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**MECHANISM OF CATALYSIS AND
INHIBITION OF *BACILLUS CEREUS* CLASS B
 β -LACTAMASE**

By

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March 1999

A thesis submitted to the University of Huddersfield in partial
fulfilment of the requirements for the degree of Doctor of Philosophy

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Abstract

Bacillus cereus class B β -lactamase (β -lactamase II) is a bacterial metalloprotein which requires zinc ions for its activity and catalyses the hydrolysis of the β -lactam amide bond of penicillins and related antibiotics. The pH dependence of k_{cat}/K_m and k_{cat} for the β -lactamase II catalysed hydrolysis of cephaloridine, benzylpenicillin and cefuroxime has been investigated and indicates that there are at least three catalytically important groups in the enzyme active site, two of pK_a 5.6 ± 0.2 and one of pK_a 9.5 ± 0.2 . Below pH 5 there is an inverse second order dependence of activity upon hydronium ion concentration, indicative of the requirement of two basic residues for catalysis. These are assigned to zinc-bound water and aspartate 90, both with pK_a of 5.6 ± 0.2 . The kinetic solvent isotope effects were investigated and found to be 1.3 ± 0.5 for k_{cat}/K_m and 1.5 ± 0.1 for k_{cat} . The effect of added methanol on the enzyme reactivity was studied and showed no influence on k_{cat} indicating that Asp90 does not act as a nucleophile to generate an anhydride intermediate. The enzyme activity, as measured by k_{cat}/K_m , was independent of zinc ion concentration from pH 6 to 7, but was augmented by increasing zinc ion concentration below pH 5. Below pH 6 the enzyme activity was found to be third order in hydronium ion concentration and a decrease in the apparent pK_a value was observed with increasing zinc ion concentration.

Several thiol compounds were synthesised and tested as *Bacillus cereus* β -lactamase II inhibitors. The kinetics and mechanism of inhibition of β -lactamase II by thiol N(2-mercaptoethyl)phenylacetamide (1), were investigated. The pH dependence of K_i shows similar pH inflections to those observed in the catalysed hydrolysis of substrates. The inhibitory effect of N-carbobenzoxy-cysteinyl amino acids was investigated and the effect of the dipeptide substituent and the stereochemistry at each centre was studied. It was found that the best inhibition was obtained with a hydrophobic side chain and that the D,D isomers exhibit the highest potency, with K_i 's in the μM region.

The possible mechanisms of action for β -lactamase II are discussed and it is concluded that the zinc ion acts as a Lewis Acid to stabilise the dianionic form of the tetrahedral intermediate and to provide a hydroxide-ion bound nucleophile. When the enzyme binds one zinc ion, the carboxylate anion of Asp90 acts as a general base to form the dianion and also, presumably, as a general acid catalyst facilitating C-N bond fission. However, when the enzyme binds two zinc ions it is proposed that the water molecule bound to the second zinc ion acts as the general acid catalyst since Asp90 is a ligand of the second zinc ion.

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Abbreviation List

CAPS	3-[Cyclohexylamino]-1-propanesulfonic acid
CAPSO	3-[Cyclohexylamino]-2-hydroxy-1-propanesulfonic acid
DCC	N,N'-Dicyclohexylcarbodiimide
DCM	Dichloromethane
DCU	N,N'-Dicyclohexylurea
DMSO	Dimethylsulfoxide
EDTA	Ethylenediaminetetraacetic Acid
ES-MS	Electrospray ionisation mass spectrometry
FT-IR	Fourier Transform Infra-Red
HEPES	N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
MES	2-[N-Morpholino]ethanesulfonic acid
MOPS	3-[N-Morpholino]propanesulfonic acid
NMR	Nuclear Magnetic Resonance
ppm	parts per million
SDS PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

TAPS	N-tris[Hydroxymethyl]methyl-3-aminopropanesulfonic acid
TMS	Tetramethylsilane
UV-Vis	Ultraviolet-Visible spectroscopy

Introduction

1. Introduction

1.1 General introduction

It has been known for a long time that microorganisms produce substances capable of inhibiting the growth of other microorganisms in order to protect themselves¹. These substances are known as antibiotics. Their discovery has had a major effect on the treatment of bacterial infections and played an important role in the development of human medicine.

The majority of antimicrobial agents either modify or inhibit the synthesis of key substances in the bacteria: some antibiotics such as sulfonamides interfere with bacterial protein synthesis, others interfere with the synthesis of bacterial DNA². The latter have proved to be very effective against bacteria, but they also carry a risk of toxicity for humans.

1.2 The β -lactam antibiotics

1.2.1 The β -lactam antibiotics

Since the discovery by Fleming in 1929 of penicillin³, by observing antibiosis between a *Penicillium notatum* mould and a culture of *Staphylococcus aureus*, the number of classes of β -lactam antibiotics continues to grow. They have been very effective, and hence, are widely used against pathogenic bacterial infections.

The β -lactam antibiotics represent a large family of compounds which can be classified into several groups according to their structure⁴. They all have a common

structural similarity, a four membered lactam ring often fused to a secondary ring structure through the nitrogen and the adjacent tetrahedral carbon atom of the β -lactam ring. The most familiar class contains the penicillins (1, Figure 1.1) (penams) with the β -lactam fused to a thiazolidine ring and another important class consists of the cephalosporins (2, Figure 1.1) (cephems) with the β -lactam fused to a dihydrothiazine ring. There are many other classes of β -lactam antibiotics which have been isolated from the fermentation broth of microbial cultures, and an even larger number have been synthesised in the laboratory. Among these are the cephamycins (3), oxacephems (4), penems (5), oxapenams (such as clavulanic acid (6)), carbapenems (such as thienamycin (7)), nocardicins (8) and monobactams (9) (Figure 1.1).

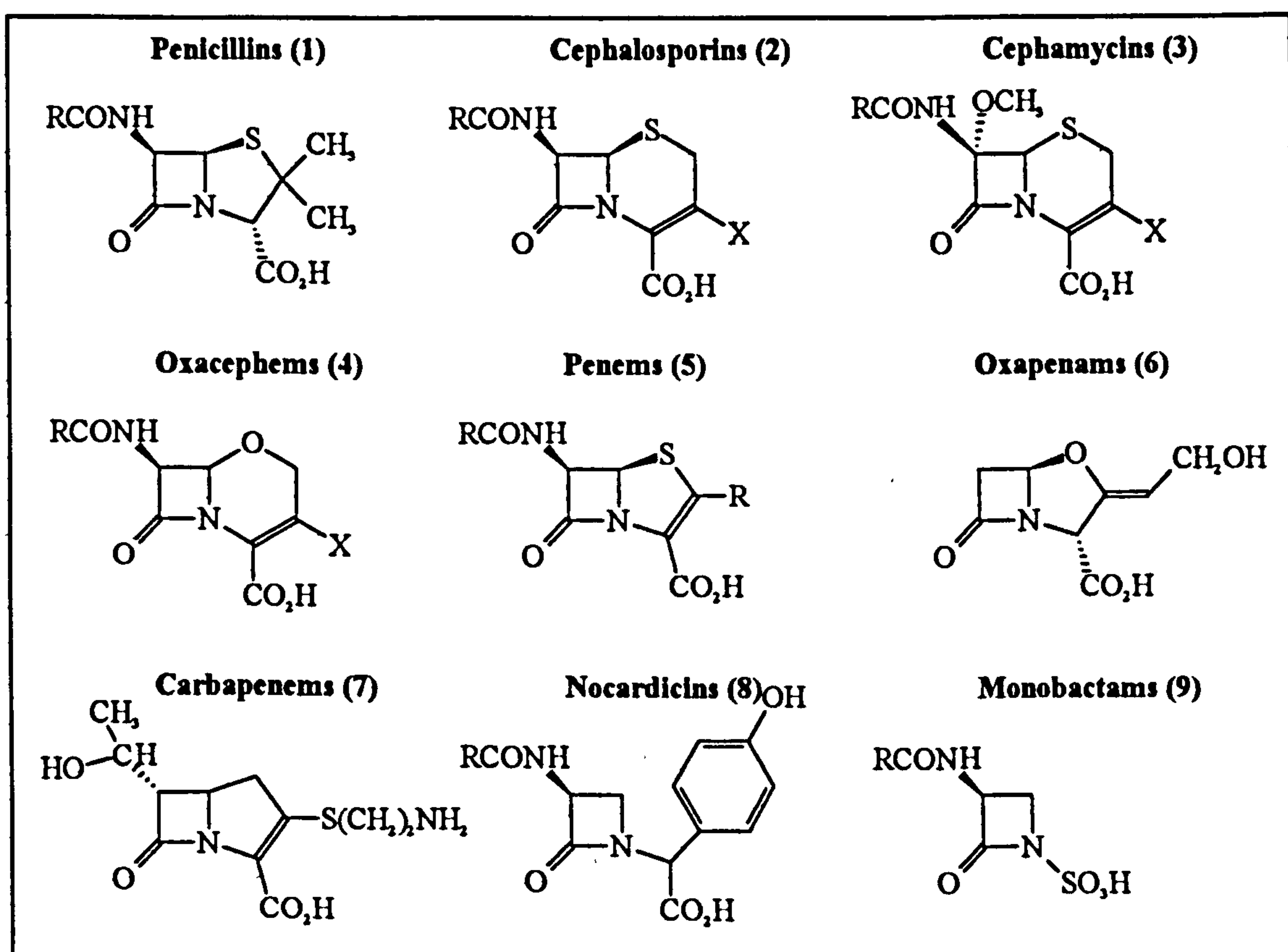


Figure 1.1 β -Lactam antibiotics

The bacterial activity of these antibiotics is often attributed to the chemical reactivity of the β -lactam ring, but the molecular recognition between the antibiotic and its receptor target enzyme is equally important.

1.2.2 The mechanism of action of β -lactam antibiotics

β -Lactam antibiotics selectively interfere with the formation of the bacterial cell wall, inducing physiological effects, leading to the death of the bacterium. One of the main reasons for the low toxicity of these antibiotics to humans is that humans don't have a cell wall..

1.2.2.1 Bacterial cell wall structure and biosynthesis

The bacterial cell wall consists of several layers¹. Rigid and porous, it provides the shape and physical protection for the cell by preventing the swelling and the subsequent rupture of the bacterial cytoplasm as a consequence of high internal osmotic pressure. Its synthesis is a major function of the bacterial cell.

Bacteria are divided into two classes according to their wall structure: Gram-positive and Gram-negative¹. The cell envelope of the Gram-negative bacteria consists of three distinct layers: the cytoplasmic (inner) membrane, the peptidoglycan layer and an outer membrane which is selectively permeable. The cell walls of Gram-positive bacteria are less complex and contain a larger amount of peptidoglycan. They are much more permeable and therefore highly susceptible to β -lactam antibiotics.

The peptidoglycan (or murein) is a rigid structural frame which consists of parallel polysaccharide chains covalently cross-linked by short peptides (Figure 1.2). The glycan is a polymer of alternating N-acetylmuramic acid (NAM) and N-acetylglucosamine (NAG) units, connected by β -1,4-glycosidic linkages. The composition of the peptide varies from bacteria to bacteria, although they commonly have the sequence L-alanyl-D-glutamyl- γ -L-X-D-alanyl, in which L-X is most often an amino carrying residue such as lysine.

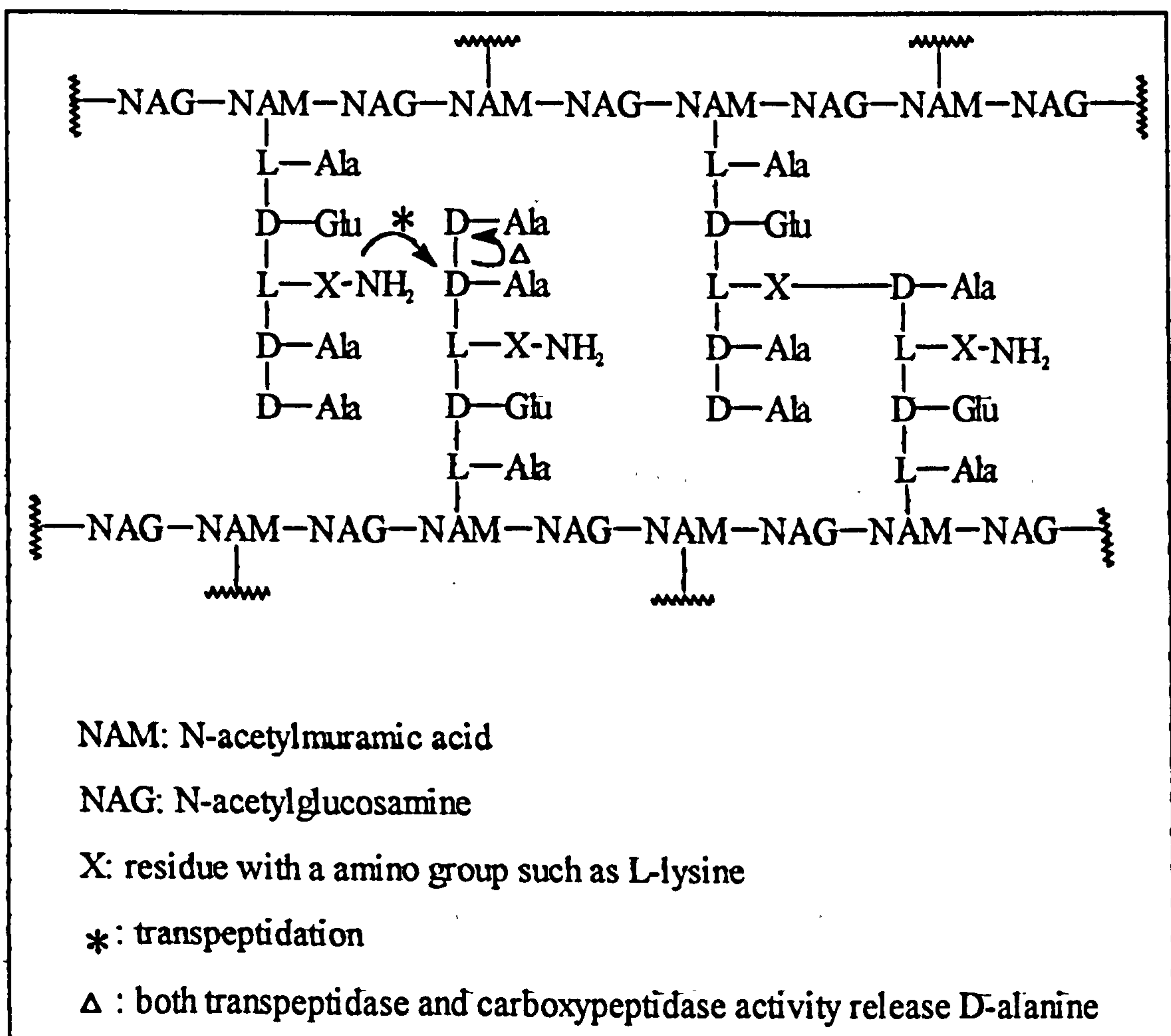


Figure 1.2 Schematic representation of the cross-linking in peptidoglycan

The biosynthesis of the peptidoglycan consists of three major steps: first the synthesis of the precursors in the cytoplasm, which are exported through the membrane in the second step using a liposoluble carrier, in the third step they are assembled to form the peptidoglycan (firstly a transglycosylation reaction which elongates the glycan strands and then transpeptidation).

The cross-linking process which occurs during the final step consists of two stages; carboxypeptidation followed by transpeptidation. It involves three enzyme activities: peptidoglycan transpeptidase, which catalyses the cross-linkage reaction; D-alanine carboxypeptidase, which hydrolyses the terminal D-alanine residue from the pentapeptide side chain and endopeptidase which hydrolyses the cross linkage between peptides⁵.

The precursor of the pentapeptide contains one additional D-alanine residue exhibiting a D-alanyl-D-alanine C terminus. This polypeptide is a substrate for the transpeptidase and the carboxypeptidase. The latter regulates the extent to which the peptidoglycan is cross-linked by clipping off the terminal D-alanine without forming a cross-link⁶. Transpeptidase reacts with its peptide substrate to form an acyl enzyme intermediate, with the elimination of D-alanine and subsequent reaction with the free amino group of a second cross-bridge leads to the formation of a cross-link and regeneration of the enzyme (Figure 1.2).

β -Lactam antibiotics are known to inhibit all three of the enzyme activities that occur in the terminal stages of peptidoglycan biosynthesis⁷. Their non toxicity

towards humans is due to the lack of molecules similar to peptidoglycan precursors, and of D-amino acids in eukaryotic cells.

1.2.2.2 Inhibition of cell wall synthesis

In spite of the fact that β -lactam antibiotics have long been employed in medicine and their mode of action extensively studied^{8,9,10,11}, the complete knowledge of their mechanism of action is still unknown. As most of the studies were performed with penicillins and cephalosporins, it is generally assumed that other β -lactam antibiotics act similarly⁶.

β -Lactam antibiotics react with a number of proteins situated at the outer surface of the plasma membrane. Studies have shown that there is no binding to intracytoplasmic components¹⁰. The mode of action of the β -lactam antibiotics is generally considered to be one of two processes, either the activation of autolytic cell wall processes or the inhibition of peptidoglycan incorporation into the cell wall¹². Two families of enzymes recognise penicillins and other β -lactam antibiotics. The first family is known as the penicillin-binding proteins or PBPs which contains the DD-peptidases which are inactivated by the β -lactams and play a major role in the late stage of the bacterial cell wall peptidoglycan synthesis. The second family contains the β -lactamases which hydrolyse the β -lactam ring.

The complexity of the reactions observed is due to the variety of PBPs in the bacterial cell membrane. There are typically three to eight distinct PBPs found in any given organism, and are all situated on the bacterial plasma membrane¹⁰. They

all appear to be serine active site enzymes and perform their catalytic cycle according to an acylation/deacylation pathway. Their physiological roles have been reviewed by Waxman and Strominger⁹.

There are two general categories of PBPs: low molecular weight (40-50 kDa), exhibiting DD-carboxypeptidase activity *in vitro*¹³, which are relatively abundant and appear to be unnecessary for the cell viability; high molecular weight PBPs (60-140 kDa) which are minor components of the cell, but are essential to its viability. Some, such as *Escherichia coli* PBPs 1A and 1B, combine transglycosylase and transpeptidase activities *in vitro*. The high molecular weight PBPs are more sensitive than the low molecular weight PBPs to both penicillins and cephalosporins⁹.

Penicillins are thought to exert their bacteriocidal effects by interfering with the synthesis of the bacterial cell wall through inhibition of the transpeptidase enzymes. The first stage of antibiotic activity is the binding of the β -lactam to the PBP, whilst the second stage is the physiological effect on the cell, induced by the formation of the enzyme-inactivated complex, such as disruption of the cell growth cycle.

Penicillin binds to and inactivates peptidoglycan transpeptidase or carboxypeptidase probably by virtue of its structural similarities to at least some of the possible conformations of the acyl-D-alanyl-D-alanine of nascent peptidoglycan strands^{9,14} (Figure 1.3). Penicillins have been described as transition state analogues of X-D-alanyl-D-alanine.

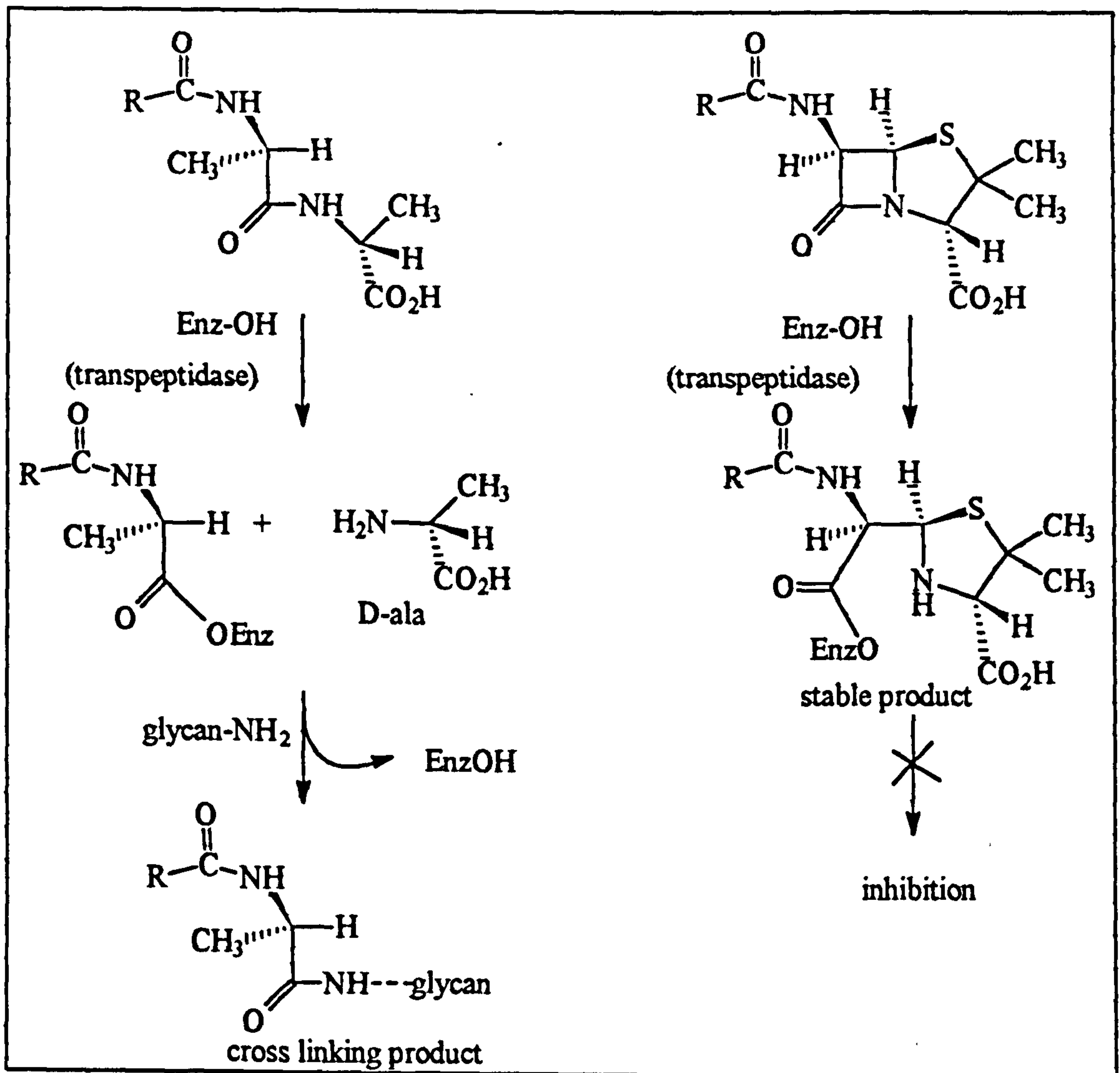


Figure 1.3 The transpeptidation reaction of N-acylated D-alanyl-D-alanine, catalysed by a transpeptidase serine enzyme, compared with the inhibition reaction with penicillin

β -Lactam antibiotics are considered to be active site-directed acylating agents. First, they bind to the enzyme reversibly, then acylate the enzyme. The β -lactam remains attached to the acyl enzyme because the leaving amino group remains covalently attached to the carboxylic acid ester thus blocking access of the amino acceptor (Figure 1.3). The acyl enzyme cannot therefore turnover and is inactivated. The catalytic pathway is described in Figure 1.4¹⁵. When the

“substrate” is penicillin, the deacylation step (k_3) is very slow, and the enzyme is immobilized as a very stable acyl enzyme complex. Cell death and lysis are known to be indirect consequences of the weakening of the peptidoglycan⁸. When S is the physiological substrate (X-D-Ala-D-Ala), the values of k_2 and k_3 are high, leading to a high turnover rate.

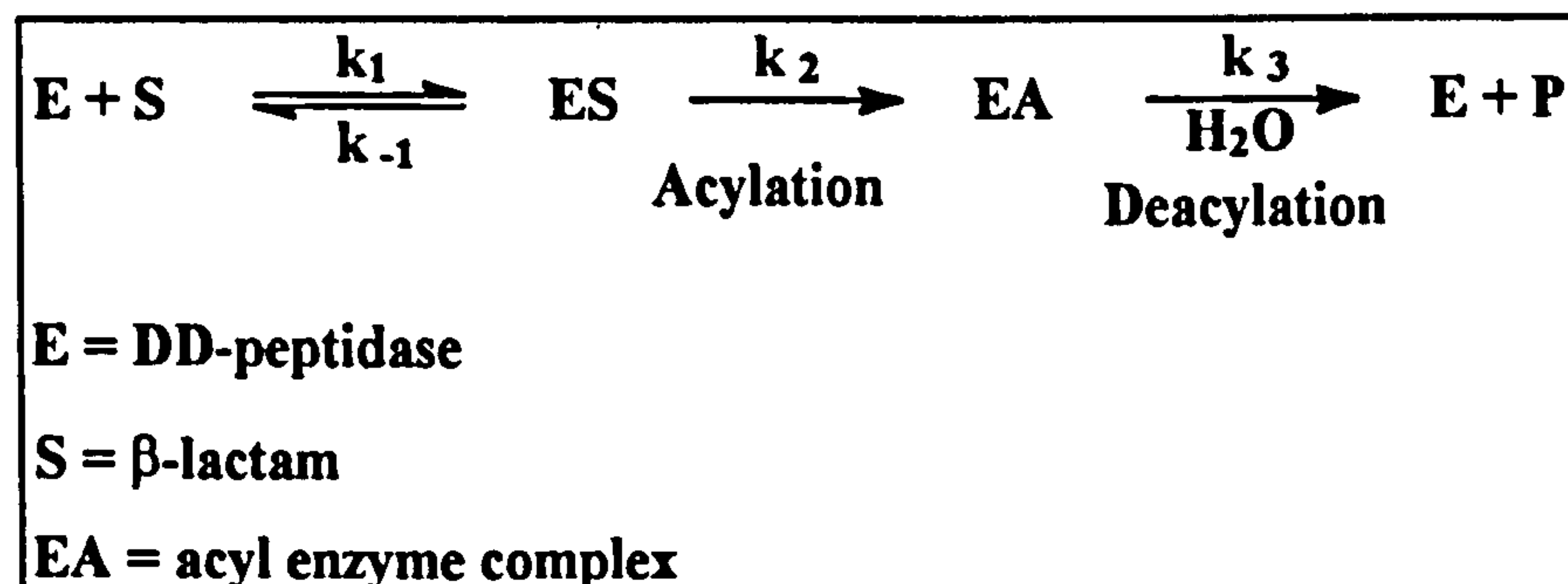


Figure 1.4 Catalytic pathway of the interaction between β -lactam and DD-peptidase

There are two essential features for the recognition of the penicillin antibiotic at the active site: the carboxyl group in the 3 position of the 5 membered ring and the β -lactam carbonyl functions⁶, the rest of the molecule bears structural similarity to the natural substrate and helps anchor the antibiotic to the active site.

1.3 Bacterial resistance to antibiotics

The extensive use of β -lactam antibiotics to fight bacterial infections has led to the emergence of resistant strains of pathogenic bacteria¹. Bacteria have found different ways to overcome the deadly action of β -lactam antibiotics. The mechanisms frequently observed are:

- efflux or active excretion of the antibiotic out of the cell using pumps;
- modification of the antibiotic target, such as modification of ribosomes, or modification of enzymes responsible for the cell wall synthesis (such as PBP mutations);
- enzymatic modification of the antibiotic making it inactive (such as the action of β -lactamases).

The production of β -lactamases represents the most widespread mechanism of bacterial resistance and poses a serious threat to the therapeutic action of the β -lactam antibiotics. They catalyse the hydrolysis of the β -lactam ring giving rise to biologically inactive products (Figure 1.5).

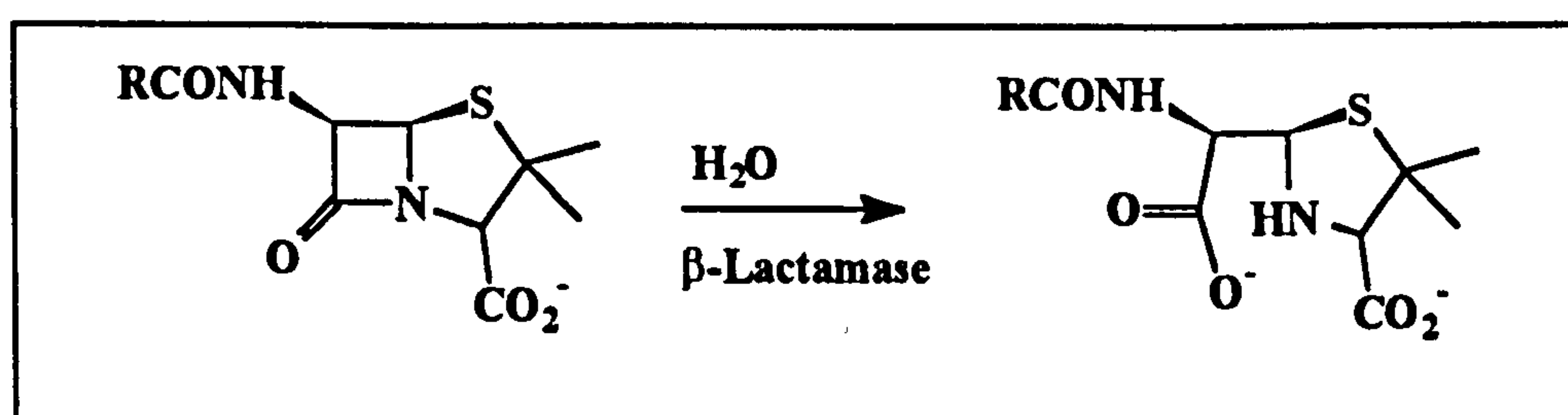


Figure 1.5 Hydrolysis of β -lactam antibiotics by β -lactamase

1.4 The β -lactamases

Fleming, as early as 1929, noticed that some groups of bacteria were not inhibited by penicillin. Later, the component responsible for this phenomenon was identified as an enzyme and named “penicillinase”¹⁶, but are generally now known as β -lactamase.

The β -lactamases appear to be produced only by bacteria and are widely distributed. About 200 enzymes have been discovered and characterised to date¹⁷.

The β -lactamase encoding genes are often found on chromosomes, transposons and plasmids, the latter allowing them to spread from bacteria to bacteria of different genera. Some bacteria are able to produce β -lactamases only when exposed to β -lactam antibiotics and this induction allows the bacterium to regulate the production of the enzymes¹⁸.

The β -lactamases may have evolved from penicillin-sensitive enzymes of cell wall biosynthesis by developing an efficient catalytic mechanism for hydrolysis of the penicilloyl-enzyme linkage (Figure 1.3, page 8). Ambler¹⁹ acknowledged the structural similarity at the active site between class A β -lactamases and some DD-peptidases, although admits that there is little evidence for a common evolutionary origin of the two types of enzymes. The suggestion of a common ancestor is, however, supported by similarities in the secondary and tertiary structures of these enzymes²⁰ and by the fact that some PBPs exhibit a weak β -lactamase activity. The

penicillin binding site in the transpeptidase is broadly similar to the penicillin hydrolysis site in the β -lactamases^{8,9,14}.

1.4.1 Description of β -lactamases

β -Lactamase enzymes (β -lactam hydrolase EC 3.5.2.6) can mechanistically be divided into two groups - serine active site enzymes, which belong to the same family as the PBPs²¹, and zinc enzymes, which require zinc ions for their activity.

The serine enzymes are classified into three groups, A, C and D, on the basis of their primary structures. The class B β -lactamases represent the zinc dependent enzyme and, being the subject of this thesis, a detailed introduction to these enzymes is offered in Section 1.5.

All four classes are produced by gram-negative bacteria, whereas gram-positive species only produce class A and B enzymes which are most often chromosome-encoded¹⁵.

1.4.2 Class A, C and D β -lactamases

The class A, C and D β -lactamases operate by a mechanism similar to the basic initial steps observed for the DD-peptidases (Figure 1.4). They act on their substrate by serine attack on the β -lactam carbonyl carbon to form an acyl enzyme intermediate. However, in the case of β -lactamases this intermediate is relatively unstable because it is hydrolysed to regenerate the enzyme. The acylation step (k_2) and deacylation step (k_3) are generally quite large and the antibiotic is rapidly hydrolysed¹⁵. The mechanism (Figure 1.6) is thought to involve a general base

deprotonating the serine residue as it attacks the carbonyl function of the β -lactam ring, forming a tetrahedral intermediate. A proton is then donated to the amine nitrogen leaving group to facilitate ring opening of the β -lactam. Deacylation may then occur by reaction with water^{22,23(a),24}.

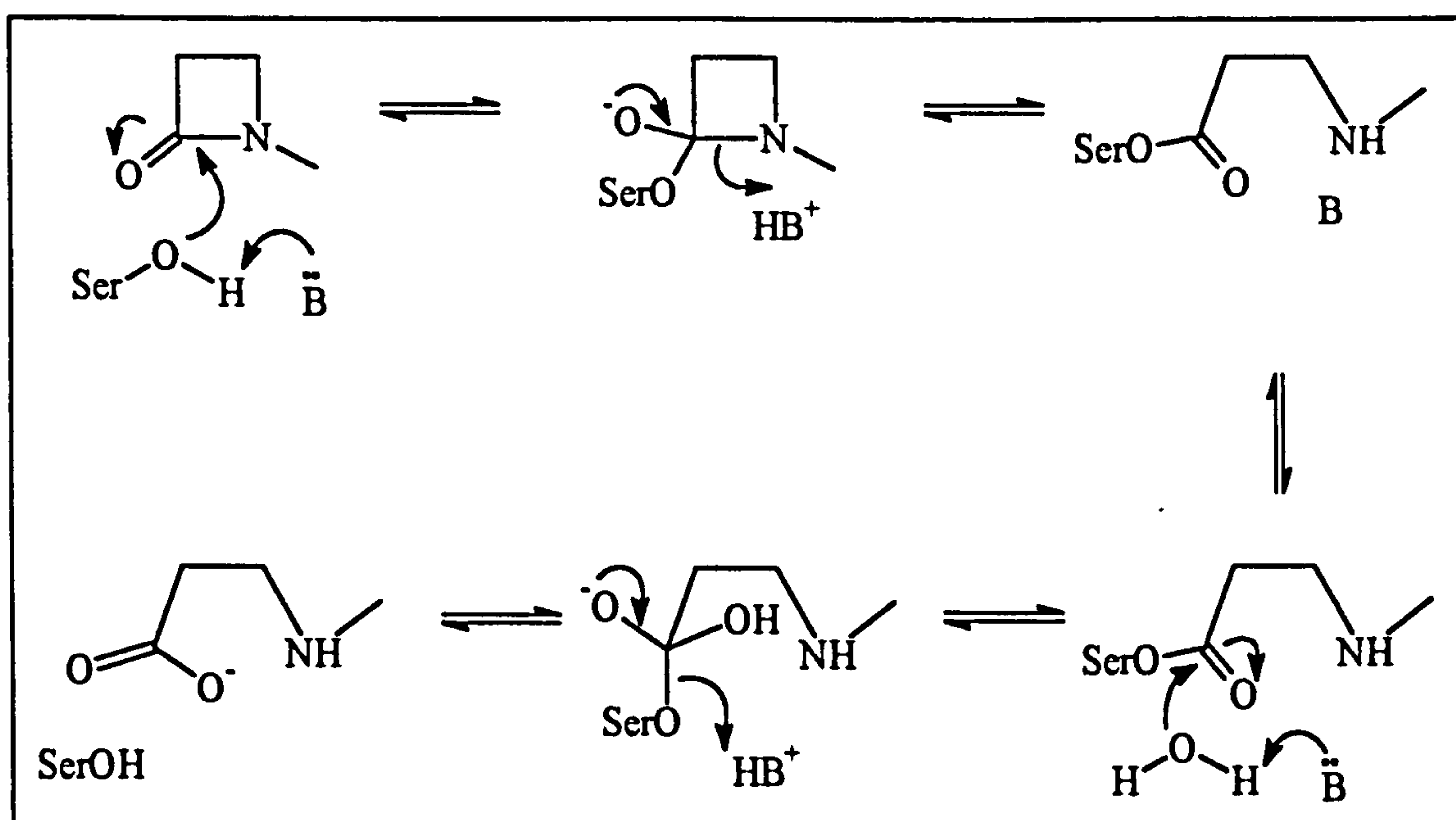


Figure 1.6 Proposed mechanism of action of serine β -lactamases²⁴

Three conserved structural elements have been found to be involved in forming the active site^{15,17}. There is evidence of an “oxyanion hole” in the active site, which is responsible for stabilising the presumed tetrahedral oxyanion intermediate^{15,20,25}.

Class A β -lactamase enzymes are found in both gram-positive (mainly chromosome-encoded) and gram-negative (mainly plasmid-encoded) bacteria²¹.

Their properties and primary structure differ considerably and they therefore represent a very heterogeneous family²⁶. They were first described as “penicillinases” for their ability to very efficiently hydrolyse penicillin. Some class

A β -lactamases can be considered as “perfect” enzymes with their best substrates having rates close to the diffusion controlled rate of encounter¹⁵. The active-site serine was identified as Ser70. The catalytic properties of these enzymes have been discussed by Matagne *et al.*²⁷.

Class C β -lactamases seem to represent a more homogeneous group. They were first described as chromosomally determined cephalosporinases¹⁶ because of a larger k_{cat} value for cephalosporins than for penicillins. It is now known that these enzymes have no particular marked preference and show very similar substrate profiles²⁶. Their structure is close to that of class A, but their sequence varies. Ser64 has been identified as being involved in the catalytic mechanism of these enzymes. The deacylation step is known to be the rate limiting step for class C β -lactamases ($k_2 \gg k_3$) (Figure 1.4)²³. In water containing methanol, the intermediate acyl enzyme can react with the alcohol to give the methyl ester of the product and the rate increases with increasing methanol concentration^{23(b)}.

Class D β -lactamases, also known as “oxacillinases” because of their ability to hydrolyse oxacillins, are very different from the other serine β -lactamases^{26,28}. They are always plasmid-encoded²³ and their kinetics with some substrates are quite complex²⁹. Very little is known about these enzymes and no tertiary structure is available yet.

Different strategies have been adopted to fight the β -lactamase mediated resistance to β -lactam antibiotics, such as the synthesis of new β -lactams which are poor substrates for many β -lactamases. Another successful approach is the production

of mechanism-based inhibitors (e.g. clavulanic acid), also known as “suicide inhibitors”³⁰. These are often β -lactams and inactivate the enzyme by acylating it but subsequently forming a stable covalent adduct³¹. The interactions between active site serine β -lactamases and mechanism-based inhibitors have been studied by Matagne *et al.*³². The potent inhibitor may be used in conjunction with good antibiotics even if they are susceptible to β -lactamase catalysed hydrolysis.

Most class A and D, but not class C, β -lactamases are inhibited by clavulanic acid²³. The incentive in pursuing the search for new inhibitors is driven by the emergence of some β -lactamases which are not inhibited by these inactivators.

1.5 The Class B β -lactamases

Class B β -lactamases are metallo-proteins which require a bivalent transition-metal ion for their activity, most often zinc. They catalyse the hydrolysis of a wide variety of antibiotics including carbapenems which generally escape the action of the active site serine enzymes. They are therefore often described as broad spectrum β -lactamases^{15,33}. They are not inhibited by clavulanic acid or other mechanisms based inhibitors of serine β -lactamases, but are inactivated by metal chelating agents such as EDTA²⁶. The catalytic properties of some well characterised class B β -lactamases have been reviewed by Felici *et al.*³⁴.

For many years the only bacterium known to produce zinc β -lactamases was the non pathogenic species *Bacillus cereus*^{38,39,40} and this enzyme was therefore considered to be clinically unimportant. Later, these metallo-enzymes were found

to be produced by at least ten different bacteria³⁵, some of which are common clinical pathogens³⁶ responsible for nosocomial infections (e.g. *Aeromonas hydrophila*, *Pseudomonas maltophilia* (L1)³⁷, *Bacteroides fragilis*). The clinical importance of these enzymes has now been re-evaluated, especially since mediated dissemination of a metallo- β -lactamase plasmid gene has been observed³⁶. Furthermore there are no clinically useful inhibitors available.

1.5.1 Class B *Bacillus cereus* β -lactamase

The first metallo- β -lactamase to be identified was from *Bacillus cereus*³⁸. Crude extracellular extracts from *B. cereus* displayed penicillinase and cephalosporinase activities, the latter being lost on purification or after exposure to EDTA and other metal chelating agents. The activity was found to be restored upon addition of zinc sulfate to the preparation³⁸. Purification and fractionation by precipitation yielded two crystalline enzymes, β -lactamase I and β -lactamase II, with penicillinase and cephalosporinase activities respectively³⁹. The term cephalosporinase did not reflect the true nature of the substrate profile of this enzyme and was replaced by the description β -lactamase II by Kuwabara *et al.*³⁹.

β -Lactamase II was found to be zinc dependent, heat stable and to have a wider range of activity than most of the β -lactamases previously described³⁸. The suggestion that the zinc was bound to the apoenzyme by one or more thiol groups was reported after it was found that several thiol reagents on β -lactamase II inhibited the zinc activation of the enzyme^{40,41}. The presence of the sole cysteine

residue in β -lactamase II was described by Davies *et al.*⁴², who also determined the molecular weight of the molecule to be 22 kDa.

The substitution of the zinc ion in β -lactamase II by other bivalent metal ions such as Co^{2+} , Cd^{2+} , Mn^{2+} gives metal-enzyme complexes which exhibit significant β -lactamase activity, the zinc enzyme being the most active, whilst Hg^{2+} and Cu^{2+} inactivate the enzyme⁴¹. Equilibrium dialysis and kinetic studies⁴¹ indicated that the enzyme could bind two zinc ions, with the second zinc binding site being of lower affinity.

Studies by ^1H NMR spectroscopy and differential tritium exchange followed by tryptic digestion of the enzyme, revealed at least 5 histidine residues in the protein^{43,44}. Three of these histidines were found to serve as ligands to the zinc ion at the first site, occupation of which is essential for activity (dissociation constant about 1 μM), a fourth histidine is thought to act as a ligand for the second binding site (dissociation constant about 24 mM)^{43,44}.

Elucidation of the amino acid sequence of β -lactamase II, showed that the enzyme consists of a simple polypeptide chain of 227 amino acids, with a molecular mass of 25 kDa^{45,46,47} and not 22 kDa as previously reported⁴². The residues involved in the active site were found to be His86, His88 and His210, and the only cysteine residue to be located at the position 168. These results were in agreement with DNA sequencing experiments⁴⁸.

In a further investigation⁴⁹ using chemical modifications (inactivation of β -lactamase II by water soluble carbodiimide in the presence of nucleophiles), it was suggested that the enzyme may contain an essential carboxylic acid involved in the catalytic mechanism, Glu37. The mechanism of action was thought to involve Glu37 acting as a general base deprotonating a water molecule, which would then attack the β -lactam ring. A pH dependence study of k_{cat}/K_m for the hydrolysis of benzylpenicillin by the Co^{2+} β -lactamase II revealed two pK_a values of 5.5 and 9.0⁵⁰. These findings supported previous results from NMR studies of zinc β -lactamase⁴³. It was suggested later, that the pK_a of 5.5 could be attributed to the zinc bound water, which could be acting as a nucleophilic group⁵⁰.

This postulated mechanism was thought to be consistent with the data obtained from cryoenzymology studies and by rapid-scanning stopped flow techniques of the Co^{2+} β -lactamase catalysed hydrolysis of substrates⁵¹, which showed that catalysis proceeded by a branched kinetic pathway with formation of non covalent enzyme-substrate complexes. The metal ion was found to be directly involved in catalysis. The mechanism of the non-enzyme catalysed hydrolysis of benzylpenicillin was studied by Page *et al.*^{52,53,54}, where the metal ion was found to initially bind to the carboxylate group and the β -lactam nitrogen, stabilising the tetrahedral intermediate and enhancing the rate of C-N cleavage. However, direct coordination of the β -lactam carbonyl oxygen to the metal ion was reported to be kinetically significant in the enzyme catalysed reaction⁵⁵.

From X-ray-crystallographic studies⁵⁶ of Cd²⁺ β -lactamase at 0.35 nm resolution, it was accepted that Glu37 was too far from the catalytic site to play the role proposed by Little *et al.*⁴⁹ and it was suggested that Glu212, which was much closer, could be involved. Two other amino acids with carboxylic acid side chains were found to lie near the active site, Asp81 and Asp90. It was later proved, by oligonucleotide mutagenesis that neither Glu37 nor Glu212 were essential for enzyme activity⁵⁷, and site-directed mutagenesis studies⁵⁸ of Asp81 and Asp90 strongly suggested that Asp90 plays an important role in catalysis, and could possibly act as a general base in the hydrolytic mechanism. By analogy with metallo-peptidase such as carboxypeptidase A⁵⁹, it was proposed that the mechanism of action involved the zinc bound water acting as a nucleophile, attacking the carbonyl group of the β -lactam ring, with Asp90 acting as the general base deprotonating the water with subsequent donation of the proton to the nitrogen of the ring to cause cleavage (Figure 1.7).

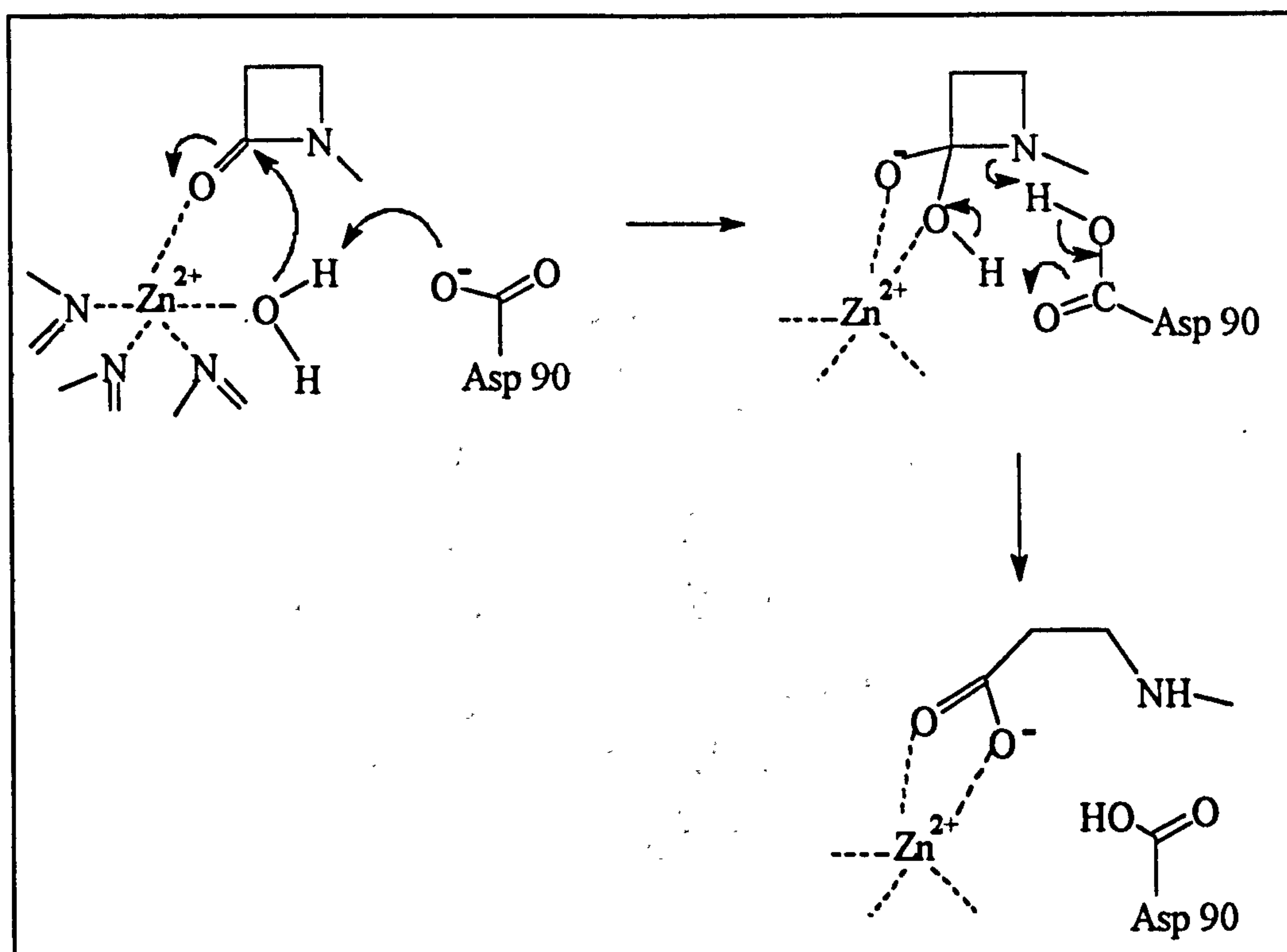


Figure 1.7 Proposed mechanism of action of β -lactamase II⁶⁰

The 3-D structure of β -lactamase II was investigated by X-ray crystallography at 2.5 Å⁶⁰ resolution (300 K) and later at 1.85 Å⁶¹ (100 K).

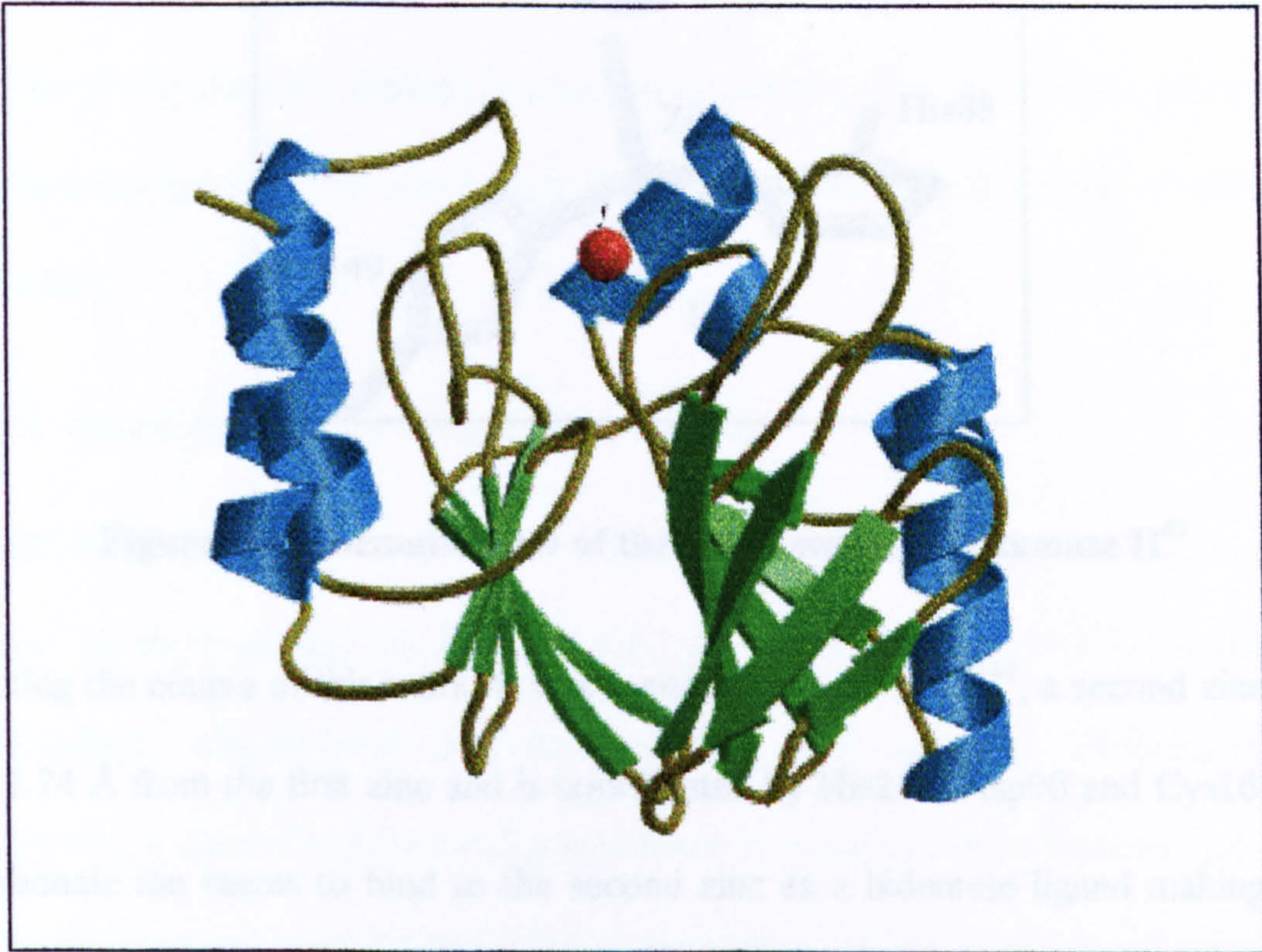


Figure 1.8 Ribbon diagram of the molecular structure of β -lactamase II⁶²

β -Lactamase II is a β sandwich structure ($\alpha\beta\beta\alpha$ protein) with helices on each external face which represents a novel type of folding (Figure 1.8). The dimensions of the molecule are about $32 \times 30 \times 28 \text{ \AA}$. Two tightly packed β -sheets are encircled by five α -helices and the active site is located at the top of the structure between the two β -sheets. Four ligands (His86, His88, His149 and a water molecule or a hydroxyl ion) are bound to the zinc which displays a tetrahedral shape (Figure 1.9).

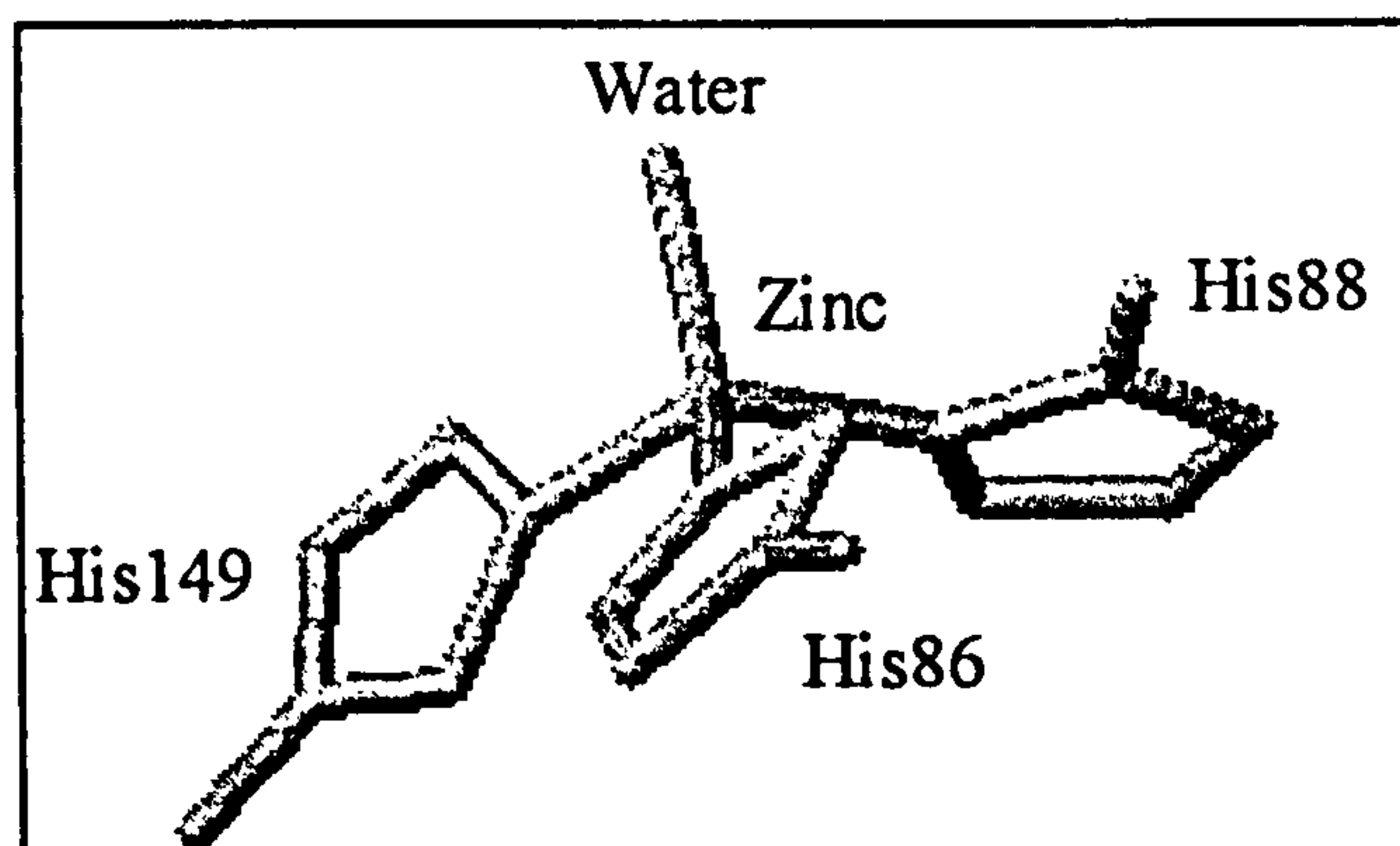


Figure 1.9 Schematic view of the metal site of β -lactamase II⁶³

During the course of this work, it was reported that, at 100 K⁶¹, a second zinc lies at 3.74 Å from the first zinc and is coordinated by His210, Asp90 and Cys168. A carbonate ion seems to bind to the second zinc as a bidentate ligand making the geometry square bipyramidal. The binding of the second zinc which was not observed at room temperature was probably favoured at low temperatures⁶¹. Comparisons between the zinc containing holoenzyme and the apoprotein (zinc-free enzyme) illustrate that in addition to its catalytic role the metal ion has a structural role⁶⁰.

Also in this work, Orellano *et al.*⁶³ confirmed that β -lactamase II binds two zinc ions. The enzyme is fully active with one zinc, however they claimed that its activity is more efficient upon binding of the second zinc after studies of the dependence of the activity on the metal concentration. They suggest a mechanism of action similar to the mechanism, described later, of the *Bacteroides fragilis* binuclear zinc β -lactamase, where the zinc1-bound water acts as a nucleophile whilst the proton donor is another water molecule bound to zinc2.

Considering the ambiguity and the uncertainty surrounding the mechanism of action of this enzyme, further investigations are required in order to more fully understand the catalytic mechanism which will assist in the design of effective inhibitors.

1.5.2 Examples of other class B β -lactamases

In addition to *Bacillus cereus* β -lactamase II, class B β -lactamases have been found to be produced by at least nine other bacterial sources³⁵: *Aeromonas hydrophila*; *Bacteroides fragilis*; *Bacillus licheniformis*; *Flavobacterium odoratum*; *Legionella gormaii*; *Pseudomonas aeruginosa*; *Pseudomonas cepacia*; *Serratia marcescens* and *Xanthomonas maltophilia*, some of which are infectious to humans³⁶.

Although all class B β -lactamases share some significant similarities in their sequences, they display structural differences which lead to different enzymological properties⁶⁴.

Class B β -lactamases from *Bacteroides fragilis* and *Aeromonas hydrophila* are described below in order to compare their structural and catalytic properties with those of *Bacillus cereus* β -lactamase II.

1.5.2.1 *Bacteroides fragilis* metallo- β -lactamases

Bacteroides fragilis is considered to be one of the most serious human pathogens⁶⁵. In 1986 Cuchural *et al.*⁶⁵ isolated a zinc- β -lactamase from two

Bacteroides fragilis strains. This enzyme is responsible for the resistance of these bacteria to a wide variety of β -lactams including imipenem (a carbapenem) and cefoxitin (a cephamycin). This enzyme is inactivated by EDTA and other metal chelating agents and the activity is restored by addition of zinc ions. It is established that metallo- β -lactamases from this species are the most potent and display very large values of k_{cat} and k_{cat}/K_m against a large number of substrates^{34,66}.

The primary sequence of *Bacteroides fragilis* zinc- β -lactamase is quite similar to β -lactamase II from *Bacillus cereus*; however, the overall amino acid identity is about 32 %⁶⁷, with the most conserved sequence being the residues surrounding the zinc binding sites such as the three histidines and the cysteine residue⁶⁸.

B. fragilis metallo- β -lactamase tightly binds two zinc ions per molecule. They are both required in catalysis for maximum activity⁶⁹. The dissociation constants of both metal binding site are thought to be lower than 10 μ M.

A recent X-ray crystallographic study of the crystal structure of the enzyme at 1.85 Å resolution⁷⁰ revealed a four layer $\alpha\beta\beta\alpha$ molecule, as observed for *B. cereus* β -lactamase II, with a binuclear zinc site situated at the edge of the $\beta\beta$ sandwich. The first zinc ion which corresponds to the high affinity zinc site in *B. cereus* β -lactamase II is bound to four ligands (His99, His101, His162, Water1) and displays a tetrahedral geometry. The second zinc ion located at 3.5 Å from the first one, has a trigonal bipyramidal geometry and is bound to Asp103, Cys181, His223 and two molecules of water (Water1, Water2) (Figure 1.10). The metal

coordination was corroborated by studies of the crystal structures of a Cd^{2+} bound enzyme and a Hg^{2+} -soaked zinc-containing enzyme⁷¹. The two zinc ions are thought to share Water1, probably as a hydroxide ion, whose binding is modulated by its interaction with an aspartate (Asp103).

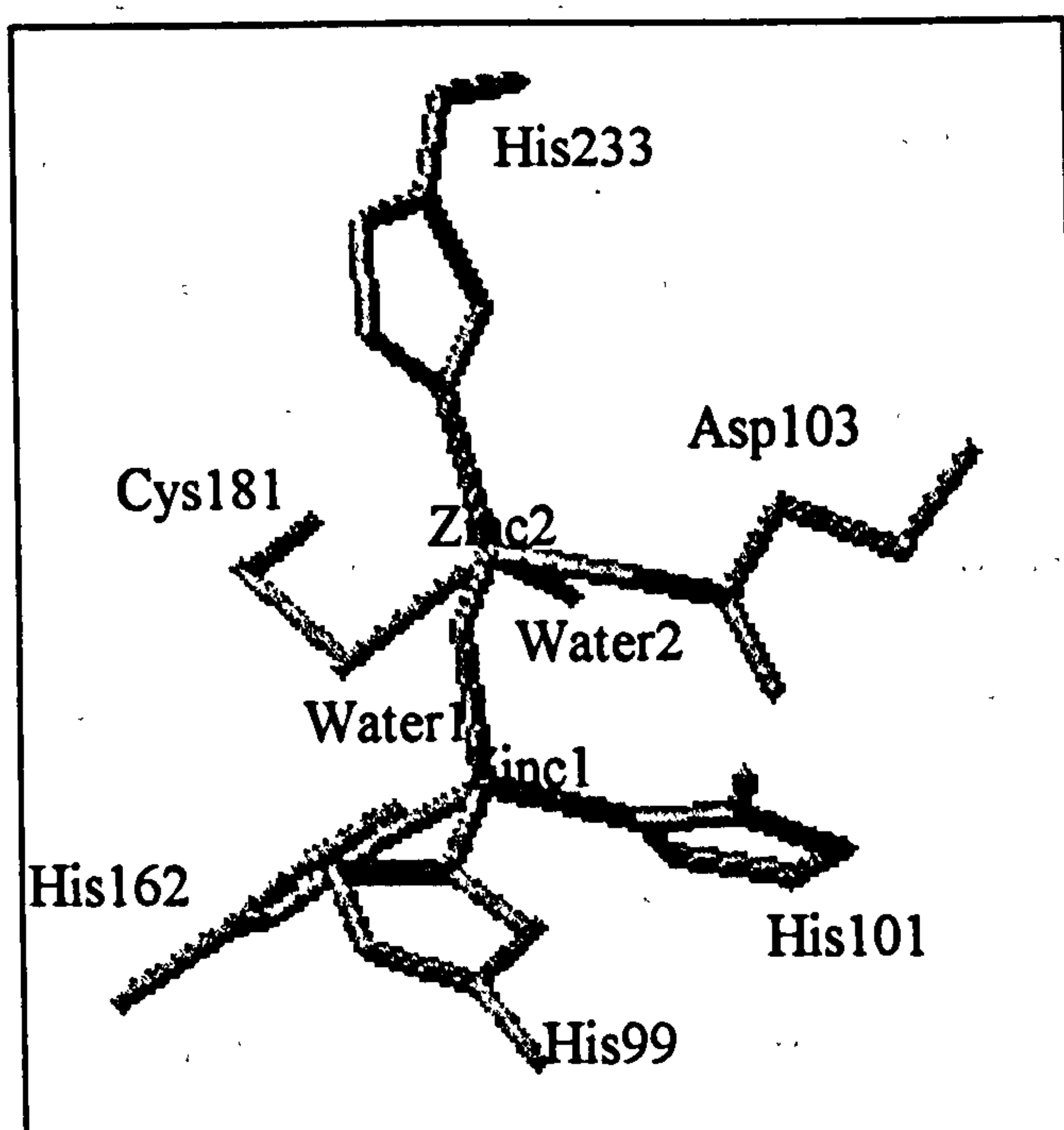


Figure 1.10 Schematic view of the metal sites of *B. fragilis* metallo- β -lactamase⁶³

It has been suggested that the catalytic mechanism involves the shared hydroxide ion acting as a nucleophile attacking the carbonyl of the β -lactam ring while Water2 could supply a proton to the nitrogen of the cleaved β -lactam^{63,71}.

