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# N-Thiolated β-Lactams: Chemistry and Biology of a Novel Class of Antimicrobial Agents for MRSA

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*N*-Thiolated β-Lactams: Chemistry and Biology

of a Novel Class of Antimicrobial Agents for MRSA

by

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A dissertation submitted in partial fulfillment
of the requirements for the degree of
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## LIST OF ABBREVIATIONS

| | |
|---|---|
| α | alpha |
| Ar | aryl |
| Ac | acetyl |
| Aco | acetoxy |
| β | beta |
| Bn | benzyl |
| Br | broad |
| Bu | butyl |
| Boc | *tert*-butyloxycarbonyl |
| bp | boiling point |
| br | broad (spectral) |
| Bz | benzoyl |
| °C | degrees Celsius |
| $^{13}C$ | carbon-13 |
| *c* | concentration (mg/ml) |
| CAN | ceric ammonium nitrate |
| Cbz | carbobenzyloxy |
| $CH_2Cl_2$ | dichloromethane |
| $Cl_2$ | chlorine gas |
| $cm^{-1}$ | wave numbers (reciprocal centimeters) |
| CSA | camphorsulfonic acid |
| D | deuterium ($^2H$) |
| DBU | 1,8-diazabicyclo[5.4.0]undec-7-ene |
| DCC | dicyclohexyl carbodiimide |
| DEAD | diethyl azodicarboxylate |
| DIBAL | diisobutylaluminum hydride |
| DMAP | 4-dimethylaminopyridine |
| DMSO | dimethylsulfoxide |
| Et | ethyl |
| $Et_3N$ | triethylamine |
| EtOAc | ethyl acetate |
| g | gram(s) |
| $^1H$ | proton |
| $^2H$ | deuterium |
| $^3H$ | tritium |
| HMDS | hexamethyldisilazide |
| hr | hour(s) |
| Hünig's base | ethyldiisopropylamine |
| Hz | hertz |
| IR | infrared |

| | |
|---|---|
| *J* | coupling-constant(s) |
| KOH | potassium hydroxide |
| LAH | lithium aluminum hydride |
| LDA | lithium diisopropylamide |
| Lawesson reagent | *para*-methoxyphenylthionophosphine sulphide dimer |
| Me | methyl |
| MeCN | acetonitrile |
| MeOH | methanol |
| mCPBA | *meta*-chloroperoxybenzoic acid |
| mg | milligram(s) |
| µg | microgram(s) |
| mM | millimoles per liter |
| mmol | millimole(s) |
| mol | mole(s) |
| MOM | methoxymethyl |
| MRSA | methicillin-resistant *Staphylococcus aureus* |
| MSSA | methicillin-susceptible *Staphylococcus aureus* |
| Ms | methanesulfonyl, mesyl |
| N | normal |
| NaOH | sodium hydroxide |
| OD | optical density |
| PCC | pyridinium chlorochromate |
| Ph | phenyl |
| PhH | benzene |
| PhMe | toluene |
| ppm | parts per million |
| NPhth | *N*-phthalimido |
| PMP | *para*-methoxyphenyl |
| RT | room temperature |
| SEM | scanning electron microscopy |
| TBAF | tetra-*n*-butylammonium fluoride |
| TBDPS | *tert*-butyldiphenylsilyl |
| TBS | *tert*-butyldimethylsilyl |
| $^{t}$Bu | *tert*-butoxycarbonyl |
| THF | tetrahydrofuran |
| TLC | thin layer chromatography |
| Tf | trifluoromethylsulfonyl, trifyl ($CF_3SO_2$) |
| TFA | trifluoroacetic acid |
| TCA | tichloroacetic acid |
| Ts | *para*-toluenesulfonyl, tosyl ($p$-$CH_3C_6H_4SO_2$) |

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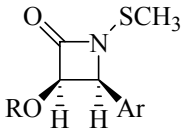
## ABSTRACT

*N*-Methylthio β-lactams (**1**) represent a promising new family of antibacterial agents whose *in vitro* activity is confined largely to S*taphylococcus* species, including multidrug-resistant forms of *S. aureus*. Originally developed in the 1980's for use as synthetic intermediates, *N*-methylthio β-lactams have recently been shown in these laboratories to possess intriguing biological properties which are addressed in Chapters I-IV. In terms of the antibacterial activities, the structural features and species specificities exhibited by these compounds are unlike those of any existing family of β-lactam drugs. The lactams seem to exert their effects intracellularly, requiring passage of the bioactive species through the cellular membrane, rather than acting extracellularly on cell wall components in the manner of penicillin and related antibiotics. The lipophilic nature of these molecules, which lack the polar side chain functionality of all other microbially-active β-lactams, suggests the compounds do not target the penicillin binding proteins within bacterial membranes. The most active members of this β-lactam class appear to be those bearing an aryl substituent (**Ar**) at $C_4$ of the ring. The synthesis and structure-activity relationship of these analogues is discussed in Chapter III. Moreover, microscopy and $^3H$ pulse-labeling studies, which are described in Chapter IV, demonstrate that *N*-methylthio β-lactams appear to be inhibitors of protein biosynthesis.




**1**

# CHAPTER I

# CLINICAL DEVELOPMENT OF NEW ANTI-MRSA ANTIBIOTICS

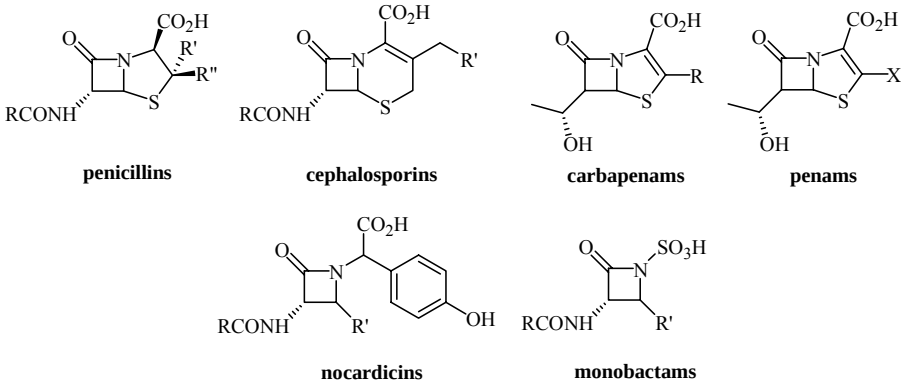
## 1.1 Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a multi-drug resistant pathogen whose insensitivity to most antibiotics has evolved over the span of five decades. Historically, methicillin, a second-generation semi-synthetic penicillin, was first introduced into hospitals in 1960 in response to the prominence of β-lactamase producing staphylococci. However, the bacteria rapidly adapted and in 1961, the first MRSA strain emerged in Cairo, Egypt.[1] As MRSA continues to evolve and becomes more resistant to current antibiotics, the need to find new therapies grows increasingly important. This chapter focuses on the recent progress towards the clinical development of new antimicrobial agents for MRSA.

## 1.2 β-Lactam Antibiotics

β-Lactams (*Figure 1.01*) remain the most widely prescribed class of antibiotics despite the continuous rise in resistance that forced the penicillins and cephalosporins to undergo multiple generations of synthetic change. *S. aureus* resistance to these antibacterials stem primarily from ring cleavage by β-lactamase and modification or overproduction of the enzymes involved in cell wall biosynthesis - penicillin-binding proteins (PBPs).[2] In recent years, structure-activity relationship (SAR) studies of semisynthetic cephalosporins and penems has identified chemical functionalities that enhance binding to low affinity transpeptidases (ie, PBP2a) and confer resistance to β-lactamase.

**Figure 1.01:** Familes of clinically important β-lactam antibiotics.

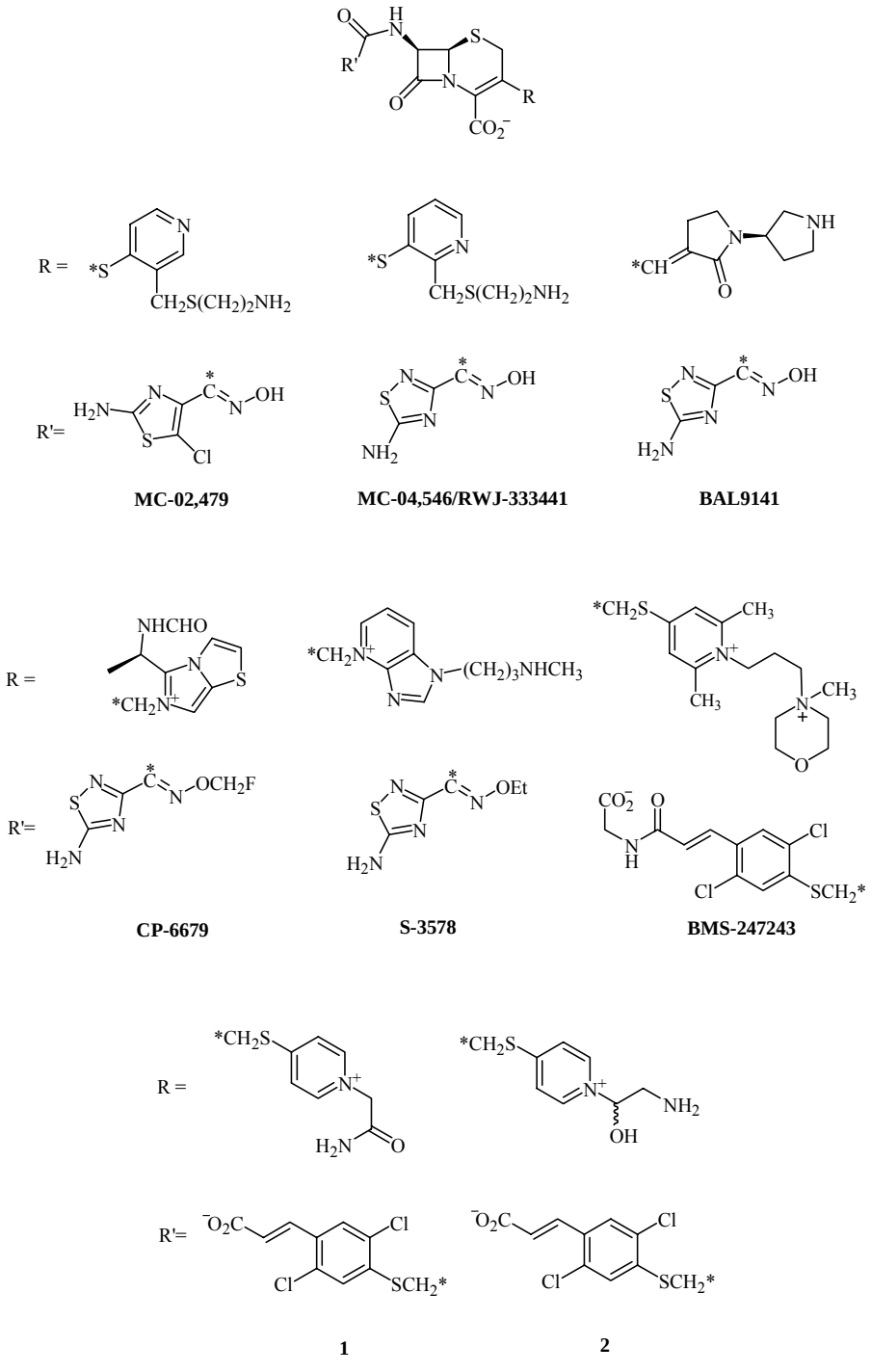



### 1.2.1 Cephalosporins

Traditional cephalosporins are poor substrates for PBP2a and are thus considered ineffective as anti-MRSA agents. During the 1990s, new semisynthetic analogues emerged possessing higher affinity for this low binding transpeptidase combined with improved stability toward β-lactamases. The cephalosporins have polar, lipophilic groups at C(3) and C(7) positions of the ring which were discovered to enhance bioactivity and chemical stability.[3] Among these, MC-02479 (RWJ-5442, Essential Therapeutics/RW

Johnson Pharmaceutical Research Institute; *Figure 1.02*, *Table 1.01*) and BAL-5788 (BASILEA Pharmaceutica AG) have since advanced to clinical trials in the US and Europe, respectively.

**Figure 1.02:** Anti-MRSA cephalosporins in clinical development.




MC-02479 is a broad spectrum cephalosporin with a C(7) 4-pyridinethiol zwitterionic moiety contributing to the lactam's activity and solubility.[4,5] MC-02479 is equipotent to vancomycin against 256

MRSA strains and the binding affinity for PBP2a is reportedly 43-fold greater than imipenem. SAR studies of the 4-pyridinethiol series also led to the discovery of MC-04546 (RWJ-333441, Essential Therapeutics/RW Johnson Pharmaceutical Research Institute; *Figure 1.02*), an analogue with enhanced serum stability lending to the cephem's above-average pharmacokinetic properties in rats.[6] BAL-5788, the water-soluble prodrug of BAL-9141 (*Figure 1.02*), is also an efficient substrate for PBP2a and effectively treated MRSA-induced endocarditis in rats.[7] Furthermore, it is stable to class A and C β-lactamases, which is attributed to the C(7) hydroxyiminoacetamido side chain.[7] Other anti-MRSA cephems bearing similar C(3) and C(7) substituents have also been reported. Currently, CP-6679 (Meiji Seika Kaisha Ltd; *Figure 1.02*) is being developed as part of an imidazothiazolium series of cephalosporins. The compound possesses 88-fold greater binding ability to PBP2a than imipenem but displays only modest *in vitro* activity against MRSA.[8] Another clinical trial candidate is S-3578 (*Figure 1.02*), a unique lipophilic cephalosporin exhibiting low serum binding (< 25%) while maintaining the therapeutic efficacy of vancomycin in rats with MRSA-induced infections.[9]

**Table 1.01:** *In vitro* and *in vivo* activities of anti-MRSA cephalosporins in clinical development.[4-9]

| | $MIC_{90}$[a] | $IC_{50}$[b] | β-lactamase stability | $ED_{50}$[c] (mg/kg) |
|---|---|---|---|---|
| MC-02479 | 2 | 0.7 | Stable | NR |
| MC-04456 | ≤ 1 | 0.5 | Stable | 1.4 |
| BAL-9141 | 2 | 0.5 | Stable | 2.4 |
| CP-6679 | 12.5 | 5.1 | NR | 12 |
| S-3578 | 4 | 4.42 | Stable | 5 to 8 |
| BMS-247243 | 5 | 0.7 | Stable | 2.0 to 3.8 |
| NB-2001 | 0.5 | NR | Not stable | NR |

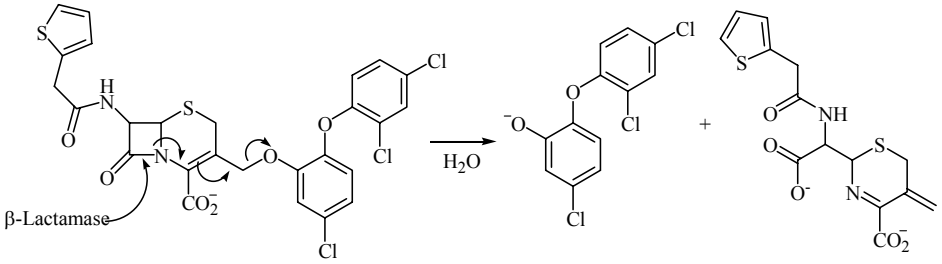
[a]Typical $MIC_{90}$ values (minimum inhibition concentration for 90% of MRSA test strains) for vancomycin and ceftriaxone are 2 and > 64 μg/ml, respectively; [b]Typical $IC_{50}$ values (concentration to inhibit 50% PBP2a activity) for imipenem are > 100 μg/ml; [c]$ED_{50}$: effective dose in 50% of mice or rats with systemic MRSA infection; NR: not reported or could not be found in the literature.

Bristol-Myers Squibb Co is investigating a thiopyridinium series of cephalosporins without a C(7) imino side chain.[10] In lieu of an iminoacetamide group is a dichlorothiophenyl acetamide conferring stability toward A to D class β-lactamases.[11] As an early lead compound from this series, BMS-247243 (Bristol-Myers Squibb Co; *Figure 1.02*) gave rise to other potent anti-MRSA cephalosporins bearing similar functionalities.[11,12] SAR was disclosed in recent publications and new candidates emerged for further pre-clinical evaluation including cephalosporins **1** and **2** (*Figure 1.02*) whose average $PD_{50}$ in mice with systemic MRSA infections is 3.9 and 0.8 mg/kg, respectively.[13]

Companies such as Cubist Pharmaceuticals Inc and BASILEA Pharmaceutica AG also boast other anti-MRSA cephalosporins in the late stages of clinical development. CAB-175 (Cubist Pharmaceuticals Inc),[14] an azomethine cephalosporin, is expected to begin human trials during the first half of 2003 for the intravenous treatment of drug-resistant bacteria. CAB-175 demonstrates bactericidal activity against MRSA while maintaining a similar safety profile to ceftriaxone. BAL-2057 (BASILEA Pharmaceutica AG), a member from a new class of narrow spectrum anti-MRSA cephalosporins, is reportedly in the final stages of optimization after displaying consistently high *in vivo* activity in animal models.[15] However, no additional information on BAL-2057 could be obtained.

Using a different chemotherapeutic approach, NewBiotics Inc is utilizing the cephalosporin NB-2001 in an enzyme-catalyzed therapeutic activation (ECTA) method for delivering the bactericide triclosan upon ring hydrolysis of the inactive prodrug (*Scheme 1.01*).[16] While extensive research on β-lactamase-dependent prodrugs has previously been conducted,[17] interest remains in ECTA as a means to circumvent the development of resistance in bacteria by controlled release of the active drug when the drug-resistant pathogen is present.

**Scheme 1.01:** Enzyme-catalyzed therapeutic activation mechanism of NB-2001.[16]

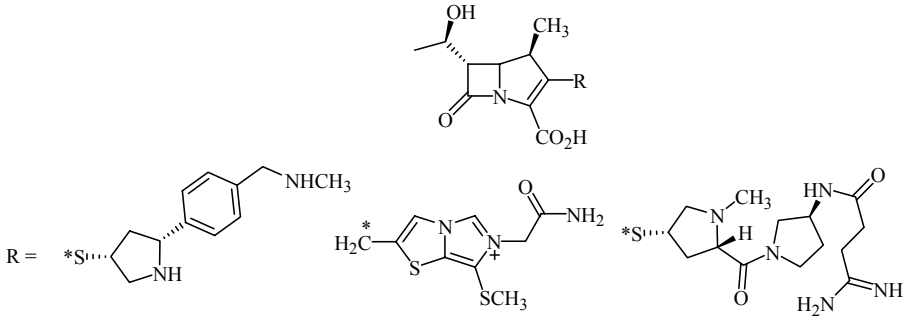



**NB2001** **triclosan**

1.2.2 Carbapenems

Although existing carbapenems are not useful drugs for MRSA, this family of antibiotics continues to draw interest because of their chemical stability towards β-lactamase. During the 1990s, anti-MRSA carbapenems[18] were being developed by Merck & Co, SmithKline Beecham plc, and Wyeth-Ayerst Research, however, investigations of the naphthosultam penems,[19] trinems,[20] and THF carbapenems,[21] respectively, were discontinued or suspended by 2001. Concurrently, J-111225 (Banyu Pharmaceutical Co Ltd), CS-023 (Sankyo Co Ltd) and CP-6509 (Meiji Seika Kaisha Ltd) are being investigated as ultra-broad spectrum 1β-methyl-carbapenems possessing activity against drug-resistant staphylococci.

**Figure 1.03:** Anti-MRSA penems in clinical development.



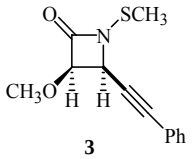

**J-111225** **CP-5609** **CS-023**

J-111225 (*Figure 1.03*), a pyrrolidinylthio-1β-methylcarbapenem, is a good inhibitor of PBP2a ($IC_{50}$ = 2.5 μg/ml) which correlates well to its *in vitro* activity against MRSA ($MIC_{90}$ = 4 μg/ml).[22] The penem possesses a *trans*-hydroxyethyl side chain that imparts β-lactamase stability and a bulky lipophilic moiety which is understood to enhance PBP2a binding. The effectiveness of J-111225 is comparable to vancomycin in mice with systemic MRSA infections ($ED_{50}$ = 5.83 mg/kg) and maintains good stability toward human renal dehydropeptidase I.[23] CS-023 (*Figure 1.03*) is an analogous broad spectrum pyrrolidinylthio-1β-methylcarbapenem being investigated as a parenteral MRSA antibiotic. The drug has a MIC of 8 μg/ml against MRSA and an $IC_{50}$ of 5.3 μg/ml to PBP2a.[24] Another extensively researched penem is CP-5609 (*Figure 1.03*), a 6,7 disubstituted imidazo[5,1*b*]thiazole-3-yl-1β-methylcarbapenem with excellent affinity for PBP2a and a MIC of 4 μg/ml against MRSA.[25]

1.2.3 *N*-Thiolated β-Lactams

*N*-Thiolated monocyclic β-lactams (*Figure 1.04*) represent a novel class of synthetic antibacterials whose mechanism of action is not inhibition of cell wall biosynthesis.[26] Additional information on the biological properties of these and related molecules is presented in chapters II-IV.

**Figure 1.04:** *N*-Methylthio β-lactams with anti-staphylococcal properties.



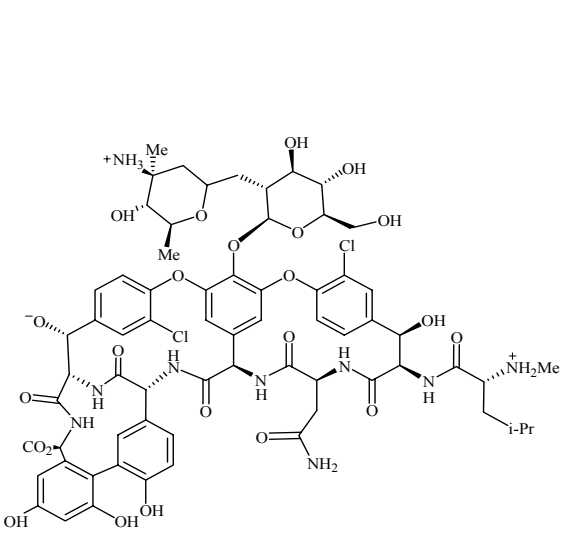
**3**

## 1.3 Peptide Antibiotics

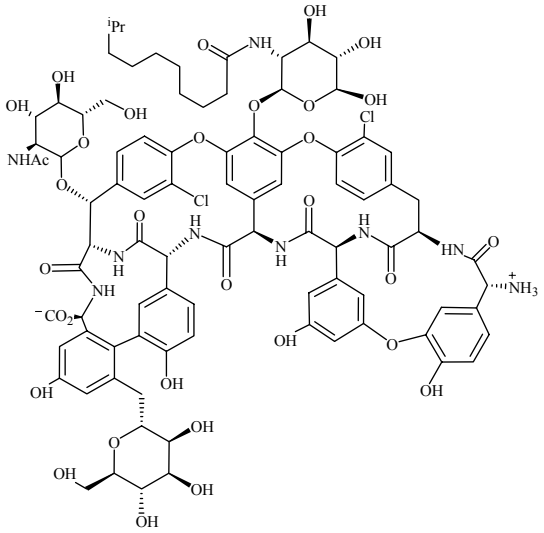
### 1.3.1 Second Generation Glycopeptides

Glycopeptides[27] (*Figure 1.05*) embody the most successful family of MRSA antibiotics and remain the drugs of choice for the treatment of hospital-borne infections. Unlike β-lactam antibiotics, glycopeptides inhibit cell wall synthesis by forming a complex with the C-terminal D-ala-D-ala of the peptidoglycan crosslinkages. Although the mechanism of action was once considered unbeatable, glycopeptide resistance emerged during the 1980s via enzyme-dependent conversion of the terminal D-alanines to D-lactates.[28] The gene encoding resistance (*vanA*, vancomycin-resistance) initially evolved in enterococci, however, in June 2002, a *S. aureus* strain harboring both the *mecA* (methicillin-resistance) and *vanA* genes was isolated from a catheter exit site of a diabetic patient in the US.[29] The recent escalation in glycopeptide-resistant bacteria counts has prompted researchers to investigate new semisynthetic analogues of vancomycin while increasing efforts to discover new natural products with enhanced bioactivity or novel modes of action.[27,30]

**Figure 1.05:** Vancomycin and teicoplanin.



**vancomycin**

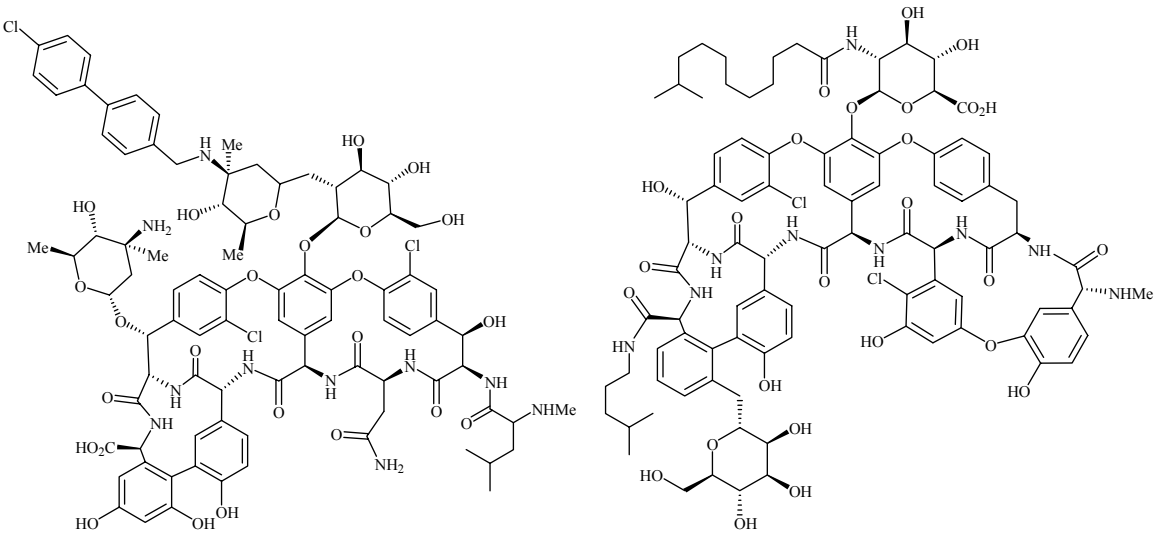


**teicoplanin**

Oritavancin (InterMune Pharm. Inc; *Figure 1.06)* is a semisynthetic glycopeptide prepared from a natural product analogue of vancomycin.[31] The peptide displays good *in vitro* activity against MRSA (*Table 1.02*) while maintaining the efficacy of vancomycin in rabbits with MRSA-induced endocarditis.[31a] After displaying a good safety profile in humans during phase I trials, oritavancin is currently in phase III trials with completion and NDA filing anticipated for January 2004. Another glycopeptide in clinical trials is dalbavancin (Biosearch Italia SpA/Versicor Inc; *Figure 1.06*, *Table 1.02*), a semisynthetic glycopeptide being developed as the first once-weekly intravenous treatment for serious infections caused by Gram-positive bacteria.[32,33] Phase I trials established dalbavancin to be well tolerated in healthy individuals and phase II studies during which hospitalized patients with skin and soft tissue infections were administered

the glycopeptide were reported as positive.[34] Biosearch is also developing ramoplanin (Biosearch Italia SpA/Genome Therapeutics Corp), a lipoglycopeptide antibiotic for use as a topical treatment for nasal carriage of MRSA.[35] However, due to the innate toxicity of ramoplanin, parenteral administration is not possible.

**Figure 1.06:** Oritavancin and dalbavancin.



**oritavancin** **dalbavancin**

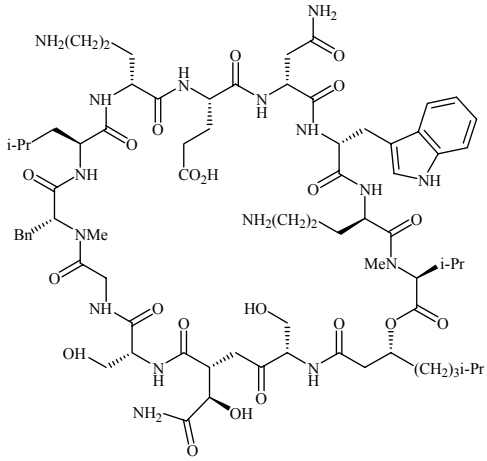
1.3.2 Lipopeptides (Daptomycin)

Daptomycin (Cubist Pharmaceuticals Inc/Gilead Sciences Inc; *Figure 1.07*) is a naturally occuring lipopeptide consisting of 13 amino acids and a decanoic acyl side chain.[36] The peptide displays rapid bactericidal activity against MRSA and the VRSA strain isolated in the US.[36a,36b] The mechanism of action for daptomycin is not entirely understood although it is known that cellular integrity is disrupted by insertion of the peptide into the plasma membrane.[36c] Daptomycin's novel mode(s) of action in bacteria should prevent the occurrence of cross-resistance and studies have shown the incidence of spontaneous-resistance is below detectable levels in *S. aureus*.[36d]

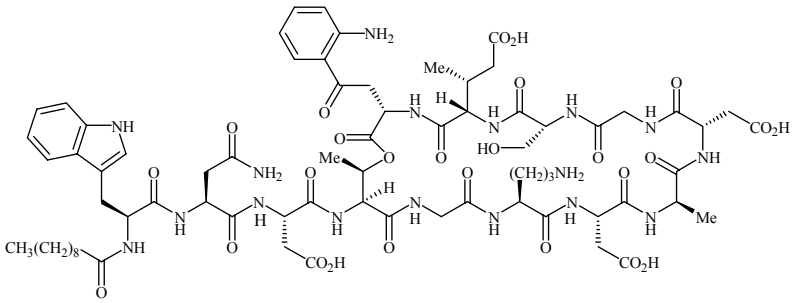
1.3.3 Depsipeptides

Wakamoto Pharmaceutical Co Ltd is developing WAP-8294A2 (*Figure 4*, *Table 1.02*), a water-soluble depsipeptide antibiotic produced by *Lysobactor* spp. WAP-8294A2 displays good *in vitro* activity against MRSA (0.78 μg/ml) and VRSA (6.25 μg/ml) while maintaining an $ED_{50}$ of 0.38 mg/kg in mice with systemic MRSA infections.[37] Shionogi & Co Ltd is also investigating two new depsipetide antibiotics in katanosin and plusbacin $A_3$.[38] The macrocycles inhibit the *in vitro* growth of MRSA at 0.39 and 1.56 μg/ml, respectively, and unlike vancomycin, katanosin and plusbacin $A_3$ reportedly block cell wall synthesis by inhibiting the formation of lipid intermediates involved in transglycosylation. *In vitro* studies have demonstrated that the incorporation of [$^{14}$C]glycine is inhibited at $IC_{50}$ values of 2.2 and 2.3 μg/ml while synthesis of nascent peptidoglycan is suppressed at $IC_{50}$s of 0.8 and 0.4 μg/ml for katanosin and plusbacin A3, respectively.[38]

**Figure 1.07:** Daptomycin and WAP-8294A2.



**daptomycin**



**WAP-8294A**

**Table 1.02:** Biological activity comparisons of oritavancin, dalbavancin and daptomycin.[31-38]

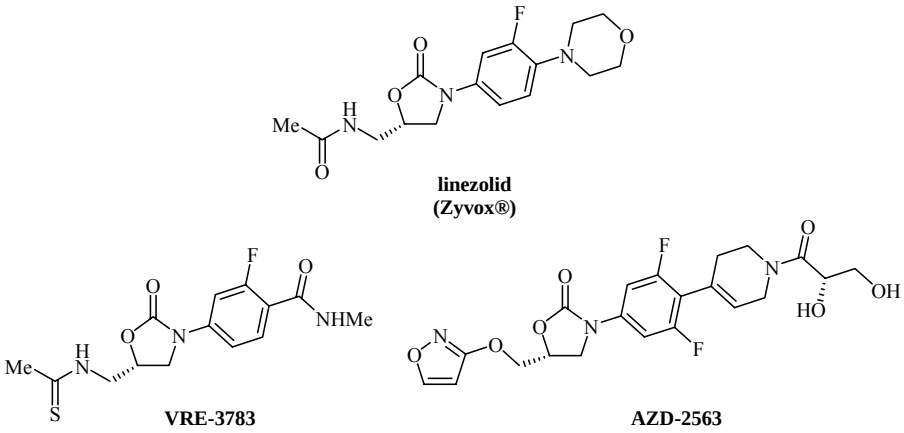
| | $MIC_{50}$[a] (μg/ml) | $MIC_{90}$[a] (μg/ml) | MIC w/serum[b] (μg/ml) | Protein binding[c] (%) | MBC[d] (μg/ml) | Terminal half-life[e] (h) |
|---|---|---|---|---|---|---|
| Oritavancin | 0.5 | 2 | 1-4 | 86-90 | 2 | 132-356 |
| Dalbavancin | 0.13 | 0.25 | 2-4 | NR | 2 | 166-212 |
| Daptomycin | 1 | 1 | NR | 90-94 | 1 | 8.5 |
| Teicoplanin | 0.5 | 2 | 2 | 90 | ≥ 2 | > 35 |
| Vancomycin | 1 | 2 | 1-2 | 30 | 2 | 6 |

[a]minimum inhibitory concentration for 50 or 90% of MRSA test strains; [b]50% bovine serum; [c]% human plasma protein binding; [d]minimum bactericidal concentration; [e]terminal half-life in humans; NR: not reported or data could not be found in the literature.

### 1.4 Oxazolidinones

Oxazolidinones are bacteriostatic antibiotics of Gram-positive bacteria that inhibit protein synthesis by binding to the 30S and 50S ribosomal subunits.[39] Linezolid (Zyvox®, *Figure 1.08*) is the only oxazolidinone in clinical use, although other promising candidates have emerged and advanced to human trials. Currently, Versicor is investigating a thioamide series of oxazolidinones that display good *in vitro* activity against MRSA ($MIC_{90}$ = 1 to 2 μg/ml).[40] VRC-3783 (Pharmacia Corp/Versicor Inc; *Figure 1.08*) and VRC-4104 (Pharmacia Corp/Versicor Inc) are two promising analogues with $ED_{50}$ values of 6.2 and 3.8 mg/kg, respectively, in a mouse septicemia model.[40b] AstraZeneca plc was developing AZD-2563 (*Figure 1.08*), an oxazolidinone with a long half-life and good activity against MRSA (MIC = 1 μg/ml), but development was discontinued by July 2002 for unknown reasons.[41]

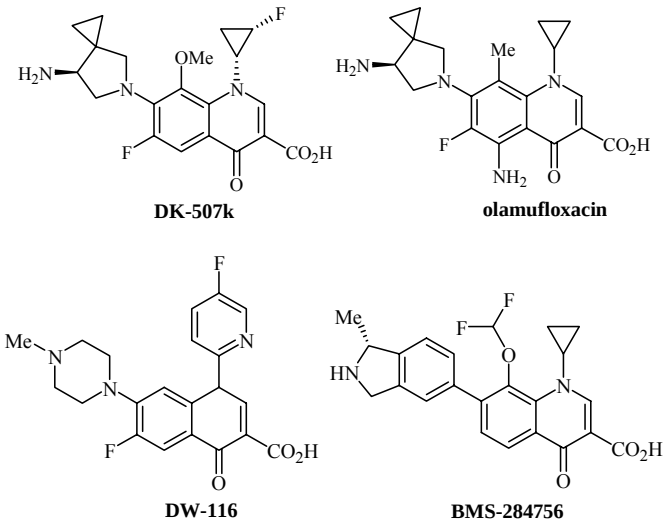
**Figure 1.08:** Linezolid, VRC-3783, and AZD-2563.




### 1.5 Quinolones, glycylcyclines and coumarin antibiotics

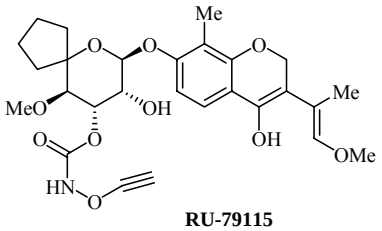
Quinolones are bactericidal agents that act on types II (DNA gyrase, negative supercoiling) and IV topoisomerase (chromosome partitioning) in *S. aureus*.[42] Several new quinolones, such as DW-116 (Dong Wha Pharmaceutical Industry Co Ltd; *Figure 1.09*),[43] olamufloxacin (Hokuriku Seiyaku KK; *Figure 1.09*),[44] and DK-507k (Daiichi Seiyaku Co Ltd, *Figure 6*)[45] have advanced to clinical trials after demonstrating strong activity against drug-resistant Gram-positive pathogens, such as MRSA ($MIC_{90}$ = 0.06, 1.56 and 0.006 μg/ml, respectively). These compounds are derived from the popular fluoroquinolone family possessing a C(6) fluorine atom that is known to substantially enhance bioactivity.[42] Des-fluoro(6) quinolones are also being investigated with BMS-284756 (ganefloxacin; Bristol-Myers Squibb Co/Toyama Chemical Co Ltd; *Figure 1.09*) at the most advanced stage in clinical development for this class .[46] Ganefloxacin is completing late phase III trials in the US, Japan and Europe with an anticipated use as a once-daily intravenous treatment for skin and soft tissue pathogens, including MRSA and fluoroquinolone-resistant *S. aureus* (MIC = ≤ 4 μg/ml).[46a] Non-fluorinated quinolones are also being developed as topical agents for SSTIs. Recently reported is T-3912 (Toyoma Chemical Co Ltd/Ferrer Internacional SA/Maruho KK), a 3-pyridinyl quinolone with potent *in vitro* activity against drug-resistant staphylococci ($MIC_{90}$ = 0.2 μg/ml).[47]

**Figure 1.09:** Anti-MRSA quinolones in clinical development.

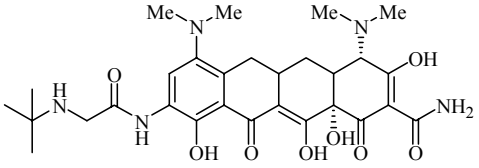


Coumarin antibiotics are another class of topoisomerase inhibitors that selectively target DNA gyrase B in *S. aureus*.[48] RU-79115 (Aventis Pharma AG; *Figure 1.10*), a coumarin related to novobiocin, displays good activity against multiple drug-resistant forms of *S. aureus* while maintaining a $PD_{50}$ of 1mg/kg in mice with induced systemic infections.[49] Glycylcyclines, although unrelated to the coumarins, are also active against multi-drug resistant strains of *S. aureus*. Tigecycline (Wyeth Research; *Figure 1.06*) was the most developed from this class of protein synthesis inhibitors, but a policy change by the US FDA had forced Wyeth to delay the phase III clinical trials.[50]

**Figure 1.10:** Anti-MRSA coumarin and glycylcycline in clinical trials.




**RU-79115**




**tigecycline (GAR-936)**

## 1.6 Conclusions

A tremendous worldwide effort to discover new anti-MRSA antibiotics is apparent. β-Lactams continue to be the most heavily researched class because of their enduring ability to fight infection while maintaining low toxicity in humans. Semisynthetic vancomycin derivatives have also gained a great deal of attention due to the long-term success of glycopeptides against MRSA. However, future therapies cannot be devised by analogue synthesis of pre-existing antibiotics alone. Compounds with novel modes of action must continue to be researched and developed as *S. aureus* continues to evolve new mechanisms and new levels of resistance to current antibiotics. In chapter III-V, the synthesis and biological properties of the novel class anti-MRSA antibiotics called "*N*-methylthio β-lactams" is discussed. The structure-activity relationships and investigations into their unknown mode of action will be described in depth for the first time.

# CHAPTER II

# CHEMISTRY AND BIOLOGICAL PROPERTIES OF *N*-THIOLATED β-LACTAMS

## 2.1 Introduction

For over 60 years, the β-lactam antibiotics have served as a powerful line of defense against bacterial infections.[51] Following the initial introduction of penicillin during World War II, a variety of other classes of β-lactam antibiotics were identified including the cephalosporins, penems, carbapenems, and monobactams (*Figure 2.01*).[52]

One of the landmark discoveries in the β-lactam field occurred in 1981, when researchers at the Takeda and Squibb laboratories isolated the first classes of N-thiolated β-lactam antibacterial agents from natural sources.[53] The Squibb scientists coined the term "monobactams" in referring to these new monocyclic β-lactams. These reports confirmed that β-lactam compounds do not necessarily require a conformationally rigid bicyclic ring system to have antibacterial capabilities, or the carboxylic acid moiety found in all other microbially-active β-lactams. These β-lactam compounds were the first to have a sulfur group attached directly to the lactam nitrogen, in the form of an N-sulfonic acid. In this chapter, a summary of the nearly 5000 β-lactam compounds that contain an N-sulfur substituent is provided. The sulfur center in these molecules can bear a diversity of residues and exist in a variety of oxidation states, as classified by the following five structure types (*Figure 2.02*). The discussion that follows is therefore divided into the following five sections:

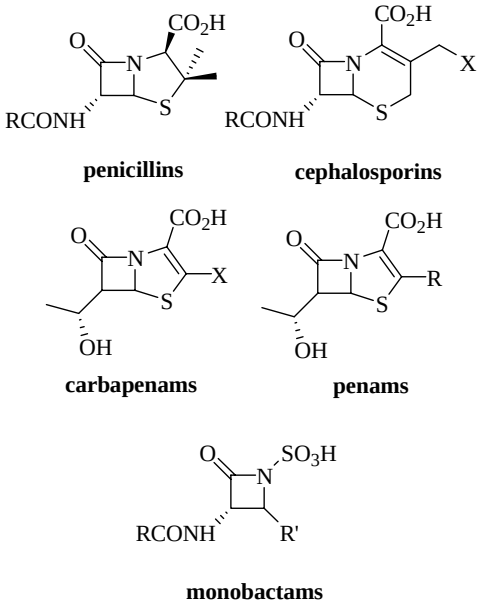
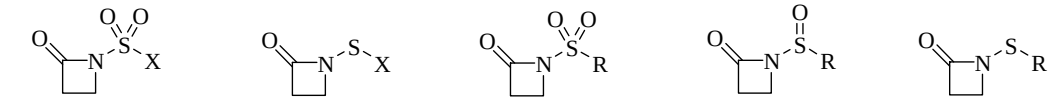



**Figure 2.01:** Classes of β-lactam antibiotics.[51]

**Figure 2.02:** Chemical classes of *N*-thiolated β-lactams.

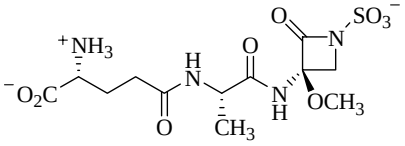



## 2.2 N-$SO_2$X β-Lactams

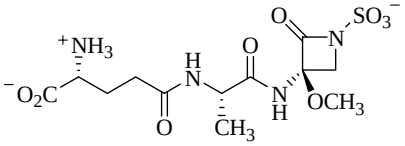
Compounds within this category include the *N*-sulfonic acids (X=OH), *N*-chlorosulfonyl lactams (X=Cl), and *N*-aryloxysulfonyl or *N*-alkyloxysulfonyl derivatives (X=OAr or OR). Each of these will be discussed separatedly in this order.

2.2.1 *N*-Sulfonic Acid β-Lactams

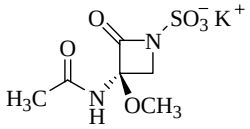
With over 3600 entries cited in the journal and patent literature, the *N*-sulfonic acid β-lactams represent the largest and most thoroughly studied class of *N*-thiolated β-lactams. The term "monobactam" is now commonly used to refer to structures containing the 2-oxoazetidine-1-sulfonic acid framework.[54] Over the past two decades, a variety of review articles have been written on the discovery and development of the monobactam antibiotics.[55]



**sulfazecin**
**(SQ 26,455)**



**isosulfazecin**



**SQ 26,180**

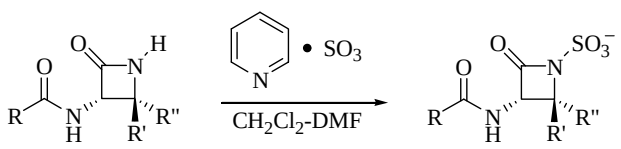
**Figure 2.03**: First monobactams discovered by American and Asian scientists. [55]

The first members of this family were described in 1981 in separate disclosures from the Takeda and Squibb laboratories.[55b] In February 1981, Takeda reported the isolation of two novel β-lactam natural products, sulfazecin and isosulfazecin (*Figure 2.03*), from Gram-negative Pseudomonas bacteria in soil. Sulfazecin was found to be active against Gram-negative bacteria but weakly active against Gram-positive microbes. Isosulfazecin, on the other hand, exhibited only weak activity against both Gram-negatives and Gram-positives.[53a]

A few months after the Takeda report appeared, Squibb[53b] laboratories published their findings on seven closely related β-lactams isolated from a diverse range of microbes, including *Acetobacterium*, *Agrobacterium*, *Chromobacterium*, *Flexibacterium*, and *Gluconobacterium* species. The compound most frequently found in these screenings, SQ 26,445, turned out to have a structure identical to that of Takeda's sulfazecin. A second lactam, SQ 26,180 seen in *Figure 2.03*, was also identified. These naturally occurring lactams proved to be highly stable to a wide range of β–lactamases, but had relatively weak antibacterial activity. These features were attributed to the low affinity the compounds have for the penicillin binding proteins (PBP's) in bacteria.[55b] Their selectivity for aerobic Gram-negative microbes warranted further study.

To be able to carry out detailed structure-activity studies, methods were soon developed to create monobactam analogues by chemically introducing or modifying the side chains on the lactam ring. The most common approach involves the *N*-thiolation of an *N*-unsubstituted lactam **1** with pyridine-sulfur trioxide complex (*Scheme 2.01*). This procedure, developed by the Takeda laboratories, works particularly well for $C_4$ unsubstituted systems (**2**, R'=R"=H), giving good yields of the *N*-sulfonated lactam as its pyridinium salt.[56] The salt can be readily exchanged for other cations (K+) or protonated to give the *N*-sulfonic acid.

**Scheme 2.01:** *N*-Sulfonation of β-lactams with pyridine-sulfur complex.[56]



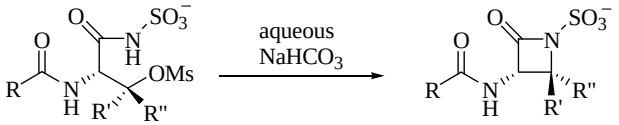
**1** **2**

The *N*-sulfonic acid moiety can also be transferred onto the ring using the powerful sulfonating agent dimethylformamide-sulfur trioxide (DMF-$SO_3$) followed by aqueous $KH_2PO_4$ workup.[57] Treatment of this product with tetrabutylammonium bisulfate and extraction with an organic solvent enables the

monobactam to be obtained as its tetrabutylammonium salt.[58] Alternatively, *N*-sulfonation can be achieved through a stepwise *N*-silylation process using trimethylsilyl chloride and triethylamine, followed by treatment of the resulting *N*-silyl lactam with trimethylsilyl chlorosulfonate ($(CH_3)_3SiOSO_2Cl$) at low temperature.[59] The silyl ester group can be hydrolyzed with $KH_2PO_4$ buffer to afford the monobactam potassium salt.

A different strategy for making monobactams is via base-promoted cyclization of a β-methanesulfonyloxyacyl sulfamate **3** in aqueous bicarbonate media, in which the stereochemistry at $C_4$ arises through an inversion process (*Scheme 2.02*).[60]

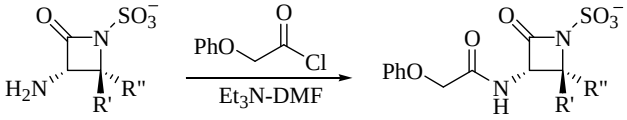
**Scheme 2.02:** Bases-promoted cyclization for synthesizing monobactams.[60]




**3** **2**

Monobactam analogues differing only in the acyl side chain can best be obtained by acylation of a 3-amino monobactamic acid **4**, as illustrated in *Scheme 2.03*. The acyl moiety can be introduced using an acyl chloride or an activated ester.[61]

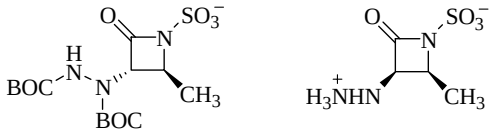
**Scheme 2.03:** Acylation of 3-amino monobactamic acids.[61]




**4** **5**

The preparation of the 3-amino monobactamic acid **4** proceeds via monobactam intermediates **6** and **7** (*Figure 2.04*), neither of which were examined for biological activity.

**Figure 2.04:** Intermediates used to synthesize 3-amino monobactamic acids.



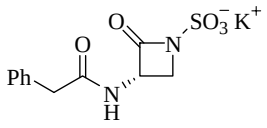

**6** **7**

Among the first series of synthetic monobactams studied were those whose side chains resembled the ones on existing β-lactam antibiotics (*Figure 2.05*). Thus, the phenylacetamidyl side chain of penicillin G gave rise to monobactams possessing a $C_3$ phenylacetamide group (as in SQ 26,324 and SQ 81,427), which were found to have increased activity against Gram-positive bacteria and enhanced affinity for PBP-1, -2, and -3. However, these compounds were still relatively weak compared to the penicillin counterpart.[62]

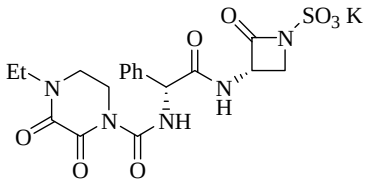
Incorporation of the uriedo moiety of the penicillin, piperacillin led to monobactams having antibacterial activity against a broader spectrum of microbes, including β-lactamase-producing strains of *Staphylococcus aureus*.[55b] The most active of the synthetic derivatives came from the attachment of an aminothiazole alkoximino side chain found in cefotaxime, a third generation cephalosporin. One of these derivatives, aztreonam (SQ 26,776), showed high affinity for PBP-3 of Gram-negative bacteria.[55b] Structure-activity studies revealed that the aminothiazole oxime is responsible for aztreonam's potent

activity against Gram-negative species, while the carboxylic acid on the oxime side chain enhances *Pseudomonas* activity.

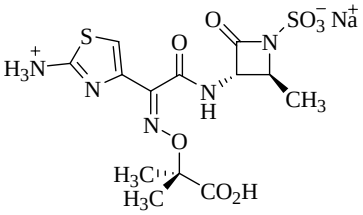
**Figure 2.05:** Synthetic monobactams with enhanced bioactivities. [55b]



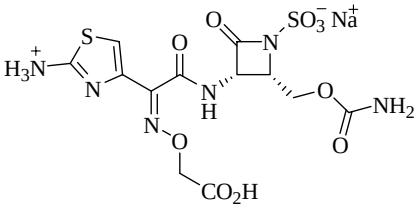
**SQ 26,324**



**SQ 81,427**



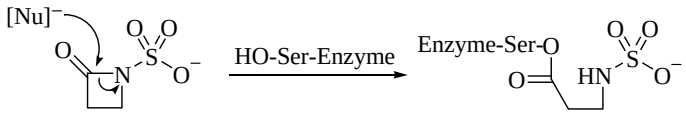
**aztreonam**
**(SQ 26,776)**



**carumonam**
**(AMA-1080)**

Like penicillin and the other β-lactam antibiotics, the monobactams inhibit bacterial growth by blocking cell wall biosynthesis. The lactams act directly on the penicillin binding proteins (PBP's), a group of membrane-bound serine transpeptidases that catalyze cell wall crosslinking.[61b] Upon binding in the active site, the compounds undergo β-lactam ring opening by acylating the catalytic serine hydroxyl group as depicted in *Scheme 2.04*. The PBP, giving a stable enzyme adduct that is catalytically inactive. By disrupting these crosslinking proteins, the β-lactams induce morphological deformities in the cell that leads to rupture of the bacterium. The *N*-sulfonic acid moiety plays a key role by activating the carbonyl of the monobactam ring to nucleophilic attack and ring opening in the enzyme active site.

**Scheme 2.04:** Mechanism of β-lactam hydrolysis by serine transpeptidases.




The ability of the monobactams to bind selectively to the bacterial PBP's depends largely on the nature of the $C_3$ and $C_4$ side chains on the β-lactam ring. The naturally occurring monobactams possess an acylamido side chain and often a methoxy group at $C_3$ of the ring. The methoxy moiety protects the ring from enzymatic hydrolysis by β-lactamases, but also decreases antibacterial activity. Other types of small polar groups at $C_3$ also reduce antibacterial activity. Monobactams of bacterial origin do not possess $C_4$ side chains, but synthetic analogues that have alkyl or heteroatom-containing residues at $C_4$ show significantly enhanced biological activity.[62] Aztreonam (SQ 26,776) and carumonam (AMA 1080; Ro 17-2301), two of the most active agents developed so far, have a methyl and carbamate group, respectively, at $C_4$.[63]

Along with enhanced stability to β-lactamases, these functionalized monobactams are highly potent against aerobic, Gram-negative bacilli such *Pseudomonas aeruginosa*, *Haemophilus influenzae* and members of the *Enterobacteriaceae* family. Aztreonam is bactericidal in its action[64] and binds selectively to PBP-3 of Gram-negative bacteria, resulting in the elongation of the cell and death through cell lysis.

However, the low affinity aztreonam has for the PBP's of Gram-positive and anaerobic microorganisms allows it to have a narrow spectrum of antimicrobial activity. Most commonly, the drug is used in the treatment of bacteremias, urinary tract infections, pelvic and intra-abdominal infections, and respiratory infections.[65] Since its activity is selective for aerobic Gram-negative bacilli, aztreonam can be combined with other antibacterial agents and be used as an alternative to aminoglycosides or penicillins in penicillin-allergic patients.

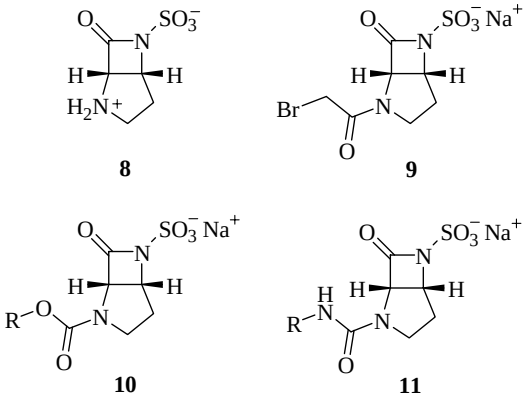
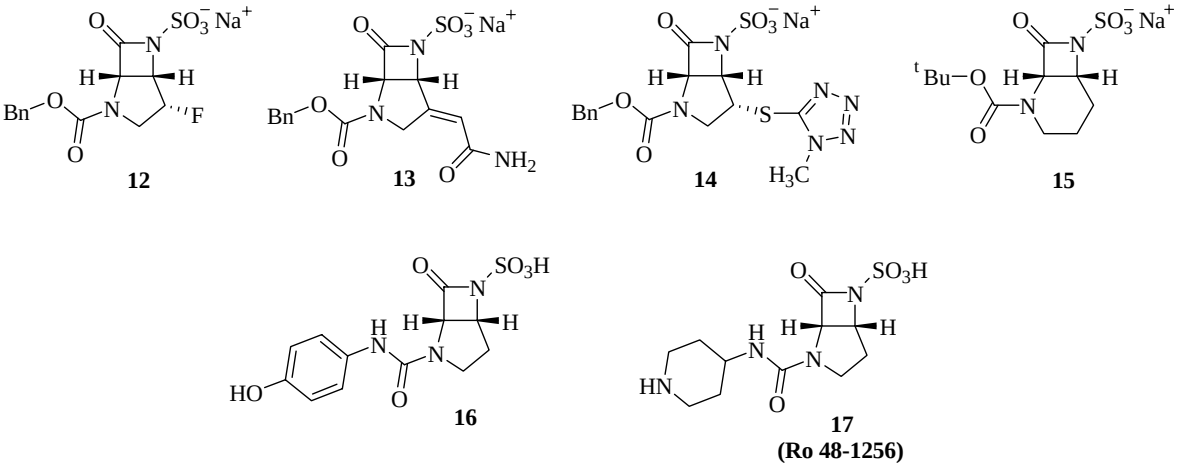


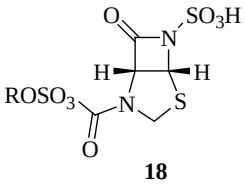

**Figure 2.06:** Bicyclic monobactam inhibitors of class C β-lactamase.[29]

A variety of bicyclic (bridged) monobactams **8-17** (*Figures 2.06* and *2.07*) have also been shown to serve as mechanism-based inhibitors of class C β-lactamases, some having half-inhibition constants down to 10 $\mu M^{-3}$.[66] The acyl-enzyme adduct formed by these compounds has a half-life toward hydrolysis up to 48 hours. This stability allowed the determination of an X-ray structure for bridged monobactam **8** covalently bound via lactam ring opening to the active site serine of *Citrobacter freundii* 1203 β-lactamase. While none of the inhibitors possess antibacterial activity, synergistic effects were observed for most of the analogues when used in combination with ceftriaxone (a third generation cephalosporin) against β-lactamase-producing strains of *Enterobacteriacea*e. Thus, using a 1:4 ratio of drug:monobactam, the MIC's could be reduced from 128 μg/mL (without inhibitor) to as low as 0.25 μg/mL for *Citrobacter freundii*. The compounds are much less effective against either class A or class B β-lactamases, and do not have significant synergies against a TEM-3 (class A β-lactamase)-producing strain of *Escherichia coli* or an 18 SH (a class C β-lactamase)-producing strain of *Pseudomonas aeruginosa*. This is probably due to the limited ability of the lactams to permeate the outer membrane of these particular microbes.[67]

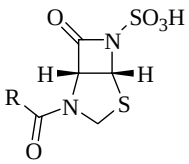
Interestingly, the disubstituted derivatives **12**, **13**, and **14**, and BOC-protected analogue **15** offered no synergistic effect in combination when used in combination with a penicillin. However, lactam **17** (Ro 48-1256) provides a good compromise between β-lactamase affinity and membrane permeability.[68] Hoffman-LaRoche AG patented a variety of related bridged monobactam derivatives **18-21** for use in liposome solutions as β-lactamase inhibitors.[69] There has also been some interest in the development of monobactams as cysteine proteinase inhibitors.[70]

**Figure 2.07:** Bicyclic monobactams **12-21**.[68-70]

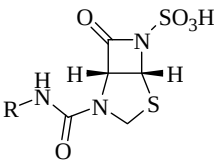




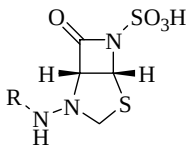
**18**



**19**



**20**

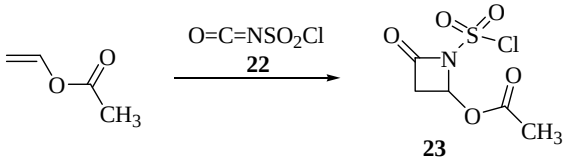


**21**

2.2.2 *N*-Chlorosulfonyl β-Lactams

*N*-Chlorosulfonyl β-lactams have been exploited primarily as reactive intermediates in synthesis, and have not been studied as bioactive molecules themselves.[71] This is undoubtedly due to their high chemical reactivity and instability in aqueous media. *Scheme 2.05* depicts the standard method for preparing *N*-chlorosulfonyl lactams by a [2+2]-cycloaddition of an olefin with chlorosulfonyl isocyanate **22**,[72] as shown for the conversion of vinyl acetate to 4-acetoxy lactam **23**.[73]

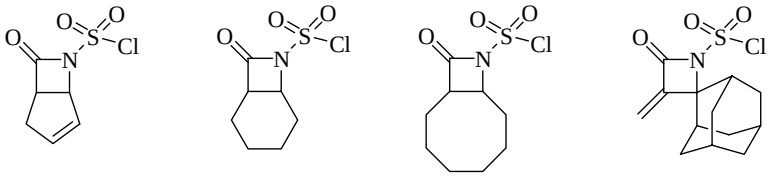
**Scheme 2.05:** Synthesis of 4-acetoxy *N*-chlorosulfonyl-azetidin-2-one **23**.[72]



**23**

The method has been applied to the synthesis of some bicyclic and multicyclic systems such as **24**-**27** (*Figure 2.08*).[74]

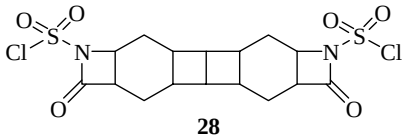
**Figure 2.08:** Bicyclic and multicyclic *N*-chlorosulfonyl β-lactams **24-27**.[75]



**24** **25** **26** **27**

The unusual dimeric compound **28** (*Figure 2.09*), made by photodimerization, has been employed in the construction of space-separated *bis*-heterocycles.[75]

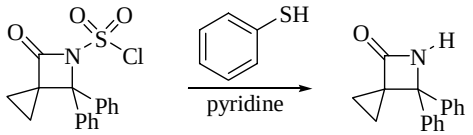
**Figure 2.09:** Dimeric *N*-chlorosulfonyl β-lactam **28**.[75]



**28**

The N-S bond in *N*-chlorosulfonyl derivatives is readily cleaved by thiol nucleophiles. Thiophenol has been shown to reduce *N*-chlorosulfonyl β-lactams to give *N*-protio β-lactams in the presence of pyridine. This was demonstrated for the deprotection of **29** as seen *vide infra*.[76]

**Figure 2.10:** Deprotection of *N*-chlorosulfonyl β-lactams with thiophenol.[76]



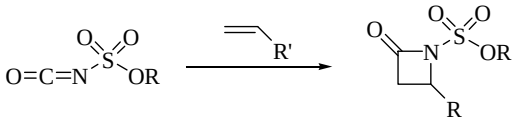

**29** **30**

Thus, while providing access to *N*-unsubstituted β-lactams, the inherently high reactivity of *N*-chlorosulfonyl β-lactams would appear to limit their usefulness as potential therapeutic agents *per se*.

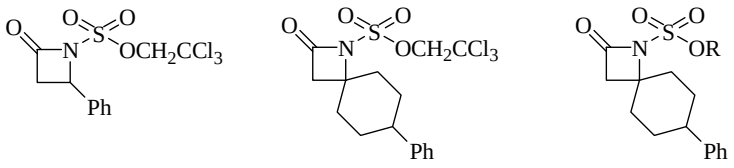
2.2.3 *N*-Aryloxysulfonyl and *N*-Alkoxysulfonyl β-Lactams

Around 30 *N*-aryloxy- and *N*-alkoxysulfonyl β-lactam derivatives have been described in the literature.These β-lactams are made by cycloaddition of an olefin with an aryloxy- or alkoxysulfonyl isocyanate **31**, as illustrated in *Scheme 2.06*. The method is effective for the synthesis of $C_3$ unsubstituted adducts such as **33**-**37** (see *Scheme 2.06*).

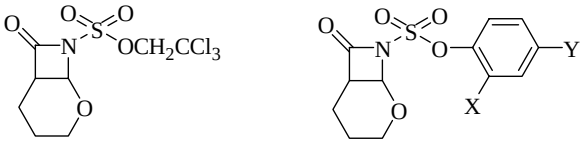
**Scheme 2.06:** Synthesis of *N*-aryloxysulfonyl and *N*-alkoxysulfonyl β-lactams.[43]




**31** **32**




**33** **34** **35**



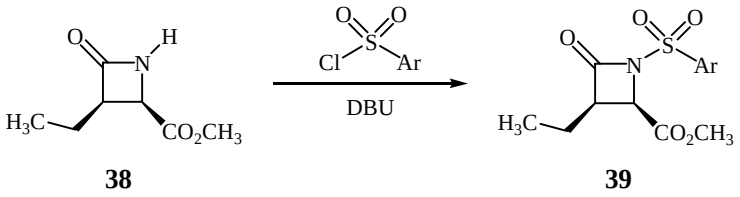

**36** **37**

Starting from the cyclic enol ether, trichloroethoxy derivative **36** and aryloxy (X=H, Y=$CH_3$; X=H, Y=$CH_3O$; X=$NO_2$, Y=H) analogues **37** have also been prepared in this manner.[77] Based on published reports, there have not been any studies or reports on whether these compounds possess biological properties.

**2.3 N-$SO_2$R β-Lactams**

Numerous articles can be found throughout the literature describing the preparation and use of *N*-sulfonyl β-lactams as intermediates in synthesis. There are several common procedures for synthesizing *N*-sulfonyl β-lactams. The most direct way is by treatment of an $N_1$ unsubstituted lactam with a sulfonyl chloride in the presence of a base. Two examples are illustrated in *Scheme 2.07*.[78]

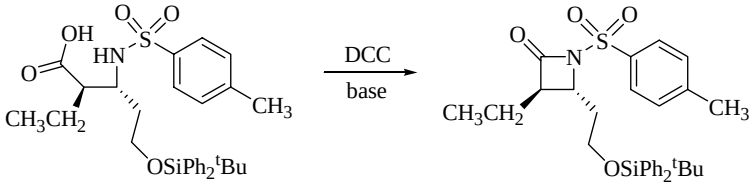
**Scheme 2.07:** Sulfonylation of *N*-protio β-lactams.[78]




**38** **39** **40** **41**

*N*-Tosyl β-lactams such as **43** have also been formed by dehydrative ring closure of a sulfonamido carboxylic acid **42** in the presence of dicyclohexylcarbodiimide (DCC) and 4-pyrrolidinopyridine (*Scheme 2.08*). This particular reaction was used in a published carbapenem synthesis.[79]

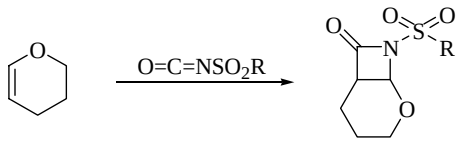
**Scheme 2.08:** Dehydrative ring closure of sulfonamido carboxylic acid **42**.[79]



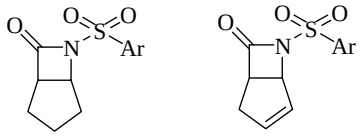

**42** **43**

Barrett demonstrated that bicyclic *N*-sulfonyl derivatives **45-47** could be prepared by olefin-isocyanate cycloaddition as depicted in *Scheme 2.09*. None of these products were examined for biological activity, however.[77]

**Scheme 2.09:** β-Lactam synthesis via olefin-isocyanate cycloaddition.[43]



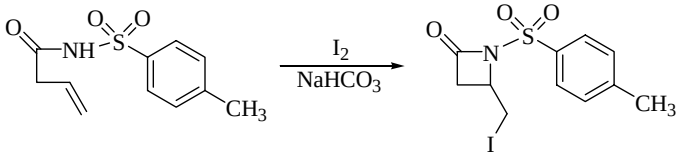

**44** **45**



**46** **47**

Biloski employed a different approach for closing the β-lactam ring of compound **49** as seen in *Scheme 2.10*. Here, alkenyl amide **48** underwent iodocyclization in aqueous base to afford the β-lactam product.[80]

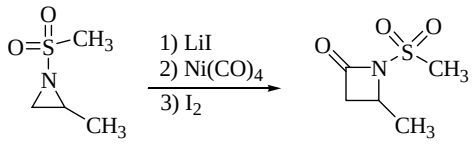
**Scheme 2.10:** β-Lactam synthesis via base-mediated iodocyclization.[80]




**48** **49**

In an isolated report, *N*-methanesulfonyl lactam **50** was prepared, albeit in poor yield, by a transition metal-mediated CO insertion of *N*-mesylaziridine as illustrated below (*Scheme 2.11*). The method was not extended to other sulfonyl substrates or to more complex systems, and would appear to be rather limited in scope.[81]

**Scheme 2.11:** β-Lactam synthesis via Ni-mediated CO insertion.[81]

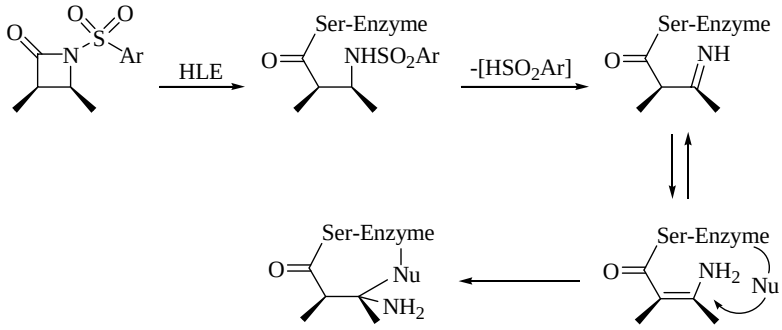



**50**

Of the nearly 600 *N*-sulfonyl β-lactams that have been reported, relatively few have been examined for biological properties. This may be related to the fact that the compounds lack the ionic residues needed for binding to bacterial PBP's, and consequently, should be devoid of antibacterial activity. However, *N*-arylsulfonyl β-lactams have been shown to be inhibitors of other serine proteases, including human leukocyte elastase (HLE).[82]

The postulated mechanism for elastase inhibition by *N*-arylsulfonyl β-lactams is depicted in *Scheme 2.12*.[78a] The sulfonyl moiety is responsible for activating the β-lactam ring towards nucleophilic ring opening by a serine residue, and then after lactam ring opening, for serving as a stable leaving group to create an imine intermediate by loss of arylsulfinic acid. It is proposed that tautomerization of the resulting imine to the enamine enables a second active site nucleophile (perhaps a histidine) to add, giving a crosslinked adduct that is highly resistant to hydrolysis. Attempts to prove the mechanism by trapping of the arylsulfinic acid were inconclusive.

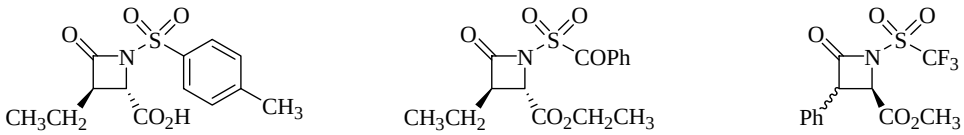
**Scheme 2.12:** Proposed mechanism of *N*-arylsulfonyl β-lactams inhibitors of human leukocyte elastase.[78a]




However, a high resolution crystal structure of a stable acyl-enzyme adduct formed between *N*-arylsulfonyl lactam **51** (*Figure 2.11*) and elastase from porcine pancreas has been reported.[83] The interesting feature found in the structure is that the ester functionality formed between the serine and lactam

moiety does not occupy the oxyanion hole in the active site. This could explain the high resistance this adduct has toward hydrolysis and release.

**Figure 2.11:** *N*-Sulfonyl β-lactams inhibitors of porcine pancreatic elastase.[78,81,83]

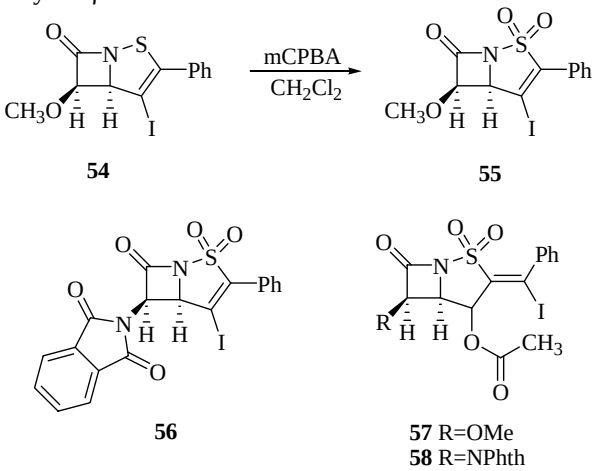


**51** **52** **53**

In addition to the *N*-arylsulfonyl compounds, *N*-acylsulfonyl lactam **52** (*Figure 2.11*) is also an inhibitor of porcine pancreatic elastase.[81] The trifluoromethylsulfonyl lactam **53** (*Figure 2.11*), however, has no affect on human leukocyte elastase activity.[78a] Along with the elastase (serine protease) activity, Merck has shown interest in using *N*-alkylsulfonyl and *N*-arylsulfonyl β-lactams as anticholesteremic agents through their inhibition of HMG-CoA synthase.[84]

Some interesting bicyclic *N*-sulfonyl β-lactams have been reported as well. The N-S-fused bicyclic lactam **55** was prepared by oxidation of sulfenamide **54** with *m*-chloroperbenzoic acid (*Scheme 2.13*).[78b-d] The compound was devoid of antibacterial activity despite its highly electrophilic β-lactam ring. However, the phthalimide analogue **56** (see *Scheme 2.13*), interestingly enough, was found to possess weak antibacterial activity against *Staphylococcus aureus* and *Vibrio cholerae*; the mechanism for this activity was not investigated.

