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Synthesis and In-Vitro Cell Viability/Cytotoxicity Studies of Novel Pyrrolobenzodiazepine Derivatives

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Synthesis and *In-Vitro* Cell Viability/Cytotoxicity Studies of Novel Pyrrolobenzodiazepine Derivatives

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A thesis submitted to the faculty of the Department of Chemistry and the Honors College at East Tennessee State University as required for the Honors-in-Discipline Bachelor of Science degree in Chemistry.

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ABSTRACT

Pyrrolobenzodiazepines (PBDs) are a group of naturally occurring compounds that were discovered in the cultures of *Streptomyces* in the 1960s. Some natural PBDs discovered in these cultures, such as anthramycin and sibiromycin, were shown to possess a broad spectrum of anti-tumor activity. Since cancer is still a leading cause of death globally, the development of novel anti-proliferative derivatives of PBDs is essential for human welfare worldwide. Further synthesis and structure-activity relationship (SAR) studies of the parent natural products and their tetracyclic analogs will lead to the discovery of drug candidates. In this work, thirteen PBD analogues were synthesized using no more than three to four synthetic steps, beginning with commercially obtainable L-proline and isatoic anhydride. The MTT assay, which is a colorimetric assay that uses 3-(4,5-Dimethylthiazol-2-Yl)-2,5-diphenyltetrazolium bromide (MTT) to assess cell metabolic activity, was initially implimented to test the *in vitro* cytotoxicity of the compounds using multiple cell lines, namely: SKBR-3, MCF-7, SKMEL-2, CaCo 2, HCT 116, and Mia Paca. Nearly all of the compounds decreased the cell viability of MCF-7 by roughly 20%. Additionally, the anti-proliferative activity of the PBD products were further evaluated by the NCI-60 Human Tumor Cell Lines Screen, which is a part of the National Cancer Institute's Development Therapeutics Program - Drug Synthesis and Chemistry Branch.

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DEDICATION

This work is dedicated to my mother and father, Carol and Kim Jarrett, for their endless encouragement, support, and selflessness to ensure that both their children and grandchildren had every opportunity to find happiness and success. Additionally, I would like to dedicate this work to the founders of the United States of America for their installation of a government which protects and encourages free speech, and ultimately scientific inquiry.

ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to my research mentor, Dr. Abbas G. Shilabin, for his outstanding guidance, patience, and encouragement throughout my undergraduate research; Dr. Shilabin taught me many invaluable lessons which I sincerely believe would have not been attainable in the standard undergraduate experience. Additionally, I would like to thank Dr. Scott Kirkby and Dr. Greg W. Bishop for serving as readers of this thesis, and those in my research group, namely Joel-Annor Gymafi, Joseph Osazee, and Pushpa Reddy for their guidance, support, and humbleness in all aspects.

I would like to thank Dr. Victoria Palau for graciously allowing me to have access to her lab at the James H. Quillen College of Medicine, and allowing me to use the cell lines she had access to for the cell viability tests of our compounds, and Crystal Whitted for instructing me in the maintenance of the cell lines, in addition to teaching me how to perform the MTT Assay. Additionally, I would like to thank the NCI-60 Human Tumor Cell Lines Screen, which is a part of the National Cancer Institute's (NCI) Development Therapeutics Program (DTP)-Drug Synthesis and Chemistry Branch for testing our compounds.

Finally, I wish to express my most sincere gratitude to East Tennessee State University, the Department of Chemistry, the Honors College, the Student-Faculty Collaborative Grant Program, and the Honors-in-Discipline Scholarship Program for allowing the completion of this research to be possible.

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CHAPTER 1

INTRODUCTION

Cancer is one of the most formidable opponents of modern healthcare. It is projected by the American Cancer Society that 39.6% of men and women will develop cancer at some point in their life time.[1] In addition, the Centers for Disease Control states that cancer is the second leading cause of death in the United States, not far behind heart disease (Figure 1).[2] Even when cancer is diagnosed early, the most modern treatments fail to completely cure the patient at times.