However, recent studies on the orthorhombic crystal structure of the *B. fragilis* metallo- β -lactamase reported by Carfi *et al.*^{72,73} at 2.0 Å resolution indicate that zinc1 is bound to His82, His84, His145 and zinc2 to Asp86, Cys164, His206 and one water molecule. The shared hydroxide is not found, but the difference between

these findings and the structure proposed by Concha *et al.*⁷⁰ could come from different crystallisation conditions.

1.5.2.2 *Aeromonas hydrophila* metallo- β -lactamases.

As early as 1970, it was found that members of the genus *Aeromonas* (a Gram-negative bacteria), were able to produce up to three different β -lactamases⁷⁴. These bacteria are responsible for a range of infections of varying clinical severity in both adults and children⁷⁴. Among the β -lactamases isolated from these strains is a class C cephalosporinase, a class D penicillinase and a class B β -lactamase, which are all chromosomally encoded. The production of these enzymes is usually inducible⁷⁴.

Aeromonas metallo- β -lactamase, unlike the other class B β -lactamases, displays a relatively specific substrate profile. It shows a poor activity against some penicillins and cephalosporins and behaves as a relatively specific carbapenemase³⁴, with only two good substrates imipenem (a carbapenem) and ampicillin (a penicillin).

A. hydrophila metallo- β -lactamase shows a significant degree of similarity with the other β -lactamases in some regions of its sequence. There are at least five regions of homology, three of them involving the ligand binding residues. From a molecular point of view this enzyme seems to be quite different from *B. cereus* and *Bacteroides fragilis* metallo- β -lactamases. Massida *et al.*⁷⁵ suggest the presence of at least 2 distinct molecular subclasses within class B β -lactamase.

A. hydrophila metallo- β -lactamase seems to be fully active with only one zinc ion per enzyme. Zinc concentration dependence studies⁷⁶ of this enzyme have shown that the enzyme binds a first zinc very tightly and that the binding of a second zinc noncompetitively inhibits the enzyme with a K_i of 46 μ M at pH 6.5. The zinc ion is bound to three ligands, two histidine and one asparagine. Unlike the *B. cereus* and the *B. fragilis* metallo- β -lactamases, the first histidine is replaced by an asparagine and this difference could explain the rather specific substrate profile of these enzymes³⁴.

1.6 Zinc-metallo-proteases.

Class B β -lactamases are members of the group of hydrolytic metallo-enzymes^{77,78} which includes carboxypeptidase A⁵⁹ (CPA) and the angiotensin converting enzyme (ACE).

Carboxypeptidase A was the first metallo-enzyme for which the high resolution structure and sequence were obtained⁷⁹ and is also one of the most extensively studied, thus serving as a prototype for zinc-peptidases. CPA is a zinc digestive protease which is produced by fish and mammalian pancreas and catalyses the hydrolysis of C-terminal amino acid residues from peptides and proteins, with a preference for hydrophobic side chains such as phenylalanine. It also catalyses the hydrolysis of similarly configured esters.

CPA is a mononuclear zinc enzyme, with a molecular weight of 34472 Da, composed of 307 amino acids⁵⁹. The zinc ion is bound to three amino acids

residues (His69, Glu72, His196) and a water molecule^{79,80,81}. The other important amino acids present in the active site are Glu270, Tyr248 and Arg145, which are hydrogen bonded to the zinc bound water.

Although extensively studied, the catalytic mechanism of CPA remains ambiguous, and different pathways have been proposed:

Early works^{79,80,82} on the mechanism of action of the enzyme involved a nucleophilic pathway with Glu270 acting as nucleophile forming an anhydride with the carbonyl of the scissile peptide bond (Figure 1.11 (1)). Although no covalent acylenzyme intermediate was detected by methanolysis experiments⁸³ and cryokinetics studies⁸⁴, recent studies on the catalytic hydrolysis of specific ester substrates^{85,86} and resolution of reaction intermediates by different spectroscopic techniques^{87,88}, support the possibility of the anhydride pathway.

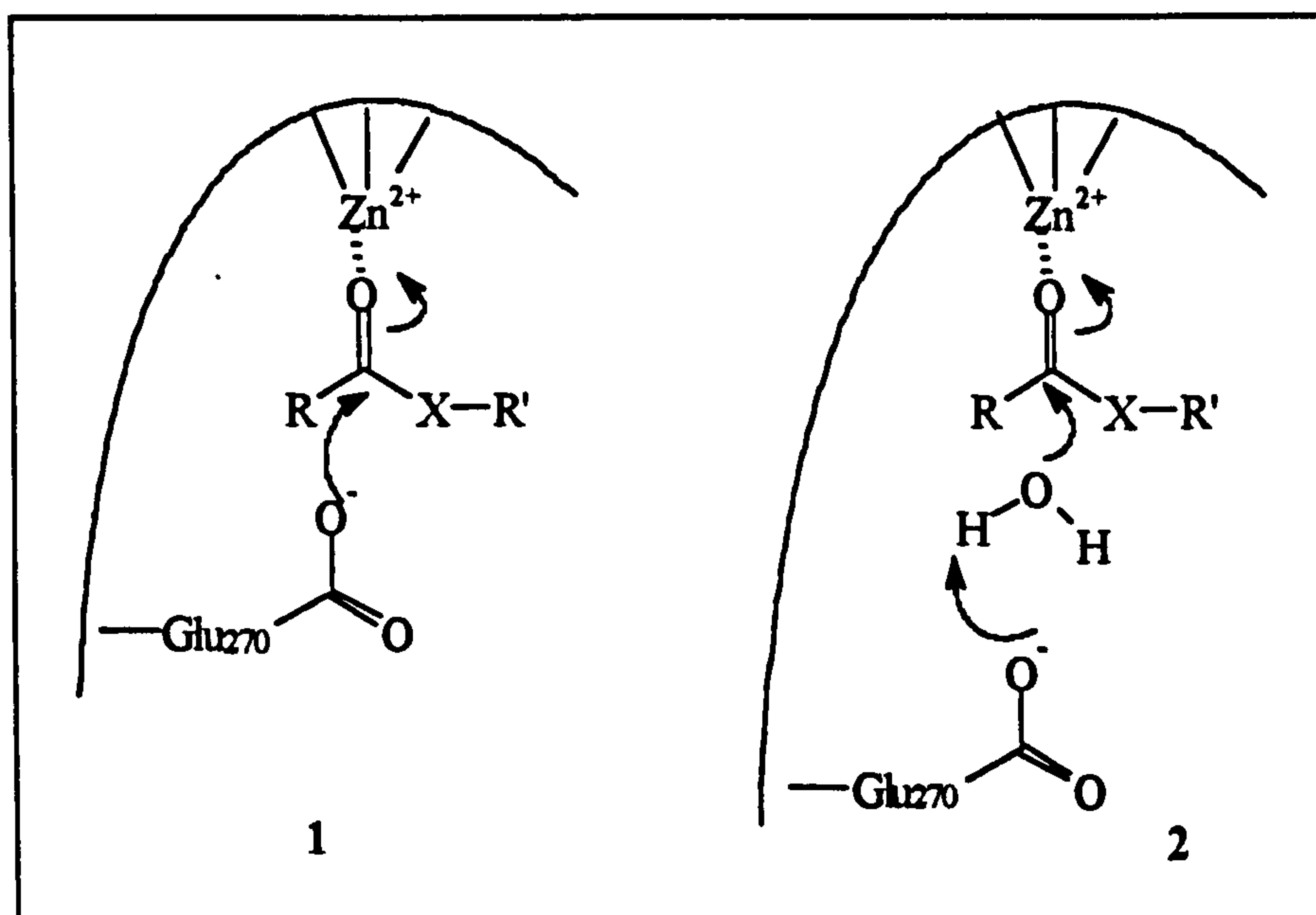


Figure 1.11 Proposed mechanisms of action of carboxypeptidase A⁸⁶

Another proposed mechanism involves Glu270 acting as a general base⁸⁹ and is thought to proceed through Glu270 assisting the attack of an external water molecule (Figure 1.11 (2)) or, more plausibly, a zinc bound water on both peptide and ester substrates⁹⁰. Peptides and esters may be hydrolysed through different mechanisms, forming distinct metallo-intermediates^{84,91}. X-ray structural studies of enzyme-substrate and enzyme-inhibitor complexes^{92,59} support the zinc bound water pathway. Figure 1.12 shows the catalytic pathway based on the mechanism proposed by Christianson *et al.*⁵⁹. The zinc bound water molecule, assisted by Glu270 acting as a general base, attacks the substrate carbonyl, thereby forming a tetrahedral intermediate. Glu270 donates a proton back to the nitrogen causing C-N bond fission and collapse of the intermediate. However, it is worth noting that the conversion of the tetrahedral intermediate to the products shown requires at least two proton transfer steps.

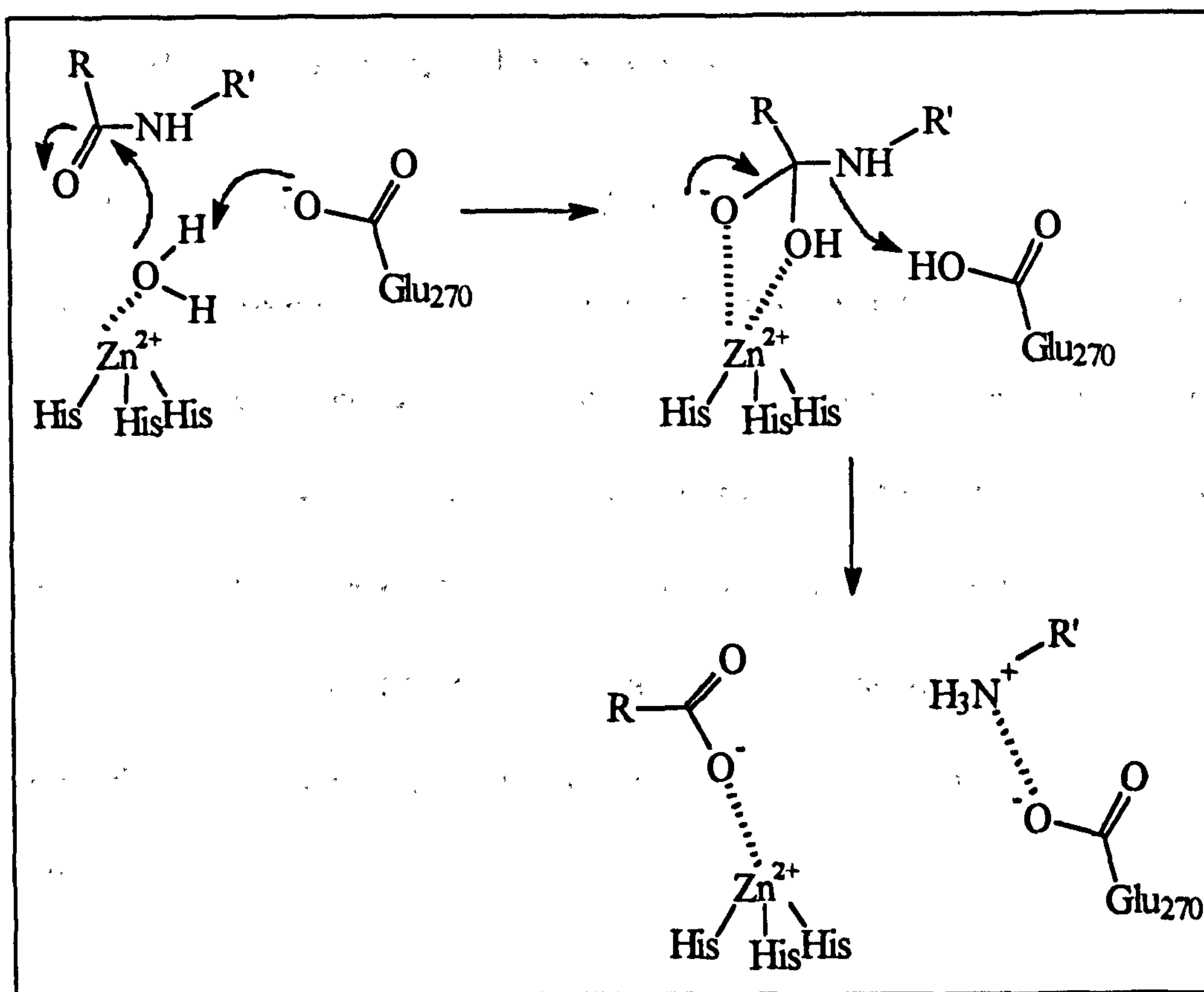


Figure 1.12 Zinc-bound water pathway in carboxypeptidase A mechanism

At present it appears that both proposed mechanisms of catalysis are plausible and recent molecular dynamic simulations⁹³ of the CPA-substrate complex do not rule out either.

1.7 Role of zinc ion in metallo-enzymes

Zinc is a II B element, with a complete *d*-shell and two additional 4s electrons and therefore readily loses two electrons and chemically combines in the +2 oxidation state⁹⁴. Zinc is an essential ion for many metallo-enzymes being required for catalytic activity, as well as having a structural role in certain proteins⁹⁵. Although replaceable by other metal ions, the zinc-enzyme complex is usually the most efficient. The importance of zinc ion in catalysis by β -lactamase II has long been recognised⁴⁰, and, in addition to its catalytic role, its structural role has also been reported⁶⁰.

The zinc ion in mono-nuclear metallo-enzymes is most often coordinated to three amino acid residues and a water molecule⁷⁷. A His residue is the most frequent ligand, although Glu, Asp, or Cys residues are also found coordinated to zinc.

Good models, such as zinc complexes of macrocyclic polyamines, are available to probe the role of the zinc ion in metallo-enzymes^{96,97}. Studies of these models suggest that the major role of the zinc ion is to act as a Lewis acid, which is also reflected by the effect of the zinc-complex on the pK_a value of the water ligand.

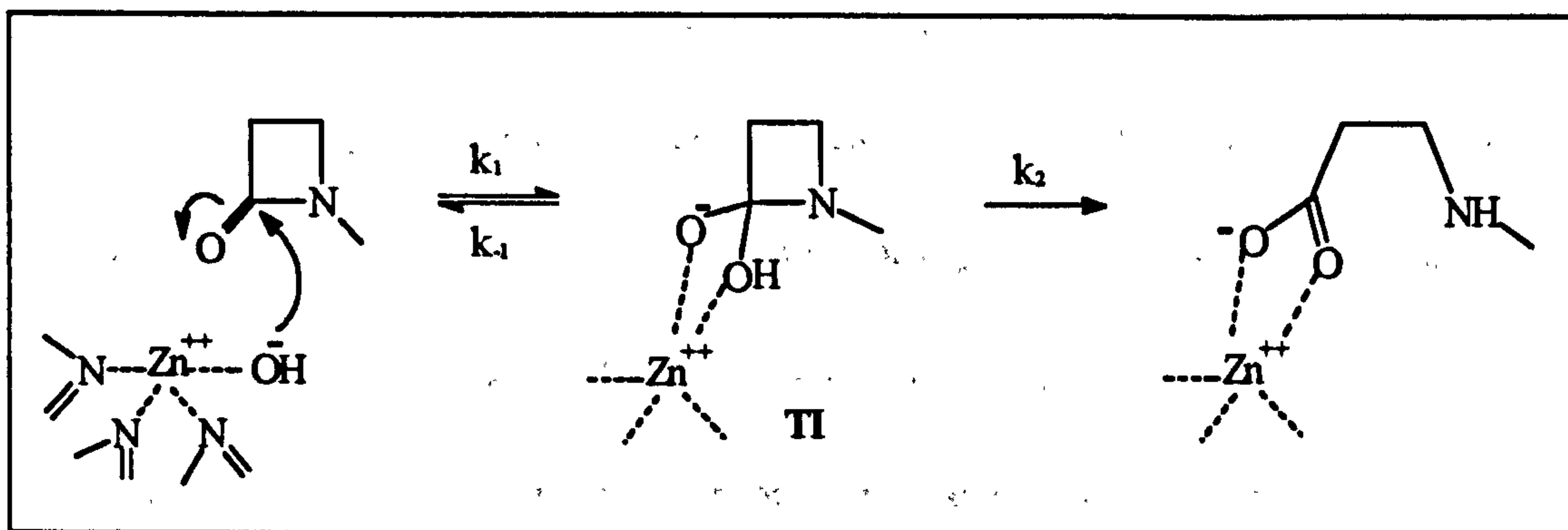
It is known that the pK_a of the zinc-bound water is determined by the electronic properties of the metal complex as a whole and by the electrostatic contributions from nearby residues^{98,99,100}. From studies on mutated binding sites in CA II (carbonic anhydrase II found in the red blood cells) the three histidine residues were found to be responsible for the electrostatic stabilization of the zinc-

hydroxide and the negatively charged transition state. These results highlight the importance of these neutrally charged ligands in maintaining the low zinc-water pK_a ¹⁰¹. The principal role proposed for the zinc complex of the carbonic anhydrase metallo-enzyme is the electrostatic stabilisation of the negatively charged transition state, which is a dominant factor for catalysis. The zinc complex is also responsible for the lowering of the pK_a of its bound water, thereby supplying a nucleophilic hydroxide ion for catalysis at neutral pH¹⁰².

It is proposed that the zinc ion in metallo-enzymes is involved in coordinating the nucleophilic water molecule at the active site, correctly positioning it for attack at the carbonyl function of the substrate and increasing its nucleophilicity by lowering its pK_a ^{63,77,94}. Reciprocally the low pK_a of the zinc bound water in metallo-enzyme is a good indication of the Lewis acidity of the zinc ion^{95, 103}. The substrate could bind directly to the metal ion, which would polarize the substrate without displacing the metal bound water resulting in a pentacoordinate intermediate⁹⁴.

Model studies of zinc complexes^{98,104} have shown that most zinc-bound hydroxide species have an efficient nucleophilicity independent of the coordination number of the zinc and of the nature of the other ligands. However, although a decrease in the pK_a of the zinc-bound water makes zinc-bound hydroxide available at lower pH it also reduces its nucleophilicity compared with the hydroxide anion. By definition, a lower pK_a acid produces a weaker conjugate base and, usually, a weaker nucleophile. Hydroxide-ion bound to zinc is not a powerful nucleophile.

Similar to carbonic anhydrase, the zinc ion in β -lactamase II is coordinated to three histidine residues and a water molecule. The role of zinc in β -lactamase II is thought to involve the supply of a hydroxide ion for nucleophilic attack on the carbonyl of the β -lactam ring and the subsequent stabilisation of the tetrahedral intermediate (TI) formed (Scheme 1-1).



Scheme 1-1

1.8 Zinc-metallo-protease inhibition

1.8.1 Metallo-protease inhibitors

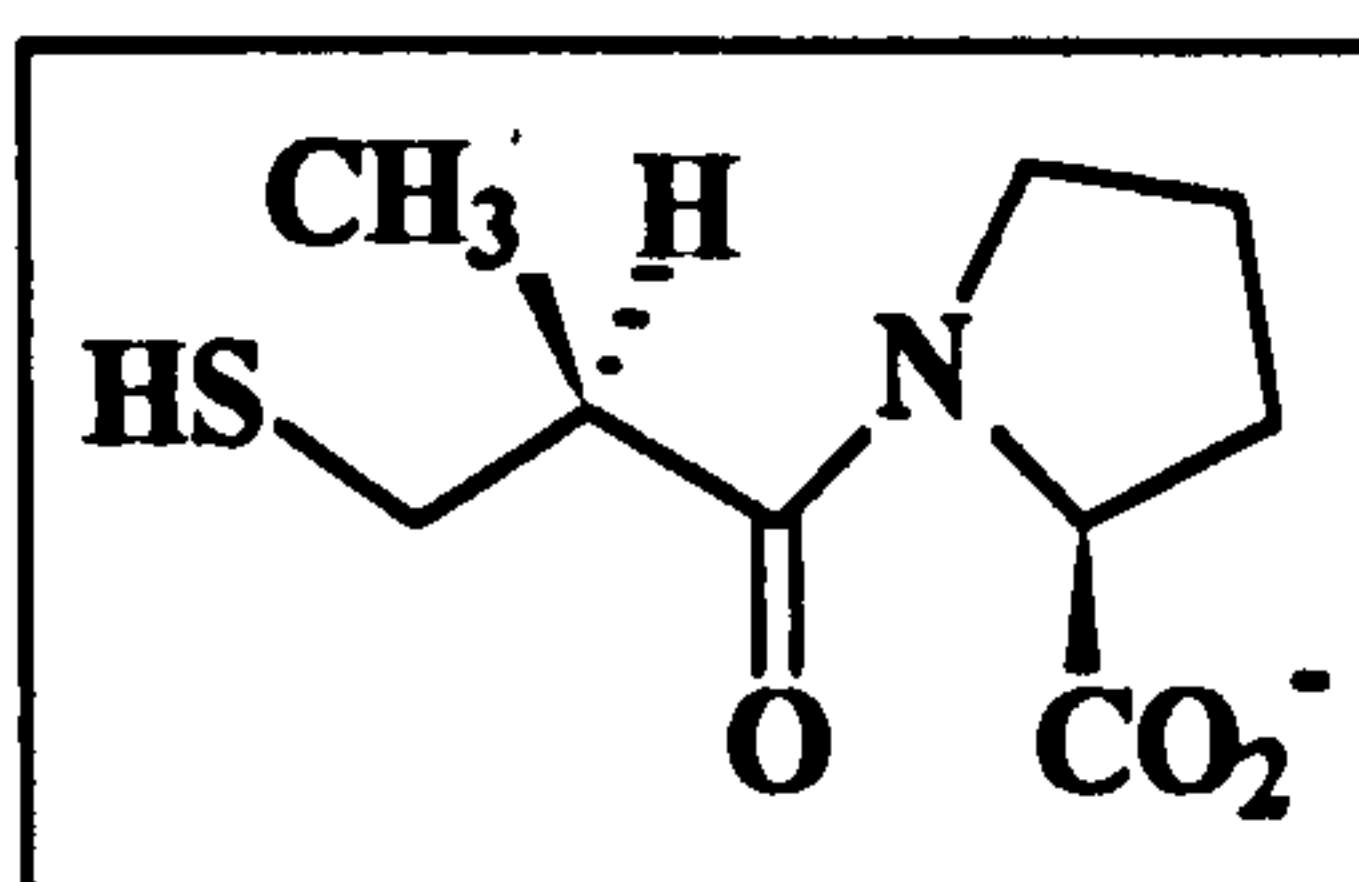
The prototypic carboxypeptidase A has been extensively used as a model for the study of metallo-enzyme inhibition¹⁰⁵.

Compounds such as phosphonates have been reported to be effective inhibitors of CPA^{106,107}, with the CBz-Phe-Val^P-(O)-Phe-enzyme complex displaying one of the strongest interactions with a K_i of 11 fM. This appears to be one of the lowest values ever measured for a K_i . These phosphonates are thought to act as transition state analogues of the general base mechanism of catalysis of CPA.

Other compounds such as 2-benzyl-3,4-epoxybutanoic acid¹⁰⁸ are known to inactivate the enzyme by reaction of Glu270 with the epoxide, forming a covalent adduct. The importance of the stereochemistry of this compound in the inhibition mechanism of this enzyme (and particularly for the development of effective inhibitors) has been highlighted¹⁰⁸.

Thiols have been reported to be good inhibitors of zinc metallo-proteases: Dithiols such as dithiotriitol inhibit efficiently the VanX enzyme which is a metallo D,D-dipeptidase responsible for the resistance of the bacterium *Enterococcus faecium* to vancomycin¹⁰⁹. Furthermore, carboxypeptidase A is competitively inactivated by compounds such as (S)-2-mercapto-2-benzylpropanoic acid¹¹⁰.

Captopril is one of the first orally effective hypotensive agents and is specially designed to inhibit the angiotensin converting enzyme (ACE), a zinc metalloprotease involved in the production of angiotensin II, a vasoconstricting agent¹¹¹. This thiol compound is active site directed, zinc coordinating and with a subnanomolar K_i value. Many other inhibitors have been synthesised on the basis of the captopril structure and its interaction with the zinc active site of ACE^{112,113}.



Captopril

The design of compounds which have a thiol (mercaptan) moiety as a zinc-binding ligand has proved to be an effective way to synthesise potent inhibitors of metallo-enzymes.

1.8.2 Zinc- β -lactamase inhibitors

One approach to the problem of resistant bacterial strains has been the development of β -lactamase inhibitors. No clinically useful inhibitors of these enzymes have been found yet, although they can be inhibited by metal chelating agents such as EDTA, and also by some mercurial compounds³⁶.

When the work reported in this thesis was started there were very few known inhibitors but over the past few years, the synthesis of several potential zinc- β -lactamase inhibitors have been reported :

Van Hove *et al.*¹¹⁴ report the synthesis of compounds A and B (Figure 1.13) which displayed weak inhibitory action of metallo- β -lactamases.

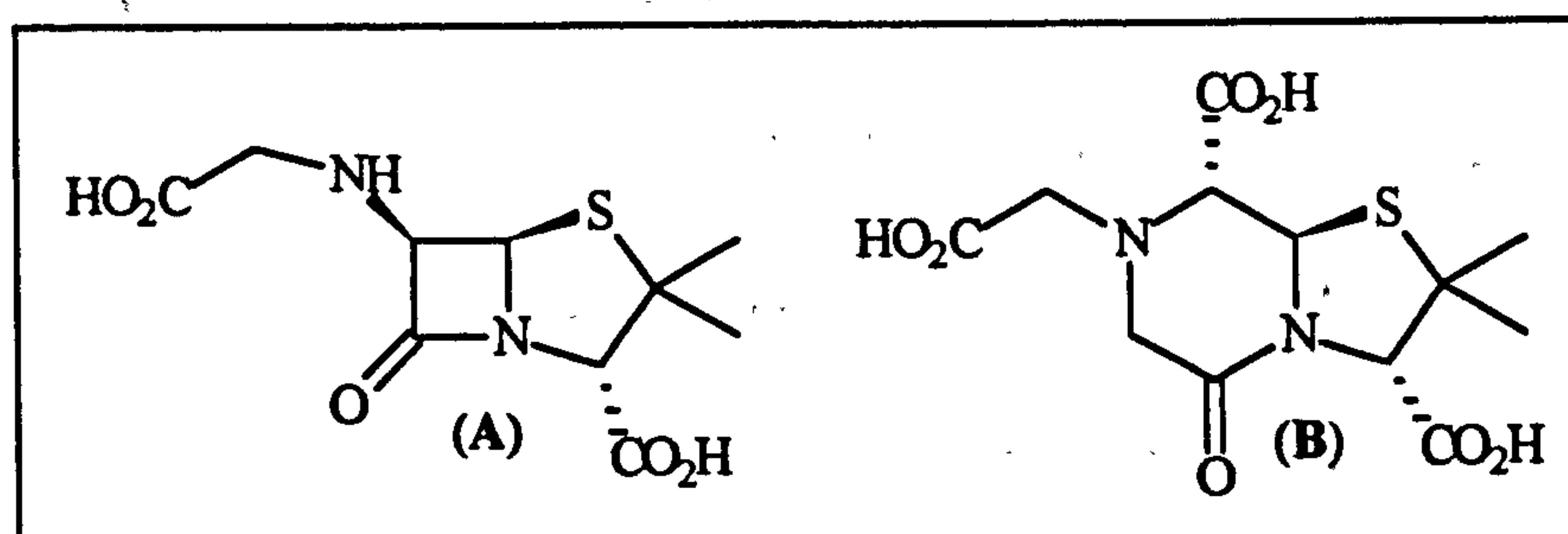


Figure 1.13

These compounds lead to a small inactivation of the β -lactamase II, which could be immediately restored upon addition of zinc sulfate to the system inhibited by (A).

Trifluoromethyl alcohols and ketones (Figure 1.14) were reported to have an inhibitory action on several metallo- β -lactamases with K_i varying from 300 to 1000 μ M for (C) and from 30 to 1000 μ M for (D) depending on the side chain R and the stereochemistry^{115,116}. Earlier studies on trifluoromethyl ketones¹¹⁷ described their interaction with carboxypeptidase A, where the compounds bind to the enzyme as a gem-diol, with one of the hydroxyl groups being coordinated to the zinc ion.

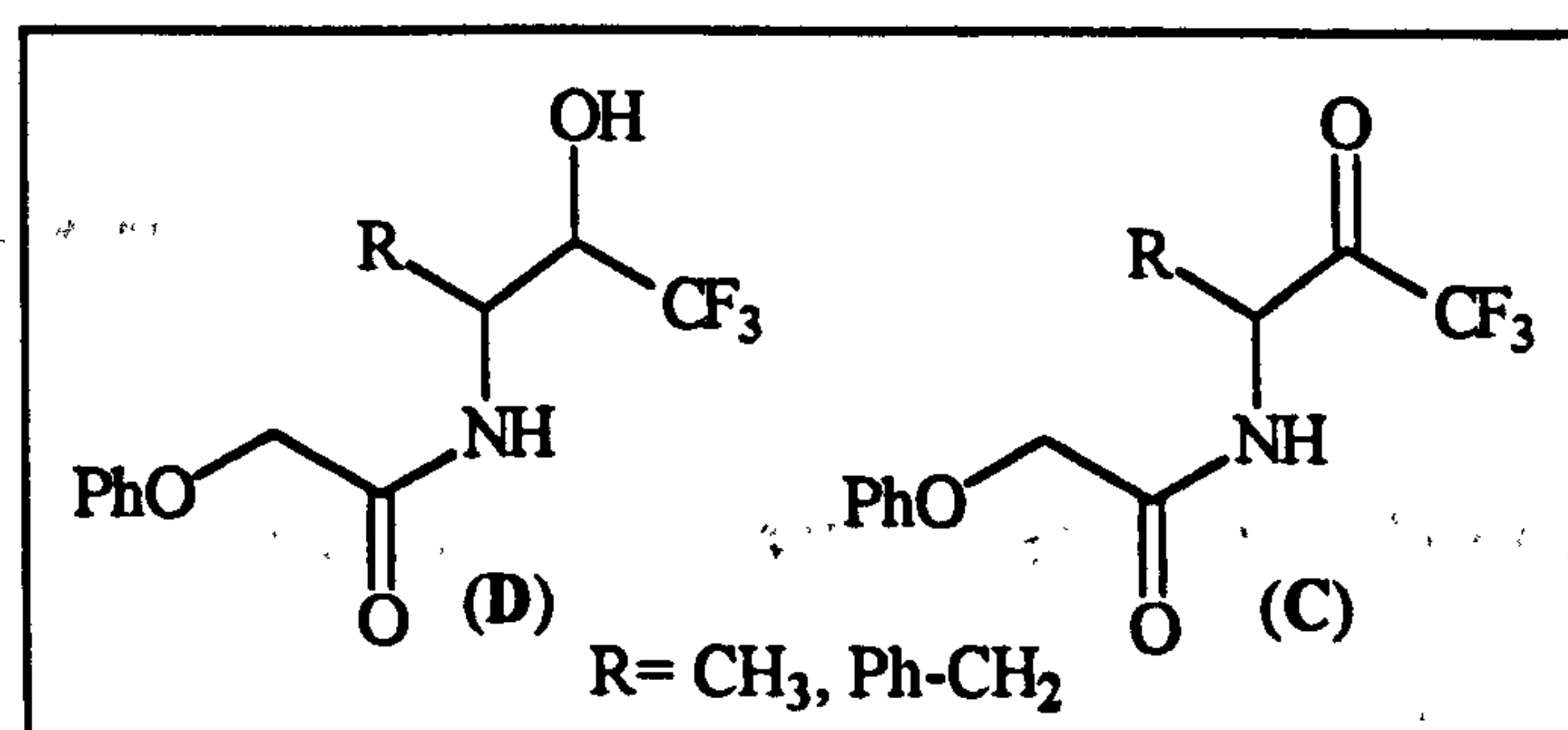


Figure 1.14

Payne *et al.*¹¹⁸ report the progressive and irreversible inhibition of metallo- β -lactamase from several bacterial strains by some mercaptoacetic acid thiol ester derivatives. The mechanism of inhibition is thought to be due to the hydrolysis by the enzyme of the inhibitor, subsequently releasing the mercaptoacetic acid component, which then forms a disulfide bound with Cys168 of the second zinc active site under the conditions of the assay. The study of the different range of potencies of these compounds against different metallo- β -lactamases indicates some differences between their active sites.

Later, Goto *et al.*¹¹⁹ confirmed the effectiveness of low molecular weight thiols such as mercaptoethanol, mercaptoacetic acid and ethylmercaptoacetate as reversible inhibitors of the binuclear zinc- β -lactamase from *Serratia marcescens*.

Recently, biphenyl tetrazoles (BPTs) were found to be potent non-peptide inhibitors of *B. fragilis* metallo- β -lactamases¹²⁰, where the tetrazole group of the compound is thought to interact directly with one of the two zinc ions of the enzyme. The inhibitory action is increased by using appropriate substituents on the molecule.

1.9 Research aims

As previously reported, the metallo- β -lactamases show little or no susceptibility to traditional β -lactamase inhibitors such as clavulanic acid and sulbactam. At present, no clinically useful inhibitor is known. None of the compounds described above act as inhibitors of all known metallo- β -lactamases. The specific inhibition observed

for enzymes from different strains highlights the structural heterogeneity of these enzymes.

Although great advances have been made in the understanding of metallo-enzyme catalysis, some areas remain unclear.

It is therefore important to characterise the mechanism of action of these enzymes.

The aim of this work is to contribute to a more detailed knowledge of the kinetics and mechanism of catalysis of metallo- β -lactamase in general through the study of the kinetic parameters and inhibition of the *Bacillus cereus* β -lactamase II. This thesis discusses the pH dependence and kinetic solvent-isotope effects, as well as the effect of the zinc concentration on the enzyme catalysis.

As seen previously, thiols tend to be good inhibitors of metallo-enzymes through coordination of the sulfur to the zinc ion. Herein is reported the synthesis of thiols as potential inhibitors, acting as analogues of the tetrahedral intermediate formed during the hydrolysis of the substrate, and the study of the kinetics and mechanisms of inhibition. These compounds are related to the primary penicillin type structure with suitable modifications to make them bind strongly to the zinc active site.

Experimental

2. Experimental

2.1 Synthesis

All solvents and reagents were used as obtained from commercial sources unless otherwise stated.

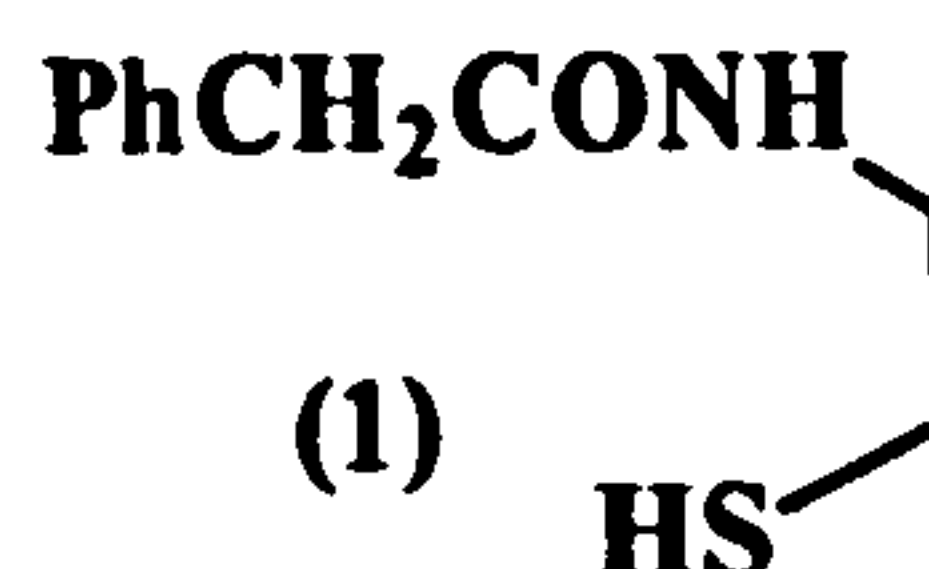
^1H and ^{13}C NMR were recorded at 270 MHz on a Bruker AC-270 spectrometer. The chemical shifts (δ_{H}) are quoted in p.p.m. and referenced to TMS. The abbreviation used are s : singlet, d : doublet, t : triplet, dd : doublet of doublet, dt : doublet of triplet, m : multiplet. The coupling constants are given in Hertz.

Infrared spectra were obtained on a Perkin Elmer 1600 Series FTIR, as nujol mulls or chloroform solutions. The absorptions are given in wavenumbers (cm^{-1}).

The melting points are not corrected and were recorded on a Gallenkamp melting point apparatus.

The mass spectra were recorded on a VG (Fisons Instruments) Quattro (II) S Q mass spectrometer. Only the mass to charge ratio of the molecular ion is reported.

2.1.1 Synthesis of *N*(2-mercaptoethyl)phenylacetamide (1)



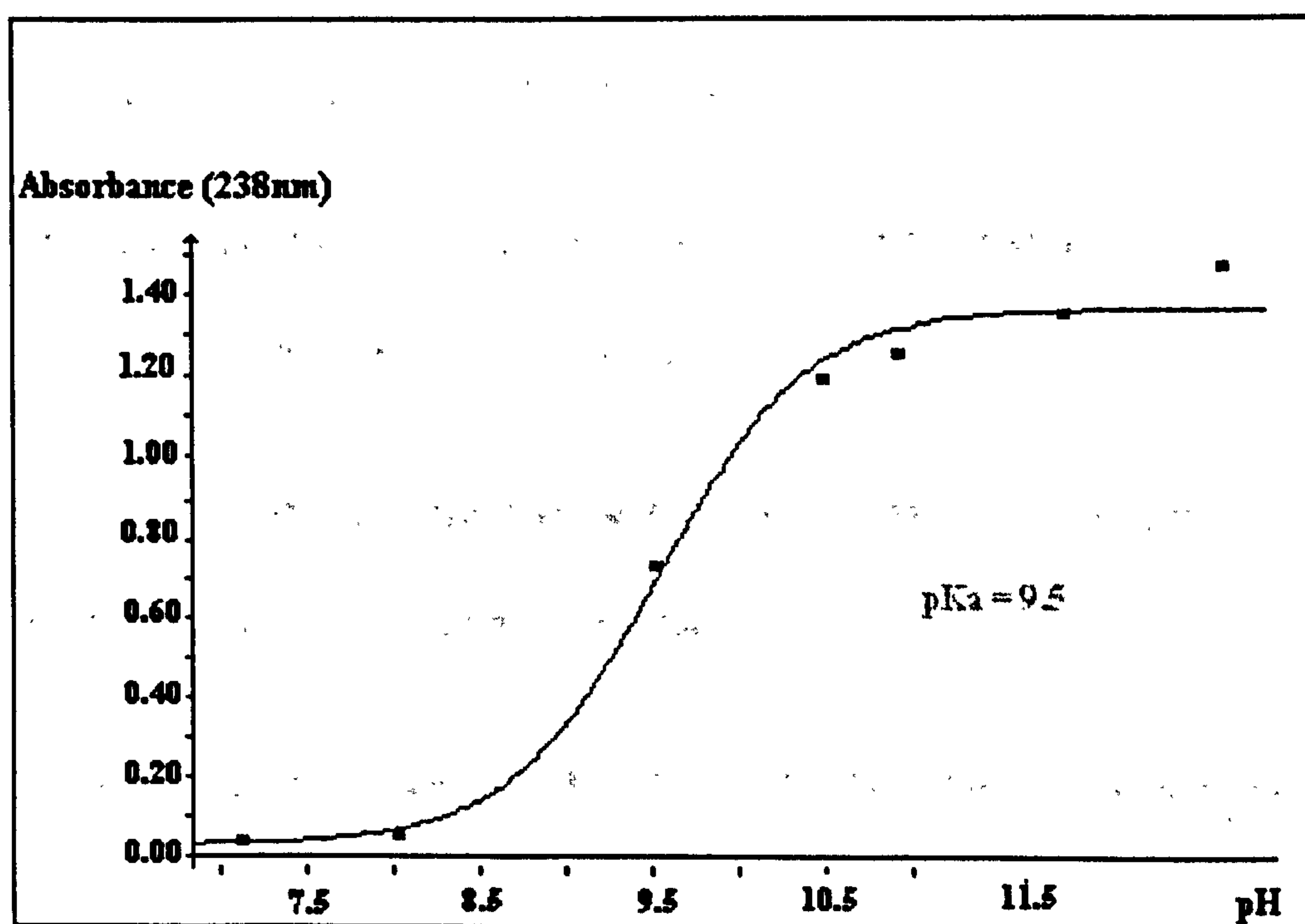
Phenylacetyl chloride (5.5 mmol) was added dropwise to a solution of cystamine (2.5 mmol) in water (40 ml) containing sodium hydroxide (11.25 mmol) and was cooled to 0 °C. The mixture was vigorously stirred. At the end of the reaction, the white precipitate formed was filtered and washed with water. The solid was recrystallised from hot methanol. The reaction was followed by TLC in chloroform/methanol/ammonia system (10:12:1, by vol). The disulfide thus obtained was reduced by adding sodium borohydride (88 mmol) to a solution of the disulfide (4.4 mmol) in ethanol/water (5:3, v/v). The mixture was heated to 90 °C and stirred for 1 hour. The reaction was stopped by cooling the mixture which was then acidified to pH 2 with dilute hydrochloric acid. After evaporation of the ethanol, the product was separated by filtration, the filtrate was extracted with chloroform to obtain the remaining thiol. The combined extract were washed with water and dried over anhydrous sodium sulfate. The solvent was removed by rotary evaporation to give a white product. The total yield was 87 %. The reaction was followed by TLC in chloroform/methanol system (99:1, v/v). After exposure of the TLC plates to iodine vapour, the thiol appeared as a white spot on a tanned background.

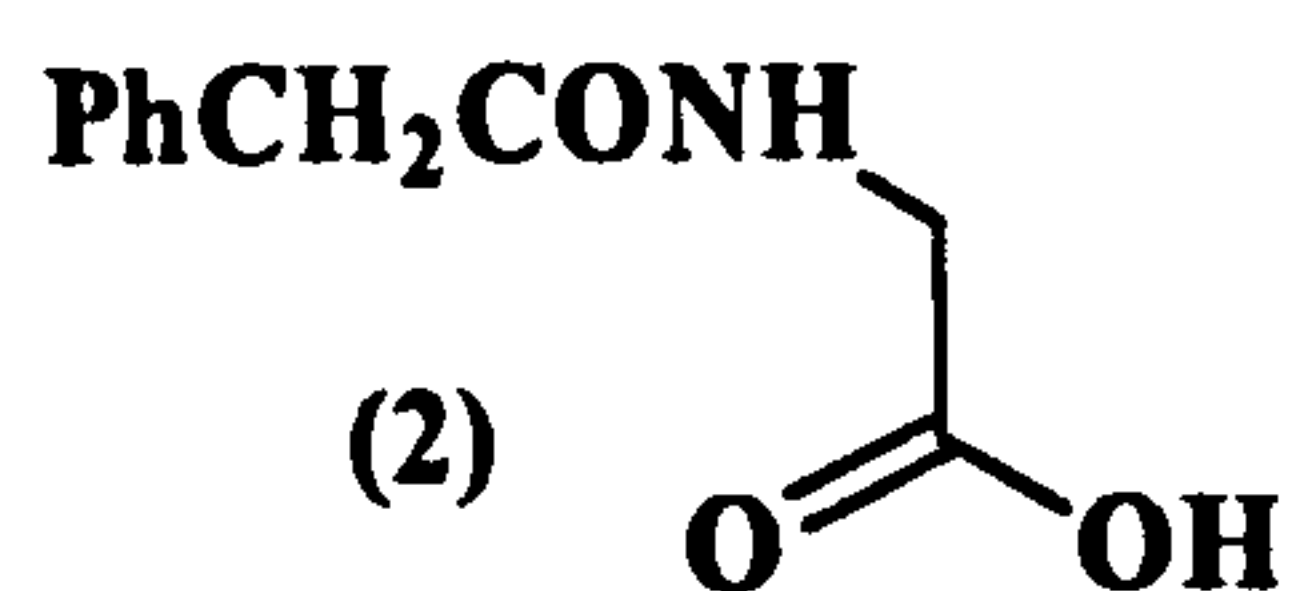
$^1\text{H-NMR}$ δ_{H} (CDCl_3) 7.30 (5 H, m, Ar-H), 5.75 (1 H, m, NH), 3.55 (2 H, s, $\text{CH}_2\text{-Ar}$), 3.35 (2 H, m, CH_2), 2.52 (2 H, dt, J 6.6 and 8.5, $\text{CH}_2\text{-S}$), 1.18 (1 H, t, J 8.5, SH).

IR (Nujol) ν_{max} 3253 (amide NH), 2544 (SH), 1637 cm^{-1} (amide CO)

mp 40-44 $^{\circ}\text{C}$: literature value 50-52 $^{\circ}\text{C}$

A pK_a of 9.5 ± 0.10 of the thiol was determined from the dependence of the absorbance of an aqueous 2.52×10^{-4} M solution at 238 nm as a function of pH, as shown below.



2.1.2 Synthesis of *N*-phenylacetylglycine (2)

Phenylacetyl chloride (10.8 mmol) was added dropwise to a solution of glycine (8.9 mmol) in water (40 ml) containing sodium hydroxide (45 mmol) and was cooled to 0 °C. The mixture was vigorously shaken. The solution was subsequently acidified with dilute hydrochloric acid (acid to congo red) and the product separated as white crystals which were filtered, washed with water, and dried under vacuum. The total yield was 67 %.

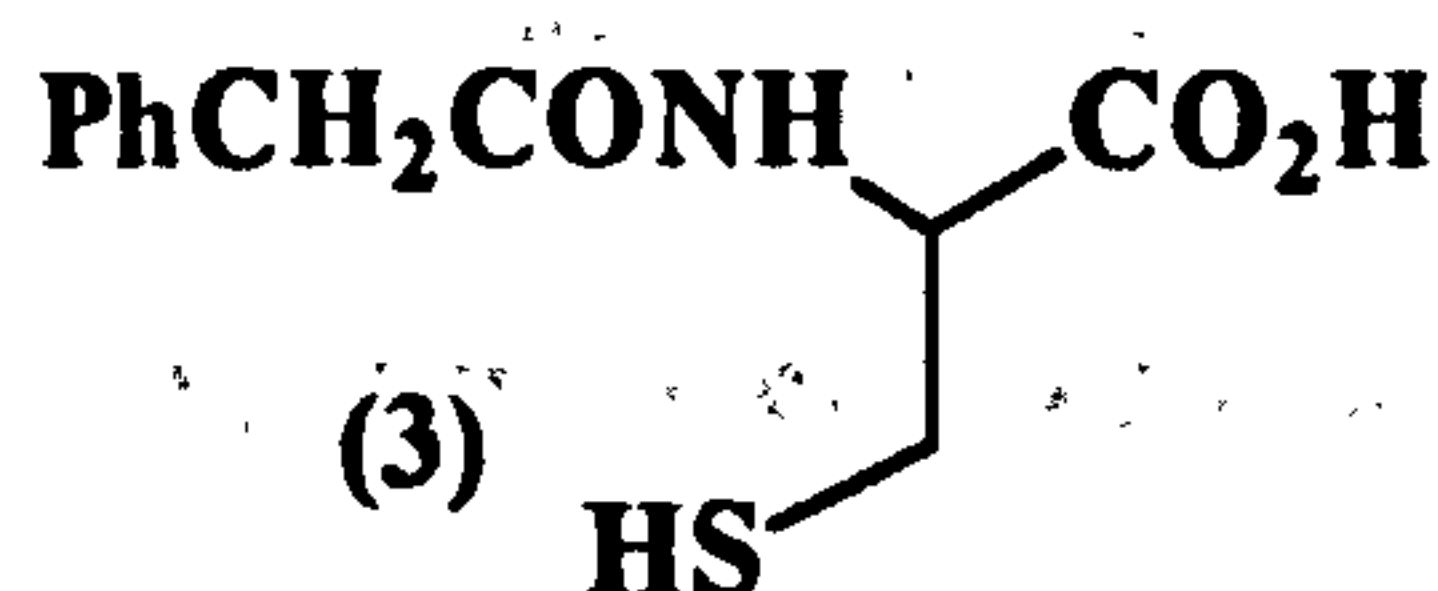
¹H-NMR δ_{H} (DMSO) 8.39 (1 H, t, *J* 5.8, NH), 7.26 (5 H, m, Ar-H), 3.77 (2 H, d, α CH₂ Gly, *J* 5.8), 3.48 (2 H, s, CH₂-Ar).

¹³C-NMR δ_{C} (DMSO) 168.8 (COOH), 168.1 (CONH), 133.6, 126.6, 125.7, 123.9 (C₆H₅), 39.5 (CH₂-Ar), 38.3 (α CH₂ Gly).

IR (nujol) ν_{max} 3373 (amide NH), 1727 (COOH), 1609 cm⁻¹ (amide CO),

mp 120-122 °C

2.1.3 Synthesis of *N*-phenylacetyl-*L*-cysteine (3)



Phenylacetyl chloride (13 mmol) was added dropwise to a solution of cystine (5.9 mmol) in water (60 ml) containing sodium hydroxide (30 mmol) and was cooled to 0 °C. The mixture was vigorously stirred. After the end of the reaction, the solution was acidified with dilute hydrochloric acid (acid to congo red) and the disulfide product separated as a white precipitate which was filtered and then dried. Yield 80 %.

¹H-NMR δ_{H} (DMSO) 8.54 (1 H, d, J 7.9, NH (cys)₂), 7.26 (5 H, m, Ar-H), 4.50 (1 H, m, α CH (cys)₂), 3.50 (2 H, s, CH₂-Ar), 3.16 and 2.95 (2 H, m, β CH₂ (cys)₂ J 4.6, J 9.5, J 13.6).

¹³C-NMR δ_{C} (DMSO) 171.87 (COOH), 170.25 (CONH), 136.0, 129.0, 128.1, 126.3 (C₆H₅), 51.3 (α CH (cys)₂), 41.9 (CH₂-Ar), 40.3 (β CH₂ (cys)₂).

IR (Nujol) ν_{max} 3319 (amide NH), 1720 (COOH), 1660 cm⁻¹ (amide CO)

ES MS [M-H]⁻: m/z 475

The disulfide was then reduced by adding sodium borohydride (100 mmol) to a solution of the disulfide (5 mmol) in ethanol/water (5:3, v/v). The mixture was

heated to 90 °C, and stirred for 1 hour. The reaction was stopped by cooling the mixture which was then acidified to pH 2 with dilute hydrochloric acid. After evaporation of the ethanol, the aqueous solution was extracted with ethyl acetate (3 x 30 ml). The combined extracts were washed with water and dried over anhydrous sodium sulfate. The solvent was removed on a rotary evaporator to give an oil which solidified. Yield 90 %.

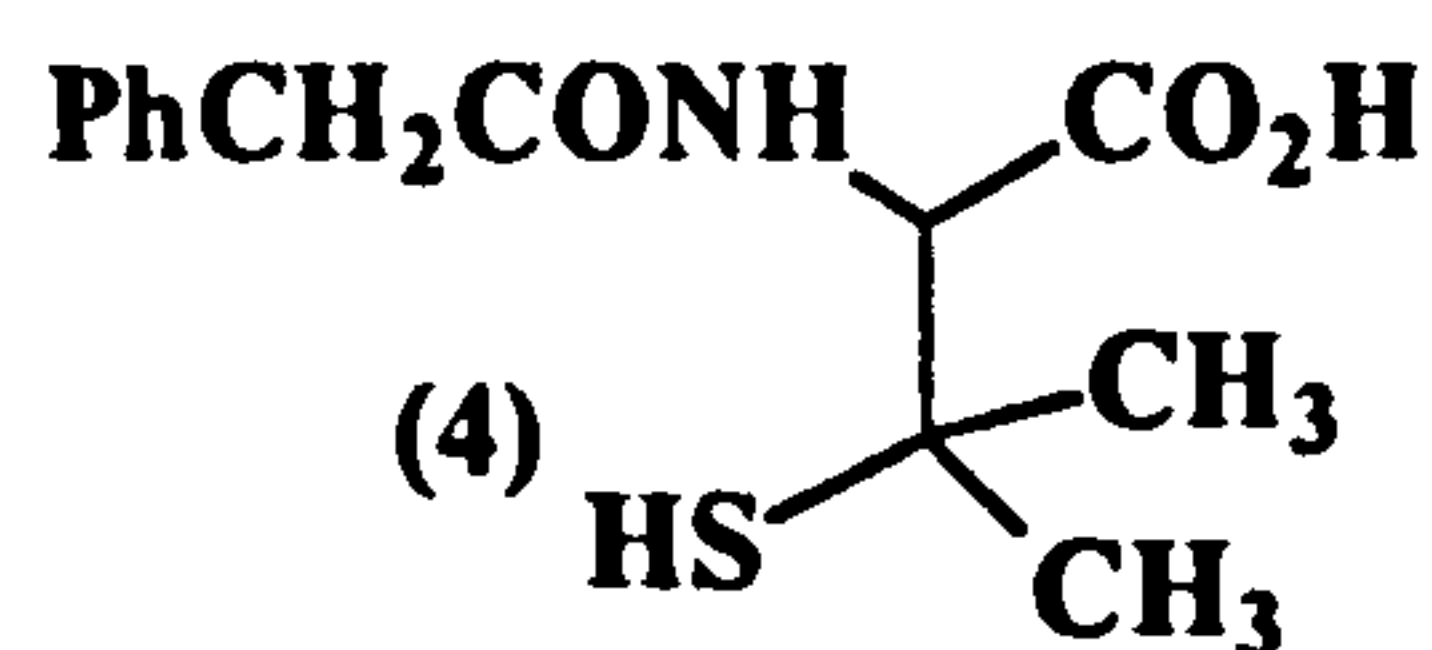
¹H-NMR δ_{H} (DMSO) 8.47 (1 H, d, J 7.8, NH cys), 7.25 (5 H, m, Ar-H), 4.40 (1 H, m, α CH cys), 3.53 (2 H, s, CH₂-Ar), 2.82 (2 H, m, β CH₂ cys), 2.45 (1 H, m, SH).

¹³C-NMR δ_{C} (DMSO) 171.6 (COOH), 170.4 (CONH), 136.3, 129.1, 128.2, 126.4 (C₆H₅), 54.5 (α CH cys), 41.9 (CH₂-Ar), 25.7 (β CH₂ cys).

IR (nujol) ν_{max} 3342 (amide NH), 2550 (SH), 1731 (COOH), 1623 cm⁻¹ (amide CO)

ES MS [M-H]⁻: m/z 238

mp 85-88 °C

2.1.4 Synthesis of *N*-phenylacetyl-DL-penicillamine (4)

Phenylacetyl chloride (0.98 mmoles) was added dropwise to a solution of penicillamine (6.7 mmoles) in water (50 ml) containing sodium hydroxide (33 mmoles) and was cooled to 0 °C. The mixture was vigorously stirred. The solution was subsequently acidified with dilute hydrochloric acid (acid to congo red) and the product separated as a white precipitate, which was filtered, washed with water and dried under vacuum. Yield 42 %.

¹H-NMR δ_H (DMSO) 8.34 (1 H, d, NH, *J* 9.2), 7.32 (5 H, m, Ar-H), 4.44 (1 H, d, αCH), 3.58 (2 H, dd, CH₂-Ar, *J*_{AB} 13.8), 2.86 (1 H, s, SH) and 1.38 and 1.36 (6 H, 2 s, γCH₃),

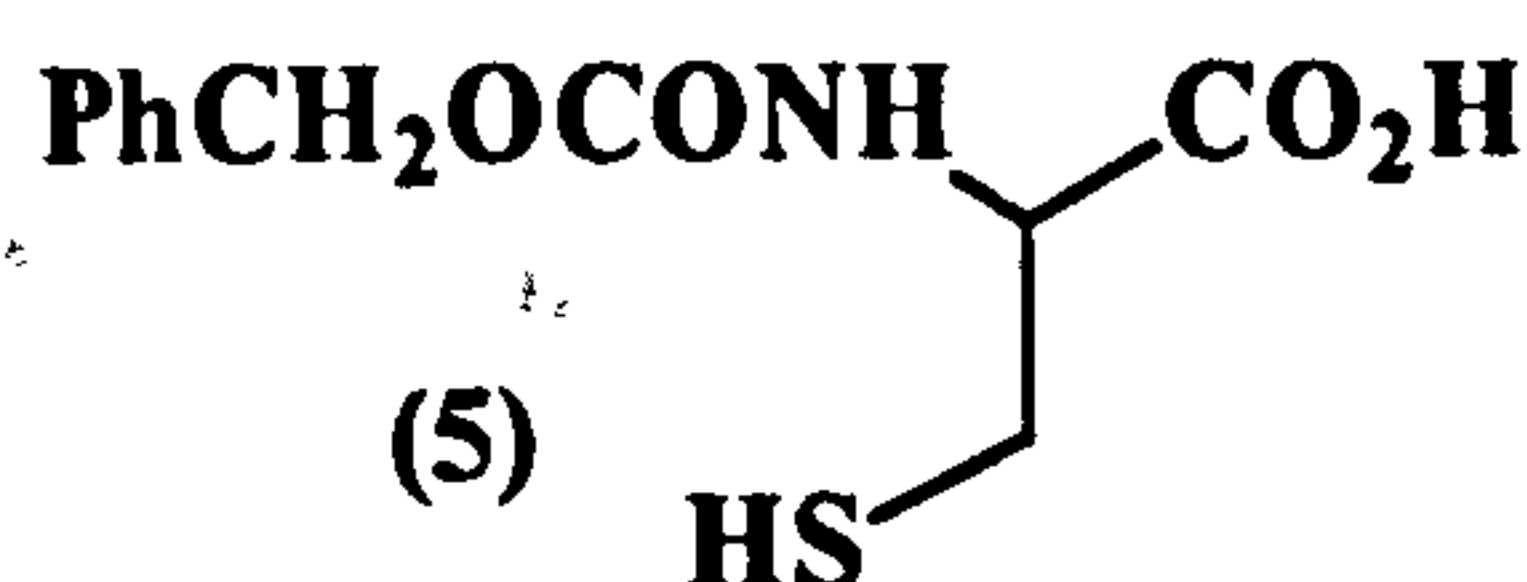
¹³C-NMR δ_C (DMSO) 171.2 (COOH), 170.3 (CONH), 136.3, 129.0, 128.1, 126.3 (C₆H₅), 61.3 (αCH), 45.5 (βC), 41.8 (CH₂-Ar), and 29.7, 29.6 (γCH₃)

IR (nujol) ν_{max} 3365 (amide NH), 2571 (SH), 1728 (COOH), 1635 cm⁻¹ (amide CO)

ES MS [M-H]⁻ m/z 266

mp 118-120 °C

2.1.5 Synthesis of *N*-carbobenzoxy-*L*-and-*D*-cysteine (5)



N,N'-Dicarbobenzoxy-cystine (*Z*-cystine) was synthesised according to the method of Du Vigneaud et Miller¹²¹.

Cystine (0.0125 moles) was dissolved in a solution of sodium hydroxide (25 ml, 1 M) and stirred at 0 °C. Carbobenzoxychloride (0.0375 moles) along with sodium hydroxide (50 ml, 2 M) were added in 10 portions during the course of 30 minutes. Stirring was continued for 1 hour after the addition of all the reactants. The mixture was allowed to warm to room temperature, washed twice with ether (30 ml), and then was immediately acidified with 6 M hydrochloric acid. The insoluble precipitate was filtered and the filtrate then extracted four times with ethyl acetate (30 ml). The precipitate was dissolved in ethyl acetate with the help of heat and combined with the ethyl acetate extracts. The solution was then washed twice with 5 % hydrochloric acid (50 ml) and twice with water (50 ml). It was then dried with anhydrous sodium sulfate and concentrated under reduced pressure. The oily residue was crystallised in chloroform, to give a white compound. Yield 50 %.

Z-Cystine

$^1\text{H-NMR}$ δ_{H} (DMSO) 7.77 (1 H, d, NH, 8.4), 7.35 (5 H, m, Ar-H), 5.07 (2 H, s, CH₂-O), 4.36 (1 H, m, α CH), 3.20 and 2.97 (2 H, m, β CH₂ J 4.1, J 10.1, J 13.7)

$^{13}\text{C-NMR}$ δ_{C} (DMSO) 172.3 (COOH), 156.2 (OCONH), 136.9, 128.4, 128.1.1, 127.9 (C₆H₅), 65.7 (CH₂-O), 53.1 (α CH), 39.6 (β CH₂)

IR (nujol) ν_{max} 3329 (amide NH), 1730 (COOH), 1655 cm⁻¹ (amide CO)

ES MS [M-H]⁻ m/z 509

The disulfide is reduced using sodium borohydride according to the method of D'Amico¹²².

Z-cystine (2.18 mmoles) was dissolved in 20 ml of ethanol. To this solution at 67-70 °C was added dropwise a solution containing 4 equivalents of sodium borohydride (8.74 mmoles) in 10 ml of ethanol. The stirred reaction mixture was heated at 75 - 80 °C for one hour. After cooling to 25 °C, the solution was added to 50 ml of iced water. To this stirred solution, 6 M hydrochloric acid was added dropwise at 0 - 10 °C until pH 3 was obtained. After evaporating under reduced pressure to remove the ethanol, the solution was extracted three times into ethyl acetate (20 ml), washed three times with distilled water and dried over anhydrous sodium sulfate. After removing the solvent under reduced pressure, 1.0 g of colourless oil was obtained. Yield 90 %.