**Scheme 2.13:** N-S-Fused bicyclic β-lactams.[78b-d]



**54** **55**

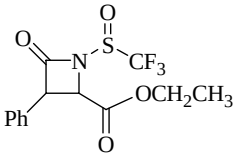
**56** **57** R=OMe
**58** R=NPhth

The highly functionalized bicyclic lactams **57** and **58** (see *Scheme 2.13*) could likewise be prepared by a similar sulfoxidation of the sulfenamides. These compounds were tested for antimicrobial properties, but did not reveal any activity against a variety of Gram-positive and Gram-negative bacteria.

### 2.4 N-SOR β-Lactams

*N*-Trifluoromethylsulfinyl lactam **59** (*Figure 2.12*) appears to be the only example of an *N*-sulfinyl β-lactam, being found in a Merck patent on the use of substituted azetidinones as anti-inflammatory and anti-degenerative agents.[85] However, there is no information about whether the compound possesses these biological properties.

**Figure 2.12:** *N*-Trifluoromethylsulfinyl β-lactam.[85]

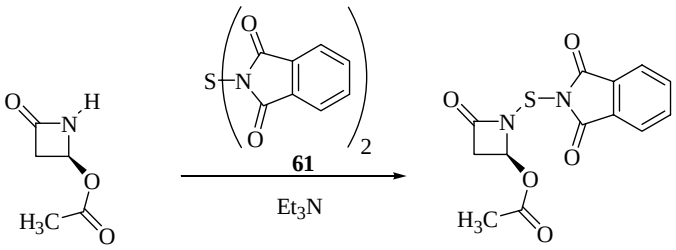


**59**

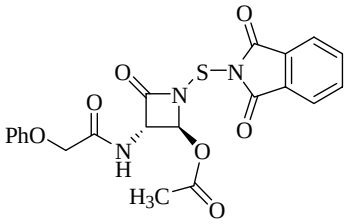
### 2.5 N-SX β-Lactams

A variety of structurally interesting molecules have been described in which an *S*-heteromoiety is attached to a β-lactam nitrogen. Typically, these compounds have been isolated as synthetic intermediates or as unexpected reaction products. Miller developed an efficient method for preparing thiophthalimide analogues such as **62** and **63** (see *Scheme 2.14*). The procedure entails the reaction of the *N*-protio lactam with sulfur *bis*-phthalimide **61** in the presence of a catalytic quantity of triethylamine.[86]

**Scheme 2.14:** Preparation of *N*-phthalimidothio β-lactams.[86]



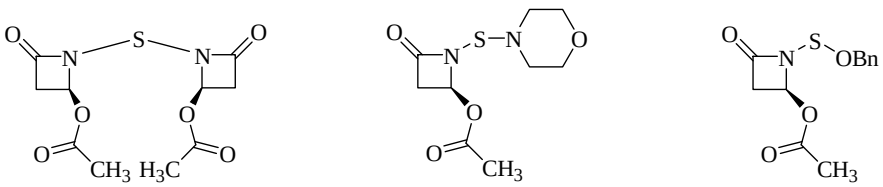
**60** **61** **62**



**63**

The sulfur linked *bis*-β-lactam adduct **64** (*Figure 2.13*) can also be prepared using this method under certain reaction conditions. Phthalimide lactam **62** is presumably an intermediate of the reaction.[86] Phthalimide lactam **62** can likewise be reacted with other nitrogen nucleophiles such as morpholine to give derivative **65**, or with benzyl alcohol in the presence of triethylamine to afford product **66** (*Figure 2.13*). No biological data was reported for any of these heterosubstituted β-lactam derivatives.

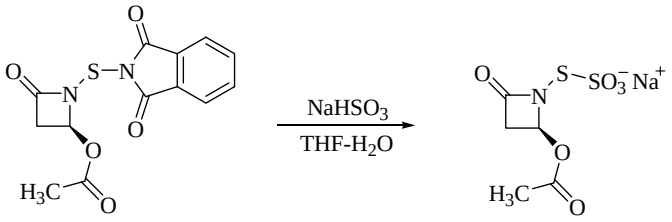
**Figure 2.13:** Heterosubstituted *N*-thiolated β-lactams **64-66**.[56]



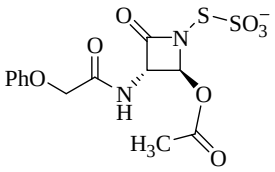
**64** **65** **66**

Miller also demonstrated that the phthalimide exchange process could be carried out using bisulfite anion as a means to prepare *N*-thiosulfuric acid derivatives such as **67** and **68** (see *Scheme 2.15*). Although only these two examples were shown, the reaction would appear to be general. Thiosulfate lactam **68** was tested for antibacterial properties but found to be inactive, which contrasts sharply with the potent antimicrobial effects of the corresponding monobactam compound (the N-$SO_3H$ analogue).[87] This confirmed the correlation between the electronegativity of the *N*-heteroatom of a monocyclic β-lactam and biological activity. Nevertheless, this dramatic difference in antibacterial activity in this case may also be due to differences in the way the molecules fit into the enzyme active site.[88]

**Scheme 2.15:** Preparation of heterosubstituted *N*-thiolated β-lactams via phthalimide exchange.[87]



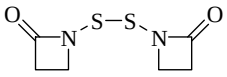

**62** **67**



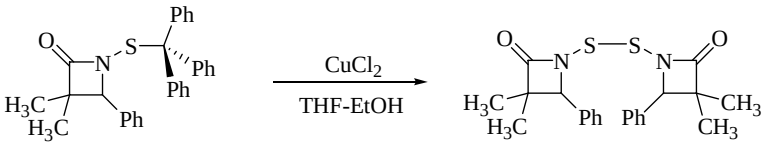
**68**

There are only two examples of disulfide-linked β-lactams of type **69** (see *Scheme 2.16*), both of which were formed as unexpected reaction products. In the first example, dimeric lactam **71** was obtained by heating *S*-trityl lactam **70** at 75°C in the presence of cupric chloride (*Scheme 2.16*).[89] The authors make no mention as to whether disulfide **71** was tested for antimicrobial activity.

**Scheme 2.16:** Synthesis of disulfide-linked β-lactams.[89]



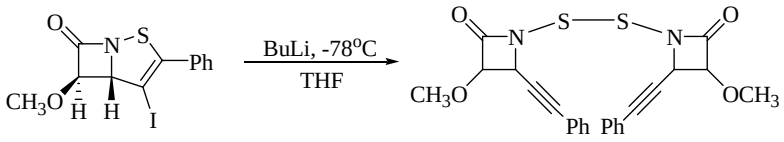
**69**




**70** **71**

Turos reported the formation of disulfide-linked lactam **72** by low temperature lithiation of vinyl iodide **54** as shown in *Scheme 2.17*. The reaction takes place via an initial metal-halogen exchange, followed by spontaneous ring-opening and dimerization. Compound **72** was screened for antibacterial properties, but found to be inactive against a wide variety of common Gram-positive and Gram-negative microbes.[78c]

**Scheme 2.17:** Synthesis of disulfide-linked lactam **72**.[78c]

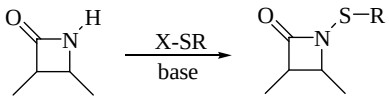



**54** **72**

## 2.6 N-SR β-Lactams

There have been a number of studies on β-lactams having an *N*-sulfenyl moiety as shown in *Scheme 2.18* for structure **74**. *N*-Sulfenyl β-lactams can be readily obtained by thiolation of an $N_1$ unsubstituted lactam **73** with an appropriate sulfenylating agent and base.

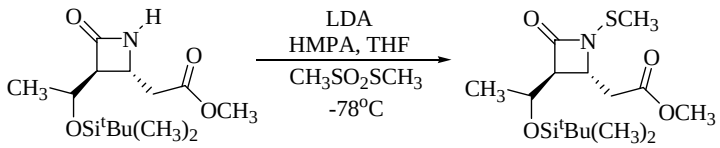
**Scheme 2.18:** Preparation of *N*-sulfenyl β-lactams.



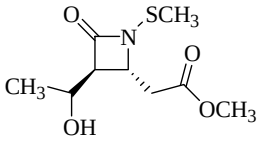

**73** **74**

Shah and Cama reported a procedure for placing a methylthio substituent onto a β-lactam nitrogen by treating the N-H precursor **75** with lithium diisopropylamide (LDA) in a mixture of hexamethylphosphoramide (HMPA) in tetrahydrofuran (THF) at low temperature, followed by trapping the amide anion with methyl methanethiolsulfonate (*Scheme 2.19*).[90]

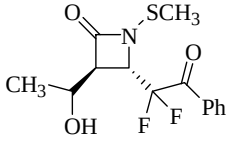
**Scheme 2.19:** *N*-Methylthiolation of β-lactams with methanethiosulfonate.[90]



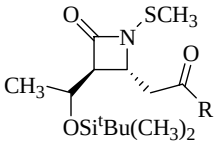

**75** **76**



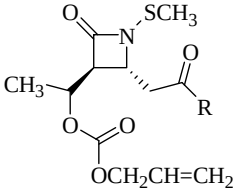
**77**



**78**



**79** R=OH
**80** R=Ph

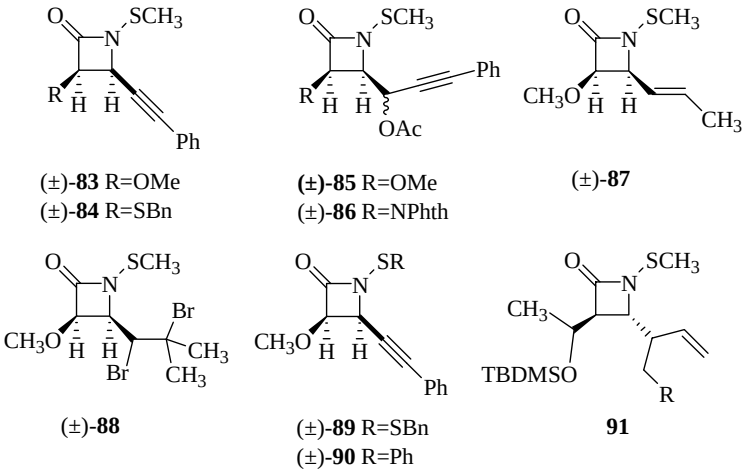


**81** R=OMe
**82** R=Ph

*N*-Methylthio lactams are stable to acid and base but can be easily deprotected with a thiolate anion. The authors showed a selection of *N*-methylthio derivatives **76**-**82** (see *Scheme 2.19*) that were synthesized, but did not report on their biological properties.

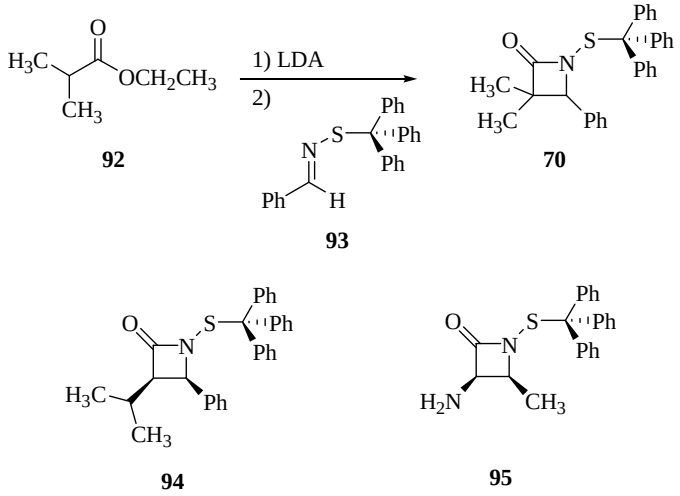
Turos has applied this same method to make *N*-alkylthio lactams **83**-**90** (*Figure 2.14*) for use as intermediates in synthesis of bicyclic β-lactams.[78b-d] For the *N*-benzylthio and *N*-phenylthio lactams **89** and **90**, respectively, the *N*-thiolation was done by trapping the amide anion with the sulfenyl chloride (generated from halogenation of the disulfide with chlorine gas). Similarly, researchers at Shionogi research laboratories prepared a series of N-methylthio lactams **91** (*Figure 2.14*) as intermediates in a carbapenem synthesis (X=F, CN, OH, $O_2CCH_3$, $OSiMe_2{}^tBu$, $OSi(CH_3)_3$, or I). None of these lactams appear to have been tested for antibacterial activity.[91]

**Figure 2.14:** Examples on *N*-methylthio monocyclic β-lactams.[78b-d]



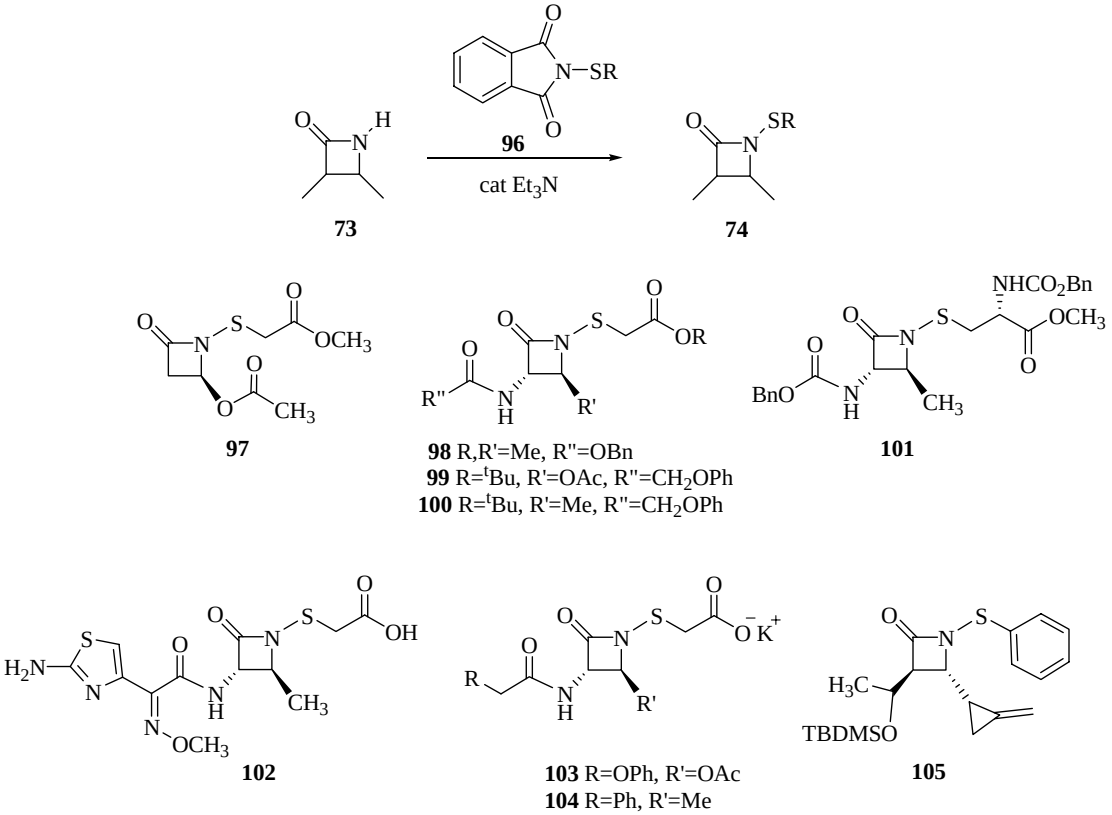

Hart introduced a procedure for preparing *S*-tritylated lactams via addition of an ester enolate to S-trityl sulfenimines such as **70**. This procedure favors formation of the *cis* β-lactam product. While the method works well for *S*-trityl lactams **70**, **94,** and **95**, attempts to apply the reaction to *S*-phenyl sulfenimines gave the *N*-unsubstituted β-lactam rather than the desired *N*-thiolated product (*Scheme 2.20*).[89]

**Scheme 2.20:** Preparation of *S*-trityl β-lactams via enolate-sulfenimine addition.[89]



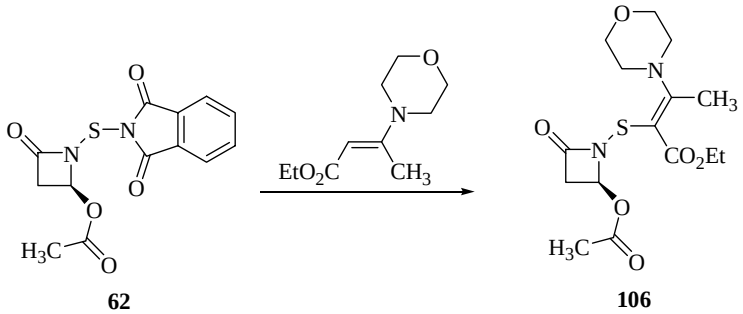
Miller reported a mild *N*-thiolation procedure that made use of *N*-thiophthalimides **96** and catalytic triethylamine. The methodology allows a large assortment of organothio groups to be introduced onto the lactam, as illustrated in the formation of ester and carboxylic acid derivatives **97**-**104** (see *Scheme 2.21*).[87] Kilburn and colleagues recently used this method to prepare *N*-phenylthio lactam **105** for studies on radical cyclizations.[92] The authors did not mention if the compound was examined for biological activity.

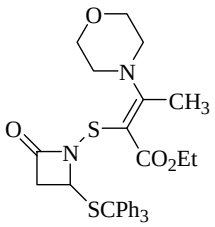
**Scheme 2.21:** Sulfenylation of *N*-protio β-lactams with *N*-thiophthalimide.[87,92]



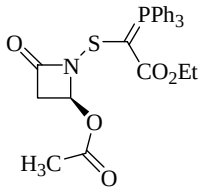

As described earlier, phthalimide-containing compounds such as **62** can be used to access a range of other *S*-derivatives. Miller showed that carbon nucleophiles readily displace the phthalimide group from **62** to deliver *N*-sulfenylated β-lactams **106**-**108** (see *Scheme 2.22*).[87]

**Scheme 2.22:** Displacement of phthalimide by a carbon nucleophile.[87]





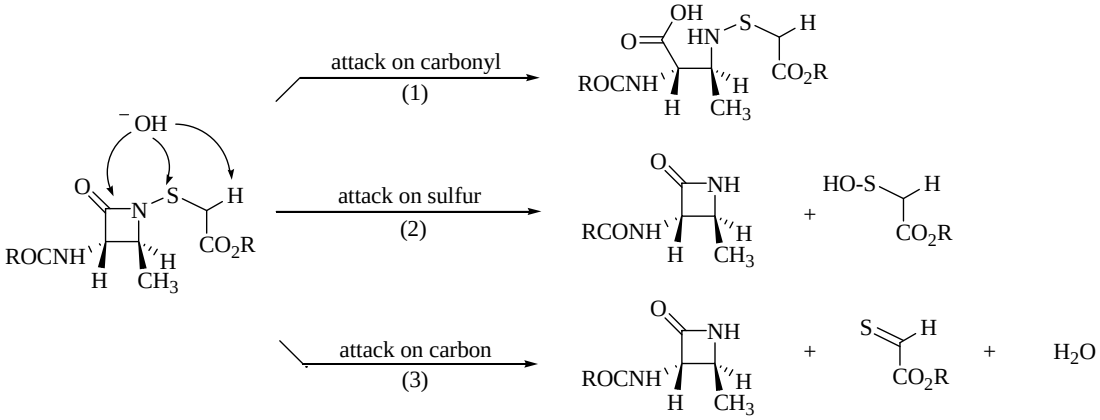
**107**



**108**

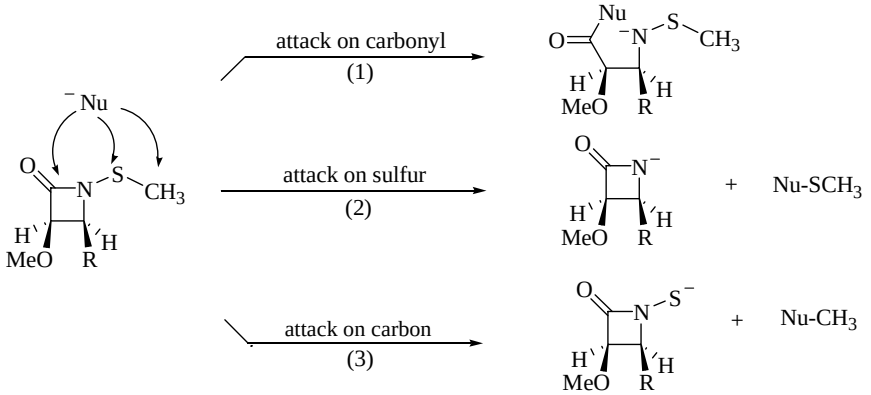
The biological studies that have been conducted on the *N*-sulfenyl β-lactams described have led to some surprising, and potentially important, findings. The first studies were those carried out by Miller that compared the synthetic N-$SCH_2CO_2H$ lactams (thiamazins) made in his laboratory to their N-$OCH_2CO_2H$ counterparts (oxamazins).[87,88,93] The oxamazins are strong antimicrobial agents, but the thiamazins were found to be devoid of antibacterial activity. It was postulated that this remarkable difference in biological properties may be due to the longer N-S bond which prevents the thiamazin binding into the active site of the transpeptidase enzyme. Miller has noted that the carbonyl stretching frequencies of the active oxamazins are consistently 10-20 $cm^{-1}$ higher than those of the inactive thiamazins, suggesting that there may possibly be an electronic basis for this difference as well. Miller also studied the stability of *S*-thioacetic acid lactams under basic conditions, and identified three decomposition pathways (*Scheme 2.23*): (1) hydroxide ion addition to the carbonyl to give the ring-opened carboxylic acid, (2) $S_N2$ attack at sulfur to cleave the N-S bond, and (3) deprotonation of the acidic carboxyl α-proton to provide the NH lactam through loss of the thioaldehyde. Miller determined that approximately 50% of the thiamazin is cleaved at the N-S bond (pathways 2 and 3) at pH 11, and the other 50% by lactam ring opening (pathway 1).[88] The thiamazins are stable below pH 10.

**Scheme 2.23:** Decomposition pathways of thiamazins by hydroxide ion.[88]




Recently, Turos has examined *N*-methylthio lactams **84-91** for antibacterial activity against a variety of common Gram-positive and Gram-negative microorganisms. Curiously, lactam **84** was found to strongly inhibit *Staphylococcus aureus*.[26a-c] The *N*-methylthio substituent of **84** is required for biological activity, since the N-H and N-$SO_2CH_3$ derivatives are totally inactive. The *S*-benzyl compound **90** also demonstrates appreciable antimicrobial activity against the same microorganisms. The antibacterial activity of these *N*-alkylthio lactams is surprising, given the total absence of any ionizable ring functionality in the molecules (required for binding to transpeptidases) and the close similarity they have in structure to the inactive thiamazins (cf. Scheme 2.23). This suggests the compounds may be operating through a different mode of action. Further investigations into this novel family of antibacterials is warranted and is the focus of chapters III-V.

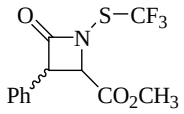
**Scheme 2.24:** Mechanisms of reactivity of *N*-thiolated β-lactams towards nucleophiles.




The *N*-methylthio β-lactams, like the thiamazins, differ from traditional β-lactams in that there are at least three sites vunerable to attack by a biological nucleophile. The types of nucleophilic reactions that could in effect provide information on the bacterial mode of action are: (1) addition to the β-lactam carbonyl, (2) substitution on the sulfur center, or (3) $S_N2$ displacement at the *N*-methylthio carbon atom (*Scheme 2.24*). Chemical studies and investgations into the intrinsic nataure of *N*-methylthio β-lactams in *S. aureus* is further detailed in chapter IV

Merck laboratories have conducted tests on *N*-trifluoromethylthio lactam **109** (*Figure 2.15*) against human leukocyte elastase; however, this lactam was found to have no inhibitory activity ($IC_{50}$ = 8 mg/mL).[78a]

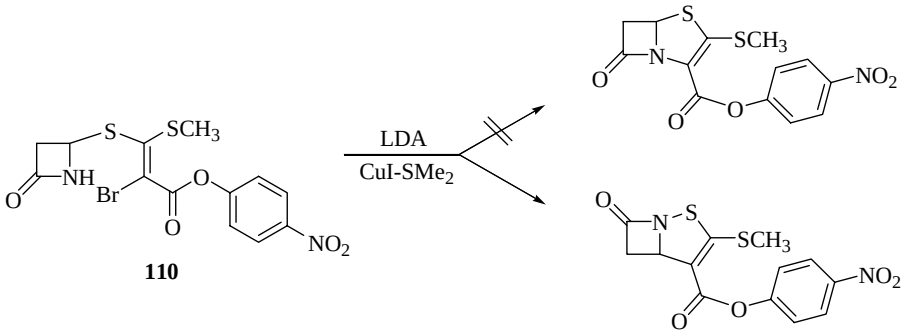
**Figure 2.15:** *N*-Trifluoromethylthio β-lactam investigated by Merck laboratories.[78a]




**109**

In addition to the monocyclic systems discussed above, there have been several reports of bicyclic compounds of this class that have the N-S bond at the site of ring fusion. The first example, *N*-fused β-lactam derivative **111**, was formed as an unexpected product during attempts to cyclize lactam **111** to the ring-closed penem (*Scheme 2.25*). The reaction occurs via a rearrangement process. No information was reported about biological testing on this adduct.[94]

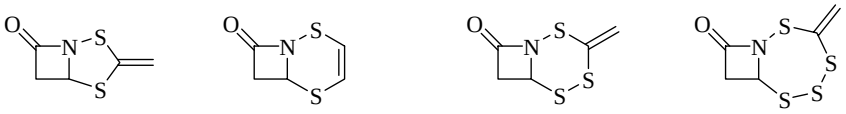
**Scheme 2.25:** Cyclization of **110** to the non-penem **111**.[94]




**110** **111**

In 1991, the Pfizer laboratories reported the synthesis of some related N-S fused lactam rings represented by structures **112** and **113** in studies on human leukocyte elastase inhibitors (*Figure 2.16*).[95]

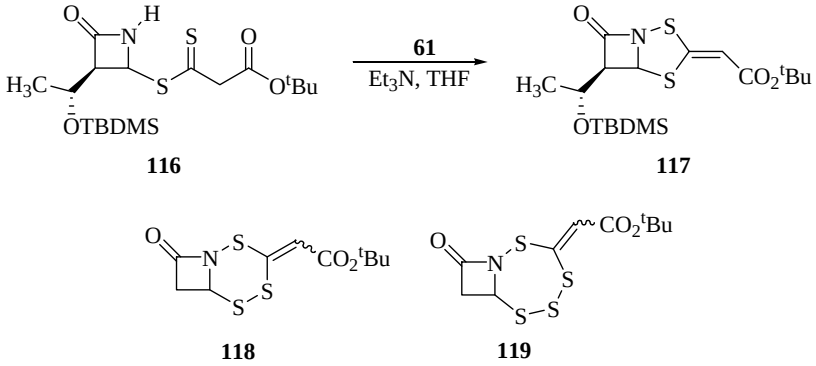
**Figure 2.16:** N-S fused bicyclic β-lactams of Pfizer.[95]



**112** **113** **114** **115**

Compound **117** was synthesized by cyclizaton of lactam **116** with sulfur *bis*-phthalimide **61** (*Scheme 2.26*). The mechanism of the ring closure involved formation of disulfide **118** and trisulfide **119** intermediates (see *Scheme 2.26*), which could be isolated and independently converted to product **117** using triphenylphosphine.

**Scheme 2.26:** Preparation of **117** using sulfur *bis*-phthalimide.[95]

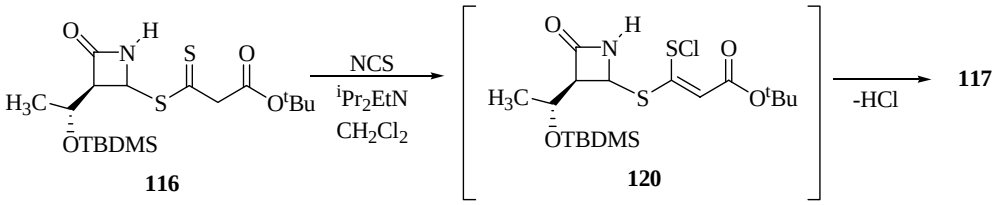



**116** **117**

**118** **119**

Alternatively, the reaction of **116** to **117** (*Scheme 2.27*) could be carried out using *N*-chlorosuccinimide (NCS) proceeding through a sulfenyl halide intermediate **120**.[95] Although only one olefin isomer is shown for **117**, the reaction in fact gave both geometric isomers which could be separated and independently examined for antibacterial activity.

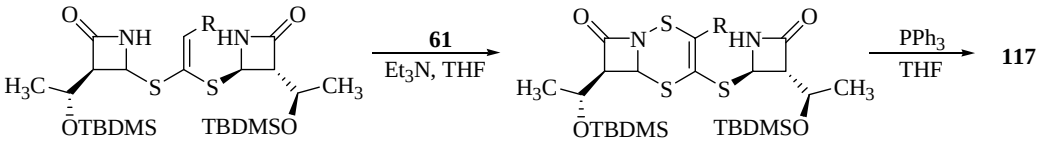
**Scheme 2.27:** Preparation of **117** using *N*-chlorosuccinimide.[95]




**116** **120**

The Pfizer group also synthesized the six-membered ring analogue **122** through a similar cyclization starting from monocyclic β-lactam **121** (*Scheme 2.28*). Product **122** was then used to prepare five- membered ring lactam **117** through a sulfur extrusion process. The conversion of **122** to **117** is thought to proceed through a fascinating, and unexpected, rearrangement process described in the paper.

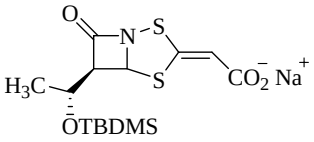
**Scheme 2.28:** Preparation of **117** using triphenylphosphine.[95]




**121** R=$CO_2{}^tBu$ **122** R=$CO_2{}^tBu$

Both geometric isomers of ester compound **117** were found to be effective inhibitors of human leukocyte elastase ($K_i$ = 1.5 μM and 4.0 μM, respectively). The sodium salt of the carboxylate, lactam **123** (*Figure 2.17*) , was prepared from ester **117** and found to be devoid of antibacterial activity. Thus, to have elastase activity, the ester rather than the anionically-charged carboxylate group is required on these lactams.[95]

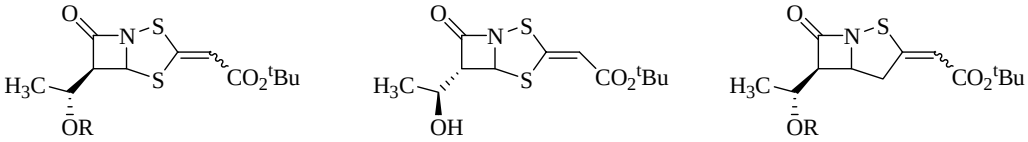
**Figure 2.17:** Human leukocyte elastase inhibitor **123**.



**123**

In a subsequent paper, Pfizer studied the elastase inhibitory properties of a series of related bicyclic lactams **124-129** (*Figure 2.18*), and compared their observed activities to their relative rates of base hydrolysis. Blocking the carbonyl with a bulky side chain as in carbonate **126** increases elastase inhibition while decreasing susceptibility to base hydrolysis. As anticipated, reversing the absolute chirality of the bicyclic ring system, as observed for lactam **127** versus **125**, completely destroys the biological activity. The study also indicated that replacement of the ring thioether sulfur atom for a carbon atom as for compounds **128** and **129** slightly increases both the rate of hydrolysis of the lactam ring and the elastase inhibition constant $K_i$. The N-S linkage makes the lactam ring more susceptible to base hydrolysis, but does not significantly affect the elastase inhibitory ability. Thus, enhancing the reactivity of the β-lactam ring does not increase the compound's potency as an elastase inhibitor, suggesting that the rate determining step in elastase inhibition is not nucleophilic attack on the β-lactam carbonyl center (*vida supra*).[96]

**Figure 2.18:** Bicyclic *N*-thiolated β-lactam elastase inhibitors **124-129**.[96]



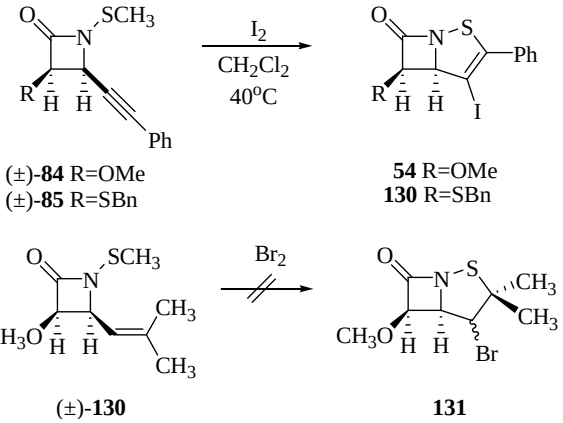
(*Z*)-**124** R=H
(*E*)-**125** R=H
(*E*)-**126** R=COBn

**127**

(*Z*)-**128** R=TBDMS
(*E*)-**129** R=H

Turos developed a synthesis of structurally related bicyclic lactams **54** and **130** by iodocyclization of unsaturated *N*-methylthio β-lactams **84** and **85** (*Scheme 2.29*).[78b-d] Attempts to construct isopenams **131** by halocyclization of alkenyl sulfenamide **130** were unsuccessful.

**Scheme 2.29:** Preparation of bicyclic lactams via halocyclization.[78b-d]




(±)-**84** R=OMe
(±)-**85** R=SBn

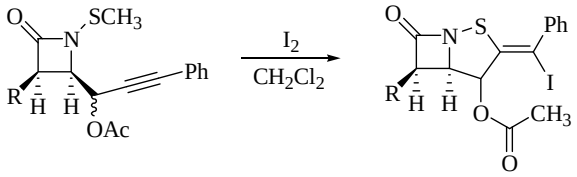
**54** R=OMe
**130** R=SBn

(±)-**130**

**131**

However, clavulanic acid-type ring systems **133**, **134**, and **135** were prepared from monocyclic *N*-methylthio precursor **85**, **86**, and **132** (*Scheme 2.30*).

**Scheme 2.30:** Halocyclization of lactams **85**, **86**, and **132**.[78b-d]




**(±)-85** R=OMe
**(±)-86** R=NPhth
**(±)-132** R=SBn

**(±)-133** R=OMe
**(±)-134** R=NPhth
**(±)-135** R=SBn

These bicyclic N-S fused compounds have a highly electrophilic β-lactam ring, yet are stable over a wide pH range (pH 1 to pH 10). Computational experiments indicate that they have about the same thermodynamic stability as the classical penicillins and penems. *Ab initio* calculations indicate that the β-lactam in these N-S fused systems is slightly more twisted due to a less highly pyramidalized nitrogen center, and have a lower LUMO energy than that of the antibiotics. Despite the structural similarities and higher electrophilicities relative to the known β-lactam drugs, lactams **54**, **131**, **133, 134**, and **135** have no antibacterial properties.[76b,76c]

## 2.7 Conclusions

The discovery of the monobactams in the early 1980's opened new avenues of investigation in β-lactam antibiotics research. This lead to exciting advances and to the introduction of aztreonam as an important clinical agent for control of Gram-negative bacterial infections. The interest in *N*-thiolated β-lactams was further heightened in the mid-1990's with the development of *N*-arylsulfonyl β-lactams as elastase inhibitors. Most recently, the discovery that *N*-methylthio β-lactams have inhibitory activity against *S. aureus* by a unique mode of action, opens the door to new investigations. As such, the focus of the following chapters will be on the investigations into the chemical and biological properties of *N*-alkylthio β-lactams.

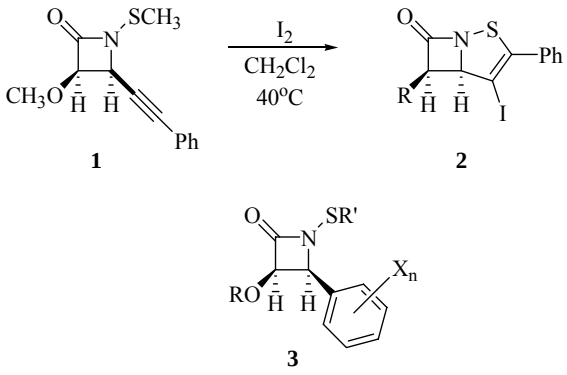
# CHAPTER III

# SYNTHESIS AND BIOLOGICAL PROPERTIES OF $C_4$ ARYL SUBSTITUTED *N*-THIOLATED β-LACTAMS

## 3.1 Introduction

As alluded to in chapters I and II, *N*-methylthio β-lactam **1,** a synthetic precursor to the clavulanic acid-type ring system **2** seen below, was identified as a substance possessing specific biological activity against *Staphylococcus aureus*.[78b-d] Although only 8 analogues of **1** have been reported previously and the antimicrobial screening ad been rather limited, the novelty of this new class of antibiotics warranted their further investigation. This chapter examines for the first time, the structure-activity relationship (SAR) of *N*-thiolated β-lactams antibiotics containing functionalized aromatic residues at the $C_4$ position. The synthesis and biological evaluation of the lactams against a panel consisting of 33 bacteria species and 10 strains of methicillin-resistant *S. aureus* (MRSA) is reported. The effect on cell growth and cell survival is also described. The information gathered from the experiments presented in this chapter can in effect be applied towards the understanding of the lactam's mode of action in bacteria.

**Scheme 3.01:** Iodocyclization of *N*-methylthio lactam **1**.

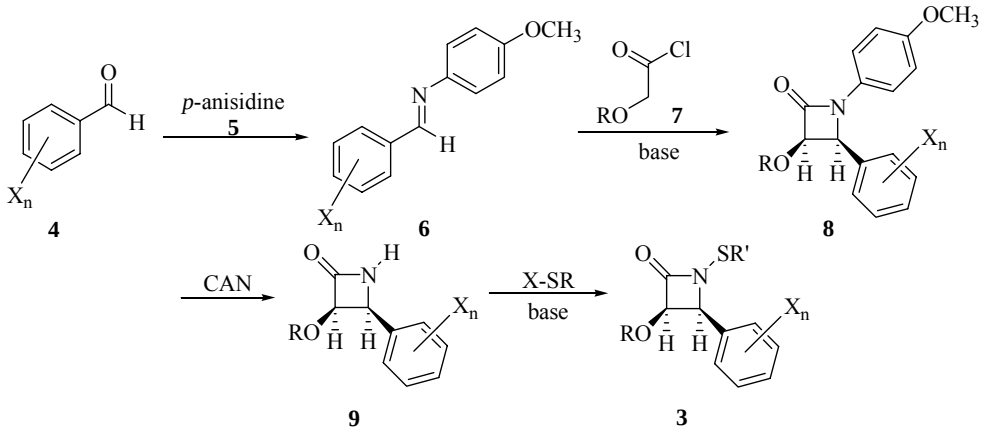



**1** **2** **3**

## 3.2 Synthesis of $C_4$ Aryl Substituted *N*-Thiolated β-Lactams

$C_4$ aryl substituted β-lactams (**3**) were prepared by the series of reactions illustrated in *Scheme 3.01*. The four stage sequence was initiated by the synthesis of *N*-(4-methoxyphenyl)imine (**6**) from aryl aldehyde **4** and *p*-anisidine. Staudinger coupling of an acid chloride (**7**) and imine by a formal [2+2] cycloaddition afforded exclusively the *cis*-(3*S*,4*R*)-substituted β-lactam **8** as a racemic mixture. Following oxidative cleavage of the *p*-anisyl residue by aqueous ceric ammonium nitrate, the *N*-protio lactam **9** was thiolated using readily available sulfenylating reagents to provide the *N*-thiolated β-lactams (**3**) in four steps. The yields of the reactions were variable depending on the substituents located at $C_3$ and $C_4$ positions of the ring and the conditions. The following section details each reaction involved in the synthesis of *N*-thiolated β-lactams.

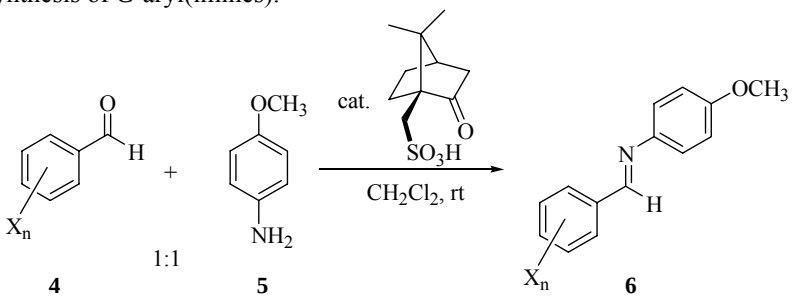
**Scheme 3.02:** General synthetic route to lactam **3**.




3.2.1 Synthesis of *C*-Aryl(imines) **6**

*N*-(4-Methoxyphenyl)imines (**6**) were prepared from the condensation of aryl aldehyde **4** and *p*-anisidine (**5**). Prior to the reaction, the crude *p*-anisidine was recrystallized in water heated to ~60°C and dried *in vacuo*.[97] While most aldehydes were used without further purification, when necessary, the acid contaminant was removed by washing with 10% sodium bicarbonate or distilling at atmospheric pressure.[97] The synthetic protocol was identical for all the mono- and multisubstituted *C*-aryl(imines). The aldehyde and *p*-anisidine were dissolved in methylene chloride and stirred at room temperature for 1-2 hrs. Approximately 1 mg of camphorsulfonic acid (CSA) was added to the mixture and heating to reflux was sometimes applied to promote the condensing of the starting materials. In most instances, conversion to the imine was completed within 1 hr and could be followed by TLC.

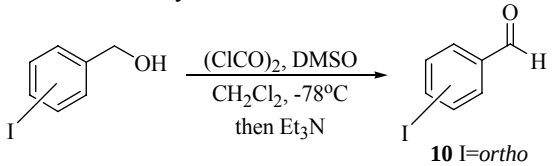
**Scheme 3.03:** Synthesis of *C*-aryl(imines).




X = H, F, Cl, Br, I, $NO_2$, $CH_3$, $OCH_3$, $O_2CR$

In cases when the aldehydes were not commercially available, the imine precursors could be generated by oxidation of the corresponding benzyl alcohols using oxalyl chloride and DMSO (*Scheme 3.04*).[98] Aldehydes synthesized by this methodology included 2-, 3-, and 4-iodobenzaldehyde (**10**-**12**).

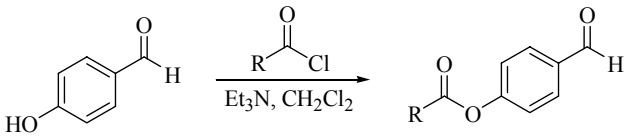
**Scheme 3.04:** Swern oxidation of iodo-benzyl alcohols.




**10** I=*ortho*
**11** I=*meta*
**12** I=*para*

Noncommercial aldeydes bearing an ester functional (**13**,**14**) were prepared by the acylation of *p*-hydroxybenzaldehyde. β-Lactams generated from **13** and **14** were used to examine the *in vitro* performance of *N*-thiolated β-lactams possessing a lipophilic, acyloxy substituent while serving as an intermediate for the preparation of a *p*-phenol derivative.

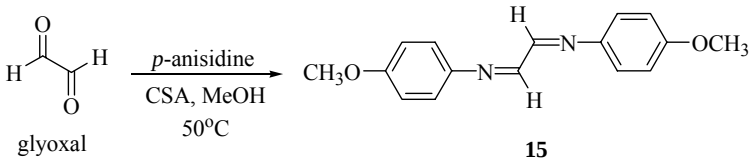
**Scheme 3.05:** Esterification of *p*-hydroxybenzaldehyde.




**13** R=$(CH_2)_4CH_3$
**14** R=$CHCH_2$

*Bis*-imine **15** was synthesized by the condensation of the dialdehyde glyoxal and *p*-anisidine, and used to generate *bis*-β-lactam analogues. Low solubility in organic solvents, however, limited the usefulness of **15** as an intermediate for making β-lactams.

**Scheme 3.06:** Synthesis of *bis*-imine **15**.

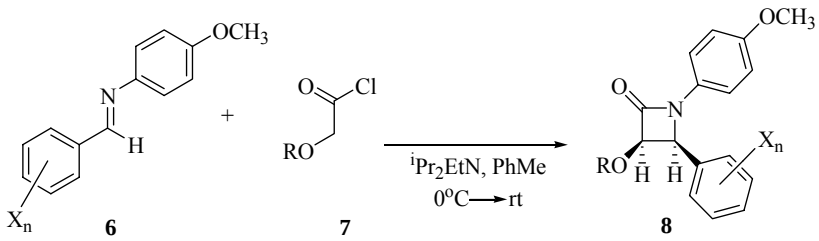



**15**

3.2.2 Synthesis of *N*-Aryl Protected β-Lactams **6** by Staudinger Coupling

The most frequently described procedure for preparing monocyclic β-lactams is Staudinger coupling. This reaction involves a formal [2+2] cycloaddition of an acid chloride (**5**) or an activated carboxylic acid[51c] to an imine (**4**, eg, Schiff base), and was the methodology employed to synthesize *N*-aryl protected β-lactams **6** (*Scheme 3.05*).

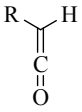
**Scheme 3.07:** Synthesis of *N*-aryl protected β-lactams by Staudinger [2+2] condensation.




**6** **7** **8**

The mechanism of β-lactam formation in the synthesis of **6** does not entail a direct acylation of the imine with an acid chloride, but rather a ketene cycloaddition. The ketene forms by deprotonation of an activated acid with a Lewis base such as ethyldiisopropylamine. If a base is absent or added after the imine and acid are combined, the ketene does not form and the cycloaddition generally occurs via direct coupling of **4** and **5** resulting in a β-lactam with a *trans* configuration.[51c] *Table 3.01* categorizes the three types of ketenes that undergo Staudinger coupling. The substituents of **4** and **5** ultimately determine the stereochemical outcome of the reaction. Acid chlorides applied to the synthesis of **6** give rise to exclusively the Bose-Evans ketene which confers the *cis* conformation when coupled with a diaryl imine (**4**).[51c] This was verified for all $C_4$ aryl analogues from the chemical shifts in the $^1H$ NMR spectra.

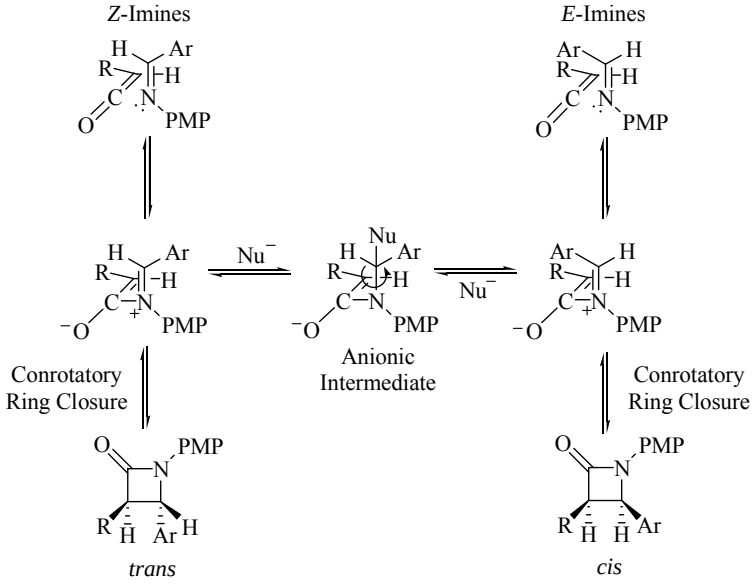
**Table 3.01:** Stereochemical outcome of the Staudinger reaction of ketenes and imines.[51c]

| |  R= | Diaryl Imines product | Alkyaryl Imine product | Glyoxalic Imines product | Imidates Imine product |
|---|---|---|---|---|---|
| Bose-Evans ketenes | OR, NHR, $N_3$, F | *cis* | *cis* | *cis* | *trans* |
| Sheehan ketenes | vinyl, Phth | *trans* | *cis* | *cis* | *trans* |
| Moore ketenes | Cl, Br, alkyl, Ar, SR $S(O)_nR$ | *trans* | *trans* | *cis* | *trans* |

*N*-Aryl imines (**4**) exist primarily in the *E*-configuration and give rise to *cis* β-lactams. Although thermodynamically less stable, kinetics allows the *cis* product to be generated via an anionic intermediate which is stabilized by the electron-rich groups of the Bose-Evans ketene. Conversely, if a *Z*-imine was used in the reaction, a *trans* β-lactam will result depending on the stability of the intermediate.

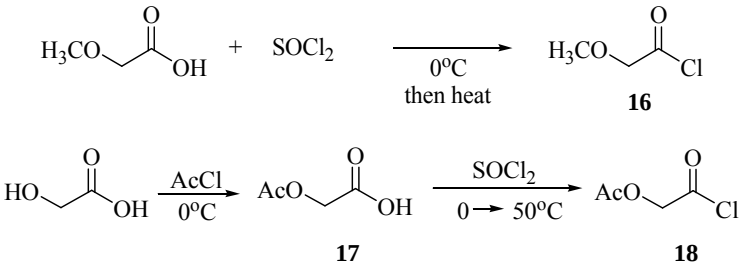
The stereochemical outcome of these reactions is rationalized by the pathway depicted in *Scheme 3.08*. Orthogonal attack of the imine on the least hindered side of the ketene carbonyl center forms a zwitterionic intermediate which can (1) interconvert via the anionic intermediate, (2) revert back to the imine and ketene, or (3) lead to *cis* and *trans* β-lactams. Both the β-lactam and imine can interconvert as well, but only if the ring opens regenerating the zwitterionic species.

**Scheme 3.08:** Ketene-imine cycloaddition.



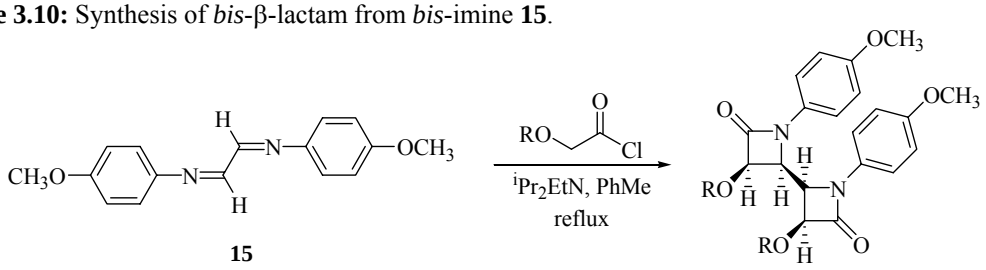

The ketene precursors (**16**) were synthesized from the corresponding carboxylic acid and thionyl chloride. This general method was used to make acid chlorides **16** and **18**. Although methoxyacetic acid was commercially available, acetoxyacetic acid (**17**) were prepared from glycolic acid and acetyl chloride in quantitative yield.

**Scheme 3.09:** Synthesis of acid chlorides **16** and **18**.




The yields of the Staudinger reaction involving acid chlorides **16** and **18** varied greatly depending on the acid chloride, solvent and temperature. Little variation was observed for the different imine derivatives or the type of base. Poor yields did however result when imine **15** was applied toward the syntheses of *bis*-β-lactam analogues.

**Scheme 3.10:** Synthesis of *bis*-β-lactam from *bis*-imine **15**.



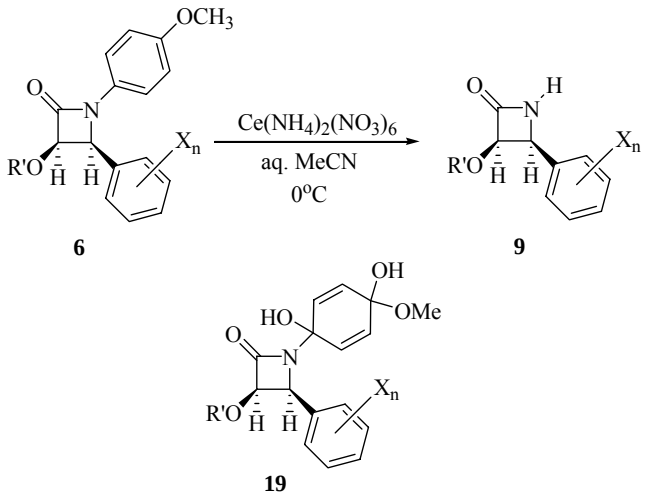

Staudinger coupling reactions typically require a tertiary amine base such as triethylamine or ethyldiisopropylamine (eg. Hünig's base). The latter was utilized for most reactions but could be replaced by triethylamine with little effect on the isolated yields. A minimum of 3 equivalents of base was needed to enable the reaction to go to completion. Mechanistically, the excess is required to (1) generate the ketene, (2) serve as a proton scavenger, and (3) act as a nucleophile ($Nu^-$) in formation of the anionic intermediate (*Scheme 3.06*).

Toluene became the solvent of choice when yields substantially increased for reactions involving acid chloride **16**. Another advantage the nonpolar medium offered was that the ammonium salt of ethyldiisopropylamine/triethylamine and sometimes the β-lactam itself were insoluble in toluene at room temperature. After filtration, the product could be cleanly isolated from the precipitated material or triturated from the concentrate of the reaction using diethyl ether or ice-cold methanol. The greatest effect on yield however, was the acid chloride component. Phenoxyacetyl chloride routinely gave the best yields followed by the methoxy- and acetoxy- counterparts. Of note, the acid chlorides and their respective ketenes were sensitive to heat and decomposed if the temperature was raised too high. Therefore, all reactions were performed at 0°C and allowed to warm to room temperature. Only in the syntheses of the *bis*-lactams analogues was reflux required, and generally gave low yields depending on the acid chloride.

3.2.3 Dearylation of β-Lactams **6** with Ceric Ammonium Nitrate

Kronenthal[99] reported a general method to remove the *p*-anisyl moiety of protected β-lactams using ceric ammonium nitrate (CAN) in aqueous acetonitrile. The same procedure was applied in the synthesis of *N*-methylthio β-lactams by the conversion of *N*-anisyl lactams **6** to *N*-protio lactams **9**. During the oxidation, the electron-rich aromatic ring permits cleavage by a radical generating species such as CAN. The reaction proceeds through intermediate **19** (*Scheme 3.11*) which decomposes to the dearylated β-lactam and benzoquinone when washed with a 5% sodium bisulfite solution.

**Scheme 3.11:** Oxidative dearylation of β-lactams **6**.



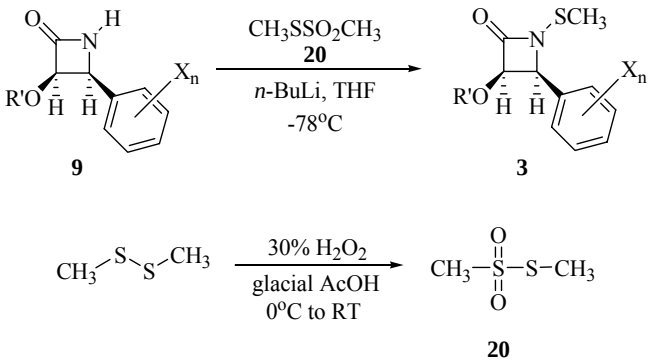

**6** **9** **19**

Deprotection of the $C_4$ aryl analogues **6** gave low to moderate yields depending on the solubility of the *N*-arylated β-lactam in acetonitrile. In most cases, the β-lactams did not dissolve and heating was required to completely solubilize the starting material. Resuspension of the heat solution in an ice water bath was necessary before the CAN solution could be added otherwise the reaction would fail. Most oxidations were complete after 30 minutes with light stirring followed by aqueous workup of the ethyl acetate extracts with sodium bicarbonate and sodium bisulfite.

3.2.4 *N*-Methylsulfenylation of β-Lactams **9**

Two methods were employed to incorporate the *N*-methylthio substituent onto the *N*-protio lactam **9**. Initially, the procedure reported by Shah and coworkers[90] was applied toward the synthesis of **3** (*Scheme 3.12*) using methylthiomesylate (**20**). Deprotonation of **9** with *n*-butyllithium at low temperature followed by lithium exchange with the methylthio moiety of **20** afforded the *N*-methylthio β-lactams in good yields. Methylthiomesylate was prepared by the oxidation of methyl disulfide with 30% hydrogen peroxide in accordance to an already established literature procedure.[90]

**Scheme 3.12:** Synthesis of *N*-methythio β-lactams and methythiomesylate.

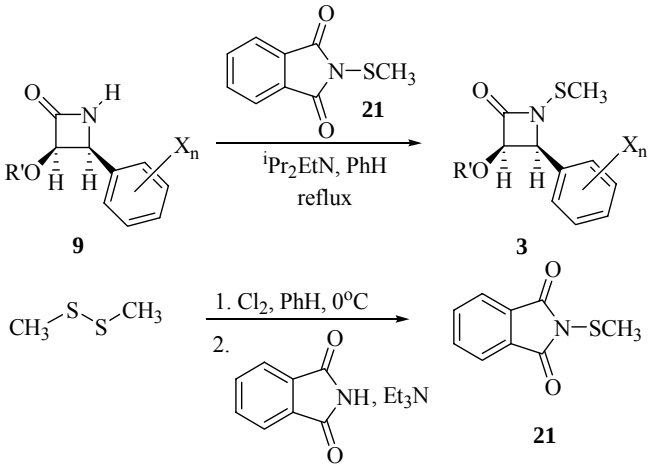



**9** **3** **20**

Another method of introducing a methythio substituent was reported by Miller using *N*-methylthiophthalimide (**21**).[86] The reaction employs a mild base such as triethylamine or Hünig's base under reflux conditions in a low boiling, nonpolar aprotic solvent. The synthesis of **21** was carried out following previously described literature procedures.[100] From methyl disulfide, chlorine gas was used to

generate the methylsulfenyl chloride *in situ* then cannulated into a flask containing 1 and 1.5 equivalents of phthalimide and triethylamine, respectively.

**Scheme 3.13:** Synthesis of *N*-methythio β-lactams and *N*-(methyl)thiophthalimide.[86,100]



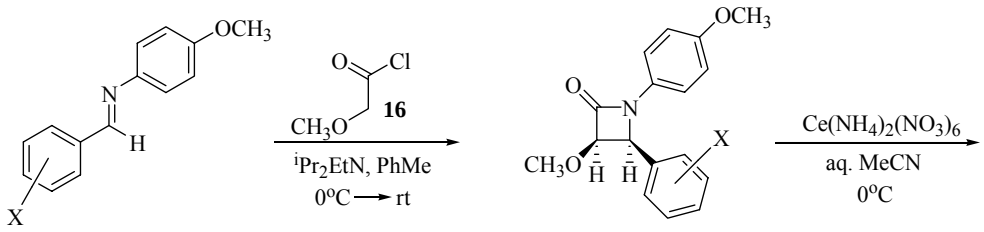

Reactions involving sulfur reagent **21** was found to be more efficient and reliable than **20** and became the method of choice for the *N*-methylthiolation of all $C_4$ aryl β-lactams.

### 3.3 The Structure-Activity Profiling of $C_4$ Phenyl Analogues

3.3.1 Synthesis and Microbiological Evaluation of **67**-**81**

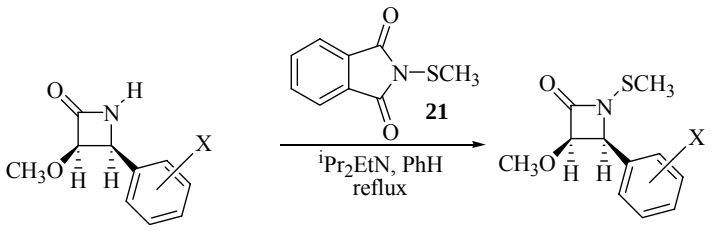
The SAR studies were initiated with the synthesis and microbial screening of monosubstituted $C_4$ aryl β-lactams **68**-**81**. As a control, an unsubstituted phenyl analogue (**67**) was included in the series for comparison. A diverse selection of functionalities and locations on the phenyl ring was chosen for the studies including: halogen (**68**-**74**), alkyl (**75**), ether (**76**), nitro (**77**,**78**), nitrile (**79**), and ester (**80**,**81**) substituents. *Scheme 3.14* illustrates the synthetic route to compounds **67**-**81** and the intermediates involved in their preparation.

**Scheme 3.14:** Synthesis of $C_4$ phenyl analogues **67**-**81**.




**22** X=H
**23** X=2-Cl
**24** X=3-Cl
**25** X=4-Cl
**26** X=4-Br
**27** X=2-I
**28** X=3-I
**29** X=4-I
**30** X=2-$CH_3$
**31** X=2-$OCH_3$
**32** X=2-$NO_2$
**33** X=3-$NO_2$
**34** X=4-CN
**35** X=4-[$O_2C(CH_2)_4CH_3$]
**36** X=4-[$O_2CCHCH_2$]

(±)-**37** X=H
(±)-**38** X=2-Cl
(±)-**39** X=3-Cl
(±)-**40** X=4-Cl
(±)-**41** X=4-Br
(±)-**42** X=2-I
(±)-**43** X=3-I
(±)-**44** X=4-I
(±)-**45** X=2-$CH_3$
(±)-**46** X=2-$OCH_3$
(±)-**47** X=2-$NO_2$
(±)-**48** X=3-$NO_2$
(±)-**49** X=4-CN
(±)-**50** X=4-[$O_2C(CH_2)_4CH_3$]
(±)-**51** X=4-[$O_2CCHCH_2$]

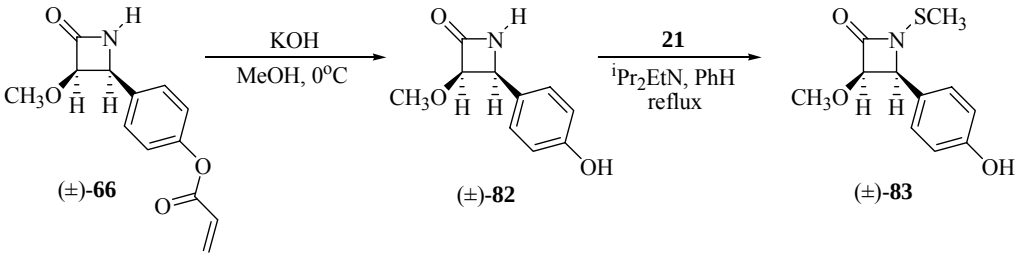



(±)-**52** X=H
(±)-**53** X=2-Cl
(±)-**54** X=3-Cl
(±)-**55** X=4-Cl
(±)-**56** X=4-Br
(±)-**57** X=2-I
(±)-**58** X=3-I
(±)-**59** X=4-I
(±)-**60** X=2-$CH_3$
(±)-**61** X=2-$OCH_3$
(±)-**62** X=2-$NO_2$
(±)-**63** X=3-$NO_2$
(±)-**64** X=4-CN
(±)-**65** X=4-[$O_2C(CH_2)_4CH_3$]
(±)-**66** X=4-[$O_2CCHCH_2$]

(±)-**67** X=H
(±)-**68** X=2-Cl
(±)-**69** X=3-Cl
(±)-**70** X=4-Cl
(±)-**71** X=4-Br
(±)-**72** X=2-I
(±)-**73** X=3-I
(±)-**74** X=4-I
(±)-**75** X=2-$CH_3$
(±)-**76** X=2-$OCH_3$
(±)-**77** X=2-$NO_2$
(±)-**78** X=3-$NO_2$
(±)-**79** X=4-CN
(±)-**80** X=4-[$O_2C(CH_2)_4CH_3$]
(±)-**81** X=4-[$O_2CCHCH_2$]

A later addition to this series was a phenolic β-lactam **83** which was synthesized by the hydrolysis of the acrylate intermediate **66**. Although *p*-hydroxybenzaldehyde was commercially available, the alcohol residue required protection for the Staudinger coupling and CAN oxidation steps. Hydrolysis of **66** afforded **82** in quantitative yield and subsequent sulfenylation of the β-lactam gave the *p*-phenol analogue in two steps from the acrylate (*Scheme 3.15*).

**Scheme 3.15:** Synthesis of $C_4$ phenolic β-lactams from **66**.




(±)-**66** (±)-**82** (±)-**83**

A diverse panel of pathogenic and nonpathogenic bacteria representing 17 genera and 33 species were used in the preliminary screening. Compounds were evaluated *in vitro* by agar diffusion (Kirby-Bauer) in accordance with the guidelines recommended by the National Committee for Clinical Laboratory Standards (NCCLS).[101] Initially, the disc variation of the test was applied to assess the susceptibility. Here, a 6 mm cellulose disc impregnated with 20 μg of the drug was placed on an agar plate inoculated with the test organism. Following incubation, the zones of growth inhibition were measured to determine the potency of the drug after 24 hrs. Another variation which gave consistent results utilizes a well instead of a disc to facilitate drug diffusion. The plates were prepared by cutting 6 mm circular holes into the inoculated medium and applying 20 μg of the test drug in dimethylsulfoxide (DMSO) to the wells. After incubation, the zone diameters were measured in millimeters to ascertain the relative potency. The results of the susceptibility test are depicted in *Table 3.03*.

The β-lactams demonstrated significant inhibitory activity against *Bacteroides*, *Bacillus*, *Micrococcus*, *Neisserria*, *Streptococcus* and *Staphylococcus* species. Peculiarly, these six genera derive from four distinct taxonomic orders defined by their genetic, morphological and metabolic traits (*Table 3.02*). Although nine genera *in toto* were found to be susceptible to the lactams, few taxonomic relationships could be used to ascertain the spectrum of activity. The class of bacteria that responded the most to the *N*-thiolated β-lactams was the "Bacilli" which consists of two orders: *Bacillales* and *Lactobacillales*. Susceptible members of this taxonomic class included species of *Bacillus*, *Staphylococcus* and *Streptococcus*. *Enterococcus Lactococcus* and *Listeria* which are also affliated with the "Bacilli" were mostly insensitive to the drugs, though. The sporadic activity among bacteria species suggests the

compounds may be acting in a different manner from traditional broad spectrum β-lactam antibiotics. Chapter IV further examines the metabolic relationships of these microbes and reasons for the randomness in the activities observed for *N*-thiolated β-lactam antibiotics.

**Table 3.02:** Comparison of microbes sensitive to *N*-thiolated β-lactam antibiotics.

| order | genera | Gram (±) | morphology | respiration | activity[a] |
|---|---|---|---|---|---|
| *Enterobacteriales* | *Salmonella* | – | rod | aerobic | weak |
| *Pasteurellales* | *Haemophilus* | – | rod | aerobic | weak |
| *Vibrionales* | *Vibrio* | – | rod | anaerobic | weak |
| *Bacillales* | *Bacillus* | + | rod | aerobic | medium |
| *Bacteroidales* | *Bacteroides* | – | rod | anaerobic | medium |
| *Lactobacillales* | *Streptococcus* | + | cocci | anaerobic | medium |
| *Neisseriales* | *Neisserria* | – | cocci | aerobic | medium |
| *Actinomycetales* | *Micrococcus* | + | cocci | aerobic | strong |
| *Bacillales* | *Staphylococcus* | + | cocci | anaerobic | strong |

[a]based on well diffusion data: strong = >20 mm; medium = 15-20 mm; weak = <15 mm.

**Table 3.03:** *In vitro* susceptibilities of bacteria to *N*-methylthio β-lactams.

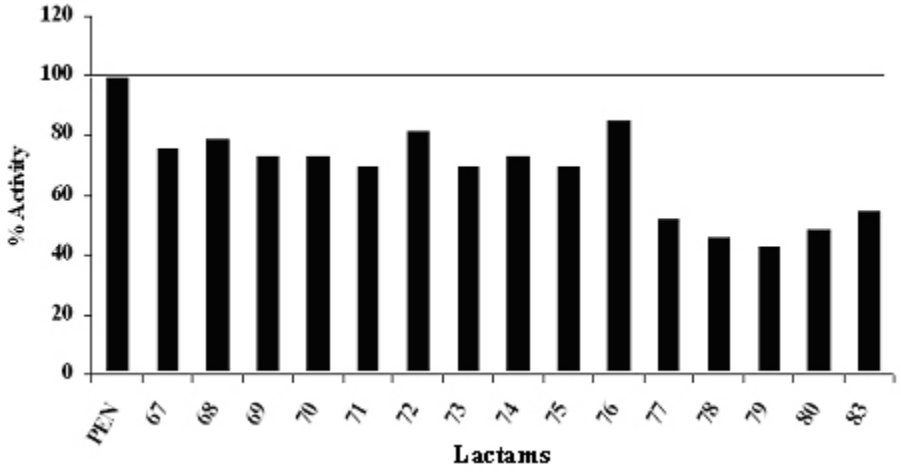
| Bacteria spp[a] | Zone of Growth Inhibition (mm)[b] | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **67** | **68** | **69** | **70** | **71** | **72** | **73** | **74** | **75** | **76** | **77** | **78** | **79** | **80** | **81** | **83** | **96** | **97** | **98** | **99** | **PEN** | **CIP** |
| *Bacteroides fragalis* | | 15 | 15 | 15 | 0 | 0 | 15 | 0 | | | | | | | | | | 0 | 13 | 0 | 0 | |
| *Bacillus anthracis* | 21 | 25 | 24 | 20 | 24 | 20 | 22 | 22 | 20 | | 18 | 10 | | 20 | 14 | | | 24 | | 20 | | 37 |
| *Bacillus cereus* | 18 | 21 | 20 | 19 | 18 | 19 | 19 | 23 | 18 | | 18 | 10 | | 15 | 13 | | | 21 | 19 | 20 | 12 | 33 |
| *Bacillus coagulans* | 14 | 20 | 20 | 20 | 18 | 13 | 15 | 22 | 15 | 13 | 17 | 0 | | 10 | 0 | | | 17 | 14 | 22 | 27 | 40 |
| *Bacillus globigii* | 14 | 19 | 18 | 18 | 20 | 17 | 14 | 20 | 15 | 14 | 16 | 0 | | 19 | 0 | | | 22 | 16 | 20 | 30 | 41 |
| *Bacillus megaterium* | 11 | 20 | 16 | 14 | 16 | 15 | 13 | 17 | 12 | 10 | 14 | 0 | | 15 | 10 | | | 21 | 14 | 20 | 41 | 40 |
| *Bacillus subtilis* | 12 | 18 | 15 | 14 | 19 | 14 | 14 | 18 | 10 | 0 | 16 | 0 | | 19 | 0 | | | 21 | 12 | 18 | 41 | 37 |
| *Bacillus thuringensis* | 15 | 20 | 19 | 17 | 20 | 17 | 17 | 19 | 15 | 13 | 17 | 0 | | 16 | 10 | | | 20 | 17 | 20 | 0 | 33 |
| *Enterobactor cloacae* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | |
| *Escherichia coli* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | |
| *Haemophilus influenzae* | | 8 | 7 | 8 | | | | | | | | | | | | | | | | | | |
| *Klebsiella pneumoniae* | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | |
| *Listeria monocytogenes* | | 0 | | | | | | | | | | | | | | | | | | | | |
| *Micrococcus luteus* | 21 | 24 | 31 | 29 | 25 | 24 | 24 | 25 | 21 | | | 22 | 21 | | | 25 | | 26 | 25 | 26 | 40 | |
| *Mycobacterium smegmatis* | | 0 | 0 | | | | | | | | | | | | | | | | | | 0 | |
| *Neisserria gonorrhoeae*[c] | | 19 | 16 | 14 | 12 | 12 | 14 | 13 | 12 | | | 12 | 12 | | | 12 | | 13 | 13 | 10 | 0 | |
| *Proteus mirabilis* | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | | | 0 | 0 | 0 | 0 | 0 | 0 | 18 | |
| *Pseudomonas aeruginosa* | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | |
| *Salmonella typhimurium* | | 8 | 10 | 10 | 8 | 0 | 0 | 0 | 0 | 0 | 0 | | | | 0 | 0 | 0 | 0 | 0 | 0 | 25 | |
| *Serratia marcescens* | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | |
| *Staphylococcus aureus* | 25 | 26 | 23 | 24 | 23 | 27 | 23 | 24 | 23 | 28 | 17 | 15 | 14 | 16 | 21 | 18 | 18 | 25 | 24 | 23 | 33 | |
| *Staphylococcus captitis* | 19 | 26 | 21 | 21 | 24 | 27 | 19 | 22 | 15 | 18 | 18 | | | | 13 | 0 | 15 | 21 | 19 | 23 | 47 | |
| *Staphylococcus cohnii* | 17 | 22 | 22 | 22 | 23 | 23 | 20 | 14 | 18 | 17 | 19 | | | | 18 | 17 | 18 | 17 | 19 | 19 | 43 | |
| *Staphylococcus epidermidis* | 31 | 29 | 25 | 23 | 30 | 29 | 23 | 28 | 25 | 20 | 18 | 20 | 20 | 20 | 20 | 17 | 20 | 24 | 25 | 23 | 50 | |
| *Staphylococcus lentus* | 12 | 18 | 18 | 18 | 15 | 17 | 18 | 15 | 0 | 10 | 16 | | | | 0 | 0 | 0 | 0 | 0 | 15 | 34 | |
| *Staphylococcus lugdunensis* | 20 | 28 | 28 | 28 | 28 | 28 | 24 | 22 | 19 | 20 | 23 | | | | 19 | 16 | 19 | 22 | 19 | 18 | 47 | |
| *Staphylococcus saprophyticus* | 17 | 20 | 21 | 18 | 19 | 23 | 18 | 18 | 15 | 13 | 15 | 15 | 14 | | 15 | 16 | 15 | 22 | 19 | 22 | 30 | |
| *Staphylococcus simulans* | 14 | 16 | 0 | 0 | 18 | 18 | 18 | 16 | 14 | 14 | 12 | 13 | 0 | | 15 | 0 | 13 | 21 | 0 | 20 | 13 | |
| *Staphylococcus warneri* | 17 | 25 | 28 | 28 | 24 | 26 | 20 | 22 | 17 | 17 | 18 | | | | 20 | 17 | 13 | 17 | 19 | 20 | 38 | |
| *Staphylococcus xylosus* | 18 | 28 | 22 | 24 | 26 | 27 | 24 | 24 | 16 | 20 | 22 | | | | 18 | 17 | 18 | 13 | 22 | 22 | 34 | |
| *Streptococcus agalactia*[d] | | 0 | 11 | 0 | 13 | 15 | | 0 | 0 | 0 | 14 | | | | 0 | 11 | 0 | 15 | 0 | 14 | | |
| *Streptococcus pyrogenes*[d] | | 14 | 11 | 12 | 14 | 14 | | 14 | 0 | 0 | 14 | | | | 0 | 21 | 14 | 16 | 10 | 14 | | |
| *Vibrio cholerae* | | | 9 | 10 | 9 | 0 | 0 | 12 | 0 | 0 | 8 | | | | 0 | 0 | 0 | 10 | 0 | 12 | 17 | |

[a]Bacteria were obtained from various commercial and noncommercial sources. See pg. 87 for more information; [b]Well diffusion on Mueller-Hinton agar; [c]Well difussion on chocolate agar; [d]Well diffusion on tryptic soy agar.

