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THE UNIVERSITY OF ALBERTA

β-LACTAMS AS β-LACTAMASE OR ELASTASE INHIBITORS

BY

CHAEUK IM

A thesis submitted to the Faculty of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy.

in

PHARMACEUTICAL SCIENCES
(Medicinal Chemistry)

FACULTY OF PHARMACY AND PHARMACEUTICAL SCIENCES

Edmonton, Alberta

FALL 1993

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UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled β-LACTAMS AS β-LACTAMASE OR ELASTASE INHIBITORS submitted by CHAEUK IM in partial fulfillment of the requirement for the degree of DOCTOR OF PHILOSOPHY in PHARMACEUTICAL SCIENCES (Medicinal Chemistry).

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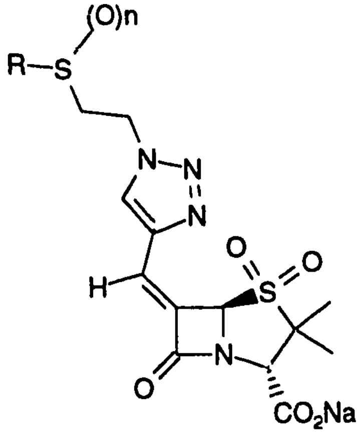
ABSTRACT

6-Exo-methylene penicillin derivatives of type (I) and (II) were synthesized and studied as possible β-lactamase inhibitors. The *in vitro* β-lactamase inhibitory activities were determined by a spectrophotometric assay using the penicillinase from *B. cereus*; cephalosporinase from *E. cloacae*; and a broad spectrum β-lactamase IIIa (R-TEM) from *E. coli*. Their activities were compared with Sulbactam and Tazobactam (YTR-830).

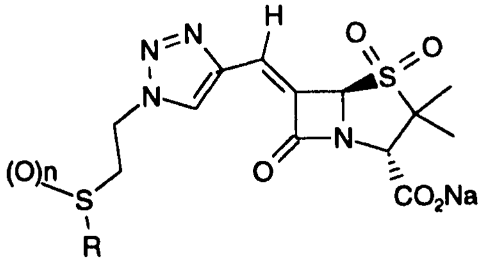
The phenylthiadiazole derivatives showed stronger activity than the methylimidazole compounds. Compounds with a sulfone (n = 2) on the side chain and the corresponding compounds with a sulfide (n = 0) were not significantly different in their activities. The phenylthiadiazole derivatives (Z-isomer; n = 0, 2) were more potent than Sulbactam and comparable to Tazobactam against the tested β-lactamases. The Z-isomers were more active than the E-isomers as previously reported in the literature for related compounds.

As a second part of this research project, 3-substituted cephalosporin derivatives (III) were prepared and studied as possible human leukocyte elastase inhibitors.

The introduction of a diphenylcyclopropyl ring at the 2-position substantially increased the inhibitory activity. The diphenylcyclopropyl group might provide a liphophilic binding site to the surface of the enzyme. The introduction of a bulky electron withdrawing group ($CO_2CH_2CH_3$) at the 4-position in the triazole ring of compounds (III) resulted in stronger activity than a small electron withdrawing group (CHO), regardless of the substituents at the 2-position in the cephalosporin.

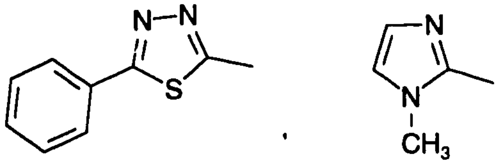


(I)

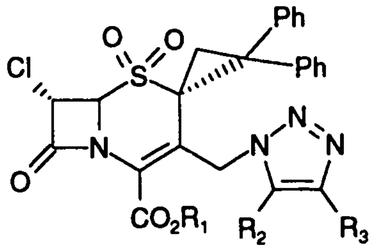


(II)

R =



n = 0 , 2



(III)

R_1 = $CH(Ph)_2$

R_2 or R_3 = H or carbonyl group

ACKNOWLEDGMENTS

The author would like to take this opportunity to express his sincere gratitude from the bottom of his heart to Dr. Ronald G. Micetich and Dr. Mohsen Daneshtalab for giving him the opportunity to carry out these two challenging projects. Their financial assistance, guidance, and encouragement during the course of his studies and advice in the preparation of this thesis are gratefully acknowledged.

The author wishes to thank the staff of Synphar Laboratories (especially, Mr. Dave Czajkowski, Dr. Samarendra Maiti, Mr. Bruce Lix, and Ms. Joanne Mclernon) for their kind advice and technical support throughout these projects and Mr. Kevin Atchison for the enzyme tests.

Finally, the author also wishes to express thanks to all members of his family for their continuous support and warm encouragement.

The author dedicates this thesis to his departed father, Youngtaek Im.

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LIST OF ABBREVIATIONS

7-ACA	7-Aminocephalosporanic acid
Ala	Alanine
6-APA	6-Aminopenicillanic acid
Asp	Aspartic acid
°C	Degrees celsius
Cys	Cysteine
DMSO	Dimethylsulfoxide
Enz	Enzyme
g	Gram
Glu	Glutamic acid
His	Histidine
HLE	Human Leukocyte Elastase
hrs.	Hours
Hz	Hertz
IR	Infra Red
Leu	Leucine
Lys	Lysine
M	Molar
Met	Methionine
mg	Milligram
μg	Microgram
μmol	Micromole
mmol	Millimole
mol	Mole
nmol	Nanomole
NMR	Nuclear Magnetic Resonance
NOE	Nuclear Overhauser Effect
PBP	Penicillin Binding Protein
Ph	Phenyl
Pro	Proline
Ser	Serine

THF	Tetrahydrofuran
Thr	Threonine
TLC	Thin Layer Chromatography
TMS	Tetramethylsilane
Val	Valine

CHAPTER 1

INTRODUCTION

1.1 β-Lactam antibiotics

β-Lactam antibiotics are antibacterial agents, which are chemically characterized by the possession of a four-membered β-lactam ring and which, in low concentration, inhibit the growth of microorganisms. They are produced by microorganisms or prepared wholly or partly by chemical synthesis. β-Lactam antibiotics can be classified into several groups according to their structure:

1.1.1 Penicillins

After Fleming observed that *Penicillium notatum* inhibited the growth of bacteria in an experimental culture dish (Fleming, 1929), many penicillins with a broader spectrum and improved pharmacokinetic properties were introduced into medical practice. These penicillins are mainly semisynthesized from 6-amino penicillanic acid (6-APA) (1), which is obtained by enzymatic deacylation of naturally occurring penicillins such as penicillin G (2) using amidases (Batchelor *et al.*, 1959), or by a chemical process.

The isoxazolyl penicillins such as cloxacillin (3), have a bulky 6-substituent to provide steric hindrance to enzymatic degradation.These

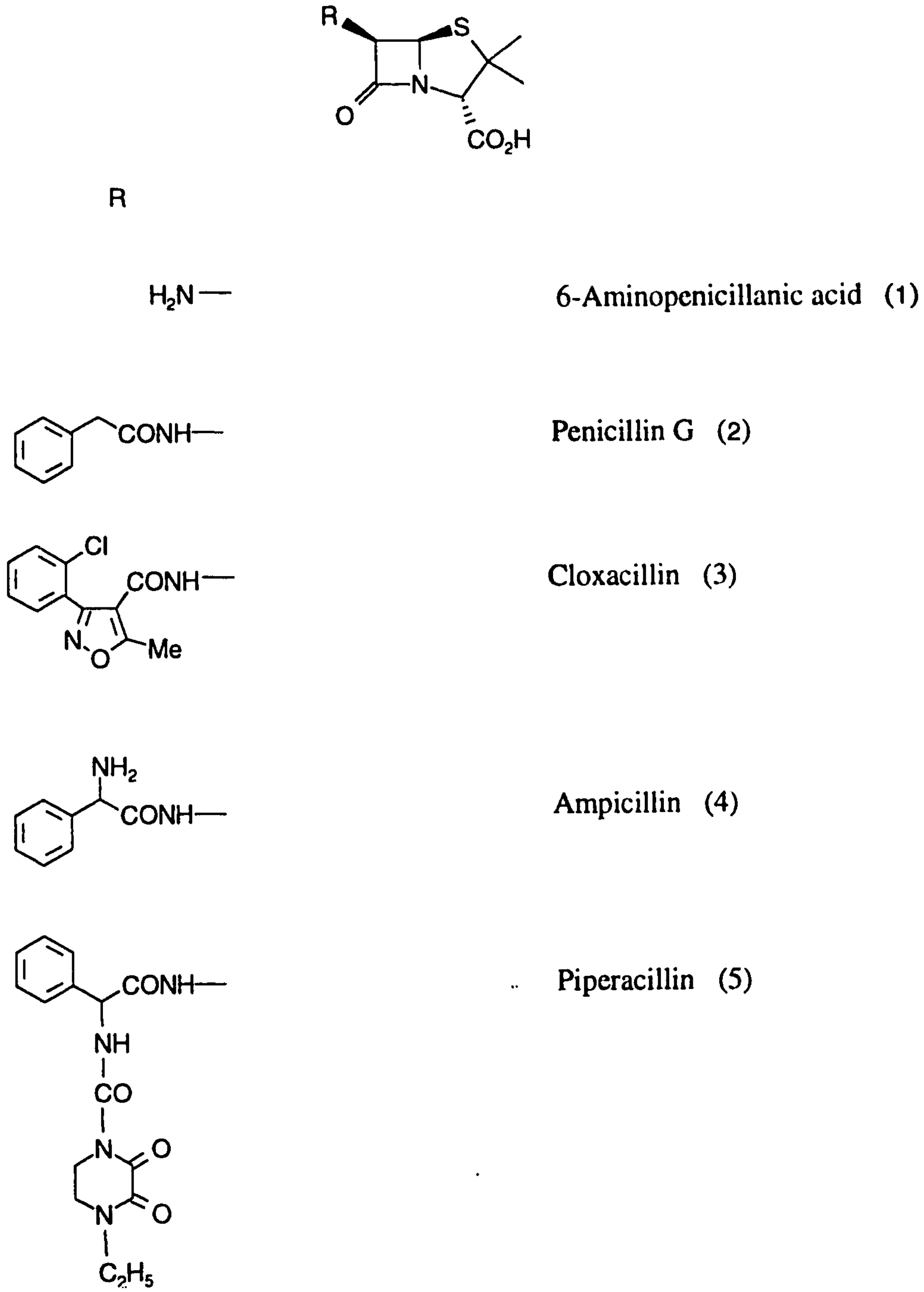


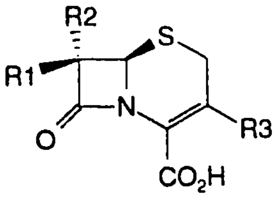
Figure 1 - 1 Structures of penicillins

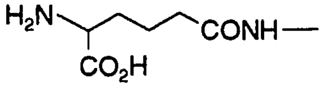
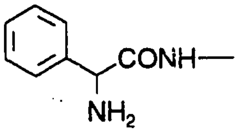
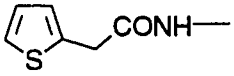
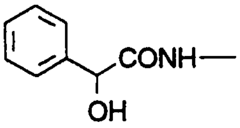
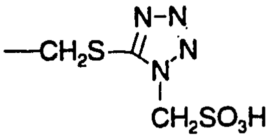
compounds have stability against the β-lactamases that hydrolyze the β-lactam ring resulting in loss of antibacterial activity. Their activity is however restricted to Gram-positive bacteria. The aminobenzylpenicillins such as ampicillin (4) (Klein *et al.*, 1963) have little stability to β-lactamases but have an extended spectrum, with activity that includes Gram-positive bacteria, and some Gram-negative bacteria such as *E. coli*, some *Proteus*, and *H. influenzae*. The acylureidopenicillins such as piperacillin (5) are generally active against Gram-positive bacteria and very active against Gram-negative bacteria, including *Ps. aeruginosa* and *Proteus vulgaris*. They are however susceptible to β-lactamases.

1.1.2 Cephalosporins

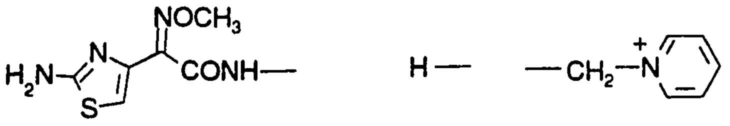
Cephalosporin C (6), a natural cephalosporin, was relatively stable to penicillinase from *Bacillus cereus* (Abraham *et al.*, 1956) and was effective against urinary tract infections (Fleming, 1963). But its clinical usefulness was limited by its generally weak antibacterial activity.

7-Aminocephalosporanic acid (7-ACA) (7), like 6-APA, is a key intermediate and has introduced thousands of new semisynthetic cephalosporins. It is prepared by a chemical process (Morin *et al.*, 1962) from cephalosporin C. In general, antibacterial properties and β-lactamase stability are associated with the substituents at the 7-position, while the groups at the 3-position determine properties such as metabolic stability and pharmacokinetic properties.

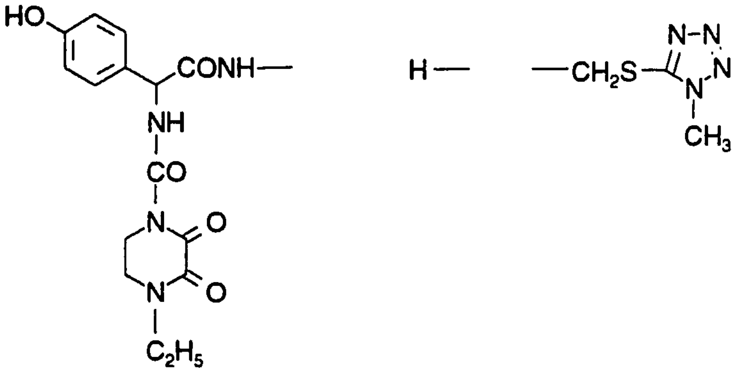


R1	R2	R3	
	H—	$—CH_2OCOCH_3$	Cephalosporin C (6)
$H_2N—$	H—	$—CH_2OCOCH_3$	7-ACA (7)
	H—	$—CH_3$	Cephalexin (8)
	H—	$—CH_2OCOCH_3$	Cephalothin (9)
	H—		Cefonicid (10)

Cefotaxime (11)



Ceftazidime (12)



Cefoperazone (13)

Figure 1 - 2 Structures of cephalosporins

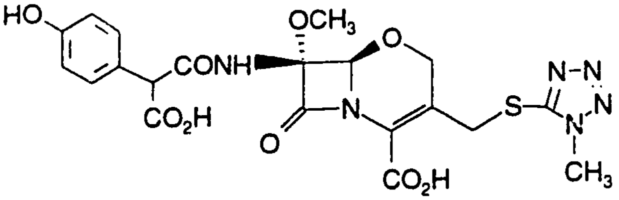
The cephalosporins have been classified into five groups according to their biological properties. Group I cephalosporins such as cephalexin (8) are orally active against *Staphylococci* and *Streptococci*, but less active against Gram-negative bacteria. They are stable against β-lactamases from Gram-positive bacteria and show some resistance to certain β-lactamases from Gram-negative bacteria.

Group II cephalosporins such as cephalothin (9) are parenterally very active against Gram-positive bacteria and moderately active against non-β -lactamase producing Gram-negative bacteria. In general, they are not sensitive to β-lactamases from Gram-positive bacteria but are sensitive to β-lactamases from Gram-negative bacteria.

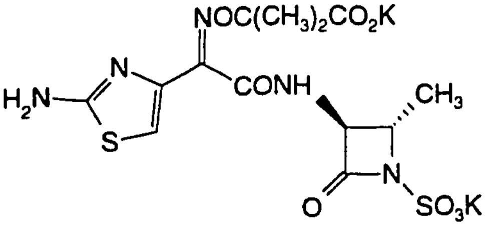
Group III cephalosporins such as cefonicid (10) are parenterally active against *Enterobacteriaceae*, *Haemophilus* and *Gonococci* and stable to β-lactamases from Gram-negative bacteria. Some of these group III cephalosporins such as cefotaxime (11) contain the aminothiazolyl acetamido group, usually substituted by a *syn*-methoxyimino group, at the 7- position and this structural feature increases their stability against β-lactamases.

Group IV cephalosporins like ceftazidime (12) are parenterally active against the *Pseudomonas* species. Some of this group, such as cefoperazone (13), have the acylureido moiety as a common feature just like the acylureido penicillins.

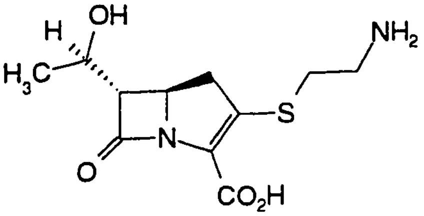
Group V cephalosporins such as moxalactam (14) are characterized by an α-methoxy group at the 7- position and this confers great β-lactamase stability to the compounds. This group is also called cephamycins.



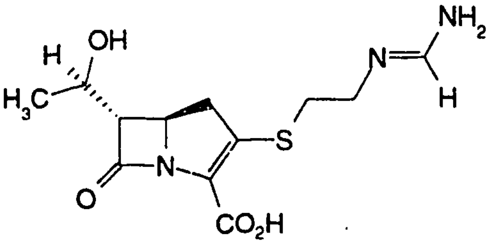
Moxalactam (14)



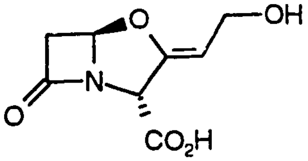
Aztreonam (15)



Thienamycin (16)



Imipenem (17)



Clavulanic acid (18)

Figure 1 - 3 Structures of other lactams

1.1.3 Other β-lactams

Monobactams were independently isolated from bacteria by Taked workers (Imada *et al.*, 1981) and a group at Squibb (Sykes *et al.*, 1985) Aztreonam (15), a chemically synthesized monobactam, showed stabilit to β-lactamases and good activity against Gram-negative bacteri including *Ps. aeruginosa*.

Thienamycin (16) from *Streptomyces cattleya*, the first carbapenen was reported by a Merk group (Albers *et al.*, 1978). It has a very broa spectrum of activity and surpasses all of the Group IV cephalosporins. Th spectrum covers both Gram-positive and Gram-negative organisn including *Ps. aeruginosa*. However, instability of thienamycin in solutio made it unsuitable for formulation as a clinical product. Fortunatel imipenem (17), the N-formimidoyl derivative, not only met th requirement of being chemically stable, but in many cases was even mor potent than thienamycin.

Clavulanic acid (18) has been isolated from *Streptomyces clavuligeru* (Howarth *et al.*, 1976) and has weak, though broad spectrum, activity a an antibiotic but it has potent inhibitory activity against a wide range of β-lactamases (Cole, 1981).

1.2 Mode of action of β-lactam antibiotics

Bacteria have a cell wall which withstands osmotic pressure. The peptidoglycan, the main structural component of the cell wall, is a branched polymer of which the backbone consists of alternating N-acetylglucosamine and N-acetylmuramic acid units crosslinked *via* the peptide side chains (Wise *et al.*, 1965) (Figure 1-4). This structure is unique to prokaryotic cells and gives an excellent selective target for β-lactam antibiotics.

The inner cytoplasmic membrane has the enzyme systems responsible for the synthesis of peptidoglycan. In Gram-positive bacteria, the cell wall consists of a cytoplasmic membrane surrounded by thick crosslinked layers of peptidoglycan, through which the β-lactam antibiotics penetrate by passive diffusion. In Gram-negative bacteria, the peptidoglycan layer is thinner and is surrounded by a complex outer membrane to form an additional penetration barrier. The access to the cytoplasmic membrane of Gram-negative cells is largely controlled by outer membrane proteins (porins) and these proteins provide passive diffusion channels for β-lactam antibiotics.

The structure of peptidoglycan and the effect of β-lactams on the crosslinking of the peptidoglycan strands were first studied by Tipper. Due to the resemblance in conformation between the D-alanyl-D-alanine and the β-lactam antibiotics , they may act as false substrates for D-alanyl-D-alanine transpeptidases (Tipper *et al.*, 1965). Transpeptidases take part in the transamination reaction to form a crosslink in the peptidoglycan

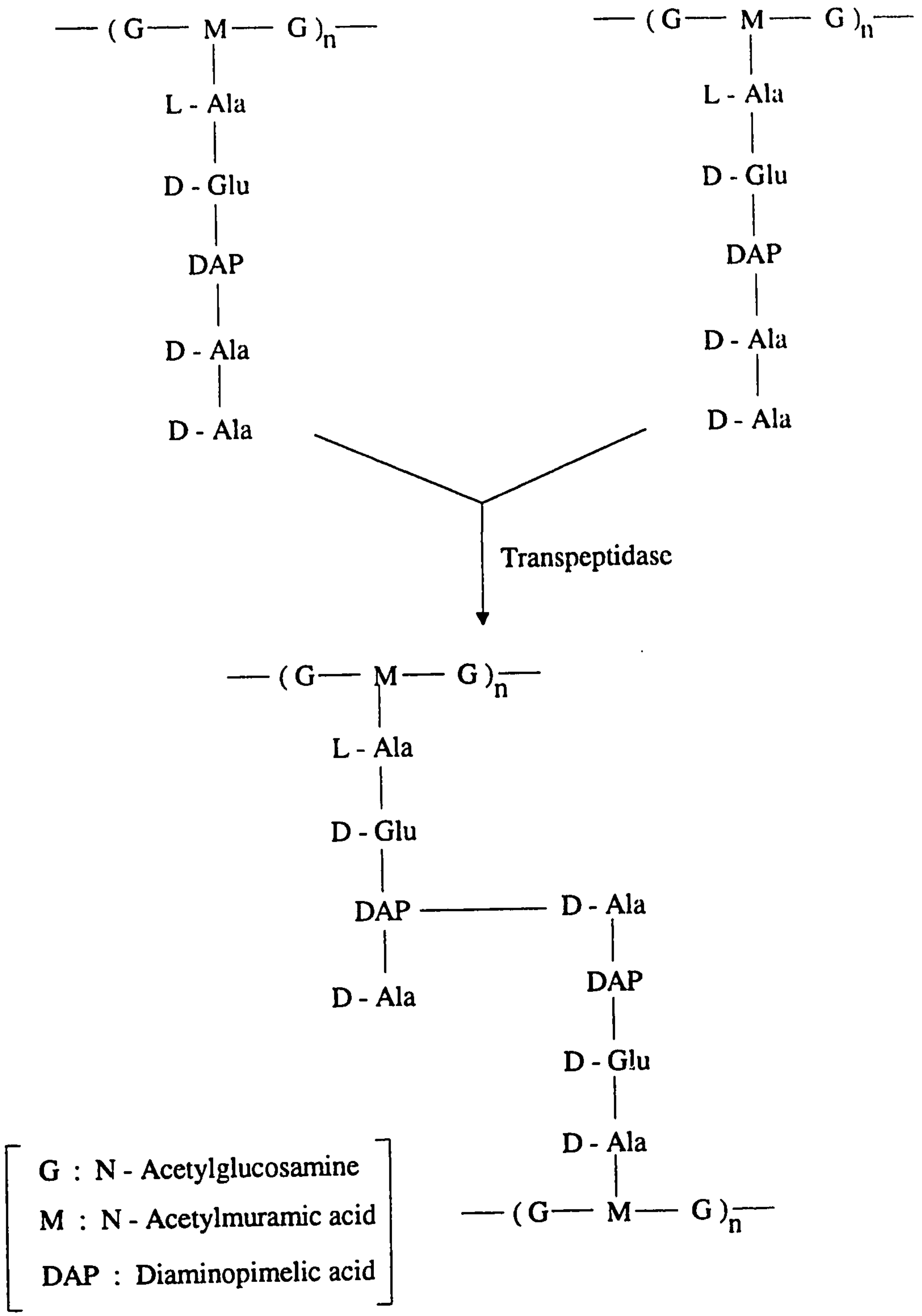


Figure 1 - 4 Structure of peptidoglycan in E. coli

resulting in changes in conformation of the enzyme and interruption of biosynthesis of peptidoglycan. (Barnett , 1973).

1.3 β-Lactamases

The mechanisms of bacterial resistance against antibiotics (Giancarlo *et al.*, 1982) include;

(a) Transformation of the antibiotic into an inactive form,

(b) Modification of targets in the cell to give low affinity for the antibiotic,

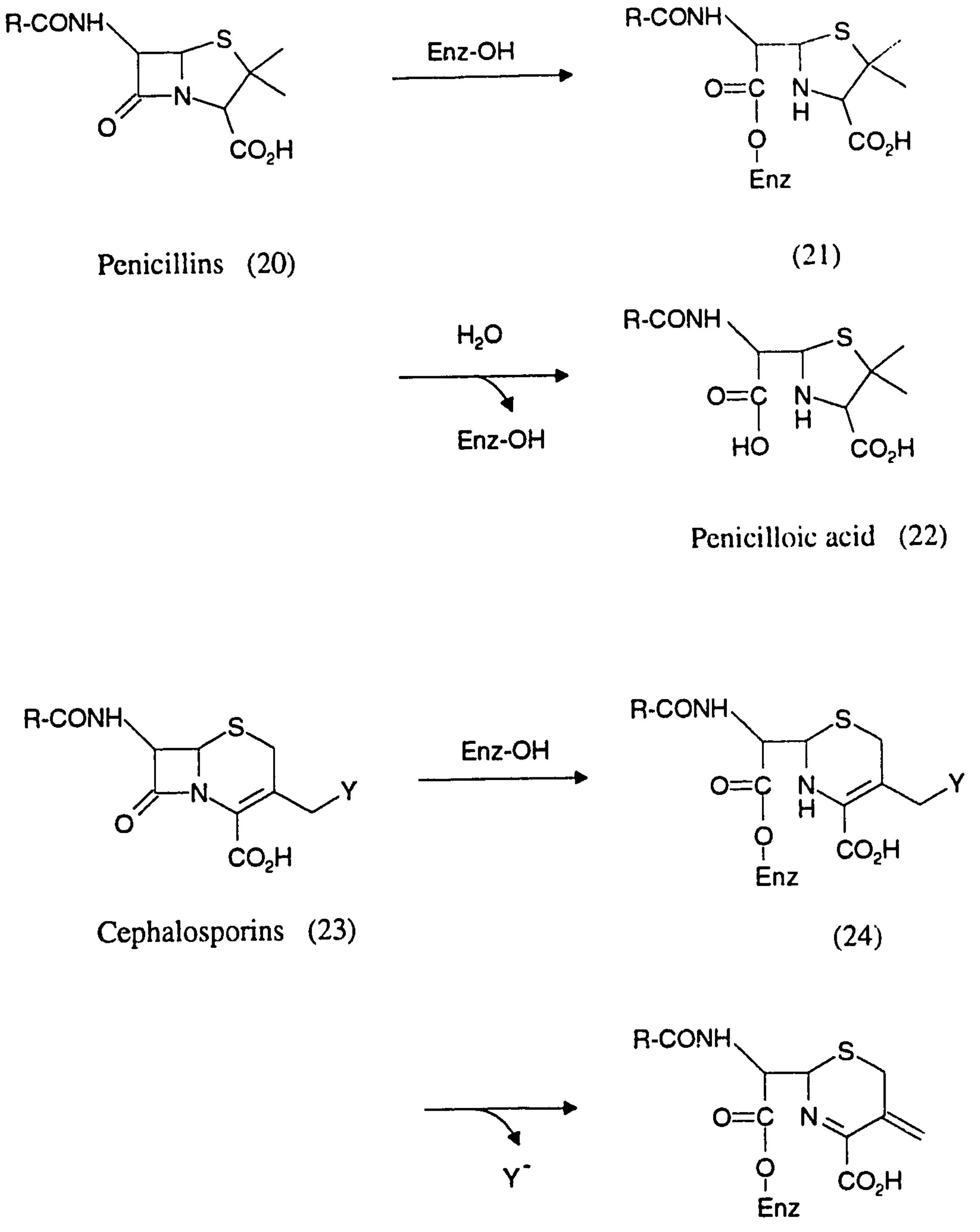
(c) Modification of the permeability of the bacteria to the antibiotic,

(d) Increased production of the targets inhibited by the antibiotic.

For β-lactam antibiotics, modification of the antibiotic by β-lactamase, which opens the four-membered ring of β-lactam to give an inactive form of acyl-enzyme, is the major resistance mechanism (Figure 1-5).

However, several other mechanisms of resistance to the β-lactams are also known. The resistance to penicillin in *Streptococcus pneumoniae* is mainly due to alteration in its PBP2 (Penicillin Binding Protein 2) and the mutant form of PBP has less affinity for penicillin.

It is known that changes in the Gram-negative outer membrane may affect the influx rate of the β-lactam and thereby the resistance level. In particular, mutational loss or modification of outer membrane proteins (porins) (e.g. OmpF and OmpC), through which β-lactams may diffuse, provides resistance against the β-lactams (Russell and Chopra, 1990).



Penicillins (20)

(21)

Penicilloic acid (22)

Cephalosporins (23)

(24)

5-Methylene-dihydrothiazine (25)

Figure 1 - 5 Interaction of β-lactams with β-lactamase

The overproduction of the low affinity PBP5 by resistant *Streptococcus faecium*, which seems to be capable of taking over the functions of the other PBPs, provides an additional resistance mechanism against β-lactams (Ghuysen *et al.*, 1989).

The production of β-lactamases was reported from Gram-positive and Gram-negative bacteria (Richmond and Sykes, 1973; Sykes and Matthew, 1976), actinomycetes (Ogawara, 1975), yeasts (Mehta and Nash, 1978), and blue-green algae (Kushner and Breil, 1977).

These β-lactamases may be chromosomally coded or carried on plasmids which may transfer the genetic material from cell to cell to spread resistance against antibiotics. Some β-lactamases are inducible, in which the production of β-lactamase is started by the exposure to certain β -lactams, so called 'inducer'. Some β-lactamases are constitutive, in which the enzymes are produced continuously even without β-lactam induction.

In Gram-positive bacteria, there is no outer membrane so that β-lactamases are secreted outside of the cell. But Gram-negative bacteria have an outer membrane so that the produced β-lactamases are located in the periplasmic space between the inner and outer membranes. In this location they are able to intercept incoming β-lactams before the antibiotics reach the target molecules bound to the cytoplasmic membrane. Therefore, comparatively small amounts of β-lactamase provide an efficient protection for the bacteria and Gram-negative bacteria have an extra defence against antibiotics, in addition to the penetration barrier of the outer membrane.

1.3.1 Classification of β-lactamases

The β-lactamases can be classified according to producers and genetic materials as follows (Neu, 1985) ;

1. Gram-positive bacteria
    - 1-1. Chromosomal ------------------------------------e.g. *Bacillus*
    - 1-2. Plasmid ----------------------e.g. *S. aureus, S. epidermidis*
2. Gram-negative bacteria
    - 2-1. Chromosomal
        - 2-1-1. Cephalosporinases
            - 2-1-1-1. Inducible -----e.g. *Enterobacter, Pseudomonas*
            - 2-1-1-2. Constitutive ----e.g. *Bacteroides, Acinetobacter*
        - 2-1-2. Penicillinases ---------------------------------e.g. *E. coli*
        - 2-1-3. Broad spectrum β-lactamases ------e.g. *Klebsiella, Bacteroides*
        - 2-1-4. Cefuroximases -------------------------e.g. *P. vulgaris*
    - 2-2. Plasmid
        - 2-2-1. Broad spectrum, but primarily penicillinases (TEM, OXA) ---e.g. *Enterobacteriaceae, Pseudomonas*

The classification proposed by Sykes and Matthew depends on the substrate, inhibitor profiles, isoelectric points, molecular weight, and genetic origin of the enzyme as follows (Sykes and Matthew, 1976) :

Type I : Enzymes active principally against cephalosporins, inhibited by isoxazolylpenicillins, not by clavulanic acid; chromosomal e.g. P99

Type II : Enzymes active predominantly against penicillins, inhibited by isoxazolylpenicillins and clavulanic acid

Type III : Enzymes equally active against penicillins and cephalosporins, inhibited by isoxazolylpenicillins and clavulanic acid; plasmid e.g. TEM, SHV

Type IV : Enzymes equally active against penicillins and cephalosporins, inhibited by clavulanic acid, not by isoxazolylpenicillins; chromosomal; e.g. K-1

Type V : Enzymes equally active against penicillins and cephalosporins, inhibited by clavulanic acid, not by isoxazolylpenicillins; plasmid;e.g. OXA, PSE

Type VI : Enzymes more active against cephalosporins than penicillins, inhibited by isoxazolylpenicillins and carbenicillin

Ambler suggested a different classification based on the amino acid sequence (Ambler, 1980). The main division of this classification is serine β-lactamases, which have an active site serine and function by a covalent acyl-enzyme mechanism. The serine β-lactamases can be further divided into three classes according to the amino acid sequences, called class A, class C, and class D.

The class A β-lactamases are a diverse class and the molecular weight of these enzymes is ~ 29,000 daltons (Ambler, 1980). The penicillinases of *S. aureus* PC1, *E. coli* TEM-1, and *E. coli* TEM-2 belong to the class A β-lactamases. α-Methylbenzyl penicilloate is the inhibitor of these enzymes (Jones *et al.*, 1989).

The cephalosporinases of *E. coli* Amp C, *P. aeruginosa*, and *Salmonella typhimurium* belong to class C β-lactamases.The structures of these enzymes resemble those of class A β-lactamases (Oefner *et al.*, 1990). The molecular weight of these enzymes is ~ 39,000 daltons (Jaurin *et al.*, 1981) and aromatic boronic acids are reported to be competitive inhibitors of these enzymes (Pazhanisamy and Pratt, 1989).

The OXA β-lactamase (Mossakowska *et al.*, 1989) is assigned to class D β-lactamases, but little has been reported about these enzyme.

Another division is metallo-β-lactamase which is referred to as class B β-lactamase. *Bacillus cereus* was found to produce zinc β-lactamase which requires zinc ions for activity (Sabath and Abraham, 1966); some other metal ions, such as cobalt and cadmium, could replace zinc ions (Davies and Abraham, 1974).The molecular weight of this enzyme is ~ 23,000 daltons (Davies and Abraham, 1974). This enzyme catalyzes the hydrolysis of nearly all β-lactams (Buckwell *et al.*, 1988) and is inhibited by chelating agents, not by the conventional β-lactamase inhibitors such as clavulanic acid.

1.4 β-Lactamase Inhibitors

The introduction of β-lactams put selective pressure on the microorganisms to overcome the antibiotics and they biosynthesized β-lactamases to defend themselves. The appearance of β-lactamase producing strains in turn applied pressure on the antibiotic researcher, who responded by introducing new antibiotics to which these strains were susceptible. This introduction of new antibiotics gave additional pressure on the microorganisms resulting in the continuous microbial resistance-new antibiotics cycle. Another way to overcome the resistance problem and break this cycle was to develop β-lactamase inhibitors, in addition to modifying β-lactams, so that they are more stable to β-lactamase.

The first significant progress came from the isolation of clavulanic acid (18) from *Streptomyces clavuligerus* (Howarth *et al.*, 1976) and olivanic acid from *Streptomyces olivaceus* (Brown *et al.*, 1977). These β-lactams are effective in enhancing the activity of β-lactamase susceptible penicillins and cephalosporins against β-lactamase producing bacteria (Reading and Cole, 1977).

Crystal structures (Kelly *et al.*, 1986; Samraoui *et al.*, 1986) and amino acid sequence comparisons (Mossakowska *et al.*, 1989; Joris *et al.*, 1988) of serine β-lactamases and bacterial cell wall DD-peptidase suggested that they are β-lactam recognizing enzymes with similar active site structure and function. It was also reported that the reverse conversion, from a β-lactamase to the DD-peptidase, has been achieved partly by the replacement of a 28 amino acid segment of the TEM β-lactamase with the corresponding region of *E. coli* DD-peptidase (Chang *et al.*, 1990). Therefore, it seems likely that the serine β-lactamases are evolutionary descendants of the DD-peptidases that catalyze the crosslinking of the peptidoglycan of bacterial cell walls.

The conclusion from the above discussion promoted the preparation of β-lactam analogues as β-lactamase inhibitors. Unfortunately there has been little progress in developing the inhibitors of class B (metallo) β-lactamase, so that active inhibitors, other than chelating agents, have not been reported yet. Serine β-lactamase (class A, C, and D) inhibitors can be classified into two major groups according to their mechanism of action as follows: acyl-enzyme forming inhibitors and transition-state analogue inhibitors.

1.4.1 Acyl-enzyme forming inhibitors

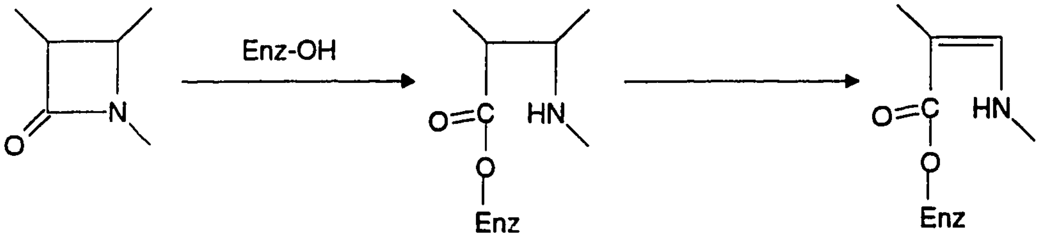
These inhibitors can be further divided into two groups according to the stability of the acyl-enzyme complex as reversible inhibitors and irreversible inhibitors.

1.4.1.1 Reversible inhibitors

Thienamycin (16) and imipenem (17) showed some activity against several serine β-lactamases and there was an indication that the free enzyme was released after interaction with these compounds (Okonogi *et al.*, 1982; Yamaguchi *et al.*, 1984). Some of the cephalosporins like moxalactam (14) also inhibit β-lactamases (Charnas *et al.*, 1988; Mazzella *et al.*, 1989).

1.4.1.2 Irreversible inhibitors

These inhibitors form a stable acyl-enzyme complex with β-lactamase and undergo rearrangement into a chemically more stable structure (29) (Figure 1-6) to inhibit the enzyme irreversibly. This group includes clavulanic acid (18), penicillanic acid sulfones, and 6-β-halopenicillanic acids.



(27) (28) (29)

Figure 1 - 6 Rearrangement of acyl-enzyme

(For full explanation, see Figure 1 - 7)

Clavulanic acid (18) has weak antibacterial activity but good β-lactamase inhibitory activity. It showed a good synergistic effect with β-lactams and protected them from inactivation (Hunter *et al.*, 1980) so that 'Augmentin' (mixture of clavulanic acid and amoxicillin) and 'Timentin' (mixture of clavulanic acid and ticarcillin) were introduced. In general, it inhibited class A β-lactamases, but not class C enzymes (Neu and Fu, 1978). However, clavulanic acid is an effective inducer of β-lactamases in *P. aeruginosa* and *E. cloacae*. Thus, there is a potential problem that clavulanic acid might decrease the activity of β-lactam antibiotics with which it is combined.

The mechanism of inhibition of β-lactamases by penicillanic acid sulfone (30) (Figure 1-7) suggested the transient inhibition by formation of (33) and the irreversible inhibition by formation of (34) (Pratt, 1989a). Penicillanic acid sulfone itself, sulbactam (30), has only weak antibacterial activity against most Gram-positive cocci, *Enterobacteriaceae*, and *Pseudomonas*. It is generally a less active inhibitor against class A β-lactamases but more active against class C enzymes than clavulanic acid (Arisawa and Then, 1982). The activity of the penicillin sulfones can be modified by changing the C-6 substituents (Hall *et al.*, 1985; Hanessian *et al.*, 1989) or C-2 substituents (Gottstein *et al.*, 1985; Tanaka *et al.*, 1988).

The tazobactam (35) was introduced and appeared to be more active than clavulanic acid against class C enzymes (Micetich *et al.*, 1987; Akova *et al.*, 1990). Hence 'Unasyn' (mixture of sulbactam (30) and ampicillin(4)) and 'Tazocine' (combination of tazobactam (35) and piperacillin (5)) were introduced. In general, the sulfone combinations seem more effective than Augmentin against chromosomal cephalosporinase while the reverse holds for plasmid mediated penicillinases.

