SYNTHESIS AND REACTIVITY OF β-SULTAMS WITH THE POTENTIAL TO ACT AS METALLO-ENZYME INHIBITORS

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Abstract

Enzyme inhibition forms the basis of much of the medicinal chemistry used in the treatment of disease. β-Sultams are cyclic sulfonamides which are both β-lactam analogues and potential pro-drugs of taurine and substituted taurines: as their hydrolysis products. Two β-sultams, their hydrolysis products and a range of dicarboxylic acids were tested as inhibitors of BcII, a metallo-β-lactamase enzyme. The two β-sultams, their hydrolysis products and some related compounds were also tested as inhibitors of glutamine synthetase following work showing that β-sultam has an effect on neurotransmission in the CNS.

A novel β-sultam, 1,2-thiazetidine-3-carboxylate-1,1-dioxide (3-carboxy-β-sultam), has been synthesised via a four-step process from L-cystine including the removal of a benzyl ester group from benzyl 3-carboxylate-β-sultam utilising sodium in liquid ammonia. The product has been characterised by NMR and MS.

The rate of hydrolysis of 3-carboxy-β-sultam was investigated using ^{1}H NMR and a pH-rate profile produced showing two hydrolysis processes on the acidic limb both of which were first order in hydronium ion concentration (k_H = 2.00 x 10^{-1} and 4.8 $M^{-1}s^{-1}$ respectively) and an alkali catalysed limb first order in hydroxide concentration, k_{OH} = 5.00 x 10^{-4} $M^{-1}s^{-1}$. The half-life of 3-carboxy-β-sultam at physiological pH is approximately 16.5 days.

The rate of hydrolysis of the unsubstituted β-sultam at acidic pH was investigated by ReactIR and shown to be first order in hydronium ion concentration though k_H was not calculated due to variations in the quality of the collected data.

Neither the 3-carboxy-β-sultam nor the unsubstituted β-sultam inhibited BcII or glutamine synthetase. D-Cysteine is a weak inhibitor of BcII, K_i = 7.5 x 10^{-3} M, and a substrate for glutamine synthetase. L-Cysteine is also a substrate for glutamine synthetase and L-cysteic acid is a very weak inhibitor of BcII.

The mechanism of BcII catalysed hydrolysis of ertapenem was investigated using ^{1}H NMR and shown to proceed via protonation of the ring opened pyrrolidine ring at C3 leading to the formation of an imine.

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List of abbreviations

6-APA	6-Aminopenicillanic acid
7-ACA	7-Aminocephalosporanic acid
Ac	Acetyl
ADP	Adenosine diphosphate
Ala	Alanine
ALS	Amyotrophic lateral sclerosis
ATP	Adenosine triphosphate
ATR	Attenuated total reflectance
Bn	Benzyl
CNS	Central nervous system
COSY	Correlation spectroscopy
d.e.	Diastereomeric excess
dec.	Decomposed
DEPT	Distortionless enhancement by polarization transfer
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
e.e.	Enantiomeric excess
EDTA	Ethylenediaminetetracetic acid
ESI-MS	Electrospray ionization mass spectrometry
Et	Ethyl
FTIR	Fourier transform infrared
GABA	γ-Amino butyric acid
Gln	Glutamine
Glu	Glutamic acid (or glutamate)
GS	Glutamine synthetase

HMBC	Heteronuclear multiple bond correlation
HSQC	Heteronuclear single quantum coherence
HPLC	High performance liquid chromatography
i-Bu	Isobutyl
IC_{50}	Half maximal inhibitory concentration (the concentration of inhibitor required to give half the miximal rate)
i-Pr	Isopropyl
IR	Infrared
J	Coupling constant
k_0	Uncatalysed rate constant (spontaneous hydrolysis)
k_{A-}	Rate constant for hydrolysis catalysed by basic form of buffer
k_{buf}	Rate constant for hydrolysis catalysed by both forms of buffer
k_{cat}	First order rate constant for the breakdown of ES
k_{cat}/K_M	Second order rate constant for enzyme activity
k_{HA}	rate constant for hydrolysis catalysed by acidic form of buffer
K_i	Rate constant for enzyme inactivation (the concentration of inhibitor required to produce half maximum inhibition)
k_{int}	First order rate constant at zero buffer concentration
K_M	The Michaelis constant (the substrate concentration at which the reaction rate is half of V_{max})
k_{obs}	Observed first order rate constant
LDA	Lithium diisopropylamide
LDH	Lactate dehydrogenase
Me	Methyl
MES	2-(N-morpholino)ethanesulfonic acid
MRSA	Methicillin resistant *Staphylococcus aureus*
NAD+	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide, reduced form
NAG	N-Acetylglucosamine

NAM	N-Acetylmuramic acid
n-Bu	Butyl
NMR	Nuclear magnetic resonance
n-Pr	Propyl
pD	Negative logarithm of deuterium ion concentration
PEP	Phospho(enol)pyruvate
Ph	Phenyl
P_i	Inorganic phosphate
PK	Pyruvate kinase
pK_a	Logarithm of acid dissociation constant
ppm	Parts per million
PTSA	Para-toluenesulfonic acid
SG	Specific gravity
ssDNA	Single stranded DNA
TBDMS	tert-butyldimethylsilyl
t-Bu	Tertiary-butyl
THF	Tetrahydrofuran
TS	Transition state
UV-Vis	Ultra violet and visible
VISA	Vancomycin intermediate *Staphylococcus aureus*
V_{max}	Maximal rate of the enzyme catalysed reaction
VRE	Vancomycin resistant *Enterococcus*
VRSA	Vancomycin resistant *Staphylococcus aureus*

Introduction

1.1 Background and Aims of Research

Since antibiotics went into general use in the mid-twentieth century bacteria have been developing strategies to fight against them so that today antibiotic resistance is a major global health concern.[1] Whilst antibiotics are still useful in the fight against the majority of bacterial infections some strains of bacteria have evolved resistance strategies so that they can no longer be controlled or killed by drugs which were once effective against them. The rate of development of new antibiotics has now been overtaken by the increase in bacterial resistance so that infections such as pneumonia, tuberculosis and post-operative wound infections are becoming increasingly resistant to current therapies and causing an increase in previously preventable fatalities.[2]

One of the most commonly used classes of drugs for treating bacterial infections is the β-lactams which includes penicillins (core structures shown in Scheme 3, page 18). Following years of misuse of antibiotics and natural evolution some bacteria have been able to develop innovative strategies for surviving against these drugs. One of the most common forms of resistance to β-lactam antibiotics is the production of an enzyme which inactivates them: β-lactamase.[3] There are two main types of β-lactamase enzyme; the most common type are those with a serine-residue at the active site with the rest being metallo-enzymes with one or two zinc ions at the active site. One way of dealing with resistance to β-lactam antibiotics is to administer the drug with a second compound or co-drug which acts as a β-lactamase inhibitor. There are a number of clinically available inhibitors for serine enzymes; the metallo-enzymes however are less well-studied and the current clinically available serine-enzyme inhibitors are inactive against them.

The development of a compound which will inhibit metallo-β-lactamase enzymes was the inspiration for this project. Work carried out by previous members of the research group has shown that substituted β-sultams act as inhibitors of a range of serine-β-lactamase enzymes.[4] In order for a compound to be of use as a drug an understanding of its stability and reactivity at physiological pH is required. The aim of this work was firstly to synthesise a new β-sultam and to determine its inhibition parameters. Secondly, work was carried out to investigate the reactivity of this new compound and the parent molecule. The final part of this project was to look at the inhibition of a metallo-β-lactamase enzyme by a range of dicarboxylic acids.

In a second project the β-sultams synthesised as metallo-β-lactamase inhibitors were tested as inhibitors of glutamine synthetase. Glutamine synthetase is an

enzyme dependent on magnesium or manganese ions and found in the central nervous system. Glutamine synthetase has an essential role in the regulation of glutamate concentration within the brain and since glutamate is an excitatory neurotransmitter the enzyme also has a vital role in regulating neurotransmission. The unsubstituted β-sultam, 1,2-thiazetidine-1,1-dioxide, has been shown to influence the effect alcohol has on the brain and so its effect on enzymes involved in neurotransmission was of interest.

The final project in this thesis is an investigation into the mechanism of hydrolysis of a β-lactam antibiotic, ertapenem, catalysed by the metallo-β-lactamase enzyme, BcII. There are ambiguities in the literature as to the site of protonation of the pyrrolidine ring following β-lactam ring opening. This investigation aimed to confirm which of the proposed mechanisms occurs.

1.2 Bacteria, Antibiotics and Resistance

1.2.1 Bacteria

There are two main types of cell; bacteria are prokaryotic cells whereas humans, animals, plants and fungi are made up of eukaryotic cells. Prokaryotes are simple cells with DNA, proteins and other water soluble components all held within one membrane. In eukaryotes the separate components are held within separate membranes within the outer cell membrane. Prokaryotes, plant cells and fungal cells also have a cell wall, whereas animal and human cells do not. Plant cell walls are made of cellulose, hemicellulose and pectin; fungus cell walls are made of chitin and bacterial cell walls are made of a layer of peptidoglycan. This peptidoglycan cell wall is the structural difference exploited by β-lactam antibiotics; for this reason it will be looked at in more detail.

The cell wall in all bacteria consists of layers of polysaccharide and protein/peptide which varies in thickness between Gram positive and Gram negative strains. The initial designation of bacteria as Gram positive or Gram negative was based on the observations of Christian Gram in 1884.[5] In studying the tissues of lungs taken from people who had died of pneumonia he wanted to be able to study the bacterial cells microsopically and so developed a new staining technique which selectively stained only certain strains of bacteria. Those which were stained were deemed Gram positive whereas those which resisted staining were Gram negative.

After the discovery of this considerable difference in bacterial cell biology much work was done to establish exactly what caused the differential staining of the various strains of bacteria. It wasn't until the 1950s though that the composition of cell walls in Gram positive and Gram negative bacteria was studied in any detail. One paper of particular interest, published in the Journal of General Microbiology in 1956, studied more than 60 strains of Gram positive bacteria and showed that, in most cases, the cell wall was made up predominantly of four sugars: glucose, galactose, glucosamine and galactosamine; and four amino acids: alanine, glutamic acid, lysine and aspartic acid.[6] By the 1960s the structure of both Gram positive and Gram negative bacteria had been studied in much more detail, in particular by electron microscopy, and the similarities and specific differences between the two types of bacteria was well established.[7]

In Gram positive bacteria the cell wall is made of multiple peptidoglycan layers up to 25 nm thick; in Gram negative bacteria there is only a single peptidoglycan layer, of around 3 nm, sandwiched between an inner and outer lipid bilayer (Figure 1).

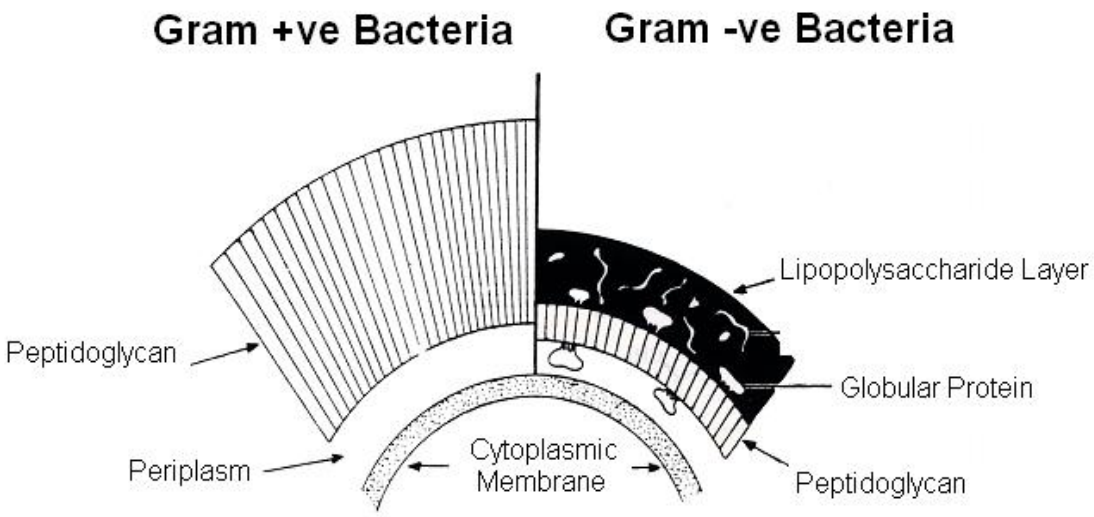
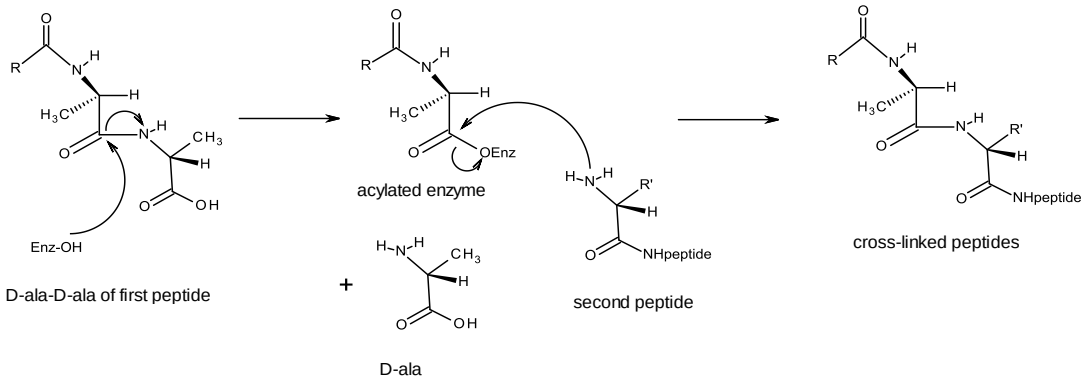


Figure 1 Cell wall structures in Gram positive and Gram negative bacteria

In both cases the polysaccharide is a NAG-NAM (N-acetylglucosamine – N-acetylmuramic acid) linear chain of the two alternating amino sugars.[8] These glycan chains are then linked together by peptide chains. Each glycan strand is initially synthesised with short amino acid chains attached to each N-acetylmuramic acid. These chains are then joined by a DD-transpeptidase enzyme which cleaves one amino acid from a chain ending with D-Ala-D-Ala residues and attaches this to either a lysine residue in Gram negative bacteria or a pentaglycine bridge on a lysine residue in Gram positive bacteria (see Scheme 1).



Scheme 1 DD-transpeptidase enzyme action in cross-linking peptidoglycan chains

This cross-linking between amino acids in different peptide chains results in a 3-dimensional structure that is strong and rigid as shown diagramatically in Figure 2.[9] Since bacteria reproduce by self-replication, in order to create a new bacterium it is necessary to replicate all the features of the cell, including the cell wall.

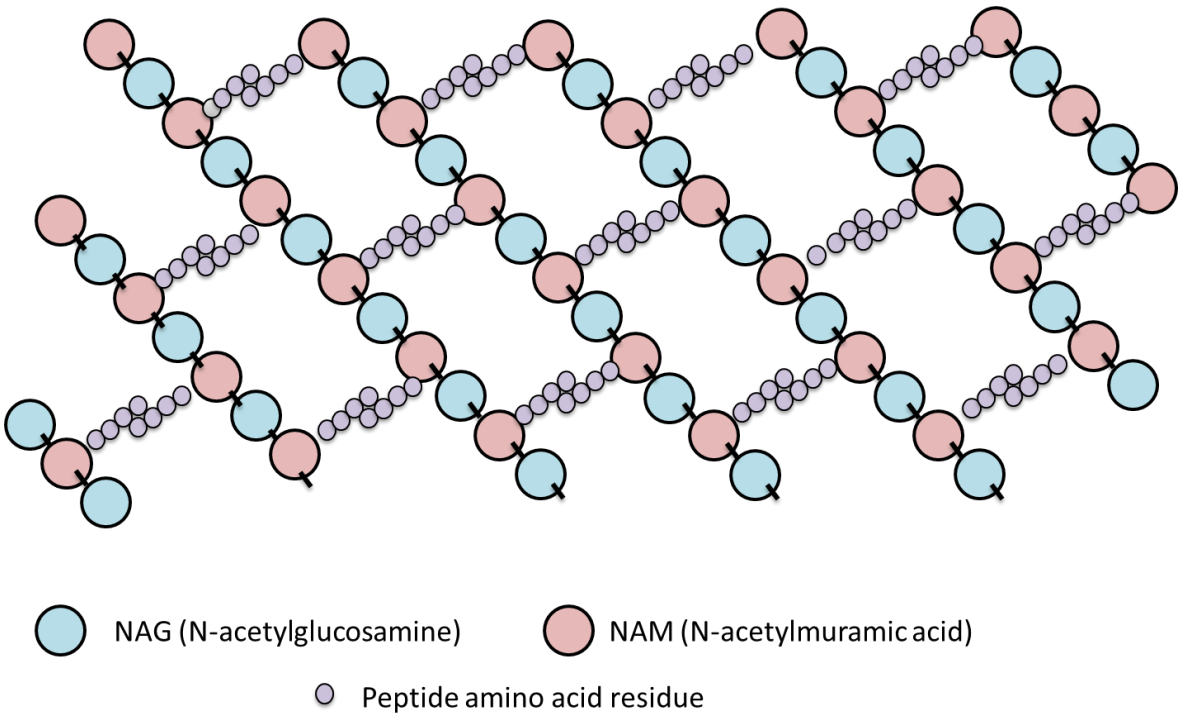
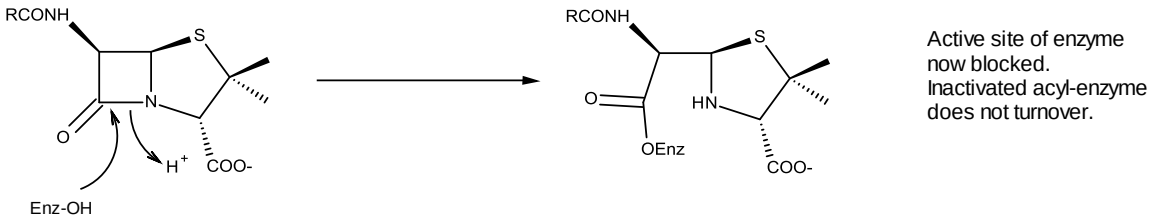


Figure 2 Structure of peptidoglycan cell wall. Shows the cross-linking of oligosaccharide chains (β-linked NAG-NAM repeats) by tetrapeptides attached to the lactic acid group of NAM via an amide bond

1.2.2 Antibiotics

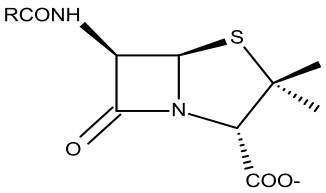
β-Lactam antibiotics are a group of anti-bacterial drugs which contain a β-lactam moiety as part of their molecular structure. The group includes penicillins, cephalosporins and carbapenems and as a group they are among the most commonly prescribed drugs in the world (core structures shown in Scheme 3).

β-Lactam antibiotics work by inhibiting the DD-transpeptidase enzyme responsible for the cross-linking of peptide chains in the synthesis of peptidoglycan (Scheme 2). The β-lactam ring with an amide side chain is a close structural analogue of D-Ala-D-Ala and so fits into the active site of the enzyme. The action of the enzyme on the β-lactam forms a stable acyl intermediate. Due to the cyclic nature of the compound the leaving group remains attached, blocking the active site and thereby inactivating the enzyme. This leaves the bacteria unable to complete the synthesis of the cell wall and leads to the batericidal nature of the drug.

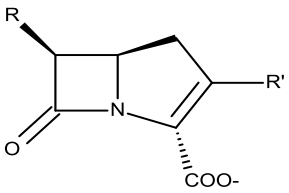


Scheme 2 Inactivation of DD-transpeptidase enzyme by penicillin

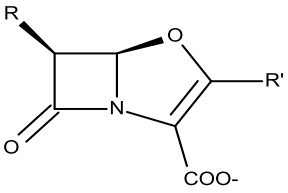
β-lactam rings fused to five-membered rings



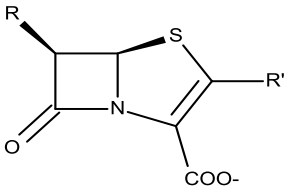
Penicillin



Carbapenem

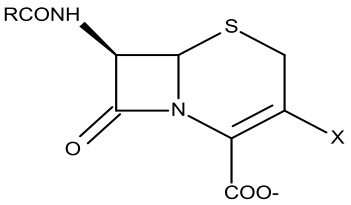


Oxapenem

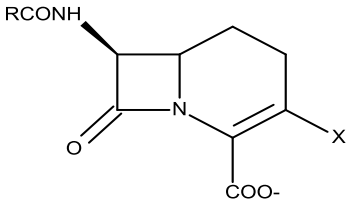


Penem

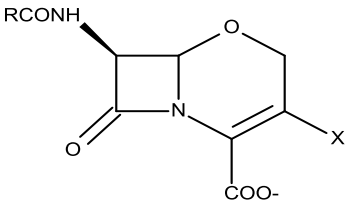
β-lactam rings fused to six-membered rings



Cephalosporin

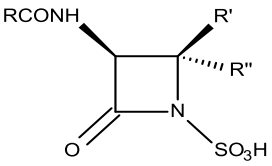


Carbacephem

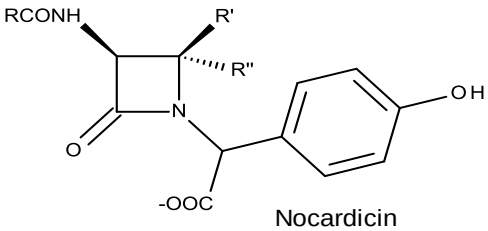


Oxacephem

Unfused β-lactam rings



Monobactam



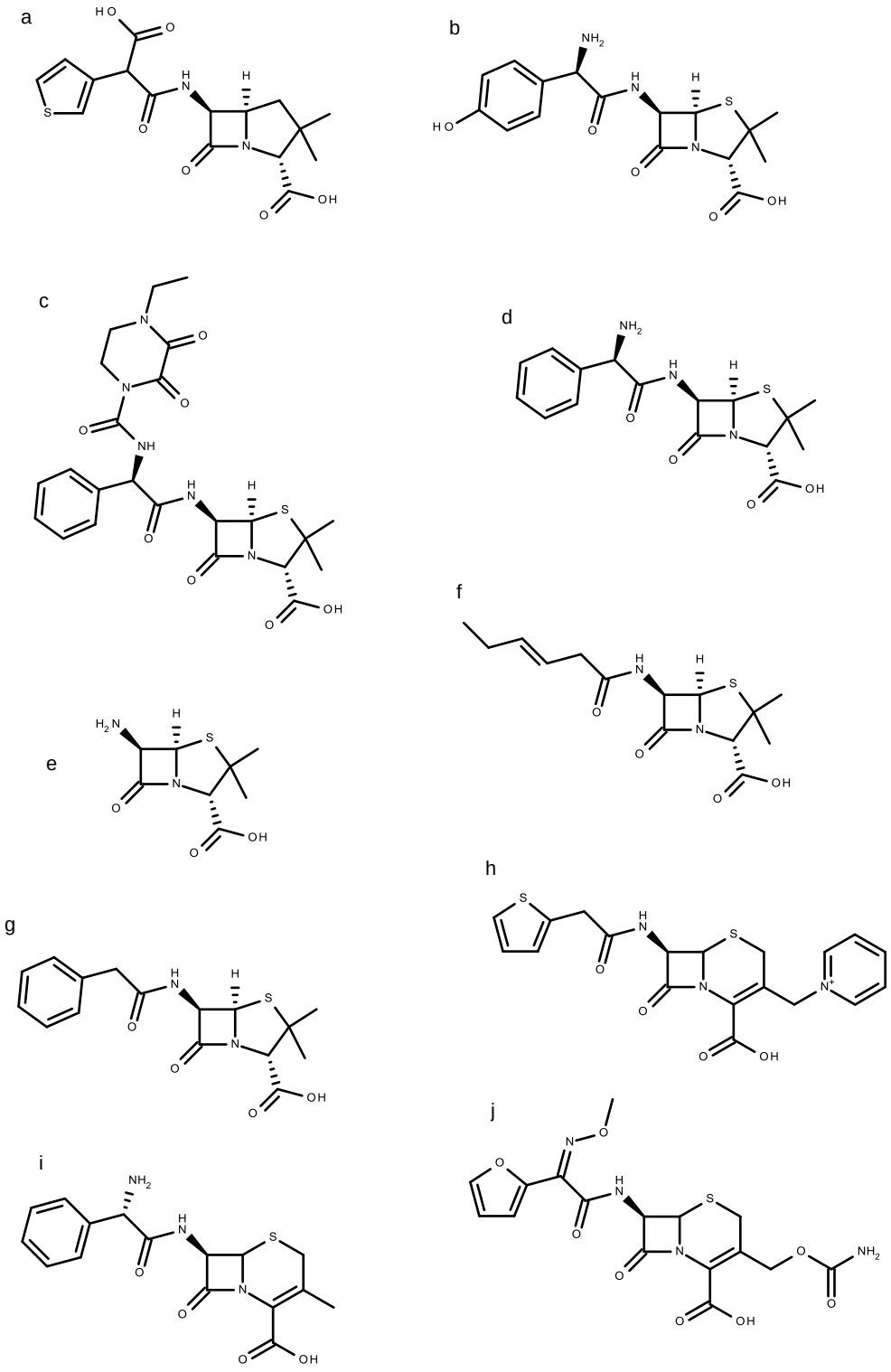
Nocardicin

Scheme 3 Structures of the main classes of β-lactam antibiotics

The antibiotic action of *Penicillium* mould was first noticed by Joseph Lister in 1871[10] and the presence of an antimicrobial agent was correctly identified by Alexander

Fleming in 1929[11]; despite these early observations the pure compound was not extracted until 1940.[12] Fermentation methods were utilised to produce useful amounts of the compound and it went into clinical usage in the mid-1940s; since then it has saved countless lives. In order to produce penicillin on a scale large enough for the antibiotic to go into wide scale use a large amount of research was conducted to optimise the process. One of the first improvements was the movement from *Penicillium notatum* (the strain from which the first penicillin compound was isolated) to *Penicillium chrysogenum*, which gave a much higher yield of bio-active compound.[13] Further improvements in the fermentation procedure have seen the introduction of phenylacetic acid to the broth medium which gives a significant increase in the yield of penicillin.[14] Precise fermentation conditions have gradually been optimised so that fermenters with a capacity of 100 000 to 300 000 gallons can now be employed to produce 40-50 grams of penicillin per litre of broth every 5-8 days. The maintenance of pH and temperature and the rates of aeration and agitation are all factors which influence the potential yield; the ideal conditions generally employed are a pH of 6.0 with the temperature maintained at 25 ^{o}C.[15] These improvements in manufacturing technology have increased the efficiency of penicillin production from 70 % to more than 90 % in 50 years and have decreased production costs from ~$350 per kilogram in 1950 to ~$15 per kilogram in 2000.[16]

Naturally occurring β-lactam antibiotics such as Penicillin F extracted from *Penicillium notatum* (Scheme 4f) and Penicillin G extracted from *Penicillium chrysogenum* (Scheme 4g) tend to have poor oral availability and can have low activity or a narrow spectrum of action. The first full synthesis of a penicillin was carried out by Sheehan in 1957.[17] One of the intermediates formed during this process was 6-aminopenicillanic acid (6-APA; Scheme 4e), the core structure of all penicillins. By adding different R groups to the amine side chain of this compound it is relatively simple to synthesise new compounds with the potential for antibiotic action.

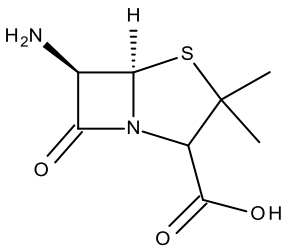


Scheme 4 Structures of some β-lactam antibiotics specifically referred to in the text: a=Ticarcillin, b=Amoxicillin, c=Piperacillin, d=Ampicillin, e=6-Aminopenicillanic acid, f=Penicillin F (2-pentenylpenicillin), g=Penicillin G (benzylpenicillin), h=Cephaloridine, i=Cephalexin and j=Cefuroxime.

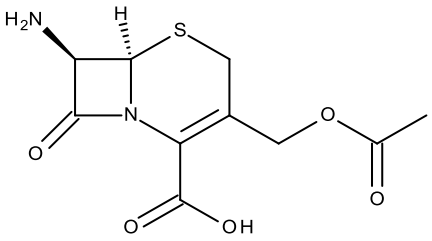
One of the first major breakthroughs in this area came in 1961 when Doyle, Nayler and Smith successfully used this technique to synthesise ampicillin, (6[D(-)-α-aminophenylacetamido]penicillanic acid, Scheme 4d) a compound which was later shown to have broad spectrum activity against both Gram positive and Gram negative bacteria.[18] One of the main advantages of this compound over previous penicillins was its oral bioavailability, a property many penicillins lack due to the acid catalysed hydrolysis of the β-lactam ring in the stomach.

Since then numerous new compounds have been synthesised and tested for antibacterial action. Some notable successes are the development of carbenicillin in 1967, amoxicillin in 1972 (Scheme 4b), piperacillin in 1978 (Scheme 4c) and ticarcillin in 1973 (Scheme 4a).[19-22] Changing the side chains on the penicillin ring can give a number of advantages: increased stability to hydrolysis, a broader spectrum of activity, increased bioavailability, increased activity and increased resistance to inactivation by β-lactamase enzymes.

The second family of naturally occurring β-lactam antibiotics is the cephalosporins. The first of these to be reported was Cephalosporin C from *Cephalosporium acremonium*, a fungus which also produces Penicillin N.[23] From this compound the cephalosporin nucleus, 7-aminocephalosporanic acid (7-ACA), was isolated and shown to be analogous to the penicillin nucleus 6-APA (Scheme 5). The main differences between the two are the replacement of the 6-APA thiazolidine ring with a modified dihydrothiazine ring in 7-ACA which also has a substituted methyl acetate group at the 3 position on the thiazine ring. As with 6-APA, modification of the 7-ACA amine side chain and methyl acetate group has resulted in the production of a number of useful antibiotic agents; the first was cephalothin in 1964[24] and other notable compounds are cephaloridine (1964)[25], cephalexin (1967)[26] and cefuroxime (1976)[27] (Scheme 4h, i and j respectively).



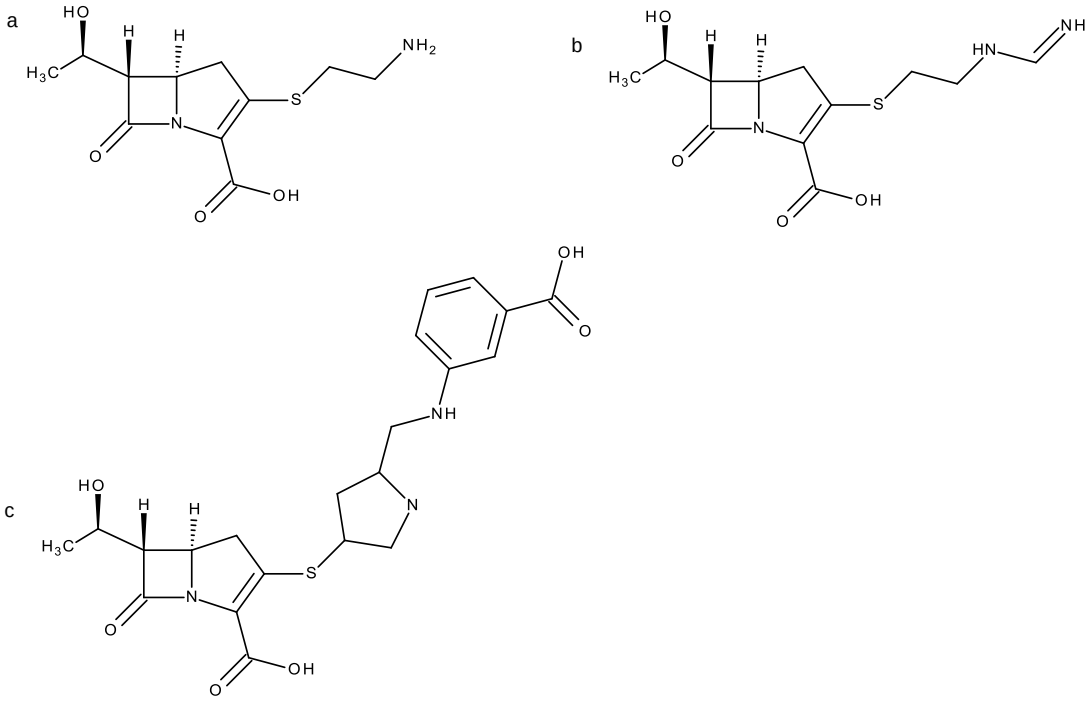
6-APA



7-ACA

Scheme 5 Core structures of penicillins and cephalosporins

Carbapenems are yet another group of β-lactam antibiotics based on natural products. The first carbapenem, thienamycin (Scheme 6a), was isolated from *Streptomyces cattleya* in 1978.[28,29] Other compounds based on the structure of thienamycin have since been synthesised and shown to have excellent antibacterial activity, particularly against a number of bacteria resistant to penicillins and cephalosporins. Imipenem (Scheme 6b) was the first synthetic carbapenem and was initially synthesised by Kropp *et al* and reported in 1985.[30] In the 1990s both meropenem[31] and panipenem[32] were developed and these were followed by the introduction of ertapenem (Scheme 6c),[33] biapenem[34] and doripenem[35] during the first part of the 21st Century.



Scheme 6 Structures of some carbapenems mentioned in the text
(a = Thienamycin, b = Imipenem, c = Ertapenem)

1.2.3 Antibiotic Resistance

Staphylococcus aureus are bacteria found naturally on the skin and in the respiratory tract and are usually harmless. In certain circumstances however, *Staphylococci* can cause skin infections such as impetigo, respiratory infections, pneumonia and meningitis. The development of penicillin had initially meant that these infections could be treated effectively, but in 1944 a sample of *Staphylococcus aureus* was discovered which was not killed by penicillin.[36] This was the first instance of clinically relevant antibiotic resistance and occurred only months after penicillin went into general use. The first methicillin resistant *Staphylococcus aureus* (MRSA) was detected in Britain in the 1960s and the strain's resistance to drugs has continued to evolve; it now shows resistance to a range of penicillins and cephalosporins as well as non-β-lactam antibiotics such as glycopeptides. Various different classes of antibiotics have been introduced over the past 100 years and for most of these some bacterial resistance mechanisms have now evolved (Figure 3).

There are four main mechanisms of antibiotic resistance present in bacteria. The best known is antibiotic modification whereby the target enzyme remains sensitive to the antibiotic but the bacterium produces another enzyme which can inactivate it prior to reaching the target. Growing numbers of bacteria demonstrate this ability, particularly via the production of β-lactamase enzymes.[37]

A second mechanism conferring resistance is the modification of the target site. Structural changes in the target molecule prevent the drug from interacting with it whilst it still maintains the ability to carry out its primary function. Resistance to cephalosporins by some species has been demonstrated by this mechanism via alterations to the structure of their transpeptidase enzymes (often referred to as penicillin binding proteins).[38]

Some bacteria have demonstrated resistance via alteration of the metabolic pathway. Sulfonamides are drugs which work by inhibiting dihydropteroate synthase, an enzyme involved in the synthesis of folic acid. Resistance to these drugs has been developed by some bacteria which have evolved the ability to take up folic acid from the environment, by-passing the need to synthesise it themselves.[39]

The last of the four main methods of antibiotic resistance is the ability of the bacteria to reduce accumulation of the drug within the cell. This may occur by the reduction

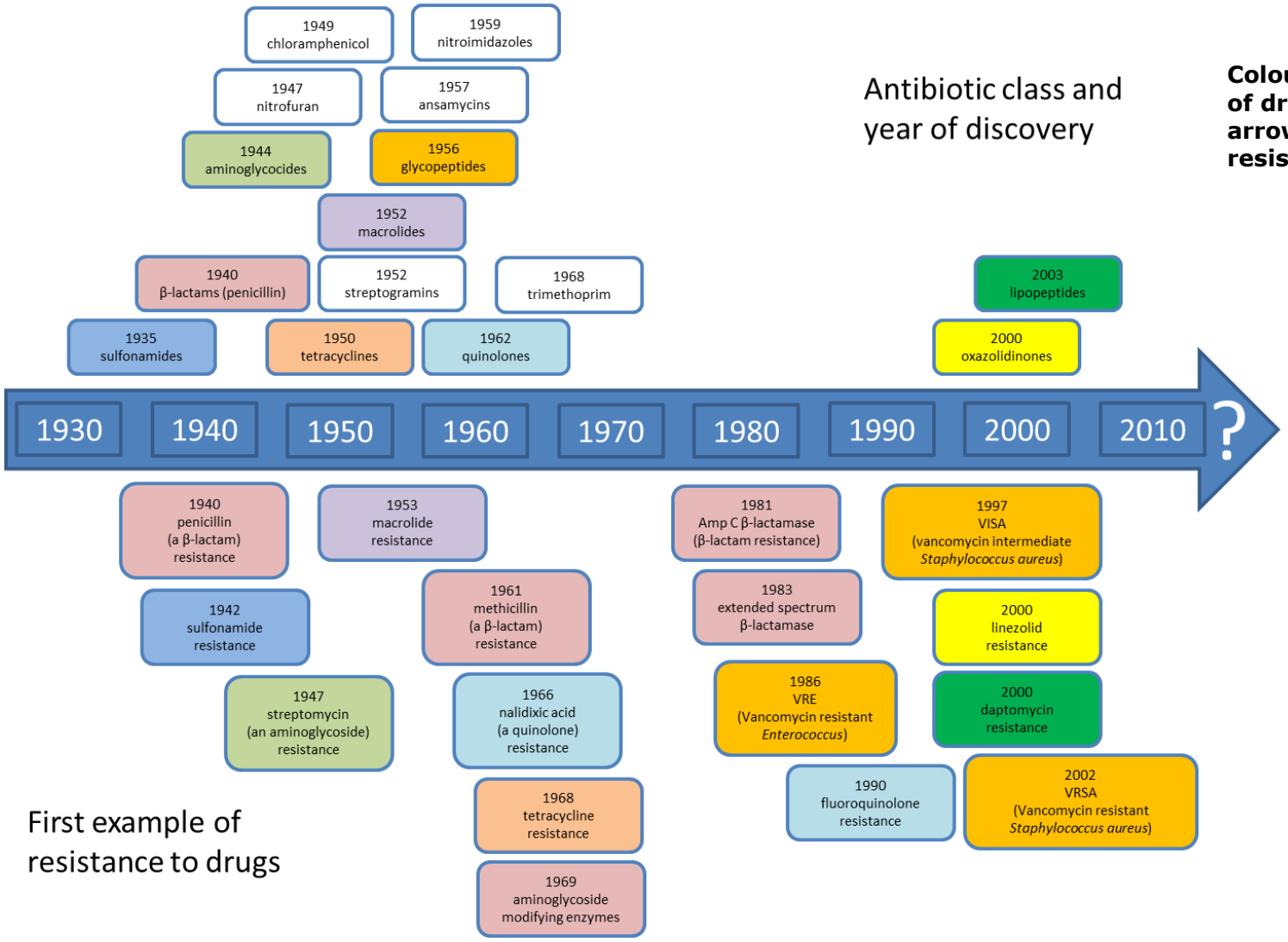


Figure 3
Colour coded diagram of drug discovery above arrow and drug resistance below arrow

Discovery references: Sulfonamides,[40] penicillins,[12] nitrofuran,[41] chloramphenicol,[42] tetracyclines,[43] streptogramins,[44] glycopeptides,[45] ansamycins,[46] nitroimidazoles,[47] quinolones,[48] trimethoprim,[49] oxazolidinones,[50] lipopeptides[51].
Resistance references: Penicillin,[52] sulfonamides,[53] streptomycin,[54] macrolide,[55] methicillin,[56] quinolones,[57] tetracycline,[58] aminoglycoside,[59] vancomycin,[60] fluoroquinolones,[61] linezolid.[62]

of cytoplasmic membrane permeability to the drug, preventing it entering the cell, or by the enhanced expression of efflux pumps which actively remove drug molecules from the cytoplasm, a method of resistance demonstrated in bacteria resistant to fluoroquinolones.[63]

The main mode of β-lactam antibiotic resistance in bacteria is via the production of a β-lactamase enzyme, a modified transpeptidase enzyme which interacts reversibly with the drug. The hydrolysis of the β-lactam ring renders the antibiotic ineffective. Some bacteria are resistant to only selected penicillins but recently strains with resistance to multiple drugs have been on the rise. The primary reason for the spread of bacterial antibiotic resistance is horizontal gene transfer. There are three main methods of horizontal gene transfer (see Table 1) all allowing antibiotic resistance genes (along with other genetic material) to be transferred from one bacterial species to another. Whilst vertical gene transfer (the passing of genetic information from parents to offspring during traditional reproduction) is the focus of the majority of genetics work regarding genetic mutations in mammals and plants, the transfer of genes by horizontal transfer methods is an extremely common form of genetic transfer in bacteria.[64]

Mode of Transfer	Type of resistance	Description
Vertical	Pre-existing	A small number of cells contain mutant DNA which confers resistance. Antibiotics kill sensitive bacteria within the colony leaving the resistant cells to multiply unhindered.
Vertical	Mutational	The introduction of a drug to a bacterial colony forces bacteria to mutate and adapt leading to drug tolerance or dependence.
Horizontal	Transmission	A bacterium can acquire genetic material which confers resistance in three ways. Transformation – the genetic information moves between cellular DNA. Transduction – the genetic information is transferred by a virus. Conjugation – a "tunnel" is formed between cells through which genetic information may pass (see Figure 4).

Table 1 Details of the modes of antibiotic resistance in bacteria

Pre-existing and mutational resistance were most probably responsible for the majority of cases of antibiotic resistance discovered in the first half of the twentieth century and, whilst posing some hazards to health at the time the constant stream of new antibiotic discoveries was able to keep infection outbreaks under control. Transmission resistance, however, is a different phenomenon and can pose a much greater health concern. Transduction and conjugation (shown diagrammatically in Figure 4) are particularly worrying due to them enabling antibiotic resistance genes to move not only from cell to cell during reproduction but also between cells, even between those from different species of bacteria.