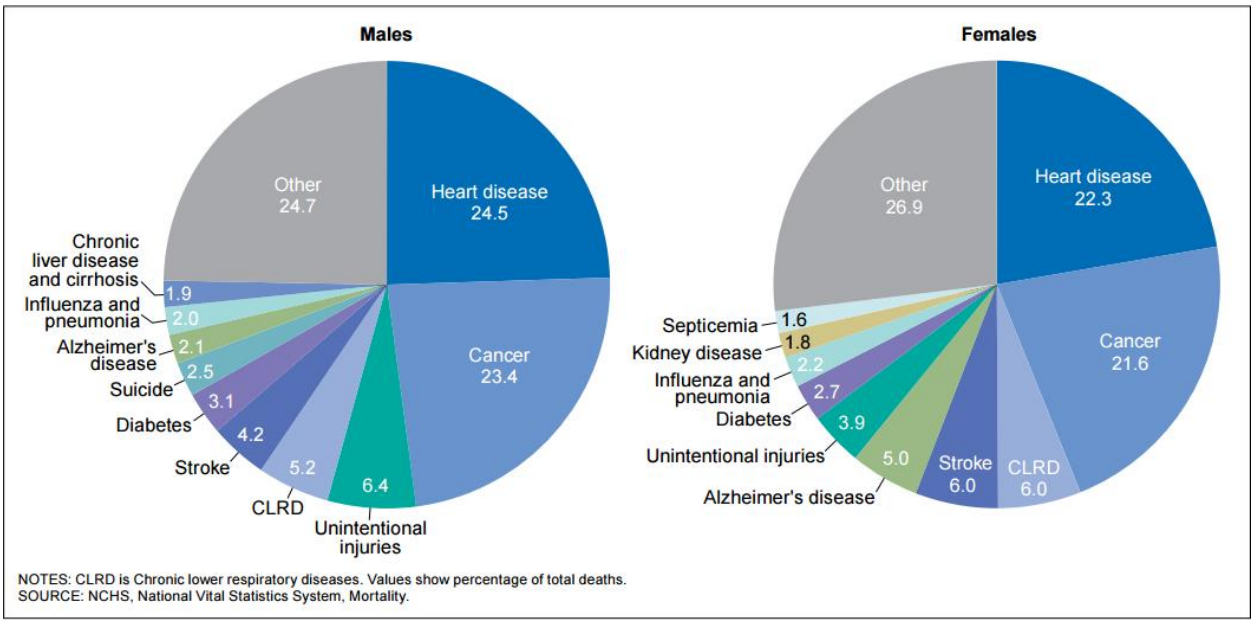


Figure 1: Leading causes of death of both men and women in 2014 as reported by the CDC.[2]

Cancer is a difficult disease to treat because cancers originating in different tissues respond differently to certain treatments; in addition, cancers that developed in the same tissue, but occur in different people, will respond to treatments differently. Therefore, to combine all of these diseases under one name, being “cancer”, can often be misleading by oversimplifying the disease; instead, it’s better to look at the different types of cancer, which are categorized by the

tissue in which they originated in, as a multitude of diseases with one common feature: uncontrolled cell division.[3]

There are three classical modes of cancer treatment: surgical removal, electromagnetic radiation therapy, and chemotherapy. These discrete modes of treatment all have their pros and cons individually; however, they are most often used together in a treatment regimen to have the most efficacious result. Surgical removal was the first method developed to treat tumors, and is often the first choice today; however, not all tumors are accessible by the surgeon, and often the tumors that are removed will grow back over time. Electromagnetic radiation therapy, which was the second method invented for cancer treatment, uses electromagnetic radiation to dismember the DNA of cancer cells, eventually causing cell death; unfortunately, electromagnetic radiation has been shown to cause cancer in healthy tissues as well. Therefore, it is critical that physicians applying this form of treatment are able to target only the cancerous cells directly.

Arguably one of the most pivotal moments for the treatment of cancer was the advent of chemotherapy. Chemotherapy was the first cancer treatment to completely cure metastatic cancer.[4] Chemotherapeutic agents work by interrupting the cell cycle of cells that are actively reproducing. Different chemotherapeutic drugs target cells at different stages of the cell cycle; understanding the mechanisms by which individual chemotherapeutic drugs work helps oncologists to predict which drug combinations would be the most effective.[5] There are a number of different classes of chemotherapeutic drugs that are divided based upon characteristics such as the mechanisms by which they inhibit cancer cell growth, their chemical structure, and their relationship to other drugs. One such group of chemotherapeutic drugs is alkylating agents, which directly damages the DNA of a cell to keep it from reproducing; these drugs may damage the DNA in all phases of the cell cycle, and are used to treat many different forms of cancer.[6]

Pyrrolobenzodiazepines

Pyrrolobenzodiazepines (PBDs) are a group of naturally occurring compounds that were first discovered in the cultures of *Streptomyces*. The first PBD molecule to be isolated and characterized was anthramycin; since then, a number of naturally occurring PBDs have been isolated from *Streptomyces*, which include sibiromycin and tomaymycin, among others.[7] Similar to other naturally occurring antibiotics used in medicine, PBDs were developed by *Streptomyces* as a means of chemical defense against invading microorganisms; however, scientists have been able to use their antibiotic properties for the treatment of cancer.

To date, thirteen natural PBD structures have been isolated from *Streptomyces* including, but not limited to, anthramycin, tomaymycin, sibiromycin, chicamycin A, and DC-81 (Figure 2).[8] Of the thirteen PBD products that have been isolated, both anthramycin and sibiromycin were shown to have broad spectrum antitumor activity against transplanted tumors.[7] As a result, these natural products were tested clinically. Anthramycin particularly was found to have significant cytotoxicity against a number of types of cancer, including gastrointestinal, breast cancers, lymphomas and sarcomas without having significantly ill effects towards red blood cells. However, like other antitumor antibiotics, the clinical use of anthramycin has been limited due to its inherent dose-limiting cardiotoxicity.[9]

