STUDIES ON THE MODE OF ACTION AND β-LACTAMASE INHIBITORY PROPERTIES OF NOVEL OXAPENEM COMPOUNDS

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Four novel oxapenem compounds were evaluated for their β-lactamase inhibitory and antibacterial properties. Two (AM-112 and AM-113) displayed intrinsic antibacterial activity with MICs of between 2 to 16μg/ml and 0.5-2μg/ml against *Escherichia coli* and methicillin-sensitive and –resistant *Staphylococcus aureus*, respectively. The isomers of these compounds, AM-115 and AM-114 did not display significant antibacterial activity.

Combination of the oxapenems with ceftazidime afforded protection against β-lactamase-producing strains, including hyperproducers of class C enzymes and extended-spectrum β-lactamase enzymes. A fixed 4μg/ml concentration of AM-112 protected a panel of eight cephalosporins against hydrolysis by class A and class C β-lactamase producers. *In vivo* studies confirmed the protective effect of AM-112 for ceftazidime against β-lactamase producing *S. aureus*, *Enterobacter cloacae* and *E. coli* strains in a murine intraperitoneal infection model.

Each of the oxapenems inhibited class A, class C and class D β-lactamases isolated from whole cells and purified by isoelectric focusing. AM-114 and AM-115 were as effective as clavulanic acid against class A enzymes. AM-112 and AM-113 were less potent against these enzymes. Class C and class D enzymes proved very susceptible to inhibition by the oxapenems. Molecular modelling of the oxapenems in the active site of the class A TEM-1 and class C P99 enzymes identified a number of potential sites of interaction. The modelling suggested that Ser-130 in TEM-1 and Tyr-150 in P99 were likely candidates for cross-linking of the inhibitor, leading to inhibition of the enzyme.

Morphology studies indicated that sub-inhibitory concentrations of the oxapenems caused the formation of round-shaped cells in *E. coli* DC0, indicating inhibition of penicillin-binding protein 2 (PBP2). The PBP affinity profile of AM-112 was examined in isolated cell membranes of *E. coli* DC0, *S. aureus* NCTC 6571, *Enterococcus faecalis* SFZ and *E. faecalis* ATCC 29213, in competition with a radiolabelled penicillin. PBP2 was identified as the primary target for AM-112 in *E. coli* DC0. Studies on *S. aureus* NCTC 6571 failed to identify a binding target. AM-112 bound to all the PBPs of both *E. faecalis* strains, and a concentration of 10μg/ml inhibited all the PBPs except PBP3.

Keywords: Oxapenems; β-lactamase; β-lactamase inhibitors; penicillin-binding proteins; computer modelling; methicillin-resistant *Staphylococcus aureus*; extended-spectrum β-lactamase; *Escherichia coli*.

In memory of my mother

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Abbreviations

Å	angstrom
A_{264}	absorbance at 264nm
A_{296}	absorbance at 296nm
A_{470}	absorbance at 470nm
AIEF	analytical isoelectric focusing
AMPS	ammonium persulphate solution
APCI	atmospheric pressure chemical ionisation
ATCC	American Tissue Culture Collection
CFU	colony forming unit
cm	centimetre
°C	degree centigrade
ddH_20	double distilled water
DNase	deoxyribonuclease
ED_{50}	50% effective dose
ESBL	extended spectrum β-lactamase
ESMIS	electrospray mass ionisation spectroscopy
EtOH	ethanol
FIC	fractional inhibitory concentration
g	gram
g	gravity
h	hour
HPLC	high pressure liquid chromatography
IC_{50}	50% inhibitory concentration
IEF	isoelectric focusing
IRT	inhibitor-resistant β-lactamase
kD	kilodaltons
K_i	inhibition constant
K_M	Michaelis constant
l	litre
µg	microgram
µl	microlitre

μM	micromole
M	molar
mA	milliamp
mg	milligram
MHA	Mueller Hinton agar
MHB	Mueller Hinton broth
MIC	minimum inhibitory concentration
min	minute
ml	millilitre
mm	millimetre
mM	millimole
MSSA	methicillin-sensitive *Staphylococcus aureus*
MRSA	methicillin-resistant *Staphylococcus aureus*
Mwt	molecular weight
NA	nutrient agar
NB	nutrient broth
NCTC	National Collection of Type Cultures
nm	nanometre
OD	optical density
OD_{470}	optical density at 470nm
PAGE	polyacrylamide-gel electrophoresis
PBP	penicillin-binding protein
pI	isoelectric point
PIEF	preparative isoelectric focusing
RNase	ribonuclease
rpm	revolutions per minute
s	second
S	substrate
[S]	substrate concentration
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
spp	species
TEMED	tetramethyl ethylamine solution
TRIS	tris(hydroxymethyl) aminomethane

TSB	tryptone soya broth
UV	ultraviolet
V	volts
VRE	vancomycin-resistant enterococci
$^{v}/_{v}$	volume per volume
V_0	initial velocity
V_{max}	maximum velocity
W	Watt
$^{w}/_{v}$	weight per volume
$^{w}/_{w}$	weight per weight

Chapter 1. Introduction

1.1 Bacterial resistance

The discovery of penicillin by Alexander Fleming in 1928, and the subsequent development of antibiotics, heralded a new era of medical treatment which has lasted for over half a century, and the success of which is now in peril. The serendipitous discovery of penicillin, a naturally produced antibiotic from *Penicillium notatum* was followed in later decades by the use of sulphonamides and the isolation and identification of cephalosporin C and other naturally produced antibiotics. The optimisation of the chemical structures of these compounds, and the synthesis of novel compounds has provided mankind with a variety of valuable, potent drugs with which to treat infections. Antibiotic therapy has decreased morbidity and mortality due to serious infections such as bacteraemia, meningitis and tuberculosis. Worldwide antibiotic consumption is increasing, and in the United States, antibiotics are the second most commonly prescribed class of drugs. Antibiotic prescribing accounts for between 30 and 50% of a hospital's drug budget, and annual expenditure on antibiotics in the US approaches seven billion dollars (Monroe and Polk 2000). In England, antibiotic prescribing reached a peak in 1995, with 44.5 million prescriptions issued, and antibacterial usage was 5.7 DDD/inhabitant/year (DDD = defined daily doses). This is equivalent to every person in England being treated with a 5.7-day course of antibiotics. Total spend on antibiotics in this year was almost £180 million. By 1999 these figures had fallen to 34.2 million prescriptions, costing £170.5 million (Anonymous 2001).

The rapid pace of discovery and development of new antibiotics has slowed. There are few compounds with a novel structure or mode of action in development. Two recently introduced compounds are the oxazolidinone, linezolid (Zyvox), and the streptogramin combination, quinupristin/dalfopristin (Synercid). Many new antibiotics are derivatives of older compounds, for example the novel quinolones gatifloxacin, moxifloxacin and gemifloxacin and the novel glycopeptide, LY-333328. The cost of developing a new antibiotic is estimated as $500 million, and takes seven to ten years (Livermore and Dudley 2000). Many pharmaceutical

companies have decided to invest research and development funds in other, more profitable therapeutic areas.

The widespread use of antibiotics in human and animal medicine has led to the emergence of antibiotic resistance in microorganisms. Bacteria resistant to the action of penicillin were observed shortly after its introduction (Abraham and Chain 1940). β-Lactamase stable antibiotics such as methicillin were introduced in the 1960s, to be followed shortly by the emergence of staphylococci resistant to its action (Jevons 1961). The glycopeptide antibiotic vancomycin, long considered to be an agent of last resort to treat serious Gram-positive infections resistant to other compounds, is no longer effective against certain enterococcal and staphylococcal strains (Uttley, *et al.* 1988; Hiramatsu, *et al.* 1997). Resistance to the newly introduced oxazolidinone and streptogramin agents, albeit isolated, has been reported (Tsiodras, *et al.* 2001; Gonzales, *et al.* 2001).

Bacterial resistance to antibiotics can arise by a number of mechanisms, both intrinsic and acquired. The outer membrane of Gram-negative bacteria such as *P. aeruginosa* represents a permeability barrier to large compounds like vancomycin, so Gram-negative bacteria are intrinsically resistant to its action. Organisms lacking a cell wall are not susceptible to antimicrobial agents such as β-lactams that act on the cell wall to exert their lethal effect. Acquired resistance mechanisms include the transfer of genes encoding for antibiotic inactivating enzymes, altered targets and decreased uptake. β-Lactamase enzymes are the most problematic antibiotic-inactivating enzymes. They are widespread in both Gram-positive and Gram-negative bacterial species, and over three hundred different enzymes have been identified (Bush 2001). β-Lactamase enzymes destroy β-lactam antibiotics by cleaving the β-lactam ring and rendering the compound inactive. Two strategies have been used to avoid this destruction: the synthesis of β-lactams resistant to hydrolysis by the enzymes (for example methicillin, flucloxacillin, third- and fourth-generation cephalosporins, carbapenems), and the development of inhibitors of the enzymes. A number of β-lactamase stable compounds have been produced since the 1960s, and since the 1980s, three β-lactamase inhibitors (clavulanic acid, sulbactam and tazobactam) have been developed and introduced into clinical practice. Despite these

measures, bacteria have evolved β-lactamases which are capable of hydrolysing 'β-lactamase stable' compounds and β-lactamases which are not inhibited by clavulanic acid and the other inhibitors. The plasmid mediated transfer of cephalosporin-hydrolysing and extended spectrum β-lactamases to strains that naturally lack these enzymes is posing a grave threat to the treatment of infections.

Examples of bacterial altered targets include the low affinity penicillin-binding proteins (PBPs) of methicillin-resistant *Staphylococcus aureus* (MRSA) and *Streptococcus pneumoniae*. These PBPs are able to carry out the normal functions of other PBPs, which have higher affinity for β-lactam antibiotics and are inhibited by the compounds. Resistance to agents like rifampicin can arise due to alteration of the RNA polymerase target, leading to decreased affinity of the antibiotic for the polymerase. Similarly, alteration of DNA gyrase, the enzyme target of quinolones can lead to resistance of the bacterium to the antibiotic (Spratt 1994).

Decreased antibiotic uptake can arise from decreased permeability of the bacterium to the drug or an efflux mechanism to actively remove the drug from the cell before it can exert its effect. Decreased permeability can be due to a decrease in the outer membrane proteins which act as porins, or mutation of those porins to prevent passage of the drug. In bacteria, antibiotic efflux pumps are typically secondary active transporters where the efflux of the drug is coupled to the downhill transport of an ion along a concentration gradient. Efflux pumps in bacteria tend to be capable of recognising a number of drugs as substrates. The SMR (Small Multidrug Resistance) superfamily of drug efflux pumps is capable of removing erythromycin, tetracycline and sulphonamides from *E. coli* (van Bambeke, *et al.* 2000). Examples of antibiotics affected by efflux mechanisms include β–lactams, erythromycin, tetracycline, quinolones, chloramphenicol and aminoglycosides (Jacoby and Archer 1991; van Bambeke, *et al.* 2000).

The incidence of bacterial resistance appears to be increasing. While this may be due in part to the increased surveillance and reporting of resistant organisms, there are clear trends which indicate that the continued use of antibiotics at the current rate will lead to problems for antimicrobial chemotherapy in the future. The widespread

use of cephalosporins, to which enterococci are tolerant, has led to the selection of enterococci as important nosocomial pathogens. The use of vancomycin to treat these organisms has ultimately led to the development of resistance to this agent. In the USA in 1989, 0.3% of enterococcal isolates were vancomycin-resistant. By 1998, this figure had increased to 17.3% (Rapp 1999). Reports of bacteraemias to the Public Health Laboratory Service (PHLS) in the United Kingdom increased every year from 1990 to 1998. In 1990 there were 31,763 reports annually. By 1998, this had increased to 51,232. The incidence of methicillin-resistance in *Staphylococcus aureus* bacteraemias reported the PHLS increased from 1.7% in 1990 to 34% in 1998. Twenty-four percent of *Enterococcus faecium* isolates were resistant to vancomycin in 1998; in 1993 this figure was 6.3% (Reacher, *et al.* 2000). From January to December 2000, 36% of *S. aureus* bacteraemias reported to the PHLS were methicillin-resistant (CDSC 2001).

1.2 *Staphylococcus aureus*

Staphylococcus aureus is a member of the genus *Micrococcus*, and is characterised by the non-planar formation of grape-like clusters of cells, typically about 1μm in size. Growth of *S. aureus* on agar gives rise to gold coloured colonies, which gave the organism its name. *S. aureus* is a Gram-positive, aerobic coccus, usually found on the skin and mucous membranes (Wilkinson 1997). Humans are a natural reservoir for *S. aureus*, with 30 to 50% of healthy adults being colonised (Lowy 1998; Livermore 2000). *S. aureus* is a commensal bacterium, becoming an opportunistic pathogen when it gains access to tissues or the bloodstream. Infections of the skin and soft tissues are frequently due to *S. aureus*, and approximately 20% of bacteraemias are due to staphylococci. Prosthetic and native valve endocarditis are often associated with *S. aureus* (Livermore 2000).

A key factor in the pathogenicity of an organism is its ability to colonise its host, invade that host and evade the host defences. Production of toxins which damage the host is also an important aspect of virulence. *S. aureus* is found in the nose, axillae and perineum in humans, as well as on dry skin and environmental surfaces (Livermore 2000). Mucin is speculated to be an important factor for binding of

S. aureus to mucous membranes (Shuter, *et al.* 1996). Once the host defences have been breached, for example after a surgical procedure, then *S. aureus* can gain access to the underlying tissues and bloodstream. *S. aureus* produces a number of adhesins (also known as microbial surface components recognising adhesive matrix molecules, MSCRAMMs) which are able to recognise plasma or extracellular matrix proteins associated with tissues or indwelling devices such as catheters. These adhesins are produced during the exponential phase of growth (Projan and Novick 1997; Lowy 1998). Adhesins are expressed on the surface of the cell and include fibronectin-binding protein, fibrinogen-binding protein, elastin-binding protein and collagen-binding protein (Patti, *et al.* 1994; Cheung, *et al.* 1995; Projan and Novick 1997; Lowy 1998; Vadaux, *et al.* 1998). Indwelling devices such as catheters can become coated with serum proteins like fibronectin and fibrinogen, serving as a locus for colonisation and subsequent infection. Mechanical damage of vasculature due to indwelling devices or natural causes can expose the underlying fibrin and elastin components, leading to bacterial thrombus formation and infections such as endocarditis (Lowy 1998).

The production of toxins and degradative enzymes deleterious to the host facilitates the invasion of *S. aureus* and penetration of tissues. These include α-toxin (haemolysin), β- and δ-haemolysin, elastase and hyaluronidase. α-Toxin causes pore formation in and lysis of red blood cells and T lymphocytes (Jonas, *et al.* 1994). β-haemolysin attacks cell membranes containing sphingomyelin (Wadstrom and Mollby 1971). δ-Haemolysin enhances the effects of β-haemolysin, but its precise role is unclear. The degradative enzymes elastase and hyaluronidase are likely to cause tissue damage which may facilitate the spread of the organism through the host (Projan and Novick 1997; Lowy 1998).

Evasion of host defences is also a key factor in the pathogenicity and virulence of *S. aureus*. The organism produces protein A, which can bind the Fc portion of IgG antibodies, preventing opsonisation of the bacterium and phagocytosis. V8 protease is a serine protease that can cleave antibodies. Proteases may also protect the bacterium from antimicrobial peptides produced by the host; cause tissue damage facilitating spread and serve as a means of obtaining nutrients. Enterotoxins, toxic

shock syndrome toxins and exofoliative toxins can modulate the immune response by causing the proliferation of T-cells and the release of cytokines and lymphokines (Dancer, *et al.* 1990; Projan and Novick 1997; Lowy 1998).

Approximately 90% of *S. aureus* strains now produce a class A β-lactamase (Jones and Pfaller 1998; Livermore 2000). The reason for this widespread dissemination of an important resistance mechanism is likely to be the widespread and continuous use of β-lactam antibiotics, both in the community and hospital setting.

Along with producing an arsenal of toxins and destructive enzymes, staphylococci have acquired a number of resistance mechanisms to protect against antibacterial destruction. The most well known of these is the production of an altered penicillin binding protein, PBP 2a (also known as PBP2'), which has low affinity for β-lactams. However, staphylococci have developed resistance mechanisms to other antibacterial agents as well. Enzymatic inactivation of antimicrobials such as aminoglycosides and chloramphenicol can occur by acetyltransferases, adenyltransferases and phosphotransferases (Shaw, *et al.* 1985; Paulsen, *et al.* 1997). Impaired transport of aminoglycosides across the cell wall can occur due to chromosomal mutation, conferring resistance to all aminoglycosides (Smith and Jarvis 1999). The target of an antibacterial agent can be altered, either by synthesis of an insensitive enzyme, for example trimethoprim-insensitive dihydrofolate reductase (Young, *et al.* 1987), or modification of the target site. The mutation of *gyrA*, the gene encoding for DNA gyrase subunit A, causes ciprofloxacin resistance and is the most common mutation in *S. aureus* (Smith and Jarvis 1999). Toxic organic cations as well as antibiotics can be removed from the cell by efflux mechanisms (Tennent, *et al.* 1989). Binding of antibacterial agents such as bleomycin to cellular proteins can inhibit the action of the compound (Paulsen, *et al.* 1997).

Genes encoding for these various resistance mechanisms are encoded on plasmids, transposons and insertion sequences. One of the most important of these is IS*431*, which contains elements encoding for resistance to aminoglycosides, antiseptics and disinfectants, heavy metals, methicillin, mupirocin, tetracycline, trimethoprim and virginiamycin (Paulsen, *et al.* 1997; Kobayashi, *et al.* 2001). Resistance genes can

also be disseminated by plasmid transfer. One or more resistance determinants can be carried by plasmids, depending on their size.

1.3 Methicillin-resistant *Staphylococcus aureus*

Staphylococcal isolates exhibiting resistance to methicillin were described soon after the introduction of methicillin into clinical practice (Jevons 1961). Methicillin is a β-lactamase stable penicillin, which has a bulky side group in the 6-position, which sterically hinders hydrolysis of the β-lactam ring. Around 60% of *S. aureus* strains at this time were producing a β-lactamase enzyme (Livermore 2000), and it was hoped that β-lactamase stable compounds such as methicillin would be effective in treating staphylococcal infections. The incidence of MRSA declined by the late 1970s and early 1980s, but has increased steadily since and between 30 and 45% of bacteraemias reported to the PHLS are caused by MRSA (Livermore 2000). In the USA, MRSA is responsible for 25% of nosocomial infections (Shopsin and Kreiswirth 2001), while 50% of isolates from intensive care units are MRSA (Chambers 2001). The spread of MRSA strains to the community has been documented and predictions of the prevalence in community isolates in the US are for 25% of strains to be MRSA within five to ten years (Chambers 2001).

Methicillin-resistant *Staphylococcus aureus* (MRSA) strains are characterised by having the *mecA* gene, which is absent in methicillin-sensitive strains. *MecA* is a 2130bp stretch of DNA believed to be of non-staphylococcal origin (de Lencastre, *et al.* 1994; Smith and Jarvis 1999). There is a *mecA* homologue in *Staphylococcus sciuri* that is very similar to *S. aureus mecA*. This *mecA* homologue appears to be native to *S. sciuri* as it has been found in numerous natural isolates of *S. sciuri* (Wu, *et al.* 1998). It is currently believed that *S. aureus mecA* is 'foreign' DNA, possibly acquired by *S. aureus* from coagulase-negative staphylococcal species (Berger-Bachi 1997).

The precise mechanisms of the transfer of the *mec* gene are not fully understood. *Mec* is chromosomally encoded and horizontal transfer is rare, occurring either by

bacteriophage transduction or transposition (Kreiswirth, *et al.* 1993). *Mec* has been transferred to three different *S. aureus* chromosome types (A, B and C), which are believed to have led to five major lineages of MRSA (Hiramatsu 1995; Livermore 2000; Chambers 2001). *Mec* is polymorphic and three types have been identified: I, II and III, differing in their size, number of insertion sequences, resistance determinants and genetic organisation (Table 1.1) (Ito, *et al.* 2001). Recent research has identified that that *mecA* is carried by a novel genetic element, SCC*mec* (staphylococcal cassette chromosome *mec*). The genes responsible for the mobilisation and transfer have been identified as *ccrAB*, cassette chromosome recombinant genes A and B. These are similar to DNA recombinases of the invertase-resolvase family. The products of *ccrAB* catalyse the excision and integration of mec into the chromosome (Katayama, *et al.* 2000).

TABLE 1.1. Differences between three types of *mec*-associated DNA. From Chambers (2001).

	mec Types		
Genetic feature	I	II	III
Size	32kb	52kb	60kb
MecA	+	+	+
mecR1-mecI	-	+	+
CcrAB	+	+	+
pUB110	-	+	-
IS*431* (number)	1	2	4
Tn*554* (number)	0	1	2

ccrAB: cassette chromosome recombinases A and B;

pUB110: integrated plasmid that encodes tobramycin and kanamycin resistance;

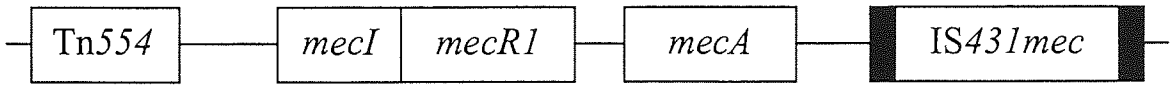
IS*431*: insertion sequence;

Tn*554*: erythromycin-resistance encoding transposons.

MecA encodes for a 78kDa penicillin-binding protein, PBP2a. This PBP2a has a reduced affinity for ß-lactam antibiotics and is a factor in methicillin resistance. Under normal growth conditions, PBP2a does not contribute to cell wall composition (Berger-Bachi 1997). PBP2a acts as a transpeptidase which takes over the function of the normal PBPs when they have been inactivated by high concentrations of ß-lactams (Reynolds and Brown 1985; de Jonge and Tomasz 1993). This PBP2a

consists of about 20% of total membrane protein, suggesting that it is catalytically inefficient (Reynolds and Brown 1985). The degree of cross-linking of cell wall muropeptides is typically around 60% under normal growth conditions - this falls to around 15% when PBP2a is involved in cell wall synthesis. This suggests that PBP2a is involved in synthesis of muropeptide dimers only (de Jonge and Tomasz 1993) and that generation of highly cross-linked oligomers by PBP2a is not possible (de Lencastre, *et al.* 1994).

FIGURE 1.1. Schematic representation of the *mec* region of MRSA illustrating *mecA* and *mec*-associated DNA. Adapted from Berger-Bachi (1998) and Chambers (1997).



The *mecA* gene is flanked by *mec*-associated DNA – the whole region is termed the mec regulon. *MecA* is regulated by *mecI*, which acts as a repressor and *mecR1* which acts as an inducer (Fig. 1.1). *MecI* and *mecR1* are located upstream of *mecA*, and separated from *mecA* by *mecA* promoter and operator sites (Chambers 1997; Grubb 1998). The *mecA* region varies between staphylococcal strains. Some strains lack the *mecI-mecR1* regulatory system and PBP2a is produced constitutively. If an inducible β-lactamase is present in a strain lacking *mecI-mecR1* then production of PBP2a is also inducible and regulation of *mecA* is carried out by the ß-lactamase regulon *blaI-blaR1* (Chambers 1997). *BlaI* and *blaR1* show 61% and 34% similarity to *mecI* and *mecR1*, respectively (Hackbarth and Chambers 1993; Berger-Bachi 1997). *BlaI-blaR1* repression of *mecA* is not as strong as that of *mecI-mecR1* (Grubb 1998). Mutation or deletion of *mecI* leads to high levels of PBP2a production (Berger-Bachi 1997).

Mec-associated DNA consists of approximately 20 to 45kb of DNA which does not code for *mecA*, *mecI* or *mecR1*. This additional DNA includes transposons, insertion sequences and plasmids. For example, Tn*554* is present which contains *ermA*, the gene encoding for inducible macrolide resistance. There is at least one copy of IS*431*, which encodes for a variety of resistance determinants. A plasmid encoding

for tobramycin resistance, pUB110, has integrated into IS*431* (Chambers 1997). Within *mec*-associated DNA, IS*431* acts as a target for homologous recombination of plasmids and transposons flanked by similar insertion sequences (Berger-Bachi 1997; Chambers 1997). IS*431* has also been found on staphylococcal plasmids as well as the chromosome, in association with genes encoding for resistance to aminoglycosides, antiseptics and disinfectants, heavy metals, tetracycline, trimethoprim and mupirocin (Paulsen, *et al.* 1997).

Methicillin-resistant *S. aureus* also produces a class A ß-lactamase, coded by the plasmid borne *blaZ* gene, and regulated by *blaI-blaR1*, located upstream of the *blaZ* gene (Hackbarth and Chambers 1993). Methicillin-resistant strains containing the *mecI-mecR1* regulon express low levels of resistance when not induced by methicillin. Mutation or deletion of *mecI* leads to high levels of resistance. However, the amount of PBP2a produced does not correlate with the degree of resistance (Murakami and Tomasz 1989; Ryffel, *et al.* 1994; Chambers 1997).

Other factors are required for methicillin resistance. These have been termed factors essential for methicillin resistance or *fem* factors. They form part of the *S. aureus* genome and are involved in peptidoglycan formation (Ryffel, *et al.* 1994). Six fem genes have been characterised, *femA* to *femF*. Mutation of *femA* or *femB* abolishes methicillin resistance completely and renders the organism more susceptible to a variety of antibiotics (Henze, *et al.* 1993; Ling and Berger-Bachi 1998). Inactivation of femB reduces the glycine content of peptidoglycan by approximately 40%, leading to reduced peptidoglycan cross-linking (Henze, *et al.* 1993). Mutation of *femC* or *femD* reduces the basal level of methicillin resistance. *FemA* and *femB* are involved in the addition of glycine residues to the pentaglycine cross-link between muramyl-pentapeptide monomers. *FemA* mutants are blocked after addition of the first glycine and *femB* mutants after addition of the third glycine (de Jonge and Tomasz 1993; Grubb 1998). *FemAB* mutants have abnormal septum formation, thickened cross walls and retarded cell separation (Berger-Bachi 1997; Berger-Bachi and Tschierske 1998). Peptidoglycan cross-bridges consist of a single glycine residue, instead of the normal pentaglycine linkage (Stranden, *et al.* 1997). *FemC* mutation affects the glutamate synthetase repressor, leading to a shortage of available glutamine in the bacteria. This results in reduced amidation of the D-Glu residue in

the pentapeptide side chain and reduction of peptidoglycan cross-linking (Berger-Bachi and Tschierske 1998). *FemD* encodes for a phosphoglucosamine mutase which catalyses the formation of glucosamine-1-phosphate from glucosamine-6-phosphate; this is the first step leading to the formation of the peptidoglycan precursor UDP-*N*-acetylglucosamine (Jolly, *et al.* 1997). *FemD* mutants have a reduced peptidoglycan precursor formation and *femF* mutation affects addition of lysine to the muramyl-pentapeptide (Ornelas-Soares, *et al.* 1994). The effect of *femE* mutation has not been defined (Berger-Bachi and Tschierske 1998; Grubb 1998).

In a given population of an MRSA strain, not all cells will be resistant to methicillin. Growth of the cells in the presence of increasing concentrations of methicillin will reveal a subset of clones that are able to grow at high methicillin concentrations, while the other cells are killed. Such analysis is termed population analysis, and it has revealed the heterogeneous nature of methicillin resistance. The small subset of clones that can grow at any concentration of methicillin are termed homogeneously resistant cells. This variation in resistance can be influenced by the growth conditions, and repeated subculture of the homogeneously resistant cells in drug-free medium results in a reversion to the heterogeneously resistant state. The reason for this variation in resistance has not been elucidated (Chambers 1997; Ehlert 1999).

The ability of MRSA to acquire resistance to antibacterial agents by genetic transfer, coupled with ß-lactamase production and reduced susceptibility to ß-lactams due to PBP2a production has produced, in less than 50 years, an organism that is resistant to virtually all commonly used agents except vancomycin and teicoplanin. The introduction of quinupristin/dalfopristin and linezolid, which have good MRSA activity, has broadened the treatment options for MRSA infections. However, isolated cases of resistance to these novel agents have been reported (see Section 1.7 below). Thus the glycopeptides still play a vital role in the treatment of multidrug-resistant MRSA infection.

1.4 Glycopeptide-intermediate *Staphylococcus aureus*

The first report of a MRSA strain with reduced susceptibility to vancomycin was in Japan in 1997. The strain isolated (Mu50) had a vancomycin MIC of 8μg/ml (Hiramatsu, *et al.* 1997). Since then, strains have been isolated in the USA and Europe (Tenover, *et al.* 2001). The Japanese strain did not carry *vanA* or *vanB* genes, so the transfer of vancomycin-resistance genes from enterococci did not occur in this strain. Such a transfer has been demonstrated experimentally (Noble, *et al.* 1992), but has yet to be identified in the clinical setting. Examination of laboratory-derived glycopeptide resistant *S. aureus* isolates showed that decreased glycopeptide sensitivity was related to increased production of PBP2 (Moreira, *et al.* 1997). Other laboratory-derived glycopeptide-resistant MRSA strains were found to have decreased resistance to methicillin and decreased peptidoglycan cross-linking that was coupled with increased vancomycin resistance (Sieradzki and Tomasz 1999). With the increasing numbers of glycopeptide-insensitive isolates being identified, some researchers have proposed a classification system for glycopeptide-intermediate *S. aureus* (GISA). Class A isolates are intermediate for both vancomycin (MIC 8 to 16μg/ml) and teicoplanin (MIC 16μg/ml). Class B isolates are intermediate for vancomycin, but susceptible to teicoplanin (MIC ≤8μg/ml). Class C isolates are susceptible to vancomycin (MIC ≤4μg/ml) but intermediate for teicoplanin (Boyle-Vara, *et al.* 2001). The workers observed a decrease in the glycopeptide MICs after 15 days passage of GISA isolates on antibiotic-free media, indicating a reversion of the GISA phenotype. In contrast to Sieradzki and Tomasz (Sieradzki and Tomasz 1999), the GISA phenotype was not associated with a decrease in methicillin resistance. Examination of the Mu50 GISA strain has revealed that this strain has a two-fold thicker cell wall and an increased amount of nonamidated muropeptides in the cell wall, compared to another GISA strain, Mu3. Mu50 is estimated to be able to bind 2.8 times more vancomycin molecules than Mu3, due to its thicker cell wall and decreased cross-linking (Hanaki, *et al.* 1998b). Both these strains displayed accelerated cell wall synthesis and turnover, compared to vancomycin-susceptible strains (Hanaki, *et al.* 1998a). The thickness of the cell wall (30 to 40 layers of peptidoglycan in Mu50, compared to about 20 in methicillin-sensitive *S. aureus*) contributed to vancomycin resistance and

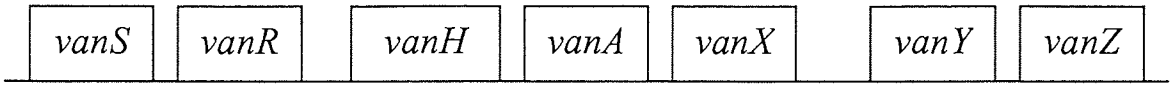
the increased production of nonamidated muropeptides enhanced vancomycin resistance. These factors are likely to be due to the accelerated cell wall synthesis system of Mu50 (Cui, *et al.* 2000).

1.5 Vancomycin-resistant enterococci

Vancomycin-resistant enterococci were first isolated in the UK in 1988 (Uttley, *et al.* 1988). There are two predominant species, *E. faecalis* and *E. faecium*. Enterococci are characterised by being resistant to a number of antibiotics, by a combination of acquired and intrinsic resistance mechanisms.

Enterococci synthesise peptidoglycan in a similar fashion to other Gram-positive organisms. The pentapeptide side chain of the nascent peptidoglycan terminates in a D-Ala-D-Ala residue. Addition of this dipeptide unit to the side chain is carried out by D-Ala-D-Ala ligase (encoded by *ddl*). The terminal D-Ala residue is cleaved during cross-linking when adjoining peptidoglycan chains are joined together. Vancomycin binds to the terminal D-Ala-D-Ala residues, preventing cleavage of the terminal D-Ala, and preventing cross-linking of the peptidoglycan chains. In vancomycin-resistant enterococci, the terminal D-Ala residue is replaced with an alternative amino acid. Vancomycin has a reduced affinity for this terminal sequence, and binds to the terminal residues with much lower affinity, resulting in resistance of the organism to vancomycin.

FIGURE 1.2. Schematic representation of the genetic organisation of the vanA operon in vancomycin-resistant enterococci. Adapted from Rice (2001).



The *vanA* gene encodes for a protein, vanA, which is a ligase of altered substrate specificity which produces a D-Ala-D-Lac dipeptide residue for addition to the peptide side chain of peptidoglycan (Bugg, *et al.* 1991; Cetinkaya, *et al.* 2000). VanH and VanX are two gene products encoded by genes located on a transposon

which carries the *vanA* gene (Fig. 1.2). VanH is a D-hydroxy acid dehydrogenase which creates a pool of D-lactate for the D-Ala-D-Lac ligase. VanX is a D,D-dipeptidase which lacks activity for D-Ala-D-Lac and reduces the availability of D-Ala-D-Ala produced by D-Ala-D-Ala ligase (Wu, *et al.* 1995). These proteins work in combination to ensure that the terminal dipeptide residue of the peptidoglycan pentapeptide side chain is D-Ala-D-Lac, thereby ensuring vancomycin-resistance. The genes encoding for these proteins are located on the *vanHAX* gene cluster, often carried on a plasmid (Arthur, *et al.* 1993), and regulated by VanR and VanS. VanS is a histidine protein kinase that is activated in the presence of vancomycin, or by the action of vancomycin on the cell wall. VanS signals VanR, the response regulator, which activates the genes *vanA, vanH* and *vanX* (Arthur, *et al.* 1997). *VanY* and *vanZ* genes have also been identified, which may contribute to vancomycin resistance.

VanB encodes for another altered-substrate ligase, which bears a 76% homology with the ligase encoded by *vanA*. As with the *vanA* protein, vanB produces a dipeptide unit for the pentapeptide side chain that consists of D-Ala-D-Lac. The operation and expression of *vanB* is augmented by genes similar to those for *vanA*, named $vanH_B$, $vanX_B$, $vanR_B$ and $vanS_B$. There are a number of different *vanB* genes, termed *vanB-1*, *vanB-2* and *vanB-3* (Dahl, *et al.* 1999). There are a similar number of *vanC* genes, among different enterococcal species, which encode for the *vanC* resistance phenotype (Clark, *et al.* 1998; Patel, *et al.* 1998). The vanC ligase catalyses the formation of a D-Ala-D-Ser dipeptide unit, which is then attached to the peptide side chain of peptidoglycan. *VanC* VRE strains exhibit a low level of resistance to vancomycin, and resistance levels vary between strains. This is believed to be due to the simultaneous production of normal D-Ala-D-Ala dipeptide units by D-Ala-D-Ala ligase, which renders the peptidoglycan cross-linking susceptible to inhibition by vancomycin. *VanD* and *vanE* genes have also been identified (Fines, *et al.* 1999; Perichon, *et al.* 2000). VanD shares 69% homology with vanA and vanB, while vanE is more closely related to vanC than other ligases (Casadewall and Courvalin 1999; Fines, *et al.* 1999).

VanA appears to be the most widely distributed phenotype amongst VRE (Clark, *et al.* 1993). The *vanA* gene is plasmid borne, and can be readily disseminated (Arthur, *et al.* 1993). The *vanB* gene is both chromosomally and plasmid located, and is not as

readily transferable. *VanD* is chromosomally located and is not transferred (Cetinkaya, *et al.* 2000).

Enterococci are relatively insensitive to β-lactam antibiotics (Krogstad and Parquette 1980; Kaye 1982). *E. faecalis* is sensitive to concentrations of ampicillin between 1 and 8μg/ml, while *E. faecium* requires concentrations of 16 to 64μg/ml to inhibit growth (Cetinkaya, *et al.* 2000). Enterococci do not produce large amounts of β-lactamase enzymes, and production is not inducible (Cetinkaya, *et al.* 2000). The insensitivity of enterococci to β-lactams is due to the production of low affinity penicillin-binding proteins (PBPs). As early as 1980, enterococcal PBPs with low affinity for penicillins and cephalosporins were identified (Georgoupapadakou and Liu 1980). The low affinity PBP was identified as being PBP5 in a *Streptococcus faecium* strain, and a similar low affinity PBP was found in *S. durans* and *S. faecalis*. Strains carrying this low affinity PBP were several hundred times less sensitive to penicillin than strains lacking the PBP (Fontana, *et al.* 1983). Later studies showed that the abundance of this low affinity PBP was related to the resistance of the strain and that loss of PBP5 from highly resistant strains resulted in hypersusceptibility to β-lactam antibiotics (Williamson, *et al.* 1985; Fontana, *et al.* 1985). In a similar manner to PBP2a in MRSA strains, PBP5 can take over the functions of the essential high-molecular-weight PBPs, which are normally essential for growth, when they have become inhibited by β-lactam antibiotics (Canepari, *et al.* 1985).

β-Lactam antibiotics are often used with aminoglycosides to treat enterococcal infections, as these two agents act synergistically. However, enterococci can become resistant to the action of aminoglycosides, either by alteration of the target site, interference with antibiotic transport or enzymatic alteration of the antibiotic (Leclercq, *et al.* 1992). Low uptake due to impaired active transport and alterations of ribosomes leading to reduced binding are due to chromosomal mutations, while production of aminoglycoside-modifying enzymes is mediated by plasmids. Reduced uptake of aminoglycosides leads to low-level resistance, while ribosomal mutation and production of modifying enzymes results in high-level resistance (Cetinkaya, *et al.* 2000).

Enterococcal infections are difficult to treat because of the intrinsic and acquired resistance mechanisms of enterococcal species. As with MRSA strains, the levels of resistance in these organisms are increasing, the strains are becoming resistant to a wider range of antibiotics, and the incidence of infections due to these organisms is increasing. Clearly, the widespread use of antibiotics is selecting organisms which are becoming increasingly difficult to treat. Novel agents such as oxazolidinones, streptogramins and experimental glycopeptides such as LY-333328 offer some hope for treatment options, but for how long their efficacy will be maintained remains to be seen.

1.6 β-lactamase enzymes

1.6.1 *Classification of β-lactamase enzymes*

β-lactamase enzymes are a diverse group of enzymes, produced by a wide variety of bacteria, differing in their catalytic properties, amino acid sequence, structure, and inhibitor profiles. To date, around 350 different enzymes have been described (Bush 2001). As such, several classification schemes have been proposed in an attempt to assemble the enzymes in an orderly fashion. Richmond and Sykes proposed a scheme in 1973 which classified the known enzymes from Gram-negative bacteria into five groups on the basis of their substrate profile (Richmond and Sykes 1973). This scheme was extended by Sykes and Matthew, incorporating isoelectric focusing data into the scheme (Sykes and Matthew 1976). The Ambler scheme was introduced in 1980 which divided β-lactamase enzymes into two groups, A and B, based on their amino acid sequence (Ambler 1980). Class A enzymes had an active site serine, while class B enzymes were metalloenzymes. Class C and class D enzymes were added to this scheme as they were identified (Jaurin and Grundstrom 1981; Ouellette, *et al.* 1987). This classification scheme is widely used as it gives a broad indication of the substrate profile of an enzyme (e.g. penicillinase, cephalosporinases), but it does not provide any further details of substrate or inhibitor profile. The most comprehensive attempt at organising the β-lactamase enzymes is the Bush-Jacoby-Medeiros scheme which attempts to correlate the substrate and

inhibitory profiles of enzymes from all bacterial sources (Bush 1989; Bush, *et al.* 1995). The Bush scheme divides enzymes into four main groups, with some subdivision of heterogeneous groups:

Group 1 enzymes are cephalosporinases, poorly inhibited by clavulanic acid. They correspond to molecular class C.

Group 2 enzymes are ß-lactamases inhibited by site-directed inhibitors (molecular classes A or D). Group 2 has been further divided into subgroups. 2a enzymes are penicillinases inhibited by clavulanic acid, and include enzymes from Gram-positive bacteria. Subgroup 2b enzymes hydrolyse both penicillins and cephalosporins and are inhibited by clavulanic acid. Subgroup 2be contains ß-lactamases capable of hydrolysing penicillins, narrow and extended spectrum cephalosporins and monobactams. 2br enzymes have a reduced affinity for ß-lactamase inhibitors. Group 2f enzymes are capable of hydrolysing carbapenems and are poorly inhibited by clavulanic acid. They contain an active site serine.

Group 3 enzymes are the metallo-ß-lactamases (molecular class B), not inhibited by clavulanic acid.

Group 4 are penicillinases not inhibited by clavulanic acid.

Table 1.2 summarises the classification schemes for bacterial β-lactamases. The Bush scheme has the advantage of providing information on the substrate and inhibitor profile of an enzyme, by virtue of the group classification. However, the classification lacks the simplicity of the Ambler scheme, and mutation of an enzyme can alter its substrate or inhibitor profile, necessitating a change to its classification, whereas the Ambler scheme classification is stable to such changes. The Ambler scheme will be used throughout this work.

TABLE 1.2 Summary of the classification schemes for bacterial β-lactamases. Adapted from Bush *et al.* (1995).

Bush-Jacoby-Medeiros group	Richmond-Sykes class	Ambler molecular class	Preferred substrates	Inhibited by clavulanic acid
1	Ia, Ib, Id	C	Cephalosporins	-
2a	Not included	A	Penicillins	+
2b	III	A	Penicillins, cephalosporins	+
2be	Not included	A	Penicillins, cephalosporins (narrow and extended spectrum), monobactams	+
2br	Not included	A	Penicillins	±
2c	II, V	A	Penicillins, carbenicillin	+
2d	V	D	Penicillins, cloxacillin	±
2e	Ic	A	Cephalosporins	+
2f	Not included	A	Penicillins, cephalosporins, carbapenems	+
3	Not included	B	Most β-lactams, including carbapenems	-
4	Not included	Not determined	Penicillins	-

1.6.2 *Class A β-lactamases*

Class A β-lactamases are commonly found in Gram-positive and Gram-negative species, including staphylococci, *Pseudomonads* and the Enterobacteriacae, frequently in clinical isolates (Yang, *et al.* 1999).They include the chromosomally encoded enzymes of *Klebsiella* spp. and *Bacteriodes* spp., as well as plasmid mediated enzymes and broad spectrum enzymes (Thomson and Smith Moland 2000). Enzymes of this class are susceptible to inhibition by clavulanic acid, tazobactam and sulbactam, the currently marketed β-lactamase inhibitors (Bush, *et al.* 1995). The widespread use of these inhibitors has led to the evolution of inhibitor resistant enzymes, termed IRT's (for inhibitor resistant TEM enzymes). While much apparent resistance to inhibitors is due to hyperproduction of susceptible enzyme which overwhelms the inhibitor (Thomson and Smith Moland 2000; Yang, *et al.* 1999), mutation of specific residues in the active site can lead to the development of inhibitor resistance (Nicolas-Chanoine 1997; Yang, *et al.* 1999). Most IRT enzymes are mutated forms of TEM-1 or TEM-2 enzymes. Comparison of the parent enzyme with IRTs has identified several residues important in contributing to inhibitor resistance. A number of IRTs have a mutation at residue 69, which is methionine in TEM-1. This residue is frequently mutated to isoleucine, leucine or valine (Chaibi, *et al.* 1999). Alteration of this residue affects the active site of the enzyme, due to the differing side chains of the mutated enzymes, which may sterically hinder the side chain of Ser-70 (Nicolas-Chanoine 1997; Chaibi, *et al.* 1999). These alterations may deform the oxyanion hole in the active site and cause a less favourable binding orientation for clavulanic acid (Nicolas-Chanoine 1997; Stapleton, *et al.* 1999). Residues such as Arg-244 are important for the action of clavulanic acid, where it activates a water molecule, leading to opening of the oxazolidine ring (Imtiaz, *et al.* 1993). Replacement of Arg-244 with a serine or cysteine would not be able to activate the water molecule due to the shorter side chain of the altered residue (Bret, *et al.* 1997; Yang, *et al.* 1999; Chaibi, *et al.* 1999; Stapleton, *et al.* 1999; Therrien and Levesque 2000). This mutation affects clavulanic acid more than tazobactam or sulbactam, as these inhibitors are believed to use a different mechanism for activation (Chaibi, *et al.* 1999). While single point mutations can confer resistance, the IC_{50} for an inhibitor (especially clavulanic acid) is higher when two or more

residues are mutated (Yang, *et al.* 1999). Substitution of Asn-276 with aspartic acid increases the K_i for clavulanic acid, possibly by affecting the electrostatic interactions with Arg-244 or by displacing the catalytically important water molecule (Nicolas-Chanoine 1997; Chaibi, *et al.* 1999). The prevalence of IRT enzymes varies between hospitals and countries, due to differing antimicrobial use and local resistance patterns. A recent study reported that 5% of *E. coli* isolates in French hospitals were resistant to amoxycillin-clavulanate, and of that 5%, between 30 and 40% of the strains had an IRT β-lactamase (Leflon-Guibout, *et al.* 2000).

Class A enzymes can also undergo mutations allowing them to hydrolyse third and fourth generation cephalosporins, previously stable to class A enzymes. Enzymes with this increased hydrolytic capacity are termed extended spectrum β-lactamase enzymes (ESBLs). The emergence of ESBLs was first observed in the 1980s when isolates of *Klebsiella* spp. and *E. coli* producing TEM-1, TEM-2 and SHV-1 enzymes were found to be resistant to cefotaxime and aztreonam. Enzymes capable of hydrolysing ceftriaxone, ceftazidime, cefepime and cefpirome have been described(Philippon *et al.* 1989). While the majority of ESBLs remain sensitive to β-lactamase inhibitors, like their parent enzymes, some have greater inhibitory resistance, for example TEM-50 from an *E. coli* clinical isolate and TEM-AQ from a *Serratia marcescens* clinical isolate (Thomson and Smith Moland 2000). There are now over 100 ESBLs described, and this group represents the fastest growing subgroup of β-lactamases (Bush and Miller 1998). The likely cause of the emergence of ESBLs is the antibiotic pressure caused by increased use of newer, later-generation cephalosporins. One study has demonstrated the emergence of an ESBL, SHV-8, over a five-month period due to treatment of an *E. coli* infection with multiple antibiotics (Rasheed, *et al.* 1997).

The majority of ESBLs are derived from TEM and SHV enzymes and can be disseminated by plasmids to organisms such as *M. morganii*, *S. marcescens* and *Citrobacter*. Other ESBLs include K1, derived from *K. oxytoca*, and PER types. K1 types are plasmid mediated and are found in a variety of strains. They are able to hydrolyse penicillins, aztreonam, cefuroxime and ceftriaxone. They are sensitive to β-lactamase inhibitors (Thomson and Smith Moland 2000). PER enzymes were

initially detected in *P. aeruginosa* and have spread by plasmids to *Acinetobacter baumanii*, *E. coli* and *K. pneumoniae*. These enzymes are sensitive to clavulanic acid (Thomson and Smith Moland 2000). Class D ESBLs have also been identified. These include derivatives of OXA-2 and OXA-10 as well as OXA-18, produced by *P. aeruginosa*. Most are capable of hydrolysing ceftazidime, and are insensitive to clavulanic acid (Philippon, *et al.* 1997; Mugnier, *et al.* 1998; Danel, *et al.* 1999; Aubert, *et al.* 2001).

To date, research suggests that ESBL enzymes remain sensitive to β-lactamase inhibitors, while IRT enzymes are not always capable of hydrolysing expanded spectrum cephalosporins (Nicolas-Chanoine 1997; Chaibi, *et al.* 1999; Stapleton, *et al.* 1999). Experimental combination of the mutations required for the ESBL phenotype with the substitutions required for the IRT phenotype did not result in expression of both phenotypes. In some cases, ESBL activity was either lost or reduced. Inhibitor resistance was not always expressed and in only one case was low- or moderate-level clavulanate resistance expressed (Stapleton, *et al.* 1999). The researchers speculated that the configuration of the active site of the ESBLs reduced the degree of the clavulanate resistance conferred. However, new enzymes (TEM-50 and TEM-AQ) have been identified which combine ESBL activity with IRT activity (Chaibi, *et al.* 1999; Thomson and Smith Moland 2000). These enzymes are likely to pose significant clinical challenges in the future.

1.6.3 *Class B β-lactamases*

Class B enzymes contain a divalent cation in the active site of the enzyme, typically zinc. The first enzyme was detected in *B. cereus* in 1966 (Sabath and Abraham 1966). The enzymes are chromosomally encoded and are found in *X. maltophilia*, *Chryseobacterium meningosepticum* and *Aeromonads* (Livermore and Woodford 2000). Acquired class B enzymes have been found in *P. aeruginosa*, *S. marcescens* and *K. pneumoniae* (Rasmussen and Bush 1997; Matagne, *et al.* 1999; Livermore and Woodford 2000). The gene for the IMP-1 enzyme can be transferred by plasmid from *P. aeruginosa*, *S. marcescens* and *K. pneumoniae* (Livermore and Woodford 2000). IMP-1 can hydrolyse carbapenems, cephalosporins, cephamycins,

oxacephamycins and penicillins (Thomson and Smith Moland 2000). The ability of noscomial pathogens like *P. aeruginosa* to produce and disseminate such enzymes is a cause for concern. The metallo-β-lactamases are able to hydrolyse a large variety of β-lactams, including carbapenems, and they are insensitive to the currently marketed β-lactamase inhibitors (Bush, *et al.* 1995). Metal chelating agents such as EDTA inhibit the enzymes (Sabath and Abraham 1966). Widespread dissemination of these enzymes by plasmidic transfer has not occurred to date even in Japan where there is widespread use of parenteral carbapenems (Bush and Miller 1998; Livermore and Woodford 2000).

1.6.4 *Class C β-lactamases*

Like class A and class D enzymes, class C enzymes contain an active site serine. They are produced by Gram-negative bacteria, principally the Enterobacteriacae but also by *Pseudomonas* and *Aeromonas* species (Matagne, *et al.* 1999; Bush, *et al.* 1995).

Class C enzymes can be chromosomally (AmpC) and plasmid encoded, and plasmid mediated transfer occurs readily between species. Species like *E. cloacae* produce inducible chromosomal enzymes (Livermore 1995) and 15-25% of *E. cloacae* strains hyperproduce these enzymes (Nicolas-Chanoine 1997). Chromosomal enzymes can be hyperproduced either by reversible induction or stable derepression (Livermore 1995; Thomson and Smith Moland 2000). Regulation of the *ampC* gene which encodes the β-lactamase is linked to the peptidoglycan biosynthesis pathway. *AmpC* is regulated by ampR, which acts as a repressor in cells not exposed to β-lactam antibiotics. Binding of UDP-muramyl-pentapeptide to AmpR causes it to act as a repressor for *ampC*. AmpD is an intracellular amidase which liberates tripeptides from the anhydromuramyl-tripeptides produced by autolytic enzymes, and returns the tripeptides to the biosynthetic pathway. In the presence of β-lactams, increased amounts of anhydromuramyl-tripeptides enter the cell via the AmpG permease. These anhydromuramyl-tripeptides displace UDP-muramyl-pentapeptide from AmpR, allowing it to induce *ampC*, leading to increased production of β-lactamase. Stable derepression occurs due to mutation of *ampD*, which leads to increased levels

of the anhydromuramyl-tripeptide degradation product. This leads to activation of AmpR and induction of *ampC* (Matagne, *et al.* 1999; Hanson and Sanders 1999). Plasmid mediated β-lactamases such as CMY and FOX are AmpC enzymes which are encoded by genes that have escaped from the chromosomes of *Citrobacter* and *Enterobacter* species (Livermore 1995). Plasmid mediated AmpC enzymes have been detected in *Klebsiella* spp., *C. freundii* and *E. coli*.

Class C β-lactamases are poorly inhibited by clavulanic acid, tazobactam and sulbactam (Bush, *et al.* 1995). The active site of class C enzymes is more open than that of class A enzymes, a factor which may account for the ability of class C enzymes to hydrolyse bulky cephalosporins (Lobkovsky, *et al.* 1993). The poorer inhibition of class C enzymes by clavulanic acid is believed to be due to the greater distance between the inhibitor and specific residues and water molecules in the active site, which prevents the opening of the oxazolidine ring (Lobkovsky, *et al.* 1993).

1.6.5 *Class D β-lactamases*

Class D enzymes were identified as a new molecular class in the late 1980s (Ouellette, *et al.* 1987; Huovinen, *et al.* 1988; Mossakowska, *et al.* 1989). They are efficient hydrolysers of oxacillin and cloxacillin, in contrast to class A and class C enzymes, and are relatively insensitive to inhibition by clavulanic acid (Bush, *et al.* 1995). Some class D OXA enzymes are capable of hydrolysing carbapenems. These enzymes are frequently found in *Acinetobacter* and *P. aeruginosa* isolates (Thomson and Smith Moland 2000; Livermore and Woodford 2000; Afzal-Shah, *et al.* 2001).

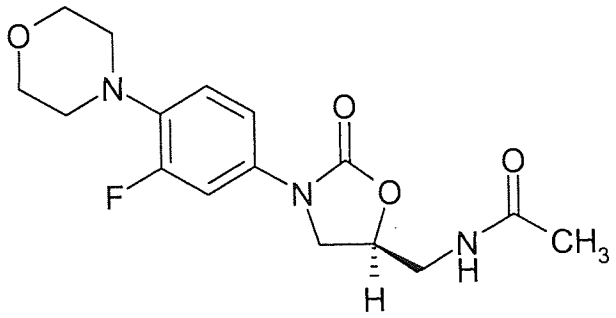
1.7 Novel antibacterial compounds

1.7.1 *Oxazolidinones*

Oxazolidinones are a class of synthetic antibacterial compounds. They were first synthesised by du Pont du Nemours & Co., Inc., with S-6213 being one of the first compounds investigated. S-6213 was found to be moderately active against

staphylococcal and streptococcal strains with MIC's ranging from 16 to 64μg/ml. It was also orally active in an *in vivo* peritonitis model (Daly, *et al.* 1988). Further screening led to the development of PNU-100592 and PNU-100766 by Upjohn Co (Brickner, *et al.* 1996). These compounds are produced in good yields with high enantiomeric purity. Initial screening of the compounds found them to be active against staphylococci (including MRSA), streptococci, enterococci, *M. tuberculosis* and Gram-negative anaerobes. Research has concentrated on these two agents, which were named eperezolid (PNU-100592) and linezolid (PNU-100766) (Fig. 1.3), with many studies reporting on their spectrum of activity. Linezolid was the first oxazolidinone antimicrobial agent to be approved by the Food and Drugs Administration (FDA) and the European Agency for the Evaluation of Medicines (EMEA). It is licensed for the treatment of pneumonia, skin and soft tissue infections caused by Gram-positive bacteria (BNF 42 2001).

FIGURE 1.3. Chemical structure of linezolid.



Linezolid has a primarily Gram-positive spectrum of activity. Numerous studies on the *in vitro* activity of linezolid against clinical isolates of Gram-positive pathogens have been carried out. These have been comprehensively reviewed by Fung *et al.* (2001). and are summarised in Table 1.3. In contrast, linezolid has limited activity against Enterobacteriacae and other Gram-negative species (Brickner, *et al.* 1996).

The mode of action of these novel synthetic compounds has yet to be fully elucidated. Studies with S-6123 demonstrated a bacteriostatic effect that was due to protein synthesis inhibition (Daly, *et al.* 1988). Lin *et al.* (1997), demonstrated that oxazolidinones have no effect on peptidyltransferase or translation termination. They postulated that oxazolidinones bind to a site on the 50S subunit, close to the binding

sites of chloramphenicol and lincomycin, near to the 30S subunit, and hence inhibit translation initiation. Further research showed that high concentrations of linezolid did not inhibit the formation of a binary complex between IF2 and fmet-tRNA, which binds to the initiation codon of mRNA at the P-site of the ribosome. Furthermore, linezolid did not inhibit the binding of mRNA to ribosomes after formation of the initiation complex. The researchers suggest that linezolid binding is partitioned between both subunits which distorts the initiator-tRNA binding site, which overlaps both subunits (Swaney, *et al.* 1998). Examination of linezolid-resistant isolates of *Halobacterium halobium* and *E. coli* have identified mutations in the central loop of domain V of 23S rRNA, suggesting that this is the binding site for the interaction of linezolid with the bacterial ribosome. The researchers proposed that linezolid and other oxazolidinones interfere with the initial interaction of fmet-tRNA with the 50S subunit during formation of the pre-initiation complex. This interference could affect the incorporation of tRNA in the 70S initiation complex, leading to inhibiton of protein synthesis (Kloss, *et al.* 1999).