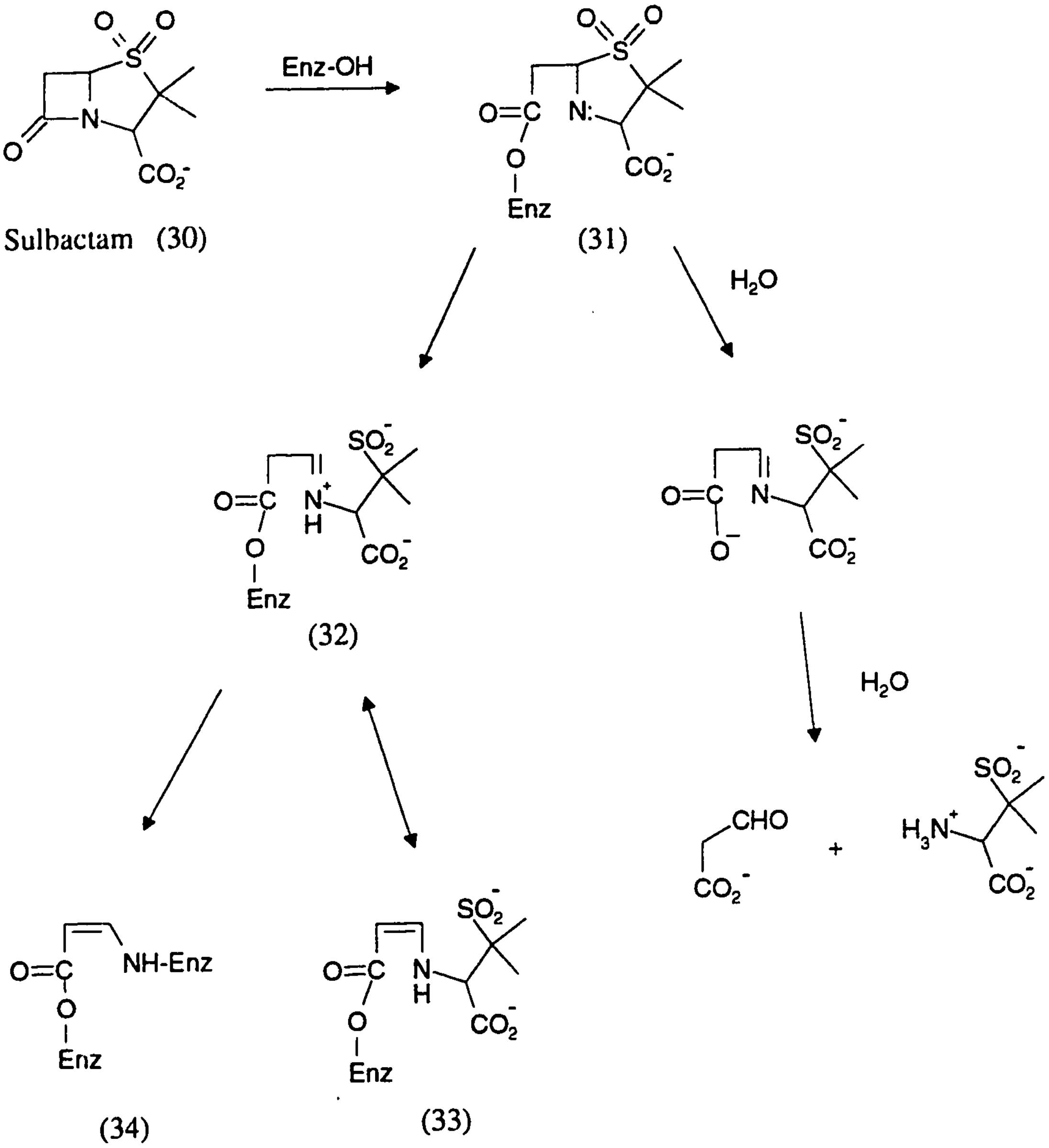
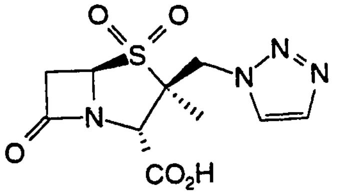
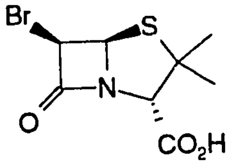


Figure 1 - 7 Inhibition mechanism of β-lactamase by penicillin sulfone

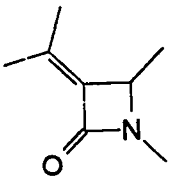
6-β-Bromopenicillanic acid (36) was the first significant inhibitor among the 6-β-halopenicillanic acids and inhibited class A β-lactamases. The α-bromo isomer was inactive to β-lactamase, but the β-bromo isomer was active (Pratt and Loosemore, 1978). 6-β-Iodopenicillanic acid is comparably active, but the chloro analogue is much less active (von Daehne, 1980; Wise *et al.*, 1981).



Tazobactam (35)



6-β-Bromopenicillanic acid (36)



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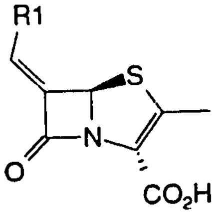


(38)

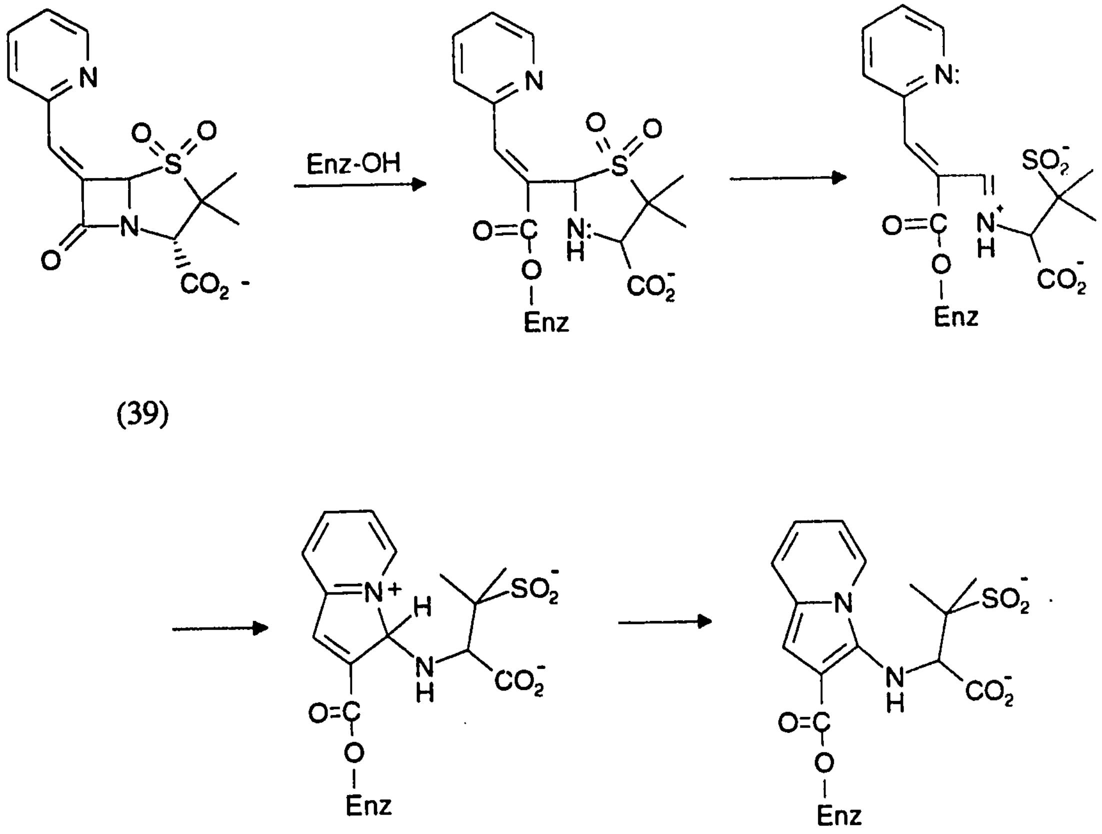
6(7)-Exo-methylene penam (cephem) structure (37) and penems (38) also inactivate β-lactamases through an acyl-enzyme intermediate. The penems (38) where R1 is a five-membered ring heterocycle (Bennett *et al.*, 1991) and especially the 1,2,3-triazole derivatives (Coleman *et al.*, 1989) are potent inhibitors of both class A and C β-lactamases. Among them, the Z-isomers are more effective inhibitors than the E-isomers (Broom *et al.*, 1990) probably due to steric hindrance of the E-isomers to interaction with the active site of the enzyme.

The combination of an exomethylene penam and sulfone gave the compound (39) which is a good inhibitor of both class A and class C β-lactamases (Chen *et al.*, 1987). The possible mechanism (Figure 1-8) suggested that interaction with the enzyme gave a hydrolytically stable bicyclic structure (40) (Chen *et al.*, 1986).

1.4.2 Transition-state analogue inhibitors

According to transition-state theory, a stable compound which resembles the transition-state structure may have an higher affinity for the enzyme than the substrate or product does. Actually such stable substances, called 'transition-state analog inhibitors', appear to be effective inhibitors against the enzyme (Polgar, 1989). They are mainly described for serine proteinases (Kraut, 1977) and only a few have been made for β-lactamases.

Borate and boronates (41) were reported to inhibit serine β-lactamases, by formation of tetrahedral adducts (42) with the active site (the serine hydroxy group) (Knap and Pratt, 1991; Crompton *et al.*, 1988). There

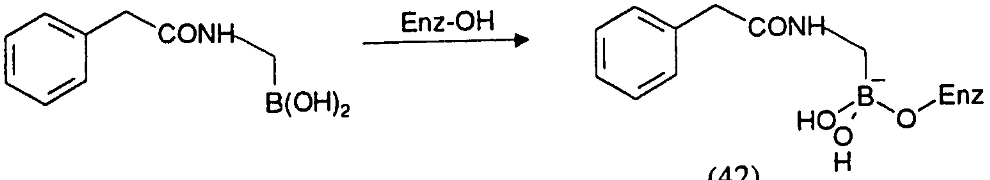


(39)

(40)

Figure 1 - 8 Possible mechanism of compound (39) as a β-lactamase inhibitor

were some reports indicating synergism between β-lactam antibiotics and boronates (Cartwright and Waley, 1984; Amicosante *et al.*, 1989). Azetidine (43) which is a penicillin derivative was reported to be a weak inhibitor (Sammes *et al.*, 1984). Phosphonate monoesters (44) showed some inhibitory activity against class A and C β-lactamases (Pratt, 1989b; Rahil and Pratt, 1991).



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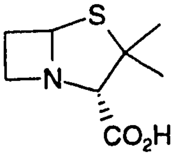
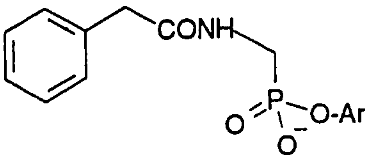


Azetidine (43)



(44)

Figure 1 - 9 Transition-state analogue inhibitors of β-lactamase

1.5 Elastase

Elastase has been defined as a group of proteinase that are able to solubilize elastin.

1.5.1 Elastin

Elastin, the elastic protein fiber, is one of the major components of connective tissues in lung, skin, and blood vessels. It is a highly cross-linked polymer of a subunit, tropoelastin, with molecular weight of 72,000 daltons (Smith *et al.*, 1972). Elastin is extremely rich in glycine, valine, alanine, and proline; but it is low in residues such as histidine and tryptophan (Yoon *et al.*, 1985).

In lung, it is found in the pulmonary interstitium and gives the property of linear expansion while retaining the ability to return to its original form (Gosline and Rosenbloom, 1985). Elastin also gives elastic property to the blood vessels. Unlike most other proteins, elastin in the blood vessel wall turns over very slowly with a half-life of years (Lefevre *et al.*, 1980). Elastin endows the skin with tissue tension to withstand considerable distortion and is codistributed with collagen, a protein providing tensile strength.

1.5.2 Distribution and classification

The presence of elastase in pancreas was first recognized in 1949 (Balo *et al.*, 1949) and the pure enzyme was isolated in 1956 from porcine pancreas (Lewis *et al.*, 1956). Elastase activity was also found in human and other animals like dog (Kokas *et al.*, 1951) and fish (Lamy *et al.*, 1961). In addition to pancreas, elastase activity was reported in many other human cells such as blood platelets (Robert *et al.*, 1970), leukocytes (Janoff *et al.*, 1968), and macrophages (Janoff and Basch, 1971). Some of the microorganisms also show elastolytic activity i.e., *Ps. aeruginosa* (Suss *et al.*, 1969) and *Streptomyces griseus* (Gertler *et al.*, 1971). Even some plant proteinases like papain show elastolytic activity (Thomas and Partridge, 1960).

The proteinases can be classified into four groups according to their key constituent and mechanism of reaction: serine proteinases, cysteine proteinases, aspartate proteinases, and metalloproteinases. Pancreatic elastase and leukocytic elastase are the member of serine proteinase group which have a serine residue in the active site. Papain and cathepsins are the cysteine proteinases and possess a cysteine residue in their active site (Mason *et al.*, 1986). Pepsin, which digests food in the stomach, is an aspartate proteinase with the aspartate residue in the active site. *Ps. aeruginosa* elastase, which has a zinc atom in the catalytic center, is a metallo-proteinases (Morihara *et al.*, 1975).

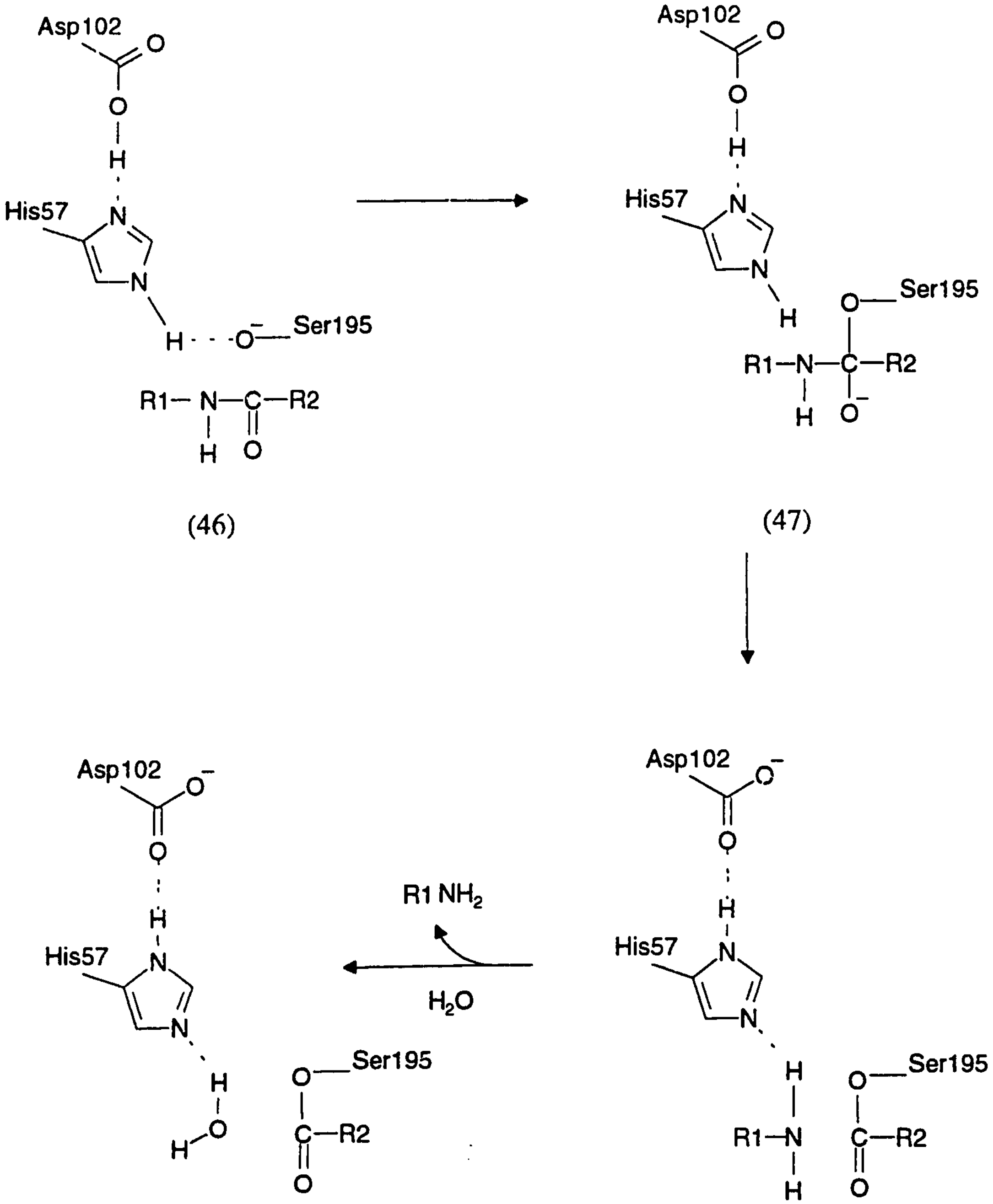
In human, leukocyte elastase and pancreatic elastase are two major serine proteinases. The human leukocyte elastase (HLE), also called human neutrophile elastase, is synthesized in the promyelocytes in the bone marrow and is packaged and released from the Golgi complex in these cells. It is a single peptide with 220 amino acid residues and is found in the granules of polymorphonuclear leukocytes, also called neutrophiles (Fouret *et al.*, 1989; Bainton *et al.*, 1971). The pancreatic elastase is stored as an inactive zymogen in the pancreas and is secreted into the intestine where it becomes activated by trypsin.

1.5.3 Mode of action of elastase

The active site area of elastase is composed of two parts: the substrate binding site which is responsible for the specificity and the catalytic site which takes part in the hydrolysis of peptide bonds. The serine and histidine residues are reported to be important in the enzyme reaction, because chemical modification of these residues resulted in the loss of catalytic activity (Brown and Wold, 1973a; Thompson and Blout, 1973).

Since these two residues are also important to α-chymotrypsin and there is a very close similarity in the structure of elastase and α-chymotrypsin, the mechanism of elastase has been inferred from that of α-chymotrypsin. In fact, x-ray crystallography showed that catalytically important serine, histidine, and aspartate residues in elastase were arranged in exactly the same manner as in α-chymotrypsin. The elastase crystals are enzymatically active so that the mechanisms deduced from crystallographic studies were also probably valid in the solution state (Shotton, 1970). Hence it may be postulated that both enzymes share a common mode of action.

The catalytic center of α-chymotrypsin is composed of three amino acid residues, i.e. histidine-57, aspartate-102, and serine-195. They are linked by hydrogen bonds and form the so-called charge relay system. According to Blow's theory (Figure 1-10), electrons are transferred from the oxygen of Ser-195 to the carbonyl group of the substrate (46); to the imidazole ring of His-57; and to the carboxyl group of Asp-102 *via* the tetrahedral intermediate (47) and release the amine part of the peptide fragment during acylation. In deacylation, electrons are, in turn, transferred from the carboxyl group of Asp-102 to the imidazole ring of His-57; to a water molecule in the media (49); and then to the oxygen of



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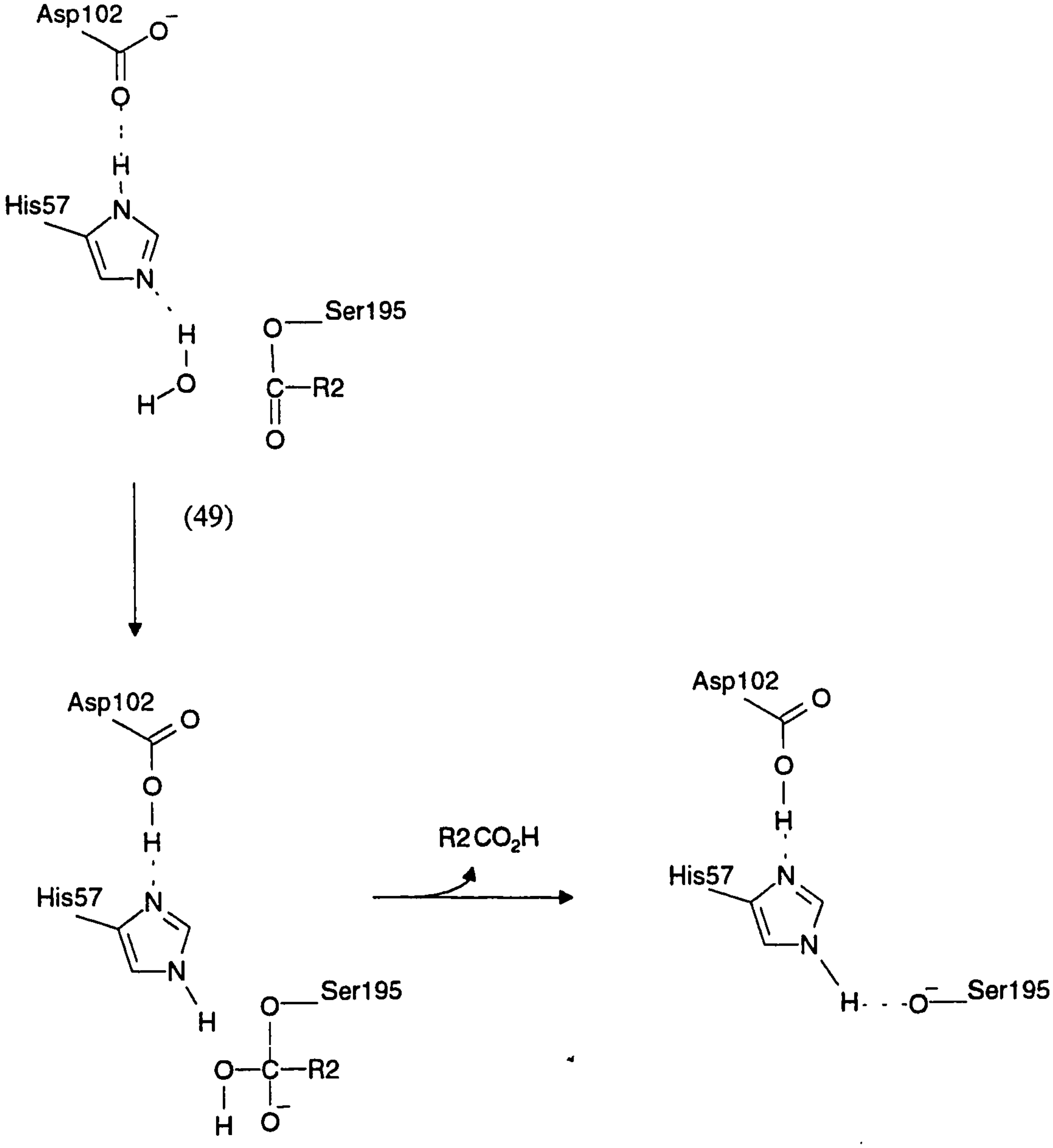


(49)

(50)

(51)

Figure 1 - 10 Charge relay system of chymotrypsin

Ser-195 to regenerate the active form of the enzyme (51) (Blow *et al.*, 1969).

In this reaction, aspartate may act as an ultimate base which receives and releases electrons during catalysis and keeps the imidazole ring properly oriented. Histidine may protect the aspartate from solvents and provide a relay for electron transfer between serine and aspartate residues.

1.5.4 Roles of elastase

1.5.4.1 Physiological roles of elastase

Human leukocyte elastase not only hydrolyzes elastin but also other substances such as collagen (Starket *et al.*, 1977), fibrinogen (Plow *et al.*, 1983), and immunoglobulin (Prince *et al.*, 1979). In addition, it cleaves complements of the immune system (Brozna *et al.*, 1977), blood coagulation factors (Schmidt *et al.*, 1975), and plasma proteinase inhibitors like anti-thrombin (Jochum *et al.*, 1981).

It hydrolyzes foreign substances such as bacteria and immunoglobulin complex, so that it is essential for phagocytosis and defense against infection by invading microorganisms. (Blondin and Janoff, 1976). The elastinolysis may be required after an acute injury of connective tissues, like a wound, as an initial step of tissue repair and it plays an important role in neutrophile migration through connective tissues (Sandhaus, 1987). Pancreatic elastases take part in digesting food. Therefore, we could expect various physiological function of elastases.

1.5.4.2 Pathological roles of elastase

Pathological processes mediated by elastase may occur as undesirable side effects of normal elastase activity.When an imbalance between elastases and their inhibitors occurs due to a deficiency of natural elastase inhibitors or abnormally high levels of elastase, severe tissue damage may occur.

Pancreatic elastase participates in pancreatitis, which occurs when pancreatic zymogens are activated and released into the circulation. Leukocyte elastase has been linked to pulmonary emphysema and rheumatoid arthritis.

Emphysema is an anatomic alteration of the lung by destructive changes of the alveolar walls. Because smoking stimulates neutrophiles to release elastase, it is the major cause of lung emphysema (Janoff, 1985). In rheumatoid arthritis, there is massive infiltration of neutrophiles into the joint space to cause a proteolytic degradation of cartilage (Barrett and Starkey, 1977). *Ps. aeruginosa* elastase attacks immunoglobulins and complement components to damage the natural defense system of the host against bacteria (Fick *et al.*, 1984).

1.6 Elastase Inhibitors

Although elastase has biologically important roles as mentioned above, its activity should be under the control of natural inhibitors to avoid tissue damage. Under normal physiological conditions, elastases are carefully regulated by compartmentalization and natural elastase inhibitors. Any elastase that reaches the circulation is quickly complexed by natural inhibitors. The complexes are cleared from the plasma by the liver and macrophages and are degraded.

The possible biological functions of elastase inhibitors are prevention and regulation of proteolysis. The elastase inhibitors may act to minimize the break down of endogenous substances and regulate the limited elastinolysis of elastase.

1.6.1 Natural elastase inhibitors

1.6.1.1 α_1-Proteinase inhibitor

α_1-Proteinase inhibitor is a plasma protein consisting of single polypeptide with 394 amino acid residues and has a methionine residue at the active site (Johnson and Travis, 1978). This inhibitor is found not only in plasma, but also in lymph and various plasma transudates including the lung alveolar lining fluid. α_1-Proteinase inhibitor is a non-specific inhibitor of serine proteinases so that it inhibits various elastases and other serine proteinases like trypsin (Delshammar *et al.*, 1976; Largman *et al.*, 1976; Beatty *et al.*, 1980). It reacts very fast with human leukocyte elastase with 1:1 binding stoichiometry (Ohlsson and olsson, 1974).

During its complex formation with elastase, a portion of the peptides in this inhibitor is cleaved and it serves as a suicide substrate for the enzymes. Hence this reaction is irreversible and the product does not dissociate into its original components. The report of correlation between deficiency of this inhibitor and emphysema (Laurell and Eriksson, 1963) is evidence for its role as an elastase inhibitor.

The main causes of emphysema are the oxidative inactivation and proteolytic inactivation of this inhibitor. Both chemical oxidation, by cigarette smoking (Janoff and Carp, 1979), and enzymatic oxidation, by neutrophil myeloperoxidase (Clark *et al.*, 1981), of this inhibitor oxidize the reactive site methionine to methionine sulfoxide derivatives (Johnson and Travis, 1979) and reduce its inhibitory activity (Beatty *et al.*, 1980).

Some other proteinases such as papain (Johnson and Travis, 1977), cathepsin L (Johnson *et al.*, 1986), *Ps. aeruginosa* elastase (Morihara *et al.*, 1984), and *S. aureus* proteinases (Potempa *et al.*, 1986) may cleave at or near the active site of this inhibitor to inactivate it.

1.6.1.2 α_2-Macroglobulin

This inhibitor is one of the major components of plasma and is biosynthesized by many cells including fibroblasts and mononuclear phagocytes. It can inactivate various proteinases including leukocyte elastase, macrophage elastase, and cathepsin L. It is actually the only known protein capable of inhibiting proteinases from all four catalytic classes i.e. serine-, cysteine-, aspartate-, and metallo-proteinases.

The proteinases hydrolyze a specific peptide bond of α_2-macroglobulin to give a conformational change to the inhibitor and this results in entrappment of the proteinases by forming covalent bonds with the inhibitor (Barrett and Starkey, 1973). One α_2-macroglobulin forms a complex with two human leukocyte elastases (Ohlsson and Olsson, 1974) and these bound enzymes retain their activities for low molecular weight substrates and inhibitors, but not for high molecular weight proteins (Bieth *et al.*, 1970).

1.6.1.3 Other natural inhibitors

Cystatins are the group of proteins that inhibit cysteine proteinases including the elastolytic enzyme cathepsin L. Cystatins A, B, and C are found in tears, seminal plasma, and saliva. Kininogens are one group of the cystatins and are found in lung and kidney where, along with α_2-macroglobulin, they control extracellular elastinolysis due to cathepsin L (Abrahamson *et al.*, 1986).

The bronchial mucous inhibitor, which contains 107 amino acids and is obtained from human secretions (Hochstrasser *et al.*, 1981), inhibits elastase to protect the lung (Thompson and Ohlsson, 1986). It is also inactivated by cigarette smoking (Carp and Janoff, 1980) and *Ps. aeruginosa* elastase (Johnson *et al.*, 1982). Eglin obtained from the leech and elasnin from a *streptomycete* are also inhibitors of elastase (Seemuller *et al.*, 1977; Ohno *et al.*, 1978).

Since human α_1-proteinase inhibitor is a potent and natural inhibitor of elastases, it would be a ideal agent against elastase related diseases. The synthesis through recombinant DNA technology (Courtney *et al.*, 1985) of mutant forms, in which methionine-358 is displaced by valine-358 to render it non-oxidizable, were reported (Rosenberg *et al.*, 1984). But these artificial α_1-proteinase inhibitors, synthesized in non-mammalian systems, lack the carbohydrate moiety and their half-lives are much shorter than that of the natural form (Travis *et al.*, 1985).

Administration of antioxidants, such as glutathione and vitamin C, is another method for preventing the inactivation of natural elastase inhibitors like α_1-proteinase inhibitor by inactivating the oxidants released from neutrophile and macrophage. The other choice might be synthetic inhibitors which fit into the active site region of the enzyme to inactivate it.

1.6.2 Synthetic elastae inhibitors

1.6.2.1 Peptides

Peptides and peptide analogues are made as substrate analogues for proteinases and can serve as inhibitors of the enzyme. Cyclic peptides that contain the active site sequence of α_1-proteinase inhibitor to mimic a three-dimensional structure, such as cyclo-(Ala-Leu-Pro-Met-Thr-Leu-3-Abz-3-Abz) (3-Abz = 3-aminobenzoyl), are reversible inhibitors of human leukocyte elastase (Powers *et al.*, 1981). Trifluoroacetyl peptides like CF_3CO-Lys-Ala-NH-C_6H_4-4-CF_3 are also reversible inhibitors of human leukocyte elastase. The trifluoroacetyl group interacts with the hydrophobic pocket near the active site of the enzyme (Dimicoli *et al.*, 1980).

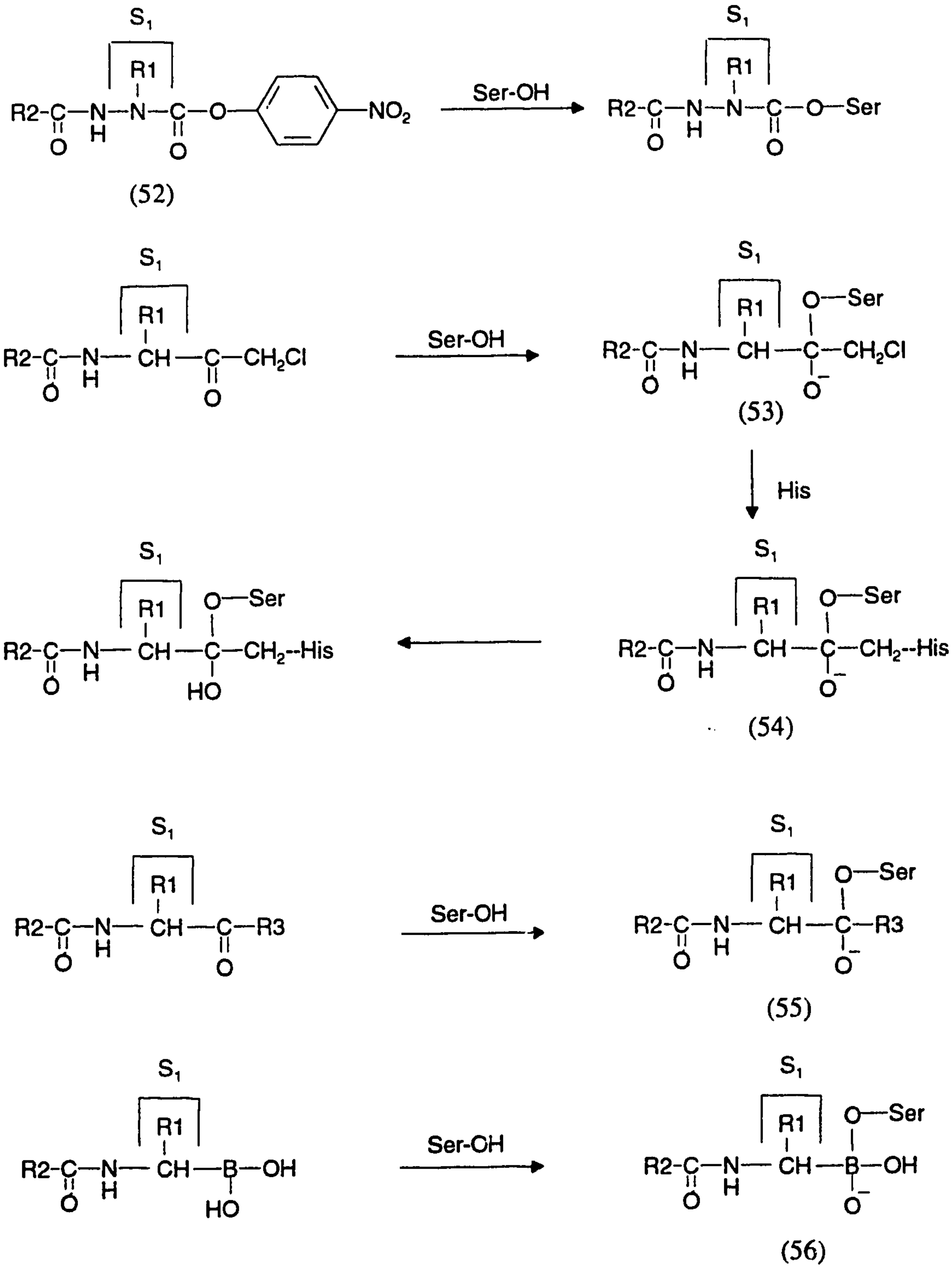
Azapeptides acylate the active site of serine (52) and inactivate human leukocyte elastase reversibly (Powers *et al.*, 1984). Peptide chloromethyl ketones like Ac-Ala-Ala-Pro-Ala-CH_2Cl (Powers, 1977) form a tetrahedral structure (53) with the enzyme and alkylate the active site histidine (54). These are irreversible inhibitors (Stein and Trainor, 1986). Peptide aldehydes and ketones are transition-state analogue inhibitors so that they form a intermediate (55) with the enzyme (Thompson, 1973). Ac-Pro-Ala-Pro-NHCH(CH_3)CHO is a potent inhibitor but its corresponding methyl ketone is much less active (Thompson and Bauer, 1979).

Since the boron atom contains a vacant 2p orbital which reacts readily with nucleophiles like -SH or -NH to form a tetrahedral boron adduct, peptide boronic acids, such as MeO-Suc-Ala-Ala-Pro-Boro-val (Boro-val : carboxylic group of valine is replaced by boronic acid), form tetrahedral adducts (56) with the active site of serine to inactivate elastase (Kettner and Shenvi, 1984).

However the potential problems of peptide-based inhibitors are poor bioavailability because of digestion and rapid metabolism by proteases.

1.6.2.2 Acylating agents

Some compounds which acylate the active site serine residue to form stable acyl-enzyme complexes, might be irreversible elastase inhibitors. Sulphonyl fluorides, such as phenylmethanesulphonyl fluoride, were reported to form an irreversibly sulfonylated enzyme (57) (Lively and Powers, 1978). Alkyl isocyanates and N-sulfocarbonyl amino acid esters also form carbamate derivatives to inhibite elastase (Brown and Wold, 1973b; Groutas *et al.*, 1985).



S_1 : Substrate binding site on the enzyme

Figure 1 - 11 Peptides as elastase inhibitors

N-Substituted saccharins (58) (Zimmerman *et al.*, 1980), benzoxazin-4-ones (59) (Teshima *et al.*, 1982), 3-alkoxy-4-chloroisocoumarins (60) (Harper and Powers, 1985), and oxazine-2,6-diones (61) (Moorman and Abeles, 1982) were reported as potent acylating inhibitors of elastases.

1.6.2.3 Mechanism-based inhibitors

These inhibitors are called suicide inhibitors or enzyme-activated inhibitors. They first interact with the active site of the enzyme to activate the reactive group in the inhibitor, which can then react with another site on the enzyme to be irreversibly inactivated. This new type of inhibitor has some advantages. Because they need to be activated by target enzyme before their inhibitory activity, this requirement makes them more selective. Most of these inhibitors interact first with the serine residue to form an acyl-enzyme and then the activated group reacts with another active site on the enzyme.

Ynenol lactones like 3-benzylynenol tetrahydrofuranone (62) acylate the active site serine residue to release the reactive allene ketones which alkylate the active site nucleophiles to give irreversibly alkylated enzymes (Tam *et al.*, 1984). Chloropyrones (Westkaemper and Abeles, 1983) and chloroisocoumarins (64) (Harper *et al.*, 1983) have a masked acid chloride group which generates an acid chloride (65) to react with histidine to form a diacyl enzyme (66) or they can be hydrolyzed to give a carboxylic acid to form a salt bridge with a protonated histidine (67). 3-Alkoxy-7-amino-4-chloroisocoumarins (68) interact with the enzyme to produce 4-

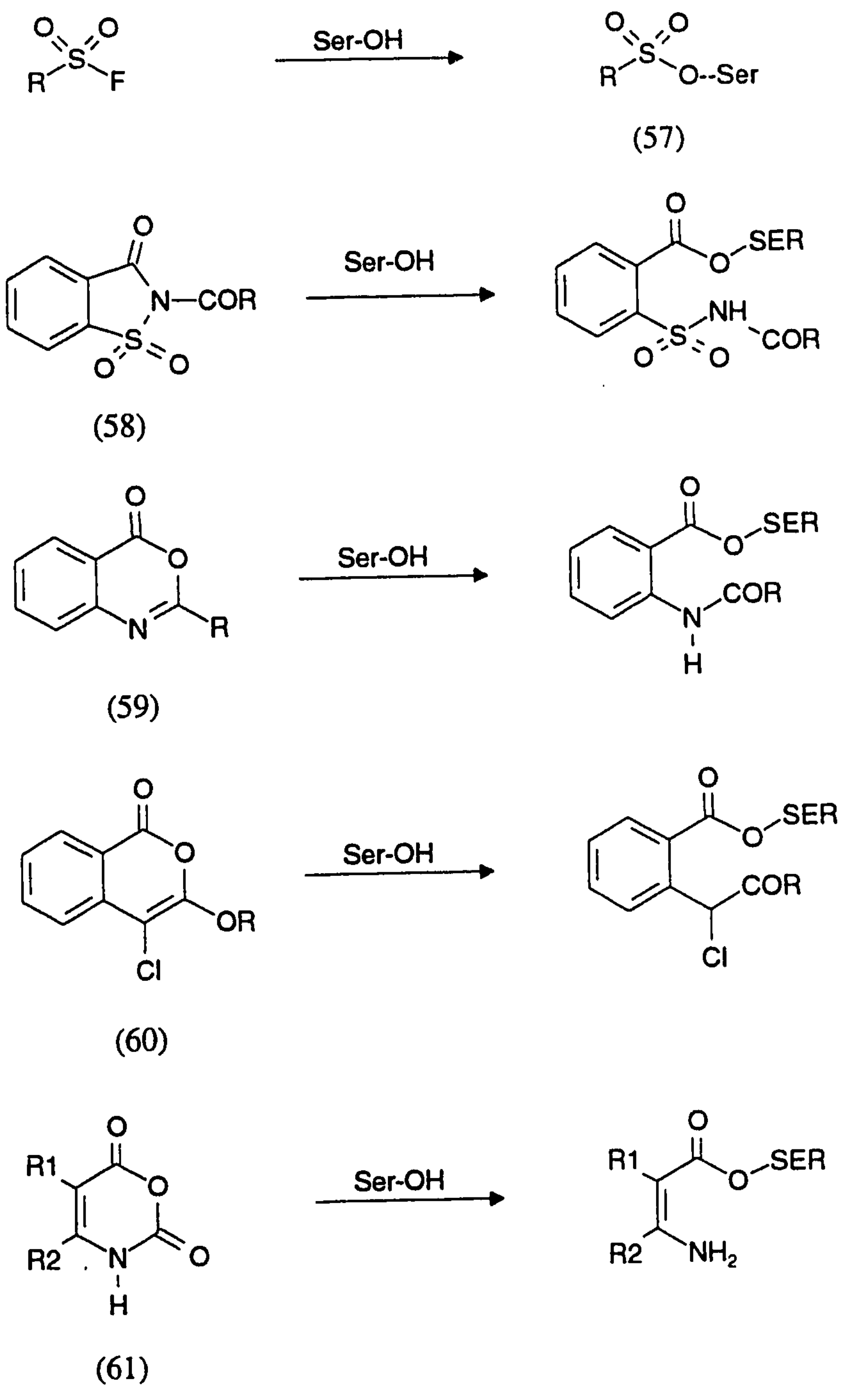


Figure 1 - 12 Mode of action of acylating agents

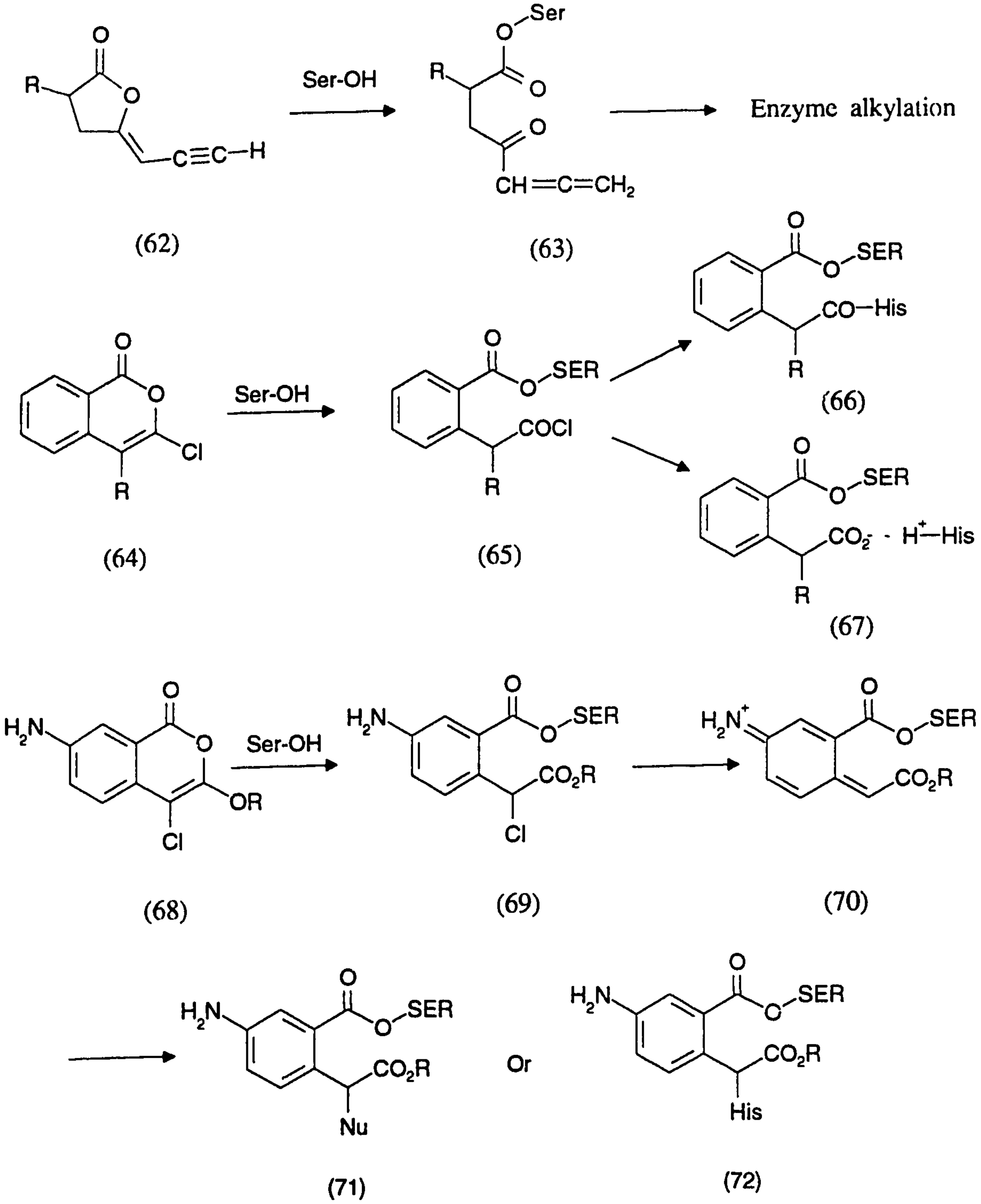


Figure 1 - 13 Mode of action of mechanism based inhibitors

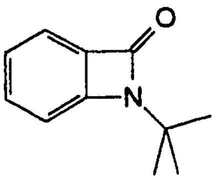
quinonimine methide (70) which can react with a nucleophile on the enzyme (71), or probably histidine (72), to form the irreversibly inhibited enzyme (Harper and Powers, 1985).

