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Synthesis and Evaluation of 1,2,4-oxadiazolidinones: The Search for A Potential Non-β-lactam β-lactamase Inhibitors.

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Synthesis and Evaluation of 1,2,4-oxadiazolidinones: The Search for A Potential Non-β-lactam β-lactamase Inhibitors

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by

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Keywords: Antibiotic resistance, β-Lactamases inhibitors, Nitrones, Cycloaddition Reaction, Oxadiazolidinones and Enzyme kinetics

ABSTRACT

Synthesis and Evaluation of 1,2,4-oxadiazolidinones: The Search for A Potential non-β-lactam β-lactamase Inhibitors

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β-lactam antibiotics have been the most widely used drug of choice to combat infectious disease caused by bacteria. Unfortunately, their effectiveness is drastically threatened by bacterial β-lactamases. β-lactamases is responsible for the resistance to most antibiotic drugs. For decades, β-lactam β-lactamases inhibitors have been used to reduce bacterial resistance; however, in this study 1,2,4-oxadiazolidinone derivatives as a non-β-lactam β-lactamases inhibitor against TEM-1 and P99 β-lactamases. The significance of oxadiazolidinone is the prominent five-membered ring scaffold in its structure, which is configurationally stable and present in other biologically active compounds such as linezolid and avibactam. Oxadiazolidinones were synthesized by treating nitrones with isocyanates. The synthesized compounds were characterized using ^{1}H and ^{13}C NMR, GC-MS, and FTIR. Afterward, they were tested using Nitrocefin as substrate to determine their effectiveness against TEM-1 and P99 serine β-lactamase. Compound **2a, 2b, 2c and 3** showed inhibition ranging from 12-38%.

DEDICATION

This work is dedicated to God almighty for His infinite wisdom, guidance and sustenance; my lovely parents, Rev. Christian E. Kalu & Pastor Charisma Kalu; Mrs. Patricia Bassey; Mr. & Mrs. Chris Ekwuribe; Mr. and Mrs. Kevin Gilpen and my adorable siblings.

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LIST OF ABBREVIATIONS

µL	microliter
Ala	Alanine
FT-IR	Fourier Transform Infra-Red
GC-MS	Gas Chromatography Mass Spectrometer
h	hours
K_2CO_3	Potassium carbonate
KBr	Potassium bromide
Kcal	kilocalories
Lys	Lysine
$MgSO_4$	Magnesium sulfate
min	minutes
mL	milliliter
mmol	millimoles
MOPS	(3-(N-morpholino) propanesulfonic acid)
Na_2SO_4	Sodium sulfate
NaCl	Sodium chloride
nm	nanometer
nM	nanomolar
NMR	Nuclear Magnetic Resonance
RT	Retention time
rt	Room temperature

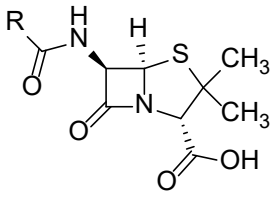
rx	reflux
Ser	Serine
TLC	Thin Layer Chromatography
UV/Vis	Ultraviolet/Visible
$CDCl_3$	Deuterated Chloroform
CH_2Cl_2	Dichloromethane
$CHCl_3$	Chloroform
cm^{-1}	Per Centimeter
DMSO-d_6	Deuterated Dimethyl sulfoxide
g	Grams
m/z	Mass to charge ratio
MeOH	Methanol
EtOH	Ethanol
$MgSO_4$	Magnesium Sulfate
MHz	Megahertz
NCF	Nitrocefin
$NH_2OH.HCl$	Hydroxylamine Hydrochloride
NH_4OH	Ammonium Hydroxide
Zn	Zinc
NH_4Cl	Ammonium Chloride
nM	Nanomolar
NMR	Nuclear Magnetic Resonance

CHAPTER 1

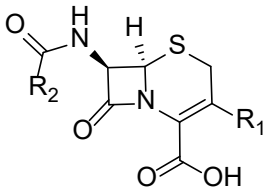
INTRODUCTION AND LITERATURE REVIEW

History of β-lactam Antibiotics

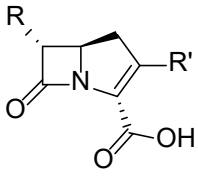
In the late nineteenth century, some French medical students studied the resistance of genus Penicillium (a fungus) to the growth of the bacteria. They could not explain if the microbial resistance was because of the presence of penicillin. In a 1929 article from St. Mary's Hospital in London, Alexander Fleming used the word "penicillin" to describe a well-defined antibacterial property.[1] Since Fleming's accidental discovery of the penicillin producing mold, years of steady progress have followed. And until today, the β-lactam group of compounds is the most successful examples of natural product application.[2] After their commercialization, a β-lactamase secreting penicillin-resistant strain of *Staphylococcus aureus* was isolated.[3,4] The use of Methicillin, a β-lactamase insensitive semi-synthetic penicillin to combat the problem of resistance, led to the evolution of another resistant strain known as methicillin-resistant *Staphylococcus aureus* (MRSA).[5]



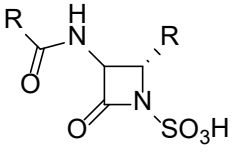
Penicillins



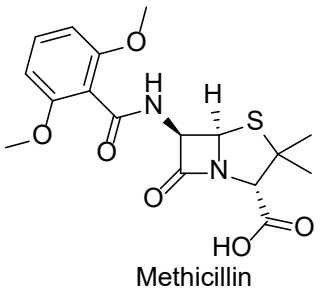
Cephalosporins



Carbapenems



Monobactams



Methicillin

Figure1: Few β-lactam Antibiotics

Most antibiotic agents have the β-lactam ring in their molecules. Bacterial resistance to this class of antibiotics is simple, as all β-lactam shared the same mode of action which is inhibition of bacterial cell wall synthesis by forming a stable covalent adduct with the active site serine residue of penicillin-binding proteins (PBPs).[6]

The PBPs are classified into two categories: the high molecular weight PBPs (HMW-PBPs) and the low molecular weight PBPs (LMW-PBPs). The HMW-PBPs are further subdivided into two groups – A and B, whereas the LMW-PBPs are subdivided into four groups based upon their tertiary structure.[7] The HMW-PBPs are the main target of the β-lactams as they are essential for cell survival. Also, HMW-PBPs are responsible for the execution of trans-glycosylation, while transpeptidation on the external side of the cytoplasmic membrane is carried out by both class A and B high molecular weight PBPs.

Some bacteria have shown significant resistance to most β-lactam antibiotics using the mechanism described below:

1. The biosynthesis of low-affinity PBPs catalyzes the transpeptidation reaction even in high doses of β-lactam antibiotics. Several Gram-positive bacteria accomplish this by mutation of residues around the active site of the PBPs hence decreasing the affinity of PBPs to β-lactam. This is commonly seen in non-β-lactamase producing Gram-negative bacteria.[7–9]
2. The production of β-lactamase can facilitate the hydrolysis of the β-lactam ring in antibiotics, and in turn transfers the plasmid, encoding with β-lactamase gene among related and unrelated species. It is an important and prevalent mechanism in Gram-negative bacteria, e.g., *Neisseria gonorrhea and Hemophilus influenza*.[8,10–12]
3. Decrease target site accessibility: Accessibility of target site plays a vital function in β-lactam drug action. The effectiveness of β-lactam depends on their ability to cross the outer membrane which is enhanced by the outer membrane protein (OMPs). Nevertheless, the bacteria eventually develop

resistance to the β-lactam through the outer membrane of the cell, leading to decreased effectiveness and increased minimum inhibitory concentration (MIC) of antibiotics. These, at most times, work with another resistance mechanism such as β-lactamase expression.[13,14]

4. Decreasing the effective number of drugs in the periplasm using efflux pumps which facilitate the export of β-lactam outside the cell. It is common among gram-negative bacteria.[14]

With the growing bacterial resistance to antibacterial drugs, there is a great need to design, synthesize and study non-β-lactam drugs that also display high specificity for the target site, low toxicity to human cells, and are not easily hydrolysable by β-lactamase.

β-lactamase Enzymes

The β-lactamase enzymes are multi-resistant to β-lactam antibiotics such as penicillin, cephalosporin, and monobactams (figure 1). These enzymes break the β-lactam ring open, which leads to deactivating the molecule's antibacterial properties. The β-lactamases were first observed and isolated by E. P. Abraham and E. Chain from Gram-negative *Bacillus Escherichia coli.*[1] It was before the clinical use of penicillin. By then, β-lactamases were not considered to be clinically relevant since penicillin was designed to treat staphylococcal and streptococcal infections and scientists could not isolate the enzymes from these Gram-positive organisms.[1,15,16]

Kirby et al. (1944) successfully isolated the penicillinase from *Staphylococcus aureus.* It alerted the emergence of a major clinical problem, as these enzymes will eventually become the major causes of bacterial resistance.[17] The production of large numbers of β-lactams, as well as the overuse of antibiotics has increased the selective pressure on disease-causing bacteria, consequently promoting the survival of organisms with multiple β-lactamase.[18,19] About 850 β-lactamases have been detected, and it is assumed

that high mutation frequency, rapid recombination, and replication rates are responsible for bacteria resistance.[20]

Classification of β-lactamase Enzymes

β-lactamases can be classified into two major categories:

1. The Ambler classes A to D, based on amino acid sequence homology, and
2. The Bush-Jacoby-Medeiros group 1 to 4 based on substrate and inhibitor profile.[21,22]

The β-lactamase are regularly grouped into four classes (A - D) based on sequence homology. The class A, C and D serine β-lactamase (SBLs) evolved from the PBP transpeptidase and are the most clinically common. They use the active serine as nucleophiles in β-lactam hydrolysis. On the other hand, the class B Metallo-β-lactamase (MBLIs) uses either a single Zn^{2+} ion or a pair of Zn^{2+} ions coordinated to His/Cys/Asp residue in their active sites.[6,22]

Based on structural analysis and amino acid sequence comparison, three prevalent active site motifs have been observed among the SBLs: motif (a) stores the nucleophilic serine needed for acylation (SXXK), motif (b) is needed for protonation of the β-lactam N4 nitrogen leaving group upon acylation (S/Y-X-N/V), and motif (c) participates in the activation of the motif (B) S/Y proton donor and in substrate recognition and oxyanion stability (K/R-T-/S-G).[23]

In this review, we will adopt the Ambler classification scheme.

Class A Serine β-Lactamases

The first β-lactamase to be observed in penicillin-resistant bacteria was the class of A β-lactamase, and they remain as the most common β-lactamase today.[24] These enzyme possesses a conserved E166 (believed to be the general base needed during hydrolytic deacylation for the activation of catalytic water), situated in a region called Ω loop.[25] Most class A serine β-lactamase are vulnerable to most β-lactamase inhibitors like clavulanate. Nevertheless, the *K. Pneumoniae carbapenemases* (KPC) showed resistance to clavulanate, and hence could probably be an exception to this inference.[26]

Class A Extended-Spectrum β-Lactamases (ESBLs)

Extended-Spectrum β-lactamase (ESBLs) are simply a class of plasmid-mediated and rapidly evolving enzymes that are currently raising a major treatment challenge in hospitalized and community-based patients.[27] They exhibit resistance to antibiotics such as the Oxyimino-Cephalosporins, Monobactam (aztreonam), and Penicillin. This class of β-lactamase is well known to be inhibited by Clavulanate.[22,28]

Experimental findings revealed that it is difficult to detect the organism that produces this enzyme because the presence of ESBLs in a bacteria cell does not often produce resistance phenotype. The National Committee for Clinical Laboratory Standards (NCCLs) recommended detection of ESBLs in *Klebsiella and E. coli* by testing if the susceptibility to ceftazidime, Cefotaxime, ceftriaxone, cefpodoxime or Aztreonam reaches the previously established thresholds for resistance synergy.[29]

Class A Serine Carbapenemases

Class A serine Carbapenemases, having two functional groups, were observed sporadically in clinical isolation since their first discovery. These β-lactamases were detected in *Enterobacter*

cloacae, Serratia marcescens, and Klebsiella spp and are susceptible to Clavulanate as single isolates.[30] Reduced susceptibility to imipenem is observed by bacteria expressing these enzymes.

Class B Metallo-β-Lactamases

They are Zn^{2+} dependent β-lactamase, and they catalyze the hydrolysis of almost all β-lactam antibiotics except monobactams.[31] Their uniqueness is because they are not inhibited by mechanism-based inhibitors such as Sulbactam, Clavulanate or Tazobactam, which are very effective against class A β-lactamase. A good example of class B Metallo-β-lactamase is the new Delhi Metallo-β-lactamase.[22,32]

Class C Serine Cephalosporinase

Serine cephalosporinase of class C is considered as an array of β-lactamase enzymes encoded in the *bla* gene of bacterial chromosomes. The bacteria expressing the gene for this class of β-lactamases is significantly resistant to penicillin and β-lactam β-lactamase inhibitors like Cefoxitin, Cefotetan, Ceftriaxone, and Cefotaxime. Nevertheless, Cloxacillin, Oxacillin, and Aztreonam are known to inhibit AmpC.[22,33]

Class D Serine Oxacillinases

They are described as oxacillinases because of their inherent ability to hydrolyze oxacillin at a lesser rate of 50%, conversely to the relatively slow hydrolysis of oxacillin by serine carbapenemases and serine cephalosporinases.[34] The class D enzymes lack the Ω loop E166 and rather involves a carboxylated lysine in an SXXK motif, which plays a dual function as the general base involved in both serine activation during acylation and in the activation of the catalytic water during hydrolytic deacylation.[25,35–37] This class of β-lactamase has a resistance mechanism against β-

lactam antibiotics. The mechanism is effective for the treatment of infectious diseases, which is caused by *Acinetobacter baumannii* and the *Enterobacteriaceae*.[38,39]

Evolution Of β-lactam and non-β-lactam β-lactamase Inhibitors

The persistent emergence of multi-drug-resistant bacteria has become a global health concern. Great effort has been made to minimize or possibly eliminate the threat posed by bacteria resistance by using new a class of β-lactam β-lactamases and non-β-lactam β-lactamase inhibitors. This could either extend the spectrum of activity or address specific bacterial resistance mechanisms that arose in the targeted bacterial population.[10,40,41]

About 65% of all prescribed antibiotics contain β-lactam – Penicillin, Cephalosporins, Carbapenems, and Monobactam.[42] The mechanism of β-lactam antibiotics (figure 2) aims at the last synthetic step in peptidoglycan (PG) biosynthesis, in which they mimic acyl D-ala-D-ala on the peptidoglycan stem in order to inhibit penicillin-binding protein (PBP) that catalyze transpeptidation of the adjacent PG strands.

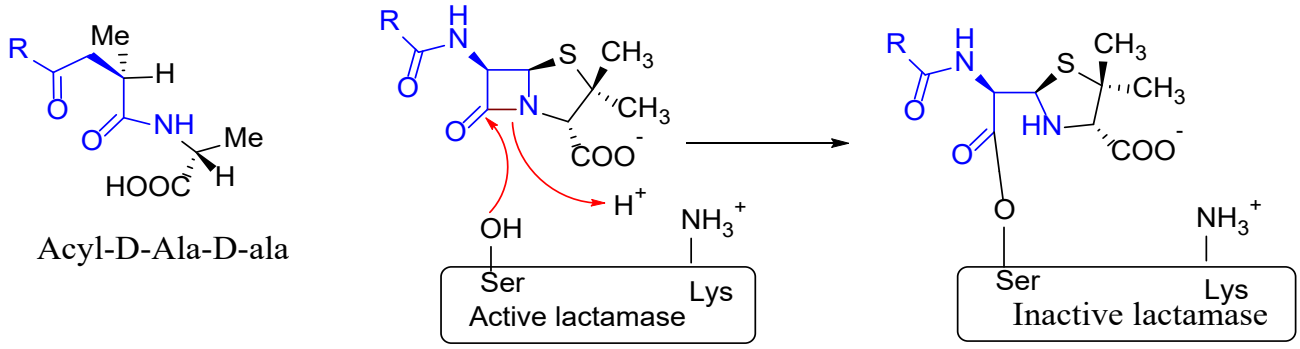


Figure 2: Mechanism of action of penicillin antibiotic drug[43]

The common mechanism of resistance to the β-lactam in Gram-negative bacteria is that β-lactamase enzymes hydrolyze the four-membered β-lactam ring, rendering it inactive (figure 3). The β-lactamase are frequently encoded on a freely movable plasmid that enhances their transmission among bacteria population. [25]

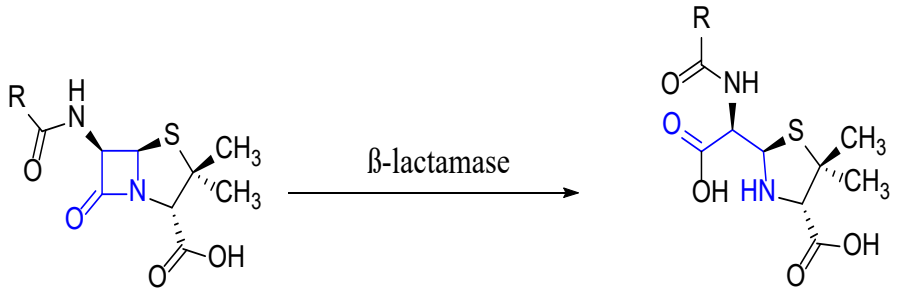


Figure 3: Mechanism of inhibition of β-lactam antibiotic by β-lactamase[44,45]

The effectiveness of some oral β-lactam antibiotics is decreasing due to the constant evolution of β-lactamase, and this has posted a great threat to human health.[25,46,47] In 1970, three β-lactam β-lactamase inhibitors (BLIs): Clavulanic acid, Sulbactam, and Tazobactam[10] (figure 4) were used to circumvent SBL mediated resistance. These inhibitors are mechanism-based covalent inactivators that form a stable acyl-enzyme intermediates with the catalytic serine β-lactam BLIs. They are preferred to others because of their low toxicity and high specificity to target cells.[6,7]

In the 1980s, the BLIs (figure 4) were used in combination with β-lactam antibiotics as a standard part of treatment to significantly reduce the minimum inhibitory concentration (MIC) against various bacteria.[48–50] Some examples of such combined drugs include Unasyn™ (ampicillin and sulbactam), Augmentin™ (amoxicillin and clavulanate), and Zosyn™ (piperacillin and tazobactam).[6,10,42,51] Despite the use of these synergistic drugs, there is still antibiotic resistance due to over-use of antibiotics by humans and in agriculture, production of inhibitor-resistant β-lactamases or enzyme hyper production.[52]

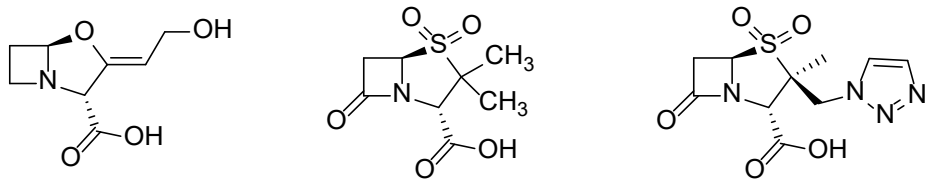


Figure 4: β-lactam mediated β-lactamase inhibitor

Conventionally, BLIs are effective against class A serine β-lactamase but ineffective to C and D SBL enzymes.[40] BLIs have no substantial antibiotic activity on their own but protect β-lactam antibiotics against destructive hydrolysis of the β-lactam ring by microorganism's β-lactamase. Currently, there are several new class A SBL (the inhibitor-resistant TEM and Complex Mutant TEM) that have drastically developed resistance to these inhibitors[10,53]. Thus, the need for a novel inhibitor is crucial.

Non-β-lactam β-lactamase Inhibitors

Non-β-lactam β-lactamase inhibitors such as Linezolid and Avibactam (figure 5) can effectively inhibit the hydrolysis of β-lactam by bacterial β-lactamase. When avibactam is used in combination with extended-spectrum Cephalosporine and Aztreonam, it forms a synergistic drug that is effective against Gram-negative bacterial infection.[54–56]

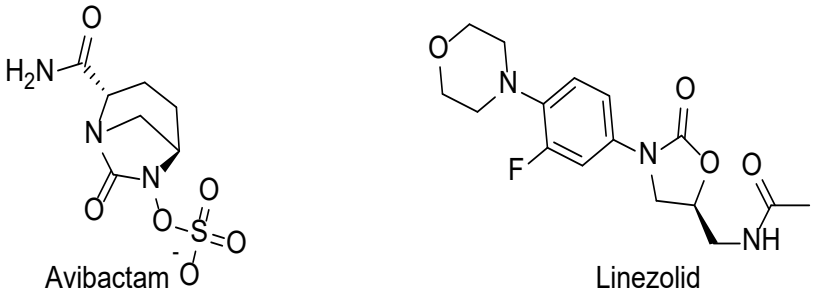


Figure 5: Some non-β-lactam based β-lactamase inhibitors[25,57]

Based on PBP inhibitors, the non-β-lactam β-lactamase can be placed into 4 categories:

- Transition state analogs
- Substrate analogs
- Covalent inhibitors
- Non-covalent inhibitors

Transition State Analogs. Transition State Analogs (TSAs) are compounds that are like the transition state of a catalyzed reaction. These compounds account for potent enzyme inhibitors that confer insight into an enzyme mechanistic behavior.[58] When enzymes bind to a substrate, they form a complex which goes through a chemical and geometric shift. The TSAs with the same shape and charge of the original transition molecule bind with the enzyme. The analog exhibits the same characteristics as the initial transition molecules, however they are still slightly different and will not lead to the required product formation. Due to this differences, they will eventually deactivate the enzyme, consequently preventing them from binding to the substrates.[59] TSA inhibitors, such as boronic acids, carbonyl compounds, and phosphates could bind to the enzyme with ease due to the affinity they have for it.[7,60] Hence, they are efficient serine β-lactamase and protease inhibitors.[10,40,41]

Substrate State Analog. Substrate State Analogs (SSAs) are compounds having a chemical structure like the substrate molecule in an enzyme-catalyzed reaction. SSAs can behave as the competitive inhibitor of an enzyme-catalyzed reaction, taking the same binding site of their analogs and consequently decreasing the substrate's efficiency. The Vmax remains the same as the intended substrate affinity decreases.[61,62] Substrate analogs react like suicide substrate by acylation of PBP active serine, just like the acylation of β-lactam.[7] An example of substrate analog includes 3-acetylpyridine adenine dinucleotide: a substrate analog of NADH,[63] bicyclic pyrazolidinones and lactivicins. The last two analogs have been observed to show clinically significant antibacterial activities and PBP inhibitors.[64–66] Jungheim & Ternansky, 1992 as cited by (Osazee, 2016) stated that bicyclic pyrazolidinones (figure 6) compounds containing a strong electron deficient group in C3 position displayed a considerable *in vitro* activity unlike others.[64]

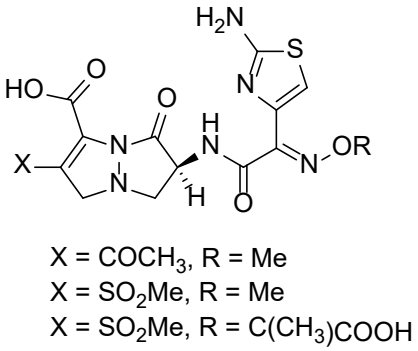


Figure 6: Bicyclic pyrazolindinones[64]

Lactivicin (LTV) (figure 7), a novel natural non-β-lactam antibiotic, was isolated in 1986 by Takeda Research group from a bacterial strain (*Empedobacter lactamgenus and Lysobacter albus*).[67–72] LTV is effective against Gram-positive bacteria.[68] Nevertheless, it is susceptible to hydrolysis by β-lactamase enzymes.[67] As a means to address the issue of susceptibility to β-lactamase, LTV derivatives

were synthesized, which increased antibacterial activity against Gram-negative bacteria and reduced its toxicity.[65,71,72]

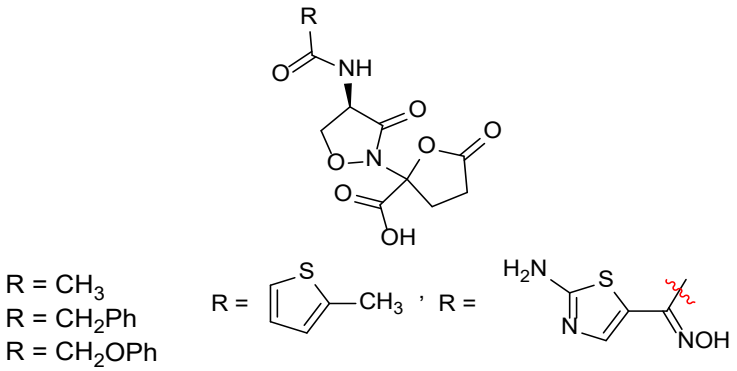


Figure 7: Lactivicin analogs[65]

Covalent Inhibitor. They are inhibitors that bind and form a bond with target enzymes. They are able to achieve this because they have a bond-forming functional group designed to react rapidly with a specific nucleophilic residue in the active site to form a permanent bond.[73] An example of covalent inhibitors include: Aspirin, Afatinib, and Rociletinib.[74] Covalent enzyme inhibitors are important biochemical substances.[74–78] Some potential benefits of covalent inhibitors are: circumventing the challenges associated with the targets having a shallow undruggable binding site, reducing the development of drug resistance due to mutation of a binding site, and high biochemical efficiency, thus resulting in lower doses and reduced off-target effects.[76]

However, the benefits are offset by some potential disadvantages like unfit for a mechanism which involves short residence time and partial inhibition, a potential risk of immune-mediated drug hypersensitivity, or less advantageous for targets that are suddenly turned over by protein synthesis.[79–81] Irrespective of their downsides, covalent inhibitors have had a significant impact on human health.[76]

Non-Covalent Inhibitors. Non-covalent inhibitors interact reversibly to the active site of PBPs with no acylation involved in the process; hence, they are regarded as highly effective inhibitors. The disadvantageous conformational changes in the active site of PBP's MRSA involved during acylation are averted.[82,83]

Some classic examples of non-covalent inhibitors are aminothiadiazol, naphthalene, sulfonamide, arylalkylidene rhodamine, arylalkylidene iminotriazolidene, anthranilic acid, and quinolones. Anthranilic acid, and quinolones was observed to be a non-covalent inhibitor of PBPs of *E.coli* and *B. subtilis*, but all active quinolones were without *in vitro* antibacterial effect against *E.coli* or *B. subtilis* by themselves.[84–89]

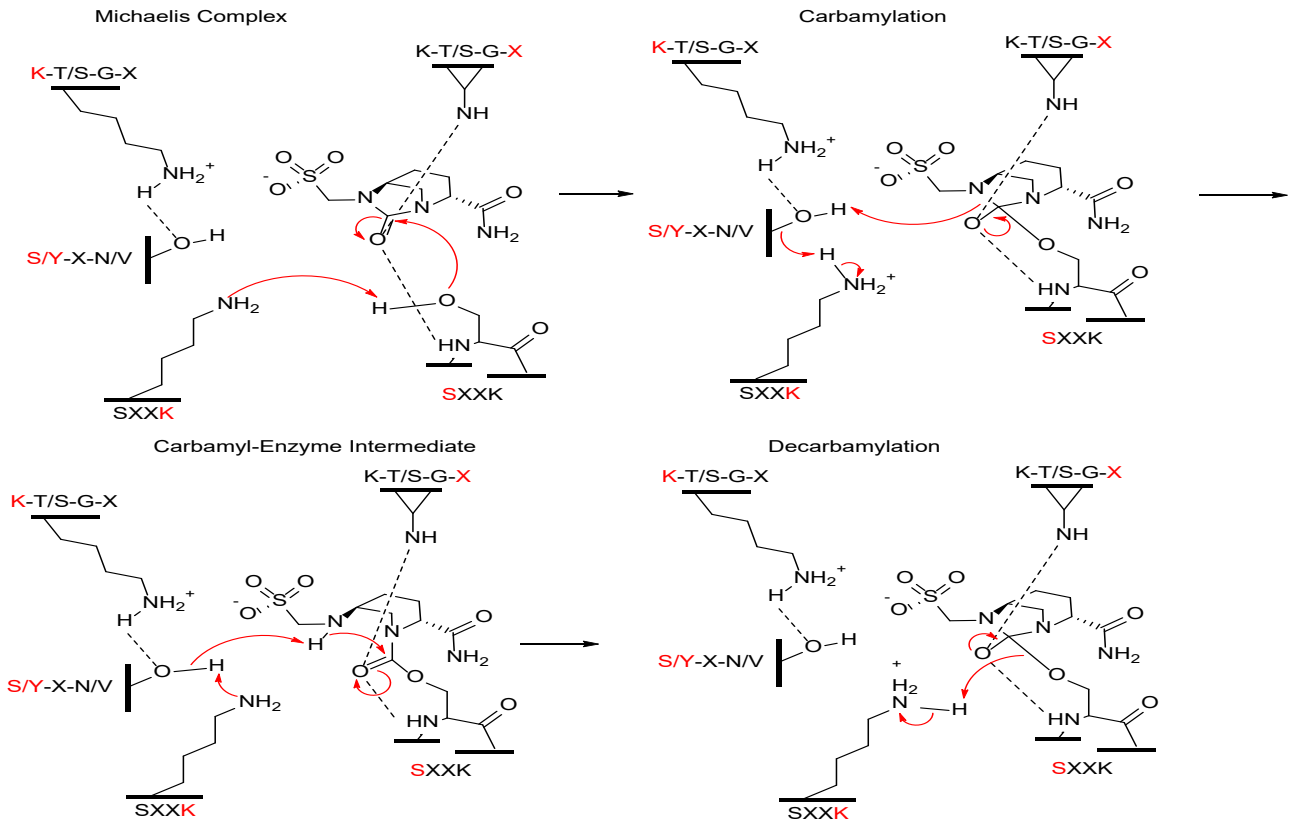
Diazabicyclooctane

In the 1990s, a novel non-β-lactam BLIs which belong to diazabicyclooctane (DBOs) class of compounds were developed.[54,90–92]. It has been proven that diazabicyclooctane avibactam is able to inhibit a broader spectrum of serine-β-lactamase than the BLI-clavulanic acid. Studies by King et al. (2015) demonstrated that 1.7 and 2.0Å resolution crystal structure of avibactam covalently attach to class D β-lactamase OXA–10 and OXA–48.