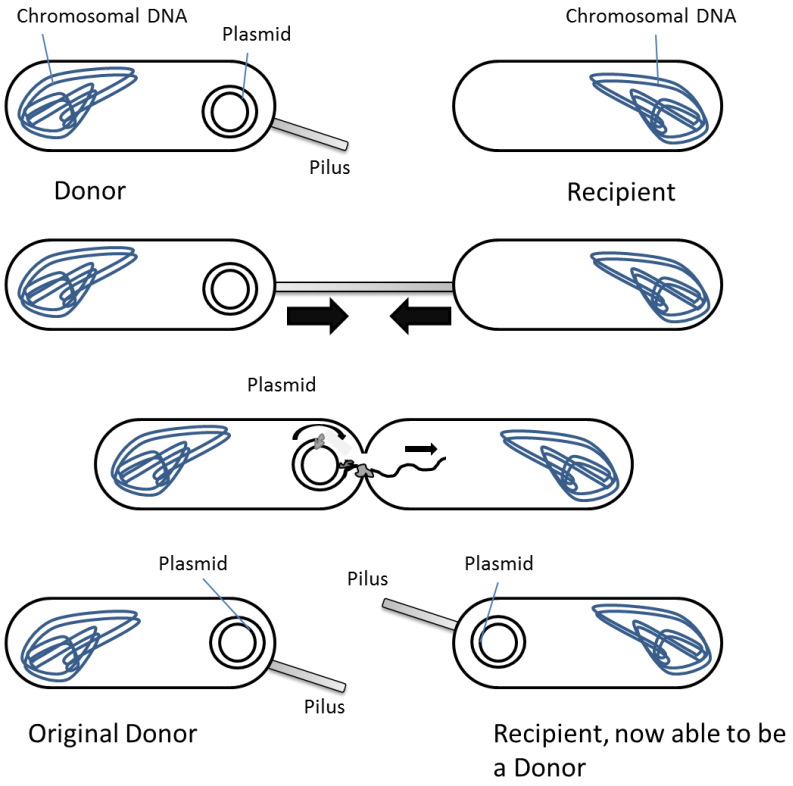


Figure 4 Transfer of genetic material between bacteria via conjugation: 1- Donor cell produces pilus. 2- Pilus attaches to recipient cell, brings the two cells together. 3- The mobile plasmid is nicked and a single strand of DNA is then transferred to the recipient cell. 4- Both cells reform their plasmids, synthesise second strands, and reproduce pili; both cells are now viable donors.

One of the most recent cases of horizontal transfer of resistance genes is that of the New Delhi metallo-β-lactamase, NDM-1, first discovered in 2008.[65] The first case of an infection by a bacteria producing NDM-1 was found in Sweden in a man who had originally contracted the infection in India. This was one of the first metallo-enzymes

shown to confer resistance to carbapenems, though some serine-carbapenemases had previously been described. Since that first discovery the enzyme has been detected in over fifteen countries around the world, from India and Sweden to the USA, UK and Japan among others.[66]

The first bacteria found to produce NDM-1 was *Klebsiella pneumoniae* but since then horizontal transfer has led to the enzyme being isolated from various other bacteria including some *Escherichia coli*, *Enterobacter cloacae* and *Salmonella enterica* strains. This rapid spread of antibiotic resistance across countries and bacterial strains is of real worldwide concern.

1.3 β-Lactamase Enzymes

The first "penicillinase" enzyme was reported in a paper in Nature by Abraham and Chain in 1940, three years before penicillin itself was in clinical use.[52] At the time it was not seen as a medical problem since it was discovered in *Bacillus (Escherichia) coli* whereas penicillin was at the time used exclusively for *Staphylococcus* infections. Kirby isolated the first β-lactamase enzyme (or penicillin inactivator) from a colony of *Staphylococcus aureus* in 1944 and there are now more than 850 distinct β-lactamase enzymes known to be produced by a wide range of both pathogenic and non-pathogenic bacteria.[36,67]

In order to more easily understand and study the ever-growing number of β-lactamase enzymes they are classified according to either their specific amino acid sequence or their affinities regarding substrates and inhibitors. The Ambler classes, A to D, classify the enzymes according to their protein structure and the amino acid sequence at the active site.[68] Classes A, C and D all have serine amino acid residues at the active site whereas class B are metallo-enzymes with one or two zinc-ions at the active site. A more recent classification (Bush-Jacoby-Medeiros classes) was established in 1995 as a way of classifying the enzymes according to their functionality.[3] This classification scheme again separates the enzymes into four main classes, 1 to 4, but this time according to their preferred substrates (penicillin, cephalosporin, carbapenem etc.) and inhibitors. A simplified description of the two classification schemes and the correlations between them can be found in Table 2.

Whilst the two classification schemes may differ in their distribution of many of the serine-β-lactamase enzymes, Ambler Class B and Bush-Jacobi-Medeiros Class 3 both contain all of the metallo-β-lactamases. For reasons of clarity and simplicity only the Ambler Classes will be referred to for the remainder of this thesis.

When Ambler originally proposed the first classification scheme for the β-lactamase enzymes they were separated into two main groups, A and B, dependent on their mechanism of action.

Ambler Class	Bush-Jacoby-Medeiros Class	Typical Substrates	Known Inhibitors	Representative Enzymes
A	2a	Penicillins		Penicillinases from Gram positive bacteria
	2b	Penicillins, Cephalosporins	Clavulanic acid	TEM-1, TEM-2, SHV-1
	2be	Penicillins, Cephalosporins, Monobactams	Clavulanic acid	TEM-3 to TEM-26, SHV-2 to SHV-6, K1 from *Klebsiella oxytoca*
	2br	Penicillins	Clavulanic acid	TEM-30 to TEM-36, TRC-1
	2c	Penicillins, Carbenicillin		PSE-1, PSE-3, PSE-4
	2e	Cephalosporins	Clavulanic acid	Inducible Cephalosporinases from *Proteus vulgaris*
	2f	Penicillins, Cephalosporins, Carbapenems	Clavulanic acid	NMC-A from *Enterobacter cloacae*, Sme-1 from *Seratia marcescens*
B	3	Most β-lactams including carbapenems	EDTA	L1 from *Xanthomonal maltophilia*, CcrA from *Bacteroides fragilis*, BcII from *Bacillus cereus*
C	1	Cephalosporins		AmpC from Gram negative bacteria, P99 from *Enterobacter cloacae,* MIR-1
D	2d	Penicillins, Cloxacillin		OXA-1 to OXA-11, PSE-2
(not assigned)	4	Penicillins		Penicillinase from *Pseudomonas cepacia*

Table 2 Comparison of the two classification systems for β-lactamase enzymes including typical substrates and representative enzymes (adapted from Bush, Jacoby and Medeiros [3])

Class A β-lactamases have a serine amino acid residue at the active site. In general they all hydrolyse penicillins, though a few are capable of hydrolysing cephalosporins, carbapenems and monobactams as well. The majority of enzymes in this class are inhibited by clavulanic acid, sulbactam and tazobactam (structures

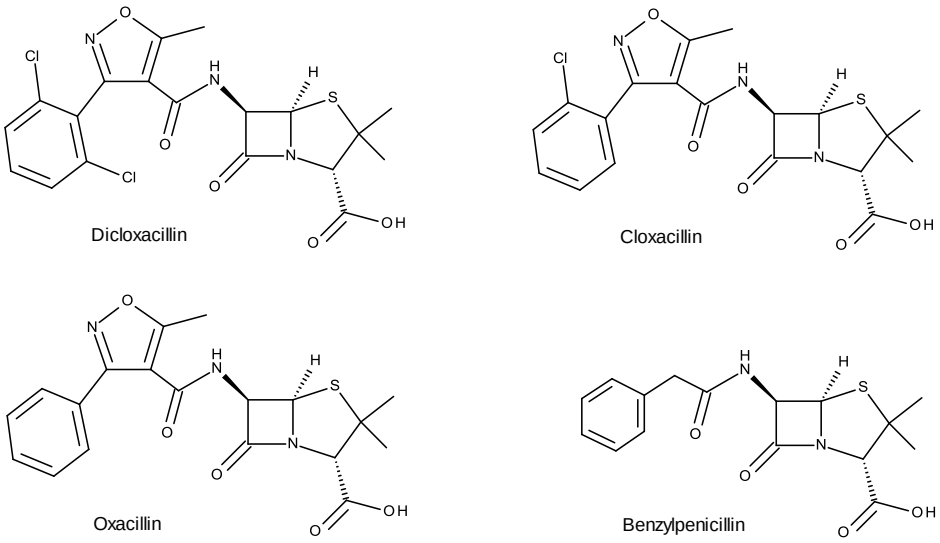
shown in Scheme 12, page 36), three clinically active β-lactam based inhibitors. The Class A β-lactamases are the most common source of resistance to β-lactam antibiotics and can be secreted by pathogenic bacteria such as *Klebsiella pneumoniae*, *Escherichia coli* and *Staphylococcus aureus* as well as non-pathogenic bacteria such as *Bacillus cereus*.

Class B β-lactamases are dependent on divalent metal ions, usually zinc, for their activity. They have a broader spectrum of activity than the serine enzymes and effectively hydrolyse most β-lactams including penicillins, cephalosporins, monobactams and carbapenems. They are not inhibited by any of the β-lactam based inhibitors of Class A enzymes and the most effective inhibitors of Class B enzymes (chelating agents such as EDTA) are not suitable for use in vivo.

The classification scheme has now been extended; Class C β-lactamases (sometimes referred to as cephalosporinases) were added to the Ambler classification system in 1981.[69] Although they are serine enzymes like Class A and are structurally similar to them they have noticeably different amino acid sequences and are more efficient at hydrolysing cephalosporins than penicillins. The best known enzyme in this class is AmpC produced by Gram negative bacteria from the *Legionella* species and *Escherichia coli* and Gram positive *Mycobacteria*. The production of this enzyme is encoded on a gene which occurs on transmissible plasmids, transferred to other bacteria by conjugation, and so there is a possibility of the enzyme being produced by any species of bacteria.[68]

The final group of enzymes in the Ambler classification system are the Class D enzymes, with this category being first suggested for the enzyme OXA-1 in 1987.[70] Jacoby *et al*. also suggested the addition of a fourth class to the Ambler system in 1988 when they sequenced a new β-lactamase, PSE-2 (a carbenicillin hydrolysing enzyme first isolated from *Pseudomonas aeruginosa*).[71] Based on the lack of structural similarity between this enzyme and enzymes from the established classes A, B and C they suggested that PSE-2 had a distinct evolutionary origin along with OXA-1 and that a new group of β-lactamases, Ambler Class D, should be established for them and similar enzymes.[71] In contrast to the other classes of β-lactamase, and in particular Class A enzymes which have been known for many years, Class D is a much newer, smaller and slightly overlooked class of β-lactamases. Like Class A and C they are active site serine enzymes but their primary structures are significantly different to those in the other groups. Whilst Class D enzymes do hydrolyse standard penicillins and cephalosporins, they are much more active against substrates from the oxacillin class of β-lactam antibiotics (Scheme 7) and are often given the

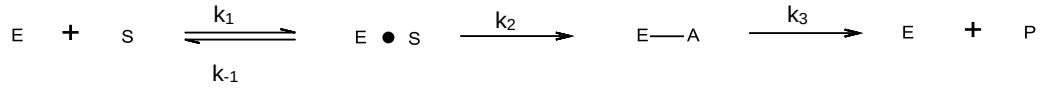
designation OXA. Most serine β-lactamases from Class A and C are inhibited by oxacillins due to the steric bulk of the side-chain so the ability of the OXA enzyme family to hydrolyse these compounds is a growing clinical concern. The other major family of Class D enzymes are the Pseudomonas specific enzymes, designated PSE, now known to be produced by *Enterobacteriaceae* as well as *Pseudomonas aeruginosa*.[72]



Scheme 7 Structures of oxacillin type β-lactam antibiotics (and benzylpenicillin for comparison)

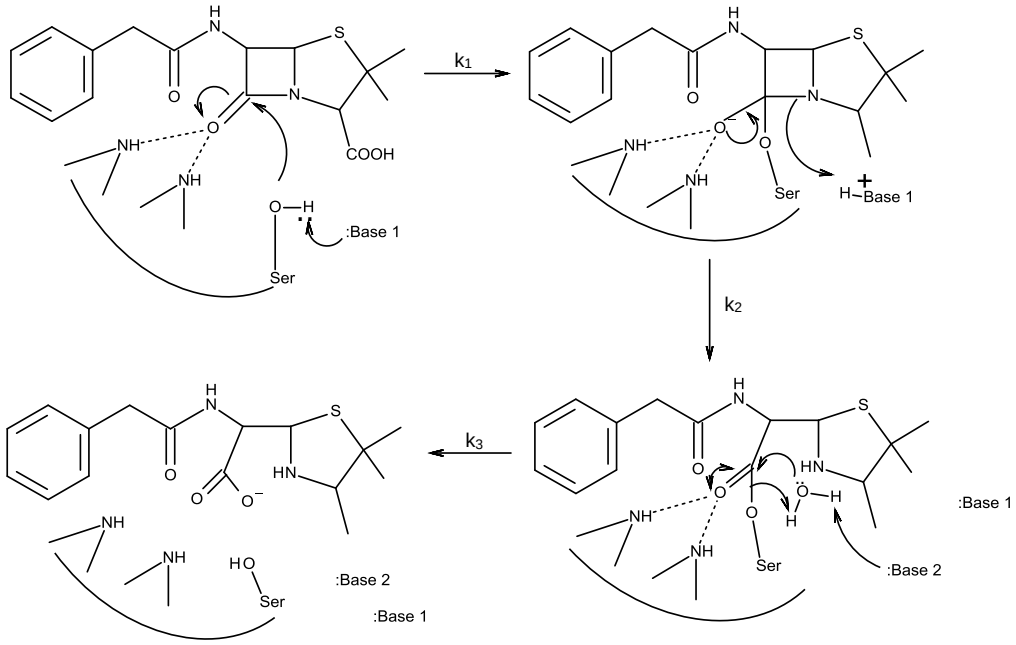
1.4 Active Site Serine β-Lactamases; Classes A, C and D

The active site serine enzymes act on β-lactam antibiotics in a similar fashion to the DD-transpeptidase enzymes; in fact they are believed to have a similar evolutionary history and both reactions can be represented by a similar schematic (Scheme 8).[73] Initial attack on the β-lactam carbonyl by the serine residue forms an acyl-enzyme intermediate. With transpeptidase enzymes this intermediate is stable and is the basis of the antibiotic action shown by β-lactam compounds. With β-lactamase enzymes however, hydrolysis can occur to release the enzyme and leave behind the inactivated antibiotic molecule (Scheme 9).



Scheme 8: Schematic representation of mechanism for DD-transpeptidase and β-lactamase enzymes with antibiotic compounds

In the case of transpeptidases k_3 is very small and so the enzyme is trapped as the acyl-enzyme intermediate. For β-lactamases, on the other hand, k_2 and k_3 are both large and so there is rapid hydrolysis of the intermediate and regeneration of the enzyme.

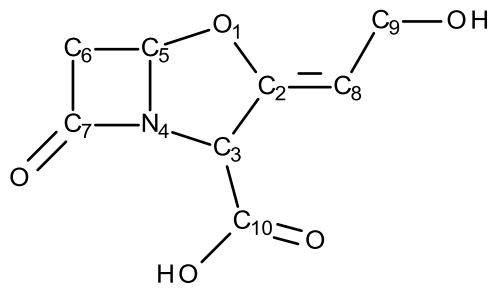


Scheme 9 Mechanism of benzylpenicillin hydrolysis by serine β-lactamases [74-76]

There are two main mechanisms for inhibition of serine-β-lactamases: mechanism based and transition state analogues. Mechanism based or suicide inhibition occurs when a substrate analogue binds to the enzyme active site but, due to modifications to the structure, an irreversible complex is formed often via reactions between the enzyme and inhibitor involving carbonyl groups or imines. Transition state analogue inhibitors work by mimicking the transition state of the normal substrate. Since enzymes work by stabilising high energy transition state intermediates, transition state mimics which do not undergo the normal reaction bind to the active site and so block it. Mechanism based inhibitors for serine-β-lactamases include clavulanic acid (administered with amoxicillin, marketed as Augmentin), sulbactam (administered with ampicillin, marketed as Unasyn) and tazobactam (administered with piperacillin, marketed as Zozyn) (see Scheme 4, page 20 for structural details of the penicillins and Scheme 12, page 36 for the structures of the inhibitor compounds).

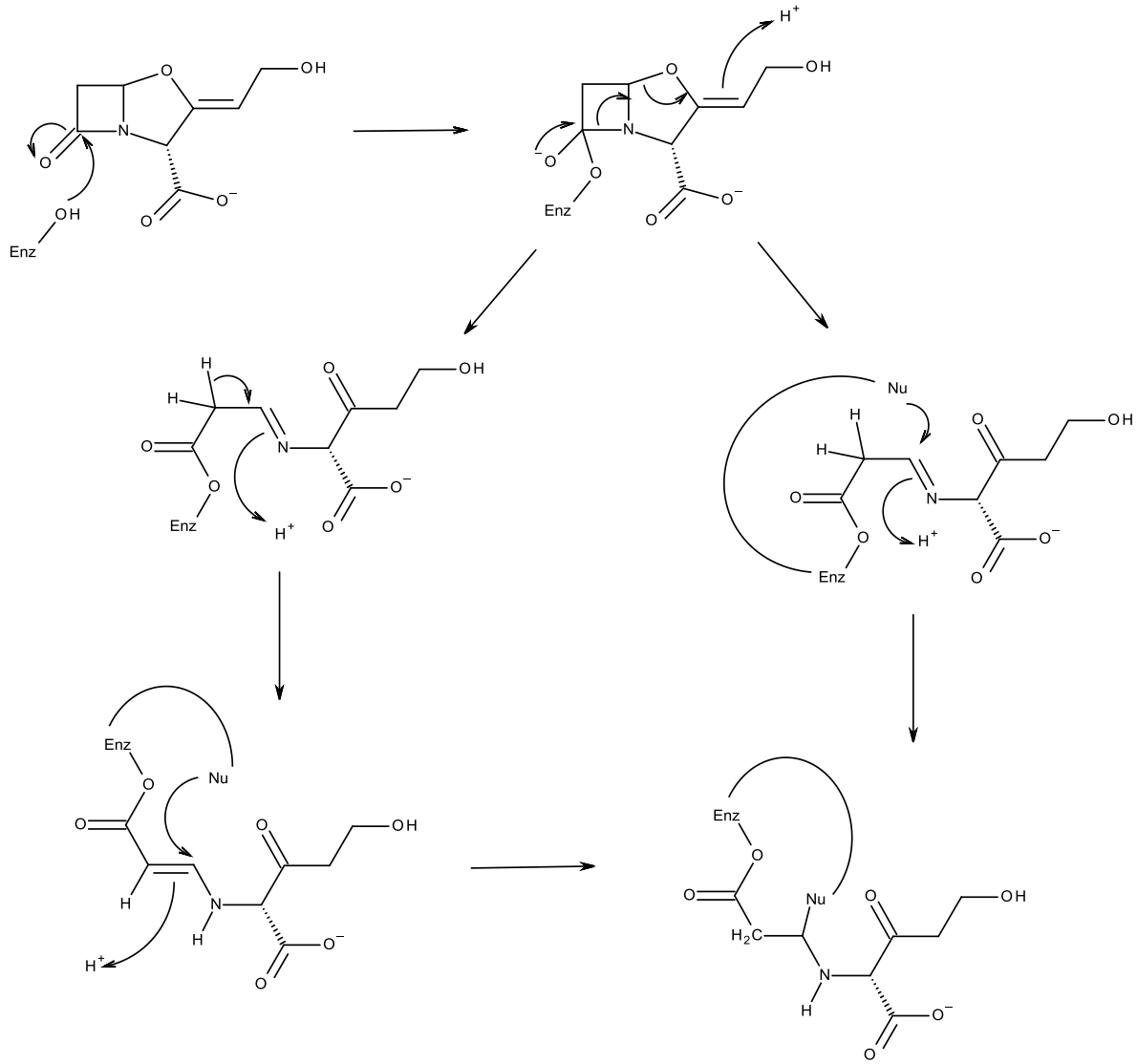
The best known and probably most widely used mechanism based inhibitor is clavulanic acid (Scheme 10).[77] Clavulanic acid was first isolated from *Streptomyces clavuligerus* by a research group at Beecham Pharmaceuticals in the mid-1970s.[78] At the time its fused β-lactam structure was different to all known β-lactams in that it has an O-containing oxazolidine ring fused to the β-lactam ring instead of the S-containing thiazolidine ring and it does not possess the acyl-amino side chain

found in penicillins and cephalosporins.[77] The group carried out extensive investigations on the compound and showed that, whilst it had little antibacterial activity on its own (only weak inhibition of DD-transpeptidase enzymes is shown in most cases), it was a potent inhibitor of the majority of β-lactamases tested from both Gram positive and Gram negative bacteria. Interestingly cephalosporinase type enzymes were poorly inhibited as were the *Bacillus cereus* enzymes BcI and BcII.[79] Whilst clavulanic acid shows very little antibacterial action on its own its ability to inhibit β-lactamase enzymes from Class A and C increases the activity of penicillin drugs (usually amoxicillin or ticarcillin, structures shown in Scheme 4, page 20) against β-lactam resistant bacterial infections when they are prescribed in a formulation together.



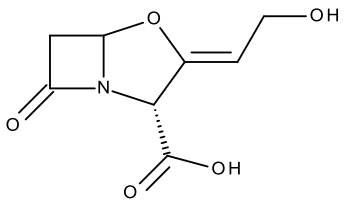
Scheme 10 Clavulanic acid (with numbered atoms)

Numerous mechanisms have been put forward for the inactivation of β-lactamase enzymes by clavulanic acid[80-82] with the two most credible being illustrated in Scheme 11.[83,84] Initially both mechanisms follow the same pathway. As a mechanism based inhibitor clavulanic acid is recognised by the enzyme and initially turned over like any other substrate. A serine residue forms an acyl-enzyme intermediate at C7 and the carbon-nitrogen bond is cleaved. The oxazolidine ring is then opened to form a keto-imine. At this point there are two main proposals for the mechanism. The first suggests protonation of the nitrogen and loss of hydrogen at C6 to form an enamine which is then attacked by a second nucleophilic serine residue to form the irreversibly inactivated complex. In the second proposal attack by a nucleophilic amino acid residue at C5 leads to the alkylation at the active site which again prevents further activity by the enzyme.[81] The inactivation of the β-lactamase enzyme by clavulanic acid allows the coadministered amoxicillin to bind to the DD-transpeptidase enzyme in the normal way, inactivating it and leading to cell death.

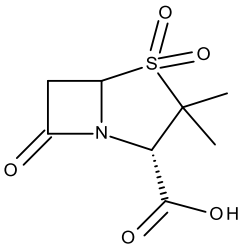


Scheme 11 Possible mechanisms for inhibition of serine-β-lactamase by clavulanic acid

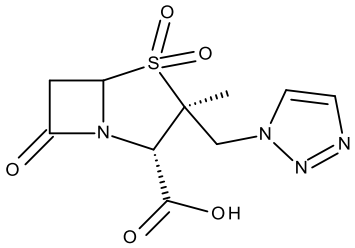
Sulbactam and tazobactam are two other clinically approved serine-enzyme inhibitors which both contain a β-lactam ring and act in a similar manner (structures shown in Scheme 12). They are also administered as co-drugs with either a penicillin or cephalosporin antibiotic.



Clavulanic acid



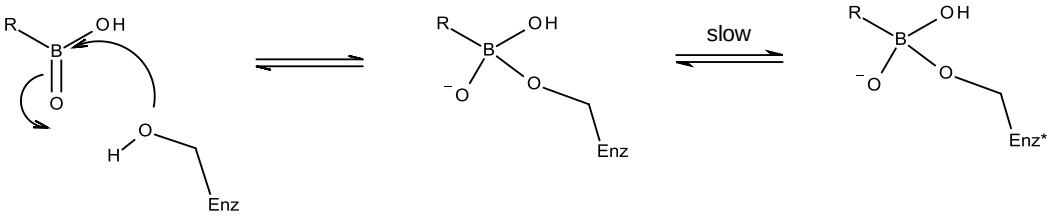
Sulbactam



Tazobactam

Scheme 12 Selected serine-β-lactamase inhibitor structures

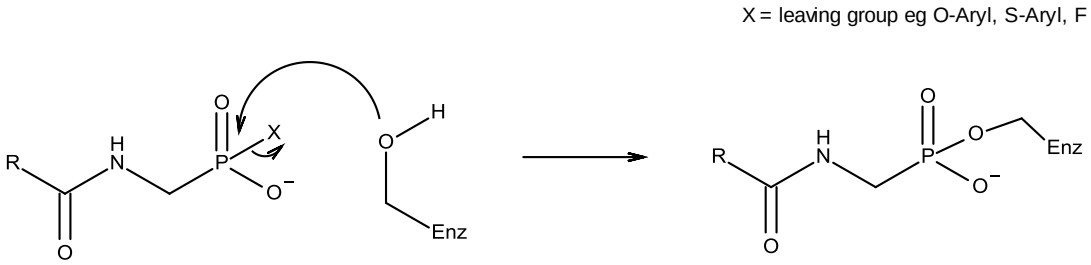
The second group of serine-enzyme inhibitors are the transition state analogues. A wide range of structurally diverse boronic acids have been shown to act as reversible inhibitors of serine-β-lactamases.[85-88] The reaction (overview in Scheme 13) involves two separate binding steps with the slower of the two leading to inhibition. This slow step involves a change in the protein conformation represented mechanistically as Enz*.[82]



Scheme 13 Mechanism of β-lactamase inhibition by boronic acids

Phosphonic acid derivatives are another family of compounds which have proven to be useful as serine-β-lactamase inhibitors.[89] This time the reaction involves phosphonylation of the enzyme and this phosphonylated enzyme has a tetrahedral conformation. Although the mechanisms vary in detail between Class A and Class C

enzymes both classes are inhibited by the compounds and an overview of the mechanism is shown in Scheme 14.[90-92]



Scheme 14 Mechanism of β-lactamase inhibition by phosphonic acid derivatives

1.5 Metallo-β-Lactamases; Class B

Class B β-lactamases are metallo-enzymes dependent on the coordination of bivalent metal ions to demonstrate activity. In general cases the preferred metal is zinc but there have been examples of cobalt, cadmium and manganese also conferring activity in a variety of enzymes.[93] The metallo-β-lactamases can be further separated into three subgroups, B1, B2 and B3; first suggested by Rasmussen and Bush in 1997 the enzymes are grouped according to amino acid sequence and substrate profile.[94]

Subclass B1 β-lactamases are all approximately 28 kDa and have three histidine residues participating in zinc and water binding at the active site. The subclass includes BcII from *Bacillus cereus* (Figure 6a); CcrA from *Bacteroides fragilis*;[95] IMP-1 from *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Serratia marscescens*[96] and BlaB from *Chryseobacterium meningsepticum*.[97] This is the largest of the three subgroups and is the best studied with crystal structures and peptide sequences being available for a large number of the enzymes in the group as well as kinetic data, substrate profiles and inhibition studies. All members of subclass B1 require two zinc ions at the active site and they efficiently catalyse the hydrolysis of almost all β-lactam compounds including those which inhibit the serine enzymes e.g. clavulanic acid and tazobactam. In Figure 5 are two images representing the CcrA metallo-β-lactamase enzyme from *Bacteroides fragilis*.[98] The first image shows the ribbon structure of the enzyme with α-helices shown in blue and β-sheets in yellow. The red spheres represent the zinc ions at the active site. In the second image the active site of the enzyme is shown as the molecular surface. A model of a benzylpenicillin molecule is shown in red, bound at the active site. The zinc ions are represented by purple spheres and the water molecules by blue spheres. This image shows the β-lactam ring clearly positioned above the di-zinc bound water molecule.

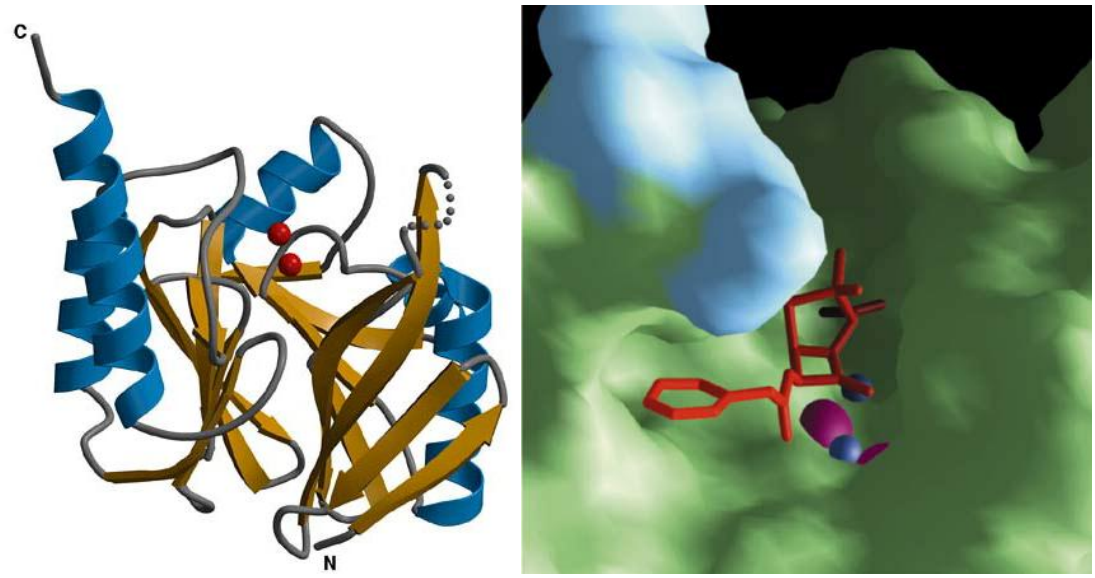
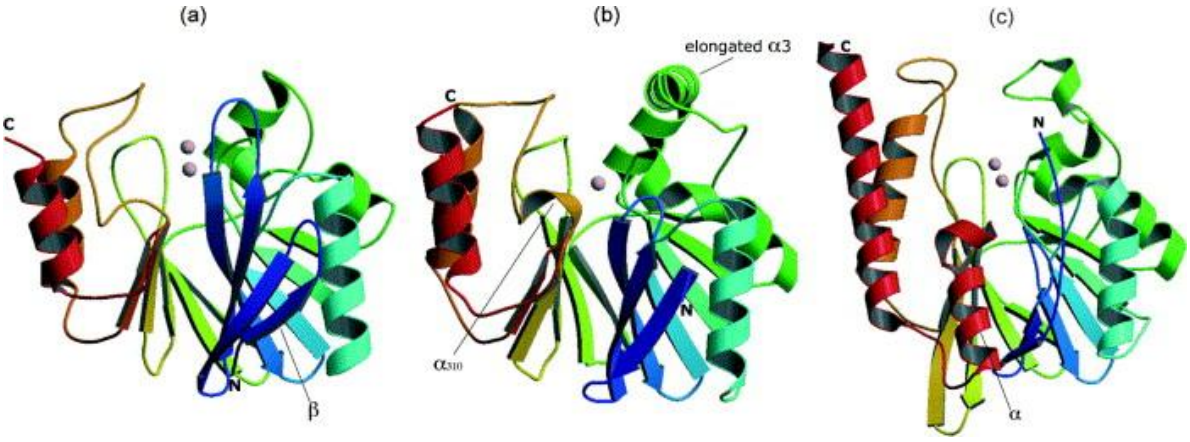


Figure 5 Representations of the metallo-β-lactamase enzyme produced by *Bacteroides fragilis* (as published by Concha *et al*)[98]

Subclass B2 β-lactamases are similar to those from subclass B1 in size and amino acid sequence but unlike the previous group they have a small substrate profile, only being truly active against carbapenems. They require only one zinc ion for activity and in many cases are actually inhibited by the binding of a second zinc. Representative enzymes for this subclass are CphA from *Aeromonas hydrophila*[99] and *Bacteroides fragilis* (Figure 6b) and Sph-I from *Serratia fonticola*.[100]



Subclass B1 Subclass B2 Subclass B3

Figure 6 Representative enzymes from the three subclasses (a) BcII from *Bacillus cereus*, (b) CphA from *Bacteroides fragilis* and (c) FEZ-1 from *Fluoribacter gormanii.*[101]

Subclass B3 is the most distinctive but least studied of the three classes. Whilst it has a similarly broad substrate profile to Class B1 its structure and amino acid sequence vary greatly from both subclasses B1 and B2. Enzymes currently assigned to subclass B3 are FEZ-1 from *Fluoribacter gormanii (*Figure 6*c)*,[102] CAU-1 from *Caulobacter crescentus*[103] and L1 from *Stentrophomonas maltophilia*.[103] Of these L1 was the first to be assigned as a subclass B3 enzyme and, unlike any of the other currently classified metallo-β-lactamases, it has been shown to be tetrameric (Figure 7).

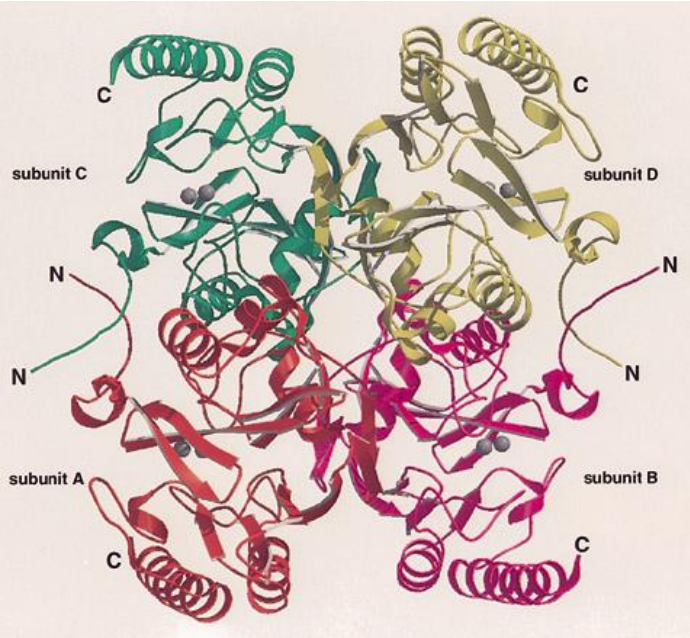
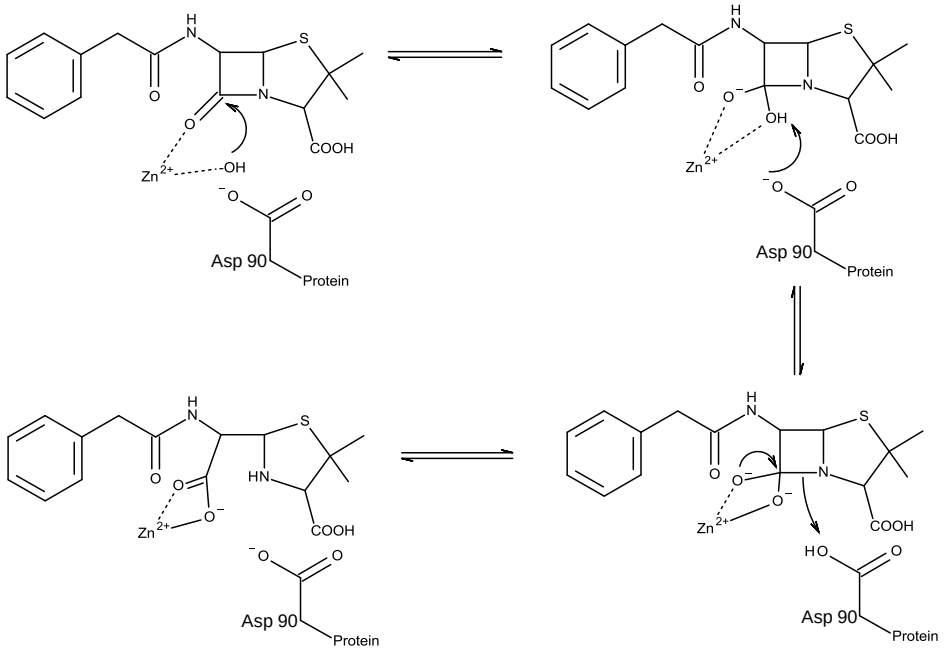


Figure 7 Tetrameric enzyme L1 from *Stentrophomonas maltophilia*[104]

1.5.1 Mechanisms of Metallo-β-Lactamases

The mechanism of action of the Class B β-lactamase enzymes, like their structures, varies greatly from that of the serine-enzymes. As with serine-enzymes the Class B enzymes vary between bacterial strains with regard to their exact amino acid sequence and tertiary structure. Several studies have been carried out to try to understand the mechanism of action of metallo-β-lactamases.[105-108] In 1998 Bounaga *et al* carried out intensive studies of the mono-zinc BcII enzyme and proposed a three-step mechanism for the catalytic hydrolysis of benzylpenicillin in which the key components were a zinc bound water molecule and an aspartic acid residue (Scheme

15).[109] In this proposal the zinc bound water molecule is deprotonated due to the action of the zinc ion acting as a Lewis acid decreasing the pK_a of the bound water. The metal bound hydroxide ion acts as a nucleophile to attack the carbon of the β-lactam carbonyl group to form a tetrahedral intermediate and the zinc acts to stabilise the negative charge formed on the carbonyl oxygen. An aspartic acid carboxylate anion then deprotonates what was the zinc bound hydroxyl to form a dianionic tetrahedral intermediate, again stabilised by the zinc ion. Protonation of the amine leaving group by the aspartic acid as the C-N bond of the β-lactam is broken completes the hydrolysis.[110] Whilst this mono-zinc BcII enzyme did show activity against benzylpenicillin further studies have now shown that the di-zinc enzyme is the one which shows most activity against β-lactam antibiotics.



Scheme 15 Mechanism of benzylpenicillin hydrolysis by mono-zinc BcII[109]

All the metallo-β-lactamases currently reported do have a striking number of similarities, particularly the di-zinc enzymes. In almost all cases Zn1 is coordinated to three histidine residues and a bridging water molecule in a tetrahedral conformation. Zn2 is coordinated to the bridging water molecule and three other amino acid residues with another molecule of water completing the trigonal

bipyrimidal arrangement. These arrangements are shown in Figure 8 where the 4-coordinated Zn1 and 5-coordinated Zn2 in *Bacillus cereus* are clearly visible.

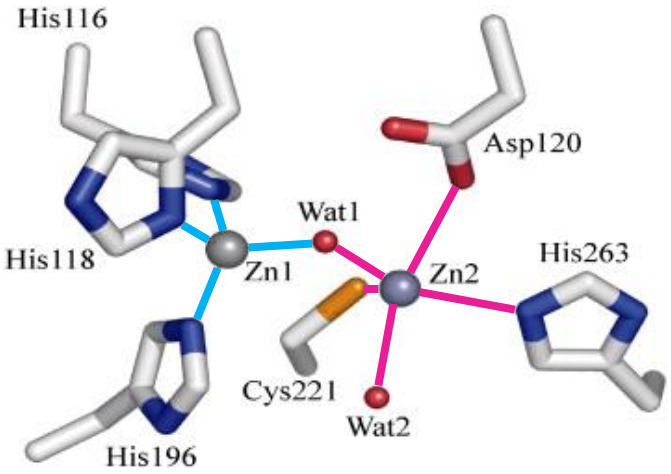
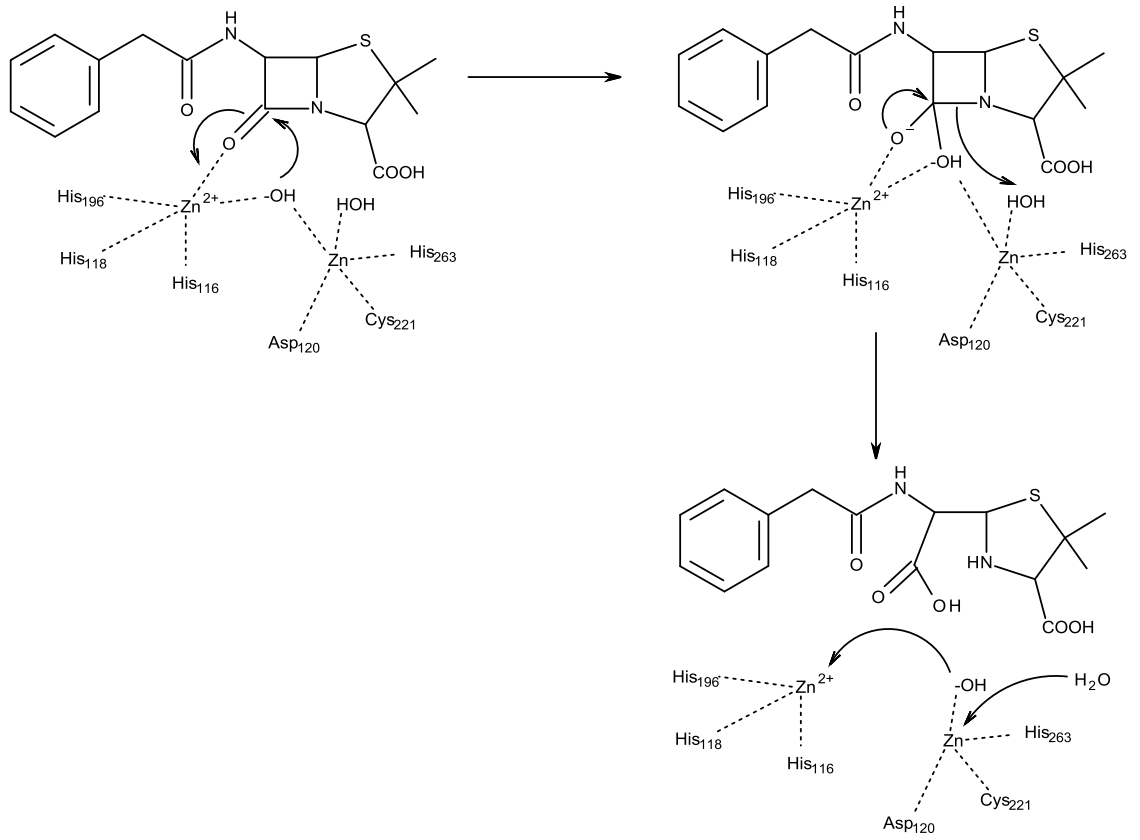


Figure 8 Dinuclear centre of *Bacillus cereus*[111]

More recently studies have shown that although some mono-zinc β-lactamases are catalytically active, for BcII the di-zinc form of the enzyme (as shown in Figure 8) is the only one relevant to β-lactam hydrolysis in vivo.[112] In fact of the three subclasses of metallo-β-lactamase Class B1 (including BcII, BlaB, IMP and VIM) and B3 (L1, FEZ-1, GOB-1 and THIN-B) both show maximum efficiency as di-zinc species but Class B2 (CphA and Sfh-I) enzymes show a reduction in activity upon binding of a second zinc ion.

The mechanism for the di-zinc metallo-β-lactamase begins with nucleophilic attack of the bridging hydroxide ion (Wat 1) on the carbonyl carbon resulting in a negatively charged tetrahedral intermediate. The zinc bound water molecule (Wat 2) can then donate a proton to the leaving nitrogen forming a hydroxide ion which replaces Wat 1 in the now vacant site. The product (hydrolysed β-lactam) can now dissociate from the enzyme active site and, once Wat 2 is replaced from the bulk solution, the enzyme is ready to catalyse hydrolysis of the next substrate molecule (Scheme 16).



Scheme 16 Mechanism of benzylpenicillin hydrolysis by di-zinc BcII[113]

1.5.2 Metallo-β-Lactamase from Bacillus cereus; BcII

The first β-lactamase enzyme which was reported to require metal ions for activity was the BcII enzyme from the bacteria *Bacillus cereus*.[114] There are two strains of the bacteria; *Bacillus cereus* 569/H/9 and *Bacillus cereus* 5/B/6 both of which produce a metallo-β-lactamase enzyme (BcII) and a serine-β-lactamase enzyme (BcI). The two metallo-enzymes are almost identical; differing only by 17 amino acid substitutions, none of which are involved in the enzyme active site.[112] Although the majority of work on BcII has been carried out on enzyme from *Bacillus cereus* 569/H/9 work done on the alternative enzyme and differences in some experimental conditions have meant that characterisation of the structure, the mechanism of action and physical measurements such as k_{cat}, K_M and zinc ion binding constants are still under debate.

