaNO_2/H_2O$,6-8 °C. **ii**. a $CH_3CO_3H/H_2O/CHCl_3$, 0 °C; b $(C_6H_5)C{=}NNH_2/CH_3CO_2H/H_2SO_4$, 0 °C. **iii.** $Zn/CH_3CN/CH_3CO_2H$, 0 °C. **iv**. 2-mercaptobenzothiazole/toluene, reflux. **v.** $CuCl_2/CH_2Cl_2$, RT. **vi**. $NaN_3/DMF/H_2O$. vii. $KMnO_4/CH_3CO_2H/H_2O$. **viii.** a vinyl acetate, 100-105 °C; b TFA

Scheme 3.4

of 3:2. The mixture was oxidized with potassium permanganate into the separable mixture of 2 β-azidomethylpenicillin-1,1-dioxide **3.14** and 3 β-azido-3α-methylcepham-1,1-dioxide **3.13**. After the separation via recrystallization with ether, 2 β-azidomethylpenicillin-1,1-dioxide was heated with vinyl acetate to give a reasonably pure product of benzhydryl tazobactam in a good yield. Deprotection was completed with TFA to give tazobactam **3.3**.

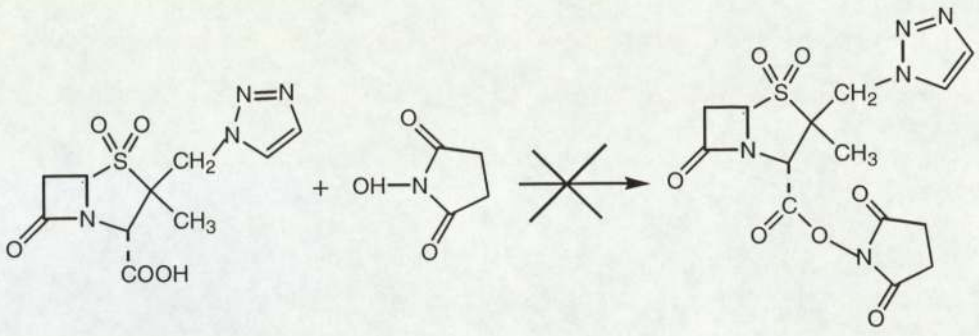
3.4 Attempts to synthesise the N-hydroxysuccinimide active ester of sulbactam



Scheme 3.5a

Attempts to make N-hydroxysuccinimide active ester of sulbactam **3.15** and tazobactam **3.16** by using the well established method (Volkmann, R A et al 1982) failed surprisingly (**Scheme 3.5a 3.5b**.)

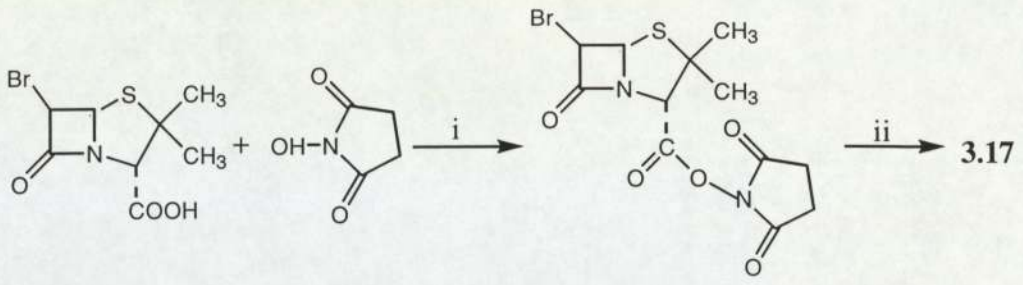
The treatment of sulbactam with N-hydroxysuccinimide (NHS) in the presence of DCC in DMF ended up with discovery of the starting materials under various conditions.



3.16

Scheme 3.5b

An alternative route to the active ester through esterification of 6-BPA with N-hydroxysuccinimide first followed by oxidation has been explored without success. However, something very interesting has been observed in the attempts. The esterification of 6-BPA with NHS successfully produced the active ester **3.18** (**Scheme 3.6**). When this active ester was treated with potassium permanganate, the only product obtained was the starting material **3.17**. In addition to the results of sulbactam resisting esterification with NHS, this result implies that the intrinsic stereochemistry of the proposed active esters, possibly the configuration between the sulfonyl group and the carboxylic acid group in sulbactam, prevents NHS from approaching the carboxylic group. In the light of the ease of formation of chloromethyl ester of sulbactam (Baltzer, B 1980), it might be concluded that the failure in esterification of sulbactam with NHS is due to the stereo-hindrance of the bulky succinimide. This has been confirmed by molecular mechanics calculation.



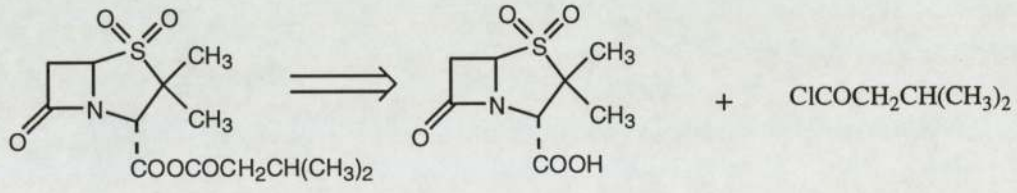
3.17 **3.18**

i. DCC/DMF; **ii** $KMnO_4/H_2SO_4$

Scheme3.6

We built up the molecules of **3.15** and **3.16** with Chem-X, then performed energetic optimization. The result showed that the structures of **3.15** and **3.16** were not reasonable as in terms of energy of the molecules as a whole and the lengths of the bonds out of normal range. Therefore these attempts were abandoned.

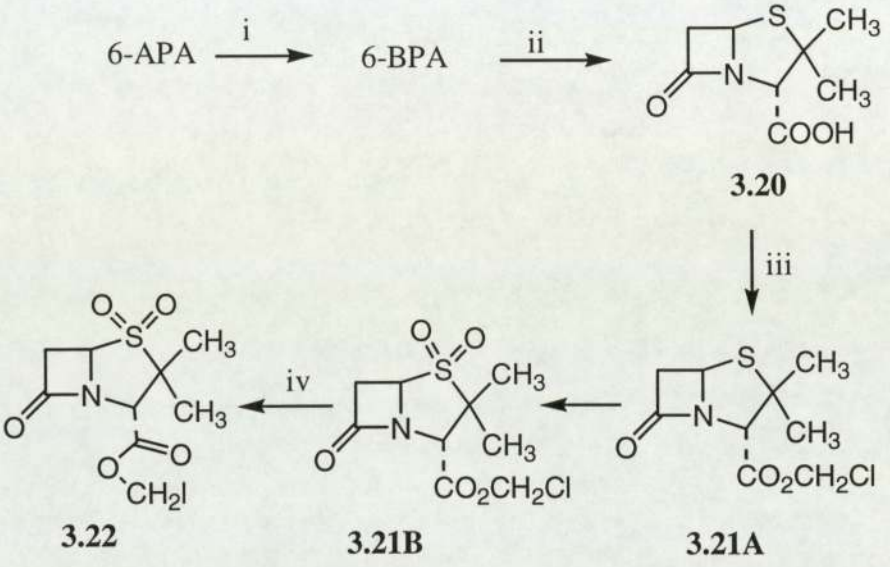
3.5 Synthesis of iodomethyl ester of sulbactam



Scheme 3.7

Iodomethyl ester of sulbactam is the key intermediate for synthesis of sultamicillin (**Scheme 3.7**) (Baltzer, B 1980). However, the literature method is not suitable to us since the key reagent, chloromethyl chlorosulfate, is not commercially available. We have successfully used chloroiodomethane, a

cheap and commercially available reagent, to replace chloromethyl chlorosulfate in the synthesis of iodomethyl ester of sulbactam in a convenient way (**Scheme 3.8**).



i. See scheme 3.3 i; **ii**. Zn dust/HCl; **iii**. a, $ClCH_2I$/TBAHS/DMF/H_2O; b, $KMnO_4$; iv. KI/Me_2CO

Scheme 3.8

Debromination of 6-BPA was furnished with Zn dust under acidic conditions to give penicillin acid **3.20**. Penicillin acid **3.20** was treated with chloroiodomethane in the presence of tetrabutylammonium hydrogen sulfate in a mixed solvent of DMF and water to give chloromethyl ester of penicillinic acid **3.21A**. Oxidation of **3.21B** with potassium permanganate was followed by substitution with potassium iodide to give **3.22**.

The reactions of **3.22** with ampicillin gave a mixture of products of sulbactam and α-amino substitution. Since this mixture has very little difference in polarity, the separation with normal methods, fast gel chromatography, failed. And we hope an alternative separation method, HPLC is being tried in the laboratory and hope to get pure sample for the biological test in the future.

Chapter 4

Bioactivity Of The Conjugates Against Bacteria And β-Lactamases

4.1 Antimicrobial Activity of Type one Conjugates-Mitozolomide and Temozolomide with β-lactam antibiotics

Determination of The Minimum Inhibitory Concentrations (MIC) of β-Lactam Conjugates 2.1 Against A Panel of Organisms.

Method:

Minimum inhibitory concentration (MIC) values were determined by a broth microdilution method carried out according to NCCLS guidelines. Test organisms were suspended in saline and the concentration adjusted to give an initial inoculum of 106 colony forming units per milliliter (CFU/ml) in Mueller-Hinton broth (MHB). Test conjugates were dissolved in a minimum of DMSO where necessary, and made up to volume in MHB. Test conjugate concentrations ranged from 0.03 ~ 62.5 μg/ml in the microtitre plate. Microtitre plates were incubated overnight at 37 °C. Growth of test organisms was observed as a button of cells at the base of the well. Tests were carried out by Dr P A Lambert of Aston University.

Results:

In an attempt to define the mode of action of the conjugates, a range of

organisms were chosen: both Gram negative and Gram positive organisms, each of which contained strains that produced β-lactamase enzymes and candidates which did not express such enzymes. Methicillin resistant and sensitive *Staphylococcus aureus* strains were included. A permeability mutant strain of *E. coli* was selected *(E. coli* DC2) along with the parent strain *E. coli* DC0. An *E. coli* strain (ESBL+) which produces an extended spectrum β-lactamase (a β-lactamase enzyme capable of hydrolysing third generation cephalosporins which are normally stable to β-lactamase) was included as well as an *Enterobacter* which produces a Group 1 lactamase which is sensitive to tazobactam *(E. cloacae* 1051E). The results of initial MIC studies are shown in Table 4.1.

None of the β-lactam conjugates were active against methicillin-resistant *Staphylococcus aureus* (MRSA) strains, either β-lactamase positive (MRSA 96-7778 and MRSA Innsbruck) or β-lactamase negative (MRSA 967992 and MRSA 96-5665). All the conjugates were active against the non-β-lactamase producing methicillin sensitive *Staph. aureus* (MSSA) strains. Conjugates containing ampicillin proved the most active against MSSA NCTC 10788 (MIC of <0.03μg/ml), while conjugates containing cephalexin were least active against this strain. All of the conjugates were very active against MSSA NCTC 10788, with MIC values ranging from <0.03-0.06μg/ml. Only the temozolamide-cephalexin conjugate **2.1b** displayed activity against the β-lactamase producing MSSA strain Hopewell.

No conjugate displayed activity against *E. coli* DC0, but all conjugates were active against the permeability mutant *E. coli* DC2, with temozolomide-ampicillin proving the most active with a MIC of 0.491μg/ml. No conjugate was active against the extended spectrum β-lactamase producing *E. coli* strain, the *E. cloacae* strain producing a Group 1 β-lactamase or the *K. pneurnoniae* candidate which also produces a β-lactamase enzyme.

Another series of MIC values were determined against the same panel of organisms, using the individual components of each conjugate to attempt to identify the active principle of the conjugate. The results are shown in Table 4.2

Neither of the two cytotoxic agents, temozolamide or mitozolamide displayed any activity against the panel of organisms. Virtually in all cases, the conjugates were less active than the parent β-lactam agents forming complexes with mitozolamide or temozolamide. The MIC values for the cephalexin conjugates are at least double those of cephalexin alone, in strains where a MIC was observed. Only in the case of ampicillin conjugates are the MIC values for ampicillin alone higher than the conjugates against the non-β-lactamase producing MSSA strains.

Table 4.1 Minimum inhibitory concentrations of β-lactam conjugates

Organism:	Concentration (~µg/ml):					
	Temozolamide -Amoxycillin	Mitozolamide -Amoxycillin	Temozolamide -Cephalexin	Mitozolamide -Cephalexin	Temozolamide -Ampicillin	Mitozolamide -Ampicillin
MRSA 96-7778*	>62.5	>62.5	>62.5	>62.5	>62.5	>62.5
MRSA innsbruck*	>62.5	>62.5	>62.5	>62.5	>62.5	>62.5
MRSA 96-7992	>62.5	>62.5	>62.5	>62.5	>62.5	>62.5
MRSA 96-5665	>62.5	>62.5	>62.5	>62.5	>62.5	>62.5
MSSA NCTC 6571	3.9	3.9	7.81	7.81	0.98	0.98
MSSA NCTC 10788	<0.03	0.06	0.06	0.06	<0.03	<0.03
MSS Hopewell*	>62.5	>62.5	15.62	>62.5	>62.5	>62.5
E. coli DC0	>62.5	>62.5	>62.5	>62.5	>62.5	>62.5
E. coli DC2	3.9	1.95	15.62	7.81	0.49	15.62
E. coli ESI3L+*	>62.5	>62.5	>62.5	>62.5	>62.5	>62.5
E. cloacae 1051E*	>62.5	>62.5	>62.5	>62.5	>62.5	>62.5
K. pneumoniae 1082E*	>62.5	>62.5	>62.5	>62.5	>62.5	>62.5

Key: * Strains producing a β-lactamase enzyme
MSSA = Methicillin-sensitive *Staphylococcus aureus*
MRSA = Methicillin-resistant *Staphylococcus aureus*
E. coli DC2 - permeability mutant of parent strain *E. coli* DC0

Table 4.2 Minimum inhibitory concentrations (MIC; μg/ml) of β-lactam antibiotics and cytotoxic agents

Organism:	Cephalexin	Ampicillin	Amoxycillin	Temozolamide	Mitozolamide
MRSA 96-7778*	>62.5	>62.5	>62.5	>62.5	>62.5
MRSA innsbruck*	>62.5	>62.5	>62.5	>62.5	>62.5
MRSA 96-7992	62.5	>62.5	>62.5	>62.5	>62.5
MRSA 96-5665	31.25	>62.5	>62.5	>62.5	>62.5
MSSA NCTC 6571	3.9	1.95	0.49	>62.5	>62.5
MSSA NCTC 10788	<0.03	<0.03	<0.03	>62.5	>62.5
MSSA Hopewell*	1.95	>62.5	>62.5	>62.5	>62.5
E. coli DC0	62.5	3.9	15.62	>62.5	>62.5
E. coil DC2	3.9	0.24	0.98	>62.5	>62.5
E. coli ESBL+*	>62.5	>62.5	>62.5	>62.5	>62.5
E.cloacae 1051E*	>62.5	>62.5	>62.5	>62.5	>62.5
K. pneurnoniae 1082E*	>62.5	>62.5	>62.5	>62.5	>62.5

Key: * Strains producing a β-lactamase enzyme
MSSA = Methicillin-sensitive *Staphylococcus aureus*
MRSA = Methicillin-resistant *Staphylococcus aureus*
E. coli DC2 - permeability mutant of parent strain *E. coli* DC0
Conor Jamieson 5th October 1999

4.2 The type two conjugates-antitumour drug mitozolomide with cephalosporins-as substrates of selected β-lactamases

The type two conjugates of antitumour drug mitozolomide with cephalosporins described in Chaper 2 were first tested to assess whether or not they are substrates of β-lactamases. PC1 (a serine type β-lactamase produced by some MRSA strains of *staphylococcus aureus*) and *β cereus* II enzyme (a metallo β-lactamase produced by *Bacillus. cereus* strains) were chosen for this study because they are commercially available. The tests were carried out by conducting experiments to measure the rate of hydrolysis of nitrocefin, a chromogenic cephalosporin, which was converted from a yellow colour to red upon hydrolysis by β-lactamases.

The experimental readings were taken by observing the colour change from yellow to red in the nitrocefin (as increase in absorbance at 429 nm) over a total run time of 1200 seconds using an ANTHOS 2001 colour photometer and enzyme kinetic software. Measurement of absorbance (A) were taken at thirty seconds intervals with a five seconds waiting time between samples. Testing against β-lactamases was carried out against controls when either the enzymes or the conjugates were excluded from the experiment. When no enzyme was added the lack of hydrolysis, no change in absorbance (A), was observed. When no conjugate was added the nitrocefin completely hydrolysed.

The first assay was carried out by assessing the hydrolytic effect of the β-lactamases on nitrocefin in the presence of the conjugates and ampicillin over

a period of twenty minutes. The second assay was carried out following the same procedure with addition of a twenty minutes pre-incubation period for the conjugates with the β-lactamases.

β-lactamase inhibitors, BRL42715B, Clavulanic acid and Sulbactam, were used as control conjugates as they were irreversible inhibitors of β-lactamases.

The experiments against PC1 and *B. cereus* II showed conjugates **2.2a-c** are competitive substrates for the β-lactamases and caused a reduction in β-lactamase activity (Table 4.3). This was demonstrated by a slower hydrolysis rate and a reduced total hydrolysis of nitrocefin.

Testing against PC1 showed conjugates **2.2a-c** to reduce the rate of nitrocefin hydrolysis and to prevent complete nitrocefin hydrolysis within the twenty-minutes sampling period. When no conjugate was added, A (Absorbance) was measured as 0.317 after 450 seconds and 0.475 after 1170 seconds. Comparisons with A measured in the presence of conjugates showed reduction in A measured after 450 seconds. This indicated a reduced rate of hydrolysis. Reductions after 1170 seconds showed a decreased total nitrocefin hydrolysis.

Interference with nitrocefin hydrolysis might be due to inhibition of the PC1 enzyme either by binding of the conjugates to the enzyme altering the active site or as more likely, by competitively occupying the enzyme active site itself. The introduction of a twenty-minutes pre-incubation period caused a reduction

in the rate of nitrocefin hydrolysis. However, in comparison with the observations in the experiments with zero pre-incubation, nitrocefin hydrolysis had occurred to a greater degree after 1170 seconds **2.2b, 2.2c**, or was continuing **2.2a**. This would indicate that pre-incubation period allowed greater binding between the conjugates and PC1. It also indicated that PC1 destroyed the conjugates to make its active site to become available again and again for hydrolysis of nitrocefin.

Table 4.3. The effect of conjugates 2.2a-c and control conjugates on nitrocefin hydrolysis by PC1 enzyme and *B. cereus* II enzyme.

	A (Absorbance)							
	PC1				*B.cereus* II enzyme			
Compd.	450 sec no PI	450 sec PI	1170 sec no PI	1170 sec PI	450 sec no PI	450 sec PI	1170 sec no PI	1170 sec PI
2.2a	0.244	0.149	0.379	0.262	0.216	0.093	0.275	0.107
2.2b	0.216	0.184	0.307	0.402	0.265	0.092	0.390	0.164
2.2c	0.242	0.216	0.378	0.405	0.214	0.109	0.314	0.165
BRL42715BB	0.093		0.103		0.102		0.103	
Clavulanic acid	0.080		0.086		0.112		0.191	
Sulbactam	0.110		0.153		0.124		0.221	
no inhibitor	0.317		0.475		0.422		0.425	
no enzyme	0.090		0.090		0.093		0.093	
	±0.02		±0.02		±0.02		±0.02	

Testing against *B. cereus* II showed that all conjugates had ability to reduce the rate and the degree of nitrocefin hydrolysis by the β-lactamases. The addition of a twenty-minutes pre-incubation period enhanced activity of all conjugates in prevention of β-lactamases hydrolysis of nitrocefin. This would indicate that irreversible binding of the conjugates to the enzyme active site occurred.

