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Synthesis and Characterization of Medicinally Privileged Aza Heterocycles

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SYNTHESIS AND CHARACTERIZATION OF MEDICINALLY PRIVILEGED AZA HETEROCYCLES

A Thesis

by

NNEOMA JAMES

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SYNTHESIS AND CHARACTERIZATION OF MEDICINALLY PRIVILEGED AZA HETEROCYCLES

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August 2024

ABSTRACT

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Four-membered cyclic amides, commonly known as β-lactams, have played a crucial role in drug discovery research since its discovery by Alexander Fleming in 1928 from Penicillium notatum. The β-lactam antibiotics are broadly effective against several bacterial infections through acylating the transpeptidase involved in cross-linking peptides to form peptidoglycan or periplasmic murein, which is a fundamental constituent of the bacterial cell wall for both the gram-positive (ten or more layers) and gram-negative (one or two layers) bacteria. Although for more than eight decades, β-lactams have been recognized as antibiotics, later, other medicinal activities of β-lactam derivatives have been reported, including lowering of high cholesterol levels, antidiabetic, and anticancer. On the other hand, synthesizing the spiro carbon center is challenging due to enormous steric hindrances and angular strain. As a part of our ongoing research on synthesizing pharmacologically relevant spiro compounds, herein we report the synthesis of spiro-centered β-lactams following the Staudinger [2+2] ketene-imine cycloaddition reaction. A small series of diversely substituted spiro-centered β-lactams has been synthesized and characterized by several spectroscopic techniques, including IR, NMR (one and two-dimensional NMR), and HRMS. Some of these spiro-β-lactams may be beneficial in future drug discovery research.

DEDICATION

This thesis is dedicated to the memory of my beloved mother, whose love, guidance, and prayers have shaped me into who I am today. To my lovely father for his unwavering support and encouragement. To my siblings for their endless support, enthusiasm and faith in my abilities. Your belief in me has been my greatest motivation, and I share this accomplishment with all of you. And to Almighty God for life and blessings.

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CHAPTER I

INTRODUCTION

The synthesis of cyclic compounds is essential in organic chemistry due to their diverse uses and applications in medicinal chemistry and materials science. Classified by the IUPAC as cyclic compounds having atoms of at least two different elements as ring members, heterocyclic compounds represent an essential branch of organic chemistry with roots in medicinal chemistry and organic synthesis. The ring structures of heterocycles are made up of elements other than carbon, with oxygen, nitrogen, and sulfur being the most common substituents (Martins et al., 2015). One remarkable structural characteristic of heterocycles, which the pharmaceutical industry continues to benefit greatly, is their capacity to exhibit substituents in defined three-dimensional representations around a core scaffold (Dua & Shrivastava, 2011). Over decades of historical development in organic synthesis, researchers have been interested in heterocyclic compounds containing sulfur and nitrogen.

Amongst heterocycles, azaheterocycles, a class of heterocyclic compounds with one or more nitrogen atoms in their rings, are essential in medicinal chemistry (Liu et al., 2023). Their versatility and structural diversity, which enable the synthesis of a wide range of compounds with various pharmacological characteristics, give them significance. Aza heterocyclic rings are excellent frameworks for drug design because nitrogen atoms enhance their interactions with biological targets through hydrogen bonding and π-π stacking interactions (Kerru et al., 2020). Among the applied branches of organic chemistry, aza heterocyclic chemistry is a distinctive and

significant class in which a large amount of research is devoted to creating new compounds and composites. Over the last twenty years, we have seen an increase in interest in these molecules. They have a wide range of uses in the chemical sciences and helped develop several organic synthesis protocols (X. Li et al., 2013). Numerous N-heterocyclic compounds in nature are building blocks of numerous molecules with significant biological roles and are a crucial component of numerous compounds that are pharmacologically active. Purines and pyrimidines are examples of N-heterocyclic compounds that make up base pairs in DNA and RNA (Walsh, 2015). The fact that azaheterocycles are involved in many pharmacological processes highlights their significance. They are essential parts of many medicinal agents, such as antivirals, antibiotics, anticancer medications, and active substances in the central nervous system (CNS). For example, aza heterocyclic structures are present in many antibacterial agents and antiviral medications for hepatitis and HIV. Aza heterocyclic compounds are the building blocks of several protein kinase inhibitors in oncology and are essential components of anticancer drugs (Akhtar et al., 2017). A cursory look at FDA databases indicates the structural importance of nitrogen-based heterocycles in pharmaceutical engineering and drug design, with nearly 75% of novel small-molecule drugs containing a nitrogen heterocycle (Al-Hazmi et al., 2024).

There are several nitrogen-containing heterocyclic rings including the three-membered heterocycles (aziridines), four-membered (azetidines and 2-azetidinones), five-membered (1,2,3-triazoles, imidazoles, pyrazoles, oxadiazoles, oxazoles, isoxazoles and thiazoles), six-membered (quinoline, Quinazoline, Pyrimidines, pyridine), seven-membered (benzodiazepine) (Singh, 2021).

Among these heterocyclic compounds, the 2-azetidinone ring system commonly known as β-lactams (four-membered cyclic amide consisting of three carbon atoms and one nitrogen atom) has become the preferred scaffold for designing numerous antibiotics and is also an essential component in organic synthesis (Kerru et al., 2020). After being discovered by Alexander Fleming in 1928 from Penicillium notatum, β-lactams antibiotics are one of the most frequently prescribed drug classes with various clinical indications (Olazarán-Santibáñez et al., 2017). The β-lactam pharmacophore is found in a vast array of molecules, both synthetic and natural, that control the global antibiotic market, as the yearly spending on these antibiotics comes to about $15 billion USD, accounting for 65% of the entire antibiotic market (Pandey & Cascella, 2024). Besides antibiotics, β-lactams have other pharmacological applications, including anticancer, cholesterol-lowering effects, anti-inflammatory, anti-diabetic, etc. The β-lactam structure can serve as a fundamental building block for the synthesis of a range of conjugates with a variety of pharmacological applications (Kerru et al., 2020).

In this study, a derivative of β-lactam was synthesized following the Staudinger [2+2] ketene-imine cycloaddition reaction.

CHAPTER II

LITERATURE REVIEW

The discovery of the first β-lactams antibiotic, Penicillin G, has marked a pivotal moment in modern medicine, leading to the development of numerous other β-lactams for treating bacterial infections. However, the clinical efficacy of β-lactams as antibiotics has been challenged by the incidence of drug resistance due to the ability of some of the bacteria to produce beta-lactamase enzymes that hydrolyze the β-lactam rings. Combining β-lactams antibiotics with beta-lactamase inhibitors has been proven to be a great way to combat antibiotic drug resistance (Lima et al., 2020).

Classes of β-lactam Antibiotics

Numerous clinical applications are possible due to the diversity of the beta-lactam class, ranging from the treatment of simple bacterial infections to the eradication of multi-drug resistant pathogens. Their effectiveness lies in their ability to inhibit bacterial cell wall synthesis, and several research is being done to combat antibody drug resistance, ensuring beta-lactams remain a cornerstone of antimicrobial therapy. Beta-lactams are classified into several groups based on their chemical structure and activities (Balsalobre et al., 2019).

Penicillin

The earliest beta-lactam antibiotics to be identified are penicillins. They are made up of a thiazolidine ring fused to a beta-lactam ring. They work well against certain gram-negative and a range of gram-positive bacteria (Pandey & Cascella, 2024). All penicillin-class antibiotics are derivatives of 6-aminopenicillins acid and feature a beta-lactam ring structure crucial for their antimicrobial activity. Penicillin classes depend on the chemical groups attached to their side chains, which primarily affect their bioavailability and extend their activity spectrum toward Gram-negative bacteria compared to penicillin G. There are four subclasses of penicillins based on their spectrum of activity: narrow-spectrum or natural penicillins, very-narrow-spectrum (or penicillinase-resistant) penicillins, extended-spectrum or aminopenicillins, and broad-spectrum or antipseudomonal penicillins. The narrow-spectrum penicillin group comprises penicillin V, penicillin G, procaine penicillin, and benzathine penicillin. The penicillinase-resistant penicillins include oxacillin, methicillin, cloxacillin, and dicloxacillin. The broad-spectrum penicillins include Ampicillin and amoxicillin. The extended-spectrum antibiotics include carbenicillin, tircacillin, and piperacillin (Zaffiri et al., 2012).

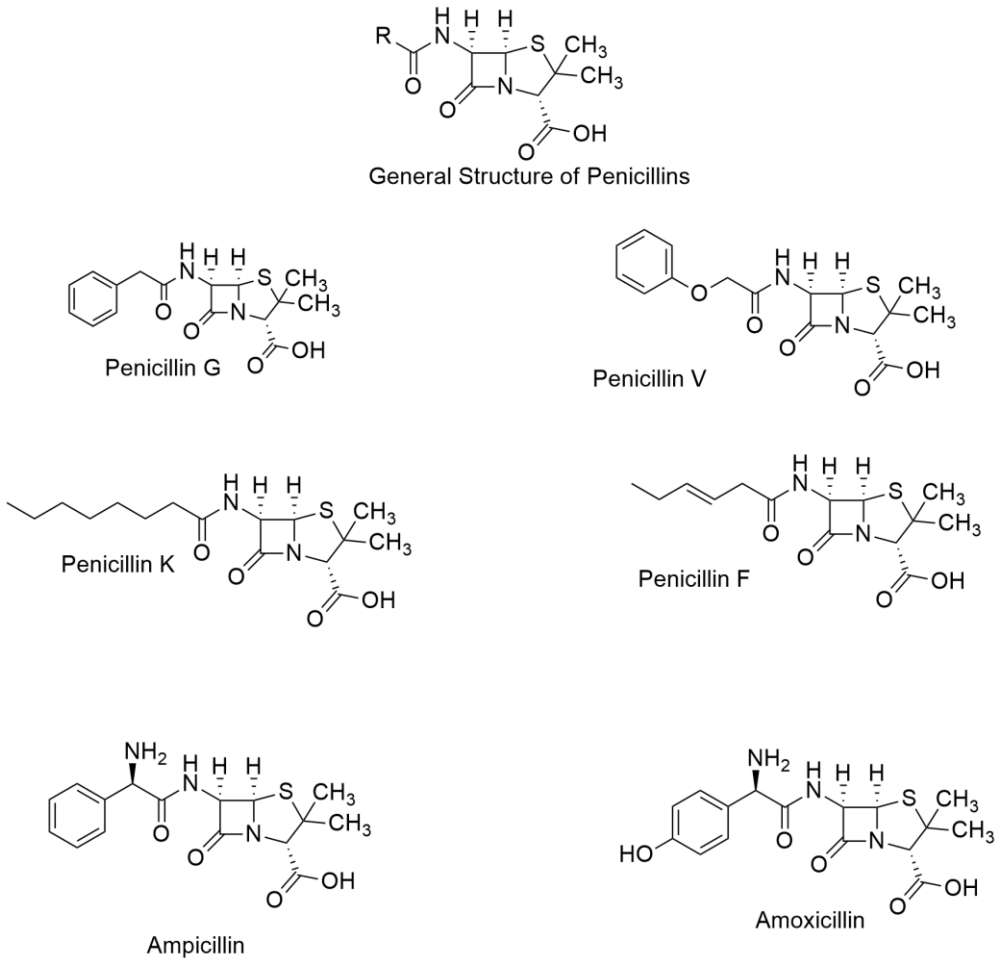


Figure 1: Examples of Penicillin Antibiotics

Cephalosporins

Cephalosporins have a beta-lactam ring fused to a dihydrothiazine ring. They are divided into generations (first, second, third, fourth, and fifth generations) based on their spectrum of activity and resistance to beta-lactamases (Pandey & Cascella, 2024). Cephalosporins are effective against common Gram-negative bacteria like Escherichia coli and nontypeable Haemophilus influenzae (ntHi), as well as methicillin-susceptible Staphylococcus aureus (MSSA). However, they are not effective against methicillin-resistant Staphylococcus aureus (MRSA) and enterococci, as these organisms are resistant to cephalosporins (Harrison & Bratcher, 2008).

Examples of cephalosporins include Cefadroxil, Cephalexin, Cefaclor, Cefprozil, Loracarbef, Cefoperazone, Cefpodoxime proxetil, etc. (Asbel & Levison, 2000).

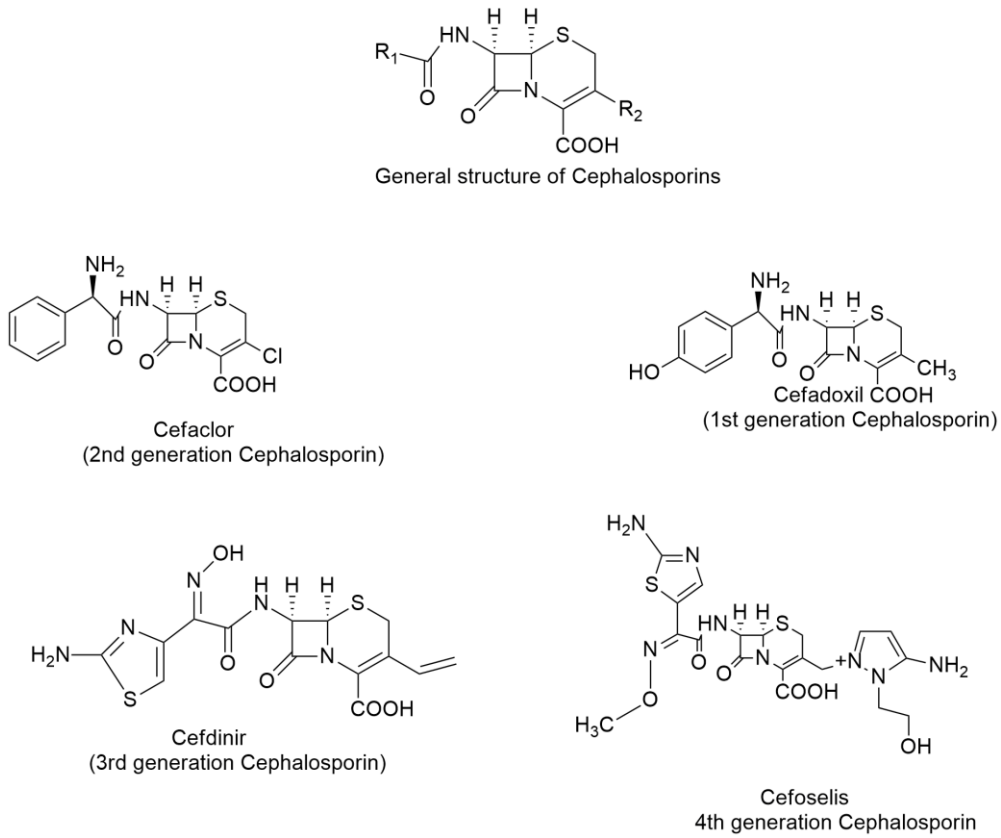


Figure 2: Examples of Cephalosporin antibiotics

Carbapenems

With a five-membered carbapenem ring fused to a beta-lactam ring, carbapenems are extremely effective broad-spectrum antibiotics. They work well against a variety of Gram-positive and Gram-negative bacteria, including strains that are resistant to multiple drugs and many beta-lactamases. Examples of carbapenems include Imipenem and Meropenem. (Knapp & English, 2001).

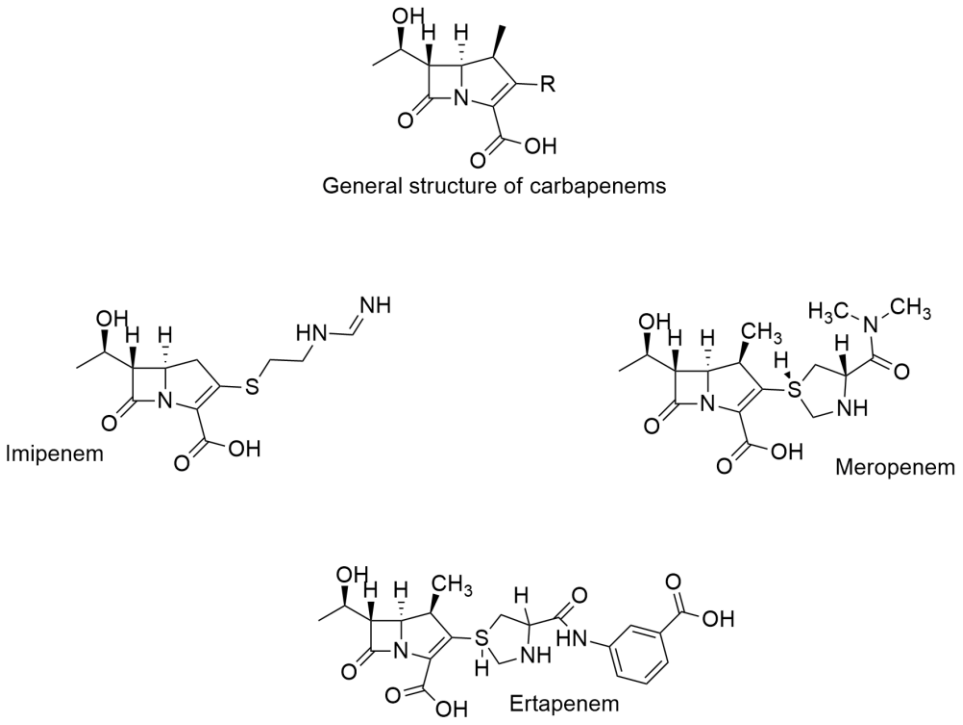


Figure 3: Examples of Carbapenem

Monobactams

Monobactams have just the beta-lactam ring and are not fused to any other ring. Aztreonam is the sole antibiotic currently available in this category. It is produced by the bacterium Chromobacterium violaceum and targets only Gram-negative aerobic bacilli (Asbel & Levison, 2000).