In 2015, avibactam was approved by the FDA, and it is currently in its phase III clinical trials as part of a combined treatment to be used with Ceftazidime to combat multi-resistant urinary tract and intra-abdominal infections.[92–94] This synergistic drug has been proven to be safe and tolerable in clinical trials, with relatively few adverse effects recorded.[54] The most prevalent resistance to this drug is the expression of new β-lactamases that are not inhibited by Avibactam.[95]

Mechanism of Action of Avibactam

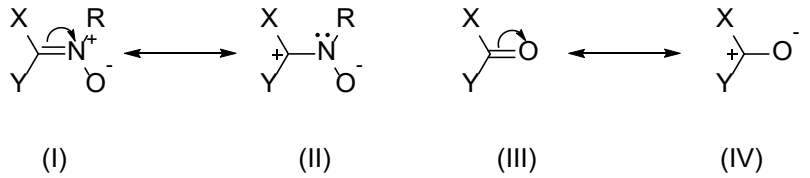
Avibactam inhibits serine β-lactamase (SBL) by forming a carbamyl linkage with the catalytic serine, which is not broken down by a hydrolytic mechanism. Rather, the decarbamylation of the avibactam occur through the recyclization of DBO fused ring structure, recovering the intact inhibitor which could either re-carbamylate the active site or be released into the solution to inhibit more SBLs[96] (scheme 1). Avibactam was not hydrolyzed by β-lactamase because the carbamyl carbon is less prone to nucleophilic attack than its ester analog.[97,98] Such stability is most likely due to the resonance effect in which the lone pair of electrons on the sp^2 hybridized N1 aligns with the carbonyl C=O P-orbital, thus increasing electron density at the electrophilic carbonyl carbon.[99] Based on the kinetic analysis of a key active site mutant for class A β-lactamase CTX-M-15, a substantiated mechanism for avibactam mediated β-lactamase inhibition was proposed.[25,43]



Scheme 1: Catalytic mechanism of avibactam based SBL inhibition (adapted from King D. et al. 2015)[25]

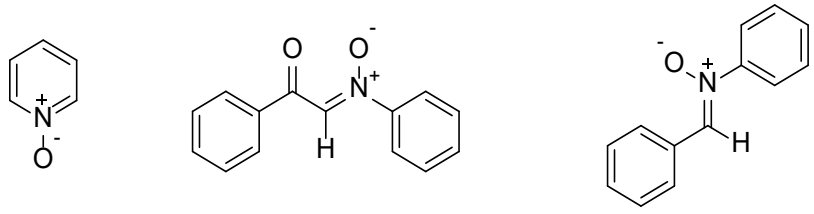
Nitrones

In 1916, Pfeiffer suggested the name "nitrones" as an abbreviation for nitrogen ketone to highlight their remarkable similarities to ketones.[100] Furthermore, Delipierre et al. (1965) observed that the similarity is due to the mesomeric effects [(i) — (ii) and (iii) — (iv)] that are present in both types of compounds, thus making nitrone [or azomethine N-oxide (i)] act like an extended carbonyl function[100] (scheme 2).



Scheme 2: Similarity of nitrones to ketones (adapted from Delipierre et al., 1965)[100]

The polarity of nitrones are influenced by the electronic effect of the substituents X, Y and R. In the case (I: X, Y, and R = alkyl) the double bond is fixed, and the positive charge is localized between the carbon and nitrogen atoms of the nitrone. The positive charge on the azomethine carbon atom (II) (scheme 2) influences most nitrone reactions.[100] There could be another case like in pyridine N-oxide (figure 8) whereby a high degree of delocalization is felt due to its aromatic character. An electron withdrawing group (X or Y) of scheme 2 will reduce electron density on the carbon, thus improving its electrophilic character. That is why C-benzoyl-N-nitrone undergoes 1,3-cycloaddition 110 times faster than CN-diphenyl nitrone[100,101] (Figure 8).



Pyridine N-oxide C-benzoyl N-nitrone CN-diphenyl nitrone

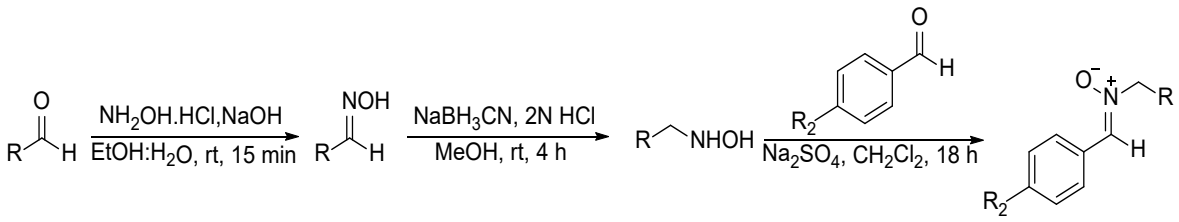
Figure 8: Some examples of nitrone

The common terms aldo- and keto-nitrones are applied occasionally to differentiate those with and without a proton on the α-carbon.[100] Nitrones can either be solid or liquid depending on the polarity of the substituent present. They are readily soluble in water except if a hydrophobic substituent such as aryl group is present. Extraction from aqueous solution by an organic solvent is only feasible after concentration of and salting out.[100] Nitrones are known to have a significant application as a spin trap in biological studies,[102] as an antibacterial agent,[103–105] and are effective in age-related diseases[106] because of the stability of the resulting nitroxide radicals.[107]

Preparation of Nitrones

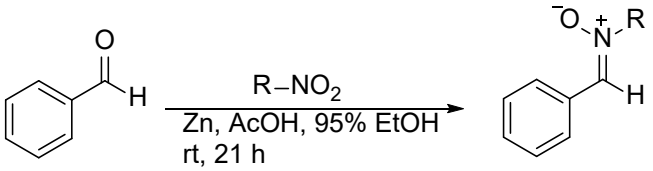
The preparation of different kinds of nitrones was reviewed by Smith in 1938 and later by Harmer and Macaluso in 1964.[108] The easiest method to synthesize nitrones involves the condensation of aldehydes with N-monosubstituted hydroxylamines,[109–111] oxidation of secondary amine, or N.N-disubstituted hydroxylamine.[112,113] These procedures give a good result but still have drawbacks, such as relatively long reaction time, the use of surplus aldehydes to reach greater conversion, and time-consuming chromatographic purification.[114]

N-Substituted Hydroxylamine. The condensation of aldehyde derivatives with N-substituted hydroxylamine (scheme 3) is the most common method for the synthesis of nitrone. However, it is limited by the availability of suitable hydroxylamine.[115,116] Generally speaking, the synthesis of nitrones works more readily with aldehydes than with ketones, although some aldehydes are prone to polymerization under alkaline conditions.[117]



Scheme 3: Synthesis of nitrones from benzaldehyde and N-substituted hydroxylamine[118]

More often, the hydroxylamine function is directly obtained in situ by zinc-dust reduction of a nitro-compound. (Scheme 4)[119] The zinc reduction of nitroalkanes was what was employed in this study. Reduction of nitro compounds go through three intermediate stages – the formation of nitroso compounds, the formation of hydroxylamine, and the formation of amine products. Interestingly, the process can be terminated at the hydroxylamine stage by various ways, among which the use of zinc in ammonium chloride in ethanol is the most common.[120]



1a : R = CH_3,
1b : R = C_2H_5

Scheme 4: Synthesis of nitrone from nitroalkanes

N, N-disubstituted Hydroxylamine. There are a good number of reagents that can oxidize both cyclic and acyclic N, N-disubstituted hydroxylamine to nitrones.[121,122] Some reactions may require the use of a suitable catalyst such as copper-ammonia complex.[123]

Gem-chloronitroso Compounds. The reaction of nitrones, obtained from gem-chloronitroso compounds, with phenyl isocyanates has been reported. [124] However, Kumar et al. (2005) reported that an attempt to synthesize gem-chloronitroso led to a low yield.[125] Similar experience occurred in this study when an attempted to synthesize nitrone from chloronitroso compounds was done. Gem-chloronitroso is generally obtained from oximes treated with elemental chlorine, or aqueous hypochlorous,[126] but frequently lead to the production of overoxidized nitro derivatives with little compounds present. Few methods are adequately selective to terminate the reaction at the nitroso phase. However, those methods involved contamination of the product, are restricted to a substrate, involves pH-dependent reaction with strict precaution, and leads to the formation of by-products, consequently having a low yield of products. Eventually, they encountered a research work on the oxidation of organic compounds with N-tert-butyl-chlorocyanamide.[127] This novel reagent was sufficiently selective to terminate the reaction at nitroso phase without being over-oxidized to nitro-compound.

Schenk et al. (1980) also reported synthesizing nitrones from nitroso compound with Grignard reagents. Their investigation on the reaction of chloronitroso adamantane with a Grignard reagent revealed that adamantylidene nitrones are formed in proportions that differ with the nature of Grignard reagent RMgX. They also noticed that a higher yield of N-methyl and N-phenyl nitrones were obtained only when the R group of the Grignard reagent was CH_3 and C_6H_5, but with all other Grignard reagents, the yield of nitrone was much lower.[124]

Spectroscopy of Nitrones

A strong absorption in 1600 cm^{-1} is characteristic of all nitrones, but the exact position of this band varies with different nitrones. It is observed that in acyclic nitrones the position of the band is slightly wider in range depending on the nature of the substituent involved.[100,124] It is difficult to state with certainty the origin of this band; some authors assign it to the C=N stretching mode[121] while others attribute it to the $C=N^{+}\rightarrow O^{-}$ group.[128] Another strong absorption in the region 1170-1280 cm^{-1} is found in all nitrones, which is thought to be due to the $N^{+}\rightarrow O^{-}$ stretching frequencies.[100] The proton NMR singlet at 3.70 ppm is attributed to the N-methyl group.[129] IR and NMR spectra analysis indicated that most nitrones were very sensitive to hydrolytic decomposition. However, the adamantylidene nitrones were less vulnerable to hydrolytic decomposition and generally more stable.[124] The NMR spectrum of the N-methyl nitrones was complicated by the existence of an incompletely resolved homo-allylic coupling across the nitrone function. Black et al. (1974) in a series of cyclic aliphatic α-methyl nitrone observed the homo-allylic coupling.[130]

Isomerism of Nitrones

Nitrones exhibiting (E)- and (Z)-isomers may interconvert.[100,108,131] They could exist either in dimeric form - aldonitrone[132,133] or in a trimeric form.[122] In contrast, at room temperature adamantylidene nitrone exits in monomeric form as deduced from their spectroscopic and chemical properties.[129,134,135] Normally, the nitrone isomers that are thermodynamically stable are those with the bulkiest group in a trans position. Aldonitrones happen to be the only known isomers to show such a trans relationship. In the case of ketonitrones with α-substituent R_1 and R_2, having a slight difference in bulkiness, both isomers may exist.[124,136,137]

More so, the calculations on barriers to rotation about C=N bond[138] demonstrated that aldonitrones are significantly more stable than ketonitrones, but under extreme conditions of cycloaddition, the aldonitrones can isomerize.[139] To circumvent the challenges associated with (E/Z) isomerism of nitrone, some cyclic nitrones were developed which permitted only single geometry about the C=N double bond, thus decreased the number of possible cycloaddition products obtained.[140] In cyclic nitrones the syn-configuration is determined by the ring, but in acyclic nitrones the double bond in the group will introduce the possibility of the existence of geometrical isomers.[141] Cyclic and acyclic nitrones are used as intermediary molecules for the preparation of medicinal products[142–145]since they can be easily synthesized and are more stable and reactive than C=N containing compounds.[146–148]

Cycloaddition Reaction

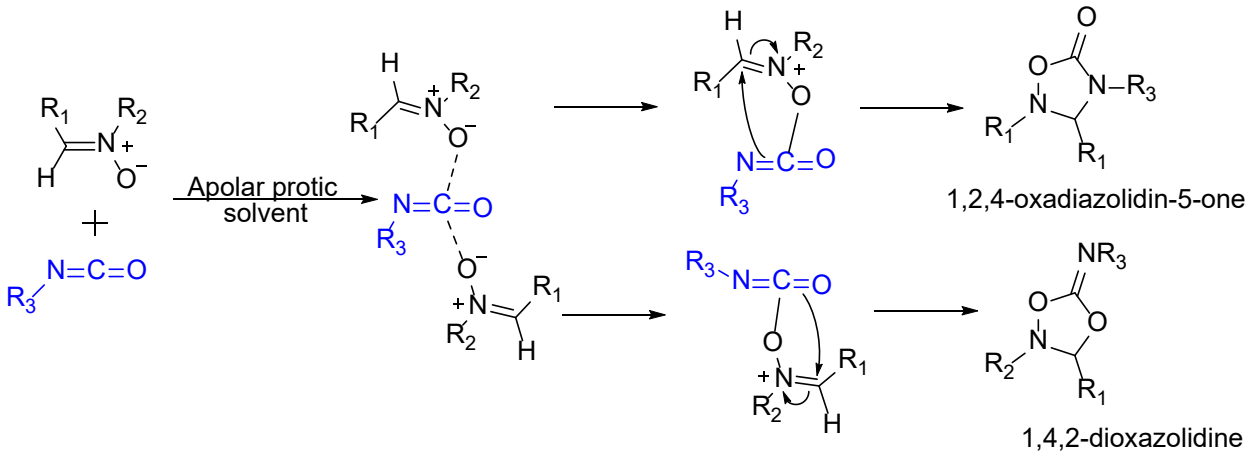
The cycloaddition reaction between phenyl isocyanate and nitrone was first published in 1894.[149] Nitrones are 1,3-dipoles with 4 pi electrons which participate in 1,3-dipolar cycloaddition reaction with different kinds of appropriate unsaturated compounds such as alkynes,[150–153] isocyanates,[154–157] isothiocyanates,[158] thiocarbonyl compounds, and phosphoranes compounds, to produce various heterocyclic five-membered ring systems.[108,148,159] The cycloadduct formed sometimes undergoes a fascinating transformation.[159,160] Nitrones are preferred as starting material of choice due to their high yield and excellent selectivity.[118] 1,3-dipolar cycloaddition is a very effective and useful reaction in generating different kinds of heterocycles with a variety of structures of high demand in pharmaceutics.[161,162]

However, it is worthy of note to state that cycloaddition reaction only occurs readily with aldonitrones. Nevertheless, stronger dipolarophiles, such as isocyanates, can react with ketonitrones.[135] Hence, treatment of nitrones with phenyl isocyanate in benzene at ambient temperature could lead to the formation of oxadiazolidinone in a very high yield.[129]

1,3-dipolar cycloaddition is important in polymer modification,[163,164] generation of Nano-structures semiconductors,[165] surface modification of ordered mesoporous carbons,[166] fluorescent single-walled Nano-tube syntheses which are used in medicine for diagnosis and controlled drug delivery,[167] and synthesis of modified DNA and RNA as molecular diagnostic tools.[168]

Mechanism of Cycloaddition

The mechanism of cycloaddition reaction of nitrones with isocyanates occurs in a concerted manner in a gas phase and in a step-wise manner in an apolar solvent. In the first step of the step-wise mechanism, the oxygen atom of the nitrone acts as a nucleophile approaching the central carbon atom of the isocyanate to form an intermediate.[169] The transition state generated is stabilized by an attractive electrostatic interaction enhanced in apolar solvent. In a typical step-wise mechanism the first step is the rate-limiting step, but the formation of 1,2,4-oxadiazolidin-5-one[170–173] or 1,4,2-dioxazolidine[174,175] is determined in the second step which is the rate-limiting step. The formation of these two products is because both the double bonds of isocyanates (C=N and C=O) were involved in the cycloaddition reaction leading to two products in scheme 5.[169]



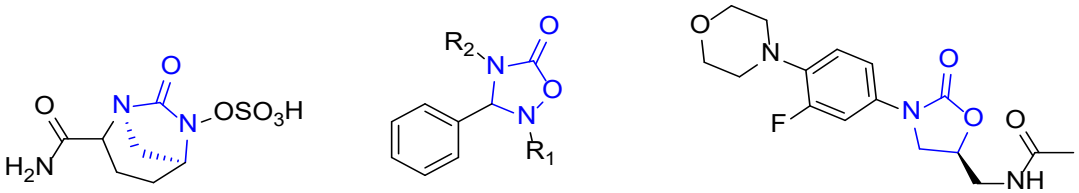
Scheme 5: Possible cycloadduct from the reaction of nitrone with isocyanates[169]

Computational studies indicate that the formation of 1,2,4-oxadiazolidin-5-one is preferred both kinetically and thermodynamically irrespective of the solvent used.[176–178] There are different opinions on what the mechanism of 1,3-cycloaddition of nitrone with isocyanate is. Some scholars considered it as a one-step, four-centered, concerted reaction that forms two new sigma bonds,[179] but not essentially at an equal rate.[101,180] A two-step mechanism having a spin-paired diradical intermediate has been posited[181,182] but not accepted. Furthermore, an alternative two-step mechanism including a zwitterionic intermediate was reported. This cannot be disregarded completely for addition reaction involving highly polarized dipolarophiles.[183,184]

The 1,2,4-Oxadiazolidinone Scaffold

Justification of Research

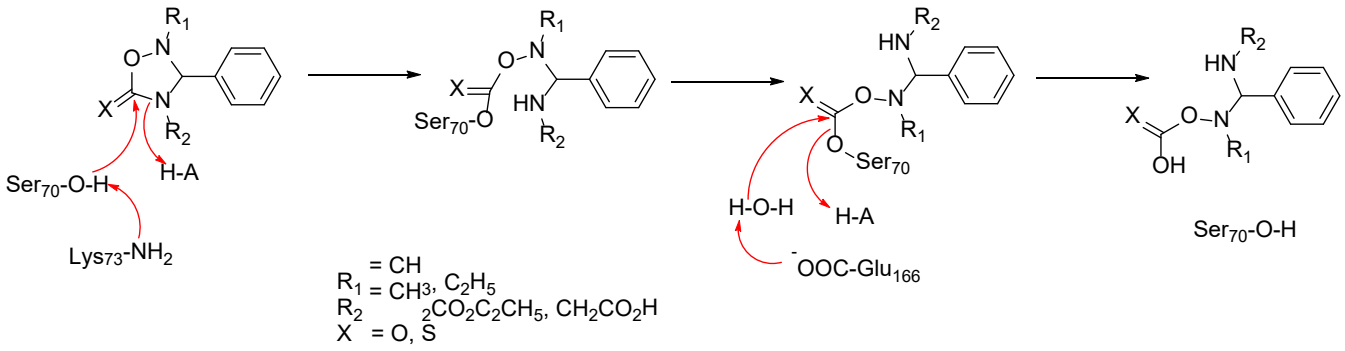
The rationale for the synthesis of oxadiazolidinone derivatives comes from the fact that oxadiazolidinones possesses a section in their scaffold that is like the active site of non-β-lactam β-lactamase inhibitors - avibactam and linezolid as shown in (scheme 6). Their structural similarity to oxazolidinones (linezolid) endows them with some advantageous features to be used as the antibiotic agent. Like the previously described of the oxazolidinones scaffold,[185–187] they are hydrolytically stable in acidic and basic conditions, and the effectiveness is due to the existence of N-aryl substitution which is observed to be significant from the isoxazoline scaffold.[57]



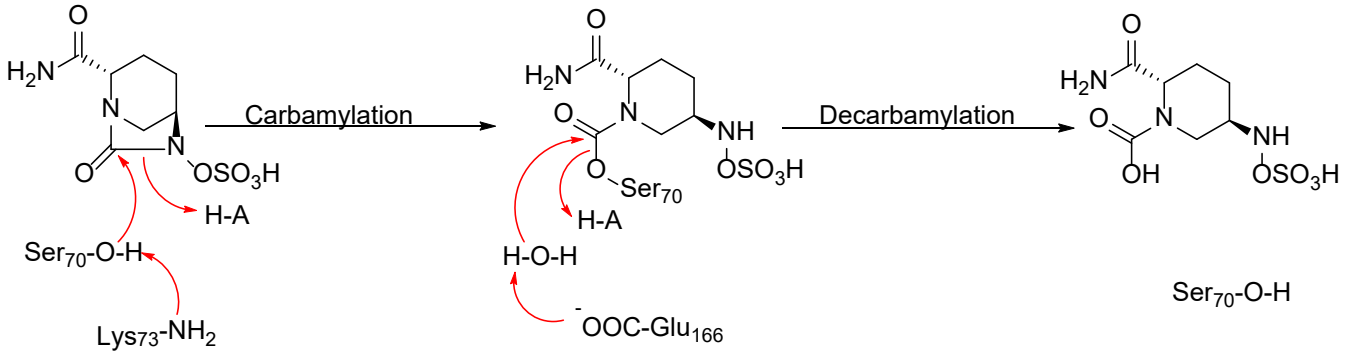
Scheme 6: The structural relationship between non-β-lactam β-lactamase inhibitors and oxadiazolidinones

Therefore, this study aims at developing an oxadiazolidinone derivatives which retain the active site and evaluate their antibiotic effects against TEM-1 and P99 serine β-lactamase. Moreover, the attack by Ser-OH function of the active site of serine β-lactamase may lead to the formation of a stable covalent bond, consequently making oxadiazolidinones an effective inhibitor of β-lactamase. Scheme 7

demonstrates the possible mechanism of action of oxadiazolidinone with respect to the mode of action of avibactam in scheme 8.



Scheme 7: Proposed mechanism of action of oxadiazolidinone analogs as a non-β-lactam β-lactamase inhibitor



Scheme 8: Mechanism of action of avibactam as a non-β-lactam β-lactamase inhibitor[43]

Ritter T. (2004) hypothesizes that although β-lactam ring in ezetimibe (figure 9) is reported to be needed, however, there is no evidence that it plays any role than a scaffold to appropriately position the pharmacophore groups.[57] Based on his hypothesis, it implies that there is a chance for the four-membered β-lactam ring to be replaced with a five- membered ring heterocycle instead, upon which substituents that are known to show antibacterial activity can be added.[57] The five-membered rings have

less angular strain which may enhance its stability and in turn prevent easy hydrolysis, a case common among the four-membered β-lactam ring.

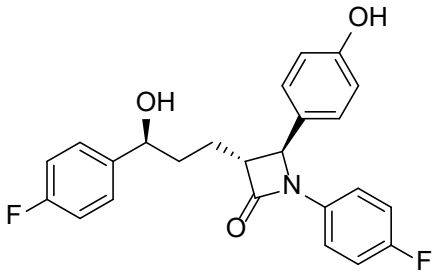


Figure 9: Structure of ezetimibe[57]

Compounds having 1,2,4-oxadizolidinone scaffold or their derivatives have been demonstrated to be biologically active.[188] For instance, 1,2,4-oxadiazolidin-3,5,-dione is present in the core structure of a herbicide like methazole and BAS-3820.[189] Furthermore, oxadiazolidinone intermediates are used to produce amidine, an important functional group amongst biological active compounds.[190–194] It was reported that N-Styrenyl amidines were conveniently synthesized from an oxadiazolidinone intermediate that underwent CO_2 elimination and Styrenyl migration. [154] By using this route to obtain N-Styrenyl amidine, they were able to circumvent the limitations associated with the conventional pinner-type methods for amidine production which involved the addition of imine to an imidate component.[195–197] Oxadiazolidinones are excluded from most literature because they lack an appropriate procedure for their synthesis in an enantiomerically pure and stable form.[57,156] This research work communicates suitable methodology to synthesize some 1,2,4-oxadiazolidinone derivatives which are described in the experimental section in chapter 2.

