

Synthesis of a small library of copper activated NNSN molecules
for the treatment of bacterial infections and cancer

by

Kayla Michelle Jamison Eschliman

B.S., Kansas Wesleyan University, 2017

AN ABSTRACT OF A DISSERTATION

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DOCTOR OF PHILOSOPHY

Department of Chemistry
College of Arts and Sciences

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Abstract

Bacterial infections are a serious health issue worldwide and the number of cases is anticipated to continue rising. The problem is especially dire when considering the increase in resistant bacteria that are becoming ever more difficult to treat. On average, someone gets an antibiotic-resistant infection every 11 seconds and every 15 seconds someone dies, according to the Centers for Disease Control and Prevention Antibiotic Resistance Threats Report 2019. Cancer is also a severe health problem, ending nearly 9 million lives worldwide each year. Copper activated drugs are of growing interest due to their ability to be used for anti-bacterial and anti-cancer applications. NNSN molecules are a novel, patented class of drugs displaying an extended thiourea motif. These drugs are synthesized by coupling a pyrazole ring and phenyl isothiocyanate together. Derivatives are produced by the various substituents added to either the pyrazole or the phenyl isothiocyanate and the placement of these substituents on the aromatic ring. The current library of NNSN compounds contains 134 derivatives. A structure activity relationship study on these compounds has been conducted to determine the best possible structures to treat *Staphylococcus aureus* infections. Despite not having any stereocenters, these small molecule drugs exhibit copper-activated reactivity toward bacterial infections (including *S. aureus* (Gram-positive), *Escherichia Coli* (Gram negative)), and *Mycobacterium tuberculosis*, and cancerous murine cell lines (Melanoma (B16F10), Glioblastoma (GL26), and Pancreatic Cancer (Pan02 and KPC)). NNSNs are non-toxic until activated by copper, the necessary concentration of which only exists naturally in the biological environment at the site of infection or within a tumor microenvironment. Copper (I/II) is an endogenous metal that is limited in the human body because of its ability to create reactive oxygen species, which are extremely damaging to bodily tissues. This presents a unique opportunity for selective activity, making the probability of harmful side effects significantly lowered. This mode of action is a theory that is strengthened by evidence determined using multiple computer modeling studies and by the calculation of copper binding constants using ultraviolet visible spectroscopy on a gradient of solutions.

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I was able to participate in this program in the summer of 2015 with Dr. Ryan Rafferty

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Dedication

This work is dedicated to the most important people in my life: my family! To my husband Steven Eschliman, mother and father Lisa and Steve St. Laurent, brother AJ and sister Allison, and maternal grandmother Maxine Ray. To my aunt Julie Cosato, cousin Kristine Hutton, and maternal grandfather Homer Gilson. To my parents in law (Heidi and Keith Eschliman, Misty and Robert Farmer, and late father-in-law Joseph Jamison), and my three sisters in law and their families, including Cindy and Charles Mood and their children Lurena and Cyrus, Samantha and Andrew Carpenter and their girls Karen, Hope, and Parker, and Beth Eschliman and Devon Friesen, along with their children Henry and Eleanor. Thank you all for supporting me through this endeavor. I also would like to thank my friends Dr. Bonnie VonHoff, Ivina Mali, Jordan Gipper, and Edruce Edouarzin, for their help and support!

Chapter 1 – Background

Antibiotic Resistance

Bacterial infections are a serious health issue worldwide and the number of cases is anticipated to continue rising. The problem is especially dire when considering the increase in resistant bacteria that are becoming ever more difficult to treat. According to the Centers for Disease Control and Prevention Antibiotic Resistance Threats Report for 2019, antibiotic resistant bacteria and fungi cause more than 2.8 million infections and 35,000 deaths per year.¹ On average, someone gets an antibiotic-resistant infection every 11 seconds and every 15 seconds someone dies.¹ The Centers for Disease Control and Prevention (CDC) has named 18 specific bacteria types as especially threatening, and placed them into three categories based on severity, as shown in **Figure 1**.¹

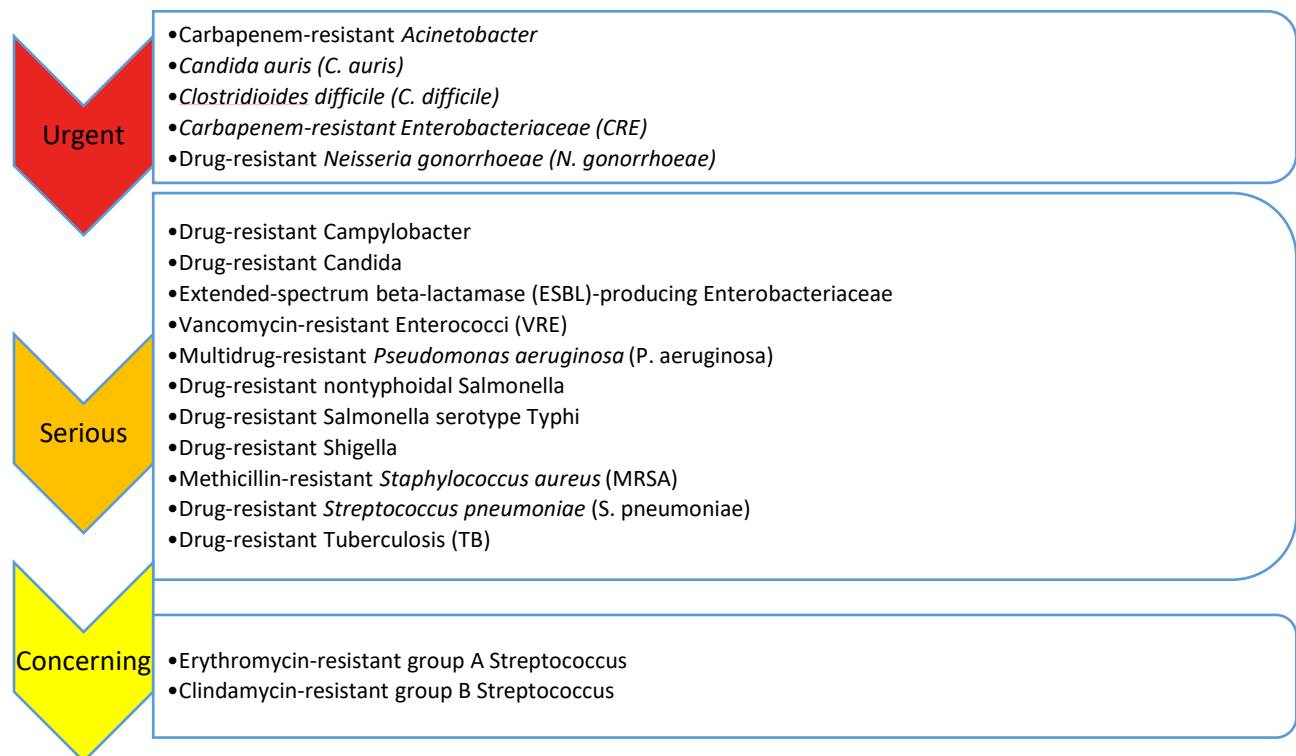


Figure 1, CDC – 18 specific bacterium types that are especially threatening, based on severity.¹

Antibiotic resistance is the ability of germs to defeat the drugs designed to kill them. This process is demonstrated by the following scenario: a patient is infected with a high number of bacteria. A small amount of them is resistant to antibiotics, as shown in orange in **Figure 2**.² The patient begins to experience symptoms and visits their physician, who prescribes an antibiotic to treat the infection. The antibiotic kills the bacteria causing the illness, but also kills the good bacteria that are meant to protect the body from further infection. Additionally, the bacteria that were resistant to the antibiotic survive, continue to grow, and reproduce until there are many bacteria causing the patient to experience illness yet again, but this time all of them have the gene for resistance to the antibiotic used. Bacteria can even transfer their resistant genes to other bacteria.² At this point, when the patient visits the physician a second time, they would likely prescribe a different antibiotic, and the process can be repeated with this drug.

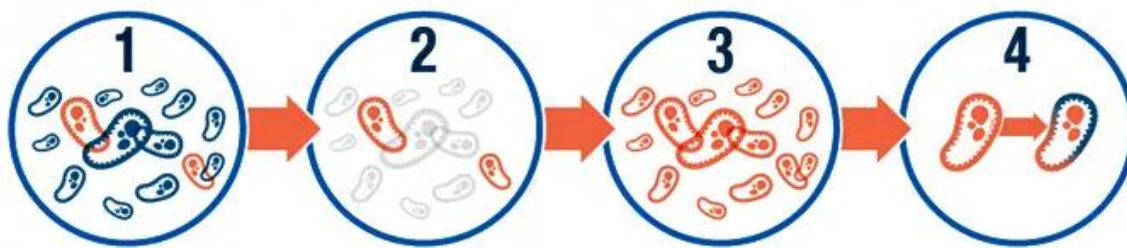


Figure 2, Antibiotic Resistance occurs when a small number of resistant bacteria (orange) survive treatment by an antibiotic drug then continue to grow and reproduce. Bacteria can even transfer their resistance to other bacteria.²

This process can be exacerbated by the fact that antibiotics are over-prescribed by physicians. A study performed by the CDC concluded that at least 30 percent of antibiotics prescribed in doctors' offices and emergency departments across the United States are unnecessary.³ These were deemed unnecessary because they were prescribed to treat respiratory conditions caused by viruses that do not respond to antibiotics. This inappropriate use has put 47 million patients at increased, unneeded risk for allergic reactions, superbugs, and the potentially deadly diarrhea, *Clostridium difficile*.³

Another issue is that patients may not finish the entire course of the antibiotic treatment because the symptoms subside. This is an extremely dangerous choice that results in the bacteria that caused the infection to survive, and these will be the ones with the greatest resistance.² This problem has been spoken out against in recent years by doctors and researchers to educate the public on the risks involved. According to the CDC, “Addressing this threat [of antibiotic resistance] requires preventing infections in the first place, slowing the development of resistance through better antibiotic use, and stopping the spread of resistance when it does develop.”¹ This means that improvements need to be made in multiple arenas, including hygiene practices, food production and food supply storage, prescribing only the necessary amounts of antibiotics for humans and animals, and adequate disinfection of surfaces in all public places (i.e.: hospitals, schools, long-term care facilities, grocery stores, etc.).¹ As mentioned in the abstract, NNSN molecules, which will be discussed further later in this chapter, can be used for the treatment of bacterial infections such as those just described and cancer.

Cancer

Cancer comprises a group of diseases that share key characteristics, namely the uncontrollable growth of abnormal cells and the ability to invade surrounding tissues. There are more than 100 different types of cancers, typically named after the region of the body in which they originate.⁴ Some cancer types lead to the formation of hard masses called tumors. However, not all cancers involve tumor formation. In fact, not all cancers show outward signs or symptoms. Ten cancers that often present in this manner include pancreatic, prostate, bladder, colon, testicular, kidney, stomach, vaginal, ovarian, and liver cancers.⁵ Asymptomatic cancer types such as this are sometimes referred to as “Silent killers.” They are extremely dangerous because the patient is unaware that there is a problem, leading to continuous growth and late-

stage diagnosis. Unfortunately, four of the previously mentioned ten cancers are also in the top 10 most common cancers, shown with red boxes in **Figure 3**.⁵

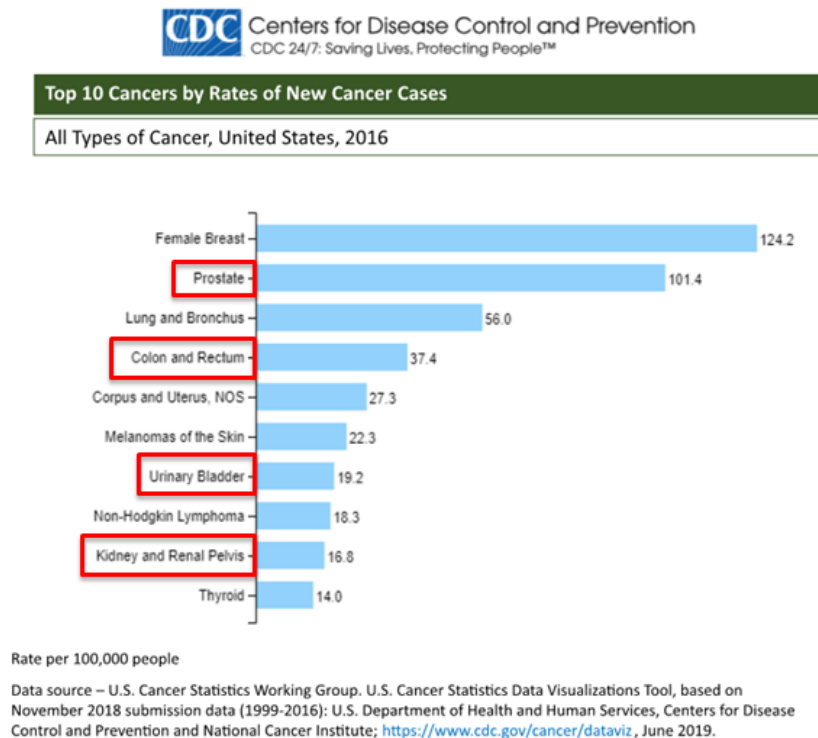


Figure 3, Top 10 Most Common Cancer Types (2016).⁵

According to the American Cancer Society: In 2021, over 1.9 million new cases of cancer were reported, resulting in nearly 608,570 people losing their life to cancer in the United States.⁶ Cancer is currently the second leading cause of death in the United States, with heart disease being the first.⁷ One of every four deaths in the United States is due to cancer. The overemphasis on United States in these statistics was purposeful – these statistics demonstrate the impact that this disease has on only one country! Cancer is a rising global epidemic, killing nearly 9 million people worldwide every year.⁸

With this problem in mind, emphasis has been put on determining how this disease occurs. There have been some risk factors identified, such as unhealthy diet, lack of exercise, smoking, consumption of alcoholic beverages, excessive sun exposure, exposure to carcinogens, and certain health conditions.⁹ It is important to maintain a healthy diet and get proper exercise

because research data has shown a correlation between being overweight and increased risk of certain cancers, such as uterine, breast, and colorectal cancers. Cigarette smoking is the foremost cause of lung cancer and furthermore secondhand smoke has been shown to have even higher concentrations of toxic chemicals than that taken in by the person smoking.⁹ Regular alcohol consumption increases the risk of liver, mouth, voice box, breast, colon, and throat cancers. Ultraviolet rays have been determined dangerous due to their ability to methylate DNA and cause cancer. Additional carcinogenic (or cancer-causing) chemicals include asbestos, nickel, cadmium, radon, vinyl chloride, benzidine, benzene. Lastly, it has also been shown that certain health conditions (Human papillomavirus, Hereditary Breast and Ovarian Cancer Syndrome, and Lynch Syndrome) increase the risk for certain cancers.⁹

There are some types of cancer can be genetic in nature, including breast, ovarian, uterine, and colorectal cancers.⁹ However, not all cancers have a genetic component. If a patient has a family history of a known genetic cancer, then they can receive a screening to determine if they have the gene that is known to contribute to the disease. Genetic screening is used to determine the presence of mutations in a DNA sample.⁹ The understanding of the genes that contribute to cancer risk is developing all the time. There are different screenings available depending on the cancer type that is suspected. The different types of genetic screening are single gene, panel testing, exome sequencing, and genome sequencing.⁹ With the knowledge obtained from the screening, a patient has the choice to have additional preventative measures. This could include receiving cancer screening sooner and/or more often than normal, such as a person with Lynch disease having a colonoscopy once every year rather than once every three years. There are also more drastic measures such as preventative surgeries to remove the organs

at risk before the cancer develops. Some examples of this are mastectomy, oophorectomy, or total gastrectomy.

Once cancer has been detected, additional tests are required to learn how advanced the cancer is. The stage of cancer development is normally denoted as stage I – IV. This includes laboratory tests, imaging (computed tomography, bone scan, positron emission tomography, ultrasound, x-rays, etc.) and a biopsy.⁹ A biopsy involves sticking a large needle into the patient to collect cells that are then studied under a microscope. The imaging studies can show any tumors that are present as well as any potential metastases. From the biopsy sample, cancer can be seen under the microscope as being made up of different types of cells. Treatments available include surgery, chemotherapy, radiation therapy, bone marrow transplant, immunotherapy, hormone therapy, targeted drug therapy and clinical trials.¹⁰ The treatment options will depend on the stage of the cancer (ex: surgery is typically only used to remove the primary tumors).

Cancer patients are at an increased risk of obtaining antibiotic resistant infections due to their compromised immunity secondary to treatments such as chemotherapy, radiation, or surgical intervention.¹¹ Bacterial infections can become very serious for these patients and lead to sepsis. Sepsis occurs when the body elicits an extreme response to the infection. Without treatment, sepsis can cause dramatic drops in blood pressure, organ failure and death. For these reasons, antibiotics are often given as a preventative measure alongside chemotherapy and surgery.¹¹ This contributes further to the over-prescription of antibiotics discussed previously. However, in this case, it is impossible to tell if the antibiotic was necessary prior to the patient developing an infection and so it is better to be safe. However, the increased use of antibiotics still contributes to the issues of antibiotic resistance by allowing more chances for bacteria to become resistant to antibiotics on the market today.

Now that the problems have been briefly explained, the discussion can begin on how to potentially counteract them. One solution to treat both bacterial infections and cancers is using copper-activated drugs. As will be discussed further in the upcoming section, copper is an endogenous metal that has potential antibacterial properties. Copper-activated drugs can harness this natural power located within the body to fight infection and cancer.

The Role of Copper

Copper was one of the first metals ever extracted and used by humans, dating back to the Chalcolithic or Copper Age, beginning around the mid-5th millennium BC, directly preceding the Bronze age. Copper was used in the production of a myriad of tools, such as chisels, razors, harpoons, cloak pins, arrows, quivers, and spearheads; furthermore, items such as plumbing pipes, helmets, statues, and figurines were also crafted from copper.¹² An alloy that revolutionized the world is known as bronze, which involves smelting together copper and tin – lead and arsenic were also involved in the early days of the Bronze Age around 3500 BC and zinc-containing alloys became popular around 1000 BC.¹²

With copper being used by humans for centuries, it became engrained in our biology. This process was sped up by historical events such as the 16th century plague; those who survived carried on the copper in their bodies. Today, copper is used in building construction (plumbing, roofing, cladding, rainwater systems, heating and cooling systems, door knobs, sinks, bathtubs, lighting, gutters), electrical and electronics (household appliances, computers, cell phones, spark plugs, electrodes, copper wiring, internet, microprocessors), transportation equipment (motors, wiring, radiators, rotors, connectors, brakes, bearings used in cars and trucks), consumer products (mattresses, fabrics, clocks, art, jewelry, musical instruments, cookware, utensils), and industrial machinery (printing, coin production).^{12,14,15,18} A pie chart

showing the uses of copper in the United States during 2019 is shown in **Figure 4**. Copper has unique properties that make it such a versatile metal for these applications, including high ductile strength, malleability, resistance to corrosion, high thermal and electrical conductivity, biofouling resistance, machinability, non-magnetic, antimicrobial activity, and demonstrated retention of important mechanical and electrical properties at cryogenic temperatures.^{13,14,15} The antimicrobial properties of copper and copper alloys can be applied to frequently touched surfaces in a hospital environment to reduce the number of infections incurred by patients during a hospital stay.¹⁸ Over time, the use of copper in all these materials has caused exposure that has led to integration and required tolerance within the bodies of humans, plants, and animals.

Uses of Copper in the United States During 2019

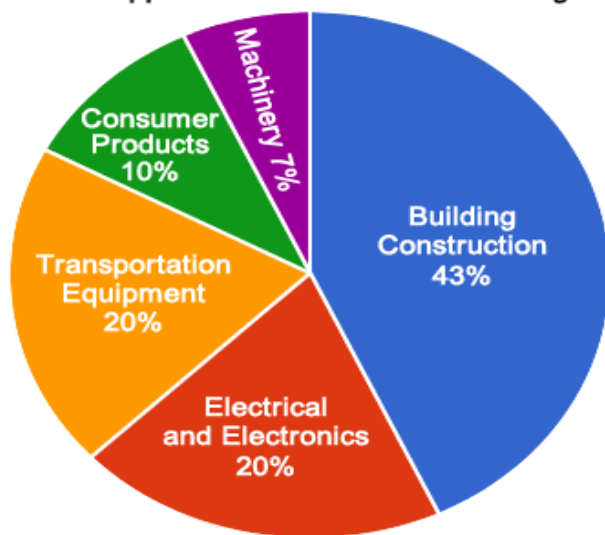


Figure 4, Pie Chart of the Uses of Copper in the United States in 2019.¹⁸

Inorganic elements such as copper, iron, and zinc all play vital roles in biological systems. In all cases, strict homeostatic regulation is required to maintain physical health. It is important to regulate copper dispersion to specific tissues only according to their fluctuating metabolic needs. Copper is important as it is involved in immune system function, processing of cholesterol, red blood cell formation, maintenance of blood vessels, nerves, healthy bones, and the development of babies in utero.^{16,17} Foods that are rich in copper include seafood and

shellfish (oysters, lobster, and spirulina), organ meats (liver and kidney), whole grains, nuts, beans, potatoes, seeds, raisins, legumes, chocolate, shiitake mushrooms, dried fruits, black pepper, yeast, and leafy greens (spinach, kale, and swiss chard).^{16,17,18} A lack of copper can lead to anemia and osteoporosis while large amounts can cause hepatitis, kidney problems, and brain disorders; it is recommended for adults over 19 to intake 900 micrograms per day of copper from dietary sources to maintain a healthy level.¹⁷

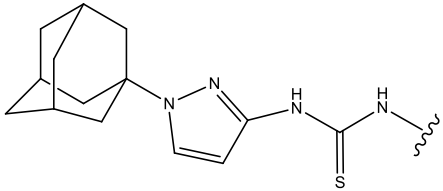
We now have an understanding that copper has multiple states of oxidation that change how it interacts with biological proteins. Cu(I) has an affinity for thiol and thioether groups, which can be found in cysteine or methionine amino acids, while Cu(II) prefers coordination with oxygen or imidazole nitrogen groups, which are found in aspartic and glutamic acid, or histidine.¹³ Copper can be damaging to tissues because intracellular copper can generate hydroxyl radicals. It is widely recognized that free radicals can damage proteins, nucleic acids, and lipids, as well as potentially interfere with the synthesis of iron-sulfur clusters. Further, copper has been shown to displace metals such as zinc from their cognate ligands in metalloproteins – this can cause serious problems like inhibition of enzymatic activity.¹³ Many bacteria can sense an elevation in copper levels and implement intracellular mobilization and export to protect themselves. In general, bacterial response to copper activates an operon (containing a transcriptional repressor, a chaperone, and a P-type exporter).¹³ In the absence of copper, transcriptional repressors work to keep this from working. However, when copper is detected by the bacteria, the repressor proteins bind to copper and release from the binding sites of their promoter DNA, allowing the operon to function.¹³ The most common cuproenzyme in bacteria, which recognizes the copper, is cytochrome c oxidase.¹³

It has been shown that bacterial copper resistance is linked to virulence in *M. tuberculosis*, *P. aeruginosa*, *Listeria monocytogenes*, and *Streptococcus pneumoniae*,¹⁹ suggesting that copper plays a key role in containing bacterial infections of this type. In bacteria-infected cells it has been shown that iron and other nutrients are withheld from the phagosomes so that the bacteria are taken up instead – other minerals, such as zinc and copper(I) are increased during this process.²⁰ Copper ions are used directly by phagocytes as an antibacterial agent,²⁰ suggesting that if the copper can infiltrate the bacteria and remain there, then the bacteria will die. The problem is that nearly all bacteria possess copper resistance mechanisms, including efflux pump(s), sequestration proteins, oxidases, transcriptional regulators, and chaperones.²⁰ However, copper (I/II) exhibits Fenton-type biochemistry at the low pH in phagolysosomes, which can damage the bacterial membranes, enabling drug uptake.²⁰ Further, in bacteria, copper (I/II) can destroy iron-sulfur clusters, which many essential enzymes depend on as cofactors.²⁰

NNSN Molecules

As previously discussed, the steady rise of multi-drug resistant bacteria is a worldwide issue that needs immediate attention. One of the direst cases is methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-sensitive *S. aureus* (MSSA) because traditional drugs struggle against this type of infection. In addition to new drugs to treat this and other infections, a new methodology for drug discovery is required if there is any hope of fighting the ever-increasing bacterial resistance. A method used to harness copper's natural antimicrobial properties is by using copper-dependent inhibitors of bacterial infections such as *S. aureus*.¹⁹ A novel concerted screening effort was employed by Dalecki et al to identify copper-dependent inhibitors of *S. aureus*.¹⁹ In this endeavor, a library containing 10,000 compounds was assayed for anti-staphylococcal activity, revealing a total of 53 copper-dependent hit molecules.¹⁹ Of the

53 hits, 45 contained thiourea structures. The most prominent hit discovered by this study was a novel family of drugs with an extended thiourea core structure, termed the NNSN motif, which made up 12 of the 45 thiourea hits.¹⁹ Of these 12 hits, 9 were purchased from ChemBridge and used by Dalecki and coworkers to conduct an early-stage structure activity relationship study – these results are shown in **Figure 5**. As predicted, there was no activity observed in the medium with no copper present. In the medium supplemented with 50 μM CuSO_4 the MIC was between 0.3 and 10 μM , confirming copper dependence. Of the analogs tested here, APT-6i performed the best, yielding excellent copper-dependent inhibition of *S. aureus* with an MIC of 0.3 μM . The MIC indicates the lowest concentration of the drug required to prevent visible growth of the bacteria. Additionally, APT-6h, APT-6g, and APT-6f all had great MIC results as all were shown to be below 5 μM .



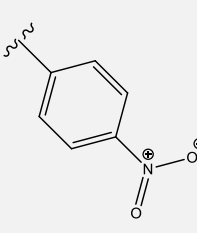
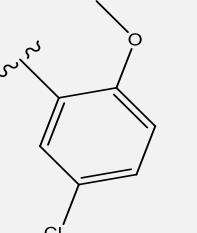
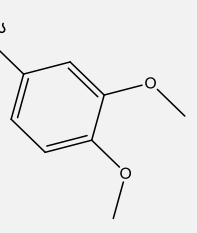
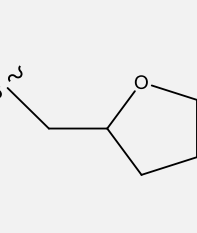
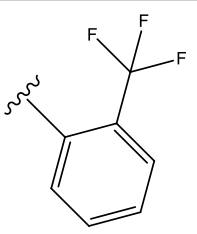
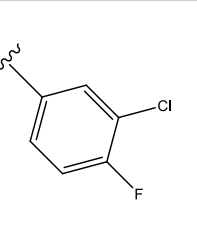
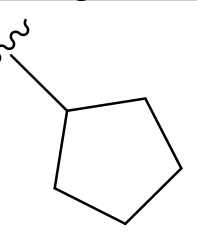
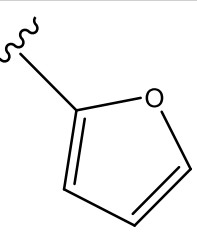
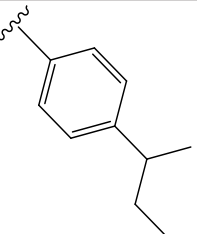
	6a	6b	6c	6d	
Structure					
MIC (μM)	10	5-10	5-10	5-10	
	6e	6f	6g	6h	6i
Structure					
MIC (μM)	2.5	2.5	1.25	0.6	0.3

Figure 5, Early-stage Structure Activity Relationship Study.¹⁹

NNSN molecules are a novel, patented class of drugs with either a classic or novel extended thiourea motif termed the NNSN motif because it contains these heteroatoms in a row, as shown in **Figure 6**. The classic motif is that which was discovered by Dalecki et al and initially tested for efficacy against *S. aureus*, as mentioned previously. The novel NNSN motif was discovered due to the synthesis of APM04, which led to unexpected isomerization. The isomer, KE03, turned out to be one of the best candidates. As it turns out, the novel NNSN motif is the most prominent isomer. Evidence of this can be found in **Table 1**, demonstrating that the yield is consistently higher for the Novel NNSN isomer than the Classic NNSN isomer.²³

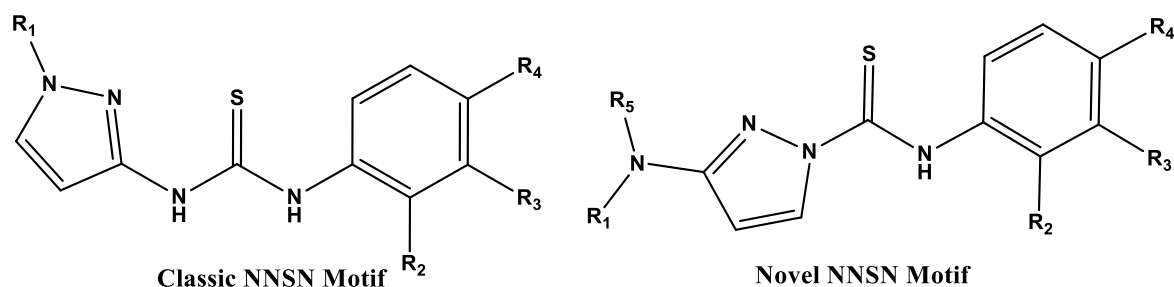


Figure 6, Classic versus Novel NNSN Motif.

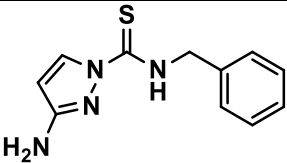
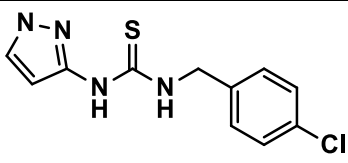
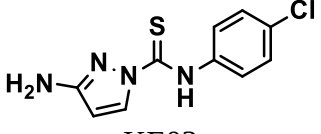
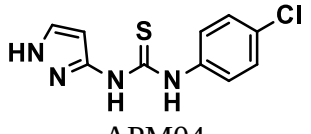
Compound	Yield %	Compound	Yield %
 APM01	65	 APM02	8
 KE03	55	 APM04	35

Table 1, Yields of Novel versus Classic NNSN Molecules.²³

Copper-activated therapies such as this have been extremely popular among researchers in recent years because of their ability to kill bacteria and cancer. Despite not having any stereocenters, these small molecule drugs exhibit copper-activated reactivity toward bacterial infections (including *S. aureus* (Gram-positive), *Escherichia Coli* (Gram negative)), and *M. tuberculosis*, and

cancerous murine cell lines (Melanoma (B16F10), Glioblastoma (GL26), and Pancreatic Cancer (Pan02 and KPC)). These drugs are synthesized by coupling a pyrazole ring and phenyl isothiocyanate together, which will be discussed further in Chapter 2. Derivatives are produced by the various substituents added to either the pyrazole or the phenyl isothiocyanate and the placement of these substituents on the aromatic ring. The current library of NNSNs contains 134 derivatives. Some of the most effective derivatives for the treatment of *E. Coli* (results discussed further in Chapter 2) are those where KE03 was combined with components of the peptide nanosponge, the synthesis of which is discussed further in the next section.

Peptide Nanosponges

Peptide nanosponges are a known delivery system that are made of biocompatible and biodegradable materials, have fast assembly and cargo loading, minimal systemic leaking during delivery, and fast release upon arrival to the active site.²² The size of these compounds can be changed as desired, from several micrometers down to 80 nm in diameter. The peptide is about 3.05 nm long and has a diameter of maximally 1.5 nm.²¹ A successful delivery agent must also avoid renal clearance, which is achieved by being larger than 6 nm in diameter.²¹ The nanosponges are non-toxic to monocytes and can be delivered to the tumor site through macrophages that have been activated by lymphokines.²²

There are two types of nanosponges that have been developed, categorized by the material being either lysine-based (A) or aspartic acid-based (B), as shown in **Figure 7**. This will change the overall charge of the system. The synthesis of these peptide nanosponges begins with the trimaleimide scaffold. This is produced in three steps, as shown in **Figure 8**. The peptide (K₂₀ or D₂₀ with cholesterol linkage in this case) is synthesized using solid phase peptide synthesis, along with the consensus sequence for executioner caspases (DEVDGC). The

completed sequence is connected to the trimaleimide linker by Michael addition in deoxygenated PBS buffer (pH 7.4).²²

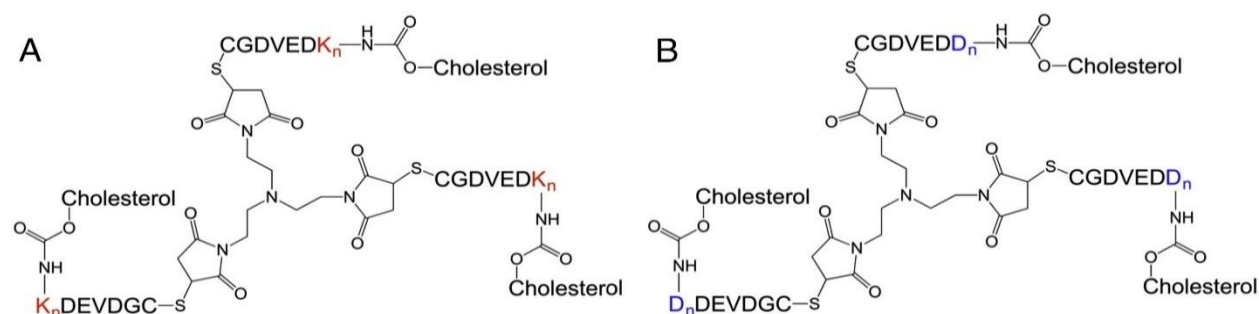


Figure 7, Nanosponge structures: (A = lysine-based) and (B = aspartic acid-based).²²

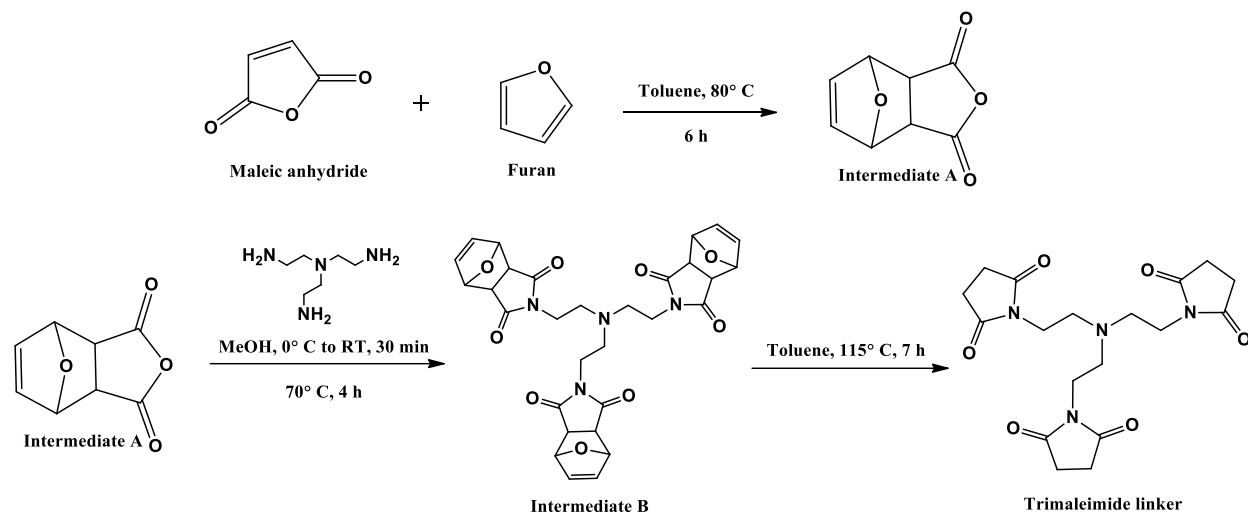


Figure 8, Trimaleimide linker synthesis.²²

Studies show that hydrophobic molecules can be incorporated inside the nanosponges.²² The nanosponge then remains stable until it reaches the site of the cancer. Once there, proteases such as caspase-3, 6 or 7 will digest the nanovesicle and release the payload selectively at the site where it is desired.²² KE03, one of the foremost NNSN molecules to date, has already been integrated into the lysine-based nanosponge structure. This molecule is named SB85, and its structure is shown in **Table 2**, in Chapter 2.

The peptide nanosponge has been characterized using Coarse-Grained Molecular Dynamics Simulations (**Figure 9**), Atomic Force Microscopy (**Figure 10**), Transmission

Electron Microscopy (**Figure 11**), Dynamic Light Scattering, and Critical Micelle Concentration (**Figure 12**). These experiments show a “sponge-like dynamic structure is spontaneously assembled, due to the formation of ion pairs, intense hydrogen bonding, and the occurrence of hydrophobic regions and water-filled nanocavities”.²² MTT Assay was performed using DK₂₀ nanosponges to measure cell viability of RAW264.7 cells, as shown in **Figure 13**. These cells were chosen because they can be used as a carrier in cell-mediated cancer therapies.²² The results of the assay show that the nanosponge is not toxic to these cells, even at 100 μ M concentration.

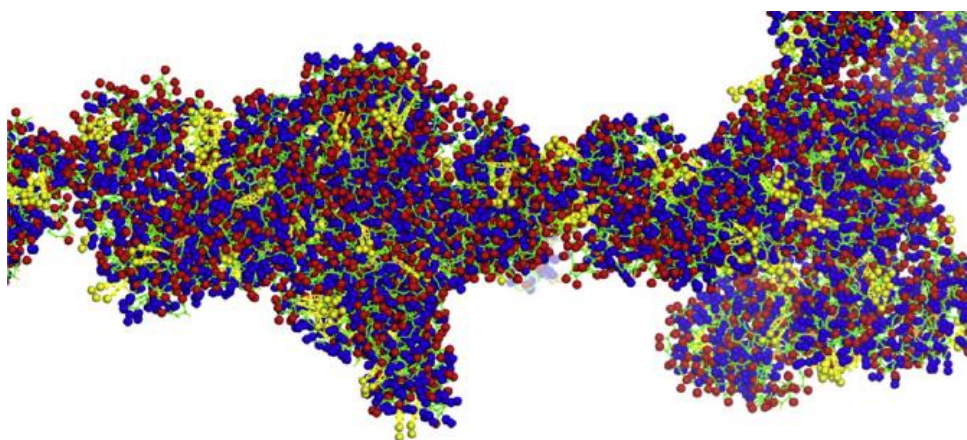


Figure 9, Coarse-Grained Molecular Dynamics Simulation of DK₂₀ nanosponge. The peptide backbone is displayed as green sticks, the Asp side chains as red balls, the Lys side chains as blue balls, and the cholesterol molecules are colored yellow.²²

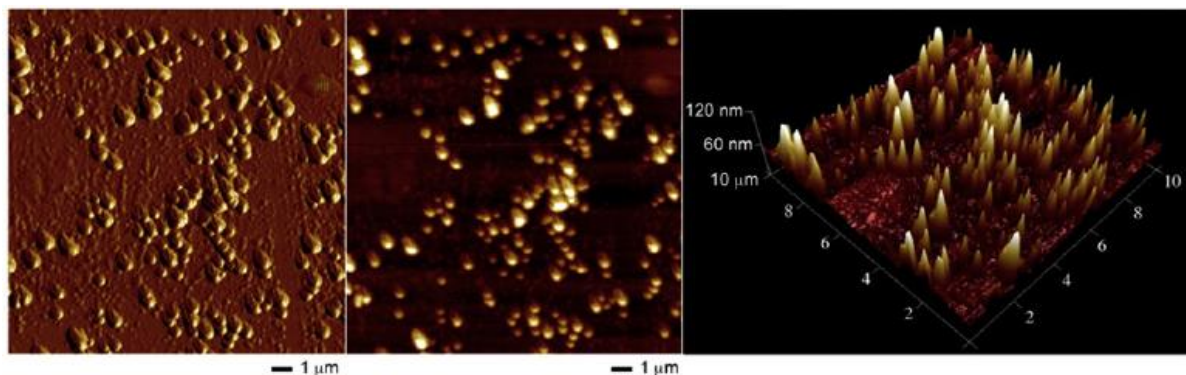


Figure 10, Atomic Force Microscopy (amplitude, phase, and 3D) of K₂₀ nanosponges.²²

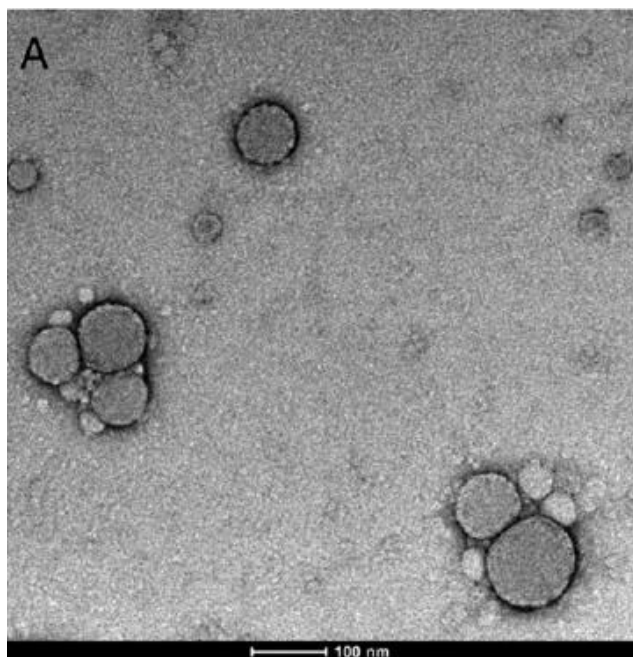


Figure 11, Transmission Electron Microscopy of K₂₀ nanosponges on graphite with uranyl acetate as positive staining.²²

The dynamic light scattering revealed that the effective diameter of the K₂₀ nanosponges was 180 ± 25 . Further, a critical concentration at which spontaneous aggregation would occur was determined in an analogous way to that of critical micelle concentration, the results of which are shown in **Figure 12**. This was accomplished by mixing (cholesterol-(K)₂₀DEV DGC)3-trimaleimide (K) and a 1:1 mixture of (cholesterol-(D)₂₀DEV DGC)3-trimaleimide (D) and (cholesterol-(K)₂₀DEV DGC)3-trimaleimide (K). The concentrations were stepwise increased in PBS buffer. The data suggests that only 0.90 g/L of type K nanosponges are required to allow spontaneous aggregation.²²

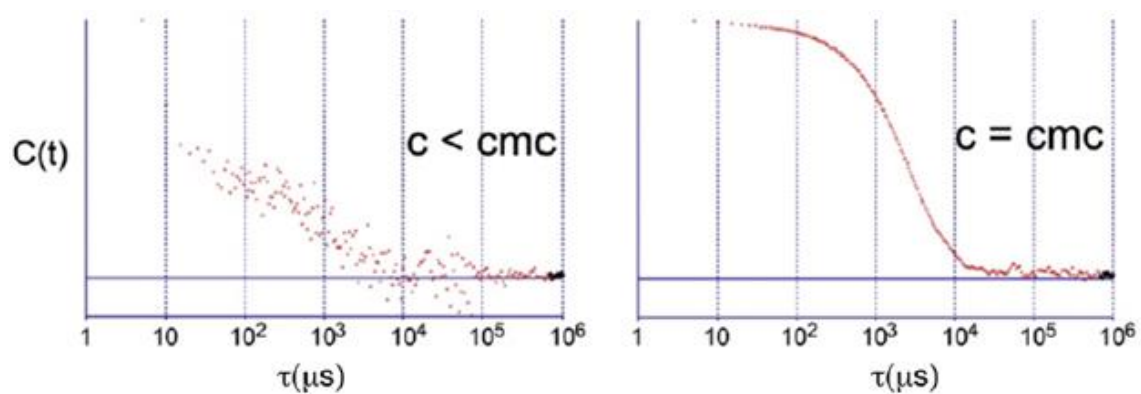


Figure 12, Correlation Curves of Dynamic Light Scattering of K₂₀ nanosponge.²²

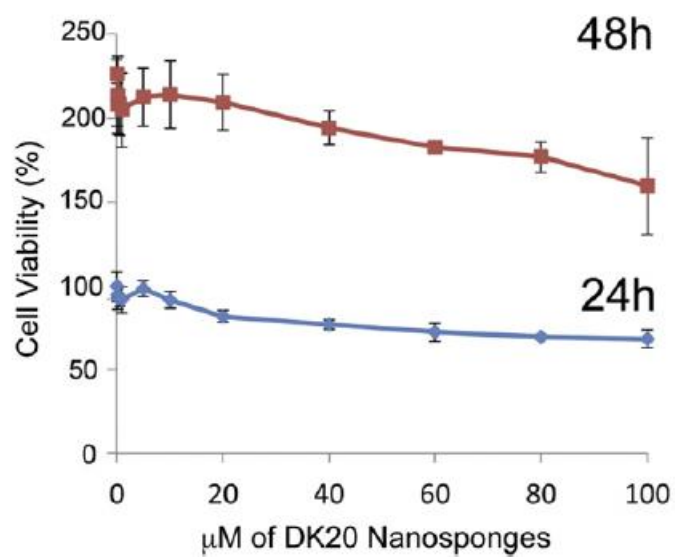


Figure 13, MTT Assay of DK₂₀ nanosponges on RAW264.7 cells.²²

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Chapter 2 – Synthesis, Characterization, Library, Biological

Activity, and SAR Study

Pyrazole Synthesis

To synthesize NNSN molecules, there are two starting materials required. These starting materials include a pyrazole ring and a phenyl isothiocyanate. Different pyrazole derivatives have been synthesized by methods such as Knorr pyrazole synthesis and solventless condensation of a 1,3-diketone and a hydrazine monohydrate derivative in the presence of a catalytic amount of $\text{H}_2\text{SO}_4\text{-SiO}_2$ at room temperature.¹ A pyrazole with the benzyl ether has proven quite effective, which was synthesized over four steps following the protocol of Knorr pyrazole synthesis followed by acetal protection of the acidic amino hydrogen, coupling with benzyl bromide and subsequent removal of the acetal.² Functional group transformations have also been performed on pyrazole rings such as methylation of 3-amino-1*H*-pyrazole, formylation followed by LAH reduction, oxidation of a methyl group to a carboxylic acid, subsequent esterification via an acid chloride, and nitration using sulfuric and nitric acids.

The first pyrazole synthesis was implemented to synthesize 3-methyl-5-phenyl-1*H*-pyrazole by dissolving 1-benzoylacetone in ethanol then adding hydrazine monohydrate (dissolved in ethanol) to the reaction at room temperature, which was then refluxed for 3 hours, as shown in **Figure 14**. No further purification was required as there was only one spot present on thin layer chromatography (TLC). This is important because TLC would reveal any side products or impurities, remaining starting materials, etc.; one spot indicates a pure product. The solvent was removed *in vacuo* using the rotary evaporator to reveal a light-yellow solid. This is a Knorr type synthesis reaction; however, it does not require acid to catalyze the reaction.

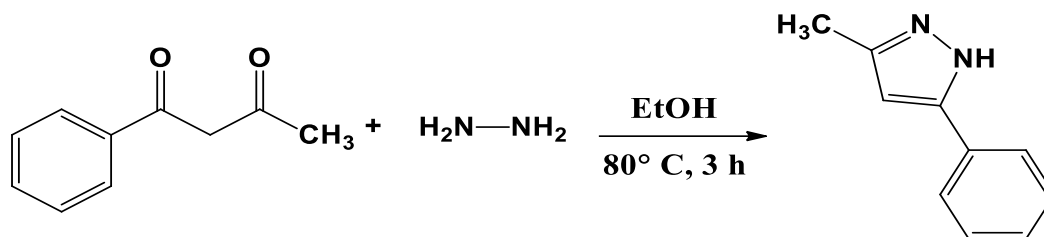


Figure 14, Synthesis of 3-methyl-5-phenyl-1H-pyrazole.

The next pyrazole synthesis to reach 1H-pyrazol-3(2H)-one follows the same procedure as above with minor modifications. Methyl-trans-3-methoxyacrylate was dissolved in ethanol and hydrazine monohydrate was added at room temperature, as shown in **Figure 15**. The reaction was refluxed under argon overnight. The solvent was removed *in vacuo* and the resulting solid was recrystallized with diethyl ether to give a light-yellow solid.

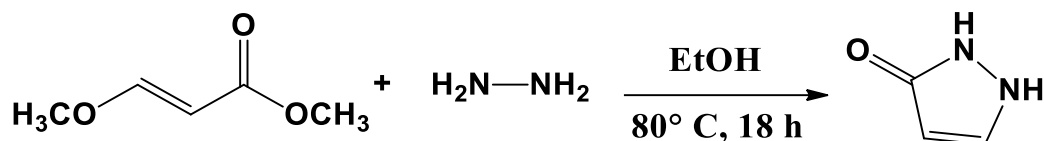


Figure 15, Synthesis of 1H-pyrazol-3(2H)-one.

1-*tert*-butyl-1H-pyrazol-3-amine was synthesized by dissolving *tert*-butylhydrazine hydrochloride in 30 mL of distilled water, which was cooled to 0° C. Potassium carbonate and sodium bicarbonate were added sequentially at 0° C. The reaction continued to stir at this temperature for 1 hour. To this clear, colorless solution was added 2-chloroacrylonitrile at 0°C. The reaction was warmed to room temperature then continued to stir for 12 hours, as shown in **Figure 16**. The resulting solution was light yellow in color – it was extracted with ethyl acetate (5 times) and the combined organic layers were washed with brine, dried over anhydrous sodium sulfate, then the solvent was removed *in vacuo*. TLC revealed multiple spots, which were separated via descending flash column chromatography using silica gel as stationary phase and a gradient mixture of dichloromethane and methanol as eluent.

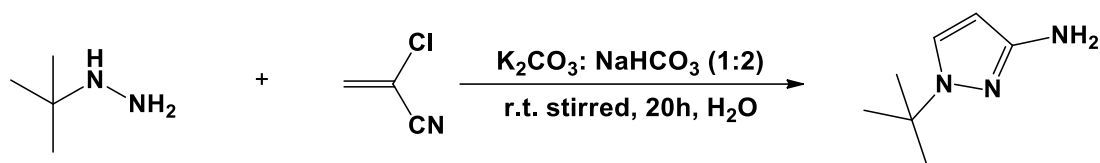


Figure 16, Synthesis of 1-tert-butyl-1H-pyrazol-3-amine.

3,5-dimethyl-1H-pyrazole and 3,4,5-trimethyl-1H-pyrazole were synthesized via a solventless sulfuric acid immobilized on silica gel ($\text{H}_2\text{SO}_4\text{-SiO}_2$) catalyzed condensation of a diketone and hydrazine at room temperature. $\text{H}_2\text{SO}_4\text{-SiO}_2$ was synthesized ahead of time according to known protocols by Dr. Man Zhang. In a mortar, 2,4-pentanedione was mixed with hydrazine monohydrate, slowly. There was a sizzling sound that occurs upon addition; the exothermic nature of the reaction can be dangerous if the hydrazine is added too quickly. Following addition, the $\text{H}_2\text{SO}_4\text{-SiO}_2$ was added, and the slurry formed was continuously stirred with a glass stirring rod until a solid formed, which took approximately 5 minutes. The solid was diluted with ethyl acetate and this mixture was filtered to collect the white solid, as shown in

Figure 17. No further purification was required for either of these compounds.

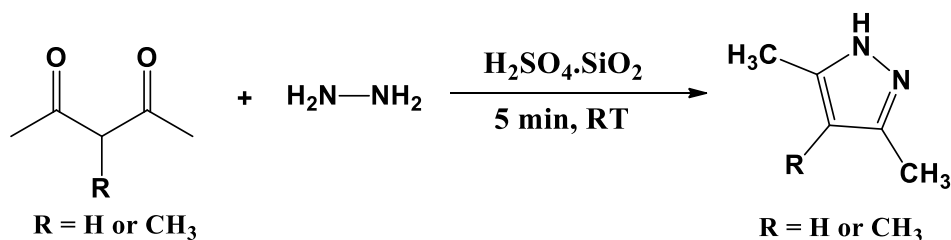


Figure 17, Synthesis of 3,5-dimethyl-1H-pyrazole (R=H) and 3,4,5-trimethyl-1H-pyrazole (R=CH₃).

3-(benzyloxy)-1H-pyrazole was synthesized over four steps following the synthesis protocol outlined in **Figure 18**. The first step is a Knorr-type synthesis where (E)-methyl 3-methoxyacrylate was dissolved in methanol and then hydrazine monohydrate was added slowly at room temperature. The reaction was then refluxed for 2 hours. The solvent was removed *in vacuo* to reveal 1H-pyrazol-3-ol as a light-yellow solid.

The next step is acetal protection of the acidic amino hydrogen so that it does not form any undesired products during the following transformations. This was done by dissolving 1H-pyrazol-3-ol in pyridine, then heating this mixture to 95° C. A mixture of acetic anhydride and pyridine was added dropwise over 20 minutes. Following addition, the reaction was stirred at 95° C for 2 hours. All volatiles were removed *in vacuo* and 200 mL of diethyl ether was added to the residue. This solution was stirred overnight at room temperature. The solid was filtered and washed with 100 mL of diethyl ether to give 1-(3-hydroxy-1H-pyrazol-1-yl)ethan-1-one as an off-white solid.

The third step is coupling with benzyl bromide via stirring a solution of 1-(3-hydroxy-1H-pyrazol-1-yl)ethan-1-one, benzyl bromide, potassium carbonate, and DMF for 10 hours at room temperature. The solvent was removed *in vacuo* then the residue was diluted with ethyl acetate and water. The organic phase was washed with brine, dried over sodium sulfate, then the volatiles removed again *in vacuo*. This crude material was purified by column chromatography to give 1-(3-(benzyloxy)-1H-pyrazol-1-yl)ethan-1-one as a colorless oil.

The final step is the removal of the acetal protecting group to yield the final product. 1-(3-(benzyloxy)-1H-pyrazol-1-yl)ethan-1-one was dissolved in methanol and tetrahydrofuran, to which 10% sodium hydroxide solution was added. This mixture was left to stir at room temperature for 5 hours. The solvent was then removed *in vacuo*. The residue was diluted using ethanol and water. The organic layer was washed with brine and dried over anhydrous sodium sulfate. This revealed the final product, 3-(benzyloxy)-1H-pyrazole, as a white solid.

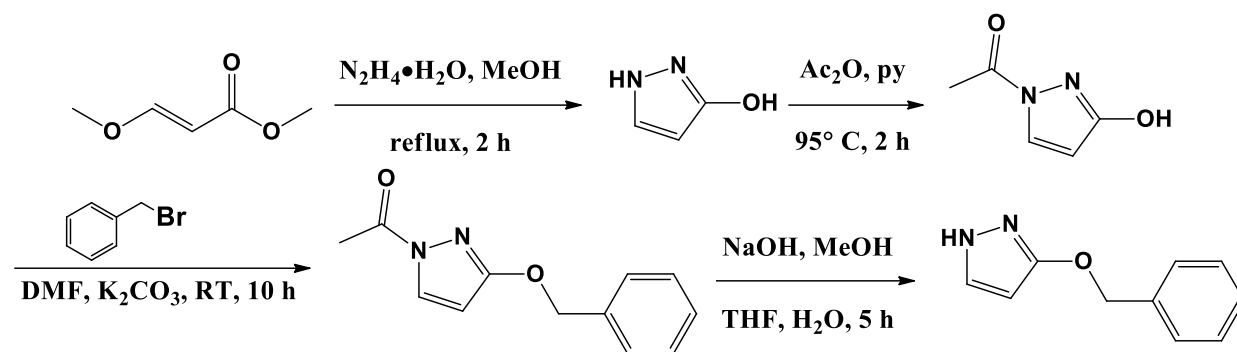


Figure 18, Synthesis of 3-(benzyloxy)-1H-pyrazole.²

N,N-dimethyl-1H-pyrazol-3-amine was synthesized by adding paraformaldehyde slowly at 0° C to a solution of 3-aminopyrazole dissolved in methanol. The reaction was then stirred at room temperature for 2 hours under argon. The mixture was again cooled to 0° C then sodium cyanoborohydride was added. Then the reaction was warmed to room temperature and continued to stir at 3 hours longer under argon, as shown in **Figure 19**. The resulting mixture was extracted three times with dichloromethane and the combined organic layers were washed with distilled water and brine, then dried over magnesium sulfate, filtered and the solvent removed *in vacuo* to yield the product as a yellow oil.

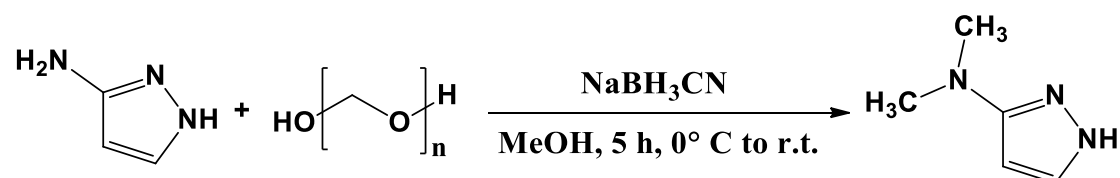


Figure 19, Synthesis of N,N-dimethyl-1H-pyrazol-3-amine.

N-methyl-1H-pyrazol-3-amine was synthesized by stirring a solution of 3-aminopyrazole and formic acid at 110° C for 12 hours under argon. The resulting mixture was concentrated to almost dryness using the rotary evaporator. The residue was washed with distilled water and dried under strong vacuum to yield *N*-(2H-pyrazole-3-yl)formamide as a white solid. This solid was dissolved in distilled tetrahydrofuran and the solution cooled to -10° C, at which point lithium aluminum hydride (LAH) was added. The reaction mixture was slowly warmed to room

temperature and continuously stirred under argon overnight, as shown in **Figure 20**. The next day, the reaction was cooled to 0° C then the reaction was quenched by adding distilled water, 15% sodium hydroxide aqueous solution, then distilled water again. The reaction mixture was then stirred at room temperature for 30 minutes and dried over magnesium sulfate, filtered, and the solvent removed *in vacuo* to yield a brown oil.

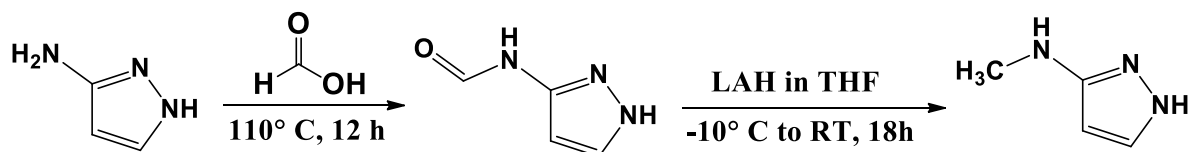


Figure 20, Synthesis of N-methyl-1H-pyrazol-3-amine.

1H-pyrazole-3-carboxylic acid was synthesis by stirring a solution of 3-methylpyrazole and water at room temperature. To this solution was slowly added a solution of potassium permanganate dissolved in water. Following the completion of this addition, the reaction was left to reflux for 4 hours, as shown in **Figure 21**. The mixture was then filtered to remove excess potassium permanganate and the filtrate (the liquid which has passed through a filter) was collected. The solvent was removed *in vacuo* and the remaining residue was placed in an ice bath. At this point, the pH was changed from 8 to 2 using a diluted solution of hydrochloric acid (2N concentration). A white precipitate begins to form in solution which was dried and collected from the flask by scraping the sides with a spatula.

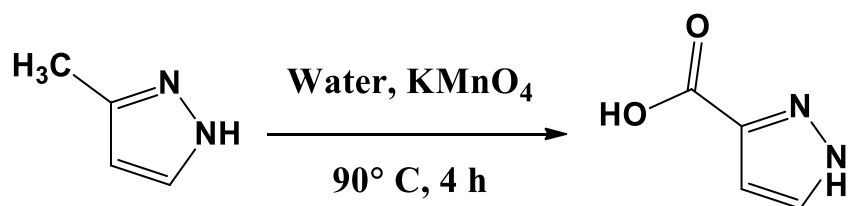


Figure 21, Synthesis of 1H-pyrazole-3-carboxylic acid.

Methyl 1H-pyrazole-3-carboxylate and Ethyl 1H-pyrazole-3-carboxylate were synthesized by esterification of 1H-pyrazole-3-carboxylic acid using thionyl chloride in the presence of an alcohol as solvent. 1H-pyrazole-3-carboxylic acid was dissolved in MeOH or EtOH, respectively, then thionyl chloride was added slowly at 0° C. The temperature was then increased to 70° C and stirred for 5 hours, as shown in **Figure 22**. The solvent was removed *in vacuo* and the residue poured into ice water and extracted with ethyl acetate. The organic layer was washed with 10% sodium bicarbonate solution followed by brine, then dried over sodium sulfate. The resulting mixture was concentrated *in vacuo* once again to reveal a white solid.

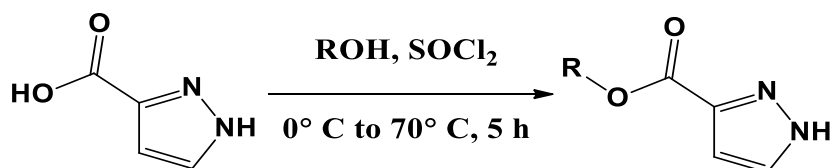


Figure 22, Synthesis of Methyl 1H-pyrazole-3-carboxylate (where R = CH₃) and Ethyl 1H-pyrazole-3-carboxylate (where R = CH₂CH₃).

4-nitro-1H-pyrazole and 3-methyl-4-nitro-1H-pyrazole were synthesized using known nitration conditions. For this, either 1H-pyrazole, or 3-methyl pyrazole, respectively, was dissolved in concentrated sulfuric acid. The solution was heated to 60° C and concentrated nitric acid was added dropwise. The reaction mixture was stirred for 90 minutes, as shown in **Figure 23**. The solution was poured into a beaker filled with ice and the product was obtained as a white solid. This was collected via gravity filtration.

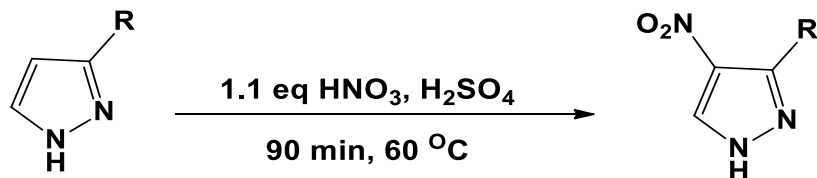


Figure 23, Synthesis of 4-nitro-1H-pyrazole (where R = H) and 3-methyl-4-nitro-1H-pyrazole (where R = CH₃).

All other pyrazoles used to reach products in the NNSN library, including 1,4-diisothiocyanatobenzene-1Hpyrazole, 1H-pyrazol-3-amine, 3-methyl-1H-pyrazole, 4-iodo-1H-pyrazol-3-amine, 1-methyl-1H-pyrazol-5-amine, 1-phenyl-1H-pyrazol-5-amine, 4-bromo-1H-pyrazole, 1H-pyrazol-4-amine, 3-(trifluoromethyl)-1H-pyrazole, 3-((4-nitrobenzyl)oxy)-1H-pyrazole, and benzo[c][1,2,5]thiadiazol-4-amine were either purchased from Fisher Scientific Inc. or synthesized by other researchers on the NNSN team. Further synthetic details can be found in Appendix A.

Phenyl Isothiocyanate Synthesis

In general, the synthesis of phenyl isothiocyanates with differing substitutions involves two steps. Firstly, a dithiocarbamate salt is produced using the appropriate aniline, base (and sometimes a solvent / other times neat) with room temperature stirring. Next, the salt is dissolved in the corresponding solvent, a desulfurization agent is added (either crystalline iodine³, or ethyl chloroformate)⁴, sometimes along with base, and allowed to stir at room temperature for 30 minutes.

1-chloro-2-isothiocyanatobenzene, 1-chloro-3-isothiocyanatobenzene, and 1-chloro-4-isothiocyanatobenzene were synthesized by the creation of dithiocarbamate salts which were treated with iodine as desulfurization agent. To prepare the dithiocarbamate salt, a solution was prepared by mixing triethylamine and carbon disulfide together. This was cooled in an ice then the chlorinated aniline was added dropwise at 0° C. This solution was warmed to room temperature and left to stir overnight. The next day, the solid was filtered and washed with 95:5 ethyl acetate/ hexane to remove any excess triethylamine. The dithiocarbamate salt was collected as a yellow solid. When this same reaction was run on the 2-chloro, 3-chloro, and 4-chloroaniline at the same exact scale simultaneously, the 3-chloro consistently gave higher yields.

A solution was then prepared by mixing the dithiocarbamate salt and sodium bicarbonate, dissolved in a 1:1 mixture of ethyl acetate and water. This solution was cooled to 0° C using an ice bath then crystalline iodine was added pinch-wise over 30 minutes at this temperature, as shown in **Figure 24**. Once the addition was completed, the organic layer (ethyl acetate) was washed with sodium thiosulfate to remove any excess iodine, water, and brine, then dried over anhydrous sodium sulfate. The solvent was removed *in vacuo*. The product was then purified using a short silica column with hexane as eluent.

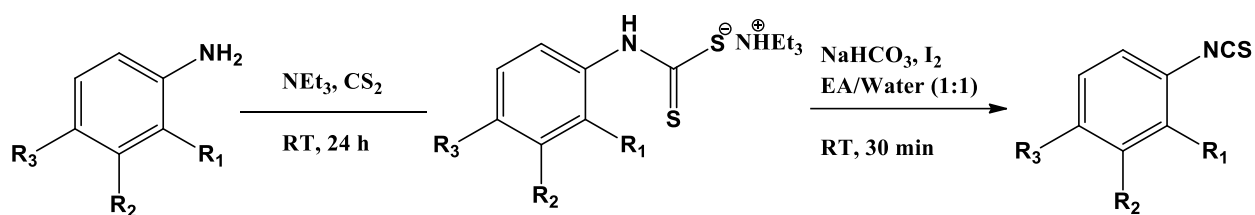


Figure 24, Synthesis of Monochlorinated Phenyl Isothiocyanates, where A = R1=Cl, R2=H, R3=H; B = R1=H, R2=Cl, R3=H; C = R1=H, R2=H, R3=Cl.

The same reaction conditions outlined above were initially attempted to reach dichlorinated isothiocyanates, however, this proved ineffective. Instead, the dithiocarbamate salts required a stronger base than triethylamine to form. Also, iodine does not work well as a desulfurization agent for dichlorinated isothiocyanates. The following is the best methodology for reaching this type of isothiocyanates.

2,3-dichlorophenylisothiocyanate, 2,4-dichlorophenylisothiocyanate, 3,4-dichlorophenylisothiocyanate, and 2,6-dichlorophenylisothiocyanate were synthesized by the creation of dithiocarbamate salts which were treated with ethyl chloroformate as desulfurization agent. To prepare the dithiocarbamate salt, 1,4-diazabicyclo[2.2. 2]octane (DABCO) and carbon disulfide were mixed and dissolved in acetone. This solution was cooled to 0° C and the dichlorinated aniline was added slowly. Following addition, the reaction was warmed to room

temperature and allowed to stir overnight. The next day, the resulting yellow / orange dithiocarbamate salt was collected via gravity filtration.

The dithiocarbamate salt was dissolved in chloroform and triethylamine was added. The solution was cooled to 0° C and ethyl chloroformate was added slowly. This was left to stir for 1 hour at this temperature, as shown in **Figure 25**. The organic layer was then washed with a dilute hydrochloric acid solution (2N concentration) then water, and then dried over anhydrous sodium sulfate. The solvent was removed in vacuo and the resulting crude product was purified via flash column chromatography to reveal a clear oil or white solid, depending on the isothiocyanate produced.

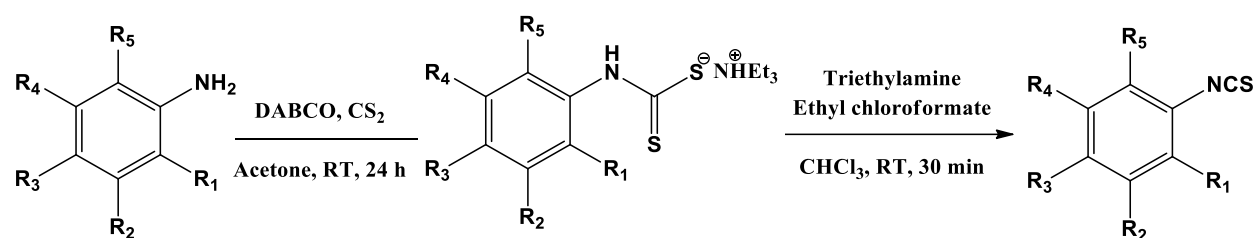


Figure 25, Synthesis of Dichlorinated Phenyl Isothiocyanates, where A = R1=Cl, R2=Cl, R3=H, R4=H, R5=H; B = R1=H, R2=Cl, R3=Cl, R4=H, R5=H; C = R1=Cl, R2=H, R3=H, R4=Cl, R5=H; D = R1=Cl, R2=H, R3=H, R4=H, R5=Cl

Any other isothiocyanates used in the creation of NNSN molecules were either purchased from Fisher Scientific Inc. or synthesized by other researchers on the NNSN team. These isothiocyanates include 2-chlorobenzyl-isothiocyanate, 3-chlorobenzyl-isothiocyanate, 4-chlorobenzyl-isothiocyanate, 1,4-diisothiocyanatobenzene-1Hpyrazole, and 2-isothiocyanato-1,3-dimethylbenzene. Further synthetic details can be found in Appendix A.

NNSN Coupling Synthesis

For the synthesis of NNSN molecules, two components are required. An aryl isothiocyanate is purchased or synthesized with the desired substituents attached, as discussed in the “Phenyl Isothiocyanate Synthesis” section. Examples of aryls showing best activity include phenyls and benzyls substituted with one or more halogens (-Cl, Br, F, etc.), nitro groups, alkoxies (such as methoxies), or alkyl groups.¹ A pyrazole ring is then purchased or synthesized with the intended substituents attached, as discussed in the “Pyrazole Synthesis” section. In this case, either substituent can be hydrogen, substituted or unsubstituted aryls, branched or unbranched alkyls or alkenyls (which may be substituted with either halogens, sulfonic acid, or nitro groups), cycloalkyls, heterocycles, or substituted or unsubstituted amines or amides.¹

Once both components have been purchased or synthesized, they are coupled together via one of seven typical procedures to produce the NNSN compound, namely Method 1 – Method 7. Following coupling, the NNSN is purified either by flash column chromatography or recrystallization. If using column chromatography, this involves loading a glass column with silica as the solid phase and the mobile phase or eluent is either hexane, ethyl acetate, dichloromethane, methanol, or a mixture of these solvents depending on the polarity of the product. Air is pushed through to speed up the process, however, depending on the compound, it can take up to several hours to finish purification through this method. Recrystallization involves taking a crude solid compound and dissolving this in a recrystallization (typically using ether or methanol in the case of NNSN molecules) by heating until the boiling point of the solvent then letting it cool slowly to room temperature then placing in an ice bath to cool further. Recrystallization works only if the proper solvent is used – the product to be purified must be highly soluble at high temperature and insoluble at low temperatures. This ensures that the

product and all impurities dissolve when heated but then only the product comes out of solution when it cools. Because of this, it is extremely important to cool slowly so that the impurities remain outside of the lattice that is formed when the solid reforms.

Method 1 involves dissolving both starting materials in DCM and stirring for 24 hours, at room temperature. 4-amino-N-(4-chlorobenzyl)-1H-pyrazole-1-carbothioamide (APM01), 3-amino-N-(4-chlorophenyl)-1H-pyrazole-1-carbothioamide (KE03), N-(2-chlorophenyl)-1H-pyrazole-1-carbothioamide (SB46), 3-amino-N-(2-chlorophenyl)-1H-pyrazole-1-carbothioamide (SB49), 3-amino-N-(3-chlorophenyl)-1H-pyrazole-1-carbothioamide (SB50), N-(3-chlorophenyl)-1H-pyrazole-1-carbothioamide (SB52), 4-amino-N-(2-chlorobenzyl)-1H-pyrazole-1-carbothioamide (SB62), N-(4-isothiocyanatophenyl)-1H-pyrazole-1-carbothioamide (SB78), 3-amino-N-(4-isothiocyanatophenyl)-1H-pyrazole-1-carbothioamide (SB79), N,N'-(1,4-phenylene)bis(1H-pyrazole-1-carbothioamide (SB80), N,N'-(1,4-phenylene)bis(3-amino-1H-pyrazole-1-carbothioamide (SB81), N-(2,3-dichlorophenyl)-1H-pyrazole-1-carbothioamide (SB87), (N-(2,4-dichlorophenyl)-1H-pyrazole-1-carbothioamide (SB88), N-(3,4-dichlorophenyl)-1H-pyrazole-1-carbothioamide (SB89), N-(2,6-dichlorophenyl)-1H-pyrazole-1-carbothioamide (SB90), 3-amino-N-(2,3-dichlorophenyl)-1H-pyrazole-1-carbothioamide (SB91), 3-amino-N-(2,4-dichlorophenyl)-1H-pyrazole-1-carbothioamide (SB92), 3-amino-N-(3,4-dichlorophenyl)-1H-pyrazole-1-carbothioamide (SB93), 3-amino-N-(2,6-dichlorophenyl)-1H-pyrazole-1-carbothioamide (SB94), and 3-amino-N-(3-chlorobenzyl)-1H-pyrazole-1-carbothioamide (SB133) were synthesized via this method, as shown in **Figure 26**. The yields of these compounds vary drastically from poor yields such as 19% to excellent yields such as 99%. This method works well for coupling unsubstituted pyrazoles and those containing amino groups.

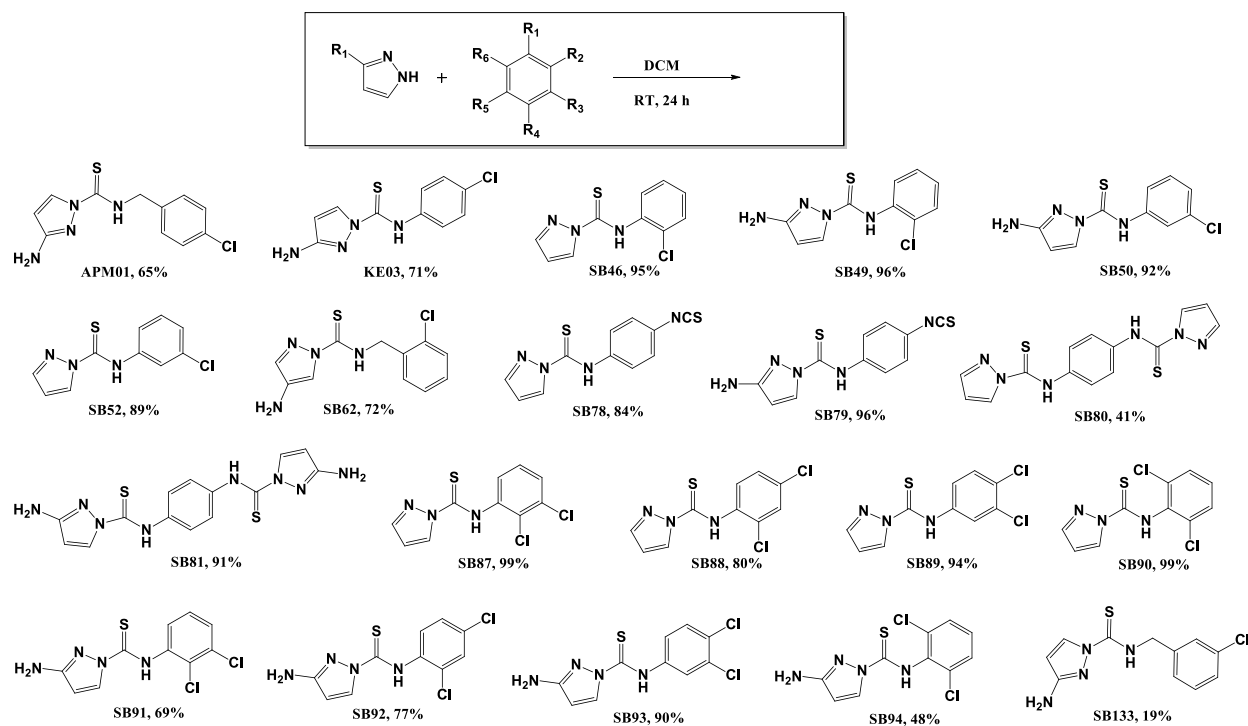


Figure 26, Synthesis of NNSN Molecules via Method 1.

Method 2 involves dissolving both starting materials in DCM and stirring for 24 hours, at reflux of 45° C. N-(2-chlorophenyl)-3-methyl-1H-pyrazole-1-carbothioamide (SB60), N-(3-chlorophenyl)-3-methyl-1H-pyrazole-1-carbothioamide (SB61), Ethyl 1-((2-chlorophenyl)carbamothioyl)-1H-pyrazole-3-carboxylate (SB69), Ethyl 1-((3-chlorophenyl)carbamothioyl)-1H-pyrazole-3-carboxylate (SB70), Methyl 1-((2-chlorophenyl)carbamothioyl)-1H-pyrazole-3-carboxylate (SB73), Methyl 1-((3-chlorophenyl)carbamothioyl)-1H-pyrazole-3-carboxylate (SB74), Ethyl 1-((4-chlorophenyl)carbamothioyl)-1H-pyrazole-3-carboxylate (SB77), N-(4-isothiocyantophenyl)-3-methyl-1H-pyrazole-1-carbothioamide (SB82), N-(2,3-dichlorophenyl)-3-methyl-1H-pyrazole-1-carbothioamide (SB97), N-(2,4-dichlorophenyl)-3-methyl-1H-pyrazole-1-carbothioamide (SB98), N-(3,4-dichlorophenyl)-3-methyl-1H-pyrazole-1-carbothioamide (SB99), and N-(2,6-dichlorophenyl)-3-methyl-1H-pyrazole-1-carbothioamide (SB100) were synthesized via this

method, as shown in **Figure 27**. The yields of these compounds also vary drastically from poor yields such as 19% to excellent yields such as 97%. This method works especially well for coupling pyrazoles that have methyl groups, methyl esters, and ethyl esters.

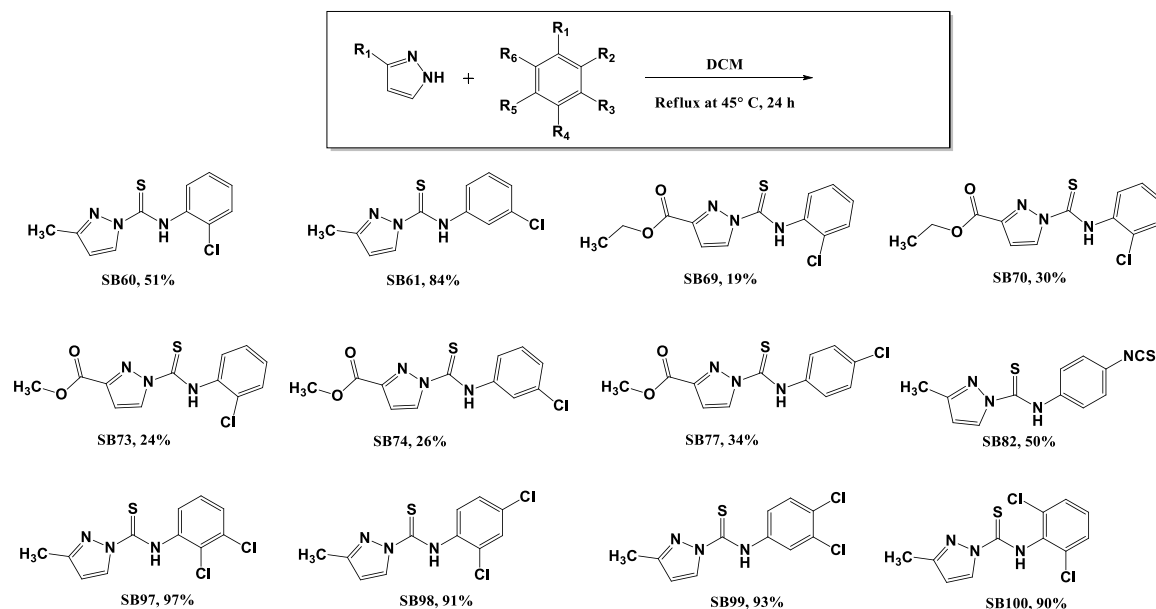


Figure 27, Synthesis of NNSN Molecules via Method 2.

Method 3 is done by dissolving both starting materials in dry tetrahydrofuran and refluxing at for 24 hours. This method was used to synthesize N-(2-chlorophenyl)-3-oxo-2,3-dihydro-1H-pyrazole-1-carbothioamide (SB132) and N-(2-chlorophenyl)-3-oxo-2,3-dihydro-1H-pyrazole-1-carbothioamide (SB133), as shown in **Figure 28**. The yields from this reaction are substrate dependent in that the SB131 shows a poor yield of 19% while SB132 shows a fair yield of 59%. This method works well for coupling 1H-pyrazol-3(2H)-one.

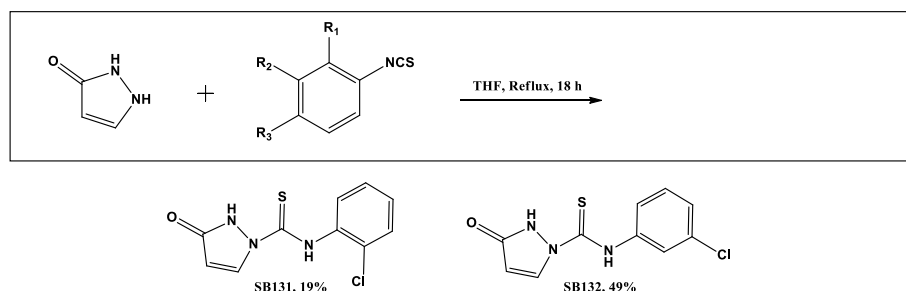


Figure 28, Synthesis of NNSN Molecules via Method 3.

Method 4 involves dissolving the pyrazole in dry tetrahydrofuran and adding anhydrous potassium carbonate and stirring for 1 hour at 50° C, then the isothiocyanate is added and the solution refluxed at 70° C for 18 hours. 3-(benzyloxy)-N-(2-chlorophenyl)-1H-pyrazole-1-carbothioamide (SB64), 3-(benzyloxy)-N-(3-chlorophenyl)-1H-pyrazole-1-carbothioamide (SB65), N-(2-chlorophenyl)-3,5-dimethyl-1H-pyrazole-1-carbothioamide (SB122), N-(3-chlorophenyl)-3,5-dimethyl-1H-pyrazole-1-carbothioamide (SB123), N-(2-chlorophenyl)-3,4,5-trimethyl-1H-pyrazole-1-carbothioamide (SB125), and N-(3-chlorophenyl)-3,4,5-trimethyl-1H-pyrazole-1-carbothioamide (SB126) were synthesized by this method, as shown in **Figure 29**. The yields of these compounds also vary drastically from poor yields such as 13% to excellent yields such as 98%. Coupling with pyrazoles with groups such as benzyl ethers, di- or tri-methylation work well with this method.

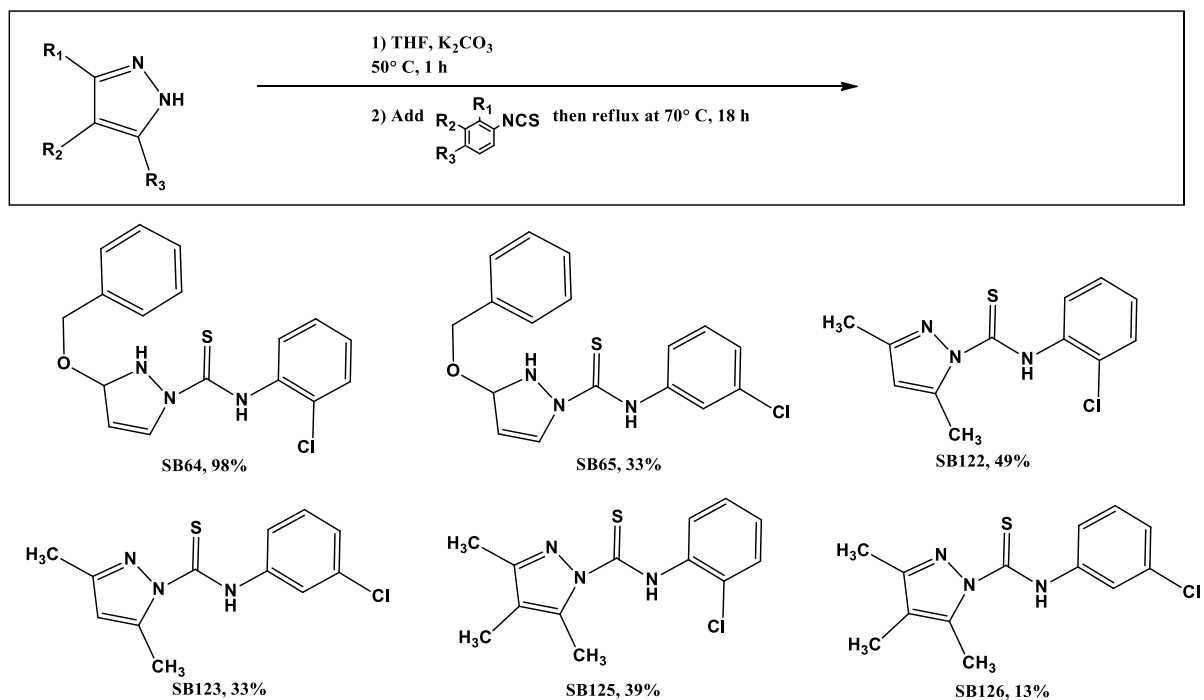


Figure 29, Synthesis of NNSN Molecules via Method 4.

Method 5 is where the pyrazole and sodium hydride are added to two separate flame-dried round bottom flasks and stirred in dry THF at 0° C for 20 minutes after which the contents

of flask A (with the base) are cannulated into flask B (with the pyrazole). The solution is stirred for 3 hours at room temperature, then the isothiocyanate added and the reaction continued to stir for 12 hours. N-(2-chlorophenyl)-4-nitro-1H-pyrazole-1-carbothioamide (SB56), N-(3-chlorophenyl)-4-nitro-1H-pyrazole-1-carbothioamide (SB59), N-(4-isothiocyanatophenyl)-4-nitro-1H-pyrazole-1-carbothioamide (SB83), N-(2,3-dichlorophenyl)-4-nitro-1H-pyrazole-1-carbothioamide (SB105), N-(2,4-dichlorophenyl)-4-nitro-1H-pyrazole-1-carbothioamide (SB106), N-(3,4-dichlorophenyl)-4-nitro-1H-pyrazole-1-carbothioamide (SB107), N-(2,6-dichlorophenyl)-4-nitro-1H-pyrazole-1-carbothioamide (SB108), N-(2-chlorophenyl)-3-methyl-4-nitro-1H-pyrazole-1-carbothioamide (SB127), and N-(3-chlorophenyl)-3-methyl-4-nitro-1H-pyrazole-1-carbothioamide (SB128) were synthesized via this method, as shown in **Figure 30**. The yields of these compounds also vary drastically from poor yields such as 20% to very good yields such as 80%. This coupling method works well with pyrazoles containing nitro groups.

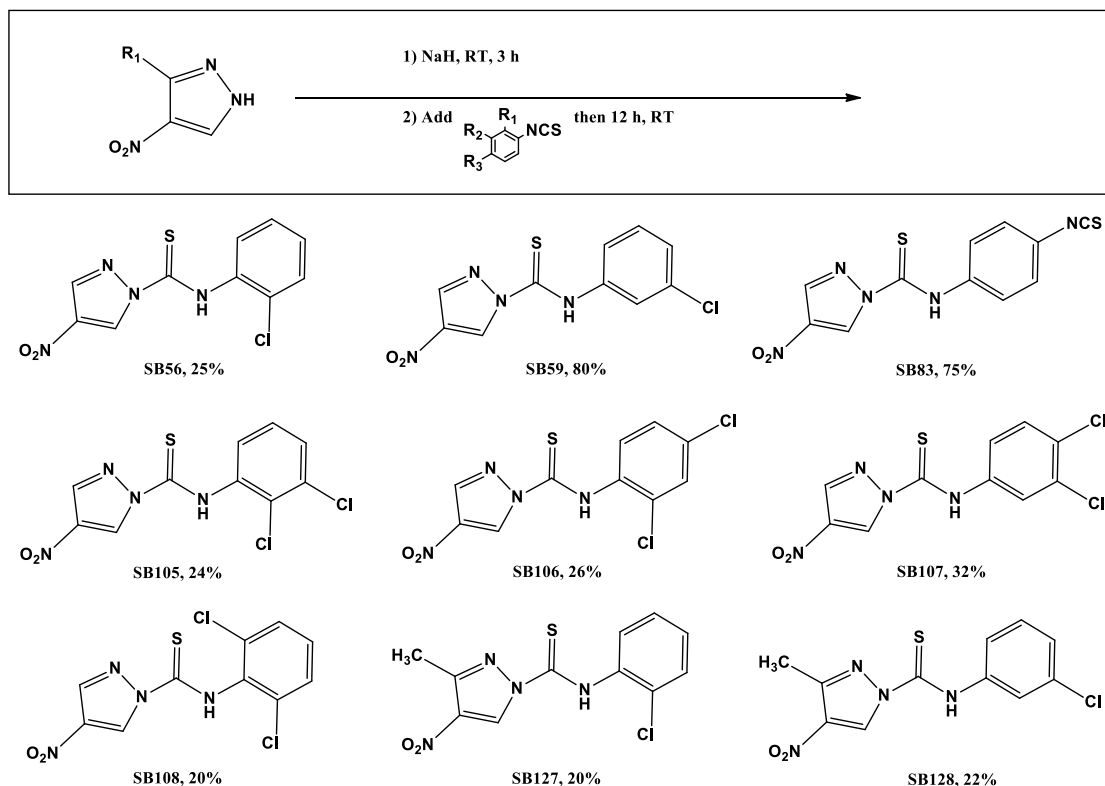
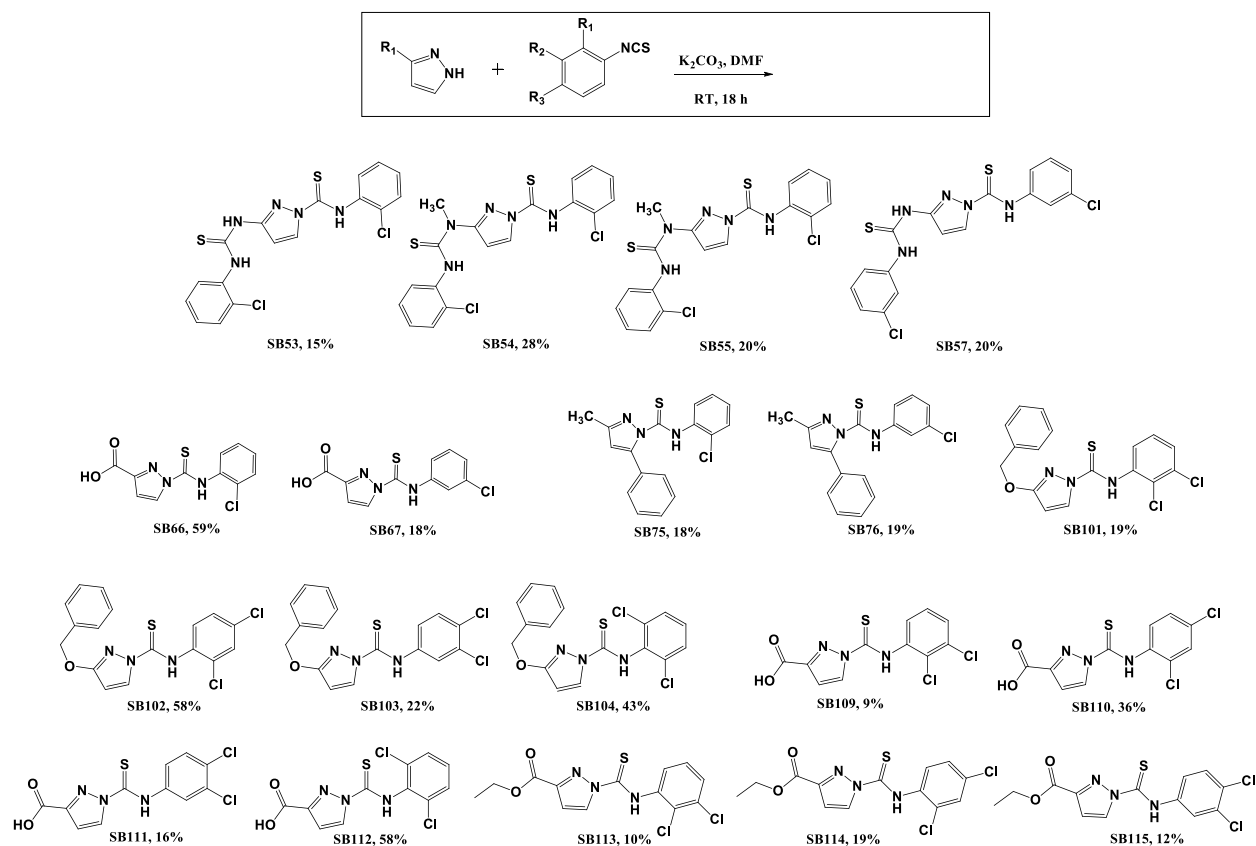


Figure 30, Synthesis of NNSN Molecules via Method 5.

Method 6 is coupling both starting materials using anhydrous potassium carbonate and N,N-dimethylformamide over 24 hours stirring at room temperature. This can be used to synthesize many different types of NNSN molecules and could be described as the most universally effective method, despite the low yields seen. N-(2-chlorophenyl)-3-(3-(2-chlorophenyl)thioureido)-1H-pyrazole-1-carbothioamide (SB53), N-(3-chlorophenyl)-3-(3-(3-chlorophenyl)-1-methylthioureido)-1H-pyrazole-1-carbothioamide (SB54), N-(2-chlorophenyl)-3-(3-(2-chlorophenyl)-1-methylthioureido)-1H-pyrazole-1-carbothioamide (SB55), N-(3-chlorophenyl)-3-(3-(3-chlorophenyl)thioureido)-1H-pyrazole-1-carbothioamide (SB57), 1-((2-chlorophenyl)carbamothioyl)-1H-pyrazole-3-carboxylic acid (SB66), 1-((3-chlorophenyl)carbamothioyl)-1H-pyrazole-3-carboxylic acid (SB67), N-(2-chlorophenyl)-3-methyl-5-phenyl-1H-pyrazole-1-carbothioamide (SB75), N-(3-chlorophenyl)-3-methyl-5-phenyl-1H-pyrazole-1-carbothioamide (SB76), 3-(benzyloxy)-N-(2,3-dichlorophenyl)-1H-pyrazole-1-carbothioamide (SB101), 3-(benzyloxy)-N-(2,4-dichlorophenyl)-1H-pyrazole-1-carbothioamide (SB102), 3-(benzyloxy)-N-(3,4-dichlorophenyl)-1H-pyrazole-1-carbothioamide (SB103), 3-(benzyloxy)-N-(2,6-dichlorophenyl)-1H-pyrazole-1-carbothioamide (SB104), 1-((2,3-dichlorophenyl)carbamothioyl)-1H-pyrazole-3-carboxylic acid (SB109), 1-((2,4-dichlorophenyl)carbamothioyl)-1H-pyrazole-3-carboxylic acid (SB110), 1-((3,4-dichlorophenyl)carbamothioyl)-1H-pyrazole-3-carboxylic acid (SB111), 1-((2,6-dichlorophenyl)carbamothioyl)-1H-pyrazole-3-carboxylic acid (SB112), Ethyl 1-((2,3-dichlorophenyl)carbamothioyl)-1H-pyrazole-3-carboxylate (SB113), Ethyl 1-((2,4-dichlorophenyl)carbamothioyl)-1H-pyrazole-3-carboxylate (SB114), Ethyl 1-((3,4-dichlorophenyl)carbamothioyl)-1H-pyrazole-3-carboxylate (SB115), Ethyl 1-((2,6-dichlorophenyl)carbamothioyl)-1H-pyrazole-3-carboxylate (SB116), Methyl 1-((2,3-

dichlorophenyl)carbamothioyl)-1H-pyrazole-3-carboxylate (SB117), Methyl 1-((2,4-dichlorophenyl)carbamothioyl)-1H-pyrazole-3-carboxylate (SB118), Methyl 1-((3,4-dichlorophenyl)carbamothioyl)-1H-pyrazole-3-carboxylate (SB119), Methyl 1-((2,6-dichlorophenyl)carbamothioyl)-1H-pyrazole-3-carboxylate (SB120), 1-((4-chlorophenyl)carbamothioyl)-1H-pyrazole-3-carboxylic acid (SB121), N-(4-chlorophenyl)-3,5-dimethyl-1H-pyrazole-1-carbothioamide (SB124), N-(2-chlorophenyl)-3-(dimethylamino)-1H-pyrazole-1-carbothioamide (SB129), N-(3-chlorophenyl)-3-(dimethylamino)-1H-pyrazole-1-carbothioamide (SB130), and N-(4-chlorophenyl)-3-(3-(4-chlorophenyl)thioureido)-1H-pyrazole-1-carbothioamide (SB134) were synthesized via this method, as shown in **Figure 31**. The yields of these compounds also vary drastically from very poor yields such as 5% to good yields such as 64%. This method does in fact show the lowest yields.



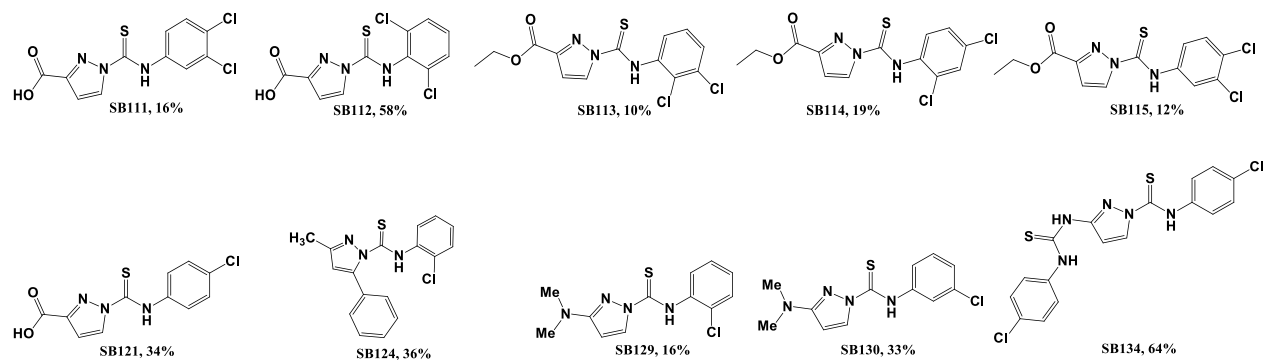


Figure 31, Synthesis of NNSN Molecules via Method 6.

Method 7 can only be used if between both starting materials a carboxylic acid and amine are both present, then they can be coupled together via 1,1'-Carbonyldiimidazole-mediated amide bond formation. 2-amino-N-(1-((4-chlorophenyl)carbamothioyl)-1H-pyrazol-3-yl)acetamide (SB84), GDVED(K)₂₀cholesterol-N-(4-chlorophenyl)-3-ureido-1H-pyrazole-1-carbothioamide (SB85), 3,3'-((((((2-aminoethyl)azanediyl)bis(ethane-2,1-diyl))bis(azanediyl))bis(carbonyl))bis(azanediyl))bis(N-(4-chlorophenyl)-1H-pyrazole-1-carbothioamide) (SB86), GDVED(K)₂₀cholesterol(linker)-N-(4-chlorophenyl)-3-ureido-1H-pyrazole-1-carbothioamide (SB96), 2-amino-N-(1-((2-chlorophenyl)carbamothioyl)-1H-pyrazol-3-yl)acetamide (SB135), 2-amino-N-(1-((3-chlorophenyl)carbamothioyl)-1H-pyrazol-3-yl)acetamide (SB136), 2-amino-N-(1-((2,3-chlorophenyl)carbamothioyl)-1H-pyrazol-3-yl)acetamide (SB137), 2-amino-N-(1-((2,4-chlorophenyl)carbamothioyl)-1H-pyrazol-3-yl)acetamide (SB138), 2-amino-N-(1-((3,4-chlorophenyl)carbamothioyl)-1H-pyrazol-3-yl)acetamide (SB139), and 2-amino-N-(1-((2,6-chlorophenyl)carbamothioyl)-1H-pyrazol-3-yl)acetamide (SB140) were synthesized via this method, as shown in **Figure 32**. The yields vary drastically as well, from poor yields such as 12% to excellent yields such as 99%.

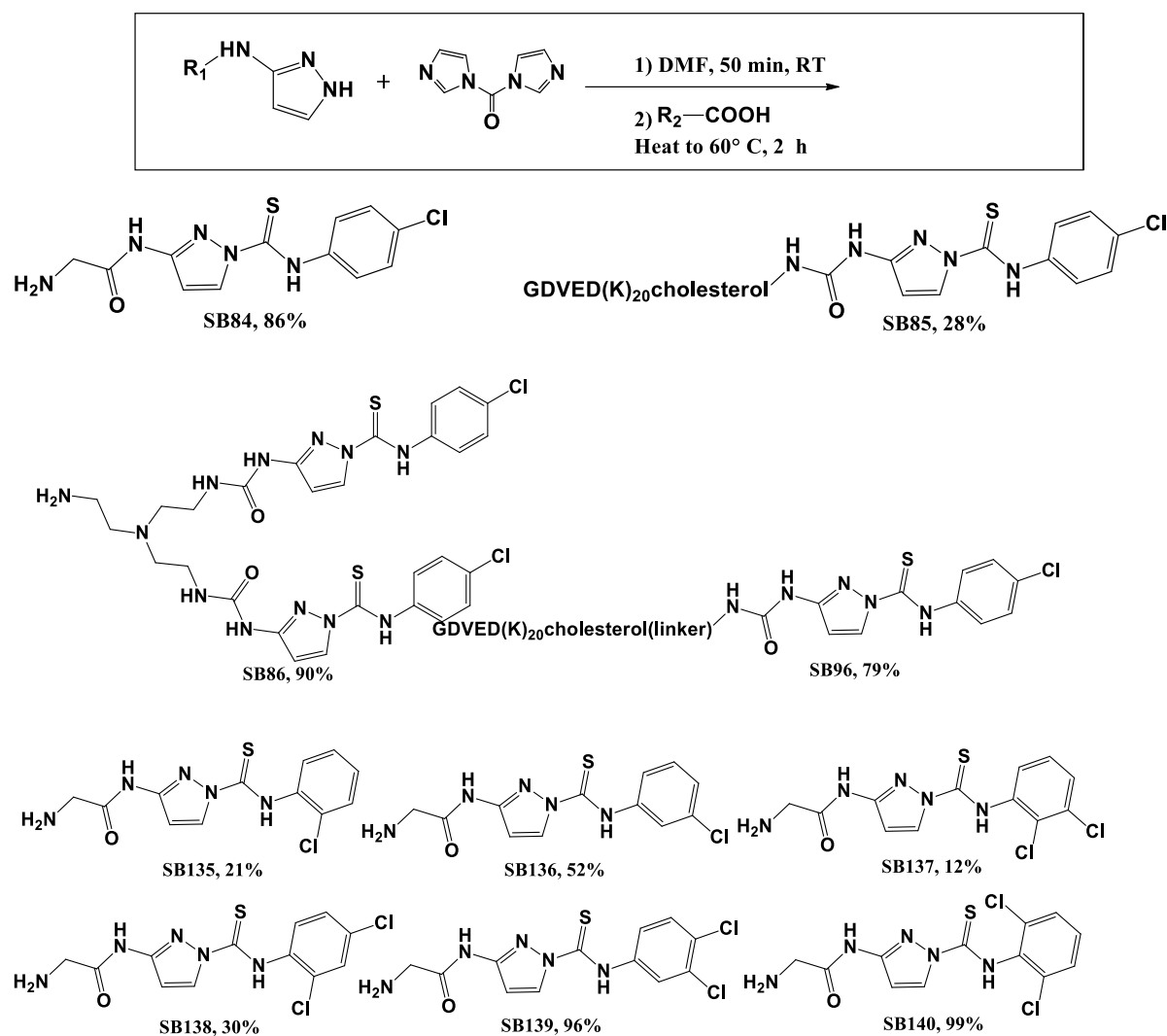


Figure 32, Synthesis of NNSN Molecules via Method 7.

This overview of the synthesis of NNSN Molecules briefly explains how these compounds were made. For full synthetic details, please see Appendix A. For full spectral characterization details, please see Appendix B.

Characterization Overview using KE03

The final NNSN products were characterized using Proton (^1H) Nuclear Magnetic Resonance (NMR) Spectroscopy, ^{13}C NMR, Infrared (IR) spectroscopy, and either High Resolution Mass Spectroscopy (HRMS) or Ultra Performance Liquid Chromatography-Mass

Spectroscopy (UPLC-MS). Proton NMR is used to elucidate the structure based on a great deal of information about molecular structure because most organic molecules contain many hydrogen atoms, and the hydrogen atoms absorb energy of different wavelengths depending on their bonding environment. Further, carbon-13 NMR spectroscopy gives information on the different environments of carbon molecules in the structure. IR spectroscopy reveals the different functional groups found within the sample. Lastly, HRMS is a highly sensitive characterization test (allows detection of analytes to the nearest 0.001 atomic mass units) that gives information about the molecular weight of the sample given. UPLC-MS is similar in that it yields information about the molecular weights within a given sample. While HRMS does not yield spectra, UPLC-MS does yield a chromatogram, a few of which are shown in **Figure 33** and **Figure 34**. For full written information on each compound characterized with this method, see either Appendix.

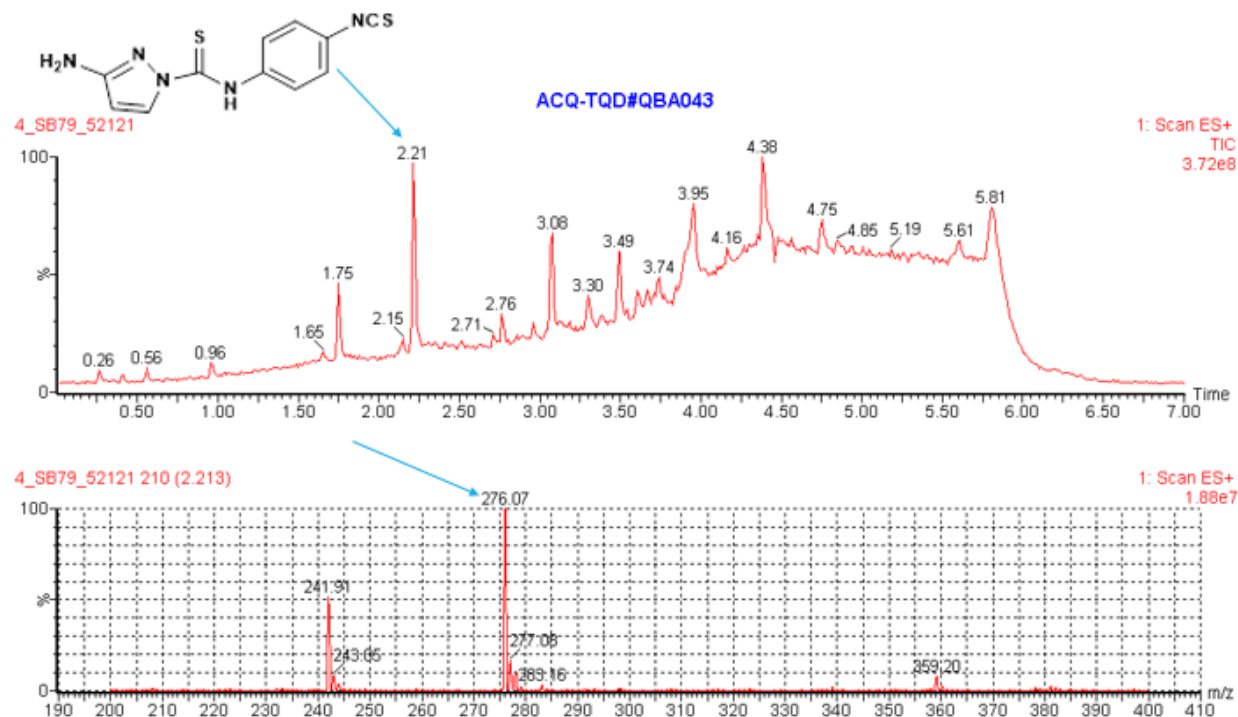


Figure 33, UPLC-MS of SB79: Flow rate 0.8 mL/min using water and acetonitrile in a gradient from 5% to 80% acetonitrile. UPLC-MS [M+1] calcd for C₁₁H₉N₅S₂ 276.35; found 276.07.

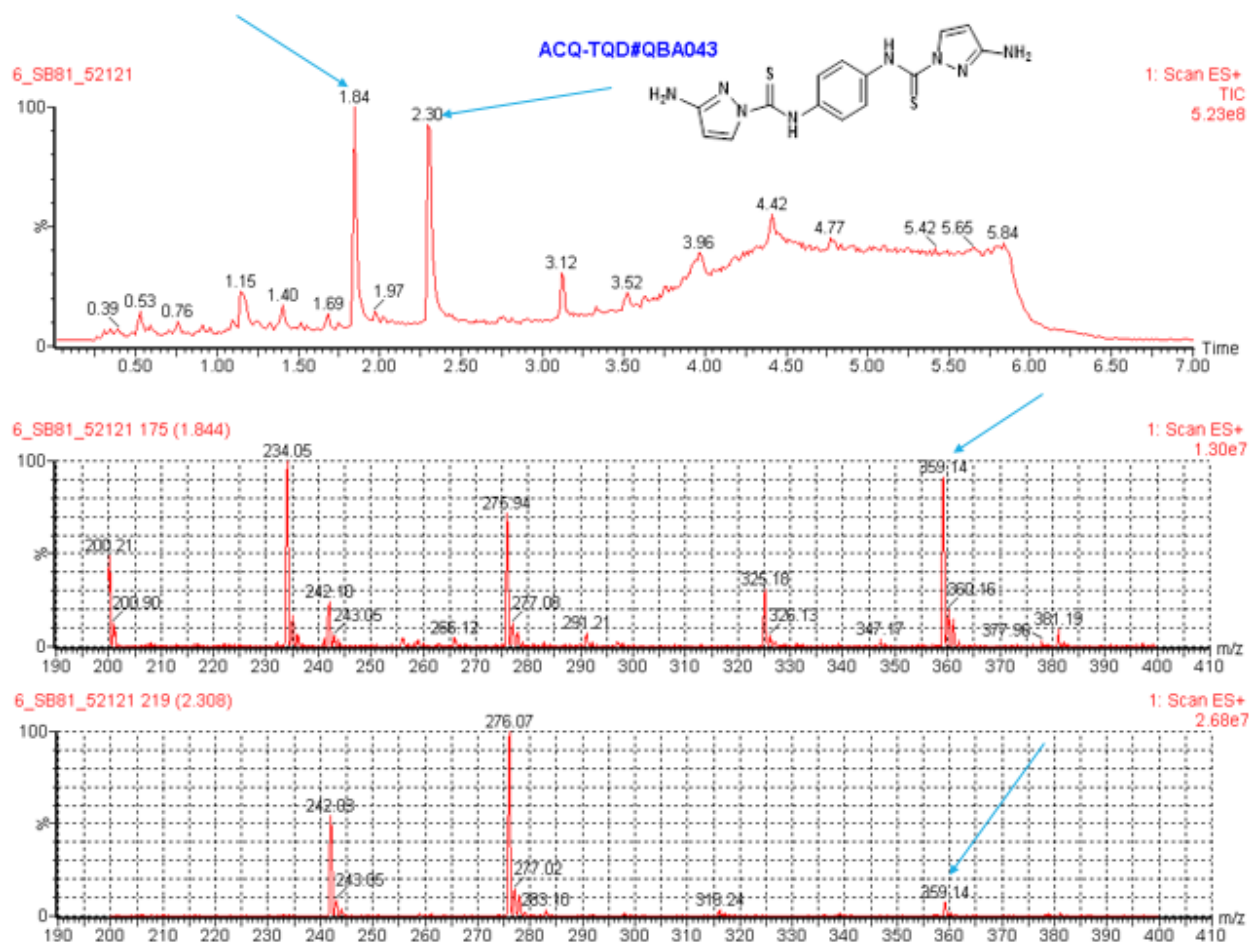


Figure 34, UPLC-MS of SB81: Flow rate 0.8 mL/min using water and acetonitrile in a gradient from 5% to 80% acetonitrile. UPLC-MS [M+1] calcd for $C_{14}H_{14}N_8S_2$ 359.44; found 359.14.

Mass spectrometry characterization is desired as it gives much needed information about the molecular weight of the sample and is needed to confirm that the desired product was formed. It would be ideal for all samples to be characterized by HRMS; however, this is a very expensive test and requires outsourcing to the University of Kansas. For this reason, some samples were run using UPLC-MS instead because this machine is readily available in the Kansas State University Chemistry Department. UPLC-MS was used to characterize 21 NNSN compounds, namely SB56, SB59, SB78, SB79, SB80, SB81, SB82, SB84, SB105, SB106, SB107, SB108, SB127, SB128, SB135, SB136, SB137, SB138, SB139, and SB140. As

can be noted in **Figure 33**, the major peak (at 2.21 minutes retention time) corresponds to the desired product mass. This is a strong indication that the desired product is present in large amounts. The same is noted in **Figure 34**, however, in this case, there are two retention times that correspond to the desired product mass, both of which are the major peaks (at 1.84 minutes and 2.30 minutes retention time, respectively). Both HRMS and UPLC-MS are highly sensitive characterization methods and meet the standards required to confirm the structures, especially when combined with the other characterization methods already discussed.

The NMR spectra for KE03 are shown in both proton and carbon-13 in **Figure 35** and **Figure 36**, respectively. For full details on NMR and IR spectra of the 88 NNSN compounds that I personally synthesized, see Appendix B.

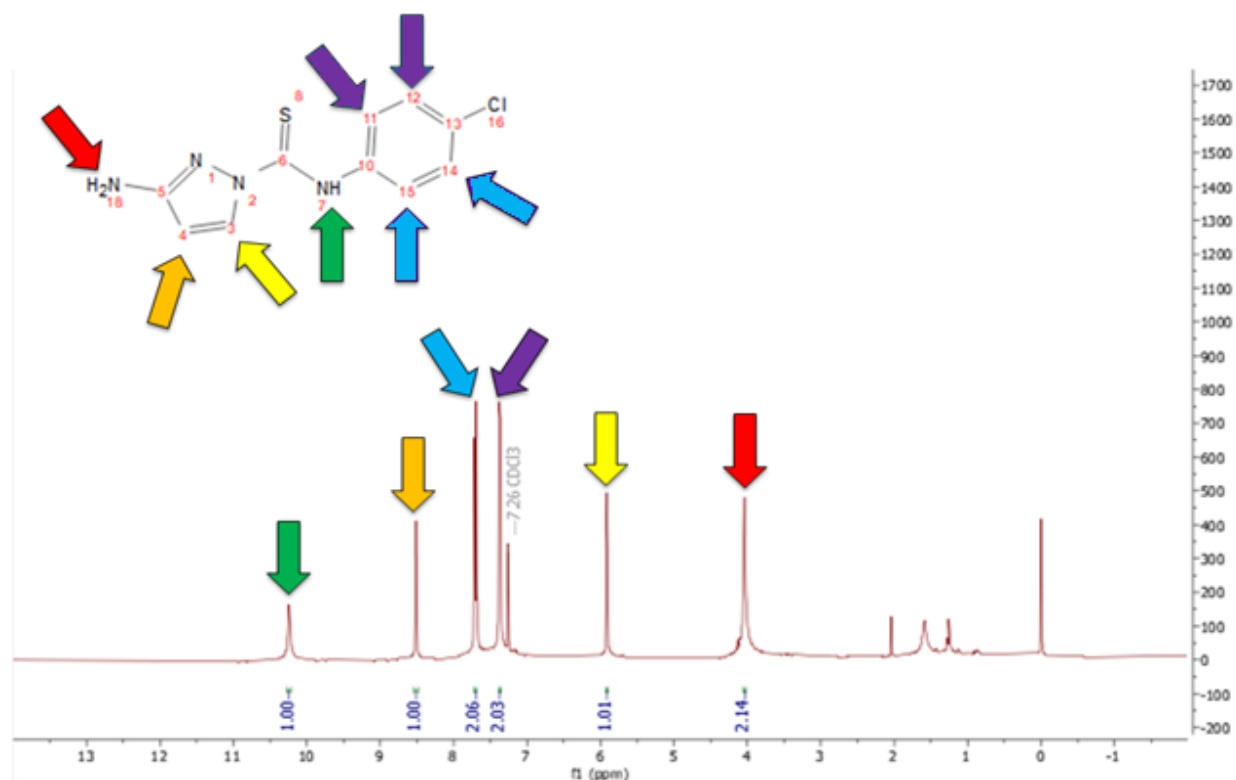


Figure 35, ^1H NMR of KE03.

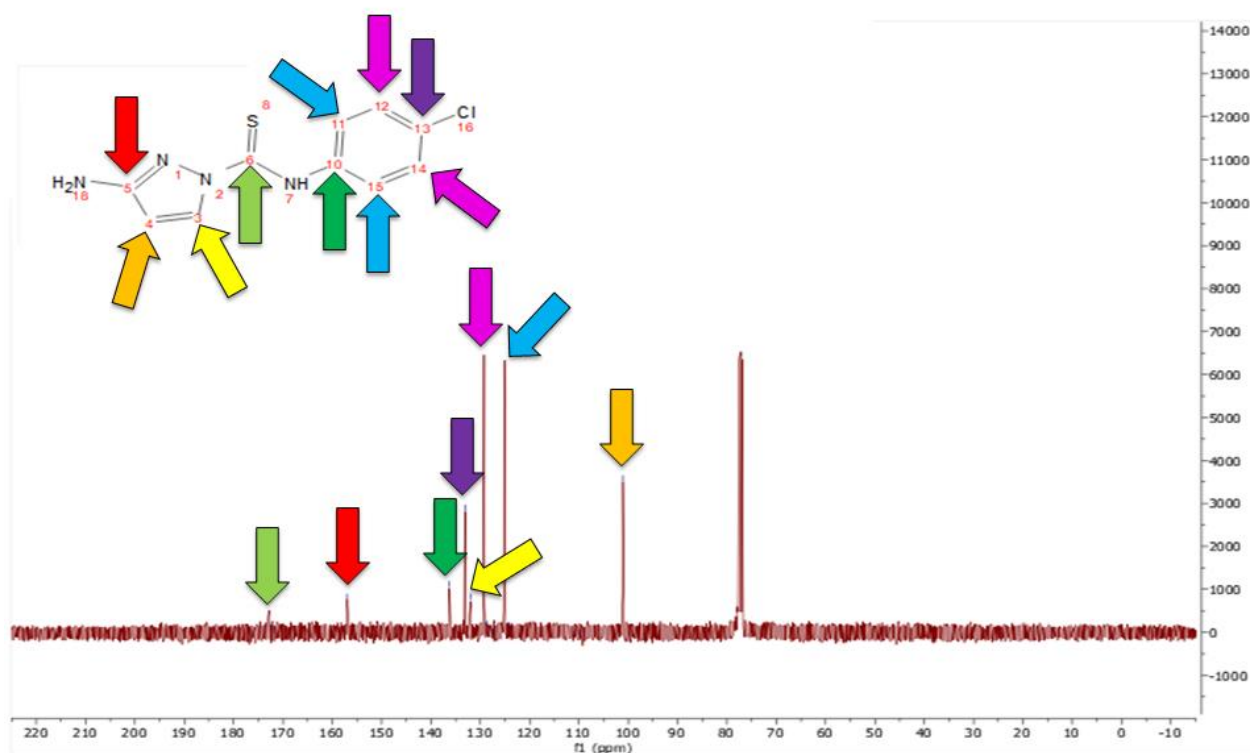
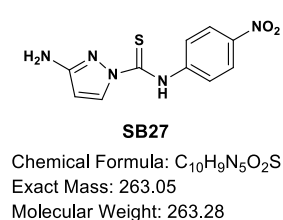
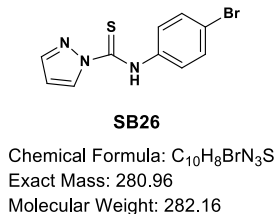
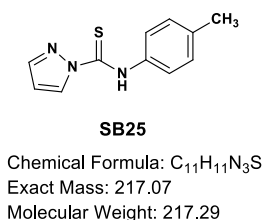
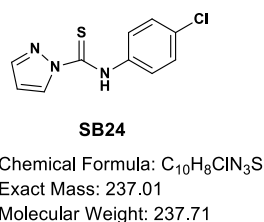
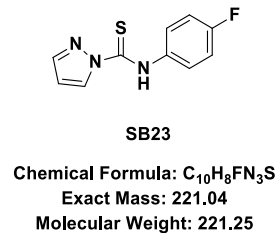
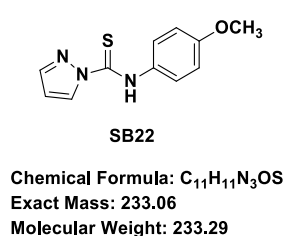
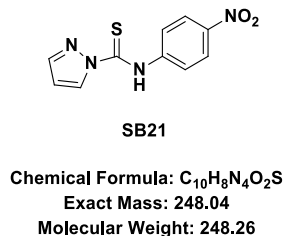
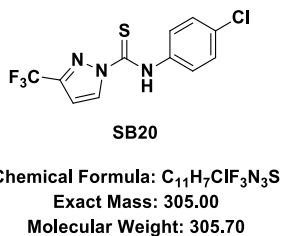
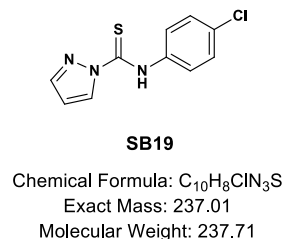
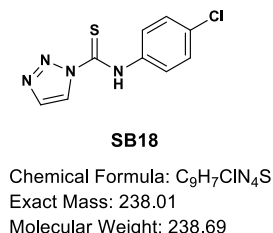
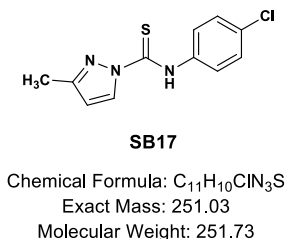
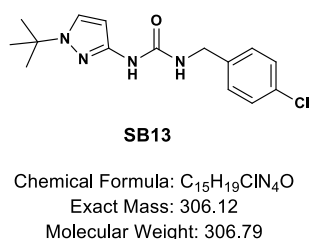
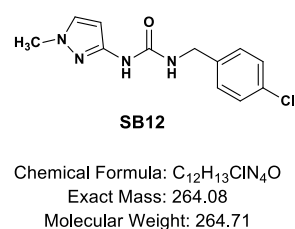
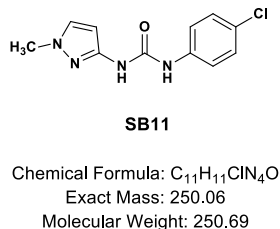
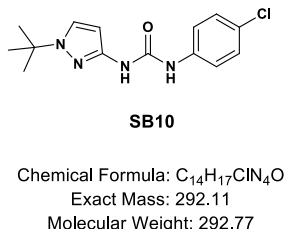
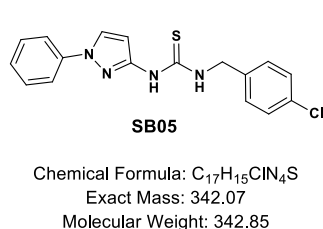
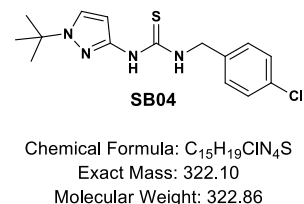
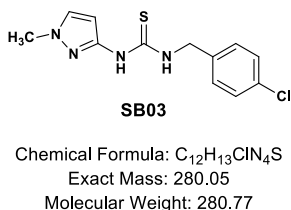
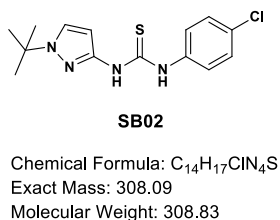
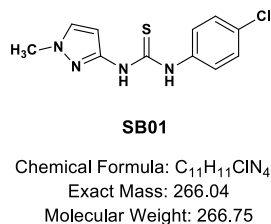
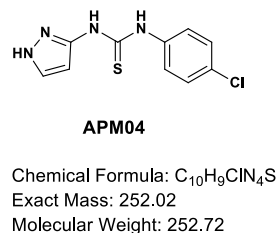
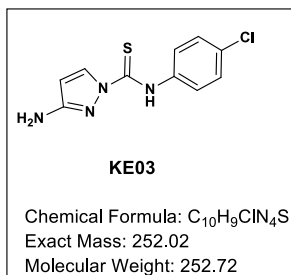
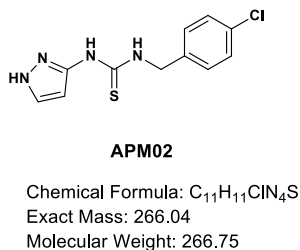
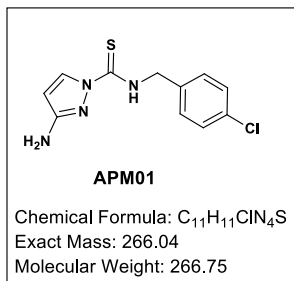
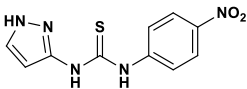


Figure 36, ^{13}C NMR of KE03.

NNSN Library

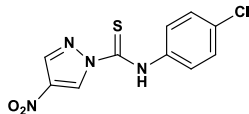
The entire NNSN Library consists of 134 derivatives, the structures of which are included in **Figure 37**. Of these, there are multiple types of compounds that have been broken down into categories. From those that I have personally synthesized (88 out of 134), marked in **Figure 37** with boxes), the categories include monochlorinated, dichlorinated, Bis-(α -chlorophenyl)thioureido-1H-pyrazole-thioamides, di-isothiocyanates, Bis-1H-pyrazole-1-carbothioamides, and some derivatives where 3-amino-N-(4-chlorophenyl)-1H-pyrazole-1-carbothioamide (KE03) was combined with the components of peptide nanosponges, which were described in the “Peptide Nanosponges” section of Chapter 1.





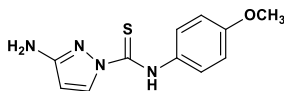
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Exact Mass: 263.05
Molecular Weight: 263.28



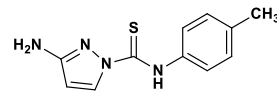
SB30

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Exact Mass: 282.00
Molecular Weight: 282.71



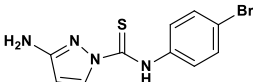
SB31

Chemical Formula: $C_{11}H_{12}N_4OS$
Exact Mass: 248.07
Molecular Weight: 248.30



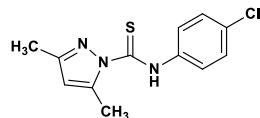
SB32

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Exact Mass: 232.08
Molecular Weight: 232.30



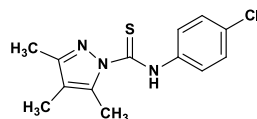
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Molecular Weight: 297.17



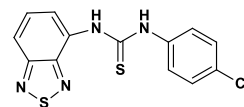
SB34

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Molecular Weight: 265.76



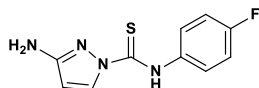
SB35

Chemical Formula: $C_{13}H_{14}ClN_3S$
Exact Mass: 279.06
Molecular Weight: 279.79



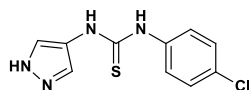
SB36

Chemical Formula: $C_{13}H_9ClN_4S_2$
Exact Mass: 320.00
Molecular Weight: 320.82



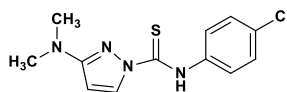
SB37

Chemical Formula: $C_{10}H_9FN_4S$
Exact Mass: 236.05
Molecular Weight: 236.27



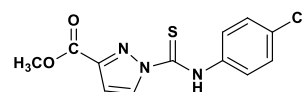
SB39

Chemical Formula: $C_{10}H_9ClN_4S$
Exact Mass: 252.02
Molecular Weight: 252.72



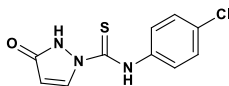
SB40

Chemical Formula: $C_{12}H_{13}ClN_4S$
Exact Mass: 280.05
Molecular Weight: 280.78



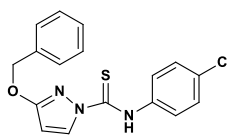
SB42

Chemical Formula: $C_{12}H_{10}ClN_3O_2S$
Exact Mass: 295.02
Molecular Weight: 295.74



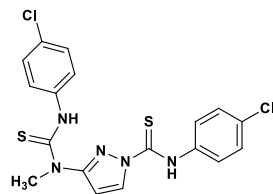
SB43

Chemical Formula: $C_{10}H_8ClN_3OS$
Exact Mass: 253.01
Molecular Weight: 253.71



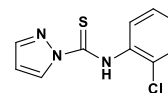
SB44

Chemical Formula: $C_{17}H_{14}ClN_3OS$
Exact Mass: 343.05
Molecular Weight: 343.83



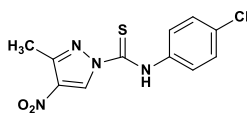
SB45

Chemical Formula: $C_{18}H_{15}Cl_2N_5S_2$
Exact Mass: 435.01
Molecular Weight: 436.38



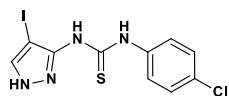
SB46

Chemical Formula: $C_{10}H_8ClN_3S$
Exact Mass: 237.01
Molecular Weight: 237.71



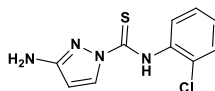
SB47

Chemical Formula: $C_{11}H_9ClN_4O_2S$
Exact Mass: 296.01
Molecular Weight: 296.73



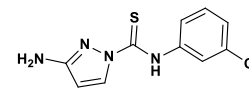
SB48

Chemical Formula: $C_{10}H_8ClN_4S$
Exact Mass: 377.92
Molecular Weight: 378.62



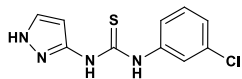
SB49

Chemical Formula: $C_{10}H_9ClN_4S$
Exact Mass: 252.02
Molecular Weight: 252.72



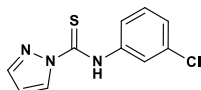
SB50

Chemical Formula: $C_{10}H_9ClN_4S$
Exact Mass: 252.02
Molecular Weight: 252.72



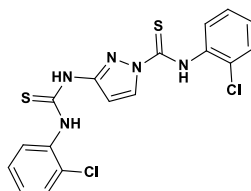
SB51

Chemical Formula: $C_{10}H_9ClN_4S$
Exact Mass: 252.02
Molecular Weight: 252.72



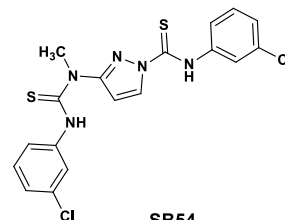
SB52

Chemical Formula: $C_{10}H_8ClN_3S$
Exact Mass: 237.01
Molecular Weight: 237.71



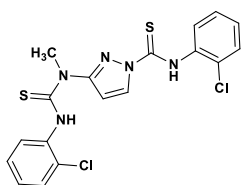
SB53

Chemical Formula: $C_{17}H_{13}Cl_2N_5S_2$
Exact Mass: 421.00
Molecular Weight: 422.35



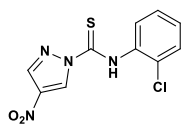
SB54

Chemical Formula: $C_{18}H_{15}Cl_2N_5S_2$
Exact Mass: 435.01
Molecular Weight: 436.38



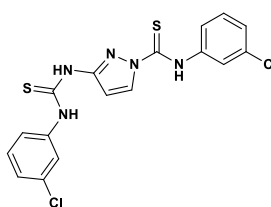
SB55

Chemical Formula: $C_{18}H_{15}Cl_2N_5S_2$
Exact Mass: 435.01
Molecular Weight: 436.38



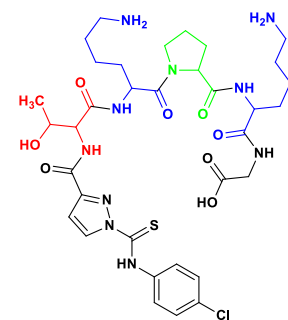
SB56

Chemical Formula: $C_{10}H_7ClN_4O_2S$
Exact Mass: 282.00
Molecular Weight: 282.71



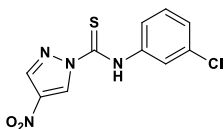
SB57

Chemical Formula: $C_{17}H_{13}Cl_2N_5S_2$
Exact Mass: 421.00
Molecular Weight: 422.35



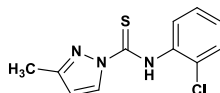
SB58

Chemical Formula: $C_{34}H_{49}ClN_{10}O_8S$
Exact Mass: 792.31
Molecular Weight: 793.33



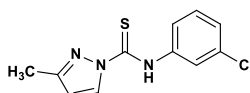
SB59

Chemical Formula: $C_{10}H_7ClN_4O_2S$
Exact Mass: 282.00
Molecular Weight: 282.71



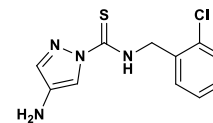
SB60

Chemical Formula: $C_{11}H_{10}ClN_3S$
Exact Mass: 251.03
Molecular Weight: 251.74



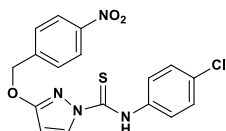
SB61

Chemical Formula: $C_{11}H_{10}ClN_3S$
Exact Mass: 251.03
Molecular Weight: 251.74



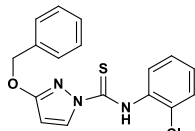
SB62

Chemical Formula: $C_{11}H_{11}ClN_4S$
Exact Mass: 266.04
Molecular Weight: 266.75



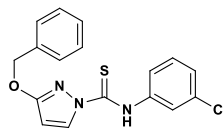
SB63

Chemical Formula: $C_{17}H_{13}ClN_4O_3S$
Exact Mass: 388.04
Molecular Weight: 388.83



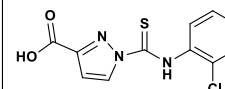
SB64

Chemical Formula: $C_{17}H_{14}ClN_3OS$
Exact Mass: 343.05
Molecular Weight: 343.83



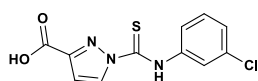
SB65

Chemical Formula: $C_{17}H_{14}ClN_3OS$
Exact Mass: 343.05
Molecular Weight: 343.83



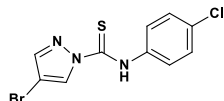
SB66

Chemical Formula: $C_{11}H_8ClN_3O_2S$
Exact Mass: 281.00
Molecular Weight: 281.72



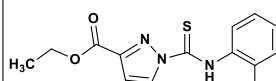
SB67

Chemical Formula: $C_{11}H_8ClN_3O_2S$
Exact Mass: 281.00
Molecular Weight: 281.72



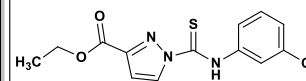
SB68

Chemical Formula: $C_{10}H_7BrClN_3S$
Exact Mass: 314.92
Molecular Weight: 316.60



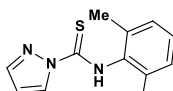
SB69

Chemical Formula: $C_{13}H_{12}ClN_3O_2S$
Exact Mass: 309.03
Molecular Weight: 309.77



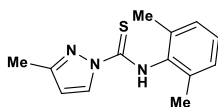
SB70

Chemical Formula: $C_{13}H_{12}ClN_3O_2S$
Exact Mass: 309.03
Molecular Weight: 309.77



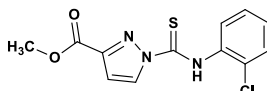
SB71

Chemical Formula: $C_{12}H_{13}N_3S$
Exact Mass: 231.08
Molecular Weight: 231.32



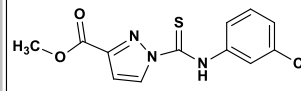
SB72

Chemical Formula: $C_{13}H_{15}N_3S$
Exact Mass: 245.10
Molecular Weight: 245.34



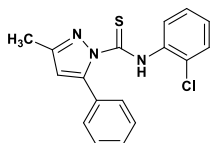
SB73

Chemical Formula: $C_{12}H_{10}ClN_3O_2S$
Exact Mass: 295.02
Molecular Weight: 295.74



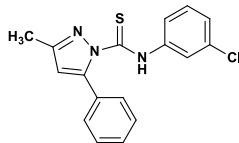
SB74

Chemical Formula: $C_{12}H_{10}ClN_3O_2S$
Exact Mass: 295.02
Molecular Weight: 295.74



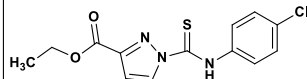
SB75

Chemical Formula: $C_{17}H_{14}ClN_3S$
Exact Mass: 327.06
Molecular Weight: 327.83



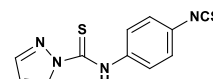
SB76

Chemical Formula: $C_{17}H_{14}ClN_3S$
Exact Mass: 327.06
Molecular Weight: 327.83



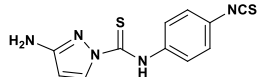
SB77

Chemical Formula: $C_{13}H_{12}ClN_3O_2S$
Exact Mass: 309.03
Molecular Weight: 309.77



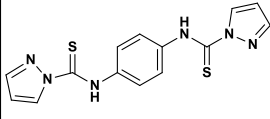
SB78

Chemical Formula: $C_{11}H_8N_4S_2$
Exact Mass: 260.02
Molecular Weight: 260.34



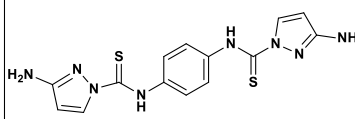
SB79

Chemical Formula: $C_{11}H_9N_5S_2$
Exact Mass: 275.03
Molecular Weight: 275.35



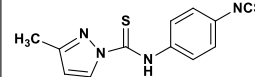
SB80

Chemical Formula: $C_{14}H_{12}N_6S_2$
Exact Mass: 328.06
Molecular Weight: 328.42



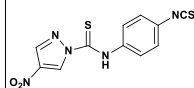
SB81

Chemical Formula: $C_{14}H_{14}N_8S_2$
Exact Mass: 358.08
Molecular Weight: 358.44



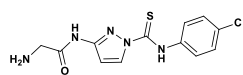
SB82

Chemical Formula: $C_{12}H_{10}N_4S_2$
Exact Mass: 274.03
Molecular Weight: 274.36



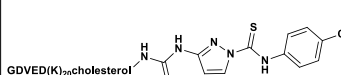
SB83

Chemical Formula: $C_{11}H_7N_5O_2S_2$
Exact Mass: 305.00
Molecular Weight: 305.34



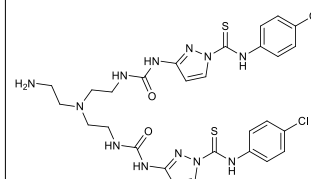
SB84

2-amino-N-(1-(4-chlorophenyl)carbamothioyl)-1H-pyrazol-3-yl)acetamide
Chemical Formula: $C_{12}H_{12}ClN_5OS$
Exact Mass: 309.05
Molecular Weight: 309.77



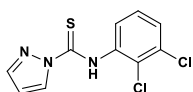
SB85

Molecular weight, assuming 2 APM03
3978.04



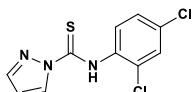
SB86

Chemical Formula: $C_{28}H_{32}Cl_2N_{12}O_2S_2$
Exact Mass: 702.16
Molecular Weight: 703.67



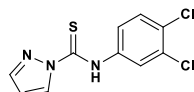
SB87

Chemical Formula: $C_{10}H_7Cl_2N_3S$
Exact Mass: 270.97
Molecular Weight: 272.15



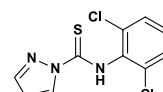
SB88

Chemical Formula: $C_{10}H_7Cl_2N_3S$
Exact Mass: 270.97
Molecular Weight: 272.15



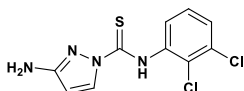
SB89

Chemical Formula: $C_{10}H_7Cl_2N_3S$
Exact Mass: 270.97
Molecular Weight: 272.15



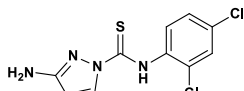
SB90

Chemical Formula: $C_{10}H_7Cl_2N_3S$
Exact Mass: 270.97
Molecular Weight: 272.15



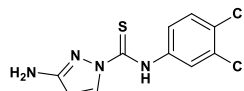
SB91

Chemical Formula: $C_{10}H_8Cl_2N_4S$
Exact Mass: 285.98
Molecular Weight: 287.17



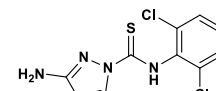
SB92

Chemical Formula: $C_{10}H_8Cl_2N_4S$
Exact Mass: 285.98
Molecular Weight: 287.17



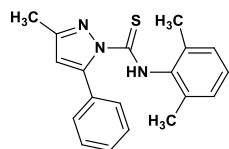
SB93

Chemical Formula: $C_{10}H_8Cl_2N_4S$
Exact Mass: 285.98
Molecular Weight: 287.17



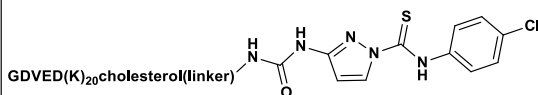
SB94

Chemical Formula: $C_{10}H_8Cl_2N_4S$
Exact Mass: 285.98
Molecular Weight: 287.17



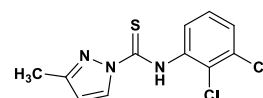
SB95

Chemical Formula: $C_{19}H_{19}N_3S$
Exact Mass: 321.13
Molecular Weight: 321.44



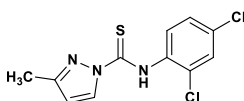
SB96

Assumed molecular weight, assuming 6 APM03
12318.68



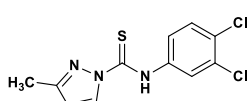
SB97

Chemical Formula: $C_{11}H_9Cl_2N_3S$
Exact Mass: 284.99
Molecular Weight: 286.18



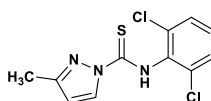
SB98

Chemical Formula: $C_{11}H_9Cl_2N_3S$
Exact Mass: 284.99
Molecular Weight: 286.18



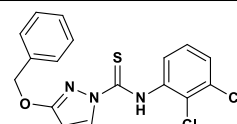
SB99

Chemical Formula: $C_{11}H_9Cl_2N_3S$
Exact Mass: 284.99
Molecular Weight: 286.18



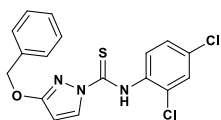
SB100

Chemical Formula: $C_{11}H_9Cl_2N_3S$
Exact Mass: 284.99
Molecular Weight: 286.18



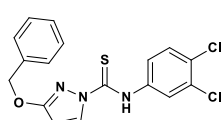
SB101

Chemical Formula: $C_{17}H_{13}Cl_2N_3OS$
Exact Mass: 377.02
Molecular Weight: 378.28



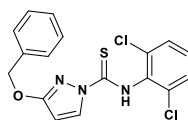
SB102

Chemical Formula: $C_{17}H_{13}Cl_2N_3OS$
Exact Mass: 377.02
Molecular Weight: 378.28



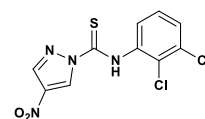
SB103

Chemical Formula: $C_{17}H_{13}Cl_2N_3OS$
Exact Mass: 377.02
Molecular Weight: 378.28



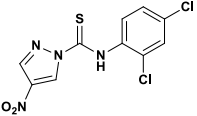
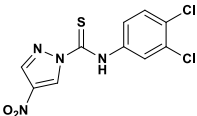
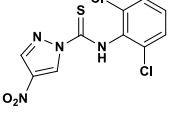
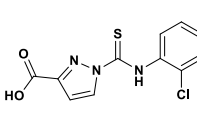
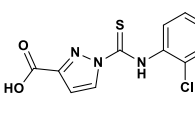
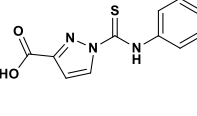
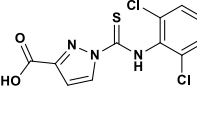
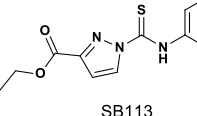
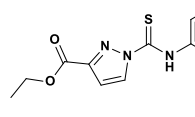
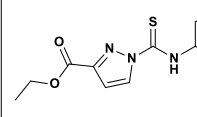
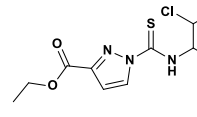
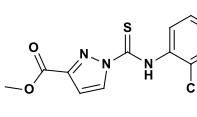
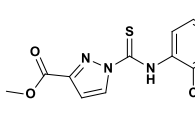
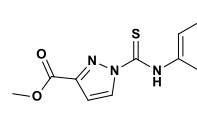
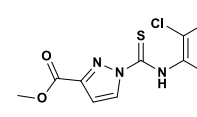
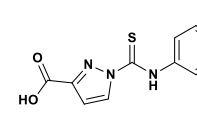
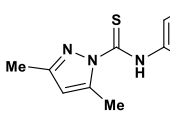
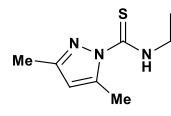
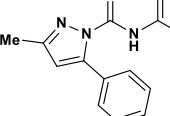
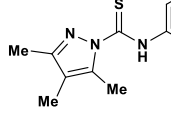
SB104

Chemical Formula: $C_{17}H_{13}Cl_2N_3OS$
Exact Mass: 377.02
Molecular Weight: 378.28



SB105

Chemical Formula: $C_{10}H_6Cl_2N_4O_2S$
Exact Mass: 315.96
Molecular Weight: 317.15

 SB106 Chemical Formula: $C_{10}H_6Cl_2N_4O_2S$ Exact Mass: 315.96 Molecular Weight: 317.15	 SB107 Chemical Formula: $C_{10}H_6Cl_2N_4O_2S$ Exact Mass: 315.96 Molecular Weight: 317.15	 SB108 Chemical Formula: $C_{10}H_6Cl_2N_4O_2S$ Exact Mass: 315.96 Molecular Weight: 317.15	 SB109 Chemical Formula: $C_{11}H_7Cl_2N_3O_2S$ Exact Mass: 314.96 Molecular Weight: 316.16
 SB110 Chemical Formula: $C_{11}H_7Cl_2N_3O_2S$ Exact Mass: 314.96 Molecular Weight: 316.16	 SB111 Chemical Formula: $C_{11}H_7Cl_2N_3O_2S$ Exact Mass: 314.96 Molecular Weight: 316.16	 SB112 Chemical Formula: $C_{11}H_7Cl_2N_3O_2S$ Exact Mass: 314.96 Molecular Weight: 316.16	 SB113 Chemical Formula: $C_{13}H_{11}Cl_2N_3O_2S$ Exact Mass: 342.99 Molecular Weight: 344.22
 SB114 Chemical Formula: $C_{13}H_{11}Cl_2N_3O_2S$ Exact Mass: 342.99 Molecular Weight: 344.22	 SB115 Chemical Formula: $C_{13}H_{11}Cl_2N_3O_2S$ Exact Mass: 342.99 Molecular Weight: 344.22	 SB116 Chemical Formula: $C_{13}H_{11}Cl_2N_3O_2S$ Exact Mass: 342.99 Molecular Weight: 344.22	 SB117 Chemical Formula: $C_{12}H_9Cl_2N_3O_2S$ Exact Mass: 328.98 Molecular Weight: 330.19
 SB118 Chemical Formula: $C_{12}H_9Cl_2N_3O_2S$ Exact Mass: 328.98 Molecular Weight: 330.19	 SB119 Chemical Formula: $C_{12}H_9Cl_2N_3O_2S$ Exact Mass: 328.98 Molecular Weight: 330.19	 SB120 Chemical Formula: $C_{12}H_9Cl_2N_3O_2S$ Exact Mass: 328.98 Molecular Weight: 330.19	 SB121 Chemical Formula: $C_{11}H_8ClN_3O_2S$ Exact Mass: 281.00 Molecular Weight: 281.72
 SB122 Chemical Formula: $C_{12}H_{12}ClN_3S$ Exact Mass: 265.04 Molecular Weight: 265.76	 SB123 Chemical Formula: $C_{12}H_{12}ClN_3S$ Exact Mass: 265.04 Molecular Weight: 265.76	 SB124 Chemical Formula: $C_{17}H_{14}ClN_3S$ Exact Mass: 327.06 Molecular Weight: 327.83	 SB125 Chemical Formula: $C_{13}H_{14}ClN_3S$ Exact Mass: 279.06 Molecular Weight: 279.79

Gram-positive or Gram-negative, and gram-negative bacteria *E. coli* can also be killed by NNSN molecules SB85 and SB96, where KE03 was coupled to K₂₀ peptide and peptide nanosponge, respectively. The MIC values of select NNSNs against these bacteria are shown in **Table 2**.