To date, isolated cases of resistance to linezolid have been identified in enterococcal infections, as a result of long (20-40 day) courses of treatment with linezolid (Gonzales, *et al.* 2001). An isolated case of a MRSA strain exhibiting resistance to linezolid has recently been described (Tsiodras, *et al.* 2001). Resistance can arise due to mutations in 23S tRNA (Kloss, *et al.* 1999; Xiong, *et al.* 2000), although the experimental frequency of spontaneous resistance is low (Kaatz and Seo 1996). It is likely that the incidence of resistance to linezolid will increase with increasing use of the drug in clinical situations.

1.7.2 *Streptogramins*

Streptogramins are natural antibiotics produced by *Streptomyces* species, which also produces pristinamycins, oestreomycins, mikamycins and virginiamycins (Allington and Rivey 2001). There are two groups, A and B. Quinupristin (group B) and dalfopristin (group A) are water- soluble derivatives of pristinamycin I_A and II_A respectively. They have recently been launched as a product called Synercid containing quinupristin and dalfopristin in a ratio of 30:70. Quinupristin/dalfopristin

is indicated for the treatment of serious Gram-positive infections where no alternative antibiotic is suitable, including hospital-acquired pneumonia, skin and soft tissue infections and infections due to vancomycin-resistant *E. faecium* (BNF 42 2001). Quinupristin/dalfopristin is active against Gram-positive strains such as staphylococci, streptococci and *E. faecium* (Table 1.3). It has activity against some Gram-negative organisms such as *Moraxella*, *Leigonella* and *Neisseria*, but lacks activity against the Enterobacteriacae and *P. aeruginosa* (Allington and Rivey 2001).

The mode of action of quinupristin/dalfopristin involves inhibition of protein synthesis. The compounds act synergistically to inhibit bacterial growth. Alone, each compound is bacteriostatic; combined the compounds are bactericidal. The mode of action of dalfopristin and other group A streptogramins is inhibition of the elongation phase in protein synthesis. Group A streptogramins have been shown to prevent attachment of tRNA molecules to both the A-site and P-site (Chinali, *et al.* 1984). This binding is reversible (Nyssen, *et al.* 1989). Group A streptogramins can bind to 50S subunits and free ribosomes, but not to ribosomes engaged in protein synthesis. Binding of AA-tRNA molecules to A-sites or P-sites of translating ribosomes confers protection against group A streptogramin binding (Chinali, *et al.* 1987).

Group B streptogramins can bind to ribosomes engaged in protein synthesis as well as free ribosomal particles (Vasquez 1975). The mode of action of group B compounds involves inhibition of peptide bond formation – resulting in formation of incomplete protein chains (Chinali, *et al.* 1988). These differing modes of action of group A and group B compounds accounts for their synergistic action, with group A compounds inhibiting the early stages of protein synthesis and group B streptogramins affecting the later stages. Also, binding of group A streptogramins increases ribosomal affinity for group B compounds (Vannuffel, *et al.* 1992).

TABLE 1.3. *In vitro* activities (MIC μg/ml) of linezolid and quinupristin/dalfopristin against selected Gram-positive bacteria.

	Linezolid[a]		Quinupristin/Dalfopristin[b]	
Organism	MIC_{90}	Range	MIC_{90}	Range
Staphylococcus aureus				
Methicillin-resistant	≤4	1-4	≤2	0.015-4
Methicillin-sensitive	≤4	0.5-4	0.006-1	0.015-4
Vancomycin-intermediate	≤4	1-4		
Enterococcus faecalis				
Vancomycin-sensitive	≤4	1-4	ND	ND
Vancomycin-resistant	≤4	2-4	ND	ND
Enterococcus faecium				
Vancomycin-sensitive	≤4	0.5-4	0.5-8	0.5-8
Vancomycin-resistant	≤4	2-4	1-16	<0.12-32
Streptococcus pneumoniae				
Penicillin-sensitive	≤1	0.25-2	1	<0.06-4
Penicillin-intermediate	≤2	0.5-2	1	<0.06-4
Penicillin-resistant	≤2	0.5-2	1	<0.12-4

[a]Adapted from Fung *et al.* (2001).

[b]Adapted from Allington and Rivey (2001).

Resistance to streptogramins can be mediated by a number of factors both intrinsic and acquired. Intrinsic resistance is commonly due to poor penetration of the compounds through the bacterial membrane. Acquired resistance mechanisms include reduced penetration of the compounds, inactivation of the compounds and alterations in the target of the compounds.

Reduced penetration has been associated with an active efflux mechanism for group A compounds. A plasmid-mediated gene, *vga*, which codes for an ATP binding protein, has been reported in *S. aureus* (located on plasmid pIP680) and coagulase-negative staphylococci (Allignet and el Solh 1995). Enzymes capable of degrading streptogramins have been identified. *S. aureus* plasmid pIP680 also contains two genes, *vat* and *vatB,* which encode streptogramin A acetyltransferase and a gene, *vgb*, which encodes for streptogramin B hydroxylase (Allignet and el Solh 1995).

The most important mechanism of resistance is due to alteration of the bacterial ribosome. This alteration affects sensitivity to macrolides, lincosamides and group B streptogramins (MLS_B group) and can either be constitutive or inducible. Constitutive resistance confers protection against the entire MLS_B group, whereas inducible resistance protects against 14- and 15-membered macrolides but not against 16-membered macrolides and group B streptogramins (Low and Nadler 1997). In *S. aureus*, genes encoding for ermA and ermB confer resistance to the MLS_B group. ErmA and ermB are methylases which dimethylate an adenine residue in 23S rRNA at the N6 position. The genes encoding for ermA and ermB (*ermA* and *ermB* respectively) are one of a number of erythromycin resistance methylase (erm) genes found in a variety of bacterial species, both Gram-negative and Gram-positive (Leclercq and Courvalin 1991). Methylation occurs at A2058 of the peptidyl transferase loop of 23S rRNA; mutations at this site can also confer resistance to group A streptogramins (Porse and Garrett 1999).

1.7.3 *Glycopeptides*

Resistance to vancomycin and teicoplanin, the clinically used glycopeptides, developed during the late 1980s among enterococcal species (Uttley *et al.* 1988). As these agents are the last line of defence against methicillin-resistant *Staphylococcus aureus* (MRSA) infections, the potential transfer of resistance from enterococcal species to staphylococcal species would raise the spectre of untreatable infections. Already, such a transfer of resistance has been demonstrated experimentally (Noble, *et al.* 1992), and staphylococci with resistance to glycopeptides have been found in clinical infections (Hiramatsu, *et al.* 1997).

With the potential limited utility of vancomycin for MRSA and enterococcal infections, efforts have focused on developing semi-synthetic glycopeptides with activity against vancomycin-resistant enterococci (VRE). LY-264826, a natural analogue of vancomycin, produced by the fungus *Amycolatopsis orientalis,* has been N-alkylated to produce derivatives with activity against VRE.

Of the novel glycopeptides, most attention has centred on LY-333328, which is an N-alkyl derivative of LY-264826 with a *p*-chlorodiphenyl side chain. One study investigated the activity of LY-333328 against VRE, vancomycin-sensitive enterococci (VSE) and methicillin-resistant staphylococcal strains. The range of MICs was between 0.12 and 2μg/ml, with LY-333328 being more active than vancomycin, teicoplanin or quinupristin/dalfopristin against VRE (Schwalbe, *et al.* 1996).

A number of reports describe the spectrum of activity of LY-333328. An investigation of Gram-positive and Gram-negative strains from 26 species reported MIC values of 2μg/ml for methicillin sensitive *S. aureus* (MSSA); 4μg/ml for MRSA; between ≤0.015 and 0.06μg/ml for *S. pneumoniae* and from 0.025 to 1μg/ml for *Streptococcus* spp., serogroups A, B C and G. MICs for vancomycin-sensitive *E. faecalis* and vancomycin-resistant *E. faecalis* were 2μg/ml and for vancomycin-sensitive *E. faecium* and vancomycin-resistant *E. faecium*, 0.25 and 4μg/ml respectively (Jones, *et al.* 1996). These results match those of a later study which found MIC's for enterococci of ≤2μg/ml, ≤0.06 for pneumococci and all 425 Gram-positive clinical isolates to be inhibited by concentrations of LY-333328 of ≤4μg/ml (Garcia-Garotte, *et al.* 1998). Another study examined the activity of LY-333328 against nosocomial Gram-positive pathogens from 12 countries. Enterococcal isolates were inhibited by concentrations of LY-333328 of 1μg/ml (Zeckel, *et al.* 2000).

Workers investigating the activity of LY-333328 against 123 isolates of enterococci, reported LY-333328 to have a MIC range of between 0.03 and 8μg/ml, highly active compared to teicoplanin (0.03-256μg/ml) and vancomycin (0.25-256μg/ml). However, vancomycin displayed greater than or equal to activity against clinical isolates of staphylococcal species (Biavasco, *et al.* 1997). Another group reported on the activity of LY-333328 on 202 strains of pneumococci, with LY-333328 having an MIC_{90} of 0.008μg/ml compared to 0.06μg/ml for teicoplanin and 0.5μg/ml for vancomycin (Fasola, *et al.* 1996).

In a rabbit model of MRSA endocarditis, LY-333328 was found to be equally as effective as vancomycin at treating the infection (Kaatz, *et al.* 1998). Other workers investigated the activity of LY-333328 in a rabbit model of aortic endocarditis with three enterococcal strains. *E. faecalis* expressing VanA or VanB phenotype or carrying no resistance genes was inoculated into the subjects. LY-333328 displayed significant activity against all three strains, while vancomycin was ineffective against the VanB phenotype and teicoplanin was inactive against the VanA phenotype strain. The researchers concluded that LY-333328 was not modified by acquired resistance to glycopeptides in *E. faecalis*, where resistant strains produce modifies peptidoglycan precursors ending in D-lactate rather than D-alanine (Saleh-Mghir, *et al.* 1999). Other workers have found that a combination of LY-333328 and gentamicin was active against *vanA* and *vanB* strains of *E. faecalis* in a rabbit model of endocarditis (Lefort, *et al.* 2000).

1.7.4 *Daptomycin*

Daptomycin is a novel cyclic lipopeptide antibiotic, first described in 1986 (Eliopoulos, *et al.* 1986). It exhibits good activity against a number of Gram-positive pathogens including staphylococci, enterococci, streptococci and corynebacteria and its activity is enhanced when calcium is present in the medium (Barry, *et al.* 2001). Studies on the mode of action of daptomycin have identified a variety of effects on bacteria, including inhibition of lipoteichoic acid biosynthesis and peptidoglycan biosynthesis (at high concentrations) (Canepari, *et al.* 1990). Daptomycin disrupts the membrane potential of the cell membrane in *S. aureus*, which may lead to inhibition of active transport and affect cell wall biosynthesis (Allen, *et al.* 1991; Alborn Jr., *et al.* 1991).

1.7.5 *Everninomicins*

Everninomicins are complex, sugar-derived antibiotics isolated from *Micromonospora carbonacae*, first described in the 1970s. One of these compounds, evernimicin (Ziracin), has undergone further evaluation, and has been shown to have activity against Gram-positive organisms. Evernimicin was found to inhibit

translocation and 50S ribosomal subunit formation in growing cells of *S. aureus* (Champney and Tober 2000). It was subsequently shown to act in the same way in Gram-negative bacteria (McNicholas, *et al.* 2000). Evernimicin has MICs against MRSA and VRE of 1μg/ml, and 0.5μ/ml against penicillin-resistant pneumococci. It is currently in Phase III testing (Bax, *et al.* 2000).

1.7.6 *Derivatives of existing agents*

Carbapenems were developed in the 1970s after the discovery of thienamycin. The first commercially developed derivative of thienamycin was imipenem, which was found to be a potent, broad-spectrum antibiotic that was resistant to hydrolysis by β-lactamase. However, *in vivo*, it was hydrolysed by renal dehydropeptidase, and is administered with the renal peptidase inhibitor, cilastatin. Introduction of a 1β-methyl substituent at the C_1 position conferred stability to renal peptidase, while retaining the antimicrobial activity of the compound. Meropenem was the first renal dehydropeptidase stable carbapenem to be introduced into clinical practice. Biapenem is another 1β-methylcarbapenem, currently in clinical trials. Other novel carbapenems in development include lenapenem (Ohtake, *et al.* 1997), CS-834 (Miyauchi, *et al.* 1997), MK-826 (Gill, *et al.* 1998) and DA-1131 (Sader and Gales 2001).

Trinems are novel β-lactams which contain a tricyclic nucleus and a structure related to that of carbapenems. Sanfetrinem is a novel trinem compound, currently being evaluated. It has good broad-spectrum activity, including activity against staphylococci, streptococci and enterococci. Activity against Enterobacteriacae is similar to that of imipenem (Singh, *et al.* 1996; Sader and Gales 2001).

Novel quinolones have been developed, including gemifloxacin, moxifloxacin and gatifloxacin. These agents have improved activity against *S. pneumoniae* compared to parent compounds such as ciprofloxacin. Gemifloxacin is active against ciprofloxacin and multidrug resistant strains of *S. pneumoniae* (Bhavnani and Ballow 2000; Bax, *et al.* 2000).

Novel derivatives of tetracyclines called glycylcyclines have been developed. One compound, GAR-936, had good activity against a variety of Gram-positive strains including enterococci, staphylococci and streptococci. All strains were inhibited by concentrations of GAR-936 of ≤2μg/ml (Boucher, *et al.* 2000). A number of glycylcyclines have been synthesised and their activity and mode of action has recently been reviewed (Sum, *et al.* 1999).

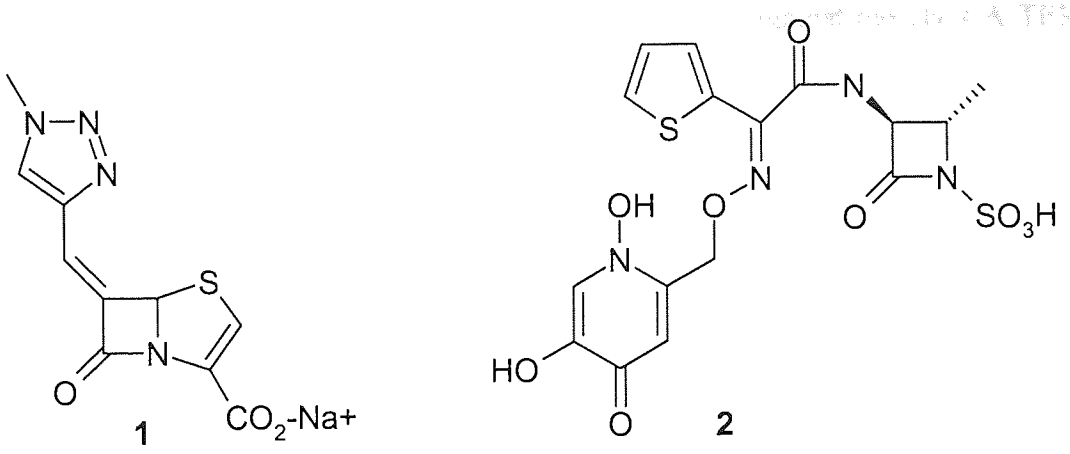
Ketolides are derivatives of the 14-membered ring structure of macrolides. Telithromycin (HMR 3647) is derived from the structure of clarithromycin, and appears to have enhanced activity against resistant Gram-positive strains. MICs against penicillin- and erythromycin-resistant pneumococci were 0.25μg/ml (Chu 1999; Bax, *et al.* 2000). Other derivatives are also being evaluated.

1.8 Novel β-lactamase inhibitors

Established β-lactamase inhibitors such as clavulanic acid, tazobactam and sulbactam are good inhibitors of class A enzymes, including ESBLs, but have poor activity against class C and class D enzymes, and lack activity against the class B metallo-β-lactamases (Bush, *et al.* 1995). There is a great need for novel inhibitors with activity against all classes of enzymes, and much research has focused on producing and testing novel β-lactamase inhibitors.

BRL 42715 is a synthetic penem which has good inhibitory activity against both class A and class C enzymes, while lacking any intrinsic antibacterial activity (Coleman, *et al.* 1989; Farmer, *et al.* 1994; Matagne, *et al.* 1995). IC_{50} values are between one and two orders of magnitude lower than those of clavulanic acid, tazobactam or sulbactam for class A, C and D enzymes (Coleman, *et al.* 1989). Further development of BRL 42715 was halted because it was chemically unstable (Farmer, *et al.* 1994), but the structure shows promise for the future development of inhibitors.

FIGURE 1.4. Chemical structures of BRL 42715 (**1**) and Syn2190 (**2**).



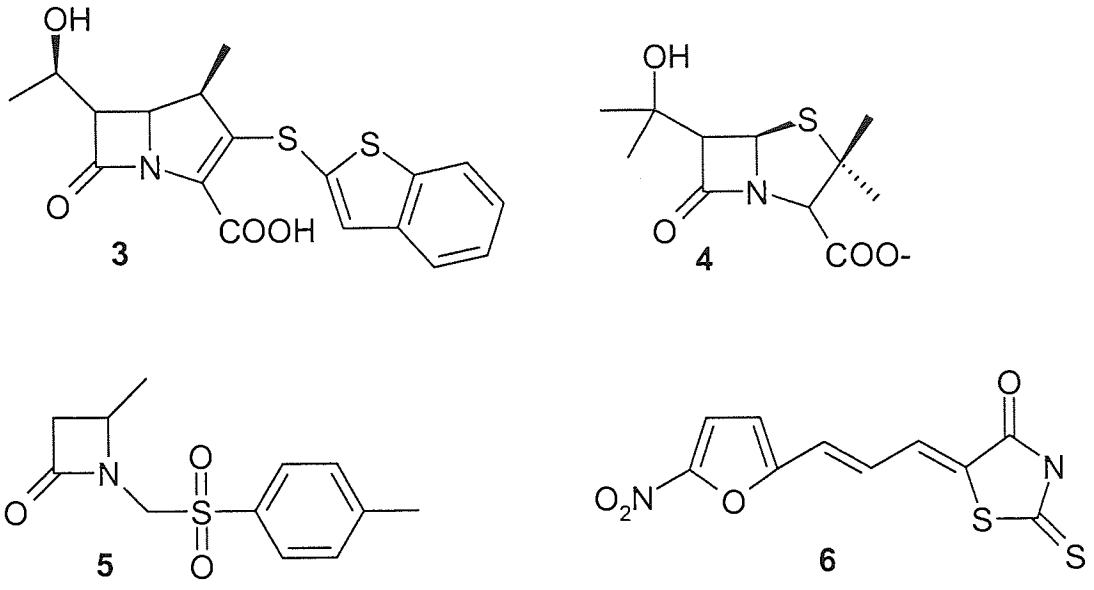
Syn2190 is another novel β-lactamase inhibitor. It is a monobactam derivative that is a potent inhibitor of class C enzymes. It had IC_{50} values between 220- and 850-fold lower than tazobactam against class C enzymes. However against class A and ESBL enzymes, tazobactam was more potent with IC_{50} values 10^3- to 10^4-fold lower than Syn2190. When combined with cephalosporins *in vitro* and *in vivo* in a murine peritonitis model and a murine urinary tract model, Syn2190 exhibited synergy with ceftazidime and cefpirome (Nishida, *et al.* 1999).

A series of 1β-methylcarbapenems have been synthesised which were active against the IMP-1 metallo-β-lactamase. This enzyme is capable of hydrolysing carbapenems and can be spread by plasmid transfer. One compound, J-110,441 (**3**), was found to be a potent inhibitor of both IMP-1 and also class C β-lactamase with K_i values of 0.0037 and 0.037μM, respectively (Nagano, *et al.* 1999). An enzyme that shares a high homology with IMP-1 is the class A NMC-A enzyme produced by *E. cloacae*. This enzyme can hydrolyse penicillins, cephalosporins and carbapenems. A series of penicillanic acid derivatives were constructed with increasingly larger side chains at the 6α position. The most effective inhibitor of the NMC-A enzyme was 6α-(hydroxypropyl)penicillanic acid (**4**) (Mourey, *et al.* 1998). A series of novel monobactams were later synthesised, some of which were rapid, irreversible inactivators of the enzyme (**5**) (Mourey, *et al.* 1999).

A series of rhodanines have been investigated as potential inhibitors of class C β-lactamases. These compounds do not share the β-lactam structure. One compound,

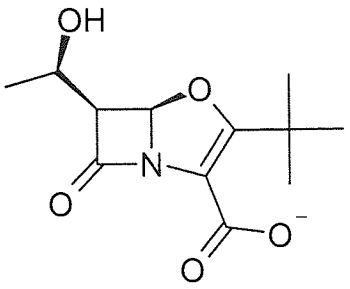
a 5-nitrofuran analogue (**6**), was found to possess selective class C inhibitory action, coupled with antibacterial action, while lacking activity against the class A TEM-1 enzyme (Grant, *et al.* 2000).

FIGURE 1.5. Chemical structures of some novel β-lactamase inhibitors.

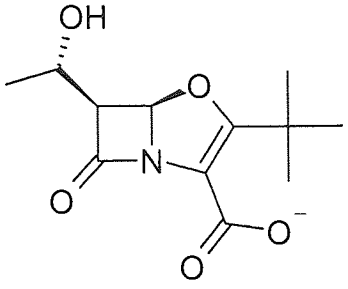


Replacement of the sulphur atom in the five membered ring of β-lactams with an oxygen led to a novel family of β-lactam compounds called oxapenems. These were first developed in the late 1970s (Pfaendler, *et al.* 1993). Unfortunately, these compounds were poorly stable and were not developed further. In the 1990s interest in oxapenems resumed and in 1993, a novel oxapenem with good activity against *S. aureus*, MRSA, anaerobes and *E. coli* was synthesised. This compound, (1'R, 5R, 6R) 2-tert-butyl-6-(1'-hydroxyethyl)oxapenem-3-carboxylate (AM-113, **7**) had increased stability compared to early oxapenems, which accounted for its antibacterial action. The side chain of the compound was similar to that of thienamycin (Pfaendler, *et al.* 1993). This compound is enantiomeric and exists in the (1'S, 5R, 6R) form as well (AM-114, **8**). Further derivatisation of the oxapenem nucleus led to the synthesis of (1'R, 5R, 6R) 3-(4-amino-1,1-dimethyl-butyl)-6-(1'-hydroxyethyl)oxapenem-3-carboxylic acid (AM-112) (**9**) and its enantiomer (1'S, 5R, 6R), AM-115 (**10**).

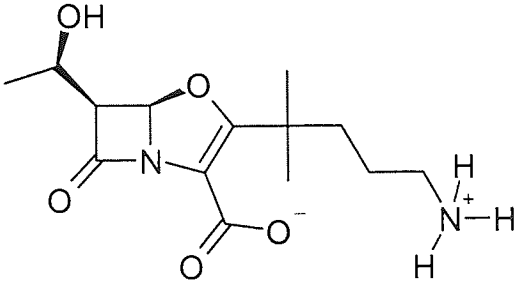
FIGURE 1.6. Chemical structures of four novel oxapenem compounds.



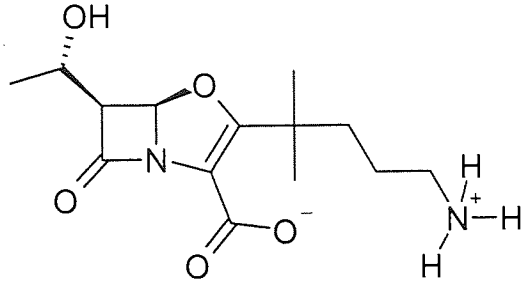
7 (AM-113)



8 (AM-114)



9 (AM-112)



10 (AM-115)

1.9 Aims of the study

A series of novel oxapenem compounds (AM-112, AM-113, AM-114 and AM-115) have recently been synthesised as potential β-lactamase inhibitors. These compounds were supplied by Amura Ltd. (Cambridge, UK) for evaluation. The aims of the study were to evaluate the antibacterial properties of the compounds against a range of organisms; to identify and quantify the β-lactamase inhibitory action of the compounds against a variety of enzymes; to identify the mode of action of the compounds against selected bacteria by penicillin-binding protein and morphology studies; to elucidate the mechanism of inhibition of β-lactamase enzymes and to evaluate the *in vivo* activity of the compounds against experimental models of infection.

Chapter 2. Oxapenems as β-lactamase inhibitors

2.1 Introduction

β-lactamase enzymes represent a serious threat to the future utility of β-lactam antibiotics for the treatment of infections. Therefore much research is focused on developing inhibitors to overcome these enzymes. The first commercially produced β-lactamase inhibitor was clavulanic acid. Clavulanic acid is a natural β-lactamase inhibitor produced by *Streptomyces* species, first described in 1976 (Brown, *et al.* 1976). Clavulanic acid lacks significant antibacterial activity alone, and is partnered with either amoxycillin (Augmentin) or ticarcillin (Timentin) when marketed commercially. Two other inhibitors are on the market, tazobactam and sulbactam. Both compounds are synthetic penicillanic acid sulphones (English, *et al.* 1978; Fisher, *et al.* 1981; Mezes, *et al.* 1982; Yamaguchi, *et al.* 1986). Sulbactam has been combined with ampicillin (Unasyn), tazobactam protects the β-lactamase labile piperacillin (Tazocin); both inhibitors lack antibacterial activity.

Studies on the oxapenem β-lactamase inhibitors were carried out in cell free assays, using isolated enzymes obtained from bacterial strains grown in the laboratory. Commercial sources of enzymes were not used due to the great expense of these enzymes and also the limited range of enzymes that were available. Isoelectric focusing was the technique used to isolate and purify the β-lactamase enzymes.

Isoelectric focusing separates a mixture of proteins on the basis of their net charge. In this respect it differs from SDS polyacrylamide gel electrophoresis, where all the proteins carry a net negative charge and are separated on the basis of their molecular weight. The isoelectric point (pI) of the protein will be the pH where the net charge on all the amino acid residues is neutral. Thus proteins, which differ in amino acid sequence, will differ in their isoelectric point. This phenomenon forms the basis of isoelectric focusing. A pH gradient is established by use of carrier ampholytes suspended in a support matrix. When an electric current is passed through the matrix, the carrier ampholytes arrange themselves in order of their pIs. Those ampholytes

with low pI migrate to the anode while those with high pIs move to the cathode. When the pH gradient is established, the net charges on the carrier ampholytes will be zero and the gradient becomes stationary. The pH gradient can be varied by choice of ampholytes – a pH gradient of 3-10 was used in these experiments. When a mixture of proteins is placed on the matrix, each protein will migrate towards the anode or cathode, depending on the net charge on the protein. The proteins migrate through the pH gradient towards the electrode until the charge on the protein becomes neutral and the protein ceases migration. This point is the isoelectric point of the protein and can be determined by measuring the pH of the matrix at that point. Any diffusion of the ampholytes or focused proteins is countered by the applied electric field.

Preparative and analytical isoelectric focusing differ both in the amount and purity of the protein mixtures applied to the matrix and the nature of the matrix. For preparative focusing of β-lactamase enzymes, a complex mixture of proteins obtained from disruption of the bacterial cell (approx. 1ml volume) is applied to a dextran (Sephadex) gel. Protein loads of up to 10g/ml of matrix can be separated by this method, although resolution is improved at lower protein loads (Winter, *et al.* 1977). Once focusing is complete, the enzyme is located by staining with nitrocefin and excised from the gel for further purification. Analytical isoelectric focusing is used to identify the isoelectric point of a purified solution of the β-lactamase enzyme. In this case, the matrix is a polyacrylamide gel, to which very small sample volumes of the solution of interest (~10μL) are placed on the surface of the gel. As with preparative focusing, the enzyme is located by staining with nitrocefin and the pI is measured using a glass electrode pH meter.

Preparative isoelectric focusing was used to obtain isolated extracts of β-lactamase enzymes from class A, class C and class D. These enzyme extracts were then used to investigate the inhibitory properties of the oxapenem compounds. Inhibitor assays were performed using nitrocefin as the substrate for the enzyme. Nitrocefin is a cephalosporin compound, first described in 1972 (O'Callaghan, *et al.* 1972). It yields a yellow coloured solution that absorbs at 217nm and 386nm. When nitrocefin is hydrolysed by β-lactamases, the breakdown product produces an intense red colour,

which absorbs at 482nm. By monitoring the absorbance of a solution of β-lactamase enzyme and nitrocefin at 492nm (the nearest absorbance filter on the Anthos Plate reader), one can determine the rate of the reaction and the K_M. If an inhibitor is added to the mixture, one can then determine the IC_{50} and the K_i of the inhibitor. These parameters were determined for each of the four oxapenem compounds and clavulanic acid for eight bacterial enzymes.

2.2 Materials and Methods

2.2.1 *Preparation of β-lactamase enzymes*

Isolated enzyme extracts were prepared from the following organisms: *E. coli* J53 SHV-5, *E. coli* J53 TEM-10, *E. coli* J53 TEM-1, *E. cloacae* 1051E P99, *S. marcescens* S2-con, *P. aeruginosa* S+A, *E. coli* J53 OXA-1 and *E. coli* J53 OXA-5. An overnight culture of the required organism was grown in MHB, supplemented with 0.1mM 6-aminopenicillanic acid (6-APA, Sigma, UK) to stimulate β-lactamase production, at 37°C. Aliquots of 50ml of the overnight culture were inoculated into 500ml volumes of fresh, pre-warmed MHB, supplemented with 0.1mM 6-APA and incubated at 37°C with vigorous shaking, until OD_{470nm}=1.0. The cells were harvested by centrifugation at 10,000rpm for ten minutes at 4°C, washed in SPB and resuspended in SPB. The cells were then disrupted by sonication using a MSE Soniprep 150 (MSE Ltd., Crawley, Sussex, UK) with a 0.5cm diameter probe operating at full power. Six ten-second cycles of sonication with constant cooling in an ice bath were required to disrupt the cells. The disrupted cells were then centrifuged at 10,000rpm for thirty minutes at 4°C to remove any whole cells and cell debris. Aliquots of the supernatant containing the crude enzyme were stored at -70°C until required.

2.2.2 *Preparative isoelectric focusing*

A rectangular glass mould measuring 23.7×10.3×0.5cm was filled with the IEF slurry, which consisted of Sephadex G-75 (Sigma) 10%, Ampholine (pH 3.5-10, LKB) 1% and ddH_20 to 100%. IEF was carried out on a LKB Bromma 2117 Multiphor II electrophoresis unit, attached to a Bio-Rad Model 250/2.5 power supply applying 200V across the gel. The cooling plate of the electrophoresis unit was maintained at 4°C by a LKB Bromma 2209 Multi Temp water bath. The cathode consisted of a strip of filter paper dipped in 1M NaOH, while the anode electrolyte

was 1M H_3PO_4. A volume of 1ml of the enzyme supernatant was applied midway along the gel, and the surface of the gel was sprinkled with Sephadex G-75 to absorb any excess liquid. Electrophoresis was carried out for eighteen hours.

The presence of the β-lactamase enzyme was detected using filter paper dipped in a dilute solution of nitrocefin, and the isoelectric point was measured using a glass electrode pH meter. Once the enzyme was located, the area of gel containing the enzyme was excised from the gel, and the enzyme eluted with 10mM sodium phosphate buffer. The eluted enzyme was stored in aliquots at -70°C until required.

2.2.3 *Analytical isoelectric focusing*

Self-poured AIEF plates were prepared according to the method described in the LKB Application Note 250 (Winter, *et al.* 1977). The glass plates and rubber spacer which form the mould were cleaned with 70%$^{v}/_{v}$ ethanol prior to assembly. The mould was first filled with ddH_20 to ensure a seal had been formed. The gel was prepared according to the formula in Table 2.1.

TABLE 2.1. Formula for Analytical Isoelectric focusing gel.

Reagent	Volume	Reagent	Volume
Acrylamide stock solution (29.1%$^{w}/_{v}$)	10ml	Bis-acrylamide stock solution (0.9%$^{w}/_{v}$)	10ml
Glycerol (87%$^{w}/_{w}$)	7ml	Ampholine 4-6	0.2ml
Ampholine 5-7	0.2ml	Ampholine 9-11	0.4ml
Ampholine 3.5-10	2.8ml	ddH_20	To 60ml

The mixture was degassed for ten minutes using a water trap. To initiate polymerisation, 1.5ml of AMPS (1%$^{w}/_{v}$) was added and mixed gently. The glass mould was filled with the mixture using a needle and syringe, and left for several hours to set. After polymerisation had occurred, the upper glass plate and rubber spacer were removed and the gel was cut to the required size and shape. The gel was placed on the cooling plate of a LKB Bromma 2117 Multiphor II electrophoresis

unit, maintained at 4°C by a LKB Bromma 2209 Multi Temp water bath. The electrophoresis unit was connected to a Bio-Rad 3000Xi power pack applying a constant voltage of 1000V across the gel for twenty-four hours. Test samples and markers were laid on the gel in 10µl aliquots on rectangular strips of filter paper. The filter papers were removed one hour after electrophoresis had begun.

After electrophoresis was complete, the gel was immersed in a fixing solution (sulphosalicyclic acid 3.46%$^{w}/_{v}$, trichloroacetic acid 11.5%$^{w}/_{v}$ in distilled water) for one hour to precipitate the proteins and wash out the ampholytes. The gel was then soaked in a destaining solution (ethanol 25%$^{v}/_{v}$, acetic acid 8%$^{v}/_{v}$ in distilled water) for thirty minutes, and then immersed for fifteen minutes at 60°C in staining solution (coomassie brilliant blue R-250 0.115%$^{w}/_{v}$ in destaining solution). The gel was destained by repeated immersion and washing in destaining solution.

2.2.4 *Determination of kinetic parameters*

The kinetic parameters K_M (Michaelis-Menten constant) and V_{max} (maximal rate of enzyme activity) for each enzyme were determined by a spectroscopic method involving the hydrolysis of nitrocefin by the β-lactamase enzyme. Aliquots of 50µl of the isolated enzyme were mixed in a microtitre plate with 50µl of SPB and maintained at 37°C. A volume of 50µl of nitrocefin was added and the absorbance of the suspension was monitored over a ten-minute period at 492nm. The concentration of the substrate (nitrocefin) was varied from 10µg/ml to 150µg/ml with respect to the enzyme concentration, which remained constant. Three replicates of each sample were measured. The raw absorbance data was plotted against time for each substrate concentration and the slope of the linear portion of this line corresponded to the initial velocity (V_0) of the enzyme reaction. The initial velocity data and substrate concentration for each enzyme were determined and entered into the enzyme kinetics template of Prism (GraphPad Prism version 3.02 for Windows, GraphPad Software, San Diego, California, USA) which plotted the data as Michaelis-Menten, Linweaver-Burk and Eadie-Hofstee plots. From these plots, the kinetic parameters K_M and V_{max} were determined.

2.2.5 *Cell-free β-lactamase inhibition studies*

Nitrocefin (Oxoid, Basingstoke, UK) was prepared and stored according to the manufacturers instructions. AM-112, AM-113, AM-114 and AM-115 were supplied by Amura Ltd (Cambridge, UK) and stored at -70°C until required. Clavulanic acid (USP reference standard) was obtained from U.S. Pharmacopeia (Rockville, MD, USA) and stored at 4°C until required. All the inhibitors were freshly prepared in 10mM sodium phosphate buffer (pH 7) prior to use. TEM-1 β-lactamase was supplied by Prof. C. Schofield (University of Oxford). All other enzymes were prepared in the laboratory as described above.

β-lactamase activity was determined by a spectrophotometric method using nitrocefin as the substrate, according to the method of O'Callaghan *et al.* (1972). The assays were carried out in flat-bottomed microtitre plates, with a final reaction volume of 150μL in each well. For the inhibition studies, the enzyme was pre-incubated with the inhibitor for fifteen minutes at 37°C prior to addition of the substrate. The absorbance at 492nm was measured for a ten-minute period after addition of the substrate using an Anthos 2001 Plate Reader (Anthos Labtech, Saltzburg, Austria). The temperature was maintained at 37°C for the course of the assay.

2.3 Results

2.3.1 *Determination of K_M and V_{max}*

Values for K_M and V_{max} for each of the enzymes were determined using the enzyme kinetics template in Prism (GraphPad Prism version 3.02 for Windows, GraphPad Software, San Diego, California, USA). Spectroscopic data for the hydrolysis of varying concentrations of nitrocefin by each enzyme was collected and plotted against time. The slope of the line plotted for each nitrocefin concentration was determined. This slope corresponded to the initial velocity (V_0) of the reaction. From this data, the enzyme kinetics template in Prism calculated the K_M and V_{max} using the Michaelis-Menten equation, the Lineweaver-Burk transformation and the Eadie-Hofstee plot. The values obtained for these parameters are listed in Table 2.2.

TABLE 2.2. Summary of K_M and V_{max} values for eight β-lactamase enzymes calculated from the hydrolysis of nitrocefin. Values were calculated by Prism (GraphPad Prism version 3.02 for Windows, GraphPad Software, San Diego, California, USA). The standard deviation was within ±5%.

Enzyme	Michaelis-Menten		Lineweaver-Burk		Eadie-Hofstee	
	K_M (μM)	V_{max} (μmol/s)	K_M (μM)	V_{max} (μmol/s)	K_M (μM)	V_{max} (μmol/s)
TEM-1	226	0.0223	630	0.0196	470	0.0125
TEM-10	71	0.0032	42	0.0027	44	0.0028
SHV-5	131	0.0007	64	0.0005	64	0.0005
P99	44	0.0038	41	0.0036	47	0.0039
S2	36	0.0095	23	0.0085	24	0.0088
S+A	10	0.0015	10	0.0015	9	0.0015
OXA-1	665	0.0521	1133	0.0583	160	0.0004
OXA-5	44	0.0004	79	0.0004	90	0.0005

The K_M value for a substrate is the concentration at which the velocity of the reaction rate is half its maximum value. It can be determined graphically from the Michaelis-Menten plot of V_0 versus substrate concentration (Fig. 2.1). However, because the maximum rate of velocity, V_{max}, of the enzyme reaction is usually extrapolated from the Michaelis-Menten plot, the value of K_M is prone to error. A

solution to this is to convert the hyperbolic Michaelis-Menten plot into a linear plot. This is done by the Lineweaver-Burk transformation, where the Michaelis-Menten equation is converted to a reciprocal form and becomes:

$$\frac{1}{V} = \frac{1}{V\,\max} + \left(\frac{K_M}{V\,\max} \times \frac{1}{[S]} \right)$$

A plot of $\frac{1}{V}$ versus $\frac{1}{[S]}$ yields a straight line with a *y*-intercept of $\frac{1}{V\,\max}$, an *x*-intercept of $-\frac{1}{K_M}$ and a slope of $\frac{K_M}{V\,\max}$ (Figure 2.2).

FIGURE 2.1. Michaelis-Menten plot of initial velocity (V_0) against substrate concentration ([S]) for *E. coli* SHV-5 β-lactamase with nitrocefin as the substrate.

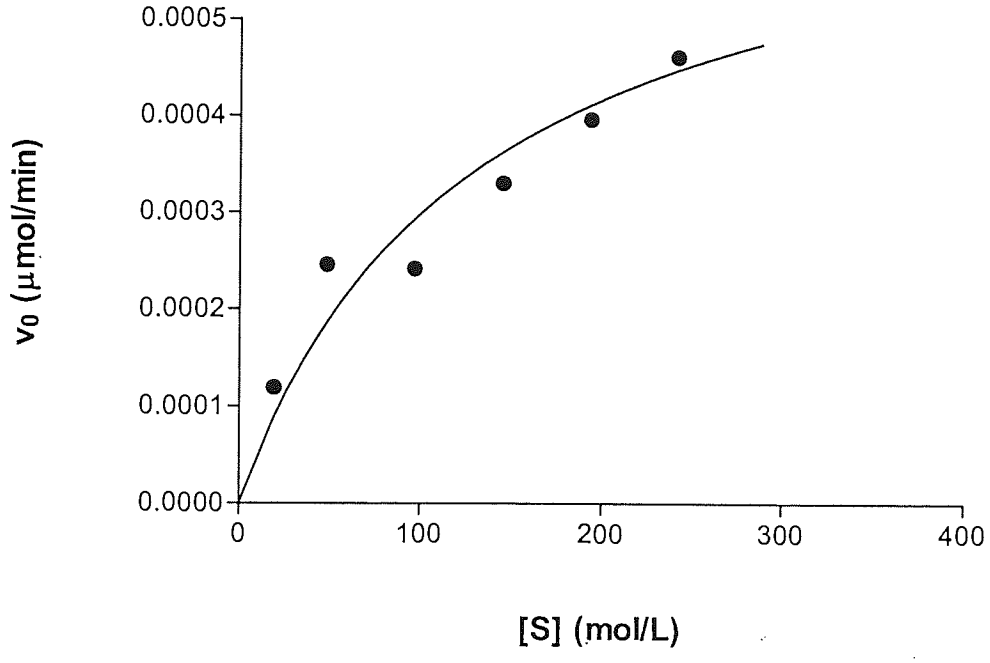
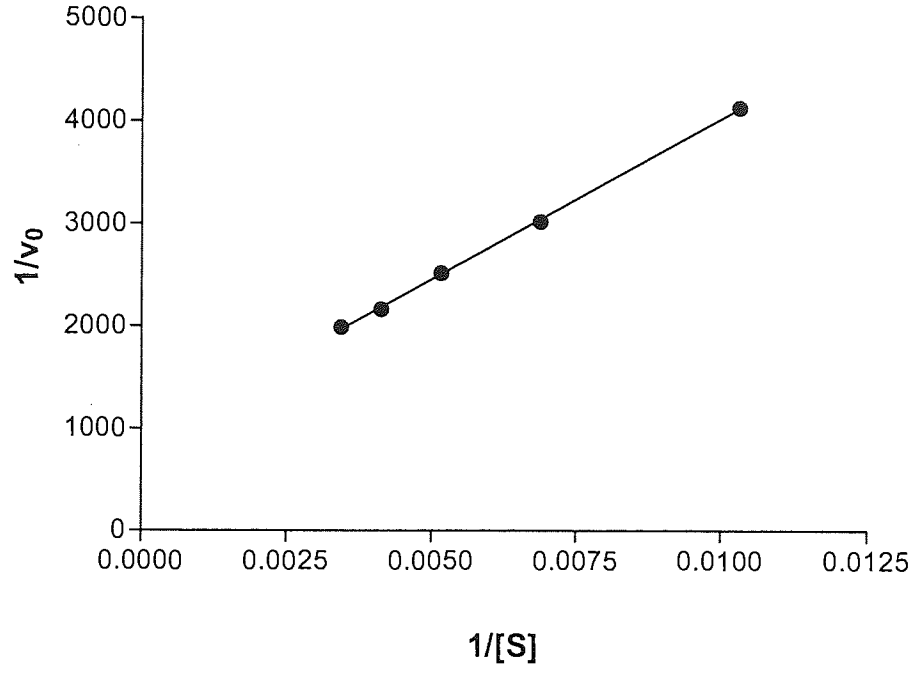
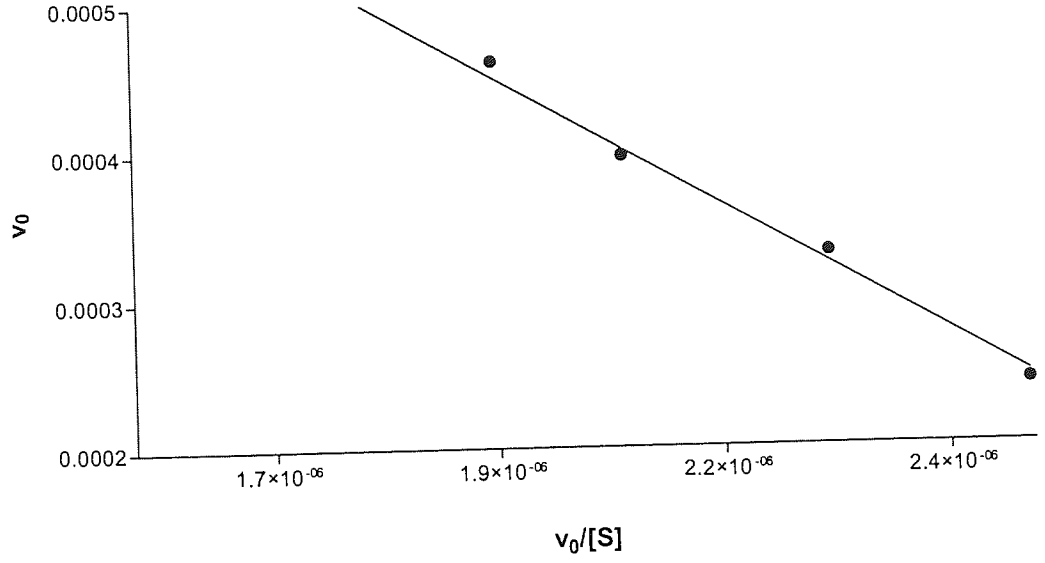


FIGURE 2.2. Lineweaver-Burk plot of $1/V_0$ against 1/[S] for *E. coli* SHV-5 β-lactamase with nitrocefin as the substrate.



The Linweaver-Burk plot has been criticised because the data at low substrate concentrations (high 1/[S]) are scattered and can exaggerate the experimental error. V_0 measurements at low substrate concentrations are less accurate. The Eadie-Hofstee plot provides another means of determining K_M and V_{max}. A graph is drawn of V_0 versus $\frac{V_0}{[S]}$ – the *y*-intercept equals the V_{max} and the slope of the line equals the K_M (Fig. 2.3). The drawback of the Eadie-Hofstee plot is that the values on the *x*-axis are not independent of those on the *y*-axis, as they contain an element of the velocity of the reaction. Thus any experimental error will appear on both axes.

FIGURE 2.3. Eadie-Hofstee plot of the initial velocity (V_0) against V_0/[S] for *E. coli* SHV-5 β-lactamase with nitrocefin as the substrate.



The K_M values listed in Table 2.1 show some variations depending on which plot was used to calculate the values. There is close agreement between the values obtained for the class C enzymes P99, S2 and S+A. However, TEM-1 and OXA-1 show wide variations between the values obtained. The K_M value calculated for an enzyme depends on a number of factors including pH, temperature and enzyme concentration. The enzymes used in this panel have not been purified to homogeneity, so the K_M values obtained for these enzymes cannot easily be compared to published values. Nevertheless, the K_M values are within the order of magnitude range that can be expected for these enzymes (Stryer 1981).

The values of K_M and V_{max} calculated by the Lineweaver-Burk transformation were used to calculate the K_i and IC_{50} values described below. This was because there was a better correlation of the data using this equation than either the Michaelis-Menten or Eadie-Hofstee equations (i.e. the r^2 value for the goodness of fit was closer to 1.0).

2.3.2 *Inhibition studies*

The oxapenem compounds were investigated for their β-lactamase inhibitory properties. Clavulanic acid was chosen for comparison. Chapter 3 presents results based on the ability of the oxapenems to inhibit β-lactamases produced by intact bacteria in MIC tests. The focus of investigation in this chapter was the inhibition of isolated β-lactamase enzymes. Eight β-lactamase enzymes were isolated from Gram-negative bacteria. The inhibitor concentration at which 50% of the enzyme activity was inhibited (IC_{50}) and the inhibition constant (K_i) were determined for clavulanic acid and the four oxapenem compounds by a spectrophotometric method using nitrocefin as the substrate. The K_i value is the dissociation constant for the enzyme-inhibitor complex. The concentration of the inhibitor was varied with respect to a constant enzyme and substrate concentration. The enzymes were pre-incubated with the inhibitor for fifteen minutes prior to addition of the substrate. Inhibition profiles for *E. coli* TEM-1 β-lactamase are shown in Figs. 2.4-2.8.

The slope of the linear portion of the graph for the control and inhibited enzymes was determined from the equation for the slope of a line:

$$m = \frac{y_2 - y_1}{x_2 - x_1}$$

This was assumed to correspond to the initial velocity (V_0) of the reaction. These values were entered into the enzyme kinetics template of Prism along with the log of the inhibitor concentration, the K_M for the enzyme and the substrate concentration. The Prism package then calculated the IC_{50} for each inhibitor by non-linear regression and the K_i values using the Cheng and Prusoff equation (Cheng and Prusoff 1973)

$$K_i = \frac{IC_{50}}{\left(1 + \frac{[S]}{K_M}\right)}$$

FIGURE 2.4. Hydrolysis of nitrocefin (50µg/ml) by *E. coli* TEM-1 β-lactamase: effect of 15 minutes pre-incubation with clavulanic acid.

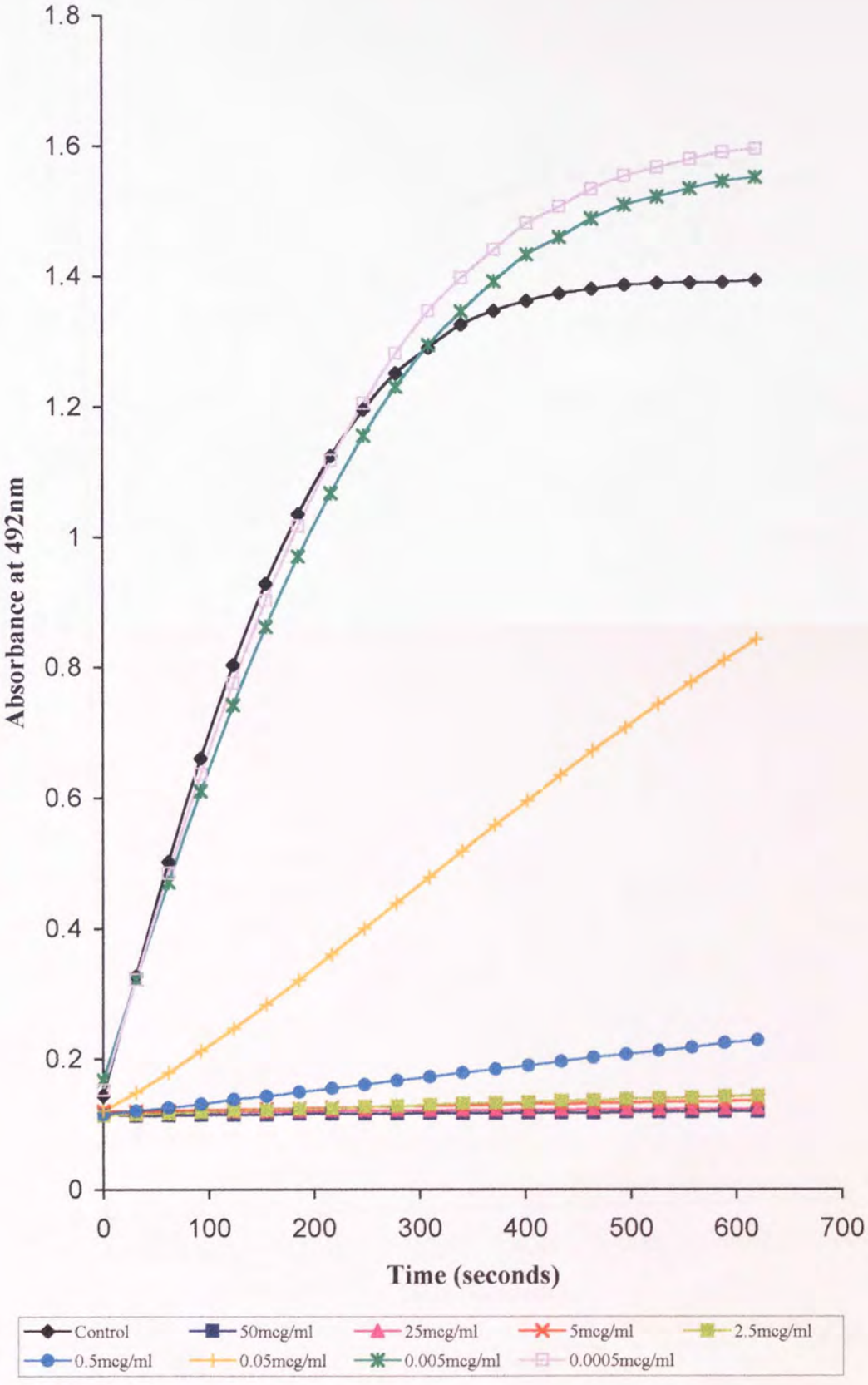


FIGURE 2.5. Hydrolysis of nitrocefin (50μg/ml) by *E. coli* TEM-1 β-lactamase: effect of 15 minutes pre-incubation with AM-112.

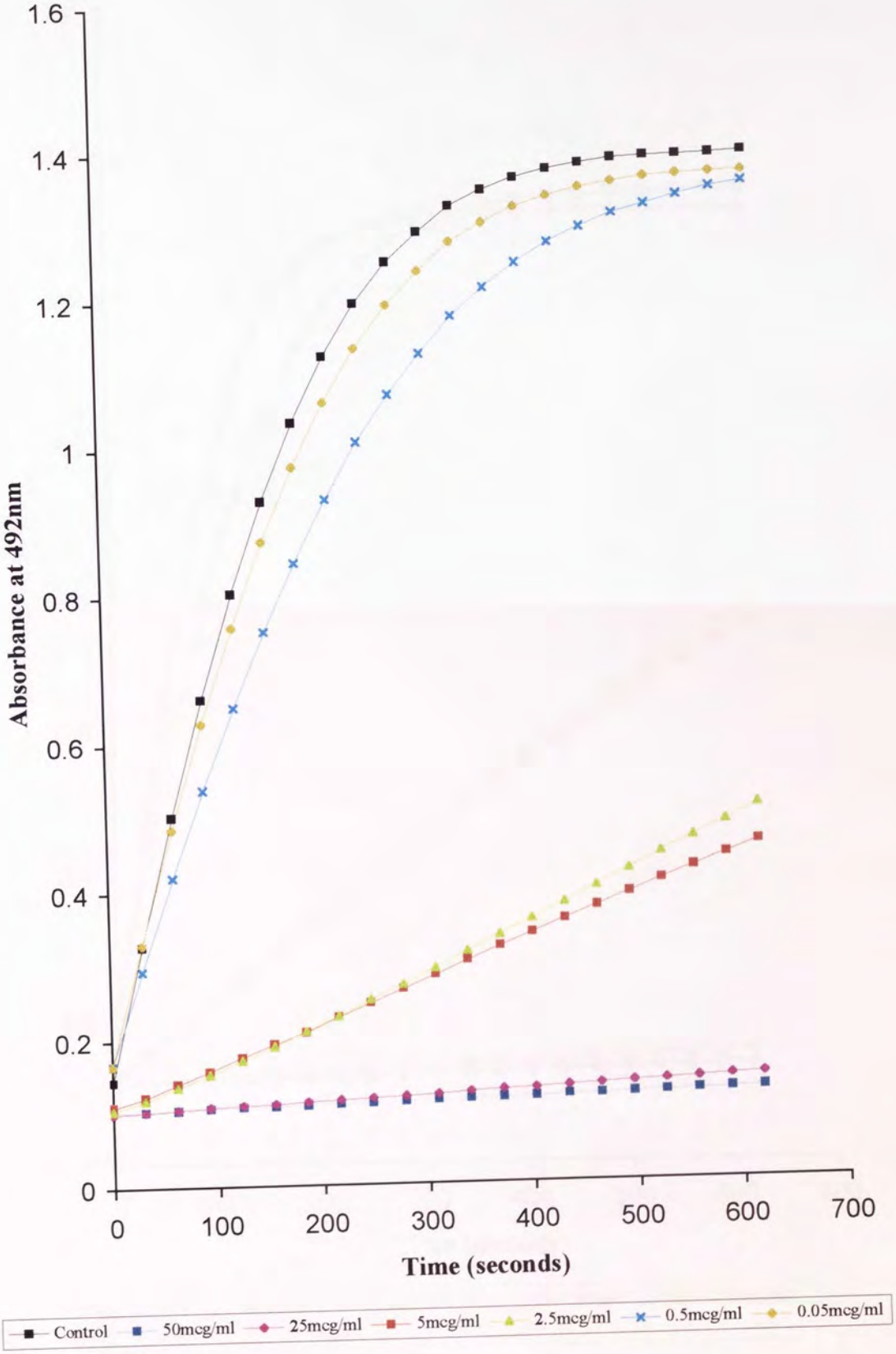


FIGURE 2.6. Hydrolysis of nitrocefin (50μg/ml) by *E. coli* TEM-1 β-lactamase: effect of 15 minutes pre-incubation with AM-113.