Chemistry of Oxadiazolidinone

A model reaction between nitrones and isocyanates revealed that the reaction depends on the nitrone structure, and it is positively enhanced by the presence of an electron donating group at the nitrone carbon atom. However, nitrones with electron-poor substituent also give rise to oxadiazolidinone in moderate yield over a long period of time upon being heated.[157] Therefore, the electron withdrawing group possibly makes the oxygen atom of nitrone less nucleophilic and reduces the probability of the nitrone-oxygen attack towards the electron-poor isocyanate group.[116,198]

The reaction of oxaziridine with isocyanate to furnish nitrone-isocyanate cycloadduct strongly rely on the electronic character of the substituent on the carbon atom of the oxaziridine, just like the nitrones.[199] Electron-rich oxaziridine with phenyl isocyanates was also rapid and progressed with some conversion to produce the corresponding bis-oxadiazolidin-5-one in thirty minutes.

Stereochemistry of Oxadiazolidinone

A meticulous examination of the 1,3-cycloaddition reaction of nitrones has been reported.[159]1,3-cycloaddition of chiral nitrones with dipolarophile such as monosubstituted alkene and phenyl isocyanate is cis-stereoselective.[200] This isomerism is because of the approach of 1,3 dipole of E/Z nitrone configuration and dipolarophile in endo or exo fashion.[139] Separately, in either case, the nitrone may approach either the *re* or *s*i face of the dipolarophile.[201]

The treatment of ethyl (1,1-diphenylmethylene)-carbamate with hydroxylamine in alkoxide ion results in the formation of 3,3-diphenyl-1,2,4-oxadiazolidin-5-one. However, when (1,1-diphenylmethylene)-carbamoyl chloride was treated with hydroxylamine, an isomeric product, 5,5-diphenyl-1,2,4-oxadiazolidin-3-one was obtained. Le Fur et al. (1981),[202] in the experiment of the two compounds as anticonvulsants in the mouse corneal electroshock method revealed that 5,5-diphenyl-

1,2,4-oxadiazolidin-3-one was 1/3, and 3,3-diphenyl-1,2,4-oxadiazolidin-5-one was 1/6 as active as 5,5-diphenylhydantoin.[203]

Structural Confirmation of Oxadiazolidinone

Goldschmidt and Beckmann, in 1890,[149] were the first to report about cycloaddition of nitrones with isocyanates. Prior to 1987, researchers were contemplating the type of cycloadduct that would be formed when nitrones undergo cycloaddition reaction with isocyanates. Nevertheless, with the aid of ^{15}N NMR spectroscopy and X-ray crystallography, it revealed that the cycloadduct synthesized from various aryl-substituted nitrones and aryl-isocyanates are substituted 1,2,4-oxadiazolidinones[204–206] and not previously reported 1,3,4-oxadiazolidinones[205] (figure 10).

1,2,4-oxadiazolidinone 1,3,4-oxadiazolidinone

Figure 10: Oxadiazolidinone scaffold numenclature[205]

Goldschmidt et al.(1890) attributed the 1,2,4-oxadiaxolidinone structure to the cycloadduct of N-benzyl-C-phenylnitrone with phenyl isocyanate on the grounds of its transformation into N-benzyl-N-phenylbenzamidine as it reacted with sodium methoxide.[206] An intensive study by Huisgen et al. (1969), on 1,3-dipolar cycloaddition, reported other nitrone-isocyanate adduct and attributed the structure as an adduct of N-methyl-C-phenylnitrone with phenyl isocyanates.[200] The low-resolution mass spectrum in which m/z fragment corresponding to the ion $PhC=N^{+}Ph$ was the most outstanding confirmation for this structure.

Preparation of Oxadiazolidinones

There are several procedures on the synthesis of 1,2,4-oxadizolidinone.[207–209] Nevertheless, it is challenging to obtain a general method for their preparation in enantiomerically pure form.[210] In the search for the synthesis of new potential drugs, the first procedure for the synthesis of enantiomerically pure oxadiazolidinone through cycloaddition of nitrone with isocyanates was developed.[57] The usefulness of isocyanate in various synthetic processes cannot be overstated as they play a vital role in cycloaddition with nitrones to generate heterocycles such as oxadiazolidinones, which has potential pharmaceutical and biological activity.[211] Isocyanates are also employed in polymer industries and academia due to their high yield and the lack of formation of by-products associated with their usage.[212] The treatment of nitrones with isocyanates in appropriate solvents furnished a stable 1,2,4-oxadiazolidin-5-one.[159,213]

In order to produce oxadiazolidinone, we must take into consideration the availability of effective methodology for their successful and convenient synthesis in optically active form.[139,214] Also, the heterocycle would have to be configurationally stable, most especially, in acidic and alkaline condition.[156]

Specific Goal

Our goal is to synthesize oxadiazolidinone derivatives (as a non-β-lactam β-lactamase inhibitor) with the potential of being used in combination with β-lactam antibiotics. This is intended to restore the effectiveness of some β-lactam antibiotic drugs that lost their potency, due to the continuous evolution of β-lactamase enzyme when used alone.

CHAPTER 2

EXPERIMENTAL SECTION

Materials

Phenyl isocyanate, ethyl isothiocyanato acetate, nitromethane, nitroethane, and benzaldehyde were purchased from Alfa Aesar Chemical Company. The deuterated solvents (DMSO-d_6 and $CDCl_3$) were bought from Sigma-Aldrich Chemical Company. Solvents (DCM, absolute ethanol, 95% ethanol, ethyl acetate, toluene, hexane, petroleum ether, diethyl ether, chloroform, Acetone, methanol, anhydrous tetrahydrofuran (THF), anhydrous dioxane, and benzene), Salts ($MgSO_4$, Na_2SO_4, NaCl, MOPS buffer), and other reagents (glacial acetic acid, hydrochloric acid, sodium hydroxide, lithium hydroxide, zinc powder and ammonium chloride) were obtained from Alfa Aesar Chemical Company. Enzyme kinetic essay (Nitrocefin (NCF), and bovine serum albumin (BSA)) used for enzyme kinetics assay were purchased from Bio Vision Incorporated. Enzymes (TEM-1 β lactamase, and P99 β lactamase) were ordered from Invitrogen and Sigma-Aldrich Chemical Company, respectively.

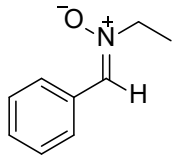
Instrumentation

The ^{1}H and ^{13}C nuclear magnetic resonance (NMR), were reported in $CDCl_3$ and DMSO-d_6 on a Joel Eclipse 400 MHz spectrometer; chemical shifts were recorded in part per million (ppm) with reference to residual signal of $CDCl_3$ and DMSO-d_6 at δ_H/δ_C 7.25/76.8 ppm and 2.50/39.5 ppm respectively; NMR data were reported as follows: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplets, dd = doublet of doublet; Coupling constant in Hz. IR analysis was carried out using Shimadzu IR prestige-21 FTIR spectrometer; absorptions were given in wavenumbers (cm^{-1}). UV/Vis absorbance and kinetic analysis were done using 8454 UV/Vis spectrometer with a PCB 1500 water Peltier system by Agilent Technologies and quartz 1000 µL cuvettes having 1 cm path length was

utilized. The melting points were ascertained using an IA9100 series. Thermo Scientific Electrothermal Digital Melting point instrument. The molecular weight of compounds was determined using a Shimadzu GC-MS - QP 2010 plus; chromatogram and spectra were recorded in 70 eV and peaks were given in percent (m/z). Chromatographic purification of the product was done on E. Merck Silica gel using a force flow of eluent at 0.3-0.5 bar pressure.[215] Concentration under reduced pressure was performed by rotary evaporation at 40-50 $^{\circ}$C in appropriate pressure. Pure compounds were subsequently dried under high vacuum.[57]

Chemistry

General procedure for the synthesis of (Z)-N-(benzylidene) ethanamine oxide (**1a**)

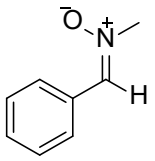


1.80 mL (25 mmol) of nitroethane, 2.80 mL (27.5 mmol) of benzaldehyde and 1.87 g (35 mmol) of ammonium chloride were added to 150 mL EtOH/H_2O (1:1) and cooled to 0 °C. 1.64 g (25 mmol) of Zn powder was added to the reaction mixture. After 24 hours, the mixture was filtered through a celite pad and washed with dichloromethane (DCM). The filtrate was extracted with dichloromethane, and the combined organic layer was dried with anhydrous sodium sulfate. After the salt was filtered off, it was concentrated and purified by vacuum distillation under reduced pressure. It was further purified with a fractional column and monitored with TLC to obtain the desired product in good amount.

Yield: 2.18 g (58.5%). **m.p.**: 48-50 °C

^{1}H-NMR (400 MHz $CDCl_3$): δ = 1.19-1.23 (t, 3H), 3.60-3.65 (q, 2H), 7.08-7.09 (q, 3H), 7.17 (s, 1H), 7.96-8.01 (q, 2H). **^{13}C-NMR** (400 MHz $CDCl_3$): δ = 13.43, 61.7 (C-N), 128.3-128.3, 130.1 (C=N), 130.6, 133.4. **IR** (KBr): $\tilde{\nu}$ (cm^{-1}) = 3052, 2927 (N=C-H), 2360, 1736, 1519 (C=N), 1448, 1322 (CNO), 1155 (N→O), 981, 756, 692. **UV** λ max ($CHCl_3$): 298, 209 nm. **GC-MS** (70 eV) m/z (%): 149 (85) [M^+], 148 (100), 120 (41), 105 (16), 91 (23), 77 (33), 65 (17), 50 (13).

General method for the preparation of (Z)-N-(benzylidene) methanamine oxide (**1b**)

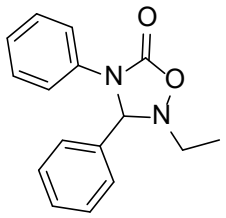


To a stirred solution of 5.10 mL of benzaldehyde (1 equiv, 50 mmol) in 319 mL of 95% ethanol was added 5.35 mL (2 equiv, 100 mmol) of nitromethane followed by 9.8 g (3 equiv, 150 mmol) of Zn powder. Finally, 18.9 mL (6 equiv, 300 mmol) of acetic acid was added drop-wise at 0 °C, and the mixture was stirred at room temperature for 24 hours. Afterwards, the solvent was rotary-evaporated, and the zinc was filtered off through a plug of celite pad eluted with DCM. The resulting liquid was purified by flash column chromatography elution of ethyl acetate with hexane (2:1) to obtain a pure nitrone after evaporation of the column fraction. 6.03 g of the product was obtained.

Yield: 6.03 g (89%). **m.p.**: 75-77 °C.

^{1}H-NMR (400 MHz $CDCl_3$) δ = 3.88 (s, 3H), 7.37 (s, 1H) 7.40-7.42 (t, 3H), 8.19-8.21 (q, 2H). **^{13}C-NMR** (400 MHz $CDCl_3$) δ = 54.4 (C-N), 128.6, 130.3 (C=N), 130.7, 135.8. **IR** (KBr): $\tilde{\nu}$ (cm^{-1}) = 3054 (N=C-H), 2360, 1596 (C=N), 1403 (CNO), 1168 (N→O), 1025, 944, 757, 676. **UV** λ max ($CHCl_3$): 217, 210 nm. **GC-MS** (70 eV) m/z (%): 135 (66) [M^+], 134 (100), 118 (21), 107 (20), 89 (16), 77 (31), 65 (14), 50 (11), 42 (21).

General procedure for the preparation of 2-ethly-3,4-diphenyl-1,2,4-oxadiazolidin-5-one (**2a**)

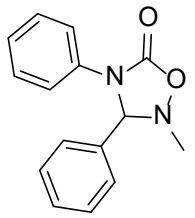


To 1.0 g (6.7 mmol) of **1a** in 2.5 mL of DCM, at room temperature, is added 0.73 mL (6.7 mmol) of phenyl Isocyanate. The solution was stirred at room temperature for 38 hours; after evaporating the solvent, it was concentrated in vacuum to obtain an off-white solid. The solid was then triturated in methanol for 3 hours, filtered off, washed with hexane, and dried in vacuum to obtain 1.35 g of the targeted compound.

Yield 1.35 g (75%). **m.p**.: 118-119 °C.

^{1}H-NMR (400 MHz $CDCl_3$) δ =1.29-1.33 (t, 3H), 3.14-3.28 (d, j=32 Hz, 2H), 5.68 (s, 1H), 7.08-7.15 (d, 2H), 7.28-7.38 (m, 8H). **^{13}C-NMR** (400 MHz $CDCl_3$) δ = 12.1, 53.6, 83.5 (benzylic carbon), 120.7, 125.3, 127.2, 129.2, 129.3, 129.9, 135.7, 136.2, 154.8 (C=O). **IR** (KBr): $\tilde{\nu}$ (cm^{-1}) = 2978, 2360, 2341, 1754 (C=O), 1598, 1500, 1456, 1378, 1222, 1130, 970, 842, 754, 694. **UV** λ max ($CHCl_3$): 241, 197 nm. **GC-MS** (70 eV) m/z (%): 267 (1.6) [M^+], 224 (53), 223 (100), 180 (57), 148 (42), 119 (87), 104 (82), 77 (96), 44 (30).

Preparation of 2-methyl-3,4-diphenyl-1,2,4-oxadiazolidin-5-one (**2b**)



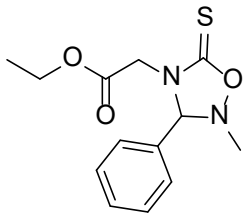
0.68 g (5 mmol) of nitrone **1b** in 20 mL dioxane was placed in 100 mL round bottom flask and 1.10 mL (10 mmol) of phenyl isocyanate was added to the mixture. The reaction mixture was heated at 60-75 ˚C for 60 minutes. The solvent was evaporated using rotary evaporator, and the product was crystallized from absolute ethanol.

Yield: 0.42 g (33%). **m.p.**: 119-121 ˚C.

^{1}H-NMR (400 MHz $CDCl_3$): δ = 3.1 (s, 3H), 5.58 (s, 1H), 7.10-7.14 (t, 1H), 7.28-7.31 (m, 4H), 7.33-7.38 (d, 5H). **^{13}C-NMR** (400 MHz $CDCl_3$): δ = 45.8, 85.6 (benzylic carbon), 120.2, 121.0, 125.5, 127.3, 129.3, 130.1, 135.2, 136.1, 154.8(C=O). **IR** (KBr): $\tilde{\nu}$ (cm^{-1}) = 3343, 3037, 2348, 1758 (C=O), 1702, 1596, 1546, 1498, 1446, 1382, 1311, 1230, 1128, 840, 754, 694. **UV** λ max ($CHCl_3$): 243,194 nm.

GC-MS (70 eV) m/z (%): 254 (29) [M^+], 207 (75). 131 (20), 88 (100), 57 (33), 43 (23).

Synthesis ethyl 2-(2-methyl-3-phenyl-5-thioxo-1,2,4-oxadiazolidin-4-yl) acetate (**2c**)

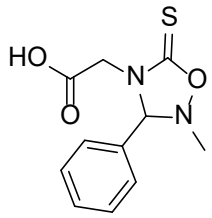


2.7 g (20 mmol) of nitrone **1b** in 40 mL dioxane was placed in 100 mL round bottom flask and 3 mL (24.2 mmol) of ethyl isothiocyanato acetate was added to the mixture. The reaction mixture was heated at 60-75 °C for 3.5 hours, and the solvent was evaporated afterward. The crude product was purified by flash column chromatography to obtain 3.7 g of a pale-yellow viscous liquid.

Yield: 3.7 g (66%). Pale yellow oil.

^{1}H-NMR (400 MHz $CDCl_3$): δ = 1.23-1.26 (t, 3H), 3.01 (s, 3H), 3.49-3.53 (d, j=16, 1H), 4.16-4.20 (q, 2H), 4.71-4.75 (d, j=16, 1H), 5.53 (s, 1H), 7.34-7.43 (dd, 5H). **^{1}H-NMR** (400 MHz $CDCl_3$): δ = 14.2, 45.1, 46.2, 62.0, 87.3 (benzylic carbon), 128.1, 129.2, 130.9, 133.0, 167.3 (C=S), 185.0 (C=O). **IR** (KBr): $\tilde{\nu}$ (cm^{-1}) = 2983, 2345, 1743, 1475, 1396, 1315, 1205, 1126, 1022, 964, 698. **UV** λ max ($CHCl_3$): 253, 209 nm. **GC-MS** (70 eV) m/z (%): 280 (2) [M^+], 244 (12), 220 (13), 147 (19), 118 (90) 91 (100), 83 (31) 55 (29).

Synthesis of 2-(methyl-3-phenyl-5-thioxo-1,2,4-oxadiazolidin-4-yl) acetic acid (**3**)



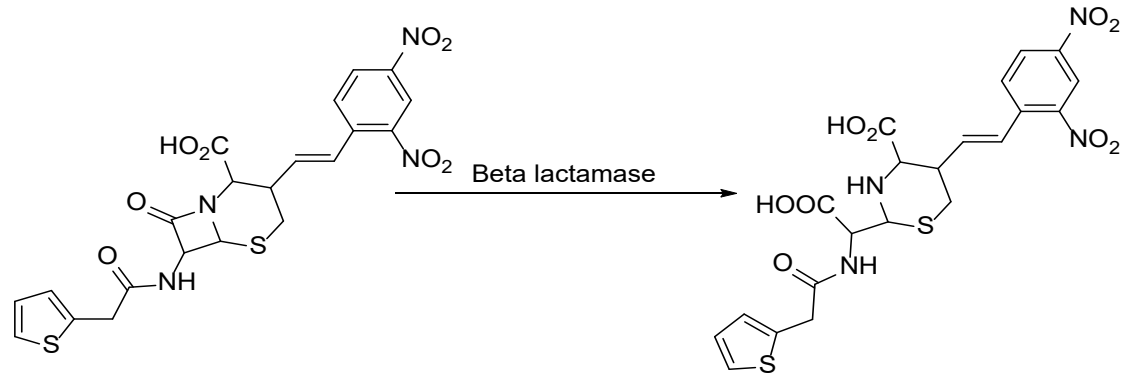
150 mg (0.52 mmol) of **2c** was dissolved in 10 mL tetrahydrofuran and 50 mg (2.1 mmol) of Lithium hydroxide in 5 mL of Water was gradually added to the solution containing the **2c**. The reaction mixture was stirred at room temperature for 30 minutes. Afterward, ammonium chloride was added, and the mixture was immediately extracted with ethyl acetate three times. The combined organic layer was dried using anhydrous sodium sulfate and the solvent removed in vacuo to obtain 100 mg of the product.

Yield: 100 mg (77%). Viscous oil.

^{1}H-NMR (400 MHz $CDCl_3$): δ =3.0 (s, 3H), 3.49-3.53 (d, j=16 Hz, 1H), 4.71-4.75 (d, j=16 Hz, 1H), 5.51 (s, 1H), 7.34-7.43 (dd, 5H), 9.98 (s, H). **^{13}C-NMR** (400 MHz $CDCl_3$): δ = 44.9, 46.2, 87.4 (benzylic carbon), 128.3, 129.5, 131.0, 132.8, 171.2 (C=S), 185.0 (C=O). **IR** (KBr): $\tilde{\nu}$ (cm^{-1}) = 2964, 2360, 1731, 1632, 1481, 1316, 1241, 1041, 700, 698. **UV** λ max ($CHCl_3$): 211, 194 nm. **GC-MS** (70 eV) m/z (%): 252 (100) [M^+], 207 (70), 185 (67), 146 (58), 133 (64), 117 (74), 91 (67), 44 (76).

Enzyme Inhibition Kinetics

Enzymes inhibition kinetics were performed on the oxadiazolidinone analogs to determine their effectiveness against β-lactamase enzymes, percentage inhibition, and enzyme residual activity. This was done using a spectrometric analysis of nitrocefin (NCF) at 485 nm at 30 °C. NCF is chromogenic in nature, and it is known to absorb light at 385 nm giving off an orange-yellow color which eventually absorbs light at a higher wavelength, due to hydrolysis by β-lactamase (scheme 9). The method used in the preparation of MOPS, BSA, substrate, and enzymes were cited from Osazee, J. 2016.[6]



Scheme 9: Hydrolysis of NCF by β-lactamase.

Preparation of MOPS stock solution (0.02 M)

8.37 g of MOPS was dissolved in 500 mL of water. Furthermore, 40 mL of the stock solution was diluted with 200 mL of water to obtain a 0.02 M solution.

Preparation of BSA (1% and 0.1%) in MOPS buffer

100 mg of BSA was dissolved in 10 mL of MOPS buffer. Then 1% of the BSA was used to dilute the enzyme. 1% of the BSA in the MOPS buffer was diluted in 9 mL of MOPS buffer to obtain 0.1% BSA, which was used for the essay.

Preparation of NCF (5 nM)

2.582 g of the substrate (NCF) was dissolved in 1 mL of 0.02 M MOPS buffer which was then diluted to 100 μM in the enzyme mixture.

Preparation of TEM-1 β-lactamase enzyme

0.56 mg/mL of the enzyme (TEM-1) was diluted to 47.5 nm using 1% BSA. 2 μL and was used for the essay to obtain 0.25 nm enzyme concentration.

Preparation of P99 β-lactamase enzyme

1.4 mg of the enzyme (P99) was dissolved in 1 mL of 1% BSA in MOPS buffer to obtain a stock solution of 300 μM. The stock solution was then diluted and used for enzyme kinetic essay.

In the essay used for enzyme kinetic analysis, NCF was observed at 100 μM, TEM-1 was at 0.25 nM, and P99 was at 0.2 nM in 20 nM MOPS buffer (pH=7.5), having 0.1% BSA in the MOPS buffer in the final volume of 600 μL. Clavulanate was used as our control at a concentration of 120 nM.
After the initial rate was observed for 3 minutes on the spectrometer, the percentage enzyme inhibition was calculated with the formula shown below:

$$\%\ \text{enzyme inhition} = \frac{\text{Initial rate of enzyme with inhibitor}}{\text{Initial rate of enzyme without inhibitor}} \times 100\%$$

Enzyme residual activity was calculated by subtracting the % enzyme inhibition from 100%.

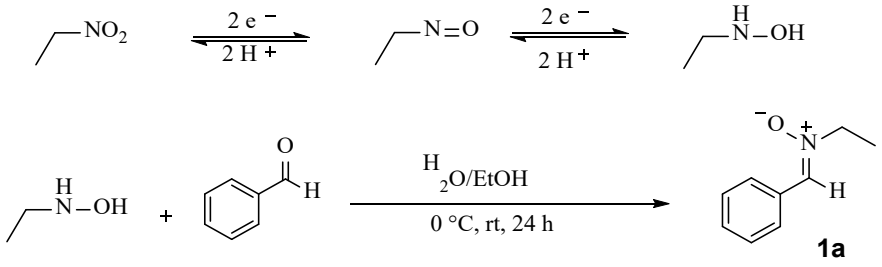
CHAPTER 3

RESULTS AND DISCUSSION

Synthesis of Nitrones

Preparation of Nitrone **1a**

(Z)-N-(benzylidene)-1-ethanamine oxide was prepared as a starting material for the synthesis of oxadiazolidinone **2a** by the reduction of nitroethane to N-substituted hydroxylamine with zinc dust in ethanol solution of ammonium chloride (NH_4Cl) followed by the condensation with benzaldehyde *in situ* to furnish nitrone **1a** without the use of nitrogen gas **(**scheme 10).[118]



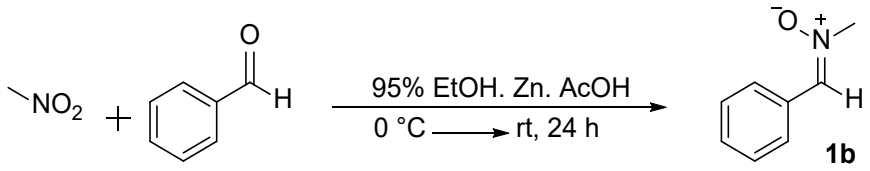
Scheme 10: Synthesis of nitrone **1a**

It was purified using vacuum distillation under reduced pressure to obtain 58% yield. The ^1H nuclear magnetic resonance (NMR) in $CDCl_3$ showed a triplet from the ($-CH_3$) group at (1.19-1.23 ppm), and a quartet from ($-CH_2$) at (3.60-3.65 ppm). The singlet at 7.17 ppm corresponds to the proton from (-N=C-H).[118] The two multiplets at (7.08-7.09 ppm) and (7.98-8.01 ppm) are signals from the benzene ring. The singlet at 1.70 ppm could be coming from the (-OH) signal of water or ethanol that was not completely removed after their use as solvents. Also, the small triplet and quartet observed at (0.91-1.00 ppm) and (3.69-3.80 ppm) are from ($-CH_3$) and ($-CH_2$) of ethanol (Appendix A1).

IR, ^{13}C NMR, and GC-MS were also used to ascertain the structure of nitrone **1a**. The IR characteristic absorption at 2927 cm^{-1}, 1519 cm^{-1}, and 1155 cm^{-1} from (-N=C-H), (C=N), and (N-O) respectively also confirms the structure of nitrone **1a**. The ^{13}C signal of (-C=N-H) at 133.4 ppm gives an insight into **1a**. In the gas chromatography and mass spectrometry (GC-MS) analysis, the parent peak was seen at a retention time (R.T) of 9.3 minutes with a peak intensity of 85% which corresponds to the exact molecular weight 149 $gmol^{-1}$ of the desired product **1a** (Appendix A2-A4).

Preparation of Nitrone **1b**

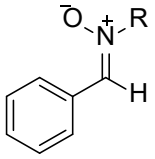
(Z)-N-benzylidenemethanamine oxide was prepared as a starting material for the synthesis of oxadiazolidinone **2b, 2c,** and **3** by the reduction of nitromethane to N-substituted hydroxylamine with zinc dust, followed by subsequent reaction with benzaldehyde *in situ* in an alcoholic solution of acetic acid (scheme 11).



Scheme 11: Preparation of nitrone **1b**

The product was purified by flash column chromatography using elution of ethyl acetate to hexane (2:1) to obtain 89% yield of the pure nitrone **1b** after rotary evaporation of column fraction. The higher yield observed for **1b,** as seen in table 1, may be because nitromethane has one carbon less than nitroethane. The shorter carbon chain length of nitromethane could have possibly enhanced their reaction with benzaldehyde.

Table 1: Nitrones, UV λ max, and infrared data



Nitrones	R	Yield (%)	m.p. (°C)	Time (h)	UV λ max (nm)	N=C-H NMR (ppm)		IR frequency (cm^{-1})	
						^{1}H	^{13}C	C=N	N-O
1a	CH_3	58	48-50	24	298	7.17	133.4	1519	1155
1b	CH_3CH_2	89	75-77	24	217	7.37	135.8	1596	1168

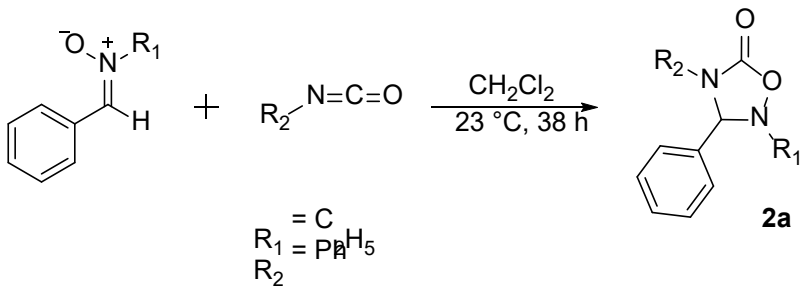
The ^{1}H NMR in $CDCl_3$ showed the (-CH_3)-proton as a singlet at 3.88 ppm. The proton is relatively deshielded since it is directly attached to an electronegative sp^2 Nitrogen. This accounts for the relatively high chemical shift (δ) for the (-CH_3)-proton of nitrone **1b** observed in appendix B1. The singlet at 7.37 ppm corresponds with the characteristic (-N=C-H)-proton signal. The benzene ring of **1b** gave off two multiplets at (7.40-7.42 ppm) and (8.19-8.20 ppm), just like nitrone **1a** (Appendix B1). The (-N=C-H) function was represented by a peak at 135.8 ppm in the ^{13}C NMR **1b**.