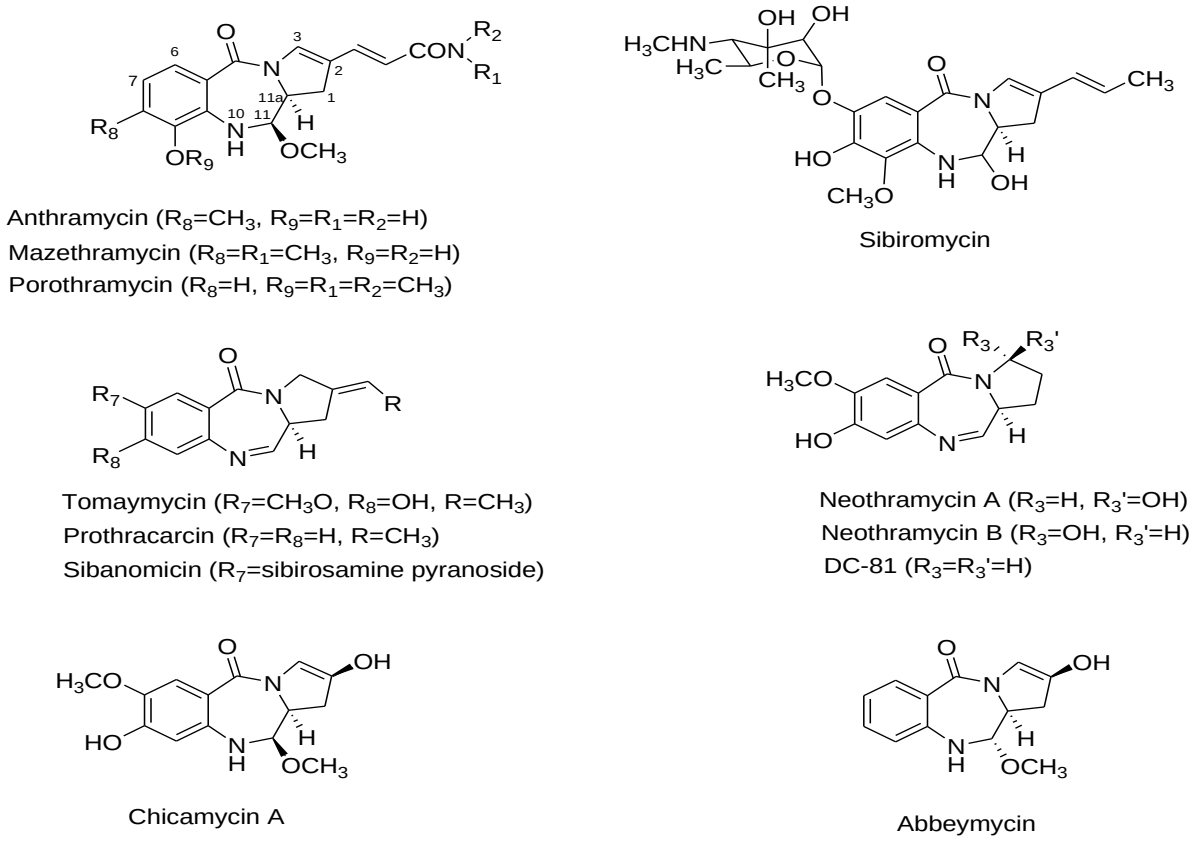


Figure 2: Naturally occurring PBDs that have been isolated from the cultures of *Streptomyces*.[8]

Pyrrolobenzodiazepines are characterized by their reoccurring tricyclic structure, which is composed of an aromatic A-ring, a 1-4-diazepin-5-one B-ring, and a pyrrolidine C-ring (Figure 3). The PBD compounds differ from one another by the location and type of substituent groups on all three rings; however, in every case, carbon C11 is electrophilic.[10] Additionally, the C11a carbon has an S-configuration, which gives the molecules a right-handed twist when viewed from the C-ring towards the A-ring.

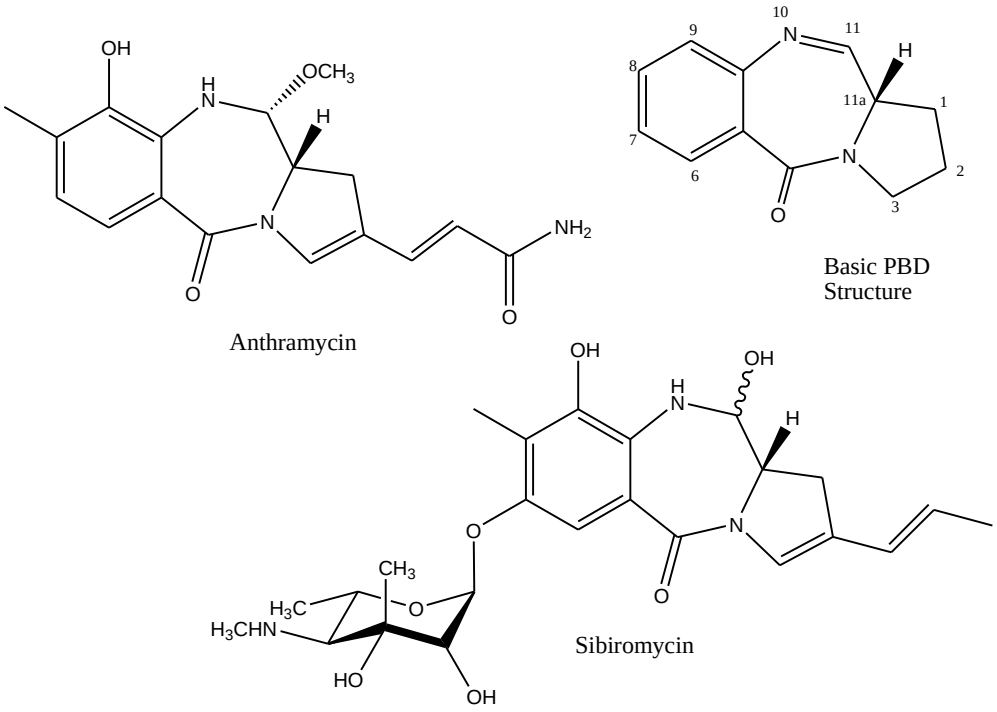
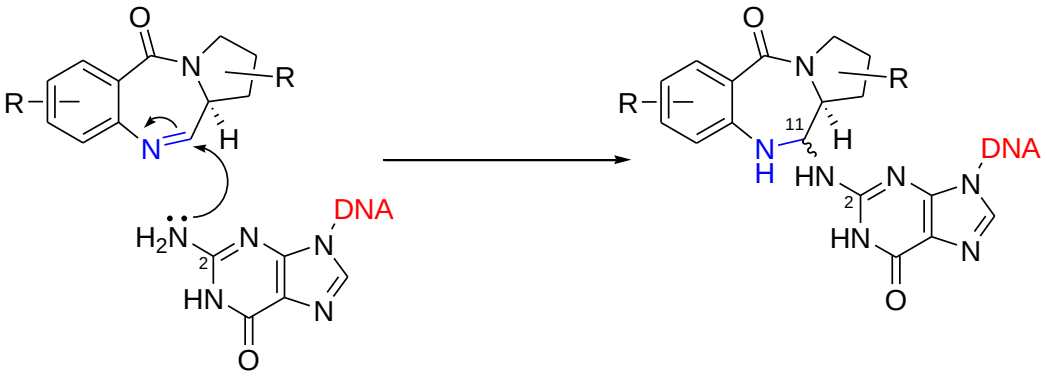


Figure 3: The core structure of PBDs; naturally occurring cytotoxic PBDs.

