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The Evaluation of Isatin 3-Hydrazone Derivatives toward Biofilm Regulation

Sarah E. Nuckolls
Longwood University

Andrew A. Yeagley

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Sarah E. Nuckolls, Andrew A. Yeagley

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Department of Chemistry and Physics
Longwood University, 201 High Street, Farmville, VA 23909

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Abstract

In the current methodology of fighting infections, antibiotics are used to eliminate the invading bacteria. A result of this process is constantly evolving antibiotic-resistant bacteria. If we hope to be more effective at preventing disease, we must shift the way we view microbial treatment. One novel method of preventing bacterial proliferation is the modulation of biofilm. Many bacteria can produce a biofilm; an extracellular substance that provides protection and antibiotic resistance to the bacteria. Attacking this biofilm and allowing the body's natural defenses to clear out the bacteria is one promising way to treat bacterial infections while avoiding adaptive bacterial mutation.

Isatin has been shown to regulate biofilm and thus exhibits properties similar to quorum sensing molecules. Additionally, isatin derived from indole which is a known quorum sensing molecule. Previous studies have shown that hydrazone and oxime derivatives of isatin have antimicrobial properties, which make them targets of interest in testing the ability of isatins to regulate biofilms. Within this study, a library of these compounds was created and tested against *E. coli, P. aeruginosa, S. aureus* to determine the OD_{600} correlation, minimum inhibitory concentration and the effect the compounds have on biofilm formation. For *E. coli* it was found that a compound was found to inhibit while others promoted. For *P. aeruginosa*, it was found that a compound was found to inhibit biofilm production, while two others were found to be promoters. Compounds were found to be biofilm inhibitors for *S. aureus*, while a compound was found to be a promoter.

Introduction

Since the discovery of penicillin in 1928, [1] the default clinical methodology when treating an infection has been to identify the bacterial pathogen responsible and subsequently cause bacterial death using an antibiotic. This routine stress on reoccurring bacterial strains has had the unexpected consequence of causing the rapid evolution of clinically significant bacteria strains. Resistance to any novel antibiotic is observed within a few years of the antibiotic's introduction into the commercial market[2].

When a colony of bacteria is dosed with an antibiotic it is susceptible to, bacterial death results. There also exists the possibility a single bacterium survives then this bacterium proliferates and spreads the mutation. Since bacteria are able to transfer genetic material easily and thus mutate quickly. This causes bacterial-resistance to an antibiotic. When this new strain of bacteria infects a host, the antibiotic that worked on its predecessor will no longer be as effective on the new strain due to its new innate resistance.[3] The speed at which bacterial evolution occurs is problematic as new antibiotics cannot be either found or synthesized as quickly as bacteria are becoming resistant to the old ones.

This emerging medical arms-race[4] is forced by the new bacterial threats that live among us today. The most well-known "superbug" is methicillin-resistant *Staphylococcus aureus* (MRSA). It is best recognized for the widespread outbreak of MRSA in 2005 within the United States.[5] The outbreak resulted in 270,000 hospitalizations and more than 18,000 deaths. The creation of this "superbug" was a

result of the laisse-faire attitude toward antibiotics within the agricultural industry. Farmers and ranchers give millions of pounds of antibiotics to livestock in order to make them grow faster and to prevent diseases. This caused a treatable strain, *Staphylococcus aureus CC398*, to develop resistance to methicillin and tetracycline, the two most common antibiotics used to treat *S. aureus* infections, which has made fighting MRSA incredibly difficult.[5]

In light of our current understanding of bacterial resistance, the traditional view of how to treat microbial infection must be set aside in order to become more effective at combating bacterial resistance. The customary approach to considering how an antibiotic should function is the first to be reconsidered. Traditional antibiotics act by hindering bacterial functions such as cell wall synthesis, DNA replication, RNA transcription, and protein synthesis which are essential for proper logarithmic growth *in vitro.*[3] Cell wall synthesis is disrupted by β-lactams and glycopeptides; β-lactams block the cross-linking of peptidoglycan by barring the peptide bond formation reaction catalyzed by transpeptidases while glycopeptides act as steric inhibitors of peptidoglycan maturation. Both of these processes reduce the cell wall's mechanical strength.[6] Quinolones obstruct DNA synthesis by forming a stable interaction complex between drug-bound topoisomerase enzyme and cleaved DNA, trapping the DNA at the cleavage stage and preventing strand rejoining.[6] The inhibition of RNA synthesis is caused by the rifamycin class of antibiotics, by inhibiting DNA-dependent transcription by binding to the subunit of a DNA-bound and actively-transcribing RNA polymerase enzyme.[6] Protein synthesis is repressed by 50S ribosome and 30S ribosome inhibitors;

50S inhibitors work by physically blocking the initiation of protein synthesis translation or translocation of peptide chain, and 30S inhibitors work by blocking the access of aminoacyl-tRNAs. These antibiotics have a history of being highly effective, yet the threat of a post-antibiotic era still looms.

The common feature amongst all these antibiotics is that they target biosynthetic processes that are only present in growing bacteria. Therefore, antibiotics that specialize in killing growing or multiplying cells cannot kill non-multiplying bacteria.[7] Contrary to traditional methods, novel treatments are aimed at disarming bacterial defenses.[2] These new molecules deactivate different types of virulence mechanisms such as toxin function, toxin delivery, bacterial adhesion, and virulence regulation. Direct inhibition of a toxin function is the basis for antitoxins for, among others, diphtheria, botulinus, and tetanus. These inhibitors focus specifically on the proteins secreted by the pathogen in question. An alternative method would be to block the downstream effects of the particular toxin by targeting the host proteins. For example, one study[8] found that the defective cAMP-activated Cl^- channel, which causes cystic fibrosis in affected individuals, can be used to block secretory diarrheas and cholera by inhibiting Cl^- channels within a host. Bacterial toxin delivery[2] can be targeted by interfering with the proper delivery of the toxin to its action site. The customary method is to create a compound which simply binds to the particular toxin in question thus inhibiting the toxin's binding ability. An example of this is the inhibition of adhesion which is a prerequisite to toxin production. Regulating the expression of virulence is attractive because it would prevent the formation of the toxin. The specific methods

used to inhibit the mechanisms for virulence factors will vary between bacterial species, however their disruption disable the bacteria's symptomatic triggers until the bacteria is dispersed by the hosts immune response. This concept of non-bactericidal regulation of infection needs to be considered as it limits the evolutionary stress that cell death causes.

As opposed to directly affecting the expression of virulence factors, influencing the biofilm stage of a bacterium's lifecycle will indirectly influence the development of virulence, as it occurs within a later stage of development. Biofilms are a good target for treatment because the main portion of a bacterium's lifecycle is spent within one and often are the cause of toxin expression, slow cell proliferation, increased genetic transfer, and inhibited host immune effectiveness. Biofilms are aggregates of bacteria within a matrix of extracellular polymeric substances (EPS) which is formed from various biopolymers. Contrary to the common notion that bacteria are only planktonic, the biofilm aggregates are stationary communities and begin as surface-attached bacteria. These communities can contain either one or multiple species of bacteria which act in concert with each other.[9] These initially adhered bacteria begin producing EPS which attracts other bacteria to the same site. While bacteria are collecting within the biofilm, the bacteria already present are proliferating new bacteria. Once the biofilm has reached maturity, the biofilm releases them into the surrounding environment, allowing them to colonize new ones.

Seeing how biofilms account for over 80% of microbial infections in humans and can contribute to heart diseases, lower respiratory diseases, and a variety of others,

their creation would seem a likely target for a new treatment strategy[10]. The biofilm matrix is over 90% of the dry mass of the biofilm and creates a skeleton for the three-dimensional structure of the biofilm, while also allowing for better adhesion to surfaces. The matrix is comprised of biomolecules such as proteins, carbohydrates, and DNA. Within the matrix individual bacterium are immobile, instead of planktonic, and held in close proximity to each other, allowing for better cell-to-cell communication and the transfer of genetic material.[11] This leads to an increased rate of developing resistance. Bacteria existing in the biofilm communities are exceedingly more resistant to standard antibiotic treatment. This is thought to be due to decreased penetration or solubility of the antibiotic into the matrix. Additionally the safety of the biofilm leads many cells to decrease in growth-rate or cease altogether persistence. This leads to a defense against antibiotics to achieve cell growth.