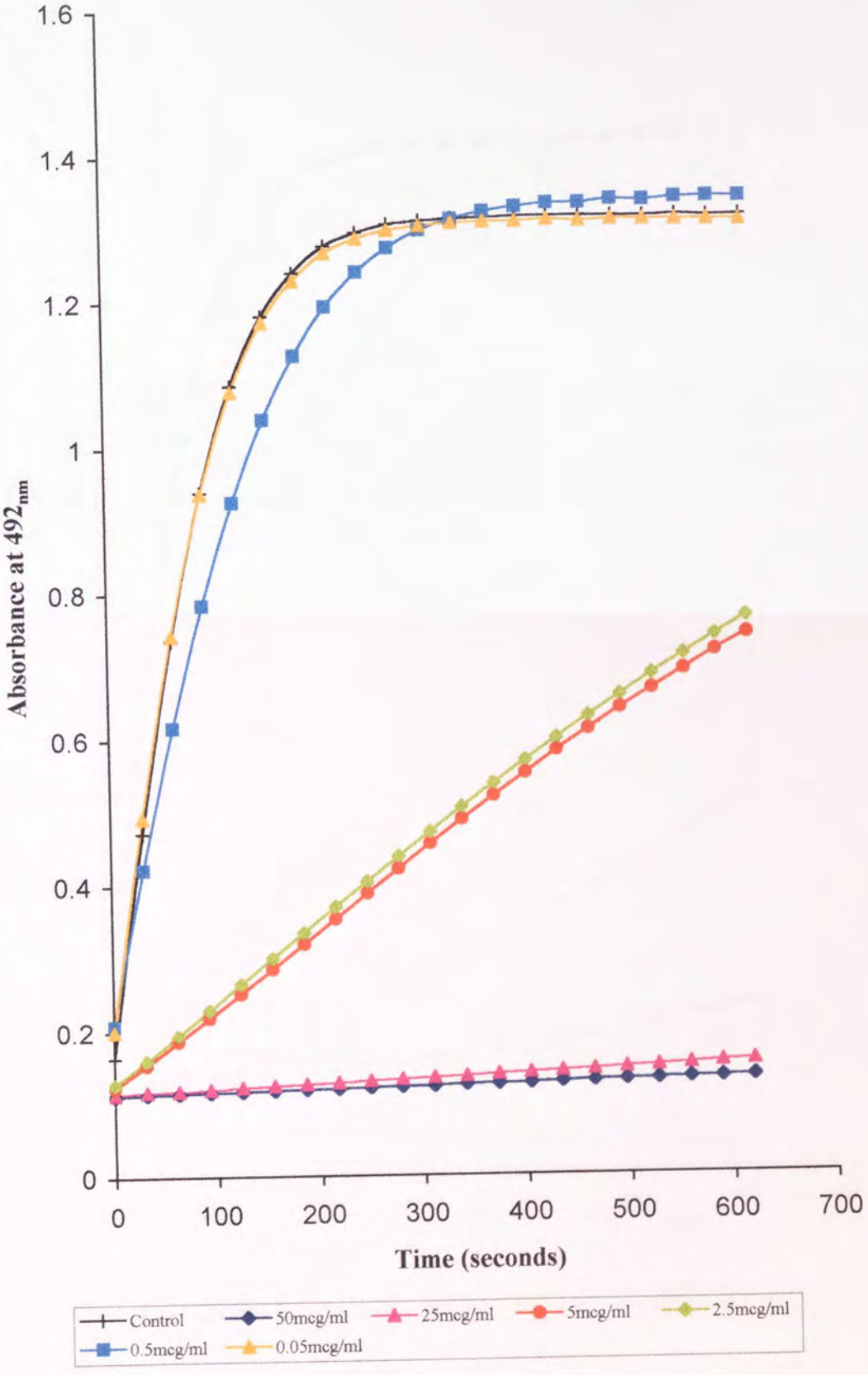


FIGURE 2.7. Hydrolysis of nitrocefin (50μg/ml) by *E. coli* TEM-1 β-lactamase: effect of 15 minutes pre-incubation with AM-114.

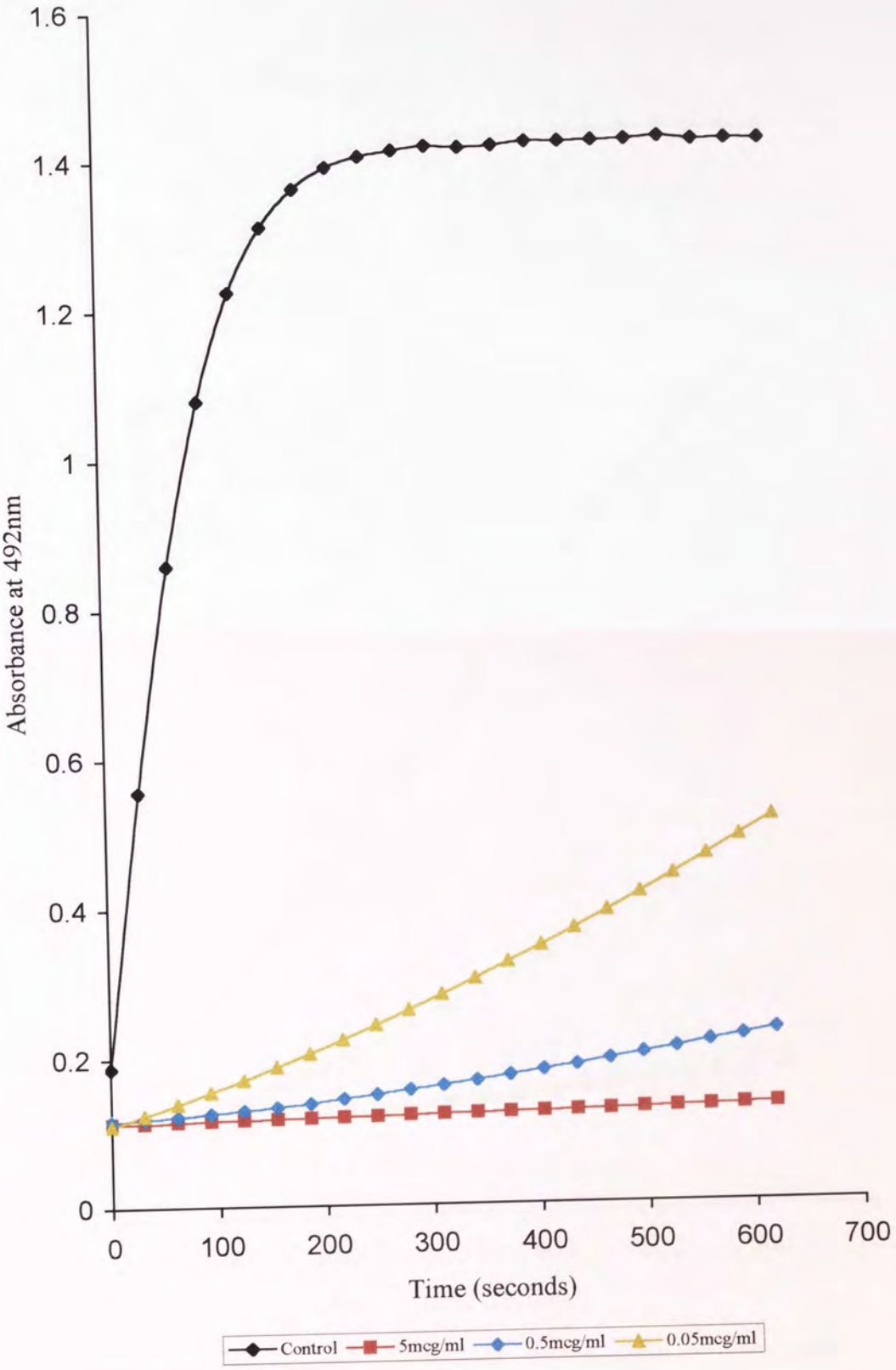


FIGURE 2.8. Hydrolysis of nitrocefin (50μg/ml) by *E. coli* TEM-1 β-lactamase: effect of 15 minutes pre-incubation with AM-115.

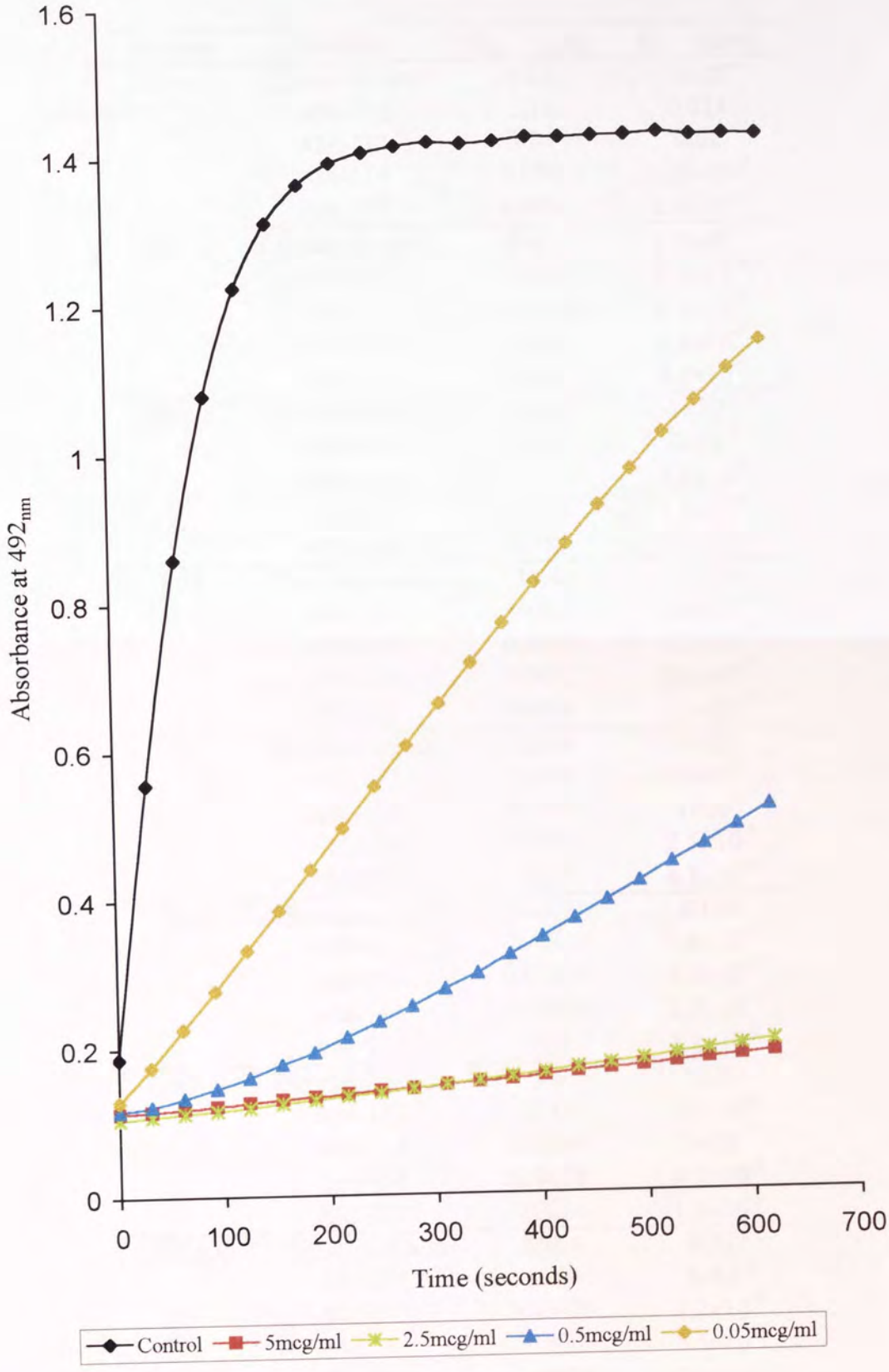


TABLE 2.3. IC_{50} and K_i values of five β-lactamase inhibitors calculated by Prism (enzyme kinetics template) for a panel of eight β-lactamase enzymes. (GraphPad Prism version 3.02 for Windows, GraphPad Software, San Diego, California, USA).

Enzyme	Inhibitor	IC_{50} (μM)	K_i (μM)
TEM-1	Clavulanic acid	0.12	7.1×10^{-4}
	AM-112	2.26	0.014
	AM-113	3.34	0.02
	AM-114	0.002	1.22×10^{-5}
	AM-115	0.004	2.4×10^{-5}
TEM-10	Clavulanic acid	0.03	2.5×10^{-5}
	AM-112	0.224	5.6×10^{-5}
	AM-113	0.0082	6.8×10^{-6}
	AM-114	0.063	5.3×10^{-5}
	AM-115	0.005	4.2×10^{-6}
SHV-5	Clavulanic acid	0.008	1×10^{-5}
	AM-112	0.16	2×10^{-4}
	AM-113	0.11	1.4×10^{-4}
	AM-114	0.012	1.5×10^{-5}
	AM-115	0.057	7.3×10^{-5}
P99	Clavulanic acid	11.4	0.009
	AM-112	0.002	1.6×10^{-6}
	AM-113	0.0018	1.5×10^{-6}
	AM-114	0.0012	9.8×10^{-7}
	AM-115	0.014	1.1×10^{-5}
S2	Clavulanic acid	326.8	0.15
	AM-112	0.067	3×10^{-5}
	AM-113	0.0023	1×10^{-6}
	AM-114	0.0055	2.5×10^{-6}
	AM-115	0.09	4.1×10^{-5}
S+A	Clavulanic acid	448.8	0.086
	AM-112	0.0019	3.6×10^{-7}
	AM-113	0.00059	1.1×10^{-7}
	AM-114	0.00014	2.7×10^{-8}
	AM-115	0.02	3.8×10^{-6}
OXA-1	Clavulanic acid	99	2.3
	AM-112	0.005	1.1×10^{-4}
	AM-113	0.00043	1×10^{-5}
	AM-114	0.0028	6.5×10^{-5}
	AM-115	0.0064	1.5×10^{-4}
OXA-5	Clavulanic acid	202.4	0.322
	AM-112	0.00067	1×10^{-6}
	AM-113	0.00079	1.2×10^{-6}
	AM-114	0.0022	3.5×10^{-6}
	AM-115	0.0054	8.6×10^{-6}

Clavulanic acid was an effective inhibitor of class A enzymes (Payne, *et al.* 1994). This activity was reflected in the results for clavulanic acid against the three class A enzymes in Table 2.3 which showed IC_{50} values of between 0.008 and 0.12μM. Clavulanic acid lacked good activity against the class D enzymes OXA-1 and OXA-5, with IC_{50}s of 99 and 202.4μM. As expected, the activity against the class C enzymes was poor; clavulanic acid was most active against P99 (IC_{50} 11.4μM), but poorly active against S2 and S+A (326.8 and 448.8μM respectively). The IC_{50} values obtained here for TEM-1, TEM-10, P99 and S2 were in the same order of magnitude as those described in a study investigating the interaction between β-lactamase inhibitors and enzymes from each molecular class (Bush, *et al.* 1993).

AM-112 exhibited a broad spectrum of activity against each of the three classes of enzyme. AM-112 was most active against the ESBL class A enzyme SHV-5 with an IC_{50} value of 0.16μM. This activity was 100-fold poorer than clavulanic acid against this enzyme. AM-112 activity against TEM-1 was approximately 20-fold poorer than clavulanic acid, and against the ESBL TEM-10 enzyme, the activity was 10-fold poorer than clavulanic acid. Nevertheless, the activity against the class A enzymes was good. Class C enzymes proved very susceptible to inhibition by AM-112. IC_{50} values against the three enzymes in the panel were between 1000 and 100,000-fold lower than those of clavulanic acid. There was also good activity against the class D enzymes.

A similar profile of activity was seen for AM-113. The activity against class A enzymes was poorest against TEM-1 (IC_{50} of 3.34μM) and most potent against the ESBL enzyme TEM-10 (IC_{50} of 0.008μM). Against both the class C and the class D enzymes, AM-113 was very active, and similarly to AM-112 had IC_{50} values between 1000 and 100,000-fold lower than clavulanic acid.

AM-114 and AM-115 both displayed potent activity against each class of enzyme. Activity against the class A enzymes was comparable to that of clavulanic acid and 1000-fold greater than either AM-112 or AM-113 against TEM-1. Activity against the class C and class D enzymes was similar to that of AM-112 and AM-113, and vastly superior to that of clavulanic acid.

2.4 Discussion

β-lactamase enzymes can be divided into four classes according to their amino acid sequence. Class A, C and D enzymes all contain serine in the active site. Class B enzymes contain a zinc ion (or ions) in the active site. Currently, no marketed β-lactamase inhibitor has any activity against class B enzymes. In this respect, the oxapenems do not differ from their established counterparts. Small-scale studies with the metallo-β-lactamase from *Bacillus cereus* demonstrated that AM-112 lacked any inhibitory activity against this enzyme (data not shown). However, while these metalloenzymes currently lack effective inhibitors, they do not yet pose the same magnitude of clinical threat as that of the class A and class C enzymes (Bush and Miller 1998).

All the β-lactamase enzymes are related and descended from a common ancestor. Class A and class D enzymes appear to be more closely related to each other than class C and class B enzymes, and vice versa (Massova and Mobashery 1998). Class A enzymes are effectively inhibited by clavulanic acid, tazobactam and sulbactam. Class C enzymes are less susceptible to inhibition by these compounds (Bush, *et al.* 1995). Class A enzymes such as TEM-1 and SHV-1 pose a clinical threat because of their propensity to mutate into extended-spectrum β-lactamases (ESBLs) which can hydrolyse a wide range of β-lactams, including antibiotics that the parent enzyme was incapable of destroying (Thomson and Smith Moland 2000). The emergence of class A enzymes which are resistant to β-lactamase inhibitors (IRT) poses a further threat to existing β-lactam compounds (Nicolas-Chanoine 1997).

Class C enzymes are capable of hydrolysing cephalosporins, a hugely important class of β-lactam antibiotics. Most class C enzymes are chromosomally produced, most commonly by Enterobacteriacae (Livermore 1995; Thomson and Smith Moland 2000). Hyperproduction of these enzymes, either by reversible induction or stable derepression can lead to highly resistant organisms. Transfer of *ampC* genes by plasmidic transfer to organisms such as *Klebsiella* spp. and *C. freundii* was first observed in the 1980s (Livermore 1995). Since then, the problem has become increasingly widespread, with huge implications for the future treatment of

infections. Thus the search for both novel antibacterial compounds which are stable to hydrolysis by class A and class C enzymes and novel inhibitors which are capable of inhibiting both these classes of enzyme is increasingly important.

Cell-free β-lactamase inhibiton assays are a commonly used method for determining the activity of inhibitors. The chromogenic cephalosporin nitrocefin provides a convenient means of observing and recording the activity of β-lactamase enzymes. The panel of enzymes used in this study was chosen to reflect the classes of enzymes that are of current significance in the clinical treatment of infections. Thus candidates from each class of the serine β-lactamases were represented. TEM-1, the parent strain of a number of ESBLs was included, along with an example of one such ESBL, TEM-10. SHV-5 was another ESBL strain included for comparison. Two class D β-lactamases, OXA-1 and OXA-5 were represented. P99 is a class C enzyme produced by *E. cloacae*; S+A is an inducible class C enzyme produced by *P. aeruginosa* and S2 is a constitutively produced class C enzyme produced by *S. marcescens*.

The results of the cell free β-lactamase inhibition experiments agree with the well established observation that clavulanic acid is a good inhibitor of class A enzymes and inhibits class C and class D enzymes to a lesser extent. The results also demonstrate that as a class, the novel oxapenems are highly effective inhibitors of the active-site serine β-lactamases. Individual profiles of activity vary between the four compounds. AM-112 and AM-115 are enantiomeric forms of the same compound, differing in the position of the C_6-hydroxyethyl substituent. Likewise, AM-113 and AM-114 differ in the position of the C_6-hydroxyethyl substituent. Both AM-112 and AM-115 have an amino-butyl side chain at position C_2, whereas AM-113 and AM-114 possess a tertiary propyl side chain at this position.

For class A enzymes, it appears that possessing a 1'S hydroxyethyl group confers enhanced activity to the oxapenem over a 1'R hydroxyethyl group, as AM-114 and AM-115 display lower IC_{50} values against class A compounds than do AM-112 or AM-113. The presence or absence of the C_2 substituent would appear to be less important. For class C enzymes, AM-113 and AM-114 are more active than AM-112

or AM-115, so it is possible that the C_2 substituent reduces the inhibitory activity of the oxapenem. The class D enzymes OXA-1 and OXA-5 give conflicting results. OXA-1 appears more susceptible to inhibition by oxapenems lacking the amino-butyl substituent at C_2 (AM-113 and AM-114) whereas the orientation of the hydroxymethyl substituent at C_6 seems more important for effective inhibition of OXA-5. Both AM-113 and AM-112 have a 1'R substituent at this position and lower IC_{50} values than AM-114 and AM-115, which have 1'S substituents. The C_2 side chain length and amino group appear less important as AM-112 and AM-115 both posses a long side chain with a terminal amino group, yet AM-112 is the most active oxapenem against OXA-5 and AM-115 is the least active. It must be stressed that these structure activity observations are drawn from a small panel of enzymes and may not be representative of each class of enzyme. Chapter 5 describes the results of molecular modelling studies carried out with the class A TEM-1 and class C P99 enzymes with each of the oxapenems.

Chapter 3. Evaluation of oxapenem antimicrobial activity

3.1 Introduction

One of the main criteria for judging the potential of a novel compound, or family of compounds is the antibacterial spectrum. *In vitro* screening of a novel compound against a panel of common bacteria is likely to be the first step of a drug evaluation process. The basis of much microbiological testing involves determination of the minimum inhibitory concentration (MIC) of a compound against an organism. The MIC is defined as the lowest concentration of a compound required to inhibit the growth of an organism. It is frequently determined in broth culture as the first tube or well containing the test compound that displays no turbidity (indicating growth of the test organism) compared to the control tube or well. A less commonly used parameter is the minimum bactericidal concentration (MBC), defined as the lowest concentration of the test compound required to kill the organism.

The focus of the work in this chapter has been to evaluate the antimicrobial activity of the novel oxapenem compounds AM-112, AM-113, AM-114 and AM-115. Broth microdilution carried out in accordance with NCCLS guidelines has been the method of choice, mainly due to the limited quantities of each oxapenem compound that have been available. The activity of the compounds in combination with other established agents, mainly cephalosporins and principally ceftazidime has been determined. Test organisms have included both Gram-positive and Gram-negative strains; a variety of β-lactamase producing strains and drug-resistant strains such as MRSA and VRE. The majority of strains were typed strains from ATCC or NCTC, although a few clinical isolates were included. Limitations of time and available compound meant that replicates of MICs have not been carried out, nor have a number of isolates of each strain been tested. The MIC data presented in this chapter is intended to be an overview of the activity of the oxapenems rather than an exhaustive evaluation of the antimicrobial spectrum of these compounds.

3.2 Materials and Methods

3.2.1 *Broth microdilution method for the determination of MIC values*

The broth microdilution method was carried out in accordance with NCCLS standards (NCCLS 2001). An overnight culture of each organism was grown on MHA plates at 37°C. A loopful of the organism was suspended in sterile saline and the optical density was adjusted to 1.0 at 470_{nm}. This gave a concentration of approximately 10^8cfu/ml. This suspension was then diluted 1:100 in sterile MHB to give a cell density of 10^6cfu/ml. Sterile U-bottomed microtitre plates (Fisher, UK) were used for the MIC tests. A volume of 50µl of sterile MHB was dispensed into each well on the plate. Test antibiotics were prepared at 2.56mg/ml in sterile 10mM SPB, and diluted 1:10 in sterile MHB to give a concentration of 256µg/ml. A volume of 50µl was dispensed into the wells of the first column, and mixed thoroughly. A 50µl aliquot was then removed from the wells in the first column and dispensed into the wells of the second column. This process was repeated across the wells of the plate. The concentration of the test antibiotic now ranged from 128µg/ml in the first column of wells to 0.06µg/ml in the twelfth well. A volume of 50µl of the test organism was then inoculated into each well of the microtitre plate. The final antibiotic concentration ranged from 64µg/ml to 0.03µg/ml. Each well was inoculated with 10^5cfu/ml. The microtitre plates were sealed with adhesive plastic film and incubated for twenty-four hours at 37°C. Growth of the test organism was seen as a "button" of cells at the bottom of the well.

3.2.1.1 *Broth microdilution in the presence of serum*

The NCCLS broth microdilution method was adapted to investigate the effects of human serum on the activity of the test compounds. The required strains were grown as previously described. MHB was prepared at single and double strength, sterilised and allowed to cool. Sterile filtered human serum (Sigma, UK) was mixed with double strength MHB in a 1:1 ratio (MHB+Serum). The microdilution procedure was

carried out as previously described using either single strength MHB or MHB+Serum.

3.2.1.2 *Broth microdilution with a fixed inhibitor concentration*

MICs were determined for a panel of organisms using a test antibiotic in combination with a fixed concentration of a β-lactamase inhibitor. The test inhibitors were prepared and dissolved in MHB at the required concentration (16μg/ml). A 50μl volume of the inhibitor solution was dispensed into all wells of each microtitre plate. An aliquot of 50μl of the test antibiotic (256μg/ml) was dispensed into all wells of the first column and double diluted across the plate, as previously described. Aliquots of 50μl of the test organism were inoculated into all wells of the microtitre plate. The final antibiotic concentration range was 64-0.03μg/ml with a fixed inhibitor concentration of 4μg/ml. The microtitre plates were sealed with adhesive plastic film and incubated for twenty-four hours at 37°C. Growth of the organism was seen as a "button" of cells at the bottom of the well.

3.2.2 *Agar dilution method for the determination of MIC values*

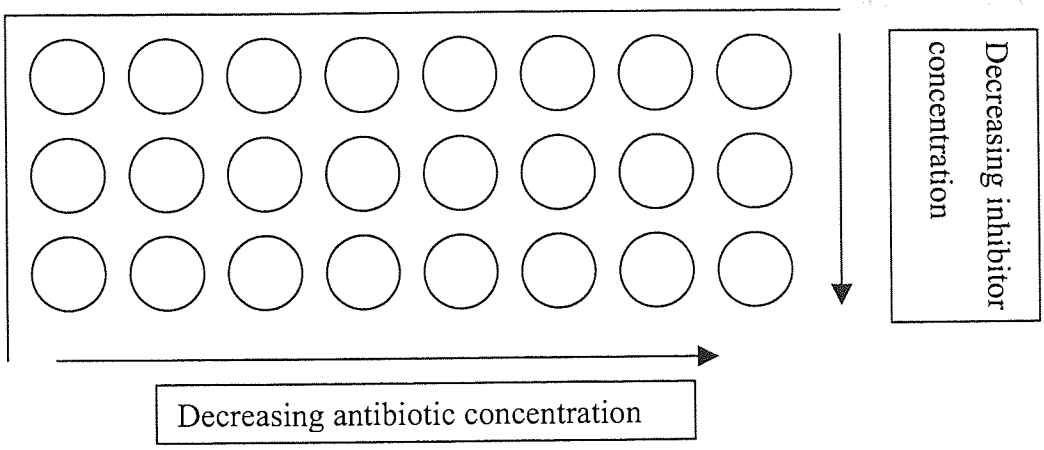
MHA was prepared, sterilised and tempered in a water bath to 45°C. For MIC studies examining the effect of pH, MHA was prepared according to the manufacturers instructions. The pH of the medium was adjusted using 1M NaOH or 1M HCl as required, prior to autoclaving. The required test organisms were grown overnight at 37°C on MHA plates. A loopful of each organism was removed from the plate and suspended in sterile saline to give an optical density of 1.0 at 470_{nm}. Each organism was then diluted 1:100 in sterile saline to give a cell concentration of 10^6cfu/ml. Test antibiotics were prepared in 10mM SPB. An aliquot of 100μl of the test antibiotic at the required concentration was dispensed into a 30ml sterile plastic universal container. A volume of 24.9ml of molten MHA was added to the universal and mixed thoroughly. The molten agar was immediately poured into a sterile petri dish and allowed to cool and set, prior to storage at 4°C until required. The concentration

range of the test antibiotics was 64-0.03μg/ml. Aliquots of 150μl of each test organism were dispensed into 36 wells of a sterile microtitre plate. A 36-pronged multipoint inoculator was sterilised with 70%$^{v}/_{v}$ ethanol and used to inoculate the surface of each agar plate. The multi-point inoculator delivered a volume of 10-20μl, thus the final inoculum was in the order of 10^5cfu/ml. The inoculated plates were incubated for twenty-four hours at 37°C, and then examined for growth.

3.2.3 *Checkerboard method for the assessment of β-lactamase inhibitors on the activity of sensitive β-lactams*

The required test organisms were grown overnight at 37°C on MHA plates. A loopful of each organism was removed from the plate and suspended in sterile saline to give an optical density of 1.0 at 470nm. Each organism was then diluted 1:100 in sterile MHB to give a cell concentration of 10^6cfu/ml. Test antibiotics and inhibitors were prepared in sterile 10mM SPB then diluted in sterile MHB to the required concentration. A volume of 50μl of sterile MHB was dispensed into each well of a U-bottomed microtitre plate. A 50μl aliquot of the test antibiotic (at 1024μg/ml) was dispensed into each well of the first column and double diluted across the plate. The antibiotic concentration range was now 512-0.125μg/ml. A volume of 50μl of the test inhibitor (at 128μg/ml) was placed in each well of the first row and double diluted down the plate. The inhibitor concentration range was now 64-0.06μg/ml. A 50μl aliquot of the test organism was then dispensed into each well of the microtitre plate. The final antibiotic concentration ranged from 128-0.06μg/ml across the plate and the inhibitor concentration ranged from 32-0.25μg/ml down the plate (Fig. 3.1). Each plate was sealed with adhesive plastic film and incubated at 37°C for twenty-four hours. Growth of the test organism was seen as a "button" of cells at the base of the well.

FIGURE 3.1. Schematic representation of microtitre plate for checkerboard method.



3.2.4 *Bioassay of AM-112 and AM-113*

An overnight culture of *Micrococcus luteus, S. aureus* NCTC 6571 and MRSA 96-7778 were grown in MHB at 37°C. Aliquots of 1ml of these cultures were inoculated into fresh, pre-warmed MHB and grown for three hours at 37°C. MHA was prepared according to the manufacturers instructions, sterilised in 120ml aliquots and tempered to 50°C. Bioassay plates (Nunc, UK) measuring 230mm by 230mm were sterilised with 70%$^{v}/_{v}$ ethanol. The molten agar at 50°C was inoculated with 1ml aliquots of each of the log phase cultures, and poured into the bioassay plates, to a depth of 3mm. Wells of approximately 5mm were cut in the set agar in a Latin square pattern. Test compounds were prepared at 100, 50, 20, 5, 2, 0.4, 0.013 and 0.0025μg/ml in 10mM SPB. A volume of 40μl of each of the test solutions was placed into the wells, with three replicates for each concentration. The bioassay plates were incubated at 37°C for twenty-four hours. Zones of inhibition were measured and plotted against the log of the concentration.

3.2.5. *Determination of the effect of serum on AM-112 and AM-113*

Bioassay plates were prepared as previously described with *S. aureus* NCTC 6571 as the test organism. AM-112 and AM-113 were prepared at 50μg/ml in 10mM SPB. The test compounds were mixed with an equal volume of either sterile-filtered human serum (Sigma, UK) or 10mM SPB and maintained at 37°C in a water bath.

Aliquots of 0.5ml were removed and filtered through Microcon YMT membrane centrifuge filters (Mwt cut-off 10kDa; Amicon Corporation, Bedford, MA, USA) immediately, and fifteen, thirty and sixty minutes, after initial mixing. The samples were centrifuged at 14,000 rpm for 15 minutes to separate the supernatant from the serum proteins. Aliquots of 40µl of the filtered samples were inoculated into the wells of the bioassay plates and incubated for twenty-four hours at 37°C. Samples were also removed at the same time points and dispensed onto bioassay plates without prior ultrafiltration and incubated as described previously. Zones of inhibition were measured after twenty-four hours.

3.3 Results

3.3.1 *Bioassay of AM-112 and AM-113*

S. aureus NCTC 6571 proved to be very sensitive to both AM-112 and AM-113, with measurable zones of inhibition across the concentration range 2-100μg/ml, so this organism was selected for the bioassay studies. Figure 3.2 shows the calibration curve for AM-112. There was a very good fit of the data to the trend line, with an R^2 value of 0.9948. The goodness-of-fit for the AM-113 calibration curve was not as good, with a R^2 value of 0.9617 (Figure 3.3).

3.3.2 *Serum stability of AM-112 and AM-113*

Using the calibration curves, it was then possible to calculate the concentrations of various unknown samples from other bioassays carried out in the presence of serum or buffer as described in the Materials and Methods section. Ultrafiltration, using Microcon eppendorf filters, was used to separate serum proteins from the supernatant for the bioassays. Samples incubated in buffer were also filtered with eppendorf filters to check whether the compounds bound to the filter. Figure 3.4 shows the effect of pre-incubation with serum on the activity of AM-112. The graph shows that there was an almost 50% loss of activity of the sample which had been filtered, whereas the sample that had not been filtered had lost less than 33% of its activity. The activity of the filtered sample was higher than that of the unfiltered sample at all points on the graph, but was especially noticeable at the start, when the concentration of the filtered sample was 19.4μg/ml, while the unfiltered sample was 13.9μg/ml.

FIGURE 3.2. Calibration curve of AM-112 against *S. aureus* NCTC 6571.

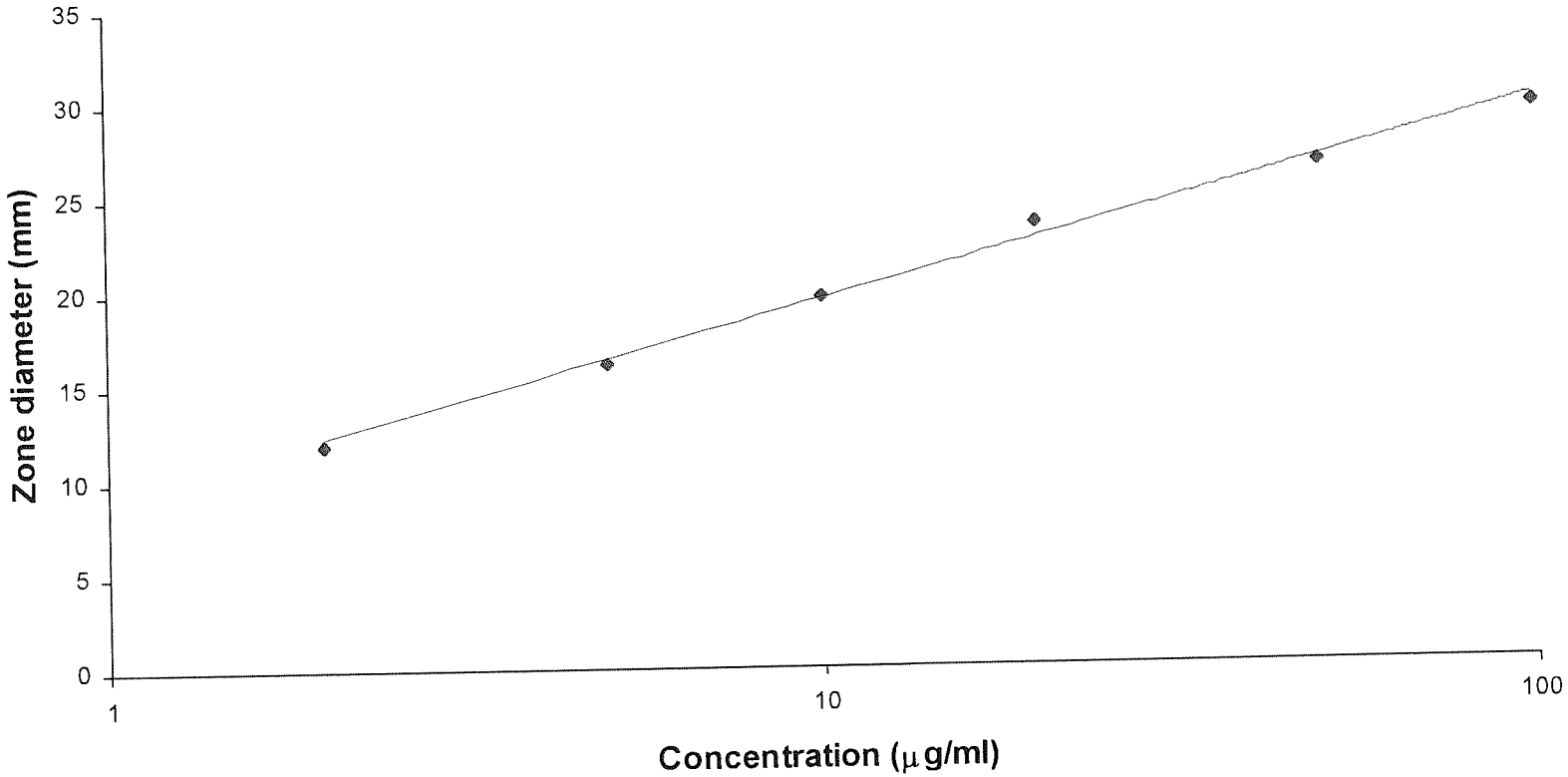


FIGURE 3.3. Calibration curve of AM-113 against *S. aureus* NCTC 6571.

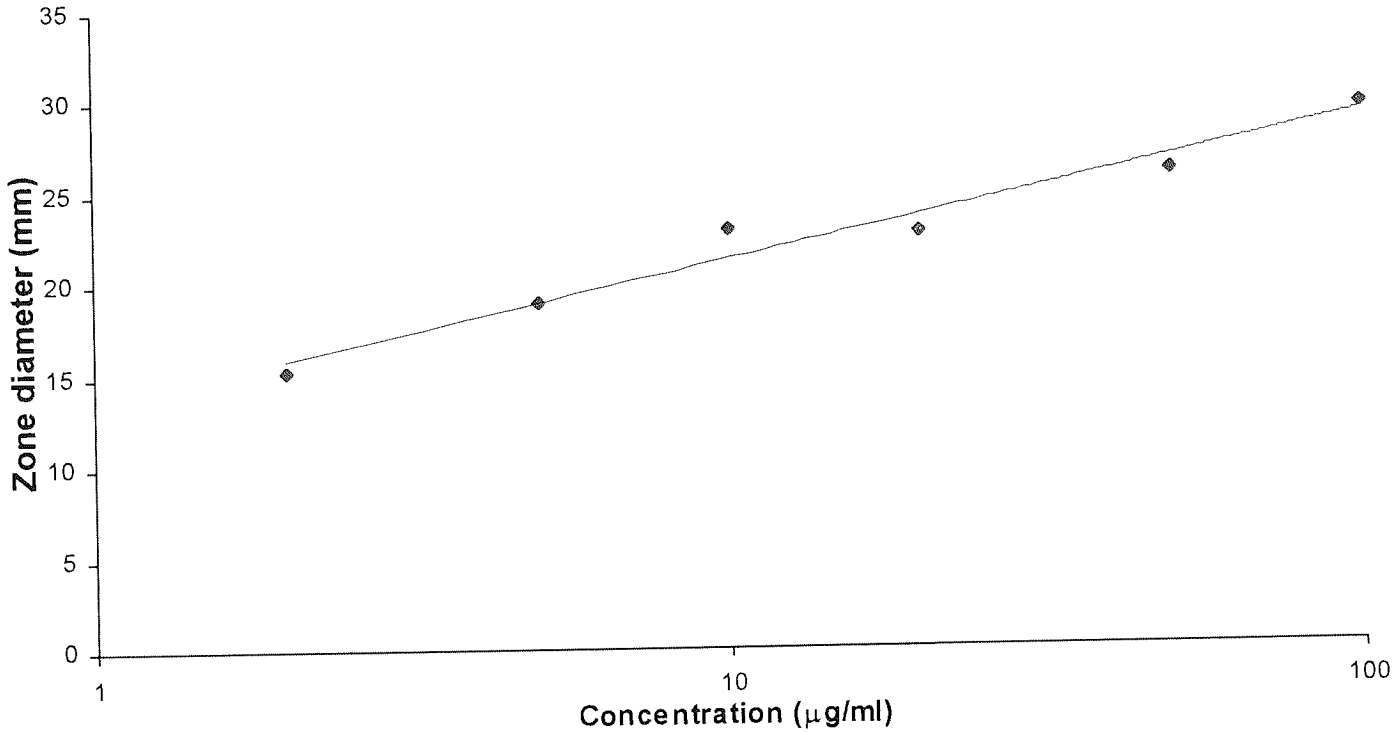


FIGURE 3.4. Effect of pre-incubation in serum, with and without ultrafiltration, on the concentration of AM-112, as determined by activity against *S. aureus* NCTC 6571

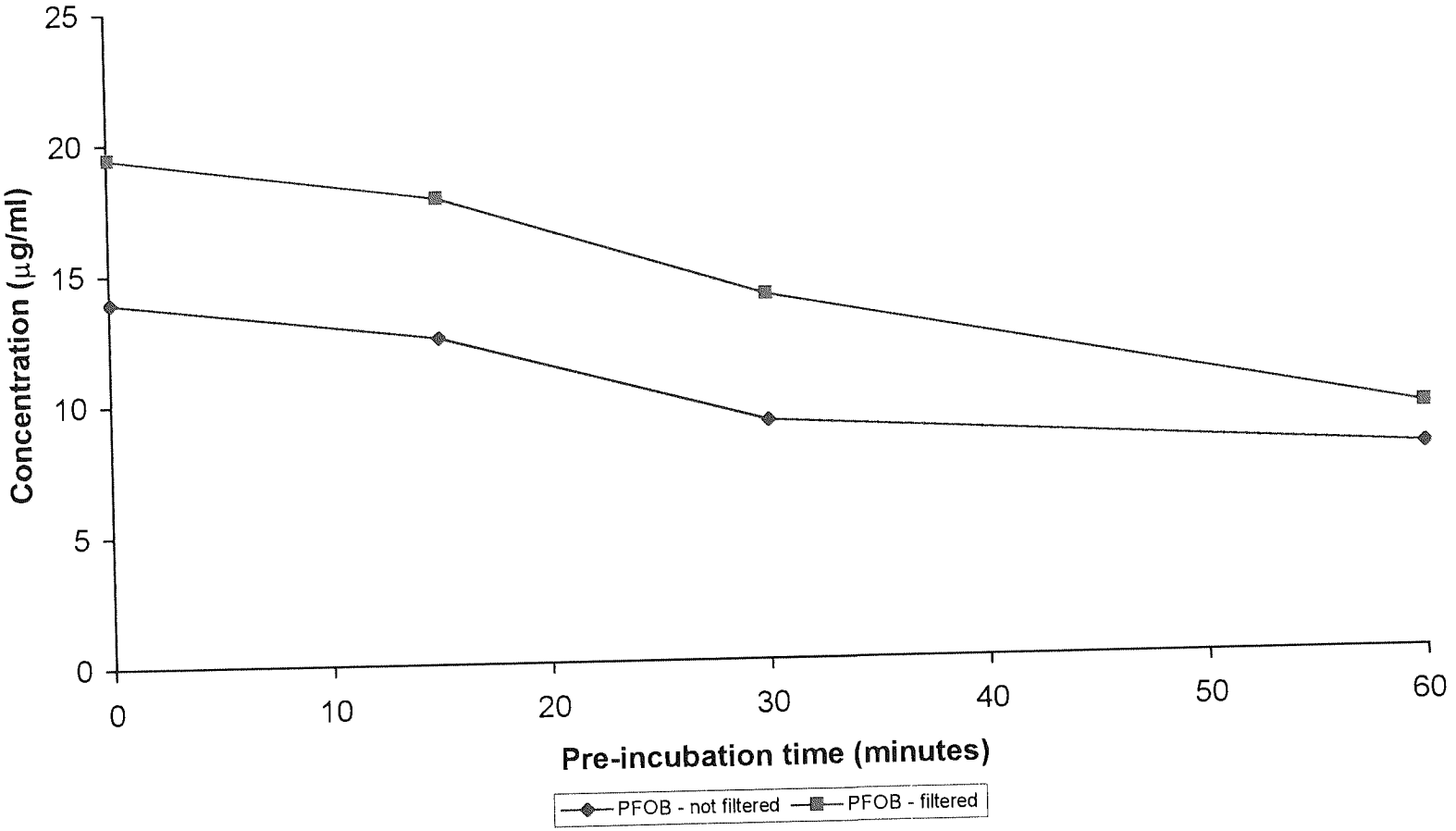


Figure 3.5 shows the dramatic loss of activity against *S. aureus* NCTC 6571 when AM-113 was pre-incubated with serum. There appears to be little difference in the activity of AM-113 whether it was ultrafiltered or not. The initial concentration of AM-113 is 25μg/ml when the sample was mixed with serum, but the measured concentration at t = 0 was only 2.8 or 2.6μg/ml. After fifteen minutes of incubation with serum, the concentration of the filtered and unfiltered samples is 0.93 and 0.47μg/ml respectively. After thirty minutes there was no measurable activity of either sample.

The results of pre-incubating AM-112 in buffer followed by ultrafiltration are shown in Figure 3.6. Pre-incubation with buffer followed by filtration results in a gradual loss of activity. There was a 20% loss of activity after three hours of pre-incubation with buffer and filtration. The sample that was not filtered shows variation in the activity assayed. An initial concentration of 20.3μg/ml dropped to 11.6μg/ml after three hours pre-incubation, despite the concentration after ninety minutes being measured as 21.2μg/ml. This variation was probably due to experimental error.

FIGURE 3.5. Effect of pre-incubation in serum, with and without ultrafiltration, on the concentration of AM-113, as determined by activity against *S. aureus* NCTC 6571

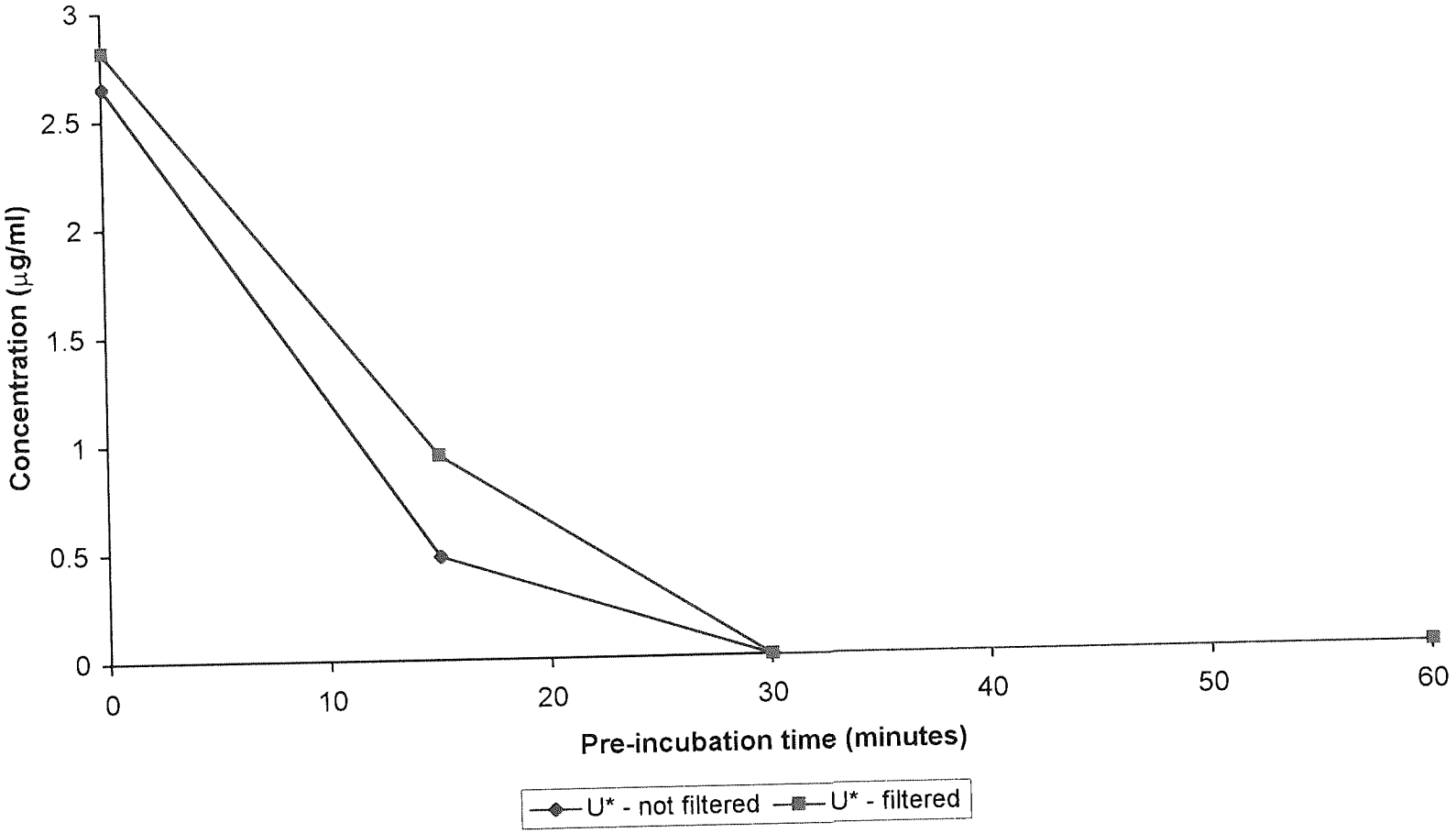
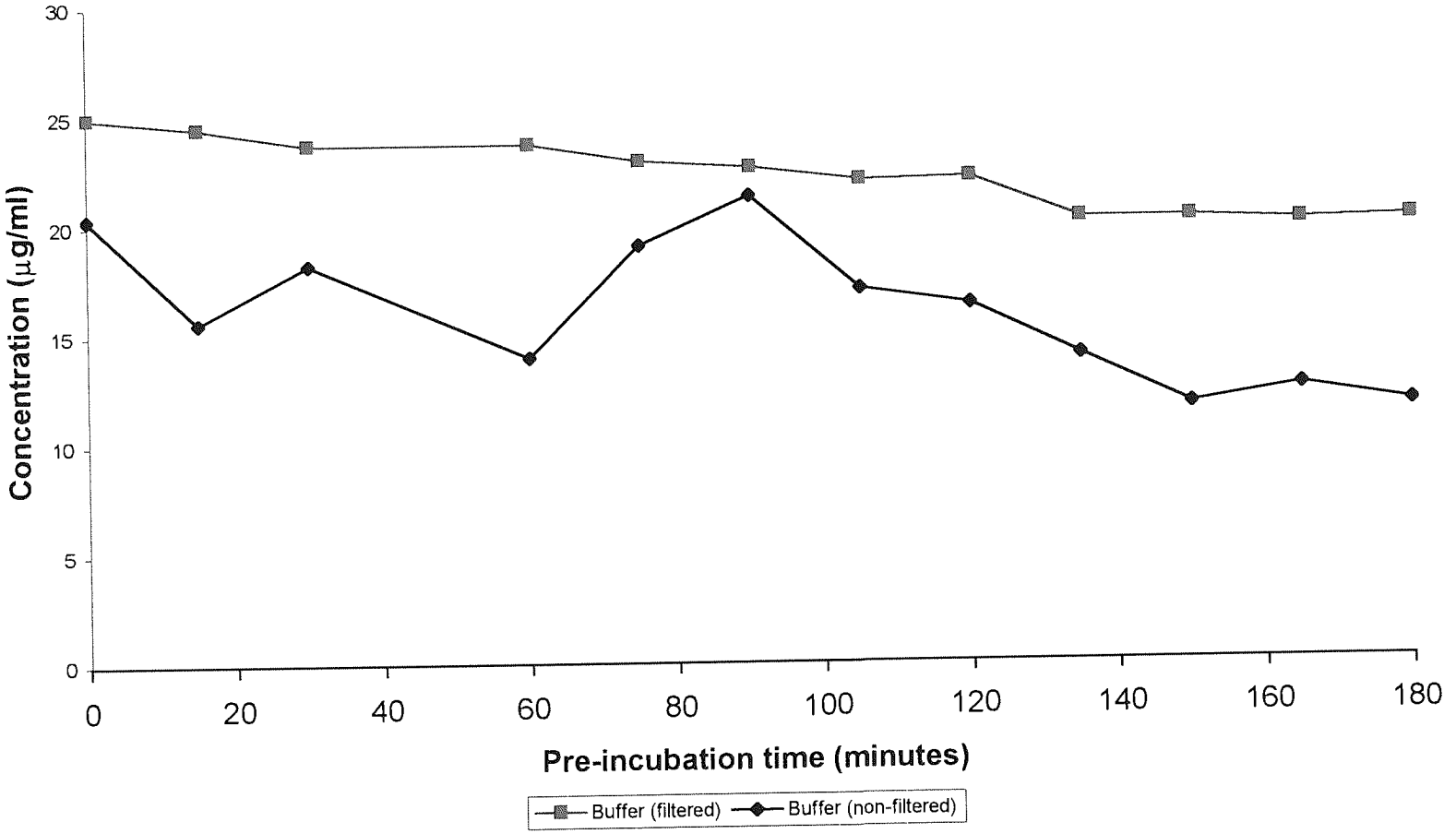
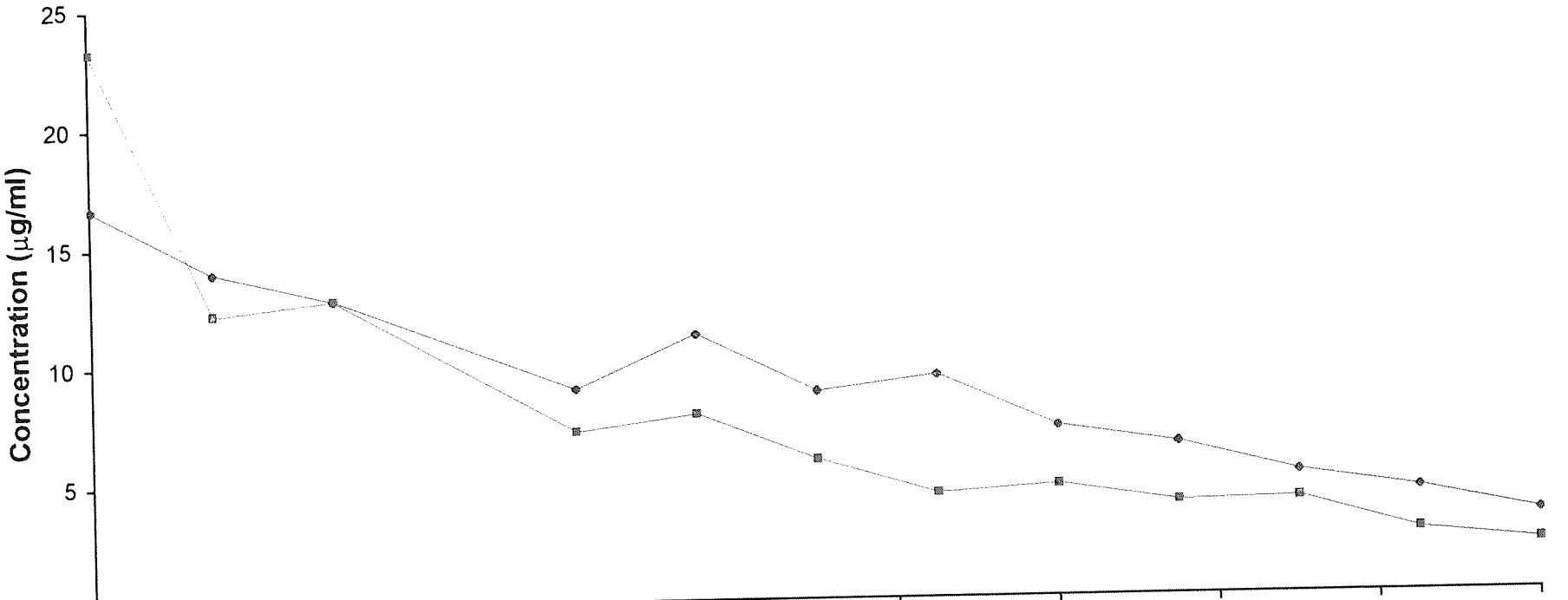


FIGURE 3.6. Effect of pre-incubation in buffer, with and without ultrafiltration, on the concentration of AM-112, as determined by activity against *S. aureus* NCTC 6571



AM-112 was incubated in serum for three hours. The loss of activity is shown in Figure 3.7. Samples that were filtered had an initial concentration of 23.2μg/ml, which fell to 12.2μg/ml after fifteen minutes, a decrease of almost 50%. After one hour, the concentration had fallen to 7.1μg/ml and after three hours the concentration was 2.1, less than 10% of the initial concentration. Unfiltered samples demonstrated a similar decay pattern. The initial concentration was lower than the filtered sample, 16.7μg/ml compared to 23.3μg/ml. The initial decay was slower than the filtered sample, after fifteen minutes the concentration had fallen to 13.9μg/ml, a decrease of 17%. The activity of the filtered sample had decreased by 50% after one hour, while after three hours the concentration was 3.35μg/ml, 20% of the initial concentration.