NNSN compounds were tested against *S. aureus* strain S. A. Newman, a clinical isolate. MIC testing was performed in the group of Prof. Dr. Frank Wolschendorf and since 2018 in the group of Prof. Dr. Olaf Kutsch in the Departments of Medicine and Microbiology at the University of Alabama at Birmingham. The procedure for MIC testing is described in detail in reference 6. A direct quote from this reference is as follows: “For the assays, compounds and the indicated metals salts were mixed in RPMI medium without phenol red (Corning) and then distributed or further diluted in 96-well plates to reach final concentrations as indicated. Metal concentrations and BCS were kept constant from well to well, at 50 μ M, while compound concentrations in DMSO were titrated in 2-fold increments down from 10 mM unless indicated otherwise. Bacteria harvested from exponentially growing cultures were first washed and diluted in RPMI and then distributed to each well for a final assay OD₆₀₀ of 0.005 ($\sim 5 \times 10^6$ cells per ml). Sterility controls and no compound controls containing either only media or media supplemented with 50 μ M CuSO₄ $\times$ 6 H₂O (Acros) were present on each challenge plate. Plates were sealed with parafilm or placed in a sealed plastic bag to reduce evaporation and edge effects and kept at 37° C overnight after which OD₆₀₀ readings were taken on a plate reader (Cytation 3, BioTek). Values were blank subtracted and normalized to the appropriate no drug controls. MIC was defined as the lowest concentration at which growth was reduced by at least 90% in comparison to the untreated controls.” (quoted verbatim from reference 6) MIC was determined by addition of resazurin, a metabolic indicator, using 10% conversion of a blank subtracted and normalized value as the cutoff.

The wavelength of $\lambda=600$ nm was selected for these assays because the light scattering from growing *S. Aureus* cultures can be detected there. Note that CuSO_4 and CuCl_2 boosted the activity of selected NNSN compounds in an identical manner. The presence copper was ascertained by complexation with bathocuproine disulfonic acid and monitored by measuring the absorption at 480 nm. Cu(II) salts were use here, because they dissolve much better in RPMI medium and can be utilized in an oxygenated atmosphere. However, copper that is taken up in an organism (mammals or bacteria) will be immediately reduced to copper (I). Staining of isolated *S. Aureus* bacteria with bathocuproine disulfonic acid failed, because there is no copper(II) in the bacteria. As shown in **Figure 38** below, *S. A. Newman* and *S. A. Lac* grow in RPMI medium in the absence and presence of $50\mu\text{M}$ of $\text{CuSO}_4 \times 6 \text{ H}_2\text{O}$.

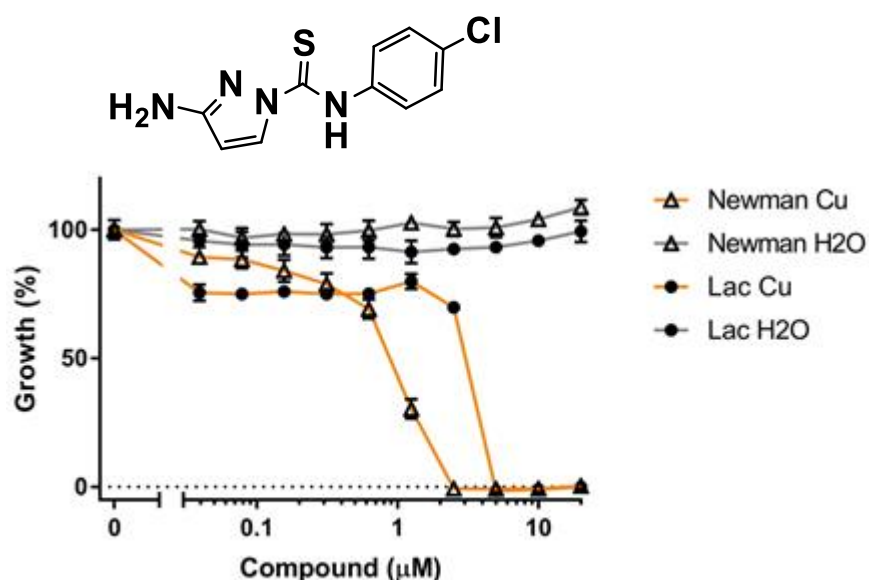
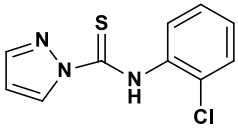
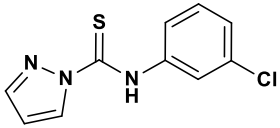
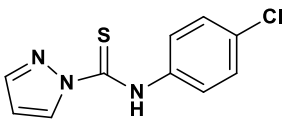
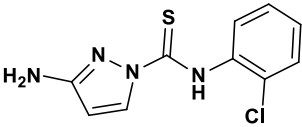
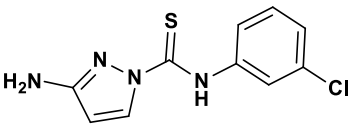
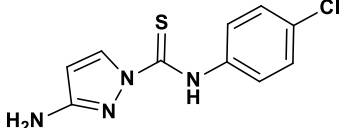
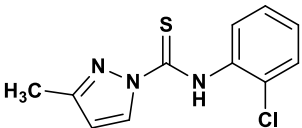
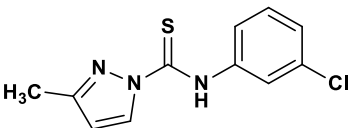
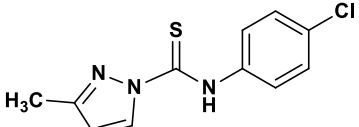
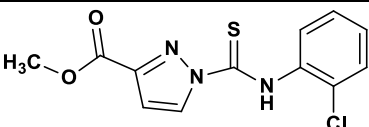
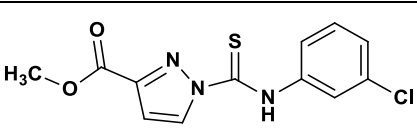
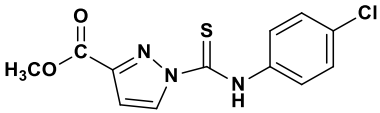
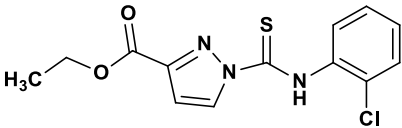
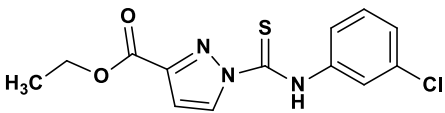
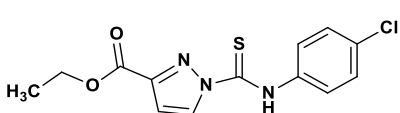
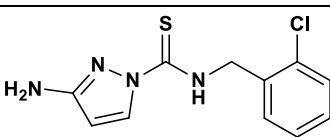
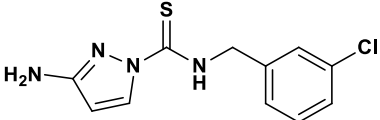
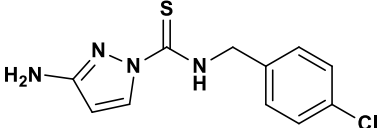
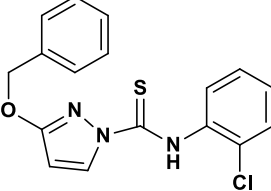
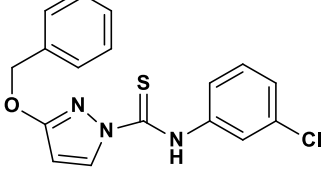
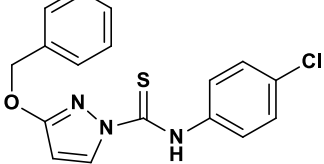
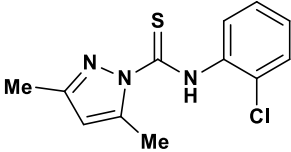
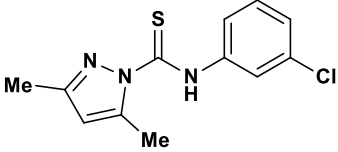
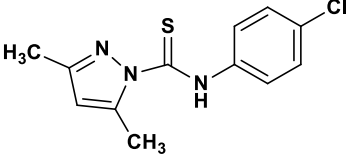
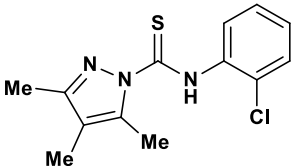
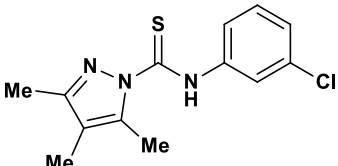
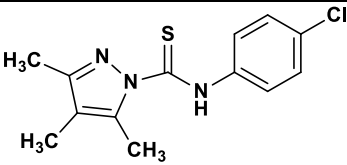
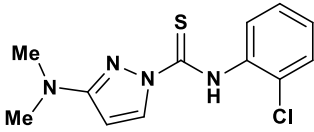
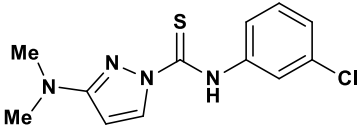
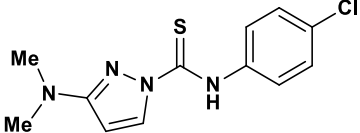
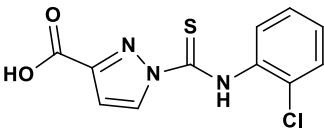
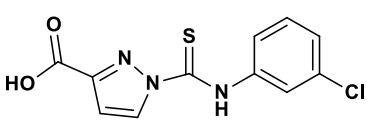


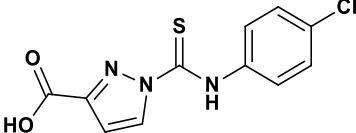
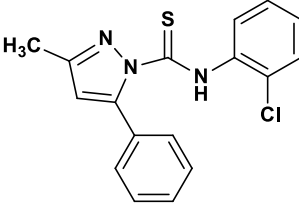
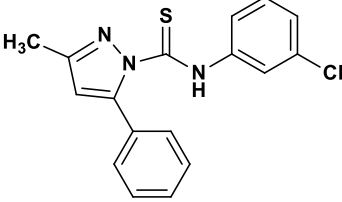
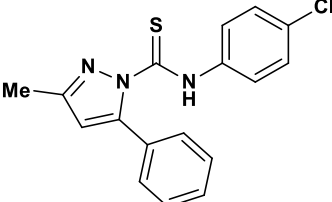
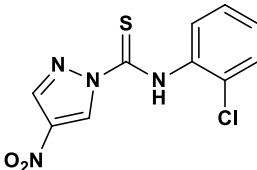
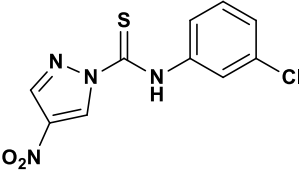
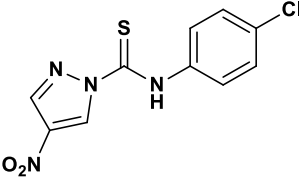
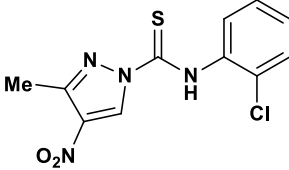
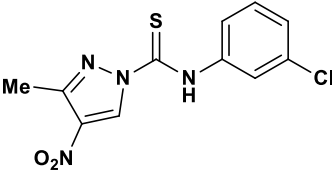
Figure 38, Dose response curves were determined by diluting 3-amino-N-(4-chlorophenyl)-1H-pyrazole-1-carbothioamide (KE03) in RPMI medium with or without $50\mu\text{M}$ copper sulfate. A logarithmic phase culture of *S. aureus* grown in Mueller Hinton broth was washed in RPMI, then diluted in fresh RPMI and added to plates for a final optical density of 0.005. The plates were incubated overnight at 37°C . Metabolic activity was determined by adding resazurin using 10% conversion of a blank subtracted and normalized value as the cutoff.

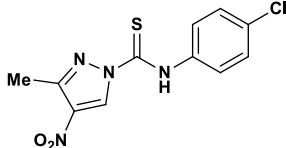
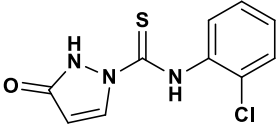
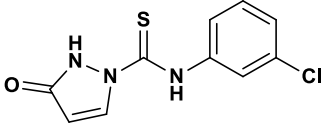
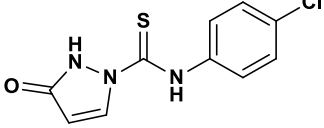
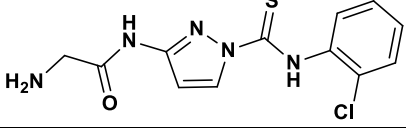
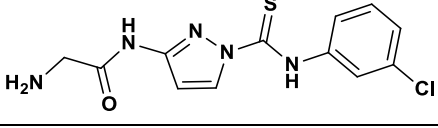
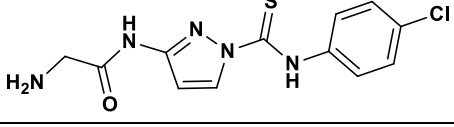
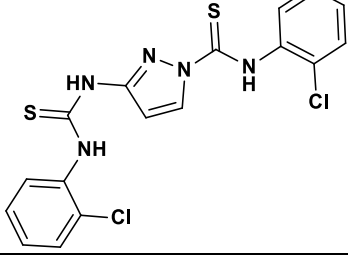
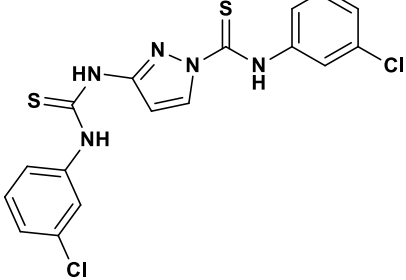
The same protocols were followed at the University of Alabama at Birmingham using *E. Coli* (W3110, lab-adjusted) strain for the MIC determinations.

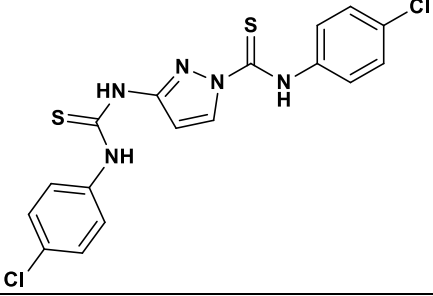
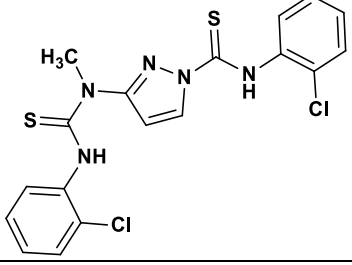
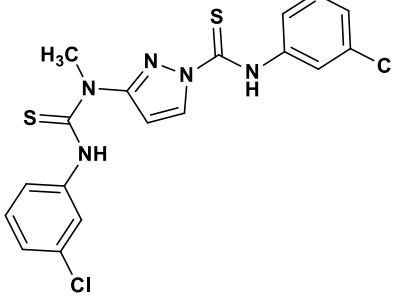
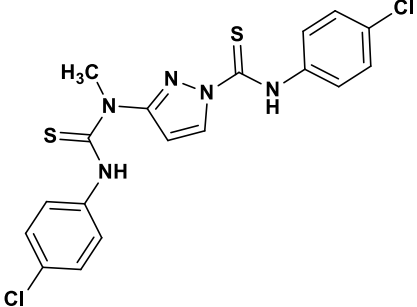
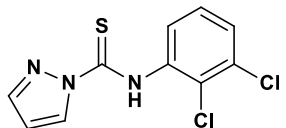
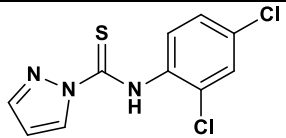
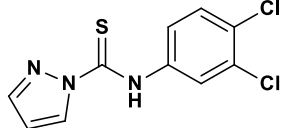
NNSN Code Name	Structure	MIC against MSSA (SA Newman)	MIC against E. coli
	SB46	0.3 μ M	--
	SB52	2.5 μ M	--
	SB24	5 μ M	--
	SB49	0.3 μ M	--
	SB50	0.15 μ M	--
	KE03	0.07 μ M	--
	SB60	1.25 μ M	--
	SB61	Pending	--
	SB17	1.25 μ M	--
	SB73	5 μ M	--
	SB74	2.5 μ M	--

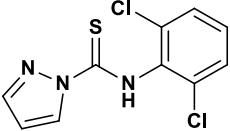
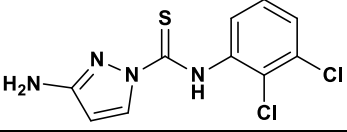
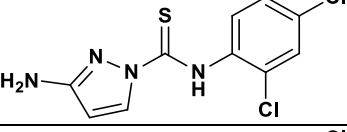
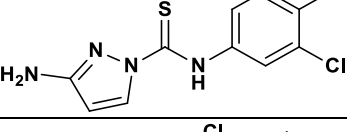
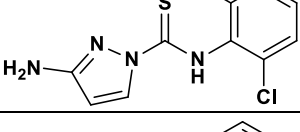
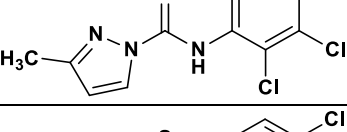
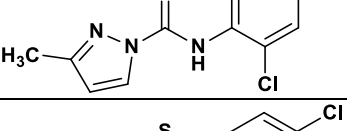
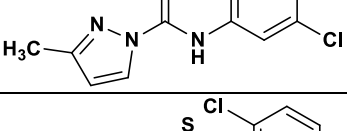
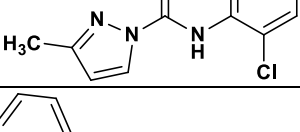
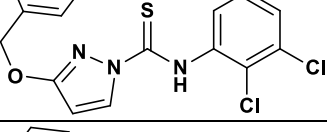
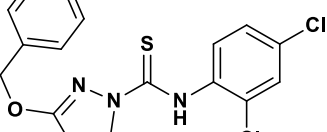
	SB42	10 μ M	--
	SB69	>10 μ M	--
	SB70	>10 μ M	--
	SB77	0.6 μ M	--
	SB62	5 μ M	--
	SB133	Pending	--
	APM01	10 μ M	--
	SB64	0.6 μ M	--
	SB65	2.5 μ M	--
	SB44	0.15 μ M	--

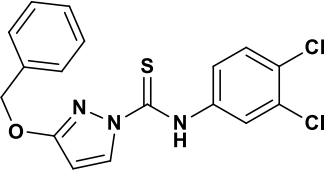
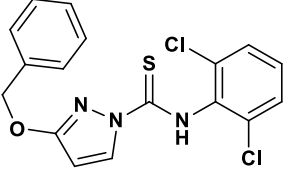
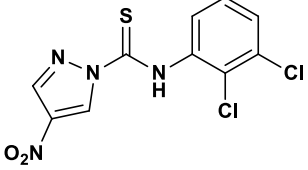
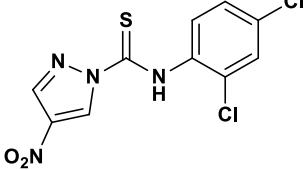
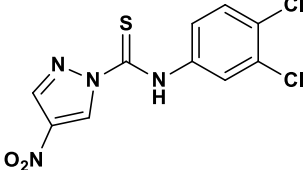
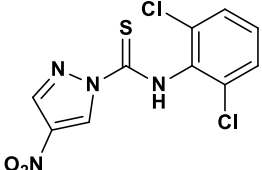
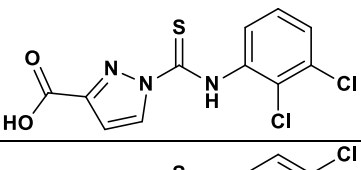
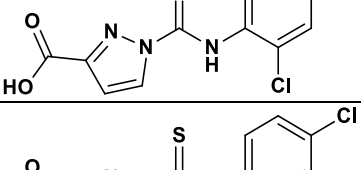
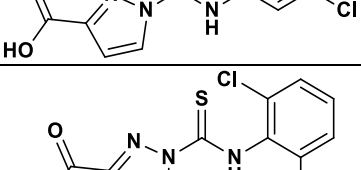
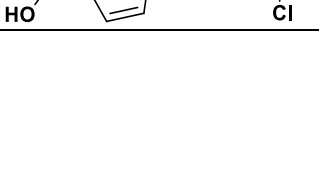
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	SB123	Pending	--
	SB34	2.66 μM	--
	SB125	>10 μM	--
	SB126	Pending	--
	SB35	>10 μM	--
	SB129	1.25 μM	--
	SB130	10 μM	--
	SB40	0.6 μM	--
	SB66	2.5 μM	--
	SB67	10 μM	--

	SB121	5 μ M	--
	SB75	>10 μ M	--
	SB76	>10 μ M	--
	SB124	>10 μ M	--
	SB56	5 μ M	--
	SB59	>10 μ M	--
	SB38	5 μ M	--
	SB127	Pending	--
	SB128	Pending	--

	SB47	2.5 μ M	--
	SB131	Pending	--
	SB132	Pending	--
	SB43	5 μ M	--
	SB135	Pending	--
	SB136	Pending	--
	SB84	2.5 μ M	>10
	SB53	0.3 μ M	--
	SB57	Pending	--

	SB134	Pending	--
	SB55	Pending	--
	SB54	Pending	--
	SB45	<0.016 μ M	--
	SB87	> 10 μ M	>10
	SB88	> 10 μ M	>10
	SB89	> 10 μ M	>10

	SB90	1.25 μ M	>10
	SB91	1.25 μ M	>10
	SB92	0.3 μ M	>10
	SB93	< 0.019 μ M	>10
	SB94	0.3 μ M	>10
	SB97	1.25 μ M	>10
	SB98	2.5 μ M	>10
	SB99	5 μ M	>10
	SB100	0.3 μ M	>10
	SB101	Pending	--
	SB102	Pending	--

	SB103	Pending	--
	SB104	Pending	--
	SB105	Pending	--
	SB106	Pending	--
	SB107	Pending	--
	SB108	Pending	--
	SB109	Pending	--
	SB110	Pending	--
	SB111	Pending	--
	SB112	Pending	--

	SB113	Pending	--
	SB114	Pending	--
	SB115	Pending	--
	SB116	Pending	--
	SB117	Pending	--
	SB118	Pending	--
	SB119	Pending	--
	SB120	Pending	--
	SB137	10 μ M	--
	SB138	1.25 μ M	--
	SB139	0.6 μ M	--

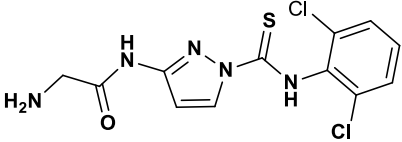
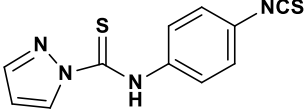
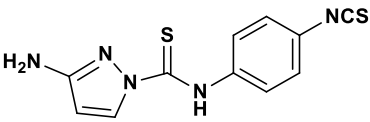
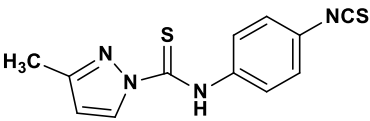
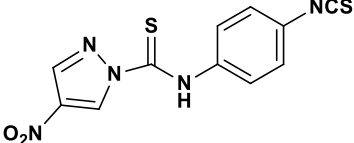
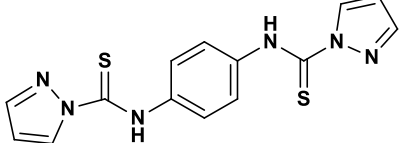
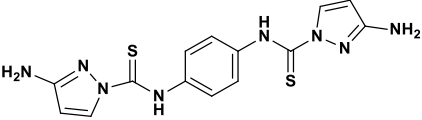
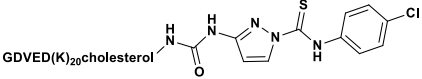
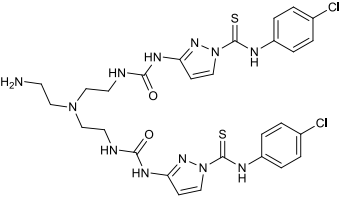
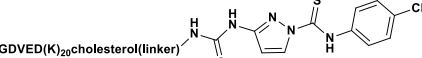
	SB140	1.25 μ M	--
	SB78	>10 μ M	--
	SB79	>10 μ M	--
	SB82	>10 μ M	>10
	SB83	>10 μ M	>10
	SB80	>10 μ M	>10
	SB81	>10 μ M	>10
	SB85	2.5 μ M	5 μ M
	SB86	>10 μ M	>10
	SB96	1.25 μ M	10 μ M

Table 2, MIC values of NNSNs against MSSA and *E. coli*.

Select NNSNs were also tested against *M. tuberculosis* (Avirulent Mtb strain mc²6206 (H37Rv Δ panCD Δ leuCD, double auxotroph) by Mitra Avishek in the Neiderwies group based in the Microbiology Department of the University of Alabama at Birmingham. The conditions involved growing the strain on copper free 7H9 liquid growth medium. The medium was supplemented with 0.2% Casamino Acids, 10% ADS (Albumin, Dextrose, Salt), 0.02% Tyloxapol, 24 μ g/ml D-Pantothenate, and 50 μ g/ml L-Leucine. The cells were then treated with the associated NNSN (either KE03, SB45, SB46, SB64, or SB79) in the absence of copper as well as in the presence of 50 μ M CuSO₄ x 7H₂O. The readout was performed using Alamar blue assay at 530 nm and 590 nm. The results obtained were IC₅₀ values, which is a quantitative measure that indicates how much of a drug is needed to inhibit, *in vitro*, a given biological process or biological component by 50%. These results are shown in **Table 3** below, showing that KE03, SB45, and SB64 could all be worthy candidates for the treatment of MTB.

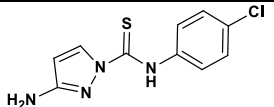
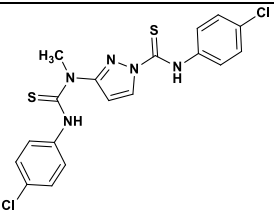
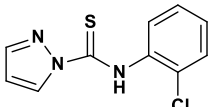
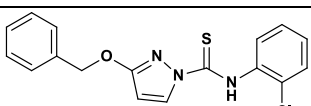
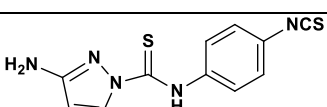
SB#	Structure	IC ₅₀ - Cu	IC ₅₀ + Cu
KE03		>12.5 μ M	0.3 μ M
SB45		>50 μ M	2 μ M
SB46		>100 μ M	10 μ M
SB64		>100 μ M	0.8 μ M
SB79		>100 μ M	>100 μ M

Table 3, IC₅₀ Values of Select NNSNs Against MTB.

Anticancer Activity

Select NNSN molecules KE03, SB40, SB45, SB46, SB57, SB64, SB77, and SB129 have proven to have activity against some cancer cells, the LC₅₀ values of which are shown in **Table 4**. Note that LC₅₀ is the concentration of the chemical that will kill 50% of the given biological process or biological component with a single exposure. The lowest concentration is desired because this will kill the cancer or inhibit bacterial growth most effectively.

To obtain the LC₅₀ values against cancerous cells, fellow Bossmann group researchers Dr. Obdulia Covarrubias-Zambrano and Ivina Mali adhered to the following protocol: 30,000 cells per well were loaded in a 96-well plate and incubated for 24 hours at 37° C. After 24 hours, the medium was aspirated from all wells. For the control wells, 150 µL of respective medium was added. Each NNSN was tested with and without CuSO₄ on these plates. For testing an NNSN with CuSO₄, 50 µM CuSO₄ was loaded into each experimental well (excluding the control wells), followed by 0 µM, 0.5 µM, 1 µM, 2 µM, 3 µM, 4 µM, and 5 µM of each NNSN (which were dissolved in 0.1% DMSO solution). These were incubated at 37° C for 24 hours. After 24 hours, the medium was aspirated from all wells (control and experimental). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added and 110 µL of medium to all wells. This was incubated at 37° C for 4 hours. After 4 hours, 10% Sodium dodecyl sulfate (SDS) in 0.01 M Hydrochloric acid (HCl) (100 µL) was added to all wells. This was incubated overnight at 37° C. The next day, all 96-well plates were read at 550 nm and 690 nm. The cell viability was then calculated using the formula (experimental well/control cells only well) x 100.⁷

The results of this study on select NNSN compounds is shown in **Table 4**. There are five different murine (mouse) cell lines that were tested, including Neural Stem Cells (NSC),

Melanoma (B16F10), Glioblastoma (GL26), and Pancreatic Cancer (Pan02 and KPC). NSCs are a non-cancerous cell line that act as a control for these experiments. As shown, there are multiple candidates that would work well against each cancer type, shown in green. For melanoma (B16F10), the best candidate is SB57 which showed a very low LC₅₀. For GL26, the best candidate was SB45. For PAN02, the best candidate was also SB45. For KPC, the best candidate was SB77, showing an LC₅₀ in the nanomolar range. Those showing dashes were not run due to the cells not being viable and ready to use at the time that these experiments were performed.

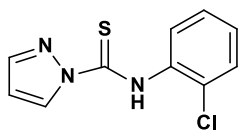
NNSN	NSC		B16F10		GL26		Pan02		KPC	
	- Cu	+ Cu	- Cu	+ Cu	- Cu	+ Cu	- Cu	+ Cu	- Cu	+ Cu
KE03	2.66	1.61	0.74	0.29	12,314	0.28	1,000	0.23	1.62	0.11
SB45	5.50	0.42	0.50	0.10	1.37	0.05	1,380	0.17	0.13	0.15
SB46	0.51	1.71	0.21	0.11	0.10	0.28	0.30	0.21	0.64	0.007
SB64	82,370	0.88	0.1	0.34	1.58	0.17	0.61	0.25	0.55	0.32
SB40	-	-	0.23	0.44	-	-	1.88	1500	0.24	1.42
SB77	-	-	274359	570531	-	-	0.17	5.14	0.0078	0.000026
SB57	-	-	278456	0.001325	-	-	0.012	2320	0.00073	1.699
SB129	-	-	6436	3260	-	-	0.0057	2.28	0.0076	2.197

Table 4, LC₅₀ values (μM) of NNSNs toward cancer cell lines.⁷

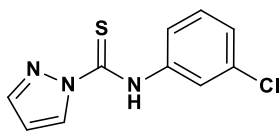
Structure Activity Relationship (SAR) Study

By considering the structure of the NNSN in comparison to the observed activity, a parallel can be drawn that can suggest which parts of the structure are most important for optimal activity. In this study, the activity of interest is the inhibition of Methicillin-sensitive *S. aureus* (MSSA), a potentially deadly bacterial strain that can lead to skin infections, pneumonia, sepsis and even death.⁷ The minimum inhibitory concentration (MIC) is the lowest concentration of a drug that prevents visible growth of a bacteria. The lower the MIC value, the more effective the

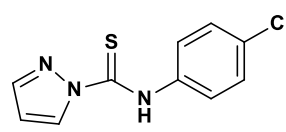
drug is inhibiting the growth of bacteria (MSSA in particular). The values marked in yellow highlight are those that are considered “fair” with an MIC of 5 μ M and those marked in green highlight are “good” with an MIC below 5 μ M.



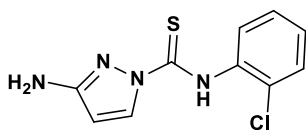
SB46, 0.3 μ M



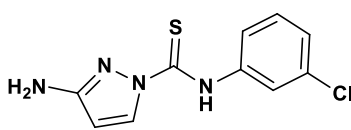
SB52, 2.5 μ M



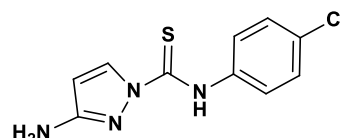
SB24, 5 μ M



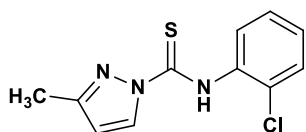
SB49, 0.3 μ M



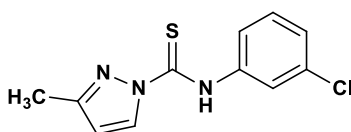
SB50, 0.15 μ M



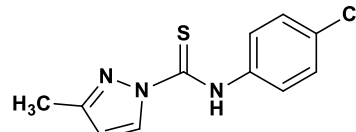
KE03, 0.07 μ M



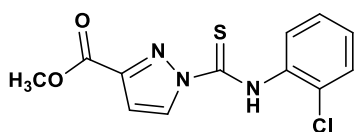
SB60, 1.25 μ M



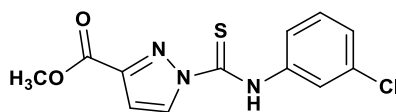
SB61, Pending



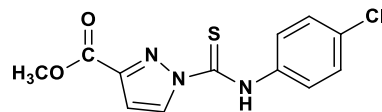
SB17, 1.25 μ M



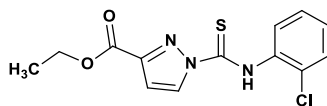
SB73, 5 μ M



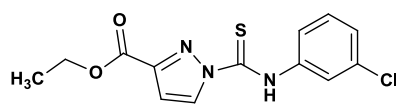
SB74, 2.5 μ M



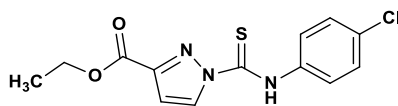
SB42, 10 μ M



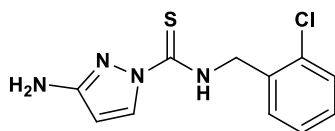
SB69, >10 μ M



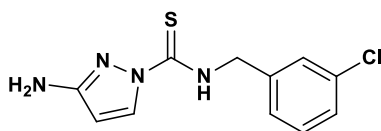
SB70, >10 μ M



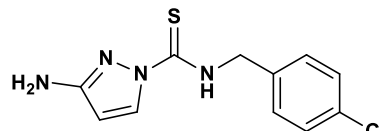
SB77, 0.6 μ M



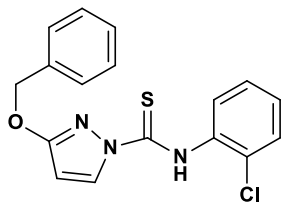
SB62, 5 μ M



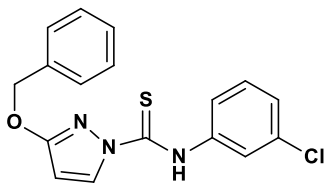
SB133, Pending



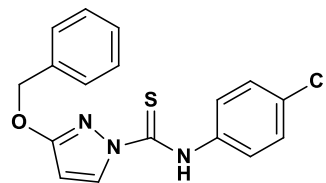
APM01, 10 μ M



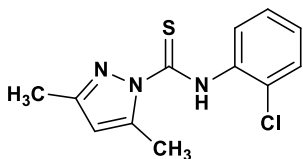
SB64, 0.6 μM



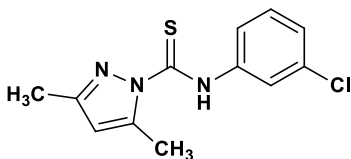
SB65, 2.5 μM



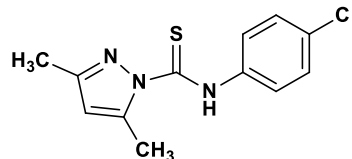
SB44, 0.15 μM



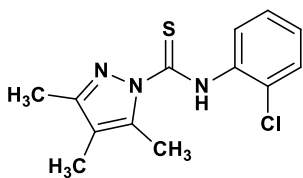
SB122, >10 μM



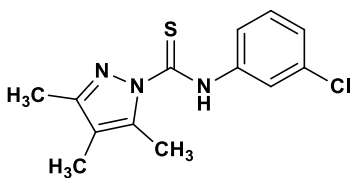
SB123, Pending



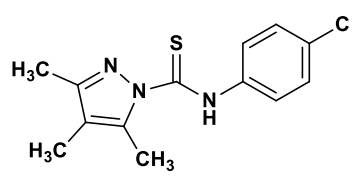
SB34, 2.66 μM



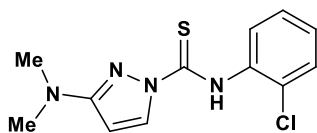
SB125, >10 μM



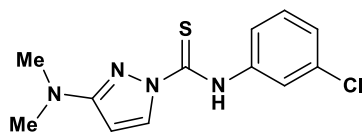
SB126, Pending



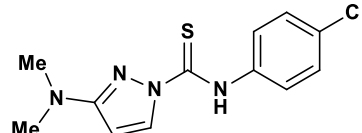
SB35, >10 μM



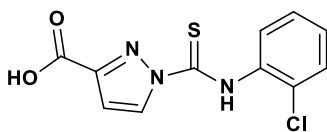
SB129, 1.25 μM



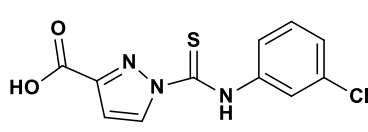
SB130, 10 μM



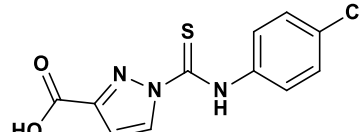
SB40, 0.6 μM



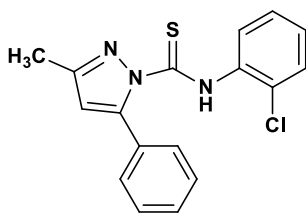
SB66, 2.5 μM



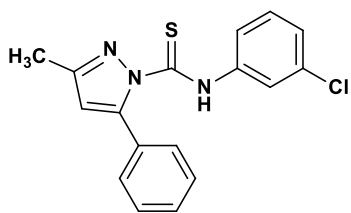
SB67, 10 μM



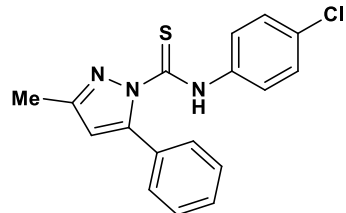
SB121, 5 μM



SB75, >10 μM



SB76, >10 μM



SB124, >10 μM

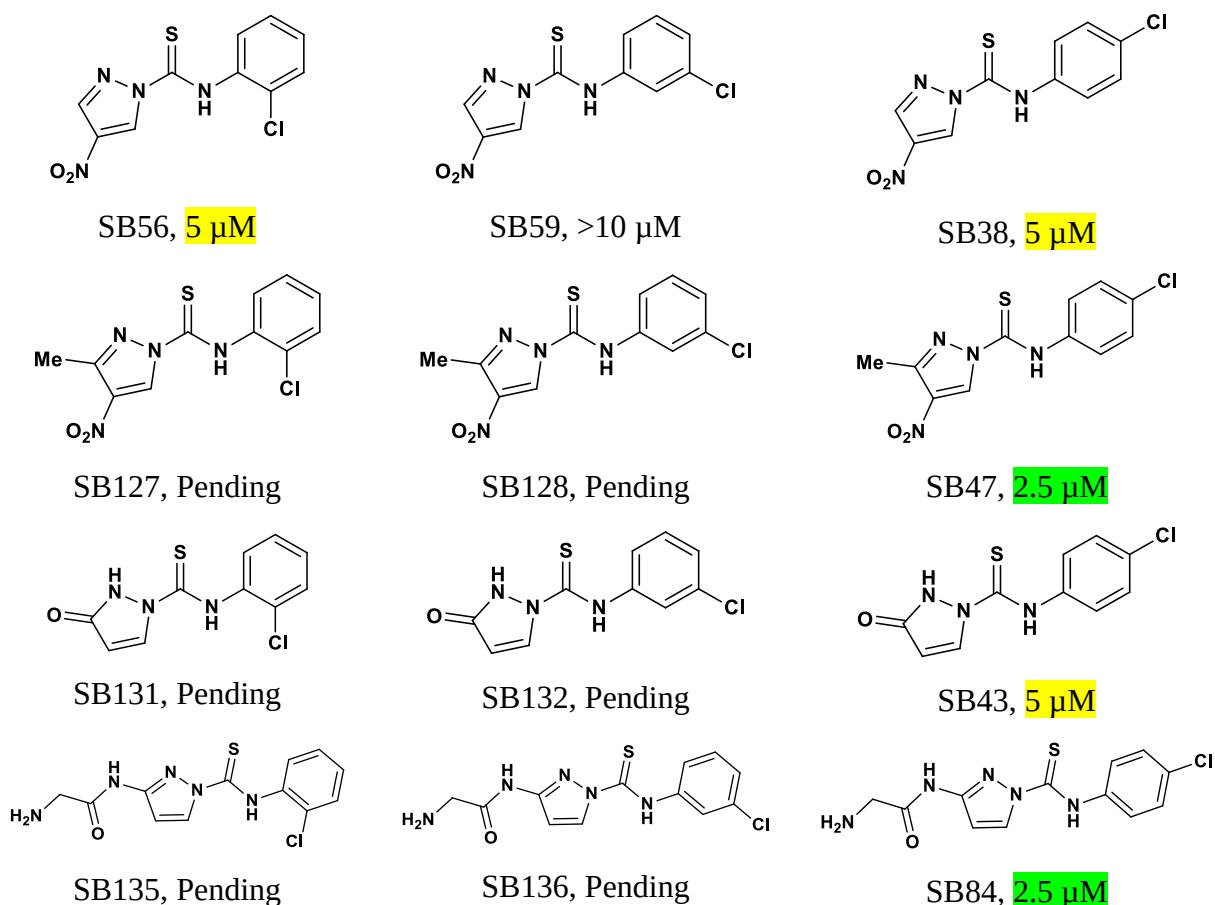


Figure 39, Structure and associated MIC values for MSSA of monochlorinated NNSNs.

	2-chloro	3-chloro	4-chloro
pyrazole	0.3	2.5	5.0
3-aminopyrazole	0.3	0.15	0.07
3-methylpyrazole	1.25	Pending	1.25
Methyl ester pyrazole	5.0	2.5	10
Ethyl ester pyrazole	>10	0.6	5.0
3-aminopyrazole + benzyl ITC	5.0	Pending	10
benzyl ether pyrazole	0.6	2.5	0.15
dimethyl pyrazole	>10	Pending	2.66
trimethyl pyrazole	>10	Pending	>10
N-dimethyl pyrazole	1.25	10	0.6
3-CA pyrazole	2.5	10	5.0
3-methyl-5-phenyl pyrazole	>10	>10	>10
4-nitropyrazole	5.0	>10	5.0
3-methyl-4-nitropyrazole	Pending	Pending	2.5
1H-pyrazol-3(2H)-one	Pending	Pending	5.0
Glycine + 3-aminopyr-NNSN	Pending	Pending	2.5

Table 5, Structure and associated MIC values against MSSA for monochlorinated NNSNs.

From the data presented in **Figure 39** and **Table 5**, it can be determined that the pyrazole substitution plays a large role in the activity. Depending on the pyrazole used, the activity can vary from 0.07 μM to $>10\ \mu\text{M}$. Aromatic substitution also plays a large role, with the movement of the chlorine between ortho (2-Cl), meta (3-Cl), and para (4-Cl) making as large a difference as 9.4 μM , like when the pyrazole is substituted with an ethyl ester group. In this case, the 2-Cl isomer shows poor activity of $>10\ \mu\text{M}$ while simply changing the chlorine to the meta position (3-Cl) increases the activity up to 0.6 μM ! This is a substantial difference that is only affected by the aromatic substitution. This indicates that out of these 38 that were tested so far, there are 18 candidates that could be used to treat MSSA. For reference, the MIC of Vancomycin (the drug of choice for the treatment of MRSA and MSSA infections today), is 2.2 μM .¹

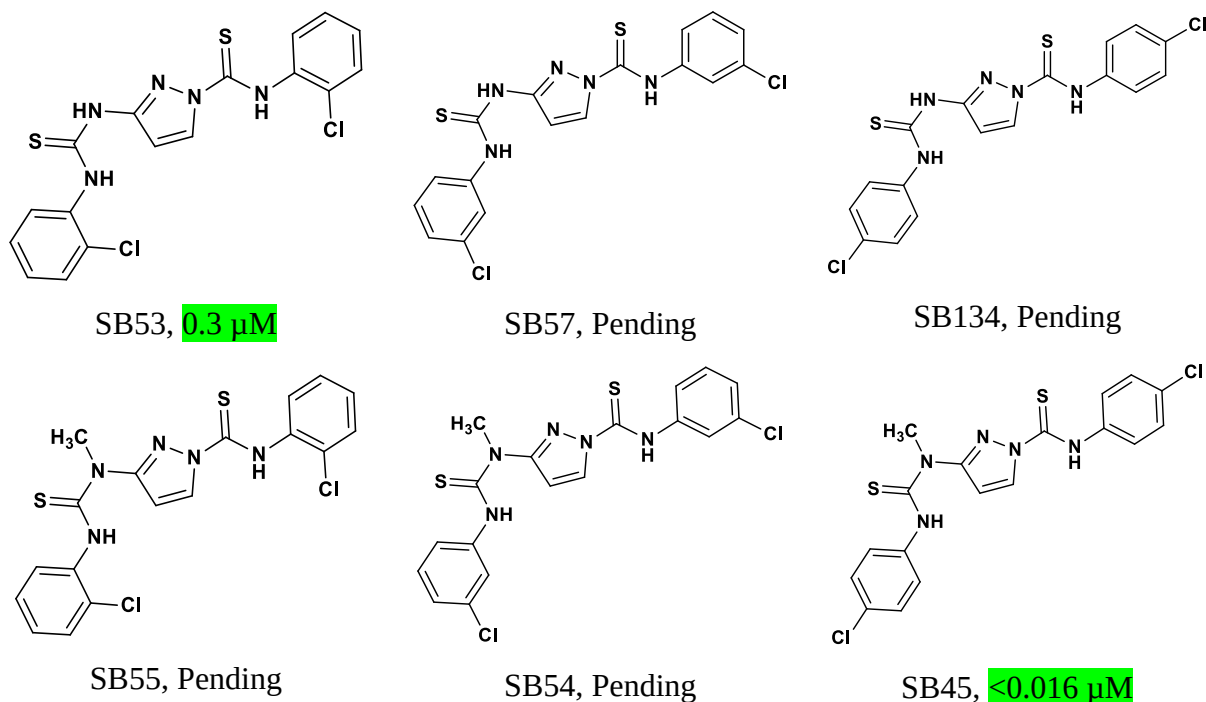
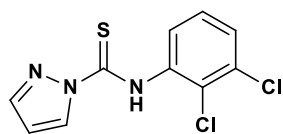


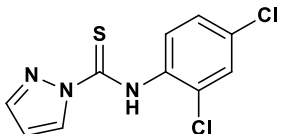
Figure 40, Structure and associated MIC values against MSSA for Bis-(x-chlorophenyl)thioureido-1H-pyrazole-thioamides.

As shown in **Figure 40**, most MIC values for the Bis-(x-chlorophenyl)thioureido-1H-pyrazole-thioamides are still pending. However, there is an expectation for these to have great

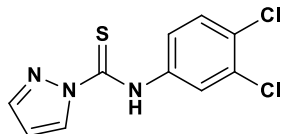
activity judging by the fact that SB45 shows the lowest MIC value of all the NNSNs that have been tested so far. This is one of only two NNSNs that show activity in the nanomolar range.



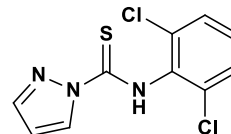
SB87, >10 μ M



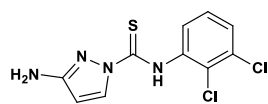
SB88, >10 μ M



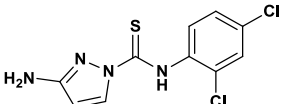
SB89, >10 μ M



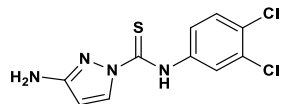
SB90, 1.25 μ M



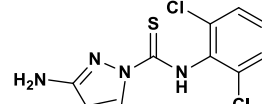
SB91, 1.25 μ M



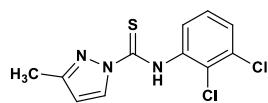
SB92, 0.3 μ M



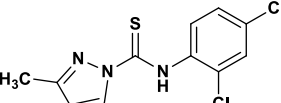
SB93, < 0.019 μ M



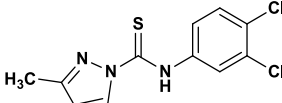
SB94, 0.3 μ M



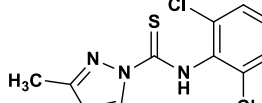
SB97, 1.25 μ M



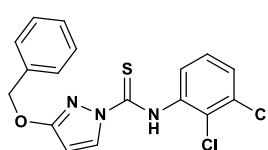
SB98, 2.5 μ M



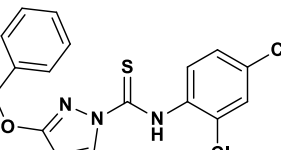
SB99, 5 μ M



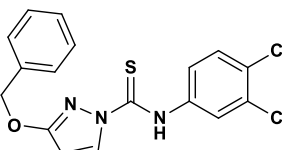
SB100, 0.3 μ M



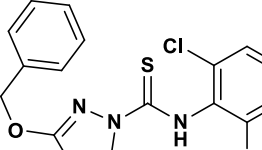
SB101, 0.3 μ M



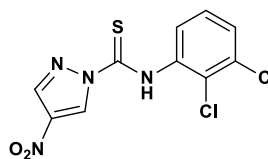
SB102, Pending



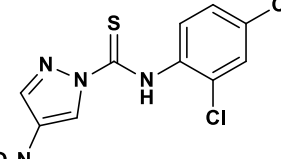
SB103, Pending



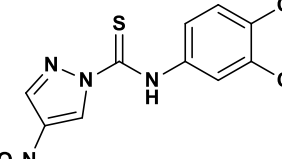
SB104, Pending



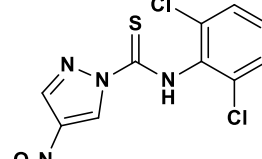
SB105, Pending



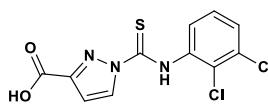
SB106, Pending



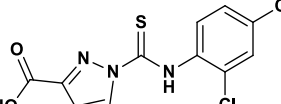
SB107, Pending



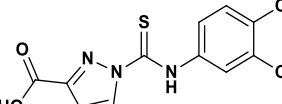
SB108, Pending



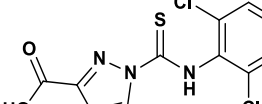
SB109, Pending



SB110, Pending



SB111, Pending



SB112, Pending

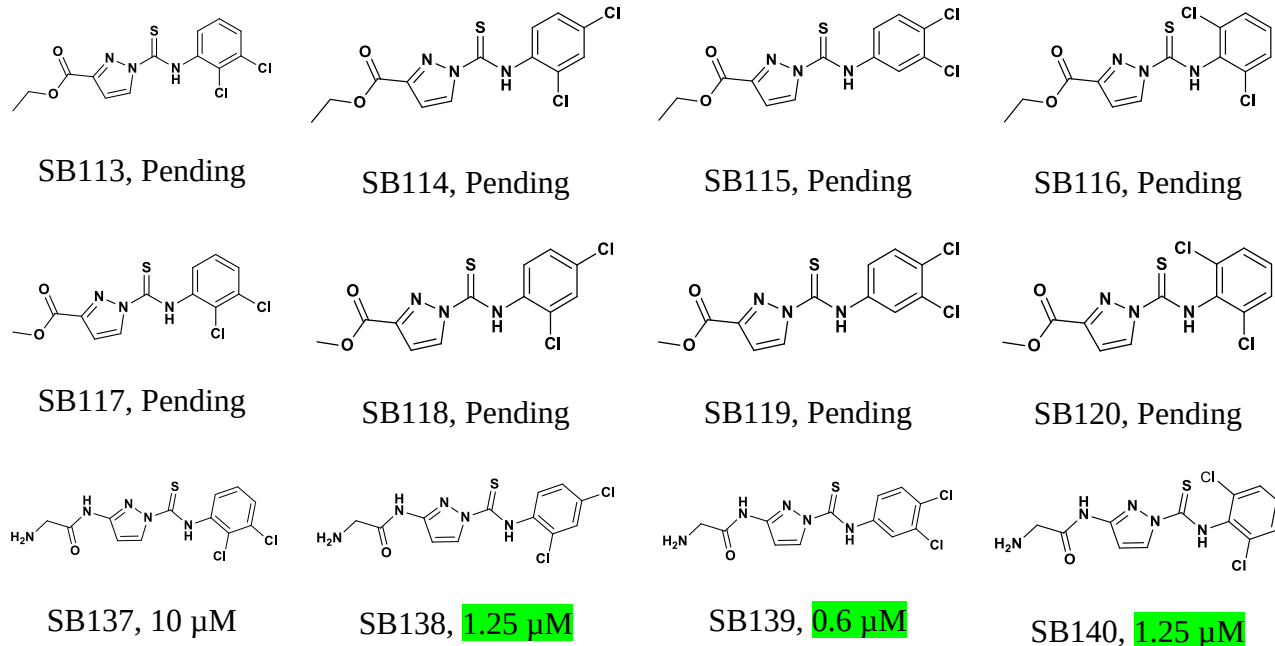


Figure 41, Structure and associated MIC values against MSSA for Dichlorinated NNSNs.

	2,3-Cl	2,4-Cl	3,4-Cl	2,6-Cl
pyrazole	>10 μ M	>10 μ M	>10 μ M	1.25 μ M
3-aminopyrazole	1.25 μ M	0.3 μ M	<0.019 μ M	0.3 μ M
3-methylpyrazole	1.25 μ M	2.5 μ M	5 μ M	0.3 μ M
ether benzyl pyrazole	Pending	Pending	Pending	Pending
4-nitropyrazole	Pending	Pending	Pending	Pending
3-CA pyrazole	Pending	Pending	Pending	Pending
ethyl ether pyrazole	Pending	Pending	Pending	Pending
methyl ether pyrazole	Pending	Pending	Pending	Pending
Glycine + 3-aminopyr-NNSN	10 μ M	1.25 μ M	0.6 μ M	1.25 μ M

Table 6, Structure and associated MIC values against MSSA for dichlorinated NNSNs.

As shown in **Figure 41** and **Table 6**, a large majority of the dichlorinated NNSNs are still pending for MIC values at this time. However, of the 12 compounds that have been tested against MSSA, 11 NNSNs have good MIC values, as marked in green, and there is also 1 compound, SB99, has an MIC of 5 μ M. All twelve of these compounds could serve as candidate drugs for the treatment of MSSA. SB93 shows an MIC in the nanomolar range, which would serve as an exceptional candidate.

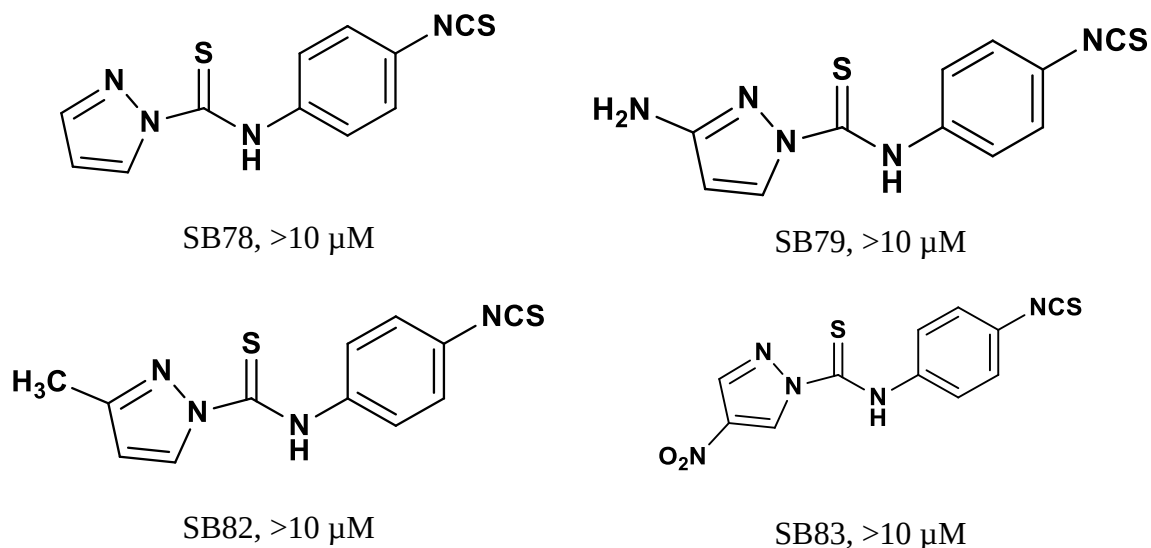


Figure 42, Structure and associated MIC values against MSSA for Diisothiocyanato NNSNs.

The idea behind the synthesis of Diisothiocyanato NNSN compounds was that because the isothiocyanate plays such an important role and because isothiocyanates themselves have been shown to have antibacterial and anti-cancer properties,⁵ it would be beneficial to synthesize some NNSNs with additional free isothiocyanates. However, upon the synthesis and subsequent testing against MSSA, none of these compounds show increased activity as shown in **Figure 42**. Unfortunately, none of these would serve as candidates for the treatment of MSSA. The same trend was seen for Bis-1*H*-pyrazole-1-carbothioamides as shown in **Figure 43**. This class was envisioned because the prior Bis NNSNs performed so well, however, no activity was seen.

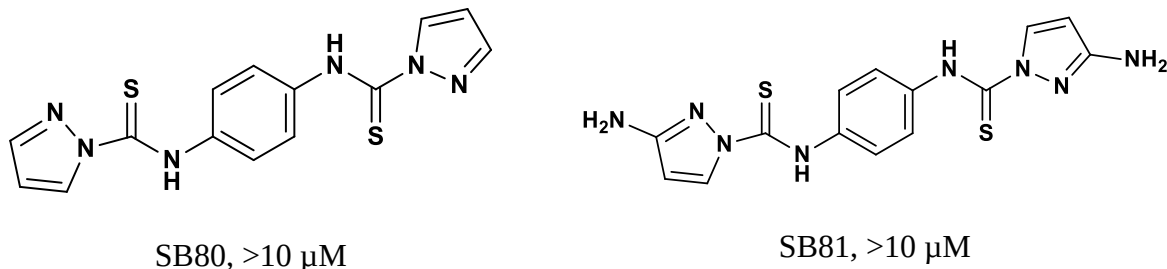


Figure 43, Structure and associated MIC values against MSSA for Bis-1*H*-pyrazole-1-carbothioamides.

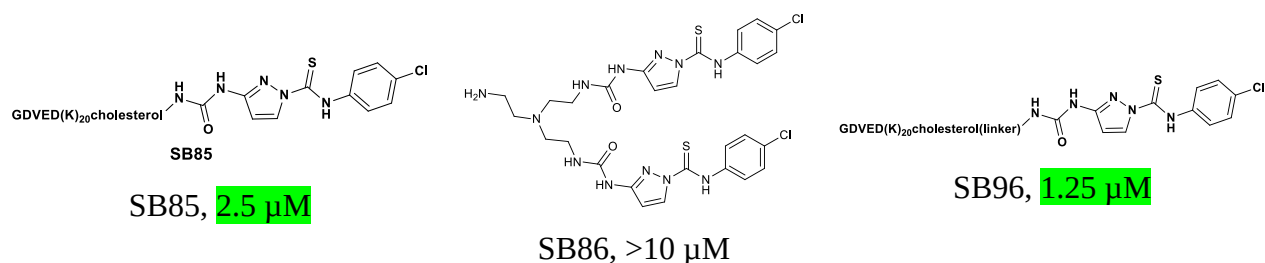


Figure 44, Structure and associated MIC values against MSSA for NNSNs combined with Nanosponge components.

As demonstrated in **Figure 44**, combining KE03 with the components of the nanosponge has a great effect on the MIC. KE03 by itself shows an MIC of 0.07 μM . Once this drug (2 equivalents) is combined with tris(2-aminoethyl)amine (1 equivalent) to form SB86, the compound loses activity completely. However, when KE03 is combined with the peptide (K)₂₀DEVGDG-cholesterol to form SB85, the MIC goes down to 2.5 μM . Once the nanosponge is completed ((K)₂₀DEVGDG-cholesterol-linker) and KE03 is bound to it, the MIC goes down again to 1.25 μM . However, note that without the nanosponge KE03 shows a better MIC. For this reason, it should not be necessary to use the nanosponge to transport KE03 as it does not increase its activity.

The overall conclusion from this SAR study is that there are two NNSN compounds that show nanomolar activity, namely SB93 and SB45, the structures of which include 3-amino-1*H*-pyrazole. Other examples of increased activity being related to 3-amino-1*H*-pyrazole include SB49, SB50, KE03, SB62, SB91, SB92, and SB94. An unsubstituted pyrazole also leads to good results, such as SB46, SB52, SB24, and SB90. 3-methyl-1*H*-pyrazole has shown some good MIC values including SB60, SB17, SB97, SB98, SB99, and SB100. Methyl and Ethyl Esters substituted in the 3-position of the pyrazole yields good MIC values in SB73, SB74 and SB77. 3-benzyl ether pyrazole also yielded good MIC values in SB64, SB65 and SB44. Di-methylated pyrazoles show good MIC values in SB34. 3-(*N*-dimethylated)-1*H*-pyrazole gave good MIC values in SB129 and SB40. 3-carboxylic acid-1*H*-pyrazole yielded good MIC values in SB66

and SB121. Combining glycine into the pyrazole also creates good drug candidates such as SB138, SB139, and SB140. This study also concludes that aromatic substitution plays a large role in the outcome of MIC. Simply changing the substitution of the chlorine atom on the aromatic ring can cause over a 10-fold increase in biological activity in some cases, such as from SB52 (4-Cl) which yielded an MIC of 5 to SB46 (2-Cl) with an MIC of 0.3. Out of the 65 total compounds that have been tested against MSSA so far, there are 34 compounds that have an MIC value less than 5 μ M and 8 with an MIC of exactly 5 μ M. This reveals a total of 42 new drug candidates that could be used to treat MSSA.

Chapter 2 References

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Chapter 3 – Mode of Action of NNSN Molecules

Theoretical Mode of Action

NNSNs are non-toxic until activated by copper, the necessary concentration of which only exists naturally in the biological environment at the site of infection or within a tumor microenvironment.¹ Copper (I/II) is an endogenous metal with potent antibacterial properties. It defends the immune system by catalyzing redox reactions. Copper is limited in the human body because of its ability to produce reactive oxygen species (ROS) which are tissue damaging. Therefore, tight homeostatic regulation is mandatory, restricting the free copper concentration in blood and healthy tissue to about 1 micromolar or less.¹

There are two main events that can cause alteration of the copper concentration, namely upregulation occurring in activated phagosomes and cancerous cells. In both cases, the copper concentration has shown to increase by 5-50 times that found in normal tissues.¹ The copper concentration and its distribution throughout cancer cells is directly related to a tumor's growth, angiogenesis, and metastasis.² The cell membranes have developed means to remove the copper through efflux pumps. The NNSN-copper complex forms an iminium-type cation, as shown in **Figure 45**, which is then able to impair numerous enzymes of the cell. Iminium cations are potent warheads because they react with multiple targets through nucleophilic addition, electron transfer, and radical reactions.¹ This effectively shuts down the efflux pumps that remove copper from the cell. The copper floods into the cell and leads to cell death.

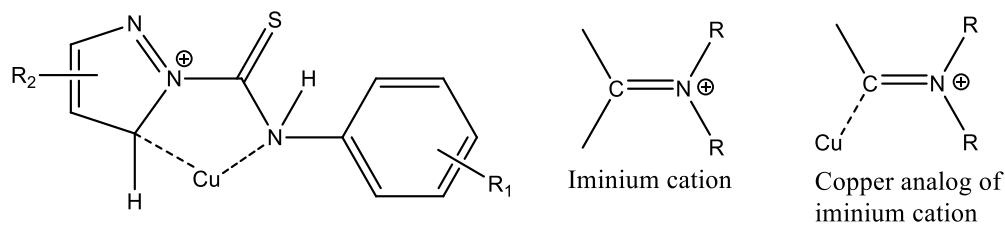


Figure 45, Structure of iminium cation formed following copper activation.¹

The early idea of how copper would bind to a general NNSN structure is also shown in **Figure 45**. This initial structure was based on the data obtained through NMR titrations (which were performed by dissolving the NNSN in deuterated acetone and adding aliquots of copper(I) bromide solution of known concentration to monitor how the structure changes).³ These titrations also observed 1:1 complexation geometry.³ However, the suggested complexation structure shown in **Figure 45** has been proven incorrect by Computer Modeling Studies, which will be discussed further later in this chapter.

This iminium cation is formed by the proposed mechanism shown in **Figure 46**. A general novel NNSN structure is shown to begin with. This undergoes resonance of the energy from the lone electron pair on the nitrogen adjacent to the C=S bond in structure I to form a double bond between the two pyrazole nitrogens as well as an iminium carbocation, and the initial double bond adjacent to the second pyrazole nitrogen is broken and a different C=C bond formed, the next double bond is also broken yielding a carbanion (negatively charged carbon atom).

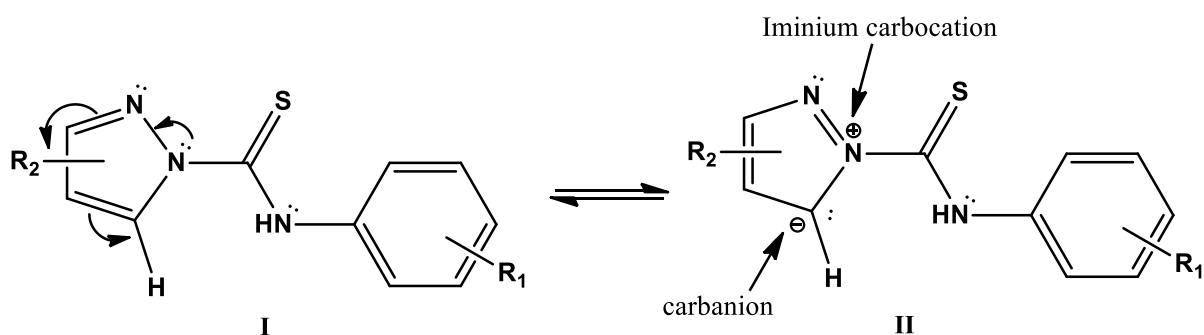


Figure 46, Proposed mechanism of iminium cation formation following copper activation.¹

Computer Modeling Studies

To investigate the interaction between various NNSN compounds and copper and understand how complexation occurs, density function calculations (DFT) calculations were carried out along with optimization and energy calculations and the structures visualized using MacMolPlt.⁴ This was done using six distinct Classic NNSN isomers, termed Isomer 1 through Isomer 6, the structure of which is shown in **Figure 47**. MacMotPlt was used to visualize the geometries of the isomers, which were then submitted for optimization.⁴ The structure with the lowest bond energy revealed through this study is Isomer 3. It is posited that the presence of a hydrogen bond between the nitrogen atom of the pyrazole ring and the hydrogen atom of the adjacent thiourea and the fact that the pyrazole ring and aromatic ring planes are oriented to minimize the amount of steric hindrance in this isomer offers stabilization.⁴

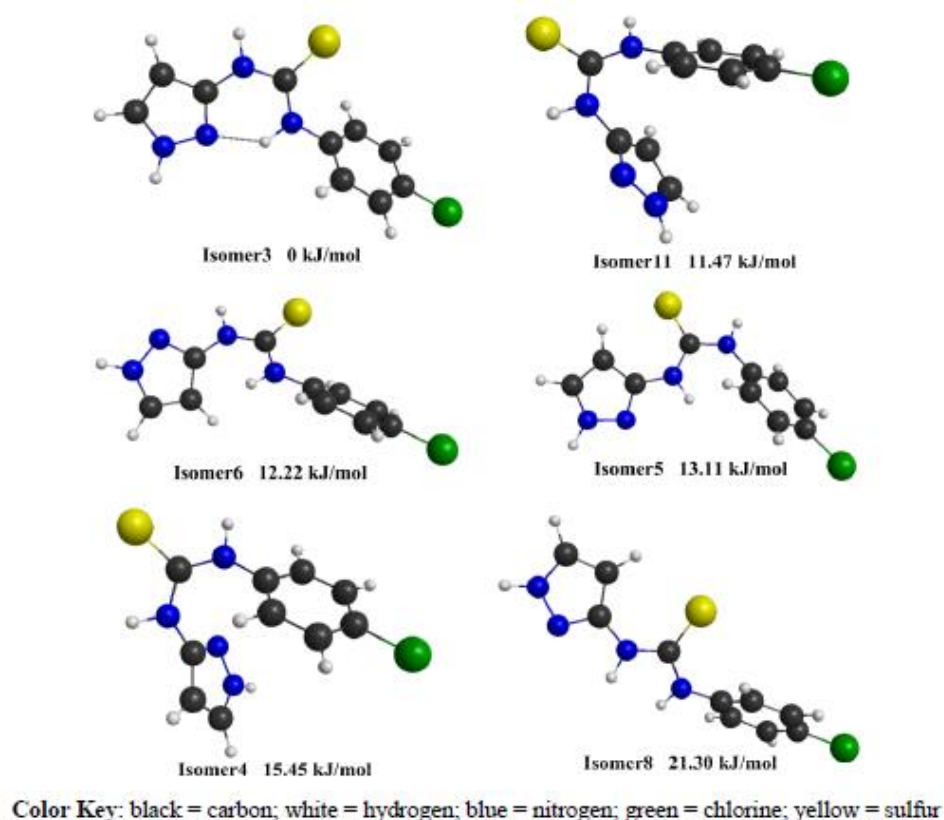


Figure 47, Structures of six NNSN isomers used in DFT studies.⁴

The geometry optimization and energy calculations were performed by Tejaswi Shrestha et al using the DFT model and basis sets of polarized double zeta (DZP) quality for hydrogen, nitrogen, carbon, and chlorine; for copper, polarized triple zeta (TZP) quality basis sets were used.⁴ Only valence electrons were considered since these are typically involved in bond interaction and all other electrons remained in a frozen core for the calculations. The optimizations were formed assuming water as solvent and modeled by the Conductor like Screening Model (COSMO) of solvation.⁴ Once the geometries were optimized using this methodology, the possible sites for copper(I) complexation on the isomers were identified and these complexes were manually created in MacMolPlt. These isomers were also submitted for optimizations and calculations to identify the most stable copper-NNSN complexes, the structures of which are shown in **Figure 48**.⁴

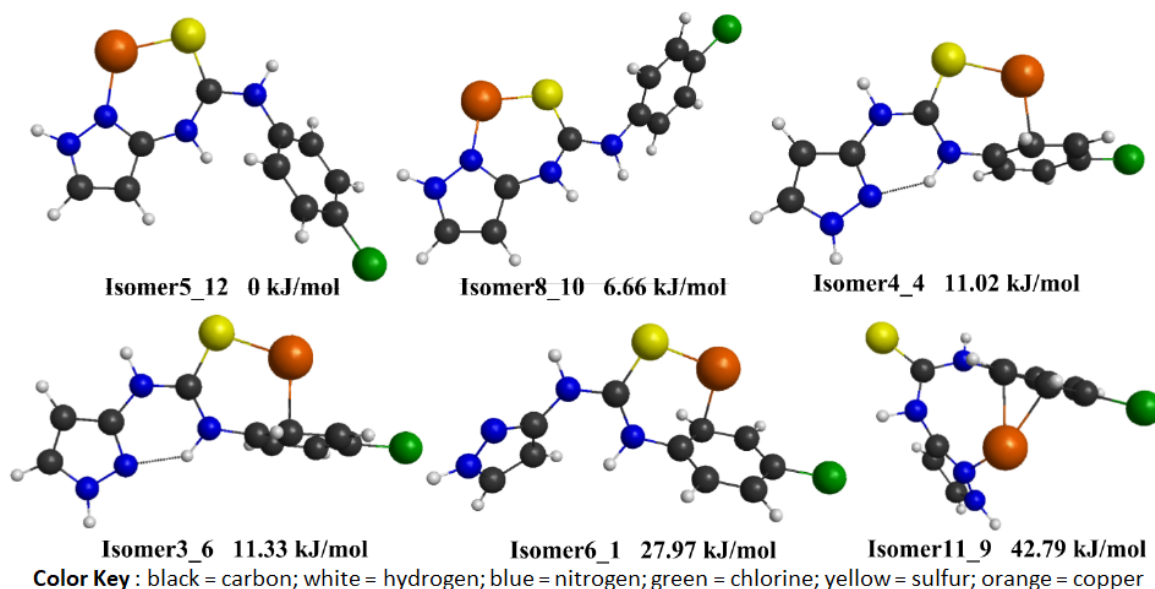


Figure 48, Geometries and relative energies of NNSNs generated using MacMolPlt.⁴

Copper (I) is the most likely electron state because although Cu (II) is usually the most stable in aqueous conditions, the microenvironment of cancerous tumors is reductive in nature.¹ Copper (I) is highly electrophilic and will be drawn to areas of high electron density. Such

possible binding sites include the lone electron pair of the nitrogen in the pyrazole ring, the lone electron pair of the sulfur in the thiourea group, and the pi electrons in the carbon ring.⁴ An interaction with the chlorine atom is not likely, since chlorine is electronegative and is not a good electron donor. Overall, this study revealed a motif of copper complexation with sulfur and either nitrogen or carbon, with the exception being isomer11_9. This isomer presents an interesting example in that it is unique to the rest of the group. The copper is shown to be accepting electrons from the aromatic ring and the nitrogen of the pyrazole ring.⁴ This causes two carbons of the aromatic ring to donate pi electrons, ultimately disturbing the symmetry of the ring. Due to this disturbance in symmetry, the isomer is less stable and shows a much higher energy.⁴ Further, the copper ion is not interacting with the sulfur atom in this isomer, while it was consistently with the other five isomers. This observation further adds to the high energy observed, since sulfur is a better ligand to complex with copper than either nitrogen or carbon.⁴ Another common theme seen in this study is that the angle of the bonds form part of a square planar geometry, which is the preferred structure for the copper (I) ion.⁴

The conclusions formed from this study led to the original complexation structure shown in **Figure 45** earlier in this chapter to be disproven. The early complexation structure, based upon NMR titration data, suggested that the copper would interact with the pyrazole -CH nearest the carbonothioyl group and with the -NH originating from the isothiocyanate. This structure is odd in that it does not suggest any interaction between the copper and areas of high electron density such as the sulfur, which will occur most of the time as shown in the computer modeling study. Other expected areas for copper to be attracted are the lone electron pair of the nitrogen in the pyrazole ring and the pi electrons in the carbon ring, of which neither are shown in this early structure determination.

We decided to collaborate further with the Aikens research group to add to the understanding about the geometry of the NNSN-copper complexes when compared to experimental UV visible spectroscopy and ^1H Nuclear Magnetic Resonance spectroscopy data. This study was performed by Gowri Kuda Singappulige, utilizing DFT calculations with B3LYP exchange correlation function. These calculations used the COSMO implicit solvent model and included long-range corrections. Further, this study was designed to ascertain the differences that appear when aromatic substitution changes were applied to the aromatic ring of the NNSN structure, particularly for a single chlorine atom in the ortho, meta, or para position. Both experimental and theoretical data was collected in the presence of and absence of copper(I).

The first step in this endeavor was to determine the local minima for the rotational isomers of ortho-Cl, as shown in **Figure 49**. As mentioned previously, planar geometry is preferred, which supports the evidence shown that Isomer_2 has the lowest bond energy of the five isomers tested due to having this geometry. Isomer_3 shows no hydrogen bonding and thus is energetically less favorable. The graph in **Figure 50** shows how the energetics of ortho-Cl, meta-Cl, and para-Cl compounds compare to one another. Isomer_2 was found to be the most energetically favorable compound despite whether the chlorine atom was in the ortho, meta, or para position. Out of all the compounds, the Para-chlorinated Isomer_2 was the most stable. Isomer_1 and Isomer_3 are energetically very different when the chlorine is in the ortho position, however, they are energetically similar when the chlorine is in the meta position. This is indicated in **Figure 50** by the green circles around the data for Isomer_1 and Isomer_3. From this data, it was decided that Isomer_2 is the most likely structure due to its low energy despite the aromatic substitution.

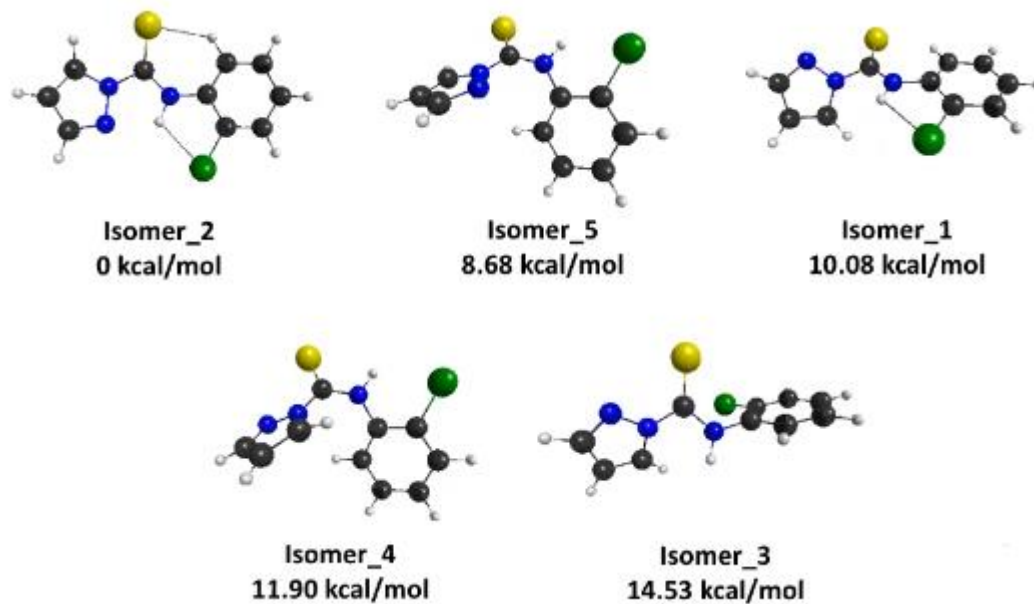


Figure 49, Local minima found for ortho-Cl (rotational isomers).

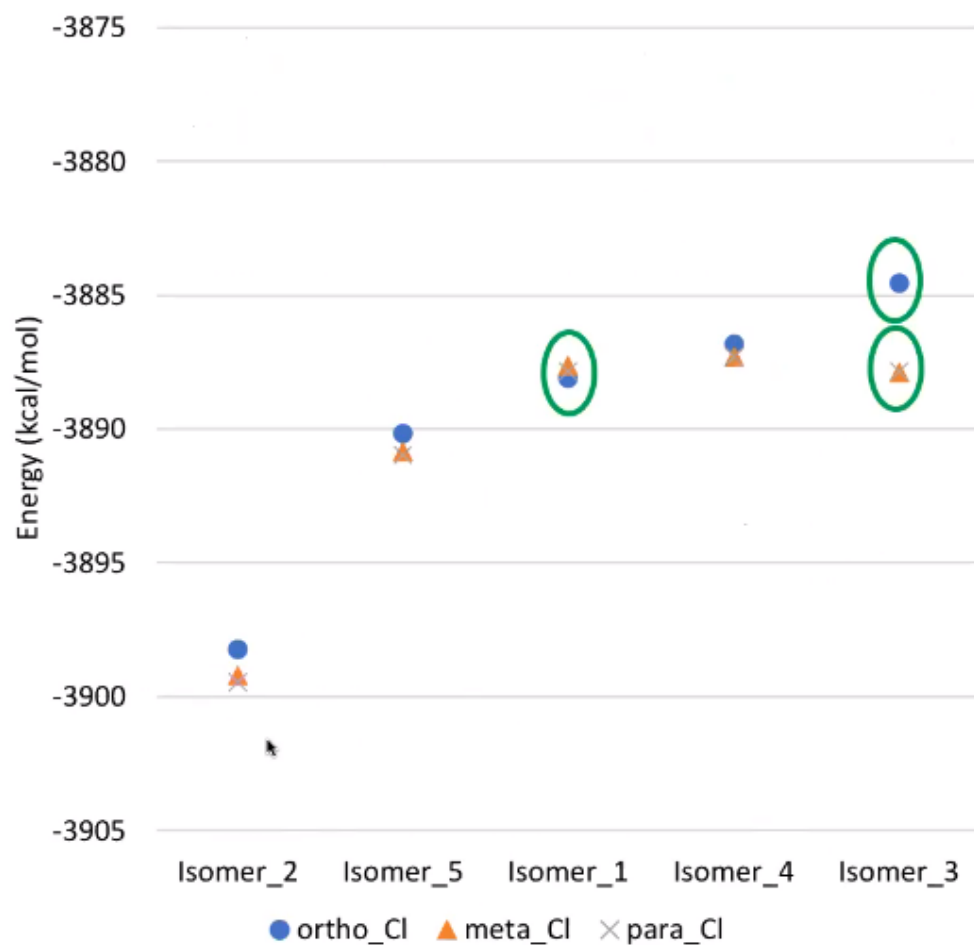


Figure 50, Energetics of Ortho-Cl, Meta-Cl, and Para-Cl Compared.

The next step was the calculations for optical spectra of the five isomers, which were directly compared to the experimental UV visible spectra, as shown in **Figure 51**. This data suggests that the spectral shapes were similar in all but the Ortho-chlorinated Isomer-3. This is hypothesized to be due to the orientation of the molecule allowing the pi electrons to interact with the sulfur. The lowest peaks calculation on the Calculated spectra for Isomer_3 was due to the electrons moving from the nitrogen between the aromatic ring and the sulfur as well as from the phenyl ring to the sulfur. The second lowest calculated peaks observed was due to electrons moving from the phenyl ring to the sulfur as well. The two main peaks that were calculated, around 325 nm and 275 nm, matched well with the first two experimental peaks.

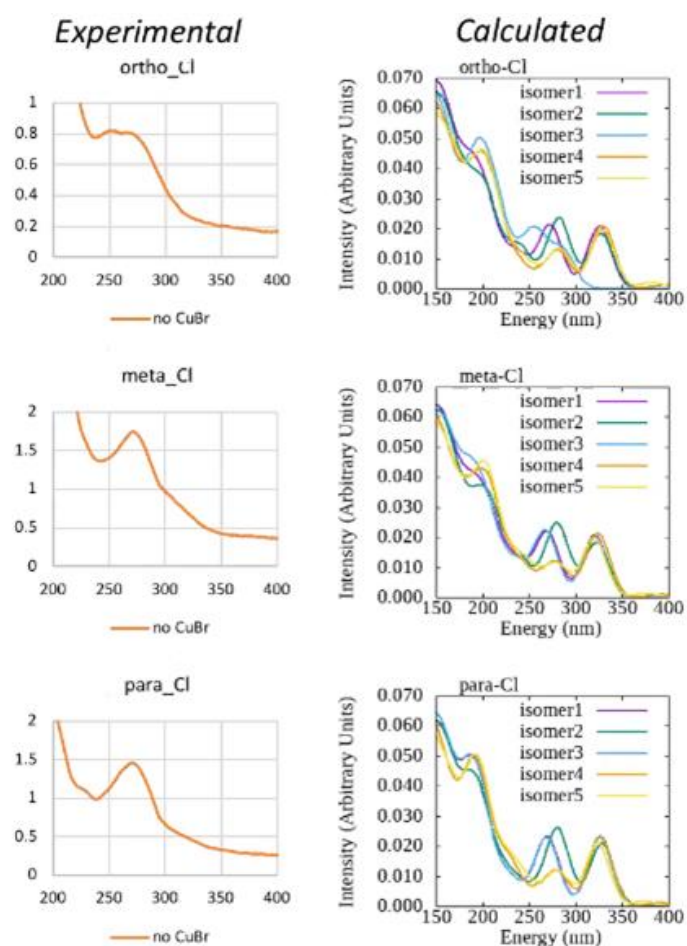


Figure 51, Calculated Optical Spectra versus Experimental UV Vis Spectra for No-Cu Isomers.

The relative energies of the five isomers bound to copper(I) were then calculated using the same methodology. The rotation around the C-N bond proved different when the compound was bound to copper. All five isomers were tested for ortho-Cl derivatives, but with the other derivatives, the focus was shifted to Isomer 1 and Isomer 3 because these were found to be the most stable. The relative energies of the tested compounds are shown in **Figure 52**. The structures of these copper-bound isomers are shown in **Figure 53**.

	ortho-Cl		meta-Cl		para-Cl	
	Relative Energy (kcal/mol)		Relative Energy (kcal/mol)		Relative Energy (kcal/mol)	
orientation	Isomer_1	Isomer_3	Isomer_1	Isomer_3	Isomer_1	Isomer_3
1	0.0	19.9	0.5	20.7	0.1	
2	9.3	10.4	0.5	0.0	9.0	0.0
3	29.3	29.3	8.6	10.0	8.6	8.5
4	27.0	0.9	8.3	0.0	8.3	0.2
5	8.4	28.1		28.1	8.3	27.6
6				37.4		37.2

Figure 52, Relative energies of copper-bound compounds.

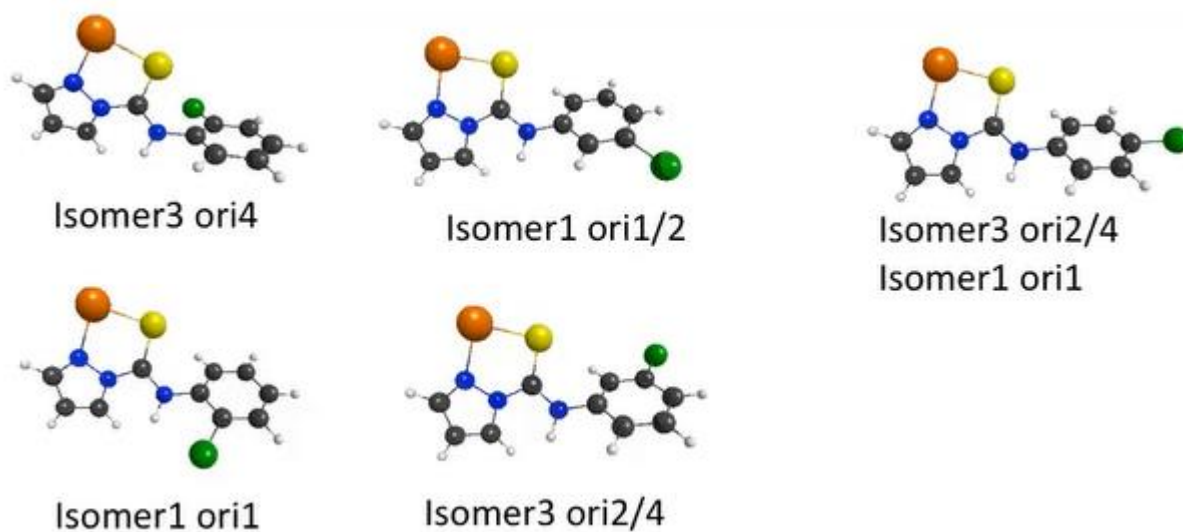


Figure 53, Structures of copper-bound isomers.

The calculations for optical spectra of the five copper-bound isomers, which were directly compared to the experimental UV visible spectra, as shown in **Figure 54**. The spectral shapes that were calculated matched the experimental spectra for all isomers, except for Isomer3-ori4. From this data, it was concluded that for ortho-Cl, the most likely isomer is Isomer1-ori1; for meta-Cl, either are possible; for para-Cl, the most likely isomer is isomer3-ori2.

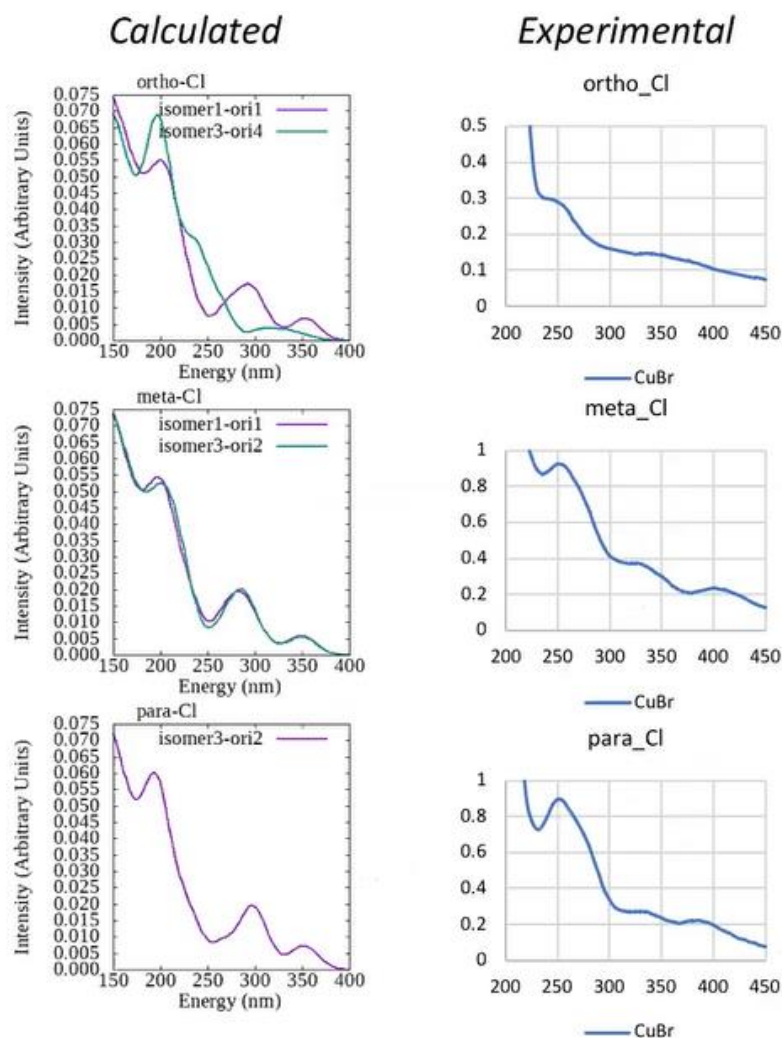


Figure 54, Calculated Optical Spectra versus Experimental UV Vis Spectra for Copper-Bound Isomers.

The conclusions drawn for this computational study confirmed a planar geometry is preferred in the structures where hydrogen bonding is possible. There was no significant difference noted in energetical stability and spectra properties when aromatic substitution

changes were applied, with the exception being for ortho-Cl. In the ortho-Cl isomers, geometrical differences led to spectra changes. The rotation around the C-N bond varied between the Cu-bound and no-Cu systems. This is believed to be due to the preference of copper to bind with the nitrogen of the pyrazole ring and sulfur. Lastly, the origin of peaks in optical spectra show significant contribution from copper.

Theoretical NMR values were also calculated by Gowri Kuda Singappulige and compared to experimental spectra for a simple NNSN structure, namely SB19. The structures of the two theoretical conformers, named Isomer 2 and Isomer 6, are shown in **Figure 55**. The experimental and theoretical NMR comparison and calculated NMR shielding is shown in **Figure 56**. This data suggests that the isomer 6 is the true confirmation of SB19. The theoretical data matches very accurately with the experimental spectra.

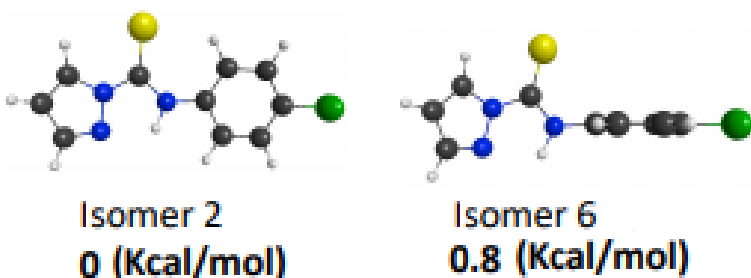


Figure 55, Structures of two theoretical isomers of SB19.

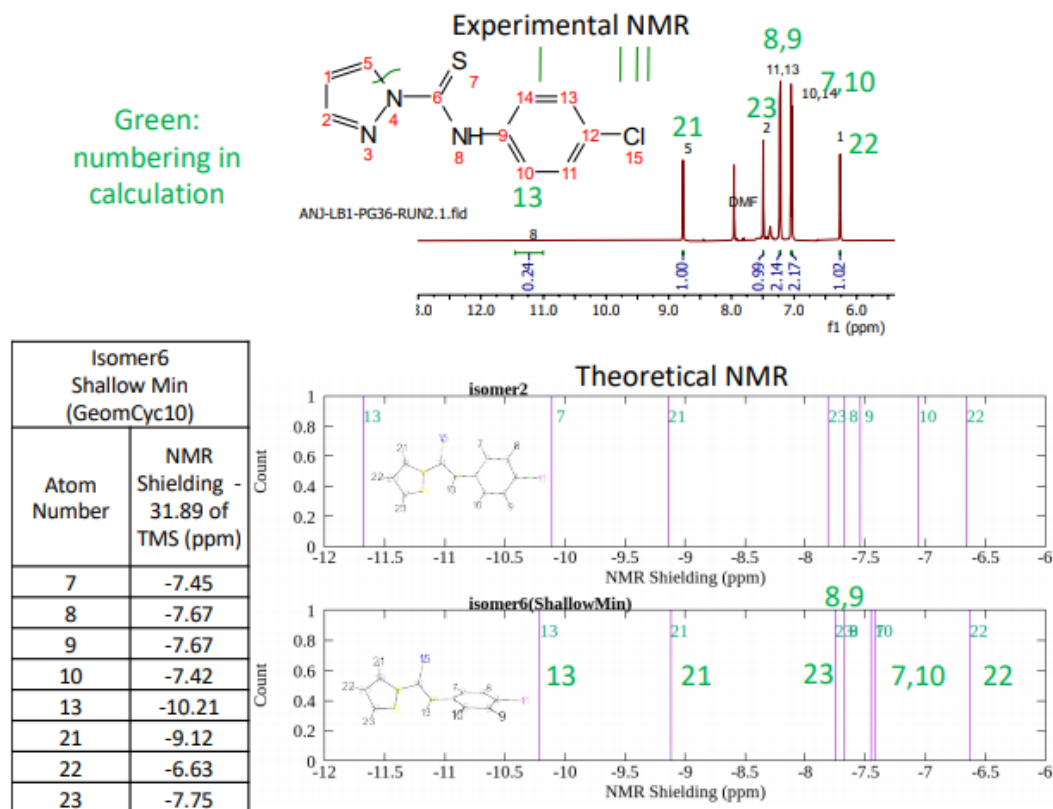


**Figure 56, Experimental versus Theoretical NMR with Calculated NMR Shielding.
Copper Binding Constant Calculations**

The first step in measuring copper binding constants is to prepare some solutions. The first solution needed for this is HEPES buffer. To make this solution, weigh out 5.5957 mg of HEPES and dilute with water to 1 liter. The pH must then be adjusted to be between 7.2 and 7.4. The next solution is Copper(I) bromide, which must have a concentration of 0.00011 M. The last solution is that containing the NNSN sample. The amount of NNSN must then be calculated

according to the equation: $\frac{1 \times 10^{-3} \text{ moles}}{1000 \text{ mL}} \times 2 \text{ mL} \times MW \left(\frac{\text{g}}{\text{mole}} \right) = \text{Theoretical Mass Needed.}$

Once this amount has been weighed, it should be dissolved in 200 microliters of methanol and sonicated until fully dissolved. Add 1800 microliters of HEPES buffer. Once these solutions have been prepared, then a concentration gradient must be made, as shown in **Figure 57**, which will be run using UV visible spectroscopy.

Blank	0.25	0.5	0.75	1.00	1.50
• 2.4 mL CuBr • 0.6 mL HEPES buffer	• 2.4 mL CuBr • 525 μL HEPES buffer • 75 μL SB63	• 2.4 mL CuBr • 450 μL HEPES buffer • 150 μL SB63	• 2.4 mL CuBr • 375 μL HEPES buffer • 225 μL SB63	• 2.4 mL CuBr • 300 μL HEPES buffer • 300 μL SB63	• 2.4 mL CuBr • 150 μL HEPES buffer • 450 μL SB63

Figure 57, Concentration Gradient for Copper Binding Constant Calculations.

Once all the samples in the concentration gradient have been run for an NNSN sample, SB63 for example, the data should form a graph like that in **Figure 58**. Ideally, a sharp, symmetrical peak will form. In some cases, more than one peak can be seen, like in **Figure 58**. If more than one peak is present, the sharpest peak is chosen. Four wavelengths covering this peak are inputted into an Excel sheet and then run on the UV 1:1 Bindfit program from Supramolecular.com. If the run is successful, then an optimized value will be generated which is considered the Copper Binding Constant for that sample. This screen is shown in **Figure 59**.

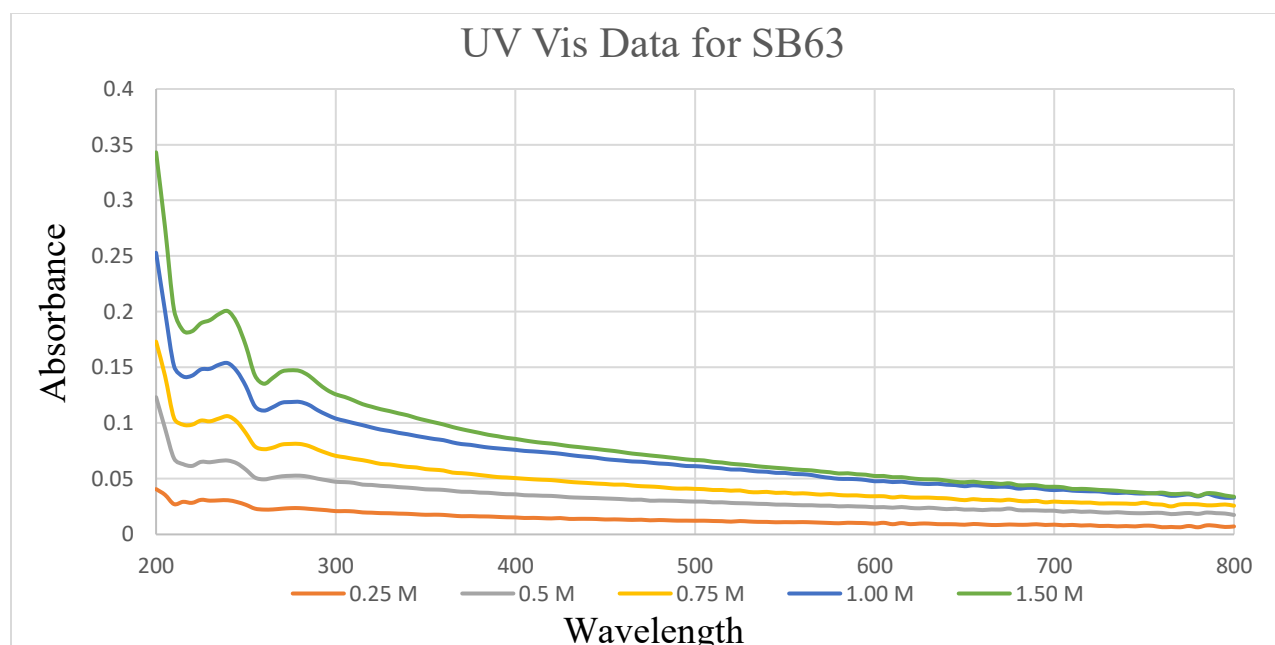


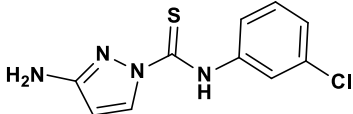
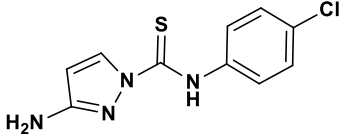
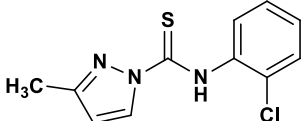
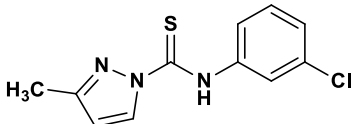
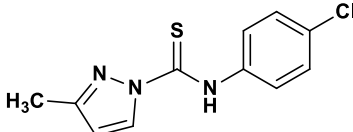
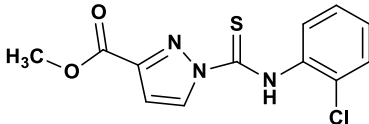
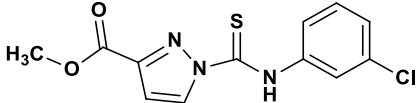
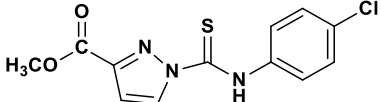
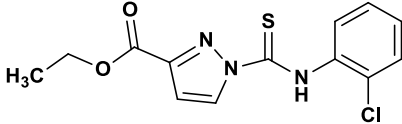
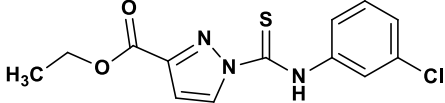
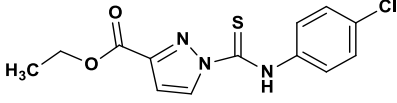
Figure 58, UV Visible Spectroscopy Data for SB63.

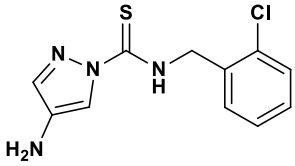
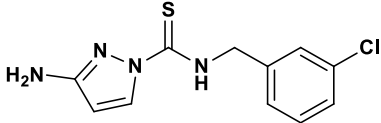
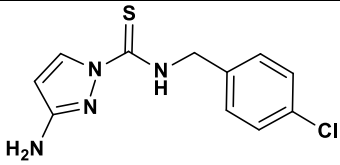
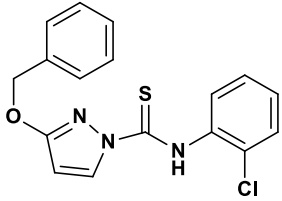
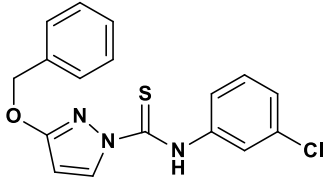
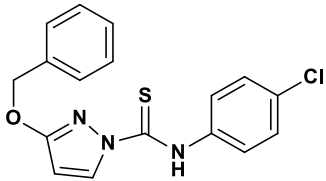
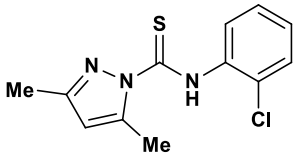
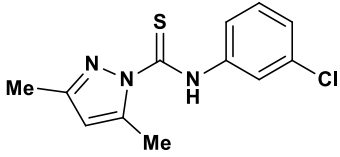
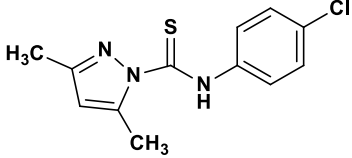


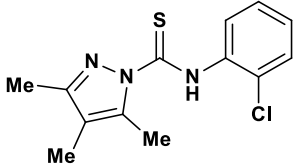
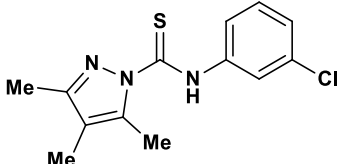
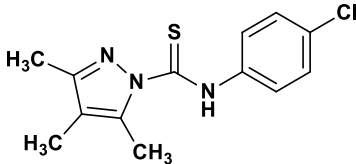
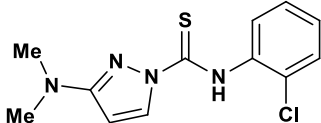
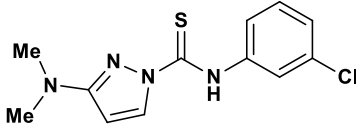
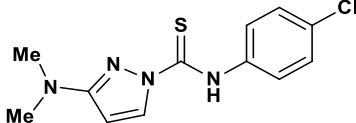
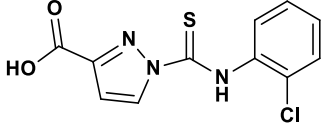
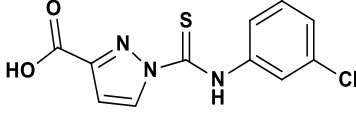
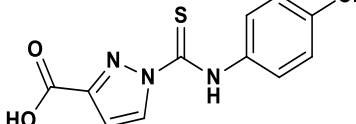
Figure 59, Screenshot of a Successful Run of UV Vis Data on Supramolecular.com's Bindfit program.

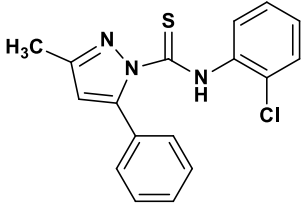
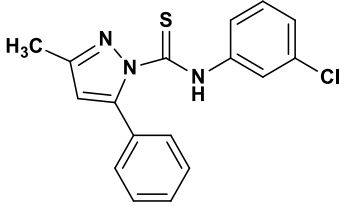
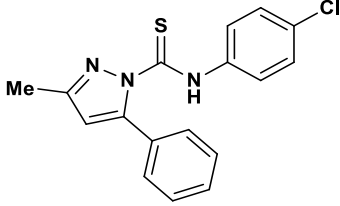
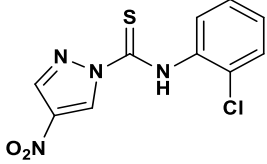
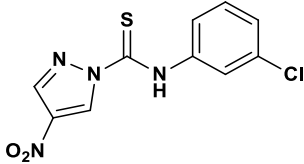
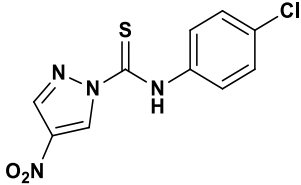
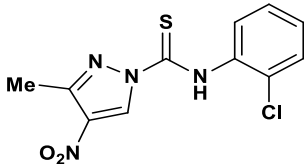
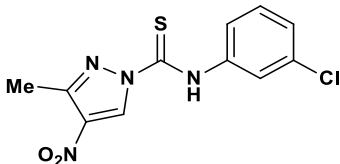
For the Copper Binding Constants, there are six categories, namely Monochlorinated, Bis-(x-chlorophenyl)thioureido-1H-pyrazole-thioamides, Dichlorinated, Diisothiocyanato, Bis-1H-pyrazole-1-carbothioamides, and NNSN-Nanosponge components. The compounds highlighted in yellow were synthesized by other researchers on the NNSN team.

Structure	SB#	Cu Binding
	SB46	8600 M
	SB52	2200 M
	SB24	2100 M
	SB49	4700 M

	SB50	1950 M
	KE03	53200 M
	SB60	1200 M
	SB61	2200 M
	SB17	54020 M
	SB73	985 M
	SB74	1128 M
	SB42	800 M
	SB69	450 M
	SB70	2550 M
	SB77	1580 M

	SB62	11943 M
	SB133	17423 M
	APM01	3200 M
	SB64	5200 M
	SB65	1879 M
	SB44	25700 M
	SB122	330 M
	SB123	2650 M
	SB34	2375 M

	SB125	520 M
	SB126	2375 M
	SB35	1280 M
	SB129	1400 M
	SB130	3050 M
	SB40	58500 M
	SB66	1400 M
	SB67	2650 M
	SB121	5500 M

	SB75	985 M
	SB76	3160 M
	SB124	550 M
	SB56	770 M
	SB59	2800 M
	SB38	1100 M
	SB127	2581 M
	SB128	5506 M

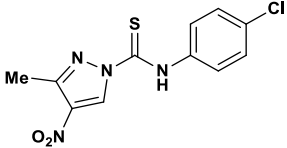
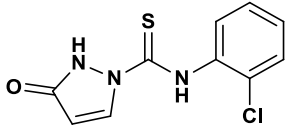
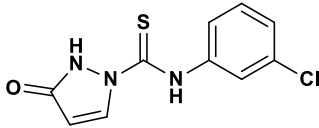
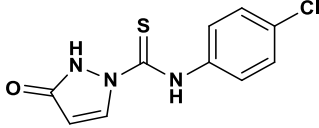
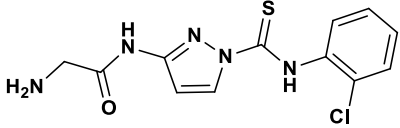
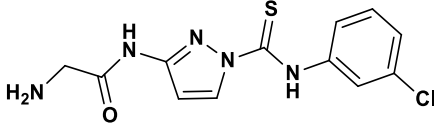
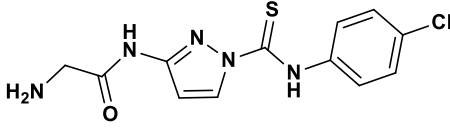
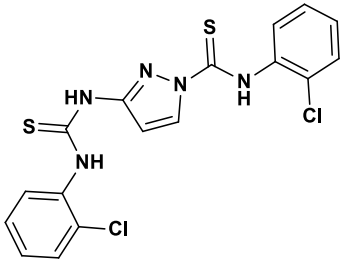
	SB47	1870 M
	SB131	2107 M
	SB132	10000 M
	SB43	51490 M
	SB135	11018 M
	SB136	7250 M
	SB84	1623 M

Table 7, Copper Binding Constants of Monochlorinated NNSN Molecules.

	SB53	13438 M

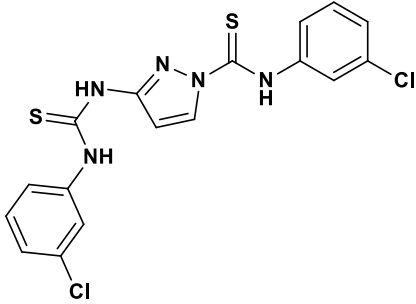
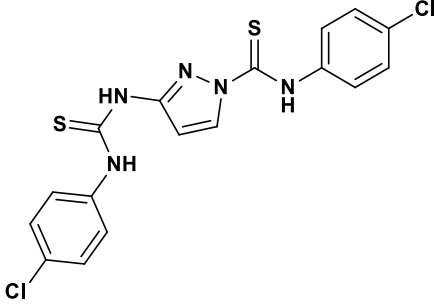
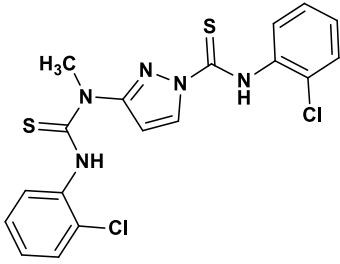
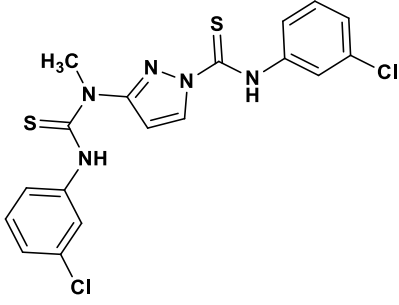
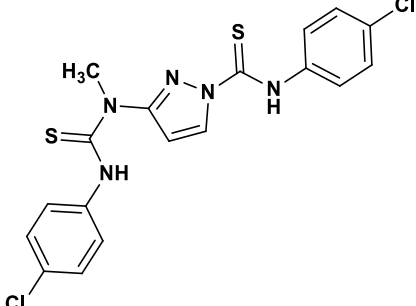
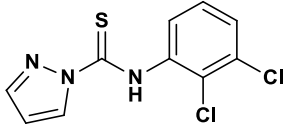
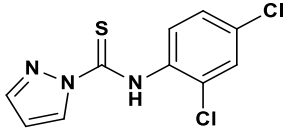
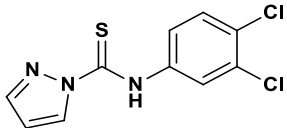
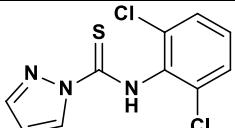
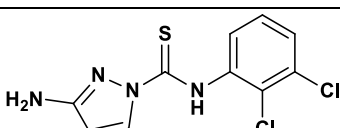
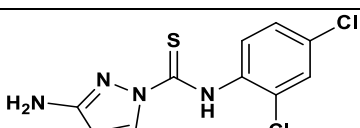
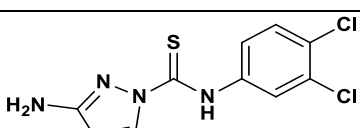
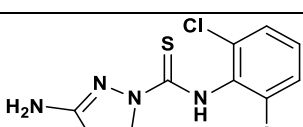
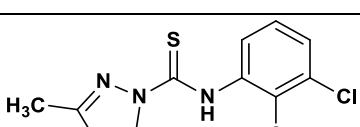
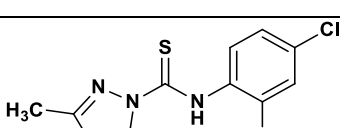
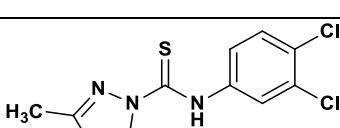
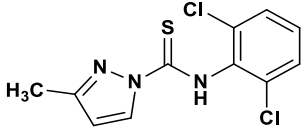
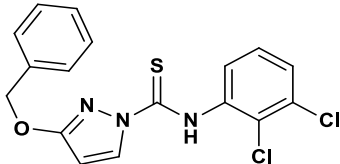
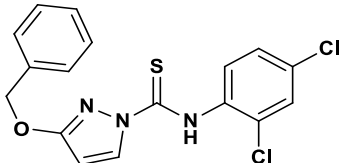
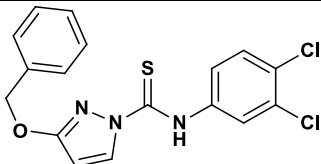
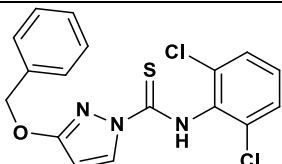
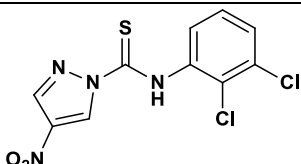
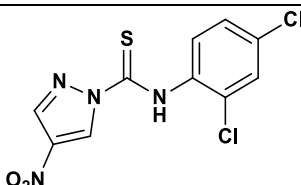
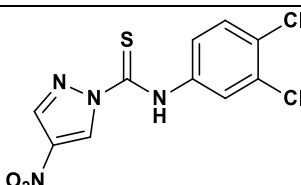
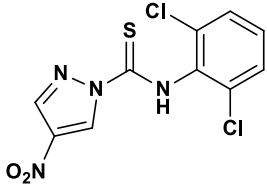
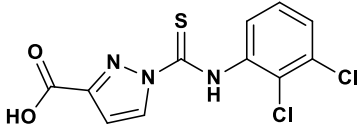
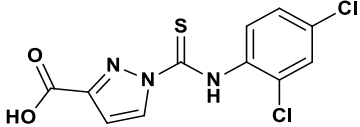
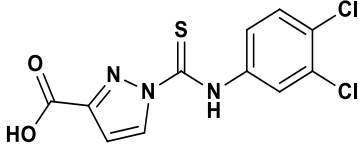
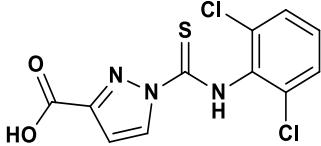
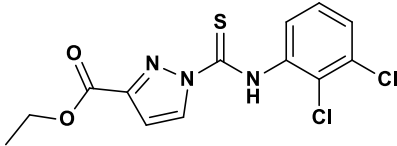
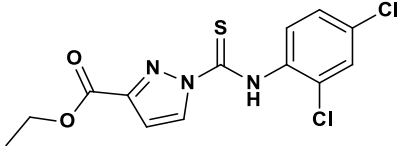
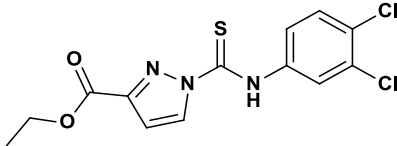
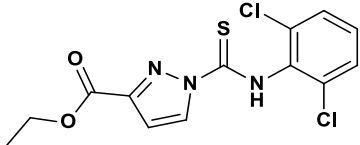
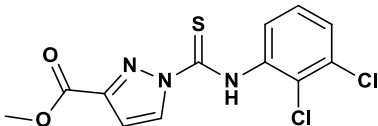
	SB57	1112 M
	SB134	33478 M
	SB55	39730 M
	SB54	5179 M
	SB45	58760 M

Table 8, Copper Binding Constants of Bis-(x-chlorophenyl)thioureido-1H-pyrazole-thioamide Derivatives.

	SB87	10806 M
	SB88	2005 M
	SB89	1996 M
	SB90	2409 M
	SB91	29025 M
	SB92	695 M
	SB93	12935 M
	SB94	229147 M
	SB97	1051 M
	SB98	1053 M
	SB99	1050 M

	SB100	1050 M
	SB101	1203 M
	SB102	1149 M
	SB103	1415 M
	SB104	1048 M
	SB105	274641 M
	SB106	186465 M
	SB107	12582 M

	SB108	5863 M
	SB109	1197 M
	SB110	1190 M
	SB111	1185 M
	SB112	3626 M
	SB113	1961 M
	SB114	240317 M
	SB115	157786 M
	SB116	7988 M
	SB117	93190 M

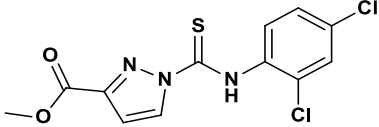
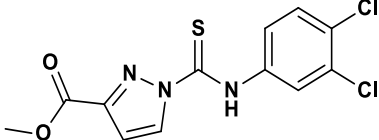
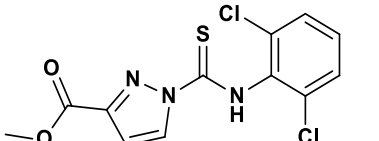
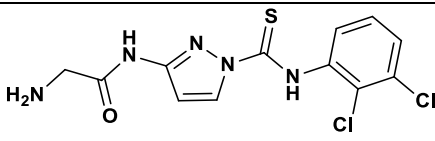
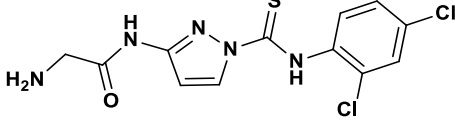
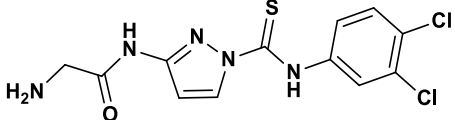
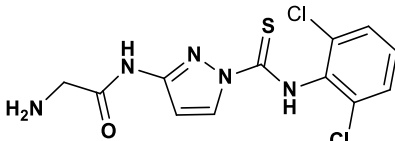
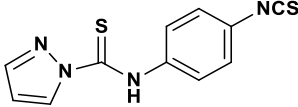
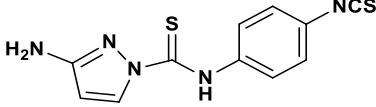
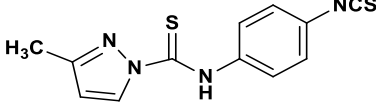
	SB118	7539 M
	SB119	130854 M
	SB120	1493 M
	SB137	6155 M
	SB138	258005 M
	SB139	8255 M
	SB140	16554 M

Table 9, Copper Binding Constants for Dichlorinated NNSNs.

	SB78	98066 M
	SB79	8382 M
	SB82	262110 M

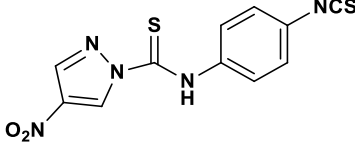
	SB83	624062 M

Table 10, Copper Binding Constants for Diisothiocyanato NNSNs.

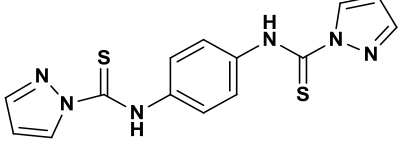
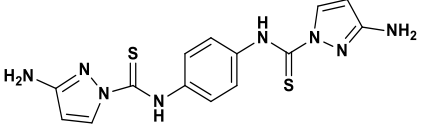
	SB80	10623 M
	SB81	2165 M

Table 11, Copper Binding Constants for Bis-1*H*-pyrazole-1-carbothioamide NNSN Derivatives.

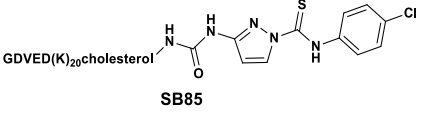
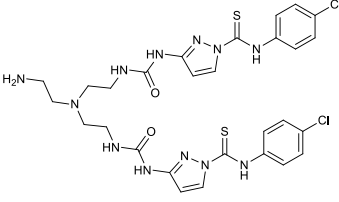
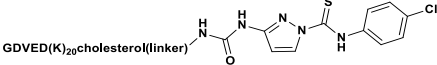
 SB85	SB85	557 M
	SB86	92985 M
	SB96	5298 M

Table 12, Copper Binding Constants for NNSNs with Peptide Nanosponges Components.

Copper Binding Trends

For the copper binding of monochlorinated NNSNs, the trend is not consistent, and changes based on the substituents attached to the pyrazole. If the substituent on the 3-position of the pyrazole is a hydrogen, benzyl ether, dimethylated amine, carboxylic acid, glycine, or a ketone, then the trend is 2-chloro > 3-chloro > 4-chloro. In the case of 3-aminopyrazole, the trend is 4-chloro > 2-chloro > 3-chloro. In the case of 3-methylpyrazole, the trend is 3-chloro > 3-chloro > 2-chloro. If the substituent on the pyrazole is either 3-methyl ester, 3-methyl-5-phenyl pyrazole, or 3-methyl-4-nitropyrazole, then the trend is 3-chloro > 2-chloro > 4-chloro. Lastly, if

the pyrazole has either 3-ethyl ester, 3,5-dimethylated, 3,4,5-trimethylated, or 4-nitro, then the trend is 3-chloro > 4-chloro > 2-chloro. The ITC affects the trend as well; NNSN compounds generated by coupling the 3-aminopyrazole to a 2-, 3-, or 4-benzyl isothiocyanate rather than 2-, 3-, or 4-phenyl isothiocyanate changes the trend. In the case of the benzyl isothiocyanates, the trend is 3-chloro > 2-chloro > 4-chloro while for the phenyl isothiocyanates, it is the exact opposite. These trends are represented in **Table 13** and **Figure 60**.

	2-chloro	3-chloro	4-chloro
pyrazole	8600	2200	2100
3-aminopyrazole	4700	1950	53200
3-methylpyrazole	1200	2200	54020
Methyl ester pyrazole	985	1128	800
Ethyl ester pyrazole	450	2550	1580
3-aminopyrazole + benzyl ITC	11943	17423	3200
benzyl ether pyrazole	5200	18729	25700
dimethyl pyrazole	330	2650	2375
trimethyl pyrazole	520	2375	1280
N-dimethyl pyrazole	1400	3050	58500
3-CA pyrazole	1400	2650	5500
3-methyl-5-phenyl pyrazole	985	3160	550
4-nitropyrazole	770	2800	1100
3-methyl-4-nitropyrazole	2581	5506	1870
1H-pyrazol-3(2H)-one	2107	10000	51490
Glycine + 3-aminopyr-NNSN	11018	7250	1623

Table 13, Copper Binding Trends for Monochlorinated NNSNs.

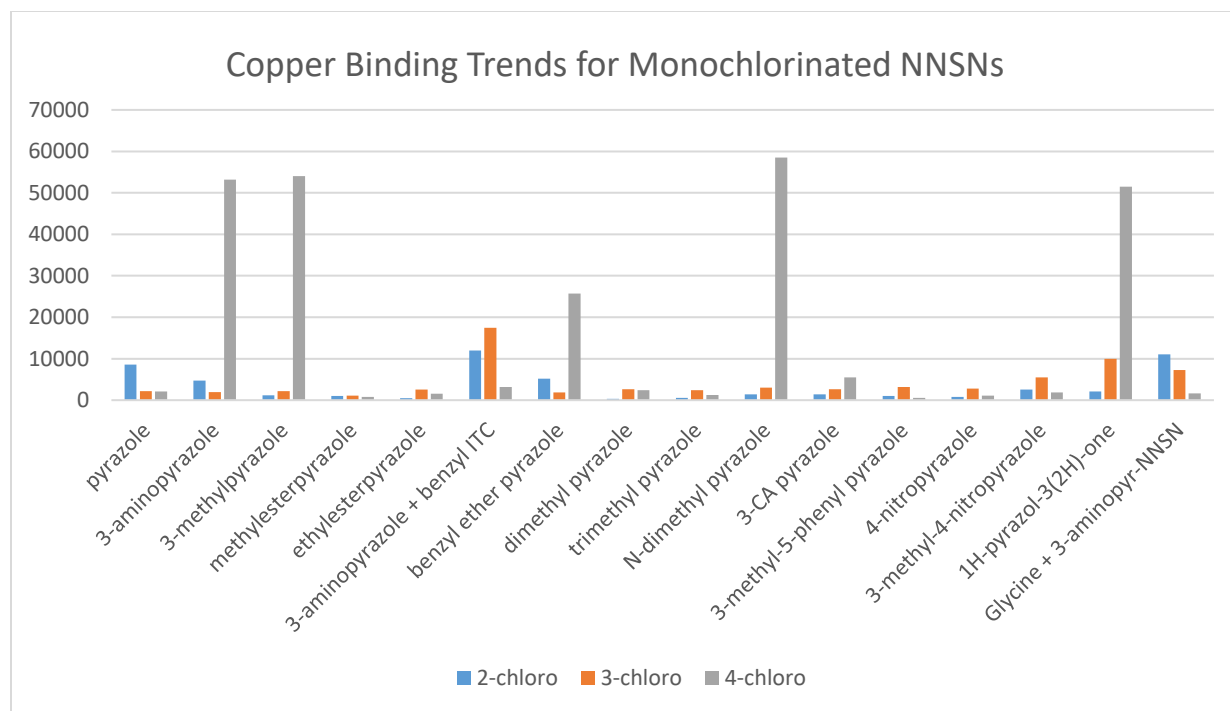


Figure 60, Bar Graph of Copper Binding Trends for Monochlorinated NNSNs.

For the Bis-(x-chlorophenyl)thioureido-1H-pyrazole-thioamide NNSN derivatives, the trend is quite clear and consistent, as seen in **Table 14** and **Figure 61**. The 4-chloro has the highest binding constant, followed by the 2-chloro and lastly the 3-chloro derivatives. Also, when one of the thioamides is methylated, the amount of copper binding increases while maintaining the same trend based on aromatic substitution. The amount of increase in copper binding depends, being about 2.95 times for 2-chloro, 4.65 times for 3-chloro, and 1.75 times for 4-chloro.

	2-chloro	3-chloro	4-chloro
NH Derivatives	13438	1112	33478
N-Me Derivatives	39730	5179	58760

Table 14, Copper Binding Trends for Bis-(x-chlorophenyl)thioureido-1H-pyrazole-thioamide Derivatives.

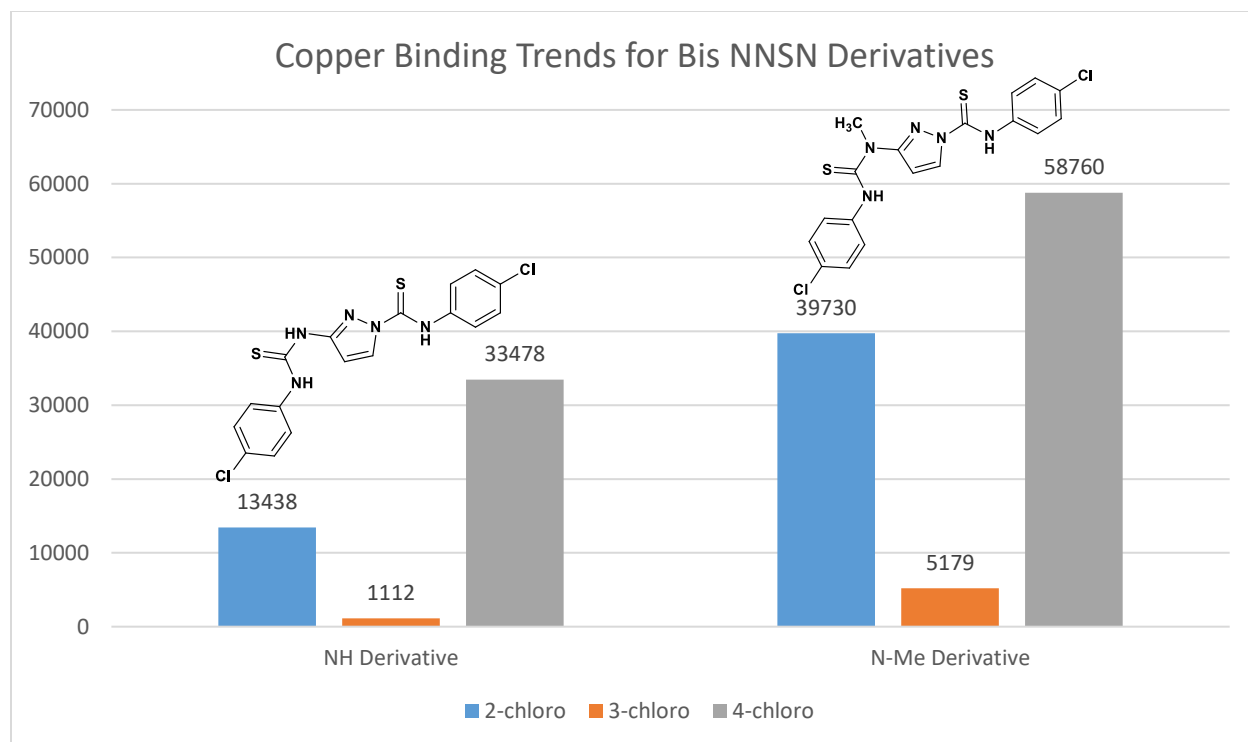


Figure 61, Bar Graph of Copper Binding Trends for Bis-(x-chlorophenyl)thioureido-1H-pyrazole-thioamide Derivatives.

For the copper binding constants of dichlorinated NNSNs, the trends are again not consistent across the board. It depends on the pyrazole used to synthesize the NNSN. If the pyrazole is unsubstituted, the trend is 2,3-Cl > 2,6-Cl > 2,4-Cl > 3,4-Cl. If a 3-aminopyrazole is used, the trend is 2,6-Cl > 2,3-Cl > 3,4-Cl > 2,4-Cl. For the 3-methylpyrazole, the trend is 2,4-Cl > 2,3-Cl > 3,4-Cl = 2,6-Cl. For either the ether benzyl pyrazole or the methyl ether pyrazole, the trend is 3,4-Cl > 2,3-Cl > 2,4-Cl > 2,6-Cl. For 3-nitropyrazole, the trend is 2,3-Cl > 2,4-Cl > 3,4-Cl > 2,6-Cl. The 3-carboxylic acid pyrazole shows a trend of 2,6-Cl > 2,3-Cl > 2,4-Cl > 3,4-Cl. Lastly, the ethyl ether pyrazole has a trend of 2,4-Cl > 3,4-Cl > 2,6-Cl > 2,3-Cl. These trends are shown in **Table 15** and **Figure 62**.

	2,3-Cl	2,4-Cl	3,4-Cl	2,6-Cl
pyrazole	10806	2005	1996	2409
3-aminopyrazole	29025	695	12935	229147
3-methylpyrazole	1051	1053	1050	1050
ether benzyl pyrazole	1203	1149	1415	1048

4-nitropyrazole	274641	186465	12582	5863
3-CA pyrazole	1197	1190	1185	3626
ethyl ether pyrazole	1961	240317	157786	7988
methyl ether pyrazole	93190	7539	130854	1493
Glycine + 3-aminopyr-NNSN	6155	258005	8255	16554

Table 15, Copper Binding Trends for Dichlorinated NNSNs.

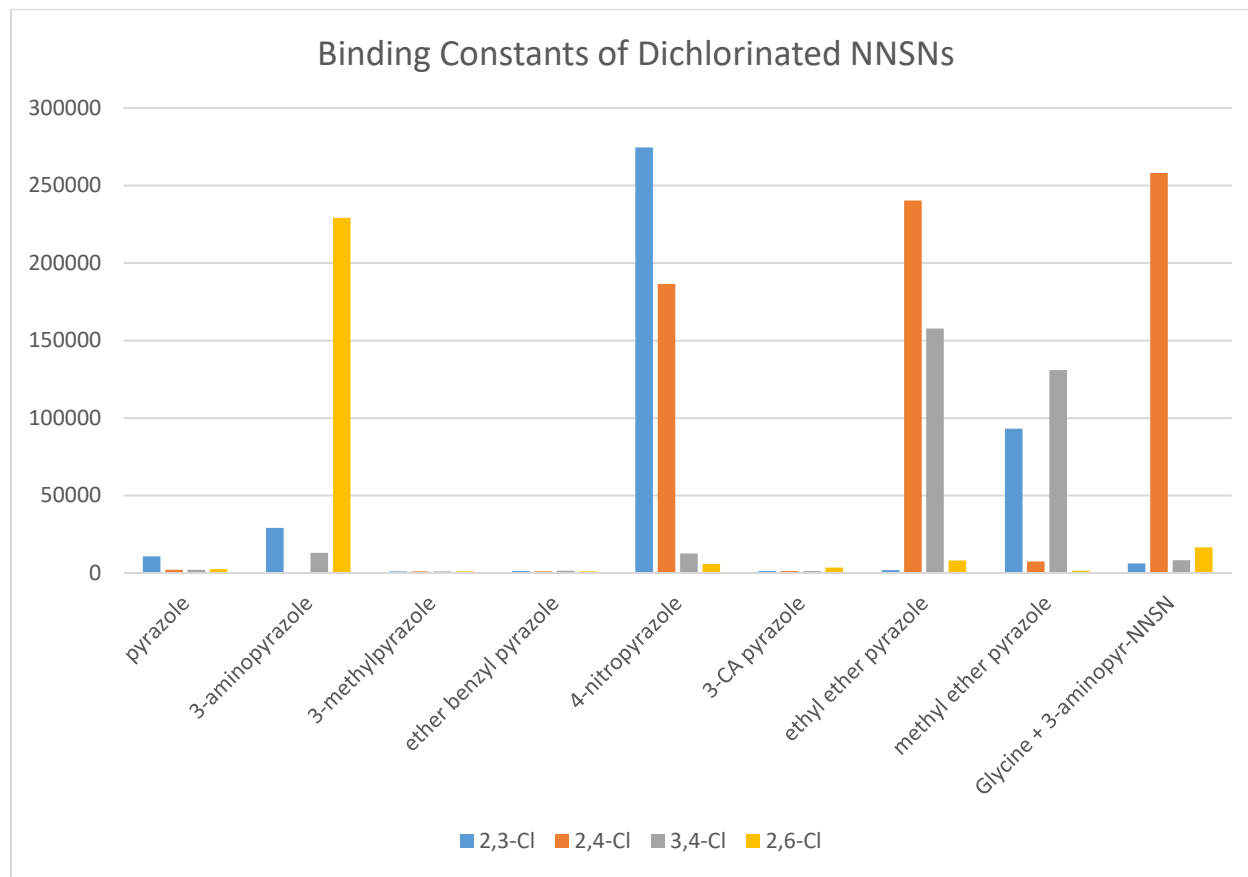


Figure 62, Bar Graph for Copper Binding Trends for Dichlorinated NNSNs.

Lastly, the trends for the Diisothiocyanato and the Bis-1H-pyrazole-1-carbothioamide NNSN derivatives are shown in **Table 16** and **Figure 63**. In this category, SB83 shows a definitive affinity for copper(I) as well as SB82 and SB78. The other compounds, namely SB79, SB80, and SB81 show a moderate affinity toward copper(I).

SB#	Cu Binding Constant
SB78	98066
SB79	8382
SB80	10623
SB81	2165
SB82	262110
SB83	624062

Table 16, Copper Binding Constants for Diisothiocyanato NNSNs (SB78, SB79, SB82, and SB83) and Bis-1H-pyrazole-1-carbothioamide (SB80 and SB81).

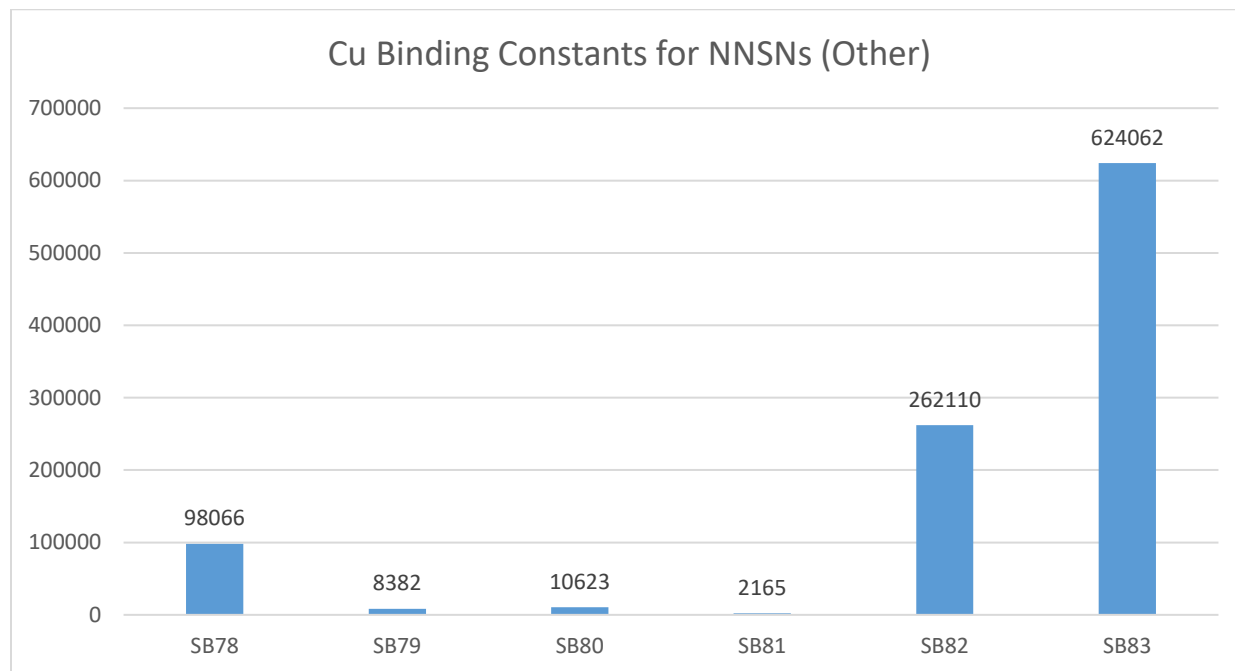


Figure 63, Bar Graph for Copper Binding Trends for Diisothiocyanato NNSNs (SB78, SB79, SB82, and SB83) and Bis-1H-pyrazole-1-carbothioamide (SB80 and SB81).

NNSNs show about 10 magnitudes lower binding constant values overall than the binding constants of Cu⁺ binding proteins. This means that the NNSNs cannot compete for the stored copper in the body through these proteins. Known chaperones include Cox11, Cox17 and Sco1 proteins for copper delivery to the CuA and CuB of CcO, CCS for copper delivery to Cu, Zn-superoxide dismutase, and Atx1 for copper delivery to the P1-type ATPase N termini.⁵ This specificity is key because it allows these drugs to be administered with less potential for side effects.

Chapter 3 References

1. Bossmann, S.; Kalubowilage, M.; Zhang, M.; Delpe-Archarige, A.; Eschliman, K.; Wolschendorf, F.; Kutsch, O.; Dalecki, A.; Narmore, W.; Kansas State University Research Foundation and The University of Alabama at Birmingham Research Foundation, USA, assignees. Compounds with copper- or zinc-activated toxicity against microbial infection, filed Sept. 20, 2018.
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5. Inesi, G. Molecular features of copper binding proteins involved in copper homeostasis <https://iubmb.onlinelibrary.wiley.com/doi/full/10.1002/iub.1590>.

Chapter 4 – Conclusion

Bacterial infections are a serious health issue worldwide and the number of cases is anticipated to continue rising. Antibiotic resistance is a serious, complicated problem due to the ability of germs to defeat the drugs that are designed to kill them. New therapies are required to combat this issue immediately. Cancer is also a massive health issue around the world that causes one in every four deaths in the United States alone. While early diagnosis leads to better prognostic outcomes, effective treatments are equally important in the treatment of cancers. Cancer and bacterial infections are interrelated in that cancer patients are at an increased risk of obtaining antibiotic resistant infections due to their compromised immunity secondary to treatments such as chemotherapy, radiation, or surgical intervention.

One solution to treat both bacterial infections and cancers is using copper-activated drugs. Copper is an endogenous metal that required strict homeostatic regulation to maintain physical health. Copper is important as it is involved in immune system function, processing of cholesterol, red blood cell formation, maintenance of blood vessels, nerves, healthy bones, and the development of babies in utero. A lack of copper can lead to anemia and osteoporosis while large amounts can cause hepatitis, kidney problems, and brain disorders; it is recommended for adults over 19 to intake 900 micrograms per day of copper from dietary sources to maintain a healthy level. Copper is strictly controlled in the blood because intracellular copper can generate hydroxyl radicals. It is widely recognized that free radicals can damage proteins, nucleic acids, and lipids, as well as potentially interfere with the synthesis of iron-sulfur clusters. Further, copper has been shown to displace metals such as zinc from their cognate ligands in metalloproteins – this can cause serious problems like inhibition of enzymatic activity.

A method used to harness copper's natural antimicrobial properties is by using copper-dependent inhibitors of bacterial infections such as *S. aureus*, such as the novel family of molecules containing an NNSN motif. NNSN molecules are a novel, patented class of drugs with either a classic or novel extended thiourea motif termed the NNSN motif because it contains these heteroatoms in a row. There is currently 134 derivatives in a small library. Despite not having any stereocenters, these small molecule drugs exhibit copper-activated reactivity toward bacterial infections (including *S. aureus* (Gram-positive), *Escherichia Coli* (Gram negative)), and *M. tuberculosis*, and cancerous murine cell lines (Melanoma (B16F10), Glioblastoma (GL26), and Pancreatic Cancer (Pan02 and KPC)). These drugs are synthesized by coupling a pyrazole ring and phenyl isothiocyanate together via one of seven typical methods; the recommended method depends on the substituents found on the pyrazole ring. Most derivatives will then require purification, either by recrystallization or flash column chromatography. These novel compounds were characterized using Proton (^1H) Nuclear Magnetic Resonance (NMR) Spectroscopy, ^{13}C NMR, Infrared (IR) spectroscopy, and either High Resolution Mass Spectroscopy (HRMS) or Ultra Performance Liquid Chromatography-Mass Spectroscopy (UPLC-MS).

Some of the most effective derivatives for the treatment of *E. Coli* (results discussed further in Chapter 2) are those where KE03 was combined with components of the peptide nanosponge. The peptide nanosponge is synthesized by beginning with the trimaleimide scaffold. This is produced in three steps, namely Diels Alder ring formation followed by reaction with tris(2-aminoethyl)amine in methanol to produce the trimaleimic acid adduct, then finally the adduct was refluxed in toluene to yield the desired product. The peptide (K₂₀ or D₂₀ with cholesterol linkage in this case) is synthesized using solid phase peptide synthesis, along with the

consensus sequence for executioner caspases (DEVDGC). The completed sequence is connected to the trimaleimide linker by Michael addition in deoxygenated PBS buffer (pH 7.4).

NNSNs were tested for biological activity on bacteria such as Methicillin-sensitive *S. aureus* (MSSA), *M. tuberculosis* (MTB), and *E. Coli*. A Structure-Activity Relationship (SAR) study was performed using the data for MSSA strain S. A. Newman, a clinical isolate that was tested for MIC of various NNSNs in the group of Prof. Dr. Frank Wolschendorf and since 2018 in the group of Prof. Dr. Olaf Kutsch in the Departments of Medicine and Microbiology at the University of Alabama at Birmingham by the procedure described on page 74. The same protocols were followed at the University of Alabama at Birmingham using *E. Coli* (W3110, lab-adjusted) strain for the MIC determinations. Select NNSNs were also tested against *M. tuberculosis* (Avirulent Mtb strain mc²6206 (H37Rv Δ panCD Δ leuCD, double auxotroph) by Mitra Avishek in the Neiderwies group based in the Microbiology Department of the University of Alabama at Birmingham.

During the SAR study, 65 total NNSN compounds were tested with various structural changes and differing aromatic substitution of the chlorine atom on the aromatic ring. Out of the 65 compounds that were tested, there were 34 compounds with an MIC under 5 micromolar and 8 compounds with an MIC of exactly 5 micromolar. Out of these 42 new drug candidates that could be used to treat MSSA (S.A. Newman), there are 2 best candidates (outlined in red) that show activity in the nanomolar range (SB45 and SB93), as shown in **Figure 64**.

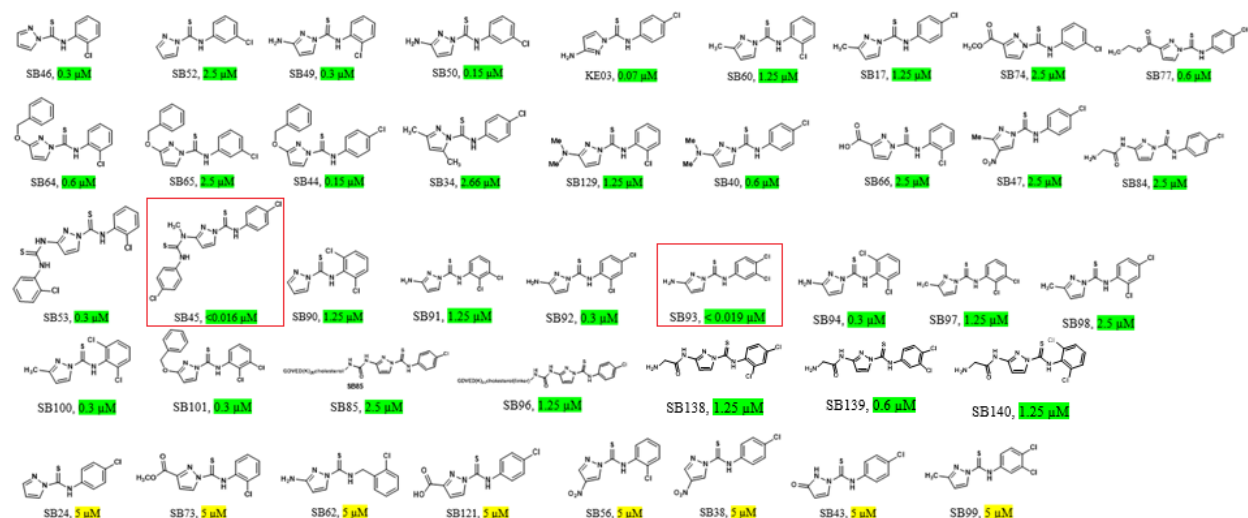


Figure 64, 42 new drug candidates that could be used to treat MSSA (S.A. Newman) with 2 best candidates (outlined in red) that show activity in the nanomolar range (SB45 and SB93).