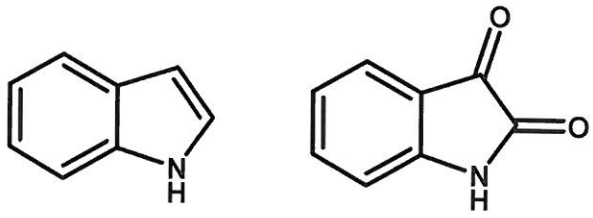
One avenue to influencing the creation of a biofilm is by inhibiting quorum sensing. Quorum sensing is the regulation of gene expression as a reaction to fluxes in cell-population density. The bacteria produce and release chemical signal molecules, known as autoinducers, and change their response to the surrounding based on the concentration of the autoinducer. Thus as cell density increases the autoinducer concentration increases and the bacteria respond. Once a minimum concentration is detected by a bacterium, the stimulus of an autoinducer leads to a change in gene expression within the bacterium. There are two main classes of autoinducers: oligopeptides and *N*-acyl homoserin lactones (AHL). The bacterial response to these two classes varies by strain; however, there is a universal autoinducer Autoinducer-2, a

furanosyl borate diester, which demonstrates biofilm up-regulation in most species of bacteria.[12] It is thought that this autoinducer is the remaining element of an initial system. These two main subclasses of autoinducers exist because while quorum sensing pathways have been kept relatively conserved through bacterial evolutions, the molecules used as signals were changed. These changes are a response to an innate proclivity for bacterial strains to reside with some strains but not with others. If one bacterial stain produces a waste product that is harmful for another, the first will not grow well in contact with the second. To avoid this, the bacteria learn to recognize the quorum sensing signals from bacteria they cannot live with as a sign to disperse the biofilm they have created and move elsewhere.[13]

Bacteria use quorum sensing to regulate a wide variety of physiological activities, including virulence and biofilm formation[13]. This allows for an opportunity to exploit such as a system, much like jamming a communication signal. The synthesis of a molecule mimicking the shape of known autoinducers, so that the synthesized molecule would be accepted by the bacterium and ultimately cause confusion. Ideally such a molecule would be a mock-autoinducer but differ enough that it would not trigger the same response that the native autoinducer does. Although the quorum sensing system is conserved for all bacteria, the signal relay mechanisms, specific chemical signals, and particular target genes involved change per species and strain. For example, a molecule which is considered an autoinducer and decreases biofilm for one bacterial strain may up-regulate the biofilm in another. Even with this complication, the signaling systems

are similar enough that an autoinducer which affects one bacterial strain can make for a good testing target for other strains and species of bacteria.

Indole (Figure 1) is a metabolite of the amino acid tryptophan and has recently been found to participate in signaling biofilm formation as an autoinducer with some involvement in quorum sensing.[14] In particular, indole is found in large quantities in at least 85 bacterial species, many of which are pathogenic[15]. This widespread abundance of indole may indicate its broad-spectrum recognition by bacterial strains in the form of a third autoinducer system or subsystem. Within *Escherichia coli* indole promotes bacterial secretion of toxins, helps maintain genetic stability, and plays a role as an active signal in metabolic control. Additionally, indole is thought to be a chemo-repellant, which is a chemical substance which decreases motility, thus affecting the bacteria's ability to colonize. The oxidized form of indole, isatin (Figure 1), is also a found within same biological systems and may also influence biofilm formation. Isatin is thought to be a downstream metabolite of indole and has been found to be a biofilm regulator within *E. coli* strains, decreasing biofilm formation four-fold for Enterohemorrhagic *Escherichia coli*.[16] The specific pathways which form endogenous isatin still require further experimental effort.



Indole **Isatin**

Figure 1: Structures of indole and isatin

Current literature has shown on the effect oxidized indole derivatives and isatin itself have on biofilm regulation. [17] It may be possible that drugs containing an isatin derivative could compete with endogenous isatin, and the derivative then influences biochemical pathways where isatin is involved.[17] Isatin derivatives have been found to act as inhibitors of apoptosis, anticonvulsants, and other antiviral, anti-bacterial, and anti-fungal agents.[18] There is proof that isatin is a bimolecular product of human cytochrome P450-catalyzed metabolism of indole and thus not a result of non-enzymatic oxidation[19]. Thus a mock-isatin derivative may interplay with biochemical processes in which isatin plays a role. We hope to confirm the pervious observations that isatin has influence on biofilm formation. The focus for this study was the development and testing of isatin and isatin derivatives focusing on the substitution of the 3-oxo carbonyl for hydrazone. Hydrazone derivatives were chosen due to their previously researched biological activity and antimicrobial properties.[20] Previously synthesized derivatives were found to have a bactericidal effect on both Gram negative and Gram positive bacteria. Compounds **1** and **2** are (Figure 2) derivatives taken from literature.[21] The addition of an anti-microbial property to isatin could be useful.

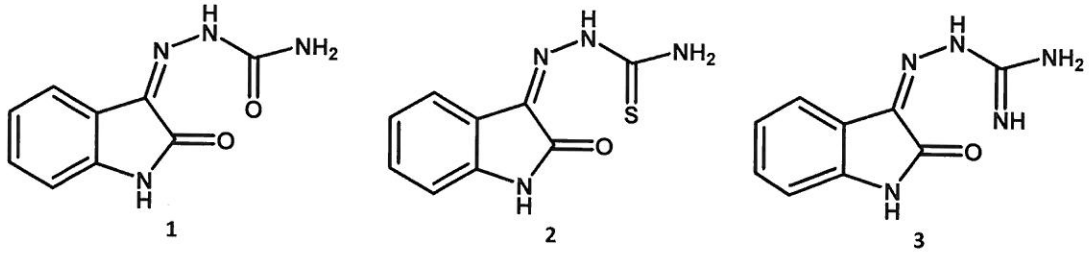


Figure 2: Structures of isatin derivatives

J. Lee, *et. al.* found isatin had an effect on biofilm formation for both *E. coli* strains and *Pseudomonas aeruginosa*[15] while hydrazone derivatives were found to have

significant antibiotic activity against *Staphylococcus aure us.*[21] In particular, compound **2** was found to be active against *Staphylococcus* bacteria at a minimum inhibitory concentration (MIC) of 204 µg/mL and compound **1** was found to be active at a MIC of 106 µg/mL for *Staphylococcus aureus.*[21] Thus, *E. coli*, *P. aeruginosa*, and *S. aureus* were chosen as targets for the created derivatives due to their ubiquity in nature and the previously stated applications.

To accurately investigate biofilm inhibition, it first has to be established whether the derivative in question is a bactericidal compound. This requires the use of minimum inhibitory concentration (MIC) assays to assess the lowest concentration at which a particular compound visually prevents growth of the test strain of bacteria. Only after this concentration is established can biofilm inhibition be investigated since effects on biofilm can only be confirmed below the compound's ability to inhibit cell growth. Inhibition assays examine how a bacteria's ability to form biofilm is affected by the addition of a compound. Providing insight into whether the compound in question could be used for the treatment of that particular bacterial species in the event of an infection. The results of this test were quantified through the use of UV – Visible spectrometry, after washing away the media containing planktonic bacteria and Gram staining the biofilm adhered to the bottom of the well. This data is expressed through the use of the half maximal inhibitory concentration (IC_{50}). An IC_{50} value denotes the concentration required to inhibit a biochemical process by half. Within these experiments the tested process was biofilm formation. This found value is used by the pharmacological industry as a unit to specify the *in vitro* efficiency of a drug.

Special considerations must be made when testing inhibition of a compound near its MIC. If the compound is bactericidal then it could be killing some of the bacteria at the start of the assay. The compound could also be bacteriostatic, thus disable the bacteria and not allow for cell proliferation. Investigating the possibility of this would require growth curve analysis, where the OD_{600} of both the dosed and pure bacterial solutions would be tested every hour. This observation of growth vs. time allows us to determine if biofilm decrease is a consequence of delayed growth.

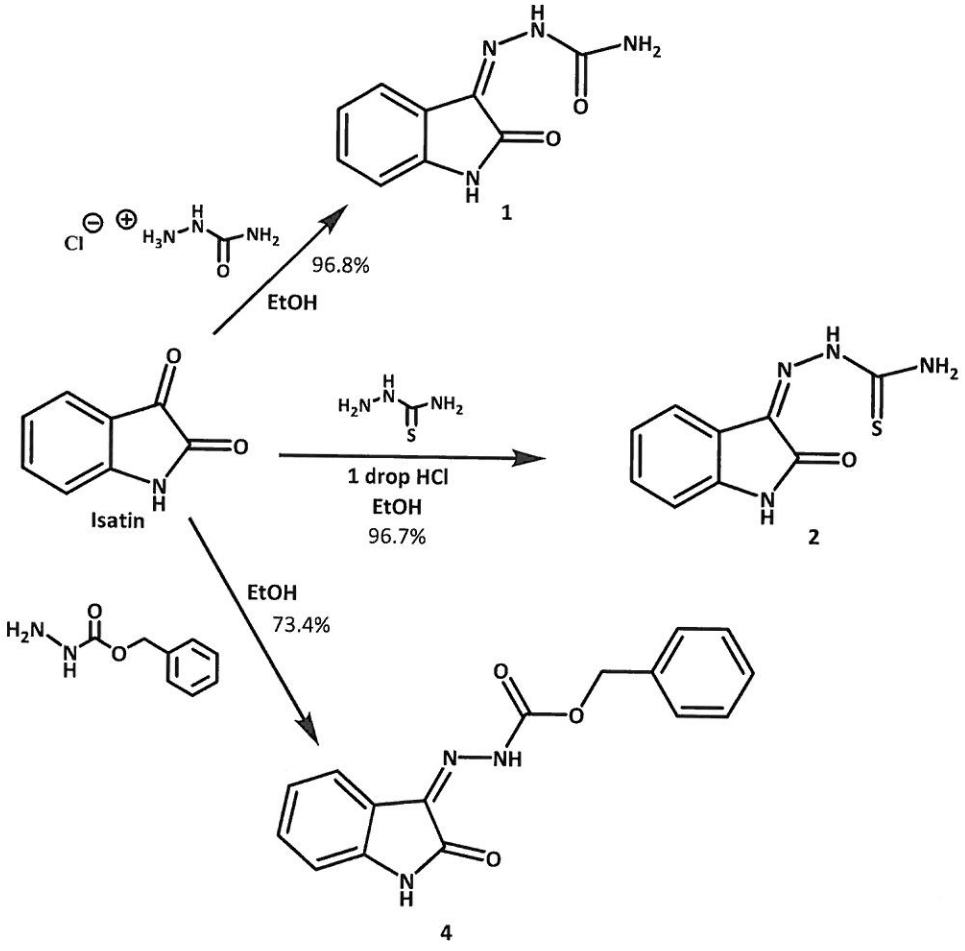
The final consideration for this investigation is how the toxicity of the compounds in question would be tested in order to determine if they are fit to be used in mammalian systems. The compounds in question would be tested against a eukaryote to determine if they are toxic within the small-scale before any further determinations are made. If it is found that the compound is toxic, a biofilm inhibitor would still have limited use within industry for such things as the coating for waste water pipes, paints, and plastic impregnation.