3.3.2 Structure-Activity Relationship of **67**-**81** and **83** Against MSSA

Based on the biological evaluation of lactams **67**-**81** and **83**, it was evident that aromatic substitution is not a prerequisite for antimicrobial activity. Lactam **67** demonstrated equal or greater propensity to inhibit the *in vitro* growth of bacteria to those containing functionalized phenyl rings (**68**-**81**,**83**). The influence of aromatic substitution on activity appeared to be moderate. An antagonistic effect was observed for analogues possessing highly polar phenyl ring residues against the 33 member panel of bacteria. Compounds containing powerful deactiving or activating groups such as nitro (**77**,**78**) and nitrile (**79**) or acyloxy (**80**,**81**) and hydroxy (**83**), respectively, were about 30% less active than **67**. Although the reason for this anomaly is unclear, the ability for a highly polarizable aromatic ring to attenuate bioactivity was evident particularly against species of *Bacillus* and *Staphylococcus*.

**Figure 3.01:** Percent relative activity of penicillin for monosubstituted analogues against methicillin-susceptible *Staphylococcus aureus* (MSSA).




No discernable differences in bioactivity could be ascertained for compounds **68**-**76**. These include derivatives that possess weak deactivating aromatic substituents like halogens (**68**-**74**) and mild-medium activating groups such as alkyl (**75**) and alkoxy (**76**). The bacteria most susceptible were *Staphylococcus* and *Micrococcus luteus*. *Bacillus* spp. was also sensitive to the drugs though not to the same extent. Location and size of the aryl ring substituent had no apparent influence on biological activity. The expectation was that increasing ring bulk would lead to erosion of bioactivity. This however, was not the case for the chloro-, bromo-, and iodo-substituted lactams **68**-**74**. As illustrated in the graph above, the percent activity of penicillin against methicillin-susceptible *Staphylococcus aureus* (MSSA; ATCC 25923) is nondiscrete for the haloaryl, tolyl, and methoxyphenyl derivatives. Although the zone sizes were slightly larger for *ortho*-substituted **68**, **72**, and **76**, the minimum inhibitory concentrations (MICs) remained at or about 10 μg/ml for MSSA.

3.3.2 Structure-Activity Relationship of **67**-**81** and **83** Against MRSA

Compounds **67**-**81** and **83** were tested against a panel of methicillin-resistant *Staphylococcus aureus* (MRSA) isolates. Eight of the ten strains were obtained from Lakeland Regional Medical Center and identified as USF652-659. Prior to screening, each of the Lakeland isolates was assayed for β-lactamase activity. Using a previously described acidimetric assay to monitor the hydrolysis of penicillin G,[102] all eight strains was verified to be β-lactamase-producing forms of *S. aureus*.

*Table 3.04* and *Figure 3.02* compare the relative effectiveness of **67**-**81** and **83** to inhibit the *in vitro* growth of MRSA. The glycopetide and current treatment of choice for MRSA-type infections, vancomycin, and penicillin were included as controls. Amazingly, the analogues retained their biological activity against forms of *S. aureus* that were resistant to penicillin. The structure-activity relationship of **67**-**81** against the multi-drug resistant strains parallel those observed in the microbiological evaluation of

MSSA. It was apparent based on these findings that *N*-thiolated β-lactams are transparent to the destructive forces of β-lactamase (eg. penicillinases) which act to neutralize the ability of penicillins and cephalosporins to impede cell wall biosynthesis. In the case of benzylpenicillin, the impact of β-lactamase on susceptibility was quite evident for the MRSA. A 50% reduction in zone size was detected against the ten strains for this "activated" β-lactam antibiotic.

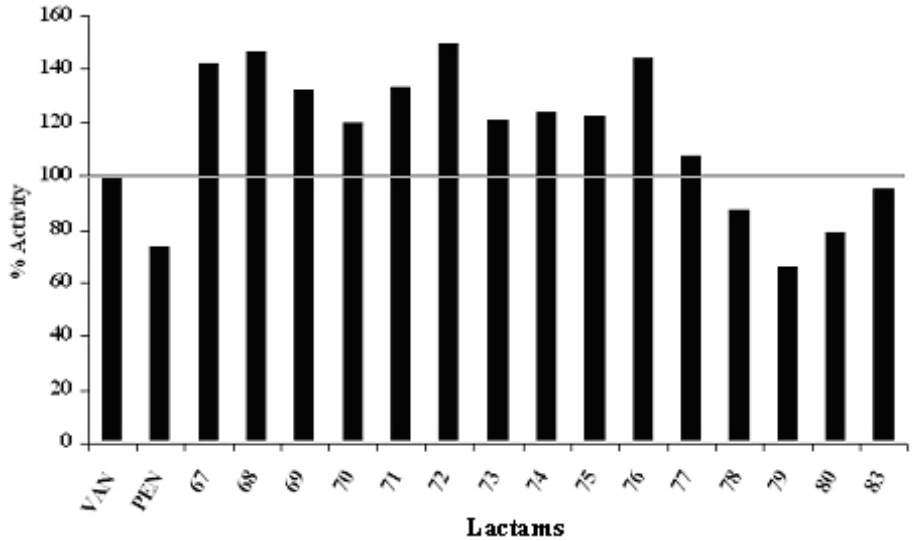
**Table 3.04** Kirby-Bauer data for analogues **67-81** and **83** against *S. aureus*.

| Strain[a] | Zone of Growth Inhibition (mm)[b] | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **67** | **68** | **69** | **70** | **71** | **72** | **73** | **74** | **75** | **76** | **77** | **78** | **79** | **80** | **81** | **83** | **PEN[c]** | **VAN[d]** |
| MSSA ATCC 25923 | 25 | 26 | 23 | 24 | 26 | 27 | 23 | 24 | 23 | 28 | 17 | 15 | 14 | 16 | 21 | 18 | 33 | |
| MRSA ATCC 33591 | 20 | 26 | 26 | 19 | 29 | 28 | 25 | 26 | 17 | 21 | 17 | | | | 20 | 14 | 15 | 20 |
| MRSA ATCC 43300 | 21 | 29 | 25 | 20 | 26 | 30 | 23 | 25 | 19 | 24 | 19 | | | | 17 | 0 | 18 | 20 |
| MRSA USF652 | 30 | 30 | 24 | 26 | 25 | 34 | 23 | 27 | 23 | 29 | 18 | 17 | 14 | 17 | 16 | 14 | 8 | 19 |
| MRSA USF653 | 30 | 29 | 28 | 27 | 27 | 29 | 23 | 24 | 27 | 32 | 23 | 22 | 16 | 17 | 15 | 19 | 15 | 18 |
| MRSA USF654 | 26 | 28 | 22 | 23 | 26 | 27 | 21 | 24 | 23 | 27 | 20 | 16 | 12 | 15 | 20 | 18 | 10 | 19 |
| MRSA USF655 | 25 | 27 | 23 | 23 | 29 | 28 | 24 | 24 | 23 | 27 | 22 | 14 | 12 | 15 | 19 | 18 | 14 | 19 |
| MRSA USF656 | 28 | 29 | 22 | 25 | 24 | 29 | 22 | 25 | 25 | 28 | 22 | 18 | 13 | 16 | 20 | 19 | 12 | 21 |
| MRSA USF657 | 27 | 29 | 22 | 23 | 27 | 28 | 25 | 24 | 23 | 27 | 20 | 18 | 10 | 16 | 21 | 19 | 12 | 18 |
| MRSA USF658 | 26 | 27 | 19 | 24 | 20 | 28 | 23 | 20 | 22 | 26 | 21 | 17 | 12 | 14 | 19 | 18 | 19 | 18 |
| MRSA USF659 | 24 | 24 | 18 | 18 | 20 | 23 | 22 | 17 | 20 | 23 | 18 | 11 | 12 | 14 | 17 | 0 | 16 | 18 |

[a]USF652-659 were obtained from Lakeland Regional Medical Center, Lakeland, FL.
[b]Kirby-Bauer well diffusion on TSA.
[c]Benzylpenicillin potassium salt (penicillin G)
[d]Vancomycin hydrochloride.

Comparison of *in vitro* susceptibilities of MRSA to *N*-methylthio β-lactams and vancomycin yielded promising results. In the graph below, the mean zone data of the ten MRSA isolates for compounds **67-80** and **83** is depicted as percentage of activity relative to vancomycin. The ability to inhibit the growth of MRSA was greatest for analogues **67-76**. The β-lactams consistently demonstrated 20-50% and 50-75% greater anti-MRSA activity than vancomycin and penicillin, respectively.

**Figure 3.02:** Percent relative activity of vancomycin for monosubstituted analogues against methicillin-resistant *Staphylococcus aureus* (MRSA).



3.3.3 Effect of Drug Amount Versus Zone Diameter

Subsequent to the screening of compounds **67-81**, a series of experiments were conducted to calculate the optimal amount of drug needed for well diffusion measurements. The results of these studies are depicted in graphs of *Figures 3.03* and *3.04*. Plots 3.03a and b draw a comparison of the relative susceptibilities of MSSA and MRSA to lactam **69** and penicillin, respectively, at equimolar amounts. The first graph reveals a linear relationship between the μgs of lactam **69** and the zone diameter generated. Against MSSA (ATCC 25923), the inhibitory activity of **69** rose steadily as the amount increased from 2.8 to 56.2 μg/well. The same trend was observed against MRSA (ATCC 43300) indicating that the compound retained full activity against the β-lactamase-producing strain at all drug loadings. Conversely, *Figure 3.03b* illustrates that the potency of penicillin G against the drug-resistant strain was precipitously lower relative to the MSSA isolate. Although the antibiotic was equally effective at all concentrations for MSSA, no anti-MRSA activity was observed until greater than 28 μg was applied to the wells. The marked decrease in susceptibility was again a clear indication of the capabilities penicillinases have to neutralize traditional β-lactams antibiotics such as penicillin G.

**Figure 3.03:** Equimolar drug loads versus zone of growth inhibition of (a) lactam **69** and (b) penicillin G against MSSA and MRSA.

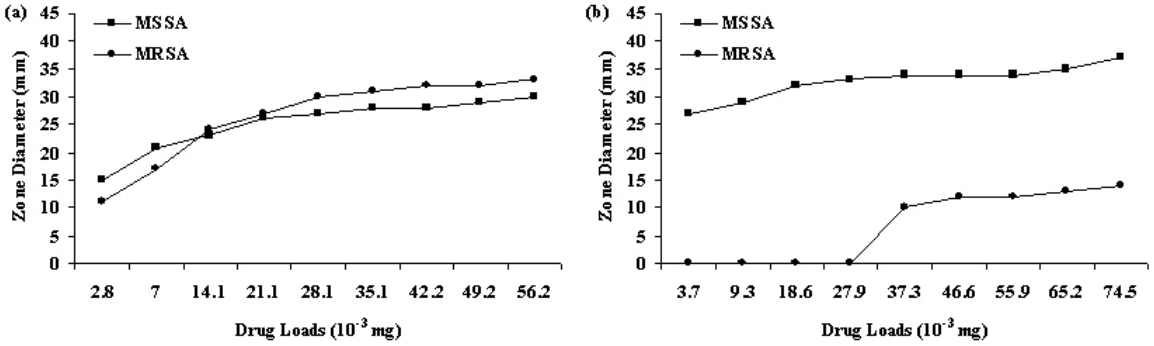
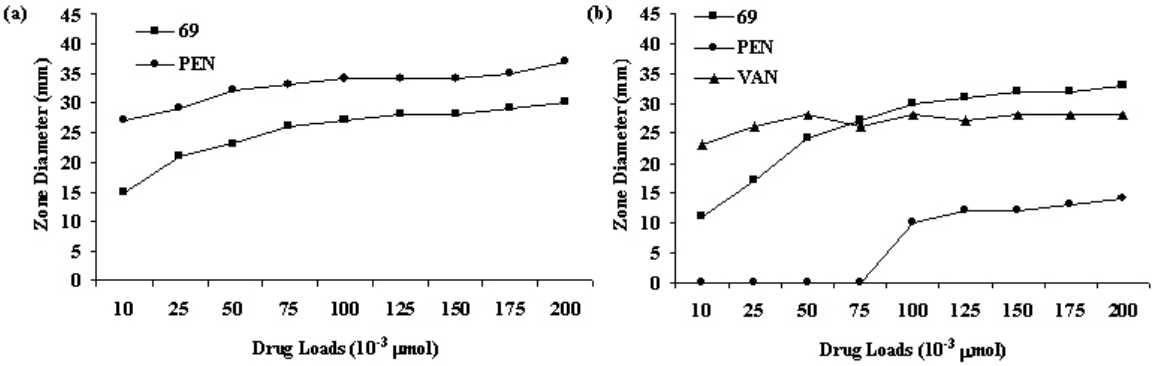



**Figure 3.04:** Equimolar drug loads versus zone of growth inhibition of lactam against (a) MSSA and (b) MRSA.




*Figures 3.04a* and *3.04b* compare the performance of **69** against MSSA and MRSA to penicillin G and vancomycin. The first plot depicts the effectiveness of the β-lactam and penicillin G at parallel concentrations against MSSA. It was discovered that compound **69** produced smaller zones than penicillin against the non-resistant *S. aureus* strain up to 200 $\mu mol^{-3}$/well (equivalent to 51.4 μg of **69**, or 64 μg of penicillin G). The ability of penicillin to yield zone sizes >25 mm even at 10 $\mu mol^{-3}$ was consistent with the lower MIC value of the antibiotic over lactam **69**. As predicted, a reversal in activities occurred when the two drugs were screened against MRSA (*Figure 3.04b*). Lactam **69** demonstrated superior ability to inhibit the *in vitro* growth of the drug-resistant strain over a 10-200 $\mu mol^{-3}$ range compared to penicillin.

Vancomycin also displayed less potency than **69** when greater than 75 μmol$^{-3}$ of compound was applied to the well.

3.3.4 Minimum Inhibitory Concentration (MIC)[101]

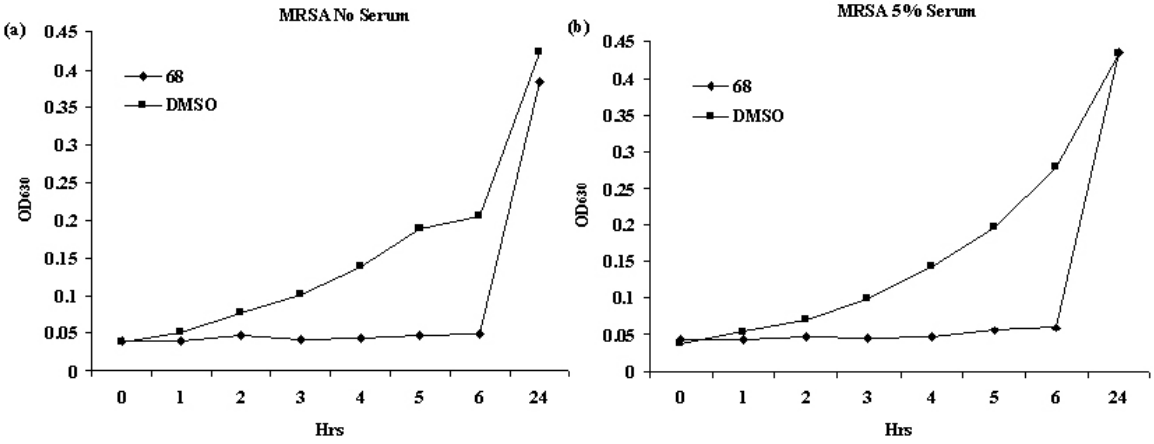
The lack of ionizable groups makes *N*-thiolated β-lactams insoluble in aqueous media, therefore minimum inhibitory concentrations (MIC) proved to be difficult to measure. Some lactams could be solubilized if a 9:1 ratio of aqueous media to dimethyl sulfoxide (DMSO) was utilized. It was reported that *S. aureus* could sustain logarithmic growth in presence of 8-10% DMSO and this was reconfirmed prior to the MIC determinations. Unfortunately, most of analogues could not be solubilized unless 15-25% DMSO was present in the growth medium.

Serial dilutions of TSB media was the initial method of choice to determine MICs though the results were neither consistent nor comparable to the potency levels seen with the raw data from the Kirby-Bauer diffusion evaluation. The agar variation, however, provided dependable results that were not obtainable by broth dilutions of the lactams. The agar media were prepared in 48 well plates and inoculated by applying a 1 μl suspension of freshly prepared cultures to the well. Following a 24 hrs incubation period, the plates were examined for growth. The lowest concentration to inhibit the visual growth of bacteria was recorded as the MIC. The MIC values for lactam **68** against MSSA (ATCC 25923) was 10 μg/ml. Though penicillin G (<1 μg/ml) was superior to **68**, the antibiotic did not retain the same effectiveness against the MRSA isolates. The MICs to inhibit 90% of the MRSA strains ($MIC_{90}$) was >125 μg/ml for penicillin G in comparison to 8 μg/ml for compound **68**.

3.3.5 *In Vitro* Activity in Blood Serum

The ability of a pharmacological agent to retain its antibacterial properties in blood serum is an important attribute if the drug were to be used clinically. Growth studies designed to examine the chemical stability of the *N*-thiolated β-lactams in the presence of serum were performed against MRSA. The graphs in *Figure 3.05* depict the efficacy of **68** to inhibit the *in vitro* growth of *S. aureus* over 24 hrs in the presence of serum. Bacterial growth was monitored during the course of the study by optical density of the cultures at 630 nm.

**Figure 3.05:** Growth studies of MRSA with and without blood serum.



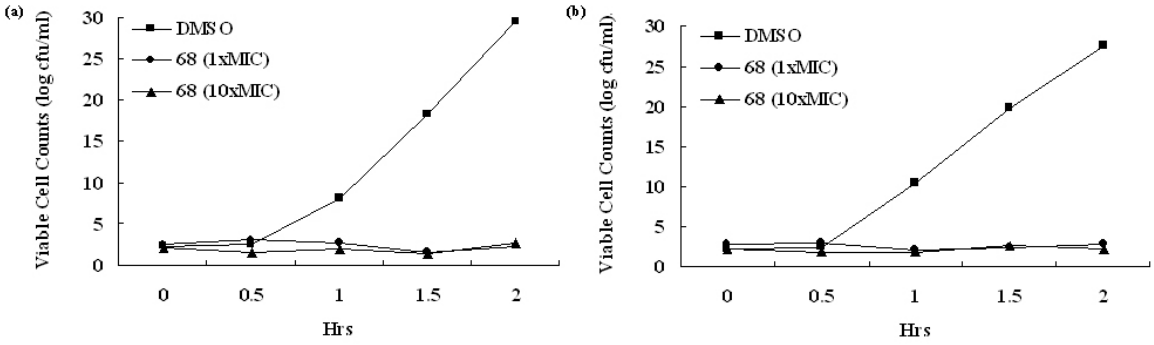

It was apparent based on these studies that the chemical components of blood serum did not affect the lactam's ability to hinder microbial growth. *Figure 3.05a* and *3.05b* are plots of the growth study performed in Mueller-Hinton broth (MHB) in the presence or absence of blood serum. In the antibiotic-free media (DMSO only), a steady progression of cellular growth was observed over 24 hrs for *S. aureus*. Conversely, bacteria viability was abruptly halted in the presence of lactam **68** at 1x MIC (8 μg/ml) after 1 hr. Over 6 hrs, the drug uniformly stunted microbial growth in the serum and serum-free MHB. These data

suggest that the *in vitro* half-life ($t_{½}$) of lactam **68** with serum extends beyond 6 hrs. Between 6 and 24 hrs though, both media were equally turbid to the control (DMSO only) suggesting that the $t_{½}$ is less than 24 hrs. It was postulated based on these finding that *N*-thiolated β-lactams could be bacteriostatic antibiotics. Intrinsically, the drugs may not be capable of causing cell death but act in a manner that suppresses growth until drug levels were low enough for the bacteria to reproduce. The next section further examines the nature of *N*-thiolated β-lactams to affect the viability of bacteria.

3.3.6 Time-Kill Studies

Antibiotics can be categorized by the way they affect the viability of bacteria. Classification as a bacteriostatic or bacteriocidal agent can provide insight on the pharmacokinetic properties such as effective dosage. Additional information on the cellular target of a new antibiotic can be extracted from the intrinsic nature of other known drugs. Apropos those antibiotics that inhibit protein synthesis (ie, chloramphenicol) or secondary metabolites (ie, sulfa drugs) have been historically considered bacteriostatic agents whereas those that disrupt the cell wall (ie, penicillin) or directly damage DNA (ie, metronidazole) are bactericidal.

**Figure 3.06:** Time-kill studies of **68** against (a) MSSA and (b) MRSA.



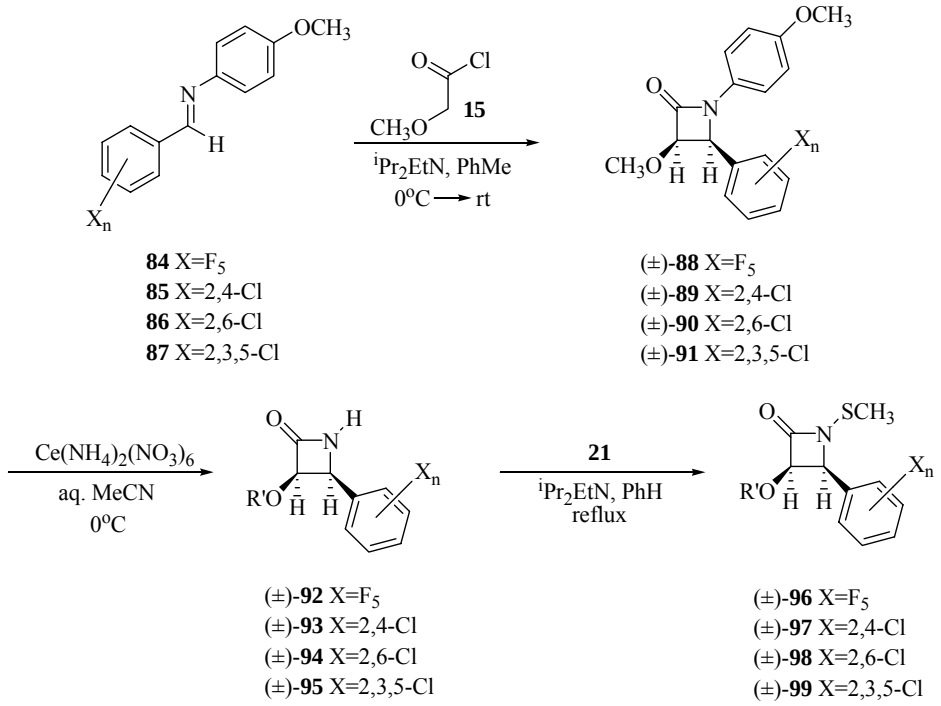

The effect of *N*-thiolated β-lactams on cell survival was examined by monitoring the growth of bacteria at high drug concentrations. Early logarithmic phase cultures of *S. aureus* were used in the study and growth was measured by viable cell counts. *Figures 3.06a* and *3.06b* depicts cell survival for MSSA and MRSA, respectively, when cultured in the absence or presence of lactam **68** over a 2 hr time frame. In the absence of lactam **68**, MSSA and MRSA grew logarithmically; in the presence of **68**, bacterial growth was immediately halted. While reproduction ceased at the MIC level of the lactam as well as at 10x MIC, the number of viable cells remains constant throughout the duration of the experiment. Consequently, bacteria growth was clearly inhibited by lactam **68**, but little to no decrease in cell population was observed. These data provide substantial evidence that even at high drug concentrations, *N*-methylthio β-lactams are bacteriostatic agents toward staphylococci.

### 3.4 Multihalogenated Phenyl Analogues and Their Biological Activities

3.4.1 Synthesis of Multihalogenated Phenyl Analogues

Follow up studies on the structure-activity relationships of $C_4$ phenyl analogues were conducted to examine the effect of multisubstitution and lipophilicity on the bioactivity of *N*-thiolated β-lactams. Ergo the multihalogenated analogues **96**-**99** were prepared (*Scheme 3.16*) in the same manner that was previously described in *Section 3.2*. The aldehydes employed to make the pentafluoro- (**84**), dichloro- (**97**,**98**), and tricholorophenyl (**99**) *N*-(methoxyphenyl)imines were obtained from commercial sources. Staudinger coupling followed by anisyl removal and *N*-thiolation of the protio β-lactams **92**-**95** with *N*-(methyl)thiophthalimide (**21**) gave **96**-**99** for screening.

**Scheme 3.16:** Synthesis of multihalogenated *N*-thiolated β-lactams.




**84** X=$F_5$
**85** X=2,4-Cl
**86** X=2,6-Cl
**87** X=2,3,5-Cl

(±)-**88** X=$F_5$
(±)-**89** X=2,4-Cl
(±)-**90** X=2,6-Cl
(±)-**91** X=2,3,5-Cl

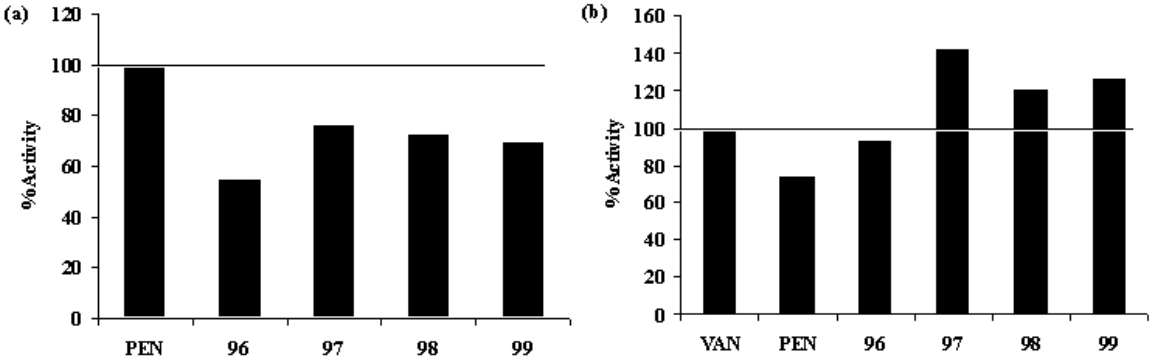
(±)-**92** X=$F_5$
(±)-**93** X=2,4-Cl
(±)-**94** X=2,6-Cl
(±)-**95** X=2,3,5-Cl

(±)-**96** X=$F_5$
(±)-**97** X=2,4-Cl
(±)-**98** X=2,6-Cl
(±)-**99** X=2,3,5-Cl

3.4.2 Antimicrobial Activity of Multihalogenated Phenyl Analogues

Multihalogenated β-lactams **96-99** demonstrated comparable bioactivities to the monosubstituted analogues **68-74** (*Table 3.02*). The spectrum of activity was again narrow which included species of *Bacillus*, *Micrococcus*, and *Staphylococcus*. The multichlorinated lactams **97-99** were equipotent to their monosubstituted counterparts **68-70** against MSSA and MRSA. The di- and trisubstituted analogues displayed about 70% the activity of penicillin against MSSA (*Figure 3.07a*) and 20-40% greater potency against 10 strains of MRSA than vancomycin (*Figure 3.07b*).

**Figure 3.07:** Percent activities of lactams **96-99** against (a) MSSA and (b) MRSA.




Surprisingly, both forms of *S. aureus* were least susceptible to the pentafluorinated derivative **96**. It was postulated before these results that the added hydrophobicity by the fluorine atoms would confer enhanced bioactivity to the β-lactams. Lipophilic substances have been known to permeate cell membranes amid greater efficiency thus increasing the intracellular bioavailibilty of the drug. This was clearly not the case for lactam **96**. Though the reason for the attenuation of potency is unknown, it is possible that repulsive forces evoked by the electronegativities of the fluorine atoms could decrease recognition and

binding of the lactam to the biological target. However, other explanations can also be put forth. More on the mechanism of action and reactivity of *N*-thiolated β-lactams will be discussed in chapter IV.

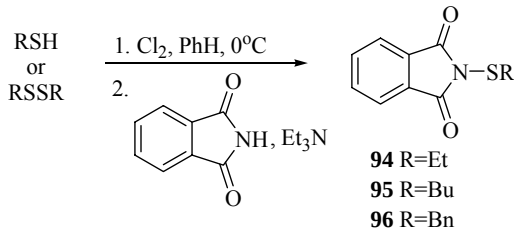
### 3.5 *N*-Sulfenylated Analogues and Their Biological Activities

Based on the data gathered from the microbial screening of **67-81**, **83**, and **96-99**, it was apparent that functionalization of the aryl ring will not improve the *in vitro* performance of the β-lactams. Though few structure-activity relationships could be established from the aryl substituted series, it was believed that modification of the sulfur-bearing substituent could have the greatest impact on biological activity. This section examines the antimicrobial properties of *N*-thiolated β-lactams having different sulfenyl substituents attached to the ring.

#### 3.5.1 Synthesis of *N*-Sulfenylated Analogues

A series of 10 other sulfenyl analogues was synthesized using *N*-thiolating reagents **94-99**.[100] As described in *Section 3.2.4*, the *N*-thiophthalimides were prepared via chlorination of the corresponding thiol or disulfide to generate the sulfenyl chloride *in situ*. The volatile liquid was then canulated into a flash containing phthalimide and triethylamine. The reactions were performed neat for 1 hr in an ice bath (*Scheme 3.17*). Reagents **94-99** were then recrystallized from methanol and used without further purification.

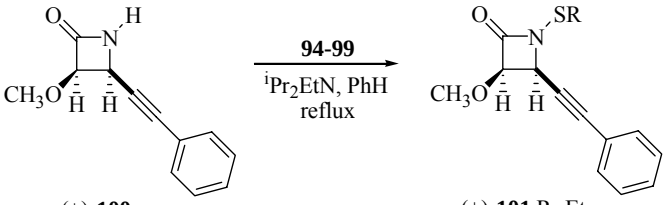
**Scheme 3.17:** Synthesis of *N*-thiophthalimide reagents **94-99**.[100]




**94** R=Et
**95** R=Bu
**96** R=Bn
**97** R=Ph
**98** R=$C_6H_{11}$
**99** R=$C_2H_4OH$

*N*-Sulfenylated derivatives of the alkynyl lactam **1** were the first to be synthesized and evaluated for antimicrobial activity. Compounds **101-106** were prepared from **100** by base-promoted thiolation using the corresponding *N*-thiophthalimide (**94-99**) (*Scheme 3.18*).

**Scheme 3.18:** Synthesis of *N*-sulfenyl analogues **101-106**.

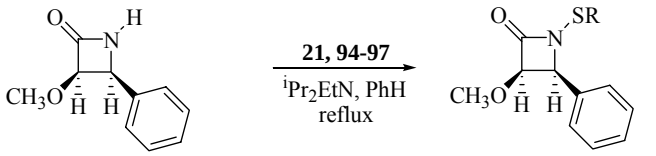



(±)-**100**

(±)-**101** R=Et
(±)-**102** R=Bu
(±)-**103** R=Bn
(±)-**104** R=Ph
(±)-**105** R=$C_6H_{11}$
(±)-**106** R=$C_2H_4OH$

Sulfenylated analogues of **67** were also synthesized from the phenyl-substituted lactam **49** using *N*-thiophthalimides **94-97** (*Scheme 3.19*).

**Scheme 3.19:** Synthesis of *N*-sulfenyl analogues **107-110**.




(±)-**49**

(±)-**67** R=Me
(±)-**107** R=Et
(±)-**108** R=Bu
(±)-**109** R=Ph
(±)-**110** R=$C_6H_{11}$

3.5.2 Antimicrobial Susceptibilities to Lactams **101-109**

A structure-activity profile could be established for β-lactams possessing larger sulfur-bearing substituents. The alkynyl and phenyl analogues, **101-106** and **107-109**, respectively, were screened against a panel of bacteria that were known to be susceptible to this drug class. In comparison to the *N*-methylthio β-lactams **1** and **67**, a 5-40% reduction in the zone diameter was detected for **101-109**. The reduced bioactivity was observed against species of *Staphylococcus*, *M. luteus*, and *N. gonorrhoeae* (*Table 3.05*). It became evident from the data that increasing the sulfur bulk decreased the biological activity of the β-lactams. This was again somewhat surprising since the hydrophobicity rose without augmenting the *in vitro* performance of the lactam. Presumably, the sulfur moiety which is the most labile group on the molecule, has direct involvement in the mode of action. Increasing the size of groups on sulfur could hinder the attack of a biological nucleophile or reduce the binding capabilities of the lactam to its cellular target.

**Table 3.05**: Kirby-Bauer data for analogues **1, 67,** and **101-109**.

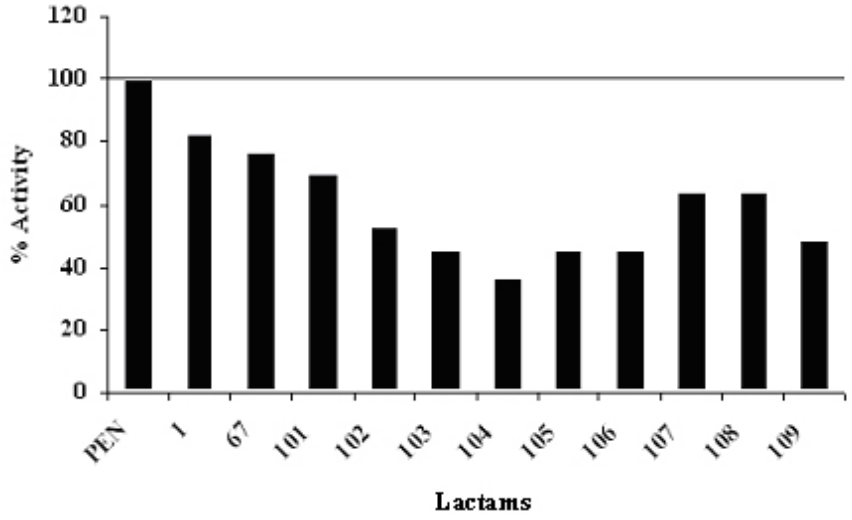
| Species/Strain[a] | Zone of Growth Inhibition (mm)[b] | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **1** | **67** | **101** | **102** | **103** | **104** | **105** | **106** | **107** | **108** | **109** | **PEN**[c] | **VAN**[d] |
| MSSA ATCC 25923 | 27 | 25 | 23 | 17 | 15 | 12 | 15 | 15 | 21 | 21 | 16 | 33 | |
| MRSA USF652 | 31 | 30 | 28 | 18 | 15 | 12 | 17 | 18 | 21 | 24 | 18 | 8 | 19 |
| MRSA USF653 | 30 | 30 | 27 | 20 | 15 | 10 | 16 | 18 | 25 | 24 | 15 | 15 | 18 |
| MRSA USF654 | 28 | 26 | 23 | 16 | 16 | 12 | 14 | 17 | 18 | 20 | 15 | 10 | 19 |
| MRSA USF655 | 27 | 25 | 24 | 17 | 17 | 13 | 14 | 15 | 20 | 22 | 16 | 14 | 19 |
| MRSA USF656 | 30 | 28 | 24 | 18 | 15 | 12 | 15 | 17 | 20 | 20 | 14 | 12 | 21 |
| MRSA USF657 | 28 | 27 | 25 | 16 | 15 | 12 | 14 | 18 | 17 | 20 | 14 | 12 | 18 |
| MRSA USF658 | 27 | 26 | 24 | 18 | 18 | 13 | 15 | 18 | 21 | 22 | 17 | 19 | 18 |
| MRSA USF659 | 24 | 24 | 22 | 16 | 15 | 13 | 16 | 18 | 20 | 20 | 15 | 16 | 18 |
| *S. epidermidis* | 30 | 31 | 23 | 18 | | 10 | 16 | 14 | 27 | 25 | 0 | 50 | |
| *S. saprophyticus* | 22 | 22 | 18 | 15 | | 10 | 0 | 13 | 16 | 19 | 10 | 30 | |
| *S. simulans* | 21 | 14 | 15 | 15 | | 10 | 8 | 12 | 11 | 18 | 0 | 13 | |
| *M. luteus* | 23 | 21 | 24 | 23 | | 13 | 15 | 17 | 21 | 16 | 16 | 40 | |
| *N. gonorrhoeae* | 13 | 14 | 10 | 8 | | 0 | 0 | 0 | 13 | 25 | 0 | 0 | |

[a]USF652-659 were obtained from Lakeland Regional Medical Center, Lakeland, FL.
[b]Kirby-Bauer well diffusion on TSA.
[c]Benzylpenicillin potassium salt (penicillin G)
[d]Vancomycin hydrochloride.

*N*-Ethylthio lactams (**101**, **107**) and the *N*-butylthio lactam **108** were the most active members from the series of sulfenyl analogues. Compared to **1** and **67**, the compounds were slightly less active against MSSA but more active than the benzyl (**103**), phenyl (**104**), and cyclohexyl (**105**) analogues (*vide infra*). A linear relationship was established between the bulk of the *S*-substituent and the observed biological activity. In general, the performance of the β-lactams declined as the length of the hydrocarbon

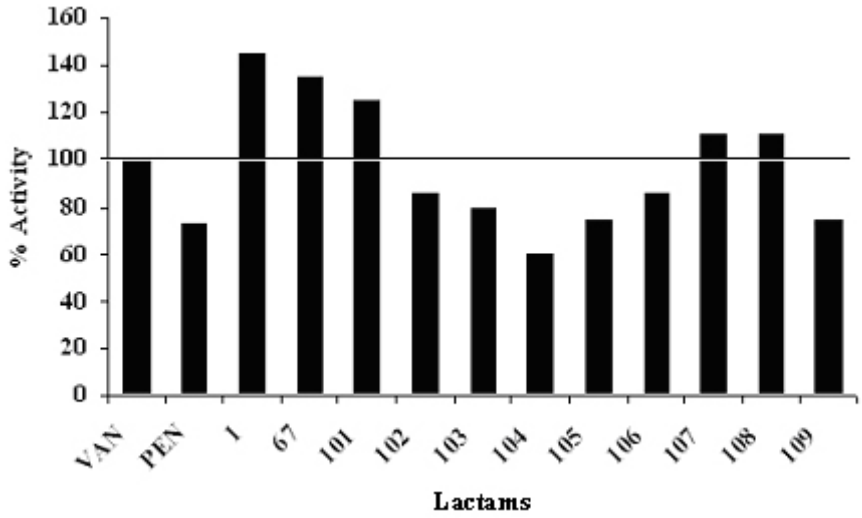
chain on sulfur increased. The addition of a heteroatom-containing side chain also had an antagonist effect on the anti-staphylococcal activity of β-lactam **106**. The reason why the β-mercaptoethanol (**106**) lactam was 30% less active to the mercaptoethane (**101**) lactam is not yet known.

**Figure 3.08:** Comparison of anti-MSSA activities of lactams **101-109** to penicillin G.




The structure-activity relationship observed for lactams **101-109** against MRSA was similar to the profile seen *vida supra* for the susceptible-form of *S. aureus*. The size of the *S*-substituent once again had the greatest impact on biological activity. All compounds appeared stable to β-lactamase given that no alteration of the antibacterial properties was detected. Most possessed between 90 and >100% the inhibitory activity than vancomycin though remained less potent than **1** and **67** against eight strains of MRSA (*Figure 3.09*).

**Figure 3.09:** Anti-MRSA activity comparison of **101-109**.

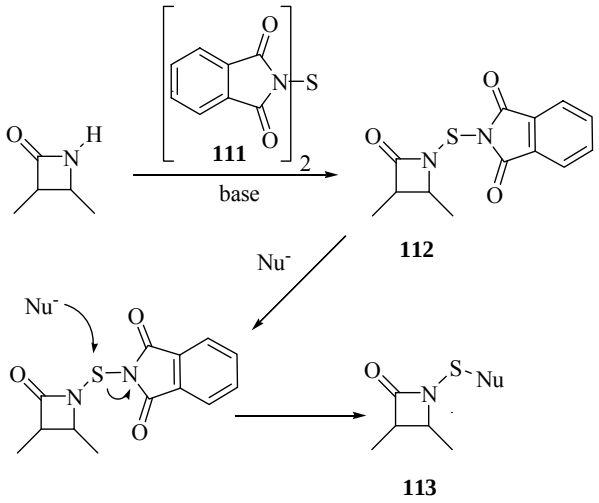



### 3.6 Antibacterial Activity of Heterosubstituted *N*-Thiolated β-Lactams

Attempts to make heterosubstituted *N*-thiolated β-lactams (**113**) were performed by a substitution reaction involving (2-oxo-1-azetidinyl)-thiophthalimides (**112**). As described in chapter II, **112** has been

reported to react with nucleophilic substances such as benzyl alcohol, morpholine, and sodium bisulfite to generate **113** in good yields (*Scheme 3.20*).[86]

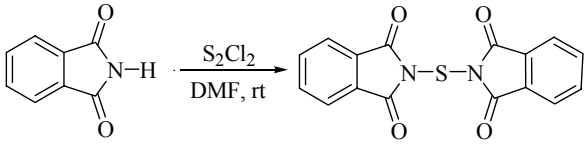
**Scheme 3.20:** Synthesis of heterosubstituted *N*-thiolated β-lactams.




**113**

Prior to the synthesis of the heterosubstituted *N*-thiolated β-lactams, *N*,*N*'-thiobisphthalimide (**111**) was prepared from the treatment of phthalimide with sulfur monochloride in dry DMF (*Scheme 3.21*).[103]

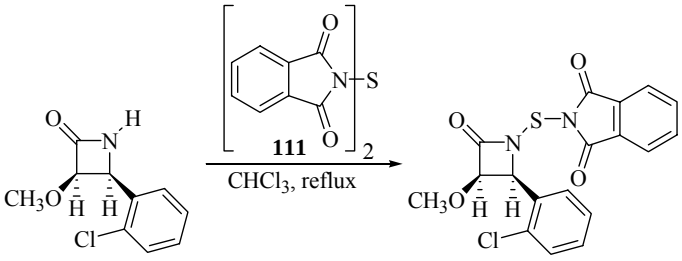
**Scheme 3.21**: Synthesis of *N*,*N*'-thiobisphthalimide (**111**).




**111**

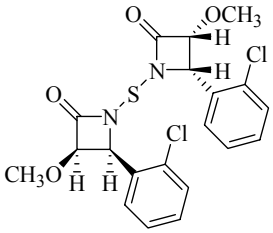
The *o*-chlorophenyl β-lactam **53** and **111** were refluxed in chloroform (*Scheme 3.22*) and the progress of the reaction was monitored by TLC. Following the disappearance of starting material, the product was purified by silica gel chromatography. Surprisingly, the product of the reaction was not **115** but the dimerized β-lactam **116** depicted in *Figure 3.10*.

**Scheme 3.22:** Reaction of **53** with *N*,*N*'-thiobisphthalimide (**111**).

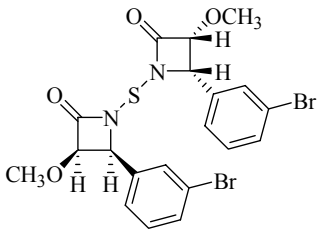



**(±)-53** **115** not isolated

**Figure 3.10:** Thiolated *bis*-β-lactam dimers **116** and **117**.



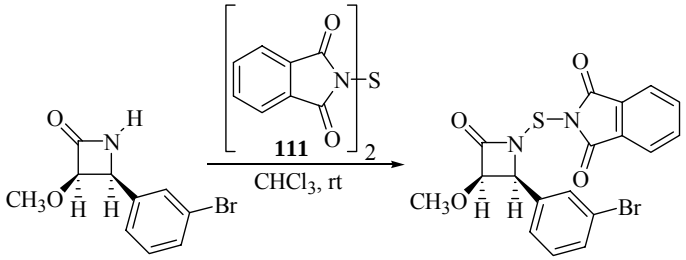
(±)-**116**



(±)-**117**

The desired intermediate was generated when the *m*-bromophenyl β-lactam **118** and **111** were reacted without heating (*Scheme 3.23*).

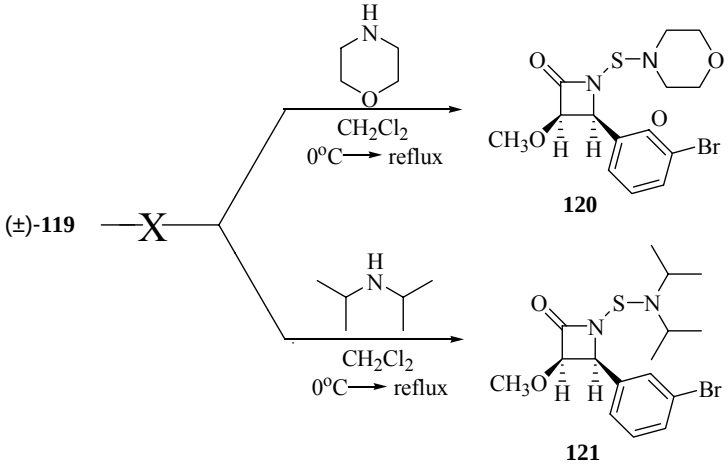
**Scheme 3.23:** Synthesis of *N*-phthalimidothio β-lactam **119**.



(±)-**118** (±)-**119**

In accordance to the previously described procedure, a 200% mole equivalent of the amine nucleophiles, morpholine and diisopropylamines were reacted with **119** at 0–25°C in attempt to generate the heterosubstituted *N*-thiolated β-lactams **120** and **121** (*Scheme 3.24*). However, the TLC indicated after 24 hrs that a reaction had not occurred. Heat was next applied and only then was the starting material consumed. Although **119** reacted under reflux, the product that formed was again a β-lactam dimer (**117**).

**Scheme 3.24:** Reaction of **119** with morpholine and diisopropylamine.



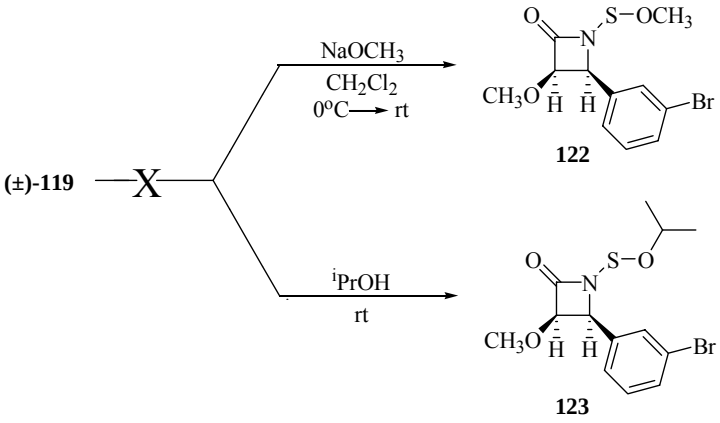
**120**

**121**

With the understanding that elevated temperature could not be used for the substitution reactions, other types of nucleophiles were attempted such as sodium methoxide and isopropanol. However, once

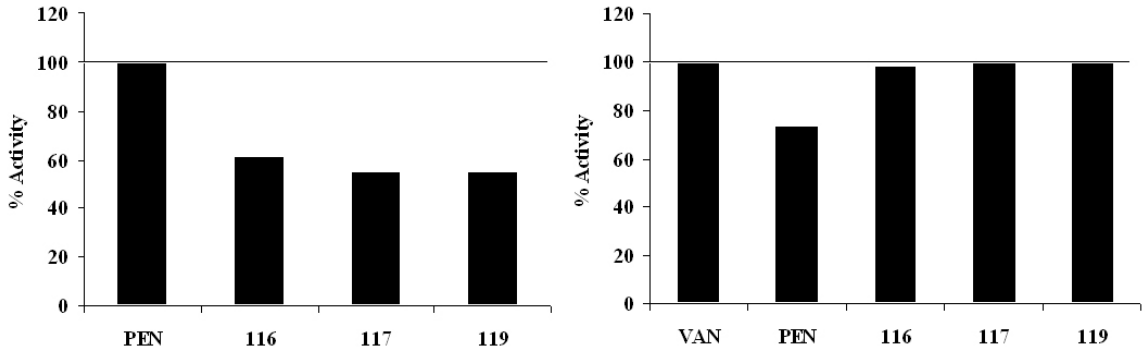
again the desired product (**122**,**123**) from the displacement of phthalimide was not formed. Either starting material or the *N*-protio β-lactam (**118**) resulted from the reaction (*Scheme 3.25*)

**Scheme 3.25:** Reaction of **119** with sodium methoxide and isopropanol.




The heterosubstituted β-lactams **116**, **117** and **119** were tested for biological activity against MSSA and MRSA. Surprisingly, the compounds were found to possess some antimicrobial properties (see *Table 3.05*). Although the lactams were less potent than penicillin for MSSA, they were approximately equal to vancomycin in efficacy against MRSA (*Figure 3.11*). These results contradict previous findings that increasing the molecular bulk on sulfur diminishes the activity of the β-lactam (*see Section 3.5*). To the contrary, **116**, **117** and **119** were more effective at eliciting an antimicrobial response than the phenyl (**104**,**109**), benzyl (**103**), and cyclohexyl (**105**) *S*-substituted analogues. Though the reason for this remains unclear, it is postulated that the sulfur of the heterosubstituted *N*-thiolated β-lactams is more electrophilic with a second nitrogen bound, making it more reactive, and thus more susceptible, to attack by a biological nucleophile.

**Figure 3.11:** Comparison of anti-MSSA and anti-MRSA activities **116**, **117**, and **119**.

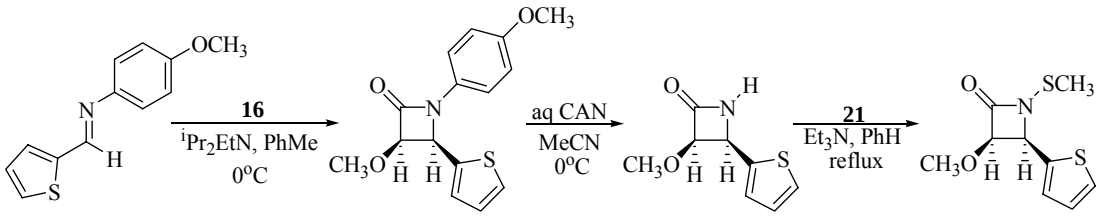



### 3.7 Additional *N*-Thiolated β-Lactam Analogues Probed For Biological Activity

Other *N*-methylthio β-lactam analogues were synthesized and probed for antibacterial activity that did not fit in the series of compounds described in *Sections 3.2-3.5*. Among these was **127** which contained a 2-thiophene substituent instead a phenyl group at the $C_4$ position of the ring (*Scheme 3.26*). The lactam was synthesized by the methodologies previously discussed and tested by well diffusion. The results are depicted in *Table 3.05*. Analysis of the zone data revealed that a $C_4$ phenyl ring is not a requirement for antibacterial activity. Lactam **127** was equipotent to the most active *N*-methylthio β-lactams despite lacking a 6-carbon member aryl substituent. These data also suggest that other heterocycles could be placed at $C_4$ of

the β-lactam and still retain biological activity. Synthesis of a 2-furan and 2-pyridine analogue was attempted, though, both decomposed during the CAN reaction.

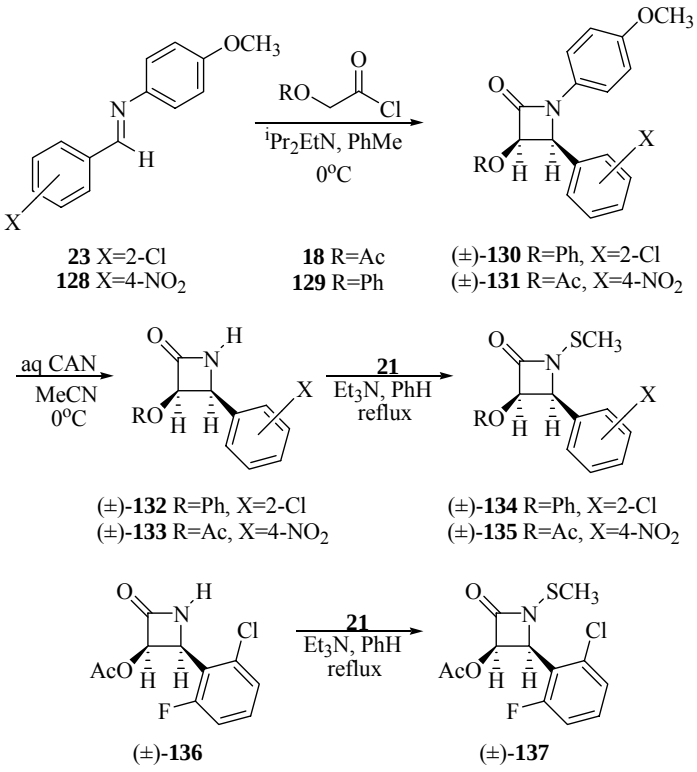
**Scheme 3.26:** Synthesis of 2-thiophene β-lactam **127**.




**124** (±)-**125** (±)-**126** (±)-**127**

Alternative substituents at the $C_3$ position of the β-lactam were examined for their influence on the biological properties (*Scheme 3.27*). Phenoxy (**134**) and acetoxy (**135**,**137**) derivatives were prepared from the corresponding acid chlorides (**18**,**129**) and tested by well diffusion. It was apparent from the zone data that the larger substituents at $C_3$ do not enhance the efficacy of the lactams (*Table 3.06*). In particularly, the phenoxy analogue of **68** displayed 45% reduced activity with the added molecular bulk.

**Scheme 3.27:** Synthesis of lactams **134**, **135**, and **137**.




**23** X=2-Cl
**128** X=4-$NO_2$

**18** R=Ac
**129** R=Ph

(±)-**130** R=Ph, X=2-Cl
(±)-**131** R=Ac, X=4-$NO_2$

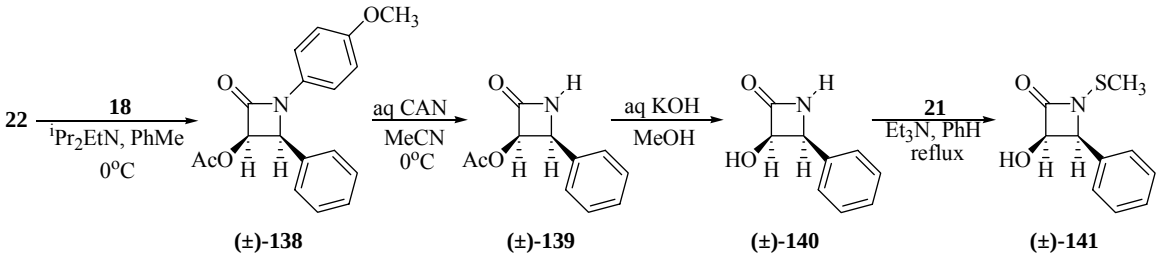
(±)-**132** R=Ph, X=2-Cl
(±)-**133** R=Ac, X=4-$NO_2$

(±)-**134** R=Ph, X=2-Cl
(±)-**135** R=Ac, X=4-$NO_2$

(±)-**136** (±)-**137**

With the implication that increasing the size of the $C_3$ substituent leads to erosion of bioactivity, a substituent smaller that methoxy was incorporated and tested against the same bacteria panel. The group assigned to the position was a hydroxy which was generated by hydrolysis of the acetoxy group in lactam **139** (*Scheme 3.28*). The zone data revealed that the hydroxy analogue **141** was about 25% less effective than the methoxy derivative **67** at inhibiting the growth of *S. aureus* (see *Tables 3.03, 3.05*).

**Scheme 3.28:** Synthesis of $C_3$ hydroxy substituted analogue.




**(±)-138** **(±)-139** **(±)-140** **(±)-141**

Based on the Kirby-Bauer data, it appeared that the antibacterial properties are influenced by the bulk and polarity of the substituent located at the $C_3$ position of the β-lactam. In this regard, it was hypothesized that MeO ≥ AcO > OH > PhO is the order by which the substituents affect the biological activity of *N*-methylthio β-lactams.

**Table 3.06:** Disc diffusion data for various *N*-thiolated β-lactams.

| Species/Strain[a] | Zone of Growth Inhibition (mm)[b] | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | **116** | **117** | **119** | **127** | **134** | **135** | **137** | **141** | **PEN[c]** | **VAN[d]** |
| MSSA ATCC 25923 | 20 | 15 | 18 | 27 | 15 | 15 | 22 | 19 | 33 | |
| MRSA USF652 | 18 | 21 | 18 | 28 | 18 | 15 | 22 | 15 | 8 | 19 |
| MRSA USF653 | 19 | 20 | 20 | 28 | 15 | 11 | 25 | 23 | 15 | 18 |
| MRSA USF654 | 19 | 20 | 22 | 27 | 15 | 11 | 20 | 18 | 10 | 19 |
| MRSA USF655 | 17 | 19 | 18 | 29 | 14 | 12 | 19 | 20 | 14 | 19 |
| MRSA USF656 | 18 | 19 | 19 | 28 | 16 | 14 | 23 | 19 | 12 | 21 |
| MRSA USF657 | 19 | 19 | 20 | 26 | 13 | 11 | 21 | 19 | 12 | 18 |
| MRSA USF658 | 18 | 16 | 15 | 26 | 15 | 14 | 21 | 18 | 19 | 18 |
| MRSA USF659 | 18 | 19 | 20 | 24 | 16 | 11 | 20 | 15 | 16 | 18 |
| *S. epidermidis* | | | | 28 | 16 | 16 | 26 | | 50 | |
| *S. saprophyticus* | | | | 20 | 14 | 13 | 19 | | 30 | |
| *S. simulans* | | | | 0 | 13 | 0 | 13 | | 13 | |
| *M. luteus* | | | | 15 | 20 | 20 | 20 | | 40 | |
| *N. gonorrhoeae* | | | | 11 | 7 | 10 | 15 | | 13 | |

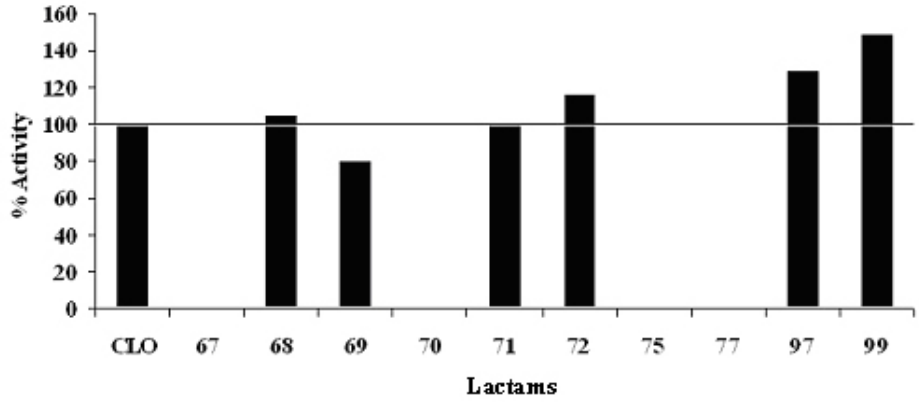
[a]USF652-659 were obtained from Lakeland Regional Medical Center, Lakeland, FL.
[b]Kirby-Bauer well diffusion on TSA.
[c]Benzylpenicillin potassium salt (penicillin G)
[d]Vancomycin hydrochloride.

### 3.8 Antifungal Properties of *N*-Thiolated β-Lactams

*N*-Thiolated β-lactams demonstrated unique antifungal properties against two members of the genus *Candida*. The compounds were tested by well diffusion on yeast-nitrogen base agar at 50 μg quantities. As a control, clotrimazole at the same amount was tested as a gauge for percent activity. The antifungal performance for various *N*-methylthio β-lactams is depicted in the charts below. Against *C. albicans* and *C. tropicalis*, a discernable structure-activity profile could be established for the $C_4$ phenyl substituted analogues. This contrasted the SAR studies for bacteria where few correlations could be detected.

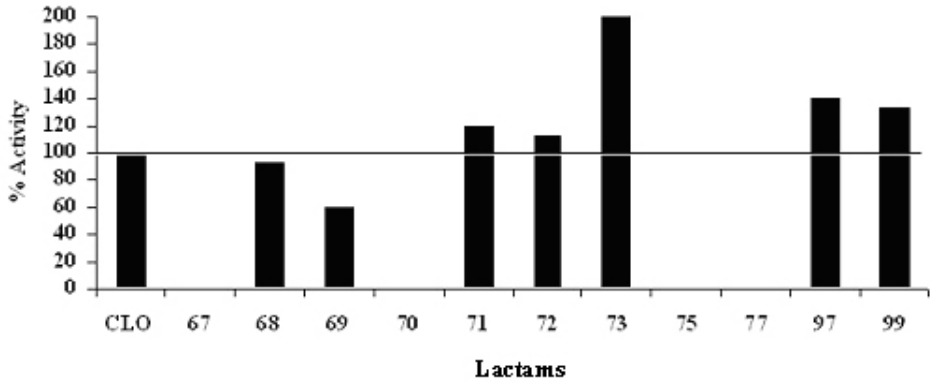
The most apparent relationship that could be ascertained for lactams possessing anti-*Candida* properties was that they all contained a haloaryl residue. The phenyl (**67**), tolyl (**75**), and 2-nitrophenyl (**77**) were completely devoid of activity suggesting that ring substitution has a critical role in drug delivery or in binding to the biological target. The position of the halogen appeared to have little effect on the *in vitro* perfomance of the lactam. The monosubstituted β-lactams were equally effective when the halogen was located *ortho*, *meta*, or *para*, although the *m*-chorophenyl analogue (**70**) was oddly found to be inactive against both *Candida* species (*Figures 3.12*, *3.11*).

**Figure 3.12:** Comparision of antifungal activities against *C. albicans*.




Another effect that was not observed against bacteria was the enhancement of biological activity when the aryl ring contained multiple chlorine atoms. The efficacy of the **97** and **99** to inhibit fungal growth was 15-40% greater than the monosubstituted lactams **68** and **69**.

**Figure 3.13:** Comparision of antifungal activities against *C. tropicalis*.




*N*-Sulfenylated β-lactams **101-109** were also screened against the *Candida* species and as expected, the activity diminished when the size of the *S*-substituent increased (data not shown).

### 3.9 Antiviral Properties of *N*-Thiolated β-Lactams

In collaboration with Professor Erik De Clercq's laboratory[114] at the Rega Institute for Medical Research in Leuven, Belgium, the antiviral properties of lactams **1**, **69**, and **75** were assessed in preinfected human HeLa (cervix carcinoma), HEL (human embroyonic lung), and Vero (African green monkey kidney) cell cultures. Prior to screening, cytotoxicity measurements were determined for the 3 cell lines and recorded as the minimum cytotoxic concentration to induce microscopic alterations in normal cell morphology (MCC). The antiviral performance of the antibiotics were subsequently assayed and expressed as the minimum concentration to reduce cytopathogenicity by 50% ($MIC_{50}$).

The antiviral activitites of the β-lactams against vesicular stomatitis (rhabdovirus), coxsackie (enterovirus), and respiratory syncytial virus (pneumovirus) in HeLa cells is shown in *Table 3.07*. For comparison, the antivirals brivudin (nucleoside mimic), (S)-DHPA (*S*-adenosylhomocysteine hydrolase inhibitor), and the ribavarin (reverse transcriptase inhibitor) were included as controls. None of the lactams

demonstrated inhibitory activities against the 3 viruses. Compounds **1**, **69**, and **75** were ineffective at subtoxic concentrations greater than 80 μg/ml in HeLa cells whose MCC value was >400 μg/ml.

**Table 3.07:** Cytotoxicity and antiviral activity of *N*-methylthio β-lactams **1**, **69**, and **75** in HeLa (cervix carcinoma) cell cultures.

| Compound | MCC[a] | $MIC_{50}$[b] | | |
|---|---|---|---|---|
| | | Vesicular stomatitis virus | Coxsackie Virus B4 | Respiratory syncytial virus |
| **1** | 400 | >80 | >80 | >80 |
| **69** | 400 | >80 | >80 | >80 |
| **75** | 400 | >80 | >80 | >80 |
| Brivudin | >400 | >80 | >80 | >80 |
| (S)-DHPA | >400 | >80 | >80 | >80 |
| Ribavirin | >400 | >80 | >80 | >80 |

[a]minimum cytotoxic concentration (μg/ml); [b]minimum inhibitory concentration (μg/ml).

Similarly, herpes simplex virus-1 (HSV-1, strain KOS), HSV-1 (strain TK$^-$ KOS ACV$^r$), HSV-2 (strain G), vaccinia virus, and vesicular stomatitis virus were insensitive to *N*-methylthio β-lactams at concentrations >16 μg/ml in HEL cells (*Table 3.08*). In Vero cells, the compounds were also inactive against parainfluenza type 3 virus, reovirus type 1, Sindbis virus, Coxsackie B4 virus, and Punta Toro virus at MICs >16 μg/ml (*Table 3.09*).

**Table 3.08:** Cytotoxicity and antiviral activity of *N*-methylthio β-lactams **1**, **69**, and **75** in HEL (Human Embryonic Lung) cell cultures.

| Compound | MCC[a] | $MIC_{50}$[b] | | | | |
|---|---|---|---|---|---|---|
| | | HSV-1 (KOS) | HSV-1 TK$^-$ KOS ACV$^r$ | HSV-2 (G) | Vaccinia virus | Vesicular stomatitis virus |
| **1** | ≥16 | >16 | >16 | >16 | >16 | >16 |
| **69** | ≥16 | >16 | >16 | >16 | >16 | >16 |
| **75** | ≥16 | >16 | >16 | >16 | >16 | >16 |
| Brivudin | >400 | 0.0256 | 80 | >400 | 3.2 | >400 |
| Ribavirin | >400 | >400 | >400 | >400 | 16 | 240 |
| ACG | >400 | 0.384 | 9.6 | 0.384 | >400 | >400 |
| DHPG | >100 | 0.0038 | 0.48 | 0.0064 | >100 | >100 |

[a]minimum cytotoxic concentration (μg/ml); [b]minimum inhibitory concentration (μg/ml).

**Table 3.09:** Cytotoxicity and antiviral activity of *N*-methylthio β-lactams **1**, **69**, and **75** in Vero cell cultures.

| Compound | MCC[a] | $MIC_{50}$[b] | | | | |
|---|---|---|---|---|---|---|
| | | Parainfluenza-3 virus | Reovirus-1 | Sindbis virus | Coxsackie virus B4 | Punta Toro virus |
| **1** | 400 | >80 | >80 | >80 | >80 | >80 |
| **69** | 400 | >80 | >80 | >80 | >80 | >80 |
| **75** | 80 | >16 | >16 | >16 | >16 | >16 |
| Brivudin | >400 | >400 | >400 | >400 | >400 | >400 |
| (S)-DHPA | >400 | 48 | 48 | >400 | >400 | >400 |
| Ribavirin | >400 | 80 | 9.6 | >400 | >400 | 16 |

[a]minimum cytotoxic concentration (μg/ml); [b]minimum inhibitory concentration (μg/ml).

# CHAPTER IV

# MODE OF ACTION OF *N*-THIOLATED β-LACTAMS

## 4.1 Introduction

Antibiotics can be grouped by at least 3 classification schemes: (1) narrow-spectrum vs. broad-spectrum, (2) bacteriostatic vs. bactericidal, and (3) intracellular vs. extracellular. In chapter III, *N*-thiolated β-lactams were identified as bacteriostatic agents possessing a narrow range of activity which implied that the biological target is internalized. Cytostatic agents usually evoke arrest of intracellular processes involving biosynthetic pathways through enzyme or secondary metabolite modification. Conversely, antibiotics whose function is to weaken the outer periphery of cells (eg, cell wall and plasma membrane) are in most cases, cytocidal (*Table 4.01*). Based on the attributes of existing antibacterials, *N*-thiolated β-lactams were believed to act in a different manner from traditional β-lactam antibiotics. The studies presented in this chapter examine the lactam's effect on cellular processes in *S. aureus* in an effort to define the mechanism of action.

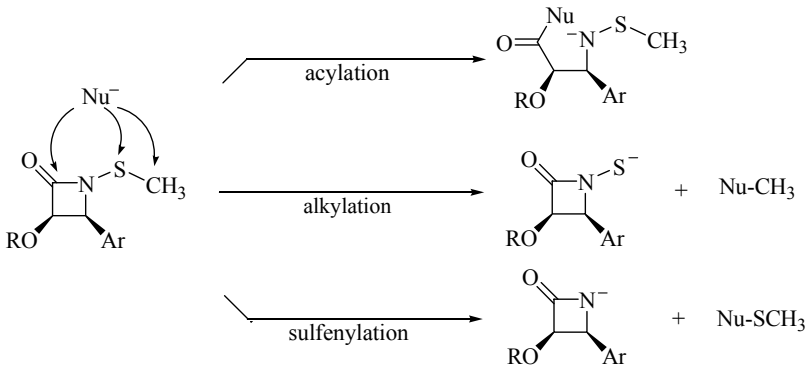
**Table 4.01:** Important classes of antibiotics and there respective intrinsic characteristics.

| | representative member(s) | bacteri-ostatic, cidal | activity spectrum | cellular target | cell process affected |
|---|---|---|---|---|---|
| I. extracellular | | | | | |
| a) β-lactams | penicillins cephalosporins | -cidal | broad | transpeptidase | cell wall |
| b) glycopeptides | vancomycin, teicoplanin | -cidal | narrow | crosslinkages in cell wall | cell wall |
| c) polymyxins | polymyxin B | -cidal | narrow | phospholipids | plasma membrane |
| d) bacitracin | bacitracin A | -cidal | narrow | lipid carrier | cell wall |
| II. intracellular | | | | | |
| a) quinolones | Cipro® | -cidal | broad | topoisomerase | DNA synthesis |
| b) ansamycins | Rifadin® (rifampicin) | -cidal | broad | RNA polymerase | mRNA synthesis |
| c) macrolides | erythromycin | -ostatic | narrow | 50S ribosome | translocation |
| d) aminoglycosides | streptomycin, gentamicin | -cidal | broad | 16S rRNA | initiation |
| e) tetracycline | minocylin | -ostatic | broad | 30S ribosome | tRNA binding |
| f) chloramphenicol | Chloromycetin® | -ostatic | broad | peptidyl transferase | tRNA charging |
| g) oxazolidinone | Zyvox® | -ostatic | narrow | 50S ribosome | initiation |

## 4.2 Probing the Modes of Action

There are at least 3 types of reactions that can occur between *N*-methylthio β-lactams and a biological nucleophile ($Nu^-$) (*Scheme 4.01*). For the β-lactam class of molecules, it is naturally assumed that nucleophilic addition occurs on the carbonyl center of the ring. As in the case of penicillins and cephalosporins, a serine residue within the active site of cell wall peptidases adds to the lactam, disabling the enzyme. Chemical studies of *N*-thiolated β-lactams have shown, however, the ring to be less prone to nucleophilic ring opening such as hydrolysis, therefore other types of reactivity were considered.