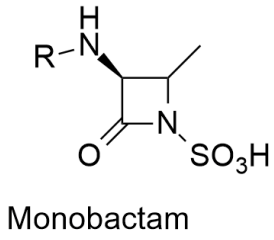


Figure 4: Example of Monobactam

β -lactamase Inhibitors

These are agents that inactivate serine beta-lactamases, which are enzymes that hydrolyze and inactivate the beta-lactam ring and they include clavulanic acid, sulbactam, tazobactam, avibactam, and vaborbactam (Wong & van Duin, 2017).

Mechanism of Action

The β-lactam antibiotics are broadly effective against several bacterial infections through acylating the transpeptidase involved in cross-linking peptides to form peptidoglycan, which is a fundamental constituent of the bacterial cell wall for both the gram-positive and gram-negative bacteria. The square ring structural feature of a β-lactam has a higher inner tension than a five- or six-membered ring thereby making β-lactam antibiotics more reactive in inactivating penicillin-binding proteins (PBPs) compared to five- or six-membered rings. Additionally, the carbonyl group offers an ideal site for nucleophilic attack during hydrolysis (Kim et al., 2023).

In 1974, Blumberg and Strominger proposed that all β-lactam antibiotics inhibit bacterial cell wall synthesis after discovering the structural similarities between penicillin and the D-Ala-D-Ala terminus of peptidoglycan (PGN) in the cell walls of Gram-positive and Gram-negative bacteria (Lima et al., 2020). Bacteria cell walls consist of PGN, which are essential for bacterial growth, development, mechanical stability, and rigidity. The PGN is a macromolecule composed of alternating units of N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) along with a crosslinking peptide component attached to MurNAc. PBPs recognize the terminal d-Ala-d-Ala dipeptide segment from one glycan chain in the model bacterium E. coli and crosslink it to the peptide of another glycan chain. The pentapeptide linked to MurNAc's d-Ala4 targets a nucleophilic attack by the catalytic serine residue in PBPs' active site.

This attack cleaves the terminal d-Ala5 and forms an acyl catalytic intermediate. A crosslink between the peptides is created when the amino group of mDAP3, or meso-2,6-diaminopimelic acid, in the pentapeptide from a different glycan strand attacks the intermediate acyl group of d-Ala4. The d-Ala-d-Ala dipeptide backbone and the core scaffold of β-lactam antibiotics share nearly the same three-dimensional structural, geometric, and stereochemical similarities. The β-lactam antibiotics' nucleophilic attack on d-Ala4 is preserved in the carbonyl group of the β-lactam rings. β-lactam antibiotics and dipeptides differ primarily in that the former has a more rigid and bulky core structure because of their core ring structures, and the latter is connected to various outside parts. The nitrogen atom of beta-lactam antibiotics with two linked rings adopts a bent conformation. One side of the ring surface is extensively exposed in this conformation, while the other side is better protected from the solvent. The convex side of the beta-lactam rings faces the active site of penicillin-binding proteins (PBPs) when they bind to them. This orientation positions the target carbonyl group of the beta-lactams for the best possible nucleophilic attack by the PBPs' catalytic serine. As a result, the beta-lactams and PBPs form covalent bonds more effectively. PBPs provide hydrogen bond donors to stabilize the acyl group of the newly bound beta-lactams on the opposite concave side of the beta-lactam rings (Kim et al., 2023).

Drug Resistance in β-lactam

Since its discovery, beta-lactam antibiotics have played a central role in fighting against bacterial infections. The ‘upgradation’ of ß -lactams from one generation to another is required for drug resistance. Due to the extensive use of these antibiotics, bacterial resistance is increasingly becoming a significant issue in both community and hospital environments. The mechanisms of bacterial resistance to β-lactams mainly fall into four categories: Inactivation by the production of beta-lactamases, decreased penetration to the target site, alteration of target site PBPs, and efflux from the periplasmic space through specific pumping mechanisms.

A large class of beta-lactamases is responsible for hydrolyzing beta-lactam antibiotics. Beta-lactamase enzyme-encoding genes are frequently found on mobile genetic elements such as transposons and plasmids, which facilitate their vertical transfer to progeny and horizontal transfer to neighboring bacteria. By hydrolyzing the carbonyl group in the beta-lactam ring which is comparable to the peptide bond in the d-Ala d-Ala dipeptide, beta-lactamases render beta-lactam antibiotics inactive (Palacios et al., 2018). Sequence analysis suggests that PBPs and b-lactamases diverged from a common ancestor. A lesser degree of b-lactam catalysis is possessed by all PBPs (Kong et al., 2010). There are various types of β-lactamases, and several classification systems have been proposed. In 1989, Bush and collaborators first attempted a functional classification, which was later refined in 1995. In 1980, Ambler's structural classification categorizes β-lactamases into four distinct molecular classes (A, B, C, and D) based on amino acid sequences. Amber class A, C, and D belong to the serine β-lactamases which perform a nucleophilic attack by a catalytic serine residue, while Amber class B belongs to the metallo-β-lactamases that have cofactor Zn2+ ions in the active site (K. Bush, 2018). The catalytic mechanism of serine beta-lactamases closely resembles that of penicillin-binding proteins (PBPs). The formation of the acyl

ester is specifically due to a nucleophilic acyl substitution, which is aided by the serine's hydroxyl group in the enzyme's active site attaching to the BLAs' β-lactam ring's carbonyl group and opening it. Subsequently, the enzyme's covalent bond with the β-lactam ring's carbonyl group is broken by a water molecule's nucleophilic attack, producing a carboxylic group. This allows the broken-down BLA to disperse and inactivate additional β-lactam molecules. Additionally, Beta-lactams may undergo decarboxylation, which would result in their total and irreversible inactivation (Alfei & Zuccari, 2022). In metallo-β-lactamases, the carbonyl group of β-lactams is directly attacked by zinc ions, coordinating a catalytic water molecule in the active site that hydrolyzes the β-lactam ring without forming an intermediate acyl form (Bahr et al., 2021).

For β-lactams to be effective, they must be able to reach their molecular targets within bacteria. Gram-negative bacteria's outer membrane acts as a selective permeability barrier, preventing access to many possible chemotherapeutic agents and making their cell wall a formidable challenge. Some hydrophobic medications, like aminoglycosides and macrolides, can get past this barrier by diffusing straight through the outer lipid bilayer (Nikaido, 2003). Hydrophilic medications, like fluoroquinolones and β-lactams, penetrate the periplasm by means of enhanced diffusion, which is facilitated by specific Outer Membrane Proteins (OMPs) known as porins. These porins, or β-barrel proteins, open a hydrophilic channel through which low molecular weight solutes can pass through the outer membrane (Pagès et al., 2008; Vergalli et al., 2020). Compounds may preferentially enter the cell through a specific channel, and the width and selectivity of the internal pores of the different porins produced by a given bacteria vary. Thus, changes in the quantity and makeup of porins in the outer membrane, resulting from deletion, mutation, or altered expression levels of porins, can have a substantial effect on the antibiotic susceptibility of bacterial isolates. This is frequently linked to additional resistance mechanisms,

like the synthesis of β-lactamase or the expression of drug efflux systems (Bahr et al., 2021; Delcour, 2009).

PBP 2_(PBP2a) of Staphylococcus aureus and PBP 2x of Streptococcus pneumoniae are two examples of altered penicillin-binding proteins that exhibit relatively low affinity toward beta-lactam antibiotics. Because these PBPs are relatively resistant to penicillins, they cannot be inactivated by them, and they can take on the roles of other PBPs once they are deactivated (Chambers, 1997).

Efflux pumps are a component of the acquired or intrinsic resistance phenotype. Various substrates can be exported by efflux pumps from the periplasmic region of the cell to the external environment. These pumps determine drug resistance to multiple drugs in different Gram-negative bacteria, particularly P. aeruginosa (Poole, 2004). Many beta-lactam antibiotics, such as cephalosporin, penicillin, tetracycline, quinolones, and chloramphenicol, can cause decreased susceptibility when the organism's outer membrane permeability decreases, and mexAmexB-OprD is upregulated (X. Z. Li et al., 2000; Srikumar et al., 2000).

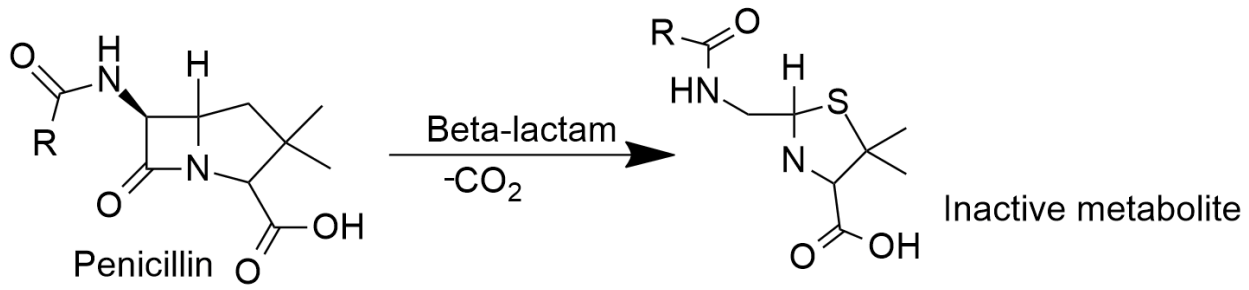


Figure 5: Mechanism of beta-lactam resistance

β-lactamase Inhibitors

The impact of beta-lactamases' hydrolytic activity can be mitigated in two main ways. The first principle is obtaining compounds that inhibit or deactivate beta-lactamases. Three inhibitors-sulbactam, clavulanic acid, and tazobactam-lactamase—are employed in clinical settings. Penicillin and all three of these substances have structural similarities. Among these compounds' characteristics are their strong affinity for β-lactamases, their comparatively longer active site occupancy than β-lactams, their distinct reaction chemistry, and their poor enzyme hydrolysis (Helfand et al., 2003; Padayatti et al., 2004). The second principle is obtaining a novel beta-lactam antibiotic with a high affinity for β-lactamases that is either not hydrolyzed by PBP or hydrolyzed poorly by it. This was the original justification for cephalosporins or extended-spectrum carbapenems. A common illustration of this idea is the creation of drugs like doripenem and ceftobiprole. Ceftobiprole is an "anti-MRSA cephalosporin" that exhibits a strong affinity for PBP2, is resistant to S. aureus penicillinase, and is active against gram-negative bacteria with beta-lactamases. Doripenem, a modified carbapenem with a 1-beta-methyl group and a sulfamoylaminomethyl substituted pyrrolidylthio group at the C2 position, exhibits extremely high activity against P. aeruginosa, Burkholderia cepacia, and Acinetobacter spp (Chen et al., 2005; von Eiff et al., 2005).

Clavulanic acid

The first combination β-lactam β-lactamase inhibitor created in 1985 for parenteral administration was ticarcillin-clavulanate. It boosts the inhibitory activity against E. coli, Proteus species, H. influenzae, Pseudomonas species, Klebsiella species, Providencia, and staphylococci that produce -lactamases. The organism's susceptibility to amoxicillin is increased when amoehilus

influenza and Neisseria gonorrhea, which are resistant to amoehilus influenza, are combined with clavulanic acid (Brogden et al., 1981; L. M. Bush & Johnson, 2000).

Tazobactam

The first preparation of piperacillin and tazobactam occurred in the United States in 1993. It is well known that piperacillin exhibits antibiotic activity against both gram-positive and gram-negative bacteria as well as anaerobes and aerobes. The combination of piperacillin and tazobactam exhibits broad-spectrum antibacterial activity against gram-positive and gram-negative bacteria, making it an effective β-lactamase inhibitor. However, this combination is ineffective against gram-negative bacillus isolates that contain AmpC β-lactamase. The combination of piperacillin and tazobactam has been shown to be useful in the treatment of several infections, including infections of the abdomen (Gin et al., 2007).

Sulbactam

Though not as effective as clavulanic acid, sulbactam is a semi-synthetic compound that can inactivate β-lactamases. It exhibits high activity against class II–IV and relatively low action against class 1β-lactamase. Certain antibiotics, such as ampicillin, have an extended and more potent antibacterial activity when combined with sulbactam. This is because the combination of sulbactam and ampicillin tends to increase the activity of the antibiotic against bacteria that are resistant to antibiotics. It was discovered that sultamicillin, a compound that contained sulbactam-ampicillin, was clinically effective in treating a wide range of infections, including those of the skin and soft tissues. It was also noted that neisseria gonorrhea infections, which are ampicillin-resistant, could be treated with a single intramuscular dose of ampicillin-sulbactam combined with probenecid (M. Ibrahim et al., 2019) (Olazarán-Santibáñez et al., 2017)

β -lactams as Anti-cancer Drugs

Cancer is the second leading cause of death in the USA, following heart disease, and it poses a significant threat to global health. Although chemotherapy is an effective treatment, some cancer cells develop resistance by altering their mechanisms of action, making them less responsive to chemotherapeutic agents (James et al., 2024). Since many anticancer medications now on the market are toxic, their effectiveness is constrained. Thus, the development of novel chemotherapeutic agents with reduced toxicity becomes imperative. For many years, bacterial infections with minimal or no toxicity have been treated with β-lactam antibiotics. It was then hypothesized that as cancer cells do not produce ß-lactamase enzymes, the beta-lactam ring's stability should be higher in tumor environments. With appropriate chemical modifications, ß-lactams should inhibit the proteins that are responsible for the proliferation, angiogenesis, and metastasis of various cancers. Several kinds of research are now being conducted to synthesize and elucidate the anticancer potentials of beta-lactams and their mechanism of action (Olazarán-Santibáñez et al., 2017). Since these compounds have a range of pharmacological properties and some have shown promising potency against drug-sensitive and drug-resistant cancer cell lines, β-lactam derivatives are flexible and appealing scaffolds for drug discovery. Therefore, the β-lactam moiety serves as a valuable model for the creation of new anticancer drugs (Zhang & Jia, 2020). Some examples of anticancer beta-lactams are shown in Table 1(Olazarán-Santibáñez et al., 2017).

Table 1: Examples of Anticancer Beta-lactams

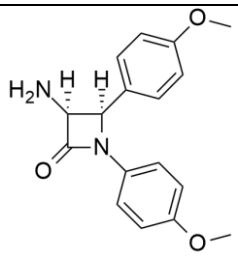
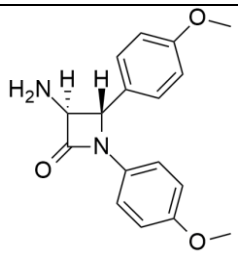
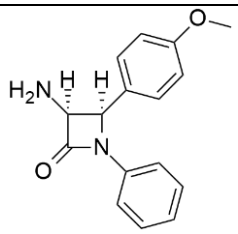
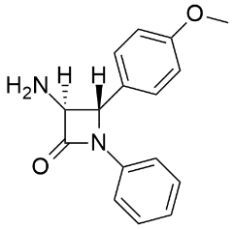
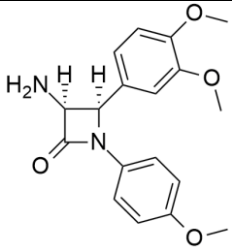
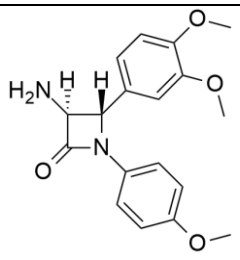
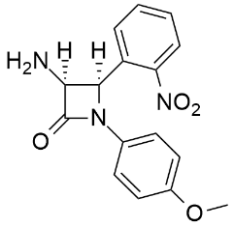
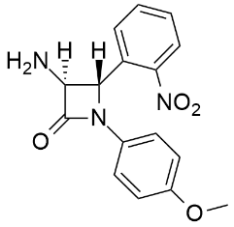
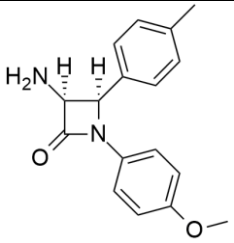
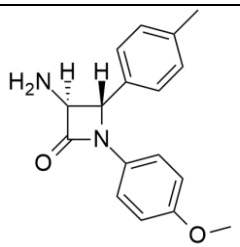
S/N	Code	Structure
1	(±)-*Cis*-3-amino-1,4-*bis*(*4*-methoxyphenyl) azetidin-2-one (1C)	
2	(±)-*Trans*-3-amino-1,4-*bis*(*4*-methoxyphenyl) azetidin-2-one (1T)	
3	(±)-*Cis*-3-amino-4-(*4*-methoxyphenyl)-1-phenyl-azetidin-2-one (2C)	
4	(±)-*Trans*-3-amino-4-(*4*-methoxyphenyl)-1-phenyl-azetidin-2-one (2T)	
5	(±)-*Cis*-3-amino-4-(3,4-dimethoxyphenyl)-1-(4-methoxyphenyl) azetidin-2-one (3C)	

Table 1: Examples of Anticancer Beta-lactams (cont.)