Results and Discussion

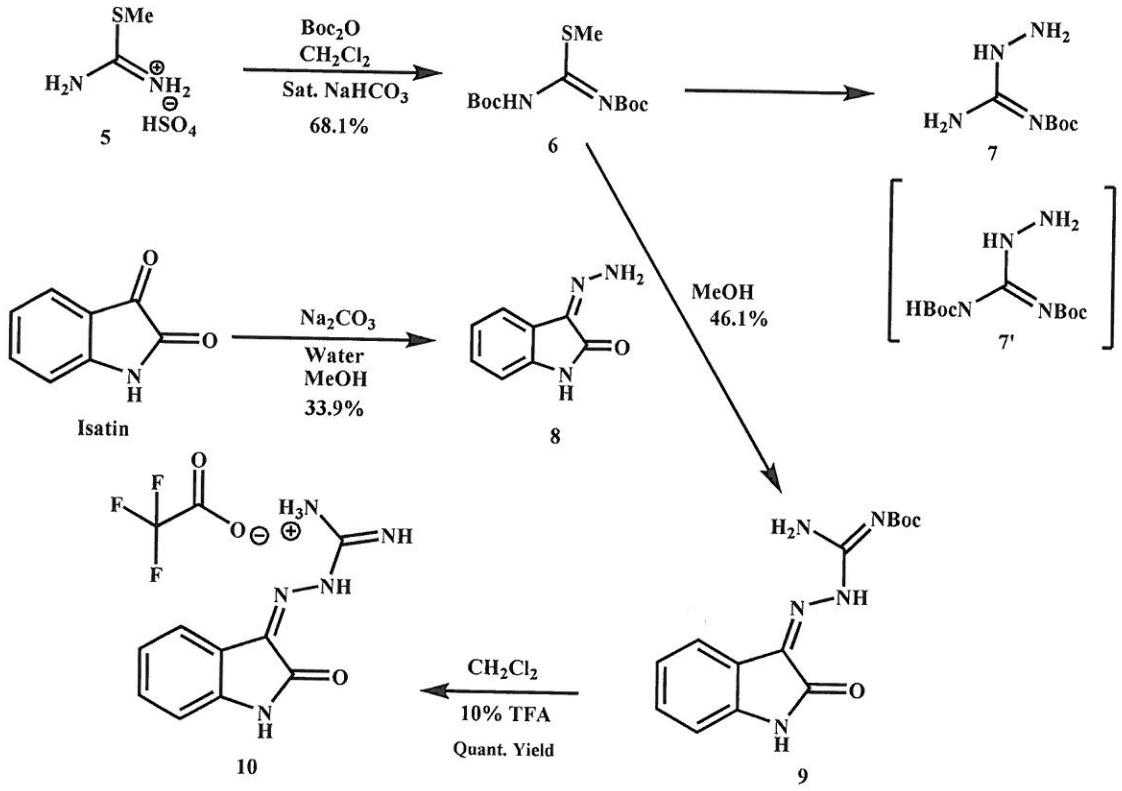
In order to investigate whether isatin or hydrazone derivatives had an effect on biofilm, the synthesis of a library of isatin derivatives was first carried out. These derivatives were then placed through biological testing by first correlating the colony counts with optical density to allow for quick bacterial concentration determination for both assays. Thereafter, the isatin compounds were investigated through the use of the MIC and biofilm inhibition assay to examine if biofilm would form with the tested compound present.

Synthesis of library

Compounds **1**, **2** and **4** were synthesized via condensation with the appropriate hydrazide derivative (Scheme 1). Thiourea derivative **2** was found to require acid catalyzed condensation. This was not necessary for **1** presumably because the hydrazide reagent was a salt. Due to multiple nitrogens, the guanidine derivative **3** could not be synthesized using the same condensation method so a di-*tert*-butoxycarbonyl (di-Boc) protection pathway was used (Scheme 2).

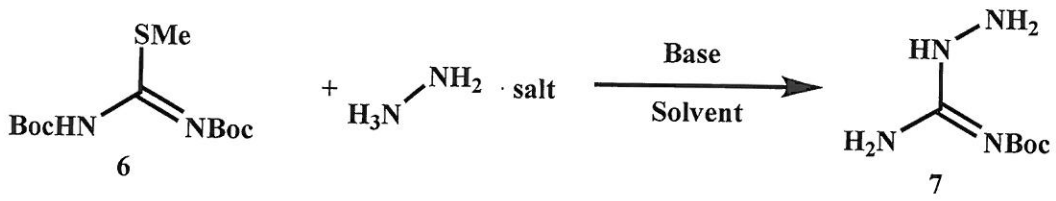


Scheme 1: Condensated derivatives of isatin



Scheme 2: Di-*tert*-buroxycarbonyl protection and guanidine derivative creation pathway

The procedure for the di-Boc protection was taken from literature to prepare intermediate **6**.[7] Multiple methods were attempted to create compound **7'** as shown in Table 1. All conditions lead to the mono-Boc **7**. Condensation of compound **7** and isatin provided more than one product towards preparation of **9**. The free hydrazone **8** was prepared and condensed with **6** to obtain compound **9**. As seen previously, one tert-butoxycarbonyl group was removed; however compound **9** appeared pure and was still carried through deprotection in acidic conditions creating the TFA salt **10**.



	Hydrazine salt	Base	Solvent
1	$\cdot H_2SO_4$	$NaHCO_3$	MeOH
2	$\cdot H_2SO_4$	Triethylamine	MeOH
3	$\cdot H_2SO_4$	$Na^{\ominus}\ ^{\oplus}OMe$	MeOH
4	· 2 HCl	$Na^{\ominus}\ ^{\oplus}OMe$	MeOH
5	$\cdot H_2SO_4$	Na_2CO_3	Water and MeOH
6	· 2 HCl	Na_2CO_3	Water and MeOH
7	$\cdot H_2SO_4$	Sat. $NaHCO_3$	CH_2Cl_2

Table 1: Reaction conditions used in attempting guanidine derivative **9**.

Biological testing

Correlation of Colony Counts and Optical Density

Colony forming units were correlated with optical density to determine the OD_{600} for approximately 5×10^5 colony forming units (CFUs) to start the MIC assay. Colony counts were completed for *E. coli* ATCC® 25922 and *P. aeruginosa* Carolina® 155250A on Luria broth agar plates, and on tryptic soy agar plates for *S. aureus* Carolina® 155554A. For *E.coli*, the count of CFUs was best observed on the plate diluted to a correlation of the OD_{600} from the parent culture and the CFUs gave an OD_{600} at 5×10^5 CFUs. Similar analysis for *P. aeruginosa* provided an OD_{600} value of 0.000005. For

S. aureus, the correlation extrapolated to an OD_{600} of 0.004. While cultures will contain some differences and error is expected, the colony count for *P. aeruginosa* was the only sample not within error of previously performed colony count data. As a result an OD_{600} of 0.0013 was used based on previous data collected within the Christian Melander lab.

Minimum Inhibitory Concentration Assay

Synthesized compounds **1**, **2**, **4**, **8**, and **10**, were tested within the concentration ranges from 15 μg/mL to 128 μg/mL with Erythromycin and Penicillin G as controls for cell death. The MIC assays for *E. coli* yielded MICs of 128 μg/mL for Erythromycin and Penicillin G, the positive controls, with no observable cell death for any of the synthesized compounds or isatin at or below 128 μg/mL. Only Erythromycin proved toxic to *P. aeruginosa* at 128 μg/mL, with no observable cell death for the synthesized compounds or the isatin. *S. aureus* proved more susceptible with observed MICs of 0.125 μg/mL for Erythromycin, 128 μg/mL for Penicillin G, 128 μg/mL for isatin, and 32 μg/mL for **4**. There was no observable cell death for any of the other synthesized compounds at the tested concentrations.

Biofilm Inhibition Assay

2-Aminobenzimidozole (2ABI) (Figure 3) was chosen as the control as it is known to inhibit biofilm formation for *P. aeruginosa* with an IC_{50} 47 μM.[22] Isatin was tested as it was the parent molecule for all the derivatives created. Both 2ABI and isatin were tested for all bacteria. For the biofilm inhibition assays for *E.coli*, compounds **1**, **2**, and **10** were tested. The compounds tested for *P. aeruginosa* were compounds **1**, **2**, **10**, and **8**. For the assays for *S. aureus* the compounds tested were compounds **1**, **2**, **10**, **4**, and **8**.