1.6.2.4 β-Lactams

β-Lactams are one of the mechanism-based inhibitors. Since β-lactams acylate bacterial serine enzymes such as bacterial cell wall transpeptidase and β-lactamase, it is not so surprising that they may also acylate serine proteinase, especially elastase. N-t-Butylbenzo-β-lactam (73) was the first β-lactam to be studied as a serine proteinase inhibitor and it showed inhibitory activity against chymotrypsin (Kaiser, 1974). The Merck group reported that thienamycin esters (74) and carbapenem esters (75) were inhibitors of human leukocyte elastase (Doherty *et al.*, 1985a; 1985b).

In particular, cephalosporins were studied as potential human leukocyte elastase inhibitors. 7α-Substituted cephalosporins showed greater activity than 7β-substituted compounds as antibiotics. This may be due to the ability of elastase to cleave L-L amino acid peptide bonds in contrast to the D-D amino acid peptide bonds cleaved by bacterial serine enzymes (Doherty *et al.*, 1986). Smaller substituents in the 7α-position give more potent activity than larger substituents. This may be due to the preference of human neutrophile elastase for amino acids with small alkyl substituents in the binding sites of its substrates.

Another interesting feature is that unlike antibiotics the carboxy ester compounds showed better activity than free carboxylic acid compounds (Doherty *et al.*, 1986). The possible explanation may be that the bacterial



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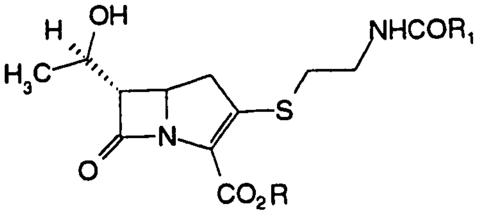

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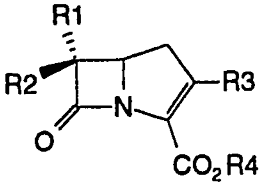
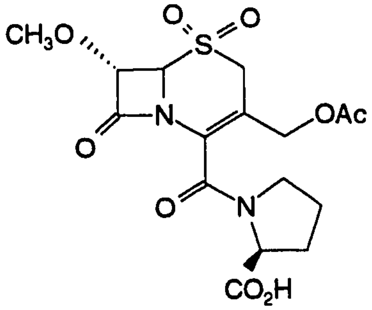


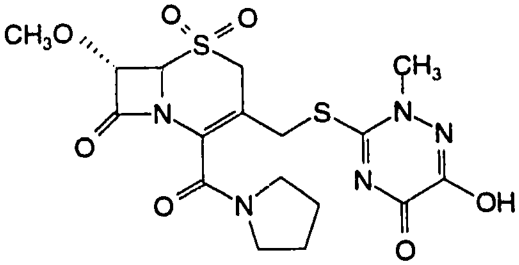
(75)

cell wall peptidases are carboxydipeptidases and β-lactamases act on the antibiotics with free carboxylic acid, while elastases prefer neutral molecules at its sites of cleavage (Bieth, 1986).

The sulfone of 7α-methoxy or chloro cephalosporanic acid esters satisfies the above requirements and shows good inhibitory activities against human leukocyte elastase. In animal and human blood models, cephalosporin derivatives like L-658,758 (76) and L-659,286 (77) reduced tissue damage by elastase in the hamster lung (Fletcher *et al.*, 1990) and showed good elastase inhibitory activity in human blood (Pacholok *et al.*,



L - 658,758 (76)



L - 659,286 (77)

1990). These studies indicate that this type of inhibitor is active in an *in vivo* environment and can be potential elastase inhibitors.

The possible mechanism of 7α-methoxy cephalosporin (78) as a elastase inhibitor involves the same initial binding step as the β-lactam antibiotics and β-lactamase inhibitors: acylation of the serine residue in the active site (Figure 1-14). The hydroxyl group of the Ser-195 reacts with the β-lactam carbonyl to form an acyl-enzyme intermediate (79). Further modification of this intermediate may lead to either (81) or (82). When Y is a good leaving group, pathway A is possible. The elimination of the leaving group leads to formation of an exocyclic methylene (80) at the 3-position. Nucleophilic attack by His-57 gives a second covalent bond between the 3-position of the inhibitor and the enzyme (81). In such cases where Y is not a leaving group, pathway B may be an alternative. The dihydrothiazine ring is then opened to give a sulfinic acid. A salt bridge

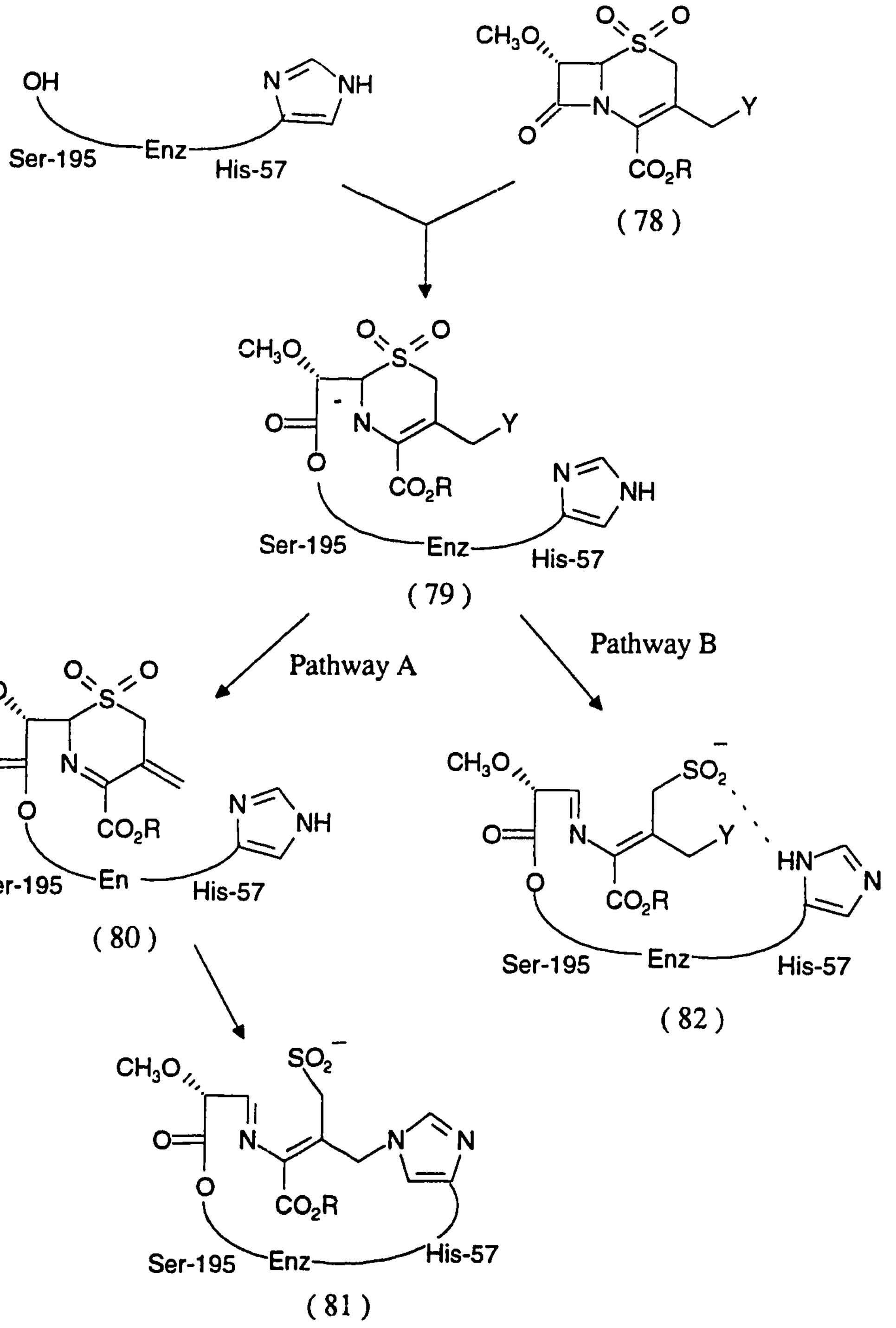


Figure 1 - 14 The possible mechanism of 7α-methoxycephalosporin as an elastase inhibitor

between the sulfinate and the enzyme (probably His-57) or a conformational change could then stabilize the inhibited enzyme (82) (Shah *et al.*, 1990)

CHAPTER 2

OBJECTIVES

The main objectives of this research were to study the synthesis and biological properties of β-lactam compounds, which would inhibit β-lactamases or human leukocyte elastase.

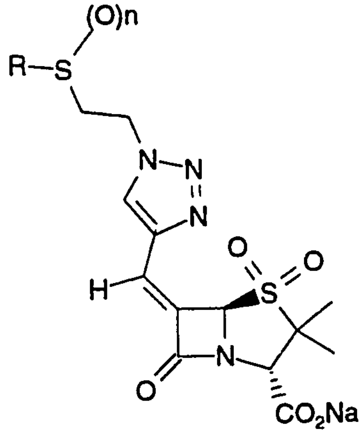
Once penicillin was discovered, many antibiotics were introduced in medical practice to cure bacterial infectious diseases. They include general classes such as β-lactams, aminoglycosides, macrolides, peptides, tetracyclines, and quinolones. Among them, the β-lactams have been widely used due to their broad spectrum of activity and low toxicity.

Their continuous usage as antiinfectious agents was, however, challenged by the β-lactamase producing bacteria. The hydrolysis of the β-lactam ring by β-lactamases is the major resistance mechanism for the β-lactam antibiotics. There are two approaches to overcome the bacterial resistance caused by β-lactamases. One is to find or prepare new β-lactam compounds which would not be susceptible to these enzymes. Another approach is the introduction of β-lactamase inhibitors in combination with the antibiotics. This would be the direct and fundamental method to overcome the β-lactamase resistance problem.

Since penicillanic acid sulfone was discovered to be a potential β-lactamase inhibitor, its derivatives were prepared and tested. The chemical modifications at the 2- or 6-position were reported to change their activities against β-lactamases. Among them, sulbactam (30) and tazobactam (35) were regarded as very promising β-lactamase inhibitors.

6-Exo-methylene penicillin (RO 15-1903) and 6-exo-methylene penem with a 1,2,3-triazole group at the 6-position (BRL-42715) showed excellent inhibitory activities. The Beecham group reported that when electron withdrawing groups were introduced at the 1-position of the triazole moiety (83), the β-lactamase inhibitory activity was improved (Broom *et al.*, 1988).

From the above observation, the combination of an exo-methylene penicillin with a 1,2,3-triazole group, sulfone, and electron withdrawing group might provide potential β-lactamase inhibitors. Therefore, compounds (84) and (85) were synthesized and studied as possible β-lactamase inhibitors.



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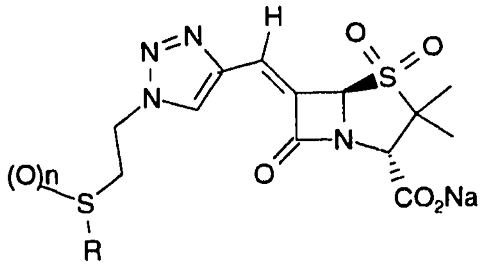

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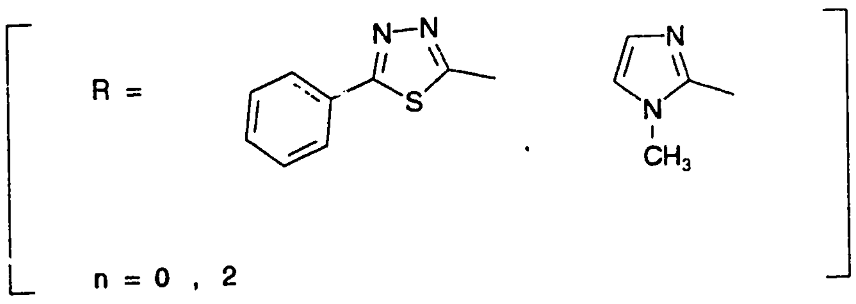
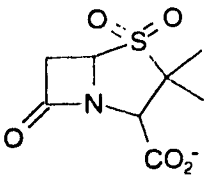
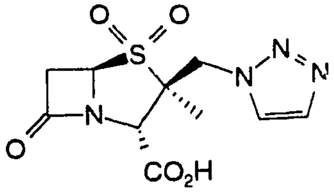




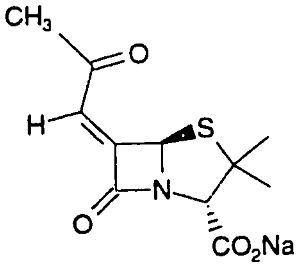
Sulbactam (30)

(Pfizer)



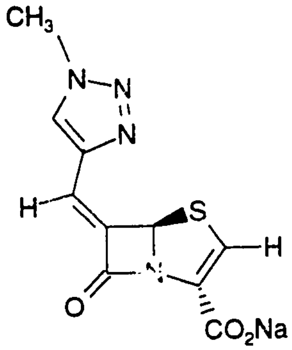
Tazobactam (35)

(Taiho)



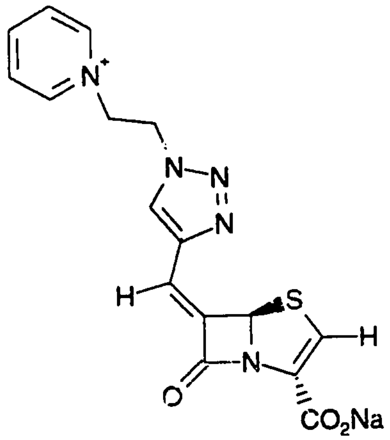
RO 15 - 1903

(Hoffmann - La Roche)



BRL 42715

(Beecham)



(83)

(Beecham)

The human leukocyte elastase is widely distributed in humans and has physiologically important roles as mentioned in the previous chapter. The imbalance between elastase and its natural inhibitors causes serious tissue damage and leads to various diseases like emphysema. However, there is no commercially available drug against human leukocyte elastase.

Since the cephalosporins could be modified to be inhibitors of human leukocyte elastase (Doherty *et al.*, 1986), these compounds have been synthesized and studied to find a potent and specific inhibitor of human leukocyte elastase. The following observations were reported by the Merck group from X-ray crystallographic studies.

The preferred sulfone at the 1-position may have two roles. It may give more efficient initial binding to the enzyme by providing hydrogen bonding sites (with Gln-192 and Val-216) and may also enhance acylation of the enzyme by facilitating β-lactam ring opening (Doherty *et al.*, 1990).

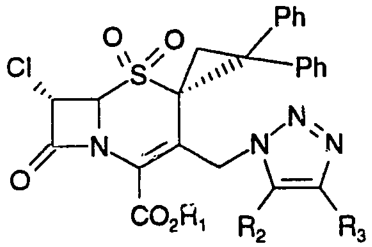
The substituents at the 2-position would extend over the surface of the enzyme providing some liphophilic binding without interaction to any particular parts of the enzyme and liphophilic substituents would increase the inhibitory activity (Hagmann *et al.*, 1989).

The inhibitory activity against human leukocyte elastase is influenced by electronic as well as steric effects of groups in the 3-position. The 3-substituent is oriented to a relatively open area on the enzyme which could accommodate a large group in this position. The activity is increased with the size and inductive electron withdrawing effect of this substituent (Shah *et al.*, 1990).

The C4-carboxyl group is close to the active site His-57 and its negative charge could attract the imidazole ring of His-57. This attraction might pull the imidazole moiety away from its optimal position in the enzyme active site and prevent the initial acylation of the inhibitor (Navia *et al.*, 1987). Thus liphophilic esters and amides showed better activity than free carboxylic acids (Finke *et al.*, 1990).

The human leukocyte elastase has a small S1 binding pocket near the active site and prefers a small α-oriented substituent such as a methoxy or a chloro group at the 7-position of the cephalosporin (Doherty *et al.*, 1990).

From these observations, 3-substituted cephalosporin derivatives (86) were prepared and studied as possible human leukocyte elastase inhibitors.



(86)

R_1 = $CH(Ph)_2$

R_2 or R_3 = H or carbonyl group

CHAPTER 3

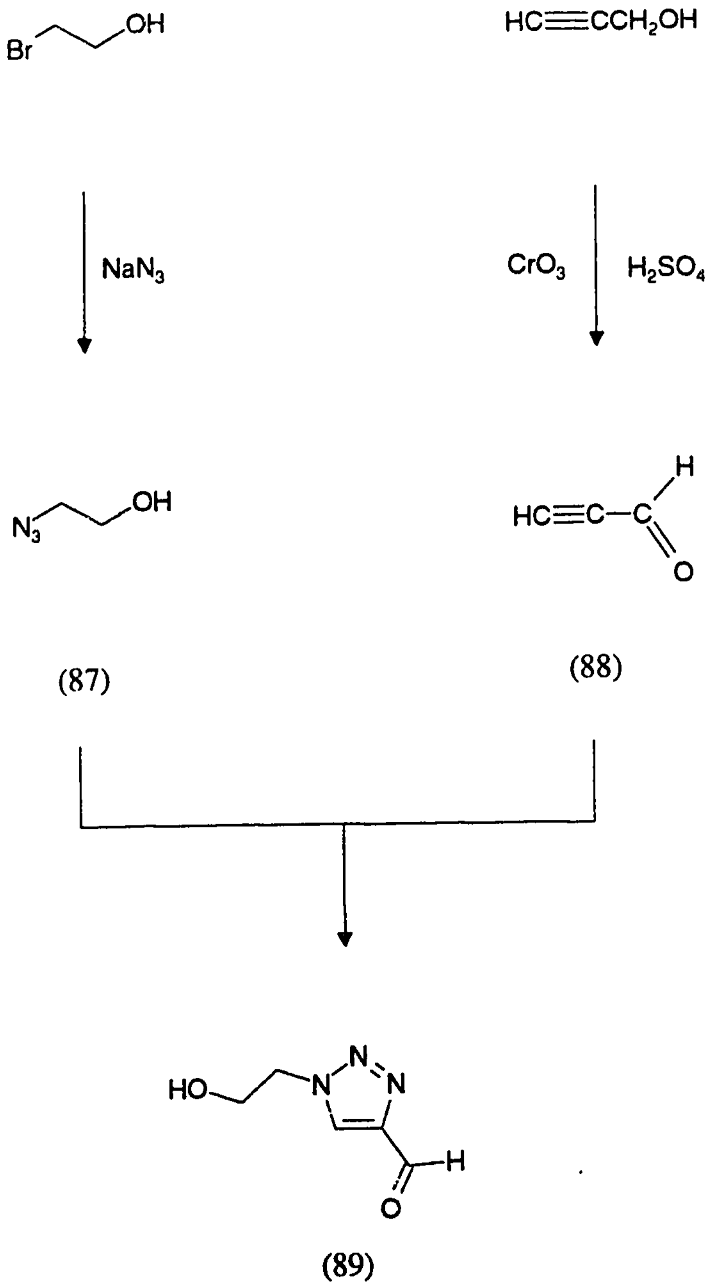
RESULTS AND DISCUSSION-1

β-LACTAMASE INHIBITORS

Synthesis of 1-(2-hydroxyethyl)-1,2,3-triazole-4-carboxaldehyde (89)

The synthesis of the compound (89) is summarized in the scheme 3-1. The 2-azidoethanol (87) was prepared in 61 % yield by reacting sodium azide in water with 2-bromoethanol in acetone at 55°C. The oxidation of propargyl alcohol with a 1.03 molar equivalent of chromium trioxide at −5 °C under 40 ~ 60 mmHg pressure in sulfuric acid, followed by distillation afforded the desired propargyl aldehyde (88) in 31 % yield, after redistillation at 53 ~ 57°C under normal pressure. The maintenance of reaction temperature and pressure was important, because the product should be distilled before it is oxidized further to the carboxylic acid.

The 1,3-dipolar cycloaddition reaction of 2-azidoethanol with propargyl aldehyde in dichloromethane gave 1-(2-hydroxyethyl)-1,2,3-triazole (89) in 69 % yield. This reaction is exothermic and without cooling the reaction mixture, the 5'-carboxaldehyde isomer is also produced. The 4'-carboxaldehyde compound was the major product and the 5'-carboxaldehyde isomer was formed at high temperature. This may be due to steric hindrance between hydroxyethyl group and the aldehyde group.



Scheme 3 - 1 Synthesis of compound (89)

Synthesis of 1-(2-substituted thioethyl)-1,2,3-triazole-4-carboxaldehydes (90 and 91)

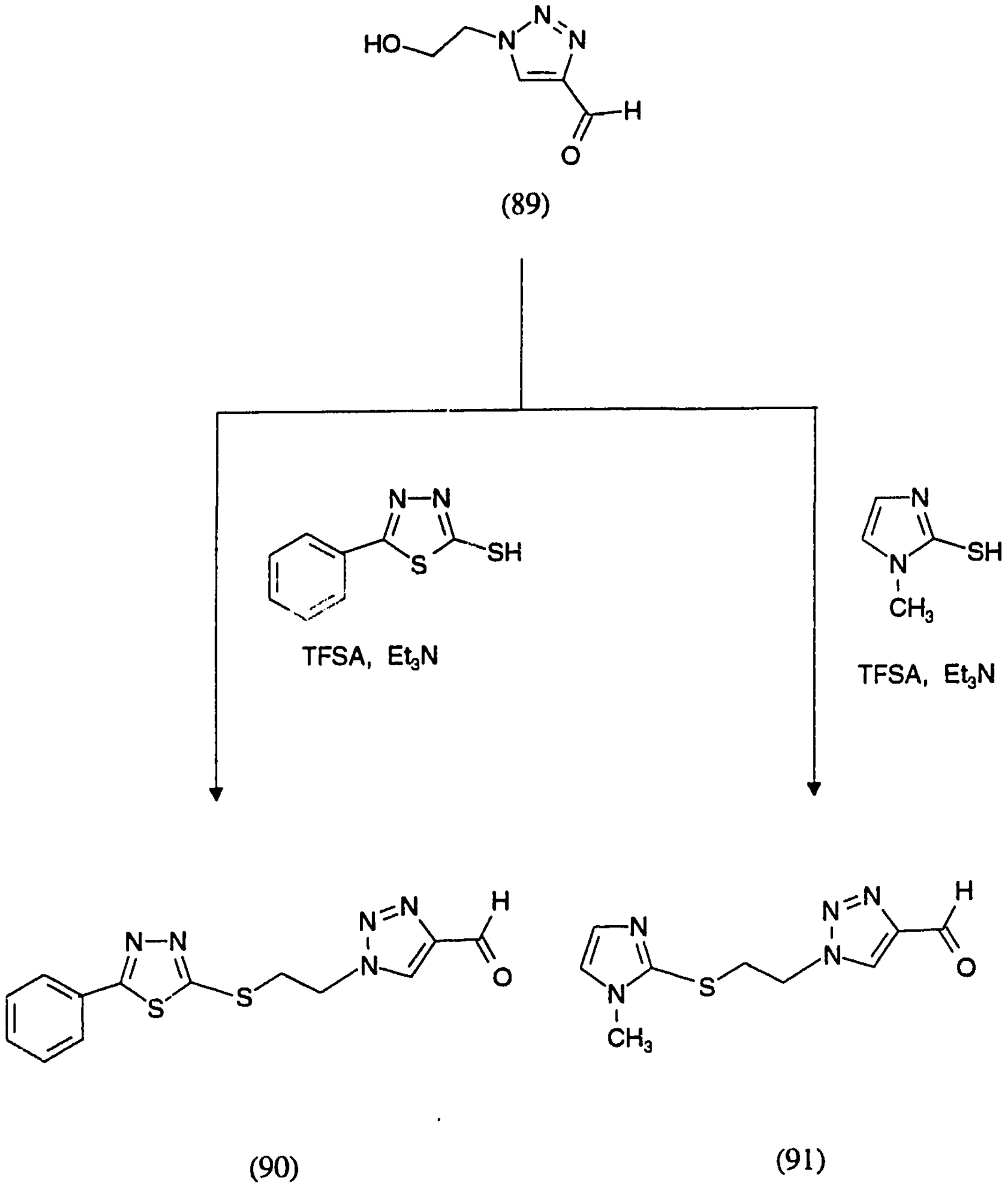
The hydroxyethyl compound (89) was first converted into the trifluorosulfonyl derivatives by treatment with one molar equivalent of trifluoromethanesulfonic anhydride in the presence of triethylamine at −15 ~ −20°C under an argon atmosphere. This reaction mixture reacted with 5-mercapto-2-phenyl-1,3,4-thiadiazole in the presence of triethylamine to yield the desired 1-(2-substituted thioethyl)-1,2,3-triazole compound (90) in 35 % yield.

Using a similar procedure described above, compound (91) was prepared from the 2-mercapto-1-methylimidazole and trifluoromethanesulfonic anhydride in the presence of triethylamine in 51 % yield. The synthesis of compound (90) and (91) is described in scheme 3-2.

Synthesis of the p-methoxybenzyl (3S,5R,6S)-6-bromo-penam-3-carboxylate-1,1-dioxide (94)

The 6-amino penicillanic acid (6-APA) was first treated with 2.5 N sulfuric acid solution and potassium bromide, then reacted with sodium nitrite at 3 ~ 5°C for 5 hrs. to give a 6α-bromo penam compound (92) in 84 % yield.

The protection of the penam carboxyl group was carried out in 76 % yield by reaction with p-methoxybenzyl alcohol and cyanuric chloride in the presence of pyridine in dichloromethane at −20 ~ −27°C to yield the p-



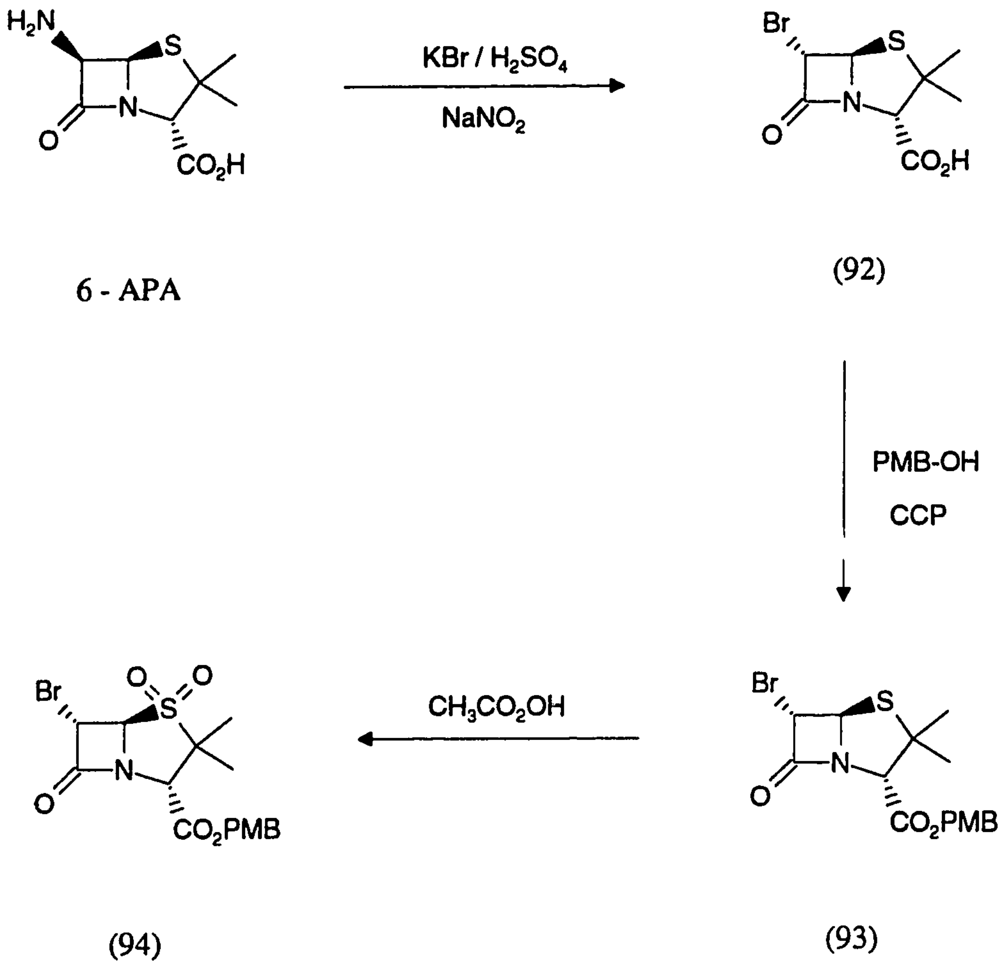
Scheme 3 - 2 Synthesis of compounds (90 and 91) methoxybenzyl ester compound (93) which could be deprotected by anhydrous aluminum chloride. The alternative choice for the protection of the carboxylic acid would be the allyl group, which could be deprotected by tetrakis(triphenylphosphine) palladium. But this catalysts is expensive and very sensitive to light and temperature, so that the p-methoxybenzyl group was chosen as the protecting group.

The oxidation of the sulfide (93) in the β-lactam ring to sulfone (94) was accomplished by reaction with 3.95 molar equivalent of peracetic acid in dichloromethane at room temperature. This reaction is exothermic and cooling was required to get a higher yield. During oxidation, the sulfone was formed via the sulfoxide which was produced within 1 ~ 2 hrs. from sulfide, but oxidation from sulfoxide to sulfone took more time to complete. The synthetic route of compound (94) is summarized in scheme 3-3.

NMR analysis of the compounds (92, 93, and 94)

The coupling constants between the C5-H and C6-H in these compounds ($J_{5,6}$) are 1.2 Hz for compound (92), 1.3 Hz for compound (93), and 1.3 Hz for compound (94). These coupling constants indicated that these protons were *trans* to each other, i.e., C5α-H and C6β-H.

The Nuclear Overhauser Effect (NOE) is an important method for probing three-dimensional structures of the molecules as well as for establishing signal assignments for the protons indistinguishable on the basis of their signal multiplicity. In simple terms, the NOE can be



PMB = p-Methoxy benzyl

CCP = Cyanuric chloride pyridine

Scheme 3 - 3 Synthesis of compound (94)

explained as follows. If two protons in a molecule are spatially proximal, i.e., R (the vector distance between them) ≤ 3A°, saturation of one of these protons with an intense rf field will cause an increase in the integrated intensity of the other and the magnitude of which will be proportional to $1/R^6$. It is therefore possible to estimate from experimentally determined NOE values not only which nuclei in a molecule are responsible for the relaxation of a particular nucleus, but also the relative distances between the interacting nuclei (Demarco and Nagarajan, 1972).

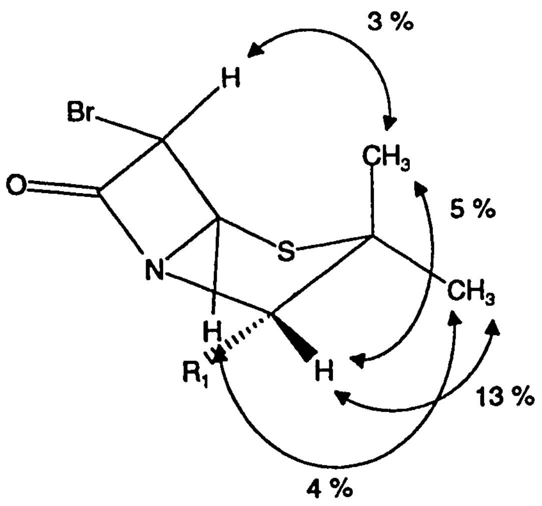
In order to obtain information regarding the thiazolidine ring conformation and to determine relative assignments for the two C2-CH_3 singlets in the NMR spectra, an NOE study of the compounds (92, 93, and 94) was undertaken. The results of this study are presented in table 3-1.

From the NOE experimental data, the following conclusions could be drawn. In compounds (92 and 93), the C3-H would be closer to the low field methyl (C2α-CH_3) than to the high field methyl (C2β-CH_3). C5-H would be close to the low field methyl, but not to the high field methyl. C6-H would be close to the high field methyl but not to the low field methyl. These observations suggest that the conformation of compounds (92 and 93) may be similar to conformation [A] in figure 3-1.

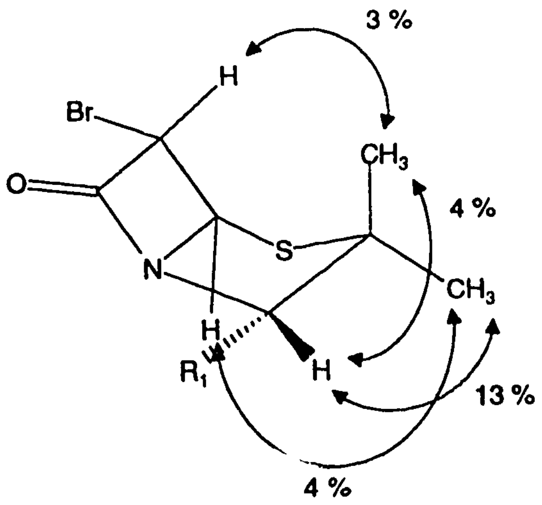
The relative assignment of the two methyl singlets in compounds (92 and 93) is based on the fact that the C6-H should be closer to the C2β-CH_3 than to C2α-CH_3 in conformation [A]. NOE between the C6-H and the high field methyl suggest that the high field methyl would be C2β-

Table (3-1) NOE data of compounds (92, 93, and 94)

Compounds	Protons irradiated (δ)	Proton observed	Intensity increased (%)
(92) ($CDCl_3$)	Low- field methyl (1.65)	C3-H	13
		C5-H	4
		C6-H	Nil
	High- field methyl (1.57)	C3-H	5
		C5-H	Nil
		C6-H	3
(93) ($CDCl_3$)	Low- field methyl (1.58)	C3-H	13
		C5-H	4
		C6-H	Nil
	High- field methyl (1.37)	C3-H	4
		C5-H	Nil
		C6-H	3
(94) ($CDCl_3$)	Low- field methyl (1.54)	C3-H	10
		C5-H	Nil
		C6-H	1
	High- field methyl (1.24)	C3-H	2
		C5-H	10
		C6-H	Nil

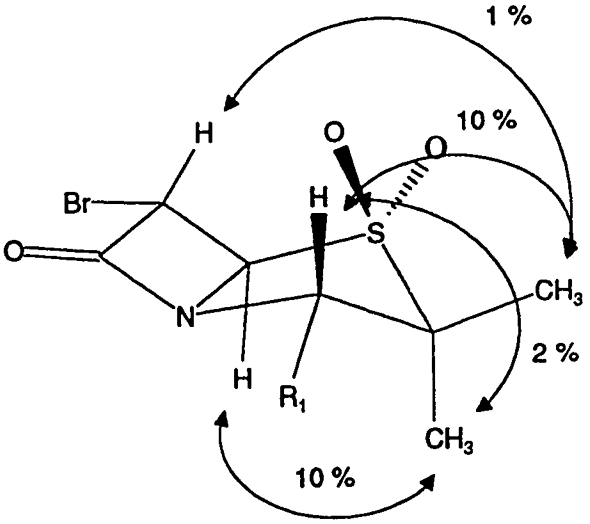


R_1 = CO_2H (92)



R_1 = CO_2PMB (93)

Conformation [A]



R_1 = CO_2PMB (94)

Conformation [B]

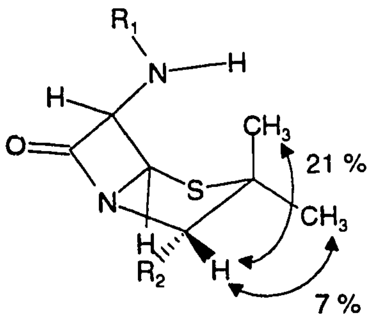
Figure 3 - 1 Thiazolidine ring conformations and NOE's in compounds (92, 93, and 94)

CH_3. But this assignment is the reverse of that of previously reported ones, even though the conformation is the same (Figure 3-2, A) (Cooper *et al.*, 1969; Demarco and Nagarajan, 1972).

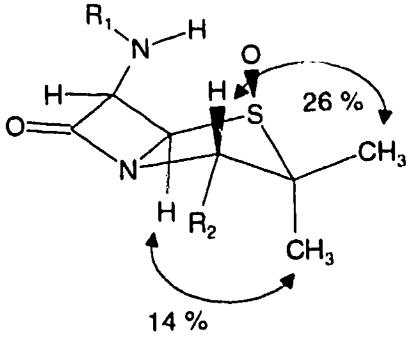
Demarco and Nagarajan mentioned that " the low field methyl group, which gives the larger NOE of the two for H-3, must correspond to C2β-CH_3, since this group, irrespective of the conformation adopted by the thiazolidine ring, is always spatially more proximal to H-3 than C2α-CH_3". They compared the NOE between the C3-H and the two CH_3 to assign C2α-CH_3 and C2β-CH_3. But C6β-H would be a better criteria to assign them. The 3 % NOE between the C6-H and the high field methyl and no NOE between the C6-H and the low field methyl strongly suggest that the high field methyl would be C2β-CH_3.