BcII is a Class B1 metallo-β-lactamase and was first reported in 1966 by Sabath and Abraham.[114] Although they did not isolate the enzyme they recognised that it was a

separate entity to the previously known BcI serine-enzyme. They showed that the mixture of the two enzymes efficiently catalysed the hydrolysis of penicillin and cephalosporin substrates in the presence of $ZnSO_4$ but in the absence of Zn^{2+} ions (after treatment with EDTA) the "penicillinase" activity was retained whilst the "cephalosporinase" activity was lost.

In 1974 Abraham succeeded in isolating BcI and BcII from *Bacillus cereus* 569/H/9 using chromatography.[115] Along with Davies and Melling he managed to show that the two enzymes were fundamentally different; the two enzymes have different molecular weights (28000 and 22000 Da respectively), very different substrate profiles (Table 3), and different amino acid sequences (and of particular note is the presence of cysteic acid in BcII which is completely absent from BcI).

Enzyme	Substrate	Rate of hydrolysis (moles of substrate/ minute/mole of enzyme)
BcI	Benzylpenicillin	2.100×10^5
BcII	Benzylpenicillin	0.800×10^5
BcII	Cephalosporin C	0.506×10^5

Table 3 Enzymatic activities of BcI and BcII isolated by Davies, Abraham and Melling[115]

This data was very important as an indication of bacterial ability to evolve new resistance strategies with *Bacillus cereus* now able to resist both penicillin and cephalosporin type antibiotics via the production of different enzymes; BcI: a serine-enzyme and BcII: a metallo-enzyme. Although BcII shows activity against both penicillins and cephalosporins it is interesting to note that its penicillin hydrolysis rate, whilst being less than that of the BcI rate, is still greater than the BcII catalysed rate of cephalosporin hydrolysis. Figure 9 shows a representation of BcII with a cephalosporin bound at the active site.

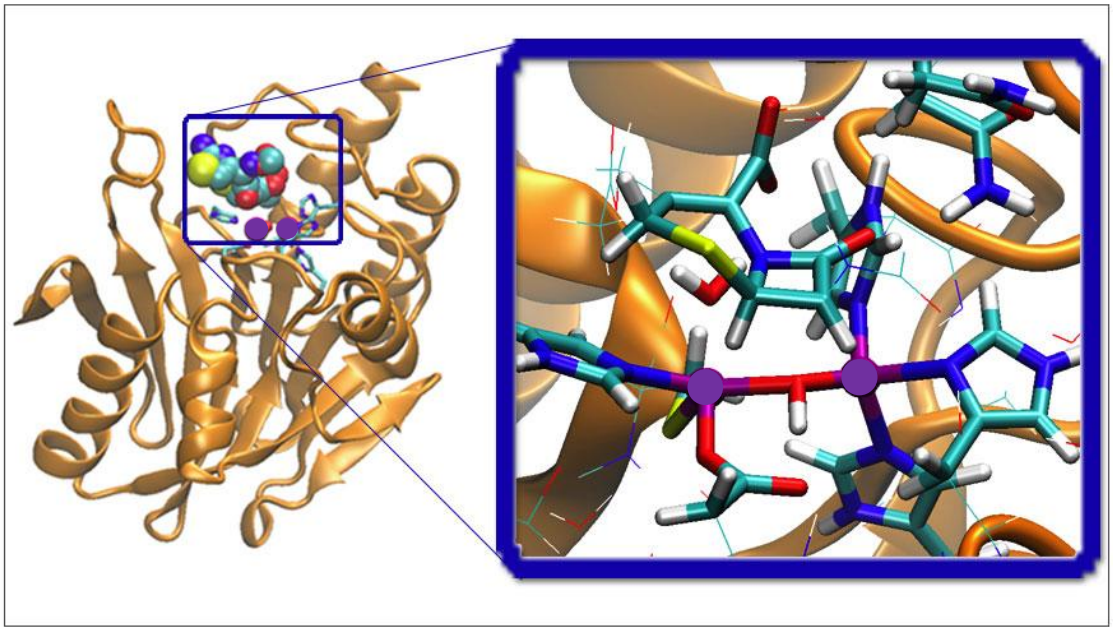


Figure 9 Representation of the BcII enzyme with cephalosporin substrate superimposed[116]

On the left of the image is a simulated representation of the BcII metallo-β-lactamase. The two purple spheres represent the two zinc ions coordinated to various amino acid residues, represented by stick models. The space filled molecule is a representative β-lactam antibiotic.

On the right is an enlarged view of the active site of the enzyme. Again the purple spheres represent the zinc ions and in this view it is possible to see the Wat 1 hydroxide ion bound between them (red oxygen and white hydrogen) and the Wat 2 water molecule (red V with white tips) which is held between the metal and the substrate. Comparison of this model structure with the mechanism in Scheme 16 shows how the arrangements of the water molecule and hydroxide ion are essential to the function of the enzyme. The β-lactam antibiotic (a cephalosporin in this case) is situated above the zinc ions with the β-lactam ring almost directly above the zinc bound hydroxide ion which initiates the mechanism. The Wat 2 water molecule is also held in an ideal position to allow protonation of the nitrogen in the second step. The amino acids involved in interactions with the zinc ions, the water molecules and the antibiotic are shown as stick representations.[116]

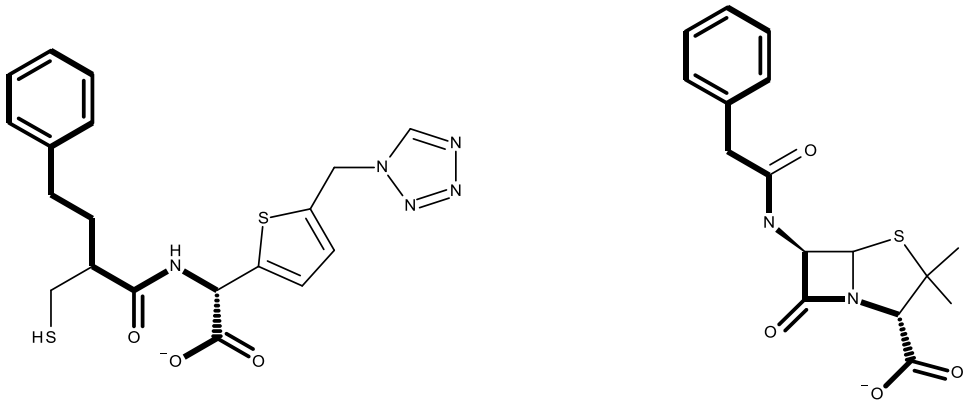
1.6 Inhibition of Class B Metallo-β-Lactamases

Various different classes of compounds have been shown to have an inhibitory effect on the rate of β–lactam hydrolysis by a range of metallo-β-lactamase enzymes. Despite the basic mechanism of catalysis being the same for most of the known metallo-β-lactamases the inhibitors which have been reported show massively different inhibitory abilities against the different enzymes.

A high percentage of the reported inhibitor compounds contain sulfur, as a thiol[117] or thioester[118] for example or mimic the structure of the natural β-lactam substrate e.g. cyclobutanones.[119]

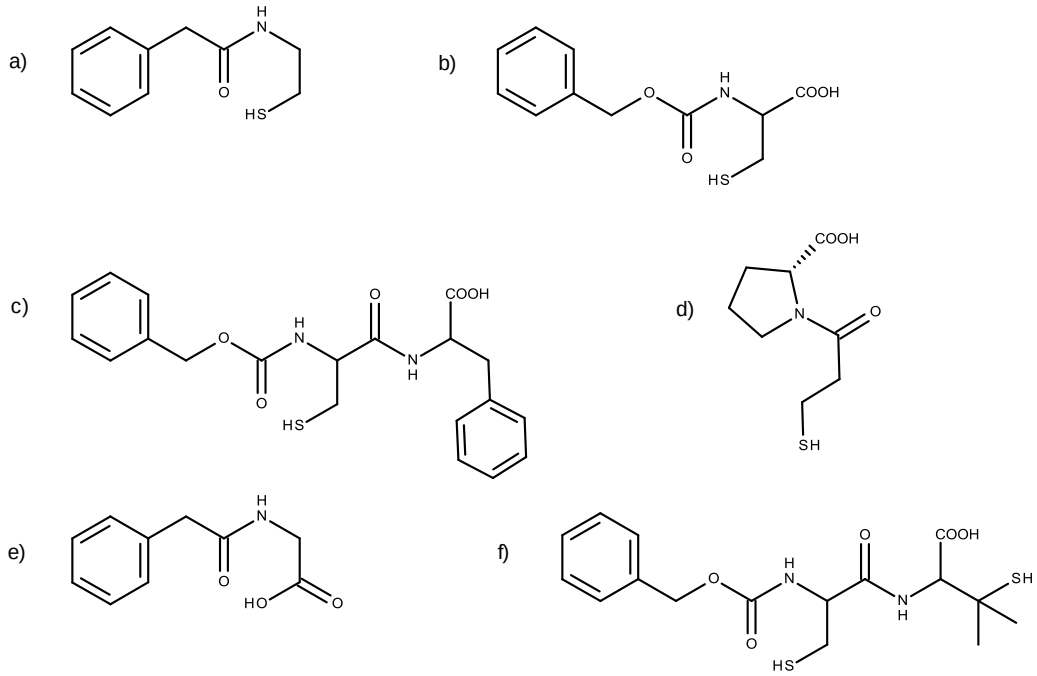
Many zinc dependent enzymes coordinate the metal ion via cysteine residues (though histidine and aspartic acid residues are also commonly found to be zinc binding amino acids) and compounds containing sulfur are known to be effective as zinc dependent enzyme inhibitors due to the mutual affinity of zinc and sulfur.[120] It is therefore a logical step to assume that sulfur containing compounds may well act as effective inhibitors of Class B β-lactamases.

One of the first reports of sulfur containing compounds being used as inhibitors of metallo-β-lactamases was made in 1999 by Nagano *et al*. They synthesised carbapenem analogues with sulfur containing side-chains most of which gave IC_{50} values of less than 10 μM against the IMP-1 enzyme from *Pseudomonas aeruginosa* using nitrocefin as the substrate.[121] The same group published further examples of this family of sulfur compounds the following year[122] when a second family of sulfur containing inhibitors, mercaptocarboxylates, were also reported by a collaborative group from SmithKline Beecham and the University of Liege.[123] Although these compounds (unlike the previous work in this area) did not contain a β-lactam ring they did show structural similarities to benzylpenicillin (Scheme 17). Whilst the kinetics of the inhibition were not specifically explored in this paper it was reported as an inhibitor and the mode of inhibitor binding was investigated using X-ray diffraction.



Scheme 17 Structural similarities between mercaptoacetic acids and benzylpenicillin (in bold)

Another family of sulfur containing inhibitors, the cysteinyl peptides, were first reported by a research group from the University of Huddersfield in 2001. A range of peptides containing a cysteine residue and a number of control compounds were investigated as inhibitors of BcII. The best inhibitors of all the compounds tested were those with a thiol group and a hydrophobic side chain α to the carboxyl terminus of the peptide. The peptide isomers with D-D configuration were the most efficient, with N-carbobenzoxy-D-cysteinyl-D-phenylalanine (Scheme 18c) giving the best K_i of approximately 3.0 μM. Captopril (Scheme 18d), a thiol containing inhibitor of the zinc dependent angiotensin converting enzyme (ACE) was shown to have moderate inhibitory activity (K_i ~ 42 μM) whilst the non-sulfur containing N-phenylacetylglycine (Scheme 18e) showed very poor activity (K_i ~1000 $\pm$ 150 μM). Another interesting result was that for N-carbobenzoxy-D-cysteinyl-D-penicillamine (Scheme 18f) which initially showed inhibitory action; however, this was discovered to be due to the dithiol nature of the compound chelating the zinc. The mono-thiol compounds (Scheme 18a-c) demonstrated their activity by displacing the zinc bound hydroxide ion (Wat 1) from the enzyme active site and therefore showed potential for clinical usage.[124]



Scheme 18 Structures of relevant cysteinyl peptides and analogues

Following on from the work of the SmithKline Beecham / University of Liege collaboration reported in 2000 the Frère group investigated some simple mercaptocarboxylic acids (thiomandelic acids) in 2001 and showed that they inhibited a broad spectrum of metallo-β-lactamases from across the three subclasses (Table 4).

Enzyme	**BcII**	**CfiA (CcrA)**	**L1**	**IMP-1**	**IMP-2**	**VIM-1**	**BlaB**	**FEZ-1**	**CphA**
Subclass	B1	B1	B3	B1	B1	B1	B1	B3	B2
K_i (μM)	0.34	0.80	0.051	0.029	0.059	0.23	0.56	0.27	144

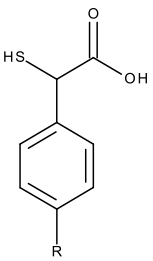


Table 4 Inhibition of a range of metallo-β-lactamases by thiomandelic acid (structure shown)

The data they collected showed that the mode of inhibition was via the thiol group binding to the zinc atoms at the active site whilst the carboxylate group was bound to an arginine residue found in most of these enzymes.[125]

Cyclobutanone mimics of β-lactam antibiotics were reported as modest inhibitors of both serine- and metallo-β-lactamases in 2010 following on from some less successful work in the 1980s.[126,127] Enzymes from all four β-lactamase classes were chosen for testing and the two best compounds (Table 5) showed IC_{50} values of less than 400 μM for each of the tested enzymes. The best inhibition was shown against the Class C enzyme GC1 which had IC_{50} values of less than 10 μM but the activity of the inhibitors against Class B enzymes was also satisfactory with activity against the representative Class B enzyme (IMP-1, a subclass B1 enzyme) having IC_{50} values of ~100-200 μM.[119]

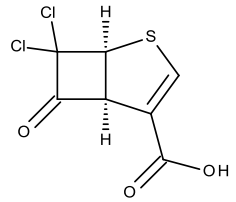
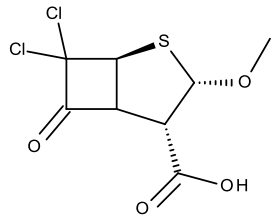
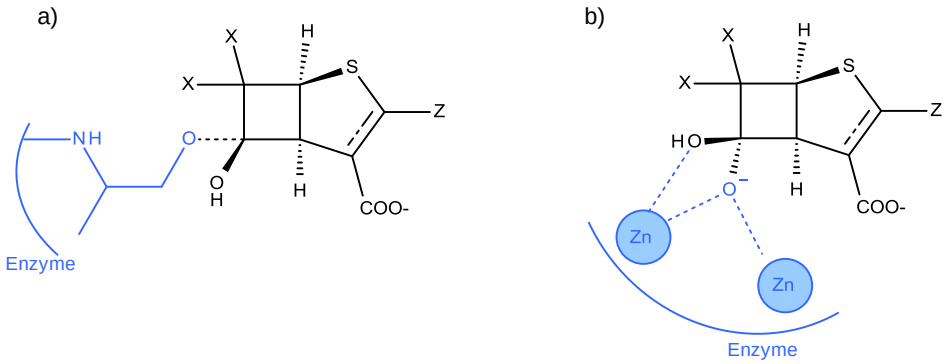
Inhibitor structure	Class A KPC-2, μM	Class B IMP-1, μM	Class C GC1, μM	Class D OXA-10, μM
	26 ± 2	213 ± 21	4.5 ± 0.3	370 ± 15
	58 ± 2	122 ± 5	6.5 ± 1.4	156 ± 6

Table 5 IC_{50} values for inhibition of β-lactamase enzymes by (1S,5S)-6,6-dichloro-7-oxo-4-thiabicyclo[3.2.0]hept-2-ene-2-carboxylic acid (top) and (2S,3S,5S)-6,6-dichloro-3-methoxy-7-oxo-4-thiabicyclo[3.2.0]heptane-2-carboxylic acid (bottom) **[119]**

Structural data for the binding of the cyclobutanones at the enzyme active site was obtained for the Class D OXA-1 enzyme. This data enabled the authors to show that the conformation of the enzyme-substrate complex was similar enough to that of β-lactam structures to confirm the binding modes shown in Scheme 19.



Scheme 19 Interactions between cyclobutanones and a) serine β-lactamases and b) metallo-β-lactamases

The use of single-stranded DNA (ssDNA) is another interesting idea for metallo-β-lactamase inhibition and it has been shown to reversibly inhibit BcII from *Bacillus cereus* 5/B/6. The initial paper on this was published in 2009 and to date no further work in this area has been reported. However, the initial data looks promising with K_i values for a 30 residue ssDNA and a 10 residue section of ssDNA being 0.92 nM and 0.31 nM respectively. Investigations with a second metallo-enzyme showed no inhibitory activity suggesting the ssDNA is not simply a chelating agent but actually interacts with the active site. Interestingly the same sequence of ssDNA also showed no activity against the serine-β-lactamase, BcI. All of this evidence suggests that the ssDNA binds the metallo-β-lactamase by interfering with the coordination of either one or both of the zinc ions at the active site. The ssDNA molecules were also shown to suppress the growth of both Gram positive and Gram negative bacteria when added to cultures in combination with the antibiotic cephalexin.[128]

Another major group of metallo-β-lactamase inhibitors are dicarboxylates, with some showing better activity than the sulfur containing compounds.[129] Various different dicarboxylates have been investigated starting with succinic acids in 2001[130] and including phthalic acid derivatives,[131] furans[132] and most recently diethyl maleates[133] all of which seem to show similar binding interactions with one carboxylate displacing the di-zinc bound water molecule (Wat 2) and the second forming a bridge between Zn2 and a lysine residue at the active site.[129] The investigations carried out on succinic acids and phthalic acids both looked at the enzyme IMP-1 from *Pseudomonas aeruginosa*, the furan investigations were computational and focussed on the BcII and L1 enzymes and for the study with diethyl maleates isolates from *Enterobateriaceae* and *Acinetobacter* with IMP, VIM or NDM enzymes were tested.

1.7 β-Sultams

1.7.1 Structure

β-Sultams are the sulfonyl analogues of β-lactams. The simplest β-sultam is the unsubstituted 1,2-thiazetidine-1,1-dioxide (Scheme 21a, page 53), but many β-sultams with substitutions have been reported. The ring may be planar or nonplanar and this depends on the nature of any substituents and their positions on the ring.[134] In either case the ring is highly strained and contains three heteroatomic bonds; C-S, S-N and N-C.[135] A crystal structure of the substituted β-sultam N-methyl-3,4-diphenyl-1,2-thiazetidine-1,1-dioxide was published in 1985.[136] This showed that the β-sultam ring was distorted relative to the β-lactam ring with a buckling of 14.7^{o} and a C-S-N bond angle of 82^{o}. The S-N bond in this β-sultam was measured at 1.643 Å which is substantially more than the 1.35 Å carbonyl C-N bond length observed in analogous β-lactams. These geometrical variations are likely to influence the chemical and enzymatic reactivity with respect to β-lactams. An interesting comparison of reactivity in β-sultams and β-lactams has been reviewed.[137] The paper commences with an overview of the chemical and biological activity and reactivity of β-lactams which includes a description of their effectiveness as mechanism based inhibitors of serine enzymes. The reactivity of β-sultams (and β-phospholactams) is also discussed with reference to the variations in rate enhancements seen between the four-membered rings and their respective acyclic counterparts. Specific reactions involving acid and base catalysed hydrolysis of β-sultams are then discussed as explained in greater detail in section 1.7.3.

1.7.2 Synthesis

Four main routes to β-sultam synthesis have been reported:

Cyclisation by S-N bond formation

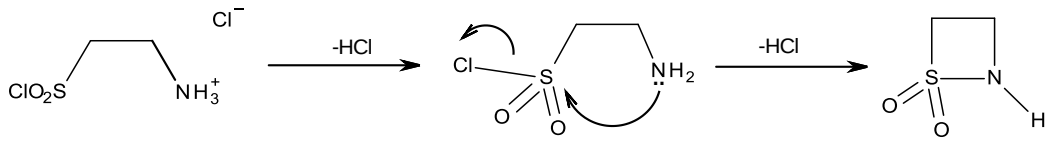
Cyclisation by C-N bond formation

[2+2] cycloadditions of sulfonimines and alkenes (C-N and C-S bond formation)

[2+2] cycloadditions of sulfenes and imines (C-C and N-S bond formation).

1.7.2.1 Cyclisation by S-N Bond Formation

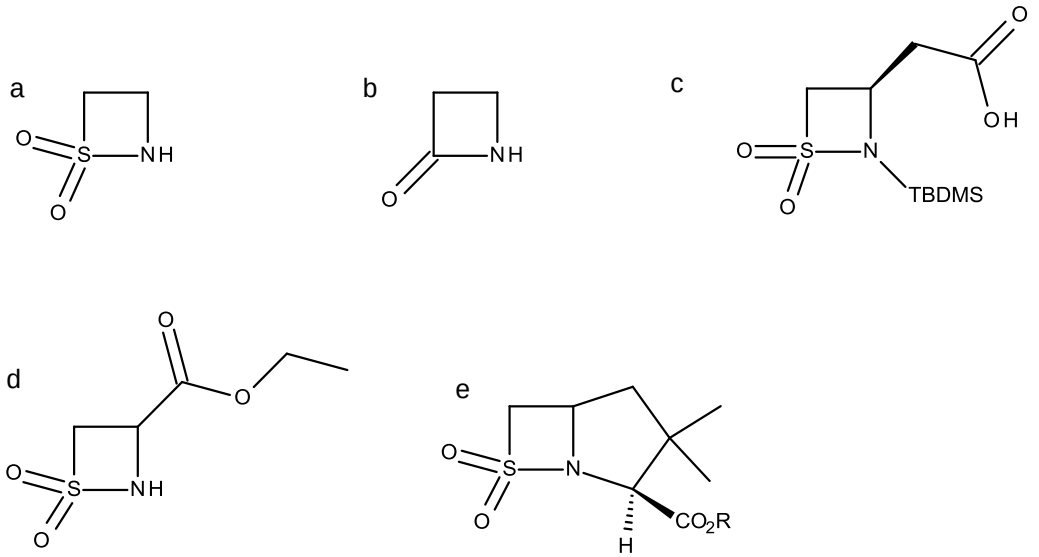
By far the most common method of β-sultam synthesis is cyclisation, and of the two possible methods for this the formation of the S-N bond is the most popular (Scheme 20).



Scheme 20 Cyclisation via S-N bond formation

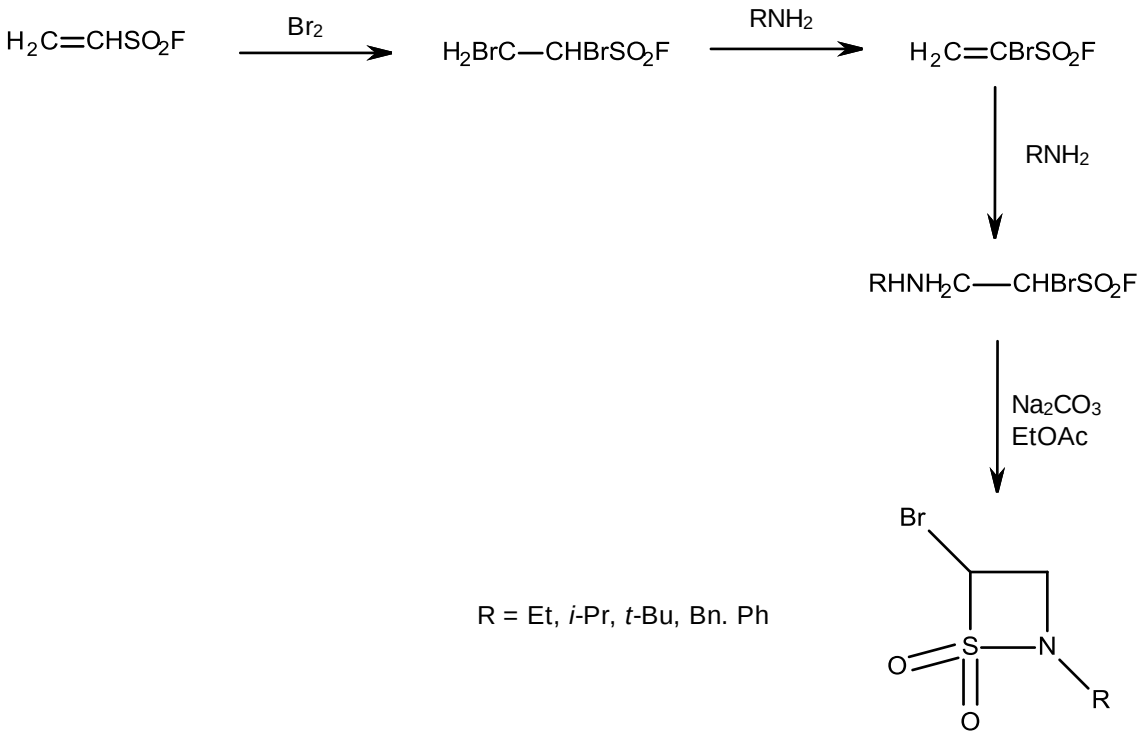
The first reported β-sultam was synthesised in this way by Baganz and Dransch in 1960 (Scheme 21d).[138] They took the modified amino acid cystine diethyl ester and reacted it with chlorine and ethanol to give the sulfonyl chloride. Neutralisation then gave the cyclised product, ethyl 1,1-dioxothiazetidine-3-carboxylate. The first reported synthesis of the unsubstituted β-sultam (Scheme 21a) was made by Le Berre in 1972 and this was carried out following a similar method.[139] Since then the same process has been used to synthesise a range of N-substituted and 3-substituted β-sultams with hypochlorous acid or phosphorus pentachloride being used to convert the taurine derivative to the sulfonyl chloride prior to neutralisation and cyclisation, usually with either sodium carbonate suspended in ethyl acetate or a solution of ammonia in chloroform.

Bicyclic β-sultams have also been synthesised in a similar manner. The first report was made in 1982 by Koller *et al.* and concerned the synthesis of some bicyclic β-sultam based penicillin analogues (Scheme 21e).[140]



Scheme 21 Structures of some β-sultams of particular interest (and b, the unsubstituted β-lactam for comparison)

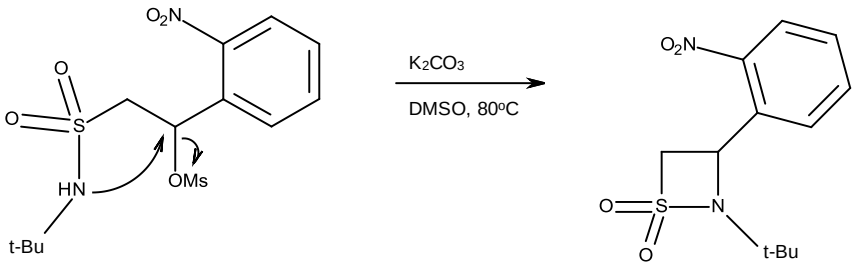
Another method for the synthesis of β-sultams by S-N bond formation is the cyclisation of fluorosulfonyl amino alkanes. This method is less popular than the chlorosulfonyl process but has been used to successfully synthesise a number of N-substituted and 4-bromo β-sultams (general reaction shown in Scheme 22).[141]



Scheme 22 N-substituted and 4-bromo β-sultams as synthesised by fluorosulfonyl amino alkane cyclisation

1.7.2.2 Cyclisation by C-N Bond Formation

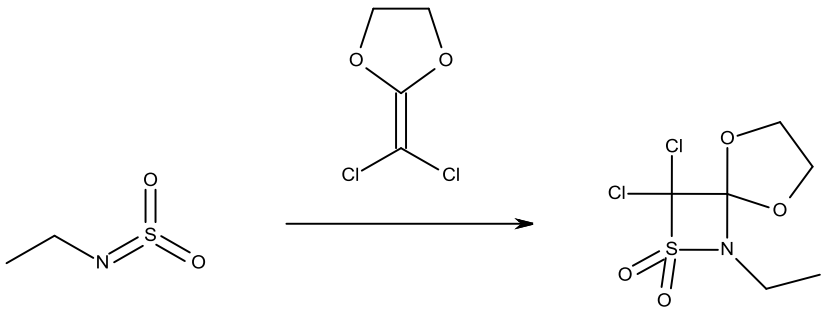
The second method of cyclisation is via C-N bond formation. The first report of this method was made in 1984 and involved the cyclisation of β-hydroxysulfonamide mesylates. This is a relatively simple reaction requiring only exposure to potassium carbonate in DMSO at 80 ^{o}C to form the substituted β-sultam. In all reported cases this method always yields an N-substituted β-sultam but is useful as a method of synthesising highly substituted β-sultams (examples shown in Scheme 23).[142]

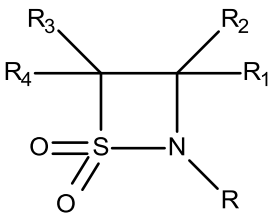


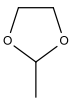
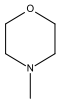
Scheme 23 β-Sultam synthesis by C-N bond formation during cyclisation
(scheme for synthesis of 2-tert-butyl-3-(o-nitrophenyl)-1,2-thiazetidine-1,1-dioxide)

1.7.2.3 [2 + 2] Cycloaddition (C-N and C-S Bond Formation)

[2+2] cycloaddition reactions have also been successfully utilised in the preparation of β-sultams. N-Sulfonylamines react with nucleophilic olefins to give 1,2-thiazetidine 1,1-dioxides. The first report of this reaction (an example of C-N and C-S bond formation) was made by Burgess and Atkins in 1967 during their research into new preparation methods for sulfonylamines. During the attempted preparation of one such compound in the presence of 2-(dichloromethylene)-1,3-dioxolane they collected and characterised the cycloaddition product; 4,4-dichloro-3,3-ethylenedioxy-2-ethyl-1,2-thiazetidine 1,1-dioxide (Scheme 24).[143]

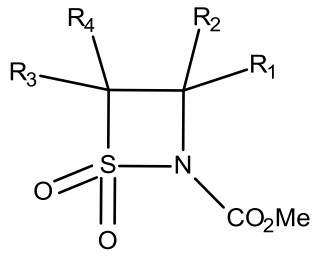




R	Et	Me	CO_2Me
R1			Ph
R2	H	H	H
R3	Cl	Me	H
R4	Cl	Me	H
Ref.	[143]	[144]	[145]

Scheme 24 Examples of compounds synthesised by [2+2] cycloaddition (scheme shows cycloaddition of 1-(sulfonylamino)ethane and 2-(dichloromethylene)-1,3-dioxolane)

The first β-sultams synthesised by this method were prepared from highly electrophilic N-sulfonylamines, such as N-sulfonylbenzamide, which reacted with ethoxyethene to form N- and 3-substituted cycloaddition products.[146] Further research into the preparation of the sulfonylamine starting materials by the group over the next few years lead to the publication of a second paper, this time specific to the cycloaddition reaction. In this paper they reported the synthesis of a wide range of β-sultams with up to three organic groups substituted at the 3- and 4-positions with a carbomethoxy group at the N-position (examples in Scheme 25).[145]

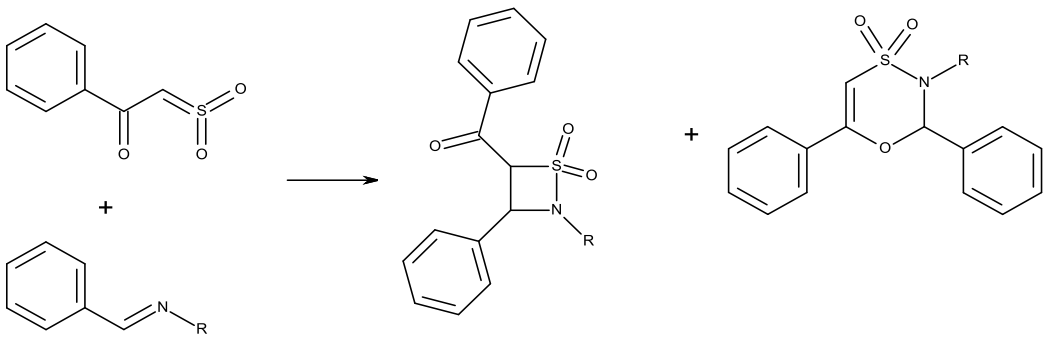


R_1	R_2	R_3	R_4
Ph	H	H	H
Ph	Ph	H	H
Me	Me	Me	H
1-pyrrolidinoyl	H	Me	Me

Scheme 25 2-carbomethoxy-1,2-thiazetidines synthesised by the method of Burgess and Williams[145]

1.7.2.4 [2 + 2] Cycloadditions (N-S and C-C Bond Formation)

The final general method of β-sultam synthesis and second example of a [2+2] cycloaddition reaction generating β-sultams (N-S and C-C bond formation) is the reaction of sulfenes with imines as shown in Scheme 26. These reactions of benzoylsulfenes with benzylidenamines generate either [2+2] or [4+2] cycloadducts. In the first paper reporting this reaction in 1970 it was shown that variation of the R group on the benzylidenamine could change the percentage of [2+2] and [4+2] yields. The presence of triethylamine was also shown to alter the percentages of the products with the [2+2] product being the only one isolated when it was excluded.[147] In a later publication the preference for 3- and 4- substituents to be in cis- geometry was shown in the synthesis of 2-methyl-4-phenyl-3-aryl-1,2-thiazetidine-1,1-dioxide by this same mechanism.[148]



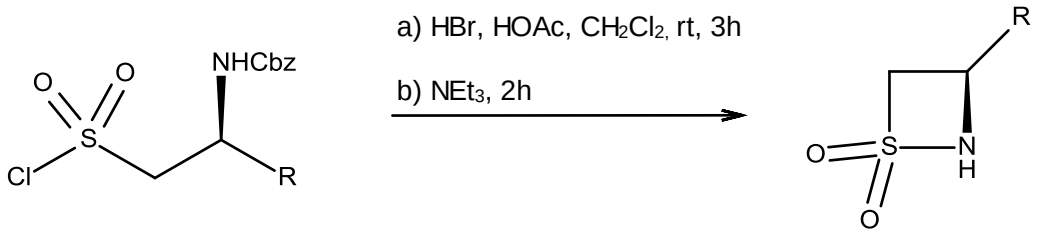
Scheme 26 [2+2] Cycloaddition reaction of 1-phenyl-2-sulfonyl-ethanone and a 1-phenyl-methanimine

1.7.2.5 Advances in β-Sultam Synthesis

Since the early investigations of β-sultams numerous advances in the synthesis of 1,2-thiazetidine-1,1-dioxides have been made. According to a review of sultam synthesis published in 2013 there has been a dramatic increase in the amount of research being carried out in this area over the past 20 years.[149] It is proposed that there may be two contributing factors for the increase. Firstly, an academic interest in methods of sultam synthesis due to a lack of efficient general reaction schemes applicable to sultams of varying ring sizes and substitution patterns. Secondly there is a growing interest in the biological activity of sultams and their potential use in both the pharmaceutical and agricultural industries.[149]

Whilst β-sultam synthesis still mainly relies on the four processes initially highlighted above, new methods do show improvements in the asymmetry and potential complexity of the structures. Asymmetry may be present within the ring or with respect to substituents on C-3 or C-4.

One of the early reports regarding the synthesis of β-sultams with internal asymmetry was made in 2002 by Enders and Wallert and used S-N bond formation for the cyclisation of an asymmetric starting material to give 3-substituted β-sultams in excellent enantiomeric purity (examples shown in Scheme 27).[135]

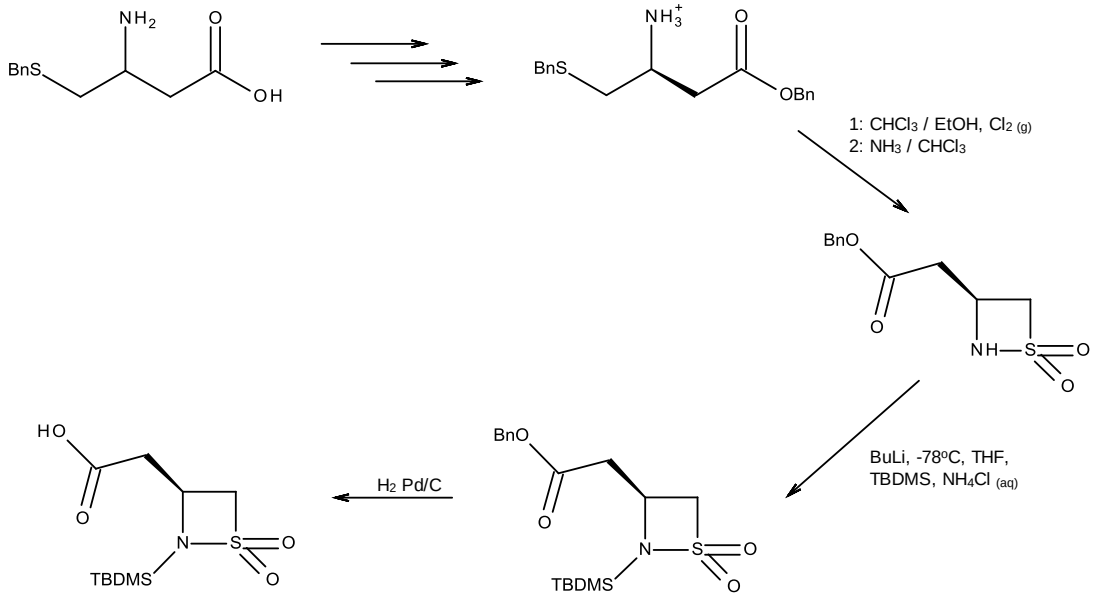


R group	Yield (%) over 2 steps	ee (%)
Et	29	≥96
n-Pr	68	≥96
i-Pr	47	≥96
n-Bu	78	≥96
$(CH_2)_2Ph$	55	≥96

Scheme 27 Examples of high enantio-purity 3-substituted β-sultams as prepared by Enders and Wallert[135]

β-Sultams with asymmetric ring substituents have also been synthesised by various methods. The variations tend to be in synthesising the acyclic precursors rather than in the method of cyclisation which always tends to be by neutralisation in base to remove HCl and formation of the N-S bond.[149-153]

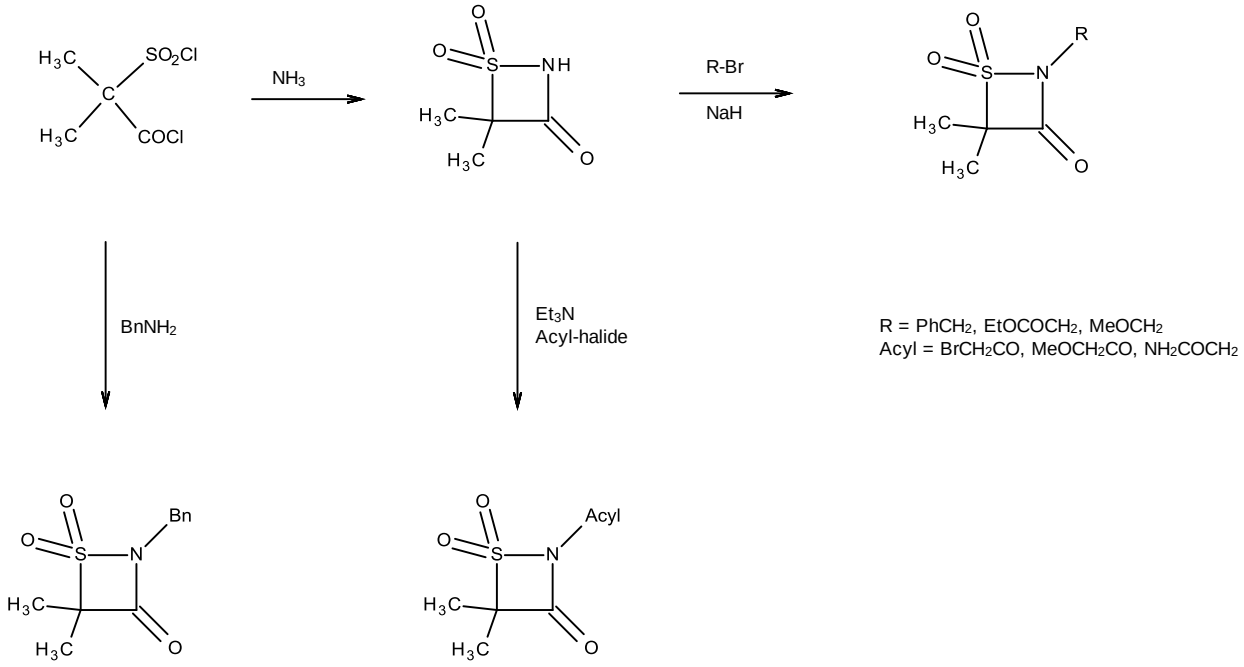
As previously demonstrated substitutions at the N-, 3- and 4-position are common either individually or in combinations of two or three. The group of Hans-Hartwig Otto in Greifswald, Germany has spent many years looking at different methods of adding reactive substituents to various positions on the β-sultam ring as well as synthesising bicyclic systems.[154-159] Of particular interest regarding this thesis is the work the group reported in 2004 regarding the synthesis of a β-sultam with acetic acid substituted at the 3 position on the ring (Scheme 21c, page 53).[160] Benzyl (R,S)-2-tert-butyldimethylsilyl-1,2-thiazetidine-1,1-dioxide was successfully synthesised from an amino acid starting material, (R,S)-S-benzyl-β-homocysteine, by oxidative chlorination followed by base-catalysed ring closure and silylation. Palladium catalysed hydrogenolysis was used to prepare the acid by ester cleavage (Scheme 28).



Scheme 28 Synthesis of N-protected 3-acetic acid β-sultam (adapted from paper [160])

Another particularly interesting piece of work by the Otto group involves the synthesis of 1,2-thiazetidin-3-one 1,1-dioxides; compounds which represent a combination of the β-sultam and β-lactam structures. Various different approaches

were used to synthesise the 4,4-disubstituted 3-oxo-β-sultams. The first was direct cyclisation of 2-chlorosulfonyl-2-methyl-propanoyl chloride with an amine, though this only proved successful in the synthesis of the N-unsubstituted compound and the N-benzyl derivative. Other derivatives were synthesised by N-alkylation of the N-unsubstituted compound via reaction with a bromoalkane and sodium hydride in DMF. N-acylated derivatives were also synthesised via reaction with either an acid halide or an anhydride with triethylamine in THF. These were particularly unstable compounds but interesting due to the presence of a diacylated and sulfonated N-atom (see Scheme 29).[154]



Scheme 29 Synthesis of 3-oxo-β-sultams

1.7.3 Reactivity

Except under highly specific conditions β-sultams undergo hydrolysis by S-N bond fission.[161] The acid and base catalysed hydrolysis is particularly rapid in comparison to both the analogous acyclic sulphonamides (which are especially stable to hydrolysis under alkaline conditions) and the analogous β-lactam compounds. As sulfonyl transfer reactions are usually much slower (~10^3 fold) than acyl transfer reactions the difference in reactivity between β-sultams and β-lactams is particularly

striking.[162] Both acid and base catalysed hydrolysis of β-sultams yield the same final product but the mechanisms do vary, and this is often reflected in the relative rates of the processes when electron donating or withdrawing substituents are present.[163]

A huge amount of work has been done by previous members of this group regarding the mechanisms and kinetics of β-sultam hydrolysis. In 2000 Baxter *et al* published a thorough report on "Reactivity and Mechanism in the Hydrolysis of β-Sultams".[163] Within this paper they reported the second order rate constants for the alkaline hydrolysis of structurally comparable β-sultams and β-lactams showing the significant rate enhancement due to the presence of a sulfonyl group rather than an acyl group (compared to the converse situation with the acyclic analogues) (data summarised in Table 6).