Select NNSNs were tested using *E. Coli* (W3110, lab-adjusted) strain for the MIC determinations. Out of the 20 select NNSNs that were tested against this strain, 2 candidates emerged. *E. Coli* is a gram-negative bacterium that is more difficult to kill, for this reason SB85 and SB96 have been marked as candidates because they show activity of 10 micromolar or lower, as shown in **Figure 65**.

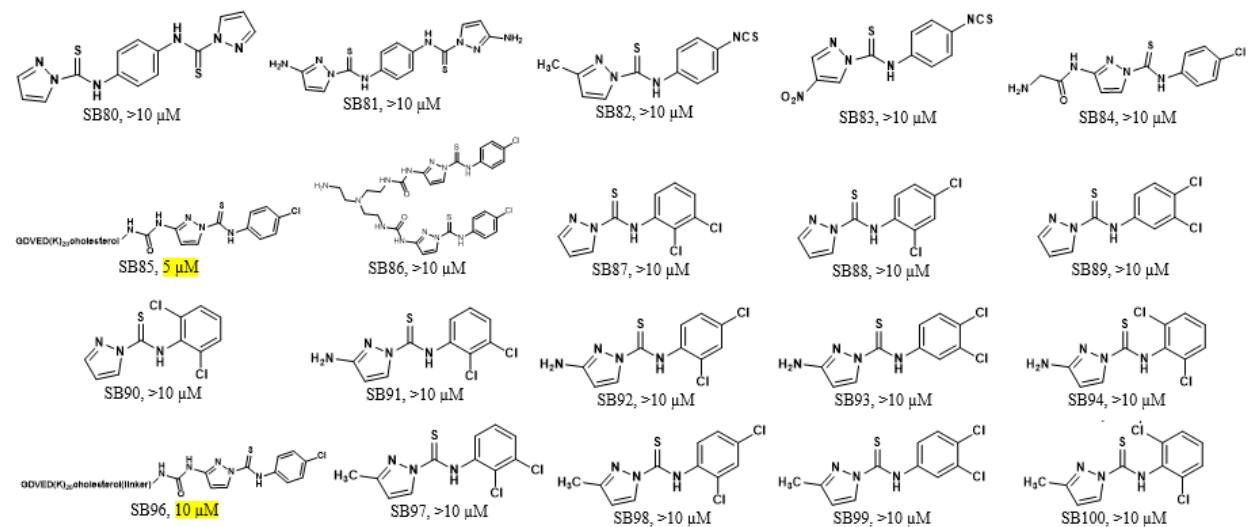


Figure 65, 2 new drug candidates emerge for the treatment of *E. Coli*.

Select NNSNs were tested against *Mycobacterium tuberculosis* at the University of Alabama at Birmingham by Mitra Avishek in the Neiderwies groups in the Microbiology department. Out of the five NNSNs that were tested, 3 candidates were found that had values of 2 micromolar or less, as shown in **Figure 66**.

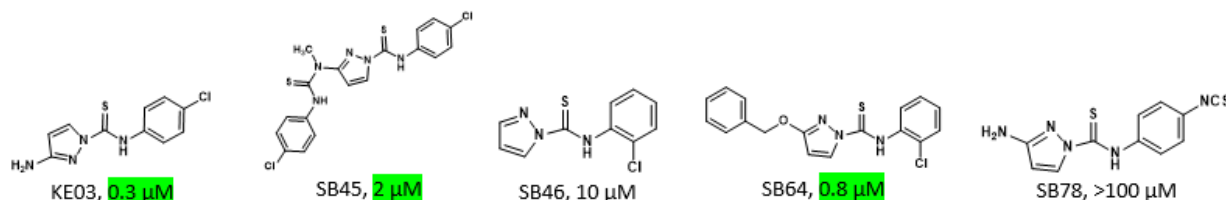


Figure 66, 3 new drug candidates found for the treatment of *Mycobacterium tuberculosis*.

Select NNSN molecules (KE03, SB40, SB45, SB46, SB57, SB64, SB77, and SB129) have proven to have activity against some cancer cells. To obtain the LC_{50} values against cancerous and noncancerous cells, fellow Bossmann group researchers Dr. Obdulia Covarrubias-Zambrano and Ivina Mali adhered to the protocol detailed on page 87. The results for the five different murine (mouse) cell lines that were tested, including Neural Stem Cells (NSC), Melanoma (B16F10), Glioblastoma (GL26), and Pancreatic Cancer (Pan02 and KPC) are shown in **Figure 67**. NSCs are a non-cancerous cell line that act as a control for these experiments. Note that LC_{50} is the concentration of the chemical that will kill 50% of the given biological component with a single exposure. The lowest concentration is desired because this will kill the cancer or inhibit bacterial growth most effectively. From these results, it was determined that for melanoma (B16F10), the best candidate is SB57 which showed a very low LC_{50} . For GL26, the best candidate was SB45. For PAN02, the best candidate was also SB45. For KPC, the best candidate was SB77, showing an LC_{50} in the nanomolar range. Those showing dashes were not run due to the cells not being viable and ready to use at the time that these experiments were performed. Also note that for most of these, the difference seen when the copper is absent versus

when it is present is rather large, like for example the results for SB57 on B16F10; when copper is absent, the value is 278,456 but in the presence of copper it dropped all the way to 0.001325!

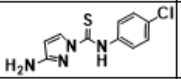
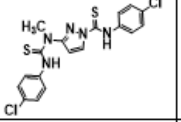
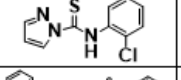
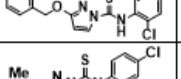
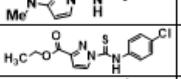
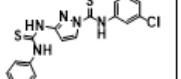
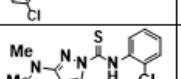
NNSN		NSC		B16F10		GL26		Pan02		KPC	
		- Cu	+ Cu	- Cu	+ Cu	- Cu	+ Cu	- Cu	+ Cu	- Cu	+ Cu
KE03		2.66	1.61	0.74	0.29	12,314	0.28	1,000	0.23	1.62	0.11
SB45		5.50	0.42	0.50	0.10	1.37	0.05	1,380	0.17	0.13	0.15
SB46		0.51	1.71	0.21	0.11	0.10	0.28	0.30	0.21	0.64	0.007
SB64		82,370	0.88	0.1	0.34	1.58	0.17	0.61	0.25	0.55	0.32
SB40		-	-	0.23	0.44	-	-	1.88	1500	0.24	1.42
SB77		-	-	274359	570531	-	-	0.17	5.14	0.0078	0.000026
SB57		-	-	278456	0.001325	-	-	0.012	2320	0.00073	1.699
SB129		-	-	6436	3260	-	-	0.0057	2.28	0.0076	2.197

Figure 67, Results for application of various NNSNs on five different murine (mouse) cell lines, including Neural Stem Cells (NSC), Melanoma (B16F10), Glioblastoma (GL26), and Pancreatic Cancer (Pan02 and KPC).

To explain the theoretical mode of action, it must be noted that NNSNs are non-toxic until activated by copper, the necessary concentration of which only exists naturally in the biological environment at the site of infection or within a tumor microenvironment. In both cases, the copper concentration has shown to increase by 5-50 times that found in normal tissues. The copper concentration and its distribution throughout cancer cells is directly related to a tumor's growth, angiogenesis, and metastasis. The cell membranes have developed means to remove the copper through efflux pumps. The NNSN-copper complex forms an iminium-type cation, which is then able to impair numerous enzymes of the cell. Iminium cations are potent warheads because they react with multiple targets through nucleophilic addition, electron

transfer, and radical reactions. This effectively shuts down the efflux pumps that remove copper from the cell. The copper floods into the cell and leads to cell death.

The early idea of how copper would bind to a general NNSN structure is shown in **Figure 68**. This initial structure was based on the data obtained through NMR titrations (which were performed by dissolving the NNSN in deuterated acetone and adding aliquots of copper(I) bromide solution of known concentration to monitor how the structure changes). These titrations also observed 1:1 complexation geometry.

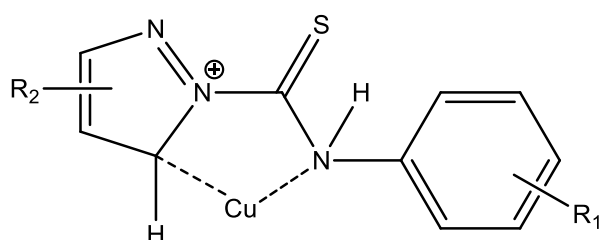


Figure 68, Initial structure was based on the data obtained through NMR titrations.

DFT calculations using the COSMO model of solvation were used to optimize the geometries of select NNSN structures and the possible sites for copper(I) complexation on the isomers were identified and these complexes were manually created in MacMolPlt. These isomers were also submitted for optimizations and calculations to identify the most stable copper-NNSN complexes. Overall, this study revealed a motif of copper complexation with sulfur and either nitrogen or carbon, with the exception being isomer11_9. This isomer presents an interesting example in that it is unique to the rest of the group. The copper is shown to be accepting electrons from the aromatic ring and the nitrogen of the pyrazole ring. This causes two carbons of the aromatic ring to donate pi electrons, ultimately disturbing the symmetry of the ring. Due to this disturbance in symmetry, the isomer is less stable and shows a much higher energy. Further, the copper ion is not interacting with the sulfur atom in this isomer, while it was consistently with the other five isomers. This observation further adds to the high energy

observed, since sulfur is a better ligand to complex with copper than either nitrogen or carbon. Another common theme seen in this study is that the angle of the bonds form part of a square planar geometry, which is the preferred structure for the copper (I) ion.

The early complexation structure based upon NMR titration data, shown in **Figure 68**, suggested that the copper would interact with the pyrazole -CH nearest the carbonothioyl group and with the -NH originating from the isothiocyanate. This structure is odd in that it does not suggest any interaction between the copper and areas of high electron density such as the sulfur, which will occur most of the time as shown in the computer modeling study. Other expected areas for copper to be attracted are the lone electron pair of the nitrogen in the pyrazole ring and the pi electrons in the carbon ring, of which neither are shown in this early structure determination.

We decided to collaborate further with the Aikens research group to add to the understanding about the geometry of the NNSN-copper complexes when compared to experimental UV visible spectroscopy and ^1H Nuclear Magnetic Resonance spectroscopy data. This study was performed by Gowri Kuda Singappulige, utilizing DFT calculations with B3LYP exchange correlation function. These calculations used the COSMO implicit solvent model and included long-range corrections. Isomer 1 was found to be the most stable for ortho-Cl and Isomer 3 was found most stable for meta-Cl and para-Cl. The relative energies of the five isomers bound to copper(I) were then calculated using the same methodology. The rotation around the C-N bond proved different when the compound was bound to copper. All five isomers were tested for ortho-Cl derivatives, but with the other derivatives, the focus was shifted to Isomer 1 and Isomer 3 because these were found to be the most stable. The calculations for optical spectra of the five copper-bound isomers, which were directly compared to the

experimental UV visible spectra. The spectral shapes that were calculated matched the experimental spectra for all isomers, except for Isomer3-ori4. From this data, it was concluded that for ortho-Cl, the most likely isomer is Isomer1-ori1; for meta-Cl, either are possible; for para-Cl, the most likely isomer is isomer3-ori2. Finally, theoretical NMR values were calculated by Gowri Kuda Singappulige and compared to experimental spectra for a simple NNSN structure, namely SB19. Out of the two conformers, the data suggests that the isomer 6 is the true confirmation of SB19. The theoretical data matches very accurately with the experimental spectra.

The conclusions drawn for this computational study confirmed a planar geometry is preferred in the structures where hydrogen bonding is possible. There was no significant difference noted in energetical stability and spectra properties when aromatic substitution changes were applied, with the exception being for ortho-Cl. In the ortho-Cl isomers, geometrical differences led to spectra changes. The rotation around the C-N bond varied between the Cu-bound and no-Cu systems. This is believed to be due to the preference of copper to bind with the nitrogen of the pyrazole ring and sulfur. Lastly, the origin of peaks in optical spectra show significant contribution from copper.

For the calculation of copper binding constants, the first step in measuring copper binding constants is to prepare some solutions, namely HEPES buffer and Copper(I) bromide [0.00011 M]. The amount of NNSN must then be calculated according to the equation shown. Once this amount has been weighed, it should be dissolved in 200 microliters of methanol and sonicated until fully dissolved, then add 1800 microliters of HEPES buffer. Upon preparation of these solutions, then a concentration gradient must be made, which will be run using UV visible spectroscopy. Once all the samples in the concentration gradient have been run for an NNSN

sample, SB63 for example, the data should form a graph. Ideally, a sharp, symmetrical peak will form. In some cases, more than one peak can be seen. If more than one peak is present, the sharpest peak is chosen. Four wavelengths covering this peak are inputted into an Excel sheet and then run on the UV 1:1 Bindfit program from Supramolecular.com. If the run is successful, then an optimized value will be generated which is considered the Copper Binding Constant for that sample.

For the copper binding of monochlorinated NNSNs, the trend is not consistent, and changes based on the substituents attached to the pyrazole. If the substituent on the 3-position of the pyrazole is a hydrogen, benzyl ether, dimethylated amine, carboxylic acid, glycine, or a ketone, then the trend is 2-chloro > 3-chloro > 4-chloro. In the case of 3-aminopyrazole, the trend is 4-chloro > 2-chloro > 3-chloro. In the case of 3-methylpyrazole, the trend is 4-chloro > 3-chloro > 2-chloro. If the substituent on the pyrazole is either 3-methyl ester, 3-methyl-5-phenyl pyrazole, or 3-methyl-4-nitropyrazole, then the trend is 3-chloro > 2-chloro > 4-chloro. Lastly, if the pyrazole has either 3-ethyl ester, 3,5-dimethylated, 3,4,5-trimethylated, or 4-nitro, then the trend is 3-chloro > 4-chloro > 2-chloro. The ITC affects the trend as well; NNSN compounds generated by coupling the 3-aminopyrazole to a 2-, 3-, or 4-benzyl isothiocyanate rather than 2-, 3-, or 4-phenyl isothiocyanate changes the trend. In the case of the benzyl isothiocyanates, the trend is 3-chloro > 2-chloro > 4-chloro while for the phenyl isothiocyanates, it is the exact opposite.

For the Bis-(x-chlorophenyl)thioureido-1H-pyrazole-thioamide NNSNs, the 4-chloro has the highest binding constant, followed by the 2-chloro and lastly the 3-chloro derivatives. Also, when one of the thioamides is methylated, the amount of copper binding increases while maintaining the same trend based on aromatic substitution. The amount of increase in copper

binding depends, being about 2.95 times for 2-chloro, 4.65 times for 3-chloro, and 1.75 times for 4-chloro.

For the copper binding constants of dichlorinated NNSNs, the trends are again not consistent across the board. It depends on the pyrazole used to synthesize the NNSN. If the pyrazole is unsubstituted, the trend is 2,3-Cl > 2,6-Cl > 2,4-Cl > 3,4-Cl. If a 3-aminopyrazole is used, the trend is 2,6-Cl > 2,3-Cl > 3,4-Cl > 2,4-Cl. For the 3-methylpyrazole, the trend is 2,4-Cl > 2,3-Cl > 3,4-Cl = 2,6-Cl. For either the ether benzyl pyrazole or the methyl ether pyrazole, the trend is 3,4-Cl > 2,3-Cl > 2,4-Cl > 2,6-Cl. For 3-nitropyrazole, the trend is 2,3-Cl > 2,4-Cl > 3,4-Cl > 2,6-Cl. The 3-carboxylic acid pyrazole shows a trend of 2,6-Cl > 2,3-Cl > 2,4-Cl > 3,4-Cl. Lastly, the ethyl ether pyrazole has a trend of 2,4-Cl > 3,4-Cl > 2,6-Cl > 2,3-Cl.

For the Diisothiocyanato and Bis-1H-pyrazole-1-carbothioamide NNSNs, SB83 shows a definitive affinity for copper(I) as well as SB82 and SB78. The other compounds, namely SB79, SB80, and SB81 show a moderate affinity toward copper(I).

NNSNs show about 10 magnitudes lower binding constant values overall than the binding constants of Cu⁺ binding proteins. This means that the NNSNs cannot compete for the stored copper in the body through these proteins. Known chaperones include Cox11, Cox17 and Sco1 proteins for copper delivery to the CuA and CuB of CcO, CCS for copper delivery to Cu, Zn-superoxide dismutase, and Atx1 for copper delivery to the P1-type ATPase N termini. NNSNs can only be activated when the body releases copper, such as in the case specifically when it is attempting to fight intruders, such as at the site of bacterial infection or cancerous tumors. This specificity is key because it allows these drugs to be administered with less potential for side effects.

Highlights

- NNSN Molecules are a novel family of copper-dependent inhibitors that were initially discovered by a novel concerted screening effort employed by Dalecki and coworkers
- A small library of 134 NNSN derivatives has been synthesized and characterized (88 derivatives by Kayla)
- These NNSNs have proven effective at killing infections such as Methicillin-sensitive *Staphylococcus aureus* (MSSA), *Mycobacterium tuberculosis* (MTB), and *Escherichia coli*; biological activity has also been demonstrated on murine cancer cell lines for melanoma, glioblastoma, and pancreatic cancers
- The mode of action for NNSNs is theorized to be due to the formation of an iminium cation upon copper complexation, which will occur selectively at the site of bacterial infection or cancerous tumor(s)
- NNSNs are uniquely selective, making the probability of harmful side effects significantly lowered
- Computer Modeling Studies have been performed to understand the interaction between NNSN compounds and copper to elucidate complexation structures
- Copper Binding Constants were calculated by running a gradient of solutions via UV visible spectroscopy then running through Supramolecular.com

Future Work

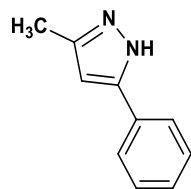
1. Receiving the data that is still marked as “Pending” in the SAR study from our collaborators would really increase our understanding of these trends and could reveal new candidates to treat MSSA.
2. The library of compounds can be increased, with more derivatives being synthesized and characterized to further understand the importance of substituents on the pyrazole ring and the role of aromatic substitution. There is still not a totally clear trend, but more data would help in this endeavor.
3. An *in vivo* study on mice would be ideal to further test the efficacy of the NNSN compounds.

Appendix A

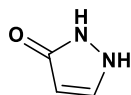
Experimental Section

Unless otherwise noted, all commercial reagents and solvents were purchased with the highest purity grade from Fisher Scientific Inc. 2-chlorobenzyl-isothiocyanate, 3-chlorobenzyl-isothiocyanate, 4-chlorobenzyl-isothiocyanate, 1,4-diisothiocyanatobenzene-1*H*-pyrazole, 1*H*-pyrazole, 3-(trifluoromethyl)-1*H*-pyrazole, 1*H*-pyrazol-3-amine, 3-methyl-1*H*-pyrazole, 2-hydroxypropane-1,2,3-tricarboxylic acid, and 1*H*-1,2,3-triazole were purchased from Aldrich and used without further purification unless specified. 4-iodo-1*H*-pyrazol-3-amine, 1-methyl-1*H*-pyrazol-5-amine, 1-phenyl-1*H*-pyrazol-5-amine, 4-bromo-1*H*-pyrazole, 1*H*-pyrazol-4-amine, 3-((4-nitrobenzyl)oxy)-1*H*-pyrazole, benzo[c][1,2,5]thiadiazol-4-amine, and 2-isothiocyanato-1,3-dimethylbenzene were synthesized by other researchers on the NNSN team. Solvents used mainly N,N-Dimethylformamide (DMF), Dichloromethane (Methylene Dichloride; DCM) and Tetrahydrofuran (THF) were distilled or purchased in high purity and anhydrous condition. Silica gel (200–300 meshes) was used for column chromatography, and TLC inspections were taken on Silica XHL TLC Plates, w/UV254, glass backed, 250um. All the reagents and products were stored under Argon atmosphere. The synthesized compounds were stored in a -20 °C freezer. ¹H (400, 600 MHz) and ¹³C (100, 151 MHz) spectra were obtained on Bruker-Ascend spectrometers. Infrared spectra were recorded with a Nicolet 380 FT-IR. HRMS (+ESI-TOF) measurements were performed using an LCT Premier instrument at the University of Kansas. UPLC-MS measurements were obtained on a Waters Acquity TQD Tandem Quadrapole Ultra Performance Liquid Chromatography Mass Spectrometry/Mass Spectrometry machine using a C18 reversed phase organic column.

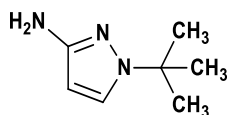
Pyrazole Synthetic Procedure



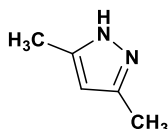
3-methyl-5-phenyl-1H-pyrazole: 1-benzoylacetone was (500 mg, 3.08 mmol) dissolved in EtOH and started to stir. While it was being stirred, (1eq) of hydrazine mixed with EtOH was added to the stirring mixture. The reaction mixture was refluxed for 3 hr. The progress of the reaction was monitored by TLC (50% EtOAc/hexane). No further purification was needed as TLC showed only single product spot. All the volatile components were removed in *vacuo* and obtained light yellow solid product (370 mg, 76%). ¹H NMR (400 MHz, DMSO-*d*₆) δ 7.73 (d, *J* = 7.6 Hz, 2H), 7.36 (t, *J* = 7.6 Hz, 2H), 7.25 (t, *J* = 7.4 Hz, 1H), 6.41 (s, 1H), 2.23 (s, 3H). ¹³C NMR (151 MHz, DMSO-*d*₆) δ 150.86, 139.94, 134.41, 129.03, 127.57, 125.40, 101.44, 10.97.



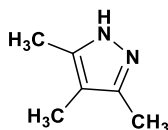
1H-pyrazol-3(2H)-one: To a solution of methyl-*trans*-3-methoxyacrylate (1.05 g, 9.03 mmol) in ethanol (10 mL) was added hydrazine hydrate (540 mg, 10.8 mmol). The reaction was kept refluxing under argon overnight. The resulting residue was concentrated to dryness and recrystallized with diethyl ether to give a light-yellow solid (770 mg, 99%). ¹H NMR (400 MHz, DMSO-*d*₆) δ 10.39 (s, 1H), 7.33 (dd, *J* = 2.3, 0.6 Hz, 1H), 5.41 (dd, *J* = 2.2, 0.6 Hz, 1H). ¹³C NMR (101 MHz, DMSO-*d*₆) δ 161.58, 89.96.



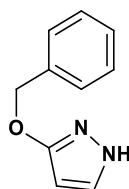
1-tert-butyl-1H-pyrazol-3-amine: A solution of 1.54 g (12.40 mmol, 1 equiv.) tert-butylhydrazine hydrochloride in 30 mL of distilled water was cooled to 0° C. Then 1.71 g (12.40mmol, 1 equiv.) K₂CO₃ and 2.08 g (24.80 mmol, 2 equiv.) NaHCO₃ were added sequentially. The mixture was kept stirring at 0°C for 1h. The resulting solution was clear and colorless. 1.09 g (12.40 mmol, 1 equiv.) 2-chloroacrylonitrile was added at 0°C. The reaction mixture was warmed to RT and then stirred for 12 h. After this time, the solution turned out to be light yellow. The solution was then extracted with ethyl acetate (5 times). The combined organic layers were washed with brine, dried over anhydrous NaSO₄, and concentrated to dryness. Descending column chromatography using silica gel as stationary phase and a gradient mixture of CH₂Cl₂ and MeOH as eluent gave 1,381 mg (yield 71%) of a brown solid. ¹H NMR (400 MHz, Chloroform-d) 6 7.23 (d, J = 2.4 Hz, 1H), 5.54 (d, J = 2.4 Hz, 1H), 3.63 (bs, 2H), 1.49 (s, 9H). ¹³C NMR (101 MHz, Chloroform-d) 6 153.69, 126.82, 92.15, 57.39, 29.70.



3,5-dimethyl-1H-pyrazole: H₂SO₄·SiO₂ was synthesized via known protocols. In a mortar, 100 mg (1.0mmol, 1 equiv.) 2,4 pentanedione were mixed with 35 mg (1.1 mmol, 1.1 equiv.) of hydrazine, followed by adding 20 mg H₂SO₄·SiO₂. The slurry was continuously ground for 5 min and then diluted with 3 mL ethyl acetate. The resulting reaction mixture was filtered, and the filter cake washed three times with ethyl acetate. The filtrate was concentrated to dryness to give 60 mg (yield 62%) a white solid. ¹H NMR (600 MHz, Chloroform-d) 6 9.09 (bs, 1H), 5.83 (s, 1H), 2.28 (d, J = 0.6 Hz, 6H). ¹³C NMR (151 MHz, Chloroform-d) 6 144.53, 104.27, 12.31.



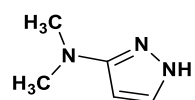
3,4,5-trimethyl-1H-pyrazole: Synthesis of 3,4,5-trimethyl-1H-pyrazole followed a similar synthetic route as 3,5-dimethyl-1H-pyrazole (directly above) to reveal a white solid (74%). ^1H NMR (400 MHz, Chloroform-*d*) δ 10.28 (bs, 1H), 2.19 (s, 6H), 1.90 (s, 3H). ^{13}C NMR (101 MHz, Chloroform-*d*) δ 142.10, 110.61, 10.87, 7.62.



3-(benzyloxy)-1H-pyrazole: This compound was synthesized over four steps, as outlined below:

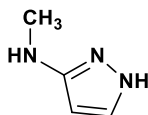
- 1) To a solution of methyl 3-methoxyacrylate (11.62 g, 100 mmol) in MeOH (15 mL) was added hydrazine monohydrate (5.5 g, 110 mmol). The mixture was then refluxed for 2 hours. All volatiles were removed in vacuo to give 1H-pyrazol-3-ol as a light-yellow solid (8 g, 95%). ^1H NMR (400 MHz, DMSO-*d*₆) δ 11.26 (s, 1H), 9.93 (s, 1H), 7.35 (s, 1H), 5.43 ppm (s, 1H).
- 2) 1H-pyrazol-3-ol (11.62 g, 100 mmol) was dissolved in pyridine (100 mL) and the mixture was heated to 95° C. A mixture of acetic anhydride (18.4 mL, 204 mmol) and pyridine (20 mL) was added dropwise over 20 minutes. Following addition, the reaction stirred at 95° C for 2 hours, then all volatiles were removed in vacuo and 200 mL of Et₂O was added to the residue. This solution was stirred overnight at RT. The solid was filtered and washed with 100 mL of Et₂O to give 1-(3-hydroxy-1H-pyrazol-1-yl)ethenone as an off-white solid (19.1 g, 78%). ^1H NMR (400 MHz, DMSO-*d*₆) δ 10.97 (s, 1H), 8.13 (s, 1H), 6.01 (s, 1H), 2.48 ppm (s, 3H).

- 3) A solution was prepared by mixing 1-(3-hydroxy-1H-pyrazol-1-yl)ethenone (3.78 g, 30 mmol), benzyl bromide (5.61 g, 33 mmol), and K₂CO₃ (5.52, 40 mmol) in DMF (100 mL). This solution was stirred at RT for 10 hours. The solvent was removed in vacuo then the residue was diluted with ethyl acetate and water. The organic phase was washed with brine, dried over Na₂SO₄, then the volatiles removed in vacuo. This crude material was purified by column chromatography to give 1-(3-(benzyloxy)-1H-pyrazol-1-yl)ethan-1-one as a colorless oil (5.5 g, 85%). ¹H NMR (400 MHz, CDCl₃) δ 8.07 (d, J=2.9 Hz, 1H), 7.46 (d, J=7.0 Hz, 2H), 7.39 (t, J=7.2 Hz, 2H), 7.35 (d, J=6.8 Hz, 1H), 6.00 (d, J=3.0 Hz, 1H), 5.29 (s, 2H), 2.61 ppm (s, 3H).
- 4) A solution was made by mixing 1-(3-(benzyloxy)-1H-pyrazol-1-yl)ethan-1-one (1.5 g, 6.94 mmol), methanol (10 mL), tetrahydrofuran (15 mL), and 10% sodium hydroxide (0.55 mL). This mixture was left to stir at room temperature for 5 hours. The solvent was then removed in vacuo. The residue was diluted using ethanol and water. The organic layer was washed with brine and dried over anhydrous sodium sulfate. This revealed a white solid (529 mg, 44%). ¹H NMR (400 MHz, DMSO-*d*₆) δ 11.89 (s, 1H), 7.50 (d, J = 2.3 Hz, 1H), 7.43 – 7.34 (m, 5H), 5.69 (d, J = 2.4 Hz, 1H), 5.12 (s, 2H). ¹³C NMR (151 MHz, DMSO-*d*₆) δ 163.30, 137.97, 130.38, 128.76, 128.23, 128.19, 89.89, 70.34.

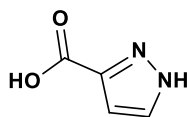


N,N-dimethyl-1H-pyrazol-3-amine: To a solution of 350 mg (4.22 mmol, 1 equiv.) 3-aminopyrazole in 10 mL of methanol at 0 °C 380 mg paraformaldehyde were added. The resulting white slurry was stirred for 2h at RT under argon. The mixture was then cooled to 0° C, followed by adding 500 mg of NaBH₃CN. The reaction mixture was continuously

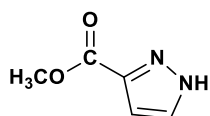
stirred under argon at RT for another 3 h. The resulting reaction mixture was extracted three times with CH₂Cl₂. The combined organic layers were washed with distilled water, brine, and then dried over MgSO₄, filtered, and concentrated to give 160 mg (yield 34%) as yellow oil. ¹H NMR (400 MHz, DMSO-*d*₆) 11.71 (bs, 1H), 7.41 (bs, 1H), 5.60 (bs, 1H), 2.71 (s, 6H).



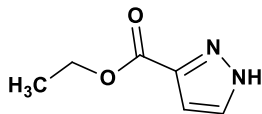
N-methyl-1H-pyrazol-3-amine: A solution of 970 mg (11.67mmol) 3-aminopyrazole in 10 mL of formic acid (90%) was stirred at 110 °C for 12 h under argon. The resulting reaction mixture distilled to almost dryness using a rotary evaporator, washed with distilled water, and dried under strong vacuum to give 471 mg (yield 36%) *N*-(2*H*-pyrazole-3-yl)formamide as a white solid. To 319 mg (2.9 mmol) of this solid in 10 mL of distilled THF at -10 °C 110 mg (2.9 mmol) of lithium aluminum hydride was added. The reaction mixture was allowed to slowly warm to RT and continuously stirred under argon overnight. After cooling to 0 °C, the reaction solution was quenched by adding 0.11 mL of distilled water, 0.11 mL of 15% NaOH aqueous solution, and again 0.33 mL of distilled water. The resulting reaction mixture was stirred at RT for 30 min, dried over MgSO₄, filtered, and concentrated to dryness to give 311 mg (yield 91%) of a brown oil. This oil was used in the subsequent reaction without further purification. ¹H NMR (400 MHz, DMSO-*d*₆) 7.25 (d, *J* = 2.1 Hz, 1H), 5.33 (d, *J* = 2.1 Hz, 1H), 2.62 (s, 3H).



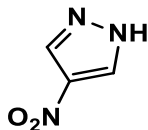
1H-pyrazole-3-carboxylic acid: A solution of 3-methyl pyrazole (765 mg, 9.32 mmol) and water (30 mL) was made and allowed to stir at room temperature. To this was slowly added a solution of potassium permanganate (3.2 g, 20.5 mmol) dissolved in water. Following addition, the reaction was left to reflux at 90° C for 4 hours. The mixture was then filtered, and the filtrate collected. The solvent was removed in vacuo and the remaining residue was placed in an ice bath. The pH was changed from 8 to 2 using 2N HCl, producing a white precipitate (1.00 g, 96%). ¹H NMR (400 MHz, DMSO-*d*₆) δ 7.72 (d, *J* = 2.2 Hz, 1H), 6.69 (d, *J* = 2.2 Hz, 1H). ¹³C NMR (101 MHz, DMSO-*d*₆) δ 163.09, 141.32, 108.35, 11.52.



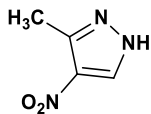
Methyl 1H-pyrazole-3-carboxylate: To a solution of 3-pyrazolecarboxylic acid (500 mg, 4.46 mmol) in MeOH (25 mL) was slowly added thionyl chloride (1.062 g, 8.92 mmol) at 0° C. After completion of the addition, the temperature of the reaction mixture was increased to 70° C and stirred for 5 hours. Methanol was removed in vacuo, and the resulting residue was poured into ice water and extracted with ethyl acetate. The organic layer was washed with 10% NaHCO₃ solution, brine, and then dried over Na₂SO₄. The resulting mixture concentrated in vacuo to reveal a white solid (116 mg, 21%). ¹H NMR (600 MHz, DMSO-*d*₆) δ 7.83 (s, 1H), 6.78 – 6.77 (m, 1H), 3.80 (s, 3H). ¹³C NMR (101 MHz, DMSO-*d*₆) δ 163.03, 107.94, 51.91.



Ethyl 1H-pyrazole-3-carboxylate: To a solution of 1H-pyrazole-3-carboxylic acid (500 mg, 4.46 mmol) and ethanol (25 mL) was slowly added thionyl chloride (1.06 g, 8.92 mmol) at 0° C. Following addition, this mixture was heated at 70° C for 5 hours. The solvent was then removed in vacuo and the resulting residue was poured into ice water. The product was then extracted with ethyl acetate and the organic layer washed with 10% sodium bicarbonate, brine, and then dried over anhydrous sodium sulfate. This revealed an off-white solid (84.8 mg, 14%). ¹H NMR (400 MHz, DMSO-*d*₆) δ 7.87 (d, *J* = 6.4 Hz, 1H), 6.75 (s, 1H), 4.27 (d, *J* = 7.1 Hz, 2H), 1.28 (d, *J* = 7.1 Hz, 3H). ¹³C NMR (101 MHz, DMSO-*d*₆) δ 162.00, 134.57, 108.12, 105.95, 60.56, 14.66, 11.42.

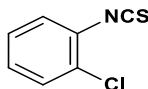


4-nitro-1H-pyrazole: Pyrazole (1.0 g, 14.69 mmol) was measured in to a 100 mL round bottom flask and dissolved in 5 mL of H₂SO₄ (after 10 min pyrazole was fully dissolved). Afterwards, the solution was heated to 60 °C and 1.1 eq of (1.46 mL) conc. HNO₃ was added dropwise followed by stirring for 90 min at 60 °C. After 90 min, the product solution was poured into 15 g of ice/water. The product was obtained as a white color solid precipitate (1,411 mg, 85%). ¹H NMR (400 MHz, Chloroform-*d*) δ 8.27 (s, 2H). ¹³C NMR (101 MHz, DMSO-*d*₆) δ 136.10, 130.02.

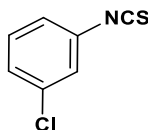


3-methyl-4-nitro-1H-pyrazole: To a RBF was added 3-methyl-1*H*-pyrazole (1 g, 14.7 mmol) and concentrated sulfuric acid (6.63 g, 67.6 mmol). The reaction was heated to 60° C and concentrated nitric acid (2.22 g, 35.3 mmol) was added slowly. The reaction continued to stir at 60° C for 2 hours. The reaction was removed from the heat and poured over ice to reveal a white precipitate. This was collected via gravity filtration (1.4171 g, 76% yield). ¹H NMR (600 MHz, DMSO-*d*₆) δ 8.25 (s, 1H), 8.05 – 8.02 (m, 1H), 2.45 (s, 3H). ¹³C NMR (151 MHz, DMSO-*d*₆) δ 142.03, 135.02, 132.78, 11.85.

Phenyl isothiocyanate Synthetic Procedure

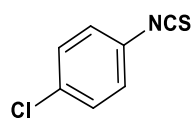


1-chloro-2-isothiocyanatobenzene: A solution was prepared by mixing triethylamine (33.7 mL, 242.6 mmol) and carbon disulfide (12 mL, 200 mmol) together and cooled in an ice bath to 0° C. To this was added 2-chloroaniline (6.14 mL, 58.6 mmol) dropwise over 30 minutes. This solution was warmed to RT and left to stir overnight. The next day, the resulting solid was filtered and washed with 95% EA/hexane to remove any excess triethylamine. ((2-chlorophenyl)amino)(λ1-sulfaneyl)methanethione was collected as a yellow solid weighing 10.3 g (58%). A solution was prepared by the addition of ((2-chlorophenyl)amino)(λ1-sulfaneyl)methanethione (4.62 g, 15.2 mmol) and sodium bicarbonate, (2.55 g, 30.4 mmol) dissolved in 100 mL of ethyl acetate/water (1:1). This solution was cooled to 0° C using an ice bath. Iodine (3.85 g, 15.2 mmol) was then added pinch-wise over a period of 30 minutes. The ethyl acetate layer was washed with sodium thiosulfate, water, and brine, and then dried over anhydrous sodium sulfate. The product was then purified with a short silica column using hexane as the solvent yielding 2.57 g of clear yellow oil (49%). ¹H NMR (400 MHz, Chloroform-*d*) δ 7.47 – 7.36 (m, 1H), 7.32 – 7.15 (m, 3H). ¹³C NMR (101 MHz, Chloroform-*d*) δ 139.06, 132.01, 130.38, 130.02, 128.21, 127.84, 126.90.



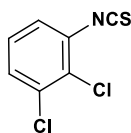
1-chloro-3-isothiocyanatobenzene: A solution was prepared by mixing triethylamine (33.7 mL, 242.6 mmol) and carbon disulfide (12 mL, 200 mmol) together and cooled in an ice bath to 0° C. To this was added 2-chloroaniline (6.14 mL, 58.6 mmol) dropwise over 30 minutes. This

solution was warmed to RT and left to stir overnight. The next day, the resulting solid was filtered and washed with 95% EA/hexane to remove any excess triethylamine. ((2-chlorophenyl)amino)(λ^1 -sulfaneyl)methanethione was collected as a yellow solid weighing 17.2 g (97%). A solution was prepared by mixing triethylamine (33.7 mL, 242.6 mmol) and carbon disulfide (12 mL, 200 mmol) together and cooled in an ice bath to 0° C. To this was added 2-chloroaniline (6.14 mL, 58.6 mmol) dropwise over 30 minutes. This solution was warmed to RT and left to stir overnight. The next day, the resulting solid was filtered and washed with 95% EA/hexane to remove any excess triethylamine. ((2-chlorophenyl)amino)(λ^1 -sulfaneyl)methanethione was collected as a yellow solid weighing 9.03 g (92%). To a solution of ((3-chlorophenyl)amino)(λ^1 -sulfaneyl)methanethione (2.15 g, 7.09 mmol), sodium bicarbonate (1.2 g, 14.2 mmol), ethyl acetate (12.5 mL), and water (12.5 mL) was added iodine crystals (1.8 g, 7.09 mmol) pinch-wise at 0° C. The ethyl acetate layer was washed with sodium thiosulfate, water, and brine, and then dried over anhydrous sodium sulfate. A column was run using hexane to yield a light-yellow oil (4.6 g, 92%). ¹H NMR (400 MHz, Chloroform-*d*) δ 7.32 – 7.22 (m, 2H), 7.26 – 7.19 (m, 1H), 7.11 (dt, *J* = 7.2, 1.9 Hz, 1H). ¹³C NMR (101 MHz, Chloroform-*d*) δ 135.34, 132.87, 130.71, 127.80, 126.11, 124.16.



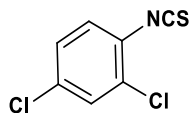
1-chloro-4-isothiocyanatobenzene: A solution was prepared by mixing triethylamine (33.7 mL, 242.6 mmol) and carbon disulfide (12 mL, 200 mmol) together and cooled in an ice bath to 0° C. To this was added 2-chloroaniline (6.14 mL, 58.6 mmol) dropwise over 30 minutes. This solution was warmed to RT and left to stir overnight. The next day, the resulting solid was filtered and washed with 95% EA/hexane to remove any excess triethylamine. ((2-

chlorophenyl)amino)(λ 1-sulfaneyl)methanethione was collected as a yellow solid weighing 17.0 g (95%). To a solution of ((4-chlorophenyl)amino)(λ 1-sulfaneyl)methanethione (14 g, 46 mmol), sodium bicarbonate (7.7 g, 92 mmol), ethyl acetate (50 mL), and water (50 mL) was added iodine crystals (10.8 g, 46 mmol) pinch-wise at 0° C. The ethyl acetate layer was washed with sodium thiosulfate, water, and brine, and then dried over anhydrous sodium sulfate. A column was run using hexane to yield a light-yellow solid (4.3 g, 45%). ^1H NMR (400 MHz, DMSO- d_6) δ 7.54 – 7.45 (m, 4H). ^{13}C NMR (101 MHz, DMSO- d_6) δ 134.95, 132.70, 130.36, 129.53, 128.81, 128.23, 125.77.

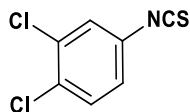


2,3-dichlorophenylisothiocyanate: To a RBF was added 1,4-diazabicyclo[2.2.2]octane (DABCO) (8.7 g, 77.8 mmol) and carbon disulfide (4.9 g, 64.8 mmol). This was dissolved in acetone (15 mL) and the solution cooled to 0° C. 2,3-dichloroaniline (4.2 g, 25.9 mmol) was added slowly. The reaction solution was warmed to RT and allowed to stir overnight. The next day, the resulting solid was filtered. ((2,3-chlorophenyl)amino)(λ 1-sulfaneyl)methanethione was collected as a yellow-orange solid weighing 8.2 g (93%). To a RBF was added (2,3-dichlorophenyl)carbamodithioate (34.2 g, 144.2 mmol), triethylamine (20.1 mL, 144.2 mmol), and chloroform (200 mL). This solution was cooled using an ice bath. Ethyl chloroformate (13.7 mL, 144.2 mmol) was added slowly at 0° C. This was left to stir for 1 hour at this temperature. The organic layer was then washed with 2N HCl and water then dried over anhydrous sodium sulfate. The solvent was removed in vacuo and the resulting solid dried under high vacuum. The crude product was purified via flash column chromatography to reveal an off-white solid (5.2494 g, 18%). ^1H NMR (600 MHz, DMSO- d_6) δ 7.55 (dd, J = 8.1, 1.5 Hz, 1H), 7.44 (dd, J = 8.1, 1.5

Hz, 1H), 7.36 (t, $J = 8.1$ Hz, 1H). ^{13}C NMR (151 MHz, $\text{DMSO}-d_6$) δ 138.48, 133.02, 130.68, 129.81, 129.58, 129.32, 126.26.

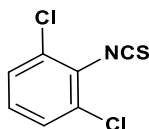


2,4-dichlorophenylisothiocyanate: To a RBF was added 1,4-diazabicyclo[2.2. 2]octane (DABCO) (8.7 g, 77.8 mmol) and carbon disulfide (4.9 g, 64.8 mmol). This was dissolved in acetone (15 mL) and the solution cooled to 0° C. 2,4-dichloroaniline (4.2 g, 25.9 mmol) was added slowly. The reaction solution was warmed to RT and allowed to stir overnight. The next day, the resulting solid was filtered. ((2,4-chlorophenyl)amino)(λ 1-sulfaneyl)methanethione was collected as a yellow-orange solid weighing 6.33 g (72%). To a RBF was added (2,4-dichlorophenyl)carbamodithioate (72 g, 303.6 mmol), triethylamine (42.3 mL, 303.6 mmol), and chloroform (500 mL). This solution was cooled using an ice bath. Ethyl chloroformate (26.9 mL, 303.6 mmol) was added slowly at 0° C. This was left to stir for 1 hour at this temperature. The organic layer was then washed with 2N HCl and water then dried over anhydrous sodium sulfate. The solvent was removed in vacuo and the resulting solid dried under high vacuum. The crude product was purified via flash column chromatography to reveal a white solid (15.7146 g, 25%). ^1H NMR (600 MHz, $\text{DMSO}-d_6$) δ 7.74 (d, $J = 2.4$ Hz, 1H), 7.51 (d, $J = 8.6$ Hz, 1H), 7.45 (dd, $J = 8.6, 2.4$ Hz, 1H). ^{13}C NMR (151 MHz, $\text{DMSO}-d_6$) δ 138.63, 133.05, 132.00, 130.10, 129.09, 128.66, 128.05.



3,4-dichlorophenylisothiocyanate: To a RBF was added 1,4-diazabicyclo[2.2. 2]octane (DABCO) (8.7 g, 77.8 mmol) and carbon disulfide (4.9 g, 64.8 mmol). This was dissolved in

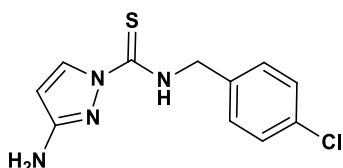
acetone (15 mL) and the solution cooled to 0° C. 3,4-dichloroaniline (4.2 g, 25.9 mmol) was added slowly. The reaction solution was warmed to RT and allowed to stir overnight. The next day, the resulting solid was filtered. ((3,4-chlorophenyl)amino)(λ1-sulfaneyl)methanethione was collected as a yellow-orange solid weighing 8.1 g (92%). To a RBF was added (3,4-dichlorophenyl)carbamodithioate (9.2 g, 38.8 mmol), triethylamine (5.42 mL, 38.8 mmol), and chloroform (50 mL). This solution was cooled using an ice bath. Ethyl chloroformate (3.69 mL, 38.8 mmol) was added slowly at 0° C. This was left to stir for 1 hour at this temperature. The organic layer was then washed with 2N HCl and water then dried over anhydrous sodium sulfate. The solvent was removed in vacuo and the resulting crude product was purified via flash column chromatography to reveal a clear oil (0.6969 g, 9%). ¹H NMR (600 MHz, DMSO-*d*₆) δ 7.68 (d, *J* = 2.5 Hz, 1H), 7.61 (d, *J* = 8.7 Hz, 1H), 7.36 (dd, *J* = 8.7, 2.5 Hz, 1H). ¹³C NMR (151 MHz, DMSO-*d*₆) δ 136.38, 132.55, 131.90, 130.95, 130.60, 128.09, 126.65.



2,6-dichlorophenylisothiocyanate: To a RBF was added 1,4-diazabicyclo[2.2. 2]octane (DABCO) (8.7 g, 77.8 mmol) and carbon disulfide (4.9 g, 64.8 mmol). This was dissolved in acetone (15 mL) and the solution cooled to 0° C. 2,6-dichloroaniline (4.2 g, 25.9 mmol) was added slowly. The reaction solution was warmed to RT and allowed to stir overnight. The next day, the resulting solid was filtered. ((2,6-chlorophenyl)amino)(λ1-sulfaneyl)methanethione was collected as a yellow-orange solid weighing 7.4 g (84%). To a RBF was added (2,6-dichlorophenyl)carbamodithioate (36.6 g, 154.3 mmol), triethylamine (15.6 mL, 154.3 mmol), and chloroform (200 mL). This solution was cooled using an ice bath. Ethyl chloroformate (16.7 mL, 154.3 mmol) was added slowly at 0° C. This was left to stir for 1 hour at this temperature.

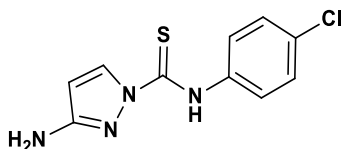
The organic layer was then washed with 2N HCl and water then dried over anhydrous sodium sulfate. The solvent was removed in vacuo and the resulting crude product was purified via flash column chromatography to reveal a clear oil (6.297 g, 20%). ^1H NMR (600 MHz, $\text{DMSO-}d_6$) δ 7.55 (dd, $J = 8.1, 1.5$ Hz, 1H), 7.44 (dd, $J = 8.1, 1.5$ Hz, 1H), 7.36 (t, $J = 8.1$ Hz, 1H). ^{13}C NMR (151 MHz, $\text{DMSO-}d_6$) δ 138.49, 133.02, 130.68, 129.81, 129.58, 129.32, 126.25.

NNSN Synthetic Procedure



APM01

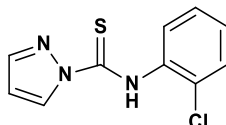
4-amino-N-(4-chlorobenzyl)-1H-pyrazole-1-carbothioamide: A solution of 3-aminopyrazole (120 mg, 1.44 mmol, 1.0 eq.), 4-chlorophenyl isothiocyanate (292 mg, 1.58 mmol, 1.1 eq.), and dichloromethane (10 mL) was stirred at room temperature for 24 hours. The solvent was removed in vacuo, purified by flash column chromatography (100% DCM to 5% DCM/MeOH), revealing a white solid (250 mg, 65%). ^1H NMR (400 MHz, DMSO- d_6) δ 10.55 (s, 1H), 7.36 (s, 2H), 7.35 (s, 2H), 7.33 (d, J = 2.4 Hz, 2H), 5.40 (d, J = 2.0 Hz, 2H), 4.77 (d, J = 4.6 Hz, 2H). ^{13}C NMR (101 MHz, DMSO- d_6) δ 176.76, 152.41, 142.01, 137.33, 132.27, 130.12, 128.91, 89.21, 96.44. FTIR, cm^{-1} : 3406, 3297 (-NH $_2$), 3247 (-NH-), 3047 (C-H aromatic), 2960, 2919 (CH pyrazole), 1605 (aromatic C=C), 1517 (aromatic C=N), 1215 (C=S), 731 (aromatic C-Cl). HRMS (+ESI-TOF) m/z : [M^+] calcd for $\text{C}_{11}\text{H}_{11}\text{ClN}_4\text{S}$ 266.03929; found 266.03930.



KE03

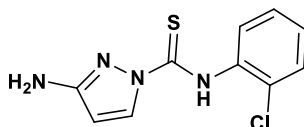
3-amino-N-(4-chlorophenyl)-1H-pyrazole-1-carbothioamide: A solution of 3-aminopyrazole (973 mg, 11.7 mmol, 1 eq.), 4-chlorophenyl isothiocyanate (2.18 g, 12.9 mmol, 1.1 eq.), and dichloromethane (10 mL) was stirred at room temperature for 24 hours. The solvent was removed in vacuo and re-crystallized with MeOH, revealing an off-white solid (2.1 g, 71%). ^1H

NMR (400 MHz, DMSO-d₆) δ 10.99 (s, J = 4.5 Hz, 1H), 8.41 (s, 1H), 7.67 (d, 2H), 7.45 (d, 2H), 6.98 (s, 1H). ¹³C NMR (400 MHz, DMSO-d₆) δ 176.30, 149.26, 137.93, 129.68, 129.08, 128.35, 125.75, 94.31. FTIR, cm⁻¹: 3471, 3460 (-NH₂), 3305 (-NH-), 3060, 3005 (C-H aromatic), 2960 (CH pyrazole), 1610, 1588 (aromatic C=C), 1516 (aromatic C=N), 1242 (C=S), 640 (aromatic C-Cl). HRMS (+ESI-TOF) m/z : [M⁺] calcd for C₁₀H₉ClN₄S 252.72326; found 252.72328.



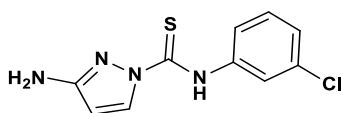
SB46

N-(2-chlorophenyl)-1H-pyrazole-1-carbothioamine: A solution of pyrazole (29.F3 mg, 0.353 mmol, 1 eq.), 2-chlorophenyl isothiocyanate (65.9 mg, 0.388 mmol, 1.1 eq.), and dichloromethane (10 mL) was stirred at room temperature for 24 hours. The solvent was removed in vacuo, purified by flash column chromatography (100% DCM to 5% DCM/MeOH), revealing a white solid (97 mg, 95%). ¹H NMR (400 MHz, Chloroform-d) δ 11.16 (s, 1H), 8.64 (dd, J = 8.2, 1.6 Hz, 1H), 7.75 (dd, J = 1.7, 0.8 Hz, 1H), 7.63 (d, J = 2.1 Hz, 2H), 7.49 (dd, J = 8.0, 1.5 Hz, 1H), 7.40 – 7.35 (m, 1H), 7.24 (dd, J = 1.5, 0.6 Hz, 1H), 6.57 (t, J = 2.1 Hz, 1H). ¹³C NMR (400 MHz, Chloroform-d) δ 142.99, 131.35, 130.38, 129.86, 128.19, 127.82, 127.61, 127.36, 126.91, 124.97, 110.20. FTIR, cm⁻¹: 3277 (-NH), 3149 (C-H aromatic), 1580 (aromatic C=C), 1516 (aromatic C=N), 1231 (C=S), 730 (aromatic C-Cl). HRMS (+ESI-TOF) m/z : [M⁺] calcd for C₁₀H₉ClN₄S 252.72326; found 252.72326.



SB49

3-amino-N-(2-chlorophenyl)-1H-pyrazole-1-carbothioamide: A solution of 3-aminopyrazole (39.1 mg, 0.471 mmol, 1 eq.), 2-chlorophenyl isothiocyanate (87.8 mg, 0.517 mmol, 1.1 eq.), and dichloromethane (10 mL) was stirred at room temperature for 24 hours. The solvent was removed in vacuo, purified by flash column chromatography (100% DCM to 5% DCM/MeOH), revealing a white solid (114 mg, 96%). ¹H NMR (400 MHz, Chloroform-d) δ 10.65 (s, 1H), 8.54 – 8.52 (m, 1H), 7.46 (dd, J = 8.0, 1.5 Hz, 2H), 7.35 (dddd, J = 8.1, 6.7, 1.5, 0.5 Hz, 1H), 7.21 – 7.17 (m, 1H), 5.94 – 5.93 (m, 1H), 4.07 (s, 4H). ¹³C NMR (400 MHz, Chloroform-d) δ 207.39, 154.21, 130.85, 128.29, 92.78, 57.57, 30.99, 29.72, 28.34. FTIR, cm⁻¹: 3420 (-NH₂), 3328 (-NH-), 3199 (C-H aromatic), 1584 (aromatic C=C), 1531 (aromatic C=N), 1239 (C=S), 745 (aromatic C-Cl). HRMS (+ESI-TOF) m/z: [M⁺] calcd for C₁₀H₈ClN₃S 237.70862; found 237.70870.

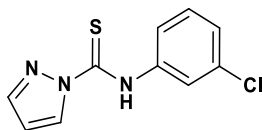


SB50

3-amino-N-(3-chlorophenyl)-1H-pyrazole-1-carbothioamide: A solution of 3-aminopyrazole (150 mg, 1.81 mmol, 1 eq.), 3-chlorophenyl isothiocyanate (337 mg, 1.98 mmol, 1.1 eq.), and dichloromethane (10 mL) was stirred at room temperature for 24 hours. The solvent was removed in vacuo, purified by flash column chromatography (100% DCM to 5% DCM/MeOH), revealing a white solid (421 mg, 92%). ¹H NMR (400 MHz, Chloroform-d) δ 10.28 (s, 1H), 8.52 (d, J = 3.0 Hz, 1H), 7.88 (t, J = 2.0 Hz, 1H), 7.64 (ddd, J = 8.1, 2.2, 1.0 Hz, 1H), 7.33 (t, J = 8.1 Hz, 1H), 7.23 (ddd, J = 8.1, 2.0, 1.0 Hz, 1H), 5.92 (d, J = 3.0 Hz, 1H), 4.05 (s, 2H). ¹³C NMR (400 MHz, Chloroform-d) δ 171.39, 156.93, 138.85, 134.67, 133.07, 130.05, 126.66, 123.68, 121.79, 101.20. FTIR, cm⁻¹: 3413 (-NH₂), 3320 (-NH-), 3242 (C-H aromatic), 2918, 2849 (CH

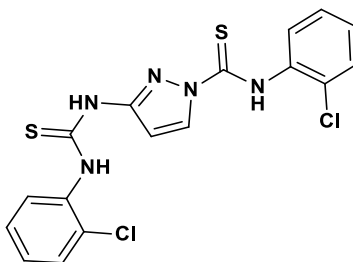
pyrazole), 1586 (aromatic C=C), 1530 (aromatic C=N), 1239 (C=S), 770 (aromatic C-Cl).

HRMS (+ESI-TOF) m/z: [M⁺] calcd for C₁₀H₉ClN₄S 252.72326; found 252.72328.



SB52

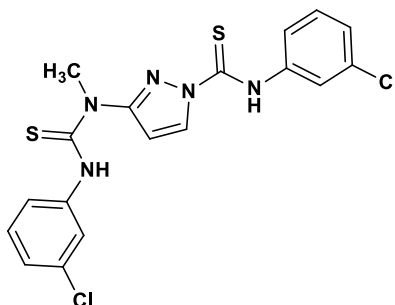
N-(3-chlorophenyl)-1H-pyrazole-1-carbothioamine: A solution of pyrazole (100 mg, 1.47 mmol, 1 eq.), 3-chlorophenyl isothiocyanate (273 mmol, 1.62 mmol, 1.1 eq.), and dichloromethane (10 mL) was stirred at room temperature for 24 hours. The solvent was removed in vacuo, purified by flash column chromatography (100% DCM to 5% DCM/MeOH), revealing a white solid (310 mg, 89%). ¹H NMR (400 MHz, Chloroform-d) δ 10.75 (s, 1H), 8.74 (dd, J = 2.8, 0.8 Hz, 1H), 7.93 (t, J = 2.0 Hz, 1H), 7.68 – 7.65 (m, 1H), 7.37 (t, J = 8.1 Hz, 2H), 7.11 (dt, J = 7.2, 2.0 Hz, 1H), 6.36 (t, J = 2.1 Hz, 1H). ¹³C NMR (400 MHz, Chloroform-d) δ 173.16, 142.80, 138.48, 134.83, 131.38, 130.71, 130.20, 127.80, 127.14, 126.11, 124.16, 123.70, 121.80, 110.23, 105.44. FTIR, cm⁻¹: 3258 (-NH-), 3093 (C-H aromatic), 1585 (aromatic C=C), 1539 (aromatic C=N), 1298 (C=S), 769 (aromatic C-Cl). HRMS (+ESI-TOF) m/z: [M⁺] calcd for C₁₀H₈ClN₃S 237.70862; found 237.70864.



SB53

N-(2-chlorophenyl)-3-(3-(2-chlorophenyl)thioureido)-1H-pyrazole-1-carbothioamide: To a solution of 3-aminopyrazole (52 mg, 0.627 mmol, 1.0 eq.) and 2-chlorophenyl isothiocyanate

(235 mg, 1.38 mmol, 2.2 eq.) in anhydrous DMF (3 mL), anhydrous potassium carbonate (286 mg, 2.07 mmol, 3.3 eq.) was added. The reaction mixture was kept stirring under argon at room temperature for 24 hours. This was filtered to remove excess K₂CO₃ and the filtrate concentrated to dryness. Descending column chromatography using silica gel as stationary phase and a gradient of DCM and MeOH as eluent gave a white solid (41 mg, 15%). ¹H NMR (400 MHz, Chloroform-*d*) δ 10.65 (s, 2H), 8.60 (dd, *J* = 8.2, 1.6 Hz, 2H), 8.53 (dd, *J* = 3.0, 0.5 Hz, 1H), 7.46 (dd, *J* = 8.0, 1.5 Hz, 2H), 7.35 (ddd, *J* = 8.2, 7.5, 1.5 Hz, 3H), 7.20 (td, *J* = 7.7, 1.6 Hz, 2H), 5.94 (dd, *J* = 3.0, 0.6 Hz, 1H). ¹³C NMR (101 MHz, Chloroform-*d*) δ 157.03, 133.08, 129.72, 127.49, 127.23, 127.17, 125.24, 101.28, 77.45. FTIR, cm⁻¹: 3420, 3327, 3199 (-NH-), 1584 (aromatic C=C), 1529 (aromatic C=N), 1239 (C=S), 744 (aromatic C-Cl). HRMS (+ESI-TOF) *m/z*: [M⁺] calcd for C₁₇H₁₃Cl₂N₅S₂ 420.99894; found 420.99827.

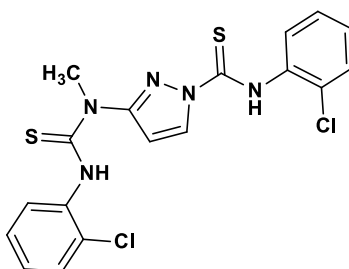


SB54

N-(3-chlorophenyl)-3-(3-(3-chlorophenyl)-1-methylthioureido)-1H-pyrazole-1-

carbothioamide: To a solution of N-methyl-1H-pyrazol-3-amine (47 mg, 0.481 mmol, 1.0 eq.) and 3-chlorophenyl isothiocyanate (179 mg, 1.06 mmol, 2.2 eq.) in anhydrous DMF (2 mL), anhydrous potassium carbonate (220 mg, 1.58 mmol, 3.3 eq.) was added. The reaction mixture was kept stirring under argon at room temperature for 24 hours. This was filtered to remove excess K₂CO₃ and the filtrate concentrated to dryness. Descending column chromatography using silica gel as stationary phase and a gradient of DCM and MeOH as eluent gave a yellow

solid (60 mg, 28%). ^1H NMR (400 MHz, DMSO-*d*₆) δ 11.73 (s, 1H), 10.51 (s, 1H), 8.75 (dt, J = 3.1, 1.6 Hz, 1H), 7.71 (q, J = 1.8 Hz, 1H), 7.57 (dq, J = 5.6, 1.7 Hz, 2H), 7.48 (td, J = 8.0, 1.4 Hz, 2H), 7.40 – 7.35 (m, 2H), 7.22 (dt, J = 7.8, 1.7 Hz, 2H), 6.74 – 6.70 (m, 1H), 3.72 (d, J = 1.5 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-*d*₆) δ 182.07, 174.70, 155.14, 142.35, 140.17, 133.84, 133.37, 132.98, 132.06, 130.95, 130.47, 128.72, 127.54, 126.50, 126.03, 125.75, 125.47, 124.86, 106.24. FTIR, cm^{-1} : 3309, 3060 (-NH-), 1589 (aromatic C=C), 1522 (aromatic C=N), 1427 (-CH₃), 1290 (C=S), 758 (aromatic C-Cl). HRMS (+ESI-TOF) m/z : [M⁺] calcd for C₁₈H₁₅Cl₂N₅S₂ 435.01459; found 435.01439.

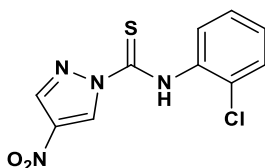


SB55

N-(2-chlorophenyl)-3-(3-(2-chlorophenyl)-1-methylthioureido)-1H-pyrazole-1-

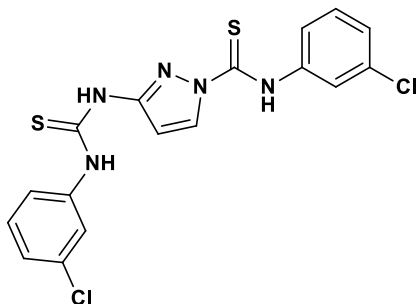
carbothioamide: To a solution of N-methyl-1H-pyrazol-3-amine (150 mg, 1.55 mmol, 1.0 eq.) and 2-chlorophenyl isothiocyanate (575 mg, 3.40 mmol, 2.2 eq.) in anhydrous DMF (3 mL), anhydrous potassium carbonate (705 mg, 5.12 mmol, 3.3 eq.) was added. The reaction mixture was kept stirring under argon at room temperature for 24 hours. This was filtered to remove excess K₂CO₃ and the filtrate concentrated to dryness. Descending column chromatography using silica gel as stationary phase and a gradient of DCM and MeOH as eluent gave a yellow solid (137 mg, 20%). ^1H NMR (400 MHz, DMSO-*d*₆) δ 11.47 (s, 1H), 9.64 (s, 1H), 7.88 (s 1H), 7.51 – 7.47 (m, 4H), 7.34 – 7.31 (m, 2H), 7.26 – 7.23 (m, 2H), 6.31 (t, J = 2.2 Hz, 1H), 3.71 (s, 3H). ^{13}C NMR (101 MHz, DMSO-*d*₆) δ 131.12, 130.50, 130.41, 130.15, 129.92, 129.76, 129.27,

128.38, 128.31, 127.98, 127.88, 127.71, 97.75, 55.60, 41.44. FTIR, cm^{-1} : 3350 (-NH), 3049, 2947 (C-H aromatic), 1704 (aromatic C=C), 1600 (aromatic C=N), 1433 (-CH₃), 1350 (C=S), 730 (aromatic C-Cl). HRMS (+ESI-TOF) m/z: [M⁺] calcd for C₁₈H₁₅Cl₂N₅S₂ 435.01459; found 435.01462.



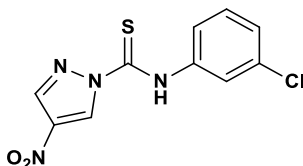
SB56

N-(2-chlorophenyl)-4-nitro-1H-pyrazole-1-carbothioamide: Two 100 mL round bottom flasks were flame dried to remove moisture. To flask A was added 4-nitro pyrazole (24 mg, 0.217 mmol, 1.0 eq.) and dry THF (10 mL). To flask B was added sodium hydride (7.6 mg, 0.318 mmol, 1.5 eq.) and dry THF (10 mL). Both flasks were allowed to stir at 0° C for 20 minutes. The mixture from flask B was cannulated into flask A using a two-headed needle. This solution was kept stirring at r.t. under argon for 3 hours. 2-chlorophenyl isothiocyanate (40 mg, 0.233 mmol, 1.1 eq.) was added to the reaction, which continued stirring for 12 hours at r.t. The reaction was concentrated to dryness to reveal a white solid (15 mg, 25%). ¹H NMR (400 MHz, Chloroform-d) δ 11.01 (s, 1H), 8.57 (dd, J = 8.2, 1.6 Hz, 1H), 7.48 (dd, J = 8.0, 1.5 Hz, 1H), 7.44 – 7.41 (m, 1H), 7.36 (td, J = 7.8, 1.5 Hz, 1H), 7.25 – 7.19 (m, 1H), 6.28 (d, J = 2.8 Hz, 1H). ¹³C NMR (400 MHz, Chloroform-d) δ 176.62, 168.38, 154.70, 146.78, 48.96. FTIR, cm^{-1} : 3112 (-NH), 2931, 2873 (C-H aromatic), 1677 (aromatic C=C), 1501 (aromatic C=N), 1311 (C=S), 735 (aromatic C-Cl). UPLC-MS [M+1] calcd for C₁₀H₇ClN₄O₂S 283.71; found 283.10.



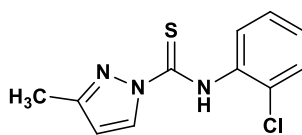
SB57

N-(3-chlorophenyl)-3-(3-(3-chlorophenyl)thioureido)-1H-pyrazole-1-carbothioamide: To a solution of 3-aminopyrazole (70 mg, 0.843 mmol, 1.0 eq.) and 3-chlorophenyl isothiocyanate (313 mg, 1.85 mmol, 2.2 eq.) in anhydrous DMF (2 mL), anhydrous potassium carbonate (384 mg, 2.78 mmol, 3.3 eq.) was added. The reaction mixture was kept stirring under argon at room temperature for 24 hours. This was filtered to remove excess K_2CO_3 and the filtrate concentrated to dryness. Descending column chromatography using silica gel as stationary phase and a gradient of DCM and MeOH as eluent gave a white solid (70 mg, 20%). ^1H NMR (400 MHz, Chloroform-*d*) δ 10.28 (s, 2H), 8.52 (d, J = 3.0 Hz, 1H), 7.88 (t, J = 2.0 Hz, 1H), 7.64 (dd, J = 8.1, 1.9 Hz, 2H), 7.34 (t, J = 8.1 Hz, 3H), 7.25 – 7.20 (m, 3H), 5.93 (d, J = 2.9 Hz, 1H). ^{13}C NMR (101 MHz, Chloroform-*d*) δ 133.08, 130.05, 126.67, 123.69, 121.80, 101.20. FTIR, cm^{-1} : 3431, 3330, 3154 (-NH-), 1661 (aromatic C=C), 1580 (aromatic C=N), 1288 (C=S), 754 (aromatic C-Cl). HRMS (+ESI-TOF) m/z : $[M]^+$ calcd for $C_{17}H_{13}Cl_2N_5S_2$ 420.99894; found 420.9989.



SB59

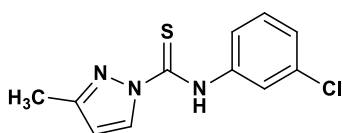
N-(3-chlorophenyl)-4-nitro-1H-pyrazole-1-carbothioamide: Two 100 mL round bottom flasks were flame dried to remove moisture. To flask A was added 4-nitro pyrazole (50 mg, 0.663 mmol, 1.0 eq.) and dry THF (10 mL). To flask B was added sodium hydride (15.9 mg, 0.663 mmol, 1.0 eq.) and dry THF (10 mL). Both flasks were allowed to stir at 0° C for 20 minutes. The mixture from flask B was cannulated into flask A using a two-headed needle. This solution was kept stirring at r.t. under argon for 3 hours. 3-chlorophenyl isothiocyanate (82 mg, 0.486 mmol, 1.1 eq.) was added to the reaction, which continued stirring for 12 hours at r.t. The reaction was concentrated to dryness to reveal a white solid (100 mg, 80%). ¹H NMR (400 MHz, DMSO-d₆) δ 10.09 (s, 1H), 7.68 – 7.65 (m, 1H), 7.40 – 7.31 (m, 4H), 7.17 (dddd, J = 7.6, 2.1, 1.4, 0.7 Hz, 1H). ¹³C NMR (400 MHz, DMSO-d₆) δ 180.36, 141.52, 133.28, 130.76, 124.92, 123.76, 122.71, 55.58, 31.37. FTIR, cm⁻¹: 3210 (-NH), 3118, 3095 (C-H aromatic), 1584 (aromatic C=C), 1441 (aromatic C=N), 1303 (C=S), 735 (aromatic C-Cl). UPLC-MS [M+1] calcd for C₁₀H₇ClN₄O₂S 283.71; found 283.23.



SB60

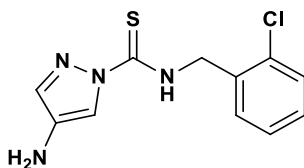
N-(2-chlorophenyl)-3-methyl-1H-pyrazole-1-carbothioamide: A solution of 3-methylpyrazole (109 mg, 1.33 mmol, 1.0 eq.), 2-chlorophenyl isothiocyanate (247 mg, 1.46 mmol, 1.1 eq.), and dichloromethane (10 mL) was refluxed at 45° C for 24 hours. The solvent was removed in vacuo and re-crystallized with MeOH, revealing a white solid (170 mg, 51%). ¹H NMR (400 MHz, DMSO-d₆) δ 11.73 (s, 1H), 8.62 (d, J = 2.7 Hz, 1H), 7.75 (t, J = 2.1 Hz, 1H), 7.61 – 7.56 (m, 1H), 7.46 – 7.40 (m, 2H), 7.34 (ddd, J = 8.1, 2.1, 1.0 Hz, 1H), 6.46 (d, J = 2.7 Hz, 1H), 2.31 (s,

3H). ^{13}C NMR (400 MHz, DMSO- d_6) δ 175.03, 153.70, 140.32, 133.23, 132.05, 130.73, 12728.71, 127.11, 126.10, 125.12, 111.54, 104.30. FTIR, cm^{-1} : 3267 (-NH), 3153, 2925 (C-H aromatic), 1589 (aromatic C=C), 1482 (aromatic C=N), 1407 (-CH₃), 1346 (C=S), 759 (aromatic C-Cl). HRMS (+ESI-TOF) m/z : [M⁺] calcd for C₁₁H₁₀ClN₃S 251.02840; found 251.02838.



SB61

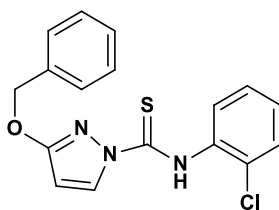
N-(3-chlorophenyl)-3-methyl-1H-pyrazole-1-carbothioamide: A solution of 3-methylpyrazole (100 mg, 1.21 mmol, 1.0 eq.), 3-chlorophenyl isothiocyanate (227 mg, 1.34 mmol, 1.1 eq.), and dichloromethane (10 mL) was refluxed at 45° C for 24 hours. The solvent was removed in vacuo and re-crystallized with MeOH, revealing a white solid (257 mg, 84%). ^1H NMR (400 MHz, DMSO- d_6) δ 11.73 (s, 1H), 8.62 (d, J = 2.7 Hz, 1H), 7.75 (t, J = 2.1 Hz, 1H), 7.62 – 7.55 (m, 1H), 7.49 – 7.40 (m, 1H), 7.34 (ddd, J = 8.0, 2.1, 1.0 Hz, 1H), 6.46 (d, J = 2.7 Hz, 1H), 2.31 (s, 3H). ^{13}C NMR (400 MHz, Chloroform- d) δ 176.89, 151.22, 141.12, 133.96, 130.59, 129.95, 129.60, 127.28, 90.18, 45.76. FTIR, cm^{-1} : 3402 (-NH), 3267, 3200 (C-H aromatic), 1596 (aromatic C=C), 1444 (aromatic C=N), 1393 (-CH₃), 1334 (C=S), 739 (aromatic C-Cl). HRMS (+ESI-TOF) m/z : [M⁺] calcd for C₁₁H₁₀ClN₃S 251.02840; found 251.02842.



SB62

4-amino-N-(2-chlorobenzyl)-1H-pyrazole-1-carbothioamide: A solution of 3-aminopyrazole (117 mg, 1.41 mmol, 1.0 eq.), 2-chlorobenzyl isothiocyanate (284 mg, 1.55 mmol, 1.1 eq.), and

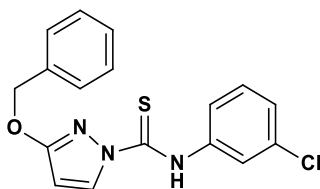
dichloromethane (10 mL) was stirred at room temperature for 24 hours. The solvent was removed in vacuo, purified by flash column chromatography (100% DCM to 5% DCM/MeOH), revealing a white solid (269 mg, 72%). ¹H NMR (400 MHz, Chloroform-d) δ 9.58 (s, 1H), 7.47 – 7.44 (m, 1H), 7.41 – 7.39 (m, 1H), 7.27 – 7.24 (m, 3H), 6.41 – 6.37 (m, 2H), 5.44 (d, J = 2.0 Hz, 1H), 4.98 (d, J = 6.0 Hz, 2H). ¹³C NMR (101 MHz, Chloroform-d) δ 176.89, 151.22, 141.12, 133.96, 130.59, 129.95, 129.60, 127.28, 90.18, 45.76. FTIR, cm⁻¹: 3413 (-NH), 3245, 3150 (C-H aromatic), 1600 (aromatic C=C), 1541 (aromatic C=N), 1308 (C=S), 775 (aromatic C-Cl). HRMS (+ESI-TOF) m/z: [M⁺] calcd for C₁₁H₁₁ClN₄S 266.03929; found 266.03929.



SB64

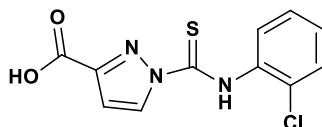
3-(benzyloxy)-N-(2-chlorophenyl)-1H-pyrazole-1-carbothioamide: 3-(benzyloxy)-1H-pyrazole (2.66 g, 15.4 mmol, 1.0 eq.) was dissolved in dry THF and transferred to a round bottom which consisted of K₂CO₃ (4.22 g, 30.6 mmol, 2.0 eq.). The reaction mixture was stirred vigorously for 1 h at 50° C. Then 2-chlorophenyl isothiocyanate (2.85 g, 16.9 mmol, 1.1 eq.) was added and refluxed at 70° C for 18 h. Potassium carbonate solid was filtered off by gravity filtration and filtrate's THF was evaporated in vacuo. Resulting crude product was purified by flash column chromatography (100% hexane to 5% ethyl acetate/hexane) to afford a light-yellow solid (5.1 g, 98%). ¹H NMR (400 MHz, DMSO-d₆) δ 11.30 (s, 1H), 8.56 (d, J = 3.0 Hz, 1H), 7.59 (ddd, J = 7.4, 5.5, 1.8 Hz, 2H), 7.53 – 7.50 (m, 2H), 7.39 (dddd, J = 15.0, 11.6, 8.4, 6.4 Hz, 5H), 6.28 (d, J = 3.0 Hz, 1H), 5.36 (s, 2H). ¹³C NMR (400 MHz, DMSO-d₆) δ 175.26, 165.05, 136.71, 136.30, 134.03, 131.39, 130.34, 130.15, 129.56, 128.93, 128.76, 128.16, 99.60, 71.20.

FTIR, cm^{-1} : 3265, 3200 (C-H aromatic), 1596 (aromatic C=C), 1513 (aromatic C=N), 1300 (C=S), 1089 (aliphatic ether), 738 (aromatic C-Cl). HRMS (+ESI-TOF) m/z : $[M^+]$ calcd for $\text{C}_{17}\text{H}_{14}\text{ClN}_3\text{OS}$ 343.05461; found 343.05458.



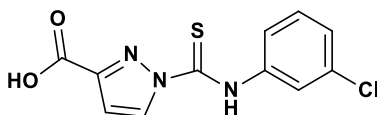
SB65

3-(benzyloxy)-1H-pyrazole (77 mg, 0.442 mmol, 1.0 eq.) was dissolved in dry THF and transferred to a round bottom which consisted of K_2CO_3 (61 mg, 0.442 mmol, 1.0 eq.). The reaction mixture was stirred vigorously for 1 h at 50°C . Then 3-chlorophenyl isothiocyanate (75 mg, 0.442 mmol, 1.0 eq.) was added and refluxed at 70°C for 18 h. Potassium carbonate solid was filtered off by gravity filtration and filtrate's THF was evaporated in vacuo. Resulting crude product was recrystallized to reveal a yellow solid (41 mg, 33%). ^1H NMR (400 MHz, DMSO-d_6) δ 8.64 (t, $J = 2.6$ Hz, 1H), 7.46 – 7.28 (m, 4H), 7.15 (t, $J = 7.9$ Hz, 2H), 7.08 – 7.01 (m, 2H), 6.82 – 6.69 (m, 2H), 5.75 (d, $J = 2.6$ Hz, 1H), 5.18 (s, 2H). ^{13}C NMR (400 MHz, DMSO-d_6) δ 163.24, 137.74, 132.61, 132.40, 129.67, 128.97, 128.80, 128.29, 124.70, 122.91, 122.29, 120.64, 92.41, 70.20. FTIR, cm^{-1} : 3314 (-NH), 3204 (C-H aromatic), 1590 (aromatic C=C), 1546 (aromatic C=N), 1372 (C=S), 1092 (aliphatic ether), 756 (aromatic C-Cl). HRMS (+ESI-TOF) m/z : $[M^+]$ calcd for $\text{C}_{17}\text{H}_{14}\text{ClN}_3\text{OS}$ 343.05461; found 343.05462.



SB66

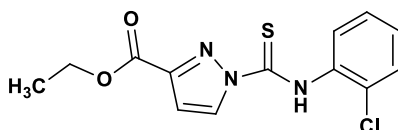
1-((2-chlorophenyl)carbamothioyl)-1H-pyrazole-3-carboxylic acid: To a solution of 1H-pyrazole-3-carboxylic acid (100 mg, 0.893 mmol, 1.0 eq.) and 2-chlorophenyl isothiocyanate (166 mg, 0.982 mmol, 1.1 eq.) in anhydrous DMF (2 mL), anhydrous potassium carbonate (246 mg, 1.78 mmol, 2.0 eq.) was added. The reaction mixture was kept stirring under argon at room temperature for 24 hours. This was filtered to remove excess K₂CO₃ and the filtrate concentrated to dryness. Descending column chromatography using silica gel as stationary phase and a gradient of DCM and MeOH as eluent gave a light-yellow solid (148 mg, 59%). ¹H NMR (400 MHz, DMSO-d₆) δ 10.81 (s, 1H), 8.37 (d, J = 3.0 Hz, 1H), 7.77 – 7.72 (m, 1H), 7.55 (dd, J = 8.0, 1.6 Hz, 1H), 7.42 – 7.28 (m, 3H), 5.96 (d, J = 3.0 Hz, 1H). ¹³C NMR (400 MHz, DMSO- d₆) δ 173.97, 159.25, 149.66, 136.19, 133.06, 130.31, 130.18, 129.96, 129.71, 129.37, 129.07, 128.80, 128.33, 127.89, 127.65, 127.39, 102.57. FTIR, cm⁻¹: 3338 (-NH), 3200 (carboxylic acid), 1563 (aromatic C=C), 1583 (aromatic C=N), 1388 (C=S), 745 (aromatic C-Cl). HRMS (+ESI-TOF) m/z: [M⁺] calcd for C₁₁H₈ClN₃O₂S 281.00257; found 281.00252.



SB67

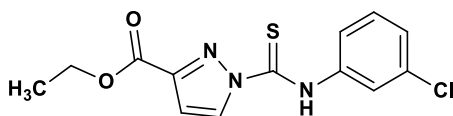
1-((3-chlorophenyl)carbamothioyl)-1H-pyrazole-3-carboxylic acid: To a solution of 1H-pyrazole-3-carboxylic acid (100 mg, 0.893 mmol, 1.0 eq.) and 3-chlorophenyl isothiocyanate (166 mg, 0.982 mmol, 1.1 eq.) in anhydrous DMF (2 mL), anhydrous potassium carbonate (246 mg, 1.78 mmol, 2.0 eq.) was added. The reaction mixture was kept stirring under argon at room temperature for 24 hours. This was filtered to remove excess K₂CO₃ and the filtrate concentrated to dryness. Descending column chromatography using silica gel as stationary phase and a gradient of DCM and MeOH as eluent gave a light-yellow solid (45 mg, 18%). ¹H NMR (400

MHz, DMSO-d₆) δ 10.07 (s, 1H), 7.97 (d, J = 7.7 Hz, 0H), 7.67 (t, J = 2.0 Hz, 1H), 7.42 – 7.33 (m, 2H), 7.20 (dt, J = 7.1, 2.0 Hz, 1H). ¹³C NMR (400 MHz, DMSO-d₆) δ 180.17, 162.79, 141.30, 133.67, 133.06, 130.90, 130.59, 124.73, 123.59, 122.55, 122.19, 118.22, 117.31, 36.26, 31.24. FTIR, cm⁻¹: 3180 (-NH), 3160 (carboxylic acid), 1583 (aromatic C=C), 1532 (aromatic C=N), 1304 (C=S), 773 (aromatic C-Cl). HRMS (+ESI-TOF) m/z: [M⁺] calcd for C₁₁H₈ClN₃O₂S 281.00257; found 281.00254.



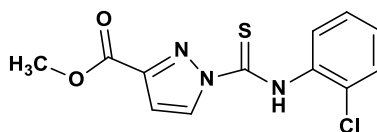
SB69

Ethyl 1-((2-chlorophenyl)carbamothioyl)-1H-pyrazole-3-carboxylate: A solution of ethyl 1H-pyrazole-3-carboxylate (50 mg, 0.357 mmol, 1.0 eq.), 2-chlorophenyl isothiocyanate (66 mg, 0.393 mmol, 1.1 eq.), and dichloromethane (10 mL) was refluxed at 45° C for 24 hours. The solvent was removed in vacuo, purified by flash column chromatography (100% DCM to 5% DCM/MeOH), revealing a white solid (21 mg, 19%). ¹H NMR (400 MHz, DMSO-d₆) δ 8.75 (s, 1H), 7.55 (dd, J = 18.1, 8.0 Hz, 1H), 7.47 (m, 1H), 7.38 (m, 1H), 6.74 (s, 2H), 4.34 (m, 1H), 4.21 (m, 2H), 1.30 (d, J = 7.0 Hz, 3H). ¹³C NMR (400 MHz, DMSO-d₆) δ 181.80, 162.61, 143.39, 136.63, 130.64, 130.48, 130.28, 130.19, 129.93, 129.71, 129.06, 128.09, 127.76, 127.67, 107.87, 60.44, 14.70. FTIR, cm⁻¹: 3243 (-NH), 3127, 2990 (C-H aromatic), 1697 (C=O), 1581 (aromatic C=C), 1516 (aromatic C=N), 1463 (-CH₃), 1396 (C=S), 758 (aromatic C-Cl). HRMS (+ESI-TOF) m/z: [M⁺] calcd for C₁₃H₁₂ClN₃O₂S 309.03388; found 309.03383.



SB70

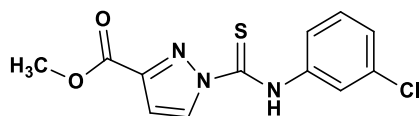
Ethyl 1-((3-chlorophenyl)carbamothioyl)-1H-pyrazole-3-carboxylate: A solution of ethyl 1H-pyrazole-3-carboxylate (50 mg, 0.357 mmol, 1.0 eq.), 3-chlorophenyl isothiocyanate (66 mg, 0.393 mmol, 1.1 eq.), and dichloromethane (10 mL) was refluxed at 45° C for 24 hours. The solvent was removed in vacuo, purified by flash column chromatography (100% DCM to 5% DCM/MeOH), revealing a white solid (33 mg, 30%). ¹H NMR (400 MHz, DMSO-d₆) δ 12.12 (s, 1H), 8.83 (d, J = 2.8 Hz, 1H), 7.63 (d, J = 2.1 Hz, 1H), 7.47 (d, J = 1.5 Hz, 3H), 7.01 (d, J = 2.7 Hz, 1H), 4.37 (d, J = 7.1 Hz, 2H), 1.34 (t, J = 7.1 Hz, 3H). ¹³C NMR (400 MHz, DMSO-d₆) δ 180.16, 162.76, 141.30, 134.35, 133.06, 131.83, 130.55, 128.49, 126.24, 125.37, 123.55, 122.50. FTIR, cm⁻¹: 3246 (-NH), 3128, 2984 (C-H aromatic), 1724 (C=O), 1636 (aromatic C=C), 1534 (aromatic C=N), 1410 (-CH₃), 1302 (C=S), 765 (aromatic C-Cl). HRMS (+ESI-TOF) m/z: [M+] calcd for C₁₃H₁₂ClN₃O₂S 309.03388; found 309.03387.



SB73

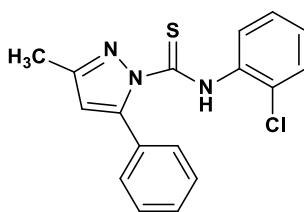
Methyl 1-((2-chlorophenyl)carbamothioyl)-1H-pyrazole-3-carboxylate: A solution of methyl 1H-pyrazole-3-carboxylate (45 mg, 0.357 mmol, 1.0 eq.), 2-chlorophenyl isothiocyanate (66 mg, 0.393 mmol, 1.1 eq.), and dichloromethane (10 mL) was refluxed at 45° C for 24 hours. The solvent was removed in vacuo, purified by flash column chromatography (100% DCM to 5% DCM/MeOH), revealing a white solid (25 mg, 24%). ¹H NMR (400 MHz, DMSO-d₆) δ 10.81 (s, 1H), 8.37 (d, J = 3.0 Hz, 1H), 7.77 – 7.72 (m, 1H), 7.55 (dd, J = 8.0, 1.6 Hz, 1H), 7.42 – 7.28 (m, 2H), 5.96 (d, J = 3.0 Hz, 1H). ¹³C NMR (400 MHz, DMSO- d₆) δ 173.97, 159.25, 149.66, 136.19, 133.06, 130.31, 130.18, 129.96, 129.71, 129.37, 129.07, 128.80, 128.33, 127.89, 127.65, 127.39, 102.57. FTIR, cm⁻¹: 3338 (-NH), 3200 (carboxylic acid), 1563 (aromatic C=C), 1583

(aromatic C=N), 1388 (C=S), 745 (aromatic C-Cl). HRMS (+ESI-TOF) m/z: [M⁺] calcd for C₁₂H₁₀ClN₃O₂S 295.01822; found 295.01824.



SB74

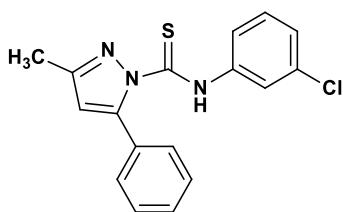
Methyl 1-((3-chlorophenyl)carbamothioyl)-1H-pyrazole-3-carboxylate: A solution of methyl 1H-pyrazole-3-carboxylate (50 mg, 0.396 mmol, 1.0 eq.), 3-chlorophenyl isothiocyanate (74 mg, 0.437 mmol, 1.1 eq.), and dichloromethane (10 mL) was refluxed at 45° C for 24 hours. The solvent was removed in vacuo, purified by flash column chromatography (100% DCM to 5% DCM/MeOH), revealing a white solid (30 mg, 26%). ¹H NMR (400 MHz, DMSO-d₆) δ 7.87 (s, 1H), 7.66 – 7.60 (m, 1H), 7.52 – 7.31 (m, 3H), 6.76 (s, 1H), 3.80 (s, 3H). ¹³C NMR (400 MHz, DMSO-d₆) δ 180.17, 163.04, 143.12, 134.34, 131.85, 130.56 (d, J = 4.0 Hz), 128.51, 126.27, 125.40, 124.73, 123.58, 122.54, 107.91. FTIR, cm⁻¹: 3446 (-NH), 3154, 3121 (C-H aromatic), 1717 (C=O), 1584 (aromatic C=C), 1363 (aromatic C=N), 1260 (C=S), 754 (aromatic C-Cl). HRMS (+ESI-TOF) m/z: [M⁺] calcd for C₁₂H₁₀ClN₃O₂S 295.01822; found 295.01820.



SB75

N-(2-chlorophenyl)-3-methyl-5-phenyl-1H-pyrazole-1-carbothioamide: To a solution of 3-methyl-5-phenyl-1H-pyrazole (161 mg, 1.02 mmol, 1.0 eq.) and 2-chlorophenyl isothiocyanate (172 mg, 1.02 mmol, 1.0 eq.) in anhydrous DMF (2 mL), anhydrous potassium carbonate (281 mg, 2.04 mmol, 2.0 eq.) was added. The reaction mixture was kept stirring under argon at room

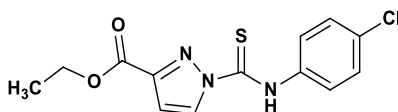
temperature for 24 hours. This was filtered to remove excess K_2CO_3 and the filtrate concentrated to dryness. Descending column chromatography using silica gel as stationary phase and a gradient of DCM and MeOH as eluent gave a light-yellow solid (60 mg, 18%). ^1H NMR (600 MHz, DMSO- d_6) δ 12.54 (s, 1H), 7.74 (d, J = 7.5 Hz, 2H), 7.44 – 7.35 (m, 2H), 7.28 (t, J = 7.9 Hz, 1H), 7.17 (dd, J = 7.9, 1.5 Hz, 1H), 7.04 – 6.98 (m, 1H), 6.78 (dd, J = 8.0, 1.5 Hz, 1H), 6.53 (td, J = 7.5, 1.5 Hz, 1H), 6.44 (s, 1H), 2.25 (s, 3H). ^{13}C NMR (400 MHz, DMSO- d_6) δ 130.21, 129.94, 129.43, 129.13, 128.12, 127.67, 126.01, 125.40, 117.24, 115.87. FTIR, cm^{-1} : 3320 (-NH), 3059 (C-H aromatic), 1571 (aromatic C=C), 1458 (-CH₃), 1312 (aromatic C=N), 1202 (C=S), 761 (aromatic C-Cl). HRMS (+ESI-TOF) m/z : $[M+]$ calcd for $C_{17}H_{14}ClN_3S$ 327.05970; found 327.05965.



SB76

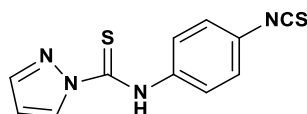
N-(3-chlorophenyl)-3-methyl-5-phenyl-1H-pyrazole-1-carbothioamide: To a solution of 3-methyl-5-phenyl-1H-pyrazole (161 mg, 1.02 mmol, 1.0 eq.) and 3-chlorophenyl isothiocyanate (172 mg, 1.02 mmol, 1.0 eq.) in anhydrous DMF (2 mL), anhydrous potassium carbonate (281 mg, 2.04 mmol, 2.0 eq.) was added. The reaction mixture was kept stirring under argon at room temperature for 24 hours. This was filtered to remove excess K_2CO_3 and the filtrate concentrated to dryness. Descending column chromatography using silica gel as stationary phase and a gradient of DCM and MeOH as eluent gave a light-yellow solid (62 mg, 19%). ^1H NMR (600 MHz, DMSO- d_6) δ 12.55 (s, 1H), 8.00 – 7.13 (m, 9H), 6.44 (s, 1H), 2.25 (s, 3H). ^{13}C NMR (400 MHz, DMSO- d_6) δ 162.79, 141.52, 133.68, 131.02, 130.89, 129.08, 127.71, 125.41, 122.13,

118.12, 117.21, 116.69, 115.87, 36.25, 31.23. FTIR, cm^{-1} : 3059 (-NH), 2852 (C-H aromatic), 1661 (aromatic C=C), 1458 (-CH₃), 1311 (aromatic C=N), 1292 (C=S), 761 (aromatic C-Cl). HRMS (+ESI-TOF) m/z: [M⁺] calcd for C₁₇H₁₄ClN₃S 327.05970; found 327.05975.



SB77

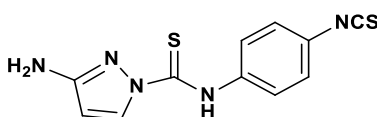
Ethyl 1-((4-chlorophenyl)carbamothioyl)-1H-pyrazole-3-carboxylate: A solution of ethyl 1H-pyrazole-3-carboxylate (81 mg, 0.580 mmol, 1.0 eq.), 4-chlorophenyl isothiocyanate (108 mg, 0.638 mmol, 1.1 eq.), and dichloromethane (10 mL) was refluxed at 45° C for 24 hours. The solvent was removed in vacuo, purified by flash column chromatography (100% DCM to 5% DCM/MeOH), revealing a white solid (61 mg, 34%). ¹H NMR (400 MHz, DMSO-d₆) δ 13.48 (s, 1H), 7.86 (t, J = 1.8 Hz, 1H), 7.57 – 7.44 (m, 3H), 7.43 – 7.35 (m, 1H), 6.74 (t, J = 2.1 Hz, 1H), 4.26 (q, J = 7.1 Hz, 2H), 1.29 (t, J = 7.1 Hz, 3H). ¹³C NMR (400 MHz, DMSO-d₆) δ 180.22, 162.60, 143.39, 140.37, 138.78, 132.70, 130.48, 130.38, 129.53, 128.88, 128.82, 128.25, 125.81, 109.27, 107.86, 61.17, 60.44, 14.71, 14.62. FTIR, cm^{-1} : 3290 (-NH), 2978 (C-H aromatic), 1729 (C=O), 1584 (aromatic C=C), 1505 (aromatic C=N), 1413 (-CH₃), 1342 (C=S), 729 (aromatic C-Cl). HRMS (+ESI-TOF) m/z: [M⁺] calcd for C₁₃H₁₂ClN₃O₂S 309.03388; found 309.03389.



SB78

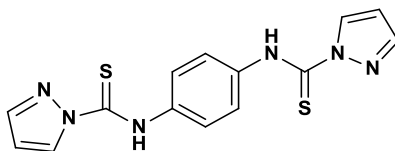
N-(4-isothiocyanatophenyl)-1H-pyrazole-1-carbothioamide: A solution of 1H-pyrazole (18 mg, 0.260 mmol, 1.0 eq.), 1,4-diisothiocyanatobenzene (50 mg, 0.260 mmol, 1.0 eq.), and dichloromethane (10 mL) was stirred at room temperature for 24 hours. The solvent was

removed in vacuo, purified by flash column chromatography, revealing a white solid (57 mg, 84%). ^1H NMR (400 MHz, DMSO- d_6) δ 11.96 (s, 1H), 8.76 (dd, J = 8.0, 2.9 Hz, 1H), 8.00 (d, J = 3.9 Hz, 1H), 7.74 (s, 1H), 7.52 (s, 4H). ^{13}C NMR (101 MHz, DMSO- d_6) δ 174.75, 143.97, 139.41, 137.89, 132.44, 129.65, 128.01, 126.61, 124.64, 110.64 (d, J = 14.9 Hz), 104.67. FTIR, cm^{-1} : 3241 (-NH), 2919, 2851 (C-H aromatic), 2087 (-NCS), 1510 (aromatic C=C), 1417 (-CH₃), 1294 (aromatic C=N), 1282 (C=S), 751 (aromatic C-Cl). UPLC-MS [$\text{M}+1$] calcd for $\text{C}_{11}\text{H}_8\text{N}_4\text{S}_2$ 261.34; found 261.05.



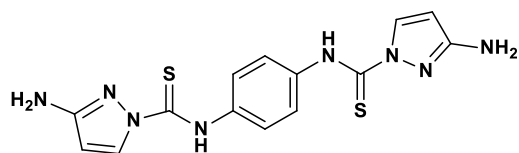
SB79

3-amino-N-(4-isothiocyanatophenyl)-1H-pyrazole-1-carbothioamide: A solution of 3-aminopyrazole (22 mg, 0.260 mmol, 1.0 eq.), 1,4-diisothiocyanatobenzene (50 mg, 0.260 mmol, 1.0 eq.), and dichloromethane (10 mL) was stirred at room temperature for 24 hours. The solvent was removed in vacuo, purified by flash column chromatography, revealing a white solid (69 mg, 96%). ^1H NMR (400 MHz, DMSO- d_6) δ 8.43 (dd, J = 4.8, 3.0 Hz, 1H), 7.78 – 7.73 (m, 1H), 7.65 (d, J = 4.0 Hz, 1H), 7.53 – 7.43 (m, 2H), 5.99 (t, J = 2.9 Hz, 1H), 5.72 (s, 2H). ^{13}C NMR (101 MHz, DMSO- d_6) δ 173.05, 159.13 (d, J = 7.2 Hz), 138.26, 136.37, 133.72, 133.11 (d, J = 5.1 Hz), 128.02, 127.49, 126.50 (d, J = 9.1 Hz), 125.36, 102.62. FTIR, cm^{-1} : 3415 (-NH), 3329, 3244 (C-H aromatic), 2082 (-NCS), 1570 (aromatic C=C), 1475 (-CH₃), 1293 (aromatic C=N), 1258 (C=S), 761 (aromatic C-Cl). UPLC-MS [$\text{M}+1$] calcd for $\text{C}_{11}\text{H}_9\text{N}_5\text{S}_2$ 276.35; found 276.07.



SB80

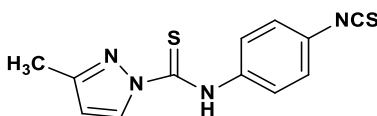
N,N'-(1,4-phenylene)bis(1H-pyrazole-1-carbothioamide): A solution of 1H-pyrazole (39 mg, 0.572 mmol, 2.2 eq.), 1,4-diisothiocyanatobenzene (50 mg, 0.260 mmol, 1.0 eq.), and dichloromethane (10 mL) was stirred at room temperature for 24 hours. The solvent was removed in vacuo, purified by flash column chromatography, revealing a white solid (35 mg, 41%). ¹H NMR (400 MHz, DMSO-d₆) δ 8.76 (ddd, J = 8.4, 2.8, 0.7 Hz, 1H), 8.03 – 7.96 (m, 1H), 7.74 – 7.70 (m, 2H), 7.53 – 7.49 (m, 4H), 6.26 (s, 2H). ¹³C NMR (101 MHz, DMSO-d₆) δ 174.75 (d, J = 4.8 Hz), 143.90 (d, J = 9.5 Hz), 137.95, 136.73, 135.22, 134.06, 132.43, 129.65, 128.01, 127.26, 126.61, 126.14, 110.62 (d, J = 13.1 Hz), 104.67. FTIR, cm⁻¹: 3275, 3120 (-NH), 1506 (aromatic C=C), 1293 (aromatic C=N), 1225 (C=S), 764 (aromatic C-Cl). UPLC-MS [M+1] calcd for C₁₄H₁₂N₆S₂ 329.42; found 327.20. UPLC-MS [M+1] calcd for C₁₄H₁₂N₆S₂ 329.42; found 327.20.



SB81

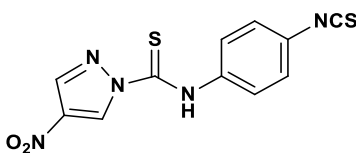
N,N'-(1,4-phenylene)bis(3-amino-1H-pyrazole-1-carbothioamide): A solution of 3-aminopyrazole (48 mg, 0.576 mmol, 2.2 eq.), 1,4-diisothiocyanatobenzene (50 mg, 0.260 mmol, 1.0 eq.), and dichloromethane (10 mL) was stirred at room temperature for 24 hours. The solvent was removed in vacuo, purified by flash column chromatography, revealing an off white solid (186 mg, 91%). ¹H NMR (400 MHz, DMSO-d₆) δ 12.82 (s, 1H), 11.39 (s, 1H), 7.71 (s, 2H), 7.50 (s, 2H), 7.25 (s, 4H), 6.26 (t, J = 2.0 Hz, 4H). ¹³C NMR (101 MHz, DMSO-d₆) δ (214.47, 212.55, 192.41, 181.79, 164.46, 162.03, 160.36, 131.74, 119.57, 104.68, 92.89, 91.10, 90.71, 74.74. FTIR, cm⁻¹: 3349 (-NH₂), 3143, 3121, (-NH), 1513 (aromatic C=C), 1290 (aromatic

C=N), 1214 (C=S), 764 (aromatic C-Cl). UPLC-MS [M+1] calcd for C₁₄H₁₄N₈S₂ 359.44; found 359.14.



SB82

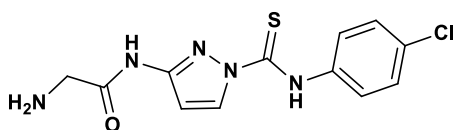
N-(4-isothiocyanatophenyl)-3-methyl-1H-pyrazole-1-carbothioamide: A solution of 3-methylpyrazole (21 mg, 0.260 mmol, 1.0 eq.), 1,4-diisothiocyanatobenzene (50 mg, 0.260 mmol, 1.0 eq.), and dichloromethane (10 mL) was stirred at 45° C for 24 hours. The solvent was removed in vacuo, purified by flash column chromatography, revealing a white solid (36 mg, 50%). ¹H NMR (400 MHz, DMSO-d₆) δ 11.81 (s, 1H), 8.64 (d, J = 2.8 Hz, 1H), 7.68 (d, J = 13.6 Hz, 2H), 7.48 (d, J = 7.7 Hz, 2H), 6.47 (d, J = 10.9 Hz, 1H), 2.32 (d, J = 3.0 Hz, 3H). ¹³C NMR (101 MHz, DMSO-d₆) δ 174.54, 139.41, 135.19, 133.00, 129.64, 128.02, 127.29, 126.93, 126.67, 126.54, 126.07, 125.92, 124.63, 124.52, 111.32, 104.10, 14.11. FTIR, cm⁻¹: 3349 (-NH₂), 3143, 3121, (-NH), 2100 (-NCS), 1513 (aromatic C=C), 1290 (aromatic C=N), 1214 (C=S), 764 (aromatic C-Cl). UPLC-MS [M+1] calcd for C₁₂H₁₀N₄S₂ 275.36; found 274.23.



SB83

N-(4-isothiocyanatophenyl)-4-nitro-1H-pyrazole-1-carbothioamide: Two 100 mL round bottom flasks were flame dried to remove moisture. To flask A was added 4-nitro pyrazole (29 mg, 0.260 mmol, 1.0 eq.) and dry THF (10 mL). To flask B was added sodium hydride (12.5 mg, 0.520 mmol, 2.0 eq.) and dry THF (10 mL). Both flasks were stirred at 0° C for 20 minutes. The mixture from flask B was cannulated into flask A using a two-headed needle. This solution was

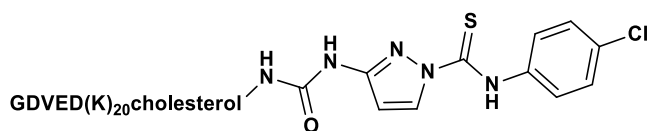
kept stirring at r.t. under argon for 3 hours. 1,4-diisothiocyanatobenzene (50 mg, 0.260 mmol, 1.0 eq.) was added to the reaction, which continued stirring for 12 hours at r.t. The reaction was concentrated to dryness to reveal a white solid (60 mg, 75%). ^1H NMR (400 MHz, DMSO- d_6) δ 9.97 (s, 1H), 8.03 (d, J = 8.9 Hz, 1H), 7.78 (d, J = 8.4 Hz, 2H), 7.36 (s, 2H), 7.15 (s, 1H). ^{13}C NMR (101 MHz, DMSO- d_6) δ 167.46, 136.36, 135.49, 133.80, 132.17, 132.08, 129.14, 125.74, 124.18, 123.71, 122.11, 67.87, 38.54, 30.89, 30.26, 28.83, 23.71, 23.48, 22.87, 14.37, 11.27. FTIR, cm^{-1} : 3390 (-NH), 3241, 2919 (C-H aromatic), 2059 (-NCS), 1477 (aromatic C=C), 1416 (-CH₃), 1271 (aromatic C=N), 1148 (C=S), 735 (aromatic C-Cl). UPLC-MS [M+1] calcd for C₁₁H₇N₅O₂S₂ 306.34; found 305.22.



SB84

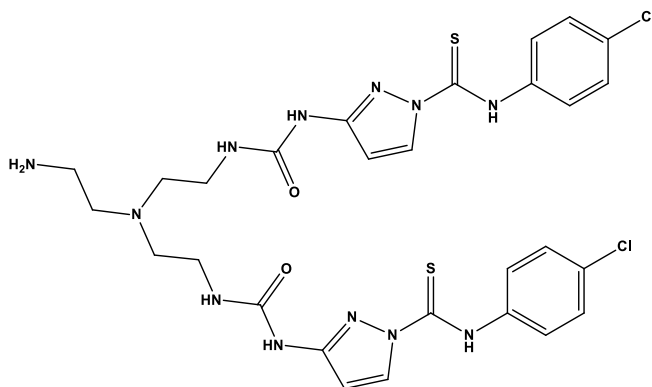
2-amino-N-(1-((4-chlorophenyl)carbamothioyl)-1H-pyrazol-3-yl)acetamide: To a RBF was added carbonyldiimidazole (29.7 mg, 0.396 mmol) and dimethylformamide (3 mL). This solution was allowed to stir at room temperature and glycine was added slowly. Following completion of this addition, the reaction was left to stir at room temperature for 50 minutes. This was then heated to 60° C and left to reflux for 2 hours. The solvent was then removed in vacuo and ice water added to the residue. A pink-colored solid precipitated, which was then collected via filtration (106 mg, 86%). ^1H NMR (400 MHz, DMSO- d_6) δ 12.70 (s, 1H), 10.81 (s, 1H), 7.75 (s, 1H), 7.68 (s, 2H), 7.42 (s, 2H), 7.05 (s, 1H), 6.05 (s, 2H). ^{13}C NMR (101 MHz, Deuterium Oxide) δ 149.71, 130.21, 128.94, 128.85, 126.29, 94.89, 40.53, 40.32, 40.11, 39.90, 39.70, 39.49, 39.28. FTIR, cm^{-1} : 3365(-NH₂), 3179, 3045 (-NH), 1575 (C=O), 1527 (aromatic

C=C), 1269 (aromatic C=N), 1205 (C=S), 755 (aromatic C-Cl). UPLC-MS [M+1] calcd for $C_{12}H_{12}ClN_5OS$ 310.77; found 310.29.



SB85

GDVED(K)₂₀cholesterol-N-(4-chlorophenyl)-3-ureido-1H-pyrazole-1-carbothioamide: To a round bottom flask was added KE03 (29 mg, 0.113 mmol, 2.0 eq.), 1,1'-Carbonyldiimidazole (18 mg, 0.113 mmol, 2.0 eq.) and DMF (2 mL). This was left to stir at room temperature under argon for 50 minutes. The peptide K20-cholesterol (196 mg, 0.0564 mmol, 1.0 eq.) was added to the reaction and refluxed at 60° C overnight. The solvent was removed in vacuo and diethyl ether was added to the residue. The resulting sticky material was lyophilized to reveal a dark yellow solid (62 mg, 28%). ¹H NMR (400 MHz, DMSO-d₆) δ 8.03 (d, J = 14.5 Hz, 2H), 7.96 (s, 3H), 2.90 (s, 8H), 1.10 (t, J = 7.0 Hz, 2H), 0.86 (s, 1H). ¹³C NMR (101 MHz, DMSO-d₆) δ 171.94, 162.79, 128.61, 121.55, 39.12, 37.44, 36.26, 31.23, 31.07, 27.08, 22.71, 19.72. FTIR, cm⁻¹: 3250, 3013, 2905 (-NH), 1675 (C=O), 1500 (C=S), 1109 (aromatic C=N), 811 (aromatic C-Cl).

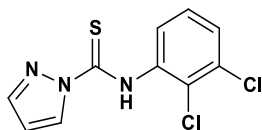


SB86

3,3'-((((((2-aminoethyl)azanediyl)bis(ethane-2,1-

diyl))bis(azanediyl))bis(carbonyl))bis(azanediyl))bis(N-(4-chlorophenyl)-1H-pyrazole-1-

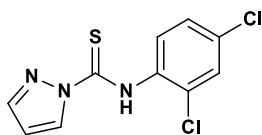
carbothioamide): To a round bottom flask was added KE03 (6 mg, 0.033 mmol, 1.0 eq.), 1,1'-Carbonyldiimidazole (32 mg, 0.198 mmol, 6.0 eq.) and DMF (3 mL). This was left to stir at room temperature under argon for 50 minutes. 2-hydroxypropane-1,2,3-tricarboxylic acid (50 mg, 0.198 mmol, 6.0 eq.) was added to the reaction and refluxed at 60° C overnight. The solvent was removed in vacuo and diethyl ether was added to the residue. The resulting material was lyophilized to reveal a yellow solid (21 mg, 90%). ¹H NMR (400 MHz, DMSO-d₆) δ 9.73 (d, J = 36.1 Hz, 6H), 8.06 – 7.91 (m, 9H), 7.64 (s, 2H), 7.49 – 7.43 (m, 4H), 7.38 – 7.30 (m, 4H), 7.01 (dt, J = 6.3, 3.0 Hz, 5H), 6.55 (d, J = 8.7 Hz, 2H). ¹³C NMR (101 MHz, DMSO-d₆) δ 161.69, 135.62, 128.95, 115.62, 53.47, 42.31, 36.26, 35.96. FTIR, cm⁻¹: 3282, 3050, 2933, (-NH), 1647 (C=O), 1419 (C=S), 1173 (aromatic C=N), 719 (aromatic C-C). HRMS (+ESI-TOF) m/z: [M⁺] calcd for C₂₈H₃₂Cl₂N₁₂O₂S₂ 703.66600; found 703.66636.



SB87

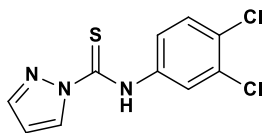
N-(2,3-dichlorophenyl)-1H-pyrazole-1-carbothioamide: A solution of 1H-pyrazole (46 mg, 0.668 mmol, 1.0 eq.), 2,3-dichlorophenylisothiocyanate (150 mg, 0.735 mmol, 1.1 eq.), and dichloromethane (10 mL) was stirred at room temperature for 24 hours. The solvent was removed in vacuo, purified by flash column chromatography, revealing a white solid (182 mg, 99%). ¹H NMR (400 MHz, DMSO-d₆) δ 11.90 (s, 1H), 8.73 (d, J = 2.8 Hz, 1H), 7.67 (ddd, J = 11.7, 8.0, 1.6 Hz, 1H), 7.55 (dd, J = 8.0, 3.9, 1.6 Hz, 1H), 7.45 (dt, J = 16.1, 8.1 Hz, 1H), 6.65 (dd, J = 2.8, 1.6 Hz, 1H), 6.26 (t, J = 2.0 Hz, 1H). ¹³C NMR (101 MHz, DMSO-d₆) δ 176.22,

144.29, 138.42, 133.00, 132.55, 132.48, 130.76, 130.49, 130.16, 130.01, 129.53, 129.38, 128.80, 126.52, 110.76, 104.66. FTIR, cm^{-1} : 3227 (-NH), 1521 (aromatic C=C), 1417 (-CH₃), 1280 (aromatic C=N), 1182 (C=S), 750, 728 (aromatic C-Cl). HRMS (+ESI-TOF) m/z : [M⁺] calcd for C₁₀H₇Cl₂N₃S 270.97377; found 270.97381.



SB88

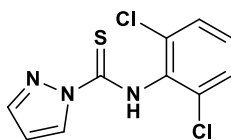
N-(2,4-dichlorophenyl)-1H-pyrazole-1-carbothioamide: A solution of 1H-pyrazole (46 mg, 0.668 mmol, 1.0 eq.), 2,4-dichlorophenylisothiocyanate (150 mg, 0.735 mmol, 1.1 eq.), and dichloromethane (10 mL) was stirred at room temperature for 24 hours. The solvent was removed in vacuo, purified by flash column chromatography, revealing a white solid (147 mg, 80%). ¹H NMR (400 MHz, DMSO-d₆) δ 8.72 (d, J = 2.8 Hz, 1H), 7.86 (d, J = 2.3 Hz, 1H), 7.79 (d, J = 2.2 Hz, 1H), 7.53 (td, J = 4.3, 3.8, 2.3 Hz, 1H), 6.65 (dd, J = 2.8, 1.6 Hz, 1H), 6.26 (t, J = 2.0 Hz, 1H). ¹³C NMR (101 MHz, DMSO-d₆) δ 176.16, 144.25, 133.18, 133.10, 132.44, 132.02, 131.72, 130.22, 129.75, 129.21, 128.84, 128.42, 128.10, 110.70, 104.65, 45.78, 44.42, 8.90. FTIR, cm^{-1} : 3271 (-NH), 1569 (aromatic C=C), 1468 (-CH₃), 1289 (aromatic C=N), 1189 (C=S), 845, 747 (aromatic C-Cl). HRMS (+ESI-TOF) m/z : [M⁺] calcd for C₁₀H₇Cl₂N₃S 270.97377; found 270.97344.



SB89

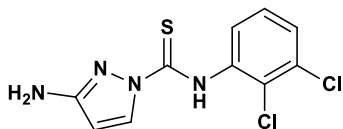
N-(3,4-dichlorophenyl)-1H-pyrazole-1-carbothioamide: A solution of 1H-pyrazole (46 mg, 0.668 mmol, 1.0 eq.), 3,4-dichlorophenylisothiocyanate (150 mg, 0.735 mmol, 1.1 eq.), and

dichloromethane (10 mL) was stirred at room temperature for 24 hours. The solvent was removed in vacuo, purified by flash column chromatography, revealing a white solid (174 mg, 94%). ^1H NMR (400 MHz, DMSO- d_6) δ 11.99 (s, 1H), 8.75 (dd, J = 2.7, 0.7 Hz, 1H), 7.82 (d, J = 2.4 Hz, 1H), 7.73 – 7.68 (m, 1H), 7.45 (dd, J = 8.6, 2.4 Hz, 1H), 6.65 (dd, J = 2.8, 1.6 Hz, 1H), 6.26 (t, J = 2.0 Hz, 1H). ^{13}C NMR (101 MHz, DMSO- d_6) δ 175.07, 144.06, 138.62, 136.13, 132.53, 132.49, 132.02, 131.12, 130.96, 130.80, 130.65, 129.29, 128.33, 127.83, 126.86, 126.45, 110.82, 104.65. FTIR, cm^{-1} : 3226 (-NH), 1581 (aromatic C=C), 1467 (-CH₃), 1218 (aromatic C=N), 1186 (C=S), 759, 726 (aromatic C-Cl). HRMS (+ESI-TOF) m/z : [M⁺] calcd for C₁₀H₇Cl₂N₃S 270.97377; found 270.97359.



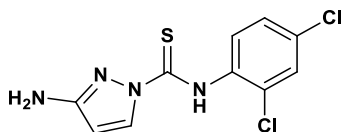
SB90

N-(2,6-dichlorophenyl)-1H-pyrazole-1-carbothioamide: A solution of 1H-pyrazole (23 mg, 0.334 mmol, 1.0 eq.), 2,6-dichlorophenylisothiocyanate (75 mg, 0.368 mmol, 1.1 eq.), and dichloromethane (10 mL) was stirred at room temperature for 24 hours. The solvent was removed in vacuo, purified by flash column chromatography, revealing a white solid (91 mg, 99%). ^1H NMR (400 MHz, DMSO- d_6) δ 10.91 (s, 1H), 8.72 (d, J = 2.6 Hz, 1H), 7.86 (d, J = 1.5 Hz, 1H), 7.63 (d, J = 8.2 Hz, 1H), 7.50 (d, J = 8.1 Hz, 1H), 7.37 (s, 1H), 6.52 (dd, J = 2.7, 1.6 Hz, 1H). ^{13}C NMR (101 MHz, DMSO- d_6) δ 140.60, 131.68, 129.67, 129.46, 128.81, 128.78, 127.64, 104.65, 46.09, 9.26, 9.23. FTIR, cm^{-1} : 3184 (-NH), 1529 (aromatic C=C), 1435 (-CH₃), 1243 (aromatic C=N), 1187 (C=S), 779, 763 (aromatic C-Cl). HRMS (+ESI-TOF) m/z : [M⁺] calcd for C₁₀H₇Cl₂N₃S 270.97377; found 270.97394.



SB91

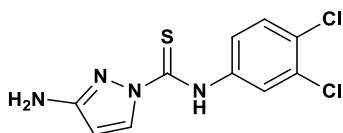
3-amino-N-(2,3-dichlorophenyl)-1H-pyrazole-1-carbothioamide: A solution of 3-aminopyrazole (55 mg, 0.668 mmol, 1.0 eq.), 2,3-dichlorophenylisothiocyanate (150 mg, 0.735 mmol, 1.1 eq.), and dichloromethane (10 mL) was stirred at room temperature for 24 hours. The solvent was removed in vacuo, purified by flash column chromatography, revealing a white solid (132 mg, 69%). ^1H NMR (400 MHz, DMSO- d_6) δ 11.04 (s, 1H), 8.02 – 7.96 (m, 1H), 7.53 (dd, J = 8.1, 1.5 Hz, 1H), 7.40 (s, 1H), 7.28 – 7.24 (m, 1H), 7.01 (t, J = 8.0 Hz, 1H), 6.74 (ddd, J = 7.8, 6.5, 1.5 Hz, 1H), 6.15 – 5.93 (m, 2H). ^{13}C NMR (101 MHz, DMSO- d_6) δ 177.58, 175.22, 153.78, 149.55, 147.21, 138.68, 132.05, 132.00, 130.24, 128.48, 128.12, 128.03, 127.92, 127.45, 117.20, 114.07, 91.05, 55.38. FTIR, cm^{-1} : 3404 (-NH₂), 3191 (-NH), 1530 (aromatic C=C), 1449 (-CH₃), 1246 (aromatic C=N), 1187 (C=S), 732, 715 (aromatic C-Cl). HRMS (+ESI-TOF) m/z : [M⁺] calcd for C₁₀H₈Cl₂N₄S 285.98467; found 285.98450.



SB92

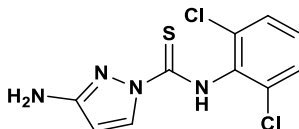
3-amino-N-(2,4-dichlorophenyl)-1H-pyrazole-1-carbothioamide: A solution of 1-aminopyrazole (46 mg, 0.668 mmol, 1.0 eq.), 2,3-dichlorophenylisothiocyanate (150 mg, 0.735 mmol, 1.1 eq.), and dichloromethane (10 mL) was stirred at room temperature for 24 hours. The solvent was removed in vacuo, purified by flash column chromatography, revealing a white solid (147 mg, 77%). ^1H NMR (400 MHz, DMSO- d_6) δ 11.04 (s, 1H), 8.09 (d, J = 8.8 Hz, 1H), 7.73 (dd, J = 10.9, 2.5 Hz, 2H), 7.46 (dd, J = 8.8, 2.5 Hz, 1H), 7.25 (s, 2H), 5.76 (s, 2H). ^{13}C NMR

(101 MHz, DMSO-d₆) δ 153.76, 149.51, 135.89, 131.60, 130.85, 130.18, 129.42, 129.13, 127.53, 91.01, 55.40, 45.78, 44.41, 8.89. FTIR, cm⁻¹: 3422 (-NH₂), 2937 (-NH), 1547 (aromatic C=C), 1473 (-CH₃), 1247 (aromatic C=N), 1125 (C=S), 747, 660 (aromatic C-Cl). HRMS (+ESI-TOF) m/z: [M⁺] calcd for C₁₀H₈Cl₂N₄S 285.98467; found 285.98428.



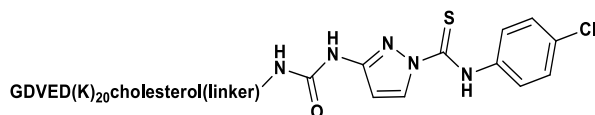
SB93

3-amino-N-(3,4-dichlorophenyl)-1H-pyrazole-1-carbothioamide: A solution of 3-aminopyrazole (46 mg, 0.668 mmol, 1.0 eq.), 2,3-dichlorophenylisothiocyanate (150 mg, 0.735 mmol, 1.1 eq.), and dichloromethane (10 mL) was stirred at room temperature for 24 hours. The solvent was removed in vacuo, purified by flash column chromatography, revealing a white solid (173 mg, 90%). ¹H NMR (400 MHz, DMSO-d₆) δ 10.92 (s, 1H), 8.15 (d, J = 2.4 Hz, 1H), 7.57 (dd, J = 8.7, 2.5 Hz, 1H), 7.26 (d, J = 2.1 Hz, 1H), 7.18 (d, J = 8.7 Hz, 1H), 6.74 (d, J = 2.6 Hz, 1H), 6.52 (dd, J = 8.7, 2.6 Hz, 1H), 5.38 (d, J = 2.1 Hz, 1H). ¹³C NMR (101 MHz, DMSO-d₆) δ 176.72, 175.21, 153.76, 149.61, 149.54, 139.59, 131.00, 130.96, 130.73, 130.58, 130.24, 127.37, 125.84, 124.59, 116.45, 114.95, 114.51, 102.86, 91.06, 55.38. FTIR, cm⁻¹: 3157 (-NH₂), 2924 (-NH), 1568 (aromatic C=C), 1490 (-CH₃), 1237 (aromatic C=N), 1187 (C=S), 748, 711 (aromatic C-Cl). HRMS (+ESI-TOF) m/z: [M⁺] calcd for C₁₀H₈Cl₂N₄S 285.98467; found 285.98464.



SB94

3-amino-N-(2,6-dichlorophenyl)-1H-pyrazole-1-carbothioamide: A solution of 1H-pyrazole (46 mg, 0.668 mmol, 1.0 eq.), 2,3-dichlorophenylisothiocyanate (150 mg, 0.735 mmol, 1.1 eq.), and dichloromethane (10 mL) was stirred at room temperature for 24 hours. The solvent was removed in vacuo, purified by flash column chromatography, revealing a white solid (92 mg, 48%). ¹H NMR (400 MHz, DMSO-*d*₆) δ 10.89 (s, 1H), 8.36 (d, *J* = 3.0 Hz, 1H), 7.56 (d, *J* = 8.1 Hz, 2H), 7.43 – 7.37 (m, 1H), 5.98 (d, *J* = 3.0 Hz, 1H), 5.72 (s, 2H). ¹³C NMR (101 MHz, DMSO-*d*₆) δ 174.89, 159.38, 134.98, 134.83, 133.17, 131.68, 130.24, 129.98, 129.69, 129.48, 128.94, 102.42. FTIR, cm⁻¹: 3157 (-NH₂), 2924 (-NH), 1568 (aromatic C=C), 1490 (-CH₃), 1237 (aromatic C=N), 1187 (C=S), 748, 711 (aromatic C-Cl). HRMS (+ESI-TOF) *m/z*: [M⁺] calcd for C₁₀H₈Cl₂N₄S 285.98467; found 285.98447.

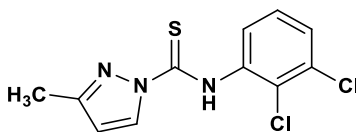


SB96

GDVED(K)₂₀cholesterol(linker)-N-(4-chlorophenyl)-3-ureido-1H-pyrazole-1-

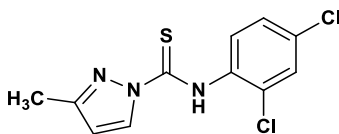
carbothioamide: To a round bottom flask was added KE03 (3 mg, 0.011 mmol, 6.0 eq.), 1,1'-Carbonyldiimidazole (2 mg, 0.011 mmol, 6.0 eq.) and DMF (2 mL). This was left to stir at room temperature under argon for 50 minutes. The peptide K20-cholesterol-linker (20 mg, 0.0018 mmol, 1.0 eq.) was added to the reaction and refluxed at 60° C overnight. The solvent was removed in vacuo and diethyl ether was added to the residue. The resulting material was lyophilized to reveal a yellow solid (18 mg, 79%). ¹H NMR (400 MHz, Deuterium Oxide) δ 7.91 (s, 1H), 7.82 (s, 1H), 7.32 (s, 1H), 7.12 (s, 3H), 4.20 (t, *J* = 7.2 Hz, 5H), 3.90 (s, 1H), 3.16 (s, 1H), 2.90 (q, *J* = 7.4, 5.6 Hz, 13H), 2.75 (s, 2H), 2.59 (s, 1H), 2.15 (s, 1H), 2.02 (s, 1H), 1.68 (s, 12H), 1.60 (s, 13H), 1.34 (s, 13H), 1.20 (s, 1H), 0.90 – 0.83 (m, 3H). ¹³C NMR (101 MHz,

Deuterium Oxide) δ 173.58, 135.28, 120.95, 53.35, 39.07, 36.87, 26.33, 22.10. FTIR, cm⁻¹: 3300 (-NH₂), 2950, 2835 (-NH), 1620 (C=O), 1576 (aromatic C=C), 1199 (aromatic C=N), 1061 (C=S), 756 (aromatic C-Cl).



SB97

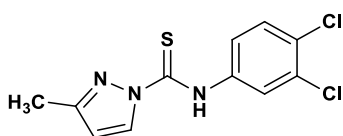
N-(2,3-dichlorophenyl)-3-methyl-1H-pyrazole-1-carbothioamide: A solution of 3-methylpyrazole (75 mg, 0.913 mmol, 1.0 eq.), 2,3-dichlorophenylisothiocyanate (205 mg, 1.0 mmol, 1.1 eq.), and dichloromethane (10 mL) was stirred at 45° C for 24 hours. The solvent was removed in vacuo, purified by flash column chromatography, revealing a white solid (254 mg, 97%). ¹H NMR (600 MHz, DMSO-d₆) δ 11.75 (s, 1H), 8.62 (d, J = 2.8 Hz, 1H), 7.66 (ddd, J = 8.2, 5.3, 1.5 Hz, 1H), 7.50 (dd, J = 8.0, 1.6 Hz, 1H), 7.46 – 7.42 (m, 1H), 6.49 (d, J = 2.8 Hz, 1H), 2.51 (p, J = 1.9 Hz, 3H). ¹³C NMR (151 MHz, DMSO-d₆) δ 175.98, 153.83, 138.52, 133.06, 133.00, 132.47, 130.75, 130.57, 130.07, 130.02, 129.55. FTIR, cm⁻¹: 3196 (-NH₂), 3150 (-NH), 1577 (aromatic C=C), 1452 (-CH₃), 1225 (aromatic C=N), 1052 (C=S), 776, 735 (aromatic C-Cl). HRMS (+ESI-TOF) m/z: [M⁺] calcd for C₁₁H₉Cl₂N₃S 284.98942; found 284.98916.



SB98

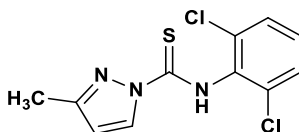
N-(2,4-dichlorophenyl)-3-methyl-1H-pyrazole-1-carbothioamide: A solution of 3-methylpyrazole (98 mg, 1.19 mmol, 1.0 eq.), 2,4-dichlorophenylisothiocyanate (268 mg, 1.31 mmol, 1.1 eq.), and dichloromethane (10 mL) was stirred at 45° C for 24 hours. The solvent was

removed in vacuo, purified by flash column chromatography, revealing a white solid (312 mg, 91%). ^1H NMR (600 MHz, DMSO- d_6) δ 8.61 (d, J = 2.7 Hz, 1H), 7.79 – 7.76 (m, 1H), 7.61 – 7.49 (m, 2H), 6.48 (d, J = 2.8 Hz, 1H), 5.98 (s, 1H), 2.33 (s, 3H). ^{13}C NMR (151 MHz, DMSO- d_6) δ 175.96, 153.81, 135.73, 133.08, 132.83, 131.82, 129.70, 128.37, 111.37, 104.07, 44.37. FTIR, cm^{-1} : 3224 (-NH₂), 2975 (-NH), 1568 (aromatic C=C), 1442 (-CH₃), 1284 (aromatic C=N), 1018 (C=S), 758, 662 (aromatic C-Cl). HRMS (+ESI-TOF) m/z : [M⁺] calcd for C₁₁H₉Cl₂N₃S 284.98942; found 284.98949.



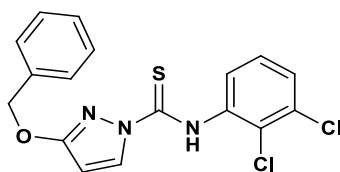
SB99

N-(3,4-dichlorophenyl)-3-methyl-1H-pyrazole-1-carbothioamide: A solution of 3-methylpyrazole (79 mg, 0.962 mmol, 1.0 eq.), 3,4-dichlorophenylisothiocyanate (216 mg, 1.06 mmol, 1.1 eq.), and dichloromethane (10 mL) was stirred at 45° C for 24 hours. The solvent was removed in vacuo, purified by flash column chromatography, revealing a white solid (257 mg, 93%). ^1H NMR (600 MHz, DMSO- d_6) δ 11.82 (s, 1H), 8.64 (d, J = 2.8 Hz, 1H), 7.98 (d, J = 2.3 Hz, 1H), 7.73 – 7.64 (m, 2H), 6.49 (d, J = 2.8 Hz, 1H), 2.51 (p, J = 1.8 Hz, 3H). ^{13}C NMR (151 MHz, DMSO- d_6) δ 174.85, 153.63, 138.72, 136.04, 133.07, 132.52, 132.03, 131.03, 130.96, 130.73, 130.63. FTIR, cm^{-1} : 3270 (-NH₂), 2930 (-NH), 1578 (aromatic C=C), 1419 (-CH₃), 1288 (aromatic C=N), 1026 (C=S), 772, 734 (aromatic C-Cl). HRMS (+ESI-TOF) m/z : [M⁺] calcd for C₁₁H₉Cl₂N₃S 284.98942; found 284.98922.



SB100

N-(2,6-dichlorophenyl)-3-methyl-1H-pyrazole-1-carbothioamide: A solution of 3-methylpyrazole (27 mg, 0.334 mmol, 1.0 eq.), 2,6-dichlorophenylisothiocyanate (75 mg, 0.367 mmol, 1.1 eq.), and dichloromethane (10 mL) was stirred at 45° C for 24 hours. The solvent was removed in vacuo, purified by flash column chromatography, revealing a white solid (86 mg, 90%). ¹H NMR (600 MHz, DMSO-d₆) δ 11.70 (s, 1H), 8.62 – 8.59 (m, 1H), 7.59 (d, J = 8.2 Hz, 1H), 7.46 – 7.37 (m, 2H), 6.50 – 6.47 (m, 1H), 2.20 (s, 3H). ¹³C NMR (151 MHz, DMSO-d₆) δ 175.98, 153.96, 134.56 (d, J = 32.4 Hz), 133.01, 131.68, 130.54, 129.46, 129.10, 111.34, 104.10, 14.13. FTIR, cm⁻¹: 3284 (-NH₂), 2984 (-NH), 1555 (aromatic C=C), 1434 (-CH₃), 1234 (aromatic C=N), 1071 (C=S), 739, 766 (aromatic C-Cl). HRMS (+ESI-TOF) m/z: [M⁺] calcd for C₁₁H₉Cl₂N₃S 284.98942; found 284.98928.

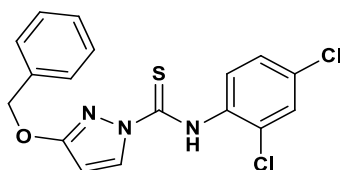


SB101

3-(benzyloxy)-N-(2,3-dichlorophenyl)-1H-pyrazole-1-carbothioamide: To a solution of 3-(benzyloxy)-1H-pyrazole (200 mg, 1.15 mmol, 1.0 eq.) and 2,3-dichlorophenyl isothiocyanate (258 mg, 1.26 mmol, 1.1 eq.) in anhydrous DMF (3 mL), anhydrous potassium carbonate (317 mg, 2.29 mmol, 2.0 eq.) was added. The reaction mixture was kept stirring under argon at room temperature for 24 hours. This was filtered to remove excess K₂CO₃ and the filtrate concentrated to dryness. Descending column chromatography using silica gel as stationary phase and a gradient of DCM and MeOH as eluent gave a light-yellow solid (82 mg, 19%). ¹H NMR (600 MHz, DMSO-d₆) δ 8.63 (d, J = 2.9 Hz, 1H), 7.96 (s, 1H), 7.53 – 7.49 (m, 2H), 7.39 (dt, J = 24.0, 7.6 Hz, 6H), 7.23 (d, J = 7.9 Hz, 1H), 5.30 (s, 2H). ¹³C NMR (151 MHz, DMSO-d₆) δ 164.22, 162.79, 137.19, 136.58, 133.35, 132.48, 131.96, 131.81, 129.37, 129.25, 128.96, 128.87, 128.66,

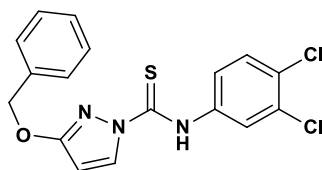
128.52, 128.23, 128.07, 127.53. FTIR, cm⁻¹: 3228 (-NH), 2877 (C-H aromatic), 1522 (aromatic C=C), 1554 (aromatic C=N), 1380 (C=S), 1045 (aliphatic ether), 773, 729 (aromatic C-Cl).

HRMS (+ESI-TOF) m/z: [M⁺] calcd for C₁₇H₁₃Cl₂N₃OS 377.01564; found 377.01560.



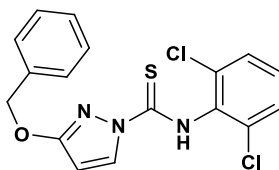
SB102

3-(benzyloxy)-N-(2,4-dichlorophenyl)-1H-pyrazole-1-carbothioamide: To a solution of 3-(benzyloxy)-1H-pyrazole (200 mg, 1.15 mmol, 1.0 eq.) and 2,4-dichlorophenyl isothiocyanate (258 mg, 1.26 mmol, 1.1 eq.) in anhydrous DMF (3 mL), anhydrous potassium carbonate (317 mg, 2.29 mmol, 2.0 eq.) was added. The reaction mixture was kept stirring under argon at room temperature for 24 hours. This was filtered to remove excess K₂CO₃ and the filtrate concentrated to dryness. Descending column chromatography using silica gel as stationary phase and a gradient of DCM and MeOH as eluent gave a light-yellow solid (253 mg, 58%). ¹H NMR (600 MHz, DMSO-d₆) δ 8.64 (d, J = 2.8 Hz, 1H), 7.52 (d, J = 2.4 Hz, 1H), 7.45 – 7.29 (m, 7H), 7.14 (d, J = 8.6 Hz, 1H), 5.92 (d, J = 2.8 Hz, 1H), 5.25 (s, 2H). ¹³C NMR (151 MHz, DMSO-d₆) δ 163.62, 162.80, 137.94, 137.51, 132.80, 128.79 (d, J = 8.5 Hz), 128.49 (t, J = 18.7 Hz), 128.21 (d, J = 6.0 Hz), 127.58 – 126.79 (m), 93.72, 89.79, 70.39 (d, J = 6.2 Hz), 51.13, 46.29, 44.85, 36.26, 31.24. FTIR, cm⁻¹: 3153 (-NH), 2936 (C-H aromatic), 1534 (aromatic C=C), 1343 (C=S), 1046 (aliphatic ether), 748, 731 (aromatic C-Cl). HRMS (+ESI-TOF) m/z: [M⁺] calcd for C₁₇H₁₃Cl₂N₃OS 377.01564; found 377.01588.



SB103

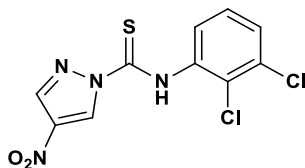
3-(benzyloxy)-N-(3,4-dichlorophenyl)-1H-pyrazole-1-carbothioamide: To a solution of 3-(benzyloxy)-1H-pyrazole (200 mg, 1.15 mmol, 1.0 eq.) and 3,4-dichlorophenyl isothiocyanate (258 mg, 1.26 mmol, 1.1 eq.) in anhydrous DMF (3 mL), anhydrous potassium carbonate (317 mg, 2.29 mmol, 2.0 eq.) was added. The reaction mixture was kept stirring under argon at room temperature for 24 hours. This was filtered to remove excess K_2CO_3 and the filtrate concentrated to dryness. Descending column chromatography using silica gel as stationary phase and a gradient of DCM and MeOH as eluent gave a light-yellow solid (95 mg, 22%). ^1H NMR (600 MHz, DMSO- d_6) δ 8.72 (d, J = 2.8 Hz, 1H), 7.95 (d, J = 4.2 Hz, 1H), 7.65 – 7.21 (m, 8H), 7.06 (dd, J = 8.6, 2.4 Hz, 1H), 5.23 (s, 2H). ^{13}C NMR (151 MHz, DMSO- d_6) δ 170.11, 163.69, 162.79, 151.60, 137.47, 133.04, 130.33 (d, J = 5.1 Hz), 129.99, 128.81, 128.34, 125.39, 124.55, 123.38, 93.63, 89.76, 70.40, 36.25. FTIR, cm^{-1} : 3138 (-NH), 2929 (C-H aromatic), 1583 (aromatic C=C), 1521 (aromatic C=N), 1349 (C=S), 1042 (aliphatic ether), 773, 693 (aromatic C-Cl). HRMS (+ESI-TOF) m/z : $[M]^+$ calcd for $C_{17}H_{13}Cl_2N_3OS$ 377.01564; found 377.01571.



SB104

3-(benzyloxy)-N-(2,6-dichlorophenyl)-1H-pyrazole-1-carbothioamide: To a solution of 3-(benzyloxy)-1H-pyrazole (75 mg, 0.431 mmol, 1.0 eq.) and 2,6-dichlorophenyl isothiocyanate (97 mg, 0.474 mmol, 1.1 eq.) in anhydrous DMF (3 mL), anhydrous potassium carbonate (119

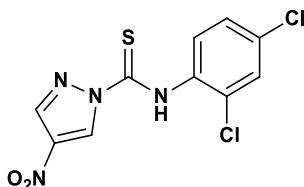
mg, 0.862 mmol, 2.0 eq.) was added. The reaction mixture was kept stirring under argon at room temperature for 24 hours. This was filtered to remove excess K_2CO_3 and the filtrate concentrated to dryness. Descending column chromatography using silica gel as stationary phase and a gradient of DCM and MeOH as eluent gave a light-yellow solid (70 mg, 43%). ^1H NMR (600 MHz, DMSO- d_6) δ 8.66 (d, J = 2.4 Hz, 1H), 7.96 (s, 1H), 7.40 (dddd, J = 55.5, 42.3, 25.1, 7.7 Hz, 9H), 5.23 (s, 2H). ^{13}C NMR (151 MHz, DMSO- d_6) δ 164.66, 163.36, 162.80, 137.66, 136.49, 132.57, 132.11, 128.78 (dd, J = 17.2, 7.9 Hz), 128.63 – 128.29 (m), 128.17 (d, J = 15.0 Hz), 125.57 (d, J = 39.7 Hz), 117.35, 99.12, 70.99, 70.27, 36.27, 31.24. FTIR, cm^{-1} : 3150 (-NH), 2950 (C-H aromatic), 1556 (aromatic C=C), 1477 (aromatic C=N), 1385 (C=S), 1093 (aliphatic ether), 782, 734 (aromatic C-Cl). HRMS (+ESI-TOF) m/z : $[M+]$ calcd for $C_{17}H_{13}Cl_2N_3OS$ 377.01564; found 377.01553.



SB105

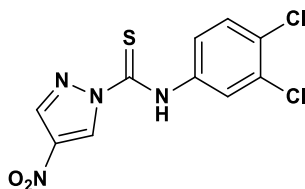
N-(2,3-dichlorophenyl)-4-nitro-1H-pyrazole-1-carbothioamide: Two 100 mL round bottom flasks were flame dried to remove moisture. To flask A was added 4-nitro pyrazole (50 mg, 0.442 mmol, 1.0 eq.) and dry THF (10 mL). To flask B was added sodium hydride (21 mg, 0.885 mmol, 1.5 eq.) and dry THF (10 mL). Both flasks were stirred at 0° C for 20 minutes. The mixture from flask B was cannulated into flask A using a two-headed needle. This solution was kept stirring at r.t. under argon for 3 hours. 2,3-dichlorophenylisothiocyanate (99 mg, 0.487 mmol, 1.1 eq.) was added to the reaction, which continued stirring for 12 hours at r.t. The reaction was concentrated to dryness to reveal a white solid (34 mg, 24%). ^1H NMR (600 MHz, DMSO- d_6) δ 9.45 (s, 2H), 8.54 (s, 1H), 7.29 – 7.21 (m, 1H), 7.16 (q, J = 7.8 Hz, 2H). ^{13}C NMR

(151 MHz, DMSO-d₆) δ 147.21, 133.25 (d, J = 33.2 Hz), 131.99, 128.49, 127.65, 127.22, 124.80, 117.18, 115.11, 114.07. FTIR, cm⁻¹: 3216 (-NH), 3150, 3081 (C-H aromatic), 2046 (-NCS), 1446 (aromatic C=C), 1408 (-CH₃), 1272 (aromatic C=N), 1151 (C=S), 775, 722 (aromatic C-Cl). UPLC-MS [M+1] calcd for C₁₀H₆Cl₂N₄O₂S 318.15; found 317.19.



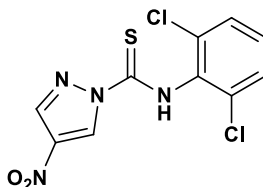
SB106

N-(2,4-dichlorophenyl)-4-nitro-1H-pyrazole-1-carbothioamide: Two 100 mL round bottom flasks were flame dried to remove moisture. To flask A was added 4-nitro pyrazole (50 mg, 0.442 mmol, 1.0 eq.) and dry THF (10 mL). To flask B was added sodium hydride (21 mg, 0.885 mmol, 1.5 eq.) and dry THF (10 mL). Both flasks were stirred at 0° C for 20 minutes. The mixture from flask B was cannulated into flask A using a two-headed needle. This solution was kept stirring at r.t. under argon for 3 hours. 2,4-dichlorophenylisothiocyanate (99 mg, 0.487 mmol, 1.1 eq.) was added to the reaction, which continued stirring for 12 hours at r.t. The reaction was concentrated to dryness to reveal a white solid (34 mg, 26%). ¹H NMR (400 MHz, DMSO-d₆) δ 9.74 (s, 2H), 7.70 (d, J = 2.4 Hz, 1H), 7.64 (d, J = 8.6 Hz, 1H), 7.44 (dd, J = 8.6, 2.4 Hz, 2H). ¹³C NMR (101 MHz, DMSO-d₆) δ 181.92, 135.85, 131.76, 131.69, 131.57, 129.46, 128.57, 128.10, 127.93, 117.80. FTIR, cm⁻¹: 3130 (-NH), 2956, 2813 (C-H aromatic), 2052 (-NCS), 1466 (aromatic C=C), 1404 (-CH₃), 1289 (aromatic C=N), 1141 (C=S), 800, 752 (aromatic C-Cl). UPLC-MS [M+1] calcd for C₁₀H₆Cl₂N₄O₂S 318.15; found 317.07.



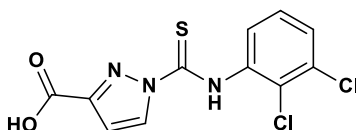
SB107

N-(3,4-dichlorophenyl)-4-nitro-1H-pyrazole-1-carbothioamide: Two 100 mL round bottom flasks were flame dried to remove moisture. To flask A was added 4-nitro pyrazole (50 mg, 0.442 mmol, 1.0 eq.) and dry THF (10 mL). To flask B was added sodium hydride (21 mg, 0.885 mmol, 1.5 eq.) and dry THF (10 mL). Both flasks were stirred at 0° C for 20 minutes. The mixture from flask B was cannulated into flask A using a two-headed needle. This solution was kept stirring at r.t. under argon for 3 hours. 3,4-dichlorophenylisothiocyanate (99 mg, 0.487 mmol, 1.1 eq.) was added to the reaction, which continued stirring for 12 hours at r.t. The reaction was concentrated to dryness to reveal a white solid (45 mg, 32%). ¹H NMR (600 MHz, DMSO-d₆) δ 8.71 – 8.63 (m, 1H), 7.88 (dd, J = 5.3, 2.5 Hz, 1H), 7.41 – 7.37 (m, 4H). ¹³C NMR (151 MHz, DMSO-d₆) δ 142.80, 135.74, 133.11, 130.54, 130.08 (d, J = 21.8 Hz), 129.66, 123.76, 122.49, 121.53, 67.48. FTIR, cm⁻¹: 3420 (-NH), 3150, 2876 (C-H aromatic), 2079 (-NCS), 1465 (aromatic C=C), 1414 (-CH₃), 1274 (aromatic C=N), 1149 (C=S), 782, 750 (aromatic C-Cl). UPLC-MS [M+1] calcd for C₁₀H₆Cl₂N₄O₂S 318.15; found 318.84.



SB108

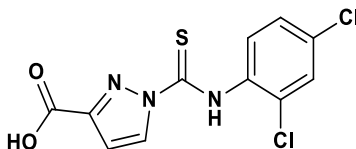
N-(2,6-dichlorophenyl)-4-nitro-1H-pyrazole-1-carbothioamide: Two 100 mL round bottom flasks were flame dried to remove moisture. To flask A was added 4-nitro pyrazole (50 mg, 0.442 mmol, 1.0 eq.) and dry THF (10 mL). To flask B was added sodium hydride (21 mg, 0.885 mmol, 1.5 eq.) and dry THF (10 mL). Both flasks were stirred at 0° C for 20 minutes. The mixture from flask B was cannulated into flask A using a two-headed needle. This solution was kept stirring at r.t. under argon for 3 hours. 2,6-dichlorophenylisothiocyanate (99 mg, 0.487 mmol, 1.1 eq.) was added to the reaction, which continued stirring for 12 hours at r.t. The reaction was concentrated to dryness to reveal a white solid (28 mg, 20%). ¹H NMR (400 MHz, DMSO-d₆) δ 7.99 (s, 2H), 7.63 (d, J = 8.2 Hz, 1H), 7.25 (d, J = 8.0 Hz, 1H), 6.61 (t, J = 8.0 Hz, 1H), 3.34 (s, 1H). ¹³C NMR (101 MHz, DMSO-d₆) δ 162.76, 135.84, 131.66, 129.51, 129.34, 128.41, 118.61, 117.31, 36.20, 31.17. FTIR, cm⁻¹: 3138 (-NH), 3120, 2929 (C-H aromatic), 1933 (-NCS), 1433 (aromatic C=C), 1408 (-CH₃), 1237 (aromatic C=N), 1149 (C=S), 768, 751 (aromatic C-Cl). UPLC-MS [M+1] calcd for C₁₀H₆Cl₂N₄O₂S 318.15; found 319.05.