In compound (94), the low field methyl would be closer to the C3-H and C6-H than the high field methyl. The high field methyl would be closer to the C5-H than the low field methyl, as shown in figure 3-1 (conformation [B]). Thus, the low field methyl would be assigned to C2β-CH_3 and the high field methyl to C2α-CH_3. Similar NOE observations and methyl assignments were reported by Cooper (Cooper *et al.*, 1969) (Figure 3-2, D).

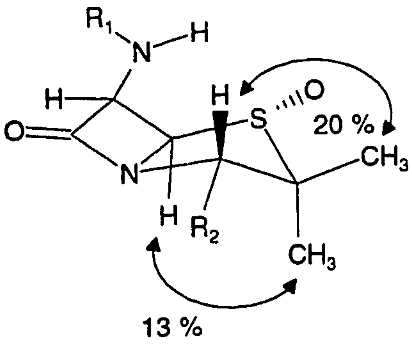
In compound (93), the CH_2 protons of the p-methoxybenzyl ester appeared as a singlet, but compound (94) showed two doublets for these protons. This may be explained as follows. Compound (93) has the conformation [A], in which the C2α-CH_3 is equatorial. In this model, the p-methoxybenzyl ester has some space for free rotation and these CH_2 protons are magnetically equivalent to give a singlet.



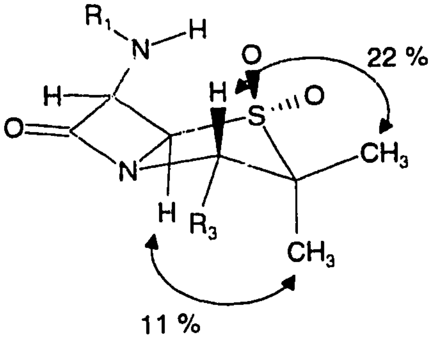
[A]



[B]



[C]



[D]

R_1 = $C_6H_5OCH_2CO$
R_2 = CO_2CH_3
R_3 = $CO_2CH_2CCl_3$

Figure 3 - 2 Thiazolidine ring conformations and NOE's in penicillin and related derivatives

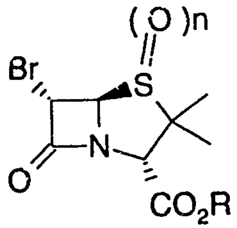
Compound (94), however, has the conformation [B], in which the C2α-CH_3 is in an axial position. When the p-methoxybenzyl ester rotates around, it would be faced steric hindrance by the C2α-CH_3 and might not rotate freely. Without free rotation, these two protons are not magnetically equivalent and would not give a singlet. This observation also supports the chair form conformation [B] for compound (94). The NMR data of compounds (92, 93, and 94) are presented in Table 3-2.

Synthesis of p-methoxybenzyl (3S,5R,6S)-6-[(1RS)-1-hydroxy-1-{1-(2-substituted thioethyl)-1,2,3-triazole-4-yl}methyl]penicillanate-1,1-dioxides (95 and 105)

The 6α-bromo penam compound (94) was first treated with methyl magnesium bromide in anhydrous tetrahydrofuran at −78°C and then reacted with 1-{2-(2-phenyl-1,3,4-thiadiazole-5-yl)thioethyl}-1,2,3-triazole-4-carboxaldehyde (90) in dichloromethane to afford the stereoisomeric mixture of the hydroxy compound (95) in 58 % yield. This isomeric mixture could not be separated by classical silica gel column chromatography.

In a large scale reaction, where more than 2.5 g of 6α-bromo penam compound (94) was used for the reaction, the possible reaction intermediate was not stable even at −78°C and decomposed before reacting with the carboxaldehyde group and resulted in a lower yield.

Table (3-2) NMR data of compounds (92, 93, and 94)



n = 0 R = H (92)
n = 0 R = PMB (93)
n = 2 R = PMB (94)

Compounds	2α-CH$_3$	2β-CH$_3$	C3-H	C5-H	C6-H	OCH$_3$	CH$_2$ of PMB
(92)	1.65	1.57	4.59	4.84 (d, 1.2)	5.41 (d, 1.2)		
(93)	1.58	1.37	4.54	4.79 (d, 1.3)	5.40 (d, 1.3)	3.81	5.13
(94)	1.24	1.54	4.41	4.67 (d, 1.3)	5.15 (d, 1.3)	3.82	5.12, 5.26 (two d, 11.5)

Using a similar method, another hydroxy compound (105) was synthesized from the 6α-bromo penam compound (94) and 1-{2-(1-methyl imidazole-2-yl)thioethyl}-1,2,3-triazole-4-carboxaldehyde (91) in 59 % yield. The synthesis of compounds (95 and 105) is illustrated in scheme 3-4. The human leukocyte elastase inhibitory activity of these compounds (95 and 105) was tested with the expectation of finding a new type of human leukocyte elastase inhibitor, but they were not active against this enzyme. Their test results are summarized in table 3-3.

Synthesis of p-methoxybenzyl (3S,5R,6S)-6-[(1RS)-1-acetoxy-1-{1-(2-substituted thioethyl)-1,2,3-triazole-4-yl}methyl]penicillanate-1,1-dioxides (96 and 106)

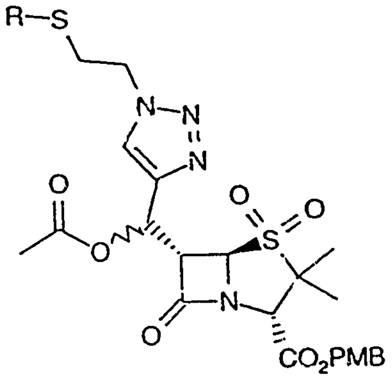
The acetoxy compound (96) was prepared in quantitative yield by the reaction of the corresponding hydroxy compound (95) with acetic anhydride in the presence of pyridine. Using a similar procedure, another acetoxy compound (106) was synthesized from the corresponding hydroxy compound (105) and acetic anhydride in 90 % yield. The synthesis of compound (96)and (106) is summarized in scheme 3-4.

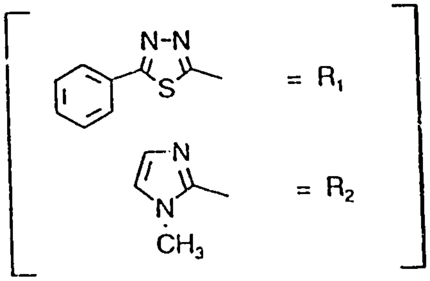
The human leukocyte elastase inhibitory activities of these compound (96 and 106) was tested and their test results are summarized in table 3-3.

NMR analysis of the compounds (95, 96, 105, and 106)

The coupling constants between the C5-H and C6-H in these

Table (3-3) Human leukocyte elastase inhibitory activity of PMB ester compounds ; IC$_{50}$ values (n molar)

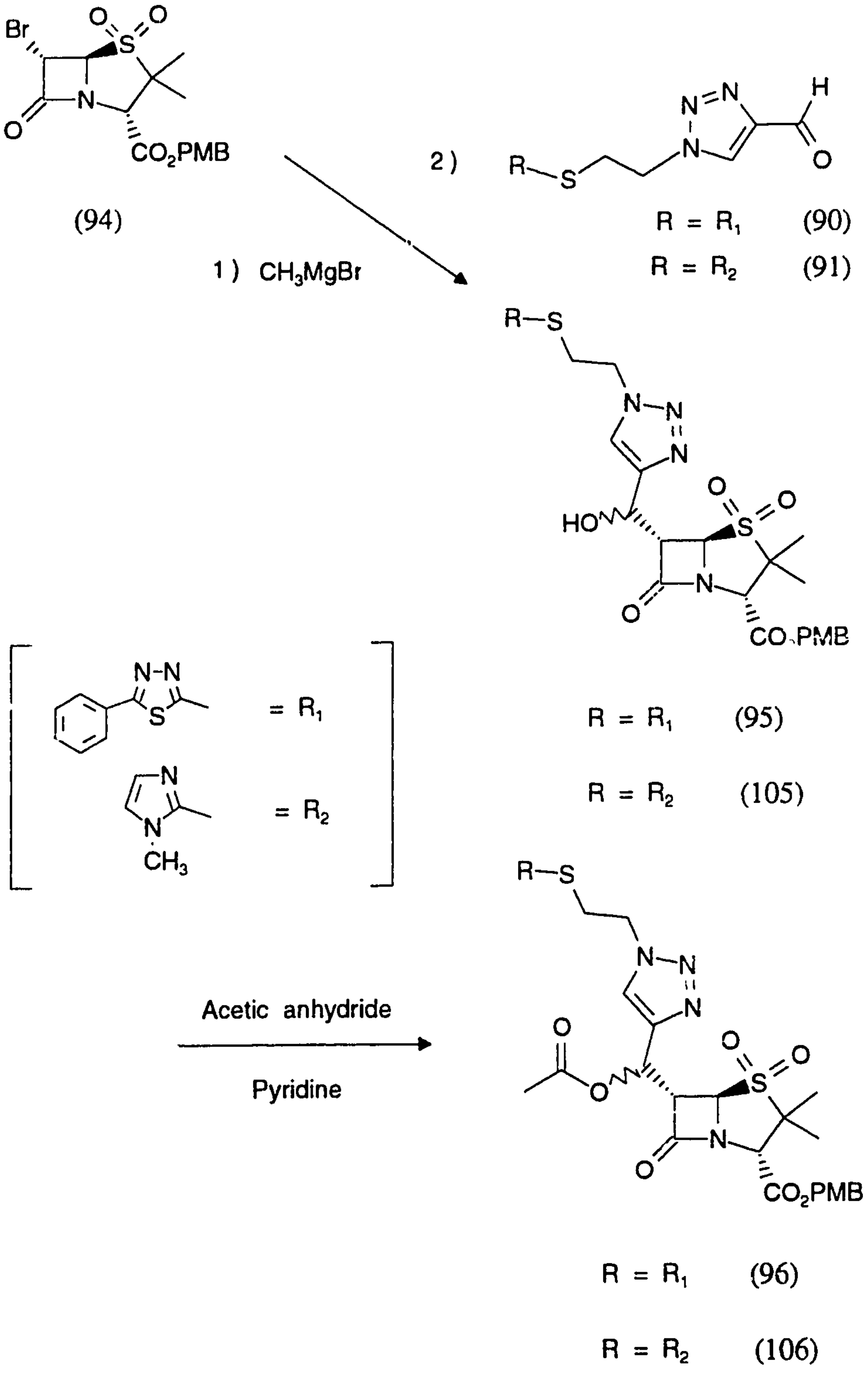




R = R$_1$ (96)

R = R$_2$ (106)

Compounds	IC$_{50}$ (nmol)	Compound	IC$_{50}$ (n molar)
(95)	> 1000	(105)	> 1000
(96)	370	(106)	220
(97)	> 1000	(107)	> 1000
(98)	> 1000	(108)	> 1000
(99)	> 1000	(109)	> 1000
(100)	> 1000	(110)	> 1000



Scheme 3 - 4 Synthesis of compounds (95, 96, 105, and 106)

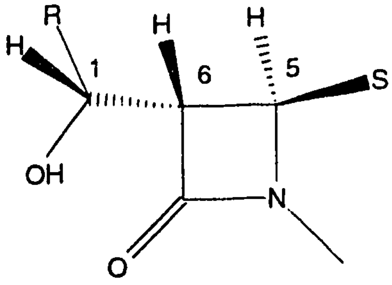
compounds suggest that these protons are *trans* to each other, i.e., C5α-H and C6β-H. The coupling constants ($J_{5,6}$) are 1.8 Hz for compound (95), 1.7 Hz for compound (96), 1.8 Hz for compound (105), and 1.9 Hz for compound (106).

This means that the compounds (95, 96, 105,and 106) could consist of two isomers (A and B) from the four possible isomers (Figure 3-3). Due to the steric hindrance of the penam β face, back (α) side attack might be the easier approach to the penam and give the *trans* compounds (A and B) as major products. Even though the minor products (C and D) which are *cis* in C5-H and C6-H could be produced, they might be obtained in such low yields that they could not be detectable in the NMR spectra.

In compound (95), the S-CH_2 of the side chain at the C-6 substituent gave a multiplet instead of a triplet. This suggested that this compound was not a pure isomer, but was a mixture of two isomers (A and B). The other protons of these isomers were indistinguishable in the 200 MHz NMR spectra.

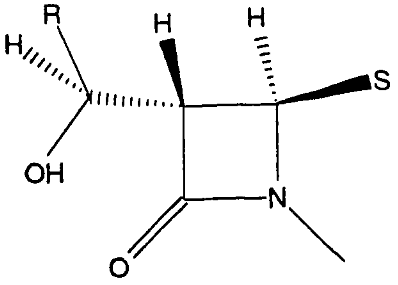
Compound (105) showed clearly in the NMR spectra that this was a mixture of two isomers (A and B). Most of the protons of these isomers showed different chemical shifts and the integrated intensities suggested that they were a mixture in the ratio of 3 : 7.

The acetoxy compound (96) also showed that the protons of the isomers were almost indistinguishable in the NMR spectra. But several broad peaks (such as the CH_2 of p-methoxybenzyl ester, C2α-CH_3, and C2β-CH_3) suggested that this compound was a mixture of two isomers.



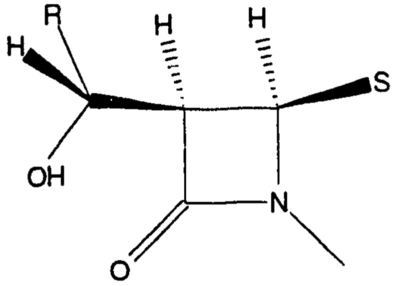
(5R, 6S, 1S)

A



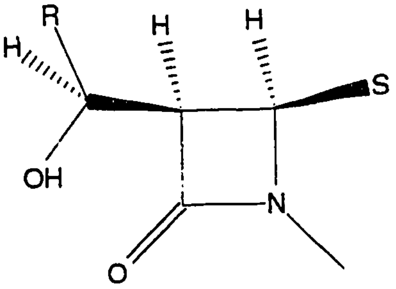
(5R, 6S, 1R)

B



(5R, 6R, 1S)

C



(5R, 6R, 1R)

D

Figure 3 - 3 The relative β-lactam chemistry of compounds (95, 96, 105, and 106)

The other acetoxy compound (106) showed that the protons of the isomers have closer chemical shift values than in the hydroxy compound (105). They were however still clearly distinguishable in many protons, such as the triazole-H, CH_3 of the acetoxy group, the $C2\alpha$-CH_3, and $C2\beta$-CH_3. The NMR data of compounds (95, 96, 105, and 106) are presented in Table 3-4.

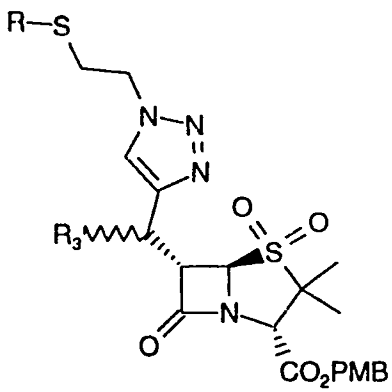
Synthesis of p-methoxybenzyl (3S,5R)-6(Z)-[1-{1-(2-substituted thioethyl)-1,2,3-triazole-4-yl}methylene]penicillanate-1,1-dioxides (97 and 107) and p-methoxybenzyl (3S,5R)-6(E)-[1-{1-(2-substituted thioethyl)-1,2,3-triazole-4-yl}methylene]penicillanate-1,1-dioxides (98 and 108)

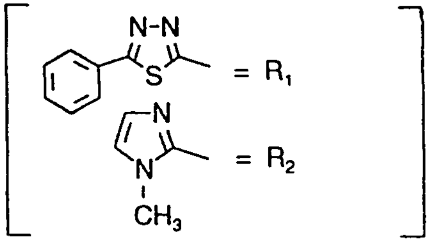
The introduction of a double bond at the 6-position of the penam sulfone was accomplished by the elimination reaction of the acetoxy compounds (96 and 106) with 1,5-diazabicyclo[4.3.0]non-5-ene in dichloromethane at –70°C.

The compound (96) produced the two isomers: Z-isomer (97) and E-isomer (98) in 50 and 35 % yield, respectively. From the compound (106), the Z-isomer (107) and E-isomer (108) were obtained in 42 and 51 % yield, respectively. The ratio of the two isomers (Z-isomer : E-isomer) was 10 : 7 from the compound (96) and 5 : 6 from the compound (106). The synthesis of compounds (97, 98, 107, and 108) is shown in scheme 3-5.

The human leukocyte elastase inhibitory activity of these compounds (97, 98, 107, and 108) was tested and their test results are summarized in

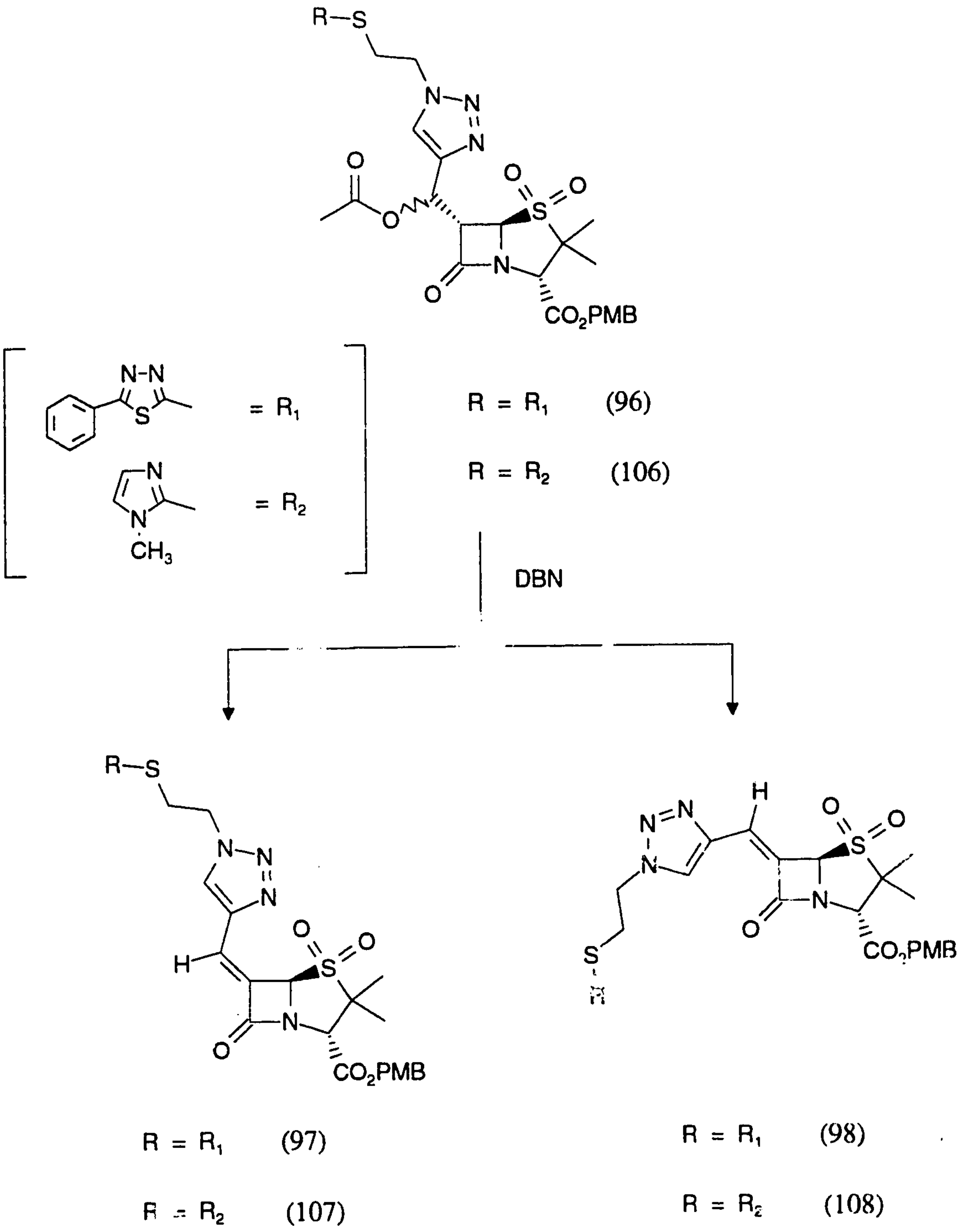
Table (3-4) NMR data of compounds (95, 96, 105, and 106)





$R = R_1$ $R_3 = OH$ (95)
$R = R_2$ $R_3 = OH$ (105)
$R = R_1$ $R_3 = OCOCH_3$ (96)
$R = R_2$ $R_3 = OCOCH_3$ (106)

Compd	2α-CH_3	2β-CH_3	CH_2 of PMB	C3-H	C5-H	C6-H	C<u>H</u>OH
(95)	1.24	1.52	5.07, 5.23 (two d, 11.7)	4.38	4.81 (d, 1.8)	4.13 (dd, 1.8, 4.4)	5.41 (d, 4.4)
(96)	1.24	1.53	5.08, 5.20 (two d, 11.7)	4.38	4.76 (d, 1.7)	4.31 (dd, 1.7, 5.0)	6.50 (d, 5.0)
(105)	1.23, 1.25 (two s)	1.52	5.08, 5.23 (two d, 11.7)	4.38, 4.46 (two s)	4.81, 4.85 (two d, 1.8)	4.12, 4.21 (two dd, 1.8, 4.6)	5.41, 5.48 (two d, 4.6)
(106)	1.24, 1.31 (two s)	1.53, 1.55 (two s)	5.08, 5.20 (two d, 11.7)	4.38, 4.40 (two s)	4.76 (d, 1.9)	4.28, (dd, 1.9, 4.9)	6.34, 6.48 (two d, 4.9)



R = R₁ (96)

R = R₂ (106)

R = R₁ (97)

R = R₂ (107)

R = R₁ (98)

R = R₂ (108)

Scheme 3 - 5 Synthesis of compounds (97, 98, 107, and 108)

table 3-3.

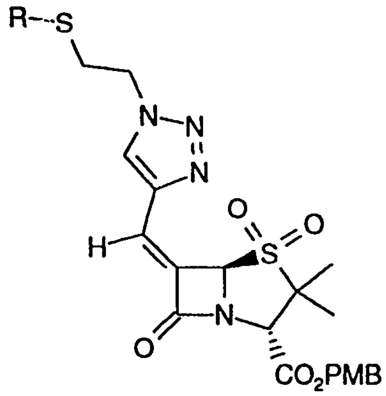
NMR analysis of the compounds (97, 98, 107, and 108)

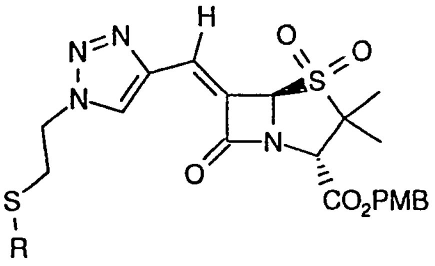
The elimination reaction of the compound (96) with DBN produced the double bond at the C-6 position and resulted in the formation of two isomers, i.e., Z-isomer and E-isomer. In the E-isomer, the 5-hydrogen of the triazole ring is close to the carbonyl group of the β-lactam ring. These carbonyl group might render some anisotropic effect (probably deshielding effect) on the 5-hydrogen of the triazole ring. In the Z-isomer, this proton is too far away to be under the anisotropic effect of the carbonyl group in the β-lactam ring.

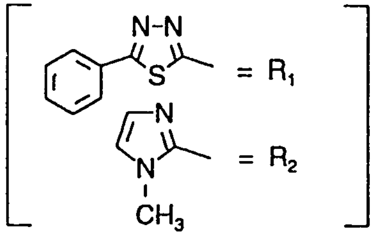
The chemical shift values of this proton in the compound (98) (δ 8.75) is at a much lower field than that of the compound (97) (δ 7.89), which is similar to the values of compound (95) (δ 7.95) and (96) (δ 7.87). This observations suggested that the compound (97) was the Z-isomer and the compound (98) was the E-isomer.

Regarding the triazole proton, the compound (107) and (108) showed a similar pattern of chemical shift values as compound (97) and (98), respectively. From this observation, the compound (107) would be assigned to the Z-isomer and (108) to E-isomer. The NMR data of compounds (97, 98, 107, and 108) are presented in table 3-5

Table (3-5) NMR data of compounds (97, 98, 107, and 108)







R = R_1 (97) R = R_1 (98)

R = R_2 (107) R = R_2 (108)

Compounds	2α-CH_3	2β-CH_3	CH_2 of PMB	C3-H	C5-H	C$\underline{H}$=C6	triazole-H
(97)	1.32	1.53	5.12, 5.28 (two d, 11.7)	4.44	5.63 (d, 1.3)	7.30 (d, 1.3)	7.89
(98)	1.30	1.53	5.12, 5.28 (two d, 11.7)	4.42	5.15	7.10	8.75
(107)	1.33	1.54	5.12, 5.28 (two d, 11.7)	4.44	5.64 (d, 1.3)	7.26 (d, 1.3)	7.91
(108)	1.31	1.54	5.12, 5.29 (two d, 11.7)	4.43	5.17	7.08	8.70

Synthesis of p-methoxybenzyl (3S,5R)-6(Z)-[1-{1-(2-substituted sulfonylethyl)-1,2,3-triazole-4-yl}methylene]penicillanate-1,1-dioxides (99 and 109)

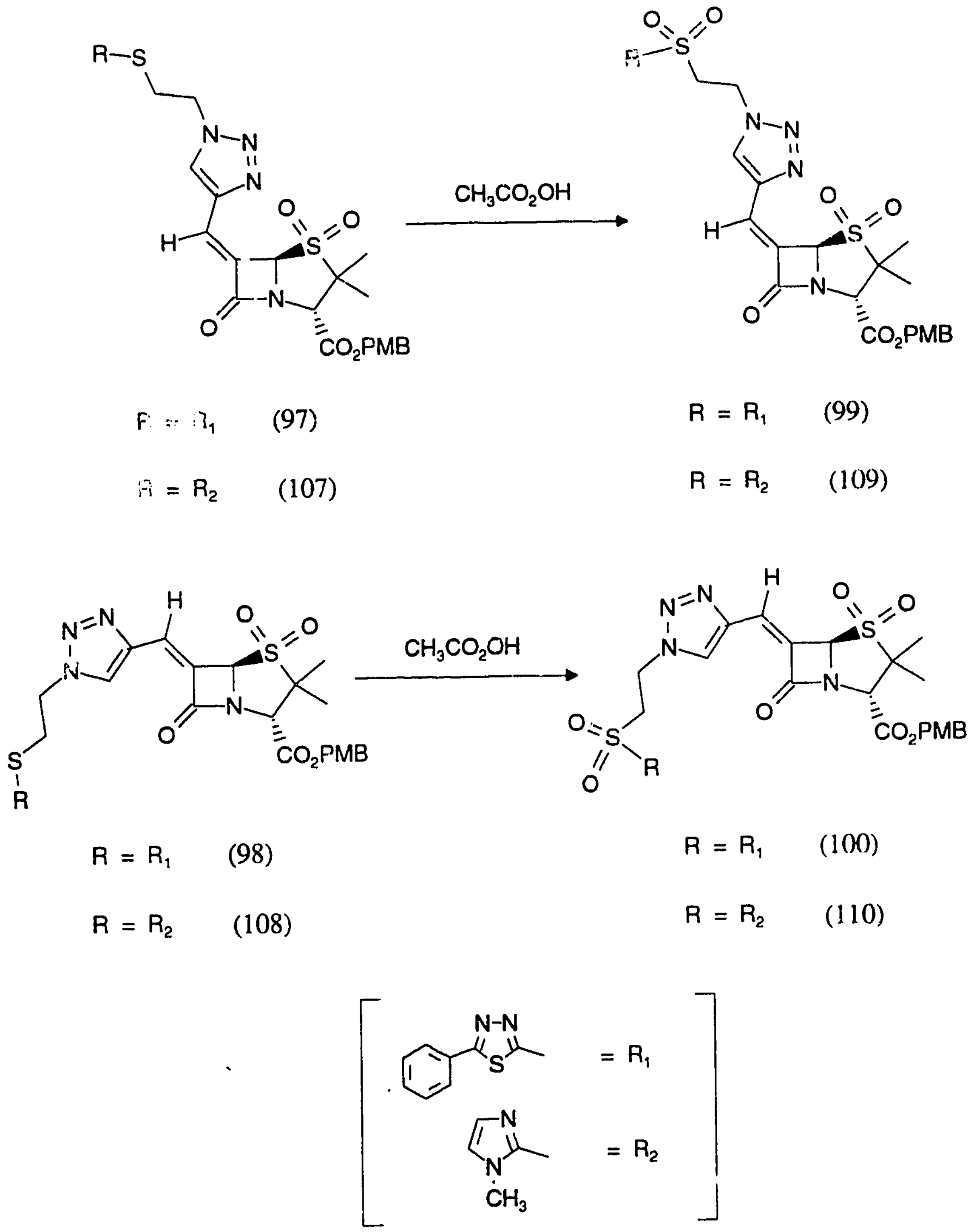
By the treatment with 2 molar equivalent of peracetic acid, the thioethyl compounds (97 and 107) in dichloromethane were converted into the corresponding sulfonylethyl compounds (99 and 109) in 88 and 86 % yield, respectively. The synthesis of compounds (99 and 109) is shown in scheme 3-6.

The human leukocyte elastase inhibitory activities of these compounds (99 and 109) were tested and their test results are summarized in table 3-3.

Synthesis of p-methoxybenzyl (3S,5R)-6(E)-[1-{1-(2-substituted sulfonylethyl)-1,2,3-triazole-4-yl}methylene]penicillanate-1,1-dioxides (100 and 110)

Using a similar method described for the compound (99), the sulfonylethyl derivatives of the E-isomer (100 and 110) were obtained from the corresponding thioethyl E-isomers (98 and 108) in 93 and 67 % yield, respectively. The synthesis of compounds (100 and 110) is presented in scheme 3-6. The NMR data of compounds (99, 100, 109, and 110) are presented in Table 3-6.

The human leukocyte elastase inhibitory activities of compounds (100 and 110) were tested and their test results are summarized in table 3-3.



R = R_1 (97)

R = R_2 (107)

R = R_1 (99)

R = R_2 (109)

R = R_1 (98)

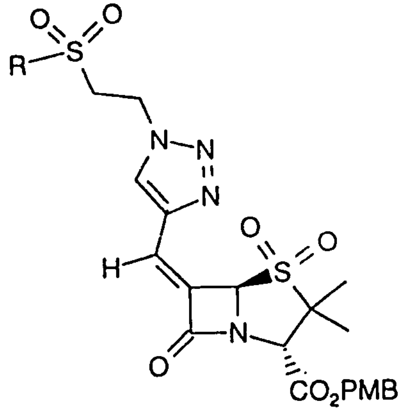
R = R_2 (108)

R = R_1 (100)

R = R_2 (110)

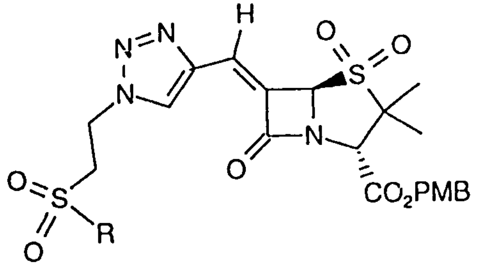
Scheme 3-6 Synthesis of compounds (99, 100, 109, and 110)

Table (3-6) NMR data of compounds (99, 100, 109, and 110)



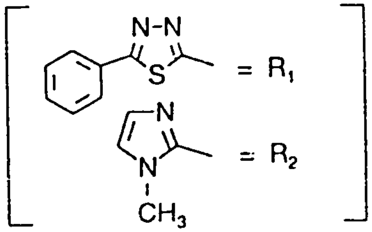
R = R$_1$ (99)

R = R$_2$ (109)



R = R$_1$ (100)

R = R$_2$ (110)



Compounds	2α-CH$_3$	2β-CH$_3$	CH$_2$ of PMB	C3-H	C5-H	C$\underline{H}$=C6	triazole-H
(99) (CDCl$_3$ + DMSO-d$_6$)	1.28	1.50	5.12, 5.28 (two d, 11.7)	4.40	5.51 (d, 1.1)	7.25 (d, 1.1)	8.11
(100) (DMSO-d$_6$)	1.31	1.45	5.15, 5.28 (two d, 11.7)	4.54	5.74	7.25	8.82
(109)	1.33	1.54	5.12, 5.28 (two d, 11.7)	4.44	5.63 (d, 11)	7.20 (d, 1.1)	7.77
(110)	1.31	1.55	5.13, 5.29 (two d, 11.7)	4.43	5.16	6.95	8.67

The data in table 3-3 indicated that only 1-acetoxy compounds (96 and 106) were relatively active and the others were not active against human leukocyte elastase. The possible explanation is that the 1-acetoxy moiety may fit into the small S1 binding pocket near the active site to show inhibitory activity. But the other compounds have no such small moiety to fit the binding pocket. Even though compounds (95 and 105) have a small moiety (-OH) in the same position, this group may be hydrogen bonded to the surrounding water and not fit into the S1 binding pocket.

Acylation of active site of enzyme is another possible explanation for mechanism of inhibitory activity of compounds (96 and 105).

Synthesis of sodium (3S,5R)-6(Z)-[1-{1-(2-substituted thioethyl)-1,2,3-triazole-4-yl}methylene]penicillanate-1,1-dioxides (101 and 111)

The p-methoxybenzyl compounds (97 and 107) were first converted into the corresponding free carboxylic compounds by deprotection with 2.5 molar equivalent of aluminum chloride in an anhydrous mixture of dichloromethane and anisole at –40 ~ –45°C under a nitrogen atmosphere for 1 hr. The adjustment of pH to 7.1 with 0.1 N-sodium hydroxide solution afforded the desired sodium salt. The very slow and careful titration of the sodium hydroxide solution was required during the pH adjustment, since the β-lactam ring is sensitive to strong base.

The resulting sodium carboxylate solution was freeze-dried to give a solid which was purified on a reverse phase column using water and acetonitrile as the eluent solution to give the pure compounds (101 and

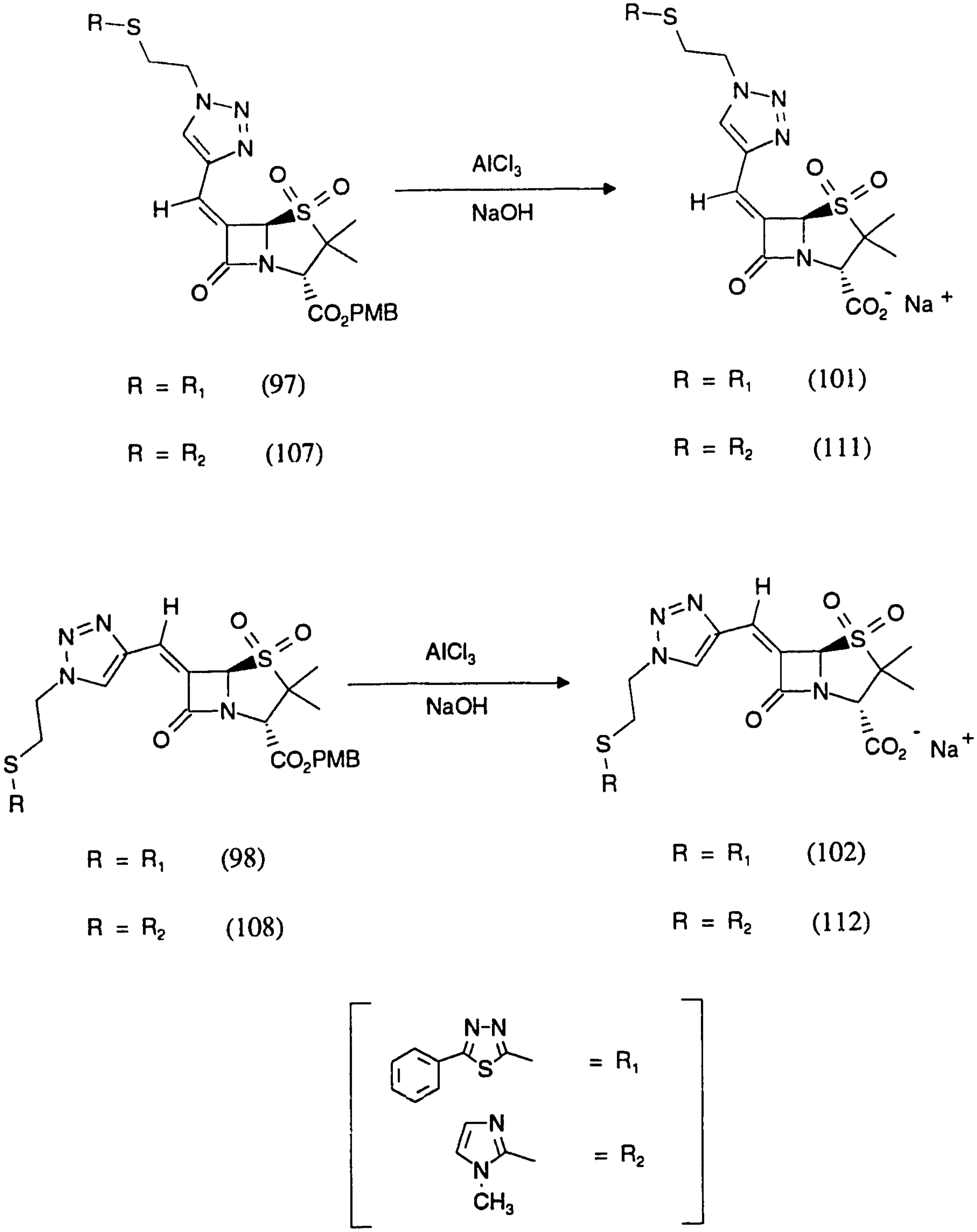
111) in 45 and 56 % yield, respectively. On a column, the residue was first washed only with distilled water to get rid of ionic impurities and then slowly increasing the percentage of acetonitrile resulted in reducing the polarity of the eluent solution and moving the product on the reverse phase column. Each portion of the eluent was monitored with reverse phase TLC and detected by ultraviolet irradiation.

Once purification was finished, the column was washed with acetonitrile and treated with distilled water and then reused again for the next purification. The synthesis of compounds (101 and 111) is illustrated in scheme 3-7. The β-lactamase inhibitory test results of compounds (101 and 111) are shown in table 3-7.

Synthesis of sodium (3S,5R)-6(E)-[1-{1-(2-substituted thioethyl)-1,2,3-triazole-4-yl}methylene]penicillanate-1,1-dioxides (102 and 112)

The sodium salts of the E-isomers (102 and 112) were obtained in 51 and 53 % yields respectively from the corresponding p-methoxybenzyl ester compounds (98 and 108) by a similar reaction as the compound (101). The synthesis of compounds (102 and 112) is shown in scheme 3-7.

The β-lactamase inhibitory test results of compounds (102 and 112) are shown in table 3-7. The NMR data of compounds (101, 102, 111, and 112) are presented in Table 3-8.