FIGURE 3.7. Effect of pre-incubation in serum, with or without ultrafiltration on the concentration of AM-112 as determined by activity against *S. aureus* NCTC 6571.



3.3.3 *Effect of serum on the activity of ceftazidime or piperacillin alone and in combination with clavulanic acid, tazobactam, AM-112 or AM-113*

Tables 3.1 and 3.2 list the minimum inhibitory concentrations of ceftazidime or piperacillin alone and in combination with a range of β-lactamase inhibitors, carried out in the presence or absence of human serum. All strains produced β-lactamase enzymes. Ceftazidime was inactive against the class C enzyme producing *E. cloacae* strains, and the extended spectrum β-lactamase producing *E. coli* strain. An MIC of 16μg/ml was recorded for the *E. coli* OXA-1 strain, while the other strains had MICs in the range <0.03-1μg/ml. A 4μg/ml concentration of clavulanic acid was effective in lowering the ceftazidime MIC against *E. coli* ESBL+ and *E. coli* OXA-1 to 0.25μg/ml, but had no effect on the *E. cloacae* strains. Tazobactam displayed a similar profile of activity to clavulanic acid. AM-112 and AM-113, when combined with ceftazidime, lower the MIC against the majority of strains, except *K. pneumoniae* K1+ and *Serratia* spp. The MIC for *E. cloacae* P99+ was lowered from >64μg/ml to 0.5μg/ml and 0.25μg/ml by AM-112 and AM-113 respectively.

The addition of 50% human serum to the medium resulted in a mixed spectrum of activity of ceftazidime and the β-lactamase inhibitors. Alone, the activity of ceftazidime was decreased against *E. coli* TEM-1, unchanged against *E. coli* ESβL+ and *E. cloacae* Hennessey, and enhanced against the other strains. The activity of the clavulanic acid and ceftazidime combination, in the presence of serum, was enhanced against the *E. coli* strains, *C. diversus* 2046E and *K. pneumoniae* K1+ and unchanged against the *E. cloacae* strains and *Serratia* spp. When combined with ceftazidime in the presence of serum, the activity of tazobactam was enhanced against all strains, except *E. cloacae* Hennessey, which remained unchanged. AM-112 and AM-113 displayed a mixed spectrum of activity when combined with ceftazidime in the presence of serum. AM-112 retained the activity against *C. diversus*, *K. pneumoniae* K1+ and *Serratia* spp., but was less active against the other strains, compared to the combination of AM-112 and ceftazidime in the absence of serum. The activity of AM-113 also varied in the presence of serum.

TABLE 3.1. Minimum inhibitory concentration (MIC) of ceftazidime alone and in combination with a fixed 4μg/ml concentration of either clavulanic acid, tazobactam, AM-112 or AM-113. MICs carried out in the presence or absence of human serum.

	MIC (μg/ml) Serum					MIC (μg/ml)				
Organism	Alone	CLAV	TAZ	AM-112	AM-113	Alone	CLAV	TAZ	AM-112	AM-113
E. coli W3110 R+	2	0.06	<0.03	0.125	<0.03	0.5	0.25	0.5	0.06	<0.03
E. coli W3110 R-	2	0.06	<0.03	0.125	0.03	16	0.25	0.125	<0.03	<0.03
E. coli ESBL+[a]	>64	0.125	0.25	32	64	>64	0.25	0.5	8	4
E. cloacae P99+	16	>64	8	32	>64	>64	>64	>64	0.5	0.25
E. cloacae Hennessy	>64	>64	>64	4	>64	>64	>64	>64	8	8
C. diversus 2046E	<0.03	0.5	<0.03	<0.03	2	1	0.125	0.25	<0.03	0.125
K. pneumoniae K1+	<0.03	0.125	<0.03	0.25	0.5	0.125	0.25	1	0.25	0.5
Serratia sp.	<0.03	0.125	<0.03	<0.03	0.125	<0.03	0.125	<0.03	<0.03	0.25

CLAV, clavulanic acid; TAZ, tazobactam.

[a] Extended spectrum β-lactamase enzyme.

TABLE 3.2. Minimum inhibitory concentration (MIC) of piperacillin alone and in combination with a fixed 4μg/ml concentration of either clavulanic acid, tazobactam, AM-112 or AM-113. MICs carried out in the presence or absence of human serum.

	MIC (μg/ml) Serum					MIC (μg/ml)				
Organism	Alone	CLAV	TAZ	AM-112	AM-113	Alone	CLAV	TAZ	AM-112	AM-113
E. coli W3110 R+	>64	32	>64	>64	>64	>64	1	<0.03	<0.03	<0.03
E. coli W3110 R-	>64	1	1	0.5	8	>64	0.06	1	0.5	<0.03
E. coli ESBL+[a]	>64	32	4	>64	>64	>64	8	16	>64	>64
E. cloacae P99+	>64	>64	>64	32	>64	>64	>64	>64	1	4
E. cloacae Hennessy	>64	>64	>64	>64	>64	>64	>64	>64	32	32
C. diversus 2046E	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64
K. pneumoniae K1+	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64
Serratia sp.	1	1	0.25	0.06	0.25	2	1	0.25	0.25	0.5

CLAV, clavulanic acid; TAZ, tazobactam.

[a] Extended spectrum β-lactamase enzyme.

The activity was decreased against *E. coli* ESβL+, the *E. cloacae* strains and *C. diversus* 2046E, unchanged against *E. coli* TEM-1 and OXA-1 and *K. pneumoniae* K1+ and enhanced against *Serratia* spp.

Piperacillin lacked activity against all the strains in the panel with MICs above 64μg/ml, except *Serratia* spp., which had an MIC of 2μg/ml. Addition of clavulanic acid reduced the MIC for the *E. coli* strains and *Serratia* spp., but had no effect on the other strains. Tazobactam followed a similar trend, with no activity against *E. cloacae* strains, *C. diversus* or *K. pneumoniae* K1+. AM-112 and AM-113 reduced the MIC of *E. cloacae* Hennessey to 32μg/ml and *E. cloacae* P99+ to 1μg/ml and 4μg/ml respectively. Both compounds lacked activity against *E. coli* ESBL+ and also *C. diversus* 2046E and *K. pneumoniae* K1+. Neither piperacillin alone or combined with any of the β-lactamase inhibitors had any activity, in the presence of serum, against *E. coli* TEM-1, the *E. cloacae* P99+ strain, *C. diversus* 2046E or *K. pneumoniae* K1+. *E. coli* OXA-1 was rendered more susceptible when piperacillin was combined with any of the β-lactamase inhibitors, with MICs in the range 0.5-8μg/ml. Lower MIC values were also obtained when piperacillin was combined with tazobactam, AM-112 or AM-113 against *Serratia* spp.

3.3.4 *Effect of pH on the activity of ceftazidime alone and in combination with clavulanic acid or AM-112*

Table 3.3 and Table 3.4 list the minimum inhibitory concentrations of ceftazidime, clavulanic acid and AM-112 against a panel of Gram-positive and Gram-negative organisms. The agents were tested alone, and in combination, in Mueller Hinton broth at pH 6.4, 7.4 and 8.4. The Mueller Hinton broth was prepared at three different pHs to determine the effect of pH on the activity of AM-112 and identify the optimum pH for activity.

Alone, ceftazidime was most active at pH 6.4, having MICs in the expected range for *E. coli* and *S. aureus*. At pH 7.4 ceftazidime was less active against all strains, while at pH 8.4 ceftazidime activity was only enhanced against *P. aeruginosa* ATCC 27853. Clavulanic acid alone lacked activity against the panel at each of the pH

levels. AM-112 also lacked activity against the panel at pH 6.4 and 8.4 and was most active at pH 7.4. This activity was weaker than expected for AM-112 against both *E. coli* and *S. aureus* strains.

When combined with clavulanic acid, AM-112 had a poorer spectrum of activity against the panel than AM-112 alone, and a slightly better spectrum than clavulanic acid alone. Combination of clavulanic acid and ceftazidime resulted in a mixed spectrum of activity against the panel at each pH level. At pH 6.4, the combination performed worse that ceftazidime alone, with an MIC against *E. coli* ATCC 35218 of >128μg/ml, compared to 2μg/ml for ceftazidime alone. However, at pH 7.4 the MIC of the combination was 16μg/ml, equal to the MIC of ceftazidime alone. At pH 8.4 the MIC for *P. aeruginosa* ATCC 27853 was 2μg/ml, compared to 128μg/ml at pH 6.4 and 16μg/ml at pH 7.4.

The combination of ceftazidime and AM-112 was less active at pH 8.4 that at either pH 6.4 or pH 7.4. There appeared to be little difference in the activity of the combination at either pH 6.4 or pH 7.4. There could be a two- to four-fold difference in the MIC for a given organism, but these differences in activity were divided between the pH levels.

TABLE 3.3. Minimum inhibitory concentration (MIC) of ceftazidime, clavulanic acid and AM-112, in Muller Hinton broth at pH 6.4, 7.4 and 8.4.

	Ceftazidime			Clavulanic acid			AM-112		
Organism	6.4	7.4	8.4	6.4	7.4	8.4	6.4	7.4	8.4
E. coli ATCC 25922	0.5	0.5	16	64	>128	>128	128	32	>128
E. coli ATCC 35218	2	16	32	>128	>128	>128	64	16	>128
MRSA Innsbruck	16	32	32	>128	>128	>128	128	8	128
S. aureus ATCC 29213	2	128	16	>128	64	>128	64	16	32
E. cloacae P99+	32	32	32	>128	64	>128	32	32	>128
E. faecalis ATCC 29212	32	64	>128	>128	>128	>128	64	32	>128
E. faecium ATCC 10547	>128	>128	>128	>128	>128	>128	32	32	128
P. aeruginosa ATCC 27853	8	16	4	>128	>128	>128	128	>128	>128

TABLE 3.4. Minimum inhibitory concentration (MIC) of ceftazidime, clavulanic acid and AM-112, in combination in Muller Hinton broth at pH 6.4, 7.4 and 8.4.

	Ceftazidime+AM-112			Ceftazidime+Clavulanic acid			Clavulanic acid+AM-112		
Organism	6.4	7.4	8.4	6.4	7.4	8.4	6.4	7.4	8.4
E. coli ATCC 25922	1	1	16	16	32	64	128	128	>128
E. coli ATCC 35218	2	0.5	32	>128	<0.06	32	128	128	>128
MRSA Innsbruck	<0.06	0.5	16	>128	16	16	128	128	>128
S. aureus ATCC 29213	0.5	1	4	16	>128	32	128	64	128
E. cloacae P99+	1	<0.06	32	>128	>128	>128	>128	64	>128
E. faecalis ATCC 29212	2	4	32	32	>128	>128	>128	>128	>128
E. faecium ATCC 10547	1	1	32	32	>128	>128	>128	>128	>128
P. aeruginosa ATCC 27853	2	1	1	>128	16	2	>128	64	128

3.3.5 *Activity of ceftazidime in combination with either clavulanic acid or AM-112 against a panel of enterococci*

TABLE 3.5. Minimum inhibitory concentration (MIC) of ceftazidime, clavulanic acid and AM-112 alone and in combination against a panel of enterococci.

Organism	MIC (μg/ml)			MIC (μg/ml)[a]	
	CAZ	CLAV	AM-112	CAZ + CLAV	CAZ + AM-112
E. faecalis Phillips	>128	>128	32	64	<0.06
E. faecalis SFZ	>128	>128	64	32	<0.06
E. faecalis NCTC 5957	>128	>128	32	64	<0.06
E. faecalis 24952	>128	>128	32	>128	<0.06
E. faecalis NCTC 7171	>128	>128	16	>128	<0.06
E. hirae ATCC 10541	>128	>128	16	>128	<0.06
VRE 300 1562[b]	>128	>128	128	>128	>128
VRE 300 1590	>128	>128	>128	>128	>128
VRE 300 1662	>128	>128	>128	>128	>128
VRE 300 2043	>128	>128	32	>128	<0.06

[a]Fixed 4μg/ml concentration of inhibitor.

[b]VRE, vancomycin-resistant enterococci.

The MICs for the panel of enterococci determined by broth microdilution are listed in Table 3.5. The panel of enterococci included six vancomycin sensitive strains and four clinical isolates, which were vancomycin resistant. Ceftazidime and clavulanic acid alone proved to be inactive against the panel. AM-112 displayed differing degrees of activity, with MICs of 16μg/ml against *E. faecalis* NCTC 7171 and *E. hirae* ATCC 10541; MICs of 32μg/ml against three strains of enterococci and VRE 300 2043 and an MIC of 64μg/ml against *E. faecalis* SFZ. AM-112 did not display activity against three VRE strains.

When ceftazidime was combined with a fixed clavulanic acid concentration of 4μg/ml, the combination reduced the ceftazidime MIC from >128μg/ml to between 32 and 64μg/ml for three vancomycin sensitive strains. There was no activity against the vancomycin resistant strains. When combined with 4μg/ml of AM-112, the activity of ceftazidime was enhanced against the six vancomycin-sensitive strains

and one vancomycin resistant strain. The MICs were lowered from >128μg/ml to <0.06μg/ml, a 2048-fold reduction. The combination lacked activity against three of the vancomycin-resistant strains.

3.3.6 *Activity of a combination of AM-112 with various antibiotics against enterococci*

TABLE 3.6. Fractional inhibitory concentration (FIC) indices of various antibiotics combined with AM-112, against four enterococcal strains.

Compound	FIC index			
	E. faecalis 56059 *vanA*[a]	*E. faecalis* 78097 *vanB*[b]	*E. faecalis* ATCC 29212	*E. hirae* ATCC 10541
Ceftazidime	1.003	0.125	0.156	0.5
Cefuroxime	1.007	0.023	0.063	0.251
Vancomycin	2	0.265	0.265	0.531
Clavulanic acid	2	2	2	2
Imipenem	1.015	2.015	0.075	0.061
Ampicillin	0.265	0.515	0.075	0.061

[a] *E. faecalis* clinical isolate expressing the *vanA* vancomycin resistance gene.

[b] *E. faecalis* clinical isolate expressing the *vanB* vancomycin resistance gene.

Table 3.6 lists the fractional inhibitory concentration (FIC) indices of various antibiotics combined with AM-112, determined against four enterococcal strains. The FIC indices were calculated from the results of checkerboard MICs determined by broth microdilution in Mueller Hinton broth. The FIC index for two drugs A and B is calculated by dividing the concentration of drug A which is the lowest inhibitory concentration in that row or column by the MIC for drug A alone. The same calculation is carried out for drug B and the two FICs are summed to yield the FIC index (Eliopoulos and Moellering Jr. 1991).

$$\left(\frac{[A]}{MIC_A}\right)+\left(\frac{[B]}{MIC_B}\right) = FIC_A + FIC_B = \text{FIC index}$$

Synergism is defined as a FIC index of ≤0.5, additivity as a FIC index of 1.0 a antagonism is defined as a FIC index equal to or above 2.0.

There was an antagonistic effect with the combination of AM-112 and clavula acid against all the strains tested, with a FIC index of 2.0 for each strain. B ceftazidime and cefuroxime had additive effects against *E. faecalis* 56059, a displayed synergy against the other enterococci. The combination of vancomycin a AM-112 was antagonistic against *E. faecalis* 56059, but synergistic against the th other strains. Imipenem was additive with AM-112 against *E. faecalis* 560 antagonistic against *E. faecalis* 78097 and synergistic against vancomycin-sensitive enterococci. Ampicillin was synergistic with AM-112 aga all the strains tested.

3.3.7 *Activity of ceftazidime and oxapenems, alone and in combination agains panel of β-lactamase producing strains*

Table 3.7 lists the minimum inhibitory concentrations of ceftazidime, AM-1 AM-113, AM-115 and AM-114 against a panel of thirty-six bacterial strains. panel of organisms consisted of both Gram-positive and Gram-negative stra β-lactamase producing strains were included in the panel, including *E. coli* SHV-5 and *E. coli* J53 TEM-10, which are extended spectrum β-lactamase (ES producers. Two enterococcal strains that are resistant to vancomycin were inclu *E. faecalis* 57097 and *E. faecalis* 78097, which express the *vanA* and *v* phenotype respectively. Ceftazidime was active against both β-lactamase non-β-lactamase producing *E. coli* strains with MICs in the range 0.125-0.25μg for non-enzyme producing strains and 0.25-2μg/ml for enzyme producers. Str producing ESBLs were more resistant to ceftazidime, with MICs between 16μg and >64μg/ml. Methicillin-sensitive staphylococci had MICs in the range 4-8μg while the methicillin-resistant strain MRSA Innsbruck had an MIC of >64μg/ml.

TABLE 3.7. Minimum inhibitory concentration (MIC) of ceftazidime (CAZ AM-112, AM-113, AM-115 and AM-114 against a panel of Gram-positive ar Gram-negative organisms.

	MIC (μg/ml)				
Organism	CAZ	AM-112	AM-113	AM-114	AM-11
E. coli J53-1	0.125	8	8	32	>64
E. coli OXA-1	0.25	8	8	32	>64
E. coli OXA-2	0.25	16	16	>64	>64
E. coli OXA-3	0.5	16	16	>64	>64
E. coli OXA-5	0.5	8	16	32	>64
E. coli SHV-1	2	8	8	32	>64
E. coli SHV-2	0.25	2	4	16	>64
E. coli SHV-3	0.125	1	2	8	>64
E. coli SHV-4	>64	16	8	32	>64
E. coli SHV-5	16	16	16	64	>64
E. coli TEM-1	0.25	16	4	16	>64
E. coli TEM-3	16	8	16	64	>64
E. coli TEM-6	>64	16	8	32	>64
E. coli TEM-9	>64	8	8	32	>64
E. coli TEM-10	>64	8	16	64	>64
E. coli PSE-4	0.125	16	16	32	>64
E. coli ATCC 35218	0.125	8	4	16	>64
E. coli ATCC 25922	0.25	8	4	32	>64
S. aureus NCTC 6571	4	1	0.5	16	2
S. aureus ATCC 29213	8	1	0.5	1	2
MRSA Innsbruck	>64	2	1	16	4
P. aeruginosa 2297-con	>64	>64	>64	>64	>64
P. aeruginosa 1407-con	>64	>64	>64	>64	>64
P. aeruginosa ATCC 27853	2	>64	>64	>64	>64
E. cloacae P99+	32	2	8	64	>64
E. cloacae Hennessey	>64	32	64	>64	>64
E. cloacae 84-con	>64	16	32	>64	>64
C. freundii C2-con	64	16	4	32	>64
S. marcescens S2-con	1	16	32	>64	>64
M. morganii M1-con	8	16	4	16	>64
E. faecalis NCTC 07171	>64	2	4	>64	>64
E. hirae ATCC 10541	>64	8	2	>64	>64
E. faecalis NCTC 5957	32	32	8	>64	>64
E. faecalis ATCC 29212	32	32	8	>64	>64
E. faecalis 56059 *vanA*	>64	>64	2	64	>64
E. faecalis 78097 *vanB*	32	>64	16	>64	>64

Constitutive β-lactamase producing *P. aeruginosa* strains had high ceftazidime MICs of >64μg/ml, while the non-enzyme producing strain *P. aeruginosa* ATCC 27853 had a much lower MIC of 2μg/ml. The constitutive enzyme producing *E. cloacae* strains and *C. freundii* C2-con had similarly high MICs of 32->64μg/ml, yet *S. marcescens* S2-con and *M. morganii* M1-con, which produce β-lactamases, had lower MICs of 1μg/ml and 8μg/ml respectively. As a group, the enterococci were relatively resistant to ceftazidime with MICs in the range 32->64μg/ml.

Of the oxapenem compounds, AM-112 and AM-113 displayed the most activity against the panel of organisms, while AM-115 and AM-114 only had activity against a few strains. AM-112 and AM-113 had MICs against *E. coli* strains in the range 1-16μg/ml, including ESBL strains, whereas AM-115 and AM-114 had MICs in the range 8->64μg/ml. Activity against staphylococci, including MRSA Innsbruck, was in the range 0.5-2μg/ml for AM-112, AM-113 and AM-115. All of the compounds were inactive against *P. aeruginosa* strains, and had mixed activity against the constitutive enzyme producing strains *E. cloacae*, *C. freundii* C2-con, *S. marcescens* S2-con and *M. morganii* M1-con. AM-112 was the most active compound against these strains. AM-113 was the most active compound against the enterococci, with MICs in the range 2-16μg/ml. Neither AM-115 nor AM-114 had any activity against the enterococci.

Table 3.8 lists the MICs of ceftazidime in combination with the oxapenem compounds in a 1:1 ratio. Overall, an equal concentration of ceftazidime and oxapenem β-lactamase inhibitor resulted in MICs against *E. coli* strains of between 0.125 and 0.25μg/ml. All the oxapenems were effective in reducing the MIC of ceftazidime against the ESBL strains, with ceftazidime and AM-114 proving to be the most active combination, with MICs in the range 1-2μg/ml.

TABLE 3.8. Minimum inhibitory concentration (MIC) of ceftazidime (CAZ) combined with AM-112, AM-113, AM-115 and AM-114 in a 1:1 ratio.

Organism	MIC (μg/ml)[a]				
	CAZ[b]	CAZ + AM-112	CAZ + AM-113	CAZ + AM-114	CAZ + AM-115
E. coli J53-1	0.125	0.25	0.125	0.125	0.125
E. coli OXA-1	0.25	0.5	0.25	0.25	0.25
E. coli OXA-2	0.25	0.25	0.25	0.25	0.25
E. coli OXA-3	0.5	0.5	0.5	0.5	0.5
E. coli OXA-5	0.5	0.25	0.5	0.25	0.25
E. coli SHV-1	2	0.25	0.25	0.25	0.25
E. coli SHV-2	0.25	0.125	0.125	0.125	0.125
E. coli SHV-3	0.125	0.03	0.125	0.03	0.03
E. coli SHV-4	>64	4	2	2	8
E. coli SHV-5	16	8	8	1	8
E. coli TEM-1	0.25	0.25	0.25	0.25	0.5
E. coli TEM-3	16	4	2	2	4
E. coli TEM-6	>64	8	8	2	4
E. coli TEM-9	>64	8	4	2	4
E. coli TEM-10	>64	8	4	2	4
E. coli PSE-4	0.125	0.25	8	0.5	0.25
E. coli ATCC 35218	0.125	0.125	0.25	0.125	0.125
E. coli ATCC 25922	0.25	0.25	0.25	0.25	0.25
S. aureus NCTC 6571	4	2	0.5	1	4
S. aureus ATCC 29213	8	4	0.5	2	4
MRSA Innsbruck	>64	0.03	0.03	0.03	0.03
P. aeruginosa 2297-con	>64	32	16	16	32
P. aeruginosa 1407-con	>64	64	16	32	64
P. aeruginosa ATCC 27853	2	4	2	2	2
E. cloacae P99+	32	4	2	4	4
E. cloacae Hennessey	>64	4	4	4	16
E. cloacae 84-con	>64	4	8	8	8
C. freundii C2-con	64	0.03	2	2	4
S. marcescens S2-con	1	0.03	0.25	0.5	0.03
M. morganii M1-con	8	1	1	0.25	1
E. faecalis NCTC 07171	>64	16	1	32	>64
E. hirae ATCC 10541	>64	16	2	>64	>64
E. faecalis NCTC 5957	32	0.03	0.25	0.03	0.03
E. faecalis ATCC 29212	32	8	4	16	16
E. faecalis 56059 *vanA*	>64	64	0.25	>64	>64
E. faecalis 78097 *vanB*	32	16	8	16	32

[a]MICs quoted refer to the concentration of both ceftazidime and the inhibitor – i.e. ceftazidime MIC 1μg/ml, 1μg/ml of inhibitor.

[b]Ceftazidime MICs repeated for comparison.

Against the staphylococci, the MICs for ceftazidime plus AM-112 or AM-113 were lowered between two and four-fold compared to ceftazidime alone, despite the MICs for ceftazidime plus AM-112 and AM-113 being higher than for AM-112 or AM-113 alone. All oxapenems were effective in reducing the MIC of ceftazidime against MRSA Innsbruck to <0.03μg/ml. The activity of ceftazidime against the enzyme producing *P. aeruginosa* strains was enhanced by the oxapenem compounds, with the MIC being reduced two to four-fold. A similar pattern was seen for the *E. cloacae* strains, with MICs being reduced up to sixteen-fold. Ceftazidime activity against *C. freundii*, *S. marcescens* and *M. morganii* was enhanced, to varying extents, by each of the oxapenems. The combination of AM-115 and ceftazidime was the most active against the enterococci, and was the only combination with activity against the strain expressing the *vanA* phenotype. AM-112 had more activity that AM-113 and AM-114 against the panel of enterococci. AM-114 was slightly more active than AM-113.

Table 3.9 lists the minimum inhibitory concentrations of ceftazidime combined with each of the oxapenems in a 2:1 ratio. The MICs quoted refer to the ceftazidime concentration, the inhibitor concentration was half that of ceftazidime. Overall, the combination of ceftazidime with AM-113 was the most effective, with lower MICs against more strains than any of the other combinations. AM-113 and ceftazidime were most active against the *E. coli* strains, with MICs in the range 0.03-0.25μg/ml. There was also good activity against the ESBL strains, with a MIC of 2μg/ml for *E. coli* TEM-10 and *E. coli* SHV-5, compared with the combination of AM-112 and ceftazidime, which has a MIC of 16μg/ml for these strains. No combination had significant activity against the enzyme producing *P. aeruginosa* strains, with MICs of 32-64μg/ml. AM-112 combined with ceftazidime was the most active against the *E. cloacae*, *C. freundii*, *S. marcescens* and *M. morganii* strains, with MICs in the range 0.5-4μg/ml.

TABLE 3.9. Minimum inhibitory concentration (MIC) of ceftazidime (CAZ) combined with AM-112, AM-113, AM-115 and AM-114 in a 2:1 ratio.

Organism	MIC (μg/ml)[a]				
	CAZ[b]	CAZ + AM-112	CAZ + AM-113	CAZ + AM-114	CAZ + AM-115
E. coli J53-1	0.125	0.125	0.125	0.25	0.125
E. coli OXA-1	0.25	0.25	0.25	0.25	0.25
E. coli OXA-2	0.25	0.25	0.25	0.25	0.125
E. coli OXA-3	0.5	0.5	0.25	0.5	0.25
E. coli OXA-5	0.5	0.25	0.25	0.25	0.03
E. coli SHV-1	2	0.125	0.125	0.25	0.125
E. coli SHV-2	0.25	0.125	0.125	0.125	0.125
E. coli SHV-3	0.125	0.125	0.03	0.03	0.03
E. coli SHV-4	>64	8	2	4	0.03
E. coli SHV-5	16	16	4	2	2
E. coli TEM-1	0.25	0.5	0.25	0.25	0.06
E. coli TEM-3	16	2	2	2	2
E. coli TEM-6	>64	4	8	2	2
E. coli TEM-9	>64	8	8	4	2
E. coli TEM-10	>64	16	8	4	2
E. coli PSE-4	0.125	0.5	0.25	0.25	0.125
E. coli ATCC 35218	0.125	0.125	0.125	0.125	0.125
E. coli ATCC 25922	0.25	0.25	0.125	0.25	0.03
S. aureus NCTC 6571	4	1	1	2	4
S. aureus ATCC 29213	8	2	1	2	4
MRSA Innsbruck	>64	2	0.125	0.06	0.03
P. aeruginosa 2297-con	>64	32	32	32	32
P. aeruginosa 1407-con	>64	64	32	32	32
P. aeruginosa ATCC 27853	2	2	4	2	2
E. cloacae P99+	32	4	4	8	8
E. cloacae Hennessey	>64	4	8	8	8
E. cloacae 84-con	>64	4	16	16	16
C. freundii C2-con	64	2	4	4	2
S. marcescens S2-con	1	0.5	0.5	0.5	1
M. morganii M1-con	8	1	0.25	0.25	1
E. faecalis NCTC 07171	>64	32	16	32	>64
E. hirae ATCC 10541	>64	16	8	>64	64
E. faecalis NCTC 5957	32	8	8	16	32
E. faecalis ATCC 29212	32	8	8	16	16
E. faecalis 56059 *vanA*	>64	32	32	>64	>64
E. faecalis 78097 *vanB*	32	8	8	16	32

[a]MICs quoted refer to ceftazidime concentration. Inhibitor concentration is half that of quoted ceftazidime concentration, i.e. ceftazidime MIC 32μg/ml, inhibitor concentration 16μg/ml.

[b]Ceftazidime MICs repeated for comparison.

As seen with the combination of ceftazidime and oxapenems in a 1:1 ratio, AM-113 and ceftazidime in a 2:1 ratio was most active against the enterococci. AM-115 and ceftazidime was the least active combination. No combination had significant activity against the *vanA* phenotype enterococcus, while AM-112 and AM-113 had MICs of 8μg/ml against the *vanB* phenotype *E. faecalis* 78097.

3.3.8 *Activity of eight cephalosporins alone and in combination with AM-112 or clavulanic acid against a panel of β-lactamase producing strains*

Each of the cephalosporins exhibited a MIC profile against the panel of bacteria. No agent was active against the *E. cloacae* strains, with the lowest MIC being 32μg/ml for ceftazidime and cefepime. There was variable activity for the cephalosporins against the extended-spectrum β-lactamase (ESBL) producing *E. coli* strains TEM-10 and SHV-5. Cefotaxime was the most active agent, with MICs of 0.5 and 1μg/ml, respectively. Ceftazidime was very sensitive to hydrolysis by *E. coli* TEM-10 with a MIC of >64μg/ml. *P. aeruginosa* ATCC 27853 was resistant to the first and second-generation cephalosporins (cefazolin, cefaclor, cefuroxime) with MICs in the range 32 to 64μg/ml.

Clavulanic acid was not effective in protecting any of the cephalosporins against destruction by the class C β-lactamases produced by the *E. cloacae* strains except cefepime, where the MIC was lowered between four and eight-fold. The ESBLs produced by *E. coli* TEM-10 and SHV-5 proved susceptible to inhibition by clavulanic acid, with resultant lowering of the MICs for each cephalosporin. There was no enhancement of the activity of the cephalosporins against *P. aeruginosa* ATCC 27853 in the presence of clavulanic acid.

TABLE 3.10. Minimum inhibitory concentrations (MICs) of eight cephalosporins alone and in combination with either AM-112 or clavulanic acid against a panel of β-lactamase producing bacteria and an enterococcus.

Organism	MIC (μg/ml)		
	Cefazolin	Cefazolin + AM-112	Cefazolin + CLAV
E. cloacae Hennessey	>64	8	>64
E. cloacae P99+	>64	<0.03	>64
E. coli SHV-5	32	<0.03	0.25
E. coli TEM-10	8	<0.03	0.25
E. coli TEM-1	64	1	0.5
P. aeruginosa ATCC 27853	>64	>64	>64
E. faecalis SFZ	32	8	16
C. diversus 2046E	>64	>64	64

Organism	MIC (μg/ml)		
	Cefaclor	Cefaclor + AM-112	Cefaclor + CLAV
E. cloacae Hennessey	>64	2	>64
E. cloacae P99+	>64	<0.03	>64
E. coli SHV-5	16	<0.03	0.125
E. coli TEM-10	32	<0.03	0.25
E. coli TEM-1	64	0.5	0.25
P. aeruginosa ATCC 27853	>64	>64	>64
E. faecalis SFZ	32	16	32
C. diversus 2046E	>64	>64	64

Organism	MIC (μg/ml)		
	Ceftriaxone	Ceftriaxone + AM-112	Ceftriaxone + CLAV
E. cloacae Hennessey	>64	0.5	>64
E. cloacae P99+	64	<0.03	64
E. coli SHV-5	2	<0.03	0.06
E. coli TEM-10	0.25	<0.03	0.06
E. coli TEM-1	0.06	0.06	0.06
P. aeruginosa ATCC 27853	32	64	>64
E. faecalis SFZ	64	0.5	32
C. diversus 2046E	>64	32	0.125

TABLE 3.10 – *Continued*

Organism	MIC (μg/ml)		
	Cefoperazone	Cefoperazone + AM-112	Cefoperazone + CLAV
E. cloacae Hennessey	>64	1	>64
E. cloacae P99+	64	<0.03	>64
E. coli SHV-5	4	<0.03	0.06
E. coli TEM-10	4	<0.03	0.06
E. coli TEM-1	>64	<0.03	0.06
P. aeruginosa ATCC 27853	4	4	8
E. faecalis SFZ	32	4	8
C. diversus 2046E	>64	>64	32

Organism	MIC (μg/ml)		
	Cefotaxime	Cefotaxime + AM-112	Cefotaxime + CLAV
E. cloacae Hennessey	64	0.5	>64
E. cloacae P99+	64	<0.03	32
E. coli SHV-5	1	<0.03	0.06
E. coli TEM-10	0.5	<0.03	0.06
E. coli TEM-1	0.25	<0.03	0.06
P. aeruginosa ATCC 27853	8	64	64
E. faecalis SFZ	2	0.06	1
C. diversus 2046E	4	0.125	0.06

Organism	MIC (μg/ml)		
	Ceftazidime	Ceftazidime + AM-112	Ceftazidime + CLAV
E. cloacae Hennessey	>64	0.5	>64
E. cloacae P99+	32	<0.03	32
E. coli SHV-5	16	<0.03	0.06
E. coli TEM-10	>64	<0.03	0.125
E. coli TEM-1	0.5	<0.03	0.125
P. aeruginosa ATCC 27853	4	2	4
E. faecalis SFZ	32	1	64
C. diversus 2046E	1	0.06	0.25

TABLE 3.10 – *Continued*

Organism	MIC (μg/ml)		
	Cefuroxime	Cefuroxime + AM-112	Cefuroxime + CLAV
E. cloacae Hennessey	>64	>64	>64
E. cloacae P99+	>64	<0.03	>64
E. coli SHV-5	8	<0.03	0.25
E. coli TEM-10	16	<0.03	2
E. coli TEM-1	8	0.25	1
P. aeruginosa ATCC 27853	>64	>64	>64
E. faecalis SFZ	64	0.5	4
C. diversus 2046E	>64	64	64

Organism	MIC (μg/ml)		
	Cefepime	Cefepime + AM-112	Cefepime + CLAV
E. cloacae Hennessey	32	0.5	8
E. cloacae P99+	64	1	8
E. coli SHV-5	32	0.03	2
E. coli TEM-10	64	0.03	0.25
E. coli TEM-1	4	0.03	0.125
P. aeruginosa ATCC 27853	4	2	8
E. faecalis SFZ	16	4	8
C. diversus 2046E	16	0.03	0.06

Organism	MIC (μg/ml)	
	AM-112	CLAV
E. cloacae Hennessey	64	32
E. cloacae P99+	16	32
E. coli SHV-5	8	32
E. coli TEM-10	16	32
E. coli TEM-1	32	32
P. aeruginosa ATCC 27853	>64	>64
E. faecalis SFZ	64	>64
C. diversus 2046E	16	32

AM-112 had a potent effect on the activity of the cephalosporins against *E. cloacae* strains. MICs were reduced up to 1024-fold against *E. cloacae* P99 and up to 128-fold against *E. cloacae* Hennessey. A similar spectrum of activity was seen against the ESBL producing *E. coli* strains and *E. coli* TEM-1. The activity of

ceftazidime and cefepime against *P. aeruginosa* ATCC 27853 was enhanced two-fold, the activity of cefotaxime and ceftriaxone was reduced while the other cephalosporins remain unchanged. AM-112 enhanced the activity of cefotaxime, ceftriaxone, cefepime and ceftazidime against *C. diversus* 2046E, but had no enhanced effect with the other cephalosporins.

3.3.9 *Activity of four cephalosporins alone and in combination with AM-112 at a 2:1 ratio and fixed 4μg/ml combination against a panel of bacterial strains*

Tables 3.11-3.14 list the MICs determined for four cephalosporins (ceftazidime, cefepime, ceftriaxone and cefoperazone) alone and in combination with AM-112 at a 2:1 ratio and also with a fixed 4μg/ml concentration of AM-112. The panel of test organisms consisted mainly of β-lactamase producing strains, although some non-enzyme producers were also included for comparison.

None of the cephalosporins had good activity against MRSA strains. Cefepime and ceftriaxone were the most active against *S. aureus* strains (MIC range 1-2μg/ml). Ceftazidime and cefoperazone were most susceptible to the ESBL producers (MIC ranges 16-64μg/ml and 4-64μg/ml respectively) while cefepime was the least susceptible (0.03-4μg/ml). Ceftriaxone was the most active cephalosporin against β-lactamase producing strains (<0.03-0.06μg/ml) and non-enzyme producers (0.03-0.25μg/ml). Cefepime had the greatest activity against the enterococcal strains (4-16μg/ml) while ceftazidime was the least active (32->64μg/ml). The Enterobacteriacae were most susceptible to cefepime (0.06-4μg/ml) and least sensitive to cefoperazone (16->64μg/ml). Only cefepime had activity against the constitutive β-lactamase producing *P. aeruginosa* strains; all the other cephalosporins had MICs above 64μg/ml.

TABLE 3.11. Minimum inhibitory concentrations (MICs) of ceftriaxone, alone and in combination with AM-112 against a panel of bacteria.

Organism	MIC (μg/ml)			
	CTX[a]	AM-112	CTX+AM-112 (2:1)[b]	CTX+AM (4μg/m
MRSA 96-7778	>64	16	32	>64
MRSA Innsbruck	>64	4	8	8
MRSA 96-7992	64	8	4	8
MRSA 96-5665	64	8	4	8
S. aureus NCTC 6571	2	0.5	1	<0.0
S. aureus ATCC 29213	2	0.5	1	<0.0
S. aureus ATCC 25923	1	0.5	0.5	<0.0
S. aureus NCTC 10788	2	0.5	0.5	<0.0
E. coli J53-1	<0.03	32	<0.03	<0.0
E. coli J53 TEM-1	0.06	16	<0.03	<0.0
E. coli J53 TEM-3	4	16	2	0.5
E. coli J53 TEM-6	0.5	8	<0.03	<0.0
E. coli J53 TEM-9	8	16	2	1
E. coli J53 TEM-10	1	32	1	0.25
E. coli J53 SHV-1	<0.03	16	<0.03	<0.0
E. coli J53 SHV-2	0.06	16	<0.03	<0.0
E. coli J53 SHV-3	0.06	4	<0.03	<0.0
E. coli J53 SHV-4	16	16	2	0.25
E. coli J53 SHV-5	1	32	<0.03	0.25
E. coli J53 OXA-1	0.06	16	<0.03	<0.0
E. coli J53 OXA-2	<0.03	16	<0.03	<0.0
E. coli J53 OXA-3	0.06	16	<0.03	<0.0
E. coli J53 OXA-5	0.06	16	<0.03	<0.0
E. coli J53 PSE-4	<0.03	32	2	<0.0
E. coli X580	0.5	2	0.5	<0.0
E. coli ATCC 25922	0.06	16	0.06	<0.0
E. coli NCTC 10418	<0.03	16	<0.03	<0.0
E. coli ATCC 35218	<0.03	64	<0.03	<0.0
E. faecalis ATCC 29212	4	>64	4	2
E. faecalis SFZ	32	>64	4	2
E. faecalis 56059	>64	>64	>64	>6
E. faecalis 78097	4	>64	4	4
E. hirae ATCC 10541	8	64	4	2
E. faecium NCTC 7171	32	64	4	2
E. cloacae P99	>64	16	16	3
E. cloacae Hennessey	>64	64	16	>6
E. cloacae 84-con	>64	64	16	>6
S. marcescens S2-con	4	64	2	1
M. morganii M1-con	8	64	8	4
C. freundii C2-con	>64	16	8	3
C. diversus 2046E	2	16	16	8
K. pneumoniae K1+	16	32	8	8
P. aeruginosa ATCC 27853	8	>64	4	1
P. aeruginosa NCTC 10662	>64	>64	8	1
P. aeruginosa 1405-con	>64	>64	>64	>6
P. aeruginosa 2297-con	>64	>64	>64	>6

[a] CTX, ceftriaxone.

[b] Ratio of ceftriaxone to AM-112 is 2:1, MIC quoted refers to ceftriaxone concentration.

[c] Fixed 4μg/ml concentration of AM-112.

TABLE 3.12. Minimum inhibitory concentrations (MICs) of ceftazidime, alone and in combination with AM-112 against a panel of bacteria.

Organism	MIC (μg/ml)			
	CAZ[a]	AM-112[b]	CAZ+AM-112 (2:1)[c]	CAZ+AM (4μg/m
MRSA 96-7778	>64	16	32	32
MRSA Innsbruck	>64	4	8	32
MRSA 96-7992	>64	8	8	16
MRSA 96-5665	>64	8	16	16
S. aureus NCTC 6571	8	0.5	4	<0.0
S. aureus ATCC 29213	8	0.5	4	<0.0
S. aureus ATCC 25923	8	0.5	1	<0.0
S. aureus NCTC 10788	4	0.5	1	<0.0
E. coli J53-1	0.25	32	0.25	<0.0
E. coli J53 TEM-1	0.5	16	0.5	0.2
E. coli J53 TEM-3	16	16	0.5	2
E. coli J53 TEM-6	>64	8	0.25	32
E. coli J53 TEM-9	>64	16	32	>64
E. coli J53 TEM-10	>64	32	16	64
E. coli J53 SHV-1	0.25	16	0.25	0.12
E. coli J53 SHV-2	0.25	16	0.125	<0.0
E. coli J53 SHV-3	0.25	4	<0.03	<0.0
E. coli J53 SHV-4	>64	16	4	16
E. coli J53 SHV-5	32	32	8	8
E. coli J53 OXA-1	0.25	16	0.25	0.2
E. coli J53 OXA-2	0.25	16	0.25	0.2
E. coli J53 OXA-3	0.5	16	<0.03	0.:
E. coli J53 OXA-5	0.25	16	<0.03	0.2
E. coli J53 PSE-4	0.25	32	0.125	0.2
E. coli X580	4	2	0.25	<0.
E. coli ATCC 25922	0.25	16	0.5	0.2
E. coli NCTC 10418	0.125	16	0.25	0.2
E. coli ATCC 35218	0.125	64	0.25	<0.
E. faecalis ATCC 29212	32	>64	8	8
E. faecalis SFZ	32	>64	16	1
E. faecalis 56059	>64	>64	>64	<0
E. faecalis 78097	32	>64	16	1
E. hirae ATCC 10541	>64	64	32	>(
E. faecium NCTC 7171	>64	64	16	3
E. cloacae P99	>64	16	8	
E. cloacae Hennessey	>64	64	16	3
E. cloacae 84-con	>64	64	32	>
S. marcescens S2-con	0.5	64	0.5	0
M. morganii M1-con	8	64	1	0
C. freundii C2-con	>64	16	8	
C. diversus 2046E	0.5	16	0.25	0
K. pneumoniae K1+	1	32	32	
P. aeruginosa ATCC 27853	2	>64	0.5	
P. aeruginosa NCTC 10662	1	>64	<0.03	
P. aeruginosa 1405-con	64	>64	64	
P. aeruginosa 2297-con	64	>64	64	

[a] CAZ, ceftazidime.

[b] AM-112 MICs repeated for comparison.

[c] Ratio of ceftazidime to AM-112 2:1. MIC quoted refers to ceftazidime concentration.

[d] Fixed 4μg/ml concentration of AM-112.

TABLE 3.13. Minimum inhibitory concentrations (MICs) of cefoperazone, alone and in combination with AM-112 against a panel of bacteria.

Organism	MIC (μg/ml)			
	CPZ[a]	AM-112[b]	CPZ+AM-112 (2:1)[c]	CPZ+AM-1 (4μg/ml)[d]
MRSA 96-7778	>64	16	4	2
MRSA Innsbruck	64	4	4	8
MRSA 96-7992	64	8	2	0.5
MRSA 96-5665	32	8	2	1
S. aureus NCTC 6571	4	0.5	0.5	<0.03
S. aureus ATCC 29213	2	0.5	0.5	<0.03
S. aureus ATCC 25923	2	0.5	0.5	<0.03
S. aureus NCTC 10788	1	0.5	0.25	<0.03
E. coli J53-1	0.125	32	0.125	<0.03
E. coli J53 TEM-1	32	16	8	32
E. coli J53 TEM-3	16	16	<0.03	1
E. coli J53 TEM-6	8	8	<0.06	1
E. coli J53 TEM-9	64	16	8	8
E. coli J53 TEM-10	8	32	2	1
E. coli J53 SHV-1	0.125	16	0.06	0.125
E. coli J53 SHV-2	0.25	16	0.06	<0.03
E. coli J53 SHV-3	0.5	4	<0.03	<0.03
E. coli J53 SHV-4	32	16	2	1
E. coli J53 SHV-5	4	32	0.06	1
E. coli J53 OXA-1	0.25	16	0.25	0.125
E. coli J53 OXA-2	64	16	2	64
E. coli J53 OXA-3	1	16	1	0.25
E. coli J53 OXA-5	0.25	16	<0.03	0.25
E. coli J53 PSE-4	4	32	1	4
E. coli X580	0.5	2	0.125	<0.03
E. coli ATCC 25922	0.25	16	0.25	0.125
E. coli NCTC 10418	0.125	16	0.06	0.03
E. coli ATCC 35218	4	64	1	1
E. faecalis ATCC 29212	8	>64	8	16
E. faecalis SFZ	16	>64	16	16
E. faecalis 56059	64	>64	32	64
E. faecalis 78097	32	>64	16	32
E. hirae ATCC 10541	4	64	8	8
E. faecium NCTC 7171	64	64	8	64
E. cloacae P99	>64	16	8	8
E. cloacae Hennessey	>64	64	16	>64
E. cloacae 84-con	>64	64	32	>64
S. marcescens S2-con	16	64	4	8
M. morganii M1-con	>64	64	32	>64
C. freundii C2-con	64	16	16	16
C. diversus 2046E	16	16	16	64
K. pneumoniae K1+	>64	32	>64	>64
P. aeruginosa ATCC 27853	4	>64	2	4
P. aeruginosa NCTC 10662	4	>64	4	4
P. aeruginosa 1405-con	>64	>64	>64	>64
P. aeruginosa 2297-con	>64	>64	>64	>64

[a] CPZ, cefoperazone.

[b] AM-112 MICs repeated for comparison.

[c] Ratio of cefoperazone to AM-112 is 2:1. MIC quoted refers to cefoperazone concentration.

[d] Fixed 4μg/ml concentration of AM-112.

TABLE 3.14. Minimum inhibitory concentrations (MICs) of cefepime, alone and i combination with AM-112 against a panel of bacteria.

Organism	MIC (μg/ml)			
	CFP[a]	AM-112[b]	CFP+AM-112 (2:1)[c]	CFP+A (4μg
MRSA 96-7778	>64	16	16	6
MRSA Innsbruck	64	4	4	
MRSA 96-7992	64	8	4	
MRSA 96-5665	64	8	4	
S. aureus NCTC 6571	2	0.5	0.5	<0
S. aureus ATCC 29213	2	0.5	0.5	<0
S. aureus ATCC 25923	1	0.5	0.5	<0
S. aureus NCTC 10788	1	0.5	0.5	<0
E. coli J53-1	1	32	<0.03	<0
E. coli J53 TEM-1	0.125	16	0.06	0.
E. coli J53 TEM-3	2	16	<0.03	0.
E. coli J53 TEM-6	<0.03	8	<0.03	<0
E. coli J53 TEM-9	4	16	2	
E. coli J53 TEM-10	2	32	1	0.
E. coli J53 SHV-1	<0.03	16	<0.03	<0
E. coli J53 SHV-2	<0.03	16	<0.03	<0
E. coli J53 SHV-3	<0.03	4	<0.03	<0
E. coli J53 SHV-4	2	16	1	0.
E. coli J53 SHV-5	0.25	32	<0.03	0.
E. coli J53 OXA-1	0.25	16	0.125	<0
E. coli J53 OXA-2	<0.03	16	<0.03	<0
E. coli J53 OXA-3	<0.03	16	<0.03	<0
E. coli J53 OXA-5	<0.03	16	<0.03	<0
E. coli J53 PSE-4	<0.03	32	<0.03	<0
E. coli X580	<0.03	2	0.125	<0
E. coli ATCC 25922	<0.03	16	<0.03	<0
E. coli NCTC 10418	<0.03	16	<0.03	<0
E. coli ATCC 35218	<0.03	64	<0.03	<0
E. faecalis ATCC 29212	4	>64	4	
E. faecalis SFZ	8	>64	4	
E. faecalis 56059	>64	>64	>64	>
E. faecalis 78097	8	>64	8	
E. hirae ATCC 10541	16	64	8	
E. faecium NCTC 7171	8	64	8	
E. cloacae P99	2	16	0.5	0
E. cloacae Hennessey	4	64	1	
E. cloacae 84-con	4	64	2	
S. marcescens S2-con	0.25	64	0.125	0
M. morganii M1-con	1	64	1	
C. freundii C2-con	1	16	0.5	
C. diversus 2046E	0.06	16	0.06	0.
K. pneumoniae K1+	1	32	1	
P. aeruginosa ATCC 27853	0.5	>64	0.5	
P. aeruginosa NCTC 10662	1	>64	<0.03	<0
P. aeruginosa 1405-con	16	>64	8	
P. aeruginosa 2297-con	4	>64	4	

[a] CFP, cefepime.

[b] AM-112 MICs repeated for comparison.

[c] Ratio of cefepime to AM-112 is 2:1. MIC quoted refers to cefepime concentration.

[d] Fixed 4μg/ml concentration of AM-112.

When the cephalosporins were combined with AM-112 in a 2:1 ratio, cefoperazon was the most active agent against MRSA strains (2 to 4μg/ml) and MSSA strain (0.25 to 0.5μg/ml). Both ceftriaxone and cefepime had the best activity against ESB producing *E. coli* strains (0.03 to 2μg/ml); ceftriaxone was the most active agains β-lactamase producers (0.03μg/ml). Cefepime was the most active agent against th non-enzyme producing *E. coli* (0.03 to 0.125μg/ml). No cephalosporin had activit against the vancomycin-resistant *E. faecalis* 56059 strain, while ceftriaxone was th most active agent against the other enterococcal strains (4μg/ml). Activity against th Enterobacteriacae was mixed; cefepime was the most effective agent (0.06 t 2μg/ml) against these strains as well as *P. aeruginosa* strains. The cephalosporir were also combined with AM-112 at a fixed 4μg/ml concentration. Cefoperazor was the most effective against the MRSA strains (0.5 to 8μg/ml). Each of th cephalosporins was equally active against *S. aureus* strains (<0.03μg/ml Ceftriaxone was the most active against Gram-negative ESBL producers (0.0 1μg/ml), while cefepime had the lowest MICs against the β-lactamase producir *E. coli* strains (0.03 to 0.06μg/ml). Both cefepime and ceftriaxone were the mo active against non-enzyme producing strains. Ceftriaxone was the most effecti agent against the enterococci (2 to 4μ/ml). Cefepime had the lowest MICs against th Enterobacteriacae (0.06 to 1μg/ml) and also against *P. aeruginosa* strains (<0.03 16μg/ml).

3.4 Discussion

The oxapenem compounds represent a novel class of β-lactam agents, which have intrinsic antibacterial activity combined with an ability to inhibit β-lactamase enzymes produced by a variety of bacterial species. The aims of the experiments in this section were to investigate the antibacterial activity of the compounds against a range of bacteria, both alone and in combination with a number of established antibacterial agents.

The activity of AM-112 and AM-113 in the presence of serum was investigated. The results indicated that AM-113 was highly unstable in the presence of serum with a complete loss of activity within thirty minutes. In contrast, AM-112 lost only between 33-50% of its activity after one hour of incubation with serum. As a result of these studies, AM-112 was selected as the candidate compound for more intensive study, but investigation of AM-113 continued on a lesser scale.

Piperacillin and ceftazidime were candidates as potential partners for novel oxapenems. The activity of a combination of piperacillin or ceftazidime and β-lactamase inhibitors was evaluated in the presence of human serum and compared to the activity of the combinations without the addition of serum. The activities of all the antibiotic-inhibitor combinations were decreased in the presence of serum, compared to the serum-free combinations. As demonstrated by the bioassays carried out in the presence of serum (Figs. 3.4-3.7), the reduction in activity of AM-112 and AM-113 would be expected. From the data it would appear that ceftazidime is a better partner for the oxapenems than piperacillin, as it has lower MICs both in the presence and absence of serum.

Of the four oxapenem compounds, AM-112 and AM-113 were active against a range of bacteria, while AM-115 and AM-114, their respective isomers, lacked significant activity against the same bacteria. For example, the AM-112 and AM-113 MIC ranges for *E. coli* were 8 to 16μg/ml and 2 to 16μg/ml respectively, while for AM-115 and AM-114 the MICs were >64μg/ml and 8 to >64μg/ml respectively. Both AM-112 and AM-113 were very active against staphylococci, both

methicillin-sensitive and resistant strains, with MICs lower than those of established agents such as ceftazidime (Table 3.7). AM-113 was also active against the Gram-positive enterococci, including *E. faecalis* 56059, which expresses the *vanA* vancomycin-resistance phenotype. The MIC against this organism was 2μg/ml.

Much work has focused on the protection afforded by the oxapenem inhibitors to existing β-lactam agents from β-lactamase enzymes produced by bacterial species. Combining β-lactamase inhibitors with β-lactam agents is a well-established strategy for enhancing the spectrum of activity of the β-lactam agent. Examples of this strategy include amoxycillin-clavulanic acid (Augmentin), piperacillin-tazobactam (Zosyn) and ampicillin-sulbactam (Unasyn). This strategy is of particular importance with the emergence of extended spectrum β-lactamase enzymes that can destroy oximino-β-lactams such as ceftazidime, cefotaxime and ceftriaxone, previously resistant to β-lactamase-mediated hydrolysis. ESBLs are normally sensitive to inhibition by β-lactamase inhibitors (Thomson and Smith Moland 2000), but inhibitor-resistant enzymes have been identified (Nicolas-Chanoine 1997). These enzymes are derived from the TEM, SHV and OXA families of enzymes (Bush and Miller 1998; Therrien and Levesque 2000). Unfortunately, none of the bacterial strains tested produced an inhibitor-resistant β-lactamase enzyme, so the activity of the oxapenems against this emerging class of enzymes could not be assessed.

Each of the oxapenem inhibitors was combined with ceftazidime in a 1:1 and 2:1 ratio to determine the protection afforded by the inhibitors to ceftazidime (Table 3.8 and Table 3.9). The panel of bacteria included Class A, Class C and Class D β-lactamase producing strains, extended spectrum β-lactamase producers and constitutive enzyme producers. Alone, ceftazidime was inactive against the ESBL+ strains, and those strains that constitutively produced a β-lactamase enzyme, with MICs in the range 16 to >64μg/ml. (Table 3.7).