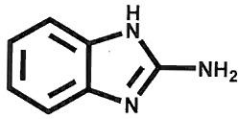


Figure 3: Structure of 2-Aminobenzimidozole (2ABI)

The biofilm inhibition assays for *E.coli* indicated that both compounds **1** and **2** inhibited biofilm with IC 50 values of 380 µg/mL and 112 µg/mL, respectively (Table2). The control, 2ABI, compound **3**, and isatin were found to promote biofilm. Conversely, 2ABI, compound **8**, and isatin were found to be biofilm inhibitors for *P. aeruginosa* with IC 50 values of 20 µg/mL, 207 µg/mL, and 12 µg/mL, respectively, while compounds **1** and **2** were found to be biofilm promoters. Compound **10** was found to not affect biofilm formation. The biofilm inhibition assays for *S. aureus* indicated that compounds **3** and **8** inhibit biofilm with IC 50 values of 51 µg/mL and 195 µg/mL, while compound **2** and isatin were found to promote biofilm production. 2ABI was found to not affect biofilm production for *S. aureus*.

Bacteria	Compound	MIC (ug/mL)[a]	IC 50 (ug/mL)	Promoter[c]
E. coli	2ABI	> 128	-	Yes
	1	> 128	380[b]	-
	2	> 128	112	-
	10	> 128	-	Yes
	Isatin	> 128	-	Yes
P. aeruginosa	2ABI	> 128	30	-
	1	> 128	-	Yes
	2	> 128	-	Yes
	8	> 128	207[b]	-
	10	> 128	-	-
	Isatin	> 128	12	-
S. aureus	2ABI	> 128	-	-
	1	> 128	-	-
	2	> 128	-	Yes
	4	32	-	-
	8	> 128	195[b]	-
	10	> 128	51	-
	Isatin	128	-	Yes

[a] 128 was the highest concentration tested. [b] extrapolated from best fit quadratic [c] biofilm increase relative to control

Table 2: Observed anti-microbial effects.

Conclusions

Within this study, procedures for the creation of isatin derivatives were developed. These derivatives have been tested against *E. coli*, *P. aeruginosa*, *S. aureus* to determine the OD_{600}, minimum inhibitory concentration and the effect the compounds have on biofilm production. Within the MIC assays, only isatin and

compound **4** were found to cause bacterial death within range of concentrations tested. Compounds **1** and **2** were found to be biofilm inhibitors for *E. coli*, while 2ABI, compound **3**, and isatin were found to be biofilm promoters. For the biofilm assays of *P. aeruginosa*, it was found that 2ABI, compound **8**, and isatin inhibit biofilm production while compounds **1** and **2** promoter production. Compound **3** was found to have no effect on the biofilm production of *E. coli*. 2ABI and compounds **3** and **8** were found to be biofilm inhibitors for *S. aureus*. Compound **2** and isatin were found to be promoters, while compounds **1** and **4** had no effect on biofilm production.

Looking to the future, in the short term, the results attained from this investigation for the biofilm assays could be repeated as to verify their accuracy and determine standard deviations. Also, the MIC assays for the compounds demonstrating bacterial death could be refined by narrowing concentration ranges closer to the found MIC. The growth curves need to be created for all observed inhibition concentrations to evaluate if any of the compounds are influencing cell growth. Another short term adjustment that could be made is to change the bacteria the compounds were tested against to give a better understanding of the compounds abilities, possibly adding Gram positive bacteria since values were improved against *S. aureus.*

As a longer term goal, compounds that have shown promise would be tested against a eukaryotic organism to test whether promising compounds would be toxic to humans. If not, then they could be tested further for potential use as a replacement for antibiotics.

Experimental

General Information

Proton nuclear magnetic resonance (^{1}H NMR) and Carbon-13 nuclear magnetic resonance (^{13}C NMR) spectra were recorded with a Varian EM 360L NMR spectrometer. The solvent used was dimethyl sulfoxide-d6. The infrared spectrum was recorded with a Thermo Scientific Nicolet iSIO. Ultraviolet/Visible (UV) values for biofilm assay were recorded with a Biotek ELx808 Multichannel Plate Reader. Melting points were recorded with a Barnstead Electromantle (EM series). The shaken incubation was performed in a VWR 1585 shaking incubator set at 37.0 °C and 200 RPM. The stationary incubation was performed in a Precision Scientific Co oven (Model 17) set to 37 °C.

Compound Synthesis

General Procedure for creation of 1 and 4

To a solution of 1 eq of isatin and EtOH (6.5 mL) was added 1 eq of respective hydrazide derivative. This mixture was refluxed at 60 °C for thirty minutes, and then allowed to recrystallize overnight. The pure solid was filtered. **1**:Yellow solid (0.481 g, 2.31 mmol, 96.8 % yield); Mp 262 – 282 °C; Rf 0.5 in 50:50 ethyl acetate/ hexanes; IR (acetone film) 3467 (w), 3300 (br), 3235 (br), 3132 (br), 2999(br), 2821(br), 1703(s), 1622(w), 1573 (s), 1463(s); ^{1}H NMR (60 Hz, DMSO-d6) δ 11.73 (1 H, s), 11.10 (1 H, s), 7.61 (2 H, d), 7.34-6.85 (6 H, m); ^{13}C NMR (15 Hz, DMSO-d6) δ 162.6, 155, 141.5, 131, 130.3, 122.2, 120.3, 120.2, 110.8. **4**: Yellow solid(0.011 g, 0.038 mmol, 73.4 % yield); Mp 220 -230 °C; Rf 0.31 in 40:60 ethyl acetate/hexanes; IR (acetone film) 3269 (br) 1735 (s) 1705 (s) 1614 (br) 1497 (s); ^{1}H NMR (60 Hz, DMSO-d6) δ 11(1 H, s), 10.74(1 H, s), 8 (1 H, d), 7.43 (6 H, s),

7.03 (2 H, d), 5.28 (2 H, d); ^{13}C NMR (15 Hz, DMSO-d6) δ 164.7, 154.7, 154.4, 143.6, 136.1, 132.5, 128.6, 128.3, 126.3, 115.5, 110.5, 67.2.

Procedure for creation of 2

To a solution of 1 eq of isatin and EtOH (6.5 mL) was added 1 eq of thiosemicarbazide and 1 drop of 6 M HCl. The mixture was refluxed at 60 °C for sixty minutes. The solid was filtered to obtain a pure yellow solid (0.18 g, 0.796 mmol, 96.7% yield). Mp 284 °C; Rf 0.42 in 50:50 ethyl acetate/hexanes; IR 3516 (br), 3420 (br), 3336 (br), 3265 (br), 3162 (br), 1701 (s), 1670 (s), 1621 (s), 1593 (s), 1583 (s), 1463 (s); ^{1}H NMR (60 Hz, DMSO-d6) δ 12.51 (1 H, s), 11.21 (1 H, s), 9.04 (1 H, s), 8.67 (1 H, s), 7.68 (1 H, d), 7.40 – 6.87 (3 H, m); ^{13}C NMR (15 Hz, DMSO-d6) δ 178.8, 162.6, 142.4, 132.1, 131.3, 122.3, 120.9, 1229.9, 111.

Procedure for creation of 8

A mixture of 1 eq of isatin, 3 eq of hydrazine sulfate salt, 1.5 eq of sodium carbonate, MeOH (5 mL), and water (5 mL) was refluxed at 60 °C for two hours and allowed to recrystallize at room temperature. The solid was vacuum filtered, then triturated in water and vacuum filtered again to obtain a pure yellow solid (0.158 g, 0.980 mmol, 33.9% yield). Mp 208 °C; Rf 0.55 in 40:60 ethyl acetate/hexanes; IR 3352 (br), 3209(br), 2834(w), 1681(s), 1655 (s), 1587(s), 1549(s), 1465(s); ^{1}H NMR (60 Hz, DMSO-d6) δ 10.68 (2 H, d), 9.65 (1 H, s), 7.45 - 6.84 (5 H, m); ^{13}C NMR (15 Hz, DMSO-d6) δ 162.8, 138.6, 127, 126.2, 122.2, 121.3, 117.4, 109.9.

Procedure for creation of 10

Di-Boc protection of methyl thiourea was carried out as per literature procedure.[23] The Di-Boc protected 1 eq of thiourea was then refluxed with 1 eq of **8** and MeOH (10 mL, 0.2 M) in an oil bath at 60 °C. Silica was added and the mixture was concentrated. Crude product was eluted at 40% ethyl acetate/60% hexanes through a silica gel column. The pure product was then stirred 10% TFA (0.5 mL) and dichloromethane (5 mL). Chloroform was then added and the reaction was concentrated to provide the solid yellow TFA salt of the product (0.096 g, 0.319 mmol, quantitative yield). Mp 195 °C; Rf 0.53 in 50:50 ethyl acetate/hexanes; IR 3428 (br) 3114 (br) 1720 (s) 1703 (s) 1638 (w) 1612 (s) 1572 (s) 1469 (s); ^{1}H NMR (60 Hz, DMSO-d6) 10.95 (1 H, s), 8.46 (6 H, s), 7.47 – 6.89 (4 H, m); ^{13}C NMR (15 Hz, DMSO-d6) 164, 157.3, 144.2, 139.5, 133.5, 126.6, 121.8, 115, 110.9.