S/N	Code	Structure
6	(±)-*Trans*-3-amino-4-(3,4-dimethoxyphenyl)-1-(*4*-methoxyphenyl) azetidin-2-one (**3T**)	
7	(±)-*Cis*-3-amino-1-(4-methoxyphenyl)-4-(2-nitrophenyl) azetidin-2-one (4C)	
8	(±)-*Trans*-3-amino-1-(*4*-methoxyphenyl)-4-(*2*-nitrophenyl) azetidin-2-one (4T)	
9	(±)-*Cis*-3-amino-1-(4-methoxyphenyl)-4-(*p*-tolyl) azetidin-2-one (5C)	
10	(±)-*Trans*-3-amino-1-(*4*-methoxyphenyl)-4-(*p*-tolyl)azetidin-2-one (5T)	

β -lactams as Prodrugs for Anticancer Chemotherapies

Numerous antitumor antibiotics approved for cancer therapy inhibit tumor cell growth by intercalating with DNA. Additionally, using beta-lactams as prodrugs has successfully enhanced the delivery of chemotherapeutic agents directly to tumor sites. The most widely used beta-lactams for prodrug-based cancer chemotherapy are cephalosporins. Because of their innate reactivity when hydrolyzed by beta-lactamase enzymes, they were chosen as prodrug candidates. The 3'-substituent is expelled because of a secondary reaction that is started by hydrolyzing the beta-lactam ring. This position may be linked to a cytotoxic component that is released when the enzyme hydrolyzes the beta-lactam ring of the cephalosporin (Kuhn et al., 2004). Moreover, cephalosporins have been utilized in the highly publicized Antibody Directed Enzyme Prodrug Therapy (ADEPT) technique, which targets anticancer drugs selectively to tumor cells. An enzyme that is covalently linked to a monoclonal antibody that is specific for a tumor cell antigen is used in the ADEPT drug delivery technique. By using this tactic, the cytotoxic agent can be delivered to target tumor cells specifically while appearing as a prodrug. The patient is first given the monoclonal antibody-enzyme immunoconjugate (mAb-enz) to achieve the site-specific generation of the cytotoxic agent. As a result, prelocalization of the mAb-enz on the targeted tumor cell surface is made possible. After that, the prodrug—the enzyme's substrate is given, causing the medication to release selectively (Bagshawe, 1987). Two cephalosporin prodrugs of mitomycin C were tested on clone 62 melanoma and H2987 lung adenocarcinoma cell lines. One of the prodrugs had cytotoxicity that was comparable to the parent drug, according to in vitro studies, while another prodrug had cytotoxicity that was 40 and 10 times lower toward clone 62 and H2987 melanoma cells, respectively (Vrudhula et al., 1997).

Apoptosis Induction

A previous study reported the synthesis of fourteen diversely substituted diastereomeric β-lactam derivatives. One of these newly synthesized cis-β-lactam derivatives (6C) was evaluated in vitro for its anticancer properties and found to be more potent in human cervical carcinoma SiHa cell lines, ten times less toxic in normal liver Chang cell lines, and six times more potent in murine melanoma B16F10 cell lines when compared to the positive control, the well-known anticancer alkaloid colchicine. The mechanism of action of the compound was found to be apoptotic celldeath through caspase activation, and its binding sites on β-tubulin were found to be like those of colchicine. Using a molecular docking assay, the molecular coupling of compound 6C on the β-tubulin colchicine site revealed binding interactions with the amionacid residues Cys 239, Lys 252, Val 323, Met 257, Lys 350, Leu 246, and Ala 248 (Olazarán-Santibáñez et al., 2017). Many synthetic N-methylthio-beta-lactam compounds have been shown to cause apoptosis in a variety of tumor cell lines, including MCF-7 and MDA-MB-231 breast cancer cells, PC-3 and DU-145 prostate cancer cells, and PCI-13 head-and-neck cancer cells (Cainelli et al., 2003). Another study reported the synthesis of 2-azitidinone derivatives from Baylis Hillman allyl amine, where one of the derivatives with the highest potency to breast cancer cells increased the expression of pro-apoptotic genes (Bax and Bid) while decreasing the expression of anti-apoptotic gene (Bcl-2) (Geesala et al., 2016).

PARP Inhibitor

Nuclear enzymes known as poly (ADP-ribose)polymerases (PARPs) polymerize poly(adenosine diphosphate-ribose) on substrate proteins that are essential for various biological processes, such as cell division, DNA repair, telomere integrity, gene transcription, and survival and death of cells. Several small molecule PARP inhibitors have been reported to show anticancer

activities in different types of cancer including pancreatic cancer (Shetu et al., 2023). To investigate the impact of adding a β-lactam moiety on the inhibitory activity of PARP enzymes, Penning et al. synthesized a series of tetrahydropyridopyridazinone PARP inhibitors for the treatment of cancer. The compound with a β-lactam moiety showed more PARP inhibitory activity, which is about 40 times more than the parent compound (Zhu et al., 2012)

AMPK Activator

It has been suggested that AMPK, a serine/threonine protein kinase conserved in eukaryotes, functions as a cellular energy sensor to control how cells respond to nutritional or environmental stress (Koh et al., 2008). An increase in the cellular AMP/ATP ratio can cause the upstream kinase STK11 to phosphorylate on Thr172, activating AMPK (Shackelford & Shaw, 2009). Numerous cancer cell types are cytotoxic to AMPK activation, suppressing tumor growth, thereby indicating AMPK as a viable target for cancer therapy. Furthermore, a variety of AMPK activators have therapeutic applications as chemotherapy agents, mediating apoptotic effects and impeding the growth of spontaneous tumors (Fruman & Edinger, 2008). Diverse derivatives of 1,4-diaryl-2-azetidinones were shown to have cytotoxic effects against duodenal adenocarcinoma cells (HuTu-80 cells) through inducing apoptosis, inhibit tubulin polymerization, arrest the cell cycle at G2/M phase (Tripodi et al., 2012).

Tubulin Polymerization Inhibitor

Numerous cellular processes rely on microtubules, which are cytoskeletal structures created by α and β tubulin heterodimers self-assembling. Numerous chemically diverse substances bind to tubulin, changing the polymerization and dynamics of microtubules in a variety of ways

(Flynn et al., 2002). An oral anti-tumor β-lactam derivative was reported to exert its effect by targeting the tubulin colchicine binding site

AKT Inhibitor

Protein kinase B (Akt), a serine/threonine kinase that is activated at two residues, Thr308 and Ser473, and is usually over-expressed or over-activated in various cancer cells (Brownawell et al., 2001). Inhibiting the activating of AKT reduces the proliferating cancer cells, thereby making Akt an attractive target for anticancer drug discovery (Wang et al., 2008). A β-lactam derivative reduced phosphorylation of AKT and GSK-3β and the significant decrease in AKT kinase activity in a concentration-dependent (Geesala et al., 2016).

Estrogen Receptor

β-Lactam derivative containing an estrogen receptor ligand and a tubulin ligand has been reported to have dual-targeting properties for both the estrogen receptor and tubulin. It was shown to induce apoptosis in MCF-7 cells and accumulation of cells in the G2/M phase (O'Boyle et al., 2014).

Medicinal Importance of Spiro Compounds

Spiro compounds are molecules containing two rings with only one shared atom. Their good balance between conformational restriction and flexibility makes them free from absorption and permeability issues. They are ideal building blocks for synthesizing novel pharmacological compounds since this structural arrangement allows for a wide range of chemical and physical properties. The biological activity, potency, and selectivity of many drugs are frequently improved by including a spiro moiety (Acosta-Quiroga et al., n.d.). Spiro compounds have a unique molecular structure where two rings share a common atom. This arrangement makes them

excellent building blocks for synthesizing novel compounds with diverse chemical and physical properties. Spiro structures are found in various synthetic and natural products, often enhancing the biological activity, potency, and selectivity of these compounds. Spiro compounds are associated with various pharmacological activities, including anticancer, antibacterial, anti-inflammatory, and antiviral properties. However, their synthesis is challenging due to a quaternary carbon, requiring specific reaction conditions and methods (Zheng & Tice, 2016). Despite being unconventional scaffolds for bioactive molecules, spirocyclic systems have been successfully incorporated into more molecules with pharmacological applications, such as antidiabetic, anticancer, and anti-Alzheimer's; in some cases, they have even been successfully developed as commercial drugs. This is due to recent advancements in the isolation and characterization of new compounds from natural products and new synthetic routes to spiro building blocks (Acosta-Quiroga et al., n.d.). Several studies have reported the biological activities of spiro compounds as anticancer agents (Bora et al., 2021; S.-W. Li et al., 2016; Reddy et al., 2015), antioxidants (Acosta-Quiroga et al., n.d.), antibacterial (N. A. Ibrahim et al., 2022; Naglaa et al., 2022), antidiabetic ((Toumi et al., 2021), anti-inflammatory ((Hafez et al., 2008), antihypertensive ((Chapman et al., 2007; Sica, 2015), insecticidal ((Yao et al., 2023; Zubair, 2023).

CHAPTER III

METHODOLOGY

Experimental

Chemicals and Apparatus

During the investigation, solvents were acquired from Fisher Scientific International Inc. (Pittsburgh, PA), while chemicals were purchased from Sigma-Aldrich, Inc. (St. Louis, MO) and VWR International (Missouri, TX). Solvents were further dried and purified by conventional methods before use. The melting point of the synthesized products was ascertained through the utilization of Barnstead Thermolyne and Electrothermal Meltemp. FT-IR spectra were recorded on a Bruker Alpha modular Platinum-ATR FT-IR spectrometer using OPUS software. The mass spectrum was determined using the Advion expression compact mass spectrometer. NMR spectra were recorded on a Bruker Ascend 600 MHz using d6-DMSO as the solvent, with the spectra generated using TopSpin software. The chemical shifts are expressed as δ values in parts per million (ppm), and the coupling constants (J) are given in hertz (Hz).

Synthesis of Spiro Beta-lactam

In this research, a novel beta-lactam derivative has been synthesized using a multistep synthetic procedure. Firstly, Imine (Schiff base) is prepared from a ketone or aldehyde and an amine. Furthermore, the Spiro beta-lactam is then synthesized from the Imine through the Staudinger reaction.

Procedure for the Preparation of Imine (Isatin + 2-Aminopyridine)

10mmol each of isatin and 2-aminopyridine were added into 250ml round bottom flask. Afterwards, 50ml dry methanol and sufficient 4Å molecular sieves were added into the same flask. Also, an air-free experimental condition was maintained throughout the reaction time under an inert argon atmosphere. Then, the reaction mixture was stirred overnight. After completion of the reaction (monitored by thin-layer chromatography), the reaction mixture was filtered through vacuum filtration using dichloromethane and methanol as the solvents for washing. After filtration, the imine (filtrate) was transferred into a 250ml round bottom flask, and the solvent was removed under reduced pressure distillation (by rotary evaporator) and dried in the desiccator for 48 hours.

Procedure for the Preparation of Imine (Isatin + p-Anisidine)

A 1:1 molar ratio of isatin and p-anisidine was dissolved in anhydrous toluene under argon gas. The solution was stirred and refluxed using a Dean-Stark separator for 4 hours, monitored to completion with thin-layer chromatography. The solvent was evaporated under reduced pressure distillation, and the pure imine was isolated through recrystallization in dichloromethane, hexane, and diethyl ether.

Procedure for the Synthesis of Acetoxy Spiro Beta-lactams via Staudinger [2+2] ketene-Imine Cycloaddition Reaction

In the 250ml round bottom flask containing the prepared imine, 70ml of dry dichloromethane (DCM) was added. Then 5.56ml of anhydrous triethylamine (TEA) was added. The reaction mixture was stirred in an ice bath for 20 minutes. Then 2.16ml of acetoxy acetyl chloride was added dropwise. The reaction mixture was stirred for 48 hours. Also, an air-free experimental condition was maintained throughout the reaction time under an inert argon atmosphere. After completion of the reaction (monitored by thin layer chromatography), the

reaction mixture was extracted with saturated brine solution, DCM, and deionized H_2O successively. The organic layer was dried over anhydrous sodium sulfate, filtered, and evaporated to obtain the crude product

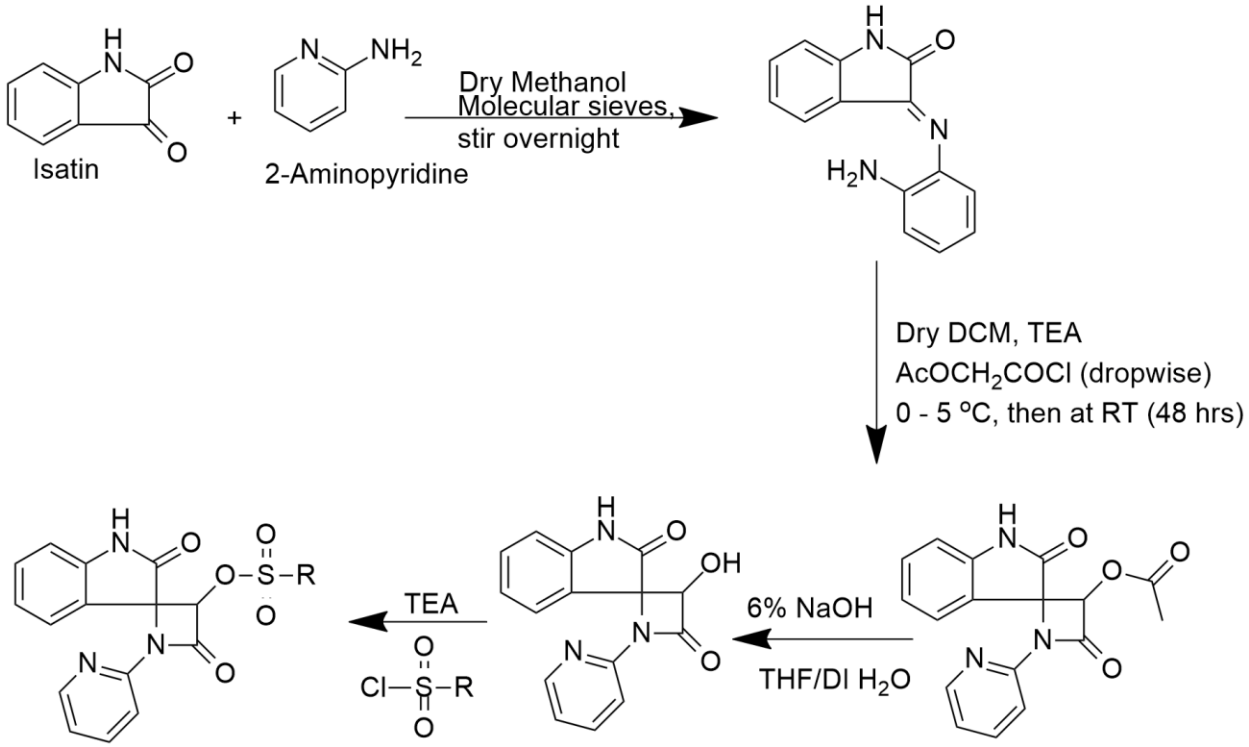


Figure 6: Reaction Mechanism for the synthesis of Beta-lactam (Isatin + 2-Aminopyridine)

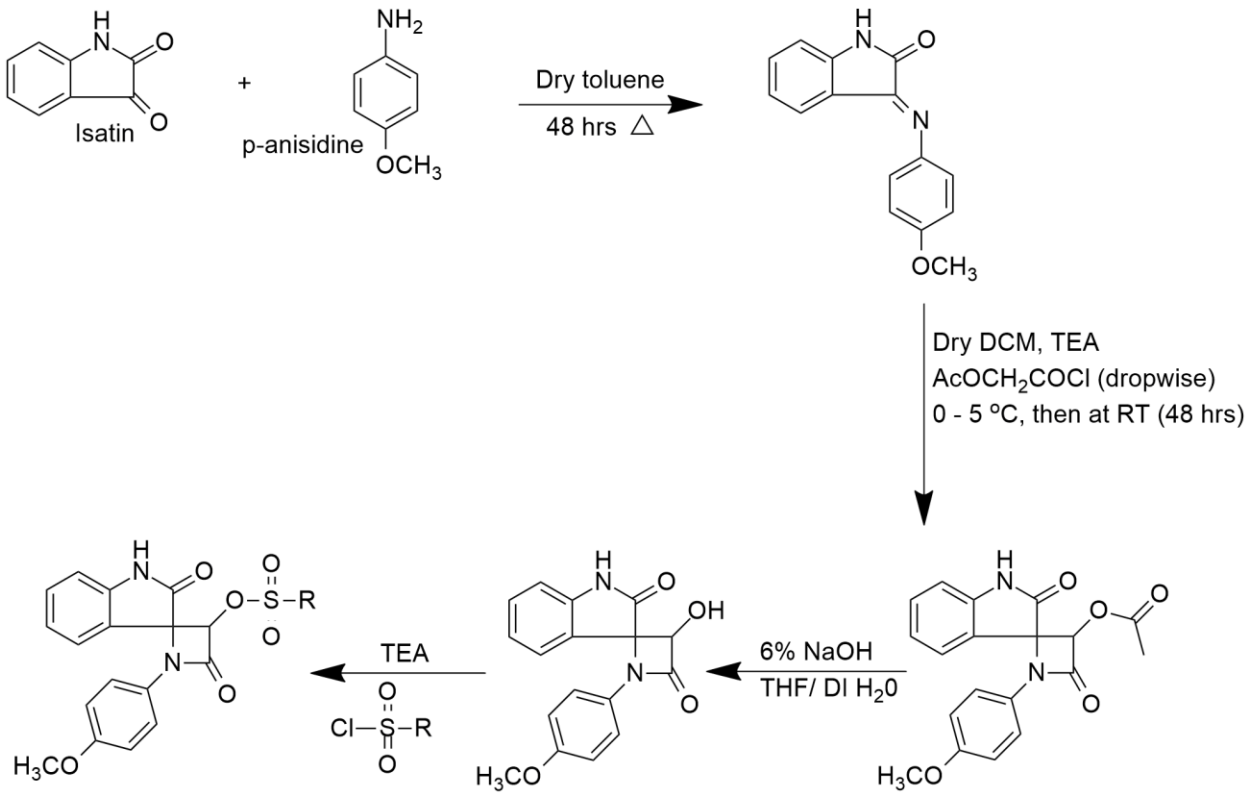


Figure 7: Reaction Mechanism for the synthesis of Beta-lactam DBRM (Isatin + p-Anisidine)

Isolation of pure product

The impure product from reaction mixture was purified through column chromatography over silica gel using ethyl acetate-hexane and methanol-ethyl acetate as solvent.