**Scheme 4.01:** Possible reactions of *N*-thiolated β-lactams.

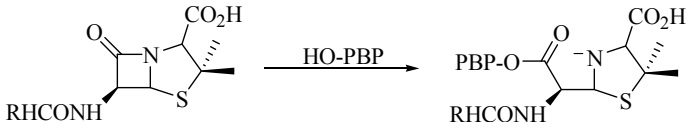



Whereas the least reactive substituents are located at the $C_3$ (-OR) and $C_4$ (-Ar) positions of the β-lactam, the sulfenyl moiety is the most vulnerable to nucleophilic attack. Alkylation is one manner by which the cellular target might be chemically altered by the *S*-methyl residue. Most alkylating drugs react with nucleotides to inhibit DNA replication or compromise the integrity of the super helices. Examples of DNA alkylating agents include leinamycin and mitomycin. The *S*-methyl moiety could as well transfer to the target to induce an inhibitory response. These 3 pathways will be discussed in *sections 4.2-4.4* presenting the evidence for or against each reaction type.

### 4.3 *N*-Thiolated β-Lactams as Acylating Agents

Deactivation of the penicillin-binding proteins (eg, transpeptidase), is the only mechanism for which β-lactam antibiotics are known to eradicate bacteria. A serine residue in the active site of the PBPs adds to the carbonyl center of penicillins and cephalosporins, forming a stable ester linkage (*Scheme 4.02*). The addition reaction occurring on the hydroxyl of serine is enhanced by ring strain and a carboxylic acid moiety located in proximity to the β-lactam carbonyl. Situated at the β-C of the lactam nitrogen, the acid functionality increases the electrophilicity of the carbonyl carbon.[51] Repositioning or eliminating the carboxylate negates the ability of the antibiotics to irreversibly bind to the PBPs.

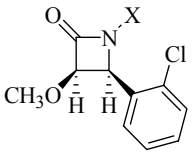
**Scheme 4.02:** Acylation of penicillin-binding proteins (HO-PBP), a serine hyrdrolase, by penicillin.[51]




The lack of an acid functionality in the *N*-methylthio β-lactams suggests that attack on the carbonyl carbon does not occur. Chemical studies of lactam **68** (*Figure 4.01*) in buffered media verified the ring's stability towards equimolar amounts of potassium hydroxide and serine. In addition, the compound was unreactive to commercial β-lactamases (eg, serine hydrolases from *B. subtilis*) and did not thwart the enzyme's ability to hydrolyze penicillin G to the corresponding β-amino acid. However, to prove

unequivocally that the *N*-thiolated β-lactam was not inhibiting the PBPs, light and electron microscopy were used to probe for damage or thinning of the cell wall in *S. aureus* treated with high concentrations of lactam **68**.

**Figure 4.01:** *N*-Protio β-lactam **53** and *N*-methylthio β-lactam **68**.

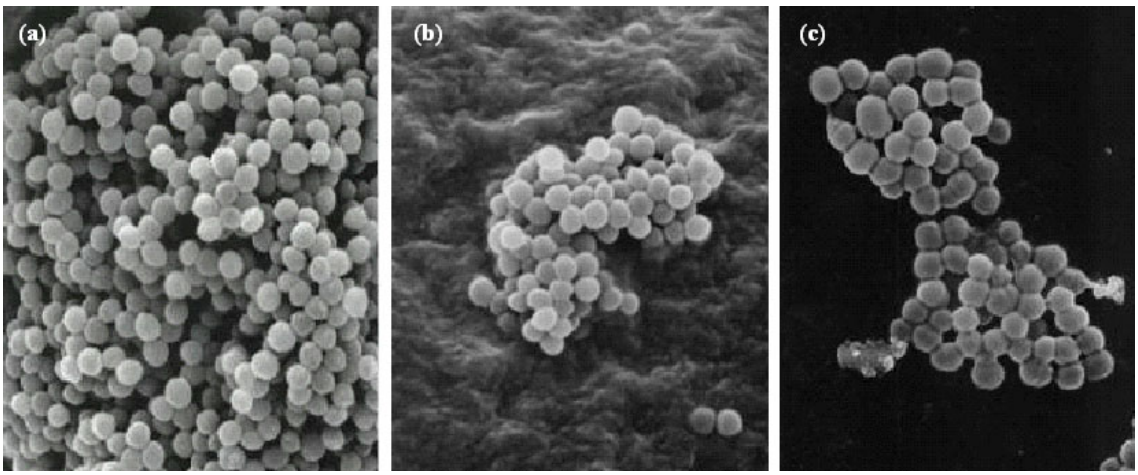



(±)-**53** X=H
(±)-**68** X=$SCH_3$

Section 4.3.1 Scanning Electron Microscopy

Scanning electron microscopy (SEM) is a technique that can be used to examine cell morphology at >10,000 magnification. Bacteria exposed to antibiotics that disrupt the cell wall (ie, β-lactams) or cytoplasmic membrane (ie, polymyxins) can be observed by SEM for physiological damage elicited by the drugs. Cultures of *S. aureus* inoculated with lactam **68** and penicillin G were inspected by SEM for changes in cell size and appearance in comparison to a culture with no antibiotic. The samples were prepared from Kirby-Bauer diffusion plates by incision of the agar along the outer zones where bacterial growth is inhibited and sublethal doses of the drugs are present. The advantage of preparing the samples from agar cultures as opposed to broth is that a concentration gradient is produced as the antibiotics difuse through the solid media, allowing microscopic examination among a range of exposure amounts.

**Figure 4.02:** Scanning electron microscopy of *S. aureus* cultured with (a) no antibiotic, (b) lactam **68**, and (c) penicillin G.

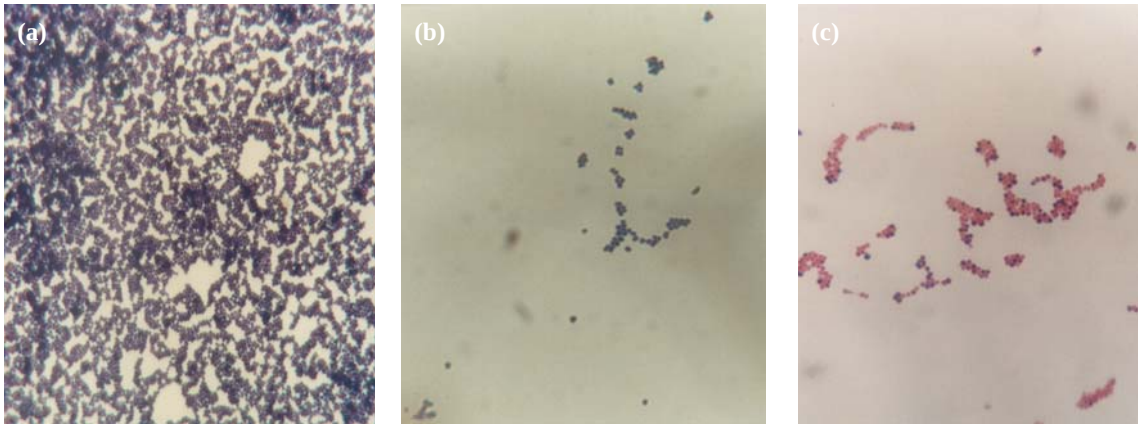



Results of the electron microscopy experiments are depicted in *Figure 4.02a-c*. The first image portrays the appearance of *S. aureus* grown with no antibiotic present. In its natural state, staphylococci grow to about 1 μm in diameter and reproduce in clusters of spherical-shaped cells. Cultures treated with lactam **68** also resembled *S. aureus* in its natural state, appearing spherical and uniform with no apparent deformities. When exposed to penicillin G, though, the bacteria were no longer uniform in size and shape (*Figure 4.02c*). The cocci appeared wrinkled, concaved, and often sheared resulting from deterioration of the cell wall. This was the first substantial evidence that *N*-thiolated β-lactams do not inhibit bacterial transpetidases nor cause rupturing of the cytoplasmic membrane in staphylococcus.

Section 4.3.2 Light Microscopy

The inhibition of cell wall biosynthesis in Gram-positive bacteria can be detected by standard light microscopy. Using the Gram-stain technique, bacteria are able to be distinguished by the thickness (eg, crosslinked peptidoglycan content) of their cell walls. The appearances of *S. aureus* exposed to (a) no antibiotic, (b) lactam **68**, and (c) penicillin G after staining is shown below. In its natural state, staphylococcus produces a thick cell wall which retains the crystal-violet stain upon decolorization with 95% ethanol (*Figure 4.03a*). When the bacterium is treated with a peptidase inhibitor, though, the Gram stain is lost due to thinning of the peptidoglycan shell. The staphylococci appear pink or red under the light microscope as a result. *Figure 4.03c* reveals the effect of penicillin G on the assimilation of the bacterial cell wall. The vast majority of cells manifested the staining characteristics of Gram-negative bacteria as a consequence of diminished peptidoglycan incorporation. The limited number of staphylococci that did retain the Gram stain in all probability came from the original inoculum before exposure to penicillin G. Mature bacteria with intact cell walls are not affected by β-lactam antibiotics and can be Gram stained.

**Figure 4.03:** Light microscopy of *S. aureus* exposed to (a) no antibiotic, (b) lactam **68**, and (c) penicillin G.




The effect on cell wall synthesis when *S. aureus* was treated with lactam **68** is shown in *Figure 4.03b*. It was quite apparent that all of the bacteria had completely intact cell walls. The staphylococci retained the crystal-violet stain indicating a high level of incorporated peptidoglycan. Based on these findings, it was concluded that *N*-thiolated β-lactams do not inhibit the formation of bacterial cell walls.

4.3.3 Model Membrane Studies

While *N*-thiolated β-lactams do not appear to inhibit formation of the cell wall, further proof was needed to establish that the compounds are not extracellular antibiotics. Microscopy experiments revealed that lactam **68** does not cause staphylococcal cells to rupture due to deterioration of the cell wall or plasma membrane. Additional evidence, however, was sought to confirm that the integrity of the plasma membrane was not being altered by the compounds.

The lipophilic nature of *N*-thiolated β-lactams might confer "detergent-like" properties enabling incorporation into biological membranes. To determine if lactam **68** can destabilize a phospholipid bilayer, model studies were performed in collaboration with Dr. Pavel Grigoriev[105] on black lipid membranes (eg, poreless membranes) prepared from commercially available long chain fatty acids in a nonpolar medium. Incorporation was monitored by electrical conductance of the lipid bilayers; increased conductivity and subsequent disintegration of the bilayer signified a disturbance in the membrane.

For the initial study, conductivity measurements indicated that lactam **68** at concentrations up to 15 μg/ml (0.06 μM from a DMSO stock solution) did not have an effect on the stability of the bilayer. These findings demonstrate that the lactam did not actively absorb into the interface of the hydrophobic membrane. Poron formation, the cause for the observed changes in conductance, would have resulted if

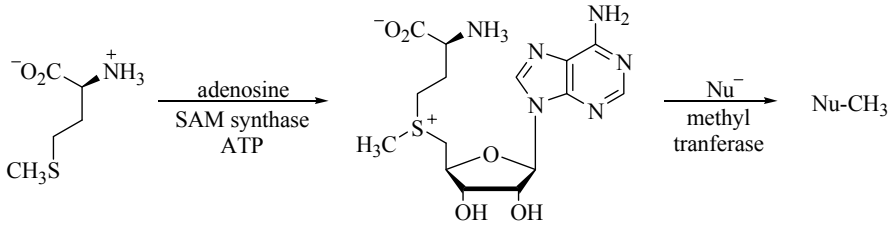
absorption had occurred. Lactam **68** was also assayed with a membrane containing model channels (porons) formed by amphotericin B. A change in conductance was observed for this anion-selective, porous bilayer, though, further experiments were needed to confirm these results. In addition, this ongoing collaboration will next examine the effect *N*-thiolated β-lactams possessing longer alkyl side chains for added lipophilicity.

### 4.4 *N*-Thiolated β-Lactams as Alkylating Agents

The ability of lactam **68** to function as a biological alkylator was next examined. Most antibiotics from the family of bioalkylating drugs have nucleic acids as a common cellular target. Hence, investigations of the methylation pathway focused on the interaction of *N*-thiolated β-lactams with supercoiled DNA and their effect on DNA strand replication.

Consideration of the alkylation mechanism derived from the *in vivo* use of methionine in cells to alter the structure and function of proteins, nucleic acids, and phospholipids. *Scheme 4.03* depicts the manner in which substrates are methylated by methionine in bacteria. Enzyme-dependent sulfenylation of adenosine with L-methionine generates *S*-adenosylmethionine which selectively dealkylates in the presence of a methyl transferase and complimentary substrate.

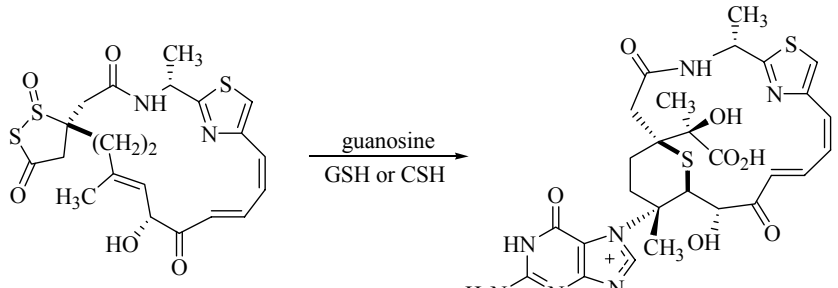
**Scheme 4.03:** Methylation of a nucleophilic substrate from the conversion of L-methionine to *S*-adenosylmethionine.




L-methionine *S*-adenosylmethionine

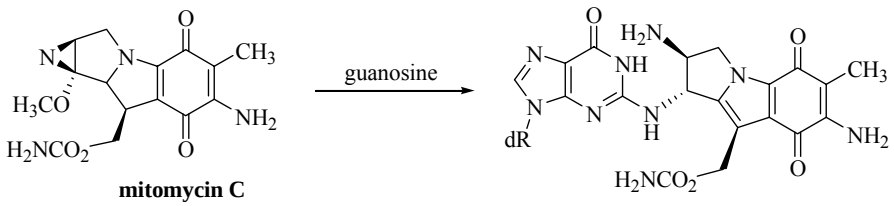
The clinical application of DNA alkylators is in cancer chemotherapy. Leinamycin[107] and mitomycin C are examples of antitumor drugs which alkylate guanosine residues in helical DNA (*Scheme 4.04*). Chemical modification of DNA bases can lead to strand breakages with apoptotic consequences. With the possibility that *N*-thiolated β-lactam could be a new member in the drug family of DNA alkylating agents, the anticancer properties of the compounds were investigated by researchers at H. Lee Moffitt Cancer Research Institute.

**Scheme 4.04:** Alkylation of guanosine by leinamycin and mitomycin C.




**leinamycin**

dR = deoxyribose; GSH = glutathione; CSH = cysteine.




4.4.1 Anticancer Properties of Lactam **68**[26d]

*N*-Methylthio β-lactam **68** demonstrated unique anticancer properties against human leukemic and solid tumor cell lines. In Jurkat T (leukemia) cells, the lactam was found to inhibit cellular mitosis and to induce apoptosis through (1) p38 mitogen-activated protein (MAP) kinase activation, (2) mitochondrial cytochrome *c* release, and (3) caspase activation. Activation of apoptosis was measured by the proficiency of caspase-3 to cleave poly(ADP-ribose) polymerase (PARP) in lysed Jurkat T cell extracts following treatment with lactam **68** (50 μM). Apoptotic commitment was observed after 4 hrs of exposure based on the fragmentation of PARP to p38. At this point, the ratio of viable to nonviable cells was 5:1. After 24 hrs, the nonviable population increased to 60% as determined by a trypan blue exclusion assay.

A rise in the S-phase population was also detected for Jurkat T cells treated with lactam **68**. Pulse-labeling experiments with $^{3}$H-thymidine confirmed that cells stalled in the S-phase had reduced DNA replicating abilities. TUNEL assays further revealed that the attenuation of DNA biosynthesis was due to strand breakages occurring prior to the attainment of S-phase.

4.4.2 DNA Cleavage Experiment

Though substantial anticancer properties were demonstrated by lactam **68**, it was unknown if the reduced viability of the cancer cells was instigated by chemical modification of DNA. Further experiments were required to verify if *N*-thiolated β-lactams are functional as nucleotide alkylators. A cell-free study was performed to detect whether a plasmid treated with an *N*-methylthio β-lactam could cause strand breakage or reduced torsion of supercoiled DNA. Plasmid pBR322 (0.5μg; Sigma Biochemicals) was incubated with lactam **68** at 37°C in sodium phosphate buffer (50 mM; pH 7.2) for 24 hrs and analyzed for fragmentation or linearization by agarose gel electrophoresis containing 1% ethidium bromide.

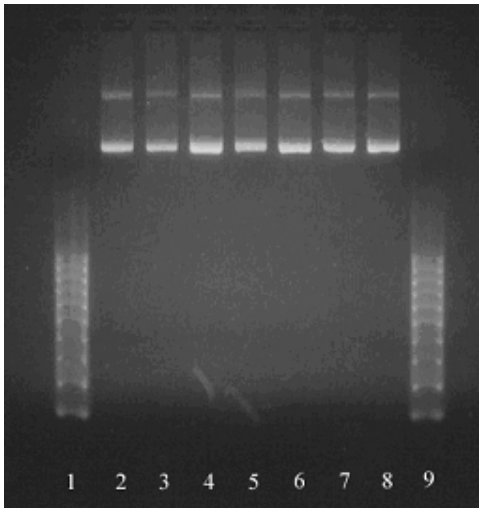



**Figure 4.04:** Supercoiled DNA treated with lactam **68** at 5-100 μM. Plasmid pBR322 (0.5μg) was incubated with lactam **68** at 37°C in sodium phosphate buffer (50 mM, pH 7.2) for 24 hrs and analyzed by agarose gel electrophoresis (ethidium bromide staining). Lane 1: marker. Lane 2: pBR322. Lane 3: pBR322 + DMSO. Lane 4. pBR322 + 5 μM **68**. Lane 5: pBR322 + 10 μM **68**. Lane 6: pBR322 + 25 μM **68**. Lane 7: pBR322 + 50 μM **68**. Lane 8: pBR322 + 100 μM **68**. Lane 9: marker.

The initial study examined the effect of lactam **68** on DNA integrity at elevated concentrations (5-100 μM). Results of this experiment are depicted by the gel in *Figure 4.04*. In lanes 2 and 3, two bands were observed for the plasmid in the absence of lactam **68**. With the supercoiled pBR322 giving rise to the

denser band, a weak band of relaxed or "nicked" DNA was formed by the electrical current and passage through the gel. Lanes 3-8 contained plasmid samples treated with various concentrations of lactam **68**. It was evident that the compound did not cause fragmentation or relaxation of the super helix. The bands were equivalent in location and illumination to those observed for the untreated plasmids (lanes 2,3) thus indicating that chemical modification of the DNA did not occur with 5-100 μM of the lactam present.

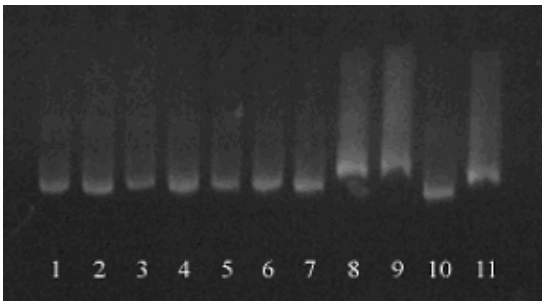



**Figure 4.05:** Supercoiled DNA treated with lactam **68** in the presence of thiols. Plasmid pBR322 (0.5μg) was incubated with lactam **68** at 37°C in sodium phosphate buffer (50 mM, pH 7.2) for 24 hrs and analyzed by agarose gel electrophoresis (ethidium bromide staining). Restriction digests conducted with EcoR1 were performed at 37°C for 1 hr. Lane 1: pBR322 + 100 μM **68**. Lane 2: pBR322 + 100 μM glutathione. Lane 3: pBR322 + 100 μM glutathione + 100 μM **68**. Lane 4: pBR322 + 100 μM DTT. Lane 5: pBR322 + 100 μM DTT + 100 μM **68**. Lane 6: pBR322 + 100 μM 2-mercaptoethanol. Lane 7: pBR322 + 100 μM 2-mercaptoethanol + 100 μM **68**. Lane 8: linearized pBR322 (EcoR1digest). Lane 9: pBR322 + EcoR1 + 100 μM **68**. Lane 10: pBR322 + DMSO. Lane 11: pBR322 + EcoR1 + DMSO.

DNA alkylators are often in a prodrug form requiring chemical or enzymatic activation before the nucleotide addition can occur. Leinamycin, for example, is transformed into a potent alkylating antibiotic by a thiol-mediated reaction with cysteine or glutathione.[109] Cells susceptible to leinamycin possess thiol-rich intracellular environments which may also be needed for *N*-thiolated β-lactams to alkylate its cellular target. A second study was performed to examine the stability of plasmid pBR322 (5 μM) after treatment of lactam **68** (100 μM) with various thiols (100 μM) (*Figure 4.05*). In lane 1, the plasmid following 24 hr exposure to lactam **68** is shown. As expected, the supercoil did not relax or linearized with the antibiotic present. DNA samples loaded in lanes 2-7 were incubated with glutathione (lane 2,3), dithiotreitol (DTT; lane 4,5), or β-mercaptoethanol (lane 6,7) as possible activators of **68**. Samples with lactam also added, are located in lanes 3,5, and 7. After 24 hrs, the superhelix of the DNA appeared unaltered by the combination of **68** and equimolar amounts of glutathione, DTT, or β-mercaptoethanol. The bands of lanes 2-7 were comparable in illumination and location to the antibiotic-free plasmid loading (lane 10).

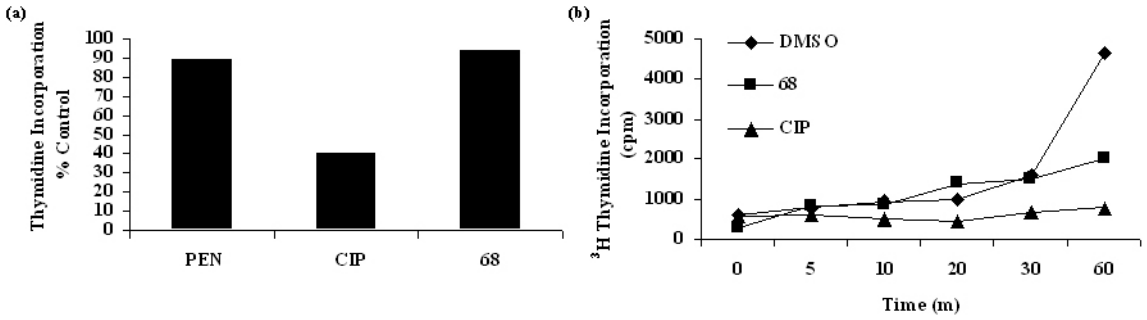
To verify the cleavability of the plasmid, pBR322 was linearize with the endonuclease, EcoR1 (Sigma Biochemicals). Samples containing the digested DNA are located in lanes 8 and 11. A loading comprised of lactam **68** and EcoR1 was applied to lane 9 to establish if the compound could function as an endonuclease inhibitor. Not surprisingly, linearization of the supercoil DNA was unhindered by the *N*-methylthio β-lactam.

### 4.4.3 Pulse-Labeling Studies of DNA Replication

Disruption of the super helix by chemical modifications of nucleotide bases can cause the arrest of DNA replication. To further substantiate that *N*-thiolated β-lactams are not alkylating drugs or affect nucleotide assimilation into double-stranded DNA, pulse-labeling experiments with $^{3}$H-thymidine were performed to monitor DNA replication in bacteria.[110] Protocols of the labeling experiment were as follows: cultures of *S. aureus* grown to early logarithmic phase ($10^{4}$ cfu/ml) were inoculated with $^{3}$H-thymidine (3 μCi/ml) and lactam **68** (20 μg/ml in DMSO from a 1 mg/ml stock; 2x MIC), penicillin G (2 μg/ml; 2x MIC), or the inhibitor of DNA replication, ciprofloxacin (30 μg/ml; 2x MIC). A sample containing only DMSO (20μl/ml) was used as a control in the study. Aliquots of 50 μl were removed at the appropriate time intervals and precipitated in trichloroacetic acid (TCA). Incorporation was measured by scintillation counts following filtration of the TCA precipitated material on glass fiber filters.

The labeling experiments revealed that lactam **68** has no effect on DNA replication in staphylococcus (*Figure 4.06*). *Figure 4.06a* depicts thymidine incorporation as a percent of the control for the three antibiotics. As expected, ciprofloxacin (CIP) was the most potent, inhibiting 65% of thymidine utilization after 30 mins. Conversely, with lactam **68** and penicillin G (PEN) present, ≥90% of the nucleotide was converted into DNA. The plot in *Figure 4.06b* further details the rate of thymidine uptake over a period of 1 hr. Again, ciprofloxan caused almost a complete and immediate cessation of DNA replication in *S. aureus*. Lactam **68** appeared not to affect thymidine incorporation up to 30 mins. Afterwards, the utilization began to temper most likely due to the halt of bacterial reproduction (see *Section 3.3.5* for growth studies).

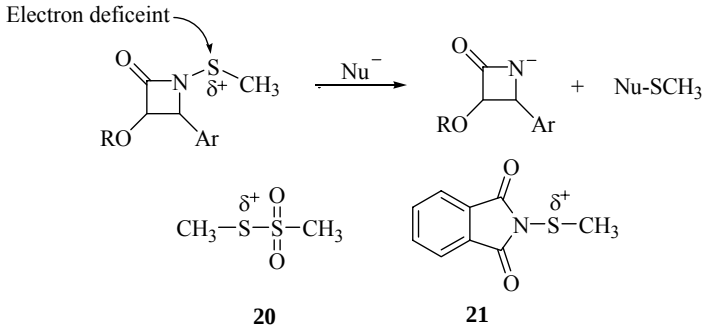
**Figure 4.06:** Thymidine incorporation in *S. aureus*.




## 4.5 *N*-Thiolated β-Lactams as Thiolating Agents

*N*-Thiolated β-lactams were discovered to have similar sulfenylating properties to the sulfur transfer reagent (**20**, **21**) involved in their preparation. An adjacent electronegative atom enhances the electrophilicity and vulnerability of the sulfurs toward nucleophilic attack (*Scheme 4.05*). Chemical studies of lactam **68** revealed that the most reactive substances towards **68** are those containing a sulfhydryl group. In buffered media, the *S*-methyl was rapidly cleaved by cysteine, glutathione (GSH), 1,4-dithio-treitol (DTT), and β-mercaptoethanol.

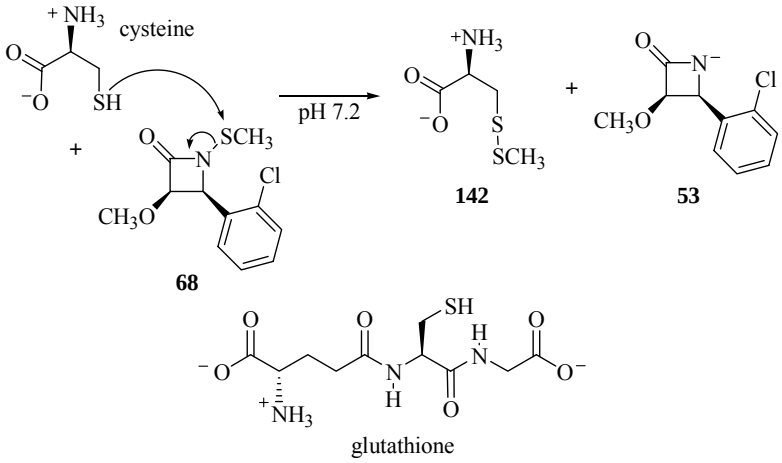
**Scheme 4.05:** Electrophilic sulfenylation of nucleophiles.




### 4.5.1 Enzyme-Binding Properties of *N*-Thiolated β-Lactams

Based on the preceding investigations, *N*-thiolated β-lactams appear to inhibit staphylococcal growth by *S*-methyl transfer to an unidentified protein or metabolite. Chemical studies involving lactam **68** and the 20 L-amino acids comprising proteins revealed that cysteine is the only component of proteins that reacts with the lactam under physiological conditions. The products of the reaction were presumably *S*-methyl-cystine (**142**) and *N*-protio lactam **53** as depicted below.

**Scheme 4.06:** Reaction of cysteine and lactam **68**.




To evaluate the *in vitro* effect of cysteine on the anti-staphylococcal properties of *N*-thiolated β-lactams, equimolar amounts of the cysteine-containing tripeptide, glutathione (see *Scheme 4.06*) and lactam **68** were added to the same well in a Kirby-Bauer diffusion experiment. Following overnight incubation, the plate inoculated with *S. aureus* was examined for growth inhibition. A zone of inhibition was absent for the well containing lactam **68** and glutathione. The antagonistic effect conferred by the tripeptide could explain the selectivity observed in the activity spectrum of *N*-methylthio β-lactams.

To further substantiate the neutralizing effect of glutathione, a plate was prepared with a 1 mg reservoir of the tripeptide located at the center and three surrounding wells containing 20 μg of the monochlorophenyl *N*-methylthio β-lactams **68**-**69** (*Figure 4.07*). The ability of glutathione to protect the bacteria from the antibiotics was again clearly evident by the presence of concaved zones between the glutathione- and lactam-containing wells. At the dimpled region of the zones, the concentration of glutathione was sufficient to deactivate the lactams allowing proliferation of the staphylococci. The protective role of glutathione likely explains the bacterial selectivity of the antibiotics. Species demonstrating the highest sensitivity to the lactams may have thiol-deficient intracellular environments. To determine if a correlation between the sulfhydryl content and biological activity exists, thiol concentrations in susceptible and nonsusceptible bacteria were measured.

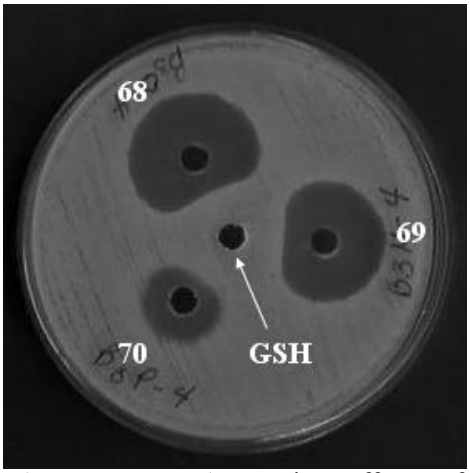
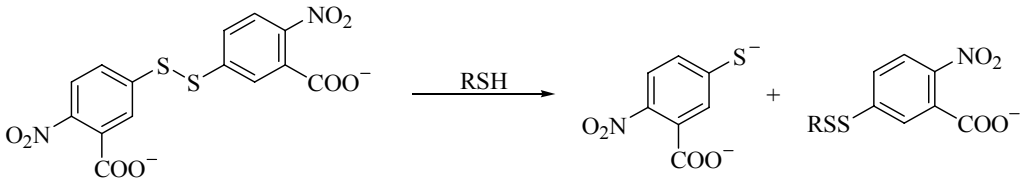



**Figure 4.07:** Antagonist effect of glutathione (GSH) on the anti-MSSA properties of *N*-thiolated β-lactams.

4.5.2 Thiol Determination in Bacteria

The concentration of sulfhydryl-containing molecules in bacteria was quantitated with Ellman's reagent, 5,5'-dithiobis(2-nitrobenzoic acid).[111] The reaction between the reagent and the thiol extracts results in cleavage of the disulfide bond and generation of the fluorophore, 2-nitro-5-thiobenzoate anion (*Scheme 4.07*). Optical density measurements ($OD_{412}$) were then taken of the samples and used to calculate the relative amount of thiols contained in the cells.

**Scheme 4.07:** Reaction of Ellman's reagent and a thiol.[111a]




Ellman's reagent — 2-Nitro-5-thiobenzoate anion (fluorescence-active)

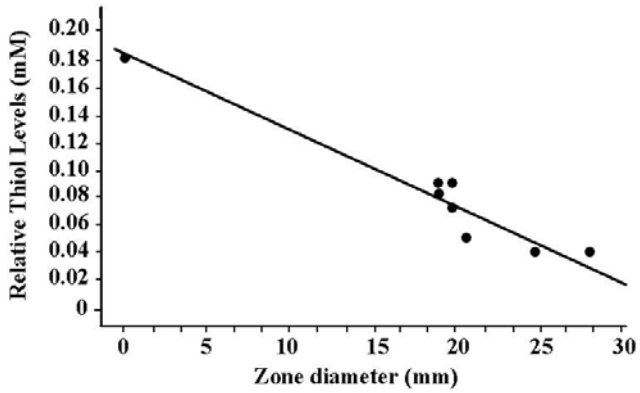
The intracellular sulfhydryl content of eight bacterial species with known susceptibilities to *N*-methylthio β-lactam **68** is depicted in *Table 4.02*. It was evident from the data that the thiol levels in bacteria were inversely proportional to the sensitivities the organisms displayed toward the antibiotic (*Figure 4.08*). The greatest concentration was discovered in *E. coli* which has been recognized in a previous report as a bacterial species maintaining a thiol-rich intracellular environment to guard against oxidants.[#] *N*-Thiolated β-lactams are entirely devoid of microbiological activity against *E. coli* and these data suggests that the microbe is protected by its proficiency to produce low molecular weight thiols.

**Table 4.02:** Correlation between relative intracellular thiol levels and susceptibility to lactam **68**.

| | ΔA | $C_o$ (mM) | zone (mm) |
|---|---|---|---|
| *Bacillus anthraces* | 0.239 | 0.04 | 25 |
| *Staphylococcus aureus* | 0.288 | 0.04 | 28 |
| *Bacillus megaterium* | 0.312 | 0.05 | 21 |
| *Bacillus cereus* | 0.412 | 0.07 | 20 |
| *Bacillus subtilis* | 0.475 | 0.08 | 19 |
| *Bacillus globigii* | 0.566 | 0.09 | 20 |
| *Bacillus niger* | 0.569 | 0.09 | 19 |
| *Escherichia coli* | 1.098 | 0.18 | 0 |

Susceptible bacteria were found to have ≤50% the total thiol content in *E. coli*. The lowest amounts were found in *B. anthraces* and *S. aureus* who also displayed the highest sensitive to lactam **68**. Additional species of *Bacillus* that displayed susceptibilities were also low producers of sulfhydryl compounds, although, the levels were slightly higher than those observed for *B. anthraces* and *S. aureus*. The zone sizes were smaller for these bacteria as well, with the implication that the elevated thiol content was responsible for reduced bioactivity.

**Figure 4.08:** Zone diameter of lactam **68** compared to relative thiol levels in select bacteria.



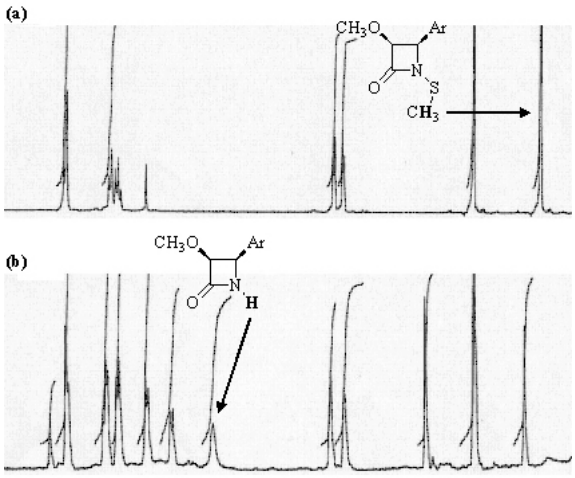
Though a correlation between sulfhydryl content and susceptibility to *N*-methylthio β-lactams could be established, the selectivity in all likelihood involves other factors. The existence, amount, and accessibility of the biological target(s) are additional factors that would influence the antimicrobial potency of the lactams

4.5.4 $^{1}$H NMR Studies

NMR studies were conducted to verify that the *N*-protio lactam is the only byproduct between the interaction of *N*-methylthio β-lactams and staphylococcal cells. The product was recovered by ethyl acetate extraction of an overnight broth culture of *S. aureus* inoculated with lactam **68**. The extracts were combined, concentrated, and analyzed by NMR. The $^{1}$H NMR spectrum of the crude isolated residue revealed the clean formation of the *N*-protio β-lactam **53** as represented by the broad singlet positioned at 6.97 ppm (*Figure 4.09b*). The loss of the *S*-methyl substituent was also confirmed by the absence the methyl proton which would give rise to a signal at 2.41 ppm (*Figure 4.09a*).

Although no attempts were made to identify the location of thio- transfer, the evidence from this and prior experiments suggest that the *S*-methyl is cleaved by a protein or metabolite following entry of the β-lactams into the cytoplasm. The *N*-protio lactam byproduct is subsequently secreted into the extracellular environment where in itself does not possess antibacterial properties. Further studies involving fluorescently-labeled or radiolabeled *N*-thiolated β-lactams are needed, however, to verify this proposed mechanism.

**Figure 4.09:** $^{1}$H NMR spectrums of (a) lactam **68** prior to inoculation of *S. aureus* and (b) lactam **68** following inoculation of *S. aureus*.




**4.6 Effects of Lactam 68 on Gene Expression**

4.6.1 Introduction

Gene expression is a biological process targeted by many families of antibiotics including: tetracyclines, macrolides, aminoglycosides, streptogramins, and oxazolidinones (see *Table 4.01*). In most cases, drugs that affect transcription or translation are bacteriostatic suggesting that one of these processes may be subject to inhibition by *N*-thiolated β-lactams. To test this hypothesis, incorporation of $^{3}$H-uridine and $^{3}$H-isoleucine into RNA and proteins, respectively, was measured in a broth culture of *S. aureus* treated with lactam **68**.

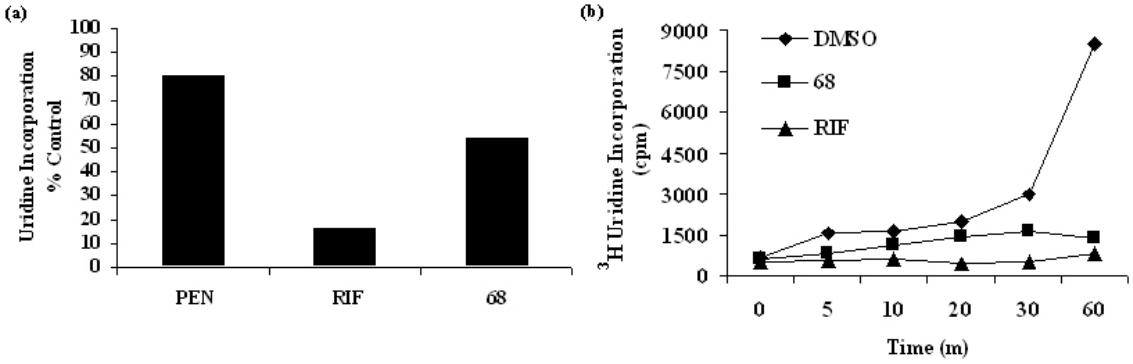
4.6.2 Pulse-Labeling Studies of RNA Assimilation

RNA labeling studies[110] with $^{3}$H-uridine were conducted to assess the influence of lactam **68** on transcription in *S. aureus*. Cultures were grown to early logarithmic phase ($10^{4}$ cfu/ml) and inoculated with $^{3}$H-uridine (3 μCi/ml) and lactam **68** (20 μg/ml in DMSO from a 1 mg/ml stock; 2x MIC), penicillin G (2 μg/ml; 2x MIC), or an inhibitor of RNA synthesis, rifampicin (2 μg/ml; 2x MIC). A sample containing only DMSO (20 μl/ml) was used as a control in the study.

*Figure 4.10* compares uridine uptake in cultures treated for 30 mins with the 3 antibiotics. As expected, penicillin G had only a moderate effect on RNA assembly into polynucleotide chains. The slight decrease was probably due to reduced cell counts associated with the bactericidal nature of the antibiotic. Conversely, rifampicin (RIF) clearly impacted the proficiency of *S. aureus* to transcribe RNA. The nucleotide usage was less than 20% of the control with the culture containing the RNA synthesis inhibitor.

Lactam **68** was also found to modulate uridine incorporation though not to the extent of rifampicin. After 30 mins, the nucleotide usage was 50% of the control suggesting that transcription may not be the primary process targeted by the lactam. Frequently, inhibition of a single biosynthetic pathway will hinder subsequent cellular processes by a negative feedback mechanism. These responses are observed in bacteria exposed to translation inhibitors. When protein synthesis stalls, the cell recognizes the accumulation of aminoacyl-tRNA and activates a feedback inhibitor to disable DNA replication, RNA synthesis, and peptidoglycan incorporation into cell walls. This was observed in *S. aureus* for chloramphenicol which obstructed RNA synthesis after 10 mins of exposure to the peptidyl transferase inhibitor (data not shown).

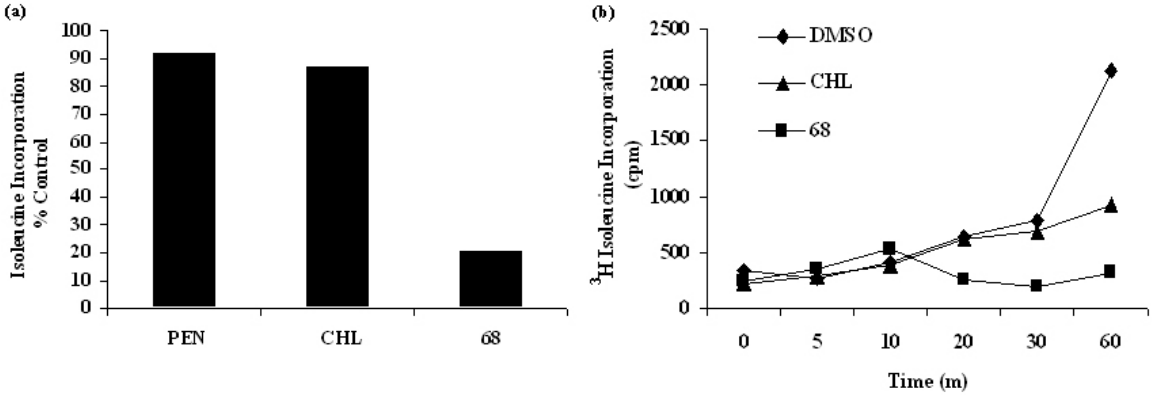
**Figure 4.10:** Uridine incorporation in *S. aureus*.




4.6.3 Pulse-Labeling Studies of Protein Synthesis

To determine if the primary role of growth inhibition by *N*-thiolated β-lactams is as a transcription inhibitor, the rate of protein synthesis was monitored by the uptake of $^{3}$H-isoleucine.[110] The experiments were conducted in the same manner previously described in *Section 4.3.3*. An overnight culture of *S. aureus* was grown to early logarithmic phase ($10^{4}$ cfu/ml) and inoculated with $^{3}$H-isoleucine (5 μCi/ml). A larger amount of the radiolabeled amino acid was required because of its lower utility compared to the DNA and RNA precursors. Isoleucine is 1 of 20 amino acids comprising proteins therefore the probability of its incorporation is less than thymidine and uridine. The cultures were then treated with lactam **68** (20 μg/ml in DMSO from a 1 mg/ml stock; 2x MIC), penicillin G (2 μg/ml; 2x MIC), or the translation inhibitor, chloramphenicol (30 μg/ml; 2x MIC). A sample containing only DMSO (20μl/ml) was once again used as a control.

**Figure 4.11:** Isoleucine incorporation in *S. aureus*.




*Figures 4.11a* and *4.11b* compare the relative rates that mRNA was translated in the presence of lactam **68**, penicillin G, and chloramphenicol (CHL). Penicillin G and chloramphenicol weakly inhibited protein synthesis in *S. aureus* after 30 mins compared to lactam **68** (*Figures 4.11a*) Based on these results, it was clearly evident that the lactam is a more powerful inhibitor of translation than transcription. The plot of *Figures 4.11b* further elaborates the propensity of the antibiotic to curtail polypeptide formation. For the initial 10 mins of the experiment, a linear progression of isoleucine incorporation was observed for the culture containing lactam **68**. Afterwards, a large dissension in protein synthesis was detected. Neither in the DNA or RNA incorporation studies was such a dramatic decline observed. Moreover, cultures treated with the lactam demonstrated a steady increase of thymidine and uridine incorporation that eventually leveled off over time. It was apparent from these results that the primary mode of action of *N*-thiolated β-lactams is to impede protein synthesis while transcription appears to be interrupted as a consequence of the translation blockage.

# CHAPTER V

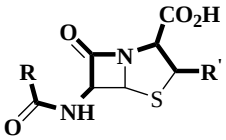
# DISCUSSION, CONCLUSIONS, AND FUTURE DIRECTIONS

## 5.1 Discussion and Conclusions

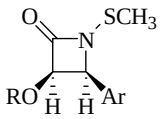
### 5.1.1 Introduction

Over the past 50 years, thousands of β-lactam antibiotics have been discovered in nature and through synthetic means. The biological target common to all members in this chemical class of antimicrobials has been the membrane-bound transpeptidases and DD-carboxypeptidases. These enzymes, collectively referred to as penicillin-binding proteins (PBPs), recognize traditional β-lactam antibiotics (ie, penicillins and cephalosporins) as the D-ala-D-ala residues in peptidoglycan (*Figure 5.01*).[55] In misrepresenting the normal substrates of PBPs, these antibacterials bind irreversibly, disabling the enzymes. Chemical structure comparison would suggest that *N*-methylthio β-lactams (**3**) do not react with the peptidases of bacterial cell walls (*Figure 5.01*). They lack the D-ala-D-ala backbone and additional binding motifs required for reactivity with the PBPs.

**Figure 5.01:** Chemical structures of penicillin and *N*-methylthio β-lactams.




penicillin




**3**

bonds in bold represent the D-ala-D-ala section recognized by PBPs.

Scientists have historically used the β-lactams discovered in nature as models to create analogs with enhanced potency and expanded activity spectrums. Seldom have researchers strayed from the concept that β-lactams must bind to a peptidase to function as an antibacterial. *N*-Methylthio β-lactams are the first novel design of a β-lactam having antimicrobial properties while not possessing PBP-binding capabilities. With an apparent intracellular mode of action, their activity spectrum contrasts to that of traditional β-lactam antibiotics amid the inclusion of yeast and cancer.

### 5.1.2 Narrow vs. Broad Spectrum

A narrow range of microbes demonstrated *in vitro* susceptibility to the inhibitory effects of *N*-methylthio β-lactams. As illustrated in *Table 5.01*, bacterial species very sensitive to the lactams were represented by 3 genera: *Bacillus*, *Micrococcus*, and *Staphylococcus*. The vast majority of bacteria were not susceptible indicating high selectivity among the 48 species that were screened. The sporadic activity displayed by *N*-thiolated β-lactams is unusual for an antimicrobial agent. Most antibiotics in clinical use possess broad spectrum activities despite being reserved for certain bacterial pathogens.

**Table 5.01:** Susceptibility comparison of *N*-methylthio β-lactams **67**-**81**, **83**, and **97**-**99**.

| Bacteria (Species:Strains) | Gram (±) | Zone of Growth Inhibition (mm) | | | | |
|---|---|---|---|---|---|---|
| | | >30 | 21-30 | 15-20 | <15 | 0 |
| *Bacteriodes* sp. (1:1) | - | | | X | X | X |
| *Bacillus* spp. (7:7) | + | | X | X | X | |
| *Enterobacter* sp. (1:1) | - | | | | | X |
| *Enterococcus* spp. (6:6) | + | | | | X | X |
| *Escherichia* sp. (1:3) | - | | | | | X |
| *Fusobacterium* spp. (6:16)[112] | - | | | | | X |
| *Haemophilus* sp. (1:3) | - | | | | X | |
| *Klebsiella* sp. (1:1) | - | | | | | X |
| *Lactococcus* sp. (1:1) | + | | | | X | X |
| *Listeria* sp. (1:2) | + | | | | X | X |
| *Micrococcus* sp. (1:1) | + | X | X | | | |
| *Mycobacterium* sp. (1:1) | - | | | | | X |
| *Neiserria* sp. (1:2) | - | | | X | X | X |
| *Peptostreptococcus* sp. (1:1)[112] | + | | | | | X |
| *Porphyromonsa* (1:2)[112] | - | | | | | X |
| *Proteus* sp. (1:1) | - | | | | | X |
| *Pseudomonas* sp. (1:1) | - | | | | | X |
| *Salmonella* sp. (1:1) | - | | | | X | X |
| *Serratia* sp. (1:1) | - | | | | | X |
| *Sporobolomyces* sp. (1:1)[113] | - | | | | | X |
| *Staphylococcus* spp. (10:21) | + | X | X | X | X | X |
| *Streptococcus* spp. (2:2) | + | | | X | X | X |
| *Vibrio* sp. (1:2) | - | | | | X | X |
| susceptibilities | | high | | medium | weak | none |

Broad spectrum antibacterials kill or inhibit a wide range (ie, genera) of Gram-positive and Gram-negative microbes exposed to the drugs. Antibiotics in clinical use that have been classified as broad spectrum include: aminoglycosides (ie, streptomycin, gentamicin), tetracyclines (ie, doxycycline), semi-synthetic penicillins (ie, ampicillin, amoxicillin), 2nd and 3rd generation cephalosporins, monobactams (ie, aztreonam), carbapenems (ie, imipenem), macrolides (ie, erythromycin), most quinolones (ie, ciprofloxacin), chloramphenicol, cycloserine, sulfonamides, and many others. Narrow spectrum bacteriocidal or bacteriostatic therapeutics are selective for either Gram-positive or Gram-negative bacteria. Glycopeptides (ie, vancomycin, teicoplanin), natural and semi-synthetic penicillins (ie, penicillin G), and oxazolidinones are used clinically to treat Gram-positive bacterial infections whereas polymyxin (ie, colisitin) and nalidixic acid are mainly effective for Gram-negative bacteria. *N*-Methylthio β-lactams appear to have a limited spectrum of activity similar to the glycopeptides. Although they possess dissimilar modes of action and cytotoxicities, *N*-methylthio β-lactam and glycopeptide antibiotics both inhibit the growth of Gram-positive bacteria deriving from the "Bacilli" class of medically important pathogens.

5.1.3 Bioactivity Spectrum

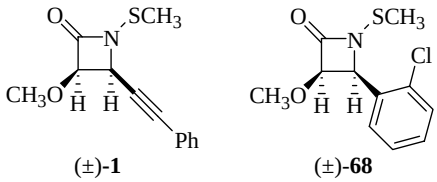
*N*-Methylthio β-lactams demonstrated a unique bioactivity spectrum that was distinct from any pre-existing class of drugs. Inhibitory effects were observed against bacterial, fungal, and cancer cells but lacking for viruses. In bacteria, the most susceptible cell-types to *N*-methylthio β-lactams were Gram-positive. Gram-negative species displayed little or no sensitivities to the lactams with the exception of *Neisserria gonorrhoeae* which had modest susceptibility towards the antibiotic. Clearly though, the bacterial species most affected by the lactams derived from the genera of *Staphylococcus*. A broad range of bioactivities was achieved by the drugs against 10 species of staphylococci. MSSA, MRSA, *S. epidermidis*,

and *S. lugdunensis* were consistently the most susceptible; *S. lentus* and *S. simulans* were consistently the least susceptible. The lactams were also effective against some species of *Bacillus*, most notably *B. anthracis* whose pathogenesis causes anthrax disease. The greatest surprise, though, were the bioactivities observed against eukaryotes, particularly yeast and cancer cells. Until the discovery of *N*-methylthio β-lactams, never have antifungal or anticancer properties been observed for a member from the β-lactam class of antibiotics. Moreover, their ability to obstruct the viability of eukaryotic pathogens further substantiates that the PBPs are probably not the primary biological target in bacteria. The potential therapeutic use of *N*-methylthio β-lactams for treating mycosis and carcinoma warranted additional screening which are presently ongoing.

5.1.4 Structure-Activity Relationship

Few structure-activity relationships were established for the *N*-thiolated β-lactam analogues. In bacteria, changes to the $N_1$, $C_3$, and $C_4$ substituents did not expand the activity spectrum nor enhance the *in vitro* performance of the lactams against a particular class or species. Increasing polarity or lipophilicity, though, reduced biological activity for bacteria susceptible to the lead compound, lactam **1** (*Figure 5.02*). Analogues which demonstrated the greatest potency contained a $C_4$ aryl ring functionalized by a weak activating or deactivating group such as methyl or chlorine, respectively. The monochlorinated lactam **68** (*Figure 5.02*) gave the best overall activity against bacteria, yeast, and cancer. Analogues with multiple chlorine atoms, however, were the most effective inhibitors of yeast proliferation. Di- and trichlorinated lactams demonstrated 15-40% greater activity than **68** and clotrimazole.

**Figure 5.02:** Structure of *N*-methylthio β-lactams **1** and **68**.




(±)-**1** (±)-**68**

5.1.5 Mechanism of Action

*N*-Methylthio β-lactams appear to inhibit protein synthesis as their primary mode of action. Substantiating this hypothesis were the results gathered from multiple chemical and biological experiments. Chemical studies demonstrated that the carbonyl carbon and the *S*-methyl carbon of the thiolated lactams are least vulnerable to nucleophilic attack which was an observation also reported for the thiamazins[#] (*Figure 5.03*). As illustrated in *Figure 5.04*, antibiotics that perform via an acylating (ie, penicillin) or alkylating (ie, mitomycin C) mechanism possess an electropositive center (δ+) at the sites of reactivity conferred by neighboring electronegative atoms. In the case of *N*-methylthio β-lactams, the highly activated, electron-deficient sulfur center is the most reactive part of the molecule due to the adjacent amide functional (*Figure 5.04*). For this reason, the ability of the lactams to acylate or alkylate cellular components in bacteria was considered remote.

Microscopy and DNA studies further validated the proposed chemical mechanism by demonstrating that the lactams do not inhibit cell wall synthesis through acylation of bacterial PBPs or alkylation of supercoiled DNA thereby causing linearization or disrupting DNA replication. Intracellular transfer of the *S*-methyl substituent appears to be the means which the viability of bacteria, yeast, and cancer cells is reduced. In bacteria, though, the lactams seem to have a cystostatic effect on growth which implies that the primary process targeted by the compounds is protein synthesis.

**Figure 5.03:** Reaction of thiamazins at pH 11.[88]

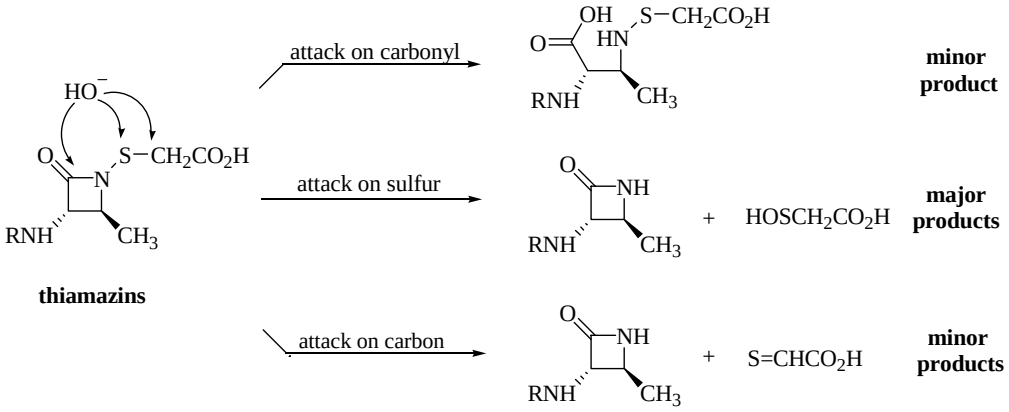
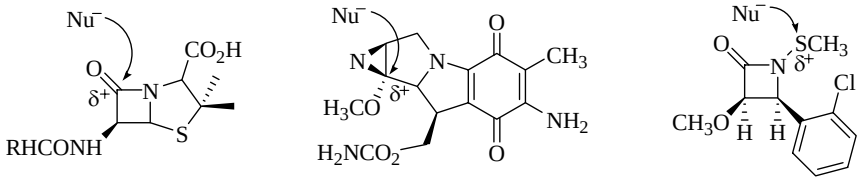



**Figure 5.04:** Site of nucleophilic attack on electropositive centers of 3 antibiotics: penicillin, mitomycin C, and *N*-methylthio β-lactam **68**.



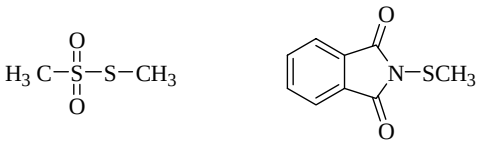

The vast majority of bacteriostatic antibiotics are inhibitors of gene expression including: aminoglycosides, tetracyclines, macrolides, and oxazolidinones. These antibacterials operate by slowing cell proliferation allowing time for the natural defenses of the body to eradicate the infectious bacteria. Reproduction and other biosynthetic processes in bacteria are usually suppressed when gene expression is blocked. This was observed in the incorporation studies with lactam **68** which showed that inhibition of protein synthesis in staphylococci caused a 50% reduction in RNA synthesis. Future studies will be dedicated to determining the precise stage at which protein assimilation is interrupted.

## 5.2 Future Directions

### 5.2.1 Structure-Activity Relationship

To help define the intracellular target of *N*-methylthio β-lactams, several key investigations were attempted or are, at present, being conducted. Additional SAR studies were performed to determine if the β-lactam ring was required for bioactivity. Methylthiomesylate (**20**) and *N*-(methylthio)phthalimide (**21**) (*Figure 5.05*) were the initial compounds screened but found to be devoid of antistaphylococcal properties. Other molecules analogous to $C_4$ aryl substituted *N*-methylthio β-lactams were, however, desired to ascertain if the azetidinone was a prerequisite for activity.

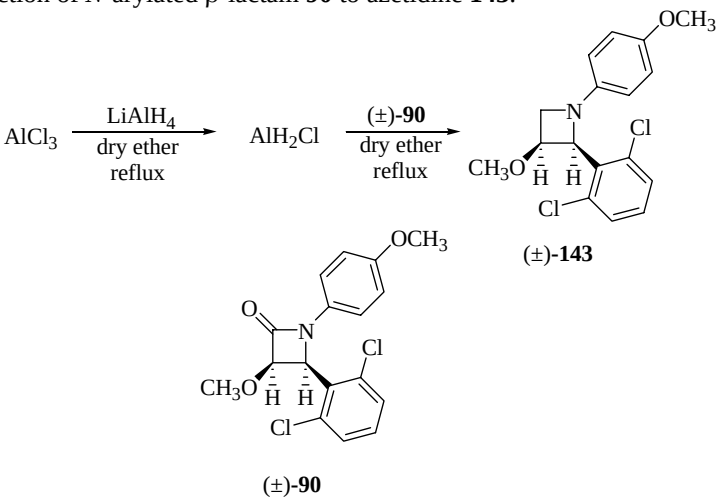
**Figure 5.05:** Methylthiomesylate (**20**) and *N*-(methylthio)phthalimide (**21**).




**20** **21**

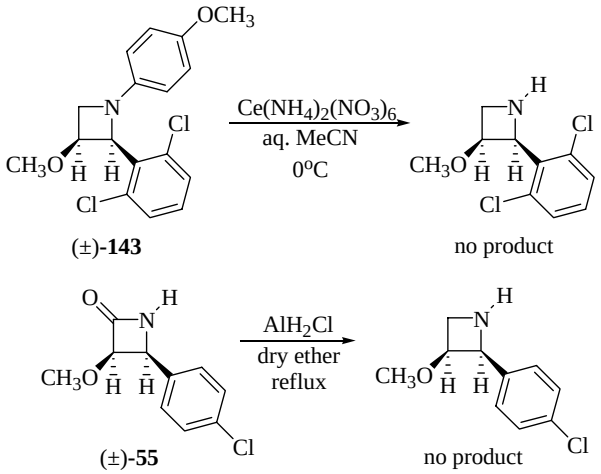
Preparation of azetidines from *N*-arylated and *N*-protio β-lactams were the first compounds pursued in these studies. A general method of reducing the carbonyl carbon of β-lactams was reported by Ojima[114] using monochlorohydroalane ($AlH_2Cl$). As depicted in *Scheme 5.01*, the metal hydride was generated *in situ* from the reduction of aluminum trichloride with lithium aluminum hydride. *N*-Anisyl lactam **90** was then added to the stirring solution of $AlH_2Cl$ and the mixture was refluxed for 4 hrs. Following aqueous workup, azetidine **143** was isolated as a yellow oil in quantitative yield.

**Scheme 5.01:** Reduction of *N*-arylated β-lactam **90** to azetidine **143**.



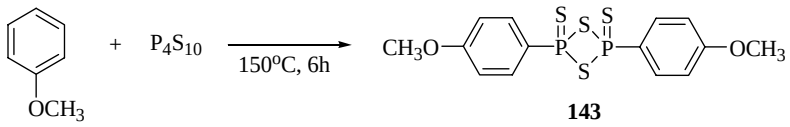

Attempts to deprotect azetidine **143** with cerium ammonium nitrate were unsuccessful. The dearylated azetidine could not be obtained from the reaction as a result of oxidative decomposition. Efforts to reduce the carbonyl carbon of *N*-protio β-lactams with $AlH_2Cl$ also failed; $^1H$ NMR spectra of the reaction following workup confirmed the presence of unconverted starting material.

**Scheme 5.02:** Reduction of *N*-arylated β-lactam **90** to azetidine **143**.




Despite the failed attempts to make *N*-methylthio azetidines, additional types of *N*-containing rings were explored. Azetidine-2-thiones (eg, thiolactams) were successfully prepared from $C_4$ aryl-substituted β-lactams using 2,4-*bis*(4-methoxyphenyl)-1,3-dithiadiphosphonate-2,4-disulfide (Lawesson's reagent).[115] Lawesson's reagent (**143**) was synthesized by refluxing anisole and phosphorous pentasulfide in a 10:1 ratio (*Scheme 5.03*).[116] After 6 hrs, the yellow precipitate was filtered, washed with chloroform/ether, and dried *in vacuo* at room temperature to prevent polymerization of the reagent.

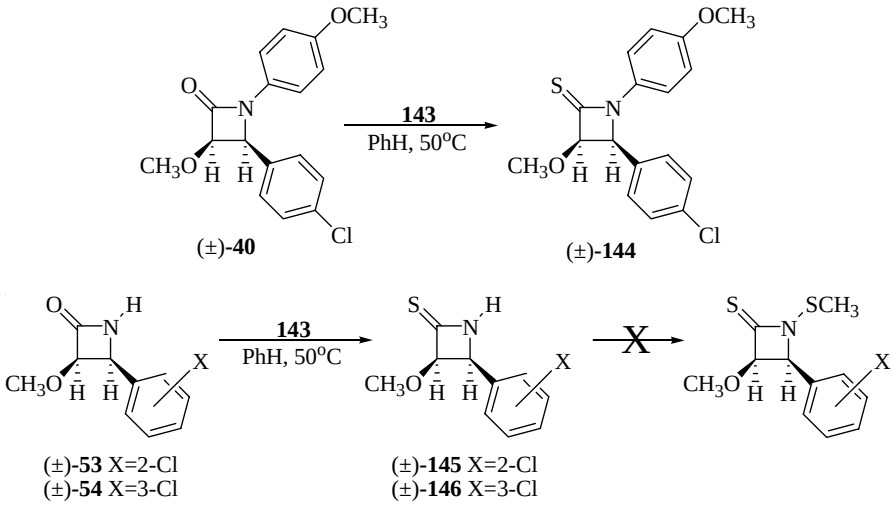
**Scheme 5.03:** Preparation of Lawesson's reagent.




**143**

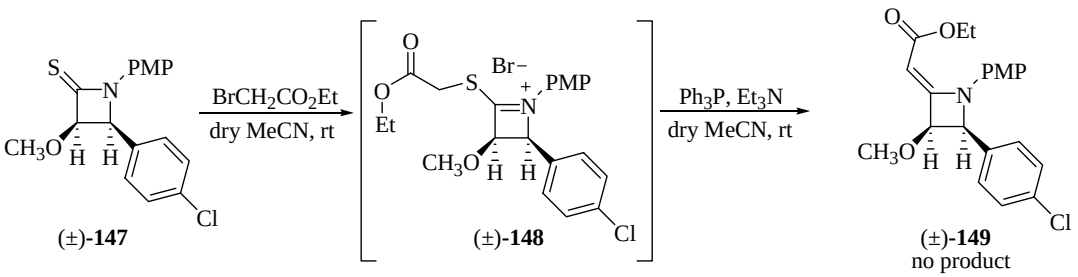
*N*-Aryl protected β-lactam **40** was then subjected to the thionation conditions using Lawesson's reagent (*Scheme 5.04*). Compounds **40** and **143** were stirred at approximately 50°C for 4 hrs followed by silica gel chromatography to give thiolactam **144** as a yellow solid. With the β-lactams proving stable to the reaction conditions, *N*-protio β-lactams **53** and **54** were converted to their respective thiolactams **144** and **145**. Attempts to prepare *N*-methylthio thiolactams were, however, unsuccessful.

**Scheme 5.04:** Synthesis of thiolactams **144**-**146**.



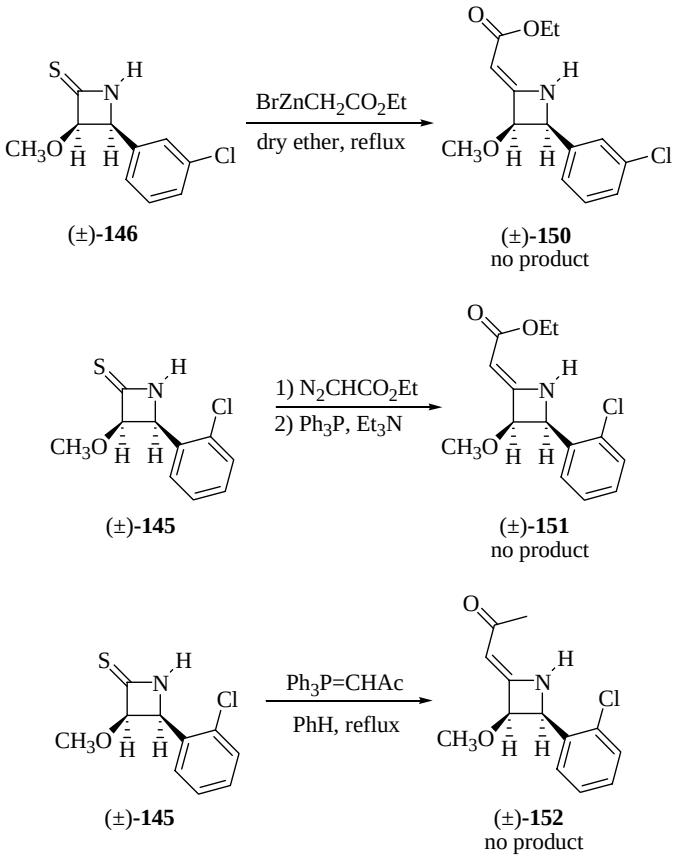

Other analogous compounds that were explored included 2-methylene azetidines. Previously, these vinylogous carbamates have been generated from thiolactams either by an Eschenmoser sulfide contraction,[117] thio-Reformasky,[118] or thio-Wittig reaction.[119] Eschenmoser's method has been the most widely used and was first attempted on the *N*-arylated thiolactam **147** (*Scheme 5.05*). Ethyl bromoacetate and **147** were stirred at room temperature to generate the thioalkyliminium salt **148** as an intermediate (not isolated).[117c] After 1 hr, triphenylphosphine was added to induce the sulfide contraction whereby, eliminating sulfur. However, the vinylgous amide **149** was not obtained, presumably due to ring strain or insufficient nucleophilicity of the thiocarbonyl.

**Scheme 5.05:** Attempted synthesis of 2-methylene azetidines from thiolactam **147** via Eschenmoser sulfide contraction.



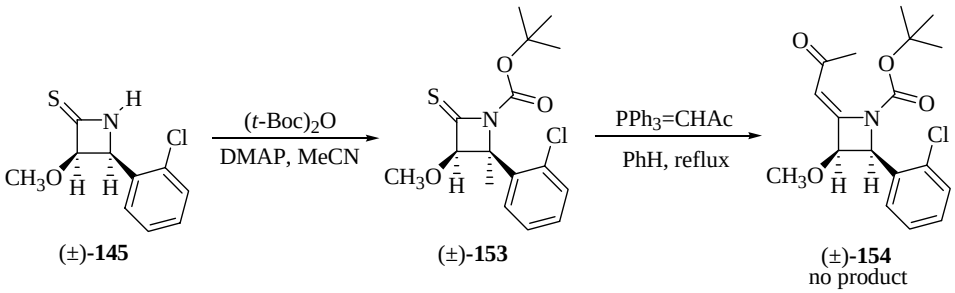
The thio-Reformansky was then tried with ethyl bromozincacetate on the *N*-protio thiolactam **146** (*Scheme 5.06*).[118] Again, the reaction produced unsatisfactory results. Conversion to the vinylogous azetidine was also attempted with ethyl diazoacetate and the stabilized ylide, (carbmethoxymethylene)triphenyl phosphine, but the desired product was not obtained (*Scheme 5.06*).

**Scheme 5.06:** Unsuccessful syntheses of vinylogous azetidines from *N*-protio thiolactams.



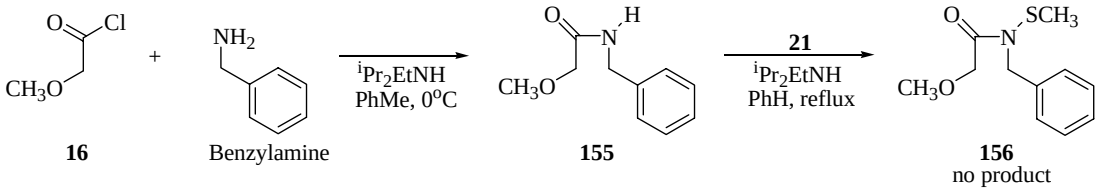

Previous reports[119b] have shown that thiolactams require an electron-withdrawing group on the amide nitrogen for the thiocarbonyl to have sufficient reactivity to nucleophilic attack. A common substituent allocated to the nitrogen atom of the thiolactam which can later be removed with hydrofluoric acid is an alkoxycarbonyl. Apropos, *N*-protio lactam **145** was acylated with di-*tert*-butylcarbonyl anhydride in the presence of catalytic base to generate *N*-Boc thiolactam **153** in quantitative yield (*Figure 5.07*). The thio-Wittig reaction was then attempted on the Boc activated compound, however, only starting material was recovered after 24 hr of reflux in benzene.

**Scheme 5.07:** Unsuccessful attempts to synthesize vinylogous azetidines from *N*-Boc thiolactams.



Synthesis of an open ring *S*-methyl amide was also attempted via the sulfenylation of amide **155**, prepared from the reaction of benzyl amine and methoyxacetyl chloride (*Scheme 5.08*). The sulfenamide **156**, a compound analogous to *N*-methylthio β-lactam **67**, could not be obtained from **155** using *N*-methylthiophthalimide (**21**).