SB109

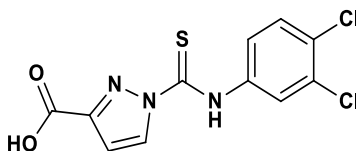
1-((2,3-dichlorophenyl)carbamothioyl)-1H-pyrazole-3-carboxylic acid: To a solution of 1H-pyrazole-3-carboxylic acid (215 mg, 1.92 mmol, 1.0 eq.) and 2,3-dichlorophenyl isothiocyanate (430 mg, 2.11 mmol, 1.1 eq.) in anhydrous DMF (3 mL), anhydrous potassium carbonate (529 mg, 3.83 mmol, 2.0 eq.) was added. The reaction mixture was kept stirring under argon at room temperature for 24 hours. This was filtered to remove excess K₂CO₃ and the filtrate concentrated to dryness. Descending column chromatography using silica gel as stationary phase and a gradient of DCM and MeOH as eluent gave a light-yellow solid (54 mg, 9%). ¹H NMR (400

MHz, DMSO-d₆) δ 8.48 (s, 2H), 7.56 (s, 1H), 7.20 (d, J = 25.3 Hz, 2H), 6.92 (s, 1H). ¹³C NMR (101 MHz, DMSO-d₆) δ 145.97, 138.77, 132.05, 128.01, 125.35, 124.36, 123.12, 121.48. FTIR, cm⁻¹: 3320 (-NH), 3150 (carboxylic acid), 1607 (aromatic C=C), 1534 (aromatic C=N), 1380 (C=S), 780, 708 (aromatic C-Cl). HRMS (+ESI-TOF) m/z: [M⁺] calcd for C₁₁H₇Cl₂N₃O₂S 314.96360; found 314.96350.



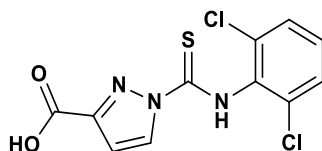
SB110

1-((2,4-dichlorophenyl)carbamothioyl)-1H-pyrazole-3-carboxylic acid: To a solution of 1H-pyrazole-3-carboxylic acid (250 mg, 2.32 mmol, 1.0 eq.) and 2,4-dichlorophenyl isothiocyanate (500 mg, 2.45 mmol, 1.1 eq.) in anhydrous DMF (3 mL), anhydrous potassium carbonate (616 mg, 4.46 mmol, 2.0 eq.) was added. The reaction mixture was kept stirring under argon at room temperature for 24 hours. This was filtered to remove excess K₂CO₃ and the filtrate concentrated to dryness. Descending column chromatography using silica gel as stationary phase and a gradient of DCM and MeOH as eluent gave a white solid (253 mg, 36%). ¹H NMR (600 MHz, DMSO-d₆) δ 9.30 (s, 1H), 8.08 (d, J = 8.8 Hz, 1H), 8.06 (s, 1H), 7.61 (s, 2H), 7.06 (dd, J = 8.6, 2.4 Hz, 1H), 6.79 (d, J = 8.6 Hz, 1H). ¹³C NMR (151 MHz, DMSO-d₆) δ 162.85, 144.41, 129.11 (d, J = 18.0 Hz), 128.56, 128.06 (d, J = 8.9 Hz), 116.72, 68.00, 51.13, 44.87, 36.28. FTIR, cm⁻¹: 3280 (-NH), 1617 (aromatic C=C), 1565 (aromatic C=N), 1127 (C=S), 772, 737 (aromatic C-Cl). HRMS (+ESI-TOF) m/z: [M⁺] calcd for C₁₁H₇Cl₂N₃O₂S 314.96360; found 314.96307.



SB111

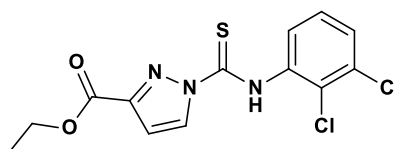
1-((3,4-dichlorophenyl)carbamothioyl)-1H-pyrazole-3-carboxylic acid: To a solution of 1H-pyrazole-3-carboxylic acid (200 mg, 1.79 mmol, 1.0 eq.) and 3,4-dichlorophenyl isothiocyanate (400 mg, 1.96 mmol, 1.1 eq.) in anhydrous DMF (3 mL), anhydrous potassium carbonate (493 mg, 3.57 mmol, 2.0 eq.) was added. The reaction mixture was kept stirring under argon at room temperature for 24 hours. This was filtered to remove excess K₂CO₃ and the filtrate concentrated to dryness. Descending column chromatography using silica gel as stationary phase and a gradient of DCM and MeOH as eluent gave a white solid (88 mg, 16%). ¹H NMR (400 MHz, DMSO-d₆) δ 10.17 (s, 1H), 9.10 (s, 1H), 7.87 (dd, J = 5.5, 2.5 Hz, 1H), 7.56 (dd, J = 29.1, 8.7 Hz, 2H), 7.44 (dd, J = 8.7, 2.5 Hz, 1H), 7.35 (dd, J = 8.8, 2.6 Hz, 1H). ¹³C NMR (101 MHz, DMSO-d₆) δ 180.24, 152.60, 140.03, 139.82, 131.52, 131.05, 130.77, 125.61, 124.32, 120.08, 119.09. FTIR, cm⁻¹: 3200 (-NH), 3050 (carboxylic acid), 1654 (aromatic C=C), 1521 (aromatic C=N), 1384 (C=S), 784, 726 (aromatic C-Cl). HRMS (+ESI-TOF) m/z: [M⁺] calcd for C₁₁H₇Cl₂N₃O₂S 314.96360; found 314.96314.



SB112

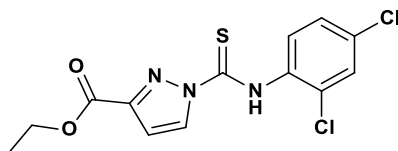
1-((2,6-dichlorophenyl)carbamothioyl)-1H-pyrazole-3-carboxylic acid: To a solution of 1H-pyrazole-3-carboxylic acid (75 mg, 0.669 mmol, 1.0 eq.) and 2,6-dichlorophenyl isothiocyanate (150 mg, 0.736 mmol, 1.1 eq.) in anhydrous DMF (3 mL), anhydrous potassium carbonate (185 mg, 1.34 mmol, 2.0 eq.) was added. The reaction mixture was kept stirring under argon at room temperature for 24 hours. This was filtered to remove excess K₂CO₃ and the filtrate concentrated to dryness. Descending column chromatography using silica gel as stationary phase and a

gradient of DCM and MeOH as eluent gave a light-yellow solid (124 mg, 58%). ^1H NMR (400 MHz, DMSO- d_6) δ 10.08 (s, 1H), 7.71 (dt, J = 10.4, 2.1 Hz, 1H), 7.45 – 7.37 (m, 2H), 7.35 – 7.30 (m, 1H), 7.20 (dt, J = 7.5, 1.8 Hz, 1H). ^{13}C NMR (101 MHz, DMSO- d_6) δ 141.28, 136.96, 132.97, 130.52, 129.73, 125.32, 124.69, 124.23, 124.17, 123.57, 122.50. FTIR, cm^{-1} : 3150 (-NH), 2925 (carboxylic acid), 1654 (aromatic C=C), 1526 (aromatic C=N), 1383 (C=S), 813, 769 (aromatic C-Cl). HRMS (+ESI-TOF) m/z : $[\text{M}^+]$ calcd for $\text{C}_{11}\text{H}_7\text{Cl}_2\text{N}_3\text{O}_2\text{S}$ 314.96360; found 314.96371.



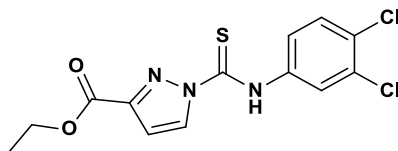
SB113

Ethyl 1-((2,3-dichlorophenyl)carbamothioyl)-1H-pyrazole-3-carboxylate: To a solution of ethyl 1H-pyrazole-3-carboxylate (255 mg, 1.82 mmol, 1.0 eq.) and 2,3-dichlorophenyl isothiocyanate (408 mg, 2.0 mmol, 1.1 eq.) in anhydrous DMF (3 mL), anhydrous potassium carbonate (502 mg, 3.64 mmol, 2.0 eq.) was added. The reaction mixture was kept stirring under argon at room temperature for 24 hours. This was filtered to remove excess K_2CO_3 and the filtrate concentrated to dryness. Descending column chromatography using silica gel as stationary phase and a gradient of DCM and MeOH as eluent gave a light-yellow solid (61 mg, 10%). ^1H NMR (400 MHz, DMSO- d_6) δ 12.11 (s, 1H), 8.84 (d, J = 2.8 Hz, 1H), 7.67 – 7.50 (m, 3H), 7.03 (d, J = 2.8 Hz, 1H), 4.39 (q, J = 7.1 Hz, 2H), 1.35 (t, J = 7.1 Hz, 3H). ^{13}C NMR (101 MHz, DMSO- d_6) δ 175.87, 161.40, 146.94, 134.09, 131.68, 129.69, 129.48, 129.23, 129.02, 111.71, 107.87, 61.74, 14.67. FTIR, cm^{-1} : 2949 (C-H aromatic), 1649 (aromatic C=C), 1412 (-CH₃), 774, 703 (aromatic C-Cl). HRMS (+ESI-TOF) m/z : $[\text{M}^+]$ calcd for $\text{C}_{13}\text{H}_{11}\text{Cl}_2\text{N}_3\text{O}_2\text{S}$ 342.99490; found 342.99465.



SB114

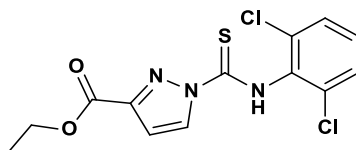
Ethyl 1-((2,4-dichlorophenyl)carbamothioyl)-1H-pyrazole-3-carboxylate: To a solution of ethyl 1H-pyrazole-3-carboxylate (300 mg, 2.14 mmol, 1.0 eq.) and 2,4-dichlorophenyl isothiocyanate (481 mg, 2.36 mmol, 1.1 eq.) in anhydrous DMF (3 mL), anhydrous potassium carbonate (591 mg, 4.28 mmol, 2.0 eq.) was added. The reaction mixture was kept stirring under argon at room temperature for 24 hours. This was filtered to remove excess K_2CO_3 and the filtrate concentrated to dryness. Descending column chromatography using silica gel as stationary phase and a gradient of DCM and MeOH as eluent gave a light-yellow solid (137 mg, 19%). ^1H NMR (400 MHz, DMSO- d_6) δ 9.75 (s, 1H), 7.72 – 7.56 (m, 1H), 7.44 (dd, J = 8.6, 2.4 Hz, 3H), 6.88 – 6.74 (m, 1H), 4.27 (q, J = 7.1 Hz, 2H), 1.29 (t, J = 7.0 Hz, 3H). ^{13}C NMR (101 MHz, DMSO- d_6) δ 181.91, 144.39, 135.85, 131.69, 131.54, 129.45, 128.57, 128.06, 127.92, 119.36, 117.82, 116.71, 14.70. FTIR, cm^{-1} : 3164 (-NH), 3123, 2924 (C-H aromatic), 1696 (C=O), 1647 (aromatic C=C), 1548 (aromatic C=N), 1444 (-CH₃), 1336 (C=S), 783, 727 (aromatic C-Cl). HRMS (+ESI-TOF) m/z : $[M]^+$ calcd for $C_{13}H_{11}Cl_2N_3O_2S$ 342.99490; found 342.99472.



SB115

Ethyl 1-((3,4-dichlorophenyl)carbamothioyl)-1H-pyrazole-3-carboxylate: To a solution of ethyl 1H-pyrazole-3-carboxylate (225 mg, 1.60 mmol, 1.0 eq.) and 3,4-dichlorophenyl

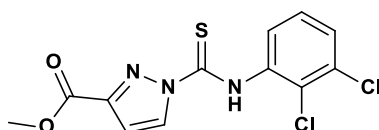
isothiocyanate (360 mg, 1.76 mmol, 1.1 eq.) in anhydrous DMF (3 mL), anhydrous potassium carbonate (442 mg, 3.20 mmol, 2.0 eq.) was added. The reaction mixture was kept stirring under argon at room temperature for 24 hours. This was filtered to remove excess K_2CO_3 and the filtrate concentrated to dryness. Descending column chromatography using silica gel as stationary phase and a gradient of DCM and MeOH as eluent gave a light-yellow solid (65 mg, 12%). ^1H NMR (400 MHz, DMSO- d_6) δ 13.67 – 13.28 (m, 1H), 10.17 (s, 1H), 7.85 (d, J = 2.4 Hz, 1H), 7.60 (d, J = 8.6 Hz, 1H), 7.44 (dd, J = 8.8, 2.5 Hz, 1H), 6.76 (s, 1H), 4.27 (q, J = 7.1 Hz, 2H), 1.29 (t, J = 7.1 Hz, 3H). ^{13}C NMR (101 MHz, DMSO- d_6) δ 180.26, 139.82, 131.05, 130.78, 130.49, 126.93, 125.63, 124.34, 107.96, 60.53, 14.69. FTIR, cm^{-1} : 3414 (-NH), 3244, 3123 (C-H aromatic), 1744 (C=O), 1647 (aromatic C=C), 1510 (aromatic C=N), 1431 (-CH₃), 1303 (C=S), 784, 727 (aromatic C-Cl). HRMS (+ESI-TOF) m/z : $[M]^+$ calcd for $C_{13}H_{11}Cl_2N_3O_2S$ 342.99490; found 342.99490.



SB116

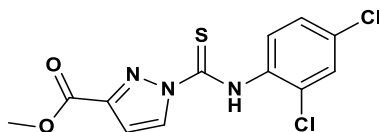
Ethyl 1-((2,6-dichlorophenyl)carbamothioyl)-1H-pyrazole-3-carboxylate: To a solution of ethyl 1H-pyrazole-3-carboxylate (75 mg, 0.536 mmol, 1.0 eq.) and 2,6-dichlorophenyl isothiocyanate (120 mg, 0.589 mmol, 1.1 eq.) in anhydrous DMF (3 mL), anhydrous potassium carbonate (148 mg, 1.07 mmol, 2.0 eq.) was added. The reaction mixture was kept stirring under argon at room temperature for 24 hours. This was filtered to remove excess K_2CO_3 and the filtrate concentrated to dryness. Descending column chromatography using silica gel as stationary phase and a gradient of DCM and MeOH as eluent gave a light-yellow solid (37 mg, 20%). ^1H NMR (400 MHz, DMSO- d_6) δ 13.48 (s, 1H), 7.86 (s, 1H), 7.66 (dd, J = 8.2, 1.5 Hz,

1H), 7.61 – 7.53 (m, 1H), 7.48 – 7.31 (m, 1H), 6.76 – 6.72 (m, 1H), 4.27 (q, J = 7.2 Hz, 2H), 1.29 (t, J = 7.1 Hz, 3H). ¹³C NMR (101 MHz, DMSO-d₆) δ 143.38, 138.38, 132.99, 130.76, 130.48, 130.03, 129.55, 128.28, 126.55, 121.34, 107.86, 60.44, 14.70. FTIR, cm⁻¹: 2984 (-NH), 2931, 2850 (C-H aromatic), 1712 (C=O), 1654 (aromatic C=C), 1501 (aromatic C=N), 1463 (-CH₃), 1384 (C=S), 767, 709 (aromatic C-Cl). HRMS (+ESI-TOF) m/z: [M⁺] calcd for C₁₃H₁₁Cl₂N₃O₂S 342.99490; found 342.99468.



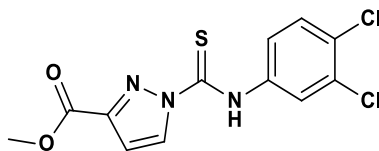
SB117

Methyl 1-((2,3-dichlorophenyl)carbamothioyl)-1H-pyrazole-3-carboxylate: To a solution of methyl 1H-pyrazole-3-carboxylate (250 mg, 1.98 mmol, 1.0 eq.) and 2,3-dichlorophenyl isothiocyanate (445 mg, 2.18 mmol, 1.1 eq.) in anhydrous DMF (3 mL), anhydrous potassium carbonate (546 mg, 3.96 mmol, 2.0 eq.) was added. The reaction mixture was kept stirring under argon at room temperature for 24 hours. This was filtered to remove excess K₂CO₃ and the filtrate concentrated to dryness. Descending column chromatography using silica gel as stationary phase and a gradient of DCM and MeOH as eluent gave a light-yellow solid (89 mg, 14%). ¹H NMR (400 MHz, DMSO-d₆) δ 9.25 (s, 1H), 8.11 – 8.02 (m, 1H), 7.40 – 7.29 (m, 3H), 3.35 (s, 3H). ¹³C NMR (101 MHz, DMSO-d₆) δ 152.47, 138.68, 138.08, 132.31, 132.16, 129.73, 129.21, 128.83, 128.56, 128.29, 124.77, 121.76, 121.36. FTIR, cm⁻¹: 2929 (C-H aromatic), 1715 (C=O), 1527 (aromatic C=C), 1502 (aromatic C=N), 1432 (-CH₃), 1300 (C=S), 767, 702 (aromatic C-Cl). HRMS (+ESI-TOF) m/z: [M⁺] calcd for C₁₂H₉Cl₂N₃O₂S 328.97925; found 328.97897.



SB118

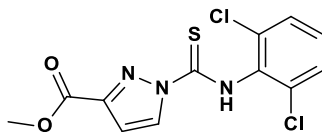
Methyl 1-((2,4-dichlorophenyl)carbamothioyl)-1H-pyrazole-3-carboxylate: To a solution of methyl 1H-pyrazole-3-carboxylate (250 mg, 1.98 mmol, 1.0 eq.) and 2,3-dichlorophenyl isothiocyanate (445 mg, 2.18 mmol, 1.1 eq.) in anhydrous DMF (3 mL), anhydrous potassium carbonate (546 mg, 3.96 mmol, 2.0 eq.) was added. The reaction mixture was kept stirring under argon at room temperature for 24 hours. This was filtered to remove excess K_2CO_3 and the filtrate concentrated to dryness. Descending column chromatography using silica gel as stationary phase and a gradient of DCM and MeOH as eluent gave a light-yellow solid (42 mg, 6%). ^1H NMR (400 MHz, DMSO- d_6) δ 13.50 (s, 1H), 9.75 (s, 1H), 7.86 (d, J = 2.3 Hz, 1H), 7.62 (dd, J = 23.6, 8.6 Hz, 1H), 7.51 (dd, J = 8.7, 2.3 Hz, 1H), 7.44 (dd, J = 8.6, 2.4 Hz, 1H), 3.80 (s, 3H). ^{13}C NMR (101 MHz, DMSO- d_6) δ 135.85, 133.10, 132.02, 131.70 (d, J = 5.6 Hz), 131.55, 130.23, 129.45, 129.21, 128.85, 128.10, 127.92. FTIR, cm^{-1} : 3430 (-NH), 3231, 2953 (C-H aromatic), 1720 (C=O), 1578 (aromatic C=C), 1520 (aromatic C=N), 1463 (-CH₃), 1303 (C=S), 764, 714 (aromatic C-Cl). HRMS (+ESI-TOF) m/z : $[M+]$ calcd for $C_{12}H_9Cl_2N_3O_2S$ 328.97925; found 328.97920.



SB119

Methyl 1-((3,4-dichlorophenyl)carbamothioyl)-1H-pyrazole-3-carboxylate: To a solution of methyl 1H-pyrazole-3-carboxylate (250 mg, 1.98 mmol, 1.0 eq.) and 2,3-dichlorophenyl isothiocyanate (445 mg, 2.18 mmol, 1.1 eq.) in anhydrous DMF (3 mL), anhydrous potassium

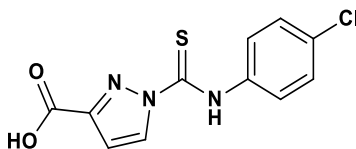
carbonate (546 mg, 3.96 mmol, 2.0 eq.) was added. The reaction mixture was kept stirring under argon at room temperature for 24 hours. This was filtered to remove excess K_2CO_3 and the filtrate concentrated to dryness. Descending column chromatography using silica gel as stationary phase and a gradient of DCM and MeOH as eluent gave a light-yellow solid (106 mg, 16%). ^1H NMR (400 MHz, DMSO- d_6) δ 10.17 (s, 1H), 7.86 (dd, J = 6.7, 2.5 Hz, 1H), 7.60 (d, J = 8.7 Hz, 3H), 7.44 (dd, J = 8.7, 2.5 Hz, 1H), 3.35 (s, 3H). ^{13}C NMR (101 MHz, DMSO- d_6) δ 180.25, 152.61, 140.04, 139.83, 131.52, 131.05, 130.78, 126.92, 125.62, 124.01, 120.09, 119.11. FTIR, cm^{-1} : 3549 (-NH), 3028, 2946 (C-H aromatic), 1714 (C=O), 1580 (aromatic C=C), 1524 (aromatic C=N), 1401 (-CH₃), 1339 (C=S), 804, 771 (aromatic C-Cl). HRMS (+ESI-TOF) m/z : [M⁺] calcd for $C_{12}H_9Cl_2N_3O_2S$ 328.97925; found 328.97941.



SB120

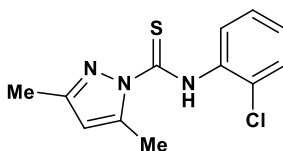
Methyl 1-((2,6-dichlorophenyl)carbamothioyl)-1H-pyrazole-3-carboxylate: To a solution of methyl 1H-pyrazole-3-carboxylate (250 mg, 1.98 mmol, 1.0 eq.) and 2,3-dichlorophenyl isothiocyanate (445 mg, 2.18 mmol, 1.1 eq.) in anhydrous DMF (3 mL), anhydrous potassium carbonate (546 mg, 3.96 mmol, 2.0 eq.) was added. The reaction mixture was kept stirring under argon at room temperature for 24 hours. This was filtered to remove excess K_2CO_3 and the filtrate concentrated to dryness. Descending column chromatography using silica gel as stationary phase and a gradient of DCM and MeOH as eluent gave a light-yellow solid (36 mg, 5%). ^1H NMR (400 MHz, DMSO- d_6) δ 13.51 (s, 1H), 7.89 – 7.85 (m, 1H), 7.63 (d, J = 8.2 Hz, 2H), 7.45 – 7.27 (m, 1H), 6.75 (t, J = 2.2 Hz, 1H), 3.79 (s, 3H). ^{13}C NMR (101 MHz, DMSO- d_6) δ 163.04, 143.12, 140.59, 131.68, 130.54, 129.57 (d, J = 21.1 Hz), 127.65, 107.91, 51.86. FTIR,

cm⁻¹: 3278 (-NH), 3125, 2921 (C-H aromatic), 1728 (C=O), 1490 (aromatic C=C), 1438 (-CH₃), 1306 (C=S), 805, 762 (aromatic C-Cl). HRMS (+ESI-TOF) m/z: [M⁺] calcd for C₁₂H₉Cl₂N₃O₂S 328.97925; found 328.97955.



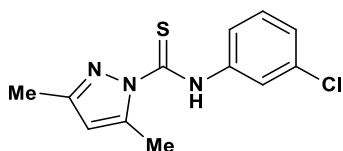
SB121

1-((4-chlorophenyl)carbamothioyl)-1H-pyrazole-3-carboxylic acid: To a solution of 1H-pyrazole-3-carboxylic acid (850 mg, 7.59 mmol, 1.0 eq.) and 4-chlorophenyl isothiocyanate (1.4 g, 8.35 mmol, 1.1 eq.) in anhydrous DMF (2 mL), anhydrous potassium carbonate (2.1 g, 15.2 mmol, 2.0 eq.) was added. The reaction mixture was kept stirring under argon at room temperature for 24 hours. This was filtered to remove excess K₂CO₃ and the filtrate concentrated to dryness. Descending column chromatography using silica gel as stationary phase and a gradient of DCM and MeOH as eluent gave a light-yellow solid (1014 mg, 34 %). ¹H NMR (400 MHz, DMSO-d₆) δ 13.45 (s, 1H), 11.98 (s, 1H), 8.81 (d, J = 2.8 Hz, 1H), 7.93 (m, 1H), 7.73 (m, 1H), 7.54 (m, 1H), 7.44 (m, 1H), 7.06 (d, J = 2.8 Hz, 1H). ¹³C NMR (400 MHz, DMSO-d₆) δ 159.36, 148.61, 138.78, 130.38, 129.36 (d, J = 6.1 Hz), 128.86 (d, J = 6.8 Hz), 128.27 (d, J = 3.7 Hz), 127.63, 125.82, 122.18, 109.98. FTIR, cm⁻¹: 3311 (-NH) 2918 (C-H aromatic), 1663 (C=O), 1524 (aromatic C=N), 1414 (-CH₃), 1302 (C=S), 729 (aromatic C-Cl). HRMS (+ESI-TOF) m/z: [M⁺] calcd for C₁₁H₈ClN₃O₂S 281.00257; found 281.00260.



SB122

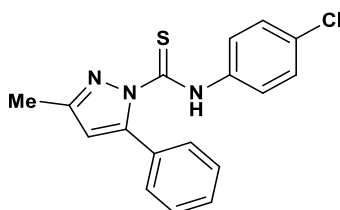
N-(2-chlorophenyl)-3,5-dimethyl-1H-pyrazole-1-carbothioamide: 3,5-dimethyl-1H-pyrazole (100 mg, 1.04 mmol, 1.0 eq.) was dissolved in dry THF and transferred to a round bottom which consisted of K₂CO₃ (287 mg, 2.08 mmol, 2.0 eq.). The reaction mixture was stirred vigorously for 1 h at 50° C. Then 2-chlorophenyl isothiocyanate (193 mg, 1.14 mmol, 1.1 eq.) was added and refluxed at 70° C for 18 h. Potassium carbonate solid was filtered off by gravity filtration and filtrate's THF was evaporated in vacuo. Resulting crude product was purified by flash column chromatography (100% hexane to 5% ethyl acetate/hexane) to afford an off-white color solid (135 mg, 49%). ¹H NMR (400 MHz, DMSO-d₆) δ 12.55 (s, 1H), 8.02 (dd, J = 7.0, 1.9 Hz, 1H), 7.83 – 7.71 (m, 1H), 7.54 – 7.23 (m, 1H), 7.05 – 6.97 (m, 1H), 6.59 – 6.51 (m, 1H), 2.61 (s, 3H), 2.25 (s, 3H). ¹³C NMR (400 MHz, DMSO-d₆) δ 151.72, 149.20, 148.15, 144.46, 137.06, 132.05, 129.13 (d, J = 9.8 Hz), 126.62, 125.40, 122.90, 115.64, 107.91. FTIR, cm⁻¹: 3319 (-NH) 2923 (C-H aromatic), 1526 (aromatic C=N), 1439 (-CH₃), 1345 (C=S), 751 (aromatic C-Cl). HRMS (+ESI-TOF) m/z: [M⁺] calcd for C₁₂H₁₂ClN₃S 265.04405; found 265.04402.



SB123

N-(3-chlorophenyl)-3,5-dimethyl-1H-pyrazole-1-carbothioamide: 3,5-dimethyl-1H-pyrazole (300 mg, 3.12 mmol, .0 eq.) was dissolved in dry THF and transferred to a round bottom which consisted of K₂CO₃ (861 mg, 6.24 mmol, 2.0 eq.). The reaction mixture was stirred vigorously for 1 h at 50° C. Then 3-chlorophenyl isothiocyanate (580 mg, 3.43 mmol, 1.1 eq.) was added and refluxed at 70° C for 18 h. Potassium carbonate solid was filtered off by gravity filtration and filtrate's THF was evaporated in vacuo. Resulting crude product was purified by flash column chromatography (100% hexane to 5% ethyl acetate/hexane) to afford an off-white color

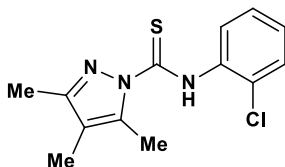
solid (282 mg, 33%). ^1H NMR (400 MHz, DMSO- d_6) δ 12.00 (s, 1H), 8.04 (ddd, J = 22.8, 8.2, 1.6 Hz, 1H), 7.60 – 7.51 (m, 1H), 7.44 – 7.13 (m, 1H), 6.78 (dd, 1H), 6.53 (td, 1H), 2.23 (s, 3H), 2.13 (d, 3H). ^{13}C NMR (400 MHz, DMSO- d_6) δ 150.89, 145.11, 143.81, 134.31, 129.93, 129.42, 128.12, 127.67, 123.65, 117.25, 115.87, 103.66. FTIR, cm^{-1} : 3328 (-NH) 2927 (C-H aromatic), 1518 (aromatic C=N), 1426 (-CH₃), 1345 (C=S), 763 (aromatic C-Cl). HRMS (+ESI-TOF) m/z : [M⁺] calcd for C₁₂H₁₂ClN₃S 265.04405; found 265.04404.



SB124

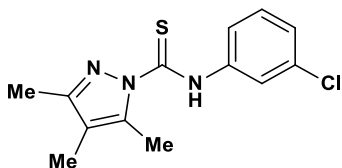
N-(4-chlorophenyl)-3,5-dimethyl-1H-pyrazole-1-carbothioamide: To a solution of 3-methyl-5-phenyl-1H-pyrazole (323 mg, 2.04 mmol, 1.0 eq.) and 4-chlorophenyl isothiocyanate (380 mg, 2.25 mmol, 1.1 eq.) in anhydrous DMF (2 mL), anhydrous potassium carbonate (563 mg, 4.08 mmol, 2.0 eq.) was added. The reaction mixture was kept stirring under argon at room temperature for 24 hours. This was filtered to remove excess K₂CO₃ and the filtrate concentrated to dryness. Descending column chromatography using silica gel as stationary phase and a gradient of DCM and MeOH as eluent gave a light-yellow solid (240 mg, 36%). ^1H NMR (400 MHz, DMSO- d_6) δ 12.57 (s, 1H), 8.02 (d, J = 7.5 Hz, 1H), 7.77 (dd, J = 23.5, 8.0 Hz, 2H), 7.38 (ddq, J = 44.7, 26.9, 8.1, 7.4 Hz, 6H), 7.04 – 6.86 (m, 1H), 6.55 (d, J = 8.3 Hz, 1H), 2.25 (s, 3H). ^{13}C NMR (101 MHz, DMSO- d_6) δ 151.73, 149.19, 148.12, 144.47, 138.76, 137.05, 132.04, 129.12, 127.72, 126.62, 125.40, 122.91, 120.32, 119.17, 115.65, 107.90, 101.70. FTIR, cm^{-1} : 3343 (-NH) 2924 (C-H aromatic), 1587 (aromatic C=N), 1517, 1450, 1399, (-CH₃), 1346 (C=S),

764 (aromatic C-Cl). HRMS (+ESI-TOF) m/z: [M⁺] calcd for C₁₂H₁₂ClN₃S 265.04405; found 265.04402.



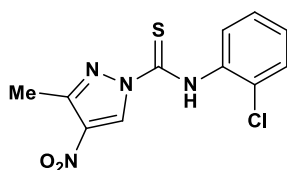
SB125

N-(2-chlorophenyl)-3,4,5-trimethyl-1H-pyrazole-1-carbothioamide: 3,4,5-trimethyl-1H-pyrazole (350 mg, 3.18 mmol, 1.0 eq.) was dissolved in dry THF and transferred to a round bottom which consisted of K₂CO₃ (878 mg, 6.36 mmol, 2.0 eq.). The reaction mixture was stirred vigorously for 1 h at 50° C. Then 2-chlorophenyl isothiocyanate (592 mg, 3.5 mmol, 1.1 eq.) was added and refluxed at 70° C for 18 h. Potassium carbonate solid was filtered off by gravity filtration and filtrate's THF was evaporated in vacuo. Resulting crude product was purified by flash column chromatography (100% hexane to 5% ethyl acetate/hexane) to afford an off-white color solid (347 mg, 39%). ¹H NMR (400 MHz, DMSO-d₆) δ 9.87 (s, 1H), 8.07 (dd, J = 8.2, 1.6 Hz, 1H), 7.60 – 7.49 (m, 1H), 7.49 – 7.34 (m, 1H), 7.20 (td, J = 7.7, 1.6 Hz, 1H), 2.89 (s, 6H), 1.91 (s, 3H). ¹³C NMR (400 MHz, DMSO-d₆) δ 150.86, 148.49, 139.37, 134.43, 130.61, 129.89 (d, J = 6.2 Hz), 129.67, 129.02, 128.38, 127.68 (d, J = 7.9 Hz), 126.05, 124.95, 122.91. FTIR, cm⁻¹: 3311 (-NH) 2976 (C-H aromatic), 1529 (aromatic C=N), 1508, 1473, 1439, (-CH₃), 1346 (C=S), 716 (aromatic C-Cl). HRMS (+ESI-TOF) m/z: [M⁺] calcd for C₁₃H₁₄ClN₃S 279.05970; found 279.05966.



SB126

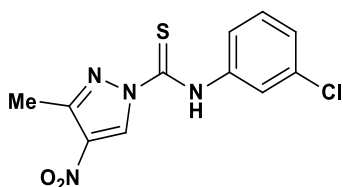
N-(3-chlorophenyl)-3,4,5-trimethyl-1H-pyrazole-1-carbothioamide: 3,4,5-trimethyl-1H-pyrazole (350 mg, 3.18 mmol, 1.0 eq.) was dissolved in dry THF and transferred to a round bottom which consisted of K₂CO₃ (878 mg, 6.36 mmol, 2.0 eq.). The reaction mixture was stirred vigorously for 1 h at 50° C. Then 3-chlorophenyl isothiocyanate (592 mg, 3.5 mmol, 1.1 eq.) was added and refluxed at 70° C for 18 h. Potassium carbonate solid was filtered off by gravity filtration and filtrate's THF was evaporated in vacuo. Resulting crude product was purified by flash column chromatography (100% hexane to 5% ethyl acetate/hexane) to afford an off-white color solid (115 mg, 13%). ¹H NMR (400 MHz, DMSO-d₆) δ 10.06 (s, 1H), 7.89 (t, J = 2.2 Hz, 1H), 7.77 – 7.63 (m, 2H), 7.40 – 7.30 (m, 2H), 7.08 – 6.96 (m, 1H), 6.49 (dt, J = 7.8, 2.5 Hz, 1H), 2.45 (s, 3H), 2.05 (s, 3H), 1.82 (s, 3H). ¹³C NMR (400 MHz, DMSO-d₆) δ 150.71 (d, J = 11.2 Hz), 141.29 (d, J = 15.6 Hz), 133.24 (d, J = 38.1 Hz), 130.87 – 130.35 (m), 124.74 (d, J = 16.9 Hz), 123.79 (d, J = 23.6 Hz), 122.63, 120.27, 119.34, 115.47, 113.44, 112.86, 109.65. FTIR, cm⁻¹: 3341 (-NH) 2921 (C-H aromatic), 1526 (aromatic C=N), 1506, 1477, 1411, (-CH₃), 1344 (C=S), 723 (aromatic C-Cl). HRMS (+ESI-TOF) m/z: [M⁺] calcd for C₁₃H₁₄ClN₃S 279.05970; found 279.05968.



SB127

N-(2-chlorophenyl)-3-methyl-4-nitro-1H-pyrazole-1-carbothioamide: Two 100 mL round bottom flasks were flame dried to remove moisture. To flask A was added 3-methyl-4-nitro pyrazole (300 mg, 2.36 mmol, 1.0 eq.) and dry THF (10 mL). To flask B was added sodium hydride (85 mg, 3.54 mmol, 1.5 eq.) and dry THF (10 mL). Both flasks were allowed to stir at 0° C for 20 minutes. The mixture from flask B was cannulated into flask A using a two-headed

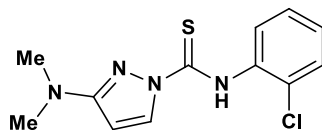
needle. This solution was kept stirring at r.t. under argon for 3 hours. 2-chlorophenyl isothiocyanate (439 mg, 2.60 mmol, 1.1 eq.) was added to the reaction, which continued stirring for 12 hours at r.t. The reaction was concentrated to dryness to reveal a white solid (143 mg, 20%). ¹H NMR (400 MHz, DMSO-d₆) δ 8.19 (s, 1H), 7.70 (d, J = 11.9 Hz, 1H), 7.41 (td, J = 18.4, 16.9, 9.1 Hz, 2H), 7.20 (d, J = 7.6 Hz, 1H), 2.90 (s, 3H). ¹³C NMR (400 MHz, DMSO-d₆) δ 162.75, 141.29, 140.72, 137.13, 133.06, 132.78, 131.02, 130.54, 124.70, 123.58, 122.52. FTIR, cm⁻¹: 3210 (-NH) 2971 (C-H aromatic), 1499, 1437, 1403, (-CH₃), 1317 (C=S), 759 (aromatic C-Cl). UPLC-MS [M+1] calcd for C₁₁H₉ClN₄O₂S 297.73; found 296.96.



SB128

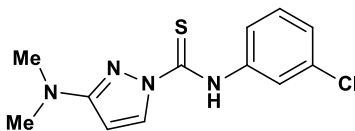
N-(3-chlorophenyl)-3-methyl-4-nitro-1H-pyrazole-1-carbothioamide: Two 100 mL round bottom flasks were flame dried to remove moisture. To flask A was added 3-methyl-4-nitro pyrazole (300 mg, 2.36 mmol, 1.0 eq.) and dry THF (10 mL). To flask B was added sodium hydride (85 mg, 3.54 mmol, 1.5 eq.) and dry THF (10 mL). Both flasks were allowed to stir at 0° C for 20 minutes. The mixture from flask B was cannulated into flask A using a two-headed needle. This solution was kept stirring at r.t. under argon for 3 hours. 2-chlorophenyl isothiocyanate (439 mg, 2.60 mmol, 1.1 eq.) was added to the reaction, which continued stirring for 12 hours at r.t. The reaction was concentrated to dryness to reveal a white solid (152 mg, 22%). ¹H NMR (400 MHz, DMSO-d₆) δ 13.66 (s, 1H), 8.39 (s, 1H), 7.70 (dt, J = 12.5, 2.0 Hz, 1H), 7.43 – 7.22 (m, 3H), 2.90 (s, 3H). ¹³C NMR (101 MHz, DMSO-d₆) δ 180.15, 162.75, 141.29, 133.06, 130.54, 124.70, 123.57, 122.52, 122.14, 118.20, 117.27. FTIR, cm⁻¹: 3261 (-NH)

2923 (C-H aromatic), 1585 (aromatic C=N), 1546, 1474, (-CH₃), 1339 (C=S), 773 (aromatic C-Cl). UPLC-MS [M+1] calcd for C₁₁H₉ClN₄O₂S 297.73; found 297.04.



SB129

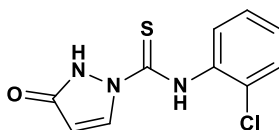
N-(2-chlorophenyl)-3-(dimethylamino)-1H-pyrazole-1-carbothioamide: To a solution of N,N-dimethyl-1H-pyrazol-3-amine (100 mg, 0.899 mmol, 1.0 eq.) and 2-chlorophenyl isothiocyanate (152 mg, 0.899 mmol, 1.0 eq.) in anhydrous DMF (2 mL), anhydrous sodium hydride (32 mg, 1.34 mmol, 1.5 eq.) was added. The reaction mixture was kept stirring under argon at room temperature for 24 hours. This was filtered to remove excess K₂CO₃ and the filtrate concentrated to dryness. Descending column chromatography using silica gel as stationary phase and a gradient of DCM and MeOH as eluent gave a light-yellow solid (40 mg, 16%). ¹H NMR (400 MHz, DMSO-d₆) δ 10.99 (s, 1H), 8.49 (d, J = 3.0 Hz, 1H), 7.77 (d, J = 7.9 Hz, 1H), 7.66 – 7.56 (m, 1H), 7.47 – 7.34 (m, 2H), 6.34 (d, J = 3.2 Hz, 1H), 2.95 (s, 6H). ¹³C NMR (101 MHz, DMSO-d₆) δ 145.13, 131.41, 129.84, 129.42, 128.60, 128.38, 128.11, 127.65, 117.51, 117.24, 115.88, 65.40, 41.44, 15.65. FTIR, cm⁻¹: 3326 (-NH) 2920 (C-H aromatic), 1594 (aromatic C=N), 1539, 1441, (-CH₃), 1359 (C=S), 746 (aromatic C-Cl). HRMS (+ESI-TOF) m/z: [M+] calcd for C₁₂H₁₃ClN₄S 280.05494; found 280.05490.



SB130

N-(3-chlorophenyl)-3-(dimethylamino)-1H-pyrazole-1-carbothioamide: To a solution of N,N-dimethyl-1H-pyrazol-3-amine (100 mg, 0.899 mmol, 1.0 eq.) and 3-chlorophenyl

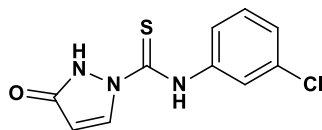
isothiocyanate (152 mg, 0.899 mmol, 1.1 eq.) in anhydrous DMF (2 mL), anhydrous sodium hydride (32 mg, 1.34 mmol, 1.5 eq.) was added. The reaction mixture was kept stirring under argon at room temperature for 24 hours. This was filtered to remove excess K_2CO_3 and the filtrate concentrated to dryness. Descending column chromatography using silica gel as stationary phase and a gradient of DCM and MeOH as eluent gave a light-yellow solid (83 mg, 33%). ^1H NMR (400 MHz, DMSO- d_6) δ 11.04 (s, 1H), 8.51 (d, J = 3.1 Hz, 1H), 7.80 (t, J = 2.1 Hz, 1H), 7.69 – 7.59 (m, 1H), 7.49 – 7.31 (m, 2H), 6.34 (d, J = 3.1 Hz, 1H), 2.96 (s, 6H). ^{13}C NMR (400 MHz, DMSO- d_6) δ 173.25, 161.41, 140.15, 133.53, 132.93, 130.44, 126.50, 125.60, 124.63, 123.58, 100.77, 81.34 – 77.76 (m). FTIR, cm^{-1} : 3413 (-NH) 2951 (C-H aromatic), 1579 (aromatic C=N), 1500, 1417, (-CH₃), 1349 (C=S), 755 (aromatic C-Cl). HRMS (+ESI-TOF) m/z : [M⁺] calcd for $C_{12}H_{13}ClN_4S$ 280.05494; found 280.05496.



SB131

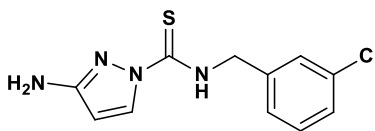
N-(2-chlorophenyl)-3-oxo-2,3-dihydro-1H-pyrazole-1-carbothioamide: A solution of 1,2-dihydro-3H-pyrazol-3-one (335 mg, 3.99 mmol, 1.0 eq.), 2-chlorophenyl isothiocyanate (741 mg, 4.39 mmol, 1.1 eq.), and tetrahydrofuran (10 mL) was refluxed at 45° C under argon for 24 hours. The solvent was removed in vacuo. Descending column chromatography using silica gel as stationary phase and a gradient mixture of DCM and MeOH as eluent gave a white solid (201 mg, 19%). ^1H NMR (400 MHz, DMSO- d_6) δ 11.12 (s, 2H), 8.51 (d, J = 3.0 Hz, 1H), 7.68 – 7.51 (m, 2H), 7.39 (dtd, J = 21.1, 7.5, 1.7 Hz, 2H), 6.08 (d, J = 3.0 Hz, 1H). ^{13}C NMR (400 MHz, DMSO- d_6) δ 175.22, 164.54, 136.27, 133.45, 131.00, 130.61, 130.05, 130.00, 129.99, 129.64,

129.23, 129.00, 127.99, 127.70, 100.40, 89.81. FTIR, cm^{-1} : 3204 (-NH), 1535 (aromatic C=N), 1309 (C=S), 748 (aromatic C-Cl).



SB132

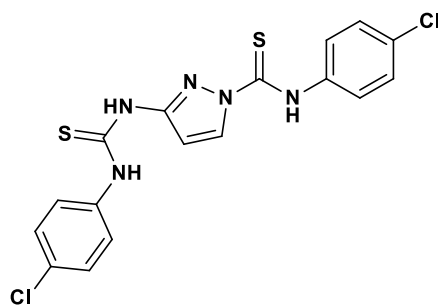
N-(3-chlorophenyl)-3-oxo-2,3-dihydro-1H-pyrazole-1-carbothioamide: A solution of 1,2-dihydro-3H-pyrazol-3-one (314 mg, 3.73 mmol, 1.0 eq.), 3-chlorophenyl isothiocyanate (694 mg, 4.11 mmol, 1.1 eq.), and tetrahydrofuran (10 mL) was refluxed at 45° C under argon for 24 hours. The solvent was removed in vacuo. Descending column chromatography using silica gel as stationary phase and a gradient mixture of DCM and MeOH as eluent gave a white solid (466 mg, 49%). ^1H NMR (400 MHz, Deuterium Oxide) δ 13.51 (s, 1H), 13.32 (s, 1H), 10.74 (d, J = 3.0 Hz, 1H), 10.01 (t, J = 2.0 Hz, 1H), 9.81 (ddd, J = 8.1, 2.0, 1.0 Hz, 1H), 9.59 (dd, J = 9.5, 1.5 Hz, 1H), 9.54 (ddd, J = 8.0, 2.1, 1.0 Hz, 1H), 8.29 (d, J = 3.0 Hz, 1H). ^{13}C NMR (400 MHz, Deuterium Oxide) δ 176.62, 166.65, 142.36, 135.68, 135.16, 133.99, 132.64, 130.66, 128.82, 128.44, 127.83, 127.54, 126.87, 102.65, 42.79, 42.58, 42.37, 42.16, 41.96, 41.75, 41.54. FTIR, cm^{-1} : 3247 (-NH) 3150 (C-H aromatic), 1527 (aromatic C=N), 1364 (C=S), 727 (aromatic C-Cl).



SB133

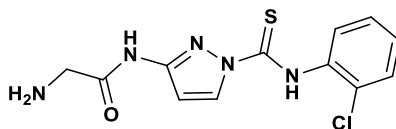
3-amino-N-(3-chlorobenzyl)-1H-pyrazole-1-carbothioamide: 1,2-dihydro-3H-pyrazol-3-one (335 mg, 3.99 mmol, 1.0 eq.), 2-chlorophenyl isothiocyanate (741 mg, 4.39 mmol, 1.1 eq.) were dissolved in dichloromethane (10 mL) was stirred at room temperature for 24 hours. The solvent was removed in vacuo. Descending column chromatography using silica gel as stationary phase

and a gradient mixture of DCM and MeOH as eluent gave a white solid (201 mg, 19%). ^1H NMR (400 MHz, DMSO- d_6) δ 11.12 (s, 2H), 8.51 (d, J = 3.0 Hz, 1H), 7.68 – 7.51 (m, 2H), 7.39 (dtd, J = 21.1, 7.5, 1.7 Hz, 2H), 6.08 (d, J = 3.0 Hz, 1H). ^{13}C NMR (400 MHz, DMSO- d_6) δ 175.22, 164.54, 136.27, 133.45, 131.00, 130.61, 130.05, 130.00, 129.99, 129.64, 129.23, 129.00, 127.99, 127.70, 100.40, 89.81. FTIR, cm^{-1} : 3204 (-NH), 1535 (aromatic C=N), 1309 (C=S), 748 (aromatic C-Cl). HRMS (+ESI-TOF) m/z : $[M]^+$ calcd for $\text{C}_{11}\text{H}_{11}\text{ClN}_4\text{S}$ 266.03929; found 266.03927.



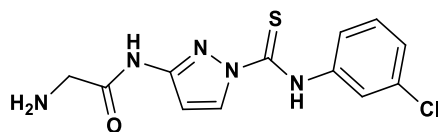
SB134

N-(4-chlorophenyl)-3-(3-(4-chlorophenyl)thioureido)-1H-pyrazole-1-carbothioamide: To a round bottom flask was added 3-amino-1H-pyrazole (250 mg, 3.01 mmol, 1.0 eq.), 4-chlorophenyl isothiocyanate (1,118 mg, 6.62 mmol, 2.2 eq.), anhydrous potassium carbonate (1,370 mg, 9.93 mmol, 3.3 eq.), and 3 mL of DMF. This was left to stir at room temperature overnight. The solvent was removed using a gentle stream of air, then the product was purified via flash column chromatography to reveal a light-yellow solid (811.5 mg, 64%). ^1H NMR (400 MHz, DMSO- d_6) δ 11.15 (s, 1H), 10.90 (s, 1H), 9.95 (s, 1H), 8.74 (d, J = 3.0 Hz, 1H), 7.54 – 7.51 (m, 4H), 7.48 – 7.44 (m, 2H), 7.41 – 7.37 (m, 2H), 6.45 (d, J = 3.0 Hz, 1H). ^{13}C NMR (101 MHz, DMSO- d_6) δ 180.21, 178.00, 174.45, 151.57, 139.05, 138.79, 137.54, 133.83, 131.88, 130.94, 129.53, 129.21, 128.99, 128.83, 128.71, 125.80, 102.68. FTIR, cm^{-1} : 3329, 3285, 3180 (-NH-), 1628 (aromatic C=C), 1582 (aromatic C=N), 1281 (C=S), 759, 726 (aromatic C-Cl).



SB135

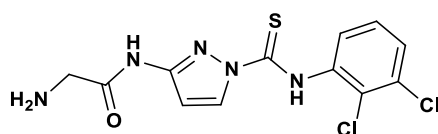
2-amino-N-(1-((2-chlorophenyl)carbamothioyl)-1H-pyrazol-3-yl)acetamide: To a RBF was added 3-amino-N-(2-chlorophenyl)-1H-pyrazole-1-carbothioamide (673 mg, 2.664 mmol), carbonyldiimidazole (432 mg, 2.664 mmol) and dimethylformamide (5 mL). This solution was allowed to stir at room temperature for 50 minutes. After which, glycine (200 mg, 2.664 mmol) was added slowly. This was then heated to 60° C and left to reflux for 2 hours. The solvent was then removed in vacuo and ice water added to the residue. A pink-colored solid precipitated, which was then collected via filtration (174 mg, 21%). ¹H NMR (400 MHz, DMSO-d₆) δ 12.72 (s, 1H), 11.79 (s, 1H), 10.95 (s, 1H), 8.06 (d, J = 8.0 Hz, 1H), 7.75 (d, J = 2.5 Hz, 1H), 7.57 – 7.51 (m, 1H), 7.37 (t, J = 7.3 Hz, 1H), 7.26 (td, J = 7.7, 1.6 Hz, 1H), 6.02 (d, J = 13.8 Hz, 1H). ¹³C NMR (151 MHz, DMSO-d₆) δ 177.40, 158.67, 149.67, 136.62, 130.19, 129.70, 129.08, 128.34, 127.64, 127.38, 102.57, 94.58. FTIR, cm⁻¹: 3188 (-NH₂), 3054, 3017 (-NH), 1576 (C=O), 1527 (aromatic C=C), 1280 (aromatic C=N), 1206 (C=S), 752 (aromatic C-Cl). UPLC-MS [M+1] calcd for C₁₂H₁₂ClN₅OS 310.77; found 310.98. UPLC-MS [M+1] calcd for C₁₂H₁₂ClN₅OS 310.77; found 310.98.



SB136

2-amino-N-(1-((3-chlorophenyl)carbamothioyl)-1H-pyrazol-3-yl)acetamide: To a RBF was added 3-amino-N-(3-chlorophenyl)-1H-pyrazole-1-carbothioamide (673 mg, 2.664 mmol), carbonyldiimidazole (432 mg, 2.664 mmol) and dimethylformamide (5 mL). This solution was

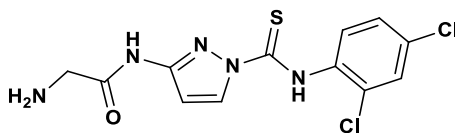
allowed to stir at room temperature for 50 minutes. After which, glycine (200 mg, 2.664 mmol) was added slowly. This was then heated to 60° C and left to reflux for 2 hours. The solvent was then removed in vacuo and ice water added to the residue. A pink-colored solid precipitated, which was then collected via filtration (432 mg, 52%). ¹H NMR (400 MHz, DMSO-*d*₆) δ 12.71 (s, 1H), 10.87 (s, 1H), 7.96 (s, 1H), 7.75 (s, 1H), 7.50 (d, *J* = 8.2 Hz, 1H), 7.40 (t, *J* = 8.0 Hz, 1H), 7.25 (d, *J* = 8.2 Hz, 1H), 6.06 (s, 1H). ¹³C NMR (151 MHz, DMSO-*d*₆) δ 213.32, 176.37, 149.84, 139.33, 132.74, 131.00, 129.55, 125.05, 123.68, 122.23, 92.22, 30.32. FTIR, cm⁻¹: 3330 (-NH₂), 3124, 3060 (-NH), 1576 (C=O), 1531 (aromatic C=C), 1273 (aromatic C=N), 1208 (C=S), 762 (aromatic C-Cl). UPLC-MS [M+1] calcd for C₁₂H₁₂ClN₅OS 310.77; found 310.98.



SB137

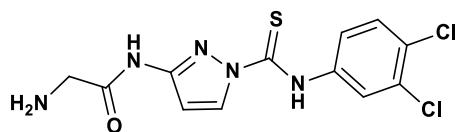
2-amino-N-(1-((2,3-chlorophenyl)carbamothioyl)-1H-pyrazol-3-yl)acetamide: To a RBF was added 3-amino-N-(2,3-dichlorophenyl)-1H-pyrazole-1-carbothioamide (287 mg, 0.999 mmol), carbonyldiimidazole (162 mg, 0.999 mmol) and dimethylformamide (5 mL). This solution was allowed to stir at room temperature for 50 minutes. After which, glycine (75 mg, 0.999 mmol) was added slowly. This was then heated to 60° C and left to reflux for 2 hours. The solvent was then removed in vacuo and ice water added to the residue. A yellow colored solid precipitated, which was then collected via filtration (41 mg, 12%). ¹H NMR (600 MHz, DMSO-*d*₆) δ 10.62 (s, 2H), 7.81 (dd, *J* = 8.2, 1.5 Hz, 2H), 7.54 (t, *J* = 8.1 Hz, 2H), 7.45 (s, 1H), 4.35 (d, *J* = 1.3 Hz, 2H), 1.25 (d, *J* = 8.5 Hz, 2H). ¹³C NMR (151 MHz, DMSO-*d*₆) δ 182.44, 171.87, 133.64, 132.65, 132.03, 131.71, 131.29, 129.08, 128.19, 128.13, 69.46, 49.86. FTIR, cm⁻¹: 3193 (-NH₂), 3056,

3025 (-NH), 1577 (C=O), 1522 (aromatic C=C), 1287 (aromatic C=N), 1163 (C=S), 768, 732 (aromatic C-Cl). UPLC-MS [M+1] calcd for C₁₂H₁₂Cl₂N₅OS 345.22; found 345.26.



SB138

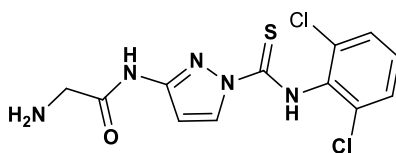
2-amino-N-(1-((2,4-chlorophenyl)carbamothioyl)-1H-pyrazol-3-yl)acetamide: To a RBF was added 3-amino-N-(2,4-dichlorophenyl)-1H-pyrazole-1-carbothioamide (287 mg, 0.999 mmol), carbonyldiimidazole (162 mg, 0.999 mmol) and dimethylformamide (5 mL). This solution was allowed to stir at room temperature for 50 minutes. After which, glycine (75 mg, 0.999 mmol) was added slowly. This was then heated to 60° C and left to reflux for 2 hours. The solvent was then removed in vacuo and ice water added to the residue. An orange colored solid precipitated, which was then collected via filtration (103 mg, 30%). ¹H NMR (600 MHz, DMSO-*d*₆) δ 12.75 (s, 1H), 11.05 (s, 1H), 8.10 (d, *J* = 8.8 Hz, 1H), 7.77 – 7.72 (m, 2H), 7.47 (dd, *J* = 8.7, 2.4 Hz, 1H), 6.01 (s, 1H). ¹³C NMR (151 MHz, DMSO-*d*₆) δ 177.34, 149.55, 147.98, 135.86, 132.30, 130.84, 130.18, 129.33, 129.23, 127.53, 94.59, 49.82. FTIR, cm⁻¹: 3410 (-NH₂), 3189, 3040, (-NH), 1590 (C=O), 1531 (aromatic C=C), 1272 (aromatic C=N), 1200 (C=S), 757, 738 (aromatic C-Cl). UPLC-MS [M+1] calcd for C₁₂H₁₂Cl₂N₅OS 345.22; found 345.01.



SB139

2-amino-N-(1-((3,4-chlorophenyl)carbamothioyl)-1H-pyrazol-3-yl)acetamide: To a RBF was added 3-amino-N-(3,4-dichlorophenyl)-1H-pyrazole-1-carbothioamide (287 mg, 0.999 mmol), carbonyldiimidazole (162 mg, 0.999 mmol) and dimethylformamide (5 mL). This solution was

allowed to stir at room temperature for 50 minutes. After which, glycine (75 mg, 0.999 mmol) was added slowly. This was then heated to 60° C and left to reflux for 2 hours. The solvent was then removed in vacuo and ice water added to the residue. An orange solid resulted (332 mg, 96%). ¹H NMR (600 MHz, DMSO-*d*₆) δ 12.73 (s, 1H), 10.94 (s, 1H), 8.15 (d, *J* = 2.5 Hz, 1H), 7.76 (t, *J* = 2.0 Hz, 1H), 7.64 (d, *J* = 8.6 Hz, 1H), 7.57 (dd, *J* = 8.7, 2.5 Hz, 1H), 6.06 (s, 1H), 4.12 (q, *J* = 5.3 Hz, 2H), 1.18 (t, *J* = 7.1 Hz, 2H). ¹³C NMR (151 MHz, DMSO-*d*₆) δ 170.84, 149.54, 139.59, 130.99, 130.74, 130.24, 125.84, 124.60, 60.24, 49.07, 21.24, 14.56. FTIR, cm⁻¹: 3338 (-NH₂), 3140, 3000, (-NH), 1569 (C=O), 1527 (aromatic C=C), 1270 (aromatic C=N), 1209 (C=S), 759, 712 (aromatic C-Cl).



SB140

2-amino-N-(1-((2,6-chlorophenyl)carbamothioyl)-1H-pyrazol-3-yl)acetamide: To a RBF was added 3-amino-N-(2,6-dichlorophenyl)-1H-pyrazole-1-carbothioamide (287 mg, 0.999 mmol), carbonyldiimidazole (162 mg, 0.999 mmol) and dimethylformamide (5 mL). This solution was allowed to stir at room temperature for 50 minutes. After which, glycine (75 mg, 0.999 mmol) was added slowly. This was then heated to 60° C and left to reflux for 2 hours. The solvent was then removed in vacuo and ice water added to the residue. An orange/red solid resulted (340 mg, 99%). ¹H NMR (600 MHz, DMSO-*d*₆) δ 12.75 (s, 1H), 11.06 (s, 1H), 7.99 (d, *J* = 8.2 Hz, 1H), 7.76 (s, 1H), 7.54 (d, *J* = 8.0 Hz, 1H), 7.40 (t, *J* = 8.1 Hz, 1H), 6.02 (s, 1H), 3.37 (s, 2H), 1.36 – 1.10 (m, 2H). ¹³C NMR (151 MHz, DMSO-*d*₆) δ 177.53, 149.56, 138.68, 132.04, 130.25, 128.33 – 127.90 (m), 127.43, 112.92, 94.63, 14.39. FTIR, cm⁻¹: 3402 (-NH₂), 3186, 3015, (-NH), 1578 (C=O), 1528 (aromatic C=C), 1283 (aromatic C=N), 1210 (C=S), 748, 735 (aromatic C-Cl).

Appendix B

Characterization of Novel NNSN Compounds

APM01

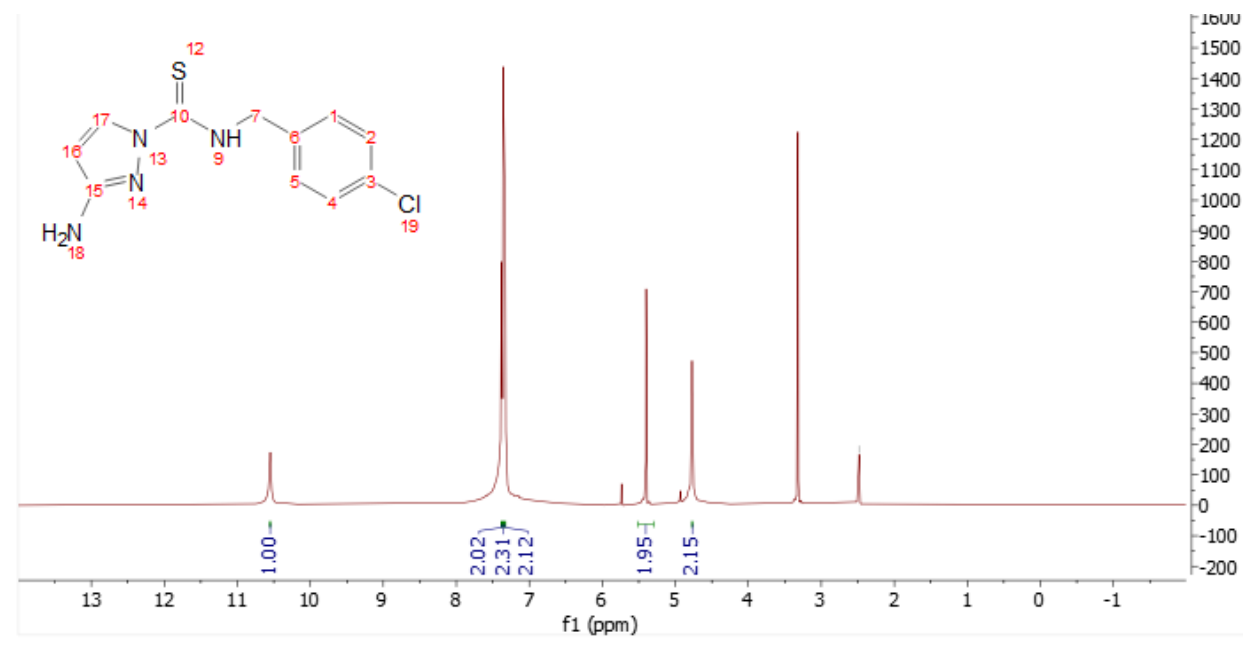


Figure 69, ¹H NMR of APM01.

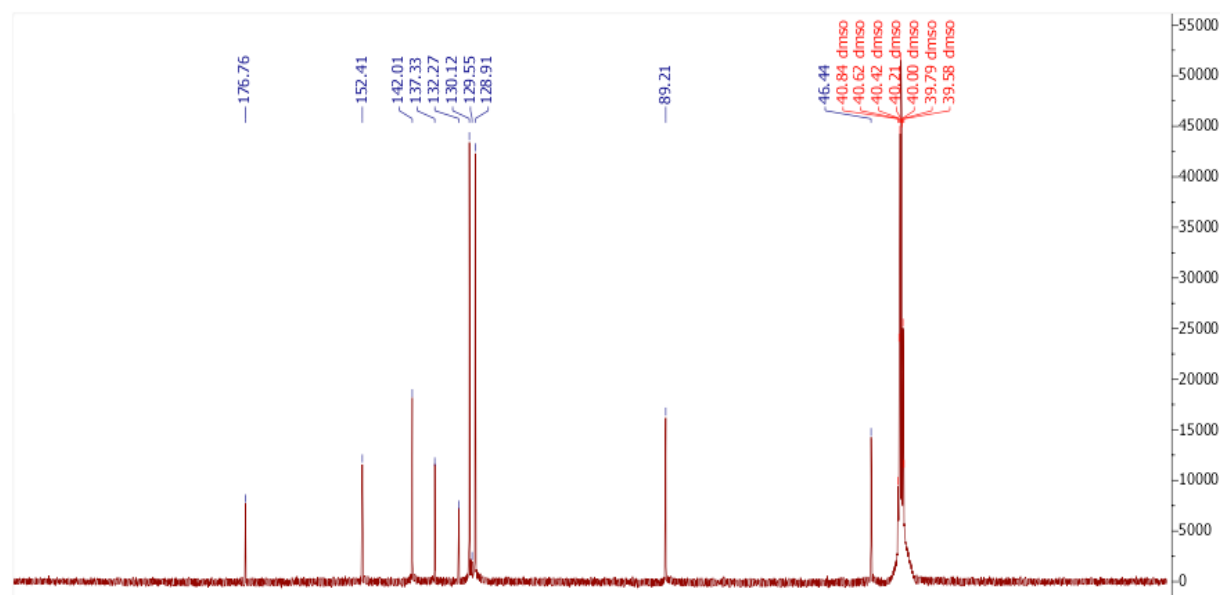


Figure 70, ¹³C NMR of APM01.

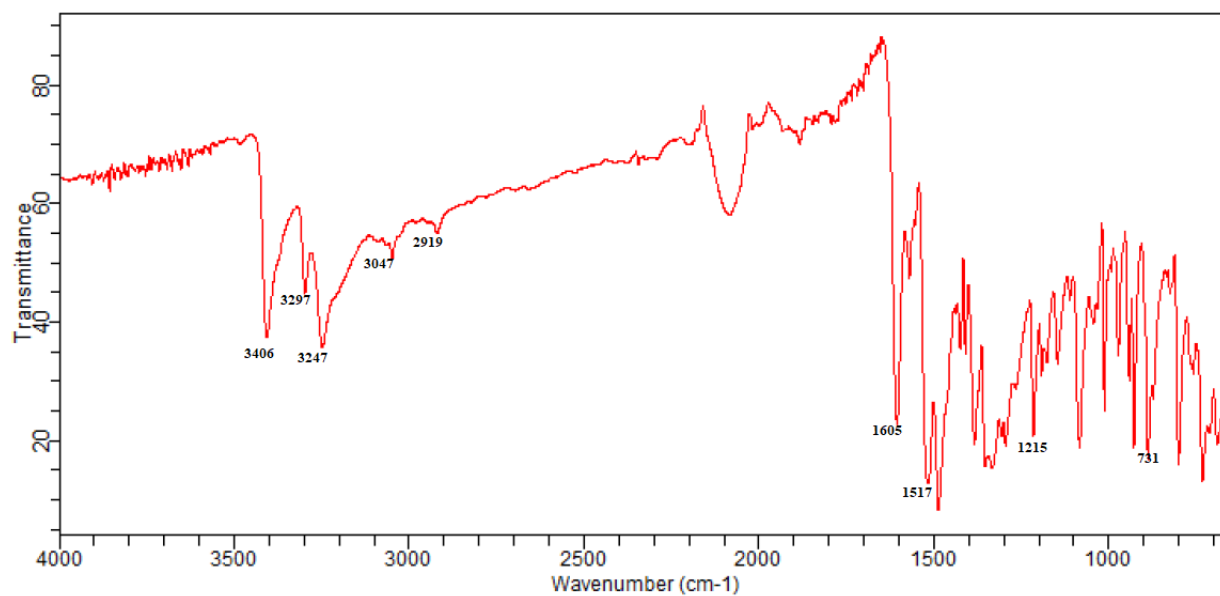


Figure 71, IR of APM01.

HRMS (+ESI-TOF) m/z: [M⁺] calcd for C₁₁H₁₁ClN₄S 266.03929; found 266.03930.

KE03

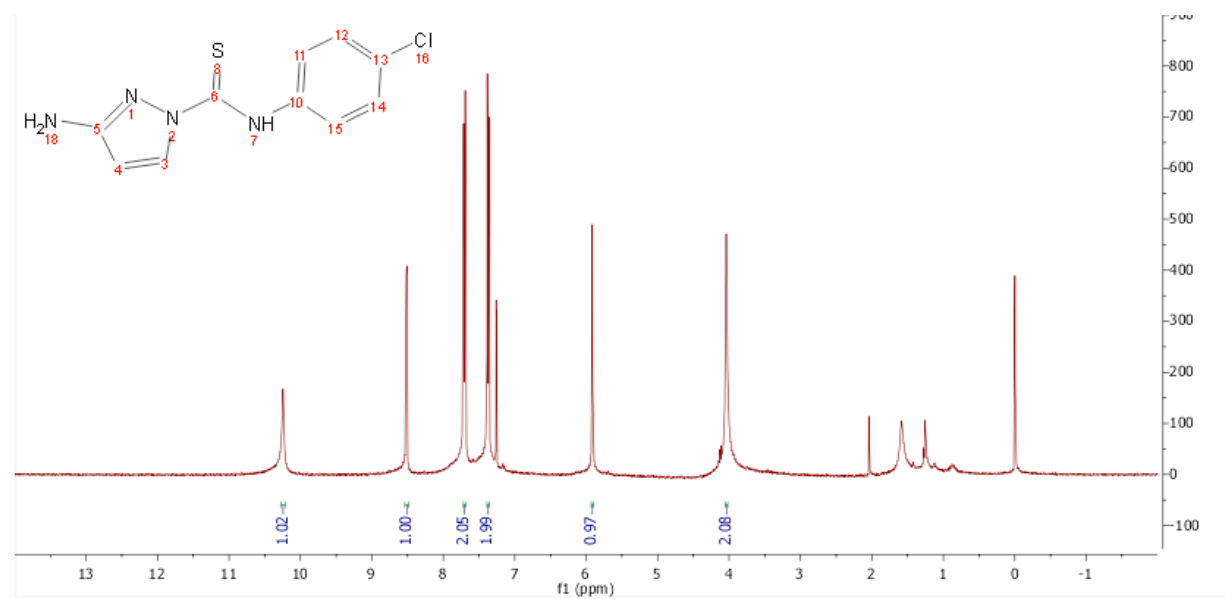


Figure 72, ¹H NMR of KE03.

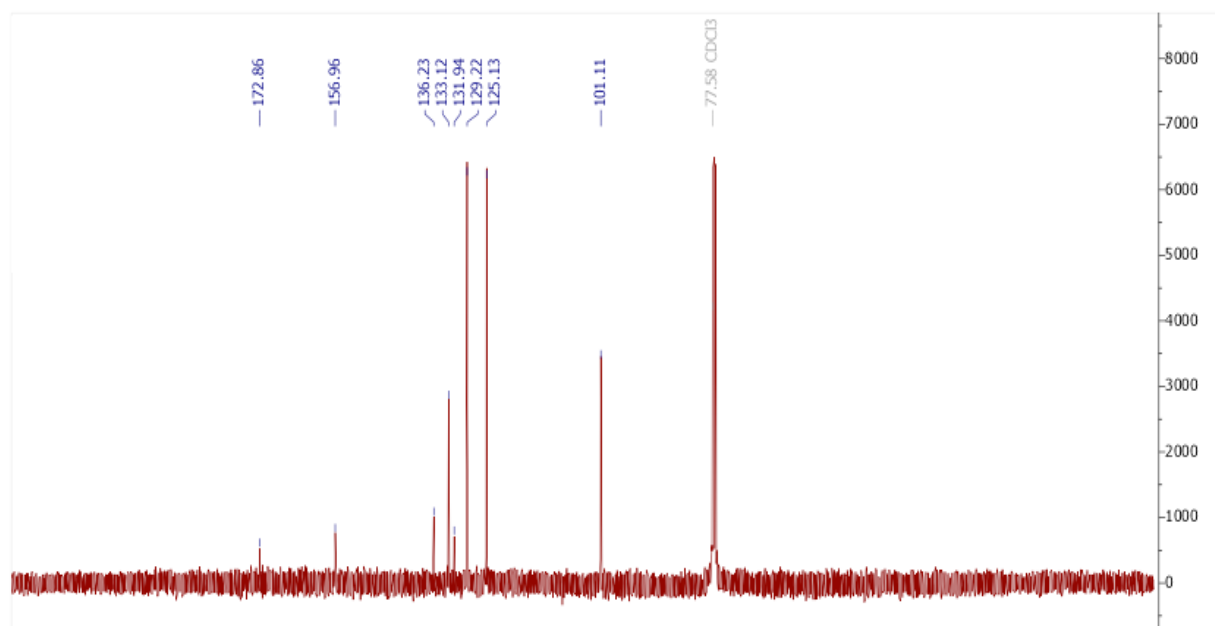


Figure 73, ^{13}C NMR of KE03.

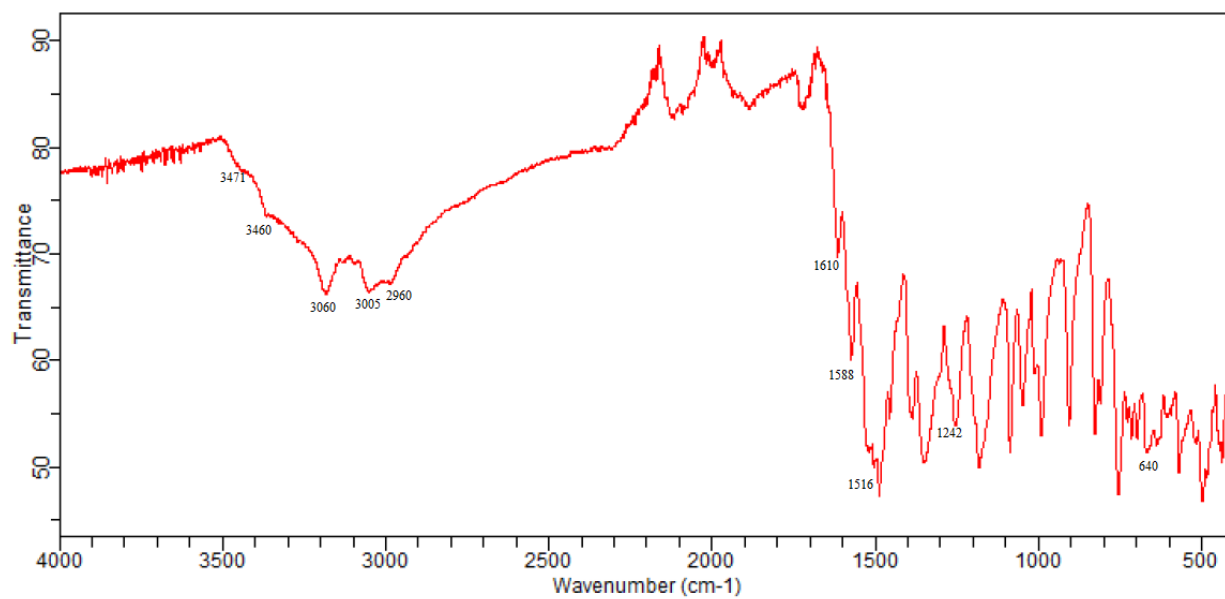


Figure 74, IR of KE03.

HRMS (+ESI-TOF) m/z : $[\text{M}^+]$ calcd for $\text{C}_{10}\text{H}_9\text{ClN}_4\text{S}$ 252.72326; found 252.72328.

SB46

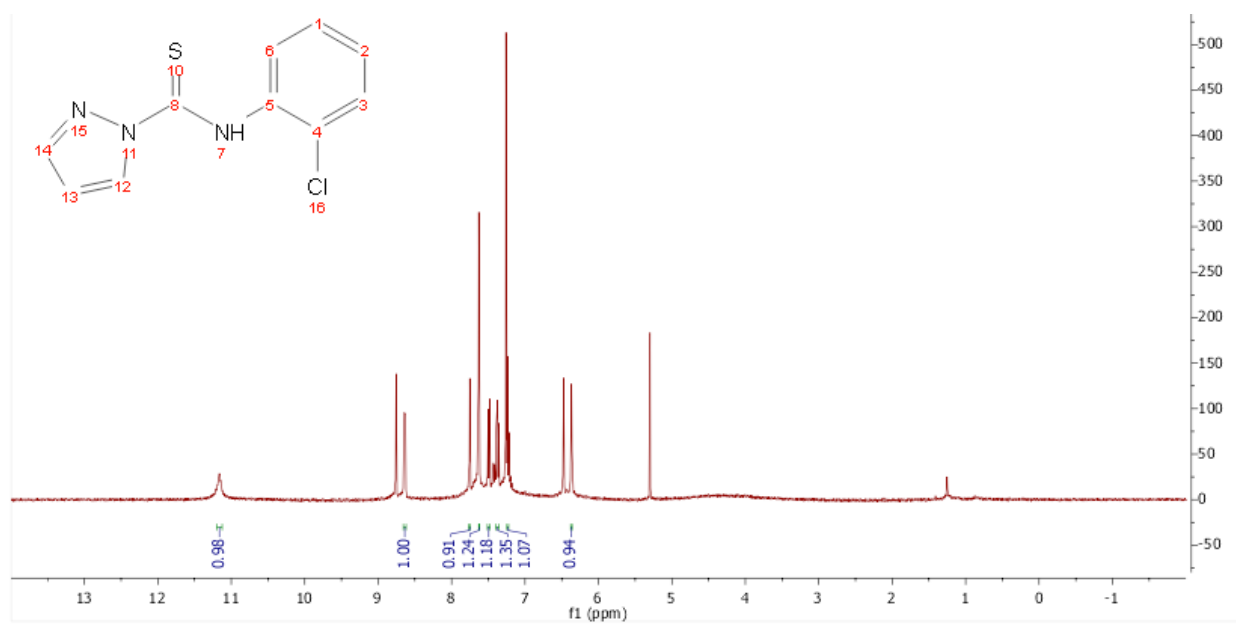


Figure 75, ¹H NMR of SB46.

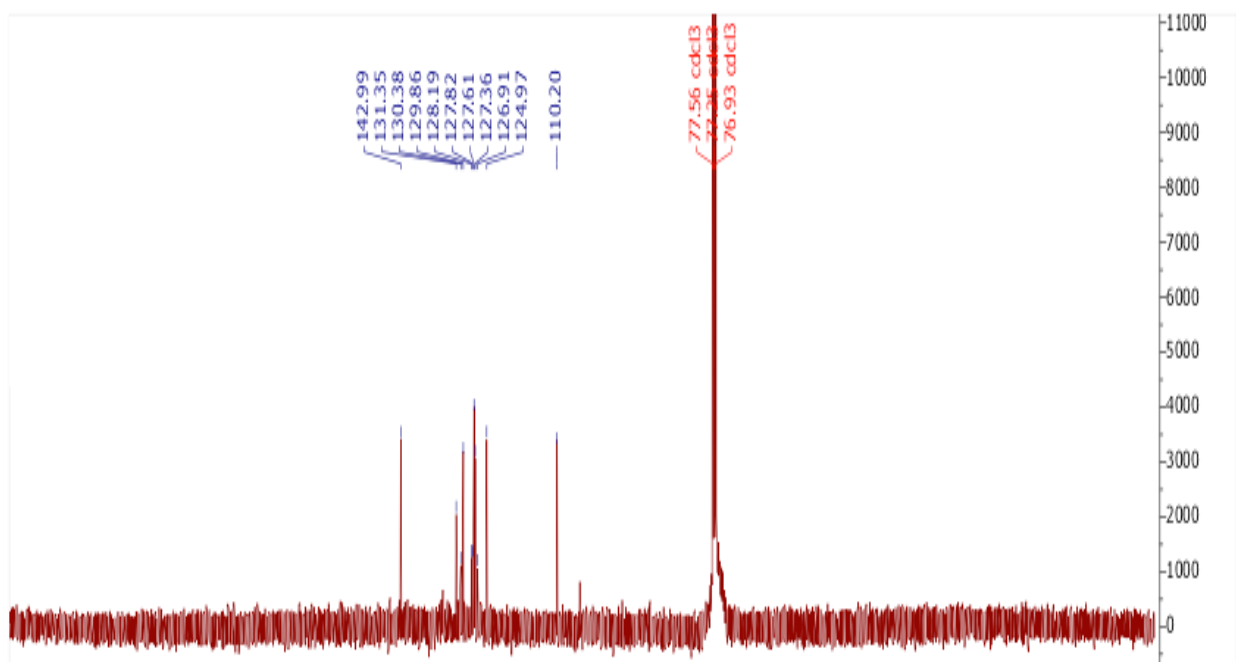


Figure 76, ¹³C NMR of SB46.

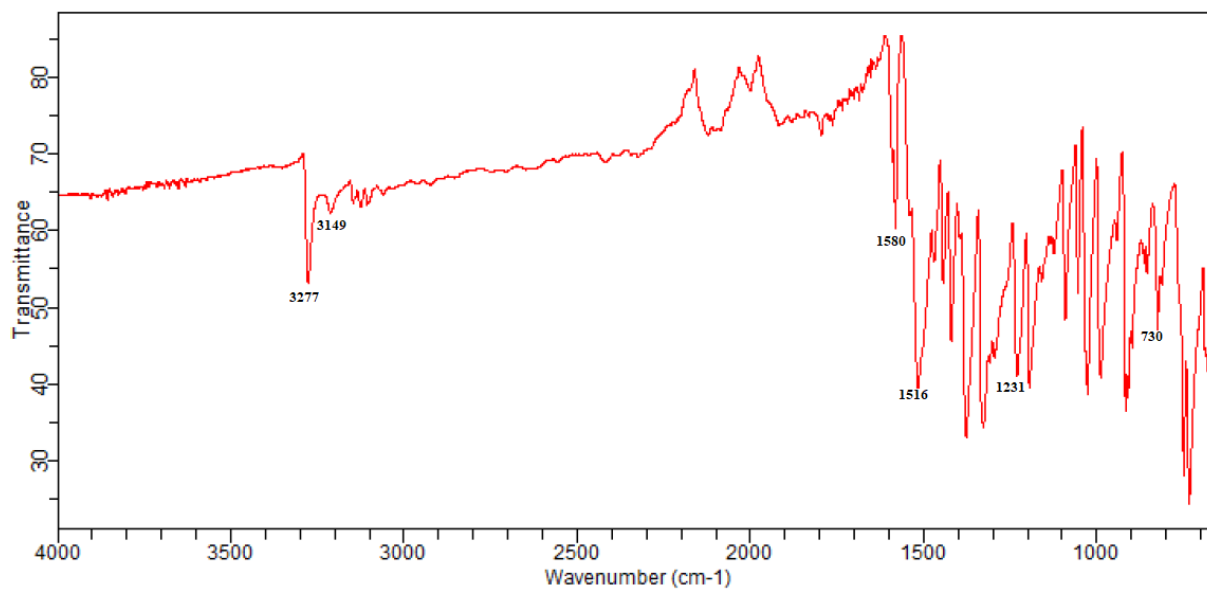


Figure 77, IR of SB46.

HRMS (+ESI-TOF) m/z : $[M]^+$ calcd for $C_{10}H_9ClN_4S$ 252.72326; found 252.72326.

SB49

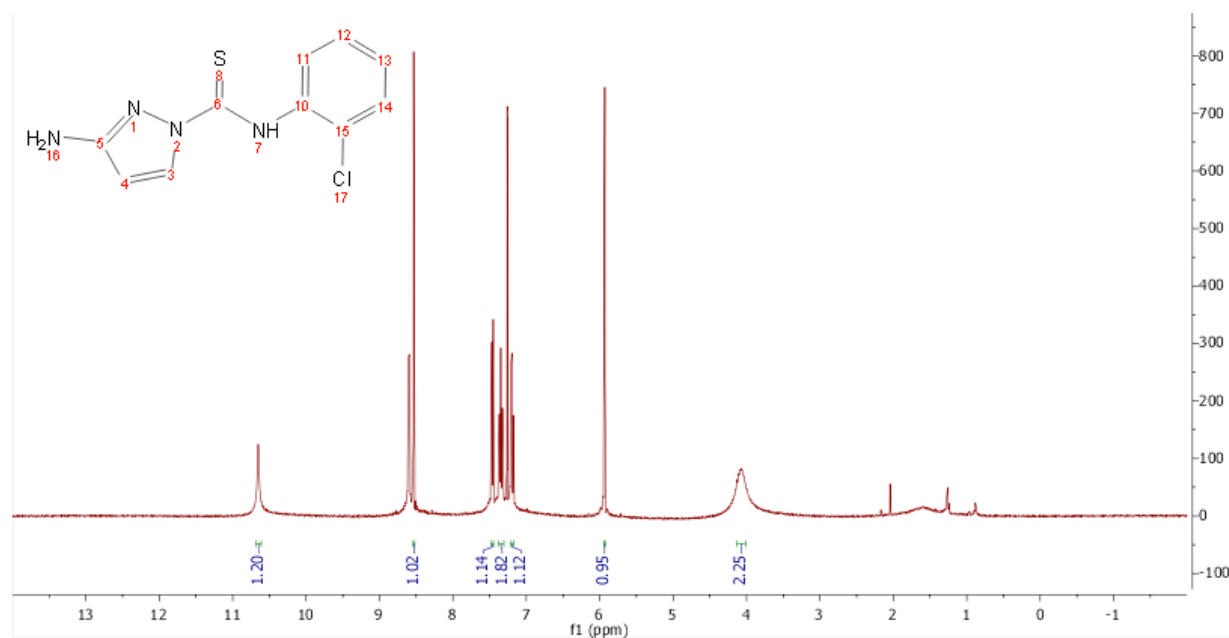


Figure 78, ^1H NMR of SB49.

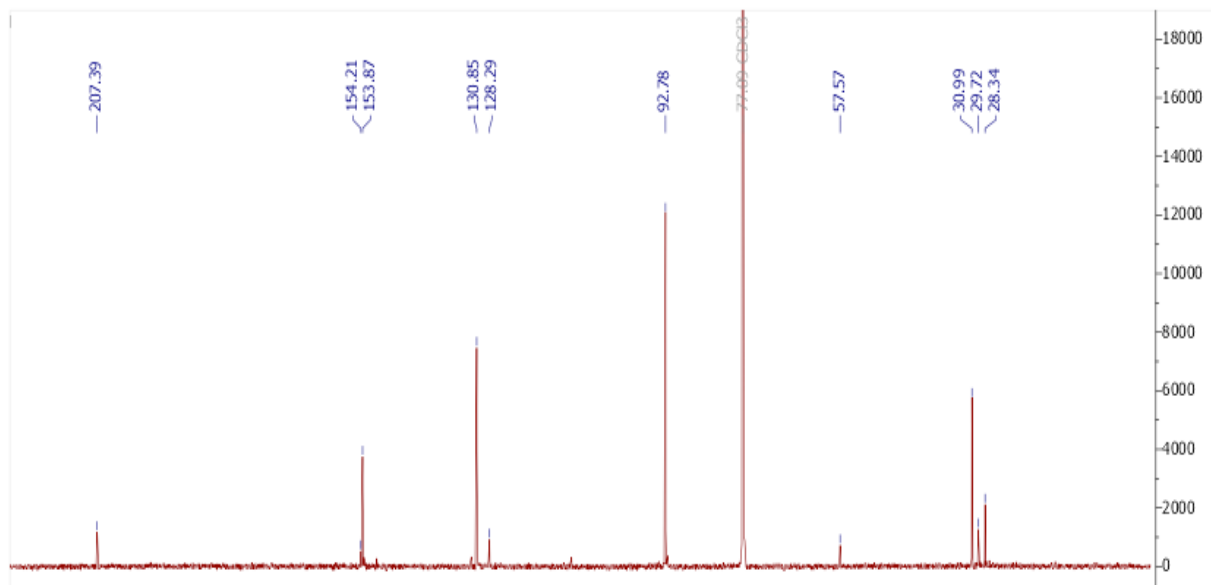


Figure 79, ¹³C NMR of SB49.

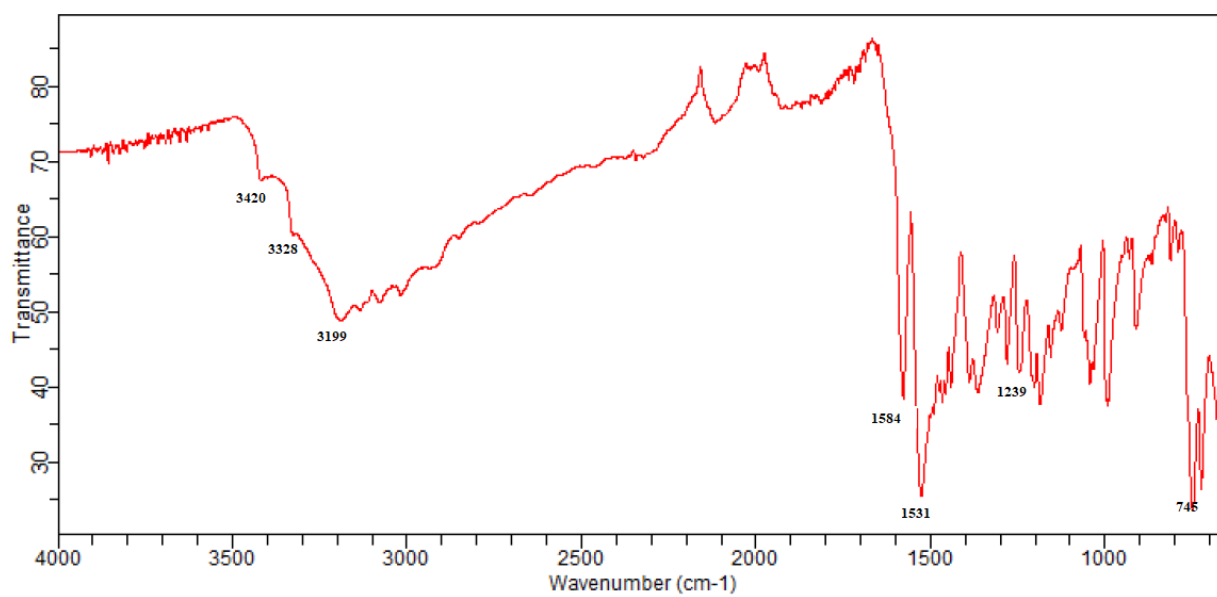


Figure 80, IR of SB49.

HRMS (+ESI-TOF) m/z: [M⁺] calcd for C₁₀H₈ClN₃S 237.70862; found 237.70870.

SB50

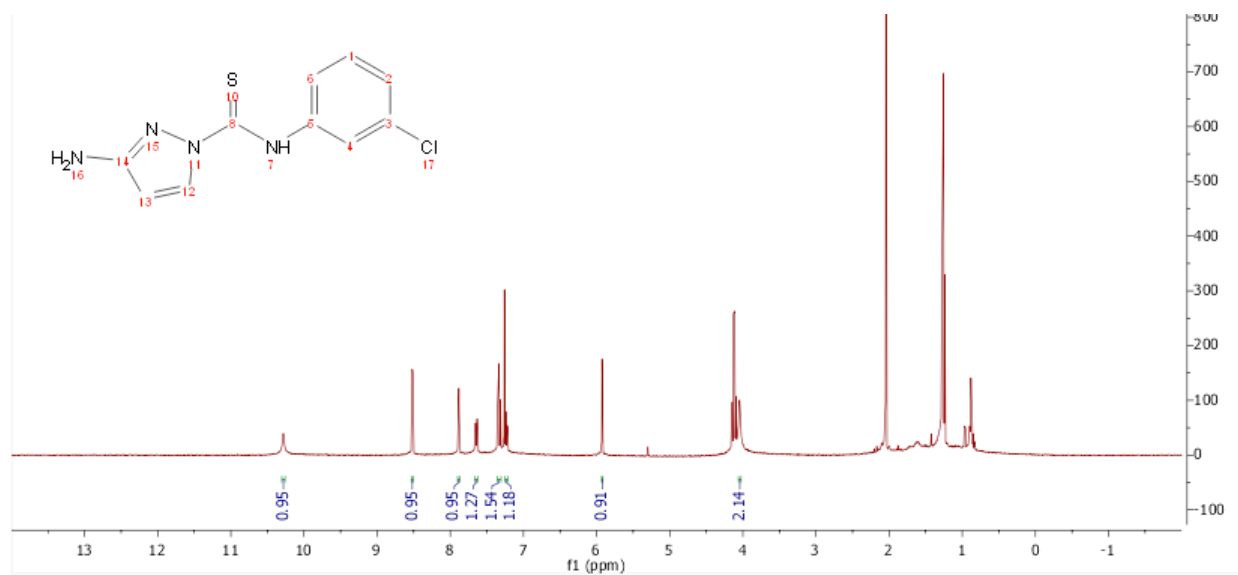


Figure 81, ¹H NMR of SB50.

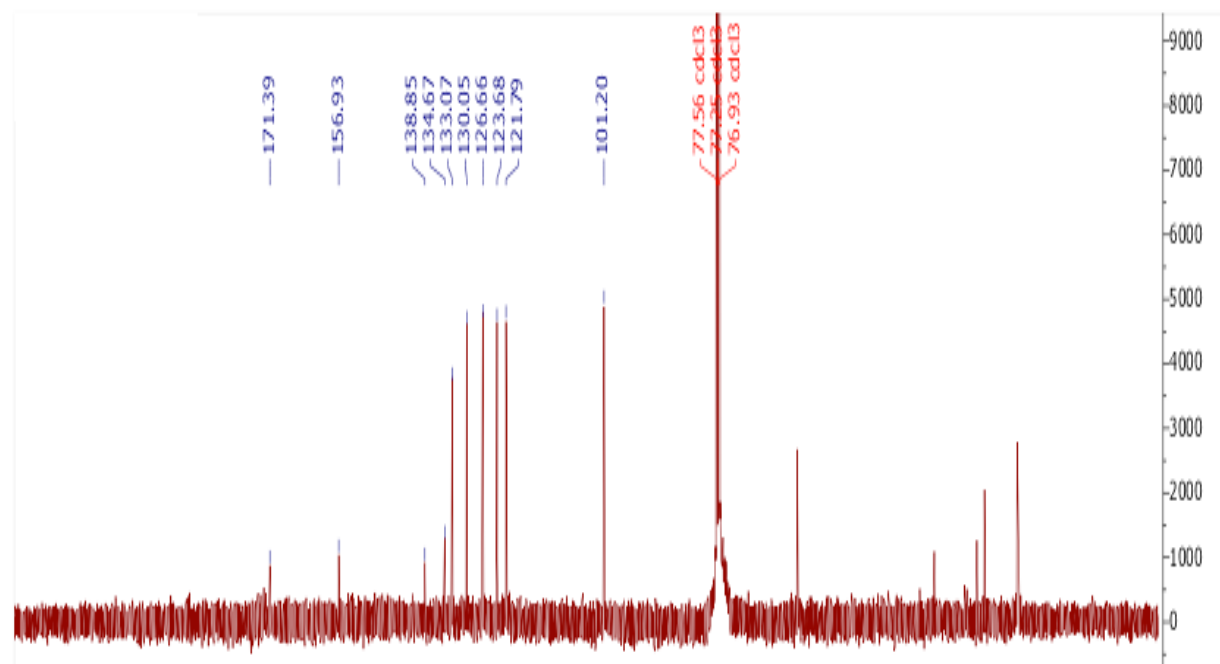


Figure 82, ¹³C NMR of SB50.

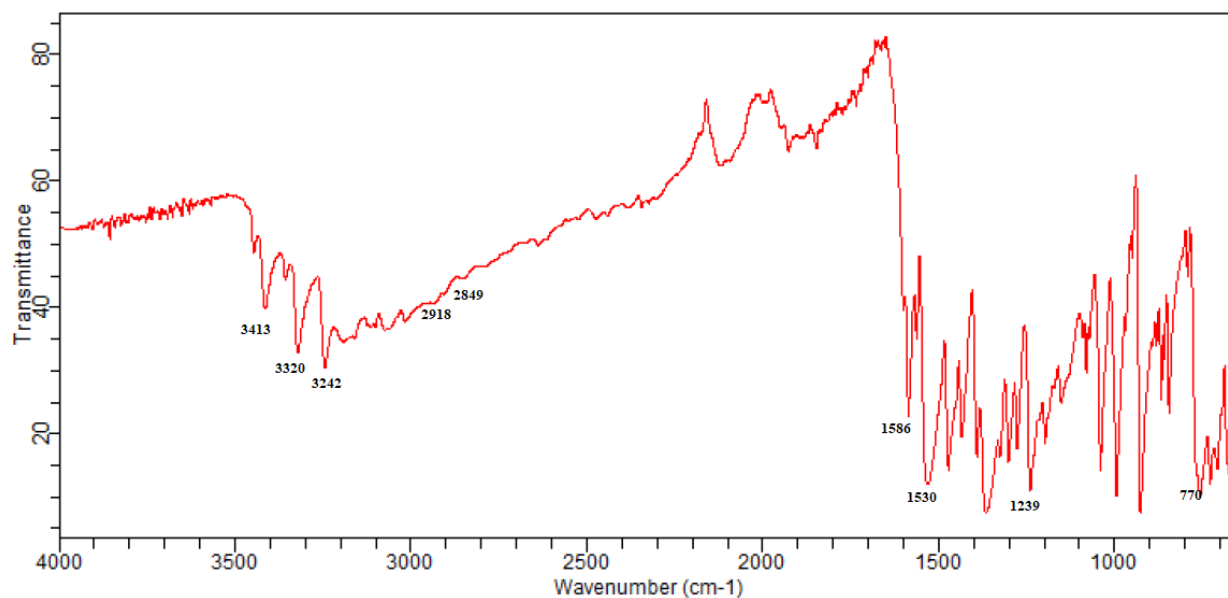


Figure 83, IR of SB50.

HRMS (+ESI-TOF) m/z: [M⁺] calcd for C₁₀H₉ClN₄S 252.72326; found 252.72328.

SB52

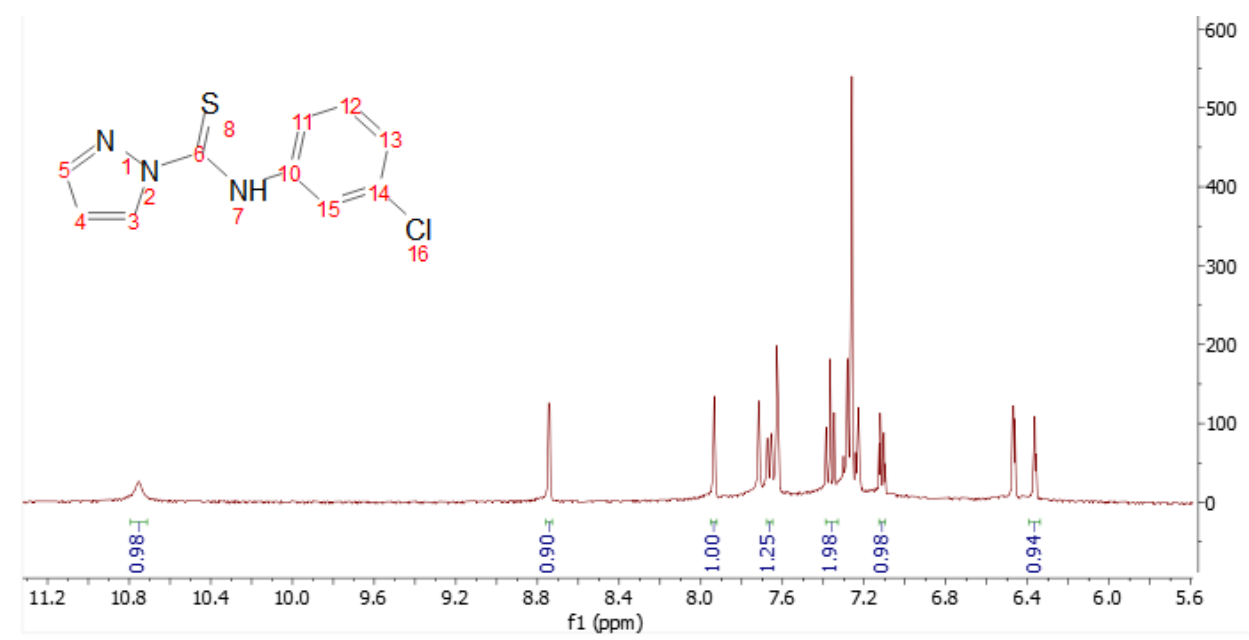


Figure 84, ¹H NMR of SB52.

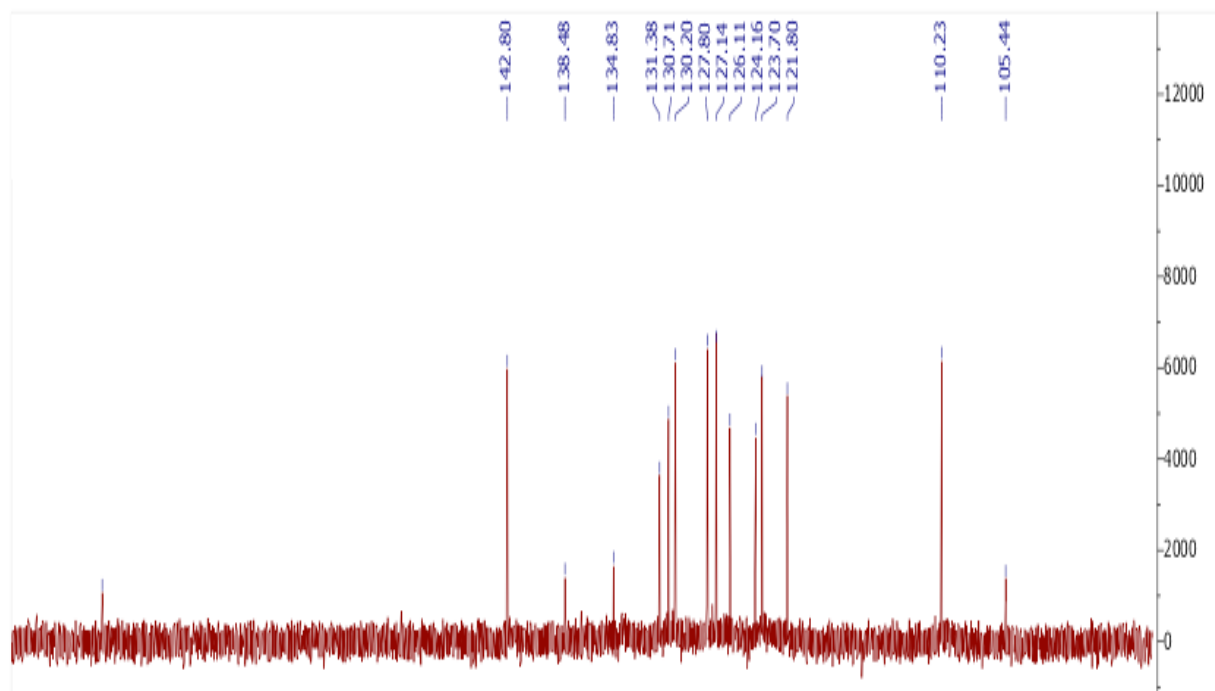


Figure 85, ^{13}C NMR of SB52.

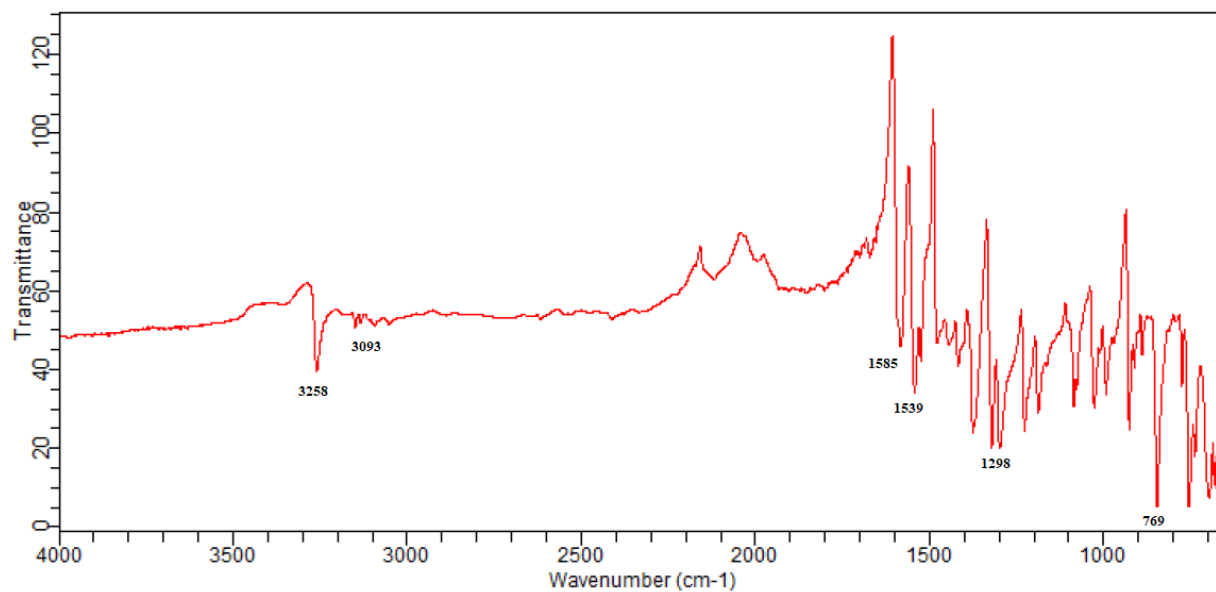


Figure 86, IR of SB52.

HRMS (+ESI-TOF) m/z : $[\text{M}^+]$ calcd for $\text{C}_{10}\text{H}_8\text{ClN}_3\text{S}$ 237.70862; found 237.70864.

SB53

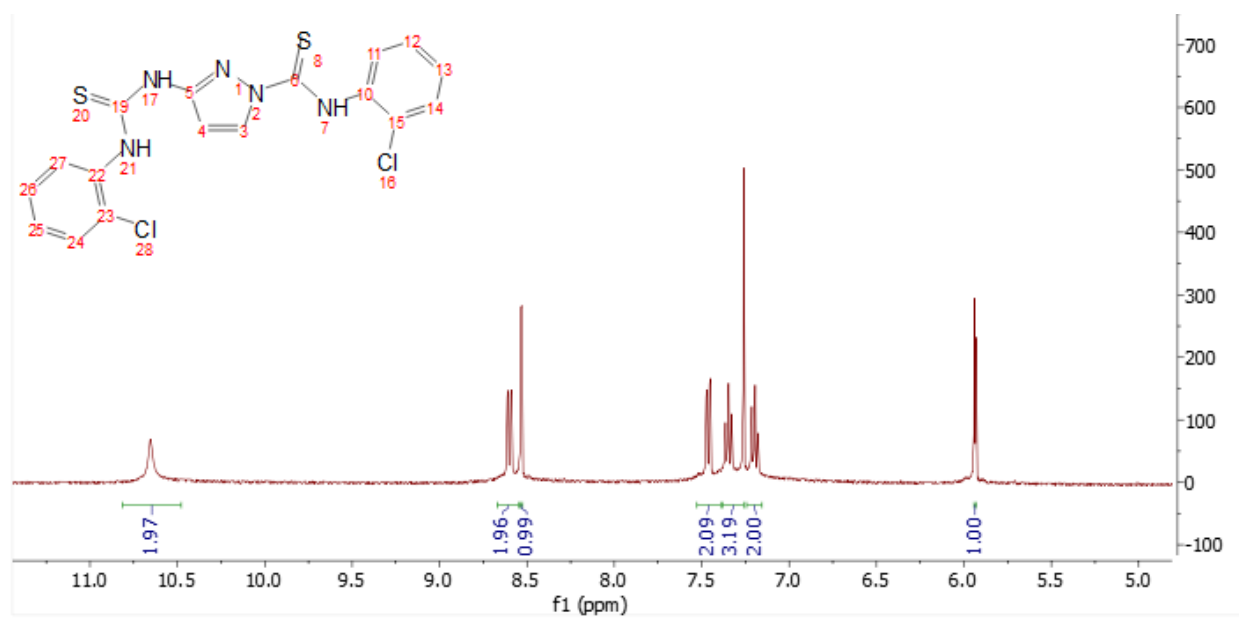


Figure 87, ^1H NMR of SB53.

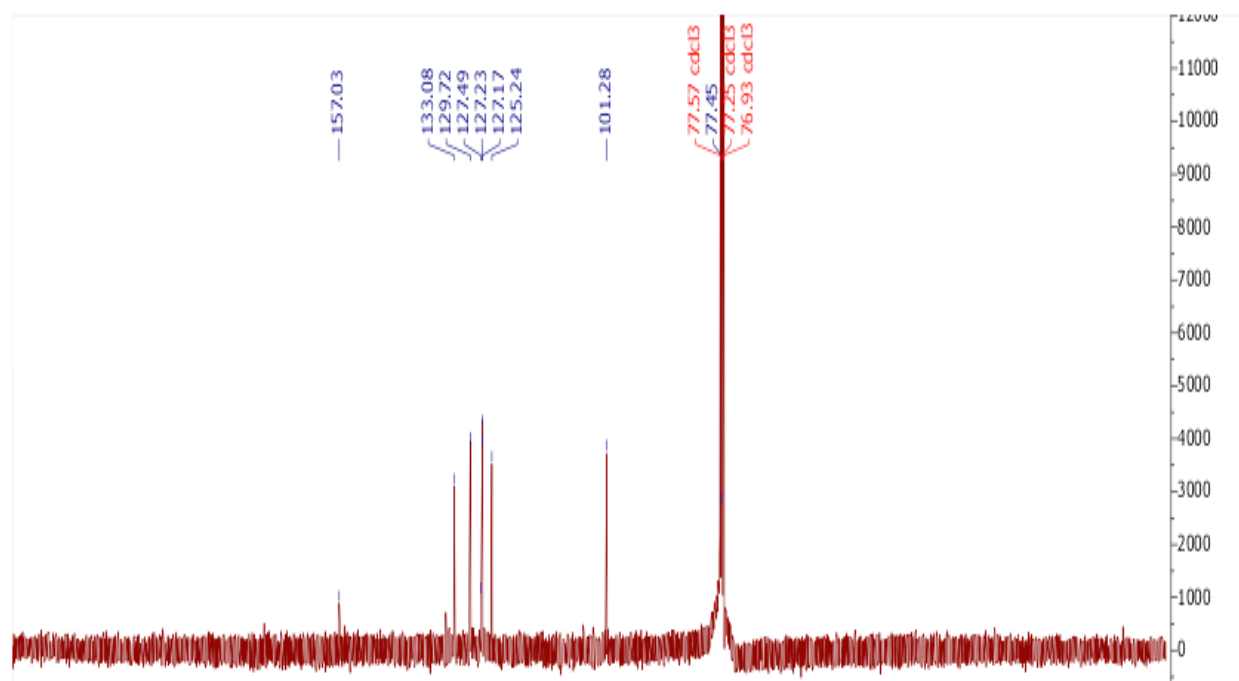


Figure 88, ^{13}C NMR of SB53.

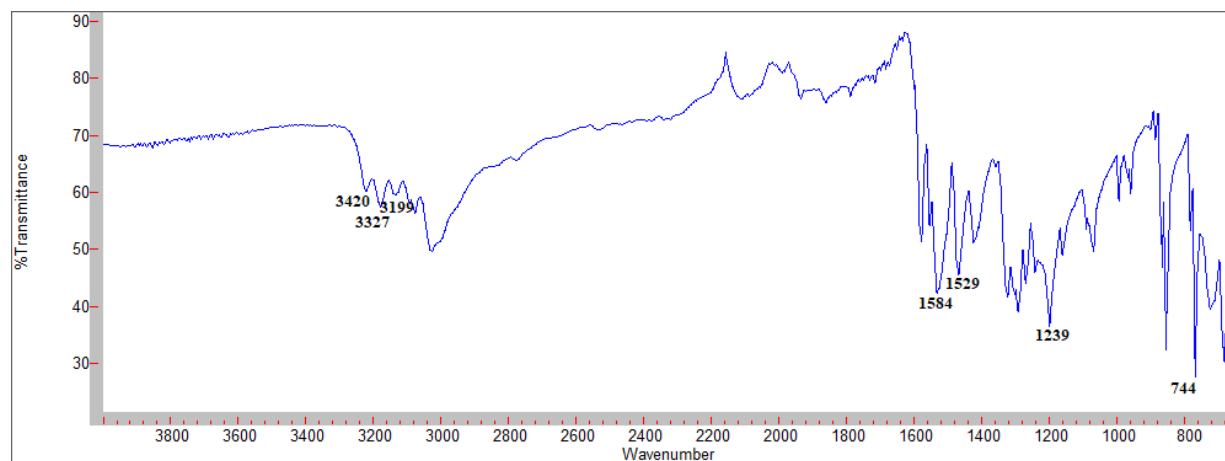


Figure 89, IR of SB53.

HRMS (+ESI-TOF) m/z: [M⁺] calcd for C₁₇H₁₃Cl₂N₅S₂ 420.99894; found 420.99827.

SB54

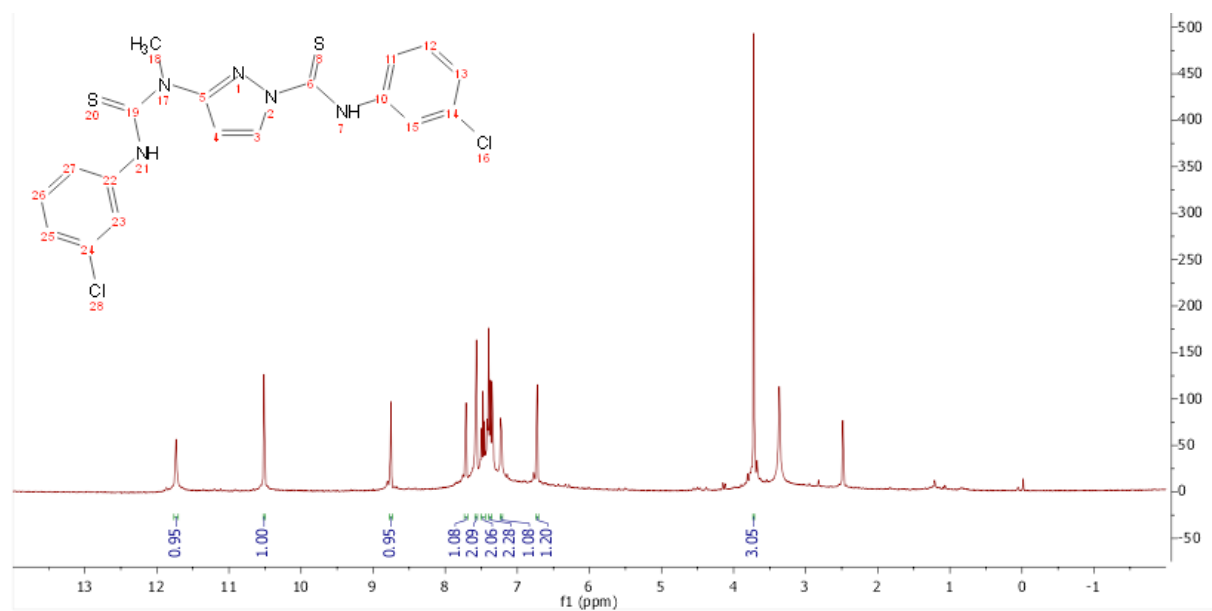


Figure 90, ¹H NMR of SB54.

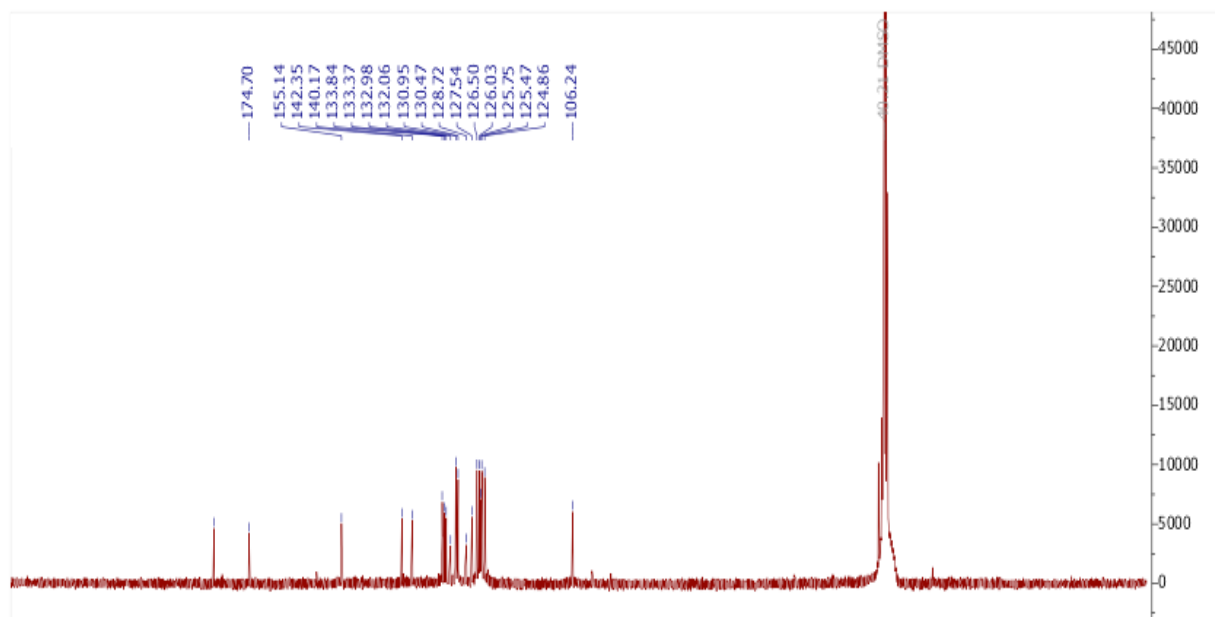


Figure 91, ¹³C NMR of SB54.

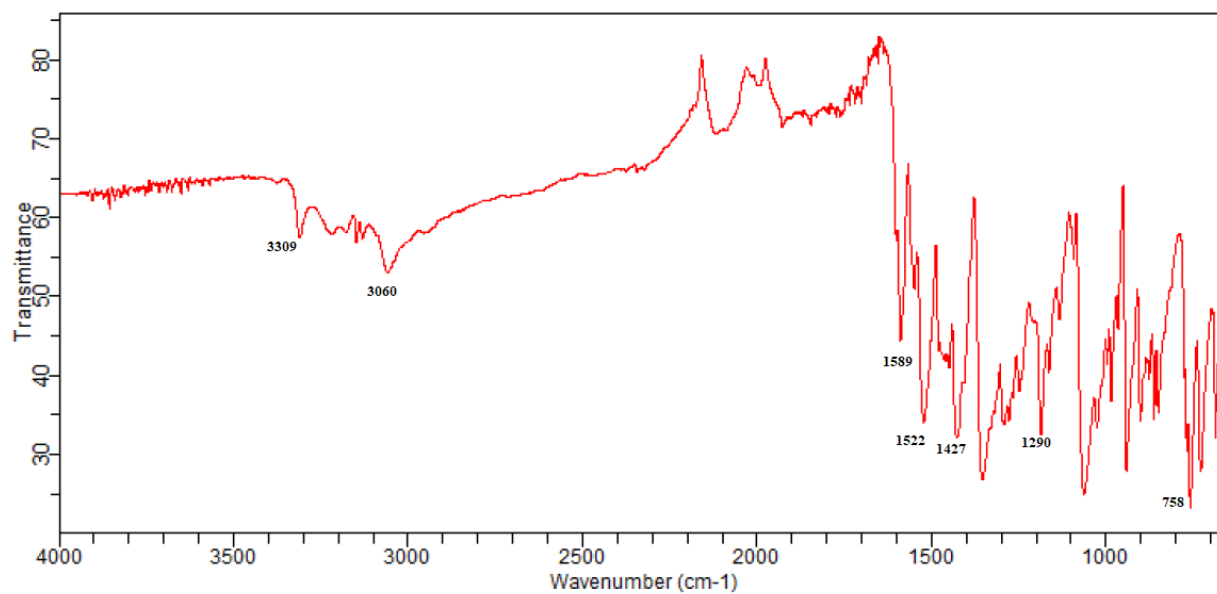


Figure 92, IR of SB54.

HRMS (+ESI-TOF) m/z: [M⁺] calcd for C₁₈H₁₅Cl₂N₅S₂ 435.01459; found 435.01439.

SB55

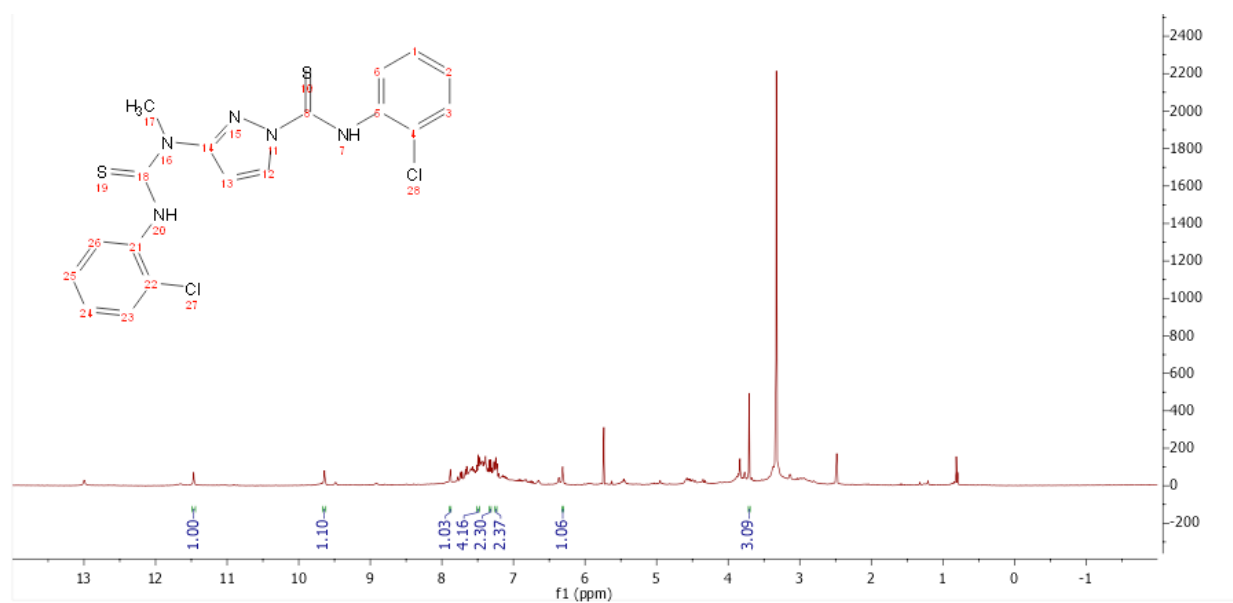


Figure 93, ¹H NMR of SB55.

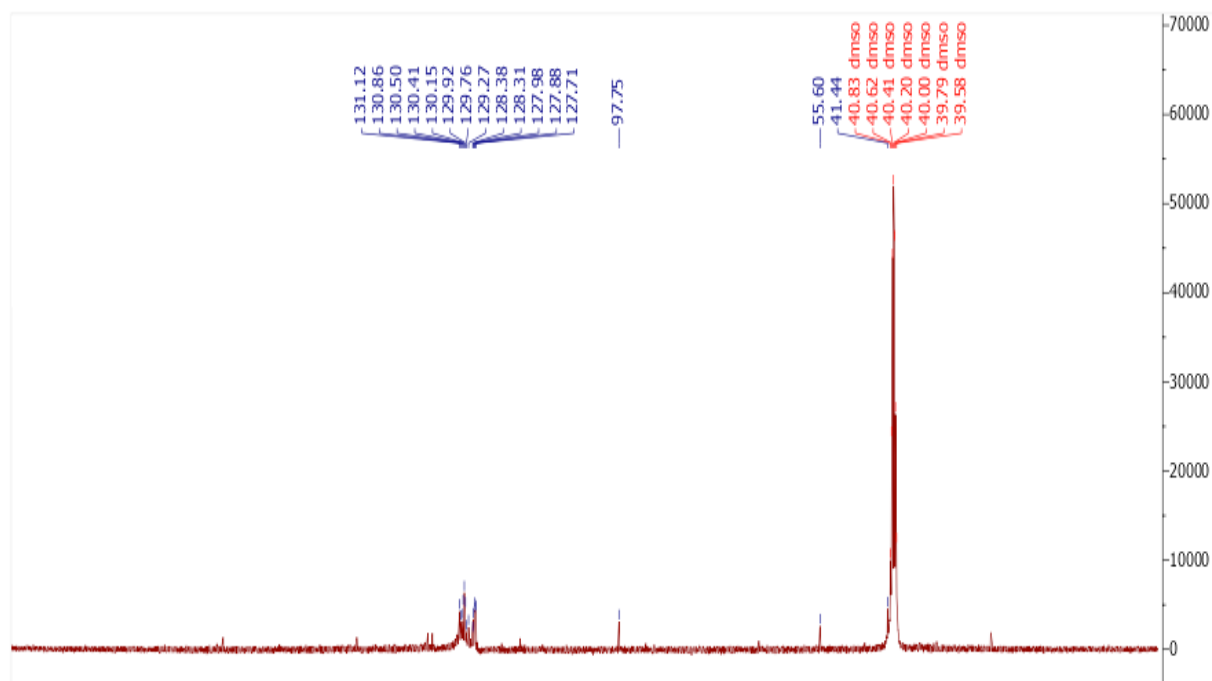


Figure 94, ¹³C NMR of SB55.

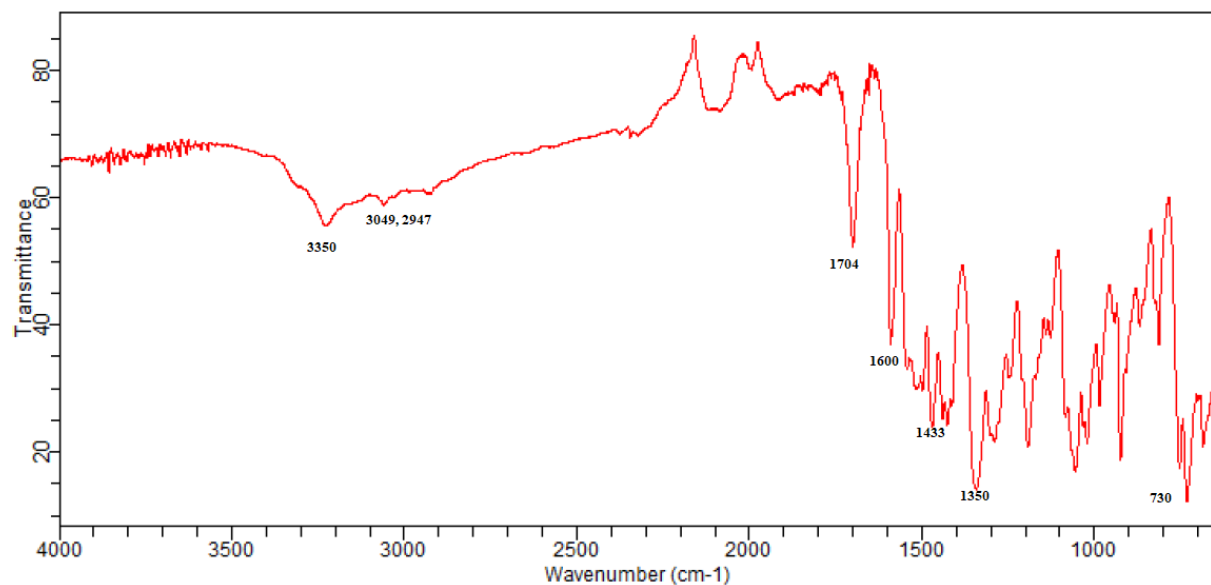


Figure 95, IR of SB55.

HRMS (+ESI-TOF) m/z: [M⁺] calcd for C₁₈H₁₅Cl₂N₅S₂ 435.01459; found 435.01462.

SB56

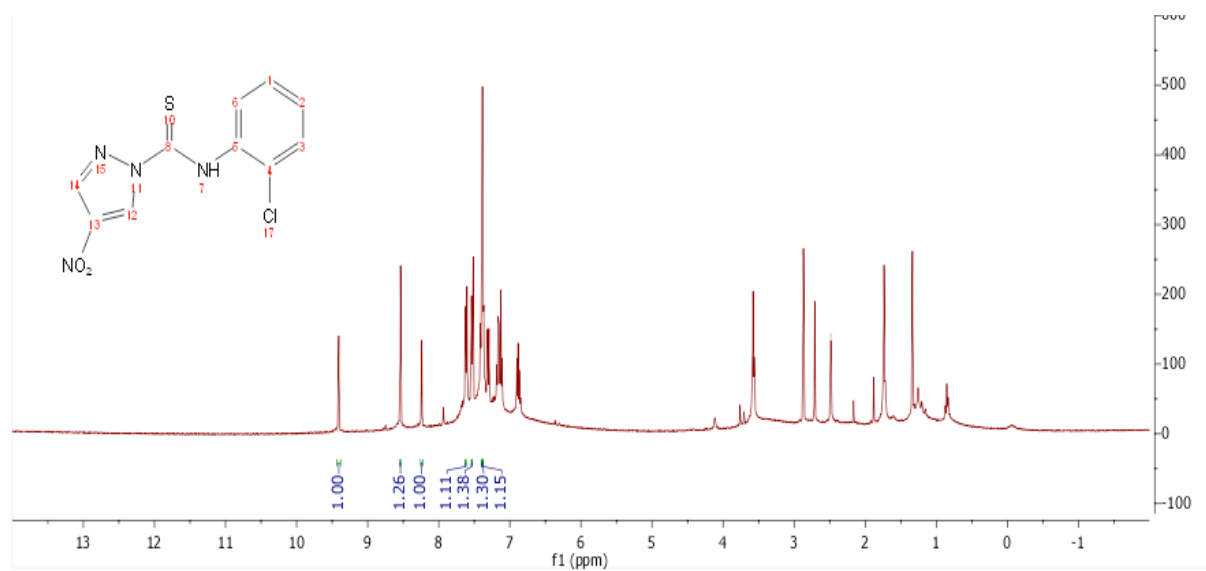


Figure 96, ¹H NMR of SB56.

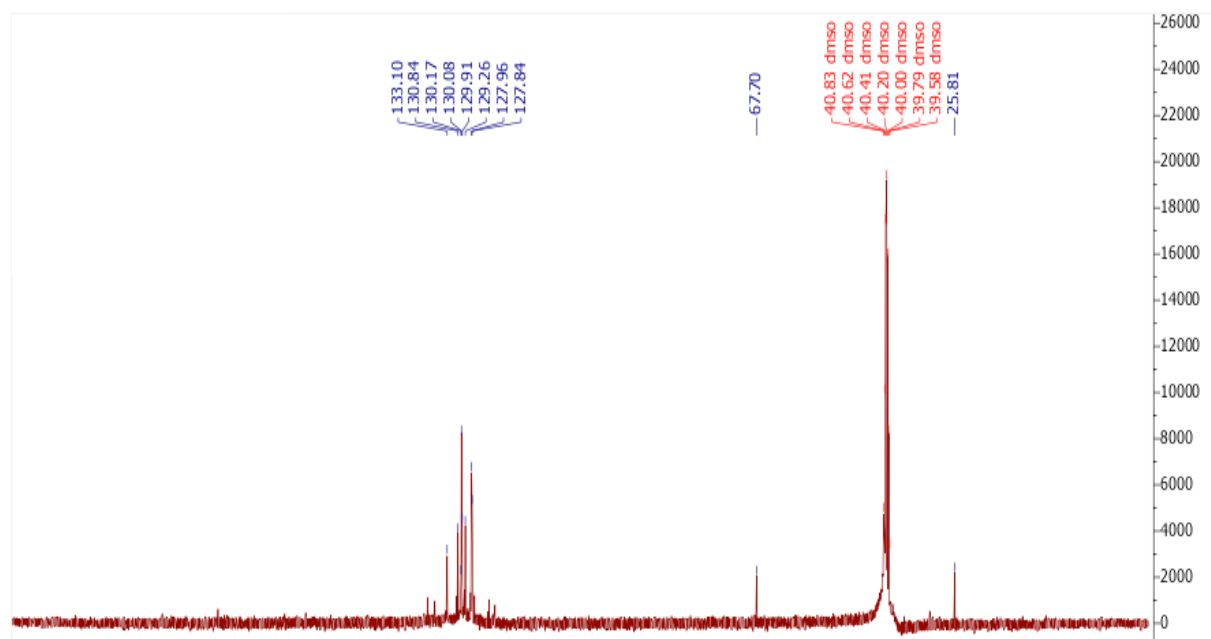


Figure 97, ^{13}C of SB56.

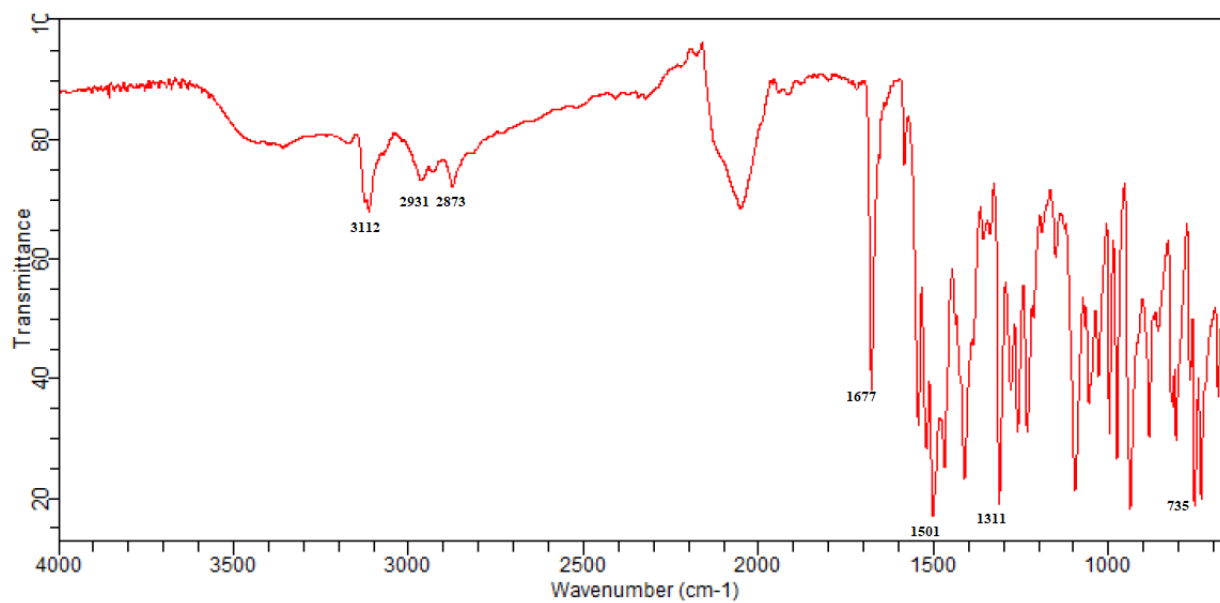


Figure 98, IR of SB56.

UPLC-MS $[\text{M}+1]$ calcd for $\text{C}_{10}\text{H}_7\text{ClN}_4\text{O}_2\text{S}$ 283.71; found 283.10.

SB57

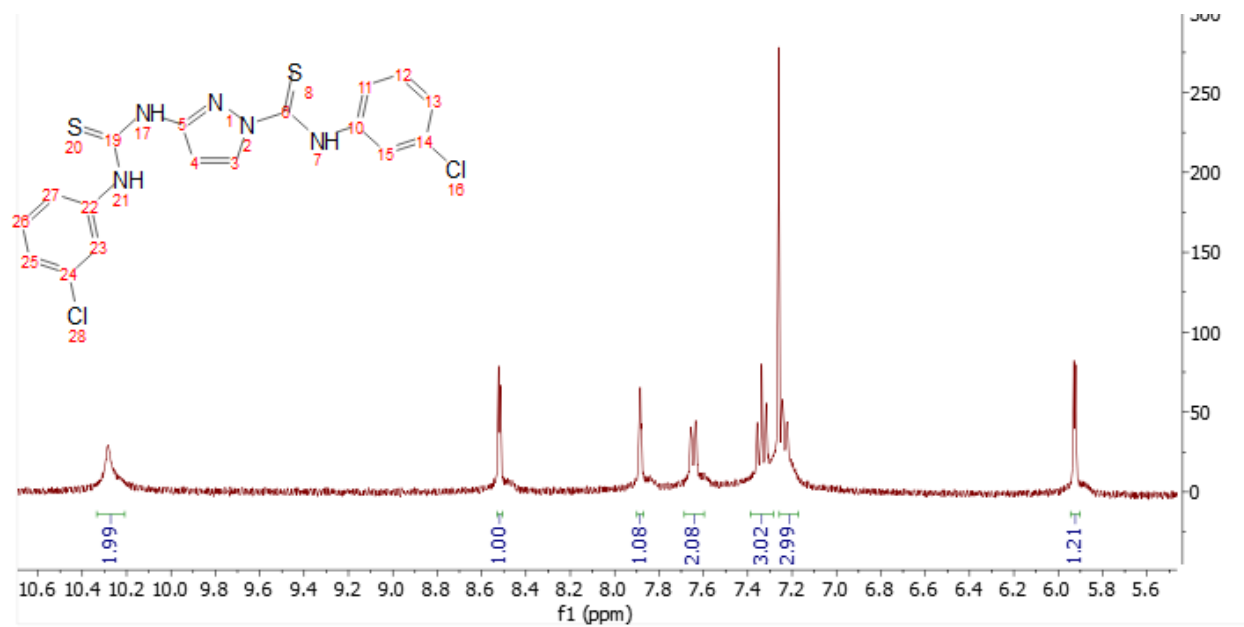


Figure 99, ^1H NMR of SB57.

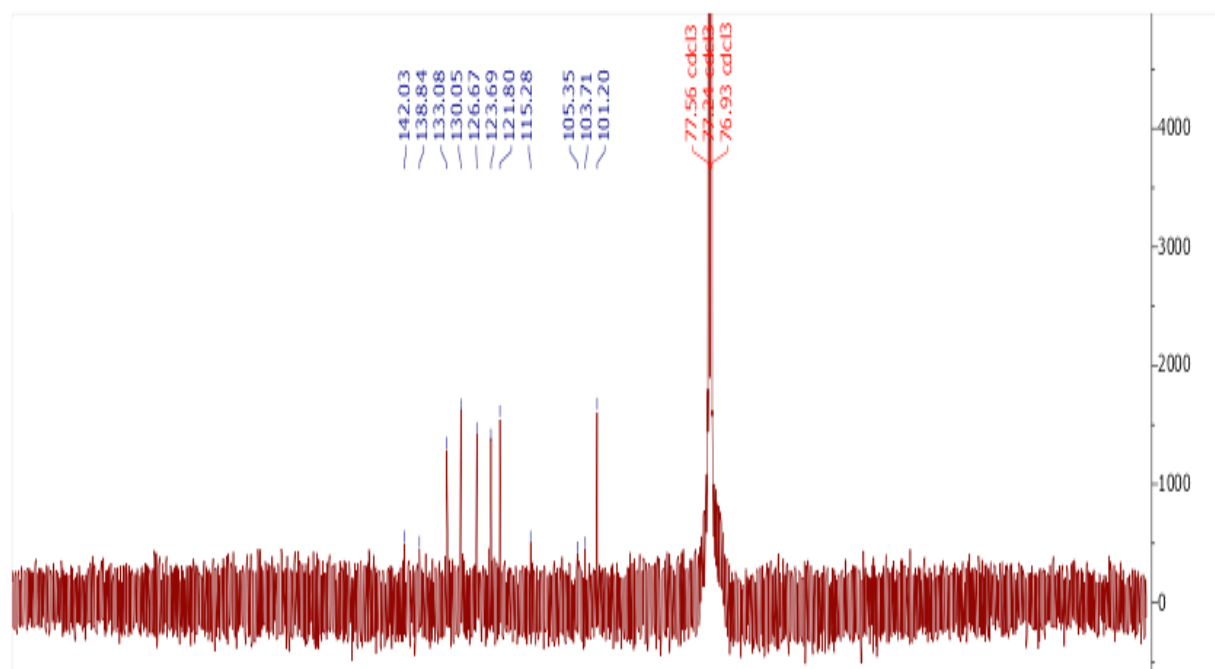


Figure 100, ^{13}C of SB57.

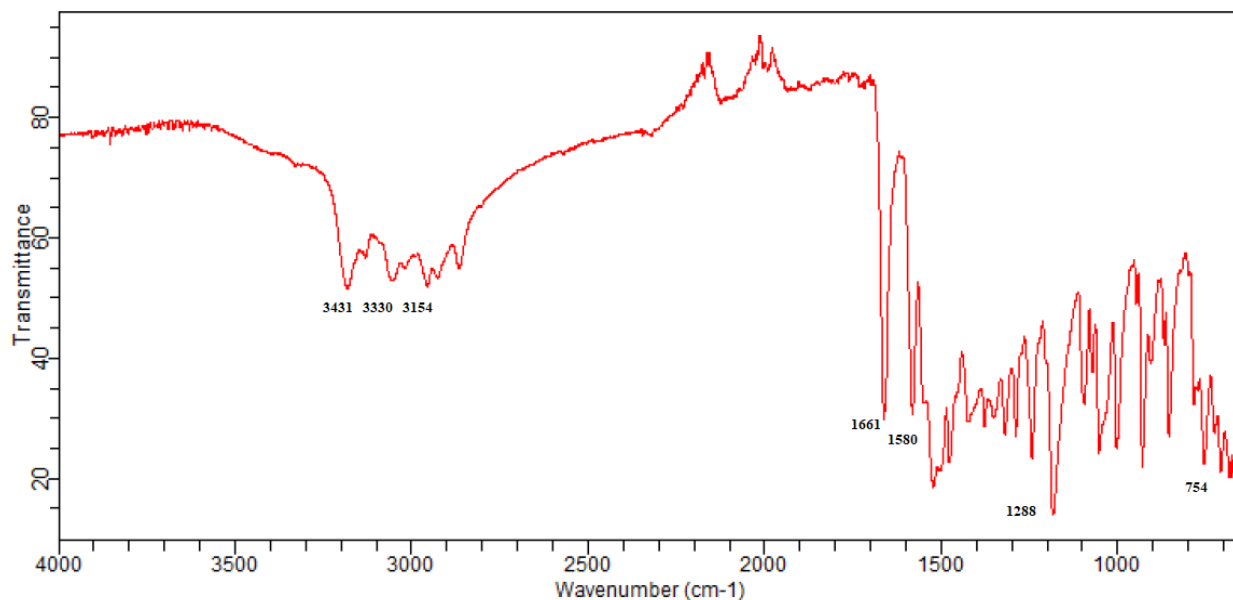


Figure 101, IR of SB57.

HRMS (+ESI-TOF) m/z: [M⁺] calcd for C₁₇H₁₃Cl₂N₅S₂ 420.99894; found 420.9989.

SB59

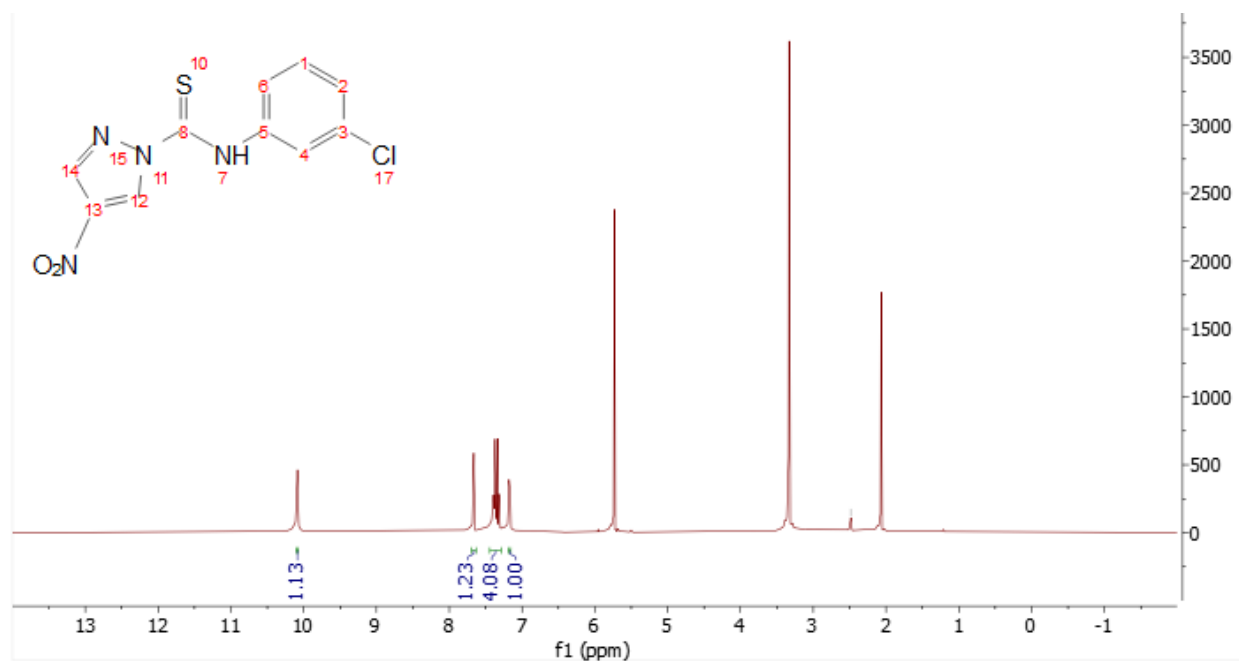


Figure 102, ¹H NMR of SB59.

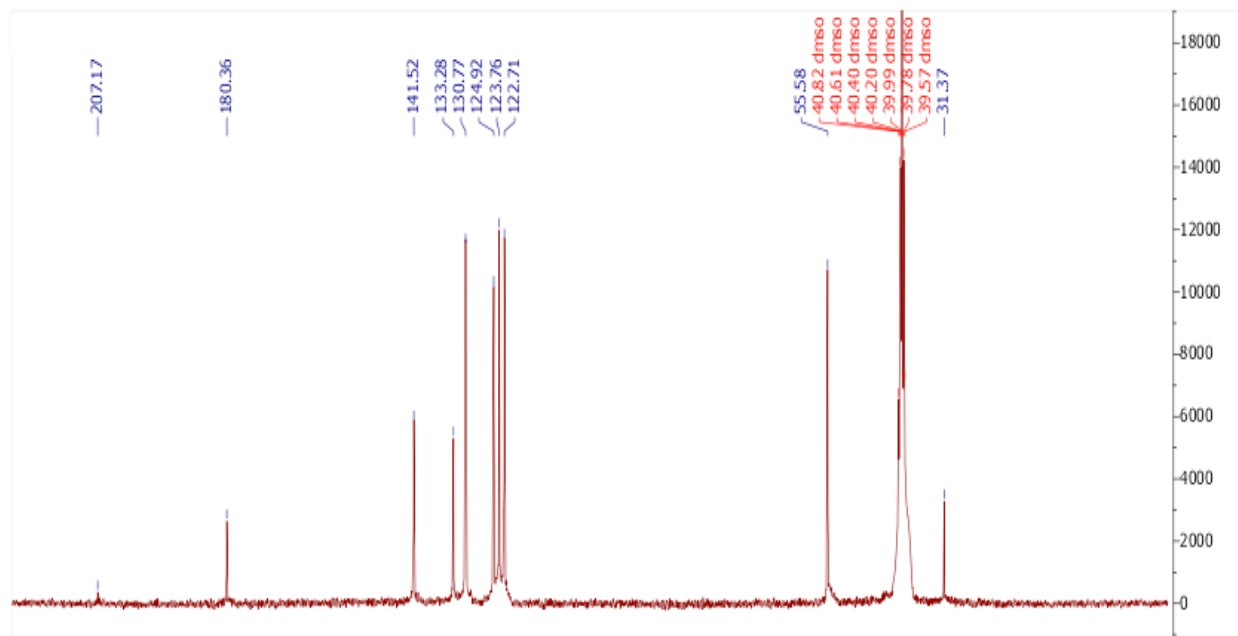


Figure 103, ^{13}C NMR of SB59.