While lacking antimicrobial activity, AM-115 and AM-114 are highly effective β-lactamase inhibitors, affording a high degree of protection to ceftazidime. MICs against ESBLs were reduced up to 32-fold and against strains such as *E. cloacae* 84-con 16-fold. All the oxapenems enhanced the activity of ceftazidime against

certain strains of enterococci, although the activity varied widely within this group. Cephalosporins are poorly active against enterococci (Georgoupapadakou and Liu 1980; Fontana, *et al.* 1990). The reduced susceptibility of enterococci is due to the presence of PBPs that have a low affinity for β-lactam antibiotics (Fontana, *et al.* 1983). It is believed that these low affinity PBPs can take over the physiological functions of other PBPs in the cell membrane when these PBPs have become inhibited by a β-lactam compound (Coyette, *et al.* 1980). Evidence supporting this finding comes from a study by Fontana *et al.* (1983) that found that *E. hirae* ATCC 9790 (formerly *S. faecium*) could grow normally when all the PBPs except PBP 5 had been inhibited. The workers also found that penicillin resistance increased with the amount of PBP 5 present in the cell. These findings have been confirmed by other researchers (Fontana, *et al.* 1985; Canepari, *et al.* 1987; Lleo, *et al.* 1987; Al-Obeid, *et al.* 1990).

Enterococcal species can have one or more of these low affinity PBPs (Williamson, *et al.* 1985), which may explain the range of sensitivity of the enterococci to different β-lactam antibiotics. Thus the activity of a β-lactam against an enterococcal species depends on its ability to bind to these low affinity PBPs (Williamson, *et al.* 1985; Amalfitano, *et al.* 1991). AM-112 has affinity for all the PBPs of *E. faecalis* SFZ and *E. faecalis* ATCC 29212 (see Chapter 5). It is likely that this affinity, combined with the action of ceftazidime on the PBPs, results in the synergistic activity against the enterococci. The other oxapenems probably share this affinity for the PBPs. The varying affinity of each oxapenem for the low affinity PBPs is likely to be the reason for the differences seen in the synergy with ceftazidime against the enterococci.

The activity of AM-112 and AM-113 can be attributed to their intrinsic antibacterial action–alone, AM-113 had MICs of 2-8μg/ml for some enterococcal strains while AM-112 had MICs of 2-32μg/ml. AM-115 and AM-114 displayed no activity. Yet when combined with ceftazidime in a 1:1 ratio, the MIC could be lowered to 0.25μg/ml for AM-114 and 0.03μg/ml for AM-115 against *E. faecalis* NCTC 5957.

The synergistic activity of the combination of AM-112 and ceftazidime against enterococci was confirmed by the data from Table 3.5. Reductions in the MIC of ceftazidime against six enterococcal strains was at least 2048-fold with AM-112 at a 4μg/ml concentration, compared to the MIC of ceftazidime alone. AM-112 was also shown to be either synergistic or additive when combined with a number of antibacterial agents against two VRE strains and two vancomycin-sensitive strains (Table 3.6).

Reducing the ratio of the oxapenem to ceftazidime from 1:1 to 2:1 did not have any clear-cut effects on the protection afforded to ceftazidime. For AM-112, halving the concentration did not significantly alter the ceftazidime MICs – the variation in MIC was only two-fold, and the variation were evenly divided between the two concentrations. AM-113 was more effective at protecting ceftazidime at a 2:1 ratio. Ceftazidime MICs were lower against sixteen strains, compared to four strains with lower ceftazidime MICs at a 1:1 ratio. This enhanced activity at lower inhibitor concentrations was principally against the *E. coli* strains. However, like AM-112, there was invariably only a two-fold difference in the MICs.

For both AM-115 and AM-114, the protection of ceftazidime was more effective at a 1:1 ratio. For AM-115, the higher concentration of inhibitor was most active against the staphylococci, constitutive-enzyme producers such as *P. aeruginosa* and *E. cloacae* strains and the enterococci. For AM-114, this activity was spread throughout the panel, including some *E. coli* strains, staphylococci and constitutive enzyme producers. The enhanced activity of AM-115 and AM-114 against enzyme producing strains, particularly constitutive producers, at a 1:1 ratio with ceftazidime is probably due to more inhibitor being available to inhibit the enzyme, resulting in greater protection of the partner β-lactam. For strains not producing a β-lactamase enzyme, higher concentrations of AM-115 and AM-114 may enhance the low level of inherent antibacterial activity by increasing binding to a low affinity target, which enhances the action of ceftazidime.

AM-113 was more active when combined with ceftazidime in a 1:2 ratio than an equal ratio, while AM-112 was equally active at either concentration. The reason for this is not clear. Perhaps the binding of AM-112 and AM-113 becomes saturated at

lower concentrations and higher concentrations have no additional antibacterial effect.

The protective effect of AM-112 or clavulanic acid at a fixed 4μg/ml concentration in combination with eight cephalosporins was investigated against a panel of β-lactamase producing strains (Table 3.10). Clavulanic acid was effective at inhibiting class A enzymes and ESBLs produced by the panel of organisms, but was not effective in protecting the cephalosporins against class C enzymes produced by organisms such as *E. cloacae* P99. AM-112 enhanced the activity of the cephalosporins against class A, class C and ESBL producing strains. AM-112 only enhanced the activity of two cephalosporins against *P. aeruginosa*, indicating the poor activity AM-112 has against this strain.

The oxapenems are a novel class of β-lactams that possess potent β-lactamase inhibitory properties. Two compounds, AM-112 and AM-113, also possess intrinsic antibacterial activity while their respective isomers, AM-115 and AM-114, lack antimicrobial potency on their own. While the spectrum of activity of AM-112 and AM-113 does include Gram-negative and Gram-positive bacteria - including MRSA and enterococcal strains - the activity of the compounds is not sufficiently high to consider their use as stand alone agents. The potent β-lactamase inhibitory properties of each of the agents have led to investigation of the ability of these compounds to protect existing agents such as cephalosporins. Of the cephalosporins, ceftazidime is the agent that has undergone most evaluation as a potential partner for an oxapenem compound. The results in this chapter demonstrate the superior protective effect of AM-112 compared to clavulanic acid for ceftazidime. This protection extends to each of class A, class C and class D enzymes as well as ESBLs.

Chapter 4. Mechanism of β-lactamase inhibition by oxapenem compounds

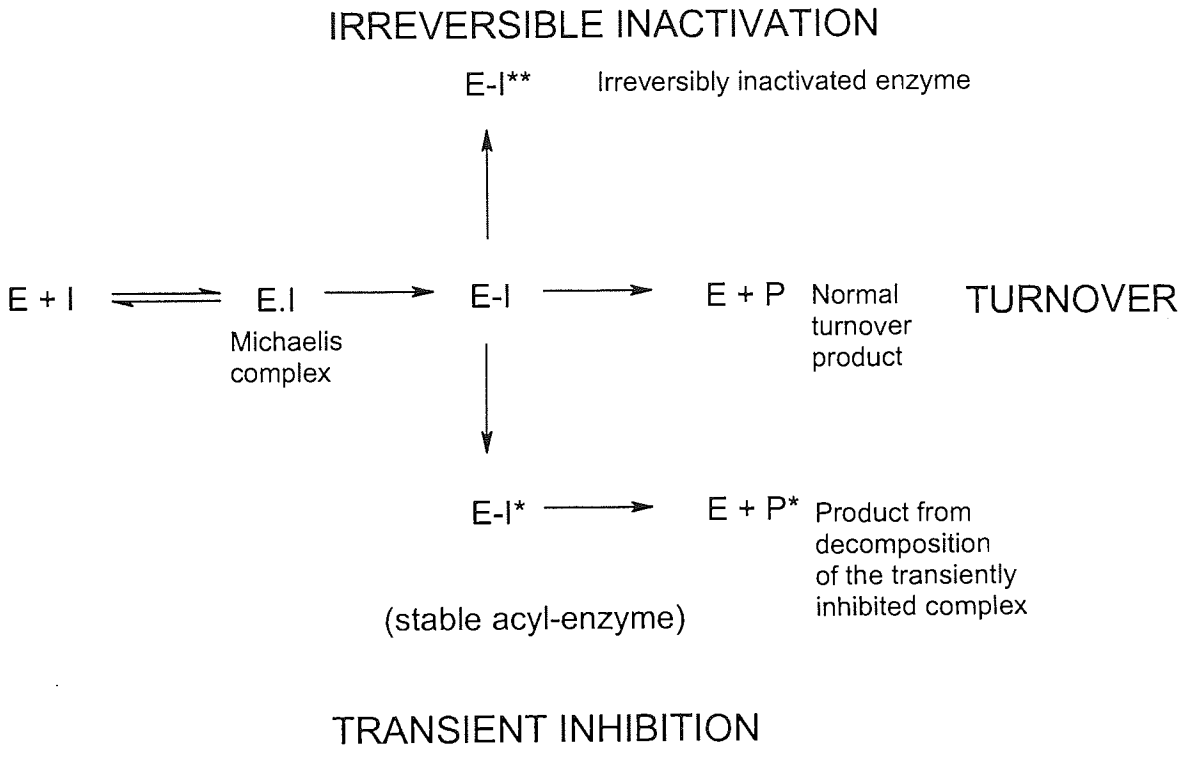
4.1 Introduction

The destruction of β-lactam antibiotics by β-lactamase enzymes is believed to occur by acylation of the β-lactam ring by a reactive nucleophile in the active site of the enzyme. This reactive nucleophile is Ser-70 in class A enzymes and Ser-64 in class C enzymes. The attack on the β-lactam ring leads to opening of the ring. Deacylation then occurs, with regeneration of the free enzyme and release of inactive penicillanic acid. β-lactamase inhibitors interfere with this process. Acylation of the inhibitor by the active site serine leads to formation of a reactive chemical species within the active site. This species can cross-link to another residue in the active site, leading to irreversible inactivation of the enzyme. The precise mechanism for inhibition of the active site by β-lactamase inhibitors has not been fully elucidated, nor have the residues that the inhibitors cross-link to been definitively assigned. Indeed, recent research suggests that cross-linking of tazobactam in the active site of a class C enzyme is not necessary for inhibition of that enzyme (Bonomo, *et al.* 2001). Much research has therefore focused on elucidating the mechanism of inactivation by β-lactamase inhibitors, mainly clavulanic acid and tazobactam, of class A and class C enzymes.

Mechanism-based β-lactamase inhibitors such as clavulanic acid, tazobactam and sulbactam are the most widely studied inhibitors of class A enzymes. None of these clinically used agents has significant activity against class C enzymes (Payne, *et al.* 1994; Bush, *et al.* 1995). There are currently no marketed agents with good activity against class C enzymes, but experimental compounds such as phosphonate inhibitors (Lobkovsky, *et al.* 1994) and the novel oxapenem, Syn2190 have been described (Nishida, *et al.* 1999).

Figure 4.1 shows the possible pathways which can be followed by β-lactamase inhibitors.

FIGURE 4.1. Illustration of the pathways that β-lactamase inhibitors can follow after binding to a β-lactamase enzyme. Adapted from Therrien and Levesque (2000).



Binding of the inhibitor leads to covalent modification of the active site of the enzyme, preventing conversion of the substrate (i.e. the inhibitor) to the product and regeneration of the enzyme. Detailed studies of the stages of enzyme inhibition have been made, in order to understand the process and hopefully to design new inhibitors with enhanced activity. A number of techniques have been used to study the interactions of established inhibitors and novel compounds with β-lactamase enzymes. Reaction of an inhibitor or substrate with the enzyme and subsequent examination of the crystal structure of the transition state analogue is a common approach for studying inhibitor or substrate binding. This technique can provide information on the orientation of the compound in the active site, whether the active site or the compound has undergone any conformational rearrangement, which residues are important for the binding of the compound, the role of solvent molecules

in the acylation and deacylation of the compound as well as the effects of mutation of residues within the active site on the binding of the inhibitor or substrate. Kinetic analysis of the interactions of the inhibitor or substrate with the enzyme can provide information of the type of inhibition caused by the inhibitor (e.g. competitive, non-competitive, reversible, irreversible), the catalytic efficiency of the enzyme for the substrate, the turnover number of the inhibitor and other kinetic parameters. Electrospray mass ionisation spectroscopy (ESMIS) is a powerful technique which allows the investigation and characterization (when coupled with high performance liquid chromatography) of fragments of the enzyme and bound inhibitor. Thus fragments of the enzyme with bound inhibitor can be detected by their mass increases compared to native enzyme. This allows identification of specific residues which are involved in binding of the inhibitor, as well as providing information on the fate of the inhibitor and the rearrangements it undergoes in the active site. The advantage of ESMIS coupled to HPLC is that it allows the real-time investigation of the binding of the inhibitor to the enzyme, and although many steps in this process occur too quickly to be detected, the information gleaned from slower steps can help to propose a reaction mechanism for such binding.

Clavulanic acid, tazobactam and sulbactam are the most widely investigated inhibitors of class A enzymes. The following description of the interaction of an inhibitor with a class A enzyme will draw on the studies performed with these compounds to describe the stages involved.

FIGURE 4.2. C-alpha trace of *E. coli* TEM-1 β-lactamase (Brookhaven Databank ID 1BTL). The position of Ser-70 and other active site residues is shown in green. α-Helices are shown in red and β-sheets in blue.

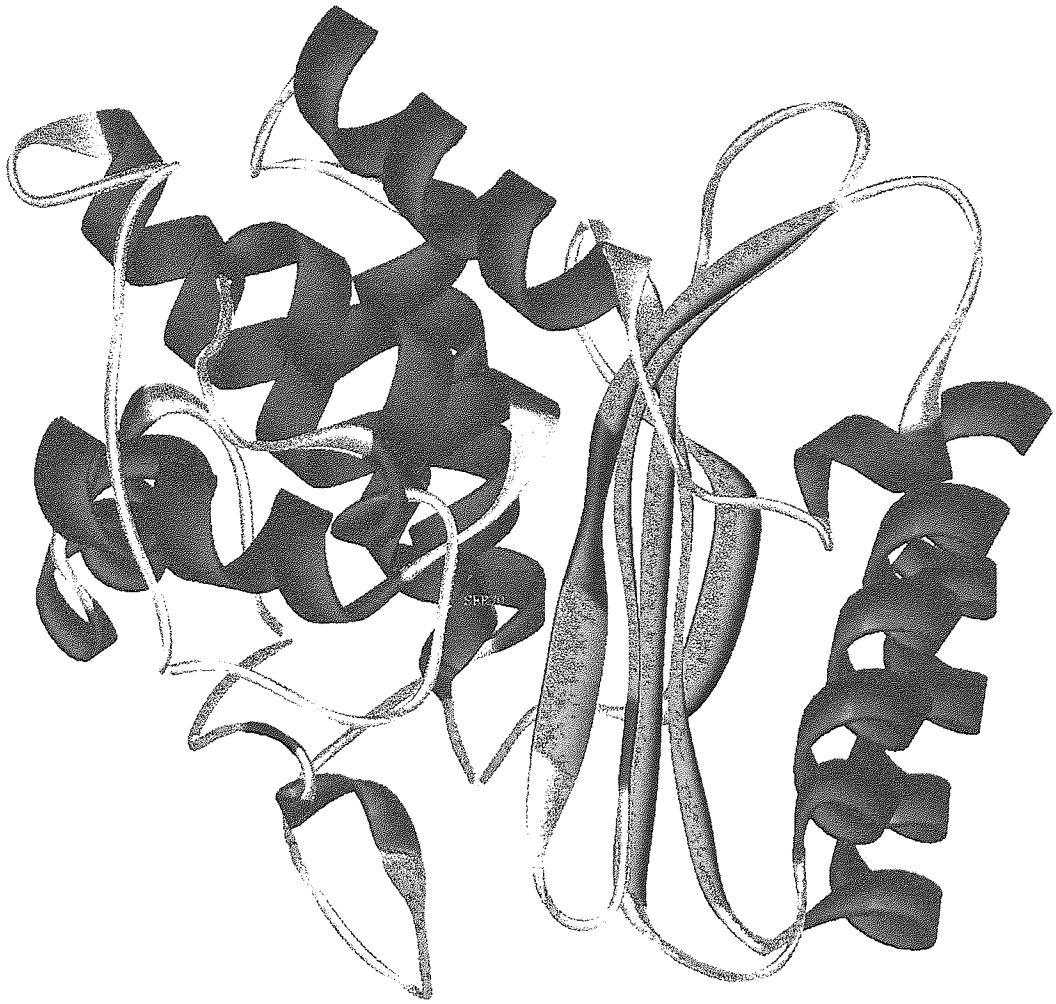
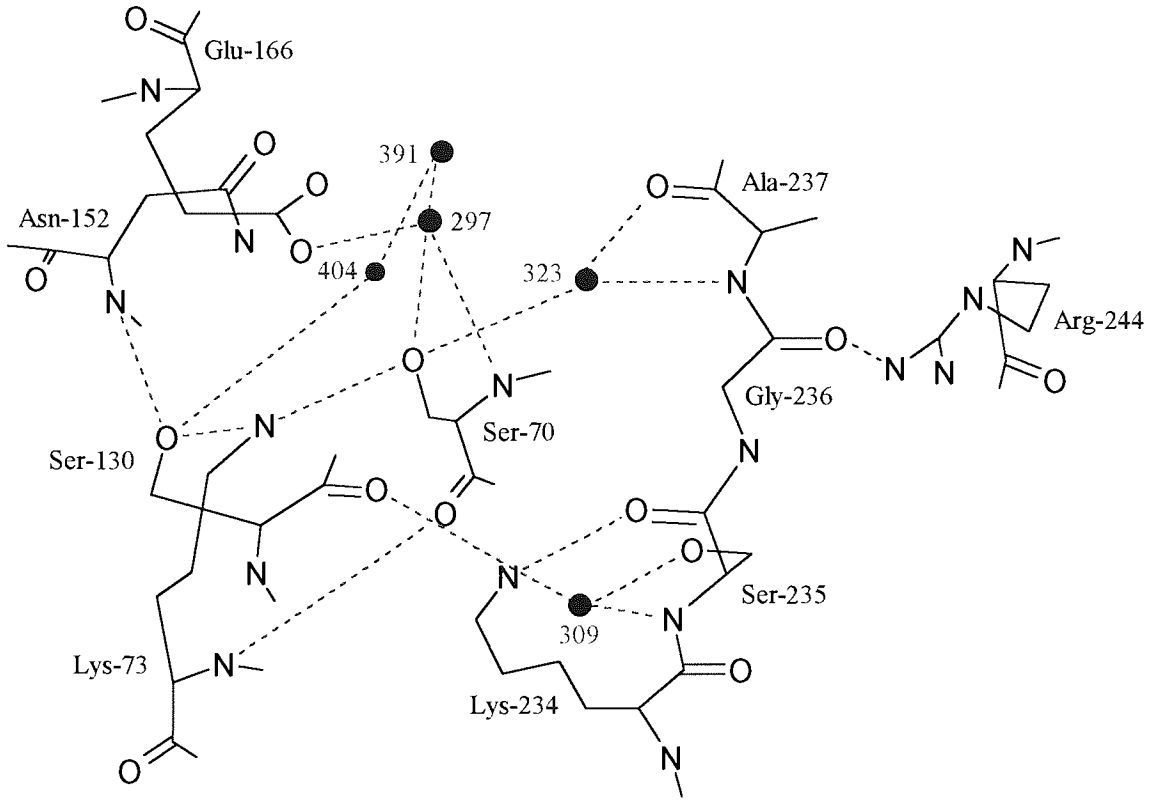


Fig. 4.2 shows the alpha carbon trace of the *E. coli* TEM-1 enzyme, while Fig. 4.3 is a schematic representation of the active site residues of the enzyme, showing the hydrogen-bonding interactions between the residues of the active site.

FIGURE 4.3. Schematic representation of the active site of the class A β-lactamase of *E. coli* TEM-1 (Brookhaven Databank ID, 1BTL). Hydrogen bonding between residues is shown in green. Water molecules are represented by black spheres. The amino acids are numbered according to the Ambler scheme.



Clavulanic acid, tazobactam and sulbactam all contain a β-lactam ring fused to a 5-membered ring. As such they are structurally similar to penicillins, which themselves are structural analogues of the C-terminal end of D-alanyl-D-alanine (Tipper and Strominger 1965). Thus the inhibitors are recognised as substrates for the enzyme and enter the active site of the enzyme. The active site of class A enzymes contain four strictly conserved residues (Ser-70, Lys-73, Ser-130 and Glu-166) recognized to be important in catalysis (Matagne, *et al.* 1999). The initial step is formation of the acyl-enzyme complex. The active site of the enzyme contains an oxyanion hole, involved in the stabilisation of the tetrahedral intermediates. This oxyanion hole is formed by the main chain amino groups of Ser-70 and Ala-237. The residue which acts as a general base is still undefined. One possible residue is the conserved residue Glu-166. This residue is believed to remove the proton from Ser-70, possibly via a water molecule which acts as a relay for the proton transfer.

The activated oxygen of Ser-70 then attacks the carbonyl carbon of the β-lactam ring. The proton removed from the hydroxyl group of Ser-70 is delivered back to the leaving nitrogen of the β-lactam along a network of hydrogen bonds involving the ε-amino groups of Lys-73 and Lys-234, another water molecule and the hydroxyl group of Ser-130, which acts as the ultimate proton donor (Matagne, *et al.* 1999). The heteroatom at the 1-position acts as a leaving group from the C_5 carbon to generate an acyclic linear molecule (Therrien and Levesque 2000). Clavulanic acid is believed to differ from sulbactam and tazobactam because it requires protonation of the nitrogen of the β-lactam ring to allow opening of the heterocyclic, whereas the penicillanic acid sulphones do not require this protonation step (Yang, *et al.* 2000). This protonation improves the efficiency of the opening of the heterocyclic ring because it transforms the enolate into an oxonium species, compared to sulbactam where the sulphone is a poor leaving group. Other researchers have observed that the distances between the C_5 atom of the inhibitor and any of the potential nucleophilic residues (Lys-73, Ser-130, Lys-234) are much greater for sulbactam than for clavulanic acid, which may account for the higher turnover number observed for sulbactam (Imtiaz, *et al.* 1994).

The degree of inhibition of the enzyme depends on the fate of the linear imine adduct formed by opening of the β-lactam and 5-membered rings. Transient inhibition of the enzyme can occur when tautomerisation of the imine to a more stable intermediate enamine occurs (Knowles 1985; Therrien and Levesque 2000)(Fig. 4.4). The fate of the imine species of any of the compounds is similar, differing only in the chemical structures cleaved from the imine. The fates of the imine species of clavulanic acid and tazobactam have been well characterised. Ser-130 plays an important role for each compound. For tazobactam, Ser-130 can interact with the imine species to form a cross-linked vinyl-ether, with the loss of a sulphonic acid fragment (Yang, *et al.* 2000; Kuzin, *et al.* 2001). A similar reaction occurs for clavulanic acid, with the loss of a portion of the heterocyclic ring (Brown, *et al.* 1996). This cross-linking irreversibly inactivates the enzyme. Regeneration of the enzyme can occur by decarboxylation and hydrolysis of the imine intermediate (Brown, *et al.* 1996; Therrien and Levesque 2000; Kuzin, *et al.* 2001). Sulbactam is believed to cross-link

to either Ser-130 or a lysine residue (either Lys-73 or Lys-234) with a similar effect (Knowles 1985; Imtiaz, *et al.* 1994; Therrien and Levesque 2000)(Fig. 4.5).

FIGURE 4.4. Simplified proposed interaction of clavulanic acid with class A β-lactamase. Adapted from Imtiaz *et al.* (1993).

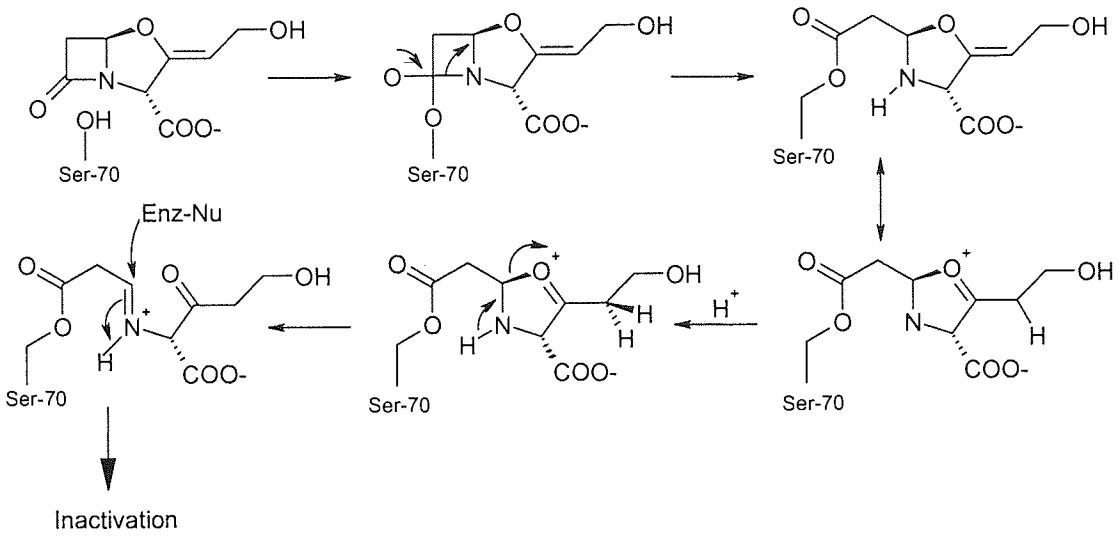
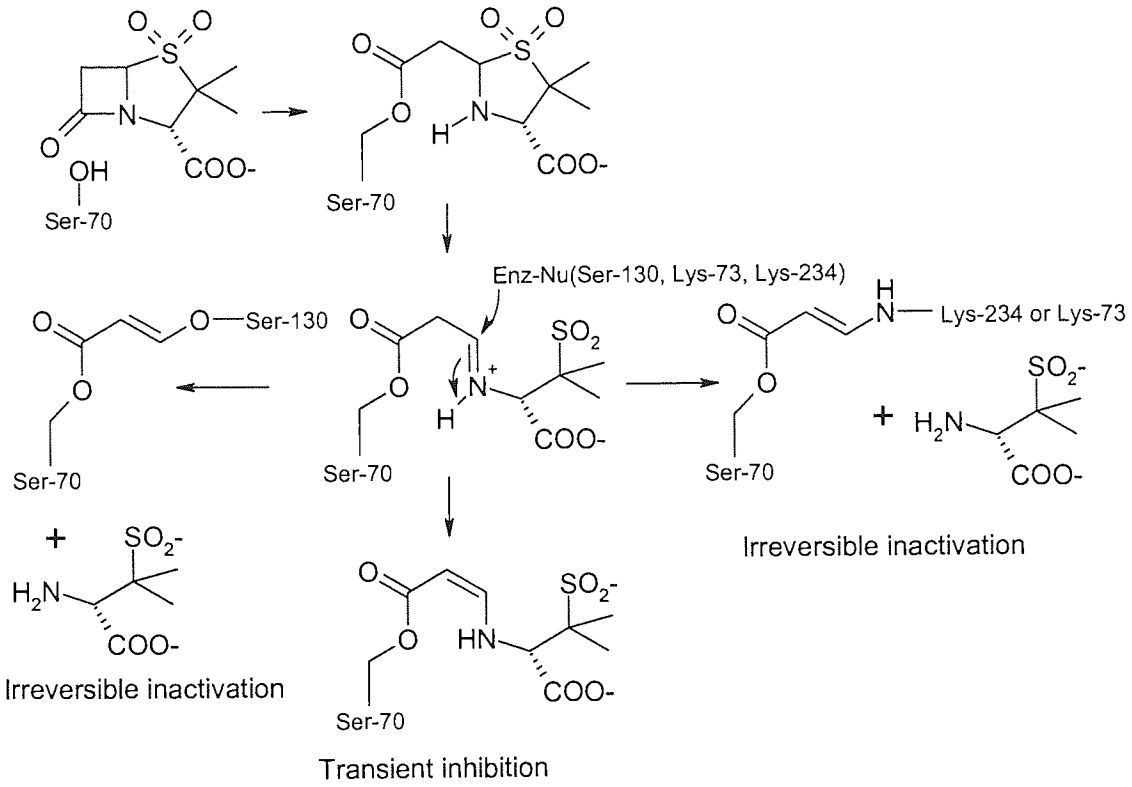


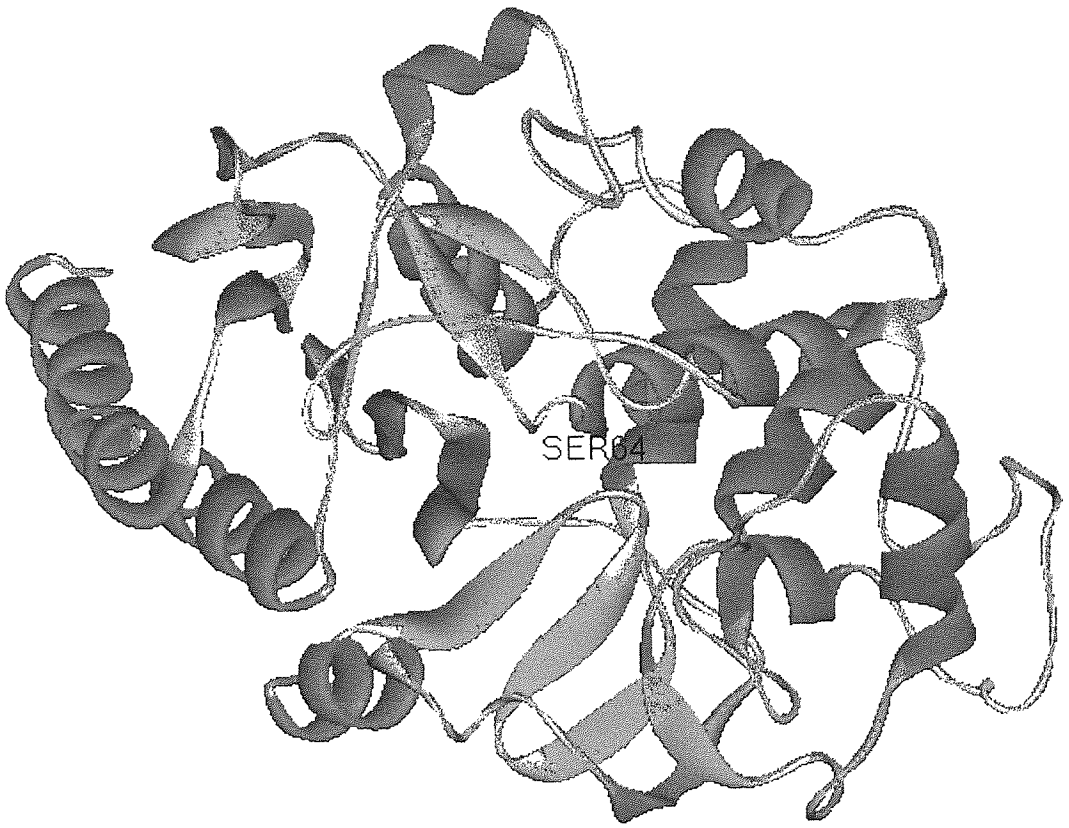
FIGURE 4.5. Proposed interaction of sulbactam with class A β-lactamase. Adapted from Imtiaz *et al.* (1994).



To date, similar studies on the interactions of inhibitors and class C enzymes have not been carried out to the same extent. Crystallographic examination of transition-state analogues has been performed on a phosphonate inhibitor (Lobkovsky, *et al.* 1994), the monobactam aztreonam (Oefner, *et al.* 1990) and a cephem sulphone (Crichlow, *et al.* 2001). These studies identified the role of Tyr-150 in the binding of the inhibitor in the active site. Tyr-150 was postulated to carry out the same role in class C enzymes that Glu-166, with a water molecule, is proposed to do for class A enzymes, namely to act as the general acid/general base catalyst for the active site (Lobkovsky, *et al.* 1994). In these crystal structures, Tyr-150 occupies the equivalent position in the active site. The sulphinate anion of the cephem sulphone is postulated to block a water molecule from attacking the acyl intermediate, preventing deacylation (Crichlow, *et al.* 2001). The binding of clavulanic acid in the active site of a class C enzyme from *E. cloacae* has been modelled. This model showed that a residue required for proton donation leading to opening of the heterocyclic ring was too distant from the carboxylic acid group of clavulanic acid. The acid group hydrogen bonded to Asn-346 and a water molecule,

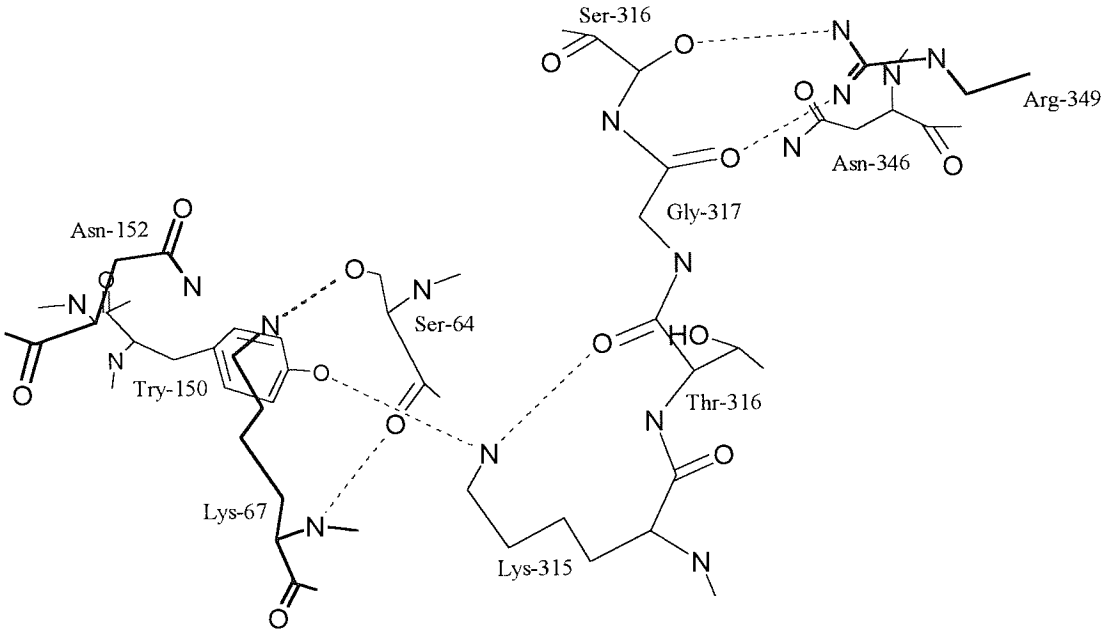
but the water molecule was not close enough to provide a proton for the ring opening step (Lobkovsky, *et al.*. 1993).

FIGURE 4.6. C-alpha trace of *E. cloacae* P99 β-lactamase (Brookhaven Databank ID 1BLS). Ser-64 and other active site residues are shown in green; the α-helices are shown in red and the β-sheets in blue.



The α-carbon trace of the *E. cloacae* P99 enzyme is shown in Fig. 4.6, while Fig. 4.7 is a schematic diagram of the active site of the P99 enzyme.

FIGURE 4.7. Schematic representation of the active site of the class C β-lactamase of *E. cloacae* P99 (Brookhaven Databank ID, 1BLS). Hydrogen bonding between the residues is shown in green. The amino acids are numbered according to the Ambler scheme.



The oxapenem compounds are effective inhibitors of both class A and class C β-lactamase enzymes, but their mechanism of action is unknown. AM-112 was selected as a candidate for study in order to investigate the mode of action against the class C β-lactamase produced by *E. cloacae* P99 using electrospray mass ionisation spectroscopy (ESMIS), atmospheric pressure chemical ionisation (APCI) and analysis of tryptic digests. In addition, each of the oxapenems was docked into the active site of a class A enzyme (TEM-1) and P99 using a molecular modelling package in an attempt to identify reasons for differences in activity of these compounds against class A and class C enzymes.

4.2 Materials and Methods

4.2.1 *Electrospray Mass Ionisation Spectroscopy (ESMIS)*

4.2.1.1 *Preparation of enzyme*

E. cloacae P99 Type IV β-lactamase was purchased from Sigma. A 50mg quantity was weighed out and dissolved in 0.5ml of ddH_20. This sample was then dialysed for 48 hours in 5L of ddH_20 at 5°C, with frequent dialysate changes, to remove the phosphate salts. The dialysed enzyme was frozen in 100μL aliquots at -70°C until required. A sample of the dialysed enzyme was analysed by SDS-PAGE to investigate the purity of the enzyme. A number of bands corresponding to a range of proteins of differing molecular weights were seen on staining with coomassie blue, suggesting that the purity of the sample was low and that a successful ESMIS experiment would be unlikely without further purification of the enzyme.

4.2.1.2 *Boronate affinity chromatography of E. cloacae P99 β-lactamase*

An attempt was made to further purify the *E. cloacae* P99 Type IV β-lactamase by boronate affinity chromatography with reference to the method of Cartwright and Waley (Cartwright and Waley 1984) and Bio-Rad Bulletin 1066 (Anonymous). A volume of 400μL of a 100mg/ml suspension of the enzyme was dialysed against 20mM triethanolamine/ 0.5M NaCl (TEA) buffer for 16 hours at 4°C. Affi-Gel 601 boronate affinity gel (Bio-Rad, Hemel Hempstead, UK) was hydrated according to the manufacturer's instructions using TEA buffer. The hydrated gel was loaded into the barrel of a 5ml syringe that was plugged with glass wool, and washed with several volumes of TEA buffer. A volume of 200μL of the dialysed enzyme was loaded onto the top of the gel and allowed to stand for 30 minutes. Unbound enzyme and debris were eluted with successive 0.5ml aliquots of TEA buffer. The absorbance of the eluted fractions was measured at 280nm, and elution continued

until the absorbance of the eluate was similar to a TEA buffer control blank. The bound enzyme was then eluted with 0.5ml aliquots of 0.5M borate/0.5M NaCl (BOR) buffer (pH 7), with the absorbance being measured as described before. Both the TEA and BOR fractions were collected and dialysed against a 5L volume of ddH_20 for 16 hours at 4°C. The dialysed fractions were then freeze-dried for 24 hours, and the residue stored at -70°C until required.

4.2.1.3 *Tryptic digestion of E. cloacae P99 β-lactamase and AM-112*

Freeze-dried aliquots of the P99 enzyme purified by affinity chromatography (approximately 0.5mg dry weight) were incubated with 1mg AM-112 in 1ml 100mM MOPS buffer, pH 7.0, containing 0.5M NaCl at 37°C for six hours. A control preparation containing enzyme but no inhibitor was also prepared. Each sample was dialysed against 2 x 1 litres of 1mM MOPS buffer, pH 7.0, containing 1mM NaCl, for 48 hours at 4°C to remove any unbound AM-112. The samples were then freeze-dried and stored at -20°C until required. It was observed that the inactivated enzyme sample yielded a pale yellow residue (λmax 420nm), whereas the control enzyme produced a white powder. The samples were dissolved in 100µl of 1% ($^{w}/_{v}$) NH_4HCO_3 and treated with 0.1mg trypsin (type IX-S; Sigma, Poole, UK) dissolved in 1mM HCl (20µl) for three hours at 37°C. The samples were diluted to 1ml in water and freeze-dried in 200µl aliquots.

The peptide fragments were separated by high performance liquid chromatography (HPLC) using a C_{18} reverse-phase silica column (Hewlett Packard) with a variable gradient elution system and detected by a variable wavelength UV monitor operating at 214nm (Hewlett Packard Series 1100). The digested samples were reconstituted in 50µl 50mM NH_4HCO_3, pH 7.8 (elution buffer A). An aliquot of 25µl was injected onto the column using a 50µl loading syringe. Gradient elution was performed over 60 minutes by changing the ratio of buffer A to buffer B (20%$^{v}/_{v}$ 50mM NH_4HCO_3, pH 7.8, in acetonitrile). After injection of the sample, the buffer conditions were maintained at 99% A and 1% B for ten minutes (1ml/min). A linear gradient was then run over 30 minutes to 1% A and 99% B. The column was then held at 99% B

for 10 minutes to wash it, followed by a return to 99% A over 10 minutes. The column was then reconditioned for 10 minutes at 99% A prior to injection of the next sample. The flow rate of the buffer was 1ml/min throughout the run. Control experiments on digested, uninhibited enzyme samples were also carried out. The peptide fragment elution was repeated under exactly similar conditions but with monitoring of the fragments at 420nm. Inhibited enzyme fractions showing a strong absorbance at this wavelength were collected and analysed by mass spectroscopy. Control enzyme fractions were also collected at the corresponding retention times.

4.2.1.4 *Running conditions of ESMIS*

Measurements were made using a Hewlett Packard HP5989 MS Engine Quadrupole mass spectrometer, coupled to a Hewlett Packard HP59987A Electrospray unit. Spectra in positive electrospray (ES+) mode were acquired using nitrogen as a nebulising and drying gas. The capilliary exit voltage (CapEx) was set at +100V. For negative electrospray (ES-) the CapEx voltage was –100V. The parameters for APCI were similar to those for ESMIS for both positive (APCI+) and negative (APCI-) modes. Scans were taken over a wide range from 95-1600 mass units. Samples were injected via flow injection analysis into a mobile phase of 90% methanol in water. The quadrupole was at 100°C and the drying gas heater at approximately 375°C.

4.2.2 Active site modelling of oxapenem inhibitors in class A and class C β-lactamase enzymes

The chemical structures of each of the oxapenems were drawn as the hydrolysed, β-lactam ring opened form using Isis™/Draw (Version 2.3, MDL Information Systems, USA) and then energy minimised using MOPAC (Version 94.1, running on a CAChe Worksystem 3.2). The files were saved in the Brookhaven Protein Databank format and loaded into the molecular modelling package Quanta (Version 98.1111; Accelrys Ltd., Cambridge, UK), running on a Silicon Graphics Octane workstation. The crystal structures of the TEM-1 and P99 enzymes were downloaded from the Brookhaven Protein Databank and viewed in Quanta. The ring opened form

of AM-112 was first positioned in the active site of each enzyme, and its position as a rigid body was manually altered to obtain a position of lowest interaction energy, as calculated by Quanta on the basis of electrostatic and Van der Waals interactions. The distance between the oxygen of the Ser-64 or Ser-70 hydroxyl group and C_6 of the β-lactam ring was maintained at 1.32Å. Once the lowest interaction energy position had been achieved a carbonyl bond was formed between the hydroxyl group of the active site serine and the C_7 of the β-lactam ring of the inhibitor. The bond length was maintained between 1.2-1.3Å, a typical C-O ester bond length (McMurry 1992). Each of the other inhibitors was then modelled in a similar orientation to that of AM-112. The acylated inhibitor-enzyme complexes were then energy-minimised without any restraints on the inhibitors, using the CHARMm function of Quanta. Energy minimisation used the steepest descents method. The hydrogen-bonding function of Quanta was then used to examine the interaction of the bound inhibitor with residues in the active site.

4.3 Results

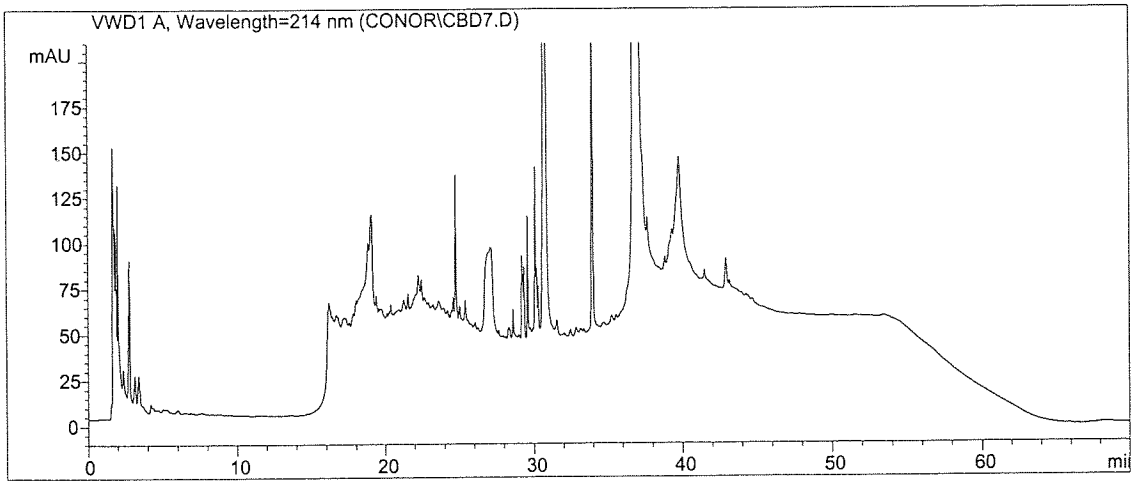
4.3.1 *Analysis of tryptic digests of P99 enzyme inhibited by AM-112*

Attempts to purify the P99 enzyme sufficiently to identify a single peak corresponding to the enzyme by ESIMS were unsuccessful. Despite passage of the enzyme through an affinity chromatography column, a number of proteins were eluted and analysis by ESIMS could not assign a peak to the enzyme.

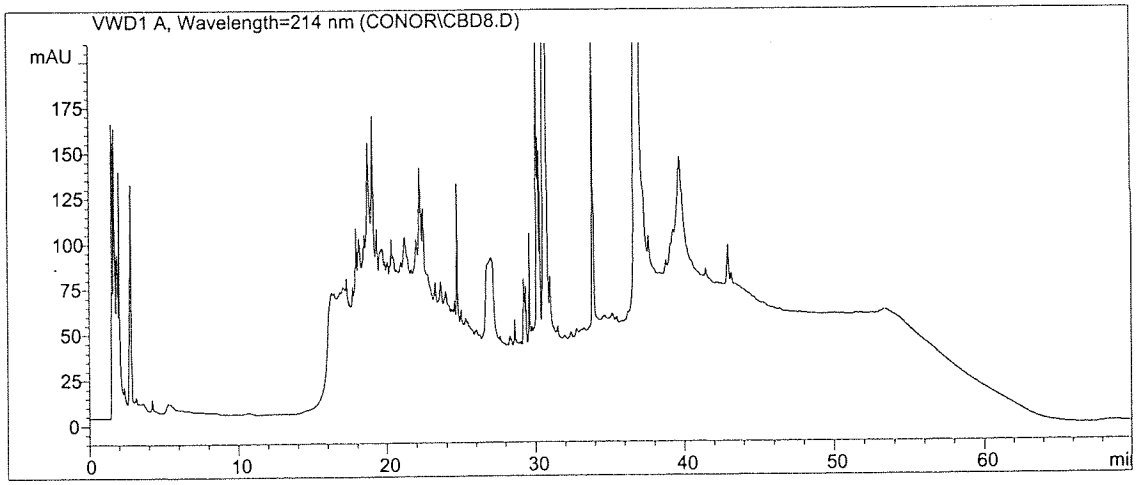
The results of the trypsin digestion of the inhibited and control enzyme samples are shown in Fig. 4.8 and Fig. 4.9. Differences in the peptide fragmentation maps of the control and inhibited enzymes are clearly seen when Fig. 4.8A is compared to Fig. 4.8B. The most marked difference is in the region with a retention time of 18-22 minutes.

FIGURE 4.8. Peptide fragmentation map after trypsin digest of (A) control untreated *E. cloacae* P99+ β-lactamase and (B) AM-112 treated enzyme monitored at 214nm.

A



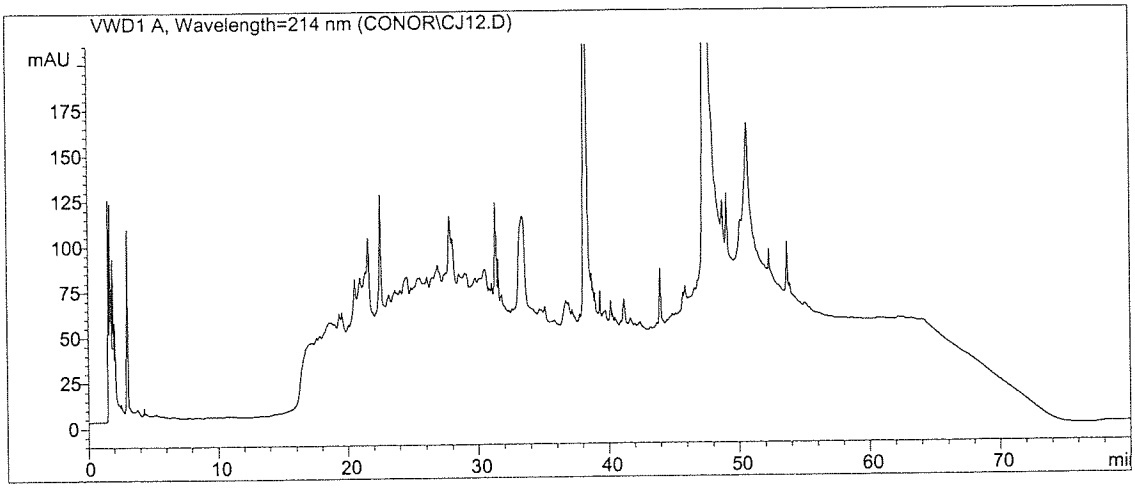
B



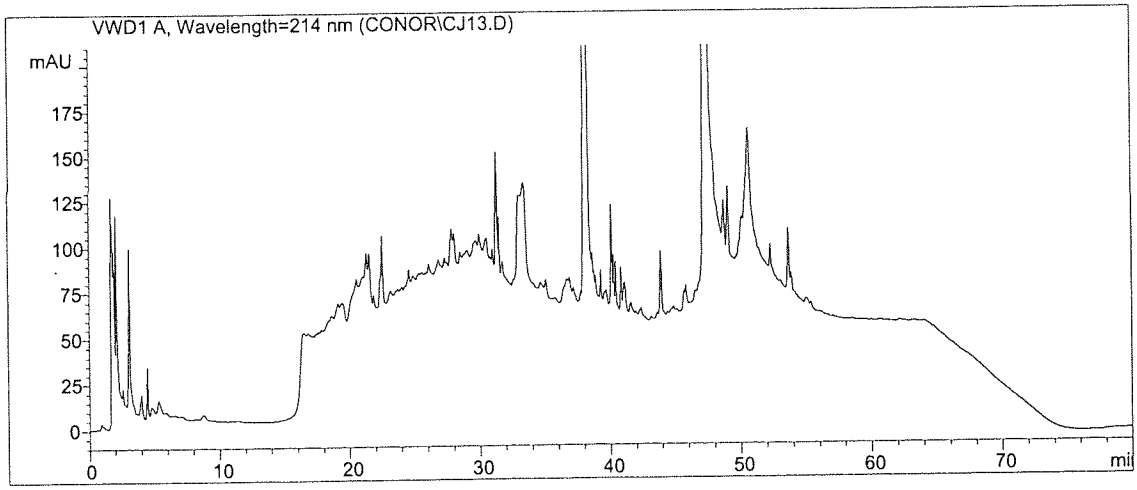
In an attempt to enhance the resolution of the peak in this area, the elution gradient was altered to produce a shallower increase in the ratio of buffer B. After 10 minutes at 1% B, the gradient was increased to 60% B over 20 minutes, then increased to 99% B over 10 minutes. The gradient was held at 99% B for 10 minutes followed by a return to 1% B over 10 minutes. The results from this altered gradient elution are shown in Fig. 4.9A (control enzyme) and Fig. 4.9B (AM-112 treated enzyme). There are some differences in the elution profile of the region 20-30 minutes, but these differences are not as pronounced as appeared in Fig. 4.8A and Fig. 4.8B. In addition the appearance of new peaks at retention time 40 min is seen in Fig. 4.9B compared to Fig. 4.9A.

FIGURE 4.9. Peptide fragmentation map after trypsin digest of (A) control untreated and (B) AM-112 treated *E. cloacae* P99+ β-lactamase monitored at 214nm.

A



B

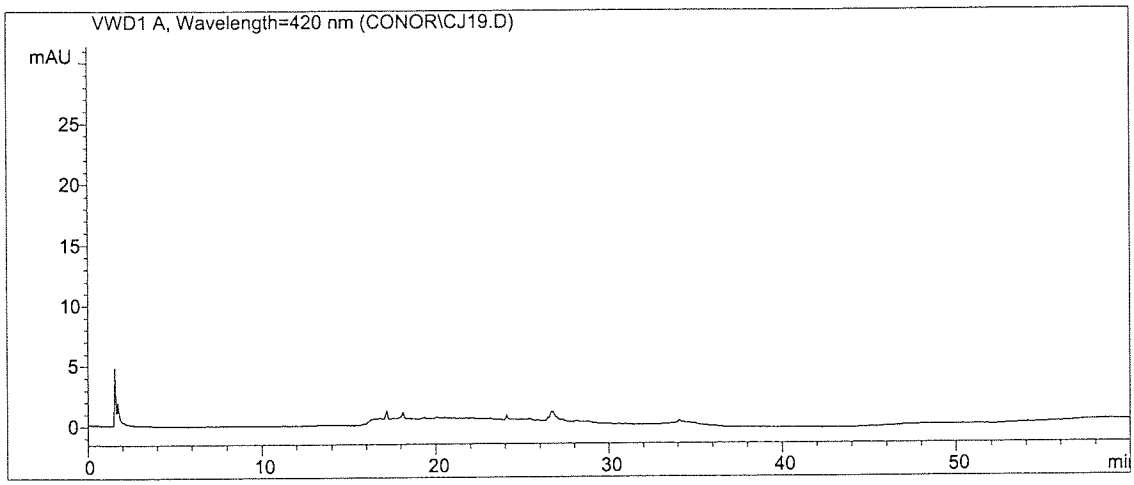


As described in Section 4.2.1.3, it was observed that after incubation of the enzyme with AM-112 and subsequent freeze-drying, a yellowish residue was formed, whereas the control enzyme formed a white residue. To investigate this further, gradient elution was performed again, with the UV detector set to 420nm in order to identify peaks that absorbed at this high wavelength. The peptide fragmentation maps at this wavelength are shown in Fig. 4.10A (control enzyme) and Fig. 4.10B (inhibited enzyme). It can be seen on the control enzyme trace (Fig. 4.10A) that there is very little absorbance at this wavelength, with the strongest absorbing peak having an absorbance of 5mAU. The inhibited enzyme trace (Fig. 4.10B) shows the appearance of a new peak with a retention time of 3 minutes, which has a much

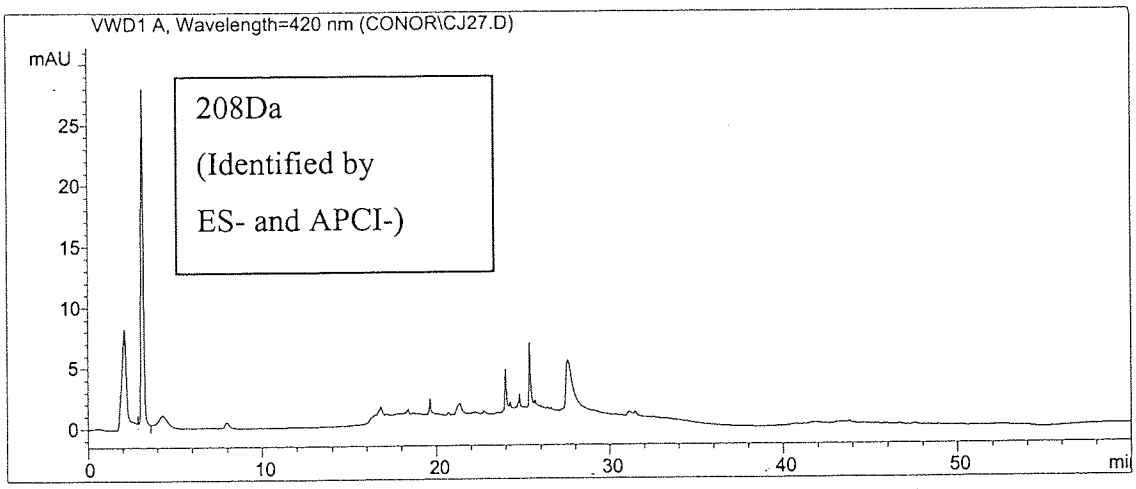
stronger absorbance of above 25mAU. There are other peaks with retention times of between 20 and 30 minutes that also have absorbance above background levels.

FIGURE 4.10. Peptide fragmentation map after trypsin digest of (A) control untreated and (B) AM-112 treated *E. cloacae* P99+ β-lactamase monitored at 420nm.

A



B



The fraction corresponding to the peak with an absorbance of 25mAU was collected from the HPLC column and subjected to analysis by ESMIS. Positive ESMIS failed to identify any peaks. Negative ESMIS and negative APCI of the inhibited enzyme peak (retention time ~3min, 25mAU) absorbing at 420nm identified a peak at 208 Da. The elution fraction of the control sample collected at the same time did not demonstrate any peaks after examination by negative ESMIS and negative APCI. Examination of the inhibited enzyme fractions showing absorbance at 420nm

(retention times between 20-30 minutes) failed to identify any peaks on negative ESMIS or APCI.