Z-Cysteine

¹H-NMR δ_H (DMSO) 7.64 (1 H, d, NH, *J* 7.9), 7.36 (5 H, m, Ar-H), 5.08 (2 H, s, CH₂-O), 4.14 (1 H, m, α CH), 2.91 and 2.75 (2 H, m, β CH₂), and 2.51 (1 H, m, SH)

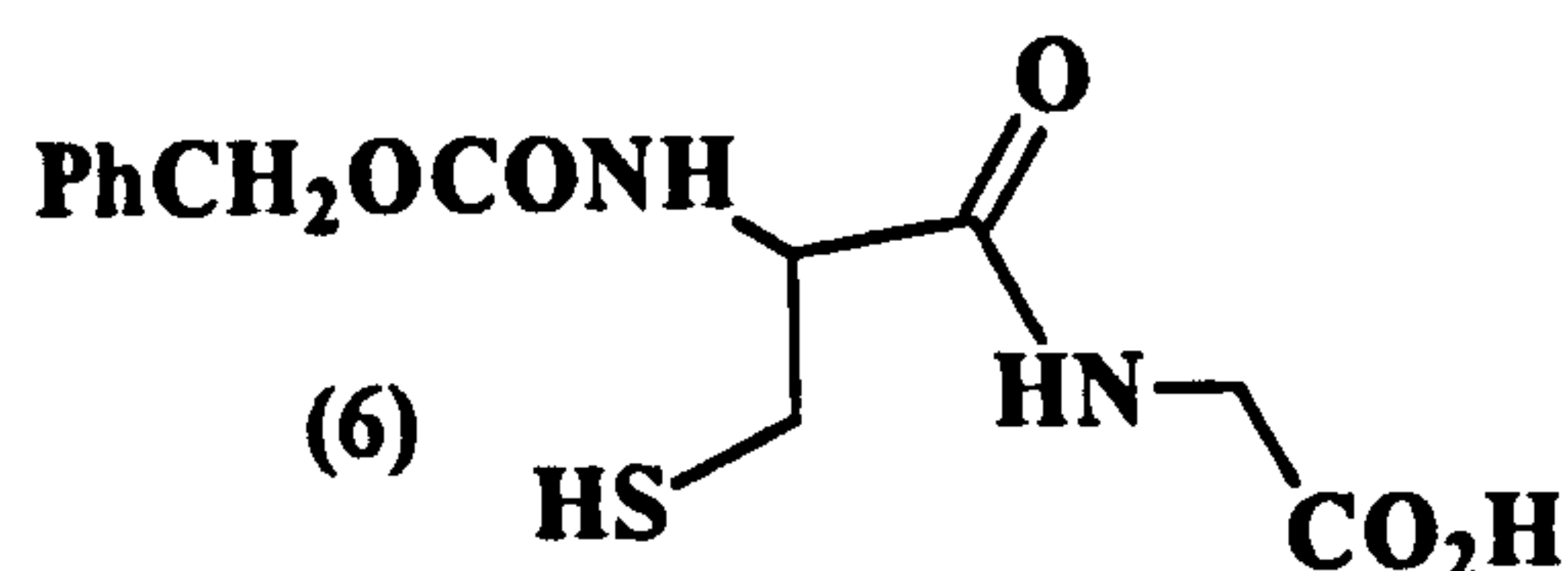
¹³C-NMR δ_C (DMSO) 172.3 (COOH), 156.2 (OCONH), 136.9, 128.5, 127.9.1, 127.8 (C₆H₅), 65.7 (CH₂-O), 56.6 (α CH), 25.6 (β CH₂)

IR (nujol) ν_{\max} 3327 (amide NH), 2571 (SH), 1719 (COOH), 1640 cm⁻¹ (amide CO)

ES MS [M-H]⁻ *m/z* 254

2.1.6 Synthesis of N-carbobenzoxy-L-cysteinyl-glycine (6)

Synthesis of N-carbobenzoxy-DL-cysteinyl-glycine



All N-carbobenzoxy-cysteinyl-amino acids were synthesised using the same procedure, which is based on the method of Vanderhaeghe and Adriaens¹²³. Details are given below, but subsequently only the analytical data are reported.

The compounds' names are abbreviated according to the following model :

(Z-Cys-GlyOMe)₂ for N,N'-dicarbobenzoxy-cystinyl-glycine methyl ester

(Z-Cys-GlyOH)₂ for N,N'-dicarbobenzoxy-cystinyl-glycine

Z-Cys-GlyOH for N-carbobenzoxy-cysteinyl-glycine

A solution of N,N'-dicarbobenzoxy-cystine (6 mmoles) in dichloromethane (50 ml), cooled to 0 °C, was treated with dicyclohexylcarbodiimide (12 mmoles), a solution of triethylamine (12 mmoles) and glycine methylester hydrochloride (12.6 mmoles) in dichloromethane (50 ml). The mixture was stirred at 0 °C for 1 hour and then for 24 hours at room temperature. The precipitated dicyclohexylurea was filtered, the filtrate washed twice with 1 M sulfuric acid (30 ml) and twice with 5 % sodium bicarbonate (30 ml). The organic layer was dried over anhydrous sodium sulfate and evaporated to dryness *in vacuo*. The residue was dissolved in acetone, the

insoluble part was removed by filtration and the solvent evaporated to give a white product. Yield 90 %.

(Z-Cys-GlyOMe)₂

¹H-NMR δ_H (DMSO) 8.50 (1 H, t, NH Gly, *J* 5.6), 7.66 (1 H, d, NH(Cys)₂, *J* 8.5), 7.36 (5 H, m, Ar-H), 5.05 (2 H, s, CH₂-O), 4.36 (1 H, m, α CH(Cys)₂), 3.85 (2 H, d, α CH₂Gly), 3.58 (3 H, s, OCH₃), 3.14 and 2.85 (2 H, m, β CH₂(Cys)₂ *J* 4.0, *J* 10.1, *J* 13.3)

¹³C-NMR δ_C (DMSO) 170.8 (CO-NHGly), 170.1 (COOCH₃), 156.1 (OCO-NH(Cys)₂), 136.9, 128.4, 127.8, 127.7 (C₆H₅), 65.6 (CH₂-O), 53.6 (OCH₃), 51.7 (α CH(Cys)₂), 40.8 (β CH₂(Cys)₂), 38.6 (α CH₂Gly)

IR (nujol) ν_{max} 3303 (amide NH), 1740 (COOMe), 1687 and 1659 cm⁻¹ (C=O).

ES MS [M+Cl]⁻ *m/z* 685

(Z-Cys-GlyOMe)₂ (5 mmoles) was dissolved in 50 ml of methanol. The mixture was cooled to 0 - 10 °C, and a solution of lithium hydroxide(10.2 mmoles) in water (17 ml) was added dropwise to the stirred solution. After 18 hours, 50 ml of water were added and the methanol was subsequently removed by evaporation. The solution was then washed twice with diethyl ether (25 ml) to remove any starting material. The aqueous phase was acidified to pH 2 with dilute hydrochloric acid and the product separated as a yellow precipitate which was filtered, washed with water, and dried *in vacuo*. Yield 70 %.

(Z-Cys-GlyOH)₂

¹H-NMR δ_H (DMSO) 8.31 (1 H, t, NH Gly, *J* 5.9), 7.63 (1 H, d, NH(Cys)₂, *J* 8.5), 7.36 (5 H, m, Ar-H), 5.05 (2 H, s, CH₂-O), 4.36 (1 H, m, α CH(Cys)₂), 3.76 (2 H, d, α CH₂Gly), 3.16 and 2.85 (2 H, m, β CH₂(Cys)₂)

¹³C-NMR δ_C (DMSO) 170.8 (CO-NHGly), 170.6 (COOH), 156.1 (OCO-NH(Cys)₂), 136.9, 128.4, 127.7, 127.2 (C₆H₅), 65.6 (CH₂-O), 53.6 (α CH(Cys)₂), 40.9 (β CH₂(Cys)₂), 40.0 (α CH₂Gly)

IR (nujol) ν_{max} 3309 (amide NH), 1729 (COOH), 1689 (amide CO), 1653 cm⁻¹ (OCONH)

ES MS [M-H]⁻ *m/z* 621, [M-312]⁻ *m/z* 310

(Z-Cys-GlyOH)₂ (2 mmoles) was dissolved in 20 ml of ethanol. To this solution at 67-70 °C was added dropwise a solution containing 4 equivalents of sodium borohydride (8.74 mmoles) in 10 ml of ethanol. The stirred reaction mixture was heated at 75 - 80 °C for one hour. After cooling to 25 °C, the solution was added to 50 ml of iced water. To this stirred solution, 6 M hydrochloric acid was added dropwise at 0 - 10 °C until pH 3 was obtained. After evaporating under reduced pressure to remove the ethanol, the solution was extracted three times into ethyl acetate, washed three times with distilled water and dried over anhydrous sodium sulphate. Removal of the solvent gave an oil (Yield 97 %). The reaction was followed by TLC in ethanol/chloroform/acetic acid system (9:2:0.5, v/v/v).

Z-Cys-GlyOH

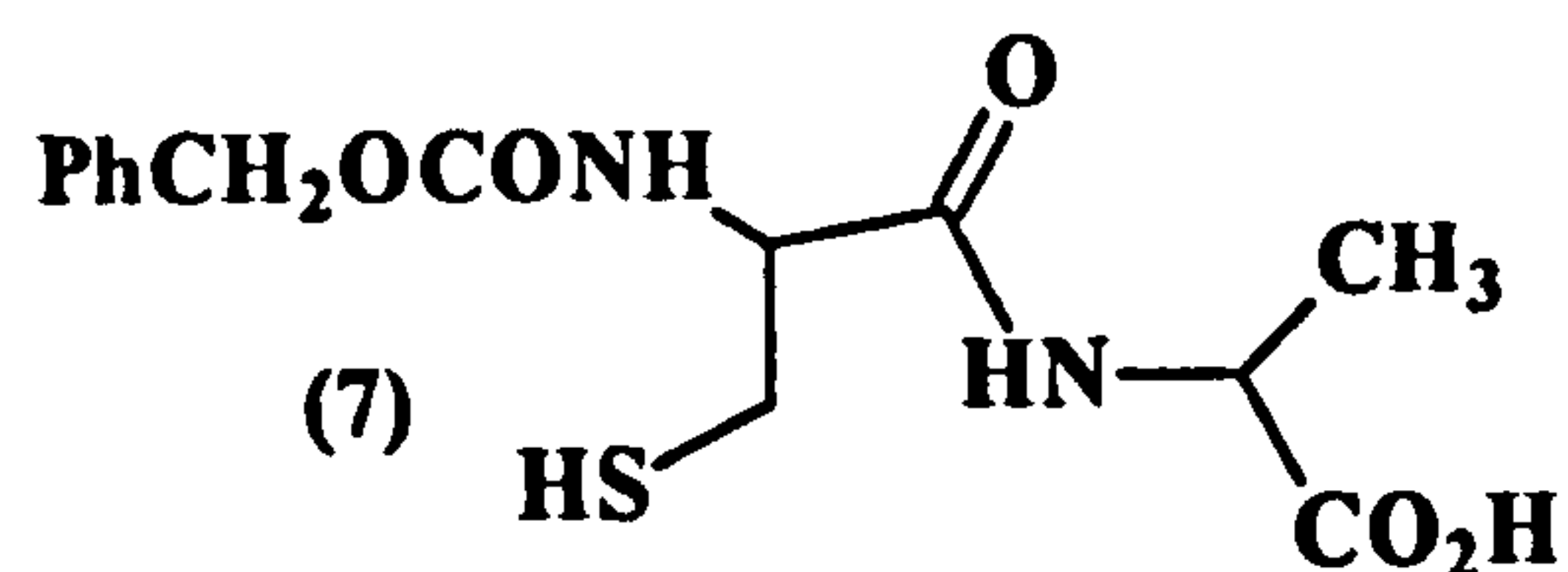
¹H-NMR δ_{H} (DMSO) 12.63 (1 H, s, COOH), 8.34 (1 H, t, NH Gly), 7.51 (1 H, d, NHCys), 7.36 (5 H, m, Ar-H), 5.05 (2 H, s, CH₂-O), 4.18 (1 H, m, α CHCys), 3.77 (2 H, m, α CH₂Gly), 2.83 and 2.67 (2 H, m, β CH₂Cys), 2.37 (1 H, t, SH *J* 8.5)

¹³C-NMR δ_{C} (DMSO) 171.3 (CO-NHGly), 170.7 (COOH), 156.2 (OCO-NHCys), 137.1, 128.5, 127.9, 127.2 (C₆H₅), 65.8 (CH₂-O), 57.4 (α CHCys), 41.0 (α CH₂Gly), 26.6 (β CH₂Cys)

IR (nujol) ν_{max} 3285, 3215 (amides NH), 2500 (SH), 1728 (COOH), 1686, 1635 cm⁻¹ (amides CO)

ES MS [M-H]⁻ *m/z* 311

2.1.7 Synthesis of *N*-carbobenzoxy-*L*-cysteinyl-*DL*-alanine (7)



(*Z*-*L*-Cys-*DL*-AlaOMe)₂

¹H-NMR δ_H (DMSO) 8.49 (1 H, m, NHAla), 7.60 (1 H, m, NH(Cys)₂), 7.36 (5 H, m, Ar-H), 5.05 (2 H, s, CH₂-O), 4.29 (2 H, m, αCHAla and αCH(Cys)₂), 3.62 (3 H, s, OCH₃), 3.11 and 2.88 (2 H, m, βCH₂(Cys)₂), 1.27 (3 H, d, βCH₃Ala *J* 7.3)

¹³C-NMR δ_C (DMSO) 172.7 (COOCH₃), 170.2 (CO-NHAla), 156.1 (OCO-NH(Cys)₂), 136.9, 128.5, 128.4, 128.1 (C₆H₅), 65.9 (CH₂-O), 53.6 (αCH(Cys)₂), 51.9 (OCH₃), 47.8 (αCHAla), 40.1 (βCH₂(Cys)₂), 17.0 and 16.8 (βCH₃Ala)

IR (nujol) ν_{max} 3420 and 3333 (amides NH), 1743 (COOMe), 1690 (amide CO), 1651 cm⁻¹ (OCONH)

ES MS [M-H]⁻ *m/z* 677

(*Z*-*L*-Cys-*DL*-AlaOH)₂

¹H-NMR δ_H (DMSO) 8.32 (1 H, m, NHAla), 7.59 (1 H, m, NH(Cys)₂), 7.35 (5 H, m, Ar-H), 5.05 (2 H, s, CH₂-O), 4.38 (1 H, m, αCH(Cys)₂), 4.23 (1 H, m, αCHAla), 3.15 and 2.87 (2 H, m, βCH₂(Cys)₂), 1.29 (3 H, m, βCH₃Ala)

^{13}C -NMR δ_{C} (DMSO) 174.1 (COOH), 170.2 (CO-NHAla), 156.2 (OCO-NH(Cys)₂), 137.0, 128.5, 128.0, 128.8 (C₆H₅), 65.8 (CH₂-O), 53.8 (α CH(Cys)₂), 47.9 (α CHAla), 40.7 (β CH₂(Cys)₂), 17.5 (β CH₃Ala)

IR (nujol) ν_{max} 3326 (amides NH), 1718 (COOH), 1669 (amide CO), 1659 cm⁻¹ (OCONH)

ES MS [M-H]⁻ m/z 649

Z-L-Cys-DL-AlaOH

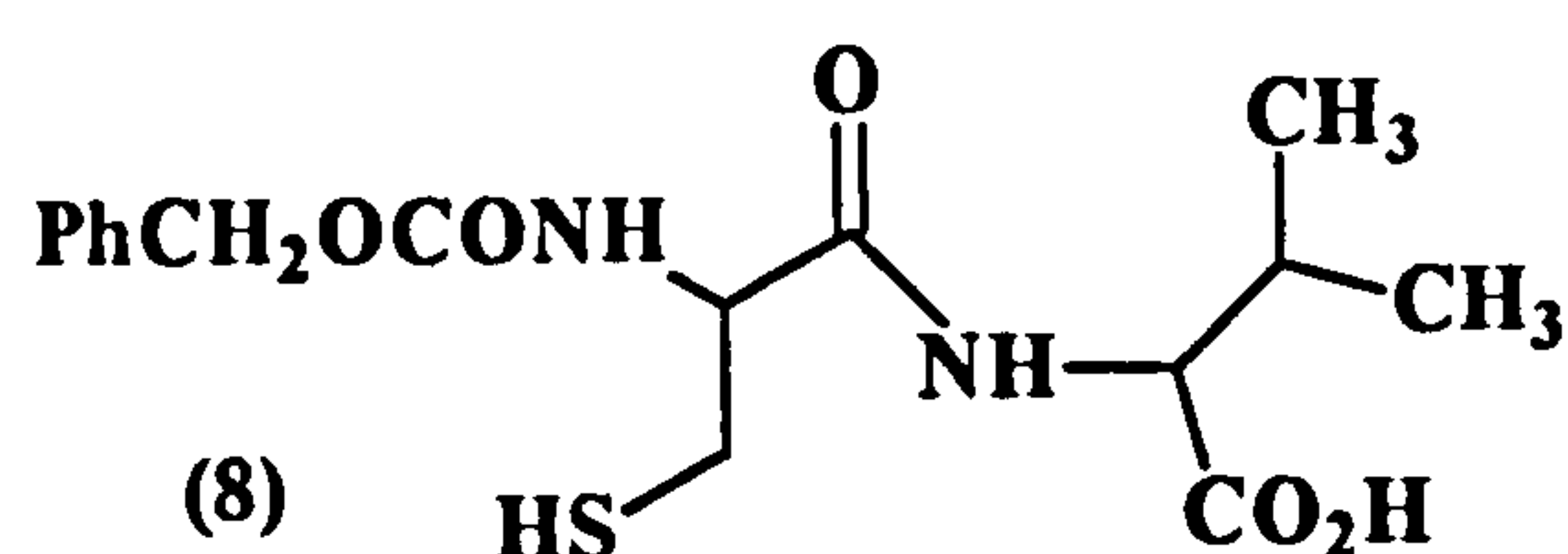
^1H -NMR δ_{H} (DMSO) 8.33 (1 H, m, NHAla), 7.44 (1 H, m, NHCys), 7.34 (5 H, m, Ar-H), 5.06 (2 H, s, CH₂-O), 4.26 (2 H, m, α CHCys and α CHAla), 2.73 (2 H, m, β CH₂Cys), 2.25 (1 H, m, SH), 1.29 (3 H, m, β CH₃Ala)

^{13}C -NMR δ_{C} (DMSO) 174.4 (COOH), 170.3 (CO-NHAla), 156.2 (OCO-NHCys), 137.2, 128.7, 128.4, 128.0 (C₆H₅), 66.0 (CH₂-O), 57.3 (α CHCys), 48.1 (α CHAla), 27.0 (β CH₂Cys), 17.7 (β CH₃Ala)

IR (nujol) ν_{max} 3413 and 3322 (amides NH), 2571 (SH), 1719 (COOH), 1671 (amide CO), 1659 cm⁻¹ (OCONH)

ES MS [M-H]⁻ m/z 325

mp 40-44 °C

2.1.8 Synthesis of *N*-carbobenzoxy-*L*-cysteinyl-*DL*-valine (8)**(*Z*-*L*-Cys-*DL*-ValOMe)₂**

¹H-NMR δ_H (DMSO) 8.30 (1 H, m, NHVal), 7.60 (1 H, m, NH(Cys)₂), 7.35 (5 H, m, Ar-H), 5.05 (2 H, s, CH₂-O), 4.40 (1 H, m, αCH(Cys)₂), 4.21 (1 H, m, αCHVal), 3.64 (3 H, s, OCH₃), 3.09 and 2.90 (2 H, m, βCH₂(Cys)₂), 2.05 (1 H, m, βCHVal), 0.86 and 0.85 (6 H, d, γCH₃Val *J* 4.6)

¹³C-NMR δ_C (DMSO) 171.8 (COOCH₃), 170.5 (CO-NHVal), 155.9 (OCO-NH(Cys)₂), 136.9, 128.4, 127.8, 127.6 (C₆H₅), 65.6 (CH₂-O), 57.5 (αCHVal), 53.7 (αCH(Cys)₂), 51.8 (OCH₃), 40.5 (βCH₂(Cys)₂), 30.1 and 29.9 (βCHVal), 19.0 and 18.1 (γCH₃Val)

IR (nujol) ν_{max} 3303 (NH), 1739 (COOMe), 1693 (amide CO), 1656 cm⁻¹ (OCONH)

ES MS [M-H]⁻ *m/z* 733

(Z-L-Cys-DL-ValOH)₂

¹H-NMR δ_H (DMSO) 8.17 (1 H, m, NHVal), 7.65 (1 H, m, NH(Cys)₂), 7.35 (5 H, m, Ar-H), 5.05 (2 H, s, CH₂-O), 4.43 (1 H, m, α CH(Cys)₂), 4.17 (1 H, m, α CHVal), 3.13 and 2.90 (2 H, m, β CH₂(Cys)₂), 2.06 (1 H, m, β CHVal), 0.86 (6 H, 3, γ CH₃Val)

¹³C-NMR δ_C (DMSO) 172.7 (COOH), 170.3 (CO-NHVal), 155.9 (OCO-NH(Cys)₂), 136.9, 128.4, 127.7, 127.1 (C₆H₅), 65.6 (CH₂-O), 57.2 (α CHVal), 53.9 (α CH(Cys)₂), 40.6 (β CH₂(Cys)₂), 30.1 (β CHVal), 19.2 and 17.9 (γ CH₃Val)

IR (nujol) ν_{\max} 3309 (NH), 1715 (COOH), 1661 cm⁻¹ (C=O)

Z-L-Cys-DL-ValOH

¹H-NMR δ_H (DMSO) 8.15 (1 H, m, NHVal), 7.60 (1 H, m, NHCys), 7.35 (5 H, m, Ar-H), 5.05 (2 H, s, CH₂-O), 4.19 (2 H, m, α CHCys and α CHVal), 2.80 (2 H, m, β CH₂Cys), 2.05 (1 H, m, β CHVal), 0.86 (6 H, 3, γ CH₃Val)

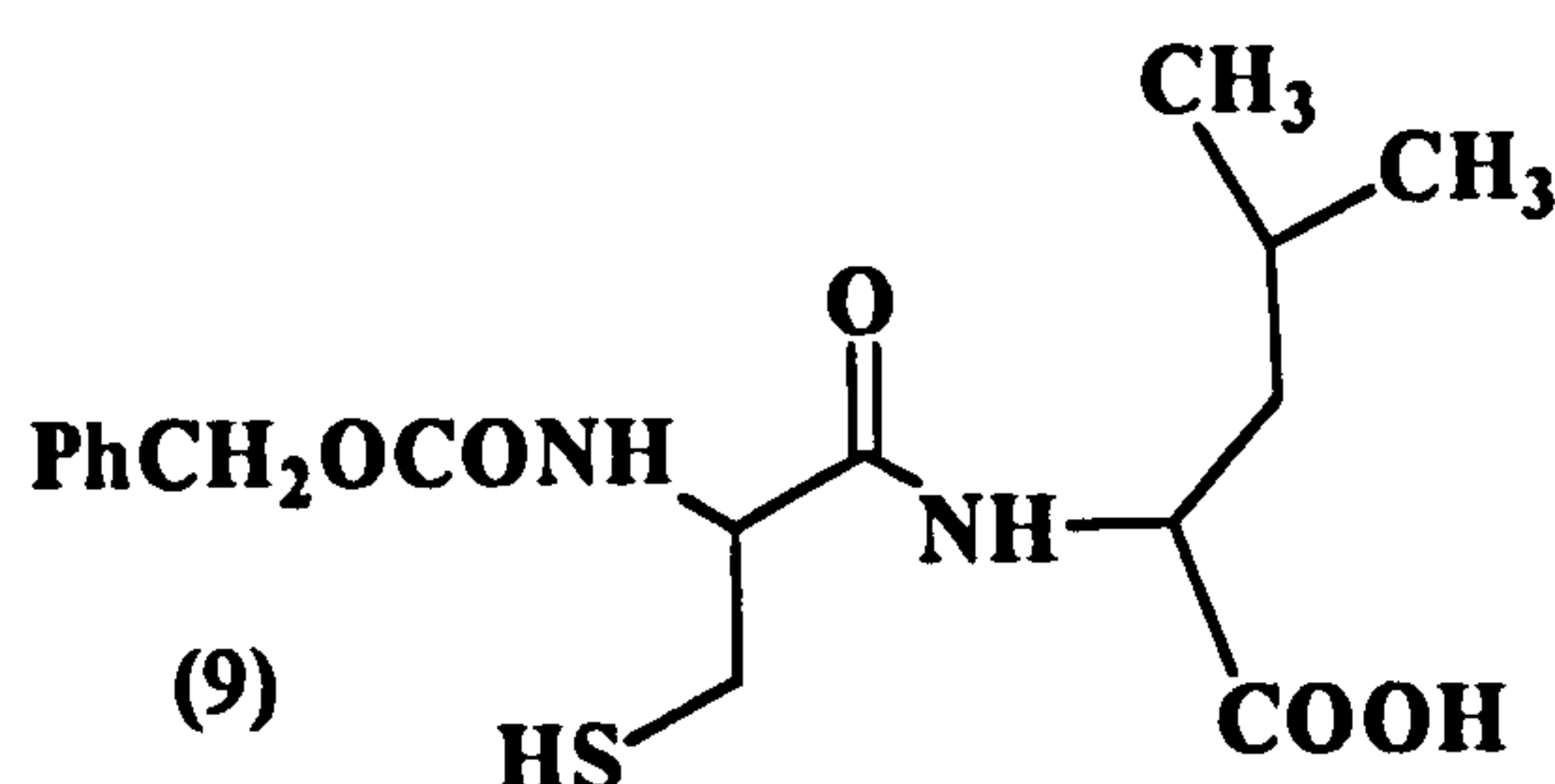
¹³C-NMR δ_C (DMSO) 172.9 (COOH), 170.5 (CO-NHVal), 156.1 (OCO-NHCys), 137.1, 128.5, 128.2, 127.9 (C₆H₅), 65.7 (CH₂-O), 57.2 (α CHVal and α CHCys), 30.2 (β CHVal), 26.9 (β CH₂Cys), 19.2 and 18.0 (γ CH₃Val)

IR (nujol) ν_{\max} 3317 (NH), 2625 (SH), 1718 (COOH), 1668 cm⁻¹ (C=O)

ES MS [M-H]⁻ m/z 353

mp 37-40 °C

2.1.9 Synthesis of *N*-carbobenzoxy-*L*-cysteinyl-*L*-leucine (9)



(*Z*-*L*-Cys-*L*-LeuOMe)₂

¹H-NMR δ_H (DMSO) 8.39 (1 H, m, NHLeu), 7.62 (1 H, m, NH(Cys)₂), 7.35 (5 H, m, Ar-H), 5.05 (2 H, s, CH₂-O), 4.33 (2 H, m, αCH(Cys)₂ and αCHLeu), 3.61 (3 H, s, OCH₃), 3.13 and 2.89 (2 H, m, βCH₂(Cys)₂), 1.55 (3 H, m, βCH₂Leu and γCHLeu), 0.85 (6 H, m, δCH₃Leu)

¹³C-NMR δ_C (DMSO) 172.7 (COOCH₃), 170.4 (CO-NHLeu), 156.0 (OCO-NH(Cys)₂), 136.9, 128.3, 127.7, 127.2 (C₆H₅), 65.6 (CH₂-O), 53.7 (αCH(Cys)₂), 51.9 (OCH₃), 50.4 (αCHLeu), 40.4 (βCH₂(Cys)₂), 39.5 (βCH₂Leu), 24.2 (γCHLeu), 22.8 and 21.3 (δCH₃Leu)

IR (nujol) ν_{max} 3266 (amides NH), 1746 (COOMe), 1703 (amide CO), 1655 cm⁻¹ (OCONH)

(Z-L-Cys-L-LeuOH)₂

¹H-NMR δ_{H} (DMSO) 8.20 (1 H, d, NHLeu J 7.9), 7.60 (1 H, d, NH(Cys)₂ J 8.1), 7.35 (5 H, m, Ar-H), 5.05 (2 H, s, CH₂-O), 4.34 (1 H, m, α CH(Cys)₂), 4.23 (1 H, m, α CHLeu), 3.13 and 2.87 (2 H, m, β CH₂(Cys)₂), 1.59 (3 H, m, β CH₂Leu and γ CHLeu), 0.86 (6 H, m, δ CH₃Leu)

¹³C-NMR δ_{C} (DMSO) 173.7 (COOH), 170.2 (CO-NHLeu), 156.0 (OCO-NH(Cys)₂), 136.9, 128.4, 128.0, 127.8 (C₆H₅), 65.6 (CH₂-O), 53.8 (α CH(Cys)₂), 50.5 (α CHLeu), 40.1 (β CH₂(Cys)₂), 39.8 (β CH₂Leu), 24.3 (γ CHLeu), 22.9 and 21.4 (δ CH₃Leu)

IR (nujol) ν_{max} 3312 (NH), 1693 (COOH), 1655 cm⁻¹ (C=O)

Z-L-Cys-L-LeuOH

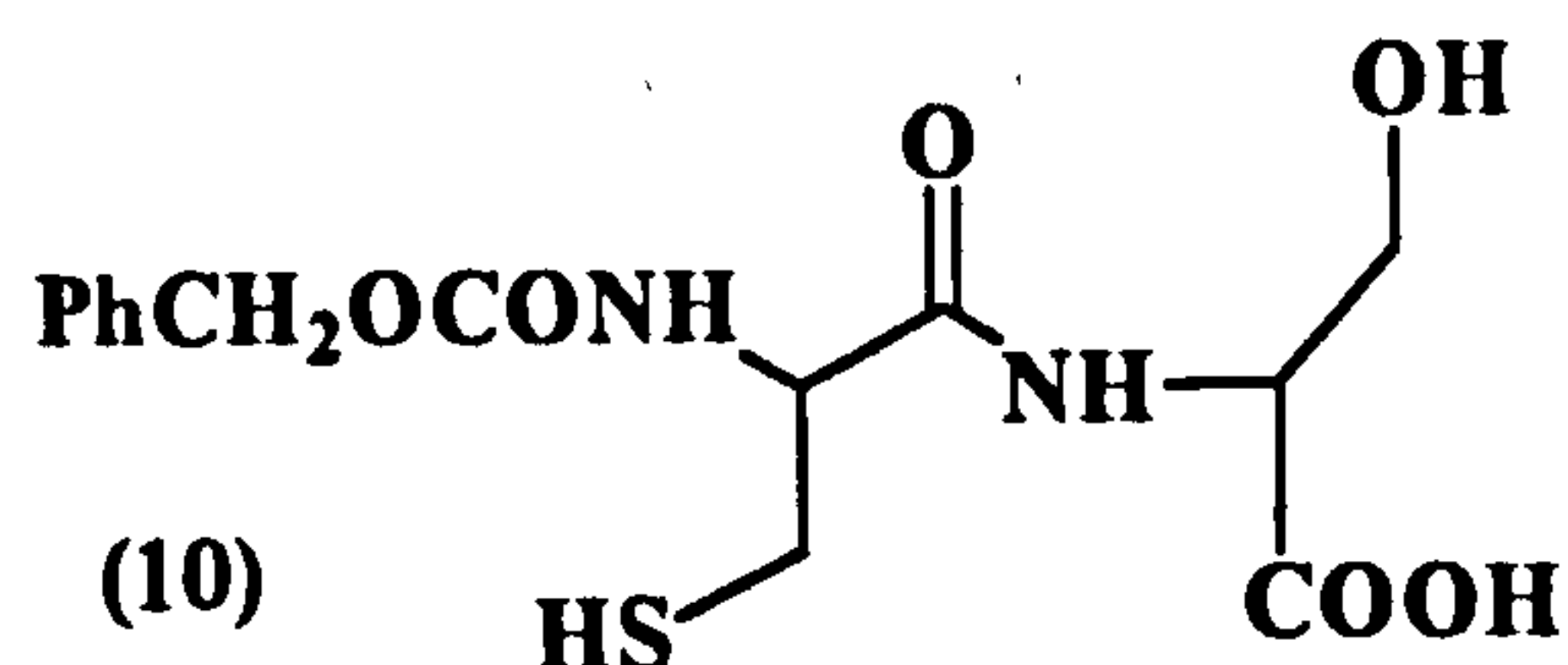
¹H-NMR δ_{H} (DMSO) 8.22 (1 H, m, NHLeu), 7.53 (1 H, m, NHCys), 7.35 (5 H, m, Ar-H), 5.05 (2 H, s, CH₂-O), 4.25 (2 H, m, α CHCys and α CHLeu), 2.85 (2 H, m, β CH₂Cys), 1.58 (3 H, m, β CH₂Leu and γ CHLeu), 0.88 (6 H, m, δ CH₃Leu)

¹³C-NMR δ_{C} (DMSO) 174.9 (COOH), 170.2 (CO-NHLeu), 156.0 (OCO-NHCys), 136.9, 128.4, 127.8, 127.7 (C₆H₅), 65.6 (CH₂-O), 57.18 (α CHCys), 50.5 (α CHLeu), 40.1 (β CH₂Leu), 26.45 (β CH₂Cys), 24.5 (γ CHLeu), 22.9 and 21.4 (δ CH₃Leu)

IR (nujol) ν_{max} 3308 (NH), 2503 (SH), 1714 (COOH), 1661 cm⁻¹ (C=O)

ES MS [M-H]⁻ m/z 361

2.1.10 Synthesis of *N*-carbobenzoxy-*L*-cysteinyl-*DL*-serine and *N*-carbobenzoxy-*DL*-cysteinyl-*L*-serine(10)



(*Z*-*L*-Cys-*DL*-SerOMe)₂, (*Z*-*DL*-Cys-*L*-SerOMe)₂

¹H-NMR δ_H (DMSO) 8.30 (1 H, m, NHSer), 7.65 (1 H, m, NH(Cys)₂), 7.36 (5 H, m, Ar-H), 5.06 (2 H, s, CH₂-O), 4.42 (2 H, m, αCH(Cys)₂ and αCHSer), 3.72 (2 H, m, βCH₂Ser), 3.63 (3 H, s, OCH₃), 3.15 and 2.88 (2 H, m, βCH₂(Cys)₂)

¹³C-NMR δ_C (DMSO) 170.8 (COOCH₃), 170.5 (CO-NHSer), 156.1 (OCO-NH(Cys)₂), 136.9, 128.4, 128.1, 127.8 (C₆H₅), 65.6 (CH₂-O), 61.2 (βCH₂Ser), 54.9 (αCHSer), 53.7 (αCH(Cys)₂), 52.0 (OCH₃), 40.5 (βCH₂(Cys)₂)

IR (nujol) ν_{max} 3505 (NH), 1738 (COOMe), 1700 (amide CO), 1651 cm⁻¹ (C=O)

ES MS [M-H]⁻ m/z 709

(Z-L-Cys-DL-SerOH)₂, (Z-DL-Cys-L-SerOH)₂

¹H-NMR δ_H (DMSO) 8.10 (1 H, m, NHSer), 7.65 (1 H, m, NH(Cys)₂), 7.35 (5 H, m, Ar-H), 5.05 (2 H, s, CH₂-O), 4.42 (1 H, m, αCH(Cys)₂), 4.28 (1 H, m, αCHSer), 3.67 (2 H, m, βCH₂Ser), 3.16 and 2.86 (2 H, m, βCH₂(Cys)₂)

¹³C-NMR δ_C (DMSO) 171.8 (COOH), 170.3 (CO-NHSer), 156.2 (OCO-NH(Cys)₂), 136.9, 128.5, 127.9, 127.8 (C₆H₅), 65.7 (CH₂-O), 61.4 (βCH₂Ser), 54.9 (αCHSer), 53.8 (αCH(Cys)₂), 40.5 (βCH₂(Cys)₂)

IR (nujol) ν_{max} 3298 (NH), 1718 (COOH), 1703 and 1650 cm⁻¹ (C=O)

ES MS [M-H]⁻ m/z 681

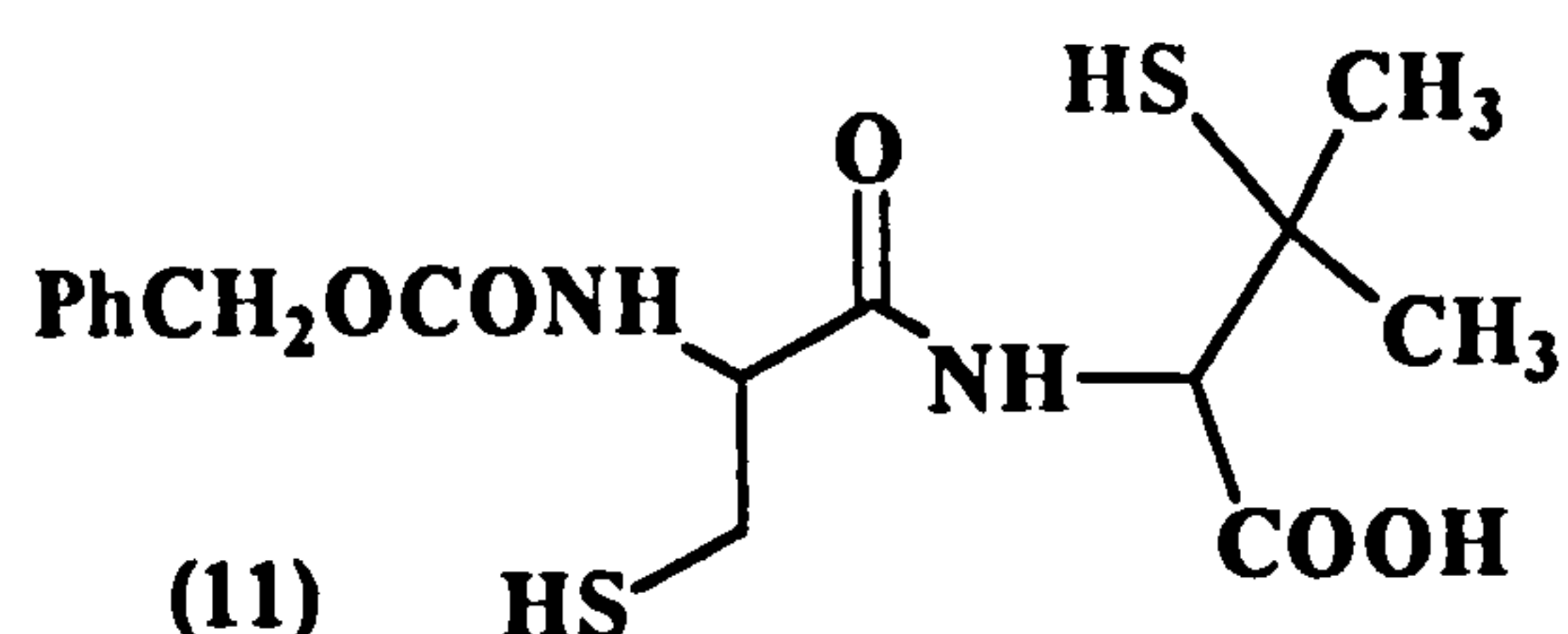
Z-L-Cys-DL-SerOH, Z-DL-Cys-L-SerOH

¹H-NMR δ_H (DMSO) 8.10 (1 H, m, NHSer), 7.53 (1 H, m, NHCys), 7.37 (5 H, m, Ar-H), 5.06 (2 H, s, CH₂-O), 4.30 (2 H, m, αCHCys and αCHSer), 3.68 (2 H, m, βCH₂Ser), 3.15 and 2.87 (2 H, m, βCH₂Cys), 2.29 (1H, m, SH)

¹³C-NMR δ_C (DMSO) 171.8, 171.7 (COOH), 170.2, 170.1 (CO-NHSer), 156.0 (OCO-NHCys), 136.9, 128.4, 127.8, 127.7 (C₆H₅), 65.6 (CH₂-O), 61.4, 61.2 (βCH₂Ser), 57.5 (αCHCys), 55.2, 55.1 (αCHSer), 26.6 (βCH₂Cys)

IR (nujol) ν_{max} 3301 (OH, NH), 2609 (SH), 1718 (COOH), 1663 (C=O)

ES MS [M-H]⁻ m/z 341

2.1.11 Synthesis of *N*-carbobenzoxy-*D*-cysteinyl-*D*-penicillamine (11)**(*Z*-D-Cys-D-PenicillamineOMe)₂**

¹H-NMR δ_H (DMSO) 8.25 (1 H, m, NHPen), 7.60 (1 H, m, NH(Cys)₂), 7.35 (5 H, m, Ar-H), 5.04 (2 H, s, CH₂-O), 4.50 (2 H, m, αCH(Cys)₂ and αCHPen), 3.63 (3 H, s, OCH₃), 3.04 and 2.73 (2 H, m, βCH₂(Cys)₂), 1.33 (6 H, m, γCH₃Pen)

¹³C-NMR δ_C (DMSO) 170.5 (COOCH₃), 169.9 (CO-NHPen), 156.0 (OCO-NH(Cys)₂), 136.9, 128.4, 127.9, 127.7 (C₆H₅), 65.6 (CH₂-O), 61.5 (αCHPen), 54.9 (αCH(Cys)₂), 51.9 (OCH₃), 45.21 (βCPen), 40.4 (βCH₂(Cys)₂), 29.5 and 24.19 (γCH₃Pen)

IR (nujol) ν_{max} 3324 (NH), 1734 (COOMe), 1675 cm⁻¹ (C=O)

ES MS [M+Cl]⁻ m/z 835

(*Z*-D-Cys-D-PenicillamineOH)₂

¹H-NMR δ_H (DMSO) 8.20 (1 H, m, NHPen), 7.57 (1 H, m, NH(Cys)₂), 7.35 (5 H, m, Ar-H), 5.04 (2 H, s, CH₂-O), 4.67 (1 H, m, αCHPen), 4.34 (1 H, m,

$\alpha\text{CH}(\text{Cys})_2$, 3.63 (3 H, s, OCH_3), 2.89 (2 H, m, $\beta\text{CH}_2(\text{Cys})_2$), 1.35 (6 H, m, $\gamma\text{CH}_3\text{Pen}$)

$^{13}\text{C-NMR } \delta_{\text{C}}$ (DMSO) 171.4 (COOH), 170.2 (CO-NHPen), 155.1 (OCO-NH(Cys)₂), 136.9, 128.4, 128.1, 127.9, (C_6H_5), 65.6 ($\text{CH}_2\text{-O}$), 59.4 (αCHPen), 53.0 ($\alpha\text{CH}(\text{Cys})_2$), 45.21 (βCPen), 40.2 ($\beta\text{CH}_2(\text{Cys})_2$), 26.8 and 19.7 ($\gamma\text{CH}_3\text{Pen}$)

IR (nujol) ν_{max} 3416 (NH), 2500 (SH), 1719 (COOH), 1671 cm^{-1} (CO)

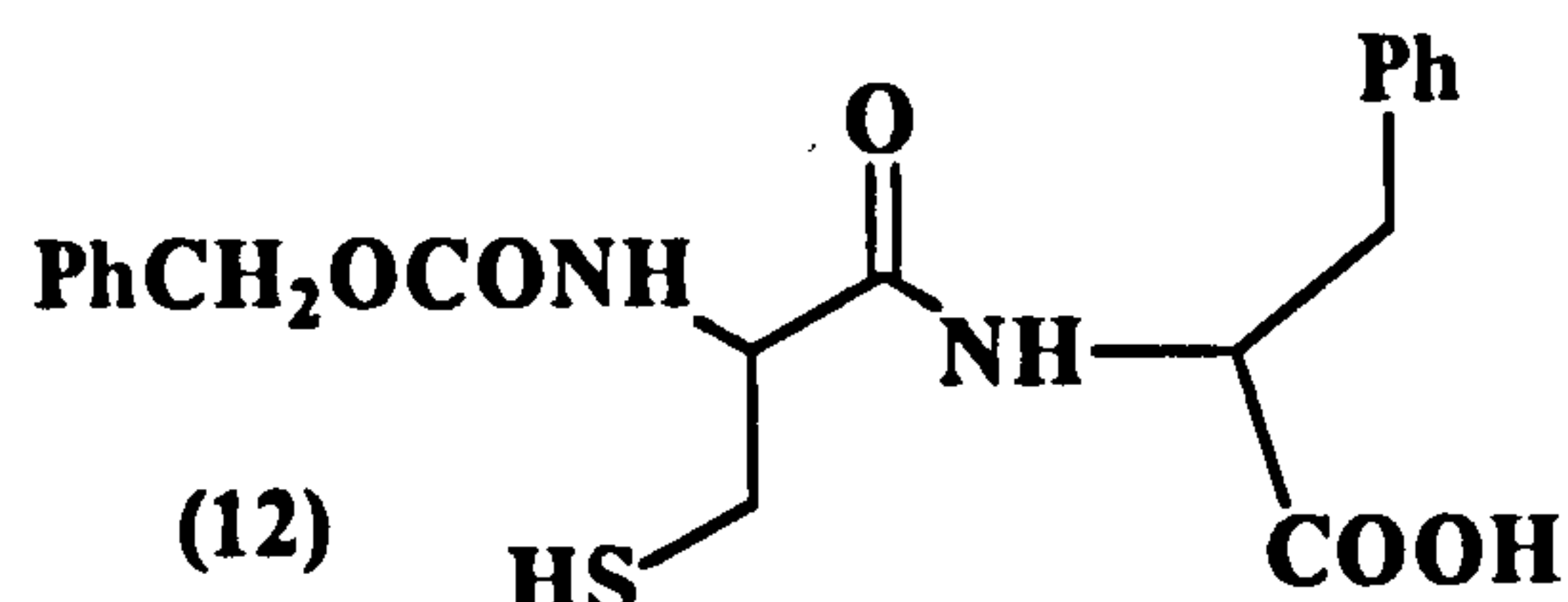
Z-D-Cys-D-PenicillamineOH

$^1\text{H-NMR } \delta_{\text{H}}$ (CDCl_3) 8.31 (1 H, d, NHPen), 7.35 (6 H, m, NHCys and Ar-H), 5.11 (2 H, s, $\text{CH}_2\text{-O}$), 4.82 (1 H, m, αCHPen), 4.68 (1 H, m, αCHCys), 2.96 (2 H, m, $\beta\text{CH}_2\text{Cys}$), 1.41 (6 H, m, $\gamma\text{CH}_3\text{Pen}$)

$^{13}\text{C-NMR } \delta_{\text{C}}$ (CDCl_3) 173.1 (COOH), 171.8 (CO-NHPen), 155.2 (OCO-NH(Cys)₂), 135.9, 128.5, 128.3, 128.1 (C_6H_5), 67.2 ($\text{CH}_2\text{-O}$), 59.5 (αCHPen), 53.6 (αCHCys), 57.0 (βCPen), 41.6 ($\beta\text{CH}_2\text{Cys}$), 26.7 and 19.8 ($\gamma\text{CH}_3\text{Pen}$)

IR (nujol) ν_{max} 3303 (NH), 2570 (SH), 1719 (COOH), 1669 cm^{-1} (CO)

ES MS $[\text{M}-3\text{H}]^-$ m/z 383 (probably disulfide bond between cysteine and penicillamine)

2.1.12 Synthesis of *N*-carbobenzoxy-cysteinyl-phenylalanine (12)**(Z-L-Cys-DL-PheOMe)₂**

¹H-NMR δ_H (DMSO) 8.50 (1 H, m, NHPhe), 7.60 (1 H, m, NH(Cys)₂), 7.35 (5 H, m, ZAr-H), 7.24 (5 H, m, PheAr-H), 5.05 (2 H, s, CH₂-O), 4.52 (1 H, m, αCHPhe), 4.34 (1 H, m, αCH(Cys)₂), 3.61 (3 H, m, OCH₃), 2.87 (4 H, m, βCH₂(Cys)₂ and βCH₂Phe)

¹³C-NMR δ_C (DMSO) 171.6 (COOCH₃), 170.2 (CO-NHPhe), 155.9 (OCO-NH(Cys)₂), 137.0, 136.9, 129.2, 129.1, 127.8, 127.0, 126.6, (ZC₆H₅ and PheC₆H₅), 65.6 (CH₂-O), 53.7 and 53.5 (αCH(Cys)₂ and αCHPhe), 52.0 and 51.9 (OCH₃), 40.4 and 40.0 (βCH₂(Cys)₂), 36.8 and 36.5 (βCH₂Phe)

IR (nujol) ν_{max} 3321 (NH), 1743 (COOMe), 1717 and 1669 cm⁻¹ (CO)

(Z-L-Cys-L-PheOMe)₂, (Z-D-Cys-D-PheOMe)₂

¹H-NMR δ_H (DMSO) 8.45 (1 H, d, NHPhe, *J* 7.9), 7.62 (1 H, d, NH(Cys)₂, *J* 8.5), 7.34 (5 H, m, ZAr-H), 7.21 (5 H, m, PheAr-H), 5.06 (2 H, s, CH₂-O), 4.53

(1 H, m, α CHPhe), 4.39(1 H, m, α CH(Cys)₂), 3.59 (3 H, s, OCH₃), 3.05 and 2.85 (4 H, m, β CH₂(Cys)₂ and β CH₂Phe)

¹³C-NMR δ_c (DMSO) 171.7 (COOCH₃), 170.4 (CO-NHPhe), 156.0 (OCO-NH(Cys)₂), 137.0, 136.9, 129.2, 128.4, 128.3, 127.9, 127.8, 127.7 (ZC₆H₅ and PheC₆H₅), 65.8 (CH₂-O), 53.8 (α CH(Cys)₂), 52.0 (α CHPhe and OCH₃), 40.4 (β CH₂(Cys)₂), 39.5 (β CH₂Phe)

(Z-L-Cys-D-PheOMe)₂, (Z-D-Cys-L-PheOMe)₂

¹H-NMR δ_H (DMSO) 8.51 (1 H, d, NHPhe, *J* 7.9), 7.56 (1 H, d, NH(Cys)₂, *J* 8.5), 7.34 (5 H, m, ZAr-H), 7.21 (5 H, m, PheAr-H), 5.04 (2 H, s, CH₂-O), 4.52 (1 H, m, α CHPhe), 4.33 (1 H, m, α CH(Cys)₂), 3.62 (3 H, s, OCH₃), 3.00 and 2.75 (4 H, m, β CH₂(Cys)₂ and β CH₂Phe)

¹³C-NMR δ_c (DMSO) 171.7 (COOCH₃), 170.3 (CO-NHPhe), 156.0 (OCO-NH(Cys)₂), 137.0, 136.9, 129.3, 128.4, 128.3, 127.9, 127.8, 127.7 (ZC₆H₅ and PheC₆H₅), 65.7 (CH₂-O), 53.6 (α CH(Cys)₂), 52.0 (α CHPhe and OCH₃), 39.9 (β CH₂(Cys)₂), 36.8 (β CH₂Phe)

(Z-L-Cys-DL-PheOH)₂

¹H-NMR δ_H (DMSO) 8.28 (1 H, m, NHPhe), 7.53 (1 H, m, NH(Cys)₂), 7.35 (5 H, m, ZAr-H), 7.21 (5 H, m, PheAr-H), 5.03 (2 H, s, CH₂-O), 4.50 (1 H, m, α CHPhe), 4.32 (1 H, m, α CH(Cys)₂), 2.90 (4 H, m, β CH₂(Cys)₂ and β CH₂Phe)

$^{13}\text{C-NMR } \delta_{\text{C}}$ (DMSO) 172.6 (COOH), 170.1 and 170.0 (CO-NHPhe), 156.0 (OCO-NH(Cys)₂), 137.3, 136.9, 129.3, 129.2, 128.4, 128.2, 127.9, 127.7, 127.1, 126.5 (ZC₆H₅ and PheC₆H₅), 65.7 (CH₂-O), 53.8, 53.6 and 53.4 ($\alpha\text{CH(Cys)}_2$ and αCHPhe), 40.0 ($\beta\text{CH}_2\text{(Cys)}_2$), 36.9 and 36.6 ($\beta\text{CH}_2\text{Phe}$)

IR (nujol) ν_{max} 3308 (NH), 1712 (COOH), 1651 cm^{-1} (CO)

ES MS [M-H]⁻ m/z 801

(Z-L-Cys-L-PheOH)₂, (Z-D-Cys-D-PheOH)₂

$^1\text{H-NMR } \delta_{\text{H}}$ (DMSO) 8.13 (1 H, d, NHPhe, J 7.8), 7.54 (1 H, d, NH(Cys)₂, J 8.7), 7.32 (5 H, m, ZAr-H), 7.22 (5 H, m, PheAr-H), 5.04 (2 H, s, CH₂-O), 4.44 (1 H, m, αCHPhe), 4.31 (1 H, m, $\alpha\text{CH(Cys)}_2$), 3.06 and 2.88 (4 H, m, $\beta\text{CH}_2\text{(Cys)}_2$ and $\beta\text{CH}_2\text{Phe}$)

$^{13}\text{C-NMR } \delta_{\text{C}}$ (DMSO) 172.4 (COOH), 169.9 (CO-NHPhe), 155.9 (OCO-NH(Cys)₂), 137.2, 136.8, 129.1, 128.3, 128.1, 127.7, 127.6, 126.4 (ZC₆H₅ and PheC₆H₅), 65.6 (CH₂-O), 53.8 ($\alpha\text{CH(Cys)}_2$), 53.4 (αCHPhe), 40.4 ($\beta\text{CH}_2\text{(Cys)}_2$), 36.5 ($\beta\text{CH}_2\text{Phe}$)

(Z-L-Cys-D-PheOH)₂, (Z-D-Cys-L-PheOH)₂

$^1\text{H-NMR } \delta_{\text{H}}$ (DMSO) 8.21 (1 H, d, NHPhe, J 8.2), 7.48 (1 H, d, NH(Cys)₂, J 8.7), 7.32 (5 H, m, ZAr-H), 7.20 (5 H, m, PheAr-H), 5.04 (2 H, s, CH₂-O), 4.48

(1 H, m, α CHPhe), 4.32 (1 H, m, α CH(Cys)₂), 3.00 and 2.74 (4 H, m, β CH₂(Cys)₂ and β CH₂Phe)

¹³C-NMR δ_c (DMSO) 172.5 (COOH), 169.9 (CO-NHPhe), 155.9 (OCO-NH(Cys)₂), 137.2, 136.8, 129.2, 128.3, 128.1, 127.8, 127.6, 126.4 (ZC₆H₅ and PheC₆H₅), 65.6 (CH₂-O), 53.7 (α CH(Cys)₂), 53.3 (α CHPhe), 39.9 (β CH₂(Cys)₂), 36.8 (β CH₂Phe)

Z-L-Cys-DL-PheOH

¹H-NMR δ_H (DMSO) 8.28 (1 H, m, NHPhe), 7.53 (1 H, m, NHCys), 7.35 (5 H, m, ZAr-H), 7.21 (5 H, m, PheAr-H), 5.04 (2 H, s, CH₂-O), 4.50 (1 H, m, α CHPhe), 4.19 (1 H, m, α CHCys), 2.90 (4 H, m, β CH₂Cys and β CH₂Phe)

¹³C-NMR δ_c (DMSO) 173.0 (COOH), 170.4 and 170.1 (CO-NHPhe), 156.3 (OCO-NHCys), 137.7, 137.3, 129.6, 128.7, 128.2, 128.0, 126.8 126.5 (ZC₆H₅ and PheC₆H₅), 66.0 (CH₂-O), 57.5 and 57.4 (α CHCys), 53.9 and 53.0 (α CHPhe), 37.3 and 37.0 (β CH₂Phe), 26.8 and 26.7 (β CH₂Cys)

IR (nujol) ν_{\max} 3314 (NH), 2570 (SH), 1719 (COOH), 1669 cm⁻¹ (CO)

ES MS [M-H]⁻ m/z 401

Z-L-Cys-L-PheOH, Z-D-Cys-D-PheOH

¹H-NMR δ_H (DMSO) 8.21 (1 H, d, NHPhe, *J* 7.7), 7.36 (6 H, m, NHCys and ZAr-H), 7.23 (5 H, m, PheAr-H), 5.04 (2 H, s, CH₂-O), 4.44 (1 H, m, α CHPhe),

4.14 (1 H, m, α CHCys), 3.00 and 2.68 (4 H, m, β CH₂Cys and β CH₂Phe), 2.25 (1H, m, SHCys)

¹³C-NMR δ_c (DMSO) 172.6 (COOH), 170.0 (CO-NHPhe), 155.9 (OCO-NH(Cys)₂), 137.3, 136.9, 129.1, 128.3, 128.1, 127.8, 127.7, 126.4 (ZC₆H₅ and PheC₆H₅), 65.5 (CH₂-O), 57.1 (α CHCys), 53.5 (α CHPhe), 36.5 (β CH₂Phe), 26.3 (β CH₂Cys)

IR Z-L-Cys-L-Phe-OH (nujol) ν_{\max} 3312 (NH), 2569 (SH), 1717 (COOH), 1692 and 1658 cm⁻¹ (CO)

IR Z-D-Cys-D-Phe-OH (nujol) ν_{\max} 3314 (NH), 2567 (SH), 1715 (COOH), 1693 and 1658 cm⁻¹ (CO)

ES MS Z-L-Cys-L-Phe-OH and Z-D-Cys-D-Phe-OH [M-H]⁻ m/z 401

mp 140-146 °C

Z-L-Cys-D-PheOH, Z-D-Cys-L-PheOH

¹H-NMR δ_H (DMSO) 8.28 (1 H, d, NHPhe, *J* 8.2), 7.34 (6 H, m, NHCys and ZAr-H), 7.23 (5 H, m, PheAr-H), 5.04 (2 H, s, CH₂-O), 4.49 (1 H, m, α CHPhe), 4.15 (1 H, m, α CHCys), 2.99 and 2.52 (4 H, m, β CH₂Cys and β CH₂Phe), 2.03 (1 H, m, SHCys)

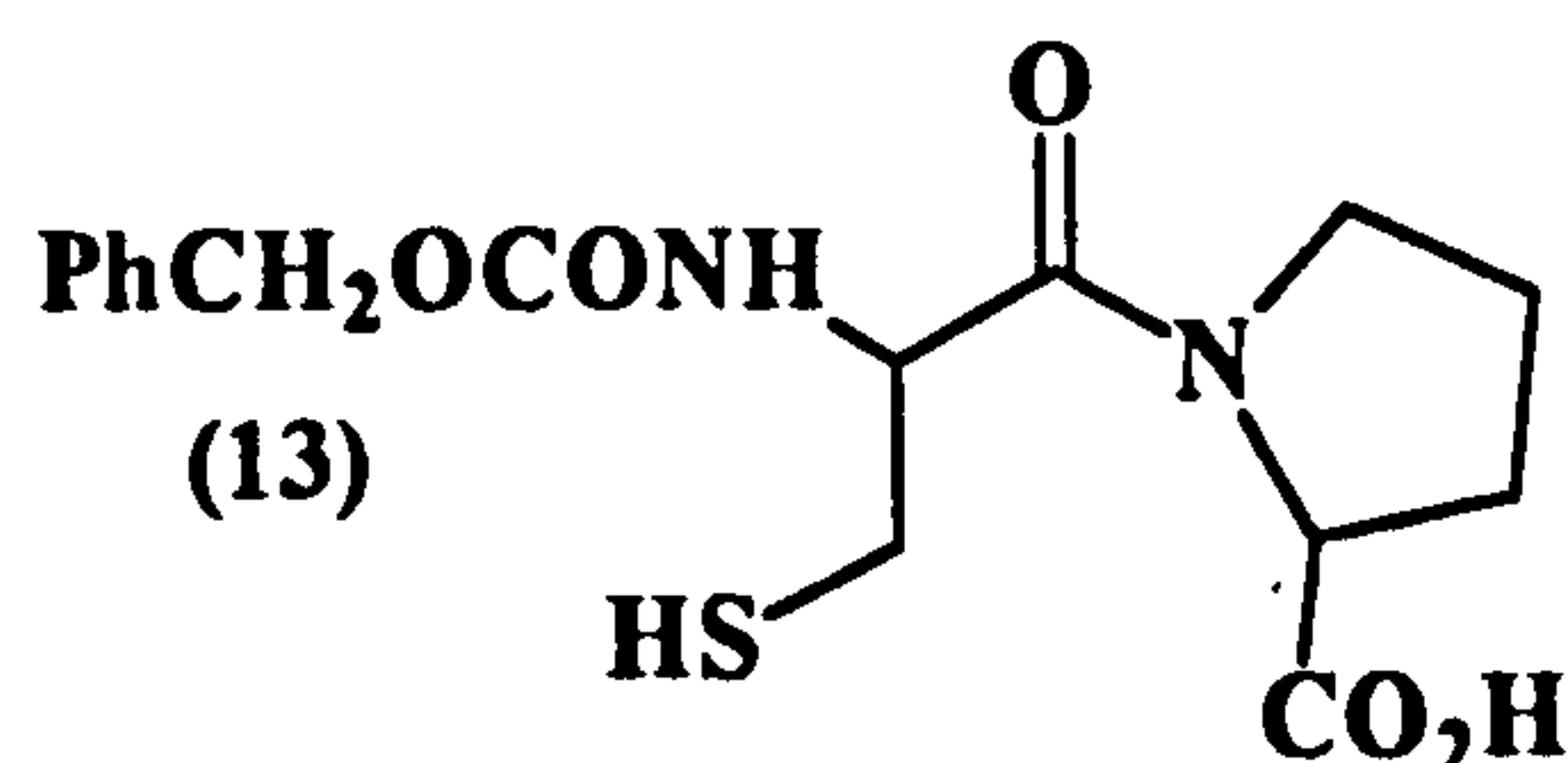
¹³C-NMR δ_c (DMSO) 172.3 (COOH), 169.7 (CO-NHPhe), 155.9 (OCO-NHCys), 137.3, 136.9, 129.2, 128.4, 128.2, 127.9, 127.8, 127.7 (ZC₆H₅ and PheC₆H₅),

65.76 (CH₂-O), 57.0 (αCHCys), 53.3 (αCHPhe), 36.9 (βCH₂Phe), 26.5 (βCH₂Cys)

IR **Z-L-Cys-D-Phe-OH** (nujol) ν_{\max} 3308 (NH), 2568 (SH), 1718 (COOH), 1669 cm⁻¹ (CO)

ES MS **Z-L-Cys-D-Phe-OH** and **Z-D-Cys-L-Phe-OH** [M-H]⁻ m/z 401

mp 118-122 °C

2.1.13 Synthesis of *N*-carbobenzoxy-*L*-cysteinyl-*DL*-proline (13)**(*Z*-*L*-Cys-*DL*-ProOMe)₂**

¹³C-NMR δ_c (DMSO) 172.6 (COOCH₃), 168.6 (CO-NHPro), 156.1 (OCO-NH(Cys)₂), 136.9, 128.4, 128.1, 127.9 (ZC₆H₅), 65.7 (CH₂-O), 58.8 (αCHPro), 51.8 (αCH(Cys)₂), 52.0 (OCH₃), 46.6 (δCH₂Pro), 39.8 (βCH₂(Cys)₂), 28.6 (βCH₂Pro), 24.5 (γCH₂Pro)

(*Z*-*L*-Cys-*DL*-ProOH)₂

¹³C-NMR δ_c (DMSO) 173.1 (COOH), 168.5 (CO-NHPro), 156.0 (OCO-NH(Cys)₂), 136.9, 128.4, 127.8, 127.7 (ZC₆H₅), 65.7 (CH₂-O), 58.8 (αCHPro), 51.7 (αCH(Cys)₂), 46.5 (δCH₂Pro), 39.8 (βCH₂(Cys)₂), 28.7 (βCH₂Pro), 24.5 (γCH₂Pro)

IR (nujol) ν_{max} 3296 (NH), 1716 (COOH), 1630 cm⁻¹ (CO)

ES MS [M-H]⁻ m/z 702

Z-L-Cys-DL-ProOH

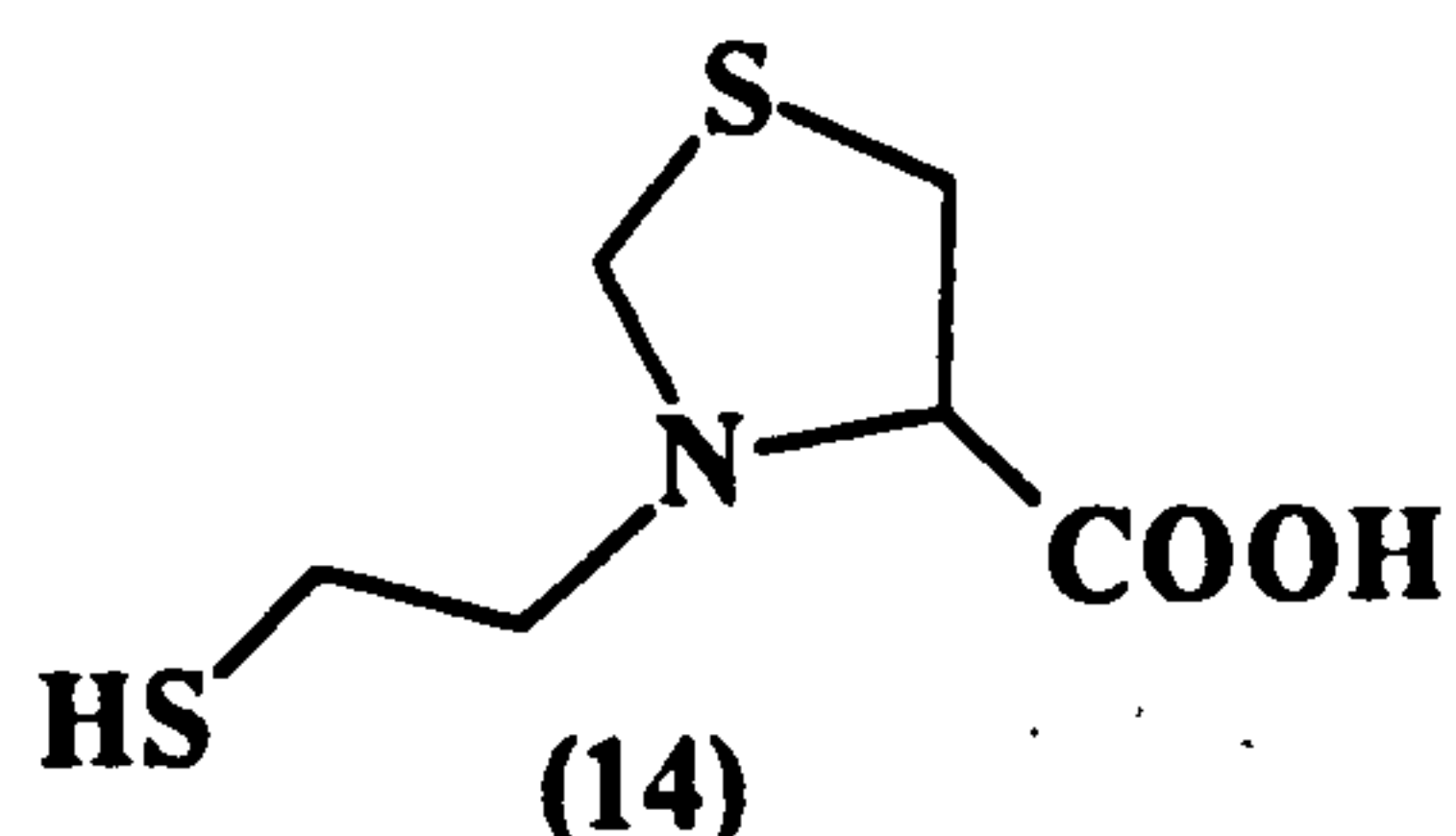
¹H-NMR δ_{H} (DMSO) 7.64 (1 H, m, NHPro), 7.34 (5 H, m, ZAr-H), 7.21 (5 H, m, PheAr-H), 5.05 (2 H, m, CH₂-O), 4.22 and 3.66 (2 H, m, α CHPro and α CHCys), 2.88, 2.70, 2.12 and 1.88 (8 H, m, β CH₂Cys and CH₂Pro)

¹³C-NMR δ_{C} (DMSO) 173.5, 173.4 (COOH), 169.1 (CO-NHPro), 156.2 (OCO-NHCys), 137.3, 127.7, 128.4, 128.1, 127.9 (ZC₆H₅), 65.9 (CH₂-O), 60.7 (α CHCys), 57.2, 56.9 (α CHPro), 46.9 (δ CH₂Pro), 29.1 (β CH₂Pro), 25.7 (β CH₂Cys), 24.4, 24.7 (γ CH₂Pro)

IR (nujol) ν_{max} 3295 (NH), 2606 (SH), 1716 (COOH), 1630 cm⁻¹ (CO)

ES MS [M-H]⁻ m/z 351

mp 60 - 65 °C

2.1.14 Synthesis of *N*-mercaptoethyl-4-*L*-thiazolidinecarboxylic acid (14)

The preparation of 4-*L*-thiazolidinecarboxylic acid methyl ester was undertaken according to the method of Ratner and Clarke¹²⁴.