When considering the interaction between PBDs and DNA, the study of DNA's helical structure is vital. Double-helical B-DNA is composed of two alternating parts: the major and minor groove.[10] The major groove is wider and deeper in comparison to the minor groove, making it more accessible to interacting molecules; in contrast, the minor groove is more shallow and narrow, causing its accessibility to be more selective. Additionally, the major grooves contain the methyl group of thymine which assists the association of the major groove with proteins. Each groove contains very specific base pair arrangements, with each base containing hydrogen bond donors and acceptors. The differences between the donor and acceptor groups of the base pairs, in addition to the size and spacing of the major and minor grooves, opens up the possibility of selectivity for drugs.

Pyrrolobenzodiazepines are cytotoxic compounds that behave as alkylating agents, forming covalent bonds with DNA. As noted previously, alkylating agents may damage DNA at

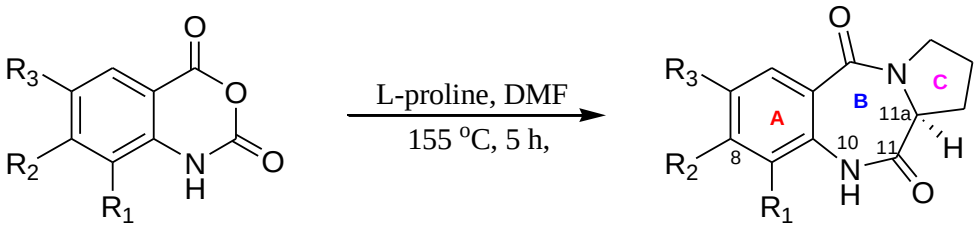
any stage of the cell cycle, and are used to treat many forms of cancer. Pyrrolobenzodiazepines exhibit an electrophilic carbon atom at the C11 position, which allows the PBDs to alkylate the nucleophilic -NH_2 group of guanine bases in the minor groove of DNA, forming an aminal bond (Scheme 1).[11] The covalent bonds that the PBDs form to the amino group of the guanine base allows the molecules to act as an adduct that blocks biological processes such as transcription and RNA polymerase progression.[12]



Scheme 1: Proposed mechanism of aminal bond formation between PBD and N^2 of guanine.[11]

As noted before, PBDs have an S-configuration at carbon C11a which gives the molecules a right-handed twist. This right-handed twist differentiates PBDs from other families of DNA-alkylating agents in that it gives the PBDs the ideal three-dimensional shape which allows them to fit snugly within the DNA double helix, enabling them to interact with the minor groove. The ability of PBDs to interact with the minor groove of DNA is essential. In fact, a synthetic PBD with R-configuration at C11a was shown to lack DNA binding affinity and in vitro cytotoxicity.[13] The minor groove is vulnerable precisely because it is normally unoccupied, due to the fact that the majority of DNA interacting molecules, such as proteins, only interact with the major groove.

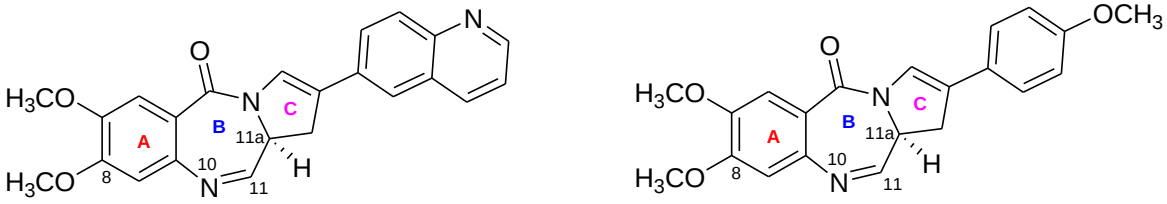
After the detection and elucidation of anthramycin's structure in 1965, its first total synthesis was reported three years later. Since the discoveries of natural PBDs that have a significant amount of cytotoxicity, chemists have synthesized many analogues with hopes of discovering new lead compounds with potent anti-proliferative activities and specific sequence recognition. [10,11] The goal of these syntheses is to introduce new substituents to the basic structure, and to ultimately enhance the cytotoxicity of the PBD molecules. An example of the synthesis of the basic structure of a PBD is shown below as Scheme 2:



Scheme 2: An example of a synthetic route of the synthesis of the core PBD structure.[14]

All natural PBDs that have been isolated from *Streptomyces* cultures are classified today as PBD monomers. Their classification as monomers is not strictly due to their existence in nature, but rather that all of the natural PBDs separated from *Streptomyces* cultures contain only one unit of the core PBD structure per molecule. Naturally, focus on the synthesis and enhancement of PBD monomers came first due to their reoccurring existence in the cell cultures of *Streptomyces*. Among the natural products, the PBDs which contained *endo-exo* unsaturation at the C2 position are the most potent; additionally, research has shown that PBD monomers produced synthetically that contain C2-unsaturation (C-ring) have enhanced *in vitro* potency.[15] More than 80 C2-aryl-substituents have been synthesized, with carbocyclic and heterocyclic C2-aryl substituents ranging from single aryl rings to fused rings systems, which has established the molecular requirements of the C-ring for *in vitro* cytotoxicity.[11] Due to the success of these

structures *in vitro*, two of them were tested *in vivo*, namely SG2738 and SG2042. SG2738 was tested on a HCT-116 human colon xenograft, but only produced a modest delay in the colon tumor growth; SG2042 was tested in six human tumor xenografts, with some antitumor activity in renal models and breast cancer models.[11,16] The structures of SG2738 and DRH417/SG2042 are shown below (Figure 4):