4.3.2 *Active site modelling of oxapenem inhibitors in class A and class C β-lactamase enzymes.*

The numbering of the atoms in the oxapenem nucleus is shown in Fig. 4.11. The energy minimised structures of the oxapenem inhibitors in the active site of the class A TEM-1 enzyme are shown in Figs. 4.12-4.15 while those of the oxapenems in the class C enzyme P99 are displayed in Figs. 4.16-4.19. Table 4.1 provides details of the distances between atoms in the inhibitor molecule and important residues in the active site for TEM-1, while Table 4.2 provides details for P99.

FIGURE 4.11. Structure of the oxapenem nucleus showing the numbering of the carbon atoms.

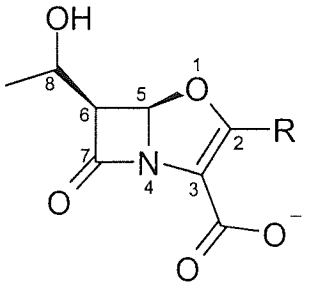


TABLE 4.1. Distances (Å) between selected active site residues of TEM-1 β-lactamase and atoms of the energy minimised acyl-enzyme complex.

	Inhibitor				
	AM-112	AM-113	AM-114	AM-115	Sulbactam[a]
Ala-237 N to C_7 of inhibitor	5.17	5.67	5.8	5.9	3.16
Ser-70 O to C_7 of inhibitor	1.3	1.28	1.32	1.3	2.98
Ser-130 O to COO^-	3.68	4.18	2.85	2.7	2.69
Ser-130 O to N_4 of inhibitor	2.92	3.14	2.52	2.88	2.92
Lys-234 N to COO^-	10.68	5.29	3.89	2.95	2.7
Ser-235 O to COO^-	2.64	2.84	4.64	3.29	2.73
Arg-244 N to COO^-	4.14	3.57	7.27	6.55	2.72
Ser-130 O to C_5 of inhibitor	3.37	3.44	2.93	3.89	-
Lys-73 N to C_5 of inhibitor	5.38	5.27	5.68	6.08	-
Lys-234 N to C_5 of inhibitor	3.42	3.18	3.37	3	-

[a]Taken from Imtiaz *et al.*, (1994).

FIGURE 4.12. Schematic representation of the energy minimised acyl-enzyme complex of AM-112 (shown in red) and *E. coli* TEM-1 β-lactamase. Hydrogen bonding between residues is shown in green. Water molecules are represented by black spheres. The amino acids are numbered according to the Ambler scheme.

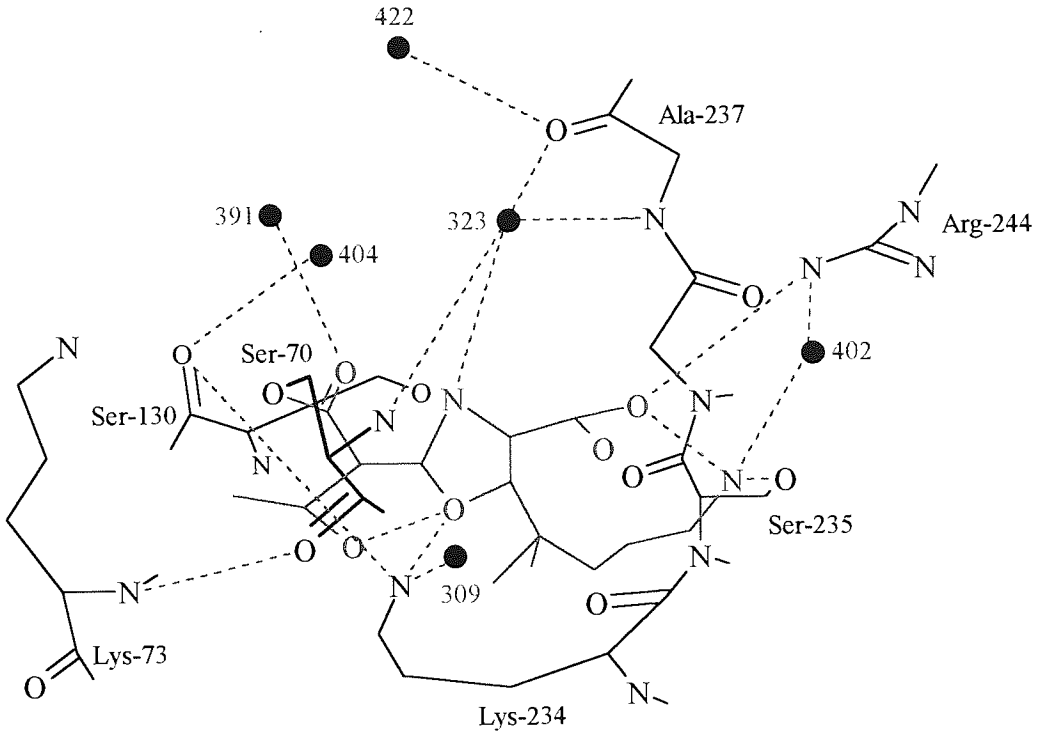


Fig. 4.12 shows the energy-minimised position of AM-112 in the active site of TEM-1. The C_7 carbonyl group of the β-lactam ring has hydrogen bonded to a solvent molecule (Wat-391). The hydroxyl group on the C_8 side chain has formed a hydrogen bond to the O_1 atom of the heterocyclic ring, which forms a bond with the amino group of Lys-234. The nitrogen of the heterocyclic ring of AM-112 has hydrogen bonded to Wat-323, which H-bonds to the main chain amino and carboxyl groups of Ala-237. The C_3 carboxylic acid group H-bonds to the terminal amino group of the C2 side chain, and also to Arg-244. The terminal amino group of the C_2 side chain forms H-bonds with the side chain hydroxyl group of Ser-235 and also with Arg-244 via a water molecule (Wat-402).

FIGURE 4.13. Schematic representation of the energy minimised acyl-enzyme complex of AM-113 (shown in red) and *E. coli* TEM-1 β-lactamase. Hydrogen bonding between residues is shown in green. Water molecules are represented by black spheres. The amino acids are numbered according to the Ambler scheme.

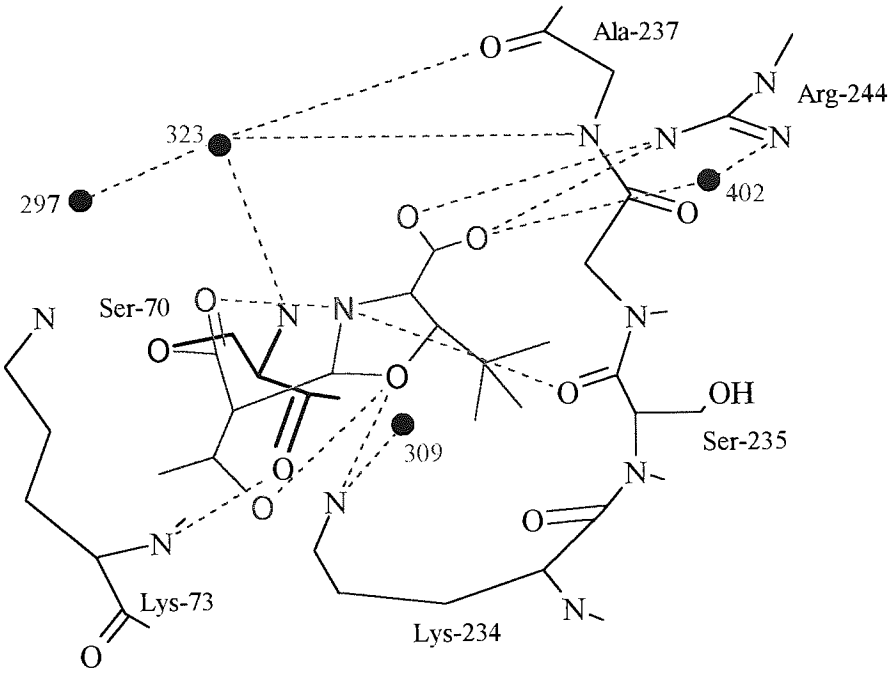
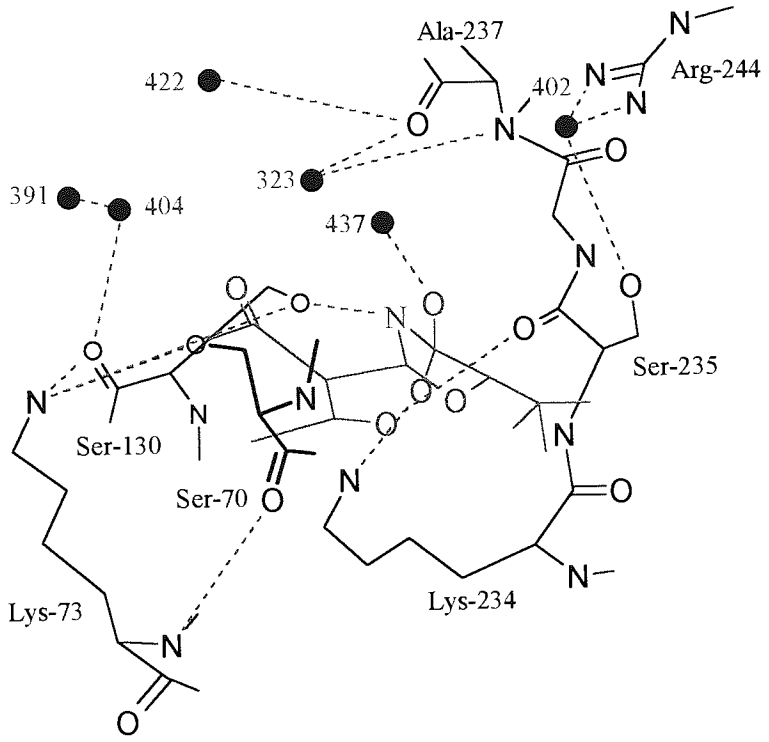


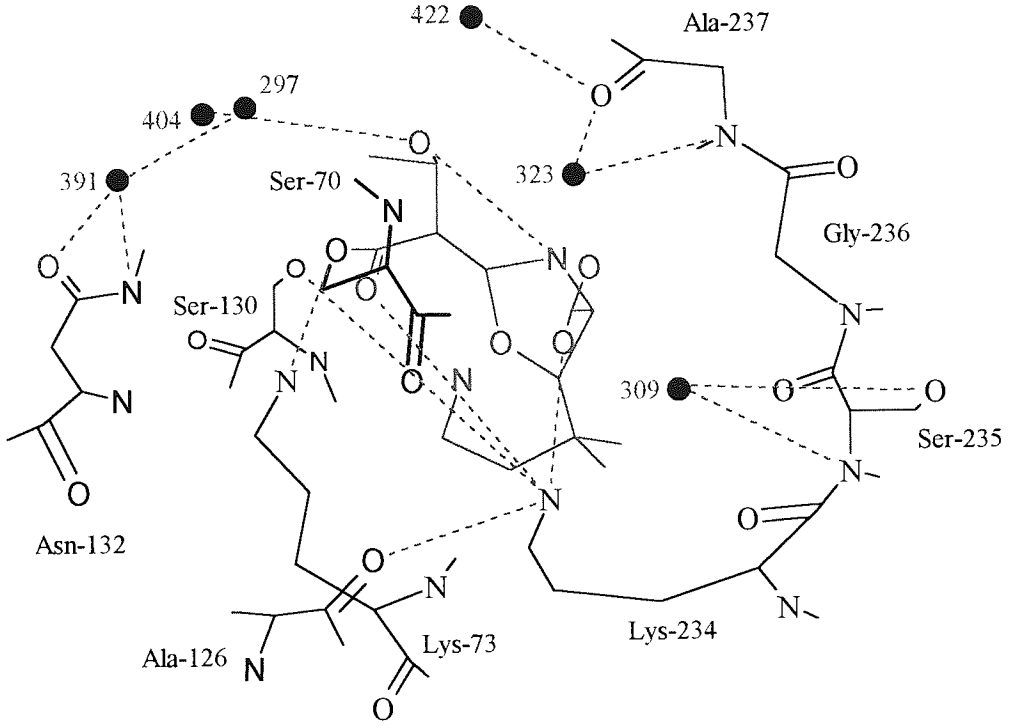
Fig. 4.13 illustrates the energy minimised acyl-enzyme complex of AM-113 and TEM-1. The carbonyl group at C_7 forms a hydrogen bond to the N_4 atom of the heterocyclic ring. The hydroxyl group of C_8 hydrogen bonds with the O_1 atom of the heterocyclic ring. The N_4 atom of this ring H-bonds to the carbonyl group of Ser-235. The terminal amino group of Lys-234 H-bonds to a solvent molecule (Wat-309) and the O_1 atom of the heterocyclic ring. The C_3 carboxylic acid group forms H-bonds with the amino residues of Arg-244 and Wat-402.

FIGURE 4.14. Schematic representation of the energy minimised acyl-enzyme complex of AM-114 (shown in red) and *E. coli* TEM-1 β-lactamase. Hydrogen bonding between residues is shown in green. Water molecules are represented by black spheres. The amino acids are numbered according to the Ambler scheme.



The energy minimised acyl-enzyme complex of AM-114 and TEM-1 is shown in Fig. 4.14. The N_4 atom of the heterocyclic ring forms a hydrogen bond to the hydroxyl group of Ser-130. One oxygen atom of the C_3 carboxylic acid group H-bonds to a solvent molecule (Wat-437), while the other oxygen atom H-bonds to the terminal amino group of Lys-234. Lys-73 forms hydrogen H-bonds with Ser-130 and Ser-70, while Arg-244 forms H-bonds with Ser-235 and Wat-402.

FIGURE 4.15. Schematic representation of the energy minimised acyl-enzyme complex of AM-115 (shown in red) and *E. coli* TEM-1 β-lactamase. Hydrogen bonding between residues is shown in green. Water molecules are represented by black spheres. The amino acids are numbered according to the Ambler scheme.



AM-115 forms fewer hydrogen bonds to residues in the active site than its isomer, AM-112 (Fig. 4.15). The carbonyl group of C_7 H-bonds to the terminal amino group of Lys-234. Lys-234 forms H-bonds with the hydroxyl group of Ser-130 and the C_3 carboxylic acid group. The N_4 atom of the heterocyclic ring forms a hydrogen bond with the hydroxyl group of the C_8 atom.

The active site of class C enzymes is more open than that of class A enzymes, a fact which might account for their ability to accommodate and hydrolyse cephalosporins, which have a larger fused-ring nucleus than the penicillins (Lobkovsky, *et al.* 1993). Consequently there appears to be fewer interactions between the oxapenem inhibitors and residues in the active site of P99 than there were for TEM-1. The energy-minimised structure for the acyl-enzyme complex of AM-112 and P99 is shown in Fig. 4.16. The amino group of the C_2 side chain formed a hydrogen bond to the hydroxyl group of Thr-316. The terminal amino group of Lys-315 bonded to the hydroxyl group of C_8 and the O_1 atom of the heterocyclic ring. An oxygen atom of the C_3 carboxylic acid group hydrogen bonded to the main chain amino group of Asn-346.

FIGURE 4.16. Schematic representation of the energy minimised acyl-enzyme complex of AM-112 (shown in red) and *E. cloacae* P99 β-lactamase. Hydrogen bonding between residues is shown in green. The amino acids are numbered according to the Ambler scheme.

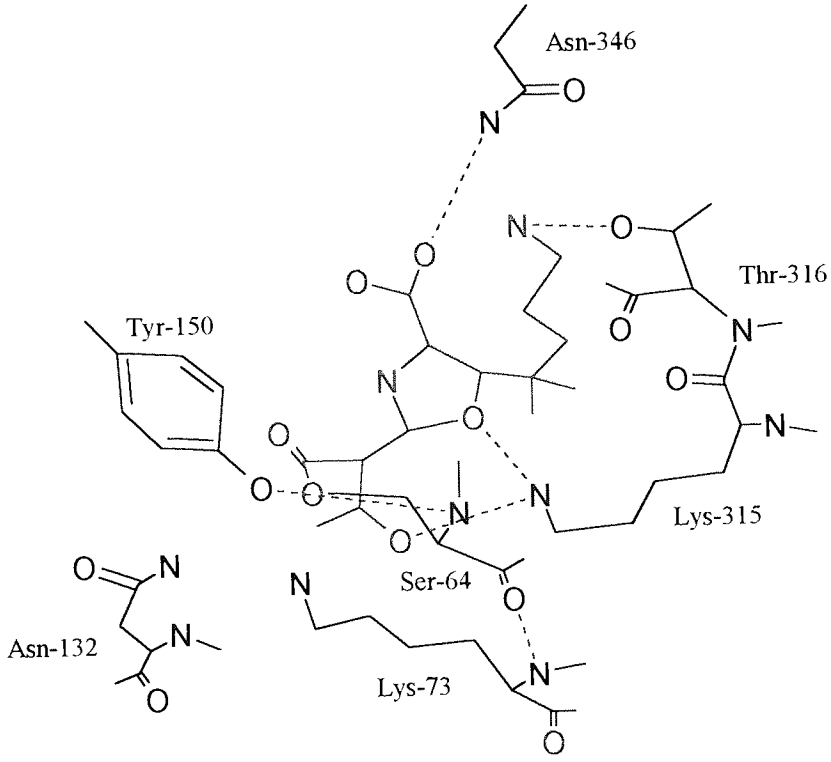


Figure 4.17 shows the energy-minimised structure of AM-113 in the active site of P99. As with AM-112, the C_3 carboxylic acid group H-bonds to Asn-346, and also H-bonds to Arg-349. The N_4 atom of the heterocyclic ring H-bonds to the carbonyl group of Thr-316, which also H-bonds to Tyr-150 via Lys-315. The C_8 hydroxyl group forms an intramolecular hydrogen bond to the O_1 atom of the heterocyclic ring.

FIGURE 4.17. Schematic representation of the energy minimised acyl-enzyme complex of AM-113 (shown in red) and *E. cloacae* P99 β-lactamase. Hydrogen bonding between residues is shown in green. The amino acids are numbered according to the Ambler scheme.

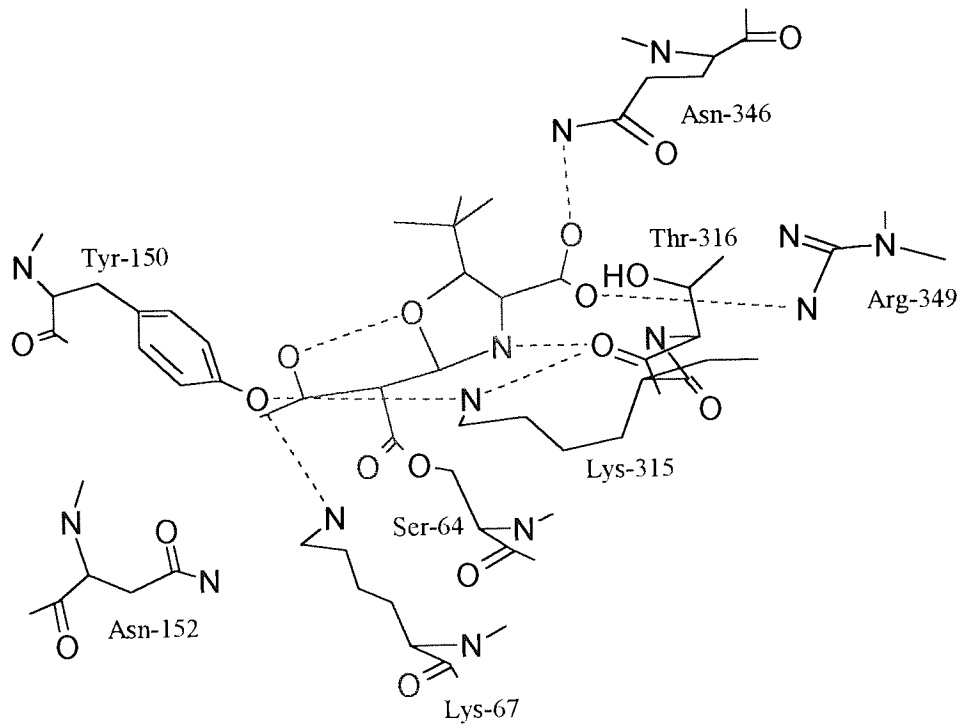
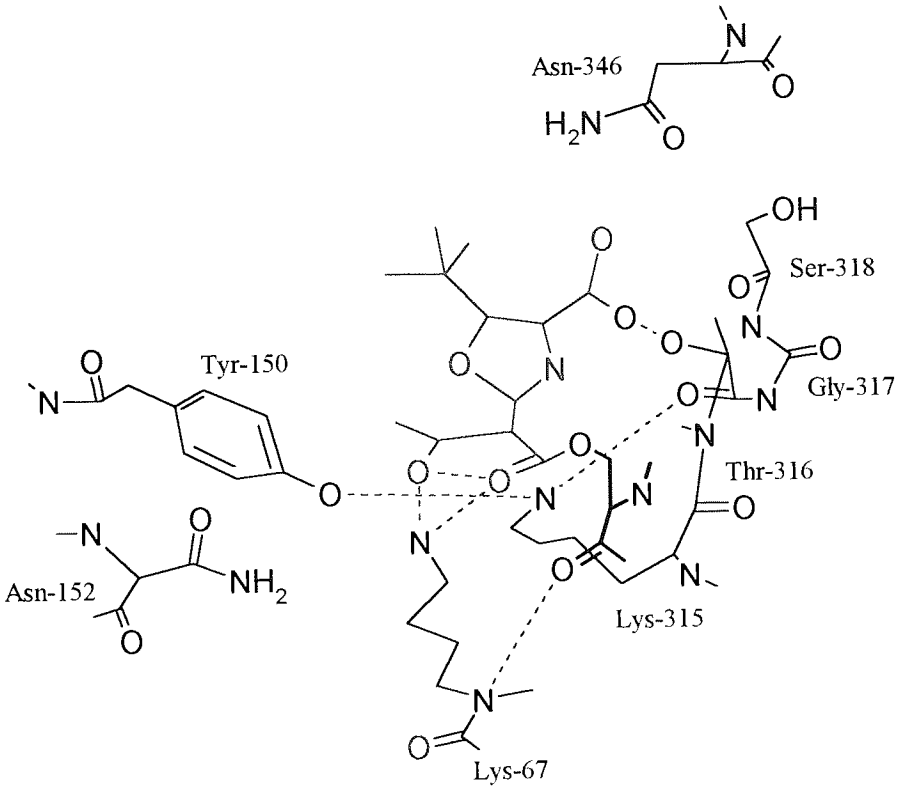


Fig. 4.18 shows the energy-minimised binding of AM-114 to P99. The amino group of Lys-67 H-bonds to the C_7 carbonyl group and the C_8 hydroxyl moiety, which also form an intramolecular hydrogen bond. The other interaction of AM-114 with the active site of P99 is the hydrogen bond formed between the C_3 carbonyl group and Thr-316.

FIGURE 4.18. Schematic representation of the energy minimised acyl-enzyme complex of AM-114 (shown in red) and *E. cloacae* P99 β-lactamase. Hydrogen bonding between residues is shown in green. The amino acids are numbered according to the Ambler scheme.



The energy-minimised structure of AM-115 in the active site of P99 is shown in Fig. 4.19. The terminal amino residue of the C_2 side chain interacts with the carbonyl group of Ser-318, the C_3 carboxylic acid group and also with the C_7 carbonyl moiety. The C_3 carboxylic acid group also H-bonds to the amino group of Ser-318. The C_8 hydroxyl group H-bonds to the carbonyl function of Thr-316.

FIGURE 4.19. Schematic representation of the energy minimised acyl-enzyme complex of AM-115 (shown in red) and *E. cloacae* P99 β-lactamase. Hydrogen bonding between residues is shown in green. The amino acids are numbered according to the Ambler scheme.

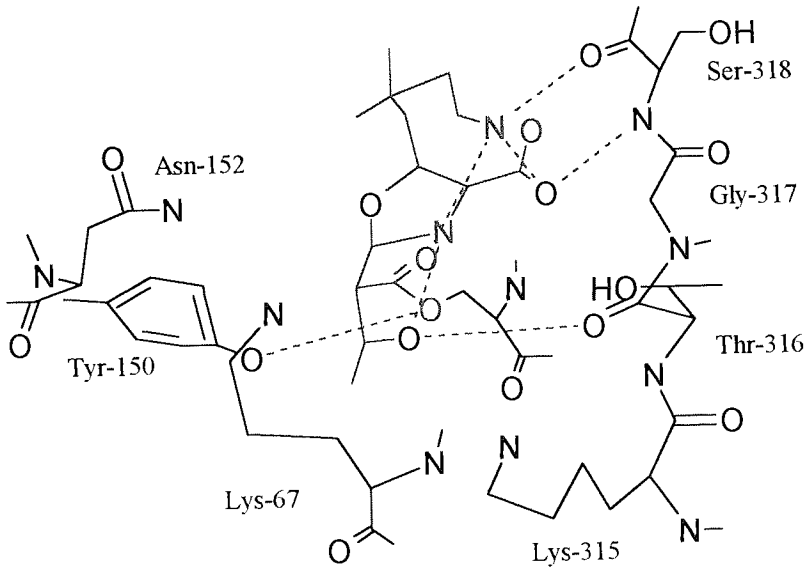


TABLE 4.2. Distances (Å) between selected active site residues of P99 β-lactamase and atoms of the energy minimised acyl-enzyme complex.

	Inhibitor			
	AM-112	AM-113	AM-114	AM-115
Ser-64 O to lactam CO	1.3	1.33	1.29	1.32
Lys-67 N to C_5	5.24	4.68	5.36	4.7
Lys-315 N to C_5	3.42	3.58	4.23	4.7
Tyr-150 O to C_5	3.15	3.32	3.18	3.71

4.4 Discussion

The commercially obtained P99 enzyme obtained from Sigma proved to contain a number of protein bands after separation by SDS-PAGE and staining with coomassie blue. The large number of proteins of differing molecular weights would have made it extremely difficult to assign any peaks arising from ESMIS to the enzyme. Thus an attempt was made to purify the enzyme by affinity chromatography. Partial purification of the enzyme was achieved by this method, giving a major band at approximately 39kDa, with a decrease in the number of contaminating protein bands seen after SDS-PAGE. ESMIS was attempted on this purified enzyme, but no peak could be assigned to the enzyme. From the amino acid sequence of the P99 enzyme (Brookhaven Protein Databank, ID 1BLS) the molecular weight of the enzyme is 39,222 Da. No peak corresponding to this size was detected by ESIMS.

Had ESMIS proved successful in detecting the enzyme, the next step would have been to react the enzyme with AM-112. After dialysis of the sample to remove unbound AM-112, the sample would have been subjected to analysis by ESMIS. This may have identified a new peak with a higher molecular weight, indicating the presence of bound AM-112 in the enzyme. From the increase in mass, it would be possible to speculate on the size of the fragment of AM-112 bound to the enzyme, and from this, propose a reaction mechanism for binding of AM-112 to the active site.

An alternative approach was used to investigate the peptide fragmentation maps obtained by HPLC after tryptic digestion of the enzyme. These show differences between the control enzyme and the sample incubated with AM-112 (Fig. 4.8A and 4.8B). However, the differences were minor and it is difficult to state that they are evidence of cross-linking of AM-112 in the active site of the enzyme. Trypsin cleaves proteins at the carboxyl end of lysine and arginine residues. Complete tryptic digestion of the P99 enzyme could potentially result in thirty peptide fragments, the shortest having three residues and the longest possessing twenty-nine amino acids. The peptide fragmentation map will necessarily be complex, especially if incomplete digestion of the P99 enzyme occurs. The central theory behind the tryptic digest of a

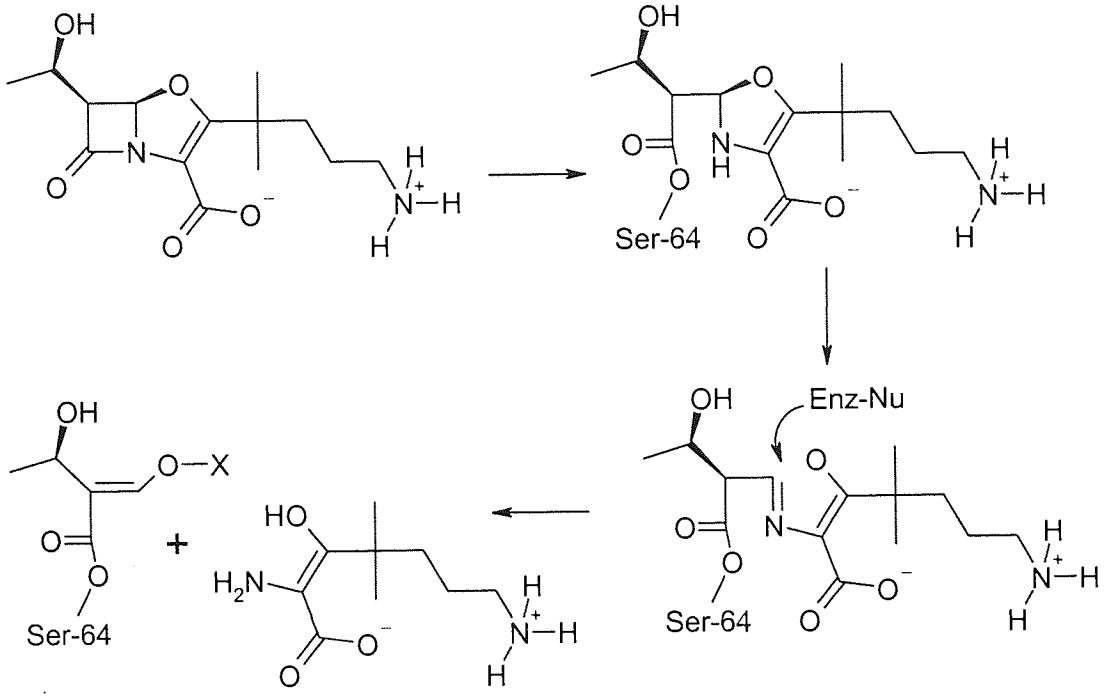
β-lactamase enzyme inhibited by a compound is that cross-linking of the inhibitor to certain residues in the active site will produce changes in the peptide fragmentation map. If the β-lactamase inhibitor does cross-link to two residues that are not available for trypsin cleavage, then one might expect that the peptide fragmentation map would show the disappearance of two peaks and the appearance of one peak, compared to the control sample. If the inhibitor cross-linked to another residue within the trypsin cleaved fragment, then one might expect to see small changes in the elution profile, but not necessarily any peaks appearing or disappearing.

Bonomo *et al.*(2001) have demonstrated such an effect with tazobactam and the class C CMY-2 β-lactamase, using tryptic digestion. The digested peptide fragments were resolved by liquid chromatography coupled to mass spectroscopy. Liquid chromatography identified peaks with slightly different retention times, while mass spectroscopy of these peaks allowed a change in mass of only 80 Da to be observed and characterised. Gledhill *et al.* (1991) used tryptic digestion and HPLC to investigate the inactivation of *B. cereus* β-lactamase I (class A) by chlorinated 6-spiroepoxypenicillins. They observed changes in the peptide elution profile of the inhibited enzyme compared to the control enzyme and formation of a chromophore at a higher wavelength by the inhibited enzyme that was not observed in the native enzyme. The interaction of tazobactam with the class A PC1 β-lactamase from *S. aureus* was investigated by Denny *et al.* (1994). No difference in the peptide fragmentation profile of the inhibited enzyme compared to the control enzyme was observed, which led the workers to speculate that had cross-linking by tazobactam occurred, that it might have occurred between Ser-70 and Lys-73, i.e. within a trypsin cleaved fragment, and thus would not be detectable by HPLC alone. ESMIS has been used to observe intermediates formed in the reaction of clavulanic acid with TEM-2 β-lactamase from *E. coli*. The authors of this study reported on four modified proteins formed after the incubation of the enzyme with clavulanic acid (Brown, *et al.* 1996). ESMIS has also been used to identify acyl-enzyme intermediates formed by the reaction of P99 β-lactamase with four β-lactam antibiotics (Aplin, *et al.* 1990).

The results of experiments carried out in this chapter cannot confidently predict the mechanism of action of AM-112-induced β-lactamase inhibition. Small differences were seen in the peptide fragment elution profile of the inhibited P99 enzyme compared to the control sample. The observation of formation of a yellow residue after dialysis and freeze-drying led to speculation that binding of AM-112 to the enzyme resulted in the formation of a new chromophore that absorbed at a higher wavelength, as suggested by the yellow colour. The chromophore could be due to a fragment of AM-112 cleaved from the molecule after acylation of the compound by the active site serine. To investigate this, inhibited and control enzyme samples were passed through the HPLC column with UV detection at 420nm, a wavelength where the amino acids of the peptide fragments would not be likely to absorb, yet a conjugated novel chromophore might. The poor reproducibility of the sample runs of both control and inhibited enzymes hindered efforts to identify peaks absorbing at the higher wavelength. Not only did the absorption and retention time of peaks vary between samples, they differed between aliquots of the same sample. This may be due to the potential lability of any chromophore formed after cross-linking. Negative ESMIS and APCI of inhibited enzyme peaks absorbing at 420nm identified a peak at 208 Da. The molecular weight of the proposed cross-linking cleaved fragment was 203 Da. The cleaved fragment is speculated to be formed after the initial acylation of the enzyme by the inhibitor and tautomerisation of the complex between the enamine and imine forms, as described for clavulanic acid, tazobactam and sulbactam. Nucleophilic attack by a residue in the active site (possibly Tyr-150 in class C enzymes) would lead to opening of the heterocyclic ring to form the linear acyclic species. A portion of the molecule would be cleaved during the formation of the cross-linked species, which leads to the irreversible inhibition of the enzyme (Fig. 4.18). A similar reaction would be expected for class A enzymes, where Ser-130 is likely to be the cross-linking residue. However, while it is feasible to speculate that a cross-linked inhibitor in the active site must result in a portion of the molecule being cleaved, it seems unlikely that such a small fragment (203Da) would be retained after 48-hours of dialysis. Similarly, if the yellow colour was due to the natural decomposition of AM-112 while maintained at 37°C for six hours, then one could expect that the decomposed fragments would be removed by dialysis. That the yellow colour is observed after dialysis suggests that it is either due to the

freeze-drying process or to some alteration of the active site after inhibiton by AM-112.

FIGURE 4.20. Simplified reaction scheme for the proposed cross-linking of AM-112 to Ser-64 and unknown residue X in the active site of a class C β-lactamase, showing the proposed cross-linking cleaved fragment.



Freeze-drying of the sample may have led to the decomposition of the inhibited enzyme and the inhibitor, resulting in the yellow colour observed. However, the fact that the eluted fraction which shows strong absorbance at 420nm compared to the control sample contains a fragment with a mass of 208Da cannot yet be explained. As already described, this mass is too large to be a cleaved fragment of the inhibitor, and too small to be a cross-linked section of the enzyme obtained after tryptic digestion. It is not a contaminant from the buffers used, as these did not produce any peaks when examined by negative ESMIS and ACPI.

The acyl-enzyme transition state analogue for each oxapenem inhibitor in the active site of the class A TEM-1 and class C P99 enzymes was modelled. There are several reasons why the acyl-enzyme intermediate was chosen for study. The most obvious reason is that crystallographic studies on the position of the bound inhibitors had not been performed. Thus there was no information on the position of any bound

inhibitor in the active site, whether in a cross-linked form or as an acyclic linear structure. Crystallographic data would have yielded important information on the residues in the active site which might be involved in the acylation and possible cross-linking of the inhibitor. The ESMIS studies carried out as described above could not identify a peak corresponding to the native enzyme. As a result further planned studies on the reaction of the inhibitors with the enzyme, as analysed by ESMIS, were not carried out. Therefore no information on possible fragments of inhibitor binding to residues could be gleaned. After entry of an inhibitor into the active site, which is regarded as being guided by specific residues in class A enzymes such as Arg-244, located near the opening of the active site, which attracts the C_3 carboxylate of the heterocyclic ring of the inhibitor, repositioning of the molecule occurs to form the Michaelis complex. This complex forms due to the interaction of Lys-234 and the substrate carboxylate, and is stabilised by hydrogen bonding with the side chain of Ser-235 or Thr-235 (Zafaralla, *et al.* 1992). Subsequent to such positioning, acylation of the inhibitor occurs. This acyl-enzyme complex was chosen for modelling because it represents a definite stage in the inhibition of the enzyme. The residues involved in forming the complex are still in debate, and the fate of the inhibitor after acylation is unknown. Thus the acyl-enzyme complex appeared to be a logical choice for modelling and could provide clues on the next stages of the inhibition of the enzyme.

Water molecules in the active site of class A enzymes play an important role in the deacylation stage of β-lactam hydrolysis. Effective inhibitors of class A enzymes have been found to displace these catalytically important molecules (Maveyraud, *et al.* 1996; Ness, *et al.* 2000). Modelling of the initial acyl-enzyme complex formed by the oxapenem inhibitors indicates that both AM-112 and AM-113 interact with Wat-309 via the terminal amino residue of Lys-234. The isomers of these compounds, AM-114 and AM-115 do not appear to interact with the solvent molecule in this position. It is possible that their binding to Ser-70 and the subsequent collapse of this tetrahedral intermediate and opening of the β-lactam ring serves to displace this solvent molecule. This would hinder the deacylation step of the enzyme, catalysed by the hydrolytic water molecule, leading to prolonged inhibition of the enzyme. This might account for the improved activity of AM-114 and AM-115 against class A enzymes, compared to AM-112 and AM-113.

The residue involved in the opening of the 5-membered ring of the inhibit leads to the formation of the linear acyclic species has not been precisely de class A enzymes, Ser-130 has been suggested to play such a role for clavul and tazobactam (Knowles 1985; Imtiaz, *et al.* 1993; Brown, *et al.* 1996; Ya 2000; Kuzin, *et al.* 2001) while Ser-130, Lys-73 or Lys-234 plays a simila sulbactam (Knowles 1985; Imtiaz, *et al.* 1994). From the energy m structures of the acyl-enzyme complexes, the C_5 atom of the inhibitor was to be between 2.9 and 3.9Å from the ε-amino group of Lys-234. For Ly distance between the ε-amino group and the C_5 atom was between 5.2 and 6 β-hydroxyl group of Ser-130 was located between 3-3.4Å from the C_5 at Ser-130 and Lys-234 are close enough to this C_5 atom to cause openi heterocyclic ring as shown in Figs. 4.5 and 4.6. The distance between Lys-7 C_5 atom is likely to be too great for any cross-linking to occur.

AM-112 and AM-115 have long side chains at the C_2 position, termin positively charged amino residue. AM-113 and AM-114 lack this long s instead having a hydrophobic tertiary butyl group at this position. It expected then that the terminal amino group of AM-112 and AM-115 cou with residues in the active site that are not available to either AM-113 or The results from the IC_{50} tests described in Chapter 2 indicate that AM- more effective at inhibiting the TEM-1 enzyme than AM-112 (IC_{50} of 0.0 AM-115, compared to 2.26μM for AM-112). The energy-minimised str AM-112 and AM-115 differ markedly in the location of the C_2 side AM-112, the side chain has remained mostly linear, and the terminal am interacts with the hydroxyl side chain of Ser-235, Wat-323 and the C_3 acid group. For AM-115, both the C_2 side chain and the C_3 carboxylic acid forced from a linear position and are looped back towards the heterocyc this position the C_2 side chain does not form any hydrogen bonds with resi active site. It is not clear why AM-115 forms this looped structure while chain of AM-112 remains linear. The position of the water molecule (Wa be significant, and may force the C_2 side chain of AM-115 to conformation. Perhaps it is due to the position of the C_8 hydroxyl group w

for AM-112 and 1'S for AM-115. The position of this hydroxyl group in relation to the O_1 atom of the heterocyclic ring and the C_3 carboxyl group may favour the looping of the C_2 side chain towards the C_8 end of the oxapenem. The energy-minimised structures are generated by the CHARMm function of Quanta and are not based on any crystallographic information. As such, the structures must be interpreted with caution. It is surprising that the position of the side chain at the C_2 position should differ so markedly between the two compounds, bearing in mind that the only difference between them is the orientation of the C_8 atom.

Both AM-113 and AM-114 lack the long side chain at the C_2 position, instead having a tertiary butyl group at this position. This is a hydrophobic side chain which probably presents a steric hindrance to residues in the active site. AM-114 is the most potent inhibitor of the TEM-1 enzyme. The hydroxyl group of Ser-130 hydrogen bonds to the N_4 atom of the heterocyclic ring. This hydroxyl moiety is also closer to the C_5 atom of AM-114 (2.93Å) than any of the other oxapenems. The hydrophobic C_2 side chain of AM-114 may have displaced the water molecule adjacent to Lys-234 (Wat-309). The same side chain in AM-113 has not displaced this solvent molecule, which interacts with AM-113 via the amino residue of Lys-234.

AM-113 and AM-114 differ in their positioning of the C_8 hydroxyl group. For AM-113 the 1'R position of this hydroxyl group allows it to form a hydrogen bond to the O_1 atom of the heterocyclic ring. The same hydroxyl residue in AM-114 is 1'S and it interacts with the Ser-235 carbonyl group. Perhaps this interaction of the C_8 hydroxyl group of AM-114 with active site of the enzyme, compared to the intramolecular interaction of the C_8 hydroxyl group of AM-113, confers enhanced stability of the cross-linked species, leading to a more profound inhibition of the enzyme. This might account for the 1000-fold difference in the IC_{50} values from AM-113 and AM-114 (3.34μM and 0.002μM respectively).

The role of Arg-244 in the inhibition of class A enzymes by clavulanic acid has been described (Imtiaz, *et al.* 1993). Arg-244 and a nearby water molecule are important for the activity of clavulanic acid, by forming hydrogen bonds to the carboxylic acid moiety. This allows the carboxylate to function as a general base, facilitating the transfer of a proton, via a water molecule, to cause opening of the oxazolidine ring

(Fig. 4.5) (Imtiaz, *et al.* 1993). This opening of the oxazolidine ring leads to cross-linking in the active site and irreversible inhibition of the enzyme. Both AM-112 and AM-113 form hydrogen bonds with Arg-244 in the active site of TEM-1. Yet both these inhibitors have poorer IC_{50} values than AM-114 and AM-115, which do not interact with Arg-244, according to the energy-minimised structures. This suggests that the interaction of AM-112 and AM-113 with Arg-244 does not confer enhanced inhibitory properties on the oxapenem, and may serve to decrease the effectiveness of the inhibition.

The active site of class C enzymes is more open than that of class A enzymes, but still contains the same conserved catalytic residues (Ser-64/Ser-70, Lys-67/Lys-73, Lys-315/Lys-234, Tyr-150/Ser-130) located in equivalent positions within 0.4Å (Lobkovsky, *et al.* 1993). The role of Arg-244 in the activity of clavulanic acid was described above. A residue with a similar function in class C enzymes is Arg-349, but this residue is not accessible to the carboxylic acid group of clavulanic acid. Instead, the carboxylic acid group forms hydrogen bonds to Asn-346 and a water molecule. This water molecule is too far away to donate a proton needed to cause opening of the heterocyclic ring and inhibition of the enzyme. This may be a reason why clavulanic acid is a poor inhibitor of class C enzymes (Lobkovsky, *et al.* 1993).

Both AM-112 and AM-113 interact with Asn-346, and AM-113 is also able to form a hydrogen bond to Arg-349. Neither AM-114 nor AM-115 form H-bonds to these residues. As with Arg-244 in the TEM-1 enzyme, it is difficult to assign significance to the interaction of AM-112 and AM-113 to Asn-346, as AM-114 lacks this interaction yet is equally potent as an inhibitor of P99.

The hydroxyl group of Tyr-150 is believed to act as the general acid/general base catalyst of the class C active site (Lobkovsky, *et al.* 1994). This residue is located between 3.15 and 3.8Å from the C_5 atom of the oxapenems. The terminal amino groups of Lys-315 and Lys-67 are located 3.4-4.7Å and 4.6-5.3Å from the C_5 atom, respectively. Based on these distances, Tyr-150 is the most likely active site residue to interact with the inhibitors, leading to cross-linking of the active site.

Each of the oxapenems, AM-112, AM-113 and AM-114 displays an almost equal potency for inhibiting the P99 enzyme (IC_{50} between 0.0012-0.002μM). AM-115 has a slightly poorer inhibitory activity, being ten-fold less potent (IC_{50} 0.014μM). As with the energy-minimised structure of AM-115 in the active site of TEM-1, in the active site of P99, AM-115 adopts a similar 'looped' conformation, with the terminal amino residue of the C_2 side chain bonding to the C_7 carbonyl group and the C_3 carboxylic acid group. The distance between C_5 and Tyr-150 is greatest for AM-115 (3.71Å) compared to the other oxapenems (3.15-3.32Å). Perhaps this distance is too great for effective cross-linking of AM-115 to occur, resulting in poorer inhibition of the enzyme.

The C_3 carboxylic acid group of each of the inhibitors interacts with the active site, albeit at different residues for each oxapenem. For AM-112, this group is oriented away from the Lys-315-Ser-318 side chain, and interacts with Asn-346. For each of the other compounds, the C_3 carboxyl group is aligned towards this side chain. AM-113 interacts with Asn-346 and Arg-349, rather than the serine or threonine residues of the 315-318 side chain. AM-114 interacts with Thr-316, while AM-115 H-bonds the main chain amino group of Ser-318. This side chain appears to be important for the alignment of the inhibitor, perhaps positioning the molecule such that the C5 atom is presented to the catalytic residue, Tyr-150.

A recent study suggests that destabilising, unfavourable interactions of inhibitors may be a factor in inhibiting class C enzymes by disrupting the active site, as opposed to favourable interactions which reduce the energy threshold for bonding and subsequent inhibition (Trehan, *et al.* 2001). The authors found that destabilising contacts were formed between Asn-152 and the $C_6\alpha$ and $C_7\alpha$ substituents of imipenem and moxalactam respectively, which prevented the deacylation of the inhibitor.

It is difficult to state whether a similar mechanism is occurring for the oxapenem inhibitors. Each of the compounds has a similar potency against the P99 enzyme, as determined by the cell-free β-lactamase inhibition studies, despite the differing interactions of each compound with the active site. As each of the acyl-enzyme

complexes was energy minimised, then any unfavourable, high energy interactions of 6α substituents with Asn-152 would not be observed.

The currently used β-lactamase inhibitors clavulanic acid, tazobactam and sulbactam are effective inhibitors of class A enzymes. Crystallographic data, results from ESMIS and liquid chromatography, coupled with molecular modelling studies indicate that these compounds inhibit the enzyme by cross-linking residues in the active site, leading to irreversible inhibition of the enzyme. It is reasonable to assume that the oxapenems act in a similar manner. Evidence from cell-free β-lactamase assays indicates that the compounds are effective inhibitors of class A, class C and class D enzymes. Modelling of the inhibitors in the active site of class A enzymes suggests that the compounds may cross-link to Ser-130 to inhibit the enzyme. This residue was the closest candidate nucleophilic residue to the target C_5 atom of the heterocyclic ring. This C_5 atom is widely accepted as being the point of cleavage of the 5-membered ring of clavulanic acid, sulbactam and tazobactam.

In class C enzymes, Tyr-150 is believed to carry out a similar general base/general acid function as Ser-130 in class A enzymes. The modelling studies of the oxapenems in the active site of P99 identified Tyr-150 as a potential site of cross-linking. This residue was located between 3.15-3.71Å from the C_5 atom of the heterocyclic ring from the results of the energy-minimisation studies.

In the absence of crystallographic data, the next logical step in these investigations would be to carry out molecular modelling on the next stage of the process of β-lactamase inhibition, that of the acyclic linear molecule, with the aim of identifying suitable residues to which cross-linking could take place.

The energy minimised structures generated by Quanta for the acyl-enzyme complexes formed by each of the inhibitors in the active site of the TEM-1 enzyme do not provide clear answers to explain the differences in activity seen between the compounds in the cell-free β-lactamase assays described in Chapter 2. Those results indicate that the position of the C_8 hydroxyl group may be an important factor in the efficiency of inhibition, as both AM-114 and AM-115 have a 1'S hydroxyl group at

this position, and both compounds were 1000-fold more potent than AM-112 and AM-113. Similarly, the effect of stereochemistry on the activity of the oxapenems against P99 has not been elucidated. The cell-free β-lactamase inhibition studies suggest that the differing stereochemistry of AM-112, AM-113 and AM-114 is not important for the inhibition of the enzyme, as all the inhibitors were equipotent. However, the energy-minimised structures generated by Quanta represent one of a number of possible orientations of the acyl-enzyme complex. The formation of the acyl-enzyme complex is only an early step in the process of β-lactamase inhibition, so it is possible that in the later stages rearrangement of the active site, and opening of the heterocyclic ring, bring into play those structural elements of the inhibitor which affect the eventual inhibition of the active site.

Chapter 5. Studies on the mode of action of novel oxapenems

5.1 Introduction

Bacterial species are bounded by a cell wall that protects the cell from the osmotic pressure of the cell contents. This internal osmotic pressure can range from 5 to 20 atmospheres as a result of solute concentration via active transport (Anonymous 1991). The cell wall consists of a layer of peptidoglycan that imparts flexibility and tensile strength. In Gram-positive bacteria the cell wall also contains teichoic acids; while in Gram-negative organisms lipoprotein, lipopolysaccharide and an outer membrane are also present.

The principal component of the cell wall, peptidoglycan, consists of a repeating macromolecule, which has three parts:

- A backbone, composed of alternating *N*-acetylglucosamine (*N*-AcGlN) and *N*-acetylmuramic acid (*N*-AcMur) joined by glycosidic linkages;
- A tetrapeptide side chain L-alanyl-D-glutamyl-L-R-D-alanyl (where L-R is a variable amino acid residue) joined by an amide bond to the D-lactyl group of *N*-AcMur;
- A cross-bridge, from the terminal D-ala residue of one tetrapeptide side chain to the penultimate residue of an adjoining tetrapeptide side chain. In many Gram-negative bacteria this cross-link is a simple peptide bond, while in Gram-positive bacteria it can be an additional short bridging stem peptide chain (e.g. in *S. aureus* it consists of a pentaglycine chain)

The third residue (L-R) in the tetrapeptide side chain can vary between bacterial species. In *E. coli* it is diaminopimelic acid, while in *Staphylococcus aureus* it is L-lysine. The synthesis of nascent peptidoglycan chains is a complex multistep process (Ghuysen 1997). The initial steps take place in the cytoplasm, where UDP-*N*-acetylglucosamine is converted into an intermediate form, UDP-*N*-acetylglucosamine-enolpyruvate by addition of a 3-carbon pyruvate

fragment from phosphoenol pyruvate to the 4 position of *N*-AcGlN. This reaction is catalysed by a pyruvate transferase enzyme, MurA. MurB then converts this intermediate to UDP-*N*-acetylmuramic acid. Five ligases then act sequentially to add L-Ala (MurC), D-Glu (MurD), diaminopimelic acid (in the case of *E.* coli; MurE) and D-Ala-D-Ala (previously joined together by Ddl; dipeptide added by MurF) to UDP-*N*-acetylmuramic acid, to form UDP-*N*-acetylmuramyl-pentapeptide. The hydrolysis of ATP to ADP and Pi provides the energy for these steps. The next step involves transfer of UDP-*N*-acetylmuramyl-pentapeptide from the uridylic carrier to the hydrophobic transmembrane lipid carrier, undecaprenyl phosphate. MraY cleaves UMP from the molecule and transphosphorylates the remaining phosphate to the phosphate group of the lipid carrier, forming undecaprenyl-pyrophosphoroyl-*N*-acetylmuramyl-pentapeptide (lipid I intermediate). Another enzyme called MurG transfers UDP-*N*-acetylglucosamine to the *N*-acetylmuramyl-pentapeptide residue of the lipid I intermediate to form the lipid II intermediate, with the release of the remaining UDP.

The final stages of peptidoglycan biosynthesis take place when the lipid II intermediate crosses the cell membrane. The lipid II intermediate is linked to the growing cell wall while still attached to the lipid carrier. Nascent peptidoglycan in the cell wall consists of uncross-linked linear glycan chains, with pentapeptide side chains on each *N*-acetylmuramic acid residue. Addition of the disaccharide pentapeptide (Lipid II intermediate) to an existing strand of peptidoglycan occurs by rupture of the phospho-*N*-acetylmuramoyl-pentapeptide linkage to the lipid carrier, followed by formation of a β-1,4 linkage between C_1 of *N*-acetylmuramic acid and C_4 of an *N*-acetylglucosamine residue on an existing peptidoglycan chain. This reaction is catalysed by a transglycosylase enzyme. The lipid carrier returns to the cytoplasmic face of the cell membrane, to participate in further rounds of precursor transport. Figure 5.1 summarises the stages involved in peptidoglycan biosynthesis.

FIGURE 5.1. Outline of the stages of peptidoglycan biosynthesis in *E. coli*. Reprodced with permission of Dr. P. A. Lambert.

Transpeptidase enzymes catalyse the final cross-linking of the glycan chains via their pentapeptide side chain to the mature peptidoglycan. The peptide bond of the terminal (fifth) D-Ala residue of the pentapeptide side chain is cleaved and a new peptide bond is formed between the fourth D-Ala residue and the free amino group of the penultimate residue (lysine or diaminopimelic acid) of a nearby tetrapeptide side chain in the pre-existing peptidoglycan in the cell wall (Ghuysen 1997). Synthesis of peptidoglycan is essential for growth of the cell, cell division and maintenance of the cell wall. DD-transpeptidases and transglycosylases are involved in synthesis of the peptidoglycan. Autolytic enzymes such as carboxypeptidases, endopeptidases and glycosidases are also involved in cell wall maintenance (Jamin, *et al.* 1995). Together, these enzymes are responsible for maintenance of the cell wall, and thus viability of the cell. These enzymes are known as penicillin binding proteins (PBPs), because of their ability to covalently bind penicillin and other β-lactams. The presence of these enzymes can be detected by labelling of the enzymes with a radiolabelled β-lactam compound, followed by separation of the proteins by SDS-PAGE and visualisation by fluorography. This was first carried out in 1975 (Spratt and Pardee 1975) and remains the standard method for identifying the PBPs of a particular organism.

Most bacterial species possess PBPs, although they vary in number, molecular weight, abundance and affinity for β-lactams between species. A representative bacterial strain will contain between four and eight PBPs with molecular sizes ranging from 120kDa to 35kDa (Georgopapadakou 1993). The highest molecular weight PBP is termed PBP 1 and the nomenclature follows in order of decreasing size. High molecular mass (HMM) PBPs generally have transpeptidase and transglycosylase activity, with low molecular mass (LMM) PBPs possessing carboxypeptidase functions (Jamin, *et al.* 1995; Ghuysen 1991). HMM PBPs have a multidomain structure, with a domain for transpeptidase activity; the other domains have not been fully elucidated (Massova and Mobashery 1998). Transpeptidase activity is located at the C-terminal, while transglycosylase activity is located at the N-terminal end of the protein (Van Heijenoort 1994). LMM PBPs are likely to be single domain structures (Massova and Mobashery 1998; Jamin, *et al.* 1995; Ghuysen 1991). The carboxypeptidase activity exhibited by LMM PBPs is believed

to be involved in the maturation of peptidoglycan and preparation of murein sacculus for new peptidoglycan synthesis (Van Heijenoort 1994). Carboxypeptidases cleave the terminal D-alanine residue from the pentapeptide side chain of the peptidoglycan unit. This prevents cross-linking of the unit to the existing peptidoglycan chain. Carboxypeptidases also cleave the carboxy-terminal peptide bond which cross-links adjacent peptidoglycan strands and thus help to control the extent of peptidoglycan cross-linking (Ghuysen 1991).