Biological Screening

General Colony Count/Optical Density Correlation Procedure

Bacteria were cultured in Mueller-Hinton broth (MHB) media for 6-8 hrs. The cultures were then diluted by 10 then an optical density at 600 nm (OD_{600}) was recorded. Bacterial solutions of concentrations at 1/2, 1/4, 1/8, and 1/16 of this solution were measured for OD_{600} values. The original bacterial cultures were then plated on agar plates (Luria broth plates for *E. coli* and *P. aeruginosa*, tryptic soy agar plates for *S. aureus*) at concentrations of 10^{-7} through 10^{-12}. These plates were then incubated for 16 hrs and plates bearing 100 or less colonies were counted. These bacterial counts were used to find the bacterial concentration (colony forming units) of the cultures. The

bacterial concentration was then plotted against the OD_{600} values to extrapolate the OD_{600} value for a solution of bacteria bearing 5×10^5 CFU mL^{-1}. This value can then be used in the MIC assays for quick determination of starting inoculates.

General Minimum Inhibitory Concentration (MIC) Procedure

Mueller-Hinton broth (MHB) was inoculated at a concentration of 5×10^5 CFU mL^{-1} with the bacteria. The inoculate was then divided into 1 mL aliquots and test compound was added to provide the highest tested concentration. 200 μL of test solutions were then added to the wells 2-7 within the first row of a 96 well polystyrene plate. Cells 1 and 8 were reserved for control lanes of inoculated solutions that were free of test compounds. Inoculated media (100 μL) was added to rows 2-12 of the 96-well polystyrene plate. The test samples were then serially diluted (100 μL transfers) down their corresponding rows. Between each transfer the samples were mixed 6-8 times by pipet. Once serial dilution was complete the plate was then sealed with Glad Press and Seal® and incubated under stationary conditions at 37 °C. After 16 hrs MIC values were recorded as the lowest concentration of the test compound that caused no visible bacterial growth.

General Biofilm Inhibition Assay Procedure

Overnight cultures of bacteria (*E. coli* in LB, *P. aeruginosa* in LB, and *S. aureus* in TSB plus glucose) were diluted to an OD_{600} of 0.01. Test compounds were then diluted into 1 mL of this bacterial solution at predetermined concentrations. A 96-well polystyrene plate was then used horizontally to add 2-100 μL water columns (columns 1 and 12), 4-100 μL control columns with no compound (columns 5-8), and 6-100 μL compound containing

lanes in decreasing dosage concentrations (columns 2-4 and 9-11). The plates were then covered with Glad Press and Seal® and incubated under stationary conditions at 37 °C for 24 hrs. The media was then discarded and planktonic bacteria were removed by a gentle water washing. Each well was then stained with 125 μL of 0.5% solution of crystal violet at room temperature for 30 mins. After thoroughly washing away the excess crystal violet with water the stained biofilm was dissolved in 200 μL of 95% ethanol. This solution was allowed to sit for 15 mins and 100 μL was transferred to the corresponding wells of a polystyrene microtiter dish. Biofilm inhibition was then quantified by measuring the OD_{630} of each well. Blank wells were used to subtract background polystyrene adhered stain.

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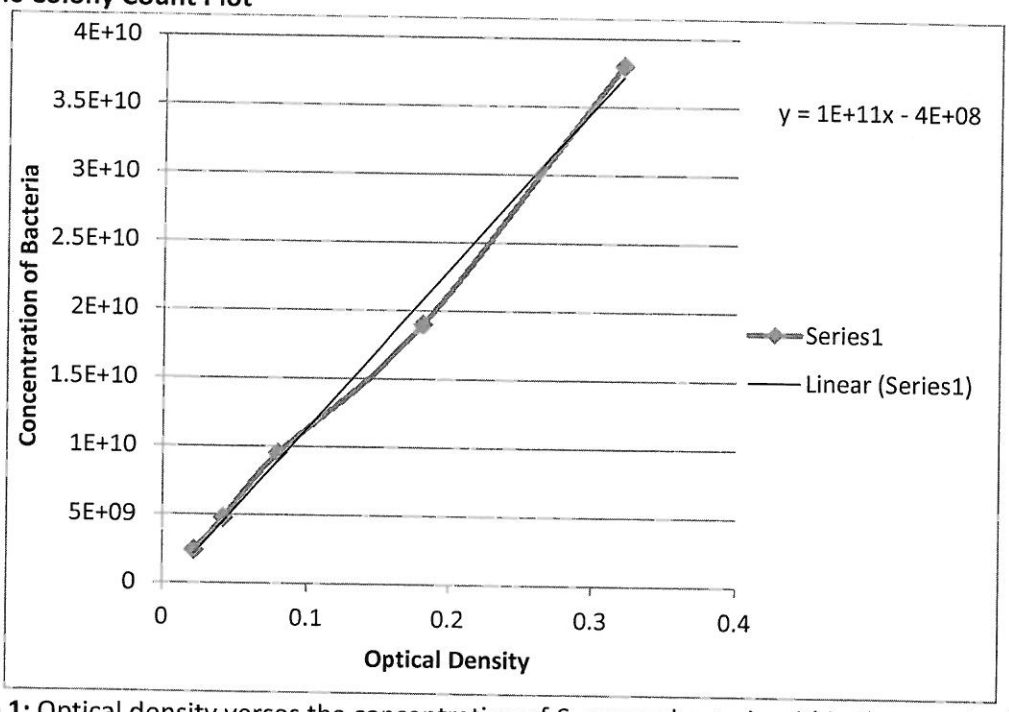
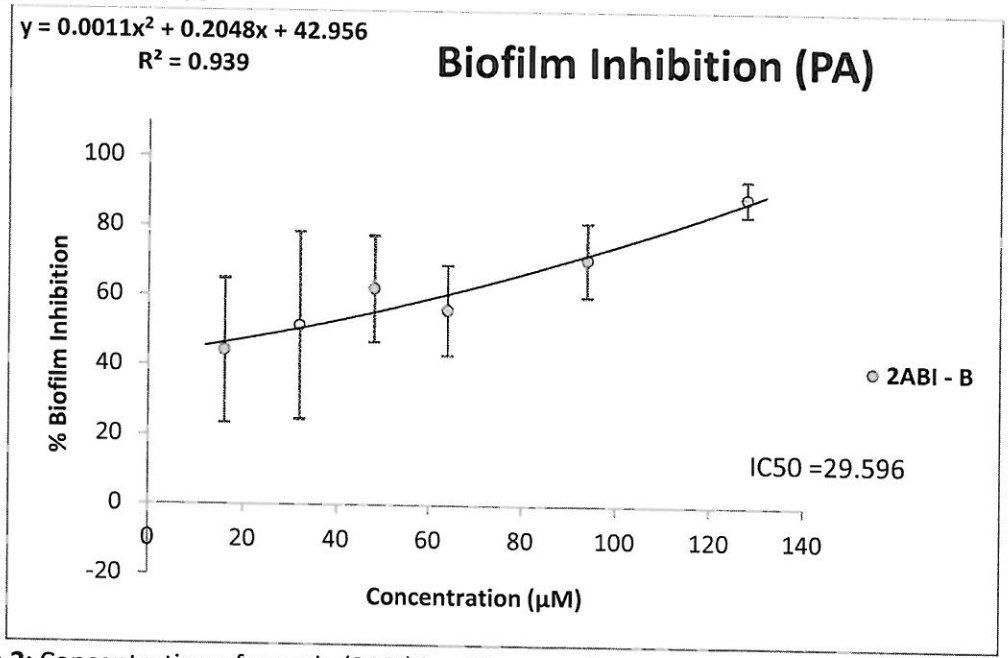
Appendix