Subsequently, the pure isolated compounds were analyzed using Fourier Transform Infrared (FTIR) spectroscopy, mass spectroscopy (MS), and nuclear magnetic resonance (NMR) spectroscopy.

CHAPTER IV

RESULT AND DISCUSSION

Characterizations

Table 2: Characteristics of Compounds

Compound ID	Melting Point	Eluting Solvent
DBRM	187.7 ºC – 190.4ºC	20% Ethyl acetate 80% Hexane
BL (Isatin + 2-Aminopyridine)	204.9 ºC – 205.6ºC	2% Methanol 98% Ethyl acetate

Characterization of Compound 1

DBRM**:** Light yellow (5.74%); m.p 187.7 ºC – 190.4ºC; IR (KBr) 1766, 1747, 1729, 1620, 1511, 1467, 1390, 1373, 1244 cm^{-1} (Figure 10); ^{1}HNMR(600MHz,DMSO) δ 10.72 (s), 7.56 (d, 7.32Hz), 7.3 (t, 7.68Hz), 7.0 (t, 7.44Hz), 6.9 (m, 7.92Hz), 6.8 (d, 8.58Hz), 5.82 (s), 5.75 (s), 2.50 (s), 2.06 (s) (Figure 11); ^{13}C NMR (150 MHz, DMSO) 10 carbon δ 20.50, 55.75, 66.97, 82.15, 111.05, 115.30, 118.86, 122.91, 123.81, 124.97, 129.46, 131.27, 142.56, 156.96, 160.91, 169.98, 172.05 (Figure 12). Chemical Formula: $C_{19}H_{16}N_2O_5$; Molecular Weight: 353.11.

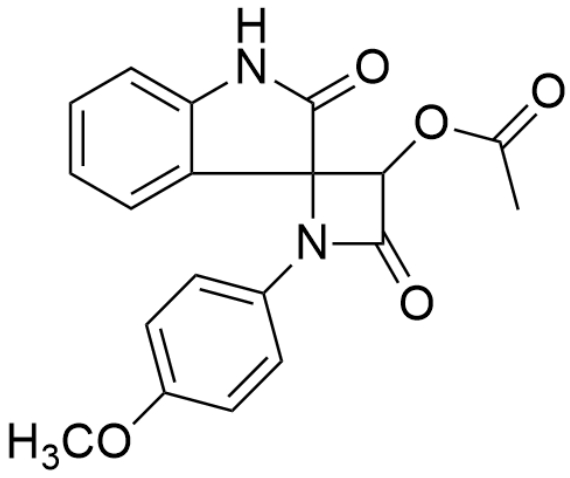


Figure 8: Structure of DBRM Beta-lactam

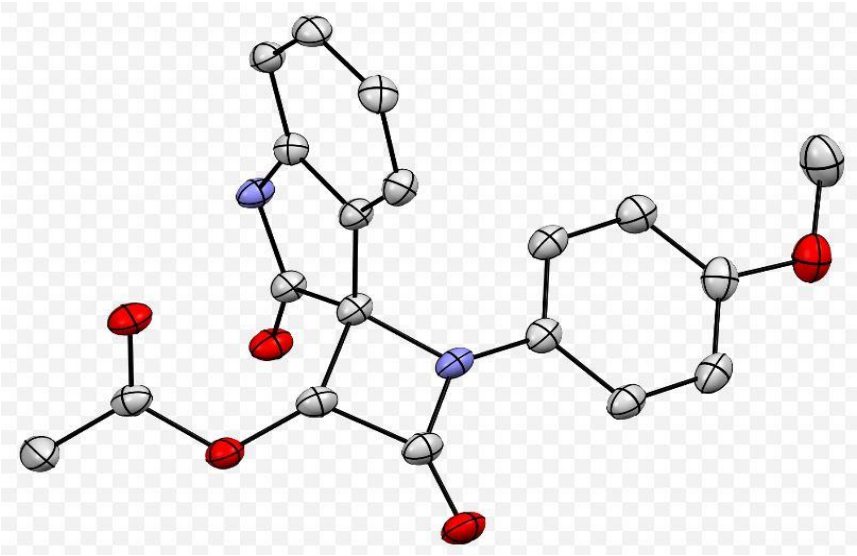


Figure 9: X-ray crystallographic (ORTEP) structure

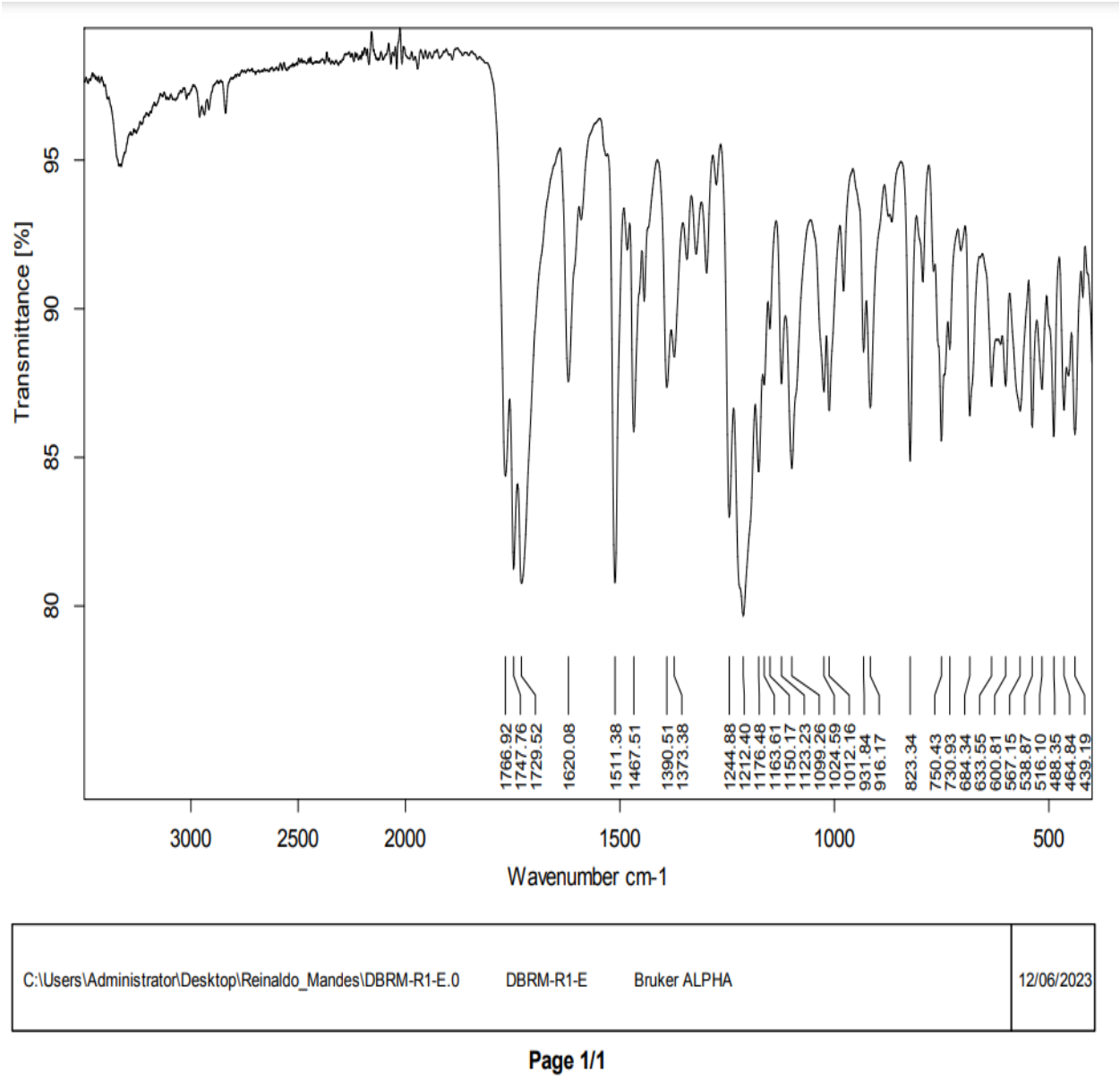


Figure 10: FT-IR of DBRM

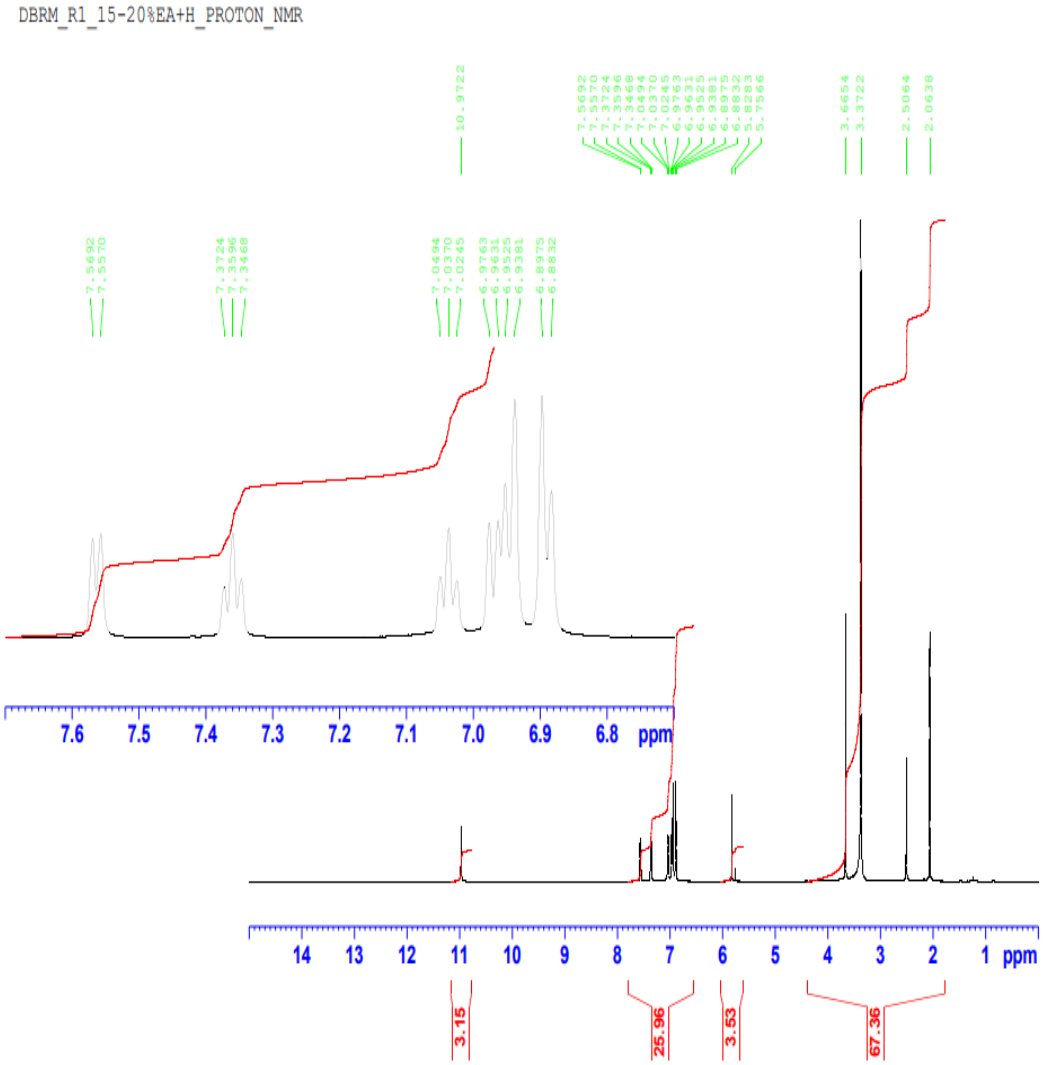


Figure 11: ^{1}H NMR of DBRM

Table 3: Different proton positions in DBRM and their chemical shifts

Chemical Shift (δ)	Peak number	J value (Hz)
10.9	Singlet	
7.5	Doublet	7.32
7.3	Triplet	7.68
7.0	Triplet	7.5
6.9	Multiplet	
6.8	Doublet	8.58
5.8	Singlet	
3.6	Singlet	
2.0	Singlet	

Table 4: Different carbon positions in DBRM and their chemical shifts

Types	Chemical shift, δ (ppm) ^{13}C NMR
C=O	172.0526
C=O	169.9851
C=O	160.9125
C(N)	156.9644
C(=)	142.5662
CH(=)	132.2762
C(=)	129.4713
CH(=)	124.9633
C(=)	123.8210
CH(=)	122.9116
CH(=)	118.8920
CH(=)	115.3090
CH(=)	111.0595
CH	82.1501
C	66.9770
OCH3	55.7504
CH3	20.5001