The difference in rate enhancements can be explained in terms of thermodynamics and transition state stabilisations. Four-membered ring systems are such strained systems that ring opening is always thermodynamically favourable. Because the opening of the ring reduces the bond strain, reactions which result in the cleavage of a ring bond in cyclic systems normally have a lower activation energy due to the increased stability of the transition state. This leads to these reactions almost always being faster than in the analogous acyclic systems. In β-lactams this rate enhancement in the hydrolysis of cyclic over acyclic compounds is only minimal and this is because the strain in the ring is not released on formation of the transition state (TS). In β-sultams the formation of the TS is accompanied by a large relief in strain which explains the significant rate enhancements seen over acyclic sulfonamides.

In β-lactams the initial state and transition state are both destabilised by ring strain since the ideal C-C-N bond angle in the initial state is ~120^{o} and in the TS it is ~109^{o} based on the preferred trigonal and tetrahedral arrangements respectively. Since the ring strain is not significantly reduced by movement to the TS there is little energetic advantage and so only minimal rate enhancement is seen.

Conversely in β-sultams the ring strain relief shown by the five-coordinate intermediate significantly enhances rates compared to acyclic systems. As with β-lactams there is significant ring strain in the initial state with an ideal C-S-N bond angle of 109^{o} in the tetrahedral arrangement. The transition state, however, shows a trigonal bipyramidal arrangement so that the new C-S-N bond angle of 90^{o} now shows the preferred geometry. In acyclic sulfonamides the formation of the TBPI does not introduce a more favoured bond angle. The difference in free energy between initial and transition states for the cyclic system is therefore reduced in

comparison to the acyclic system and the rate enhancements observed accounted for.[164]

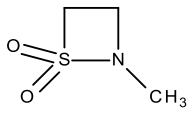
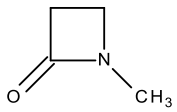
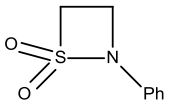
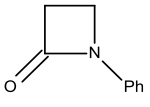
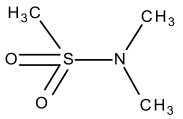
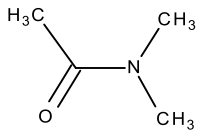
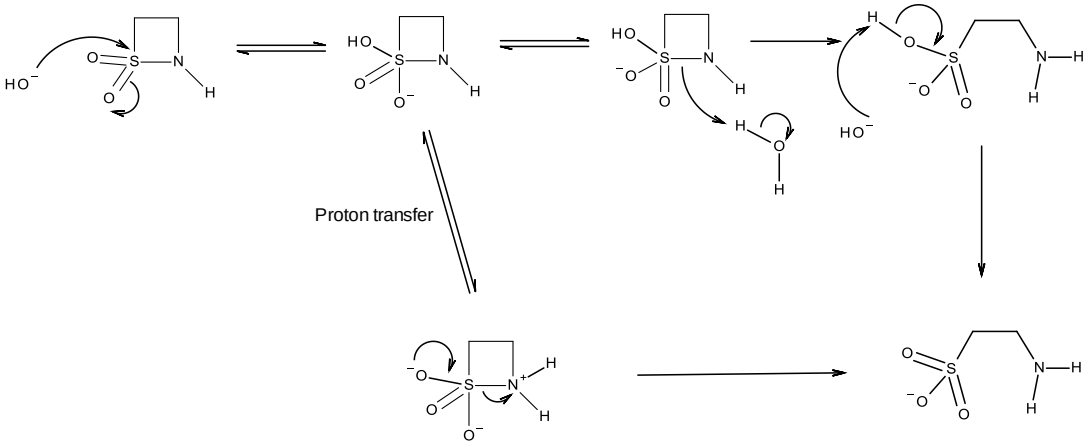
	Sulfonyl analogue	Carbonyl analogue	Relative rates ($k_{OH\ sulfonyl}$ / $k_{OH\ carbonyl}$)
			2.31×10^{2}
k_{OH} $M^{-1}s^{-1}$	1.41×10^{-2}	6.1×10^{-5}	
			1.47×10^{3}
k_{OH} $M^{-1}s^{-1}$	5.69	3.87×10^{-3}	
			8.70×10^{-5}
k_{OH} $M^{-1}s^{-1}$	$<2 \times 10^{-9}$	2.3×10^{-5}	
Relative rates in cyclic compounds ($k_{OH\ alkyl}$ / $k_{OH\ aryl}$)	2.47×10^{-3}	1.58×10^{-2}	

Table 6 Second order rate constants for the alkaline hydrolysis of some analogous acyl and sulfonyl compounds (adapted from [163])

The effect of pH on the rate of both acid and base catalysed hydrolysis of β-sultams has also been studied in some depth by previous members of this group. pH rate profiles for both the N-methyl- and N-phenyl-β-sultams show a high reactivity towards both acid and base hydrolysis with an apparent lack of a pH independent (or spontaneous) hydrolysis reaction. The N-methyl-β-sultam shows a minimum rate at approximately pH 8 whereas the minimal rate for the N-phenyl-β-sultam is seen at around pH 6. Also the rate of hydrolysis of the N-phenyl-β-sultam is considerably

more rapid at pH 12 than that of the N-methyl-β-sultam (k_{obs} = 6.67 x 10^{-2} s^{-1} and 1.41 x 10^{-4} s^{-1} respectively).[165] Through their studies on the effect of N-substituents on the rate of hydrolysis the group has shown that electron withdrawing substituents on nitrogen increase the rate of the base catalysed reaction and, along with various other pieces of data, they were able to convincingly prove the mechanism of alkaline hydrolysis and show that it proceeds via the trigonal bipyrimidal intermediate previously mentioned.[134,163] Of interest here though are the two possible mechanisms of bond cleavage after the formation of the trigonal bipyramidal anionic intermediate. One route shows a unimolecular ring-opening mechanism via proton transfer and the second shows a bimolecular process involving a water molecule (Scheme 30). Both methods are kinetically viable and the actual route taken may be influenced by the nature and number of substituents on the ring.



Scheme 30 Mechanism of the alkaline catalysed hydrolysis of β-sultams

Acid catalysed hydrolysis is a much more facile process showing only one viable mechanism (Scheme 31). Initial protonation of the β-sultam nitrogen is followed by unimolecular S-N bond fission. The hydrolysis reaction then proceeds via an unstable sulfonylium ion which is trapped by water to give the zwitterionic product. In contrast to the base catalysed process with acid catalysed hydrolysis N-alkyl-β-sultams are more reactive than N-aryl-β-sultams (Table 7) but the differences in rate are not so dramatic.[134,163]

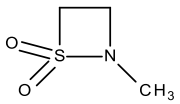
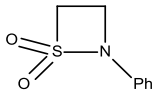
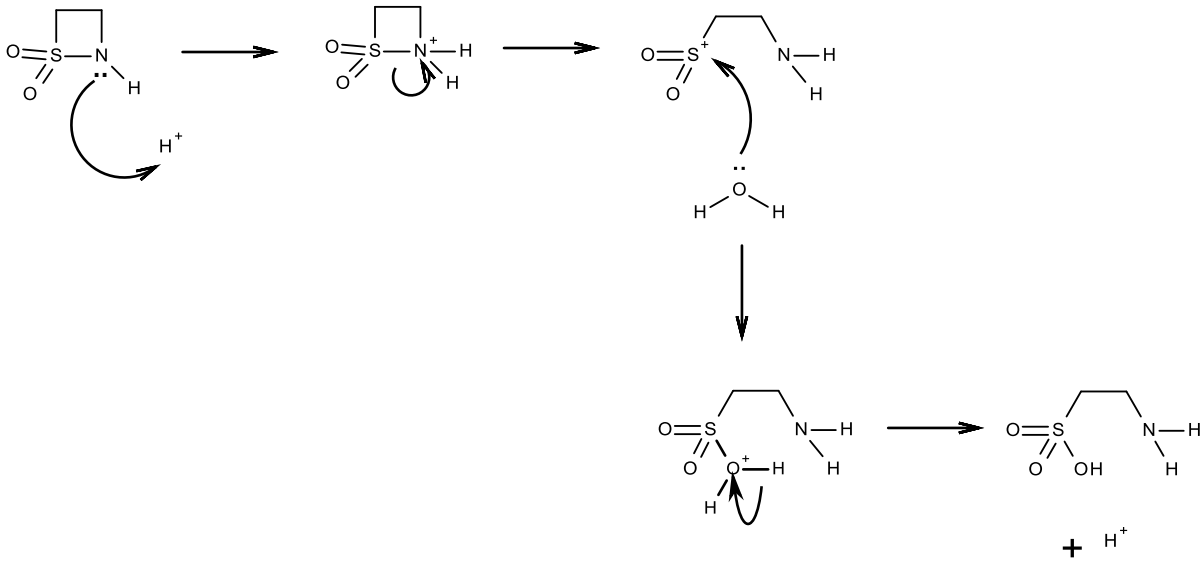
	N-alkyl β-sultam	N-aryl β-sultam	
			Observed rate difference (k_{alkyl} / k_{aryl})
k_H $M^{-1}s^{-1}$	2.64	5.63×10^{-2}	46.89
k_{OH} $M^{-1}s^{-1}$	1.41×10^{-2}	5.69	2.48×10^{-3}

Table 7 Comparison of acid and base catalysed reaction rates for N-alkyl and N-aryl β-sultams



Scheme 31 Mechanism of the acid catalysed hydrolysis of β-sultams

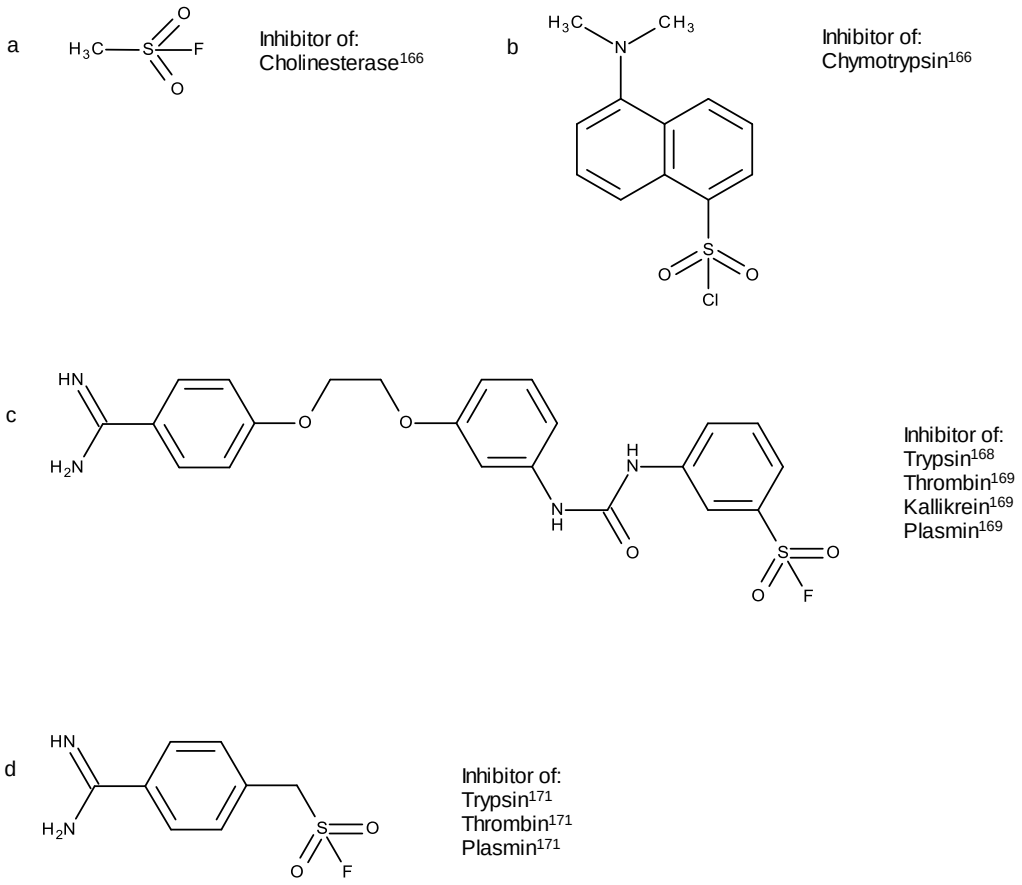
The reactivity of 3-oxo-β-sultams (molecules which are both β-sultams and β-lactams) has also been investigated with regard to the mechanism and rate of hydrolysis. In an early report by the group concerning the hydrolysis of N-benzyl-4,4-dimethyl-3-oxo-β-sultam compared to an N-acyl-β-sultam product analysis showed that for 3-oxo-β-sultams nucleophilic attack occurred at the sulfonyl centre

and hydrolysis proceeded via expulsion of a carboxamide leaving group.[161] In a later paper the second order rate constants for alkaline hydrolysis for a range of N- and 4-substituted 3-oxo-β-sultams were shown to be ~10^3 – 10^5 $M^{-1}s^{-1}$ with the fastest reaction being the hydrolysis of the previously reported N-benzyl-4,4-dimethyl-3-oxo-β-sultam with a k_{OH} of 1.83 x 10^5 $M^{-1}s^{-1}$. In all cases the hydrolysis proceeded via attack at the sulfonyl centre leading to S-N not C-N bond fission which is consistent with previous observations that β-sultams are 10^2 to 10^3-fold more reactive than β-lactams towards alkaline hydrolysis.[166]

1.7.4 Sulfonyl Compounds as Enzyme Inhibitors

As previously mentioned β-sultams are cyclic compounds which can be formed by the cyclisation of taurines: 2-carbon systems with terminal sulfonyl and amide groups. The first report of sulfonyl compounds being used as enzyme inhibitors was published in 1963, a paper which discussed a range of organic sulfonyl fluorides as inhibitors of acetylcholinesterase, α-chymotrypsin and trypsin.[167] This paper was followed a year later by one which showed phenylmethanesulfonyl fluoride to be an irreversible inhibitor of α-chymotrypsin via sulfonylation of an active site serine residue.[168]

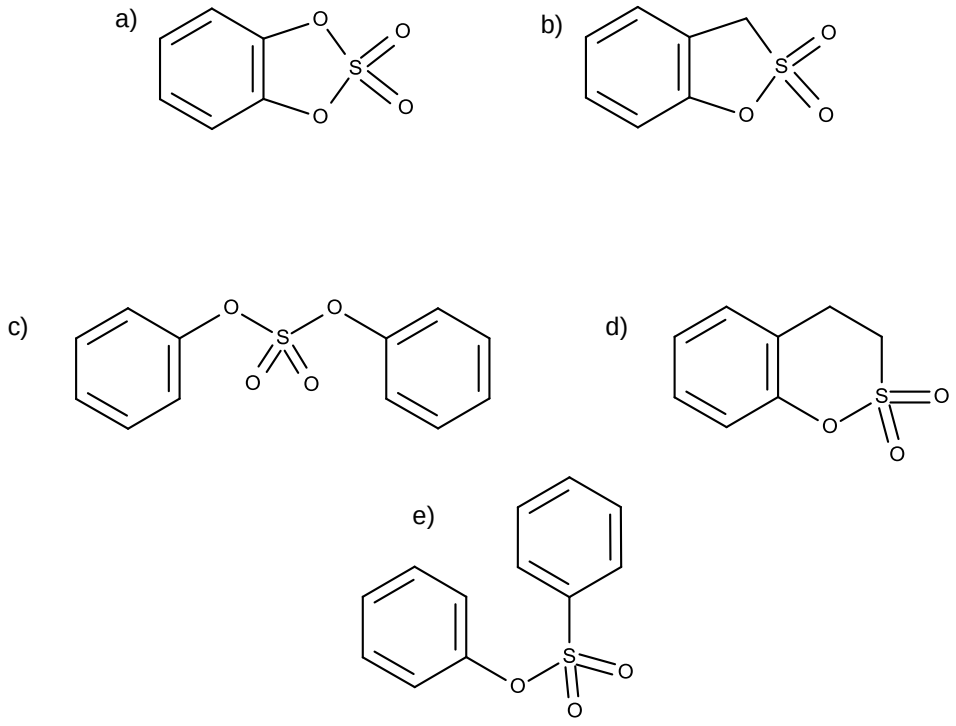
This work was continued over the next two decades with benzenesulfonyl fluorides being developed as inhibitors of a range of enzymes (trypsin[169], thrombin,[170] and kallikrein[171] among others). The reactivity of aromatic sulfonyl fluorides was also developed with the addition of a positively charged benzamidine or pyridinium group to the benzene ring, increasing the reactivity. As part of this work (p-amidinophenyl)methanesulfonyl fluoride was synthesised and shown to be an irreversible inhibitor of serine proteases, particularly bovine trypsin and human thrombin.[172] Structures of some of the sulfonyl compounds investigated are shown in Scheme 32.



Scheme 32 Structures of serine protease inhibitors
(**a**=methanesulfonyl fluoride, **b**=1-dimethylaminonaphthalene-5-sulfonyl chloride, **c=**(p-[m(m-fluorosulfonylphenylureido)phenoxyethoxy] benzamidine and **d**=(p-amidinophenyl)methanesulfonyl fluoride)

Cyclic sulfonates and sulfate esters have also been investigated with respect to their reactivity and enzyme inhibition abilities. Five membered cyclic sulfate esters such as catechol cyclic sulfate (Scheme 33a) and 2-hydroxy-α-toluenesulfonic acid sultone (Scheme 33b) have been shown to have rates of hydrolysis 10^7 and 10^6 times faster than the acyclic analogue diphenyl sulfate (Scheme 33c) respectively.[173] Conversely six-membered systems (Scheme 33d) whilst having a hydrolysis rate faster than that of an acyclic analogue (Scheme 33e) by one order of magnitude showed no real rate enhancement like that in the five-membered system which has a second order rate constant for reaction 10^4 times higher than the six-membered system.[174] This work

on cyclic system rate enhancements follows on from that carried out on phosphate esters in the 1950s and 1960s.[175]



Scheme 33 Cyclic and acyclic sultone structures (Ref [173,176])

Sulfonamides were reported as inhibitors of carbonic anhydrase in 1940 and have been the subject of much work right up to the present day.[177-180] Although much of the work still centres around inhibition of carbonic anhydrase, sulfonamides have also been shown to inhibit other enzymes such as tyrosine phosphatase,[181] a mutant isocitrate dehydrogenase found in leukaemia cancer cells[182] and aggrecanase, an enzyme targeted with regard to osteoarthritis treatment.[183]

1.7.5 β-Sultams as Enzyme Inhibitors

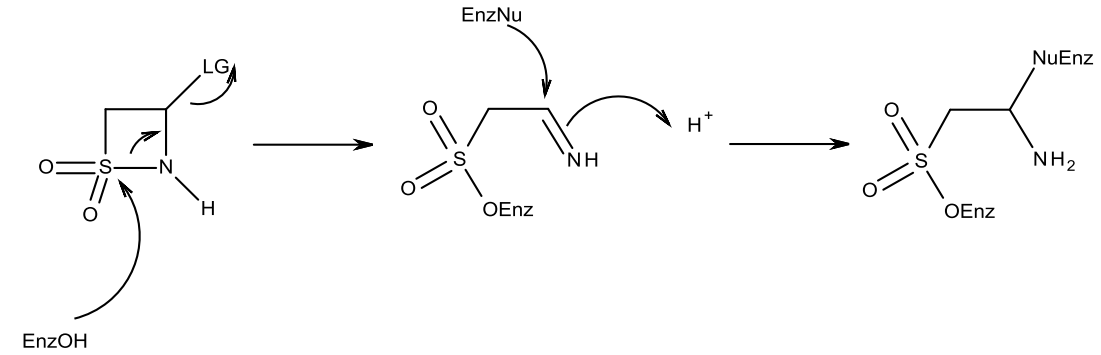
In 2001 the inactivation of the serine protease enzymes by N-benzoyl-β-sultam was first reported[184] and this was followed in 2003 by reports of N-acyl-β-sultams inactivating the same elastase enzyme.[185] Two years later N-acyl-β-sultams were shown to inhibit the *Streptomyces* R61 DD-transpeptidase enzyme by way of

sulfonylating the active site serine residue.[186] In another paper in the same year these same N-acyl-β-sultams were shown to inactivate the Class C β-lactamase P99 from *Enterobacter cloacae*.[187] This paper also looked at the action of analogous β-lactams on the same enzyme. The β-lactams were shown to be substrates for the enzyme with hydrolysis taking place via an acylation-deacylation process. Conversely the β-sultams inactivated the enzyme by sulfonylation of the active site serine residue forming a sulfonate ester which then underwent C-O bond fission. ESI-MS investigations showed that elimination of the sulfonate anion led to the formation of a dehydroalanine residue.

β-Sultams have the potential to act as inhibitors of β-lactamases via either the mechanism based or transition state analogue route. Mechanism based inhibition of serine-β-lactamases is possible with β-sultams which have a leaving group at the 3-position or where there is a leaving group on an N-acyl substituent (Scheme 34).

Scheme 34 Representative structures of β-sultams with the potential to inhibit serine β-lactamase enzymes

In a similar manner to β-lactam type inhibitors (clavulanic acid or sulbactam for example) the attack of the enzyme at the sulfonyl centre (the carbonyl centre in the β-lactams) and loss of the leaving group results in formation of an electron-deficient imine or iminium ion which could be attacked by a second nucleophilic amino acid residue at the active site. This would trap the enzyme and cause irreversible inhibition (see Scheme 35).

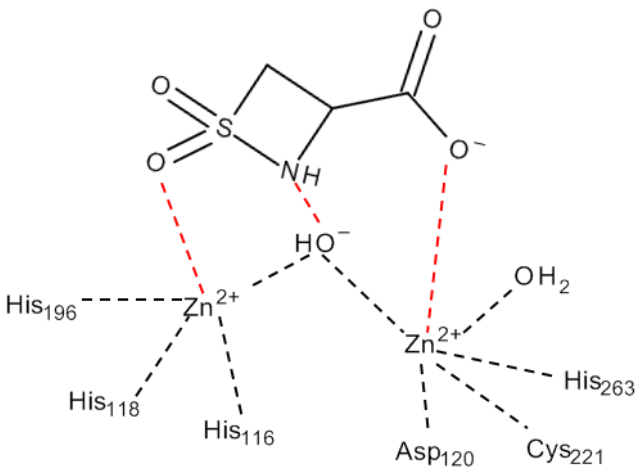


Scheme 35 Mechanism based inhibition of serine β-lactamase enzymes by β-sultams

Transition state analogue inhibition could be achieved with metallo-β-lactamases due to the tetrahedral sulfonyl group which imitates the sp^3 intermediate formed by enzyme attack at the carbonyl centre of β-lactam antibiotics during normal enzyme action. This could be especially true upon ionisation of the β-sultam to generate an anion suitable for complexing to Zn^{2+}. Non-covalent interactions between the zinc ion of metallo-enzymes and the sulfonyl group can potentially lead to transition state analogue inhibition. A similar result is also possible via interaction of the sulfonyl centre with the oxyanion hole of serine enzymes.

Scheme 36 Zinc interactions with a representative β-sultam and β-lactam transition state demonstrating potential for β-sultams to act as transition state analogues

It is proposed that 1,2-thiazetidine-3-carboxylate-1,1-dioxide (3-carboxy-β-sultam) has the potential to act as a transition state analogue of the Class B β-lactamase BcII via interactions of the zinc ion (Zn1) with the sulfonyl group as shown above. Further interactions should occur between the amine and zinc bound hydroxide ion (Wat 1) and also between the second zinc ion (Zn2) and the carboxylate group as demonstrated in Scheme 37.

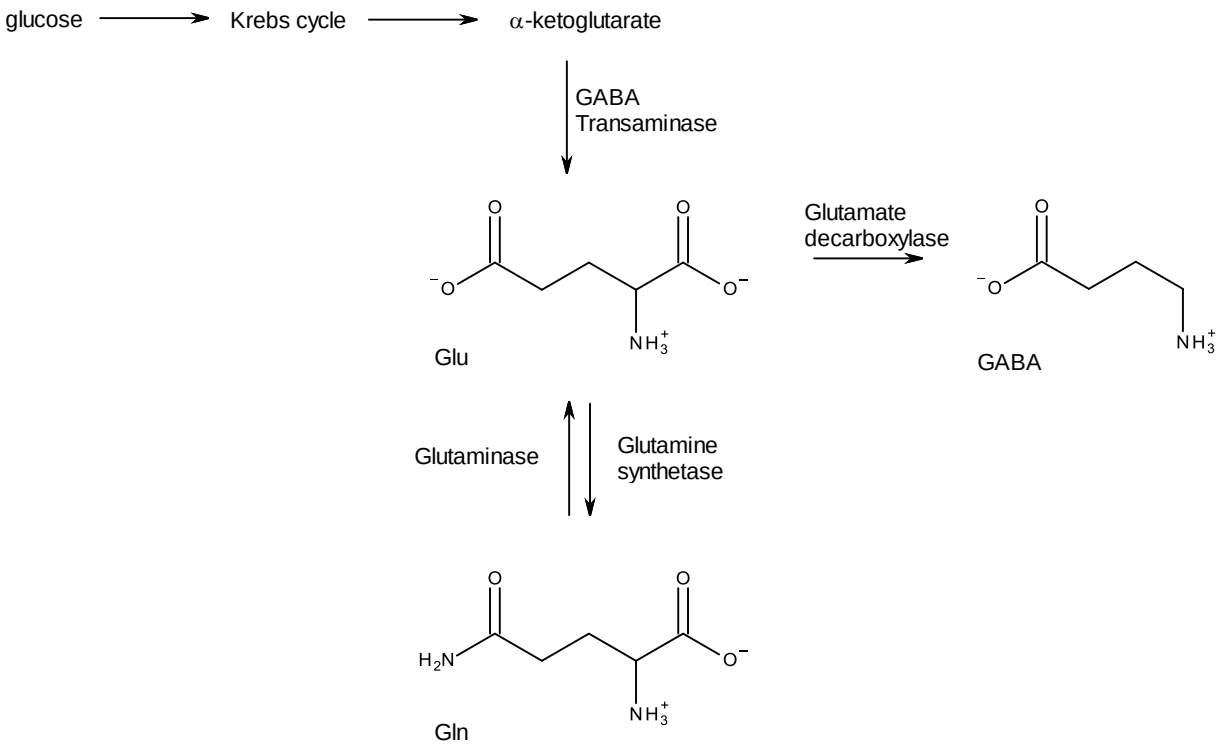


Scheme 37 Potential interactions between the active site of BcII and 3-carboxy-β-sultam

1.8 Glutamine Synthetase

1.8.1 The Roles and Regulation of Glutamic Acid in The Human Body

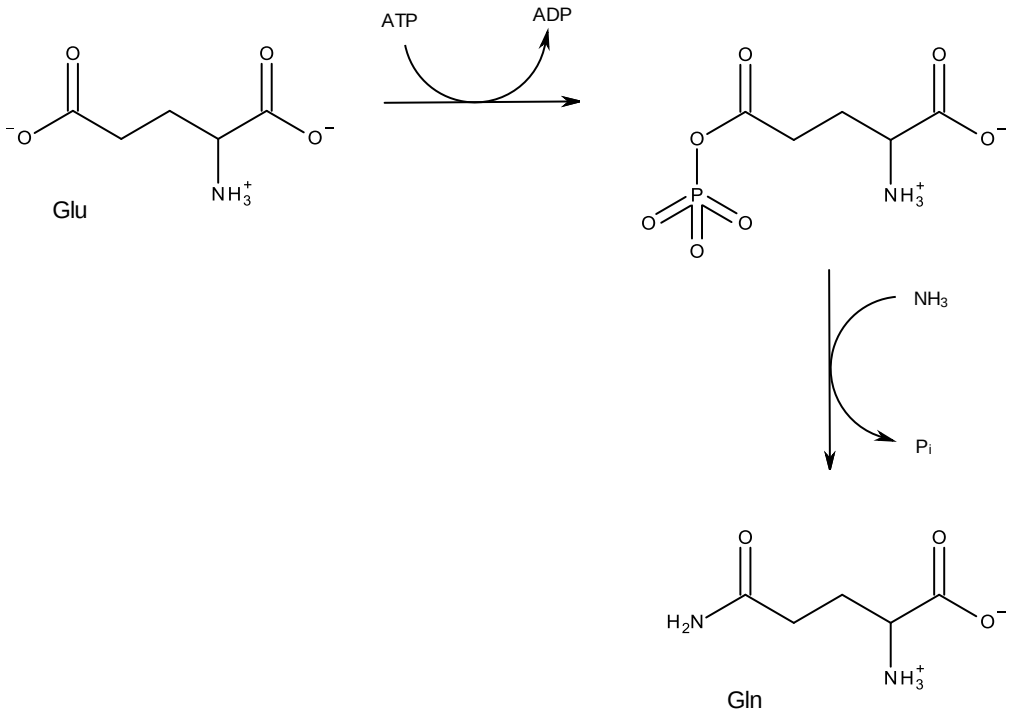
Glutamic acid, or glutamate, is a non-essential, proteinogenic amino acid which also has a role in the human body as an excitatory neurotransmitter. It is non-essential as it can be synthesised within the body via the synthesis and degradation pathway detailed in Scheme 38. The inhibition of any of the enzymes along this pathway will necessarily cause a change in the concentration of glutamic acid.



Scheme 38 Synthesis and degradation of glutamic acid (Glu)

Glutamine synthetase (GS) is an important enzyme in this pathway and plays a vital role in the brain, kidneys and liver. Within the brain this enzyme is involved in the regulation of glutamate and the metabolism of nitrogen by catalysing the synthesis of glutamine. This is an ATP-dependent pathway which involves the phosphorylation of the side chain carboxylate group to yield ADP and an acyl-phosphate intermediate.

This intermediate then reacts with ammonia to form glutamine and inorganic phosphate (as shown in Scheme 39).



Scheme 39 Overview of glutamine synthetase catalysed formation of glutamine from glutamic acid

1.8.2 The Role of Glutamic Acid in Neurodegeneration

Glutamate is the major excitatory neurotransmitter within the central nervous system (CNS).[188] The concentrations of glutamate within the CNS are approximately 1000-fold higher than those of the neurotransmitters dopamine, noradrenaline and serotonin.[189] Glutamate has been shown to be an excitotoxic agent under certain conditions; high concentrations of glutamate can cause excessive stimulation of glutamate receptors causing the death of cells which express these receptors.[188] The concentration of glutamate in the extracellular space can be controlled by glutamate transporters which transport glutamate across cellular membranes. This is important as the removal of glutamate from the extracellular space is the major method of terminating an excitatory signal. Alterations in the expression of glutamate

transporters and the accompanying changes to extracellular glutamate concentrations have been implicated in the following diseases (among others):

Amyotrophic lateral sclerosis (ALS)[190-192]

Alzheimer's disease[193,194]

Huntington's disease[195]

Parkinson's disease[189]

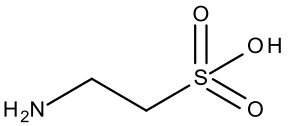
Epilepsy.[195]

1.8.3 Glutamic Acid Analogues (and Associated Compounds) as Enzyme Inhibitors and CNS Drugs

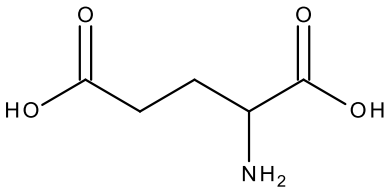
Numerous analogues of glutamate and other compounds in the glutamic acid synthesis/degradation pathway have been investigated as potential inhibitors of key enzymes in the CNS and as potential drugs for the treatment of a range of diseases, particularly those of a neurological nature (some structures are shown in Scheme 40). Numerous studies have also been carried out on glutamine synthetase from sources other than the human CNS; enzymes from rat brain and liver, sheep brain and even pea plants have all been investigated.

L-Methionine sulfoximine is structurally very similar to L-glutamic acid and has been shown to act as a convulsant. It irreversibly inhibits glutamine synthetase via tight binding of the phosphorylated methionine sulfoximine and ADP at the active site.[196] Another structural analogue of glutamic acid which inhibits glutamine synthetase (from pea plants) is tabtoxinine-β-lactam. This is a particularly interesting structure which both mimics the structure of glutamate and contains a β-lactam ring, yet it does not inhibit DD-transpeptidase enzymes or β-lactamases.[197] Taurine, the hydrolysis product of β-sultam, is a glutamic acid analogue which has been implicated as a potential therapeutic agent. It has been shown to have an effect in the treatment of neurodegenerative diseases such as Parkinson's disease and alcohol-induced brain damage possibly due to its anti-inflammatory actions.[198] Some compounds structurally related to taurine have also been reported to be useful as drugs which can reduce the craving for alcohol in recovering post-detoxification alcoholics[199-201] Acamprosate (N-acetyl homotaurine or 3-acetamidopropane-1-sulfonic acid) is one such compound; marketed under the trade name Campral it is

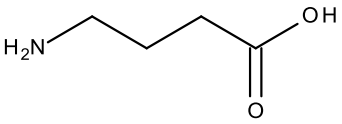
used to treat alcohol dependancy.[202] Other reports suggest that taurine can prevent neurodegeneration in the elderly, particularly that associated with Alzheimer's disease.[203,204] Gabapentin is an anticonvulsant used in the treatment of epilepsy. It is a GABA analogue (rather than a glutamate analogue) but has been shown to have effects on the enzymes in the metabolic pathways of both GABA and glutamate.[205]



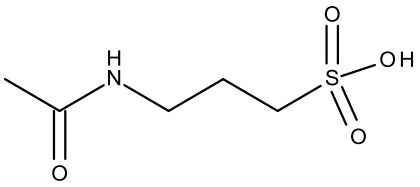
Taurine



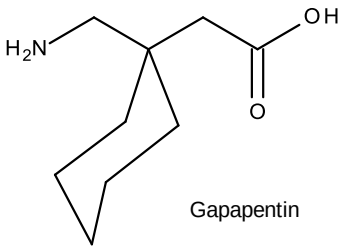
Glutamic acid



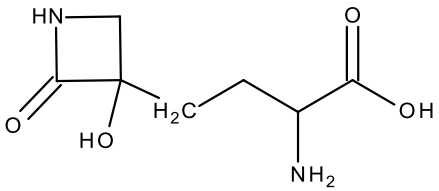
GABA



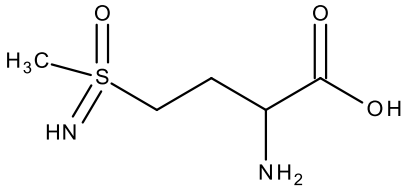
Acamprosate



Gapapentin



Tabtoxinine-β-lactam

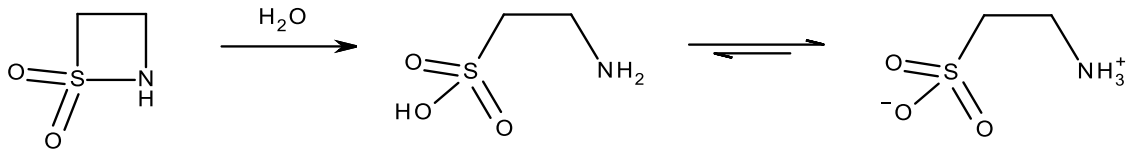


L-Methionine sulfoximine

Scheme 40 Structure of taurine, glutamic acid and related compounds

1.8.4 β-Sultams as Pro-Drugs for Treating Neurodegenerative Diseases

As has been previously discussed in section 1.7.3 the hydrolysis of the unsubstituted β-sultam (1,2-thiazetidine-1,1-dioxide) yields taurine, a compound which is zwitterionic in aqueous solution (Scheme 41).



Scheme 41 Hydrolysis of β-sultam to yield taurine

The administration of taurine as a drug however, is not a facile process. The zwitterionic nature of the compound makes it highly lipophobic and hydrophilic and this decreases the ability of taurine to diffuse across cell membranes so that it is poorly absorbed from the gut.[206] The charged nature of the molecule also reduces the amount of taurine which can be taken across the blood-brain barrier. In order for taurine to be a useful drug in the treatment or prevention of neurodegeneration it is necessary to find a method of getting it into the brain and to the site of action. Two possible ways of facilitating this are:

Producing taurine analogues with lower hydrophilicity

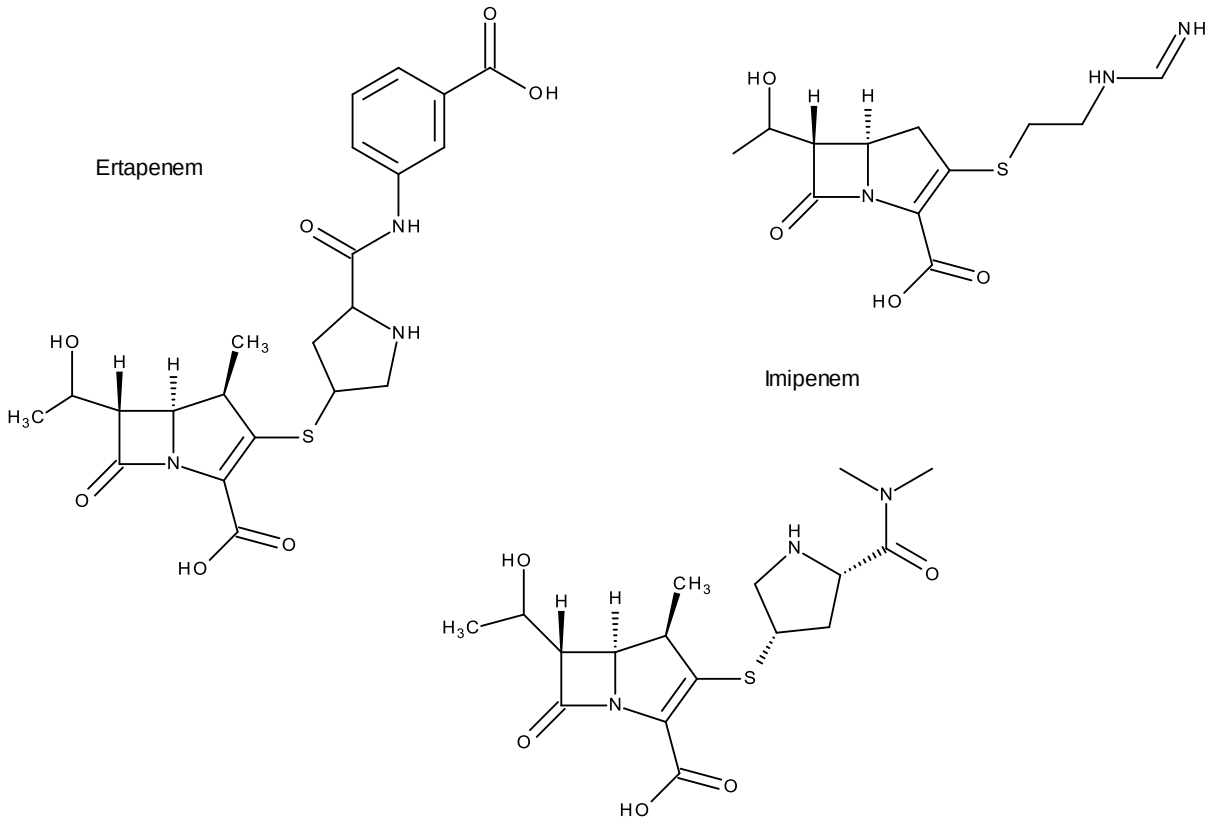
Producing a pro-drug which in some way releases taurine into cells once it has crossed into the brain.

β-Sultams could be ideal pro-drugs for taurine analogues if their half-lives at physiological pH are of an order which allows delivery of the pro-drug to the site of action prior to hydrolysis. Previous work by Ward *et al*. has studied the effects of β-sultam and taurine on the inflammatory response and rate of glutamate release both in cells and in rats.[198] Their work showed that β-sultam had an enhanced ability to reduce the inflammatory response over taurine (its hydrolysis product) both *in vitro* and *in vivo*. It also had an effect on the amount of glutamate release, and again this was a greater effect than with taurine. It is suggested that the increased lipophilicity of the β-sultam over taurine increases the uptake across cell membranes and that

the use of β-sultams as pro-drugs for taurine analogues may be of significant benefit in the treatment of neurodegenerative conditions. Enzymes within the CNS are potentially targets for these compounds and their activity as inhibitors of glutamine synthetase is therefore of interest.

1.9 Mechanism of Ertapenem Hydrolysis by the Metallo-β-Lactamase enzyme BcII

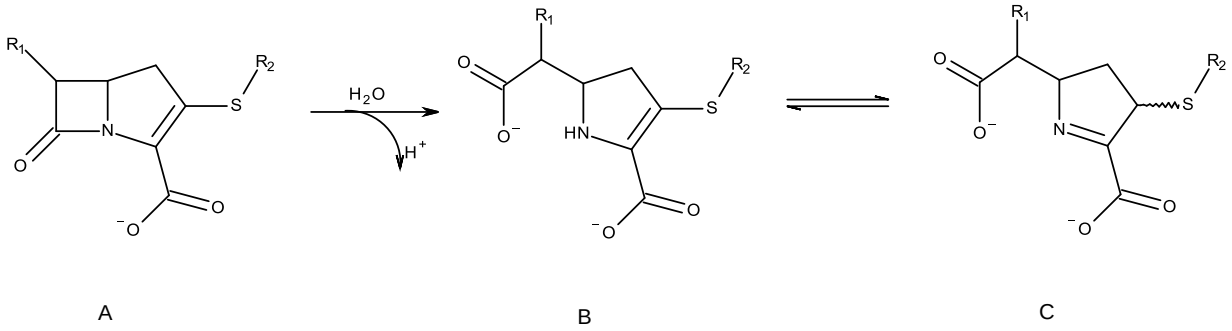
Ertapenem is a carbapenem antibiotic with a similar mode of action to imipenem and meropenem (Scheme 42). In this age of increasing resistance of bacteria to β-lactam antibiotics the carbapenems are of vital importance as a last line of attack as currently most bacteria are still susceptible to their activity. As with all drugs an understanding of their stability and the mechanism of their action is of great importance.



Scheme 42 Structures of representative carbapenem antibiotics

The stability of ertapenem at a range of pHs in aqueous solutions was investigated by Zajac *et al.* and they published pH rate profiles for the hydrolysis of ertapenem at temperatures from 30 – 60 ^{o}C.[207] Tioni *et al.* studied imipenem hydrolysis and specifically looked at the formation of a reaction intermediate in the hydrolysis by BcII.[208] They used various techniques to characterise the intermediate and concluded

that protonation during hydrolysis at the active site of the enzyme occurred initially on nitrogen (Scheme 43B), but that tautomerisation may lead to the formation of the imine product (Scheme 43C).