R = R$_1$ (97)

R = R$_2$ (107)

R = R$_1$ (101)

R = R$_2$ (111)

R = R$_1$ (98)

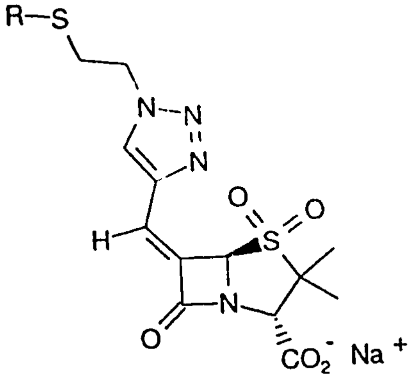
R = R$_2$ (108)

R = R$_1$ (102)

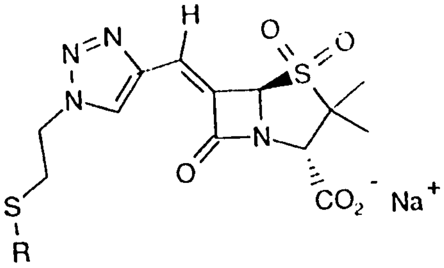
R = R$_2$ (112)

Scheme 3 - 7 Synthesis of compounds (101, 102, 111, and 112)

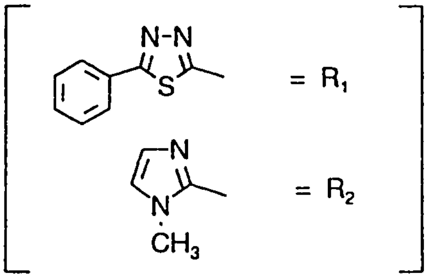
Table (3-7) β-Lactamase inhibitory activity of compounds (101, 102, 111, and 112) ; IC_{50} values (μ molar)



R = R_1 (101)
R = R_2 (111)



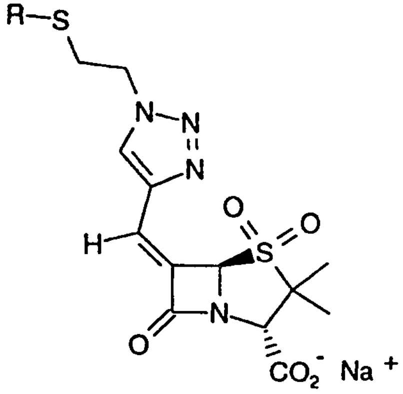
R = R_1 (102)
R = R_2 (112)

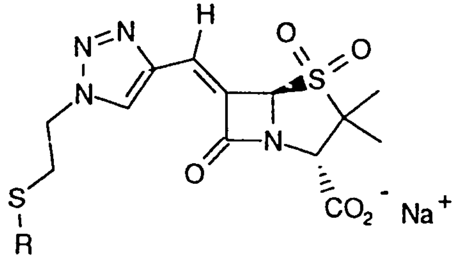


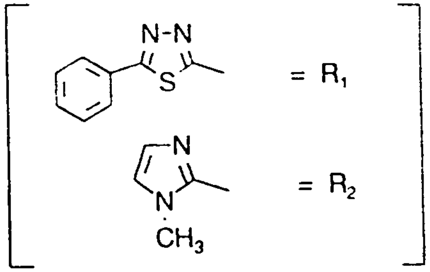
Compounds	Penicillinase	TEM (broad spectrum)	Cephalosporinase
(101)	5.5	0.045	0.40
(102)	> 100	1.63	16.4
(111)	> 100	0.47	0.38
(112)	>> 100	> 20	19.6
Tazobactam	0.31 (50.6 %)*	0.076 (84.8 %)*	2.4 (55.3 %)*
Sulbactam	23 (44.3 %)*	4.9 (88.9 %)*	20 (63.2 %)*

*Percentage inhibition under standard assay

Table (3-8) NMR data of compounds (101, 102, 111, and 112)







R = R₁ (101)
R = R₂ (111)

R = R₁ (102)
R = R₂ (112)

Compounds	2α-CH₃	2β-CH₃	C3-H	C5-H	CH=C6	CH₂N	SCH₂	triazole-H
(101) (DMSO-d_6)	1.40	1.46	3.87	5.78	7.40	4.90 (t, 5.9)	3.90 (t, 5.9)	8.46
(102) (DMSO-d_6)	1.37	1.46	3.81	5.51	7.09	4.95 (t, 6.2)	3.90 (t, 6.2)	8.83
(111) (DMSO-d_6)	1.38	1.44	3.78	5.73 (d, 1.2)	7.35 (d, 1.2)	4.68 (t, 6.3)	3.50 (t, 6.3)	8.39
(112) (DMSO-d_6)	1.38	1.46	3.82	5.54	7.10	4.73 (t, 6.3)	3.51 (t, 6.3)	8.76

Synthesis of sodium (3S,5R)-6(Z)-[1-{1-(2-substituted sulfonyl ethyl)-1,2,3-triazole-4-yl}methylene]penicillanate-1,1-dioxides (103 and 113)

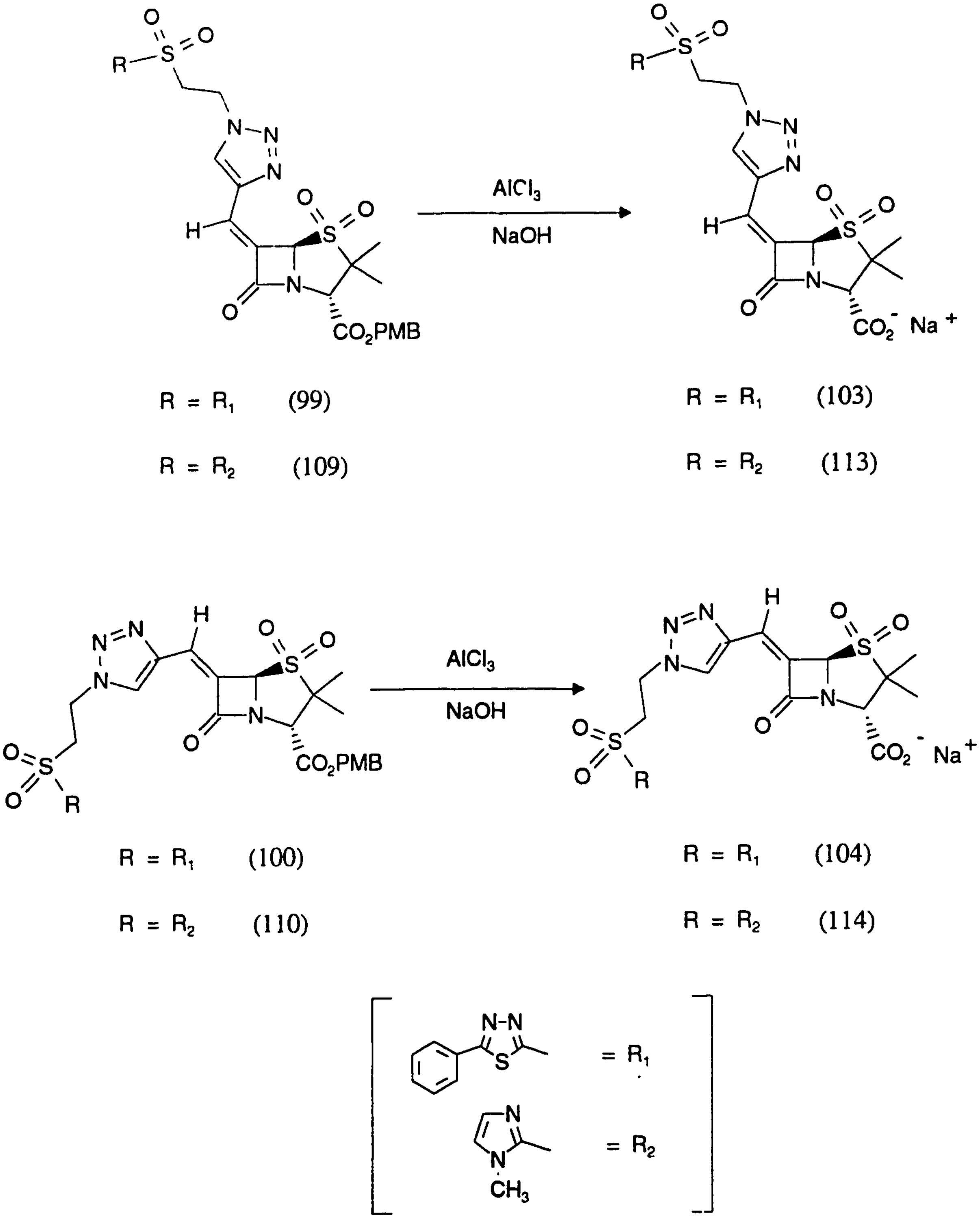
Analogously, the sodium salts of the sulfonylethyl compounds (103 and 113) were prepared in 63 and 52 % yields respectively, from the corresponding p-methoxybenzyl ester compounds (99 and 109) using a similar procedure for the compound (101). The synthesis of compounds (103 and 113) is shown in scheme 3-8.

The β-lactamase inhibitory test results of compounds (103 and 113) are summarized in table 3-9.

Synthesis of sodium (3S,5R)-6(E)-[1-{1-(2-substituted sulfonylethyl)-1,2,3-triazole-4-yl}methylene]penicillanate-1,1-dioxides (104 and 114)

Similarly, the sodium salts of the sulfonylethyl compounds (104 and 114) were synthesized in 48 and 39 % yields respectively, from the corresponding p-methoxybenzyl ester compounds (100 and 110) using a similar procedure for the compound (101). The synthesis of compounds (104 and 114) is presented in scheme 3-8.

The β-lactamase inhibitory test results of compounds (104 and 114) are shown in table 3-9. The NMR data of compounds (103, 104, 113, and 114) are presented in Table 3-10.



R = R$_1$ (99)

R = R$_2$ (109)

R = R$_1$ (103)

R = R$_2$ (113)

R = R$_1$ (100)

R = R$_2$ (110)

R = R$_1$ (104)

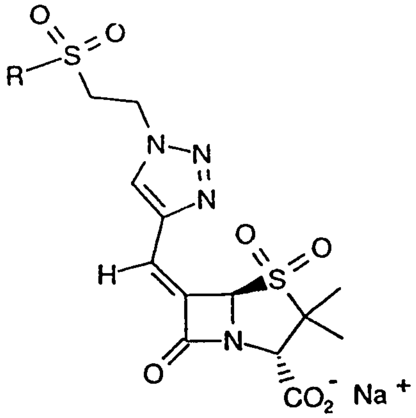
R = R$_2$ (114)

= R$_1$

= R$_2$

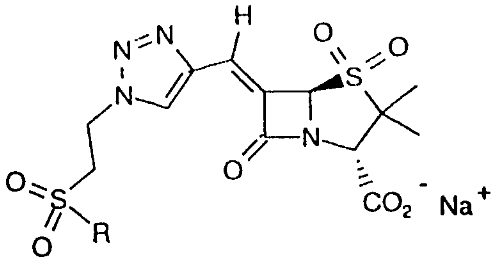
Scheme 3 - 8 Synthesis of compounds (103, 104, 113, and 114)

Table (3-9) β-Lactamase inhibitory activity of compounds (103, 104, 113, and 114) ; IC_{50} values (μ molar)



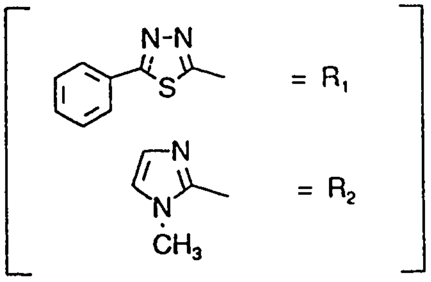
R = R_1 (103)

R = R_2 (113)



R = R_1 (104)

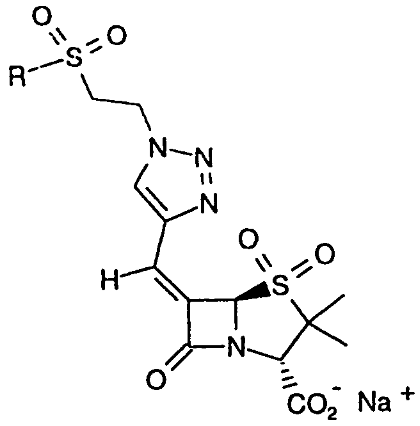
R = R_2 (114)



Compounds	Penicillinase	TEM (broad spectrum)	Cephalosporinase
(103)	4.6	0.30	0.066
(104)	>> 100	> 20	13.5
(113)	> 100	1.08	0.24
(114)	>> 100	> 20	> 20
Tazobactam	0.31 (50.6 %)*	0.076 (84.8 %)*	2.4 (55.3 %)*
Sulbactam	23 (44.3 %)*	4.9 (88.9 %)*	20 (63.2 %)*

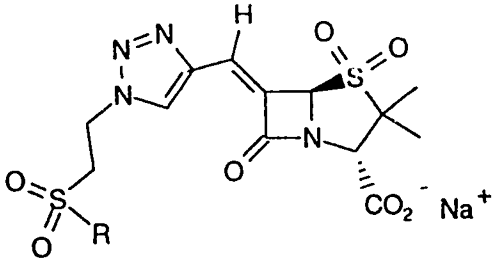
*Percentage inhibition under standard assay

Table (3-10) NMR data of compounds (103, 104, 113, and 114)



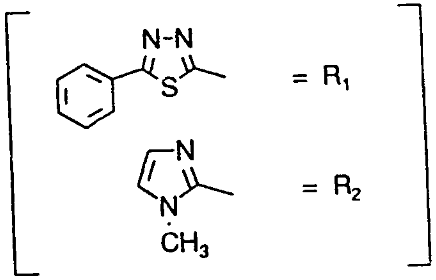
R = R₁ (103)

R = R₂ (113)



R = R₁ (104)

R = R₂ (114)



Compounds	2α-CH$_3$	2β-CH$_3$	C3-H	C5-H	CH=C6	CH$_2$N	SCH$_2$	triazole-H
(103) (DMSO-d$_6$)	1.38	1.45	3.92	5.66	7.39	5.01 (t, 6.3)	4.57 (t, 6.3)	8.45
(104) (DMSO-d$_6$)	1.37	1.45	3.80	5.46	7.00	5.05 (t, 6.2)	4.55 (t, 6.2)	8.82
(113) (DMSO-d$_6$)	1.38	1.44	3.78	5.69 (d, 0.9)	7.32 (d, 0.9)	4.84 (t, 6.4)	4.21 (t, 6.4)	8.33
(114) (DMSO-d$_6$)	1.38	1.46	3.83	5.54	7.05	4.90 (t, 6.2)	4.21 (t, 6.2)	8.71

Structure - activity relationships

From the observed data, the following structure-activity relationships can be drawn.

1) The phenylthiadiazole compounds showed stronger activity than the methylimidazole compounds against all three β-lactamases. The phenyl ring may provide a hydrophobic binding site with the enzymes.

2) The Z-isomers were much more active than the E-isomers as previously reported. The possible explanation is that the side chain in the E-isomers is close to the carbonyl group of the β-lactam ring which causes steric hindrance to interaction with the active site of the enzymes.

3) Compounds with a sulfone on the side chain were slightly more active against cephalosporinase than the sulfide compounds.

4) Compounds with a sulfide on the side chain were more active against TEM β-lactamases than the sulfone compounds.

The activity of compounds (101 and 103) were comparable to that of tazobactam. Compound (103) was much more active against cephalosporinase than tazobactam. Compound (101) had similar activity against TEM β-lactamases as tazobactam. All compounds were less active against penicillinase than tazobactam.

Compounds (101 and 103) were more active against penicillinase than sulbactam but the others were less active against penicillinase. In general, the newly synthesized compounds were more active against cephalosporinase and TEM β-lactamases than sulbactam.

CHAPTER 4

RESULTS AND DISCUSSION-2

HUMAN LEUKOCYTE ELASTASE INHIBITORS

Synthesis of (6R,7R)-7-amino-3-azidomethyl-3-cephem-4-carboxylic acid (115)

The (6R,7R)-7-amino-3-acetoxymethyl-3-cephem-4-carboxylic acid (7-ACA) was first treated with a 1.1 molar equivalent of sodium bicarbonate and the pH was adjusted to 6.5 with 10 % sodium hydroxide solution. Sodium azide was added to the resulting reaction mixture which was heated to 60°C for 6.5 hrs. This reaction afforded the corresponding sodium 3-azidomethyl carboxylate compound which was then acidified to pH 2.0 with hydrochloric acid solution to provide a precipitate. This solid was filtered and dried over anhydrous P_2O_5 overnight to give the 3-azidomethyl carboxylic compound (115) in 62 % yield.

The coupling constant between the C6-H and C7-H ($J_{6,7}$ = 5.1 Hz) indicated that these protons were in a *cis* relationship, i.e., C6α-H and C7 α-H.

Synthesis of diphenylmethyl (6R,7R)-7-amino-3-azidomethyl-3-cephem-4-carboxylate (116) and diphenylmethyl (4R,6R,7R)-7-amino-3-azidomethyl-2-cephem-4-carboxylate (117)

The reaction of the 3-cephem carboxylic compound (115) with diphenyldiazomethane gave a mixture of the diphenylmethyl ester compounds, which were separated by silica gel column chromatography to afford the 3-cephem (116) and the 2-cephem (117) in 32 and 41 % yield, respectively.

The coupling constant between the C6-H and C7-H in these compounds (116 and 117) indicated that these protons were in a *cis* relationship, i.e., C6α-H and C7α-H. The coupling constant ($J_{6,7}$) of compound (116) is 5.2 Hz and that of compound (117) is 4.1 Hz.

Synthesis of diphenylmethyl (6R,7S)-3-azidomethyl-7-chloro-3-cephem-4-carboxylate (118) and diphenylmethyl (4R,6R,7S)-3-azidomethyl-7-chloro-2-cephem-4-carboxylate (119)

The chlorination of 7β-amino-3-cephem compound (116) was accomplished by treatment with hydrochloric acid solution and sodium nitrite to give the 7α-chloro-3-cephem compound (118) in 62 % yield. The 7α-chloro-2-cephem compound (119) was obtained from 7β-amino-2-cephem compound (117) in 34 % yield using the same method.

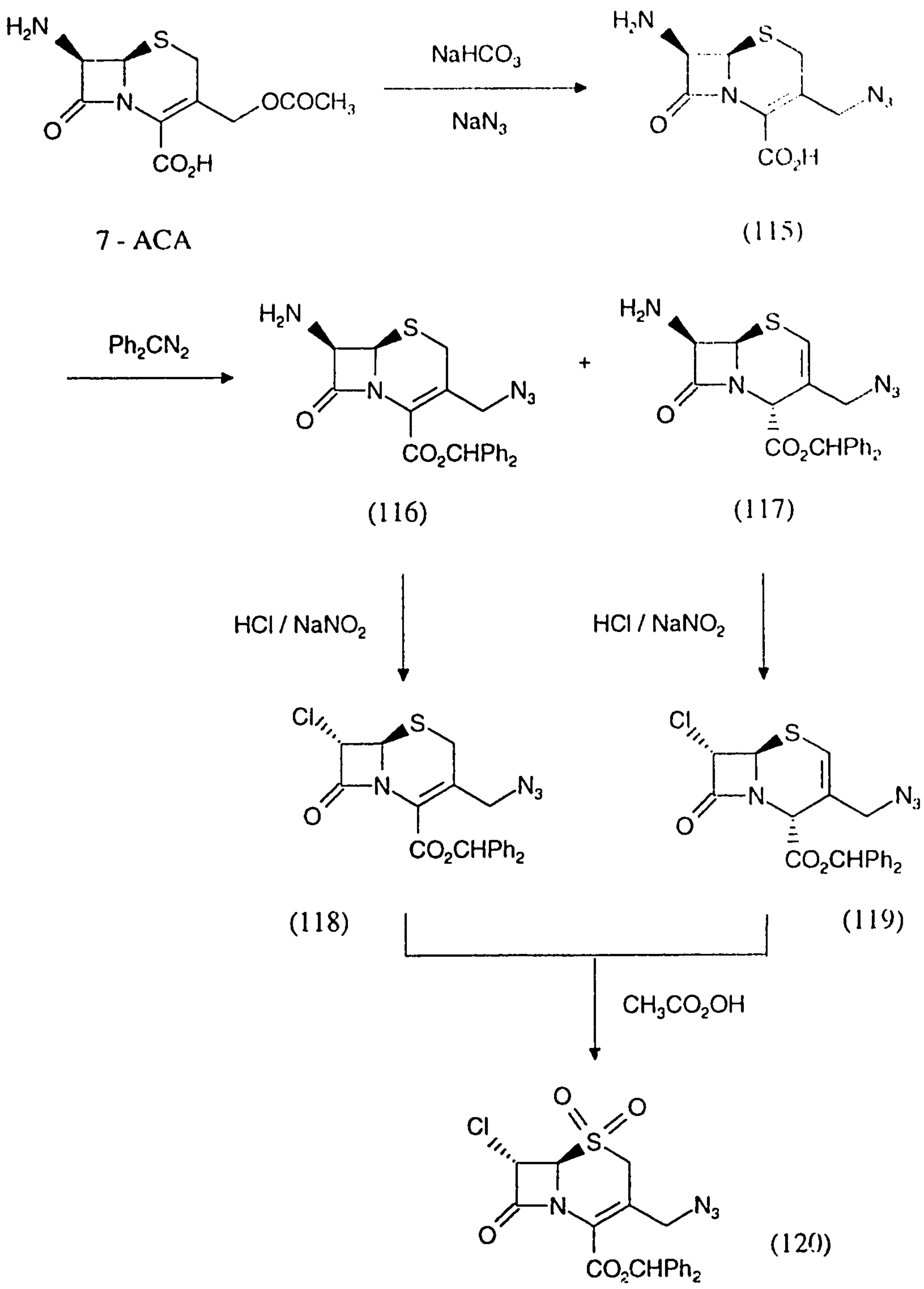
Compound (118) had a small coupling constant between the C6-H and C7-H ($J_{6,7}$ = 1.3 Hz). It suggested that these protons were in a *trans* relationship to each other, i.e., C6α-H and C7β-H. In compound (119), however, this coupling constant was so small that the peaks for C6-H and C7-H overlapped into a singlet.

Synthesis of diphenylmethyl (6R,7S)-3-azidomethyl-7-chloro-3-cephem-4-carboxylate-1,1-dioxide (120)

The oxidation of the sulfide to sulfone in the β-lactam ring was carried out by reaction of the 3-cephem compound (118) with peracetic acid to give the corresponding 3-cephem sulfone derivative (120) in 62 % yield. Similarly, the 2-cephem compound (119) was oxidized to the 3-cephem sulfone (120) in 53 % yield. The synthesis of the compound (120) from 7-ACA is illustrated in scheme 4-1.

The small coupling constant ($J_{6,7}$ = 1.7 Hz) suggests that the C6-H and C7-H are in a *trans* relationship to each other, i.e., C6α-H and C7β-H. There are two doublets for the C2-H_2 at δ 3.81 and 4.03. The higher field doublet (δ 3.81) would be for the C2β-H and the lower field doublet (δ 4.03) for C2α-H. This assignment is supported by the following observations. First, the lower field doublet has a lower height and broader peak than that of the higher field doublet. It may be due to the very small coupling between the C2α-H and C6α-H, which is too small to be measured in the 200 MHz NMR. Second, the C6α-H shows a multiplet instead of a doublet due to this small coupling.

There is a 1,3-*cis*-diaxial relationship between the C2α-H and C6α-H and this unique long range coupling has been previously reported to exist



Scheme 4 - 1 Synthesis of compound (120)

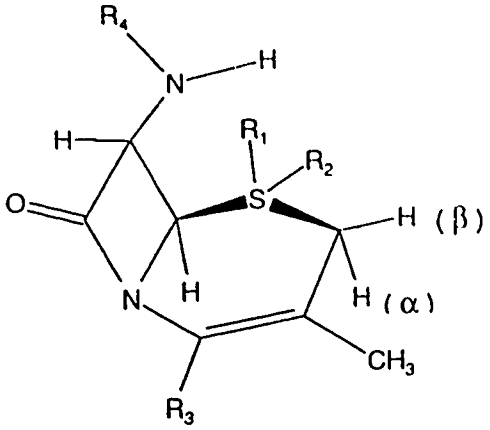
in 3-cephem-β-sulfoxides. Although the origin of this coupling is not apparent, it is believed that the β-sulfoxide bond is in some way responsible for the transfer of spin coupling information from the C2α-H to C6α-H (Cooper *et al.*, 1970). These observations were also found in compounds (121, 124, 127, 130, and 133).

NMR analysis of 2-cephems and 3-cephems

In compounds (117 and 119), the C3-CH_2 peaks are singlets , but the compounds (115, 116, 118, and 120) show two doublet peaks for these protons. These observations may be explained as follows.

Hodgkin and Maslen reported that the C2, C3, C4, N5, and C4-carbonyl group lay in a plane in sodium cephalosporin C (Hodgkin and Maslen, 1961). Cooper also proposed a similar conformation for the 3-cephem compounds (Figure 4-1) (Cooper *et al.*, 1970). With this stereochemical arrangement, the C3-CH_2 and C4-carboxyl group are close in co-plane and may interact sterically and / or electronically. So it may prevent free rotation of the C3-CH_2N_3 bond. This results in magnetically non-equivalent protons for C3-CH_2 which have two doublet peaks in its NMR spectra. A similar observation was supported by Green (Green *et al.*, 1965). NOE measurements on compounds (116, 118, and 120) support the above conformation.

From the NOE data (Table 4-1), the following conclusions are drawn. In compounds (116 and 118), the C6-H would be close to the low field C2-H and CH_2N_3 would be close to high field C2-H. These observations



R_1 = R_2 = Lone pair electrons
R_1 = Lone pair electrons , R_2 = O
R_2 = Lone pair electrons , R_1 = O

R_3 = $CO_2CH_2CCl_3$
R_4 = $COCH_2OPh$

Figure 4 - 1 Possible dihydrothiazine ring conformation in 3 - cephems

suggest that the conformation of compounds (116 and 118) may be similar to the conformation in Figure 4-1. The assignment of two C2-H doublets is based on the fact that the C6-H should be close to C2α-H and the CH_2N_3 should be close to C2β-H. 2 % NOE between the C6-H and the lowfield C2-H and 4% NOE between CH_2N_3 and the high field C2-H indicate that the lowfield C2-H could be assigned to C2α-H and the high field C2-H to

C2β-H. A similar assignment was previously reported (Demarco and Nagarajan, 1972).

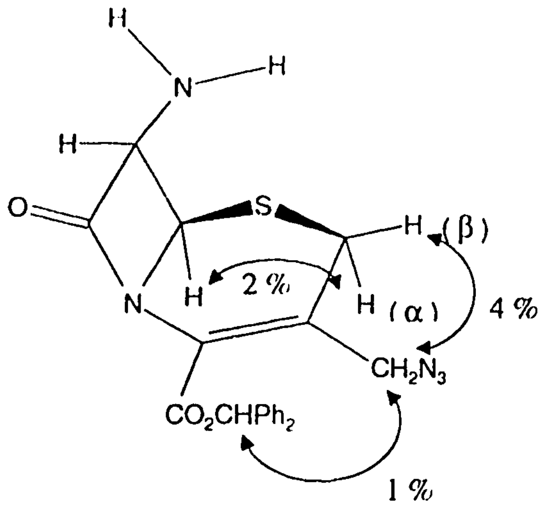
NOE data of compound (120) were similar to compounds (116 and 118) so that the conformation and assignment of the C2-H would be similar. The very small coupling between the C2α-H and the C6α-H also support this conformation. The NOE data and the dihydrothiazine ring conformation of compounds (116, 118, and 120) are shown in Table 4-1 and Figure 4-2. The NMR data of compounds (115,116,118,and 120) are presented in table 4-2.

Cooper suggested that the 2-cephem compounds had a different conformation as shown in figure 4-3 (Cooper *et al.*, 1970). With this model, the dihydrothiazine ring does not lie in a plane and the C3-azidomethyl group and C4-carboxyl group would have less steric interaction than in the 3-cephems. This may permit free rotation of the C3-CH_2N_3 bond and give a singlet for the C3-CH_2 in compounds (117 and 119). The NOE data of compounds (117 and 119) support this conformation (Figure 4-4). Similar NOE were reported by Cooper (Cooper *et al.*, 1970).

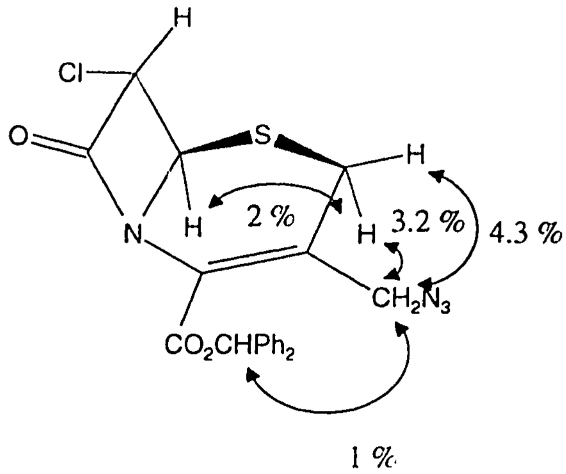
Another strong support for this chair form conformation is from the coupling constant between the C2-H and the C4-H . $J_{2,4}$ is 1.3 Hz for compound (117) and 0.9 Hz for compound (119). These allylic couplings have been also reported by Cooper (Cooper *et al.*, 1970). Since the magnitude of this coupling constant is dependent on the angle (ϕ) between the plane of the double bond (i.e., C2-C3=C4 plane) and adjacent C-H bond, the allylic coupling constant involving the C2-H and C4-H in 2-

Table (4-1) NOE data of compounds (116, 118, and 120)

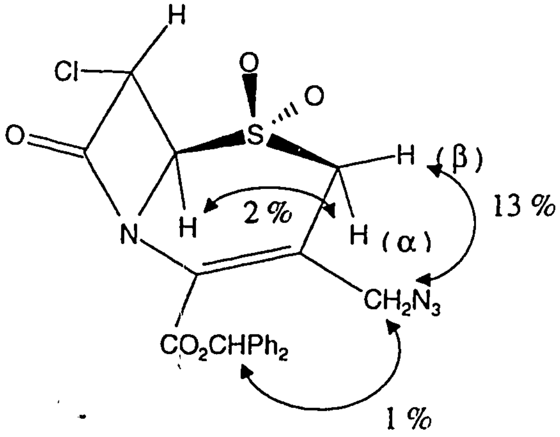
Compounds	Irradiated proton	Observed protons	Increased intensity (%)
(116) ($CDCl_3$)	CH_2N_3	High-field C2-H (3.41)	4
		CO_2CH	1
	C6-H	Low-field C2-H (3.57)	2
(118) ($CDCl_3$)	CH_2N_3	Low-field C2-H (3.51)	3.2
		High-field C2-H (3.35)	4.3
		CO_2CH	1
	C6-H	Low-field C2-H (3.51)	2
(120) ($CDCl_3$)	CH_2N_3	High-field C2-H (3.81)	13
		CO_2CH	1
	C6-H	Low-field C2-H (4.03)	2



(116)



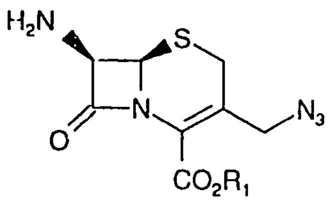
(118)



(120)

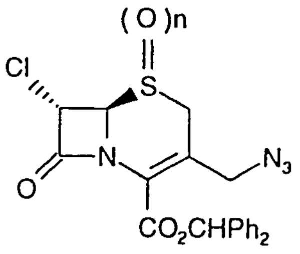
Figure 4 - 2 Dihydrothiazine ring conformation and NOE data of compounds (116, 118, and 120)

Table (4-2) NMR data of compounds (115, 116, 118, and 120)



R_1 = H (115)

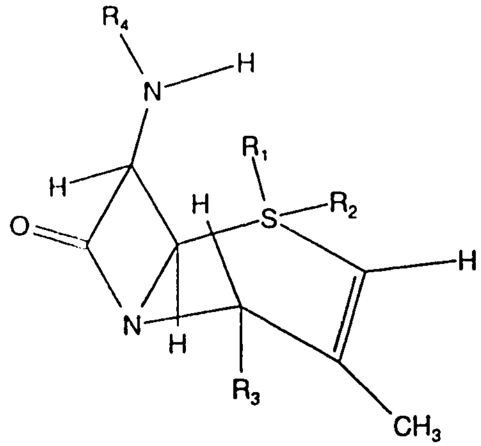
R_1 = $CHPh_2$ (116)



n = 0 (118)

n = 2 (120)

Compounds	C2α-H	C2β-H	CO_2CH	C6-H	C7-H	C3-CH_2
(115) (DMSO-d_6)	3.63 (d, 18.1)	3.42 (d, 18.1)		4.99 (d, 5.1)	4.81 (d, 5.1)	3.89, 4.37 (two d, 13.3)
(116)	3.57 (d, 18.4)	3.41 (d, 18.4)	6.98	4.96 (d, 5.2)	4.87 ~ 4.72 (m)	3.96, 4.23 (two d, 13.9)
(118)	3.51 (d, 18.1)	3.35 (d, 18.1)	6.97	4.69 (d, 1.3)	4.76 (d, 1.3)	3.92, 4.18 (two d, 14.1)
(120)	4.03 (d, 18.1)	3.81 (d, 18.1)	6.96	4.79 (m)	5.33 (d, 1.7)	4.15, 4.27 (two d, 14.9)



R_1 = R_2 = Lone pair electrons

R_1 = Lone pair electrons , R_2 = O

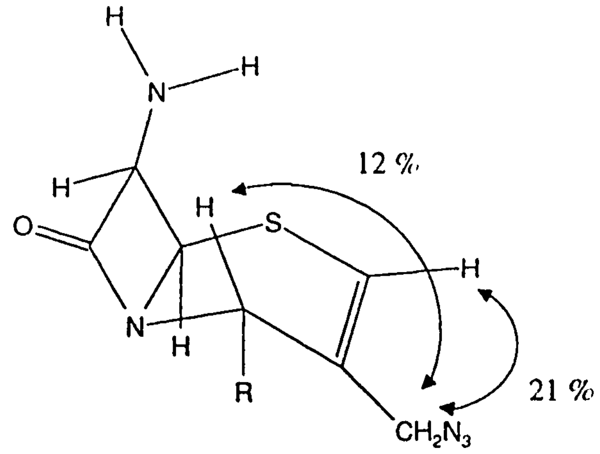
R_2 = Lone pair electrons , R_1 = O

R_3 = $CO_2C(CH_3)_3$

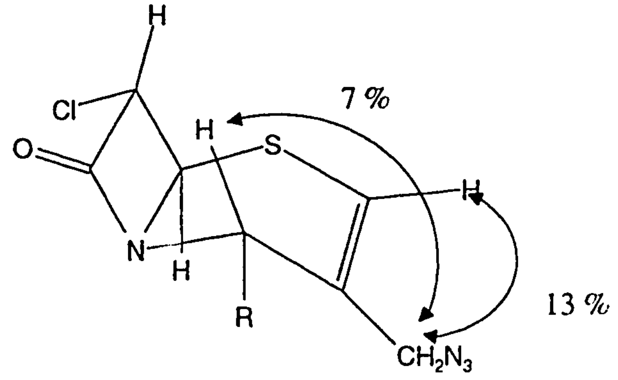
R_4 = $COCH_2OPh$

Figure 4 - 3 Possible dihydrothiazine ring conformations in 2 - cephems

cephems would be stereospecific (Figure 4-5). In allylic coupling, the π electrons of the double bond apparently help to transmit the spin information from one nucleus to the other. When all nuclei are co-planar, i.e., ϕ = 0° or 180° (Figure 4-6, B), there is no interaction of the allylic C-H bond orbital with the π system so that the allylic coupling constant would be 0 Hz. However, when the allylic C-H bond is perpendicular to



(117)



(119)

R = CO_2CHPh_2

Figure 4 - 4 Dihydrothiazine ring conformation and NOE data of compounds (117 and 119)

the C=C plane (as is the π electrons), i.e., φ = 90° (Figure 4-6, A), the interaction between the π electrons and the C-H bond orbital would reach the maximum value and the allylic coupling constant reaches 3 Hz (Pavia *et al.*, 1979). Thus, the observed coupling constants between the C2-H and the C4-H in compounds (117 and 119) not only support the above conformation of the dihydrothiazine ring but also confirm the previously assigned β-configuration for the C4-H, i.e., C4β-H (Van Heyningen and Ahern, 1968). If the C4-H has an α-configuration, φ would be close to 180 ° and the coupling constant should be smaller than 1.3 and 0.9 Hz. The NMR data of compounds (117and 119) are shown in table 4-3.

Synthesis of diphenylmethyl (6R,7S)-7-chloro-3-(1,2,3-triazole-1-yl)methyl-3-cephem-4-carboxylate-1,1-dioxide (121)

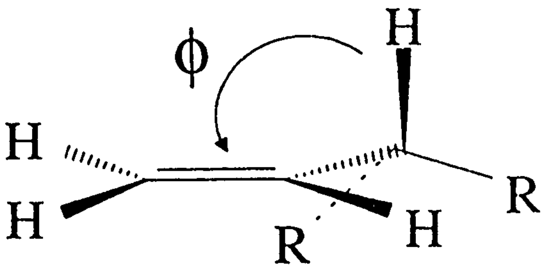
The 1,3-dipolar cycloaddition reaction of the 3-azidomethyl-3-cephem compound (120) with acetylene was performed in a steel-bomb to afford the 3-(1,2,3-triazole)-3-cephem compound (121) in 59 % yield.

The human leukocyte elastase inhibitory activity of this compound (121) was tested and the result is presented in table 4-4.

Synthesis of diphenylmethyl (6R,7S)-7-chloro-3-(substituted-1,2,3-triazole-1-yl)methyl-3-cephem-4-carboxylate-1,1-dioxides

(124, 127, 130, and 133)

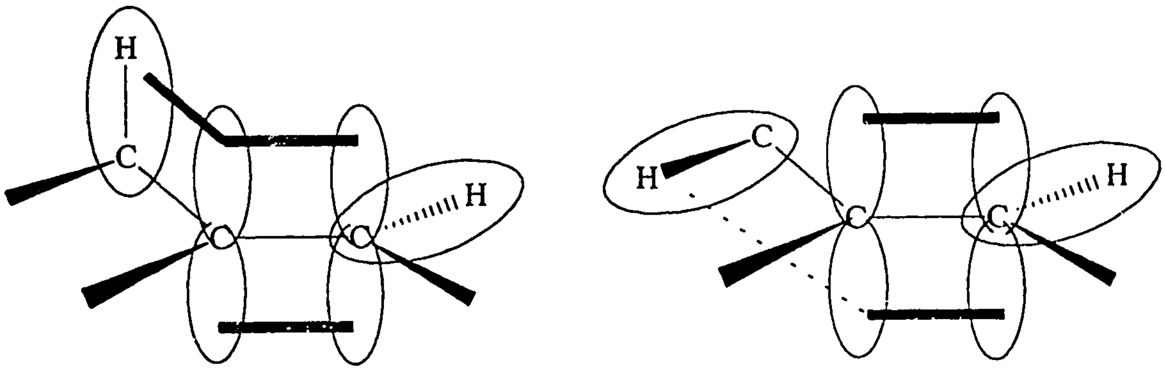
The 3-azidomethyl-3-cephem compound (120) in benzene was reacted



$J < 0.5$ Hz for $20° > \phi > 170°$

$J = 1.3 \sim 3.0$ Hz for $60° < \phi < 110°$

Figure 4 - 5 The allylic coupling and angle (φ) between the plane of the double and adjacent C - H bond



[A]

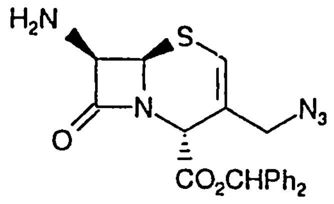
[B]

Maximum Interaction

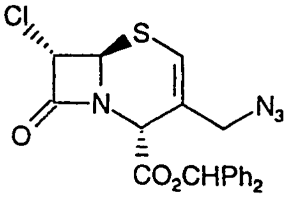
Minimum Interaction

Figure 4 - 6 Geometric arrangements that maximize and minimize allylic coupling

Table (4-3) NMR data of compounds (117 and 119)



(117)



(119)

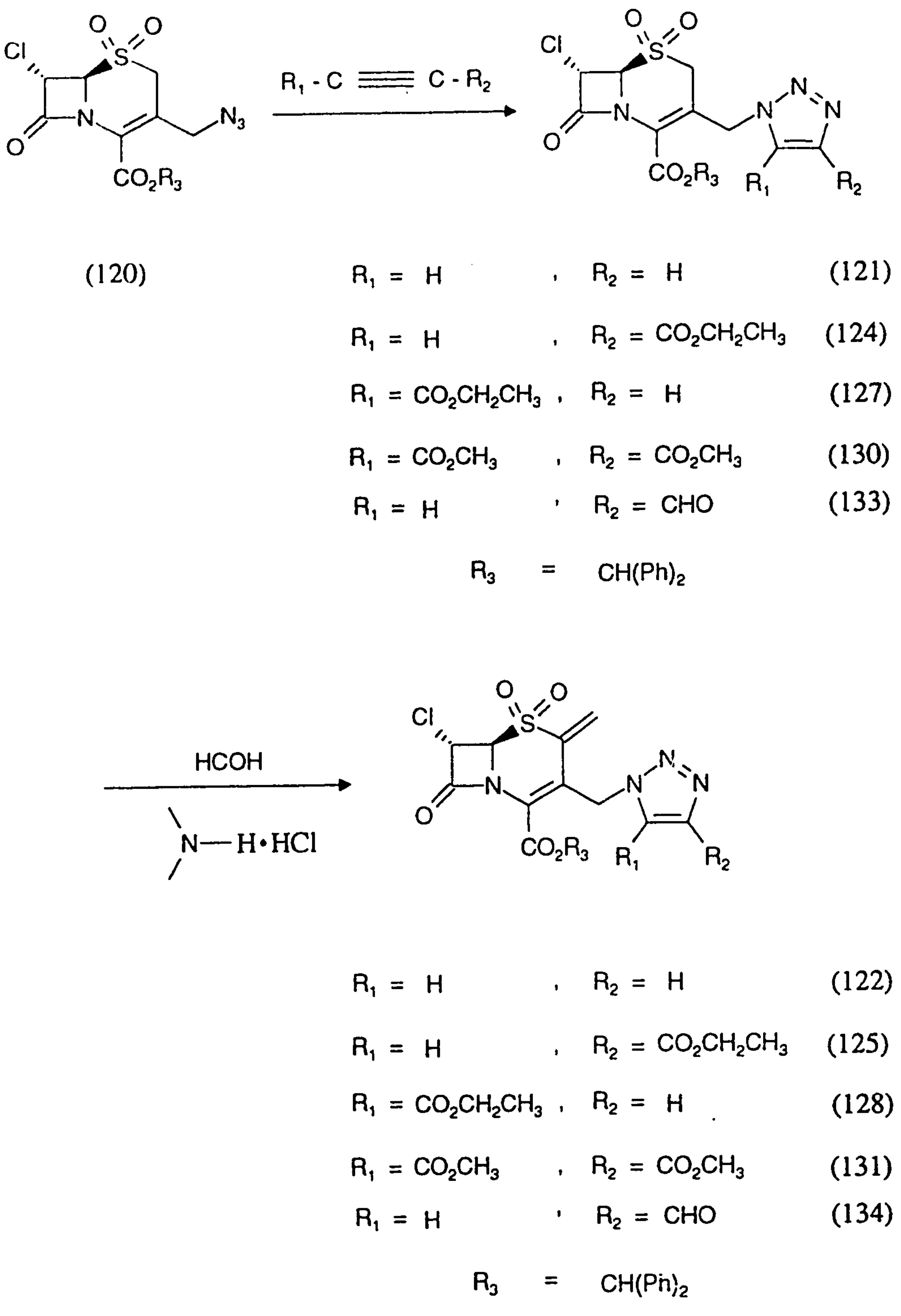
Compounds	C4-H	C3-CH$_2$	C2-H	CO$_2$CH	C6-H	C7-H
(117)	5.10 (d, 1.3)	3.85	6.38 (d, 1.3)	6.89	5.18 (d, 4.1)	4.60 (m)
(119)	5.14 (d, 0.9)	3.87	6.30 (d, 0.9)	6.90	4.77 (br. s)	5.03 (br. s)

with ethylpropiolate at 80 ~ 90°C in a pressure bottle to give a mixture of the corresponding 3-(substituted-1,2,3-triazole)-3-cephem compounds which were separated by silica gel column to afford the 4-ethoxycarbonyl triazole derivative (124) and the 5-ethoxycarbonyl triazole derivative (127) in 56 % and 34 % yields, respectively. The 5'-isomer was in lower yield than the 4'-isomer probably due to bulkiness of diphenylmethyl ester group or the steric hindrance between the ethoxycarbonyl group and the 3-azidomethyl group during the 1,3-dipolar cycloaddition reaction.