The degree in reduction of nitrocefin hydrolysis by the conjugates **2.2a-c** against *B. cereus* II was observed far greater than those against PC1. The results showed A which were between ±0.003 of the mean A seen in the control (no enzyme) after 450 seconds i. e. virtually no nitrocefin hydrolysis. After 1170 seconds, these figures rose to +0.071, +0.026 and +0.014 above the control level for **2.2a** and **2.2b**, respectively. This was a proof that these conjugates either inhibited the *B. cereus* II enzyme or interacted with it to prevent the active site from hydrolysing action for a period of twenty minutes given a twenty-minutes pre-incubation period.

Against PC1 and *B.cereus II* enzyme, BRL42715B, clavulanic acid and sulbactam were effective agents since it was known that they are broken down by class B metallo β-lactamases.

4.3 Activity against bacteria of the type two conjugates of antitumour drug mitozolomide with the cephalosporin

Materials and Methods.

Initial testing for anti-bacterial activity

Initial testing for activity was carried out on *S. aureus* NCTC6571, (a penicillin sensitive strain). 20 µl of each of the conjugate **2.2a-f** (concentration of 5 mg/ml dissolved in DMSO) was placed in wells in nutrient agar plates which had been surface inoculated with *S. aureus* NCTC6571. Ampicillin (concentration of 5 mg/ml dissolved in water) was included as a control. Zones of inhibition were measured after 16 hours incubation at 37 °C.

Testing for activity against β-lactamase producing bacteria.

Tests were then carried out to assess the activity of the conjugates **2.2a-f** using ampicillin as a control against bacteria,

1) producing a serine type β-lactamase enzyme and

2) producing a metallo type β-lactamase enzyme.

The serine β-lactamase producing bacteria used were MRSA NH278, MRSA NH424, MRSA NH123 and MRSA 24-7882. The metallo β-lactamase producing bacteria were *B. cereus* 4 and *B. cereus* 5.

Applying the same techniques as above, nutrient agar was used as the growth medium inoculated with the bacterium then the test conjugates added to wells in the plates. Zones of inhibition were measured after 16 hours incubation at 37 °C.

Testing to assess whether the activity of conjugates **2.2a-f** was due to inhibition of β-lactamase enzymes or was a cellular effect.

Assays were carried out for the conjugates **2.2a-f** against isolated enzymes of type PC1 (Serine β-lactamase type enzyme produced by some MRSA strains of S. *aureus*) and against B. *cereus II* a β-lactamase enzyme produced by B. *cereus* strains). The enzymes were obtained from the Centre For Applied Microbiology and Research, Porton Down, Salisbury, Wiltshire.

Calibration experiments were carried out to identify the optimum concentrations of enzyme and nitrocefin to give a protocol that gave interpretable results. The aim was to produce a result where the colour change associated with nitrocefin hydrolysis was slow enough that accurate initial readings could be taken and fast enough that in the absence of any interference, complete hydrolysis occurred after between 400 and 800 seconds. The experimental readings were taken by observing the yellow → red colour change in the nitrocefin (as increase in absorbance at 429 nm) over a total run time of 1200 seconds using an ANTHOS 2001 colour photometer and enzyme kinetic software. Measurements of optical density (OD) were taken at thirty seconds intervals with a five seconds waiting time between samples.

A 0.1 M potassium phosphate buffer was used to maintain a pH of 7.0. The calibration experiments used the enzymes PC1 and *B-cereus* II at varied concentration and nitrocefin at varied volume of phosphate buffer to make a total volume of 200 µl.

The following protocol was decided upon:

- 5 µl of Nitrocefin added to each well.
- 10 µl of the conjugate into each well.
- 20 µl of enzyme added to each well.
- 165 µl of 0.1 M Phosphate buffer (buffered to pH7) added to each well.

This protocol was decided upon because these values gave the optimum time course for measurement obtainable where the same protocol could be followed by both β-lactamase enzyme types.

The protocol was then re-run in the presence of each of the conjugates **2.2a-f** (10 μl of 5 mg/ml stock solution). In addition, a control with just nitrocefin and each of the conjugates **2.2a-f** was run.

The first assay was carried out by assessing the hydrolytic effect of the β-lactamase enzymes on nitrocefin in the presence of the conjugates **2.2a-f** and ampicillin between twenty minutes. A second assay was carried out following the same techniques but with a twenty minutes pre-incubation period for the conjugate with the β-lactamase enzyme. The experiment was carried out on a microtitre plate.

The experiment was carried out using a multi-tip pipette to add the enzyme and buffer mix to the conjugates **2.2a-f** and then to the nitrocefin. This ensured that when making measurements all conjugates **2.2a-f** had equal exposure times to the enzymes.

Experiments with varied incubation periods.

Further experiments were carried out with varied pre-incubation periods. Ampicillin, conjugates **2.2d** and **2.2e** were tested.

Pre-incubation periods of 60, 40, 20, 10, 5 and zero minutes were used. Following pre-incubation, measurements for OD were taken as previously described.

Experiments with varied drug concentration.

Calculation of MICs for the conjugates **2.2a-f** against MRSA strain NH424 and *B. cereus* 5.

Calculation of the MICs for the conjugates **2.2a-f** and ampicillin was carried out on microtitre plates. Initial dilution and sterilisation of the conjugates was required. This was achieved by dilution of all the conjugates in water to make solutions of 250µl/ml concentration. Sterility was achieved by filtration using a non-polar sterile filter.

Further dilution with sterile water was needed to produce solutions of 125 µl/ml strength.

MICs were calculated by measuring the growth found at varied concentration of inhibitor. Inhibitor concentrations of 1, 2, 4, 8, 16, 32, and 64 µl/ml were tested for signs of bacterial growth (Table 4.4)

Table 4.4 Conjugates were tested according to the following protocol

Concentration/well µl/ml	Volume of broth (Double Strength)	Volume of inhibitor added.	Volume water added
64	100 µl	51.2 µl (250 µl/ml)	48.8 µl
32	100 µl	25.6 µl (250 µl/ml)	74.4 µl
16	100 µl	12.8 µl (250 µl/ml)	87.2 µl
8	100 µl	6.4 µl (250 µl/ml)	93.6 µl
4	100 µl	6.4 µl (125 µg/ml)	93.6 µl

2	100 µl	3.2 µl (125 µg/ml)	96.8 µl
1	100 µl	1.6 µl (125 µg/ml)	98.4 µl

Growth levels were observed after 16 hours and MICs determined.

Calculation of MICs for the conjugate against a selection of 33 strains of bacterium.

Measurement of MICs for ampicillin and conjugates **2.2a, 2.2b. 2.2d, 2.2e** and **2.2f** against 33 strains of bacteria were made using a multi point inoculation technique.

Agar plates were made up which contained test conjugates at a given concentration. The concentrations used were 64, 32, 16, 8, 4, 2, and 1 µl/ml. Obtained from a sterile stock solution of 2.5 mg/ml. Mixing was achieved by adding the test conjugates to a clean plate dish then added 20 ml of warm nutrient agar. The volumes of test conjugates added were as follows:

Table 4.5 The volumes of test conjugates added

Concentration required (µg/ml)	Volume of stock solution needed (conc. 2.5 mg/ml)	Volume Agar added to each plate.
1	8 µl	20 ml
2	16 µl	20 ml
4	32 µl	20 ml
8	64 µl	20 ml
16	128 µl	20 ml
32	256 µl	20 ml
64	512 µl	20 ml

Each plate was then inoculated using a multi point inoculator with 33 strains stored in a 6 by 6 matrix in individual wells of a microtitre plate. The micro-organisms were prepared by Ms K. Tims (see below).

Table 4.6 Organisms used for multi point MIC testing

Well Number	Strain	Designation	Infection usually caused
1	Escherichia coli	W3310 R-	UTI Pathogens
2	*Escherichia coli*	W3310 R+	
3	*Escherichia coli*	1286	
4	Escherichia coli	1278 B	
5	*Enterobacter cloacae*	NCTC 11582	
6	*Enterobacter cloacae*	NCTC 11579	
7	*Serratia marcescens*	CDC	
8	*Serratia marcescens*	4444	
9	*Serratia marcescens*	S1221	
10	*Serratia marcescens*	PINK	
11	*Pseudomonas aeruginosa*	PA01	Burns and lung
12	*Pseudomonas aeruginosa*	PA01 DR	infections
13	*Pseudomonas aeruginosa*	Z799	
14	*Pseudomonas aeruginosa*	Z799/61	
15	*Stenotrophomonas maltophilia*	M. FOLAN	
16	*Stenotrophomonas maltophilia*	1	
17	*Klebsiella pneumoniae*	DL1	
18	*Staphylococcus haemolyticus*	O'NEILL	UTI pathogen
19	*Bacillus subtilis*	11	Not pathogenic
20	*Bacillus*	7	cause infection
21	*Bacillus aureus*	4	Food poisoning
22	*Bacillus aureus*	5	
23	*Bacillus aureus*	6	
24	*Enterococcus faecalis*	EBH1	UTI infections
25	*Enterococcus faecalis*	DOCKER	
26	*Enterococcus faecium*	NCTC 7171	
27	*Staphylococcus epidermidis*	NCTC 11047	Skin infections
28	*Staphylococcus epidermidis*	CANAVAN	

29	*Staphylococcus epidermidis*	MILLER	
30	*Staphylococcus epidermidis*	HEDGES	
31	*Staphylococcus aureus* MRSA	NH 123	
32	*Staphylococcus aureus* MRSA	24-7882	
33	*Staphylococcus aureus* MRSA	96-5665	

Experiments to show any possible synergistic activity between conjugates **2.2a-f** and ampicillin.

Experiments were carried out to assess whether the conjugate would behave synergistically in conjugation with ampicillin.

Initial experiments were carried to test for possible synergistic effects between each of **2.2a-f** and ampicillin. These tests were carried out by inoculating agar plates with 0.1 ml inoculum (inoculums used were MRSA NH424 and *B. cereus* 5), then adding 10 µl of substrate to wells in the agar. Wells in the agar were located approximately twenty millimeter apart. Synergy was determined as occurring when a greater degree of growth inhibition was observed where the ampicillin and conjugate diffused into the same area of the plate.

Upon interpretation of these results, further experiments were carried out to provide quantitative data concerning the degree of synergy. Experiments were carried out on a microtitre plate to test for synergy at varied concentration of ampicillin and conjugates **2.2d** and **2.2e.**

Table 4.7 Concentrations of ampicillin and conjugates added to each well

Amp = 64 Conjugate = 64	Amp = 64 Conjugate = 32	Amp = 64 Conjugate = 16	Amp = 64 Conjugate = 8	Amp = 64 Conjugate = 4	Amp = 64 Conjugate = 2	Amp = 64 Conjugate = 1
Amp = 32 Conjugate = 64	Amp = 32 Conjugate = 32	Amp = 32 Conjugate = 16	Amp = 32 Conjugate = 8	Amp = 32 Conjugate = 4	Amp = 32 Conjugate = 2	Amp = 32 Conjugate = 1
Amp = 16 Conjugate = 64	Amp = 16 Conjugate = 32	Amp = 16 Conjugate = 16	Amp = 16 Conjugate = 8	Amp = 16 Conjugate = 4	Amp = 16 Conjugate = 2	Amp = 16 Conjugate = 1
Amp = 8 Conjugate = 64	Amp = 8 Conjugate = 32	Amp = 8 Conjugate = 16	Amp = 8 Conjugate = 8	Amp = 8 Conjugate = 4	Amp = 8 Conjugate = 2	Amp = 8 Conjugate = 1
Amp = 4 Conjugate = 64	Amp = 4 Conjugate = 32	Amp = 4 Conjugate = 16	Amp = 4 Conjugate = 8	Amp = 4 Conjugate = 4	Amp = 4 Conjugate = 2	Amp = 4 Conjugate = 1
Amp = 2 Conjugate = 64	Amp = 2 Conjugate = 32	Amp = 2 Conjugate = 16	Amp = 2 Conjugate = 8	Amp = 2 Conjugate = 4	Amp = 2 Conjugate = 2	Amp = 2 Conjugate = 1
Amp = 1 Conjugate = 64	Amp = 1 Conjugate = 32	Amp = 1 Conjugate = 16	Amp = 1 Conjugate = 8	Amp = 1 Conjugate = 4	Amp = 1 Conjugate = 2	Amp = 1 Conjugate = 1

Ampicillin was added to wells in columns down the plate at varied concentration. The conjugates were added to wells in rows across the plate at varied concentration.

RESULTS.

Initial testing for anti-bacterial activity.

Table 4.8 Zones of inhibition against S. *aureus* (6571)

Conjugate tested	Zone diameter.(mm)
Ampicillin	47
2.2a	12
2.2b	18
2.2c	15
2.2d	21
2.2e	26
2.2f	25

These results showed that all the conjugates had activity against a non-β-lactamase producing organism.

Testing for activity against β-lactamase producing bacteria.

Table 4.9 Zones of inhibition (mm) against various strains of β-lactamase producing bacteria.

Conjugate tested	Mean Zone Diameter (mm)					
	MRSA NH278	MRSA NH424	MRSA NH123	MRSA 24-7882	*B. cereus* 4	*B. cereus* 5
Amp.	13	11	19	14	35	34
2.2a	13	14	12	12	17	25
2.2b	13	13	12	13	14	24
2.2c	11	11	11	10	9	24
2.2d	16	15	16	17	22	30
2.2e	18	17	18	14	30	36
2.2f	15	15	15	16	25	34

These results showed activity in the conjugates **2.2a-f** against β-lactamase producing bacteria. The control, ampicillin should ideally not have shown any inhibition against β-lactamase producing organisms.

Testing to assess whether the activity of the conjugates was due to inhibition of β-lactamase enzymes or was a cellular effect.

The results from these experiments showed that all **2.2a-f** conjugates cause a reduction in β-lactamase enzyme (PC1 and *B. cereus* II) activity. This was demonstrated by a slower hydrolysis rate and a reduced total hydrolysis of nitrocefin.

The effectiveness of inhibition against the *B. cereus* II enzyme was greatly increased by the inclusion of a twenty-minute pre-incubation period for conjugates **2.2a-e.** None of the conjugates showed similar efficacy for the PC1 enzyme. Pre-incubation did not show the increase in inhibition seen with the *B. cereus* II enzyme.

Ampicillin caused a reduction in nitrocefin hydrolysis. Addition of a pre-incubation period for ampicillin led to an increase in nitrocefin hydrolysis.

Overleaf are tables for conjugates **2.2a-f** and ampicillin showing the effect of their addition to both the PC1 and *B. cereus* II enzymes on the hydrolysis of

nitrocefin. The effect of pre-incubating the enzyme with the conjugates for twenty minutes is also shown.

Table 4.10 Results against the PC1 β-lactamase enzyme

Time	Absorbance(OD) measured at 492 nm			
	450 sec no PI.	450 sec PI.	1170 sec no PI.	1170 sec PI.
2.2a	0.216	0.184	0.307	0.402
2.2b	0.238	0.156	0.367	0.283
2.2c	0.235	0.151	0.387	0.354
2.2d	0.244	0.149	0.379	0.262
2.2e	0.242	0.216	0.378	0.405
2.2f	0.293	0.249	0.426	0.432
No inhibitor	0.317	0.317	0.475	0.475
Ampicillin	0.216	0.189	0.393	0.412
No enzyme	0.090 +/- 0.02	0.090 +/- 0.02	0.093 +/- 0.02	0.093 +/- 0.02

Table 4.11 Results against the *B. cereus* II β-lactamase enzyme

Time	Absorbance (OD) measured at 492 nm			
	450 sec no PI.	450 sec PI.	1170 sec no PI.	1170 sec PI.
2.2a	0.265	0.092	0.390	0.164
2.2b	0.256	0.087	0.363	0.199
2.2c	0.234	0.127	0.330	0.228
2.2d	0.216	0.093	0.275	0.107
2.2e	0.214	0.109	0.314	0.165
2.2f	0.149	0.190	0.217	0.323
No inhibitor	0.422	0.422	0.425	0.425
Ampicillin	0.283	0.221	0.355	0.398
No enzyme	0.090 +/- 0.02	0.090 +/- 0.02	0.093 +/- 0.02	0.093 +/- 0.02

PI. symbolises Pre-incubation.

Experiments with different incubation periods.

Table 4.12 Observed Optical Densities for Ampicillin, and Inhibitors 2.2d and 2.2e against PC1. (N.B. the figures across the top of the table refer to pre-incubation time used)

	OD at 492 nm				
Time	60	40	20	10	5
Ampicillin					
0 sec.	0.107	0.120	0.118	0.139	0.131
450 sec.	0.580	0.537	0.623	0.554	0.523
1170 sec.	0.631	0.563	0.659	0.578	0.540
Inhibitor 2.2d					
0 sec.	0.070	0.070	0.073	0.076	0.079
450 sec.	0.313	0.290	0.389	0.447	0.483
1170 sec.	0.526	0.531	0.541	0.518	0.531
Inhibitor 2.2e					
0 sec.	0.078	0.077	0.076	0.088	0.088
450 sec	0.488	0.484	0.536	0.545	0.566
1170 sec.	0.526	0.522	0.542	0.559	0.543

Table 4.13 Observed Optical Densities for Ampicillin, and Inhibitors 2.2d and 2.2e against *B. cereus* II. (N.B. The figures across the top of the table refer to pre-incubation time used)

	OD at 492 nm				
Time	60	40	20	10	5
Ampicillin					
0 sec.	0.173	0.182	0.193	0.185	0.203
450 sec.	0.567	0.506	0.521	0.551	0.452
1170 sec.	0.629	0.535	0.562	0.594	0.471
Inhibitor 2.2d					
0 sec.	0.135	0.116	0.119	0.125	0.111
450 sec.	0.568	0.532	0.531	0.483	0.381
1170 sec.	0.587	0.518	0.525	0.517	0.488
Inhibitor 2.2e					
0 sec.	0.102	0.091	0.104	0.113	0.096
450 sec	0.540	0.521	0.531	0.553	0.535
1170 sec.	0.571	0.549	0.572	0.567	0.557

Experiments with varied inhibitor concentration.

No change in the rate and extent of nitrocefin hydrolysis with varied orotic acid concentration was observed against the *B. cereus* II enzyme.

Table 4.14 Calculation of MICs for the conjugates **2.2a-f** against MRSA NH424 and B. *cereus* 5

	OD at 492 nm					
Time	10	8	6	4	2	1
450 sec.	0.406	0.402	0.414	0.435	0.393	0.396
1170 sec.	0.395	0.389	0.400	0.419	0.379	0.389

MICs were measured for **2.2a-f** against MRSA NH424 and *B. cereus* 5. The results showed only **2.2e** to show effective inhibition of MRSA NH424, *B. cereus* 5 was inhibited by conjugates **2.2b, 2.2e** and **2.2f.**

Table 4.15 MICs for all conjugates 2.2a-f against MRSA NH424 and *B. cereus* 5

	MIC (mm)						
Conjugate	Amp.	2.2a	2.2b	2.2c	2.2d	2.2e	2.2f
MRSA 424	64	64	32	64	32	16	32
B. cereus 5	32	16	32	64	32	4	8

Calculation of MICs for conjugates **2.2a-f** against a selection of 33 strains of bacterium.