Structural elucidation was further done using IR and GC-MS. The IR spectrum showed a (-N=C-H) stretch at 3054 cm^{-1}, (-C=N) stretch at 1596 cm^{-1}, (-C=N-O) at 1403 cm^{-1}, and (N→O) stretch at 1168 cm^{-1} confirming the structure of **1b**[129,139]. The GC-MS showed a parent peak at a retention time of 8.4 minutes with a peak area of 66.10% indicating the exact molecular weight of 135 $gmol^{-1}$ for nitrone **1b** (Appendix B2-B4).

Synthesis of 1,2,4-oxadiazolidinones

Synthesis of 1,2,4-oxadiazolidin-5-one **2a**

The treatment of nitrone **1a** with commercially available phenyl isocyanate in dichloromethane (DCM) precedes through cycloaddition to afford 78% yield of 2-ethyl-3,4-diphenyl-1,2,4-oxadiaxolidin-5-one (**2a**) after trituration in methanol for 3 hours.[57,124,139,156,157] (scheme 12).



Scheme 12: Synthesis of oxadiazolidinone **2a**.

Phenyl isocyanate was used because it is a good dipolarophile, and it plays a vital role in cycloaddition to generate heterocycles with biological activity. Furthermore, their usage enhances the production of compounds in high yield without byproducts.[211]

Oxadiazolidinone **2a** was obtained as a colorless solid and characterized by using ^{1}H, and ^{13}C NMR, IR, and mass spectrometry. ^{1}H NMR in $CDCl_3$ showed a triplet at (1.29-1.33 ppm) from ($-CH_3$) group. A doublet at (3.13-3.28 ppm, j=11.3 Hz)[216] from ($-CH_2$) was observed instead of a quartet due to the germinal coupling effect of each proton in the ($-CH_2$) on one another. The (-C-H)-protons of the two benzene rings were observed as multiplets at 7.08-7.18 ppm and 7.28-7.38 ppm.

To demonstrate the cycloaddition that occurred,[116] a section of ^{1}H NMR of **1a** is compared with the corresponding section of ^{1}H NMR of **2a** in (figure 11). The singlet of (-N=C-H)-proton in nitrone **1a** at 7.17 ppm is seen after cycloaddition as a singlet of oxadiazolidinone **2a** at 5.68 ppm (Appendix C1).

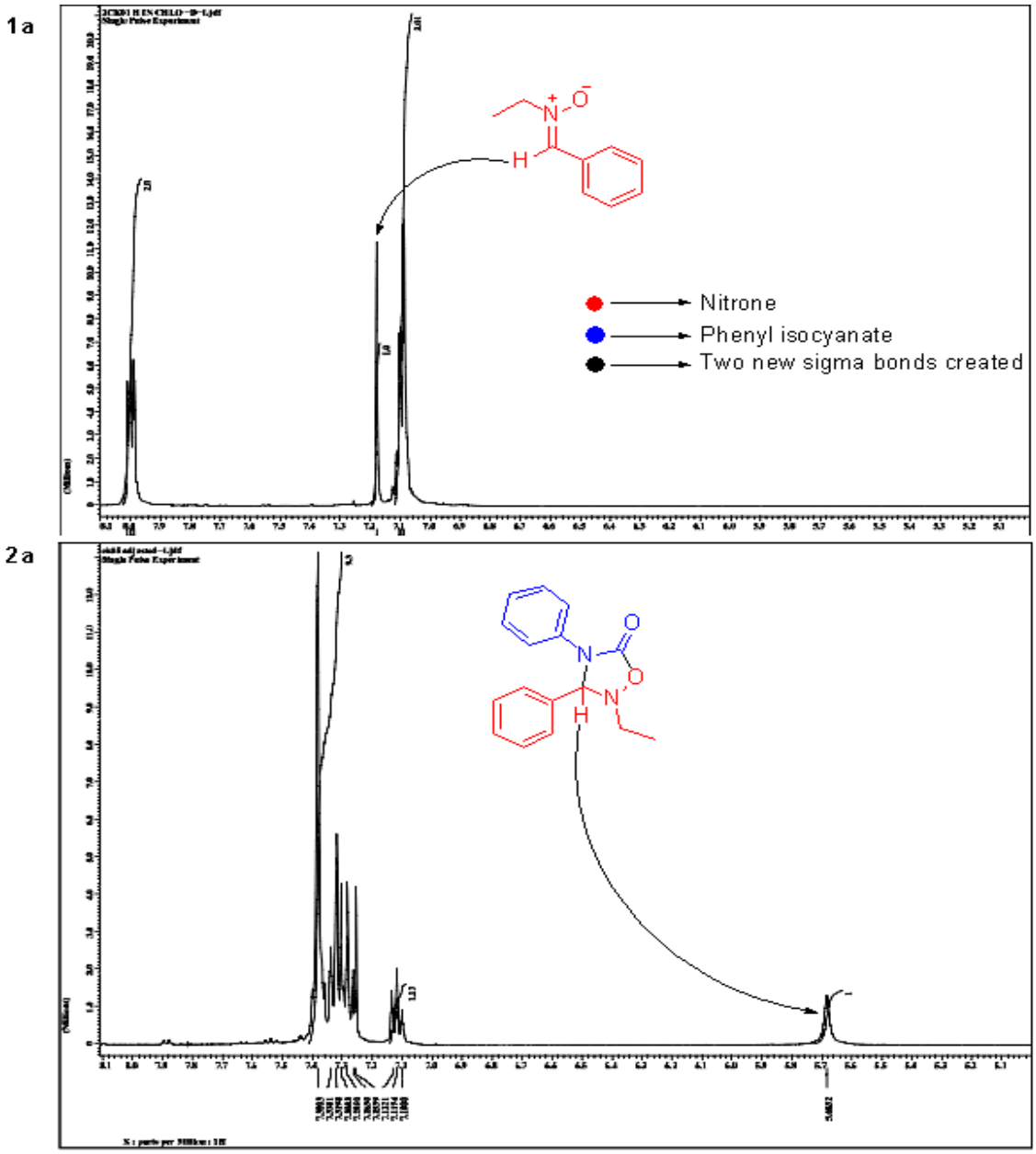
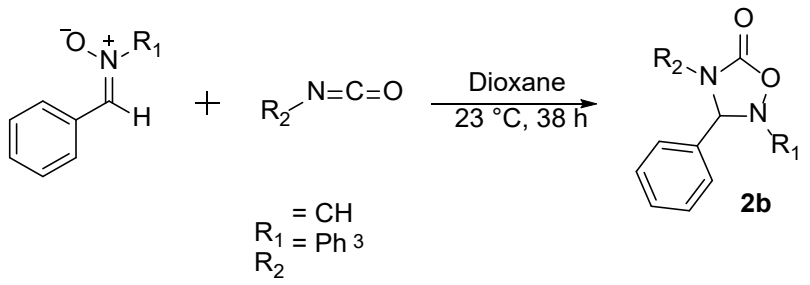


Figure 11: ^{1}H NMR spectra of **1a** and corresponding oxadiazolidinone **2a**

The ^{13}C NMR peaks at 154.8 and 83.5 ppm correspond to the signals from (-C=O) and the benzylic carbon, respectively. The UV λ max analysis revealed that **2a** absorbs at 241 and 197 nm. In the IR spectrum, the signal at 1155cm^{-1} of N→O vibration of nitrone **1a** drastically decreased while a strong signal at 1754 cm^{-1} of C=O vibration appeared, confirming the formation of the five-membered ring structure of **2a** (figure 11). Also, the absence of (-N=C=O) absorption at 2300 cm^{-1} in the IR spectrum of **2a** distinctively indicates that all phenyl isocyanate was consumed.[199] GC-MS showed a peak at RT of 16.6 minutes giving off a molecular peak of 267 gmol^{-1} for **2a** (Appendix C2-C4).

Synthesis of 1,2,4-oxadiazolidin-5-one **2b**

2-methyl-3,4-diphenyl-1,2,4-oxadiazolidin-5-one (**2b**) was synthesized through 1,3-dipolar cycloaddition of nitrone **1b** with phenyl isocyanate using the same procedure applied to produce **2a**. However, in this reaction dioxane was used as the solvent to obtain a 33% yield of the pure **2b** (scheme 13).



Scheme 13: Synthesis of oxadiazolidinone **2b**

^{1}H NMR in $CDCl_3$ showed a singlet from (-CH_3) group at 3.04 ppm and the benzylic proton at 5.58 ppm which is in line with literature data.[198] The (-CH) of the two benzene rings of **2b** gave a multiplets at (7.10-7.18 ppm) and (7.28-7.38 ppm), just like the benzene rings of **2a**. The ^{13}C NMR of (-

N=C-H) signal at 135.8 ppm in nitrone **1b** appeared at 85.6 ppm as the benzylic carbon for 2-methyl-3,4-diphenyl-1,2,4-oxadiazolidin-5-one (**2b**) (figure 12).

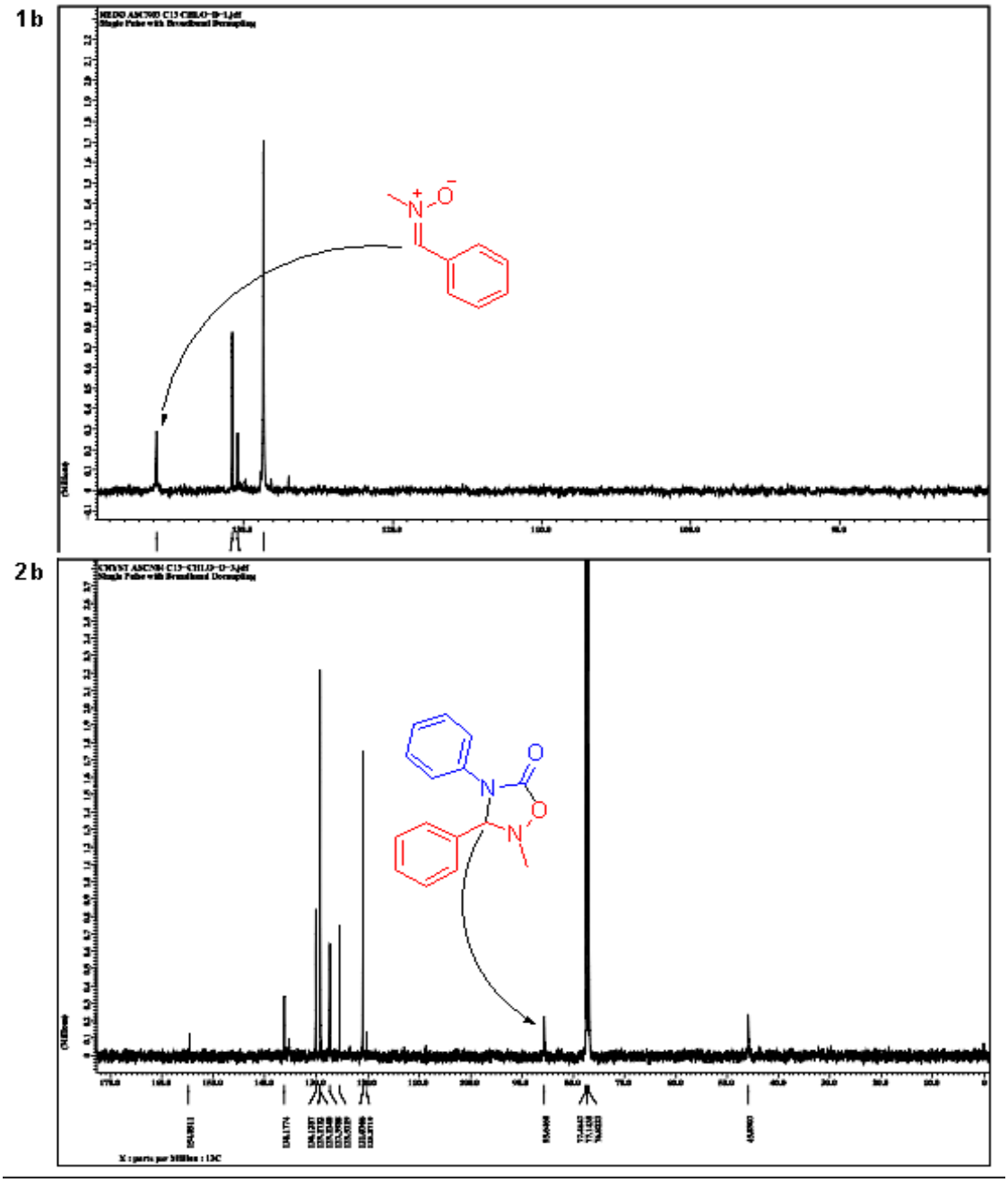


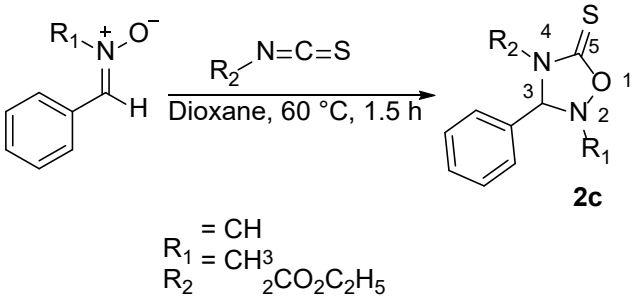
Figure 12: ^{13}C NMR spectra of **1b** and corresponding cycloadduct **2b**

The transformation from sp^2 carbon in nitrone **1b** to sp^3 carbon in **2b** may be responsible for the shielding towards the upfield region. The ^{13}C signal at 154.8 ppm corresponding to the presence of (-C=O) shows a stretching at 1754 cm^{-1} in the IR spectrum, hence confirming the structure for **2b**. UV λ max indicates that **2b** absorbs at 243 and 194 nm. In the GC-MS analysis, the exact molecular weight of 254 $gmol^{-1}$ indicating **2b** was seen at a retention time of 14.2 minutes with a peak intensity of 28.49% (Appendix D1-D4).

Synthesis of ethyl 2-(2-methyl-3-phenyl -5-thioxo-1,2,4-oxadiazolidin-4-yl) acetate (**2c**)

2c was obtained in good yield when nitrone **1b** was treated with commercially available ethyl isothiocyanato acetate in dioxane at 60 °C for 3.5 hours. Just like other analogs, the structure of **2c** was deduced from their ^1H and ^{13}C NMR, IR, and GC-MS analysis.

The ^1H NMR in $CDCl_3$ showed two sharp singlets: from ($-N-CH_3$) at 3.01 ppm and from the characteristic benzylic proton at 5.53 ppm. The ($-CH_2$) attached to C=O and N4 gave off two doublets, resulting from the germinal coupling at (3.48-3.54 ppm and 4.71-4.75 ppm, j=16 Hz). The two multiplets at (1.23-1.28 ppm) and (4.16-4.22 ppm) correspond to ($-CH_3$) and ($-CH_2$), respectively. The benzene ring appeared as a doublet of doublet at 7.34-7.43 ppm. The singlet at 2.01 is possibly from the ($-CH_3$) of ethyl acetate used for flash column chromatography (Appendix E1).

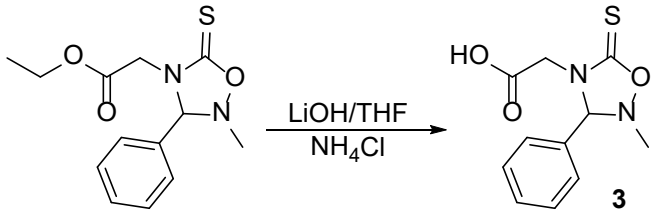


Scheme 14: Synthesis of compound **2c**

The ^{13}C NMR signal of **2c** at 185.0 and 167.3 ppm corresponds to (-C=O) and (-C=S) functionality, respectively. The C=O is more deshielded than C=S because oxygen in the carbonyl group is more electronegative than Sulphur in the thionyl group. The strong (-C=O) absorption at 1743 cm^{-1} and (-C=S) at 1475 cm^{-1} in the IR spectrum further confirms the five-membered 2-ethyl-(2-methyl-3-phenyl-5-thioxo-1,2,4-oxadiazolidin-4-yl) acetate. The GC-MS of **2c** shows a peak at 11.1 minutes giving a molecular weight of 280 $gmol^{-1}$ corresponding to the exact molecular weight of **2c** (Appendix E2-E4).

Synthesis of 2-(2-methyl-3-phenyl-5-thioxo-1,2,4-oxadiazolidin-4yl) acetic acid (**3)**

The ester functional group (-$COOCH_2CH_3$) in **2c** was hydrolyzed to carboxylic (-COOH) functionality in **3** by the treatment of **2c** with lithium hydroxide (LiOH) in tetrahydrofuran (THF). The pH of the solution was adjusted with ammonium chloride (NH_4Cl), and the crude product was extracted with ethyl acetate to obtain compound **3** in 77% yield.



Scheme 15: Synthesis of compound **3**

Compound **3** was characterized by ^1H and ^{13}C NMR, IR, and GC-MS. To demonstrate that the five-membered heterocycle **3** was retained and hydrolysis was successful, a section of the ^1H NMR spectrum of **2c** in $CDCl_3$ is compared with the corresponding ^1H NMR spectrum of **3** in figure 13.

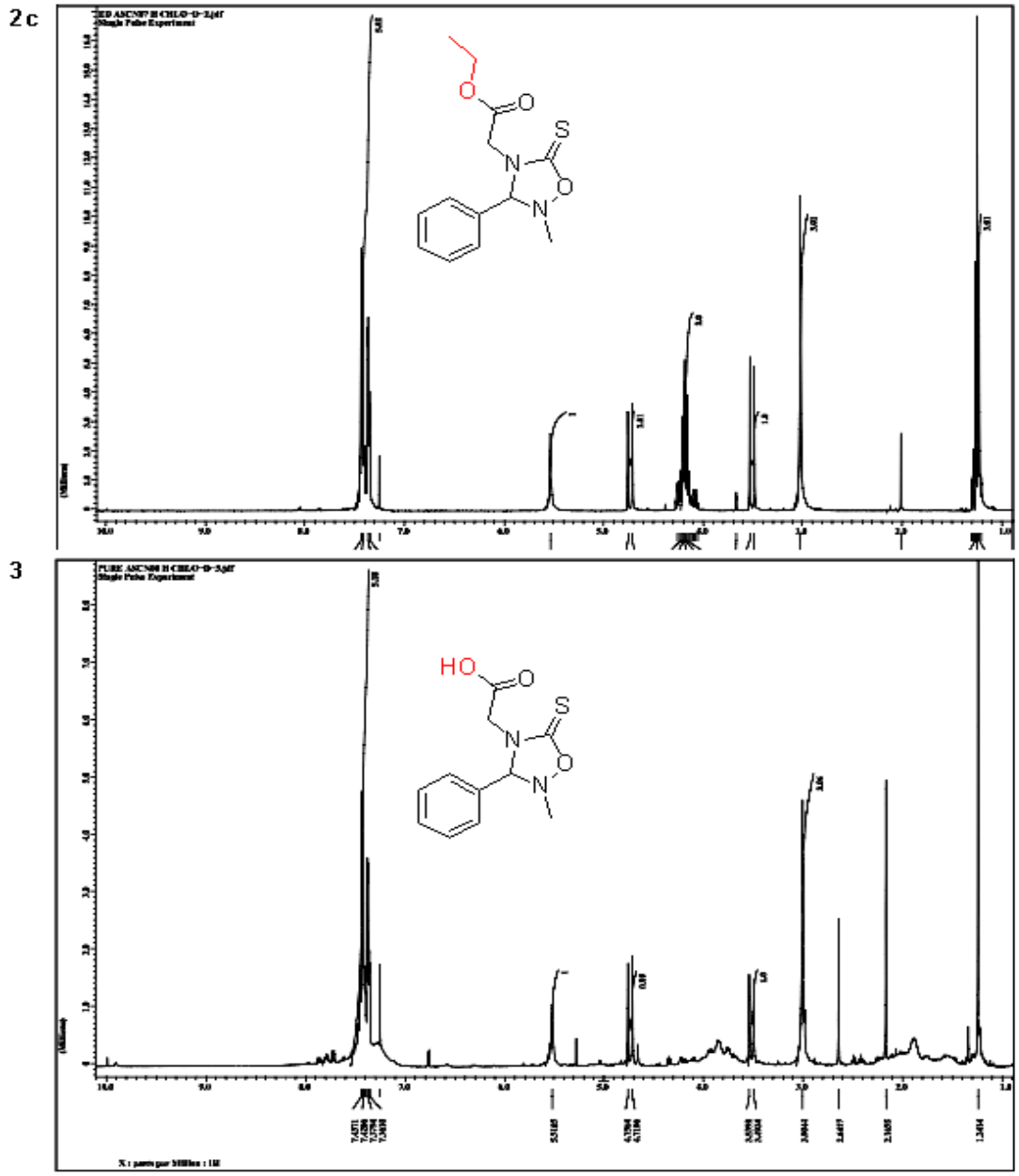


Figure 13: ^{1}H NMR of **2c** and corresponding cycloadduct **3** indicating successful hydrolysis

The signals from (-CH_3) and (-CH_2) of **2c** at (1.23-1.28 ppm) and (4.16- 4.20 ppm) diminished in **3**. Also, the appearance of a singlet at 9.98 ppm corresponds with the proton from (-OH) of carboxylic group. The illustration explained in figure 13 indicates successful hydrolysis of the ester to carboxylic acid (Appendix F1). Furthermore, to illustrate that the five-membered scaffold of **3** was retained in the

process of hydrolysis, the ^{13}C NMR spectrum of **2c** is compared with the corresponding ^{13}C NMR of **3** in figure 14.

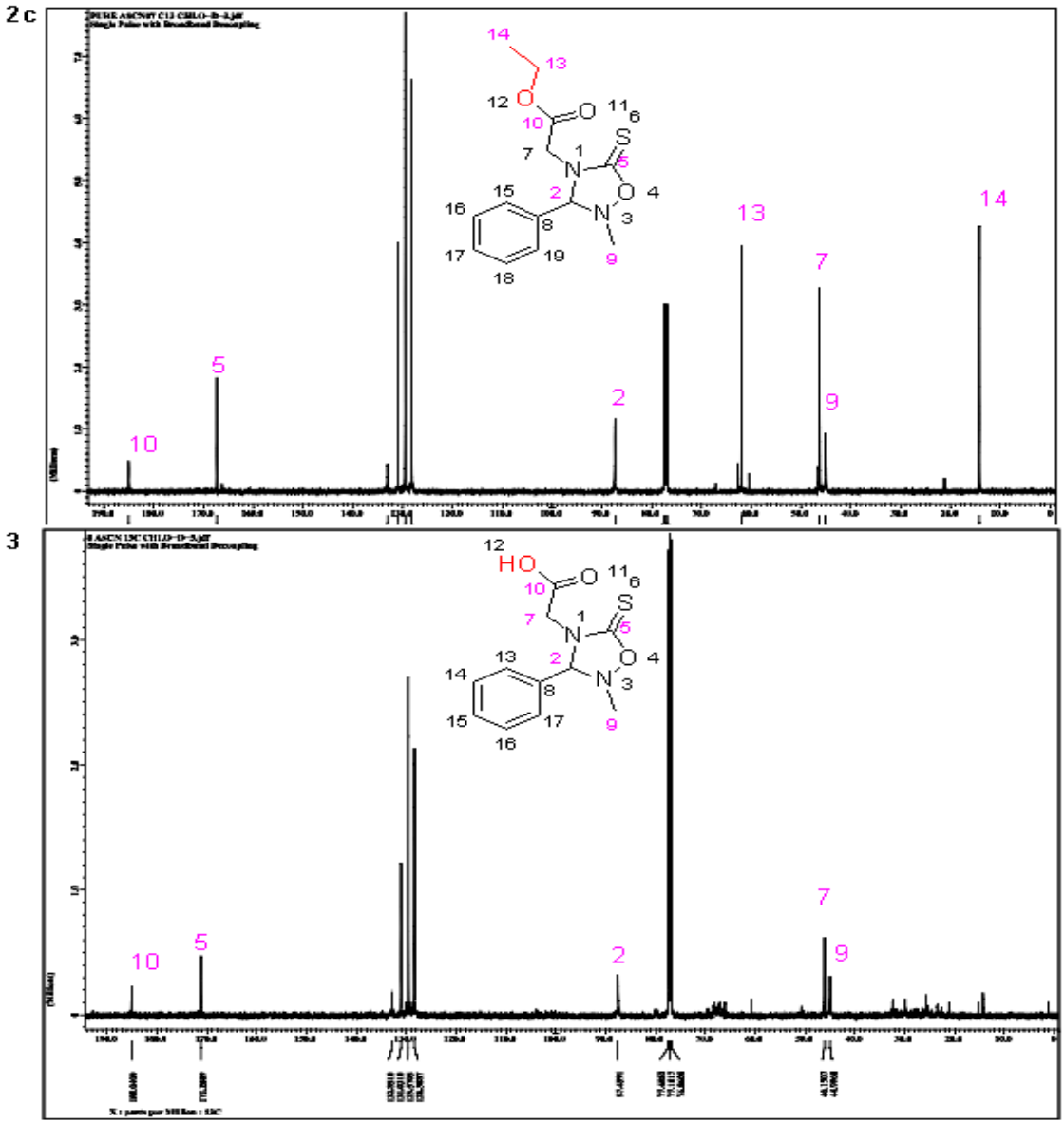


Figure 14: ^{13}C NMR of **2c** with the corresponding product of hydrolysis **3**.

The signals from (-C=O) at 185.0 ppm and (-C=S) at 170 ppm that is observed in both spectra (figure 14) indicates that the five-membered scaffold in **2c** and **3** are still intact. The ^{13}C signals from (-

CH_2-N4) at 46.15 ppm and (-N2-CH_3) at 44.99 ppm are still intact (Figure 15). The IR stretch at 1731 cm^{-1} and 1481 cm^{-1} in table 2 corresponds with C=O and C=S vibration respectively.