The 3-(4,5-dimethoxycarbonyl-1,2,3-triazole)-3-cephem compound (130) was similarly obtained in 85 % yield by the reaction with dimethylacetylene dicarboxylate in 85 % yield.

Analogously, the 3- (4- carboxaldehyde - 1,2,3 - triazole)- 3- cephem compound (133) was synthesized in 45 % yield from the reaction with propargyl aldehyde. Since propargyl aldehyde is not stable at high temperature and in presence of light, the reaction was performed at room temperature and the pressure bottle was covered with aluminum foil, with the hope of increasing the yield. The result was not successful, since the yield was 42 %.

From the TLC analysis, this reaction yielded only 4'-carboxaldehyde compound (133), but none of the 5'-carboxaldehyde isomer. The possible explanation of this result is that bulkiness of diphenylmethyl ester group or the steric hindrance between the aldehyde group and 3-azidomethyl group may prevent the formation of the 5'-isomer in this cycloaddition reaction. The synthesis of the triazole compounds (121, 124, 127, 130, and 133) is shown in scheme 4-2.



(120)

R_1 = H , R_2 = H (121)
R_1 = H , R_2 = $CO_2CH_2CH_3$ (124)
R_1 = $CO_2CH_2CH_3$, R_2 = H (127)
R_1 = CO_2CH_3 , R_2 = CO_2CH_3 (130)
R_1 = H , R_2 = CHO (133)

R_3 = $CH(Ph)_2$

R_1 = H , R_2 = H (122)
R_1 = H , R_2 = $CO_2CH_2CH_3$ (125)
R_1 = $CO_2CH_2CH_3$, R_2 = H (128)
R_1 = CO_2CH_3 , R_2 = CO_2CH_3 (131)
R_1 = H , R_2 = CHO (134)

R_3 = $CH(Ph)_2$

Scheme 4 - 2 Synthesis of triazole compounds and exomethylene compounds

The human leukocyte elastase inhibitory activity of these compounds (124, 127, 130, and 133) was tested and the results are summarized in table 4-4. The unsubstituted triazole compound (121) showed the lowest inhibitory activity among the tested compounds. Introduction of an electron withdrawing group on the triazole ring enhanced the inhibition. The 5-ethoxycarbonyl triazole compound (127) had little increased activity over the 4-ethoxycarbonyl triazole compound (124).

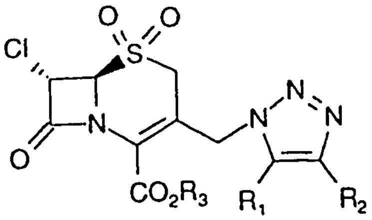
NMR analysis of the compounds (121, 124, 127, 130, and 133)

As mentioned for compound (120), the C2α-H in these compounds shows broader peaks at the lower field than the C2β-H; and the C6α-H is a multiplet due to the long range coupling between C2α-H and C6α-H.

The compounds (121, 124, and 133) do not have a bulky group at the 5-position in the triazole ring which would prevent free rotation around the C3-CH_2 bond by steric interaction and hence show near singlet peaks for the C3-CH_2 protons. However compounds (127 and 130) have bulky ester groups at the 5-position in the triazole ring and this would result in some restriction to free rotation, resulting in two doublets for the C3-CH_2 protons.

The compounds (124 and 127) are isomers of each other. The two doublets and lower field shifts of the C3-CH_2 protons (probably due to 5'-carbonyl anisotropic effect) in the compound (127) provide a clue to distinguish it from its isomer (124). Thus, compound (127) would be assigned as the 5-ethoxycarbonyl triazole derivative and compound (124)

Table (4-4) Human leukocyte elastase inhibitory activity of compounds (121, 124, 127, 130, and 133)



R_1 = H , R_2 = H (121)

R_1 = H , R_2 = $CO_2CH_2CH_3$ (124)

R_1 = $CO_2CH_2CH_3$, R_2 = H (127)

R_1 = CO_2CH_3 , R_2 = CO_2CH_3 (130)

R_1 = H , R_2 = CHO (133)

R_3 = $CH(Ph)_2$

Compounds	IC_{50} (n molar)
(121)	290
(124)	97.5
(127)	78.3
(130)	162
(133)	128

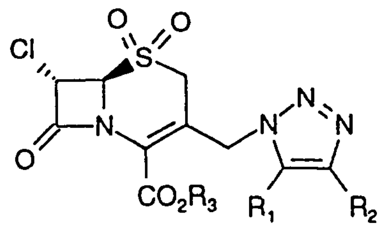
as the 4-ethoxycarbonyl triazole derivative. These observations could be used also for the assignment of structure to compound (133) as the 4-formyl triazole derivative not as a 5-formyl triazole derivative. The NMR data of compounds (121, 124, 127, 130, and 133) are presented in table 4-5.

Synthesis of diphenylmethyl (6R,7S)-7-chloro-2-methylene-3-(substituted-1,2,3-triazole-1-yl)methyl-3-cephem-4-carboxylate-1,1-dioxides (122, 125, 128, 131, and 134)

The 3-(substituted-1,2,3-triazole)-3-cephem compounds (121, 124, 127, 130, and 133) in dioxane were first treated with a 3 molar equivalent of dimethylamine-HCl and t-BuOH at room temperature, followed by the addition of an excess amount of formaldehyde, and then heated to 60 ~ 65 °C for 2 hrs. This reaction yielded the correponding 2-exomethylene compounds (122, 125, 128, 131, and 134) in 62, 55, 59, 45, and 33 % yields, respectively. The synthesis of the 2-exomethylene compounds (122, 125, 128, 131, and 134) is depicted in scheme 4-2.

The human leukocyte elastase inhibitory activity of these compounds (122, 125, 128, 131, and 134) was tested and their results are listed in table 4-6. The bulky substituent at the 4- or 5- position in the triazole ring increased the inhibitory activity (125 and 128). But the small substituent at the 4'- and 5'- position (131) or 4'- position (134) reduced the inhibition.

Table (4-5) NMR data of compounds (121, 124, 127, 130, and 133)



R_1 = H , R_2 = H (121)

R_1 = H , R_2 = $CO_2CH_2CH_3$ (124)

R_1 = $CO_2CH_2CH_3$, R_2 = H (127)

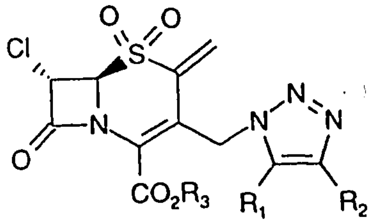
R_1 = CO_2CH_3 , R_2 = CO_2CH_3 (130)

R_1 = H ' R_2 = CHO (133)

R_3 = $CH(Ph)_2$

Compounds	R_1	R_2	C2α-H	C2β-H	$C3-CH_2$	C6-H	C7-H
(121)	H	H	4.01 (d, 18.6)	3.76 (d, 18.6)	5.24	4.84 (m)	5.31 (d, 1.5)
(124)	H	CO_2Et	4.13 (d, 18.2)	3.83 (d, 18.2)	5.24	4.95 (m)	5.30 (d, 1.6)
(127)	CO_2Et	H	3.73 (d, 17.9)	3.46 (d, 17.9)	5.49, 6.13 (two d, 15.9)	4.74 (m)	5.31 (d, 1.5)
(130)	CO_2CH_3	CO_2CH_3	3.98 (d, 19.3)	3.78 (d, 19.3)	5.47, 5.99 (two d, 15.8)	4.86 (m)	5.38 (d, 1.6)
(133)	H	COH	4.10 (d, 18.0)	3.91 (d, 18.0)	5.24, 5.25 (two s)	4.84 (m)	5.33 (d, 1.8)

Table (4-6) Human leukocyte elastase inhibitory activity of compounds (122, 125, 128, 131, and 134)



R_1 = H , R_2 = H (122)

R_1 = H , R_2 = $CO_2CH_2CH_3$ (125)

R_1 = $CO_2CH_2CH_3$, R_2' = H (128)

R_1 = CO_2CH_3 , R_2 = CO_2CH_3 (131)

R_1 = H , R_2 = CHO (134)

R_3 = $CH(Ph)_2$

Compounds	IC_{50} (n molar)
(122)	139
(125)	75.1
(128)	124
(131)	274
(134)	175

NMR analysis of the compounds (122, 125, 128, 131, and 134)

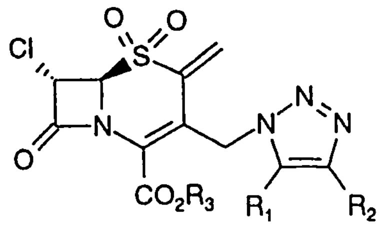
The C3-CH_2 protons of all these compounds (122, 125, 128, 131, and 134) show two doublets which indicate some restriction in the free rotation of the C3-CH_2 bond by the C2=CH_2 group. The C3-CH_2 protons of compounds (128 and 131) are shifted to a lower field probably due to the anisotropic effect rendered by the 5-carbonyl group of the triazole ring. The NMR data of compounds (122, 125, 128, 131, and 134) are presented in table 4-7.

Synthesis of diphenylmethyl (2R,6R,7S)-7-chloro-2-spiro(2,2-diphenyl)cyclopropyl-3-(substituted-1,2,3-triazole-1-yl)methyl-3-cephem-4-carboxylate-1,1-dioxides (123, 126, 129, 132, and 135)

The 2-methylene-3-(substituted-1,2,3-triazole)-3-cephem compounds (122, 125, 128, 131, and 134) were reacted with diphenyldiazomethane at room temperature to give the corresponding spiropyrazoline intermediates which through losing nitrogen yielded the corresponding 2-spiro(2,2-diphenyl)cyclopropyl-3-cephem compounds (123, 126, 129, 132, and 135) as a final products in 32, 61, 65, 53, and 47 % yields, respectively.

Once the diazomethane compound was added to the exomethylene derivatives, bubbling was observed within a few seconds which indicated the loss of nitrogen gas. During purification on the silica gel column, the unreacted diphenyldiazomethane released nitrogen gas and gave bubbles in the column, which disturbed the column purification. To separate the

Table (4-7) NMR data of compounds (122, 125, 128, 131, and 134)



R_1 = H , R_2 = H (122)

R_1 = H , R_2 = $CO_2CH_2CH_3$ (125)

R_1 = $CO_2CH_2CH_3$, R_2 = H (128)

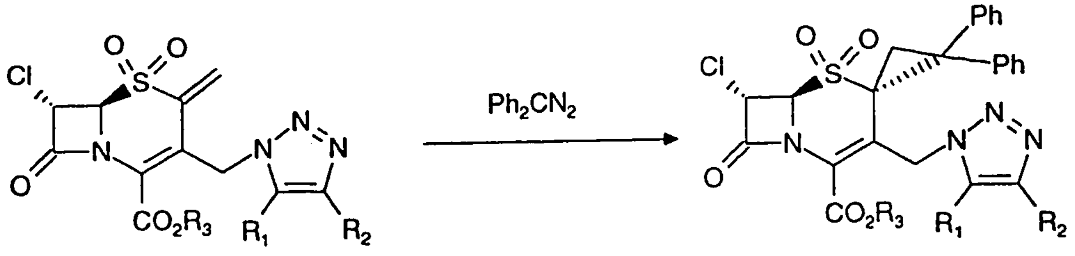
R_1 = CO_2CH_3 , R_2 = CO_2CH_3 (131)

R_1 = H , R_2 = CHO (134)

R_3 = $CH(Ph)_2$

Compounds	R_1	R_2	C2=CH_2	C3-CH_2	C6-H	C7-H
(122)	H	H	6.76	5.13, 5.55 (two d, 15.3)	4.96 (d, 1.8)	5.41 (d, 1.8)
(125)	H	CO_2Et	6.65, 6.73 (two d, 3.0)	5.18, 5.49 (two d, 15.3)	5.00 (d, 1.7)	5.42 (d, 1.7)
(128)	CO_2Et	H	6.24, 6.63 (two d, 2.8)	5.59, 6.10 (two d, 15.5)	4.99 (d, 1.7)	5.40 (d, 1.7)
(131)	CO_2CH_3	CO_2CH_3	6.34, 6.71 (two d, 2.9)	5.45, 5.98 (two d, 15.4)	5.00 (d, 1.7)	5.40 (d, 1.7)
(134)	H	COH	6.72, 6.80 (two d, 3.0)	5.19, 5.55 (two d, 15.2)	4.97 (d, 1.8)	5.42 (d, 1.8)

unreacted diazomethane compound before column purification, the reaction mixture was dissolved in ethyl acetate and an excess amount of hexane was added to precipitate the product, while the unreacted diazomethane remained in solution. The precipitated solid was filtered and purified by silica gel column chromatography to obtain the pure product. The synthesis of 2-spiro(2,2-diphenyl)cyclopropyl compounds (123, 126, 129, 132, and 135) is illustrated in scheme 4-3.



R_1 = H , R_2 = H (123)

R_1 = H , R_2 = $CO_2CH_2CH_3$ (126)

R_1 = $CO_2CH_2CH_3$, R_2 = H (129)

R_1 = CO_2CH_3 , R_2 = CO_2CH_3 (132)

R_1 = H , R_2 = CHO (135)

R_3 = $CH(Ph)_2$

Scheme 4 - 3 Synthesis of 2 - spiro(2,2-diphenyl)cyclopropyl compounds

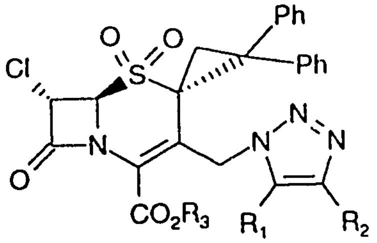
The human leukocyte elastase inhibitory activity of these compounds (123, 126, 129, 132, and 135) was tested and the results are presented in table 4-8. The introduction of an electron withdrawing group to the triazole ring generally reduced the inhibitory activity. Substitution at the 5-position in the triazole ring (129 and 132) resulted in comparable activity to the unsubstituted triazole compound (123). But substitution at the 4-position in the triazole ring (126 and 135) decreased inhibition substantially.

NMR analysis of the compounds (123, 126, 129, 132, and 135)

The cycloaddition reaction of diphenyl diazomethane to the double bond of the exomethylene compound is stereospecific. The addition reaction took place at the *exo* double bond ($C2=CH_2$), not at the *endo* double bond (C3=C4). It may be due to the bulkiness of the diphenyl diazomethane to approach the *endo* double bond. This cycloaddition reaction gave spiropyrazoline compounds, which were very sensitive to light and / or heat and rapidly lost nitrogen gas (N_2) to produce the C2-spirocyclopropyl group.

Regarding stereoselectivity of the bulky diphenyl diazomethane, the less hindered α-face attack would be the easier approach to the 2-exomethylene cephem and lead to the 2-spirocyclopropyl cephem with 2R configuration as a major end product. The hindered β-face attack could give the 2S configuration as a minor end product (Jaszberenyi *et al.*, 1988). From the spots of TLC analysis, there was one predominant

Table (4-8) Human leukocyte elastase inhibitory activity of compounds (123, 126, 129, 132, and 135)



R_1 = H , R_2 = H (123)

R_1 = H , R_2 = $CO_2CH_2CH_3$ (126)

R_1 = $CO_2CH_2CH_3$, R_2 = H (129)

R_1 = CO_2CH_3 , R_2 = CO_2CH_3 (132)

R_1 = H , R_2 = CHO (135)

R_3 = $CH(Ph)_2$

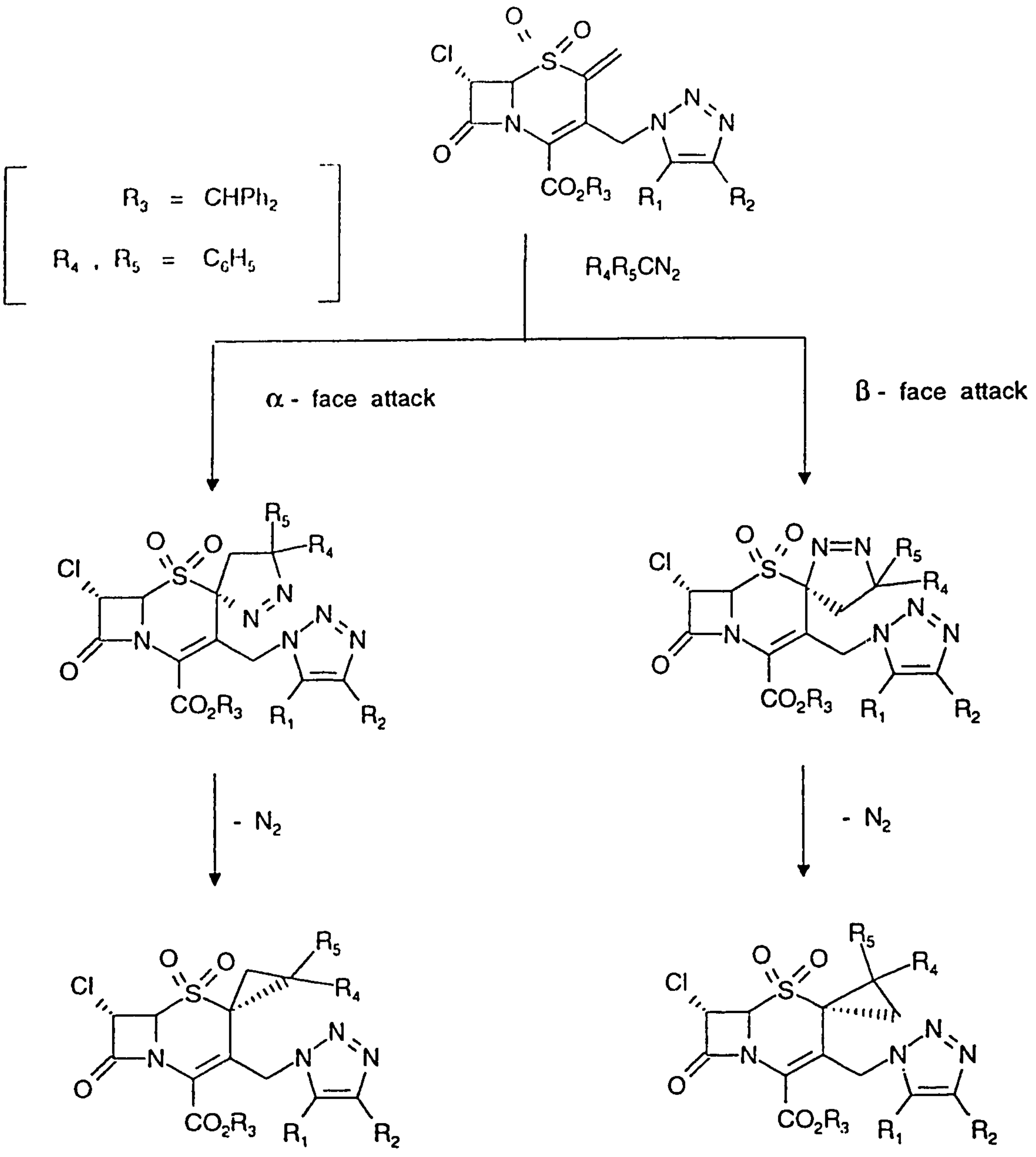
Compounds	IC_{50} (n molar)
(123)	15.3
(126)	48.6
(129)	17.7
(132)	19.9
(135)	63.0

product (2R-configuration) and the other possible product (2S-configuration) was negligible even though it could be prepared as a minor product (Scheme 4-4).

There are two possible conformations about the dihydrothiazine ring. One is the *endo*-conformation and another is the *exo*-conformation (Figure 4-7). In case of the *endo*-conformation, the C7β-H is close to the cyclopropane hydrogens and the presence of a NOE between these protons might be expected. In the *exo*-conformation, these protons are separated too far to expect it. NOE was not observed in the experiment and this lack of a NOE supported the *exo*-conformation in compound (123.)

There are also two possible configuration for 2-spiro(2,2-diphenyl) cyclopropane; the 2S configuration and the 2R configuration (Figure 4-8). In the 2S configuration, the C6α-H is close to the cyclopropyl hydrogens so that a NOE between these protons could be expected. In the 2R conformation, however, they are separated too far to observe a NOE. The absence of a NOE between the C6α-H and cyclopropyl hydrogens indicated that compound (123) is in the 2R conformation. These results led to a 2R-*exo* conformation for compound (123) (Figure 4-9 and Table 4-9). A similar observations were also reported by Jaszberenyi (Figure 4-10).

The C3-CH_2 protons of all these compounds (123, 126, 129, 132, and 135) show two doublets and there would be prevention of free rotation of the C3-CH_2 bond by the bulky group of 2-spiro (2,2-diphenyl) cyclopropane. The NMR data of compounds (123, 126, 129, 132, and 135) are presented in table 4-10.



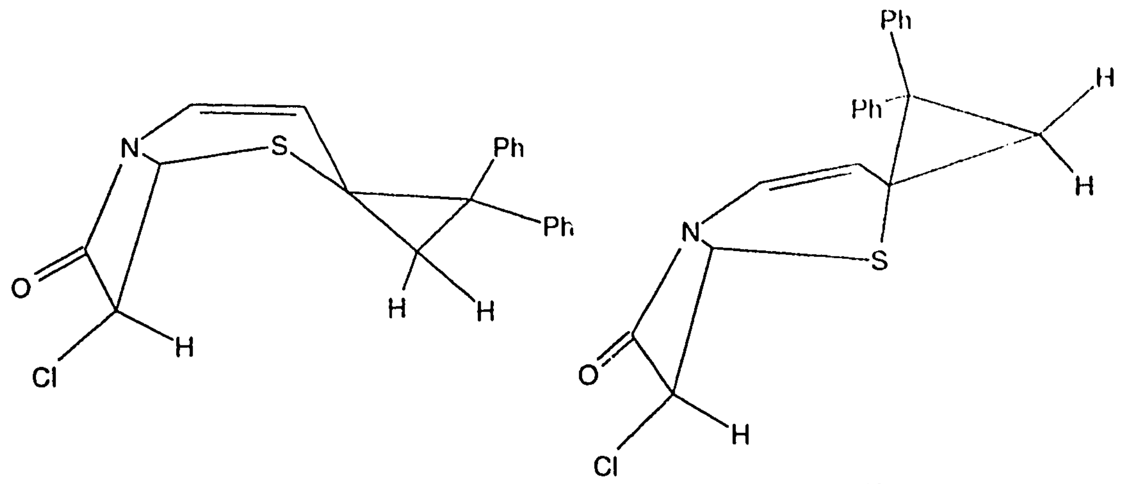
< 2R - Configuration >

Major product

< 2S - Configuration >

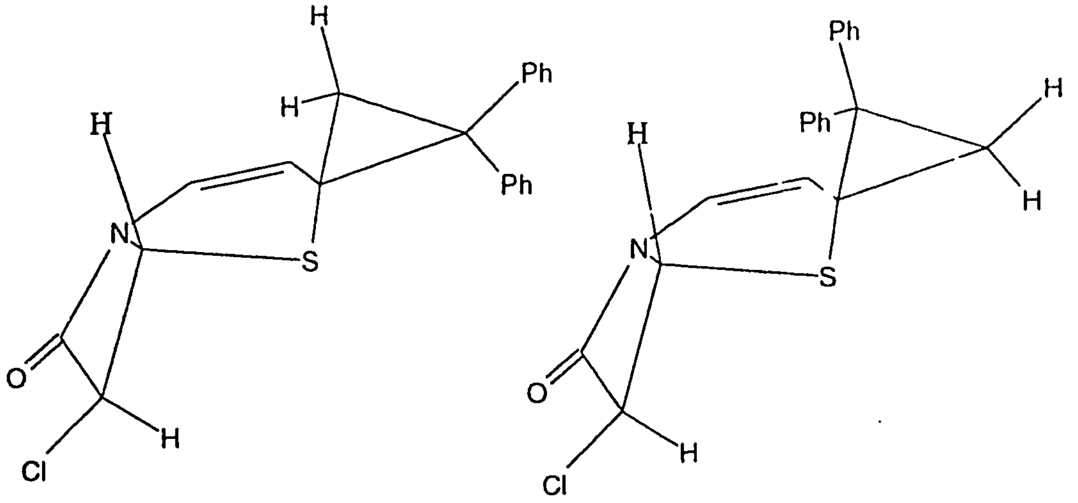
Minor product

Scheme 4 - 4 The possible products of 2 - spirocyclopropyl cephems



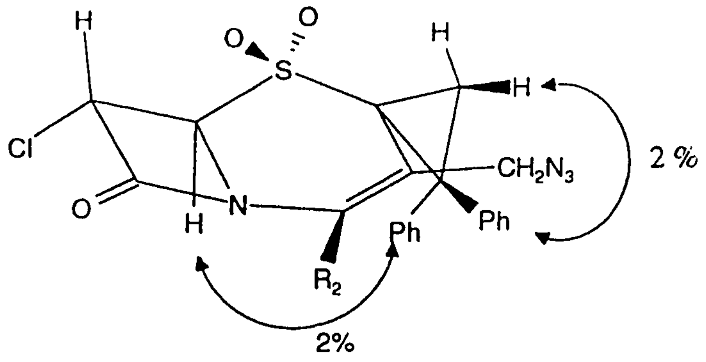
< endo >   < exo >

Figure 4 - 7 The possible structures for 2 - spirocyclopropylcephems with different dihydrothiazine conformations.



< 2S - exo >   < 2R - exo >

Figure 4 - 8 The possible exo conformations of 2 - spiro(2,2 - diphenyl)cyclopropyl cephems



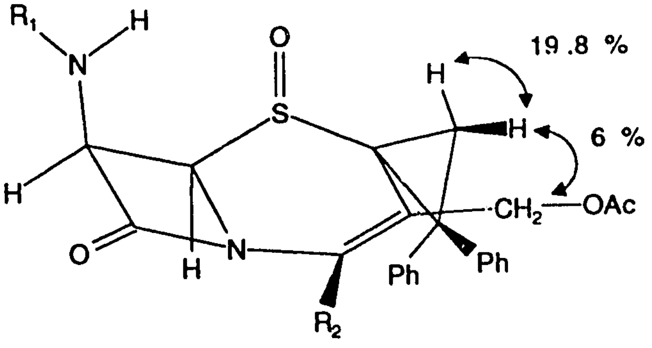
R_2 = CO_2CHPh_2

< 2R - exo >

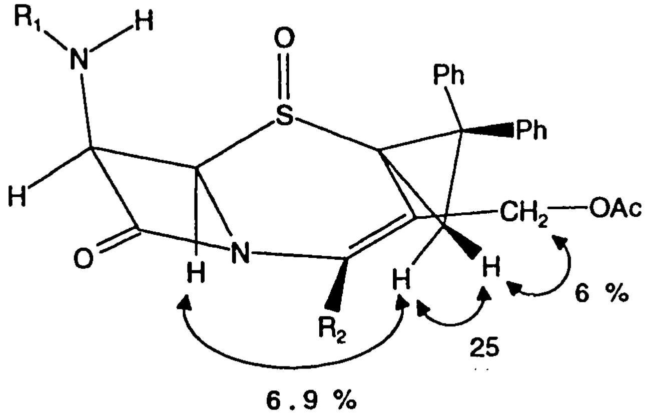
Figure 4 - 9 The possible structure of compound (123)

Table (4-9) NOE data of compound (123)

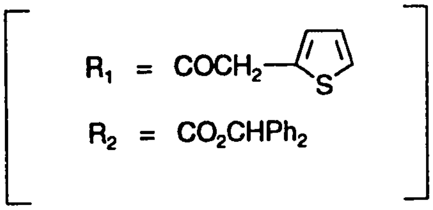
Compound	Irradiated proton	Observed protons	Intensity increased (%)
(123) ($CDCl_3$)	Cyclopropyl-H_2	Phenyl-protons	2
	C6-H	Phenyl-protons	2
		Cyclopropyl-H_2	Nil
	C7-H	Cyclopropyl-H_2	Nil



< 2R - exo >



< 2S - exo >

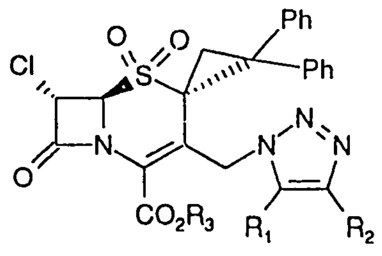


R_1 = $COCH_2$—

R_2 = CO_2CHPh_2

Figure 4 - 10 The NOE data for Benzhydryl 7β - (2 - thien - 2 - yl) acetamido - 2 - spiro (2,2, - diphenyl) cyclopropyl - 3 - acetoxymethyl -3 - cephem - 4 - carboxylate -1- (β) - oxide

Table (4-10) NMR data of compounds (123, 126, 129, 132, and 135)



R_1 = H , R_2 = H (123)
R_1 = H , R_2 = $CO_2CH_2CH_3$ (126)
R_1 = $CO_2CH_2CH_3$, R_2 = H (129)
R_1 = CO_2CH_3 , R_2 = CO_2CH_3 (132)
R_1 = H , R_2 = CHO (135)

R_3 = $CH(Ph)_2$

Compounds	R_1	R_2	Cyclopropyl-CH_2	C3-CH_2	C6-H	C7-H
(123)	H	H	2.63, 2.81 (two d, 7.8)	3.51, 4.11 (two d, 15.4)	5.08 (d, 2.1)	5.28 (d, 2.1)
(126)	H	CO_2Et	2.65, 2.85 (two d, 7.9)	3.52, 4.12 (two d, 15.4)	5.09 (d, 2.1)	5.29 (d, 2.1)
(129)	CO_2Et	H	2.44, 3.06 (two d, 7.7)	3.76, 4.75 (two d, 15.7)	5.02 (d, 2.0)	5.27 (d, 2.0)
(132)	CO_2CH_3	CO_2CH_3	2.74, 3.17 (two d, 8.1)	3.51, 4.61 (two d, 15.6)	5.02 (d, 2.0)	5.26 (d, 2.0)
(135)	H	COH	2.66, 2.89 (two d, 7.2)	3.52, 4.15 (two d, 15.3)	5.08 (d, 1.9)	5.29 (d, 1.9)

Structure - activity relationships

From the observed data, the following structure - activity relationships could be drawn.

The substitution of the triazole ring with electron withdrawing groups, such as esters and aldehyde, on the compounds which have no substitution at the 2- position, increased the inhibitory activity against human leukocyte elastase. But the reverse observations were found on the compounds which have a diphenylcyclopropyl group at the 2- position and mixed results were obtained from the compounds which have a 2-exomethylene group.

In compounds (122 and 125), which have no substitution at the triazole ring or ester group at the 4- position of the triazole ring, the introduction of a 2-exomethylene group at the 2- position increased the inhibition against human leukocyte elastase. But the reverse observations were found in the compounds (128, 131, and 134) where a ester was substituted at the 5- or 4- and 5- position of the triazole ring or an aldehyde group was joined to the 4- position of the triazole ring.

The introduction of a diphenylcyclopropyl ring at the 2- position substantially increased the inhibitory activity against human leukocyte elastase as expected. The diphenylcyclopropyl group might provide some lipophilic binding site to the surface of the enzyme.

The introduction of a bulky electron withdrawing group ($CO_2CH_2CH_3$) at the 4- position in triazole ring (124, 125, and 126) showed stronger activity than a small electron withdrawing group (-CHO) (133, 134, and 135), regardless of the substituents at the 2- position in the 3-cephem.

It was generally known that stronger inhibitory activity was observed with the order diphenylcyclopropyl > exomethylene > no substitution at the 2- position (unpublished data at Synphar).

CHAPTER 5

EXPERIMENTAL

Melting points were determined with a Thomas Hoover capillary melting point apparatus and are uncorrected. The infrared (IR) spectra were taken on a Shimadzu IR-460 spectrometer. Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker AC-200-F spectrometer using tetramethylsilane as an internal standard in deuterochloroform ($CDCl_3$) or dimethylsulfoxide-d_6 (DMSO-d_6). Fast atom bombardment mass spectra (FAB-MS) were recorded with Kratos AEI-MS-9 (modified) mass spectrometer in the Department of Chemistry, University of Alberta. Microanalyses were performed with CHN ethyl acetate-1108 Elemental Analyzer at Synphar Laboratories. Thin layer chromatography (TLC) was performed with Sigma silica coated glass plates (T-6270, with 254 nm fluorescent indicator, 250 μm layer thickness) and the spots were visualized with an ultraviolet (UV) detector, Spectroline Model CM-10. The reverse phase thin layer chromatography (TLC) was performed with Analtech RPS-F (250 micron layer thickness). Column chromatography was performed using silica gel (Merck type 7734, 70 ~ 230 mesh), unless otherwise noted. The reverse phase column chromatography was performed in column packed with a Prepex 40 ~ 63 C18 from Phenomenex.

5.1 Experiments of β-Lactamase Inhibitors

2-Azidoethanol (87)

This compound was synthesized using similar method of the reference (Boyer and Hamer, 1955). To a stirred solution of sodium azide (83.22 g, 1.28 mol) in water (340 ml), 2-bromoethanol (79.99 g, 0.64 mol) and acetone (240 ml) were added at room temperature. The mixture was gently refluxed at 55°C for 24 hrs. The solvent was removed under reduced pressure and the aqueous layer was extracted with dichloromethane. The organic layer was dried over anhydrous sodium sulfate and concentrated under reduced pressure to give the residue. The crude product was purified by vacuum distillation to give compound (87) (33.94 g, 61 %) as a liquid: b.p.$_{40\ \mathrm{mmHg}}$: 75 ~ 76°C (reported b.p.$_{40\ \mathrm{mmHg}}$: 75°C); IR ($CHCl_3$): 2115 cm^{-1}; ^1H NMR ($CDCl_3$), δ: 2.19 (H, br s, O$\underline{H}$, exchangeable with D_2O), 3.42 (2H, t, J=5.0 Hz, C$\underline{H}_2$O), 3.78 (2H, m, NC$\underline{H}_2$). NMR spectra agreed with the assigned structure.

Propargyl aldehyde (88)

This compound was synthesized using same method of the reference (Sauer, 1963). A 3000 ml three-necked round-bottomed flask was fitted with a thermometer, dropping funnel, mechanical overhead-stirrer, and an exit tube attached to two traps and two cold fingers in series. To a stirred solution of propargyl alcohol (192.60 g, 3.44 mol), concentrated sulfuric acid (225 ml), and water (550 ml) in the flask which was cooled to −5°C, a mixture of chromium trioxide (353.57 g, 3.54 mol), concentrated sulfuric acid (227 ml), and water (675 ml) were added slowly over 5 hrs.using a dropping funnel, while the pressure was maintained at 40 ~ 60 mmHg and the reaction temperature was maintained at 2 ~ 10°C. After complete addition of the chromium trioxide mixture, the cooling bath was removed and the reaction mixture was permitted to warm to room temperature, while the pressure was lowered to 10 ~ 15 mmHg to distill the remaining product. The condensates of traps and cold fingers were combined and redistilled under nitrogen atmosphere at normal pressure using a fraction column to give the compound (**88**) (56.59 g, 31 %) as a liquid: b.p.: 53 ~ 57°C (reported b.p.: 54 ~ 57°C); IR ($CHCl_3$): 2100, 1671 cm^{-1}; ^1H NMR ($CDCl_3$), δ: 3.55 (H, s, C$\underline{H}$), 9.22 (H, s, formyl-H). NMR spectra agreed with the assigned structure.

1-(2-Hydroxyethyl)-1,2,3-triazole-4-carboxaldehyde (89)

To a stirred solution of 2-azidoethanol (**87**, 25.50 g, 0.29 mol) in dry dichloromethane (50 ml) at an ice bath temperature, propargyl aldehyde (**88**, 18.99 g, 0.35 mol) in dry dichloromethane (50 ml) was added over 1 hr. using a dropping funnel. After complete addition of the aldehyde solution, dropping funnel was replaced with a water-cooled condenser. The reaction mixture was stirred at room temperature for 20 hrs. and concentrated under reduced pressure to give a sticky purple oil. The residue was purified on a silica gel column using hexane : ethyl acetate (1:3 v/v) solution as the eluant to give the compound (**89**) (28.65 g, 69 %) as a solid: m.p.:74 ~ 75°C; IR ($CHCl_3$): 1696, 1521 cm^{-1}; ^1H NMR ($CDCl_3$), δ: 2.46 (H, br s, $O\underline{H}$, exchangeable with D_2O), 4.07 ~ 4.17 (2H, m, $OC\underline{H}_2$), 4.60 (2H, t, J=4.9 Hz, $C\underline{H}_2N$).8.28 (H, s, triazole-$\underline{H}$), 10.13 (H, s, formyl-H); Exact mass calcd. for $C_5H_7N_3O_2$: 141.0537; Found (HRMS): 141.0532.

1-{2-(2-Phenyl-1,3,4-thiadiazole-5-yl)thioethyl}-1,2,3-triazole-4-carboxaldehyde (90)

Triethylamine (7.17 g, 70.86 mmol) was added to a stirred solution of 1-(2-hydroxyethyl)-1,2,3-triazole-4-carboxaldehyde (**89**, 10.00 g, 70.86 mmol) in dry dichloromethane (250 ml) and the mixture was cooled at −15 ~ −20°C under an argon atmosphere. After 40 minutes, trifluoromethane sulfonic anhydride (20.00 g, 70.86 mmol) was slowly added to the reaction mixture and stirred at −15 ~ −20°C for an additional 3.5 hrs. To the solution of 5-mercapto-2-phenyl-1,3,4-thiadiazole (13.77 g, 70.86 mmol) in dry dichloromethane (130 ml), triethylamine (7.17 g, 70.86 mmol) was added and the mixture was stirred for 2 hrs. This thiadiazole solution was added to the reaction mixture of triazole dropwise at −15 ~ −20°C for 40 minutes and stirred at room temperature overnight.The reaction mixture was extracted with dichloromethane and washed with 20 % sodium bicarbonate solution and brine. The organic layer was dried over anhydrous sodium sulfate and concentrated under reduced pressure.

The crude product was chromatographed on silica gel column with dichloromethane : ethyl acetate (4:1 v/v) solution as the eluant to give the compound (**90**) (7.87 g, 35 %) as a solid: m.p.: 160 ~ 162°C; IR ($CHCl_3$): 1699, 1523 cm^{-1}; ^1H NMR ($CDCl_3$), δ: 3.89 (2H, t, J=6.3 Hz, $SC\underline{H}_2$), 5.02 (2H, t, J=6.3 Hz, $C\underline{H}_2N$), 7.49 ~ 7.52 and 7.87 ~ 7.92 (5H, m, $\underline{Ph}$), 8.23 (H, s, triazole-$\underline{H}$), 10.16 (H, s, formyl-H). Anal. calcd. for $C_{13}H_{11}N_5OS_2$: C, 49.19; H, 3.49; N, 22.07. Found: C, 49.05; H, 3.22; N, 21.84.