**Scheme 5.08:** Unsuccessful synthesis of noncyclic thioamide **156**.

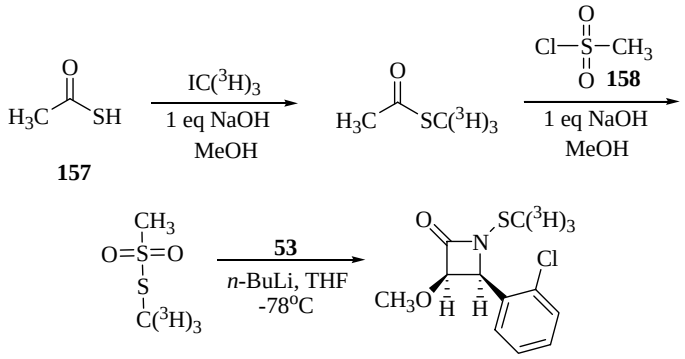



**16** Benzylamine **155** **156** no product

5.2.2 Elucidation of the Biological Target

Elucidation of the biological target is the primary focus of current investigations. To ascertain the cellular binding site of the lactams, the *S*-methyl residue will be labeled with tritium which can be tracked inside the bacteria. The proposed synthetic route to the radiolabeled lactam is depicted in *Scheme 5.09*. Thioacetic acid (**157**) is alkylated with commercially available [$^3$H]methyl iodide then subsequently cleaved by mesyl chloride (**158**) under basic conditions to provide [$^3$H]methylthiomesylate (**159**). Afterwards, the mesylate shall be used to sulfenylate lactam **53** to give the [$^3$H]*N*-methylthio β-lactam for the probing studies.

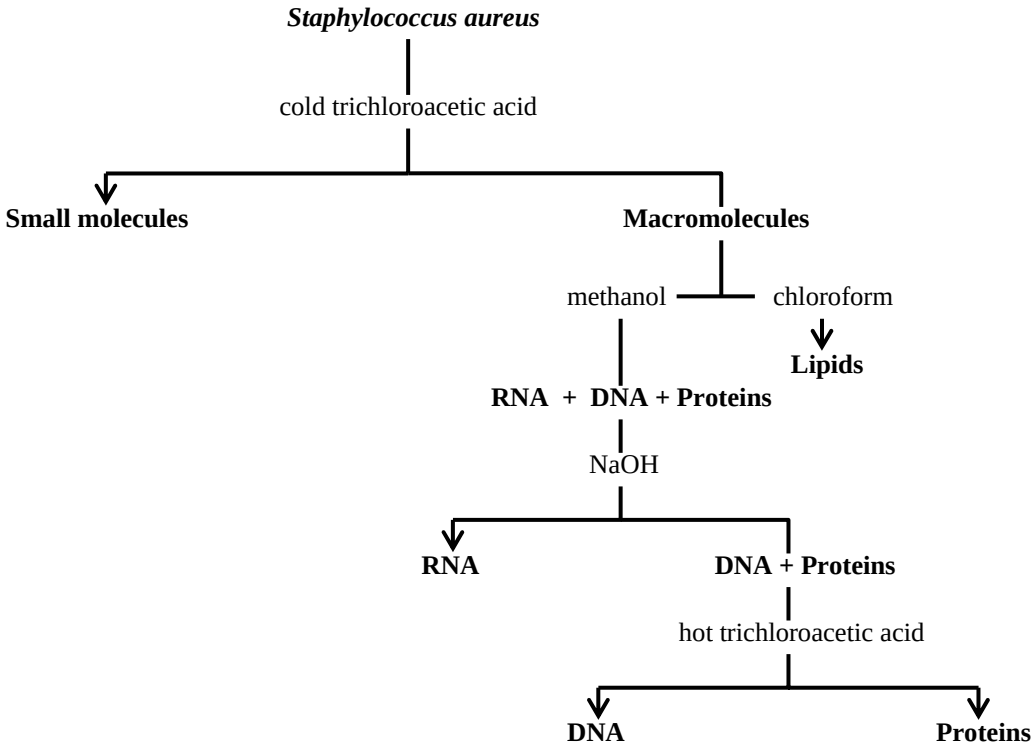
**Scheme 5.09:** Proposed synthetic route to [$^3$H]-labeled lactam **160**.




**157** **159** **160**

The [$^3$H]*S*-methyl will be traced by fractionation of *S. aureus* into its subcellular components following treatment with **168**. The flow diagram in *Figure 5.04* depicts the manner by which the fractionation will proceed.[120] A $10^8$ cfu/ml suspension of *S. aureus* in buffer will be treated with the [$^3$H]lactam for 30 mins. The labeled cells are then harvested, lysed, and fractioned by chemical means (*Figure 5.04*). Radiation levels will next be measured to determine if the *S*-methyl is bound to a metabolite, RNA, DNA, a protein, or a lipid. Afterwards, the fraction(s) containing the label can be further analyzed to resolve the molecular size of the biological target.

**Figure 5.06:** Cell fractionation of *S. aureus*.




In conclusion, this thesis has traced the development of the project from a curious and unexpected discovery of an *N*-methylthio β-lactams having antibacterial properties to a potentially valuable class of new antibiotics. The remaining research and the focus of current investigations is to design additional analogues with enhanced potency (eg, lower MICs) and greater stability to glutathione. Furthermore, experiments to identify the cellular target will continue to be conducted to validate the novelty of these antibiotics and bring them to the forefront as a new weapon for the treatment of MRSA.

# CHAPTER VI

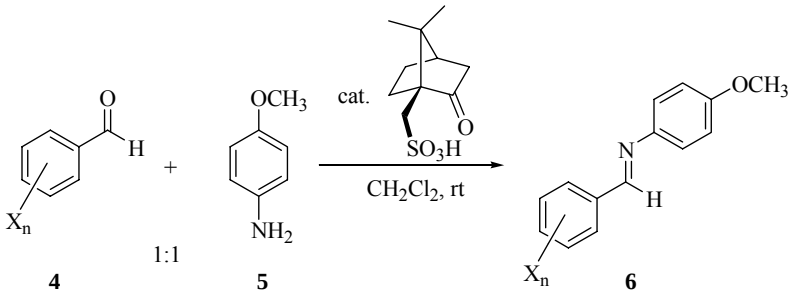
# MATERIALS AND METHODS

## 6.1. Synthetic Procedures

All reagents needed for the synthesis of *N*-thiolated β-lactams were purchased from Sigma-Aldrich Chemical Company and used without further purification. Solvents were obtained from Fisher Scientific Company. Products purified by flash chromatography used J.T. Baker flash chromatography silica gel (40 μm). NMR spectra were recorded in $CDCl_3$. $^{13}C$ NMR spectra were proton decoupled, but not fluorine decoupled, therefore, some signals in the spectra are split by $J_{C\text{-}F}$-coupling.

### 6.1.2 Preparation of *N*-Anisylimines

***N*-Anisylimines (6):** Aldehyde (1 mmol), *p*-methoxyaniline (1 mmol), and 1 mg camphorsulfonic acid (cat.) were dissolved in dichloromethane and stirred at room temperature for 1 hr. Afterwards, an appropriate amount of magnesium sulfate was added and the mixture was filtered. The solvent was then removed *in vacuo* and purified by silica gel chromatography or recrystallized from methanol.

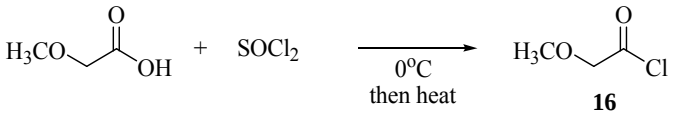



**4** **5** **6**

X = H, F, Cl, Br, I, $NO_2$, $CH_3$, $OCH_3$, $O_2CR$

### 6.1.3 Preparation of Acid Chlorides

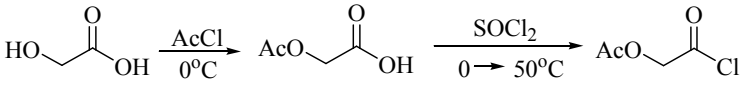
**Methoxyacetyl chloride (18):** To a 250 ml flame dried round bottom flask containing methoxyacetic acid (31.38 g, 0.35 mol) was added thionyl chloride (41.4 g, 0.35 mol) dropwise while stirring for 0.5 hr at 0°C. The ice bath was remove and the mixture reacted for 12 hr at 30°C. The pale yellow solution was then distilled to provide pure acid chloride (bp 112-113 °C).




**16**

**Acetoxyacetyl chloride (18):** To a 250 ml round bottom flask containing glycolic acid (10 g, 0.13 mol) was added acetyl chloride (20 g, 0.26 mol) dropwise at 0°C. The ice bath was removed and the resulting slush dissolves within 30 min after the acetyl chloride addition. After 1 hr, a white precipitate forms. Benzene (50 ml) was added and the reaction stirred for an additional 12 hr. The solid was then filtered and washed with benzene and ice cold methanol to give pure product **17** in 80% yield (mp 62-63°C). Afterwards, thionyl chloride (31.8 g, 0.24 mol) was added dropwise at 0°C to a 250 ml flask containing

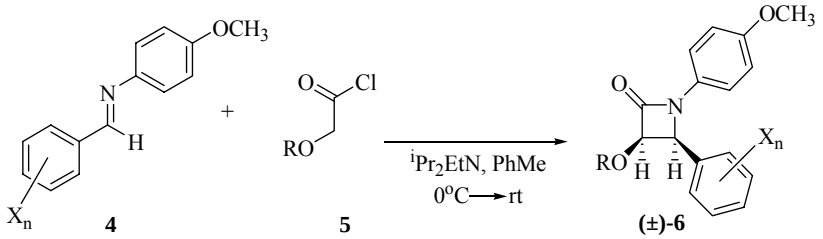
acid **17** (24.6 g, 0.24 mol) while stirring. The ice bath was then removed and the resulting slush was stirred overnight at 30°C. After 12 hr, the liquid was distilled *in vacuo* to give the acetoxyacetyl chloride in 60% yield from glycolic acid.




**17** **18**

### 6.1.4 Preparation of *N*-4-Anisyl Azetidin-2-ones

***N*-4-Anisyl azetidin-2-ones (6):** Imine (1 mmol) and *N*,*N*-ethyldiisopropylamine (3 mmol) or triethyl amine (3 mmol) were added to a 250 ml round bottom flask containing dry dichloromethane or toluene (50 ml per g of imine) and the mixture was chilled in an ice bath to 0°C. Acid chloride (1.1 mol) in dry toluene (50 ml per g acid) was added dropwise (5 ml per min) to the stirring solution. Following addition of the acid chloride, the flask was removed from the ice bath and the reaction monitored by thin layer chromatography. After 1 hr, the solution was gently heated overnight if imine was still present. The solvent was then removed *in vacuo* and the crude product was redissolved in ethyl acetate. After $dH_2O$ extraction, the organic layer was dried with magnesium sulfate, filtered, and the crude material was concentrated *in vacuo*. Diethyl ether or methanol was then added and the solution chilled to precipitate the β-lactam. When silca gel chromatography was required for purification, separation was achieved with ethyl acetate and hexanes mixtures.




**4** **5** **(±)-6**

**(±)-(3S,4R)-4-(phenyl)-3-methoxy-1-(4-methoxyphenyl)azetidin-2-one (37):** white solid; mp 168-170 °C; $^1$H NMR (250 MHz, $CDCl_3$): 7.33-7.16 (m, 5H), 7.19 (d, 2H, *J* = 9.0 Hz), 6.71 (d, 2H, *J* = 9.0 Hz), 5.11 (d, 1H, *J* = 4.8 Hz), 4.74 (d, 1H, *J* = 4.8 Hz), 3.66 (s, 3H), 3.11 (s, 3H); $^{13}$C NMR (63 MHz, $CDCl_3$): δ 163.7, 156.3, 133.3, 130.5, 128.5, 118.7, 114.3, 84.8, 61.8, 58.4, 55.4.

**(±)-(3S,4R)-4-(2-chlorophenyl)-3-methoxy-1-(4-methoxyphenyl)azetidin-2-one (38):** white solid; mp 183-184 °C; IR (neat) 1747 cm$^{-1}$ (C=O); $^1$H NMR (250 MHz, $CDCl_3$): δ 7.43 (d, 1H, *J* = 7.4 Hz), 7.29-7.19 (m, 5H), 6.80 (d, 2H, *J* = 9.0 Hz), 5.61 (d, 1H, *J* = 4.8 Hz), 4.89 (d, 1H, *J* = 4.8 Hz), 3.73 (s, 3H), 3.27 (s, 3H); $^{13}$C NMR (63 MHz, $CDCl_3$): δ 163.6, 156.4, 133.2, 131.2, 130.3, 129.6, 128.9, 127.0, 118.6, 114.1, 84.9, 59.1, 58.8, 55.4.

**(±)-(3S,4R)-4-(3-chlorophenyl)-3-methoxy-1-(4-methoxyphenyl)azetidin-2-one (39):** white solid; mp 189-191 °C; $^1$H NMR (250 MHz, $CDCl_3$): δ 7.28-7.21 (m, 4H), 7.17 (d, 2H, *J* = 8.9 Hz), 6.72 (d, 2H, *J* = 9.0 Hz), 5.09 (d, 1H, *J* = 4.7 Hz), 4.74 (d, 1H, *J* = 4.7 Hz), 3.68 (s, 3H), 3.15 (s, 3H); $^{13}$C NMR (63 MHz, $CDCl_3$): δ 163.5, 156.4, 134.5, 132, 130.3, 129.3, 128.8, 118.7, 114.3, 84.7, 61.1, 58.5, 55.4.

**(±)-(3S,4R)-4-(4-chlorophenyl)-3-methoxy-1-(4-methoxyphenyl)azetidin-2-one (40):** white solid; mp 136-138 °C; IR (neat) 1731 cm$^{-1}$ (C=O); $^1$H NMR (250 MHz, $CDCl_3$): δ 7.28-7.21 (app m, 4H), 7.17 (d, 2H, *J* = 8.9 Hz), 6.72 (d, 2H, *J* = 9.0 Hz), 5.09 (d, 1H, *J* = 4.7 Hz), 4.74 (d, 1H, *J* = 4.7 Hz), 3.68 (s, 3H), 3.15 (s, 3H) 2.39 (s, 3H); $^{13}$C NMR (63 MHz, $CDCl_3$): δ 163.5, 156.4, 134.5, 132.0, 130.3, 129.3, 128.8, 118.7, 114.3, 84.7, 61.1, 58.5, 55.5.

**(±)-(3S,4R)-4-(4-bromophenyl)-3-methoxy-1-(4-methoxyphenyl)azetidin-2-one (41):** white solid; mp 154-156 °C; $^{1}$H NMR (250 MHz, $CDCl_3$): δ 7.45 (d, 2H, *J* = 8.4 Hz), 7.23-7.14 (AB m, 4H) 6.73 (d, 2H, *J* = 9.0 Hz), 5.01 (d, 1H, *J* = 4.7 Hz), 4.75 (d, 1H, *J* = 4.8 Hz), 4.79 (d, 2H, *J* = 4.7 Hz), 3.66 (s, 3H), 3.16 (s, 3H); $^{13}$C NMR (63 MHz, $CDCl_3$): δ 163.5, 156.4, 132.5, 131.8, 130.3, 129.6, 122.7, 118.7, 114.4, 84.7, 61.2, 58.5, 55.4.

**(±)-(3S,4R)-4-(2-iodophenyl)-3-methoxy-1-(4-methoxyphenyl)azetidin-2-one (42):** white solid; mp 130-132 °C; $^{1}$H NMR (250 MHz, $CDCl_3$): δ 7.84 (d, 1H, *J* = 7.8 Hz), 7.28-7.15 (m, 4H), 7.00 (t, 1H, *J* = 7.8 Hz), 6.76 (d, 2H, *J* = 9.0 Hz), 5.40 (d, 1H, *J* = 4.8 Hz), 4.84 (d, 1H, *J* = 4.8 Hz), 3.69 (s, 3H), 3.24 (s, 3H); $^{13}$C NMR (63 MHz, $CDCl_3$): δ 163.5, 156.4, 139.4, 135.4, 130.3, 130.1, 128.9, 128.3, 118.7, 114.4, 98.5, 84.8, 66.1, 59.4, 55.4.

**(±)-(3S,4R)-4-(3-iodophenyl)-3-methoxy-1-(4-methoxyphenyl)azetidin-2-one (43):** white solid; $^{1}$H NMR (250 MHz, $CDCl_3$): δ 7.67 (s, 1H), 7.62 (d, 1H, *J* = 8.0 Hz), 7.29 (d, 1H, *J* = 7.7 Hz), 7.17 (d, 2H, J = 9.0 Hz), 7.04 (t, 1H, *J* = 7.8 Hz), 6.73 (d, 2H, *J* = 9.0 Hz), 5.02 (d, 1H, *J* = 4.8 Hz), 4.73 (d, 1H, *J* = 4.8 Hz), 3.68 (s, 3H), 3.16 (s, 3H); $^{13}$C NMR (63 MHz, $CDCl_3$): δ 137.8, 136.8, 130.3, 118.7, 114.4, 84.4, 61.0, 55.4.

**(±)-(3S,4R)-4-(2-methylphenyl)-3-methoxy-1-(4-methoxyphenyl)azetidin-2-one (45):** white solid; mp 132-134 °C; IR (neat) 1734 $cm^{-1}$ (C=O); $^{1}$H NMR (250 MHz, $CDCl_3$): δ 7.23-7.08 (m, 6H), 6.75 (d, 2H, *J* = 8.9 Hz), 5.31 (d, 1H, *J* = 4.9 Hz), 4.82 (d, 1H, *J* = 4.9 Hz), 3.69 (s, 3H), 3.20 (s, 3H) 2.39 (s, 3H); $^{13}$C NMR (63 MHz, $CDCl_3$): δ 163.9, 156.3, 135.8, 131.3, 130.7, 130.4, 128.1, 127.1, 126.1, 118.7, 114.3, 84.4, 60.0, 55.4.

**(±)-(3S,4R)-4-(2-methoxyphenyl)-3-methoxy-1-(4-methoxyphenyl)azetidin-2-one (46):** white solid; mp 117-118 °C; IR (neat) 1743 $cm^{-1}$ (C=O); $^{1}$H NMR (250 MHz, $CDCl_3$): δ 7.23 (m, 3H), 7.13 (app d, 1H, *J* = 7.0 Hz), 6.85 (t, 2H, *J* = 6.85 Hz), 6.73 (d, 2H, *J* = 9.0 Hz), 5.52 (d, 1H, *J* = 4.8 Hz), 4.76 (d, 1H, *J* = 4.8 Hz), 3.85 (s, 3H), 3.67 (s, 3H), 3.16 (s, 3H); $^{13}$C NMR (63 MHz, $CDCl_3$): δ 164.0, 157.3, 156.2, 130.7, 129.3, 128.2, 121.4, 120.6, 118.7, 114.3, 110.2, 85.8, 58.6, 56.4, 55.4 (d).

**(±)-(3S,4R)-4-(2-nitrophenyl)-3-methoxy-1-(4-methoxyphenyl)azetidin-2-one (47):** white solid; mp 143-145 °C; $^{1}$H NMR (250 MHz, $CDCl_3$): δ 8.16 (app d, 1H, *J* = 9.1 Hz), 7.54-7.37 (ABm, 3H), 7.22 (d, 2H, *J* = 9.0 Hz), 6.78 (d, 2H, *J* = 9.0 Hz), 5.82 (d, 1H, *J* = 5.1 Hz), 4.95 (d, 1H, *J* = 5.1 Hz), 3.71 (s, 3H), 3.30 (s, 3H); $^{13}$C NMR (63 MHz, $CDCl_3$): δ 164.3, 156.6, 148.1, 133.9, 130.6, 130.4, 129.3, 129.0, 125.4, 118.5, 114.5, 85.5, 59.6, 59.5, 55.4.

**(±)-(3S,4R)-4-(3-nitrophenyl)-3-methoxy-1-(4-methoxyphenyl)azetidin-2-one (48):** yellow solid; $^{1}$H NMR (250 MHz, $CDCl_3$): δ 8.27 (s, 1H), 8.22 (d, 1H, *J* = 8.2 Hz), 7.72 (d, 1H, *J* = 7.7 Hz), 7.56 (d, 1H, *J* = 7.9 Hz), 7.22 (d, 2H, *J* = 9.0 Hz) 6.79 (d, 2H, *J* = 9.0 Hz), 5.30 (d, 1H, *J* = 4.8 Hz), 4.87 (d, 1H, *J* = 4.8 Hz), 3.74 (s, 3H), 3.25 (s, 3H); $^{13}$C NMR (63 MHz, $CDCl_3$): δ 163.3, 156.6, 148.3, 136.0, 133.9, 129.9, 129.7, 123.7, 123.7, 123.0, 118.6, 114.5, 84.8, 60.8, 58.7, 55.4.

**(±)-(3S,4R)-4-(4-cyanophenyl)-3-methoxy-1-(4-methoxyphenyl)azetidin-2-one (49):** white solid; mp 138-139°C; $^{1}$H NMR (250 MHz, $CDCl_3$): δ 7.60 (d, 2H, *J* = 8.1 Hz), 7.43 (d, 2H, *J* = 8.1 Hz), 7.13 (d, 2H, *J* = 8.9 Hz), 6.72 (d, 2H, *J* = 8.9 Hz), 5.18 (d, 1H, *J* = 4.6 Hz), 4.79 (d, 2H, *J* = 4.7 Hz), 3.67 (s, 3H), 3.16 (s, 3H); $^{13}$C NMR (63 MHz, $CDCl_3$): δ 163.1, 156.6, 139.2, 132.3, 130.0, 128.7, 118.6, 118.4, 114.5, 112.5, 85, 61.1, 58.7, 55.4.

**(±)-(3S,4R)-4-(4-hexanoyloxyphenyl)-3-methoxy-1-(4-methoxyphenyl)azetidin-2-one (50):** white solid; mp 91-93 °C; $^{1}$H NMR (250 MHz, $CDCl_3$): δ 7.40 (d, 2H, *J* = 8.4 Hz), 7.26-7.24 (m, 4H), 7.11 (d, 2H, *J* = 8.3 Hz), 6.78 (d, 2H, *J* = 8.4 Hz), 5.17 (d, 1H, *J* = 4.6 Hz), 4.80 (d, 1H, *J* = 4.7 Hz), 3.74 (s, 3H), 3.12 (s, 3H), 2.54 (t, 2H, *J* = 7.5 Hz), 1.74 (app q, 2H), 1.23 (m, 4H), 0.82 (app t, 3H); $^{13}$C NMR (63 MHz, $CDCl_3$): δ 172.1, 163.6, 156.3, 150.9, 130.7, 130.4, 128.9, 121.8, 118.7, 114.3, 84.7, 61.2, 58.4, 55.4, 34.4, 31.2, 24.5, 22.3, 13.9.

**(±)-(3S,4R)-4-(4-propenoyloxyphenyl)-3-methoxy-1-(4-methoxyphenyl)azetidin-2-one (51):** white solid; mp 93-94 °C; $^1$H NMR (250 MHz, $CDCl_3$): δ 7.36 (d, 2H, *J* = 8.5 Hz), 7.20 (d, 2H, *J* = 7.0 Hz), 7.11 (d, 2H, *J* = 8.5 Hz), 6.72 (d, 2H, *J* = 9.0 Hz), 6.53 (d, 1H, *J* = 17.3 Hz), 6.24 (dd, 1H, *J* = 17.3, 10.4 Hz), 6.00 (d, 1H, *J* = 10.3 Hz), 5.13 (d, 1H, *J* = 4.7 Hz), 4.74 (d, 1H, *J* = 4.7 Hz), 3.67 (s, 3H), 3.14 (s, 3H); $^{13}$C NMR (63 MHz, $CDCl_3$): δ 164.2, 163.6, 156.4, 150.7, 132.8, 130.9, 130.4, 129.0, 127.7, 121.7, 118.7, 114.3, 84.8, 61.2, 58.5, 55.4.

**(±)-(3S,4R)-4-(2,4-dichlorophenyl)-3-methoxy-1-(4-methoxyphenyl)azetidin-2-one (89):** white solid; mp 124-126 °C; $^1$H NMR (250 MHz, $CDCl_3$): δ 7.41 (s, 1H), 7.19-7.15 (m, 4H), 6.76 (d, 2H, *J* = 8.9 Hz), 6.78 (d, 2H, *J* = 9.0 Hz), 5.52 (d, 1H, *J* = 4.8 Hz), 4.83 (d, 1H, *J* = 4.9 Hz), 3.70 (s, 3H), 3.26 (s, 3H); $^{13}$C NMR (63 MHz, $CDCl_3$): δ 163.5, 156.5, 134.7, 133.8, 130.1, 129.9, 129.8, 129.4, 127.4, 118.6, 114.5, 84.5, 59.2, 58.3, 55.4.

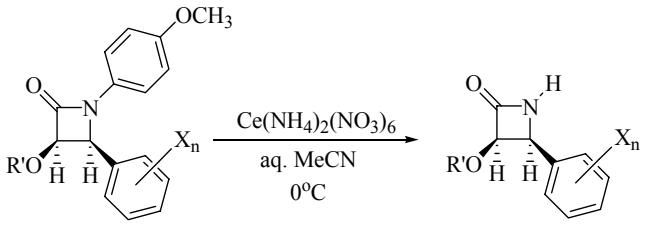
**(±)-(3S,4R)-4-(2,6-dichlorophenyl)-3-methoxy-1-(4-methoxyphenyl)azetidin-2-one (90):** crystal; mp 172-174 °C; $^1$H NMR (250 MHz, $CDCl_3$): δ 7.34 (d, 1H, *J* = 7.3 Hz), 7.20-7.10 (m, 4H), 6.73 (d, 2H, *J* = 8.9 Hz), 5.93 (d, 1H, *J* = 4.9 Hz), 4.87 (d, 1H, *J* = 4.9 Hz), 3.67 (s, 3H), 3.24 (s, 3H); $^{13}$C NMR (63 MHz, $CDCl_3$): δ 163.2, 156.4, 136.2, 131.1, 130.9, 129.9, 128.6, 117.8, 114.4.

**(±)-(3S,4R)-4-(2,3,5-trichlorophenyl)-3-methoxy-1-(4-methoxyphenyl)azetidin-2-one (91):** white solid; IR (neat) 1756 cm$^{-1}$ (C=O); $^1$H NMR (250 MHz, $CDCl_3$): δ 7.46 (app s, 1H), 7.23 (d, 2H, *J* = 8.9 Hz), 7.15 (app s, 1H), 6.83 (d, 2H, *J* = 8.9 Hz), 5.57 (d, 1H, *J* = 4.9 Hz), 4.91 (d, 1H, *J* = 4.9 Hz), 3.76 (s, 3H), 3.36 (s, 3H); $^{13}$C NMR (63 MHz, $CDCl_3$): δ 163.4, 156.7, 135.2, 134.0, 133.2, 130.0, 129.9, 129.8, 127.0, 118.5, 114.6, 84.9, 59.5, 59.2, 55.4.

**(±)-(3S,4R)-4-(2-thiophenyl)-3-methoxy-1-(4-methoxyphenyl)azetidin-2-one (125):** white solid; mp 150-153 °C; $^1$H NMR (250 MHz, $CDCl_3$): δ 7.27 (m, 3H), 7.12 (d, 1H, *J* = 2.8 Hz), 6.97 (dd, 1H, *J* = 4.9, 3.7 Hz), 6.73 (d, 2H, *J* = 9.0 Hz), 5.40 (d, 1H, *J* = 4.5 Hz), 4.76 (d, 1H, *J* = 4.6 Hz), 3.68 (s, 3H), 3.24 (s, 3H); $^{13}$C NMR (63 MHz, $CDCl_3$): δ 163.2, 156.4, 136.5, 130.4, 127.7, 126.8 (d), 125.9, 118.8 (d), 114.3, 84.9, 58.6, 57.7, 55.4.

### 6.1.5 Dearylation of *N*-Anisyl Azetidin-2-ones

**Dearylated azetidin-2-ones (9):** To a solution of *N*-anisyl β-lactam **6** (1 mmol) in acetonitrile (25 ml) at 0 °C was added 25 ml of an aqueous solution of ammonium cerium (IV) nitrate (3 mmol) over 5 min. The reaction was stirred for 25 min then quenched with 50 ml of water. The solution was extracted 3x with ethyl acetate (25 ml) and the combined organic layers were washed twice with aqueous 5% sodium bicarbonate, once with aqueous 5% sodium bisufite, and once with brine. Follwing treatment with magnesium sulfate and filtration, the solvent was removed *in vacuo* and the crude material was purified by silica gel chromatography.




**6** **9**

**(±)-(3S,4R)-4-(phenyl)-3-(methoxy)azetidin-2-one (52):** white solid; mp 68-70 °C; $^1$H NMR (250 MHz, $CDCl_3$): δ 7.31-7.19 (m, 4H), 6.47 (bs, 1H), 4.76 (d, 1H, *J* = 4.6 Hz), 4.67 (dd, 1H, *J* = 4.5, 2.8 Hz), 3.11 (s, 3H); $^{13}$C NMR (63 MHz, $CDCl_3$): δ 168.3, 134.2 (d), 129, 128.5, 86.5, 58.2, 57.5.

**(±)-(3S,4R)-4-(2-chlorophenyl)-3-(methoxy)azetidin-2-one (53):** white solid; mp 94-95 °C; IR (neat) 3275 $cm^{-1}$ (N-H), 1770 $cm^{-1}$ (C=O); $^1H$ NMR (250 MHz, $CDCl_3$): δ 7.47 (d, 1H, *J* = 6.7 Hz), 7.39-7.27 (m, 3H), 6.97 (bs, 1H), 5.25 (d, 1H, *J* = 4.5 Hz), 4.83-4.82 (app m, 1H), 3.26 (s, 3H); $^{13}C$ NMR (63 MHz, $CDCl_3$): δ 168.5, 133.7, 133.1, 132.9, 129.2, 128.2, 126.9, 86.9, 59.0, 55.9.

**(±)-(3S,4R)-4-(3-chlorophenyl)-3-(methoxy)azetidin-2-one (54):** white solid; mp 110-111 °C; IR (neat) 3196 $cm^{-1}$ (N-H), 1762 $cm^{-1}$ (C=O); $^1H$ NMR (250 MHz, $CDCl_3$): δ 7.30-7.20 (m, 4H), 6.58 (bs, 1H), 4.76 (d, 1H, *J* = 4.5 Hz), 4.69-4.67 (app m, 1H), 3.13 (s, 3H); $^{13}C$ NMR (63 MHz, $CDCl_3$): δ 168.1, 137.9, 129.6, 128.5, 127.7, 125.8, 86.7, 58.3, 57.6.

**(±)-(3S,4R)-4-(4-chlorophenyl)-3-(methoxy)azetidin-2-one (55):** white solid; mp 121-123 °C; $^1H$ NMR (250 MHz, $CDCl_3$): δ 7.36-7.27 (m, 4H), 6.86 (bs, 1H), 4.82 (d, 1H, *J* = 4.5 Hz), 4.72-4.70 (m, 1H), 3.15 (s, 3H); $^{13}C$ NMR (63 MHz, $CDCl_3$): δ 168.1, 134.2, 129.0, 128.5, 86.6, 58.2, 57.5.

**(±)-(3S,4R)-4-(4-bromophenyl)-3-(methoxy)azetidin-2-one (56):** white solid; mp 65-67 °C; $^1H$ NMR (250 MHz, $CDCl_3$): δ 7.45 (d, 2H, *J* = 8.4 Hz), 7.19 (d, 2H, *J* = 7.9 Hz), 6.39 (bs, 1H), 4.76 (d, 1H, *J* = 4.5 Hz), 4.70 (app m, 1H), 3.13 (s, 3H); $^{13}C$ NMR (63 MHz, $CDCl_3$): δ 168.4, 149.6, 134.7, 131.4, 129.3, 122.3, 116.1, 86.4, 58.2, 57.6.

**(±)-(3S,4R)-4-(2-iodophenyl)-3-(methoxy)azetidin-2-one (57):** white solid; mp 133-138 °C; IR (neat) 3227 $cm^{-1}$ (N-H), 1761 $cm^{-1}$ (C=O); $^1H$ NMR (250 MHz, $CDCl_3$): δ 7.77 (d, 1H, *J* = 8.0 Hz), 7.35-7.33 (m, 2H), 6.99 (dd, 1H, *J* = 8.0, 4.4 Hz), 6.16 (bs, 1H), 5.02 (d, 1H, *J* = 4.5 Hz), 4.79 (dd, 1H, *J* = 4.5, 3.4 Hz), 3.23 (s, 3H); $^{13}C$ NMR (63 MHz, $CDCl_3$): δ 168.0, 139.2, 137.9, 129.8, 128.2, 98.2, 86.8, 62.7, 59.3.

**(±)-(3S,4R)-4-(3-iodophenyl)-3-(methoxy)azetidin-2-one (58):** brown solid; mp 63-65 °C; $^1H$ NMR (250 MHz, $CDCl_3$): δ 7.64-7.60 (m, 2H), 7.28 (d, 1H, *J* = 7.6 Hz), 7.06 (t, 1H, *J* = 7.7 Hz), 6.37 (bs, 1H), 4.73-4.68 (m, 2H), 3.14 (s, 3H); $^{13}C$ NMR (63 MHz, $CDCl_3$): δ 167.9, 138.0, 137.4, 136.5, 130.0, 129.9, 94.2, 86.7, 58.4, 57.4.

**(±)-(3S,4R)-4-(2-methylphenyl)-3-(methoxy)azetidin-2-one (60):** brown solid; mp 110-111 °C; $^1H$ NMR (250 MHz, $CDCl_3$): δ 7.35-7.32 (m, 1H), 7.19-7.11 (m, 3H), 6.53 (bs, 1H), 4.98 (d, 1H, *J* = 4.7 Hz), 4.77 (dd, 1H, *J* = 4.6, 3.1 Hz), 3.19 (s, 3H), 2.26 (s, 3H); $^{13}C$ NMR (63 MHz, $CDCl_3$): δ 168.8, 135.6, 134, 130.1, 127.8, 126.1, 126, 86.1, 58.3, 55.7, 19.1.

**(±)-(3S,4R)-4-(2-methoxyphenyl)-3-(methoxy)azetidin-2-one (61):** brown solid; mp 81-86 °C; $^1H$ NMR (250 MHz, $CDCl_3$): δ 7.33-7.19 (AB m, 2H), 6.97-6.94 (app t, 1H), 6.82 (d, 1H, *J* = 8.2 Hz), 6.15 (bs, 1H), 5.17 (d, 1H, *J* = 4.6 Hz), 4.72 (dd, 1H, *J* = 4.6, 3.1, Hz), 3.79 (s, 3H), 3.16 (s, 3H); $^{13}C$ NMR (63 MHz, $CDCl_3$): δ 168.9, 156.9, 128.9, 127.4, 124.2, 120.5, 109.9, 86.7, 58.6, 55.3, 53.5.

**(±)-(3S,4R)-4-(2-nitrophenyl)-3-(methoxy)azetidin-2-one (62):** white solid; mp 153-150 °C; $^1H$ NMR (250 MHz, $CDCl_3$): δ 8.12 (d, 1H, *J* = 8.1 Hz), 7.64-7.56 (m, 2H), 7.47-7.42 (m, 1H), 7.09 (bs, 1H), 5.50 (d, 1H, *J* = 4.7 Hz), 4.89-4.86 (m, 1H), 3.26 (s, 3H); $^{13}C$ NMR (63 MHz, $CDCl_3$): δ 168.8, 147.7, 134.0, 132.7, 128.7, 125.1, 87.2, 59.5, 56.6.

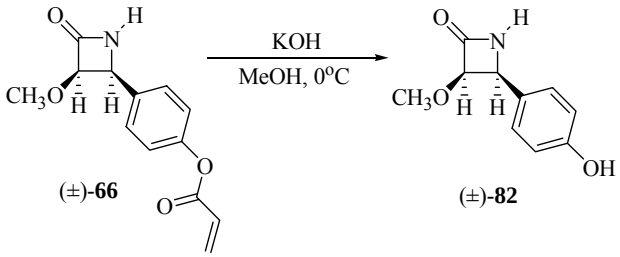
**(±)-(3S,4R)-4-(3-nitrophenyl)-3-(methoxy)azetidin-2-one (63):** white solid; mp 87-90 °C; $^1H$ NMR (250 MHz, $CDCl_3$): δ 8.17-8.14 (m, 2H), 7.67 (d, 1H, *J* = 7.5 Hz), 7.67 (t, 1H, *J* = 7.8 Hz), 6.47 (bs, 1H), 4.93 (d, 1H, *J* = 4.5 Hz), 4.67 (app m, 1H), 3.17 (s, 3H); $^{13}C$ NMR (63 MHz, $CDCl_3$): δ 168.0, 148.2, 138.2, 133.7, 129.4, 123.4, 122.6, 86.8, 58.5, 57.3.

**(±)-(3S,4R)-4-(4-cyanophenyl)-3-(methoxy)azetidin-2-one (64):** brown oil; $^1H$ NMR (250 MHz, $CDCl_3$): δ 7.67 (d, 2H, *J* = 8.2 Hz), 7.50 (d, 2H, *J* = 8.3 Hz), 6.88 (bs, 1H), 4.94 (d, 1H, *J* = 4.8 Hz), 4.81 (dd, 1H, *J* = 4.5, 2.9 Hz), 3.20 (s, 3H).

**(±)-(3S,4R)-4-(4-hexanoyloxyphenyl)-3-(methoxy)azetidin-2-one (65):** white solid; mp 58-60 °C; $^1$H NMR (250 MHz, $CDCl_3$): δ 7.39 (d, 2H, *J* = 8.5 Hz), 7.10 (d, 2H, *J* = 8.5 Hz), 6.33 (bs, 1H), 4.84 (d, 1H, *J* = 4.5 Hz), 4.76 (dd, 1H, *J* = 4.5, 2.8 Hz), 3.17 (s, 3H), 2.55 (t, 2H, *J* = 7.5 Hz), 1.78-1.72 (m, 2H), 1.42-1.36 (m, 4H), 0.93 (t, 3H, *J* = 6.8 Hz); $^{13}$C NMR (63 MHz, $CDCl_3$): δ 172.7, 171.6, 168.5, 151.1, 133.6, 129.2, 121.9, 87.0, 60.8, 58.5, 57.9, 34.7, 31.6, 31.6, 25.0, 22.7, 21.4, 14.6, 14.3.

**(±)-(3S,4R)-4-(4-propenoyloxyphenyl)-)-3-(methoxy)azetidin-2-one (66):** white solid; mp 94-96 °C; $^1$H NMR (250 MHz, $CDCl_3$): δ 7.33 (d, 2H, *J* = 8.4 Hz), 7.09 (d, 2H, *J* = 8.4 Hz), 6.55 (d, 1H, *J* = 17.3 Hz), 6.26 (dd, 1H, *J* = 17.3, 10.4), 6.00 (d, 1H, *J* = 10.4 Hz), 4.78 (d, 1H, *J* = 4.5 Hz), 4.68-4.66 (m, 1H), 3.11 (s, 3H); $^{13}$C NMR (63 MHz, $CDCl_3$): δ 168.0, 164.5, 150.5, 133.2, 132.9, 128.8, 127.7, 121.5, 116.0, 86.6, 58.2, 57.5.

**(±)-(3S,4R)-4-(4-hydroxyphenyl)-3-(methoxy)azetidin-2-one (82):** Acrylate β-lactam (99 mg, 0.4 mmol) was dissolved in 4 ml of methanol in a 3-neck flask and place in a 0$^o$C ice bath. A potassium hydroxide (44.8 mg, 0.8 mmol) dissolved in 4 ml of a 50% methanol solution was added dropwise over 1 min and allow to stir for an additional 15 min . The reaction was quenched with 10 ml of d$H_2O$ and the red solution was extracted 3x with 10 ml of ethyl acetate. The organic layer was dried with sodium sulfate then concentrated *in vacuo*. The product was recrystallized from chloroform giving a brown solid in 84% yield. mp 174-179 °C; IR (neat) 3330 cm$^{-1}$ (O-H), 1729 cm$^{-1}$ (C=O); $^1$H NMR (250 MHz, DMSO-$d_6$) δ 7.38 (bs, 1H), 8.43 (bs, 1H), 7.03 (d, 2H, *J* = 8.8 Hz), 6.66 (d, 2H, *J* = 8.5 Hz), 4.60 (d, 1H, *J* = 4.5 Hz), 4.55 (dd, 1H, *J* = 4.5, 2.3 Hz), 2.89 (s, 3H); $^{13}$C NMR (63 MHz, DMSO-$d_6$): δ 167.9, 157.4, 129.1, 127.5, 115.2, 86.5, 57.4, 56.4.




**(±)-(3S,4R)-4-(2, 3, 4, 5, 6-pentafluorophenyl)-3-(methoxy)azetidin-2-one (92):** pale solid; mp 153-155 °C; $^1$H NMR (250 MHz, $CDCl_3$): δ 6.64 (s, 1H), 5.19 (d, 1H, *J* = 4.7 Hz), 4.86 (dd, 1H, *J* = 4.6, 2.0 Hz), 3.42 (s, 3H).

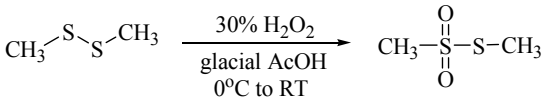
**(±)-(3S,4R)-4-(2,4-dichlorophenyl)-3-(methoxy)azetidin-2-one (93):** pale solid; mp 114-116 °C; IR (neat) 3196 cm$^{-1}$ (N-H), 1762 cm$^{-1}$ (C=O); $^1$H NMR (250 MHz, $CDCl_3$): δ 7.39-7.35 (m, 2H), 7.26 (app d, 1H, *J* = 9.3 Hz), 6.85 (bs, 1H), 4.82-4.78 (app m, 1H), 3.26 (s, 3H); $^{13}$C NMR (63 MHz, $CDCl_3$): δ 168.7, 134.8, 133.9, 132.8, 129.6, 127.7, 87.3, 59.6, 55.9.

**(±)-(3S,4R)-4-(2,3,5-trichlorophenyl)-3-methoxyazetidin-2-one (95):** white solid; mp 163-166 °C; $^1$H NMR (250 MHz, $CDCl_3$): δ 7.39 (s, 1H), 7.30 (s, 1H), 6.82 (bs, 1H), 5.15 (d, 1H, *J* = 4.6 Hz), 4.82-4.79 (m, 1H), 3.28 (s, 3H); $^{13}$C NMR (63 MHz, $CDCl_3$): δ 168.1, 137.5, 133.8, 133.1, 129.6, 129.5, 126.6, 87.0, 59.5, 56.3.

**(±)-(3S,4R)-4-(2-thiophenyl)-3-methoxyazetidin-2-one (126):** white solid; mp 85-88 °C; $^1$H NMR (250 MHz, $CDCl_3$): δ 7.35 (d, 1H, *J* = 5.0 Hz), 7.11 (d, 1H, *J* = 3.0 Hz), 7.05-7.02 (m, 1H), 6.49 (bs, 1H), 5.12 (d, 1H, *J* = 4.3 Hz), 5.12 (dd, 1H, *J* = 4.0, 2.8 Hz), 3.28 (s, 3H); $^{13}$C NMR (63 MHz, $CDCl_3$): δ 167.6, 139.2, 126.9, 126.7, 126.3, 86.6, 58.4, 54.0.

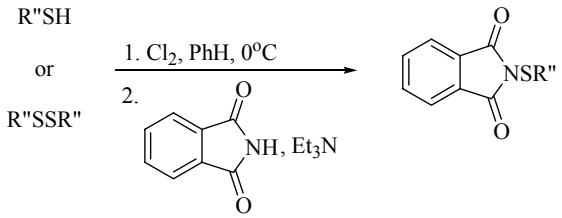
### 6.1.6 Preparation of Sulfur-Transfer Reagents

**Methyl methanethiolsulfonate (20):** Methyl disulfide (14.1 g, 160 mmol) was dissolved in 60 ml of glacial acetic acid and place in an ice water bath. Hydrogen peroxide (34 g of 30% solution) was added slowly to the mixture without stirring over 20 min. The solution was stirred for an additional 30 min at room temperature then slowly warmed to 50°C for 2 hr to destroy the excess peroxide. After testing for the presence of peroxide by starch-iodide paper, the glacial acetic acid was removed under vacuum. The residue was treated with 50 ml of sat. sodium bicarbonate and extracted with ethyl acetate. After drying over anhydrous magnesium sulfate, the solvent is removed and the yellow oil distilled. bp 60-70°C (0.3 torr); $^1$H NMR (250 MHz, $CDCl_3$): δ 2.65 (s, 3H), 3.22 (s, 3H).



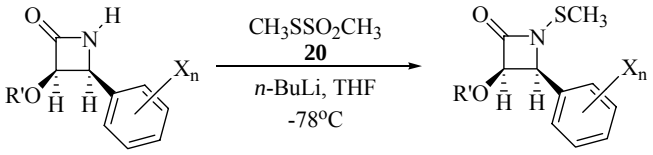

**20**

***N*-(Organothio)phthalimide:** In dry benzene containing thiol (1 mmol) or disulfide (1 mmol), chlorine gas (2 mmol in benzene) was added dropwise at 0°C while stirring. After 1 hr, the sulfenyl chloride solution was canulated into a flask containing phthalimide (1 mmol) and *N*,*N*-ethyldiisopropylamine (1.5 mmol) then stirred for an additional 2 hr. Afterwards, the solid material was filtered, wash with water and *n*-heptane, and recrystallized from methanol to give *N*-(organothio)phthalimide in good yields.



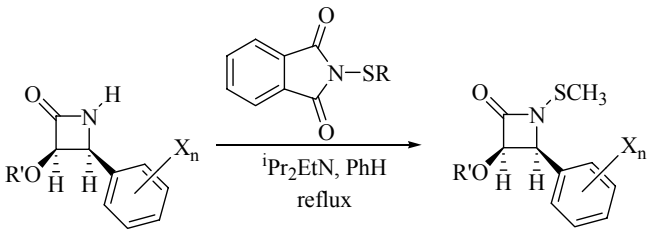

### 6.1.7 Preparation of *N*-Thiolated Azetidin-2-ones

***N*-Thiolated azetidin-2-ones (3):** To a solution of **9** (1 mmol) in dry THF at -78 °C was added *n*-butylithium (1.1 mmol). After 30 min, methyl methanethiosulfonate **20** (1 mmol) was added and the mixture was stirred for 12 hr with warming to room temperature. The mixture was poured into 5% aqueous ammonium chloride and extracted 3x with dichloromethane. The organic layers were dried with magnesium sulfate, filtered, and concentrated *in vacuo*. Flash chromatography of the crude material with ethyl acetate/hexanes mixtures gave *N*-methylthio β-lactams **3**.




**9** **3**

***N*-Thiolated azetidin-2-ones (3):** Lactam **9** (1 mmol) and *N*-(organothio)phthalimide (1 mmol) were dissolved in dry benzene with a catalytic amount of ethyldiisopropylamine. Following reflux for 1 hr, the mixture was concentrated *in vacuo* and the residue was purified by silica gel chromatography to give the *N*-methylthio β-lactams **3**.




**9** **3**

**(±)-(3S,4R)-4-(phenyl)-3-methoxy-1-(methylthio)azetidin-2-one (67):** white solid; mp 51-54 °C; $^1$H NMR (250 MHz, $CDCl_3$): 7.31 (m, 5H), 4.76 (d, 1H, *J* = 4.9 Hz), 4.72 (d, 1H, *J* = 4.9 Hz), 3.08 (s, 3H), 2.29 (s, 3H).

**(±)-(3S,4R)-4-(2-chlorophenyl)-3-methoxy-1-(methylthio)azetidin-2-one (68)** white crystal; mp 71-73 °C; IR (neat) 1756 cm$^{-1}$ (C=O); $^1$H NMR (250 MHz, $CDCl_3$): δ 7.35 (d, 1H, *J* = 7.4 Hz), 7.24 (m, 3H), 5.29 (d, 1H, *J* = 4.9 Hz), 4.80 (d, 1H, *J* = 4.9 Hz), 3.16 (s, 3H), 2.40 (s, 3H); $^{13}$C NMR (63 MHz, $CDCl_3$): δ 170.4, 133.8, 131.4, 129.6, 128.9, 126.8, 86.7, 62.7, 58.9, 21.8.

**(±)-(3S,4R)-4-(3-chlorophenyl)-3-methoxy-1-(methylthio)azetidin-2-one (69):** white crystal; mp 73-75 °C; IR (neat) 1740 cm$^{-1}$ (C=O); $^1$H NMR (250 MHz, $CDCl_3$): δ 7.30-7.29 (m, 3H), 7.21 (s, 1H), 4.73 (app s, 2H), 3.14 (s, 3H), 2.33 (s, 3H); $^{13}$C NMR (63 MHz, $CDCl_3$): δ 170.6, 136.1, 134.8, 130.1, 129.5, 129.4, 127.4, 87.0, 66.0, 58.9, 22.6.

**(±)-(3S,4R)-4-(4-chlorophenyl)-3-methoxy-1-(methylthio)azetidin-2-one (70):** white crystal; mp 62-66 °C; IR (neat) 1751 cm$^{-1}$ (C=O); $^1$H NMR (250 MHz, $CDCl_3$): δ 7.33 (d, 2H, *J* = 8.5 Hz), 7.25 (d, 2H, *J* = 8.5 Hz), 4.73 (app s, 2H), 3.13 (s, 3H), 2.31 (s, 3H); $^{13}$C NMR (63 MHz, $CDCl_3$): δ 170.2, 134.9, 130.2, 128.6, 86.5, 65.5, 58.4, 22.1.

**(±)-(3S,4R)-4-(4-bromo-phenyl)-3-methoxy-1-(methylthio)azetidin-2-one (71):** white solid; mp 80-83 °C; IR (neat) 1761 cm$^{-1}$ (C=O); $^1$H NMR (250 MHz, $CDCl_3$): δ 7.48 (d, 2H, *J* = 8.1 Hz), 7.18 (d, 2H, *J* = 8.4 Hz), 4.72 (app s, 2H), 3.13 (s, 3H), 2.31 (s, 3H); $^{13}$C NMR (63 MHz, $CDCl_3$): δ 170.6, 133.0, 132.0, 131.0, 123.5, 86.9, 66.0, 58.9, 22.6.

**(±)-(3S,4R)-4-(2-iodophenyl)-3-methoxy-1-(methylthio)azetidin-2-one (72):** white crystal; mp 62-65 °C; IR (neat) 1756 cm$^{-1}$ (C=O); $^1$H NMR (250 MHz, $CDCl_3$): δ 7.80 (d, 1H, *J* = 7.8 Hz), 7.30 (t, 1H, *J* = 7.5 Hz), 7.15 (t, 1H, *J* = 8.0 Hz), 7.00 (t, 1H, *J* = 7.5 Hz), 5.09 (d, 1H, *J* = 5.0 Hz), 4.80 (d, 1H, *J* = 4.8 Hz), 3.82 (s, 3H), 2.41 (s, 3H); $^{13}$C NMR (63 MHz, $CDCl_3$): δ 170.7, 139.9, 136.2, 130.6, 129.3, 128.6, 99.8, 87.1, 70.4, 59.6, 22.2.

**(±)-(3S,4R)-4-(3-iodophenyl)-3-methoxy-1-(methylthio)azetidin-2-one (73):** white crystal; mp 97-99 °C; IR (neat) 1745 cm$^{-1}$ (C=O); $^1$H NMR (250 MHz, $CDCl_3$): δ 7.65 (m, 2H), 7.27 (d, 1H, *J* = 7.7 Hz), 7.08 (t, 1H, *J* = 8.0 Hz), 4.73-4.67 (AB m, 2H), 3.13 (s, 3H), 2.32 (s, 3H); $^{13}$C NMR (63 MHz, $CDCl_3$): δ 170.6, 138.4, 138.2, 136.4, 130.5, 128.5, 94.5, 87, 65.8, 58.3, 22.6.

**(±)-(3S,4R)-4-(4-iodophenyl)-3-methoxy-1-(methylthio)azetidin-2-one (74):** white solid; mp 102-105 °C; IR (neat) 1766 cm$^{-1}$ (C=O); $^1$H NMR (250 MHz, $CDCl_3$): δ 7.65 (d, 2H, *J* = 8.3 Hz), 7.03 (d, 2H, *J* = 8.3 Hz), 4.70 (app s, 2H), 3.10 (s, 3H), 2.29 (s, 3H); $^{13}$C NMR (63 MHz, $CDCl_3$): δ 170.1, 137.5, 133.2, 130.7, 94.9, 86.4, 65.6, 58.4, 22.1.

**(±)-(3S,4R)-4-(2-methylphenyl)-3-methoxy-1-(methylthio)azetidin-2-one (75):** white solid; mp 80-81 °C; IR (neat) 1745 cm$^{-1}$ (C=O); $^1$H NMR (250 MHz, $CDCl_3$): δ 7.27-7.21 (m, 4H), 5.11 (d, 1H, *J* = 5.0 Hz), 4.85 (d, 1H, *J* = 5.0 Hz), 3.21 (s, 3H), 2.45 (s, 3H), 2.37 (s, 3H).

**(±)-(3S,4R)-4-(2-methoxyphenyl)-3-methoxy-1-(methylthio)azetidin-2-one (76):** white solid, mp 120-123°C; $^1$H NMR (250 MHz, $CDCl_3$): δ 7.27-7.24 (app m, 1H), 7.19-7.15 (m, 1H), 6.95 (t, 1H, *J* = 7.5 Hz), 6.86 (d, 1H, *J* = 8.2 Hz), 5.27 (d, 1H, *J* = 4.9 Hz), 4.72 (d, 1H, *J* = 4.9 Hz), 3.80 (s, 3H), 3.12 (s, 3H), 2.37 (s, 3H); $^{13}$C NMR (63 MHz, $CDCl_3$): δ 170.9, 157.7, 129.5, 128.5, 121.5, 120.4, 110.3, 86.5, 60.2, 58.5, 55.4, 21.8.

**(±)-(3S,4R)-4-(2-nitrophenyl)-3-methoxy-1-(methylthio)azetidin-2-one (77):** yellow solid; $^1$H NMR (250 MHz, $CDCl_3$): δ 8.12 (d, 1H, *J* = 8.2 Hz), 7.66-7.63 (m, 1H), 7.50-7.41 (m, 2H), 5.49 (d, 1H, *J* = 5.1 Hz), 4.93 (d, 1H, *J* = 5.2 Hz), 3.23 (s, 3H), 2.43 (s, 3H).

**(±)-(3S,4R)-4-(4-cyanophenyl)-3-methoxy-1-(methylthio)azetidin-2-one (79):** yellow solid; mp 88-90 °C; $^1$H NMR (250 MHz, $CDCl_3$): δ 7.64 (d, 2H, *J* = 8.0 Hz), 7.40 (d, 2H, *J* = 8.0 Hz), 4.79 (ABm, 2H), 3.14 (s, 3H), 2.37 (s, 3H).

**(±)-(3S,4R)-4-(4-propenoyloxyphenyl)-3-methoxy-1-(methylthio)azetidin-2-one (81):** white solid; mp 87-88 °C; $^1$H NMR (250 MHz, $CDCl_3$): δ 7.29 (d, 2H, *J* = 8.4 Hz), 7.08 (d, 2H, *J* = 8.4 Hz), 6.50 (d, 1H, *J* = 17.3 Hz), 6.21 (dd, 1H, *J* = 17.1, 10.3 Hz), 5.92 (d, 1H, *J* = 10.4), 4.75 (d, 1H, *J* = 4.8 Hz), 4.69 (d, 1H, *J* = 4.8 Hz), 3.05 (s, 3H), 2.26 (s, 3H);$^{13}$C NMR (63 MHz, $CDCl_3$): δ 170.3, 164.2, 150.9, 132.9, 131.1, 129.9, 127.7, 121.4, 86.5, 65.5, 58.3, 22.0.

**(±)-(3S,4R)-4-(4-hydroxyphenyl)-3-methoxy-1-(methylthio)azetidin-2-one (83):** white solid; mp 119-123 °C; $^1$H NMR (250 MHz, $CDCl_3$): δ 7.18 (d, 2H, *J* = 9.0 Hz), 6.81 (d, 2H, *J* = 9.0 Hz), 5.73 (bs, 1H), 4.69 (app s, 2H), 3.11 (s, 3H), 2.28 (s, 3H); $^{13}$C NMR (63 MHz, $CDCl_3$): δ 170.9, 168.5, 130.4, 124.9, 115.4, 65.9, 58.3, 22.1.

**(±)-(3S,4R)-4-(2, 3, 4, 5, 6-pentafluorophenyl)-3-methoxy-1-(methylthio)azetidin-2-one (96):** white solid; mp 72–74 °C; $^1$H NMR (250 MHz, $CDCl_3$): δ 5.15 (d, 1H, *J* = 5.1 Hz), 4.81 (d, 1H, *J* = 5.1 Hz), 3.35 (s, 3H), 2.35 (s, 3H).

**(±)-(3S,4R)-4-(2,4-dichlorophenyl)-3-methoxy-1-(methylthio)azetidin-2-one (97):** yellow crystal; mp 102-105 °C; IR (neat) 1772 $cm^{-1}$ (C=O); $^1$H NMR (250 MHz, $CDCl_3$): δ 7.37 (s, 1H), 7.28 (d, 1H, *J* = 8.3 Hz), 7.16 (d, 1H, *J* = 8.2 Hz), 5.24 (d, 1H, *J* = 4.6 Hz), 4.79 (d, 1H, *J* = 4.6 Hz), 3.19 (s, 3H), 2.40 (s, 3H); $^{13}$C NMR (63 MHz, $CDCl_3$): δ 170.5, 135.3, 134.9, 130.6, 130.3, 129.9, 127.7, 87.1, 62.6, 59.4, 22.2.

**(±)-(3S,4R)-4-(2,6-dichlorophenyl)-3-methoxy-1-(methylthio)azetidin-2-one (98):** pale yellow solid; mp 77-80 °C; IR (neat) 1771 $cm^{-1}$ (C=O); $^1$H NMR (250 MHz, $CDCl_3$): δ 7.23 (t, 1H, *J* = 8.1 Hz), 5.72 (d, 1H, *J* = 5.3 Hz), 4.90 (d, 1H, *J* = 5.1 Hz), 3.30 (s, 3H), 2.44 (s, 3H); $^{13}$C NMR (63 MHz, $CDCl_3$,): δ 168.3, 131.1, 130.1, 128.8, 88.0, 62.9, 59.2, 21.6.

**(±)-(3S,4R)-4-(2,3,5-trichlorophenyl)-3-methoxy-1-(methylthio)azetidin-2-one (99):** yellow crystal; mp 90-94 °C; IR (neat) 1761 $cm^{-1}$ (C=O); $^1$H NMR (250 MHz, $CDCl_3$): δ 7.44 (s, 1H), 7.11 (s, 1H), 5.24 (d, 1H, *J* = 5.0 Hz), 4.83 (d, 1H, *J* = 5.0 Hz), 3.26 (s, 3H), 2.45 (s, 3H); $^{13}$C NMR (63 MHz, $CDCl_3$,): δ 170.4, 135.8, 134.4, 133.5, 129, 127.6, 87.2, 63.4, 59.7, 22.2.

**(±)-(3S,4R)-4-(phenyl)-3-methoxy-1-(butylthio)azetidin-2-one (108):** colorless oil; $^1$H NMR (250 MHz, $CDCl_3$): δ 7.34-7.27 (m, 5H), 4.74-4.71 (m, 2H), 3.09 (s, 3H); $^{13}$C NMR (63 MHz, $CDCl_3$): δ 171.0, 133.6, 128.9, 128.8, 128.3, 86.3, 66.9, 58.3, 38.2, 30.8, 21.5, 13.6.

**(±)-(3S,4R)-4-(phenyl)-3-methoxy-1-(phenylthio)azetidin-2-one (109):** white solid; mp 58-60 °C $^1$H NMR (250 MHz, $CDCl_3$): δ 7.37-7.23 (m, 8H), 4.88-4.83 (AB m, 2H), 3.18 (s, 3H).

**(±)-(3S,4R)-4-(phenyl)-3-methoxy-1-(cyclohexylthio)azetidin-2-one (110):** oil; $^1$H NMR (250 MHz, $CDCl_3$): δ 7.38-7.26 (m, 5H), 4.86-4.82 (m, 2H), 3.02 (s, 3H), 2.89-2.85 (m, 1H), 1.98-1.94 (app m, 1H),

1.75 (app s, 3H), 1.35-1.19 (m, 6H); $^{13}$C NMR (63 MHz, $CDCl_3$): 171.3, 133.5, 128.9, 128.8, 128.3, 86.2, 67.6, 58.3, 49.5, 32.2, 30.9, 25.6, 25.4.

**(±)-(3S,4R)-4-(2-thiophenyl)-3-methoxy-1-(methylthio)azetidin-2-one (127):** oil; $^1$H NMR (250 MHz, $CDCl_3$): δ 7.43 (d, 1H, *J* = 5.0 Hz), 7.21 (d, 1H, *J* = 2.9 Hz), 7.06 (dd, 1H, *J* = 5.0, 2.9 Hz), 5.08 (d, 1H, *J* = 4.7 Hz), 4.82 (d, 1H, *J* = 4.8 Hz), 3.32 (s, 3H), 2.30 (s, 3H).

**(±)-(3S,4R)-4-(2,6-dichlorophenyl)-3-phenoxy-1-(methylthio)azetidin-2-one (134):** white solid; mp 114-116 °C; $^1$H NMR (360 MHz, $CDCl_3$): δ 7.39 (d, 1H, *J* = 7.8 Hz), 7.33-7.23 (m, 7H), 6.92 (t, 1H, *J* = 7.4 Hz), 6.79 (d, 2H, *J* = 7.9 Hz), 5.59 (app s, 2H), 2.49 (s, 3H); $^{13}$C NMR (90 MHz, $CDCl_3$): δ 167.0, 140.0, 133.6, 133.2, 129.7, 129.5, 128.5, 127.0, 122.6, 119.5, 83.9, 55.6.

**(±)-(3S,4R)-4-(4-nitrophenyl)-3-acetoxy-1-(methylthio)azetidin-2-one (135):** white solid; $^1$H NMR (360 MHz, $CDCl_3$): δ 8.26 (d, 2H, *J* = 8.7 Hz), 7.47 (d, 2H, *J* = 8.7 Hz), 5.96 (d, 1H, *J* = 5.0 Hz), 5.15 (d, 1H, *J* = 5.0 Hz), 2.49 (s, 3H), 1.75 (s, 3H); $^{13}$C NMR (90 MHz, $CDCl_3$): δ 182.0, 168.9, 167.8, 148.5, 140.3, 129.7, 123.8, 81.1, 65.1, 22.3, 19.6.

**(±)-(3S,4R)-4-(2-chloro-6-fluorophenyl)-3-acetoxy-1-(methylthio)azetidin-2-one (137):** yellow solid; 1787, 1745 cm$^{-1}$ (C=O); $^1$H NMR $^1$H NMR (250 MHz, $CDCl_3$): δ 7.35 (ddd, 1H, *J* = 8.4, 6.3, 2.1 Hz), 6.91 (t, 2H, *J* = 8.7 Hz), 5.95 (d, 1H, *J* = 5.2 Hz), 5.39 (d, 1H, *J* = 5.2 Hz), 2.38 (s, 3H), 1.81 (s, 3H); $^{13}$C NMR (63 MHz, $CDCl_3$): δ 168.9, 167.9, 162.2 (dd, app $J_{C\text{-}F}$ = 250, 6.8 Hz), 131.4 (t, app $J_{C\text{-}F}$ = 10.7 Hz), 112.0, 111.7, 108.8 (t, app $J_{C\text{-}F}$ = 14 Hz), 77.6, 56.8, 21.7, 19.9.

**(±)-(3S,4R)-4-(phenyl)-3-hydroxy-azetidin-2-one (141):** brown oil; $^1$H NMR (250 MHz, $CDCl_3$): δ 7.46-7.26 (m, 5H), 5.17 (d, 1H, *J* = 5.3 Hz), 4.96 (d, 1H, J = 5.0 Hz), 2.44 (s, 3H).

### 6.2 Microbiological Test Procedures

The following bacteria were used for the antimicrobial evaluation of *N*-thiolated β-lactams: *Bacillus anthracis* (Sterne strain), *Bacillus cereus* (ATCC 14579), *Bacillus coagulans* (USF 546), *Bacillus globigii* (Department of Defense Reagents Program), *Bacillus megaterium* (ATCC 14581), *Bacillus subtilis* (19569), *Bacillus thuringensis* (ATCC 10792), *Bacteroides fragalis* (obtained from Smith-Kline Laboratory), *Candida albicans* (clinical isolate), *Candida tropicalis* (clinical isolate), *Enterobacter cloace* (environmental isolate, USF510), *Enterococcus gallinarium* (ATCC 49573), *Enterococcus faecalis* (ATCC 19433), *Enterococcus casseliflavus* (ATCC 700327), *Enterococcus durans* (ATCC 6056), *Enterococcus avirum* (ATCC 14025), *Enterococcus saccharolyticus* (ATCC 43076), *Escherichia coli* (ATCC 23590), *Haemophilus influenzae (*USF 561), *Klebsiella pneumoniae* (USF 512), *Lactococcus lactis* (ATCC 11454), *Listeria monocytogenes* (ATCC 19115), *Micrococcus luteus* (environmental isolate, USF681), *Niesserria gonnorheae* (obtained from the Tampa Branch State Laboratory, β-lactamase positive, USF 662), *Pseudomonas aeruginosa* (ATCC 15442), *Salmonella typhimurium* (obtained from University of South Florida Medical Clinic, USF 515), *Serratia marcescens* (ATCC 29634), *Staphylococcus aureus* USF525 (ATCC 25923) *Staphylococcus aureus* USF652-658 (obtained from Lakeland Regional Medical Center, β-lactamase positive), *Staphylococcus epidermidis* (environmental isolate, USF528), *Staphylococcus saprophyticus* (ATCC 35552), *Staphylococcus simulans* (ATCC 11631), *Staphylococcus capitis* (ATCC 35661), *Staphylococcus cohnii* (ATCC 35662), *Staphylococcus lentus* (ATCC 700403), *Staphylococcus lugdunensis* (ATCC 700328), *Staphylococcus xylosus* (ATCC 29971), *Streptococcus pyrogenes*, *Streptococcus agalactiae*, *Vibrio cholerae* (biotype E1 Tor Ogawa, cholera toxin positive, CDC E5906),

#### 6.2.1 Antimicrobial Susceptibility Test

**Culture preparation:** From a freezer stock in tryptic soy broth (Difco Laboratories, Detroit, MI) and 20% glycerol, a culture of each organism was grown on tryptic soy agar (TSA) plates (Becton-Dickinson Laboratories, Cockeysville, MD) at 37°C for 24 hours. A $10^8$ suspension was then made in sterile phosphate buffered saline (pH 7.2) and swabbed across fresh TSA plates.

**Disc method:** From each 1mg/ml stock solution in dimethyl sulfoxide (DMSO), sterile 6mm paper discs (Becton-Dickinson Laboratories, Cockeysville, MD) were impregnated with 20 µl of the test compounds. At this concentration, the microliter quantity is equivalent to the micrograms in solution. The discs were allowed to dry in a biohazard safety hood then placed onto the inoculated TSA plates. The plates were incubated for 24 hours at 37°C and the antimicrobial susceptibilities were determined by measuring the zones of growth inhibition around each disc.

**Well method:** A $10^8$ standardized cell count suspension was then made in sterile phosphate buffered saline (pH 7.2) and swabbed across fresh TSA plates. Circular wells (6 mm in diameter) were cut into the inoculated plates and 20 µL of a 1 mg/ml stock solution of the test lactam in dimethylsulfoxide (DMSO) was pipetted into the wells. The plates were incubated for 24 hours at 37°C and the antimicrobial susceptibilities were determined by measuring the zones of growth inhibition around each well.

### 6.2.2 MIC Calculations

**Media preparation:** The minimum inhibitory concentrations were determined by the agar plate dilution (need reference). The test media were prepared in 24 well plates (Costar 3524, Cambridge, MA) by adding a known concentration of the test drug in DMSO together with a solution of Mueller-Hinton II agar (Becton-Dickinson Laboratories, Cockeysville, MD) for a total volume of 1 ml in each well. Calculations of the overall concentration of antibiotic in the wells were standardized by measuring from a 1mg/ml stock solution of the test drug. At this concentration, the microliter quantity is equivalent to the micrograms in solution. The amount of agar solution added to the wells was determined by subtracting 1000 µl from the quantity of test drug in each well to give a combined volume of 1 ml. Following preparation of the well plates, the media were allowed to solidify at room temperature for 24 hours before inoculation.

**Inoculation:** From an 24 hour culture of each organism on tryptic soy agar (TSA) plates (Becton-Dickinson Laboratories, Cockeysville, MD), the staphylococcal strains were grown overnight in 5 ml of tryptic soy broth (Difco Laboratories, Detroit, MI) at 37°C. One microliter of each culture was then applied to the appropriate well of agar and incubated at 37°C overnight. After 24 hr, the MICs were determined by examining the wells for growth.

### 6.2.3 Growth Studies

Overnight cultures of the test strains were grown to logarithmic phase in MHB. An inoculum of $10^6$ cfu/ml was added to fresh MHB and grown for 1 hr at 35°C while shaking. The test compounds diluted to 1, 5, or 10 times the MICs in DMSO was applied to each tube. Viable cell counts were determined by plating adequate dilutions of each culture. The plates were incubated and colony counts were taken after 24 hrs. Turbidity measurements were determined by transferring 0.2 ml aliquots of culture to a 48-well plate (Costar 3524, Cambridge, MA) and optical density readings taken at 630 nm with a Bio-Tek EL800 plate reader.

### 6.2.4 Metabolism studies

To a fresh $10^6$ cfu/ml suspension of *S. aureus* (ATCC 25923) in 9 ml of sterile saline was added 1 ml of a 400 µM solution of lactam **68** in DMSO. After 1 hr, 10 ml of $dH_2O$ was added and the solution was extracted 3 times with 5 ml of ethyl acetate. The organic layers were combined, dried with magnesium sulfate, and the solvent was removed under reduced pressure. The residue was dissolved in 500 µl of $CDCl_3$ and the chemical structure was elucidated by $^1H$ NMR.

### 6.2.5 Scanning Electron Microscopy (SEM) Experiments

**Sample preparation:** Samples were prepared from sections of agar taken from the disk-diffusion experiment. Sterile 6 mm diameter paper discs (Becton-Dickinson Laboratories, Cockeysville, MD) were

impregnated with 20 μg of penicillin-G potassium salt (Sigma Chemical Co, St. Louis, MO) and **68** from their 1 mg/ml stock solutions in DMSO. From a 24-hour culture of *Staphylococcus aureus* USF525 (ATCC 25923) grown on tryptic soy agar (TSA) plates (Becton-Dickinson Laboratories, Cockeysville, MD), a $10^8$ suspension was made in sterile phosphate buffered saline (pH 7.2) and swabbed across two separate TSA plates. The discs were placed on the inoculated plates and incubated for 24 hours at 37° C. After 24 hours, sections of agar in areas containing the division between the zones of inhibition and lawn of bacteria were cut out and placed into Petri dishes for the SEM preparation.

**SEM preparation:** The agar sections were flooded with 10 ml of a pre-made glutaraldehyde-osmium fixative. After 1 hour, the sections were removed and washed 3 times with 0.1 M sodium cacodylate. The samples were then sequentially submerged in 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, and 100% ethanol for periods of 5 minute. Following alcohol treatment, the sections were placed into hexamethyl disilazane (HMDS) for chemical drying. The samples were next mounted onto stubs and coated with gold/palladium by a Pelco Model 3 Sputter Coater.

**Scanning electron microscopy.** The morphology of the cells was examined with a *Novascan 30* scanning electron microscope.

### 6.2.6 Light Microscopy Experiment

**Slide preparation:** Sterile 6 mm paper discs (Becton-Dickinson Laboratories, Cockeysville, MD) containing 20 ug of B3o-4, penicillin-G potassium salt (Sigma Chemical Co, St. Louis, MO) and vancomycin hydrochloride (Abbott Laboratory, Chicago, IL) were placed on TSA plates innoculated by *S. aureus* USF525 (ATCC 25923) from an overnight culture. The plates were incubated at 37° C for 4-5 hours or until the zones of inhibition were visible. Glass 24 x 60mm coverslips (Corning Glass works, Corning, NY) were then gently pressed across the zones to adhere the bacteria. Following heat fixing over a Bunsen burner flame, the coverslips were flooded by Gram crystal violet stain (Becton Dickinson Laboratories, Cockeysville, MD) for one minute. The coverslips were rinsed with water and flooded by Gram iodine (Difco Laboratories, Detroit, MI) for one minute then decolorized by adding 95% ethanol dropwise until the crystal violet no longer flowed off the coverslips. The coverslips were rinsed again with water and counterstained with Gram safranin (Difco Laboratories, Detroit, MI) for one minute. They were then thoroughly rinsed with water, blotted dry, and mounted on glass microscope slides (Fisher Scientific, Pittsburgh, PA). The slides were view with a Nikon LABPHOT Type: 104 bright-field light microscope.