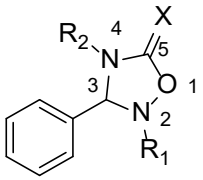


Figure 15: Oxadiazolidinone scaffold

Table 2: Physical properties and spectroscopic data of oxadiazolidinone derivatives

Compound	substituents			m.p. (°C)	Yield (%)	^1H and ^{13}C NMR (ppm)		IR (cm^{-1})	
	R_1	R_2	X			Ph-C-H	Ph-C-H	C=O	C=S
2a	C_2H_5	Ph	O	118-119	75	5.68	83.5	1754	
2b	CH_3	Ph	O	119-121	33	5.58	85.6	1758	
2c	CH_3	$CH_2CO_2C_2H_5$	S	Oil	66	5.53	87.3	1743	1475
3	CH_3	CH_2CO_2H	S	Oil	77	5.51	87.4	1712	1483

In the GC-MS analysis of **3**, the parent peak was observed at a retention time of 12.3 minutes with a peak area of 100% which corresponded to the exact molecular weight of **3.** (Appendix F2-F4).

Biological Activity

In vitro Cytotoxicity Evaluation of Oxadiazolidinone **2a**

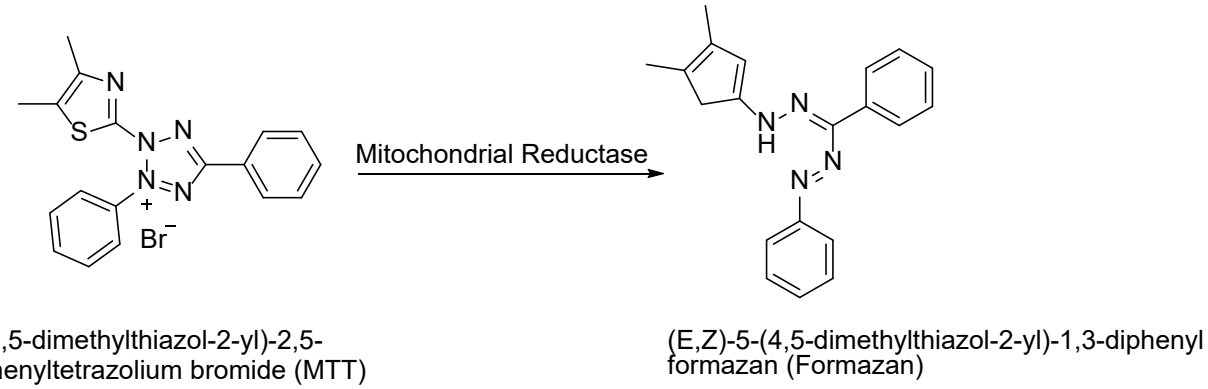
The MTT assay was used to test the in vitro cytotoxicity of **2a** on cancer cell lines obtained from the American Tissue Type Culture Collection (ATTC). These cell lines include IGROV1, OVCAR-4, HS 578T, BT-549, A498, UO-31, 786-0, CAKI-1, UACC-62, SK-MEL-28, SNB-19, SF-268, PC-3, SW-620, and EKVX, and they differ by tissue type and origin of the cells. The result of the analysis revealed that the highest inhibition was observed against renal cancer (table 2).

Table 3: The cytotoxicity essay (%) of **2a** on relative control (100%) against IGROV1, OVCAR-4, HS 578T, BT-549, A498, UO-31, 786-0, CAKI-1, UACC-62, SK-MEL-28, SNB-19, SF-268, PC-3, SW-620, and EKVX

	Cancer types	Cell line	Growth%	% Inhibition
1	Ovarian Cancer	IGROV1	95.59	4.41
		OVCAR-4	95.84	4.16
2	Breast Cancer	HS 578T	93.06	6.94
		BT-549	99.50	0.50
3	Renal Cancer	A498	82.27	17.73
		UO-31	86.72	13.33
		786-0	95.23	4.77
		CAKI-1	94.52	5.48
4	Melanoma	UACC-62	93.93	6.07
		SK-MEL-28	99.89	0.11

5	CNS Cancer	SNB-19	98.91	1.09
		SF-268	96.56	3.44
6	Prostate Cancer	PC-3	98.18	1.82
7	Colon Cancer	SW-620	97.45	2.55
8	Non-Small Cell lung Cancer	EKVX	97.38	2.62

The MTT Assay was done by adding 5-diphenyl-tetrazolium bromide to a solution of cancer cells. The cells that are active will metabolize the MTT solution and form a formazan crystal (scheme 15).



Scheme 16: Metabolism of MTT by active cancer cell to form Formazan crystals

The crystals are dissolved in a solution and their optical density measured at 570 nm with the aid of a spectrophotometer. The effectiveness of **2a** can be determined because there is a direct relationship between the optical density of the formazan crystal and the number of active cells left in the solution.

Enzyme Inhibition Kinetics

Enzyme inhibition kinetics were performed to determine the percentage of inhibition and residual activity against TEM-1 and P99 β-lactamases in 30 mM MOPS buffer. 3 µL of the enzyme was used for the assay. The initial rate is evident on how active the enzyme is. The higher the rate, the more active the enzyme. If the inhibitor is present, the activity is expected to decrease, like with clavulanate (figure 16). The percentage inhibition and residual activities of the enzymes after incubation with oxadiazolidinone analogs (**2a, 2b, 2c, and 3**) as inhibitors in the presence of chromogenic substrate NCF is shown in table 3.

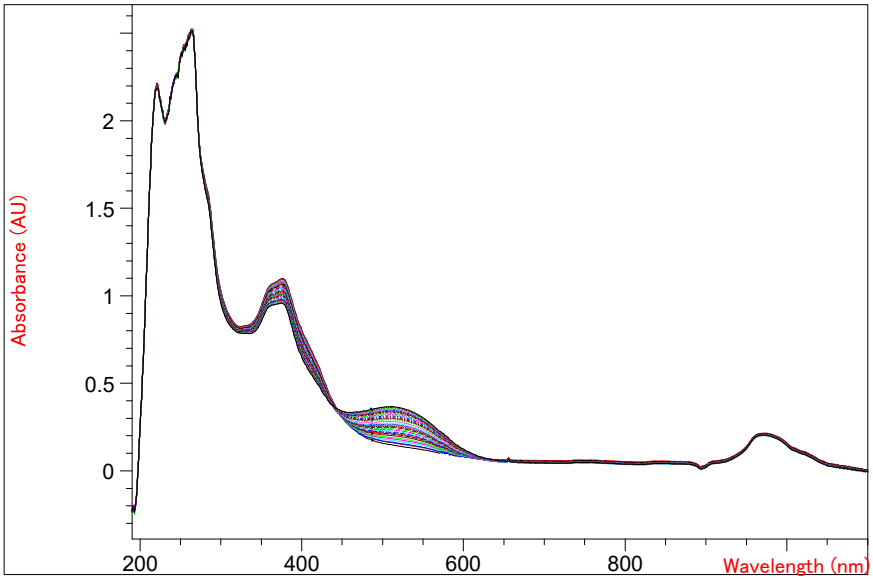


Figure 16: Hydrolysis of the substrate, NCF by TEM-1 β-lactamase

Table 4: Residual Activity and Percent Inhibition of TEM-1 for 3 minutes, 30 °C for 3 minutes Utilizing Potential Synthesized Inhibitors*

	Compound	Molecular Weight (g/mol)	Initial Rate $V_o \pm SD$ (ΔA, sec^{-1}) × 10^{-3}	Initial Rate +Inhibitor $V_o \pm SD$ (ΔA, sec^{-1}) × 10^{-3}	Residual Activity (%)	% Inhibition
1	**2a**	268.31	1.6870 ± 0.01531	1.2930 ± 0.03163	76.47	23.53
2	**2b**	254.28	2.9367 ± 0.26697	2.1840 ± 0.34975	74.37	25.63
3	**2c**	280.00	1.159 ± 0.01139	1.0139 ± 0.02758	87.48	12.52
4	**3**	252.29	2.0411 ± 0.01252	1.2465 ± 0.01698	61.07	38.93
5	**3** (P99)	252.29	7.1434 ± 0.15520	5.5067 ± 0.15981	77.09	22.91

Final concentration & volume of Enzyme (TEM-1) = 3 µL (0.45 nM),
Substrate (NCF) = 12 uL (100 µM)
0.1% BSA in MOPS buffer = 562 µL (0.02 mM, pH – 7.5)
Inhibitor (in 3% ACN) = 20 µL (500 µM)

The result obtained from the enzyme kinetic inhibition reveals that **2a-2c** displayed 12-26% inhibition against TEM-1. The relatively low percentage inhibition observed could be because they were not sufficiently soluble in the buffer solution used for the enzyme kinetic analysis, hence making the synthesized inhibitors inaccessible to the active site. Also, there is a tendency for steric hindrance, due to the substituent on the scaffolds of **2a-2c** to prevent part of the molecule from interacting appropriately with the active serine site of TEM-1 β-lactamases. The incorporation of substituent known to show great activity[57] and enhance solubility (e.g. -COOH, $-SO_3H$, -OH etc.) is most likely to improve the inhibitory activity of the synthesized inhibitors against β-lactamases. Therefore, compound **3** was synthesized to resolve the problem of steric hindrance and probably enhance the interaction between the active site of

the enzyme and the inhibitor. The enzyme kinetic inhibition analysis of **3** showed a significant increase to 38.9% against TEM-1 but 22% inhibition against P99.

CHAPTER 4

CONCLUSION AND FUTURE WORK

Conclusion

In this research work oxadiazolidinone derivatives (**2a, 2b, 2c, and 3**), as a non-β-lactam β-lactamase inhibitor, were evaluated for their inhibitory activity against TEM-1 and P99 serine β-lactamase after they were prepared using commercially available isocyanate derivatives (phenyl isocyanates and ethyl isothiocyanato acetate) with synthesized nitrone **1a** and **1b**. The tolerance for substituents on N2 and N4 position of oxadiazolidinone scaffold makes it easier for lots of functional groups to be incorporated. Thus, these compounds could be having useful synthetic application in pharmaceutical industries.

The synthesized nitrones were: (z)-N-(benzylidene)-1-ethanamine oxide (**1a**), and (z)-N-benzylidenemethanamine oxide (**1b**). The oxadiazolidinone derivatives were: 2-ethyl-3,4-diphenyl-1,2,4-oxadiazolidin-5-one (**2a**), 2-methyl-3,4-diphenyl-1,2,4-oxadiazolidin-5-one (**2b**), ethyl 2-(2-methyl-3-phenyl-5-thioxo-1,2,4-oxadiazolidin-4-yl) acetate (**2c**), 2-(2-methyl-3-phenyl-5-thioxo-1,2,4-oxadiazolidin-4-yl) acetic acid (**3**).

After successful synthesis and characterization of the compounds, an MTT Essay was used to test the in vitro cytotoxicity of oxadiazolidinone **2a** on cancer cell lines such as IGROV1, OVCAR-4, HS 578T, BT-549, A498, UO-31, 786-0, CAKI-1, UACC-62, SK-MEL-28, SNB-19, SF-268, PC-3, SW-620, and EKVX. Compound **2a** had more activity on renal cancer, decreasing the cell viability of 786-0 by about 18%. The activity on other cell lines ranged from 4-14%.

Also, the enzyme kinetic inhibition data revealed that compound **2a**-**2b** showed an inhibition against TEM-1 ranging from 12-26% which was increased to 38.9% when derivative **3** was synthesized and evaluated on the same TEM-1. The poor solubility of all synthesized compounds in the MOPS

buffer, and the steric hindrance due to the presence of a bulky substituent on the oxadiazolidinone scaffold could be the reason for the relatively low inhibitory activity of the synthesized inhibitors (**2a – 2c, and 3**) against the serine β-lactamase.

Future Work

The incorporation of functional groups (e.g. -OH, SO_3H, $-CONH_2$ etc.) with a known inhibitory history against TEM-1, P99 and other types of β-lactamases will be our focus. Also, a molecular docking to determine the possible interaction of oxadiazolidinone derivatives with the active site amino acid residue of TEM-1 will be carried out, and lastly the synthesized compounds will be subjected to Lipinski's rule of 5 to ascertain their drug likeliness.

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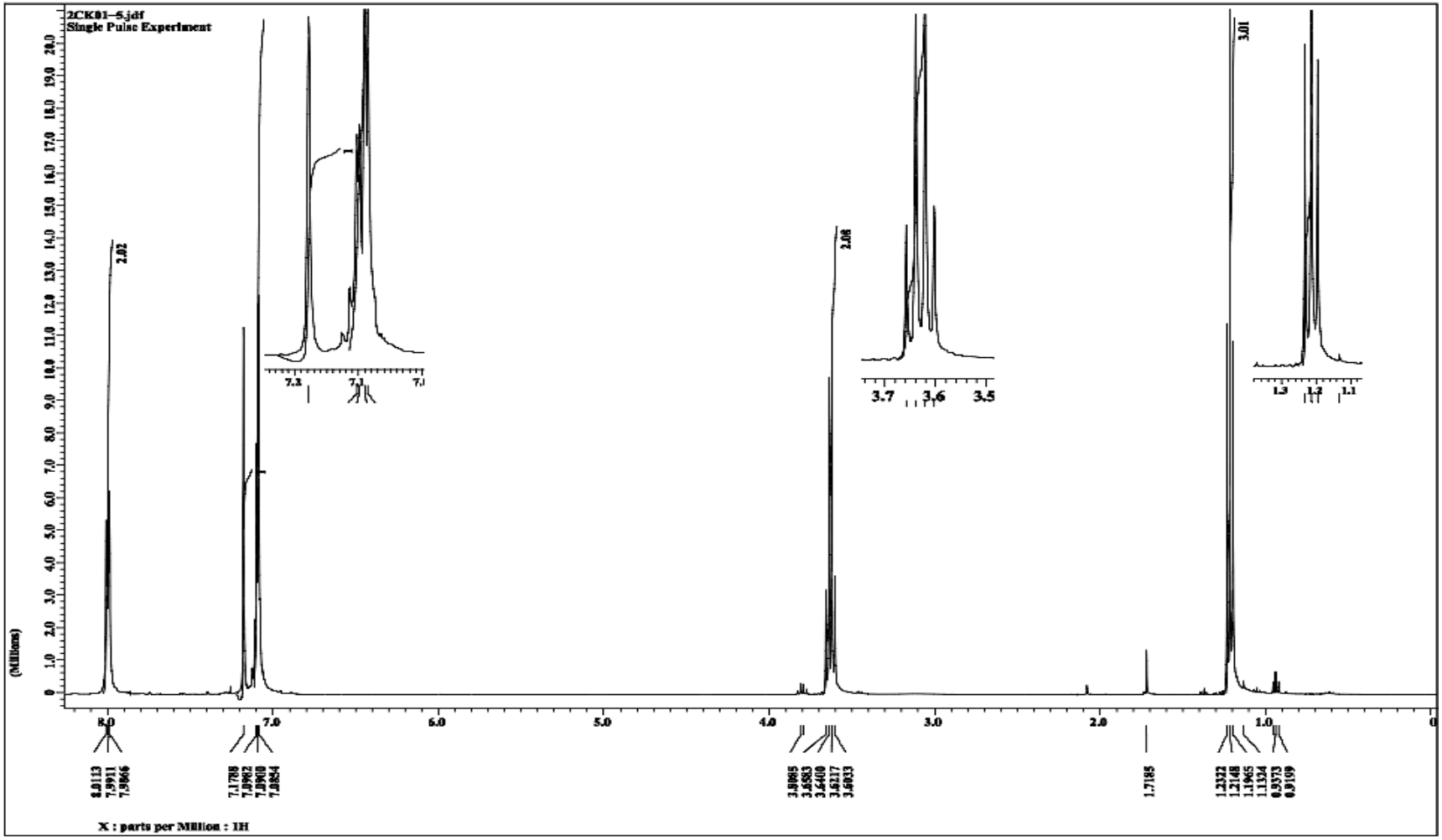
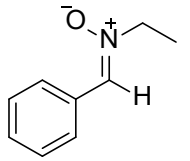
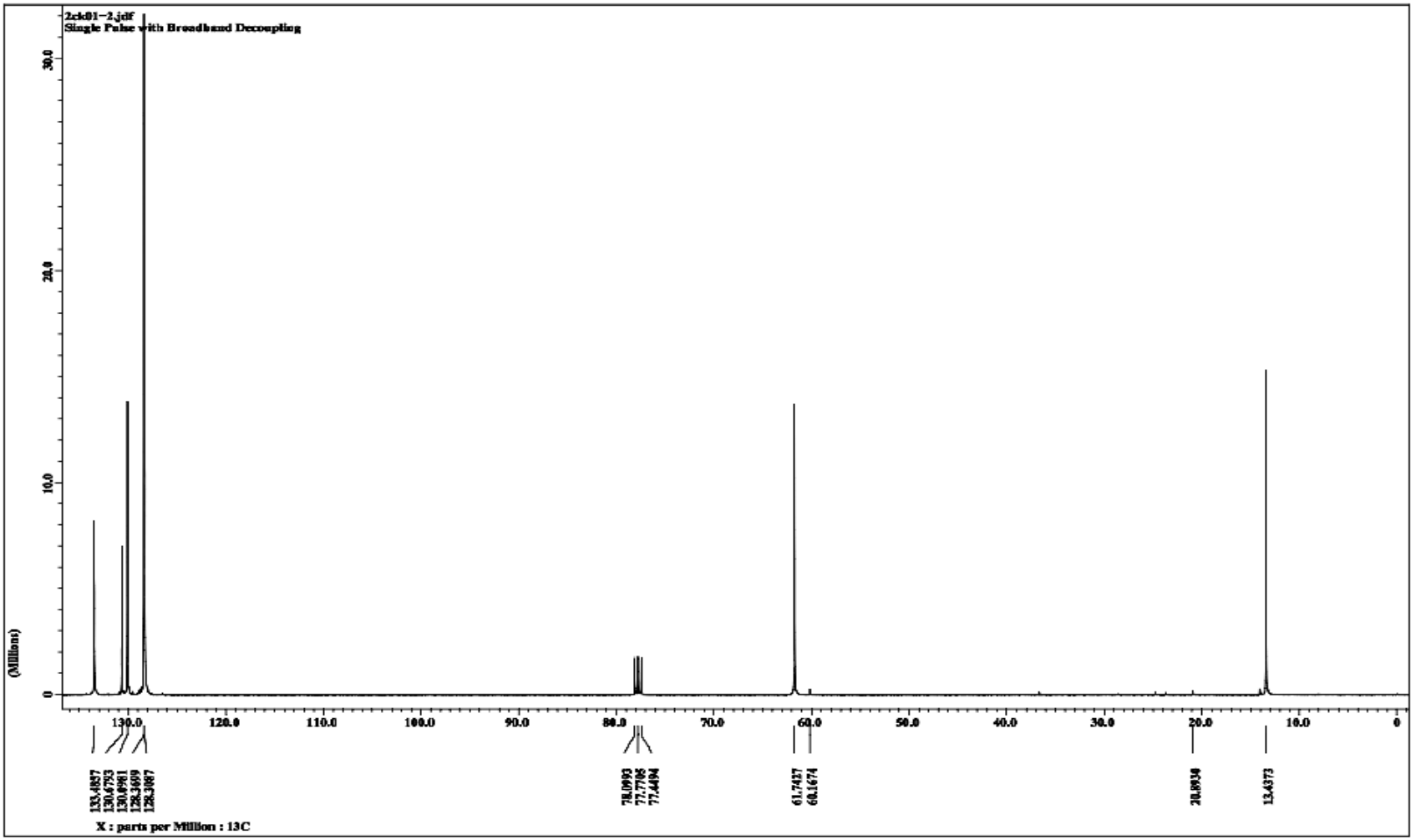
APPENDICES

Appendix A1: ^{1}H NMR Spectrum for Compound **1a** in $CDCl_3$

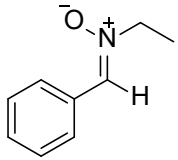


Appendix A2: ^{13}C NMR Spectrum for Compound **1a** in $CDCl_3$

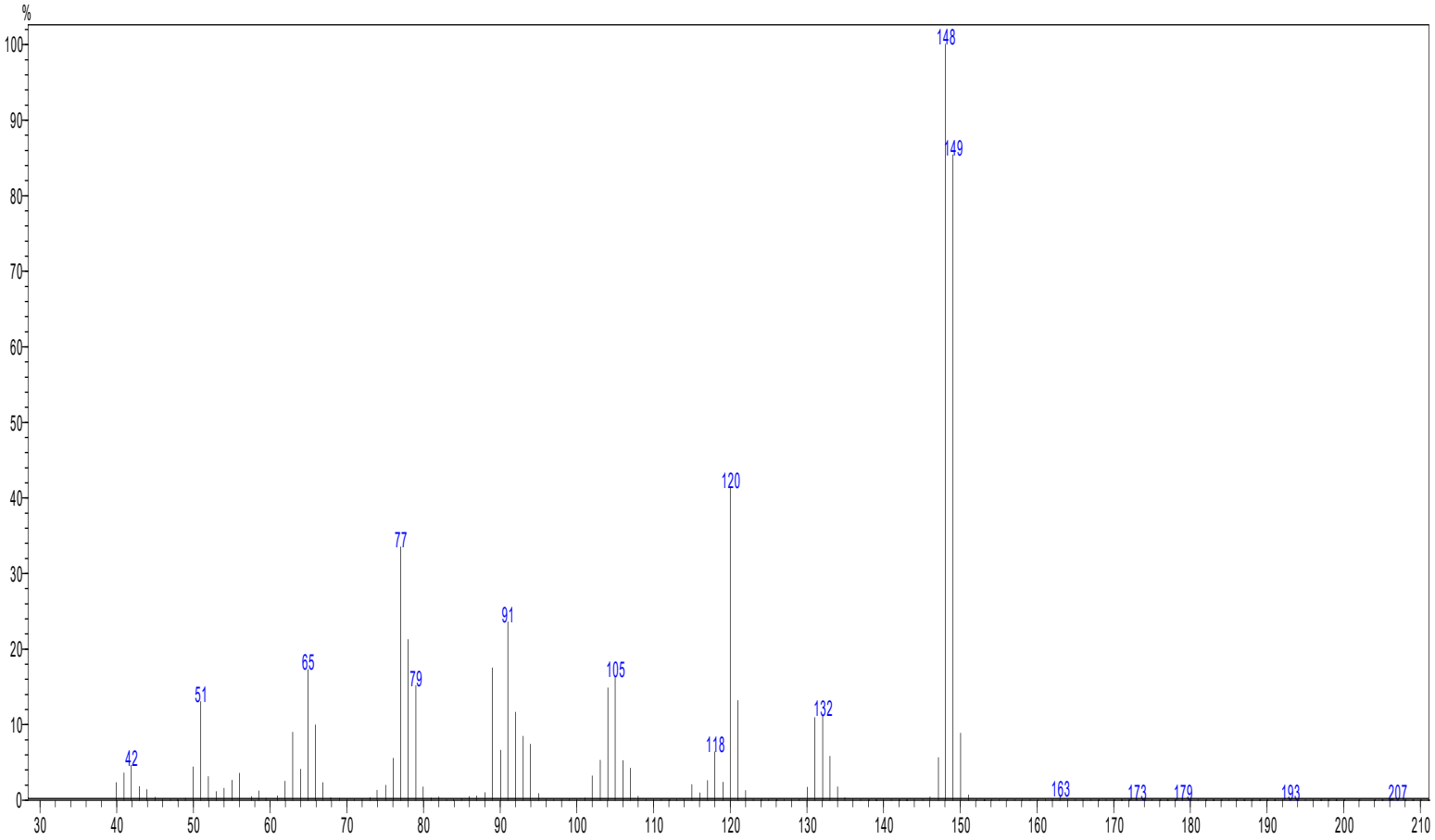




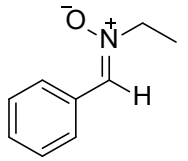
Appendix A3: GC-MS Spectrum for Compound **1a** in Acetone