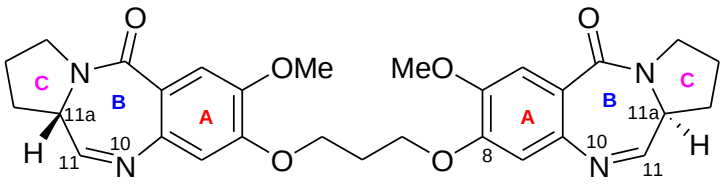
SG2738 DRH417/SG2042

Figure 4: The structures of two cytotoxic synthetic PBD monomers, SG2738 and DRH417/SG2042.

The second class of the synthetic PBDs are PBD dimers. These synthetic molecules are named dimers because they contain two complete units of the core PBD structure (two monomers). The concept behind synthesizing PBD dimers was to generate molecules which contained the PBD antitumor characteristics, but also to span greater length of DNA; with the increased length of the molecules and the active constituents of the core PBD structure on both ends, the dimers were predicted to form cross-links between the DNA strands. Cross-links such as those proposed to be formed by PBD dimers have the ability to seal the interweaving strands of DNA together, thus preventing the process of replication and ultimately cell growth. DNA cross-linking agents such platinum drugs and nitrogen mustards are widely used in modern chemotherapeutic regimens.[17]

Near the end of the 20th century, a PBD dimer known as DSB-120 was formed by linking two DC-81 monomer PBDs through their aromatic A-ring phenol C8-positions.[10] In comparison

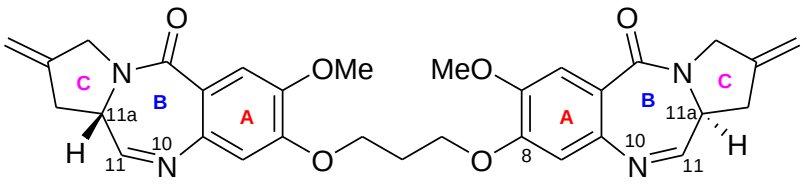
to its PBD monomer DC-81, the dimer revealed an increased DNA binding affinity. The dimers improvement in biological activity has been attributed to its irreversible interstrand cross-linking ability on DNA due to the presence of its two active sites. DSB-120 was shown to be highly cytotoxic *in vitro*; however, the potential drug's *in vivo* studies were not promising.[17] The structure of DSB-120 is shown below (Figure 5):



DSB-120

Figure 5: The structure of DSB-120, a PBD dimer which showed enhanced DNA binding affinity as compared to its monomer analogue.

Due to the success of DSB-120 *in vitro*, a second category of PBD dimers were synthesized, being modeled after the structure of the natural product tomaymycin, which contains unsaturation at the C2 position. As previously noted, all of the natural products that contain unsaturation at the C2 position were shown to have the highest amount of cytotoxicity *in vitro*. Due to the efforts to synthesize PBDs which shared the characteristics of the relatively successful natural PBD products, a dimer by the name of SJG-136 was synthesized and was shown to be significantly more cytotoxic than DSB-120, *in vitro* (Figure 6).[18] As with DSB-120, the increased cytotoxicity of SJG-136 is attributed to its enhanced ability to form DNA crosslinks due two active sites. As a result of impressive *in vitro* studies, SJG-136 was tested thoroughly *in vivo* against 10 human tumor xenografts, including melanomas, breast cancer, colon cancer, and lung cancer. The SJG-136 compound shown to be active (tumor mass reduction) against all 10 models.[19]



SJG136

Figure 6: The structure of the highly successful PBD SJG 136.[7]

Due to the impressive and extensive data collected regarding SJG-136's activity both *in vitro* and *in* vivo, the potential drug entered clinical Phase I testing against advanced solid tumors in 2004.[7] During Phase I of the clinical trials, different dosing schedules were tested to determine the best mode of dosing for the drugs. During the most effective dosing schedule, SJG-136 had promising results. For instance, a patient with malignant melanoma achieved stable disease while being treated with SJG-136; also, a patient with leiomyosarcoma had stable disease for 18 weeks.[7] In addition, a patient with ovarian carcinoma achieved a 62% decrease in tumor size, lasting for eight months. Due to the success of SJG-136 during Phase I clinical trials, the compound has recently progressed to Phase II.

The success of the particular PBD dimer SJG-136, currently in Phase II clinical trials, has once again ignited interest in developing new PBDs. However, the syntheses of PBD dimers are generally lengthier and more difficult than the syntheses of PBD monomers, which has limited the scope of structural diversification of the dimers.[11] Therefore, there has been an increasing interest in progressing PBD monomers to clinical trials. There are still structural areas of the PBD monomers left uncovered by scientists. Most research as gone into the development and characterization of different substituents on the C-ring of the monomers. However, there is still much research to be done on the relationship between chemical substituents and cytotoxic activity in regards to both the A and B rings.