Scheme 43 Tautomerisation of carbapenem (imipenem core structure) after hydrolysis

Our studies of ertapenem aim to confirm that protonation actually occurs at C3 of the pyrrolidine ring to form the product shown in Scheme 43C.

1.10 Instrumental Techniques – React-IR

React-IR is a relatively new technique first introduced in the early 1990's. It can be used to monitor a reaction *in situ* and in real time without the need for removing samples or for further sample preparation. Computer software is available to allow the monitoring of specific characteristic wavenumbers producing graphs which show the change in absorption due to disappearance of starting materials, appearance and disappearance of intermediates or the production of the final products.

The individual spectra produced during a React-IR experiment are the same as those produced in a standard FT-IR experiment. The molecules in the solution absorb the IR radiation at frequencies which correspond to the vibrational frequencies of the bonds within them. The use of a Fourier transformation allows these absorbances to be displayed as a frequency spectrum specific to the solution at that moment in time.

In a React-IR instrument (Figure 10) the IR source, interferometer, beam splitter and detector are all housed within the main unit. The optical path of the IR radiation is from the source along the probe, through an optical crystal and back up the probe to the detector in the main unit.

Figure 10 React-IR spectrometer

The infrared light passes through the crystal via total internal reflection with multiple reflections. This total internal reflection causes an evanescent wave which penetrates 0.5 to 2 μm into the sample. This is known as attenuated total reflectance (ATR) and allows extremely small samples (for example, one drop of < 0.1 ml) to be analysed by IR spectroscopy. In order for an evanescent wave to form, the crystal must be made of a material with a higher refractive index than the sample solution. Materials

often used as ATR crystals are germanium, zinc selenide or, as in the case of our instrument, diamond.

As with other types of spectroscopy the Beer-Lambert Law ($A = \varepsilon cl$) can be applied to infrared spectroscopy.[209] The law states that the absorbance (A) of a solution is equal to the concentration (c) of the solution multiplied by the path-length (l) of the sample and its molar absorptivity (ε). In the case of solutions containing multiple components it is necessary to find a wavelength (or in this case wavenumber) where only one component shows an absorbance. In this way it is possible to monitor the concentration of a species in solution using ReactIR if a unique absorption band for that species can be found. In the case of a reaction where there is a change in functional group this can be quite easy using ReactIR, and it is an advantage of ReactIR over UV-Vis spectroscopy where no chromophores are created or removed.

ReactIR was chosen as the technique to follow the hydrolysis of β-sultam to yield taurine. Sulfur-oxygen double bonds tend to absorb IR radiation between 1050 and 1450 cm^{-1}. The exact wavenumber of the absorption depends on the functionality of the double bond (part of a sulfone, sulfonic acid, sulphate etc.) and on the presence of other functional groups on the molecule. In this case preliminary work showed that the β-sultam S=O bond absorbed at 1301 cm^{-1} and the taurine S=O peak was found at 1200 cm^{-1} when in an aqueous solution. A plot of the absorbances at these wavenumbers with respect to time could be fitted to a first order rate law and exponential plots would give the pseudo-first-order rate constants for hydrolysis at low pH.

Experimental

2.1 Synthesis

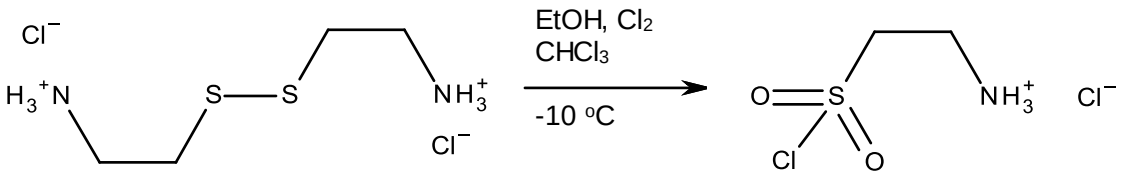
2.1.1 General

Solvents and reagents were obtained from commercial sources (Sigma Aldrich or Fisher Scientific) and used as obtained unless otherwise stated.

NMR spectra were recorded on a 400MHz Bruker Ascend spectrometer (unless stated) and chemical shifts (δ_H or δ_C) are quoted in ppm. The abbreviations used are br: broad, s: singlet, d: doublet, t: triplet, m: multiplet, dd: doublet of doublets, dt: doublet of triplets. Coupling constants (*J*) are quoted in Hertz. Infrared spectra were obtained on a Thermo Nicolet 380 FT-IR spectrometer as neat samples and the absorption peaks are quoted as wavenumbers (cm^{-1}). Mass spectra were recorded on an Agilent 6530 Accurate Mass Q-TOF LC-MS fitted with an Agilent 1290 Infinity Autosampler. Melting points were recorded on a Gallenkamp melting point apparatus.

2.1.2 Synthesis of 1,2-thiazetidine-1,1-dioxide (β-sultam)

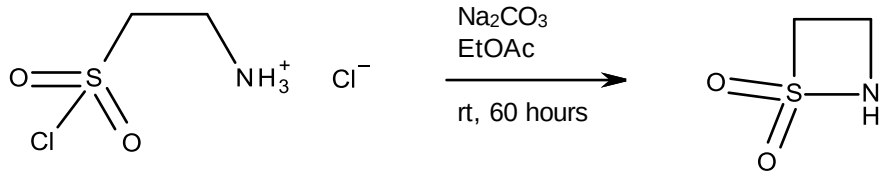
Synthesis of 2-aminoethanesulfonyl chloride hydrochloride (taurine sulfonyl chloride) [2]



[1] [2]

2-(2-Aminoethyldisulfanyl)ethanamine dihydrochloride (cystamine dihydrochloride) [1] (17.38g, 77.0mmol) was suspended in chloroform (400ml) and ethanol (200ml). Chlorine gas was passed into the suspension at -10 °C for 2 hours until saturation (noted by a permanent green colouration). The system was purged with nitrogen, dry diethyl ether (100ml) was added and the solution stirred at room temperature for 2 hours. The white precipitate was recovered by vacuum filtration and washed with dry diethyl ether (2 x 25ml) to yield a white crystalline solid (24.9g, 138mmol, 89%). This was then used directly in the next step without further analysis.

Synthesis of 1,2-thiazetidine-1,1-dioxide (β-sultam) [3]



[2] [3]

2-Aminoethanesulfonyl chloride hydrochloride (taurine sulfonyl chloride) [2] (8.500g, 47.0mmol) was added to anhydrous sodium carbonate (20.93g, 194mmol) in ethyl acetate (250 ml) and stirred at room temperature for 60 hours. The reaction mixture was filtered through celite and the solvent removed by reduced pressure rotary evaporation at 30 ^{o}C to afford a white crystalline solid (1.11g, 10.4mmol, 22%).

m.p. 52-54 ^{o}C (literature value 53 ^{o}C).[139]

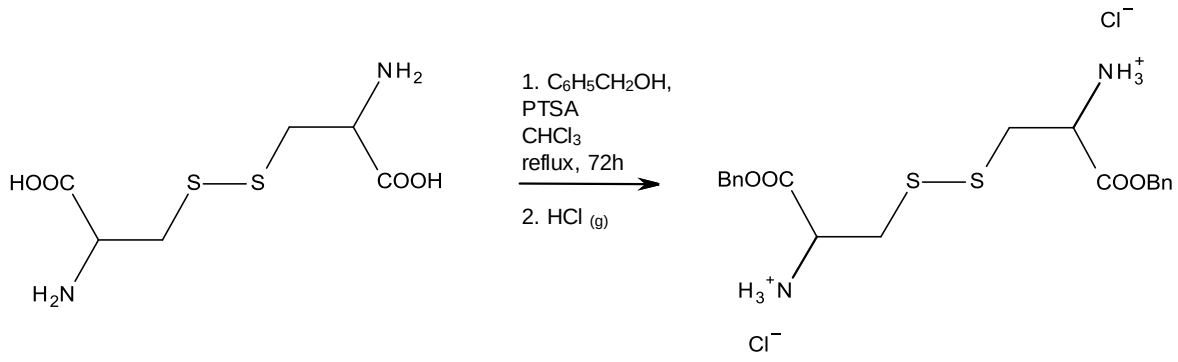
IR υ_{max} (cm^{-1}) (Neat): 3308, 2958, 2021, 1298, 1260, 1150 (**S=O**), 799.

^{1}H NMR δ ($CDCl_3$): 5.58 (1H, br s, **NH**), 4.32 (2H, dt, *J1.78 & 6.96*, **CH_2**-SO_2), 3.39 (2H, dt, *J3.87 & 6.96*, **CH_2**-NH). (literature values 3.39 (2H, dt, *J4 & 7*, **CH_2**NH), 4.32 (2H, dt, *J2 & 7*, SO_2**CH_2**), 5.53 (1H, bs, **NH**)).[210]

^{13}C NMR δ ($CDCl_3$): 60.91 (**CH_2**-SO_2), 28.08 (**CH_2**-NH). (literature values 26.8, 60.6).[210]

2.1.3 Synthesis of 1,2-thiazetidine-3-carboxylate-1,1-dioxide (3-carboxy-β-sultam)

Synthesis of benzyl 2-amino-3[(2-amino-3-benzylcarboxy-propyl)disulfonyl] propanoate dihydrochloride (L-cystine dibenzyl ester dihydrochloride) [5]



[4] [5]

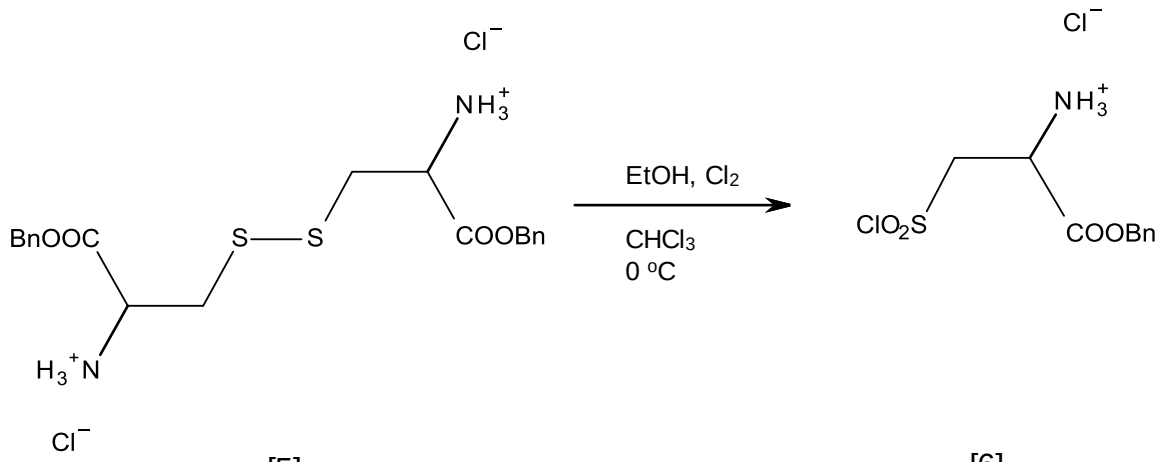
2-Amino-3[(2-amino-3-carboxy-3-propyl)disulfanyl]propanoic acid (L-cystine) [4] (20.39g, 85.0mmol), 4-methylbenzenesulfonic acid (para-tolusenesulfonic acid) (39.95g, 210mmol) and phenyl methanol (benzyl alcohol) (107.6g, 995mmol) were added to chloroform (500ml) and heated to reflux (105 ^{o}C) under modified Dean and Stark conditions for 72 hours. The solution was cooled to room temperature and poured onto diethyl ether (800ml). The precipitate was collected by gravity filtration, dissolved in saturated sodium hydrogen carbonate solution (800ml), extracted with diethyl ether (4 x 200ml) and dried over sodium sulfate. Hydrogen chloride gas was passed through the solution until precipitation was complete. The white solid was recovered by gravity filtration and allowed to air dry (18.6g, 38.0mmol, 44%).

m.p. 165-169 ^{o}C (dec.); (lit. 166 ^{o}C dec.)[211]

IR υ_{max} (cm^{-1}) (Neat): 3365, 3032-2594 (**N-H**), 1756 + 1737 (**C=O**), 1600, 1561, 1537, 1489, 1270, 1248, 1203, 1125, 1098, 1061, 901, 823, 727, 695. (literature values 3100-2500 (**NH**), 1755, 1735 (**CO**))[156]

^{1}H NMR δ (d_6-DMSO): 8.88 (6H, br s, 2x **NH_3**), 7.43 (10H, m, 2x **C_6H_5**), 5.23 (4H, m, 2x **CH_2**Ph), 4.45 (2H, s, 2x CH_2**CH**COO), 3.34 (4H, m, 2x S**CH_2**CH).

Synthesis of benzyl 2-amino-3-(chlorosulfanyl)propanoate hydrochloride [6]



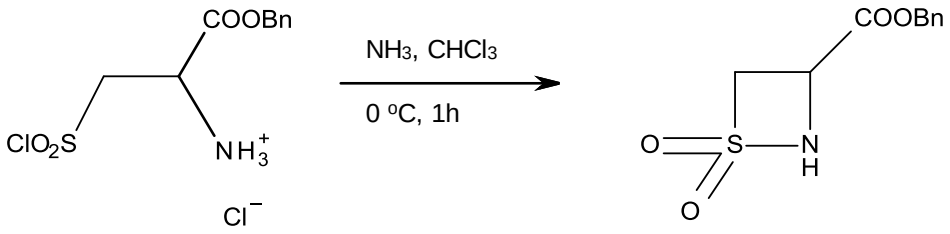
[5] [6]

Benzyl 2-amino-3[(2-amino-3-benzylcarboxy-propyl)disulfanyl]propanoate dihydrochloride (L-cystine dibenzyl ester dihydrochloride) [5] (6.900g, 14.0mmol) was suspended in chloroform (50ml) and ethanol (25ml) at 0 °C. Chlorine gas was passed through the solution to saturation (noted by a permanent green colouration). Cold dry diethyl ether (50ml) was added and the system purged with nitrogen. The flask was stored overnight at 4 °C. The precipitate was recovered by vacuum filtration and washed with ethyl acetate (2 x 50ml) to afford the product as a fine white powder (6.40g, 21.3mmol, 75%).

IR υ_{max} (cm^{-1}) (Neat): 3100-2600 (**N-H**), 2975, 1739 (**C=O**), 1534, 1385, 1369, 1244, 1166 (**O=S=O**), 1083, 1034, 901, 877, 737, 695, 520. (literature values 3100-2600 (**NH**), 1750, (**CO**), 1385, 1170 (**SO_2**)).[156]

^{1}H NMR δ (d_6-DMSO): 8.41 (~2H, br s, **NH_3^+**), 7.44 (5H, m, **C_6H_5**), 5.24 (2H, dd, **CH_2**Ph), 4.49 (2H, m, **CH_2SO_2Cl**), 3.03 (1H, m, **CH**NH_2). (literature values 3.0 (m, 2H, **3-H_2**), 4.40 (m, 1H, **2-H**), 5.20 (AB, 2H, **Ar-CH_2**), 7.40 (s, 5H, **ArH**), 8.55 and 14.4 (br, m, 3H, **NH_3^+**)).[156]

Synthesis of benzyl 1,2-thiazetidine-3-carboxylate-1,1-dioxide (3-benzyl carboxylate β-sultam) [7]



[6] [7]

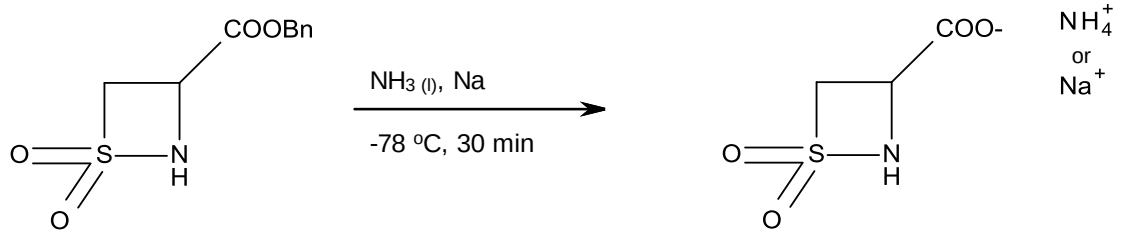
Benzyl 2-amino-3-(chlorosulfanyl)propanoate hydrochloride [6] (2.340g, 7.80mmol) was suspended in chloroform (40ml) and stirred vigorously at 0 °C. A solution of saturated ammonia in chloroform (40ml) was added slowly and the resulting solution stirred for 1 hour. The solution was concentrated by reduced pressure rotary evaporation, ethyl acetate (20ml) added and washed with distilled water (2 x 125ml) until the organics were clear. The solvent was removed by reduced pressure rotary evaporation to leave a yellow oil which solidified on cooling to yield a waxy pale yellow solid (0.98g, 4.07mmol, 52%).

IR υ_{max} (cm^{-1}) (Neat): 3283 (**N-H**), 1745 (**C=O**), 1306, 1288, 1204, 1145 (**S=O**), 1074, 739, 732, 696, 674, 464. (literature values 3320 (**NH**), 1740, (**CO**), 1320, 1300, 1155 (**SO_2**)).[156]

^{1}H NMR δ ($CDCl_3$): 7.45 (5H, m, **C_6H_5**), 6.12 (0.75H, br s, **NH**), 5.33 (2H, s, **CH_2**Ph), 4.63 (1H, dd, *J = 8.92 & 13.06*, C**H^A**$H^B SO_2$), 4.44 (1H, dd, *J = 4.27 & 13.06*, CH^A**$H^B SO_2$**), 4.27 (1H, dd, *J = 4.27 & 8.92*, **CH**NH). (literature values 4.15 (dd, *J = 10.2 Hz, 2 Hz* 1H, **3-H**), 4.32 (dd, *J = 13 Hz, 2 Hz*, 1H, **4-H**), 4.57 (dd, *J = 13 Hz, 10.2 Hz*, 1H, **4-H'**), 5.22 (s, 2H, **Ar-CH_2**), 7.37 (m, 5H, **ArH**)).[156]

^{13}C NMR δ ($CDCl_3$): 183 (**C**OO), 141 (**Ar-C**), 129 (**Ar-C**H), 128 (**Ar-C**H), 127 (**Ar-C**H), 64 (**CH_2**-SO_2), 52 (**CH**-NH).

Synthesis of 1,2-thiazetidine-3-carboxylate-1,1-dioxide (3-carboxy-β-sultam) [8]



[7] [8]

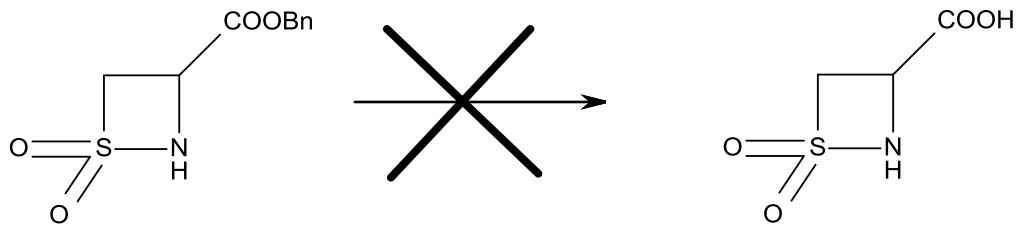
Benzyl-1,2-thiazetidine-3-carboxylate-1,1-dioxide (363.1mg, 1.50mmol) was dissolved in liquid ammonia (35ml) at -78 °C under a nitrogen atmosphere. Sodium metal (50.09mg, 2.18mmol) was added until the solution just remained deep blue. Ammonium chloride (37.34mg, 0.70mmol) was added to dissipate the blue colour and the ammonia was allowed to evaporate under a stream of nitrogen to yield a white solid (450.12mg, includes NH_4Cl salt).

^{1}H NMR δ (D_2O): 4.57 (1H, dd, *J8.78 & 13.96*, C**H**^{A}H^{B}SO_2), 4.23 (1H, dd, *J5.34 & 13.96*, CH^{A}**H**^{B}SO_2), 3.98 (1H, dd*, J5.34 & 8.78*, **CH**NH).

^{13}C NMR δ (D_2O): 181 (**C**OOH), 64 (**CH_2**-SO_2), 43 (**CH**-NH).

m/z (LC-MS-QTOF) 149.97 $(M-H)^-$, 105.98 (M-COOH)-.

2.1.4 Attempted syntheses of 1,2-thiazetidine-3-carboxylate-1,1-dioxide (3-carboxy-β-sultam) [8]



[7] [8]

Attempt 1: Catalytic Hydrogenolysis

5% palladium on carbon (29.10mg) was added to a bubble column and purged with nitrogen for 5 minutes. 3-Benzyl carboxylate β-sultam (301.4mg, 1.25mmol) was dissolved in methanol (10ml), added to the column and purged for a further 10 minutes. Hydrogen gas was bubbled through the solution for 1 hour at a flow rate of 40 ml/min. The catalyst was removed by filtering through celite and the methanol removed by rotary evaporation to yield a white powder.

Attempt 2: Catalytic Hydrogenolysis

10% palladium on carbon (35.68mg) was added to a bubble column and purged with nitrogen for 5 minutes. 3-Benzyl carboxylate β-sultam (316.8mg, 1.31mmol) was dissolved in methanol (25ml), added to the column and purged for a further 10 minutes. Hydrogen gas was bubbled through the solution for 2 hours at a flow rate of 40 ml/min. The catalyst was removed by filtering through celite and the methanol removed by rotary evaporation to yield a white powder.

Attempt 3: Transfer Hydrogenolysis

10% palladium on carbon was added to a flask and purged with nitrogen for 5 minutes. 3-Benzyl carboxylate β-sultam (139.9mg, 0.58mmol) was dissolved in ethanol (29ml) and added to the flask with 1,4-cyclohexadiene (530µl, 449mg, 5.6mmol) and stirred under a nitrogen atmosphere at 20 ^{o}C for 24 hours. The

catalyst was removed by filtering through celite and the solvents removed by rotary evaporation to yield a white powder.

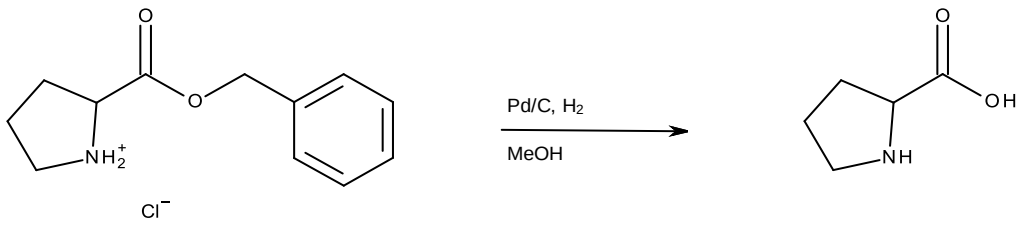
Attempt 4: Base Catalysed Hydrolysis

3-Benzyl carboxylate β-sultam (116.5mg, 0.483mmol) was dissolved in 1M NaOH (5ml) and stirred for 10 minutes. Half the solution was removed and the remainder allowed to stir for a further 30 minutes. Upon removal from the reaction flask both samples were treated as follows: the solution was extracted with DCM (2 x 1.5ml), the aqueous portion was acidified with 2M HCl (1ml), extracted into diethyl ether (2 x 1ml) and the solvent removed by rotary evaporation.

Attempt 5: Base Catalysed Hydrolysis

3-Benzyl carboxylate β-sultam (931mg, 3.86mmol) was dissolved in 1.0M NaOH (9.5ml) and stirred for 2 minutes at 0 ^{o}C. The benzyl alcohol formed was extracted with DCM (3 x 6ml). 25ml DCM was added to the aqueous portion which was acidified with dropwise addition of 2.0M HCl until the solution reached pH 1. The organic layer was separated and a further extraction of the aqueous portion carried out with DCM (8ml). These two organic solutions were combined and the solvent removed by reduced pressure rotary evaporation.

2.1.5 Debenzylation of proline benzyl ester hydrochloride



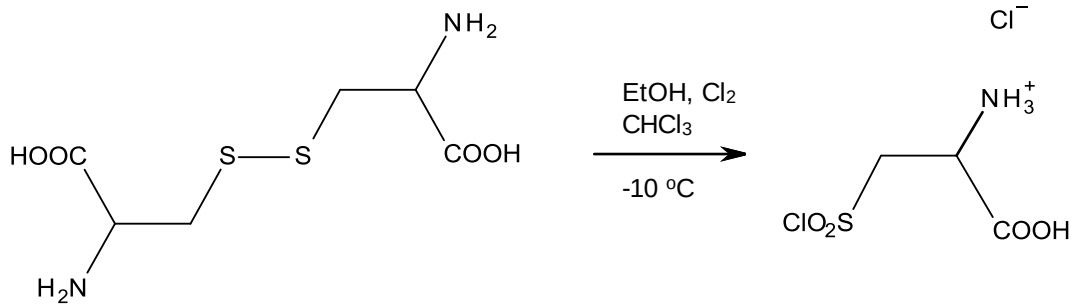
10% palladium on carbon (28.10mg) was added to a bubble column and purged with nitrogen for 5 minutes. Proline benzyl ester (232.6mg, 0.97mmol) was dissolved in methanol (10ml), added to the column and purged for a further 10 minutes. Hydrogen gas was bubbled through the solution for 2 hours at a flow rate of 30 ml/min. The catalyst was removed by filtering through celite and the methanol removed by rotary evaporation to yield an orange oil.

IR υ_{max} (cm-1) (Neat): 3382, 2958, 1622, 1553, 1450 (**C-N**), 1376 (**C-H**), 1318, 12901, 1266, 1171, 1086, 1038, 988, 946, 910, 898.

^{1}H NMR δ (D_2O): 4.27 (1H, m, **CH**-COO^-), 3.22 (1H, m, **CH^{A}**H^{B}-NH), 3.20 (1H, m, CH^{A}**H^{B}**-NH), 2.54 (1H, m, **CH^{C}**-H^{D}-CHCOO), 2.25 (1H, m, CH^{C}-**H^{D}**-CHCOO), 1.91 (2H, m, **CH_2**). (literature values 4.13, 3.42, 3.34, 2.35, 2.07, 2.00).[212]

2.1.6 Attempted cyclisation of 2-amino-3-chlorosulfonyl-propanoic acid

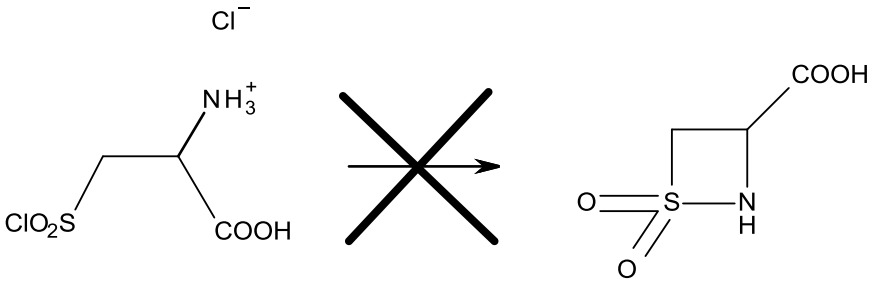
Synthesis of 2-amino-3-chlorosulfonyl-propanoate hydrochloride (L-cysteine sulfonyl chloride)



2-Amino-3[(2-amino-3-carboxy-3-propyl)disulfanyl]propanoic acid (L-cystine) (4.75g, 19.8mmol) was suspended in ethanol (18ml) and chloroform (30ml). Chlorine gas was passed into the suspension at -10 ^{o}C for 2 hours until saturation (noted by a permanent green colouration). The system was purged with nitrogen and the solution stirred at room temperature for 1 hours. Dry diethyl ether (30ml) was added and the solution stored at 4 ^{o}C overnight. The pale yellow precipitate was recovered by gravity filtration and washed with dry diethyl ether (2 x 25ml) (4.14g, 30.6mmol, 77%).

^{1}H NMR δ (D_2O): 4.44 (1H, dd, *J4.5 & 8.3*, **CH**-NH_3^+), 3.60 (1H, dd, *J4.5 & 15.2*, C**H**^{A}H^{B}-SO_2Cl), 3.28 (1H, dd, *J8.3 & 15.2*, CH^{A}**H**^{B}-SO_2Cl).

Synthesis of 1,2-thiazetidine-3-carboxylate-1,1-dioxide (3-carboxy-β-sultam) [8]



2-amino-3-(chlorosulfanyl)propanoate hydrochloride (L-cysteine sulfonyl chloride) (2.12g, 9.51mmol) and sodium carbonate (3.44g, 32.4mmol) were ground together and suspended in ethyl acetate (80ml). The suspension was stirred at room temperature for 24 hours. The solid residue was recovered by gravity filtration as an off white solid (531mg, 3.52mmol, 37%).

Analysis of the solid product by ^{1}H NMR indicated that the required compound had not been prepared as there were no doublet of doublet peaks in the region from 5 to 3 ppm. Further analysis to identify what had been made was not carried out.

2.2 Kinetic studies

2.2.1 Solutions and Buffers

Hydrochloric acid solutions were prepared from AnalaR grade hydrochloric acid 1.18 SG ~37 %. Solutions of deuterium chloride and sodium deuteroxide were prepared by diluting DCl (20 wt % in D_2O, 99.5 % D) and NaOD (30 wt %, 99+ % D) with D_2O (99.9 % D). AnalaR grade reagents were used where available in the preparation of buffers and Ultra-Pure Deionised water at 18.2 Ω was used throughout. The ionic strength of buffers was maintained at 1.0 M using AnalaR grade potassium chloride in all cases. Where buffers were required for use in NMR studies the appropriate masses of buffer salts and potassium chloride were dissolved in a minimum quantity of D_2O, freeze-dried and re-dissolved in the appropriate volume of D_2O just prior to use.

The buffers used were chloroacetic acid/chloroacetate (pK_a = 2.88), acetic acid/acetate (pK_a = 4.76), phosphate (pK_a = 2.15, 7.2, 12.33) and carbonate (pK_a = 10.25).

2.2.2 pH Measurements

The pH of aqueous buffers was measured at the start and end of each reaction to ensure no significant change in pH had occurred. The pH of buffer solutions was measured using a Metrohm 751 GPD Titrino instrument fitted with an Ag/AgX electrode. Calibration was carried out using standard buffers of pH 9.2, 7.0 and 4.0 at 25 ^{o}C prior to each use.

For deuterated buffers the pH was measured upon preparation as above and at the end of the reaction using a Sigma-Aldrich Micro pH Combination Electrode calibrated as previously stated. For deuterated solutions pD is quoted as pH meter reading +0.4.

2.2.3 Determination of the Acid-Catalysed Rate of Hydrolysis of 1,2-thiazetidine-1,1-dioxide (β-sultam) using ReactIR

The kinetics of the acid-catalysed hydrolysis of β-sultam were followed by monitoring the decrease in reactant concentration at 1301 cm^{-1} (S=O stretch) and the increase in product concentration, monitored at 1200 cm^{-1} (S=O stretch). IR spectra were recorded at regular intervals on a Mettler Toledo ReactIR 4000 fitted with a 6.3mm AgX Di-Comp Probe in the range 3000 – 650 cm^{-1} and the reaction followed until no further change in absorbance at either wavenumber was observed.

Reaction concentrations of β-sultam were between 0.05 mM and 0.20 mM.

Reactions were started by addition of acid or buffer solution (1.5 ml) to a stirring solution of β-sultam (10 mg – 40 mg) in deionised water (0.25 ml).

Pseudo-first order rate constants were calculated using Microsoft Excel from exponential plots of corrected absorbance versus time. pH rate profiles and buffer catalysis rates were all calculated by curve plotting in Excel software.

2.2.4 Determination of the Rate Constants for the Hydrolysis of 1,2-thiazetidine-3-carboxylate-1,1-dioxide (3-carboxy-β-sultam) using ^{1}H NMR

The rates of hydrolysis of 3-carboxy-β-sultam were followed using ^{1}H NMR spectra recorded on a Bruker Ascend 400 spectrometer at 20 ^{o}C. Hydrolysis of the S-N bond is accompanied by a decrease in the chemical shift of the three ring protons. The change in integral for each of the protons was plotted against time to give a rate related to that proton and the average of these gave the overall rate for that reaction. The concentration of 3-carboxy-β-sultam was between 0.210 and 0.248 M.

Pseudo-first order rate constants were calculated using Microsoft Excel from exponential plots of integration value versus time. pH rate profiles and buffer catalysis rates were all calculated by curve plotting in Excel software.

2.3 Enzyme Studies

2.3.1 Inhibition of BcII

2.3.1.1 Solutions and Equipment

The enzyme used was the Class B β-lactamase enzyme, BcII, from *Bacillus cereus* 569/H which was kindly supplied by Dharmit Mistry (University of Huddersfield) as solutions in MES buffer at pH 6.5 and at known concentrations between 2 and 6 mM. It was diluted prior to use with pH 7.1 HEPES buffer containing 0.1 μM $ZnSO_4$. Buffers were prepared daily using AnalaR reagents where available and Ultra-Pure Deionised water at 18.2 Ω was used throughout. The ionic strength of buffers was maintained at 1.0 M using AnalaR potassium chloride or sodium chloride.

All UV-Vis experiments were carried out on a Cary 4000 UV-Vis spectrometer using quartz cells at 30 ^{o}C with a water circulator and peltier system to maintain the temperature.

2.3.1.2 Reactions

25 μl stock penicillin solution was added to a quartz cell containing 2.5 ml pH 7.1 HEPES buffer solution (0.1 M, I= 1.0 M, $[Zn^{2+}]$ = $1x10^{-6}$ M) and equilibrated at 30 ^{o}C. Inhibitor solution was added to the cell with an appropriate volume of ultra-pure water to maintain a constant volume across all experiments. The experiment was initiated by addition of enzyme solution (20 μl of known concentration between 1.3×10^{-6} and 7.4×10^{-6} M). The final concentrations within the cell were:

Penicillin 0.12 - 1.10 mM

Inhibitor 1.0 μM – 25.0 mM

Enzyme 1.0×10^{-8} – 5.5×10^{-8} M

Hydrolysis of benzylpenicillin was followed by measuring the decrease in absorbance at 230 nm as a function of time.

2.3.1.3 Michaelis-Menten Kinetics

Previous publications have shown that the action of BcII on benzylpenicillin follows standard Michaelis-Menten kinetics (Equation 1) and the kinetic constants, k_{cat}, K_M and k_{cat}/K_M were determined using this equation and its derivations as described below.

$$\boldsymbol{rate = \frac{k_{cat}\,[E][S]}{K_M + [S]}} \qquad \textbf{(Equation 1)}$$

Below saturation, when [S] << K_M, the curves were fitted to a simple first order rate law to obtain the pseudo-first-order rate constant k_{obs}, which were shown to be first order in enzyme concentration and independent of substrate concentration. Above saturation, when [S] >> K_M, the curves were fitted to a zero order rate law to give the V_{max} for the reaction. The second order rate constant $\frac{k_{cat}}{K_M}$ was obtained by dividing k_{obs} by enzyme concentration; k_{cat} was obtained by dividing V_{max} by enzyme concentration.

In order to best analyse the kinetics the data was also fitted to the following linear forms of the Michaelis-Menten equation:

Lineweaver-Burk: $\boldsymbol{\frac{1}{rate} = \frac{K_M}{V_{max}}\frac{1}{[S]} + \frac{1}{V_{max}}}$

Hanes-Woolf: $\boldsymbol{\frac{[S]}{rate} = \frac{[S]}{V_{max}} + \frac{K_M}{V_{max}}}$

Eadie-Hofstee: $\boldsymbol{rate = -K_M\,\frac{rate}{[S]} + V_{max}}$

A Lineweaver-Burk plot gives $^1/_{V_{max}}$ (and hence k_{cat}) as the y intercept and $^{-1}/_{K_M}$ as the x intercept. Because it is a double reciprocal plot, of $^1/_{rate}$ versus $^1/_{[S]}$, any errors in measurement are increased and so the parameters calculated in this way are not very reliable.

Hanes-Woolf plots can again be used to determine values of K_M and V_{max}. A plot of $^{[S]}/_{rate}$ against [S] gives the x intercept as $-K_M$ and the slope as $^1/_{V_{max}}$.

The Eadie-Hofstee equation is represented graphically as a plot of rate versus $^{rate}/_{[S]}$ which yields V_{max} as the y intercept and $-K_M$ as the slope.

When plotting data in Microsoft Excel software for initial analysis the Hanes-Woolf version of the Michaelis-Menten equation gives easiest access to the parameters k_{cat} and K_M and is most reliable so was used most often when looking at BcII inhibition.

2.3.1.4 Inhibition studies

The kinetic parameters were determined from the second-order rate constant, $\frac{k_{cat}}{K_M}$. Inhibition constants, K_i, were calculated using the equation for competitive inhibition (Equation 2) which can be rearranged to give Equation 3.

$$\boldsymbol{rate = \frac{k_{cat}[E][S]}{[S]+K_M[\frac{K_i+[I]}{K_i}]}} \qquad \textbf{(Equation 2)}$$

$$\boldsymbol{\left[\frac{K_M}{k_{cat}}\right]_I = \left[\frac{K_M}{k_{cat}}\right]_0 + \left[\frac{K_M}{k_{cat}}\right]_0 \left[\frac{[I]}{K_i}\right]} \qquad \textbf{(Equation 3)}$$

By plotting $\left[\frac{K_M}{k_{cat}}\right]_I$ against [I] the value of $-K_i$ is given by the intercept on the inhibitor concentration axis.

2.3.2 Inhibition of Glutamine Synthetase

2.3.2.1 Solutions and Equipment

L-Glutamine synthetase from *Escherichia coli W* was obtained from Sigma Aldrich as a lyophilised powder containing >30% protein and having an activity of 100 - 400 units/mg protein. The enzymes required for the coupled assay (details in section 2.3.2.2) were pyruvate kinase and L-lactic dehydrogenase from rabbit muscle obtained from Sigma Aldrich as a buffered aqueous glycerol solution containing 900-1400 units/ml LDH and 600 - 1000 units/ml PK. All other reagents were obtained from commercial sources as AnalaR grade products where available and used as

supplied. Buffer solutions and solutions of ATP, PEP and NADH were prepared fresh in all cases. Ultra-Pure Deionised water at 18.2 Ω was used throughout.

2.3.2.2 Reactions

The rate of conversion of glutamate to glutamine catalysed by glutamic acid was monitored by means of the coupled assay detailed in Scheme 44.

Three cells were prepared simultaneously according to the following procedure to act as a blank cell, control cell and an inhibition cell. Phospho(enol)pyruvate (0.1 ml) and β-NADH (0.06 ml) were added to a quartz cell containing 2.6 ml reaction cocktail (imidazole buffer at pH 7.1, glutamate, ATP, magnesium chloride, potassium chloride and ammonium chloride) and incubated at 37 ^{o}C until the absorbance at 340 nm was constant. Pyruvate kinase/L-lactic dehydrogenase solution (0.04 ml) was then added and the absorbance at 340 nm again monitored until it was constant at 37 ^{o}C. The following reagents were then added to the three cells:

Blank: 0.2 ml ultra-pure water

Control: 0.1 ml ultra-pure water and 0.1 ml glutamine synthetase solution

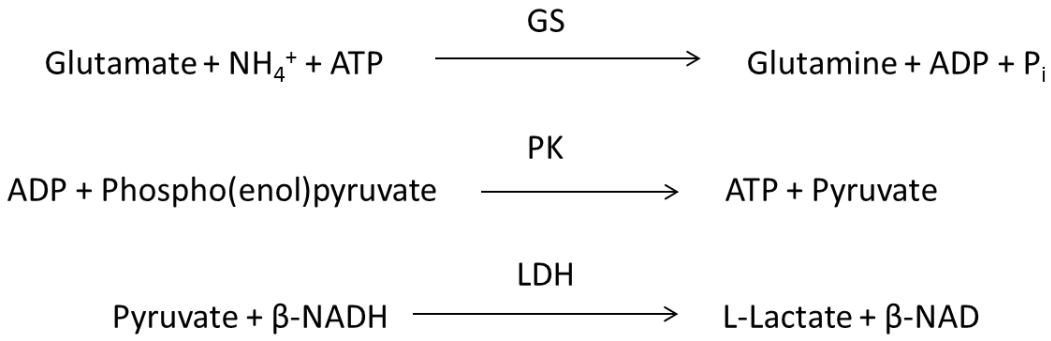
Inhibition: 0.1 ml inhibitor solution and 0.1 ml glutamine synthetase solution.

The final concentrations within each cell were:

Imidazole	34.1 mM
Sodium glutamate	102 mM
ATP	8.50 mM
PEP	1.10 mM
Magnesium chloride	60.0 mM
Potassium chloride	18.9 mM
Ammonium chloride	45.0 mM
β-NADH	0.250 mM
PK	28.0 units
LDH	40.0 units

Experimental

Inhibitor	1.00×10^{-4} – 1.00×10^{-1} M
GS	0.400 – 0.800 units



Scheme 44 Glutamine synthetase coupled assay

The concentrations of PK and LDH were kept high so that the rate of conversion of glutamate to glutamine was the overall rate limiting reaction. The oxidation of β-NADH was followed by measuring the decrease in absorbance at 340 nm as a function of time. This was taken to be equivalent to the rate of glutamate conversion to glutamine.

2.3.2.3 Calculations

The glutamine synthetase catalysed conversion of glutamate to glutamine is a reaction which follows Michaelis-Menten kinetics, as described in section 2.3.1.3.

The effects of any inhibitor on the rate of this reaction were determined by the comparison of initial rates (where [S] >> K_M) so that the initial rate was equal to V_{max} when the curves were fitted to a zero order rate law. The kinetic parameters k_{cat} and K_i were not specifically calculated. K_M values were calculated from Hanes-Woolf plots so that comparisons could be made between control rates and inhibitor reaction rates, and between rates of reaction with different inhibitor concentrations.