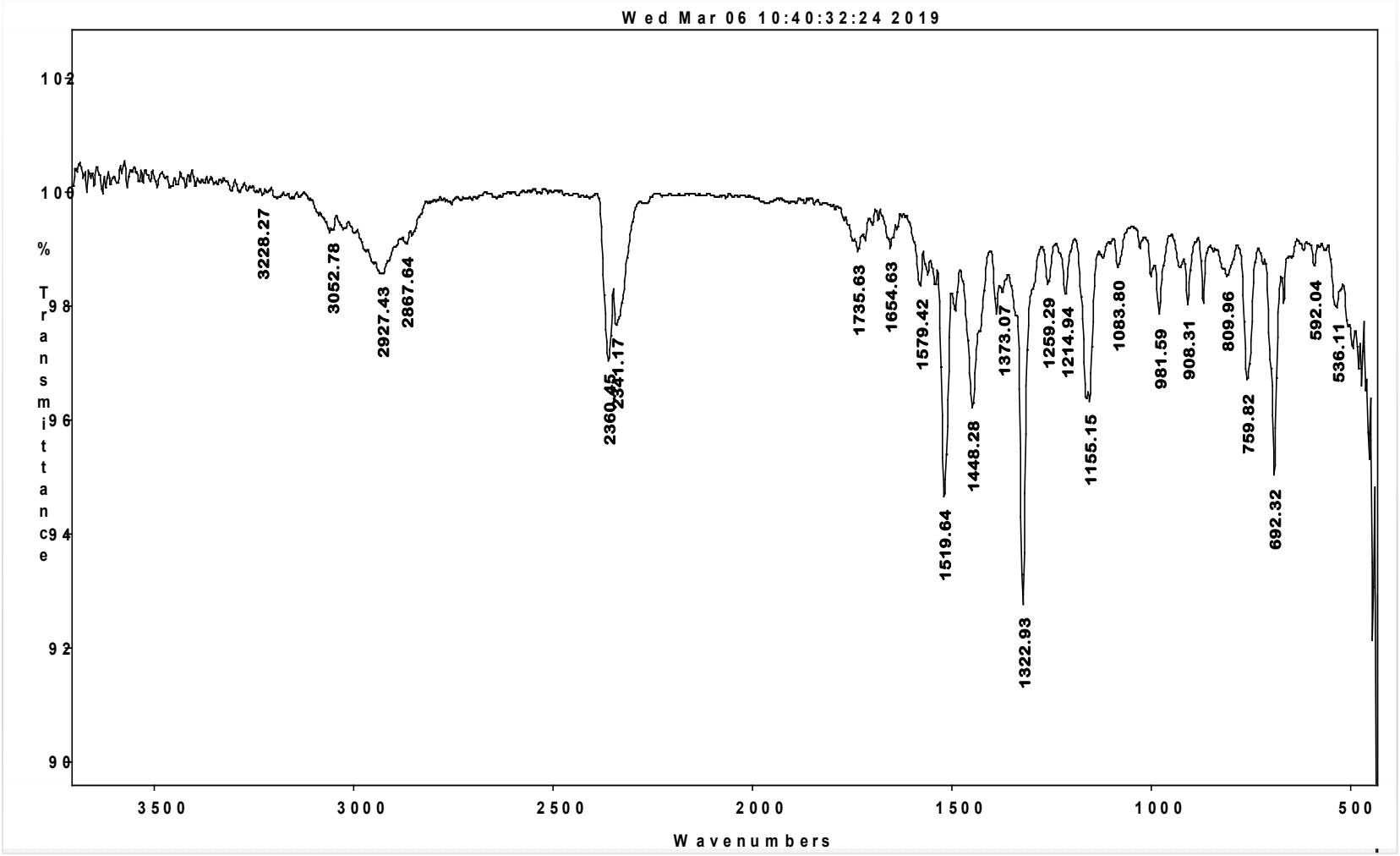


# of Peaks 434
Raw Spectrum 9.343 (scan: 1050)
Background No Background Spectrum
Exact mass 149.05

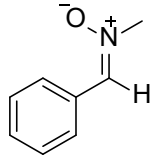


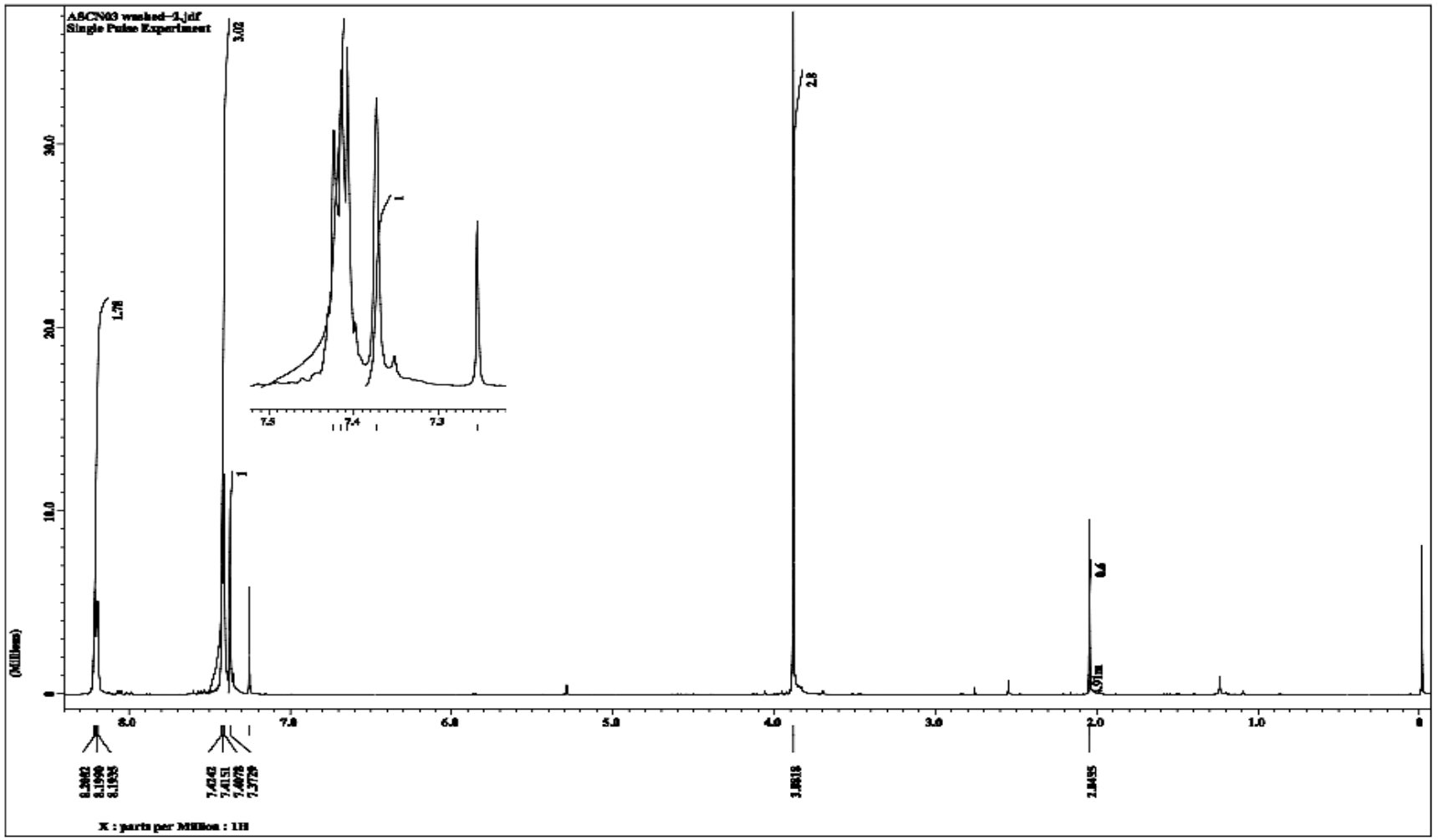
Appendix A4: IR Spectrum for Compound **1a** in Chloroform



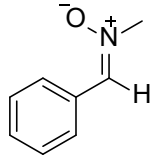


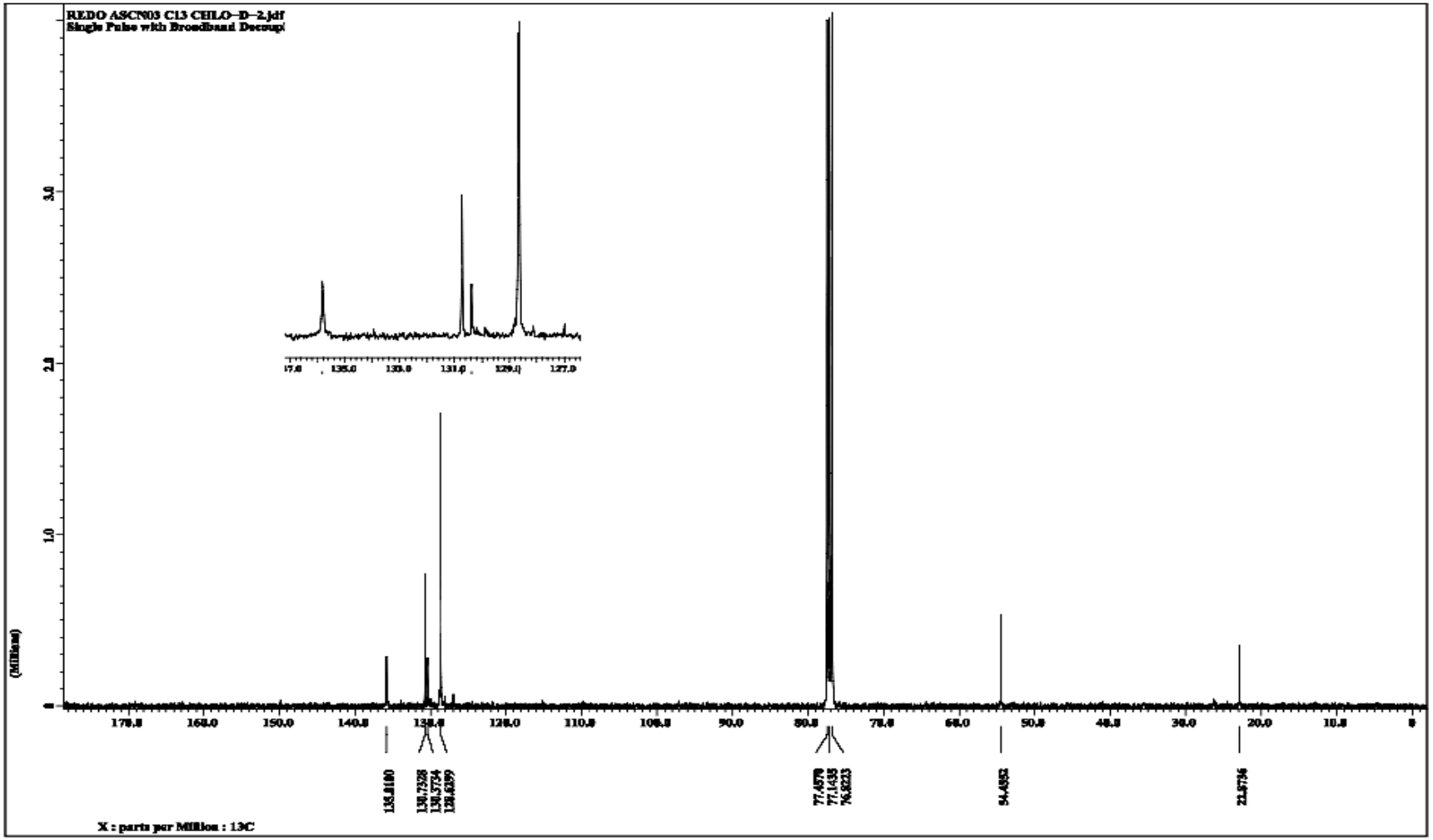
Appendix B1: ^{1}H NMR Spectrum for Compound **1b** in $CDCl_3$



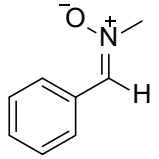


Appendix B2: ^{13}C NMR Spectrum for Compound **1b** in $CDCl_3$

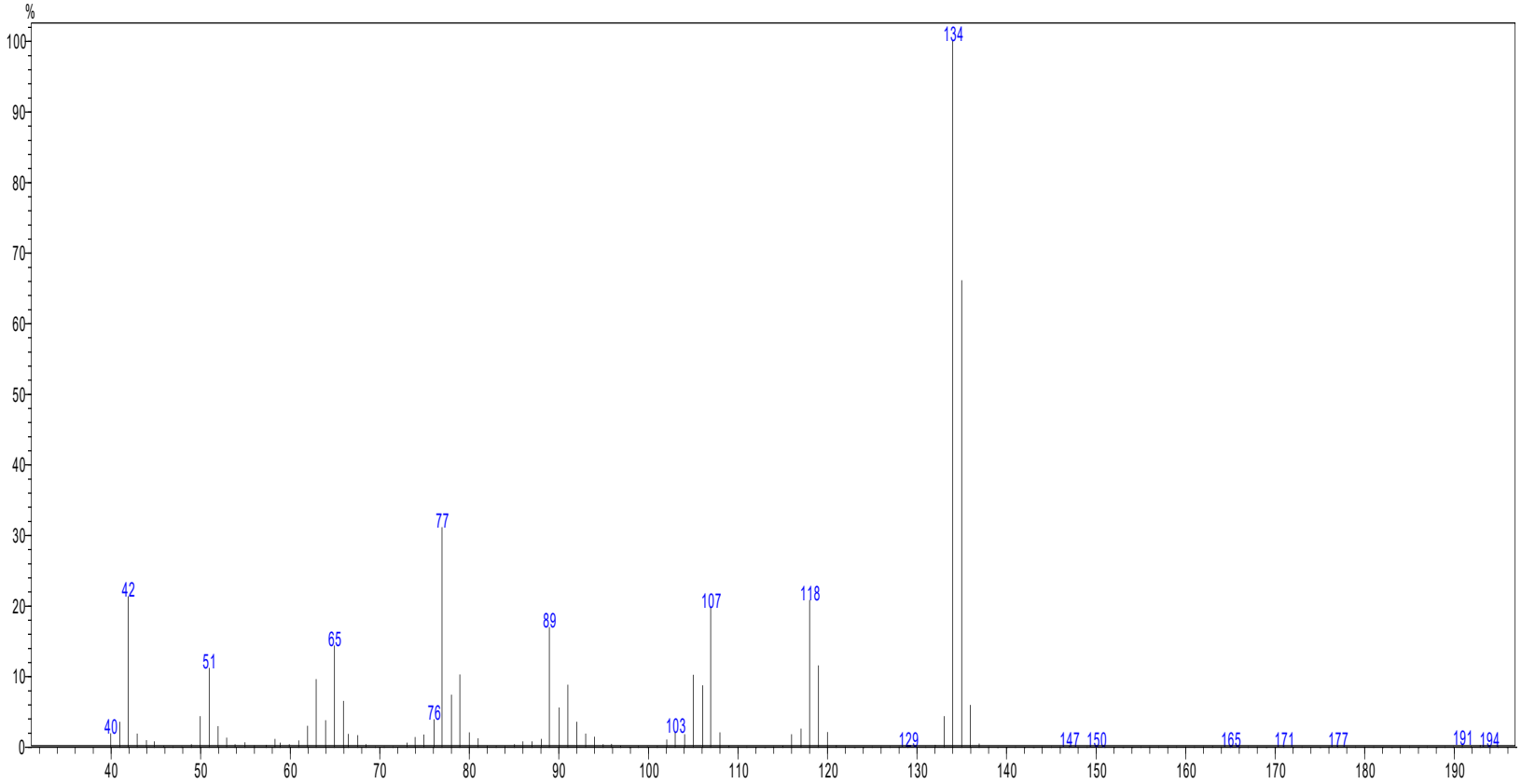




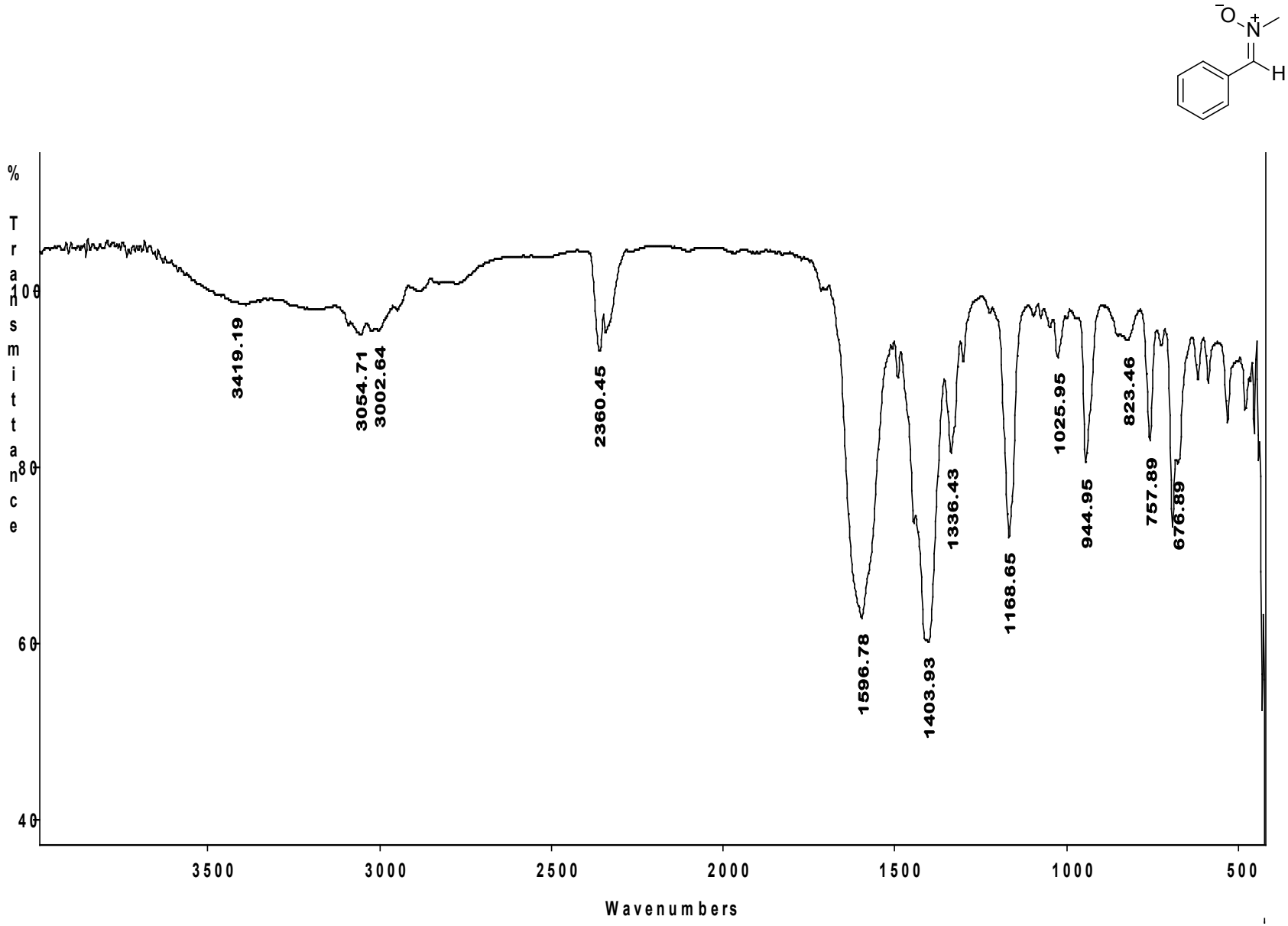
Appendix B3: GC-MS Spectrum for Compound **1b** in Acetone



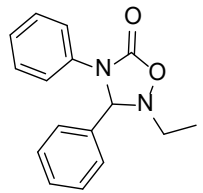
# of Peaks	434
Raw Spectrum	8.439 (scan: 778)
Background	No Background Spectrum
Exact mass	135.00

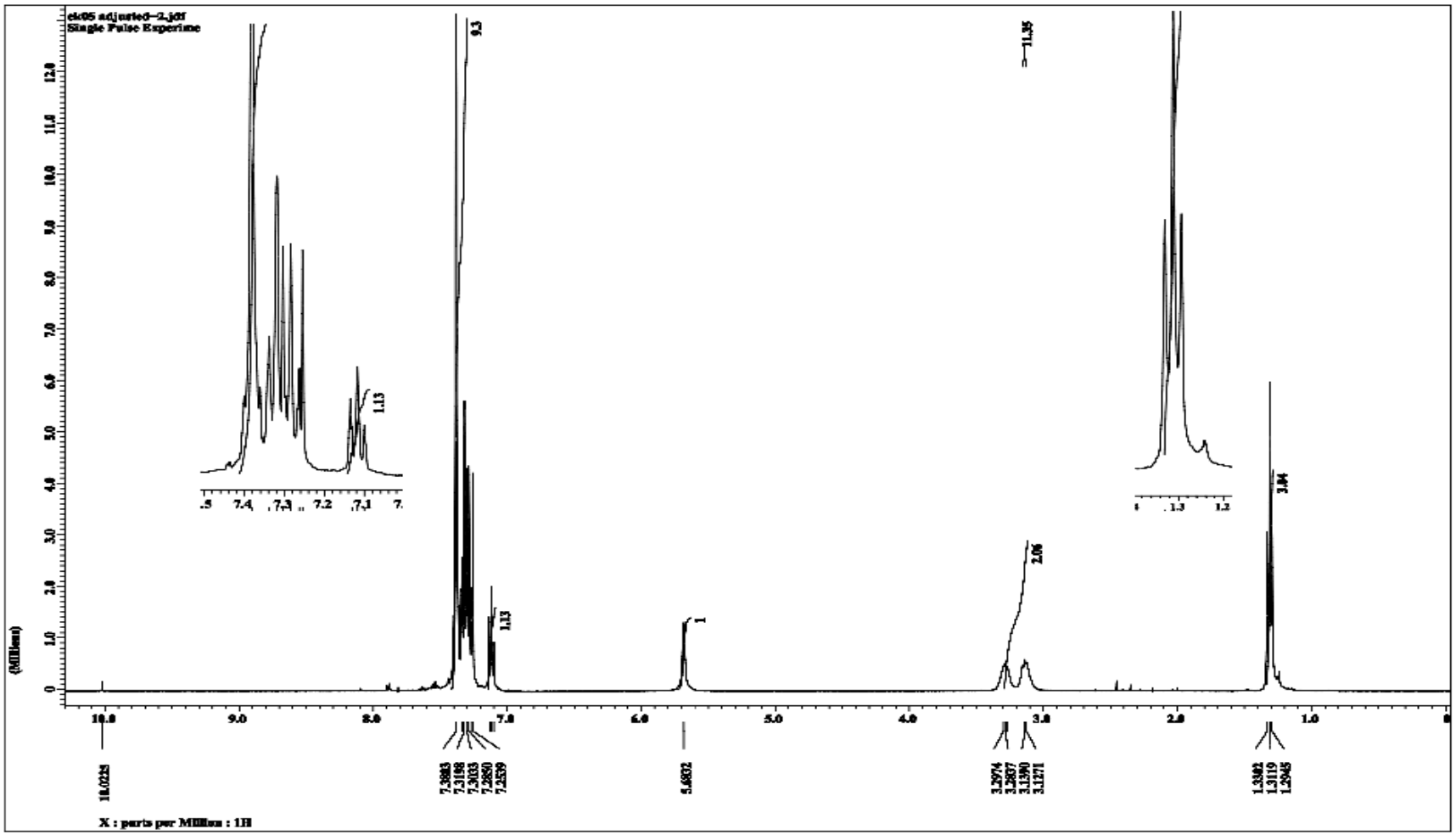


Appendix B4: IR Spectrum for Compound **1b** in Chloroform

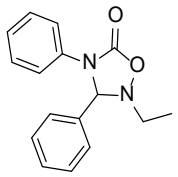


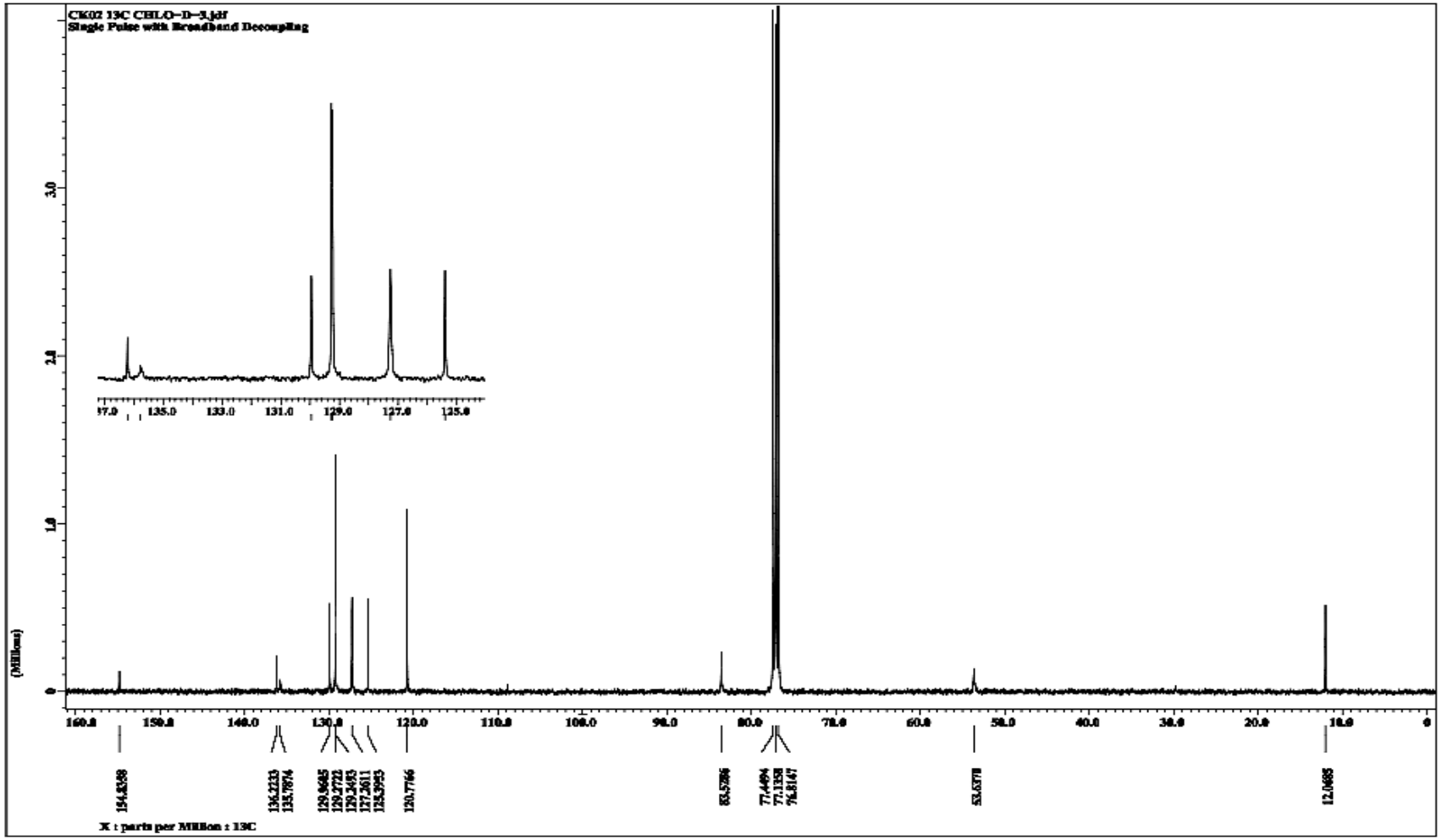
Appendix C1: ^{1}H NMR Spectrum for Compound **2a** in $CDCl_3$



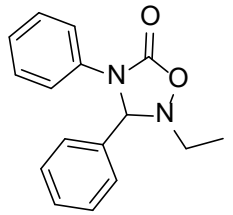


Appendix C2: ^{13}C NMR Spectrum for Compound **2a** in $CDCl_3$

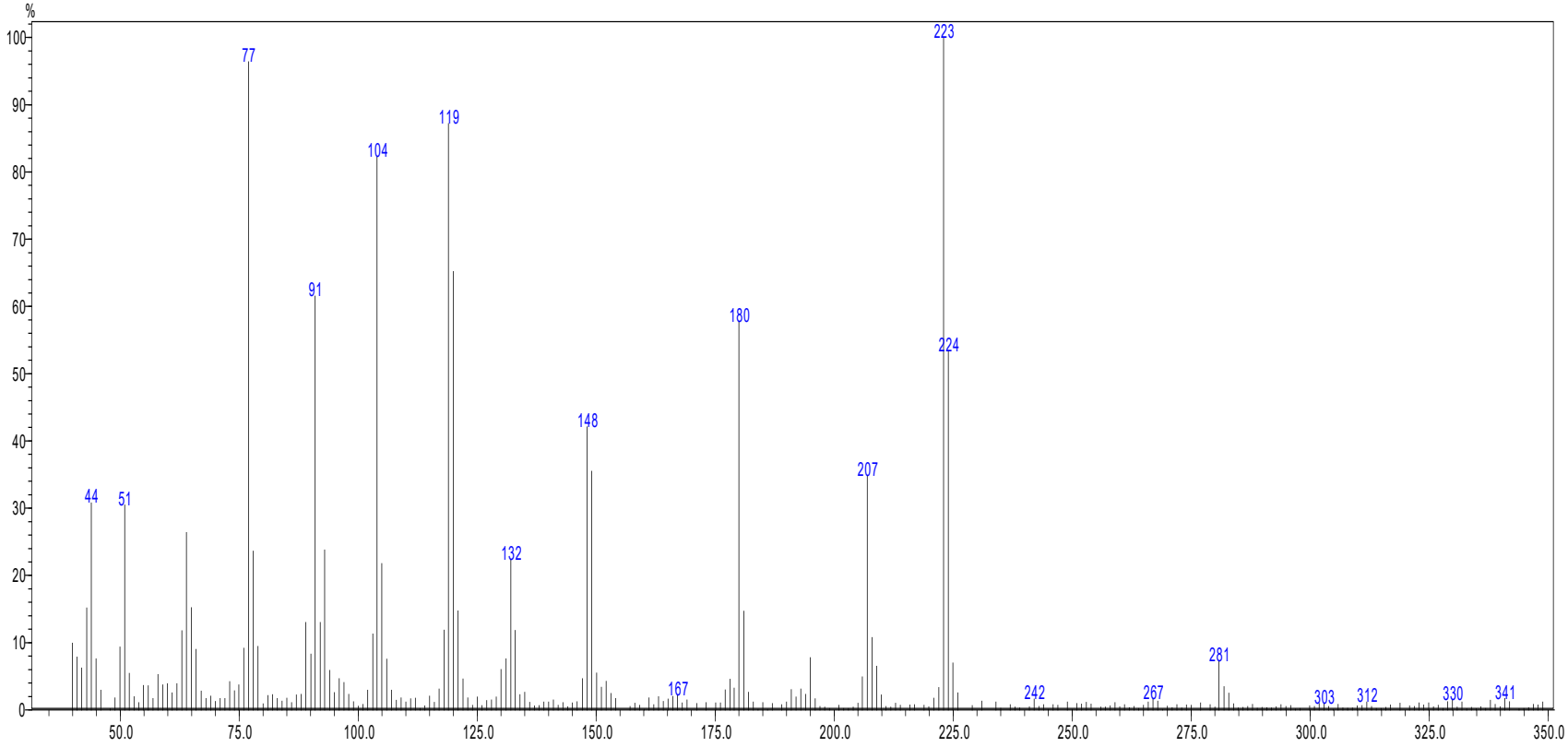




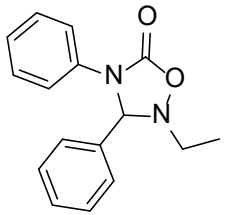
Appendix C3: GC-MS Spectrum for Compound **2a** in Acetone