CHAPTER 2

EXPERIMENTAL

Materials & Methods

All reactions proceeded from the commercially available L-proline and isatoic anhydride. Unless otherwise stated, all reagents and solvents were obtained from commercial vendors and utilized without further purification. ^{1}H-NMR, ^{13}C-NMR, GC-MS and IR spectroscopy were the techniques used for the characterization of the synthesized compounds. A JEOL-NMR Eclipse-400 MHz spectrophotometer provided all NMR data and spectra. The high frequency position conversion gave the different chemical shifts of all peaks in parts per million (ppm) with the coupling constants value (J) reported in Hz. The splitting patterns of resonance were also described as follows: singlet (s), doublet (d), doublet of doublet (dd), doublet of doublet of doublet (ddd), triplet (t), quartet (q), and multiplet (m). A Mattson Genesis II FT-IR spectrometer was also used for all IR spectra. Cambridge Melt-Temp device provided melting point readings, but without correction for the synthesized compounds. A Shimadzu GC-MS 2010 System provided the relative abundances of the smaller ion fragments from the molecular ion of all compounds. Other common chromatographic techniques such as thin layer chromatography and column chromatography were also employed in the purification of all synthesized compounds. Compounds **5** and **6** were synthesized according to previously reported methods.[30c]

Experimental Procedures

(S)-1,2,3,11a-tetrahydro-5H-benzo[e]pyrrolo[1,2-a][1,4]diazepine-5,11(10H)-dione (**1**)

Dilactam (**1**) was synthesized according to the previously reported methods with some modifications.[14] A suspension of isotaic anhydride (10.0 g, 61.34 mmol) and L-proline (7.06 g, 61.34 mmol) in N,N-dimethylformamide (DMF) (50 mL) was heated to 155 ˚C for 5 h. After cooling to room temperature, the solvent was removed in vacuo and the residue was taken up in

water. The precipitate was collected and dried to give an off-white solid. Recrystallization from acetone/DMF (10:1 v/v) afforded 10.87 g (82%) of pure Compound **1** as off-white crystals. Yield: 82.0%; m.p: 223-225 ^{o}C; ; $[\boldsymbol{\alpha}]_{\boldsymbol{D}}^{\mathbf{25}}$ = + 512^{o} (c 0.5, MeOH); ^{1}H-NMR (400 MHz, DMSO-d6): δ = 1.80 (d, J = 9.9 Hz, 1H), 1.85-2.07 (m, 2H), 3.39-3.52 (m, 1H), 3.51-3.67 (m, 1H), 4.02-4.21 (m, 1H), 7.07-7.17 (m, 1H), 7.17-7.27 (m, 1H), 7.51 (ddd, J = 8.6, 6.8, 1.3 Hz, 1H), 7.78 (dd, J = 7.9, 1.6 Hz, 1H), 10.52 (s, 1H); ^{13}C-NMR (100 MHz, DMSO-d6): δ = 23.6 (C-2), 26.3 (C-1), 47.4 (C-3), 56.8 (C-11a), 121.8, 124.4, 127.1, 130.8, 132.6, 136.9, 165.0 (CO), 171.3 (CO); IR (KBr); 3222 (N-H), 3206, 2955, 2918, 2850, 1691 (C=O), 1680 (C=O), 1621, 1551, 1536, 1479, 1443, 1412, 1385, 1285, 1259, 1179, 759, 701, 615; GC-MS (70 eV) *m/z* (%): 216 (10) [M+], 119 (14), 92 (20), 70 (100), 64 (10).

(S)-11-thioxo-1,2,3,10,11,11a-hexahydro-5H-benzo[e]pyrrolo[1,2-a][1,4]diazepin-5-one (**2**)

A mixture of Compound **1** (21.60 g, 100 mmol) and Lawesson's reagent (20.2 g, 50 mmol) in THF (400 mL) was stirred over night at room temperature. Evaporation of solvent in vacuo gave a crude solid yellow residue. The solid was washed with toluene and further washed with cold toluene to afford 20.2 g (87%) of pure Compound **2** as yellow solid.[14,23] Yield: 87.0%; m.p.: 272–274 ^{o}C ; $[\alpha]_{D}^{25}$ = + 762 ^{o} (*c* 0.5, $CHCl_3$); ^{1}H-NMR (400 MHz, DMSO-d6): δ = 1.83–1.98 (m, 1H), 1.98–2.15 (m, 2H), 2.88 (d, *J* = 5.9 Hz, 1H), 3.31–3.51 (m, 3H), 3.53–3.60 (m, 1H), 4.28 (d, *J* = 6.2 Hz, 1H), 7.29 (d, *J* = 8.1 Hz, 1H), 7.32–7.39 (m, 1H), 7.58 (td, *J* = 7.7, 1.2 Hz, 1H), 7.83 (dd, *J* = 7.7, 1.5 Hz, 1H); ^{13}C-NMR (100 MHz, DMSO-d_6): δ = 23.2 (C-2), 29.5 (C-1), 47.4 (C-3), 60.3 (C-11a), 122.3, 126.2, 128.3, 130.8, 132.7, 137.0, 164.7 (CO), 202.5 (CS); IR (KBr): 3125 (N-H), 3094, 3063, 3024, 2974, 1620 (C=O), 1579, 1523, 1478, 1452,

1418, 1381, 1272, 1193, 1166, 1145, 1103, 1069, 1055, 887, 833, 817, 786, 755, 695, 664, 625; GC-MS (70 eV) *m/z* (%): 232 (7) [M^+], 108 (6), 70 (100), 68 (6).