Sample Colony Count Plot



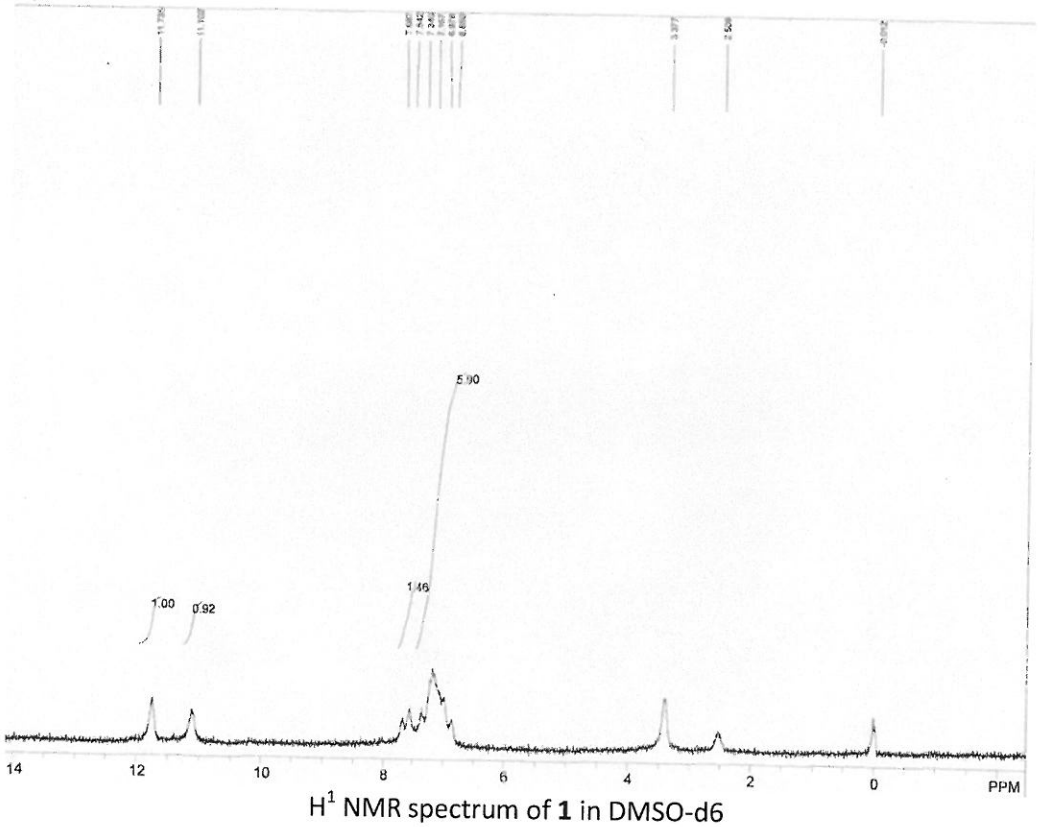
Graph 1: Optical density verses the concentration of *S. aureus* bacteria within the sample, found with colony count. OD_{600} was found to be 0.004.

Sample Inhibition Plot

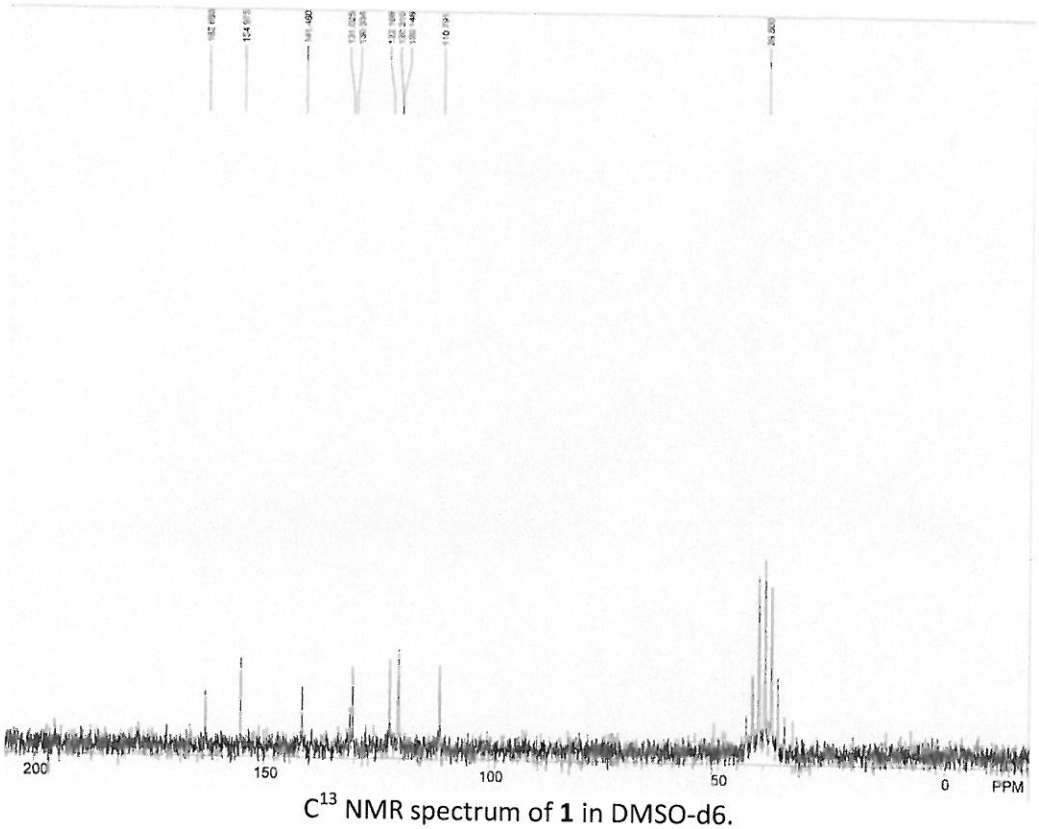


Graph 2: Concentration of sample (2ABI) in µM verses the percent biofilm inhibiton of bacterial sample (*P. Aeruginosa*). The IC 50 value for this plot was found to be 29.596