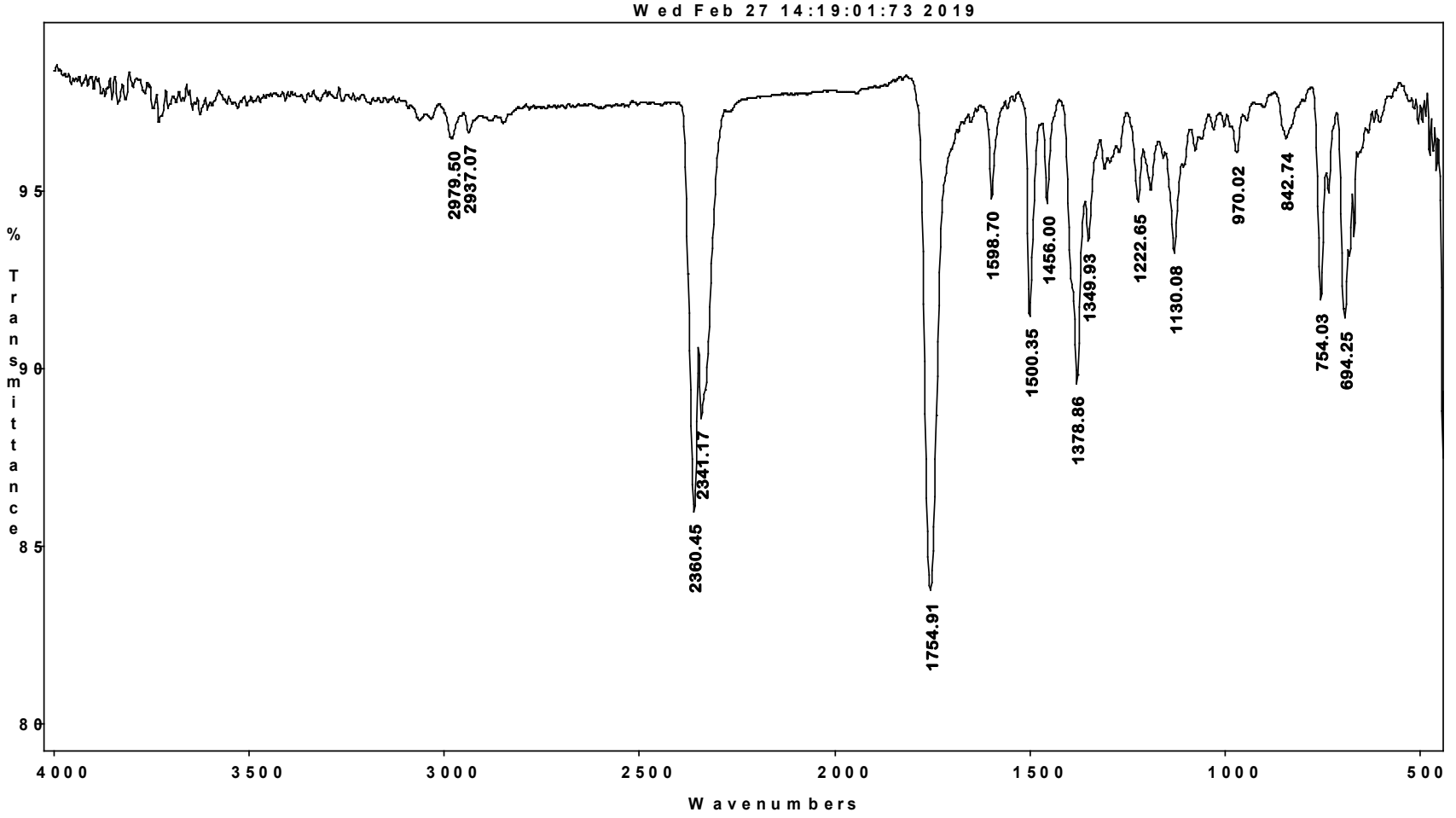


# of Peaks 409
Raw Spectrum 16.636 (scan: 1949)
Background No Background Spectrum
Exact mass 267.00

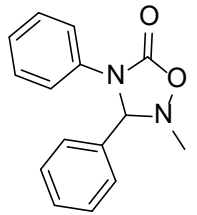


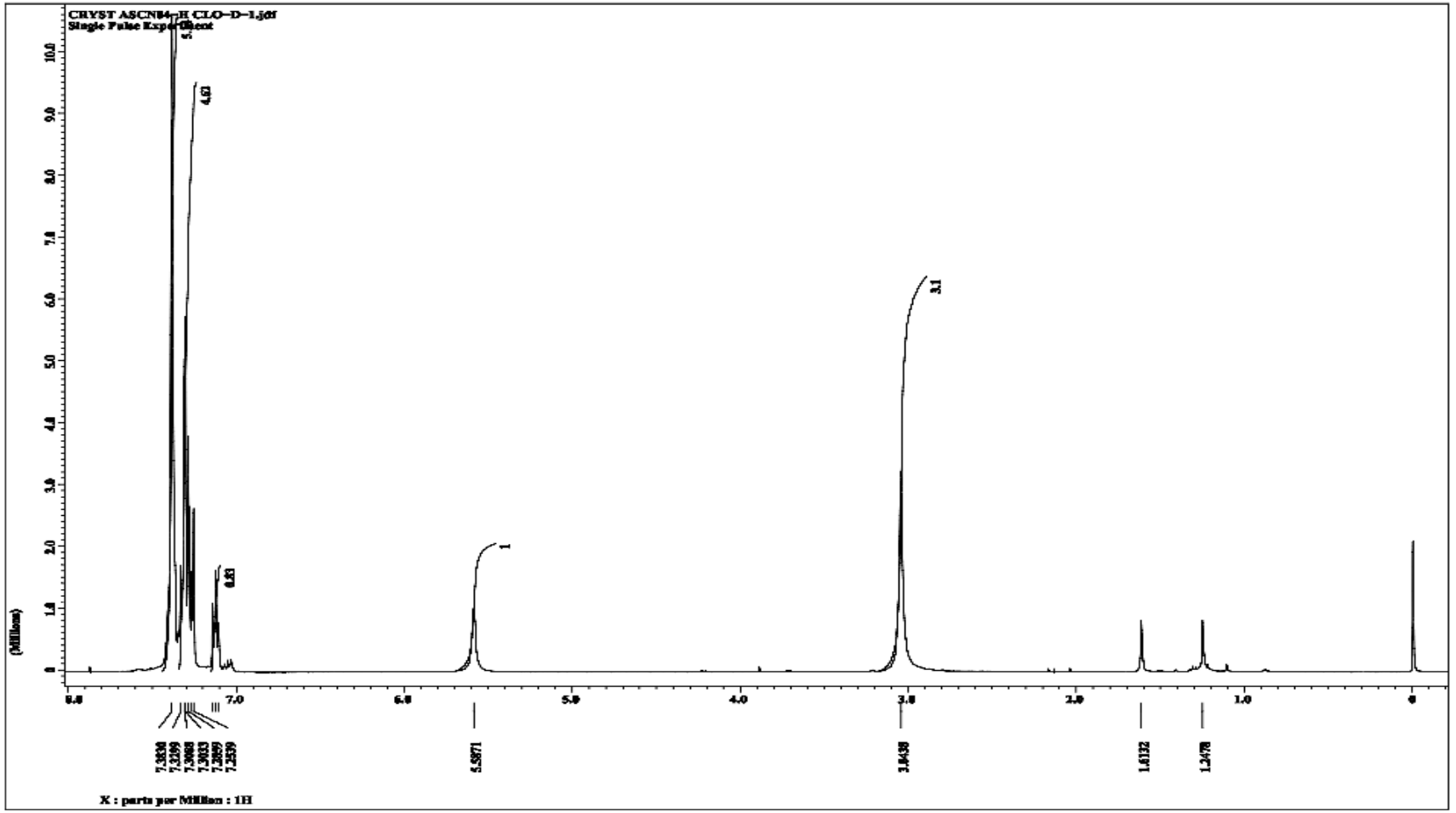
Appendix C4: IR Spectrum for Compound **2a** in Chloroform



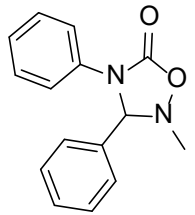


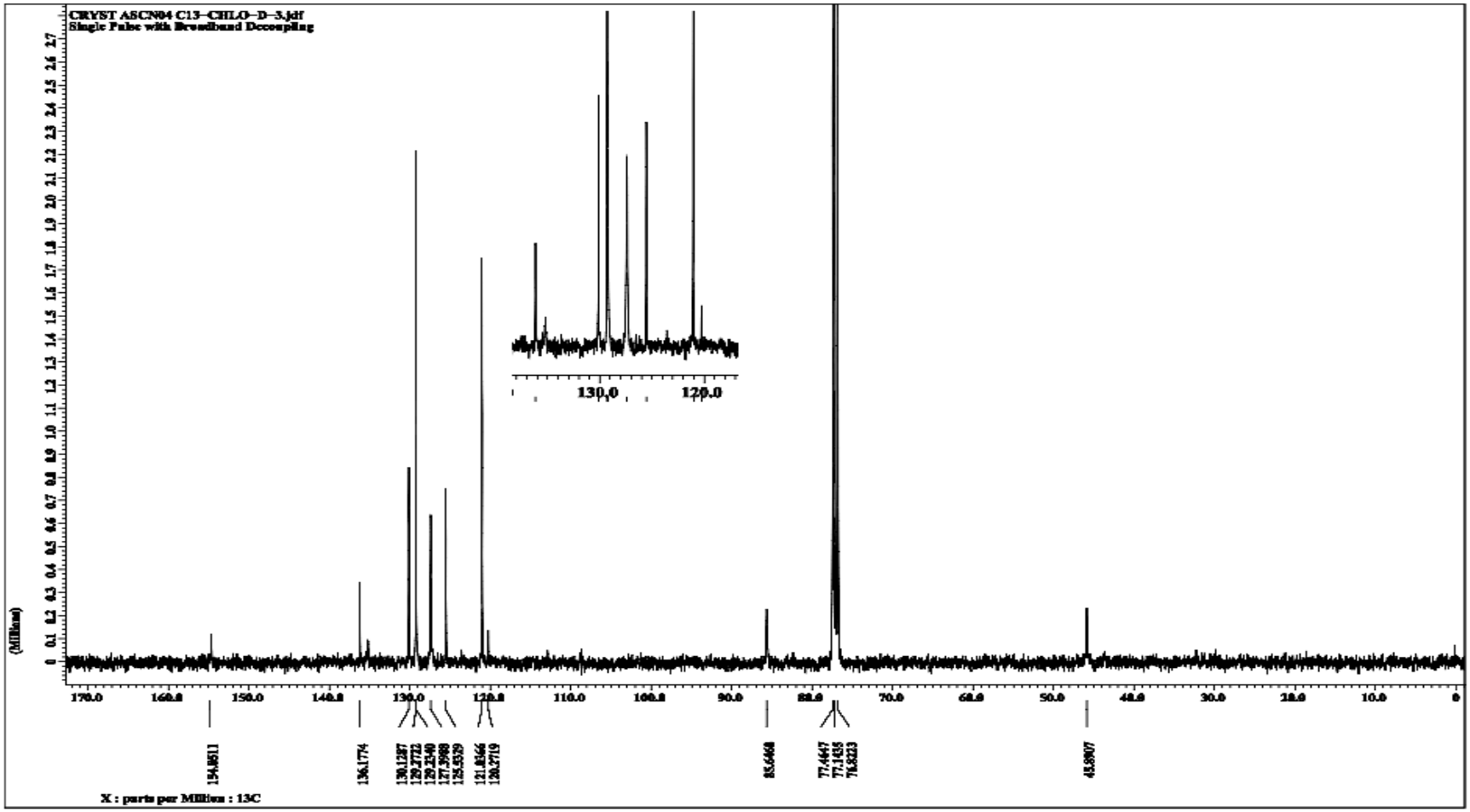
Appendix D1: ^{1}H NMR Spectrum for Compound **2b** in $CDCl_3$



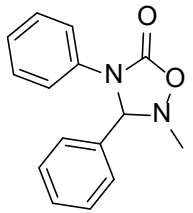


Appendix D2: ^{13}C NMR Spectrum for Compound **2b** in $CDCl_3$

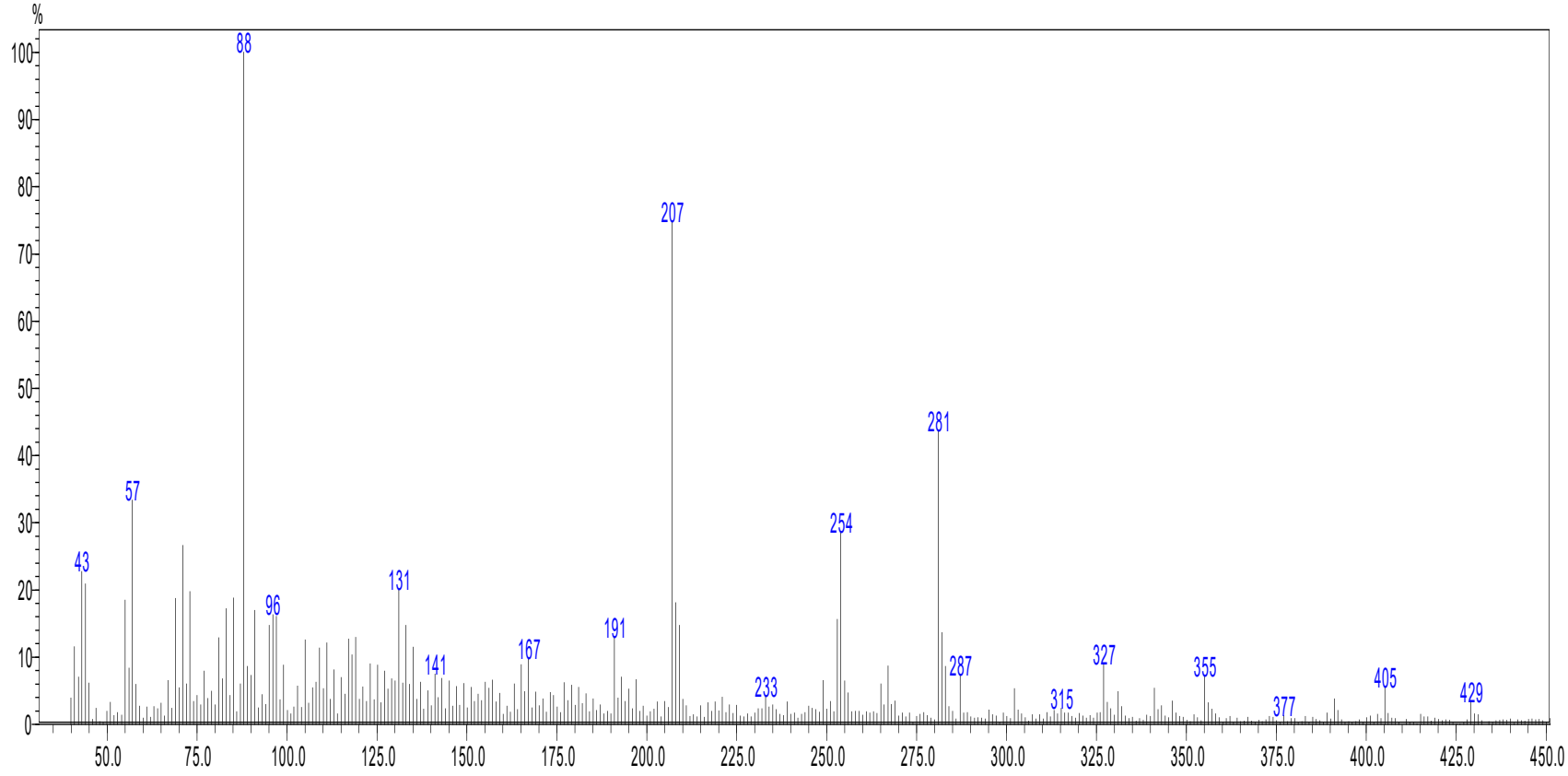




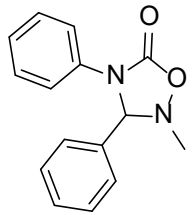
Appendix D3: GC-MS Spectrum for Compound **2b** in Acetone

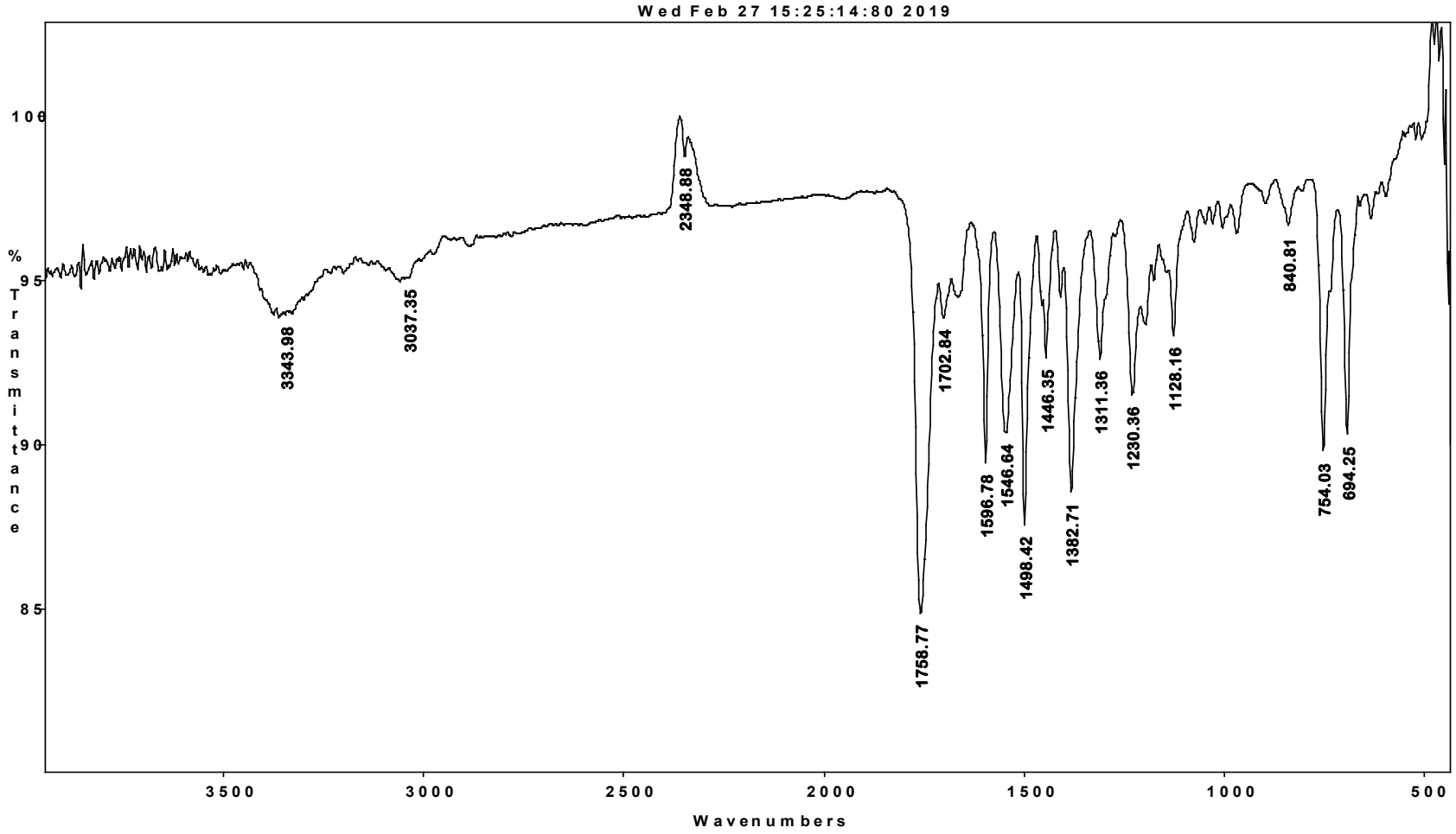


# of Peaks 455
Raw Spectrum 14.170 (scan: 1311)
Background No Background Spectrum
Exact mass 254

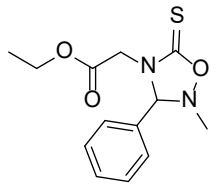


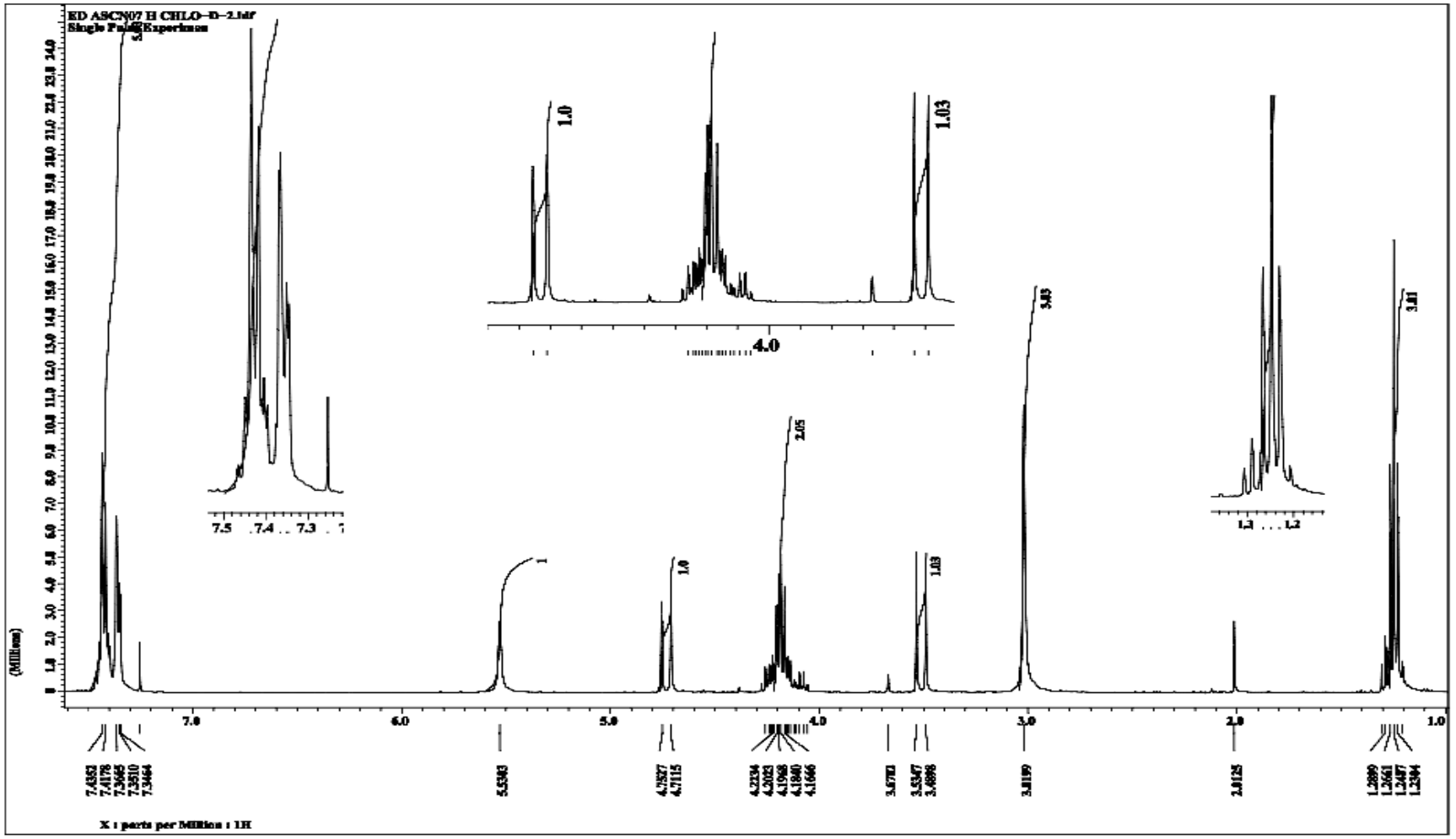
Appendix D4: IR Spectrum for Compound **2b** in Chloroform