PBPs are often classified as essential or non-essential. Essential PBPs are defined as those without which the cell is not viable, whether by inactivation by β-lactam antibiotics or by deletion or inactivation of the encoding gene. They are believed to carry out vital functions in peptidoglycan biosynthesis or other physiological processes and their disruption leads to the death of the cell. Essential PBPs usually have a high molecular mass (60-120kDa) (Georgopapadakou 1993). Non-essential PBPs are considered to be those enzymes whose functions are complementary to the viability of the cell. Inhibition of these enzymes does not, by itself, lead to cell death. The function of the PBPs and their role in the cell cycle has been mostly studied in *Escherichia coli*. *E. coli* has seven PBPs that are well defined and accepted. Recent research has identified twelve proteins in *E. coli* that bind penicillin, although some of these are not classical PBPs (Denome, *et al.* 1999). Table 5.1 lists the PBPs of *E. coli* and their possible function.

The PBPs of numerous organisms have been identified, including *S. aureus*, *S. pneumoniae* and *P. aeruginosa*. However, *E. coli* remains the model of choice for elucidating the role of PBPs in the physiology of the cell. Recent research on mutants of *E. coli* that have deletions in their PBP profile has attempted to define the roles of the PBPs in cellular viability, characteristics and peptidoglycan biosynthesis. The researchers found that mutants which had eight of a possible twelve PBPs deleted remained viable. It seems that certain PBPs can take over the biochemical role of other PBPs when they are deleted from the cell, termed the 'theory of equivalent substitution' (Young 2001). Loss of PBP 1a and 1b are lethal to the cell. However, all other possible mutant combinations remained viable. It appears that there is a subtle interplay between PBPs with regard to cell wall synthesis and cell physiology, which has not yet to date been fully elucidated.

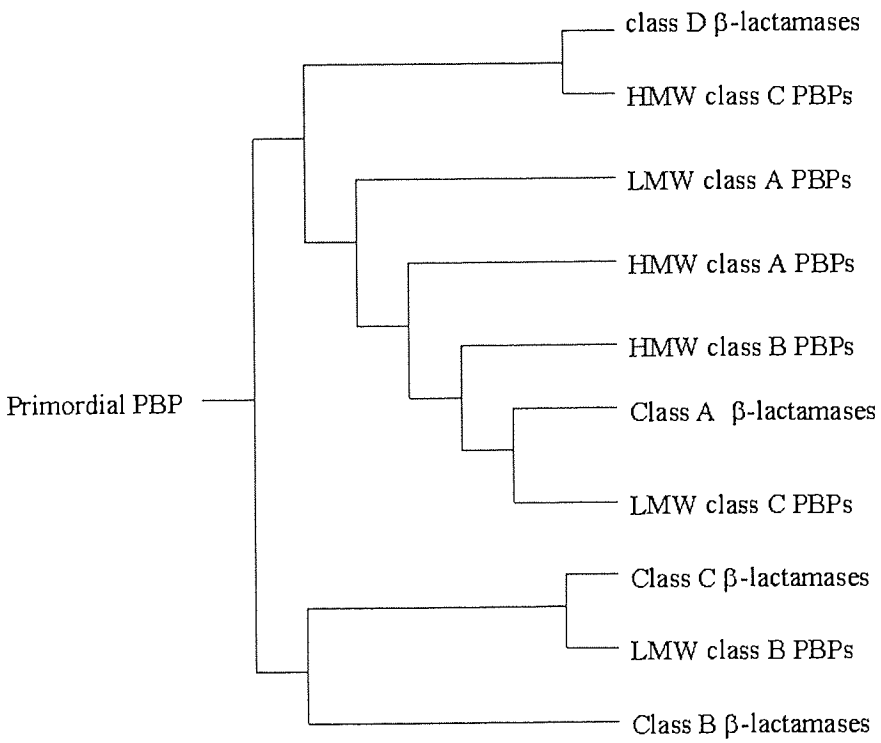
TABLE 5.1. The penicillin-binding proteins of *E. coli*, their molecular mass, enzymic activity and proposed physiological role.

PBP	Approximate molecular mass (kDa)	Enzymic activity	Physiological role
HMM PBPs			
1a	92	Transpeptidase and transglycolase	Wall elongation
1b	90	Transpeptidase and transglycolase	Wall elongation
2	66	Transpeptidase	Shape determination
3	60	Transpeptidase	Septation
LMM PBPs			
4	44	Carboxypeptidase and transglycolase	Maturation and recycling
5	42	Carboxypeptidase	Regulation of cross-linking
6	40	Carboxypeptidase	Regulation of cross-linking

HMM, high molecular mass.
LMM, low molecular mass.

Bacterial PBPs and β-lactamase enzymes are closely related. Both enzyme species are believed to arise from a common ancestor, a primordial PBP. A recent review provided details of the multiple-sequence alignment of 77 different PBPs and 73 different β-lactamases (Massova and Mobashery 1998). The results were displayed in a dendrogram showing the evolution of both PBPs and β-lactamases from a common, primordial ancestor. β-lactamase enzymes were classified as class A, B, C or D, while PBPs were divided into HMM and LMM PBPs, with three classes (A-C) for each group. The β-lactamases and PBPs are classified according to their amino acid sequence. Members of a given class are related in their primary structure, but can differ in their physiological function (Ghuysen 1991). It can be seen from Fig. 5.2 that certain groups of PBPs and β-lactamases are closely related.

FIGURE 5.2. Kinship of PBPs and β-lactamases, as identified by multiple sequence alignment. Taken from Massova and Mobashery (Massova and Mobashery 1998). (LMM, low molecular weight; HMM, high molecular weight.)



Following multiple-sequence alignment, the authors further analysed the sequence alignment for each cluster of the dendrogram. Several similarities between PBPs and

β-lactamases are apparent. Both enzymes contain an active-site serine and this catalytic serine is followed by lysine two residues apart. A glycine residue is strictly conserved in an area called locus III. Both enzymes undergo acylation of the active-site serine residue. The authors speculate that the conserved Ser-X-X-Lys sequence and Gly in locus III are the minimal requisites for the acylation step of the substrate.

The principal difference between PBPs and β-lactamases is that β-lactamase enzymes have acquired the ability to undergo a deacylation step after formation of the acyl-enzyme species, by hydrolysis of the acyl-enzyme species. This is facilitated by a hydrolytic water molecule in the active site that is activated by Glu-166 in class A and Tyr-150 in class C β-lactamases (Matagne, *et al.* 1999). β-Lactamase enzymes also have alterations to the structure of the active site which disfavours transient acylation of peptidoglycan during cross-linking. PBPs exclude water from the active site by a combination of the reaction mechanism and the structure of the active site. Exclusion of water prevents hydrolytic activity breaking down the peptidoglycan substrate after acylation to the active-site serine. Instead, a nucleophilic amine residue on the tetrapeptide side chain of the peptidoglycan can form a peptide bond with the terminal D-Ala residue of the newly synthesised peptidoglycan monomer. This transpeptidase activity leads to the formation of the polymeric peptidoglycan chain, rather than cleavage of the peptidoglycan into small molecules by hydrolysis due to the presence of a water molecule (Koch 2000).

While the biochemical roles of the different PBPs remain to be precisely elucidated, the effects of inhibition of certain PBPs by β-lactams, or deletion of the genes for these PBPs in mutant strains, are clearly defined and apparent on examination of the morphology of the cells. Again, *E. coli* remains the most widely studied model for determining the effects of PBP inhibition on morphology.

The effect of penicillin on the morphology was first observed soon after the introduction of penicillin, when filamentation of *E. coli* cells was observed. It was not until the 1970s that further research began to elucidate the effects of penicillin and other β-lactams on morphology. This work coincided with the identification of

PBPs by radiolabelling, separation by SDS-PAGE and visualisation by fluorography (Spratt and Pardee 1975; Spratt 1975).

Inhibition of the HMM PBPs of *E. coli* (1a, 1b, 2 and 3) can lead to morphological alterations in the cell. Inhibition of both PBP1a and PBP1b together causes spheroplast formation followed by rapid lysis of the cells (Tamaki, *et al.* 1977). The inhibition of PBP2 leads to spheroplast formation, which proves lethal after several generations (Spratt 1977; Tybring and Melchior 1975). Filamentation of the cells is caused by inhibition of PBP3 (Spratt and Pardee 1975; Spratt 1975). From these morphological responses to PBP inhibition, it was possible to assign physiological roles to each of the PBPs. Thus PBP1a and 1b are transpeptidases involved in cell elongation. PBP2 is involved in maintenance of the rod shape of *E. coli*, as disruption of its role leads to 'round form' cells. PBP3 is involved in septum formation of dividing cells – its inhibition leads to filament formation.

By observing the morphology of *E. coli* cells treated with established β-lactams, or novel compounds, important information about the PBP affinity and profile of a compound can be established. Observation of changes in morphology brought about by the lowest concentration of compound tested can determine the primary PBP for which that compound has affinity. For example, ceftazidime has a high affinity for PBP3 in *E. coli*, followed by PBP1a and then PBP1b. Thus the morphological changes observed are initial filament formation (due to PBP3 inhibition) followed by spheroplast formation and lysis (PBP1a and 1b inhibition) (Hayes and Orr 1983). So morphological studies can provide initial information about the PBP affinity of a compound and also its PBP profile, i.e. the order in which the PBPs are inhibited.

The next stage of the process for identifying the PBP profile of a compound is to isolate the PBPs, label them with a radioactive β-lactam in competition with the test compound, separate the PBPs by SDS-PAGE and visualise them by fluorography. This will provide more detail about the PBP affinity and profile of the compound. In the case of ceftazidime, it was found that a concentration of 0.06mg/L was sufficient to inhibit the binding of the radiolabel to PBP3 by 50%. Binding of the radiolabel to PBP1a and 1b was reduced by 50% by concentrations of 0.9mg/L and 3.4mg/L respectively (Hayes and Orr 1983).

The aims of the work presented in this chapter were to investigate the morphological effects each of the oxapenem compounds exerted on cells of *E. coli* DC0. Further to these studies, the binding of AM-112 to PBPs of *E. coli* DC0, *E. faecalis* SFZ, *S. aureus* NCTC 6571 and MRSA 96-7778 was investigated.

5.2 Materials and Methods

5.2.1 *Morphology studies*

An overnight culture of *E. coli* DC0 was grown in MHB at 37°C. This culture was used to inoculate fresh, pre-warmed MHB and incubated until the OD_{550nm}=0.2. The test compound was added to give the required concentration (range was typically 0.25 to 16μg/ml), and the cells were incubated with shaking at 37°C for two hours. Volumes of 400μl were removed and microcentrifuged at 13,000 rpm for 10 minutes to produce a pellet. A sterile loopful of the pellet was spread on a clean glass slide and allowed to air-dry. The dried slides were then stained with acridine orange stain (0.24%$^{w}/_{v}$ in 2%$^{v}/_{v}$ acetic acid) for ten seconds. Excess stain was removed with filter paper. Slides were then immersed in 2%$^{v}/_{v}$ methanol 0.34%$^{w}/_{v}$ sodium chloride for ten seconds, followed by a final rinse in 0.34%$^{w}/_{v}$ sodium chloride. Slides were air-dried again, then examined by fluorescence microscopy (Zeiss Axioskop) at ×1000 magnification using an oil immersion lens. Images were captured using a Zeiss Axioskop digital camera attached to the microscope.

5.2.2 *Penicillin binding protein studies*

5.2.2.1 *Preparation of cell membranes*

Membranes of *E. coli* DC0 were prepared according to the method of Spratt (Spratt 1977). An overnight culture of *E. coli* was grown in MHB at 37°C. Aliquots of 20ml of this culture were inoculated into three 500ml flasks of sterile pre-warmed MHB and grown for five hours with aeration and vigorous shaking until the optical density of the culture was 1.0 at 470nm. Cells were harvested by centrifugation at 10,000 rpm for 10 minutes at 4°C and washed in ice-cold 10mM sodium phosphate buffer (SPB). Cells were then resuspended in ice-cold SPB containing 0.14M 2-mercaptoethanol. Cells were broken by sonication at maximum power using a

MSE-Soniprep sonicator (MSE Ltd., Crawley, Sussex, UK). Six cycles of 30s of sonication with constant cooling in an ice bath were required to lyse the cells. Unbroken cells were removed by centrifugation at 10,000 rpm for 10 minutes at 4°C. The supernatant was ultracentrifuged (Beckman L-8 Centrifuge, UK) at 35,000 rpm for one hour at 4°C to pellet the membranes. Membranes were suspended in SPB and stored at -70° until required.

Staphylococcus aureus membranes

Preparation of *S. aureus* membranes NCTC 6571 and methicillin-resistant *Staphylococcus aureus* 96-7778 (MRSA 96-7778) followed a similar procedure to those of *E. coli*. After harvesting the cells by centrifugation and washing in SPB, cells were resuspended in 100ml of lysing buffer which contained 1mM magnesium chloride, 2mg DNase and 3mg of lysostaphin. Cells were incubated in this lysing buffer for one hour at 37°C, followed by washing in ice-cold SPB. Cells were then sonicated as described above. Unbroken cells were removed by centrifugation at 10,000 rpm for 10 minutes at 4°C. The supernatant was ultracentrifuged at 35,000 rpm for one hour at 4°C. Membranes were resuspended in SPB and stored at -70°C.

Enterococcus faecalis membranes

A lysing buffer was also used to break the cells of *E. faecalis* SFZ. Cells were harvested, centrifuged and washed as described before. Cells were then suspended in 100ml a lysing buffer containing 1mM magnesium chloride, 500 units of mutanolysin, 10mg of lysozyme and 0.5mg of DNase at 4°C for 16 hours with gentle agitation. Cells were then washed in ice-cold SPB and membrane harvesting was carried out as described for *E. coli*.

5.2.2.2 *Penicillin-binding protein assay*

The procedure for the penicillin binding protein assay is essentially that as described by Bryan and Godfrey (1991). Minor modifications were made to the procedures in an attempt to obtain the best results. For example, the concentration of acrylamide in the running gel of the SDS-PAGE was reduced to 7.5%$^{w}/_{v}$ to improve separation of

the protein bands. The Mini-Protean III slab electrophoresis system (Bio-Rad, UK) was used to visualise the proteins of *E. coli* and *S. aureus*, whereas the Protean II slab gel electrophoresis system was used to improve the resolution of the *E. faecalis* proteins and also those of *E. coli*. Information received from Dr. R. Hakenbeck (Universitat Kaiserslautern, Kaiserslautern, Germany), led to an increased incubation time for the ^{3}H-benzylpenicillin and the membranes. Trial and error determined the optimum amount of ^{3}H-benzylpenicillin required to visualise the proteins and also the optimum time required to visualise the bands by autoradiography.

5.2.2.3 *Mini-Protean III slab gel electrophoresis system*

A volume of 5µl of membrane proteins were added to 45µl of SPB. A 5µl aliquot of the required concentration of the test compound was added to the membrane suspension, while 5µl of SPB was used for the control membranes. The membranes were incubated with the test compound for 30 minutes at 37°C. A 4µl aliquot of ^{3}H-benzylpenicillin (10-30Ci/mmol) was then added to the membranes and allowed to incubate for 90 minutes at 37°C. A volume of 5µl of ice-cold benzylpenicillin (120mg/ml) was then added to stop the reaction and incubated at room temperature for 20 minutes. 25µl of sample buffer (0.5%$^{w}/_{v}$ SDS, 16.2% 0.5M Tris (pH 6.8), 16.2% glycerol, 1.62% 2-mercaptoethanol, 0.01%$^{w}/_{v}$ bromophenol blue in distilled water) was then added and the samples were boiled in a water bath for three minutes to denature the proteins. Aliquots of 20µl of each sample were loaded into the wells of a 10 lane Mini-Protean III gel (Bio-Rad, UK). Electrophoresis was carried out at 200V for approximately 45 minutes or until the blue band of the sample buffer had reached the bottom of the gel. The minigel was removed, then stained with coomassie brilliant blue R-250 (0.1%$^{w}/_{v}$ in 20%$^{v}/_{v}$ methanol 10%$^{v}/_{v}$ acetic acid) and destained with 20%$^{v}/_{v}$ methanol 10%$^{v}/_{v}$ acetic acid. This procedure allowed a quick check on the separation of the proteins. The gel was then soaked in Amplify (Amersham, UK) for 30 minutes and dried on a Bio-Rad Gel drier (Bio-Rad, UK) for 2 hours. A single 18x24cm sheet of Hyperfilm MP (Amersham, UK) X-ray film was pre-flashed using a Sensitize flash unit (Amersham, UK) and laid in contact with the

dried gel. The film was exposed to the gel for 2 weeks at -70°C. The film was developed using Kodak developer and fixed using Kodak fixer.

5.2.2.4 *Protean II slab gel electrophoresis system*

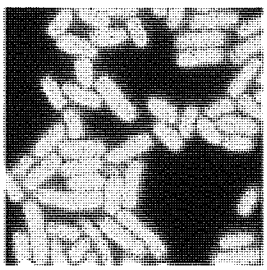
A volume of 12µl of membrane suspension was mixed with 88µl of SPB. An aliquot of 5µl of the required concentration of the test compound (or SPB for the control) was added to the membrane suspension and incubated for 30 minutes at 37°C. A 5µl volume of ^{3}H-benzylpenicillin was added and incubated at 37°C for 90 minutes. A 20µL aliquot of ice-cold benzylpenicillin (120mg/ml) was added to stop the reaction and incubated for 20 minutes at room temperature. A volume of 70µl of sample buffer was added to the membranes and the samples were boiled for three minutes in a water bath. Each lane of the gel was loaded with 40µl of sample. Electrophoresis was carried out at 200V for three to four hours, or until the sample buffer had reached the bottom of the gel. The gel was stained with coomassie blue stain for four hours and destained overnight for 16 hours. The gel was then soaked in Amplify for 30 minutes prior to drying on a Bio-Rad gel drier for six hours. Autoradiography was carried out as described above.

5.3 Results

5.3.1 *Morphology studies*

The morphological responses of *E. coli* DC0 cells to AM-112, AM-113, AM-114 and AM-115 are shown in Figs 5.3-5.6. The first figure in each sequence shows control cells which were not incubated with any compound, but were subjected to the same slide preparation and staining process as treated cells. Each figure is shown at an equal magnification.

FIGURE 5.3. *E. coli* DC0 cells treated with various concentrations of AM-112 for two hours. (a) Control; (b) 0.25μg/ml; (c) 0.5μg/ml; (d) 1μg/ml; (e) 2μg/ml; (f) 4μg/ml; (g) 8μg/ml.



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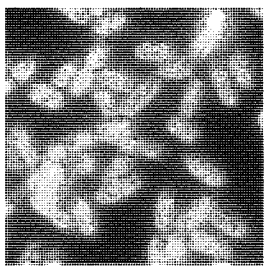

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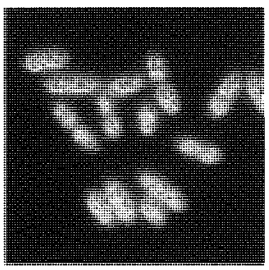

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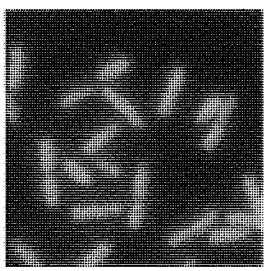

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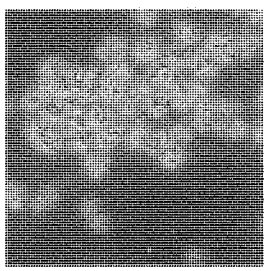

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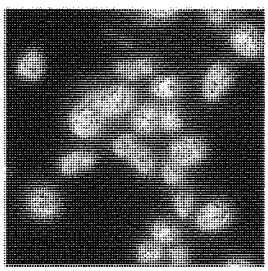

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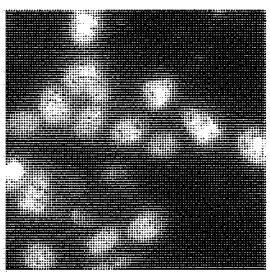
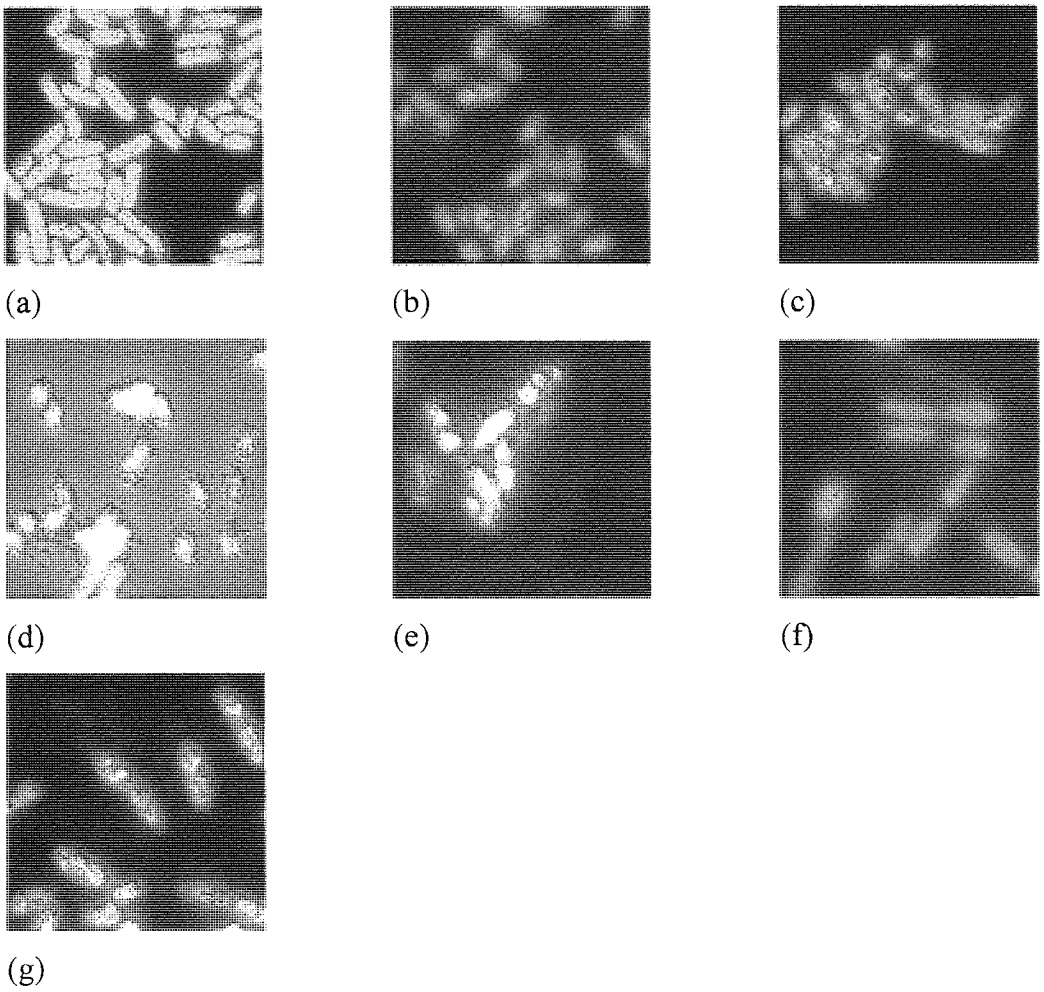


(g)

FIGURE 5.4. *E. coli* DC0 cells treated with various concentrations of AM-113 for two hours. (a) Control; (b) 0.25μg/ml; (c) 0.5μg/ml; (d) 1μg/ml; (e) 2μg/ml; (f) 4μg/ml; (g) 8μg/ml.

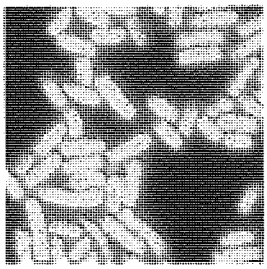


(a) (b) (c)

(d) (e) (f)

(g)

FIGURE 5.5. *E. coli* DC0 cells treated with various concentrations of AM-114 for two hours. (a) Control; (b) 0.25μg/ml; (c) 0.5μg/ml; (d) 1μg/ml; (e) 2μg/ml; (f) 4μg/ml; (g) 8μg/ml.



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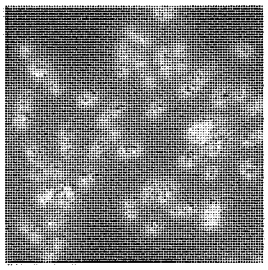

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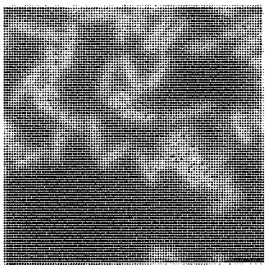

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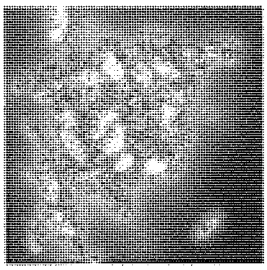

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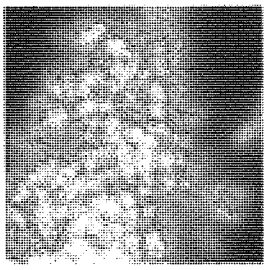
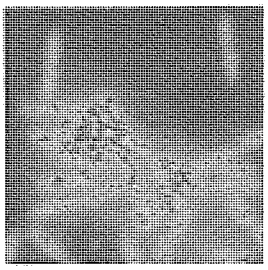
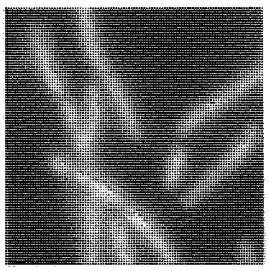


(e)

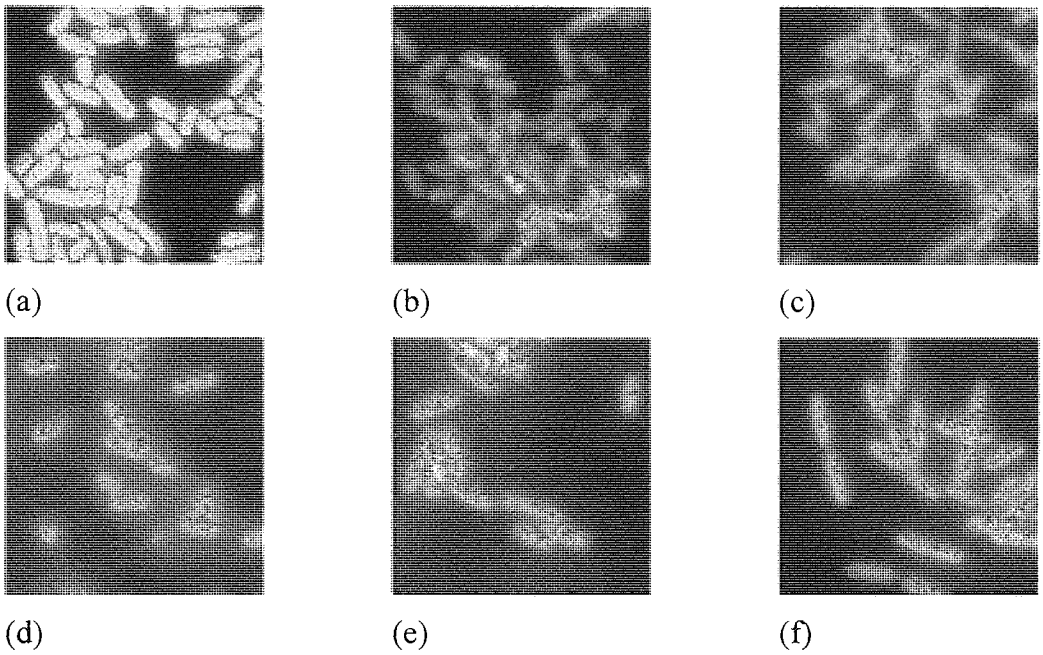


(f)



(g)

FIGURE 5.6. *E. coli* DC0 cells treated with various concentrations of AM-115 for two hours. (a) Control; (b) 0.25μg/ml; (c) 0.5μg/ml; (d) 1μg/ml; (e) 4μg/ml; (f) 8μg/ml.



(a) (b) (c)

(d) (e) (f)

The primary effect of AM-112 on *E. coli* DC0 cells was 'round form' formation, indicating inhibition of PBP2. This effect was observed at the lowest concentration of 0.25μg/ml, after two hours incubation. With increasing AM-112 concentrations, the size of the cells increased, and at the highest concentrations tested (4 and 8μg/ml), the cells had an irregular appearance. Isolated cells displayed other morphological responses such as filament and 'rabbit ears' formation; however the overall trend was swelling of the cells. AM-113, at low concentrations (0.25μg/ml and 0.5μg/ml), caused swelling of the cells, suggesting inhibition of PBP2. Higher concentrations (1 to 8μg/ml) caused elongation of the cells, which is likely to be due to inhibition of PBP3. Low concentrations of AM-114 displayed a similar pattern to the low concentrations of AM-113 i.e. swelling and elongation. At 8μg/ml, marked elongation of the cells had occurred, suggesting pronounced inhibition of PBP3. As with the other oxapenems at concentrations of 1μg/ml and below, AM-115 caused swelling of the cells, due to inhibition of PBP2. At concentrations of 4 and 8μg/ml, elongation of the cells occurred.

5.3.2 *Penicillin binding protein assay*

Initial results for the autoradiography proved disappointing. Overloading of the gel with protein led to staining and streaking of the protein bands and little or no resolution of the proteins. Overexposure of the X-ray film led to blackening of the developed film, underexposure resulted in blank X-ray films. To prevent the X-ray film sticking to the dried gel during exposure, cling film was placed between the two layers. However, it was only realised later that removal of the cling film in the dark room resulted in static discharge, and the light emitted caused local blackening of the film. The wavy lines seen in Figure 5.7 are an example of this. Some autoradiographs have random areas of blackening, possibly due to the scintillant in the Amplify solution concentrating during the drying process.

The autoradiograph obtained for the penicillin binding proteins of *E. coli* using the Protean II slab gel electrophoresis system is shown in Figure 5.7. The autoradiograph obtained by the Mini-Protean III slab gel electrophoresis system is shown in Figure 5.8.

FIGURE 5.7. Autoradiograph of the penicillin binding proteins of *E. coli* DC0, separated by Protean II slab gel electrophoresis. Membranes bound with ^{3}H-benzylpenicillin in competition with AM-112. AM-112 concentrations (μg/ml) are shown along the top of the gel.

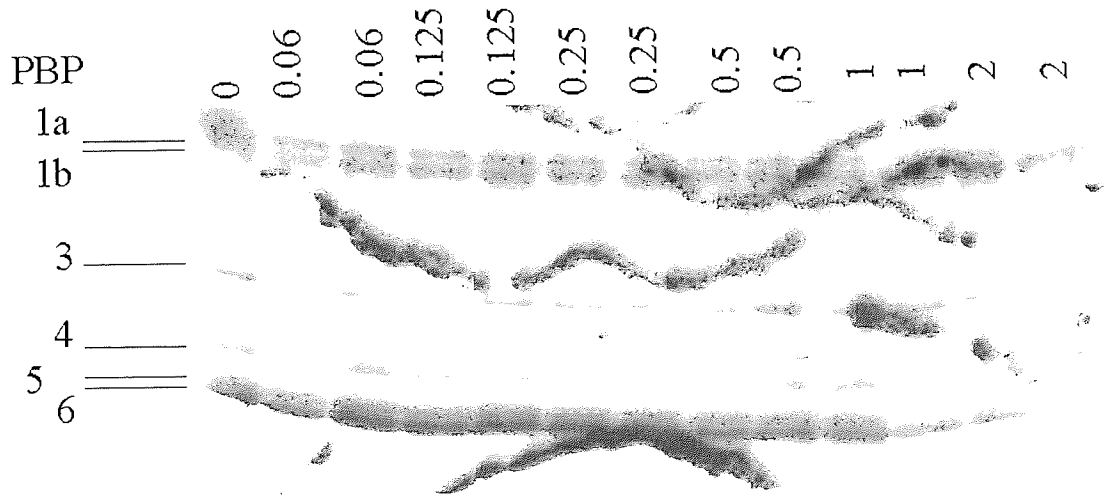


FIGURE 5.8. Autoradiograph of the penicillin binding proteins of *E. coli* DC0, separated by Mini-Protean III slab gel electrophoresis. Membranes bound with ^{3}H-benzylpenicillin in competition with AM-112. AM-112 concentrations (μg/ml) are shown along the top of the gel.

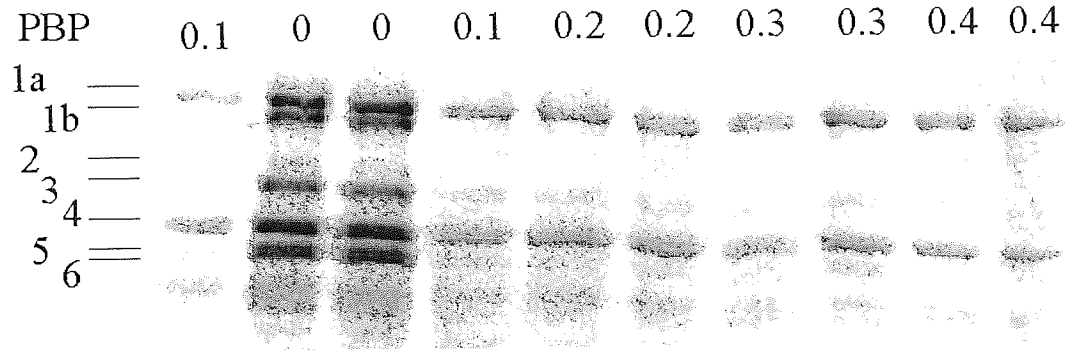


Figure 5.8 provides the clearest indication of the PBP profile of AM-112 in *E. coli* DC0. PBP 2 was poorly bound by the radiolabel and is just visible in the control lanes of the gel. At a concentration of 0.1μg/ml AM-112, the binding of the radiolabel to PBP2 was completely inhibited, while binding to PBP 1b, 3, 5 and 6 were all reduced. PBP1b was inhibited by AM-112 at 0.3μg/ml. At 0.4μg/ml, the radiolabel binding to PBP 3, 5 and 6 was inhibited, while binding to PBP 1a and 4 was slightly reduced.

The Protean II slab gel electrophoresis system was used to try and improve the resolution of the *E. faecalis* membrane proteins. Personal communication from Dr. I. N. Simpson (Amura Ltd., Cambridge, UK) and Dr. R. Hakenbeck suggested that a larger gel size and decreased concentration of acrylamide in the running gel were essential to adequately separate the proteins of *E. faecalis*. The result for this gel is seen in Figures 5.9, while Figure 5.10 shows the autoradiograph obtained by the Mini-Protean III system. Some degree of resolution is seen with both systems, with at least three bands being visible.

FIGURE 5.9. Autoradiograph of the penicillin binding proteins of *E. faecalis* SFZ, separated by Protean II slab gel electrophoresis. Membranes bound with ^{3}H-benzylpenicillin in competition with AM-112. AM-112 concentrations (μg/ml) are shown along the top of the gel.

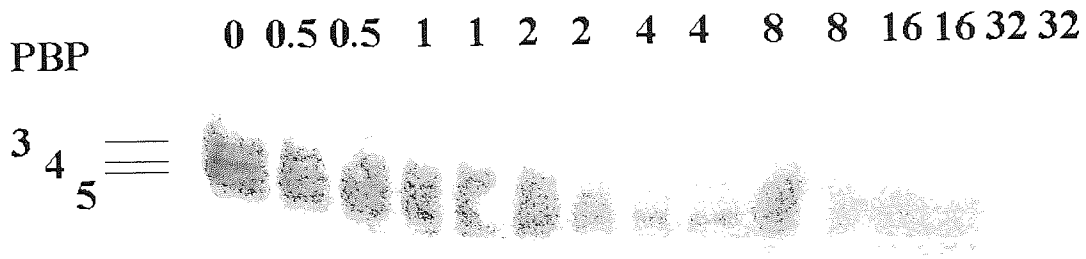
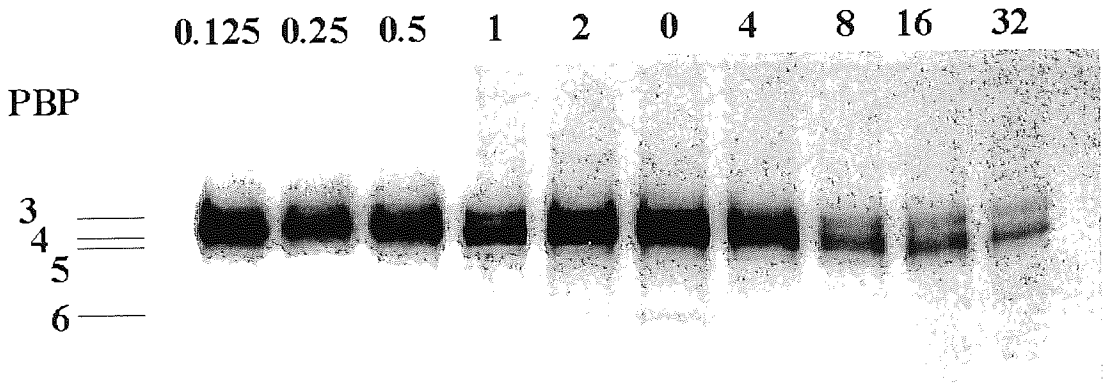


FIGURE 5.10. Autoradiograph of the penicillin binding proteins of *E. faecalis* SFZ, separated by Mini-Protean III slab gel electrophoresis. Membranes bound with ^{3}H-benzylpenicillin in competition with AM-112. AM-112 concentrations (μg/ml) are shown along the top of the gel.



PBP studies were carried out on behalf of Amura Ltd. by Dr. R. Hakenbeck, Universitat Kaiserslautern, Kaiserslautern, Germany. Dr. Hakenbeck examined the PBP profile of two strains of enterococci – *E. faecalis* ATCC 29212 and *E. faecalis* SFZ, the strain tested at Aston University. Dr. Hakenbeck compared the PBP profile of AM-112 in both strains, as well as that of clavulanic acid and imipenem for *E. faecalis* SFZ. The results are shown in Figs. 5.11-5.12.

FIGURE 5.11. Autoradiography of penicillin binding proteins of *E. faecalis* ATCC 29212 and *E. faecalis* SFZ. PBPs labelled with ^{3}H-benzylpenicillin in competition with AM-112. Reproduced with permission of Dr. R. Hakenbeck.

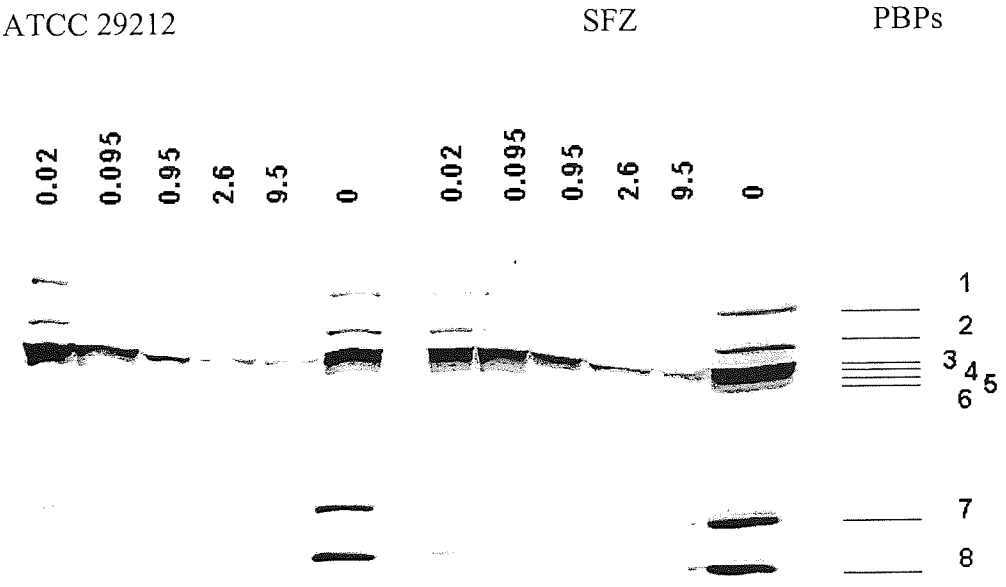
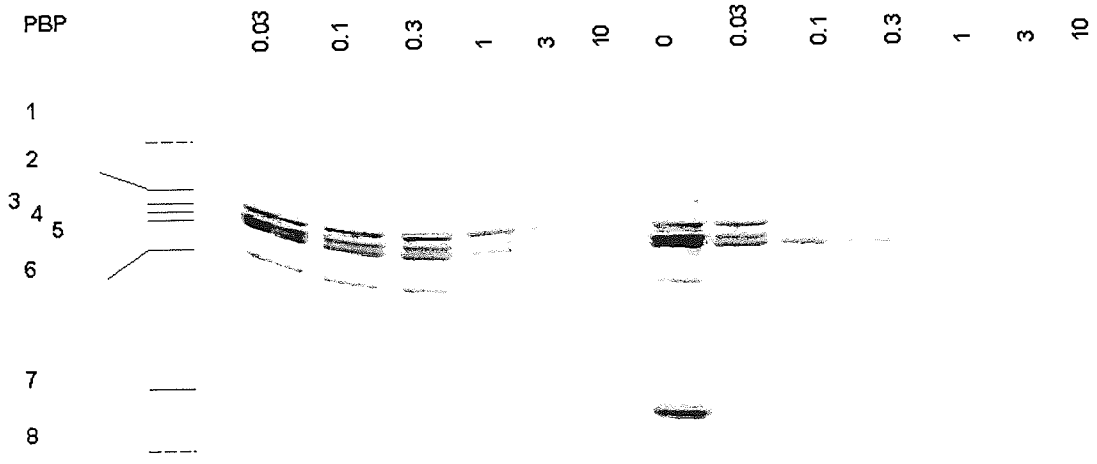


FIGURE 5.12. Autoradiography of penicillin binding proteins of *E. faecalis* SFZ. PBPs labelled with ^{3}H-benzylpenicillin in competition with AM-112 (left hand lanes) or imipenem (right hand lanes). Reproduced with permission of Dr. R. Hakenbeck.



AM-112 had affinity for enterococcal PBPs and inhibited all the PBPs except PBP3 at 9.5μg/ml. At the lowest concentration tested, 0.03μg/ml, binding to PBP7 and PBP8 was inhibited. PBP1 and PBP2 were inhibited by a concentration of 0.1mg/ml, while PBP4, 5 and 6 were inhibited at 2.6μg/ml of AM-112. Imipenem was very active against the enterococcal PBPs, with all PBPs being inhibited in the range 0.1-0.3μg/ml.

FIGURE 5.13. Autoradiograph of the penicillin binding proteins of *S. aureus* NCTC 6571 separated by Mini-Protean III slab gel electrophoresis. Membranes bound with ^{3}H-benzylpenicillin in competition with AM-112. AM-112 concentrations (μg/ml) are shown along the top of the gel.

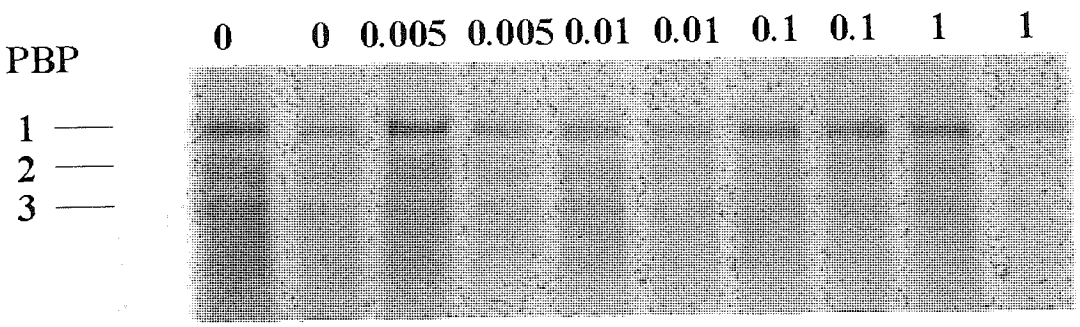
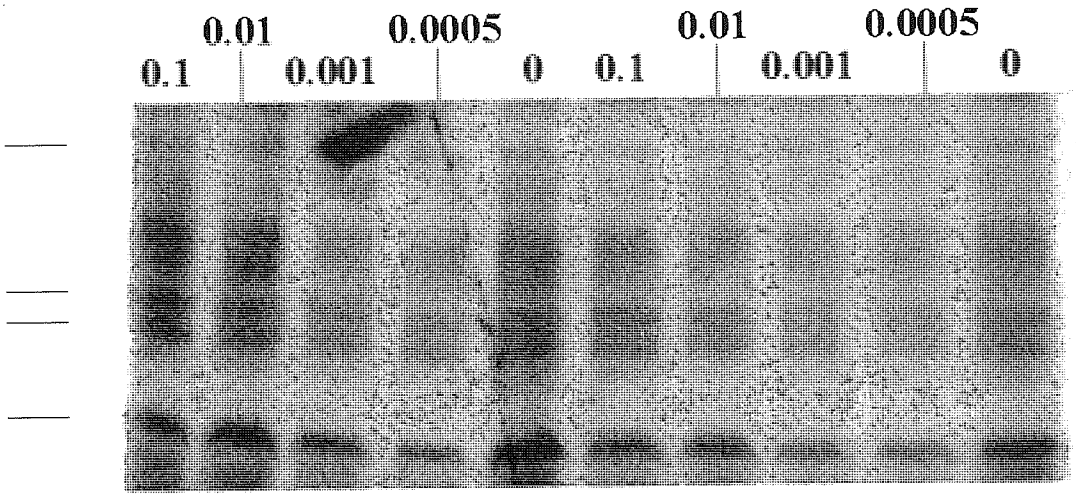


FIGURE 5.14. Autoradiograph of the penicillin binding proteins of MRSA 96-7778 separated by Mini-Protean III slab gel electrophoresis. Membranes bound with ^{3}H-benzylpenicillin in competition with AM-112. AM-112 concentrations (μg/ml) are shown along the top of the gel.



Figures 5.13 and 5.14 show the PBPs of *S. aureus* NCTC 6571 and MRSA 96-7778 respectively. The resolution on both gels is poor – three bands are visible on the *S. aureus* gel, but on the MRSA gel there are no sharp bands visible.

5.4 Discussion

The results of the morphological studies indicate that the oxapenems, as a class, have the highest selective affinity for PBP2. The effects of inhibiting this PBP were seen at the lowest concentration of each compound. In this respect, the compounds differ from ceftazidime which has PBP3 as its primary PBP target for *E. coli* DC0 (Hayes and Orr 1983). As such, the combination of an oxapenem and ceftazidime is likely to be complementary to the PBP profile of ceftazidime and may account for the enhanced activity of the combination compared to ceftazidime alone.

Two gel systems were utilised in the PBP studies. The Mini-Protean III gel electrophoresis system has the advantages of being easy to use, allows rapid separation of the proteins (45 minutes, compared to 3-4 hours for the Protean II system), and requires a smaller sample volume and thus less radiolabel. However, the resolution of the protein bands can be inferior to that of the Protean II system, especially for the PBPs of *E. faecalis*. Personal communication from Dr. I. Simpson and Dr. R. Hakenbeck had suggested that a large gel system was required to fully separate the PBPs of *E. faecalis*. Despite using the Protean II gel system, poor resolution of the *E. faecalis* PBPs was obtained. As a result of this, Dr. Hakenbeck separated the proteins using her own protocol. The resolution of the proteins was far superior to that obtained in this laboratory and the results are displayed in Figures 5.12 and 5.13.

The results for the morphology studies are consistent with the observation from the PBP studies that the primary target of AM-112 in *E. coli* is PBP2. The appearance of round forms of the cells at 0.25μg/ml (Figure 5.3(b)) matches the inhibition of PBP2 by concentrations of AM-112 above 0.1μg/ml (Figure 5.9). The PBP profile showed that the next PBP to be inhibited was PBP1b, at a concentration of 0.3μg/ml. However, the morphological response to PBP1b inhibition is lysis of the cell, which was not seen in the morphological studies until the AM-112 concentration reached 4μg/ml. It is likely that the outer membrane of *E. coli* DC0 presents a permeability barrier to AM-112, accounting for the higher concentration required to inhibit the PBP in whole cells, compared to isolated membranes. It is also possible that PBP1a,

which is only slightly inhibited by AM-112 at 4μg/ml, could take over the transpeptidation role of PBP1b allowing the cell to continue to cross-link nascent peptidoglycan and prevent cell lysis.

The oxapenems appear to have the highest selective affinity for PBP2 in *E. coli*. Further PBP labelling studies could confirm this for each of the compounds. Lack of time and the cost of the radiolabel meant that PBP analysis for AM-113, AM-114 and AM-115 were not carried out. It is interesting to observe that concentrations of between 0.25-8μg/ml of AM-114 and AM-115 result in morphological responses in *E. coli* cells, yet both of these compounds have MICs for *E. coli* strains of around 64μg/ml. AM-114 causes filamentation of *E. coli* cells after two hours at 8μg/ml, and lower concentrations cause swelling of the cells. Perhaps the inhibition of the PBPs is transient, or insufficient to kill the cells. It is possible that over the course of 24 hours at 37°C, the rate of breakdown of AM-114 and AM-115 is greater than the rate of cell killing due to PBP inhibition. Time-kill studies would provide information on the rate of kill (if any) of the compounds and the time at which the treated cells recovered and began to grow.

The autoradiographs supplied by Dr. Hakenbeck provide evidence of the affinity of AM-112 for enterococcal PBPs and complement the observation of synergy between AM-112 and ceftazidime against enterococcal strains, as described in Chapter 3. A concentration of 10μg/ml of AM-112 inhibited all the PBPs except PBP3 of *E. faecalis* SFZ and *E. faecalis* ATCC 29213. Most enterococcal strains produce one or more PBPs which have low affinity for β-lactam compounds (Georgoupapadakou and Liu 1980; Williamson, *et al.* 1985), which explains the poor activity of cephalosporins such as ceftazidime against these strains. It is likely that PBP3 in these strains is a low-affinity PBP for AM-112. In contrast, PBP5 appears to be the low-affinity target for imipenem – it is the last PBP to be inhibited (at 1μg/ml), with PBP3 being inhibited at 0.3μg/ml. The expression of low-affinity PBPs can depend on the physiological status of the organism and on factors such as temperature; the function of each PBP is not constant (Fontana, *et al.* 1983). In *E. faecium* strains, PBP5 is the low affinity target and the cell can survive with all other PBPs except PBP5 fully saturated by penicillin. Inhibition of cell growth only occurs when PBP5

becomes saturated by penicillin (Fontana, *et al.* 1983). The synergistic activity of AM-112 when combined with ceftazidime against enterococci may be due to the enhancement by AM-112 of the PBP inhibition profile of ceftazidime and in particular, to the inhibition of low-affinity PBPs against which ceftazidime is not normally active.

Due to the pronounced activity of AM-112 against staphylococcal strains, including MRSA strains, an attempt was made to determine the PBP profile of AM-112 against *S. aureus* NCTC 6571 and MRSA 96-7778. The results of the initial studies are shown in Figure 5.14 and 5.15. As can be seen, these studies were not successful in resolving the PBPs into distinct bands. Three bands are visible on the *S. aureus* autoradiograph, which are likely to correspond to the three of the five known PBPs of *S. aureus*. For the MRSA strain, one would expect to see the same three bands, in the same position, with an additional band, PBP2a, located between PBP2 and PBP3. PBP2a is the low affinity PBP that accounts for the antibiotic resistance of MRSA strains. The MRSA autoradiograph is poorly resolved and appears to show bands in different areas to that of the *S. aureus* autoradiograph. Due to the poor resolution of the autoradiograph, further conclusion about these autoradiographs cannot be drawn. However, due to the potent activity of AM-112 and AM-113 against staphylococcal strains, it would be expected to see inhibition of radiolabel binding to PBPs on the autoradiograph. For the MRSA strain, there should be decreased binding to PBP2a, the low affinity PBP, as well as inhibition of the other PBPs. For methicillin-sensitive strains, inhibition of the high molecular weight PBPs 1, 2 and 3 would be expected, as these are the essential PBPs in *S. aureus*.

Morphology studies and PBP analysis can provide important information on the mode of action of compounds. The oxapenem compounds contain a β-lactam ring and are therefore expected to share the mode of action of established β-lactam compounds i.e. inhibition of the transpeptidation step of peptidoglycan synthesis. The results from this chapter demonstrate that oxapenems have a high affinity for PBP2 in *E. coli*. AM-112 binds to enterococcal PBPs and at high concentrations inhibits the low-affinity PBPs. The PBP affinity of AM-112 against *S. aureus* and MRSA was not elucidated, but the MIC results for AM-112 and AM-113 against

these strains suggest that these compounds have high affinity for staphylococcal PBPs, including the low-affinity resistance PBP2a of MRSA strains.

Chapter 6. *In vivo* studies on AM-112

6.1 Introduction

The *in vitro* evaluation of the antibacterial spectrum of a novel agent is an important first step in the investigation of a lead compound. Basic microbiological screening to determine the minimum inhibitory concentration (MIC) of the test compound against a panel of relevant pathogenic organisms is a pre-requisite for further study. The oxapenems are a group of related novel compounds, some of which have been found to have intrinsic antibacterial action, and all of which possess potent broad-spectrum β-lactamase inhibitory properties. The ability of the compounds to inhibit β-lactamase has been investigated (Chapter 2). The basic antimicrobial spectra of the compounds alone and as partners with cephalosporin antibiotics, principally ceftazidime, have been established (Chapter 3). The next stage in the evaluation of the oxapenems is to assess their activity *in vivo* with an appropriate animal model of infection.

Initial *in vivo* studies on novel compounds typically involve mouse or rat models of infection, where the efficacy of the test compound is compared to agents established for use in treating such infections. Many models of infection are used, including pneumonia, urinary tract infections, endocarditis and catheter-related infections. Infection is caused by the introduction of a large bacterial inoculum into the target organ or system. Treatment in the form of the test compound or an established agent, is then administered orally, parenterally, intramuscularly or subcutaneously, as appropriate. One or more cycles of treatment may be given, depending on the type of infection. The efficacy of the test compound compared to the established agent may be measured by a reduction in the bacterial flora recovered from the site of the infection or by the survival of the treated animals over a defined time period. In the United Kingdom, animal testing is subject to strict control by the Secretary of State, acting in accordance with the Animals (Scientific Procedures) Act 1986.

A project licence (PPL 40/1769) was granted in respect of an application by Dr. P. Lambert to develop and run a murine thigh lesion model at the biomedical research

facility at Aston University. The thigh lesion model was chosen because it is a rapid test (only requiring 6-10 hours), it provides the potential for generating information to rank the effectiveness of established and novel agents in treating the infection and it causes minimum stress to the animals, compared with more invasive infection models. The objective of the model is to use the reduction in bacterial numbers produced by the agents in the mouse thigh infection test as a measure of antibiotic activity. The test thus demonstrates activity of the compounds *in vivo*, enabling a ranking of activity in comparison with established agents.

A more serious form of infection can be studied using the murine intraperitoneal model, where pathogenic bacteria are introduced into the peritoneal cavity. Such an infection is likely to be rapidly fatal if not treated with antibiotics. This chapter presents results from such an infection model, evaluating AM-112, carried out by Glaxo SmithKline (Verona, Italy) and Biosearch Italia (Gerenzano, Italy) on behalf of Amura Ltd. Both the thigh lesion model and the peritonitis model have the advantage of being easy to run, using small, inexpensive animals which are easy to keep and handle.

In vivo results can provide important information on the efficacy of a test compound in treating a bacterial infection. *In vivo* conditions differ greatly from those in a laboratory MIC test and may provide a greater challenge to the test compound. Pharmacokinetic and pharmacodynamic factors will affect the activity of a compound. Host enzyme systems may destroy the compound or render it inactive, or convert it to a more active metabolite. Potential toxicity problems associated with the compound may come to light. However, *in vivo* results must be interpreted with caution. The applicability of the model infection in a small mammal or primate to a similar infection in a human must be considered. Model systems of infection are often highly artificial, and may not be realistic in terms of the aetiology and pathogenesis of the corresponding human infection. The differences between mammalian biological systems are obvious, and due consideration must be given before extrapolating the results from one system to another. Nonetheless, animal models of infection provide useful and vital information on the *in vivo* characteristics of novel compounds, and are an important stage in the evaluation of novel compounds.