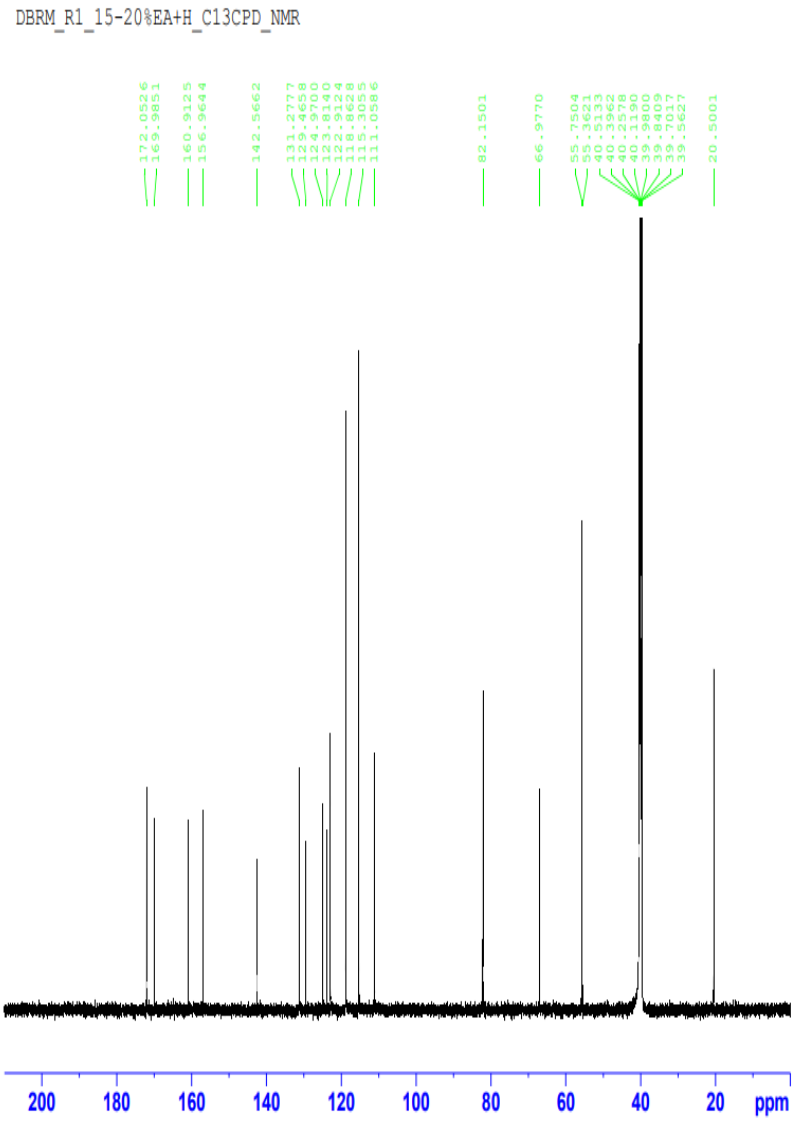


Figure 12: ^{13}C NMR of DBRM

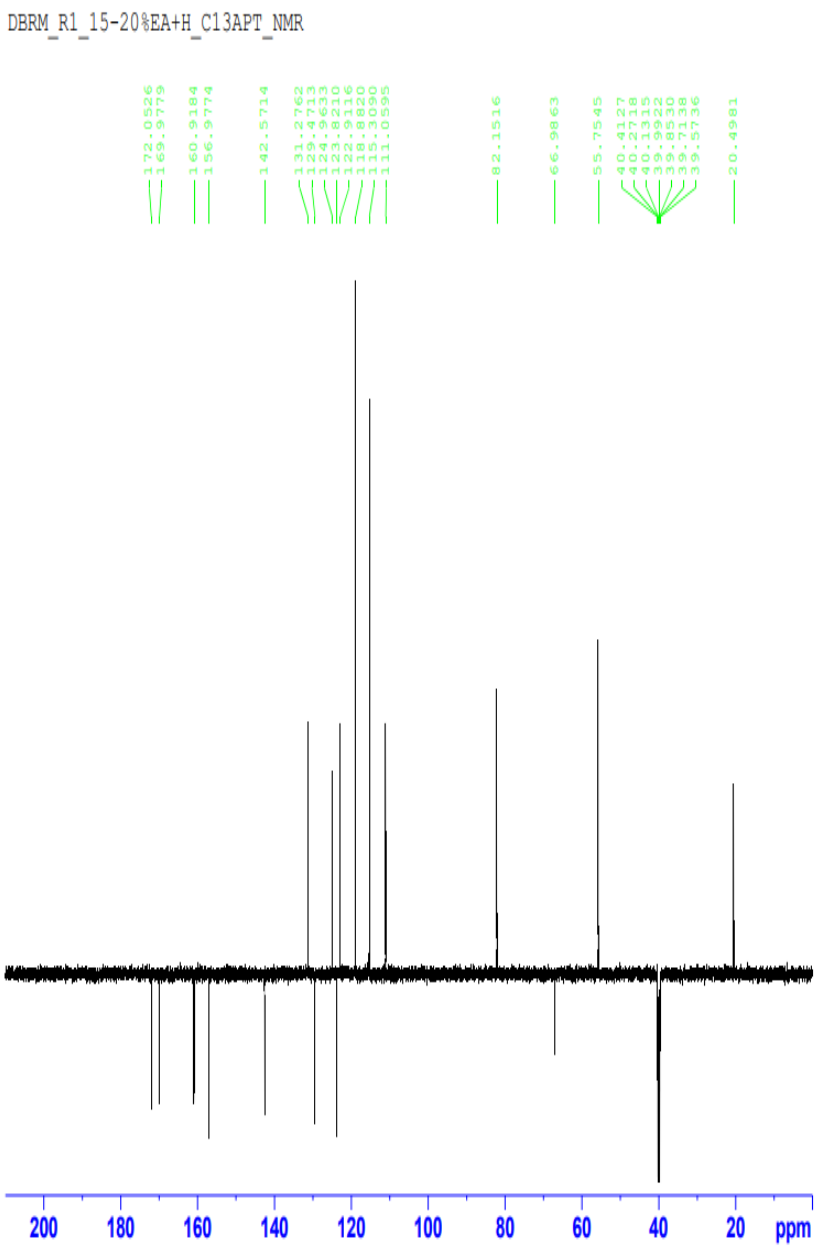


Figure 13: ^{13}C APT NMR of DBRM

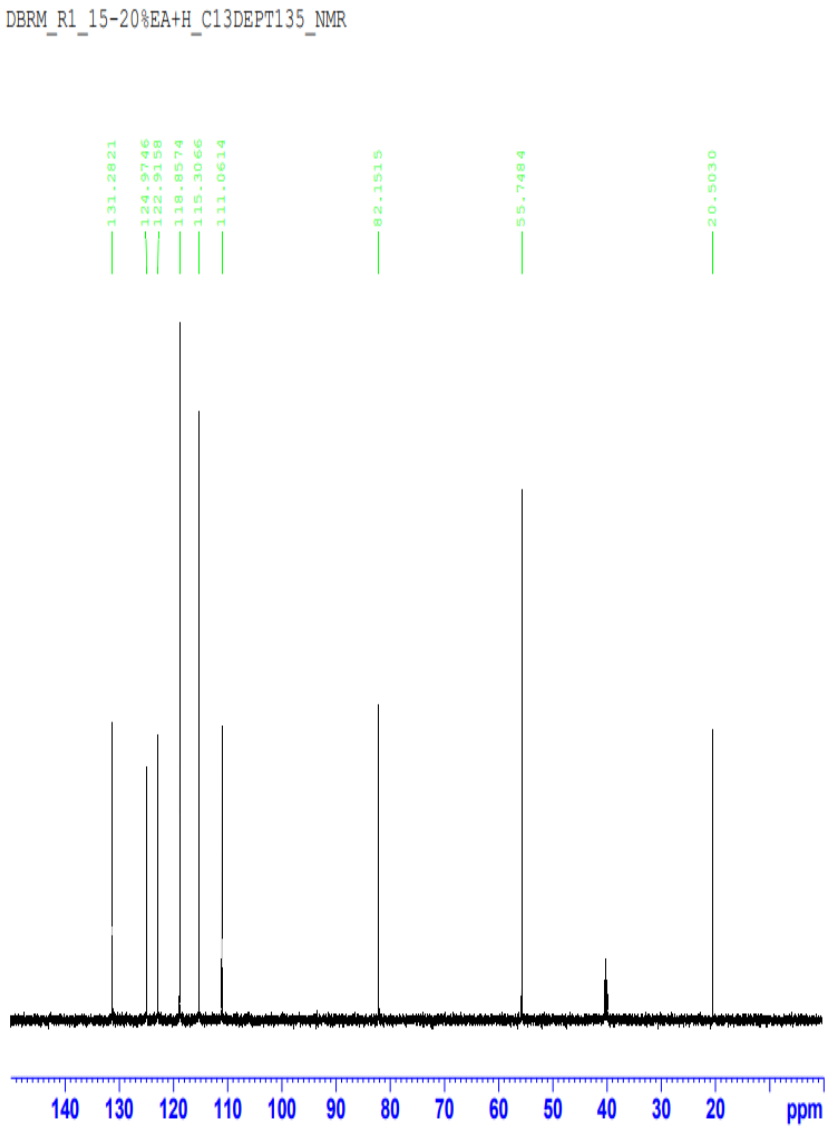


Figure 14: ^{13}C DEPT135 NMR of DBRM

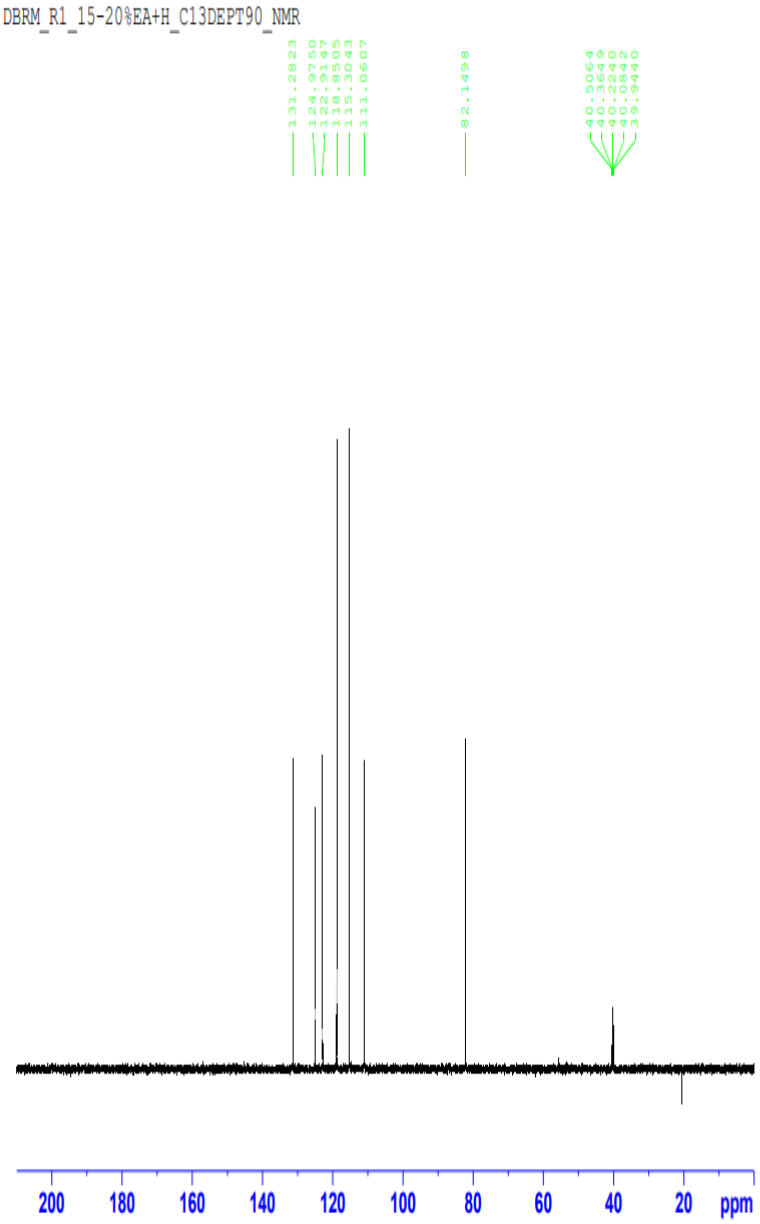


Figure 15: ^{13}C DEPT90 NMR of DBRM

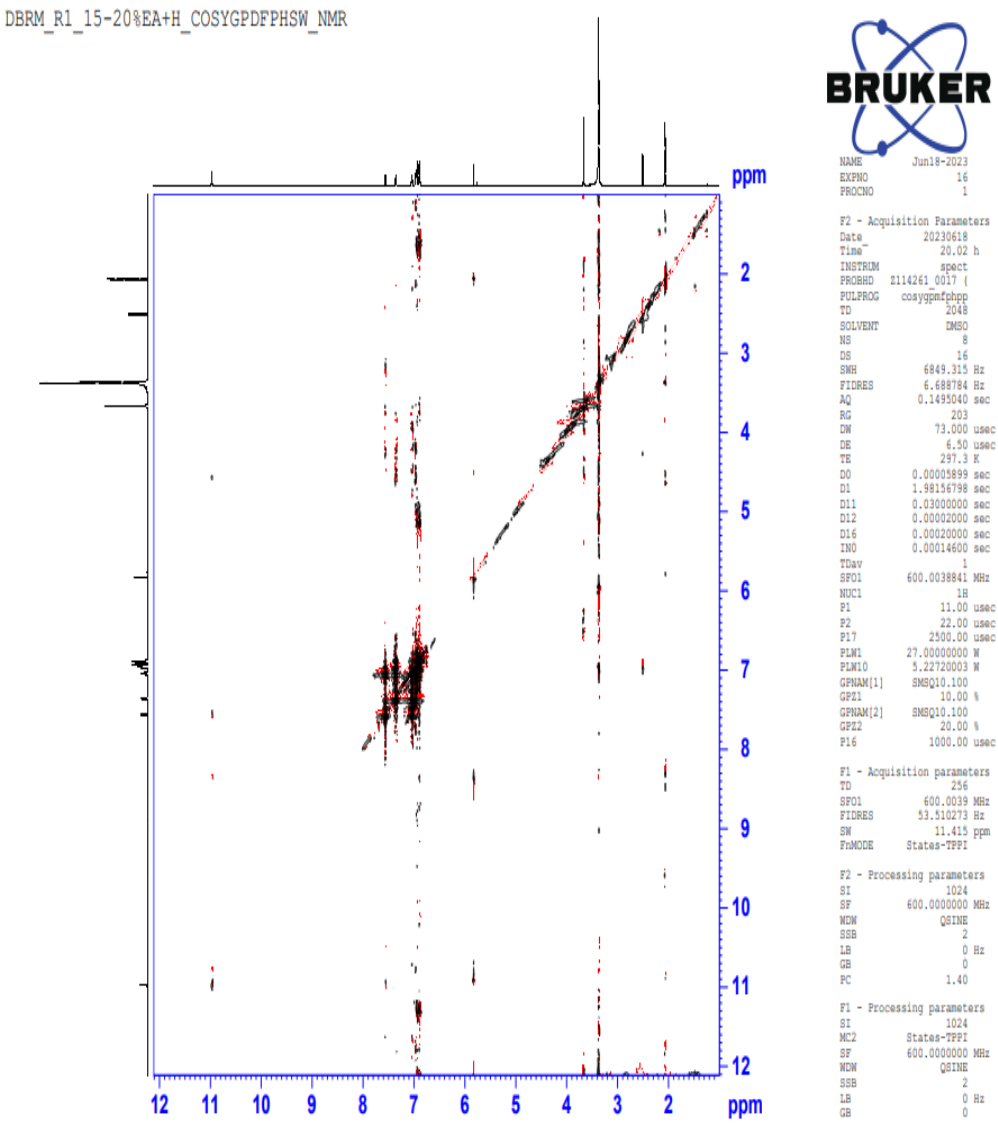


Figure 16: COSY NMR of DBRM

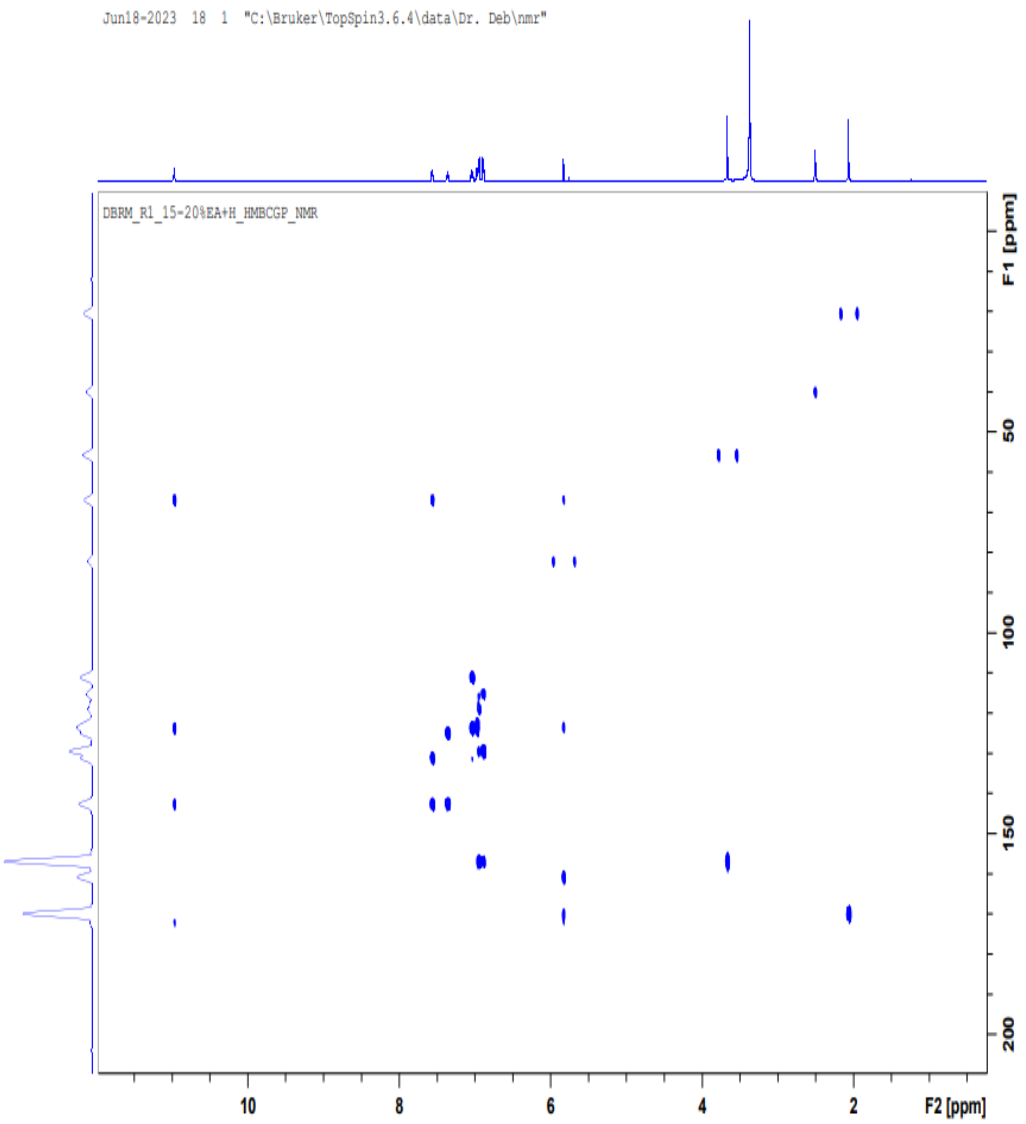


Figure 17: HMBCGP NMR of DBRM

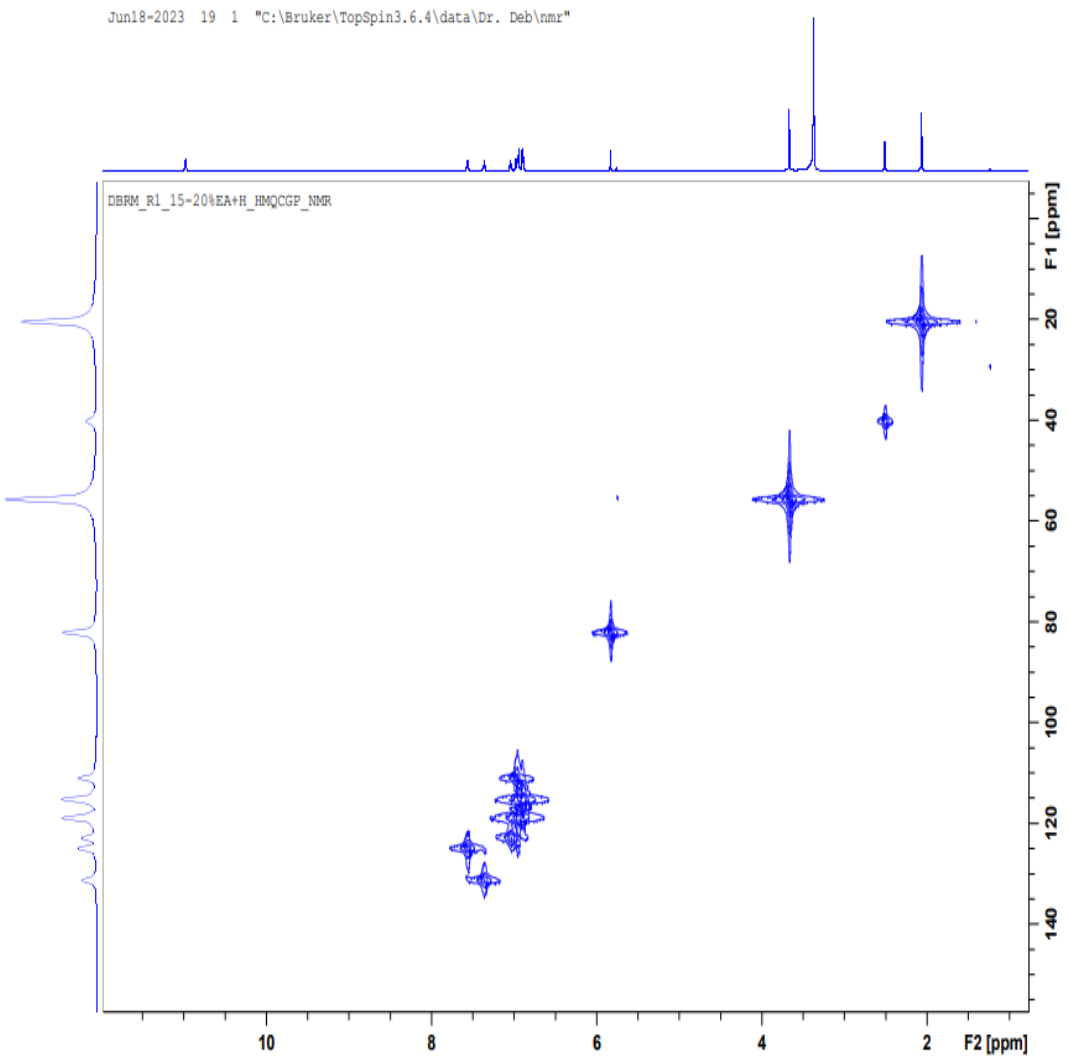


Figure 18: HMQCGP NMR of DBRM

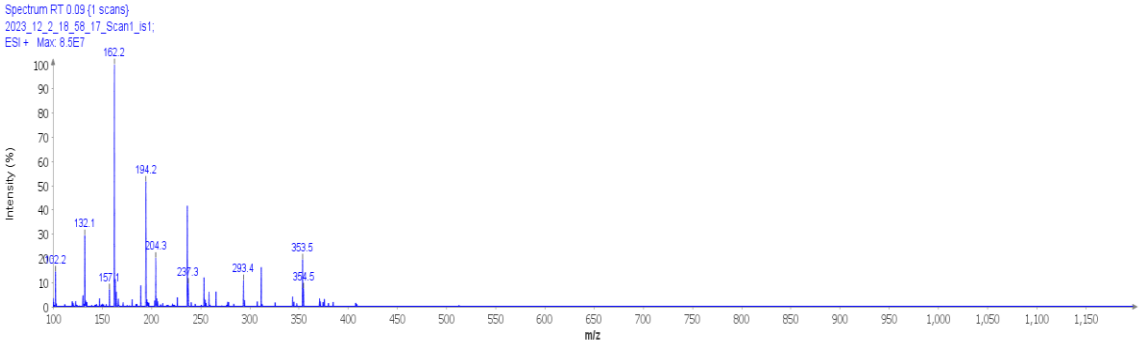


Figure 19: Mass ESI+ spectrum of DBRM

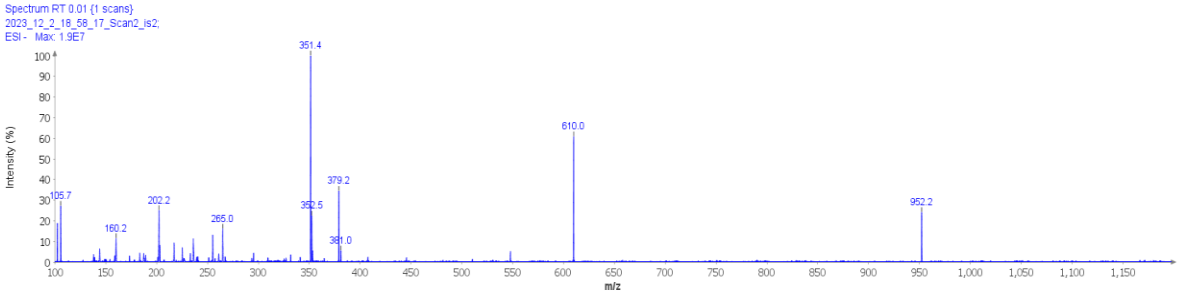


Figure 20: Mass ESI- spectrum of DBRM

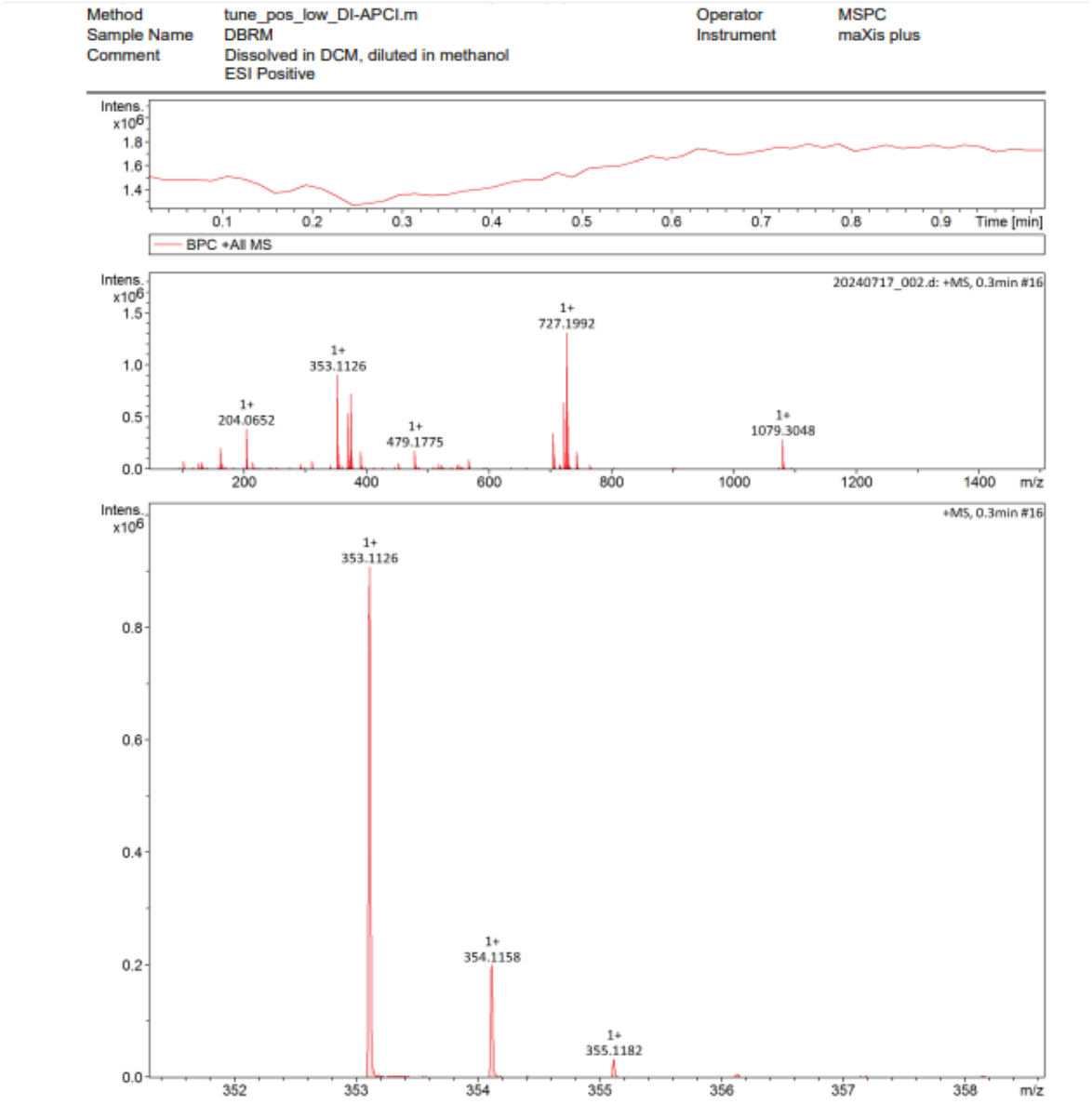


Figure 21: High-Resolution Mass spectrum of DBRM