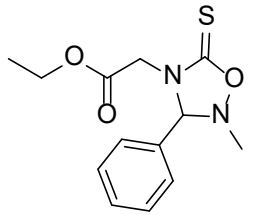


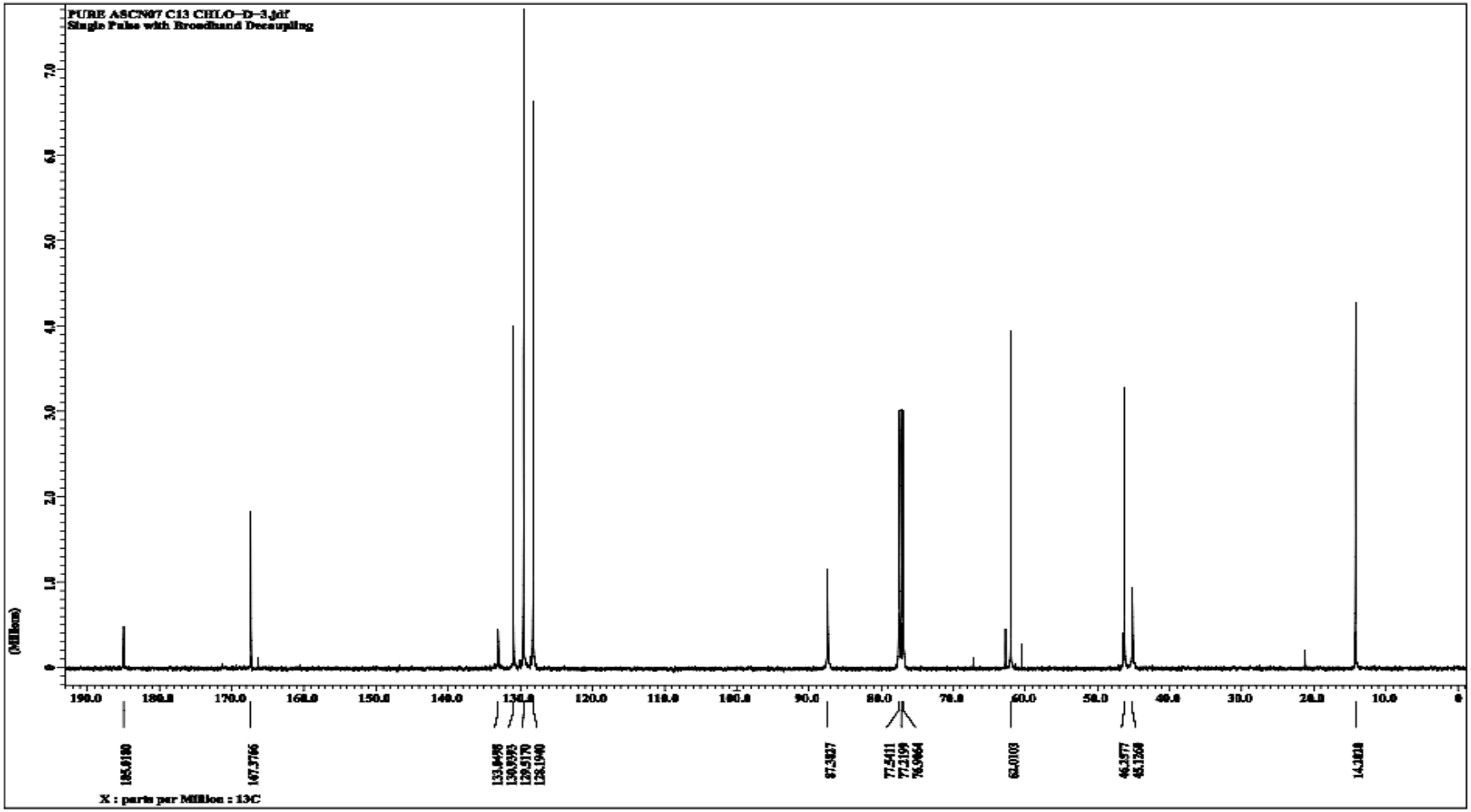
Appendix E1: ^{1}H NMR Spectrum for Compound **2c** in $CDCl_3$



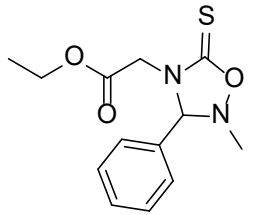


Appendix E2: ^{13}C NMR Spectrum for Compound **2c** in $CDCl_3$

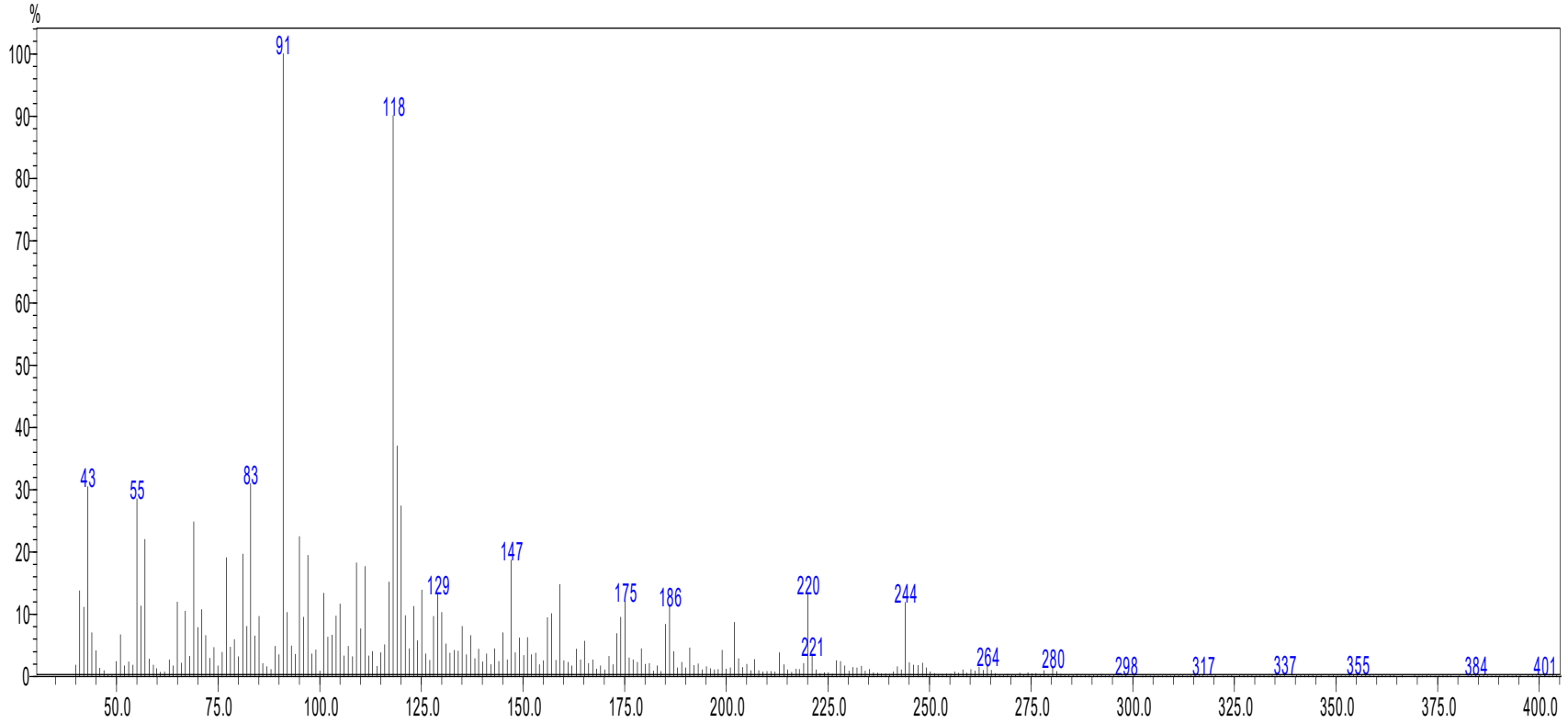




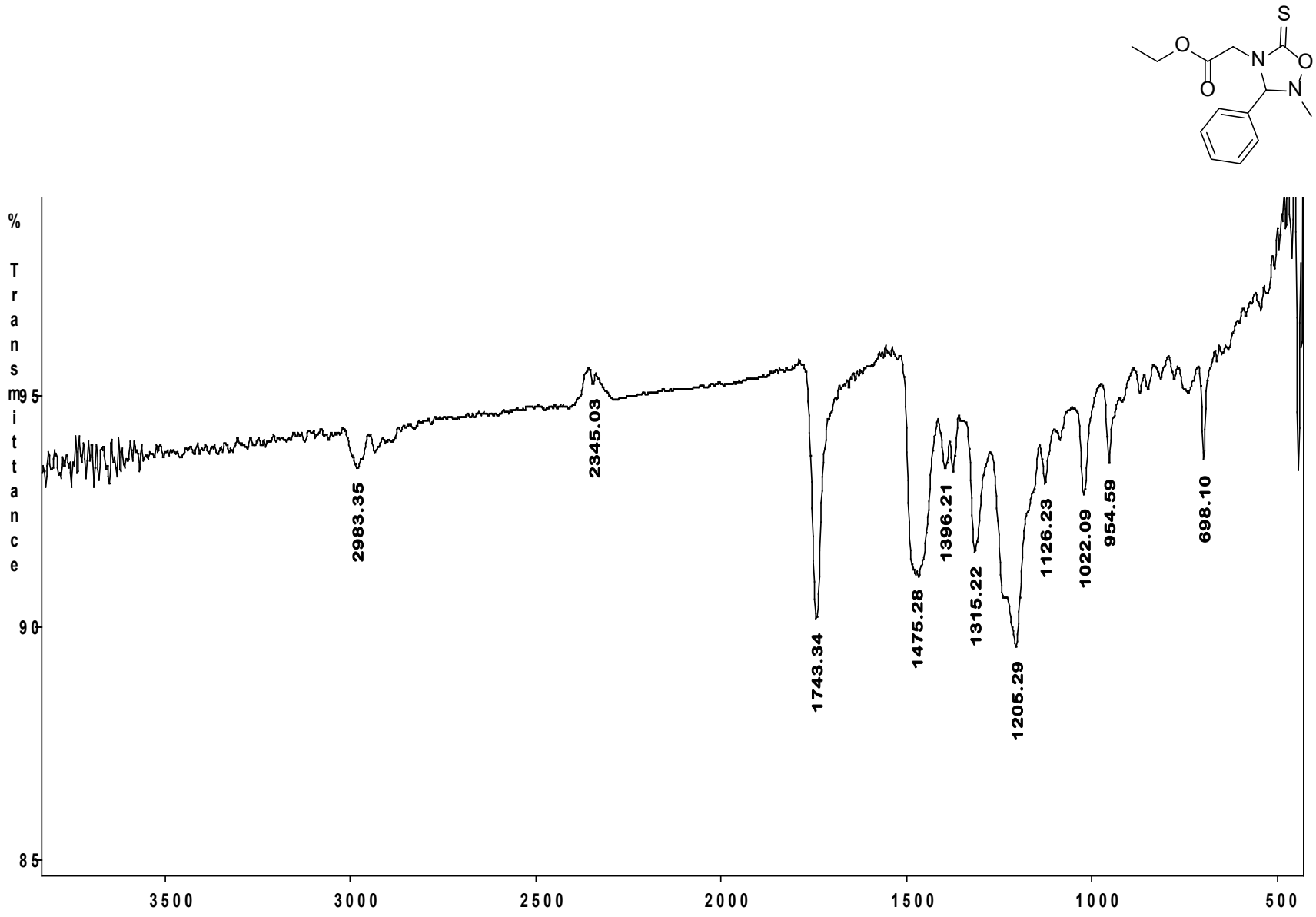
Appendix E3: GC-MS Spectrum for Compound **2c** in Acetone



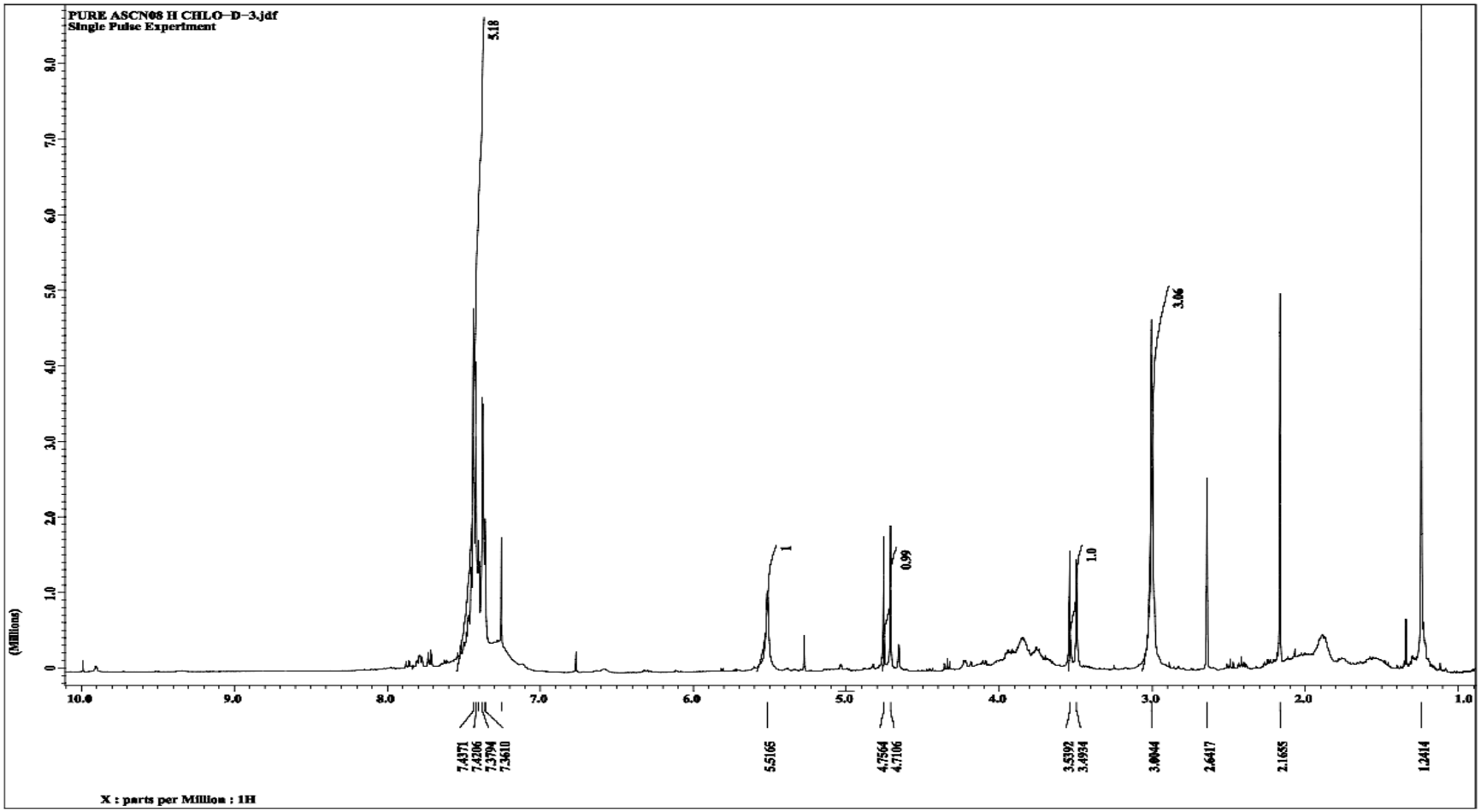
# of Peaks 436
Raw Spectrum 11.139 (scan: 878)
Background No Background Spectrum
Exact mass 280



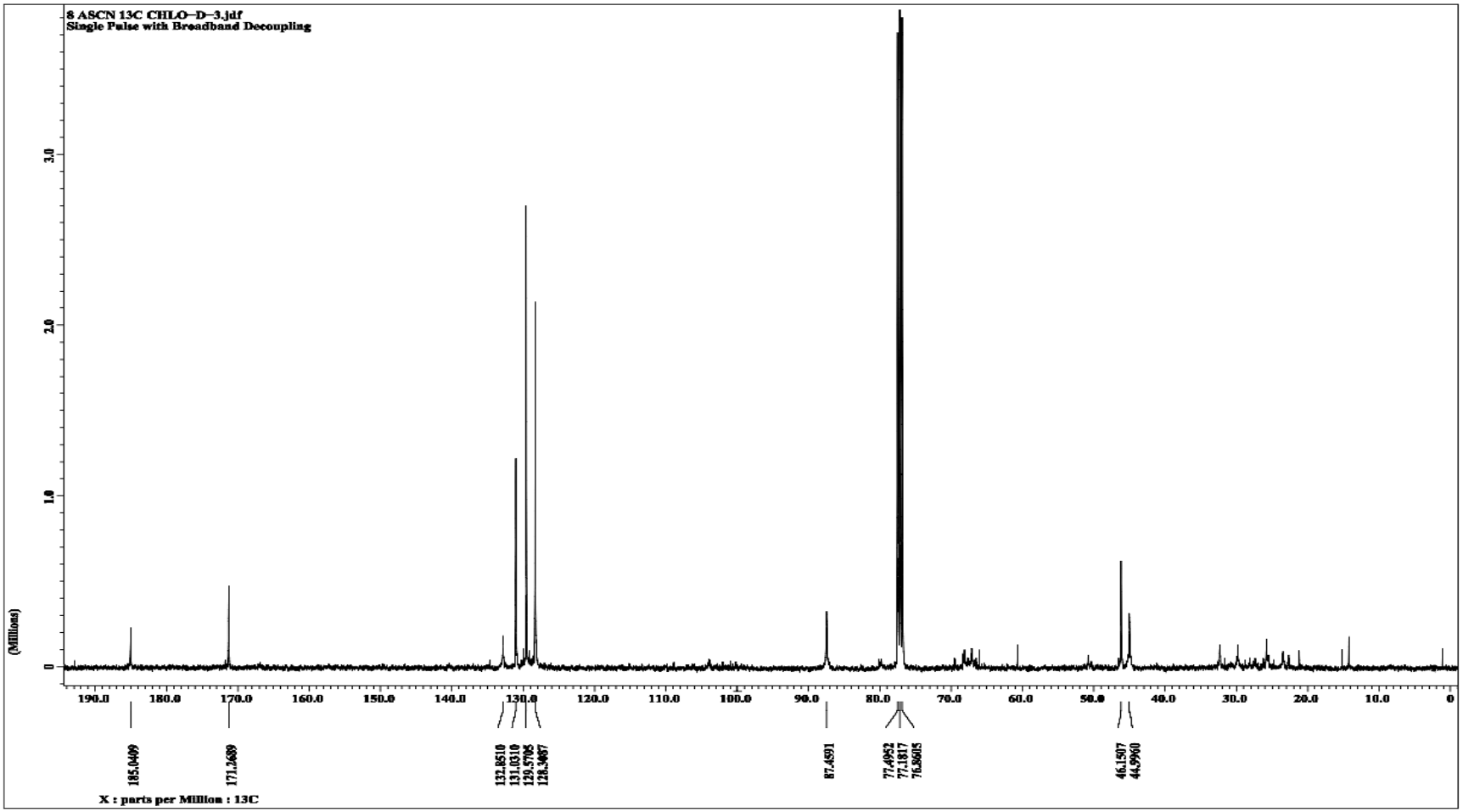
Appendix E4: IR Spectrum for Compound **2c** in Chloroform



Appendix F1: 1H NMR Spectrum for Compound 3 in $CDCl_3$

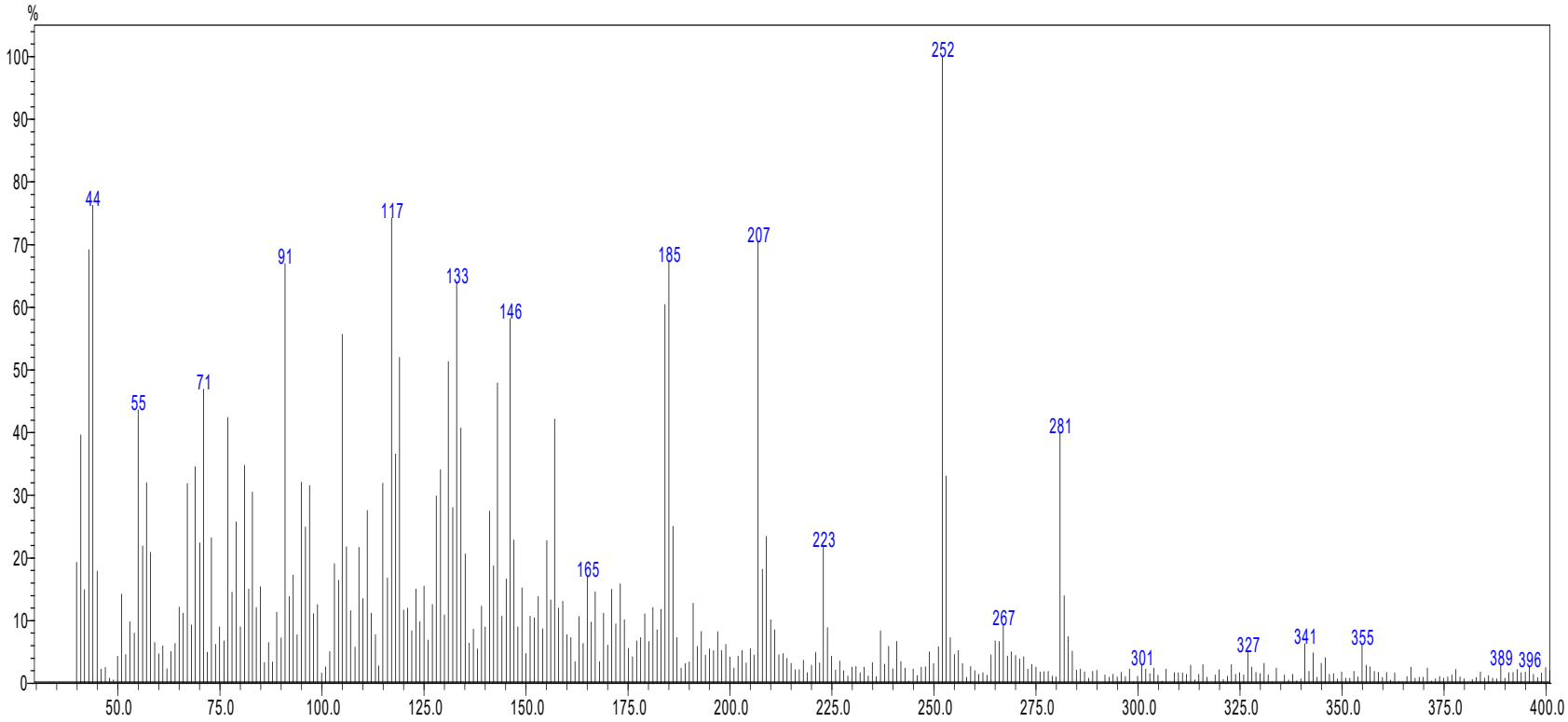


Appendix F2: ^{13}C NMR Spectrum for Compound **3** in $CDCl_3$

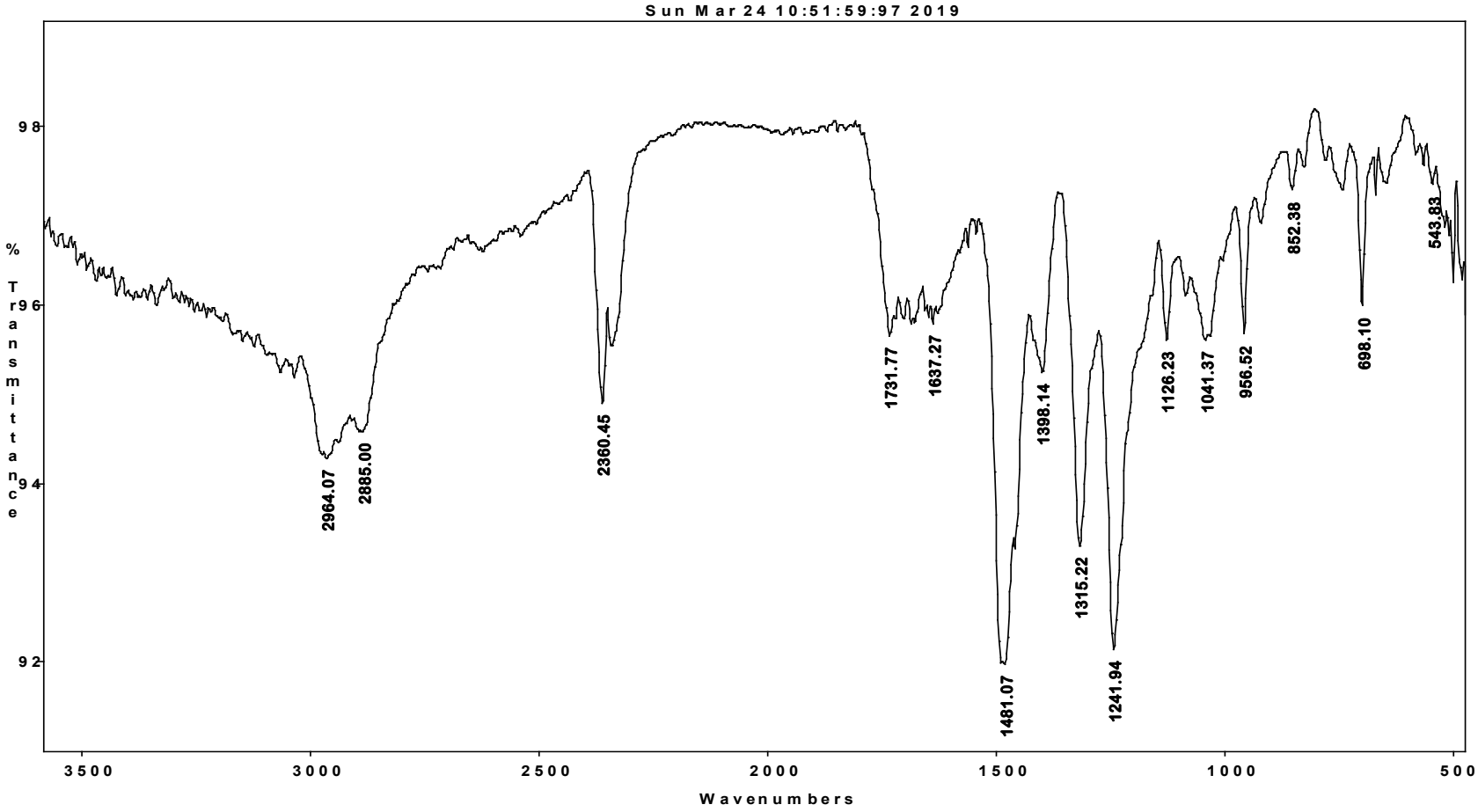


Appendix F3: GC-MS Spectrum for Compound **3** in Acetone

# of Peaks 452
Raw Spectrum 12.371 (scan : 1054)
Background No Background Spectrum
Exact mass 252.10



Appendix F4: IR Spectrum for Compound **3** in Chloroform



VITA

CHIMDI EKE KALU

Education:	Master of Science in Chemistry, May 2019 East Tennessee State University, Johnson City, TN Bachelor of Science in Chemistry. Dec. 2011 Cross River State University of Technology, Calabar, Nigeria
Professional Experience:	Graduate Assistant, East Tennessee State University, Johnson City, TN, Jan. 2017 – Dec. 2018
Presentations:	Chimdi Kalu, Noah Lyon and Abbas G. Shilabin "Synthesis, Evaluation and Biological Significance of 1, 2, 4-Oxadiazolidinone: The search for non-β-lactam β-lactamase inhibitor." 70th Southeast Regional Meeting of American Chemical Society held in Augusta, GA, November 1st, 2018. Chimdi Kalu, Austin Miller and Abbas G. Shilabin "Synthesis of 1, 2, 4-Oxadiazolidinone analogs." Appalachian Student Research Forum held in Millennium Center, Johnson City TN. 5th April 2018.
Activities and Community Involvement:	Served at Good Samaritan Christmas food pantry, Young Adult Calvary Church Johnson City, TN, Dec. 2018

Volunteered during Martin Luther (Jr) day, ETSU Gospel Choir

Jan. 2017

Environmental clean-up, Graduate Professional Student

Association (ETSU), April 2017

Assisted first aid for attendees during Guyana 50th Anniversary

May 2016