Table 4.16 MICs observed for conjugates 2.2a 2.2b, 2.2d, 2.2e, and 2.2f and ampicillin against 33 strains of bacterium

Well Number	Strain	MIC μg/ml					
		Amp	2.2a	2.2b	2.2d	2.2e	2.2f
1	*Escherichia coli*	>64	>64	>64	>64	>64	>64
2	*Escherichia coli*	>64	>64	>64	>64	>64	>64
3	*Escherichia coli*	>64	>64	>64	>64	>64	>64
4	*Escherichia coli*	>64	>64	>64	>64	>64	>64
5	*Enterobacter cloacae*	>64	>64	>64	>64	>64	>64
6	*Enterobacter cloacae*	>64	>64	>64	>64	>64	>64
7	*Serratia marcescens*	64	1	1	8	16	8
8	*Serratia marcescens*	64	1	64	64	>64	>64
9	*Serratia marcescens*	1	1	1	16	4	1
10	*Serratia marcescens*	1	1	1	8	>64	1
11	*Pseudomonas aeruginosa*	1	1	>64	32	>64	1
12	*Pseudomonas aeruginosa*	64	>64	>64	>64	>64	>64
13	*Pseudomonas aeruginosa*	1	2	32	8	64	>64
14	*Pseudomonas aeruginosa*	1	4	16	8	64	>64
15	*Stenotrophomonas maltophilia*	1	16	64	4	>64	64
16	*Stenotrophomonas maltophilia*	1	4	64	4	64	64
17	*Klebsiella pneumoniae*	8	>64	32	8	1	>64
18	*Staphylococcus haemolyticus*	32	>64	>64	>64	>64	>64
19	*Bacillus subtilis*	1	>64	64	64	>64	>64
20	*Bacillus*	1	>64	64	32	>64	64
21	*Bacillus aureus*	1	32	32	16	4	4
22	*Bacillus aureus*	1	8	64	8	32	>64
23	*Bacillus aureus*	64	64	64	>64	>64	>64
24	*Enterococcus faecalis*	1	>64	64	>64	>64	>64
25	*Enterococcus faecalis*	1	>64	>64	>64	>64	>64
26	*Enterococcus faecium*	1	>64	64	16	>64	>64

27	*Staphylococcus epidermidis*	1	8	8	8	16	2
28	*Staphylococcus epidermidis*	2	8	16	16	32	
29	*Staphylococcus epidermidis*	32	>64	>64	64	>64	>64
30	*Staphylococcus epidermidis*	1	64	1	4	32	64
31	*Staphylococcus aureus* MRSA	1	>64	64	16	8	>64
32	*Staphylococcus aureus* MRSA	1	32	32	16	16	>64
33	*Staphylococcus aureus* MRSA	8	64	>64	32	64	>64

These results show MICs ≥ 64 µl/ml which infers no discernible anti-microbial activity against many of the organisms tested. Ampicillin was more effective than any of the conjugates.

Table 4.17 Percentage of organisms inhibited by ≤ 16 µl/ml concentration

Conjugate	Percentage active at ≤ 16 µl/ml
Ampicillin	64
2.2a	36
2.2b	21
2.2c	49
2.2d	21
2.2e	15

Experiments to show any possible synergistic activity between **2.2a-f** and ampicillin.

Table 4.18 Initial experiments to test for synergy between conjugates and ampicillin, against NH 424

Ampicillin	Zone diameter	Conjugate	Zone diameter	Synergy
Ampicillin	8	2.2a	12	X
Ampicillin	10	2.2b	10	X
Ampicillin	11	2.2c	12	-
Ampicillin	10	2.2d	14	X
Ampicillin	8	2.2e	16	X
Ampicillin	12	2.2f	15	-
Ampicillin	11	Ampicillin	9	X

Table 4.19 Initial experiments to test for synergy between conjugates and ampicillin, against *B. cereus* 5

Ampicillin	Zone diameter	Conjugate	Zone diameter	Synergy
Ampicillin	32	**2.2a**	22	X
Ampicillin	35	**2.2b**	32	X
Ampicillin	27	**2.2c**	22	+
Ampicillin	35	**2.2d**	22	+
Ampicillin	36	**2.2e**	20	X
Ampicillin	35	**2.2f**	30	X
Ampicillin	34	Ampicillin	32	X

- X = no synergy, - = possible synergy, + = synergy.

Table 4.20 Growth inhibition of *B. cereus* 5 observed at varied concentrations of conjugate 2.2e and ampicillin

	Conc. Of Inhibitor							
		64	32	16		4	2	1
	64	X	X	X	X	X	X	X
Conc.	32	X	X	X	X	X	X	X
Of	16	X	X	+	+	+	+	+
Ampicillin	8	X	X	+	+	+	+	+
	4	X	+	+	+	+	+	+
	2	X	+	+	+	+	+	+
	1	X	+	+	+	+	+	+

Table 4.21 Growth inhibition of *B. cereus* 4 observed at varied concentrations of conjugate d and ampicillin

	Conc. Of Inhibitor							
		64	32	16	8	4	2	1
	64	X	X	X	X	X	X	X
Conc.	32	X	X	X	X	X	+	X
Of	16	X	X	X	X	+	+	+
Ampicillin	8	X	X	X	X	+	+	+
	4	X	X	+	+	+	+	+
	2	X	+	+	+	+	+	+
	1	X	+	+	+	+	+	+

- X signifies NO growth.
- + signifies growth.

Chapter 5

Discussion And Conclusions

5.1 The type one conjugates 2.1 a - f

The β-lactam conjugates are a unique class of compounds, combining a β-lactam antibiotic with a cytotoxic agent, with the aim of enhancing the spectrum of activity of the parent β-lactam agent. Presumably the rationale was to combine the specificity of the β-lactam agent with the toxic properties of the cytotoxic agent to provide a selectively toxic antibacterial mode of action.

Unfortunately the results of the MIC tests against a range of Gram positive and Gram negative organisms were disappointing. The conjugates failed to display activity against MRSA strains, and other bacterial strains which produce β-lactamase enzymes. It could be speculated that the conjugates would inhibit β-lactamase enzymes by the toxic activity of the cytotoxic agent as the β-lactam agent was bound to the serine group of the β-lactamase. This does not appear to be the case, judging from the results. Strains which produce a β-lactamase enzyme appear to destroy the β-lactam group, while the cytotoxic moiety lacks antibacterial activity.

Any antibacterial activity of the conjugates appears to be due to the presence

of the β-lactam group, rather that due to a synergistic and selectively toxic mechanism of the conjugate. This view is confirmed by the superior MIC values of the β-lactam agents alone compared to those of the conjugates. Perhaps this reduced activity of the conjugates is due to the cytotoxic group reducing the penetration and effectiveness of the β-lactam group. The cytotoxic agent does not protect the β-lactam agent from β-lactamase hydrolysis, nor does it augment the spectrum of the activity of β-lactam agent.

5.2 The type two conjugates 2.2a-f

The initial testing for anti-bacterial activity showed that all the conjugates do show antibacterial activity. This is the result that was expected since all them have β-lactam structures and are based upon the cephalosporin structure as outlined previously (see introduction).

The conjugates were not as effective as ampicillin in killing / inhibiting the growth of *Staphylococcus aureus* NCTC6571. Conjugates **2.2d, e** and **f** showed slightly greater activity than **2.2a, b** and **c.** This can only be due to variation in the stereochemistry of these conjugates influencing the availability of the β-lactam ring.

Testing against β-lactamase producing organisms showed decreased activity for ampicillin against MRSA strains. This was as expected and due to hydrolysis of the β-lactam ring or due to the altered target (peptidoglycan synthesising enzyme) found in MRSA strains. The observed inhibition effects

were possibly due to the high concentration of ampicillin used in this experiment. The conjugates also showed a reduced level of growth inhibition compared to against *Staphylococcus aureus* NCTC6571. However, bar the MRSA NH123 strain, all conjugates **2.2d, e** and **f** showed greater inhibition of growth than ampicillin (i.e. against the MRSA NH278, MRSA N424 and MRSA 24-7882 strains). Conjugates **2.2a, b** and **c** were shown to have activity that was either similar or less than ampicillin against all four strains.

The conclusion from this is that the addition of the temozolomide structure to cephalosporin does provide protection against attack by serine type β-lactamase enzymes.

The experiments carried out against *Bacillus cereus* 4 and *Bacillus cereus* 5 showed ampicillin to be twice as effective as against the MRSA strains. Conjugates **2.2d, e and f** were equally as effective against *Bacillus cereus* 5. No explanation can be made from these experiments.

Testing against β-lactamase enzymes was carried out against controls where either the enzyme or the conjugates was excluded from the experiment. The lack of hydrolysis no change in observed optical density (OD) seen when no enzyme is added to the nitrocefin shows that the change in OD observed is not due to the addition of the conjugates (red line on graphs). When conjugate is added the nitrocefin is completely hydrolysed. This is shown by the top curve in the graphs (green line on graphs in **Fig 5.1**) with a plateau relating to complete hydrolysis (OD measured as 0.426 after 510 seconds).

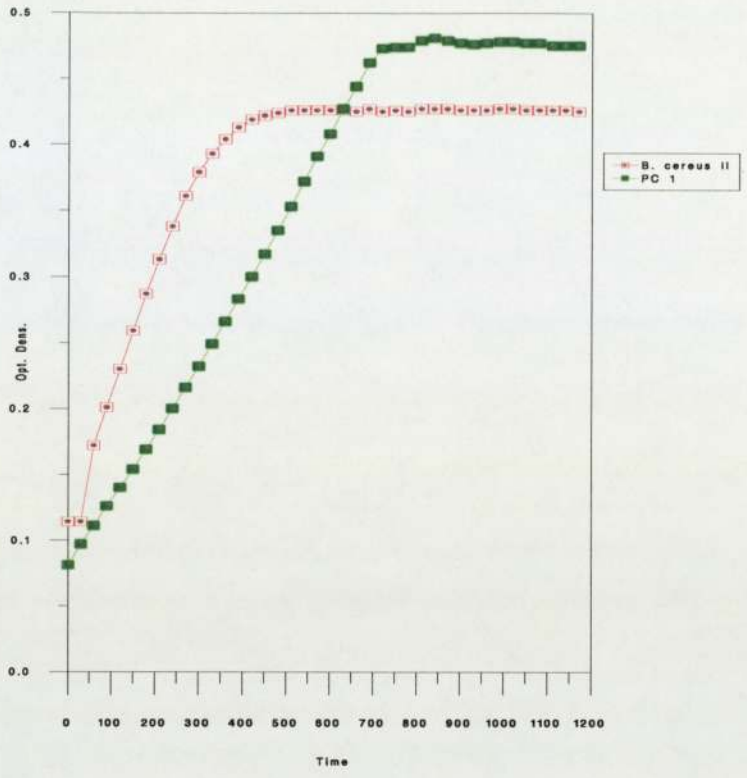


Fig 5.1 Calibration graphs for the enzyme assays with nitrocefin.

Testing against PC1 showed all type two conjugates to reduce the rate of nitrocefin hydrolysis and to prevent complete nitrocefin hydrolysis within the twenty-minute sampling period.

When no conjugate was added, OD was measured as 0.317 after 450 seconds and 0.475 after 1170 seconds. Comparisons with ODs measured in the presence of conjugate show reductions in OD measured after 450 seconds. This indicates a reduced rate of hydrolysis. Reductions after 1170 seconds show a decreased total nitrocefin hydrolysis.

Interference with nitrocefin hydrolysis may be due to inhibition of the PC1 enzyme either by binding of the conjugate to the enzyme altering the active site or as is more likely, due to competitive occupation of the enzyme active site itself.

The introduction of a twenty-minute pre-incubation period causes a reduction in the rate of nitrocefin hydrolysis. However, compared to that seen with zero pre-incubation, nitrocefin hydrolysis has occurred to a greater degree after 1170 seconds (conjugates **2.2a, e** and **f**), or is still occurring (conjugates **2.2b**, **c** and **d**). This would indicate that pre-incubation allows greater binding between the conjugate and PC1. It also indicates that they are destroyed by PC1 allowing the active site of PC1 to become available again to hydrolyse nitrocefin.

These results alone taken on their own do not show these conjugates to be effective inhibitors of the serine type β-lactamase enzyme PC1.

Testing against *B. cereus* II showed all conjugates to reduce the rate and degree of nitrocefin hydrolysis (following the strategy employed for PC1). The addition of a twenty minute pre-incubation period caused a further decrease in nitrocefin hydrolysis for all the type two conjugates except conjugate **2.2f** which caused increased nitrocefin hydrolysis (compared to with zero pre-incubation). This would indicate that irreversible binding of the conjugates to the enzyme active site occurs.

The decrease in nitrocefin hydrolysis was far greater for conjugates **2.2a-e** than was seen with the PC1 enzyme. The results for conjugates **2.2a, b** and **d** show ODs which are between +/- 0.003 of the mean OD seen in the control (no enzyme) after 450 seconds i.e. virtually no nitrocefin hydrolysis. After 1170 seconds, these figures rose to +0.071, +0.026 and +0.014 above the control level for conjugates **2.2a, b** and **d** respectively. This is proof that these conjugates either inhibit the *B. cereus* II enzyme or interact with it to prevent the active site from hydrolysing action for a period of twenty minutes given a twenty-minute pre-incubation period.

Ampicillin was included as a further control since this conjugate is known to be destroyed by β-lactamase enzymes and therefore should not show any effect on nitrocefin hydrolysis.

The observed result of decreased nitrocefin hydrolysis in the presence of ampicillin with both *B. cereus* II and PC1 was not expected. This can however be explained as due to the ampicillin taking up the enzyme active sites for the hydrolysis of its own β-lactam ring therefore competing with nitrocefin for the enzyme active site and slowing down the rate of nitrocefin hydrolysis. Pre-incubation causes increased total nitrocefin hydrolysis since more of the ampicillin was hydrolysed before nitrocefin was added therefore there was less competition for enzyme active sites over time. However pre-incubation allows the active sites to become more fully occupied at t = zero therefore the initial rate of nitrocefin is less.

Varying the pre-incubation time (i.e. pre-incubation times of 5, 10, 20, 40 and 60 minutes) with the PC1 enzyme did have an effect on the rate and degree of nitrocefin hydrolysis. When ampicillin was added, pre-incubation led to an increased rate of nitrocefin hydrolysis and increased total hydrolysis. Inhibitors **2.2d** and **e** caused a decreased rate of nitrocefin hydrolysis and decreased total hydrolysis as the pre-incubation time increased. Compared to ampicillin, pre-incubation for five minutes had little or no effect. However with pre-incubation times of 20, 40 and 60 minutes, **2.2d** and **e** showed far greater inhibition of hydrolysis than ampicillin.

The ampicillin result can be explained as per the previous text. The results for **2.2d** and **e** indicate that allowing a longer period of time for the enzyme and conjugate **2.2d** and **e** to mix increases the likelihood of an interaction or binding between the enzyme and conjugates leading to decreased enzyme activity.

The effect of varied pre-incubation time against the *B. cereus* II enzyme show that for ampicillin the effects are similar i.e. pre-incubation leads to increased nitrocefin hydrolysis. For conjugates **2.2d** and **e**, increasing the pre-incubation period also increases nitrocefin hydrolysis. This indicates that these conjugates do not bind to the *B. cereus* II enzyme active site in the same way that conventional inhibitors (i.e. clavulanic acid) bind to serine type enzymes.

The measurement of MICs against MRSA 424 and *B. cereus* 5 showed conjugate **2.2e** to inhibit the growth of MRSA 424 at a concentration of 16

μg/ml. Ampicillin showed little inhibition as expected. Against the *B. cereus* 5, MICs of 4, 8 and 16 μg / ml were recorded for conjugates **2.2e, f** and a respectively. Conjugates **2.2e, f** showed the most favorable results when taking both organisms into account.

Testing against 33 strains of which 21 (64 %) were shown to be ampicillin sensitive showed the conjugates tested to be active. In the majority of cases where the conjugates showed activity, ampicillin was equally active. Exceptions to this were *Serratia marcescens* (7) which was resistant to ampicillin but was susceptible to all the conjugates tested and *Serratia marcescens* (8) which was also ampicillin resistant but attacked by conjugate **2.2a**. These results do show that conjugate **2.2a** has potentially good anti-microbial activity at levels low enough for it to be considered for further investigation and that they imply activity against ampicillin resistant strains does exist.

Against MRSA NH 424 the evidence of synergy between ampicillin and conjugates was weak. Conjugates **2.2c** and **f** only, showed signs of possible synergy. Against *B. cereus* 5, conjugates **2.2c** and **d** gave results regarding synergy which warranted further investigation.

Conjugates **2.2d** and **e** were tested using the chequer board technique. Conjugate **2.2e** did not show any synergistic activity (as before).N.B. Conjugate **2.2e** was tested for synergy since it had shown good activity against *B. cereus* 5 and MRSA NH424. Conjugate **2.2d**, in conjunction with

ampicillin, did however show inhibition of growth at concentrations at lower drug concentrations (8 μg / ml for both conjugate **2.2d** and ampicillin). This result showed reductions in the concentrations of both conjugate **2.2d** and ampicillin needed to inhibit growth (previously 32 μg / ml for both conjugates).

Comparisons between enzyme inhibition by the conjugates and inhibition by known β-lactamase inhibitors can be made. Against the PC1 enzyme, none of the six conjugates produce enzyme inhibition comparable with sulbactam, clavulanic acid or BRL42715B. This indicates that as drugs for use against organisms producing the PC1 β-lactamase enzyme, these conjugates are of little value. It is probable that similar resistance would be observed with these conjugates against other enzymes with similar structures within the same class.

Comparisons against the same drugs using the *B. cereus* II enzyme show that with no pre-incubation , the conjugates show little activity. The results observed show greater nitrocefin hydrolysis than those for clavulanic acid and sulbactam which are known to be ineffective against metallo β-lactamase enzymes. Pre-incubation increases the effectiveness of all the conjugates. However only conjugate **2.2b** and **d** produce greater degrees of inhibition than sulbactam and clavulanic acid and are therefore worth further consideration as enzyme inhibitors. Both conjugates show similarity in the R group at R1. Possibly this is the structure which enhances the conjugates activity to β-lactamase metallo enzymes.

The link between the ability of these conjugates to inhibit β-lactamase activity and anti-microbial activity is sketchy and not provable if based upon these results alone. It has been shown however that conjugate **2.2d** is the most effective conjugate out of the six conjugates tested for inhibiting the *Bacillus cereus* II enzyme and is also the most effective of the six conjugates at inhibiting growth in the multi point MIC test. The fact that conjugate **2.2b** has activity against the *B cereus* II enzyme but not against the organisms used in the multi point MIC test shows that the addition to the R1 position is potentially the more important group regarding enzyme interaction.

In conclusion, all of the conjugates inhibit the β-lactamase enzymes PC1 and *Bacillus cereus* II to a certain degree. Activity against the PC 1 enzyme may be due to the provision of an alternative β-lactamase ring to hydrolyse thus slowing nitrocefin hydrolysis. Activity against the *B. cereus* II enzyme with pre-incubation for conjugates **2.2b** and **2.2e** (with the Ph-CH_2-CO-NH group in the R1 position) may be due to the mechanism of inhibition proposed (see introductory text).

Comparisons with the control conjugates show none of the conjugates to be as effective as BRL42715B at inhibiting β-lactamase enzymes. Therefore the approach used in creating this conjugate may be a more beneficial route to follow in the search for new antibiotics and inhibitors of β-lactamase enzymes.

Conjugate **2.2d** has been shown to have antimicrobial activity and to produce a degree of synergy when combined with ampicillin.