6.2 Materials and Methods

6.2.1 *Development of a murine thigh lesion model*

An overnight culture of MRSA 96-7778 was grown in Mueller Hinton broth at 37°C with vigorous shaking. A volume of 1ml of this culture was inoculated into sterile pre-warmed MHB and grown at 37°C with shaking until the OD470nm reached 1.0. An aliquot of 0.1ml of this suspension was added to 0.9ml of sterile saline (0.9%w/v) to give a final cell concentration of 10^7CFU/ml. A volume of 0.1ml was the inoculum for the thigh lesion model (Fantin, *et al.* 1991; Gudmundsson and Erlendsdottir 1999).

Six male albino MF-1 mice (weight 35g +/- 5%) were selected and assigned to the control group (2 mice) or the treated group (4 mice). Each mouse was chemically anaesthetised with Hypnorm (fentanyl citrate 0.135mg/ml and fluanisone 10mg/ml), diluted 1:10 in sterile saline, 0.1ml injected intraperitoneal. After five minutes, the right thigh of each mouse was shaved and 0.1ml of the bacterial suspension was injected into the thigh muscle. After one hour, the mice were displaying signs of ill health, with listless movement and fur standing proud of the body. One hour after inoculation the mice were treated with vancomycin or sterile saline. Control mice received 0.1ml sterile saline injected subcutaneously (sc) at the scruff of the neck. The vancomycin treated mice were dosed at 10, 20, 30 and 40mg/kg respectively, injected in a volume of 0.1ml sc at the scruff of the neck. Six hours after inoculation of the bacterial suspension the mice were sacrificed by cervical dislocation. The skin of each right thigh was removed and the thigh muscle along with the thighbone was excised. The muscle was removed from the bone and placed in 5ml sterile saline and stored at 5°C until required. Each thigh muscle was removed and homogenised for one minute (Silverson, Chesham, UK; 25mm head operating at 50% power setting). The homogeniser was washed then sterilised by immersion and operation in 70% ethanol between samples. Homogenised samples were diluted in sterile saline, plated out on MHA and incubated at 37°C for 48 hours. Colonies were counted and expressed as number of colony forming units per thigh.

6.2.2 *In vivo efficacy of AM-112 alone and in combination with ceftazidime in a murine intraperitoneal infection model*

The activity of AM-112 alone and in combination with ceftazidime (1:4 and 1:7) was determined in a murine intraperitoneal infection model (Di Modugno, *et al.* 1997; Frimodt-Moller, *et al.* 1999). Three pathogenic strains were used, each of which expresses a β-lactamase enzyme - *S. aureus* 3816 (class A), *E. coli* SHV-5 (class A, ESBL) and *E. cloacae* P99 (class C). The method for each organism differed, so each will be described separately.

6.2.2.1 *S. aureus* 3816

An overnight culture of *S. aureus* 3816 was grown in Mueller-Hinton broth (MHB) at 37°C. A volume of 1ml of this stationary phase culture was inoculated into fresh, pre-warmed MHB and maintained at 37°C with vigorous shaking until growth had reached the exponential phase. Aliquots of this culture were suspended in 6% hog gastric mucin to give a final cell concentration of between 10^6 and 10^7CFU/ml. Male ICR mice (20-22g) were infected with 0.1ml of the *S. aureus* 3816 inoculum by intraperitoneal injection. There were ten mice per antibiotic dose level, and each experiment was carried out on two occasions. Ceftazidime and AM-112, alone and in combination (4:1 and 7:1) were administered by subcutaneous injection to the scruff of the neck. A volume of 0.1ml was administered fifteen minutes post-infection. AM-112 alone was also administered orally via a syringe and blunt cannula. The mice were allowed access to food and water as normal and the survival of the mice was observed over four days. Mice surviving after four days were sacrificed by cervical dislocation.

6.2.2.2 *E. cloacae* P99

An overnight culture of *E. cloacae* P99 was grown in MHB at 37°C. A volume of 1ml of this culture was inoculated into fresh, pre-warmed MHB and grown with vigorous shaking at 37°C until the culture had reached exponential phase. Aliquots

of this culture were diluted in 6% hog gastric mucin to give a cell concentration of 10^7CFU/ml. Male CD1 mice (20-22g) were infected with *E. cloacae* P99 by intraperitoneal injection of 0.1ml of the inoculum. Ceftazidime, clavulanic acid and AM-112 were administered alone, and clavulanic acid or AM-112 in a 1:1 and 4:1 ratio with ceftazidime. The compounds were administered intravenously, at six dose levels, one and five hours post-infection to groups of five mice per dose level. The mice were allowed access to food and water as normal. Survival was monitored over four days. Any surviving mice were sacrificed by cervical dislocation after four days.

6.2.2.3 *E. coli* SHV-5

An overnight culture of *E. coli* SHV-5 was grown in MHB at 37°C. A volume of 1ml of this culture was inoculated into fresh, pre-warmed MHB and grown with vigorous shaking at 37°C until the culture had reached exponential phase. Aliquots of this culture were diluted in mucin (6%w/v) to give a cell concentration of 10^8CFU/ml. Female ICR mice (20-22g, Harlan Sprague Dawley, Indianapolis, USA) were infected with *E. coli* SHV-5 by intraperitoneal injection of 0.1ml of the inoculum. Ceftazidime and AM-112, alone and in a 1:1 and 2:1 ratio of ceftazidime to AM-112 were administered by subcutaneous injection to the scruff of the neck. The antibiotics were administered in a 0.1ml volume, one hour post-infection. Five mice were used per dose level. The mice were allowed access to food and water as normal. Survival was monitored over four days. Any surviving mice were sacrificed by cervical dislocation after four days. The ED_{50} values were calculated by the Probit method.

6.2.3 *Stability of oxapenems to renal peptidase*

These experiments were carried out by Dr. P. A. Lambert and are reproduced with permission. AM-112, AM-114 and AM-115 were supplied by Amura and stored at -70°C. Imipenem (European Pharmacopoeia standard) was obtained from the Council of Europe (Strasbourg, France) and stored at -70°C. Meropenem trihydrate was obtained from Astra Zeneca Pharmaceuticals (Cheshire, UK) and stored at -70°C. All samples were allowed to reach room temperature (20°C) before weighing.

Leukotriene D4 hydrolase, a renal peptidase prepared from porcine kidney was obtained from Sigma (Poole, Dorset, UK) and stored at -70°C. One unit of activity of this enzyme was reported to produce 1μmole of D-phenylalanine from Gly-D-Phe per min at pH 8.0 at 37°C (Littlewood, *et al.* 1989)

Portions (2-3mg) of AM-112, AM-114, AM-115, imipenem and meropenem were accurately weighed and dissolved in water to give 1mg/ml solutions. These were immediately diluted in 0.01M Tris-HCl buffer, pH 7.4 to give solutions containing 0.1 mg/ml AM-112, AM-114 and AM-115, and 0.05 mg/ml imipenem and meropenem. Duplicate samples (4ml) were placed in glass tubes maintained at 37°C in a heating block (Techne Dri-Block DB-2A, Cambridge, UK). To one of each duplicate tubes was added 0.04ml of renal peptidase stock solution (1 unit/ml) to give a final concentration of 0.01units/ml. Absorbance values of each solution were recorded over 3 hours at 37°C in 1cm path length quartz cuvettes for imipenem and meropenem, while values for the oxapenems were recorded over four hours. 262nm was used for AM-112, AM-114 and AM-115, while 296nm was used for imipenem and meropenem.

6.2.4 *Serum protein binding of AM-112*

The degree of protein binding of AM-112 to human, mouse, rat and monkey serum was determined by ultrafiltration. Human serum was obtained from the UK Blood Transfusion Service. Mouse and rat serum were purchased from Sigma (Poole, Dorset, UK) and Huntingdon Research Centre (Alconbury, Cambridge, UK). Monkey serum (marmoset) was obtained from Huntingdon Research Centre. AM-112 was prepared at 10 and 100mg/ml in each of the different sera, and also in phosphate-buffered saline (PBS) at 10, 20 and 100mg/ml. All solutions were stored on ice and processed as quickly as possible to minimise any loss due to thermal degradation. Binding was determined by ultrafiltration of 1ml volumes of each sample in Centrifree Micropartition devices (Millipore Corporation, Bedford, MA, USA), for 10 minutes at 1000*g*. The filters were 14mm YMT membranes with a molecular weight cut-off of 30kDa. Drug concentrations in the supernatant were determined by high performance liquid chromatography (HPLC). The HPLC column

(100 × 4.6mm) was packed with Hypersil 5 ODS and the mobile phase was methanol: water: phosphoric acid (25: 74: 1). The flow rate was 1.5ml/min. The eluate was monitored by ultraviolet absorption at 280nm. A quantity of 10μL of the supernatant was injected onto the column. The concentration of AM-112 was determined by the peak height, with reference to known AM-112 concentrations.

The percentage binding of AM-112 to serum proteins was determined from the formula:

$$\frac{\text{(Concentration in PBS ultrafiltrate – concentration in serum ultrafiltrate)}}{\text{Concentration in PBS ultrafiltrate}} \times 100$$

6.3 Results

6.3.1 *Murine thigh lesion model*

The initial inoculum for each mouse was determined by dilution of the inoculum suspension. Table 6.1 lists the average values, calculated from dilution of the samples, of the number of colony forming units present in each mouse thigh.

TABLE 6.1. Colony forming units (CFU) per thigh. Initial inoculum 7.2×10^6. Average of 5 replicates per thigh.

Sample	Average Log_{10} CFU/thigh
Control 1	5×10^7
Control 2	1.25×10^8
Vancomycin (10mg/kg)	9.4×10^7
Vancomycin (20mg/kg)	5.8×10^7
Vancomycin (30mg/kg)	3.3×10^6
Vancomycin (40mg/kg)	5.3×10^6

The results in Table 6.1 show that the bacterial inocula in both control mouse thighs grew over the time period of the experiment. The variability of the *in vivo* results is demonstrated by the control mice, where the inoculum increased ten-fold in one animal and 100-fold in the other. Growth of the inoculum was also seen in the mice treated with 10 and 20mg/kg of vancomycin. Reductions in the inoculum were seen on treatment with 30 and 40mg/kg of vancomycin, although the reduction was greater for 30mg/kg than for 40mg/kg.

The results of this experiment demonstrate the limitations of the small sample size used in the study, and also of the terms of the animal licence granted by the Home Office in respect of this study (survival studies could not be carried out) and the resources needed to carry out a larger scale study. With regard to these limitations, no further animal work was carried out at Aston University. *In vivo* work was commissioned by Amura Ltd., and carried out by Huntingdon Life Sciences and Biosearch Italia. The results of the *in vivo* work were kindly supplied by Dr. I.

Simpson (Amura Ltd.) and form the basis of the *in vivo* work described herein. The results of the *in vivo* work were presented at the 41st Interscience Conference on Antimicrobial Agents and Chemotherapy (ICAAC) in Chicago, USA in December 2001 (Poster numbers 385 and 386).

6.3.2 *In vivo efficacy of AM-112 alone and in combination with ceftazidime in murine infection models*

TABLE 6.2. Comparative efficacy of ceftazidime (CAZ) and AM-112, alone and in combination, *in vivo* against *S. aureus* 3816.

Antibiotic	Initial inoculum (CFU/ml)	Route of administration	ED_{50} (mg/kg)
CAZ	3×10^7	Subcutaneous	18.3
	8×10^8	Subcutaneous	22.6
AM-112	3×10^7	Oral	28.3
	3×10^7	Subcutaneous	2.7
	8×10^6	Subcutaneous	2.6
CAZ:AM-112 (4:1)	8×10^6	Subcutaneous	4.8 + 1.2
CAZ:AM-112 (7:1)	8×10^6	Subcutaneous	7 + 1

The results in Table 6.2 show that alone, ceftazidime had poor activity against *S. aureus* 3816. This is not unexpected, as third generation cephalosporins are poorly active against Gram-positive organisms. AM-112 was very active against *S. aureus* 3816 when administered subcutaneously. The ED_{50} was ten-fold higher when the compound was given orally. When the two agents were combined in a 4:1 ratio of ceftazidime to AM-112, the ED_{50} was lowered four-fold compared to ceftazidime alone and halved compared to AM-112 alone. These results suggest that the superior activity of the combination of ceftazidime and AM-112 compared to ceftazidime alone was due to the significant activity of AM-112 against *S. aureus* 3816, rather than any additive effect of the two agents combined.

TABLE 6.3. Comparative efficacy of ceftazidime (CAZ), clavulanic acid (CLAV) and AM-112, alone and in combination, *in vivo* against *E. cloacae* P99.

Antibiotic	Initial inoculum (CFU/ml)	ED_{50} (mg/kg)
Pilot experiment		
CAZ	2×10^7	>200
AM-112	2×10^7	20
CAZ:AM-112 (1:1)	2×10^7	2
Secondary experiments		
CAZ	2.2×10^7	>100
CLAV	1.6×10^7	>100
CAZ:CLAV (1:1)	1.6×10^7	44
CAZ:CLAV (4:1)	9.1×10^7	>100 + 25
CAZ	2.2×10^7	>100
AM-112	2.2×10^7	19
CAZ:AM-112 (1:1)	2.2×10^7	2
CAZ:AM-112 (2:1)	2.2×10^7	4 + 2
CAZ:AM-112 (4:1)	2.2×10^7	11 + 3

Both ceftazidime and clavulanic acid were poorly active against *E. cloacae* P99 when administered alone. The ED_{50} for ceftazidime was between 100 and 200mg/kg, and clavulanic acid had an ED_{50} of >100mg/kg. The poor activity of both compounds was likely to be due to the high level of the class C β-lactamase produced by *E. cloacae* P99. Ceftazidime is susceptible to hydrolysis by this enzyme and clavulanic acid is a poor inhibitor of class C enzymes. Addition of clavulanic acid in a 4:1 ratio did not lower the ceftazidime ED_{50}, but a higher concentration of clavulanic acid (1:1 ratio) lowered the ceftazidime ED_{50} to 44mg/kg.

AM-112 had moderate activity against *E. cloacae* after intravenous administration. The ED_{50} for AM-112 alone was 19mg/kg. Incorporation of AM-112 as the minor component in 1:1, 2:1 or 4:1 combinations with ceftazidime resulted in the lowering of the ED_{50} of ceftazidime at least 32-fold, and that of AM-112 eight-fold. This enhancement of the activity of ceftazidime is likely to be due to the potent β-lactamase inhibitory properties of AM-112.

TABLE 6.4. Comparative efficacy of ceftazidime (CAZ) and AM-112, alone and in combination, *in vivo* against *E. coli* SHV-5.

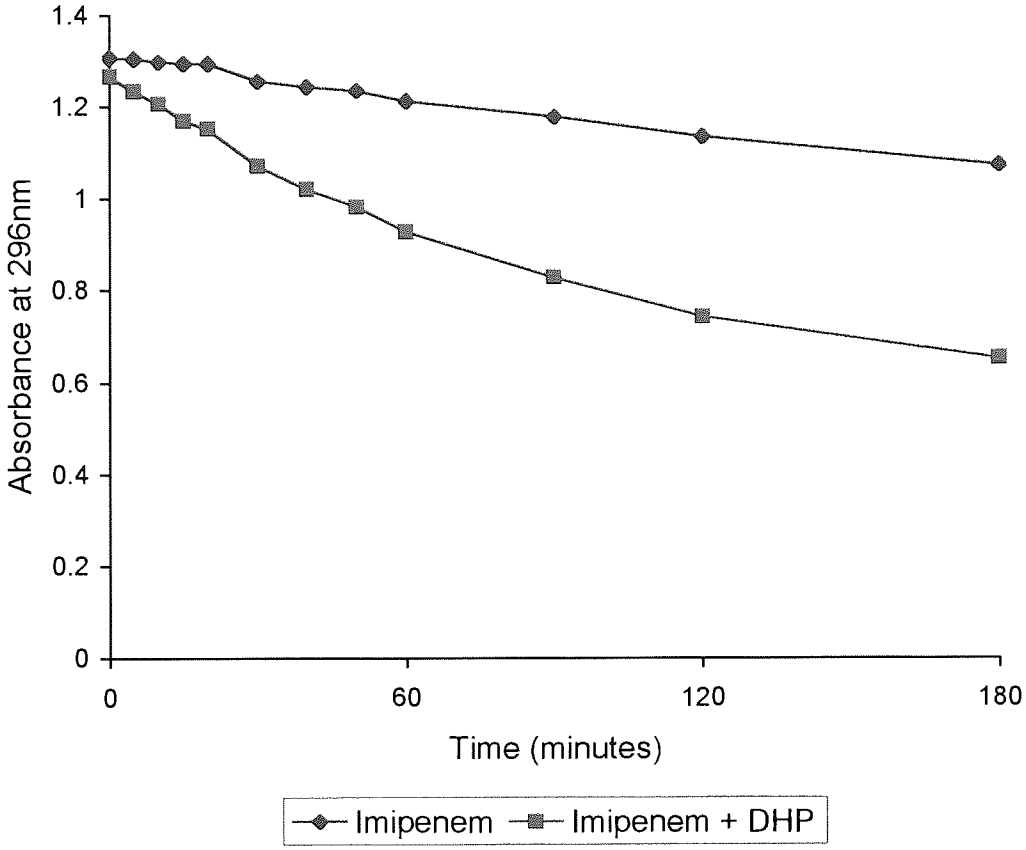
Antibiotic	Initial inoculum (CFU/ml)	ED_{50} (mg/kg)
CAZ	6×10^8	16
AM-112	6×10^8	72
CAZ:AM-112 (1:1)	6×10^8	2
CAZ:AM-112 (2:1)	6×10^8	2 + 1

E. coli SHV-5 produces an extended-spectrum class A β-lactamase enzyme which is capable of hydrolysing third and fourth generation cephalosporins. This probably accounts for the moderately high ED_{50} for ceftazidime against this organism (16mg/kg). AM-112 was only weakly active against *E. coli* SHV-5 *in vivo*, with an ED_{50} of 72mg/kg. Combination of ceftazidime and AM-112 resulted in marked synergy. A combination of each of the agents in both a 2:1 and 1:1 ratio lowered the ED_{50} of ceftazidime eight-fold, while the ED_{50} of AM-112 was dramatically reduced 32-fold. As with *E. cloacae* P99, the synergy between AM-112 and ceftazidime is likely to be due to the effective inhibition of the β-lactamase enzyme by AM-112.

6.3.3 *Stability of oxapenems to renal peptidase*

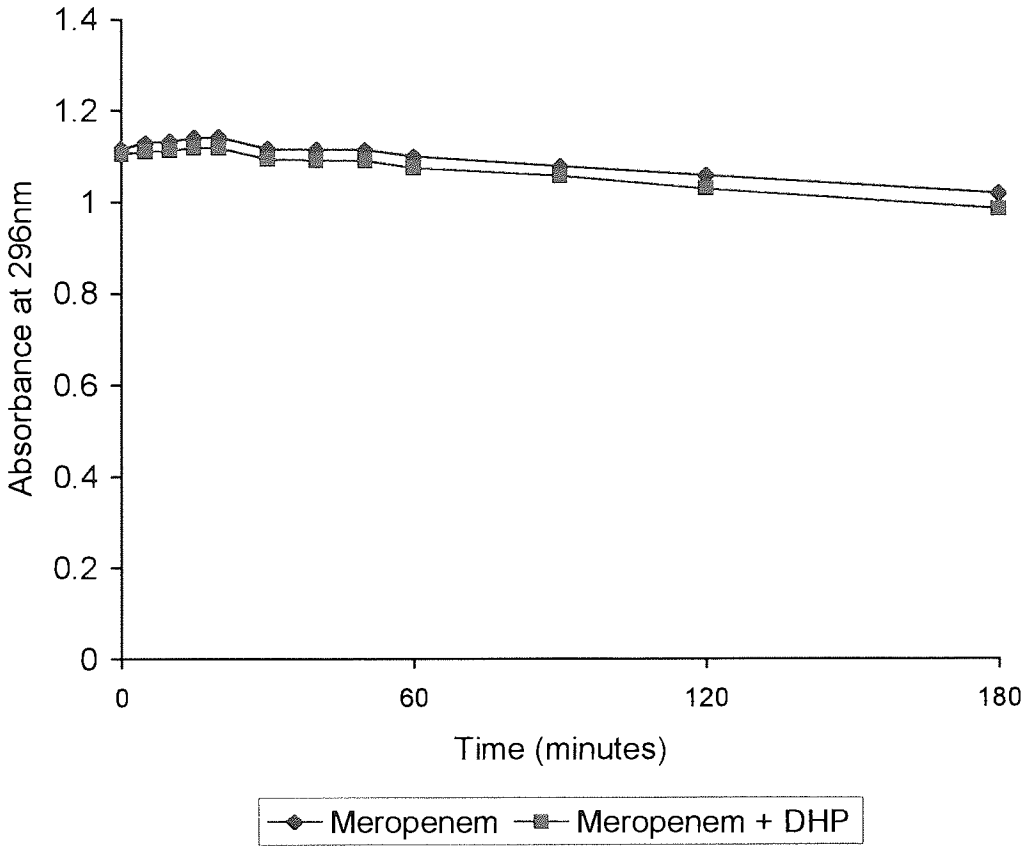
The stability of AM-112, AM-114 and AM-115 was compared to that of imipenem and meropenem, and monitored by an ultraviolet absorption assay. Figs. 6.1-6.5 show the results of these assays measured over a three- or four-hour time period.

FIGURE 6.1. Stability of imipenem (0.05mg/ml) to hydrolysis by porcine renal peptidase (DHP), monitored at 296nm over three hours at 37°C.



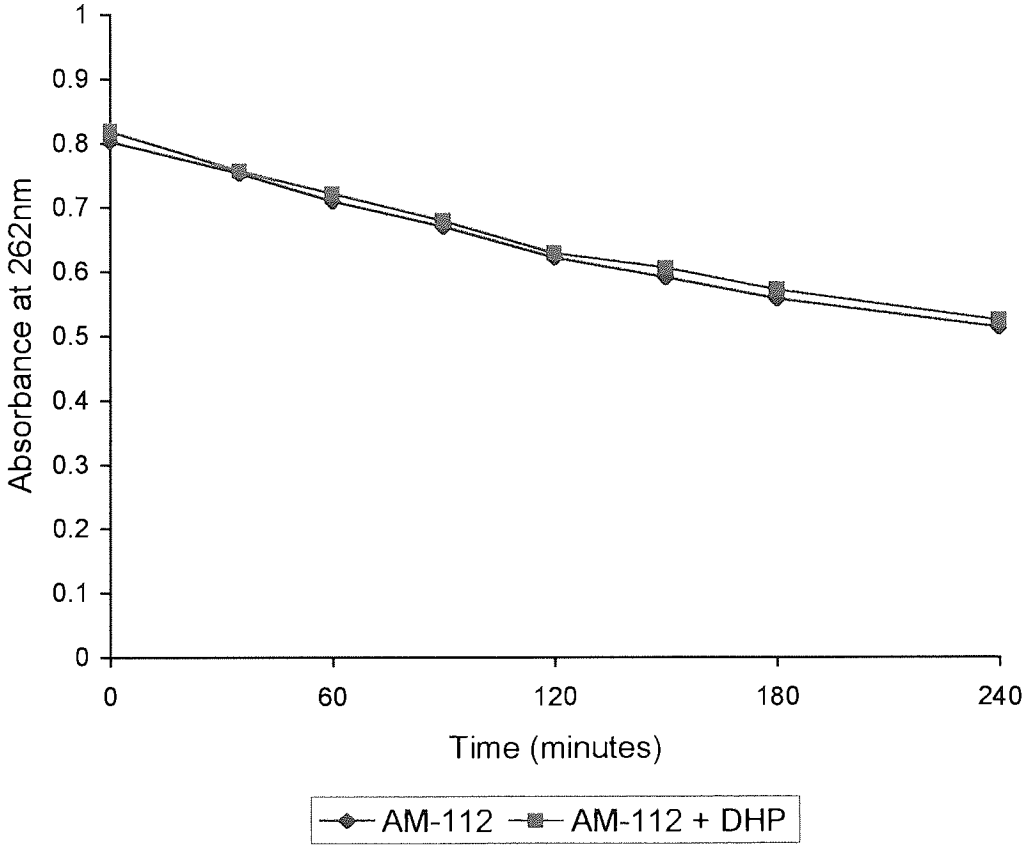
Imipenem showed an increased rate of hydrolysis in the presence of the renal peptidase compared with the hydrolysis in the absence of the enzyme. The peptidase-catalysed rate of hydrolysis of imipenem, calculated from the initial slope of the graph, was calculated to be 0.0007 μmoles/min under the conditions of the assay. In the absence of renal peptidase there was a slow rate of hydrolysis, indicating the instability of imipenem in Tris-HCl buffer at pH7.4.

FIGURE 6.2. Stability of meropenem (0.05mg/ml) to hydrolysis by porcine renal peptidase (DHP), monitored at 296nm over three hours at 37°C.



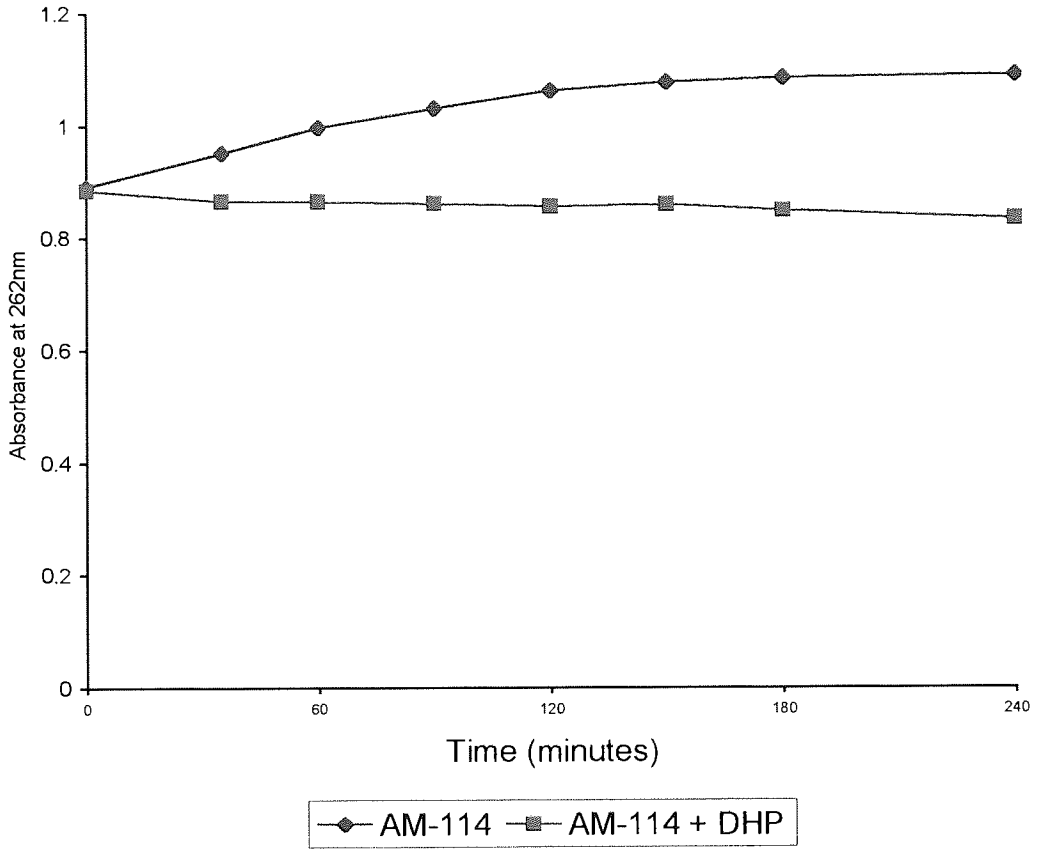
Meropenem was not sensitive to the peptidase, it still showed a slight decrease in absorbance at 296nm in the presence of the renal peptidase, which was equivalent to that of meropenem alone. In both samples, there was a slight increase in the absorbance over the initial 20 to 30 minutes, followed by a gradual decline in the absorbance over the three-hour duration of the experiment.

FIGURE 6.3. Stability of AM-112 (0.1mg/ml) to hydrolysis by porcine renal peptidase (DHP), monitored at 262nm over four hours at 37°C.



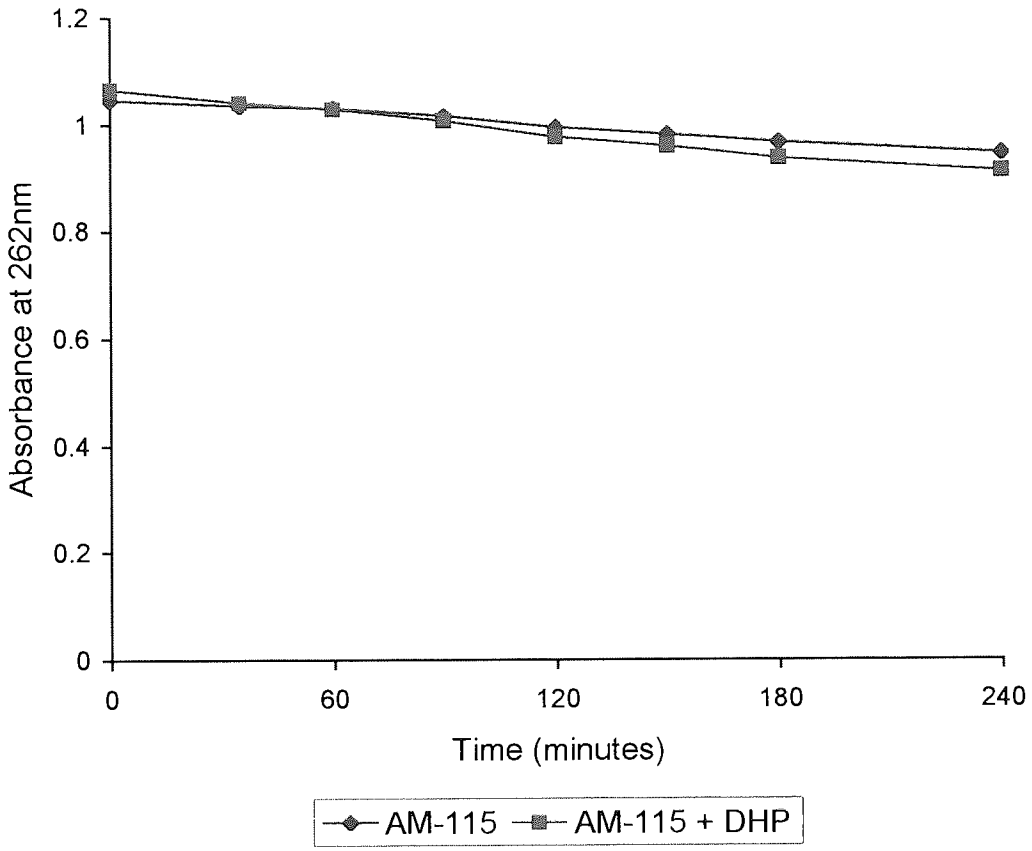
AM-112 showed a steady reduction in absorbance between 0 and 240 minutes, indicating a small amount of hydrolysis in this period, indicating some chemical instability. The rate of hydrolysis was not increased by the presence of renal peptidase confirming resistance to the enzyme.

FIGURE 6.4. Stability of AM-114 (0.1mg/ml) to hydrolysis by porcine renal peptidase (DHP), monitored at 262nm over four hours at 37°C.



AM-114 showed a slight increase in absorbance at 262nm over 240 minutes in the absence of renal peptidase. By contrast when the enzyme was present there was a slight reduction in absorbance over 240 minutes indicating some sensitivity to the peptidase.

FIGURE 6.5. Stability of AM-115 (0.1mg/ml) to hydrolysis by porcine renal peptidase (DHP), monitored at 262nm over four hours at 37°C.



AM-115 showed a slight reduction in absorbance over 240 minutes that was independent of the presence of the peptidase. This suggests stability to the peptidase.

The results show that imipenem is hydrolysed by the renal peptidase, while meropenem is stable to the enzyme. This is in agreement with previous stability studies on these carbapenems (Matsuda, *et al.* 1985; Fukasawa, *et al.* 1992; Hikida, *et al.* 1992). AM-112 is slightly unstable but resistant to renal peptidase. AM-115 is more stable than AM-112 and also resistant to peptidase. AM-114 is stable (or hydrolyses to a compound with greater absorbance at 262nm) but somewhat sensitive to renal peptidase.

6.3.4 *Serum protein binding of AM-112*

Table 6.5 lists the values obtained from the serum binding studies on AM-112.

TABLE 6.5. Protein binding of AM-112 to sera from various species, determined by ultrafiltration and HPLC assay.

Species	At 10mg/ml (%)	At 100mg/ml (%)	Mean (%)
Mouse	23.6	20.6	22.1
Rat	27.9	26.4	27.2
Monkey	9.3	9.7	9.5
Human	14.9	10.9	12.9

The results of the serum binding studies show good correlation between the two concentrations of AM-112 tested for each species. Serum binding was lowest in monkey serum (9.5%), followed by human serum (13%) and highest in rat serum (27%).

6.4 Discussion

The experiments described in this chapter aimed to establish the *in vivo* efficacy of AM-112 and determine factors which may affect any such efficacy. Murine infection models represent a useful means of determining the efficacy of compounds. There are several models that are frequently used including the thigh lesion model and the intraperitoneal infection model. Testing on laboratory animals is strictly regulated and subject to specific approval from the Home Office. An animal licence was granted to carry out experimental work on mice, specifically for the thigh lesion model. The terms of the licence precluded any survival studies, and the duration of the experiment was only permitted to last six hours before the mice had to be sacrificed. The welfare of the laboratory animals is an important factor in the consideration of an application for an animal licence.

The attempt to develop a murine thigh lesion model at Aston was not entirely successful. As described, the animal licence restricted the extent and scope of the experiment. In protocols used by other groups, the experimental animals are rendered neutropenic by injections of cyclophosphamide several days prior to the experiment, and the experiment lasts 2-3days (Gudmundsson and Erlendsdottir 1999). The adapted protocol followed at Aston was in response to the limitations imposed by the animal licence. The time limit meant that few animals could be handled by the animal house technician, which restricted the sample size for the experiment. The importance of the sample size was demonstrated in the recovered CFU/thigh in the control mice, which differed ten-fold, and also in the variability seen in the recovered CFU/thigh in the vancomycin treated animals. The process involved in removing and homogenising the thigh muscle was technically difficult and may have been a source of experimental error. It would be easier to simply score the animals on survival after a defined number of days and calculate an ED_{50} value for the treatment. Nonetheless, the murine thigh lesion model proved to be a valid model of infection. The inoculated organism did multiply within the thigh muscle, and the animals showed signs of ill health after six hours, consistent with a major infection. Treatment of the animals with subcutaneous vancomycin did reduce the inoculum at the higher doses

(30 and 40mg/kg). It is likely that a larger sample size would make this experimental model more robust and provide valid information on the efficacy of test compounds. Availability of dedicated animal test facilities at Glaxo SmithKline and at Biosearch Italia in Italy meant that it was more cost effective and easier to conduct further *in vivo* work there. The results of the murine peritonitis models were supplied by Dr. I. Simpson and are reproduced with the permission of Amura Ltd. The scope of the experiments was greater than those carried out at Aston, because the survival of the mice over four days was observed. An intraperitoneal infection is a serious, life-threatening condition if untreated. Survival of the control, untreated mice is likely to have lasted only one or two days. Only mice that would have been treated with a sufficiently high concentration of antibiotics to maintain blood levels above the MIC for a sufficient period to control the infection would be likely to survive until the end period of the experiment.

The murine intraperitoneal infection models were carried out using three different pathogenic organisms: *S. aureus* 3816, a β-lactamase producing staphylococcus; *E. cloacae* P99, which produces a class C β-lactamase in high amounts and *E. coli* SHV-5, which produces a class A extended spectrum β-lactamase (ESBL). Ceftazidime and AM-112 were tested alone and in varying combinations, and clavulanic acid was included for comparison against *E. cloacae* P99. Ceftazidime is a third generation cephalosporin which is more active against Gram-negative species than Gram-positive species. AM-112 has a good spectrum of activity against staphylococcal species, including MRSA strains. These differing activities were reflected in the MICs for the two compounds against *S. aureus* 3816, which were 16μg/ml for ceftazidime and 1μg/ml for AM-112. When administered subcutaneously to infected mice, the ED_{50} for ceftazidime was between 18.3 and 22.6mg/kg. In line with the MIC, AM-112 was much more active against the staphylococcus, with an ED_{50} of 2.6-2.7mg/kg. The bioavailability of AM-112 was much poorer after oral administration, and the ED_{50} was ten-fold higher than via the subcutaneous route. It is likely that passage through the gastro-intestinal tract results in loss of activity of AM-112, whether through hydrolytic inactivation by the gastro-intestinal contents or poor absorption from the gastro-intestinal tract. Combination of ceftazidime and AM-112 in a 7:1 ratio halved the concentration of

both components required for the ED_{50}, compared to either agent alone. Increasing the proportion of AM-112 to 1:4, reduced the concentration of ceftazidime required to a quarter of that required for efficacy by ceftazidime alone. These results show that the combination of ceftazidime and AM-112 appeared to be additive, but that the perceived additivity was primarily due to the potent activity of AM-112 against *S. aureus* 3816. A concentration of 1mg/kg of AM-112, combined with 4.8mg/kg of ceftazidime was as effective as 1mg/kg of AM-112 and 7mg/kg of ceftazidime. The ED_{50} values for both ceftazidime and AM-112 were similar to the MIC for each compound.

E. cloacae P99 is a hyperproducer of a class C β-lactamase. As such, it was not sensitive to ceftazidime (MIC 32μg/ml) or clavulanic acid (MIC 64μg/ml) alone and only moderately sensitive to AM-112 (MIC 8-16μg/ml). The ED_{50} values for ceftazidime and clavulanic acid alone were 100-200mg/kg and >100mg/kg respectively. AM-112 had moderate activity at 19-20mg/kg. Combination of clavulanic acid and ceftazidime lowered the ED_{50} of both agents, with the ED_{50} decreasing as the proportion of clavulanic acid increased. The combination of AM-112 and ceftazidime was synergistic. An eight-fold reduction in the ED_{50} of each agent was observed when the ratio of AM-112 to ceftazidime was 1:1 and 2:1. Even at a 4:1 ratio, the ED_{50} of each agent was reduced four-fold. The combination of the two agents was synergistic, probably due to the effective inhibition of the P99 β-lactamase by AM-112, which would protect the activity of ceftazidime. These results reflect the *in vitro* results for ceftazidime and AM-112 in a 1:1 and 2:1 ratio, and demonstrate that the synergy is maintained *in vivo*.

E. coli SHV-5 is an ESBL producing strain. The organism was moderately sensitive to ceftazidime and AM-112 (MICs ~16μg/ml). The ED_{50} for ceftazidime closely matched the MIC, but this was not the case for AM-112, where the ED_{50} was 72mg/kg. As with *E. cloacae* P99, the combination of ceftazidime and AM-112 was synergistic, with the ED_{50} for ceftazidime reduced 8-fold and that of AM-112 up to 32-fold, at both 1:1 and 2:1 ratios.

The results of these intraperitoneal murine infection models demonstrate that the effective inhibition of class A, class C and ESBL β-lactamases by AM-112, observed in cell-free β-lactamase inhibition studies, is maintained *in vivo*. AM-112 is effective in protecting ceftazidime from the hyperproduced class C enzyme produced by *E. cloacae* P99 and the ESBL produced by *E. coli* SHV-5. AM-112 also extends the antimicrobial spectrum of ceftazidime to include *S. aureus* 3816. These *in vivo* results confirm the in vitro findings that AM-112 and the other oxapenems are effective at protecting the activity of ceftazidime and other cephalosporins and also extend their spectrum of activity.

The extent of protein binding in marmoset and human serum was approximately 10-13%, while in mouse and rat serum the protein binding was between 22-27%. The binding of AM-112 to human serum proteins is lower than that of imipenem, which is approximately 20% (Wise, *et al.* 1981) and ceftazidime, which is approximately 17% (Harding, *et al.* 1981). The degree of serum binding of AM-112 could be influenced by the nature of the polarity of the C_2 side-chain, as is the case with penams (Gootz, *et al.* 1988).

Renal peptidase is an important factor in the inactivation of carbapenems *in vivo*, such that imipenem must be administered with a renal peptidase inhibitor, cilastatin, when used clinically. Meropenem is stable to hydrolysis by renal peptidase and is administered without an inhibitor. As the oxapenems share structural similarities to the carbapenems, it was important to investigate the stability of AM-112, AM-114 and AM-115 to hydrolysis by renal peptidase. The results in Figs. 6.1 and 6.2 confirm that imipenem is susceptible to hydrolysis by porcine renal peptidase, while meropenem is stable to such hydrolysis. The results for the oxapenems (Figs. 6.3-6.5) show that AM-112 and AM-115 are resistant to hydrolysis by porcine renal peptidase, but slightly unstable in solution over four hours, while AM-114 is slightly unstable in solution and slightly sensitive to hydrolysis by porcine renal peptidase. A source of human renal peptidase was not available during the course of this study, so the stability of the oxapenems to this enzyme could not be determined. This is an important consideration for future research.

Chapter 7. Concluding discussion

Bacterial resistance to antibiotics is an increasing problem. One of the principal mechanisms of this resistance is the production of antibiotic inactivating enzymes. The spread of genes encoding for these enzymes, by plasmids and transposons, between bacterial species and to species normally lacking in these enzymes is posing a grave threat to the future clinical utility of our existing drugs. The most problematic resistance enzyme is bacterial β-lactamase, which is produced by Gram-positive and Gram-negative species. β-lactamases are capable of rapidly inactivating susceptible β-lactam antibiotics such as penicillins and cephalosporins. As a counter-measure, β-lactamase stable compounds have been developed (for example flucloxacillin) and newer cephalosporins and carbapenems are resistant to hydrolysis by these enzymes. However, the increasing use of these compounds has resulted in the evolution of enzymes which are capable of inactivating these previously-stable compounds. The use of β-lactamase inhibitors to protect susceptible β-lactams from hydrolysis has been a successful strategy to overcome bacterial resistance due to β-lactamase production. The emergence of inhibitor-resistant β-lactamase enzymes has been documented, but to date these enzymes have not become widespread in the bacterial population. Existing agents (clavulanic acid, tazobactam and sulbactam) are effective inhibitors of class A enzymes, but are not active against class C enzymes (Bush, *et al.* 1995). Third and fourth generation cephalosporins are susceptible to hydrolysis by class C enzymes, and effective inhibitors of these enzymes are urgently needed.

The oxapenem compounds are effective inhibitors of β-lactamase enzymes. Cell-free β-lactamase assays were carried out on enzymes isolated from bacteria. Enzymes from class A, class C and class D were isolated by lysis of the cells and purification of the enzyme by isoelectric focusing. Nitrocefin was used as the substrate. From these spectroscopic assays, values for the IC_{50} and K_i for each compound against each of the eight enzymes were determined. These values were compared against the established β-lactamase inhibitor, clavulanic acid. Clavulanic acid was an effective inhibitor of class A enzymes, with IC_{50}s ranging from 0.008-0.12μM. AM-114 and AM-115 were also highly active against class A enzymes, with IC_{50}s in the range

0.002-0.06. AM-112 and AM-113 were the least active compounds against the class A enzymes. All of the oxapenems were highly active against class C and class D enzymes, in contrast to the poor activity of clavulanic acid against these enzymes.

Modelling of the inhibitors in the active site of the class A β-lactamase TEM-1 and the class C enzyme P99 was carried out using the Quanta modelling program. Examination of the energy-minimised acyl-enzyme complexes of the oxapenems in the active site of TEM-1 revealed a number of differences between the compounds. Both AM-112 and AM-113 interacted with a water molecule (Wat-309) in the active site, whereas AM-114 and AM-115 did not. Water molecules are known to be important in the deacylation step of β-lactam hydrolysis (Matagne, *et al.* 1999), and this may account for the poorer *in vitro* inhibiton of TEM-1 by AM-112 and AM-113 compared to the other compounds. In addition, the tertiary butyl group at C_2 of AM-114 appeared to displace this water molecule. The ability of the inhibitor to cross-link between catalytic residues in the active site, thus inhibiting the enzyme is an important factor in the activity of an inhibitor (Therrien and Levesque 2000). Ser-130 has been identified as an important catalytic residue in the active site of class A enzymes (Massova and Mobashery 1997). Of all the oxapenems, the C_5 atom of AM-114 was located closest to this residue. AM-114 also formed a hydrogen bond with the hydroxyl group of Ser-235, which may stabilise the cross-linked species and enhance the inactivation of the enzyme. The results for the class C β-lactamase of *E. cloacae* P99 were more difficult to interpret. The *in vitro* assays indicated that AM-112, AM-113 and AM-114 had an almost equal potency against the enzyme, while AM-115 was ten-fold less active. The C_5 atom of AM-115 was located furthest from the hydroxyl side chain of Tyr-150, which is believed to be the catalytic counterpart of Ser-130 in class A enzymes. Perhaps this distance between the inhibitor and the active site residue is less favourable for cross-linking of the inhibitor in the active site. Attempts to identify the sites of interaction of AM-112 with *E. cloacae* P99 β-lactamase by tryptic digestion of the inhibited enzyme, separation of the fragments by HPLC and characterisation of the fragments by ESMIS proved unsuccessful.

AM-112 and AM-113 had intrinsic antibacterial action, while their enantiomers AM-115 and AM-114 lacked activity. Of the four oxapenem compounds, AM-112 and AM-113 were active against a range of bacteria, while AM-115 and AM-114 lacked significant activity against the same bacteria. AM-112 and AM-113 MIC ranges for *E. coli* were 2 to 16μg/ml, while for AM-115 and AM-114 the MICs were 8 to >64μg/ml respectively. Both AM-112 and AM-113 were very active against staphylococci, both methicillin-sensitive and resistant strains, with MICs lower than those of established agents such as ceftazidime. AM-113 was also active against the Gram-positive enterococci, including *E. faecalis* 56059, which expresses the *vanA* vancomycin-resistance phenotype. The MIC against this organism was 2μg/ml. However, the activity of AM-112 and AM-113 against Gram-positive and Gram-negative organisms was not sufficient to justify their use as single agents. In addition, AM-113 proved to be rapidly inactivated in the presence of human serum. The potent β-lactamase inhibitory properties of the oxapenems, especially their activity against class C enzymes, led to their evaluation as partner agents for cephalosporins sensitive to hydrolysis by class C and ESBL enzymes. AM-112 was more effective than clavulanic acid (at a fixed 4μg/ml concentration) at protecting eight cephalosporins against a panel of class A and class C enzyme producing strains. AM-114 and AM-115 protected ceftazidime at a 1:1 ratio, AM-113 was most effective at a 1:2 ratio with ceftazidime while AM-112 was equally effective at a 1:1 or 1:2 ratio with ceftazidime against a panel of 36 bacterial strains which included class A and class C enzyme producers, MRSA strains and Enterobacteriacae. The efficacy of AM-112 in protecting ceftazidime *in vitro* was also seen in *in vivo* models of infection. AM-112 was effective in protecting ceftazidime against a β-lactamase producing strain of *S. aureus*, a class C hyperproducing strain of *E. cloacae* P99 and the ESBL producing *E. coli* SHV-5 in murine intraperitoneal infections.

The mode of antibacterial action of the oxapenems appears to be the inhibition of penicillin-binding proteins. This is in agreement with the mode of action of established β-lactam compounds. Examination of the morphology of *E. coli* DC0 cells treated with each of the oxapenems indicated that at lowest concentrations of each compound, the cells assumed a round form, compared to the rod shaped form of the untreated cells. Results from the PBP affinity studies indicated that in *E. coli*

DC0 PBP2 was the initial target of AM-112, in competition with ^{3}H-benzylpenicillin. While studies conducted at Aston were unsuccessful at identifying the PBP target in *S. aureus* and *E. faecalis* SFZ, results from Dr. R. Hakenbeck at Universitat Kaiserslautern (Kaiserslautern, Germany) showed that AM-112 had affinity for all enterococcal PBPs, but that PBP3 was only inhibited at the highest concentration of AM-112 tested.

Further research on these compounds is warranted. Full kinetic evaluation of the β-lactamase inhibitory action of the compounds is a priority. Testing the protective effect of the compounds for cephalosporins against a large battery of clinical isolates of β-lactamase producing Gram-positive and Gram-negative strains will help to identify the most suitable partner for AM-112, which is the lead compound. Crystallographic and affinity labelling studies on the compounds will shed further light on their mode of β-lactamase inhibition. The synergistic action of AM-112 with ceftazidime against vancomycin-resistant enterococci, described in this report, warrants further investigation and could prove to be highly significant in light of the growing threat posed by these organisms.

In summary, the data presented in this report indicates that the novel oxapenem β-lactam compounds have intrinsic antimicrobial action and are highly active β-lactamase inhibitors. The compounds inhibit class A, class C, class D β-lactamases and ESBLs, and thus have a broader spectrum of action than clavulanic acid, tazobactam or sulbactam. The mechanism of β-lactamase inactivation has not been defined, but is likely to involve cross-linking of the compounds in the active site of the enzyme, possibly to Ser-130 in class A enzymes and Tyr-150 in class C enzymes. Each of the compounds is effective in protecting ceftazidime from hydrolysis by β-lactamase producing strains, and the future use of these compounds is likely to be as partner agents for established β-lactams which are becoming susceptible to destruction by class C and extended spectrum β-lactamases. The mode of action of the oxapenems is due to inhibition of penicillin-binding proteins, and PBP2 is the target of action in *E. coli*. Further development of these compounds as therapeutic agents in the control of infectious diseases is warranted.

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Publications

Presented at 41st Interscience Conference on Antimicrobial Agents and Chemotherapy (ICAAC), Chicago, USA, December 2001:

Protection of cephalosporins by oxapenem AM-112, a novel, broad-spectrum β-lactamase inhibitor.
C. E. Jamieson, P.A. Lambert and I. N. Simpson.

AM-112, a novel oxapenem β-lactamase inhibitor with unexpected synergistic activity with ceftazidime against Enterococci, including some vancomycin-resistant isolates.
C. E. Jamieson, P.A. Lambert, R. Hakenbeck and I. N. Simpson.

Protection of ceftazidime against class A and class C β-lactamases by oxapenems.
C. E. Jamieson, P.A. Lambert and I. N. Simpson.

Affinity of oxapenem AM-112 for *Escherichia coli* PBPs and effect upon morphology.
C. E. Jamieson, P.A. Lambert and I. N. Simpson.

Presented at 12th European Congress of Clinical Microbiology and Infectious Diseases (ECCMID), Milan, Italy, April 2002:

Oxapenems: Unexpected synergistic activity with other β-lactams against *Enterococcus* spp.
C. E. Jamieson, P. A. Lambert, D. F. J. Brown, R. Hakenbeck, C. J. Urch and I. N. Simpson.

AM-112 (PFOB): Chemistry and biological activity of a novel, broad spectrum, β-lactamase inhibitor.
I. N. Simpson, C. J. Urch, C. E. Jamieson and P. A. Lambert.

XOB: A novel, orally absorbed, inhibitor of Class A and D β-lactamases
C. E. Jamieson, P. A. Lambert and I. N. Simpson.

Appendix 1. List of strains.

Organism	Strain	Origin
MRSA	96-7778	Clinical isolate from City Hospital, Birmingham (CHB); phage typed by PHLS, Colindale
MRSA	Innsbruck	Strain used by Pfaendler et al., (1993)
S. aureus	NCTC 6571	"Classic" Oxford strain
S. aureus	ATCC 29213	ATCC typed strain
S. aureus	ATCC 25923	ATCC typed strain
S. aureus	NCTC 10788	NCTC typed strain
E. faecalis	ATCC 25922	ATCC typed strain
E. faecalis	ATCC 29212	ATCC typed strain
E. faecalis	NCTC 5957	NCTC typed strain
E. faecalis	SFZ	Endocarditis isolate, Heartlands Hospital, Birmingham (HHB)
E. faecalis	Phillips	Endocarditis isolate, HHB
E. faecalis	24952	Clinical isolate, University of Newcastle Dental School
E. faecalis	56059	Clinical isolate, Addenbrookes Hospital, Cambridge (AHC) expressing *vanA* phenotype
E. faecalis	78097	Clinical isolate, AHC, expressing *vanB* phenotype
VRE	300 1562	Endocarditis isolate, Queen Elizabeth Hospital, Birmingham (QEHB)
VRE	300 1590	Endocarditis isolate, QEHB
VRE	300 1662	Endocarditis isolate, QEHB
VRE	300 2043	Endocarditis isolate, QEHB
E. hirae	ATCC 10541	ATCC typed strain
E. faecium	NCTC 7171	NCTC typed strain
E. coli	J53-1	Public Health Laboratory Service (PHLS) parent strain for plasmid encoded β-lactamases. Obtained from Dr. D. Livermore.
E. coli	OXA-1	PHLS transconjugant strain, carries plasmid encoded OXA-1 β-lactamase (Class D)
E. coli	OXA-2	PHLS transconjugant strain, carries plasmid encoded OXA-2 β-lactamase (Class D)
E. coli	OXA-3	PHLS transconjugant strain, carries plasmid encoded OXA-3 β-lactamase (Class D)
E. coli	OXA-5	PHLS transconjugant strain, carries plasmid encoded OXA-5 β-lactamase (Class D)

Organism	Strain	Origin
E. coli	SHV-1	PHLS transconjugant strain, carries plasmid encoded SHV-1 β-lactamase (Class A)
E. coli	SHV-2	PHLS transconjugant strain, carries plasmid encoded SHV-2 β-lactamase (Class A)
E. coli	SHV-3	PHLS transconjugant strain, carries plasmid encoded SHV-3 β-lactamase (Class A)
E. coli	SHV-4	PHLS transconjugant strain, carries plasmid encoded SHV-4 β-lactamase (Class A)
E. coli	SHV-5	PHLS transconjugant strain, carries plasmid encoded SHV-5 extended-spectrum β-lactamase (ESBL) (Class A)
E. coli	TEM-1	PHLS strain, carries plasmid encoded TEM-1 β-lactamase (Class A)
E. coli	TEM-3	PHLS transconjugant strain, carries plasmid encoded TEM-3 β-lactamase (Class A)
E. coli	TEM-6	PHLS transconjugant strain, carries plasmid encoded TEM-6 β-lactamase (Class A)
E. coli	TEM-9	PHLS transconjugant strain, carries plasmid encoded TEM-9 β-lactamase (Class A)
E. coli	TEM-10	PHLS transconjugant strain, carries plasmid encoded TEM-10 ESBL (Class A)
E. coli	PSE-4	PHLS transconjugant strain, carries plasmid encoded PSE-4 β-lactamase (Class A)
E. coli	ATCC 35218	ATCC typed strain
E. coli	ATCC 25922	ATCC typed strain
E. coli	1850E DC0	K12 strain, Glaxo SmithKline
E. coli	X580	Clinical isolate, Oxford
E. coli	NCTC 10418	NCTC typed strain
E. coli	W3110 R+	Contains RP1 resistance plasmid encoding for TEM-1 β-lactamase
E. coli	W3110 R-	Lacks RP1 resistance plasmid
E. coli	ESBL+	Clinical isolate, CHB, expresses an ESBL
E. cloacae	1051E	Glaxo SmithKline strain, hyperproducer of P99 β-lactamase (Class C)
E. cloacae	1194E Hennessey	Glaxo SmithKline strain, hyperproducer of AmpC β-lactamase (Class C)

Organism	Strain	Origin
E. cloacae	84-con	PHLS strain, constitutive derepressed AmpC β-lactamase producer (Class C)
C. diversus	2046E	Glaxo SmithKline strain
C. freundii	C2-con	PHLS strain, constitutive derepressed AmpC β-lactamase producer (Class C)
Serratia sp.	1324E	Glaxo SmithKline strain
S. marcescens	S2-con	PHLS strain, constitutive derepressed AmpC β-lactamase producer (Class C)
M. morganii	M1-con	PHLS strain, constitutive derepressed AmpC β-lactamase producer (Class C)
K. pneumoniae	K1+	Clinical isolate, Indonesia
P. aeruginosa	2297-con	PHLS strain, constitutive derepressed AmpC β-lactamase producer (Class C)
P. aeruginosa	1407-con	PHLS strain, constitutive derepressed AmpC β-lactamase producer (Class C)
P. aeruginosa	ATCC 27853	ATCC typed strain
P. aeruginosa	NCTC 10662	NCTC typed strain

Key: MRSA, methicillin-resistant *Staphylococcus aureus*

VRE, vancomycin-resistant enterococcus