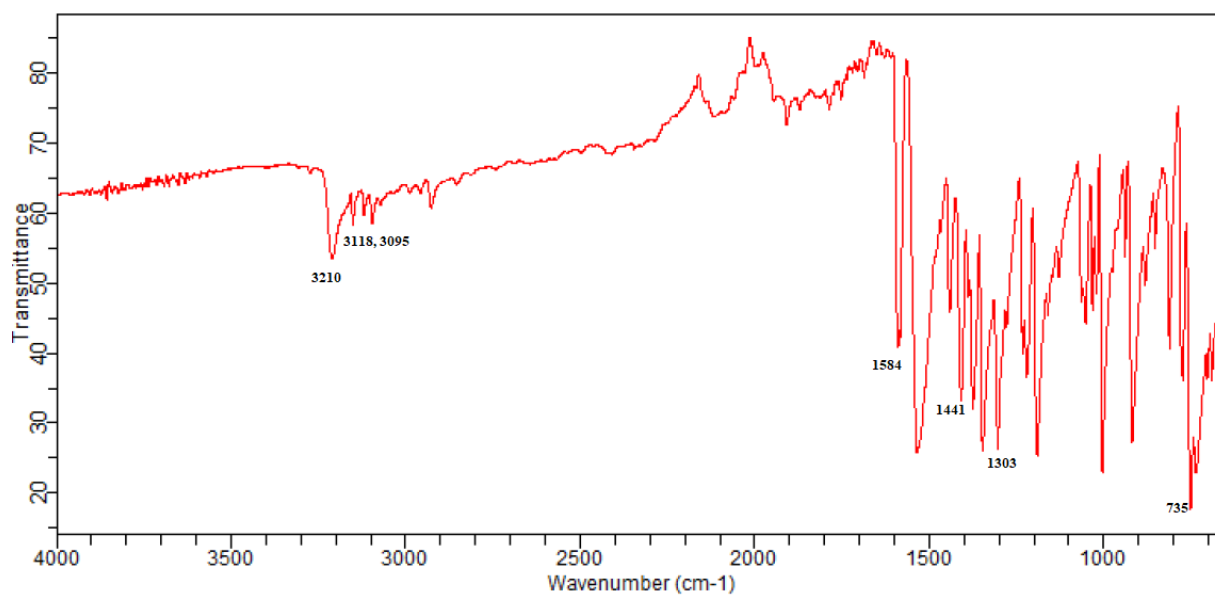


Figure 104, IR of SB59.

UPLC-MS $[M+1]$ calcd for $\text{C}_{10}\text{H}_7\text{ClN}_4\text{O}_2\text{S}$ 283.71; found 283.23.

SB60

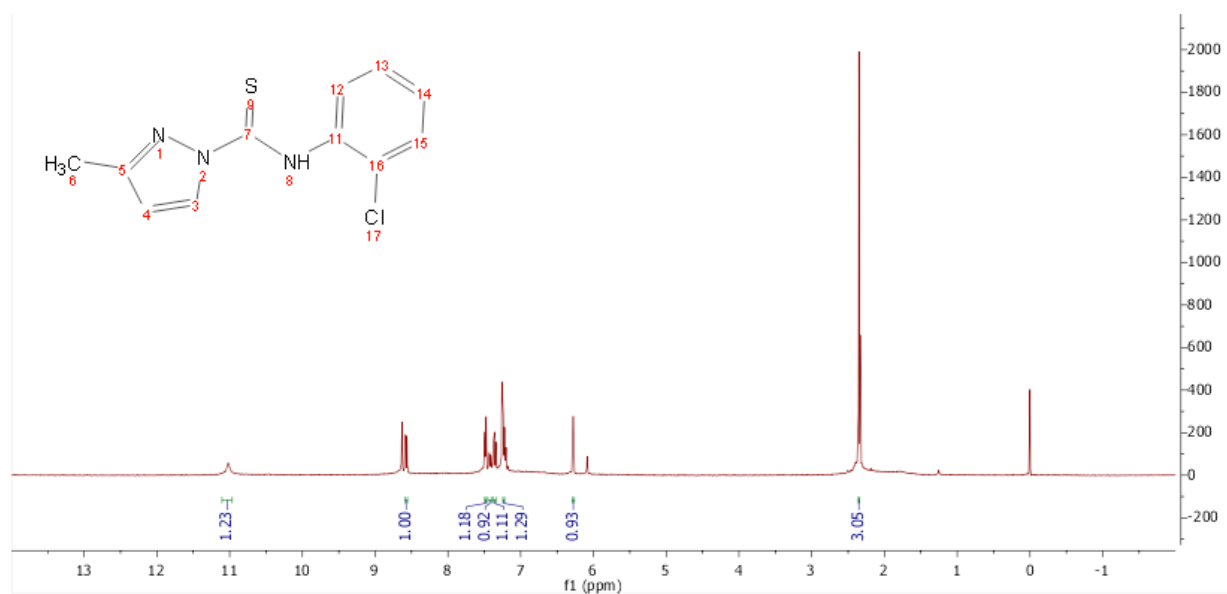


Figure 105, ¹H NMR of SB60.

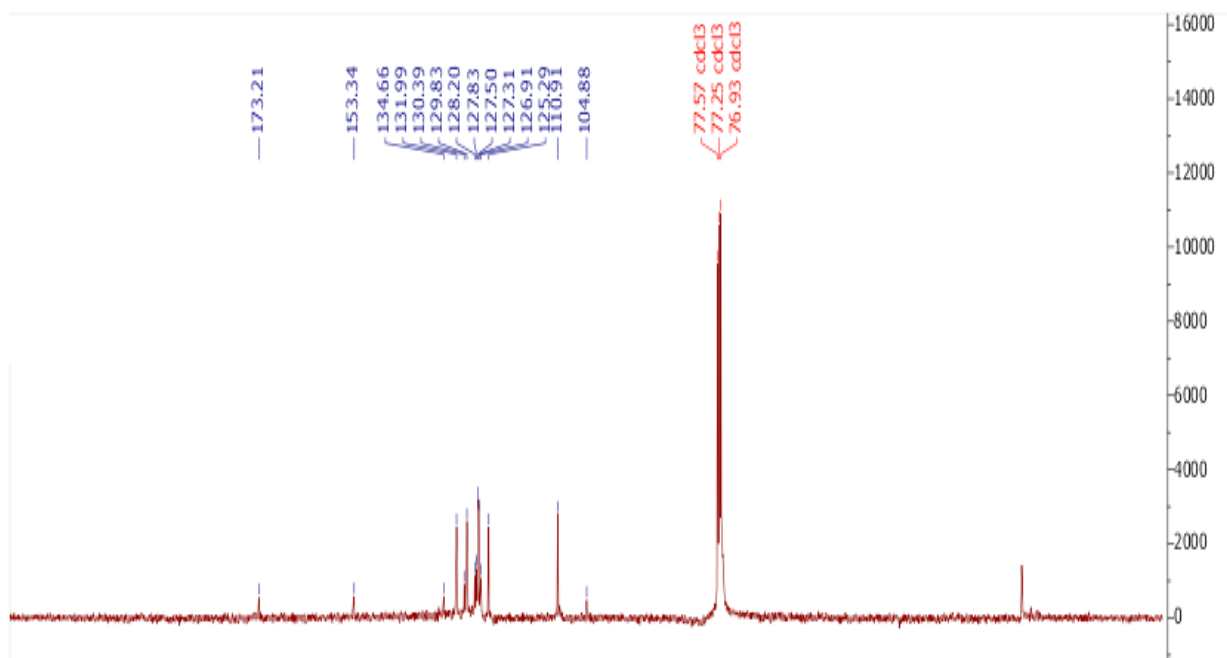


Figure 106, ¹³C NMR of SB60.

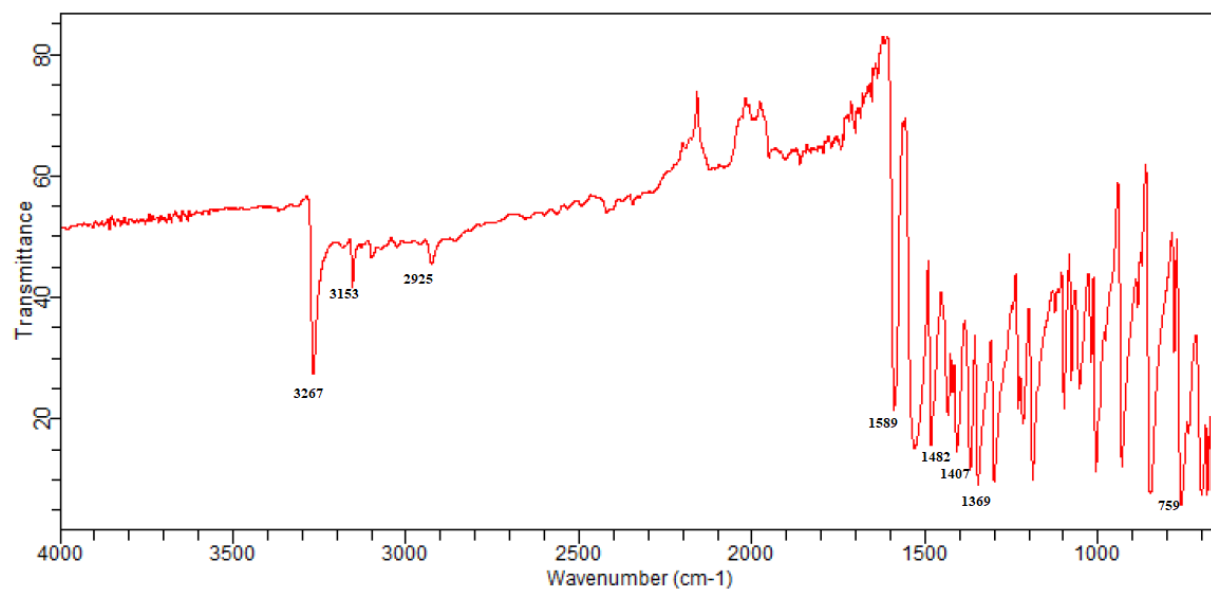


Figure 107, IR of SB60.

HRMS (+ESI-TOF) m/z : $[M]^+$ calcd for $C_{11}H_{10}ClN_3S$ 251.02840; found 251.02838.

SB61

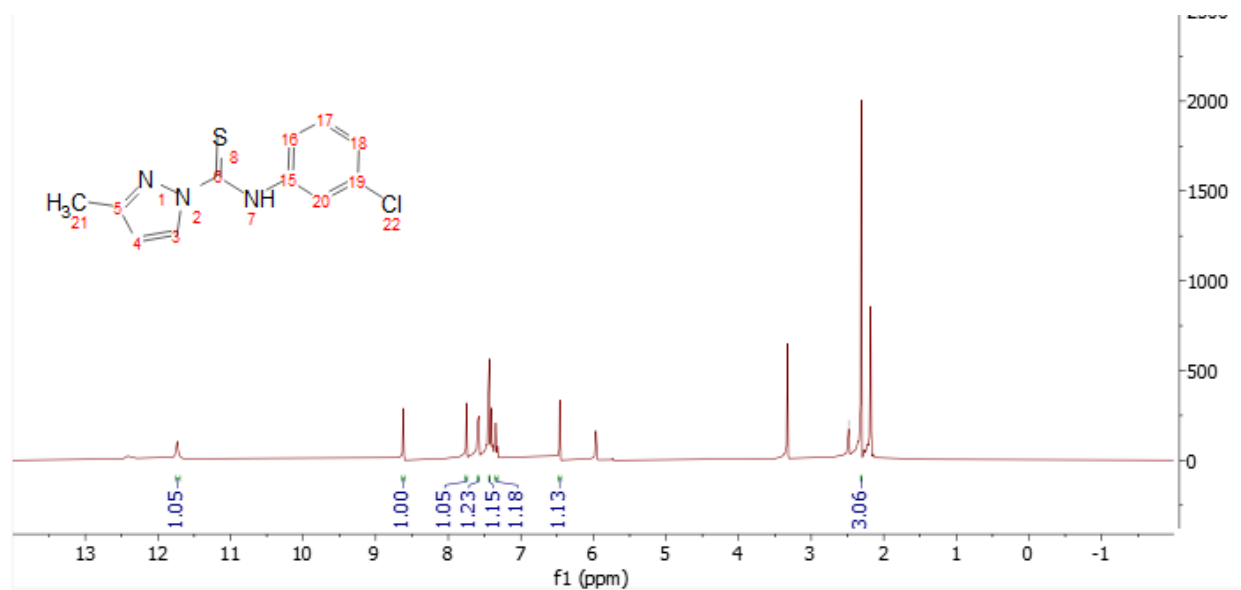


Figure 108, ¹H NMR of SB61.

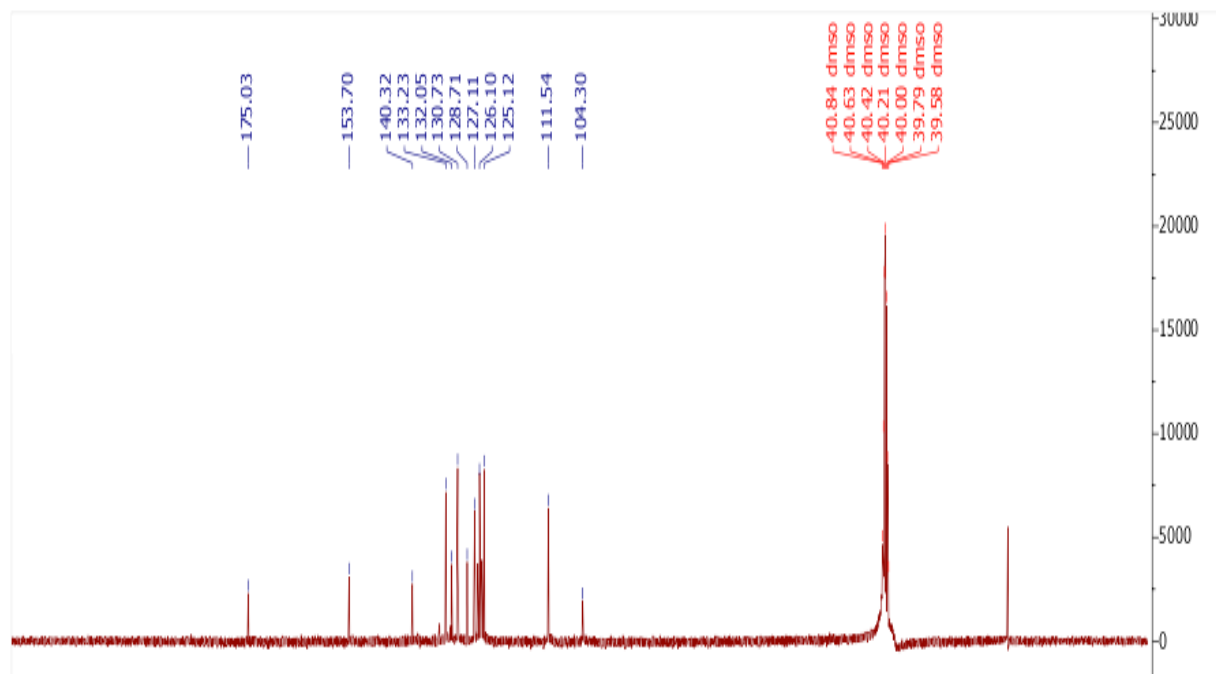


Figure 109, ^{13}C NMR of SB61.

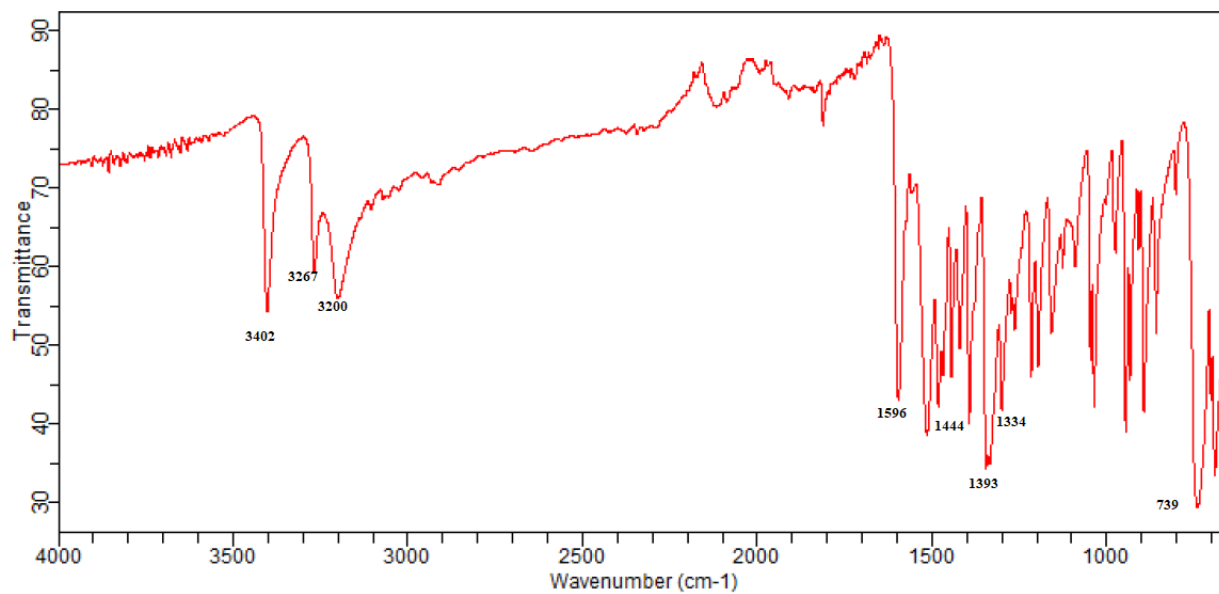


Figure 110, IR of SB61

HRMS (+ESI-TOF) m/z: [M⁺] calcd for C₁₁H₁₀ClN₃S 251.02840; found 251.02842.

SB62

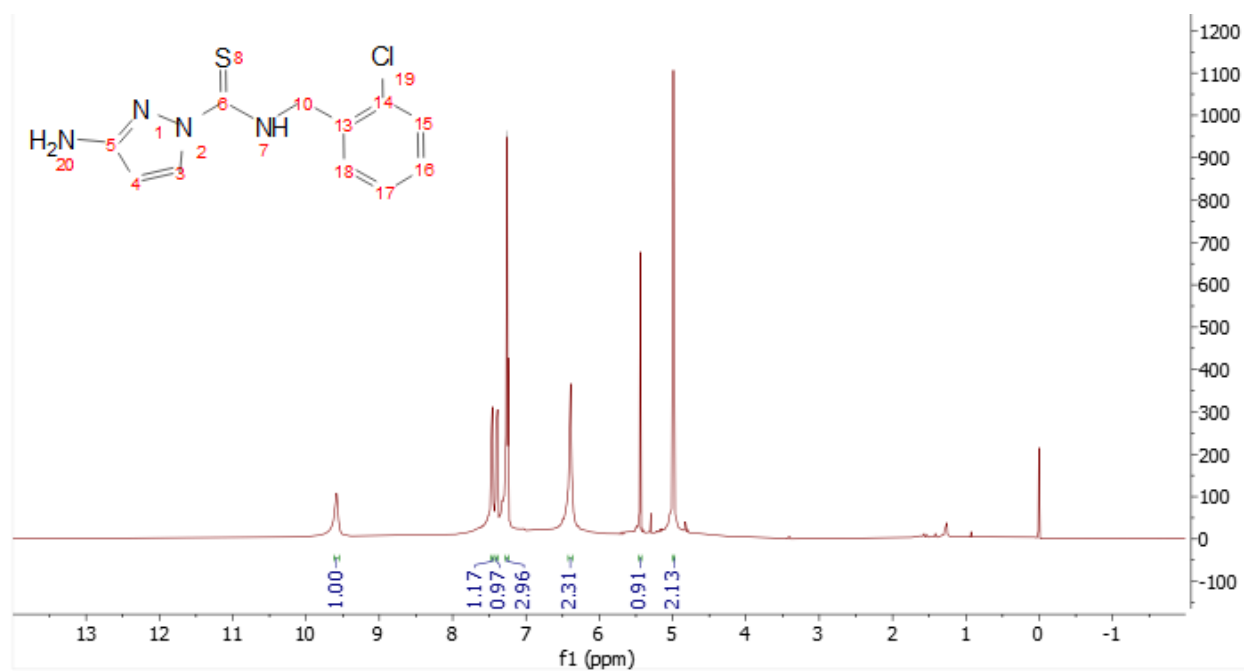


Figure 111, ^1H NMR of SB62.

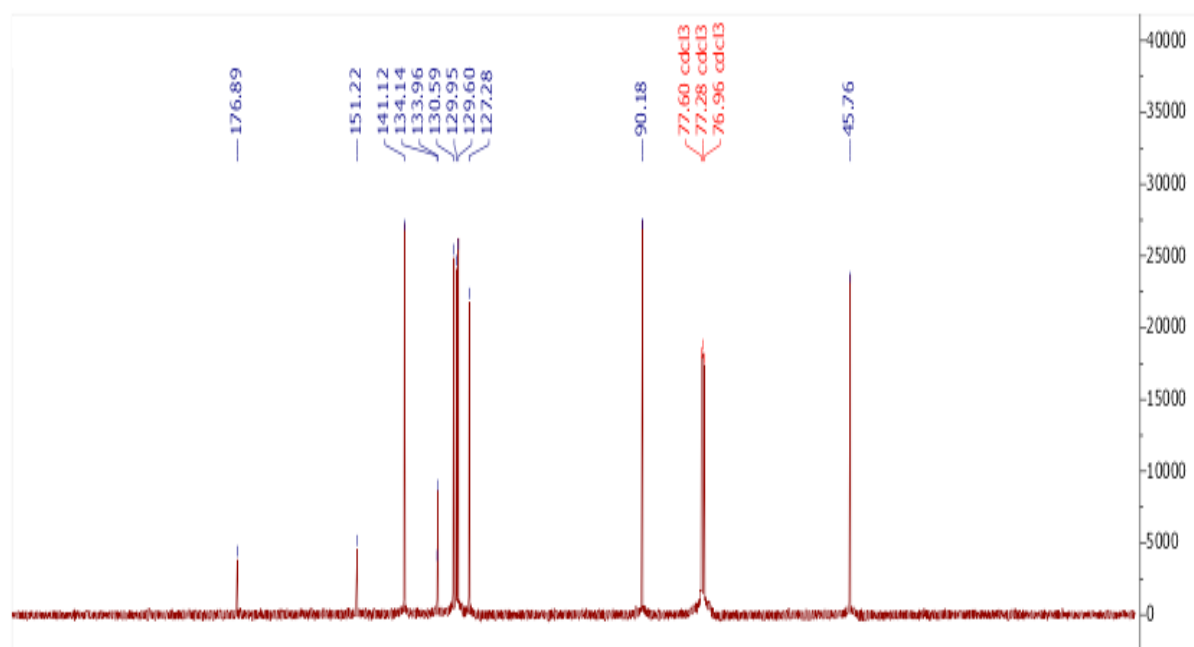


Figure 112, ^{13}C NMR of SB62.

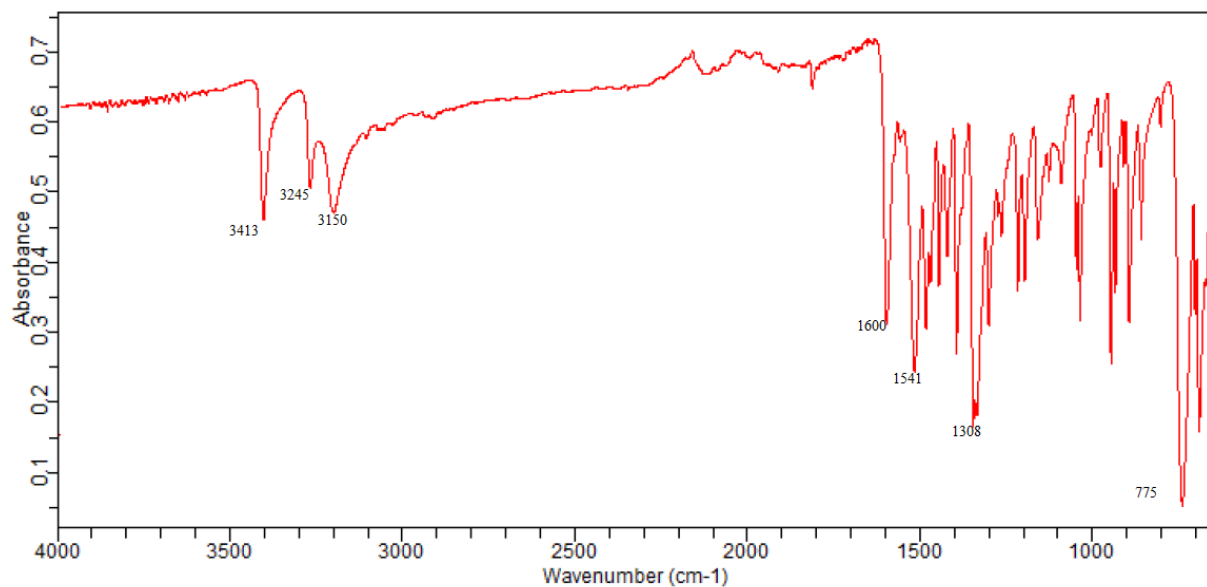


Figure 113, IR of SB62.

HRMS (+ESI-TOF) m/z: [M⁺] calcd for C₁₁H₁₁ClN₄S 266.03929; found 266.03929.

SB64

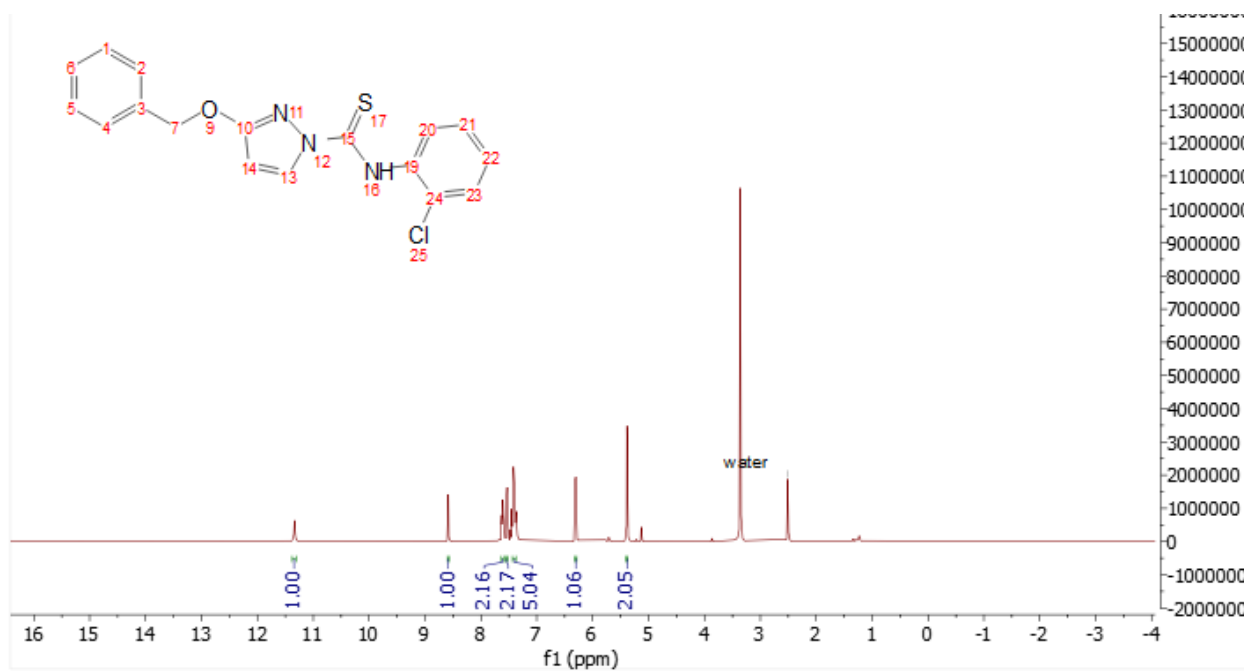


Figure 114, ¹H NMR of SB64.

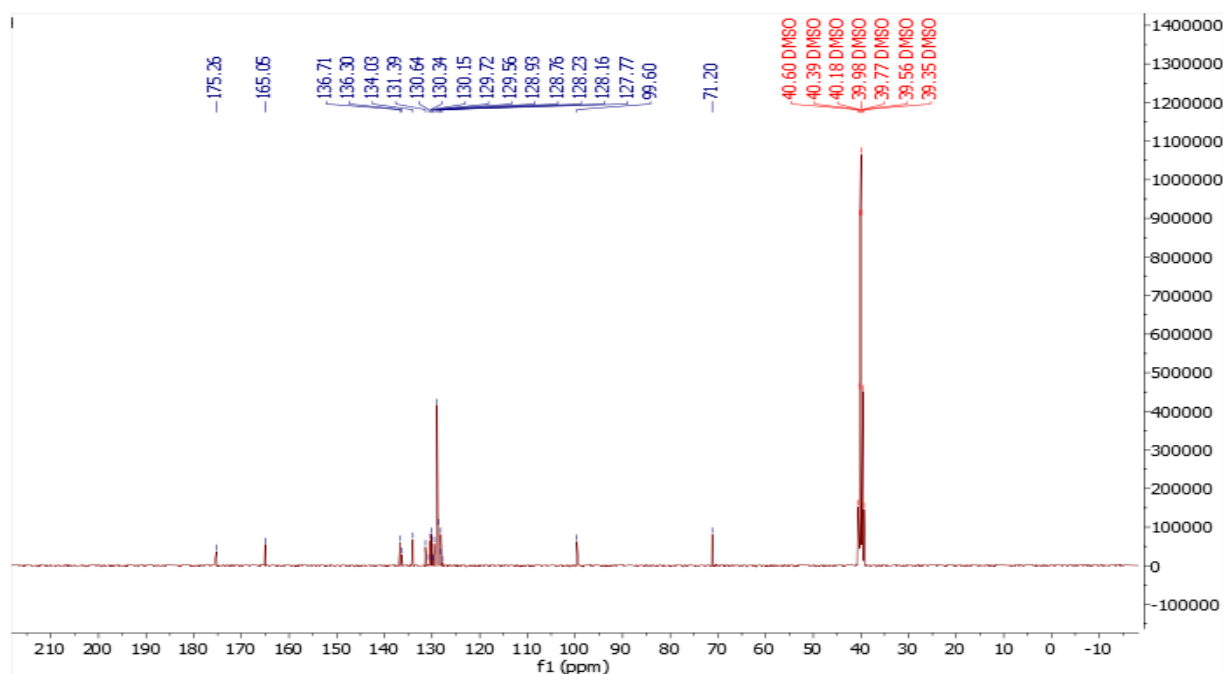


Figure 115, ^{13}C NMR of SB64.

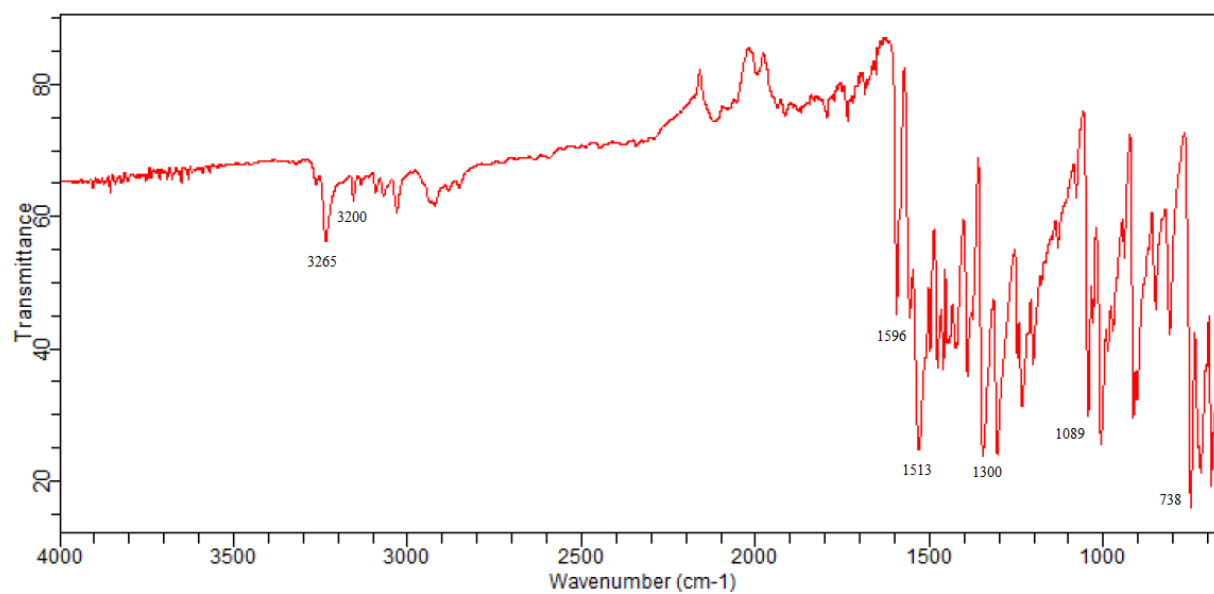


Figure 116, IR of SB64.

HRMS (+ESI-TOF) m/z : $[M]^+$ calcd for $\text{C}_{17}\text{H}_{14}\text{ClN}_3\text{OS}$ 343.05461; found 343.05458.

SB65

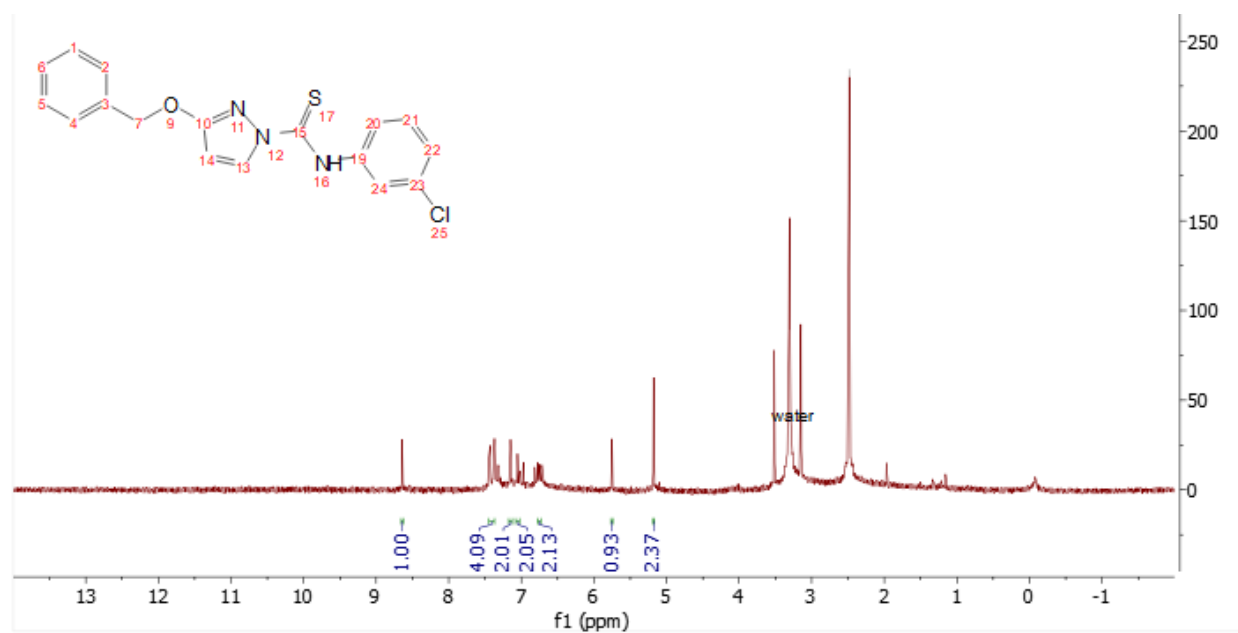


Figure 117, ^1H NMR of SB65.

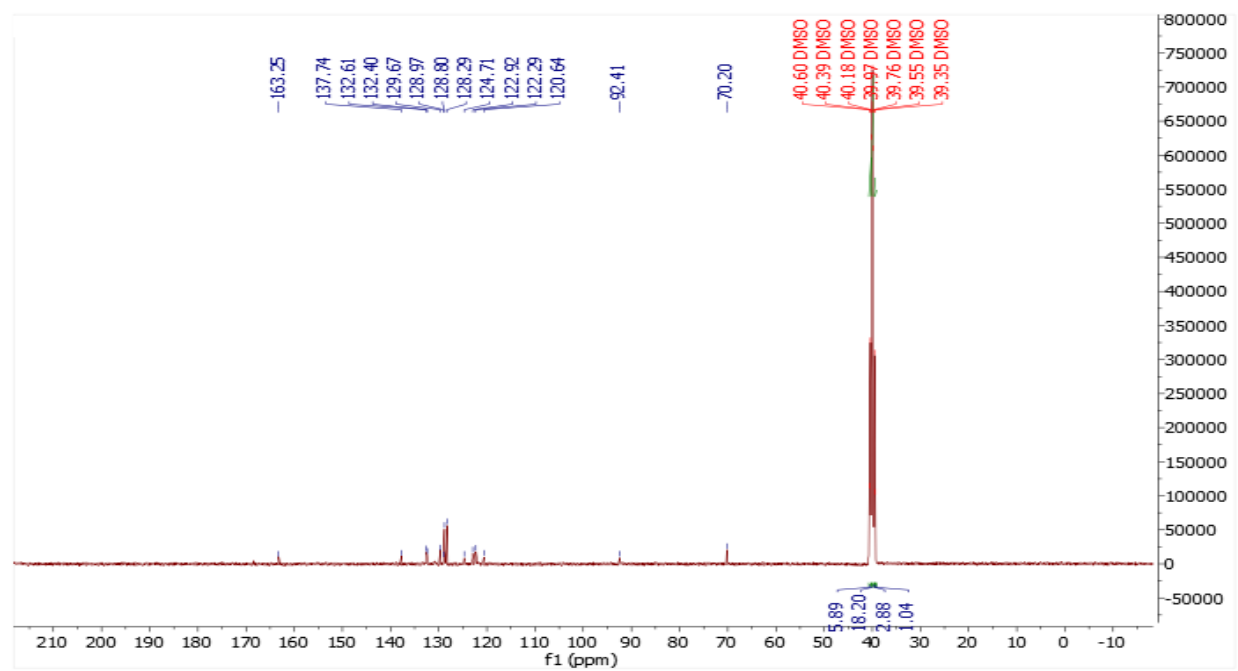


Figure 118, ^{13}C NMR of SB65.

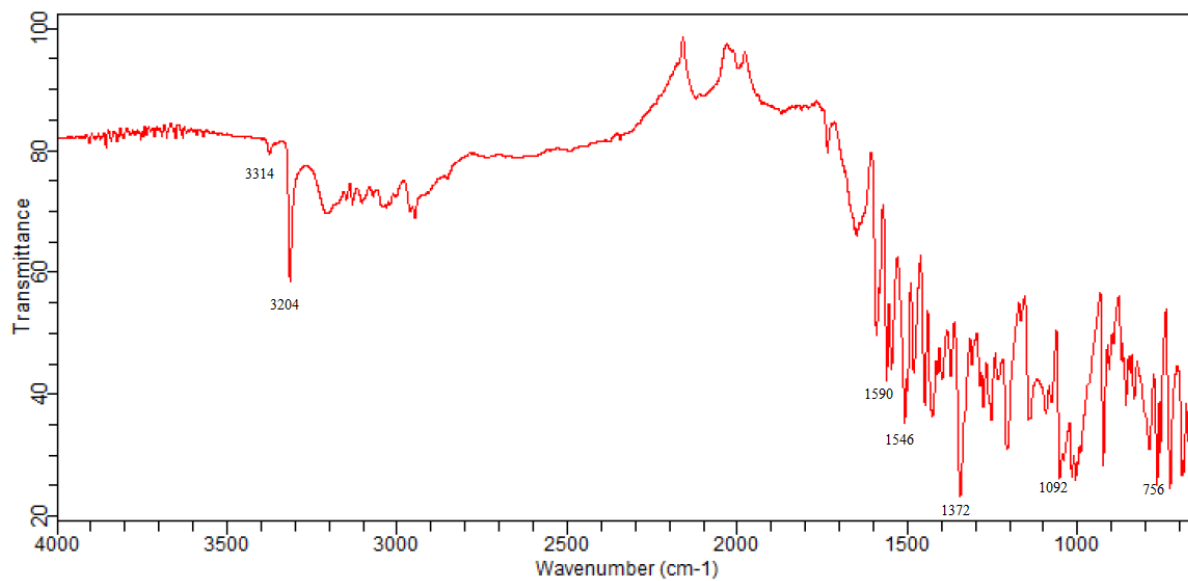


Figure 119, IR of SB65.

HRMS (+ESI-TOF) m/z: [M⁺] calcd for C₁₇H₁₄ClN₃OS 343.05461; found 343.05462.

SB66

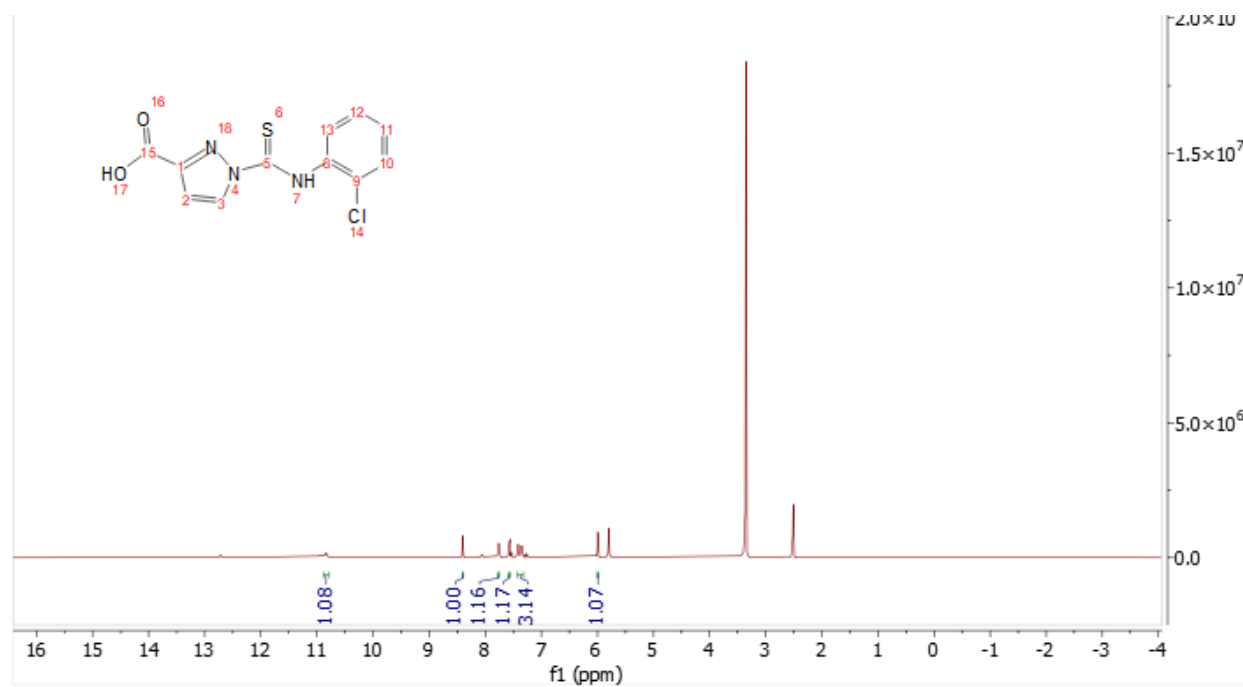


Figure 120, ¹H NMR of SB66.

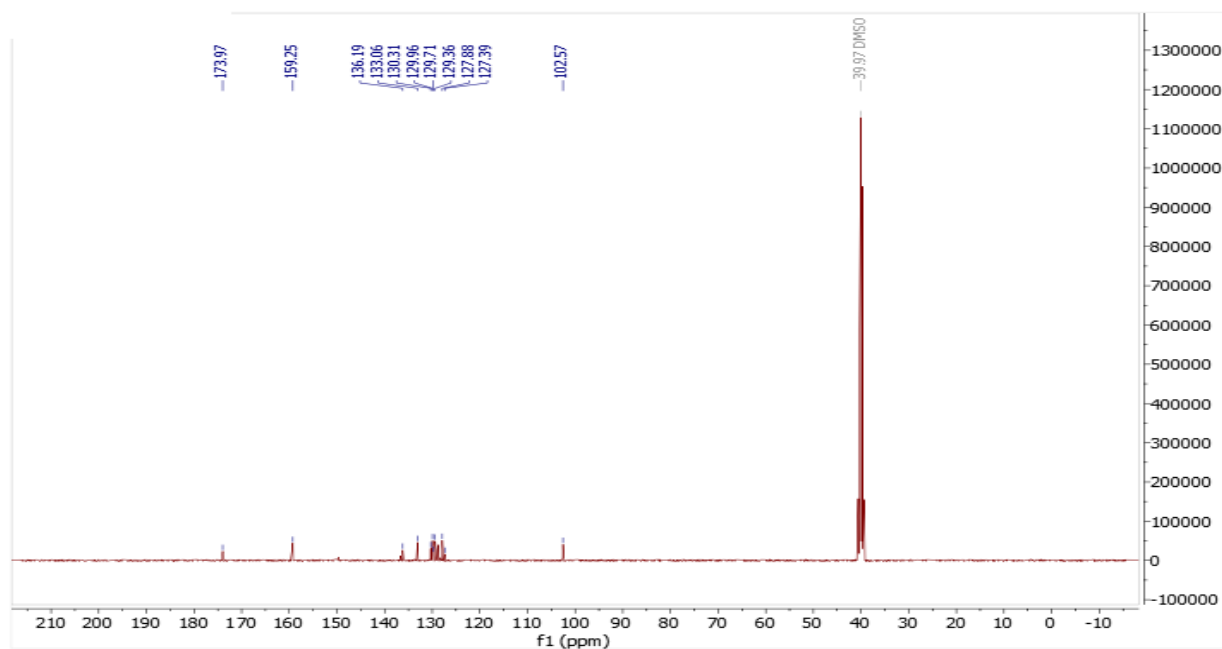


Figure 121, ¹³C NMR of SB66.

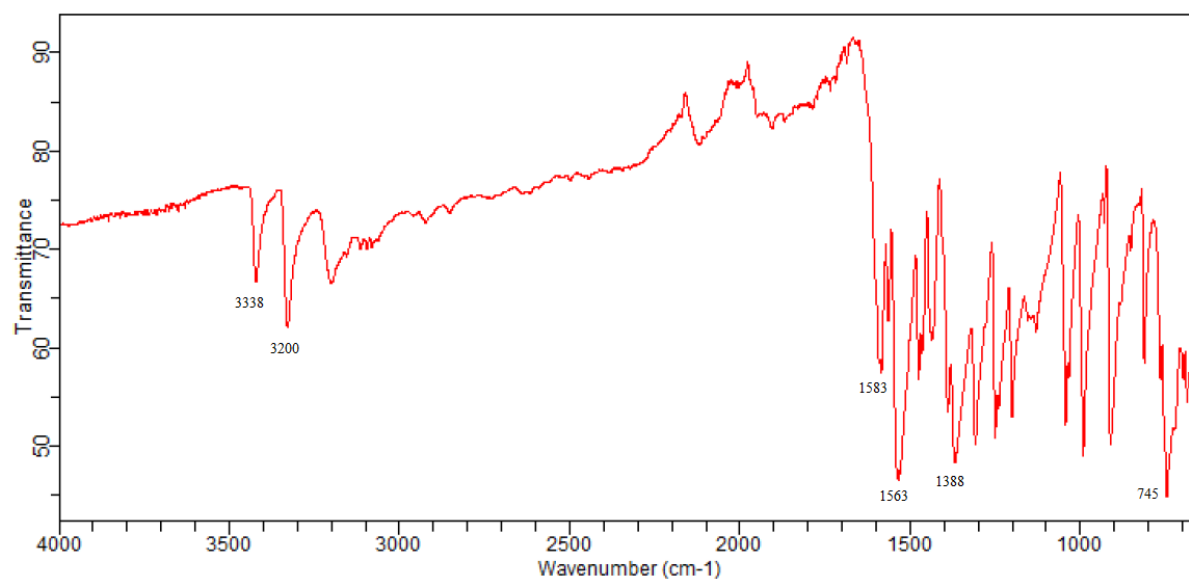


Figure 122, IR of SB66.

HRMS (+ESI-TOF) m/z: [M⁺] calcd for C₁₁H₈ClN₃O₂S 281.00257; found 281.00252.

SB67

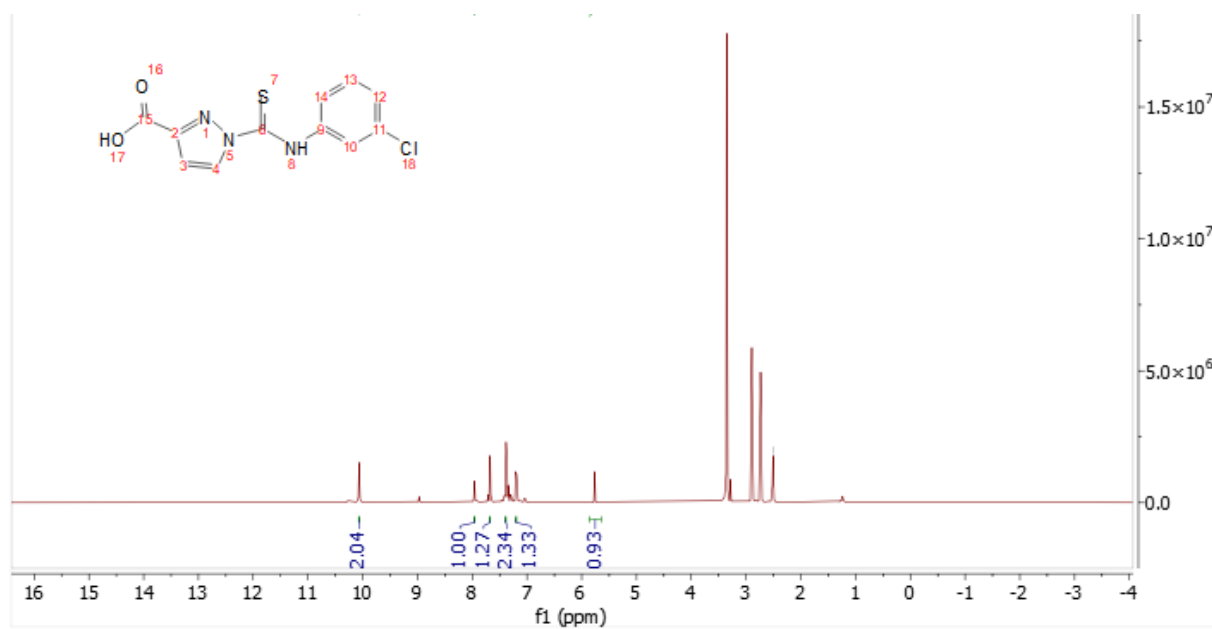


Figure 123, ¹H NMR of SB67.

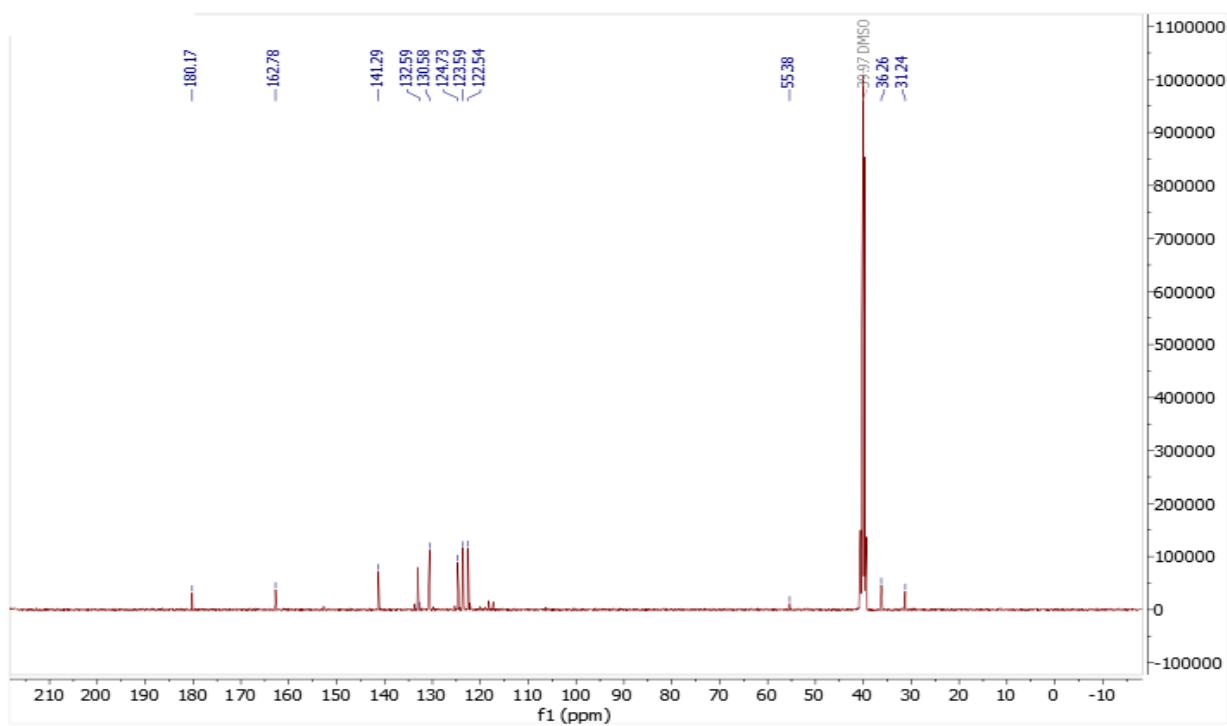


Figure 124, ¹³C NMR of SB67.

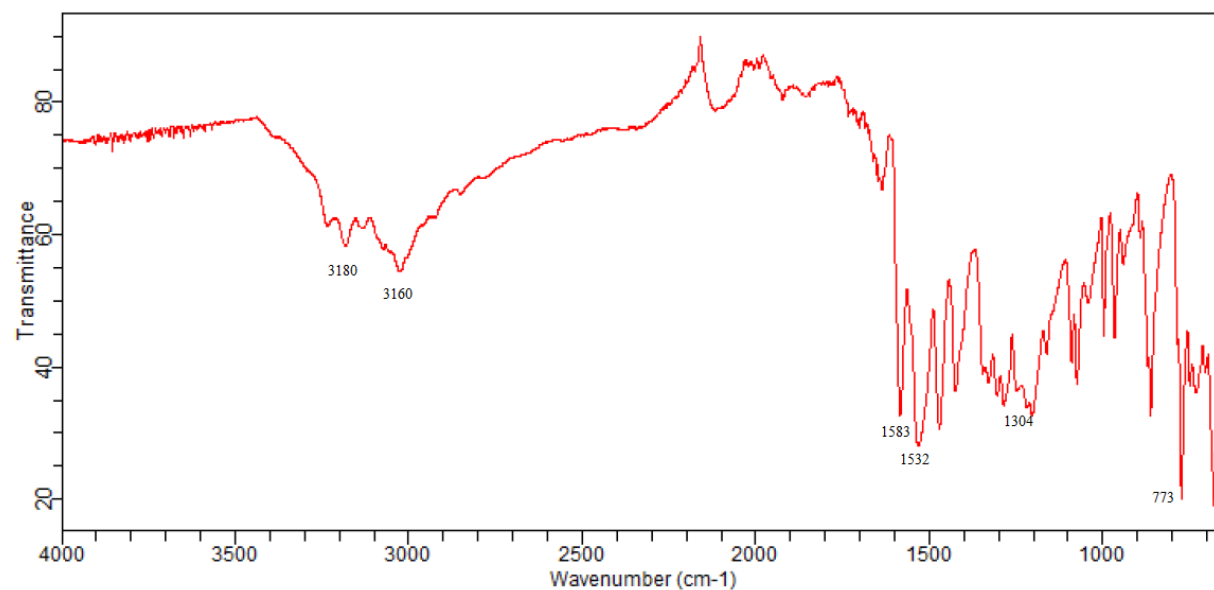


Figure 125, IR of SB67.

HRMS (+ESI-TOF) m/z: [M⁺] calcd for C₁₁H₈ClN₃O₂S 281.00257; found 281.00254.

SB69

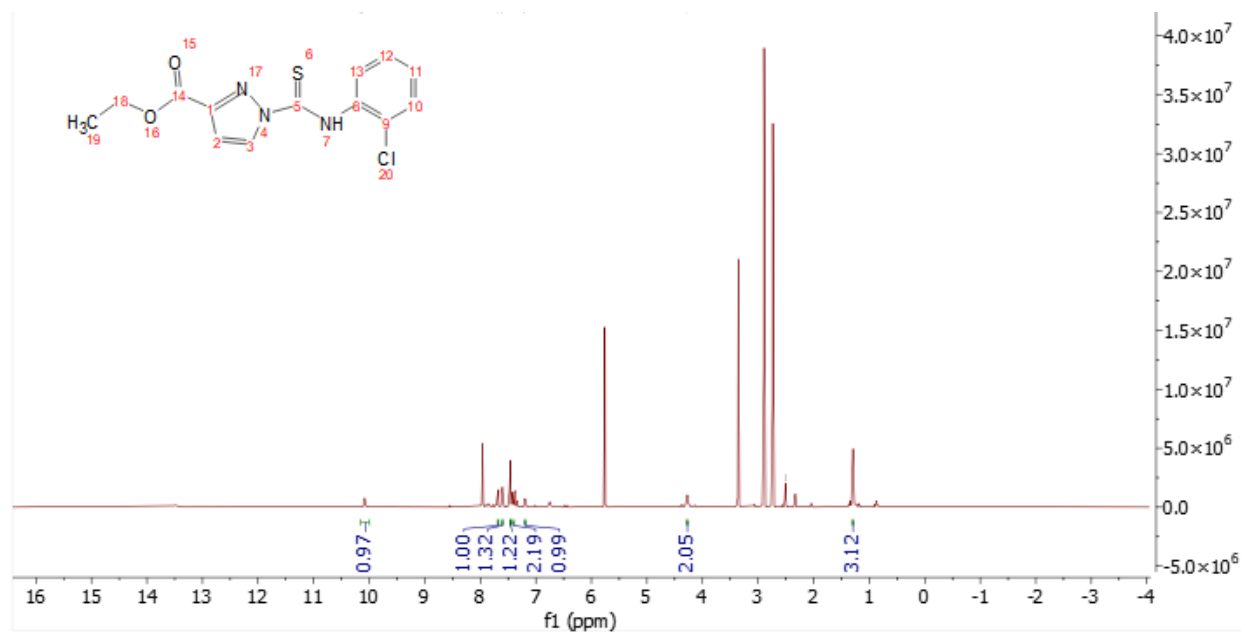


Figure 126, ¹H NMR of SB69.

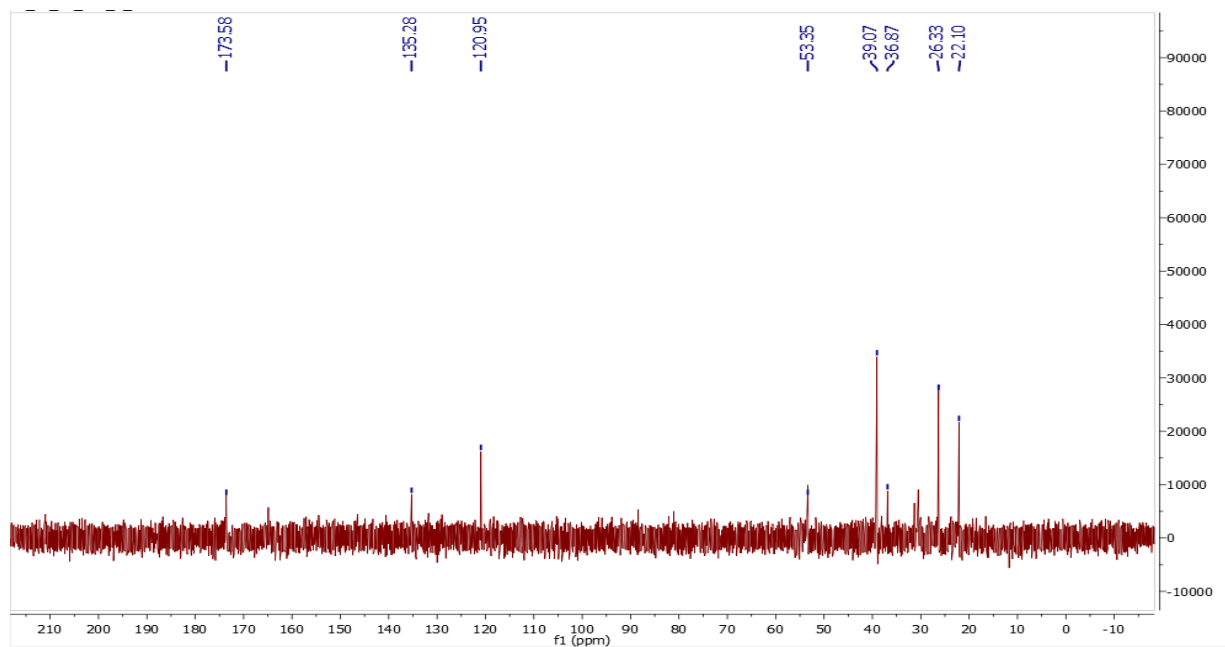


Figure 127, ¹³C NMR of SB69.

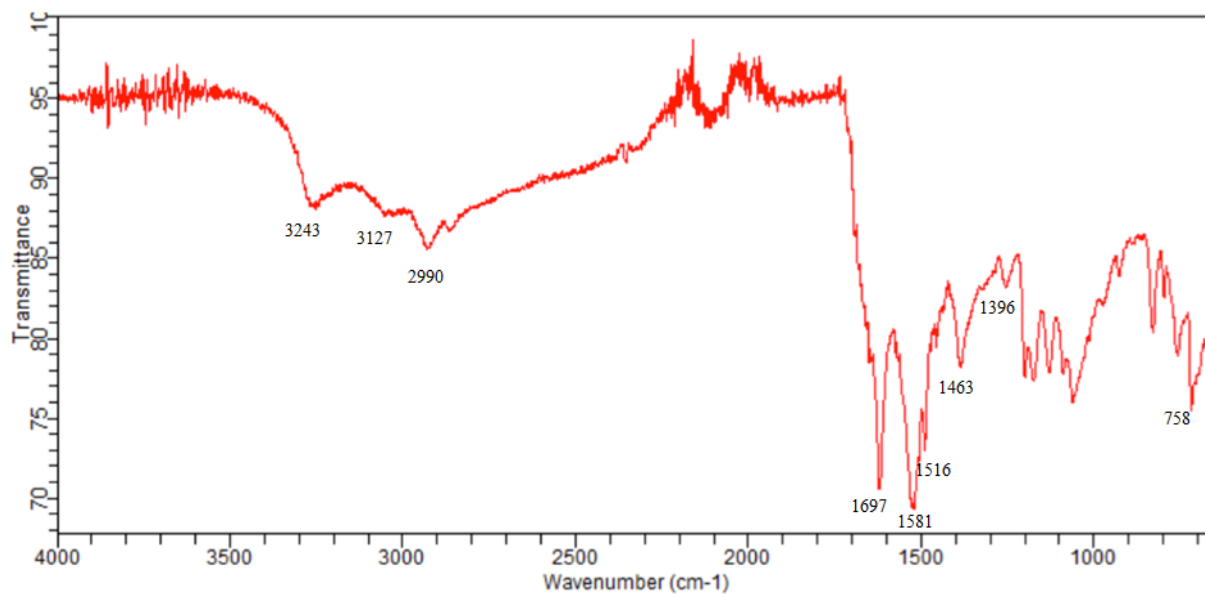


Figure 128, IR of SB69.

HRMS (+ESI-TOF) m/z: [M⁺] calcd for C₁₃H₁₂ClN₃O₂S 309.03388; found 309.03383.

SB70

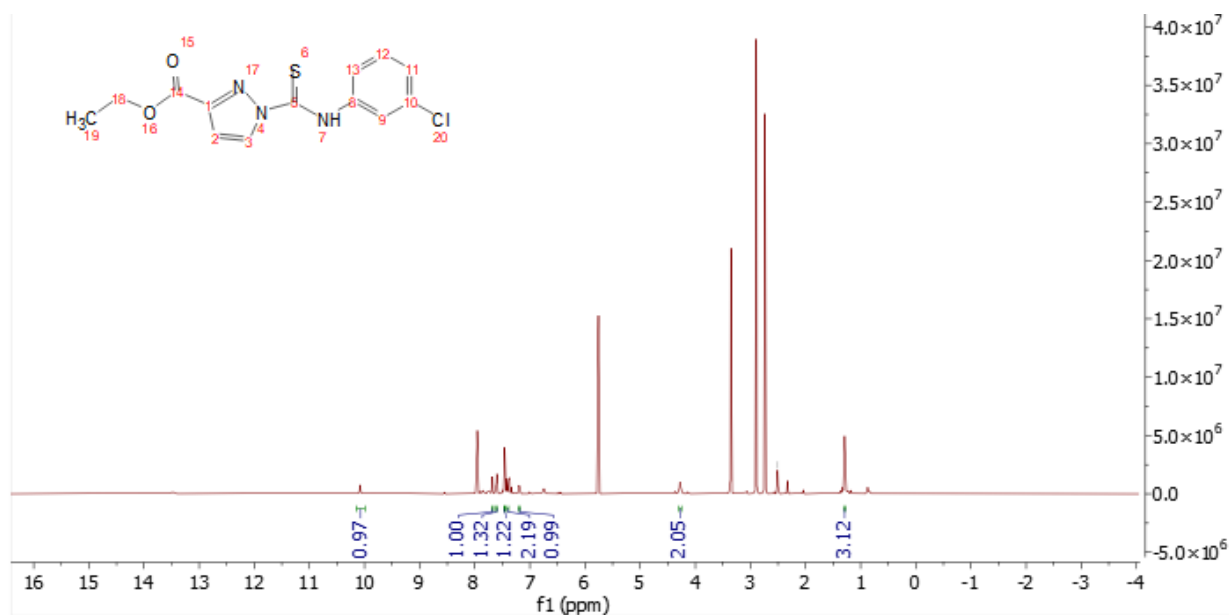


Figure 129, ¹H NMR of SB70.

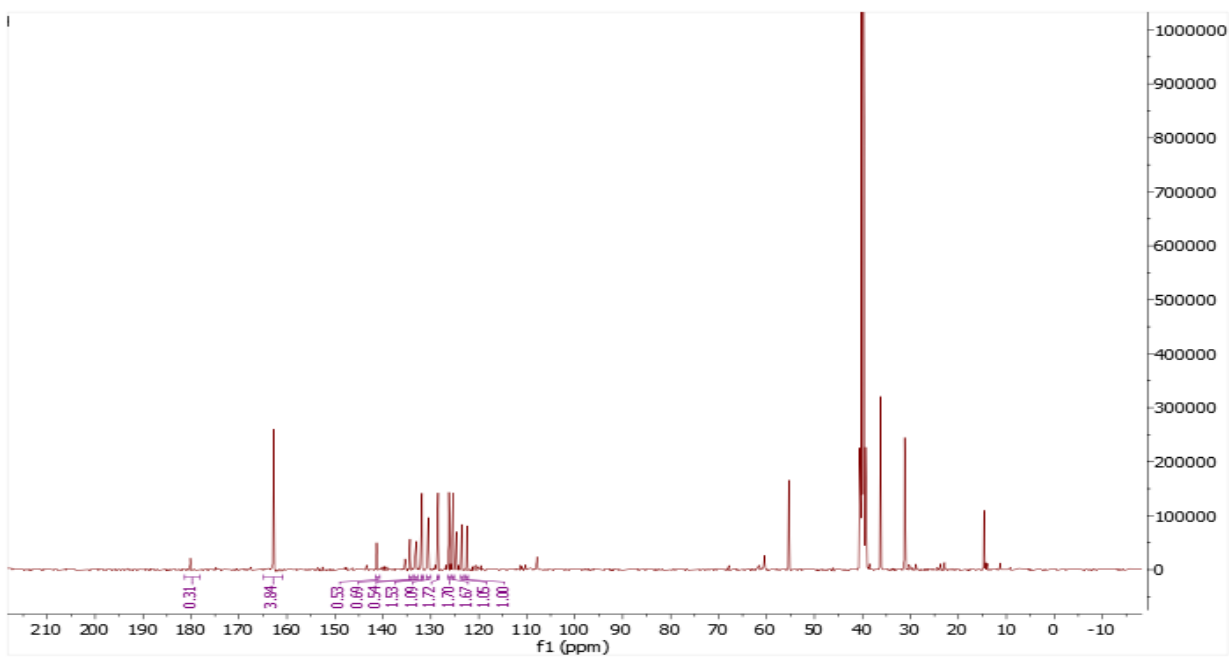


Figure 130, ¹³C NMR of SB70.

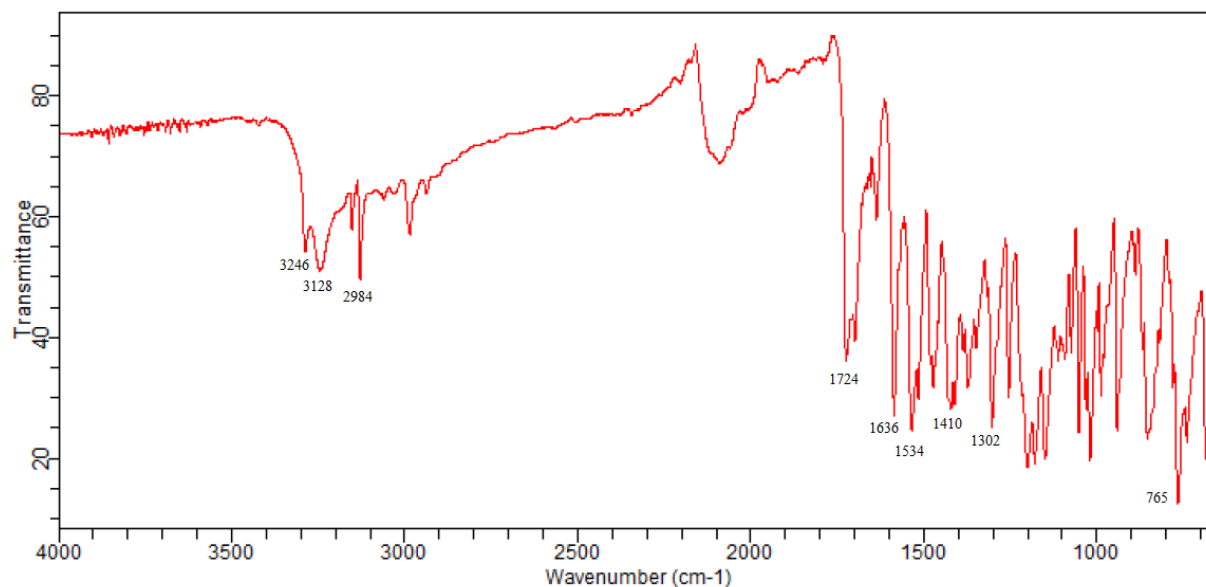


Figure 131, IR of SB70.

HRMS (+ESI-TOF) m/z: [M⁺] calcd for C₁₃H₁₂ClN₃O₂S 309.03388; found 309.03387.

SB73

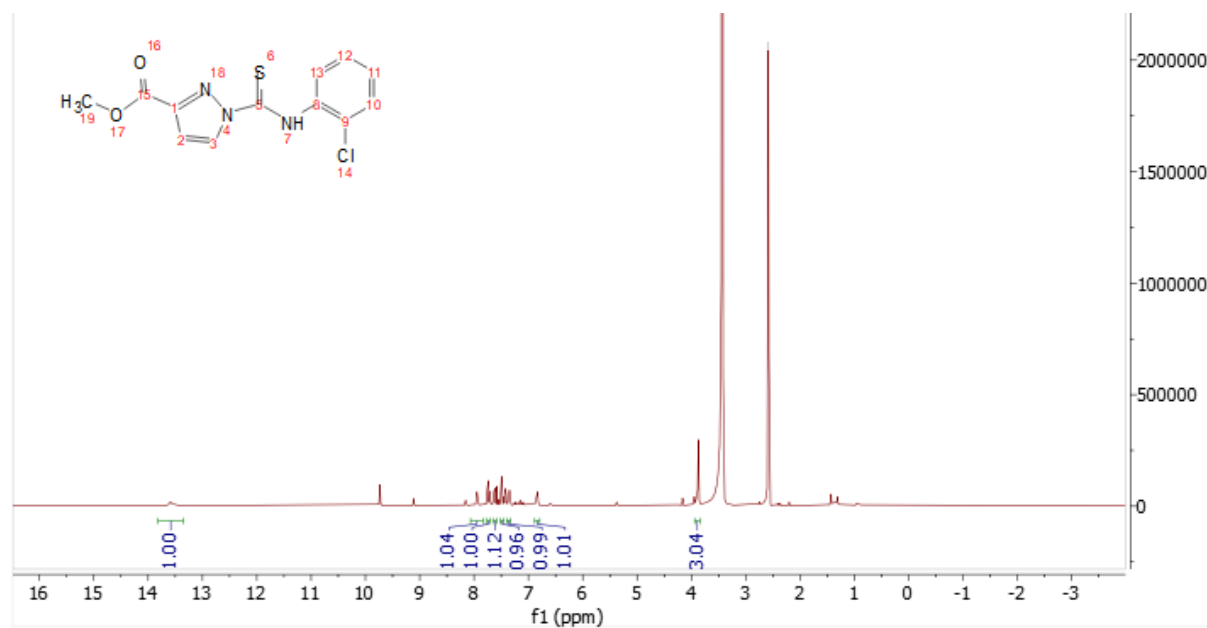


Figure 132, ¹H NMR of SB73.

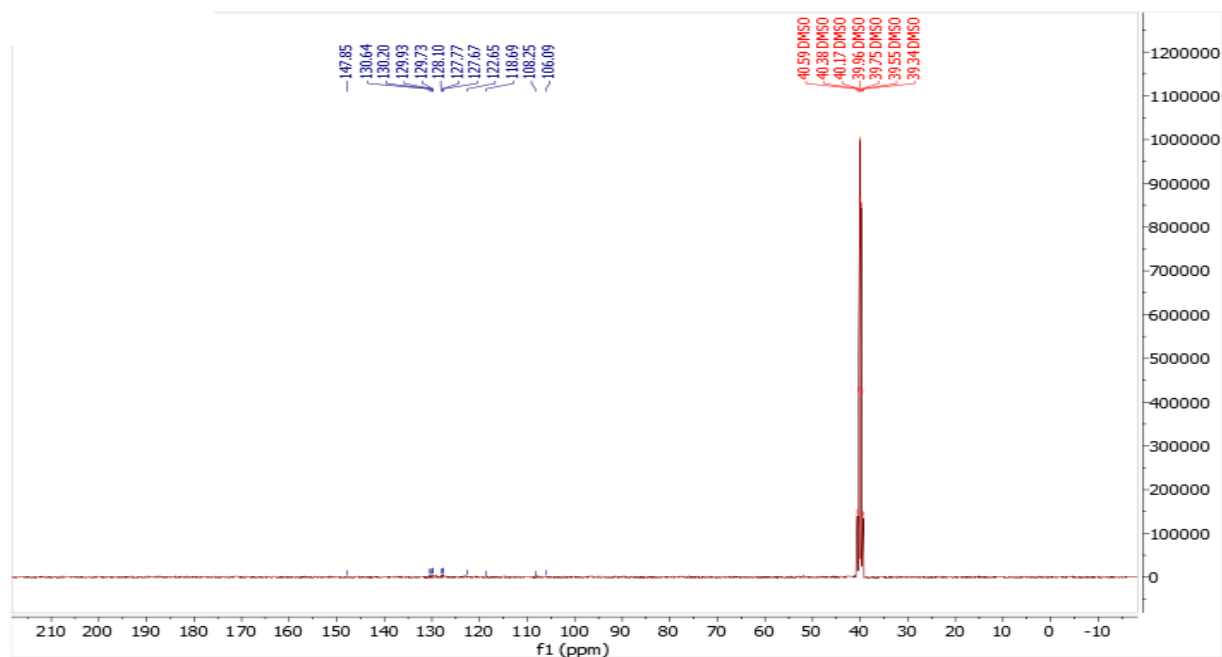


Figure 133, ^{13}C NMR of SB73.

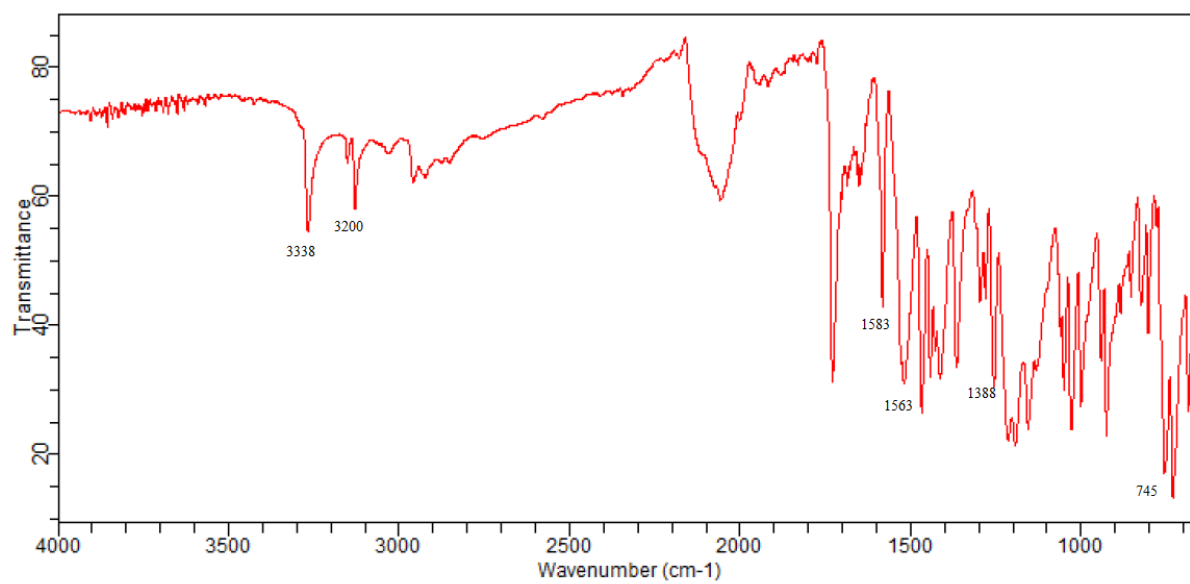


Figure 134, IR of SB73.

HRMS (+ESI-TOF) m/z : $[M]^+$ calcd for $\text{C}_{12}\text{H}_{10}\text{ClN}_3\text{O}_2\text{S}$ 295.01822; found 295.01824.

SB74

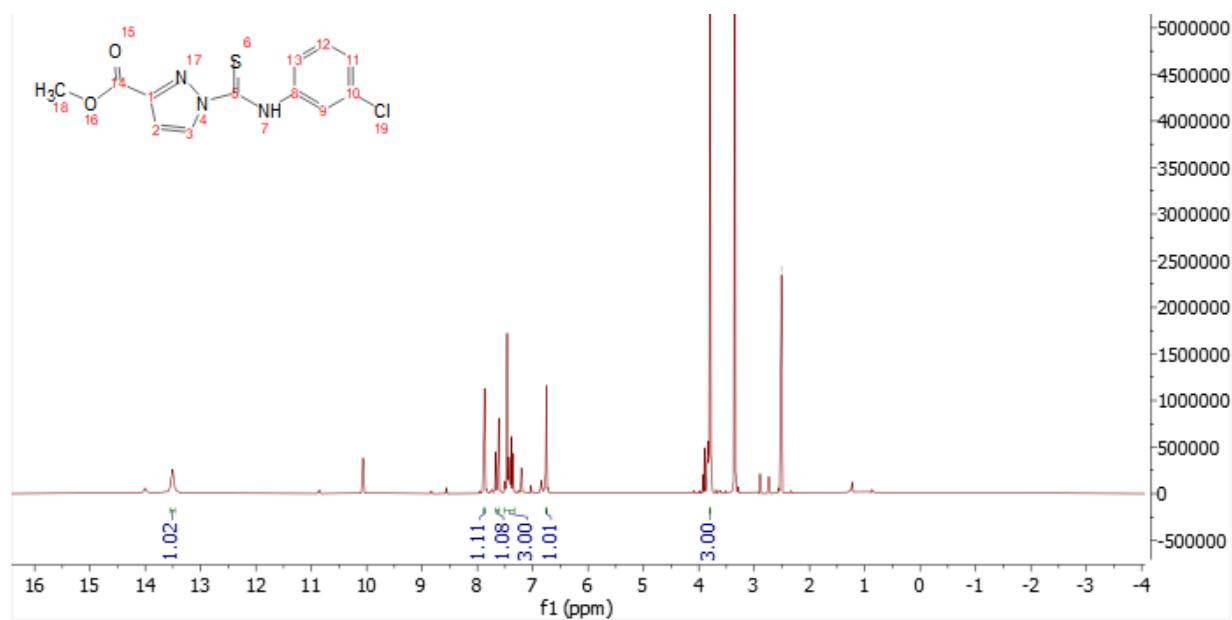


Figure 135, ^1H NMR of SB74.

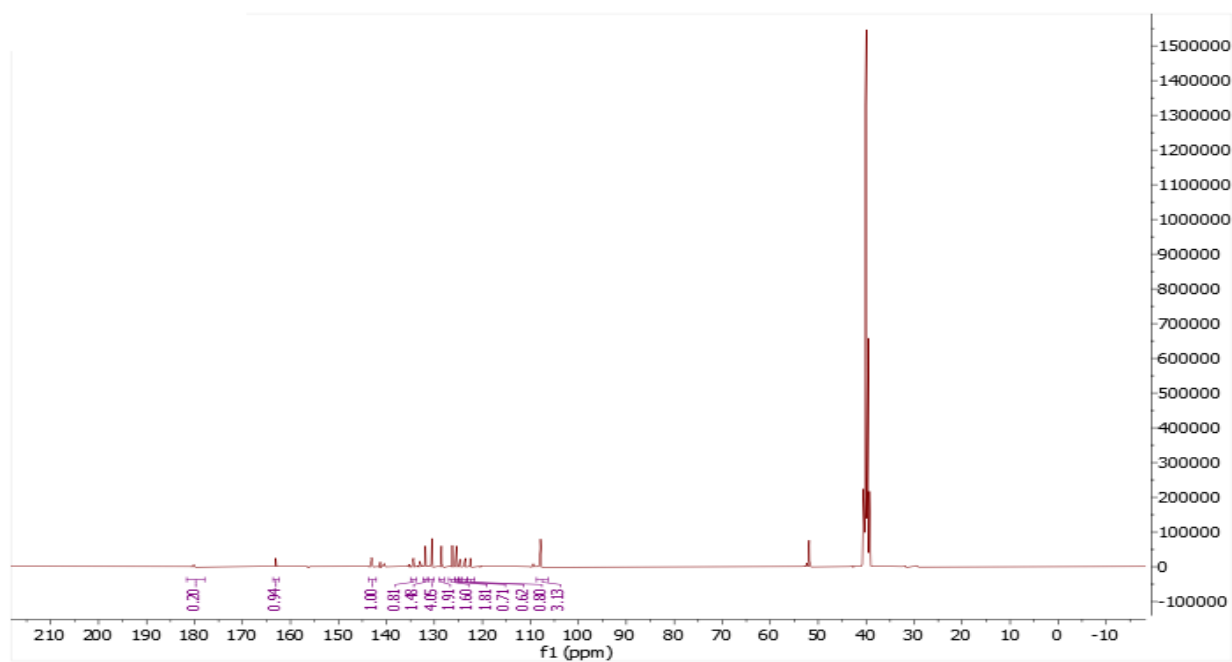


Figure 136, ^{13}C NMR of SB74.

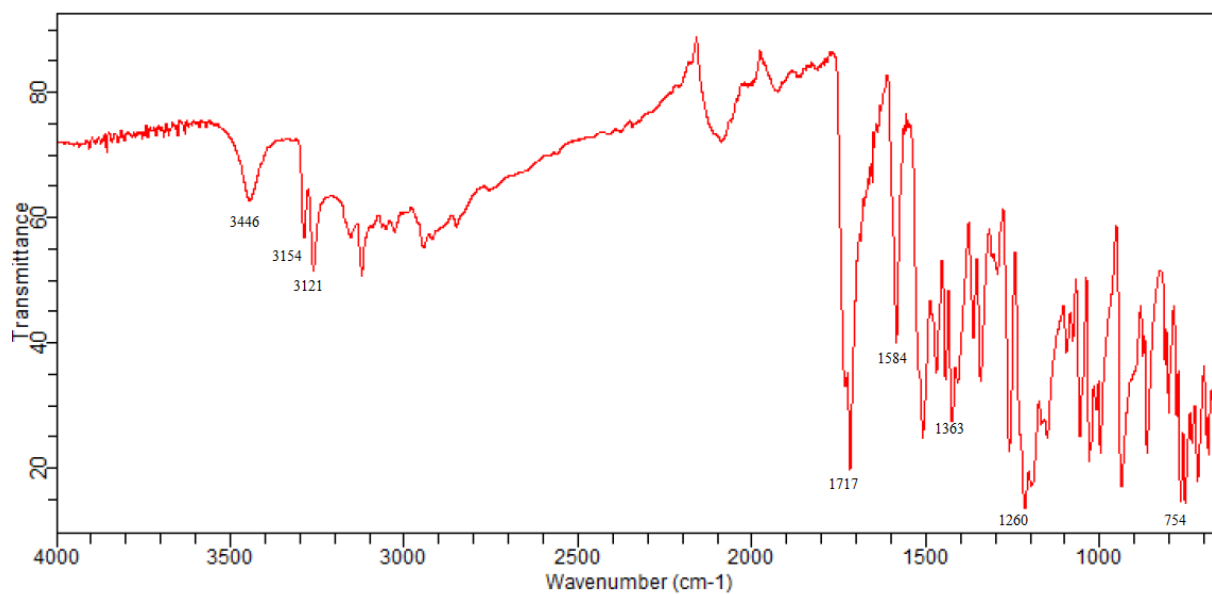


Figure 137, IR of SB74.

HRMS (+ESI-TOF) m/z: [M⁺] calcd for C₁₂H₁₀ClN₃O₂S 295.01822; found 295.01820.

SB75

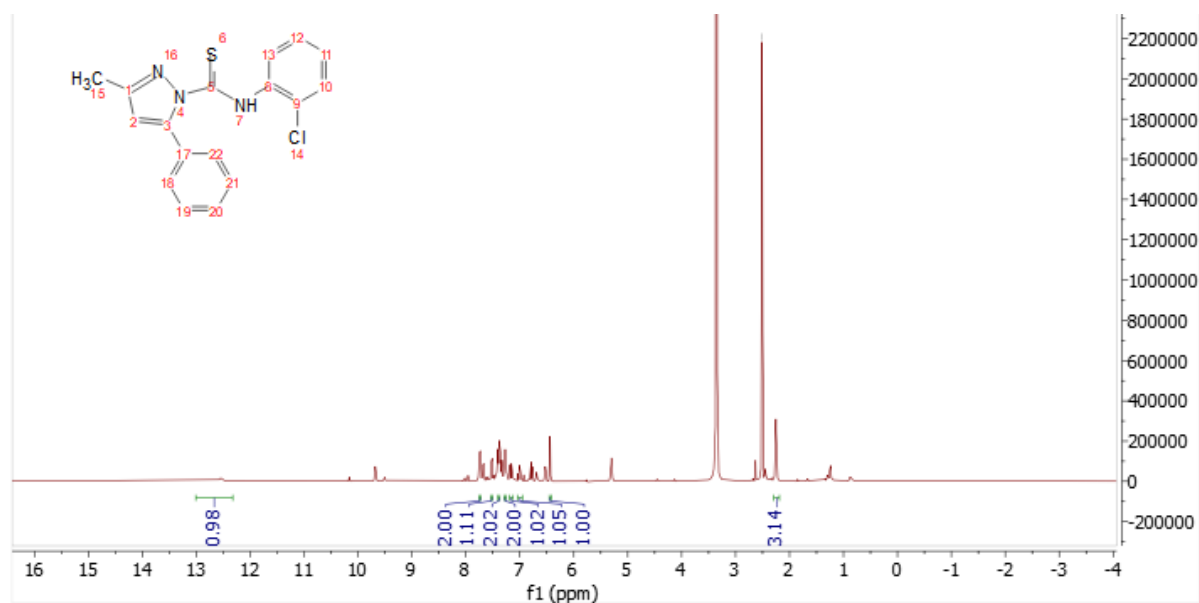


Figure 138, ¹H NMR of SB75.

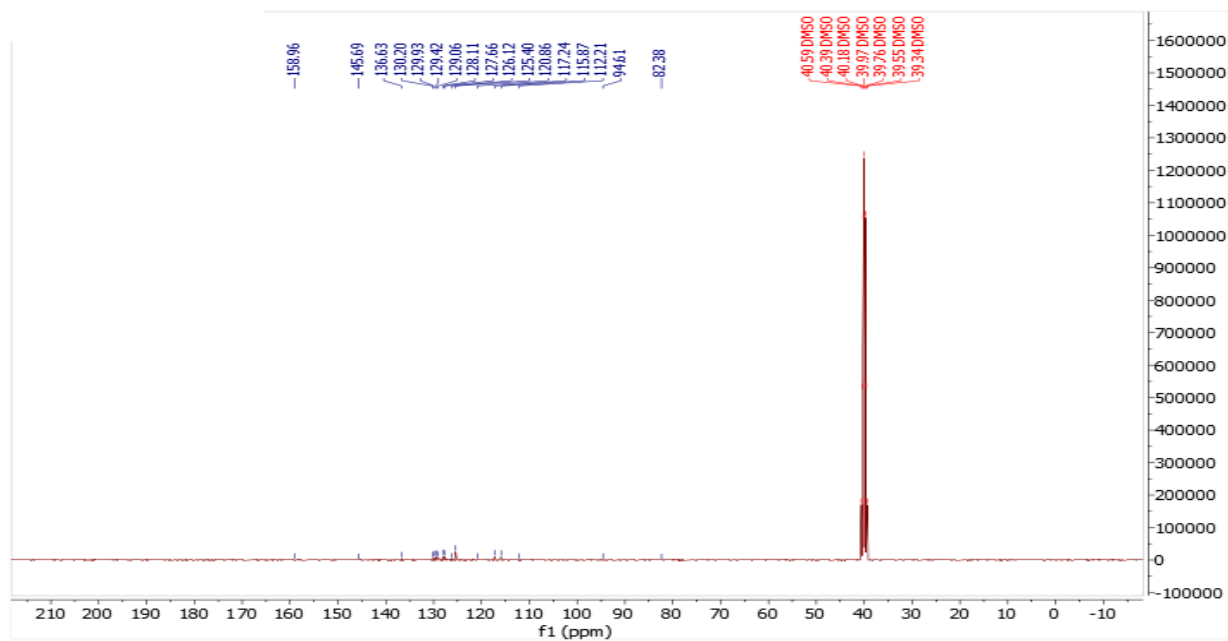


Figure 139, ^{13}C NMR of SB75.

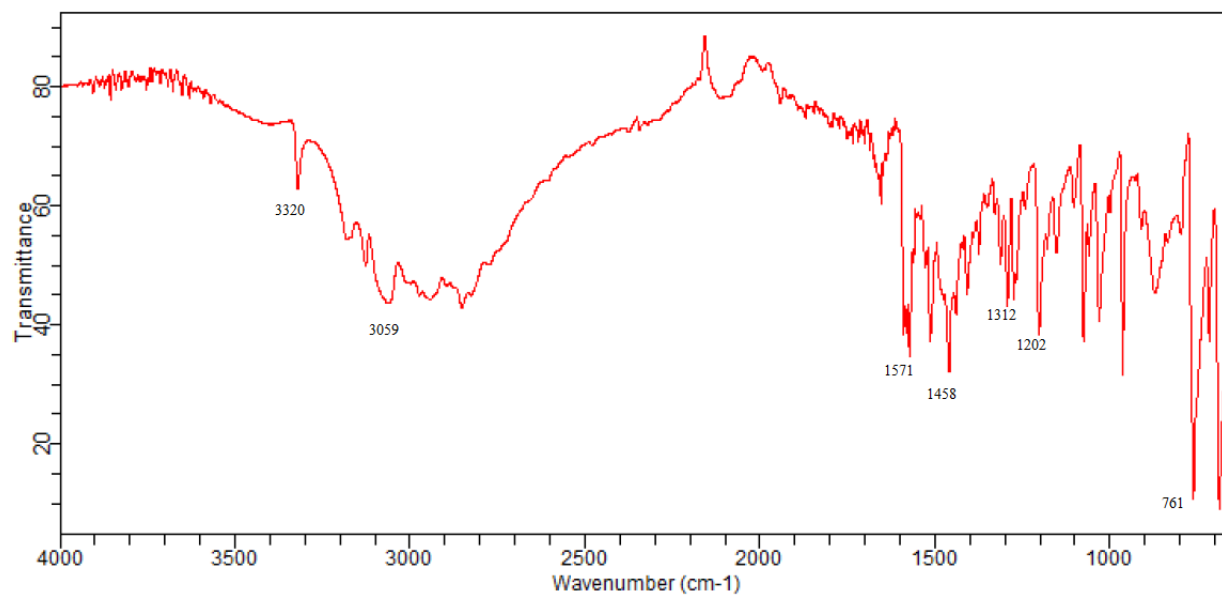


Figure 140, IR of SB75.

HRMS (+ESI-TOF) m/z : $[\text{M}^+]$ calcd for $\text{C}_{17}\text{H}_{14}\text{ClN}_3\text{S}$ 327.05970; found 327.05965.

SB76

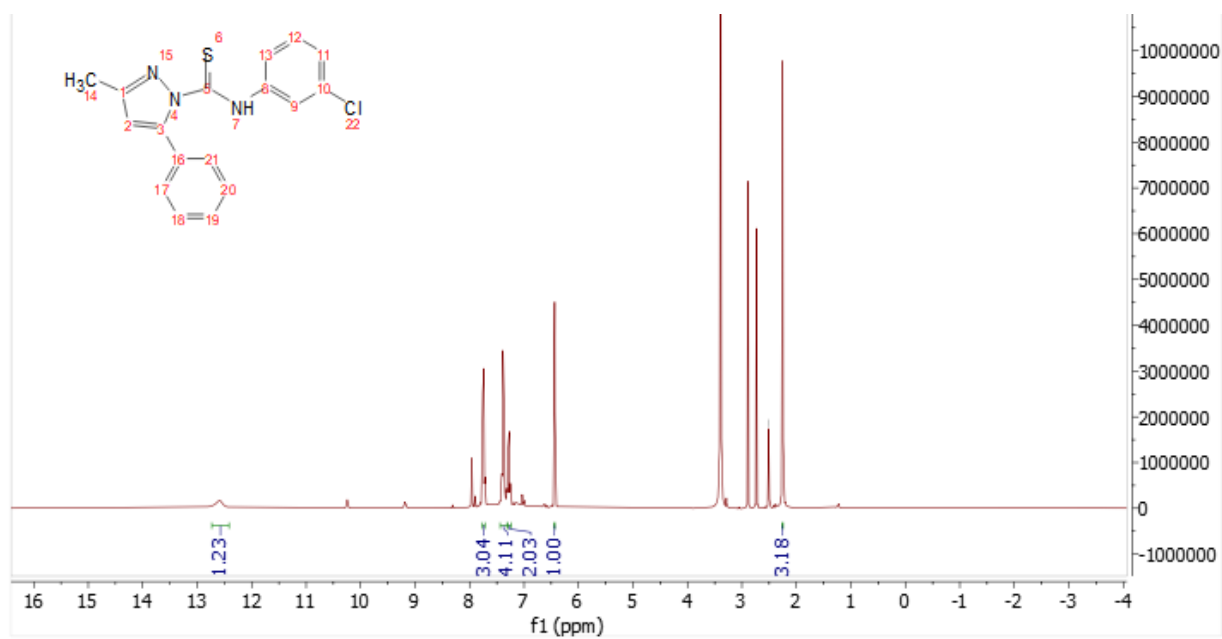


Figure 141, ^1H NMR of SB76.

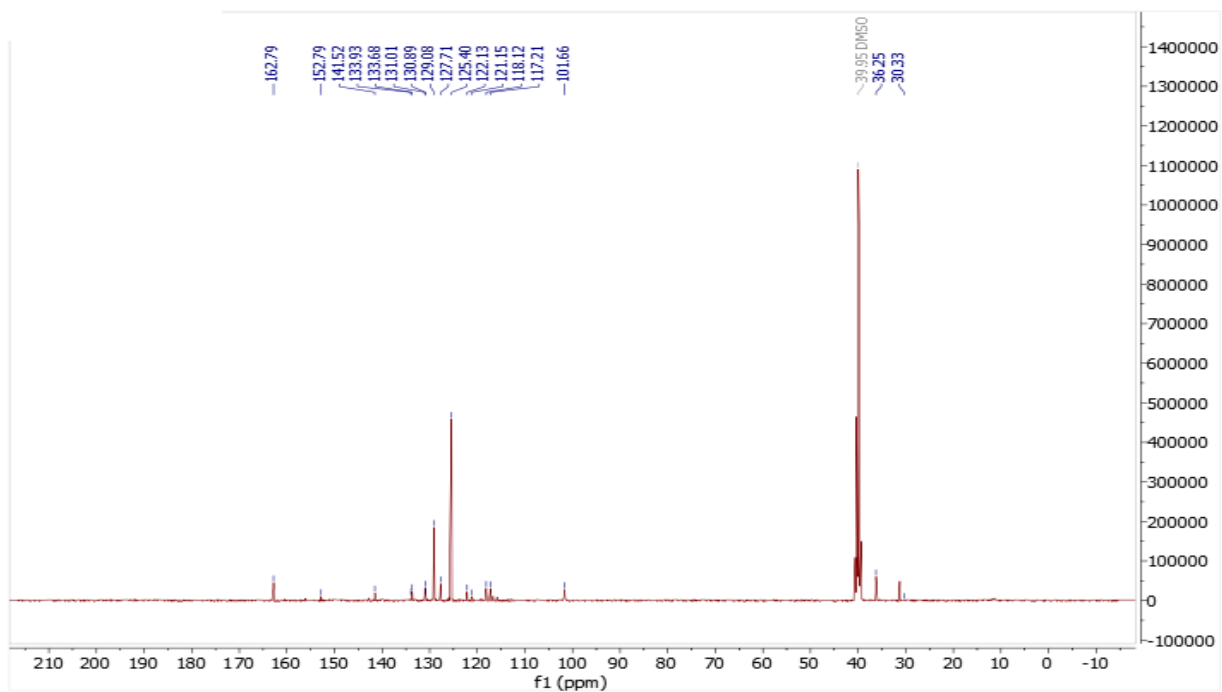


Figure 142, ^{13}C NMR of SB76.

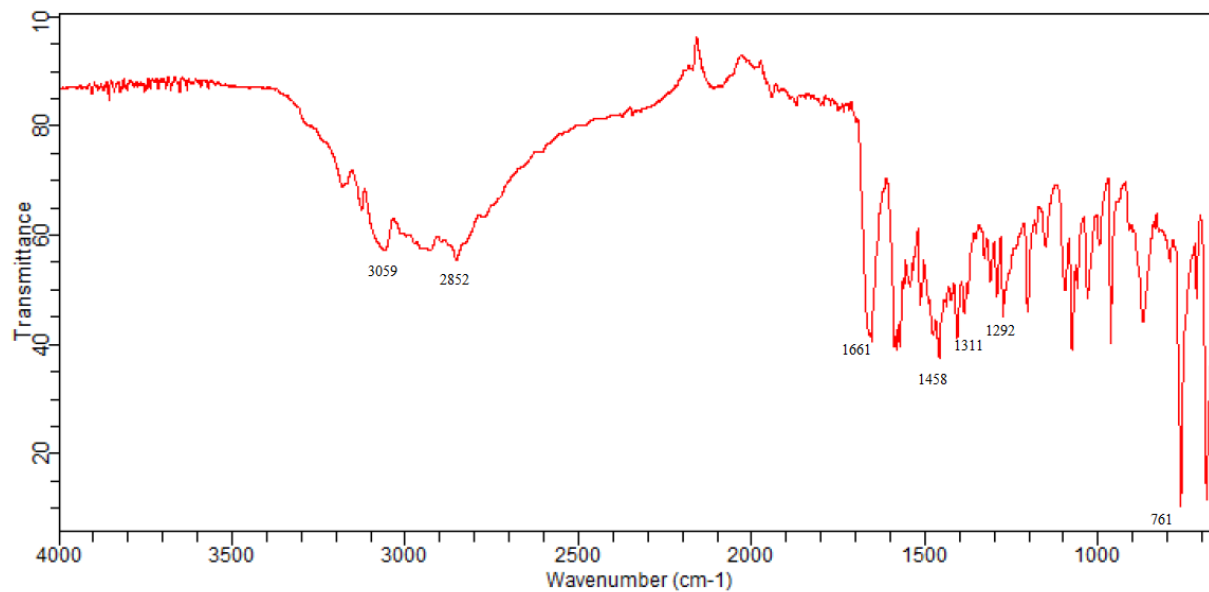


Figure 143, IR of SB76.

HRMS (+ESI-TOF) m/z: [M⁺] calcd for C₁₇H₁₄ClN₃S 327.05970; found 327.05975.

SB77

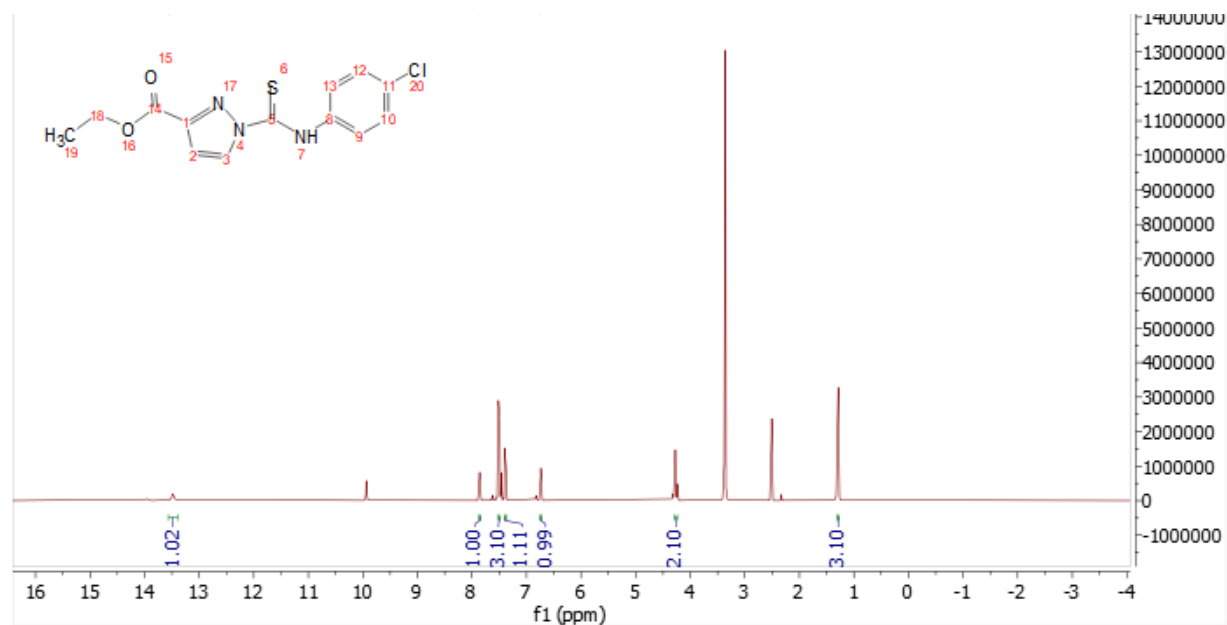


Figure 144, ¹H NMR of SB77.

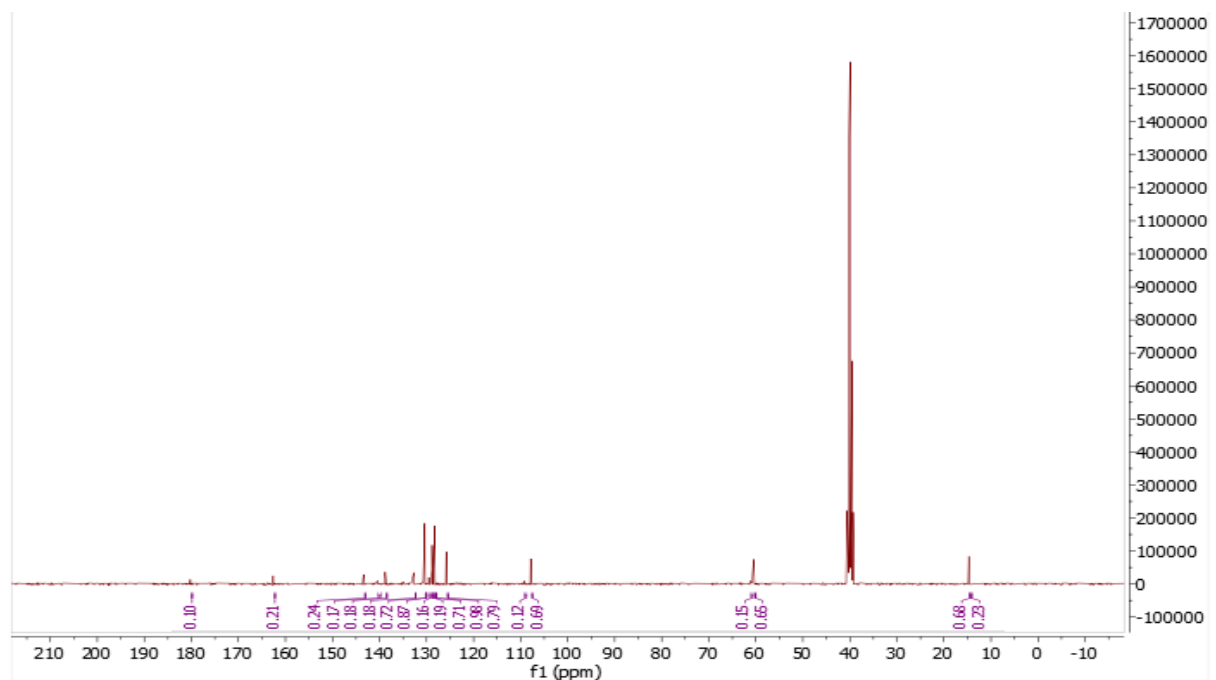


Figure 145, ^{13}C NMR of SB77.

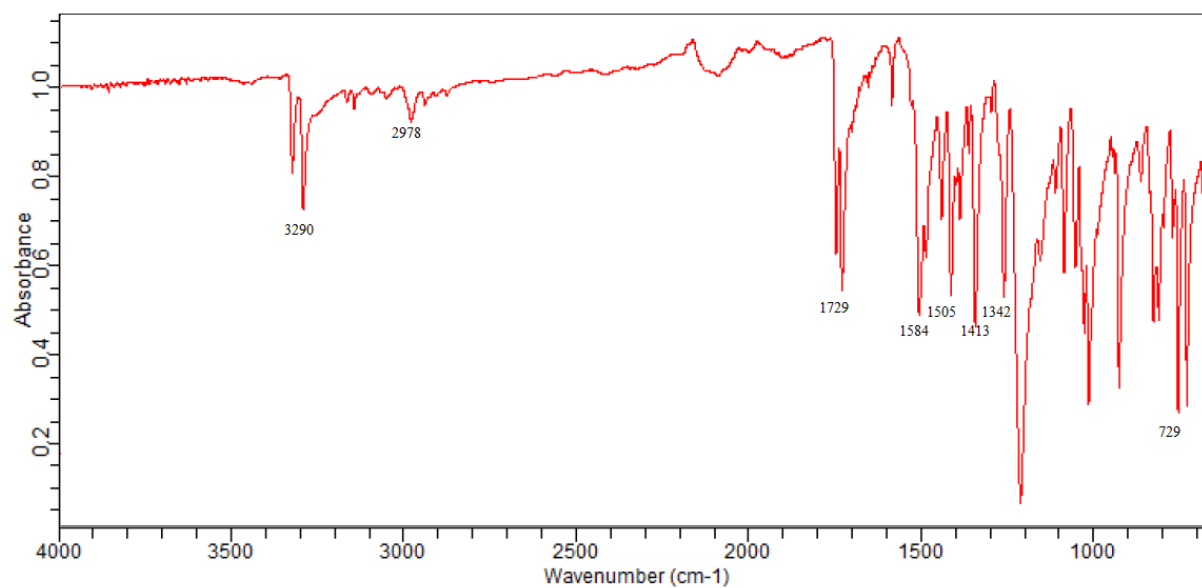


Figure 146, IR of SB77.

HRMS (+ESI-TOF) m/z : $[\text{M}^+]$ calcd for $\text{C}_{13}\text{H}_{12}\text{ClN}_3\text{O}_2\text{S}$ 309.03388; found 309.03389.

SB78

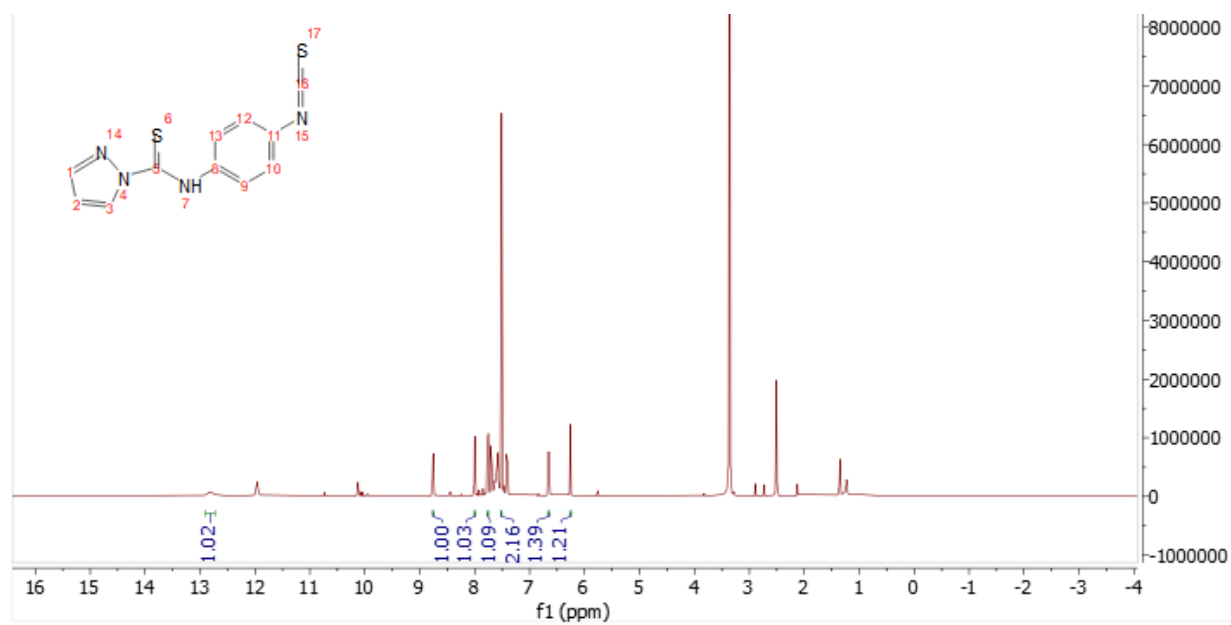


Figure 147, ^1H NMR of SB78.

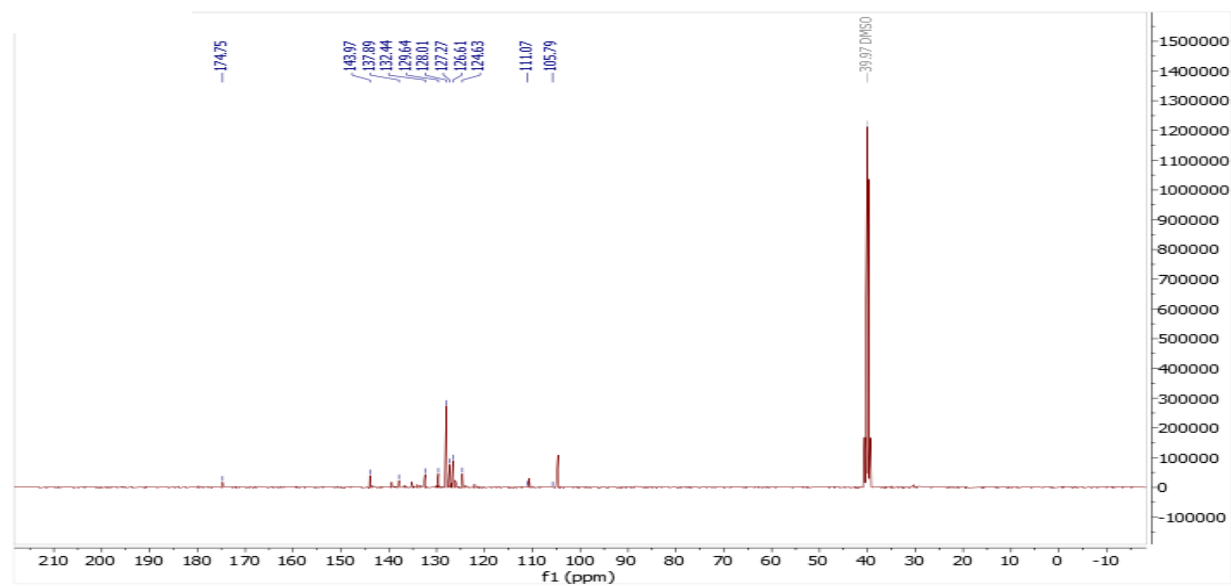


Figure 148, ^{13}C NMR of SB78.

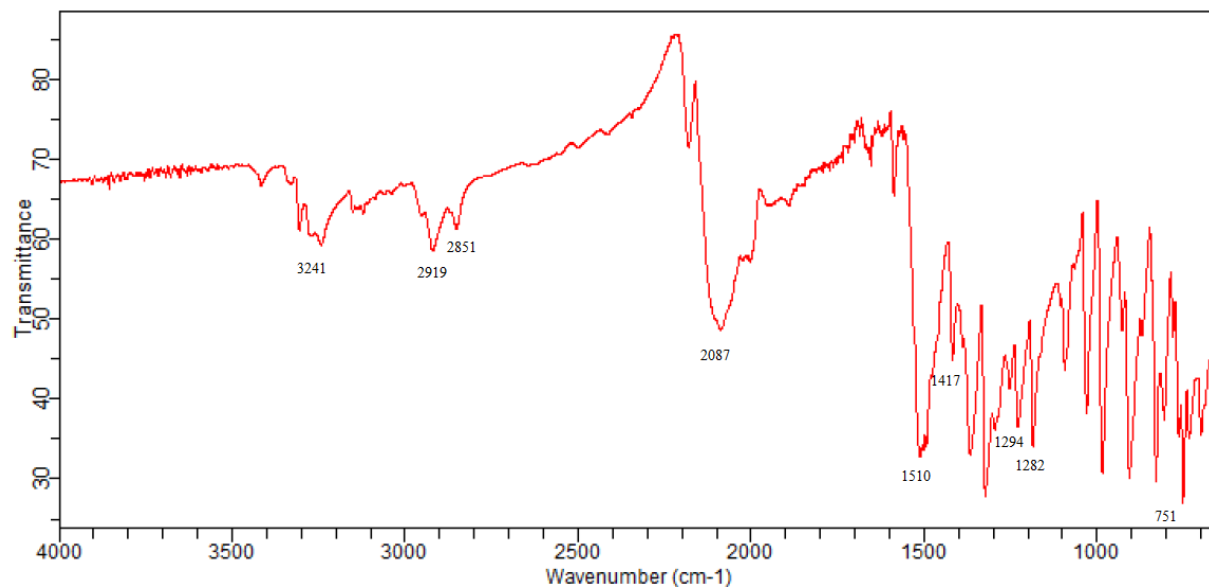


Figure 149, IR of SB78.

UPLC-MS [M+1] calcd for C₁₁H₈N₄S₂ 261.34; found 261.05.

SB79

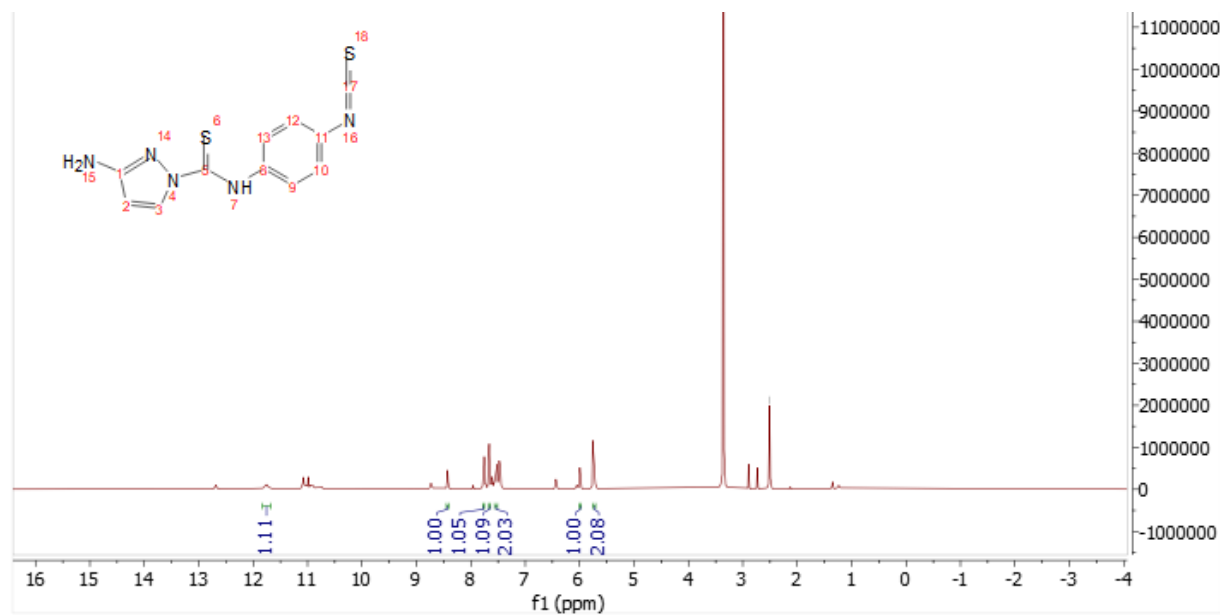


Figure 150, ¹H NMR of SB79.

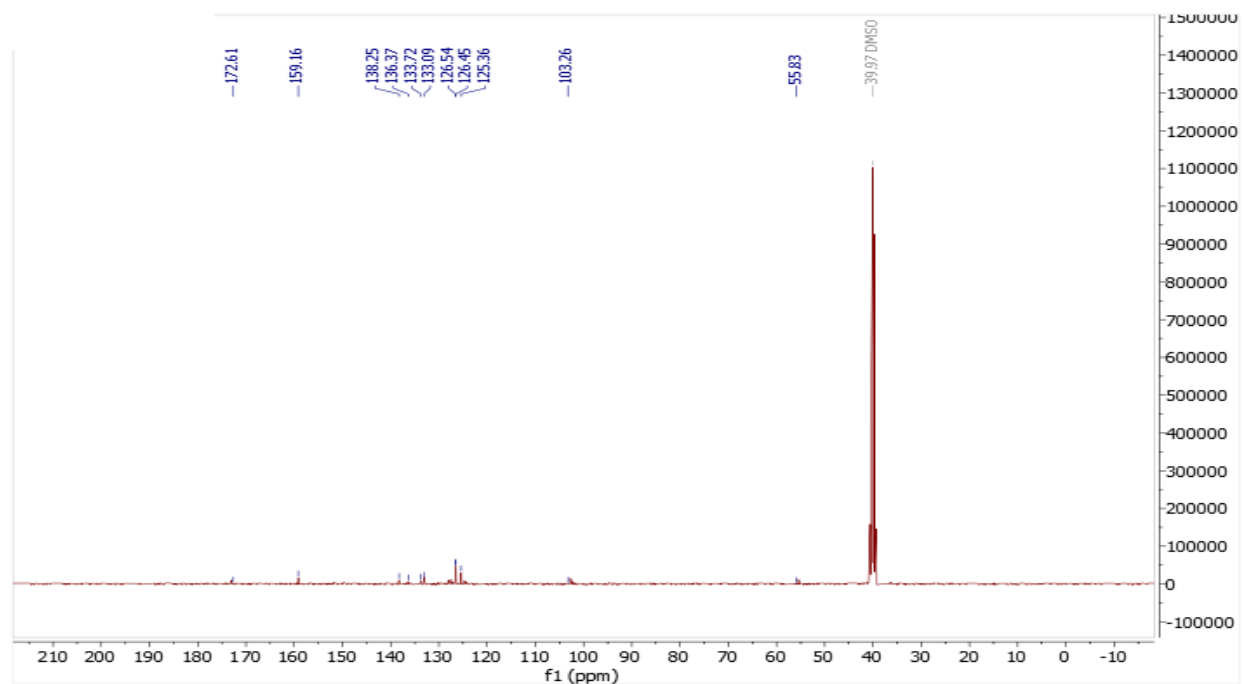


Figure 151, ¹³C NMR of SB79.

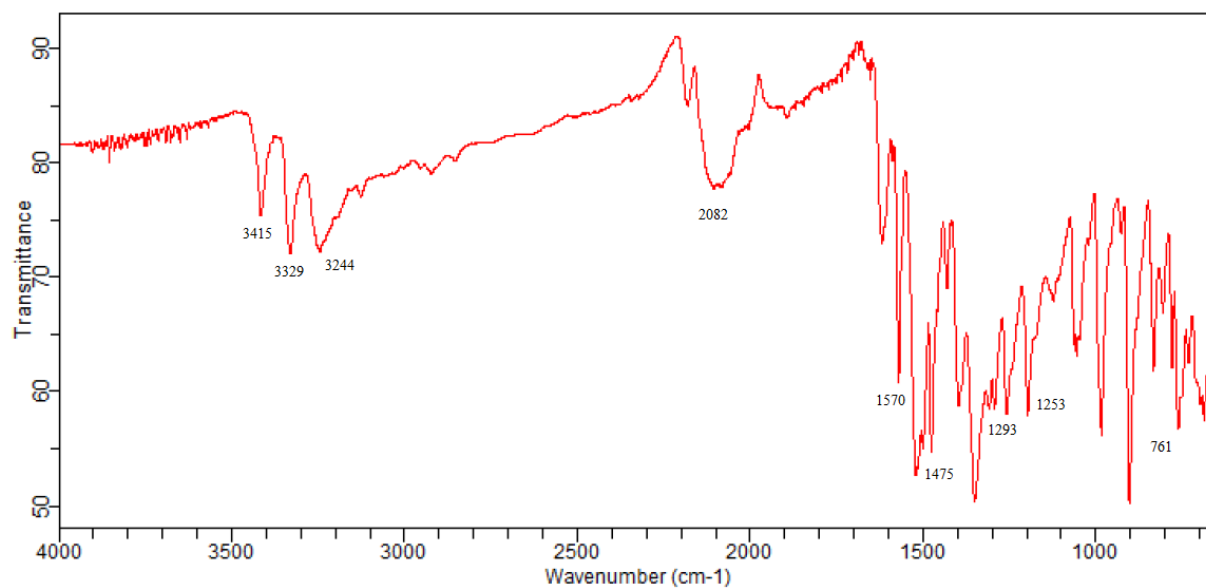


Figure 152, IR of SB79.

UPLC-MS [M+1] calcd for C₁₁H₉N₅S₂ 276.35; found 276.07.

SB80

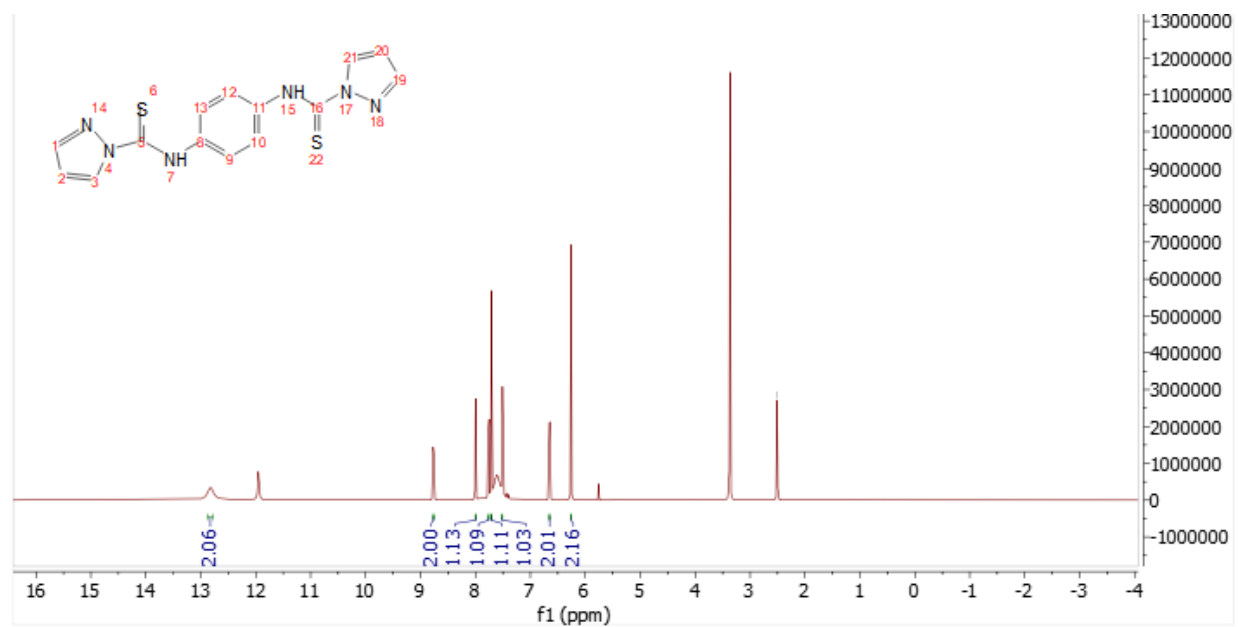


Figure 153, ^1H NMR of SB80.

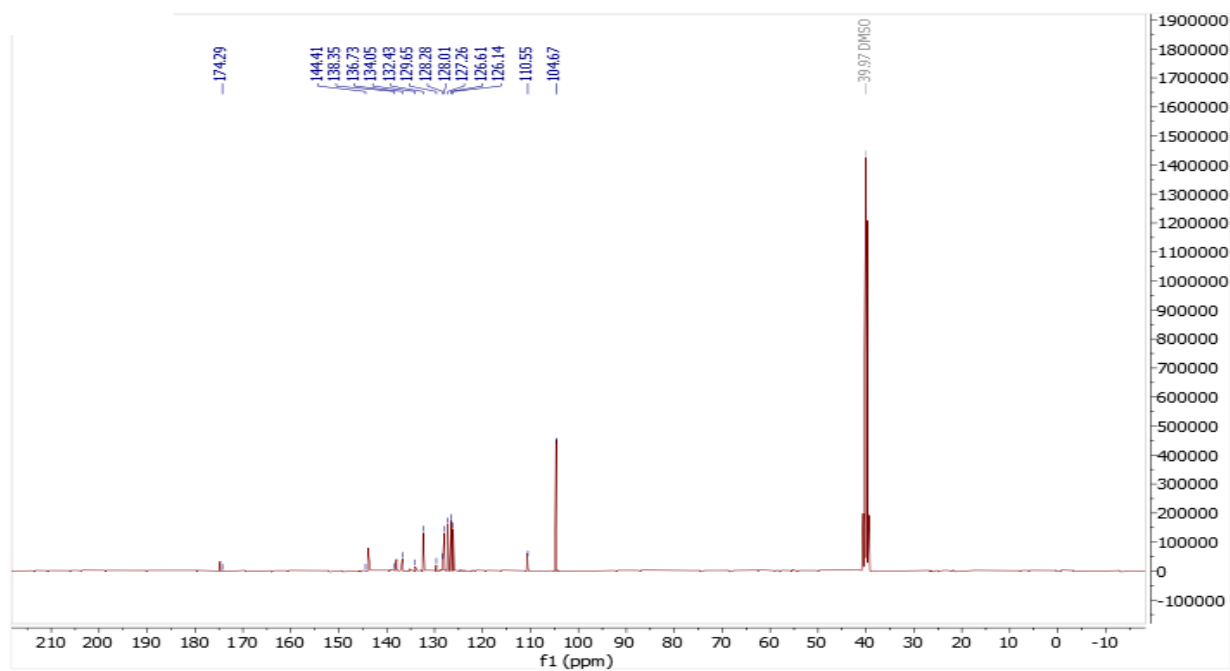


Figure 154, ^{13}C NMR of SB80.

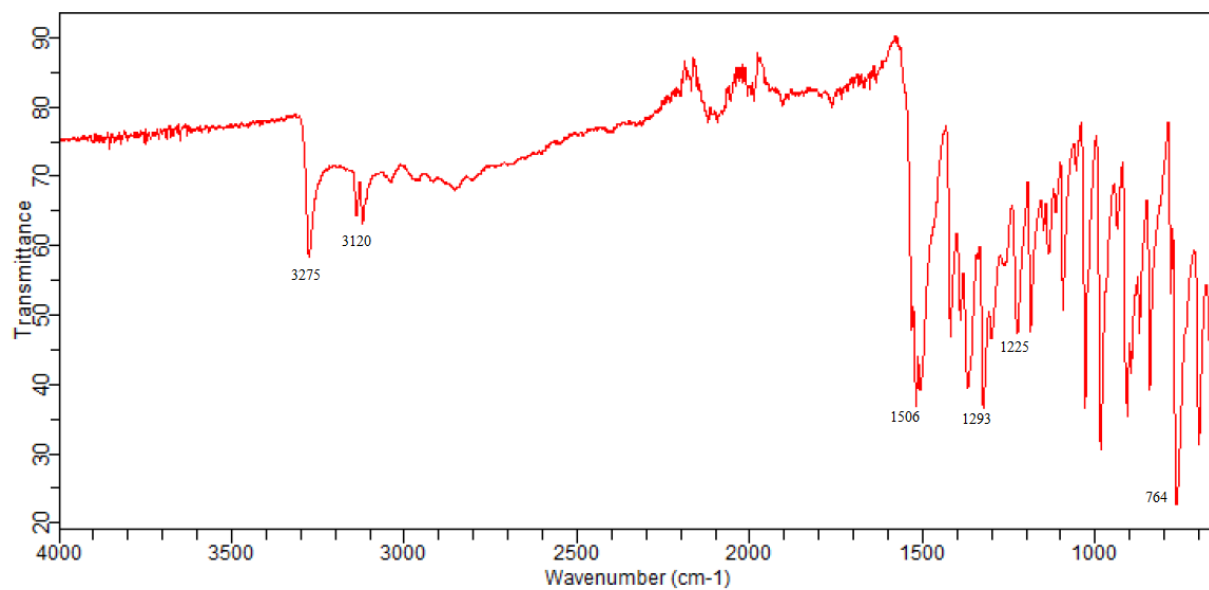


Figure 155, IR of SB80.

UPLC-MS [M+1] calcd for C₁₄H₁₂N₆S₂ 329.42; found 327.20.

SB81

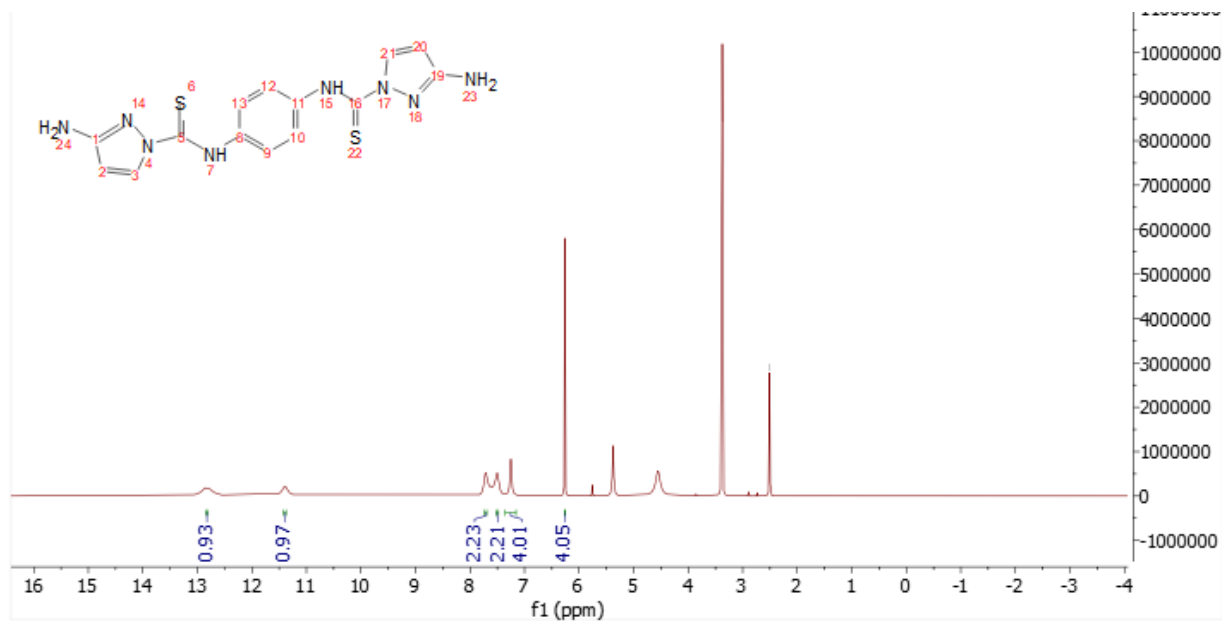


Figure 156, ¹H NMR of SB81.

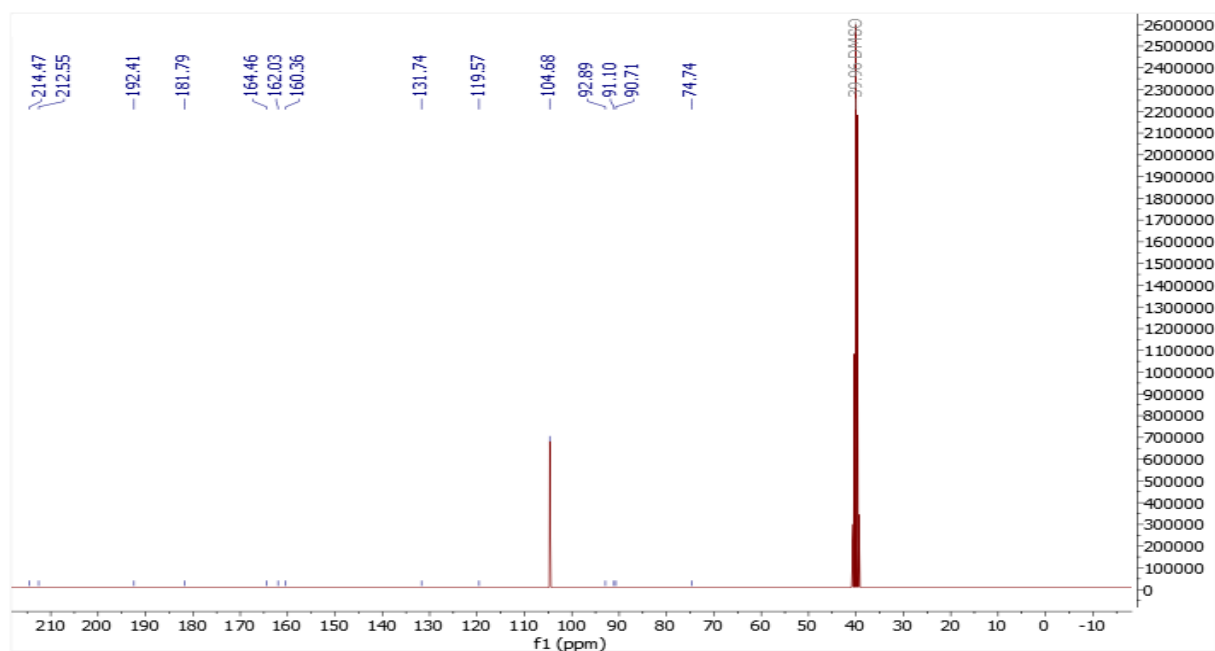


Figure 157, ¹³C NMR of SB81.

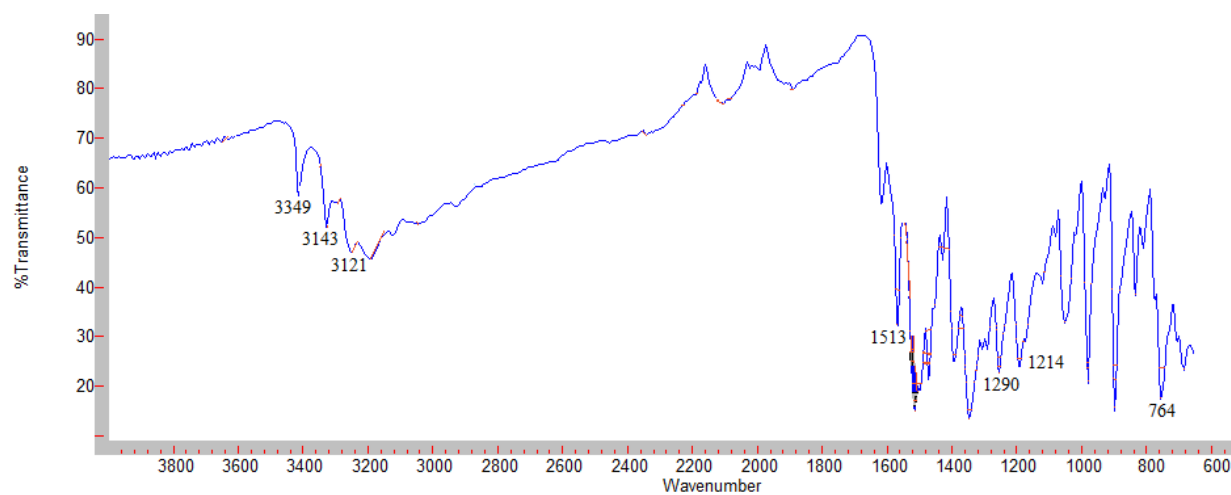


Figure 158, IR of SB81.

UPLC-MS [M+1] calcd for C₁₄H₁₄N₈S₂ 359.44; found 359.14.

SB82

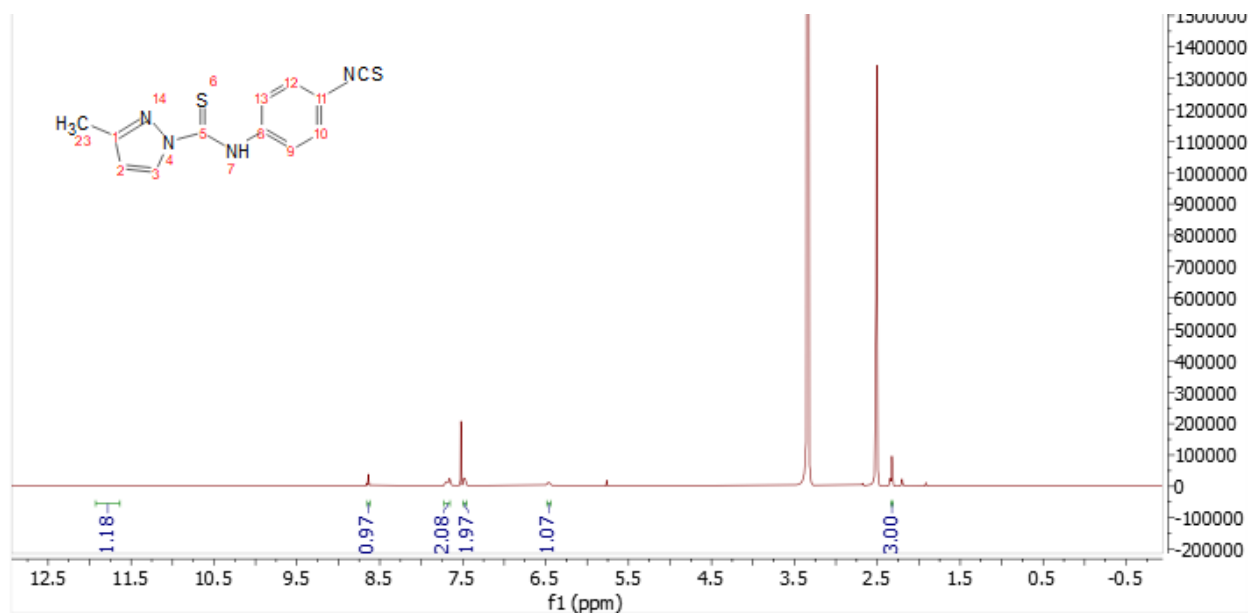


Figure 159, ¹H NMR of SB82.

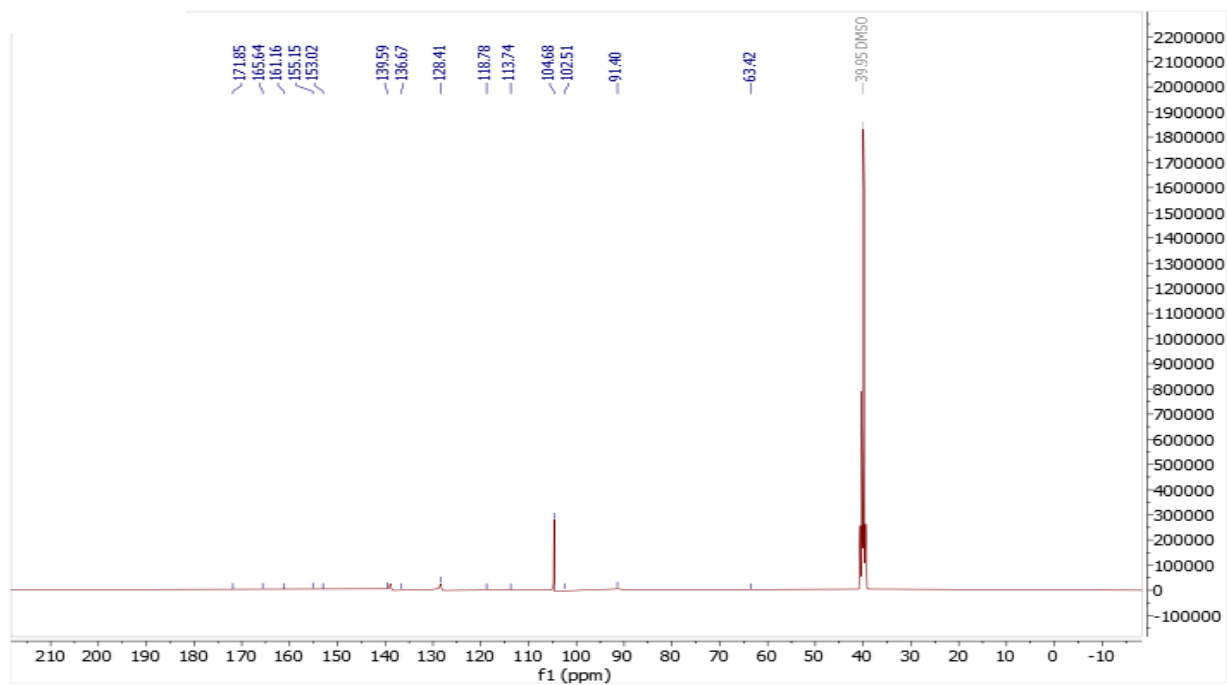


Figure 160, ¹³C NMR of SB82.

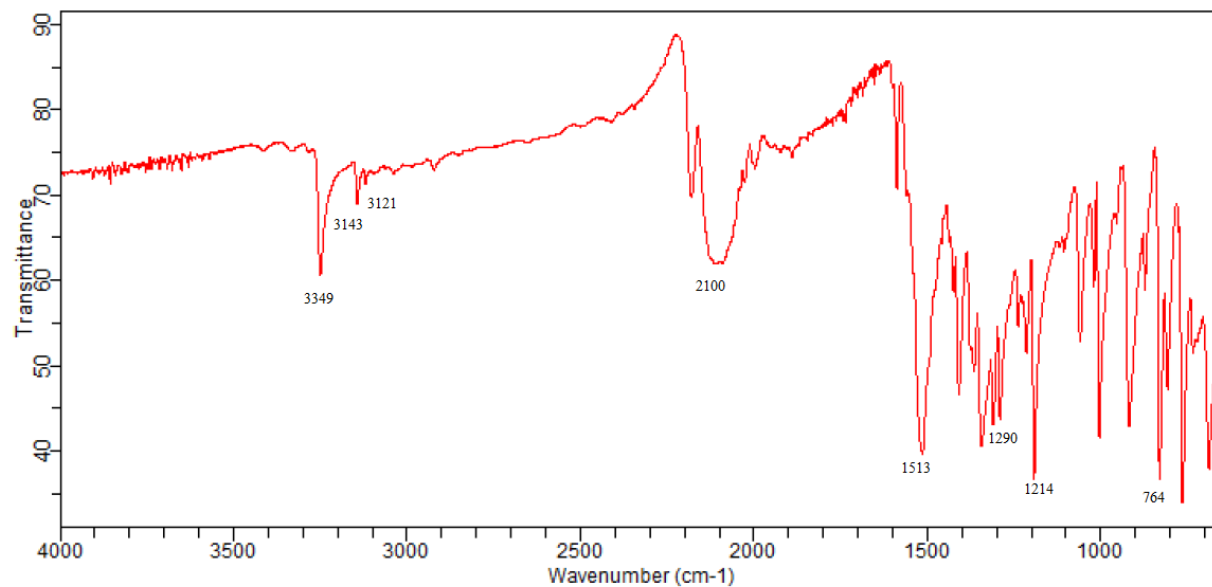


Figure 161, IR of SB82.

UPLC-MS [M+1] calcd for C₁₂H₁₀N₄S₂ 275.36; found 274.23.