The fact that these conjugates have activity against β-lactamase enzymes (*B. cereus* II) shows that these molecules do interact with β-lactamase enzymes. If the temozolomide molecule is released as has been previously proposed, these conjugates cause the release of a cytotoxic anti-cancer agent in close proximity to the β-lactamase enzyme.

5.3 Conclusion

Dual-action conjugates in which a β-lactam antibiotic with a cytotoxic agent (**2.1a-f**) and a β-lactam antibiotic with β-lactamase inhibitor (**2.2a-f**) have been obtained. The antibacterial activity of the conjugates has been tested against a panel of bacteria including several β-lactamase producing strains. The results of (**2.1a-f**) conjugates demonstrated little synergistic effect against bacteria (Table 4.1), while (**2.2a-f**) demonstrated some synergistic effect against bacteria (Table 4.15 and Table 4.16). The specific conjugate (**2.2d**) has been shown to have antimicrobial activity and to produce a degree of synergy when combined with ampicillin. Rendering it is the best choice out of the conjugates tested for the future investigation.

An optimistic search continues for an anti-cancer drug with dual-action strategies. If cancer cells can be labeled with a β-lactamase enzyme, the cephalosporin molecule would potentially bind to the enzyme causing release

of the temozolomide molecule in the proximity of cancer cells, i.e. the anti-cancer drug would be targeted specifically at cancerous cells. A study is needed on this aspect.

Chapter 6

Experiments

6.1 General Methods

NMR spectra were recorded on a Bruker AC250 Spectrometer at ^{1}H (250.1 MHz) and ^{13}C (62.9MHz). Chemical shifts are downfield of tetramethylsilane. Mass spectroscopic analysis was carried out on a Hewlett Packard 5989B MS engine with an HP 5998A API Electrospray LC/MS interface; the LC being an HP1100 system with autosampler. Infrared spectra were recorded on a Mattson 3000 FTIR Spectrometer. Solid samples were prepared as KBr discs and liquids as thin films between sodium chloride plates. Melting points were determined on Gallenkamp apparatus and are uncorrected. Flash column chromatography was performed using Sorbsil C60 silica gel. TLC was carried out using aluminium backed Merck Silica Cel 60 F_{254} plates and visualised under UV (254 nm). Potassium permanganate was used where appropriate to develop TLC plates.

6.2 Chemical Synthesis

6.2.1 Synthesis of 3-(2-Chloroethyl)-4-oxo-imidazo[5,1-d]-1,2,3,5-tetrazine-8-carboxylic acid 2.5

To mitozolomide **2.3** (1 g, 4.1 mmol) mixed with H_2SO_4 (6 ml) and stirred until the solid was completely dissolved. The reaction solution was cooled in an ice bath and the solution of sodium nitrite (1 g, 14.5 mmol) in (4 ml) of water was added dropwise, then continued stirring at room temperature 20 °C for over night. The reaction solution was poured onto ice water (300 ml) and precipitated product was filtered, washed with water (3 × 100 ml) and brine (5 × 20 ml), concentrated under *vacuum* to give the title **2.5** as white solid yield 950 mg; (96.6%) TLC (MeOH-EtOAc 2:1); mp 166-167 °C; IR (KBr disc): $^{\upsilon}$max 3611, 3483 (N-H), 3094 (C-H), 3000-2500 (O-H), 1768 (C=O), 1720 (C=O), 1563 (C-Cl) cm^{-1}; ^{1}H NMR [DMSO-d_6]: δ 4.01(t, 2H C*H*$_2$), 4.63(t, 2H C*H*$_2$Cl), 8.87(s, 1H *H*-6); ^{13}C NMR [DMSO-d_6]: δ 41.46, 50.15(CH_2CH_2Cl), 129.79, 135.90 (C-8, C-8a), 130.4 (C-6), 138.97, 161.72 (2 ×CO); Its spectroscopic and analytical properties identical to an authentic sample (Alexander, R P et al 1991).

6.2.2 3-methyl-4-oxo-imidazo[5,1-d]-1,2,3,5-tetrazine-8-carboxylic acid (temozolomide acid) 2.6

To a solution of 3-methyl-4-oxo-imidazo[5,1-d]-1,2,3,5-tetrazine-8-carboxamide **2.4** (temozolomide 1 g, 10.25 mmol) in (6ml) of H_2SO_4 was added dropwise sodium nitrite (1 g, 28.99 mmol) in the water (4 ml). The mixture was stirred at room temperature 20 °C for overnight. The reaction solution was poured in ice water (200 ml), the precipitated product was filtered, washed with ether (2 × 10ml) and water (2 × 20 ml), then dried *in vacuum* to give white solid **2.6** yield 812 mg (65 %); TLC (MeOH-EtOAc 1:2): R_f=0.44, mp 191-193 °C; IR (KBr disc): $^{\upsilon}$max 3519 (N-H), 3087 (C-H),

3000-2500 (O-H), 1764 (C=H), 1695 (C=O) 1554, 1481, 1189 cm^{-1}; ^{1}H NMR [DMSO-d_6]: δ 3.86(s, 3H CH_3); 8.80(s, 1H *H*-6); ^{13}C.NMR [DMSOd_6]: δ 36.19 (CH_3), 128.42, 136.7 (C-8, C-8a), 130.53 (C-6), 139.25, 161.59 (2 ×CO).

6.2.3 3-(2-Chloroethyl)-4-oxo-imidazo[5,1-d]-1,2,3,5-tetrazine-8-carboxylic acid, N-hydroxy succinimide ester 2.7

A solution of **2.5** (487 mg, 2 mmol), N-hydroxysuccinimide (330 mg, 2 mmol) and DCC (457 mg, 2.2 mmol) in dry dimethylformamide (10 ml) was stirred at room temperature for 8 hours. After dicyclohexylurea was removed by filtration and the solution was concentrated under high *vacuum*, the residue mixed with propan-2-oL (10 ml) and stirred for 30 minutes. The ester product was filtered out and dried under high *vacuum* to give 340 mg (50 %) of the title **2.7**; TLC (MeOH-EtOAc 2:1); mp 216-219 °C; IR (KBr disc): $^{\upsilon}$max 3324, 2925, 2847, 2362, 2338, 1623, 1577 cm^{-1}; ^{1}H NMR [DMSO-d_6]: δ 2.90(s 4H), 4.02 (t. 2H CH_2), 4.70 (t. 2H CH_2), 9.07 (s, 1H 6'-*H*); ^{13}C NMR [DMSO-d_6]: δ 25.63, 41.28, 50.70, 121.59, 131.31, 138.30, 138.59, 156.33, 170.26; m/z 340 ($C_{11}H_9N_6ClO_5$).

6.2.4 6.2.4 3-Methyl-4-oxo-imidazo[5,1-d]-1,2,3,5-tetrazine-8-carboxylic acid, N-hydroxy succinimide ester 2.8

A solution of **2.6** (560 mg, 2.87 mmol), N-hydroxysuccinimide (330 mg, 2.87 mmol) and DCC (752 mg, 3.64 mmol) in dry dimethylformamide (14 ml) was stirred at room temperature for 8 hours. After dicyclohexylurea was removed by filtration and the solution was concentrated under high *vacuum* to give

white solid **2.8** yield 503 mg (60 %); TLC (MeOH-EtOAc 1:2); mp 219-221 °C; IR (KBr disc): $^{\upsilon}$max 3505, 3324, 3112, 2925, 1742 cm^{-1}; ^{1}H NMR [DMSO-d_6]: δ 2.88(d, 4*H*), 3.92(s, 3H C*H*3), 9.03(s, 1H *H*-6).

6.2.5 6.2.5 α-[(3-Methyl-4-oxo-imidazo[5,1-d]-1,2,3,5-tetrazine-8-carboxyl)amino benzyl] penicillin 2.1a

To a solution of **2.8** (86 mg, 0.29 mmol), ampicillin (117 mg, 0.29 mmol) in dry DMF (3 ml) in an ice water bath was added dropwise triethylamine (0.05 ml, 0.33 mmol). The mixture was warmed up to 30 °C and stirred for 4 hours while temperature gradually came down to ambient. To the reaction solution was added 10 ml of ice water and then was extracted with ethyl acetate (3 × 20 ml). The aqueous layer was adjusted to the pH 1-2 with 1M HCl and ice and extracted with ethyl acetate (3 × 30 ml). The combined organic layer washed with brine (5 × 20 ml) and concentrated under *vacuum* to give white solid **2.1a** yield 58 mg (37 %); TLC (MeOH-EtOAc 2:1); mp 179-188 °C; IR (KBr disc): $^{\upsilon}$max 3343, 3127, 3058, 3031, 2928, 2856, 2360, 1751, 1670 cm^{-1}; ^{1}H NMR [DMSO-d_6]: δ 1.39(s, 3H CH_3), 1.53(s, 3H CH_3), 1.98(s, 1H), 3.87(s, 3H CH_3), 4.20(s, 1H), 5.41(d, 1H 3-*H*), 5.59(dd, 1H 7-*H*), 5.95(d, 1H 6-*H*), 7.32(dd, 3H aromatic-*H*), 7.49(d, 2H aromatic-*H*), 8.60(d, 1H *H*N), 8.89(s, 1H 6'-*H*), 9.38(d, 1H *H*N); ^{13}C NMR [DMSO-d_6]: δ 20.80, 24.50, 26.61, 30.31, 33.39, 36.35, 55.02, 58.24, 59.79, 63.75, 67.08, 70.34, 126.76, 128.49, 129.18, 134.86, 138.24, 139.11, 158.53, 168.90, 169.66, 173.10; m/z=527 ($C_{22}H_{22}N_8O_6S$).

7-{D-2-(3-methyl-4-oxo-imidazo[5,1-d]-1,2,3,5-tetrazine-8-carboxyl) amino-2-phenylacetamido)-3-methyl-8-oxo-5-thia-azabicyclo [4, 2, 0]oct-2-ene-2carboxylic acid 2.1b

To a stirred solution of cephalexin (347 mg, 1 mmol) and triethylamine (0.15 ml, 1mmol) in (4 ml) of dry DMF at an ice bath was added **2.8** (292 mg, 1 mmol). The mixture was stirred at room temperature 22-25 °C for 22 hours. Water (10 ml) was added into the reaction solution, then extracted with ethyl acetate (4 × 30 ml). To the aqueous layer was added small amount of ice, adjusted the pH to 1-2 with 1M HCl and extracted with ethyl acetate (4 × 40 ml). The combined organic phase was washed with brine (5 × 30 ml), dried and concentrated under high *vacuum* to give white solid **2.1b** yield 179 mg (34 %); TLC (MeOH-EtOAc 1:2); mp 250-256 °C; IR (KBr disc): $^{\upsilon}$max 3324, 3276, 3125, 3058, 2925, 2847, 2362, 2338, 1752, 1671 cm^{-1}; ^{1}H NMR [DMSO-d_6]: δ 1.98(s, 3H C*H*$_3$), 3.92 (s, 3H C*H*$_3$), 5.00(d, 1*H*), 5.86(dd, 1*H*), 5.90(d. 1*H*), 7.35(dd, 3H aromatic-*H*), 7.50 (d, 2H aromatic-*H*), 8.59(d, 1H *H*N), 8.87 (s, 1H *H*N), 9.50(d, 1H *H*-6); ^{13}C NMR [DMSO-d_6]: δ 19.42, 24.50, 28.89, 30.74, 33.38, 36.37, 55.25, 57.00, 58.47, 122.62, 126.77, 128.00, 128.53, 128.89, 129.19, 129.76, 134.87, 138.22, 139.11, 158.54, 163.75, 170.29; m/z=525 ($C_{22}H_{20}N_8O_6S$).

α-[(3-Methyl-4-oxo-imidazo[5,1-d]-1,2,3,5-tetrazine-8-carboxyl)amino-*p*-hydroxy benzyl] penicillin 2.1c

To a stirred solution of **2.8** (146 mg, 0.5 mmol) in (4 ml) of dry DMF amoxicillin (182 mg, 0.5 mmol) was added, followed by addition of triethylamine (0.15ml, 1 mmol) dropwise in an ice water bath. The mixture was warmed up to 30 °C and stirred for 4 hours. The reaction solution was poured into ice water (30 ml). The mixture was adjusted to the pH 1-2 with 1M HCl, extracted with ethyl acetate (4 × 20 ml), washed with brine (5 × 20 ml), and concentrated under high *vacuum* to give the title **2.1c** as white solid, yield 188 mg (69 %); TLC (MeOH-EtOAc 2:1); mp 196-235 °C; IR (KBr disc): $^{\upsilon}$max 3359, 3127, 3029, 2928, 2849, 2358, 2338, 1744, 1662 cm^{-1}; ^{1}H NMR [DMSO-d_6]: δ 1.42(s, 3H C*H*$_3$), 1.56(s, 3H C*H*$_3$), 1.97(s, 1H), 3.87(s, 3H C*H*$_3$), 4.19(s, 1H 3-*H*), 5.41(d, 1H 7-*H*), 5.57(dd, 1H 6-*H*), 5.83(d, 1H C*H*), 6.73(d, 2H aromatic-*H*), 7.27(d, 2H aromatic-*H*), 8.47(d, 1H *H*N), 8.85(d, 1H 6'-*H*), 9.23(d, 1H *H*N), 9.42(s, 1H *H*O); ^{13}C NMR [DMSO-d_6]: δ 24.49(C*H*), 25.34, 26.64, 30.12(C*H*), 33.37, 36.34, 46.01, 58.07, 63.77, 67.04, 70.37, 115.19, 128.06, 128.46, 129.25, 134.78, 139.11, 157.08, 158.41, 168.96, 170.12, 173.34; m/z=543 ($C_{22}H_{22}N_8SO_7$)

7-{D-2-[3-(2-chloroethyl)-4-oxo-imidazo[5,1-d]-1,2,3,5-tetrazine-8-carboxyl]amino-2-phenylacetamido)-3-methyl-8-oxo-5-thia-azabicyclo [4, 2, 0]oct-2-ene-2-carboxylic acid 2.1d

To a stirred solution of cephalexin (173 mg, 0.5 mmol) and **2.7** (170 mg, 0.5 mmol) in 4 ml of dry DMF was added triethylamine (0.075 ml, 0.5mmol) dropwise under cooling of an ice water bath. The mixture was warmed up to 30 °C and stirred for 4 hours. A small amount of ice water was added and the

pH was adjusted to 1-2 with 1M HCl, then extracted with ethyl acetate (3 × 30 ml). The combined ethyl acetate was washed with brine (5 × 20 ml), dried and concentrated under high *vacuum* to give the title compound **2.1d** as white solid, yielded 202 mg (71 %); TLC (MeOH-EtOAc 1:2); mp 182-187 °C; IR (KBr disc): $^{\upsilon}$max δ 3372, 3127, 3062, 2927, 2851, 2358, 2344, 1750, 1668 cm^{-1}; ^{1}H NMR [DMSO-d_6]: δ 1.91(s, 3H C*H*$_3$), 4.05(t, 2H C*H*$_2$Cl), 4.65(t, 2H C*H*$_2$), 5.00(d, 1H), 5.68(dd, 1H C*H*), 5.90(d, 1H), 7.38(dd, 3H, aromatic-*H*), 7.50(d, 2H, aromatic-*H*), 8.64(d, 1H *H*N), 8.94(s, 1H 6'-*H*), 9.54(d, 1H *H*N); ^{13}C NMR [DMSO-d_6]: δ 19.41, 28.88, 30.81, 35.83, 41.47, 50.18, 55.32, 56.98, 58.45, 122.61, 126.77, .128.00, 128.53, 129.59, 129.76, 134.31, 138.14, 138.98, 158.44, 162.38, 163.48, 163.74, 170.24; m/z=574 ($C_{23}H_{21}N_8O_6Cl$ 573).

α-[(3-(2-chloroethyl)-4-oxo-imidazo[5,1-d]-1,2,3,5-tetrazine-8-carboxyl) aminobenzyl] penicillin 2.1e

To a stirred solution of **2.7** (170 mg, 0.5 mmol) and ampicillin (202 mg, 0.5 mmol) in dry DMF (4 ml) cooled in an ice water bath was added triethylamine (0.07 ml, 0.5 mmol). The mixture was warmed up to 30 °C and stirred for 4 hours. When TLC showed the reaction was completed, ice water (10 ml) was added to the solution and was adjusted to the pH 1-2 with 1M HCl, then extracted with ethyl acetate (3 × 30 ml) washed with brine (5 × 20 ml). The combined organic layer concentrated under high *vacuum* to give the title compound **2.1e** as white solid, yield 175 mg (61 %); TLC (MeOH-EtOAc 1:2); mp 145-153 °C; IR (KBr disc): $^{\upsilon}$max 3365, 2928, 2362, 1752, 1661 cm^{-1};

^{1}H NMR [DMSO-d_6]: δ 1.39(s, 3H C*H*$_3$), 1.53(s, 3H C*H*$_3$), 4.01(t, 2H C*H*$_2$), 4.21(s, 1H 3-*H*), 4.66(t, 2H C*H*$_2$), 5.42(d, 1H 7-*H*), 5.58(m, 1H 6-*H*), 5.95(d, 1H C*H*), 7.49(dd, 4H aromatic-*H*), 8.64(d, 1H N*H*), 8.94(s, 1H 6'-*H*), 9.41(d, 1H N*H*); ^{13}C NMR [DMSO-d_6]: δ 24.50, 26.60, 30.32, 33.38, 41.47, 50.17, 55.08, 58.23, 59.79, 63.76, 67.08, 70.31, 126.76, 127.91, 128.48, 129.61, 134.31, 138.17, 138.98, 158.42, 168.89, 169.61, 173.09; m/z =575 ($C_{23}H_{23}N_8O_6ClS$ 575).

α-[(3-(2-chloroethyl)-4-oxo-imidazo[5,1-d]-1,2,3,5-tetrazine-8-carboxyl) amino-*p*-hydroxy benzyl] penicillin 2.1f

To a stirred solution of **2.7** (170 mg, 0.5 mmol) and amoxicillin (182 mg, 0.5 mmol) in 4 ml of dry DMF cooled in an ice water bath was added trietylamine (0.075 ml, 0.5 mmol) dropwise. The mixture was warmed up to 30 °C and stirred for 4 hours. The reaction solution was poured into ice water (30 ml) and was adjusted to the pH 1-2 with 1M HCl. Extract with ethyl acetate (4 × 20 ml), wash with brine (5 × 20 ml) and concentrate under high *vacuum* to give white solid **2.1f** yield 192 mg (65 %); mp 138-142 °C; IR (KBr disc): $^{\upsilon}$max 3742, 3370, 3121, 3023, 2928, 2851, 2358, 2344, 1748, 1664 cm^{-1}; ^{1}H NMR [DMSO-d_6]: δ 1.41(s, 3H C*H*$_3$), 1.55(s, 3H C*H*$_3$), 1.98 (s, 1H), 4.02(t, 2H C*H*$_2$Cl), 4.20(s,1H 3-*H*), 4.64(t, 2H C*H*$_2$), 5.42(d, 1H 7-*H*), 5.59(m, 1H 6-*H*), 5.83(d, 1H C*H*), 6.74(d, 2H aromatic-*H*), 7.29(d, 2H aromatic-*H*), 8.94(s, 1H 6'-*H*), 9.28(d, 1H N*H*), 9.47(s, 1H *H*O); ^{13}C NMR [DMSO-d_6]: δ 24.50, 25.36, 26.65, 30.16, 33.38, 41.46, 47.54, 50.16, 54.56, 58.08, 59.80, 63.76,

67.06, 70.37, 115.18, 128.06, 129.93,139.00, 157.09, 158.28, 168.94, 170.08, 173.33; m/z = 591 ($C_{23}H_{23}N_8O_7ClS$ 591.0).