### 6.2.7 DNA Cleavage Assay

To 17 μl of sodium phosphate buffer (50 mM, pH 7.4) was added 0.5 μg of pBR322 (ICN Biomedicals Inc, Aurora, OH) in 200 μl microfuge tubes. 2 μL of **2a** at 5, 10, 25, 50, and 100 μM concentrations in DMSO were added and the samples were vortexed then incubated at 37°C. After 24 hrs, 2 μl of Blue/Orange 6X Loading Dye (Promega Corp., Madison, WI) were added to 8 μl aliquots of the DNA mixtures. The samples were loaded on a 1.2% agarose gel (1.8 g medium EEO agarose; 150 ml TAE; 1 μg/ml ethidium bromide) and horizontal electrophoresis was performed at 80 V/10 cm for 2.5 hrs in TAE (40 mM Tris-acetate, pH 7.8, 1 mM EDTA). The agarose gel was visualized and photographed under UV transillumination. The same procedure was used in experiments conducted with glutathione, dithiothreitol (DTT), and 2-mercaptoethanol. For the enzyme digest samples, 20 μl of sterile $dH_2O$, 2 μl of 100 μM **2a**, 2 μl of restriction enzyme buffer H (90 mM Tris-HCl, 10 mM $MgCl_2$, 50 mM NaCl, pH 7.5), 1 μg of pBR322, and 2 μl EcoR1 were added sequentially to a 1.5 ml microfuge tube and incubated in a 37°C water bath for 1 hr.

### 6.2.8 Determination of Thiol Levels in Bacteria

**Preparation of cell extracts:** Cells from a culture grown at 37°C in LB broth were harvested by centrifugation (3100 rpm for 5 min) once an $OD_{650}$ of 1.0 to 1.5 nm was achieved. The liquid media is removed and 10 ml of sterile $dH_2O$ is added to the pellet. Following centrifugation (3100 rpm for 5 min),

the water was then removed. One milliliter of warm (60°C) 50% aqueous acetonitrile containing 25 mM methanesulfonic acid is added to the tube and vortexed at maximum speed for 1 min. The foamy cell extracts were transferred to a 1.5 ml centrifuge tube and incubated in a 60°C water bath for 15 min. The protein and the cellular debris are pelleted by centrifugation for 5 min at 8000 rpms.. The acid supernant fraction is then removed and analyzed by thiol titration with Ellman's reagent.

**Reagent preparation:** 39.6 mg of DTNB is dissolve in 10 ml of 0.5 M potassium phosphate buffer at pH 7.2.

**Measurement of thiol levels:** 0.4 ml of the acid thiol extract is transferred to in a 1 ml cuvette followed by the addition of 0.5 ml of DNTB. The absorbance of the bright yellow solution was measured at 412 nm after 2 mins using $dH_2O$ as a blank. Thiol levels ($C_o$) are then calculated by the following equation:

$C_o = [\Delta A_{412} / 13{,}600]D$

$D$ = dilution factor

$\Delta A_{412} = A_{cuvette1} - A_{cuvette2}$

where: $A_{cuvette1}$ = Absorbance of 0.5 ml of DNTB + 0.4 ml of thiol acid extract

$A_{cuvette2}$ = Absorbance of the 0.5 ml of DNTB + 0.4 ml of $dH_2O$.

**6.2.9 Macromolecule Synthesis:**

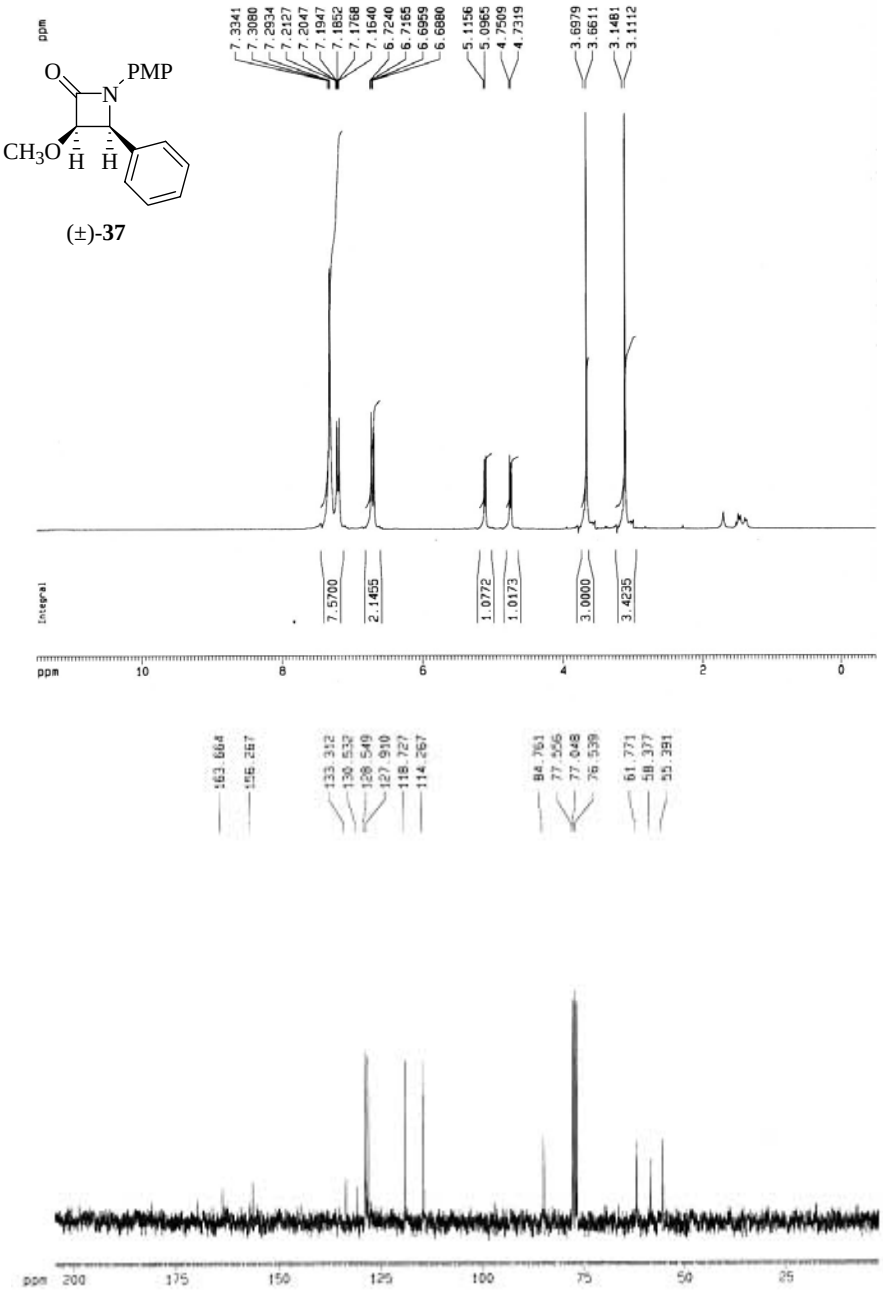
The effects of lactam **68** on DNA, RNA, and protein synthesis in *S. aureus* ATCC 25923 was determined by measuring the respective incorporations of [methyl-$^3$H]thymidine, [5-$^3$H]uridine, or L-[4,5-$^3$H]isoleucine (Amersham Life Science). Ciprofloxacin, rifampcin, and chloramphenicol were used as controls for the inhibition of DNA, RNA, and protein synthesis, respectively. Radioactive precursors were added to early logarithmic-phase *S. aureus* (3 μCi of [methyl-$^3$H]thymidine, 3 μCi of [5-$^3$H]uridine, or 5 μCi of L-[4,5-$^3$H]isoleucine) in Luria Broth in the presence or absence of an antibiotic at 2x MIC.

To assess the effect on DNA, RNA, and protein synthesis, 50-μl samples were removed from each reaction tube are the designated time intervals (5, 10, 20, 30, 45, and 60 min) and precipitated in 1 ml of ice cold 10% trichloroacetic acid. After 1 hr, the samples were filtered through glass fiber filters (GF/A; Whatman), washed with 2 ml of ice-cold 5% trichloroacetic acid and 2 ml of ice-cold 95% ethanol, and dried at room temperature overnight. The dried filters were placed in 10 ml vials containing 7 ml of counting fluid (Cytoscint, ICN International). Radioactivity was measured by liquid scintilation (Beckman Instruments).

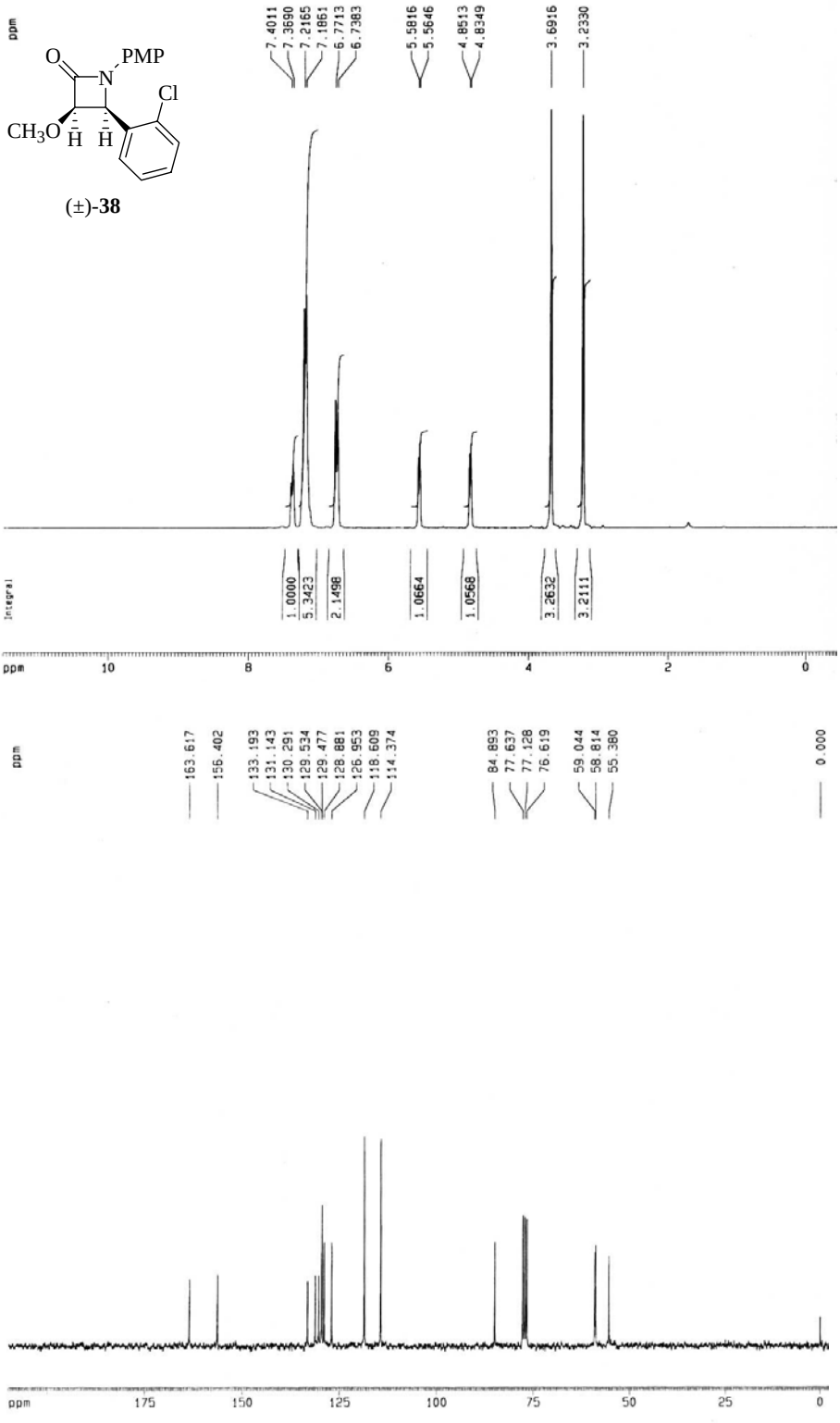
# CHAPTER VII

## $^{1}H$ and $^{13}C$ NMR SPECTRA

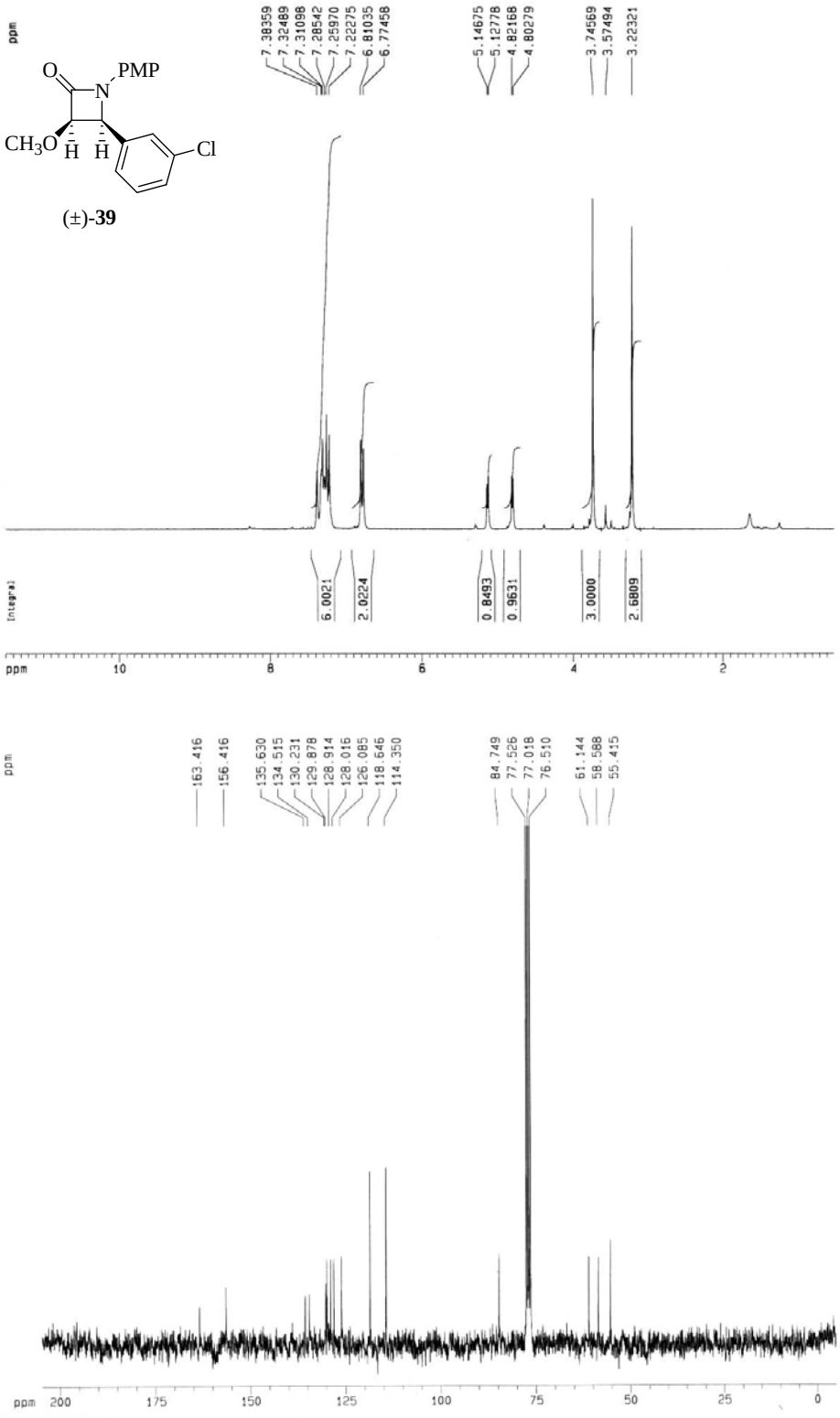
**Spectrum 7.01:** $^{1}H$ NMR (250 MHz, $CDCl_3$) and $^{13}C$ NMR (63 MHz, $CDCl_3$) of β-lactam **37**.



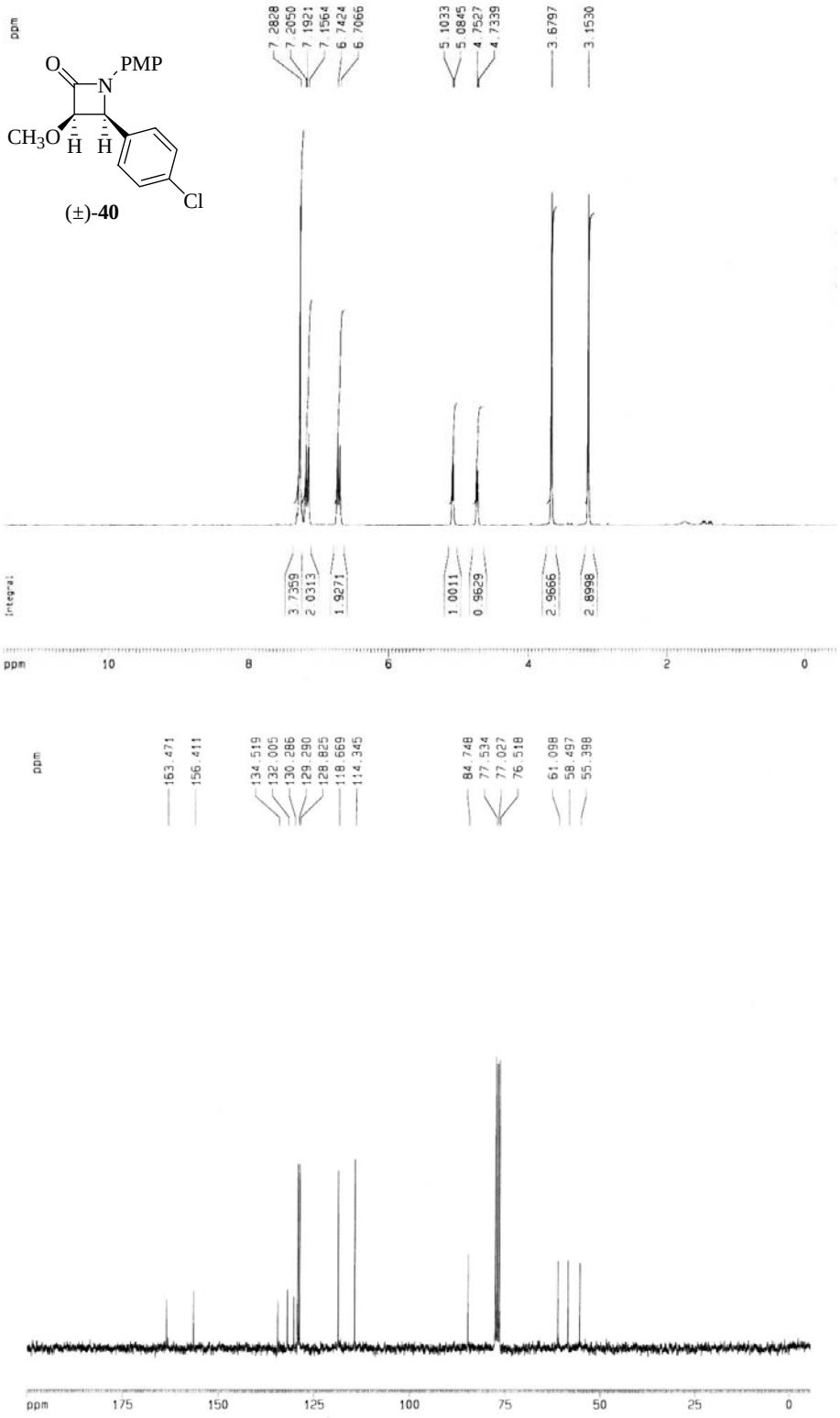
**Spectrum 7.02:** $^{1}H$ NMR (250 MHz, $CDCl_3$) and $^{13}C$ NMR (63 MHz, $CDCl_3$) of β-lactam **38**.



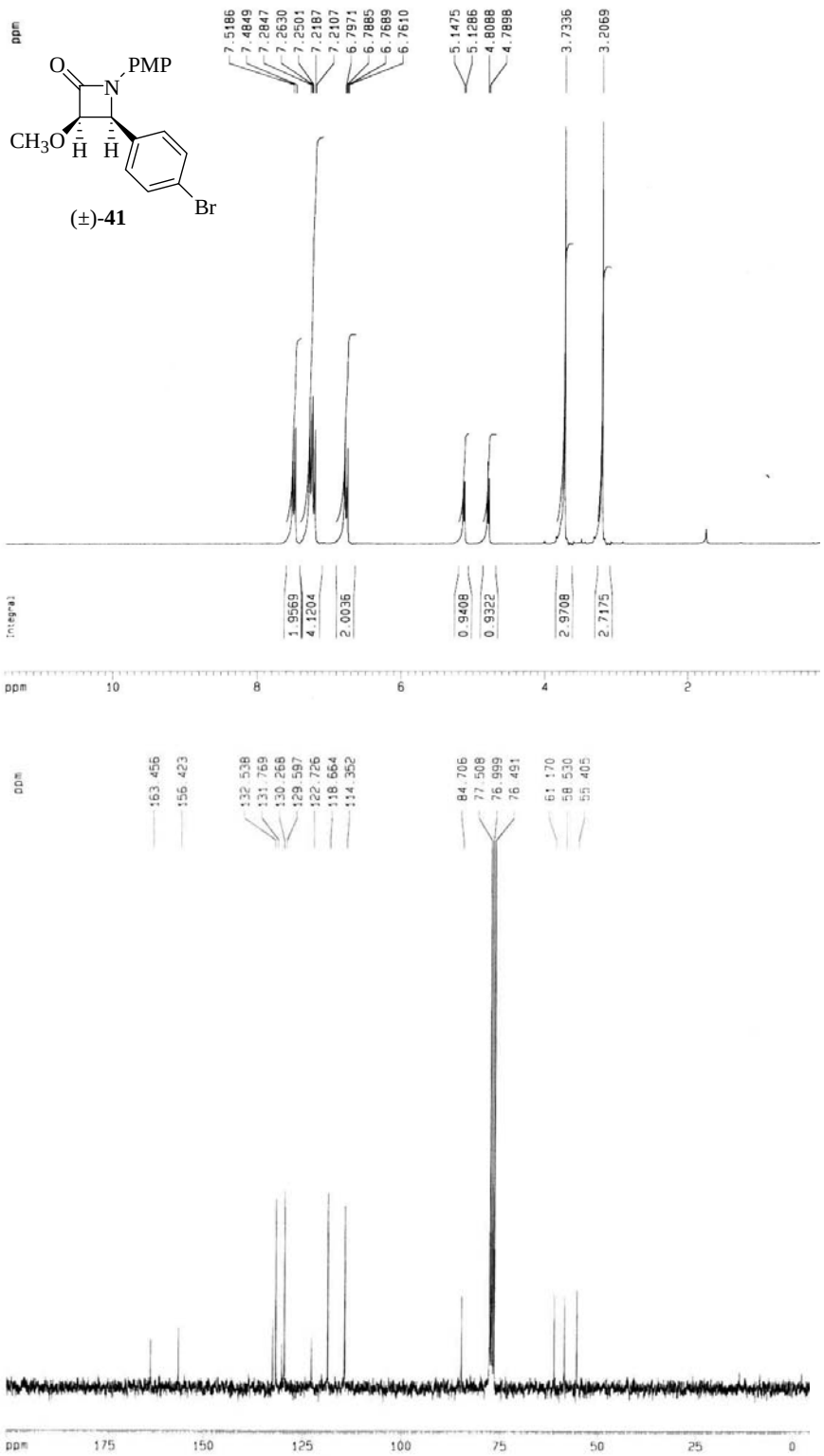
**Spectrum 7.03:** $^{1}H$ NMR (250 MHz, $CDCl_3$) and $^{13}C$ NMR (63 MHz, $CDCl_3$) of β-lactam **39**.



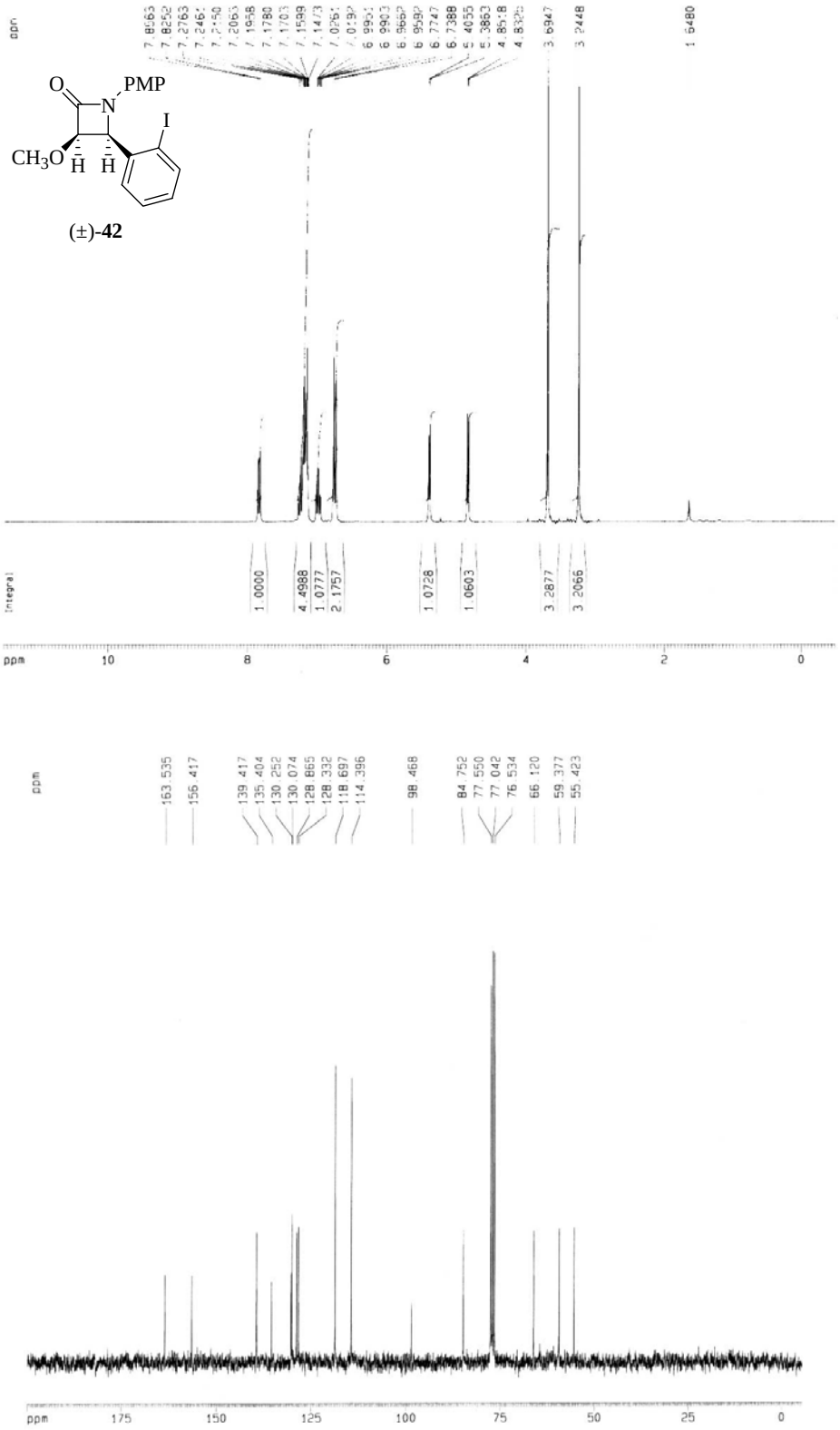
**Spectrum 7.04:** $^{1}H$ NMR (250 MHz, $CDCl_3$) and $^{13}C$ NMR (63 MHz, $CDCl_3$) of β-lactam **40**.



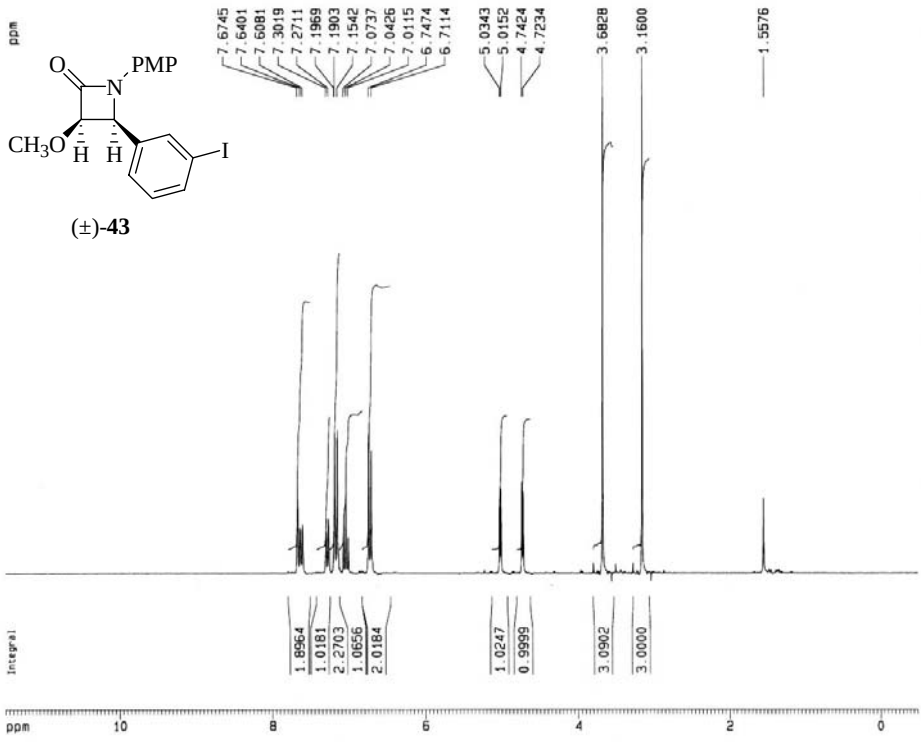
**Spectrum 7.05:** $^{1}H$ NMR (250 MHz, $CDCl_3$) and $^{13}C$ NMR (63 MHz, $CDCl_3$) of β-lactam **41**.



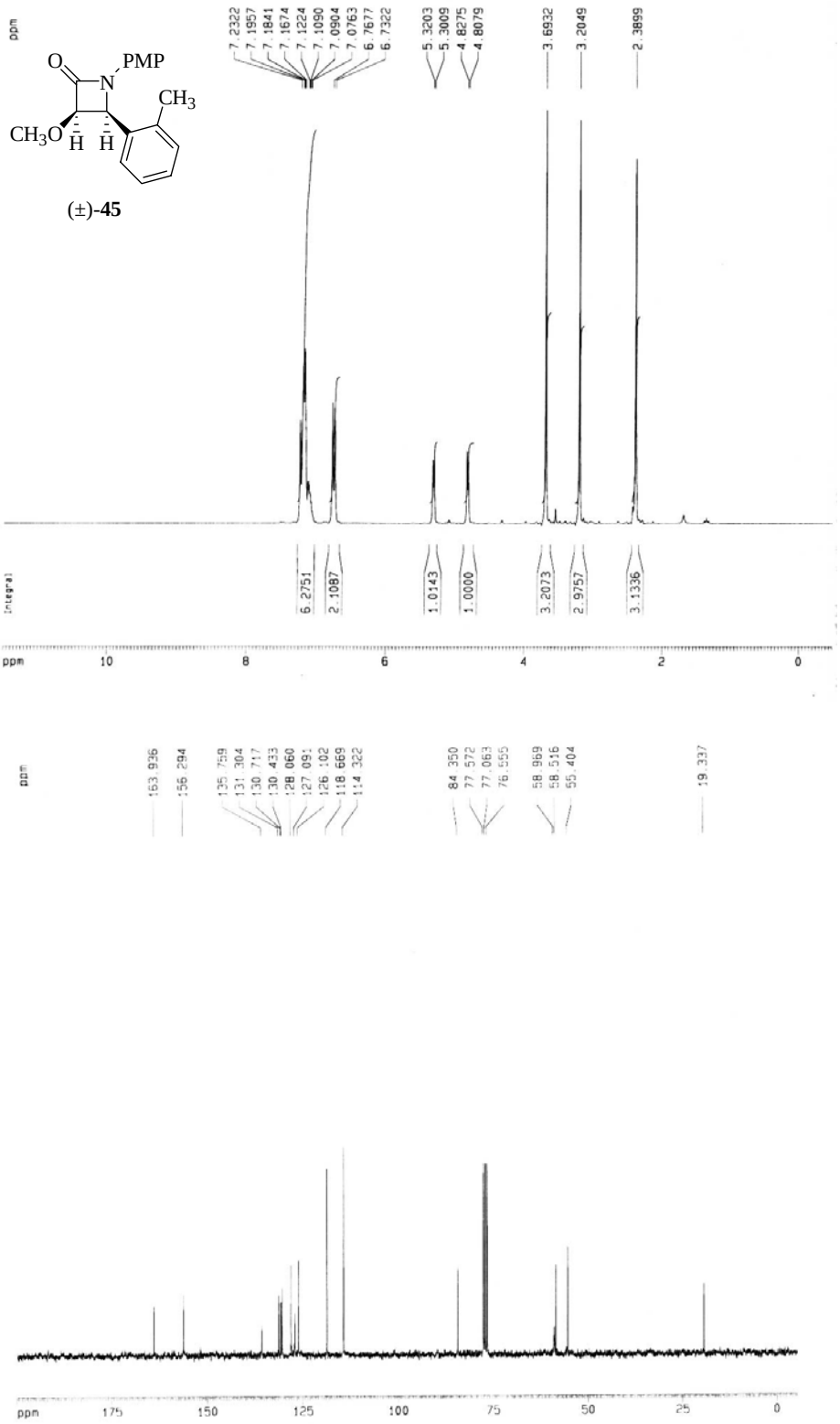
**Spectrum 7.06:** $^{1}H$ NMR (250 MHz, $CDCl_3$) and $^{13}C$ NMR (63 MHz, $CDCl_3$) of β-lactam **42**.



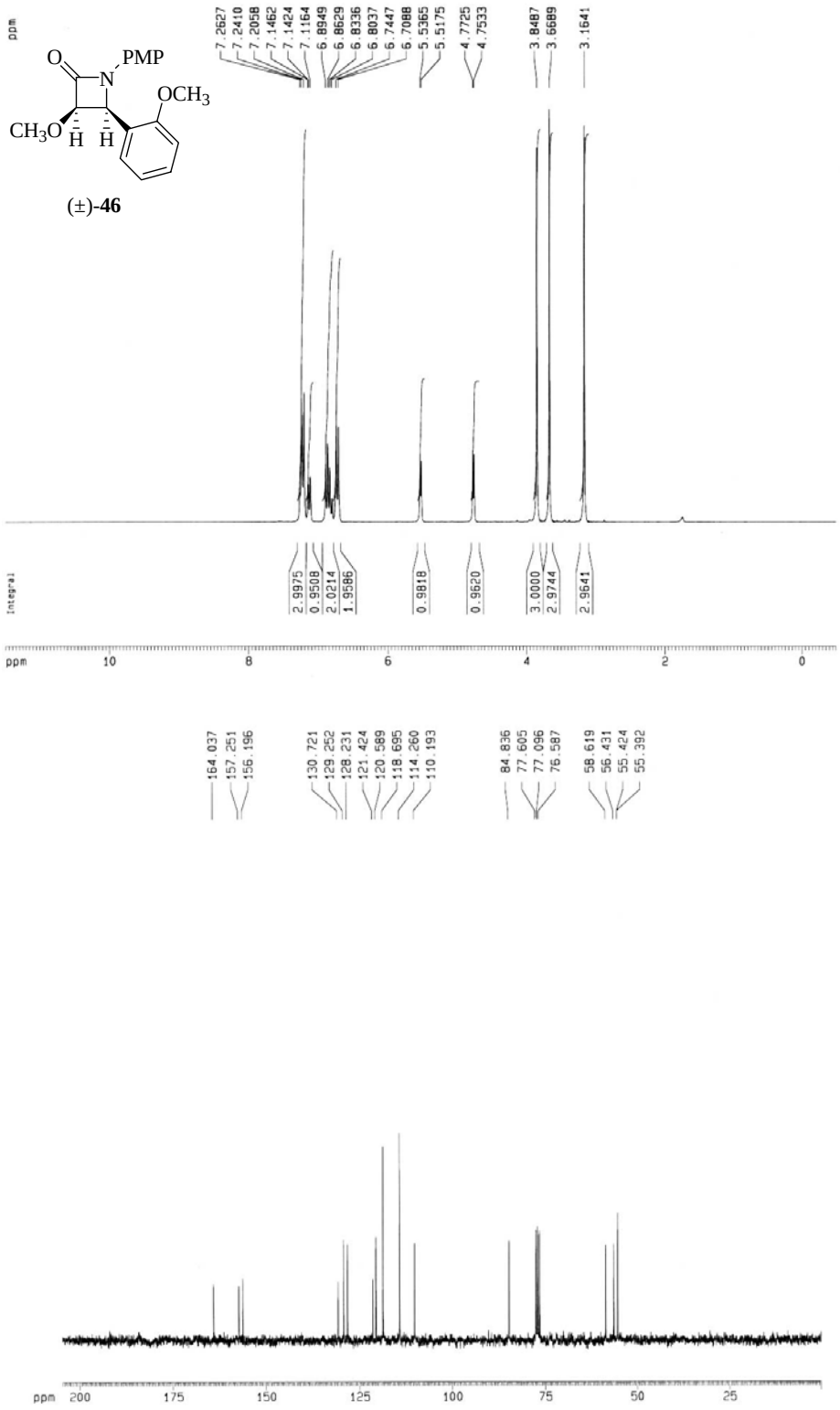
**Spectrum 7.07:** $^{1}H$ NMR (250 MHz, $CDCl_3$) of β-lactam **43**.



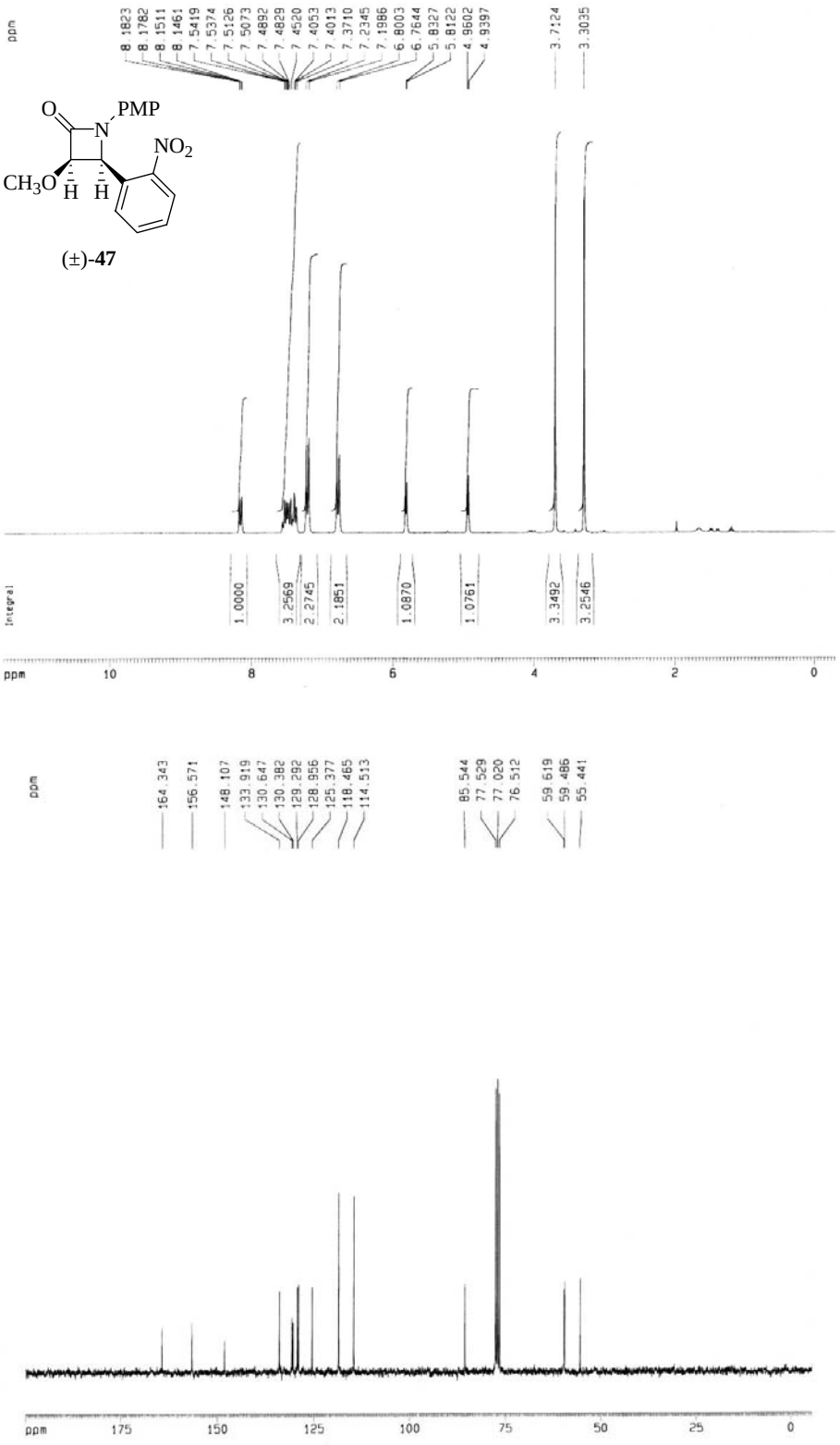
**Spectrum 7.08:** $^{1}H$ NMR (250 MHz, $CDCl_3$) and $^{13}C$ NMR (63 MHz, $CDCl_3$) of β-lactam **45**.



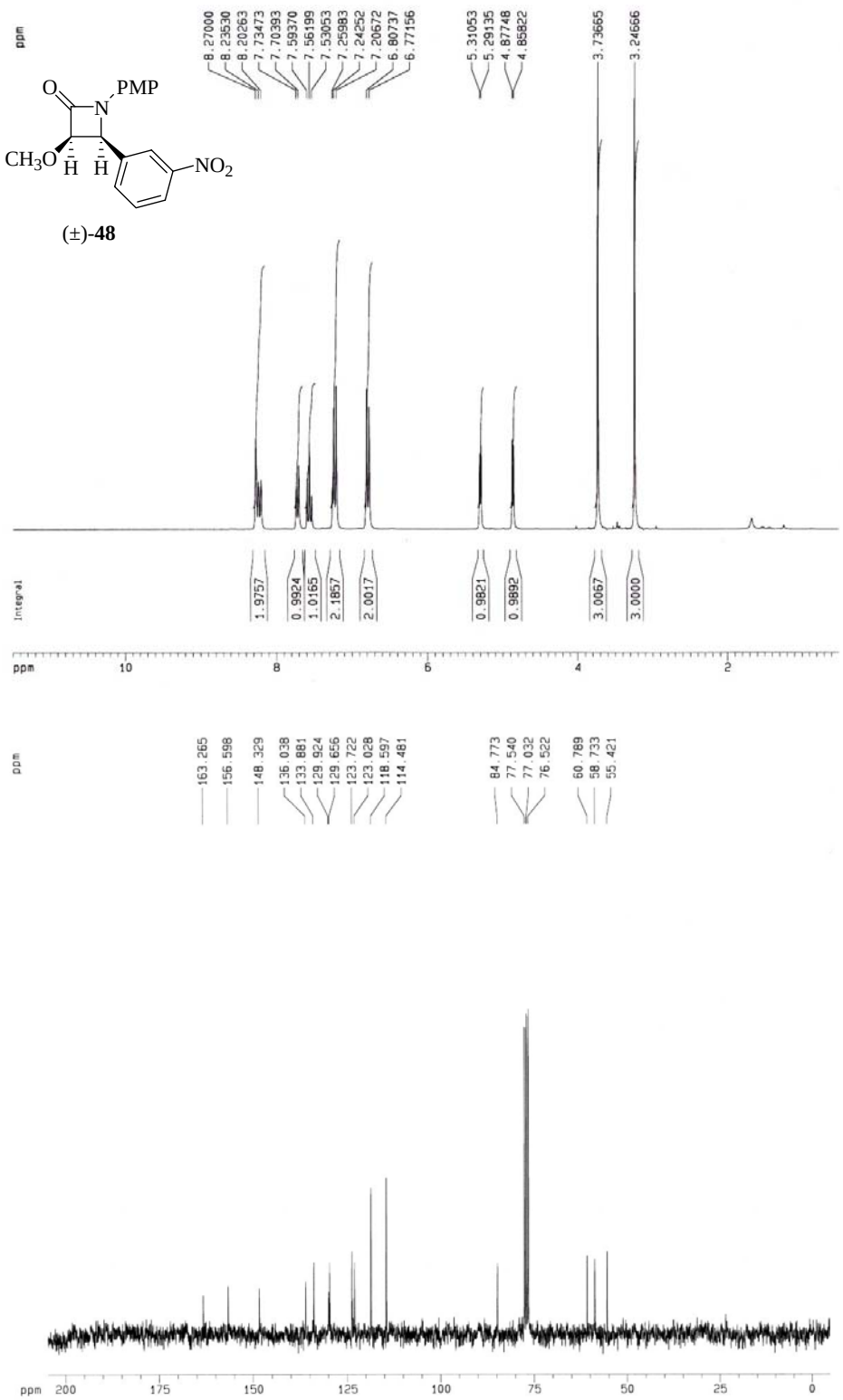
**Spectrum 7.09:** $^{1}H$ NMR (250 MHz, $CDCl_3$) and $^{13}C$ NMR (63 MHz, $CDCl_3$) of β-lactam **46**.



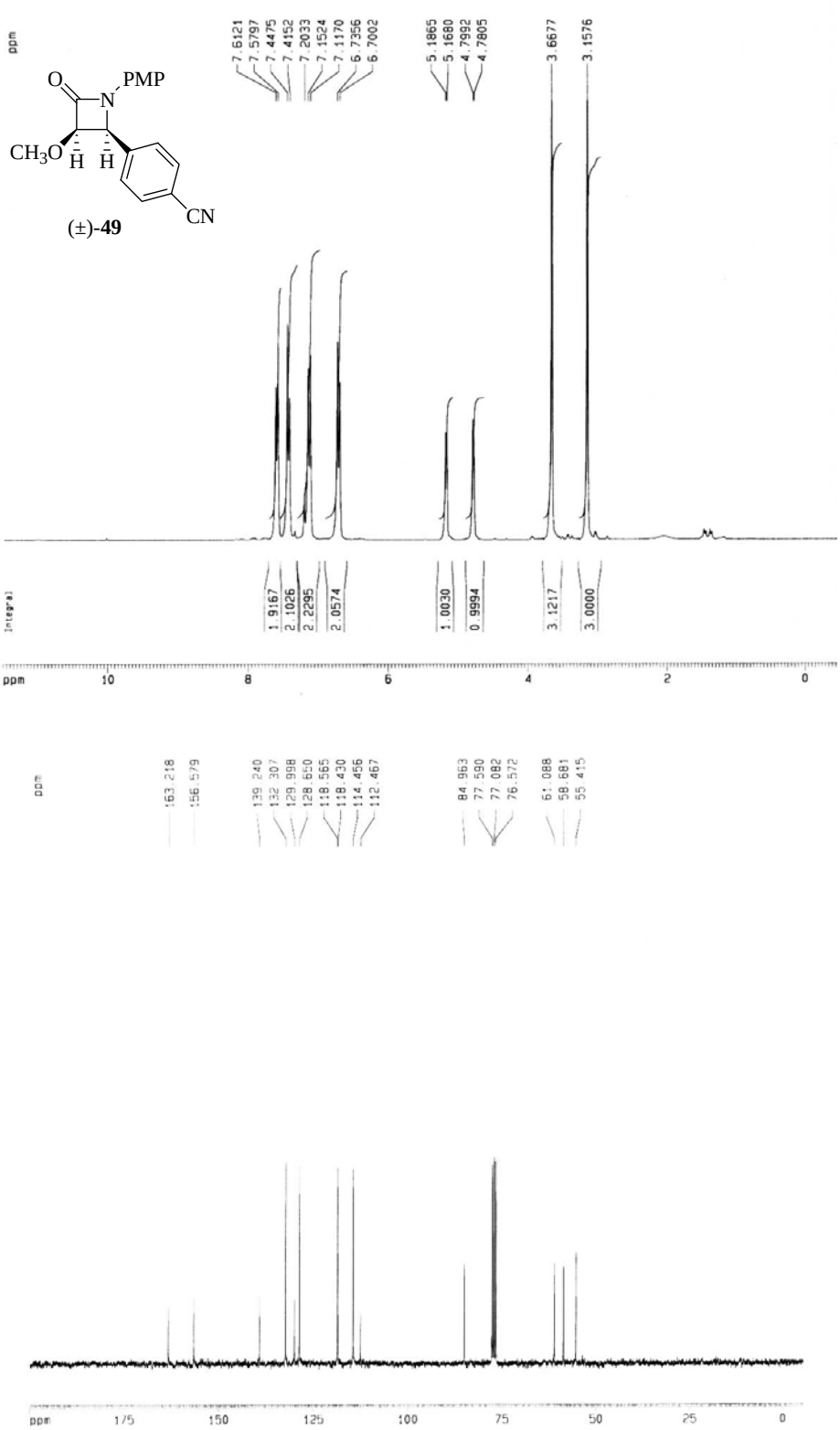
**Spectrum 7.10:** $^{1}H$ NMR (250 MHz, $CDCl_3$) and $^{13}C$ NMR (63 MHz, $CDCl_3$) of β-lactam **47**.



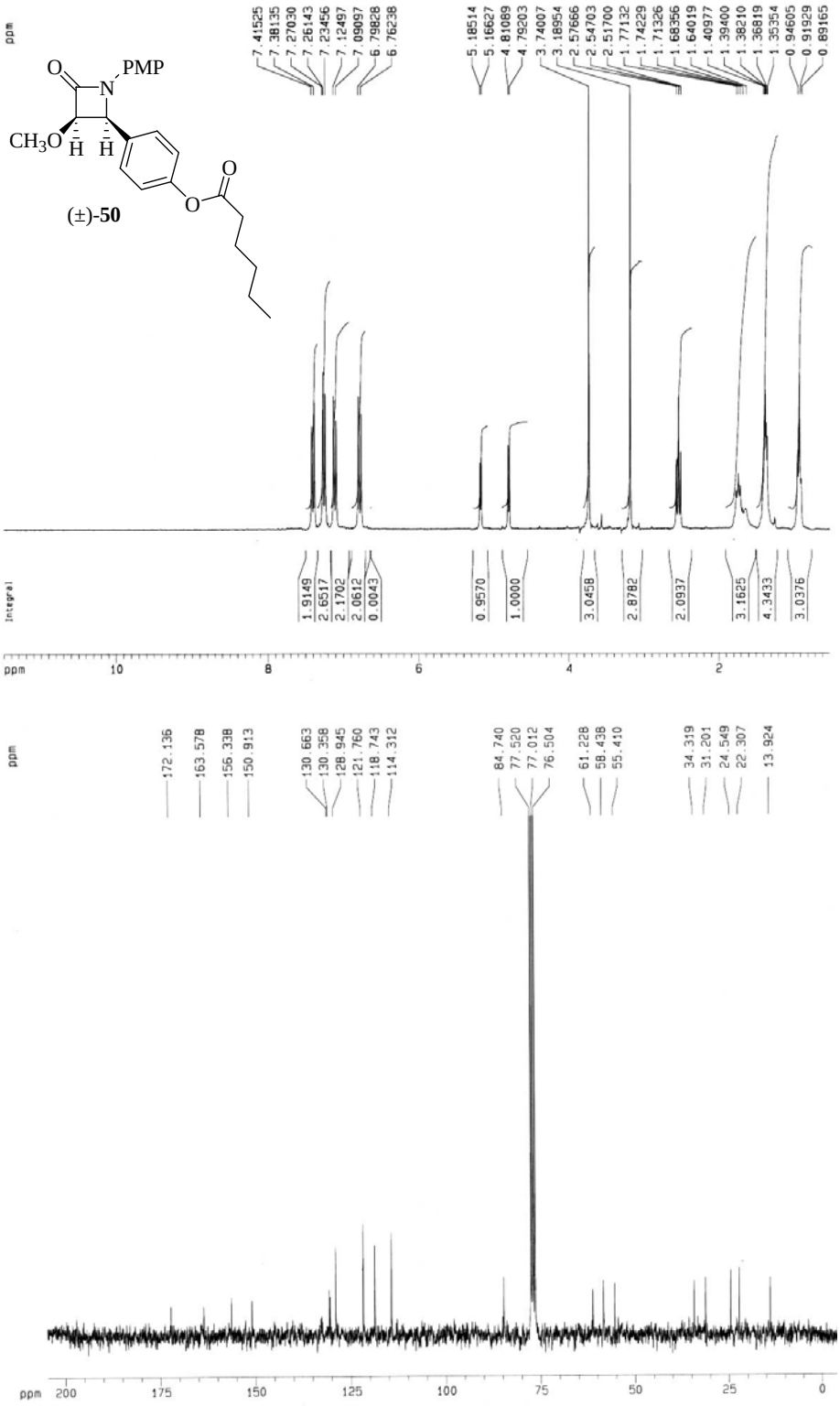
**Spectrum 7.11:** $^{1}H$ NMR (250 MHz, $CDCl_3$) and $^{13}C$ NMR (63 MHz, $CDCl_3$) of β-lactam **48**.



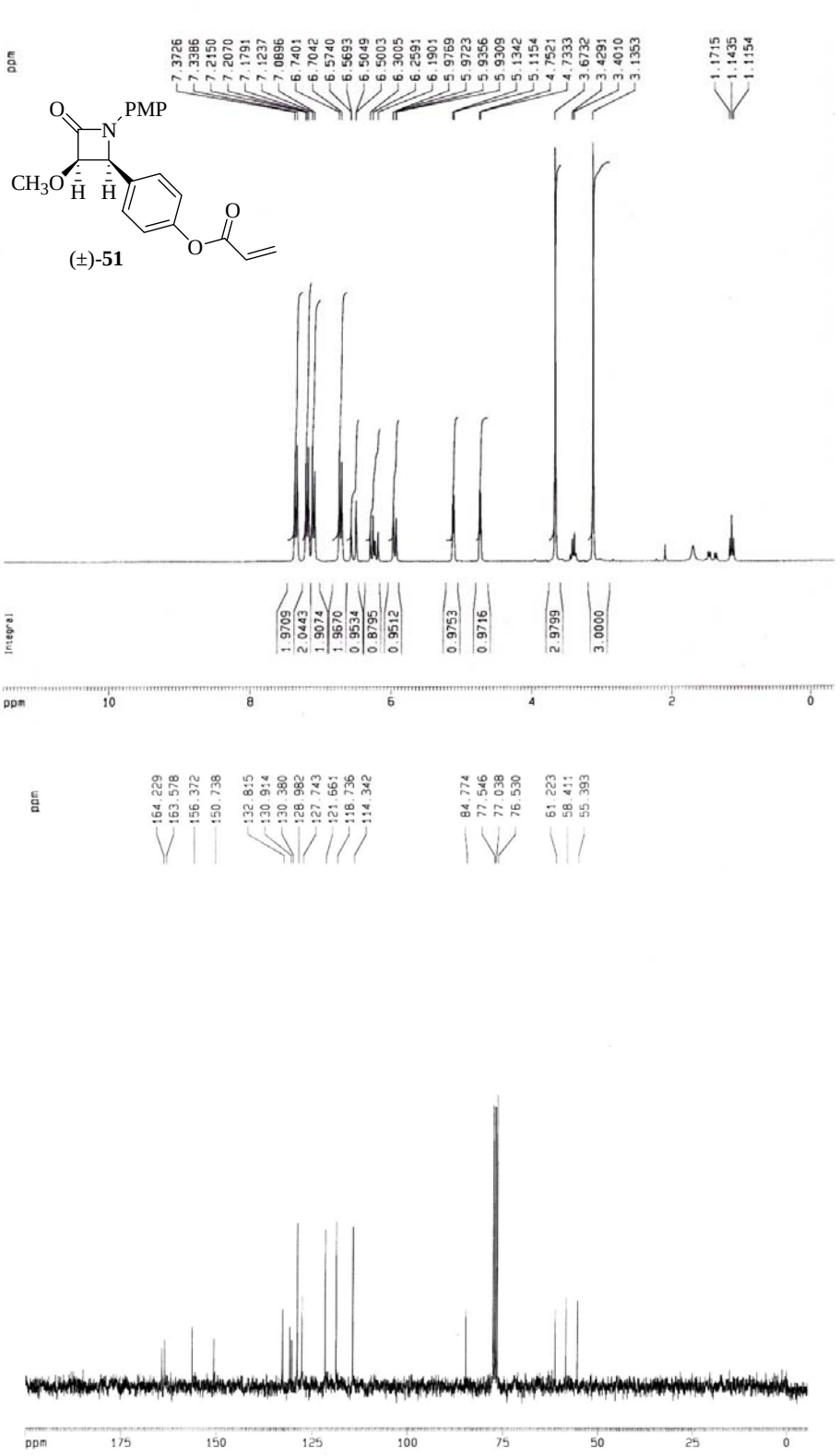
**Spectrum 7.12:** $^{1}H$ NMR (250 MHz, $CDCl_3$) and $^{13}C$ NMR (63 MHz, $CDCl_3$) of β-lactam **49**.



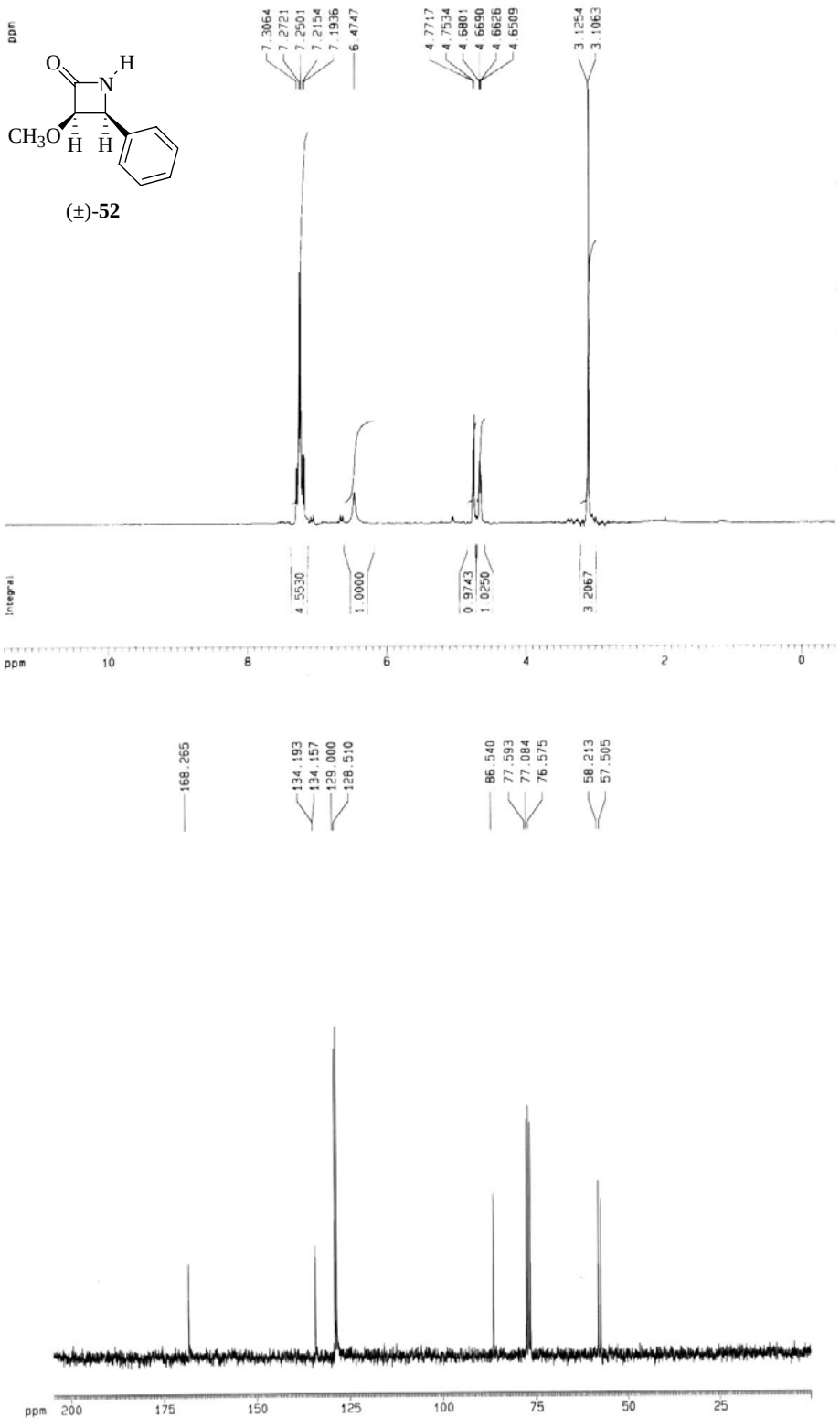
**Spectrum 7.13:** $^{1}H$ NMR (250 MHz, $CDCl_3$) and $^{13}C$ NMR (63 MHz, $CDCl_3$) of β-lactam **50**.



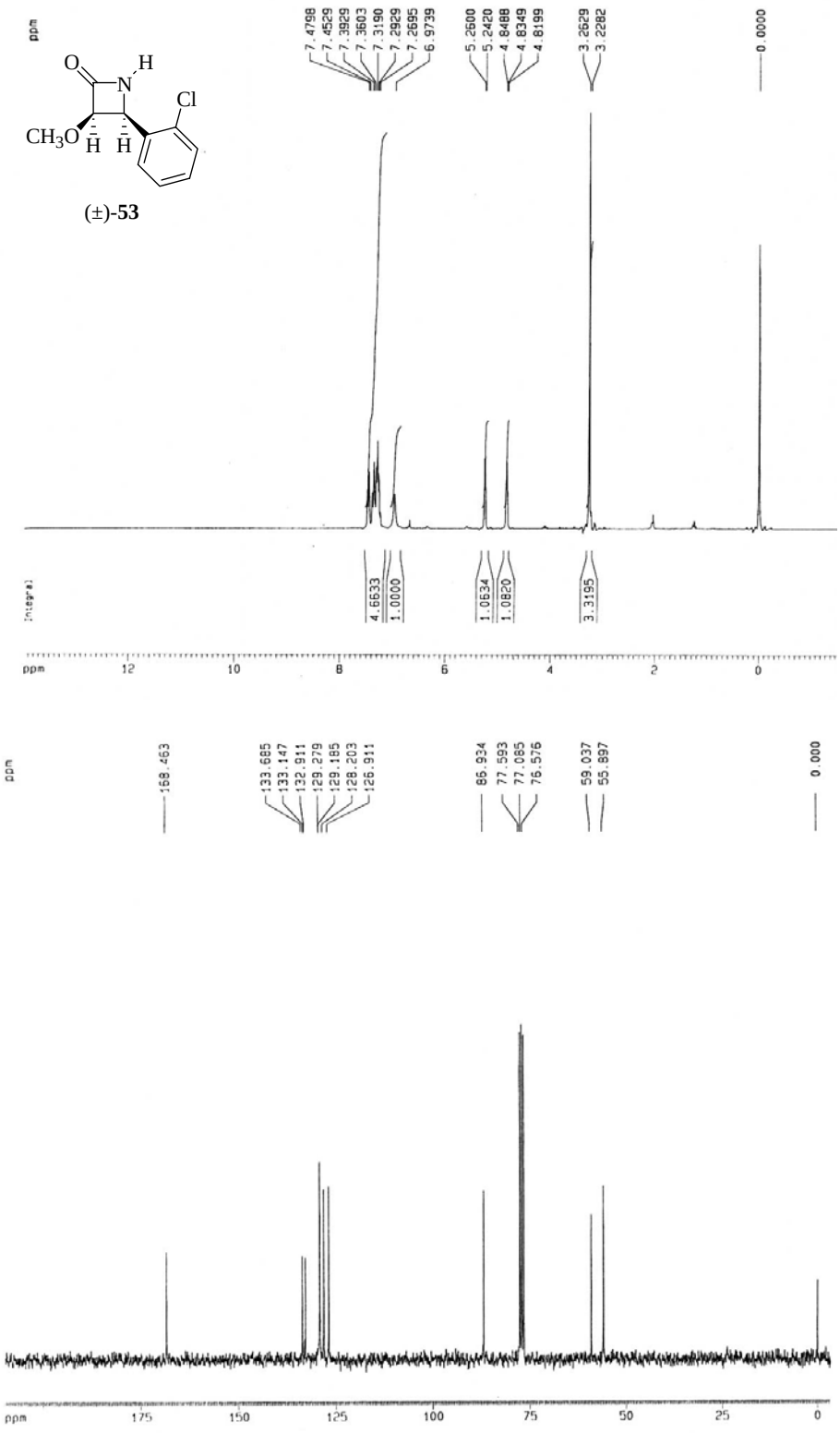
**Spectrum 7.14:** $^{1}H$ NMR (250 MHz, $CDCl_3$) and $^{13}C$ NMR (63 MHz, $CDCl_3$) of β-lactam **51**.



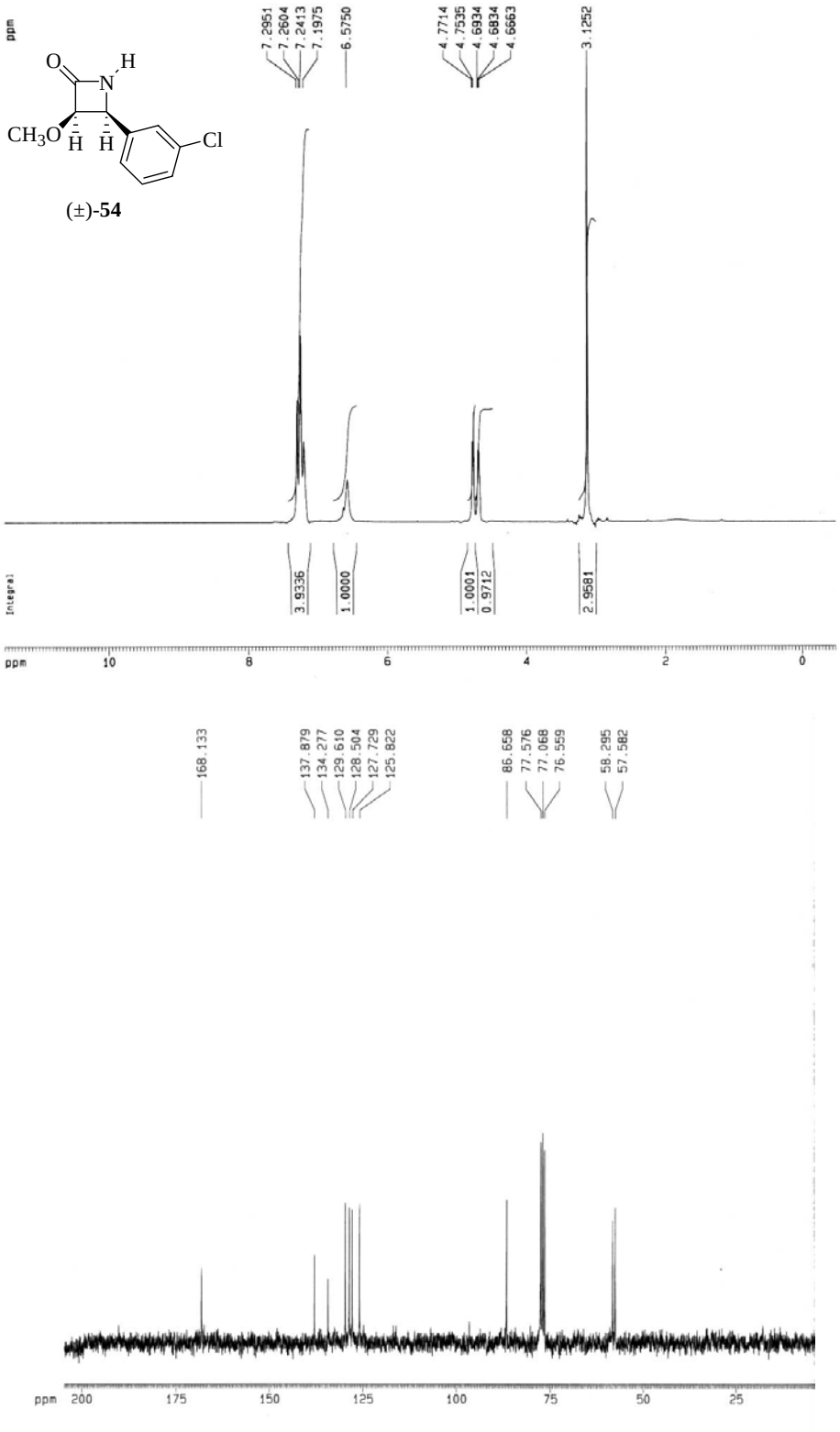
**Spectrum 7.15:** $^{1}H$ NMR (250 MHz, $CDCl_3$) and $^{13}C$ NMR (63 MHz, $CDCl_3$) of β-lactam **52**.