SB83

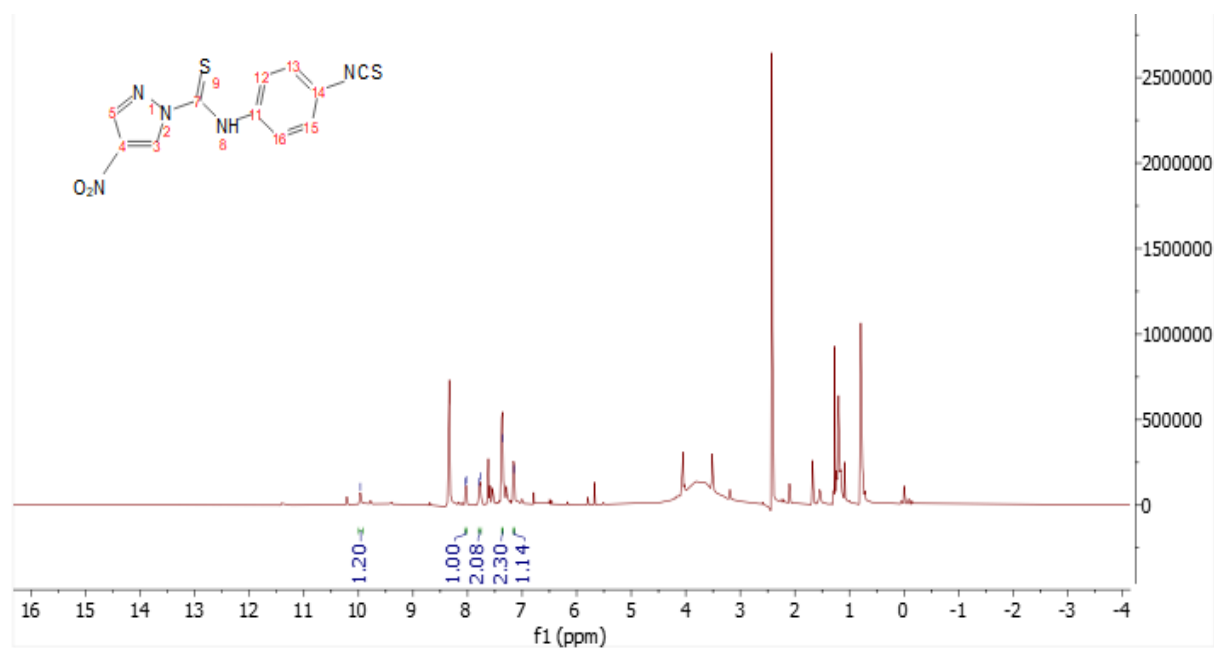


Figure 162, ¹H NMR of SB83.

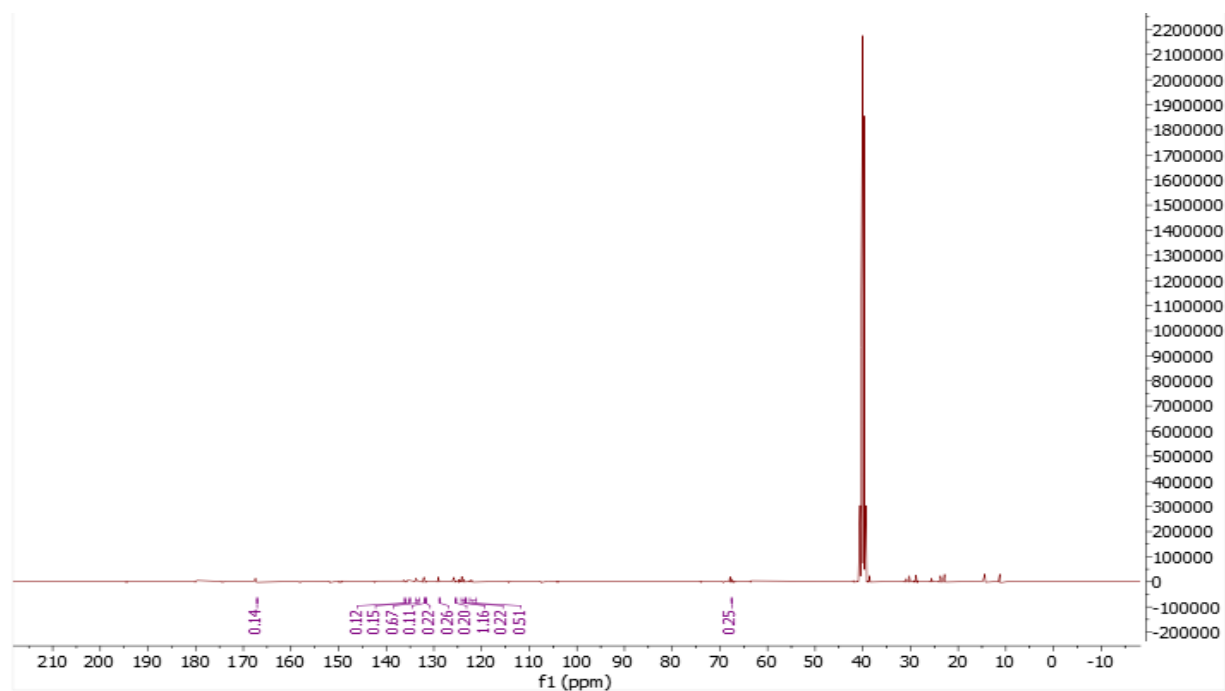


Figure 163, ¹³C NMR of SB83.

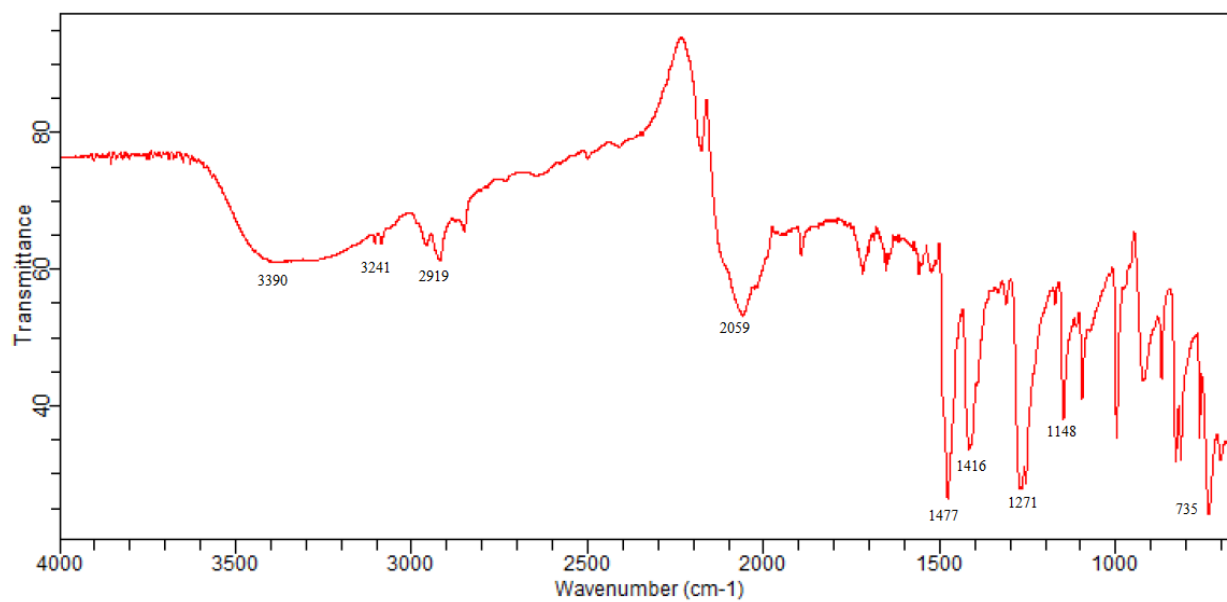


Figure 164, IR of SB83.

UPLC-MS [M+1] calcd for C₁₁H₇N₅O₂S₂ 306.34; found 305.22.

SB84

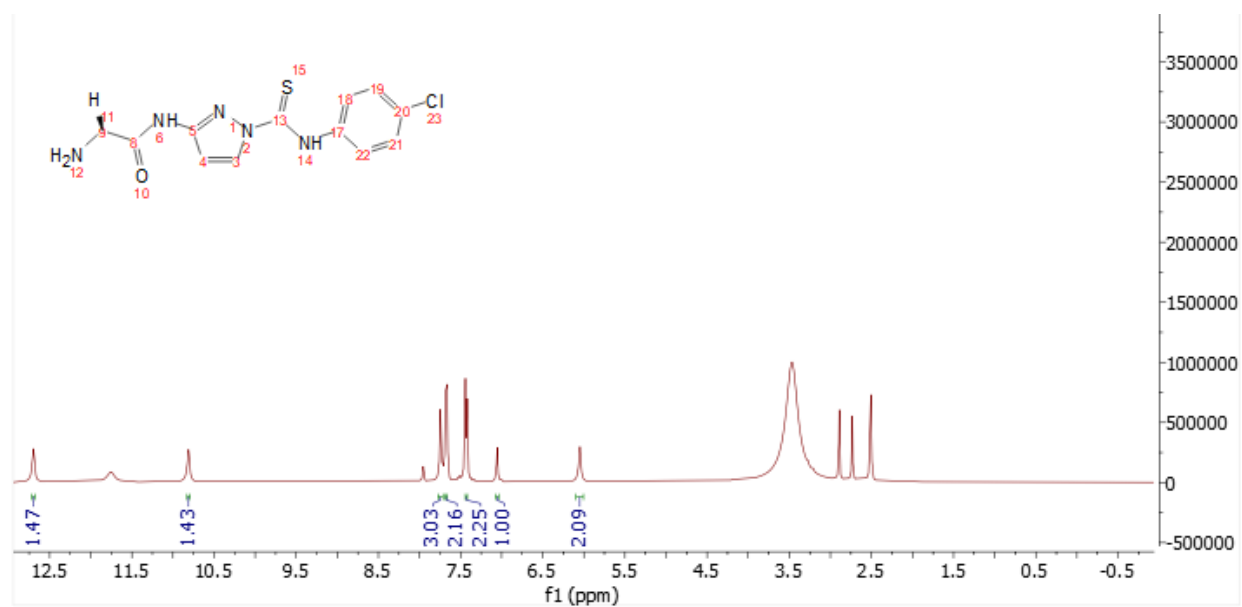


Figure 165, ^1H NMR of SB84.

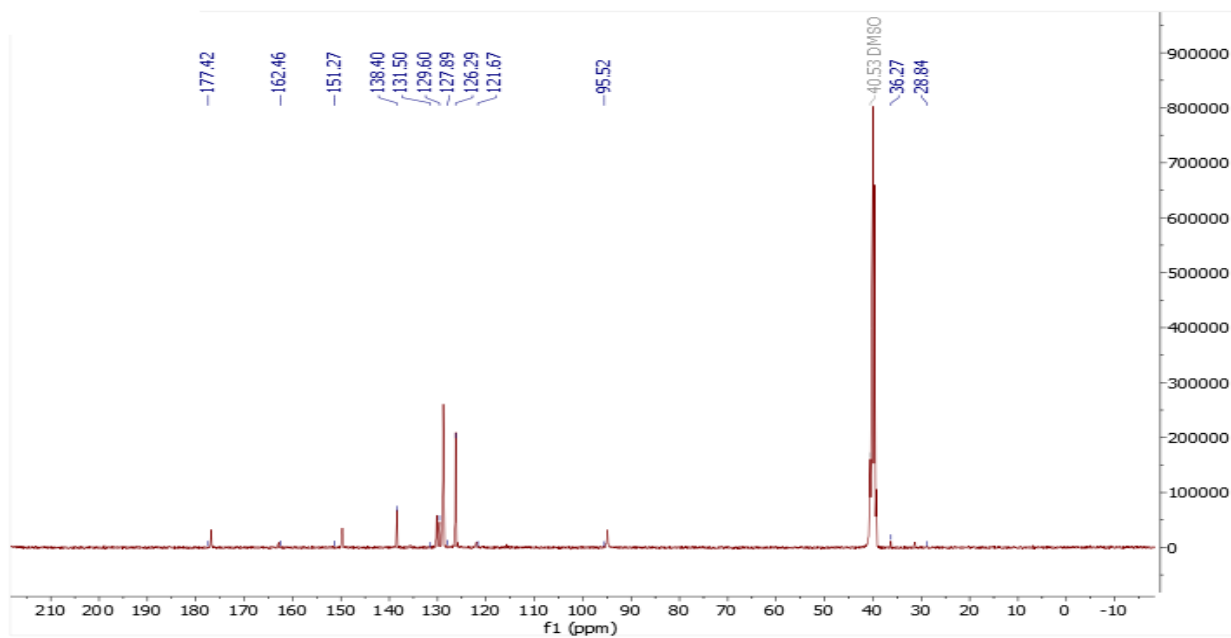


Figure 166, ^{13}C NMR of SB84.

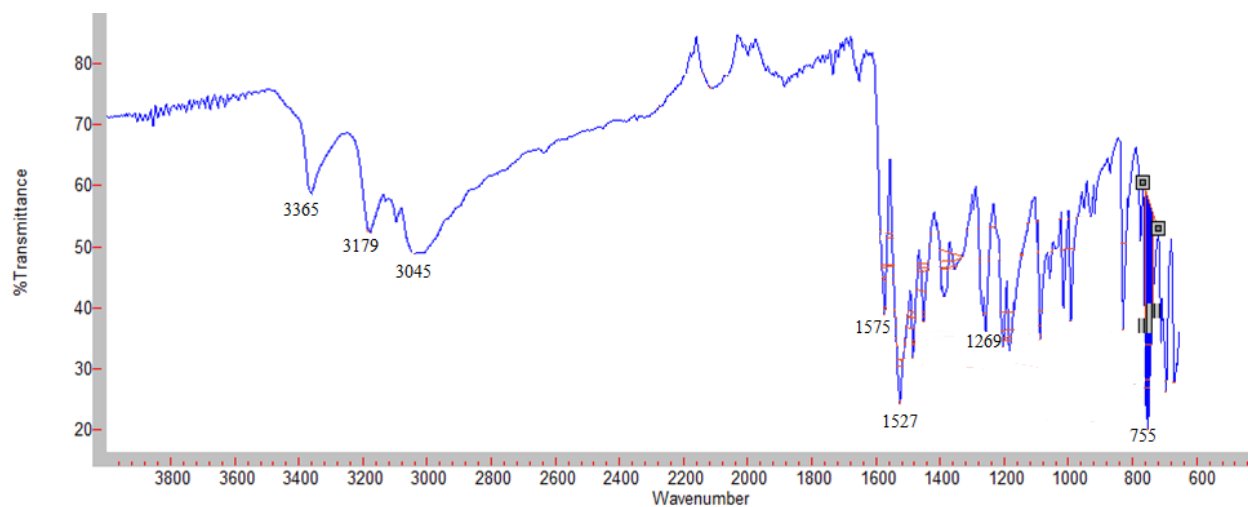


Figure 167, IR of SB84.

UPLC-MS [M+1] calcd for C₁₂H₁₂ClN₅OS 310.77; found 310.29.

SB85

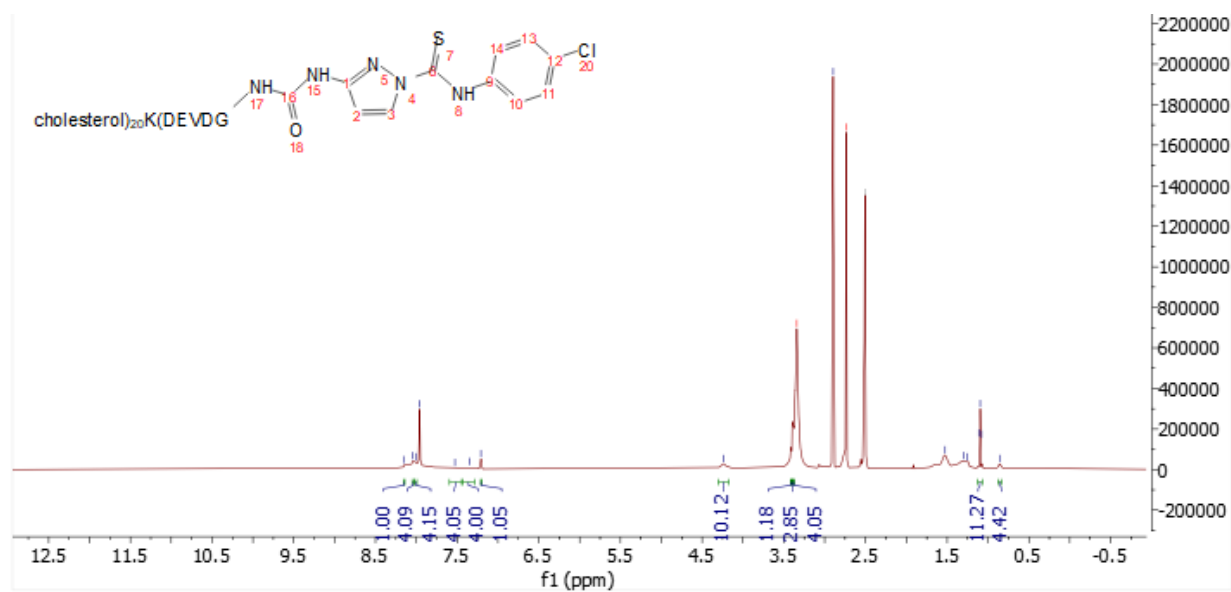


Figure 168, ¹H NMR of SB85.

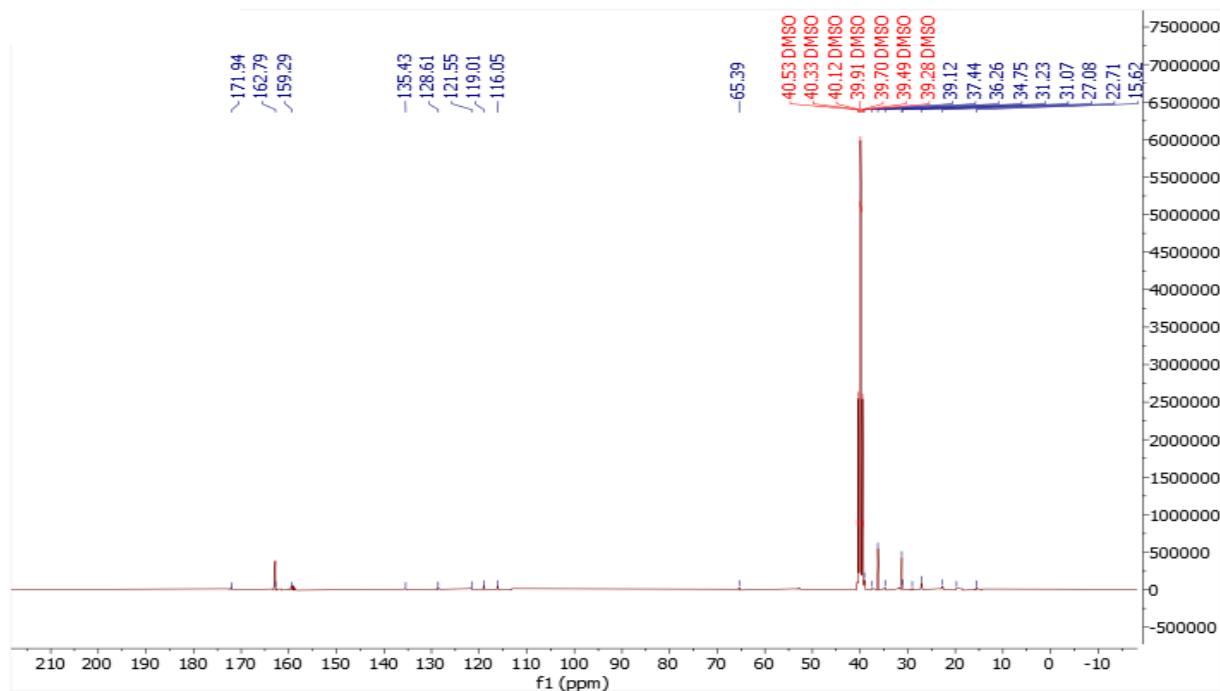


Figure 169, ¹³C NMR of SB85.

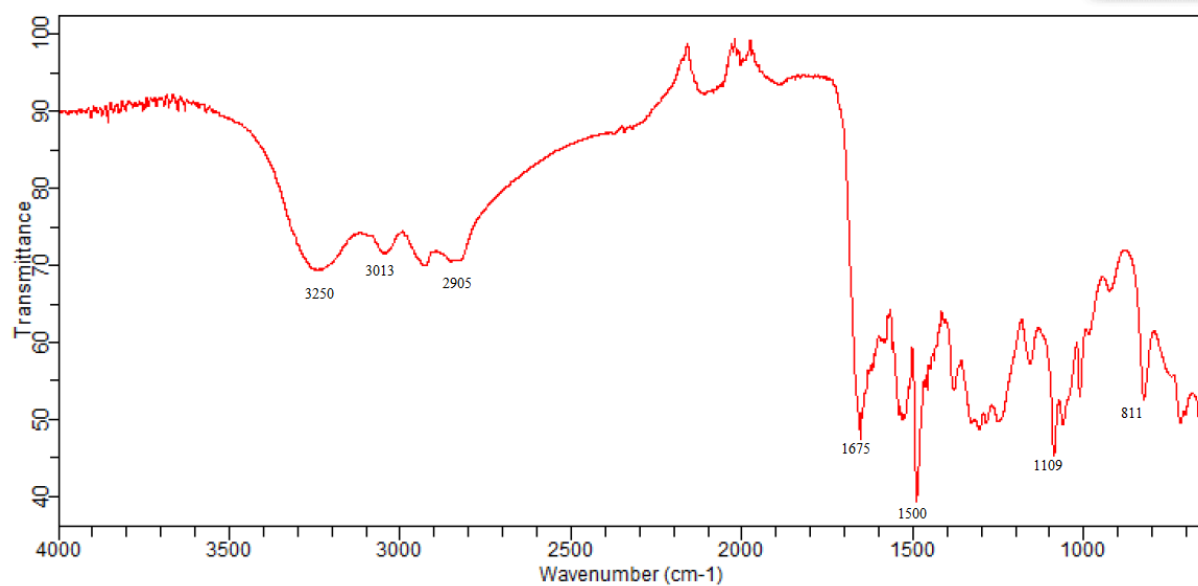


Figure 170, IR of SB85.

SB86

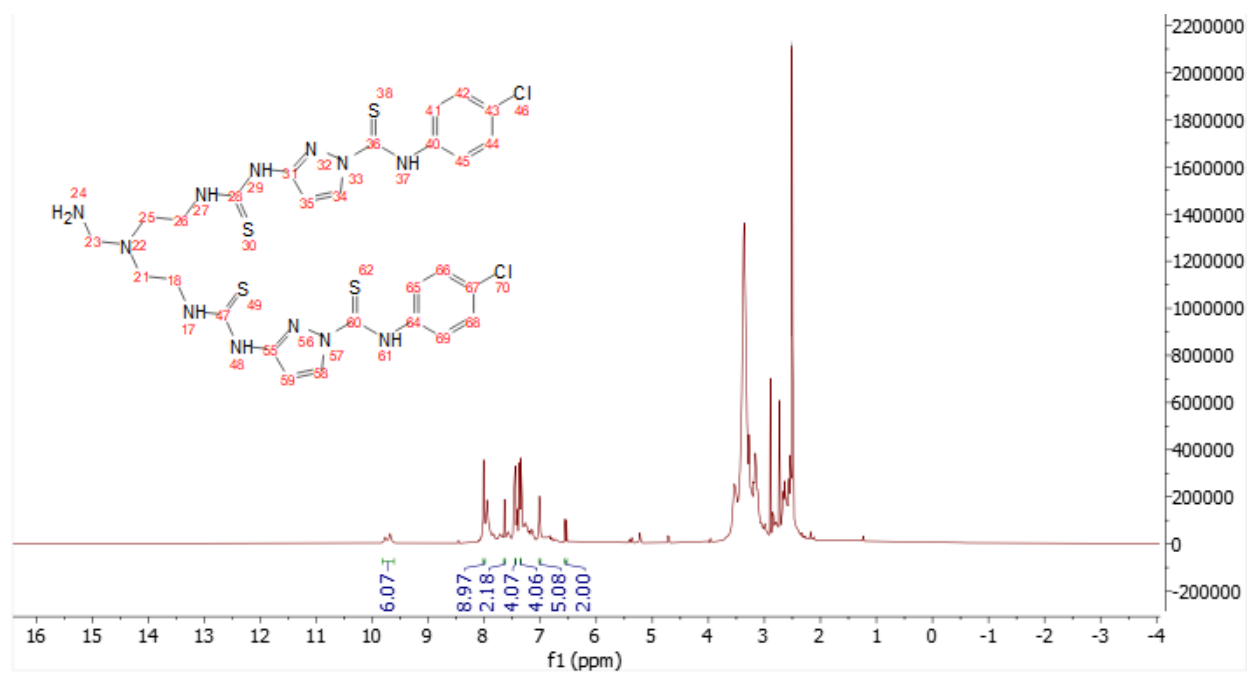


Figure 171, ¹H NMR of SB86.

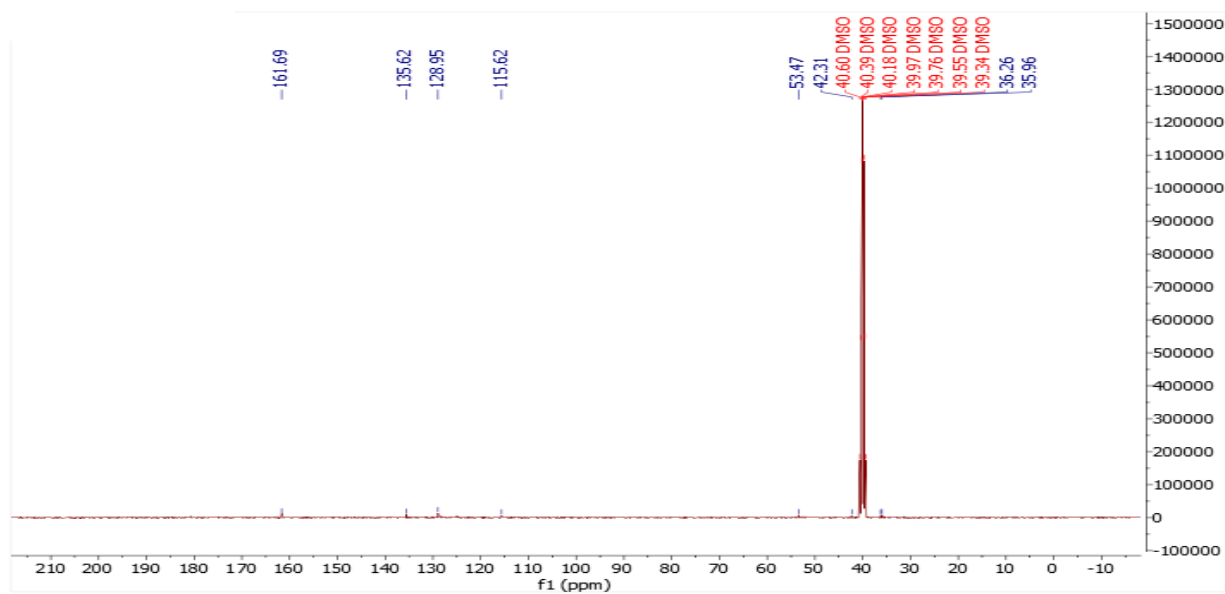


Figure 172, ¹³C NMR of SB86.

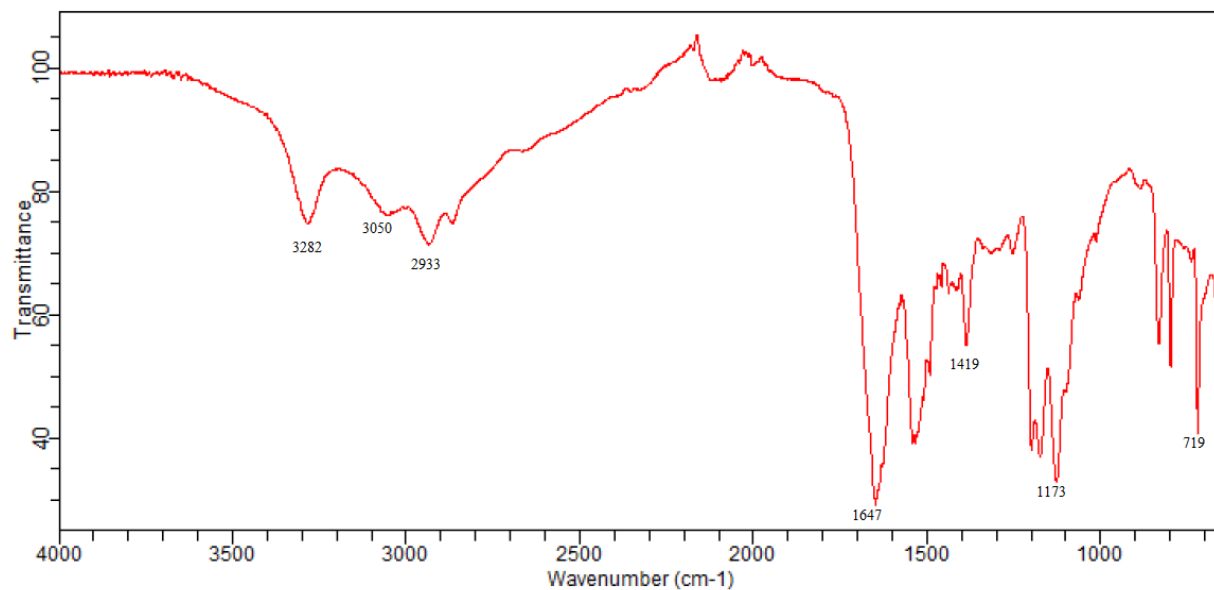


Figure 173, IR of SB86.

HRMS (+ESI-TOF) m/z: [M⁺] calcd for C₂₈H₃₂Cl₂N₁₂O₂S₂ 703.66600; found 703.66636.

SB87

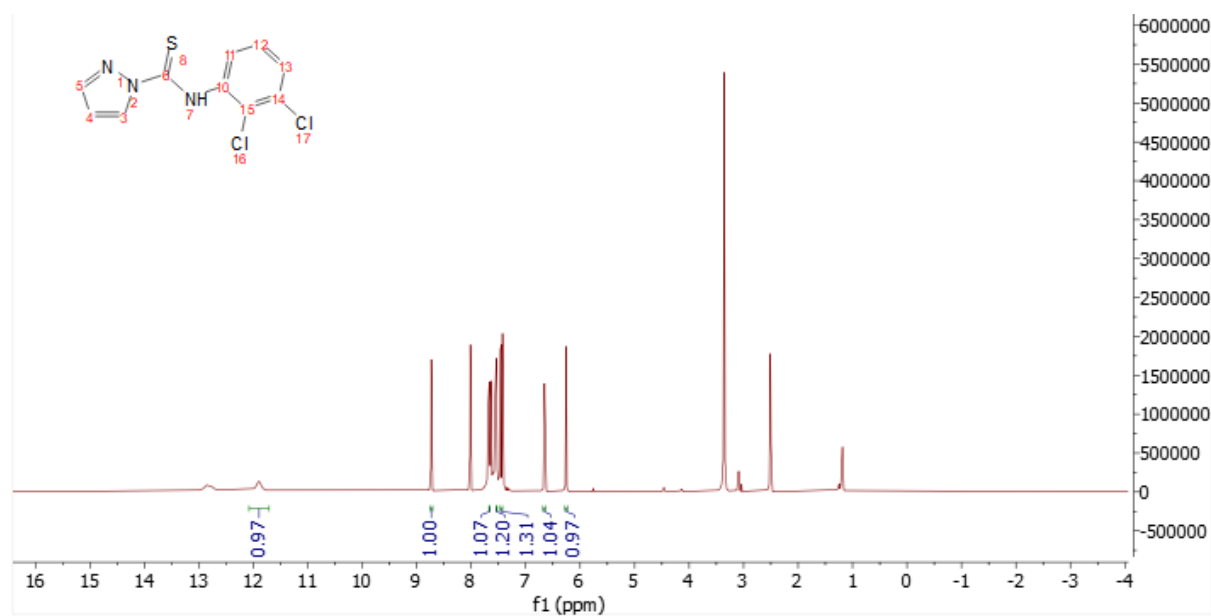


Figure 174, ¹H NMR of SB87.

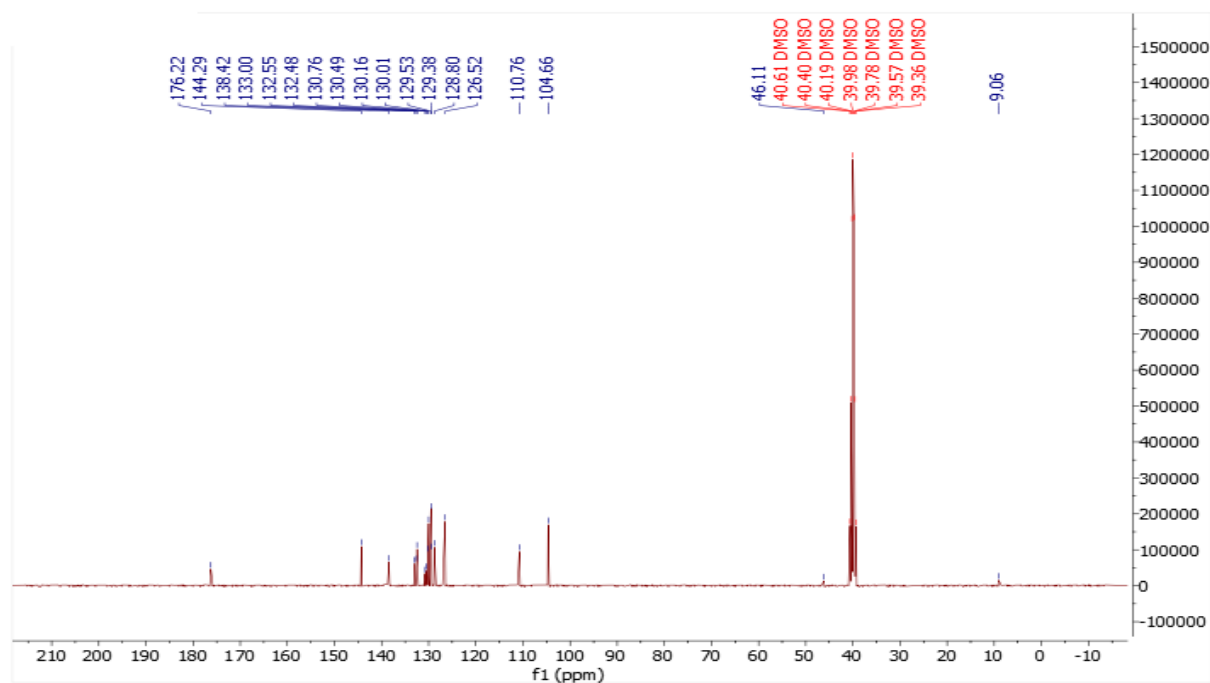


Figure 175, ^{13}C NMR of SB87.

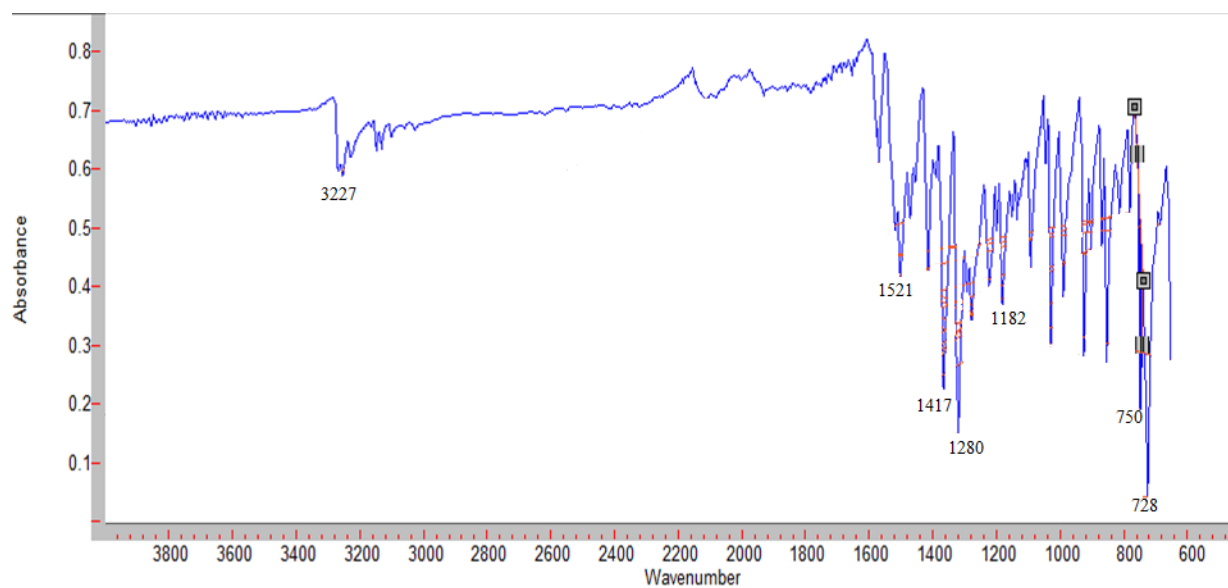


Figure 176, IR of SB87.

HRMS (+ESI-TOF) m/z : $[M+]$ calcd for $\text{C}_{10}\text{H}_7\text{Cl}_2\text{N}_3\text{S}$ 270.97377; found 270.97381.

SB88

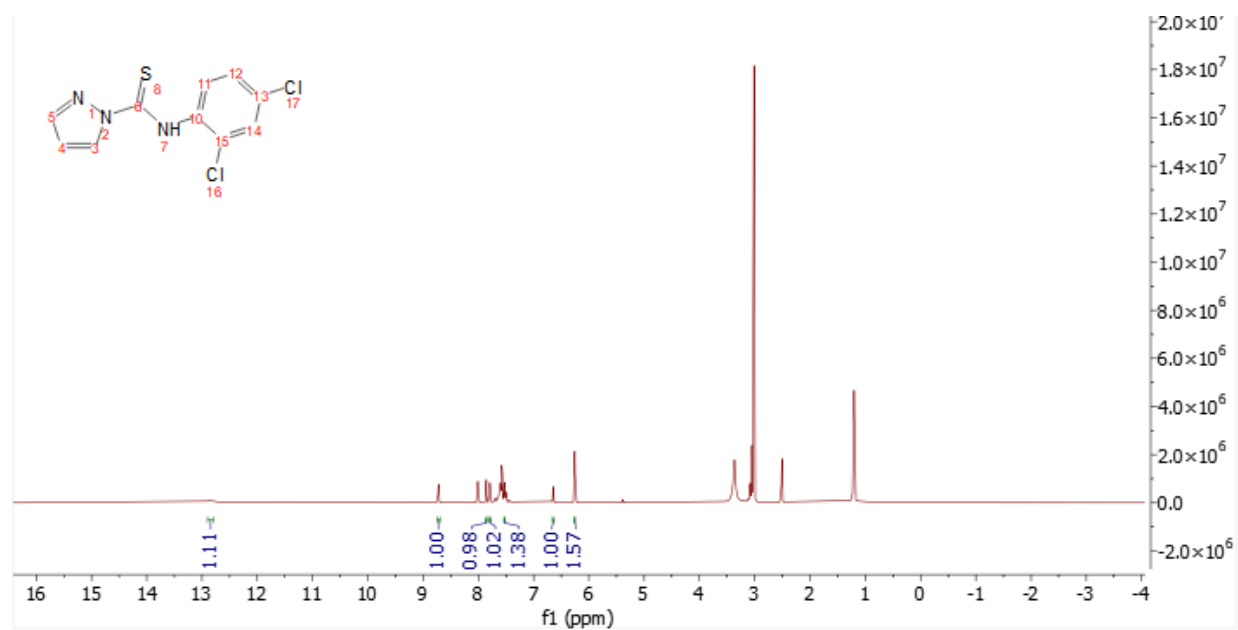


Figure 177, ¹H NMR of SB88.

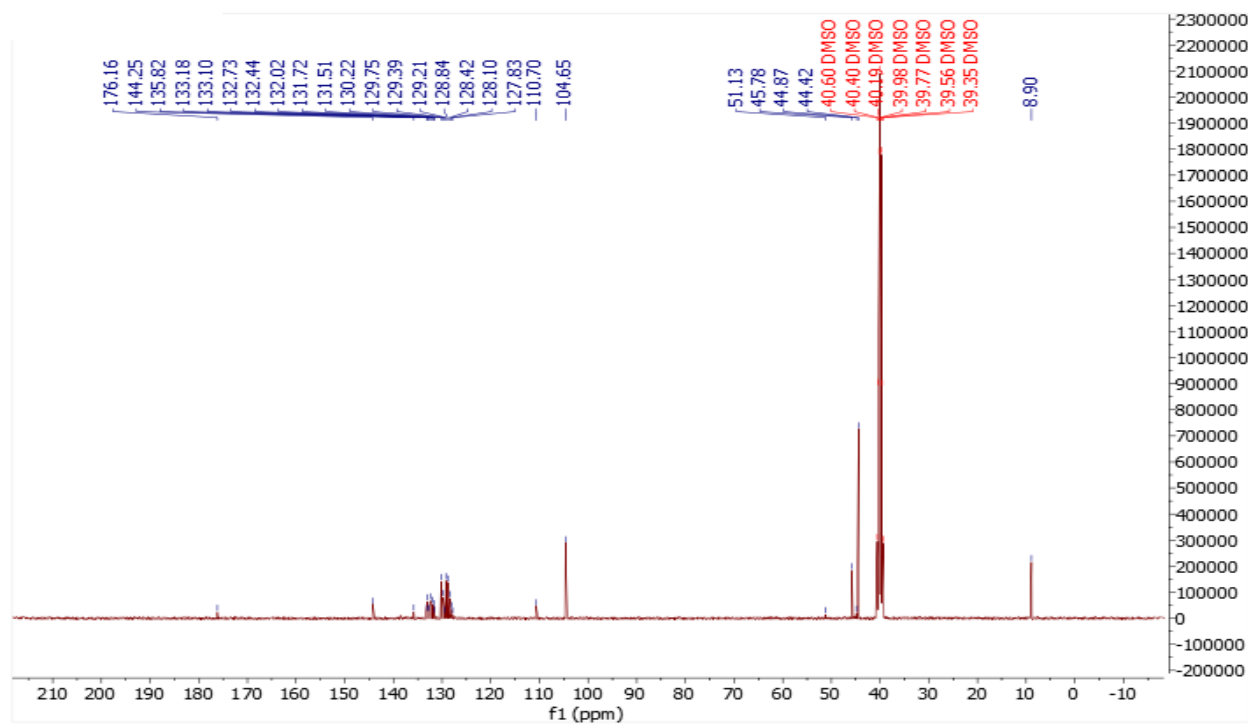


Figure 178, ¹³C NMR of SB88.

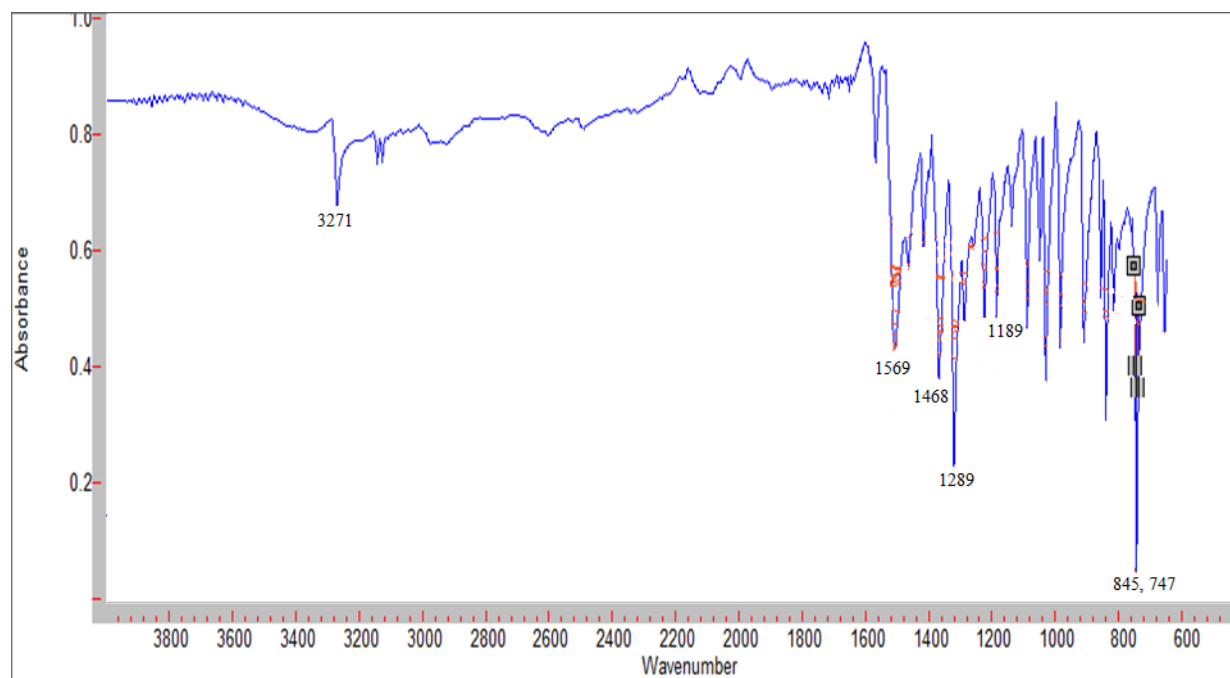


Figure 179, IR of SB88.

HRMS (+ESI-TOF) m/z: [M⁺] calcd for C₁₀H₇Cl₂N₃S 270.97377; found 270.97344.

SB89

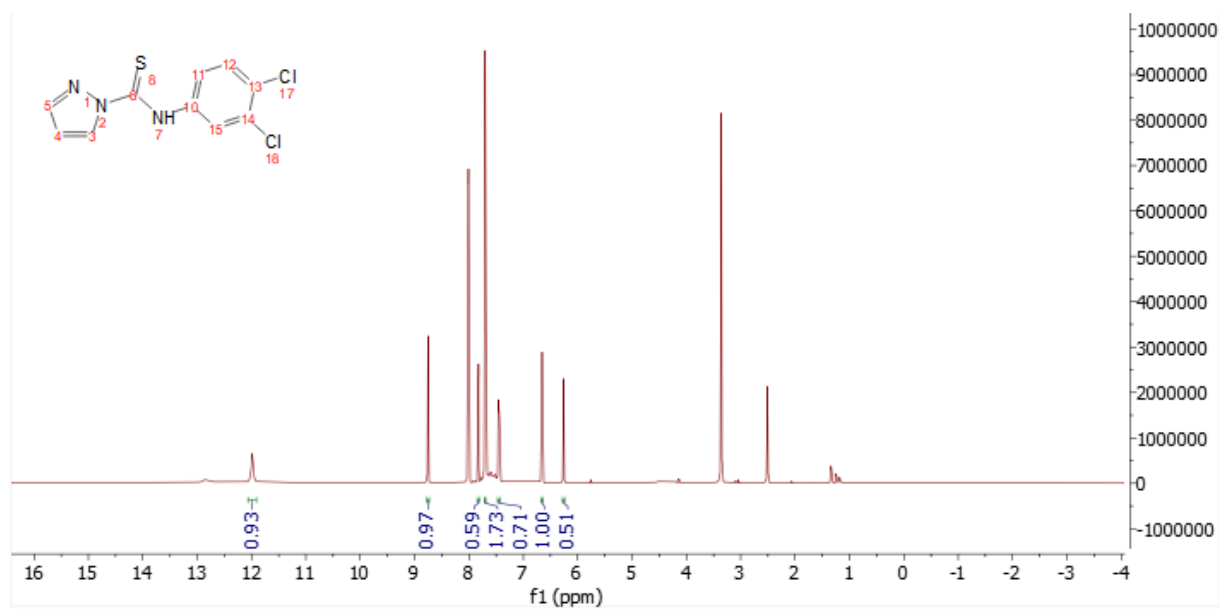


Figure 180, ¹H NMR of SB89.

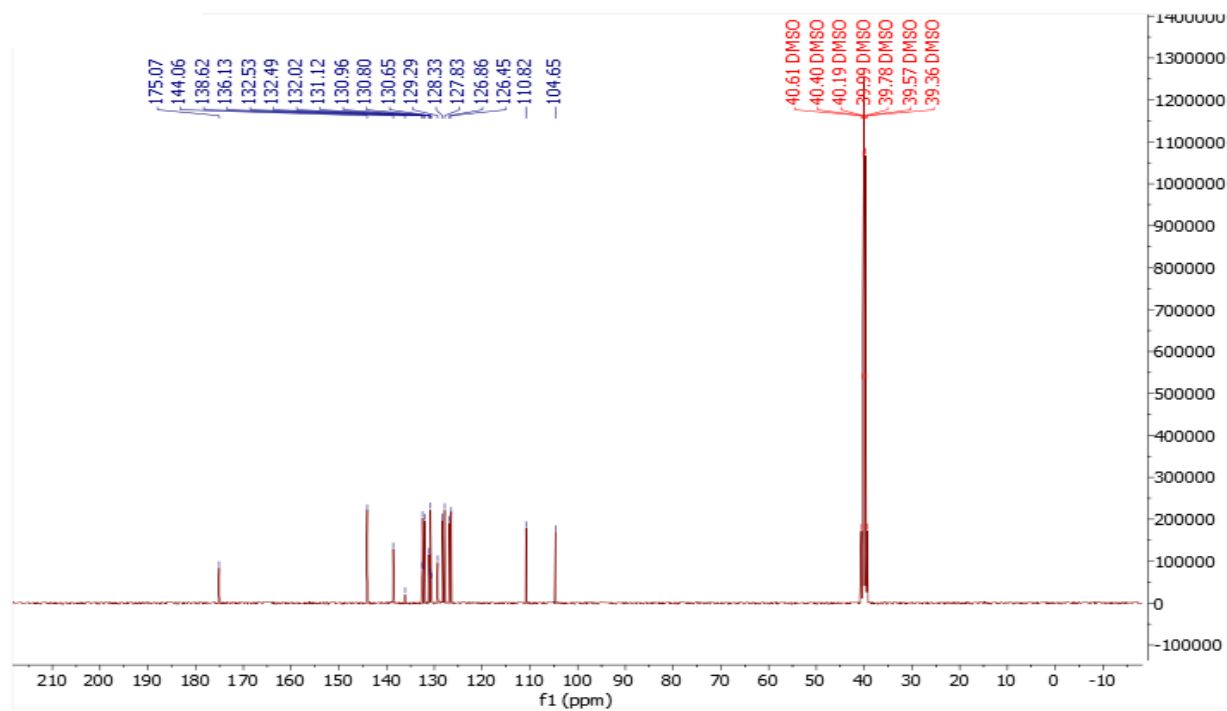


Figure 181, ¹³C NMR of SB89.

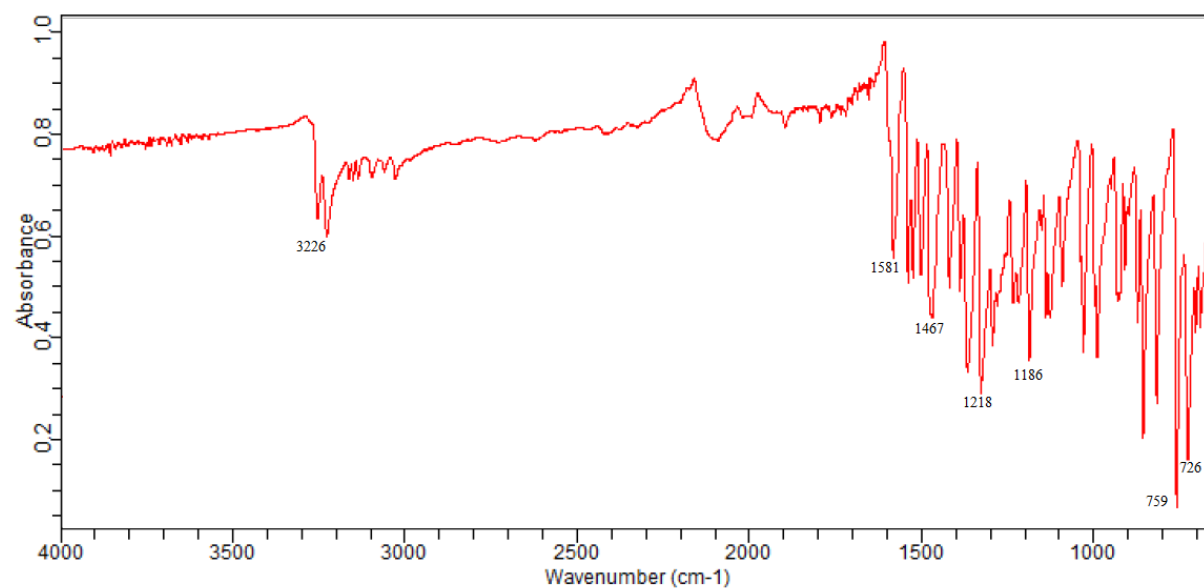


Figure 182, IR of SB89.

HRMS (+ESI-TOF) m/z: [M⁺] calcd for C₁₀H₇Cl₂N₃S 270.97377; found 270.97359.

SB90

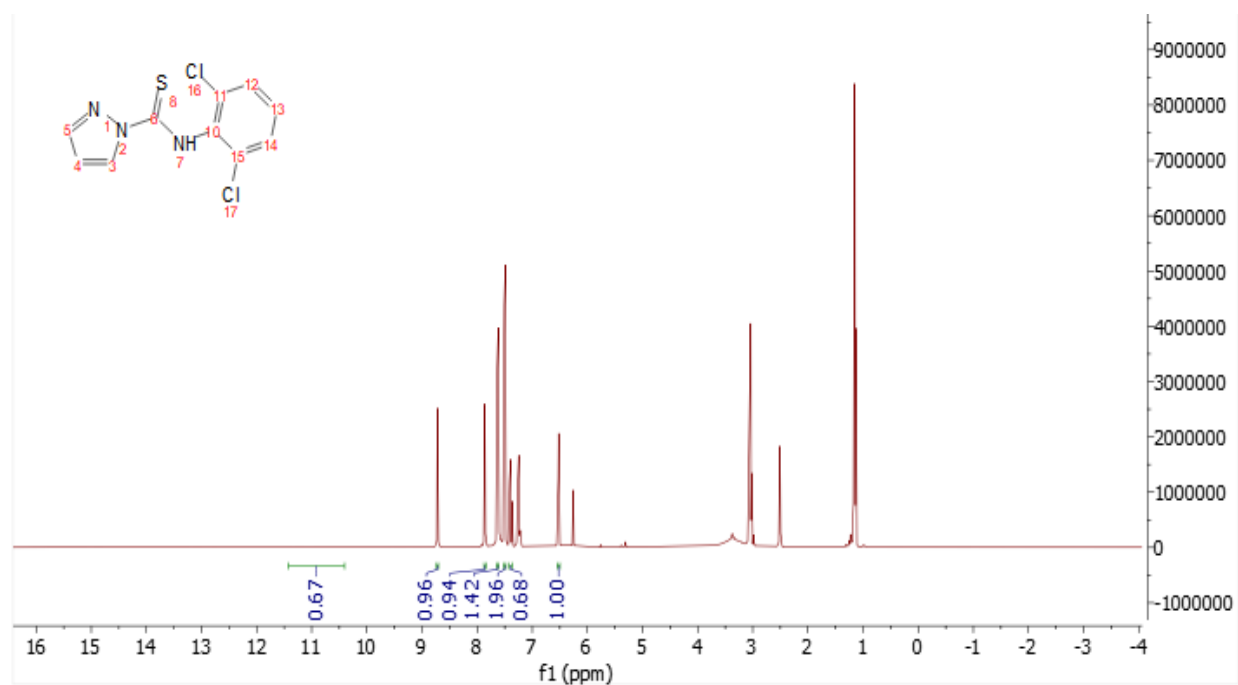


Figure 183, ^1H NMR of SB90.

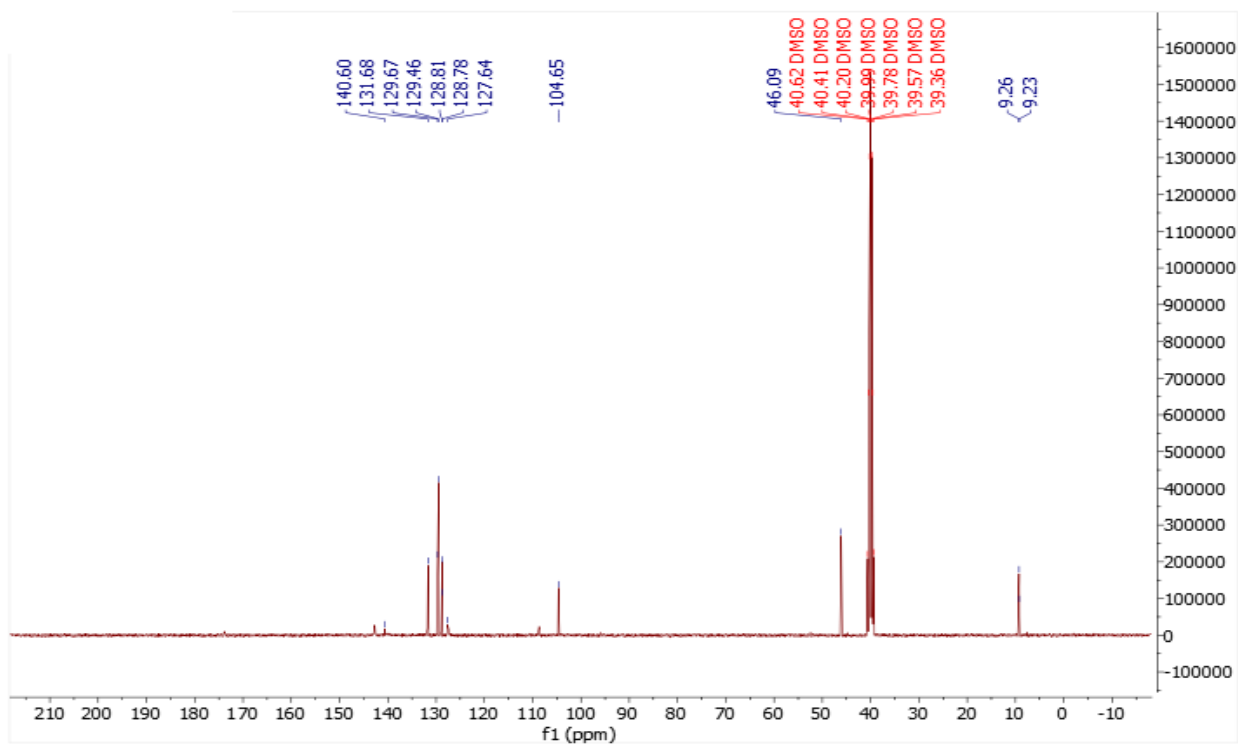


Figure 184, ^{13}C NMR of SB90.

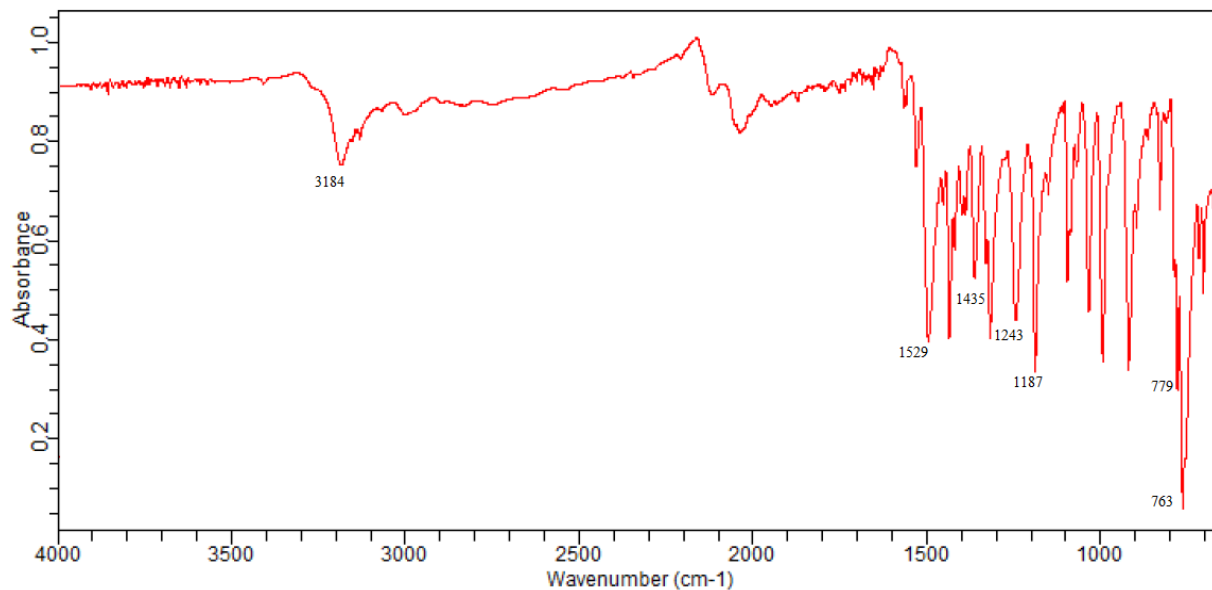


Figure 185, IR of SB90.

HRMS (+ESI-TOF) m/z: [M⁺] calcd for C₁₀H₇Cl₂N₃S 270.97377; found 270.97394.

SB91

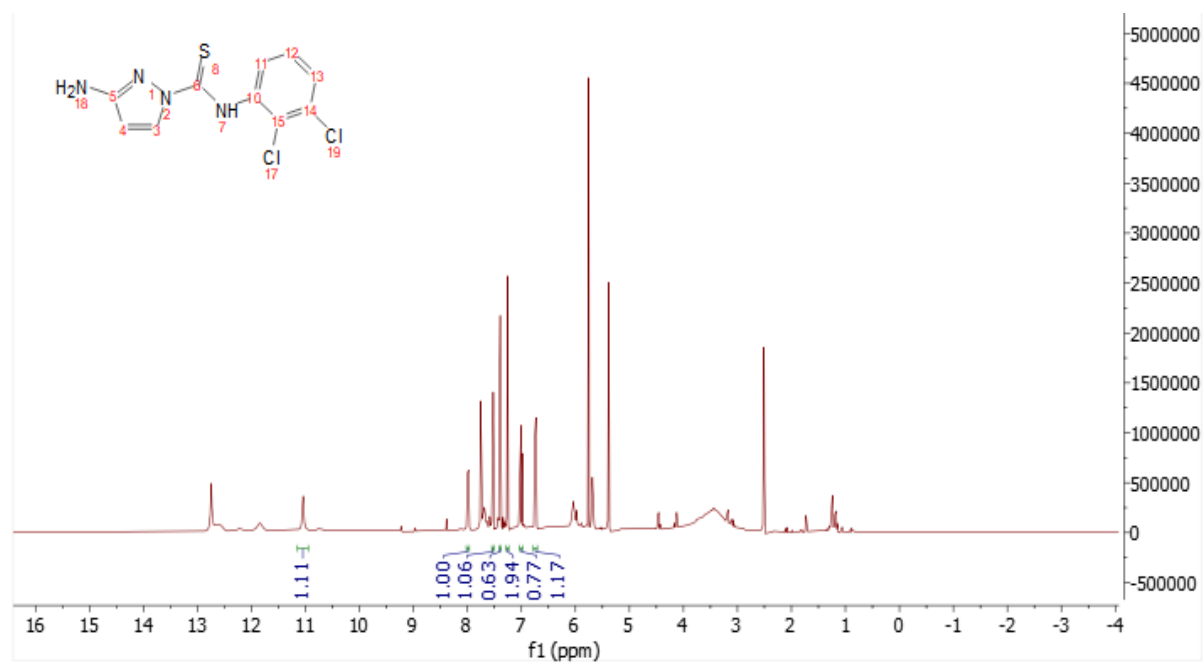


Figure 186, ¹H NMR of SB91.

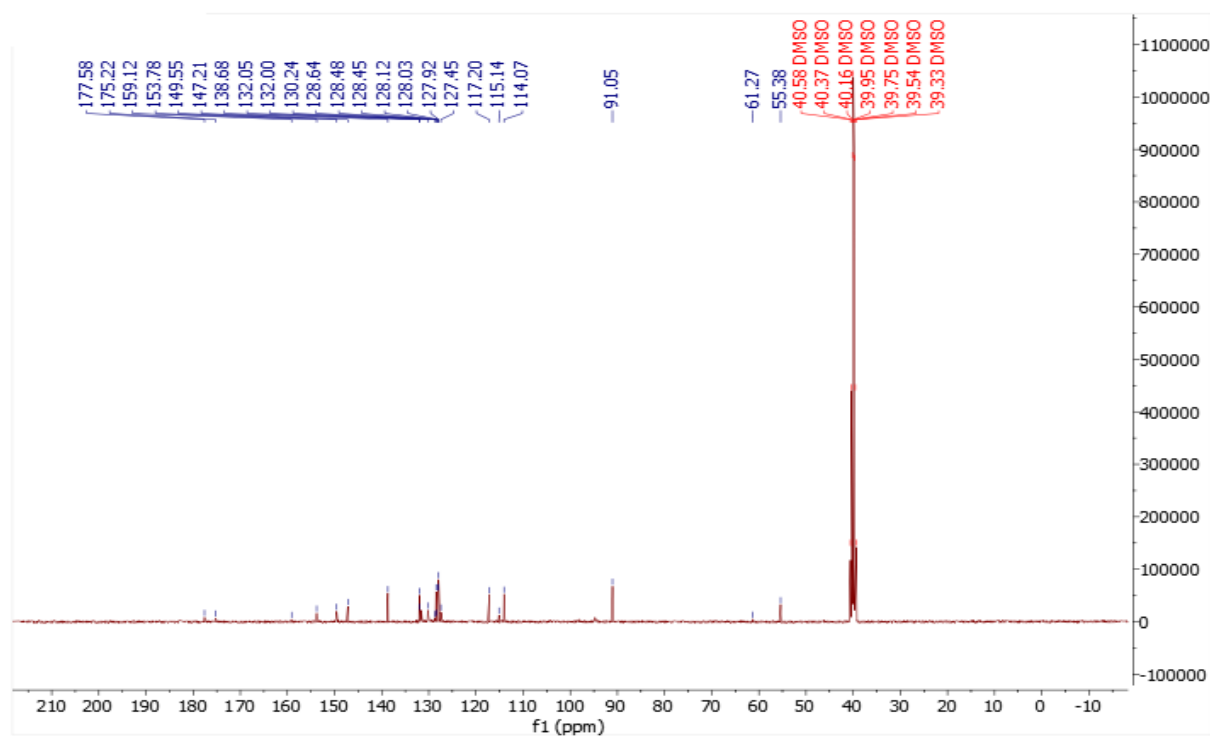


Figure 187, ¹³C NMR of SB91.

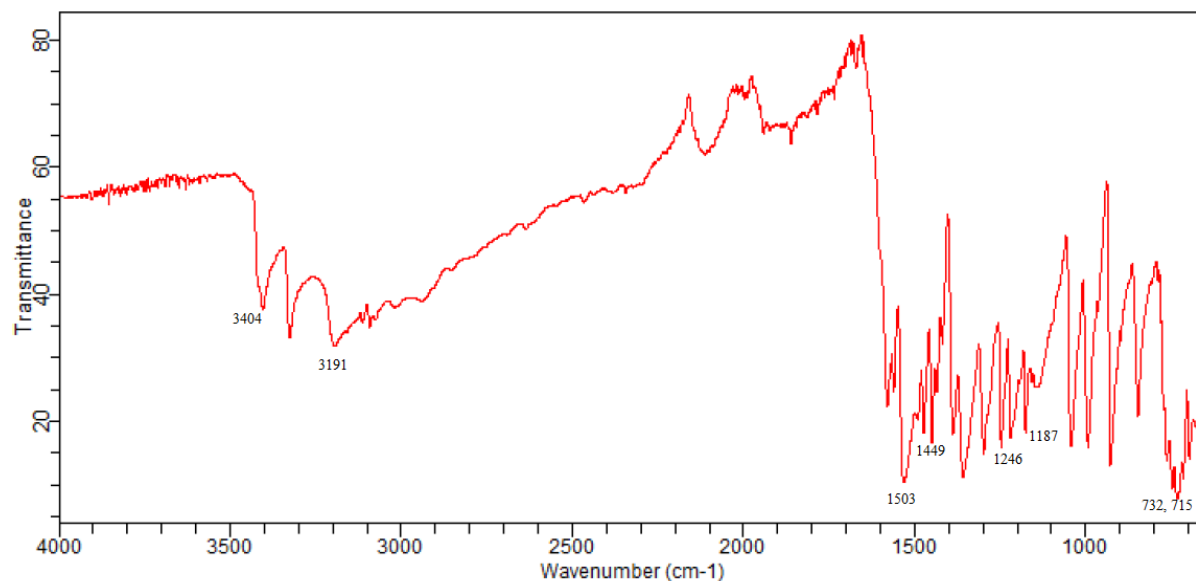


Figure 188, IR of SB91.

HRMS (+ESI-TOF) m/z: [M⁺] calcd for C₁₀H₈Cl₂N₄S 285.98467; found 285.98450.

SB92

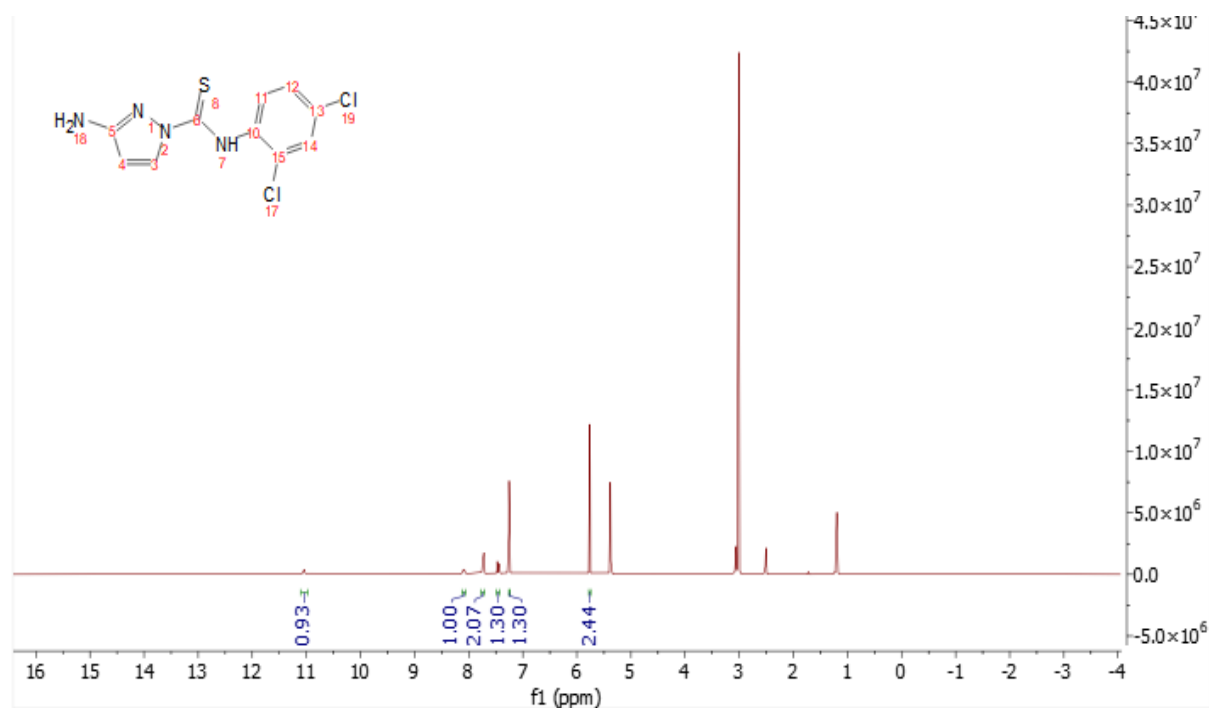


Figure 189, ¹H NMR of SB92.

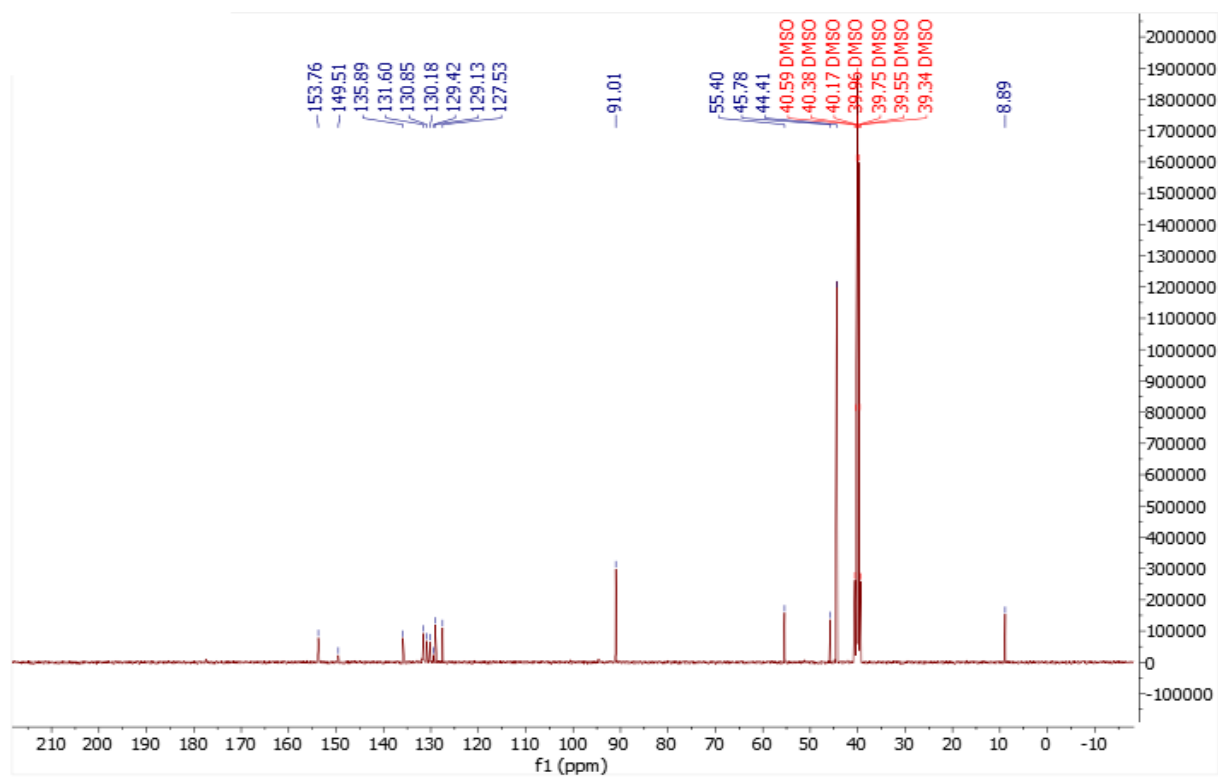


Figure 190, ¹³C NMR of SB92.

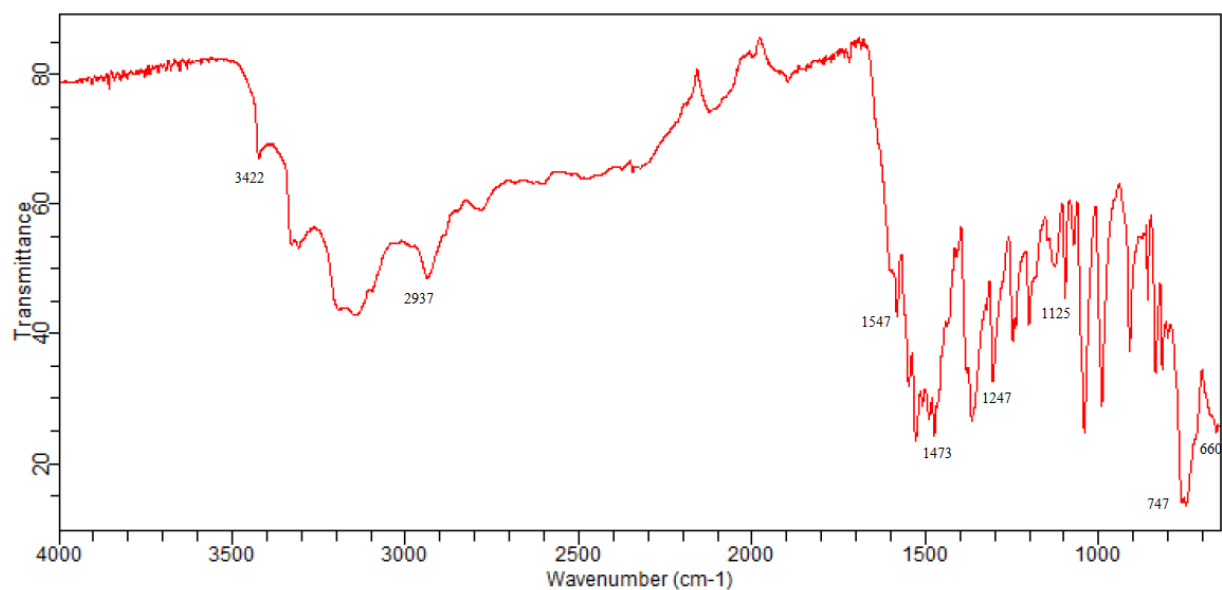


Figure 191, IR of SB92.

HRMS (+ESI-TOF) m/z: [M⁺] calcd for C₁₀H₈Cl₂N₄S 285.98467; found 285.98428.

SB93

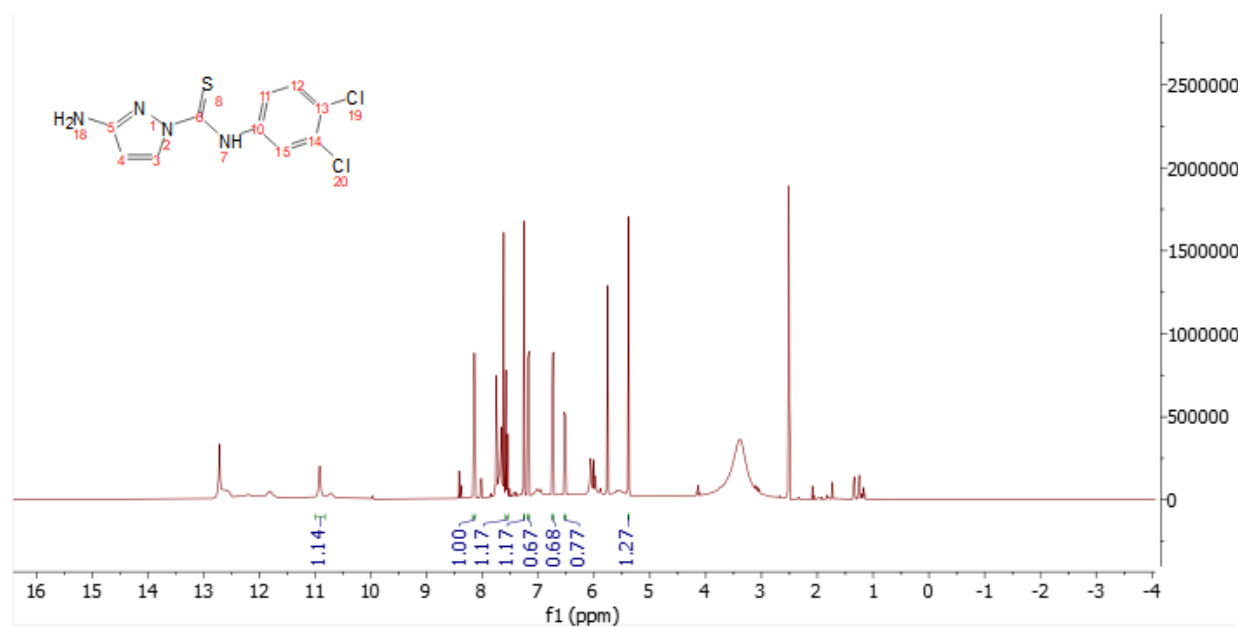


Figure 192, ¹H NMR of SB93.

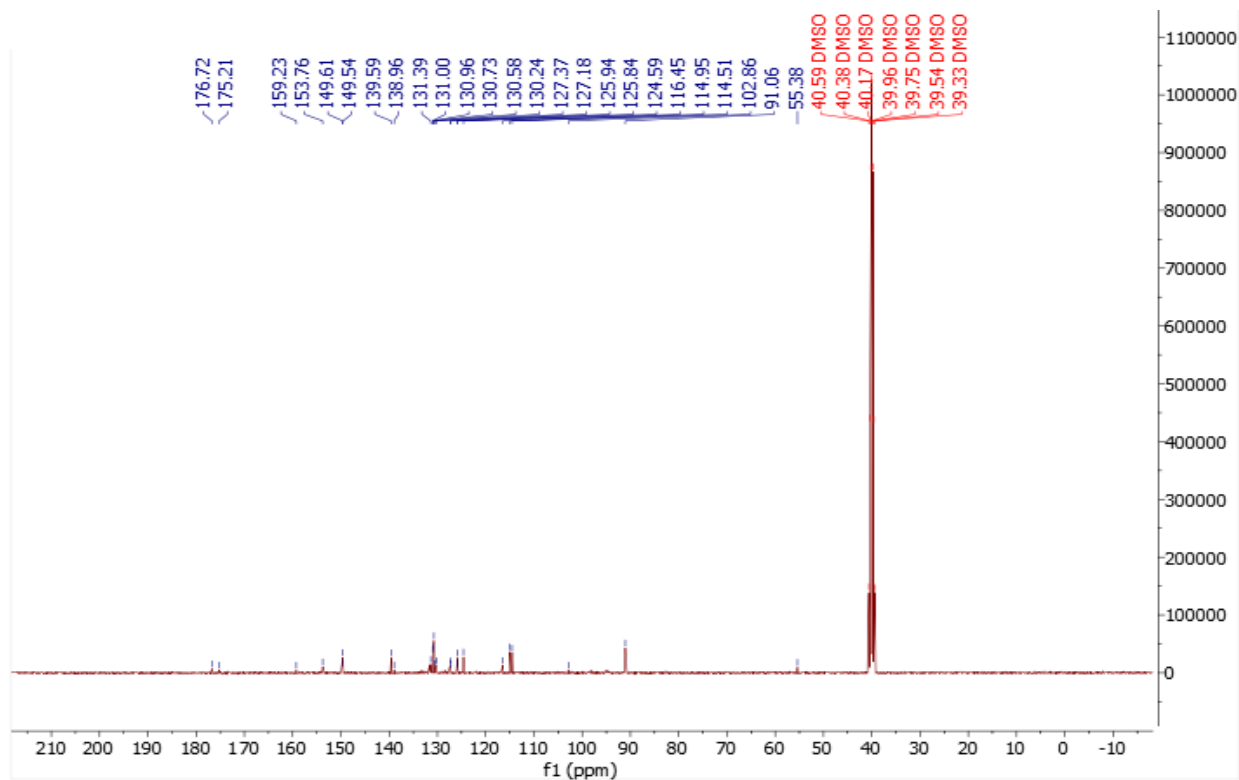


Figure 193, ¹³C NMR of SB93.

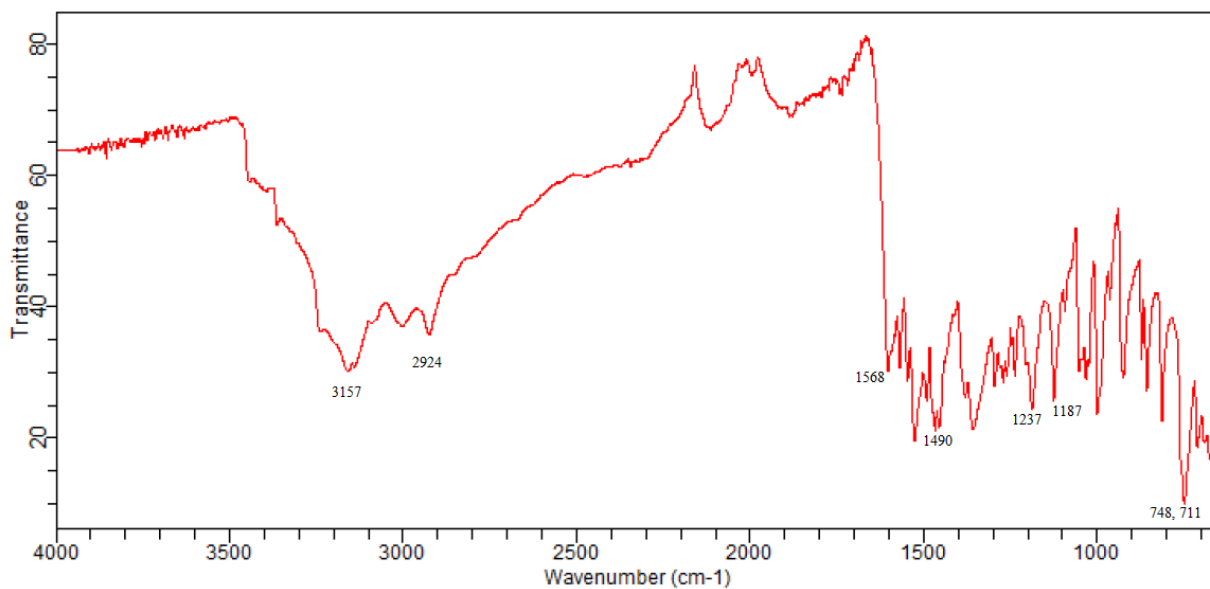


Figure 194, IR of SB93.

HRMS (+ESI-TOF) m/z: [M⁺] calcd for C₁₀H₈Cl₂N₄S 285.98467; found 285.98464.

SB94

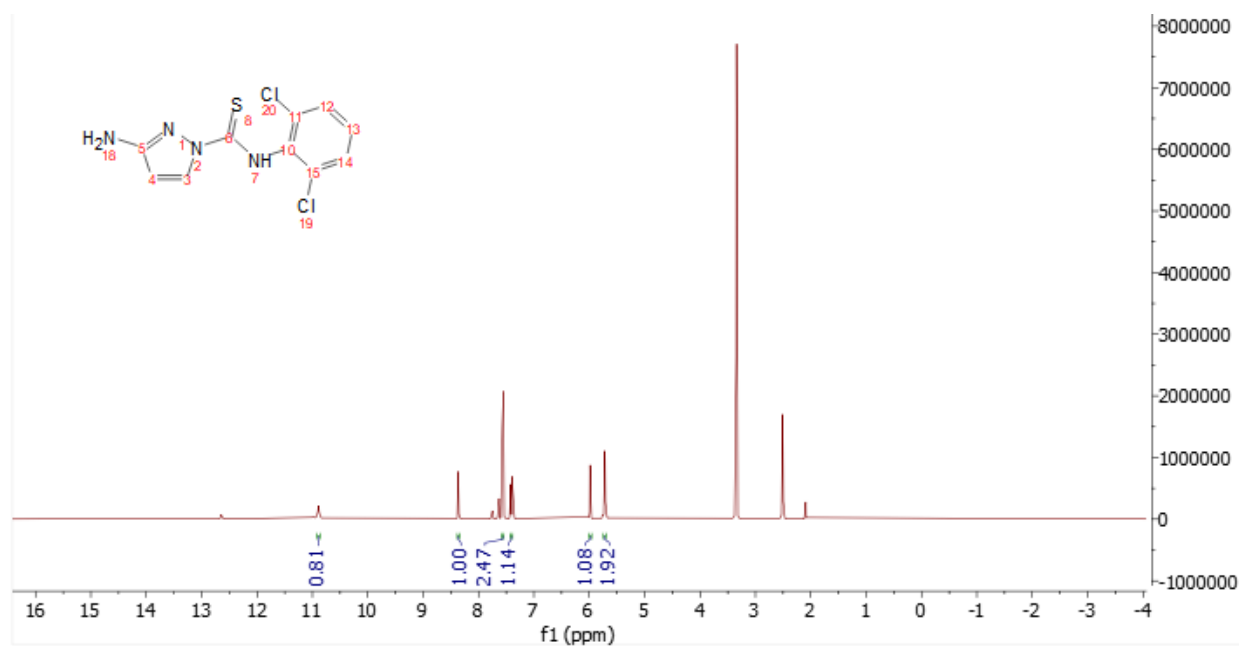


Figure 195, ¹H NMR of SB94.

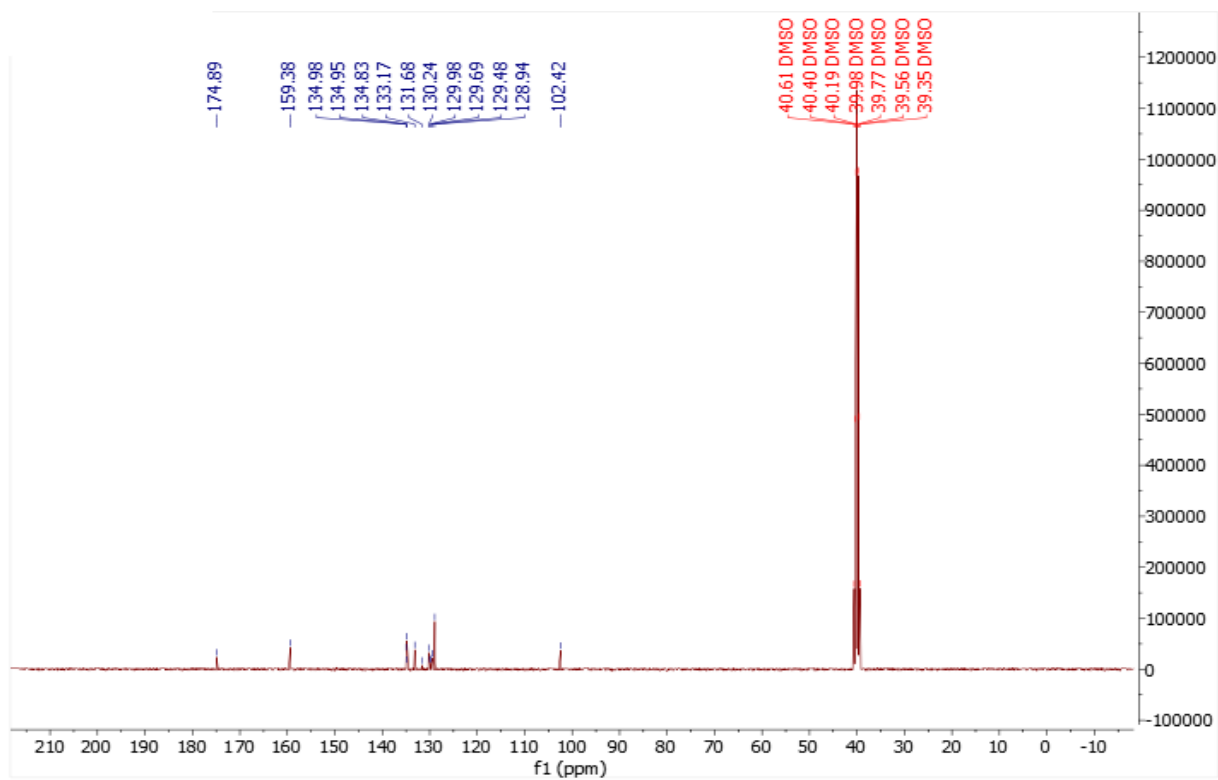


Figure 196, ¹³C NMR of SB94.

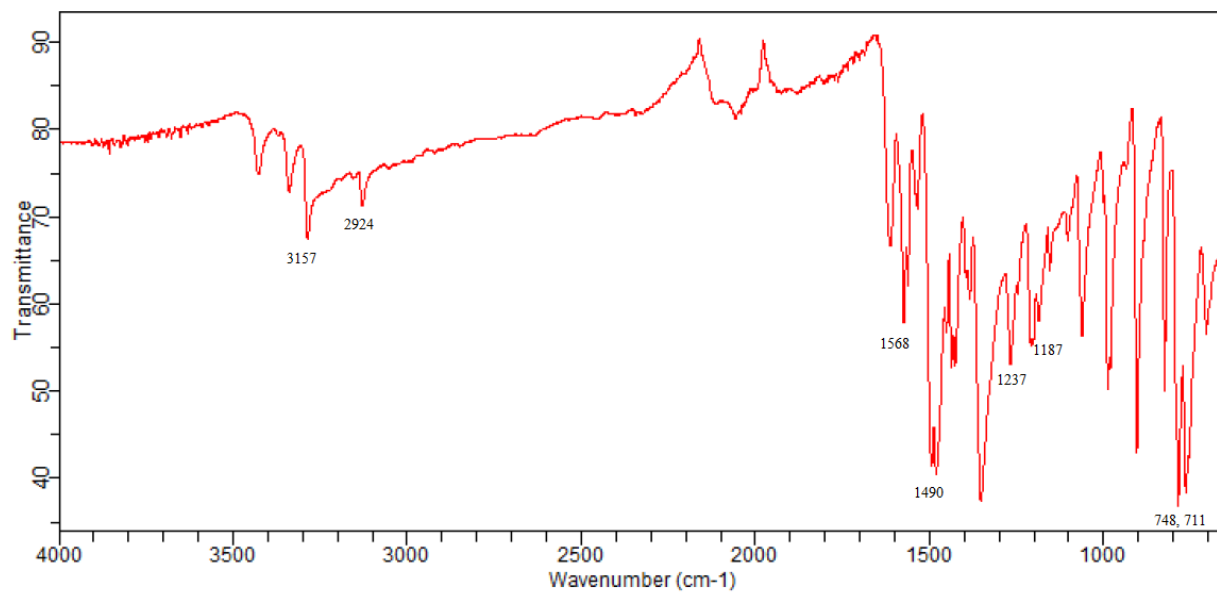


Figure 197, IR of SB94.

HRMS (+ESI-TOF) m/z: [M⁺] calcd for C₁₀H₈Cl₂N₄S 285.98467; found 285.98447.

SB96

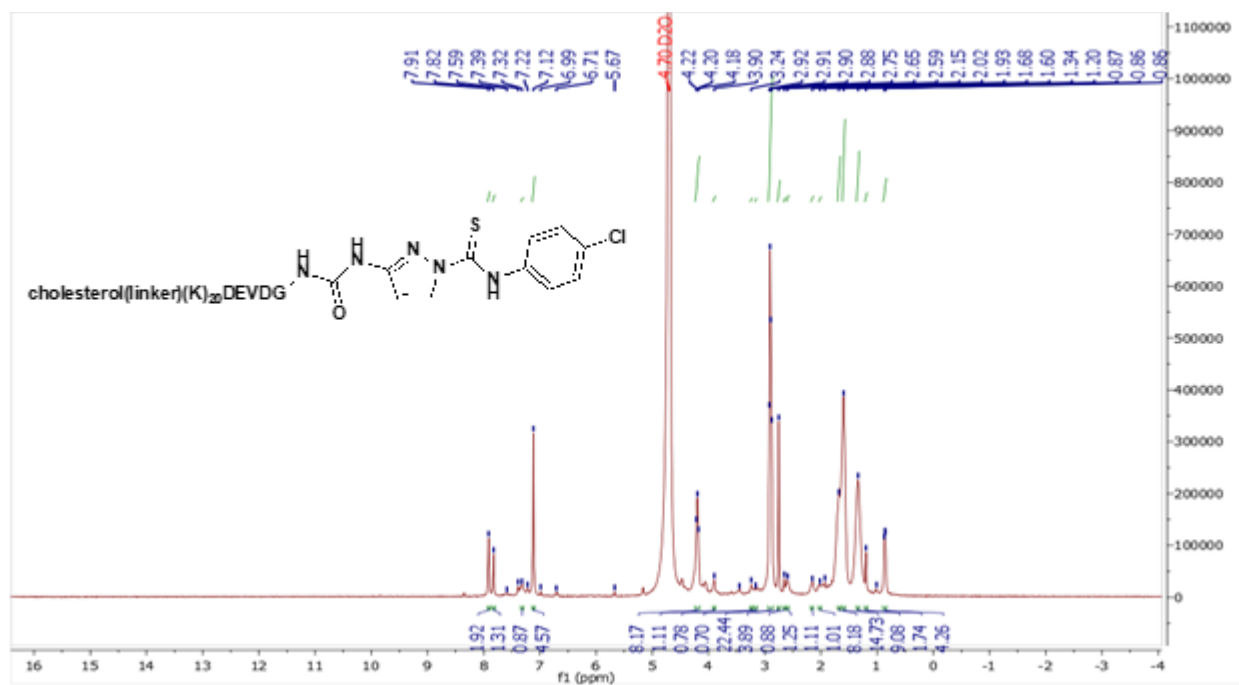


Figure 198, ¹H NMR of SB96.

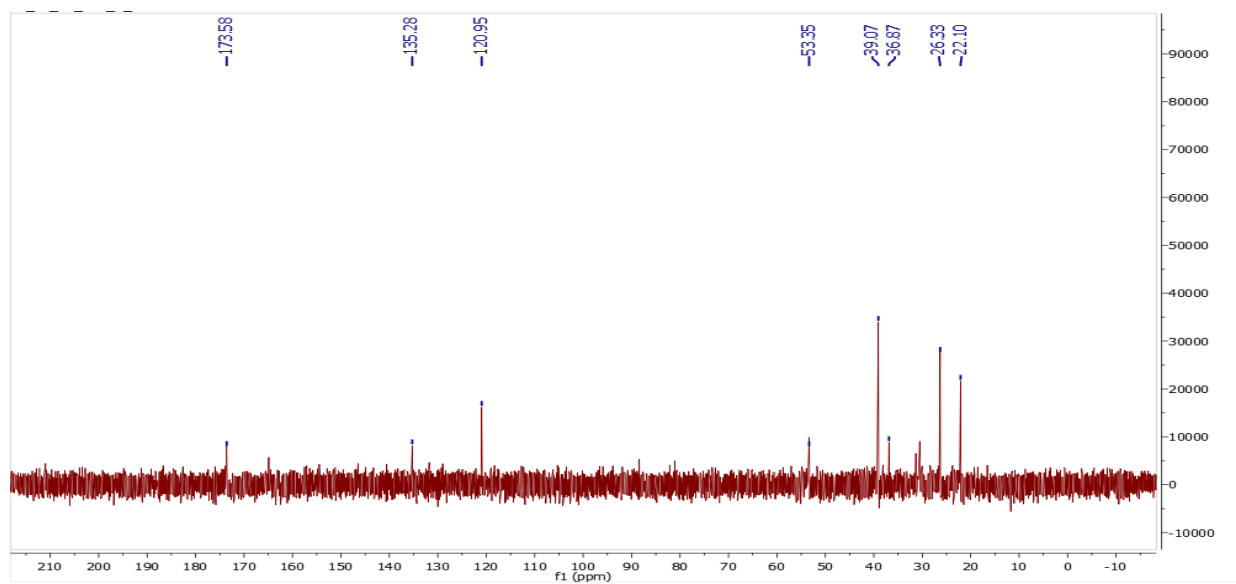


Figure 199, ^{13}C NMR of SB96.

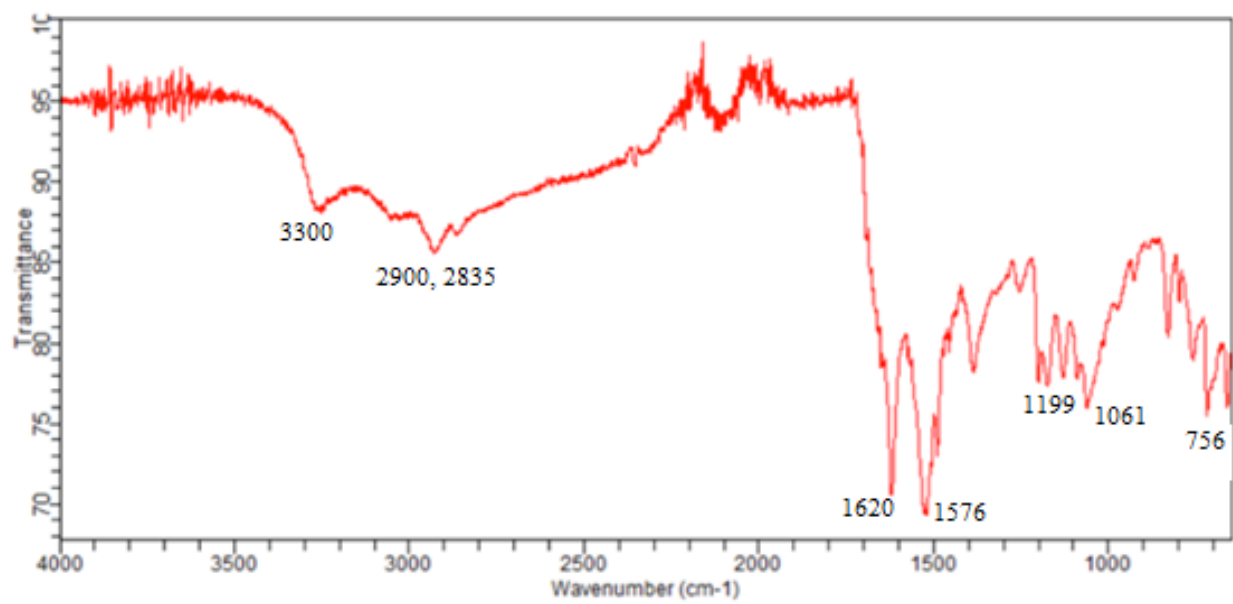


Figure 200, IR of SB96.

SB97

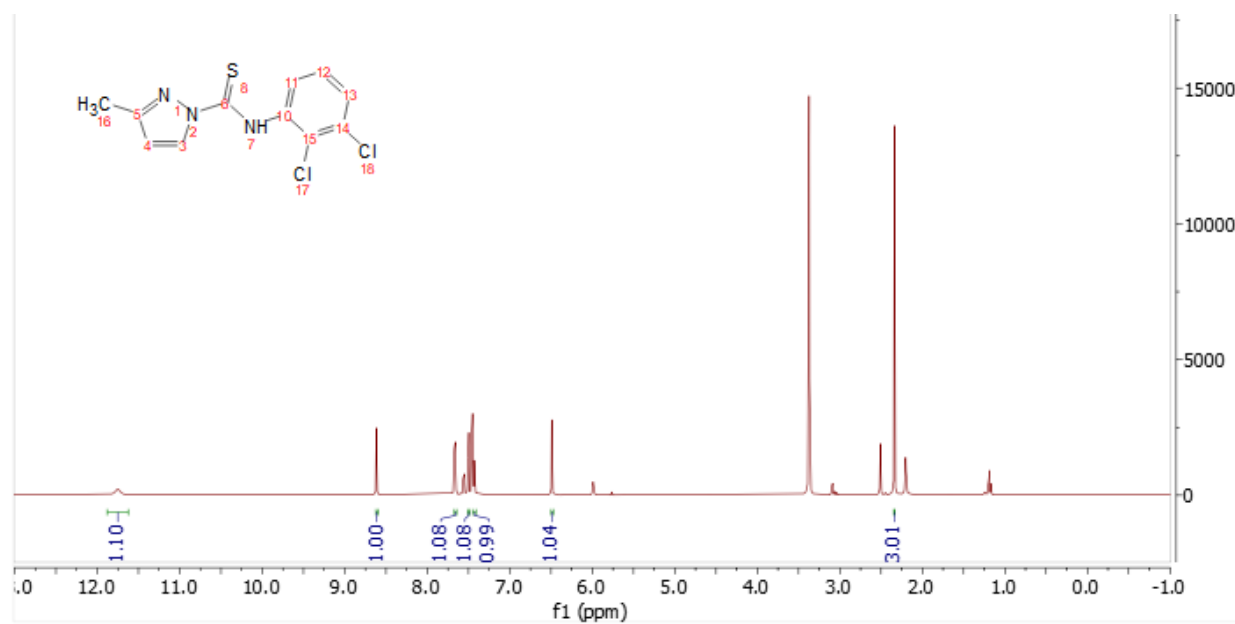


Figure 201, ¹H NMR of SB97.

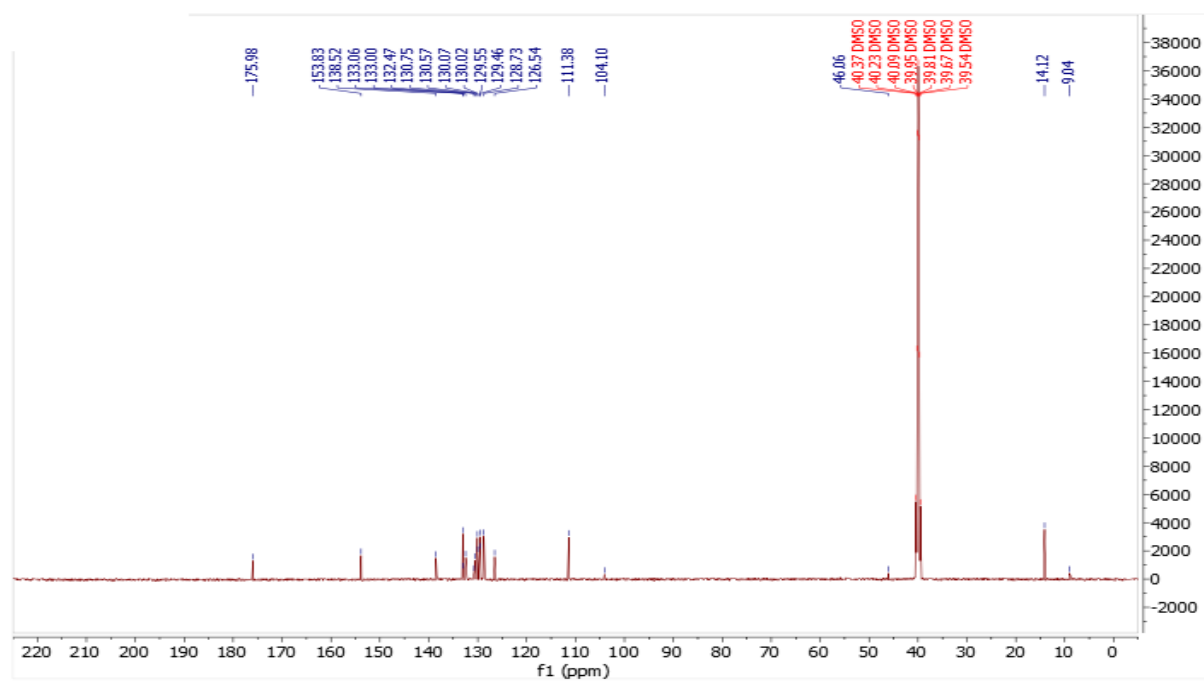


Figure 202, ¹³C NMR of SB97.

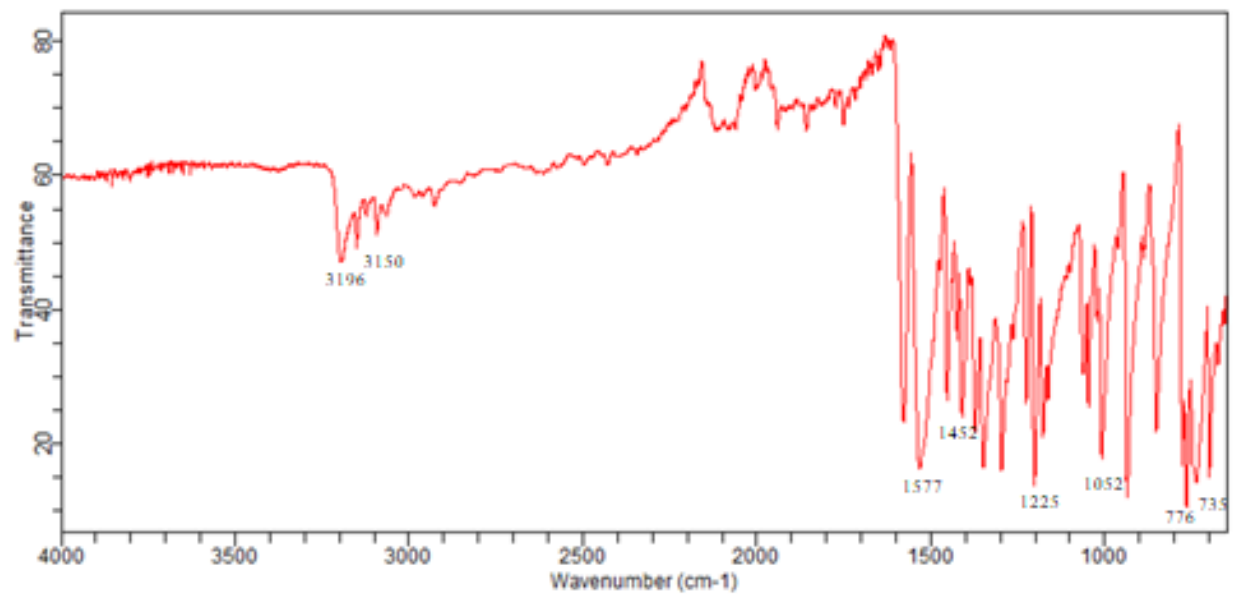


Figure 203, IR of SB97.

HRMS (+ESI-TOF) m/z: [M⁺] calcd for C₁₁H₉Cl₂N₃S 284.98942; found 284.98916.

SB98

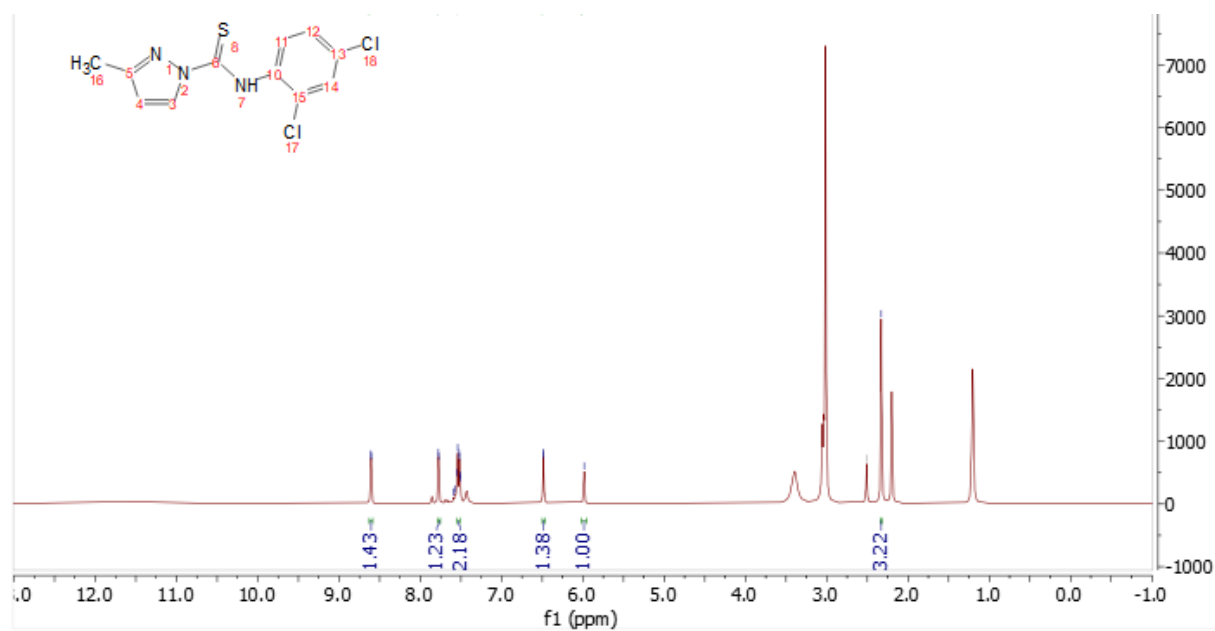


Figure 204, ¹H NMR of SB98.

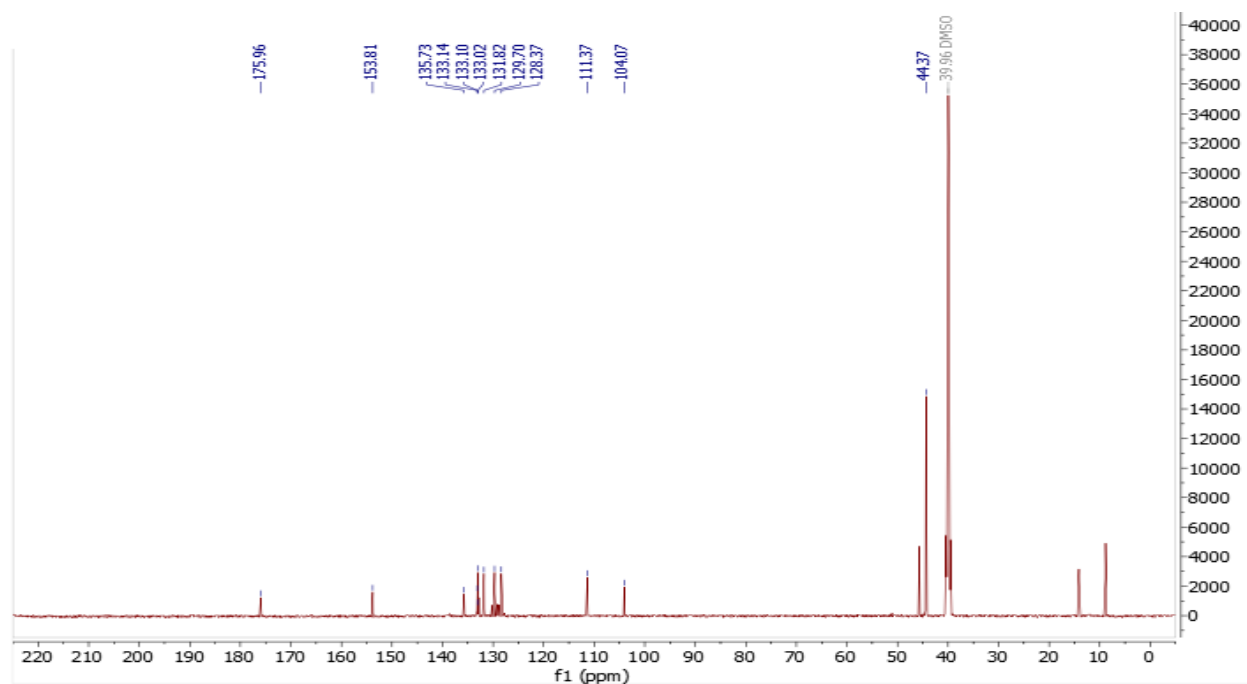


Figure 205, ¹³C NMR of SB98.

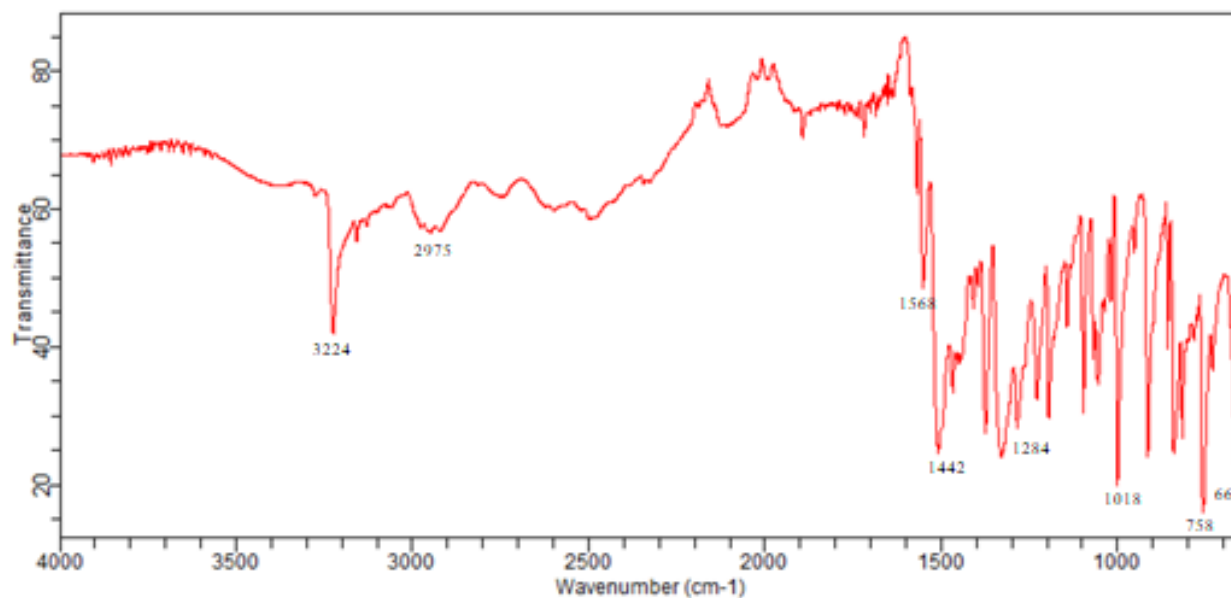


Figure 206, IR of SB98.

HRMS (+ESI-TOF) m/z: [M⁺] calcd for C₁₁H₉Cl₂N₃S 284.98942; found 284.98949.

SB99

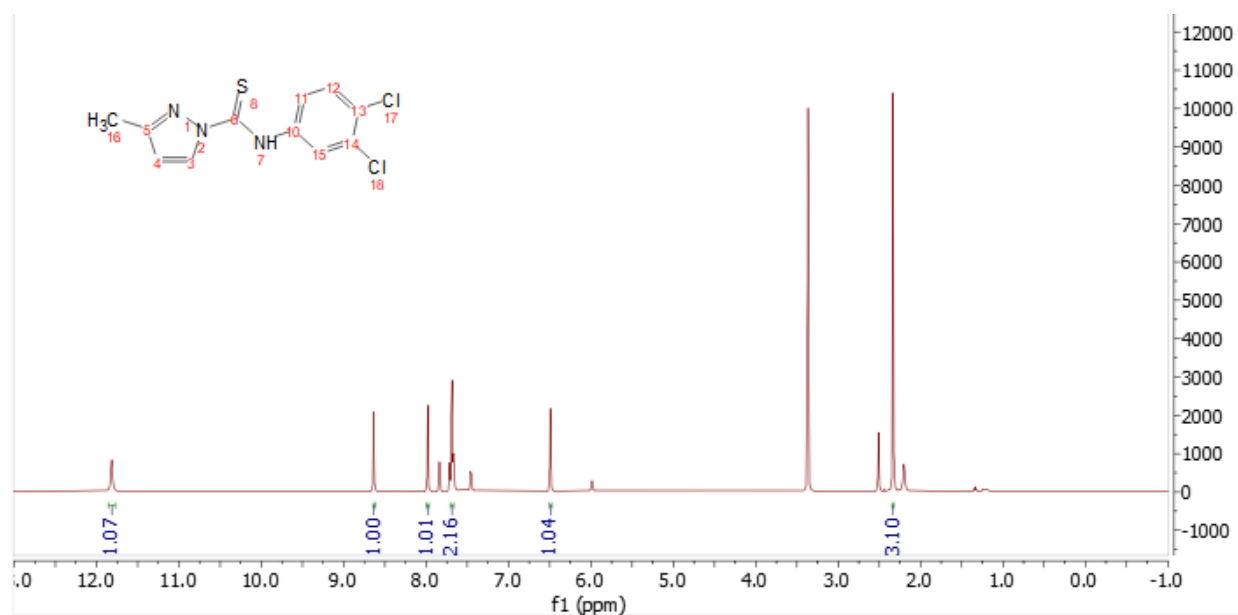


Figure 207, ¹H NMR of SB99.

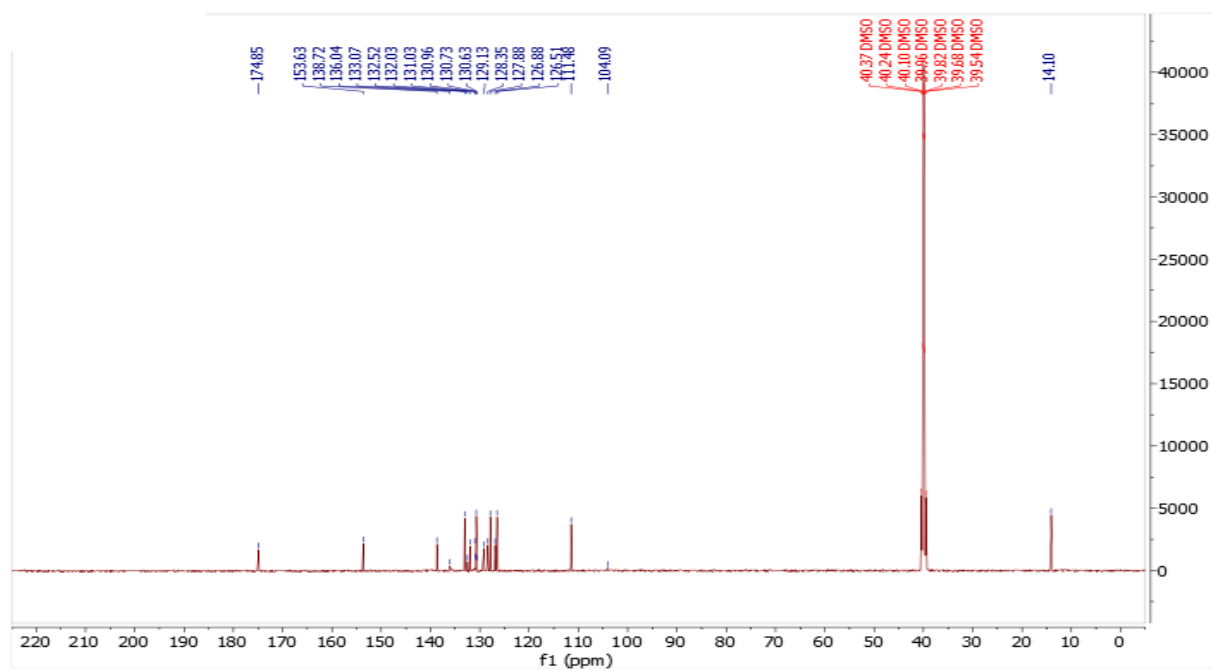


Figure 208, ¹³C NMR of SB99.

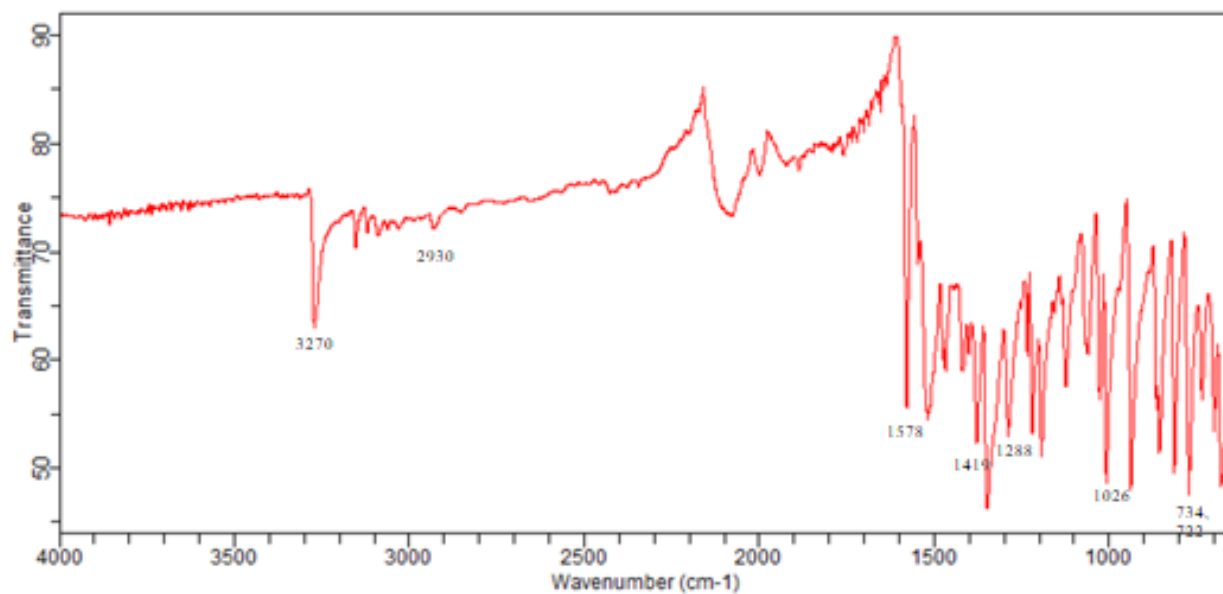


Figure 209, IR of SB99.

HRMS (+ESI-TOF) m/z: [M⁺] calcd for C₁₁H₉Cl₂N₃S 284.98942; found 284.98922.

SB100

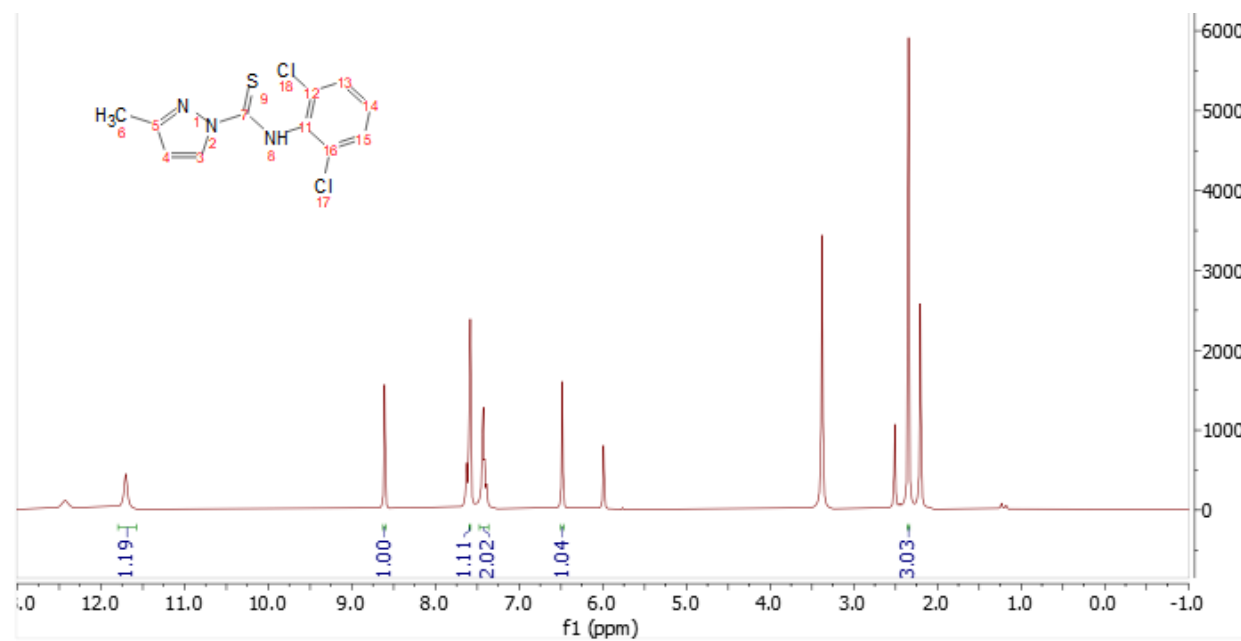


Figure 210, ¹H NMR of SB100.

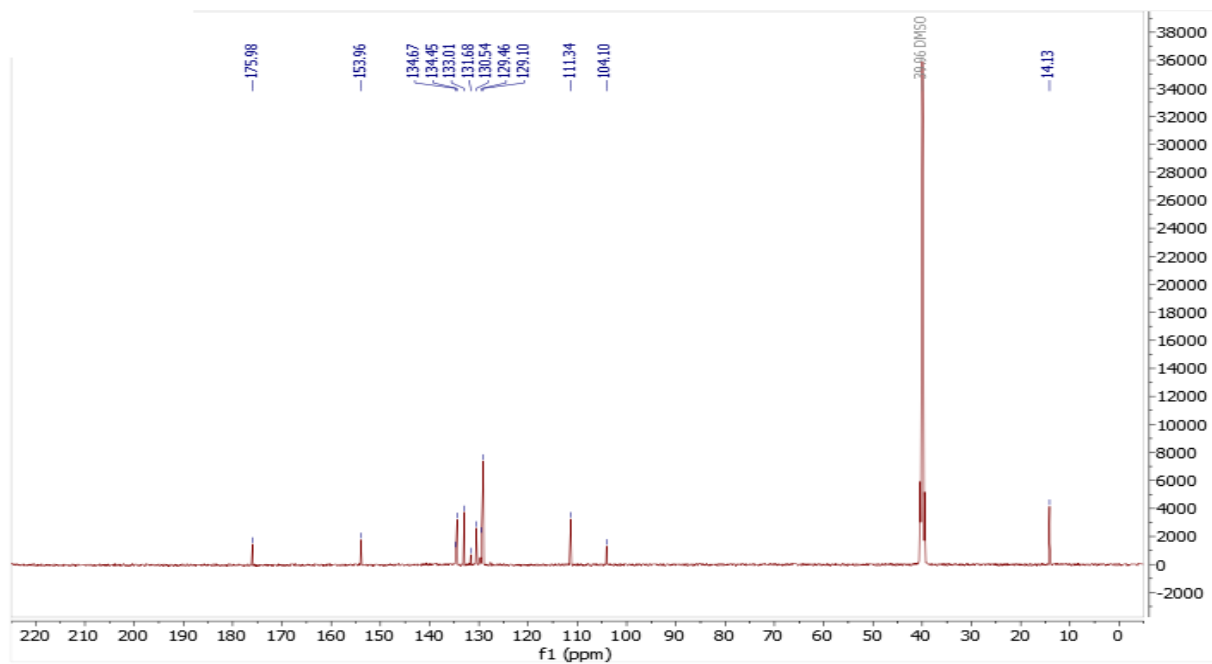


Figure 211, ¹³C NMR of SB100.

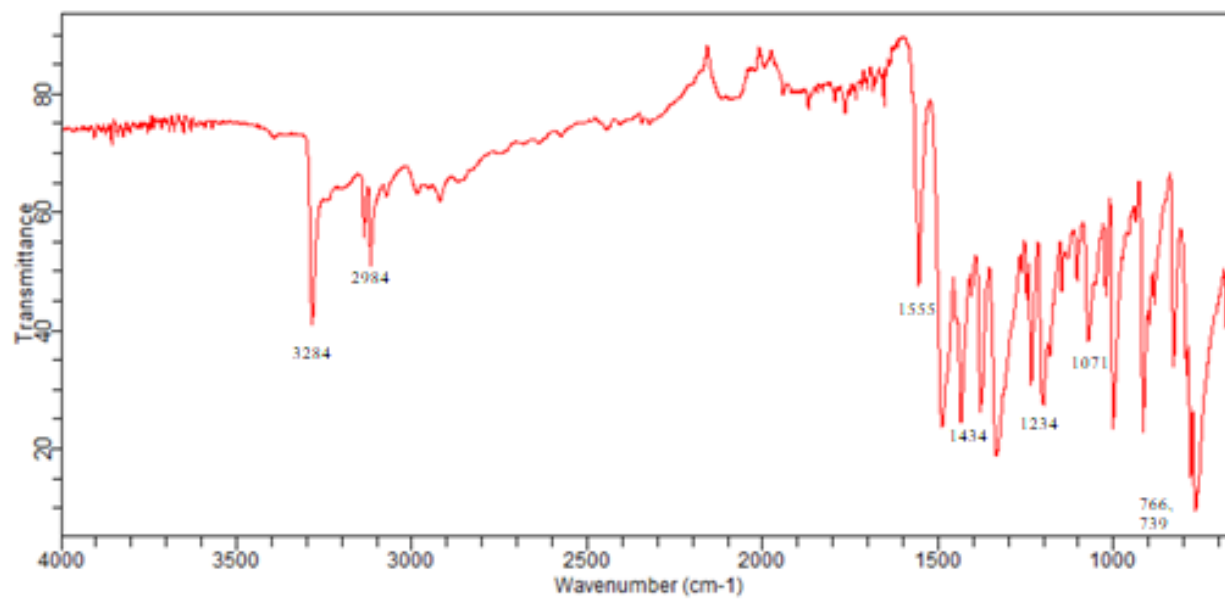


Figure 212, IR of SB100.

HRMS (+ESI-TOF) m/z: [M⁺] calcd for C₁₁H₉Cl₂N₃S 284.98942; found 284.98928.

SB101

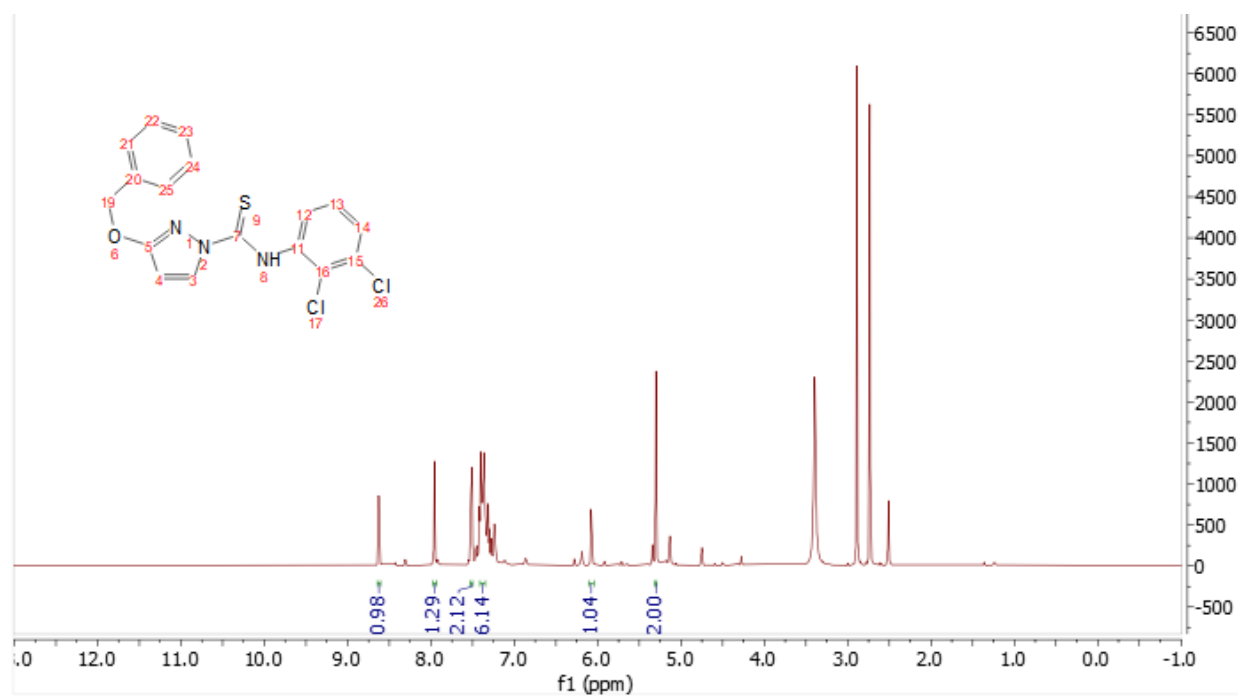


Figure 213, ¹H NMR of SB101.

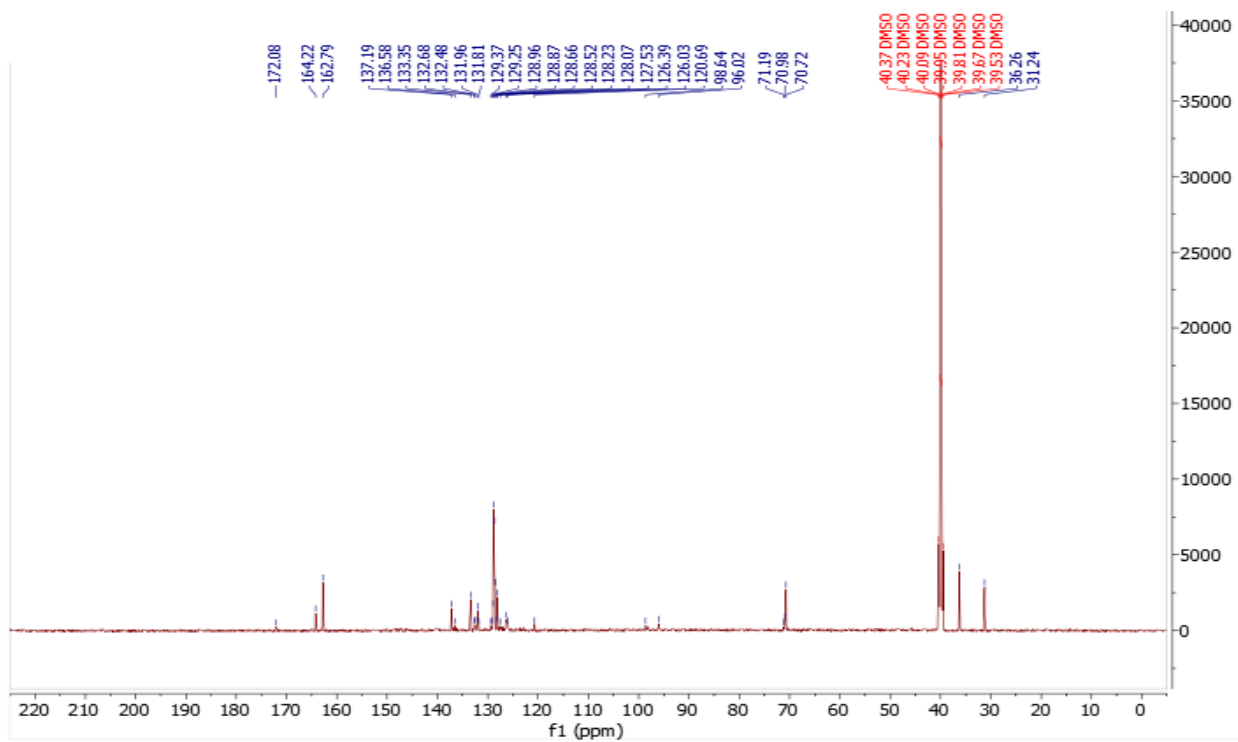


Figure 214, ¹³C NMR of SB101.

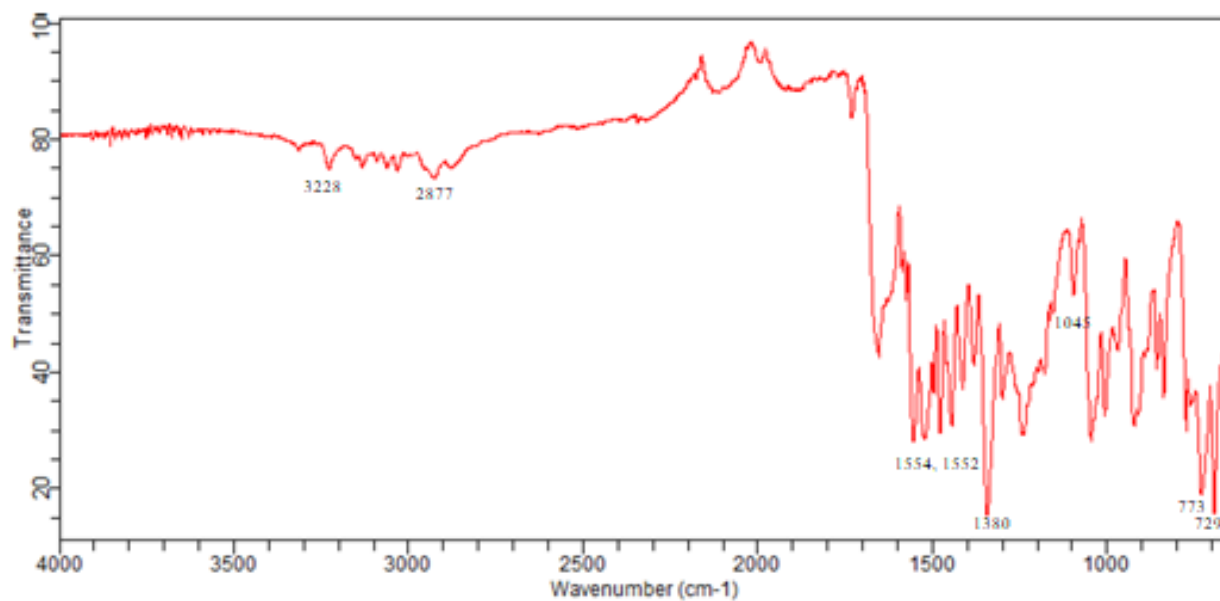


Figure 215, IR of SB101.

HRMS (+ESI-TOF) m/z: [M⁺] calcd for C₁₇H₁₃Cl₂N₃OS 377.01564; found 377.01560.

SB102

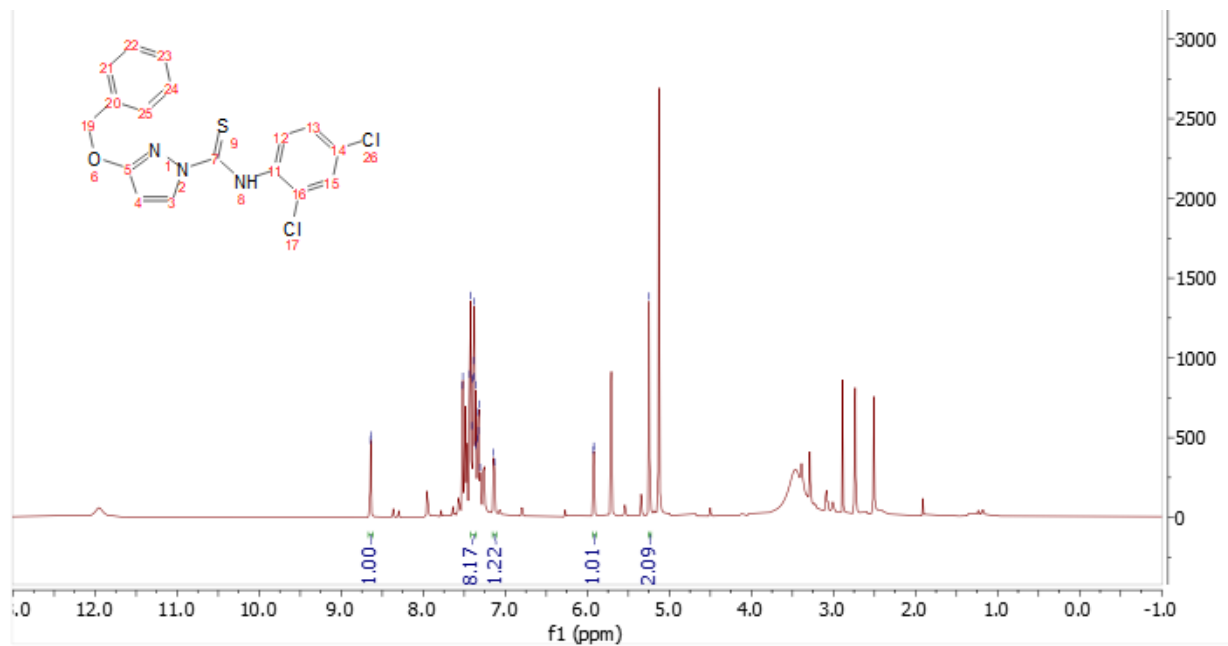


Figure 216, ¹H NMR of SB102.

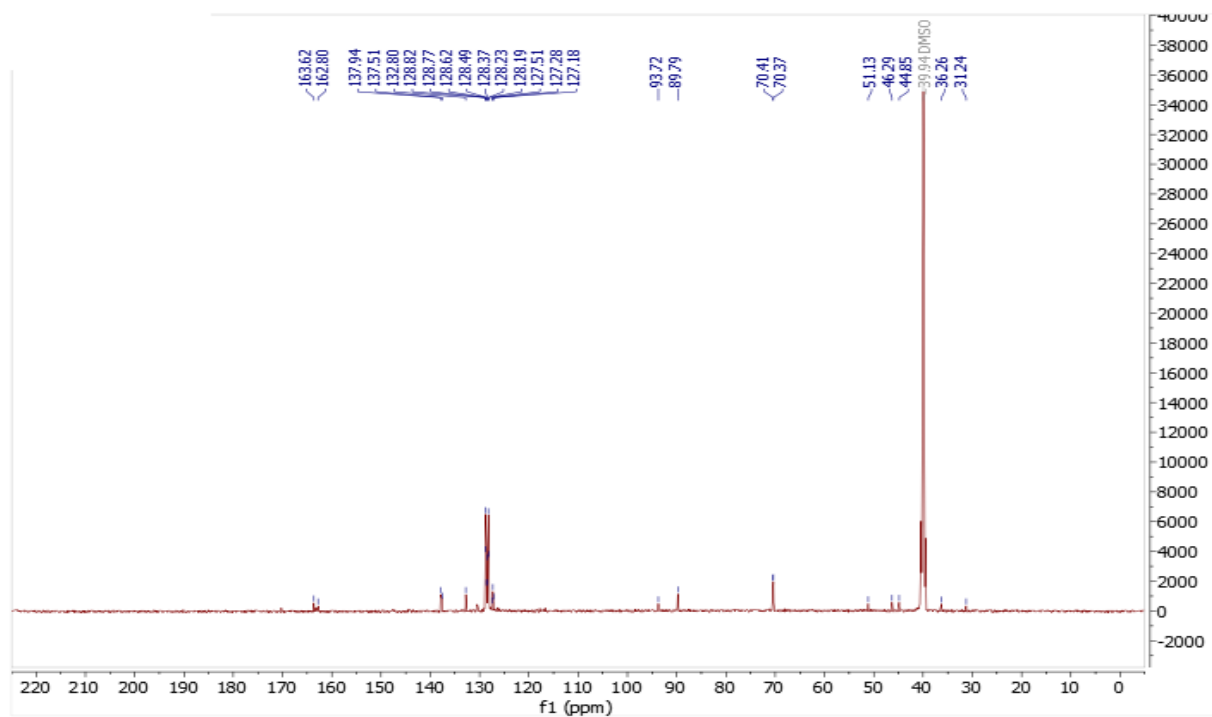


Figure 217, ^{13}C NMR of SB102.

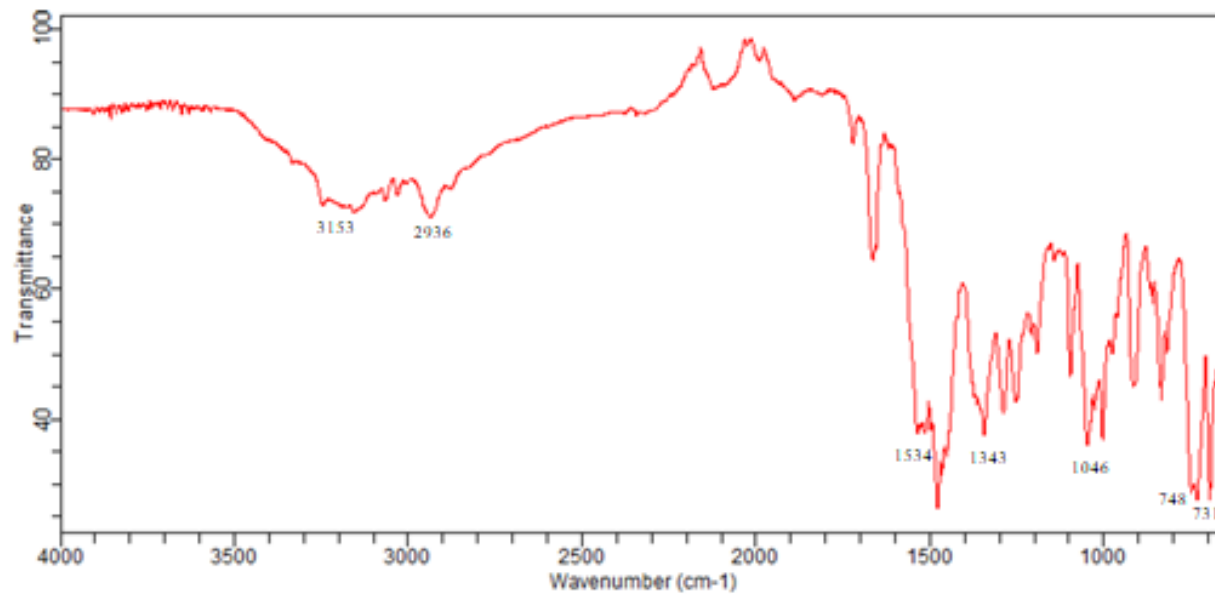


Figure 218, IR of SB102.

HRMS (+ESI-TOF) m/z : $[M]^+$ calcd for $\text{C}_{17}\text{H}_{13}\text{Cl}_2\text{N}_3\text{OS}$ 377.01564; found 377.01588.