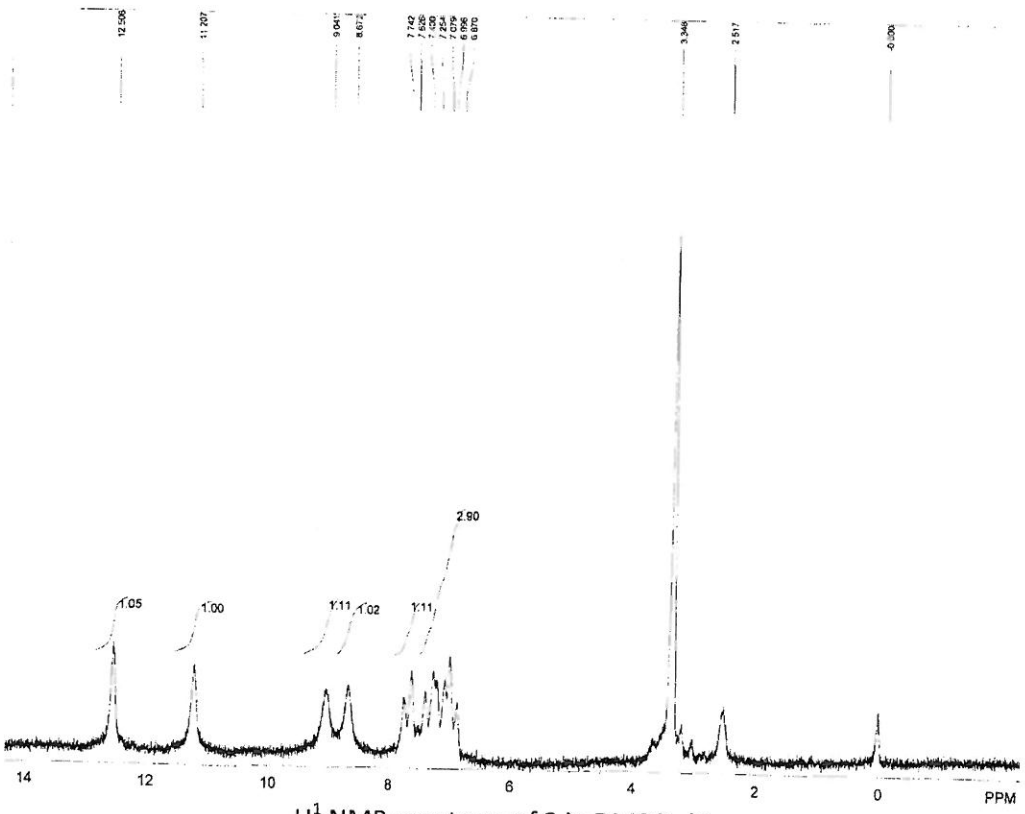
NMR Spectrum



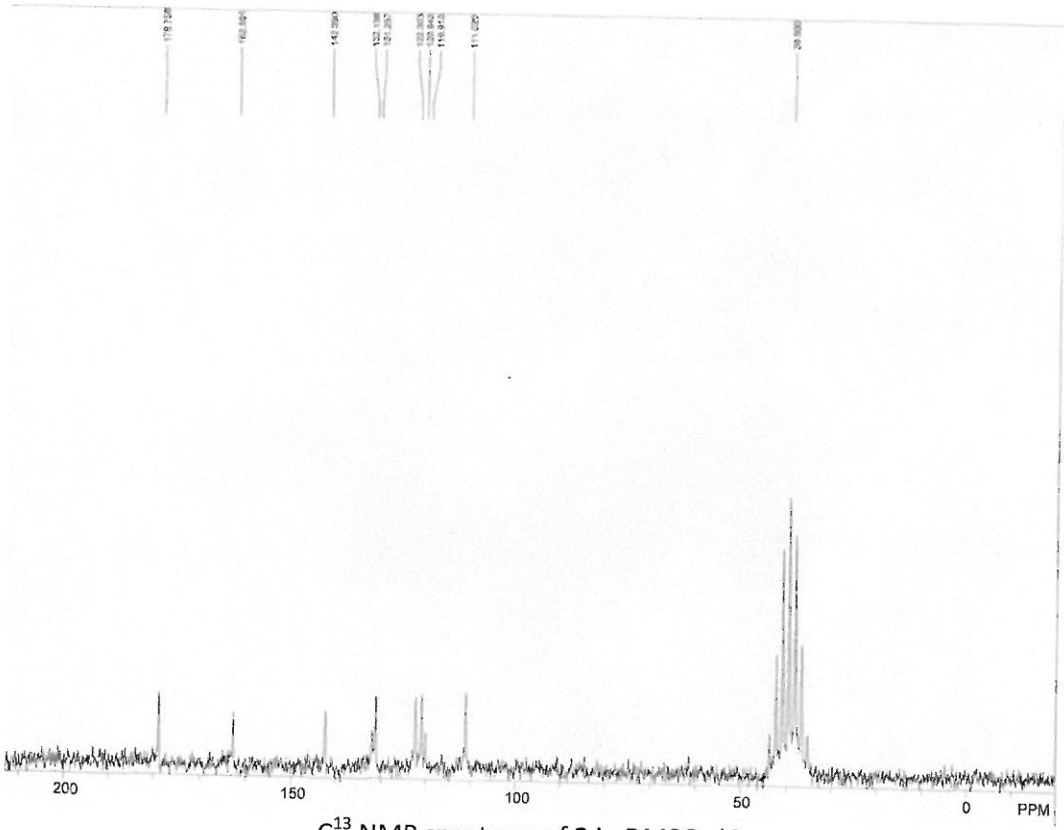
H^1 NMR spectrum of **1** in DMSO-d6



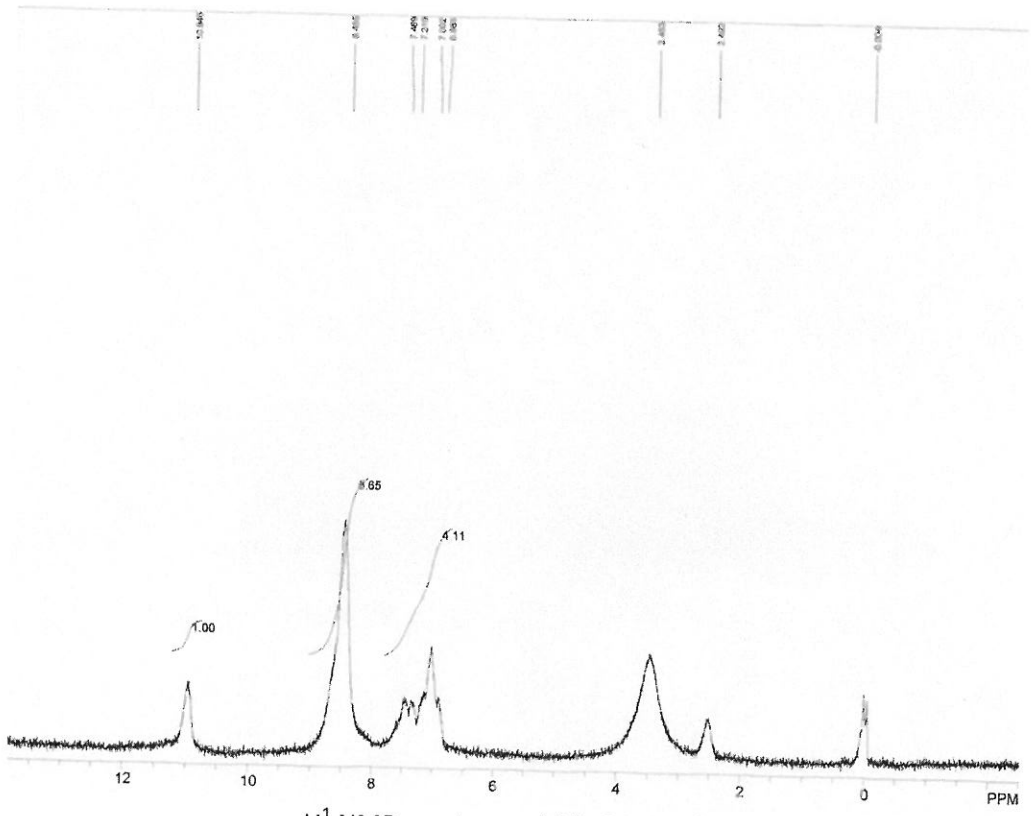
C^{13} NMR spectrum of **1** in DMSO-d6.



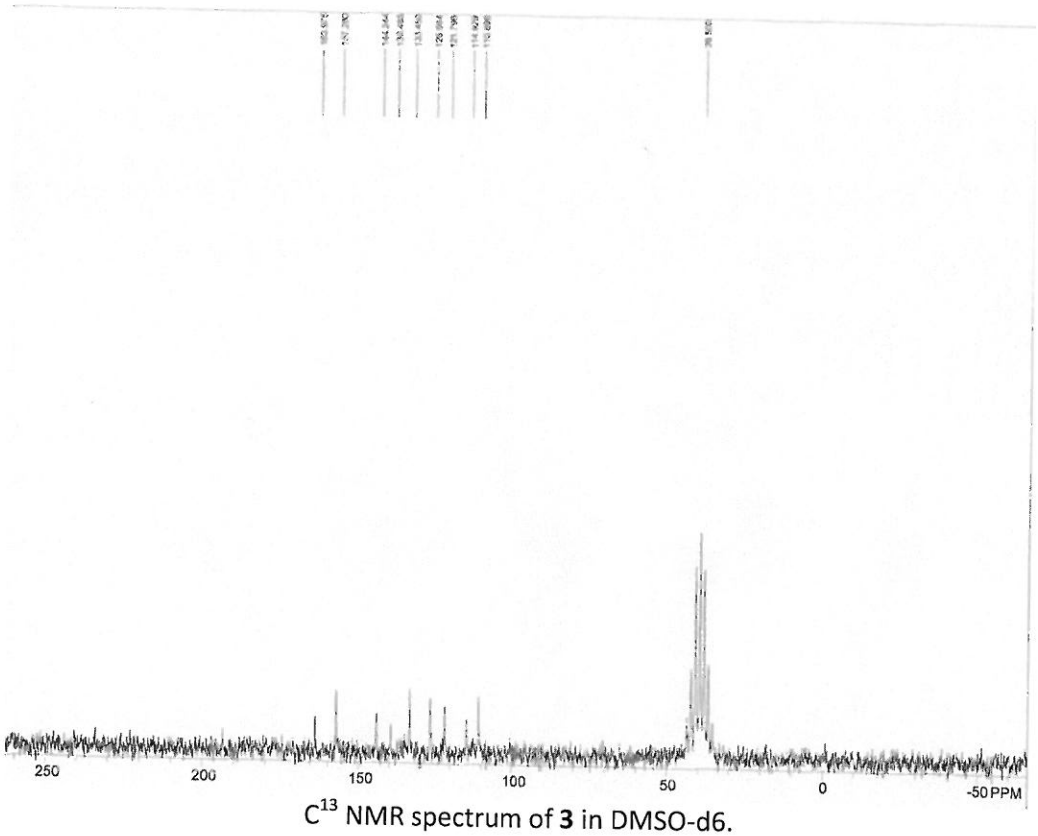
H^1 NMR spectrum of **2** in DMSO-d6



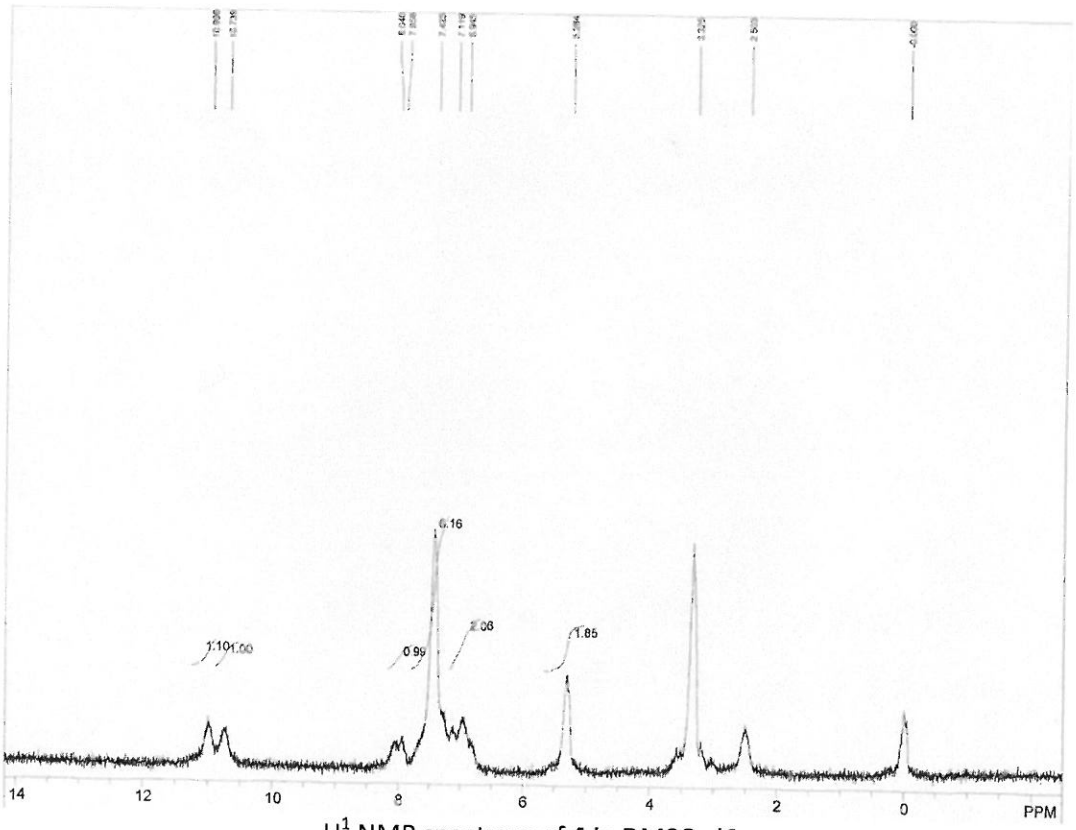
C^{13} NMR spectrum of **2** in DMSO-d6.