A solution of 4-*L*-thiazolidinecarboxylic acid (0.093 moles) in 100 ml of methanol which had been saturated with dry hydrogen chloride, was refluxed for one hour. The solution was then evaporated under reduced pressure to obtain the hydrochloride of the methyl ester as a white solid, yield 94 %. To a suspension of the ester hydrochloride (0.087 moles) in 5 ml of water, covered with about 30 ml of ether, anhydrous potassium carbonate was added slowly in excess (1.2 mole-equivalent). The ether layer was separated and extraction of the aqueous layer with ether was repeated twice. The combined organic layers were dried over anhydrous sodium sulfate and the ether was evaporated under reduced pressure to obtain a colorless oil which was purified further by distillation under reduced pressure. Yield 70 %.

¹H-NMR δ_H (DMSO) 4.13 and 4.04 (2 H, dd, γCH_2 , J_{AB} 9.0), 4.02 (1 H, m, αCH), 3.64 (3 H, s, OCH_3), 3.33 (1 H, m, NH), 3.04 and 2.84 (2 H, two q, βCH_2 , J_{AB} 10.3 J_{AX} 6.7 J_{BX} 6.0)

$^{13}\text{C-NMR } \delta_{\text{c}}$ (DMSO) 171.7 (COOCH_3), 64.8 (αCH), 52.2 (βCH_2), 52.0 (OCH_3), 36.0 (γCH_2)

IR (nujol) ν_{max} 3311 (NH) and 1740 cm^{-1} (COOMe)

A mixture of equimolar amount of 4-L-thiazolidinecarboxylic acid methyl ester (0.061 moles) and ethylenesulfide (0.061 moles) in a glass lined bomb was heated for eighteen hours at 85°C . The unreacted ethylene sulfide was then evaporated under reduced pressure to give a colorless oil, with a ratio of 3 to 1 of product/starting material. Overall yield 80 %. Attempts to separate the product from the starting material were not successful at this stage and further reactions were followed without further purification.

$^1\text{H-NMR } \delta_{\text{H}}$ (DMSO) 4.30 (1 H, m, αCH), 4.01 (2 H, m, γCH_2), 3.63 (3 H, s, OCH_3), 3.08 and 2.80 (2 H, m, βCH_2), 2.60 (4 H, m, 2 CH_2), 2.36 (1 H, t, SH, J 7.3)

$^{13}\text{C-NMR } \delta_{\text{c}}$ (DMSO) 173.1 (COOCH_3), 69.5 (αCH), 58.8 (γCH_2), 56.9 (CH_2), 52.1 (OCH_3), 31.9 (βCH_2), 23.2 (CH_2SH)

IR (nujol) ν_{max} 2552 (SH), 1736 cm^{-1} (COOMe)

ES MS $[\text{M}+\text{H}]^+$ m/z 208

The product was dissolved in 50 ml of methanol. The mixture was cooled to $0 - 10^\circ\text{C}$, (10.2 mmoles) of lithium hydroxide in water (17 ml) was added dropwise to the stirred solution. After 18 hours reaction 50 ml of water were

added, and the methanol was subsequently removed by evaporation. The aqueous solution was then extracted with ethyl acetate. The aqueous layer was then acidified to pH 1 with dilute hydrochloric acid and extracted twice with ethyl acetate. The pH of the aqueous solution was then adjusted to pH 7 and the water was removed by lyophilisation. The product was then dissolved in methanol. The insoluble fraction was filtered and acetone was added to the methanol. A yellow product precipitated upon addition of the acetone and was filtered. NMR of the precipitate revealed the presence of compound (14) as well as others such as the 4-L-thiazolidinecarboxylic acid.

Attempt to separate the mixture by column chromatography was not successful as the compounds decomposed. The mixture was solubilised in ethanol and addition of chloroform induced the formation of a yellow precipitate. NMR of the precipitate showed that the main compound was the disulfide of compound (14). However ESMS of the product showed the presence of other compounds (results not shown).

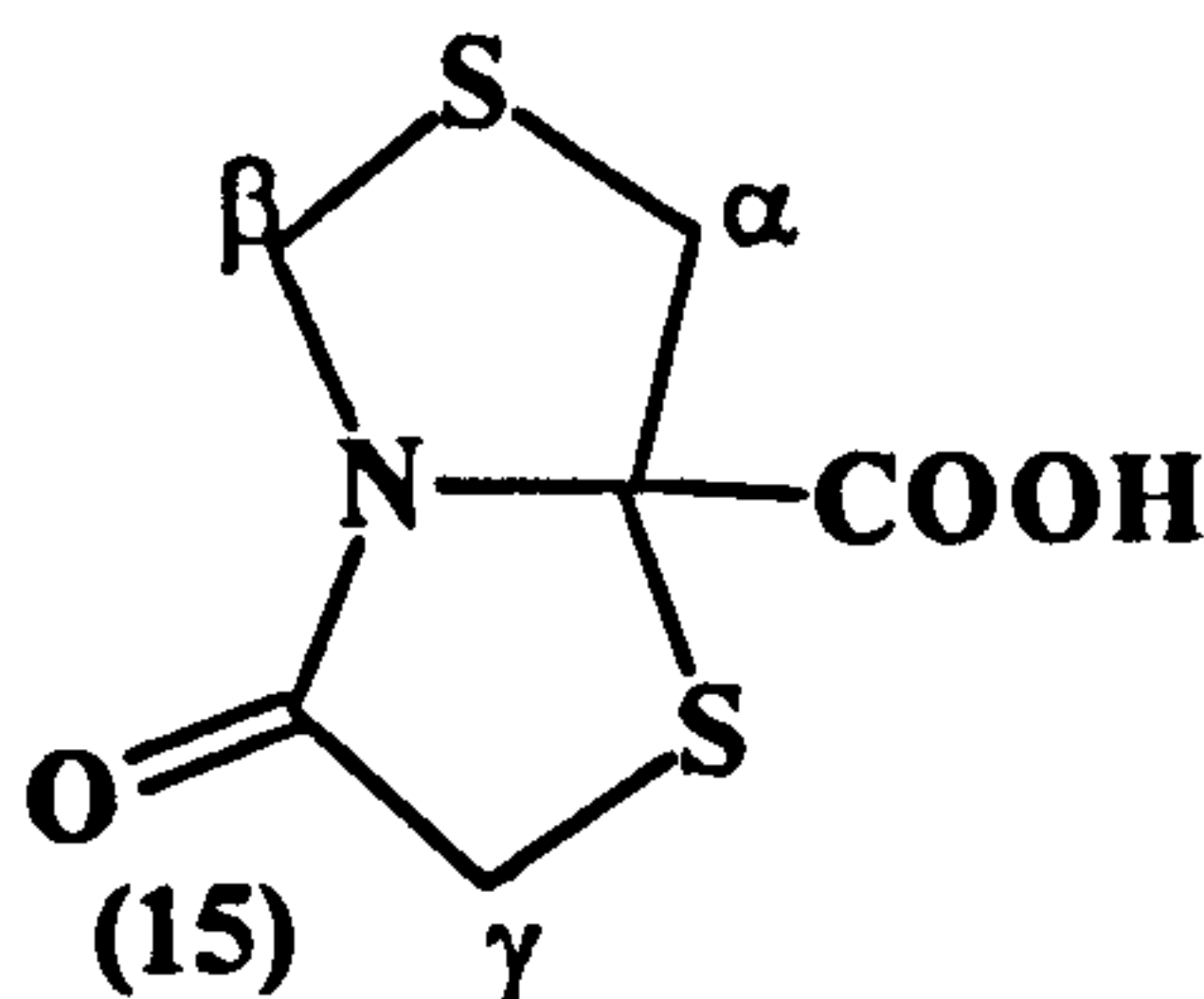
$^1\text{H-NMR } \delta_{\text{H}} (\text{D}_2\text{O})$ 4.76 and 4.33 (2 H, two d, CH_2 , J_{AB} 10.4), 4.59 (1 H, m, CH), 3.80, 3.69, 3.58, 3.40 and 3.11 (6 H, m, CH_2)

$^{13}\text{C-NMR } \delta_{\text{C}} (\text{D}_2\text{O})$ 170.3 (COOH), 70.3 (CH), 58.1 (CH_2), 54.4 (CH_2), 33.0 (CH_2), 32.4 (CH_2)

ES MS $[2\text{M}+\text{H}]^+$ m/z 385

The product was reduced using sodium borohydride. The yellow precipitate was dissolved in 20 ml of ethanol. To this solution at 67 - 70 °C was added dropwise to a solution containing 4 equivalents of sodium borohydride in 10 ml of ethanol. The stirred reaction mixture was heated at 75 - 80 °C for one hour. After cooling to 25 °C, the solution was added to 50 ml of iced water. To this stirred solution, 6 M hydrochloric acid was added dropwise at 0 - 10 °C until pH 3 was obtained. The acidic solution was then evaporated under reduced pressure. The mixture proved to be very difficult to separate and pure (14) was not obtained. No further studies on the compound were carried out.

2.1.15 Synthesis of 3-oxoperhydro[1.3]thiazolo[4.3-b][1.3]thiazole-7a-carboxylic acid (15)



A solution of dithiodiglycolic acid (6 mmoles) in dichloromethane (50 ml), cooled to 0 °C, was treated with dicyclohexylcarbodiimide (12 mmoles) and with a solution of 4-L-thiazolidinecarboxylic acid methyl ester (12.6 mmoles) in dichloromethane (50 ml). The mixture was stirred at 0 °C for 1 hour and then for 24 hours at room temperature. The precipitated dicyclohexylurea was filtered, the filtrate was washed twice with 1 M sulfuric acid (30 ml) and twice with 5 % sodium bicarbonate (30 ml). The organic layer was dried over anhydrous sodium sulfate and evaporated to dryness in vacuo. The residue was dissolved in acetone, the insoluble part was removed by filtration and the solvent evaporated to give a white product. The product of the reaction (5 mmoles) was dissolved in 50 ml of methanol. The mixture was cooled to 0 - 10 °C, a solution of lithium hydroxide (10.2 mmoles) in water (17 ml) was added dropwise to the stirred solution. After 18 hours, 50 ml of water were added, and the methanol was subsequently removed by evaporation. The solution was then washed twice with diethyl ether (20 ml) to remove any starting material. The aqueous phase was acidified to pH 2

with dilute hydrochloric acid and the product separated as a yellow precipitate which was filtered, washed with water, and dried *in vacuo*. Yield 60 %.

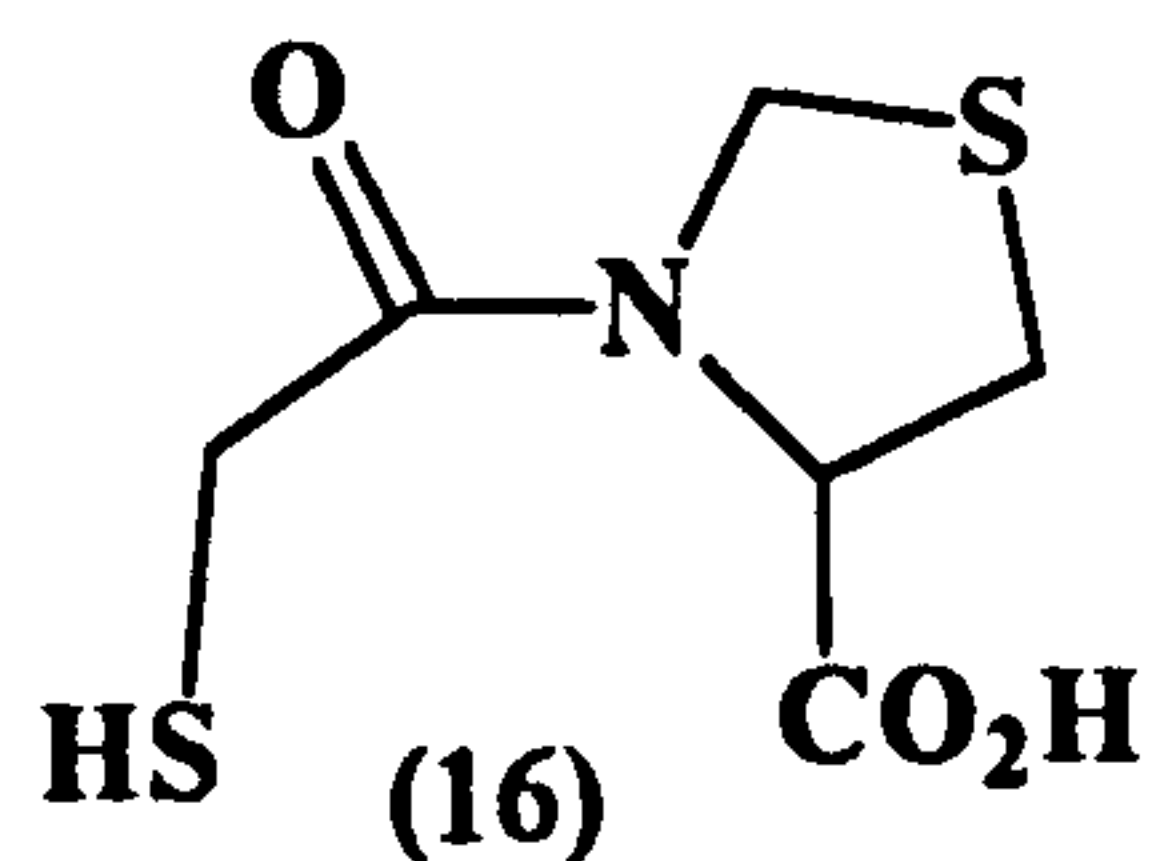
$^1\text{H-NMR}$ δ_{H} (DMSO) 4.66 and 4.06 (2 H, two d, γCH_2 , J_{AB} 8.7), 4.05 and 3.88 (2 H, two d, βCH_2 , J_{AB} 15.5), 3.55 and 3.12 (2 H, two d, αCH_2 J_{AB} 10.7)

$^{13}\text{C-NMR}$ δ_{C} (DMSO) 170.4 (COOH), 169.4 (CO-NH), 73.8 (Cq), 43.6 (γCH_2), 38.7 (αCH_2), 36.3 (βCH_2)

IR (nujol) ν_{max} 1734 (COOH), 1646 cm^{-1} (CONH)

ES MS $[\text{M-H}]^-$ m/z 204, $[2\text{M-H}]^-$ m/z 409

mp 206-208 °C

2.1.16 Synthesis of *N*-mercaptoacetyl-4-thiazolidinecarboxylic acid (16)

The procedure was based on the method of Oya *et al.*¹²⁵.

Benzoylation of thioglycolic acid

To 50 ml of 1 M sodium hydroxide and 20 ml of ether, thioglycolic acid (33 mmoles) and benzoyl chloride (39 mmoles) were added. While the temperature was kept between 0 and 5 °C, 5.4 g of potassium bicarbonate was added in 4 portions over a period of 10 minutes with vigorous stirring. The reaction was left stirring for a further 20 minutes at room temperature. The ether layer was removed and the solution extracted again with ether (2 x 20 ml). The solution was then acidified with 6 M hydrochloric acid and the white precipitate filtered off and washed with water to remove all unreacted thioglycolic acid. Yield 80 %. The product was used without further purification.

¹H-NMR δ_H (DMSO) 7.75 (5 H, m, C₆H₅), 3.91 (2 H, s, CH₂)

¹³C-NMR δ_C (DMSO) 190.2 (CO-S), 169.6 (COOH), 134.0, 129.2, 129.1, 127.1, 126.(C₆H₅), 31.53 (CH₂)

mp 88 - 90 °C

S-benzoylmercaptoacetic acid (27 mmol) was dissolved in 20 ml of dichloromethane, cooled to 0 °C and was treated with dicyclohexylcarbodiimide (27 mmol). After 10 minutes 4-L-thiazolidinecarboxylic acid (1 equivalent) was added to the solution. The mixture was stirred at room temperature for 2 hours. The precipitated dicyclohexylurea was filtered off and the filtrate washed twice with sodium bicarbonate (30 ml). The aqueous solution was acidified with 6 M hydrochloric acid. The separated oil was extracted into ethyl acetate. The extracts were washed with water (3 x 10 ml), dried over anhydrous sodium sulfate and concentrated under reduced pressure to give a yellow oil. Yield 20 %. The product was used without further purification.

ES MS [M-H]⁻ m/z 310

S-benzoylmercaptoacetyl-4-thiazolidine carboxylic acid (3 mmol) was treated with 28 % aqueous ammonia (30 ml) and the mixture was stirred for 1 hour at room temperature. The excess ammonia was removed *in vacuo* and the by-product, benzamide was extracted into ethyl acetate (3 x 10 ml). The aqueous layer was acidified with dilute hydrochloric acid and extracted with ethyl acetate (3 x 20 ml). The extracts were washed with water until neutral pH, dried over anhydrous sodium sulfate and concentrated *in vacuo* to give a colorless oil. Yield 50 %. The product was purified by preparative TLC (ethyl acetate/chloroform, 2:1). The NMR of the isolated fraction showed that the sample was very impure.

2.2 Kinetic Studies

2.2.1 Material

Deionised water was used for preparation of buffers and other aqueous solutions. D₂O, NaOD, DCl were obtained from Goss Scientific Ltd. Cephaloridine was supplied by Glaxochem Research group. Most buffers, benzylpenicillin, and cefuroxime were purchased from Sigma.

The buffers used were acetate (pK_a 4.75), MES (pK_a 6.15), MOPS (pK_a 7.20), TAPS (pK_a 8.40), CAPSO (pK_a 9.60), hexafluoroisopropanol (pK_a 9.30) and CAPS (pK_a 10.4). The ionic strength was maintained at a constant level with sodium chloride or potassium chloride (AnalaR grade).

Kinetic studies were carried out using the class B β -lactamase enzyme from *Bacillus cereus* 569/H which was kindly supplied by Professor Jean-Marie Frère, University of Liège, Belgium. The enzyme was isolated using the following procedure:

The strain used was BL21/pET-BcII, the part of the gene coding for the mature protein was cloned after the T7 promoter. The vector used was pET9a. The gene was introduced between the NdeI and BamHI sites. The size of the plasmid is equal to 5.2 kbases.

Production of the enzyme

The bacteria was grown on a liquid LB media (Lennox L Broth Base) containing 50 µg/ml Kanamycin. An overnight preculture of BL21/pET-BcII at 37 °C under orbital agitation was prepared. The culture (30 ml) was inoculated in fresh media (1 l) containing the selection agent (kanamycin 50 µg ml⁻¹). The culture was incubated at 37 °C under orbital agitation. When the culture reached an optical density of 0.6 at a wavelength of 600 nm, isopropyl-β-D-thiogalactopyranoside (IPTG) was added (1 mM final concentration), which induced the production of BcII. The culture was then incubated for a further three hours at 37 °C.

Purification

The culture was centrifuged at 5000 rpm for 15 minutes. The supernatant was discarded and the pellet resuspended in Hepes buffer (50-100 ml, 10 mM, pH 7.5, 50 µM ZnCl₂, 1/20 to 1/10 of the culture volume). The cells were disrupted by French press, sonication or cell disrupter. The cell debris were removed and the solution was centrifuged for 30 minutes at 20000 g. The supernatant was dialyzed overnight against 100 volume of Hepes buffer (same composition). After dialysis, the solution was loaded on a S-Sepharose fastflow column (Pharmacia, LKB Biotechnology) previously equilibrated with the Hepes buffer. After an extensive washing of the column with the buffer, the elution was carried out with a linear gradient of NaCl in the same buffer. The salt concentration at the end of the gradient was 0.6 M at which the enzyme was eluted. The fractions containing the

enzyme were pooled and concentrated by ultrafiltration (Amicon). The purity of the sample was evaluated by SDS-PAGE and specific activity. The yield of this step was around 95 % and the purity was about 90 %. Where a more homogeneous solution was required the sample was dialyzed overnight against the Hepes buffer in order to eliminate the salt. The dialysed sample was then applied on a Mono S-Sepharose HR5/5 column (Pharmacia, LKB Biotechnology) previously equilibrated with the Hepes buffer. After washing, elution of the enzyme was performed using a linear salt gradient, with the same solution used for the first purification step. The active fractions were pooled and concentrated. The second purification step gave a pure and homogenous enzyme solution.

2.2.2 Equipment

UV-Vis wavelength scans were performed using a Cary 1/3 (Varian) spectrophotometer. The temperature was kept constant at 30 °C with a water circulator and a peltier system.

The pHs of solutions were measured using either a JENWAY 3020 or a Beckman Φ 40 pH meter fitted with Ag/AgCl, or Hg/Hg₂Cl₂ Russel Semi-micro electrode, which were calibrated prior to use.

2.2.3 Kinetic assays

Generally, 25 μ l of a stock solution of substrate was injected into 2.5 ml of buffer solution (0.05M, I = 1 M, 30 °C, unless otherwise stated) and stirred. 25 μ l of a

stock solution of enzyme was then added to the system. In general, the substrate and enzyme concentrations in the cell were 0.12 to 1.1 mM and 0.04-2.0 μ M respectively. The experiment was repeated by adding more substrate at the end of the reaction.

Hydrolysis of the substrate was followed by measuring the decrease in absorbance, at 260 nm for cephaloridine and cefuroxime, and at 230 nm for benzylpenicillin, as a function of time. Unless otherwise stated, for stability reasons, zinc sulfate was added to the stock solution of enzyme, at a concentration which was 10 fold greater than the enzyme concentration.

Assuming that enzyme catalysis follows standard Michaelis-Menten kinetics, the kinetic constants were generally measured by following the entire course of the reaction. Below saturation, $[S] \ll K_m$, the curves were fitted to a simple first order rate law to obtain the pseudo-first-order rate constants k_{obs} which were shown to be first order in enzyme concentration and independent of substrate concentration. The second-order rate constant k_{cat}/K_m was obtained by dividing k_{obs} by the concentration of enzyme used. Complete time curves were studied because of increased accuracy and there was no evidence of either product inhibition or enzyme denaturation. Repeat kinetics runs by injecting more substrate into the cell after the first reaction was complete gave identical pseudo-first-order rate constants. Furthermore, rate constants obtained from initial rate studies also gave identical rate constants. Above saturation, when the substrate concentration was too high for complete UV assays, the initial rates were determined with low

substrate concentrations and analysed according to the Hanes equation below. Hanes plot was preferred over other treatments plots such as Lineweaver-Burk because it does not give such misleading values of experimental errors¹²⁶.

$$\frac{[S]}{v} = \frac{[S]}{V_{\max}} + \frac{K_m}{V_{\max}}$$

Unless otherwise indicated, the reported rate constants are within 5 % of the stated value.

Curve fittings were achieved using ENZFITTER (Elsevier Biosoft, Cambridge, UK) or SCIENTIST (MicroMath Scientific Software, Utah, USA) software.

2.2.3.1 Effects of buffer concentration and ionic strength on β -lactamase II activity

The effect of the buffer concentration [over the range 0.010 - 0.200 M] on the β -lactamase II catalysed hydrolysis of cephaloridine, was measured with MOPS and phosphate buffers at 30 °C and pH 7.0. The enzyme concentration used was 0.1 μ M and substrate cephaloridine 0.14 mM. The ionic strength of the buffer was maintained at 1.0 M by varying the concentration of sodium chloride.

The effect of the ionic strength [over the range 0.17 - 1.50 M] on the β -lactamase II catalysed hydrolysis of cephaloridine, was measured at 30 °C and pH 7.0 with MOPS and phosphate buffer (0.01M). The enzyme concentration used was 0.1 μ M

and substrate cephaloridine 0.13 mM. The ionic strength of the buffer was kept constant by adjusting with the calculated amount of sodium chloride.

2.2.3.2 *pH/pD dependence of enzyme activity*

The pH dependence of the rate of the β -lactamase II catalysed hydrolysis of cephaloridine, benzylpenicillin and cefuroxime was studied. The buffers used were acetate (pH 4.0-5.3), MES (pH 5.6-6.5), MOPS (pH 7.0-8.0), TAPS (pH 8.0-9.4), CAPSO (pH 9.1-10.0), hexafluoroisopropanol (pH 9.0-10.5) and CAPS (pH 10.1-11) at 30°C, 0.05 M and $I = 1.0$ M (NaCl). The concentration of enzyme used was 0.04-2.0 μ M and the concentration of zinc was 0.48-40 μ M. The substrate concentration was 0.12 mM for cephaloridine, 1.10 mM for benzylpenicillin and 0.28 mM for cefuroxime. The zinc concentration used was 0.8-40 μ M.

For solvent kinetic isotope effect experiments, the buffers were prepared in D₂O, and adjusted with NaOD or DCl. The pD was measured using the following equation : $pD = pH \text{ meter reading} + 0.40^{127}$.

2.2.3.3 *Effect of zinc concentration on the β -lactamase II activity*

The effect of zinc concentrations on the rate (k_{cat}/K_m) of the β -lactamase II catalysed hydrolysis of benzylpenicillin was studied over a range of pH [4 - 7]. The enzyme activity was determined by monitoring the β -lactamase II catalysed hydrolysis of benzylpenicillin (0.9 mM) at 30 °C, in buffer solution (0.05 M, $I = 1$ M) containing various concentrations of zinc sulfate. The stability of the

substrate was tested, prior to the assay, in the presence of the metal ion concentration used for the enzymatic assays.

The zinc ion content of the β -lactamase II stock solution was determined by atomic absorption spectrometry on a Perkin Elmer AAnalyst 100 Atomic Absorption Spectrometer. The hollow cathode lamp wavelength was set at 213.9 nm, with a current of 7 mA, and a slit width of 0.7 nm. Zinc nitrate solutions of different concentrations were used as standards.

2.2.3.4 Methanolysis Studies

The effect of the methanol concentration on the first order rate constant k_{cat} for the β -lactamase II catalyses hydrolysis of cefuroxime was studied at pH 7.0 (MOPS buffer 0.05M, ionic strength 1M (KCl), 30 °C). The cefuroxime concentration used was 0.29 mM, the enzyme concentration was 0.05 μ M and the methanol concentration varied from 0-1 M. The experiment was repeated at pH 7.0 (MOPS buffer 0.05M, ionic strength 1M (KCl), 30 °C), methanol concentration 1 M, with 8.1 mM cefuroxime and 2 μ M enzyme. The hydrolysis product was analysed with mass spectrometry. The mixture was extracted twice with ethyl acetate after adjusting the pH to 2.5 with 0.1 M hydrochloric acid. Ethyl acetate was evaporated and the product analysed by ESMS. No methyl ester of the substrate was detected.

2.2.3.5 Inhibition Studies

The compounds synthesised for this work and described in the first part of the experimental section were tested as inhibitors of β -lactamase II. Due to solubility problems in aqueous solution, the stock solutions of the compounds were prepared in ethanol.

Unless otherwise stated, 25 μ l of solution of inhibitor at a given concentration, were added to 2.5 ml of buffer containing 25 μ l of a given concentration of substrate. The stability of the substrate in the presence of inhibitor was checked prior to the injection of the enzyme to the system. The time dependence of the inhibition was studied either by incubating the enzyme with inhibitor for different times before adding the substrate or by adding more substrate at the end of the reaction. Zinc sulfate was also added to the mixture in order to investigate metal chelating reactions, and restoration of the enzyme activity. The reproducibility was checked by repeating the assay with different concentrations of inhibitor.

The kinetic parameters were determined from the second-order rate constant k_{cat}/K_m . Inhibition constants K_i were calculated using the equations for competitive inhibition:

$$\text{Rate} = \frac{k_{cat} \cdot [E] \cdot [S]}{[S] + K_m \left(\frac{K_i + [I]}{K_i} \right)}$$

$$\left(\frac{k_{cat}}{K_m}\right)_I = \left(\frac{k_{cat}}{K_m}\right)_O \cdot \left(\frac{K_i}{[I] + K_i}\right)$$

Equation 2.1

where $\left(\frac{k_{cat}}{K_m}\right)_I$ = second-order rate constant with inhibitor

and $\left(\frac{k_{cat}}{K_m}\right)_O$ = second-order rate constant without inhibitor

if $\alpha = \left(\frac{k_{cat}}{K_m}\right)_I / \left(\frac{k_{cat}}{K_m}\right)_O$ then $\alpha = \left(\frac{K_i}{[I] + K_i}\right)$

and $K_i = \left(\frac{\alpha}{1 - \alpha}\right) \times [I]$

$$\left(\frac{K_m}{k_{cat}}\right)_I = \left(\frac{K_m}{k_{cat}}\right)_O + \left(\frac{K_m}{k_{cat}}\right)_O \left(\frac{[I]}{K_i}\right)$$

Equation 2.2

Values of K_i were also obtained directly from the transformation of Equation

2.1 to Equation 2.2 and by plotting $\left(\frac{K_m}{k_{cat}}\right)_I$ against $[I]$ to give an intercept on

the inhibitor concentration axis of - K_i .

Results and discussion

3. Results and discussion

3.1 Dependence of enzyme activity on buffer concentration and ionic strength.

The effect of buffer species on enzyme kinetics has been previously reported¹²⁸ and can lead to inactivation or activation of the enzyme due to the reaction of the buffer with the substrate or the enzyme. Some proteins denature in pure water and require a moderate ionic strength for the stability of its active conformation^{129,130} and so, the effect of salt concentration is also important¹³¹.

A careful evaluation of these parameters on the enzyme catalysis is therefore necessary. The effect of buffer concentration and ionic strength on the second-order rate constant k_{cat}/K_m for the β -lactamase II catalysed hydrolysis of cephaloridine was studied with both MOPS and phosphate buffers, whilst keeping all other variables constant.

3.1.1 Effect of buffer concentration on β -lactamase II activity

The effect of buffer concentration on the second-order rate constant, k_{cat}/K_m , for the β -lactamase II catalysed hydrolysis of cephaloridine was studied with MOPS and phosphate buffers, pH 7.0, 30 °C, at a constant ionic strength of 1.0 M (maintained with NaCl). The values of k_{cat}/K_m are listed in Table 3-1.

Table 3-1 Effect of buffer concentration on (k_{cat}/K_m) for the β -lactamase II catalysed hydrolysis of cephaloridine, pH 7.0, 30 °C, enzyme 0.1 μ M

<i>Buffer Concentration (M)</i>	<i>MOPS k_{cat}/K_m ($M^{-1} \cdot s^{-1}$)</i>	<i>Phosphate k_{cat}/K_m ($M^{-1} \cdot s^{-1}$)</i>
0.010	8.02×10^4	7.77×10^4
0.025	8.56×10^4	6.81×10^4
0.050	7.92×10^4	6.66×10^4
0.075	7.37×10^4	6.08×10^4
0.100	6.47×10^4	4.21×10^4
0.150	6.22×10^4	4.61×10^4
0.200	6.05×10^4	-

Figure 3.1 shows graphically the influence of the buffer concentration on k_{cat}/K_m for β -lactamase II catalysed hydrolysis of cephaloridine. The activity of the enzyme is influenced by the buffer concentration at fixed pH. An increase in the concentration of both MOPS (o) and phosphate (+) buffers induces a decrease in the rate of hydrolysis: a 50 % decrease in activity is observed when using 0.15 M phosphate buffer, compared with that at zero buffer concentration. The effect of the buffer concentration on the rate of hydrolysis is more pronounced with phosphate than with MOPS buffer, which may be due to a small inhibitory action of this buffer on the enzyme activity. Such effects have already been observed for carboxypeptidase which is similarly inhibited by phosphate ions¹²⁸. It is difficult to propose a definitive physical explanation for this decrease in activity, but it does

demonstrate the importance of controlling buffer concentration when making comparisons in activity under different sets of conditions.

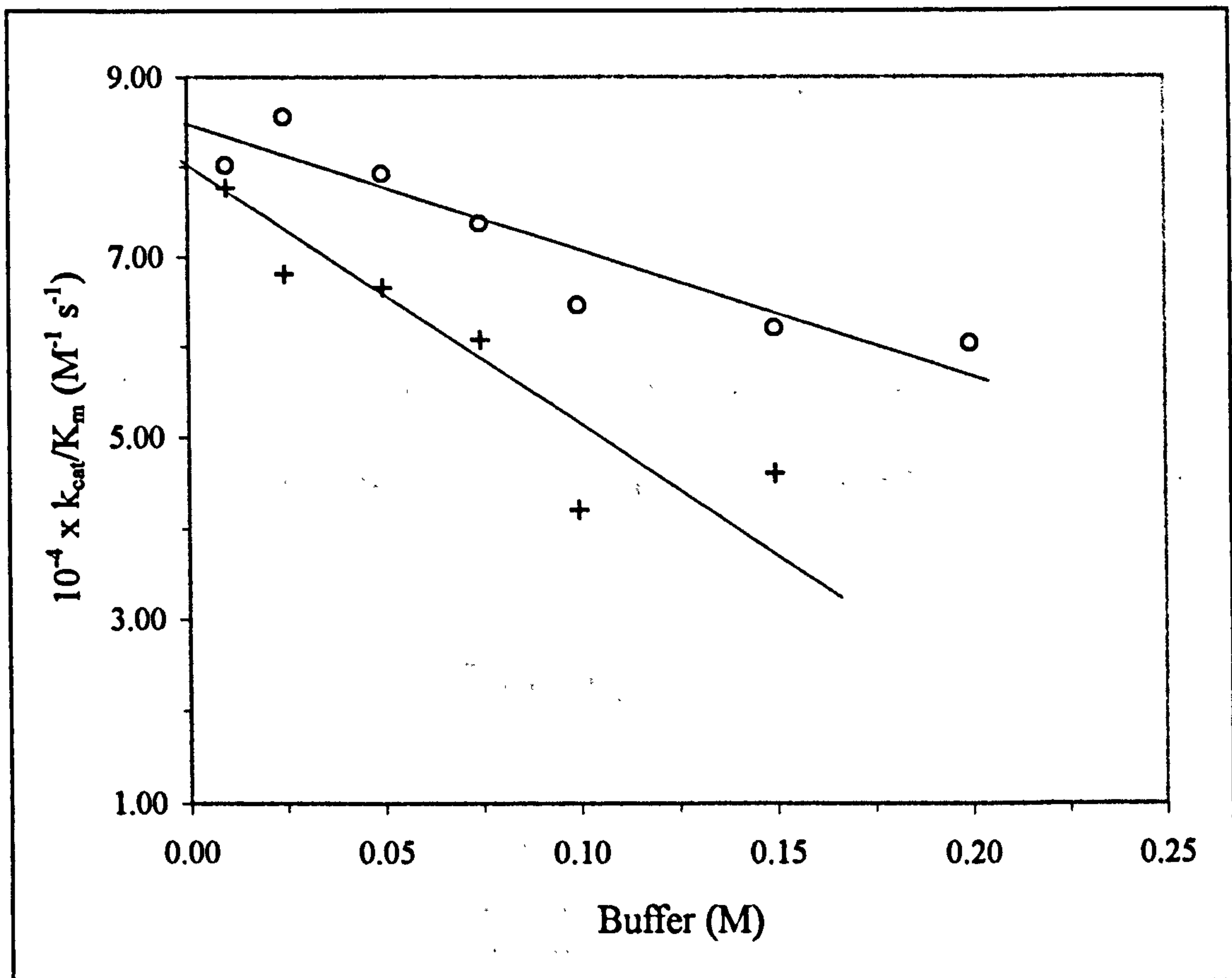


Figure 3.1 Dependence on buffer concentration for the rate constant (k_{cat}/K_m) of the β -lactamase II catalysed hydrolysis of cephaloridine (o MOPS, + phosphate), pH 7.0, 30 °C, enzyme 0.1 μM

3.1.2 Effect of ionic strength on β -lactamase II activity

The ionic strength dependence of the β -lactamase II activity with cephaloridine as the substrate has been studied using MOPS and phosphate buffers (0.01 M), at pH 7.0 and at 30 °C, over the range 0.15-1.50 M ionic strength, varied with NaCl. The results are shown in Table 3-2.

Table 3-2 Effect of ionic strength on the rate constant (k_{cat}/K_m) for β -lactamase II catalysed hydrolysis of cephaloridine, pH 7.0, 30 °C, enzyme 0.1 μ M

<i>Ionic Strength (M)</i>	<i>Buffer MOPS $k_{cat}/K_m (M^{-1} \cdot s^{-1})$</i>	<i>Buffer Phosphate $k_{cat}/K_m (M^{-1} \cdot s^{-1})$</i>
0.17	4.73×10^4	3.81×10^4
0.50	5.48×10^4	4.63×10^4
1.00	7.01×10^4	6.51×10^4
1.50	7.43×10^4	8.11×10^4

The effect of varying the ionic strength with NaCl on the second-order rate constant (k_{cat}/K_m) of β -lactamase II catalysed hydrolysis of cephaloridine is shown graphically in Figure 3.2. The activity of the enzyme increases with increasing ionic strength. A two fold increase in the value of the second-order rate constant k_{cat}/K_m is observed when increasing the ionic strength from 0.01 to 1.0 M. Many enzymes have been found to be influenced by the ionic strength, which can affect their

stability and solubility as well as their catalytic activity^{130,132,133}. The influence of ionic strength is complex and it is not very clear exactly how the reaction rate is affected, because several factors may be involved:

Non specific salt effects as counterions - the addition of salts reduces the interaction between charged species¹³⁴, the presence of counterions around charged groups weakens these interactions and may, for example, change the binding interactions between the enzyme and substrate or even increase the availability of the enzyme by decreasing binding of the enzyme to the charged glass cell¹³⁵.

Specific salt effects on protein folding: increases in ionic strength usually alter primarily electrostatic interactions in enzyme such as salt bridges or functionally linked ion binding¹³⁶. A high ionic strength may stabilise the enzyme through changes in the charge distribution involved in maintaining the active conformation of the protein^{137,138,139}. Salts may also affect the solubility of the enzyme (salting in/salting out effect on enzyme)^{140,141}.

Ionic strength may also favour substrate-enzyme interaction by decreasing the solubility of the substrate in the salted buffer.

The effect observed here is probably a generalized salt effect rather than a specific ion binding since it is relatively small. The effect of the NaCl concentration on the activity of *A. hydrophilia* metallo- β -lactamase seems different from that observed here for *B. cereus* β -lactamase II. For the former enzyme, a decrease in k_{cat}/K_m

values was observed when increasing NaCl concentrations⁷⁶, but this effect was not explained.

Based on the results of these studies, most subsequent experiments were undertaken using MOPS buffer at 0.05 M concentration and at constant ionic strength of 1 M.

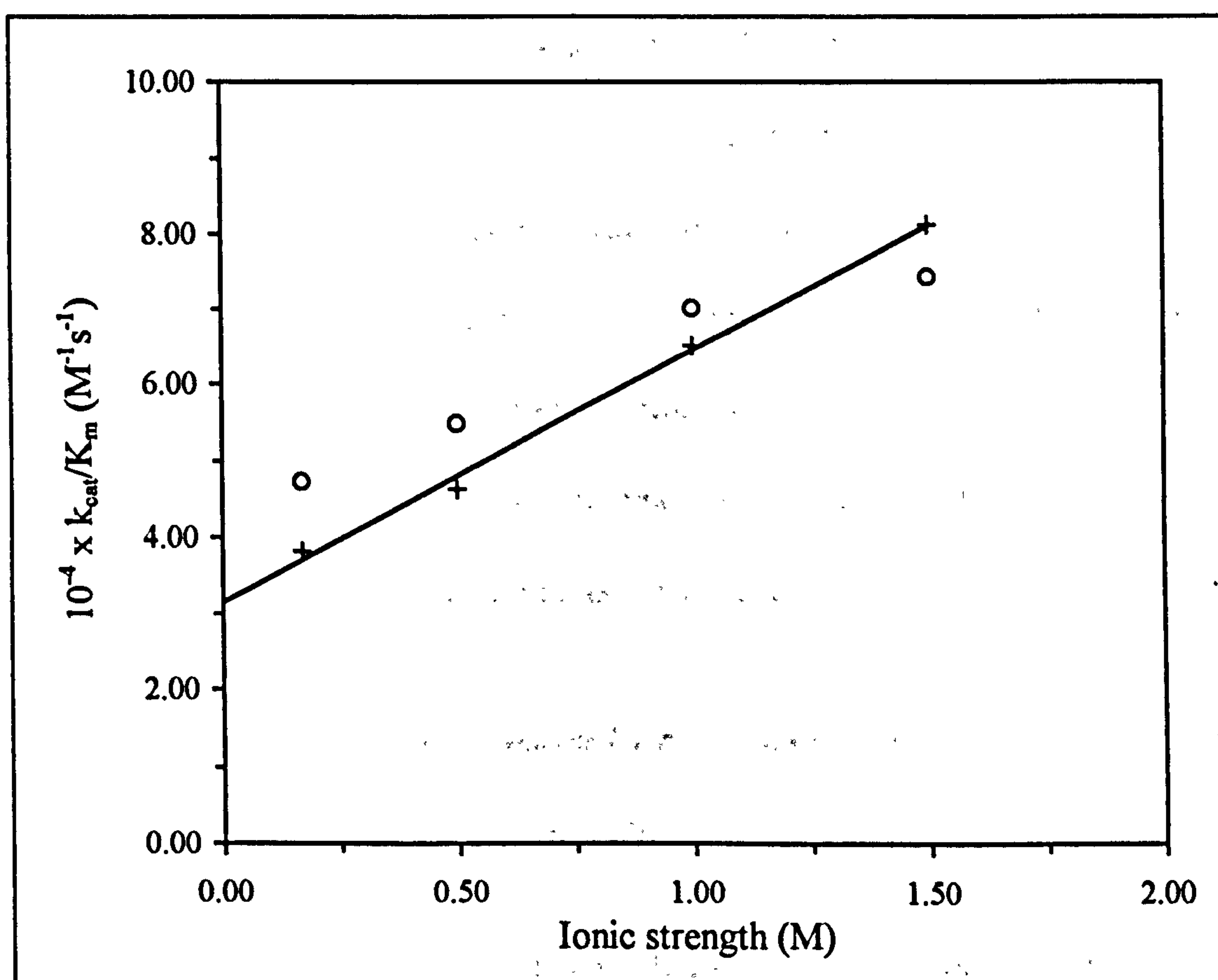


Figure 3.2 Dependence on ionic strength for the rate constant (k_{cat}/K_m) of the β -lactamase II catalysed hydrolysis of cephaloridine (o MOPS, + phosphate), pH 7.0, 30 °C, enzyme 0.1 μM

3.2 pH dependence of the rate of hydrolysis

In order to help determine the catalytic mechanism of β -lactamase II, the pH dependence of kinetic parameters was measured over the pH range of 4-11. The pH dependencies of k_{cat}/K_m for the β -lactamase II catalysed hydrolysis of cephaloridine and benzylpenicillin are shown in Figure 3.3 and Figure 3.4, respectively. The pH dependence of k_{cat} for the β -lactamase II catalysed hydrolysis of cefuroxime is shown in Figure 3.5. To ensure enzymic activity at extreme pH values, the enzyme concentrations used were high at these pH values and subsequently the zinc concentration was also higher and the concentration varied along the pH rate profile. The interpretation of the pH rate profile was made difficult at this stage as the effect of the zinc concentration on the pH rate profile (see chapter 3.4, page 131) was not taken into account at the time of these studies.

3.2.1 pH dependence of the second-order rate constant (k_{cat}/K_m) of hydrolysis of cephaloridine catalysed by β -lactamase II

The values of k_{cat}/K_m for the β -lactamase II catalysed hydrolysis of cephaloridine were determined by observing the first-order disappearance of the substrate at different pHs (Table 3-3).

Table 3-3 pH dependence of k_{cat}/K_m for the β -lactamase II catalysed hydrolysis of cephaloridine at 30 °C and ionic strength 1.0 M (NaCl). Enzyme concentration 0.1-2 μ M, zinc concentration 2-40 μ M

<i>pH</i>	$k_{cat}/K_m (M^{-1}.s^{-1})$	<i>pH</i>	$k_{cat}/K_m (M^{-1}.s^{-1})$
4.23	1.33×10^2	5.25	3.77×10^4
4.23	1.82×10^2	5.25	3.77×10^4
4.47	1.24×10^3	5.60	4.88×10^4
4.55	1.15×10^3	6.00	5.47×10^4
4.67	1.52×10^3	6.50	5.99×10^4
4.75	6.55×10^3	7.00	5.60×10^4
4.78	1.44×10^3	7.50	5.73×10^4
4.78	1.84×10^3	8.00	5.83×10^4
4.78	2.15×10^3	8.50	6.31×10^4
5.04	2.45×10^3	9.23	6.37×10^4
5.05	8.36×10^3	9.52	4.59×10^4
5.06	4.94×10^3	9.75	2.61×10^4
5.06	4.25×10^3	10.00	1.92×10^4
5.06	7.14×10^3	10.24	1.34×10^4
5.24	1.29×10^4	10.50	8.31×10^3

The values of $\log k_{cat}/K_m$ are plotted as a function of the pH in Figure 3.3. The pH dependence of $\log k_{cat}/K_m$ for the β -lactamase II catalysed hydrolysis of

cephaloridine is bell-shaped, with a slope of 2 below pH 5.5 and a slope of -1 above pH 9.5. These results indicate that there are at least three catalytically essential ionisations of the free enzyme, two acidic groups with very similar pK_a values on the acidic pH limb and one base on the basic pH limb.

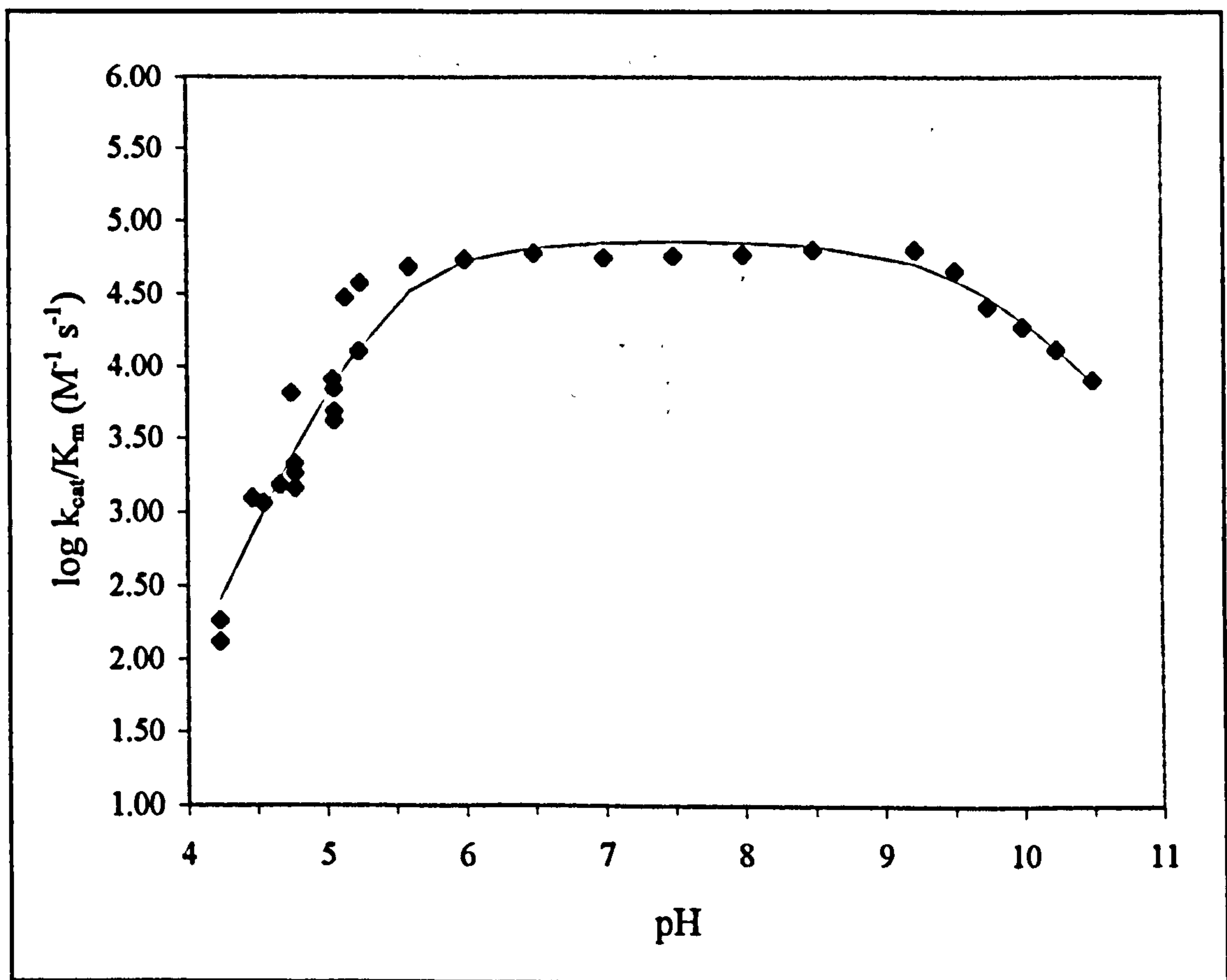


Figure 3.3 Dependence on pH for the second-order rate constant ($\log k_{\text{cat}}/K_m$) of the β -lactamase II catalysed hydrolysis of cephaloridine, 30 °C, ionic strength 1.0 M (NaCl)

The calculated kinetic parameters and the apparent ionisation constants are shown in Table 3-4. It was not possible to distinguish between pK_{a1} and pK_{a2} , the two ionisation constants corresponding to the acid limb of the pH-rate profile, from the experimental data.

Table 3-4 Kinetic parameters of the pH dependence of the β -lactamase II catalysed hydrolysis of cephaloridine, at 30 °C and ionic strength 1.0 M (NaCl)

<i>Substrate</i>	<i>$k_{cat}/K_m (M^{-1} s^{-1})$</i>	<i>$pK_{a1}=pK_{a2}$</i>	<i>pK_{a3}</i>
Cephaloridine	$6.76 (\pm 0.4) \times 10^4$	$5.64 (\pm 0.05)$	$9.64 (\pm 0.1)$

3.2.2 pH dependence of the second-order rate constant (k_{cat}/K_m) of hydrolysis of benzylpenicillin catalysed by β -lactamase II

The pH dependency of k_{cat}/K_m for the β -lactamase II catalysed hydrolysis of benzylpenicillin was studied over the pH range 4.2-10.5 (Table 3-5).

Table 3-5 pH dependence of k_{cat}/K_m for the β -lactamase II catalysed hydrolysis of benzylpenicillin at 30 °C and ionic strength 1.0 M (NaCl).

Enzyme concentration 0.1-2 μ M, zinc concentration 2-40 μ M

<i>pH</i>	$k_{cat}/K_m (M^{-1}.s^{-1})$	<i>pH</i>	$k_{cat}/K_m (M^{-1}.s^{-1})$
4.25	2.59×10^2	8.00	3.67×10^5
4.50	9.74×10^2	8.50	2.96×10^5
5.00	3.20×10^3	9.00	2.60×10^5
5.25	3.40×10^4	9.23	2.64×10^5
5.25	1.94×10^4	9.40	1.61×10^5
5.60	1.22×10^5	9.51	1.60×10^5
5.65	1.17×10^5	9.52	1.88×10^5
5.79	1.35×10^5	9.75	1.16×10^5
6.00	3.88×10^5	9.85	9.67×10^4
6.00	1.29×10^5	10.00	8.40×10^4
6.50	3.62×10^5	10.07	4.72×10^4
7.00	3.24×10^5	10.24	4.42×10^4
7.50	4.0×10^5		

The plot of $\log k_{\text{cat}}/K_m$ against pH for the β -lactamase II catalysed hydrolysis of benzylpenicillin as the substrate (Figure 3.4) is also a bell-shaped curve with again a slope of 2 below pH 6 and a slope of -1 above pH 9. The pK_a values measured $pK_{a1} = pK_{a2} = 5.83 \pm 0.05$ and $pK_{a3} = 9.30 \pm 0.1$ (Table 3-6) were very similar to those obtained for the β -lactamase II catalysed hydrolysis of cephaloridine. The similar observations for two different substrates indicate that an intrinsic property of the enzyme is being reflected. The kinetic parameter k_{cat}/K_m changes with the ionisation states of the free enzyme. In order to make comparisons with the ionisation dependency of the enzyme-substrate complex, a substrate was chosen which easily saturates the enzyme making it easy to measure the value of k_{cat} .

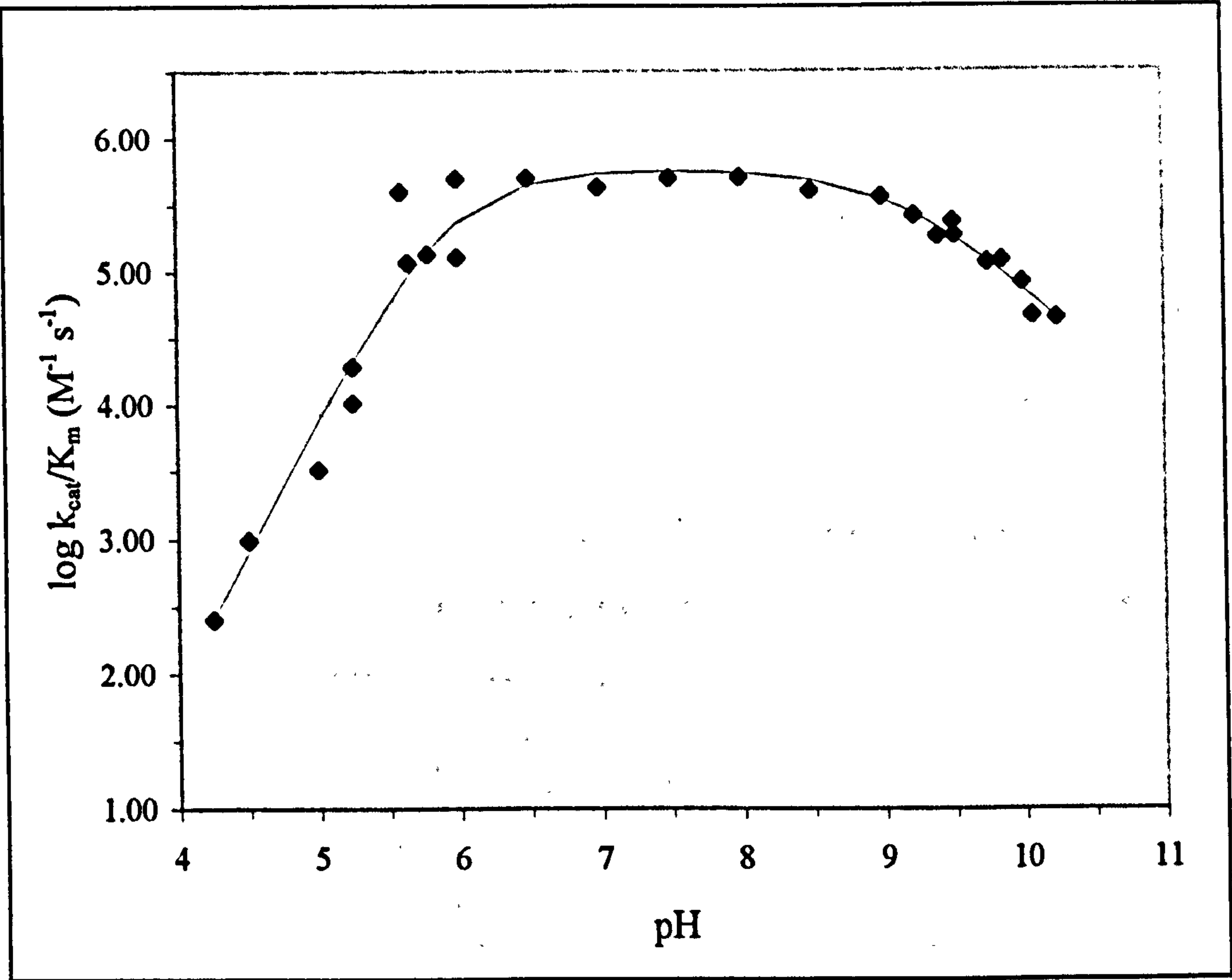


Figure 3.4 Dependence on pH for the rate constant ($\log k_{cat}/K_m$) of the β -lactamase II catalysed hydrolysis of benzylpenicillin, 30 °C, ionic strength 1.0 M (NaCl)

Table 3-6 Kinetic parameters of the pH dependence of the β -lactamase II catalysed hydrolysis of benzylpenicillin, at 30 °C and ionic strength 1.0 M (NaCl)

Substrate	k_{cat}/K_m ($M^{-1} s^{-1}$)	$pK_{a1}=pK_{a2}$	pK_{a3}
Benzylpenicillin	$4.27 (\pm 0.6) \times 10^5$	$5.83 (\pm 0.05)$	$9.30 (\pm 0.1)$

3.2.3 pH dependence of the first-order rate constant (k_{cat}) of hydrolysis of cefuroxime catalysed by β -lactamase II

The pH dependence of k_{cat} for the β -lactamase II catalysed hydrolysis of cefuroxime was investigated (Table 3-7). As the K_m value for the β -lactamase II catalysed hydrolysis of cefuroxime was quite small ($45 \pm 5 \mu\text{M}^{34}$), k_{cat} values were easily determined from initial rate measurements under saturation conditions.

Table 3-7 pH dependence of k_{cat} for the β -lactamase II catalysed hydrolysis of cefuroxime at 30 °C and ionic strength 1.0 M (NaCl). Enzyme concentration 0.1-2 μM , zinc concentration 2-40 μM

<i>pH</i>	$k_{cat} (s^{-1})$	<i>pH</i>	$k_{cat} (s^{-1})$
4.29	0.03	5.95	17.48
4.52	0.12	6.05	39.66
4.75	0.33	6.14	23.82
5.04	2.76	6.25	25.42
5.28	5.78	6.50	42.44
5.51	7.89	7.01	33.04
5.77	14.02	7.49	26.58
5.77	10.89	8.00	44.30
5.77	13.15	8.48	45.70
5.84	33.30	9.02	39.90
5.84	28.50	9.50	35.28
5.84	24.60	10.00	21.80
5.95	16.90	10.50	6.92
5.95	18.80		

Figure 3.5 shows the plot of $\log k_{\text{cat}}$ against pH from the data in Table 3-7. A bell-shaped curve was obtained with again a slope of 2 at low pH and a slope of -1 at high pH. The pH dependence of k_{cat} reflects the ionisation of the enzyme-substrate complex which appears, from these results, to be governed by the same ionisations as those in the free enzyme.

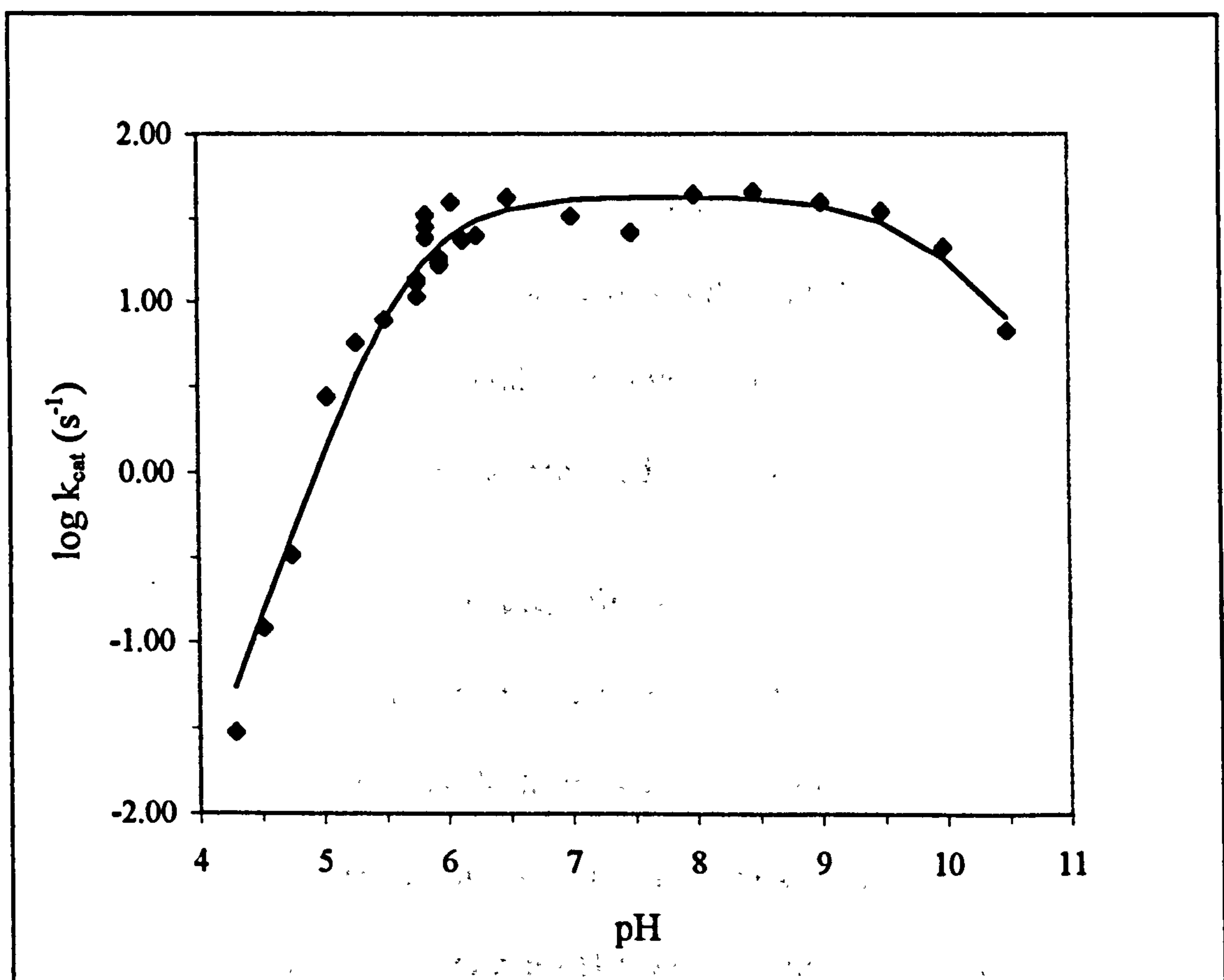


Figure 3.5 Dependence on pH for the rate ($\log k_{\text{cat}}$) of the β -lactamase II catalysed hydrolysis of cefuroxime, 30 °C, ionic strength 1.0 M (NaCl)

The calculated kinetic parameters and corresponding ionisation constants are shown Table 3-8.