Characterization of compound 2

BL (Isatin + 2-Aminopyridine): Yellow solid (83mg, 25.70%); m.p 204.9 ^{o}C – 205.6^{o}C; IR (KBr) 1746, 1680, 1651, 1634, 1603, 1584 cm^{-1} (Figure); ^{1}H NMR (600 MHz, d_6- DMSO) 11.9 (s, H), 8.5 (d, J = 8.22Hz, H), 7.9 (d, J = 5.46Hz, H), 7.8 (d, J= 7.68Hz, H), 7.7 (brs, H), 7.6 (t, J= 7.74 Hz, H), 4.7 (s, H), 7.2 (t, J =7.5Hz, H), 6.7 (brs, H). ^{13}C NMR (150 MHz, DMSO) 15 Carbon δ 20.95, 63.25, 112.33, 112.39, 119.84, 120.17, 123.69, 134.22, 135.29, 139.84, 140.39, 142.41, 156.45, 167.01, 168.74, 170.18, 199.29 ppm. Chemical Formula: $C_{17}H_{13}N_3O_4$; Molecular Weight: 323

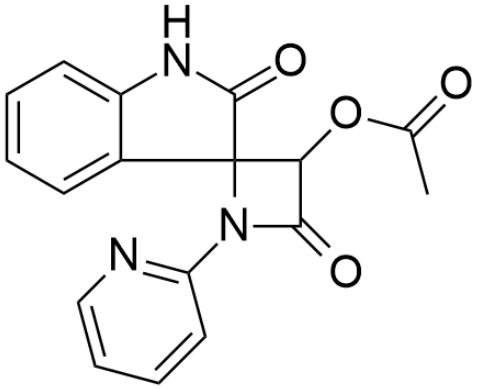


Figure 22: Structure of BL (Isatin + 2-Aminopyridine)

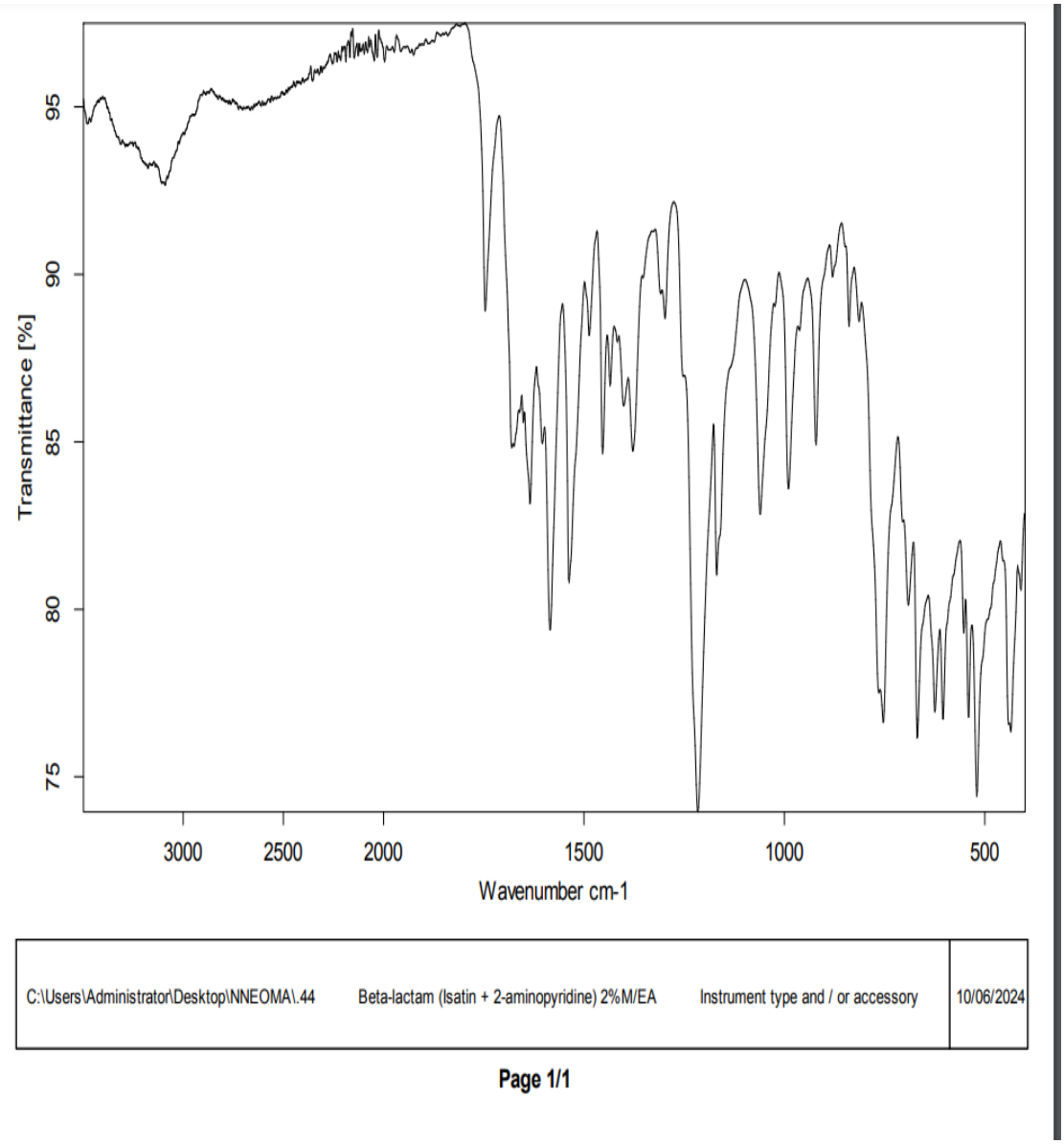


Figure 23: FT-IR of BL (Isatin + 2-Aminopyridine)