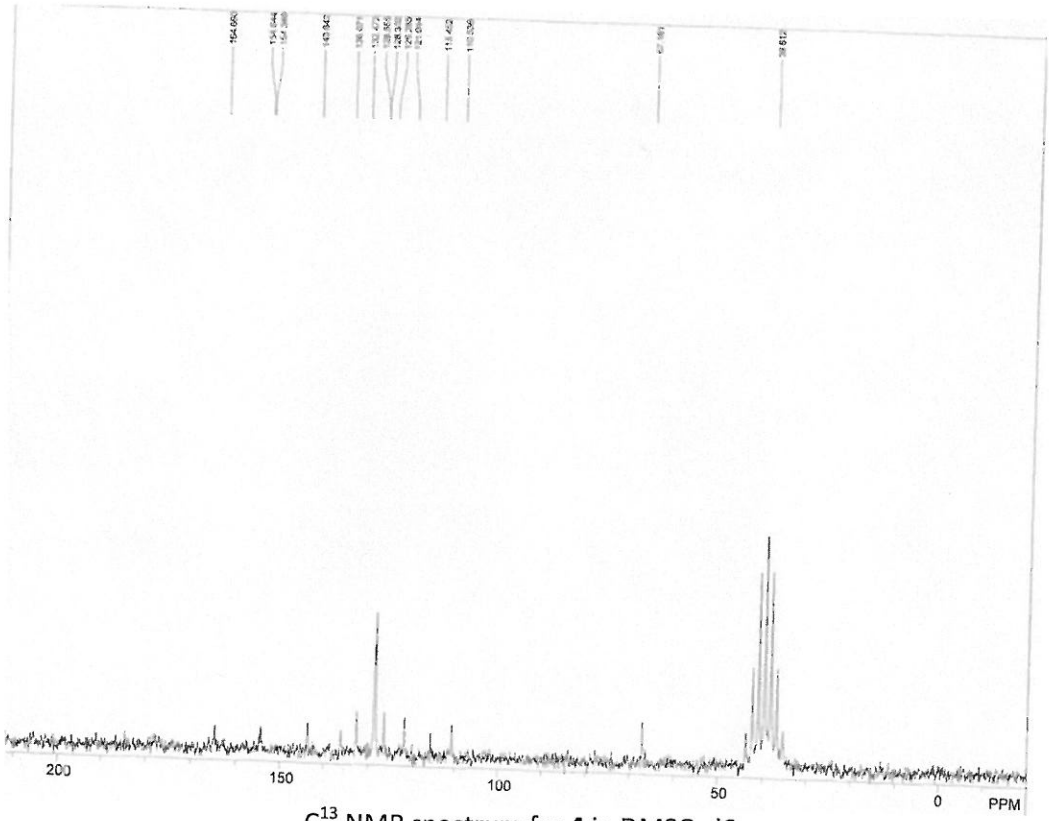
H^1 NMR spectrum of **3** in DMSO-d6.



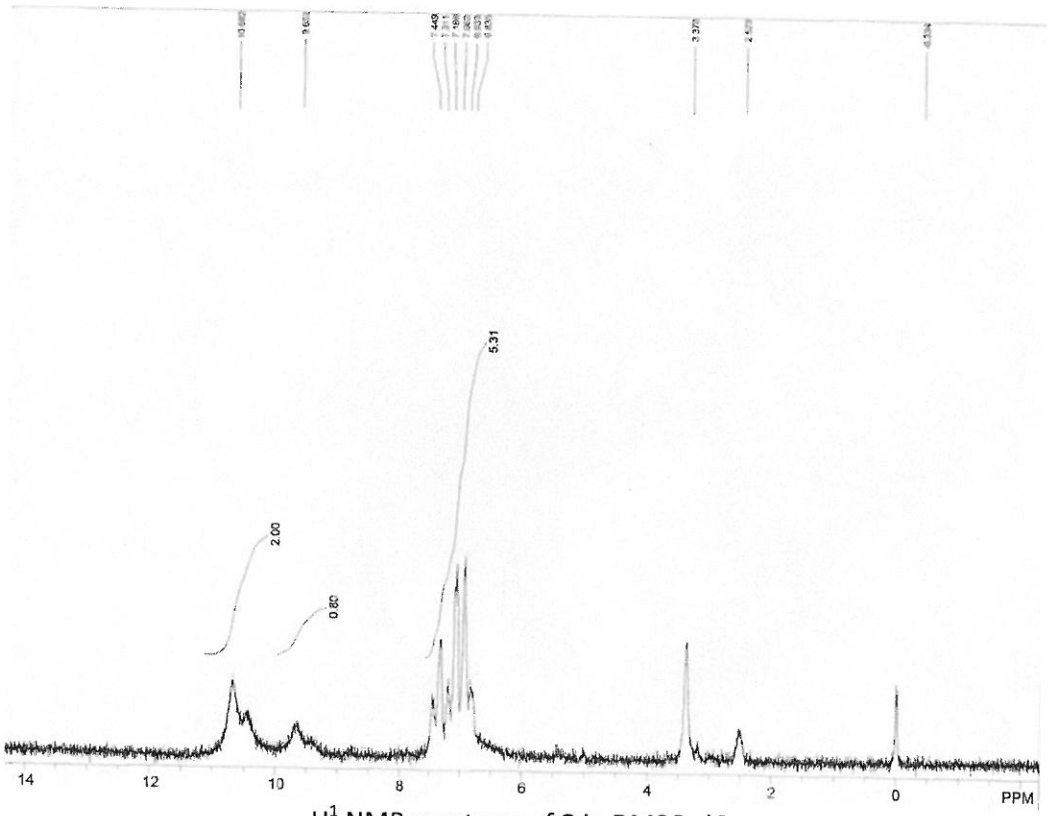
C^{13} NMR spectrum of **3** in DMSO-d6.



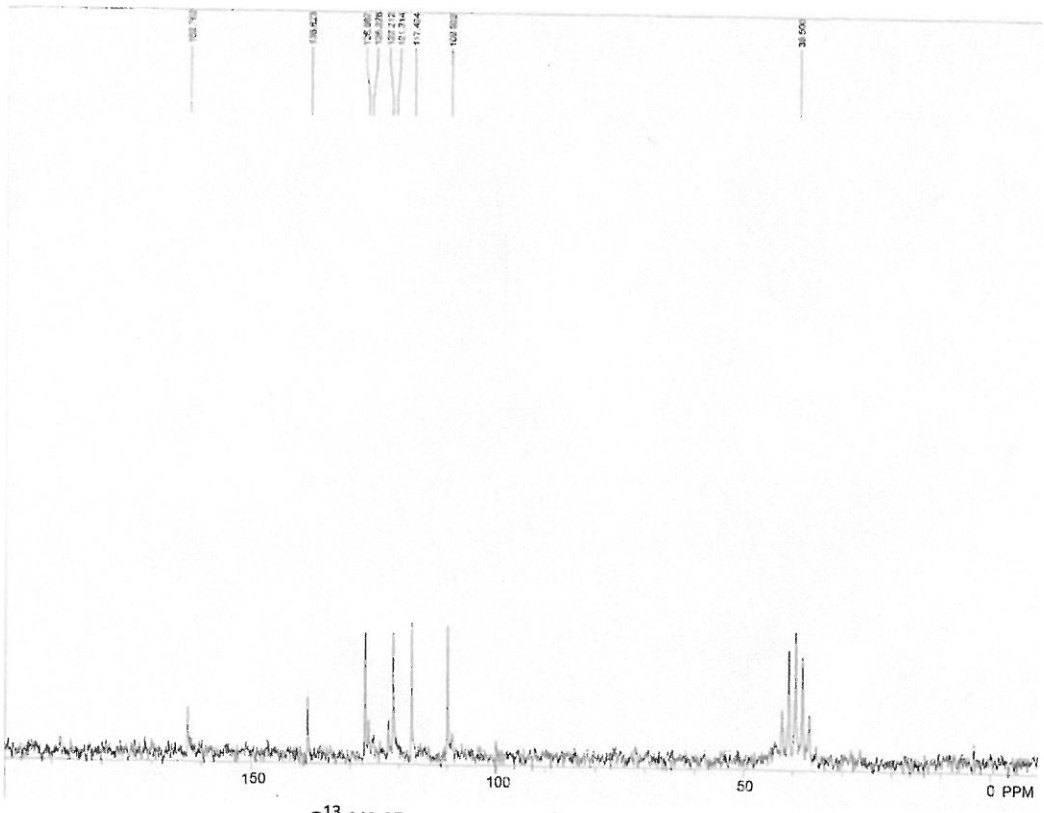
H^1 NMR spectrum of **4** in DMSO-d6.



C^{13} NMR spectrum for **4** in DMSO-d6.



H^1 NMR spectrum of **8** in DMSO-d6.



C^{13} NMR spectrum of **8** in DMSO-d6.