(S,E)-11-hydrazono-1,2,3,10,11,11a-hexahydro-5H-benzo[e]pyrrolo[1,2-a][1,4]diazepin-5-one (**7**)

A solution of Compound **2** (1.16 g, 5.0 mmol) in ethanol (20 mL) was added 98% hydrazine monohydrate (0.75 g, 15.0 mmol) and stirred for 15 h at room temperature. The solvent was removed in vacuo and the residue was taken up in water. The precipitate was collected, dried and washed with diethyl ether to afford 1.14 g (99%) of Compound **7** as off-white solid.[27] Yield: 99.0%; m.p: 178–180 °C; $[\boldsymbol{\alpha}]_{\boldsymbol{D}}^{\mathbf{25}}$ = + 552 ° (*c* 0.5, MeOH); ^{1}H-NMR (400 MHz, $CDCl_3$): δ = 1.61–2.08 (m, 4H), 2.65–2.82 (m, 1H), 3.57–3.69 (m, 1H), 3.71–3.84 (m, 1H), 4.17–4.30 (m, 1H), 6.85 (d, *J* = 8.1 Hz, 1H), 7.09 (t, *J* = 7.7 Hz, 1H), 7.32–7.40 (m, 1H), 7.90 (dd, *J* = 7.9, 1.3 Hz, 1H); ^{13}C-NMR (100 MHz, $CDCl_3$): δ = 23.4 (C-2), 26.1 (C-1), 47.3 (C-3), 55.5 (C-11a), 119.7, 123.0, 125.4, 131.5, 132.5, 137.8, 152.2, 166.2 (CO); IR (KBr): 3484, 3282, 3240, 3161, 2967, 2949,2871, 2814, 2422, 2358, 2336, 1957, 1854, 1784, 1766, 1726, 1689, 1658, 1619, 1563, 1539, 1529, 1479, 1442, 1384, 1323, 1272, 1202, 1076, 943, 903, 825.

(S)-11,12,13,13a-tetrahydro-9H-benzo[e]pyrrolo[1,2-a][1,2,4]triazolo[3,4-c][1,4]diazepine-3,9(2H)-dione (**8**)

A solution of Compound **7** (0.69 g, 3.0 mmol) and 1,1-carbonyldiimidazole (3.89 g, 24.0 mmol) in dioxane (25 mL) was refluxed for 24 hours. The solvent was removed in vacuo and the crude product was purified by flash gel column chromatography using gradient elution. The ratios of solvent mixture used were EtOAc/Hexane (1:1, 2:1, 4:1 v/v) and a final elution with only Ethyl acetate (EtOAc). Recrystallization from EtOAc/Hexane (4:1 v/v) afforded 0.76 g

(99%) of pure Compound **8** as white crystals. Yield: 99.0%; m.p: 238–240 °C; $[\boldsymbol{\alpha}]_{\boldsymbol{D}}^{\mathbf{25}}$ = + 136 ° (*c* = 0.5, DMSO); ^{1}H-NMR (400 MHz, $CDCl_3$): δ = 1.99–2.22 (m, 2H), 2.23–2.37 (m, 1H), 2.86 (ddd, *J* = 16.3, 6.8, 3.7 Hz, 1H), 3.64–3.75 (m, 1H), 3.81–3.92 (m, 1H), 4.53 (dd, *J* = 8.4, 3.3 Hz, 1H), 7.42–7.51 (m, 1H), 7.59–7.67 (m, 1H), 7.90–7.98 (m, 1H), 8.03 (dd, *J* = 7.9, 1.6 Hz, 1H), 9.90 (s, 1H); ^{13}C-NMR (100 MHz, $CDCl_3$): δ = 23.5 (C-2), 26.0 (C-1), 47.7 (C-3), 51.7 (C-11a), 123.0, 127.8, 129.0, 130.1, 131.9, 132.3, 153.9 (CO), 164.7 (CO); IR (KBr): 3435, 3375, 3183, 3091, 3000, 2909, 2878, 2814, 2326, 1711, 1611, 1574, 1490, 1466, 1412, 1343, 1285, 1239, 1202, 1167, 1118, 1072, 1024, 979, 926, 912, 825, 786, 758, 709, 656, 616; GC-MS (70 eV) *m/z* (%): 256 (60) [M^+], 102 (26), 90 (38), 43 (100).

(13aS)-3-mercapto-11,12,13,13a-tetrahydro-9H-benzo[e]pyrrolo[1,2-a][1,2,4]triazolo[3,4 -c][1,4]diazepin-9-one (**9**)

A solution of Compound **7** (0.23 g, 1.0 mmol) and 1,1-thiocarbonyldiimidazole (1.42 g, 8.0 mmol) in dioxane (20 mL) was refluxed for 24 h. The solvent was removed in vacuo and the crude product was purified by flash gel column chromatography using gradient elution. The ratios of solvent mixture used were Acetone/Hexane (10:1, 20:1 v/v). Recrystallization from EtOAc/Hexane (4:1 v/v) afforded 0.217 g (80%) of pure Compound **9** as brown crystals. Yield: 80.0%; m.p: >230 °C (dec.); $[\alpha]_{D}^{25}$ = + 37.5 ° (*c* 0.4, $CHCl_3$); ^{1}H-NMR (400 MHz, $CDCl_3$): δ = 1.62–1.99 (m, 1H), 2.03–2.28 (m, 2H), 2.29–2.45 (m, 1H), 2.89 (tt, *J* = 9.9, 3.4 Hz, 1H), 3.63–3.78 (m, 1H), 3.81–3.97 (m, 1H), 4.55 (dd, *J* = 8.4, 2.9 Hz, 1H), 7.50–7.60 (m, 1H), 7.63–7.77 (m, 1H), 8.04 (dd, *J* = 7.7, 1.5 Hz, 1H), 8.39 (d, *J* = 8.1 Hz, 1H); ^{13}C-NMR (100 MHz, DMSO-d_6): δ = 23.6 (C-2), 26.7 (C-1), 47.5 (C-3), 51.6 (C-11a), 126.2, 129.1, 130.6,

130.8, 131.2, 131.4, 153.1, 164.0 (CO), 167.7 (CS); IR (KBr): 3735, 3280, 3174, 3127, 3096, 3060, 2989, 2944, 2880, 2827, 2415, 2359, 2331, 1958, 1760, 1723, 1686, 1611, 1542, 1488, 1410, 1344, 1310, 1286, 1216, 1112, 1043, 1003, 973, 921, 892, 876, 825, 756, 704, 664, 638, 622, 610; GC-MS (70 eV) *m/z* (%): 272 (100) [M^+], 116 (23), 90 (34), 70 (33).