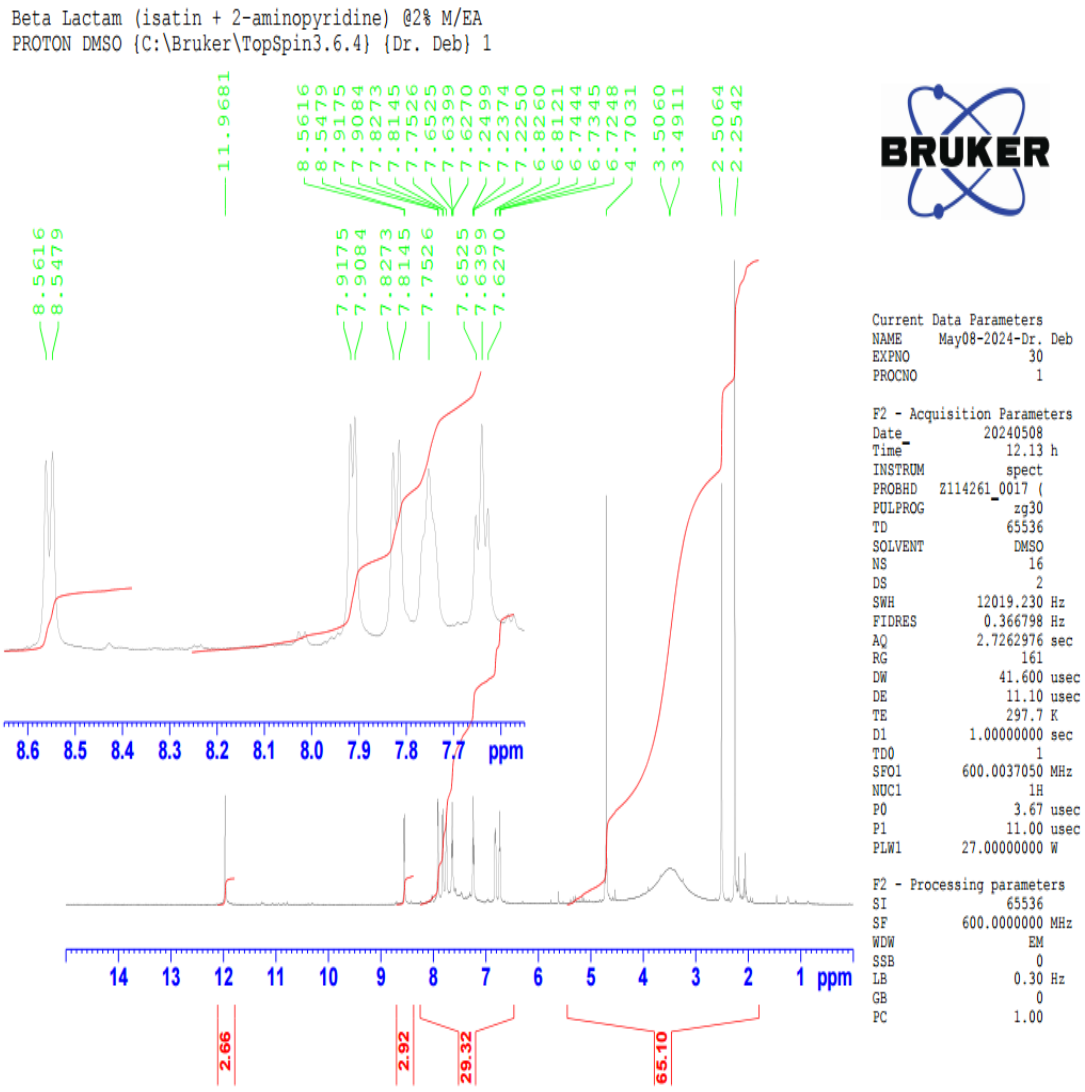


Figure 24: ^{1}H NMR of BL (Isatin + 2-Aminopyridine)

Table 5: Different proton positions in BL (Isatin + 2-Aminopyridine) and their chemical shifts

Chemical Shift (δ)	Peak number	J value (Hz)
11.9	singlet	
8.5	doublet	8.22
7.9	doublet	5.46
7.8	doublet	7.68
7.7	broad singlet	
7.6	multiplet	
7.2	multiplet	
4.7	Singlet	
2.2	singlet	

Table 6: Different carbon positions in BL (Isatin + 2-Aminopyridine) and their chemical shifts

Type	Chemical shift, δ (ppm) ^{13}C NMR
C=O	170.1852
C=O	168.7472
C=O	167.0182
C(N)	156.4509
CH(=)	142.3558
C(=)	140.3928
CH(=)	139.8403
CH(=)	135.2861
CH(=)	134.2264
CH(=)	123.6925
CH(=)	120.1885
CH(=)	112.40
C	63.2564
CH3	20.9595

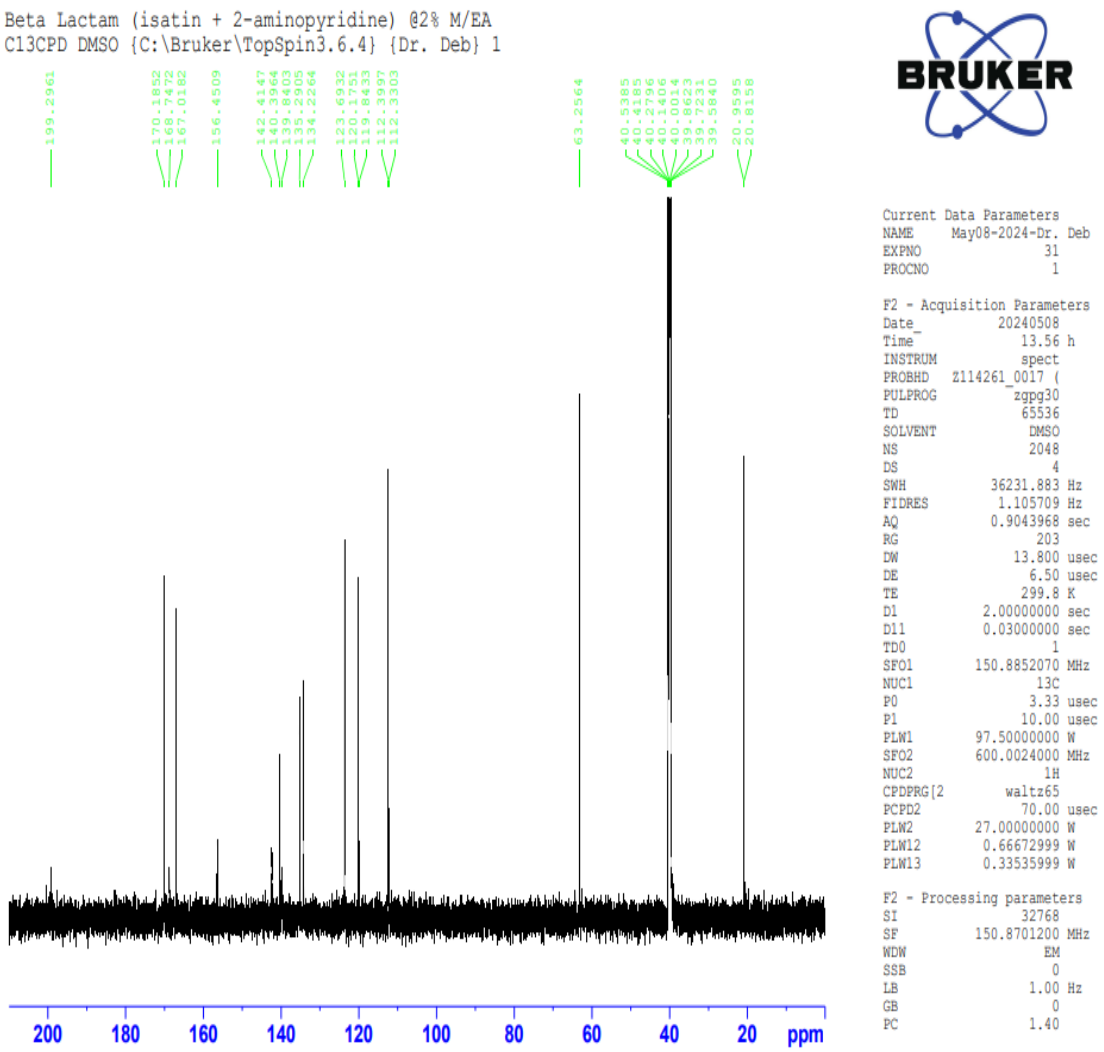


Figure 25: ^{13}C CPD of BL (Isatin + 2-Aminopyridine)

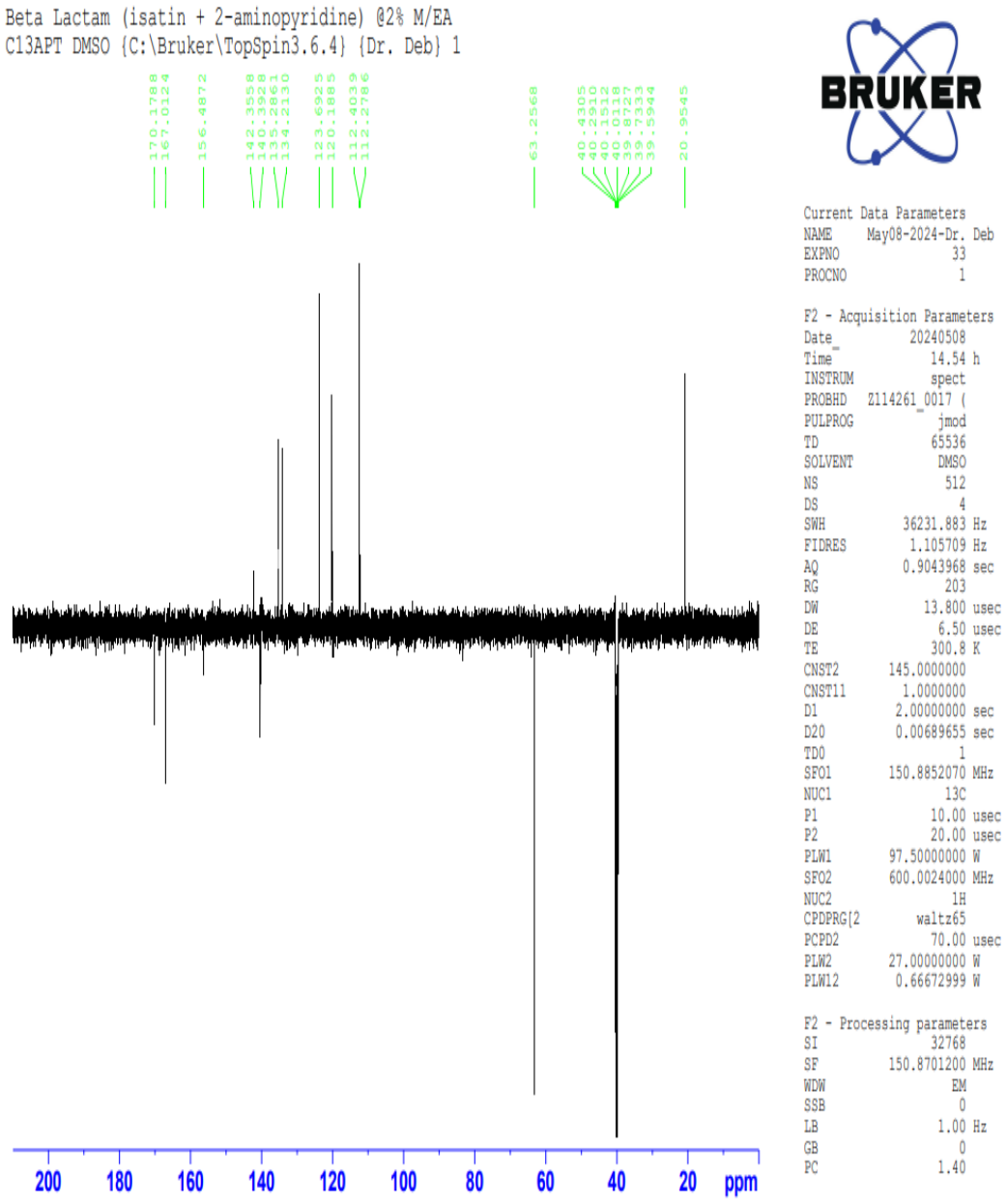


Figure 26: ^{13}C APT of BL (Isatin + 2-Aminopyridine)

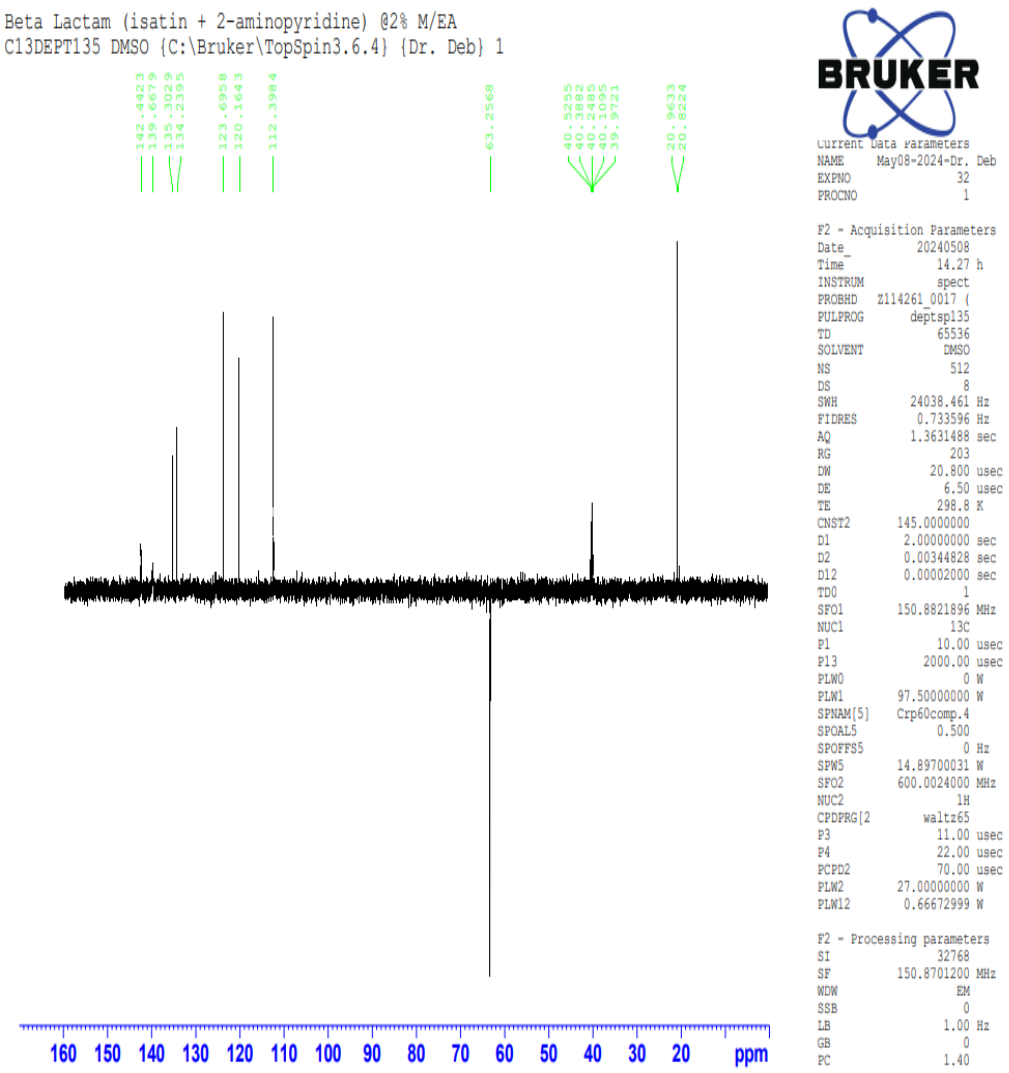


Figure 27: ^{13}C DEPT 135 of BL (Isatin + 2-Aminopyridine)

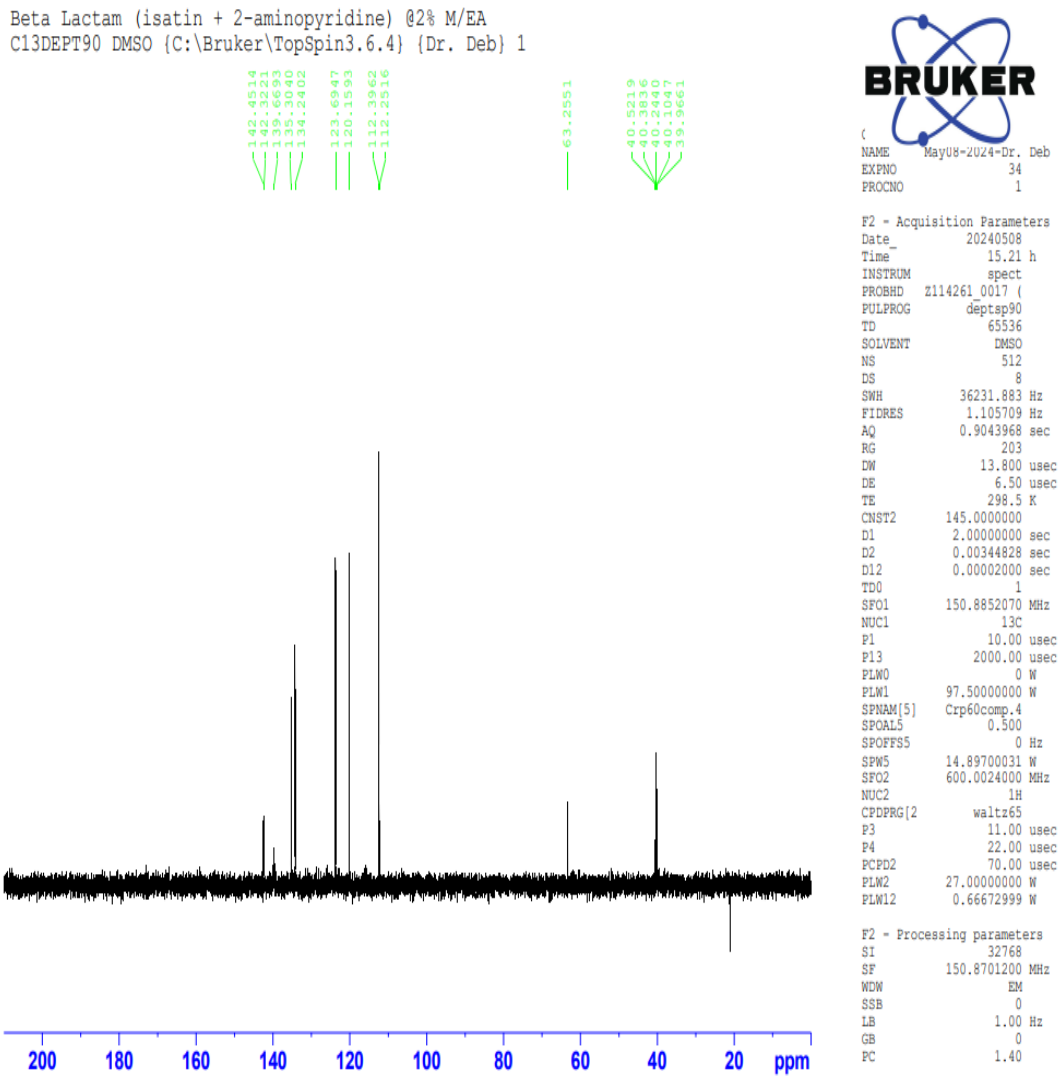


Figure 28: ^{13}C Dept 90 of BL (Isatin + 2-Aminopyridine)