Attempted synthesis of 6-[3-(2-chloroethyl)-4-oxo-imidazo[5,1-d]-1,2, 3,5-tetrazine-8-carboxyl]aminopenicillanic acid 2.1g

To a stirred solution of 6-APA (216 mg, 1 mmol) and tricthylamine (0.15 ml, 1 mmol) in 6 ml of dry DMF was added **2.7** (340.7 mg, 1 mmol) at 0-5 °C. The mixture was warmed up to 30 °C and stirred for over night. Water (10 ml) was added to the reaction solution, extracted with ethyl acetate (4 × 30 ml). Small amount of ice was added to the aqueous layer and adjusted to pH 1-2 with 1M HCl, then extracted with ethyl acetate (4 × 40 ml). The combined organic layer was concentrated under high *vacuum* to give **2.1g**. TLC (CH_2Cl_2-MeOH 4:1); ^1H NMR showed no sign of the desired product. The experiment was failed.

6.2.6 8-carbamoyl-3, 4-dihydro-4-oxo-imidazo [5,1-d] -1,2,3,5- tetrazine-3-ylacetic acid 2.9

Ethyl 8-carbamoyl-3,4-dihydro-4-oxo-imidazo[5,1-d]-1,2,3,5-tetrazine-3-ylacetate (680 mg,) was suspended in water (1 0 ml) and HCl (10 ml). The mixture was stirred for 4h at 40-45 °C. The reaction solution was poured in ice (100 ml) and precipitated product was filtered out, washed with water (3 × 50 ml) followed by ether, then concentrated under *vacuum* to obtain the titled **2.16** 140 mg.

6.2.7 N-[8-carbamoyl-3,4-dihydro-4-oxo-imidazo[5,1-d]-1,2,3,5-tetrazine-3-acetate]-succinimide 2.10

A solution of 8-carbamoyl-3,4-dihydro-4-oxo-imidazo[5,1-d]-1,2,3,5-tetrazine-3-ylacetic acid **2.9** (342 mg, 1.43 mmol), N-hydroxycuccieumide (165 mg, 1.43 mmol) and DCC (326 mg, 1.43 mmol) in dry dimethylformamide (9 ml) was stirred under cooling of ice water for 40-50 minutes, then warmed up to 30 °C to stirred for further 1h. Solid dicyclhexylurea was filtered out and concentrated under high *vacuum.* Propanol-2-ol (20 ml) was added to the residue. The solid was filtered out and washed with ether (2 × 20 ml), the filtrate was concentrated under high *vacuum* to give the title compound **2.10** ^{1}H NMR [D_2O]: δ 2.73 (5*H*), 3.41, 5.2 (1*H*), 5.77 (2*H*), 7.81 (s, 1H N*H*), 7.95 (s, 1H N*H*), 8.98 (s, 1H 6-*H*).

6.2.8 α-[N-(8-carbamoyl-3,4-dihydro-4-oxo-imidazo[5,1-d]-1,2,3,5-tetrazine-3-acetyl)]-amino-p-hydroxy benzyl] penicillin 2.1h

A solution of amoxicillin (182 mg, 0.5 mmol), N-[8-carbamoyl-3, 4-dihydro-4-oxo-imidazo[5,1-d]-1,2,3,5-tetrazine-3-acetate]-succinimide **2.10** (168 mg, 0.5 mmol) and triethylamine (0.75 ml, 0.5 mmol) in dry DMF (5 ml) cooled in an ice water bath was stirred for 4 hours while allowing the reaction temperature gradually rise to 10-15 °C. Then keep stirring at room temperature for 2 hours and then at 30 °C for 1 hour. Water (10 ml) was added to the reaction solution and extracted with ethyl acetate (4 × 30 ml). Small amount of ice was added to aqueous layer and adjusted pH to 1-2 with 1M HCl, then extracted with ethyl acetate (4 × 20 ml). The combined organic layer was concentrated under high *vacuum* to give the titled **2.18**. TLC (EtOAc MeOA

2:1); ^{1}H NMR [D_2O]: δ 1.42(s. 3H C*H*$_3$), 1.56(s. 3H C*H*$_3$), 4.19(s. 1H), 5.09(s. 1H), 5.45(s. 1H), 5.6(AB-q.2H), 6.75 (d. 2H), 7.3(d.2H), 8.9(s. 1H 6-*H*), 9.2 (AB-q 1H), 9.44 (s. 1H).

7-{D-2-[N-(8-carbamoyl-3,4-dihydro-4-oxo-imidazo[5,1-d]-1,2,3,5-tetrazine-3-acetyl]amino-2-phenylacetamido}-3-methyl-8-oxo-5-thia-azabicyclo[4, 2, 0]oct-2-ene-2-carboxylic acid 2.1i

N-[8-Carbamoyl-3,4-dihydro-4-oxo-imidazo[5,1-d]-1,2,3,5-tetrazine-3-acetate]-succinimide **2.10** (0.265 mg, 0.78mmol) cephalexin (0.27 mg, 0.78 mmol) and trietylamine (0.12 ml, 0.78 mmol) in dry DMF (5ml) was mixed at 0 °C, then warmed up to 30 °C and stirred for 4 hours. The reaction solution was poured into ice water (30 ml), then adjusted to pH 1-2 with 1M HCl. The precipitate was filtered out and dried under *vacuum* to give white solid the title compound **2.1i**. ^{1}H NMR [D_2O]: δ 1.98(s, 3H) 2.7(s. 1H), 5.64(m, 1H), 5.76(d, 1H), 7.44(m, 5H), 7.75(s, 1H N*H*), 7.95(d, 1H N*H*), 8.89(s, 1H 6'-*H*), 9.26(d, 1H), 9.44(d, 1H).

α-[N-(8-carbamoyl-3,4-dihydro-4-oxo-imidazo[5,1-d]-1,2,3,5-tetrazine-3-acetyl] aminobenzyl] penicillin 2.1j

N-[8-Carbamoyl-3,4-dihydro-4-oxo-imidazo[5,1-d]-1,2,3,5-tetrazine-3-acetate]succinimide **2.10** (304 mg, 0.9 mmol), trietylamine (0.13 ml, 0.9 mmol), ampicillin (363 mg, 0.9 mmol) and dry DMF (6 ml) was mixed at 0 °C. Allow the reaction temperature gradually rising to ambient and stirred for 1 hour, then warm up to 30 °C and stirred for 1.5 hours. The reaction solution

was poured into ice water (30 ml) and was adjusted pH to 1-2 with 1M HCl. Extract with ethyl acetate (4 × 20 ml), dry over $MgSO_4$, concentrate under hight *vacuum* to give the titled **2.1j** ^{1}H NMR [D_2O]: δ 1.37(s, 3H CH_3), 1.51(s, 3H CH_3), 4.19(s, 1H), 5.11(s, 2H), 5.37(d, 1H), 5.51(m, 1H), 5.75(d, 1H), 7.40(m, 3H), 8.82(s, 1H 6'-*H*), 9.26(dd, 2H).

6.2.9 Synthesis of allyl 3-hydroxymethyl-3-cephem-2-carboxylate 2.15

3-Hydroxymethyl-3-cephem-2-carboxylic acid **2.14** were prepared following the literature method (Cocker, J D et al 1966). To a solution of 3-hydroxymethyl-3-cephem-2-carboxylic acid **2.14** (1.69 mmol) in DMF (9 ml) and 1,4-dioxane (6 ml) was added sodium bicarbonate (3.38 mmol.) followed by allyl bromide (2.54 mmol.). The resulting solution was refluxed for 1 hour, then cooled to room temperature. The reaction solution was partitioned between ethyl acetate (100 ml) and brine (100 ml). The organic phase was washed with water (2 × 20 ml) and brine (2 × 20 ml), dried over anhydrous magnesium sulfate. Evaporation and flash chromatography (ethyl acetate : hexane 1:2) gave the title compound **2.15**. The following compounds were prepared by the same method.

Allyl 3-hydroxymethyl-7-phenylacetamido-3-cephem-2-carboxylate 2.15a

From **2.14a**, the cephalosporin **2.15a** (25.6%) was recovered as a white solid and had mp 106~108 °C. IR (KBr): 3417, 3309 (NH, OH), 3081, 2968 (CH), 1784 (β-lactam C=O), 1751, 1353 (ROC=O), 1660, 1531 (NHC=O), 1409, 1178, 695. ^{1}H-NMR ($CDCl_3$): 3.61 (s, 2 H, $PhCH_2$), 4.10, 4.20 (AB_q, J = 13.4

Hz, CH_2OH), 4.66 (d, J = 5.8 Hz, 2 H, CH_2CH=CH$_2$), 5.08 (s, 1 H, H-2), 5.23 (d, J = 3.9 Hz, H-6), 5.28-5.38 (m, 2 H, CH$_2$CH=CH_2), 5.59 (dd, J = 3.9, 8.5 Hz, H-7), 5.83-5.98 (m, 1 H, CH$_2$CH=CH$_2$), 6.27 (s, 1 H, H-4), 7.07 (d, J = 8.5 Hz, HN), 7.26-7.33 (m, 5 H, H-Ph); ^{13}C-NMR ($CDCl_3$): 42.8, 64.2, 66.7 (CH$_2$CH=CH$_2$, CH$_2$OH, PhCH$_2$), 49.7, 53.5, 60.3 (C-2, C-6, C-7), 117.37 (CH$_2$CH=CH$_2$)), 119.6 (CH$_2$CH=CH$_2$), 123.8 (C-3), 127.2, 128.7, 129.3, 130.8 (C-4, 5 x C-Ph), 134.1 (C-Ph), 164.6, 167.1, 171.8 (3 x CO).

Allyl 3-hydroxymethyl-7-(2-thienacetamido)-3-cephem-2-carboxylate 2.15b

Same procedure as before, **2.15b** was made (47.8%) as a yellow foam and had mp and spectroscopic properties identical to the reference reported (Jungheim L N et al 1993).

Allyl 3-hydroxymethyl-7-phenoxyacetamido-3-cephem-2-carboxylate 2.15c

Same procedure as before, **2.15c** was made (47.8%) as a yellow foam. mp 102~103 °C; IR(KBr) 3417, 3309 (NH, OH), 3081, 2968 (CH), 1784 (β-lactam C=O), 1751, 1353 (ROC=O), 1660, 1531 (NHC=O), 1409, 1178, 695 cm^{-1}. ^{1}H-NMR ($CDCl_3$): 4.22, 4.31 (AB$_q$, J = 13.2 Hz, CH$_2$OH), 4.59 (s, 2 H, PhOCH$_2$), 4.71 (m, 2 H, CH_2CH=CH$_2$), 5.18 (s, 1 H, H-2), 5.34 (d, J = 4.1 Hz, H-6), 5.33-5.43 (m, 2 H, CH$_2$CH=CH_2), 5.78 (dd, J = 4.0, 9.1 Hz, H-7), 5.87-6.00 (m, 1 H, CH$_2$CH=CH$_2$), 6.36 (s, 1 H, H-4), 6.94-7.10, 7.19-7.48 (2 x m, 5 H, H-Ph).^{13}C-NMR($CDCl_3$): 49.8, 53.2, 59.4 (C-2, C-6, C-7), 64.6, 66.7, 67.0 (CH$_2$CH=CH$_2$, CH$_2$OH, PhOCH$_2$), 114.7, 117.6, 124.0, 129.7, 130.8 (C-4, 5 x

CH-Ph, $CH_2CH=CH_2$), 119.7 ($CH_2CH=CH_2$), 122.2 (C-3), 156.8 (C-Ph), 164.1, 167.1, 168.6 (3 x CO).

6.2.10 Synthesis of 7-substituted 2-(allyloxy)carbonyl-3-cephem-3-methyl, 3-(2-chloroethyl) -4-oxoimidazo[5,1-d][1,2,3,5]tetrazin-8-carboxylate 2.16a-2.16c): General procedure

2.15 (0.257 mmol) and **2.5** (0.257 mmol.) dissolved in the mixture solution of DCM (8 ml) and *N,N*-diisopropylethylamine (0.514 mmol.) at room temperature. PyBOP (0.257 mmol.) was added in one portion at 0 °C. After stirring for a reaction period of 1~3 hours, the mixture was diluted with DCM, washed with brine and dried over anhydrous magnesium sulfate. Evaporation and flash chromatography (ethyl acetate: hexane 2:1) yielded the title compounds as yellowish powder. The followings are physicochemical data for **2.16a-2.16c**.

2-(Allyloxy)carbonyl-7-phenylacetamido-3-cephem-3-methyl, 3-(2-chloroethyl)-4-oxo-imidazo[5,1-*d*][1,2,3,5]tetrazin-8-carboxylate 2.16a

Prepared from **2.15a** and **2.5**, yielded **2.16a** of 41.4%, mp 85~88 °C; IR(KBr): 3450, 3411 (N-H), 3128, 3027, 2956 (C-H), 1774 (β-lactam C=O), 1741, 1240 (ROC=O), 1674, 1527 (-NHC=O), 1458, 1168, 734 (C-Cl) cm^{-1}; ^{1}H-NMR($CDCl_3$): 3.65 (s, 2 H, $PhCH_2$), 4.01 (t, J = 6.0 Hz, 2 H, CH_2CH_2Cl), 4.68 (d, J = 6.0 Hz, 2 H, $CH_2CH=CH_2$), 4.78 (t, J = 6.0 Hz, 2 H, CH_2CH_2Cl), 5.05 (AB_q, J = 12.6 Hz, 2 H, CH_2O), 5.23-5.52 (m, 4 H, H-2, H-6, $=CH_2$), 5.66 (dd, J = 4.0, 8.6 Hz, 1 H, H-7), 5.83-5.96 (m, 1 H, $CH_2CH=CH_2$), 6.53 (d, J = 8.6 Hz, 1 H, H-N), 6.61 (s, 1 H, H-4), 7.26-7.36 (m, 5 H, H-Ph), 8.48 (s, 1 H, H-6'); ^{13}C-NMR($CDCl_3$): 40.6, 43.1, 50.4 (CH_2CH_2Cl, CH_2Ph), 66.4, 66.9

($CH_2CH=CH_2$, CH_2O), 49.8, 53.4, 60.4 (C-2, C-6, C-7), 118.4 (C-3), 119.8 ($CH_2CH=CH_2$), 123.1, 127.5, 128.9, 129.2, 129.3, 130.7 (5 x CH-Ph, CH-6', CH-4, $CH_2CH=CH_2$), 129.2, 133.8, 135.5, 138.3 (C-Ph, C-8', C-8a', C=O), 159.6, 164.1, 166.6, 171.2 (4 x C=O); *m/z*: 513 , 481, 437, 371 (100%).

2-(Allyloxy)carbonyl-7-(2-thien-2-yl)acetamido-3-cephem-3-methyl, 3-(2-chloroethyl)-4-oxoimidazo[5,1-*d*][1,2,3,5]tetrazin-8-carboxylate 2.16b

Prepared from **2.15b** and **2.5,** yielded **2.16b** of (53%), mp 92 °C (dec.); IR(KBr): 3450, 3411 (N-H), 3116, 3039, 2960 (C-H), 1774 (β-lactam C=O), 1745, 1240 (ROC=O), 1682, 1527 (-NHC=O), 1458, 1169, 740 (C-Cl) cm^{-1}; ^{1}H-NMR($CDCl_3$): 3.88 (s, 2 H, CH_2CO), 4.02 (t, J = 6.0 Hz, 2 H, CH_2CH_2Cl), 4.69 (d, 2 H, J = 5.9 Hz, $CH_2CH=CH_2$), 4.80 (t, J = 6.0 Hz, 2 H, CH_2CH_2Cl), 5.07 (AB_q, J = 12.7 Hz, 2 H, CH_2O), 5.25-5.53 (m, 4 H, H-2, H-6, $=CH_2$), 5.69 (dd, J = 4.0, 8.8 Hz, 1 H, H-7), 5.83-5.96 (m, 1 H, $CH_2CH=CH_2$), 6.55 (d, J = 8.8 Hz, 1 H, H-N), 6.63 (s, 1 H, H-4), 7.00, 7.29 (2 x m, 3 H, H-thiophene), 8.50 (s, 1 H, H-6'); ^{13}C-NMR($CDCl_3$): 37.0, 40.6, 50.3 (CH_2CH_2Cl, CH_2CO), 66.4, 66.9 ($CH_2CH=CH_2$, CH_2O), 49.8, 53.4, 60.3 (C-2, C-6, C-7), 118.5 (C-3), 119.9 ($CH_2CH=CH_2$), 123.0, 126.1, 127.4, 127.8, 130.7 (3 x CH-thiophene, CH-6', CH-4, $CH_2CH=CH_2$), 129.1, 134.6, 135.5, 138.3 (C-thiophene, C-8', C-8a'), 159.6, 163.9, 166.6, 169.8 (4 x CO); *m/z*: 519, 487 (100%), 377.

2-(Allyloxy)carbonyl-7-phenoxyacetamido-3-cephem-3-methyl 3-(2-chloroethyl)-4-oxoimidazo[5,1-*d*][1,2,3,5]tetrazin-8-carboxylate 2.16c

Prepared from **2.15c** and **2.5, yielded 2.16c** of (36.5%), mp 85 °C (dec.); IR(KBr): 3450, 3411 (N-H), 3128, 3041, 2939 (C-H), 1774 (β-lactam C=O),

1747, 1238 (ROC=O), 1683, 1527 (-NHC=O), 1490, 1172, 760 (C-Cl) cm^{-1}; ^{1}H-NMR($CDCl_3$): 4.03 (t, *J* = 6.0 Hz, 2 H, CH_2CH_2Cl), 4.59 (s, 2 H, CH_2CO), 4.72 (d, *J* = 5.8 Hz, 2 H, $CH_2CH=CH_2$), 4.81 (t, *J* = 6.0 Hz, 2 H, CH_2CH_2Cl), 5.12 (AB_q, *J* = 12.6 Hz, 2 H, CH_2O), 5.30-5.41 (m, 4 H, H-2, H-6, $=CH_2$), 5.88 (dd, *J* = 4.1, 8.1 Hz, 1 H, H-7), 5.83-5.96 (m, 1 H, $CH_2CH=CH_2$), 6.68 (s, 1 H, H-4), 6.95-7.10, 7.32-7.39 (2 x m, 5 H, H-Ph), 8.52 (s, 1 H, H-6'); ^{13}C-NMR($CDCl_3$): 40.6, 50.3 (CH_2CH_2Cl, CH_2CO), 49.8, 53.1, 59.5 (C-2, C-6, C-7), 66.4, 66.8, 66.9 ($CH_2CH=CH_2$, CH_2O, CH_2CO), 118.5 (C-3), 119.7 ($CH_2CH=CH_2$), 114.6, 122.1, 122.8, 129.2, 129.6, 130.7 (5 x CH-Ph, CH-6', CH-4, $CH_2CH=CH_2$), 128.6, 135.5, 138.3, (CO, C-8', C-8a'), 156.8 (C-Ph), 159.6, 163.6, 166.7, 168.4 (4 x CO); *m/z*: 570, 529, 497 (100%), 387.

(6R,7R)-2-(Allyloxy)carbonyl-7-phenylacetamido-3-cephem-3-methyl 3-methyl-4-oxoimidazo[5,1-d] [1,2,3,5]tetrazin-8-carboxylate 2.16d.