SB103

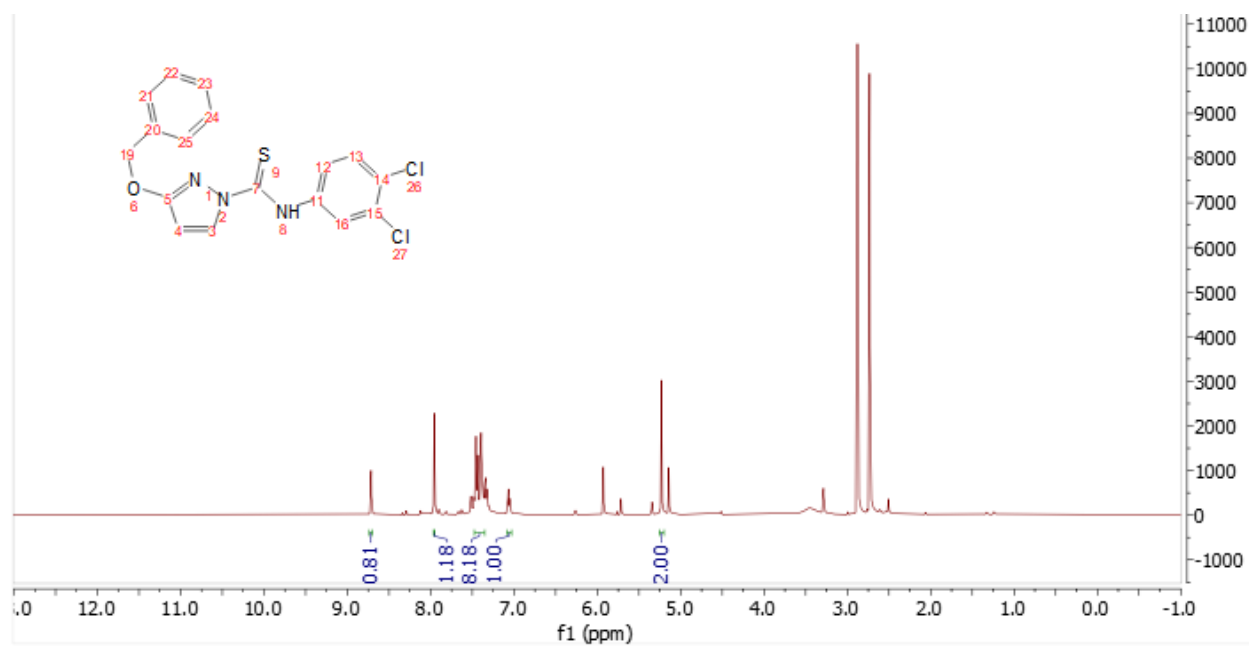


Figure 219, ^1H NMR of SB103.

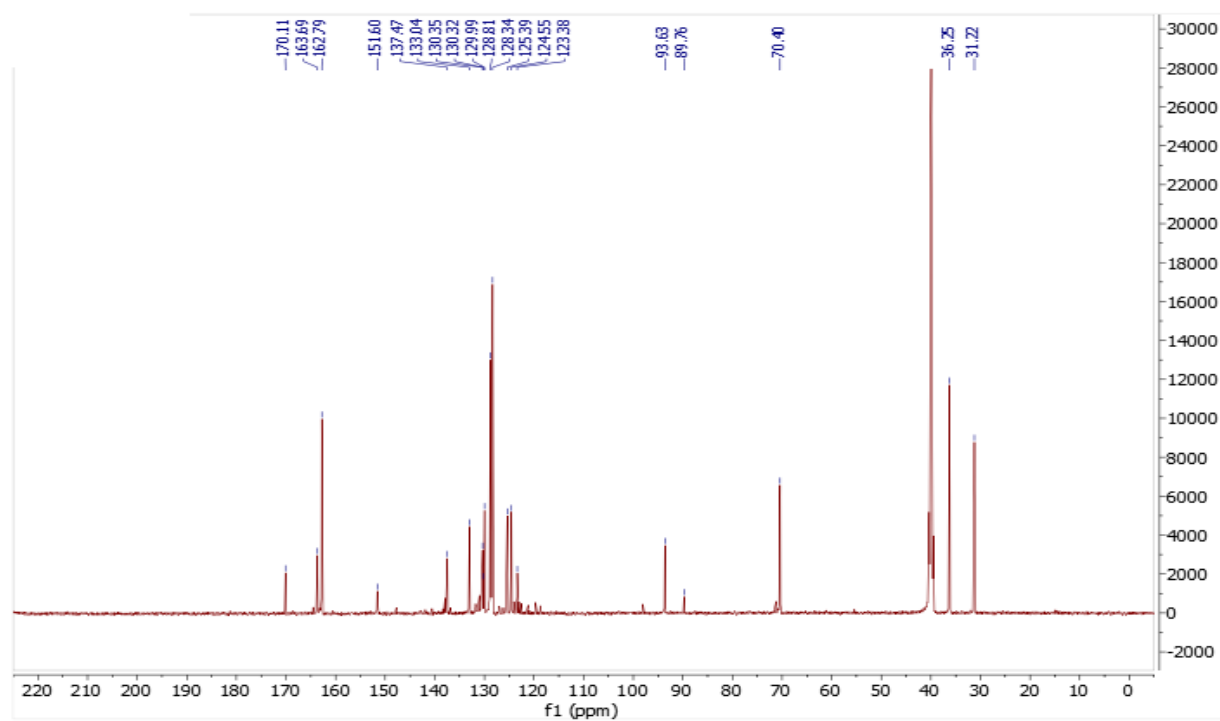


Figure 220, ^{13}C NMR of SB103.

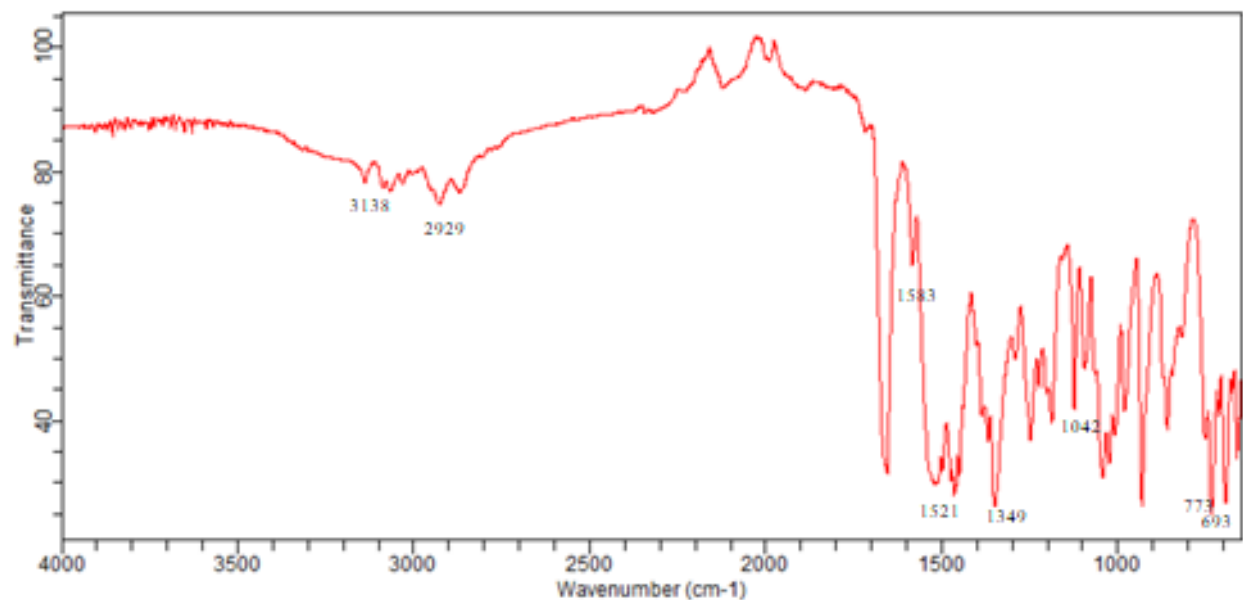


Figure 221, IR of SB103.

HRMS (+ESI-TOF) m/z: [M⁺] calcd for C₁₇H₁₃Cl₂N₃OS 377.01564; found 377.01571.

SB104

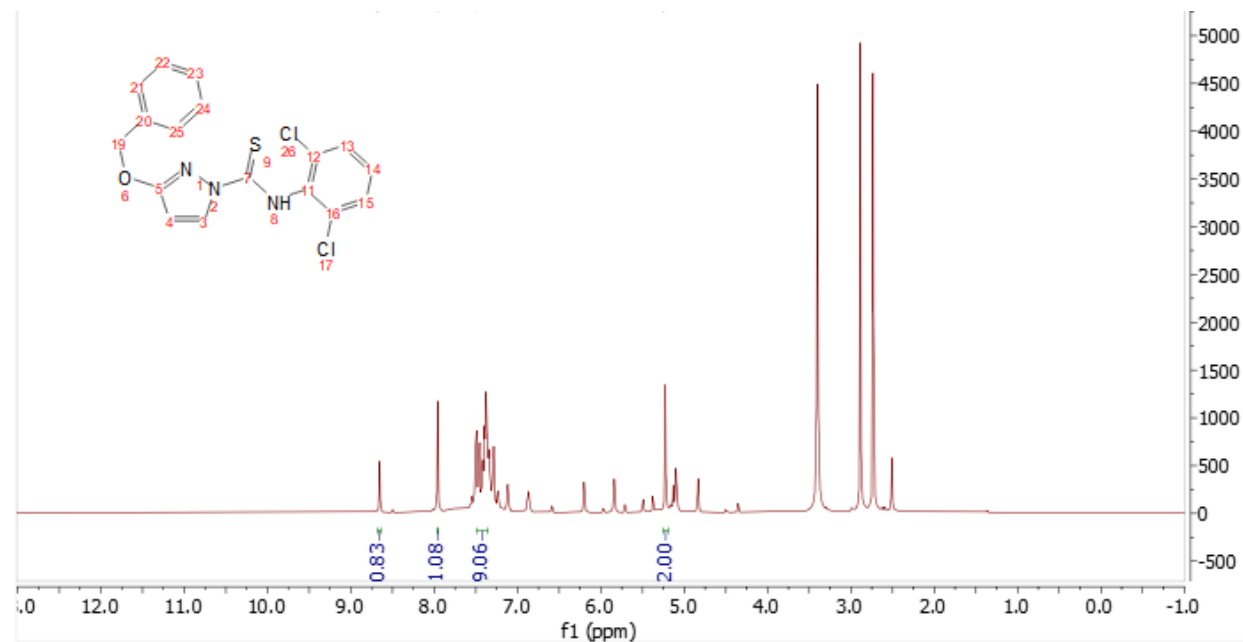


Figure 222, ¹H NMR of SB104.

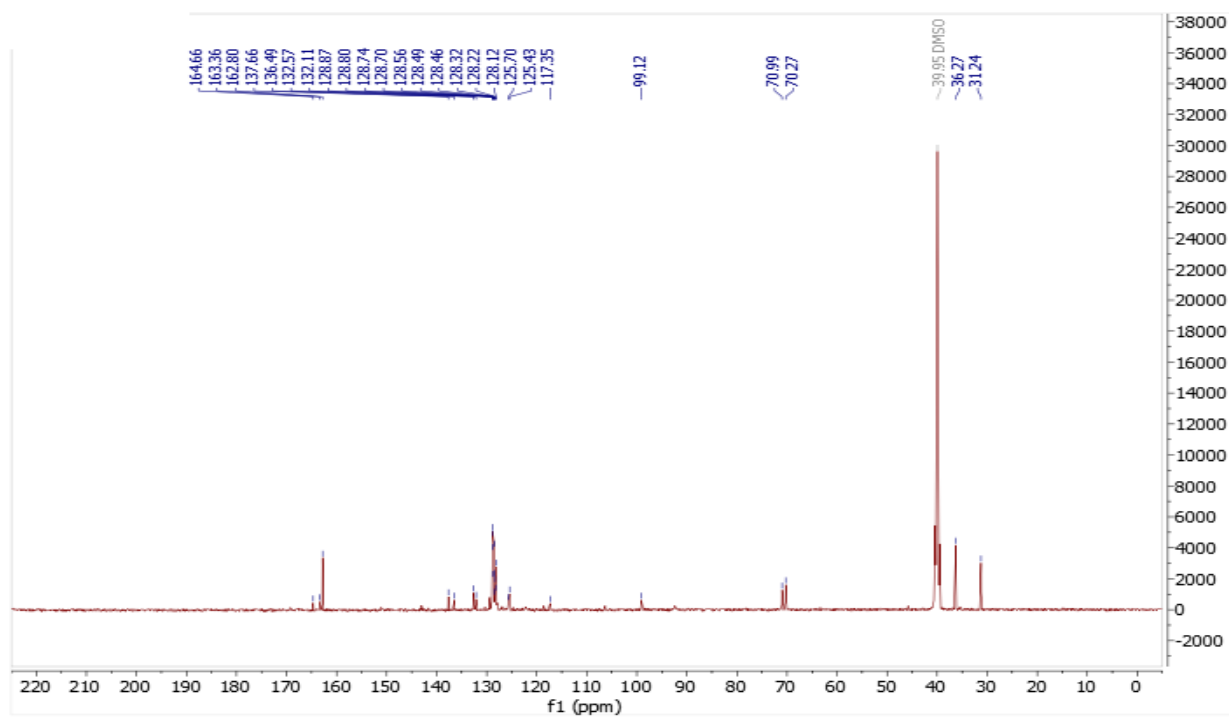


Figure 223, ^{13}C NMR of SB104.

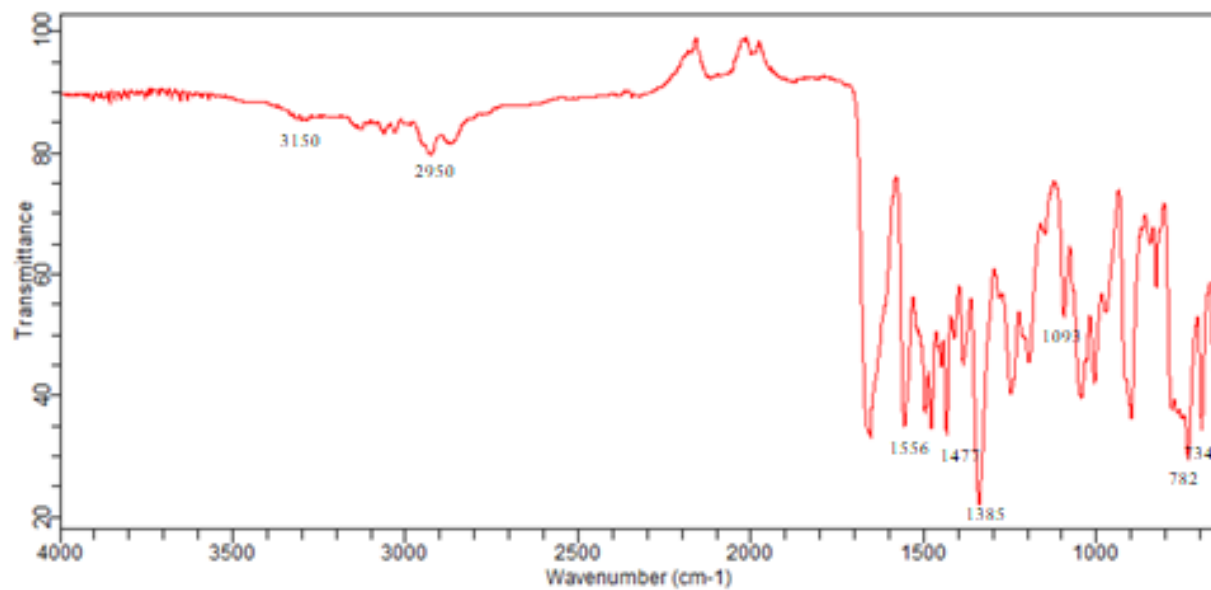


Figure 224, IR of SB104.

HRMS (+ESI-TOF) m/z : $[M]^+$ calcd for $\text{C}_{17}\text{H}_{13}\text{Cl}_2\text{N}_3\text{OS}$ 377.01564; found 377.01553.

SB105

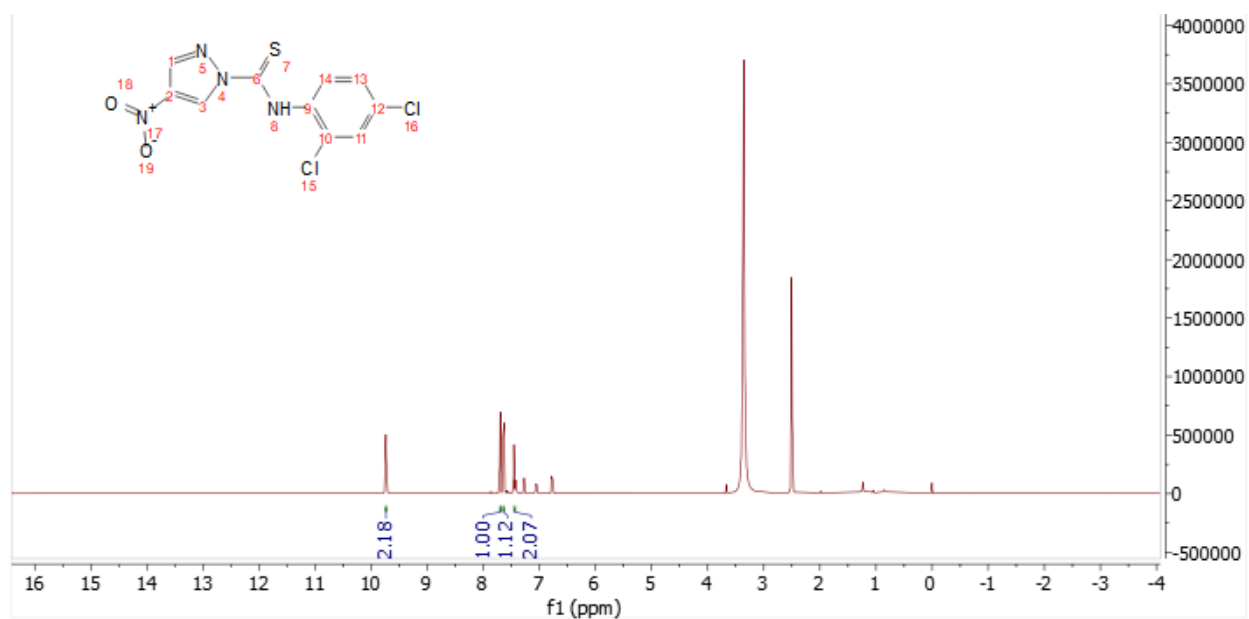


Figure 225, ^1H NMR of SB105.

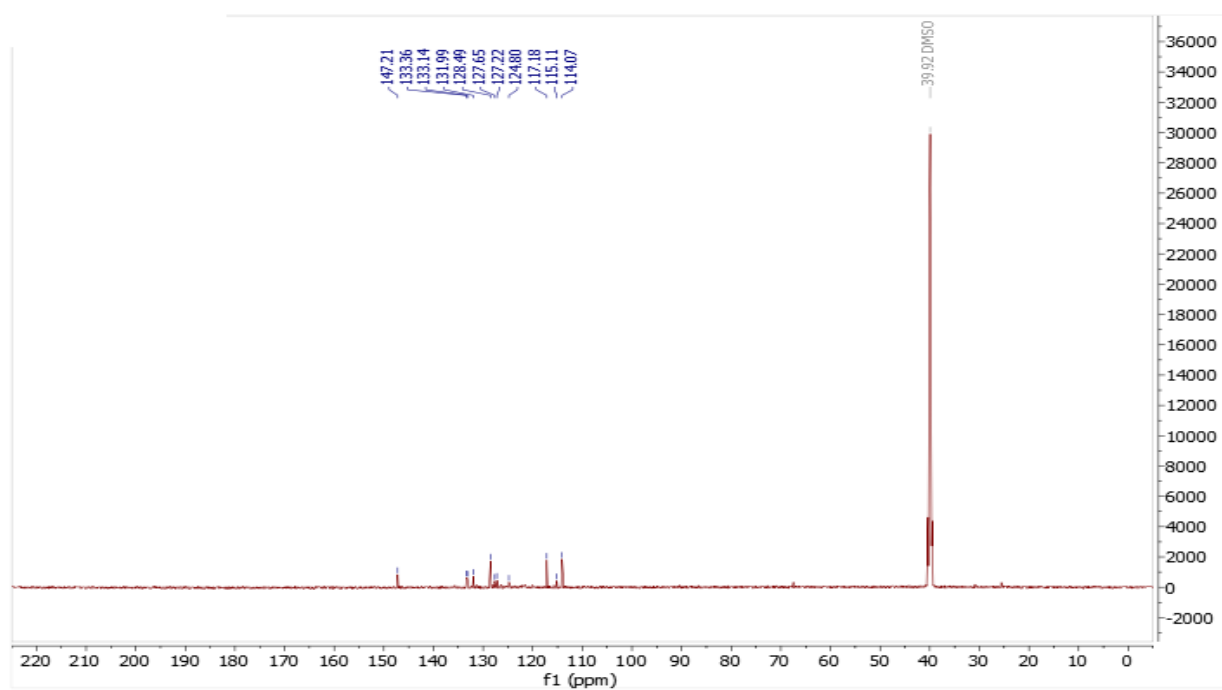


Figure 226, ^{13}C NMR of SB105.

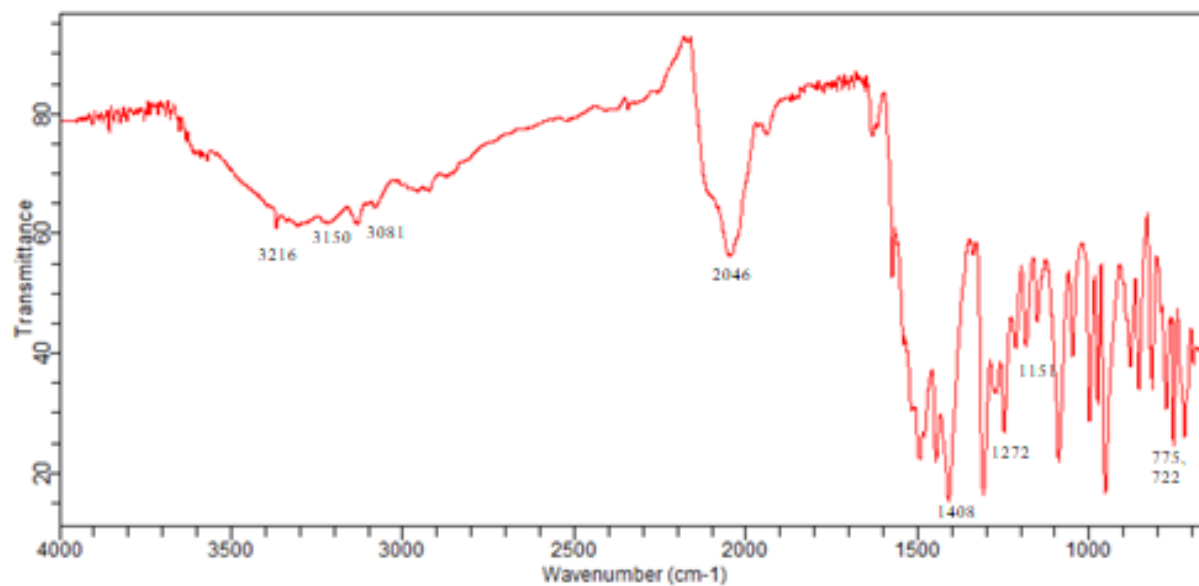


Figure 227, IR of SB105.

UPLC-MS [M+1] calcd for C₁₀H₆Cl₂N₄O₂S 318.15; found 317.19.

SB106

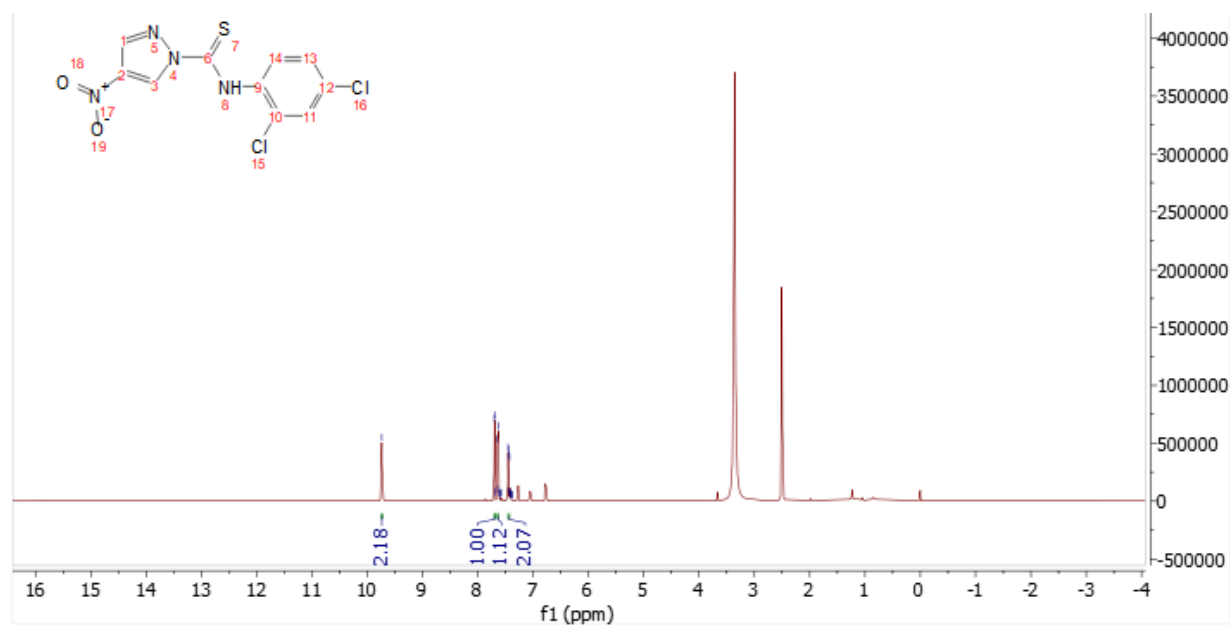


Figure 228, ¹H NMR of SB106.

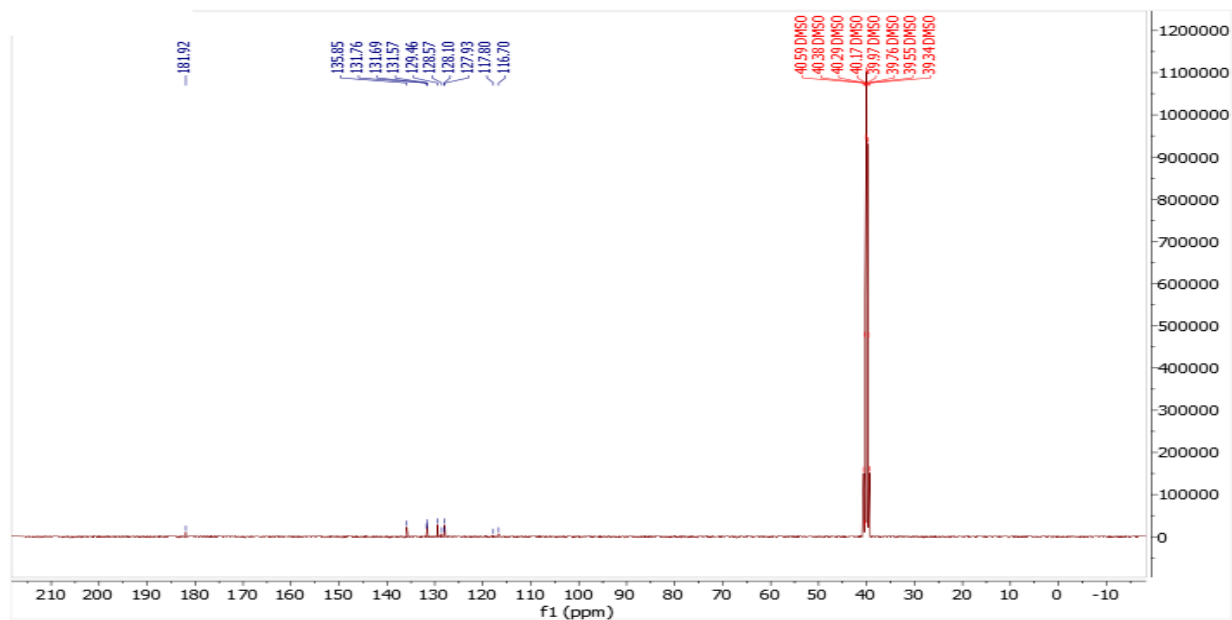


Figure 229, ¹³C NMR of SB106.

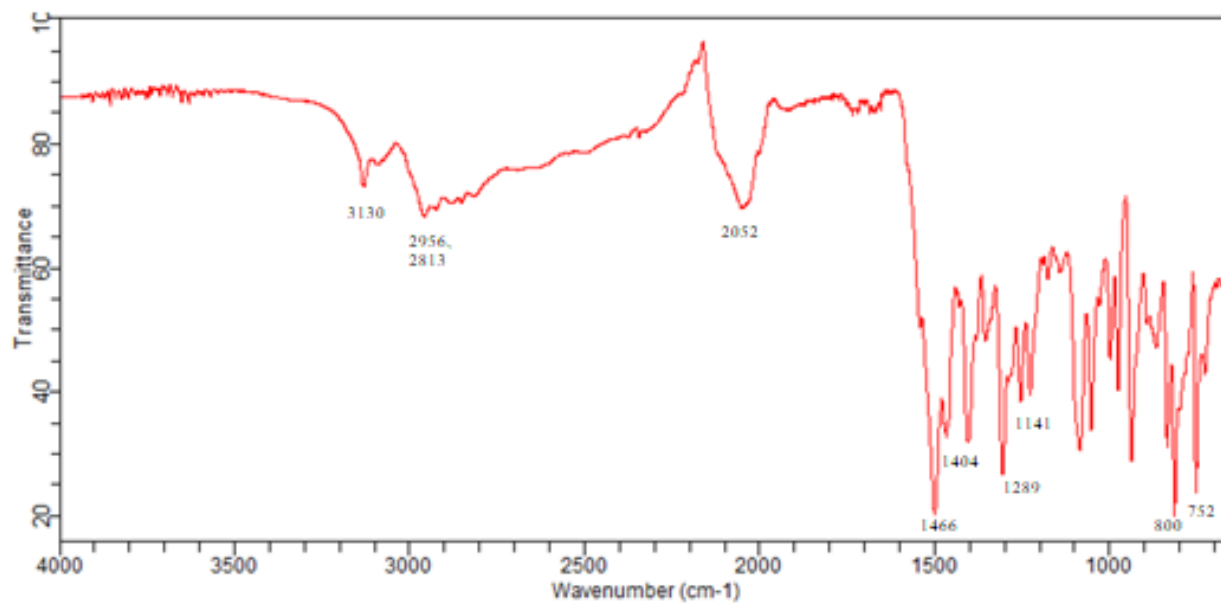


Figure 230, IR of SB106.

UPLC-MS [M+1] calcd for C₁₀H₆Cl₂N₄O₂S 318.15; found 317.07.

SB107

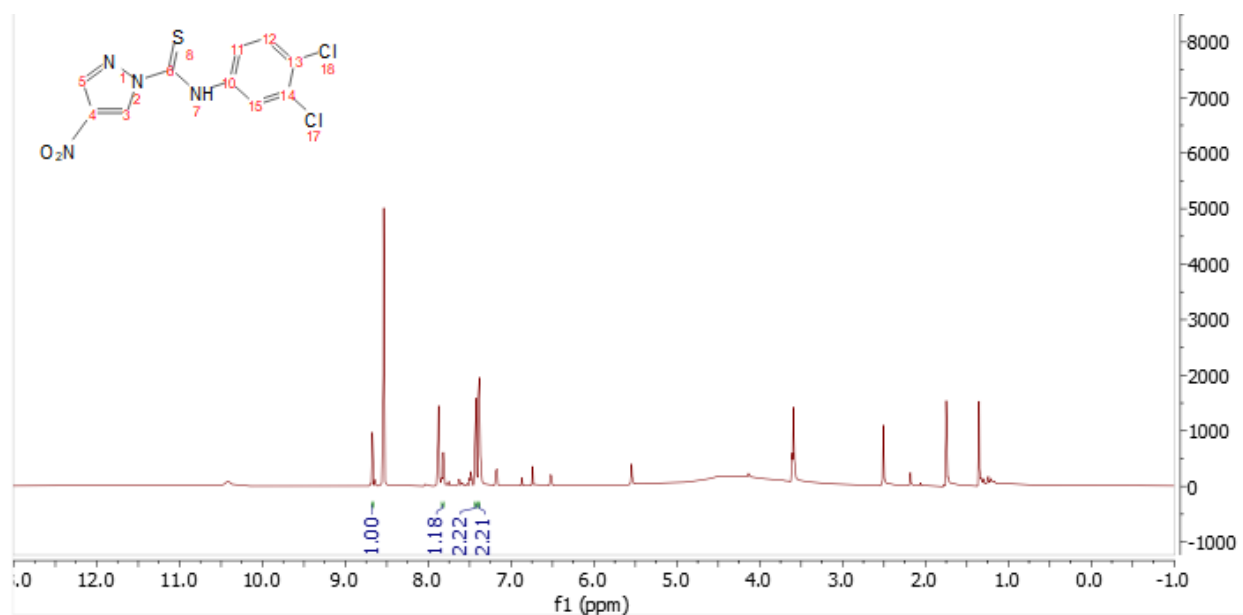


Figure 231, ¹H NMR of SB107.

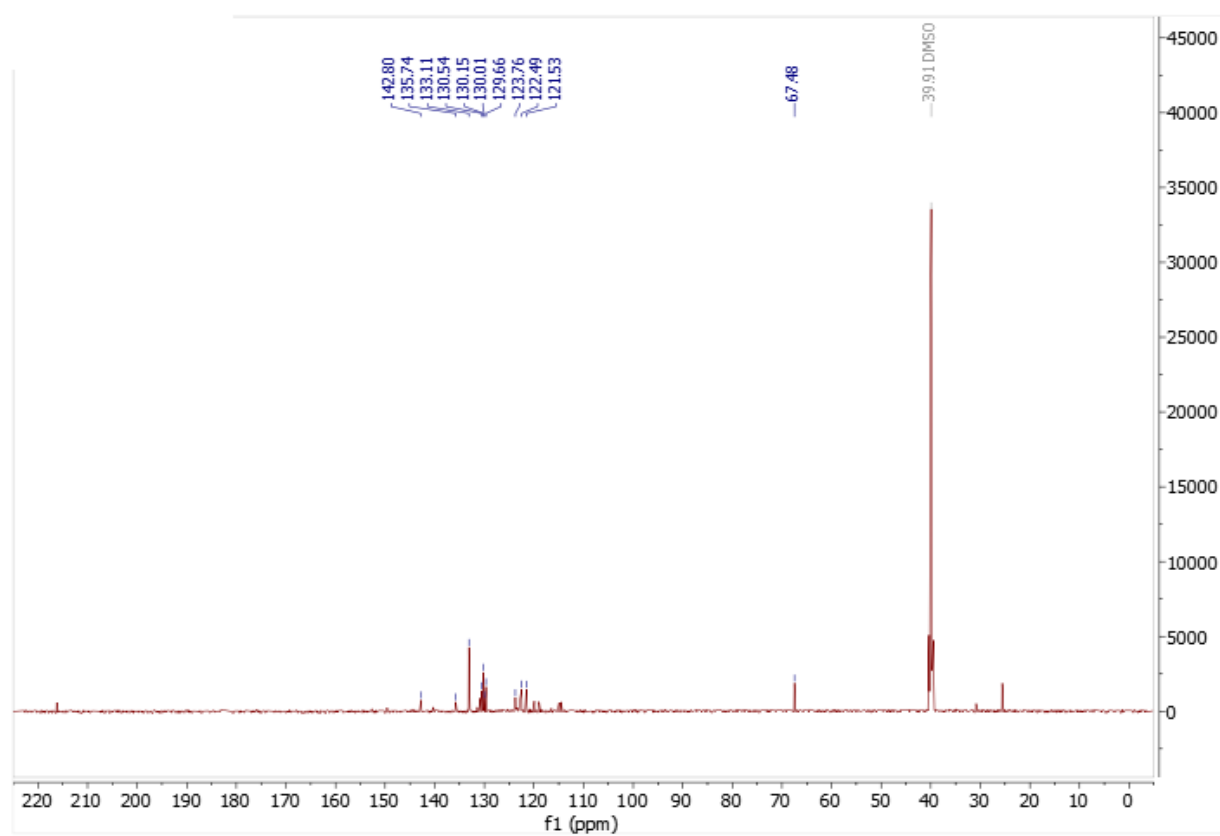


Figure 232, ¹³C NMR of SB107.

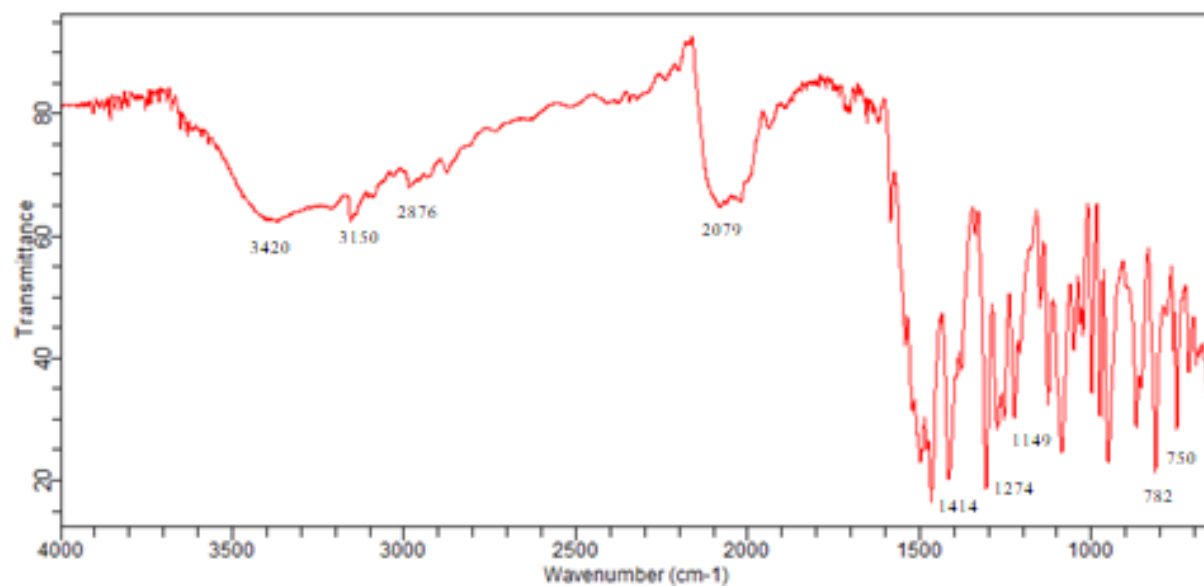


Figure 233, IR of SB107.

UPLC-MS [M+1] calcd for C₁₀H₆Cl₂N₄O₂S 318.15; found 318.84.

SB108

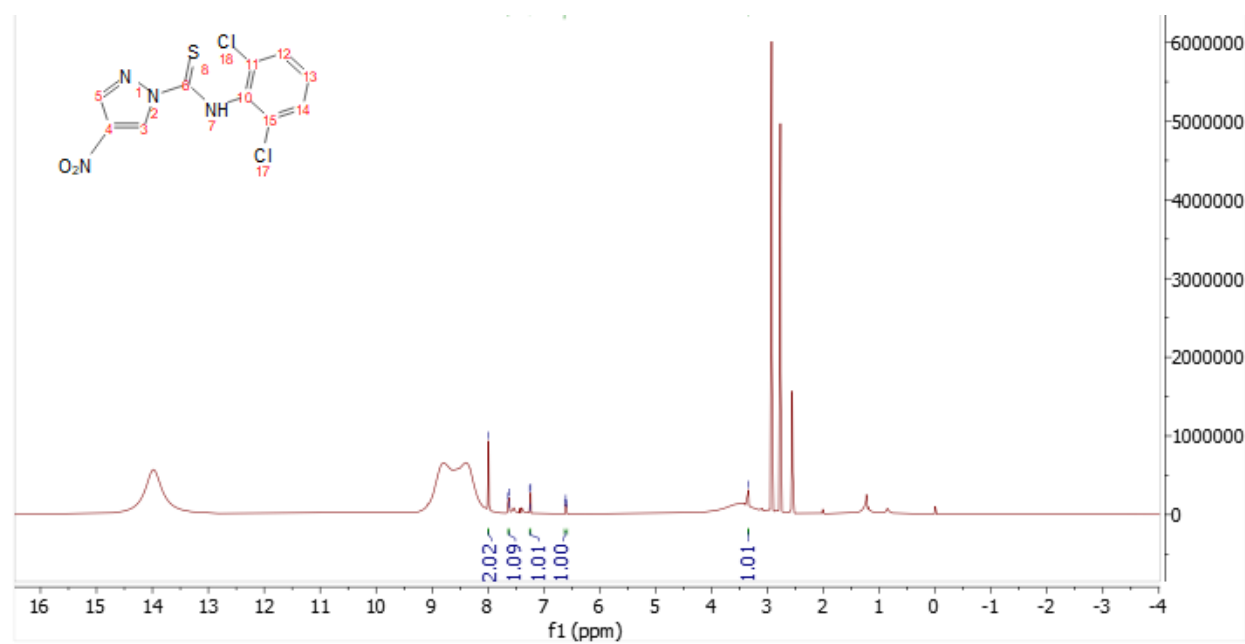


Figure 234, ¹H NMR of SB108.

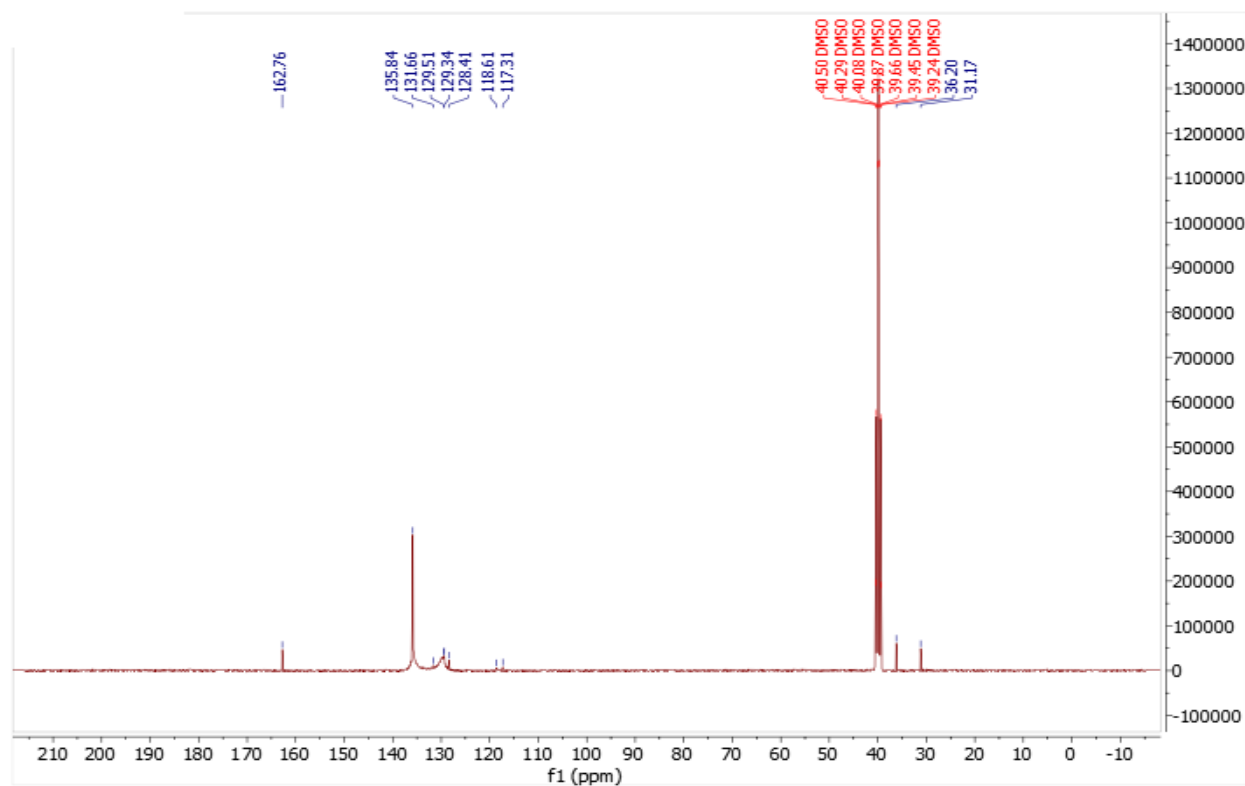


Figure 235, ^{13}C NMR of SB108.

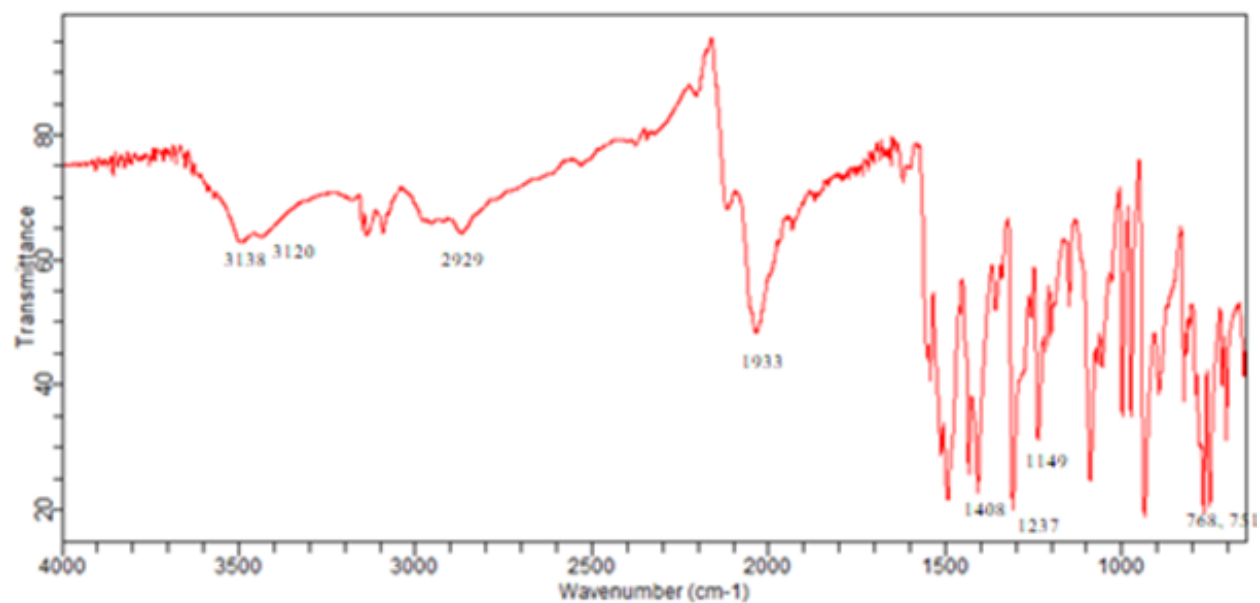


Figure 236, IR of SB108.

UPLC-MS $[\text{M}+1]$ calcd for $\text{C}_{10}\text{H}_6\text{Cl}_2\text{N}_4\text{O}_2\text{S}$ 318.15; found 319.05.

SB109

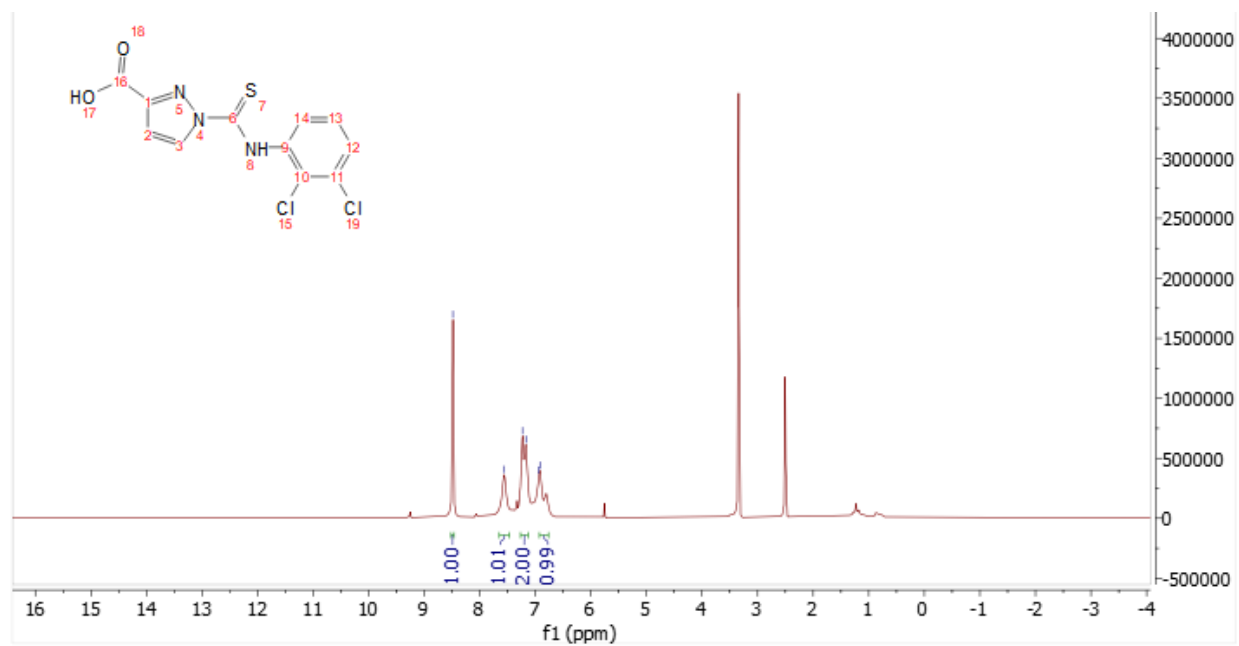


Figure 237, ^1H NMR of SB109.

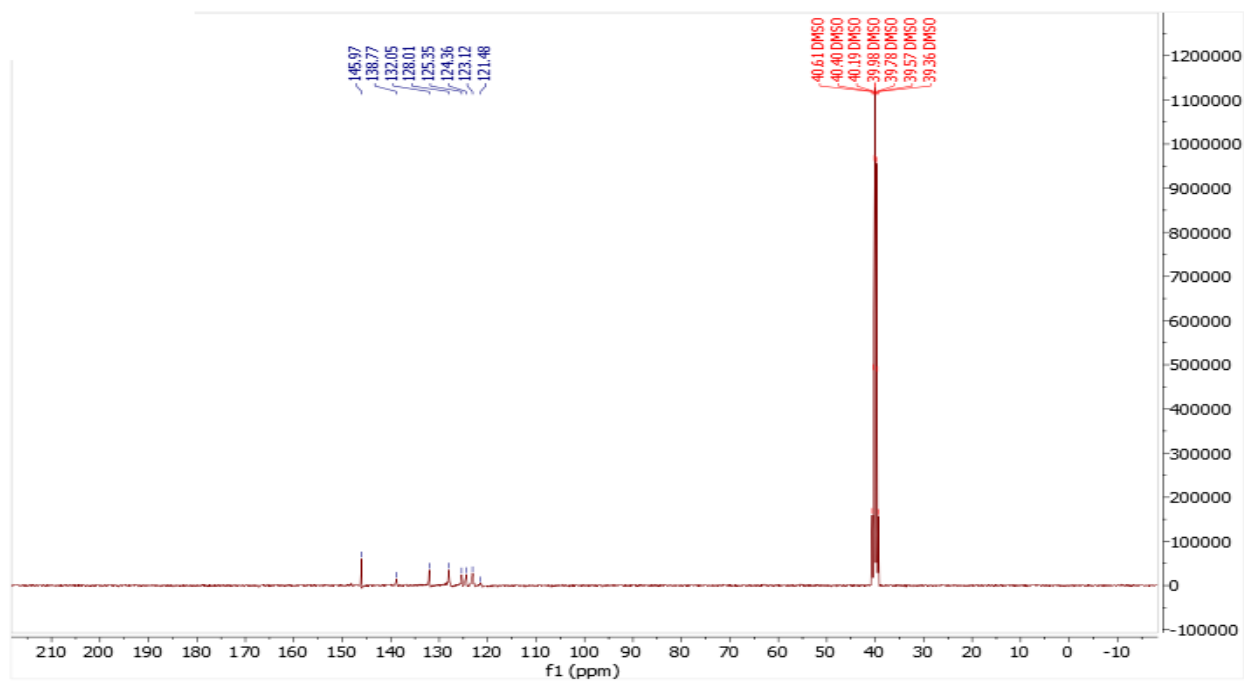


Figure 238, ^{13}C NMR of SB109.

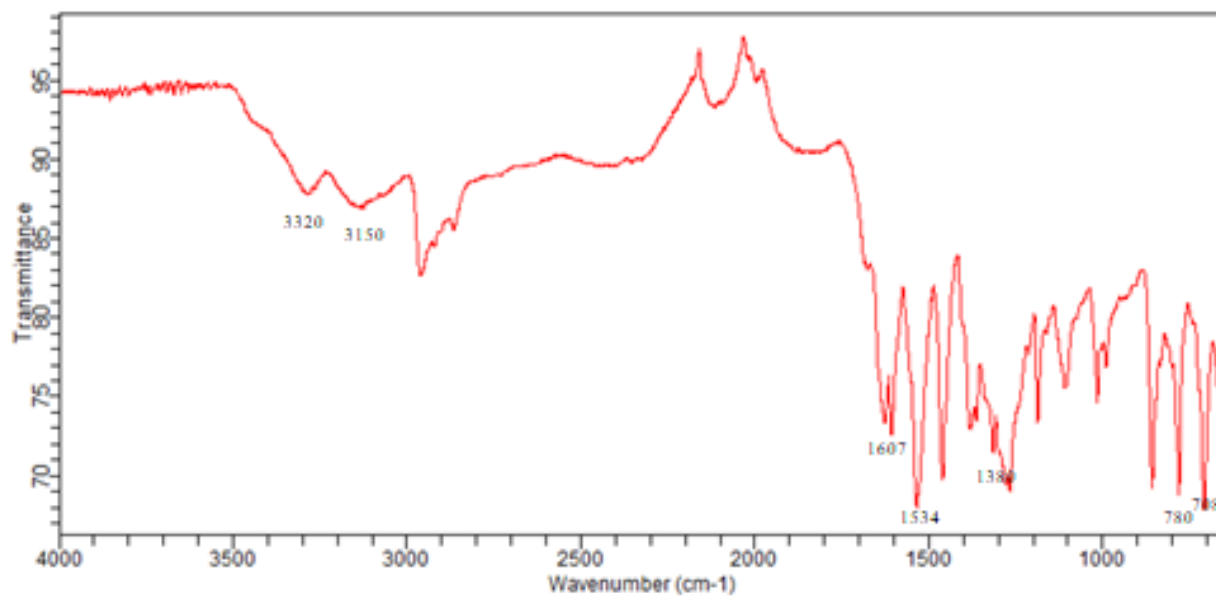


Figure 239, IR of SB109.

HRMS (+ESI-TOF) m/z: [M⁺] calcd for C₁₁H₇Cl₂N₃O₂S 314.96360; found 314.96350.

SB110

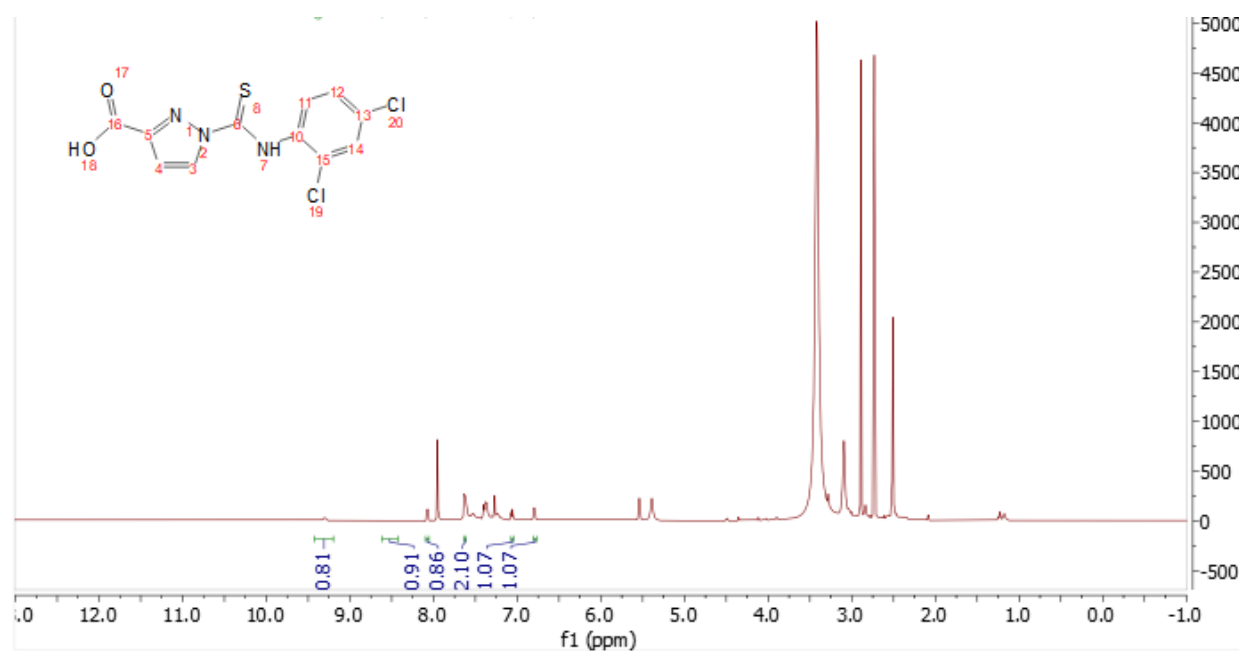


Figure 240, ¹H NMR of SB110.

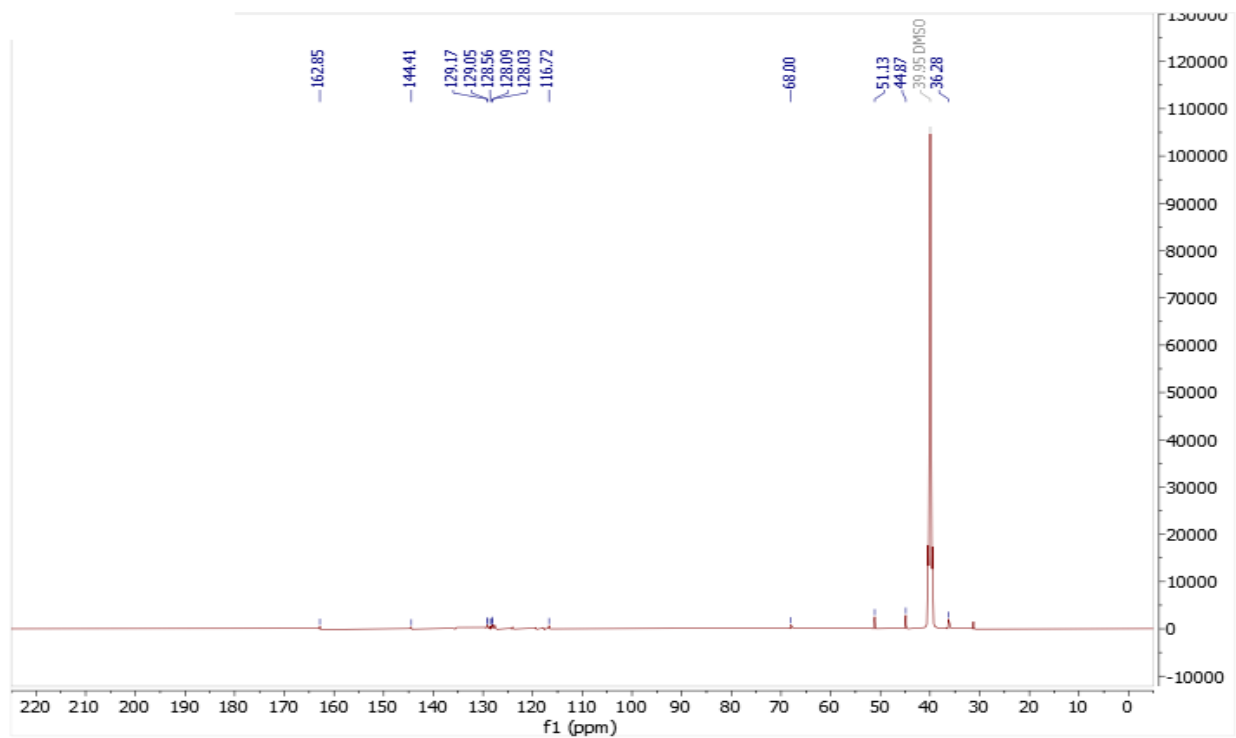


Figure 241, ^{13}C NMR of SB110.

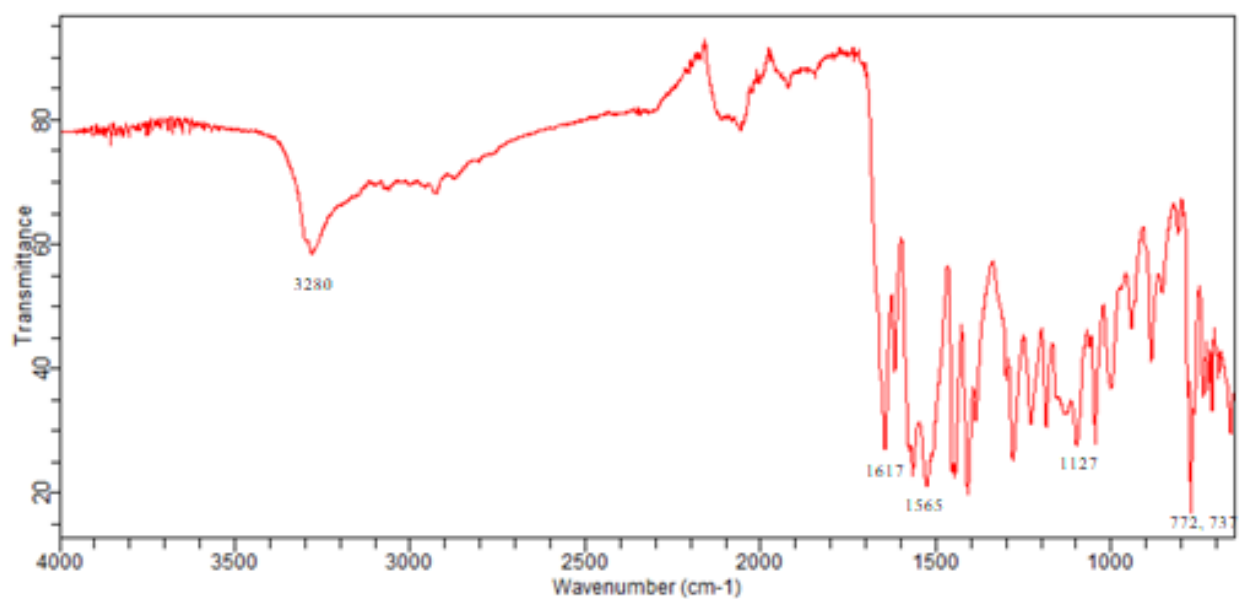


Figure 242, IR of SB110.

HRMS (+ESI-TOF) m/z : $[M]^+$ calcd for $\text{C}_{11}\text{H}_7\text{Cl}_2\text{N}_3\text{O}_2\text{S}$ 314.96360; found 314.96307.

SB111

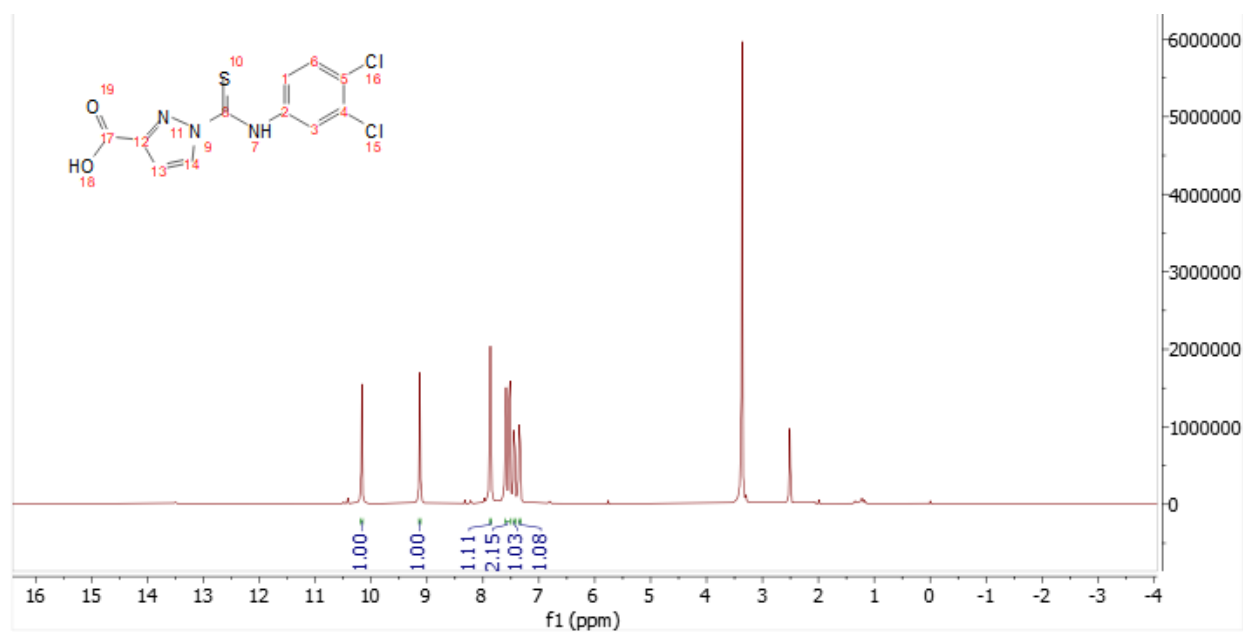


Figure 243, ¹H NMR of SB111.

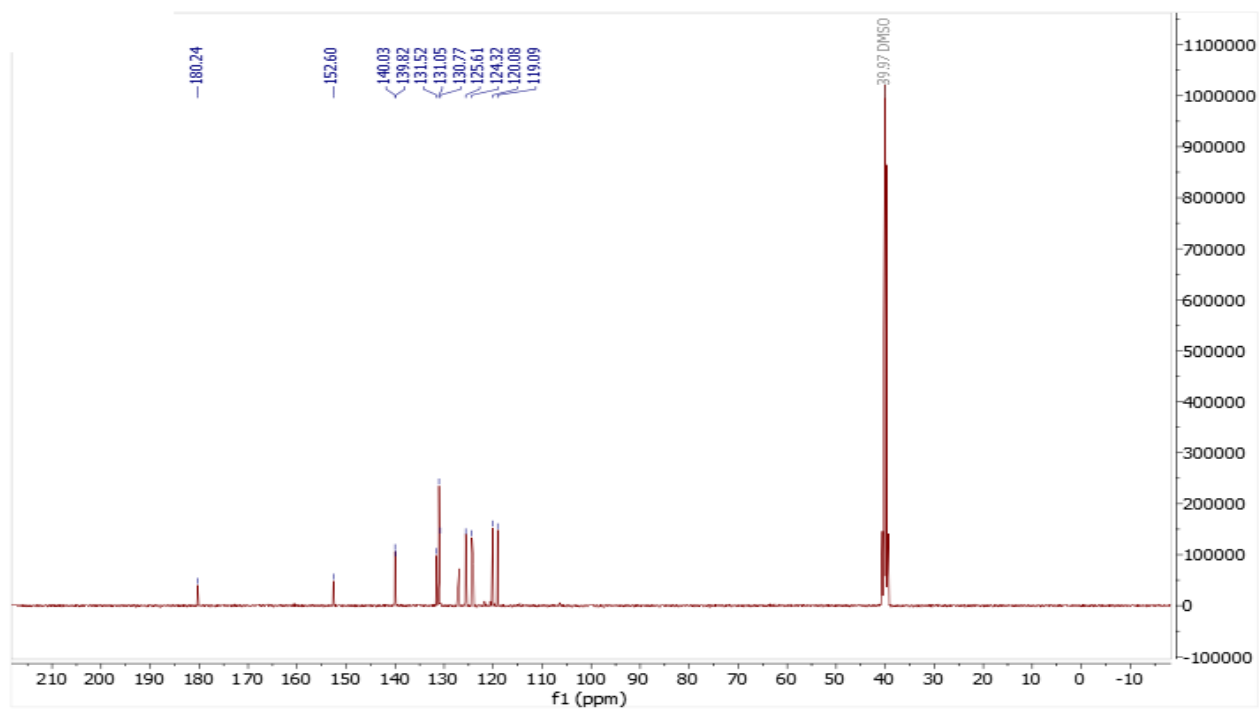


Figure 244, ¹³C NMR of SB111.

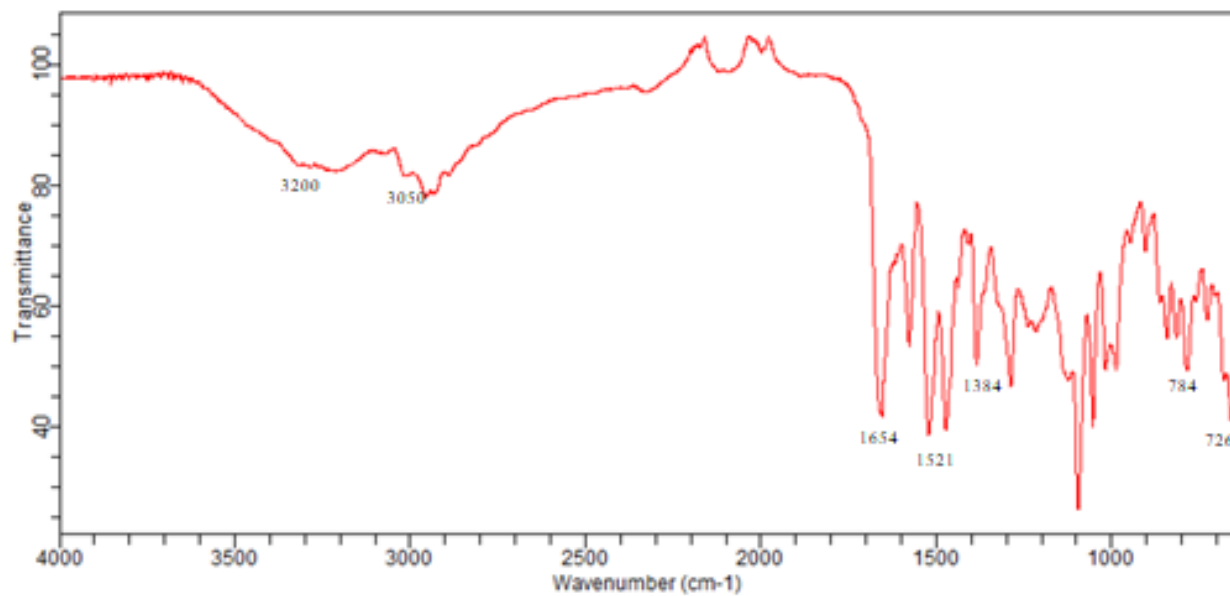


Figure 245, IR of SB111.

HRMS (+ESI-TOF) m/z: [M⁺] calcd for C₁₁H₇Cl₂N₃O₂S 314.96360; found 314.96314.

SB112

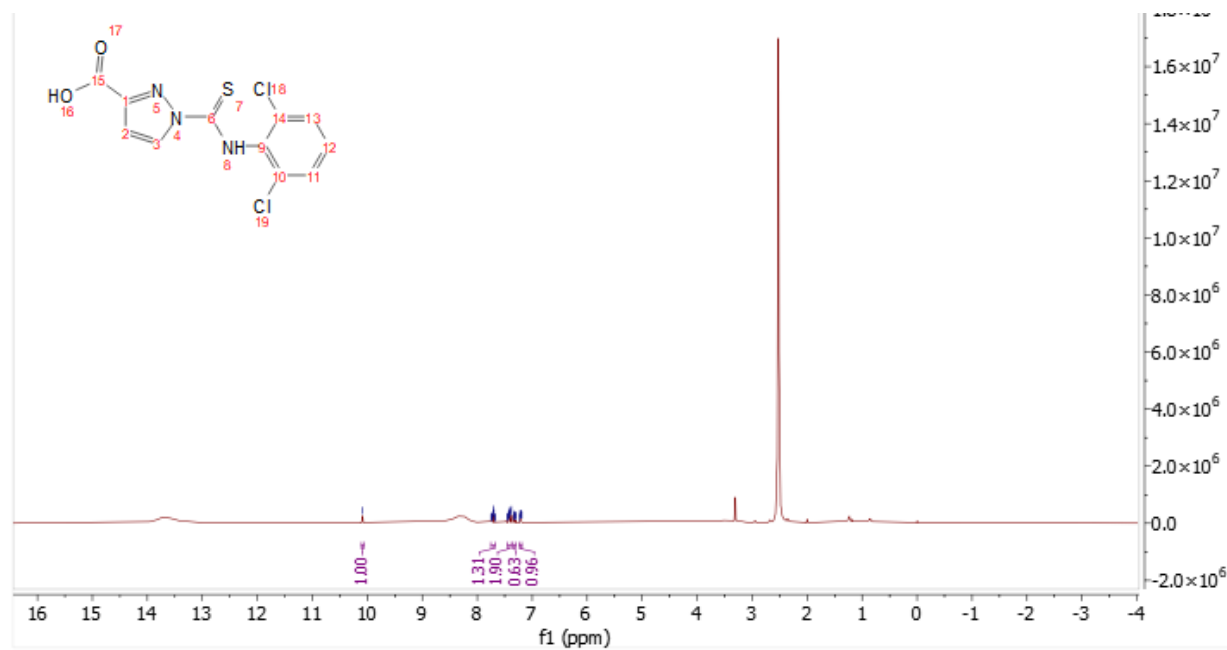


Figure 246, ¹H NMR of SB112.

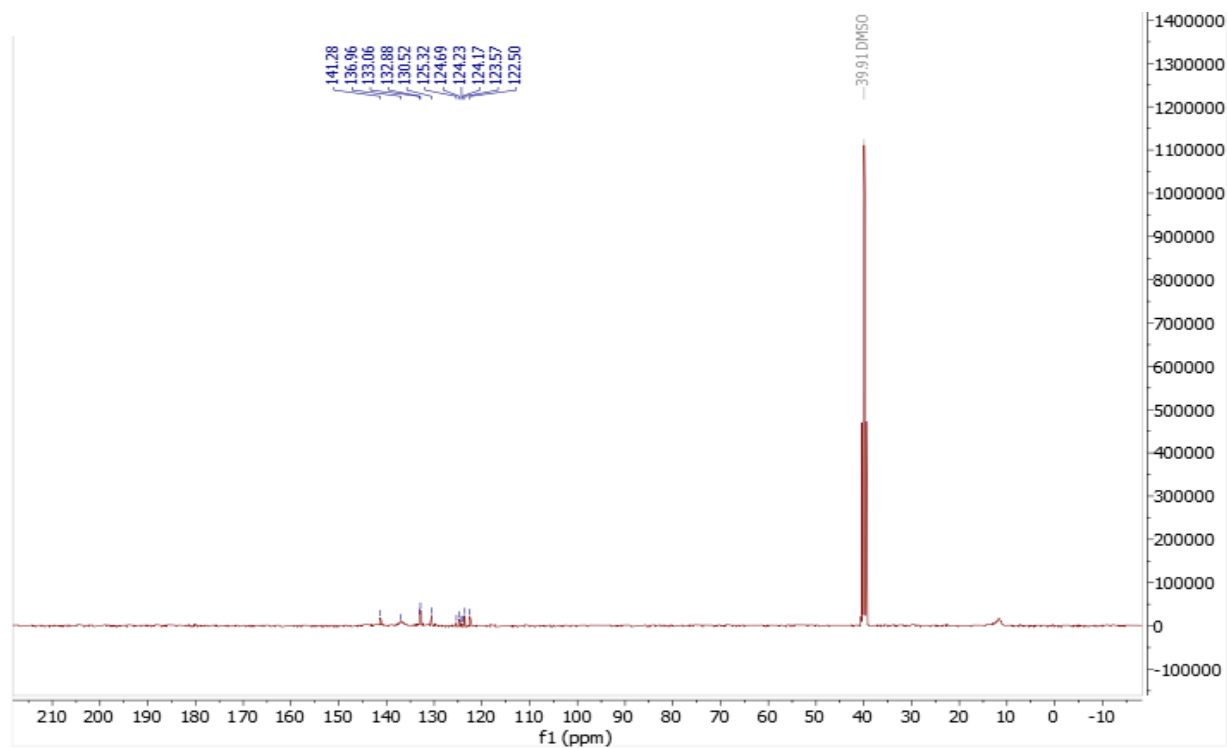


Figure 247, ¹³C NMR of SB112.

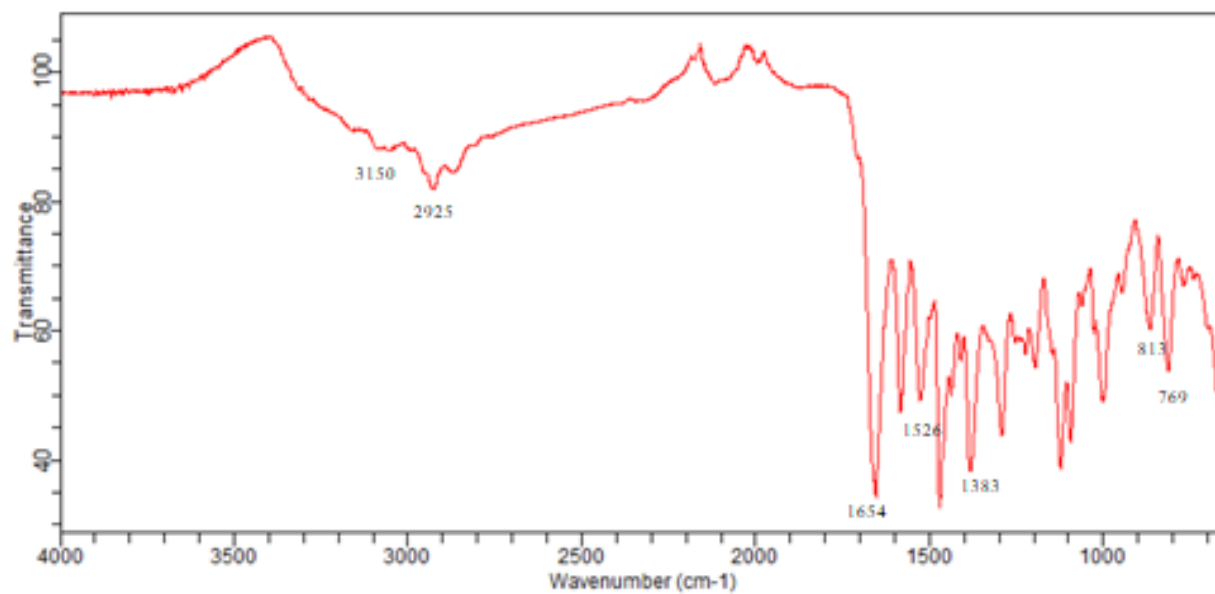


Figure 248, IR of SB112.

HRMS (+ESI-TOF) m/z: [M⁺] calcd for C₁₁H₇Cl₂N₃O₂S 314.96360; found 314.96371.

SB113

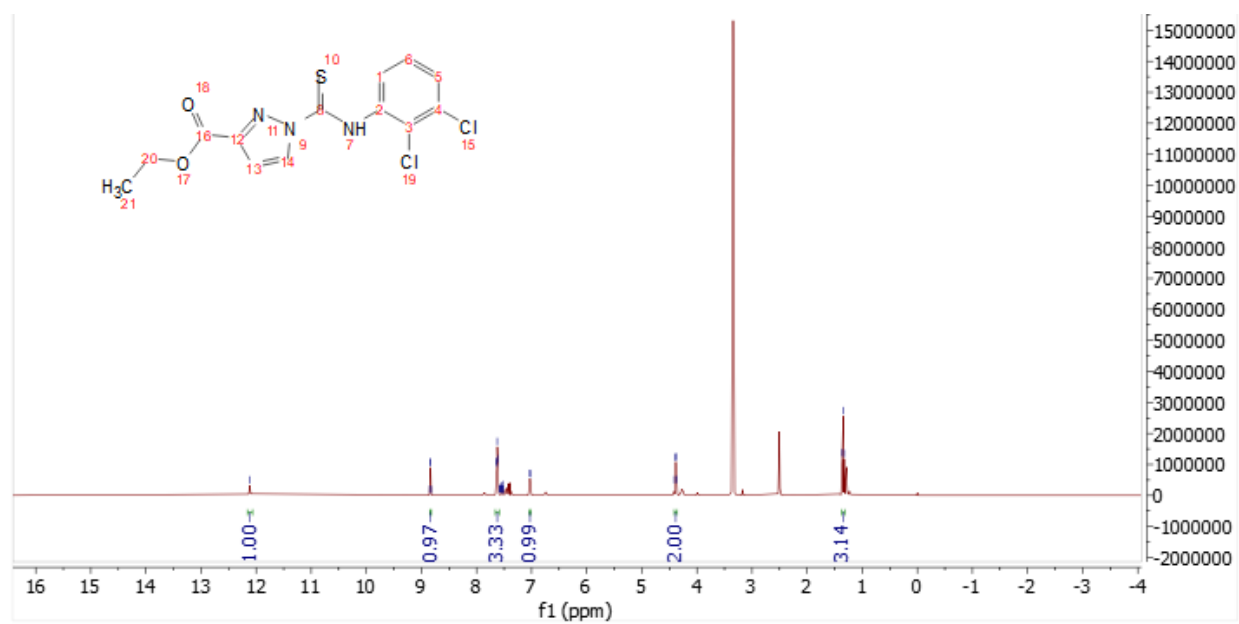


Figure 249, ¹H NMR of SB113.

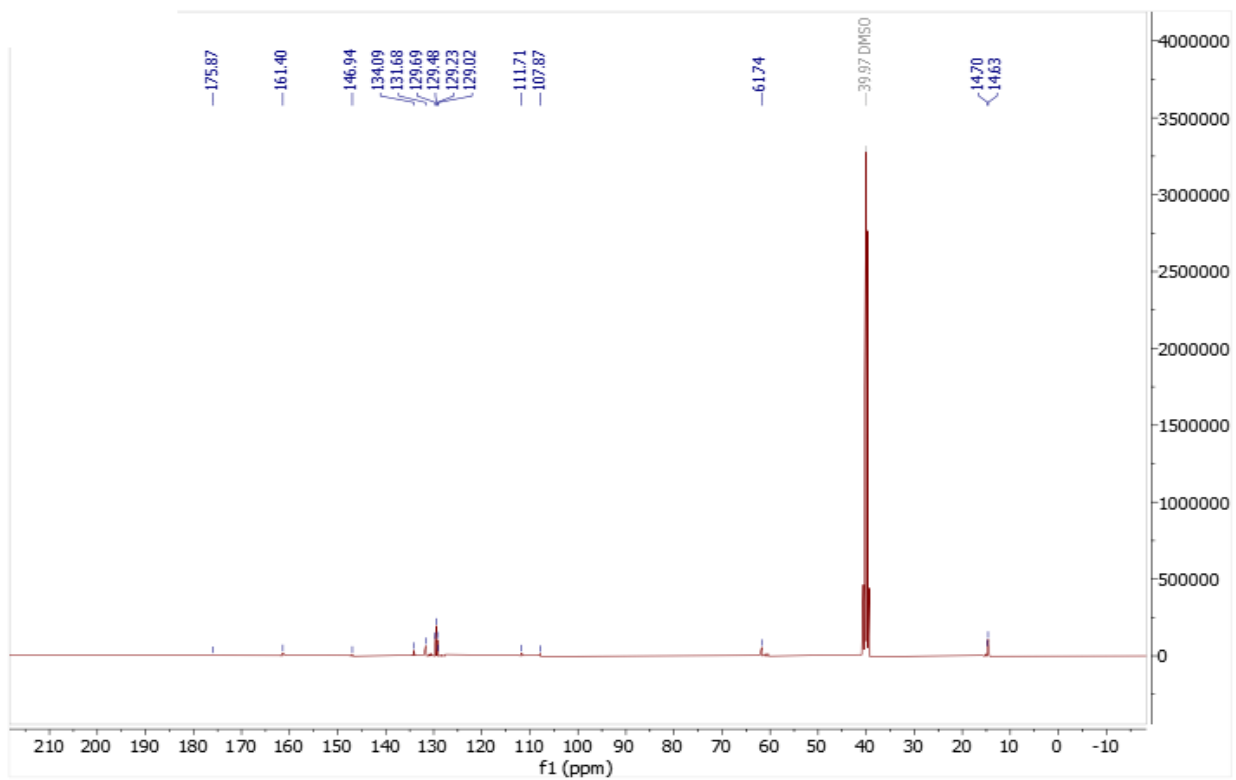


Figure 250, ¹³C NMR of SB113.

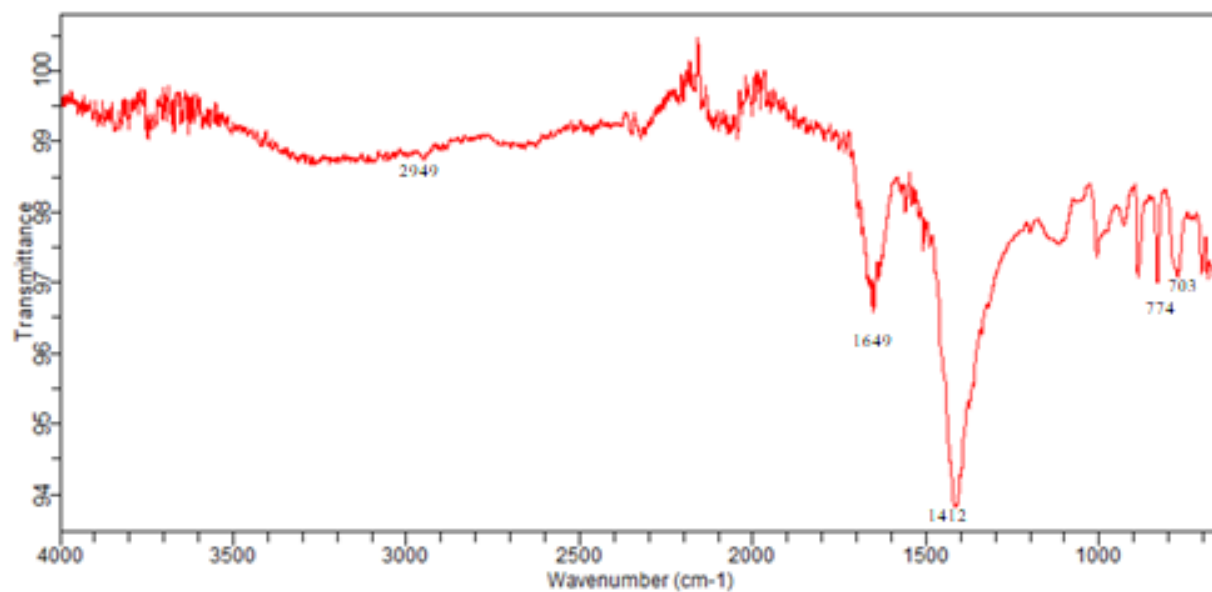


Figure 251, IR of SB113.

HRMS (+ESI-TOF) m/z: [M⁺] calcd for C₁₃H₁₁Cl₂N₃O₂S 342.99490; found 342.99465.

SB114

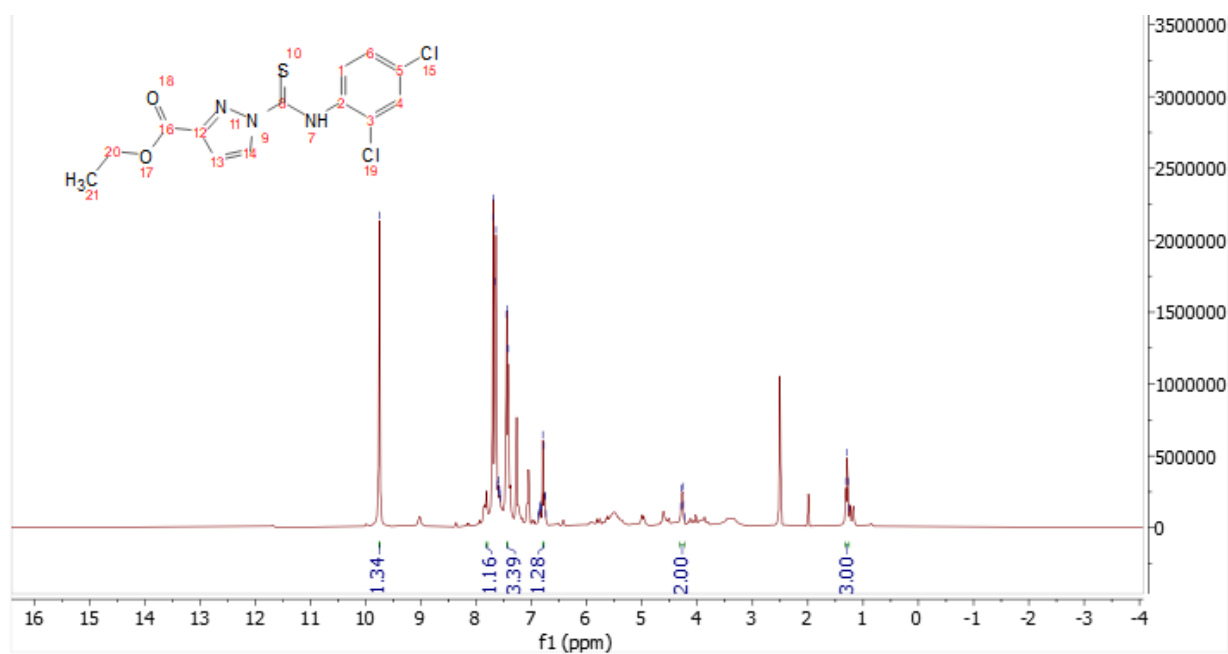


Figure 252, ¹H NMR of SB114.

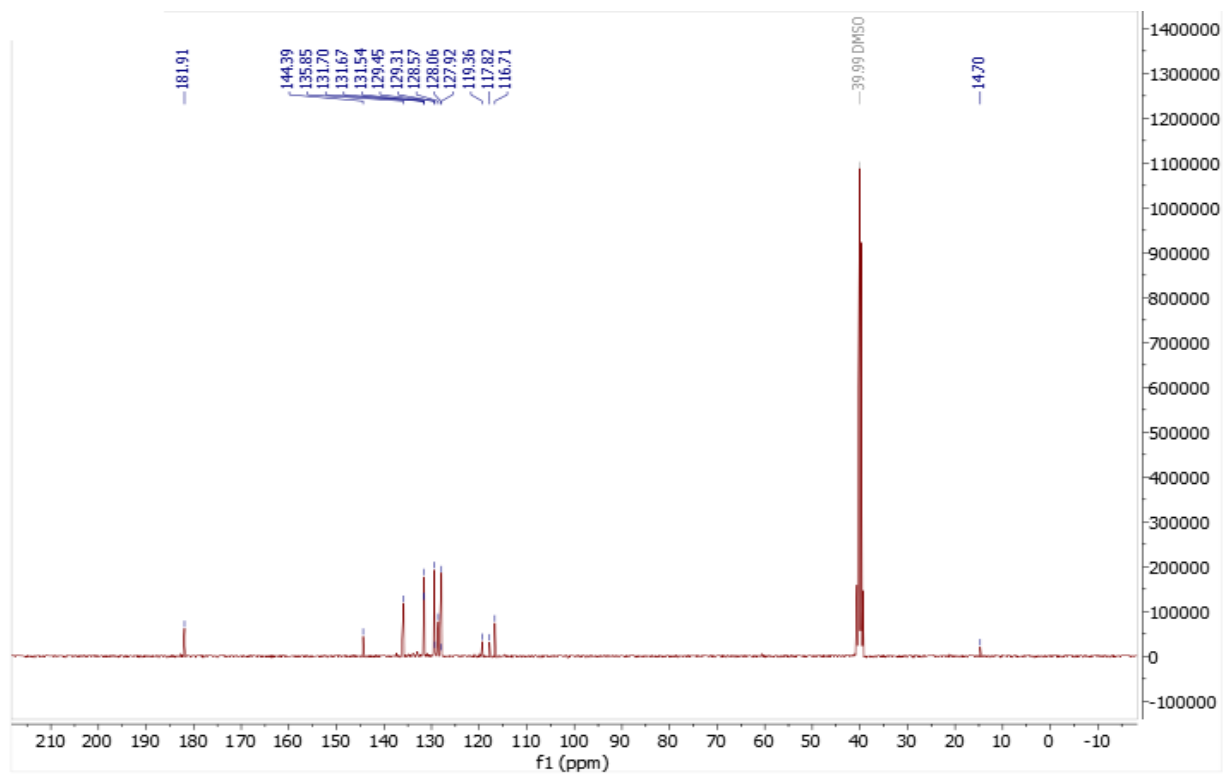


Figure 253, ^{13}C NMR of SB114.

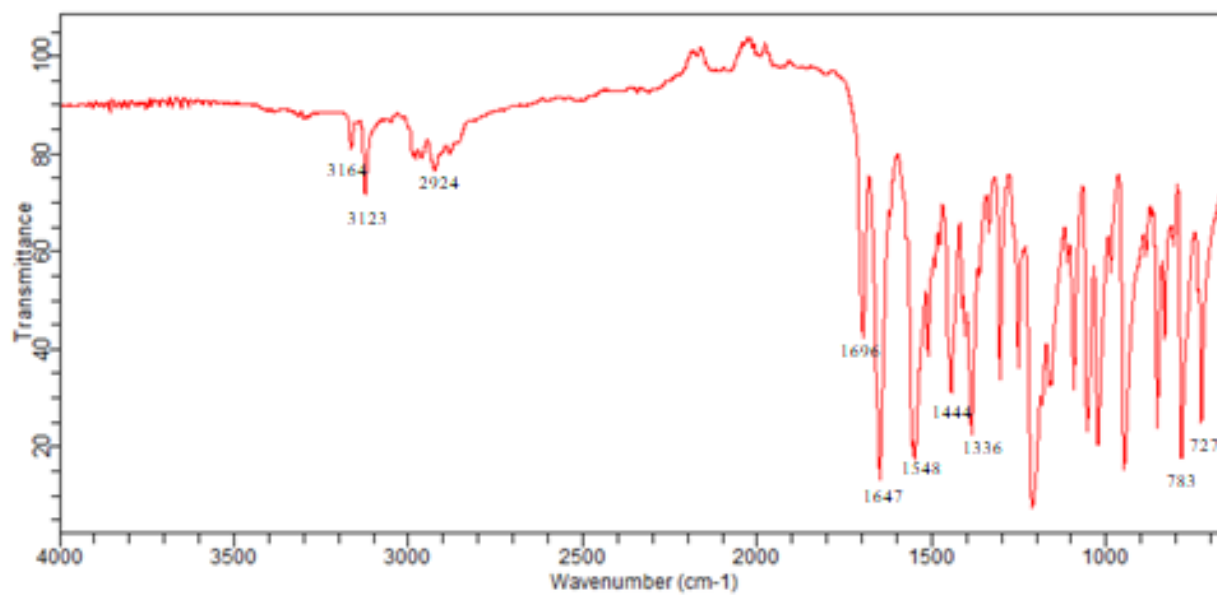


Figure 254, IR of SB114.

HRMS (+ESI-TOF) m/z : $[M]^+$ calcd for $\text{C}_{13}\text{H}_{11}\text{Cl}_2\text{N}_3\text{O}_2\text{S}$ 342.99490; found 342.99472.

SB115

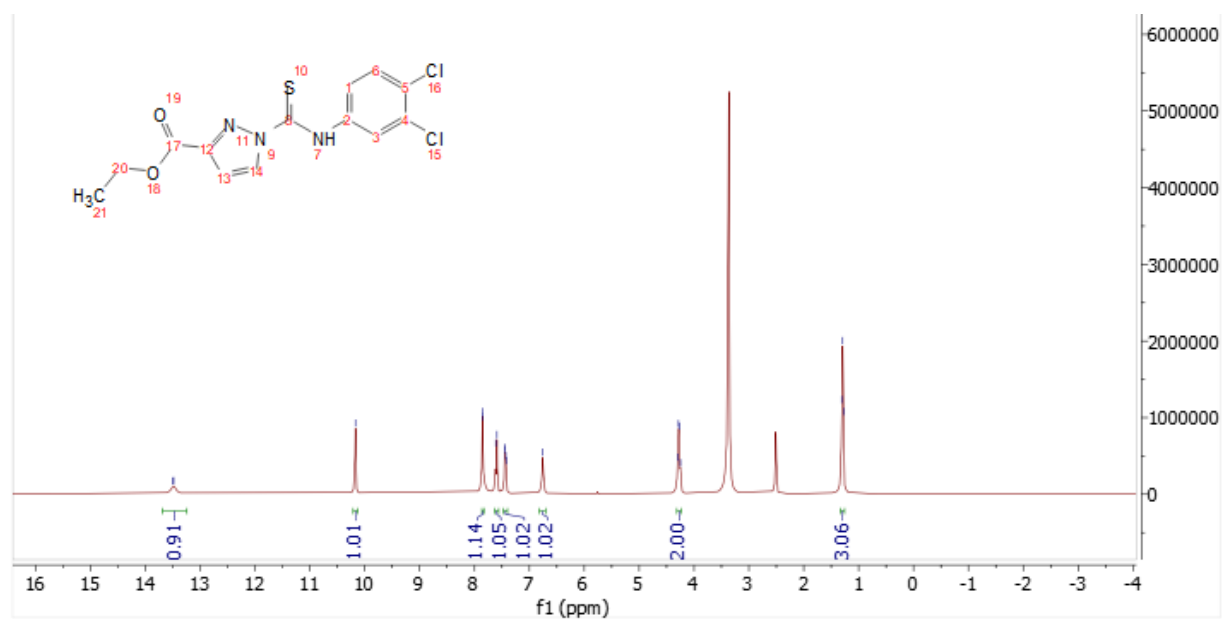


Figure 255, ¹H NMR of SB115.

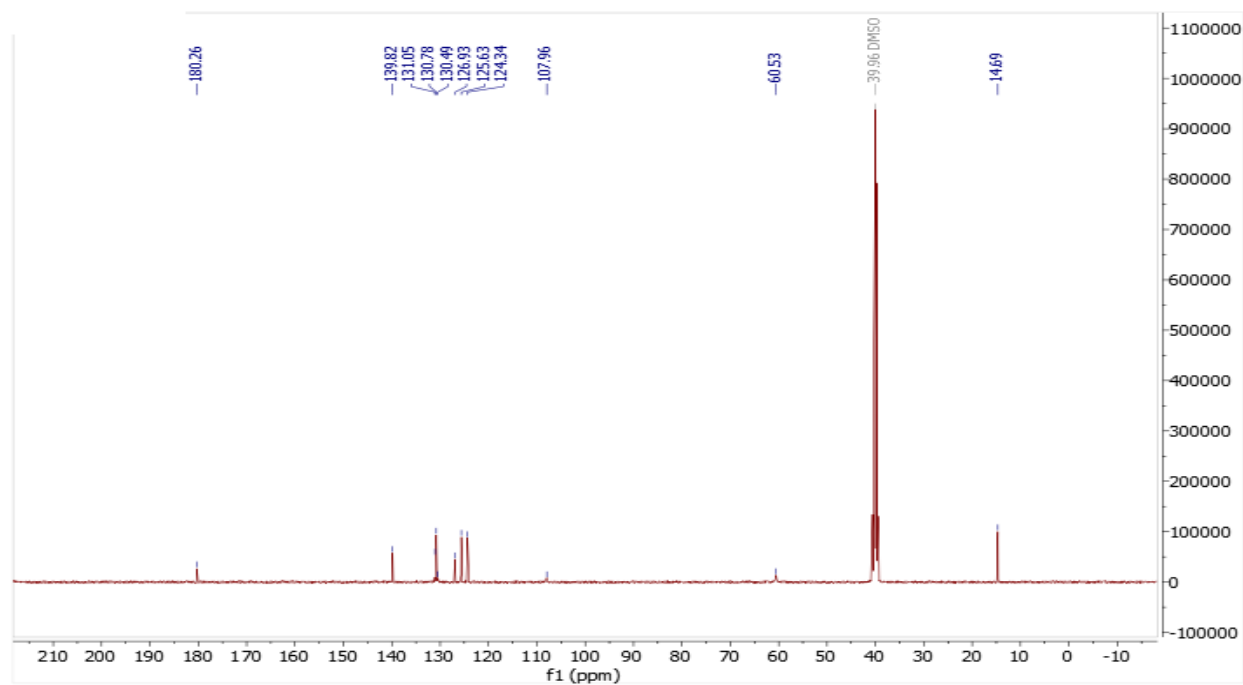


Figure 256, ¹³C NMR of SB115.

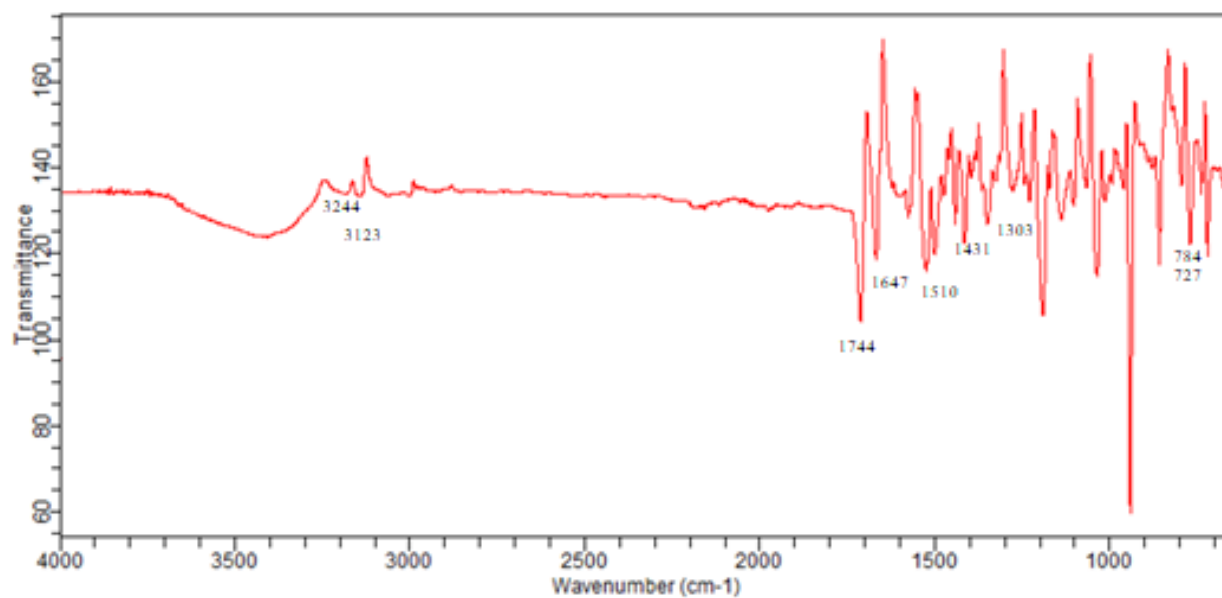


Figure 257, IR of SB115.

HRMS (+ESI-TOF) m/z: [M⁺] calcd for C₁₃H₁₁Cl₂N₃O₂S 342.99490; found 342.99490.

SB116

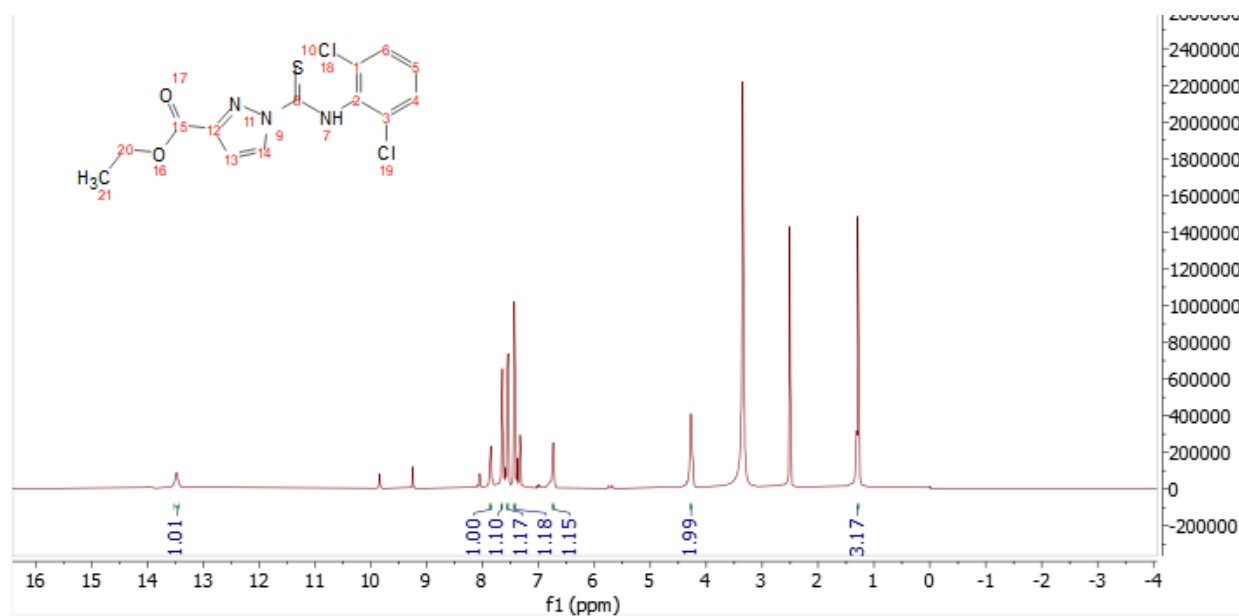


Figure 258, ¹H NMR of SB116.

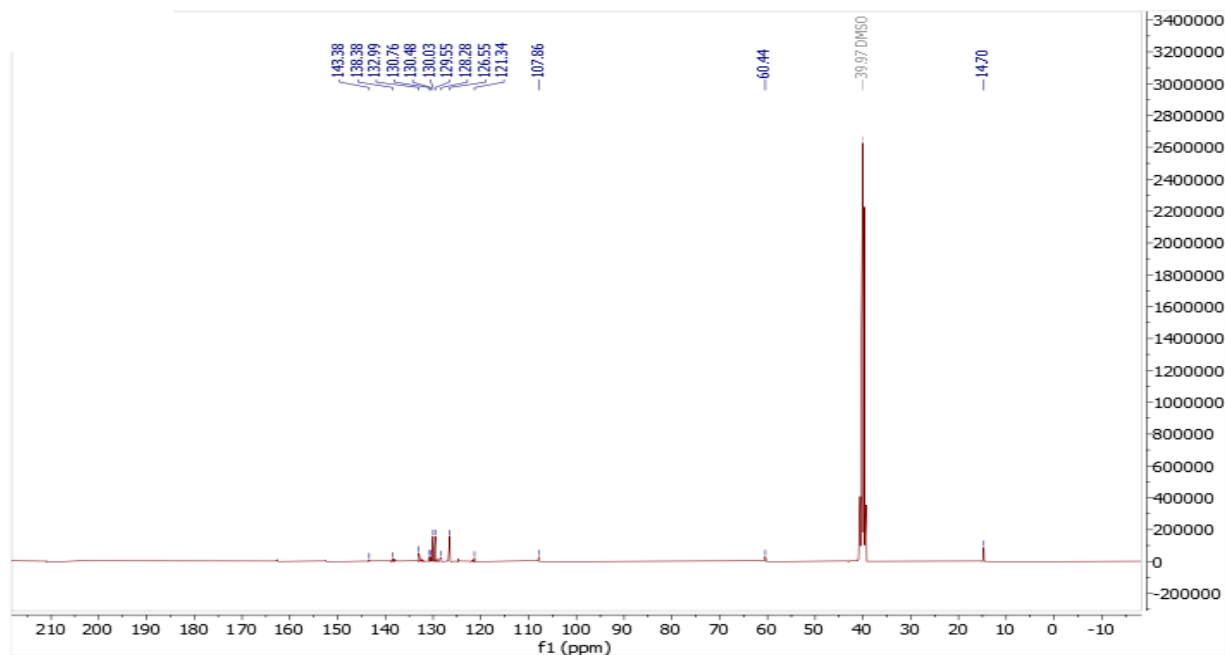


Figure 259, ^{13}C NMR of SB116

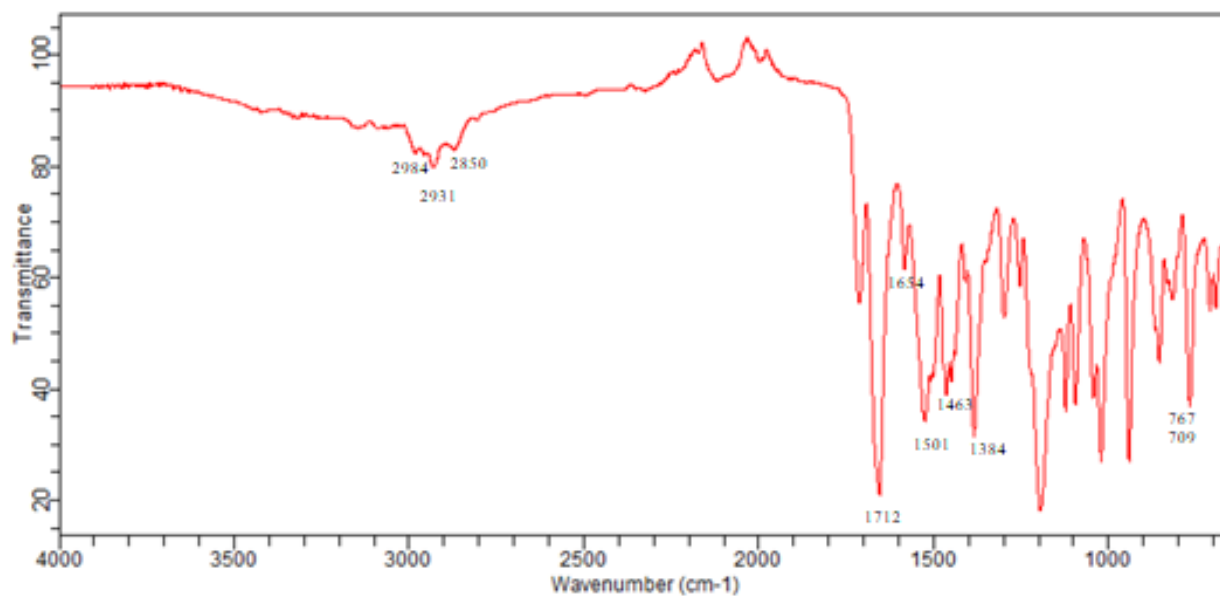


Figure 260, IR of SB116.

HRMS (+ESI-TOF) m/z : $[M]^+$ calcd for $\text{C}_{13}\text{H}_{11}\text{Cl}_2\text{N}_3\text{O}_2\text{S}$ 342.99490; found 342.99468.

SB117

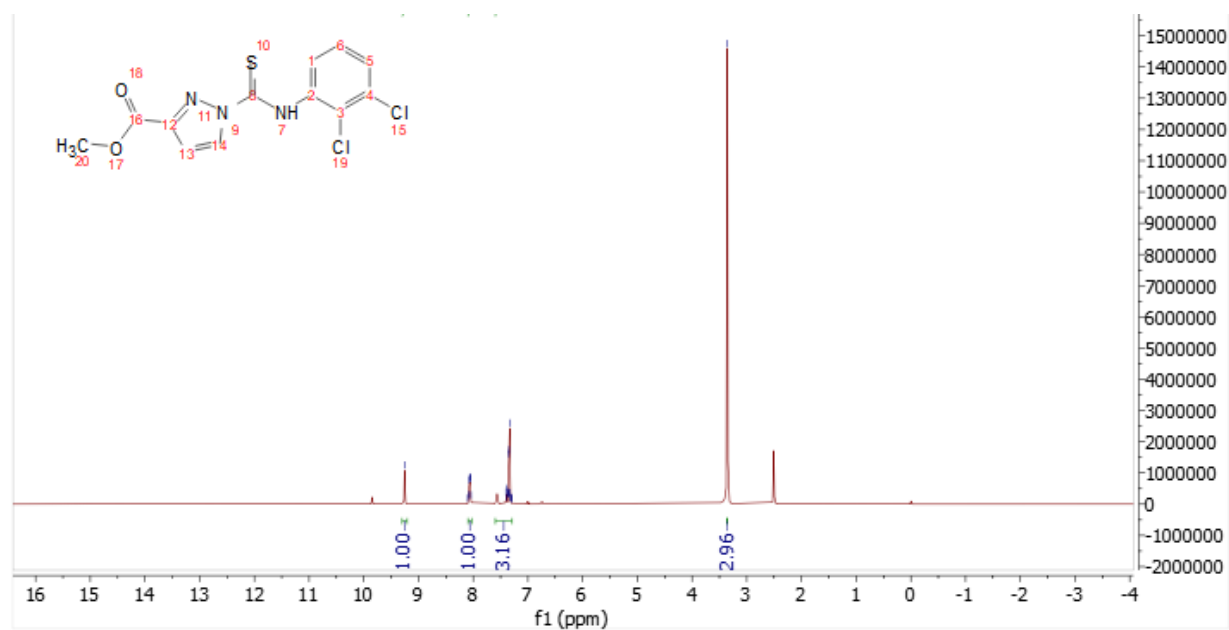


Figure 261, ¹H NMR of SB117.

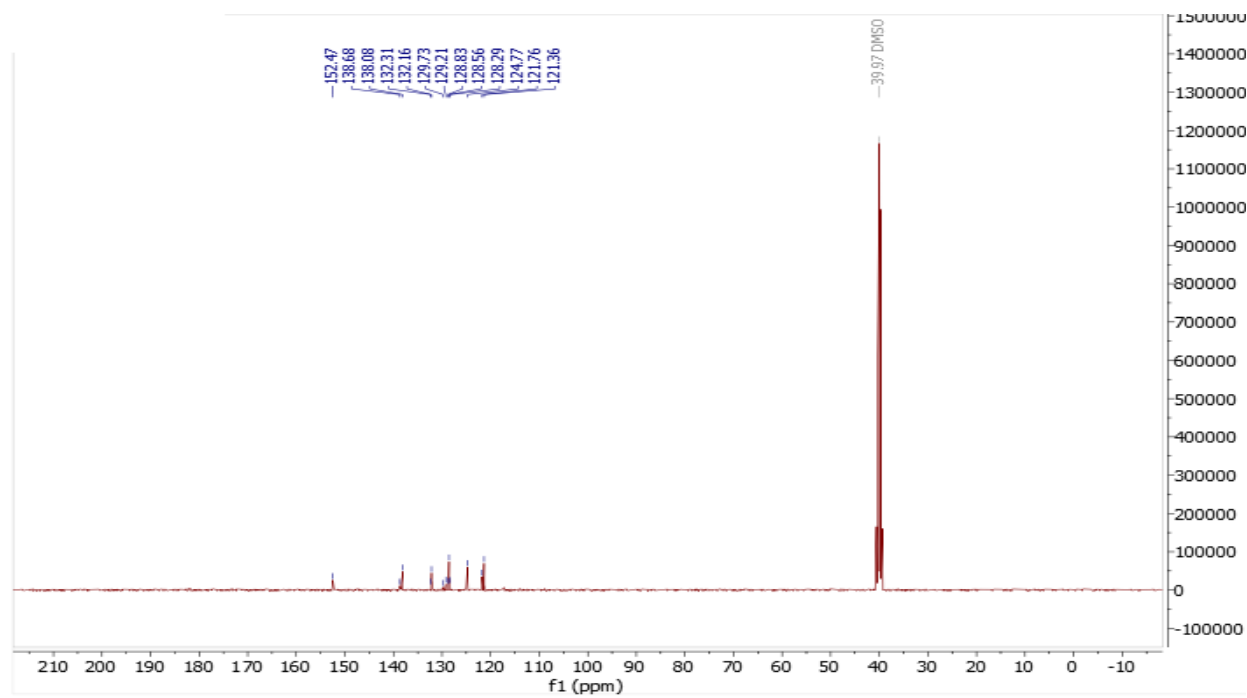


Figure 262, ¹³C NMR of SB117.

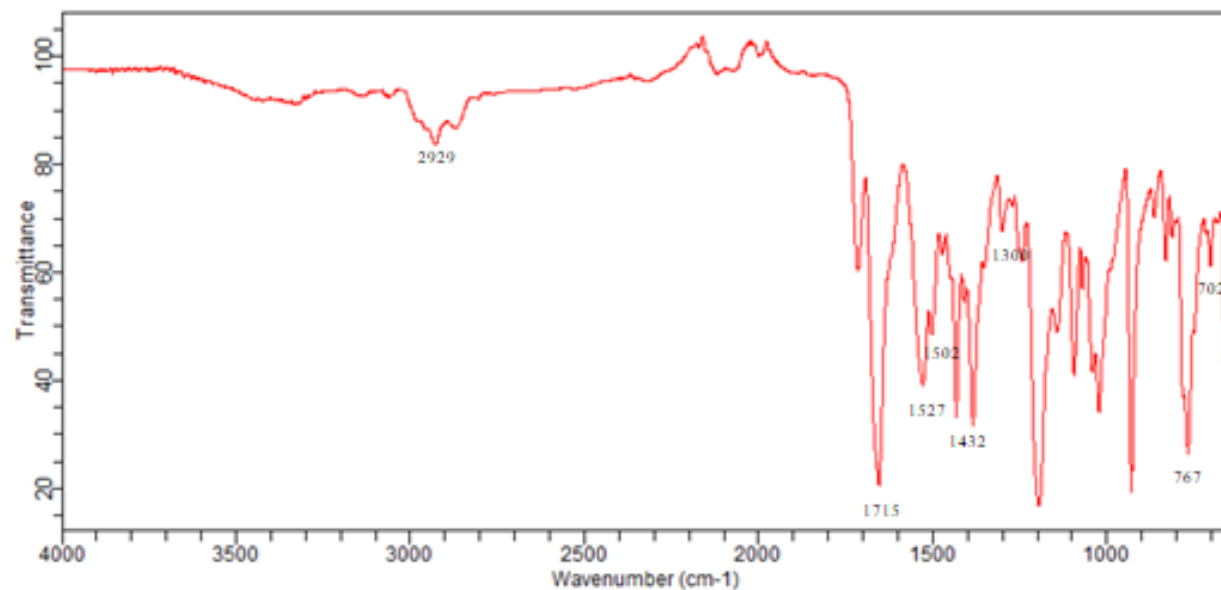


Figure 263, IR of SB117.

HRMS (+ESI-TOF) m/z: [M⁺] calcd for C₁₂H₉Cl₂N₃O₂S 328.97925; found 328.97897.

SB118

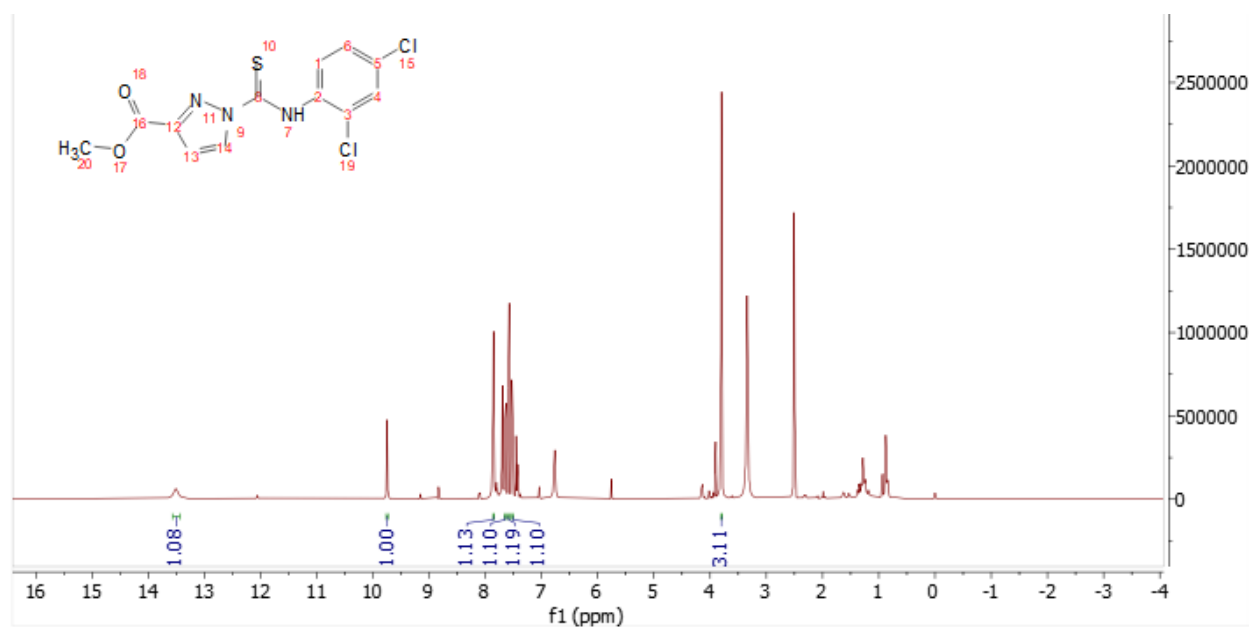


Figure 264, ¹H NMR of SB118.

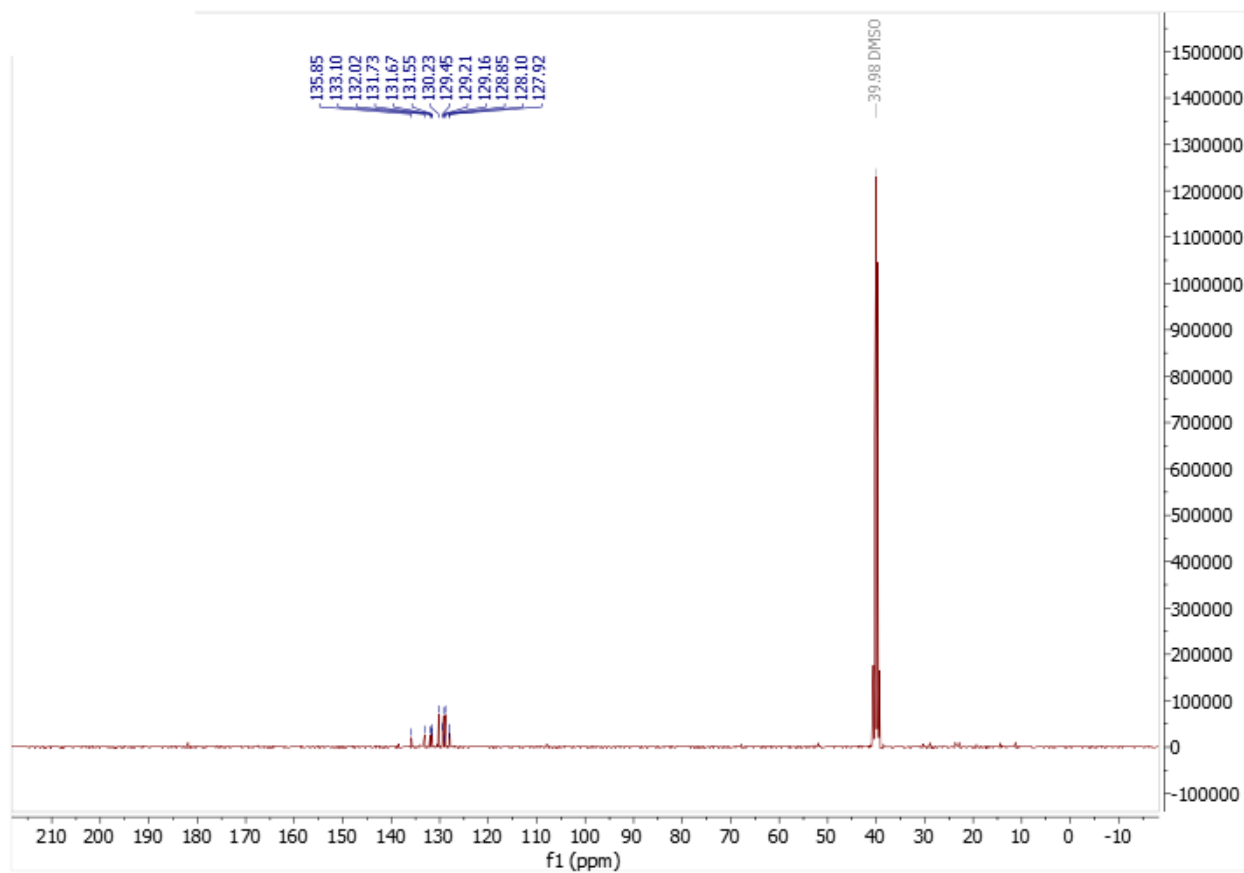


Figure 265, ¹³C NMR of SB118.

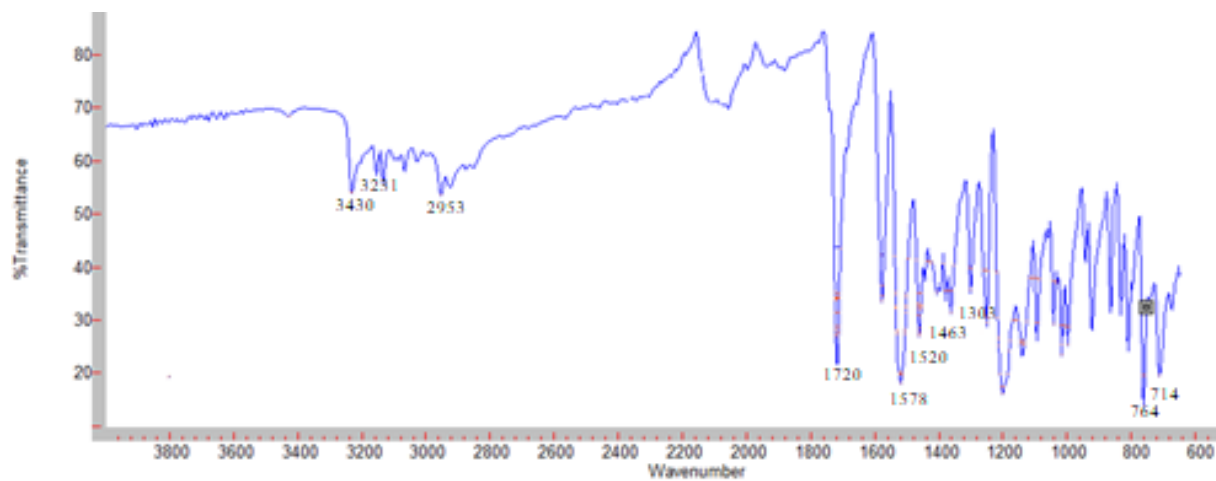


Figure 266, IR of SB118.

HRMS (+ESI-TOF) m/z: [M⁺] calcd for C₁₂H₉Cl₂N₃O₂S 328.97925; found 328.97920.

SB119

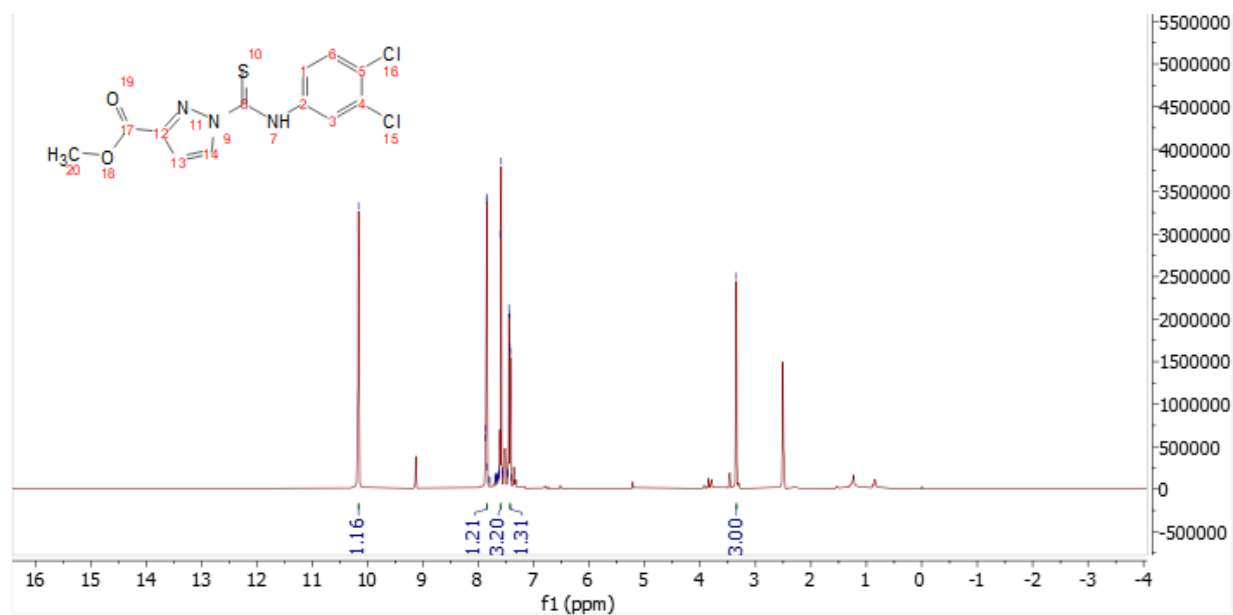


Figure 267, ¹H NMR of SB119.

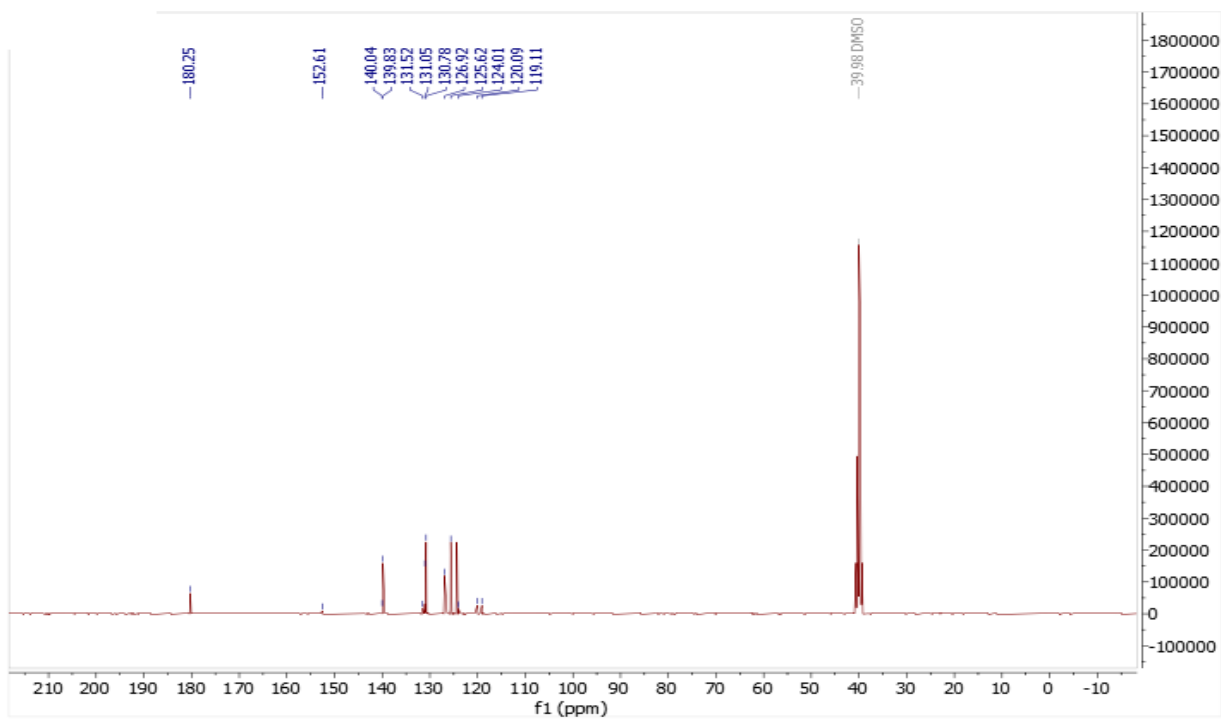


Figure 268, ¹³C NMR of SB119.

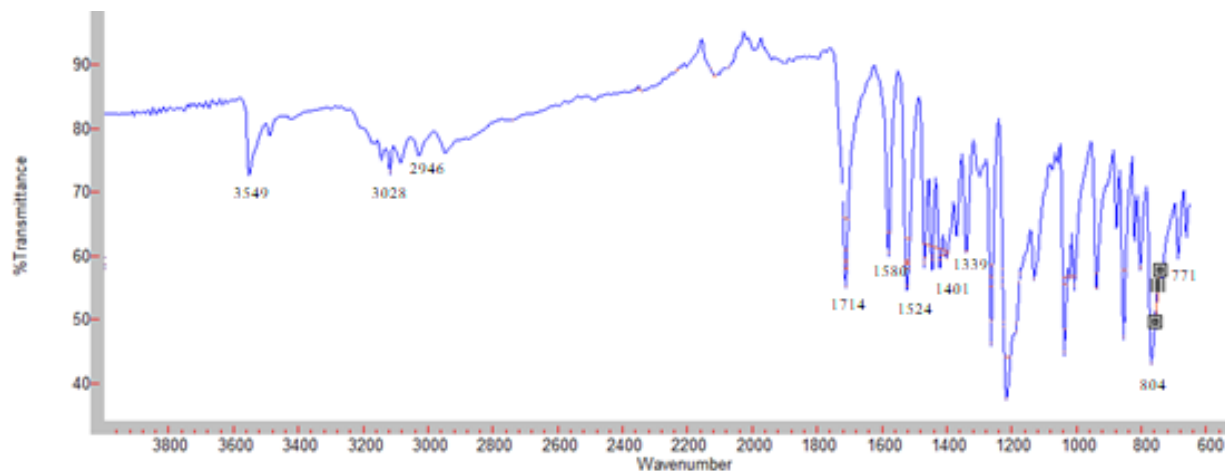


Figure 269, IR of SB119.

HRMS (+ESI-TOF) m/z: [M⁺] calcd for C₁₂H₉Cl₂N₃O₂S 328.97925; found 328.97941.

SB120

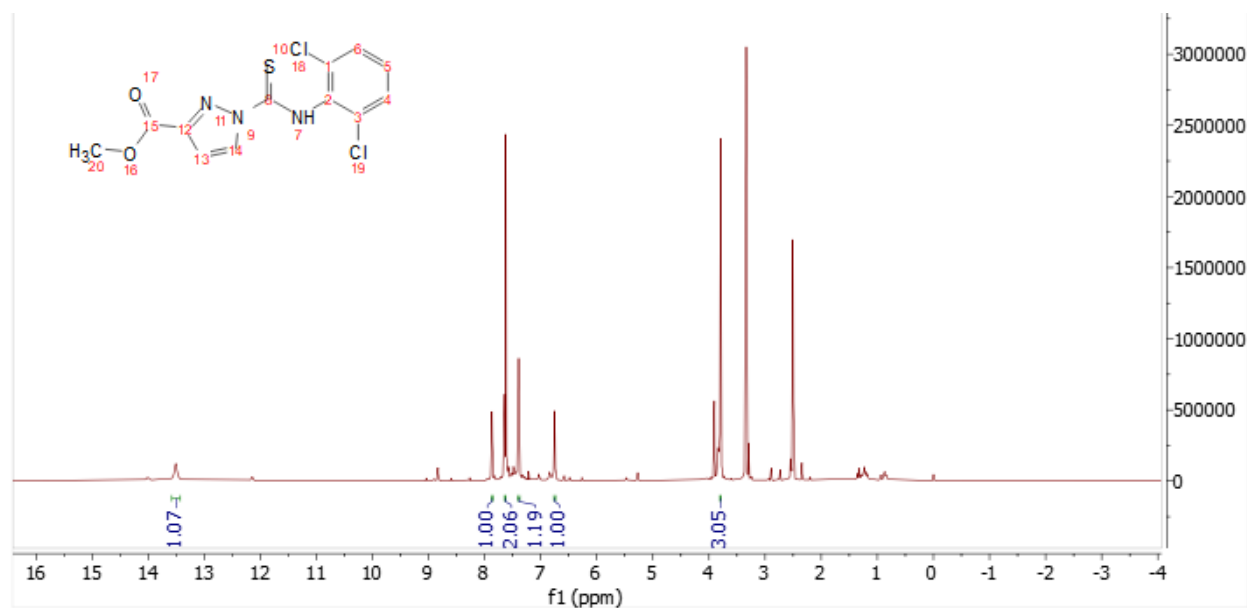


Figure 270, ¹H NMR of SB120.

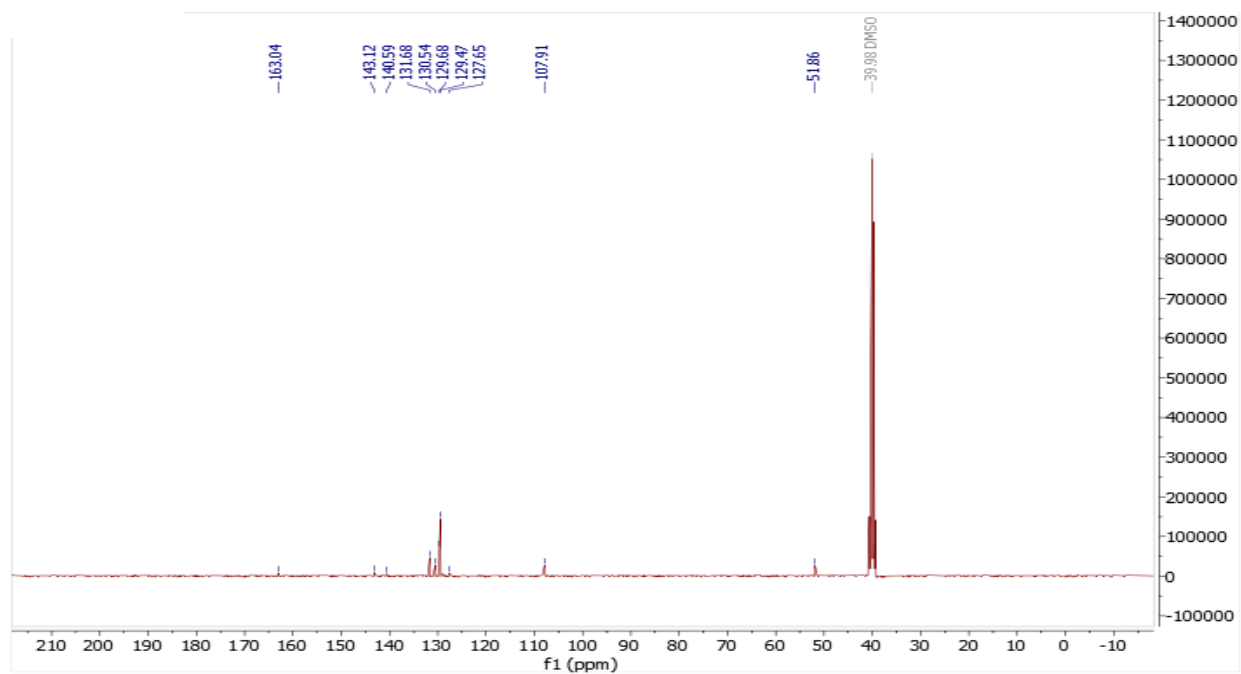


Figure 271, ¹³C NMR of SB120.

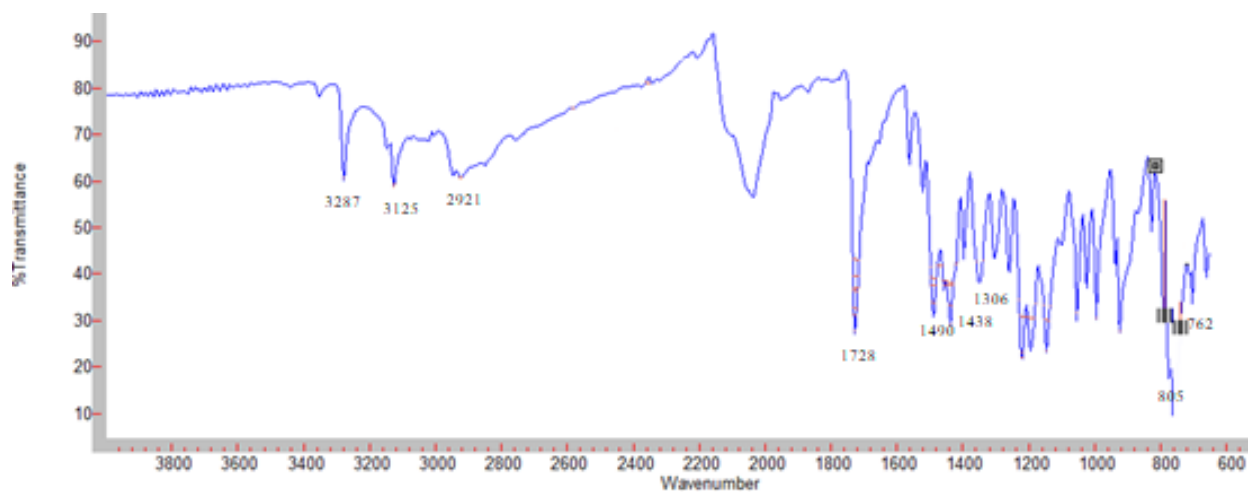


Figure 272, IR of SB120.

HRMS (+ESI-TOF) m/z: [M⁺] calcd for C₁₂H₉Cl₂N₃O₂S 328.97925; found 328.97955.

SB121

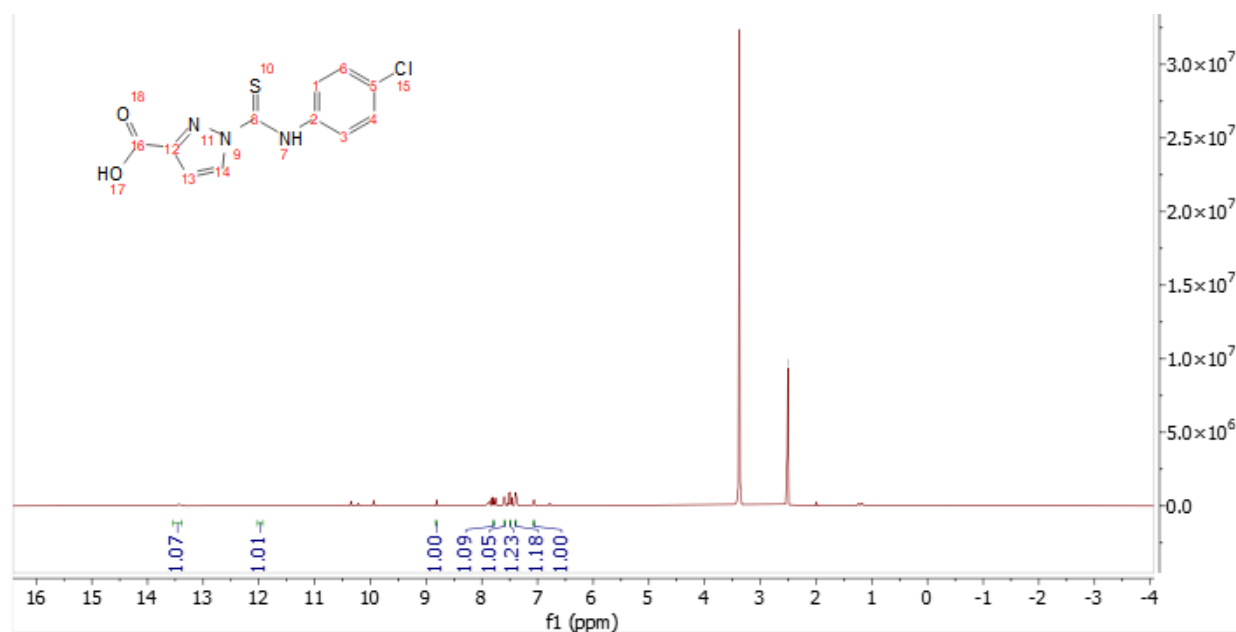


Figure 273, ¹H NMR of SB121.

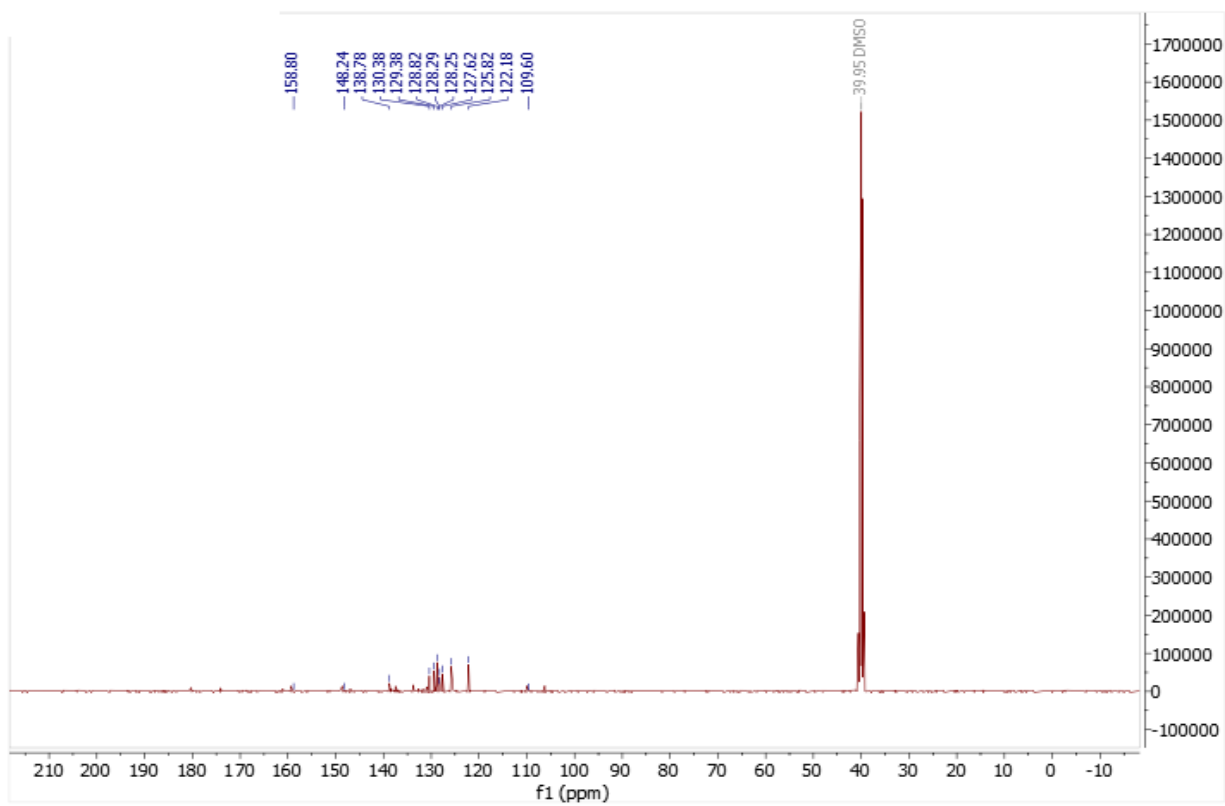


Figure 274, ¹³C NMR of SB121.