Table 3-8 Kinetic parameters of the pH dependence of the β -lactamase II catalysed hydrolysis of cefuroxime, at 30 °C and ionic strength 1.0 M (NaCl)

<i>Substrate</i>	$k_{cat} (s^{-1})$	$pK_{a1}=pK_{a2}$	pK_{a3}
Cefuroxime	35 (\pm 5)	5.73 (\pm 0.05)	9.87 (\pm 0.1)

3.2.4 Discussion

The pH dependency of k_{cat} reflects the ionisation of the enzyme-substrate complex, but more precisely those of the amino acids residues of the enzyme involved in the rate-determining step in the overall reaction¹⁴², whereas the pH dependence of k_{cat}/K_m reflects the reactions of uncomplexed substrate or enzyme.

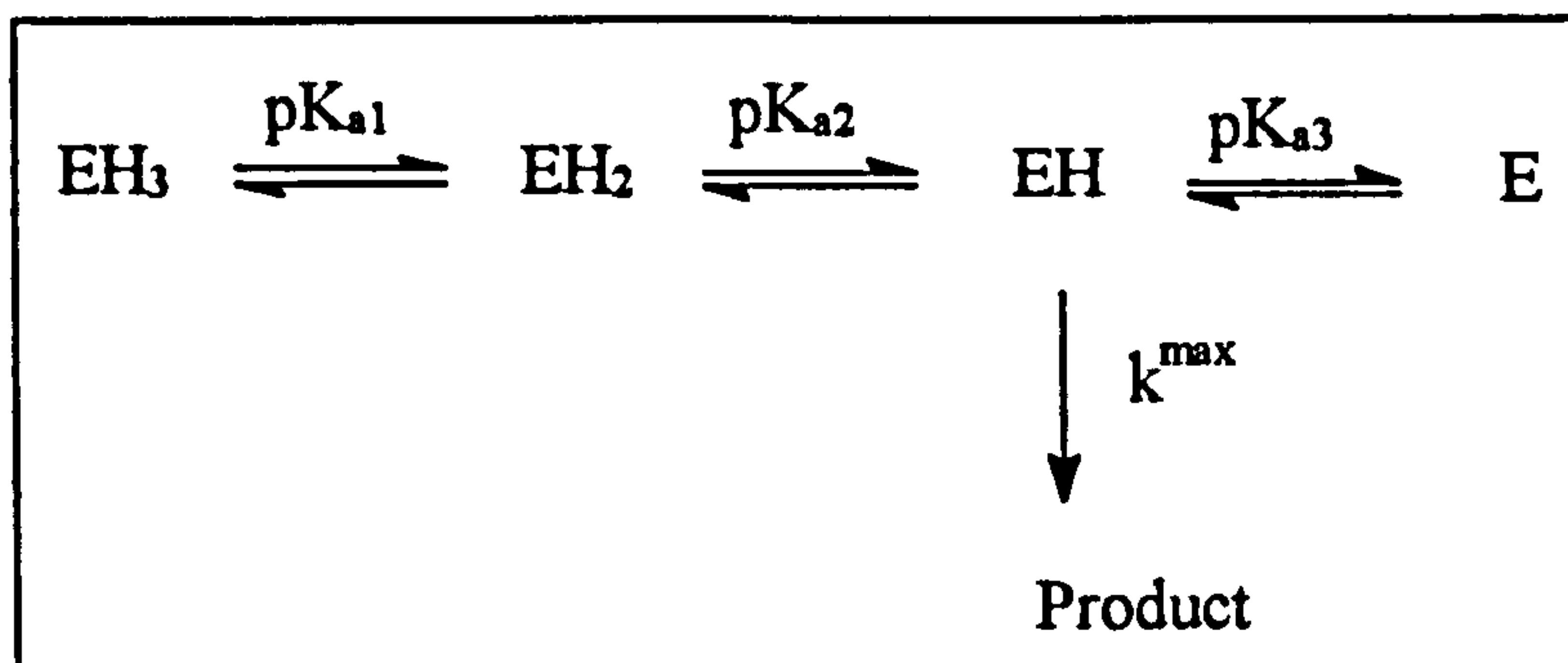
The pH dependence of $\log k_{cat}/K_m$ for the hydrolysis of cephaloridine and benzylpenicillin are shown Figure 3.3 (page 98) and Figure 3.4 (page 102). The pH dependence of $\log k_{cat}$ for the hydrolysis of cefuroxime is shown in Figure 3.5 (page 104). They all display a characteristic bell-shaped curves. Both k_{cat} and k_{cat}/K_m are pH independent between pH 6 and 9. The logarithmic plots decrease with a slope of -1.0 at higher pH values but, suprisingly, with a slope of 2.0 at lower pH values. However, slopes of 2 for pH-rate profiles have been observed for other enzymes at low pH¹⁴³.

The fact that similar behaviour is seen for three different substrates and for both $k_{\text{cat}}/K_{\text{m}}$ and k_{cat} shows that this is an intrinsic property of the enzyme and not the substrate.

These results indicate the presence of at least three ionisable groups essential for enzyme activity, two acidic groups on the low pH side with very similar pK_{a} values indiscernible from 5.6 ± 0.2 , and one on the high pH side with a pK_{a} of 9.5 ± 0.2 . The observed pK_{a} values must necessarily be the intrinsic pK_{a} values of groups on the enzyme, since the same pK_{a} values are obtained from the pH-rate profile of the hydrolysis of different substrates. Furthermore, identical pK_{a} values were measured from the pH dependence of K_{i} for the thiol (1) (chapter 3.6.2.1), which further corroborates these findings.

To ensure that the rate constant measured was not compromised by loss of activity at low pHs (due to enzyme degradation or denaturation), the stability of the enzyme was determined at these pHs. The enzyme loses no more than 10 % of the original activity when repeated hydrolysis reaction are performed, by adding more substrate after completion of the first hydrolysis. Furthermore, the enzyme was left at pH 4.0 for the corresponding time to that required for complete hydrolysis of the substrate, and its activity was then checked by changing the pH to 7.0. The rate constants measured for hydrolysis were identical to those obtained under normal conditions at pH 7.0. Circular dichroism spectra were recorded with the β -lactamase II at pH 4.5¹⁴⁴, which showed no change indicative of apparent denaturation of the enzyme at this pH.

The scheme for the ionisation of the free enzyme which is consistent with the pH rate profiles is shown in Scheme 3-1, where the active form of the enzyme is EH.



Scheme 3-1

Below saturation the observed pseudo first order rate constant, k_{obs} , varies with pH according to Equation 3.1, which generates a bell-shaped pH-rate dependency. At high pH, K_{a1} and K_{a2} are much greater than (H^+) and k_{obs} is given by $k^{\max}/(1+K_{a3}/(\text{H}^+))$. The inflection at high pH thus corresponds to K_{a3} . At low pH, two inflections should theoretically be seen and at pHs below pK_{a1} the rate constant would show an inverse second order dependence on (H^+) concentration. A logarithmic plot of k_{obs} against pH would thus show a slope of 2 at low pH. However, if pK_{a1} and pK_{a2} are close then it is difficult to experimentally distinguish between them.

$$k_{\text{obs}} = \frac{k^{\max}}{(\text{H}^+) / K_{a2} + (\text{H}^+)^2 / K_{a1}K_{a2} + 1 + K_{a3} / (\text{H}^+)}$$

Equation 3.1

To illustrate the difficulty in distinguishing two close pK_a values, theoretical curves were calculated using Equation 3.1, with different ΔpK_a s and plotted in Figure 3.6,

Figure 3.7 and Figure 3.8. It is clear from these plots that even when the two low pK_a values are separated by two pK_a units it is not easy to evaluate the two values accurately.

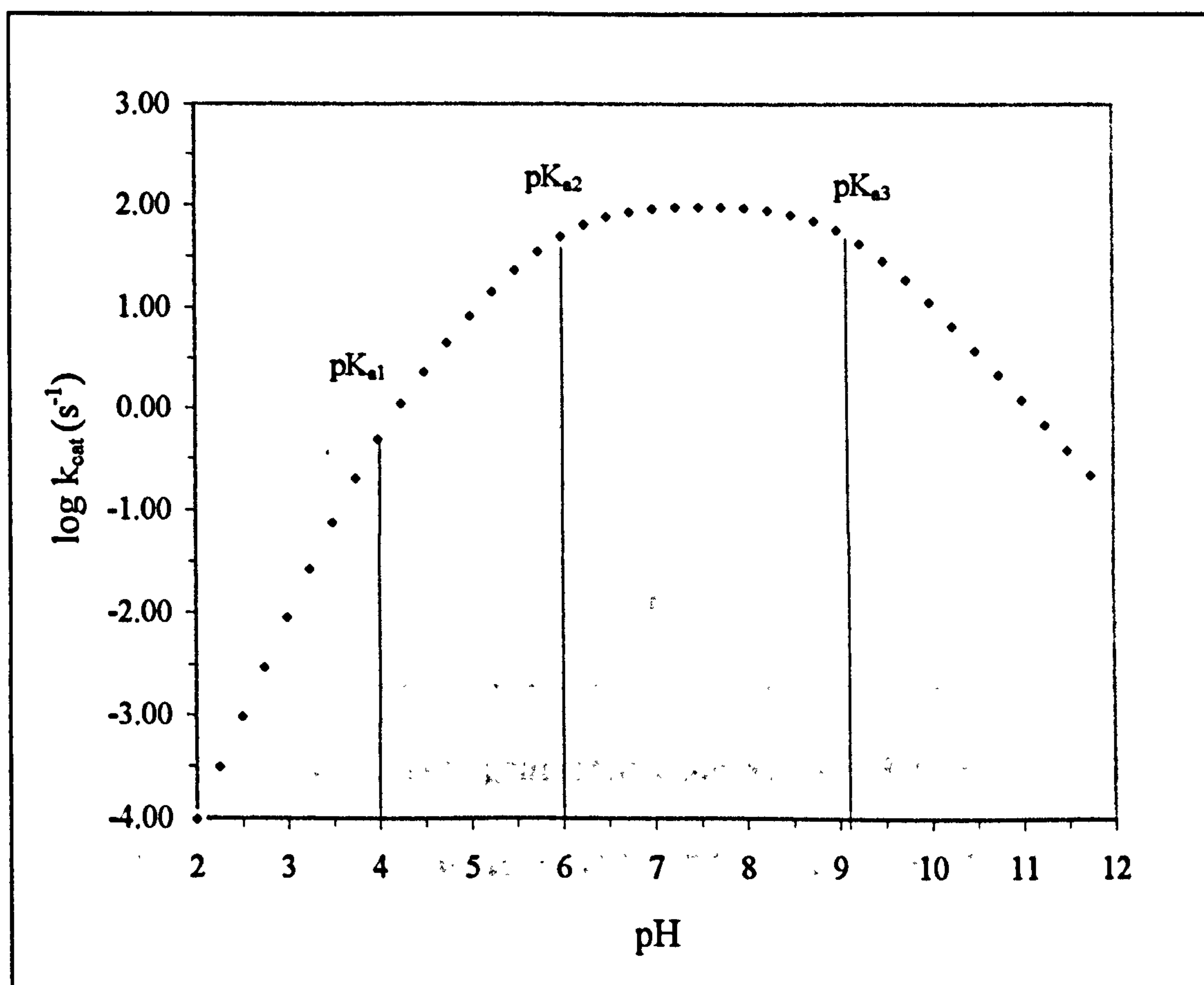


Figure 3.6 Theoretical curves generated from Scheme 3-1 and Equation 3-1.

Parameters used: $pK_{a1} = 4$, $pK_{a2} = 6$, $pK_{a3} = 9.1$, $\log k^{max} = 2.0$

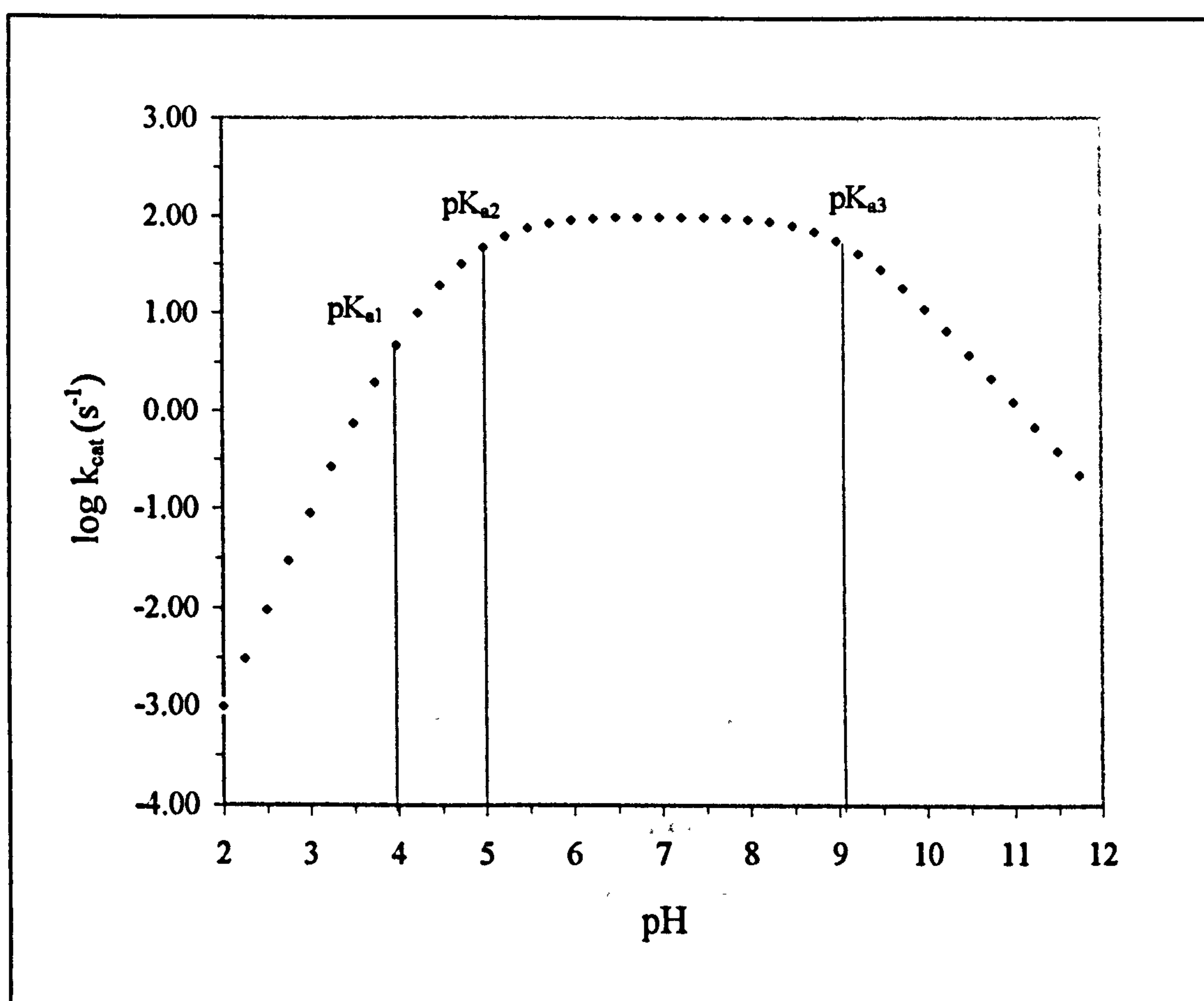


Figure 3.7 Theoretical curves generated from Scheme 3-1 and Equation 3-1.

Parameters used: $\text{pK}_{\text{a}1} = 4$, $\text{pK}_{\text{a}2} = 5$, $\text{pK}_{\text{a}3} = 9.1$, $\log k^{\text{max}} = 2.0$

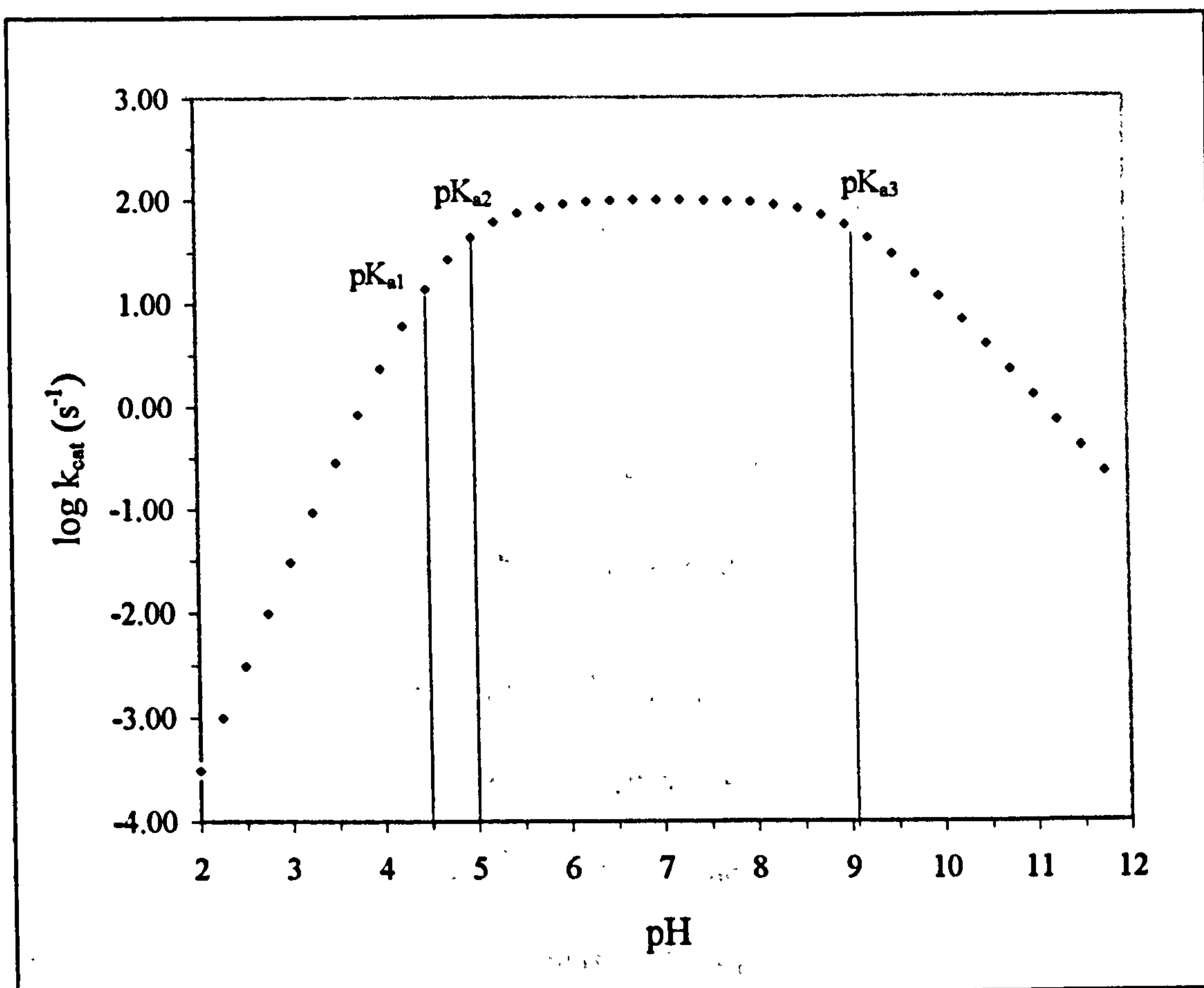


Figure 3.8 Theoretical curves generated from Scheme 3-1 and Equation 3-1.
Parameters used : $\text{pK}_{\text{a}1} = 4.5$, $\text{pK}_{\text{a}2} = 5$, $\text{pK}_{\text{a}3} = 9.1$, $\log k^{\text{max}} = 2.0$

The above plots demonstrate that if an enzyme has two catalytically important acid residues, the pK_{a} values are not easily distinguished unless separated by at least two pH units.

In the case of the β -lactamase II the activity of the enzyme is linked to the ionisation of two acidic groups both with pK_{a} values indistinguishable from 5.6 and of one basic group with a pK_{a} of 9.5. The assignment of these pK_{a} values to specific active site residues is still a matter of debate and is discussed below.

Earlier studies on pH dependence of the reaction of synthetic substrates with carboxypeptidase A, showed ionisation of two groups in the free enzyme, one with a pK_a of 6.4 which was attributed to a Glu, and the other one with a pK_a value of 9.4 which was assigned to zinc-bound water^{79,80}. However, mechanistic investigations led to different assignments for these inflections, with the low pK_a value being assigned to the zinc bound water^{145,146,147,90} and the high pK_a value to the Tyr 248 phenol group or to the His 196 residue^{148,149}.

The pK_a of hydrated zinc is about 9.6¹⁵⁰, however, it is known that coordination of a water molecule to a metal centre in enzymes significantly lowers its pK_a , with reported values as low as 5-6^{151,152,153}, this is mainly due to the environment of the zinc ion in the enzyme and to the Lewis acid properties of the metal.

Carbonic anhydrase is a mono zinc metallo-enzyme which catalyses the reversible hydration of CO_2 to HCO_3^- . The zinc ion is coordinated by three histidine residues and a water molecule which has a pK_a of about 6^{153,154,155,156}.

The pK_a of the zinc-bound water in the binuclear zinc metallo- β -lactamase from *B. fragilis* is reported to be 6.2 for the free enzyme¹⁰⁸. However upon binding to the substrate, this pK_a value is thought to be reduced to 4.2, mainly because of a more favourable deprotonation of the zinc-bound water to generate a negative charge which is better stabilised by the positive charge of the two zinc ions⁶⁰.

By analogy with the other metallo-enzymes, we propose that one of the acid groups responsible for the low pK_a value (5.6) observed for the pH dependence of

β -lactamase II catalysed hydrolysis of β -lactams reflects ionization of the zinc-bound water. This proposal is substantiated by the pH dependency of the inhibition of β -lactamase II by thiol (1) reported later in chapter 3.6.2.1. The other low pK_a value may reflect the ionisation of an essential amino acid residue.

Aspartate 90 is a highly conserved amino-acid in metallo β -lactamase sequences⁶⁰, which is located in the active site at 2.6 Å from the zinc-bound water of the β -lactamase II⁵⁸. It has been suggested that it has a catalytic role as a general base⁶⁰. It is therefore likely that the second pK_a value of 5.6 reflects the ionisation of this amino acid residue.

The identity of the acidic group with the high pK_a value of 9.5 on the enzyme has not been established.

The mechanistic deductions made from these pH-rate profiles are discussed later, as are alternative interpretations.

3.3 Kinetic solvent isotope effects

Kinetic solvent isotope effects were determined for the second-order rate constant (k_{cat}/K_m) of the β -lactamase II catalysed hydrolysis of various β -lactams. The pD-rate profile for cephaloridine and benzylpenicillin are shown in Figure 3.9 and Figure 3.10 respectively.

3.3.1 pD dependence of the second-order rate constant (k_{cat}/K_m) of hydrolysis of cephaloridine catalysed by β -lactamase II

Values of k_{cat}/K_m for the β -lactamase II catalysed hydrolysis of cephaloridine were determined at different pD values (Table 3-9). The plot of $\log k_{cat}/K_m$ is shown in Figure 3.9. The curve is bell-shaped and, similar to the pH dependence of k_{cat}/K_m , there is a second-order dependence of the rate constant on the (D^+) concentration at low pD. The kinetic parameters obtained and ionisation constants are shown in Table 3-10.

Table 3-9 pD dependence of k_{cat}/K_m for the β -lactamase II catalysed hydrolysis of cephaloridine at 30 °C and ionic strength 1.0 M (NaCl). Enzyme concentration 0.1-2 μM , zinc concentration 2-40 μM

<i>pD</i>	<i>k_{cat}/K_m (M⁻¹ s⁻¹)</i>	<i>pD</i>	<i>k_{cat}/K_m (M⁻¹ s⁻¹)</i>
4.77	4.47×10^2	8.01	7.26×10^4
5.04	2.67×10^3	8.21	8.03×10^4
5.25	1.47×10^4	8.47	7.29×10^4
5.56	1.78×10^4	8.58	8.01×10^4
5.68	3.27×10^4	8.99	8.27×10^4
5.98	5.50×10^4	9.48	4.37×10^4
6.47	8.51×10^4	10.05	3.25×10^4
6.96	7.29×10^4	10.60	1.85×10^4
7.47	7.33×10^4	11.03	8.76×10^3
7.47	7.73×10^4	11.52	4.41×10^3

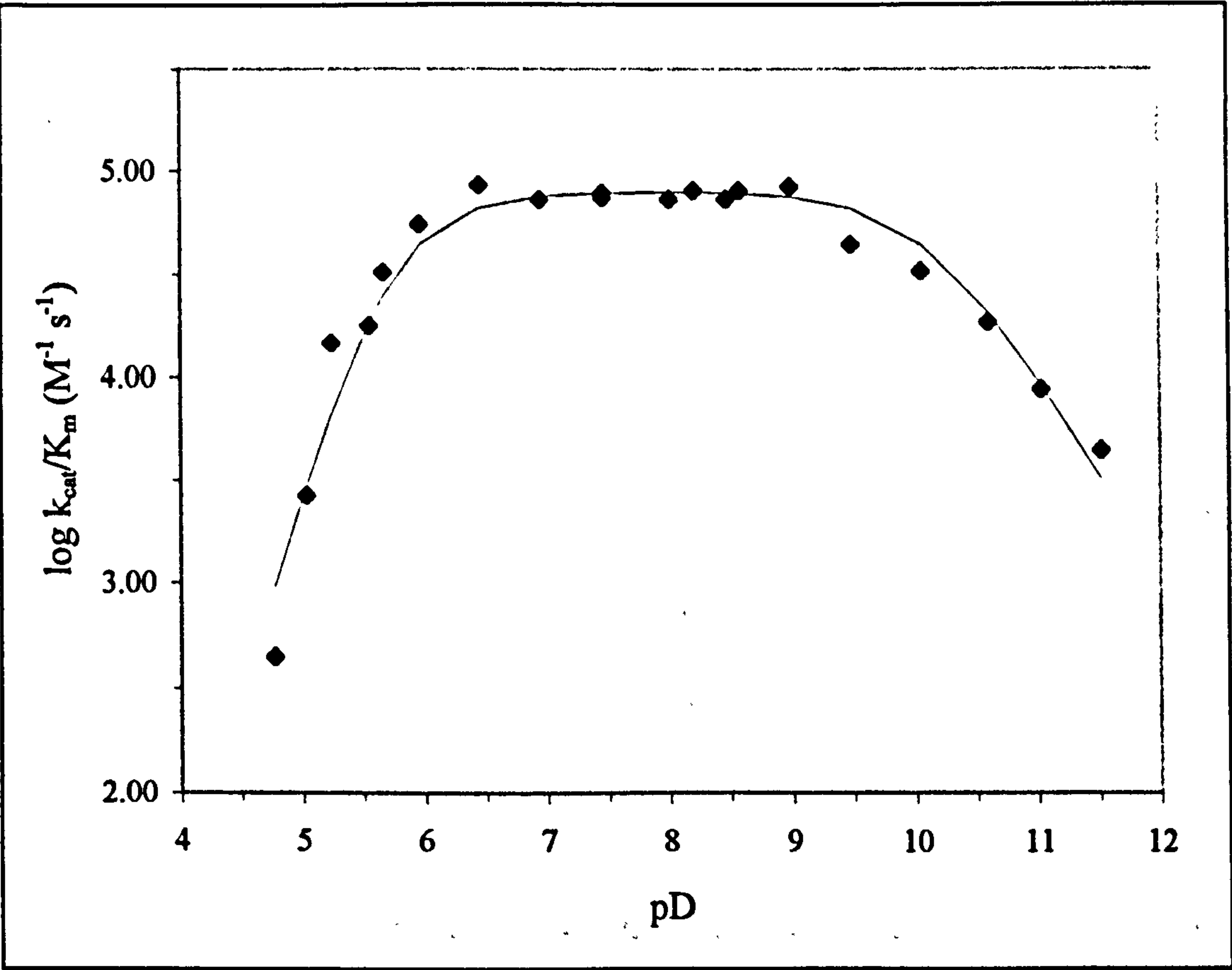


Figure 3.9 Dependence on pD for the second-order rate constant ($\log k_{cat}/K_m$) of the β -lactamase II catalysed hydrolysis of cephaloridine, 30 °C, ionic strength 1.0 M (NaCl)

Table 3-10 Kinetic parameters of the pD dependence of the β -lactamase II catalysed hydrolysis of cephaloridine, at 30 °C and ionic strength 1.0 M (NaCl)

Substrate	$k_{cat}/K_m (M^{-1} s^{-1})$	$pK_{a1}=pK_{a2}$	pK_{a3}
cephaloridine	$7.94 (\pm 0.05) \times 10^4$	$5.70 (\pm 0.05)$	$10.14 (\pm 0.1)$

3.3.2 *pD* dependence of the second-order rate constant (k_{cat}/K_m) of hydrolysis of benzylpenicillin catalysed by β -lactamase II

The *pD* dependence of k_{cat}/K_m for the β -lactamase II catalysed hydrolysis of benzylpenicillin was also investigated (Table 3-11). The results are presented in Figure 3.10 as a plot of $\log k_{cat}/K_m$ against *pD*. The curve is bell-shaped and the *pD* dependency is similar to that observed for cephaloridine, there is a slope of 2 at low *pD* indicating the second-order dependence on hydronium ion concentration of the rate at low *pD* and a slope of -1 at high *pD*. The results are shown in Table 3-12.

Table 3-11 *pD* dependence of k_{cat}/K_m for the β -lactamase II catalysed hydrolysis of benzylpenicillin at 30 °C and ionic strength 1.0 M (NaCl)
Enzyme concentration 0.1-2 μ M, zinc concentration 2-40 μ M

<i>pD</i>	k_{cat}/K_m ($M^{-1} s^{-1}$)	<i>pD</i>	k_{cat}/K_m ($M^{-1} s^{-1}$)
4.77	7.21×10^2	8.47	2.40×10^5
5.04	2.43×10^3	8.47	2.35×10^5
5.04	2.61×10^3	8.99	2.49×10^5
5.25	1.30×10^4	8.99	2.59×10^5
5.56	7.01×10^4	9.48	2.44×10^5
5.98	1.14×10^5	9.48	3.05×10^5
6.47	1.47×10^5	10.05	1.23×10^5
6.96	1.81×10^5	10.05	1.61×10^5
7.47	1.66×10^5	10.60	5.86×10^4
7.47	1.76×10^5	11.03	2.43×10^4
8.01	1.65×10^5	11.52	4.13×10^3
8.01	1.53×10^5		

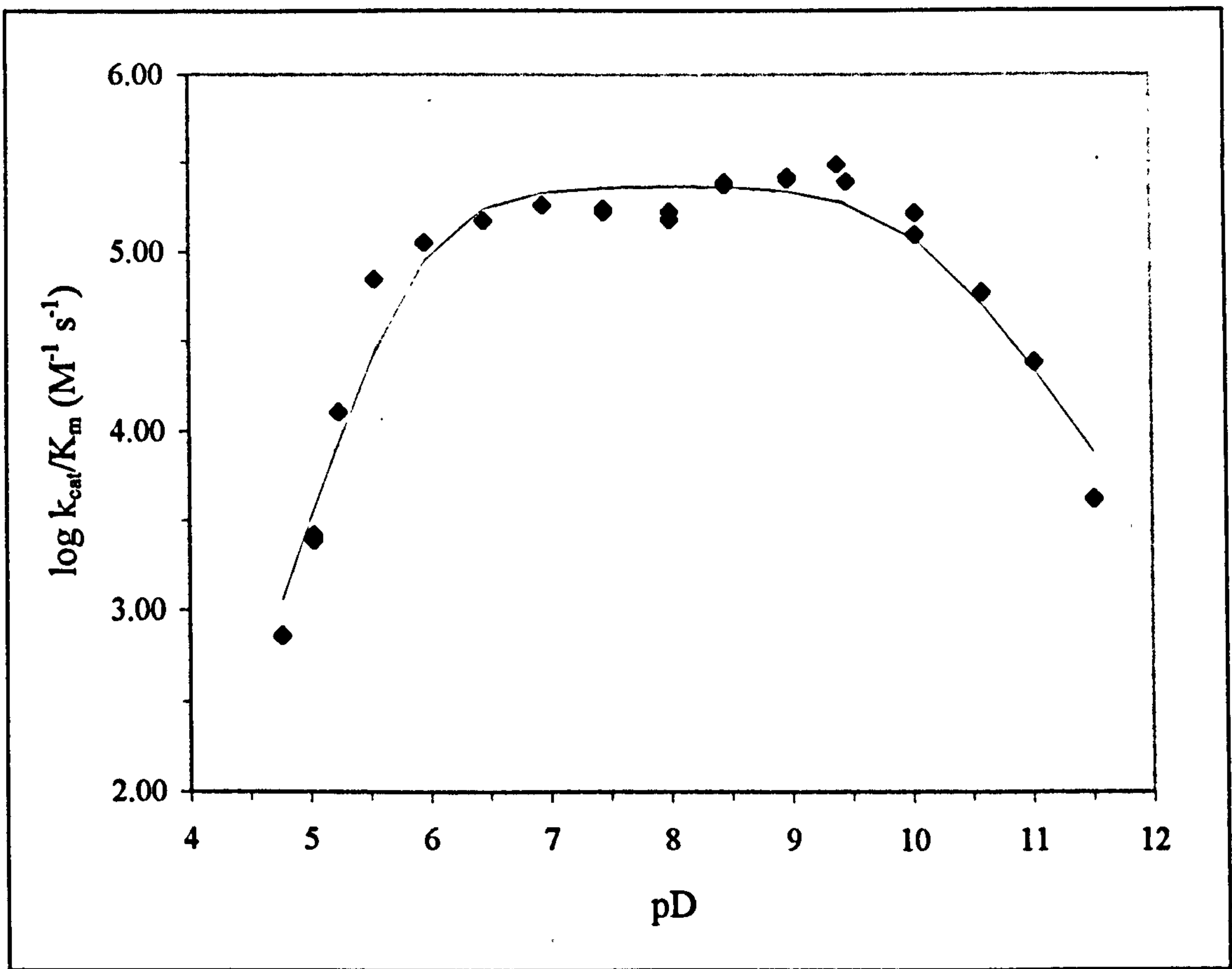


Figure 3.10 Dependence on pD for the rate constant ($\log k_{cat}/K_m$) of the β -lactamase II catalysed hydrolysis of benzylpenicillin, 30 °C, ionic strength 1.0 M (NaCl)

Table 3-12 Kinetic parameters of the pD dependence of the β -lactamase II catalysed hydrolysis of benzylpenicillin, at 30 °C and ionic strength 1.0 M (NaCl)

Substrate	$k_{cat}/K_m (M^{-1} s^{-1})$	$pK_{a1}=pK_{a2}$	pK_{a3}
Benzylpenicillin	$2.34 (\pm 5) 10^5$	$5.91 (\pm 0.05)$	$10.04 (\pm 0.1)$

3.3.3 Determination of the kinetic parameters for the β -lactamase II catalysed hydrolysis of β -lactams in H_2O and D_2O

3.3.3.1 Cephaloridine

The kinetics parameters K_m and k_{cat} for the β -lactamase II catalysed hydrolysis of cephaloridine were determined in H_2O and D_2O from linear plots of substrate concentration versus substrate / initial rate (Hanes plot), and analysed according to the Hanes equation below:

$$\frac{[S]}{v} = \frac{[S]}{V_{max}} + \frac{K_m}{V_{max}}$$

$\Delta[A]/\Delta t$ values were measured at different substrate concentrations, and dividing $\Delta[A]/\Delta t$ by $\Delta\epsilon$ gave the initial rates. The results are listed in Table 3-13 for H_2O and Table 3-14 for D_2O . At concentrations of cephaloridine above 1×10^{-3} M some product inhibition was observed. A decrease in the rate of hydrolysis was observed when repeated hydrolyses were performed. Therefore the cephaloridine concentrations used for the kinetic parameters determination were kept below 10^{-3} M.

The results were plotted and are shown in Figure 3.11 for H_2O and Figure 3.12 for D_2O . Using the Hanes plot and fitting the results to a linear regression gave the slope equal to $1/V_{max}$ and the intercept equal to K_m/V_{max} . The kinetic parameters are listed in Table 3-15 (page 122).

Table 3-13 Initial rates measured for the β -lactamase II catalysed hydrolysis of cephaloridine in H₂O at 30 °C, pH 7.0, ionic strength 1.0 M (NaCl), enzyme concentration 0.1 μ M, zinc concentration 2 μ M

$\Delta\epsilon (M^{-1} cm^{-1})$	<i>Cephaloridine (M)</i>	<i>initial rate (M s⁻¹)</i>	<i>substrate/initial rate (s)</i>
$\Delta\epsilon_{260}=9444 \pm 645$	1.00×10^{-5}	5.725×10^{-8}	1.747×10^2
$\Delta\epsilon_{260}=9444 \pm 645$	2.00×10^{-5}	1.338×10^{-7}	1.495×10^2
$\Delta\epsilon_{260}=9444 \pm 645$	2.88×10^{-5}	1.956×10^{-7}	1.472×10^2
$\Delta\epsilon_{260}=9444 \pm 645$	4.80×10^{-5}	2.722×10^{-7}	1.763×10^2
$\Delta\epsilon_{260}=9444 \pm 645$	7.68×10^{-5}	4.707×10^{-7}	1.632×10^2
$\Delta\epsilon_{260}=9444 \pm 645$	1.00×10^{-4}	7.069×10^{-7}	1.415×10^2
$\Delta\epsilon_{260}=9444 \pm 645$	2.00×10^{-4}	1.256×10^{-6}	1.592×10^2
$\Delta\epsilon_{260}=9444 \pm 645$	2.88×10^{-4}	1.440×10^{-6}	2.000×10^2
$\Delta\epsilon_{280}=4141 \pm 306$	4.80×10^{-4}	2.625×10^{-6}	1.829×10^2
$\Delta\epsilon_{280}=4141 \pm 306$	7.68×10^{-4}	2.852×10^{-6}	2.693×10^2
$\Delta\epsilon_{290}=1990 \pm 40$	1.00×10^{-3}	3.880×10^{-6}	2.577×10^2

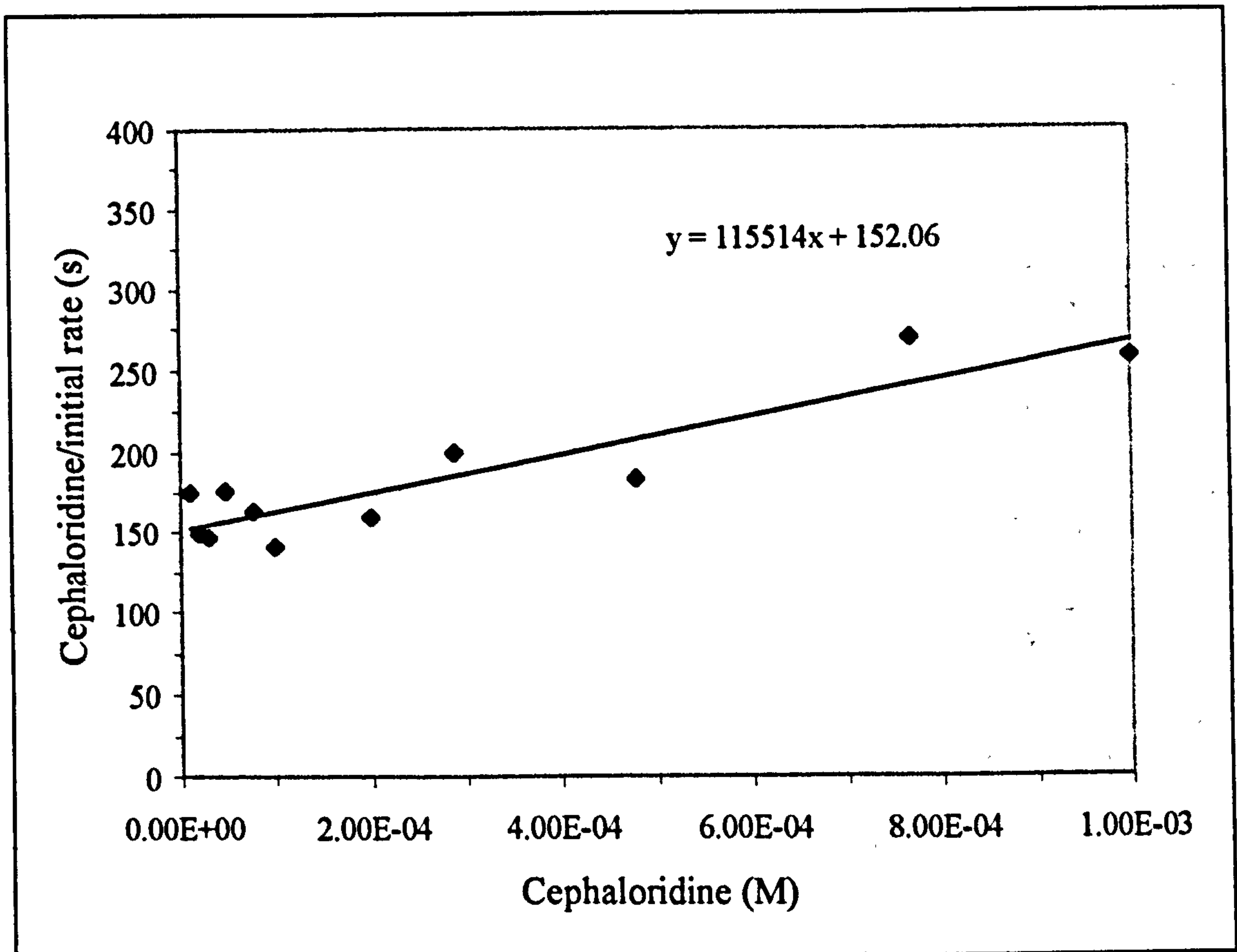


Figure 3.11 Hanes plot for the β -lactamase II catalysed hydrolysis of cephaloridine in H_2O , pH 7.0, 30 °C, ionic strength 1.0 M (NaCl)

Table 3-14 Initial rates measured for the β -lactamase II catalysed hydrolysis of cephaloridine in D₂O at 30 °C, pD 7.0, ionic strength 1.0 M (NaCl), Enzyme concentration 0.1 μ M, zinc concentration 2 μ M

$\Delta\epsilon (M^{-1} cm^{-1})$	<i>Cephaloridine (M)</i>	<i>initial rate (M s⁻¹)</i>	<i>substrate/initial rate (s)</i>
$\Delta\epsilon_{260}=8057 \pm 300$	1.00×10^{-5}	7.587×10^{-8}	1.318×10^2
$\Delta\epsilon_{260}=8057 \pm 300$	2.88×10^{-5}	2.184×10^{-7}	1.319×10^2
$\Delta\epsilon_{260}=8057 \pm 300$	4.80×10^{-5}	3.399×10^{-7}	1.412×10^2
$\Delta\epsilon_{260}=8057 \pm 300$	7.68×10^{-5}	5.120×10^{-7}	1.501×10^2
$\Delta\epsilon_{260}=8057 \pm 300$	$1.00E \times 10^{-4}$	7.232×10^{-7}	1.383×10^2
$\Delta\epsilon_{260}=8057 \pm 300$	2.00×10^{-4}	1.437×10^{-6}	1.392×10^2
$\Delta\epsilon_{280}= 4284 \pm 140$	2.88×10^{-4}	1.720×10^{-6}	1.674×10^2
$\Delta\epsilon_{280}= 4284 \pm 140$	4.80×10^{-4}	2.400×10^{-6}	2.000×10^2
$\Delta\epsilon_{290}= 1708 \pm 34$	7.68×10^{-4}	3.049×10^{-6}	2.519×10^2

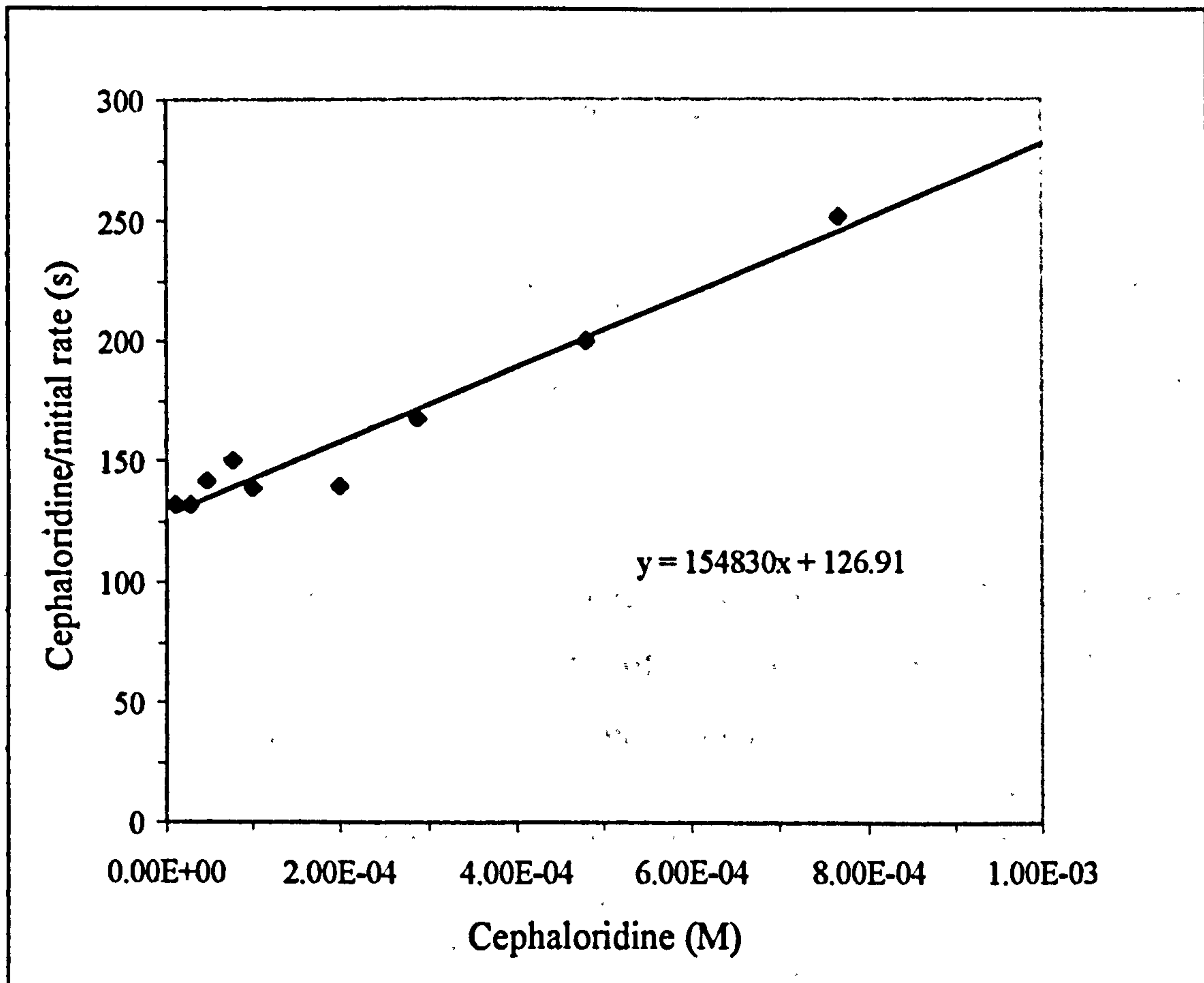


Figure 3.12 Hanes plot for the β -lactamase II catalysed hydrolysis of cephaloridine in D_2O , pD 7.0, 30 °C, ionic strength 1.0 M (NaCl)

Table 3-15 Kinetic parameters for the β -lactamase II catalysed hydrolysis of cephaloridine in H_2O and D_2O at 30 °C, pH/pD 7.0.

<i>Solvent</i>	$1/V_{max} (M^{-1} s)$	$K_m/V_{max} (M s)$	$k_{cat} (s^{-1})$	$K_m (M)$
H_2O	115514	152	86.6	1.3×10^{-3}
D_2O	154830	127	64.6	0.82×10^{-3}

3.3.3.2 Benzylpenicillin

The kinetic parameters for the β -lactamase II catalysed hydrolysis of benzylpenicillin were also determined in H_2O and D_2O from linear plots of substrate concentration versus substrate/initial rate (Figure 3.13 for H_2O and Figure 3.14 for D_2O) and analysed according to the Hanes equation. The results are listed in Table 3-18 (126).

Table 3-16 Initial rates measured for the β -lactamase II catalysed hydrolysis of benzylpenicillin in H_2O at 30 °C, pH 7.0, ionic strength 1.0 M (NaCl), Enzyme concentration 0.1 μ M, zinc concentration 2 μ M

$\Delta\epsilon (M^{-1} cm^{-1})$	<i>Benzylpenicillin (M)</i>	<i>initial rate (M s⁻¹)</i>	<i>Substrate/initial rate (s)</i>
$\Delta\epsilon_{230} = 967 \pm 50$	5.20×10^{-4}	7.44×10^{-6}	69.89
$\Delta\epsilon_{230} = 967 \pm 50$	6.94×10^{-4}	9.87×10^{-6}	70.33
$\Delta\epsilon_{230} = 967 \pm 50$	8.67×10^{-4}	1.21×10^{-5}	71.83
$\Delta\epsilon_{230} = 967 \pm 50$	1.00×10^{-3}	1.05×10^{-5}	95.24
$\Delta\epsilon_{230} = 967 \pm 50$	1.04×10^{-3}	1.34×10^{-5}	77.69
$\Delta\epsilon_{233} = 954 \pm 45$	1.08×10^{-3}	1.22×10^{-5}	88.82
$\Delta\epsilon_{237} = 642 \pm 22$	1.45×10^{-3}	1.48×10^{-5}	98.31
$\Delta\epsilon_{233} = 954 \pm 45$	1.62×10^{-3}	1.39×10^{-5}	116.97
$\Delta\epsilon_{233} = 954 \pm 45$	2.18×10^{-3}	1.46×10^{-5}	148.77
$\Delta\epsilon_{237} = 642 \pm 22$	2.60×10^{-3}	2.47×10^{-5}	105.26
$\Delta\epsilon_{248} = 172 \pm 10$	3.63×10^{-3}	2.12×10^{-5}	170.99
$\Delta\epsilon_{248} = 172 \pm 10$	4.00×10^{-3}	2.88×10^{-5}	139.08
$\Delta\epsilon_{248} = 172 \pm 10$	4.35×10^{-3}	2.58×10^{-5}	168.80
$\Delta\epsilon_{248} = 172 \pm 10$	6.40×10^{-3}	2.53×10^{-5}	252.76
$\Delta\epsilon_{248} = 172 \pm 10$	6.00×10^{-3}	2.70×10^{-5}	222.30
$\Delta\epsilon_{248} = 172 \pm 10$	9.00×10^{-3}	2.77×10^{-5}	325.03

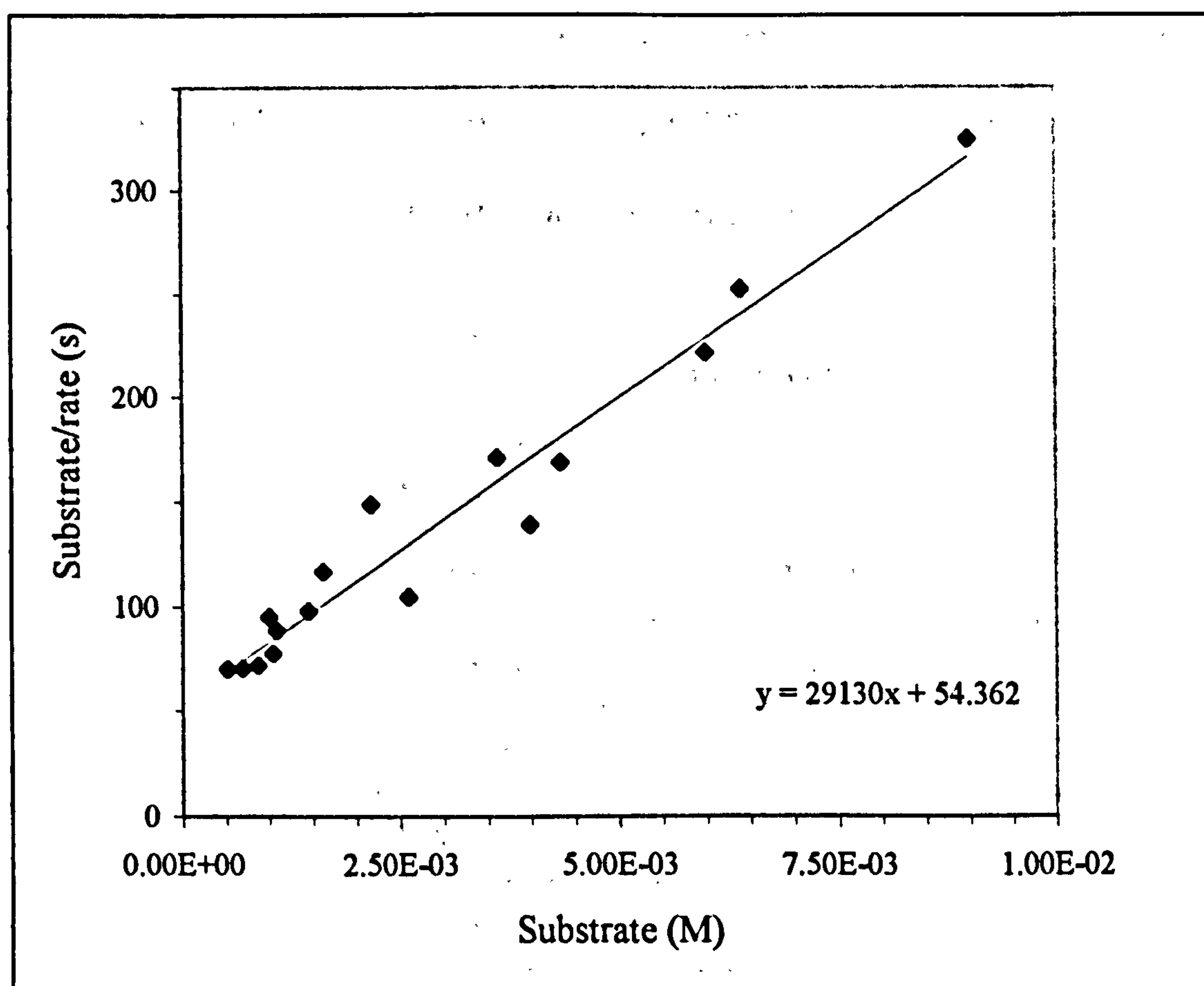


Figure 3.13 Hanes plot for the β -lactamase II catalysed hydrolysis of benzylpenicillin in H_2O , pH 7.0, 30 °C, ionic strength 1.0 M (NaCl)

A similar experiment was repeated in D₂O

Table 3-17 Initial rates measured for the β -lactamase II catalysed hydrolysis of benzylpenicillin in D₂O at 30 °C, pD 7.0, ionic strength 1.0 M (NaCl)
Enzyme concentration 0.1 μ M, zinc concentration 2 μ M

$\Delta\epsilon\ (M^{-1}\ cm^{-1})$	<i>Benzylpenicillin (M)</i>	<i>initial rate (M s⁻¹)</i>	<i>Substrate/initial rate (s)</i>
$\Delta\epsilon_{230}= 1022 \pm 24$	5.20×10^{-4}	7.16×10^{-6}	72.66
$\Delta\epsilon_{230}= 1022 \pm 24$	6.94×10^{-4}	9.67×10^{-6}	71.74
$\Delta\epsilon_{230}= 1022 \pm 24$	8.67×10^{-4}	1.09×10^{-5}	79.69
$\Delta\epsilon_{230}= 1022 \pm 24$	1.04×10^{-3}	1.19×10^{-5}	87.77
$\Delta\epsilon_{237}= 642 \pm 22$	1.00×10^{-3}	9.75×10^{-6}	102.56
$\Delta\epsilon_{237}= 642 \pm 22$	2.00×10^{-3}	1.27×10^{-5}	157.48
$\Delta\epsilon_{248}= 182 \pm 9$	4.00×10^{-3}	1.72×10^{-5}	232.29
$\Delta\epsilon_{248}= 182 \pm 9$	6.40×10^{-3}	1.98×10^{-5}	323.07

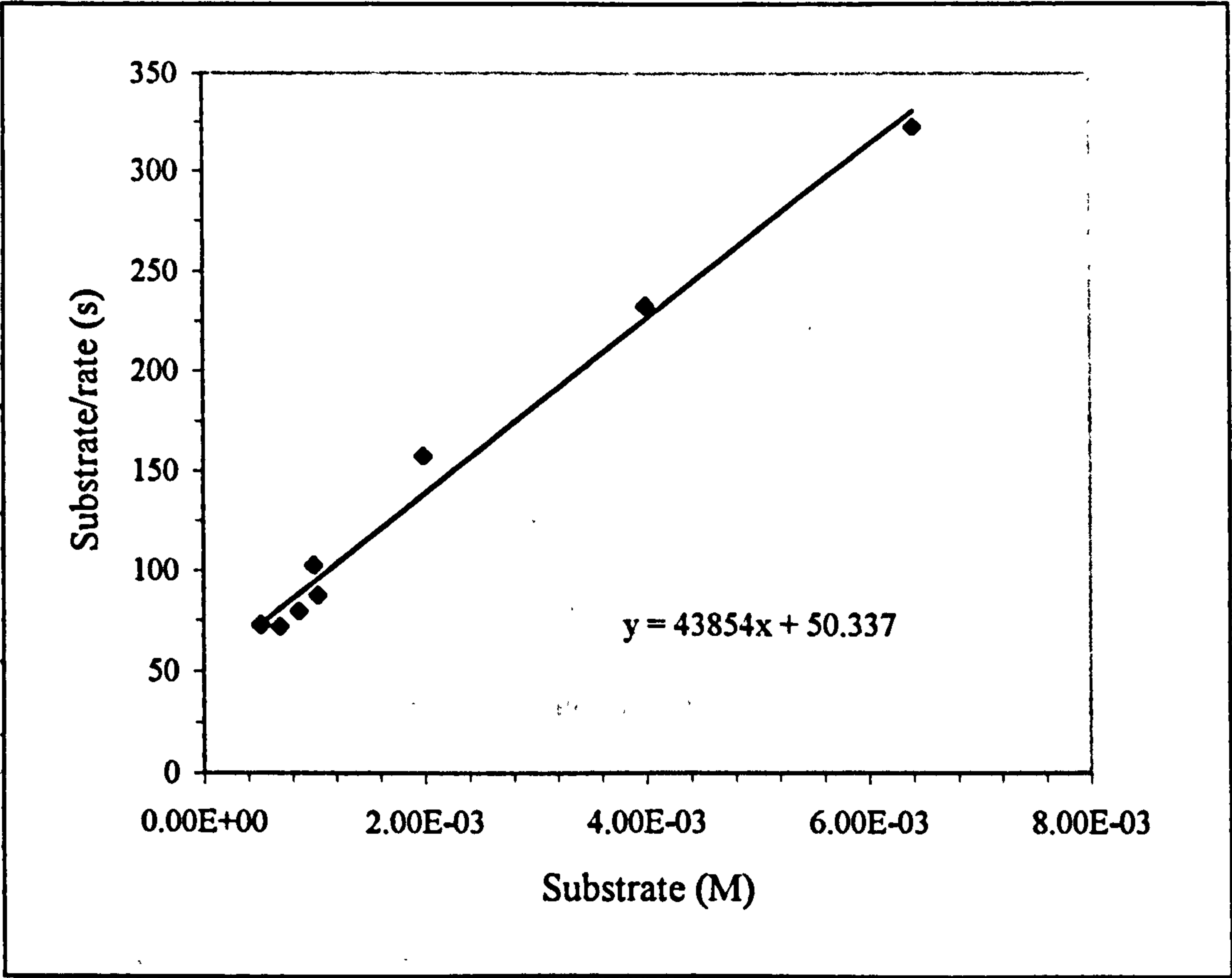


Figure 3.14 Hanes plot for the β -lactamase II catalysed hydrolysis of benzylpenicillin in D₂O, pD 7.0, 30 °C, ionic strength 1.0 M (NaCl)

Table 3-18 Kinetic parameters for the β -lactamase II catalysed hydrolysis of benzylpenicillin in H₂O and D₂O at 30 °C, pH/pD 7.0

<i>Solvent</i>	$1/V_{max} (M^{-1} s)$	$K_m/V_{max} (M s)$	$k_{cat} (s^{-1})$	$K_m (M)$
H ₂ O	2.913 x 10 ⁴	54.36	343	1.87 x 10 ⁻³
D ₂ O	4.385 x 10 ⁴	50.34	228	1.15 x 10 ⁻³

3.3.3.3 Cefuroxime

As K_m for the β -lactamase II catalysed hydrolysis of cefuroxime is low, $45 \pm 5 \mu\text{M}$ ³⁴, it was easy to work at substrate concentrations as high as 6 times K_m for k_{cat} determination. The initial rates were measured at pH and pD 7.0 and k_{cat} was determined by dividing the initial rates by the enzyme concentration. Values of k_{cat}/K_m were determined by following the entire course of the hydrolysis of $23.9 \mu\text{M}$ of cefuroxime at $\lambda = 260 \text{ nm}$ at pH and pD 7.0. A good pseudo-first order rate constant k_{obs} was measured which, when divided by the enzyme concentration, gave k_{cat}/K_m .

Table 3-19 k_{cat} and k_{cat}/K_m for the β -lactamase II catalysed hydrolysis of cefuroxime in H_2O and D_2O at pH /pD 7.0, 30 °C, ionic strength 1.0 M (KCl)

<i>Solvent</i>	$k_{cat} (\text{s}^{-1})$	$k_{cat}/K_m (\text{M}^{-1} \text{s}^{-1})$
pH 7.0	35 ± 5	$7.74 \pm 1 \times 10^5$
pD 7.0	22 ± 3	$6.31 \pm 0.7 \times 10^5$

3.3.4 Discussion of the kinetic solvent isotope effects

To determine the kinetic solvent isotope effects on the β -lactamase II kinetic parameters and ionisation constants, the pH dependencies of k_{cat}/K_m were determined in D_2O for the β -lactamase II catalysed hydrolysis of cephaloridine and benzylpenicillin (Figure 3.9 and Figure 3.10 respectively, pages 115 and 117). Solvent kinetic isotope effects on k_{cat} , were also determined by measuring the initial rate of the β -lactamase II catalysed hydrolysis of cephaloridine, benzylpenicillin and cefuroxime in D_2O , the data were analysed using Hanes plots (Figure 3.11, Figure 3.12, Figure 3.13 and Figure 3.14, page 120, 122, 124 and 126). The kinetic parameters and ionisation constants in H_2O and D_2O and the solvent isotope effects are summarized in Table 3-20.