From **2.15a** and **2.6** (77.1%) had mp 123-125 °C (dec.); IR(KBr): 3288, 3122 (N-H), 3083, 3031, 2956 (C-H), 1774 (β-1actam C=O), 1737, 1248 (ROC=O), 1654, 1537 (-NHC=O), 1457, 1326, 1172, 1052, 950 cm^{-1}; ^{1}H-NMR($CDCl_3$): 3.67 (s, 2 H, $PhCH_2$-), 4.08 (s, 3 H, CH_3), 4.68 (d, J = 5.8 Hz, 2 H, $CH_2CH=CH_2$), 5.05 (AB_q, J = 12.7 Hz, 2 H, CH_2O), 5.27-5.38 (m, 4 H, H-2, H-6, $=CH_2$), 5.68 (dd, J = 4.0, 8.7 Hz, 1 H, H-7), 5.83-5.99 (m, 1 H, $CH_2CH=CH_2$), 6.40 (d, J = 8.7 Hz, 1 It, H-N), 6.60 (s, H-4), 7.27-7.40 (m, 5 H, H-Ph), 8.46 (s, 1 H, H-6'); ^{13}C-NMR($CDCl_3$): 36.7 (CH_3), 43.2 (CH_2Ph), 49.8, 53.4, 60.4 (C-2, C-6, C-7), 66.4, 66.9 ($CH_2CH=CH_2$, CH_2O), 118.5 (C-3), 119.8 ($CH_2CH=CH_2$), 122.9, 127.6, 129.0, 129.3, 130.7 (5 x CH-Ph, CH-6', CH-4, $CH_2CH=CH_2$), 128.3, 128.6, 133.6, 138.3 (C-Ph, C-8', C-8a', C=O),

159.7, 164.0, 166.6, 171.0 (4 x C=O); *m/z:* 566 $[M+H]^+$, 481,437, 371 (100%).

(6R,7R)-2-(Allyloxy)carbonyl-7-(2-thien-2-yl)acetamido-3-cephem-3-methyl 3-methyl-4-oxoimidazo[5,1-d] [1,2,3,5]tetrazin-8-carboxylate 2.16e.

From **2.15b** and **2.6** (55%) had mp 94-95 °C (dec.); IR(KBr): 3288, 3122 (N-H), 3041, 2956 (C-H), 1780 (β-1actam C=O), 1747, 1242 (ROC=O), 1679, 1542 (NHC=O), 1457, 1327, 1164, 945 cm^{-1}; ^{1}H-NMR($CDCl_3$): 3.88 (s, 2 H, CH_2CO), 4.09 (s, 3 H, CH3), 4.69 (d, J = 6.0 Hz, 2 H, $CH_2CH=CH_2$), 5.07 (AB_q, J = 12.7 Hz, 2 H, CH_2O), 5.25-5.39 (m, 4 H, H-2, H-6, $=CH_2$), 5.70 (dd, J = 4.0, 8.7 Hz, 2 H, H-7), 5.86-5.97 (m, 1 H, $CH_2CH=CH_2$), 6.46 (d, J = 8.7 Hz, 2 H, H-N), 6.62 (s, 1 H, H-4), 7.01, 7.29 (2 x m, 3 H, H-thiophene), 8.48 (s, 1 H, H-6'); ^{13}C-NMR($CDCl_3$): 36.7 (CH_3), 37.0 (CH_2CO), 49.8, 53.4, 60.4 (C-2, C-6, C-7), 66.4, 66.9 ($CH_2CH=CH_2$, CH_2O), 118.6 (C-3), 119.9 ($CH_2CH=CH_2$,), 122.9, 125.9, 127.4, 127.7; 130.8 (3 x CH-thiophene, CH-6', CH-4, $CH_2CH=CH_2$,), 131.1, 134.7, 136.0, 138.4 (C-Ph, C-8', C-8a', C=O), 159.8, 164.0, 166.6, 170.0 (4 x C=O); m/z: 574 , 519, 487, 443, 377 (100%).

(6R,7R)-2-(Allyloxy)carbonyl-7-phenoxyacetamido-3-cephem-3-methyl 3-methyl-4-oxoimidazo[5,1-d][1,2,3,5]tetrazin-8-carboxylate 2.16f.

From **2.15c** and **2.6** (23.6%) had mp 75-76 °C (dec.); IR(KBr): 3288, 3122 (N-H), 3072, 3036, 2948 (C-H), 1776 (β-lactam C=O), 1741, 1246 (ROC=O), 1683, 1523 (NHC=O), 1490, 1462, 1172, 1049, 949 cm^{-1}; ^{1}H-NMR($CDCl_3$): 4.07 (s, 3 H, CH_3), 4.57 (s, 2 H, CH_2CO), 4.70 (d, J= 5.8 Hz, 2 H,

$CH_2CH=CH_2$), 5.08 (AB_q, J= 12.7 Hz, 2 H, CH_2O), 5.28-5.39 (m, 4 H, H-2, H-6, $=CH_2$), 5.78 (dd, J = 4.0, 9.0 Hz, 1 H, H-7), 5.87-6.00 (m, 1 H, $CH_2CH=CH_2$), 6.65 (s, 1 H, H-4), 6.90-7.07, 7.29-7.36 (2 x m, 5 H, H-Ph), 7.52 (d, J = 9.0 Hz, 1 H, H-N), 8.47 (s, 1 H, H-6'); ^{13}C-NMR($CDCl_3$): 36.7 (CH_3), 49.8, 53.2, 59.6 (C-2, C-6, C-7), 66.4, 66.9, 67.0 ($CH_2CH=CH_2$, CH_2O, CH_2CO), 118.6 (C-3), 119.9 ($CH_2CH=CH_2$,), 114.5, 114.7, 122.2, 122.8, 129.7, 130.7 (5 x CH-Ph, CH-6', CH-4, $CH_2CH=CH_2$,), 128.7, 134.5, 138.3 (C-8', C-8a', CO), 156.8 (C-Ph), 159.7, 163.8, 166.7, 168.4 (4 x CO); m/z: 582$[M+H]^+$, 497, 453, 387 (100%).

6.2.11 Synthesis of 7-Substituted 2-(allyloxy)carbonyl-5-sulfoxide-2-cephem-3-methyl, 4-oxoimidazo[5,1-d][1,2,3,5]tetrazin-8-carboxylate 2.17a-2.17c: General procedure

A solution of one of **2.16a, 2.16b or 2.16c** (0.5 mmol) in dry DCM (2 ml) was cooled to 5 °C in the ice-bath and treated dropwise with of the solution of *m*-CPBA (57~85%, 0.5 mmol) in DCM (1 ml). The reaction mixture was stirred overnight at room temperature and TLC (ethyl acetate) showed no starting material and one main product. The precipitate was collected by filtration to give the title compounds as yellowish solids. The following are physicochemical data for **2.17a**, **2.17b** and **2.17c**.

2-(Allyloxy)carbonyl-7-phenylacetamido-5-sulfoxide-2-cephem-3-methyl, 3-(2-chloro-ethyl)-4-oxoimidazo[5,1-*d*][1,2,3,5]tetrazin-8-carboxylate 2.17a.

From **2.16a,** yielded **2.17a** of 41.8%, mp 134~136 °C(dec.); IR (KBr): 3446, 3278 (N-H), 3109, 3025, 2960 (C-H), 1783 (β-lactam C=O), 1724, 1241 (ROC=O), 1651, 1525 (NHC=O), 1462, 1165, 1043 (S-O), 844, 734 (C-Cl) cm^{-1}; ^{1}H-NMR((DMSO-d_6): 3.54, 3.70 (q, *J* = 13.9 Hz, 2 H, $PhCH_2$-), 4.02 (t, *J* = 6.0 Hz, 2 H, -C*H*$_2$$CH_2Cl$), 3.70, 4.09 (q, *J* = 14.3 Hz, 2 H, H-4), 4.66 (t, *J* = 6.0 Hz, 2 H, -CH_2C*H*$_2$Cl), 4.80 (d, *J* = 6.0 Hz, 2 H, -C*H*$_2$$CH=CH_2$), 4.97 (d, *J* = 4.6 Hz, H-6), 4.99, 5.52 (q, *J* = 13.4 Hz, 2 H, -CH_2O), 5.24-5.45 (m, 2 H, -$CH_2CH=CH_2$), 5.88 (dd *J* = 4.6, 8.2 Hz, 1 H, H-7), 5.92-5.98 (m, 1 H, -$CH_2CH=CH_2$), 7.26-7.31 (m, 5 H, H-Ph), 8.49 (d, *J* = 8.6 Hz, 1 H, H-N), 8.94 (s, H-6'); ^{13}C-NMR(DMSO-d_6): 41.4, 45.6, 50.4 (C-4, -*C*H_2*C*H_2Cl, -CH_2Ph), 58.3, 66.5 (C-6, C-7), 64.0, 66.4 (-*C*$H_2CH=CH_2$, -CH_2O-), 118.7 (-$CH_2CH=CH_2$), 119.7, 125.0, 135.8, 136.5, 138.8 (C-2, C-3, C-Ph, C-8', C-9', CO), 126.6, 128.3, 129.1, 130.2, 131.7 (5 x CH-Ph, C-6', -$CH_2CH=CH_2$), 160.4, 163.2, 164.6, 171.1 (4 x CO); *m/z*: 538(10), 497 (100), 453 (20), 387 (80), 339 (50), 203 (70).

2-(Allyloxy) carbonyl-5-sulfoxide-7-(2-thien-2-yl)acetamido-2-cephem-3-methyl, 3-(2-chloroethyl)-4-oxoimidazo[5,1-*d*][1,2,3,5]tetrazin-8-carboxylate 2.17b.

From **2.16b**, yielded **2.17b** of 60%, mp 164~166 °C (dec.); IR (KBr): 3446, 3278 (N-H), 3102, 3028, 2958 (C-H), 1792 (β-lactam C=O), 1724, 1246 (ROC=O), 1652, 1531 (NHC=O), 1458, 1390, 1190, 1033 (S-O), 942, 740 (C-Cl) cm^{-1}; ^{1}H-NMR((DMSO-d_6): 3.70, 4.09 (q, *J* = 18.1 Hz, 2 H, H-4), 3.85 (q, *J* = 15.3 Hz, 2 H, -CH_2CO-), 4.02 (t, *J* = 5.9 Hz, 2 H, -C*H*$_2$$CH_2Cl$), 4.66 (t, *J* =

5.9 Hz, 2 H, -CH_2CH_2Cl), 4.78 (d, J = 5.1 Hz, 2 H, -$CH_2CH{=}CH_2$), 4.97 (d, J = 4.5 Hz, H-6), 4.99, 5.50 (q, J = 13.4 Hz, 2 H, -CH_2O), 5.23-5.45 (m, 2 H, -$CH_2CH{=}CH_2$), 5.88 (dd J = 4.5, 8.4 Hz, 1 H, H-7), 5.92-5.98 (m, 1 H, -$CH_2CH{=}CH_2$), 6.93, 7.35 (2 x m, 3 H, H-thiophene), 8.49 (d, J = 8.4 Hz, 1 H, H-N), 8.92 (s, H-6'); ^{13}C-NMR(DMSO-d_6): 35.9, 41.6, 45.8, 50.6 (C-4, -CH_2CH_2Cl, -CH_2-thiophene), 58.5, 66.7 (C-6, C-7), 64.0, 66.6 (-$CH_2CH{=}CH_2$, -CH_2O-), 118.9 (-$CH_2CH{=}CH_2$), 120.0, 125.1, 130.4, 136.6, 137.0, 139.0 (C-2, C-3, C-thiophene, C-8', C-9', CO), 125.4, 126.7, 127.0, 131.9 (3 x CH-thiophene, C-6', -$CH_2CH{=}CH_2$), 160.1, 160.6, 161.6, 164.7, 170.4 (4 x CO); *m/z*: 638 (M+1, 10), 503 (100), 475 (20), 393 (70), 345 (25).

2-(Allyloxy) carbonyl-7-phenoxyacetamido-5-sulfoxide-2-cephem-3-methyl, 3-(2-chloro-ethyl)-4-oxoimidazo[5,1-*d*][1,2,3,5]tetrazin-8-carboxylate 2.17c.

From **2.16c**, yielded **2.17c** of 41.8%, mp 121~124 °C (dec.); IR (KBr): 3446, 3278 (N-H), 3098, 3025, 2956, 2927 (C-H), 1791 (β-lactam C=O), 1747, 1246 (ROC=O), 1691, 1527 (NHC=O), 1496, 1164, 1054 (S-O), 754 (C-Cl) cm^{-1}; ^{1}H-NMR((DMSO-d_6): 3.77, 4.17 (q, J = 18.6 Hz, 2 H, H-4), 4.02 (t, J = 6.0 Hz, 2 H, -CH_2CH_2Cl), 4.65 (t, J = 6.0 Hz, 2 H, -CH_2CH_2Cl), 4.69 (s, 2 H, $PhOCH_2$-), 4.79 (d, J = 5.2 Hz, 2 H, -$CH_2CH{=}CH_2$), 5.00, 5.49 (q, J = 13.4 Hz, 2 H, -CH_2O-), 5.07 (d, J = 4.6 Hz, H-6), 5.24-5.45 (m, 2 H, -$CH_2CH{=}CH_2$), 5.87-5.95 (m, 1 H, -$CH_2CH{=}CH_2$), 6.10 (dd J = 4.6, 9.7 Hz, 1 H, H-7), 695, 7.30 (2 x m, 5 H, H-Ph), 8.20 (d, J = 9.7 Hz, 1 H, H-N), 8.94 (s, H-6'); ^{13}C-NMR(DMSO-d_6): 41.6, 45.5, 50.6 (C-4, -CH_2CH_2Cl), 58.0, 66.3 (C-6, C-7), 64.0, 66.6, 66.8 (-$CH_2CH{=}CH_2$, -CH_2O-, -CH_2OPh), 119.0 (-

$CH_2CH=CH_2$), 120.2, 125.0, 133.6, 136.8, 139.0 (C-2, C-3, C-8', C-9', CO), 128.1, 129.0, 129.8, 130.9, 131.9 (5 x CH-Ph, C-6', $-CH_2CH=CH_2$), 157.5 (C-Ph), 160.1, 165.0, 166.3, 168.4 (4 x CO); *m/z*: 578 (20), 513 (50), 403 (100), 359 (20), 219 (40).

(5S,6R,7R)-2-(Allyloxy)carbonyl-7-phenylacetamido-5-sulfoxide-2-cephem-3-methyl 3-methyl-4-oxoimidazo[5,1-d][1,2,3,5]tetrazin-8-carboxylate 2.17d

From **2.16d**, yielded **2.17d** (36.5%) had mp 146-149 °C (dec.); IR (KBr): 3280, 3104 (N-H), 3025, 2952 (C-H), 1789 (β-1actam C=O), 1722, 1243 (ROC=O), 1646, 1527 (NHC=O), 1462, 1396, 1165, 1045 (S-O), 944 cm^{-1}; ^{1}H-NMR(DMSO-d_6): 3.53, 3.69 (q, J = 13.9 Hz, 2 H, $PhCH_2$-), 3.69, 4.08 (q, J = 18.6 Hz, 2 H, H-4), 3.88 (s, 3 H, $-CH_3$), 4.79 (d, J = 5.4 Hz, 2 H, $-CH_2CH=CH_2$), 4.95 (d, J = 4.6 Hz, H-6), 4.98, 5.49 (q, J= 13.6 Hz, 2 H, $-CH_2O$), 5.23-5.47 (m, 2 H, $-CH_2CH=CH_2$), 5.88 (dd J = 4.6, 8.3 Hz, 1 H, H-7), 5.94-6.01 (m, 1 H, $-CH_2CH=CH_2$), 7.24-7.29 (m, 5 H, H-Ph), 8.48 (d, J = 8.3 Hz, 1 H, H-N), 8.98 (s, H-6'); ^{13}C-NMR(DMSO-d_6): 35.9 (CH_3), 40.9, 45.0 (C-4, $-CH_2Ph$), 57.8, 65.9 (C-6, C-7), 63.3, 65.8 ($-CH_2CH=CH_2$, $-CH_2O$-), 118.2 ($-CH_2CH=CH_2$), 119.2, 124.4, 135.3, 136.4, 138.3 (C-2, C-3, C-Ph, C-8', C-9', CO), 126.0, 127.8, 128.6, 131.2 (5 x CH-Ph, C-6', $-CH_2CH=CH_2$), 159.4, 159.9, 164.0, 170.5 (4 x CO); *m/z:* 497 (50), 453 (10), 387 (100).

(5S,6R,7R)-2-(Allyloxy)carbonyl-5-sulfoxide-7-(2-thien-2-yl)acetamido-2-cephem-3-methyl 3-methyi-4-oxoimidazo[5,1-d][1,2,3,5]tetrazin-8-carboxylate 2.17e.

From **2.16e**, yielded **2.17e** (54%) had mp 172-175 °C (dec.); IR (KBr): 3280, 3120 (N-H), 3035, 2954 (C-H), 1785 (β-1actam C=O), 1728, 1243 (ROC=O), 1648, 1529 (NHC=O), 1458, 1396, 1165, 1047(S-O), 950 cm^{-1}; ^{1}H-NMR(DMSO-d_6): 3.70, 4.09 (q, J = 18.8 Hz, 2 H, H-4), 3.79, 3.90 (q, J = 15.4 Hz, 2 H, -CH2CO-), 3.87 (s, 3 H, -CH3), 4.79 (d, d = 5.5 Hz, 2 H, -CH2CH=CH2), 4.97, 5.49 (q, J = 13.4 Hz, 2 H, -CH_2O), 4.98 (d, J = 4.6 Hz, H-6), 5.23-5.45 (m, 2 H, -$CH_2CH=CH_2$), 5.89 (dd J = 4.6, 8.4 Hz, 1 H, H-7), 5.94-6.00 (m, 1 H, -$CH_2CH=CH_2$), 6.94, 7.36 (2 x m, 3 H, H-thiophene), 8.49 (d, J = 8.4 Hz, 1 H, H-N), 8.86 (s, H-6'); ^{13}C-NMR(DMSO-d_6): 34.9, 45.8 (C-4, -CH_2-thiophene), 36.7 (CH_3), 58.5, 66.7 (C-6, C-7), 64.0, 66.6 (-$CH_2CH=CH_2$,-CH_2O-), 119.0 (-$CH_2CH=CH_2$), 120.1, 125.1, 126.1, 137.0, 137.2, 139.1 (C-2, C-3, C-thiophene, C-8', C-9', CO), 125.4, 126.8, 127.0, 129.7, 131.9 (3 x CH-thiophene, C-6', -$CH_2CH=CH_2$), 160.2, 160.6, 164.7, 170.4 (4 x CO); *m/z:* 503 (20), 475(10), 459(15), 393(100).

(5S,6R,7R)-2-(Ally1oxy)carbonyl-7-phenoxyacetamido-5-sulfoxide-2-cephem-3-methyl 3-methyl-4-oxoimidazo[5,l-d][l,2,3,5]tetrazin-8-carboxylate 2.17f

From **2.16f**, yielded **2.17f** (23.6%) had mp 143-145 °C (dec.); IR (KBr): 3280, 3104 (N-H), 3009, 2966 (C-H), 1792 (β-1actam C=O), 1717, 1245 (ROC=O), 1697, 1527 (NHC=O), 1561, 1299, 1181, 1047 (S-O), 944 cm^{-1}; ^{1}H-NMR((DMSO-d_6): 3.76, 4.17 (q, J = 18.6 Hz, 2 H, H-4), 3.88 (s, 3 H, -CH_3), 4.69 (s, $PhOCH_2$-), 4.79 (d, J = 5.2 Hz, 2 H, -$CH_2CH=CH_2$), 4.99, 5.49 (q, J = 13.4 Hz, 2 H, -CH_2O), 5.06 (d, J = 4.6 Hz, H-6), 5.24-5.39 (m, 2 H, -$CH_2CH=CH_2$), 5.91-6.02 (m, 1 H, -$CH_2CH=CH_2$), 6.10 (dd *J*= 4.6, 9.7 Hz, 1

H, H-7), 6.95, 7.30 (m, 5 H, H-Ph), 8.20 (d, J = 8.3 Hz, 1 H, H-N), 8.88 (s, H-6'); ^{13}C-NMR(DMSO-d6): 36.6 (CH_3), 45.4, 63.9, 66.6 (C-4, -CH_2Ph, $CH_2CH=CH_2$, -CH_2O-), 58.0, 66.3 (C-6, C-7), 119.0 (-$CH_2CH=CH_2$), 120.2, 124.9, 133.6, 137.1,139.1 (C-2, C-3, C-8', C-9', CO), 114.8, 128.1,129.0, 130.9, 131.9 (5 x CH-Ph, C-6', -$CH_2CH=CH_2$), 157.4 (C-Ph), 160.2, 165.0, 1663, 168.4 (4 x CO); m/z: 513(15), 403(100).