**Spectrum 7.16:** $^{1}H$ NMR (250 MHz, $CDCl_3$) and $^{13}C$ NMR (63 MHz, $CDCl_3$) of β-lactam **53**.

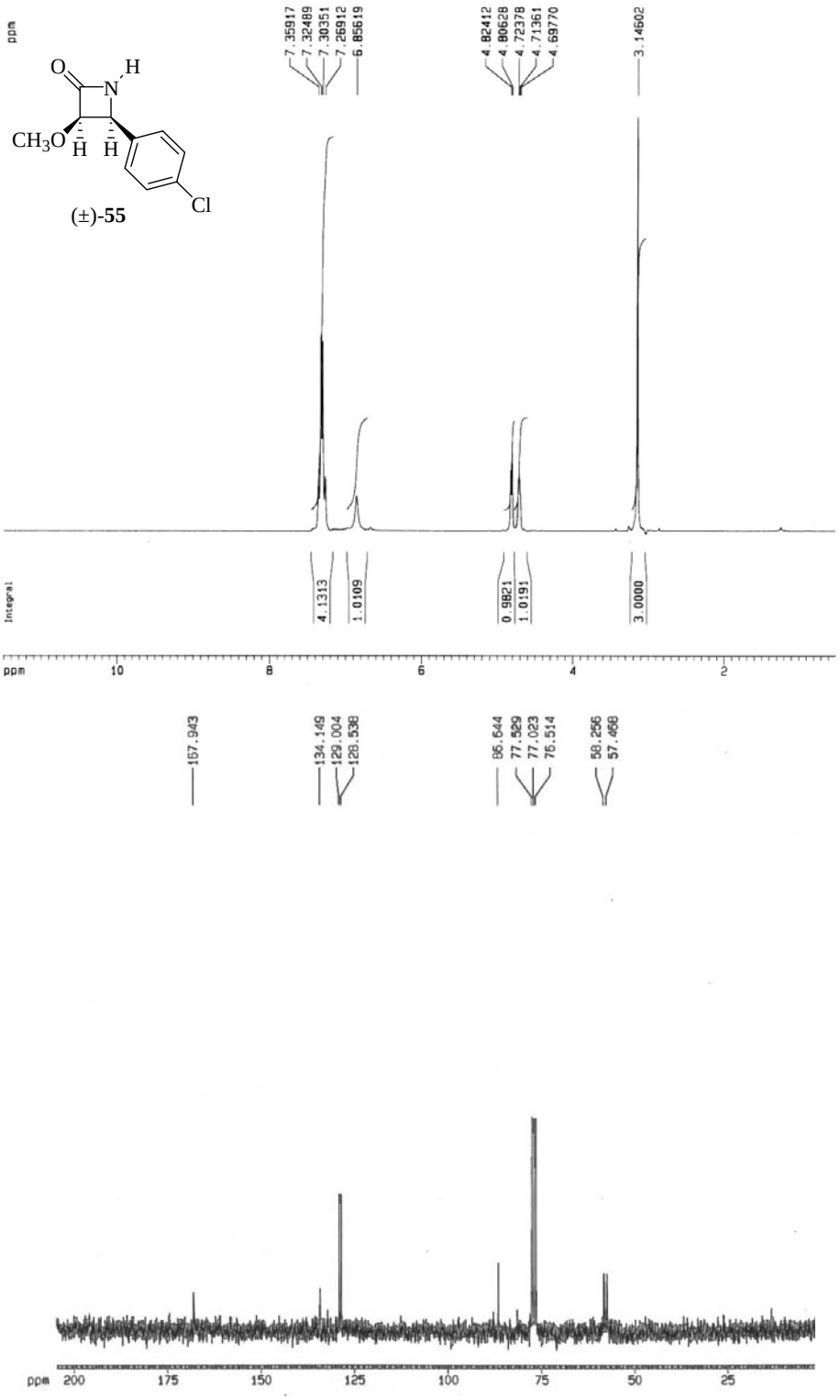


**Spectrum 7.17:** $^{1}H$ NMR (250 MHz, $CDCl_3$) and $^{13}C$ NMR (63 MHz, $CDCl_3$) of β-lactam **54**.



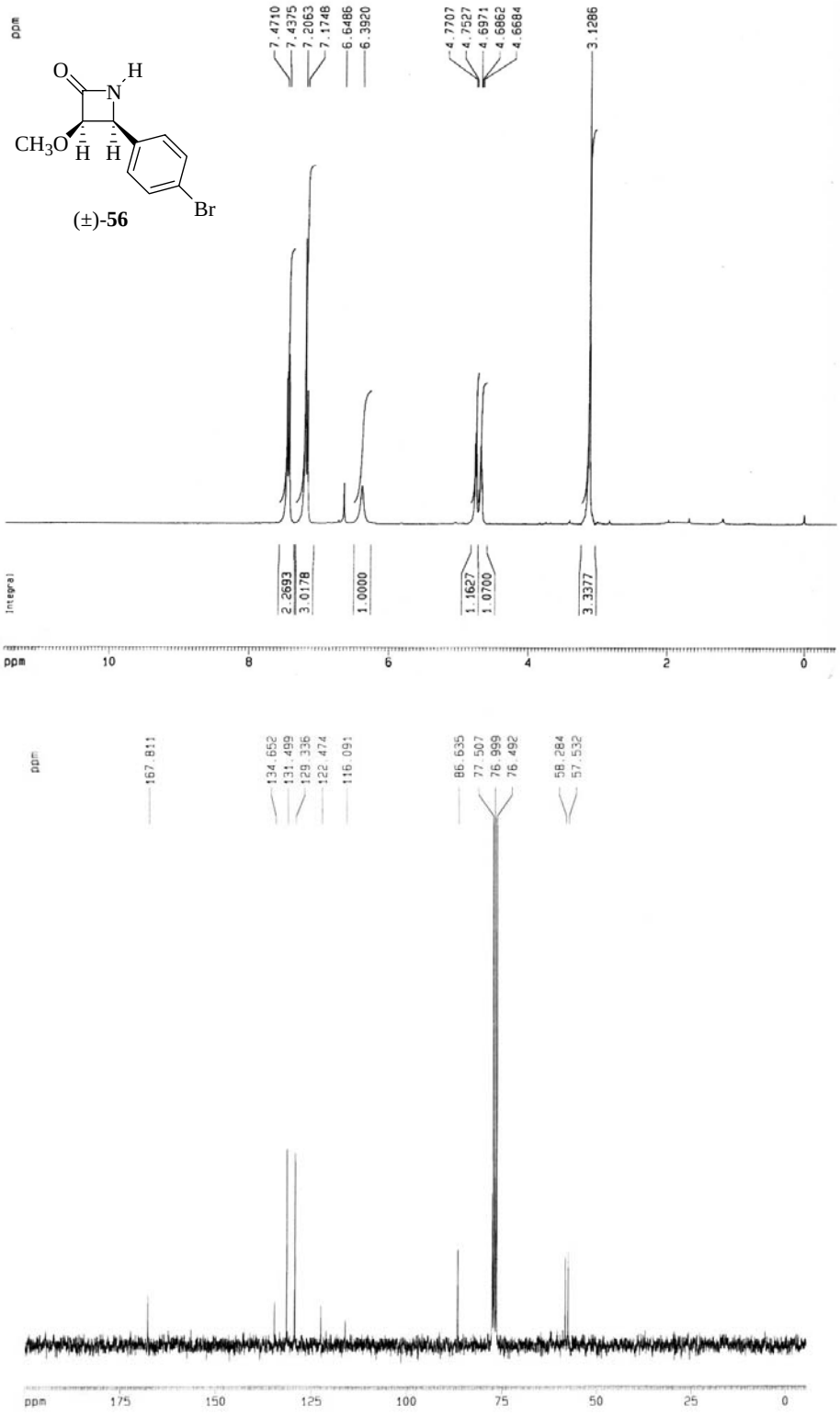

(±)-**54**

**Spectrum 7.18:** $^{1}H$ NMR (250 MHz, $CDCl_3$) and $^{13}C$ NMR (63 MHz, $CDCl_3$) of β-lactam **55**.

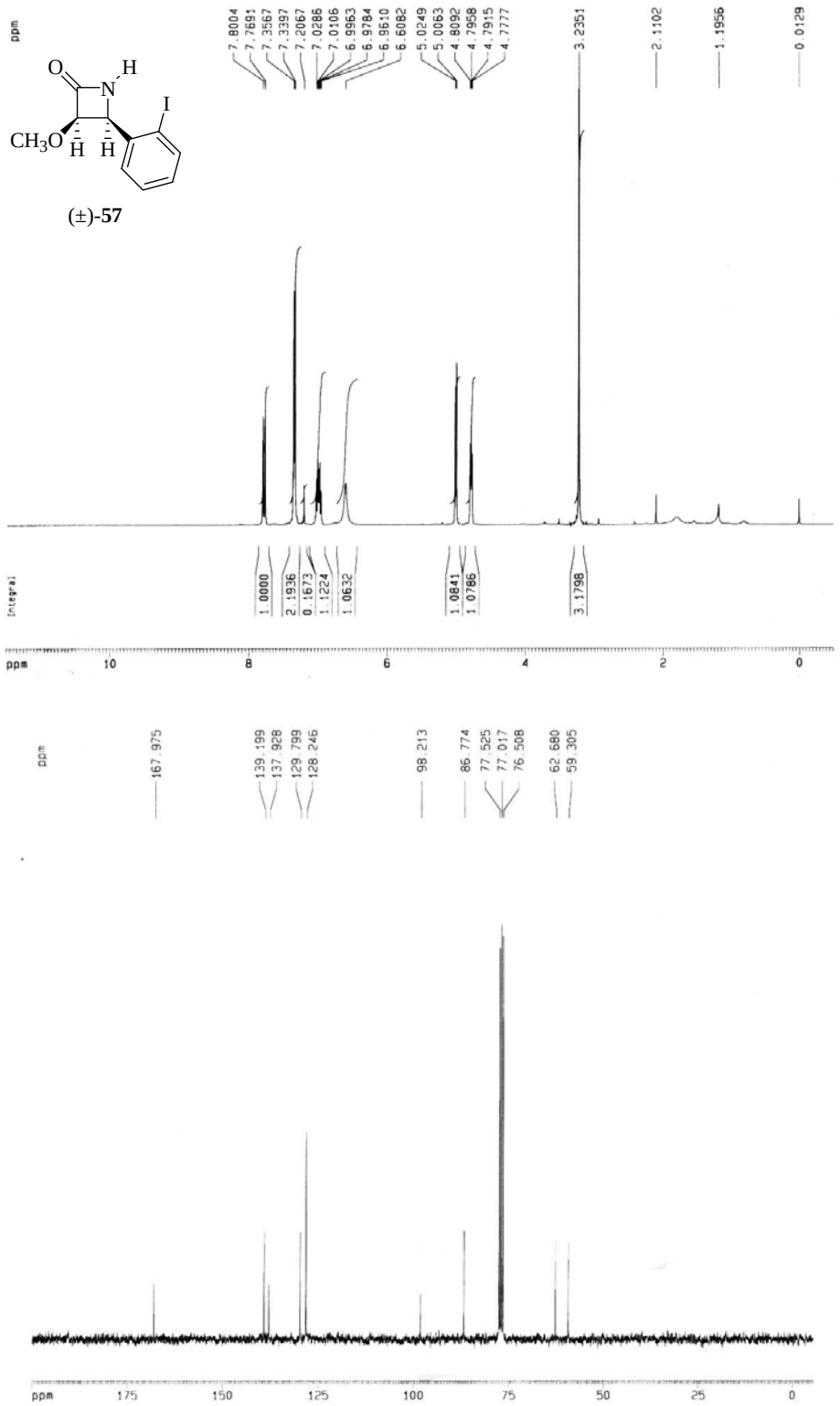



(±)-**55**

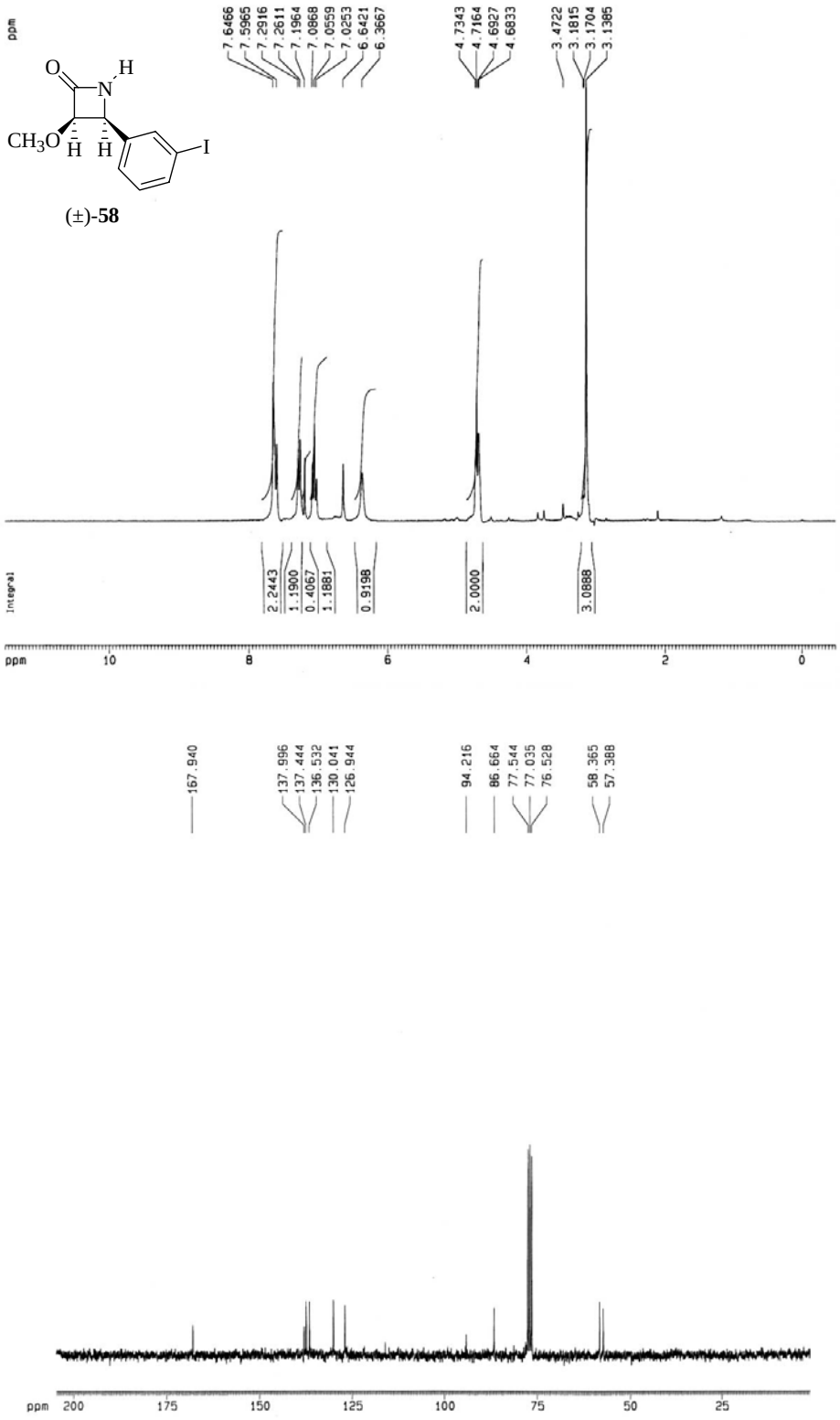
**Spectrum 7.19:** $^{1}H$ NMR (250 MHz, $CDCl_3$) and $^{13}C$ NMR (63 MHz, $CDCl_3$) of β-lactam **56**.



**Spectrum 7.20:** $^{1}H$ NMR (250 MHz, $CDCl_3$) and $^{13}C$ NMR (63 MHz, $CDCl_3$) of β-lactam **57**.

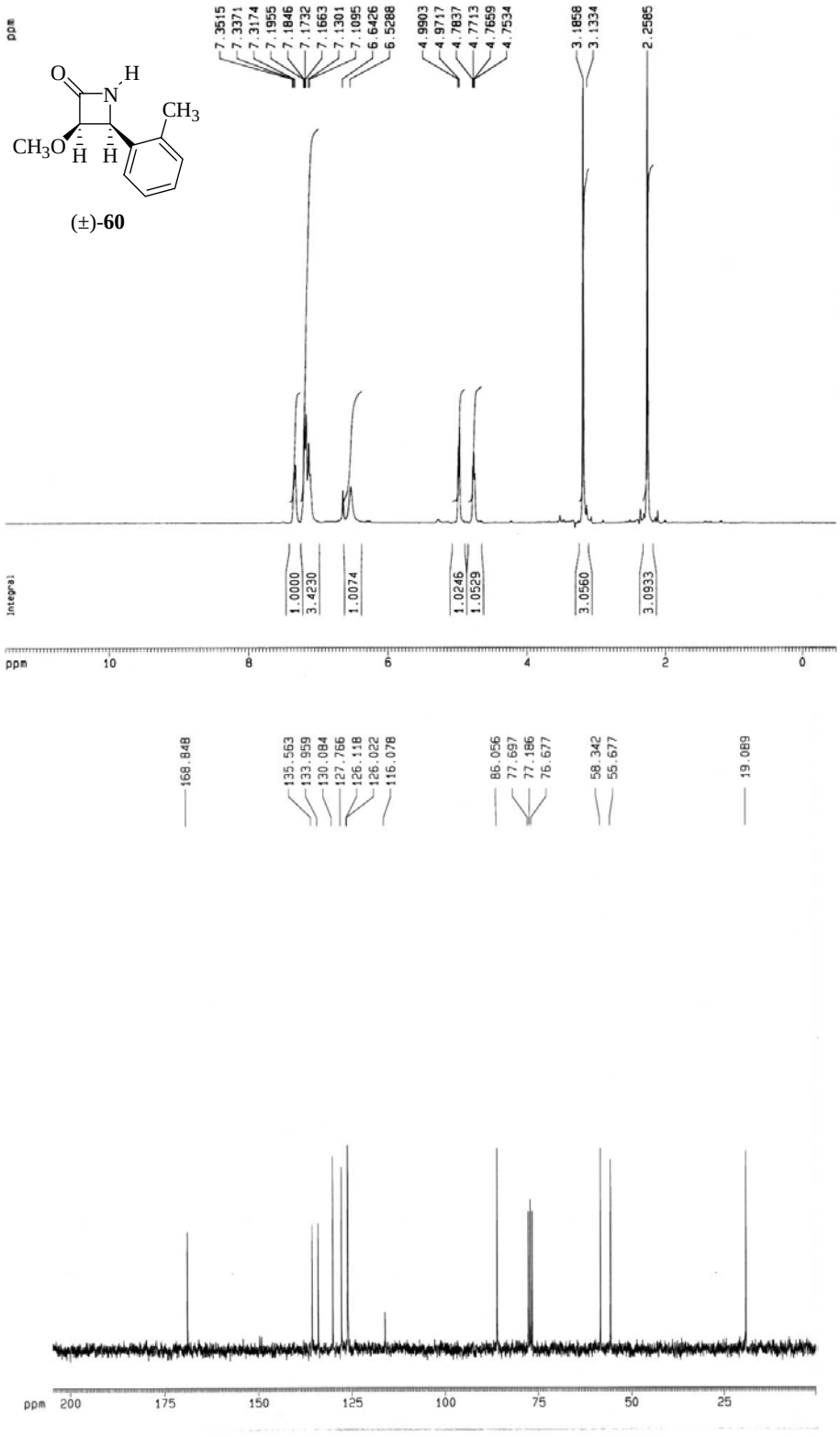


**Spectrum 7.21:** $^{1}H$ NMR (250 MHz, $CDCl_3$) and $^{13}C$ NMR (63 MHz, $CDCl_3$) of β-lactam **58**.

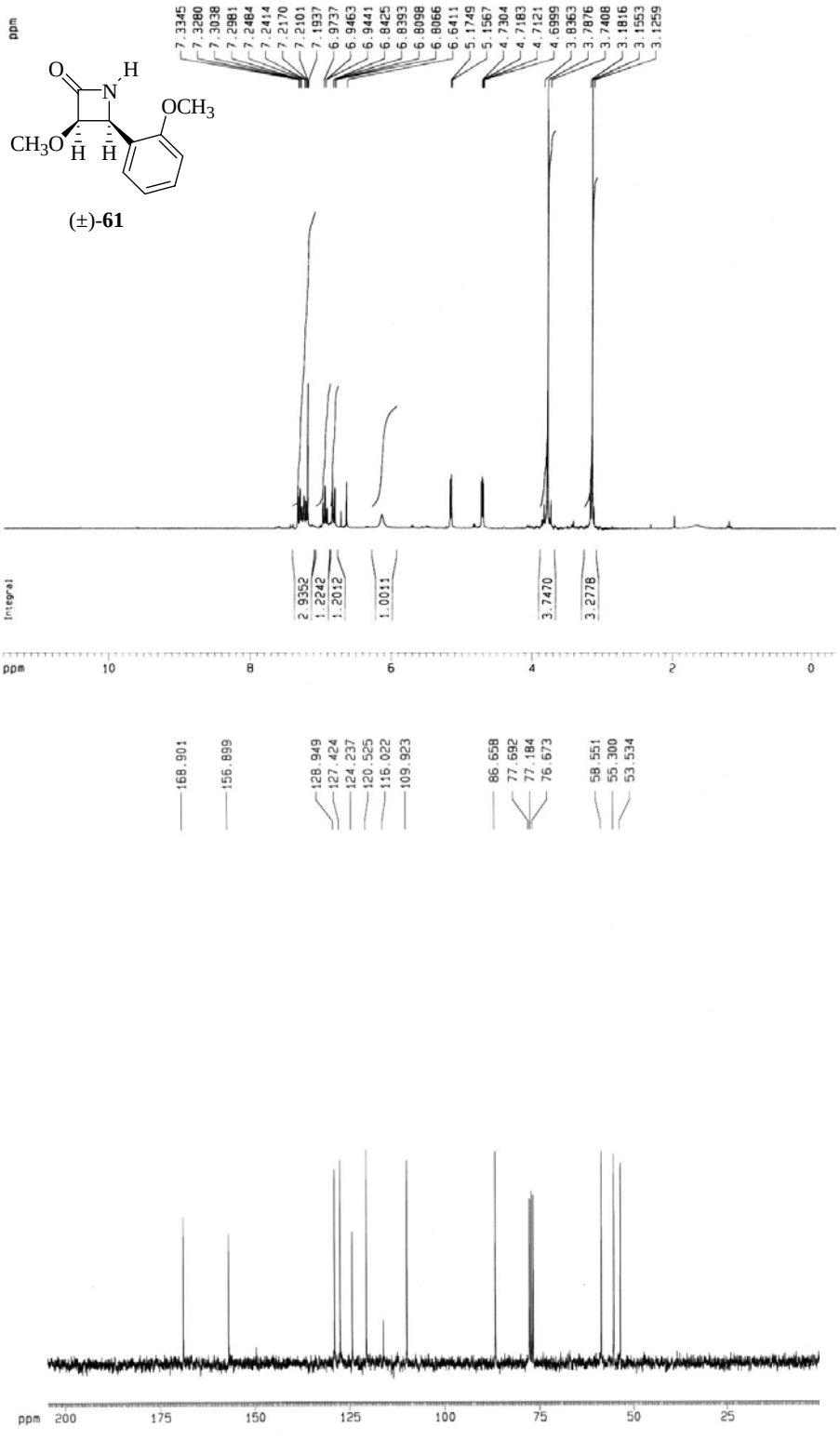



(±)-**58**

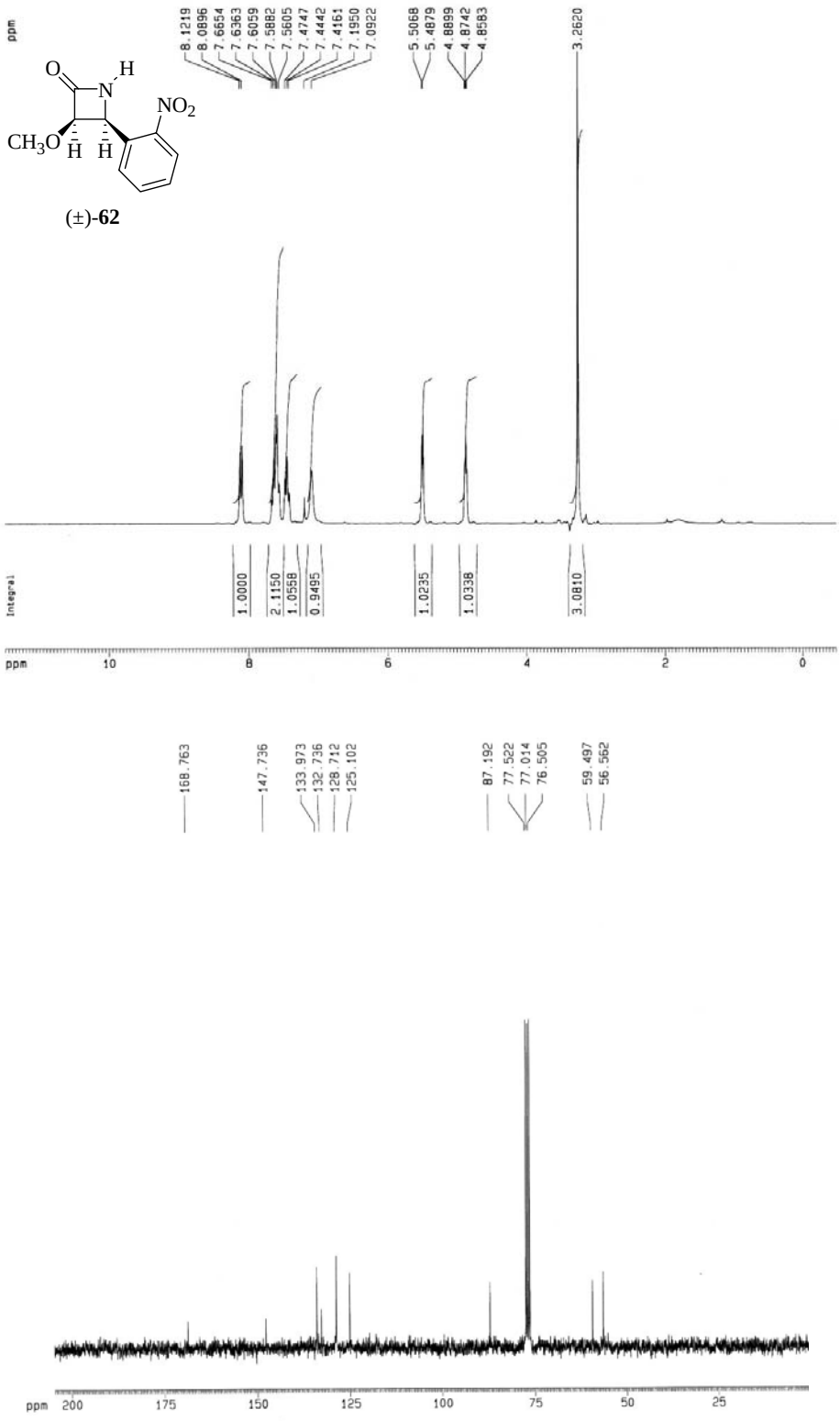
**Spectrum 7.22:** $^{1}H$ NMR (250 MHz, $CDCl_3$) and $^{13}C$ NMR (63 MHz, $CDCl_3$) of β-lactam **60**.



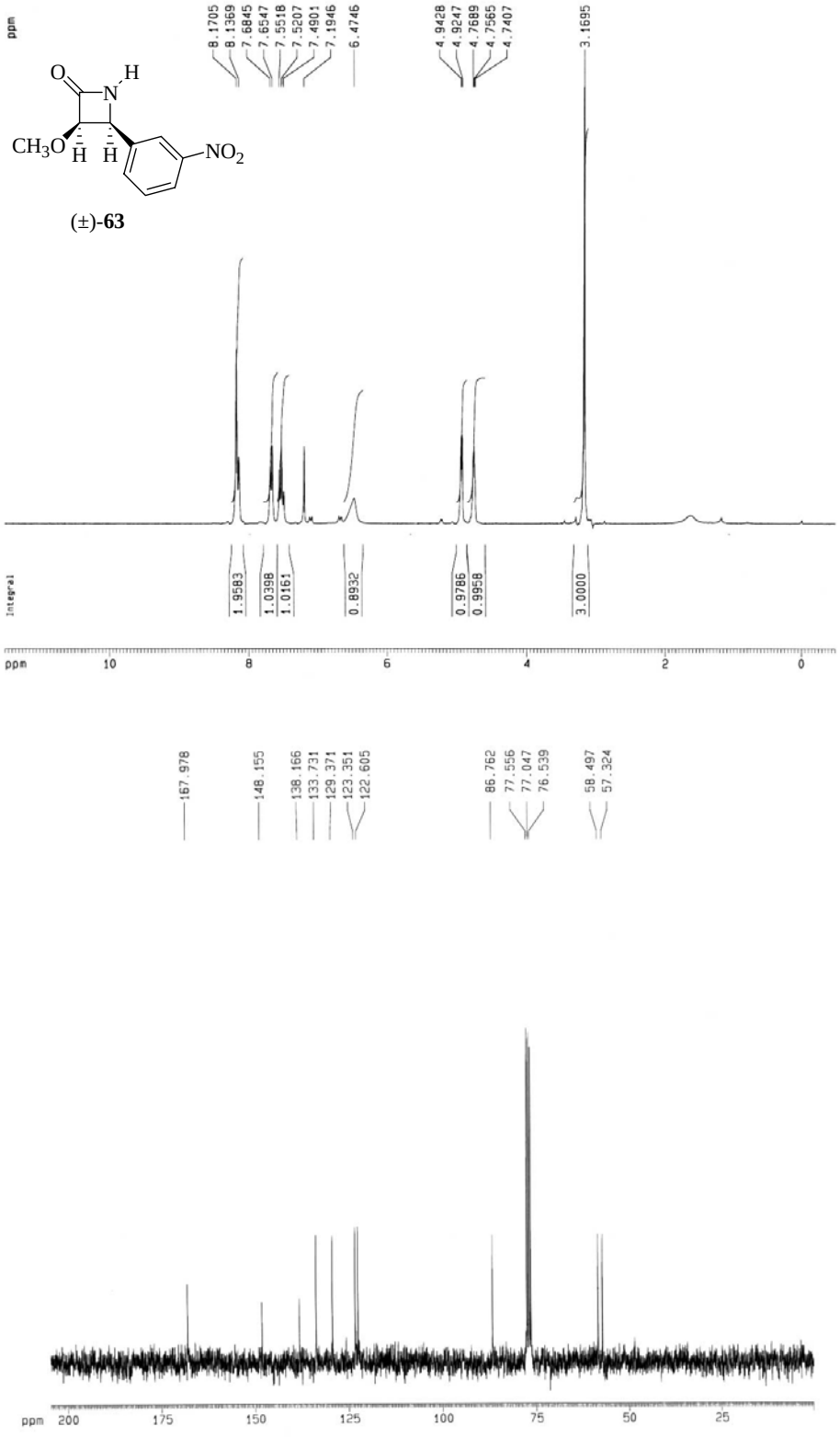
**Spectrum 7.23:** $^{1}H$ NMR (250 MHz, $CDCl_3$) and $^{13}C$ NMR (63 MHz, $CDCl_3$) of β-lactam **61**.



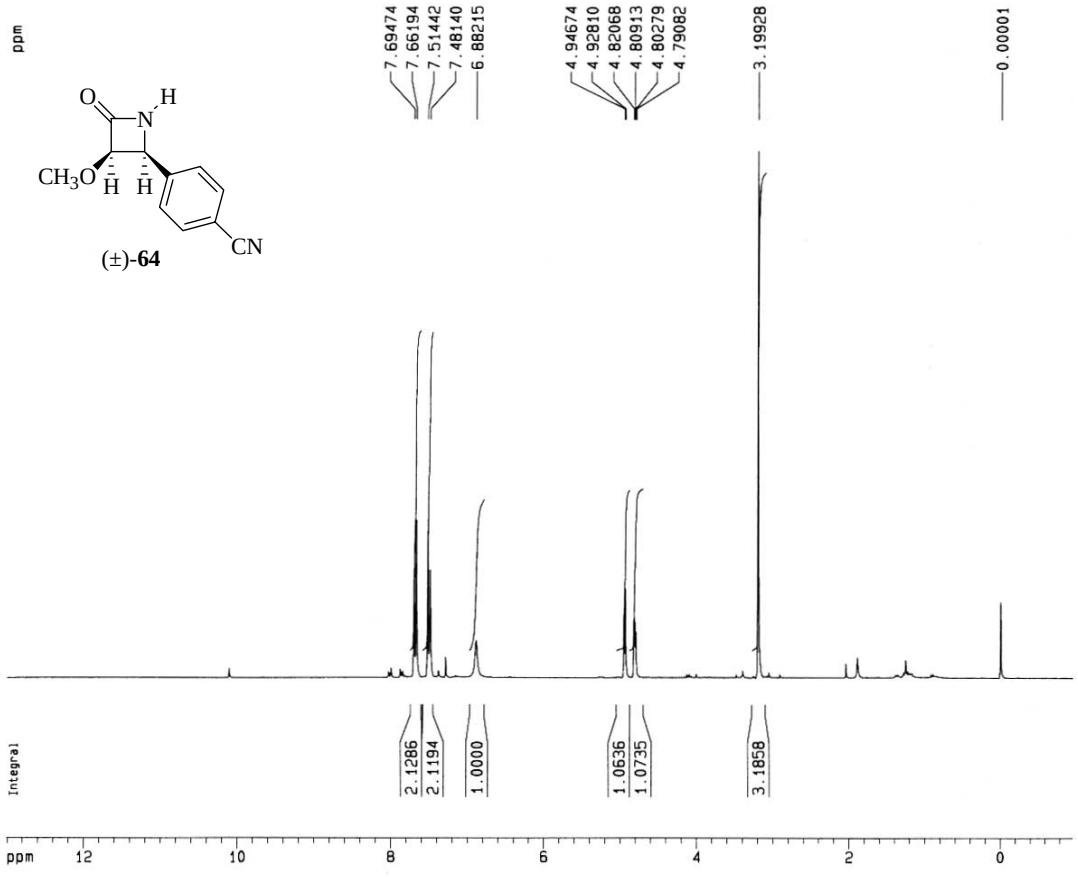
**Spectrum 7.24:** $^{1}H$ NMR (250 MHz, $CDCl_3$) and $^{13}C$ NMR (63 MHz, $CDCl_3$) of β-lactam **62**.



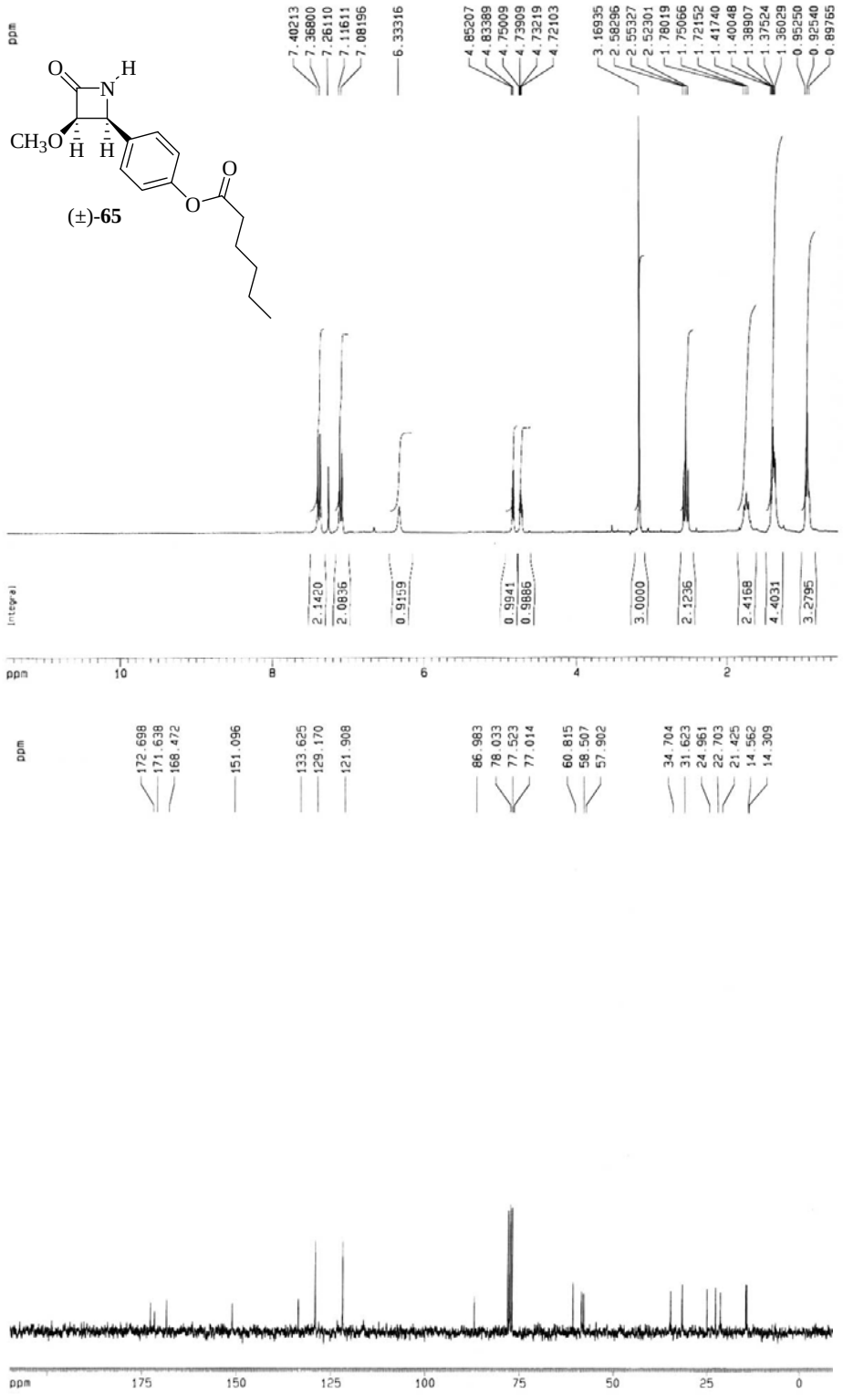
**Spectrum 7.25:** $^{1}H$ NMR (250 MHz, $CDCl_3$) and $^{13}C$ NMR (63 MHz, $CDCl_3$) of β-lactam **63**.



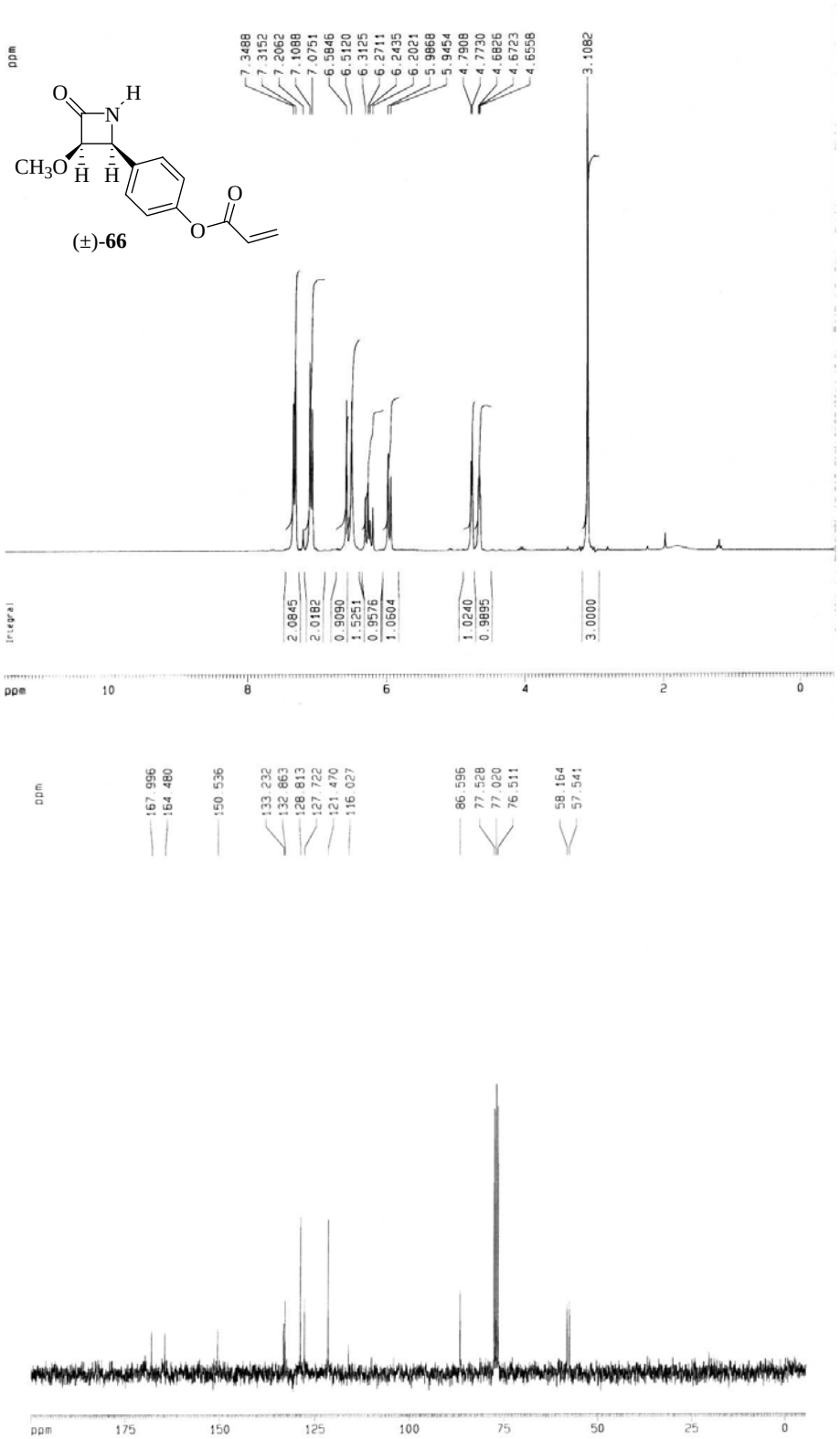
**Spectrum 7.26:** $^{1}H$ NMR (250 MHz, $CDCl_3$) of β-lactam **64**.



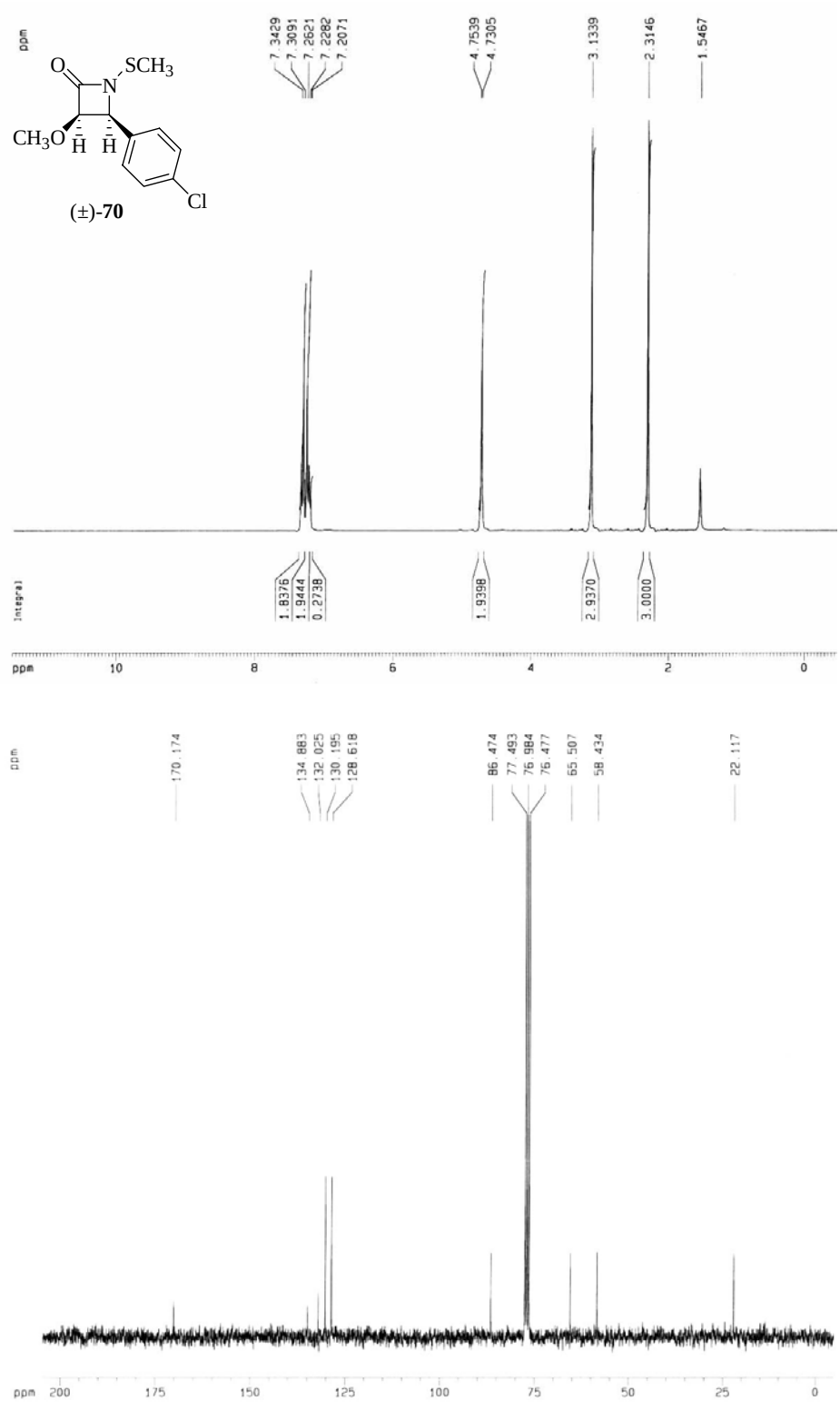
**Spectrum 7.27:** $^{1}H$ NMR (250 MHz, $CDCl_3$) and $^{13}C$ NMR (63 MHz, $CDCl_3$) of β-lactam **65**.



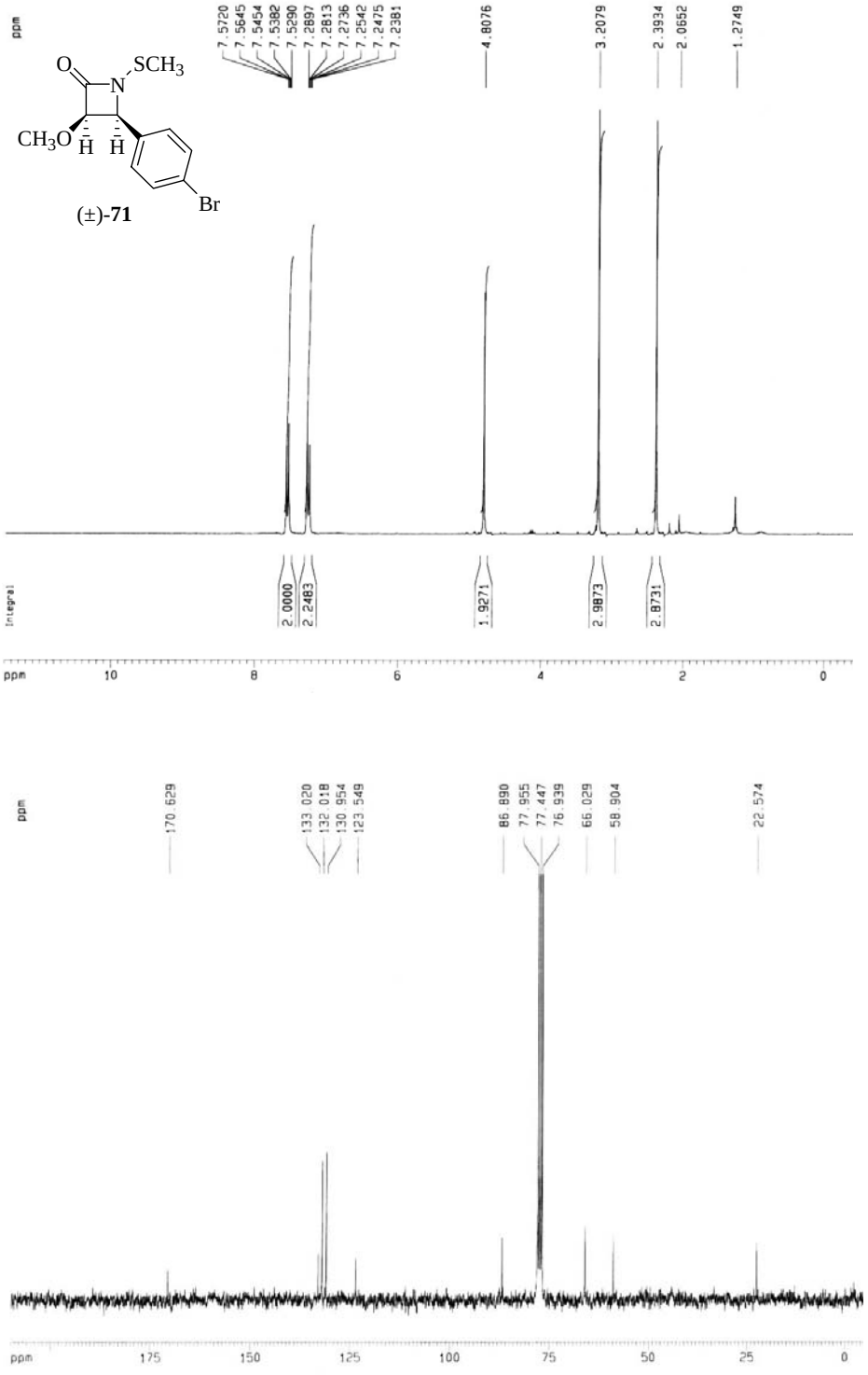
**Spectrum 7.28:** $^{1}H$ NMR (250 MHz, $CDCl_3$) and $^{13}C$ NMR (63 MHz, $CDCl_3$) of β-lactam **66**.



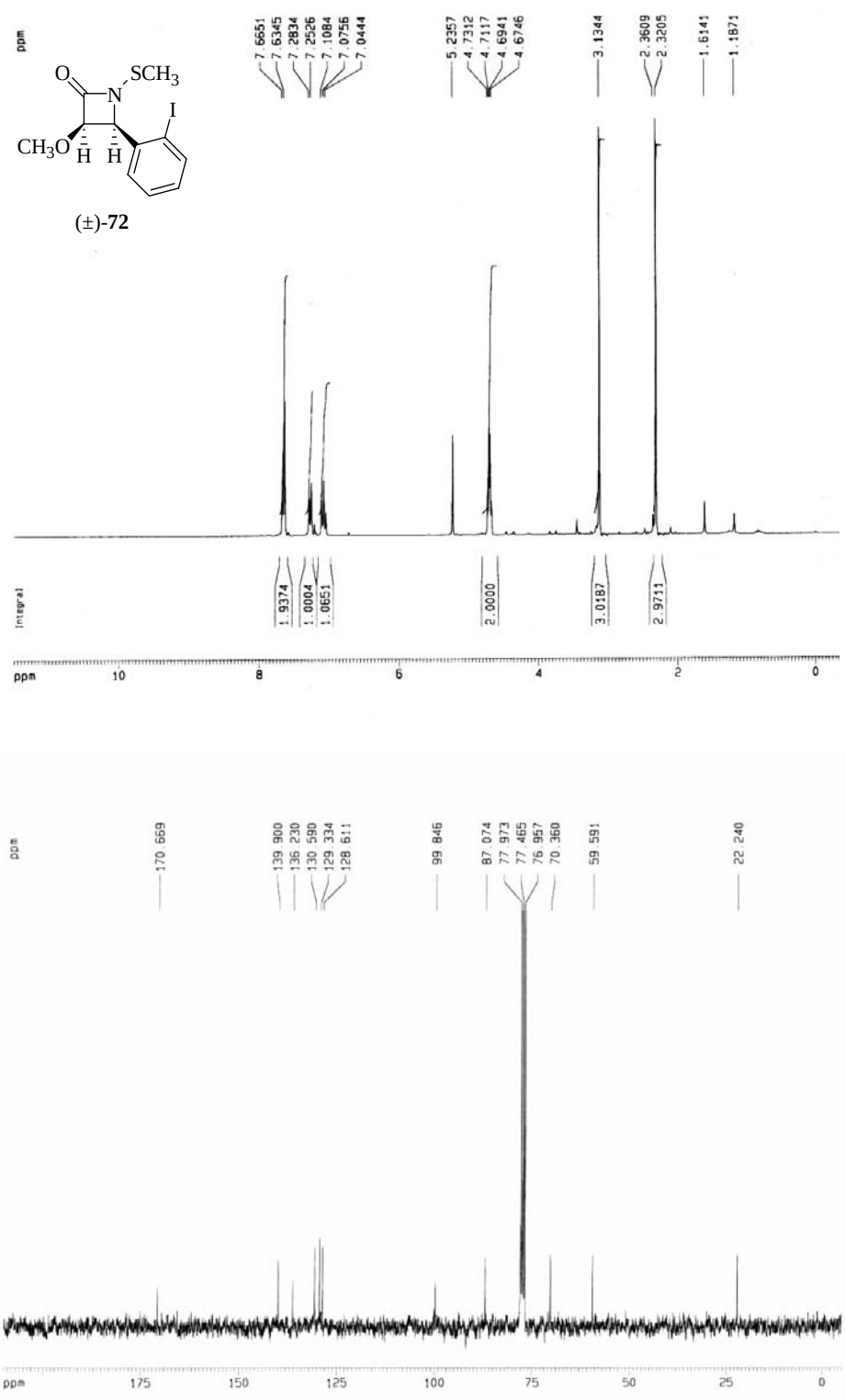
**Spectrum 7.29:** $^{1}H$ NMR (250 MHz, $CDCl_3$) and $^{13}C$ NMR (63 MHz, $CDCl_3$) of β-lactam **70**.



**Spectrum 2.30:** $^{1}H$ NMR (250 MHz, $CDCl_3$) and $^{13}C$ NMR (63 MHz, $CDCl_3$) of β-lactam **71**.

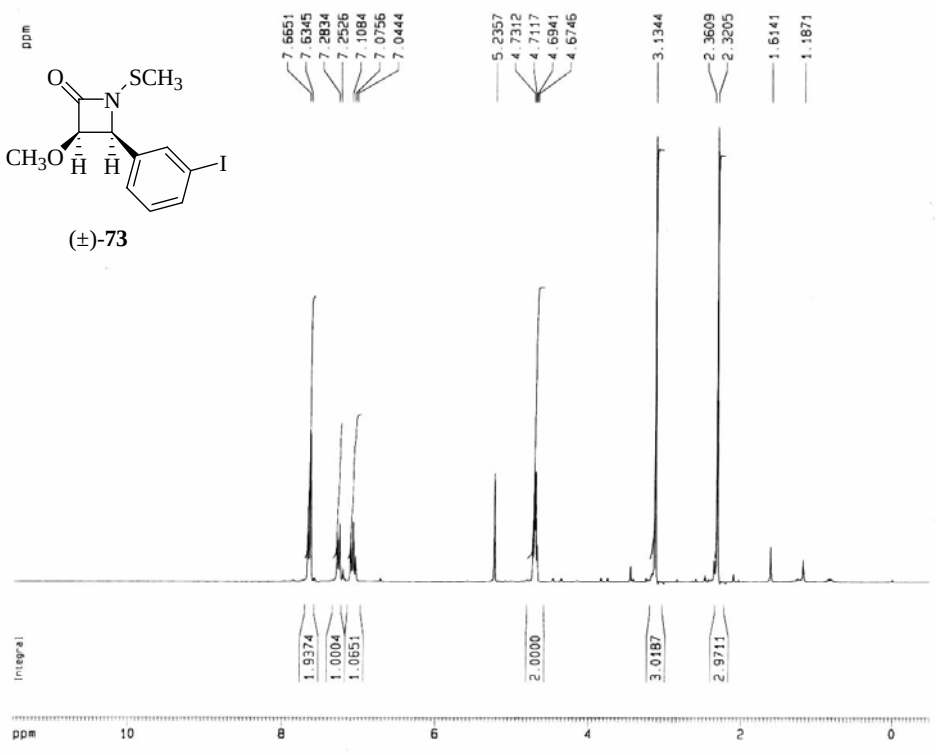


**Spectrum 7.31:** $^{1}H$ NMR (250 MHz, $CDCl_3$) and $^{13}C$ NMR (63 MHz, $CDCl_3$) of β-lactam **72**.

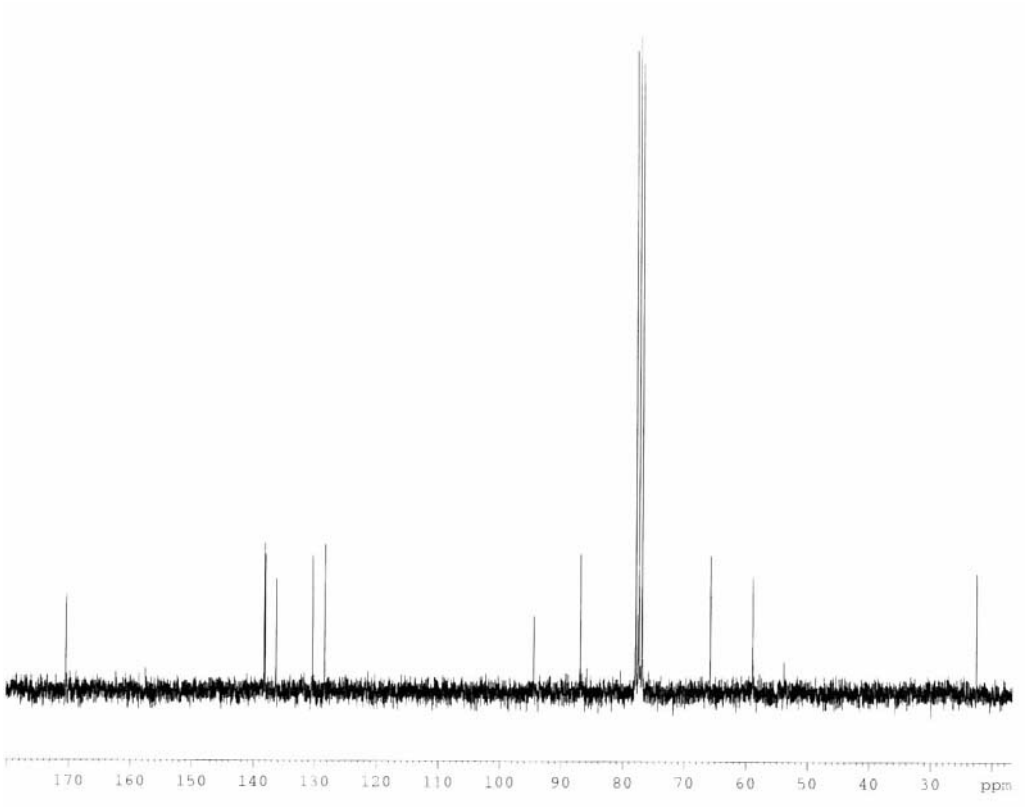



(±)-**72**

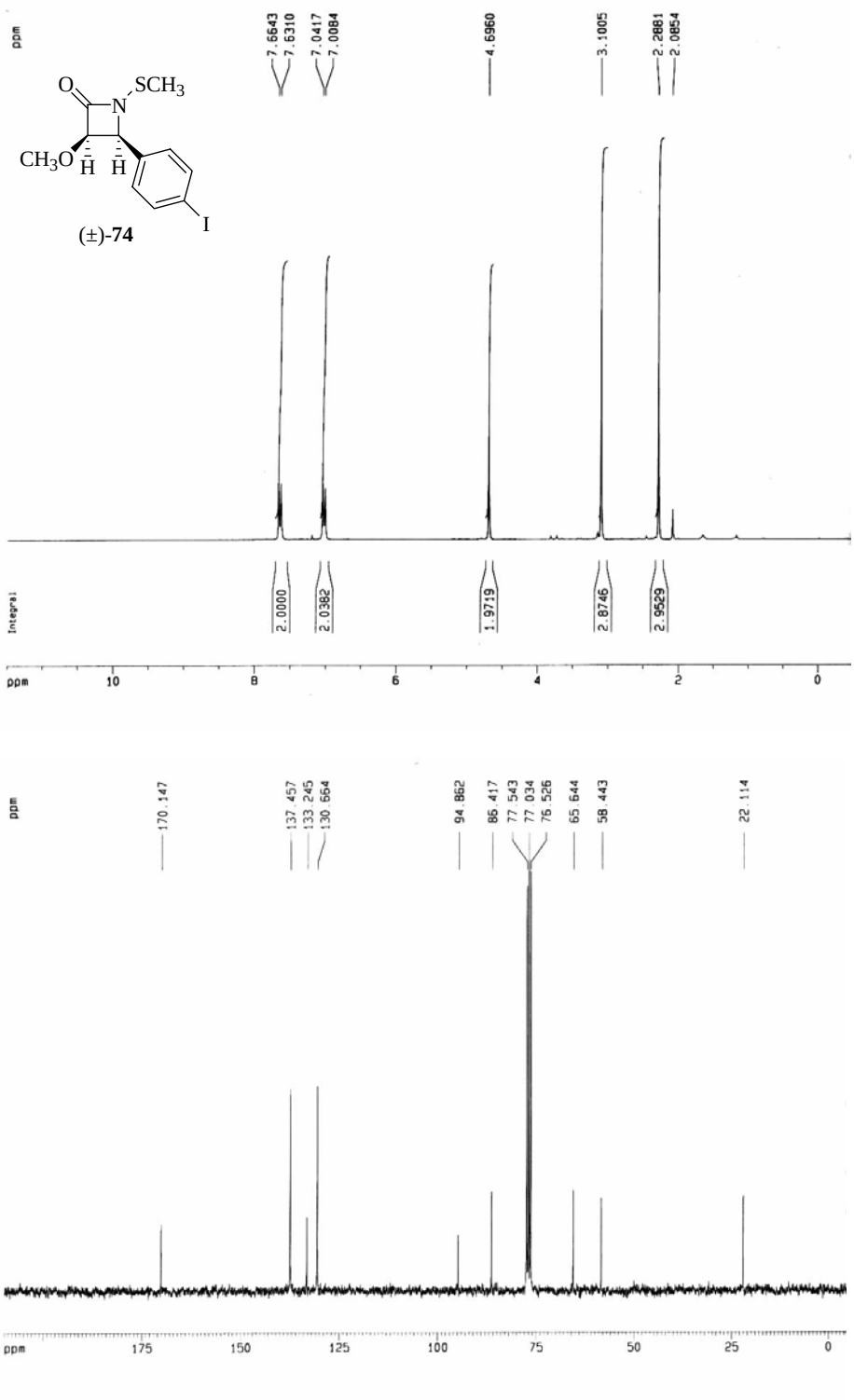
**Spectrum 7.32:** $^{1}H$ NMR (250 MHz, $CDCl_3$) and $^{13}C$ NMR (63 MHz, $CDCl_3$) of β-lactam **73**.



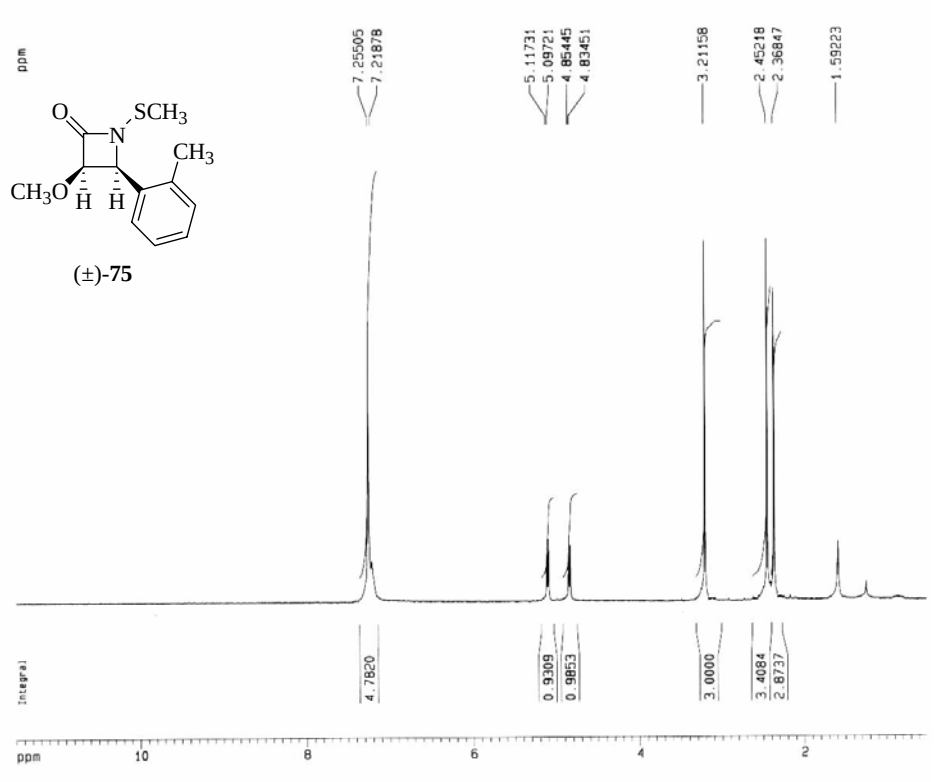

(±)-**73**



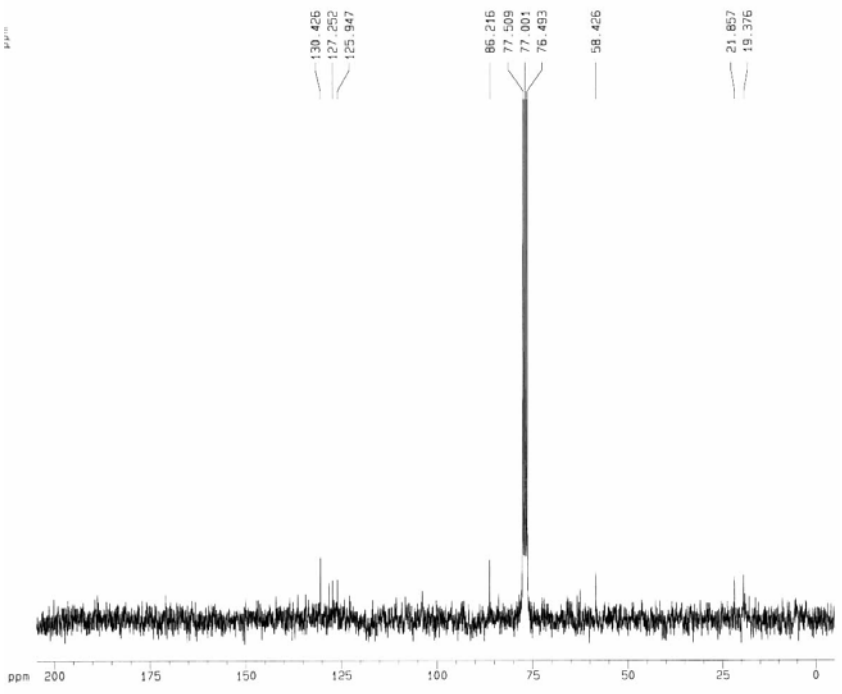
**Spectrum 7.33:** $^{1}H$ NMR (250 MHz, $CDCl_3$) and $^{13}C$ NMR (63 MHz, $CDCl_3$) of β-lactam **74**.



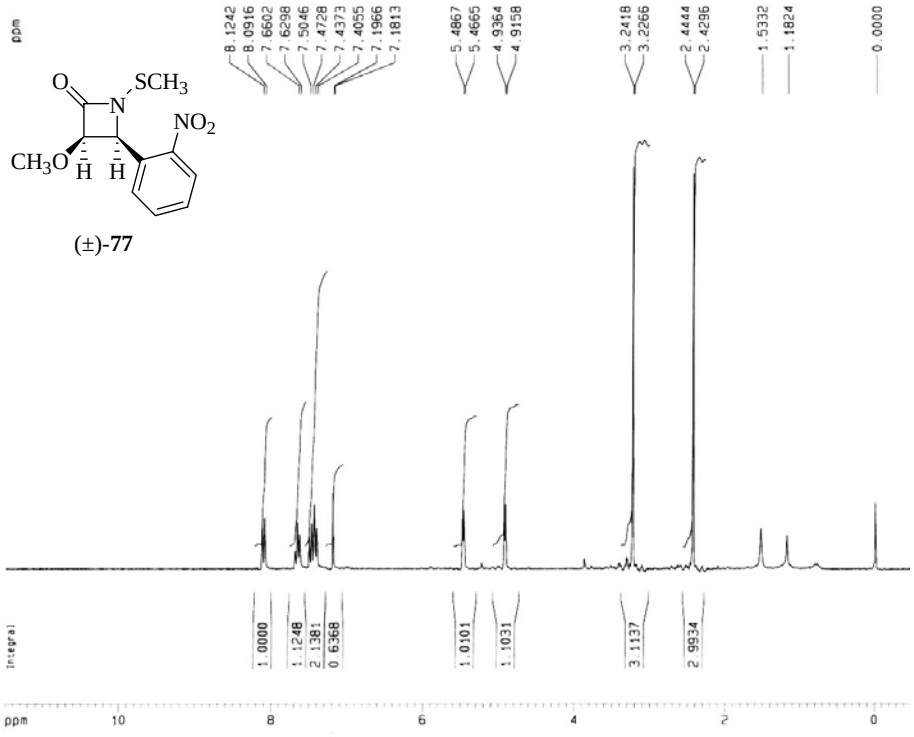
**Spectrum 7.34:** $^{1}H$ NMR (250 MHz, $CDCl_3$) and $^{13}C$ NMR (63 MHz, $CDCl_3$) of β-lactam **75**.