Table 3-20 Kinetic parameters for the β -lactamase II catalysed hydrolysis of β -lactams

	<i>Benzylpenicillin</i>	<i>Cephaloridine</i>	<i>Cefuroxime</i>
$pK_{a1} = pK_{a2} (H_2O)$	5.83 ± 0.05	5.46 ± 0.05	5.73 ± 0.1
$pK_{a1} = pK_{a2} (D_2O)$	5.91 ± 0.05	5.70 ± 0.05	-
$\Delta pK_{a1}^{H_2O/D_2O}$	+ 0.08	+ 0.24	
$pK_{a3} (H_2O)$	9.30 ± 0.1	9.64 ± 0.1	9.87 ± 0.1
$pK_{a3} (D_2O)$	10.04 ± 0.1	10.14 ± 0.1	-
$\Delta pK_{a3}^{H_2O/D_2O}$	+ 0.74	+ 0.50	
$(k_{cat}/K_m) H_2O M^{-1} s^{-1}$	$4.27 (\pm 0.6) \times 10^5$	$6.58 (\pm 0.4) \times 10^4$	$7.74 (\pm 1) \times 10^5$
$(k_{cat}/K_m) D_2O M^{-1} s^{-1}$	$2.34 (\pm 0.5) \times 10^5$	$7.87 (\pm 0.05) \times 10^4$	$6.31 (\pm 0.7) \times 10^5$
$\frac{(k_{cat}/K_m)^{H_2O}}{(k_{cat}/K_m)^{D_2O}}$	1.82	0.85	1.23
$k_{cat}^{H_2O} s^{-1}$	336 ± 10	87 ± 5	35 ± 5
$k_{cat}^{D_2O} s^{-1}$	232 ± 10	65 ± 5	22 ± 3
$k_{cat}^{H_2O} / k_{cat}^{D_2O}$	1.4	1.3	1.6

Changing the solvent from H_2O to D_2O alters the pK_a of acidic groups - generally the pK_a in D_2O is about 0.3 to 0.4 higher than in H_2O ¹²⁷. There is also the possibility of a primary kinetic isotope effect if a bond to hydrogen is broken in the rate-limiting step. Interpreting observed kinetic solvent isotope effects on the rate

of enzyme catalysed reactions usually assumes that all ionisable protons of the enzyme are rapidly exchanged in D₂O.

The pD dependence of the rate constants (both k_{cat}/K_m and k_{cat}) of the β -lactamase II catalysed hydrolysis of β -lactams, pD below 5, shows an inverse second order dependence on deuterium-ion concentration. The slope of $\log k_{\text{cat}}/K_m$ against pD being 2.0. As with the studies in H₂O, it was not possible to separate the two low pK_a values. The similar behaviour of the enzyme in H₂O and D₂O again indicates that the observations reflect an intrinsic property of the enzyme. The increase in pK_{a3} of about 0.6 ± 0.2 in D₂O is in the range expected for a weak acid. It was hoped that the change in solvent to D₂O may have a differential effect on pK_{a1} and pK_{a2} and thus make it easier to identify the individual pK_as. Unfortunately, this was not the case and it is not clear whether the apparent change in pK_{a1} and pK_{a2} is real or simply an experimental consequence of their close values.

A relatively small kinetic solvent isotope effect was observed on k_{cat}/K_m and k_{cat} .

$\frac{(k_{\text{cat}}/K_m)^{\text{H}_2\text{O}}}{(k_{\text{cat}}/K_m)^{\text{D}_2\text{O}}}$ was 1.82 for the hydrolysis of benzylpenicillin, 0.85 for cephaloridine and 1.23 for cefuroxime and $k_{\text{cat}}^{\text{H}_2\text{O}} / k_{\text{cat}}^{\text{D}_2\text{O}}$ was 1.4 for benzylpenicillin, 1.5 for cephaloridine and 1.6 for cefuroxime.

The mechanistic significance of these solvent kinetic isotope effects is discussed in chapter 3.7.

3.4 Zinc dependence of β -lactamase II activity

3.4.1 Determination of the zinc concentration of the β -lactamase II stock solution.

The zinc content of the stock solution to the β -lactamase II was measured by atomic absorption. It was found that the molar ratio of zinc to enzyme in the stock solution was two to one. Thus an experiment conducted with 1.0×10^{-8} M enzyme and no added zinc ion actually contained 2.0×10^{-8} M Zn (II).

Because the rates were so slow at low pH and to ensure that the enzyme catalysed reaction was being observed, higher concentrations of β -lactamase II were used. Subsequently, the lowest zinc concentration attained was restricted by the enzyme concentration used.

3.4.2 Zinc dependence of the β -lactamase II activity at pH 7.0

The dependence of the second-order rate constant (k_{cat}/K_m) for the β -lactamase II catalysed hydrolysis of cephaloridine on the zinc ion concentration was examined at pH 7.0, 30 °C and at constant ionic strength of 1 M (NaCl), the results are summarized in Table 3-21. It is important to note that the zinc concentrations used in this study are not found in physiological conditions.

Increasing the zinc ion concentration from 10^{-7} M to 10^{-3} M does not strongly affect the rate (k_{cat}/K_m) of the catalysed hydrolysis of cephaloridine at pH 7.0 as only a 30 % increase is observed in the rate for a 10^4 fold change in metal-ion concentration.

Table 3-21 Effect of zinc concentration on the rate constant (k_{cat}/K_m) of the β -lactamase II catalysed hydrolysis of cephaloridine (0.15 mM) at pH 7.0, 30 °C, ionic strength 1.0 M (NaCl), enzyme concentration 0.1 μ M

<i>Zinc concentration (M)</i>	<i>k_{cat}/K_m ($M^{-1} s^{-1}$)</i>
2.0×10^{-7}	7.54×10^4
3.0×10^{-7}	7.96×10^4
1.2×10^{-6}	7.39×10^4
1.0×10^{-5}	8.42×10^4
1.0×10^{-4}	9.19×10^4
1.0×10^{-3}	10.28×10^4

Many zinc proteases are inhibited by excess zinc, such as thermolysin, ACE, carboxypeptidase A⁷⁶. However, *B. cereus* β -lactamase II is reported to be fully active with one zinc ion, and the binding of a second zinc ion of lower affinity slightly increases its activity⁷⁶.

In order to determine the importance of zinc concentration on β -lactamase II activity, the zinc content of the enzyme stock solution was measured. The zinc content of the enzyme stock solution was found to be two moles of zinc for one mole of enzyme and it could therefore be argued that the results listed in Table 3-

21 are not valid as two zinc are already present with the enzyme. However, it was reported in early studies^{43,44} that the β -lactamase II binds two zinc, one zinc being tightly bound with a dissociation constant of 1 μ M and the second loosely bound with a dissociation constant of 24 mM. Because of the low affinity of the second zinc for the enzyme, it can be assumed that only one zinc is bound to the enzyme in the stock solution at neutral pH, therefore validating the results of the studies of the effect of the zinc concentration on the enzyme activity.

3.4.3 Effect of the zinc concentration of the β -lactamase II activity at low pH

The pH dependence of the rate constant (k_{cat}/K_m) for the β -lactamase II catalysed hydrolysis of benzylpenicillin was studied at different zinc concentrations (Table 3-22) at low pH. To ensure that the effect of zinc concentration observed on the enzyme catalysed reaction was not due to any background reaction, the experiment was performed for a fixed zinc concentration and pH with two different enzyme concentrations. Identical results were obtained showing that the effect on the enzyme catalysed reaction was independent of the enzyme concentration.

Figure 3.15 shows the effect of zinc ion concentration on β -lactamase II activity at different pH values, whilst Figure 3.16 shows the pH-dependence of β -lactamase II activity in the presence of different zinc concentrations at low pH.

Table 3-22 k_{cat}/K_m of β -lactamase II catalysed hydrolysis of benzylpenicillin at different zinc ion concentrations over pH range 4 - 6 at 30 °C, ionic strength 1.0 M (NaCl). Enzyme concentration 0.1-6 μ M

Zinc concentration (M)	$1.0 \times 10^{-2} \text{ M}$	$1.0 \times 10^{-3} \text{ M}$	$1.0 \times 10^{-4} \text{ M}$
pH	$k_{cat}/K_m \text{ (M}^{-1} \text{ s}^{-1}\text{)}$	$k_{cat}/K_m \text{ (M}^{-1} \text{ s}^{-1}\text{)}$	$k_{cat}/K_m \text{ (M}^{-1} \text{ s}^{-1}\text{)}$
5.98	5.13×10^5	4.71×10^5	5.15×10^5
5.44	4.81×10^5	4.20×10^5	4.36×10^5
5.49	4.76×10^5	4.27×10^5	4.56×10^5
5.05	3.70×10^5	4.09×10^5	3.26×10^5
4.51	3.01×10^5	1.34×10^5	1.01×10^4
4.25	1.01×10^5	2.31×10^5	1.25×10^3
4.03	2.86×10^4	2.90×10^3	3.30×10^2

Zinc concentration (M)	$1.0 \times 10^{-5} \text{ M}$	$1.0 \times 10^{-6} \text{ M}$
pH	$k_{cat}/K_m \text{ (M}^{-1} \text{ s}^{-1}\text{)}$	$k_{cat}/K_m \text{ (M}^{-1} \text{ s}^{-1}\text{)}$
5.98	4.33×10^5	3.85×10^5
5.71	-	3.216×10^5
5.53	-	1.625×10^5
5.49	3.24×10^5	-
5.44	2.98×10^5	-
5.27	2.76×10^5	-
5.21	-	1.420×10^4
5.05	8.87×10^4	2.51×10^3
4.51	7.54×10^2	-
4.25	2.02×10^2	-

The effect of zinc ion concentration on the second-order rate constant k_{cat}/K_m was studied over the pH range 4-6 and was found to be pH dependent (Figure 3.15). The enzyme activity greatly decreased below pH 5.5 when lowering the zinc concentration and increased upon the addition of zinc. No observable activity was seen at pH 4.0 with zinc concentrations lower than 10^{-4} M, but increasing the zinc concentration restored the enzyme activity. The effect of zinc concentration on the enzyme activity, however, decreased as the pH increased such that enzyme activity becomes independent of the zinc concentration above pH 6.

The plot of the rate constant ($\log k_{cat}/K_m$) of the β -lactamase II catalysed hydrolysis of benzylpenicillin against the logarithm of zinc concentration (Figure 3.15) shows a first order dependence on the zinc concentration, as the slopes at the different pHs are unity.

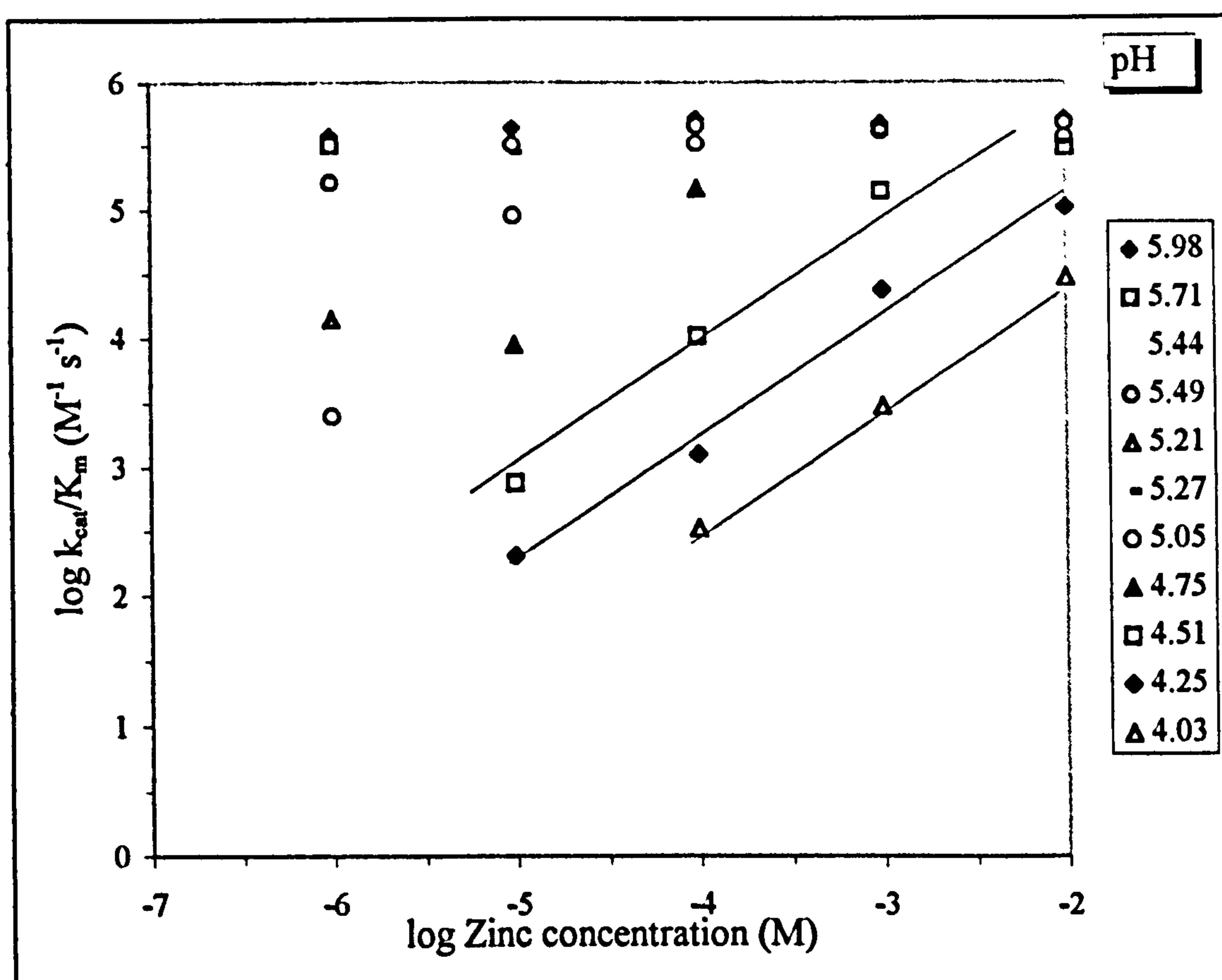


Figure 3.15 Plot of log zinc concentration against the rate constant ($\log k_{cat}/K_m$) of the β -lactamase II catalysed hydrolysis of benzylpenicillin over the pH range 4-6

The results in Table 3-22 were also plotted as the logarithm of k_{cat}/K_m against pH (over the pH range 4 - 6) for different zinc concentrations (Figure 3.16). This plot shows that the enzyme activity is retained at low pH when increasing the zinc concentration as the enzyme is still active at pH 4.0 when using zinc concentrations above 10^{-4} M. The pH-rate profile observed decrease with a slope of 3 at low pH values. The other notable feature of Figure 3.16 is that the apparent pK_a shifts from about 6 at low zinc ion concentration to about 4.5 with 0.01 M metal-ion.

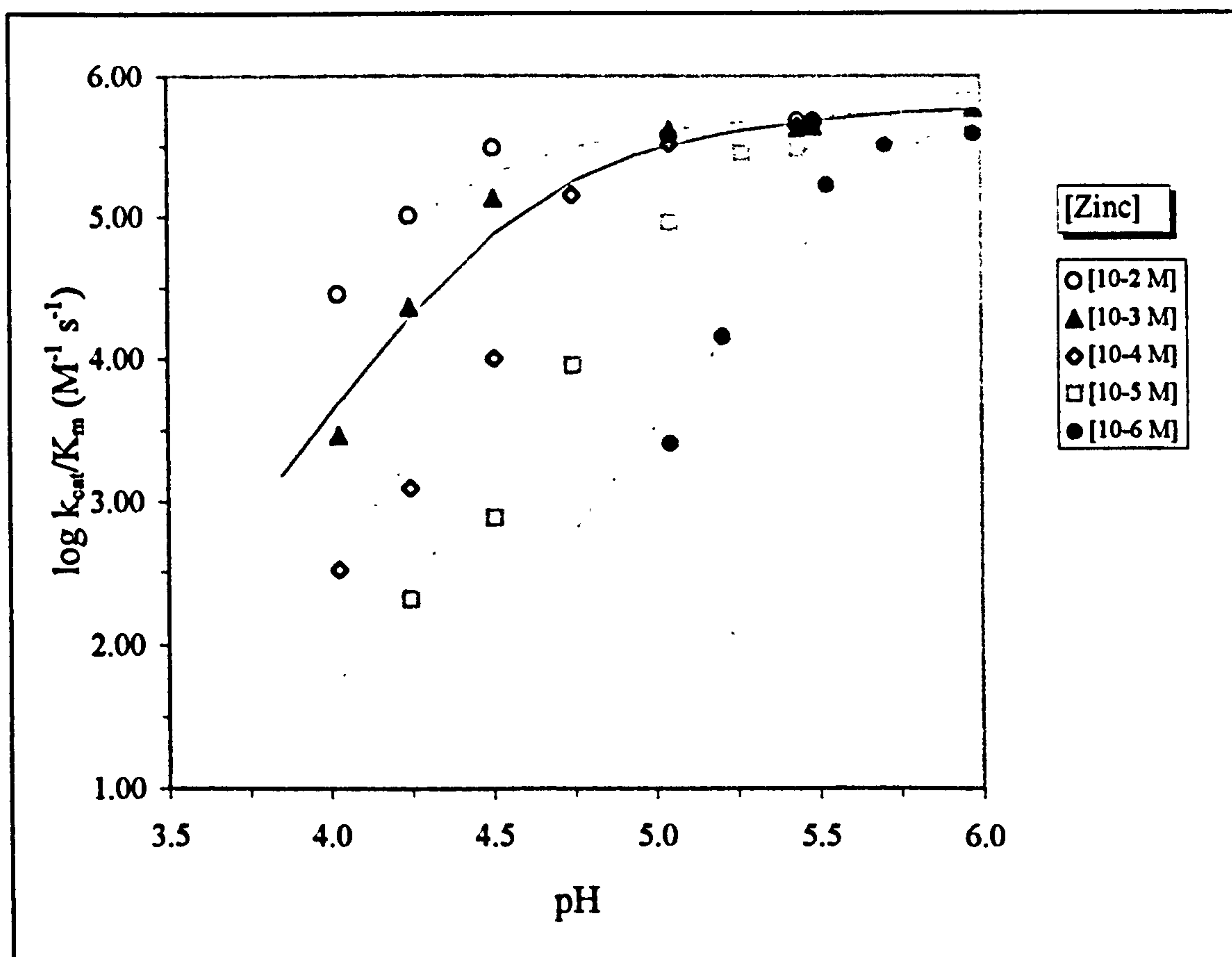
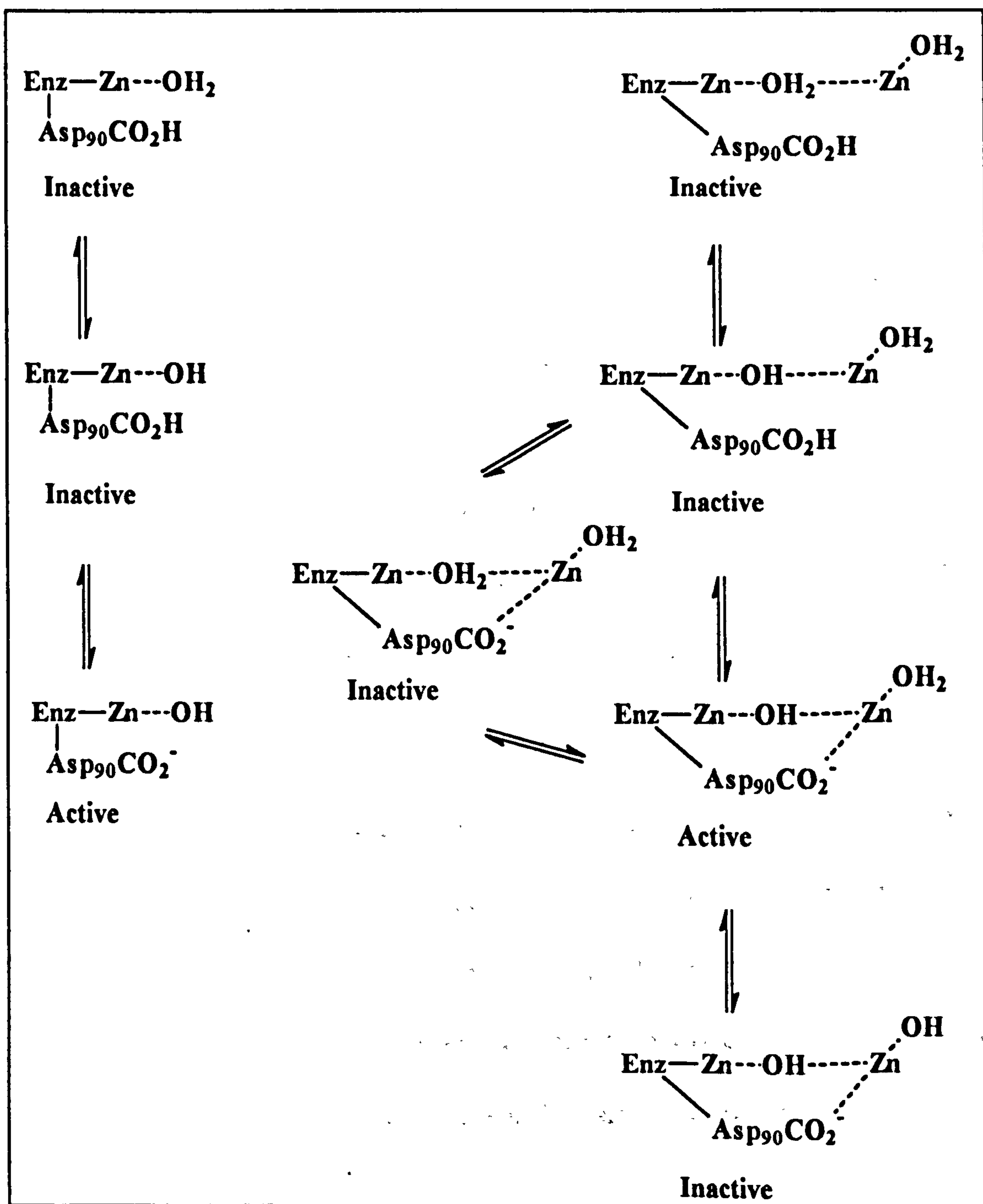


Figure 3.16 pH- $\log k_{\text{cat}}/K_m$ profile for the β -lactamase II catalysed hydrolysis of benzylpenicillin at different zinc concentrations

It can be seen from the plot above that the enzyme activity at low pH for the different zinc concentrations is third order in hydronium ion concentration. This result suggest that in addition to the ionisation of the two acidic groups previously reported in the pH-rate profile discussion, we could also be observing the effect of competition between the hydronium ion and the tightly bound zinc for the enzyme metal binding ligands, therefore loosing the zinc upon decreasing pH.

Another possible explanation for this effect could be the binding of a second zinc ion at low pH upon increase of the zinc concentration. The binding of the second

zinc could have a structural or a catalytic role. Analogy with other binuclear β -lactamases may suggest a fairly direct interaction between the two metal ions.



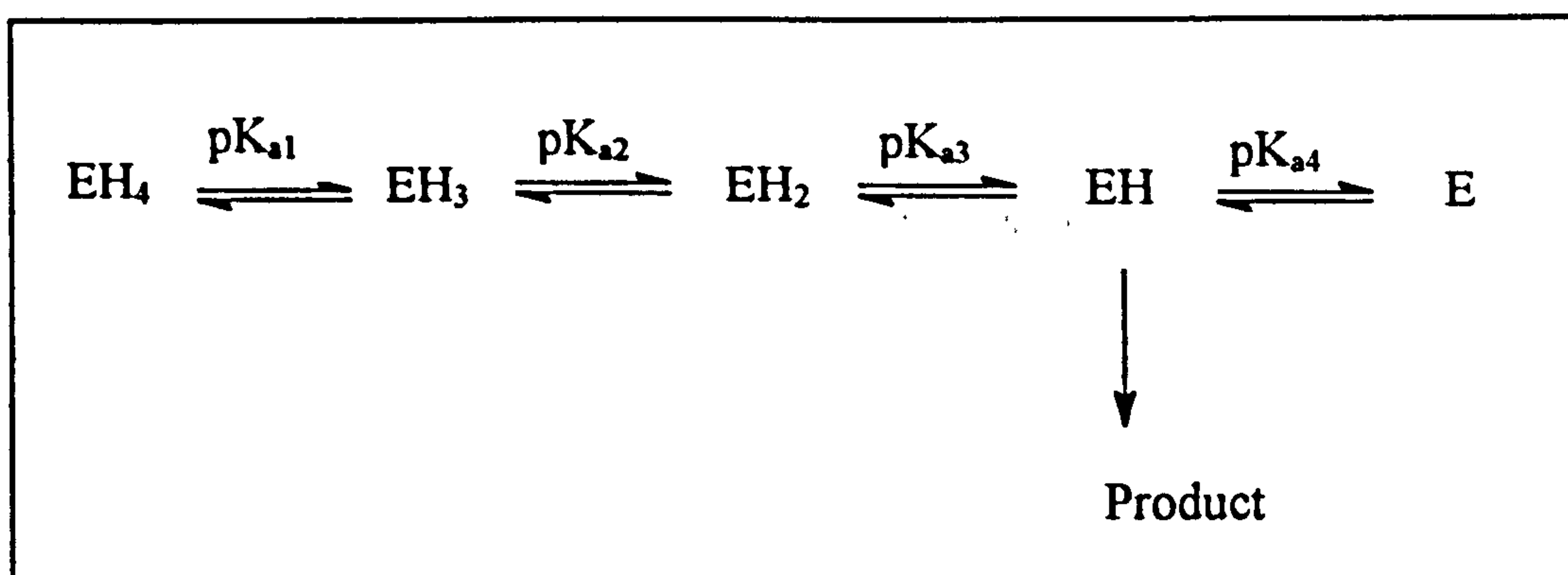
Scheme 3-2

Scheme 3-2 shows the possible ionisations involved when the enzyme binds one or two zinc ions.

When the enzyme is mononuclear, enzyme activity is observed when the two important groups zinc-bound water and Asp90 are in their deprotonated forms. The apparent pK_a values of these two group have been previously determined during the pH-rate profile studies and were found to be indiscernible from 5.6. Therefore below pH 5.6, protonation of these groups generates an inactive enzyme.

When the enzyme binds two zinc ions, it is again inactive when the two catalytically important groups, zinc-bound water and Asp90 are in their protonated forms. The second metal ion binds weakly as its coordination is modulated by the active site and K_d for the second zinc was found to be 24 mM^{43,44}. Increasing zinc concentrations drives the equilibrium toward the binuclear enzyme. The binding of the second zinc lowers the apparent pK_a value of the catalytically important groups and so generates active enzyme at lower pH than in the mononuclear case.

Assuming that three ionisations of the enzyme shown in Scheme 3-3, pK_{a1} , pK_{a2} and pK_{a3} , are indiscernible from each other, then curve fitting of the pH-rate profile was achieved using Equation 3.2 and are plotted in Figure 3.16. This allows us to calculate the apparent pK_a on the acidic limb for the pH-rate constant ($\log k_{cat}/K_m$) profiles of the β -lactamase II catalysed hydrolysis of benzylpenicillin at different zinc concentrations.



Scheme 3-3

$$k_{\text{obs}} = \frac{k^{\text{max}}}{(2 \times (\text{H}^+) / K_{a2}) + (\text{H}^+)^3 / K_{a3}K_{a1}K_{a2} + 1 + K_{a4} / (\text{H}^+)}$$

Equation 3.2

The results are summarized in Table 3-23 and show that the apparent acidic pK_a value of the enzyme is significantly affected by the zinc concentration decreasing from 5.91 at low zinc concentration to 4.47 for 10^{-2} M zinc.

Table 3-23

<i>Zinc concentration (M)</i>	<i>Calculated apparent pK_a values</i>
1.0×10^{-2}	4.47 ± 0.1
1.0×10^{-3}	4.76 ± 0.1
1.0×10^{-4}	5.12 ± 0.1
1.0×10^{-5}	5.46 ± 0.1
1.0×10^{-6}	5.91 ± 0.2

The same effect of the zinc concentration on the enzyme activity at low pH has been previously reported with carboxypeptidase A¹⁵⁷ and also with matrilysin¹⁵⁸, a matrix metallo-proteinase whose pH rate-dependence at low pH is measured with excess zinc chloride to ensure maximal activity. In these cases, the metal ion pH dependence is thought to be due to a loss of the catalytically important zinc ion upon lowering of the pH¹⁵⁷.

Valladares *et al.*⁷⁶ report the inactivation of *A. hydrophila* metallo- β -lactamase upon addition of a second zinc to the normally monozinc enzyme. A pure noncompetitive inhibition is observed with a K_i of 46 μ M. This phenomenon is reversible upon dialysis of the enzyme against a zinc free buffer. This enzyme requires only one zinc for full catalytic activity. They also reported that the metal-ion dependency of the activity was strongly pH dependent.

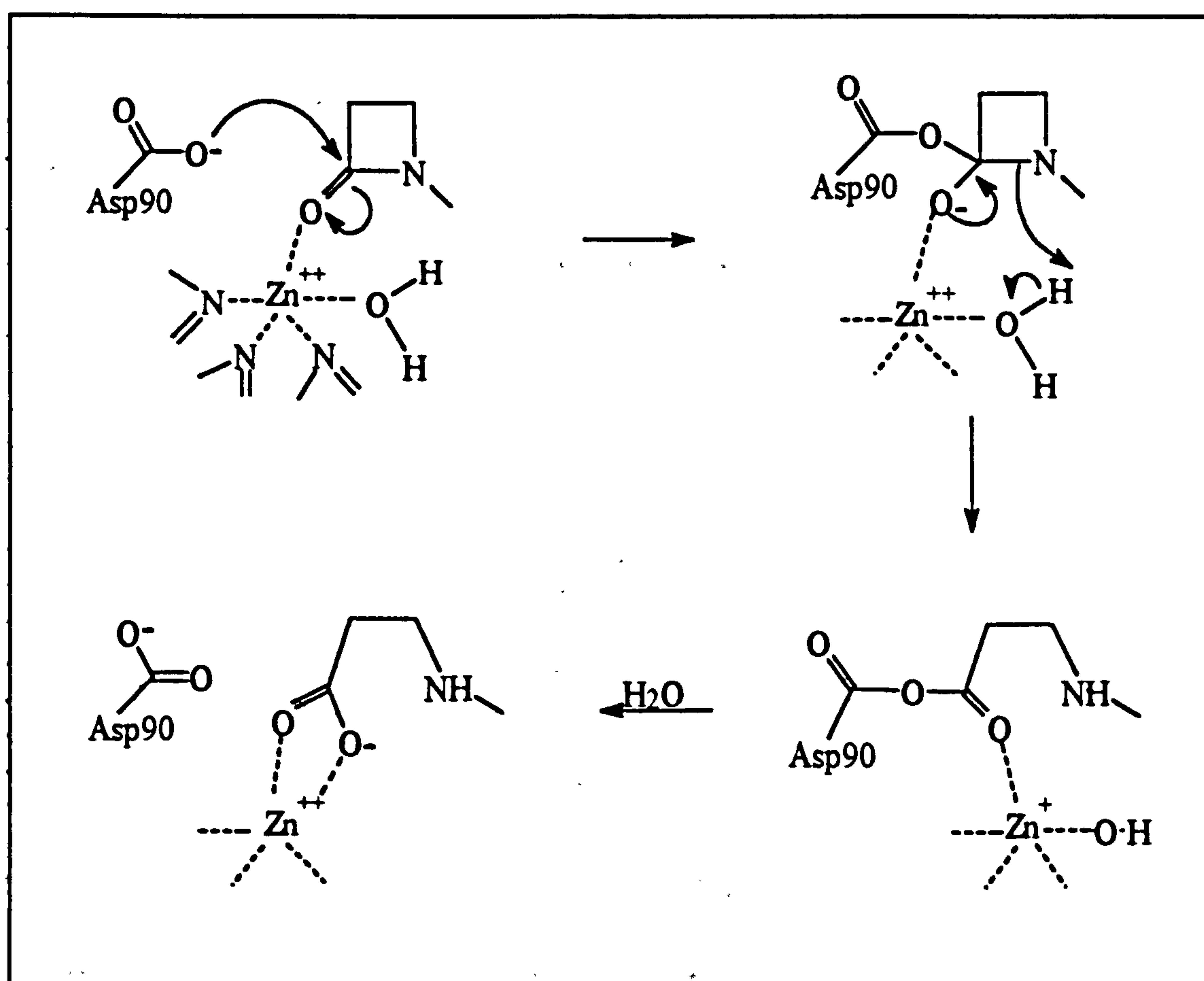
Carboxypeptidase A is also similarly inhibited by an excess of zinc in the pH range 7-9¹⁵⁹, such inhibition is thought to be due to the binding of a second zinc ion to the enzyme active site.

The influence of the zinc concentration on enzyme activity is very different for the *B. fragilis* metallo- β -lactamase for which the binding of both zinc ions is required for full catalytic activity⁷³. However, for some *B. fragilis* strains, the inhibition of the metallo- β -lactamase activity occurs as the concentration of zinc sulfate was increased beyond the concentration which allowed maximum activity¹⁶⁰. The binding of a second zinc has been reported not to be essential by Carfi *et al.*⁷³ for most class B β -lactamases and it was therefore suggested that the tightly bound zinc2 in *B. fragilis* metallo- β -lactamases was the result of an evolutionary phenomenon⁷³.

From our study of the effect of excess zinc on the β -lactamase II activity, the second zinc ion was found not to be important for catalysis at physiological pH. However its binding at low pH restores the enzyme activity.

3.5 Methanolysis

Site directed mutagenesis studies on β -lactamase II have found Aspartate 90 to be kinetically important⁵⁸. A possible mechanism for β -lactamase II catalysis is that the hydrolysis of the β -lactam proceeds through nucleophilic attack of the aspartate group forming an anhydride intermediate (Scheme 3-4).



Scheme 3-4 Nucleophilic catalysis by Asp 90

Methanol is a better nucleophile than water toward many substrates and could react with the anhydride intermediate, forming the methyl ester in preference to the hydrolysis product.

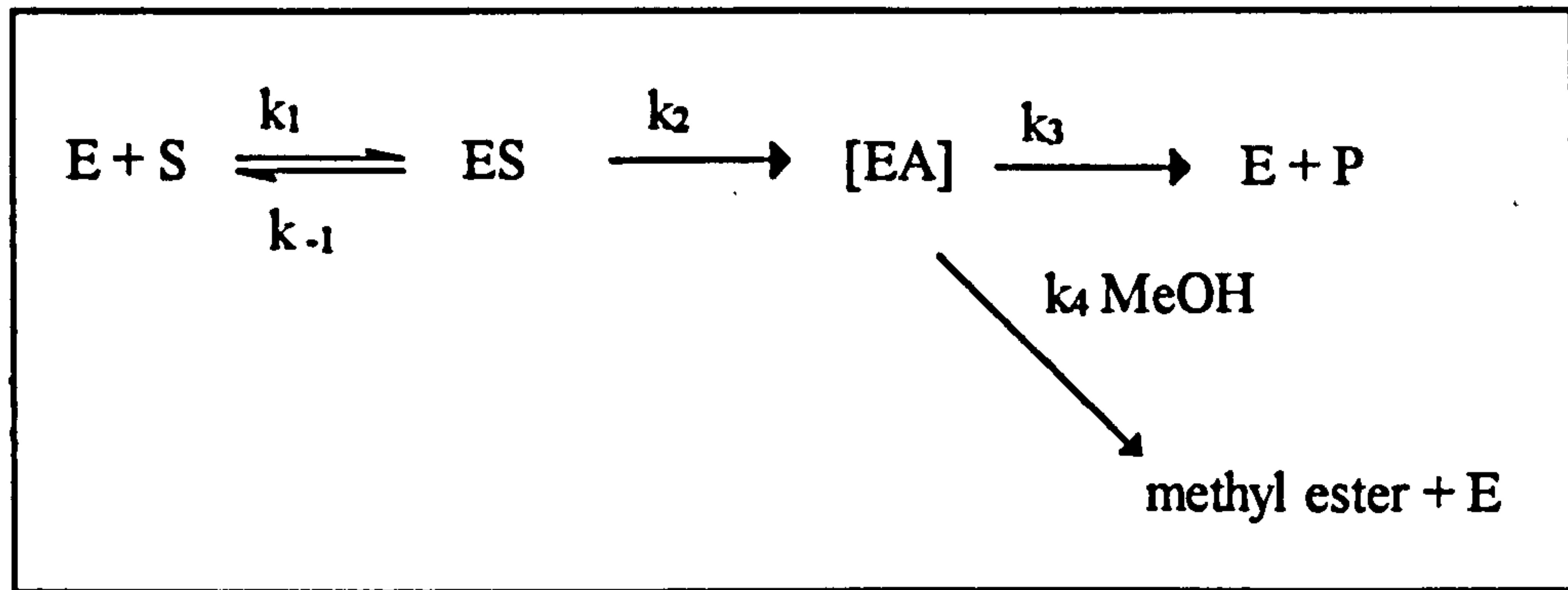
The effect of methanol concentration on the rate constant, k_{cat} , of the β -lactamase II catalysed hydrolysis of cefuroxime was investigated at pH 7.0. Cefuroxime was used as a substrate because of its small K_m value³⁴ ($45 \pm 5 \mu\text{M}$) when compared with benzylpenicillin and cephaloridine, and k_{cat} was easily measured under saturation conditions. The results are presented in Table 3-24.

Table 3-24 Effect of methanol concentration on the rate constant (k_{cat}) of the β -lactamase II catalysed hydrolysis of cefuroxime at pH 7.0, 30 °C and ionic strength 1.0 M (NaCl). Enzyme concentration 0.02 μM , zinc concentration 0.4 μM

<i>Methanol concentration (M)</i>	<i>k_{cat} (s^{-1})</i>
0.00	29.0
0.50	29.1
0.75	28.3
1.00	28.9

If the catalytic mechanism involves the formation of an enzyme-anhydride intermediate [EA] the overall process can be represented as in Scheme 3-5 and k_{cat} would be given by Equation 3.3. In the presence of methanol, both hydrolysis and methanolysis could occur competitively as a function of methanol concentration. If $k_2 \gg k_3 + k_4(\text{MeOH})$ the rate of anhydride formation is faster than its breakdown and k_{cat} would be given by $k_3 + k_4(\text{MeOH})$, the rate of degradation of the anhydride. In this case k_{cat} would increase with the methanol concentration as the intermediate

accumulates. If $k_3 + k_4(\text{MeOH}) \gg k_2$, then k_{cat} would reflect the rate of formation of the anhydride intermediate and therefore would be unaffected by the methanol concentration.



Scheme 3-5

$$k_{\text{cat}} = \frac{k_2 k_3 k_4 (\text{MeOH})}{k_2 + k_3 + k_4 (\text{MeOH})}$$

Equation 3.3

Table 3-24 summarizes the results of this experiment. No effect due to the methanol concentration was observed and there was no detectable enzyme catalysed formation of a methyl ester. This is indicative, but not proof, that the anhydride mechanism does not occur.

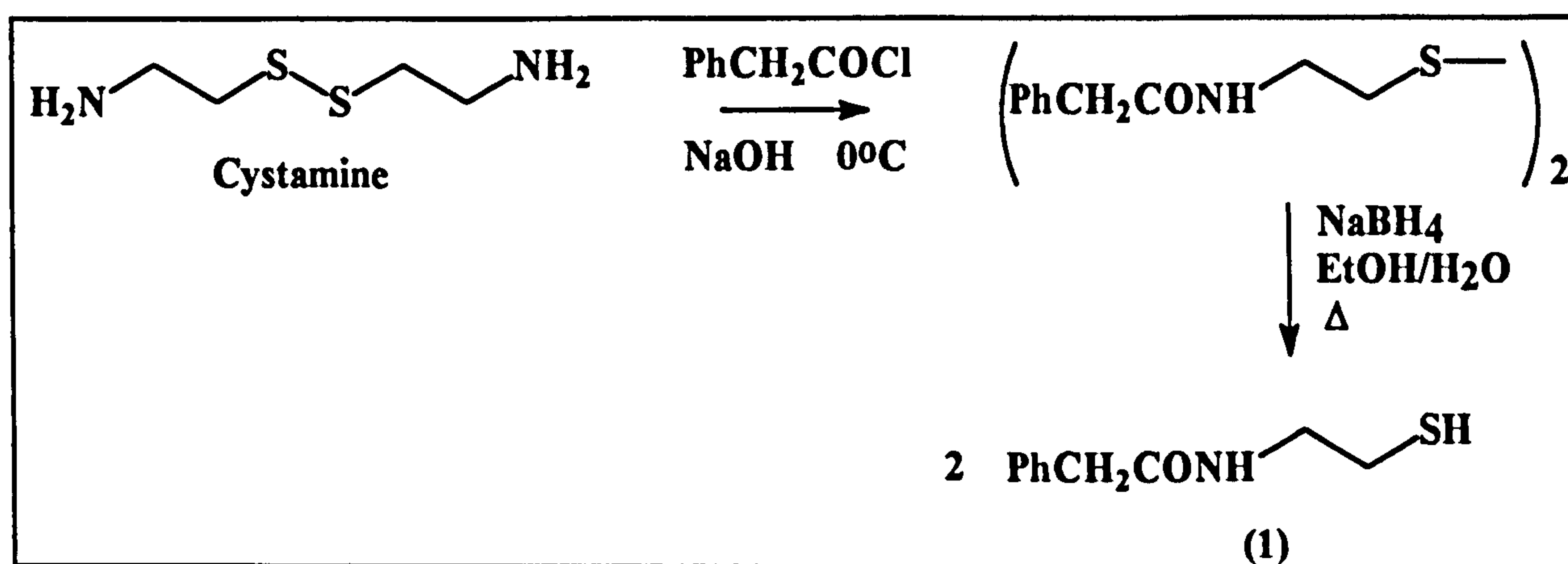
3.6 Inhibition studies

3.6.1 Synthesis

As previously reported, an efficient strategy to prepare metalloenzyme inhibitors was to design compounds with a thiol group, which would be a better chelating agent to the active site than the substrate.

Here, we report the main synthetic scheme followed for the preparation of the compounds tested as inhibitors during this work.

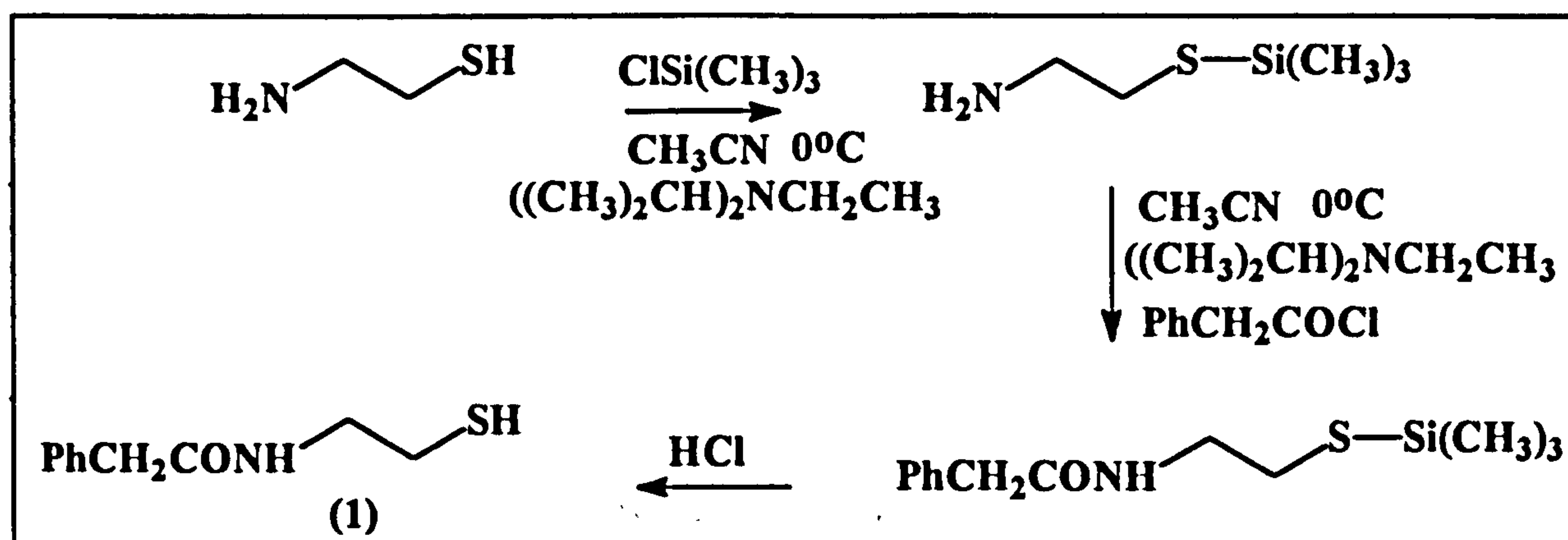
N(2-mercaptoethyl)phenylacetamide (1) was prepared according to Scheme 3-6.



Scheme 3-6 Synthesis of N(2-mercaptoethyl)phenylacetamide (1)

Cystamine was reacted with phenylacetyl chloride under Schotten-Baumann conditions. The final product (1) was obtained by reduction of the disulfide to the thiol with sodium borohydride.

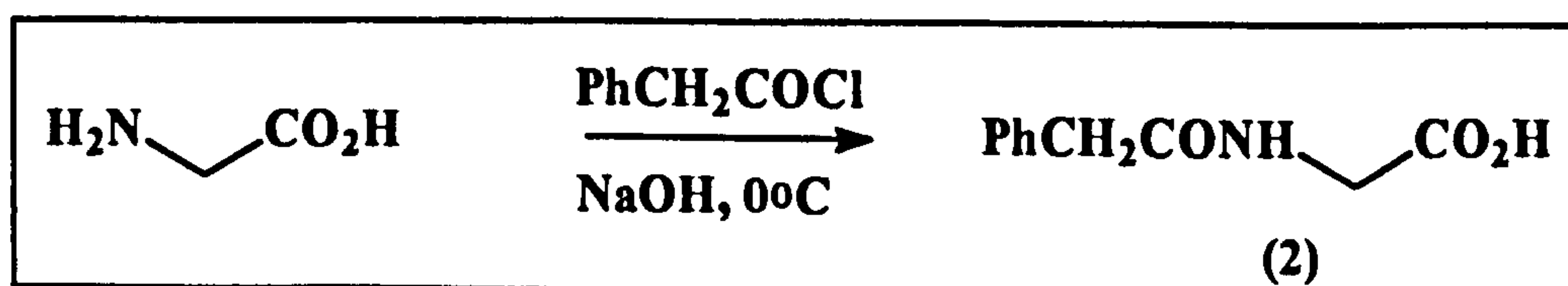
The synthesis of this compound has been previously reported in a patent concerning the preparation of N-protected 2-aminoethanethiols¹⁶¹. The synthesis proceeds via the silyl protected sulfur intermediate of the 2-aminoethanethiol (Scheme 3-7).



Scheme 3-7 Synthesis of N(2-mercaptoethyl)phenylacetamide (1)

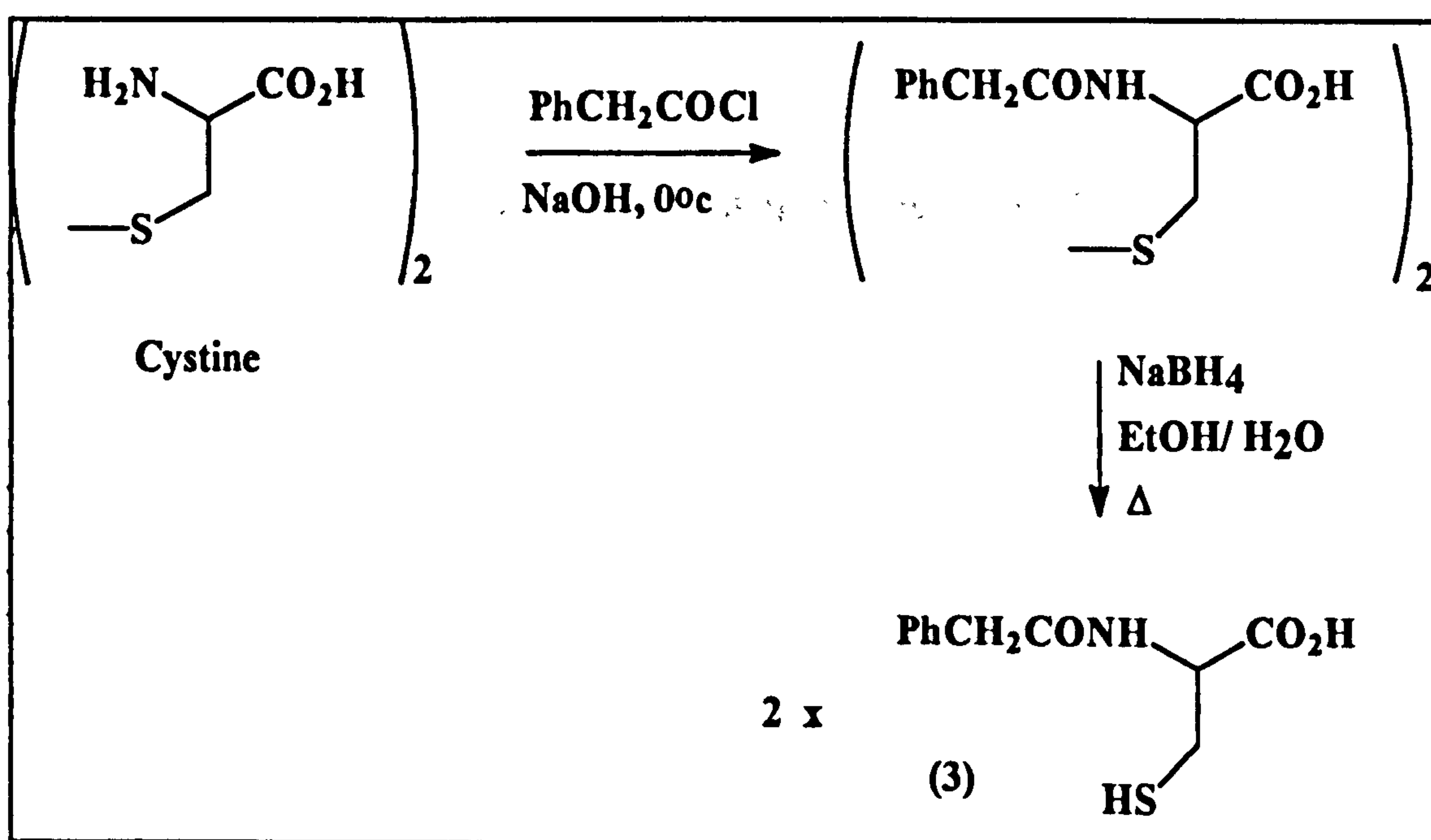
2-Aminoethanethiol was reacted with trimethylchlorosilane in the presence of base, the product formed was then acylated with phenylacetyl chloride. The thiol was subsequently deprotected by hydrolysing the silyl intermediate with dilute aqueous acid (yield 95 %).

N-Phenylacetyl glycine (2) was synthesised by reacting glycine with phenylacetyl chloride under Schotten-Baumann conditions (Scheme 3-8). The reaction was straightforward and a good yield was obtained.



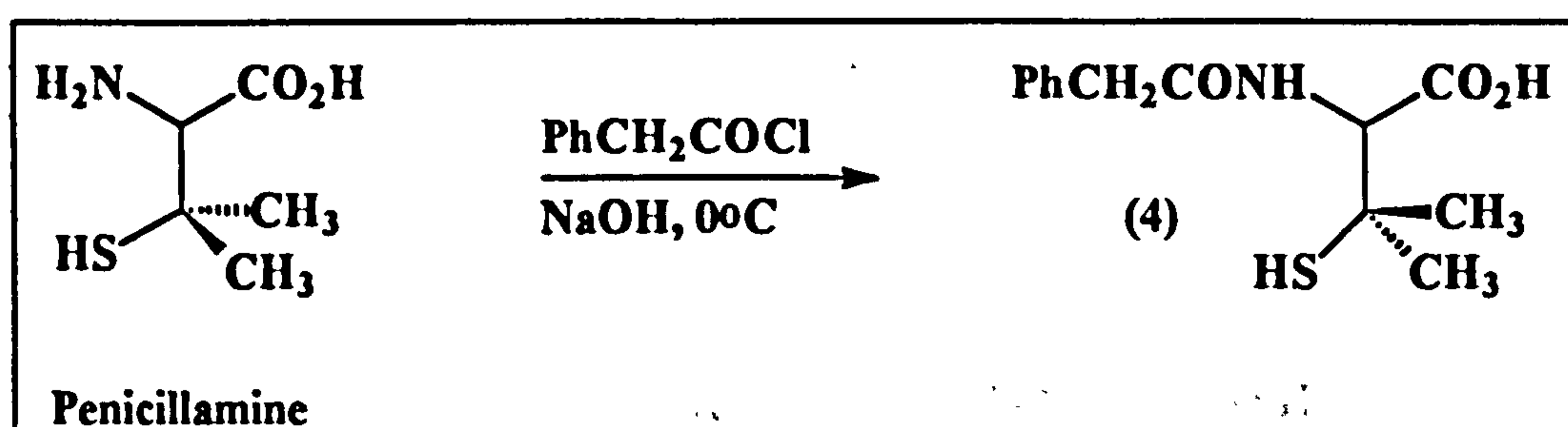
Scheme 3-8 Synthesis of N-phenylacetyl glycine (2)

N-Phenylacetyl-cysteine (3) was synthesised according to the route shown in Scheme 3-9. Reaction of cystine with phenylacetyl chloride produced N,N'-phenylacetylcystine which was subsequently reduced to the corresponding thiol (3) with sodium borohydride. The reduction of the disulfide to the thiol was confirmed by ^{13}C -NMR analysis, as a change in the chemical shift for the βCH_2 was observed going from 40 ppm for the disulfide to 26 ppm for the thiol (this was the case for all the thiols synthesised during this work).



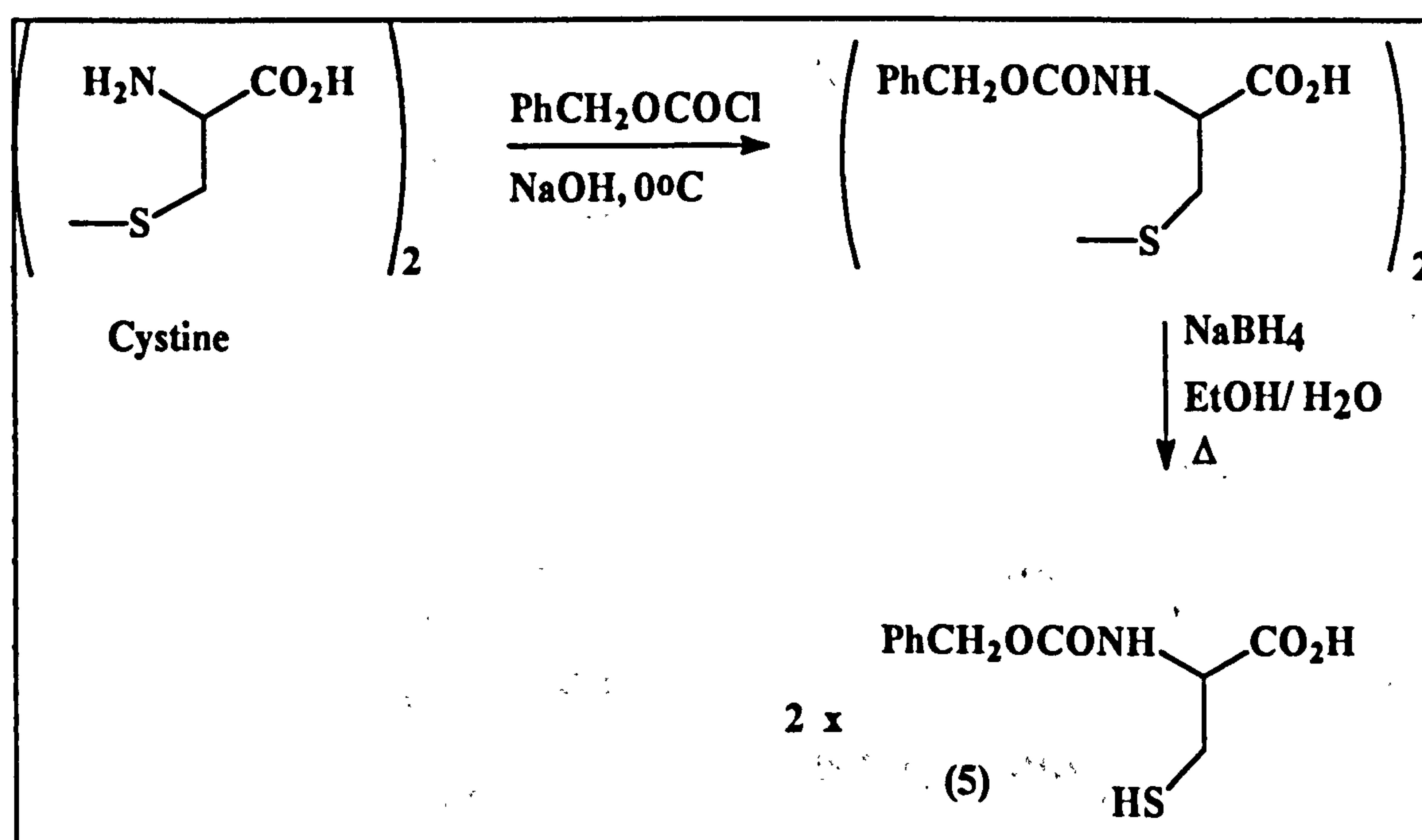
Scheme 3-9 Synthesis of N-phenylacetyl-cysteine (3)

N-Phenylacetyl-penicillamine (4) was obtained by reacting penicillamine with phenylacetyl chloride under Schotten-Baumann conditions (Scheme 3-10). There was no need to protect the thiol group during the reaction because of the steric hindrance around it.



Scheme 3-10 Synthesis of N-phenylacetyl-penicillamine (4)

Because of the difficulties encountered when attempting to prepare N-phenylacetylcysteinyl dipeptides, peptides with N-carbobenzoxy side chains were used for the following syntheses. N-Carbobenzoxy-cysteine (5) was prepared by reacting cystine with carbobenzoxy chloride and subsequently reducing the disulfide to the thiol using sodium borohydride (Scheme 3-11).



Scheme 3-11 Synthesis of N-carbobenzoxy-cysteine (5)

Scheme 3-12 shows the protocol used for the preparation of the N-carbobenzoxy-cysteinyl-amino acids: N-carbobenzoxy-cysteinyl-glycine (6), carbobenzoxy-cysteinyl-alanine (7), N-carbobenzoxy-cysteinyl-valine (8), N-carbobenzoxy-cysteinyl-leucine (9), N-carbobenzoxy-cysteinyl-serine (10), N-carbobenzoxy-cysteinyl-penicillamine (11), N-carbobenzoxy-cysteinyl-phenylalanine (12) and N-carbobenzoxy-cysteinyl-proline (13) shown in Figure 3.17.

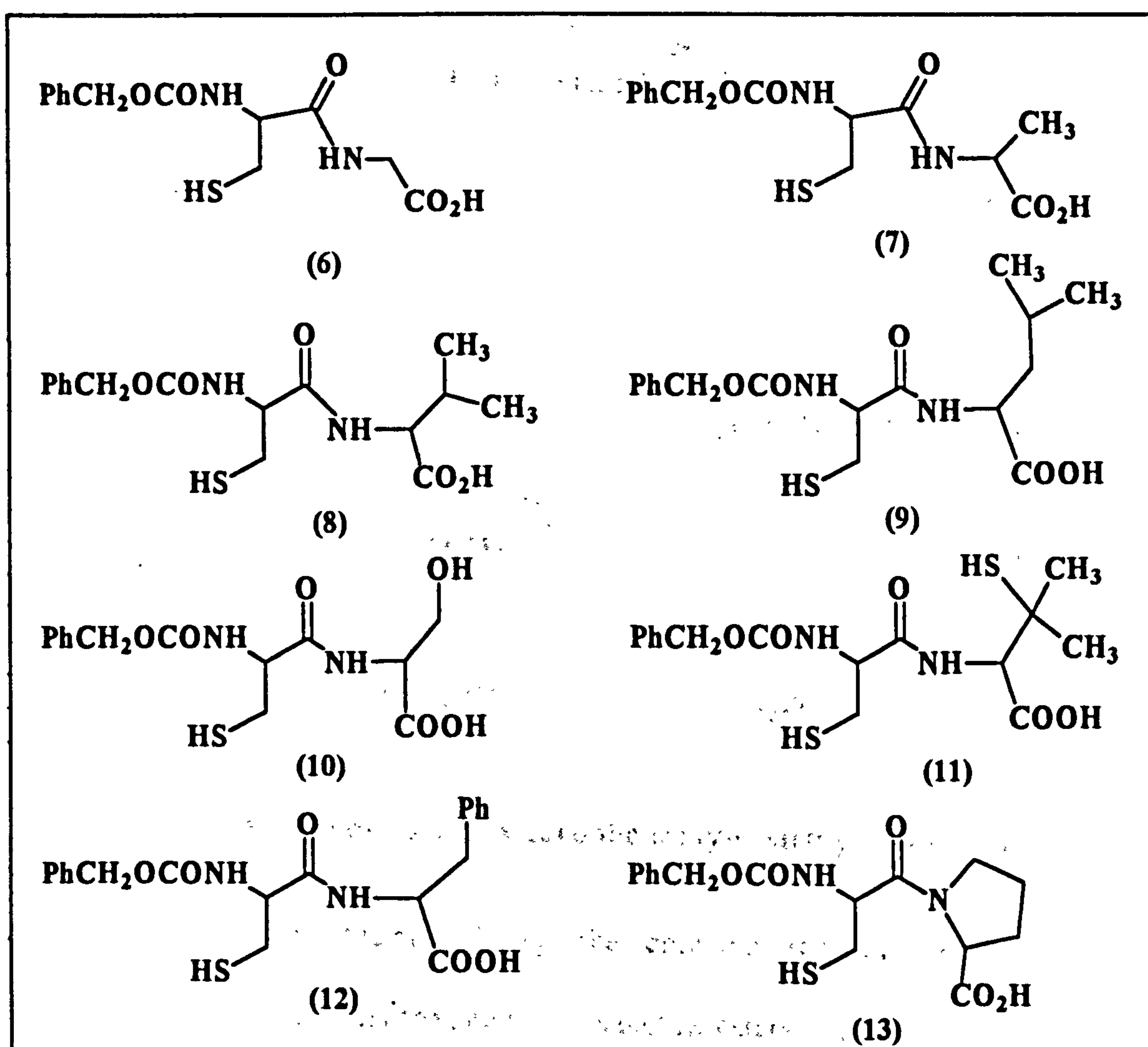
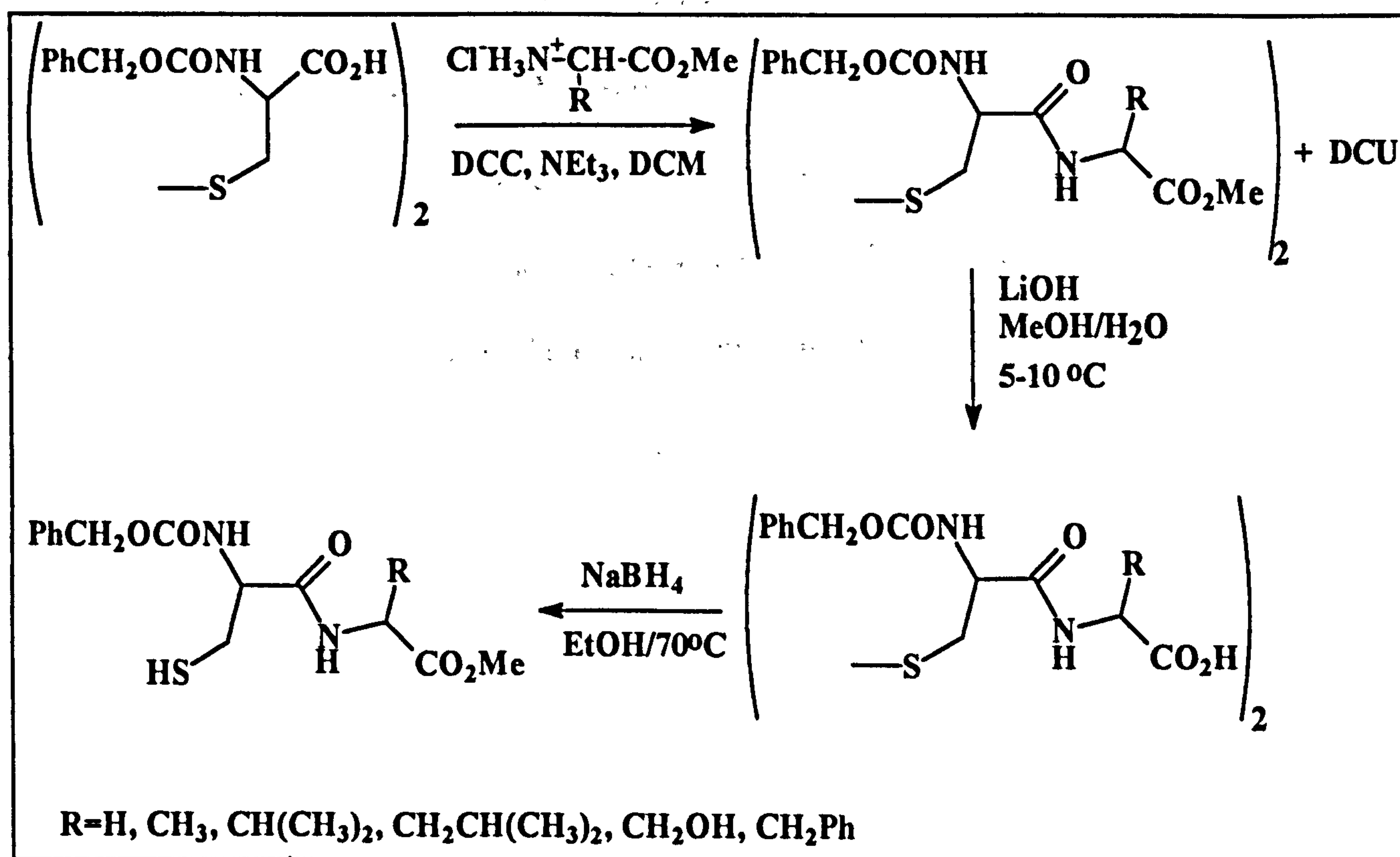


Figure 3.17

The dipeptides were prepared by coupling N,N'-dicarbobenzoxy-cystine with a series of amino acid methyl esters in the presence of dicyclohexylcarbodiimide. The methyl esters were subsequently deprotected by hydrolysis with lithium hydroxide. In the next step, the reduction of the disulfide with sodium borohydride led to the corresponding thiols.