2.4 Mechanistic Study of BcII Catalysed Hydrolysis of Ertapenem

2.4.1 Solutions and Buffers

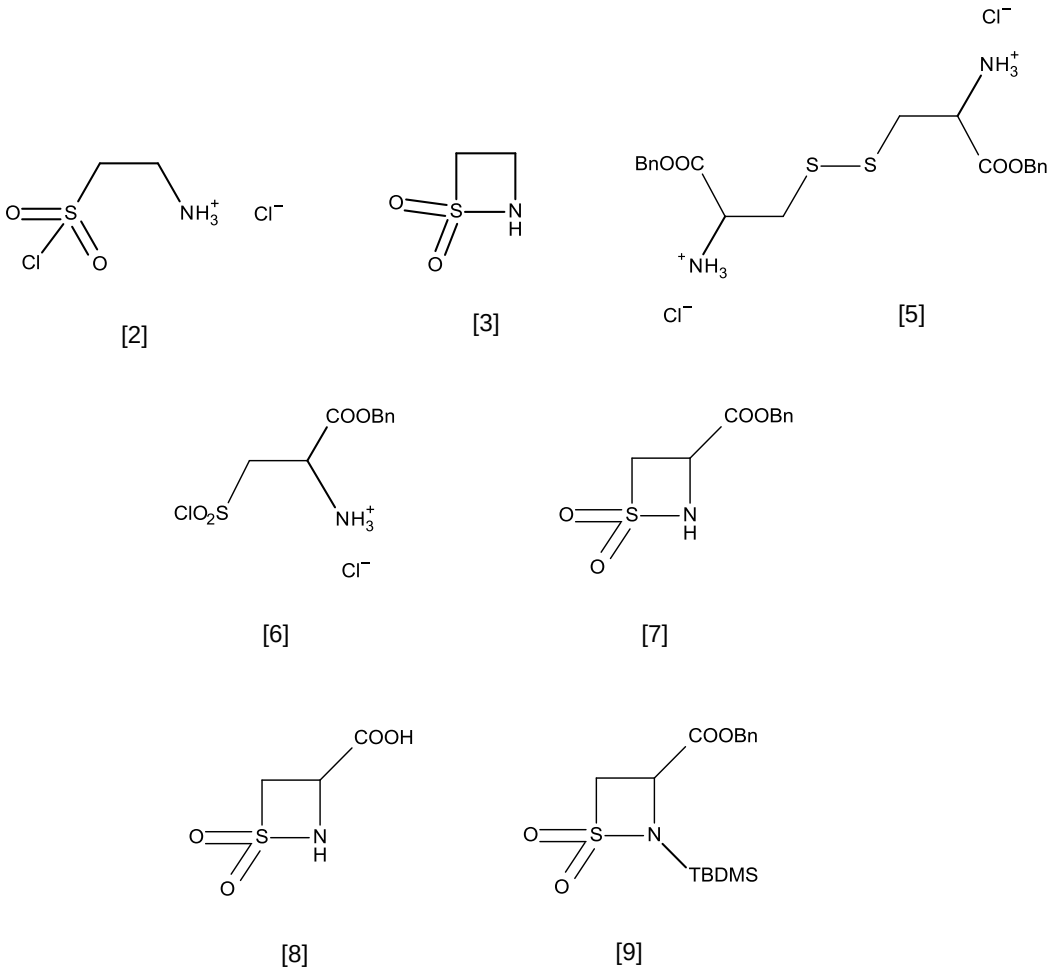
Ertapenem was kindly provided by Merck & Co. BcII was provided by Dharmit Mistry as a solution in MES buffer at pH 6.5 at a fixed concentration between 2 and 6 mM and was used as supplied. Phosphate buffer (0.1 M, pH 7.0, I = 1.0 M) was prepared in deuterium oxide, freeze dried and redissolved in D_2O just prior to use.

2.4.2 Mechanistic studies

The mechanism of BcII catalysed hydrolysis of ertapenem was investigated using NMR spectroscopy with spectra which were recorded on a Bruker Ascend 400 NMR spectrometer. Ertapenem (13.7 mg, 0.288 μmol) was dissolved in deuterated phosphate buffer (0.7 ml) and the solution monitored by NMR for 48 hours to ensure no uncatalysed hydrolysis took place. BcII (5.0 μl) was added to the solution and this solution was monitored for a further 12 hours. ^{1}H, ^{13}C, DEPT, COSY, HSQC and HMBC spectra of the compound before and after hydrolysis by BcII were compared to propose the mechanism of ring cleavage for the β-lactam ring.

Results and Discussion

3.1 Synthesis



Scheme 45 Compounds synthesised and/or discussed in this section

Compounds 2, 3, 5, 6 and 7 have been previously reported and were synthesised following the procedures suggested either by former members of the group or, in the case of compound 7, by Otto *et al*.[156,210] In all cases analytical data for the products synthesised corresponded to that published. ^{13}C NMR data for compound [7] has not previously been reported; the spectrum showed that the carboxyl carbon gave a peak at 181.6 ppm, there were also peaks at 140.7, 129.1, 127.6 and 127.3 ppm for the aromatic carbons, the benzyl CH_2 carbon appeared at 67.3 ppm and those at 63.9 and 51.9 ppm were peaks for the β-sultam ring carbons (CH_2 and CH respectively).

Compound 8 is a novel compound; previous work only reported isolation of the N-substituted version.[156,210] Following the methods for the de-esterification of the N-substituted compound [9] suggested by both Rigoreau[210] and Schwenkkraus[156] initial attempts to form the 3-carboxy-β-sultam were made using hydrogenolysis. When preliminary reactions were not successful further attempts were made using different conditions to those originally suggested (following the general procedures set out in the Experimental section, pages 89-90). The alternative solvents used were methanol, ethanol, benzyl alcohol and ethyl acetate; alternative catalysts (5% or 10% palladium on carbon) at different catalyst loadings were utilised; the time period of the reaction was increased (1 hour to 2 days) and the flow rate of hydrogen was monitored and increased (10 ml/min to 40 ml/min) but in all cases the product isolated was either the starting material (under mild conditions, short reaction time etc.) or the ring opened β-sultam. Ester cleavage was seen in a few attempts, but only when accompanied by S-N bond cleavage.

In order to validate these methods of de-esterification and to confirm the activity of the catalysts a sample of proline benzyl ester hydrochloride was submitted to some of the reaction conditions described above (as detailed in the Experimental section, page 91). ^{1}H NMR of the isolated product from this reaction confirmed that the benzyl ester group had been removed and that the techniques used were viable. It is therefore concluded that 3-benzyl carboxylate β-sultam [7] is not susceptible to de-esterification by hydrogenolysis without simultaneous S-N cleavage and ring-opening, and so an alternative method was sought.

As a means of trying to overcome the problems encountered with the previous reactions it was decided to try a transfer hydrogenation route to remove the benzyl ester group (details in the Experimental section, page 89). Transfer hydrogenation utilising 1,4-cyclohexadiene as the hydrogen source is less susceptible to catalyst poisoning and benzyl esters can be selectively and efficiently deprotected by this method.[213,214] Catalyst poisoning was suspected as being one reason why catalytic hydrogenolysis had been unsuccessful and so this method initially presented as a viable alternative. Unfortunately, as with the previous reactions, no debenzylated β-sultam was recovered after several attempts. Analysis of the recovered solid in each case showed that whilst the β-sultam ring remained intact the benzyl ester group was still present (determined by the appearance of a benzyl CH_2 peak at 5.2 ppm on the ^{1}H NMR spectrum). Previous work on the removal of benzyl ester groups by transfer hydrogenation concerning amino acids and peptides has also encountered problems with sulfur containing compounds, particularly S-benzylcysteine.[214]

The next method implemented in the attempt to synthesise compound [8] was base catalysed hydrolysis. The rationale to this method was the expectation that hydrolysis of the ester would be faster than that of the β-sultam because the latter becomes pH independent at high pH as the NH ionises. Compound [7] was dissolved in 0.1 M sodium deuteroxide (NaOD) and the reaction was monitored by ^{1}H NMR. Cleavage of the benzyl ester was too fast to observe; the benzyl CH_2 protons had shifted from 5.2 ppm, characteristic of an ester, to 4.6 ppm indicating the formation of benzyl alcohol during the time taken to collect the spectrum. Initial studies showed that the β-sultam ring remained intact with S-N bond cleavage not noticeable until after about 20 minutes. Attempts were therefore made to recover the 3-carboxy-β-sultam from the aqueous reaction mixture by initially removing the benzyl alcohol. The aqueous solution was extracted with deuterated chloroform ($CDCl_3$) and both phases were subjected to NMR analysis. The organic phase showed the presence of the benzyl alcohol which had been extracted into the $CDCl_3$ along with a relatively small amount of the β-sultam [8] or starting ester [7]. The aqueous solution showed the presence of a residual amount of benzyl alcohol with a higher proportion of [8], some of which had undergone hydrolysis and S-N bond fission.

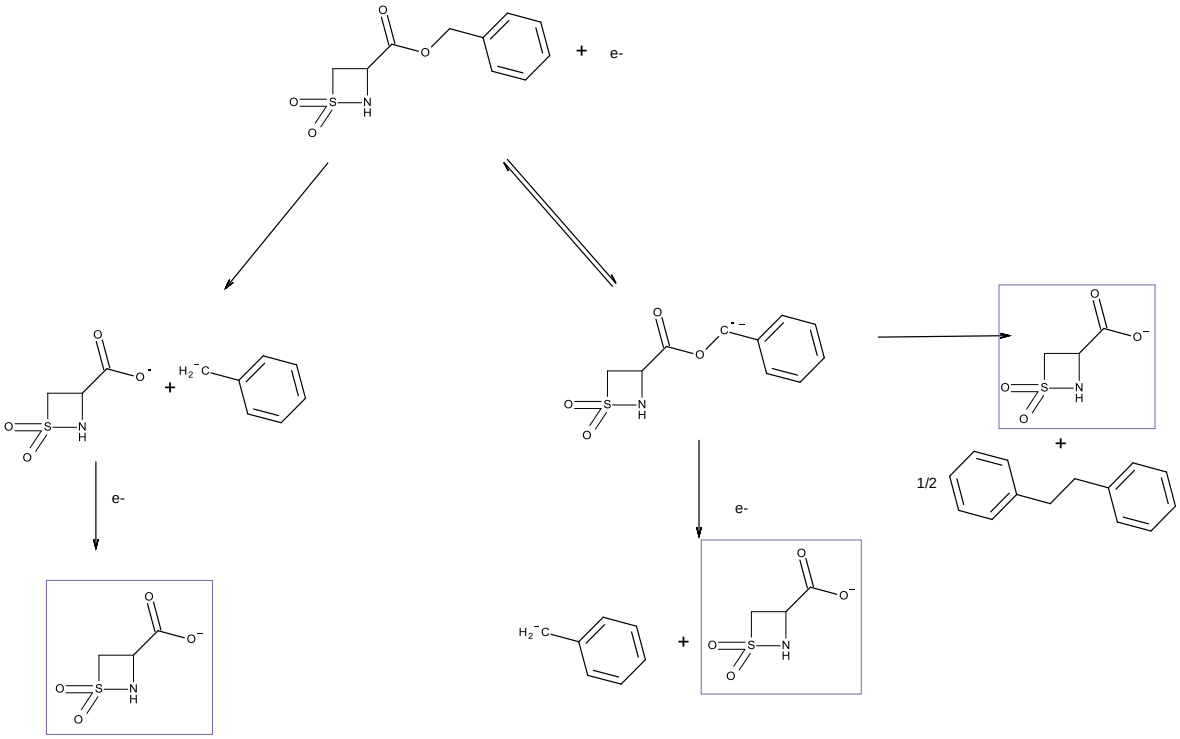
In order to improve the chance of recovering the β-sultam [8] intact benzyl-3-carboxylate β-sultam [7] was hydrolysed in 0.1 M NaOD, this time at low temperature ($\leq$ 0 ^{o}C). The extraction into deuterated chloroform was carried out rapidly and NMRs of both solutions run immediately (aqueous before organic). Again the spectra suggested that hydrolytic removal of the benzyl group had been achieved and that the benzyl alcohol produced had been taken into the organic phase whilst the majority of the β-sultam remained in the aqueous solution. Under these conditions more of the β-sultam ring remained intact and only a small amount of hydrolysis had occurred. Having established that the 3-carboxy-β-sultam could be prepared in this manner the next step in the synthesis was to isolate the product from solution. Attempts were made to acidify the aqueous solution in order to protonate the acid group and facilitate its extraction into an organic solvent; however, ^{1}H NMR showed that the hydrolysis of the S-N bond occurred on acidification. This discovery prompted studies of the pH dependence of hydrolysis of the 3-carboxy-β-sultam as discussed in section 3.2.3.

Since the benzyl ester protecting group was proving difficult to remove and the 3-carboxy-β-sultam was proving difficult to isolate from aqueous solution it was decided to attempt the direct cyclisation of 2-amino-3-chlorosulfonyl-propanoic acid. The 2-amino-3-chlorosulfonyl-propanoic acid was synthesised by reaction of L-cystine with hypochlorous acid (produced *in situ* by the reaction of chlorine and

ethanol in chloroform). The product was characterised by ^{1}H NMR; although no literature reference for this was found, comparison to the NMR of L-cystine and similarities with the ^{1}H NMR of the benzyl ester analogue and L-cysteic acid suggested that the reaction had proceeded as expected and the product was used in the second step. The cyclisation of 2-amino-3-chlorosulfonyl-propanoic acid was attempted initially using the same conditions as for the benzyl protected compound (neutralisation with a saturated solution of ammonia in chloroform) and secondly using the milder conditions utilised in the synthesis of the unsubstituted β-sultam (sodium carbonate suspension in ethyl acetate). In the first reaction the product was a brown solid which showed no ^{1}H NMR peaks characteristic of either the starting material or the desired β-sultam. The second reaction yielded a white solid. ^{1}H NMR of this compound showed numerous peaks between 1 and 5 ppm, some of which were due to contamination by residual solvent. There were three peaks between 2.7 and 4.2 ppm which showed as doublet of doublets as expected for the ABX system in the substituted β-sultam. The A and B proton peaks at 2.8 and 3.0 ppm however were at much lower chemical shifts than expected for the β-sultam and so it was concluded that cyclisation had not occurred. The presence of the ABX splitting can still be accounted for by looking at a Newman projection of the ring-open molecule (as discussed in section 3.2.1).

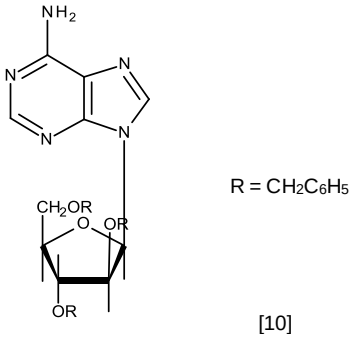
The final and only successful method attempted for the de-esterification of benzyl-3-carboxylate β-sultam [7] and isolation of 3-carboxy-β-sultam [8] was the use of sodium in liquid ammonia; reagents best known for their use in the Birch reduction of aromatic rings.

There are precedents for the use of sodium and liquid ammonia to remove benzyl esters and other functional groups present as N-, S- or carboxyl protecting groups, with the first reports involving amino acids and peptides dating back to the 1930's.[215-217] However, there appear to be very few specific examples of this method of ester removal yielding an acid, the normal products of the reaction being alcohols, diketones and aldehydes. There was no evidence for the presence of any of these functional groups in our studies. Presumably this reductive method involves electron transfer from Na and proceeds via a radical mechanism (Scheme 46).



Scheme 46 Possible mechanisms for the debenzylation of [7] by sodium in liquid ammonia (adapted from [218])

Kharasch *et al.* showed carboxylic acids to be produced as side products in this reaction,[219] yet all indications from our studies imply that the carboxylic acid is the major product. The protocol which was followed for the reaction was taken from a paper concerned with the simultaneous removal of three benzyl protecting groups (though these were benzyl ether groups, not benzyl esters) from 9-(2'-3'-5'-tri-O-benzyl-β-D-arabinofuranosyl)adenine [10].[220] In 2001 Ramos *et al*. also used this method for removing O-benzyl protecting groups, though again as ethers, not esters.



R = $CH_2C_6H_5$

[10]

The reaction to remove the benzyl ester group was carried out as described in the experimental section. After evaporation of the liquid ammonia a white powder

remained of which a sample was dissolved in D_2O and analysed by NMR. The ^1H NMR spectrum was studied for evidence of the benzyl ester, bibenzyl (1,2-diphenylethane) or toluene. In fact, as can be seen in the spectrum in Figure 11, there were no peaks in the aromatic region of the spectrum. There are, however, three doublet of doublet peaks in the region between 4 and 5 ppm, characteristic of the 3-substituted β-sultam ring protons. A small amount of the hydrolysis product was also indicated and there were various other unidentified peaks in the region below 4 ppm.

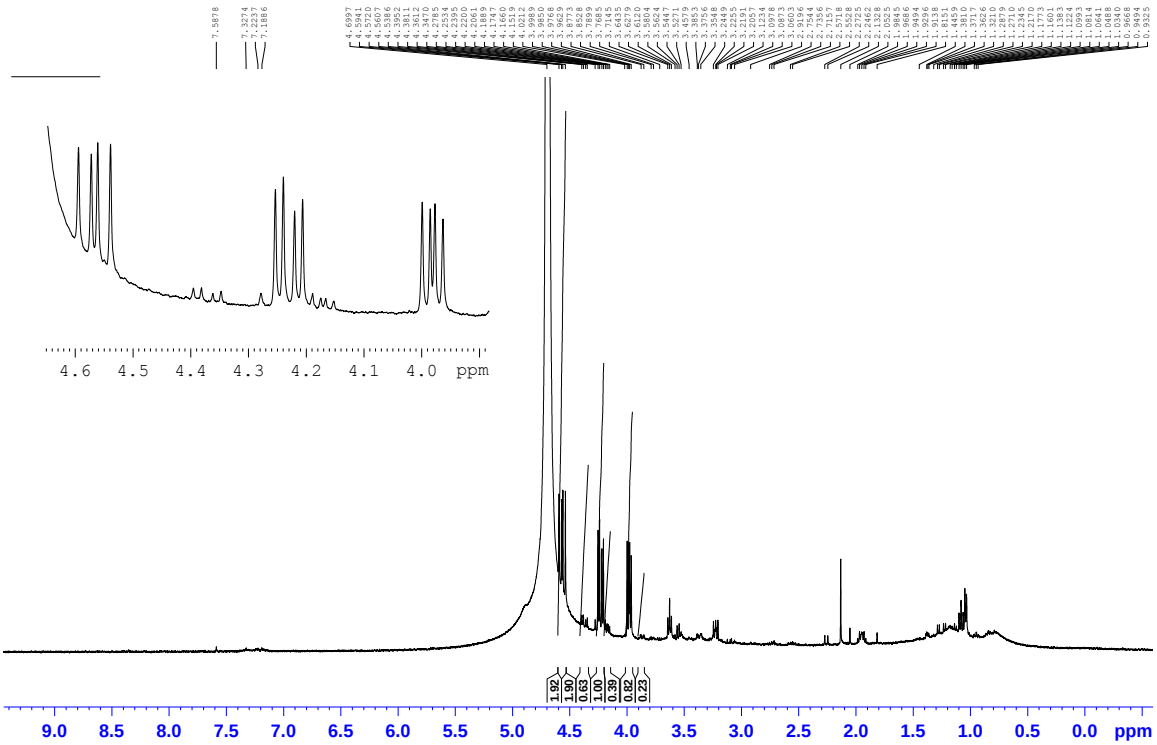
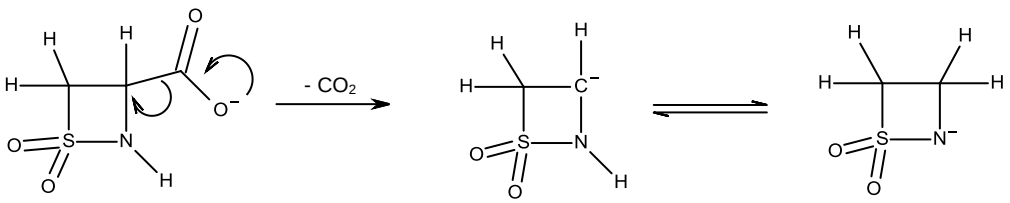


Figure 11 ^1H NMR of 3-carboxy-β-sultam with expansion of the three doublet of doublets characteristic of the β-sultam ABX system

A sample of the crude solid was subjected to mass spectrometry and this showed two major peaks; one at m/z 150 and the second at m/z 106 (Figure 12). The peak at m/z 150 relates to the desired product (accurate mass 149.97033, calculated mass 149.986652) and the one at m/z 106 relates to the decarboxylated product (accurate mass 105.98151, calculated mass 105.996276) as shown in Scheme 47. The isotope patterns for the molecular anion were in-keeping with those predicted. Although the sample masses were not exactly the same as the calculated masses

they are close enough to confirm the structure and any deviance from the actual mass may be due to the low mass of the molecule.



Scheme 47 Structures of the two ions suggested by mass spectrometry and the mechanism of decarboxylation

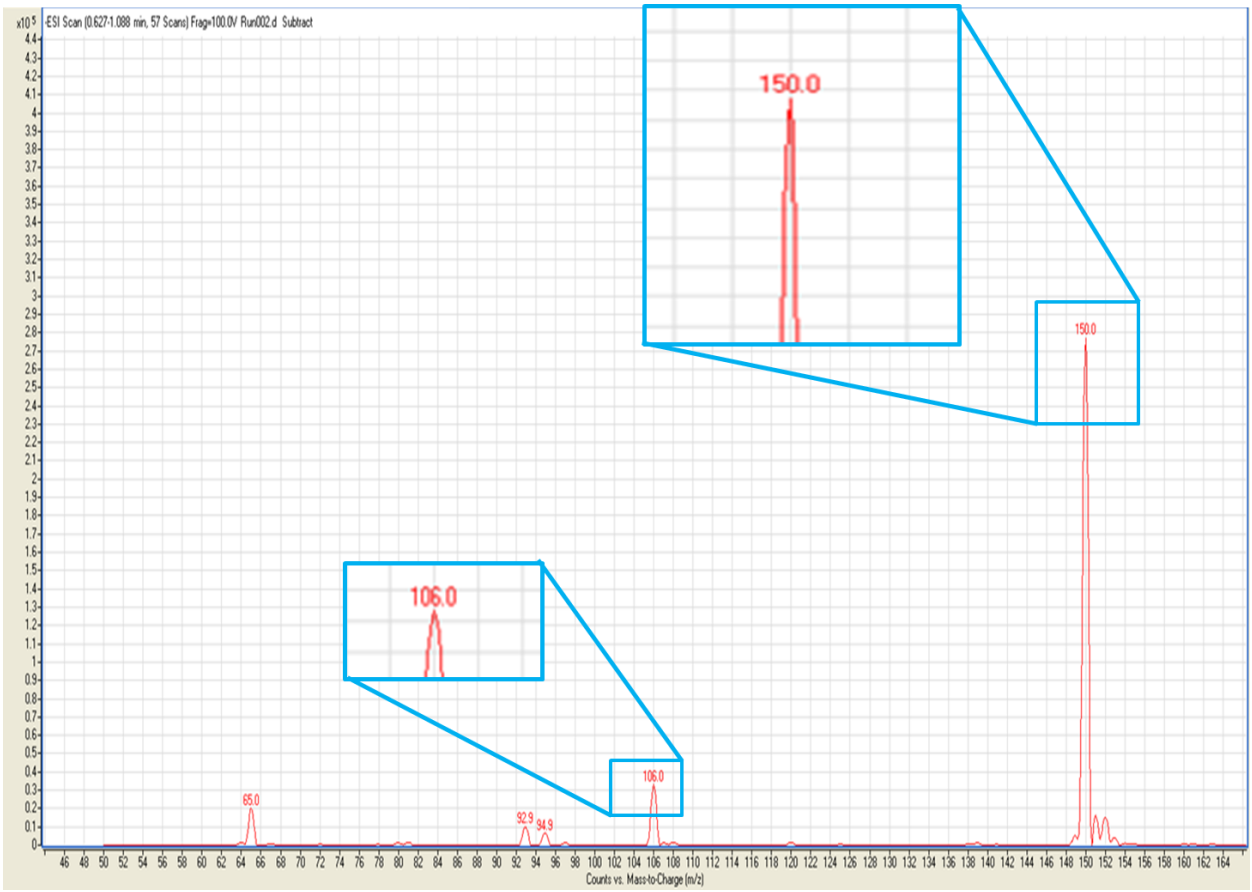
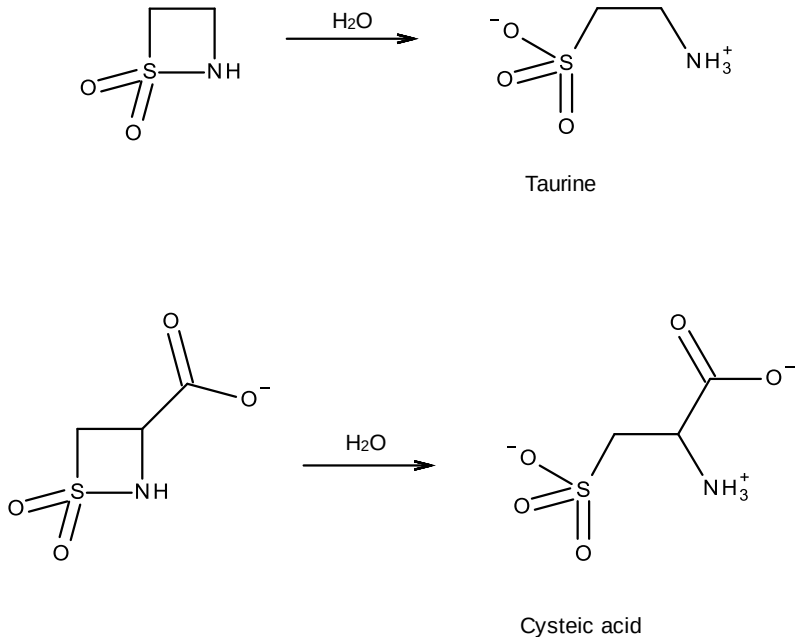


Figure 12 Mass spectrum for 3-carboxy-β-sultam

3.2 Kinetics

3.2.1 Overview of Hydrolysis

It is well established that β-sultam hydrolysis occurs by exclusive S-N bond fission (Scheme 48).[163]



Scheme 48 Hydrolysis of β-sultams (unsubstituted and the 3-carboxy derivative)

The unsubstituted β-sultam undergoes acid catalysed hydrolysis to yield taurine (characterised by comparison to a standard IR spectrum of taurine) and the 3-carboxy-β-sultam undergoes both acid and base catalysed hydrolysis to yield cysteic acid (characterised by comparison to an NMR spectrum of a standard sample of cysteic acid; see Figure 13).

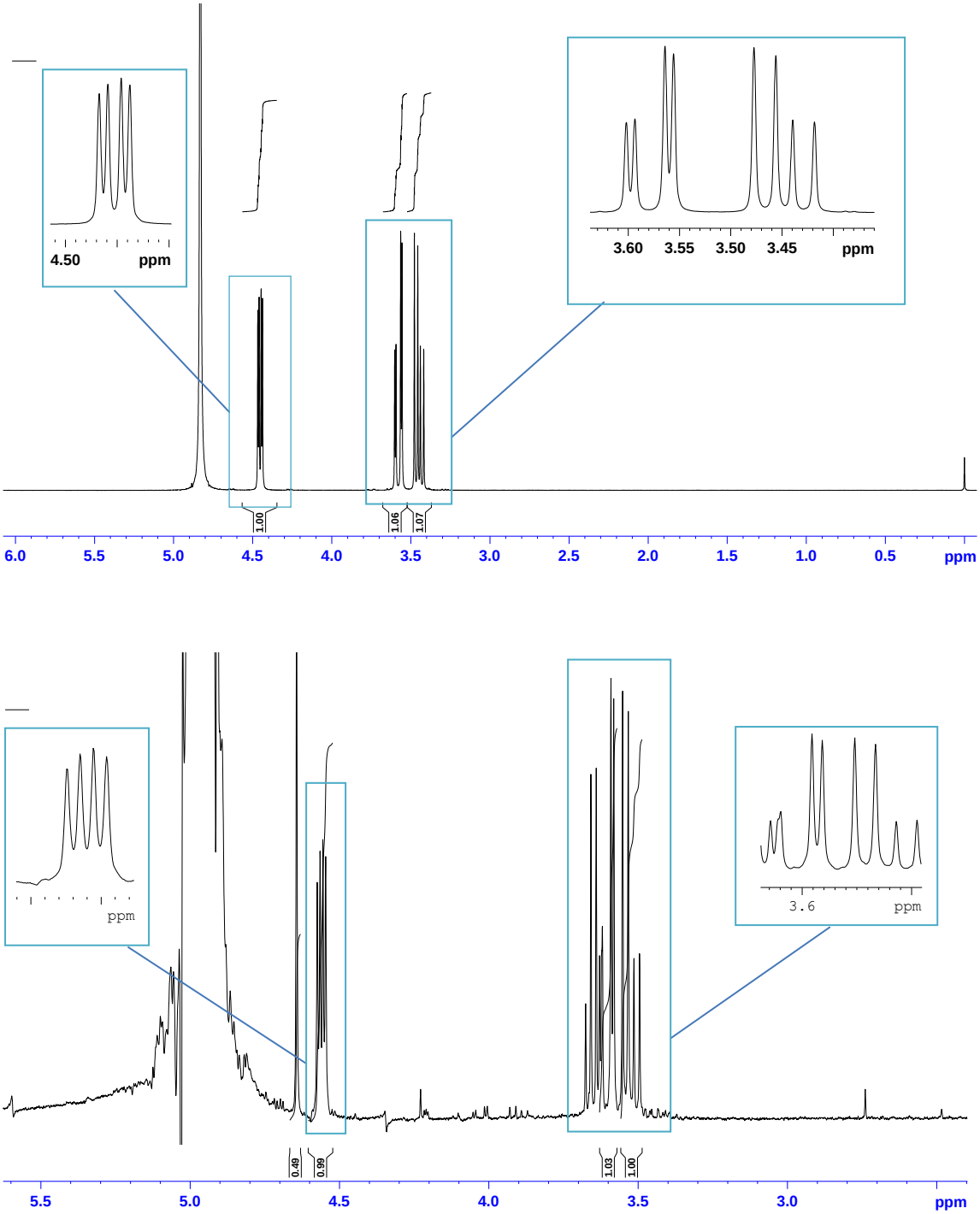


Figure 13 NMR of standard cysteic acid (top) and hydrolysed 3-carboxy-β-sultam (bottom) with expansions of key peaks

A study of the relative hydrolysis rates (previously mentioned in Section 3.1, page 105) of the ester group and the β-sultam S-N bond of the benzyl ester derivative [7] was carried out using ^{1}H NMR. The initial spectrum obtained directly after the 3-benzyl carboxylate β-sultam had been dissolved in 1M NaOD showed that the ester

bond had been cleaved whilst the S-N bond was still intact. After 16 hours about 60% of the S-N bonds had been cleaved to yield cysteic acid with three distinct peaks being present possibly indicating hydrogen bonding interactions between the sulfonate and amine groups fixing the conformation of the molecule and retaining the ABX splitting patterns (Figure 14). Upon addition of acid, however, this structure is no longer particularly favoured so that the ^{1}H NMR at very low pH (<pH 1) shows only two peaks in a ratio 2:1 for the CH_2 and CH groups respectively. This is presumably due to the protonation of the sulfonate group reducing the hydrogen bonding and therefore allowing free rotation of the carbon-carbon bond.

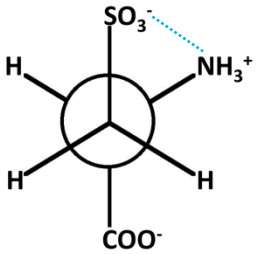


Figure 14 Newman projection of cysteine sulfonic acid

3.2.2 Hydrolysis of the Unsubstituted β-Sultam [3]

The rates of acid catalysed hydrolysis for the unsubstituted β-sultam (1,2-thiazetidine-1,1-dioxide, [3]) were established from data collected by ReactIR using a combination of chloroacetate buffers (pH 2.3 – 3.5) and dilute hydrochloric acid (pH 1.5 and 2.0). Initial reactions were carried out using formic acid; however, this decomposed in the presence of the substrate to release bubbles of CO_2 which disrupted the probe.

Full spectra were collected every 15 seconds from 3000 - 650 cm^{-1} and plotted in an overlay fashion as seen in Figure 15. The peaks of interest are those at 1300 cm^{-1} (β-sultam S=O) and 1230 cm^{-1} (due to taurine S=O). The initial spectrum is represented by the deep blue line showing high β-sultam concentration and low taurine concentration.

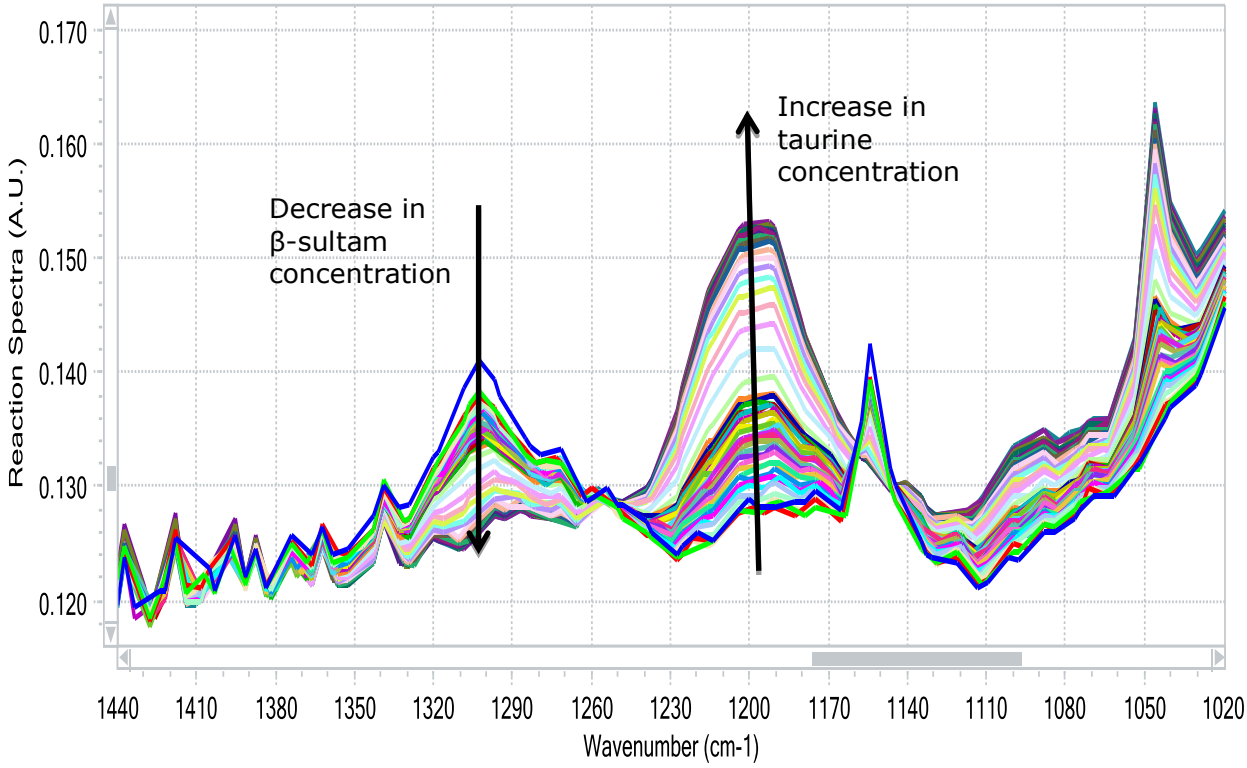


Figure 15 Overlay of IR spectra for the hydrolysis of β-sultam [3]

In its simplest form the general rate law for the buffer catalysed hydrolysis of any substrate is usually given by:

$$k_{obs} = k_H[H^+] + k_{HA}[HA] + k_{A-}[A^-] + k_0 + k_{OH}[OH^-] \quad \textbf{(Equation 4)}$$

The catalytic coefficient for a specific buffer system, k_{buf}, is equivalent to the two buffer terms which may contribute to the rate law:

$$k_{buf} = k_{HA}[HA] + k_{A-}[A^-] \quad \textbf{(Equation 5)}$$

Previous studies of N-substituted β-sultams have shown there to be no significant pH independent hydrolysis and no evidence was found for a spontaneous hydrolysis of the unsubstituted β-sultam [3].[163] Given this assumption the rate law for the hydrolysis of β-sultam at acidic pH therefore becomes:

$$k_{obs} = k_H[H^+] + k_{buf} \quad \textbf{(Equation 6)}$$

In order to determine the individual constants for the rate law, k_H and k_{buf}, the rate of hydrolysis of β-sultam was measured at a range of pHs in chloroacetate buffer at constant pH and ionic strength and varying concentrations of buffer under pseudo first-order conditions. The observed rate constants, k_{obs}, for hydrolysis of 1,2-thiazetidine-1,1-dioxide (β-sultam [3]) increased linearly with increasing total buffer concentration. This indicates the buffer is also contributing to the overall rate of the reaction (as suggested by Equation 4). By plotting the observed pseudo-first-order rate constants for hydrolysis against the total buffer concentration two important pieces of information can be determined (Figure 16). Extrapolation of each line gives the intercept on the y axis, k_{int}, which corresponds to the rate constant at zero buffer concentration. This can be used to calculate $k_{H;}$ the rate constant for specific acid catalysis. The slopes of the lines in Figure 16 give k_{buf} at each pH which is the total contribution to the rate law by both the undissociated chloroacetic acid and the chloroacetate anions in solution (Table 8).

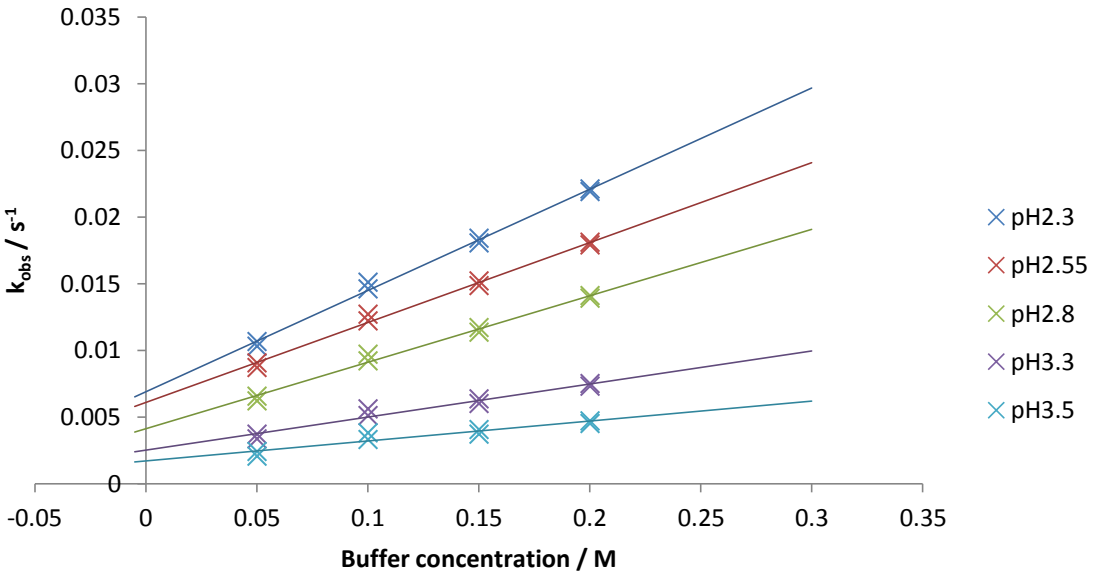


Figure 16 Plot of the observed pseudo-first order rate constant for the hydrolysis of β-sultam [3] as a function of total chloroacetate buffer concentration at the pH indicated by the key

pH	2.3	2.55	2.8	3.3	3.5
k_{int} (s^{-1})	$6.9 \times 10^{-3} \pm 3.45 \times 10^{-4}$	$6.1 \times 10^{-3} \pm 3.05 \times 10^{-4}$	$4.1 \times 10^{-3} \pm 2.05 \times 10^{-4}$	$2.5 \times 10^{-3} \pm 1.25 \times 10^{-4}$	$1.7 \times 10^{-3} \pm 8.50 \times 10^{-5}$
k_{buf} ($M^{-1}s^{-1}$)	$7.59 \times 10^{-2} \pm 3.80 \times 10^{-3}$	$5.99 \times 10^{-2} \pm 3.00 \times 10^{-3}$	$4.99 \times 10^{-2} \pm 2.50 \times 10^{-3}$	$2.49 \times 10^{-2} \pm 1.25 \times 10^{-4}$	$1.49 \times 10^{-2} \pm 7.45 \times 10^{-4}$

Table 8 Values of parameters derived from Figure 15

A graph of k_{buf} as a function of the fraction of free base, α, at each pH studied gives the individual second order rate constants for catalysis by the acidic and basic components of the buffer, k_{HA} and k_{A-}, at α = 0.0 and α = 1.0 respectively (Equation 7) (Figure 17).

$$k_{buf} = (1 - \alpha)k_{HA} + \alpha k_{A-} \qquad \textbf{(Equation 7)}$$

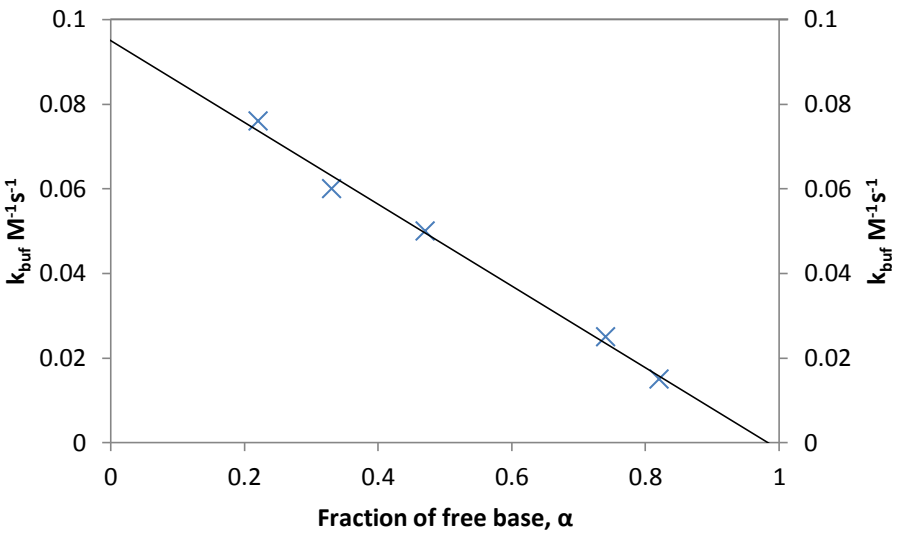
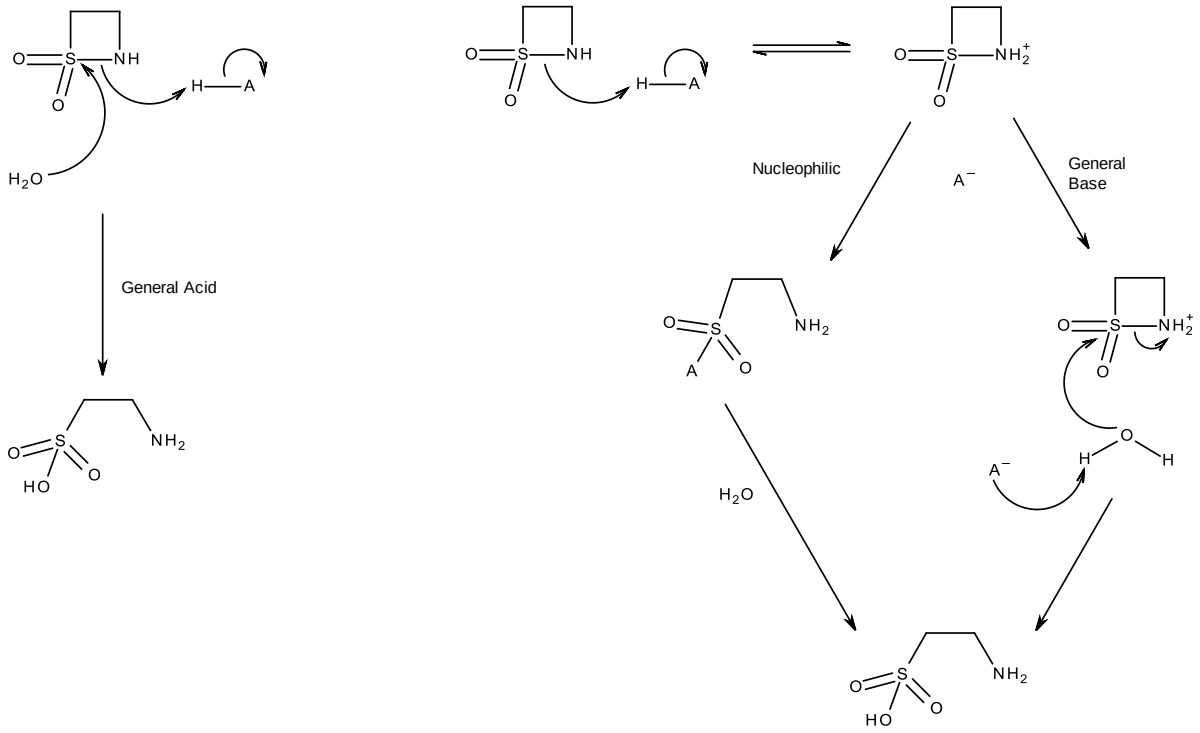


Figure 17 Plot of k_{buf} vs α (fraction of free base) for the chloroacetate buffer catalysed hydrolysis of the unsubstituted β-sultam

The intercept at α = 1.0 is indistinguishable from zero, so no rate constant for catalysis of β-sultam hydrolysis by chloroacetate anions, k_{A-}, can be determined. The second order rate constant for catalysis by chloroacetic acid, k_{HA}, is given by the intercept at α = 0.0 as 9.51×10^{-2} $M^{-1}s^{-1}$.