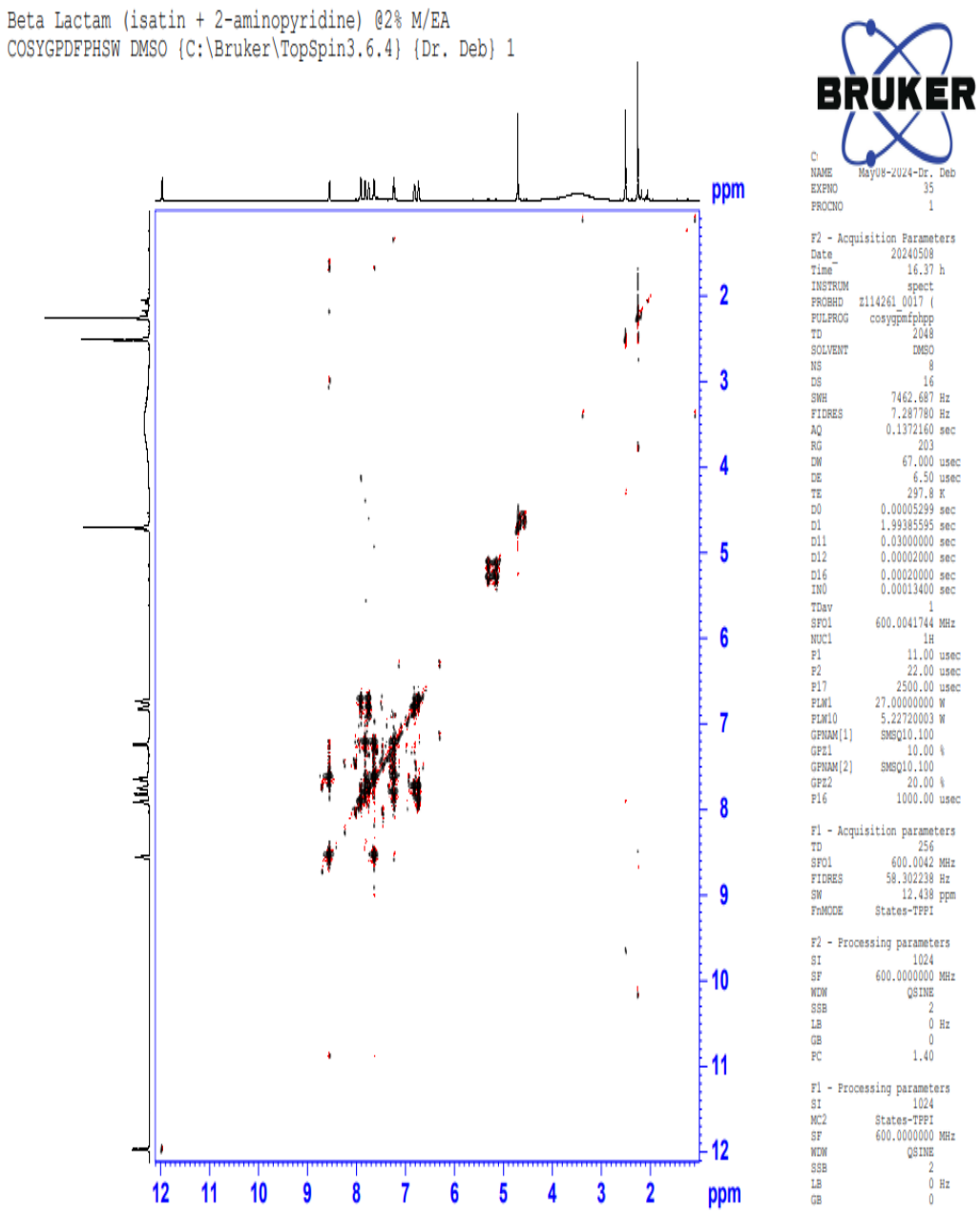


Figure 29: COSY NMR of BL (Isatin + 2-Aminopyridine)

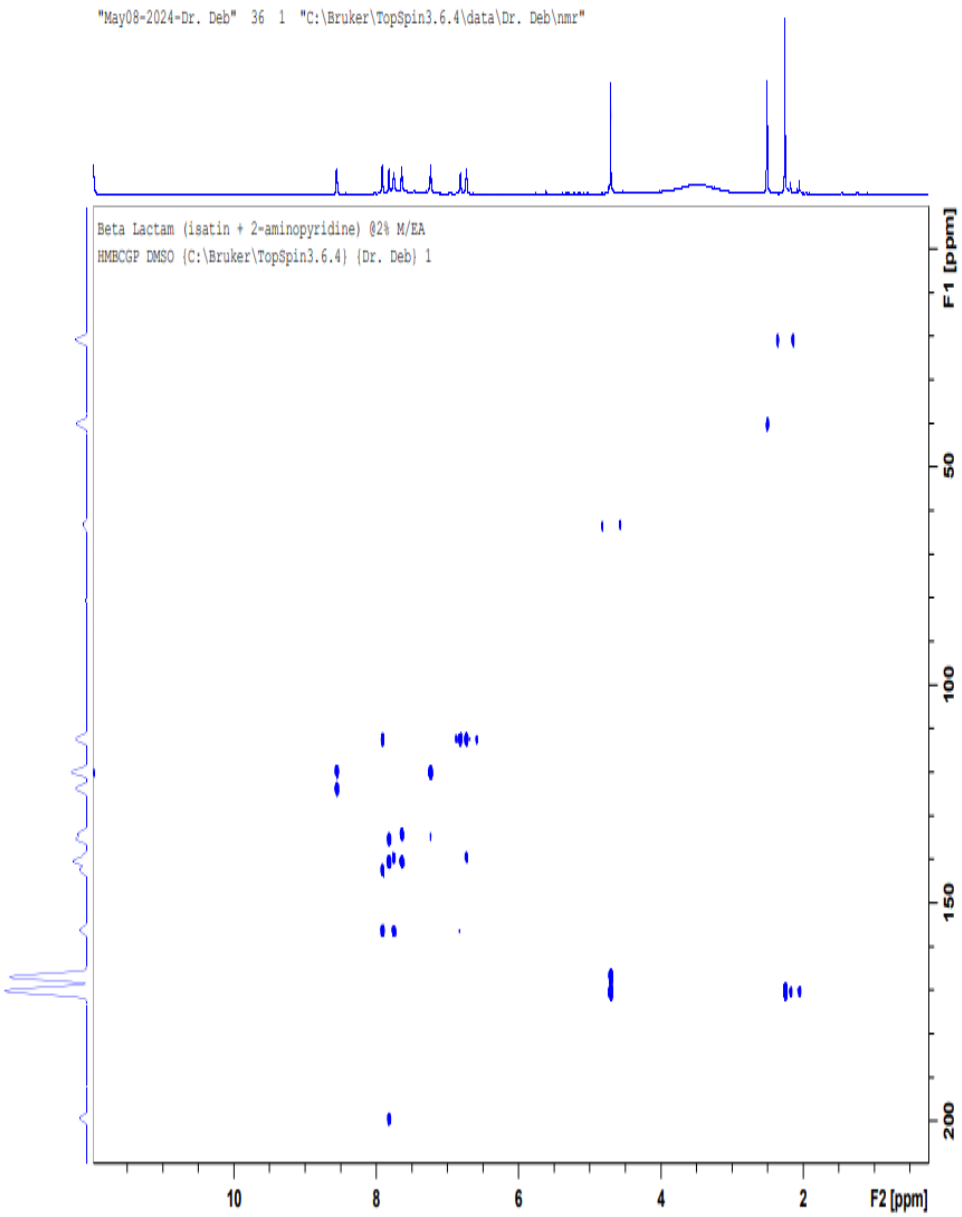


Figure 30: HMBCGP NMR of BL (Isatin + 2-Aminopyridine)

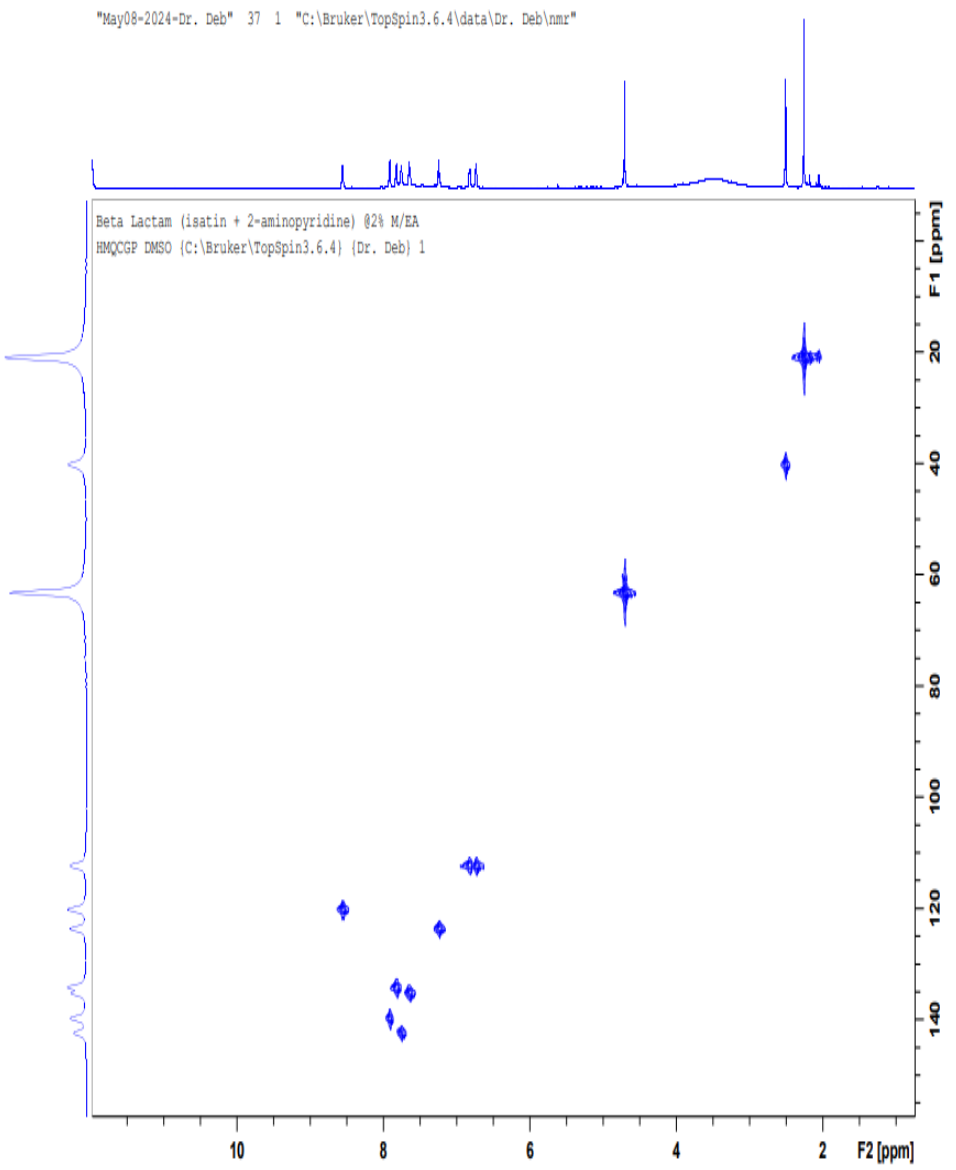


Figure 31: HMQCGP NMR of BL (Isatin + 2-Aminopyridine)

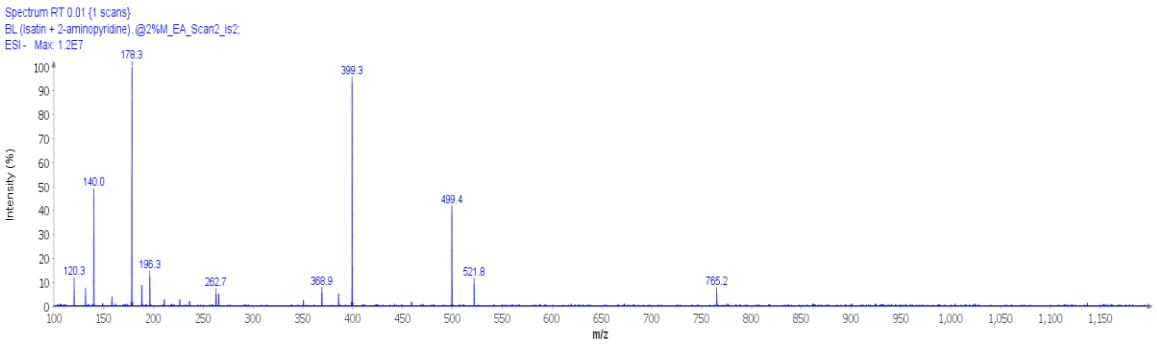


Figure 32: Mass ESI- spectrum of BL (Isatin + 2-Aminopyridine)

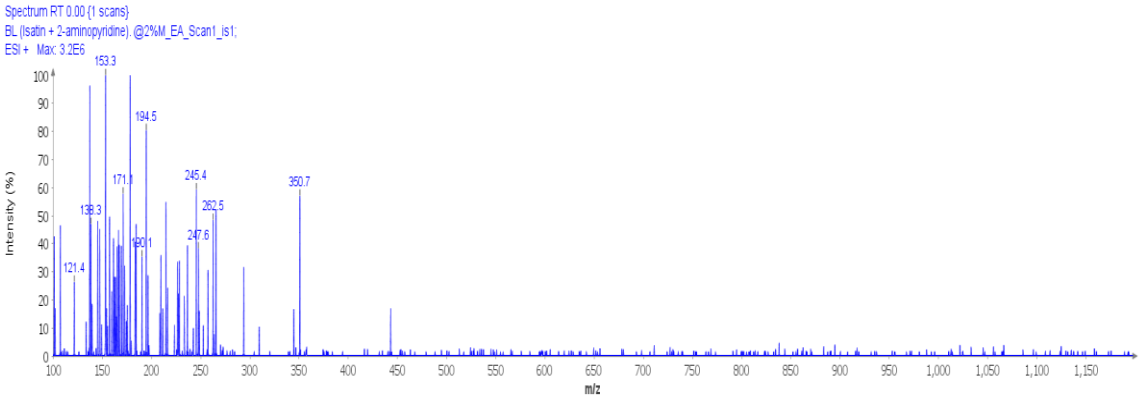


Figure 33: Mass ESI+ spectrum of BL (Isatin + 2-Aminopyridine)

CHAPTER V

CONCLUSION

Despite the availability of many chemotherapeutic drugs, most lack the ability to selectively target cancer cells. Beta-lactam is a promising candidate in this context, as it has demonstrated antibacterial properties and other biological effects. These characteristics encourage the development of more potent and selective chemotherapeutic agents derived from beta-lactams. Since their discovery, beta-lactam antibiotics have been crucial in combating bacterial infections. However, developing new generations of beta-lactams is necessary to address drug resistance, primarily caused by bacterially produced beta-lactamase enzymes that hydrolyze the highly strained beta-lactam ring. We hypothesize that since cancer cells do not produce beta-lactamase enzymes, the stability of the beta-lactam ring should be greater in tumor environments. With suitable chemical modifications, beta-lactams should inhibit proteins responsible for the proliferation, angiogenesis, and metastasis of various cancers, including hepatobiliary-pancreatic carcinomas (HPCs). HPCs include hepatocellular carcinoma (HCC), biliary tract cancers (BTCs), and pancreatic cancer (PanCa), which are extremely challenging to treat and manage.

This is the first report on the synthesis of spiro-centered β-lactams. The compounds were successfully characterized using FT-IR, MS, NMR, and X-ray. The biological activity of this newly synthesized spiro β-lactam will be evaluated. Further research and exploration are needed to fully understand its biological activities, mechanism of action, and potential applications in drug development.

Future aspect

In the future, we want to do the chemical modification of the synthesized spiro beta-lactams (hydroxylation and sulfonation) to increase its selectivity, activity, solubility, and binding affinity. Hydroxylation of our synthesized compounds helps to increase the ability of the compounds to form hydrogen bonds with receptors. Also, *in vitro* and *in vivo* studies of the compounds will be conducted on different cancer cells to identify anticancer effects.

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VITA

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