6.2.12 Synthesis of 7-Substituted 2-carboxyl-5-sulfoxide-2-cephem-3-methyl, 3-(2-chloroethyl)-4-oxoimidazo[5,1-d][1,2,3,5]tetrazin-8-carboxylate 2.2a, 2.2b and 2.2c: General method

A solution of one of **2.17a, 2.17b** or **2.17c** (0.174 mmol) in dry DMF (2 ml) was cooled to 5 °C in a ice-bath and stirred with triphenylphosphine (0.0435 mmol.) and tetrabis(triphenylphosphine)palladium (0.00174 mmol.) for 3 hours at 5~8 °C. TLC (ethyl acetate : acetic acid 2:1) showed no starting material and one main product. The yellow solution was concentrated under reduced pressure and the crude product was purified by dry-column flash chromatography to give the title compounds as a yellowish solid. The following are physicochemical data for **2.17a, 2.17b** and **2.17c.**

2-Carboxyl-7-phenylacetamido-5-sulfoxide-2-cephem-3-methyl, 3-(2-chloroethyl)-4-oxoimidazo[5,1-*d*][1,2,3,5]tetrazin-8-carboxylate 2.2a

From **2.17a**, yielded **2.2a** of 38.8%, mp 194 °C. IR (KBr): 3450, 3297 (N-H), 3128, 3027, 2960 (C-H), 1781 (β-lactam C=O), 1749, 1243 (ROC=O), 1724, 1648, 1529 (-NHC=O), 1458, 1164, 1047 (S-O), 734 (C-Cl) cm^{-1}; ^{1}H-NMR((DMSO-d_6): 3.53, 3.69 (AB_q, J = 13.9 Hz, 2 H, $PhCH_2$), 3.66, 4.03 (AB_q, J =

13.9 Hz, 2 H, H-4), 4.02 (t, J = 5.9 Hz, 2 H, CH_2CH_2Cl), 4.65 (t, J = 5.9 Hz, 2 H, CH_2CH_2Cl), 4.92 (d, J = 4.9 Hz, H-6), 4.96, 5.59 (AB_q, J = 13.2 Hz, 2 H, CH_2O), 5.81 (dd, J = 4.9, 8.2 Hz, 1 H, H-7), 7.21-7.30 (m, 5 H, H-Ph), 8.45 (d, J = 8.2 Hz, 1 H, H-N), 8.93 (s, 1 H, H-6'); ^{13}C-NMR(DMSO-d_6): 41.6, 45.6, 50.5, 64.4 (C-4, CH_2CH_2Cl, CH_2Ph, CH_2O), 58.4, 66.5 (C-6, C-7), 126.7, 128.5, 129.3 (5 x CH-Ph, C-6'), 126.8, 130.4, 136.0, 136.5, 139.0 (C-2, C-3, C-Ph, C-8', C-8a', C=O), 160.1, 162.3, 164.4, 171.2 (4 x C=O); *m/z* 355, 203 (100%)(Found: C, 40.2; N, 14.4; H 4.5; $C_{23}H_{20}ClN_7O_8S{\cdot}5H_2O$ requires C, 40.5; N, 14.4; H 4.4).

2-Carboxyl-5-sulfoxide-7-(2-thien-2-yl)acetamido-2-cephem-3-methyl, 3-(2-chloroethyl)-4-oxoimidazo[5,1-*d*][1,2,3,5]tetrazin-8-carboxylate 2.2b

From **2.17b**, yielded **2.2b** of 71.5%, mp >200 °C (dec.). IR (KBr): 3450, 3297 (N-H), 3128, 3027, 2964 (C-H), 1781 (β-lactam C=O), 1761, 1242 (ROC=O), 1654, 1610, 1565 (-NHC=O), 1454, 1172, 1035 (S-O), 786 (C-Cl) cm^{-1}; ^{1}H-NMR((DMSO-d_6): 3.48, 3.70 (AB_q, J = 18.1 Hz, 2 H, H-4), 3.78, 3.89 (AB_q, J = 15.1 Hz, 2 H, CH_2CO), 4.01 (t, J = 5.9 Hz, 2 H, CH_2CH_2Cl), 4.64 (t, J = 5.9 Hz, 2 H, CH_2CH_2Cl), 4.82 (d, J = 4.9 Hz, H-6), 4.97, 5.69 (AB_q, J = 13.1 Hz, 2 H, CH_2O), 5.65 (dd, J = 4.9, 8.6 Hz, 1 H, H-7), 6.94, 7.36 (2 x m, 3 H, H-thiophene), 8.31 (d, J = 8.6 Hz, 1 H, H-N), 8.90 (s, 1 H, H-6'); ^{13}C-NMR(DMSO-d_6): 0, 41.6, 45.4, 50.5 (C-4, CH_2CH_2Cl, CH_2Ph, CH_2O), 57.9, 66.2 (C-6, C-7), 125.3, 126.7, 128.8 (3 x CH-thiophene, C-6'), 126.9, 135.0, 136.4, 137.1, 139.0 (C-2, C-3, C-thiophene, C-8', C-8a', C=O), 160.5, 161.3, 163.3, 170.2 (4 x C=O); *m/z* 391, 295, 209 (100%) (Found: C, 41.4; N, 16.4; H 3.5; $C_{21}H_{18}ClN_7O_8S_2{\cdot}H_2O$ requires C, 41.1; N, 16.0; H 3.3).

2-Carboxyl-7-phenoxyacetamido-5-sulfoxide-2-cephem-3-methyl 3-(2-chloroethyl)-4-oxoimidazo[5,1-*d*][1,2,3,5]tetrazin-8-carboxylate 2.2c

From **2.17c**, yielded **2.2c** of (3.6%), mp 150~151 °C (dec.). IR (KBr): 3420, 3297 (N-H), 3128, 3082, 2962 (C-H), 1783 (β-lactam C=O), 1758, 1250 (ROC=O), 1724, 1698, 1526 (-NHC=O), 1492, 1159, 1033 (S-O), 754 (C-Cl) cm^{-1}; ^{1}H-NMR((DMSO-d_6): 3.54, 3.81 (AB_q, J = 17.7 Hz, 2 H, H-4), 4.02 (t, J = 5.6 Hz, 2 H, CH_2CH_2Cl), 4.65 (t, J = 5.6 Hz, 2 H, CH_2CH_2Cl), 4.67 (s, 2 H, $PhOCH_2$), 4.93 (d, J = 4.6 Hz, H-6), 5.00, 5.68 (AB_q, J = 13.0 Hz, 2 H, CH_2O), 5.85 (dd, J = 4.6, 9.7 Hz, 1 H, H-7), 6.96, 7.30 (2 x m, 5 H, H-Ph), 8.14 (d, J = 9.7 Hz, 1 H, H-N), 8.93 (s, 1 H, H-6'); ^{13}C-NMR(DMSO-d_6): 41.6, 45.1, 50.5, 65.8 (C-4, CH_2CH_2Cl, CH_2OPh, CH_2O), 57.5, 65.7 (C-6, C-7), 114.9, 121.8, 129.8 (5 x CH-Ph, C-6'), 127.3, 130.3, 136.4, 139.0 (C-2, C-3, C-8', C-8a', C=O), 157.4 (C-Ph), 160.4, 163.6, 168.3, 172.5 (4 x C=O); *m/z* 547, 363, 295, 263 (100%) (Found: C, 42.8; N, 15.2; H 3.8; $C_{23}H_{20}ClN_7O_9S{\cdot}2H_2O$ requires C, 43.0; N, 15.3; H 3.9).

2-Carboxyl-7-phenylacetamido-5-sulfoxide-2-cephem-3-methyl, 3-methyl-4-oxoimidazo[5,1-*d*] [1,2,3,5]tetrazin-8-carboxylate 2.2d

From **2.17d**, the target compound **2.2d** was prepared (40.9%), mp 182 ~ 183 °C (dec.), IR v (KBr)/cm^{-1}: 3280, 3128 (N-H), 3031, 2954, 2929 (C-H), 1783 (β-1actam C=O), 1749, 1245 (ROC=O), 1720, 1646, 1533 (-NHC=O), 1051 (S-O), 953, ^{1}H- NMR (DMSO-d_6) δ ppm: 3.53, 3.68 (ABq, J = 13.9 Hz, 2 H, $PhCH_2$), 3.64, 4.02 (ABq, J = 18.5 Hz, 2 H, H-4), 3.88 (s, 3 H, CH_3), 4.92 (d, J

= 4.7 Hz, H-6), 4.96, 5.57 (ABq, J = 13.2 Hz, 2 H, CH_2O), 5.81 (dd, J = 4.7, 8.2 Hz, 1 H, H-7), 7.21-7.30 (m, 5 H, H-Ph), 8.44 (d, J= 8.2 Hz, 1 H, H-N), 8.87 (s, 1 H, H-6'), ^{13}C- NMR (DMSO-d_6) δ ppm: 36.7 (CH_3), 41.6, 45.6, 64.3 (C-4, CH_2Ph, CH_2O), 58.4, 66.5 (C-6, C-7), 126.2, 129.7, 136.0, 137.2, 139.1 (C-2, C-3, C-Ph, C-8', C-8a', C=O), 126.8, 128.5, 129.3, 129.7 (5 x CH-Ph, C-6'), 160.2, 162.4, 164.4, 171.3 (4 x C=O), Molecular Formula: $C_{22}H_{19}N_7O_8S$ $4H_2O$, Element Analysis Found (Calcd.): C 42.7 (43.0) H 4.7 (4.4) N 15.7 (16.0)

2-Carboxyl-5- sulfoxide-7-[2-thien-2-yl)acetamido]-2- cephem-3-methyl 3-methyl-4-oxoimidazo[5,1-*d*] [1,2,3,5]tetrazin-8-carboxylate 2.2e

From **2.17e**, the target compound **2.2e** was prepared (32.2%), mp 179 ~ 181 °C (dec.), IR v (KBr)/cm^{-1}: 3280, 3128 (N-H), 3031, 2954, 2929 (C-H), 1781 (β-lactam C=O), 1760, 1243 (ROC=O), 1720, 1634, 1533 (-NHC=O), 1039 (S-O), 808, ^{1}H- NMR (DMSO-d_6) δ ppm: 3.68, 4.02 (ABq, J= 17.4 Hz, 2 H, H-4), 3.79, 3.89 (ABq, J= 14.8 Hz, 2 H, CH_2CO), 3.93 (s, 3 H, CH_3), 4.79 (d, J = 4.8 Hz, H-6), 4.95, 5.67 (ABq, J = 12.7 Hz, 2 H, CH_2O), 5.58 (dd, J = 4.8, 8.6 Hz, 1 H, H-7), 6.94, 7.36 (m, 5 H, H-thiophene), 8.28 (d, J = 8.6 Hz, 1 H, H-N), 8.84 (s, 1 H, H-6'), ^{13}C- NMR (DMSO-d_6) δ ppm: 35.8, 45.2, 66.0 (C-4, CH_2Ph, CH_2O), 36.4 (CH_3), 57.7, 66.2 (C-6, C-7), 125.1, 126.4, 126.7 (3 x CH-thiophene, C-6'), 129.4, 134.7, 136.9, 139.0 (C-2, C-3, C-thiophene, C-8', C-8a', C=O), 160.4, 163.1, 170.0, 175.8 (4 x C=O), Molecular Formula: $C_{20}H_{17}N_7O_8S_2$ $6H_2O$, Element Analysis Found (Calcd.): C 36.4 (36.6) H 4.5 (4.4) N 14.8 (14.9)

2-Carboxyl-7-phenoxyacetamide-5-sulfoxide-2-cephem-3-methyl 3-methyl-4-oxoimidazo[5,1-*d*] [1,2,3,5]tetrazin-8-carboxylate 2.2f

Form **2.17f**, the target compound **2.2f** was prepared (60.1%), mp 207 ~ 208 °C (dec.), IR v (KBr)/cm^{-1}: 3280, 3128 (N-H), 3031, 2954, Z, 2921 (C-H), 1782 (β-1actam C=O), 1751, 1245 (ROC=O), 1720, 1616, 1564 (-NHC=O), 1407, 1051 (S-O), 956, ^{1}H- NMR (DMSO-d_6) δ ppm: 3.53, 3.78 (ABq, J = 19.1 Hz, 2 H, H-4), 3.88 (s, 3 H, CH_3), 4.67 (s, 2 H, $PhOCH_3$), 4.91 (d, J = 4.7 Hz, H-6), 4.97, 5.65 (ABq, J= 12.4 Hz, 2 H, CH_2O), 5.84 (dd, J = 4.7, 9.7 Hz, 1 H, H-7), 7.21-7.30 (m, 5 H, H-Ph), 8.12 (d, J = 9.7 Hz, 1 H, H-N), 8.85 (s, 1 H, H-6'), ^{13}C- NMR (DMSO-d_6) δ ppm: 36.6 (CH_3), 45.0, 66.7 (C-4, CH_2OPh, CH_2O), 57.4, 65.7 (C-6, C-7), 114.9, 121.8, 128.9 (5 x CH-Ph, C-6'), 126.2, 129.7, 136.0, 138.1, 139.2 (C-2, C-3, C-8', C-8a', C=O), 157.4 (C-Ph), 160.2, 163.5, 168.3, 172.5 (4 x C=O), Molecular Formula: $C_{22}H_{19}N_7O_8S$ $3H_2O$, Element Analysis Found (Calcd.): C 42.9 (43.2) H 4.2 (4.1) N 15.7 (16.0)

6.2.13 6α-Bromopenicillanic Acid (6-BPA) 3.6

To 2.5N sulfuric acid (116 ml) cooled with stirring at 10 °C was added 6-amino penicillanic acid (6-APA) (10 g, 46 mmol), followed by potassium bromide (27.8 g, 233 mmol) then ethanol (92.6 ml, 95 %). The mixture was allowed to cool to 6 °C, then sodium nitrite (4.9 g, 71 mmol) in water (24 ml) was added dropwise within 20 minutes. The reaction solution was stirred for 3.5 hours at 6-8 °C. The reaction solution was extracted with chloroform (2 × 100, 4 × 50, 3 × 50 ml) and combined chloroform was washed with cold brine

(2 × 100 ml), then dried over $MgSO_4$ and concentrated under *vacuum* to give 6α-bromopenicillanic acid **3.6** (11.31 g, 87 %), a sticky white foam. TLC (MeOH:EtOAc 1:2): R_f=0.37; ^{1}H NMR [$CDCl_3$]: δ 1.59 (s, 3H C*H*$_3$); 1.70 (s, 3 H C*H*$_3$), 4.61 (s, 1H 3-*H*), 4.90 (d, 1H 5α-*H*), 5.43 (d, 1 H 6β-*H*), 9.85 (s, br.), 1 H COO*H*), ^{13}C NMR [$CDCl_3$]: δ 25.7, 33.3, 48.9, 64.8, 69.6, 70.2, 167.5, 172.1; MS m/z=280 [M^+].

Benzhydryl 6α-Bromopenicillanate-1-oxide 3.7

To a solution of **3.6** (18.38 g, 66 mmol) in chloroform (150 ml) and water (65 ml) at 0 °C was added peracetic acid (13.09g, 69 mmol) dropwise in 20 minutes. After stirred for further 30 minutes, at 0 °C benzophenone hydrazone (13.9 g, 70 mmol) and potassium iodide (4.32 ml, of 1% aqueous solution) was added followed first by peracetic acid (12.43 g, 65 mmol) dropwise in 40 minutes, then by 10 % sulfuric acid (5.2 ml). The reaction mixture was stirred at 0 °C for 1 hour then at room temperature for 1 hour. The chloroform layer was separated and the aqueous layer was back extracted with chloroform (2 × 50 ml). The combined organic layers was washed with cold water (200 ml), and stirred for 30 minutes with saturated sodium hydrogen carbonate (150 ml) at 10 °C. The chloroform layers was washed with brine, dried over $MgSO_4$ and concentrated under *vaccum* to give benzhydryl 6a-Bromopenicillanate-1-oxide **3.7** 28.17 g, (93 %), as a yellow foam; TLC (EtOAc:hexane1:2): R_f=0.4; mp 65-70 °C; IR (KBr disc): $^{\upsilon}$max 1793, 1752 cm^{-1}; ^{1}H NMR [$CDCl_3$]: δ 0.9 (s, 3H, C*H*$_3$); 1.68 (s, 3H, C*H*$_3$), 4.66 (s, 1H, 3-*H*), 5.12, 5.04 (AB-q, 2H, 5α-*H*

and 6β-*H*), 7.01 [s, 1H, $(C_6H_5)_2$ C*H*], 7.40 (m, 10H $_{arom}$), ^{13}C-NMR [$CDCl_3$]: δ 17.7, 19.7, 38.6, 65.2, 73.6, 76.4, 76.9, 77.4, 78.8, 79.1, 126.7, 127.6, 128.2, 128.5, 128.58, 128.6,138.8, 138.5, 165.8, 166.3, 193.6.

Benzhydryl 6,6-Dihydropenicillanate-1-oxide 3.8

To a stirred mixture of **3.7** (7.6 g, 16 mmol) in acetonitrile (124 ml) and glacial acetic acid (17.4 ml) at 0 °C was added zinc dust (5.4 g, 81 mmol) in 40 minutes, then the mixture was stirred at 0 °C for an additional 3 hours. After filtration through celite, filtrates was concentrated under reduced pressure. The residue was poured into ice water (50 ml) and extracted with chloroform (3 × 20 ml). The combined extracts was washed with water, then stirred for 30 minutes with saturated sodium hydrogen carbonate. Wash again with brine, dry over $MgSO_4$ and concentrate *in vaccum* to give 5.87 g of the product **3.8** as yellow foam. Purification by flash chromatography with mobile phase of ethyl acetate/hexane first 1:15 v/v then 2:1 v/v afford 1.8 g of a white solid product **3.8** (33 %); TLC (EtOAc:Hexane 2:1): R_f=0.36; mp. 148-151 °C; IR (KBr disc): $^{\upsilon}$max 3064, 2967, 1791, 1754 cm-^{1}; ^{1}H NMR [$CDCl_3$]: δ 0.90 (s, 3H, C*H*$_3$); 1.65 (s, 3H, C*H*$_3$), 3.29 (d, 2H, 6-*H*), 4.50 (s, 1H, 3-*H*), 4.88 (t, 1H, 5a-*H*), 7.01 [s, 1H, $(C_6H_5)_2$C*H*], 7.37 (m, 10H$_{arom}$); ^{13}C NMR [$CDCl_3$]: δ 18.0, 20.0, 30.8, 35.9 (CH_2), 65.3, 70.7, 73.8, 78.5, 126.6, 127.6, 128.1, 128.4, 128.5,128.6, 138.6, 139.0, 167.3, 170.5; MS m/z=383 [M]

Unsymmetrical Disulfides 3.9

To a solution of **3.8** (10.33 g, 27 mmol) and 2-mercaptobenzothiazole (4.51 g, 27 mmol) in toluene (250 ml) was stirred and heated under reflux, using a Dean-Stark trap, temperature remained at 135 –140 °C for 4 hours. Toluene was removed under reduced pressure. For the complete removal of toluene, the sticky light brown residue was dissolved in dichloromethane (20 ml) and precipitated by addition of a large volume of hexane under ice-bath and the hexane layers decanted off three times. The residue was dissolved in ether (250 ml) and cooled, the precipitated solid was filtered off, the filtrate was evaporated *in vaccum* to yield a yellow foam **3.9** 12.16g (85 %). TLC (EtOAc hexane 1:4); R_f=0.4; mp 74-81 °C; IR. (KBr disc): $^{\upsilon}$max 1768, 1739 cm-^{1}; ^{1}H NMR [$CDCl_3$]: δ 1.91 (s, 3H, C*H*$_3$); 3.44-3.12 (t, 2H,C*H*$_2$-CO), 4.99 (s, 1H, C*H*-COO), 5.10, 4.87 (2 br.2H, C=C*H*$_2$), 5.34 (dd, 1H, 4-*H*), 6.87 [s, 1H $(C_6H_5)_2CH$], 7.87-7.13 (m, 14H $_{arom}$), m/z=533 [M^+].