There is only one previous mention of the investigation of the rate of β-sultam hydrolysis in chloroacetate buffers to be found and this relates to a study of the effect of carboxylic acid catalysed hydrolysis of N-benzyl-β-sultam.[137] The value of

k_{HA} for this reaction is quoted as 7.61 x 10^{-2} $M^{-1}s^{-1}$, which is of the same order of magnitude as that of the unsubstituted β-sultam. The proposed mechanism for buffer catalysis in this paper was specific acid-nucleophilic catalysis and this is one possibility for the mechanism for the hydrolysis of the unsubstituted β-sultam also. The general acid catalysed hydrolysis of [3] could be due to several possible mechanisms as seen in Scheme 49. Both mechanisms, the concerted general acid catalysis and stepwise specific acid catalysis followed by nucleophilic or general base catalysis, show equivalent rate laws (Equation 8).



Scheme 49 Possible mechanisms of catalysis by chloroacetic acid in the hydrolysis of β-sultam [3]

$$\boldsymbol{Rate} = [\boldsymbol{S}][\boldsymbol{HA}] \equiv [\boldsymbol{SH^+}][\boldsymbol{A^-}] \qquad \textbf{(Equation 8)}$$

The intercepts of the rate-buffer plots (Figure 16), k_{int}, increase with decreasing pH indicative of an acid catalysed pathway. Pseudo first order rate constants for hydrolysis of [3] were also determined in solutions of hydrochloric acid. By combining the data from buffer catalysis experiments (k_H values) with these values a

pH rate profile for the hydrolysis of the unsubstituted β-sultam [3] at low pH is produced (Figure 18).

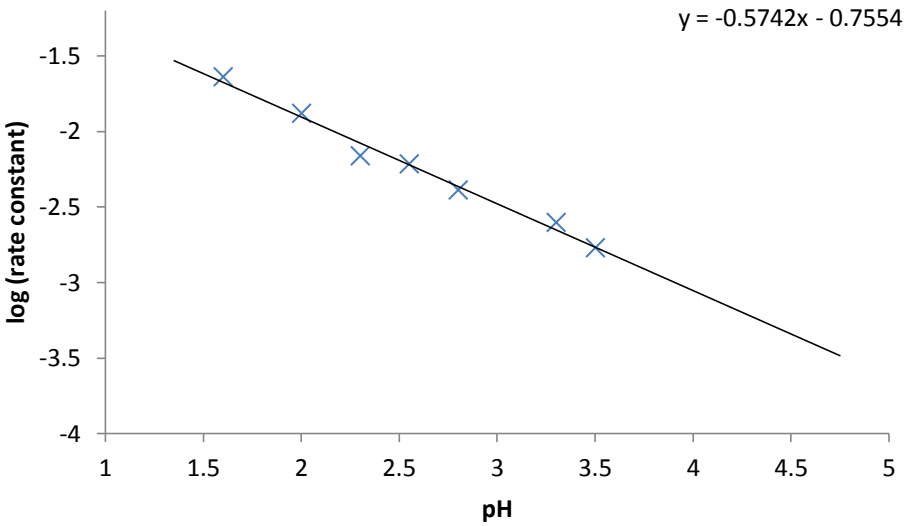


Figure 18 pH rate profile for acid catalysed hydrolysis of unsubstituted beta-sultam

Unusually the slope of the plot of log k_{obs} / log k_{int} against pH is 0.6, whereas a simple acid catalysed reaction would be expected to generate a slope of 1.0. This non-first order dependence on $[H^+]$ is also seen by the variation in the values of $k_{obs}/[H^+]$ and $k_{int}/[H^+]$ (Table 9). It is difficult to explain this and may be due to error in the novel experimental method for determining the rates using the React-IR probe. It seems unlikely that this is due to partial formation of the conjugate acid of [3] or to the partial occurrence of a pH-independent pathway.

pH	1.6	2.0	2.3	2.6	2.8	3.3	3.5
$k_{obs}/[H^+]$	1.67	1.32	1.42	2.23	2.72	5.39	6.01

Table 9 Values of $k_{obs}/[H^+]$ over pH range of study

3.2.3 Hydrolysis of 3-Carboxy-β-Sultam

The rates of hydrolysis of 3-carboxy-β-sultam [8] at various pH were established using data collected by pre-saturation ^{1}H NMR spectra taken at appropriate time intervals. Low pH/D experiments were carried out in deuterium chloride (DCl) solutions and higher pH/D experiments were carried out in sodium deuteroxide (NaOD) solutions. For mid-range pH experiments various deuterated buffers were used (as detailed in Table 10) at a range of concentrations in order to establish and eliminate any contribution from buffer catalysis.

Buffer	pH range (measured) *(pD range) (calculated)*	Concentrations utilised (M)
Phosphate	2.99 - 3.55 (3.39 – 3.95)	0.50, 0.25, 0.10
Acetate	4.78 - 5.32 (5.18 – 5.72)	0.50, 0.25, 0.10
Phosphate	6.59 - 8.01 (6.99 – 8.41)	0.50, 0.25, 0.10
Carbonate	9.69 (10.09)	0.50, 0.25, 0.10

Table 10 Details of deuterated buffers employed in the study of 3-carboxy-β-sultam hydrolysis rates

As with previous work on the hydrolysis of the unsubstituted β-sultam, plots of k_{obs} versus buffer concentration gave k_{int} (the rate constant for hydrolysis at zero buffer concentration) and k_{buf} (the contribution to the rate law by all components of the buffer solution; k_{HA} and k_{A^-}) (Equation 5). These plots can be seen for a range of buffers in Figure 19 showing the increase in observed pseudo-first order rate

constant with increasing buffer concentration indicative of buffer catalysis, either by the acidic or basic component of the buffer. The values of k_{int} are shown in Table 11 where it can be seen that the value of the rate constant increases as the pH decreases. There is a greater amount of scatter in the data than would be preferred.

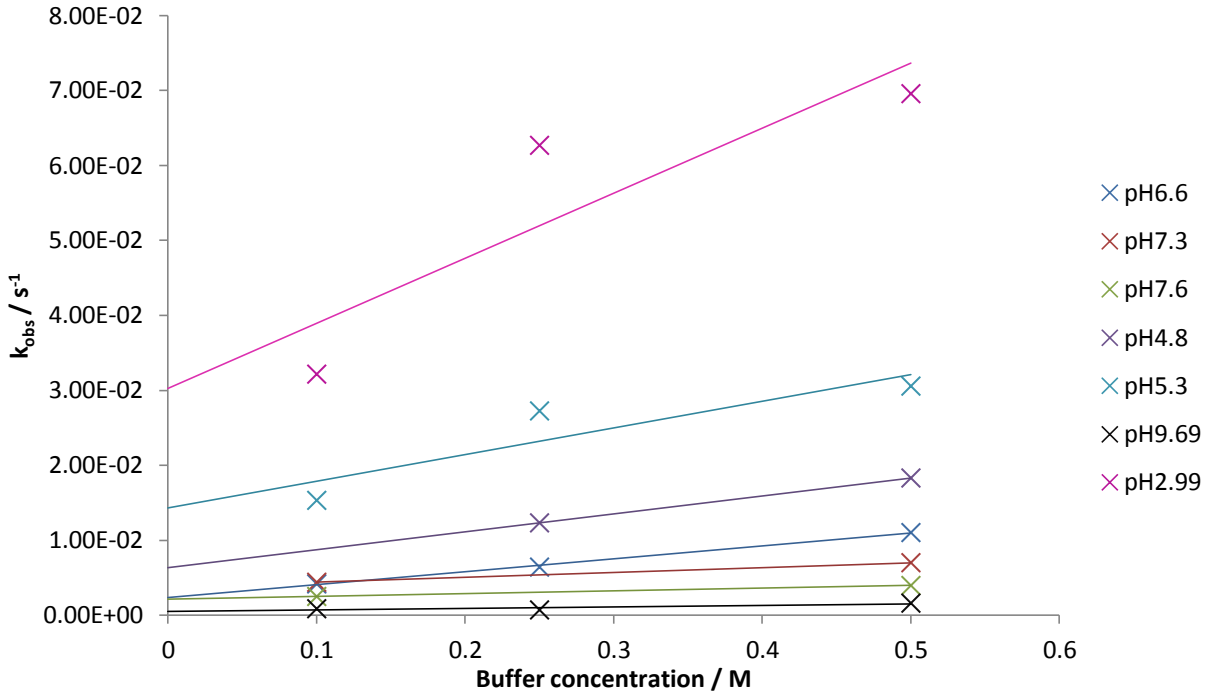


Figure 19 Plot of the observed pseudo-first order rate constants for the hydrolysis of 3-carboxy-β-sultam [8] as a function of total buffer concentration (measured pH indicated by the key)

pD	3.39	5.20	5.70	7.00	7.70	8.00	10.09
k_{int} (s^{-1})	3.02 x 10^{-2}	6.3 x 10^{-3}	1.43 x 10^{-2}	2.3 x 10^{-3}	3.7 x 10^{-3}	2.1 x 10^{-3}	5.00 x 10^{-4}

Table 11 Values of rate constants (k_{int}) for buffers, determined from Figure 19

In order to establish which form of the buffer is responsible for catalysis it is necessary to carry out experiments at a range of pHs in the same buffer system. Experiments were carried out in phosphate buffer (at pKa = 7.2) at three pHs. Plots of k_{obs} against the total buffer concentration for these experiments gave two important pieces of information about the rates of catalysis. The intercept on the y axis, k_{int}, corresponds to the rate constant at zero buffer concentration and is the

observed first-order rate constant for buffer independent hydrolysis. The slope of the graph, k_{buf}, is equivalent to the contribution to the rate law by both buffer species; the dihydrogen phosphate monoanion and the monohydrogen phosphate dianion. A plot of k_{buf} against α, the fraction of free base, gave intercepts of k_{HA} when α = 0.0 and k_{A^-} when α = 1.0. These are the second order rate constants for catalysis by the acidic ($H_2PO_4^-$) and basic (HPO_4^{2-}) components of the buffer respectively. As with the chloroacetate buffer catalysed hydrolysis of the unsubstituted β-sultam this plot (seen in Figure 20) shows that the kinetically relevant species for the phosphate buffer catalysis (pKa = 7.2) of 3-carboxy-β-sultam is the acidic phosphate monoanion, $H_2PO_4^-$ and that there is no significant catalysis by the dianion HPO_4^{2-}. Although there is only limited data (three points) it is still possible to determine the value of the rate constant k_{HA} from the graph which is equal to 2.243 x 10^{-2} $M^{-1}s^{-1}$.

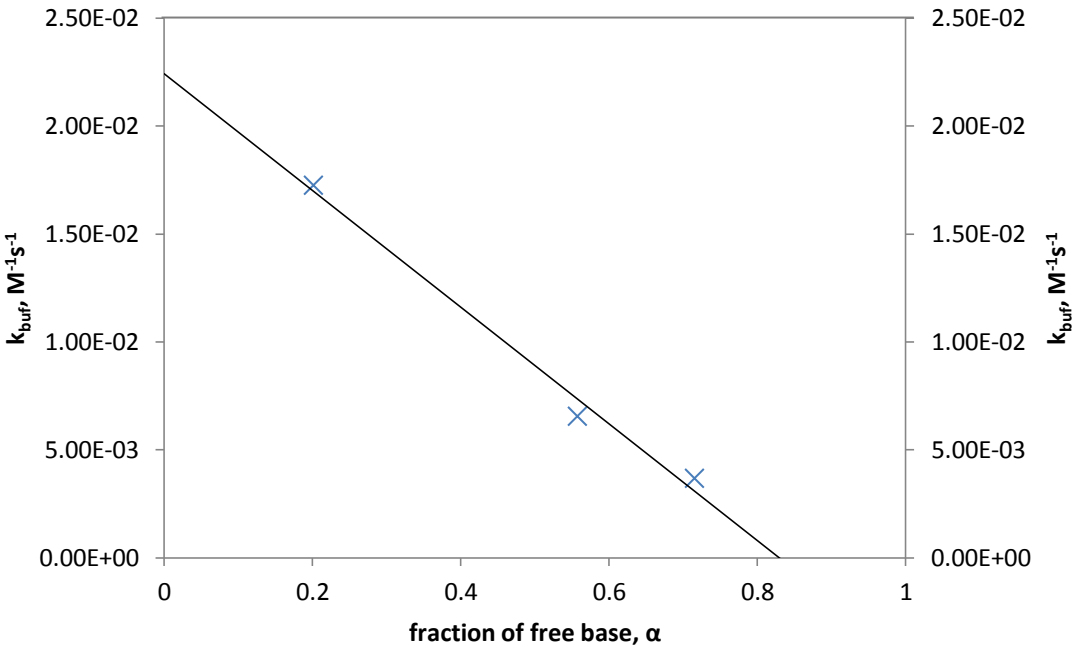


Figure 20 Plot of k_{buf} vs α (fraction of free base) for the phosphate buffer catalysed hydrolysis of the 3-carboxy-β-sultam

In carbonate buffer systems (pK_a = 10.25) data is only available for one pH, pH 9.69, and so the contribution of the individual buffer species to the catalytic coefficient k_{buf} can only be estimated via Equation 9 where α is equal to the fraction of the buffer present in the basic form (CO_3^{2-}). This assumes that k_{A^-} is zero so that it

is again the acidic form of the buffer (HCO_3^-) which is responsible for catalysis. The plot of k_{obs} gives us the values for k_{int} and k_{buf} of 4.944 x 10^{-4} and 2.036 x 10^{-3} $M^{-1}s^{-1}$ respectively. At pH 9.69 α is equal to 0.234 and so the second order rate constant for catalysis by the hydrogen carbonate anion, k_{HA} is calculated to be 6.46 x 10^{-4} $M^{-1}s^{-1}$.

$$\frac{k_{buf}}{1-\alpha} = k_{HCO_3^-} \qquad \textbf{(Equation 9)}$$

By combining the data for the hydrolysis of 3-carboxy-β-sultam in carbonate buffer (k_{int}) with that for the hydrolysis of 3-carboxy-β-sultam in sodium deuteroxide a graph of log k_{obs} versus pH gives a straight line with gradient ~ 1 confirming the first order dependence of the rate on hydroxide ion concentration (Figure 21).

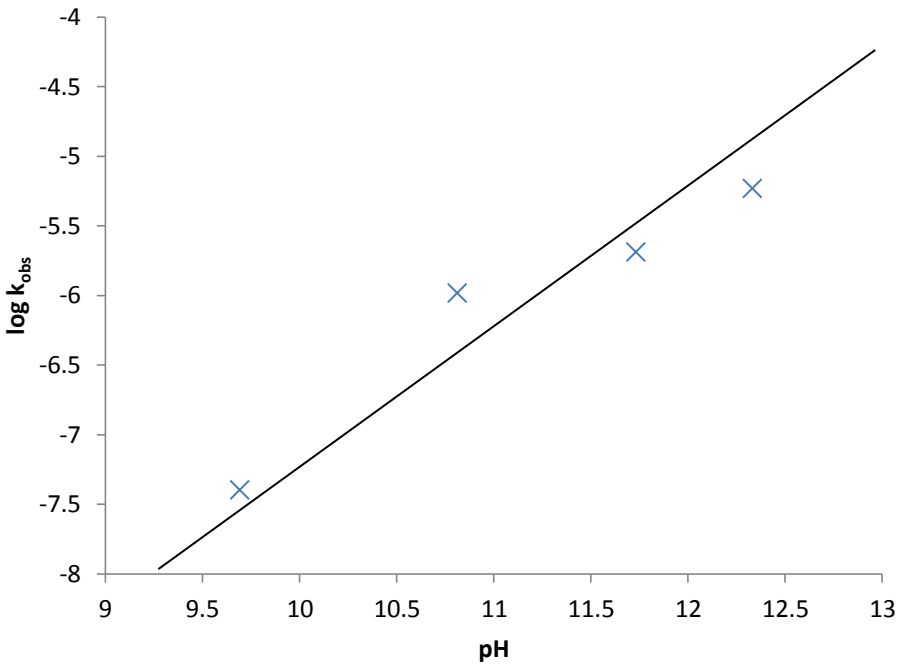


Figure 21 Plot of log_{10} k against pH for the alkaline hydrolysis of 3-carboxy-β-sultam

At lower pH, when the rate constants for hydrolysis in deuterated hydrochloric acid were added to those for hydrolysis in buffer below pH 10 the graph of log k versus pH shown in Figure 22 was produced. Attempts to fit all of the data to one line of gradient = 1 were not successful and so two individual lines were instead used to best-fit the data.

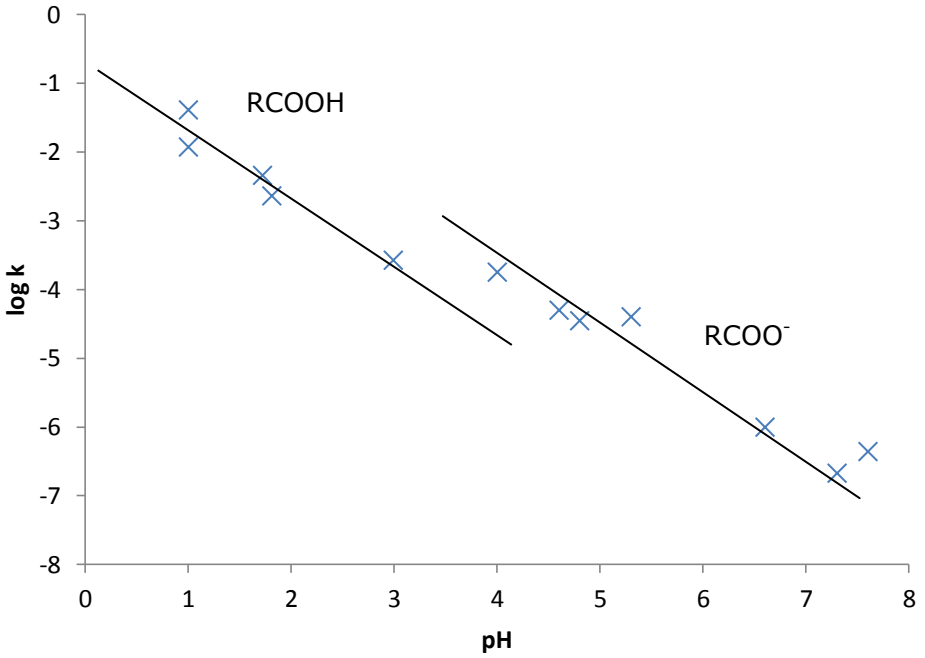


Figure 22 Plot of $\log_{10}$ k against pH showing two distinct hydrolysis mechanisms at acidic pH

This indicated that there were two contributions to the rate law for the hydrolysis of 3-carboxy-β-sultam, one at lower pH (1-4) for the hydrolysis of the β-sultam with a protonated acid group with the one at slightly higher pH (4-8) being for the hydrolysis of the carboxylate anion (Figure 22). In order to establish the rate equation for the hydrolysis of 3-carboxy-β-sultam [8] it was therefore necessary to determine the pK_a of this acid proton.

In order to accurately determine all parameters for the hydrolysis of 3-carboxy-β-sultam the experimental data was fitted to the rate equation (Equation 10) using Microsoft Excel. All of the collected rate data (acid and alkaline hydrolysis) was then plotted on one graph with the line calculated from the sum of the individual rate terms (Figure 23) (Table 12).

$$\boldsymbol{Rate = k_H K_a[S] + k'_H K_a[S] + k_0 + k_{OH}[S][OH^-]} \qquad \textbf{(Equation 10)}$$

This equation is kinetically equivalent to Equation 11 by substitution of specific substrates and accounting for ionisation at the relevant pK_a.

$$\boldsymbol{Rate} = \boldsymbol{k_H}[\boldsymbol{H^+}][\boldsymbol{RCOOH}] + \boldsymbol{k'_H}[\boldsymbol{H^+}][\boldsymbol{RCOO^-}] + \boldsymbol{k_0}[\boldsymbol{RCOO^-}] + \boldsymbol{k_{OH}}[\boldsymbol{RCOO^-}][\boldsymbol{OH^-}]$$

(Equation 11)

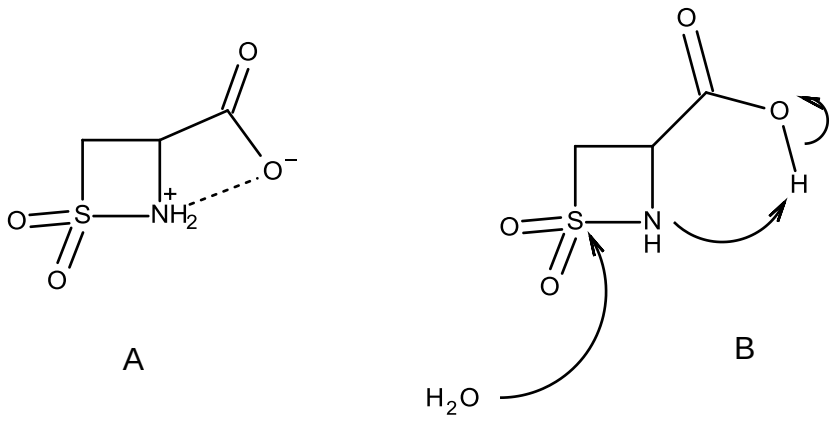
The second term in Equation 11 is kinetically ambiguous and could be due to hydrolysis of [RCOOH]:

$$\mathbf{RCOOH} \underset{}{\overset{\mathbf{K_a}}{\rightleftharpoons}} \mathbf{RCOO^-} + \mathbf{H^+}$$

$$\boldsymbol{K_a} = \frac{[\boldsymbol{RCOO^-}][\boldsymbol{H^+}]}{[\boldsymbol{RCOOH}]}$$

$$\boldsymbol{k'_H}[\boldsymbol{H^+}][\boldsymbol{RCOO^-}] = \boldsymbol{k'_H K_a}\,[\boldsymbol{RCOOH}]$$

The second order rate constant for the acid catalysed hydrolysis of [8] with dissociated carboxylic acid group [$RCOO^-$] is 4.80 $M^{-1}s^{-1}$ which is 24 fold greater than that for [8] with an undissociated carboxylic acid [RCOOH]. This rate enhancement is potentially due to the enhanced stability of the conjugate acid (Scheme 50A). The rate enhancement seen is not as large as would be expected for the potential increase in stability shown and this could be due to intramolecular general acid catalysis with the protonated form of the compound (Scheme 50B).



Scheme 50 Potential mechanisms of rate enhancement with dissociated 3-carboxy-β-sultam [8]

By manipulating the constants in Equation 10 it was possible to arrive at a line which fitted the experimental data; these values are shown in Table 12 and the final graph is in Figure 23.

	k_H, $M^{-1}s^{-1}$	k'_H $M^{-1}s^{-1}$	pK_a (K_a, M)	k_0 s^{-1}	k_{OH} $M^{-1}s^{-1}$
Value from data fitting	$2.00 \times 10^{-1} \pm 1.00 \times 10^{-2}$	$4.80 \pm 2.40 \times 10^{-1}$	4.5 (3.162×10^{-5})	$1.00 \times 10^{-8} \pm 5.00 \times 10^{-10}$	$5.00 \times 10^{-4} \pm 2.50 \times 10^{-5}$

Table 12 Rate equation constants/parameters for the hydrolysis of 3-carboxy-β-sultam

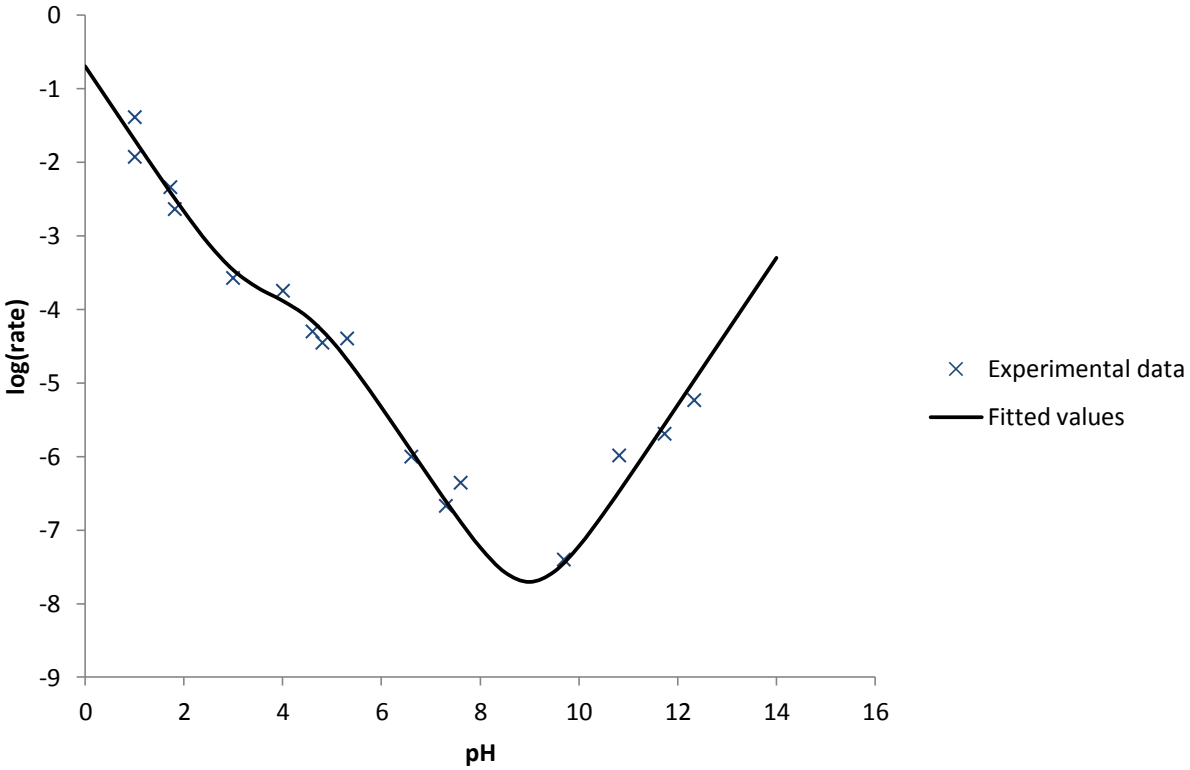


Figure 23 pH-rate profile for 1,2-thiazetidine-3-carboxylate-1,1-dioxide
Line calculated from rate equation; points are from experimental data.

Taking the rate as 4.89×10^{-7} $M^{-1}s^{-1}$ at pH 7 the half-life of 3-carboxy-β-sultam at physiological pH can be calculated and is shown to be approximately 16.4 days.

3.3 Inhibition Studies

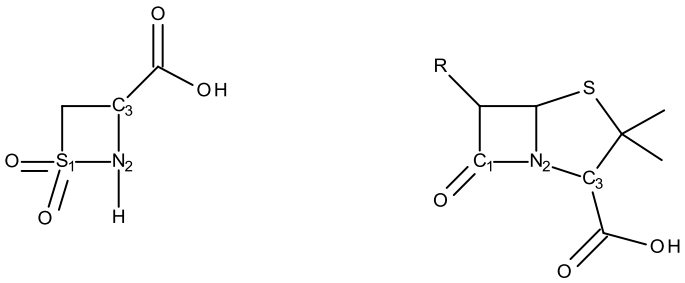
3.3.1 Inhibition of BcII

BcII is the di-zinc Class B1 metallo-β-lactamase enzyme produced by *Bacillus cereus* 569/H. Few compounds have been discovered to be inhibitors of the enzyme, particularly inhibitors which are specific only to BcII and do not interact with other metallo-enzymes such as carbonic anhydrase. BcII is an efficient catalyst of β-lactam ring hydrolysis with a k_{cat}/K_M value of 4.5 x 10^5 $M^{-1}s^{-1}$ for benzylpenicillin (k_{cat} = 680 s^{-1}, K_M = 1.5 x 10^{-3} M).[110]

3.3.1.1 β-Sultams and their Analogues as BcII Inhibitors

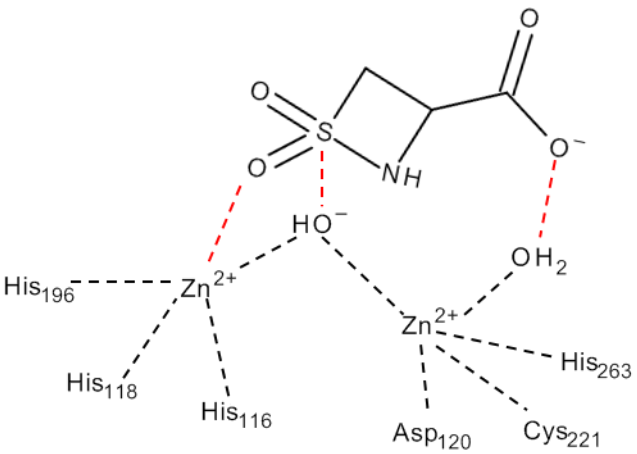
All β-lactamase enzymes, including BcII are efficient catalysts for the hydrolysis of β-lactam antibiotics, all of which have a carboxylic acid group at the three position of the fused ring. This structure is important for recognition by the enzyme active site as it is an analogue of the D-Ala-D-Ala backbone recognised by the DD-transpeptidase enzymes which β-lactam antibiotics inhibit.

3-Carboxy-β-sultam [8] was chosen as a potential inhibitor of BcII as possibly the sulfonyl centre could interact with Zn1 and the carboxylate with Zn2. Scheme 51 compares the structures of the 3-carboxy-β-sultam and a generic penicillin. This shows the position of the carboxylic acid group relative to the sulfonyl and carbonyl groups respectively in each compound. Although both structures are named with the carboxylate functionality at the 3-position this is not exactly the same position on both molecules, though they are both two bond lengths from the carbonyl/sulfonyl group.



Scheme 51 Numbering of 3-carboxy-β-sultam and penicillin to show carboxylate functionality at 3-position

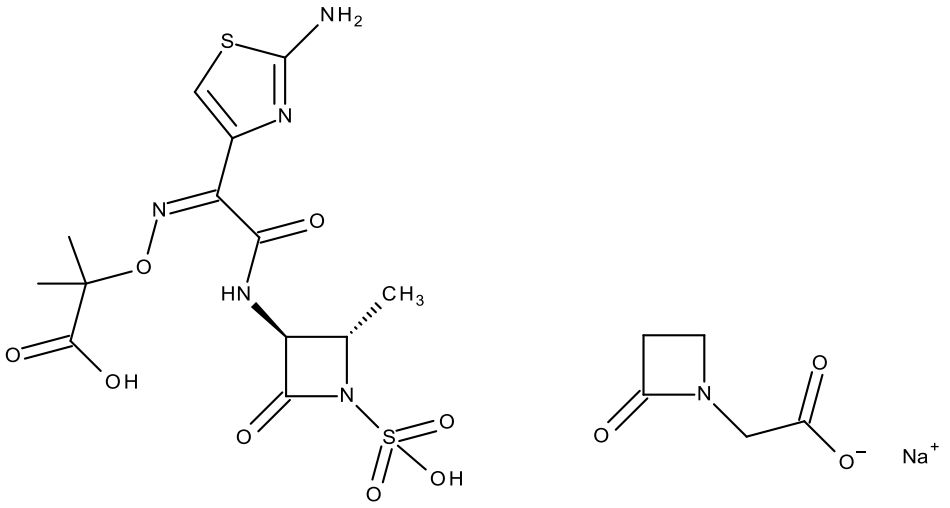
Due to the similar geometry shown between the two structures (Scheme 51) it was hoped that the β-sultam would interact with the active site zinc ions and zinc bound water molecules as shown in Scheme 52.



Scheme 52 Possible mode of interaction between 3-carboxy β-sultam and BcII

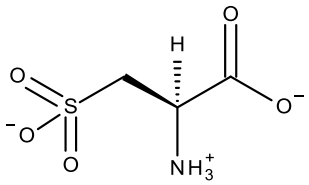
Experimental data showed that 3-carboxy-β-sultam is not a substrate for BcII β-lactamase, and neither is the unsubstituted β-sultam. Equally, neither compound showed inhibitory action against BcII despite the structural similarity between the two compounds and the β-lactam moeity of the normal substrate. It is possible that the lack of steric bulk and functional groups on the test compounds reduced the recognition by the active site. There are comparable studies in the literature for work done on the inhibition of BcII by analogous β-lactam compounds.[221] Although BcII

has a broad substrate profile, including penicillins, cephalosporins and carbapenems, it has been shown that it does not efficiently catalyse the hydrolysis of monobactams. Aztreonam (Scheme 53) is a representative monobactam which binds to BcII but is not hydrolysed by it. The azetidinone shown in Scheme 53B is a β-lactam with an N-acetoxy substituent showing similar functionality to 3-carboxy-β-sultam which does not bind to BcII at all. The suggestion in this paper was that a lack of other substituents may contribute to the lack of binding. It would be interesting to investigate 3-carboxy-β-sultams with substituents at the 4-position to see if the addition of extra fucnctionality affected the inhibitory action.

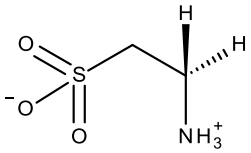


Scheme 53 Structures of monobactams, aztreonam (A) and 2-oxoazetidinyl-acetate sodium salt (B)

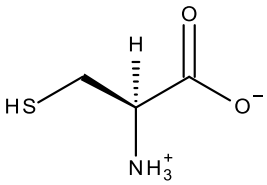
1,2-Thiazetidine-1,1-dioxide has been shown to be a potentially useful pro-drug, hydrolysing in situ to yield taurine.[198] Following on from this it seemed logical to study the hydrolysis products of the two β-sultams and their analogues as potential inhibitors of BcII. The compounds chosen were taurine, L-cysteic acid, L-cysteine and D-cysteine (based on their structural relevance and commercial availability) (Scheme 54). Taurine (the hydrolysis product of β-sultam) and L-cysteine (the thiol analogue of the hydrolysis product of 3-carboxy-β-sultam) showed no effect on the activity of BcII in the hydrolysis of benzylpenicillin (results for taurine shown in Figure 24).



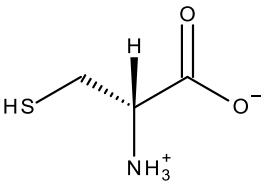
L-Cysteic acid



Taurine



L-Cysteine



D-Cysteine

Scheme 54 Hydrolysis products of β-sultams and their analogues

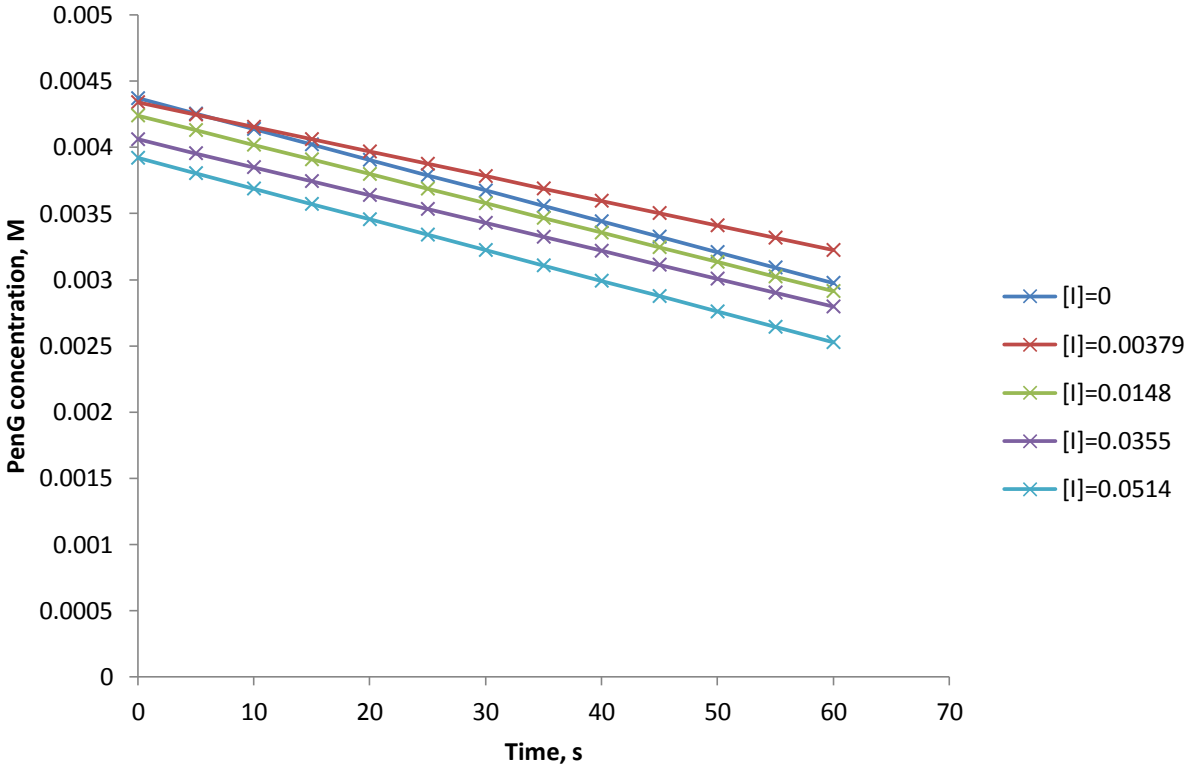


Figure 24 Effect on the rate of the BcII catalysed hydrolysis of benzylpenicillin by taurine

In contrast to the β-sultams, L-cysteic acid did have an effect on the rate of benzylpenicillin hydrolysis by BcII. It can be seen in Figure 25 that the rate of hydrolysis decreases as the concentration of inhibitor increases. The concentrations required to have this effect are very high (inhibitor concentration twenty fold higher than substrate concentration) in order to see the rate of hydrolysis approaching zero.

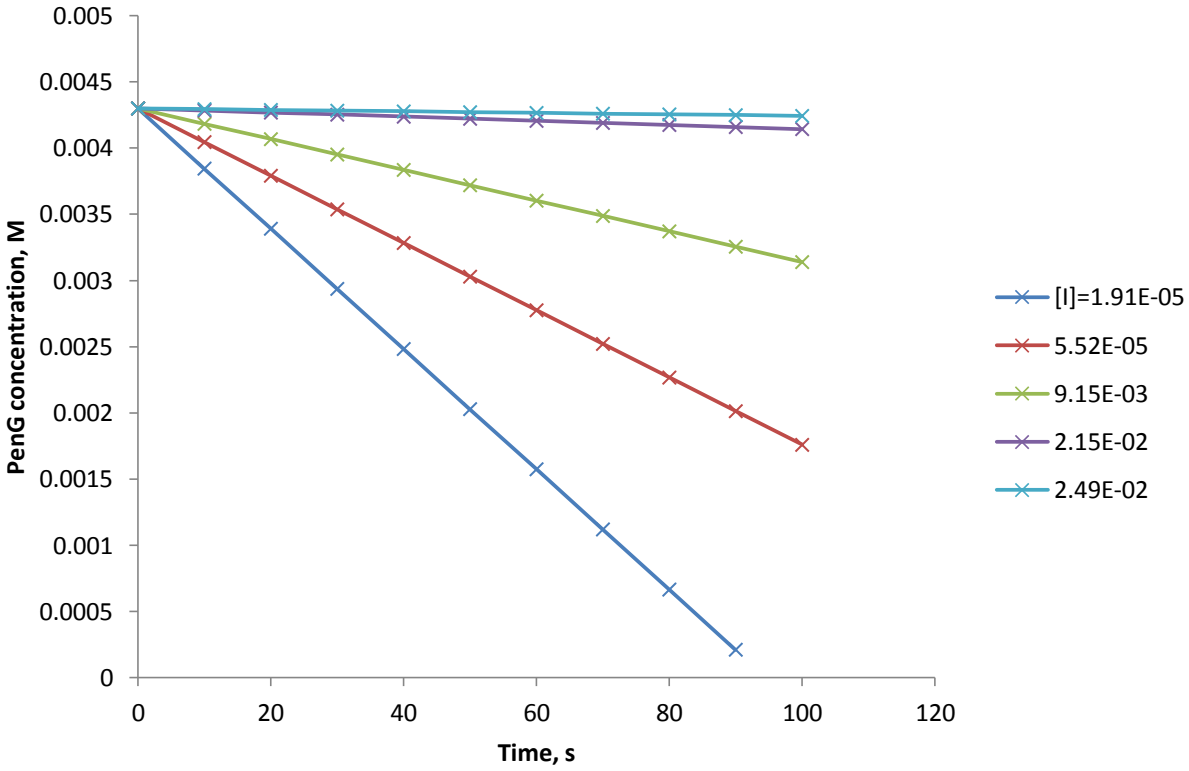


Figure 25 L-Cysteic acid inhibition of BcII

D-Cysteine was also shown to have an effect on the rate of BcII catalysed benzylpenicillin hydrolysis. In this case the inhibitor concentrations required were not quite so high and D-cysteine was shown to have a K_i value at pH 7 of 7.5 x 10^{-3} M (Figure 26).