Scheme 3-12 Synthesis of N-carbobenzoxy-cysteiny-amino acids

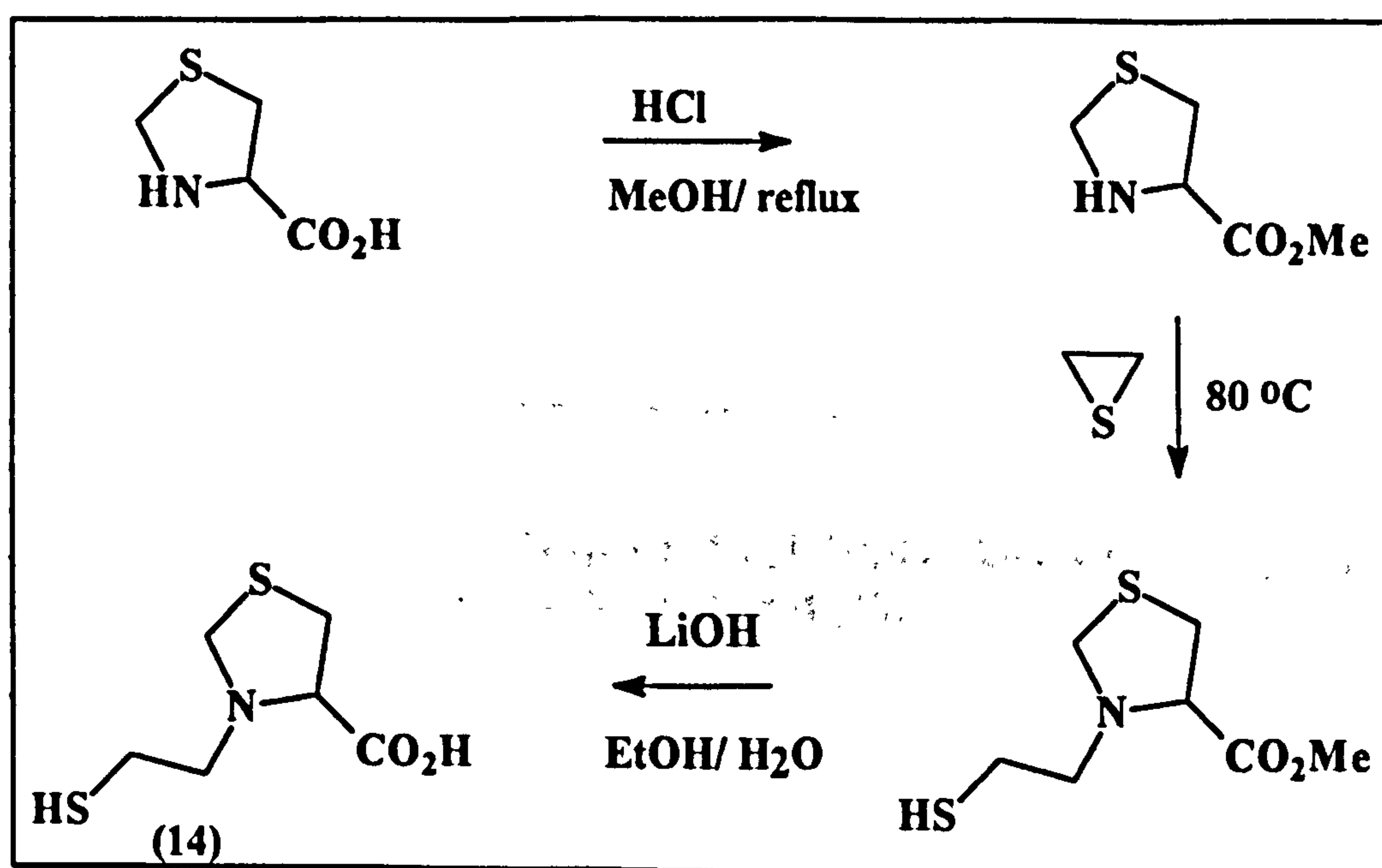
Good results were obtained during the coupling reaction between N,N'-dicarbobenzoxy-cystine and the amino acids methyl esters (Yield 80 - 95 %).

The hydrolysis step with lithium hydroxide led to 50 - 80 % yield for most of the dipeptide hydrolyses except for (Z-Cys-PheOH)₂ for which very low yields were obtained (< 20 %). This was mainly due to the poor solubility of (Z-Cys-PheOMe)₂.

in methanol. Attempts to improve the yield were made using different solvents during the hydrolysis procedure without significant success.

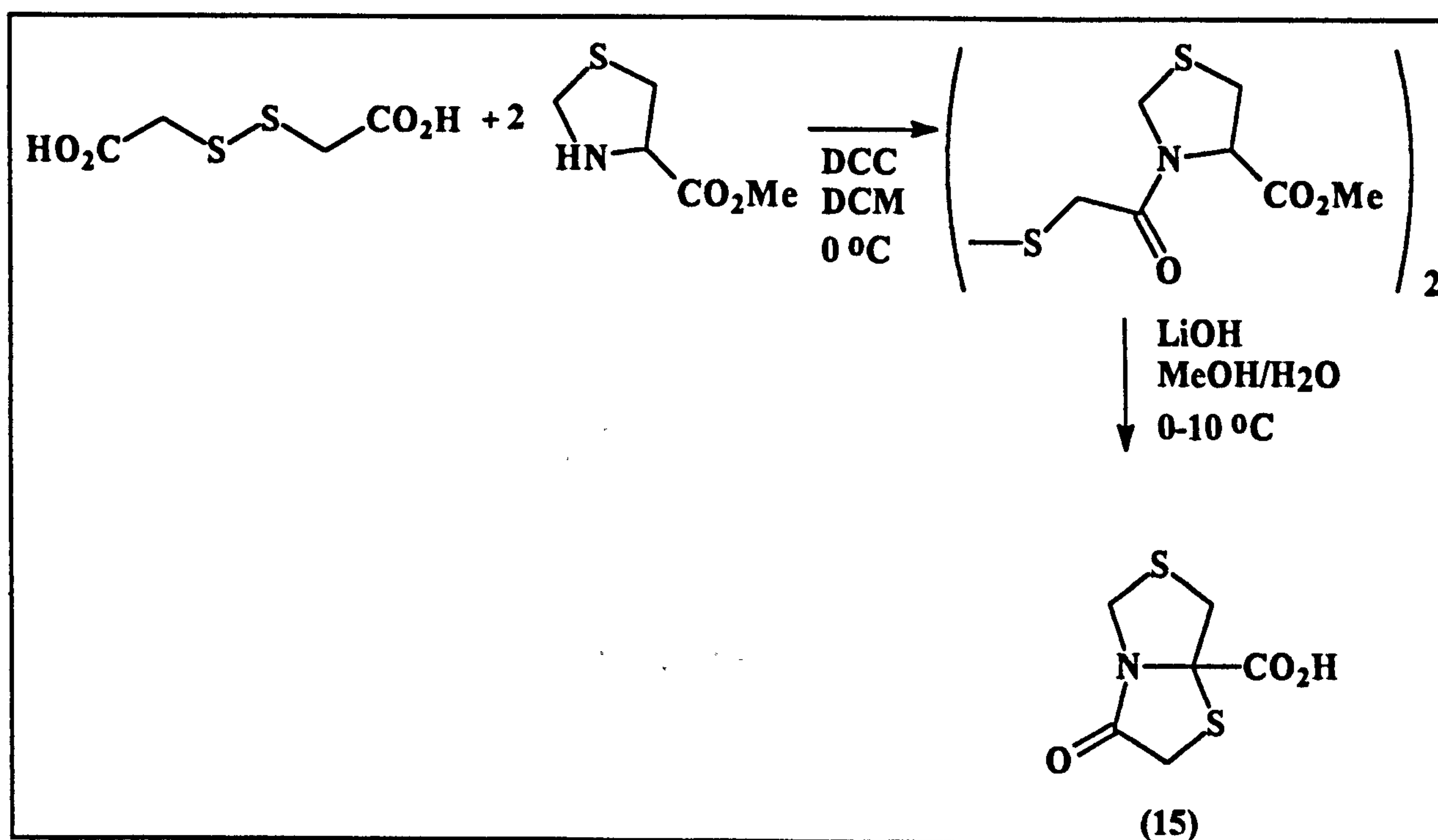
Reduction of the disulfide dipeptides to the corresponding thiols gave good yields (> 95 %). N-carbobenzoxy-cysteinyl-penicillamine (11) was obtained as the reduced compound and immediately tested as an inhibitor. However, oxidation occurred over a month as shown by NMR analysis and ESMS also showed that a disulfide bond was formed. The ^{13}C NMR showed no change in the chemical shift of $\text{CH}_2\text{-SH}$ on the cysteinyl side chain but the βC carbon of penicillamine side chain changed from 45 ppm to 57 ppm and so it was concluded that an intramolecular disulfide bond between the cysteine thiol and the penicillamine thiol was probably formed.

N-mercaptoethyl-4-L-thiazolidinecarboxylic acid (**14**) was synthesised via the route shown in Scheme 3-13. 4-thiazolidinecarboxylic acid was protected as the methyl ester in the first step, then thioethylation of the methyl-4-L-thiazolidinecarboxylate amine with ethylene sulfide gave the N-mercaptoethyl-4-L-thiazolidinecarboxylic acid methyl ester. At this stage a mixture of product/starting material was obtained (3:1). The methyl ester was subsequently hydrolysed using lithium hydroxide. Several attempts to separate the product (**14**) from the 4-thiazolidinecarboxylic acid were made, such as liquid chromatography, to purify the product. Unfortunately, the compounds decomposed on the column. The product was finally isolated as a yellow precipitate. The ^{13}C NMR showed that the majority of the compound was the disulfide of (**14**) but ESMS revealed the presence of other compounds. The disulfide was reduced using sodium borohydride but no further studies were made on the compound.



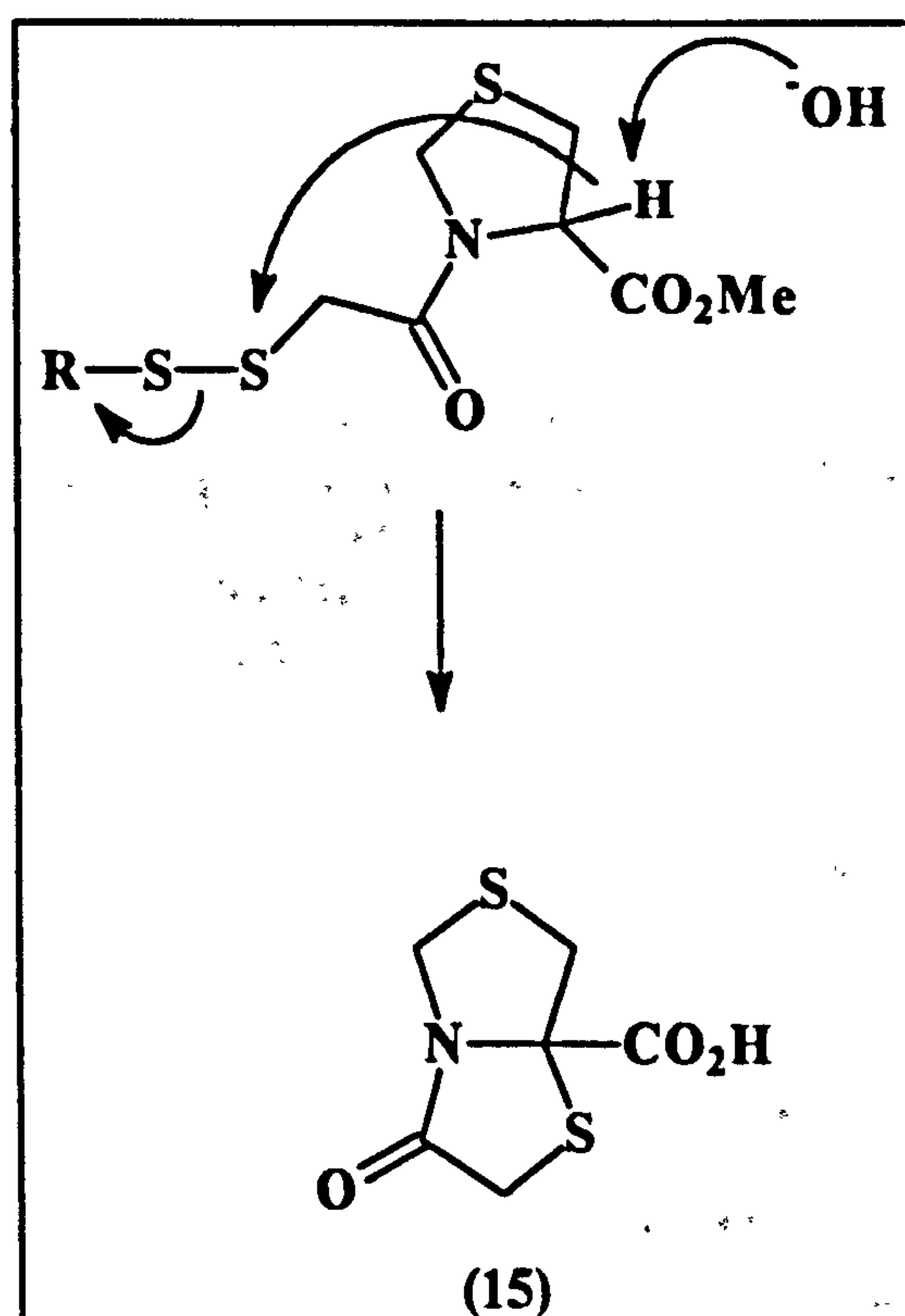
Scheme 3-13 Synthesis of N-mercaptoethyl-4-thiazolidinecarboxylic acid (**14**)

3-Oxoperhydro[1.3]thiazolo[4.3-b][1.3]thiazole-7a-carboxylic acid (15) was obtained when attempting to prepare N-mercaptoacetyl-4-thiazolidinecarboxylic acid (16) following the route shown in Scheme 3-14. Dithioglycolic acid was coupled with 4-thiazolidinecarboxylic acid methyl ester in the presence of dicyclohexylcarbodiimide. The product of the reaction was then hydrolysed with lithium hydroxide and the main product isolated was 3-oxoperhydro[1.3]thiazolo[4.3-b][1.3]thiazole-7a-carboxylic acid (15).



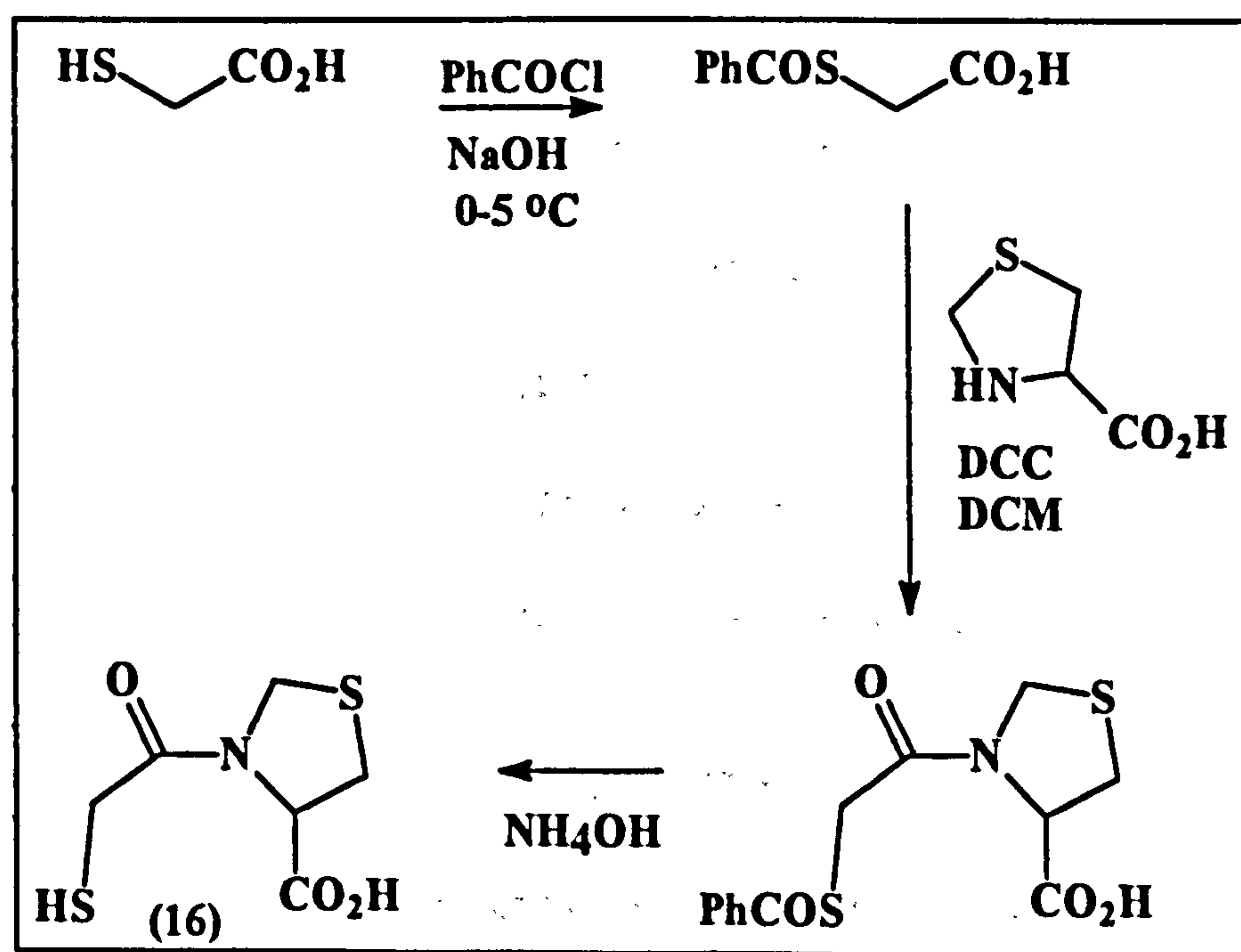
Scheme 3-14 Synthesis of 3-oxoperhydro[1.3]thiazolo[4.3-b][1.3]thiazole-7a-carboxylic acid (15)

The compound (15) is thought to be produced according to the mechanism outlined in Scheme 3-15. The product of the coupling reaction undergoes cyclisation when treated with lithium hydroxide. The cyclisation reaction may occur faster than the hydrolysis of the methyl ester.



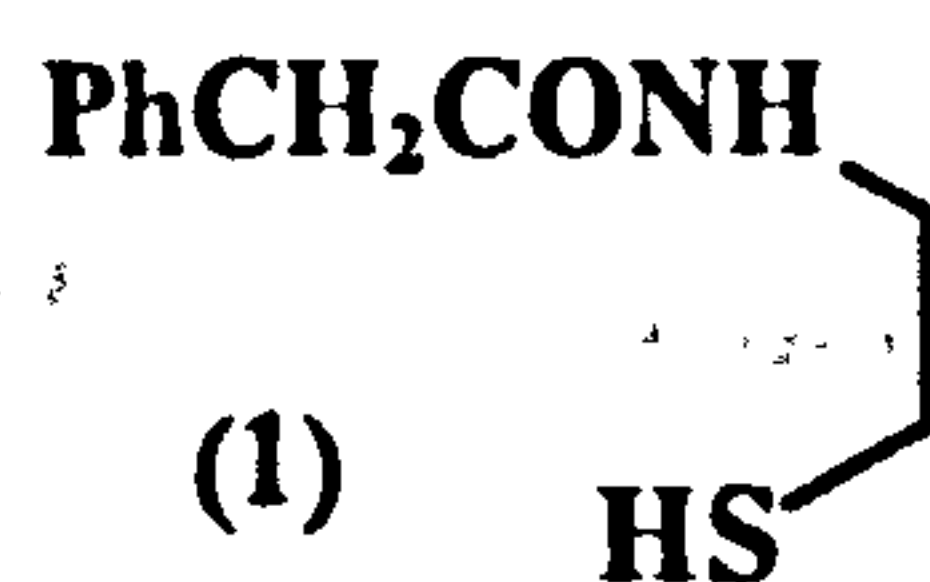
Scheme 3-15

After failing to prepare N-mercaptoacetyl-4-thiazolidinecarboxylic acid (16) (Scheme 3-14) a different route was followed (Scheme 3-16). Mercaptoacetic acid was first S-benzoylated with benzoyl chloride under Schotten-Baumann conditions. The protected carboxylic was then coupled with 4-thiazolidinecarboxylic acid. The thiol was deprotected with NH_4OH , and attempts were made to purify the product by preparative TLC. The purification was not successful, as revealed by the NMR of the product isolated.



Scheme 3-16 Synthesis of N-mercaptoacetyl-4-thiazolidinecarboxylic acid (16)

3.6.2 Inhibition studies with *N*(2-mercaptoethyl)phenylacetamide (1)



It is known that compounds like captopril interact with the zinc ion in the active site of the metallo-enzyme ACE¹⁶². Its design was based on a substrate related structure with the introduction of a thiol group to induce coordination to the zinc ion of the enzyme¹⁶³. Thiol compounds have been found to be good inhibitors of metallo-enzymes^{108,164} and, by analogy, potential inhibitors of β -lactamase II have been synthesised and tested against β -lactamase II during this work. They are all related to the primary structure of the substrate benzylpenicillin and most of them contain a thiol group which is a potential ligand for the metal ion.

The effect of varying the concentration of *N*(2-mercaptoethyl)-phenylacetamide (1) on the rate (k_{cat}/K_m) of the β -lactamase II catalysed hydrolysis of cephaloridine was studied and the results are shown in Table 3-25.

Table 3-25 Effect of thiol (1) on the rate (k_{cat}/K_m) of the β -lactamase II catalysed hydrolysis of cephaloridine at pH 7.0, 30 °C and ionic strength 1.0 M (NaCl). Enzyme concentration 0.1 μ M, zinc concentration 2 μ M

<i>Thiol (1) (M)</i>	<i>k_{cat}/K_m ($M^{-1} s^{-1}$)</i>
0.00 x 10 ⁻⁴	6.68 x 10 ⁴
1.00 x 10 ⁻⁴	3.25 x 10 ⁴
2.00 x 10 ⁻⁴	2.23 x 10 ⁴
3.00 x 10 ⁻⁴	1.74 x 10 ⁴
4.00 x 10 ⁻⁴	1.44 x 10 ⁴
5.00 x 10 ⁻⁴	1.15 x 10 ⁴
6.00 x 10 ⁻⁴	1.01 x 10 ⁴

The results were plotted as k_{cat}/K_m against inhibitor concentrations (Figure 3.18) and assuming competitive inhibition, the curve was fitted to the Michaelis-Menten equation for competitive inhibition with $S \ll K_m$ Equation 3.4.

$$\left(\frac{k_{cat}}{K_m}\right)_I = \left(\frac{k_{cat}}{K_m}\right)_O \cdot \left(\frac{K_i}{[I] + K_i}\right)$$

Equation 3.4

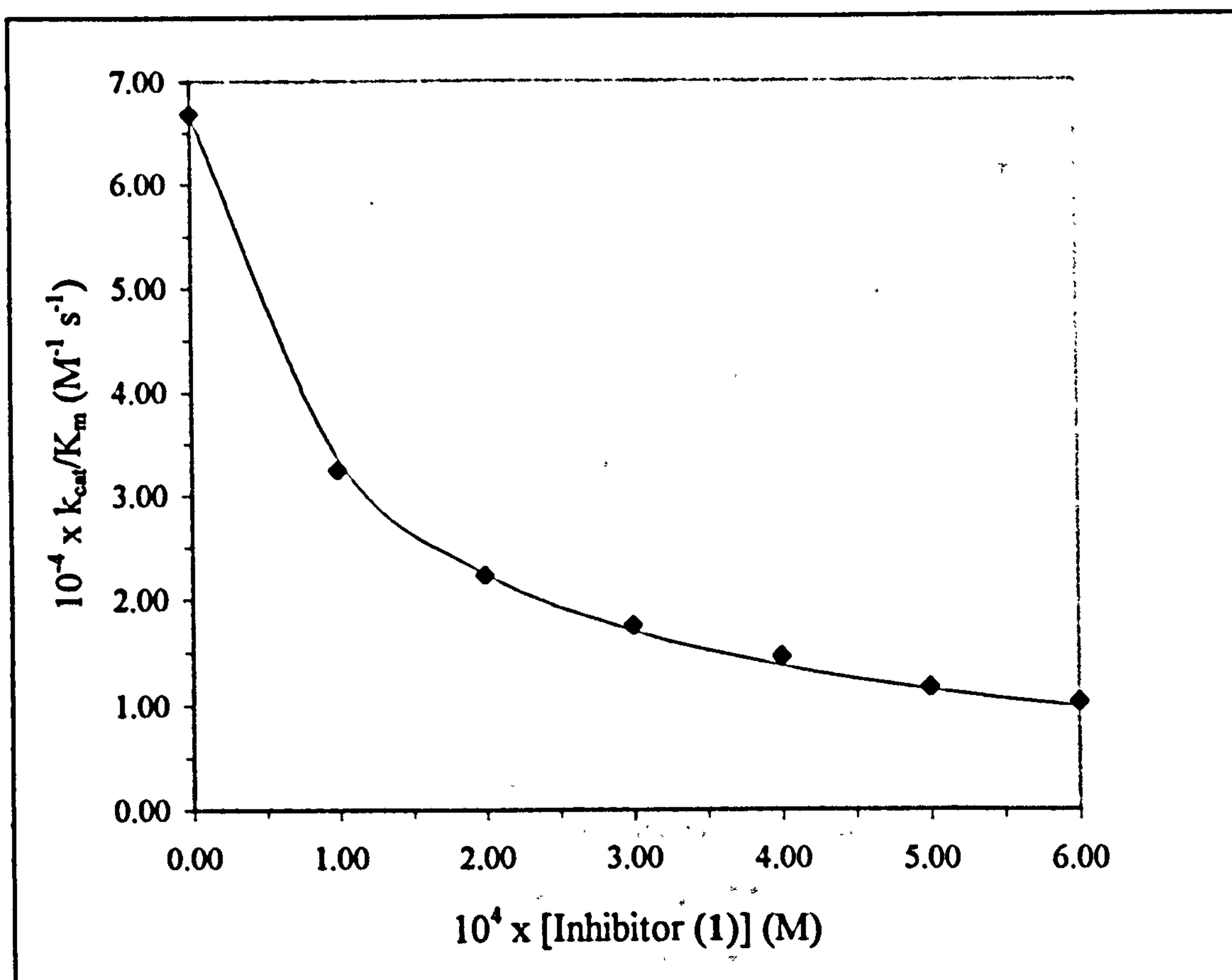


Figure 3.18 Effect of the N(2-mercaptoethyl)-phenylacetamide (1) concentration on k_{cat}/K_m for the β -lactamase II catalysed hydrolysis of cephaloridine. (The squares are experimental values and the solid line is calculated from the Equation 3.4).

When the results were plotted as K_m/k_{cat} against inhibitor concentrations (Figure 3.19), a linear relationship was obtained. This linear relationship between the two variables can be described by the Equation 3.5 which is indicative of competitive or mixed inhibition.

$$\left(\frac{K_m}{k_{\text{cat}}}\right)_i = \left(\frac{K_m}{k_{\text{cat}}}\right)_o + \left(\frac{K_m}{k_{\text{cat}}}\right)_o \left(\frac{[I]}{K_i}\right)$$

Equation 3.5

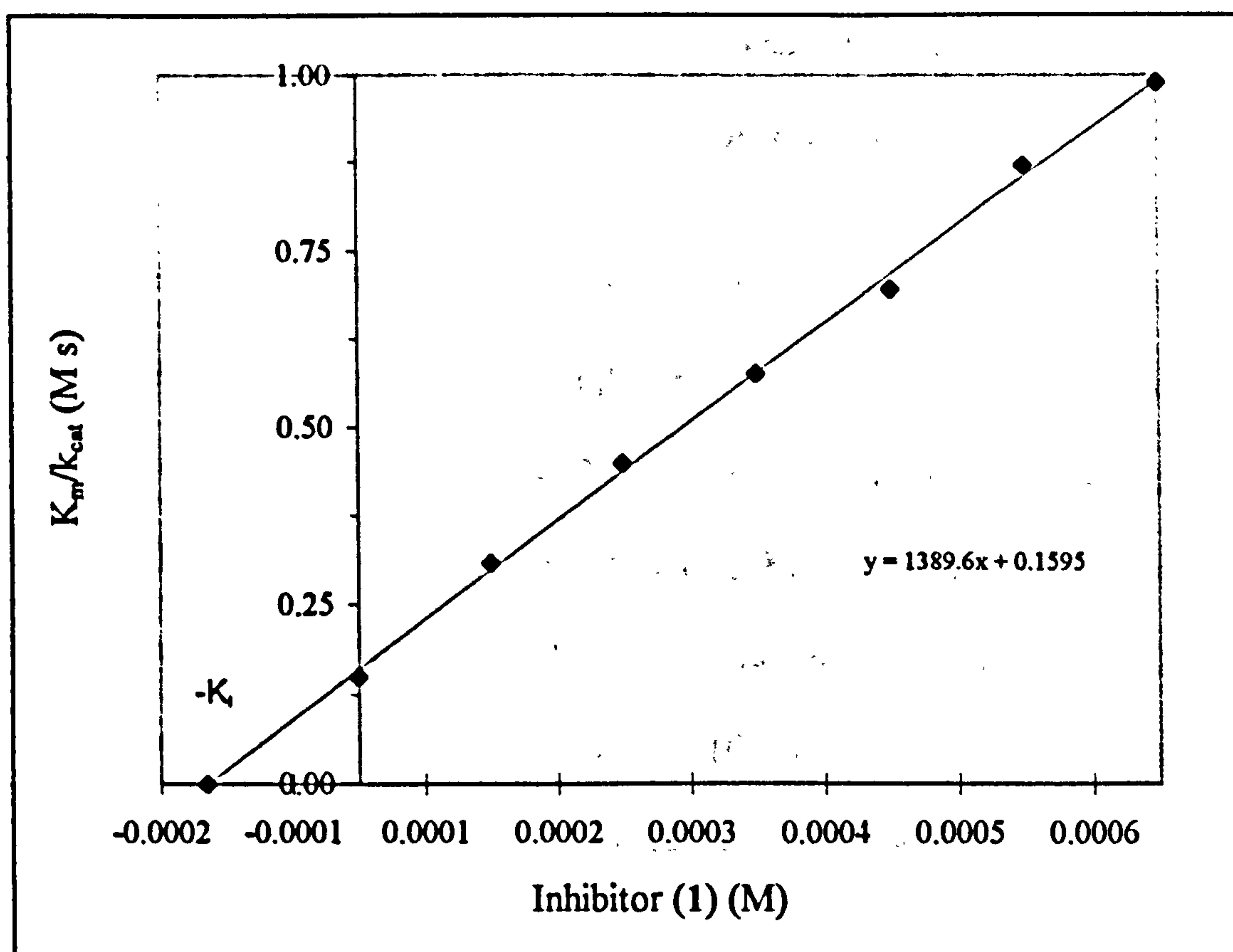


Figure 3.19 Plot of the N(2-mercaptoethyl)-phenylacetamide (1) concentration against K_m/k_{cat} for the β -lactamase II catalysed hydrolysis of cephaloridine

The plot of K_m/k_{cat} versus the inhibitor concentration is a straight line, from which K_i was extrapolated as the intercept on the x axis ($-K_i$).

From this study the N(2-mercaptoethyl)phenylacetamide (1) was found to behave as a competitive inhibitor of the β -lactamase II with a $K_i = 100.0 \mu\text{M}$, as the results obtained agreed with that obtained from Equation 3.4 for competitive inhibition, although a repeat of this experiment at different substrate concentrations would be ideal to verify this.

This inhibitor was chosen for more detailed mechanistic studies.

3.6.2.1 *pH dependence of pK_i for the β -lactamase II catalysed hydrolysis of β -lactams inhibited by N(2-mercaptoethyl)phenylacetamide (1)*

The inhibitory action of N(2-mercaptoethyl)phenylacetamide (1) on the β -lactamase II catalysed hydrolysis of β -lactams was studied as a function of pH. The inhibitor concentration used was 2×10^{-4} M. The substrate concentration being below K_m , the second-order rate constant k_{cat}/K_m was measured with and without inhibitor at different pH values. The K_i values were deduced from the Michaelis-Menten equation for competitive inhibition (Equation 3.4). The results are given in Table 3-26 as pK_i ($-\log K_i$) against pH for the studies of the hydrolysis of cephaloridine.

Table 3-26 The pH dependence of the inhibition dissociation constant (pK_i) of N(2-mercaptoethyl)phenylacetamide (1) for the β -lactamase II catalysed hydrolysis of cephaloridine at 30 °C and ionic strength 1.0 M (NaCl). Enzyme concentration 0.1-2 μ M, zinc concentration 2-40 μ M

<i>pH</i>	<i>pK_i (M)</i>	<i>pH</i>	<i>pK_i (M)</i>
4.47	3.10	7.00	4.08
4.73	3.42	7.50	4.13
4.78	3.67	8.00	4.19
5.04	3.80	8.50	4.17
5.06	3.65	9.51	4.12
5.25	4.11	9.75	3.95
5.60	4.03	10.00	3.80
6.00	4.07	10.24	3.64
6.50	4.09	10.50	3.54

The results were plotted as the pK_i versus pH, and are shown in Figure 3.20.

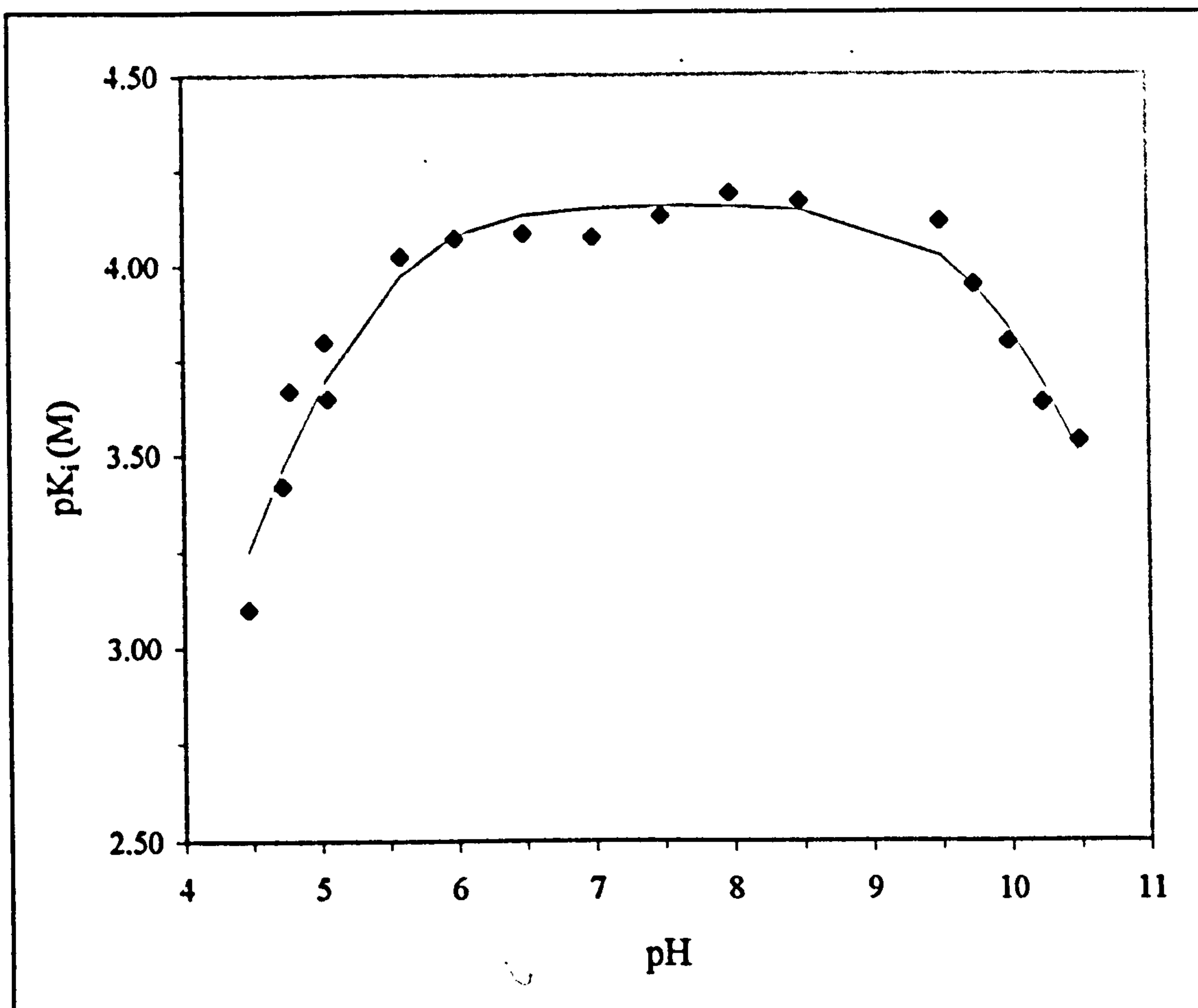


Figure 3.20 pH dependence of the inhibition constants (pK_i) of N(2-mercaptoethyl)phenylacetamide (1) for the β -lactamase II catalysed hydrolysis of cephaloridine

As shown on the above plot the curve is bell-shaped with slopes of 1, 0 and -1, indicating that the binding of the inhibitor to the enzyme is tightest between pH 6 and 9 and also pH independent. The affinity of the inhibitor for the enzyme falls off on the extremes of the profile as K_i increases (pK_i decrease) at high and low pHs.

Assuming that ionisation processes occur at the inflection points, the expression of K_i as a function of pH can be given by Equation 3.6.

$$K_{app} = \frac{K_i}{1 + (H^+)/K_{a1} + K_{a2}/(H^+)}$$

Equation 3.6

From a best fit calculation (solid line on the graph) using the equation above, approximate pK_a s were determined (Table 3-27)

Table 3-27

<i>Substrate</i>	<i>pK_{a1}</i>	<i>pK_{a2}</i>	<i>K_i (μM)</i>
Cephaloridine	5.32	9.93	67.4

In order to demonstrate that the K_i values obtained were independent of the nature of the substrate used to monitor enzyme activity, the pH dependence of pK_i for the inhibition of β -lactamase II by thiol (1) was also studied with benzylpenicillin as a substrate. Second-order rate constants were measured with and without inhibitor at different pH and the results are listed in Table 3-28 as pK_i at various pH values.

Table 3-28 The pH dependence of the inhibition dissociation constant (pK_i) of N(2-mercaptoethyl)phenylacetamide (1) for the β -lactamase II catalysed hydrolysis of benzylpenicillin at 30 °C and ionic strength 1.0 M (NaCl). Enzyme concentration 0.1-2 μ M, zinc concentration 2-40 μ M

pH	$pK_i(M)$	pH	$pK_i(M)$
5.25	3.64	9.00	4.11
5.60	3.97	9.23	4.24
5.65	3.55	9.40	4.06
5.79	3.76	9.51	4.05
6.00	4.43	9.52	4.17
6.00	3.64	9.75	4.01
6.50	4.38	9.85	4.04
7.00	4.35	10.00	3.91
7.50	4.43	10.07	3.86
8.00	4.41	10.24	3.68
8.50	4.37	10.50	3.69

The results were plotted in Figure 3.21 which gave a bell-shaped curve and similar to the pK_i -pH profile observed for cephaloridine, slopes of 1, 0, -1 were seen for this substrate also.

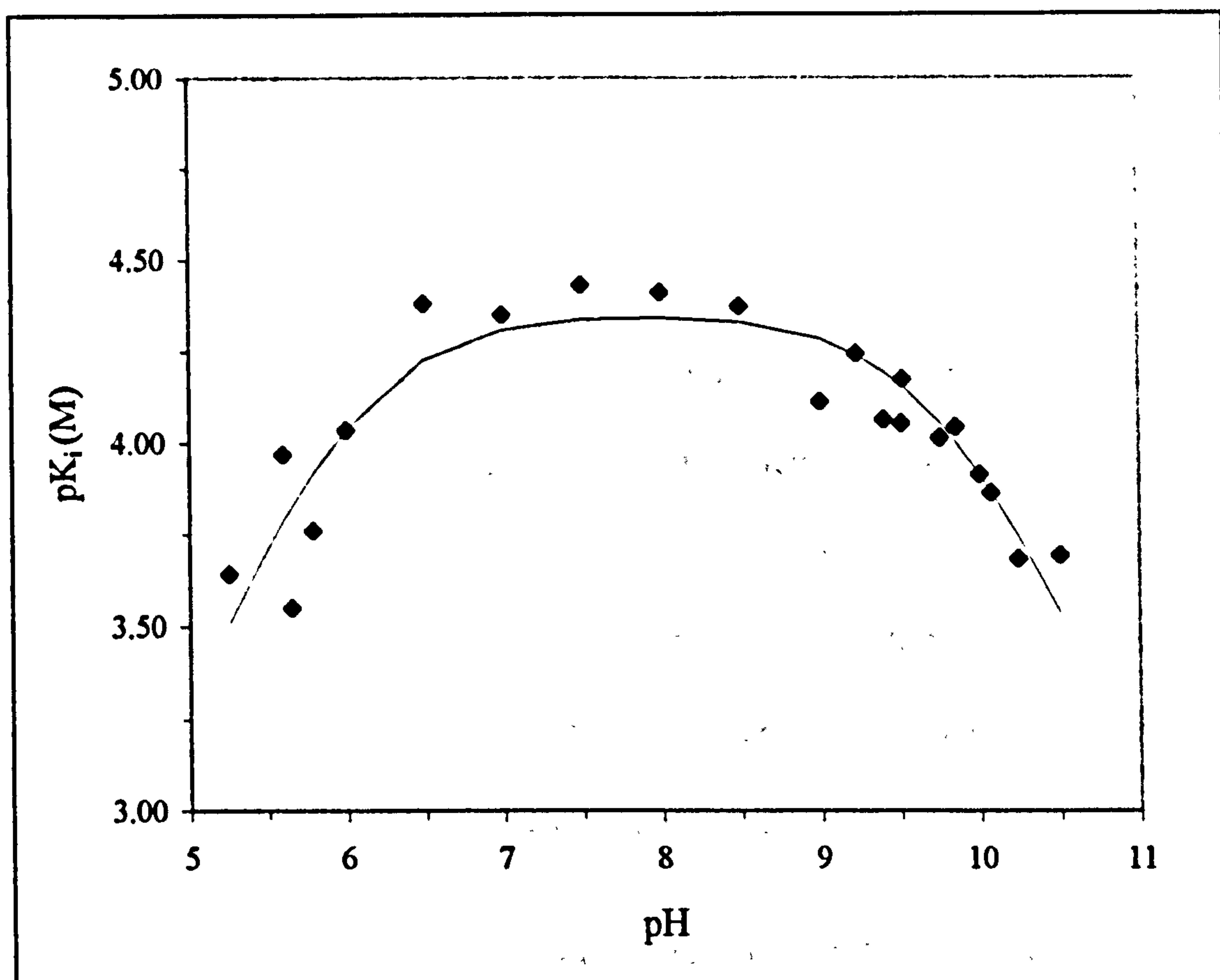


Figure 3.21 pH dependence of the inhibition constants (pK_i) of N(2-mercaptoethyl)phenylacetamide (1) for the β -lactamase II catalysed hydrolysis of benzylpenicillin

Fitting the curve to the theoretical Equation 3.6, the kinetic parameters were determined from the inflection points and are shown in Table 3-29.

Table 3-29

Substrate	pK_{a1}	pK_{a2}	$K_i (\mu M)$
Benzylpenicillin	6.03	9.75	44.4

3.6.2.2 Discussion

Inhibition constants represent pure equilibrium binding of a compound to the enzyme, the K_i -pH profiles inflections should therefore reflect intrinsic pK_a values¹⁴². By contrast, pK_a values obtained from kinetic studies may be the result of kinetic ambiguities such as changes in rate limiting step. It is therefore of interest to compare enzyme ionisation constants obtained by the two methods. The inhibition constants should of course, be independent of the substrate used to monitor enzyme activity. This was confirmed by using both benzylpenicillin and cephaloridine as the substrate for β -lactamase II activity.

Inhibition constants K_i were measured as a function of pH for the inhibition of the β -lactamase II activity by N(2-mercaptoethyl)phenylacetamide (1). The pH-dependence of pK_i for the β -lactamase II catalysed hydrolysis of both cephaloridine and benzylpenicillin (Figure 3.20 and Figure 3.21), have a characteristic bell-shaped curve with a slope of 1 at lower pH values and a slope of -1 at higher pH values. It shows that the binding is maximal and pH independent between the two inflection points, with diminished enzyme affinity at the pH extremes.

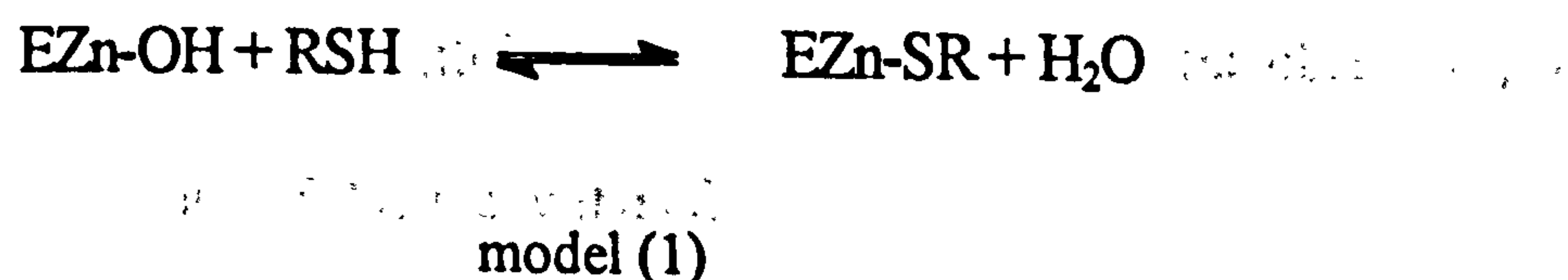
It is interesting to note that the acid limb of the pK_i -pH profile shows a definite slope of 1 in contrast to the value of 2.0 exhibited in the rate-pH profiles. This clearly indicates that the slope of 2.0 observed in the kinetic studies is a catalytic phenomenon and not the result of some form of enzyme degradation e.g. loss of

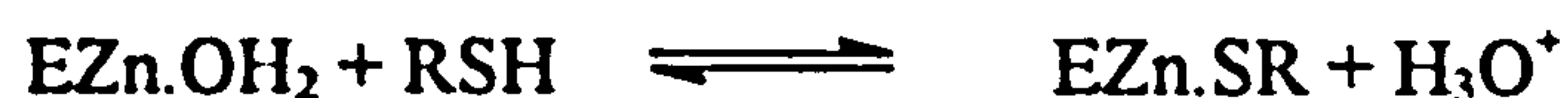
the enzyme bound zinc ion. Clearly, the binding of the inhibitor is affected by just one ionisation at low pH whereas catalytic activity is controlled by two ionisations.

The pH dependence of K_i for both substrates, show similar pH inflections to those observed in the β -lactamase II catalysed hydrolysis of substrates. The calculated pK_a values of 5.66 ± 0.4 and 9.84 ± 0.1 , derived from the inhibition studies, are similar, within error, to those obtained for catalysis 5.67 ± 0.2 and 9.60 ± 0.3 , which confirms that the values obtained from the pH dependence studies of the rate of hydrolysis of β -lactams are true equilibrium ionisations and not kinetic values.

The pK_a of the thiol group of N(2-mercaptoethyl)phenylacetamide (1) was determined by spectrophotometric titration to be 9.5 ± 0.1 , so the predominant species at neutral pH is the undissociated RSH.

The thiol of (1) is most likely to be a ligand for the zinc ion in the enzyme active site. Assuming that there is displacement of the zinc-bound water by the thiol when inhibition occurs¹⁰⁸, many binding possibilities arise as a function of the zinc-bound water pK_a . The activity of the enzyme is linked to the ionisation of three essential groups in the active site, with two pK_a values of 5.6 and one of 9.5, each of which could be assigned to the zinc-bound water. Models (1), (2) and (3) represent the different modes of binding possible.





model (2)



model (3)

However, only one mode of binding, model (1), can explain the observed invariance of K_i with pH between 6 and 9, with the neutral thiol binding to the enzyme active site in which the water ligand is deprotonated giving pH independent binding between 6 and 9.

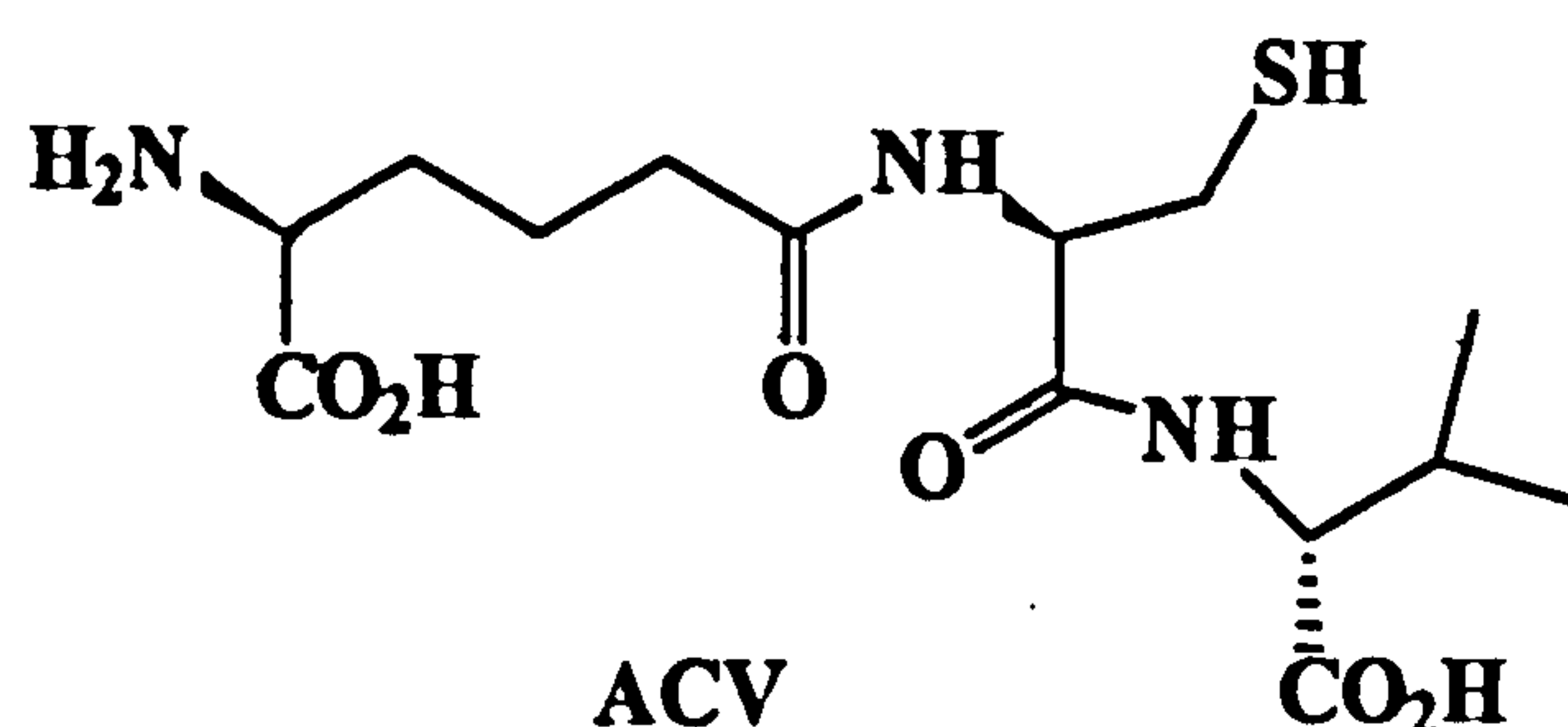
Alternative binding models (2) and (3) would generate pH dependency of the binding of the thiol. Assuming that the ionisation of the zinc-bound water correspond to the high pK_a value of 9.5, then below pH 9 the undissociated form would be predominant and binding to the thiol would cause the liberation of the hydronium ion and therefore be pH dependent (model 2).

The binding of the thiolate anion to the undissociated zinc-bound water would also be pH dependent as the concentration of the thiolate would vary with the pH (model 3).

It is therefore deduced that model (1) most probably represents the mode of binding of the thiol inhibitor (1) with β -lactamase II, and explains the observed pH profile for pK_i . The results of this study thus favour a low pK_a value of 5.6 for the zinc-bound water.

3.6.3 Structural dependence of thiol inhibition

The compounds synthesised during this work as potential inhibitors of the β -lactamase II are shown in Table 3-30. It is known that the precursor of penicillin synthesis is a tripeptide containing a cysteine residue, L- δ -(α -aminoadipoyl)-L-cysteiny-D-valine (ACV)¹⁶⁵. Based on this acyclic structure and by analogy with benzylpenicillin itself, various thiol compounds differing in their side chain structure have been synthesised and tested as inhibitors of β -lactamase II.



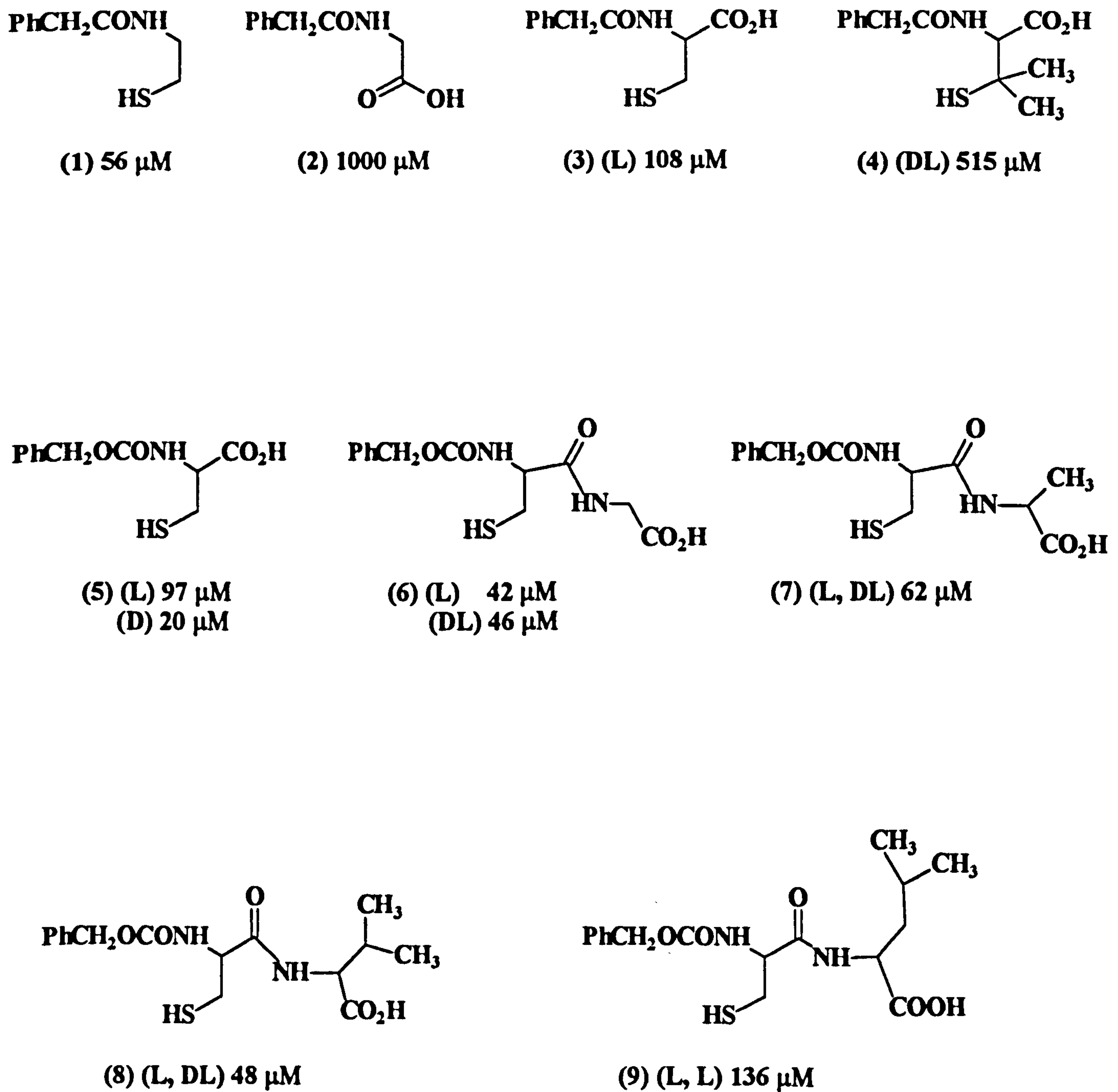
In order to determine whether any of the inhibition observed was due to metal chelation and depletion, most of the experiments were performed initially without added zinc and repeated with an excess of zinc sulfate, typically 2×10^{-4} M. The inhibition constant, K_i , for each inhibitor was estimated based on the equation below assuming competitive inhibition.