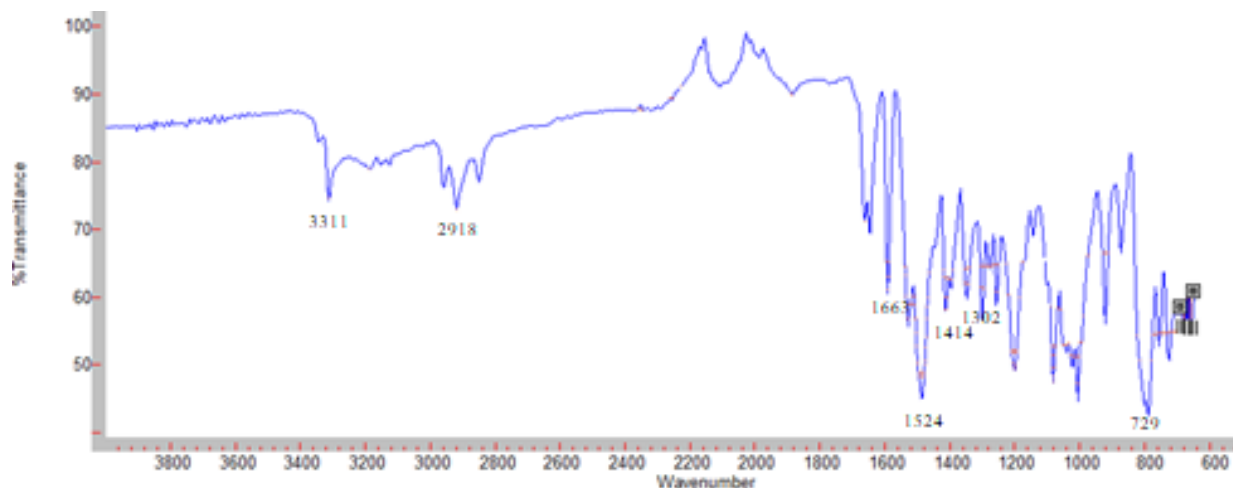


Figure 275, IR of SB121.

HRMS (+ESI-TOF) m/z : $[M]^+$ calcd for $C_{11}H_8ClN_3O_2S$ 281.00257; found 281.00260.

SB122

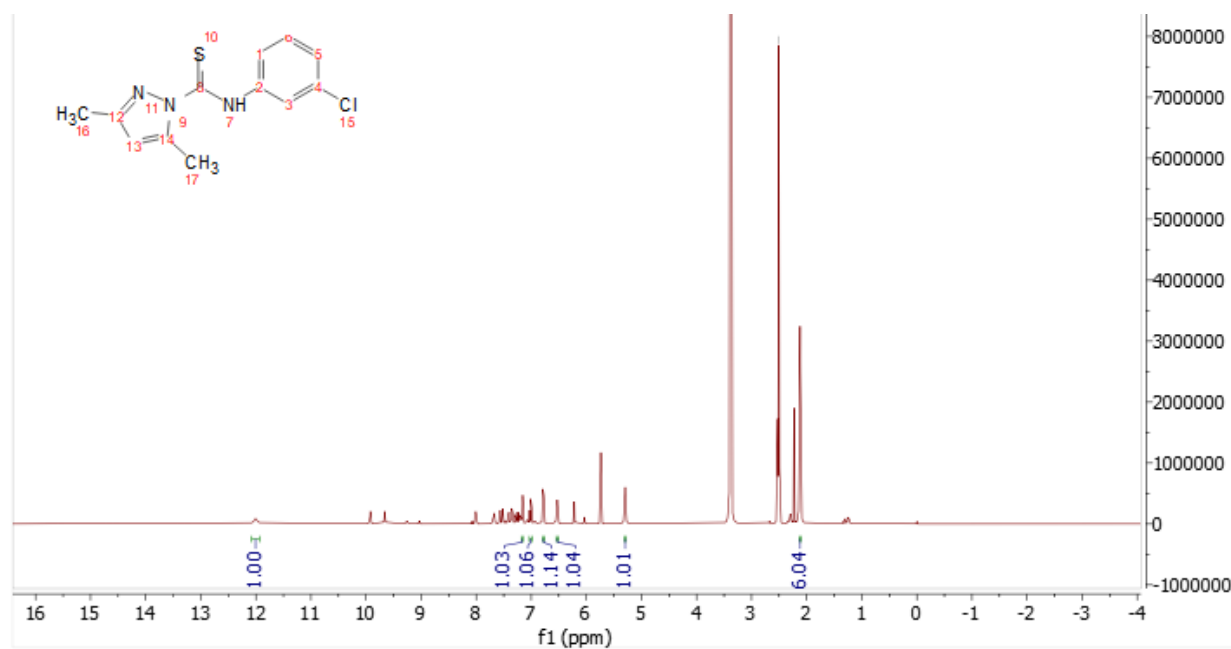


Figure 276, ¹H NMR of SB122.

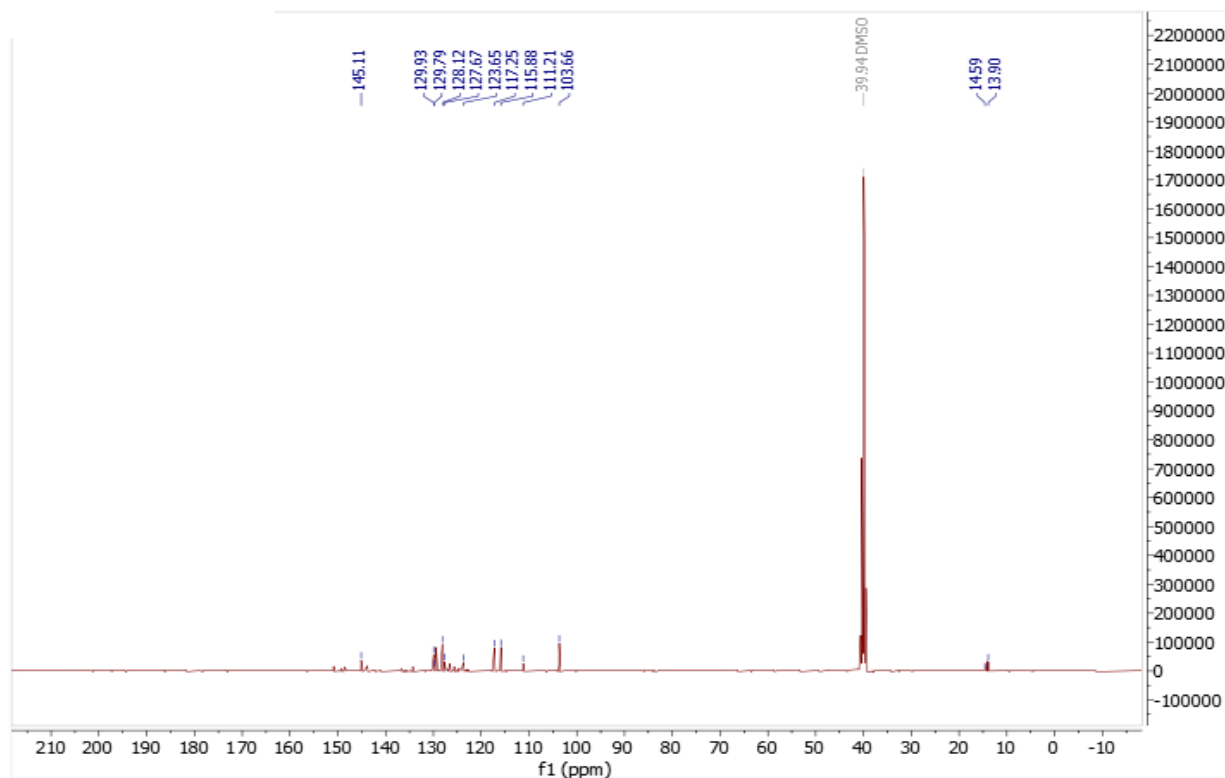


Figure 277, ^{13}C NMR of SB122.

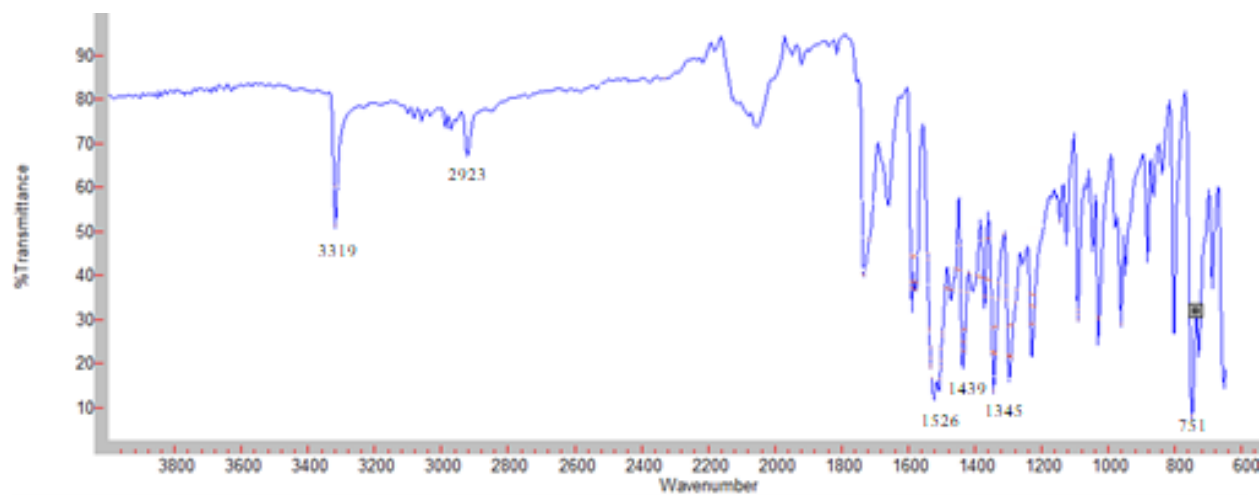


Figure 278, IR of SB122.

HRMS (+ESI-TOF) m/z : $[M]^+$ calcd for $\text{C}_{12}\text{H}_{12}\text{ClN}_3\text{S}$ 265.04405; found 265.04402.

SB123

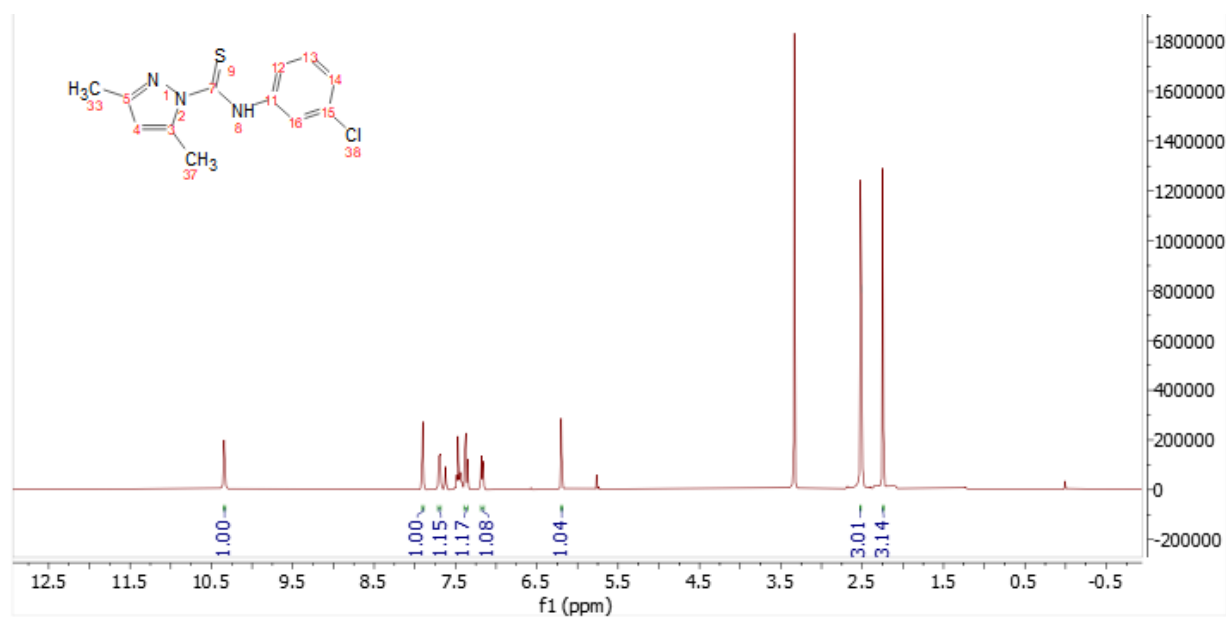


Figure 279, ¹H NMR of SB123.

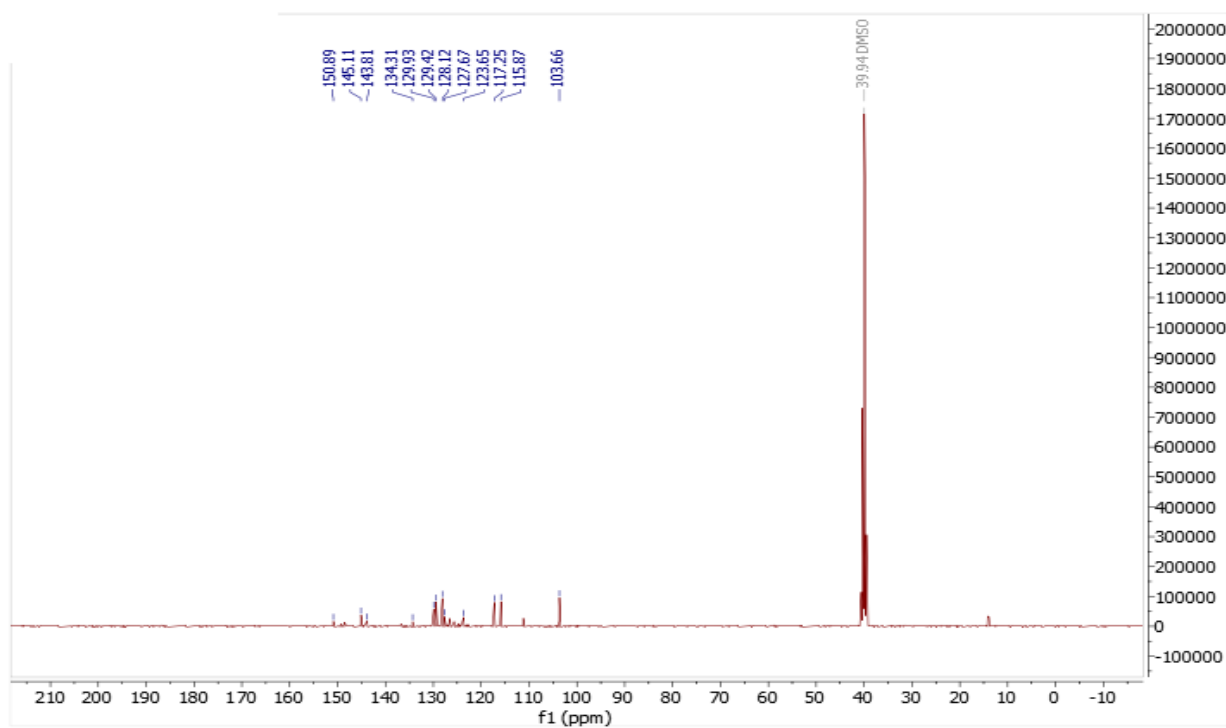


Figure 280, ¹³C NMR of SB123.

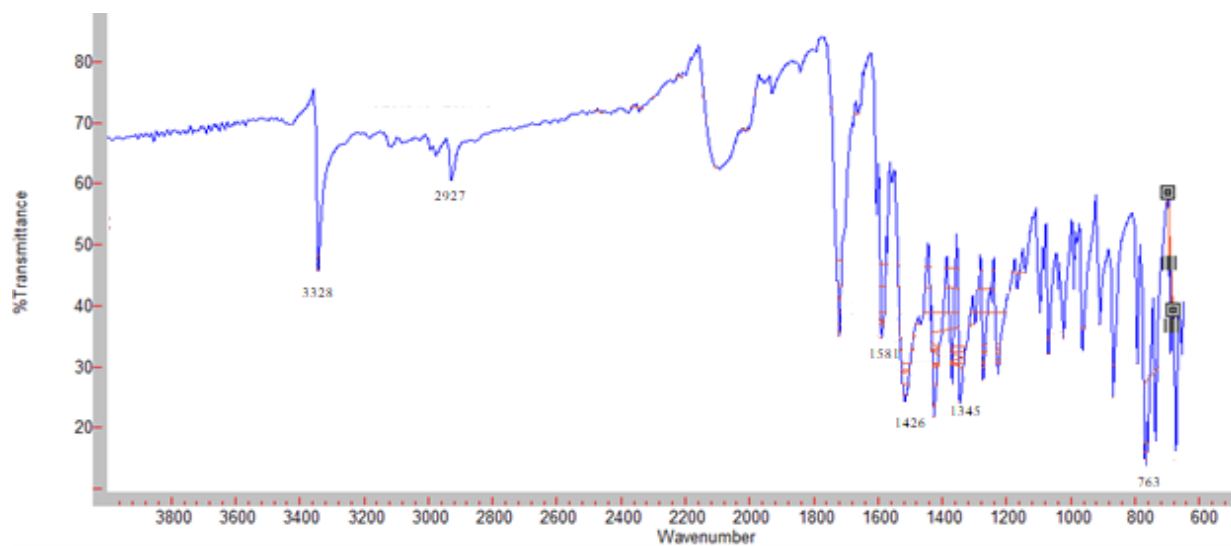


Figure 281, IR of SB123.

HRMS (+ESI-TOF) m/z: [M⁺] calcd for C₁₂H₁₂ClN₃S 265.04405; found 265.04404.

SB124

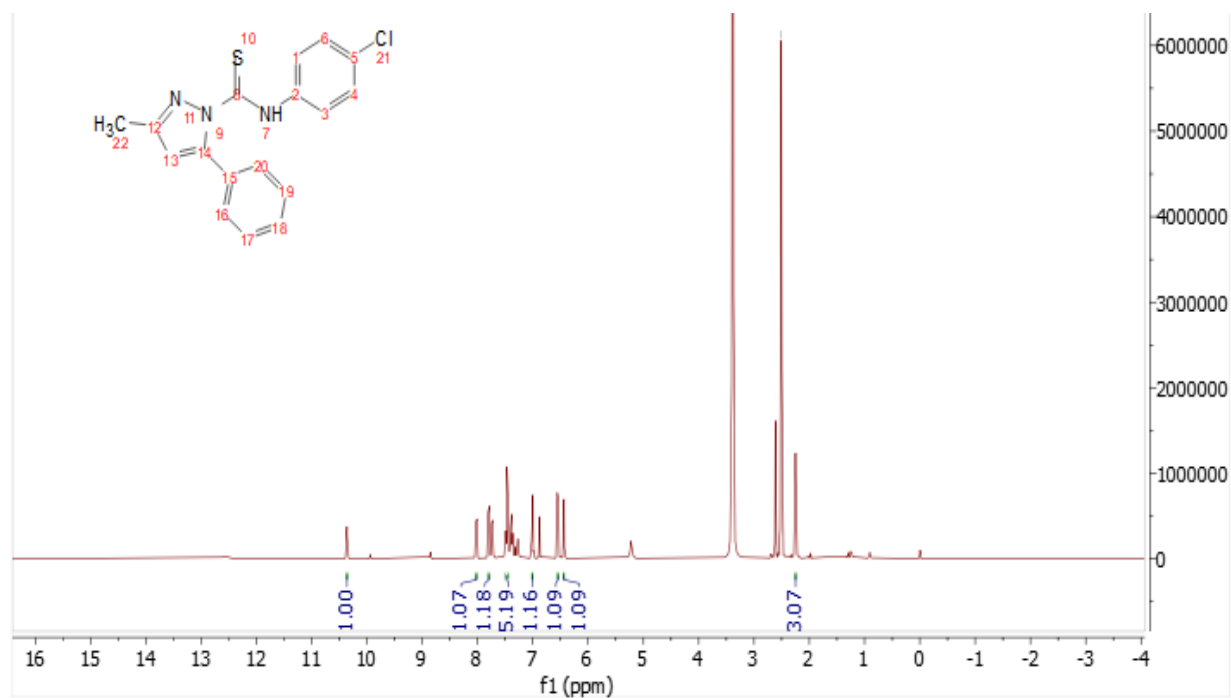


Figure 282, ¹H NMR of SB124.

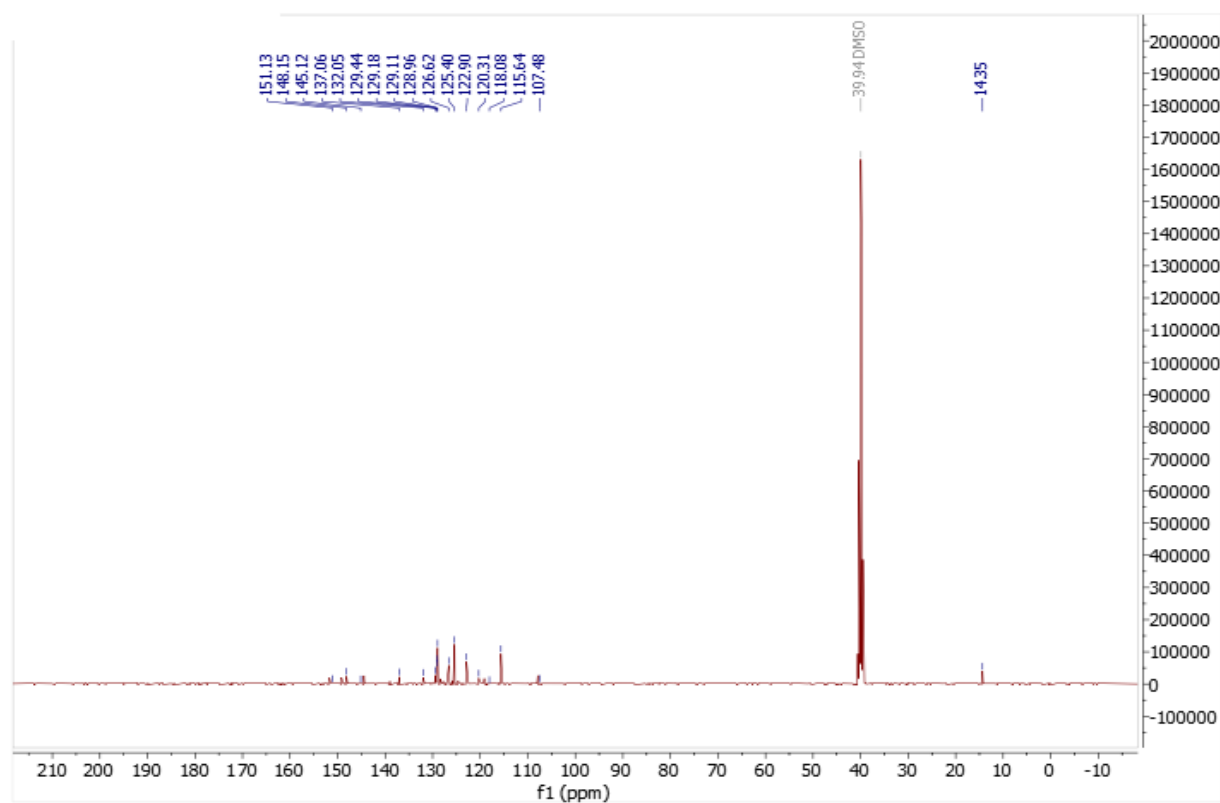


Figure 283, ^{13}C NMR of SB124.

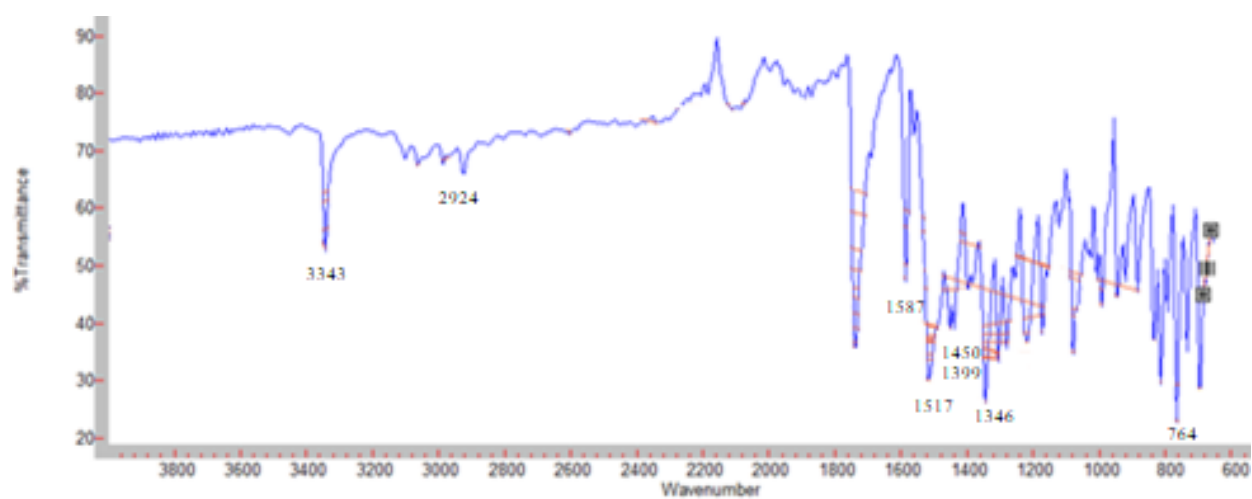


Figure 284, IR of SB124.

HRMS (+ESI-TOF) m/z : $[M]^+$ calcd for $\text{C}_{12}\text{H}_{12}\text{ClN}_3\text{S}$ 265.04405; found 265.04402.

SB125

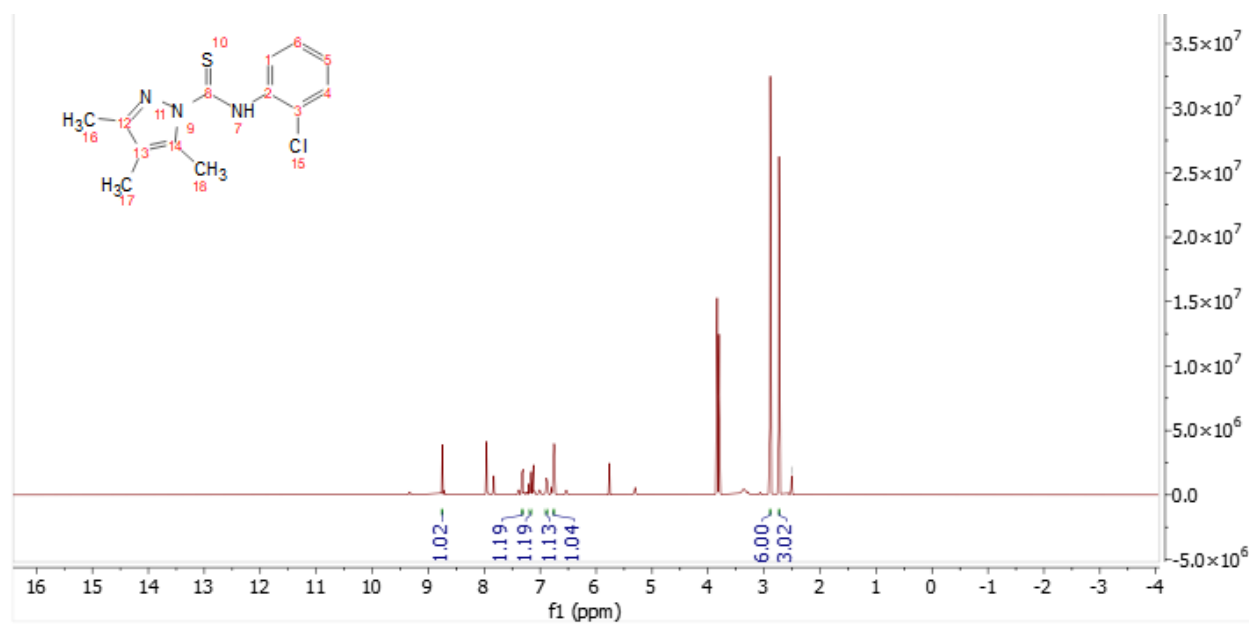


Figure 285, ¹H NMR of SB125.

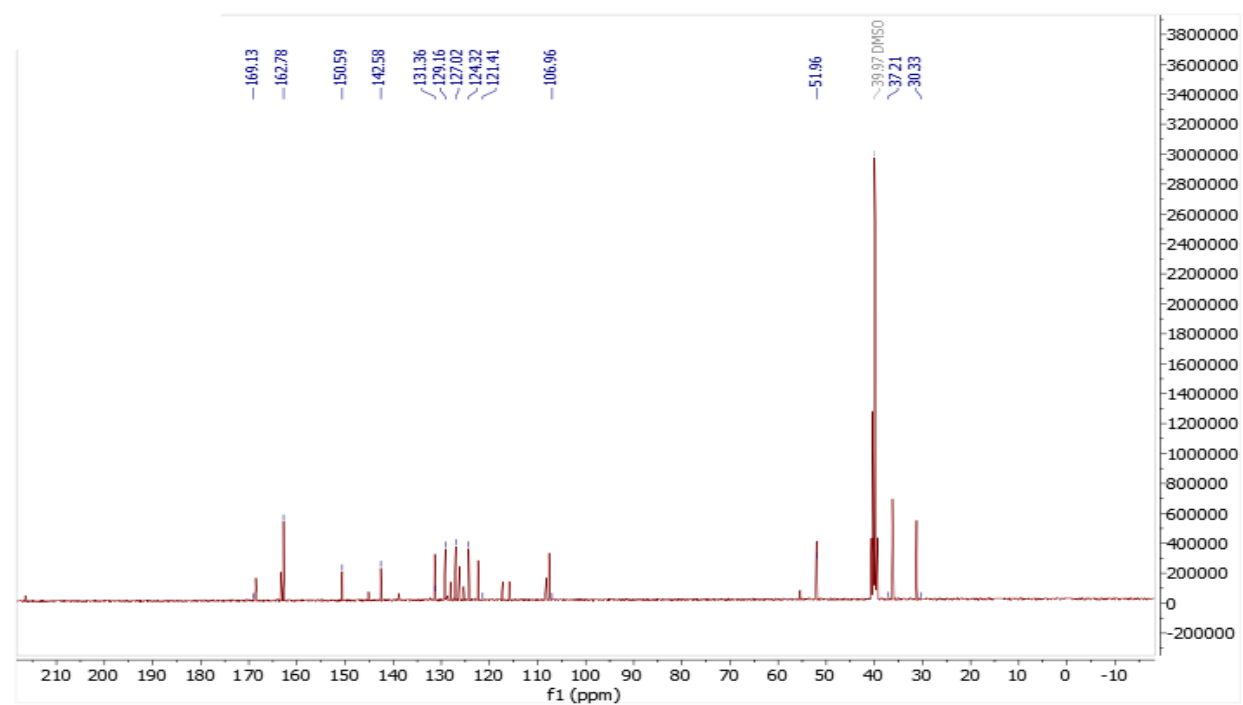


Figure 286, ¹³C NMR of SB125.

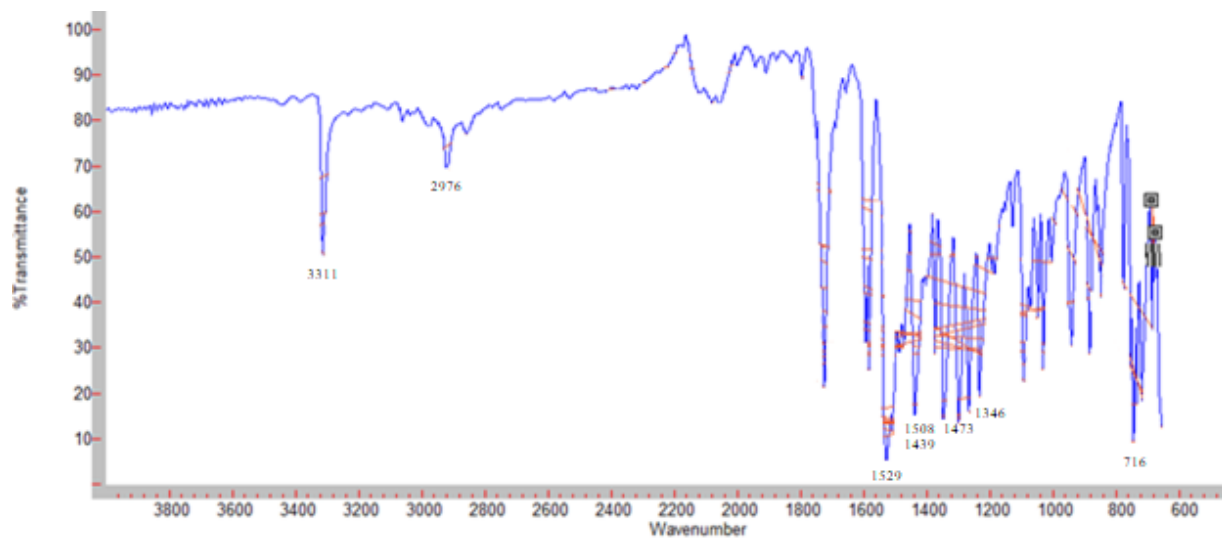


Figure 287, IR of SB125.

HRMS (+ESI-TOF) m/z: [M⁺] calcd for C₁₃H₁₄ClN₃S 279.05970; found 279.05966.

SB126

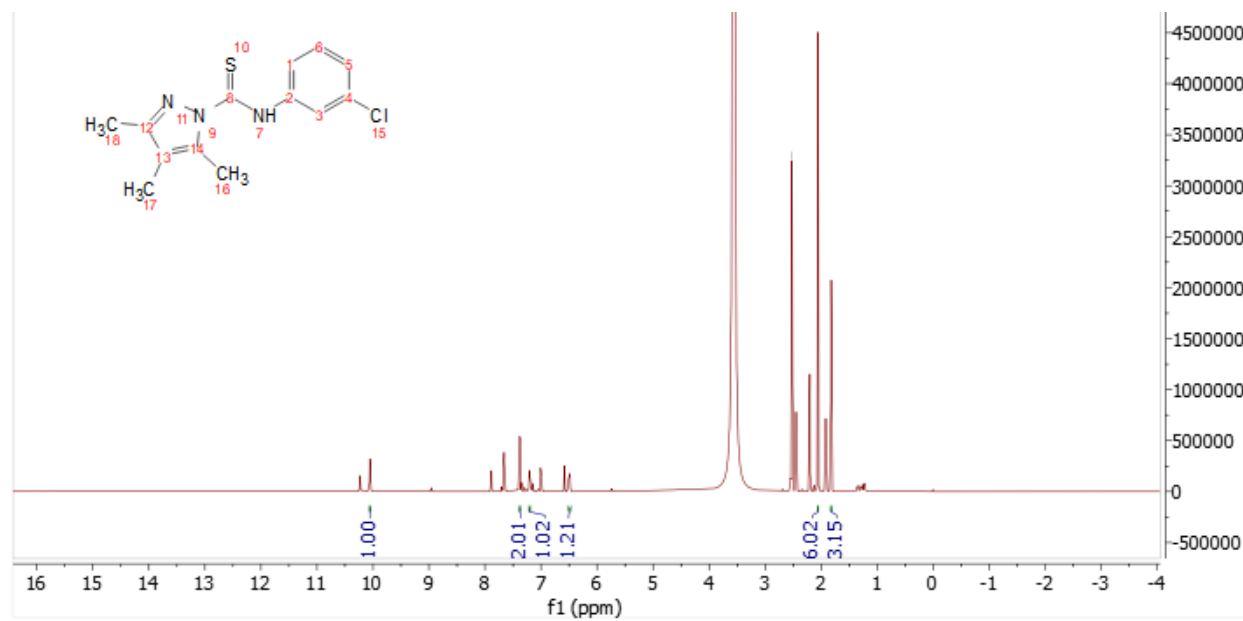


Figure 288, ¹H NMR of SB126.

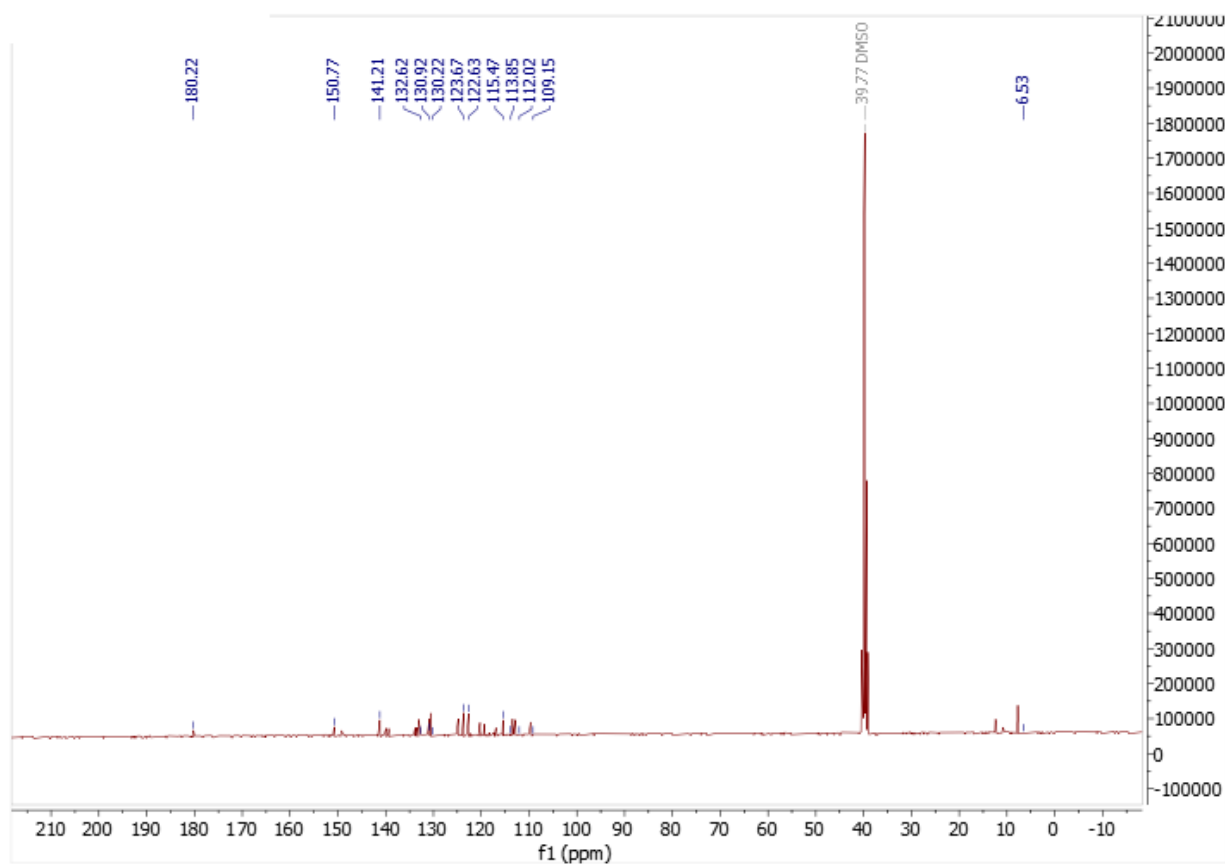


Figure 289, ¹³C NMR of SB126.

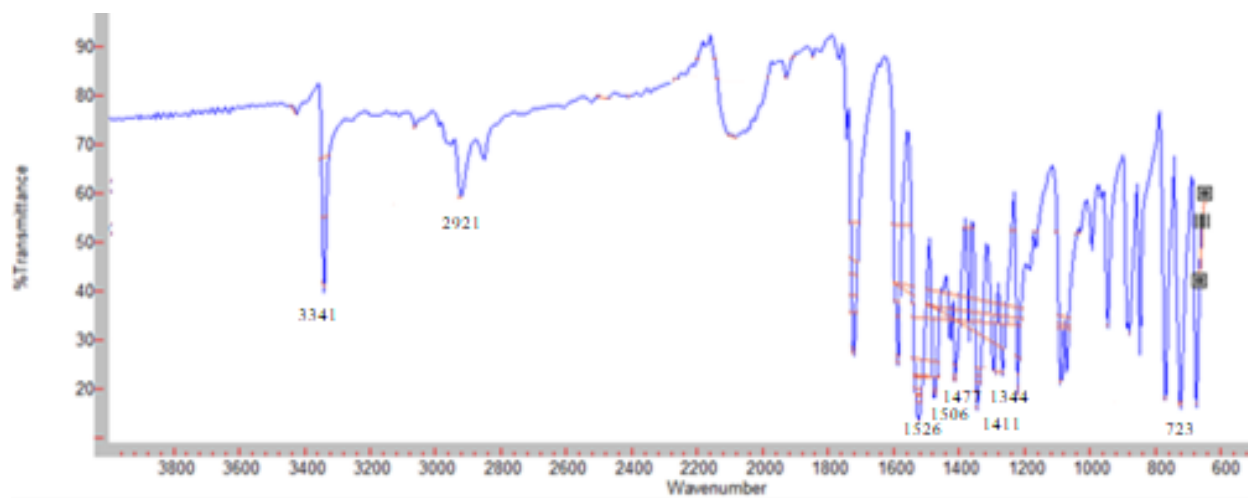


Figure 290, IR of SB126.

HRMS (+ESI-TOF) m/z: [M⁺] calcd for C₁₃H₁₄ClN₃S 279.05970; found 279.05968.

SB127

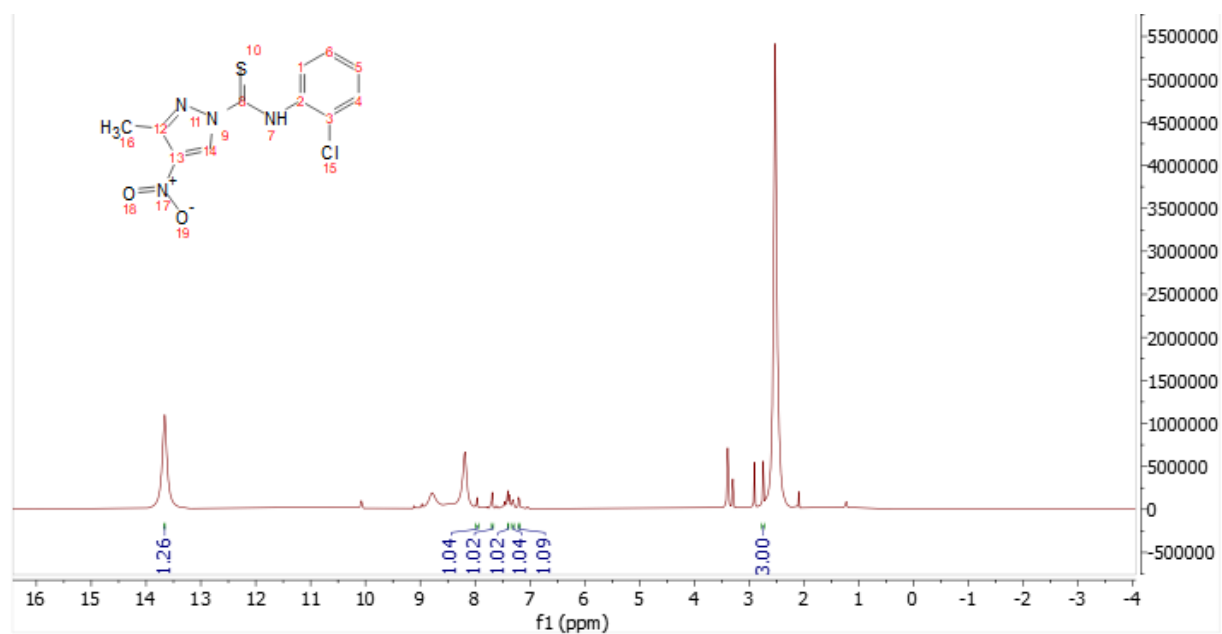


Figure 291, ^1H NMR of SB127.

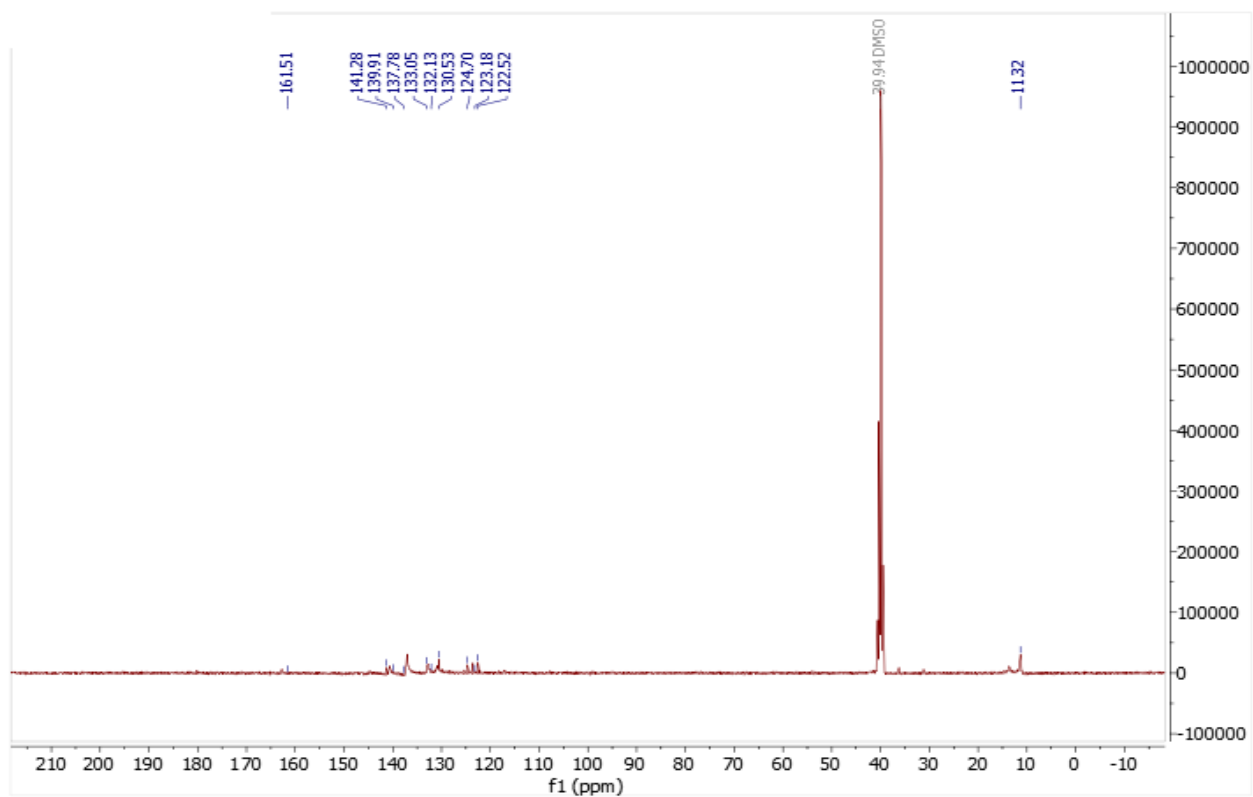


Figure 292, ^{13}C NMR of SB127.

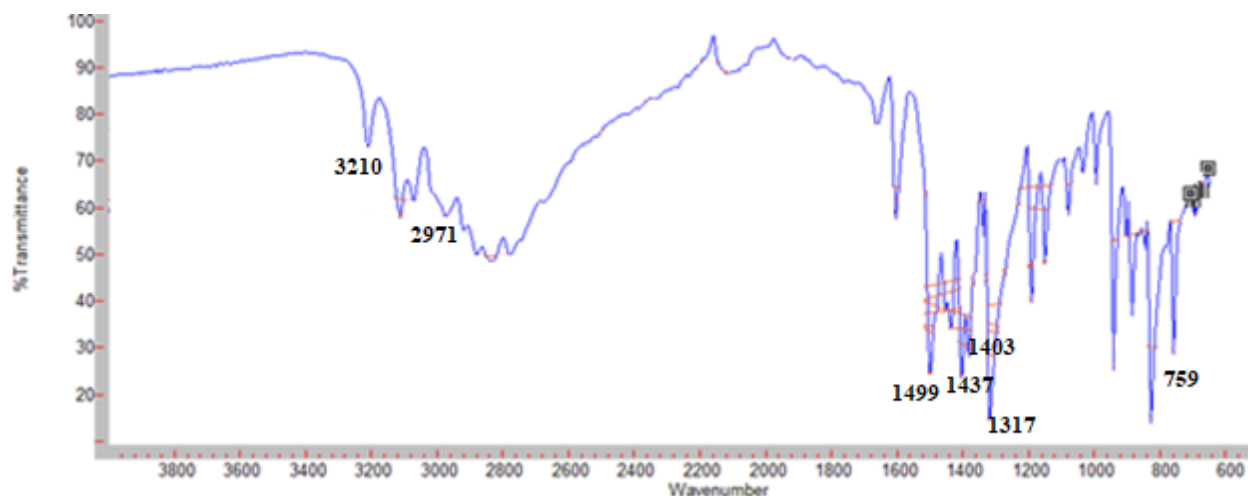


Figure 293, IR of SB127.

UPLC-MS [M+1] calcd for C₁₁H₉ClN₄O₂S 297.73; found 296.96.

SB128

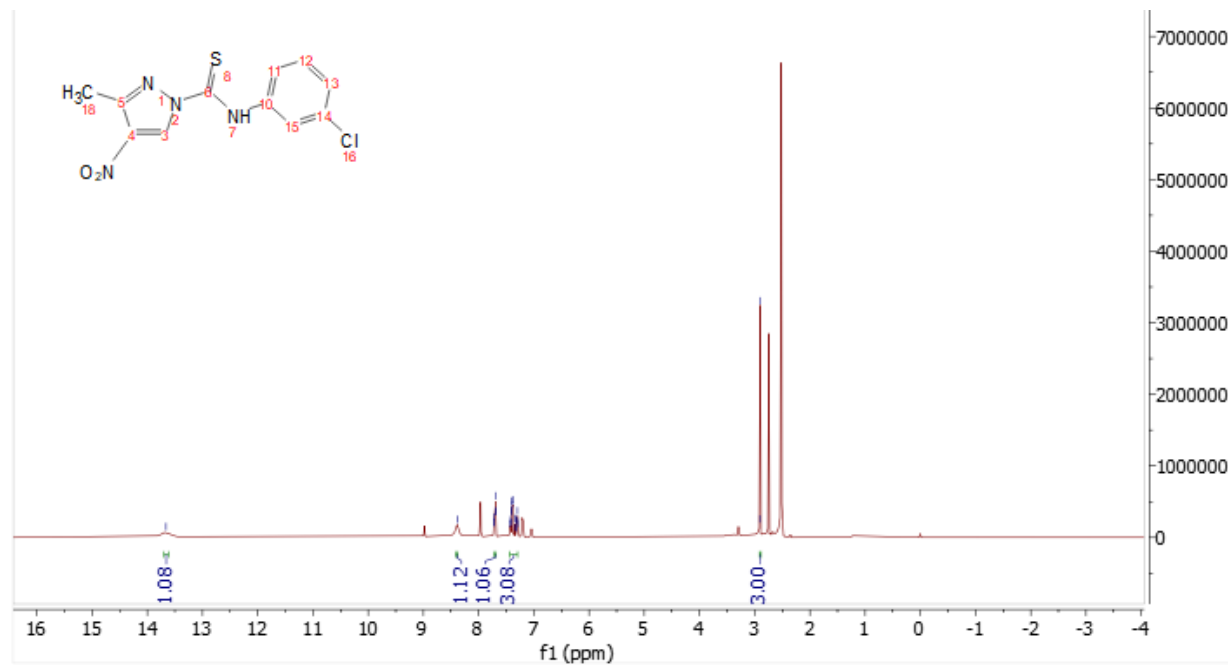


Figure 294, ¹H NMR of SB128.

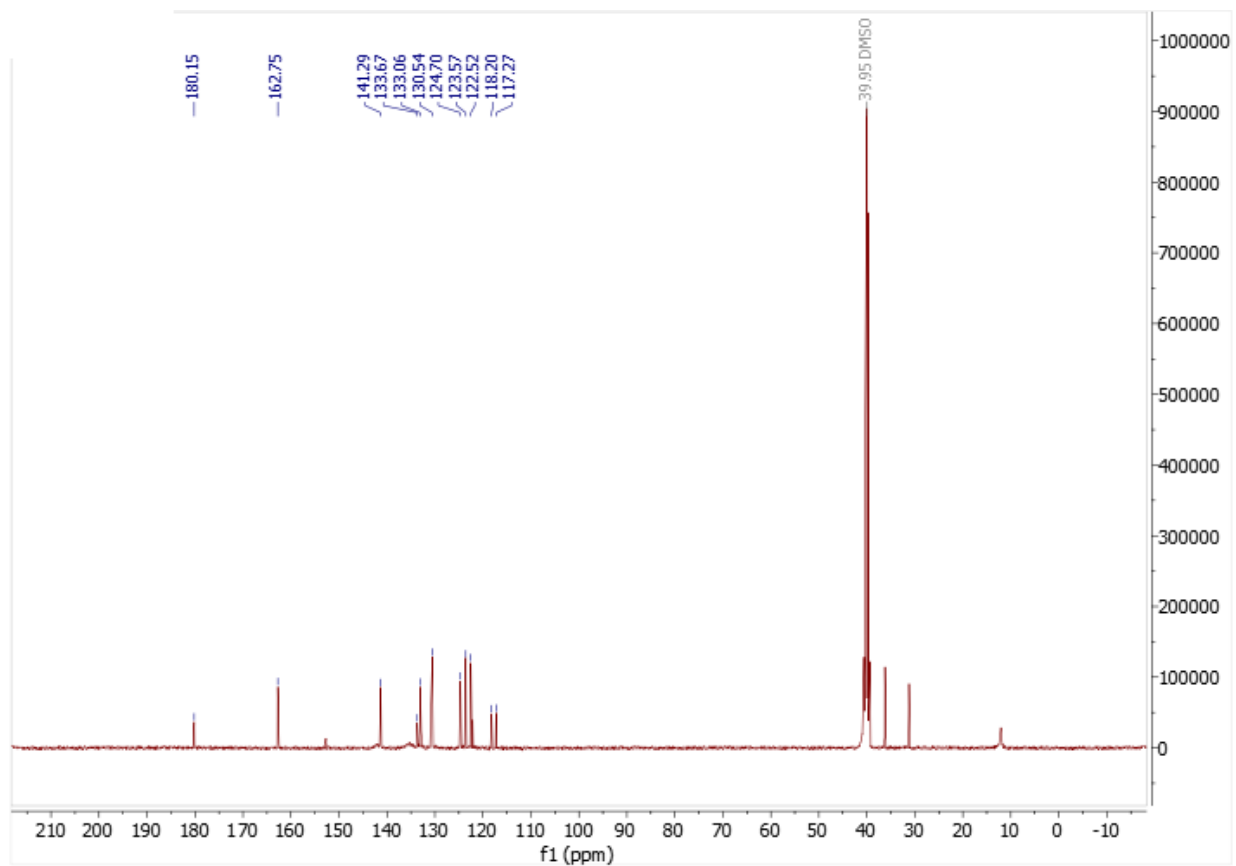


Figure 295, ¹³C NMR of SB128.

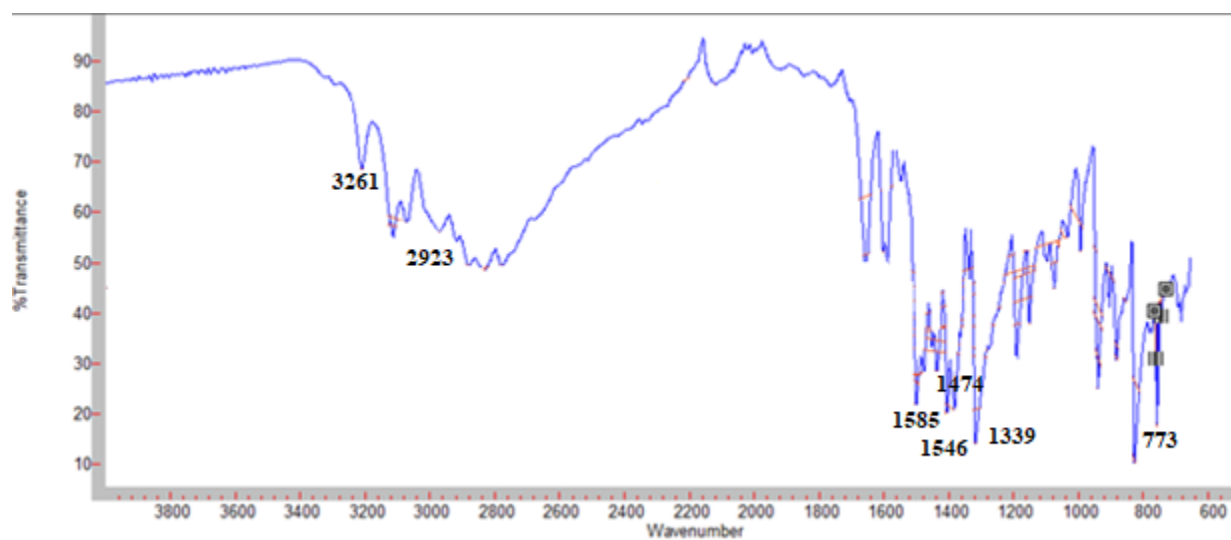


Figure 296, IR of SB128.

UPLC-MS [M+1] calcd for C₁₁H₉ClN₄O₂S 297.73; found 297.04.

SB129

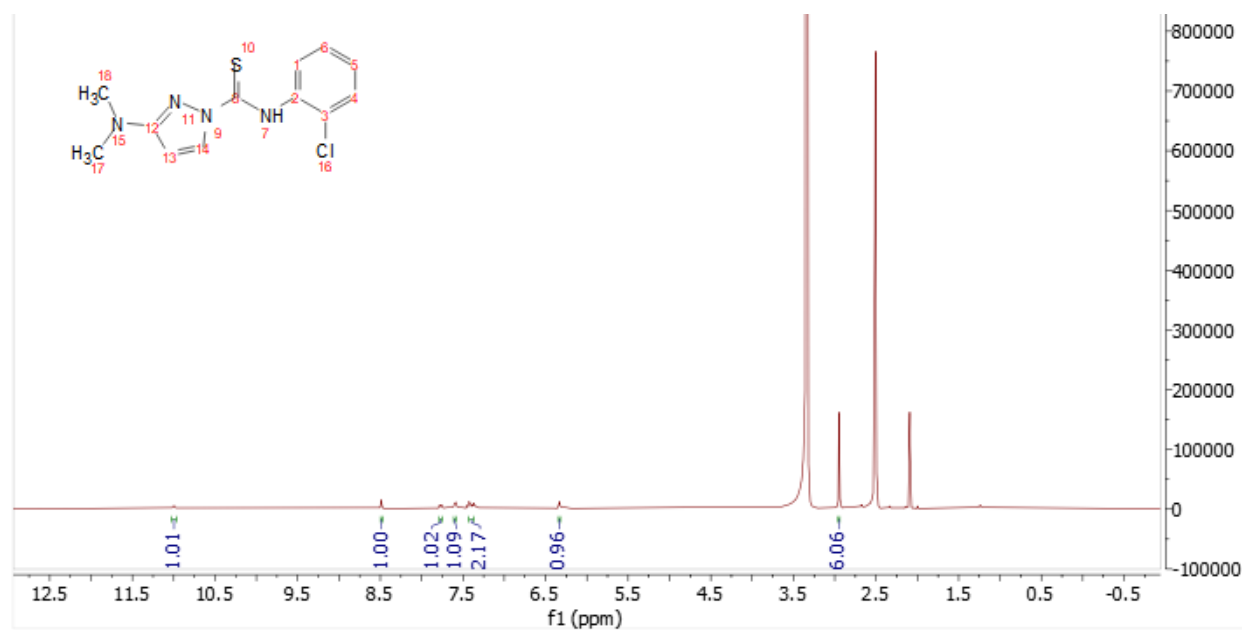


Figure 297, ¹H NMR of SB129.

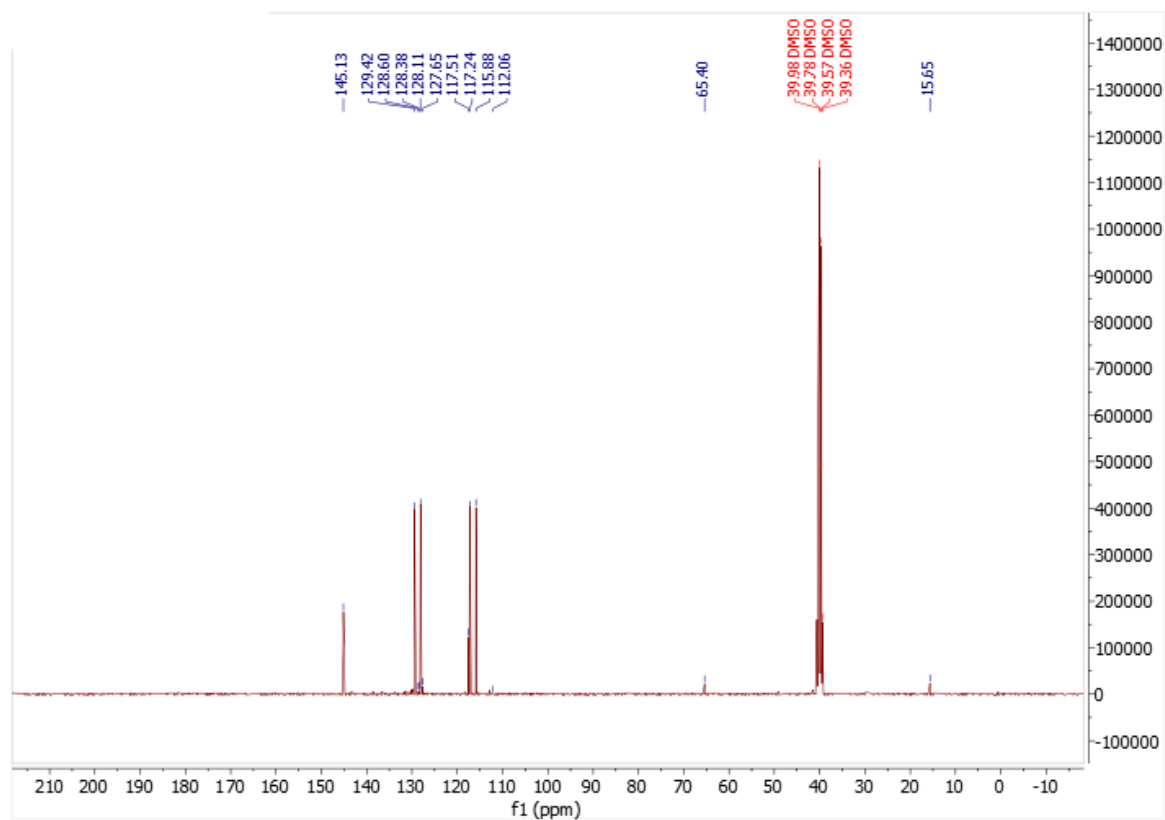


Figure 298, ¹³C NMR of SB129.

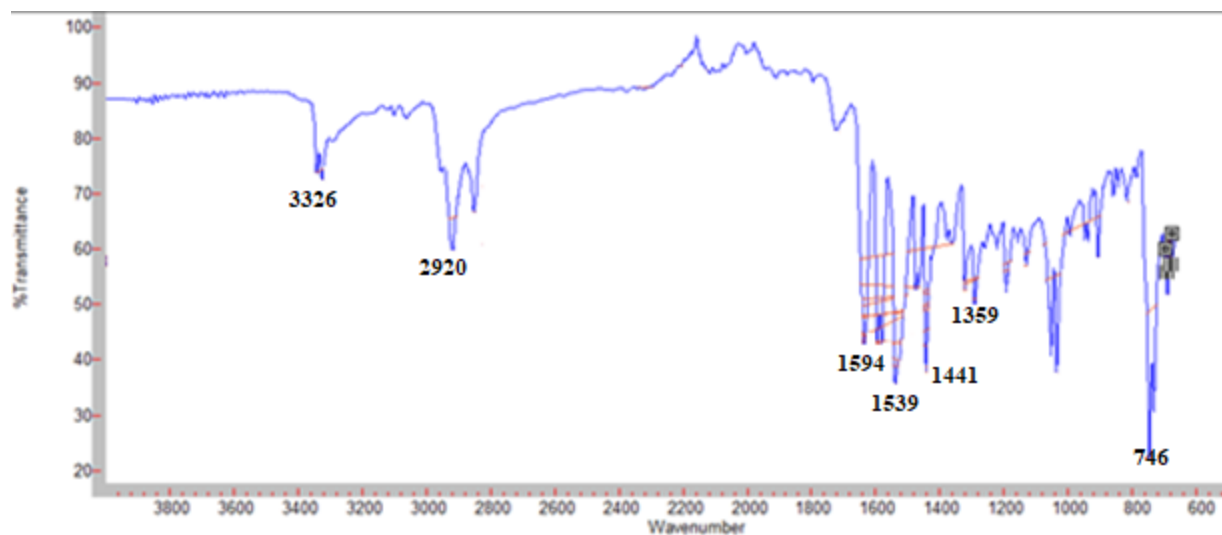


Figure 299, IR of SB129.

HRMS (+ESI-TOF) m/z: [M⁺] calcd for C₁₂H₁₃ClN₄S 280.05494; found 280.05490.

SB130

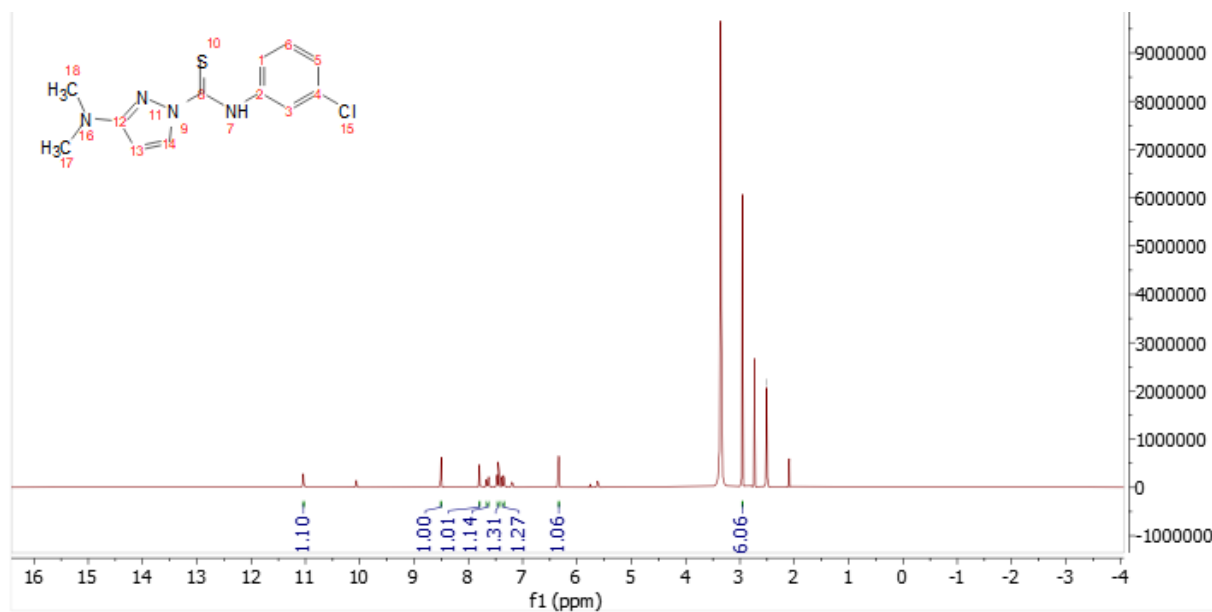


Figure 300, ¹H NMR of SB130.

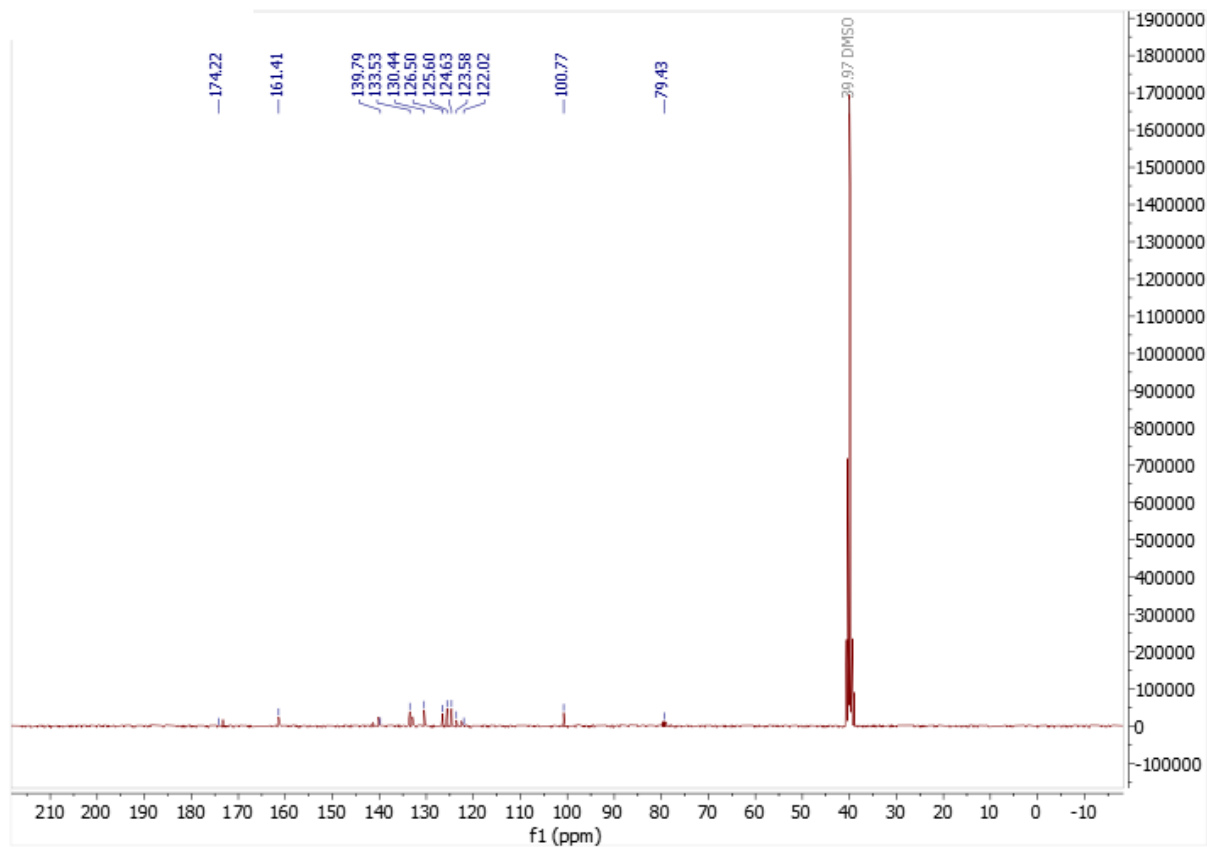


Figure 301, ^{13}C NMR of SB130.

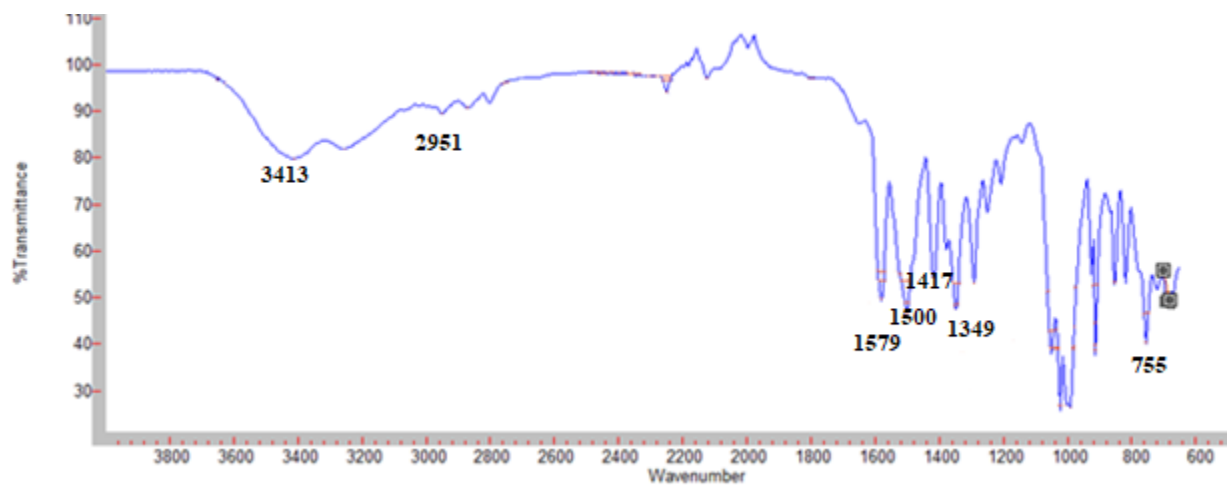


Figure 302, IR of SB130.

HRMS (+ESI-TOF) m/z : $[M]^+$ calcd for $\text{C}_{12}\text{H}_{13}\text{ClN}_4\text{S}$ 280.05494; found 280.05496.

SB131

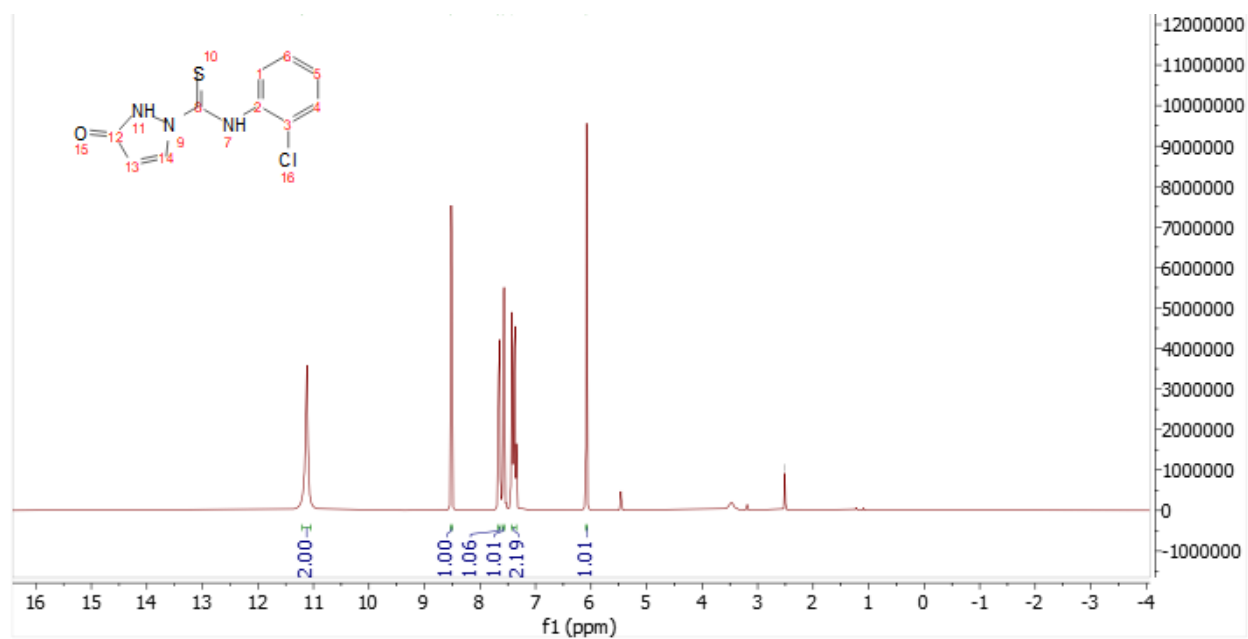


Figure 303, ¹H NMR of SB131.

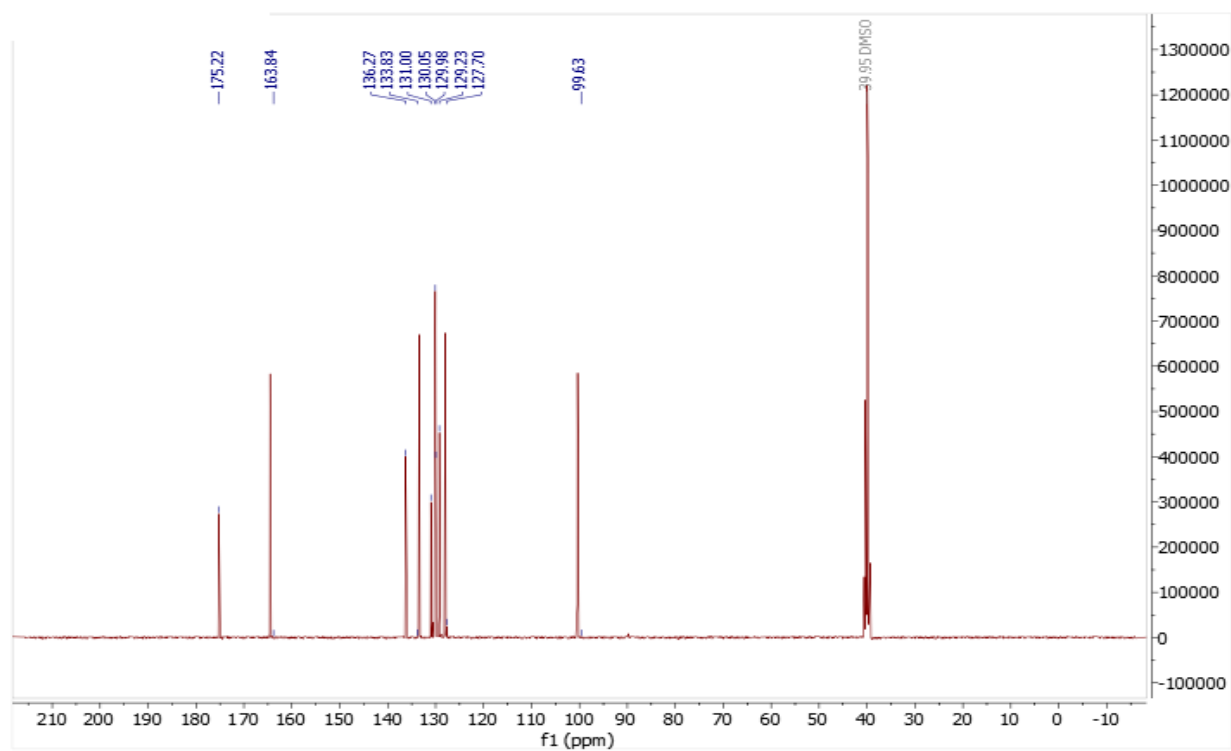


Figure 304, ¹³C NMR of SB131.

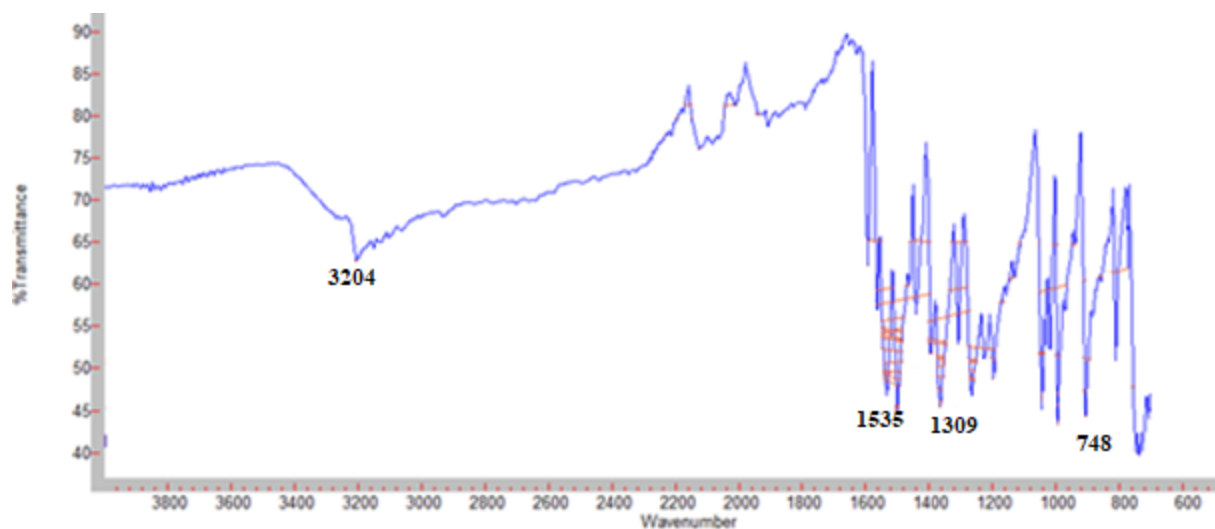


Figure 305, IR of SB131.

SB132

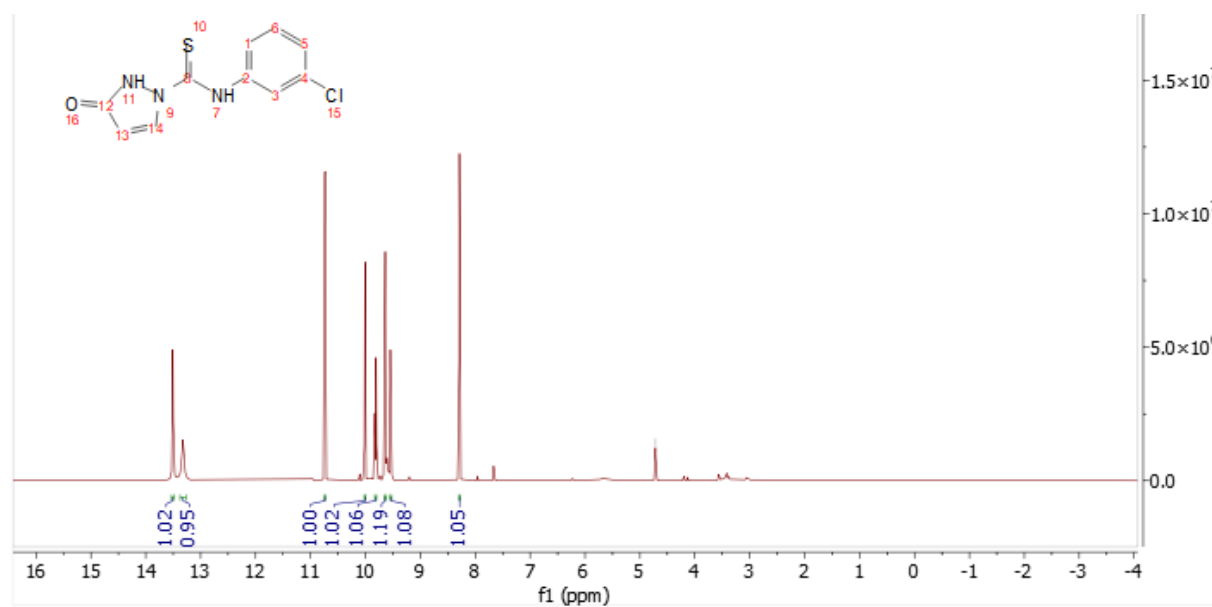


Figure 306, ¹H NMR of SB132.

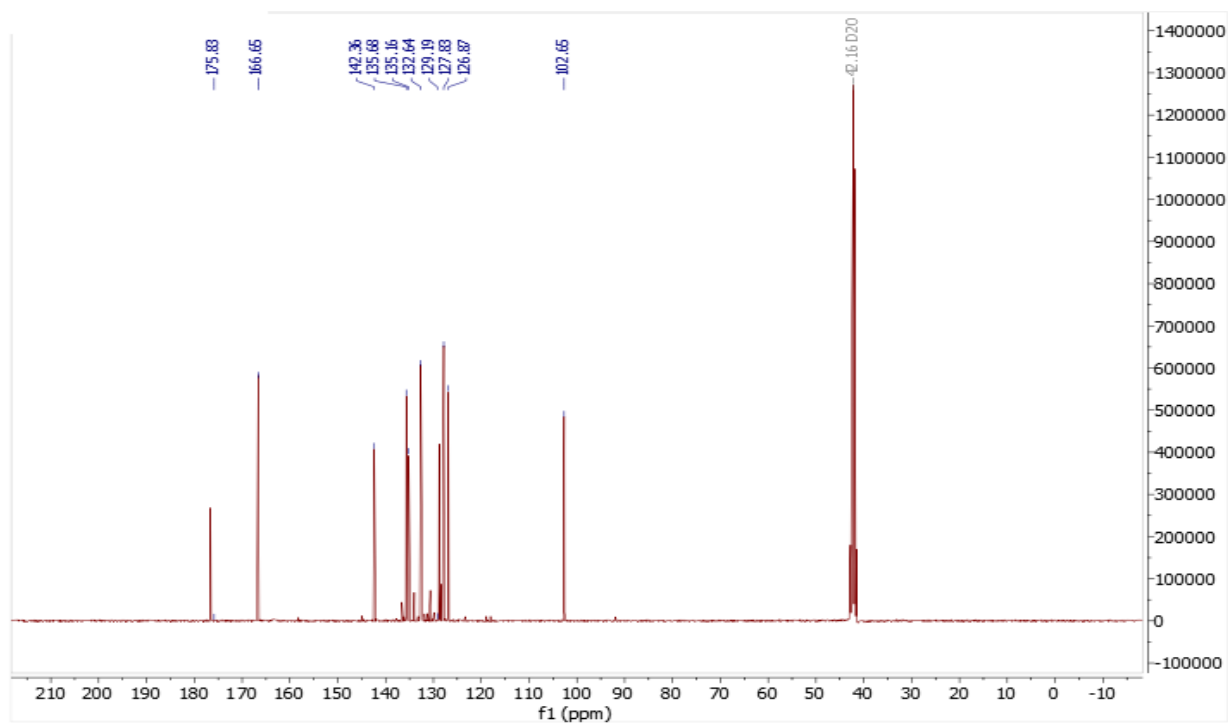


Figure 307, ¹³C NMR of SB132.

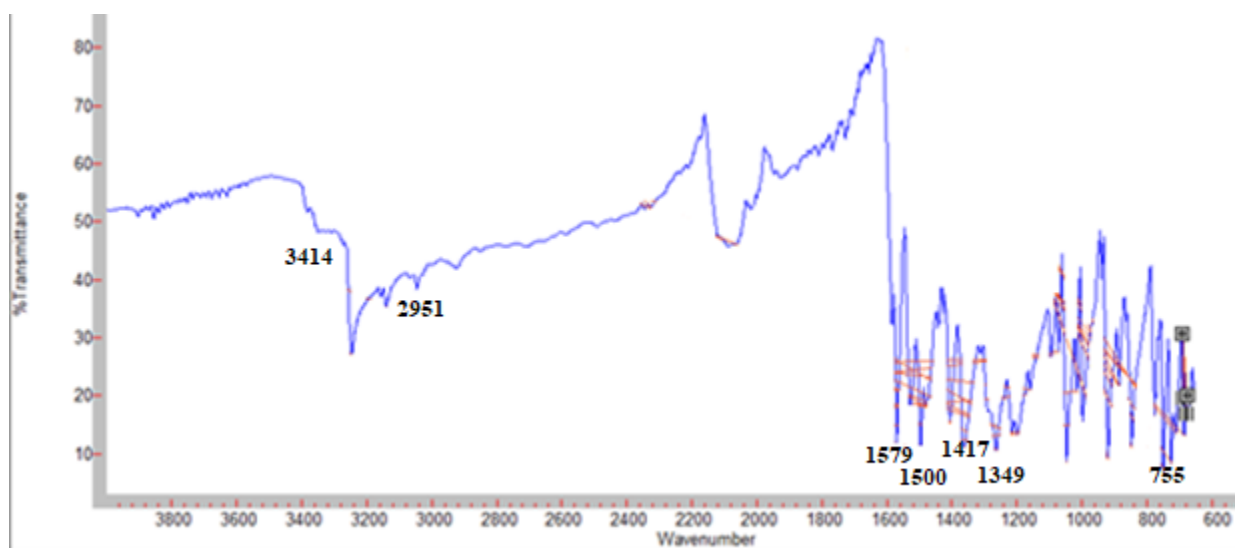


Figure 308, IR of SB132.

SB133

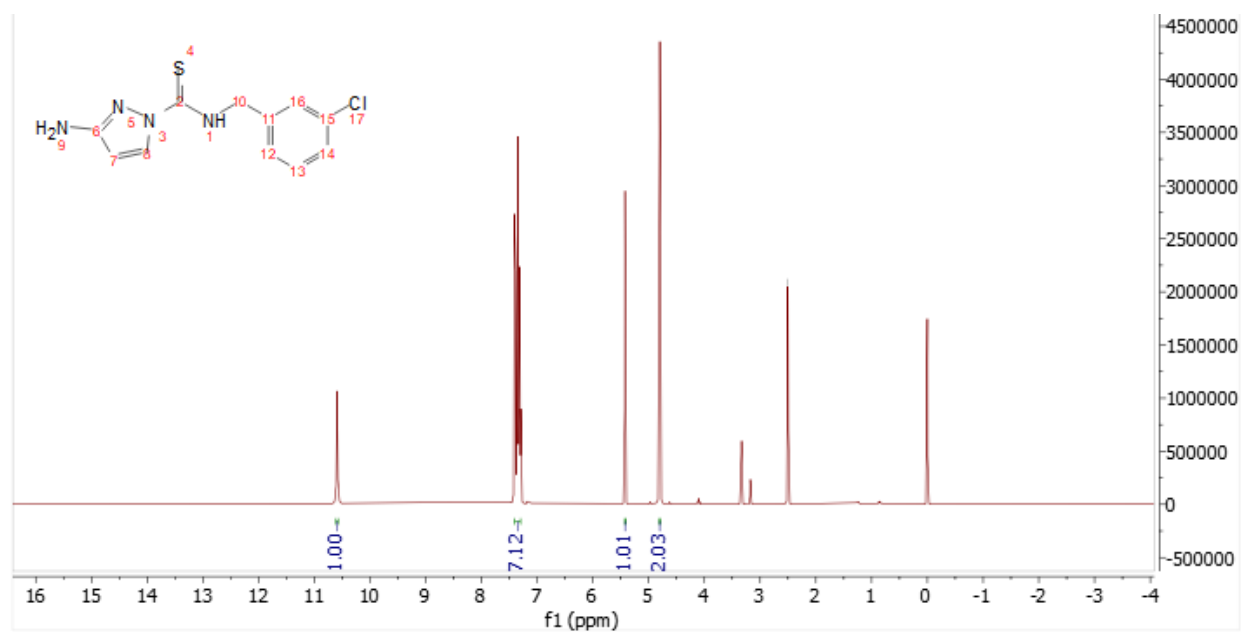


Figure 309, ¹H NMR of SB133.

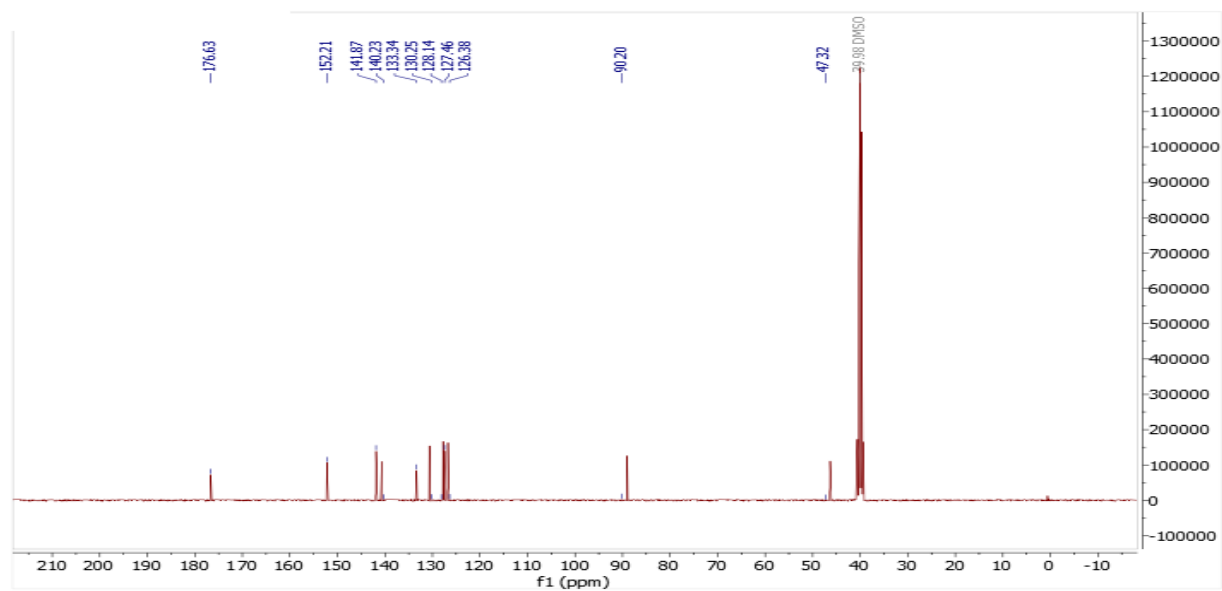


Figure 310, ¹³C NMR of SB133.

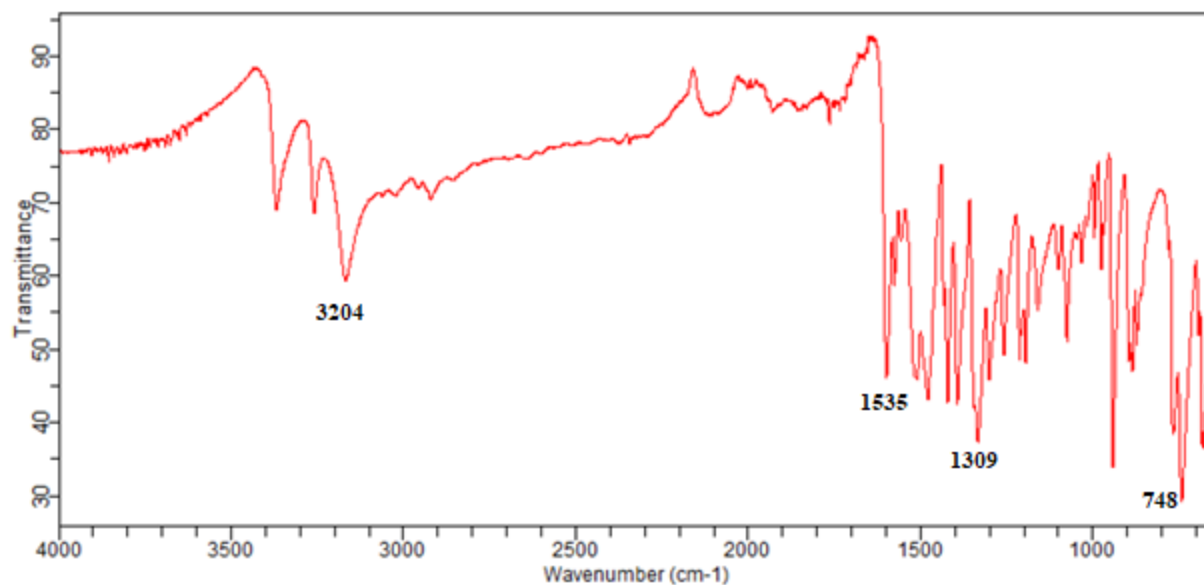


Figure 311, IR of SB133.

HRMS (+ESI-TOF) m/z: [M⁺] calcd for C₁₁H₁₁ClN₄S 266.03929; found 266.03927.

SB134

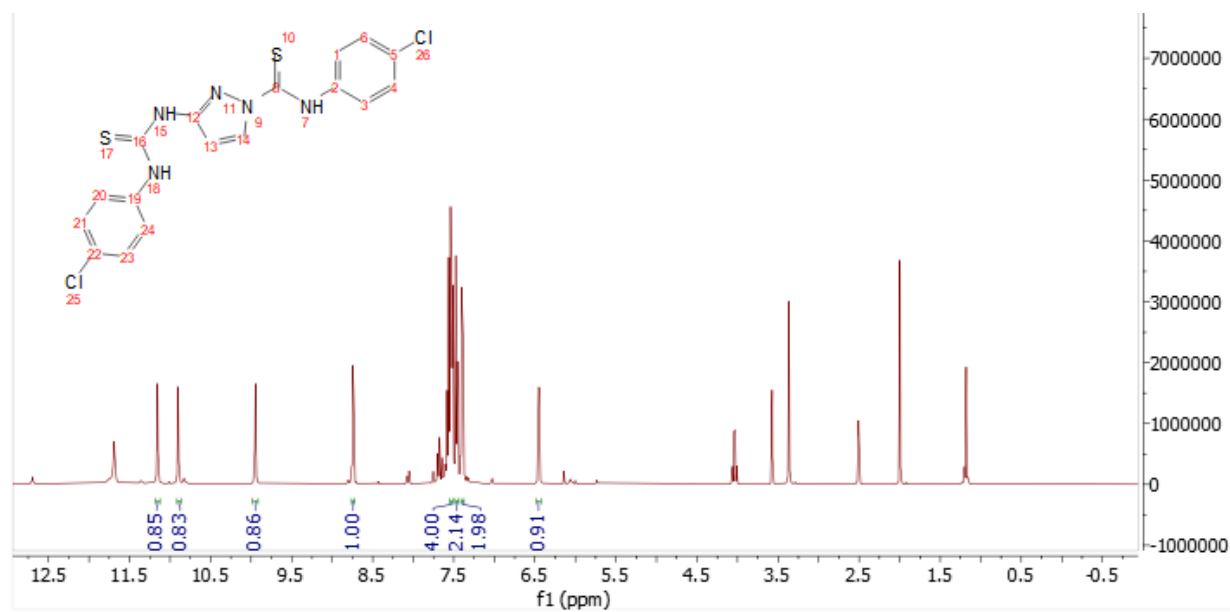


Figure 312, ¹H NMR of SB134.

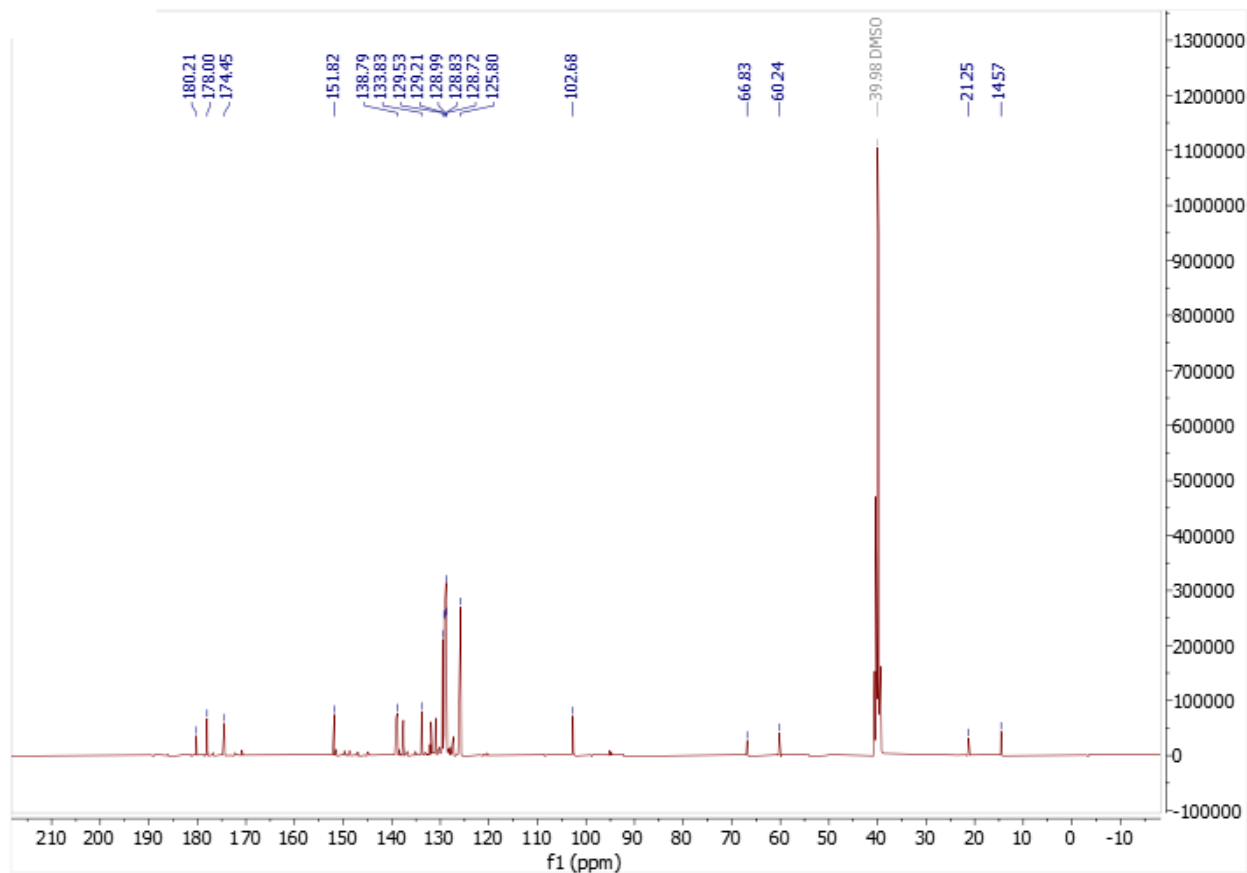


Figure 313, ¹³C NMR of SB134.

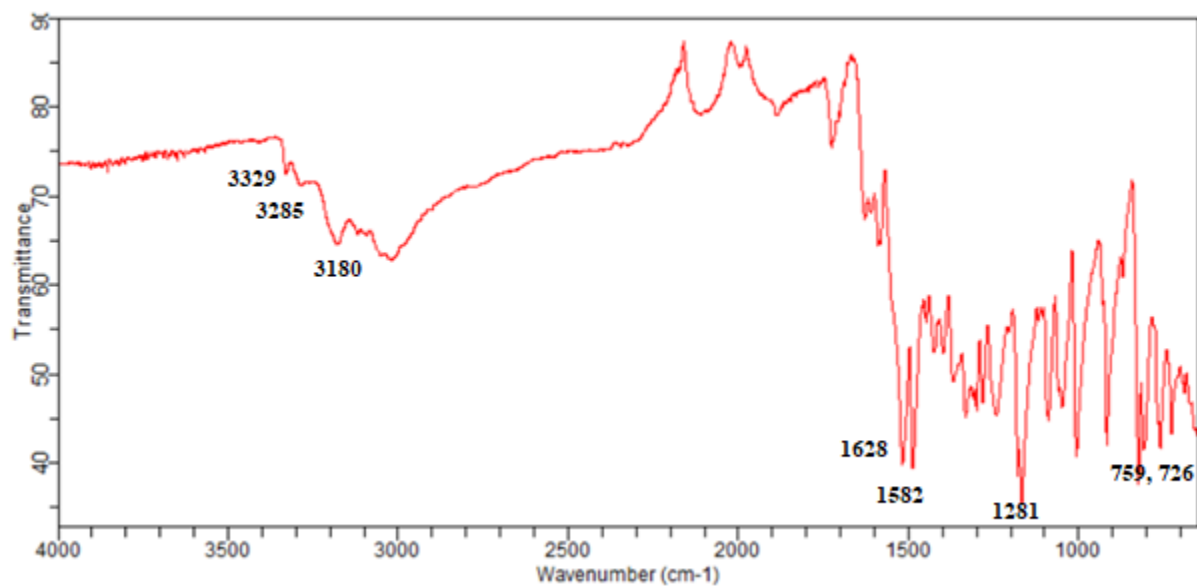


Figure 314, IR of SB134.

SB135

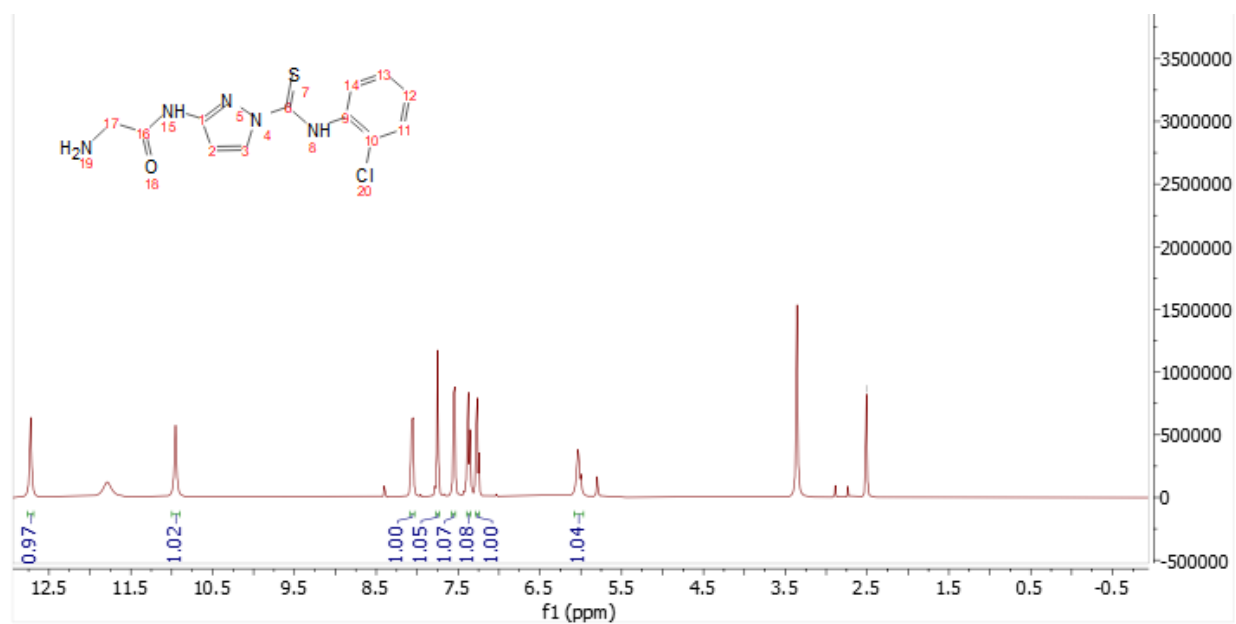


Figure 315, ¹H NMR of SB135.

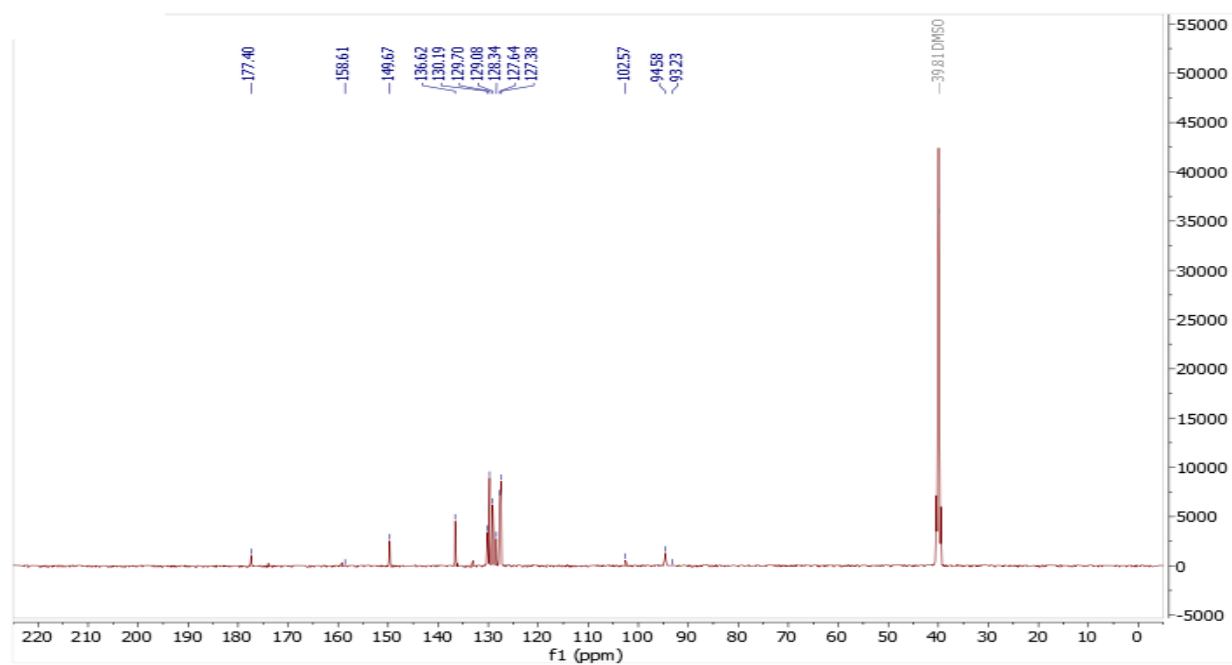


Figure 316, ¹³C NMR of SB135.

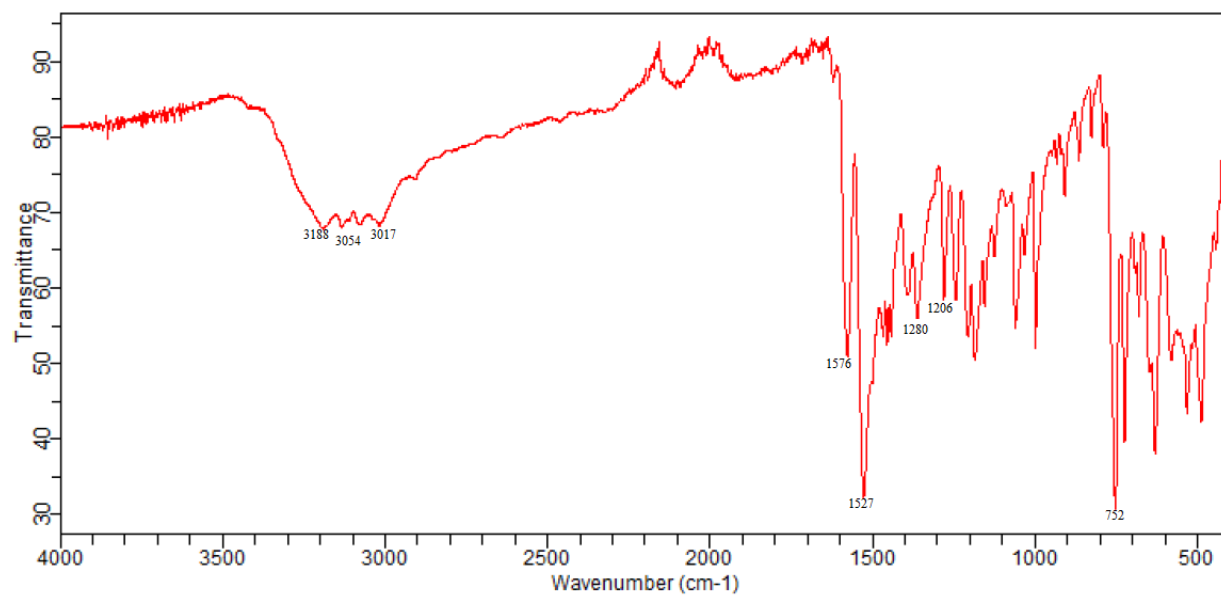


Figure 317, IR of SB135.

UPLC-MS [M+1] calcd for C₁₂H₁₂ClN₅OS 310.77; found 310.98.

SB136

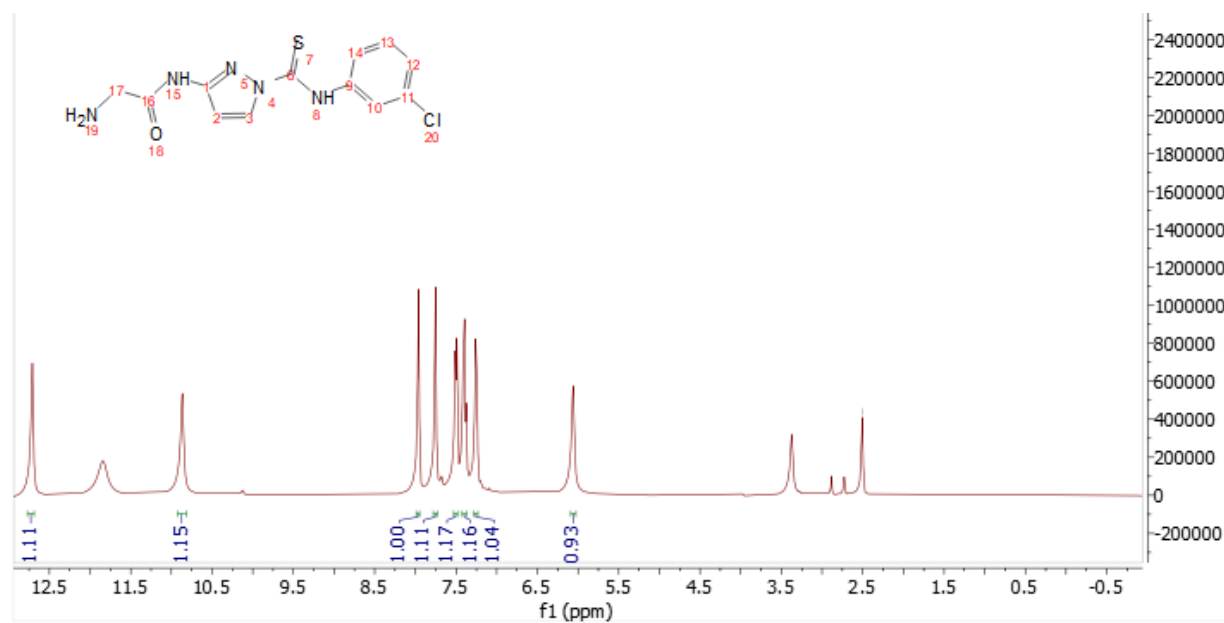


Figure 318, ¹H NMR of SB136.

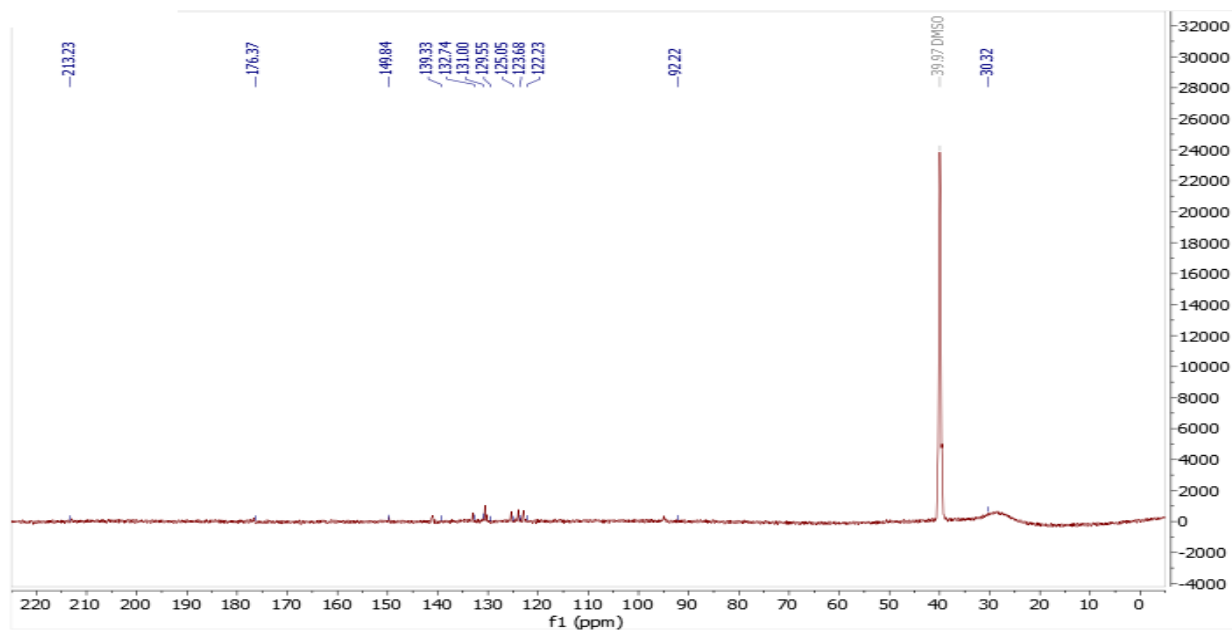


Figure 319, ^{13}C NMR of SB136.

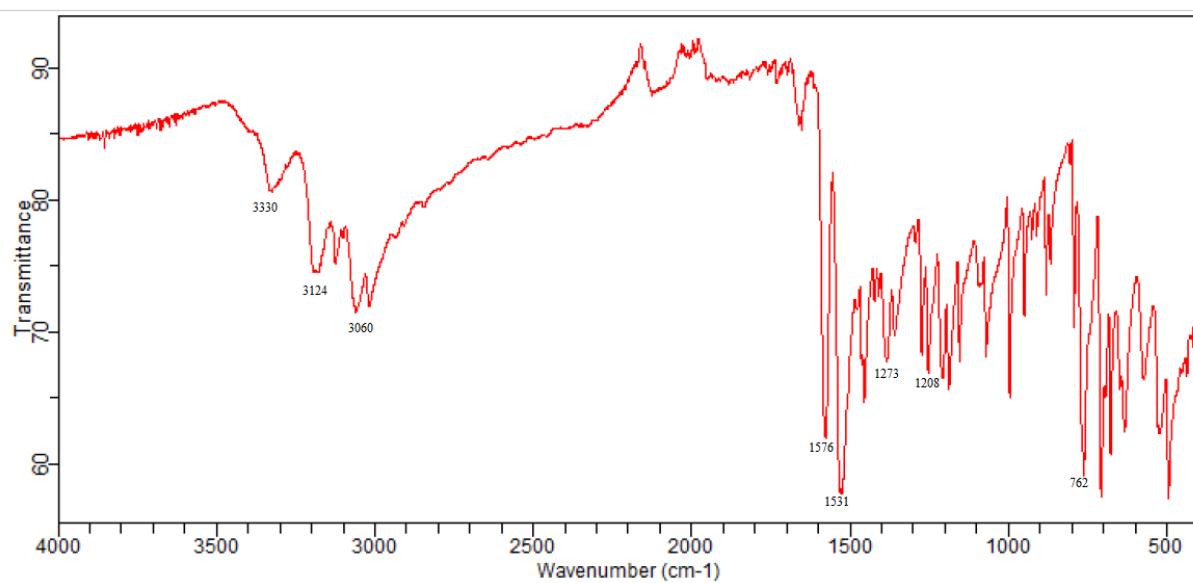


Figure 320, IR of SB136.

UPLC-MS $[\text{M}+1]$ calcd for $\text{C}_{12}\text{H}_{12}\text{ClN}_5\text{OS}$ 310.77; found 310.98.

SB137

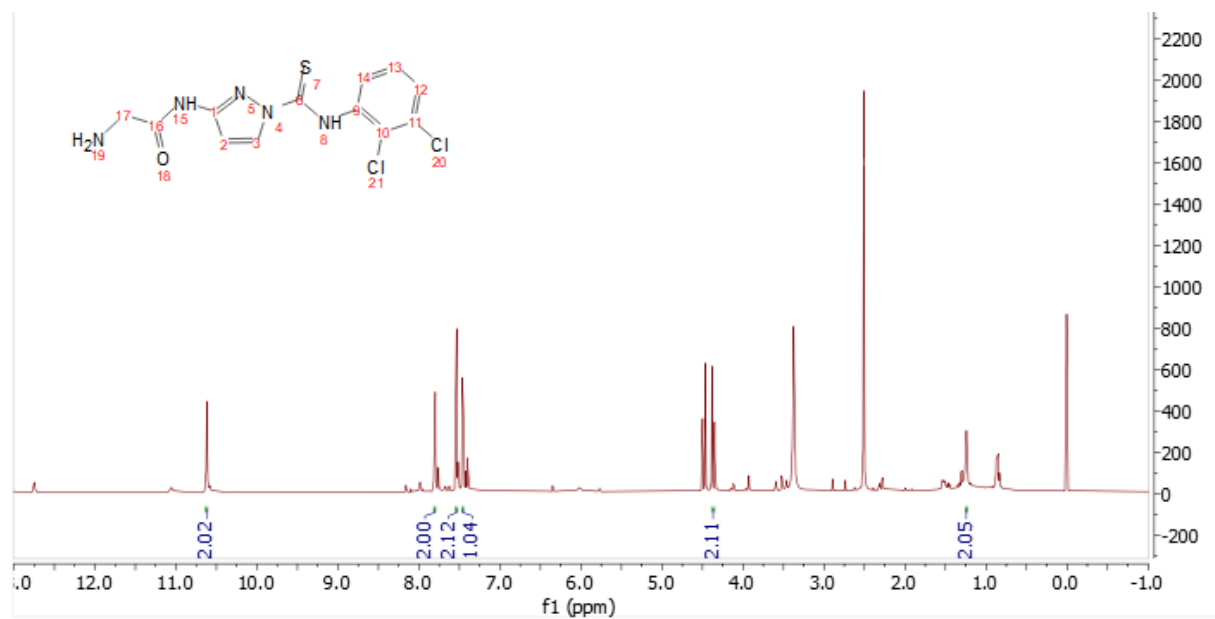


Figure 321, ^1H NMR of SB137.

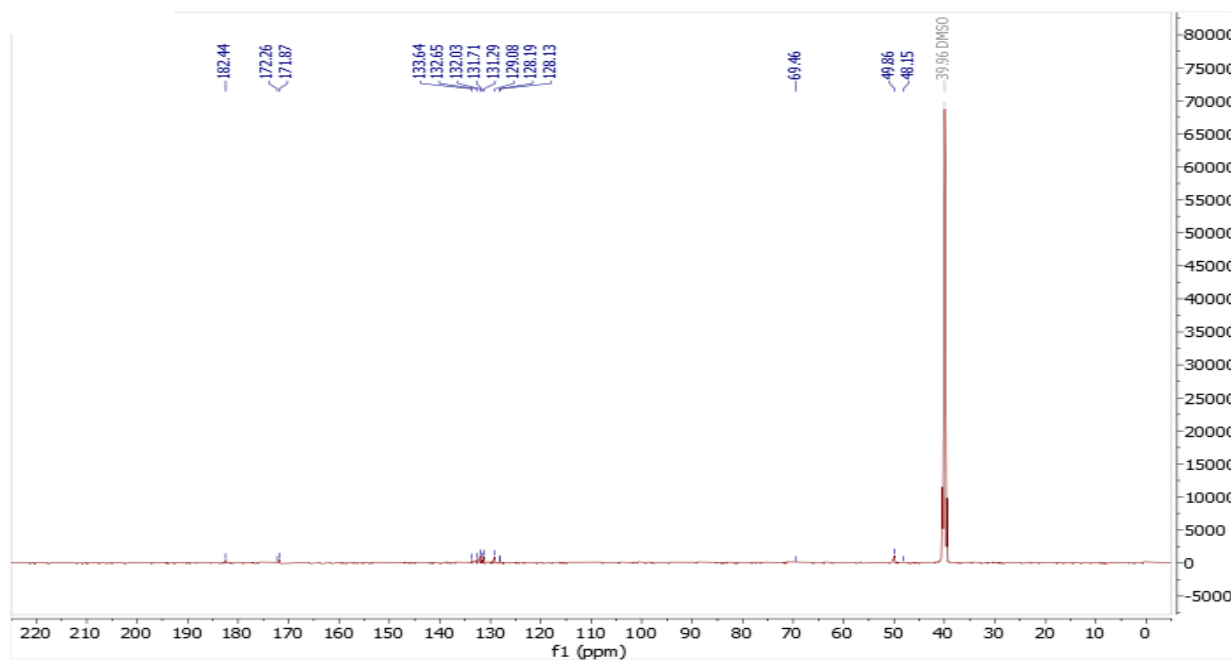


Figure 322, ^{13}C NMR of SB137.

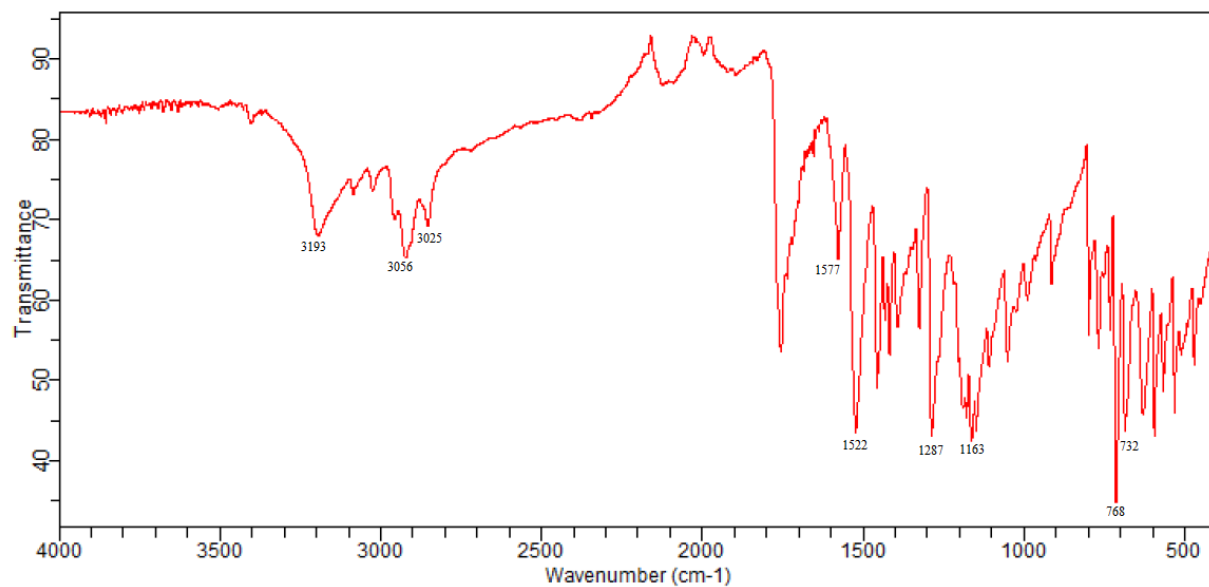


Figure 323, IR of SB137.

UPLC-MS [M+1] calcd for C₁₂H₁₂Cl₂N₅OS 345.22; found 345.26.

SB138

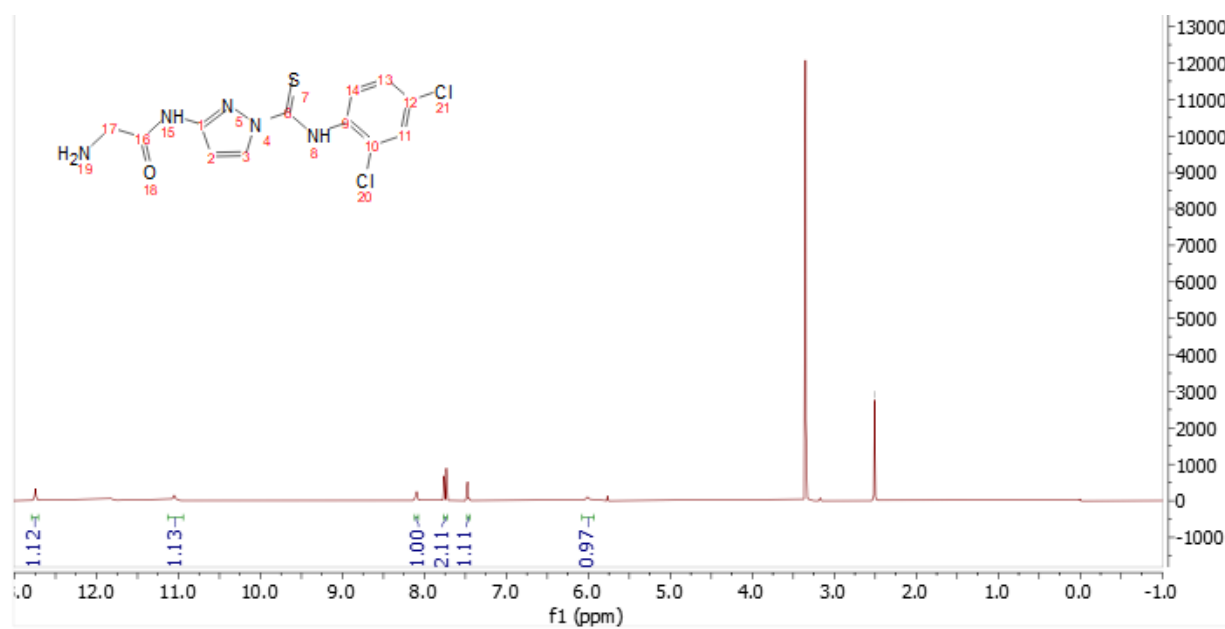


Figure 324, ¹H NMR of SB138.

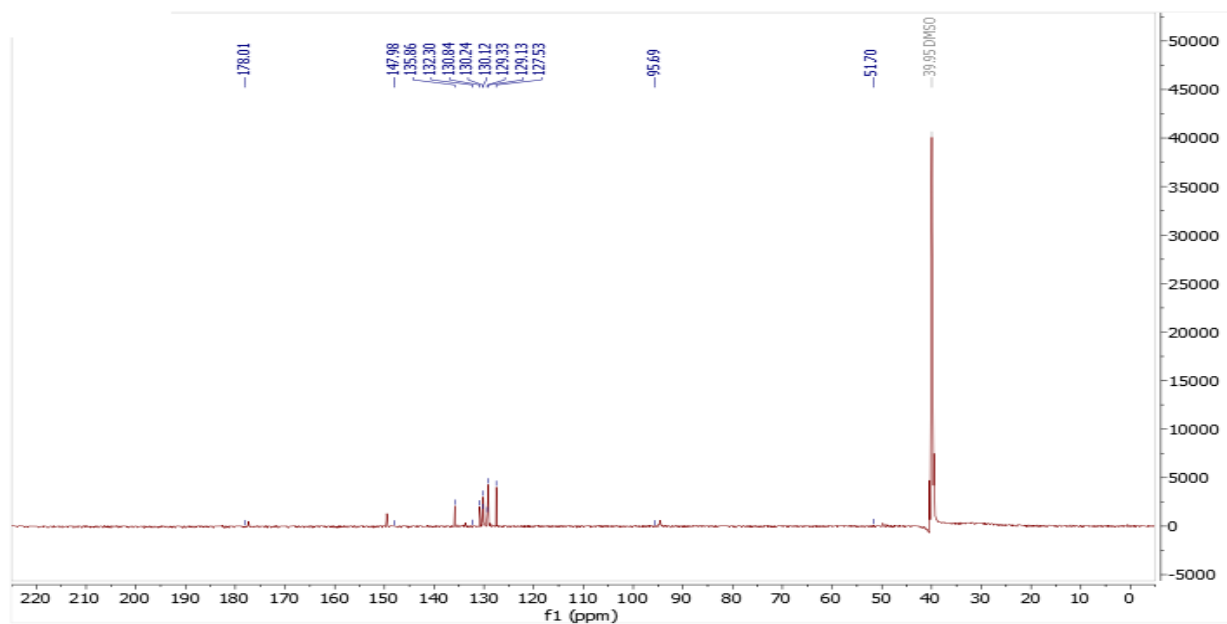


Figure 325, ¹³C NMR of SB138.

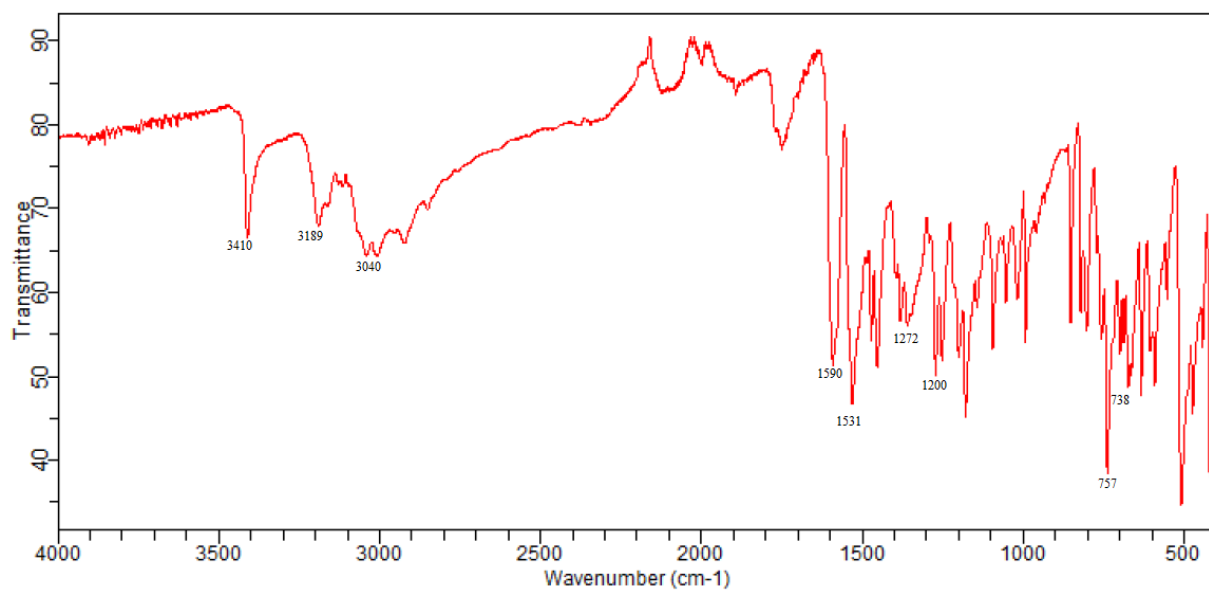


Figure 326, IR of SB138.

UPLC-MS [M+1] calcd for C₁₂H₁₂Cl₂N₅OS 345.22; found 345.01.

SB139

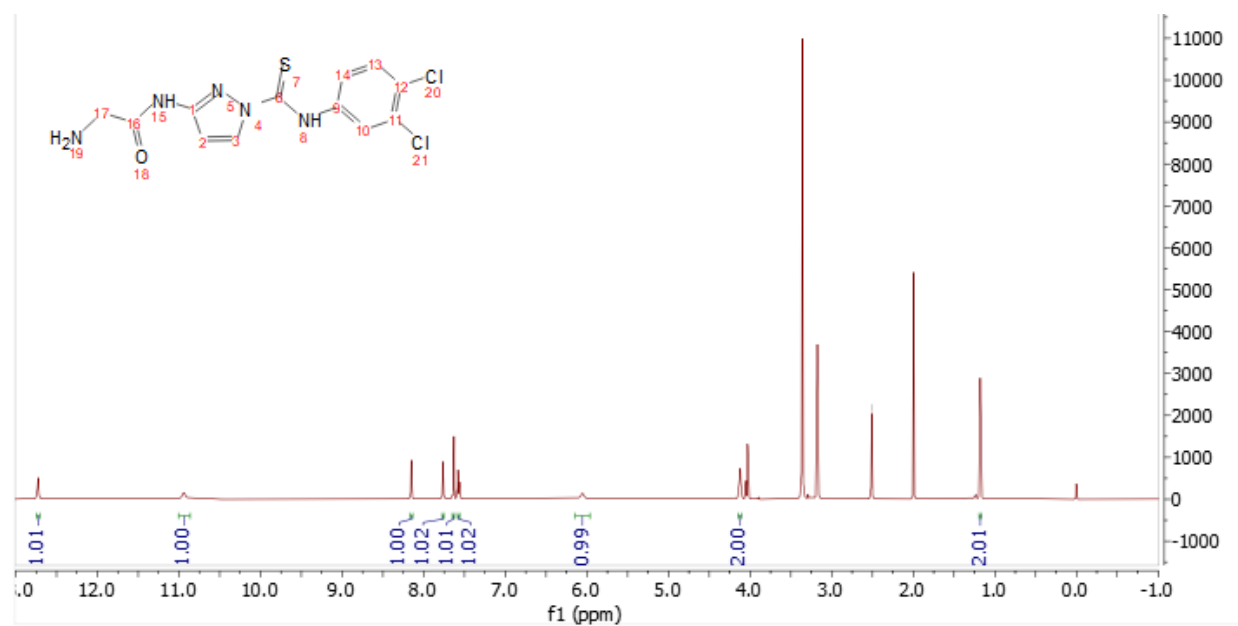


Figure 327, ¹H NMR of SB139.

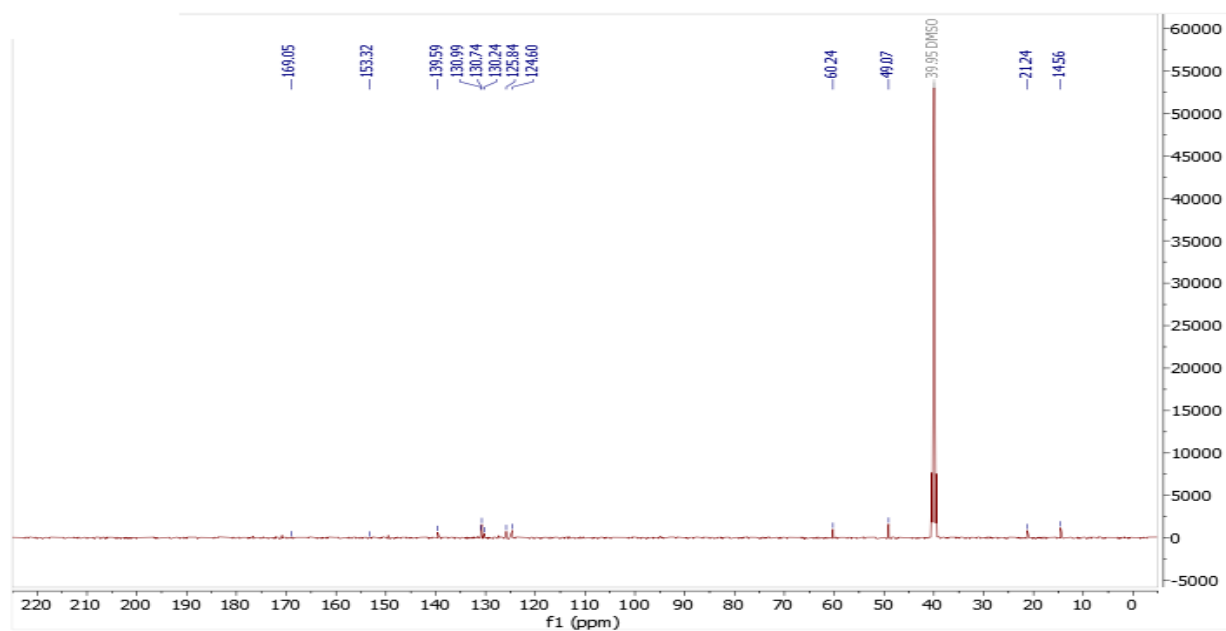


Figure 328, ¹³C NMR of SB139.

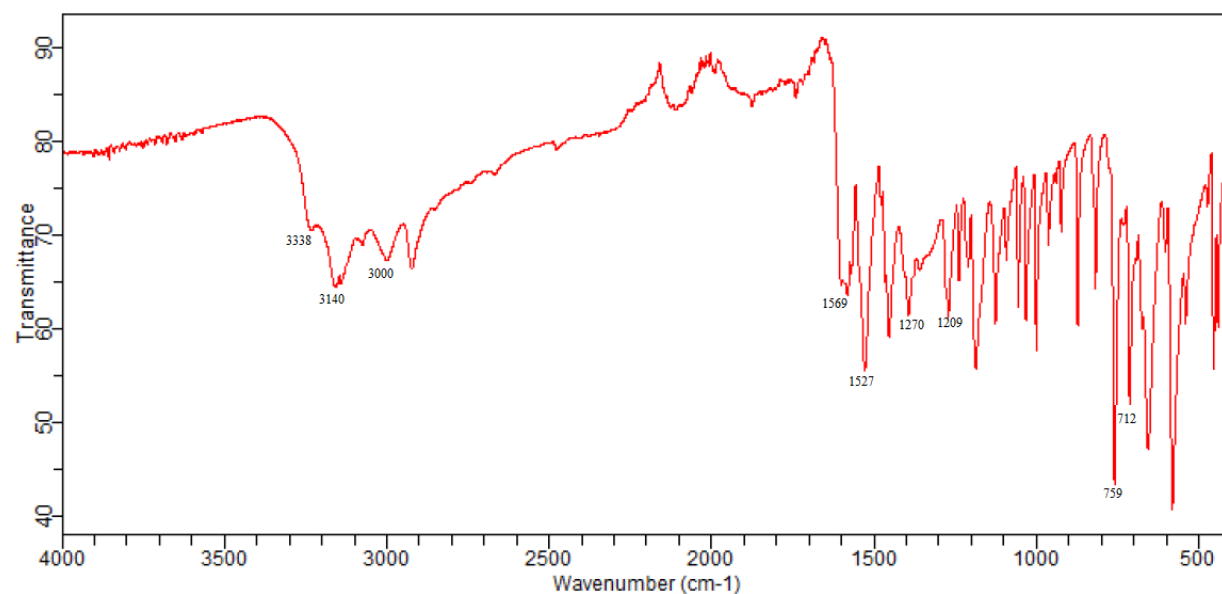


Figure 329, IR of SB139.

UPLC-MS [M+1] calcd for C₁₂H₁₂Cl₂N₅OS 345.22; found 345.33.

SB140

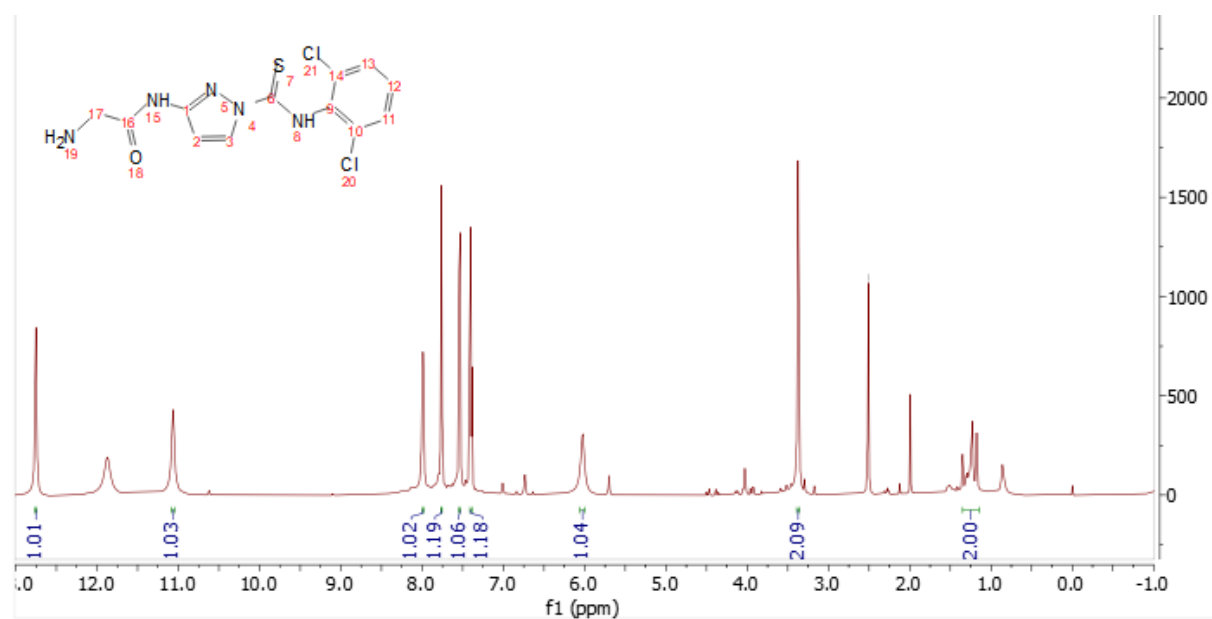


Figure 330, ¹H NMR of SB140.

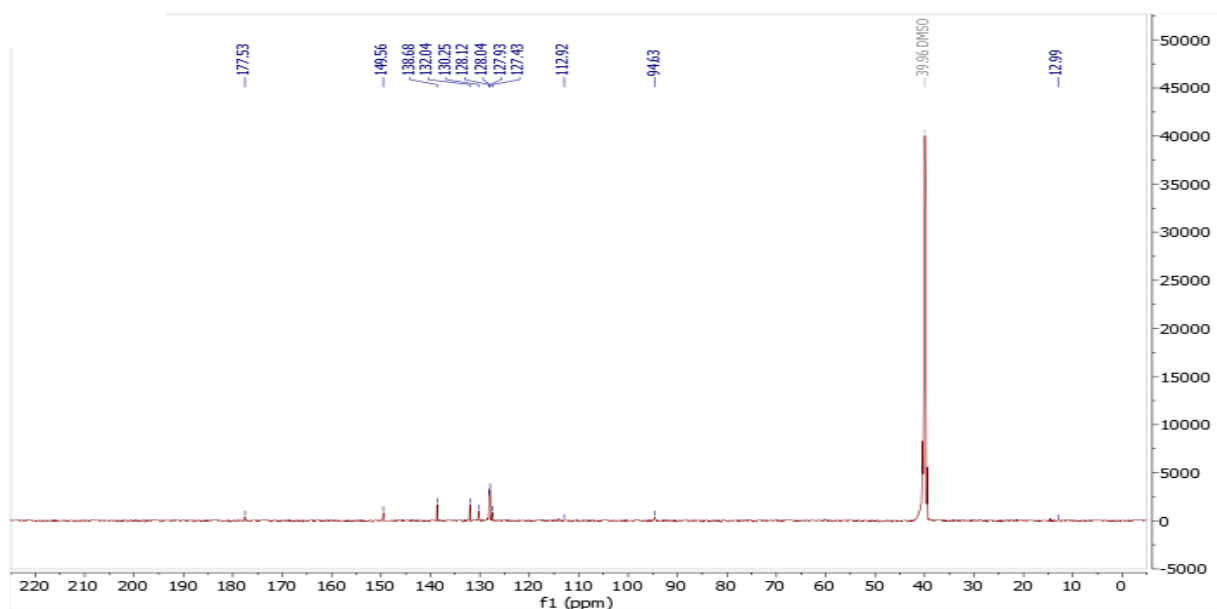


Figure 331, ¹³C NMR of SB140.

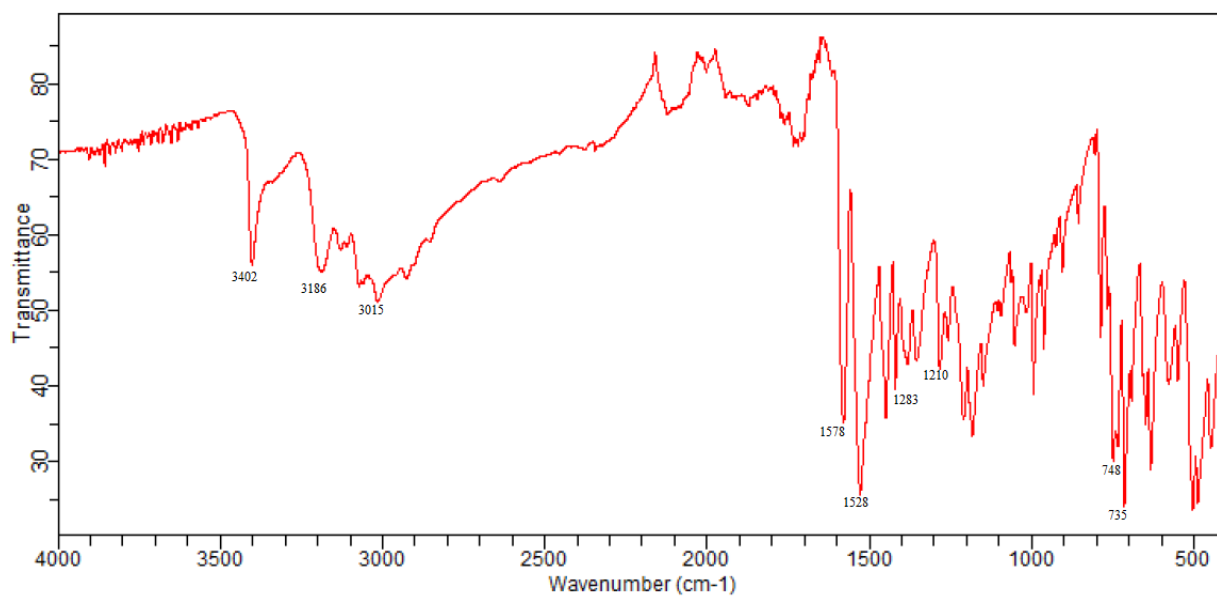


Figure 332, IR of SB140.

UPLC-MS [M+1] calcd for C₁₂H₁₂Cl₂N₅OS 345.22; found 345.01.