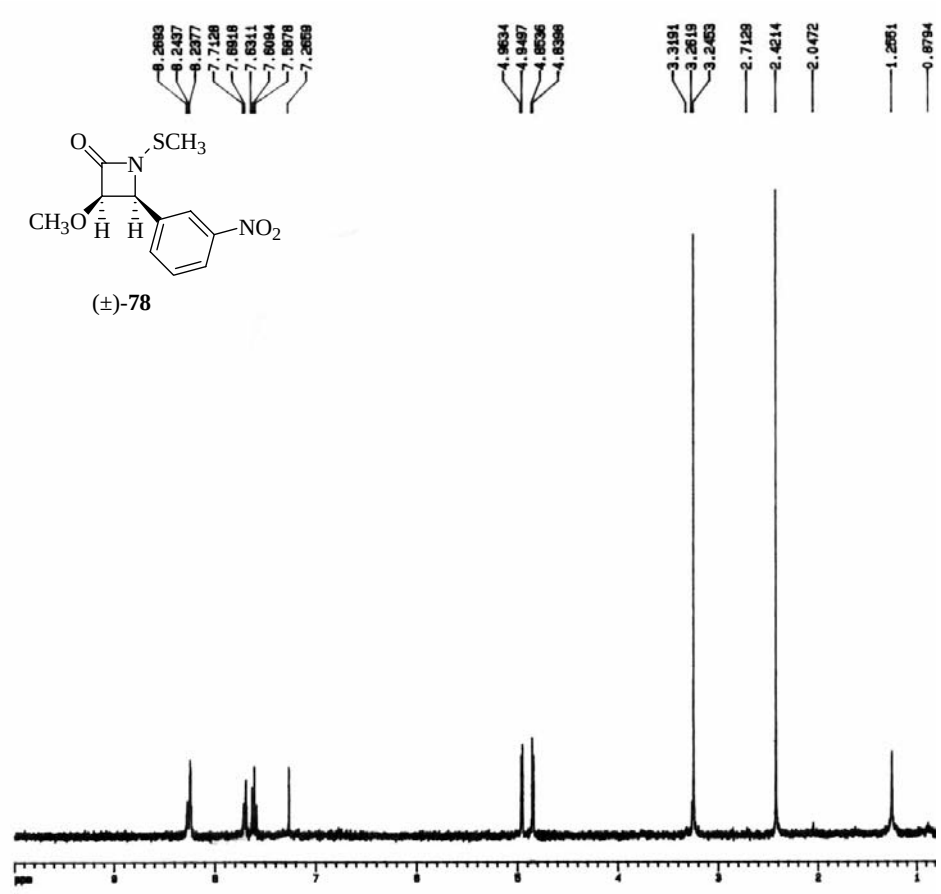

(±)-**75**



**Spectrum 7.36:** $^{1}H$ NMR (250 MHz, $CDCl_3$) of β-lactam **77**.

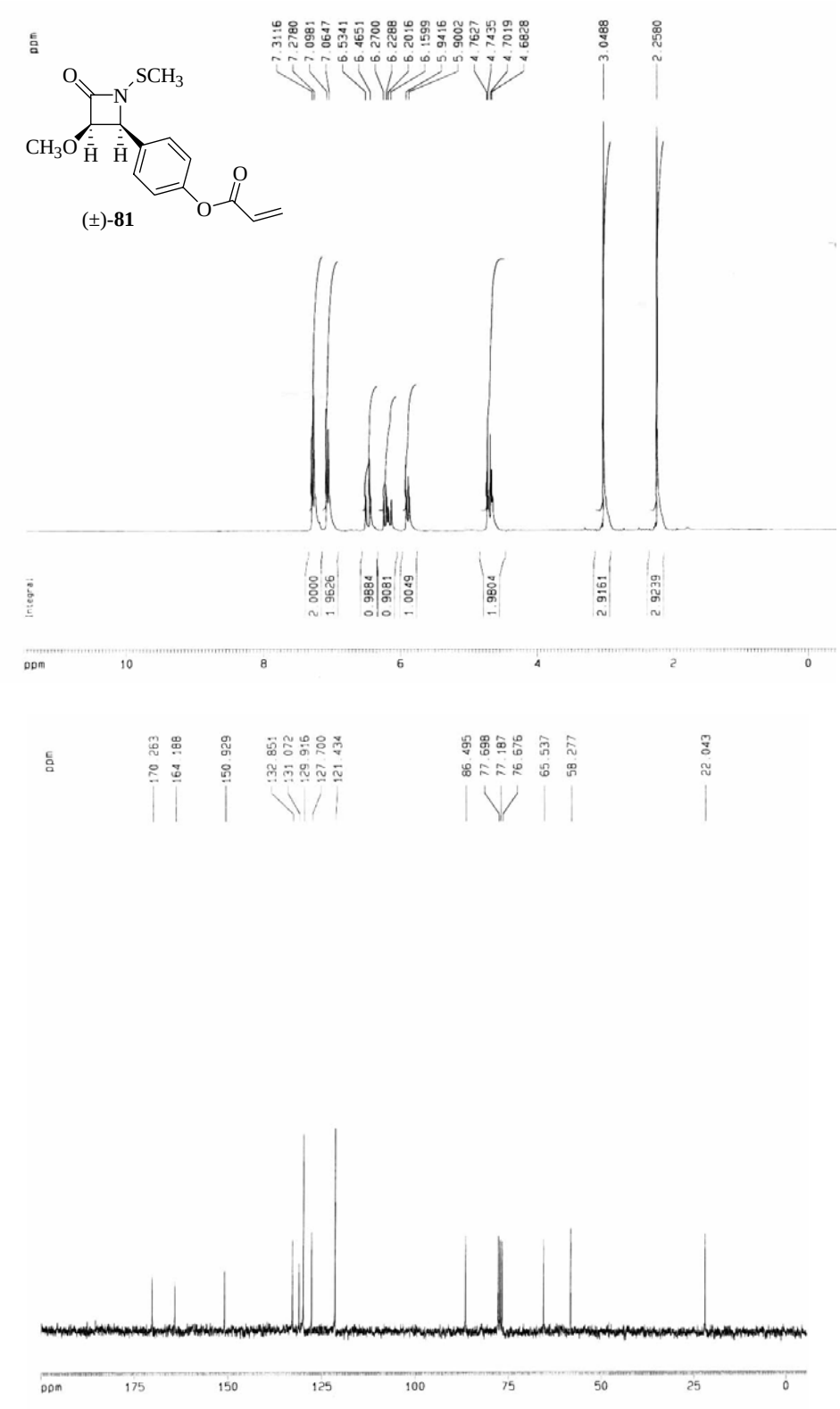


**Spectrum 7.37:** $^{1}H$ NMR (360 MHz, $CDCl_3$) of β-lactam **78**.

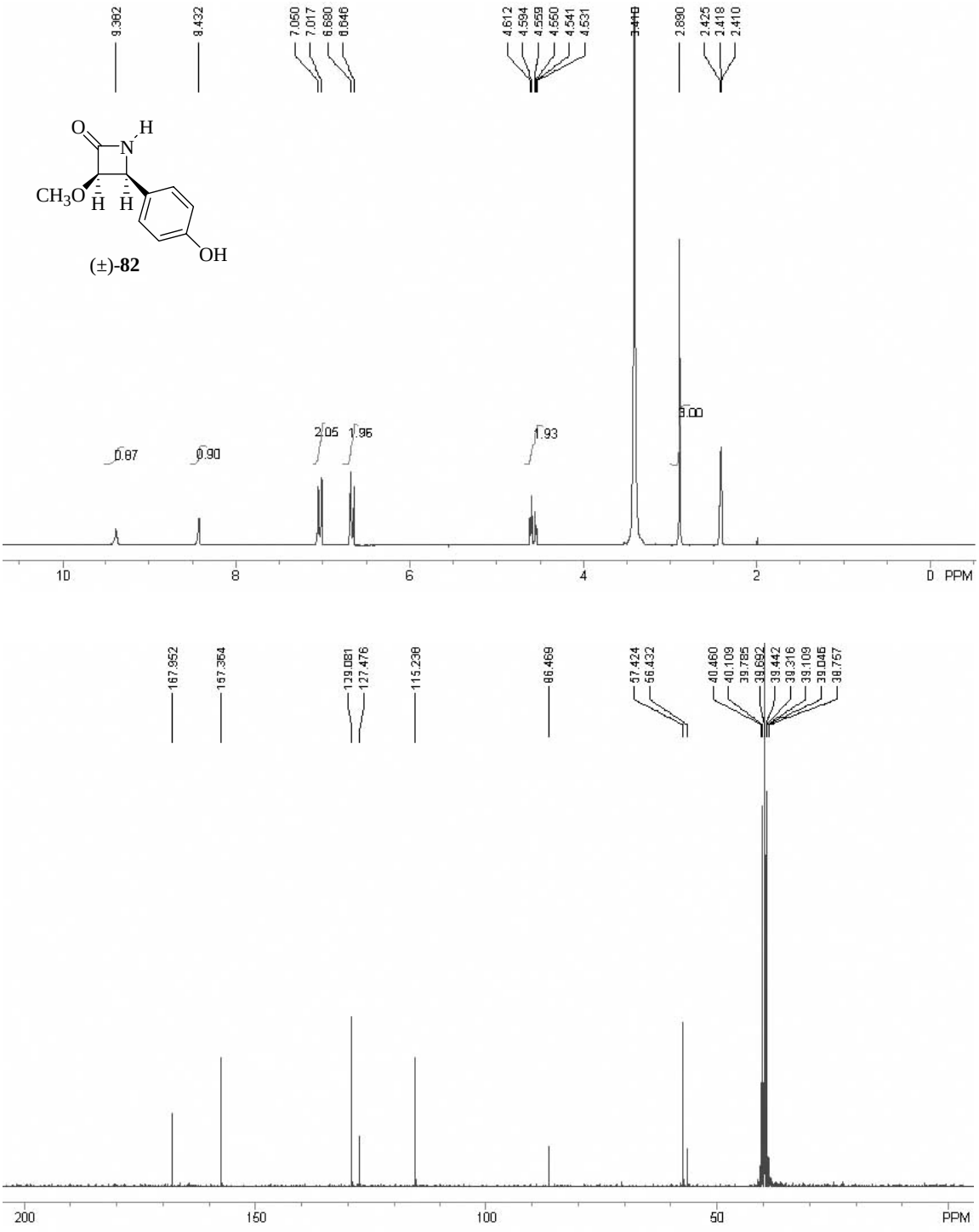



(±)-**78**

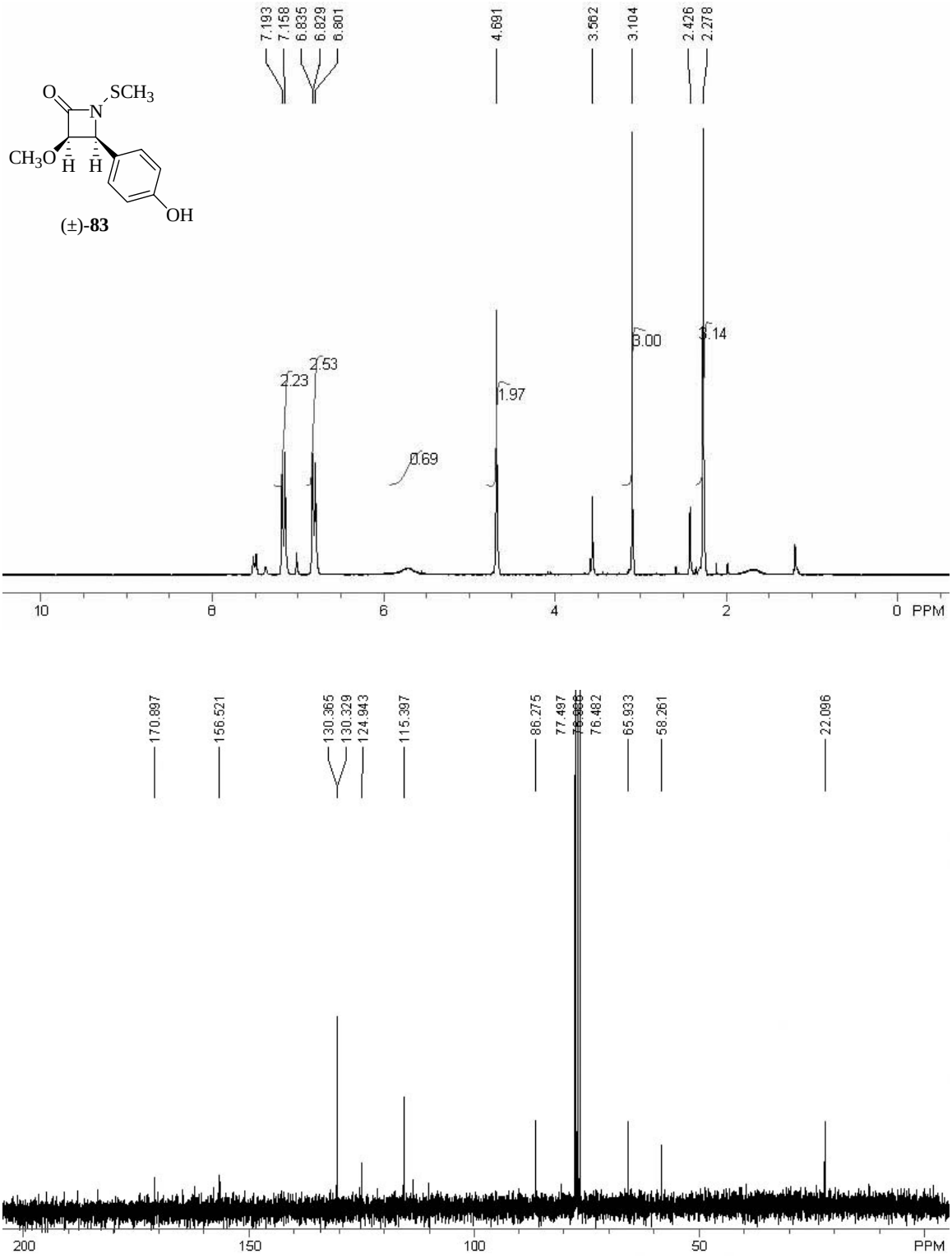
**Spectrum 7.38:** $^{1}H$ NMR (250 MHz, $CDCl_3$) and $^{13}C$ NMR (63 MHz, $CDCl_3$) of β-lactam **81**.



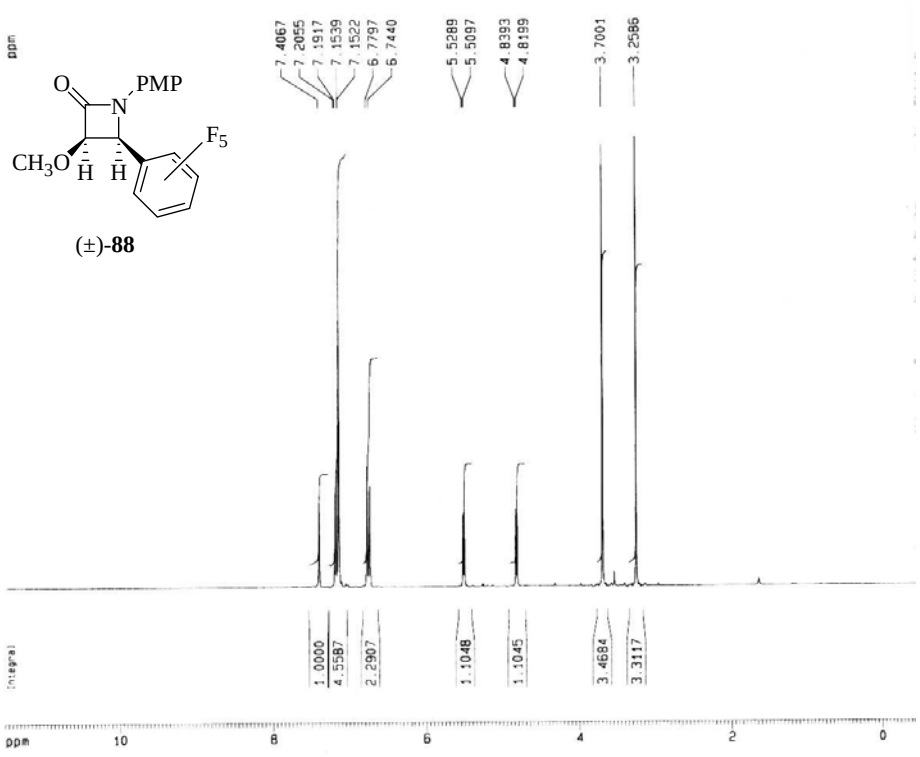
**Spectrum 7.39:** $^{1}H$ NMR (250 MHz, $CDCl_3$) and $^{13}C$ NMR (63 MHz, $CDCl_3$) of β-lactam **82**.



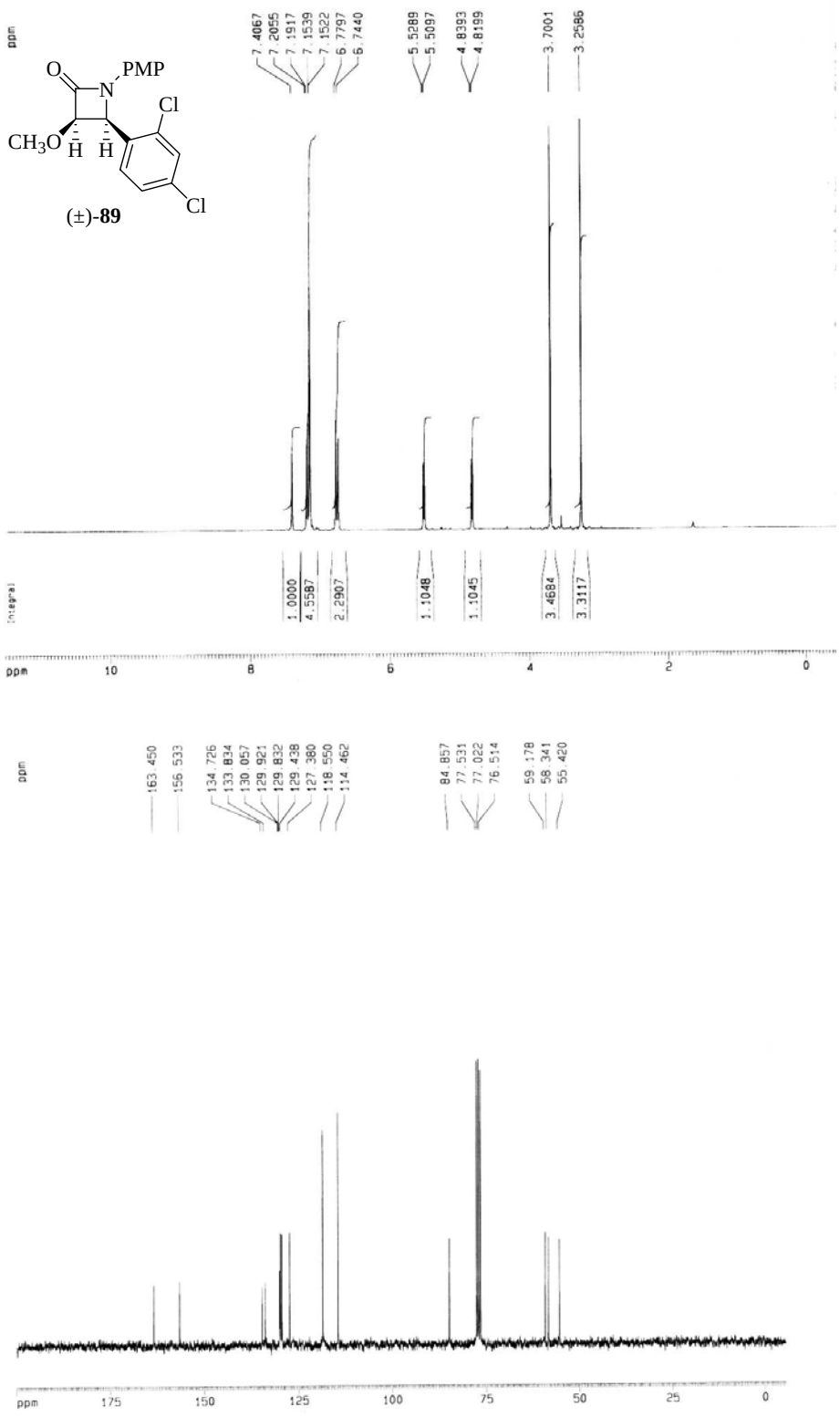
**Spectrum 7.40:** $^{1}H$ NMR (250 MHz, $CDCl_3$) and $^{13}C$ NMR (63 MHz, $CDCl_3$) of β-lactam **83**.



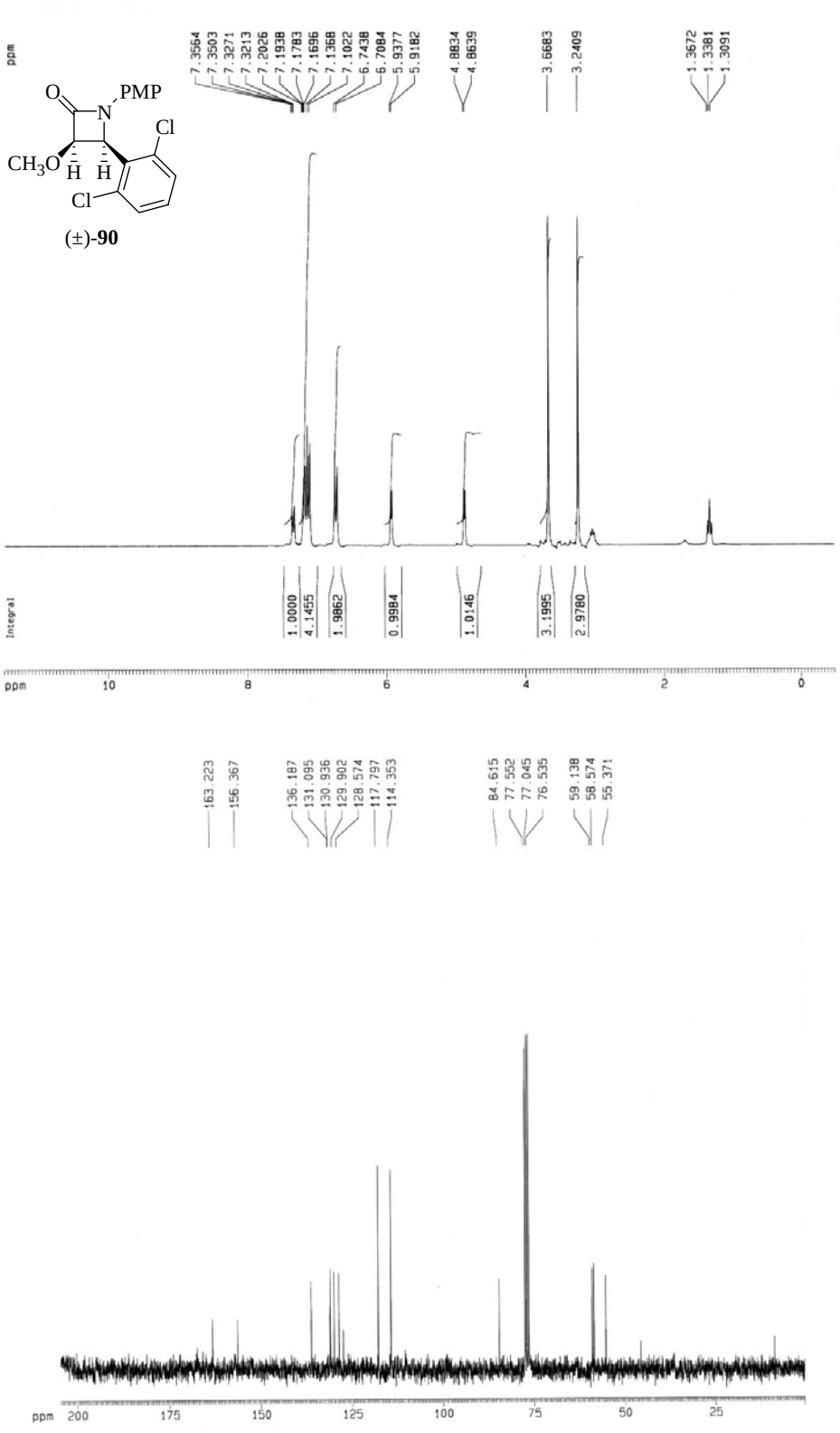
**Spectrum 7.41:** $^{1}H$ NMR (250 MHz, $CDCl_3$) of β-lactam **88**.



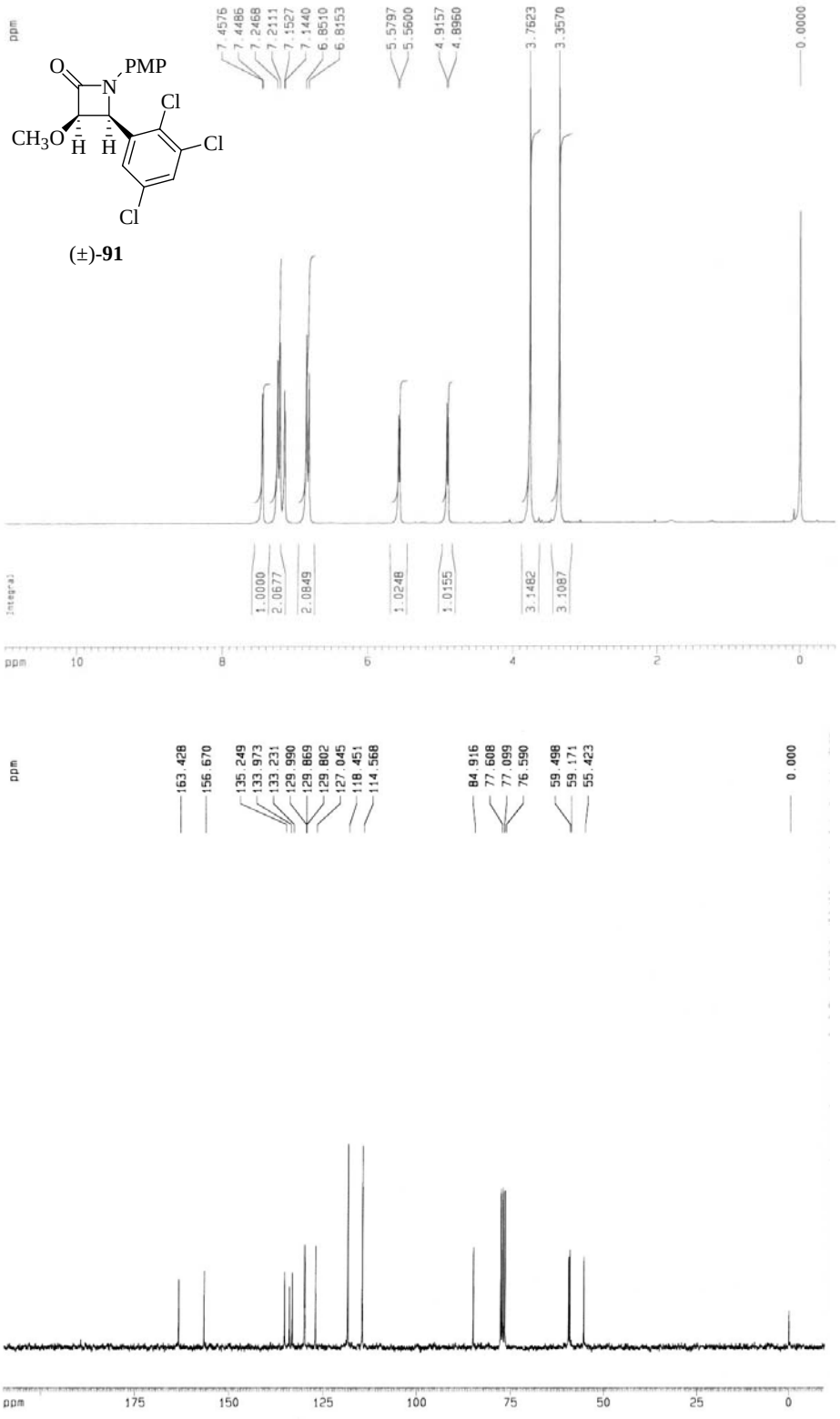
**Spectrum 7.42:** $^{1}H$ NMR (250 MHz, $CDCl_3$) and $^{13}C$ NMR (63 MHz, $CDCl_3$) of β-lactam **89**.



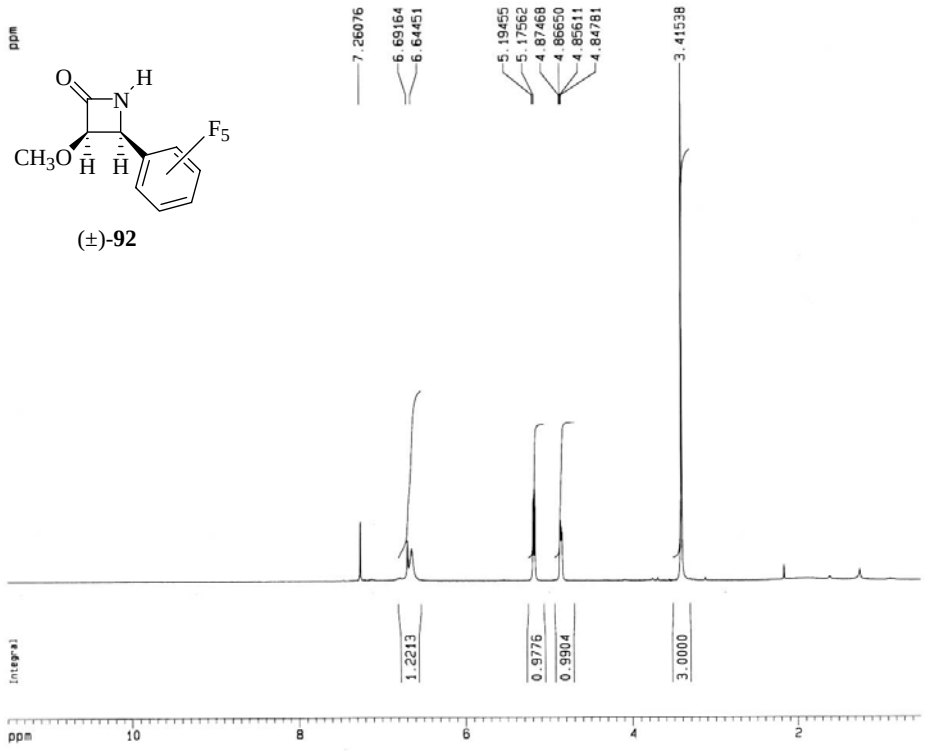
**Spectrum 7.43:** $^{1}H$ NMR (250 MHz, $CDCl_3$) and $^{13}C$ NMR (63 MHz, $CDCl_3$) of β-lactam **90**.



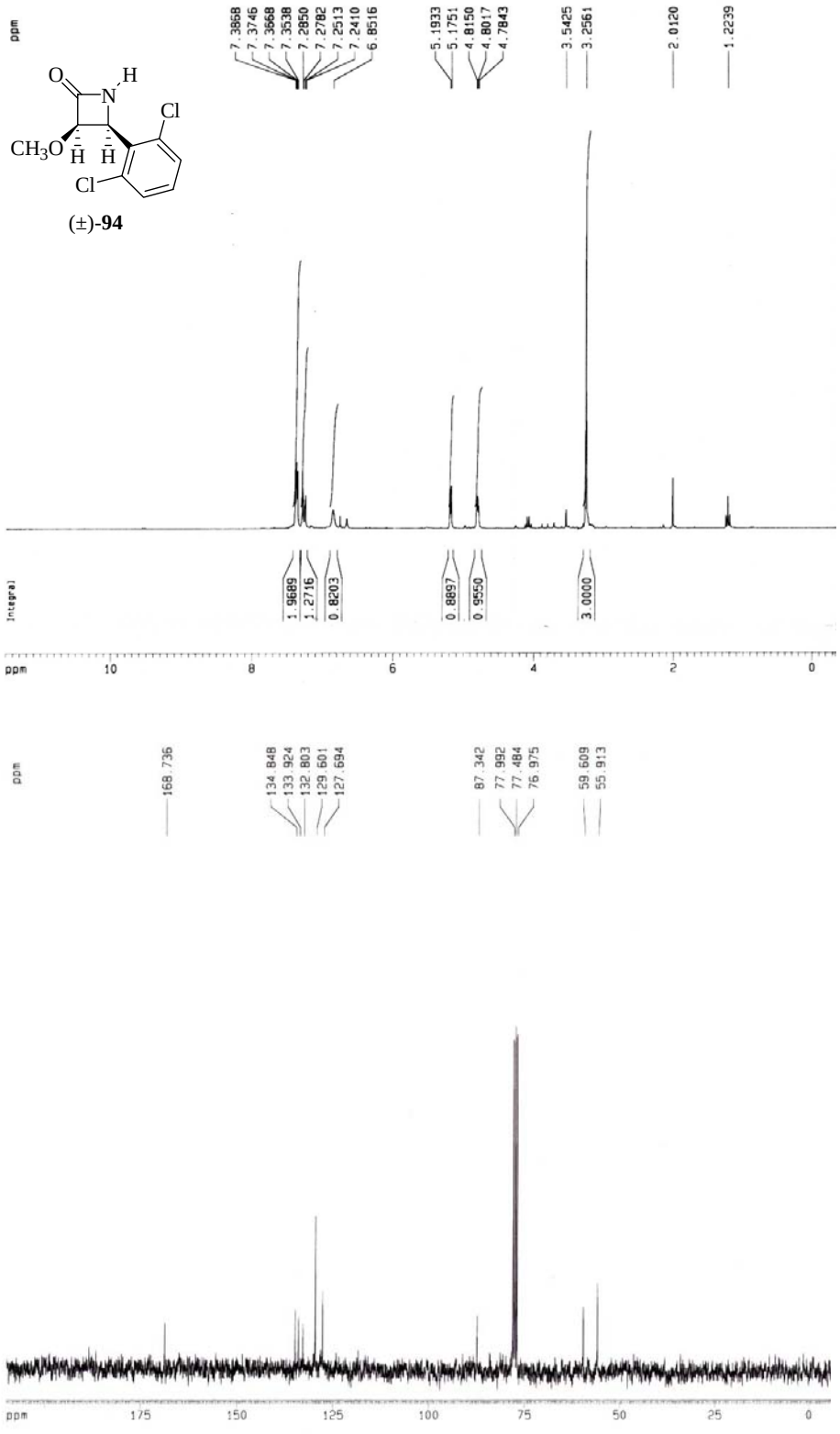
**Spectrum 7.44:** $^{1}H$ NMR (250 MHz, $CDCl_3$) and $^{13}C$ NMR (63 MHz, $CDCl_3$) of β-lactam **91**.



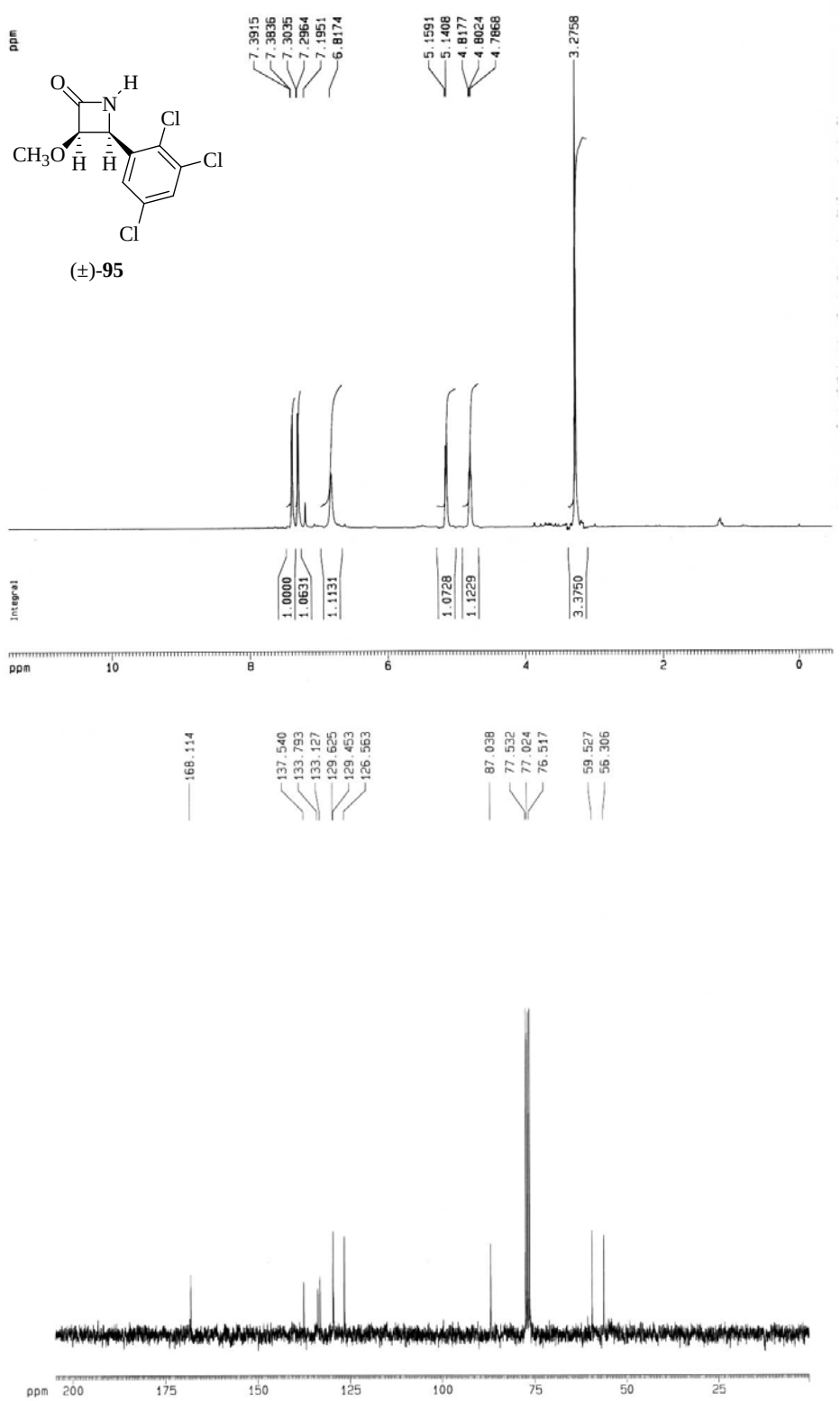
**Spectrum 7.45:** $^{1}H$ NMR (250 MHz, $CDCl_3$) of β-lactam **92**.



**Spectrum 7.46:** $^{1}H$ NMR (250 MHz, $CDCl_3$) and $^{13}C$ NMR (63 MHz, $CDCl_3$) of β-lactam **94**.

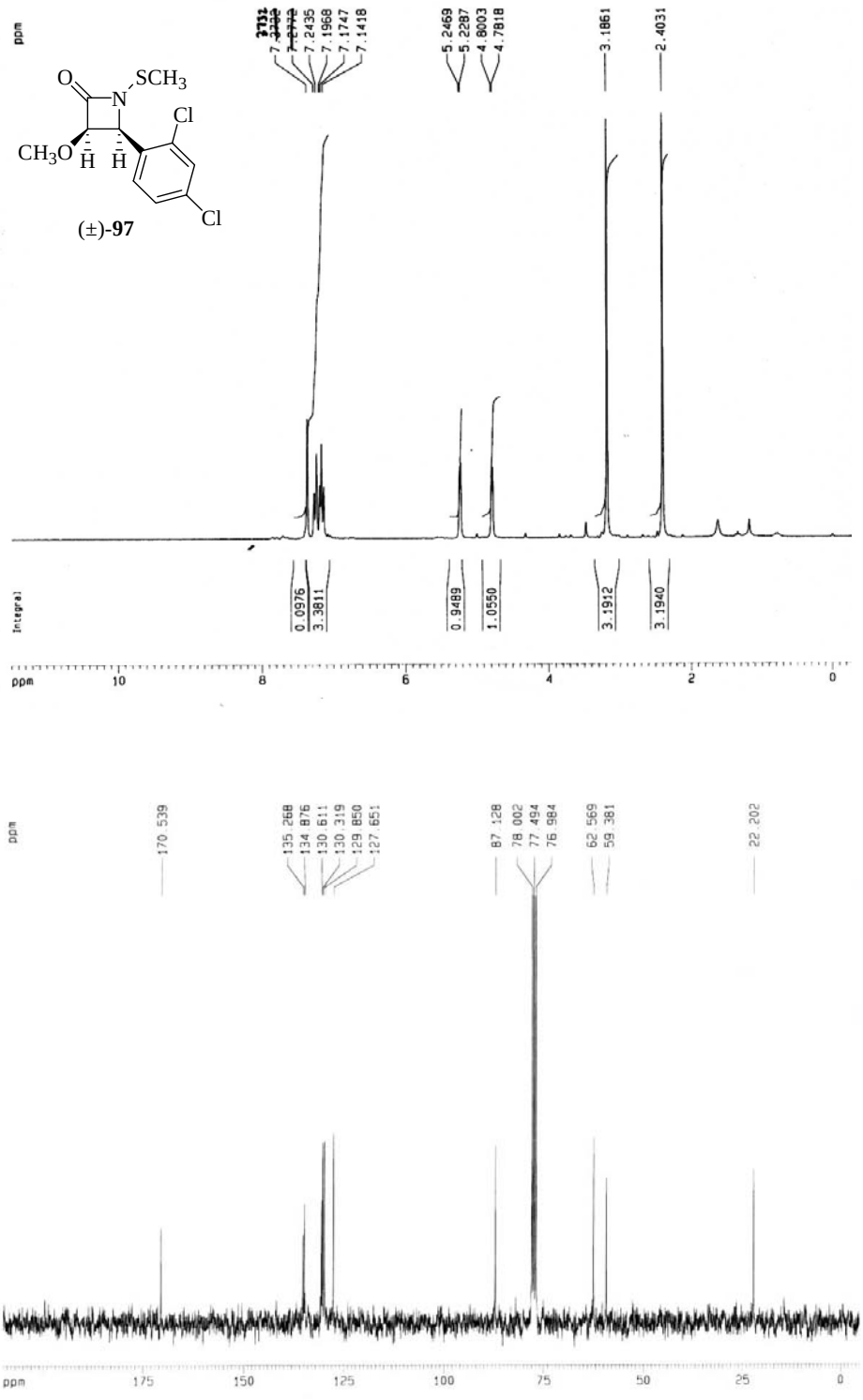


**Spectrum 7.47:** $^{1}H$ NMR (250 MHz, $CDCl_3$) and $^{13}C$ NMR (63 MHz, $CDCl_3$) of β-lactam **95**.

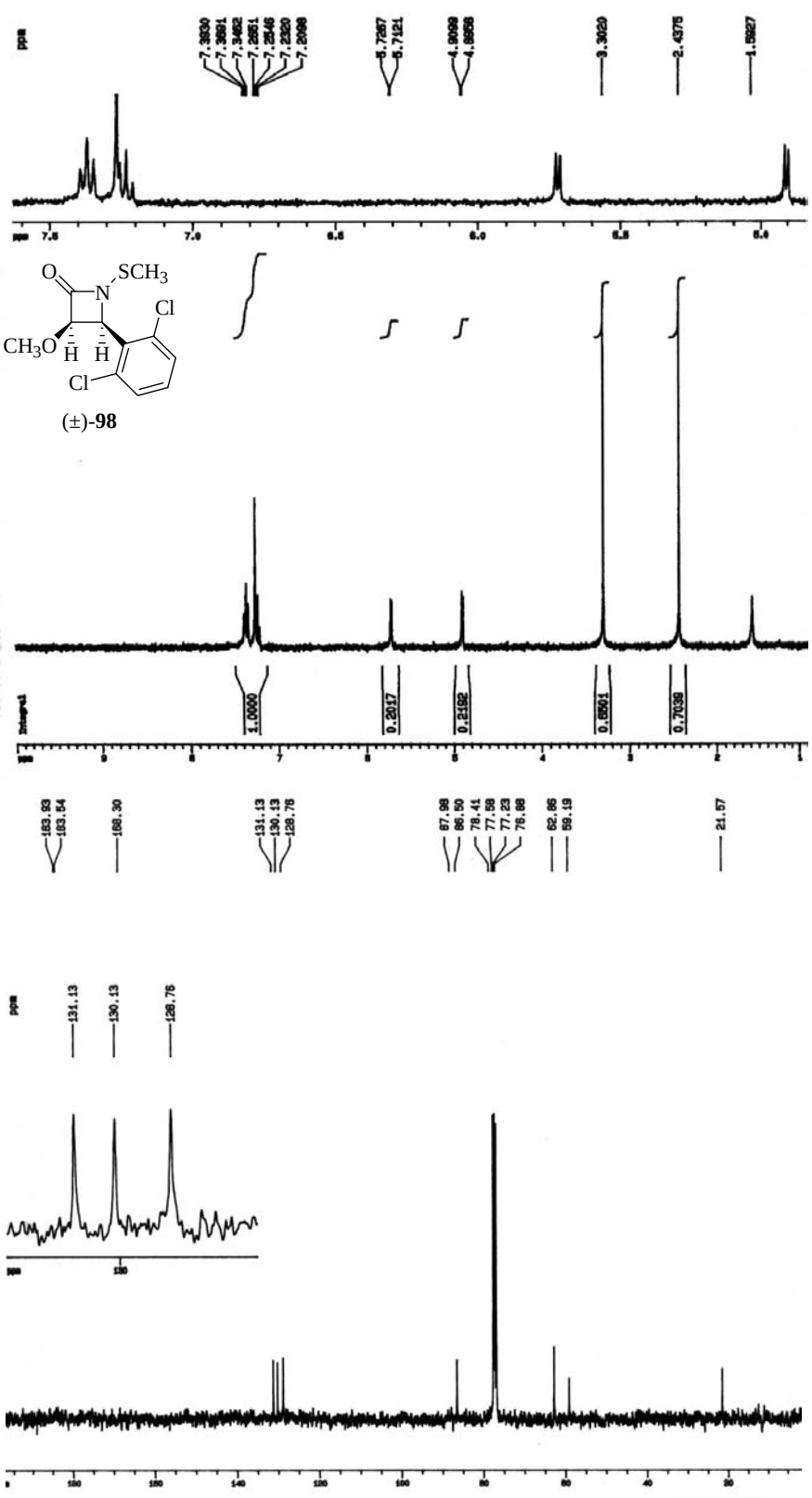



(±)-**95**

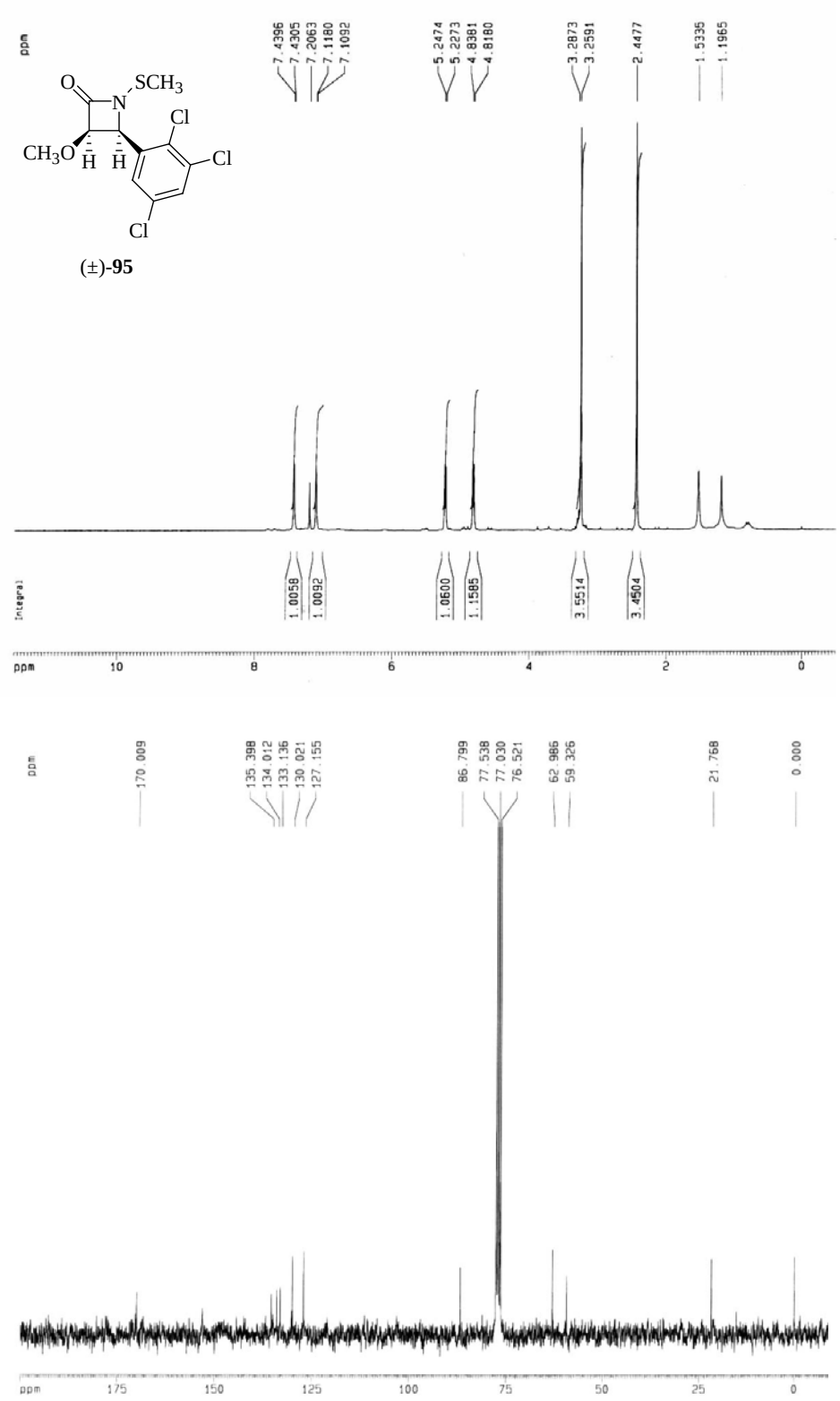
**Spectrum 7.48:** $^{1}H$ NMR (250 MHz, $CDCl_3$) and $^{13}C$ NMR (63 MHz, $CDCl_3$) of β-lactam **97**.



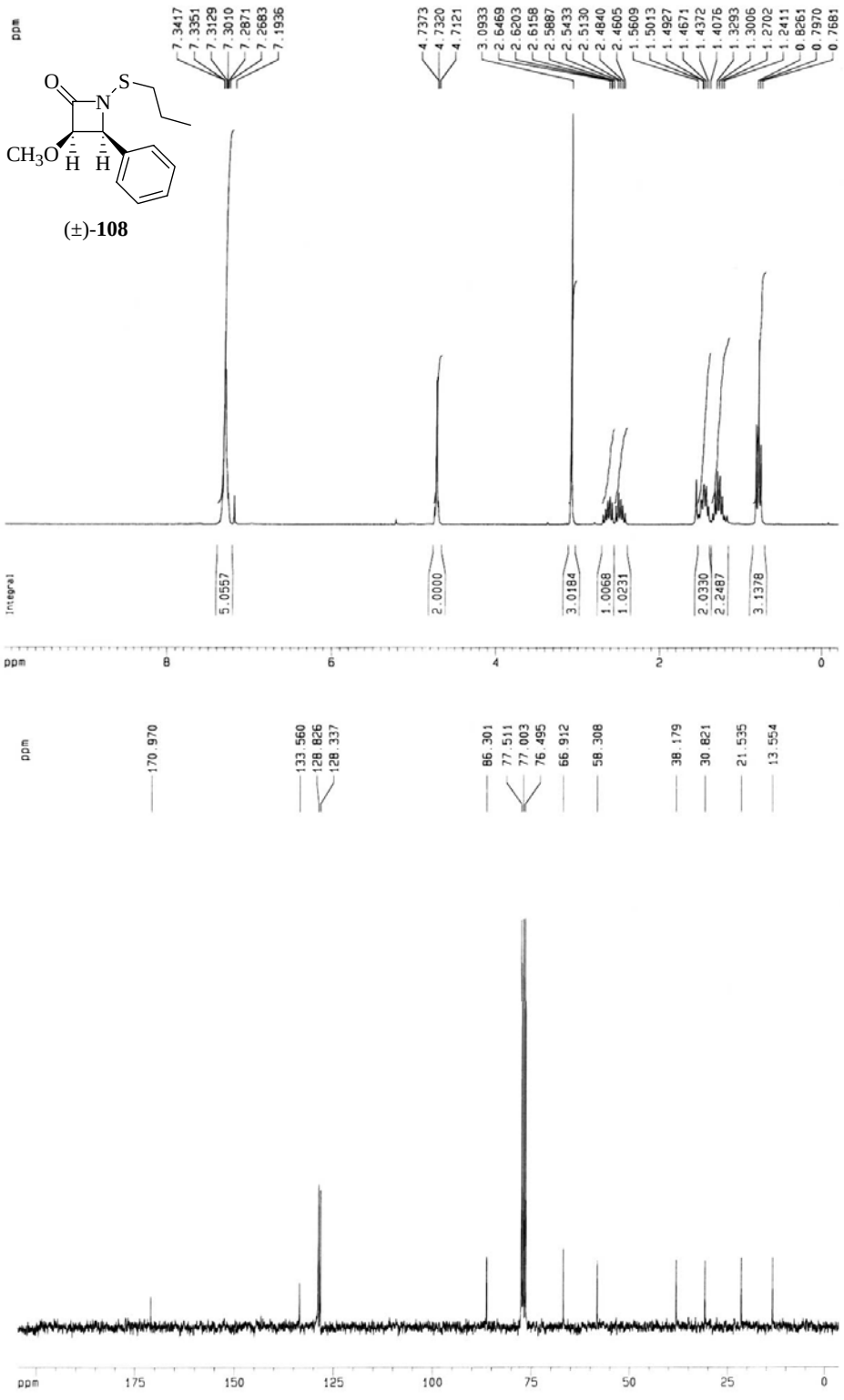
**Spectrum 7.49:** $^{1}H$ NMR (360 MHz, $CDCl_3$) and $^{13}C$ NMR (90 MHz, $CDCl_3$) of β-lactam **98**.



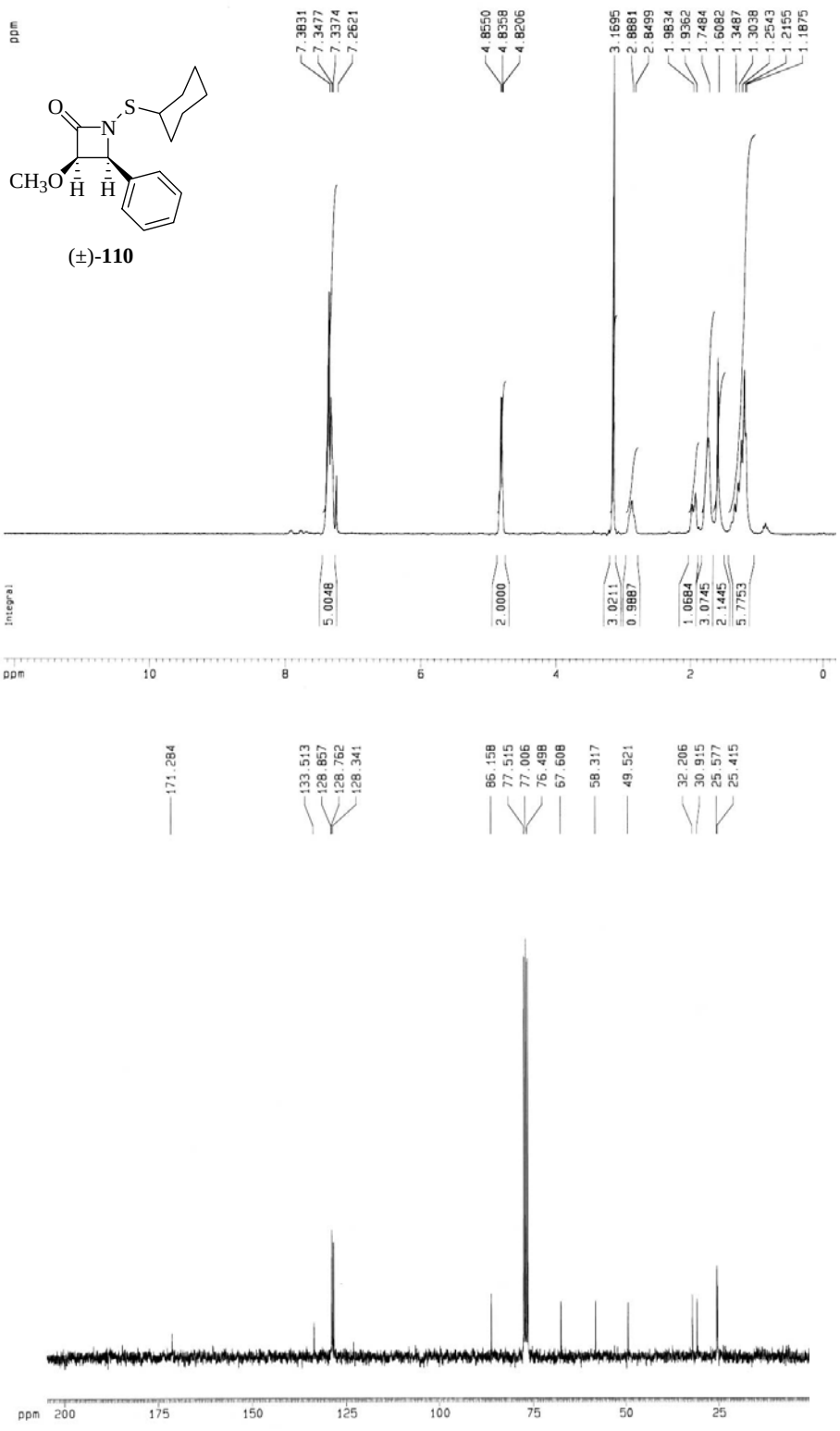
**Spectrum 7.50:** $^{1}H$ NMR (250 MHz, $CDCl_3$) and $^{13}C$ NMR (63 MHz, $CDCl_3$) of β-lactam **99**.



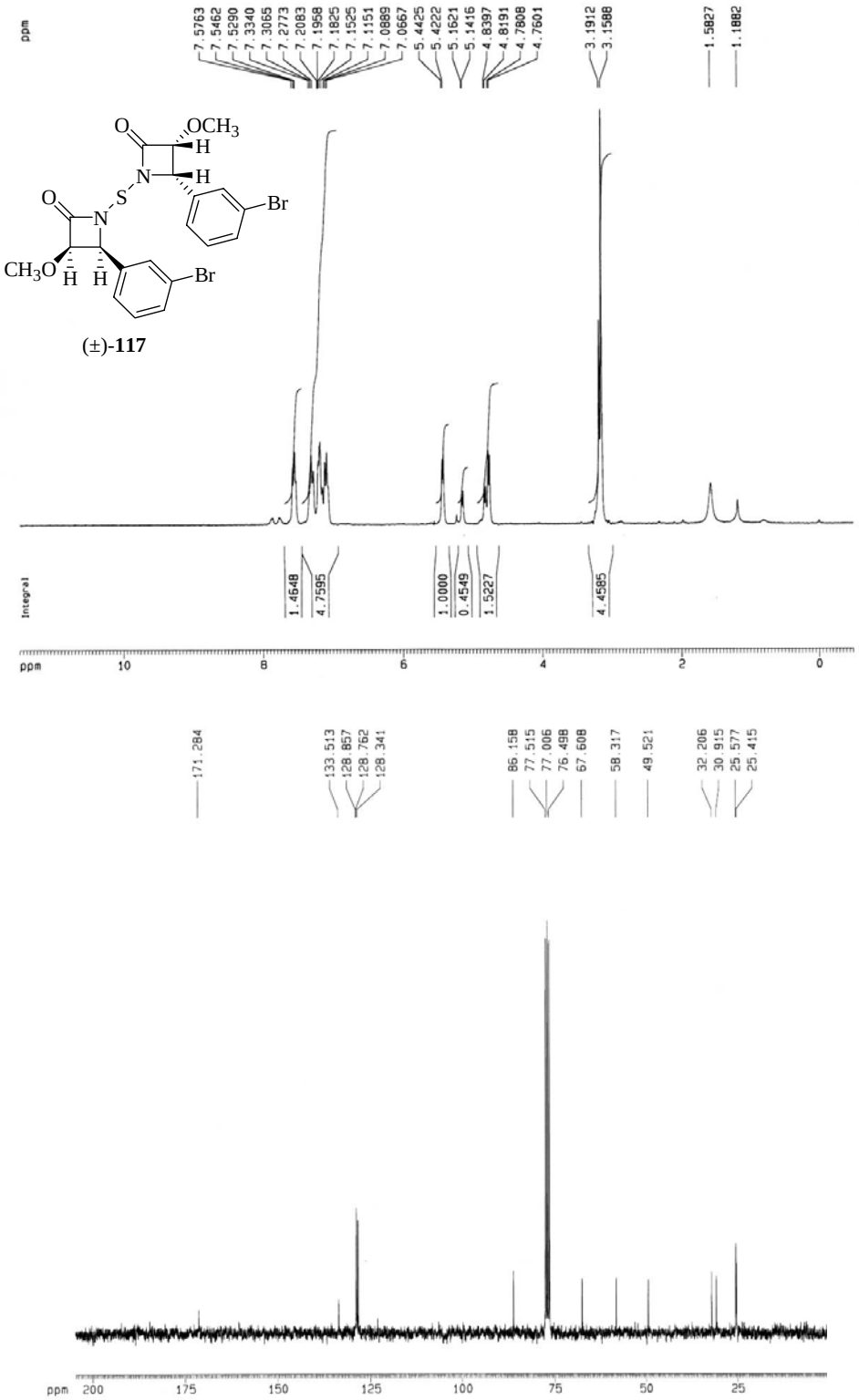
**Spectrum 7.51:** $^{1}H$ NMR (250 MHz, $CDCl_3$) and $^{13}C$ NMR (63 MHz, $CDCl_3$) of β-lactam **108**.



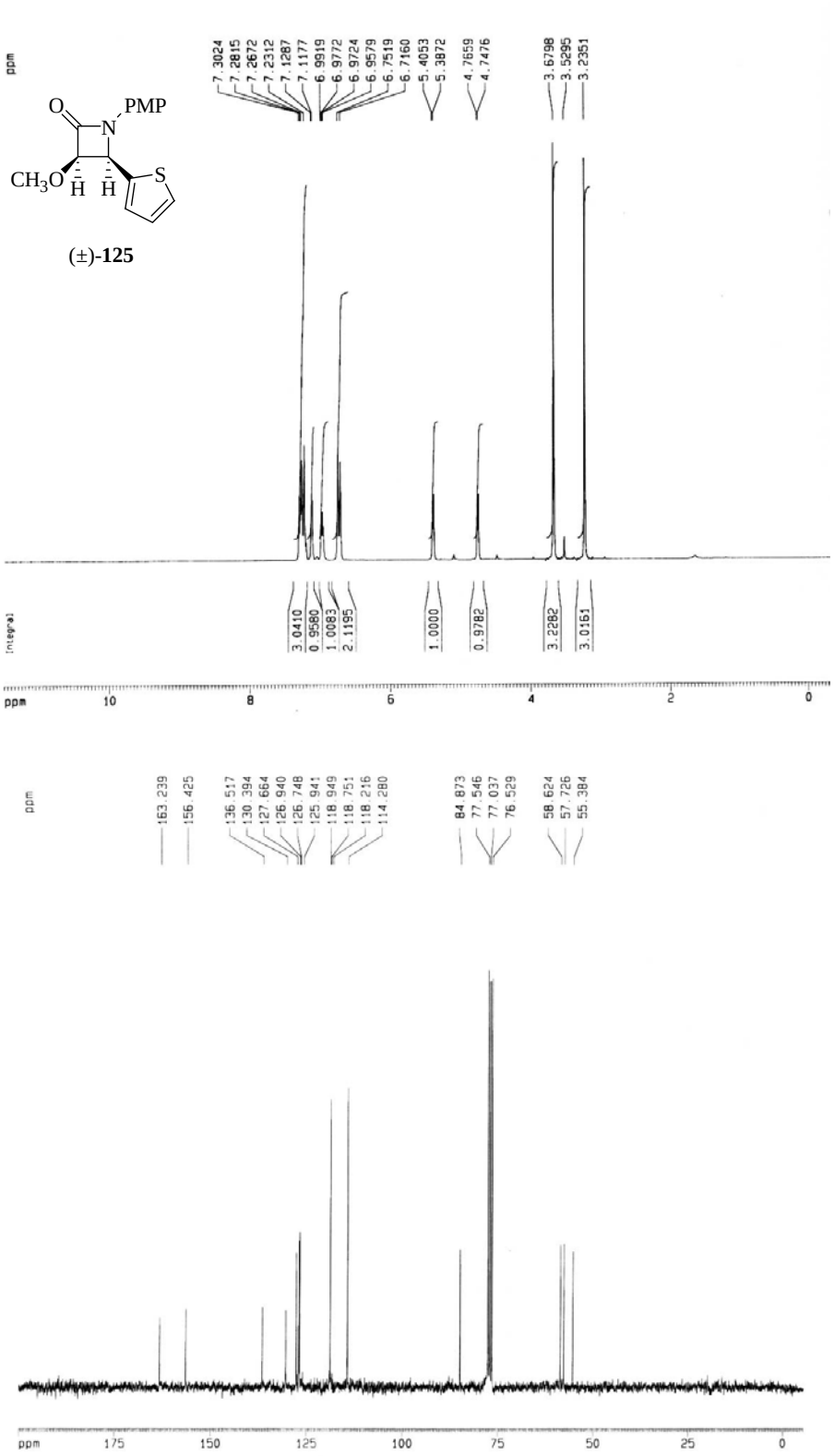
**Spectrum 7.52:** $^{1}H$ NMR (250 MHz, $CDCl_3$) and $^{13}C$ NMR (63 MHz, $CDCl_3$) of β-lactam **110**.



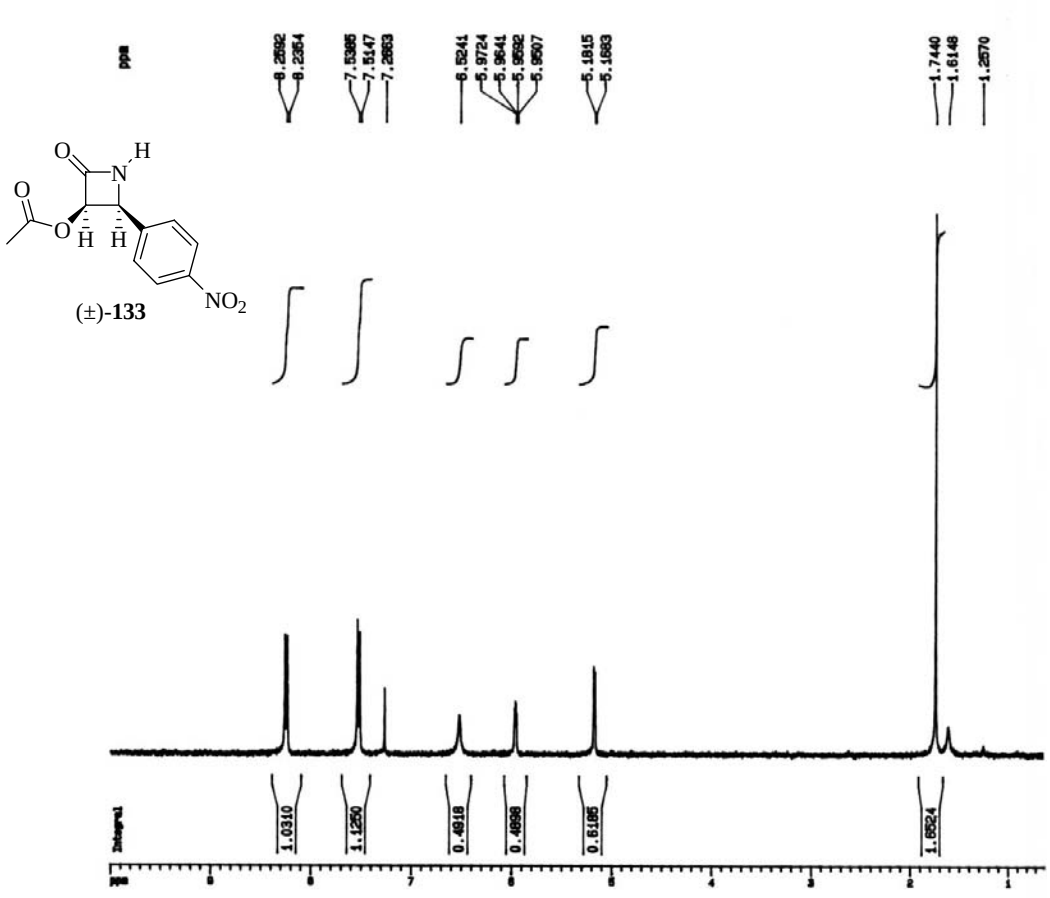
**Spectrum 7.53:** $^{1}H$ NMR (250 MHz, $CDCl_3$) and $^{13}C$ NMR (63 MHz, $CDCl_3$) of β-lactam **117**.



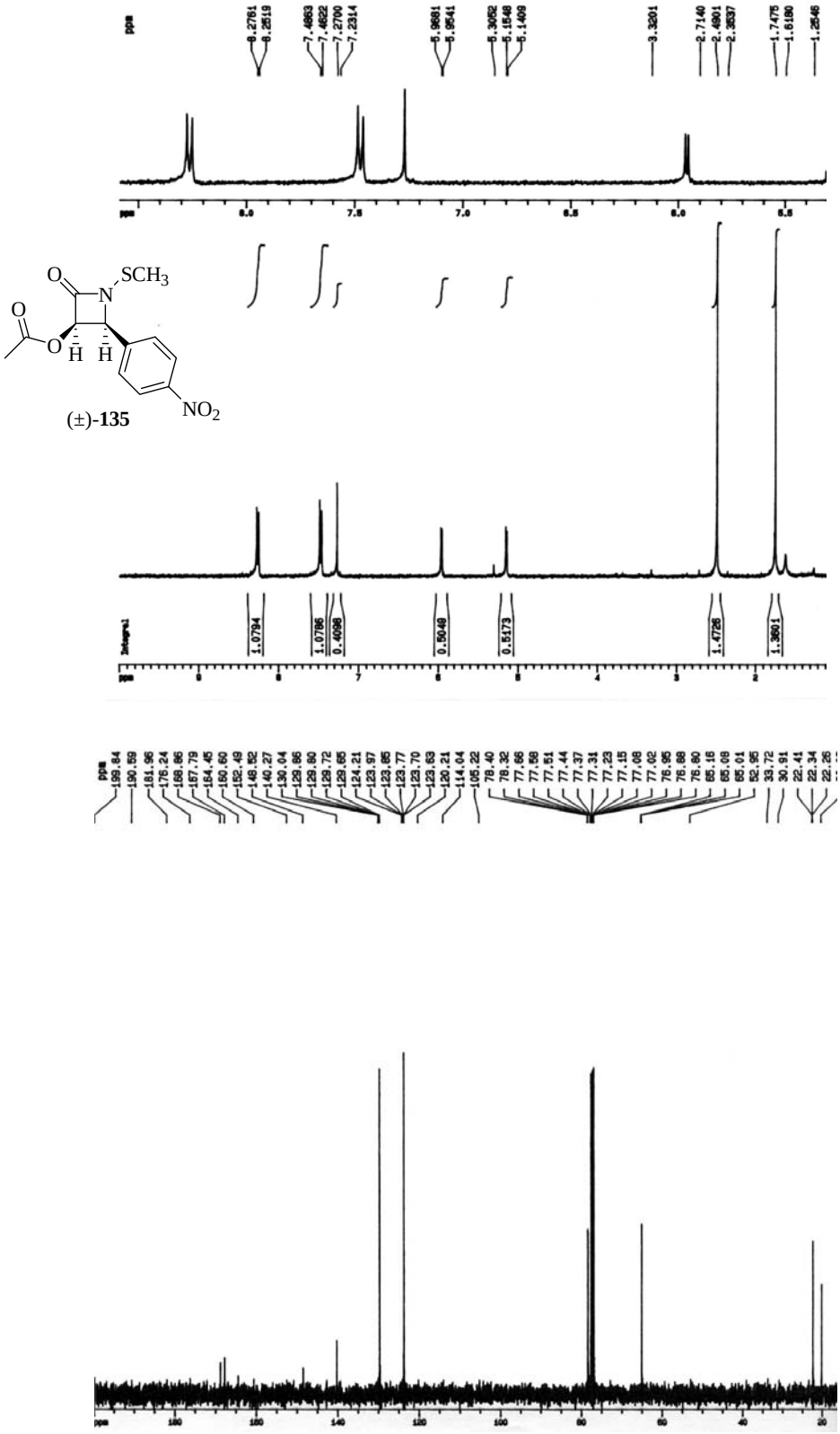
**Spectrum 7.54:** $^{1}H$ NMR (250 MHz, $CDCl_3$) and $^{13}C$ NMR (63 MHz, $CDCl_3$) of β-lactam **125**.



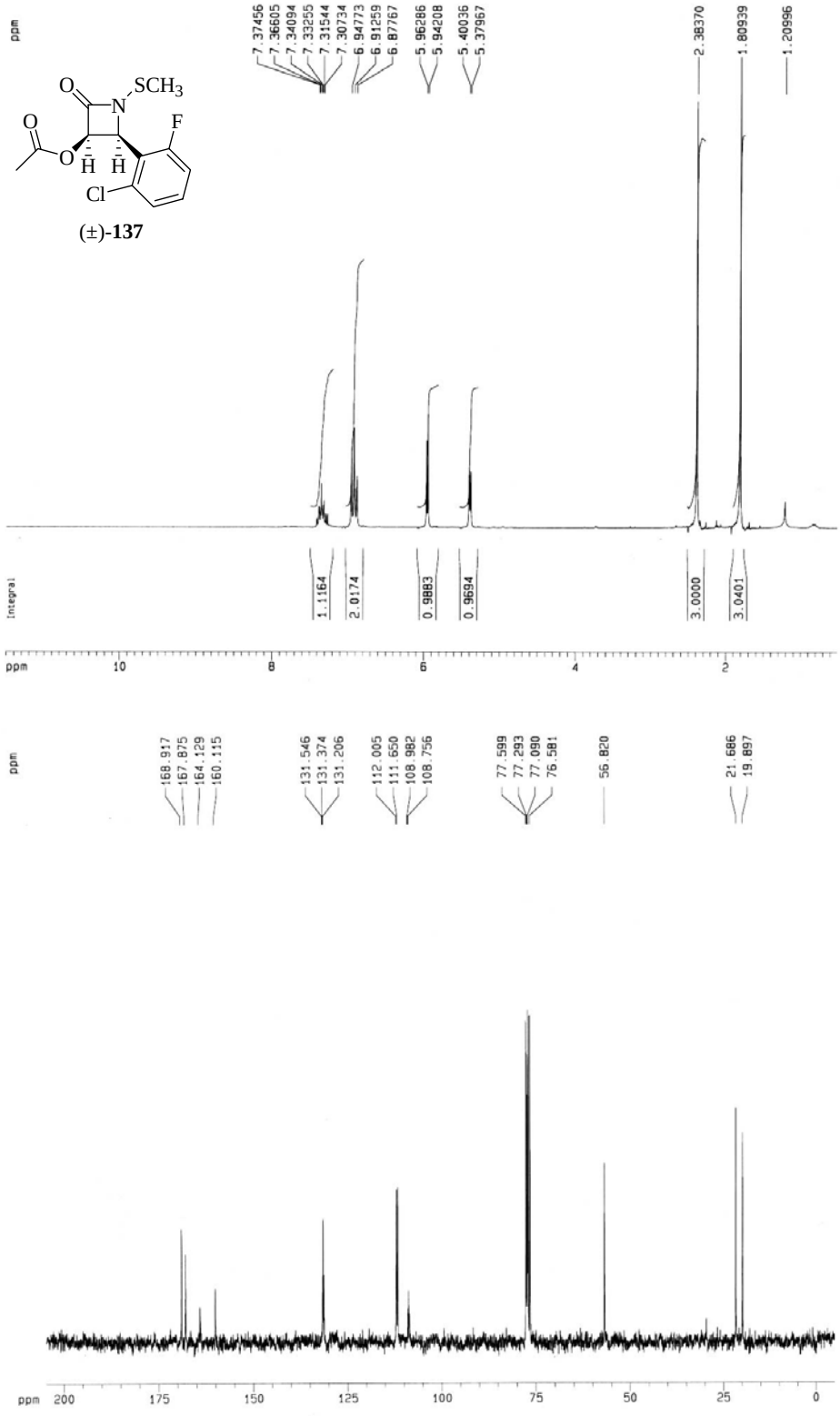
**Spectrum 7.55:** $^{1}H$ NMR (360 MHz, $CDCl_3$) of β-lactam **133**.



**Spectrum 7.56:** $^{1}H$ NMR (360 MHz, $CDCl_3$) and $^{13}C$ NMR (90 MHz, $CDCl_3$) of β-lactam **135**.



**Spectrum 7.57:** $^{1}H$ NMR (250 MHz, $CDCl_3$) and $^{13}C$ NMR (63 MHz, $CDCl_3$)of β-lactam **137**.



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Timothy Long received his bachelor's degree in biology at the University of South Florida, Tampa, FL. During his senior year, Timothy began research in the synthetic laboratory of Professor Edward Turos. Despite his interest in biology, he greatly enjoyed performing synthesis which ultimately led him to pursue a doctorate in chemistry. Currently, Timothy continues to conduct research where his interest lie in solving problems at the interface of biology and chemistry.