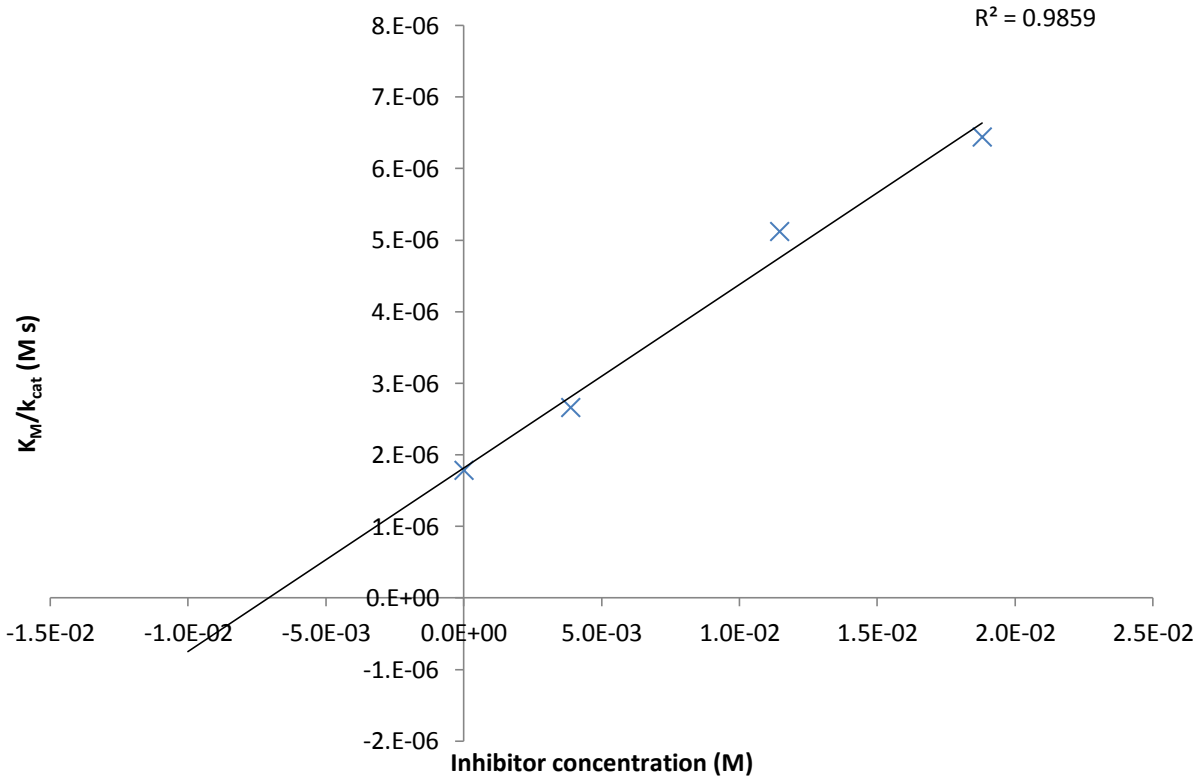


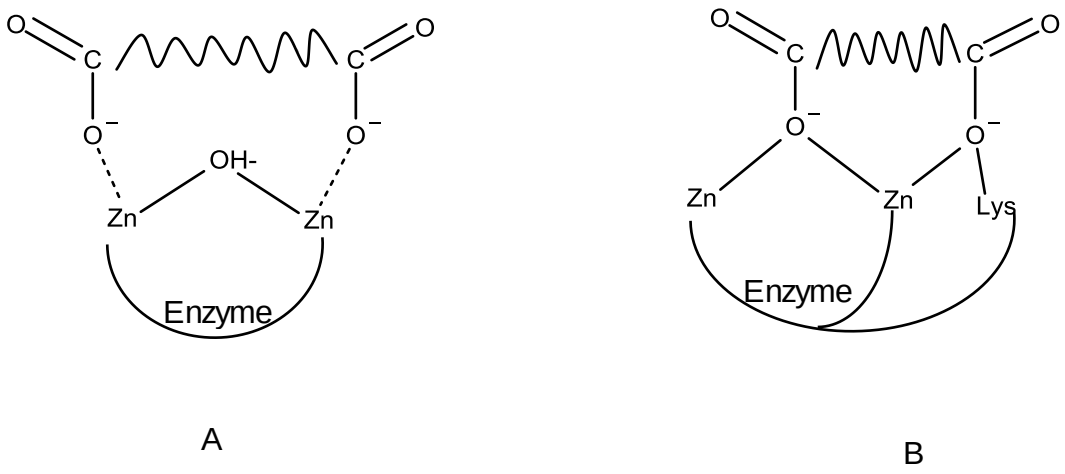
Figure 26 Inhibition of BcII by D-Cysteine

Compound	**Effect on activity of BcII**
β-Sultam	No effect
3-Carboxy-β-Sultam	No effect
Taurine	No effect
L-Cysteic Acid	Very weak inhibition
L-Cysteine	No effect
D-Cysteine	K_i = 7.5 x 10^{-3} M

Table 13 Summary of results for the inhibition of BcII by β-sultams and their analogues

3.3.1.2 Dicarboxylic Acids as BcII Inhibitors

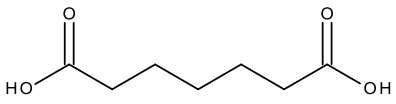
The second group of compounds chosen as potential inhibitors of BcII were dicarboxylic acids. One possibility of the interactions between the two dicarboxylates and the two active site zinc ions is demonstrated in Scheme 55A. Derivatives of succinic and phthalic acids have been shown to inhibit another Class B1 enzyme, IMP-1, via the displacement of the di-zinc bound water molecule, Wat1, by one carboxylate and the forming of a bridge by the second carboxylate between Zn2 and a lysine residue at the active site as seen in Scheme 55B.[129]



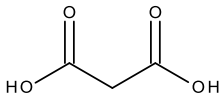
Scheme 55 Potential interactions between dicarboxylic acids and BcII

The following dicarboxylic acids were tested as potential inhibitors of the BcII catalysed hydrolysis of benzylpenicillin:

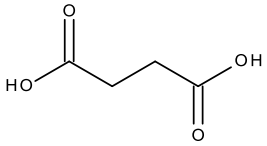
Pimelic acid [10], malonic acid [11], succinic acid [12], oxalic acid [13], cyclopentane 1,2-dicarboxylic acid [14] and 2,3-norbornanedicarboxylic acid [15] (structures in Scheme 56).



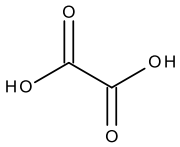
pimelic acid [10]



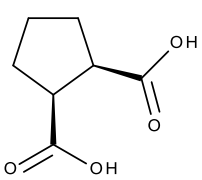
malonic acid [11]



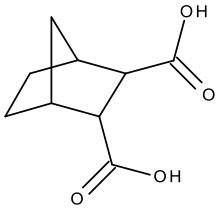
succinic acid [12]



oxalic acid [13]



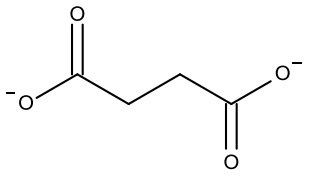
cyclopentane-1,2-dicarboxylic acid [14]



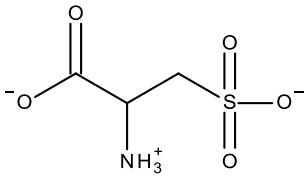
2,3-norbornanedicarboxylic acid [15]

Scheme 56 Structures of dicarboxylic acids tested as inhibitors of BcII

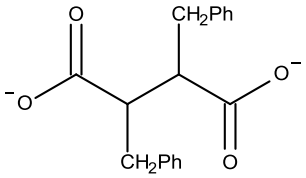
In all cases the ability of the compound to inhibit the hydrolysis of benzylpenicillin by BcII was determined by comparison of the initial rate of substrate hydrolysis in the presence of inhibitor to the control rate (with no inhibitor present). In all cases the initial rates of the reactions were of the same order with the average rate in the presence of a dicarboxylic acid being $11.2 \times 10^{-3} \pm 1.3 \times 10^{-3}$ s^{-1} compared to the control rate of 9.2×10^{-3} s^{-1}. No change in pH was noted between the start and end of any experiment. This is in sharp contrast to the results seen for L-cysteic acid which, other than the amino group and the substitution of a carboxylic for a sulfonic acid group is structurally similar to the dicarboxylic acids, particularly succinic acid (Scheme 57). Previous work has shown that IMP-1 is inhibited by disubstituted succinic acids (dibenzyl-succinic acid shown in Scheme 57) via interactions between the two carboxylate groups with Wat1 and Zn2 at the active site. It appears that whilst the substituents are not involved in the enzyme-inhibitor interactions they may be involved in recognition for the active site. This suggestion is strengthened when looking at the difference in IC_{50} values for the S,S, R,R and R,S stereoisomers of dibenzylsuccinic acid which are 0.0027, 0.21 and 200 μM respectively.



Succinic acid



L-Cysteic acid



Dibenzyl succinic acid

Scheme 57 Structures of L-cysteic acid and succinic acids

The difference in activity seen between L-cysteic acid and succinic acid with BcII may also be a function of better substrate recognition for L-cysteic acid due to the presence of the amine group. It is not clear why this may be, but it may be due to hydrogen bonding or electrostatic interactions between the molecule and amino acid residues at the active site. Another possibility is that it may be due to the reduction in free rotation of the carbon backbone due to intramolecular hydrogen bonding interactions fixing the L-cysteic acid in a preferential conformation.

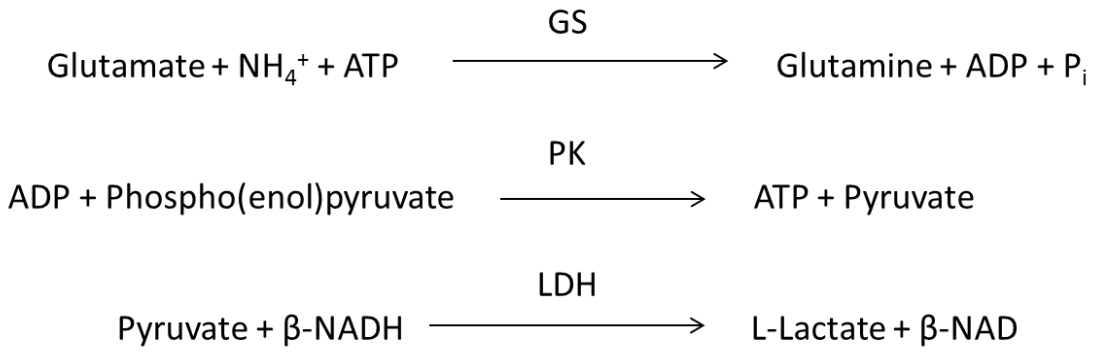
3.3.2 Inhibition of Glutamine Synthetase

Glutamine synthetase is a polymeric enzyme requiring two metal ions (either Mg^{2+} or Mn^{2+}) at each active site. It effectively catalyses the formation of glutamine from glutamate and ammonia; ATP is required to activate the glutamate carboxylate group via phosphorylation.

Initial attempts to monitor the rate of glutamine production were carried out using HPLC. Calibration was carried out for glutamate and glutamine in the presence of ATP. The reaction was set up with glutamate, ammonium salt, magnesium salt, ATP and enzyme in imidazole buffer solution at pH 7.1 and 1.0 M ionic strength maintained by addition of potassium chloride. Samples of the solution were removed at set intervals and quenched in 1.0 M sodium hydroxide solution to denature the enzyme and stop the reaction. The production of ADP by the reaction was an issue as it acted as an inhibitor of the enzyme and so only a limited number of enzyme turnovers could be monitored. Despite multiple attempts to modify the procedure no progress was made and this lead to the use of a coupled assay.

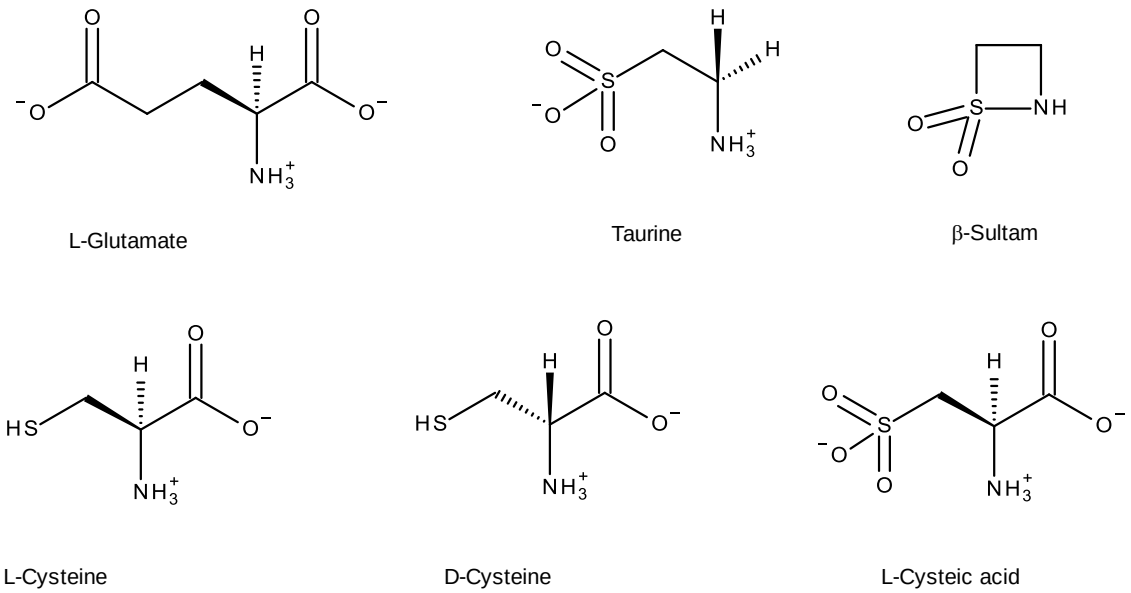
The coupled assay utilised for the glutamine synthetase (GS) reaction was that detailed in the experimental (and shown again in Scheme 58). This procedure has been widely used for investigations involving glutamine synthetase.[222-224]

Kinetic measurements of the biosynthetic activity of the enzyme were performed at 37 ^{o}C in a coupled assay with pyruvate kinase (PK) and lactate dehydrogenase (LDH). For every molecule of glutamate which is converted to glutamine, one molecule of ATP is converted to ADP. The production of ADP can be measured by the following procedure: ADP is converted into ATP by the reaction with phospho(enol)pyruvate, catalysed by PK; the pyruvate produced by this reaction is then reduced by NADH (catalysed by LDH) to produce lactate and NAD+. Thus the rate of reduction in concentration of NADH measured spectrophotometrically at 340 nm is representative of the rate of conversion of glutamate to glutamine. In each experiment a blank (with no GS enzyme present) was run to account for the background rate of NADH oxidation. A second cell was also set up as a control to measure the rate of GS activity under "normal" conditions. The third cell contained all other reactants and enzymes along with the potential inhibitor.



Scheme 58 Coupled assay utilised in the UV-Vis monitoring of the glutamine synthetase reaction

The compounds tested as inhibitors of glutamine synthetase were selected either due to their structural similarity to the normal substrate (glutamate) or because they were potential taurine (or taurine analogue) pro-drugs following work carried out by Ward *et al*. showing taurine had a potential effect on neurotransmission in the brain.[198] The structures of these potential inhibitors are shown in Scheme 59 and a summary of the results is shown in Table 14.



Scheme 59 Structures of compounds investigated as potential inhibitors of glutamine synthetase (and glutamate for comparison)

As can be seen from the sample data shown for β-sultam inhibition of glutamine synthetase in Figure 27 there is no significant difference in the rate of NADH oxidation in the presence of increasing concentrations of inhibitor. Similar results were also obtained for reactions involving taurine.

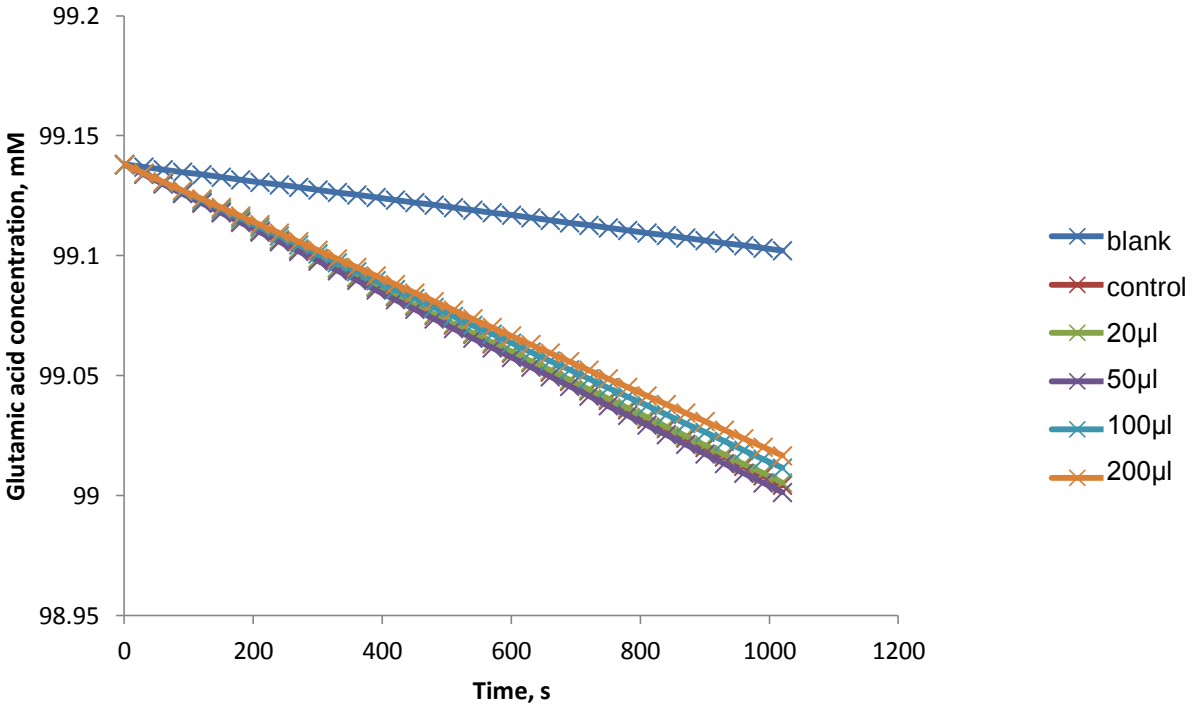


Figure 27 Effect of β-sultam on the rate of glutamine production catalysed by GS (stock inhibitor concentration = 1.5 M)

The rate enhancements seen in the case of L- and D-cysteine are possibly due to these compounds acting as substrates for the enzyme, GS. The increased concentration of substrate leads to an increase in the rate of ADP production and hence an increase in the rate of NADH oxidation. An assay was run with cysteine in the absence of glutamate and this confirmed the substrate action by following the decrease in the concentration of NADH.

L-Cysteic acid initially showed a similar rate enhancement to that seen with D- and L-cysteine (Figure 28). This was not however due to the compound being a substrate but rather the action of the compound catalysing the oxidation of NADH. This is assumed to be via an acid-catalysis mechanism but further investigations into this were not carried out.

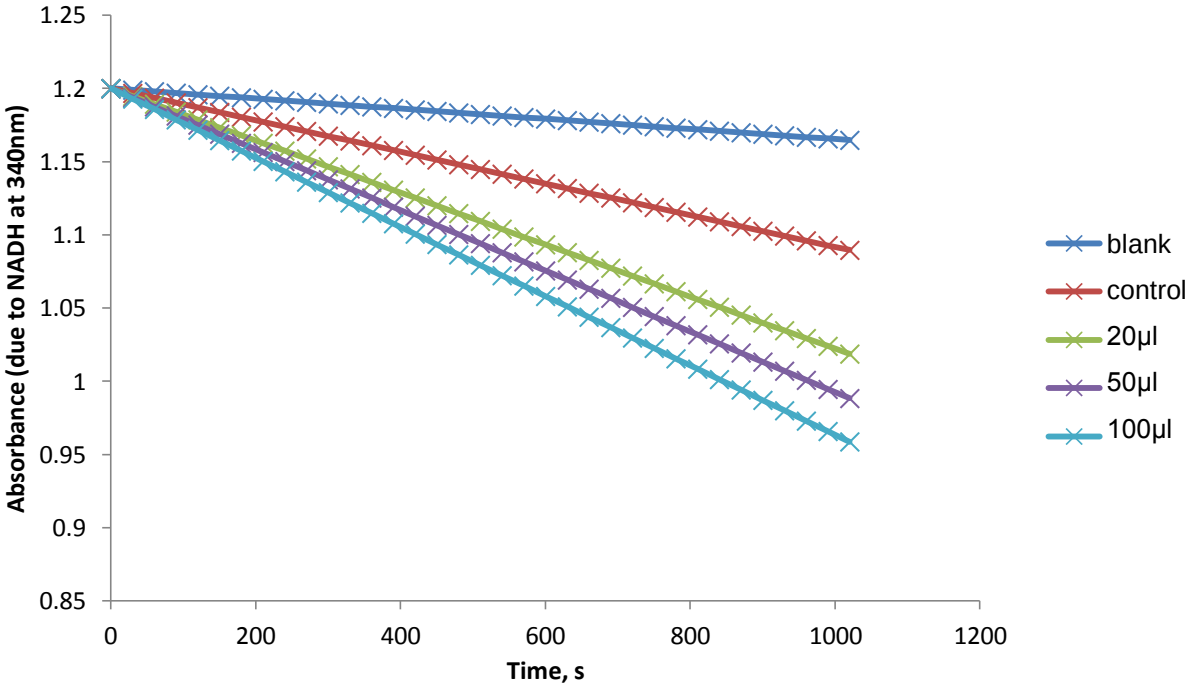


Figure 28 Apparent rate enhancements due to L-cysteic acid (stock inhibitor concentration = 0.5M)

Compound	**Effect on activity of glutamine synthetase**
β-Sultam	No effect
3-Benzyl Carboxylate β-Sultam	No effect
3-Carboxy-β-Sultam	No effect
Taurine	No effect
L-Cysteic Acid	No effect
L-Cysteine	Rate enhancement
D-Cysteine	Rate enhancement

Table 14 Summary of results regarding glutamine synthetase inhibition

Further work in this area is required to fully explain many of the observations. Testing of L- and D-cysteine as substrates of each of the three assay enzymes and product analysis would show why the rate enhancements seen occurred. They may be due to phosphorylation by GS producing ADP, or reduction by LDH and NADH going to NAD+. Another avenue of interest is to test the potential inhibitors against other neurotransmitter enzymes such as glutamate decarboxylate or GABA transaminase.

3.4 Mechanism of Ertapenem Hydrolysis by BcII

^{1}H, ^{13}C, DEPT, COSY, HSQC and HMBC NMR spectra of ertapenem were collected and used to assign all ^{1}H NMR peaks to the structure (Scheme 60) as shown in Figure 29, with assignments in Table 15.

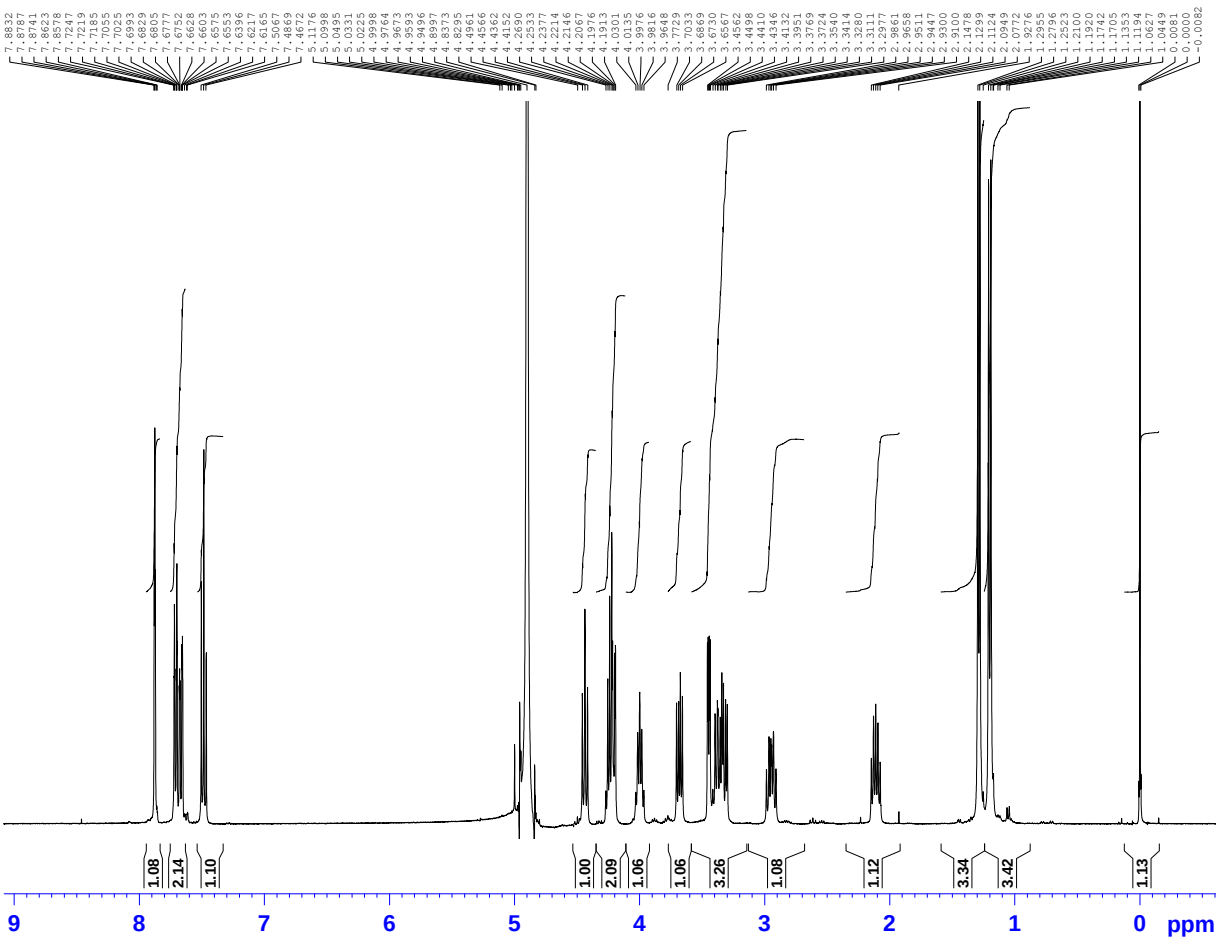
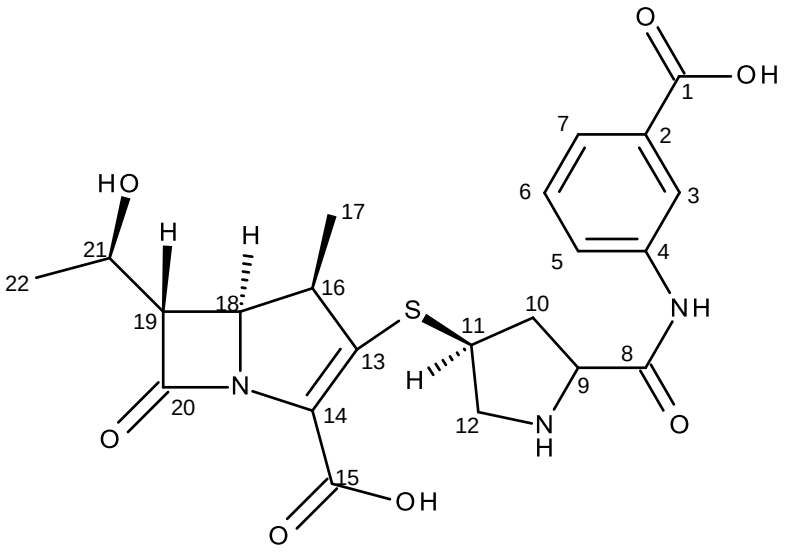


Figure 29 ^{1}H NMR spectrum of ertapenem in pH 7 deuterated phosphate buffer

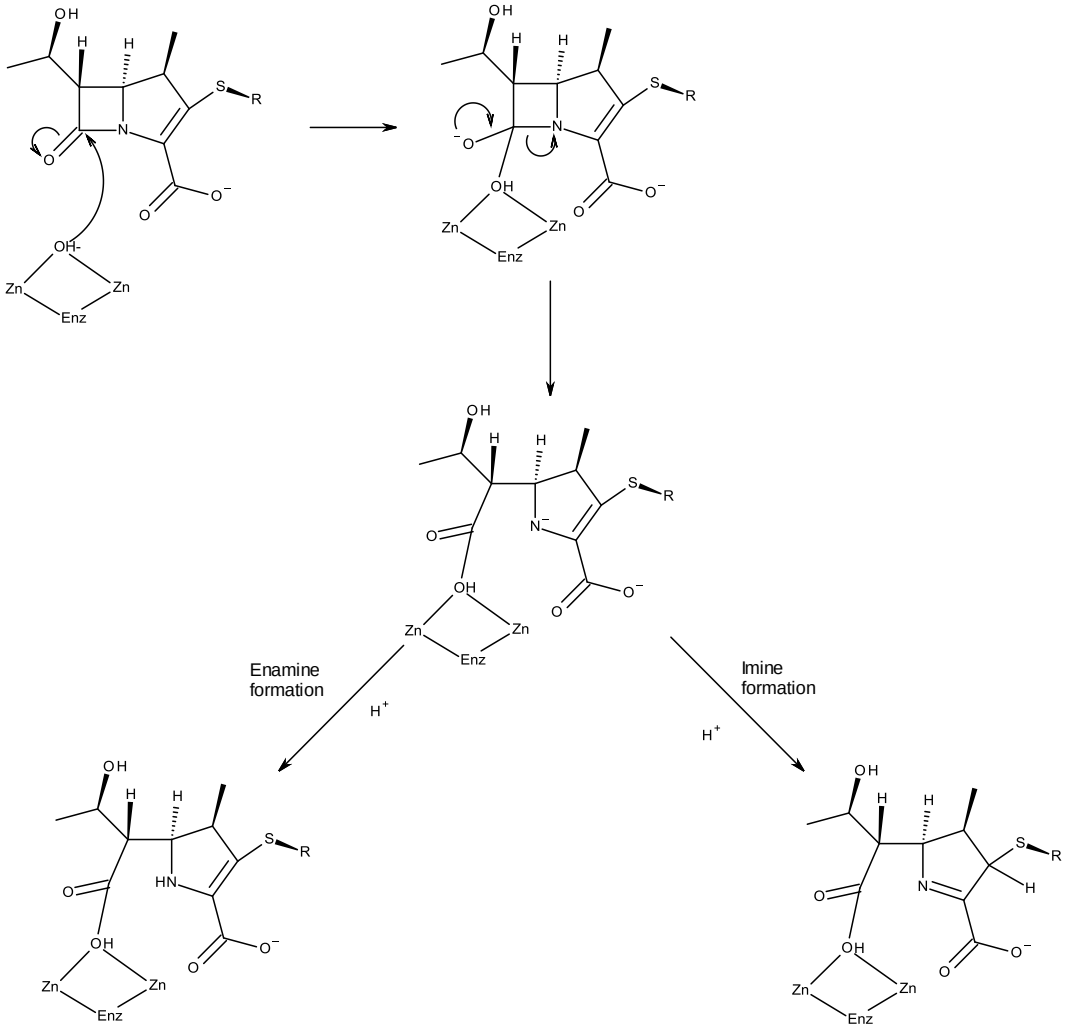


Scheme 60 Ertapenem with carbons numbered for assignment to NMR spectra

Peak shift (ppm)	Splitting	Number of hydrogens	Coupling constants (Hz)	Position
7.88	Singlet	1		3
7.71	Doublet	1	7.6	5
7.67	Doublet	1	7.6	7
7.49	Triplet	1	7.8	6
4.44	Triplet	1	8.0	9
4.25	Doublet	1	5.98	18
4.21	Doublet of doublets	1	2.6 + 9.1	21
4.00	Quintet	1	6.5	11
3.68	Doublet of doublets	1	6.5 + 11.9	12
3.45	Doublet of doublets	1	2.5 + 6.2	19
3.37	Triplet	1	8.2	16
3.32	Doublet of doublets	1	5.2 + 11.9	12
2.95	Doublet of triplets	1	7.9 + 14.3	10
2.21	Doublet of triplets	1	7.1	10
1.29	Doublet	3	6.2	22
1.20	Doublet	3	7.3	17

Table 15 Assignment of ^{1}H NMR peaks

Initial attack of the BcII enzyme on the β-lactam carbonyl (carbon-20) of ertapenem leads to the cleavage of the C-N bond of the β-lactam ring. The aim of this investigation was to prove whether protonation then occurred on the β-lactam nitrogen (via enamine formation) or on carbon-13 (via imine formation) (Scheme 61).



Scheme 61 BcII hydrolysis of ertapenem could lead to formation of an imine or enamine

Comparison of the ^{1}H NMR spectrum from after ertapenem hydrolysis to that in Figure 29 shows several differences in key peaks. There are two new sets of doublets at 1.19 and 0.72 ppm which relate to small shifts of the methyl protons (carbons 22 and 17). There are, however, more significant changes in the peaks related to the protons attached to the β-lactam and dihydropyrrole rings. There are significant

changes in the chemical shift of the protons on carbons 16, 18 and 19 associated with the changes in functionality in this area of the molecule (Table 16).

Chemical shift after BcII hydrolysis (ppm)	Chemical shift before hydrolysis (ppm)	Splitting	Carbon number	Change in ppm (from pre-hydrolysis)
2.62	4.25	Doublet	18	-1.63
4.32	3.45	Doublet of doublets	19	+0.87
2.55	3.37	Triplet	16	-0.82

Table 16 Chemical shift changes for ring protons during hydrolysis

The decrease in chemical shift for the C-16 proton suggests a movement from the enamine to imine structure. Further evidence of this mechanism was derived from studying the 2D NMR spectra.

From the HSQC spectrum (Figure 30) there is a correlation between the protons at 1.05 ppm and the carbon at 14 ppm. These are assigned to the methyl group (carbon-17) and correlations between these protons and other carbons can be established by the use of an HMBC spectrum. The protons at 1.05 ppm show three cross-peaks by HMBC (Figure 31) which can relate to carbons 16 (2 bond lengths), 18 and 13 (both three bond lengths away). The cross-peak observed at 43 ppm is due to interaction of H-17 and C-16. The second cross-peak at 73 ppm is between H-17 and C-18. The third cross-peak has to be between H-17 and C-13 and is seen at 57 ppm. There is no peak in the ^{1}H NMR spectrum for this position (nor can a cross-peak be found on the HSQC spectrum) due to deuterium incorporation. This is further evidence for the formation of the imine rather than protonation of the nitrogen. If the enamine had formed then C-13 would be expected to give a peak at around 139 ppm (as in the spectrum from before hydrolysis). The drop to 57 ppm and lack of signal on the HSQC spectrum indicates deuterium incorporation at this position during hydrolysis.

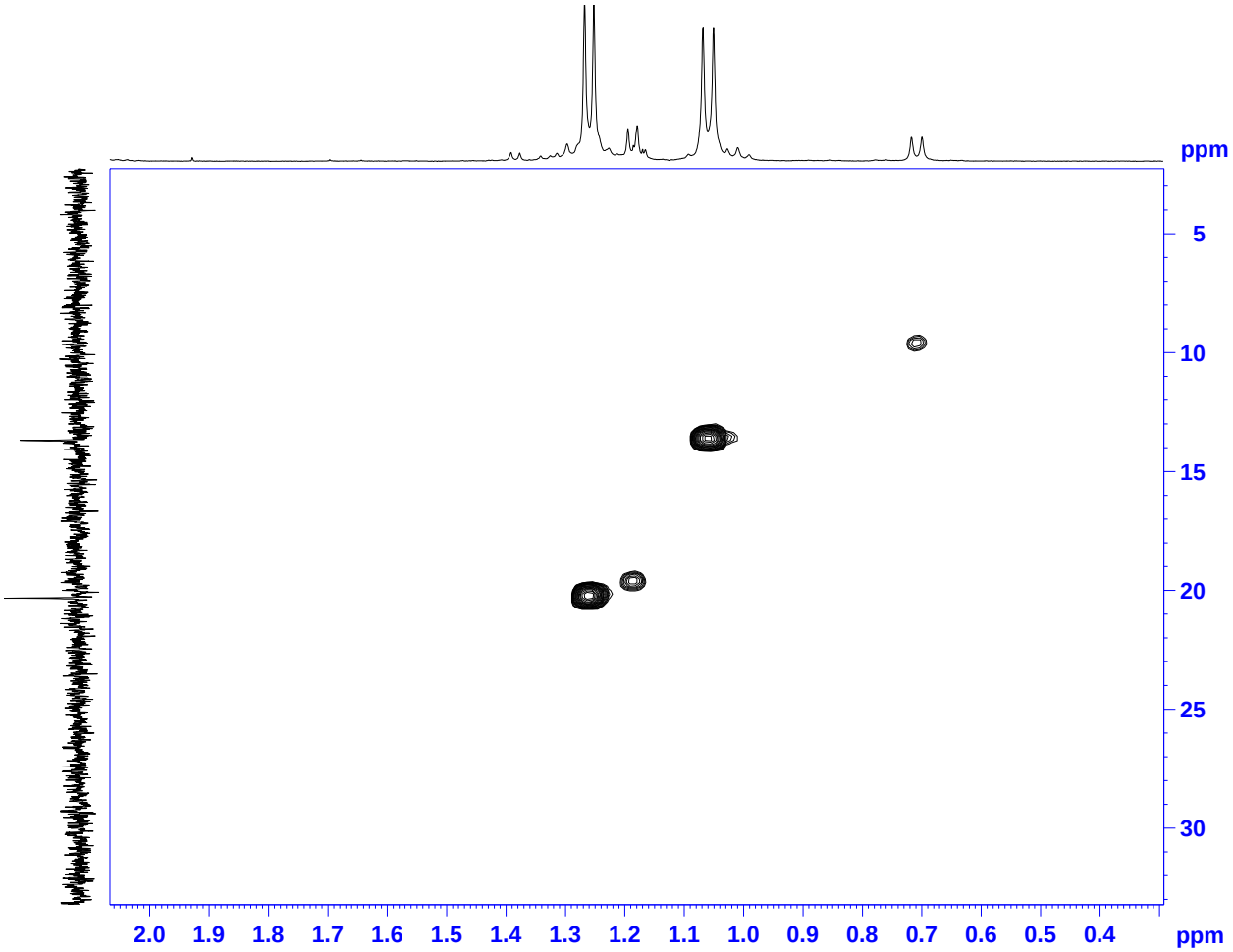


Figure 30 HSQC spectrum of BcII hydrolysed ertapenem

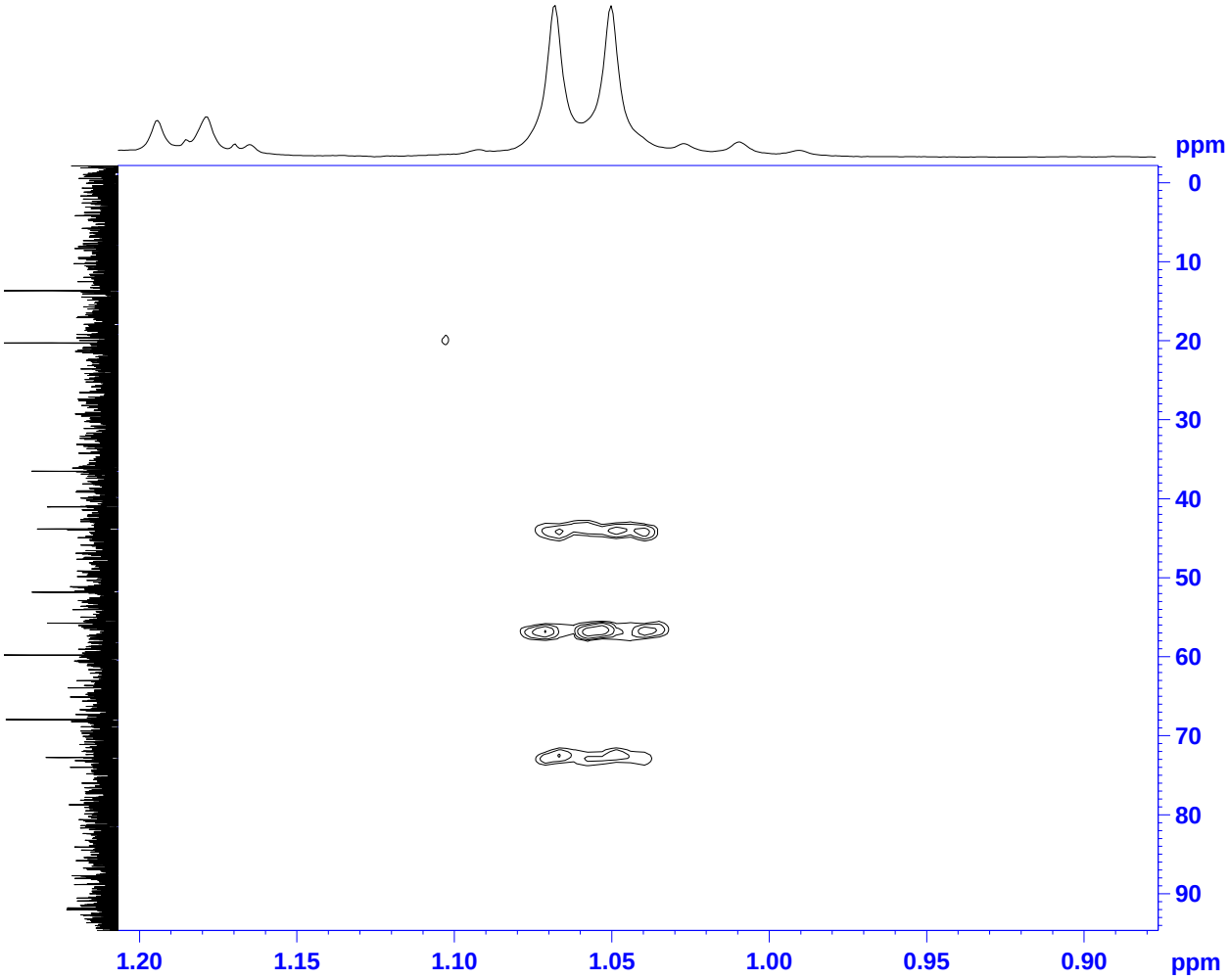


Figure 31 HMBC spectrum of BcII hydrolysed ertapenem

Conclusion

A new β-sultam, 3-carboxylate-1,2-thiazetidine-1,1-dioxide was synthesised by the debenzylation of 3-benzyl-1,2-thiazetidine-1,1-dioxide using sodium metal in liquid ammonia.

Chloroacetate buffer catalyses the hydrolysis of the unsubstituted β-sultam via the acidic form of the buffer with a rate constant of 9.66×10^{-2} $M^{-1}s^{-1}$.

3-carboxy-β-sultam undergoes acid and base catalysed hydrolysis and the acid group has a pK_a of 4.5 ($K_a = 3.162 \times 10^{-5}$).

D-Cysteine is a weak inhibitor of BcII with a K_i value of 7.5×10^{-3} M.

None of the β-sultams tested (the unsubstituted β-sultam, 3-benzyl-β-sultam or 3-carboxy-β-sultam) acted as inhibitors of BcII or GS.

Dicarboxylic acids did not show inhibition of BcII at millimolar concentrations. Both L- and D-Cysteine were shown to be substrates of GS.

Ertapenem is hydrolysed by BcII to yield an imine functionality in the dihydropyrrole ring.

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