1-{2-(1-Methylimidazole-2-yl)thioethyl}-1,2,3-triazole-4-carboxaldehyde (91)

Using the same procedure described for compound (**90**), compound (**91**) was prepared in 51 % yield from the 1-(2-hydroxyethyl)-1,2,3-triazole-4-carbox aldehyde (**89**, 10.00 g, 70.86 mmol), 2-mercapto-1-methylimidazole (8.09 g, 70.86 mmol), and trifluoromethanesulfonic anhydride (20.00 g, 70.86 mmol) in the presence of triethylamine. It is a solid: m.p.: 135 ~ 136°C; IR ($CHCl_3$): 1697, 1529 cm^{-1}; ^1H NMR ($CDCl_3$), δ: 3.51 (2H, t, J=6.3 Hz, $SC\underline{H}_2$), 3.60 (3H, s, $C\underline{H}_3$), 4.86 (2H, t, J=6.3 Hz, $C\underline{H}_2N$), 6.96 (H, d, J=1.3 Hz, imidazole-C4-$\underline{H}$), 7.07 (H, d, J=1.3 Hz, imidazole-C5-$\underline{H}$), 8.34 (H, s, triazole-$\underline{H}$), 10.15 (H, s, formyl-H). Anal. calcd. for $C_9H_{11}N_5OS$: C, 45.55; H, 4.67; N, 29.52. Found: C, 45.58; H, 4.58; N, 29.33.

(3S,5R,6S) 6-Bromo-penam-3-carboxylic acid (92)

This compound was synthesized using same method of the reference (Micetich *et al.*, 1986). To a stirred solution of 2.5N H_2SO_4 (1150 ml) in an ice bath, 6-aminopenicillanic acid (100 g, 0.46 mol), KBr (275.16 g, 2.31 mol), and ethanol (96 %, 900 ml) were added sequentially at 3 ~ 5°C. A solution of sodium nitrite (48.80 g, 0.71 mol) in water (230 ml) was added dropwise to the reaction mixture over 2 hrs. and kept stirring at 5 ~ 8°C for an additional 5 hrs. The reaction mixture was extracted with dichloromethane and washed with brine. The organic layer was dried over anhydrous sodium sulfate and concentrated under reduced pressure to give the compound (92) (109.3 g, 84 %) as a sticky foam: IR ($CHCl_3$), 1790, 1724 cm^{-1}; ^1H NMR ($CDCl_3$), δ: 1.57 (3H, s, 2β-C$\underline{H}_3$), 1.65 (3H, s, 2α-C$\underline{H}_3$), 4.59 (H, s, C3-$\underline{H}$), 4.84 (H, d, J=1.2 Hz, C5-$\underline{H}$), 5.41(H, d, J=1.2 Hz, C6-$\underline{H}$), 10.13 (H, br s, $CO_2\underline{H}$). The spectral data of compound (92) was identical with those of the reference.

p-Methoxybenzyl (3S,5R,6S)-6-bromo-penam-3-carboxylate (93)

This compound was synthesized using similar method of the reference (Coulton *et al.*, 1987). To a stirred solution of (3S,5R,6S)-6-bromo-penam-3-carboxylic acid (92, 109.30 g, 0.39 mol) in dry dichloromethane at −78°C under nitrogen atmosphere, p-methoxybenzyl alcohol (64.69 g, 0.47 mol) and pyridine (185.17 g, 2.34 mol) were added. A solution of cyanuric chloride (97.13 g, 0.53 mol) in dry dichloromethane (950 ml) was added dropwise to the reaction mixture which was stirred at −20 ~ −27°C for 3.5 hrs. and at 0°C for additional 2.5 hrs. The reaction mixture was poured into 5000 ml of 2.5N H_2SO_4 solution and extracted with dichloromethane, then washed with brine. The organic layer was dried over anhydrous calcium chloride and concentrated under reduced pressure to give a residue, which was purified on a silica gel column using hexane : ethyl acetate (5:1 v/v) solution as the eluant to give the compound **(93)** (119.36 g, 76 %) as an oil. IR ($CHCl_3$), 1793, 1741 cm^{-1}; ^1H NMR ($CDCl_3$), δ: 1.37 (3H, s, 2β-C$\underline{H}_3$), 1.58 (3H, s, 2α-C$\underline{H}_3$), 3.81 (3H, s, OC$\underline{H}_3$) 4.54 (H, s, C3-$\underline{H}$), 4.79 (H, d, J=1.3 Hz, C5-$\underline{H}$), 5.13 (2H, s, C$\underline{H}_2$), 5.40 (H, d, J=1.3 Hz, C6-$\underline{H}$), 6.86 ~ 6.93 and 7.29 ~ 7.33 (4H, m, $\underline{Ph}$). The spectral data of compound **(93)** was identical with those of the reference.

p-Methoxybenzyl (3S,5R,6S)-6-bromo-penam-3-carboxylate-1,1-dioxide (94)

To a stirred solution of p-methoxybenzyl (3S,5R,6S)-6-bromo-penam-3-carboxylate (**93**, 118.63 g, 0.30 mol) in dichloromethane (500 ml), peracetic acid (32 w/v %, 281.7 ml, 1.19 mol) was added dropwise at 0°C and the mixture was stirred at room temperature for 50 hrs. The reaction mixture was washed with water, 5 % $NaHCO_3$ solution, and brine. The organic layer was dried over anhydrous sodium sulfate and concentrated to give a residue, which was chromatographed on a silica gel column eluting with hexane : ethyl acetate (4:1v/v) solution to give the compound

(94) (63.25 g, 49 %) as a solid: m.p.: 110 ~ 112°C (reported m.p.: 81 ~ 83 °C); IR ($CHCl_3$): 1809, 1757, 1321 cm^{-1}; ^1H NMR ($CDCl_3$), δ: 1.24 (3H, s, 2α-C$\underline{H}_3$), 1.54 (3H, s, 2β-C$\underline{H}_3$), 3.82 (3H, s, OC$\underline{H}_3$), 4.41 (H, s, C3-$\underline{H}$), 4.67 (H, d, J=1.3 Hz, C5-$\underline{H}$), 5.15 (H, d, J=1.3 Hz, C6-$\underline{H}$), 5.12 and 5.26 (2H, two d, J=11.5 Hz, C$\underline{H}_2$), 6.87 ~ 6.94 and 7.28 ~ 7.35 (4H, m, $\underline{Ph}$). The spectral data of compound **(94)** was identical with those of the reference (Hanessian and Alpegiani, 1989).

p-Methoxybenzyl (3S,5R,6S)-6-[(1RS)-1-hydroxy-1-{1-(2-{2-phenyl-1,3,4-thiadiazole-5-yl}thioethyl)-1,2,3-triazole-4-yl}methyl] penicillanate-1,1-dioxide (95)

The solution of p-methoxybenzyl (3S,5R,6S)-6-bromo-penam-3-carboxylate-1,1-dioxide (**94,** 1.00 g, 2.31 mmol) in dry tetrahydrofuran (25 ml) was treated with CH_3MgBr (3 mol solution in ether, 0.93 ml, 2.78 mmol) and stirred at −78°C for 15 minutes under an argon atmosphere. To this reaction mixture, a solution of 1-{2-(2-phenyl-1,3,4-thiadiazole-5-yl)thioethyl}-1,2,3-triazole-4-carboxaldehyde (**90,** 0.73 g, 2.31 mmol) in dry dichloromethane (40 ml) was added and stirred at −78°C for 10 hrs. The reaction was quenched by adding saturated NH_4Cl solution and extracted with ethyl acetate. The organic layer was washed with brine, dried over anhydrous sodium sulfate, and concentrated. The residue was chromatographed on a silica gel column with $CHCl_3$: ethyl acetate (1:1v/v) as the eluant to give the stereoisomeric mixture of compound **(95)** (0.90 g, 58 %) as a solid: m.p.:133 ~ 134°C; IR ($CHCl_3$): 1784, 1748, 1314 cm^{-1}; ^1H NMR ($CDCl_3$), δ: 1.24 (3H, s, 2α-C<u>H</u>$_3$), 1.52 (3H, s, 2β-C<u>H</u>$_3$), 2.32 (H, br s, O<u>H</u>), 3.81 (3H, s, OC<u>H</u>$_3$) 3.85 ~ 3.89 (2H, m, SC<u>H</u>$_2$), 4.13 (H, dd, J1=1.8 Hz, J2=4.4 Hz, C6-<u>H</u>), 4.38 (H, s, C3-<u>H</u>), 4.81 (H, d, J1=1.8 Hz, C5-<u>H</u>), 4.90 (2H, t, J=6.3 Hz, C<u>H</u>$_2$N), 5.07 and 5.23 (2H, two d, J=11.7 Hz, C<u>H</u>$_2$ of PMB), 5.41 (H, d, J2=4.4 Hz, C<u>H</u>OH), 6.86 ~ 6.91 and 7.28 ~ 7.32 (4H, m, <u>Ph</u> of PMB), 7.47 ~ 7.50 and 7.85 ~ 7.90 (5H, m, <u>Ph</u>), 7.95 (H, s, triazole-<u>H</u>). Anal. calcd. for $C_{29}H_{30}N_6O_7S_3$: C, 51.92; H, 4.51; N, 12.53. Found: C, 51.53; H, 4.22; N, 12.50.

p-Methoxybenzyl (3S,5R,6S)-6-[(1RS)-1-acetoxy-1-{1-(2-{2-phenyl-1,3,4-thiadiazole-5-yl}thioethyl)-1,2,3-triazole-4-yl}methyl] penicillanate-1,1-dioxide (96)

Pyridine (2.18 g, 27.55 mmol) was added slowly to a stirred solution of p-methoxybenzyl (3S,5R,6S)-6-[(1RS)-1-hydroxy-1-{1-(2-{2-phenyl-1,3,4-thiadiazole-5-yl}thioethyl)-1,2,3-triazole-4-yl}methyl]penicillanate-1,1-dioxide (**95**, 1.54 g, 2.30 mmol) in dry tetrahydrofuran (50 ml) at 0°C under an argon atmosphere. After 15 minutes, acetic anhydride (2.34 g, 22.96 mmol) was added to the reaction mixture and stirred at room temperature for 51 hrs. The reaction mixture was extracted with dichloromethane and washed sequentially with 1N-HCl solution, 5 % $NaHCO_3$ solution, and brine. The organic layer was dried over anhydrous sodium sulfate and concentrated. The residue was purified on a silica gel column using hexane : ethyl acetate (1:1v/v) as the eluant to give the stereoisomeric mixture of compound (**96**) (1.53 g, 93 %) as a solid: m.p.:166 ~ 168°C; IR ($CHCl_3$): 1798, 1746, 1319 cm^{-1}; ^{1}H NMR ($CDCl_3$), δ: 1.24 (3H, s, 2α-$C\underline{H}_3$), 1.53 (3H, s, 2β-$C\underline{H}_3$), 2.10 (3H, s, $OCOC\underline{H}_3$), 3.81 (3H, s, $OC\underline{H}_3$) 3.85 (2H, t, J=6.4 Hz, $SC\underline{H}_2$), 4.31 (H, dd, J1=1.7 Hz, J2=5.0 Hz, C6-$\underline{H}$), 4.38 (H, s, C3-$\underline{H}$), 4.76 (H, d, J1=1.7 Hz, C5-$\underline{H}$), 4.91 (2H, t, J=6.4 Hz, $C\underline{H}_2N$), 5.08 and 5.20 (2H, two d, J=11.7 Hz, $C\underline{H}_2$ of PMB), 6.50 (H, d, J2=5.0 Hz, $C\underline{H}OH$), 6.87 ~ 6.93 and 7.27 ~ 7.31 (4H, m, $\underline{Ph}$ of PMB), 7.46 ~ 7.53 and 7.87 ~ 7.92 (5H, m, $\underline{Ph}$), 7.87 (H, s, triazole-$\underline{H}$). Anal. calcd. for $C_{31}H_{32}N_6O_8S_3$: C, 52.23; H, 4.53; N, 11.79. Found: C, 52.30; H, 4.41; N, 11.68.

p-Methoxybenzyl (3S,5R)-6(Z)-[1-{1-(2-{2-phenyl-1,3,4-thiadiazole-5-yl} thioethyl)-1,2,3-triazole-4-yl}methylene]penicillanate-1,1-dioxide (97) and p-Methoxybenzyl (3S,5R)-6(E)-[1-{1-(2-{2-phenyl-1,3,4-thiadiazole-5-yl} thioethyl)-1,2,3-triazole-4-yl}methylene]penicillanate -1,1-dioxide (98)

1,5-Diazabicyclo[4.3.0]non-5-ene (95 %, 1.34 g, 10.27 mmol) was added to a stirred solution of p-methoxybenzyl (3S,5R,6S)-6-[(1RS)-1-acetoxy-1-{1-(2-{2-phenyl-1,3,4-thiadiazole-5-yl}thioethyl)-1,2,3-triazole-4-yl}methyl]penicillanate-1,1-dioxide (**96**, 7.32 g, 10.27 mmol) in dry dichloromethane (100 ml) and the mixture was stirred at –70°C under an argon atmosphere for 20 minutes. The reaction was quenched by adding water and extracted with dichloromethane. The organic layer was washed with brine, dried over anhydrous sodium sulfate, and concentrated. The residue was chromatographed on a silica gel column using $CHCl_3$: ethyl acetate : hexane (1:3:2 v/v) as the eluant to give the compound (97) (3.35 g, 50 %) and compound (98) (2.35 g, 35 %) as a solid. Compound (97): m.p.:153 ~ 154°C; IR ($CHCl_3$): 1777, 1746, 1318 cm^{-1}; ^1H NMR ($CDCl_3$), δ: 1.32 (3H, s, 2α-C$\underline{H}_3$), 1.53 (3H, s, 2β-C$\underline{H}_3$), 3.82 (3H, s, OC$\underline{H}_3$) 3.86 (2H, t, J=6.4 Hz, SC$\underline{H}_2$), 4.44 (H, s, C3-$\underline{H}$), 4.94 (2H, t, J=6.4 Hz, C$\underline{H}_2$N), 5.12 and 5.28 (2H, two d, J=11.7 Hz, C$\underline{H}_2$ of PMB), 5.63 (H, d, J=1.3 Hz, C5-$\underline{H}$), 7.30 (H, d, J=1.3 Hz, C$\underline{H}$=C6), 6.89 ~ 6.93 and 7.31 ~ 7.35 (4H, m, $\underline{Ph}$ of PMB), 7.48 ~ 7.51 and 7.86 ~ 7.91 (5H, m, $\underline{Ph}$), 7.89 (H, s, triazole-$\underline{H}$). Anal. calcd. for $C_{29}H_{28}N_6O_6S_3$: C, 53.36; H, 4.32; N, 12.88. Found: C, 53.32; H, 4.30; N, 12.98. And compound (98) : m.p.:174 ~ 176°C; IR ($CHCl_3$): 1766, 1747, 1318 cm^{-1}; ^1H NMR ($CDCl_3$), δ: 1.30 (3H, s, 2α-C$\underline{H}_3$), 1.53 (3H, s, 2β-C$\underline{H}_3$), 3.82 (3H, s, OC$\underline{H}_3$) 3.91 (2H, t, J=6.2 Hz, SC$\underline{H}_2$), 4.42 (H, s, C3-$\underline{H}$), 4.96 (2H, t, J=6.2 Hz, C$\underline{H}_2$N), 5.12 and 5.28 (2H, two d, J=11.7 Hz, C$\underline{H}_2$ of PMB), 5.15 (H, s, C5-$\underline{H}$), 7.10 (H, s, C$\underline{H}$=C6), 6.88 ~ 6.93 and 7.30 ~ 7.35 (4H, m, $\underline{Ph}$ of PMB), 7.47 ~ 7.51 and 7.88 ~ 7.92 (5H, m, $\underline{Ph}$), 8.75 (H, s, triazole-$\underline{H}$). Anal. calcd. for $C_{29}H_{28}N_6O_6S_3$: C, 53.36; H, 4.32; N, 12.88. Found: C, 53.25; H, 4.15; N, 12.76.

p-Methoxybenzyl (3S,5R)-6(Z)-[1-{1-(2-{2-phenyl-1,3,4-thiadiazole-5-yl}sulfonylethyl)-1,2,3-triazole-4-yl}methylene]penicillanate-1,1-dioxide (99)

To a stirred solution of p-methoxybenzyl (3S,5R)-6(Z)-[1-{1-(2-{2-phenyl-1,3,4-thiadiazole-5-yl}thioethyl)-1,2,3-triazole-4-yl}methylene] penicillanate-1,1-dioxide (**97**, 0.90 g, 1.38 mmol) in dichloromethane (25 ml), peracetic acid (32 %, 0.21 g, 2.76 mmol) was added dropwise at 0°C and the mixture was stirred at room temperature for 68 hrs. The reaction mixture was extracted with dichloromethane and washed with water, 5 % $NaHCO_3$ solution, and brine. The organic layer was dried over anhydrous sodium sulfate and concentrated. The residue was purified on a silica gel column using dichloromethane : ethyl acetate (8:1v/v) as the eluant to give the compound (**99**) (0.83 g, 88 %) as a solid: m.p.:188 ~ 189°C; IR ($CHCl_3$): 1777, 1746, 1321 cm^{-1}; ^1H NMR ($CDCl_3$ with DMSO-d_6), δ: 1.28 (3H, s, 2α-C$\underline{H}_3$), 1.50 (3H, s, 2β-C$\underline{H}_3$) , 3.83 (3H, s, OC$\underline{H}_3$) 4.33 (2H, t, J=6.4 Hz, SC$\underline{H}_2$), 4.40 (H, s, C3-$\underline{H}$), 5.06 (2H, t, J=6.4 Hz, C$\underline{H}_2$N), 5.12 and 5.28 (2H, two d, J=11.7 Hz, C$\underline{H}_2$ of PMB), 5.51 (H, d, J=1.1 Hz, C5-$\underline{H}$), 7.25 (H, d, J=1.1 Hz, C$\underline{H}$=C6), 6.89 ~ 6.93 and 7.32 ~ 7.36 (4H, m, $\underline{Ph}$ of PMB), 7.51 ~ 7.60 and 7.96 ~ 8.00 (5H, m, $\underline{Ph}$), 8.11 (H, s, triazole-$\underline{H}$). Anal. calcd. for $C_{29}H_{28}N_6O_8S_3$: C, 50.86; H, 4.12; N, 12.28. Found: C, 50.36; H, 3.87; N, 12.16.

p-Methoxybenzyl (3S,5R)-6(E)-[1-{1-(2-{2-phenyl-1,3,4-thiadiazole-5-yl}sulfonylethyl)-1,2,3-triazole-4-yl}methylene]penicillanate-1,1-dioxide (100)

Using the procedure described for compound (99), compound (100) was prepared from the p-methoxybenzyl (3S,5R)-6(E)-[1-{1-(2-{2-phenyl-1,3,4-thiadiazole-5-yl}thioethyl)-1,2,3-triazole-4-yl}methylene] penicillanate-1,1-dioxide (98, 0.90 g, 1.38 mmol) and peracetic acid (32 %, 0.21 g, 2.76 mmol) in 93 % yield as a solid: m.p.:163 ~ 164°C; IR ($CHCl_3$): 1773, 1750, 1325 cm^{-1}; ^1H NMR (DMSO-d_6), δ: 1.31 (3H, s, 2 α-$C\underline{H}_3$), 1.45 (3H, s, 2β-$C\underline{H}_3$), 3.77 (3H, s, $OC\underline{H}_3$), 4.54 (H, s, C3-$\underline{H}$), 4.55 (2H, t, J=6.2 Hz, $SC\underline{H}_2$), 5.07 (2H, t, J=6.2 Hz, $C\underline{H}_2N$), 5.15 and 5.28 (2H, two d, J=11.7 Hz, $C\underline{H}_2$ of PMB), 5.74 (H, s, C5-$\underline{H}$), 7.25 (H, s, $C\underline{H}$=C6), 6.93 ~ 6.99 and 7.37 ~ 7.41 (4H, m, $\underline{Ph}$ of PMB), 7.56 ~ 7.67 and 8.05 ~ 8.09 (5H, m, $\underline{Ph}$), 8.82 (H, s, triazole-$\underline{H}$); FAB-MS *m/z* 685 (M+H).

Sodium (3S,5R)-6(Z)-[1-{1-(2-{2-phenyl-1,3,4-thiadiazole-5-yl} thioethyl)-1,2,3-triazole-4-yl}methylene]penicillanate-1,1-dioxide (101)

[General Procedure A]

Anhydrous aluminum chloride (0.31 g, 2.32 mmol) was added to a stirred solution of the p-methoxybenzyl (3S,5R)-6(Z)-[1-{1-(2-{2-phenyl-1,3,4-thiadiazole-5-yl}thioethyl)-1,2,3-triazole-4-yl}methylene] penicillanate-1,1-dioxide (97, 0.60 g, 0.92 mmol) in an anhydrous mixture of dichloromethane (6 ml) and anisole (25 ml) at –40 ~ –45°C under a nitrogen atmosphere. After 1 hour, the reaction was quenched by adding water and the pH was adjusted to pH 7.1 with 0.1 N-NaOH solution. The reaction mixture was freeze-dried to give a solid, which was purified on reverse phase column using water : acetonitrile (10 : 1 v/v) as the eluant to give the compound **(101)** (0.23 g, 45 %) as a solid: IR (Nujol): 1770 cm^{-1}; ^1H NMR (DMSO-d_6), δ: 1.40 (3H, s, 2α-C$\underline{H}_3$), 1.46 (3H, s, 2β-C$\underline{H}_3$), 3.87 (H, s, C3-$\underline{H}$), 3.90 (2H, t, J=5.9 Hz, SC$\underline{H}_2$), 4.90 (2H, t, J=5.9 Hz, C$\underline{H}_2$N), 5.78 (H, s, C5-$\underline{H}$), 7.40 (H, s, C$\underline{H}$=C6), 7.55 ~ 7.59 and 7.90 ~ 7.94 (5H, m, $\underline{Ph}$), 8.46 (H, s, triazole-$\underline{H}$); FAB-MS *m/z* 555 (M+H).

Sodium (3S,5R)-6(E)-[1-{1-(2-{2-phenyl-1,3,4-thiadiazole-5-yl} thioethyl)-1,2,3-triazole-4-yl}methylene]penicillanate-1,1-dioxide (102)

According to the procedure A described for compound **(101)**, compound **(102)** was prepared from the p-methoxybenzyl (3S,5R)-6(E)-[1-{1-(2-{2-phenyl-1,3,4-thiadiazole-5-yl}thioethyl)-1,2,3-triazole-4-yl} methylene]penicillanate-1,1-dioxide (**98**, 0.60 g, 0.92 mmol) and anhydrous aluminum chloride (0.31 g, 2.32 mmol) in an anhydrous mixture of dichloromethane (10 ml) and anisole (30 ml) in 51 % yield as a solid: IR (Nujol): 1759 cm^{-1}; ^1H NMR (DMSO-d_6), δ: 1.37 (3H, s, 2α-C$\underline{H}_3$), 1.46 (3H, s, 2β-C$\underline{H}_3$), 3.81 (H, s, C3-$\underline{H}$), 3.90 (2H, t, J=6.2 Hz, SC$\underline{H}_2$), 4.95 (2H, t, J=6.2 Hz, C$\underline{H}_2$N), 5.51 (H, s, C5-$\underline{H}$), 7.09 (H, s, C$\underline{H}$=C6), 7.56 ~ 7.59 and 7.90 ~ 7.95 (5H, m, $\underline{Ph}$), 8.83 (H, s, triazole-$\underline{H}$); FAB-MS *m/z* 555 (M+H).

Sodium (3S,5R)-6(Z)-[1-{1-(2-{2-phenyl-1,3,4-thiadiazole-5-yl} sulfonylethyl)-1,2,3-triazole-4-yl}methylene]penicillanate-1,1-dioxide (103)

Using the procedure A described for compound (**101**), compound (**103**) was prepared from the p-methoxybenzyl (3S,5R)-6(Z)-[1-{1-(2-{2-phenyl -1,3,4-thiadiazole-5-yl}sulfonylethyl)-1,2,3-triazole-4-yl}methylene] penicillanate-1,1-dioxide (**99**, 0.26 g, 0.38 mmol) and anhydrous aluminum chloride (0.13 g, 0.97 mmol) in an anhydrous mixture of dichloromethane (20 ml) and anisole (20 ml) in 63 % yield as a solid: IR (Nujol): 1750 cm^{-1}; ^1H NMR (DMSO-d_6), δ: 1.38 (3H, s, 2α-C$\underline{H}_3$), 1.45 (3H, s, 2β-C$\underline{H}_3$), 3.92 (H, s, C3-$\underline{H}$), 4.57 (2H, t, J=6.3 Hz, SC$\underline{H}_2$), 5.01 (2H, t, J=6.3 Hz, C$\underline{H}_2$N), 5.66 (H, s, C5-$\underline{H}$), 7.39 (H, s. C$\underline{H}$=C6), 7.62 ~ 7.68 and 8.07 ~ 8.12 (5H, m, $\underline{Ph}$), 8.45 (H, s, triazole-$\underline{H}$); FAB-MS *m/z* 587 (M+H).

Sodium (3S,5R)-6(E)-[1-{1-(2-{2-phenyl-1,3,4-thiadiazole-5-yl} sulfonylethyl)-1,2,3-triazole-4-yl}methylene]penicillanate-1,1-dioxide (104)

Using the procedure A described for compound (**101**), compound (**104**) was prepared from the p-methoxybenzyl (3S,5R)-6(E)-[1-{1-(2-{2-phenyl -1,3,4-thiadiazole-5-yl}sulfonylethyl)-1,2,3-triazole-4-yl}methylene] penicillanate-1,1-dioxide (**100**, 0.66 g, 0.96 mmol) and anhydrous aluminum chloride (0.32 g, 2.40 mmol) in an anhydrous mixture of dichloromethane (30 ml) and anisole (30 ml) in 48 % yield as a solid: IR (Nujol): 1759 cm^{-1}; ^1H NMR (DMSO-d_6), δ: 1.37 (3H, s, 2α-C$\underline{H}_3$), 1.45 (3H, s, 2β-C$\underline{H}_3$), 3.80 (H, s, C3-$\underline{H}$), 4.55 (2H, t, J=6.2 Hz, SC$\underline{H}_2$), 5.05 (2H, t, J=6.2 Hz, C$\underline{H}_2$N), 5.46 (H, s, C5-$\underline{H}$), 7.00 (H, s, C$\underline{H}$=C6), 7.58 ~ 7.70 and 8.05 ~ 8.12 (5H, m, $\underline{Ph}$), 8.82 (H, s, triazole-$\underline{H}$); FAB-MS *m/z* 587 (M+H).

p-Methoxybenzyl (3S,5R,6S)-6-[(1RS)-1-hydroxy-1-{1-(2-{1-methyl imidazole-2-yl}thioethyl)-1,2,3-triazole-4-yl}methyl]penicillanate-1,1-dioxide (105)

According to the process described for compound **(95)**, the stereoisomeric mixture of compound **(105)** was prepared from p-methoxybenzyl (3S,5R,6S)-6-bromo-penam-3-carboxylate-1,1-dioxide (**94**, 2.50 g, 5.78 mmol) in dry tetrahydrofuran (100 ml), 1-{2-(1-methyl imidazole-2-yl)thioethyl}-1,2,3-triazole-4-carboxaldehyde (**91**, 1.37 g, 5.78 mmol) in dry dichloromethane (150 ml), and CH_3MgBr (3 mol solution in ether, 2.31 ml, 6.94 mmol) in 59 % yield as a solid: m.p.:76 ~ 78°C; IR ($CHCl_3$): 1791, 1752, 1324 cm^{-1}; ^1H NMR ($CDCl_3$), δ: 1.23 and 1.25 (3H, two s, 2α-C$\underline{H}_3$), 1.52 (3H, s, 2β-C$\underline{H}_3$), 1.72 (H, br s, O$\underline{H}$), 3.48 (2H, m, SC$\underline{H}_2$), 3.59 and 3.60 (3H, two s, NC$\underline{H}_3$), 3.81 (3H, s, OC$\underline{H}_3$), 4.12 and 4.21 (H, two dd, J1 =1.8 Hz, J2 =4.6 Hz, C6-$\underline{H}$), 4.38 and 4.46 (H, two s, C3-$\underline{H}$), 4.68 (2H, t, J=6.3 Hz, C$\underline{H}_2$N), 4.81 and 4.85 (H, two d, J1 =1.8 Hz, C5-$\underline{H}$), 5.08 and 5.23 (2H, two d, J=11.7 Hz, C$\underline{H}_2$

of PMB), 5.41 and 5.48 (H, two d, J2 =4.6 Hz, C<u>H</u>OH), 6.87 ~ 6.91 and 7.28 ~ 7.32 (4H, m, Ph of PMB), 6.93 (H, d, J=1.1 Hz, imidazole-C4-H), 7.03 (H, d, J=1.1 Hz, imidazole-C5-H), 7.89 and 7.98 (H, two s, triazole-H); FAB-MS *m/z* 591 (M+H).

p-Methoxybenzyl (3S,5R,6S)-6-[(1RS)-1-acetoxy-1-{1-(2-{1-methyl imidazole-2-yl}thioethyl)-1,2,3-triazole-4-yl}methyl]penicillanate-1,1-dioxide (106)

The compound (**106**) was obtained from p-methoxybenzyl (3S,5R,6S)-6-[(1RS)-1-hydroxy-1-{1-(2-{1-methylimidazole-2-yl} thioethyl)-1,2,3-triazole-4-yl}methyl]penicillanate-1,1-dioxide (**105**, 0.61 g, 1.03 mmol), pyridine (0.98 g, 12.39 mmol), and acetic anhydride (1.05 g, 10.33 mmol) using the procedure described for compound (**96**). It was obtained in 90 % yield as a solid: m.p.:59 ~ 60°C; IR ($CHCl_3$): 1796, 1749, 1326 cm^{-1}; ^1H NMR ($CDCl_3$), δ: 1.24 and 1.31 (3H, two s, 2α-CH_3), 1.53 and 1.55 (3H, two s, 2β-CH_3), 2.05 and 2.10 (3H, two s, $OCOCH_3$) , 3.50 (2H, t, J=6.4 Hz, SCH_2), 3.58 (3H, s, NCH_3), 3.82 (3H, s, OCH_3), 4.28 (H, dd, J1 =1.9 Hz, J2 =4.9 Hz, C6-H), 4.38 and 4.40 (H, two s, C3-H), 4.73 (2H, t, J=6.4 Hz, CH_2N), 4.76 (H, d, J1=1.9 Hz, C5-H), 5.08 and 5.20 (2H, two d, J=11.7 Hz, CH_2 of PMB), 6.34 and 6.48 (H, two d, J2=4.9 Hz, CHOH), 6.87 ~ 6.92 and 7.28 ~ 7.32 (4H, m, Ph of PMB), 6.95 (H, d, J=1.0 Hz, imidazole-C4-H), 7.04 and 7.06 (H, two d, J=1.0 Hz, imidazole-C5-H), 7.75 and 7.85 (H, two s, triazole-H). Anal. calcd. for

$C_{27}H_{32}N_6O_8S_2$: C, 51.25; H, 5.10; N, 13.28. Found: C, 51.21; H, 4.68; N, 13.17.

p-Methoxybenzyl (3S,5R)-6(Z)-[1-{1-(2-{1-methylimidazole-2-yl} thioethyl)-1,2,3-triazole-4-yl}methylene]penicillanate-1,1-dioxide (107) and p-Methoxybenzyl (3S,5R)-6(E)-[1-{1-(2-{1-methylimidazole-2-yl} thioethyl)-1,2,3-triazole-4-yl}methylene]penicillanate-1,1-dioxide (108)

To a stirred solution of p-methoxybenzyl (3S,5R,6S)-6-[(1RS)-1-acetoxy-1-{1-(2-{1-methylimidazole-2-yl}thioethyl)-1,2,3-triazole-4-yl} methyl]penicillanate-1,1-dioxide (**106**, 6.23 g, 9.85 mmol) in dry dichloromethane (100 ml), 1,5-diazabicyclo[4.3.0]non-5-ene (95 %, 1.29 g, 9.85 mmol) was added and the mixture was stirred at –68°C under a argon atmosphere for 20 minutes. The reaction was quenched by adding water and then extracted with dichloromethane. The organic layer was washed with brine, dried over anhydrous sodium sulfate, and concentrated. The residue was chromatographed on a silica gel column using $CHCl_3$: CH_3COCH_3 (2:1 v/v) as the eluant to give the compound (**107**) (2.36 g, 42 %) and the compound (**108**) (2.87 g, 51 %) as solids. Compound (**107**): m.p.:65 ~ 67°C; IR ($CHCl_3$): 1776, 1750, 1324 cm^{-1}; ^1H NMR ($CDCl_3$), δ: 1.33 (3H, s, 2α-$C\underline{H}_3$), 1.54 (3H, s, 2β-$C\underline{H}_3$), 3.44 ~ 3.52 (2H, m, $SC\underline{H}_2$), 3.56 (3H, s, $NC\underline{H}_3$), 3.82 (3H, s, $OC\underline{H}_3$) 4.44 (H, s, C3-$\underline{H}$), 4.75 (2H, t, J=6.1 Hz, $C\underline{H}_2N$), 5.12 and 5.28 (2H, two d, J=11.7 Hz, $C\underline{H}_2$ of PMB), 5.64 (H, d, J=1.3 Hz, C5-$\underline{H}$), 6.87 ~ 6.93 and 7.29 ~ 7.35 (4H, m, $\underline{Ph}$ of PMB), 6.93 (H, d, J=1.2 Hz, imidazole-C4-$\underline{H}$), 7.03

(H, d, J=1.2 Hz, imidazole-C5-H), 7.26 (H, d, J=1.3 Hz, CH=C6), 7.91 (H, s, triazole-H); FAB-MS *m/z* 573 (M+H). And Compound **(108)**: m.p.:143 ~ 144°C; IR ($CHCl_3$): 1773, 1753, 1325 cm^{-1}; ^1H NMR ($CDCl_3$), δ: 1.31 (3H, s, 2α-CH_3), 1.54 (3H, s, 2β-CH_3), 3.56 (3H, s, NCH_3), 3.57 (2H, t, J=6.3 Hz, SCH_2), 3.82 (3H, s, OCH_3) 4.43 (H, s, C3-H), 4.75 (2H, t, J=6.3 Hz, CH_2N), 5.17 (H, s, C5-H), 5.12 and 5.29 (2H, two d, J=11.7 Hz, CH_2 of PMB), 6.87 ~ 6.95 and 7.30 ~ 7.37 (4H, m, Ph of PMB), 6.93 (H, d, J=1.2 Hz, imidazole-C4-H), 7.06 (H, d, J=1.2 Hz, imidazole-C5-H), 7.08 (H, s,CH=C6), 8.70 (H, s, triazole-H). Anal. calcd. for $C_{25}H_{28}N_6O_6S_2$: C, 52.43; H, 4.93; N, 14.68. Found: C, 52.54; H, 4.73; N, 14.71.

p-Methoxybenzyl (3S,5R)-6(Z)-[1-{1-(2-{1-methylimidazole-2-yl} sulfonylethyl)-1,2,3-triazole-4-yl}methylene]penicillanate-1,1-dioxide (109)

The above compound **(109)** was prepared from p-methoxybenzyl (3S,5R)-6(Z)-[1-{1-(2-{1-methylimidazole-2-yl}thioethyl)-1,2,3-triazole-4-yl}methylene]penicillanate-1,1-dioxide **(107**, 1.50 g, 2.62 mmol) and peracetic acid (32 %, 0.40 g, 5.26 mmol) in 86 % yield according to the procedure described for compound **(99)** and it was obtained as a solid: m.p.:89 ~ 91°C; IR ($CHCl_3$): 1783, 1750, 1325 cm^{-1}; ^1H NMR ($CDCl_3$), δ: 1.33 (3H, s, 2α-CH_3), 1.54 (3H, s, 2β-CH_3), 3.82 (3H, s, OCH_3), 3.89 (3H, s, NCH_3), 4.09 (2H, t, J=6.1 Hz, SCH_2), 4.44 (H, s, C3-H), 4.82 ~ 5.04 (2H, m, CH_2N), 5.12 and 5.28 (2H, two d, J=11.7 Hz, CH_2 of

PMB), 5.63 (H, d, J=1.1 Hz, C5-H), 6.89 ~ 6.93 and 7.31 ~ 7.35 (4H, m, Ph of PMB), 7.00 (H, s, imidazole-C4-H), 7.01 (H, s, imidazole-C5-H), 7.20 (H, d, J=1.1 Hz, CH=C6), 7.77 (H, s, triazole-H); FAB-MS *m/z* 605 (M+H).

p-Meth[illegible] (3S,5R)-6(E)-[1-{1-(2-{1-methylimidazole-2-yl} sulfony[illegible] [illegible]riazole-4-yl}methylene]penicillanate-1,1-dioxide (110)

The above compound (**110**) was obtained from p-methoxybenzyl (3S,5R)-6(E)-[1-{1-(2-{1-methylimidazole-2-yl}thioethyl)-1,2,3-triazole-4-yl}methylene]penicillanate-1,1-dioxide (**108**, 2.02 g, 3.53 mmol) and peracetic acid (32 %, 0.54 g, 7.10 mmol) in 67 % yield using the procedure described for compound (**99**) and it was obtained as a solid: m.p.:148 ~ 150°C; IR ($CHCl_3$): 1773, 1751, 1325 cm^{-1}; ^1H NMR ($CDCl_3$), δ: 1.31 (3H, s, 2α-CH_3), 1.55 (3H, s, 2β-CH_3), 3.83 (3H, s, OCH_3), 3.93 (3H, s, NCH_3), 3.99 ~ 4.31 (2H, m, SCH_2), 4.43 (H, s, C3-H), 4.99 (2H, t, J=6.6 Hz, CH_2N), 5.16 (H, s, C5-H), 5.13 and 5.29 (2H, two d, J=11.7 Hz, CH_2 of PMB), 6.89 ~ 6.93 and 7.31 ~ 7.36 (4H, m, Ph of PMB), 6.95 (H, s,CH=C6), 7.01 (2H, s, imidazole-C4-H and imidazole-C5-H), 8.67 (H, s, triazole-H). Anal. calcd. for $C_{25}H_{28}N_6O_8S_2$: C, 49.66; H, 4.67; N, 13.90. Found: C, 49.68; H, 4.49; N, 13.62.

Sodium (3S,5R)-6(Z)-[1-{1-(2-{1-methylimidazole-2-yl}thioethyl)-1,2,3-triazole-4-yl}methylene]penicillanate-1,1-dioxide (111)

Using the procedure A described for compound (**101**), the above compound (**111**) was obtained from p-methoxybenzyl (3S,5R)-6(Z)-[1-{1-(2-{1-methylimidazole-2-yl}thioethyl)-1,2,3-triazole-4-yl}methylene] penicillanate-1,1-dioxide (**107**, 0.80 g, 1.40 mmol) and anhydrous aluminum chloride (0.47 g, 3.53 mmol) in an anhydrous mixture of dichloromethane (6 ml) and anisole (24 ml) in 56 % yield as a solid: IR (Nujol): 1769 cm^{-1}; ^{1}H NMR (DMSO-d_6), δ: 1.38 (3H, s, 2α-C$\underline{H}_3$), 1.44 (3H, s, 2β-C$\underline{H}_3$), 3.50 (2H, t, J=6.3 Hz, SC$\underline{H}_2$), 3.53 (3H, s, NC$\underline{H}_3$), 3.78 (H, s, C3-$\underline{H}$), 4.68 (2H, t, J=6.3 Hz, C$\underline{H}_2$N) , 5.73 (H, d, J=1.2 Hz, C5-$\underline{H}$), 6.97 (H, d, J=1.0 Hz, imidazole-C4-$\underline{H}$), 7.24 (H, d, J=1.0 Hz, imidazole-C5-$\underline{H}$), 7.35 (H, d, J=1.2 Hz, C$\underline{H}$=C6), 8.39 (H, s, triazole-$\underline{H}$); FAB-MS *m/z* 475 (M+H).

Sodium (3S,5R)-6(E)-[1-{1-(2-{1-methylimidazole-2-yl}thioethyl)-1,2,3-triazole-4-yl}methylene]penicillanate-1,1-dioxide (112)

According to the procedure A described for compound (**101**), compound (**112**) was prepared from p-methoxybenzyl (3S,5R)-6(E)-[1-{1-(2-{1-methylimidazole-2-yl}thioethyl)-1,2,3-triazole-4-yl}methylene] penicillanate-1,1-dioxide (**108**, 0.80 g, 1.40 mmol) and anhydrous aluminum chloride (0.47 g, 3.53 mmol) in an anhydrous mixture of dichloromethane (6 ml) and anisole (24 ml) in 53 % yield as a solid: IR

(Nujol): 1751 cm^{-1}; ^1H NMR (DMSO-d_6), δ: 1.38 (3H, s, 2α-C$\underline{H}_3$), 1.46 (3H, s, 2β-C$\underline{H}_3$), 3.51 (2H, t, J=6.3 Hz, SC$\underline{H}_2$), 3.54 (3H, s, NC$\underline{H}_3$), 3.82 (H, s, C3-$\underline{H}$), 4.73 (2H, t, J=6.3 Hz, C$\underline{H}_2$N) , 5.54 (H, s, C5-$\underline{H}$), 6.96 (H, d, J=1.1 Hz, imidazole-C4-$\underline{H}$), 7.10 (H, s, C$\underline{H}$=C6), 7.23 (H, d, J=1.1 Hz, imidazole-C5-$\underline{H}$), 8.76 (H, s, triazole-$\underline{H}$); FAB-MS *m/z* 475 (M+H).