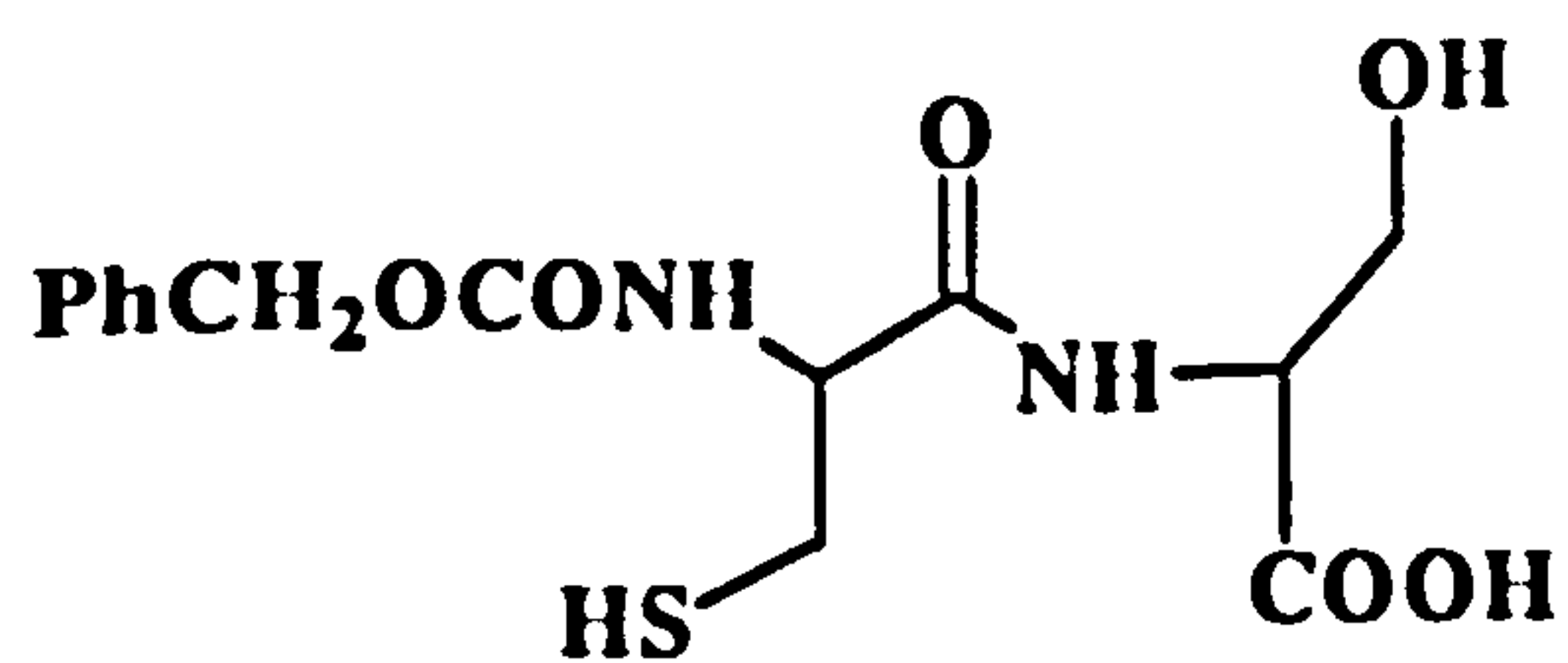
$$\text{Rate} = \frac{k_{\text{cat}} \cdot [\text{E}] \cdot [\text{S}]}{[\text{S}] + K_m \left(\frac{K_i + [\text{I}]}{K_i} \right)}$$

The results of this study are summarized in Table 3-30

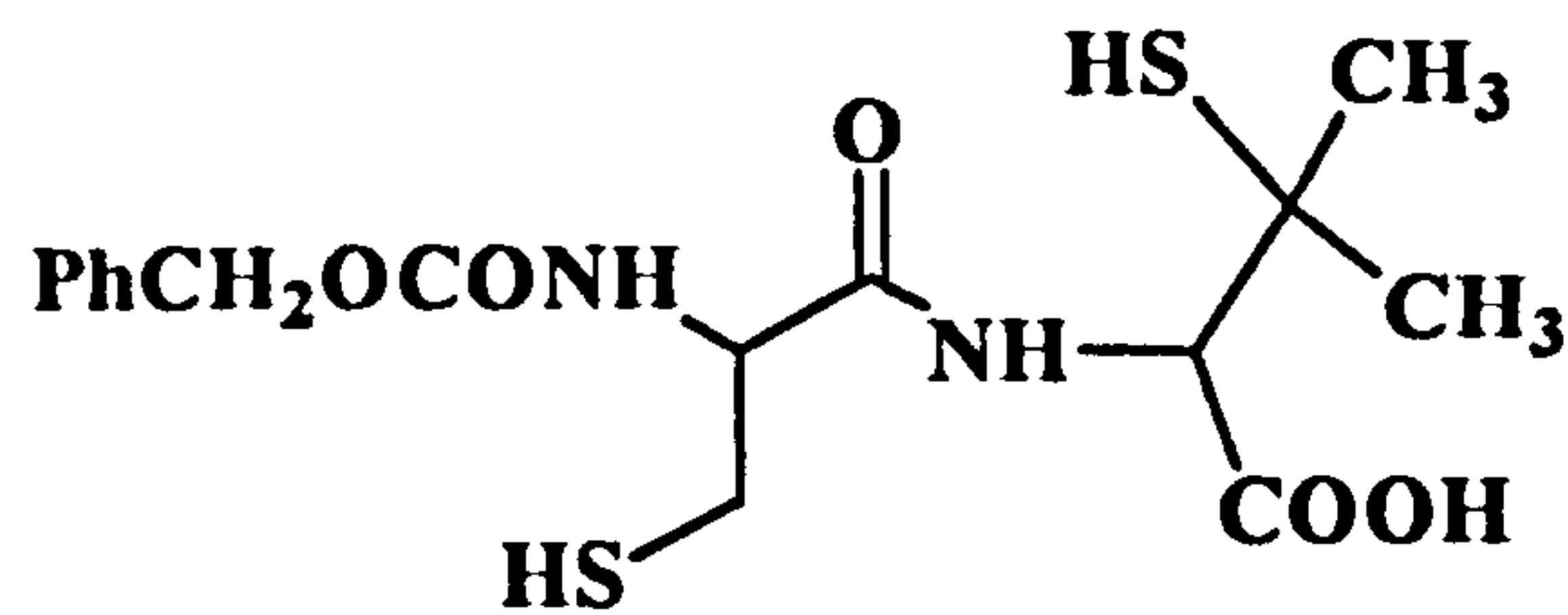
Table 3-30 Inhibitor dissociation constants for the β -lactamase II catalysed hydrolysis of β -lactams, at 30 oC pH 7.0 and ionic strength 1.0 M (NaCl) Enzyme concentration 0.1 μ M

<i>Compounds</i>	<i>K_i μM (ZnSO₄ 2 x 10⁻⁶ M)</i>	<i>K_i μM (ZnSO₄ 2 x 10⁻⁴ M)</i>
N(2-mercaptoethyl)phenylacetamide (1)	56.2 \pm 11	
N-phenylacetyl-glycine (2)	1000 \pm 150	
N-phenylacetyl-L-cysteine (3)	108 \pm 3	285 \pm 5
N-phenylacetyl-DL-penicillamine (4)	515 \pm 38	465 \pm 169
N-carbobenzoxy-L-cysteine (5)	97 \pm 5	
N-carbobenzoxy-D-cysteine (5)	20.1 \pm 1.6	30.8 \pm 1.3
N-carbobenzoxy-L-cysteinyl-glycine (6)	42.2 \pm 10	40.7 \pm 10
N-carbobenzoxy-DL-cysteinyl-glycine (6)	46.3 \pm 0.1	
N-carbobenzoxy-L-cysteinyl-DL-alanine (7)	62.0 \pm 9.3	81.5 \pm 10
N-carbobenzoxy-L-cysteinyl-DL-valine (8)	48.3 \pm 2.3	85.5 \pm 1.5
N-carbobenzoxy-L-cysteinyl-L-leucine (9)	135.5 \pm 17	173 \pm 1
N-carbobenzoxy-L-cysteinyl-DL-serine (10)	72.5 \pm 0.5	107 \pm 3
N-carbobenzoxy-DL-cysteinyl-L-serine (10)	83.8 \pm 4	118 \pm 3
N-carbobenzoxy-D-cysteinyl-D-penicillamine (11)	no hydrolysis	107 \pm 13
N-carbobenzoxy-L-cysteinyl-DL-phenylalanine (12)	17.3 \pm 0.2	22.0 \pm 1.0
N-carbobenzoxy-L-cysteinyl-L-phenylalanine (12)	26.9 \pm 0.5	40.8 \pm 3
N-carbobenzoxy-L-cysteinyl-D-phenylalanine (12)	9.3 \pm 0.4	14.7 \pm 1
N-carbobenzoxy-D-cysteinyl-L-phenylalanine (12)	11.0 \pm 1	22.0 \pm 1
N-carbobenzoxy-D-cysteinyl-D-phenylalanine (12)	3.0 \pm 0.1	11.0 \pm 0.3
N-carbobenzoxy-L-cysteinyl-DL-proline (13)	95.0 \pm 1.5	140 \pm 1
3-oxoperhydro[1.3]thiazolo[4.3-b][1.3]thiazole-7a-carboxylic acid (15)	181	
2,3-dimercaptosuccinic acid		
2,3-dimercapto-1-propane sulfonic acid.	no hydrolysis	activity restored
Sodium hydrogen sulfide		
Enalapril	5.69 x 10 ⁴	
Captopril	41.6 \pm 9	88.6 \pm 14

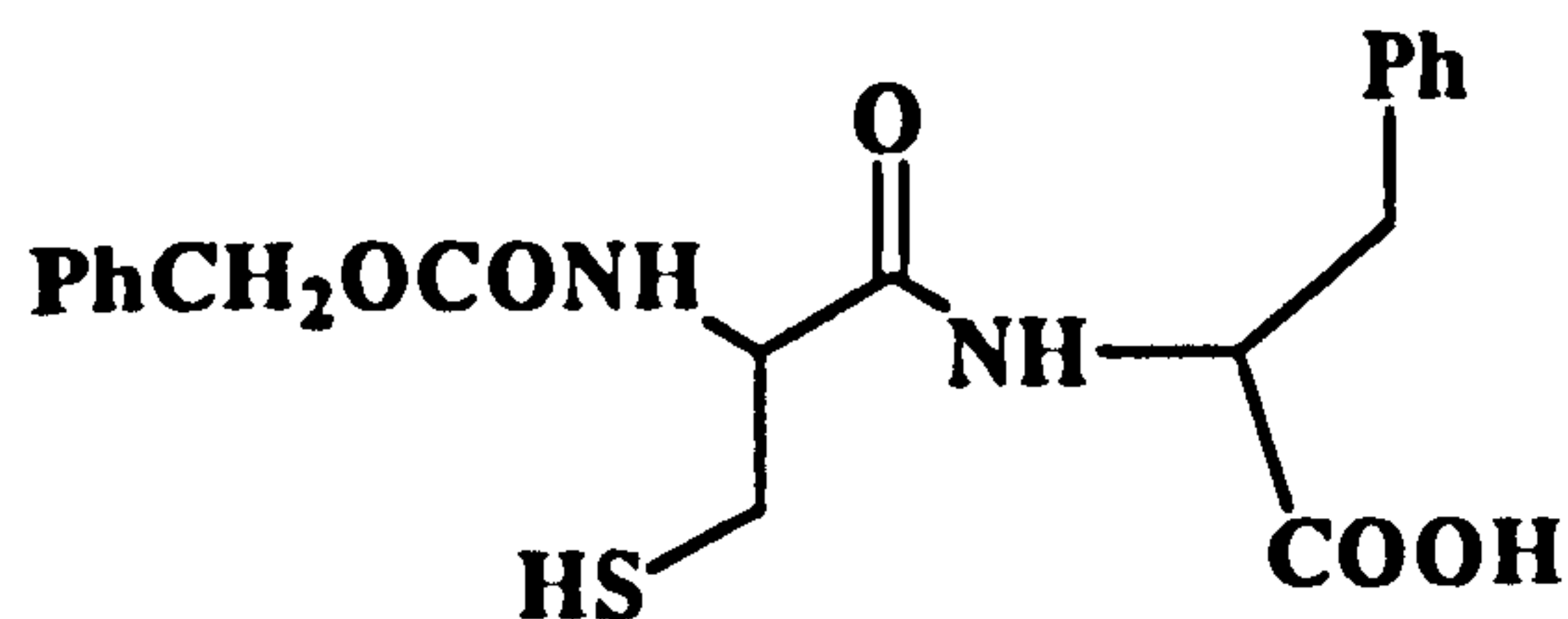




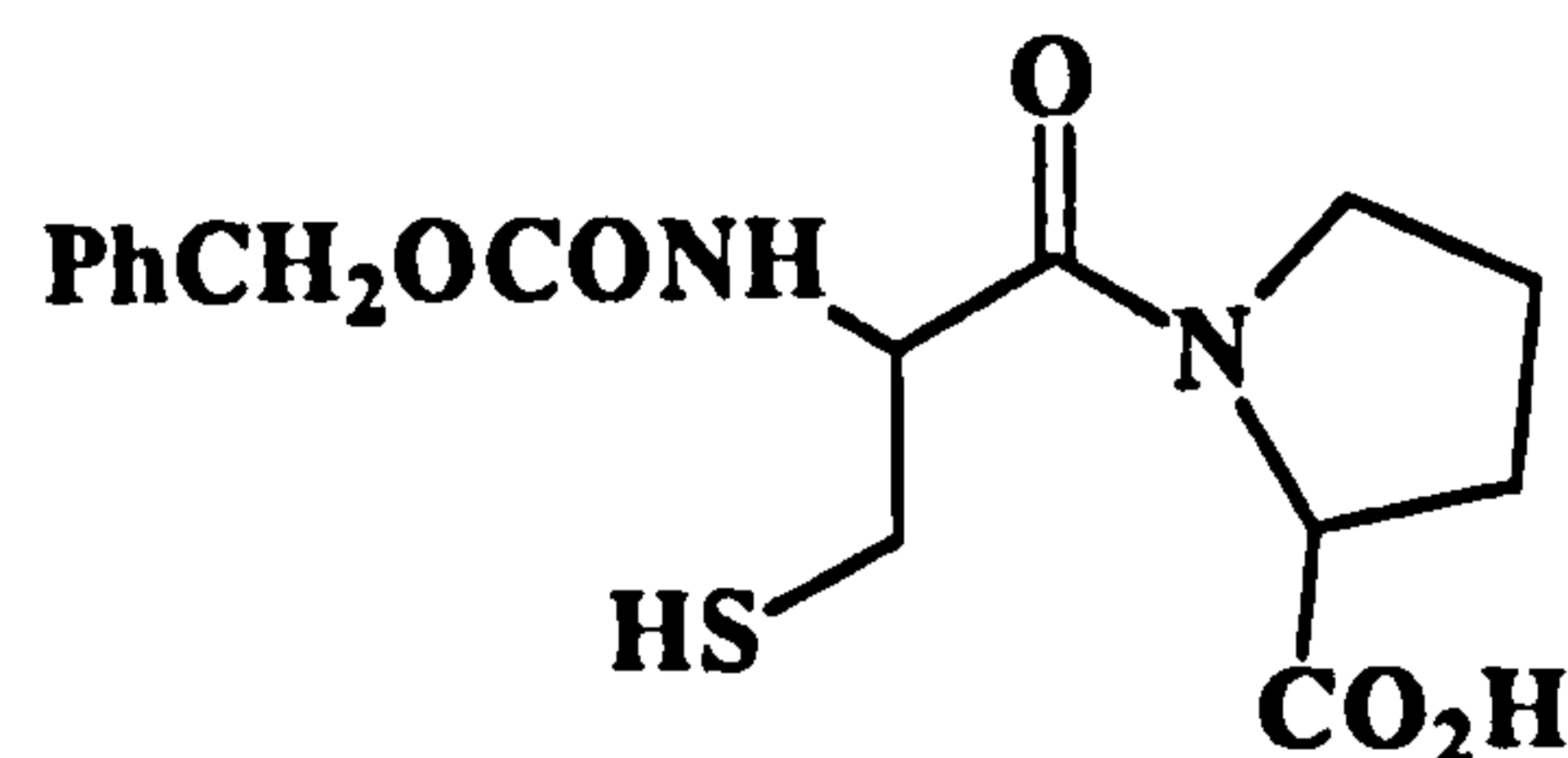
(10) (L, DL) 73 μ M
(DL, L) 84 μ M



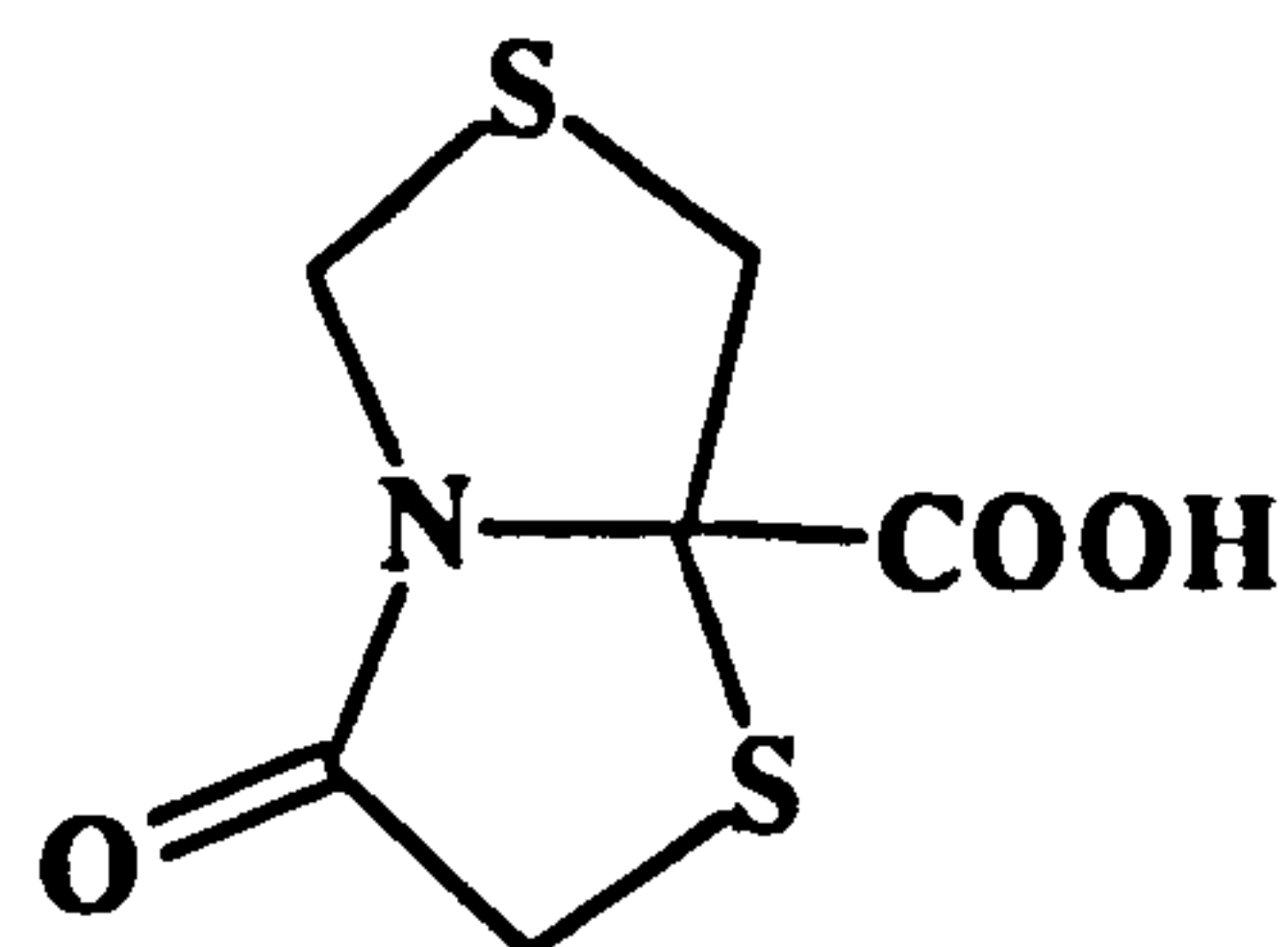
(11) deactivates by chelation



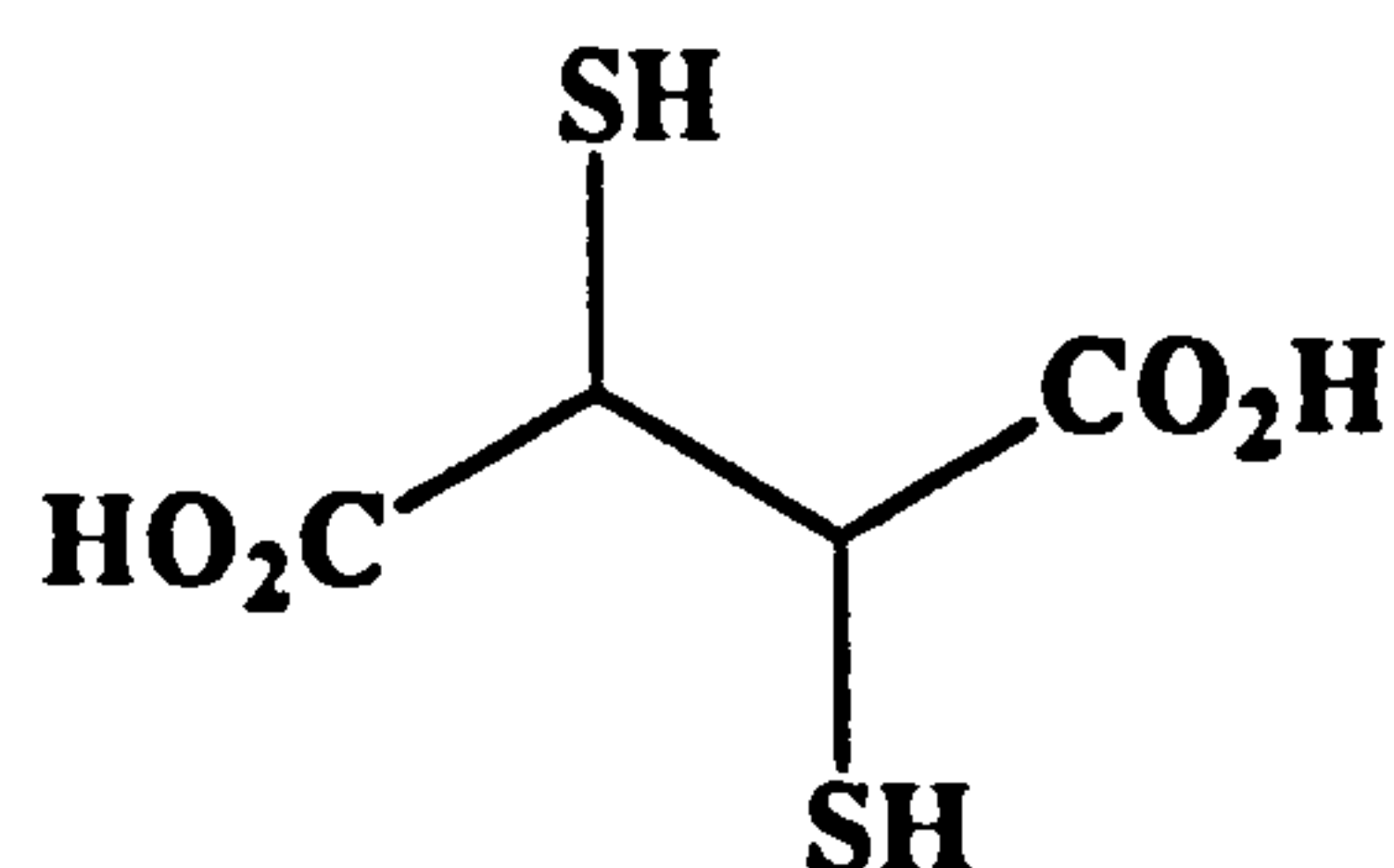
(12) (L, DL) 17 μ M
(L, L) 26 μ M
(L, D) 9 μ M
(D, L) 11 μ M
(D, D) 3 μ M



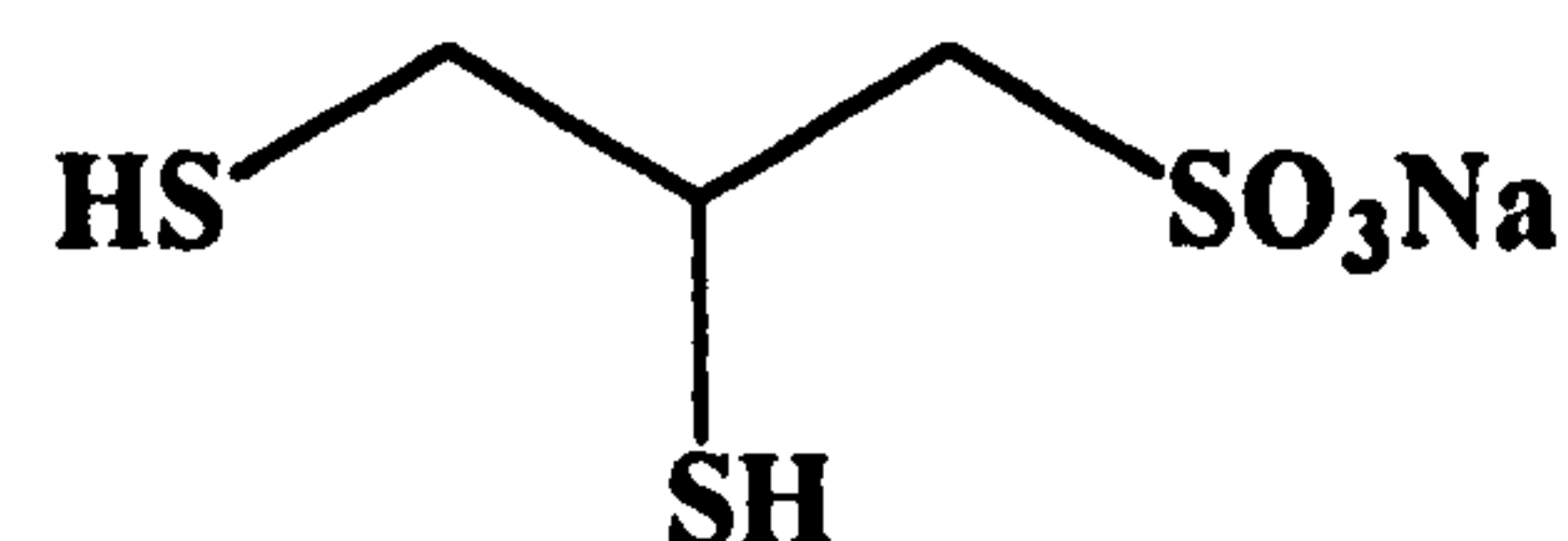
(13) (L, DL) 95 μ M



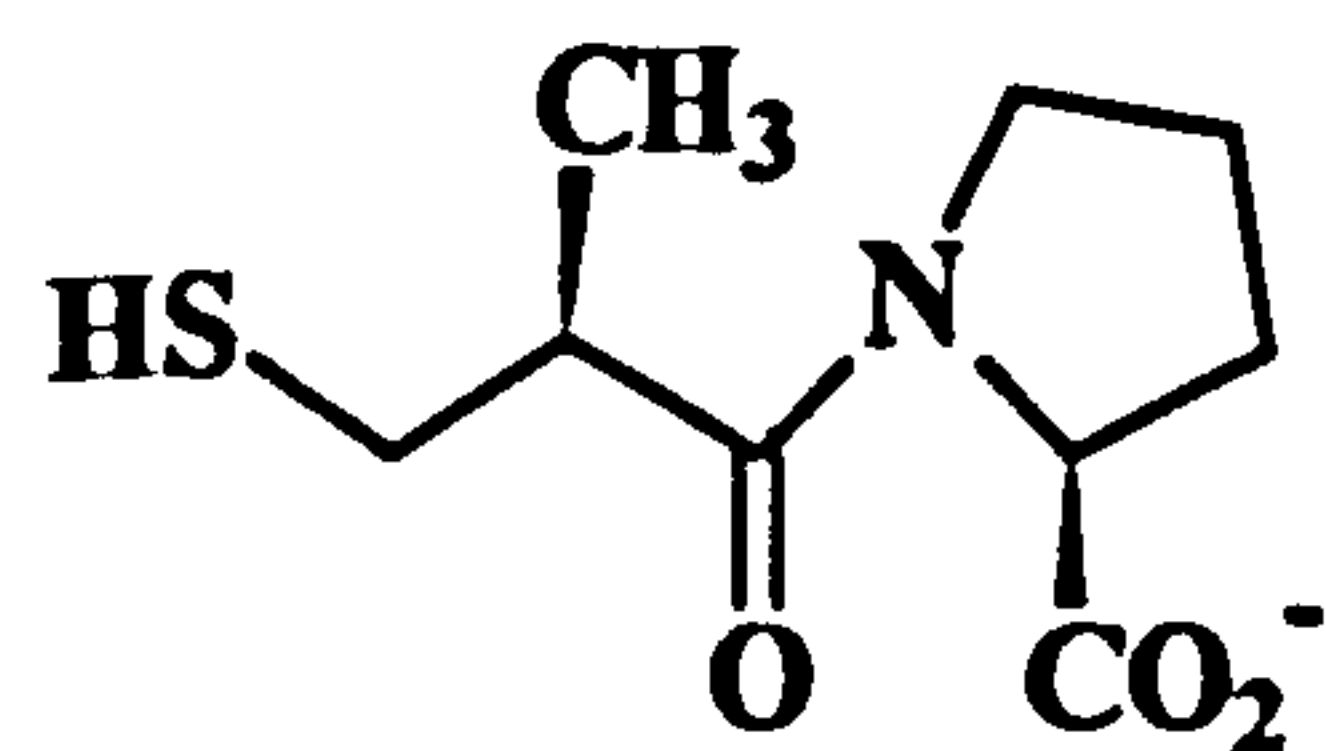
(15) 181 μ M



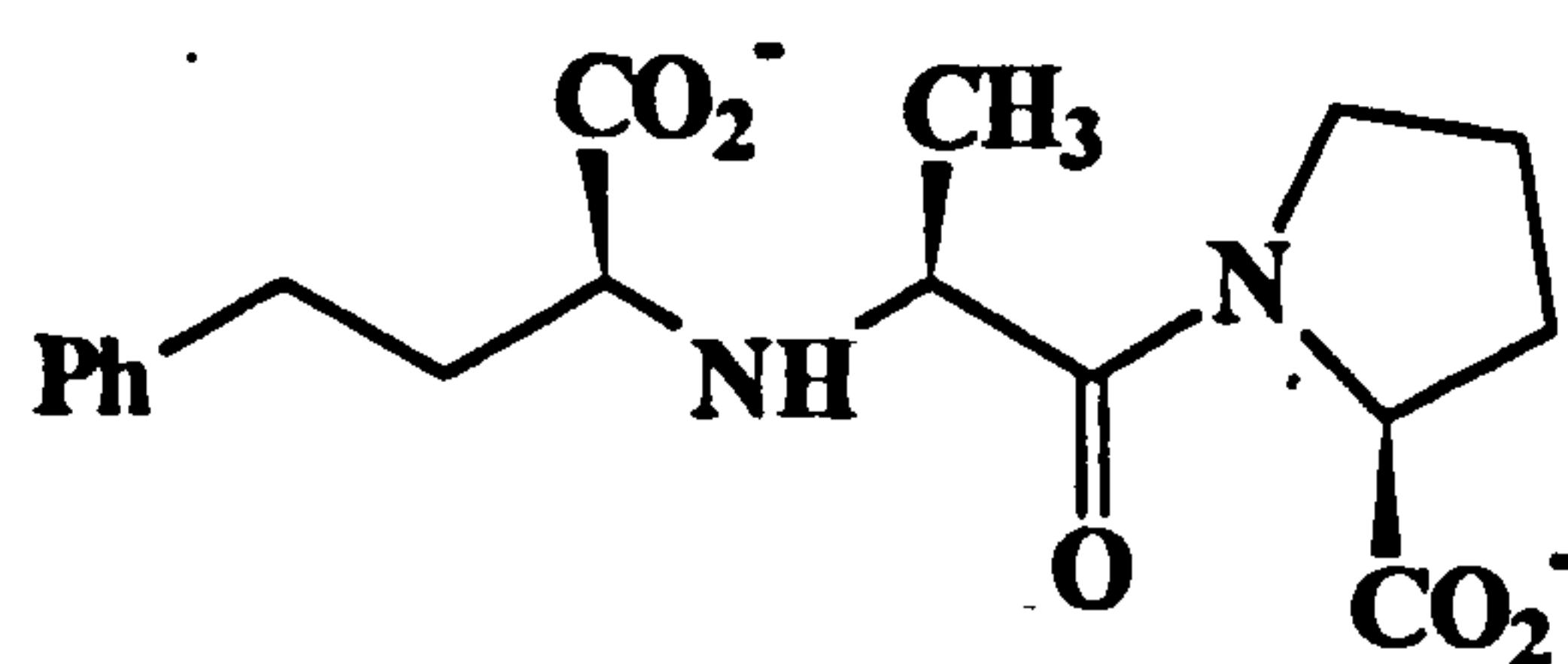
deactivates by chelation



deactivates by chelation



Captopril 42 μ M



Enalapril 5.7 x 10⁴ μ M

The compounds tested exhibited various potencies against β -lactamase II. The first thiol examined was N(2-mercaptoethyl)phenylacetamide (1) the inhibitory action of which has been previously discussed. The thiol itself is necessary for inhibition, N-phenylacetyl glycine (2) is a very poor inhibitor. The addition of a carboxylic acid residue to the basic neutral thiol structure (1) to give N-phenylacetyl-L-cysteine (3) slightly decreases potency, suggesting that a negatively charged carboxylate anion in this position does not facilitate binding. There is an order of magnitude decrease in binding when two gem-dimethyl groups are α -substituted to the thiol, as in N-phenylacetyl-DL-penicillamine (4). This again is indicative of some selectivity and suggests that thiol co-ordination is important and can be sterically inhibited.

There is only a slight difference between the K_i values measured for N-carbobenzoxy and N-phenylacetyl side chains. N-phenylacetyl-L-cysteine (3) and N-carbobenzoxy-L-cysteine (5) have similar K_i values of 108 μ M and 97 μ M, respectively.

The effect of substituents on the inhibition by the thiol dipeptides was investigated in various N-carbobenzoxy-cysteinyl amino acids. The effect on K_i upon increasing the size of the R group from CH_3 in N-carbobenzoxy-L-cysteinyl-DL-alanine (7) (K_i of 62 μ M) to $\text{CH}(\text{CH}_3)_2$ in N-carbobenzoxy-L-cysteinyl-DL-valine (8) (K_i of 42 μ M) shows an increase in affinity for the enzyme active site. When the R group is $\text{CH}_2\text{-Ph}$ as in N-carbobenzoxy-L-cysteinyl-DL-phenylalanine (12), the K_i of 17 μ M indicates a more potent inhibitor which may be due to the hydrophobic effect of the aromatic group. N-Carbobenzoxy-L-cysteinyl-serine (10) with a K_i of 72.5

μM displays a lower degree of inhibition than N-carbobenzoxy-L-cysteinyl-DL-alanine (7), which indicates that increasing the polarity of the side chain does not improve the inhibition. N-carbobenzoxy-L-cysteinyl-DL-proline (13) (K_i 95.0 μM) was less active than the other dipeptides which is probably due to the restricted flexibility of the C-N peptide bond. It seems that the best inhibition is obtained for compounds with a hydrophobic side chain.

Most of the compounds which inhibited β -lactamase II were not chelating agents, as the enzyme activity was not restored upon the addition of zinc ions. However, the enzyme was completely inactivated by N-carbobenzoxy-D-cysteinyl-D-penicillamine (11). No hydrolysis of the substrate was observed when the enzyme was submitted to different concentrations of this dithiol. The activity was, however, mostly recovered upon addition of excess zinc indicating that the dithiol was acting as a chelating agent and removed the zinc ion from the protein.

Other dithiol compounds have been tested and they exhibit a similar phenomenon against β -lactamase II. Such a phenomenon has been observed when testing 2,3-dimercaptosuccinic acid, 2,3-dimercapto-1-propanesulfonic acid and also sodium hydrogen sulfide against β -lactamase II. These compounds completely inhibited the enzyme activity at the concentration of inhibitor used, the activity was however restored upon addition of excess zinc.

Captopril and enalapril are potent inhibitors of the zinc-dependent angiotensin converting enzyme (ACE). Any potential medicinal use of inhibitors of β -lactamase II would obviously require the inhibitor to act selectively and, particularly, not to

inhibit ACE. It is equally of interest to test whether ACE inhibitors have an effect on β -lactamase II. Captopril was found to be a good inhibitor of β -lactamase II with a K_i of 41.6 μ M and repeating the assay with excess zinc did not restore the enzyme activity (K_i 88.6 μ M). However enalapril which does not contain a thiol group, did not inhibit the enzyme. This result highlights the importance of the thiol group in the design of β -lactamase II inhibitors. This was also illustrated by the fact that N-phenylacetyl-glycine (2) was not a good inhibitor of β -lactamase II, with a K_i of 1 mM.

Whilst attempting to synthesise the compound N-mercaptoacetyl-4-thiazolidinecarboxylic acid (16), the interesting 3-oxoperhydro[1.3]thiazolo[4.3-b][1.3]thiazole-7a-carboxylic acid (15) was obtained. This compound was also tested against β -lactamase II and found to be a weak inhibitor with an inhibition constant of 181 μ M. This compound obviously does not contain an ionisable thiol group, but, nonetheless, it is a better inhibitor than the simple carboxylic acid N-phenylacetyl-glycine (2) (K_i 1 mM).

There are two asymmetric centres in the dipeptides synthesised and the effect of the stereochemistry at each centre on inhibition was independently varied. For example, for the D-enantiomer of the N-carbobenzoxy-cysteine (5) was five times more potent than the L-enantiomer. N-Carbobenzoxy-L-cysteinyl-glycine (6) displays the same degree of inhibition as its racemic mixture (DL). Similar inhibitory potencies were obtained with N-carbobenzoxy-L-cysteinyl-DL-serine (10) and N-carbobenzoxy-DL-cysteinyl-L-serine (10). N-carbobenzoxy-L-

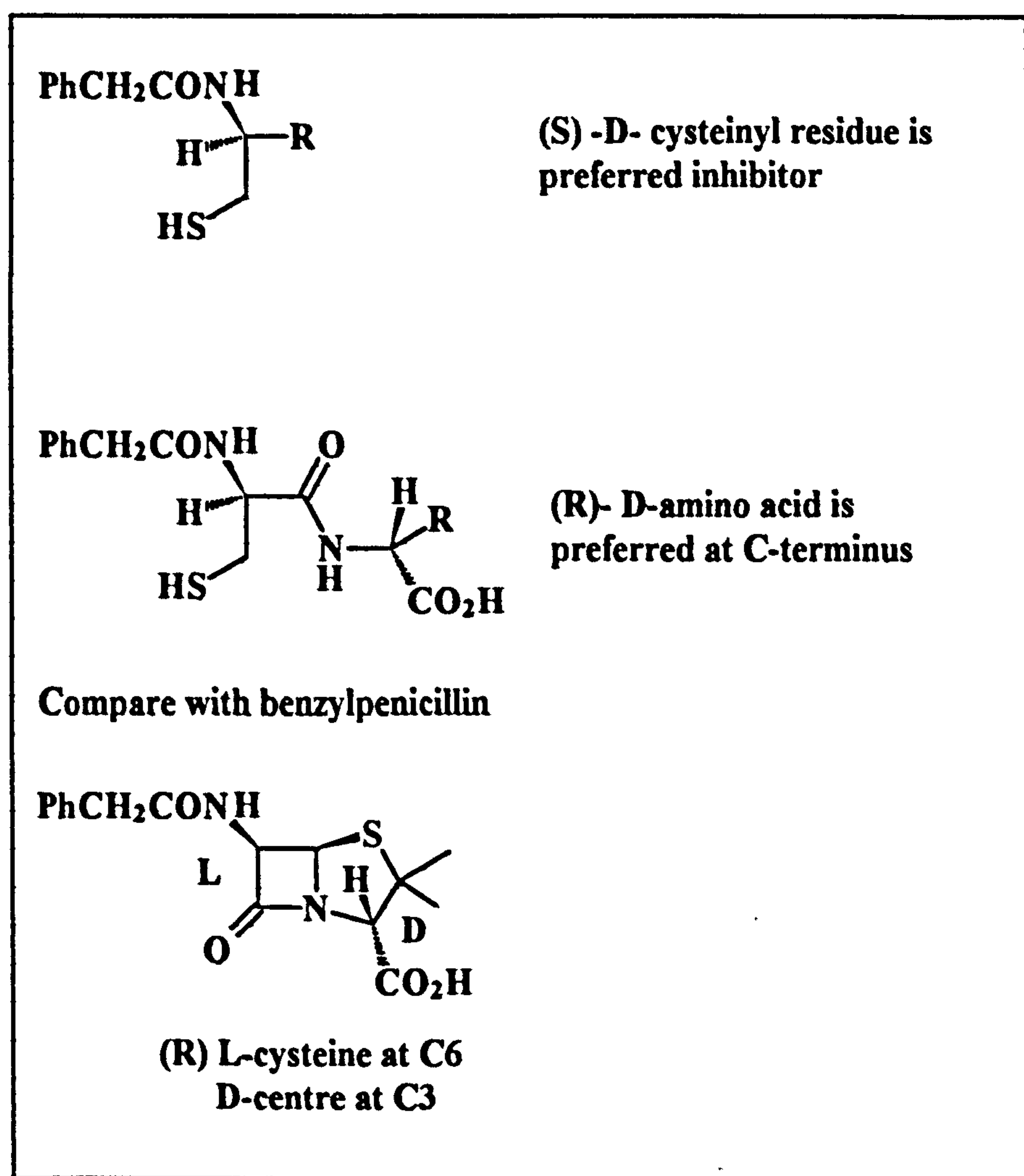
cysteinyl-L-leucine (9) with a K_i 135 μ M was the poorest inhibitor compared with the other dipeptides.

Four diastereoisomers of N-carbobenzoxy-cysteinyl-phenylalanine (12) were synthesised and tested as inhibitors of β -lactamase II. The N-carbobenzoxy-L-cysteinyl-D-phenylalanine (12) and N-carbobenzoxy-D-cysteinyl-L-phenylalanine (12), showed the same degree of inhibition whilst N-carbobenzoxy-D-cysteinyl-D-phenylalanine (12) isomer, was three times more effective. It was found that the N-carbobenzoxy-L-cysteinyl-L-phenylalanine is the weaker inhibitor of the four isomers.

From these results, we can state that thiol compounds effectively inhibit β -lactamase II. It can be concluded that the best interactions with the enzyme are obtained with compounds with a thiol residue and a hydrophobic side chain such as N-carbobenzoxy-cysteinyl-phenylalanine (12). Furthermore, they exhibit some selective stereochemistry, as the D,D isomers of N-carbobenzoxy-cysteinyl-amino acids exhibit the highest potency. N-Carbobenzoxy-D-cysteinyl-D-phenylalanine (12) with a K_i of 3.0 μ M is the most efficient *in vitro* inhibitor of β -lactamase II synthesised during this work.

These results indicate that β -lactamase II requires a D configuration at the cysteinyl amino acid centre, as shown by comparing the inhibitory effect of N-carbobenzoxy-L-cysteine (5) (K_i = 97 μ M) with N-carbobenzoxy-D-cysteine (K_i = 20 μ M). Good inhibition also requires a D configuration at the second amino acid centre as the best inhibition was obtained with the D,D isomer of N-carbobenzoxy-

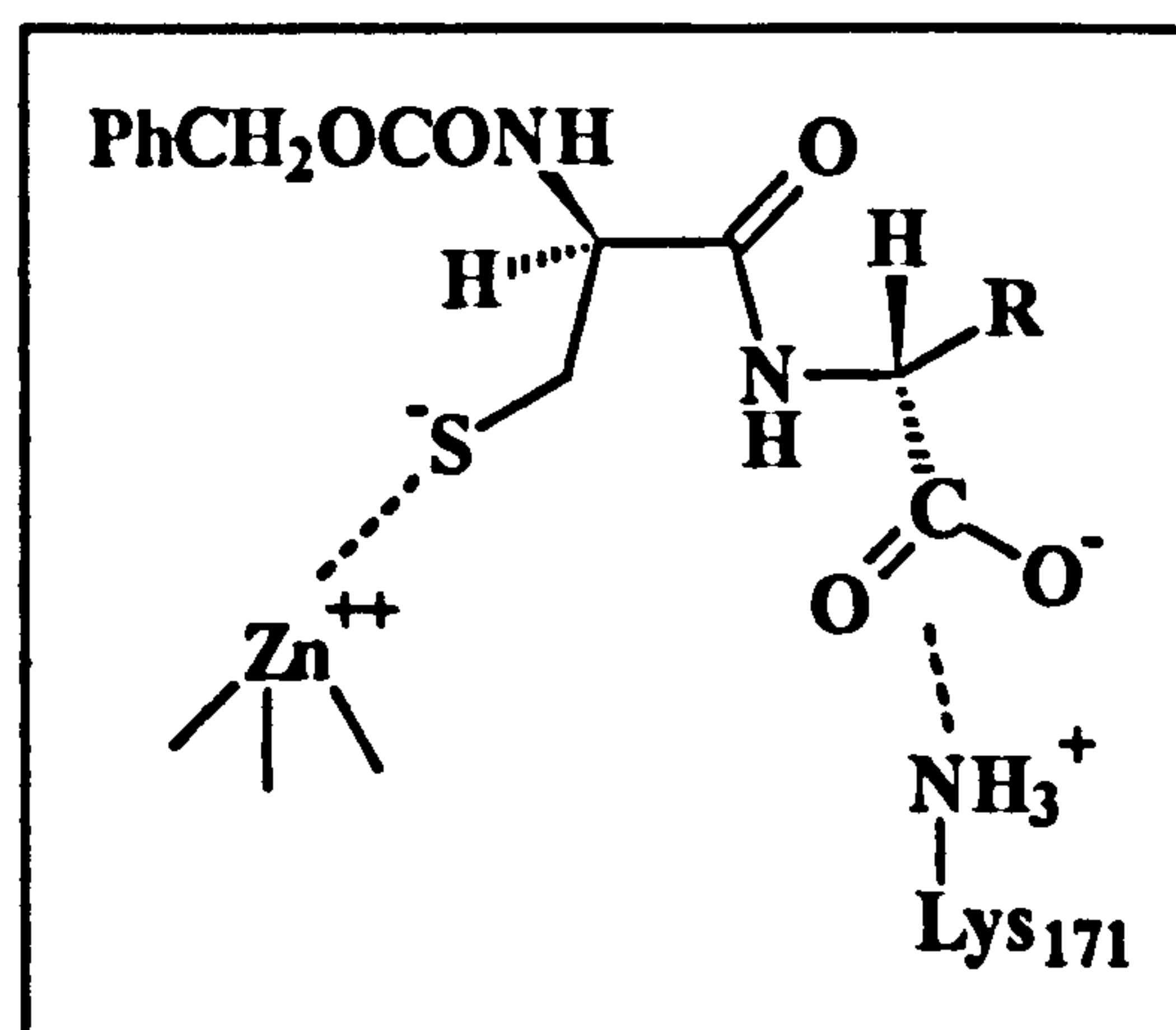
cysteinyl-phenylalanine (12) (K_i 3.0 μ M). However, the enzyme does tolerate an L configuration at both centres when combined with a D configuration. Scheme 3-17 shows the stereochemistry of the two asymmetric centres of a D,D isomer of N-carbobenzoxy-cysteinyl amino acid and a comparison made with the stereochemistry of benzylpenicillin as a substrate.



Scheme 3-17

Since the enzyme stereoselectively hydrolyses the substrate benzylpenicillin which has three asymmetric centres, it is expected that the inhibitor has to have the right stereochemistry in order to be effective. The proposed docking of the N-

carbobenzoxy-D-cysteinyl-D-amino acid inhibitor in the active site is shown in Scheme 3-18. The result of the inhibition study shows that there is discrimination in the binding of inhibitors and is further evidence that binding occurs to the zinc ion of the active site. The case of the inhibition of a binuclear zinc β -lactamase II has also to be considered where the thiol could be coordinated to both zinc ions.



Scheme 3-18 Proposed model for the binding of the thiol dipeptides to the active site of β -lactamase II

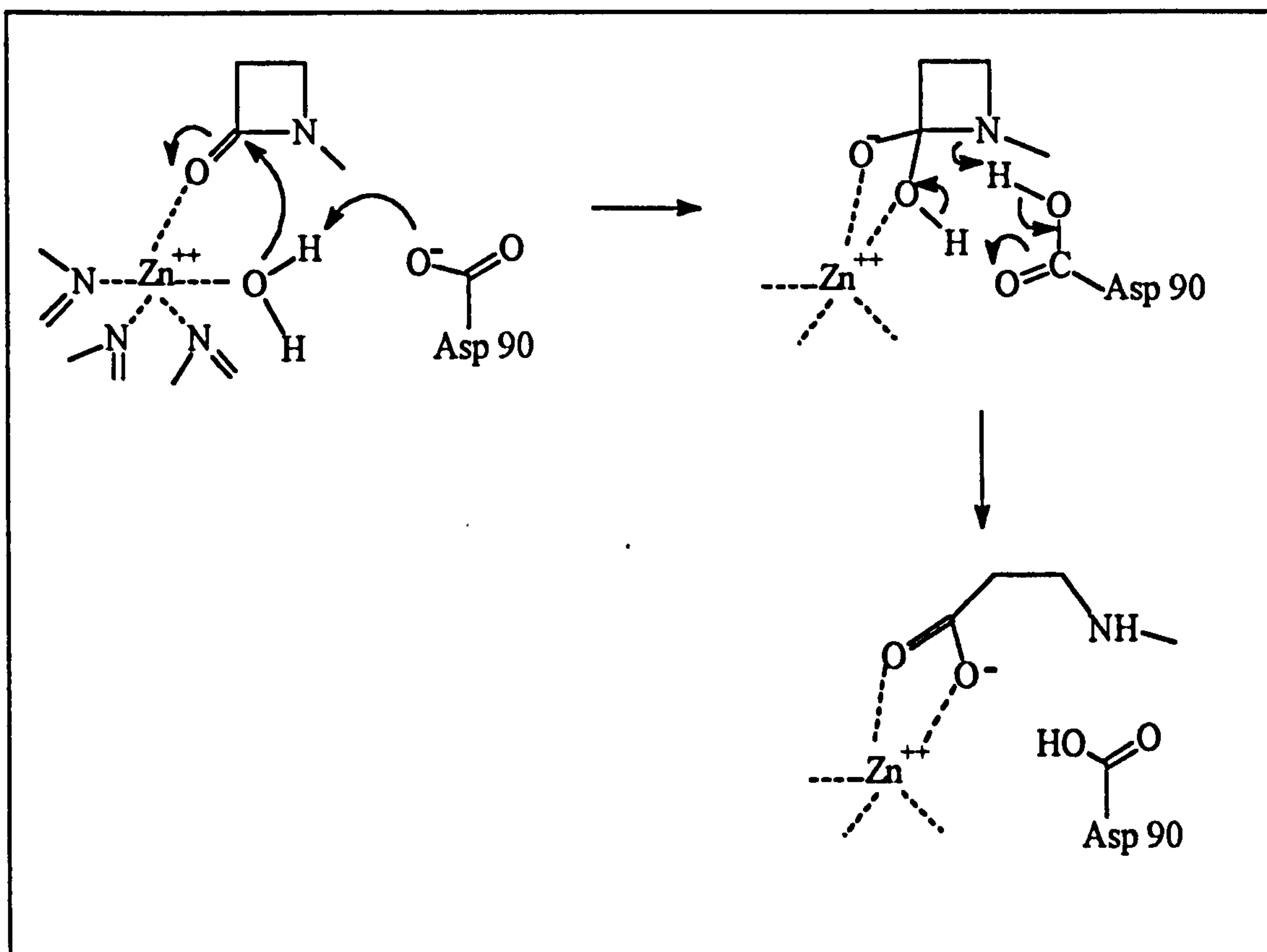
3.7 Mechanism of catalysis of β -lactamase II

As stated in the introduction, the mechanistic studies on carboxypeptidase A usually serve as models for other metallo-enzymes^{89,90}. Many pathways for the mechanism of catalysis of this enzyme have been proposed⁹³, such as the anhydride pathway^{86,87,88} where Glu270 acts as a nucleophile and attacks the carbonyl function of the peptide substrate. The other mechanism proposed involves Glu270 acting as a general base, deprotonating an external water molecule or a zinc-bound water molecule concerted with attack on the substrate^{89,90}.

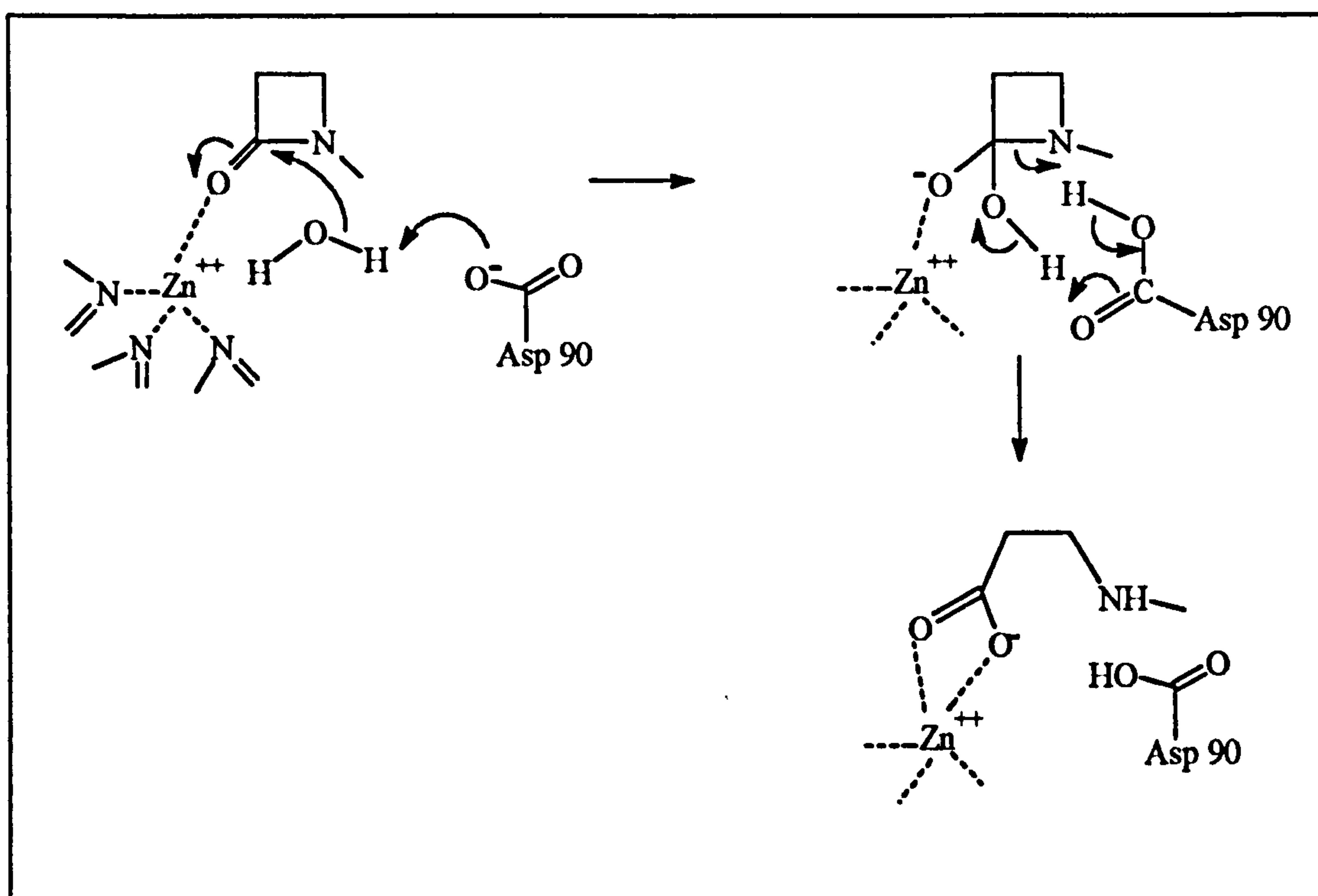
Similar to carboxypeptidase A, an anhydride pathway is possible for β -lactamase II action, where Asp90 would act as a nucleophile attacking the carbonyl function of the β -lactam ring, leading to the formation of an anhydride intermediate (Scheme 3-4, page 143). This pathway is, however, probably ruled out by the methanolysis studies. There was no effect of the methanol on the enzyme catalysis, and no methyl ester product was detected, whereas such effect could have been expected if an anhydride intermediate was involved.

Earlier studies of the mechanism of action of β -lactamase II⁴⁹, suggested a mechanism involving Asp 90 acting as a general base to promote nucleophilic attack of a zinc-bound water (Scheme 3-19) or, as for carboxypeptidase, of an external water molecule on the substrate (and Scheme 3-20). The activated water attacks the carbonyl carbon of the β -lactam ring to form a tetrahedral intermediate.

The intermediate then collapses when a proton is donated to the nitrogen by Asp90 which is acting as a general acid.



Scheme 3-19



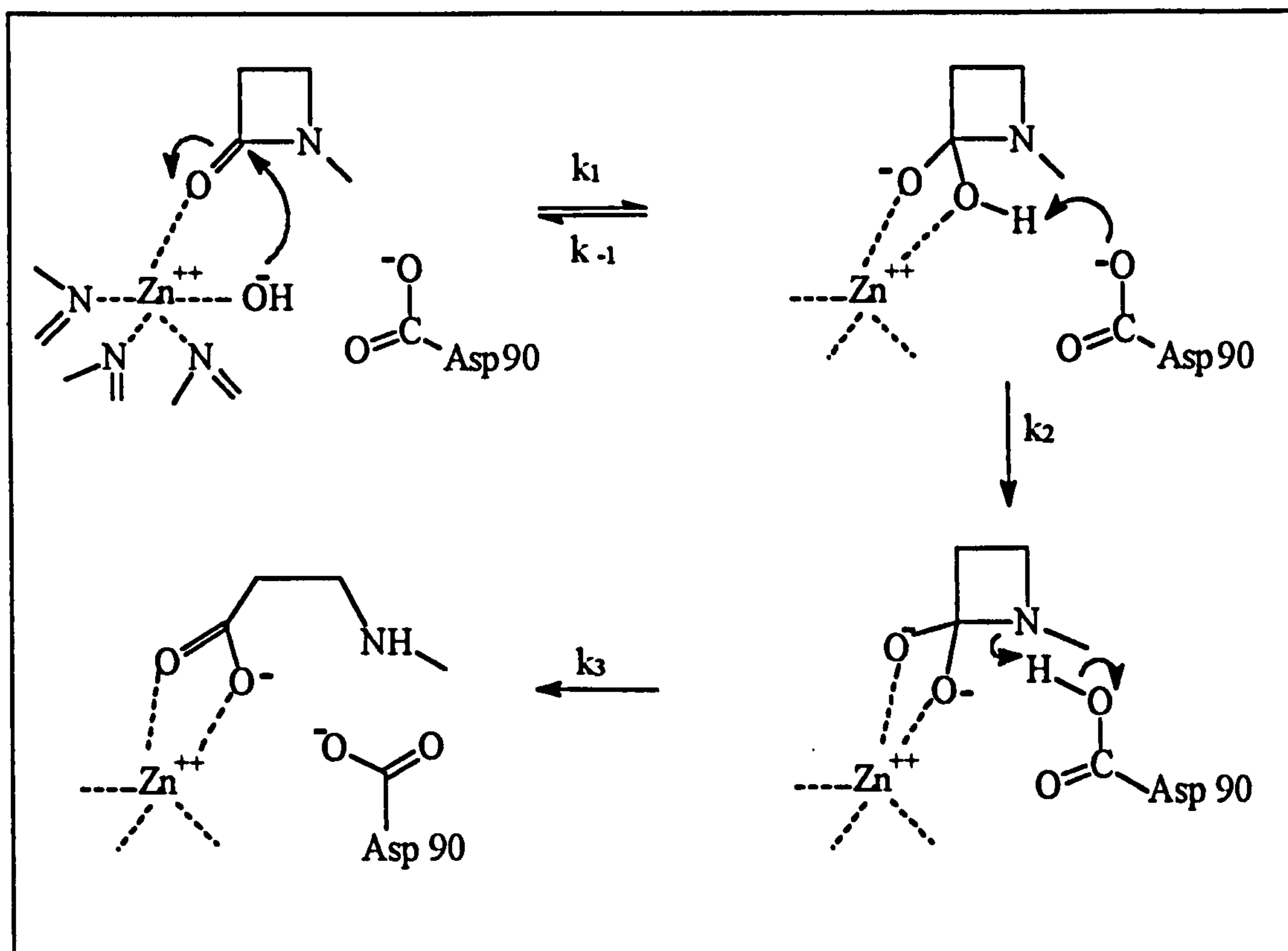
Scheme 3-20

However, the pH dependence studies of catalysis for the β -lactamase II conducted during this work, have shown that the enzyme activity was linked to the ionisation of at least three essential groups in the active site, two of which have a pK_a value of 5.6 and were attributed to the zinc-bound water and Asp90. Thus at physiological pH we would expect these two residues to be unprotonated. Kinetic solvent isotope experiments revealed that, $k_{cat}/K_m^{H_2O} / (k_{cat}/K_m)^{D_2O}$ was 1.82 for the hydrolysis of benzylpenicillin, 0.85 for cephaloridine and 1.23 for cefuroxime and $k_{cat}^{H_2O} / k_{cat}^{D_2O}$ was 1.5 ± 0.1 for all three substrates. These are relatively small kinetic solvent isotope effects, whereas the mechanisms proposed in Scheme 3-19 and Scheme 3-20 involve rate-limiting proton transfer from the zinc-bound water, which should generate significant kinetic isotope effects of 2 to 3.

Finally, from the results of the mechanistic study of the β -lactamase II, the mechanisms which meet the above considerations, the pK_a of 5.6 for the zinc-bound water and Asp90 residue and relatively small kinetic solvent isotope effects, is shown in Scheme 3-21 when the enzyme binds only one zinc ion and in Scheme 3-22 when the enzyme binds two zinc ions.

When considering the case of the mononuclear β -lactamase II (Scheme 3-21), the enzyme activity requires the two catalytically important groups, the zinc-bound water and Asp90, to be in their deprotonated form. The zinc-bound hydroxide ion attacks the carbonyl of the β -lactam ring to form a tetrahedral intermediate. Formation of the tetrahedral intermediate results in a significant build up of negative charge on both oxygens, which would be stabilised by the zinc ion. Ring opening of the β -lactam is not an easy process and the reaction will probably be reversible regenerating the β -lactam. The collapse of the tetrahedral intermediate needs therefore to be assisted by protonation of the nitrogen of the β -lactam. Ring opening will also be facilitated if the tetrahedral intermediate is deprotonated during the process¹⁶⁶, therefore creating a dianionic species. Asp90 could act as a base and deprotonates the tetrahedral intermediate. The newly protonated Asp90 will then assist the breakdown of the intermediate by general acid-catalysis, donating a proton to the β -lactam nitrogen. Asp90 in the mononuclear enzyme would therefore function as a proton shuttle^{73,167}. Either k_2 or k_3 in Scheme 3-21 could be rate-limiting.

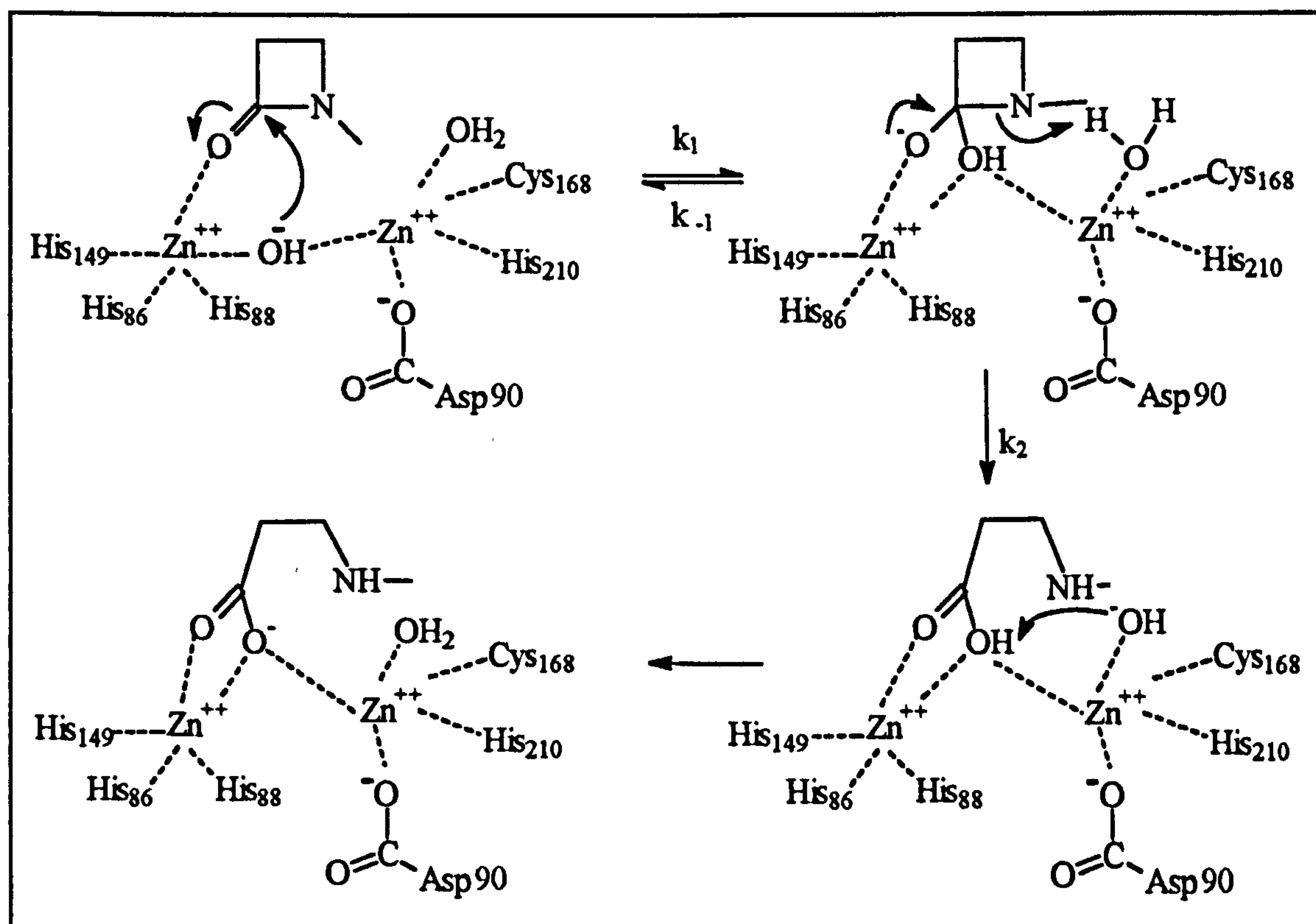
The small kinetic solvent isotope effects observed are compatible with the proposed mechanism: in the first step, where the zinc-bound hydroxide attacks the carbonyl function of the β -lactam ring and as OD^- is a stronger nucleophile than OH^- , we expect an inverse kinetic solvent isotope effect whereas the k_2 and k_3 steps would be associated with a primary kinetic solvent isotope effects.



Scheme 3-21

However, when considering the case of the enzyme binding two zinc ions, Asp90 is one of the five ligands of the second zinc ion along with Water1, Cys168, His 210 and another water molecule⁶¹ and therefore cannot perform its role as a proton shuttle. A possible mechanism in this case is shown in Scheme 3-22. The zinc-bound hydroxide acts as the nucleophile and attacks the β -lactam carbonyl. The

tetrahedral intermediate formed would then collapse upon protonation of the nitrogen of the β -lactam by the water molecule bound to the second zinc ion. Asp90 obviously cannot perform this role as it is bound to the second zinc. It has been previously suggested that water co-ordinated to zinc is a good general acid catalyst⁵³.



Scheme 3-22

Such a mechanism is in accord with the recent crystallography studies of the β -lactamase II by Fabiane *et al*¹⁶⁷. As previously reported the enzyme active site contains two zinc ions, one of high affinity and the other one of low affinity. A water molecule is reported to bridge the two zinc ions in series with Asp90 but unlike binuclear zinc peptidase whose bridging water is at equal distance between

the two zinc ions, Water1 in β -lactamase II lies very much closer to zinc1 than zinc2 and this distance is not affected by the positioning of zinc2.

The zinc-bound hydroxide (at neutral pH), which is polarized and positioned by the two zinc ions in a binuclear zinc enzyme or one zinc ion in a mono zinc form, performs the attack on the carbonyl of the β -lactam ring. However, the hydroxide when co-ordinated to two zinc ions would be expected to be a very weak nucleophile. The zinc1 is also found to have a stabilising action on the oxyanion intermediate formed during the catalysis. The role of the second zinc is not exactly understood although its binding to the enzyme at low pH seems to help restore the enzyme activity.

Bacillus cereus β -lactamase II seems to be able to function as a mono nuclear zinc enzyme as well as a binuclear zinc enzyme. Such flexibility and adaptability may well be the explanation for the large substrate profile displayed by β -lactamase II. It would be interesting to continue these studies in order to distinguish between the mononuclear and the binuclear zinc enzyme, and to investigate further the dissociation of zinc ions at low pH.

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