Benzhydryl 2β-Chloromethyl-2α-methyl-6 6-dihydropenicillanate 3.10

A mixture of **3.9** (4.22 g, 8 mmol) and copper (II) chloride (1.28 g, 10 mmol) in dichloromethane (64 ml) was stirred at room temperature for 4 hours. The mixture was filtered through celite and washed thoroughly with dichloromethane. The filtrate was washed with aqueous sodium hydrogen carbonate and brine (2 × 100 ml) and dried with $MgSO_4$. The light yellow dichloromethane solution was rapidly filtered through a small bed of silica and the solvent evaporated *in vaccum* to yield a light yellow foam **3.10** 2.65 g (83

%); TLC (EtOAc hexane 1:4): R_f=0.4; IR. (KBr disc): $^{\upsilon}$max 1764,1741 cm^{-1}; ^{1}H NMR [$CDCl_3$]: δ 1.36 (s, 3H, CH_3); 3.19-3.12 (dd, 1H, *H*-6), 3.64 (s, 2H, CH_2Cl), 3.68 (dd, 1H, *H*-6); 5.17 (s, 1H *H*-3); 5.45 (dd, 1H, *H*-5), 6.97 [s, 1H, $(C_6H_5)_2$ *CH*], 7.41 (m, $10H_{arom}$); ^{13}C NMR [$CDCl_3$]: δ 21.3, 30.8, 48.9(CH_2), 52.9(CH_2), 61.3, 64.6, 76.5, 78.3, 121.2, 122.6, 125.2, 126.5, 126.9, 127.3, 128.1, 128.31, 128.5, 128.6, 138.9, 166.3, 170.9.

Benzhydryl 2β-Azidomethyl-2α-methyl-6,6-dihydropenicillanate 3.11 and Benzhydryl 3β-Azido-3α-methyl-7,7-dihydrocephalosporanate 3.12

A solution of **3.10** (2.65 g, 6.6 mmol) in dimethylformamide (64 ml) was cooled with stirring to 5-10 °C and sodium azide (2.56 g, 39 mmol) was added, followed by water (21 ml). The mixture was stirred at room temperature for 3 hours. The solution was removed *in vaccum* and residue was diluted with ice-cold water (200 ml). The resultant mixture was extracted with ethyl acetate (3 × 50 ml). The combined extract was washed with water (8 × 50 ml). The extract was dried with $MgSO_4$ and the solvent evaporated *in vaccum* to yield a yellow foam **3.10** and **3.11** 2.034 g, (76 %); TLC (EtOAc hexane 1:4): R_f=0.6; IR (KBr disc): $^{\upsilon}$max 1769, 1741 cm^{-1}; ^{1}H NMR [$CDCl_3$]: δ 1.29 (s, 3H, CH_3); 3.15 (dd, 1H, *H*-6), 3.53 (s, 1H, CH_2N_3), 3.69-3.61 (dd, 1H, *H*-6), 4.91 (s, 1H, *H*-3), 5.44 (dd, 1H, *H*-5), 6.99 [s, 1H, $(C_6H_5)_2$ *CH*], 7.51 (m. $10H_{arom}$), 118 ^{13}C NMR [$CDCl_3$]: δ 15.96, 23.18, 39.28,(CH_2) 40.06

(CH_2), 52.94(CH_2), 54.37 (CH_2), 55.95, 60.02, 62.46, 64.25, 79.66, 121.24, 126.54, 128.78, 128.84.061

Benzhydryl 2β-Azidomethyl-2α-methyl-6, 6-dihydropenicillanate-1, 1-Dioxide 3.13 and Benzhydryl 3β-Azido-3α-methyl-7, 7-dihydrocephalosporanate-1,1-Dioxide 3.14

A mixture of **3.11**and **3.12** (4.3 g, 10 mmol) was dissolved in glacial acetic acid (220 ml). To this solution, water (29 ml) was added and potassium permanganate (4.0 g, 25 mmol) was added portion-wise for 40 minutes the mixture was stirred at room temperature for 3 hours. The excess of permanganate was decomposed by the dropwise addition of hydrogen peroxide and the mixture poured into ice-cold water (200 ml). The precipitated solid was filtered off and dissolved in dichloromethane (100 ml). The aqueous layer was saturated with sodium chloride and back extracted with dichloromethane (2 × 50 ml). The combined organic extracts were washed with saturated sodium hydrogen carbonate (2 × 50 ml), followed by brine (2 × 50 ml), and dried with $MgSO_4$. Concentration under *vacuum* gives the crude mixture 3.1 g as a light yellow foam, which was dissolved in ether (100 ml), refluxed 30 minutes and cooled to 5-10 °C. The precipitated white solid was pure **3.13**, and was filtered through celite, and concentration of the filtrate gives a sticky white foam which on crystallization from toluene/hexane affords pure **3.14**, as a white solid yield 1.095 g (35 %); TLC (EtOAc hexane 1:2): R_f=0.6; mp 67-78 °C; IR (KBr disc): $^{\upsilon}$max 2105,1798,1756 cm^{-1}; ^1H NMR

[$CDCl_3$]: δ 1.22 (s, 3H CH_3); 3.49-3.44 (dd, 1H H-6), 3.56-3.47 (dd, 1H H-6), 3.86 (AB-q, 2H CH_2N_3), 4.60 (s, 1H H-3), 4.64-4.61(dd, H-5), 6.94 [s, 1H $(C_6H_5)_2CH$], 7.35 (m, $10H_{arom}$), 131 ^{13}C NMR [$CDCl_3$]: δ 15.9, 39.3 (CH_2), 52.9 (CH_2), 60.0, 62.4, 65.0, 76.4, 77.4, 79.3, 125.2, 126.7, 127.5, 128.1, 128.4, 128.6, 128.8, 128.9, 138.1, 138.3, 165.5, 169.7.130 m/z=391.

Tazobactam 3.3

Benzhydryl 2β-azidomethyl-2α-methyl-6, 6 dihydropenicillanate-1,1-dioxide **3.14** (300 mg, 0.68 mmol) in vinyl acetate (15 ml) and DMF (15 ml) was stirred in a sealed reactor at 100 -105 °C for 36 hours. The reaction was rapidly filtered through a small bed of silica and washed with chloroform and product was concentrated under *vacuum* to give the desired compound as brown sticky benzhydryl 2β-[(1,2,3-triazol-1-yl)methyl]-2-α-methylpenam -3a-carboxylate 1,1-dioxide yield 310 mg 98 %. Without purification, it was used in the following reation. To a solution of benzhydryl 2β-[(1,2,3-triazol-1-yl)methyl]-2-α-methylpenam -3a-carboxylate 1,1-dioxide (253 mg, 0.5 mmol) in anisole (2 ml) at 0 °C, TFA (0.4 ml) was added dropwise for 10 minutes and the mixture was stirred at 0-(-5) °C for 1.5 hours and at room temperature for 0.5 hour. After addition of isopropyl ether (10 ml), the mixture was stirred at 0 °C for 10 minutes. The precipitated solid was collected by filtration and dried under *vacuum*. The desired product **3.3** was obtained as a brown solid 0.105 mg (64 %); ^1H NMR [D_2O]: δ 1.45 (s, 3*H*),

3.43-3.76 (ABX system 1*H*), 4.50 (s, 1*H*), 5.02-5.06 (m, 1*H*), 5.29 (Abq, 2*H*), 7.83 (s, 1*H*), 8.85 (s, 1*H*) same to the literature data.

6α-Bromopenicillanic Acid (6-BPA) 3.6

6-Aminopenicillanic acid 6-APA (10 g, 46 mmol) was added to 2.5 N sulfuric acid (116 ml) cooled at 10 °C with stirring, followed by potassium bromide (27.82 g, 230 mmol), then ethanol (92.6 ml, 95%). The mixture was kept between 6-9 °C with stirring. Sodium nitrite (4.9 g, 71 mmol) water (24 ml) solution was added dropwise within 20 minutes. Keep at this temperature and stir for further 3.5 hours. The mixture was extracted with chloroform (2 × 100, 4 × 50, 3 × 50 ml) and combined chloroform was washed with cold brine (2 × 100 ml), dried over $MgSO_4$ and concentrated *in vaccum* to give 6α-Bromopenicillanic Acid **3.6** 11.31 g (87 %), a sticky foam; TLC (MeOH:EtOAc 1:2): R_f=0.37; ^{1}H NMR [$CDCl_3$]: δ 1.59 (s, 3H C*H*$_3$); 1.70 (s, 3H C*H*$_3$), 4.61 (s, 1H 3-*H*), 4.90 (d, 1H 5α-*H*), 5.43 (d, 1H 6β-*H*), 9.85 (br, s, 1H COO*H*), 81 ^{13}C NMR [$CDCl_3$]: δ 25.7, 33.3, 48.9, 64.8, 69.6, 70.2, 167.5, 172, same to literature data.

6α-Bromopenicillanic acid 1,1-Dioxide 3.6a

To the methylene chloride solution (146 ml) of **3.6** (10.2 g, 36.45 mmol) placed in a 500 ml flask with a mechanical stirrer was added water (54.7 ml), then 3N sodium hydroxide (about 11 ml) dropwise over a period of 10-15 minutes until pH stable at 7.0. The aqueous layer was separated and the

organic layer was extracted with water (100 ml). The combined aqueous layers were placed in a 1000 ml three necked, round-bottomed flask with a mechanical stirrer and cooled at –5 °C. A premixed solution of potassium permanganate (9.5 g, 60.1 mmol), 85 % phosphoric acid (3.28 ml) and water (109.5 ml) was added over a period of 20 minutes until the oxidation was complete as indicated by the persistence of the dark purple permanganate colour. The pH of this reaction solution remained at 6.2. Ethyl acetate (91.2 ml) was added, and the pH of the reaction solution was adjusted to pH 1-2 with 6N hydrochloric acid (about 27.4 ml). To this bi-phase solution was added dropwise a 1M sodium bisulfite solution (46 ml) over a period of 15 minutes while the temperature was kept below 10 °C, and the pH was maintained at 1-2 by addition of 6N hydrochloric acid (11 ml). The aqueous layer was then saturated with sodium chloride, and the two phases were separated. The aqueous solution was extracted with ethyl acetate (2 × 100 ml), and the combined organic extracts were washed with brine (2 × 100 ml), dried over $MgSO_4$, concentrated under *vacuum* to give **3.6a** as a yellow foam yield 8.17 g (72 %); ^{1}H NMR [$CDCl_3$]: δ 1.53 (s, 3-H C*H*$_3$); 1.67(s, 3-H C*H*$_3$), 4.76 (s, 3*H*), 5.21 (s, 5α-*H*), 158 ^{13}C NMR [$CDCl_3$]: δ 14.0, 18.2, 19.9, 21.0. 39.7, 60.8, 63.0, 63.09, 68.6.173

Sulbactam 3.2

To a stirred and ice-cold solution of **3.6a** (2.37 g, 7.59 mmol) in acetonitrile (40 ml) and glacial acetic acid (5.54 ml) was added zinc dust (2.46 g, 37.63 mmol) over 5-10 minutes. The mixture was stirred in ice-bath for an additional

3 hours. Filtration through a celite layer to remove solid residue, the combined filtrates were concentrated under reduced pressure, then residue was poured into ice-cold water (100 ml). Adjusted pH to 1-2 with 6N hydrochloric acid (10 ml), then extracted with ethyl acetate (3 × 30 ml). The combined organic extracts were dried over $MgSO_4$ and concentrated *in vacuum* to give **3.2** as a light yellow solid, yield 0.545 g (31 %). IR (KBr disc): $^{\upsilon}$max 1776, 1760 cm^{-1}; ^{1}H NMR [DMSO-d_6]: δ 1.33 (s, 3H C*H*$_3$); 1.44 (s, 3H C*H*$_3$), 3.22 (dd, 6-*H*), 3.62 (dd, 6-*H*), 4.2 (s, 1H 3-*H*), 5.00 (t, 1H 5-*H*), 175 ^{13}C NMR [DMSO-d_6]: δ 17.69, 19.47, 37.17, 37.84, 38.18, 38.52, 38.85, 39.19, 39.52, 39.86, 60.49, 62.23, 62.53, 168.26, 172.45. 177 m/z=234 (M^+).

6.2.14 N-Hydroxysuccinimide 6α-Bromopenicillanate ester 3.18

A solution of **3.6** (1.35 g, 4.82 mmol) and N-hydroxysuccinimide (0.573 g, 4.98 mmol) in DMF (13.5 ml) was cooled in an ice-bath and treated dropwise with a solution of dicyclohexylcarbodiimide (1.08 g, 5.23 mmol) in DMF (1.5 ml) over 5 minutes. The ice was allowed to gradually melt and temperature arises to room temperature, then the mixture stirred for 21 hours. The mixture was cooled in an ice water bath for a while and solid dicyclohexylurea removed with filtration. Addition of (30 ml) 2-propanol to the filtrate precipitated the product as a white solid **3.18**, yield 1.1 g (60 %); ^{1}H NMR [$CDCl_3$]: δ 1.71 (s, 3H CH_3); 1.72 (s, 3H CH_3), 2.99 (t, 4-H), 4.87 (d, 2H 3-H 6β-H), 5.44 (s, 1H 5-H; ^{13}C NMR [$CDCl_3$]: δ 25.48(CH_2), 25.59, 33.29, 49.02, 65.17, 68.11, 70.31, 162.51, 167.10, 168.16.

6,6-Dihydropenicillanic Acid 3.20

To a solution of **3.6** (3.2 g, 11.44 mmol) in ethyl acetate (14 ml) cooled in an ice-bath, was added with stirring iced water (54 ml) followed by careful addition of 4N sodium hydroxide until the solid was completely dissolved at pH 4.0. Keep reaction temperature between 0-6 °C, add half a amount of zinc dust (5.3 g, 81.07 mmol) within 15 minutes small portion with stirring while maintained pH at 3.5 with addition of 4N hydrochloric acid. After stirring for15 minutes, the remaining zinc dust was added in one portion. Continue stirring in ice-bath for 2 hours, filter through celite, separate the organic phase and the aqueous phase, saturate the aqueous phase with sodium chloride and then extract with ethyl acetate (2 × 20 ml). The combined organic layers were washed with brine, dried over $MgSO_4$ and concentrated *in vaccum* to give the product **3.20** as a yellow foam yield 1.37 g (60 %); IR ($CDCl_3$): $^{\upsilon}$max 2973, 2930, 2358, 2338, 1735, 1646 cm^{-1}; ^1H NMR [$CDCl_3$] δ 1.59 (s, 3H C*H*$_3$); 1.73, (s, 3H C*H*$_3$) 3.62, 3.13(dd, 2H 6-*H*), 4.5 (s, 1H 3-*H*), 5.31(t, 1H, 5-*H*), 7.29 (m, 10H$_{arom}$); ^{13}C NMR ($CDCl_3$) δ 14.04, 20.96, 26.68, 30.83, 45.71 (CH_2), 60.23, 60.50 (CH_2),65.49, 70.08, 77.19; 194 m/z=200 (M^+).

6.2.15 Chloromethyl penicillanate 3.21A

A solution of **3.20** (0.2 g, 1mmol) in DMF (3 ml) with sodium hydrogen carbonate (0.086 g, 1mmol) and chloroiodomethane (0.5 ml) was stirred at 35-38 °C for 38 hours, then poured into ice water 10 ml, acidified to pH 4-5, and extracted with ether. After washed with saturated sodium hydrogen carbonate water solution and brine, the ether extract was dried over $MgSO_4$ and

concentrated *in vacuum* to give **3.21A** in a yellow foam, yield 0.09 g (36 %). ^{1}H NMR [$CDCl_3$]: δ 1.55 (s, 3H C*H*$_3$), 1.87 (s, 3H C*H*$_3$), 3.10 (dd, 1H 6-*H*), 3.62 (dd, 1H 6-*H*), 4.50 (s, 1H 3-*H*), 5.30 (dd, 1H 5-*H*), 5.77 (ABq, 2H OC*H*$_2$Cl); 191 ^{13}C NMR [$CDCl_3$] δ 14.09, 26.64, 30.89, 31.52, 45.96(CH_2), 46.25(CH_2), 60.30, 68.88(CH_2), 69.63, 166.31, 167.98, 172.42. 198.

Chloromethyl penicillanate 1,1-dioxide 3.21B

To a stirred solution of **3.21A** (0.343 g, 1.37 mmol) in isopropanol (5 ml) was added hydrogen peroxide (0.343 ml), followed by 0.5M aqueous sodium tungstate (VI) (0.09 ml). After a few minutes, the temperature of the reaction solution rose to about 60-80 °C, then slowly decreased. The solution was left at room temperature for 18 hours. Then the solution was poured into iced water, extracted with ethyl acetate (2 × 15 ml), washed with brine, dried over $MgSO_4$, and concentrated *in vacuum* to give **3.21B** as a yellow foam yield 0.15 g (39 %); ^{1}H NMR.[$CDCl_3$]: δ 1.48 (s, 3H, C*H*$_3$), 1.65 (s, 3H, C*H*$_3$), 3.50 (dd, 2H, 6-*H*), 4.45 (s, 1H, 3-*H*), 4.66 (d, 1H 5-*H*), 5.96 (ABq, 2H OC*H*$_2$Cl); ^{13}C NMR [$CDCl_3$] δ 18.20, 18.37, 20.00, 20.24, 38.18(CH_2), 53.03, 60.83, 60.96, 62.59, 63.09, 69.27, 165.36, 167.37, 170.66, 170.73.

Attempted synthesis of Iodomethyl penicillanate 1,1-dioxide 3.22

A solution of chloromethyl penicillanate 1,1-dioxide **3.21B** (0.169 g, 0.6 mmol) and potassium iodide (0.193 g, 0.9 mmol) in acetone (4 ml) was stirred

at room temperature for 18 hours. The resulting suspension was cooled to 0-5 °C and its apparent pH value was adjusted from 3.0 to 7.2 by addition of saturated aqueous sodium hydrogen carbonate with stirring. Titration with 0.5M aqueous sodium thiosulfate, water (1 ml) was added dropwise to the stirred mixture to precipitate colourless crystals. The crystals were filtered off, washed with acetone water 1:1 (10 ml), isopropanol (5 ml), and ether (5 ml), and dried to yield 89mg product, which ^{1}H NMR failed to match literature data of **3.22.**

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