Sodium (3S,5R)-6(Z)-[1-{1-(2-{1-methylimidazole-2-yl}sulfonylethyl)-1,2,3-triazole-4-yl}methylene]penicillanate-1,1-dioxide (113)

Using the procedure A described for compound (**101**), compound (**113**) was obtained from p-methoxybenzyl (3S,5R)-6(Z)-[1-{1-(2-{1-methyl imidazole-2-yl}sulfonylethyl)-1,2,3-triazole-4-yl}methylene] penicillanate-1,1-dioxide (**109**, 0.80 g, 1.32 mmol) and anhydrous aluminum chloride (0.44 g, 3.30 mmol) in an anhydrous mixture of dichloromethane (6 ml) and anisole (24 ml) in 52 % yield as a solid: IR (Nujol): 1764 cm^{-1}; ^1H NMR (DMSO-d_6), δ: 1.38 (3H, s, 2α-C$\underline{H}_3$), 1.44 (3H, s, 2β-C$\underline{H}_3$), 3.78 (H, s, C3-$\underline{H}$), 3.86 (3H, s, NC$\underline{H}_3$), 4.21 (2H, t, J=6.4 Hz, SC$\underline{H}_2$), 4.84 (2H, t, J=6.4 Hz, C$\underline{H}_2$N) , 5.69 (H, d, J=0.9 Hz, C5-$\underline{H}$), 7.09 (H, s, imidazole-C4-$\underline{H}$), 7.32 (H, d, J=0.9 Hz, C$\underline{H}$=C6), 7.44 (H, s, imidazole-C5-$\underline{H}$), 8.33 (H, s, triazole-$\underline{H}$); FAB-MS *m/z* 507 (M+H).

Sodium (3S,5R)-6(E)-[1-{1-(2-{1-methylimidazole-2-yl}sulfonylethyl)-1,2,3-triazole-4-yl}methylene]penicillanate-1,1-dioxide (114)

Using the procedure A described for compound (**101**), compound (**114**) was prepared from p-methoxybenzyl (3S,5R)-6(E)-[1-{1-(2-{1-methyl imidazole-2-yl}sulfonylethyl)-1,2,3-triazole-4-yl}methylene] penicillanate-1,1-dioxide (**110**, 0.40 g, 0.66 mmol) and anhydrous aluminum chloride (0.22 g, 1.65 mmol) in an anhydrous mixture of dichloromethane (25 ml) and anisole (25 ml) in 39 % yield as a solid: IR (Nujol): 1760 cm^{-1}; ^{1}H NMR (DMSO-d_6), δ: 1.38 (3H, s, 2α-C$\underline{H}_3$), 1.46 (3H, s, 2β-C$\underline{H}_3$), 3.83 (H, s, C3-$\underline{H}$) ,3.87 (3H, s, NC$\underline{H}_3$), 4.21 (2H, t, J=6.2 Hz, SC$\underline{H}_2$), 4.90 (2H, t, J=6.2 Hz, C$\underline{H}_2$N), 5.54 (H, s, C5-$\underline{H}$), 7.05 (2H, s, C$\underline{H}$=C6 and imidazole-C4-$\underline{H}$), 7.44 (H, s, imidazole-C5-$\underline{H}$), 8.71 (H, s, triazole-$\underline{H}$); FAB-MS *m/z* 507 (M+H).

5.2 Experiments of Elastase Inhibitors

(6R,7R)-7-Amino-3-azidomethyl-3-cephem-4-carboxylic acid (115)

This compound was synthesized using similar method of the reference (Montavon and Reiner, 1988). To (6R,7R)-7-amino-3-acetoxymethyl-3-cephem-4-carboxylic acid (54.46 g, 0.20 mol), water (1000ml) and sodium bicarbonate (18.48 g, 0.22 mol) were added. The mixture was stirred at room temperature for 1 hr. The pH was adjusted to pH 6.5 with 10 % sodium hydroxide solution. Sodium azide (95 % , 27.37g, 0.40 mol) was added to the reaction mixture followed by acetone (700 ml), then heated to 60°C for 6.5 hrs. and kept stirring overnight at room temperature. The solvent was removed under reduced pressure. The reaction mixture was cooled in an ice bath and acidified to pH 2.0 with concentrated hydrochloric acid solution. The solid was filtered and washed with distilled water, then dried over anhydrous P_2O_5 overnight to give the compound (**115**) (31.56 g, 62 %) as a solid: m.p. (dec.) : 207 ~ 210°C; IR (Nujol): 2090, 1793 cm^{-1}; ^1H NMR (DMSO-d_6), δ: 2.03 (2H, s, $\underline{H}_2N$), 3.42 (H, d, J=18.1 Hz, C2β-$\underline{H}$), 3.63 (H, d, J=18.1 Hz, C2α-$\underline{H}$), 3.89 and 4.37 (2H, two d, J=13.3 Hz, C3-C$\underline{H}_2$), 4.81 (H, d, J=5.1 Hz, C7-$\underline{H}$), 4.99 (H, d, J=5.1 Hz, C6-$\underline{H}$). The spectral data agreed with the assigned structure.

Diphenylmethyl (6R,7R)-7-amino-3-azidomethyl-3-cephem-4-carboxylate (116) and Diphenylmethyl (4R,6R,7R)-7-amino-3-azidomethyl-2-cephem-4-carboxylate (117)

To a solution of (6R,7R)-7-amino-3-azidomethyl-3-cephem-4-carboxylic acid (**115**, 5.00 g, 19.59 mmol) in dimethyl sulfoxide (90 ml), dichloromethane (120 ml) and diphenyldiazomethane (5.33 g, 27.44 mmol) were added at room temperature. The reaction mixture was stirred at room temperature for 88 hrs. and water was added. The organic layer was separated and the water layer was extracted with dichloromethane. The combined organic layer was washed with brine, dried over anhydrous sodium sulfate, and concentrated. The residue was purified on a silica gel column using dichloromethane : ethyl acetate (4:1 v/v) as the eluant to give the compound (**116**) (0.79 g, 10 %) and compound (**117**) (2.67 g, 32 %) as solids. Compound (**116**): m.p.: 158 ~ 160°C; IR ($CHCl_3$): 2110, 1780, 1727 cm^{-1}; ^1H NMR ($CDCl_3$), δ: 1.79 (2H, br. d, J=8.5 Hz , $\underline{H}_2N$), 3.41 (H, d, J=18.4 Hz, C2β-$\underline{H}$), 3.57 (H, d, J=18.4 Hz, C2α-$\underline{H}$), 3.96 and 4.23 (2H, two d, J=13.9 Hz, C3-$C\underline{H}_2$), 4.87 ~ 4.72 (H, m, C7-$\underline{H}$), 4.96 (H, d, J=5.2 Hz, C6-$\underline{H}$), 6.98 (H, s, $CO_2C\underline{H}$), 7.29 ~ 7.45 (10H, m, $\underline{Ph}_2$). The compound (**116**) was previously reported (Heymes and Lutz, 1979) and spectral data agreed with the assigned structure. Compound (**117**): m.p.: 103 ~ 105°C; IR ($CHCl_3$): 2110, 1768, 1746 cm^{-1}; ^1H NMR ($CDCl_3$), δ: 1.82 (2H, br. s, $N\underline{H}_2$), 3.85 (2H, s, C3-$C\underline{H}_2$), 4.60 (H, m, C7-$\underline{H}$), 5.10 (H, d, J=1.3 Hz, C4-$\underline{H}$), 5.18 (H, d, J=4.1, C6-$\underline{H}$), 6.38 (H, d, J=1.3 Hz, C2-

$\underline{H}$), 6.89 (H, s, $CO_2C\underline{H}$), 7.34 ~ 7.40 (10H, m, $\underline{Ph}_2$); FAB-MS *m/z* 422 (M+H).

Diphenylmethyl (6R,7S)-3-azidomethyl-7-chloro-3-cephem-4-carboxylate (118)

To a stirred solution of diphenylmethyl (6R,7R)-7-amino-3-azidomethyl-3-cephem-4-carboxylate (**116**, 0.71 g, 1.68 mmol) in $CHCl_3$ (20 ml), ethanol (95 %, 3.5 ml), water (2.7 ml) and concentrated hydrochloric acid solution (2.7 ml) were added. To the reaction mixture, sodium nitrite (0.16 g, 2.32 mmol) was added portionwise at 0°C. The reaction mixture was stirred at 0°C for 3.5 hrs. and poured into a saturated sodium chloride solution. The mixture was extracted with $CHCl_3$. The combined organic layer was washed with brine, dried over anhydrous sodium sulfate, and concentrated. The residue was chromatographed on a silica gel column using hexane : ethyl acetate (2:1 v/v) as the eluant to give the compound (**118**) (0.46 g, 62 %) as a sticky foam: IR ($CHCl_3$): 2110, 1798, 1730 cm^{-1}; ^1H NMR ($CDCl_3$), δ: 3.35 (H, d, J=18.1 Hz, $C2\beta$-$\underline{H}$), 3.51 (H, d, J=18.1 Hz, $C2\alpha$-$\underline{H}$), 3.92 and 4.18 (2H, two d, J=14.1 Hz, C3-$C\underline{H}_2$), 4.69 (H, d, J=1.3 Hz, C6-$\underline{H}$), 4.76(H, d, J=1.3 Hz, C7-$\underline{H}$), 6.97(H, s, $CO_2C\underline{H}$), 7.22 ~ 7.46 (10H, m, $\underline{Ph}_2$); FAB-MS *m/z* 441 (M+H).

Diphenylmethyl (4R,6R,7S)-3-azidomethyl-7-chloro-2-cephem-4-carboxylate (119)

Using a similar method as described for compound (**118**), compound (**119**) was prepared from the diphenylmethyl (4R,6R,7R)-7-amino-3-azidomethyl-2-cephem-4-carboxylate (**117**, 1.00 g, 0.24 mmol), sodium nitrite (0.23 g, 3.33 mmol), and concentrated hydrochloric acid solution (3.73 ml) in 34 % yield as a sticky foam: IR ($CHCl_3$): 2110, 1788, 1745 cm^{-1}; ^1H NMR ($CDCl_3$), δ: 3.87 (2H, s, C3-C$\underline{H}_2$), 4.77 (H, br. s, C6-$\underline{H}$), 5.03 (H, br. s, C7-$\underline{H}$), 5.14 (H, d, J=0.9 Hz, C4-$\underline{H}$), 6.30 (H, d, J=0.9 Hz, C2-$\underline{H}$) 6.90 (H, s, $CO_2C\underline{H}$), 7.25 ~ 7.43 (10H, m, $\underline{Ph}_2$); FAB-MS *m/z* 441 (M+H).

Diphenylmethyl (6R,7S)-3-azidomethyl-7-chloro-3-cephem-4-carboxylate-1,1-dioxide (120)

Peracetic acid (32 %, 0.28 g, 3.68 mmol) was added dropwise to a stirred solution of diphenylmethyl (6R,7S)-3-azidomethyl-7-chloro-3-cephem-4-carboxylate (**118**, 0.18 g, 0.41 mmol) in dichloromethane (7 ml) at 0°C and stirred at room temperature for 48 hrs. The reaction mixture was washed with water, 5 % $NaHCO_3$ solution, and brine. The organic layer was dried over anhydrous sodium sulfate and concentrated. The residue was purified on a silica gel column using hexane : ethyl acetate (2:1 v/v) as the eluant to give the compound (**120**) (0.12 g, 62 %) as a solid: m.p.: 131 ~ 133°C; IR ($CHCl_3$): 2115, 1815, 1730, 1343 cm^{-1}; ^1H

NMR ($CDCl_3$), δ: 3.81 (H, d, J=18.1 Hz, C2β-H), 4.03 (H, d, J=18.1 Hz, C2α-H), 4.15 and 4.27 (2H, two d, J=14.9 Hz, C3-CH_2), 4.79 (H, m, C6-H), 5.33 (H, d, J=1.7 Hz, C7-H), 6.96(H, s, CO_2CH), 7.31 ~ 7.45 (10H, m, Ph_2); FAB-MS *m/z* 473 (M+H).

Using a similar procedure, compound **(120)** was also prepared from diphenylmethyl(4R,6R,7S)-3-azidomethyl-7-chloro-2-cephem-4-carboxylate (**119**, 0.23 g, 0.52 mmol) and peracetic acid (32 %, 0.36 g, 4.70 mmol) in 53 % yield.

Diphenylmethyl (6R,7S)-7-chloro-3-(1,2,3-triazole-1-yl)methyl-3-cephem-4-carboxylate-1,1-dioxide (121)

A solution of diphenylmethyl (6R,7S)-3-azidomethyl-7-chloro-3-cephem-4-carboxylate-1,1-dioxide (**120**, 6.20 g, 13.11 mmol) in dimethoxyethane (30 ml) was transferred to a steel-bomb and weighted the mass. The solution was flushed with nitrogen gas at - 78°C and the nitrogen gas inlet tube was replaced with an acetylene gas inlet tube. The acetylene gas was passed into the reaction mixture at - 78°C for 20 minutes. The steel-bomb was removed from the dry ice bath and weighted the total mass. Acetylene (10.80 g, 414.81 mmol) was transferred to the reaction vessel. The steel-bomb was closed and heated at 85 ~ 90°C for 21 hrs. After cooling to room temperature, the reaction mixture was washed with water and brine. The organic layer was dried over anhydrous sodium sulfate and concentrated. The residue was chromatographed on a silica gel column eluting with hexane : ethyl acetate (1:2 v/v) as eluant to give compound **(121)** (3.83 g, 59 %) as a solid: m.p.:91 ~ 93°C; IR ($CHCl_3$): 1815, 1730, 1343 cm^{-1}; ^1H NMR ($CDCl_3$), δ: 3.76 (H, d, J=18.6 Hz, C2β-H), 4.01 (H, d, J=18.6 Hz, C2α-H), 4.84 (H, m, C6-H), 5.24 (2H, s, C3-CH_2), 5.31 (H, d, J=1.5 Hz, C7-H), 6.99 (H, s, CO_2CH), 7.31 ~ 7.46 (10H, m, Ph_2), 7.51 and 7.66 (2H, two d, J=0.8 Hz, triazole-H); FAB-MS *m/z* 499 (M+H).

Diphenylmethyl (6R,7S)-7-chloro-2-methylene-3-(1,2,3-triazole-1-yl) methyl-3-cephem-4-carboxylate-1,1-dioxide (122)

[General Procedure B]

To a stirred solution of diphenylmethyl (6R,7S)-7-chloro-3-(1,2,3-triazole-1-yl)methyl-3-cephem-4-carboxylate-1,1-dioxide (**121**, 3.00 g, 6.01 mmol) in dioxane (28.5 ml), dimethylamine-HCl (1.47 g, 18.04 mmol) and t-BuOH (7.1 ml)were added and stirred for 20 minutes at room temperature. Formaldehyde (37 %, 1.7 ml, 20.95 mmol) was added to the reaction mixture and heated at 60 ~ 65°C for 2 hrs. The reaction mixture was concentrated and the residue was dissolved in dichloromethane. The mixture was washed with water and brine. The organic layer was dried over anhydrous sodium sulfate and concentrated. The residue was purified on a silica gel column using dichloromethane : ethyl acetate (5 : 1 v/v) as the eluant to give compound **(122)** (1.90 g, 62 %) as a solid: m.p.:158 ~ 160°C; IR ($CHCl_3$): 1811, 1724, 1335 cm^{-1}; ^1H NMR ($CDCl_3$), δ: 4.96 (H, d, J=1.8 Hz, C6-H), 5.13 and 5.55 (2H, two d, J=15.3 Hz, C3-CH_2) 5.41 (H, d, J=1.8 Hz, C7-H), 6.76 (2H, s, C2 =CH_2), 7.05(H, s, CO_2CH), 7.30 ~ 7.42 (10H, m, Ph2), 7.50 and 7.59 (2H, two d, J=0.9 Hz, triazole-H); FAB-MS *m/z* 511 (M+H).

Diphenylmethyl (2R,6R,7S)-7-chloro-2-spiro(2,2-diphenyl) cyclopropyl-3-(1,2,3-triazole-1-yl)methyl-3-cephem-4-carboxylate-1,1-dioxide (123)

[General Procedure C]

Diphenyldiazomethane (1.00 g, 5.15 mmol) was added portionwise to a solution of diphenylmethyl (6R,7S)-7-chloro-2-methylene-3-(1,2,3-triazole-1-yl)methyl-3-cephem-4-carboxylate-1,1-dioxide (**122**, 1.60 g, 3.13 mmol) in dichloromethane (40 ml) and stirred at room temperature for 1 hour. The reaction mixture was concentrated and the residue was dissolved in ethyl acetate. Hexane was added to the mixture to precipitate the crude product. The solid was purified on a silica gel column using hexane : ethyl acetate (1:1v/v) as the eluant to give the compound (**123**) (0.67 g, 32 %) as a solid: m.p.:147 ~ 149°C; IR ($CHCl_3$): 1814, 1732, 1343 cm^{-1}; ^1H NMR ($CDCl_3$), δ: 2.63 and 2.81 (2H, two d, J=7.8 Hz, cyclopropyl-H_2), 3.51 and 4.11 (2H, two d, J=15.4 Hz, C3-CH_2), 5.08 (H, d, J=2.1 Hz, C6-H), 5.28 (H, d, J=2.1 Hz, C7-H), 7.07 (H, s, CO_2CH), 7.23 ~ 7.56 (20H, m, Ph_4), 7.65 and 7.91 (two H, two d, J=0.9 Hz, two triazole-H); FAB-MS *m/z* 677(M+H).

Diphenylmethyl (6R,7S)-7-chloro-3-(4-ethoxycarbonyl-1,2,3-triazole-1-yl)methyl-3-cephem-4-carboxylate-1,1-dioxide (124) and Diphenylmethyl (6R,7S)-7-chloro-3-(5-ethoxycarbonyl-1,2,3-triazole-1-yl)methyl-3-cephem-4-carboxylate-1,1-dioxide (127)

To a stirred solution of diphenylmethyl (6R,7S)-3-azidomethyl-7-chloro-3-cephem-4-carboxylate-1,1-dioxide (**120**, 1.80 g, 3.81 mmol) in dry benzene (18 ml) in a pressure tube, ethylpropiolate (0.75 g, 7.57 mmol) was added and heated to 80 ~ 90°C for 21 hrs. under a nitrogen atmosphere. The reaction mixture was concentrated and the residue was dissolved in dichloromethane. The mixture was washed with water and brine. The organic layer was dried over anhydrous sodium sulfate and concentrated. The residue was chromatographed on a silica gel column using dichloromethane : ethyl acetate (10:1 v/v) as the eluant to give the compound (**124**) (1.21 g, 56 %) and compound (**127**) (0.74 g, 34 %) as solids. Compound (**124**): m.p.:98 ~ 101°C; IR (CHCl$_3$). 1822, 1730, 1347 cm^{-1}; ^{1}H NMR (CDCl$_3$), δ: 1.37 (3H, t, J=7.1 Hz, CH$_3$), 3.83 (H, d, J=18.2 Hz, C2β-H), 4.13 (H, d, J=18.2 Hz, C2α-H), 4.37 (2H, q, J=7.1 Hz, CH$_2$), 4.95 (H, m, C6-H), 5.24 (2H, s, C3-CH$_2$), 5.30 (H, d, J=1.6 Hz, C7-H), 6.98 (H, s, CO$_2$CH), 7.28 ~ 7.52 (10H, m, Ph$_2$), 8.11 (H, s, triazole-H); FAB-MS *m/z* 571 (M+H). And compound (**127**): m.p.:84 ~ 86 °C; IR (CHCl$_3$): 1816, 1728, 1345 cm^{-1}; ^{1}H NMR (CDCl$_3$), δ: 1.35 (3H, t, J=6.8 Hz, CH$_3$), 3.46 (H, d, J=17.9 Hz, C2β-H), 3.73 (H, d, J=17.9 Hz, C2α-H), 4.28 (2H, q, J=6.8 Hz, CH$_2$), 4.74 (H, m, C6-H), 5.31 (H, d, J=1.5 Hz, C7-H), 5.49 and 6.13 (2H, two d, J=15.9 Hz, C3-CH$_2$), 7.00

(H, s, $CO_2C\underline{H}$), 7.30 ~ 7.49 (10H, m, $\underline{Ph}_2$), 8.13 (H, s, triazole-$\underline{H}$); FAB-MS *m/z* 571 (M+H).

Diphenylmethyl (6R,7S)-7-chloro-2-methylene-3-(4-ethoxy carbonyl-1,2,3-triazole-1-yl)methyl-3-cephem-4-carboxylate-1,1-dioxide (125)

According to the procedure B described for compound (**122**), compound (**125**) was prepared from diphenylmethyl (6R,7S)-7-chloro-3-(4-ethoxy carbonyl-1,2,3-triazole-1-yl)methyl-3-cephem-4-carboxylate-1,1-dioxide (**124**, 0.16 g, 0.28 mmol), dioxane (1.8 ml), dimethylamine-HCl (0.07 g, 0.86 mmol), t-BuOH (0.45 ml), and formaldehyde (37 %, 0.15 ml, 1.85 mmol) in 55 % yield as a solid: m.p.:96 ~ 98°C; IR ($CHCl_3$), 1823, 1731, 1340 cm^{-1}; ^1H NMR ($CDCl_3$): δ: 1.39 (3H, t, J=7.1 Hz, $C\underline{H}_3$), 4.39 (2H, q, J=7.1 Hz, $C\underline{H}_2$), 5.00 (H, d, J=1.7 Hz, C6-$\underline{H}$), 5.42 (H, d, J=1.7 Hz, C7-$\underline{H}$), 5.18 and 5.49 (2H, two d, J=15.3 Hz, C3-$C\underline{H}_2$), 6.65 and 6.73 (2H, two d, J=3.0 Hz, C2 =$C\underline{H}_2$), 7.03 (H, s, $CO_2C\underline{H}$), 7.28 ~ 7.45 (10H, m, $\underline{Ph}_2$), 8.22 (H, s, triazole-$\underline{H}$); FAB-MS *m/z* 583 (M+H).

Diphenylmethyl (2R,6R,7S)-7-chloro-2-spiro(2,2-diphenyl) cyclopropyl-3-(4-ethoxycarbonyl-1,2,3-triazole-1-yl)methyl-3-cephem-4-carboxylate-1,1-dioxide (126)

Using the procedure C described for compound (**123**), compound (**126**) was obtained from diphenylmethyl (6R,7S)-7-chloro-2-methylene-3-(4-

ethoxycarbonyl-1,2,3-triazole-1-yl)methyl-3-cephem-4-carboxylate-1,1-dioxide (**125**, 0.09 g, 0.15 mmol) and diphenyldiazomethane (0.11 g, 0.57 mmol) in 61 % yield as a solid: m.p.:138 ~ 140°C; IR ($CHCl_3$): 1818, 1733, 1344 cm^{-1}; ^1H NMR ($CDCl_3$), δ: 1.37 (3H, t, J=7.2 Hz, $C\underline{H}_3$), 2.65 and 2.85 (2H, two d, J=7.9 Hz, cyclopropyl-$\underline{H}_2$), 3.52 and 4.12 (2H, two d, J=15.4 Hz, C3-$C\underline{H}_2$), 4.51 (2H, m, $C\underline{H}_2$), 5.09 (H, d, J=2.1 Hz, C6-$\underline{H}$), 5.29 (H, d, J=2.1 Hz, C7-$\underline{H}$), 7.07 (H, s, $CO_2C\underline{H}$), 7.25 ~ 7.50 (20H, m, $\underline{Ph}_4$), 8.40 (H, s, triazole-$\underline{H}$); FAB-MS *m/z* 749 (M+H).

Diphenylmethyl (6R,7S)-7-chloro-2-methylene-3-(5-ethoxy carbonyl-1,2,3-triazole-1-yl)methyl-3-cephem-4-carboxylate-1,1-dioxide (128)

Using the procedure B described for compound (**122**), compound (**128**) was prepared from diphenylmethyl (6R,7S)-7-chloro-3-(5-ethoxycarbonyl-1,2,3-triazole-1-yl)methyl-3-cephem-4-carboxylate-1,1-dioxide (**127**, 0.10 g, 0.18 mmol), dioxane (1.8 ml), dimethylamine-HCl (0.04 g, 0.53 mmol), t-BuOH (0.45 ml), and formaldehyde (37 %, 0.05 ml, 0.62 mmol) in 59 % yield as a solid: m.p.:80 ~ 82°C; IR ($CHCl_3$): 1821, 1730, 1337 cm^{-1}; ^1H NMR ($CDCl_3$), δ:1.37 (3H, t, J=7.1 Hz, $C\underline{H}_3$), 4.33 (2H, q, J=7.1 Hz, $C\underline{H}_2$), 4.99 (H, d, J=1.7 Hz, C6-$\underline{H}$), 5.40 (H, d, J=1.7 Hz, C7-$\underline{H}$), 5.59 and 6.10 (2H, two d, J=15.5 Hz, C3-$C\underline{H}_2$), 6.24 and 6.63 (2H, two d, J=2.8 Hz, C2 =$C\underline{H}_2$), 6.93 (H, s, $CO_2C\underline{H}$), 7.27 ~ 7.40 (10H, m, $\underline{Ph}_2$), 8.08 (H, s, triazole-$\underline{H}$); FAB-MS *m/z* 583 (M+H).

Diphenylmethyl (2R,6R,7S)-7-chloro-2-spiro(2,2-diphenyl)cyclopropyl-3-(5-ethoxycarbonyl-1,2,3-triazole-1-yl)methyl-3-cephem-4-carboxylate-1,1-dioxide (129)

According to the procedure C described for compound (**123**), compound (**129**) was obtained from diphenylmethyl (6R,7S)-7-chloro-2-methylene-3-(5-ethoxycarbonyl-1,2,3-triazole-1-yl)methyl-3-cephem-4-carboxylate-1,1-dioxide (**128**, 0.06 g, 0.10 mmol) and diphenyl diazomethane (0.15 g, 0.77 mmol) in 65 % yield as a solid: m.p.:122 ~ 124°C; IR ($CHCl_3$): 1814, 1731, 1344 cm^{-1}; ^1H NMR ($CDCl_3$), δ: 1.25 (3H, t, J=7.1 Hz, $C\underline{H}_3$), 2.44 and 3.06 (2H, two d, J=7.7 Hz, cyclopropyl-$\underline{H}_2$), 3.76 and 4.75 (2H, two d, J=15.7 Hz, C3-$C\underline{H}_2$), 4.0[illegible] 4.23 (2H, m, $C\underline{H}_2$), 5.02 (H, d, J=2.0 Hz, C6-$\underline{H}$), 5.27 (H, d, J=2.0 Hz, C7-$\underline{H}$), 6.96 (H, s, $CO_2C\underline{H}$), 7.15 ~ 7.55 (20H, m, $\underline{Ph}_4$), 8.01 (H, s, triazole-$\underline{H}$); FAB-MS *m/z* 749 (M+H).

Diphenylmethyl (6R,7S)-7-chloro-3-(4,5-dimethoxycarbonyl-1,2,3-triazole-1-yl)methyl-3-cephem-4-carboxylate-1,1-dioxide (130)

Utilizing a similar method for compound (**124**), compound (**130**) was prepared from diphenylmethyl (6R,7S)-3-azidomethyl-7-chloro-3-cephem-4-carboxylate-1,1-dioxide (**120**, 1.50 g, 3.17 mmol) and dimethylacetylene dicarboxylate (1.13 g, 7.87 mmol) in 85 % yield as a solid: m.p.:96 ~ 98° C; IR ($CHCl_3$): 1817, 1730, 1346 cm^{-1}; ^1H NMR ($CDCl_3$), δ: 3.78 (H, d, J=19.3 Hz, C2β-$\underline{H}$), 3.98 (H, d, J=19.3 Hz, C2α-$\underline{H}$), 3.92 and 4.04 (two

3H, two s, two triazole-$CO_2C\underline{H}_3$), 4.86 (H, m, C6-$\underline{H}$), 5.38 (H, d, J=1.6 Hz, C7-$\underline{H}$), 5.47 and 5.99 (2H, two d,J=15.8 Hz, C3-$C\underline{H}_2$), 7.03 (H, s, $CO_2C\underline{H}$), 7.25 ~ 7.55 (10H, m, $\underline{Ph}_2$); FAB-MS *m/z* 615 (M+H).

Diphenylmethyl (6R,7S)-7-chloro-2-methylene-3-(4,5-dimethoxy carbonyl-1,2,3-triazole-1-yl)methyl-3-cephem-4-carboxylate-1,1-dioxide (131)

Using the procedure B described for compound (**122**), compound (**131**) was obtained from diphenylmethyl (6R,7S)-7-chloro-3-(4,5-dimethoxy carbonyl-1,2,3-triazole-1-yl)methyl-3-cephem-4-carboxylate-1,1-dioxide (**130**, 0.22 g, 0.36 mmol), dioxane (1.8 ml), dimethylamine-HCl (0.09 g, 1.07 mmol), t-BuOH (0.45 ml), and formaldehyde (37 %, 0.16 ml, 1.97 mmol) in 45 % yield as a solid: m.p.:93 ~ 95°C; IR ($CHCl_3$): 1818, 1731, 1340 cm^{-1}; ^1H NMR ($CDCl_3$), δ: 3.91 and 3.98 (two 3H, two s, two triazole-$CO_2C\underline{H}_3$), 5.00 (H, d, J=1.7 Hz, C6-$\underline{H}$), 5.40 (H, d, J=1.7 Hz, C7-$\underline{H}$), 5.45 and 5.98 (2H, two d, J=15.4 Hz, C3-$C\underline{H}_2$), 6.34 and 6.71 (2H, two d, J=2.9 Hz, C2 =$C\underline{H}_2$), 6.91 (H, s, $CO_2C\underline{H}$), 7.29 ~ 7.40 (10H, m, $\underline{Ph}_2$); FAB-MS *m/z* 627 (M+H).

Diphenylmethyl (2R,6R,7S)-7-chloro-2-spiro(2,2-diphenyl) cyclopropyl-3-(4,5-dimethoxycarbonyl-1,2,3-triazole-1-yl)methyl-3-cephem-4-carboxylate-1,1-dioxide (132)

With the procedure C described for compound (**123**), compound (**132**) was prepared from the diphenylmethyl (6R,7S)-7-chloro-2-methylene-3-(4,5-dimethoxycarbonyl-1,2,3-triazole-1-yl)methyl-3-cephem-4-carboxylate-1,1-dioxide (**131**, 0.12 g, 0.19 mmol) and diphenyl diazomethane (0.16 g, 0.82 mmol) in 53 % yield as a solid: m.p.:132 ~ 134°C; IR ($CHCl_3$): 1815, 1731, 1345, cm^{-1}; ^1H NMR ($CDCl_3$), δ: 2.74 and 3.17 (2H, two d, J=8.1 Hz, cyclopropyl-$\underline{H}_2$), 3.51 and 4.61 (2H, two d, J=15.6 Hz, C3-$C\underline{H}_2$), 3.68 and 3.92 (two 3H, two s, two triazole-$CO_2C\underline{H}_3$), 5.02 (H, d, J=2.0 Hz, C6-$\underline{H}$), 5.26 (H, d, J=2.0 Hz, C7-$\underline{H}$), 6.94 (H, s, $CO_2C\underline{H}$), 7.28 ~ 7.49 (20H, m, $\underline{Ph}_4$); FAB-MS *m/z* 793 (M+H).

Diphenylmethyl (6R,7S)-7-chloro-3-(4-carboxaldehyde-1,2,3-triazole-1-yl)methyl-3-cephem-4-carboxylate-1,1-dioxide (133)

Propargyl aldehyde (0.29 g, 5.37 mmol) was added to a solution of diphenylmethyl (6R,7S)-3-azidomethyl-7-chloro-3-cephem-4-carboxylate-1,1-dioxide (**120**, 2.13 g, 4.50 mmol) in dry benzene (20 ml) in a pressure bottle and stirred at room temperature for 2 days under a nitrogen atmosphere. The reaction mixture was concentrated and the residue was dissolved in dichloromethane. The mixture was washed with water and brine. The organic layer was dried over anhydrous sodium sulfate and concentrated. The residue was purified on a silica gel column using hexane : ethyl acetate (2:1 v/v) as the eluant to give 0.95 g of the unreacted compound (**120**) and the compound (**133**) (0.55 g, 42 %) as a solid: m.p.:102 ~ 105°C; IR ($CHCl_3$): 1816, 1728, 1704, 1346 cm^{-1}; ^1H NMR ($CDCl_3$), δ: 3.91 (H, d, J=18.0 Hz, C2β-$\underline{H}$), 4.10 (H, d, J=18.0 Hz, C2α-$\underline{H}$), 4.84 (H, m, C6-$\underline{H}$), 5.24 and 5.25 (2H, two s, C3-C$\underline{H}_2$), 5.33 (H, d, J=1.8 Hz, C7-$\underline{H}$), 6.98 (H, s, $CO_2C\underline{H}$), 7.31 ~ 7.45 (10H, m, $\underline{Ph}_2$), 8.14 (H, s, triazole-$\underline{H}$), 10.10 (H, s, formyl-$\underline{H}$); FAB-MS *m/z* 527 (M+H).

Diphenylmethyl (6R,7S)-7-chloro-2-methylene-3-(4-carbox aldehyde-1,2,3-triazole-1-yl)methyl-3-cephem-4-carboxylate-1,1-dioxide (134)

According to the procedure B described for compound (**122**), compound (**134**) was prepared from diphenylmethyl (6R,7S)-7-chloro-3-(4-carbox aldehyde-1,2,3-triazole-1-yl)methyl-3-cephem-4-carboxylate-1,1-dioxide (**133**, 1.18 g, 2.24 mmol), dioxane (10 ml), dimethylamine-HCl (0.55 g, 6.68 mmol), t-BuOH (2 ml), and formaldehyde (37 %, 0.64 ml, 7.89 mmol) in 33 % yield as a solid. The product was purified by recrystalization with hexane and ethyl acetate : m.p.:119 ~ 121°C; IR ($CHCl_3$): 1817, 1732, 1700, 1340 cm^{-1}; ^1H NMR ($CDCl_3$), δ: 4.97 (H, d, J=1.8 Hz, C6-$\underline{H}$), 5.19 and 5.55 (2H, two d, J=15.2 Hz, C3-C$\underline{H}_2$), 5.42 (H, d, J=1.8 Hz, C7-$\underline{H}$), 6.72 and 6.80 (2H, two d, J=3.0 Hz, C2 =C$\underline{H}_2$), 7.03 (H, s, $CO_2C\underline{H}$), 7.24 ~ 7.52 (10H, m, $\underline{Ph}_2$), 8.22 (H, s, triazole-$\underline{H}$), 10.06 (H, s, formyl-$\underline{H}$); FAB-MS *m/z* 539 (M+H).

Diphenylmethyl (2R,6R,7S)-7-chloro-2-spiro(2,2-diphenyl) cyclopropyl-3-(4-carboxaldehyde-1,2,3-triazole-1-yl)methyl-3-cephem -4-carboxylate-1,1-dioxide (135)

Using the procedure C described for compound (**123**), compound (**135**) was obtained from diphenylmethyl (6R,7S)-7-chloro-2-methylene-3-(4-carboxaldehyde-1,2,3-triazole-1-yl)methyl-3-cephem-4-carboxylate-1,1-dioxide (**134**, 0.39 g, 0.72 mmol) and diphenyldiazomethane (0.28 g, 1.44 mmol) in 47 % yield as a solid: m.p.:140 ~ 142°C; IR ($CHCl_3$): 1813, 1733, 1706, 1345, cm^{-1}; ^1H NMR ($CDCl_3$) δ: 2.66 and 2.89 (2H, two d, J=7.2 Hz, cyclopropyl-H_2), 3.52 and 4.15 (2H, two d, J=15.3 Hz, C3-CH_2), 5.08 (H, d, J=1.9 Hz, C6-H), 5.29 (H, d, J=1.9 Hz, C7-H), 7.07 (H, s, CO_2CH), 7.26 ~ 7.55 (20H, m, Ph_4), 8.47 (H, s, triazole-H), 10.08 (H, s, formyl-H); FAB-MS *m/z* 705 (M+H).

5.3 Biological section

5.3.1 *In Vitro* β-Lactamase Inhibitory Assays

The following three enzymes were used for testing β-lactamase inhibitory activities: Penicillinase (penicillinase class II from *Bacillus cereus*. purchased from Sigma); Cephalosporinase (penicillinase class I from *Enterobacter cloacae*, purchased from Sigma); Broad spectrum TEM enzyme (β-lactamase class IIIa from *Escherichia coli*, purchased from Boehringer). The inhibitory potency was determined as the concentration of the test compound (IC_{50}, μ molar) required to give 50 % inhibition of the enzyme.

5.3.1.1 Penicillinase and Cephalosporinase

The IC_{50} values were determined by a spectrophotometric method as follows; 2.875 ml of phosphate buffer solution (20 mmol KH_2PO_4 and 30 mmol Na_2HPO_4; pH 7.0), 0.015 ml of enzyme in phosphate buffer solution, and 0.050 ml of inhibitor in phosphate buffer solution were added to a 3 ml cuvette (quartz). The mixture was preincubated for 10 minutes at 30°C, then added 0.060 ml of substrate in phosphate buffer solution to start enzymatic reaction. The decrease in absorbance (A/min.) at 233 nm (for penicillin G) or 260 nm (for cephaloridine) was measured for 130 seconds. The data obtained between 10 and 130 seconds were used for the calculation. The inhibitors were added in a certain range of concentrations (x). For a control, 0.050 ml of phosphate buffer solution was added instead of inhibitor solution. The % inhibition (I) was calculated by the following equation;

$$I\ (\%) = \frac{Ao - Ax}{Ao} \times 100$$

where Ao is the rate of decrease in absorption at the inhibition concentration zero and Ax is the rate of decrease in absorption at the inhibition concentration (x)

The % Inhibition (I) was plotted against inhibitor concentration (x) and the IC_{50} values were determined from the resulting calibration curve.

5.3.1.2 Broad spectrum TEM enzyme

The procedure for TEM enzyme was the same as that for penicillinase and cephalosporinase except for the followings; The 2.880 ml of phosphate buffer solution was used instead of 2.875 ml and 0.100 ml of enzyme in phosphate buffer solution was used instead of 0.015 ml.

Table 5 - 1 The amount of enzyme and substrate for each assay

Enzyme	Amount	Substrate	Amount
Penicillinase	0.2 Unit*	Penicillin G	0.071 mg
Cephalosporinase	0.003 Unit*	Cephaloridine	0.042mg
TEM enzyme	0.2 Unit*	Penicillin G	0.071 mg

* 1 Unit of the enzyme hydrolyzes 1 μ mol of substrate per minute in pH 7.0 at 25°C.

5.3.2 *In Vitro* Human leukocyte elastase Inhibitory Assays

The human leukocyte elastase (HLE) from white blood cells was used for testing elastase inhibitory activities. The inhibitory potency was determined as the concentration of the test compound (IC_{50}, n molar) required to give 50 % inhibition of 1 μg/ml of HLE. The enzyme was purchased from Athens Research and Technology Company. MeO-Succinyl-Ala-Ala-Pro-Val-pNa (pNa : p-nitroanilide) was used as a substrate and purchased from Sigma.

The IC_{50} values were determined by a spectrophotometric method as follows; The enzyme was dissolved in 50 mmol sodium acetate solution containing 400 mmol sodium chloride (pH 5.3) and the concentration of enzyme was adjusted to 0.001 m molar (6.072 units/ml). The substrate was dissolved in 10 % DMSO and the concentration was adjusted to 14 m molar.

0.075 ml of inhibitor solution in 10 % DMSO, 0.500 ml of phosphate buffer solution (0.45 mmol KH_2PO_4, 10.8 mmol $Na_2HPO_4 \cdot 12\ H_2O$, and 562 mmol NaCl; pH 7.6), 0.390 ml of distilled water, and 0.010 ml of enzyme solution were added to a cuvette (quartz). The mixture was preincubated for 10 minutes at 30°C, then added 0.025 ml of substrate solution which was also prewarmed at 30°C. The increase in absorbance at 410 nm was caused by the hydrolytic release of p-nitroanilide from synthetic substrate and monitored for 130 seconds. The data obtained between 10 and 130 seconds were used for the calculation. The inhibitors were added in a certain range of concentration (x), including [illegible] as a control.

The same procedures were followed for the calculations of % Inhibition (I) and the determination of IC_{50} values from plot, as β-lactamase inhibitory assay. 0.061 units of enzyme and 0.207 mg of substrates were used for each assay.

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