5,6-Dihydro-4H-3-thia-6a,11b-diazabenzo[g]cyclopenta[e]azulene-1,7-dione (**13**)

To a solution of Compound **2** (0.464 g, 2.0 mmol) in anhydrous THF (40 mL) was added freshly distilled α-bromoacetyl chloride (0.39 g, 2.4 mmol). The mixture was stirred for 15 h at room temperature under nitrogen and then quenched by addition of saturated sodium bicarbonate ($NaHCO_3$) (20 mL). After extraction with chloroform (2 × 20 mL), the combined organic layers were dried over Na_2SO_4, and the solvent was removed under reduced pressure. The crude residue was subjected to flash silica gel column chromatography using EtOAc/Hexane (4:1 v/v) as eluent and the solvent was evaporated under reduced pressure to give crude yellow solids.[14] The crude solid was purified by recrystallization from Ethanol/Water mixture to afford 0.20 g (75%) of pure Compound **13** as yellow needle-like crystals. Yield: 75.0%; m.p: 165–167 ^oC; ^1H-NMR (400 MHz, $CDCl_3$): δ = 1.98–2.05 (m, 2H), 2.67 (t, *J* = 8.0 Hz, 2H), 3.83 (s, 2H,), 3.90–3.94 (m, 2H,), 7.28–7.32 (m, 1H), 7.44 (dd, *J* = 8.2, 1.0 Hz, 1H), 7.51–7.55 (m, 1H), 8.00 (dd, *J* = 8.0, 1.6 Hz, 1H); ^{13}C-NMR (100 MHz, $CDCl_3$): δ = 20.8 (C-5), 31.1 (C-4), 35.8 (C-2), 50.1 (C-6), 115.5, 124.3, 124.9, 127.2, 128.5, 133.3, 133.6, 138.9, 165.4 (CO), 172.7 (CO); IR (KBr): 3398, 3357, 3282, 2944, 2880, 2790, 2419, 2385, 2350, 2323, 1856, 1818, 1779, 1724, 1690, 1582, 1482, 1404, 1384, 1358, 1334, 1217, 1126, 1092, 871, 835, 776, 708, 659, 636.

X-ray Crystallography

A large colorless prism was cut (0.10 x 0.15 x 0.23 mm^3) and centered on the goniometer of a Rigaku Oxford Diffraction Gemini E diffractometer operating with MoKα radiation. The data collection routine, unit cell refinement, and data processing were carried out with the program CrysAlisPro[25]. The Laue symmetry and systematic absences were consistent with the monoclinic space groups *I*2 and *I*2/*m*. As the sample was known to be enantiomerically pure, the acentric space group, *I*2, was chosen to give *Z*=4 and *Z*'=1. The absolute configuration could not be determined because of anomalous dispersion effects. The structure was solved using SHELXS-2014[26] and refined using SHELXL-2014[26] via Olex2[27]. The final refinement model involved anisotropic displacement parameters for non-hydrogen atoms and a riding model for all hydrogen atoms.

Cell Culture Maintenance

The cells lines that originated in multiple tissue types were obtained were obtained from the American Tissue Type Culture Collection (ATCC), including: breast (SK-BR-3, MCF-7), colon (Caco-2, HCT 116), melanoma (SKMEL-2), and pancreatic (Mia Paca) cell lines. The tumor cell lines were grown in their respective medium according to ATCC instructions. Each medium was supplemented with 10% serum and penicillin/streptomycin. Cell lines were allowed to reach 75% confluency before treatment with the novel pyrrolobenzodiazepines or negative control (DMSO at a final maximum concentration of 0.01%).

The cell lines obtained from the ATCC were stored in liquid nitrogen until ready to use. Once the compounds were synthesized and ready to be tested, each cell line was allowed to thaw at room temperature, respectively. They were then transferred to a centrifuge tube, diluted by 5 mL of their respective media (listed on the ATTC website), and centrifuged for 3 minutes. The

supernatant fluid was then removed, and the pellet was re-suspended in 10 mL of its media, and mixed thoroughly using a pipette. Finally, the solution was transferred to a 25-cm^2 vented flask, and placed in an incubator. The incubator maintained an atmosphere of 37 ^oC and 5% carbon dioxide.

The media inside the culture flask was exchanged with fresh media every few days in order to remove dead cells and provide fresh nutrients. The cells were allowed to grow until they reached 75% confluency. Once cells were 75% confluent, the media was removed from the flask and 1.5 mL of trypsin was added in order to lift the cells from the flask. The solution of cells was then transferred to a centrifuge tube, and diluted with 5 mL of media. Again, the solution was centrifuged for 3 minutes until all of the cells formed a pellet in the bottom of the tube. The supernatant fluid was then removed and replaced with 10 mL of media, and the pellet was mixed well into solution.

MTT Assay

The first method used to test the in vitro cytotoxicity of our compounds was the MTT Assay. For our tests, we used 48-well plates. 1 mL of each cell line's respective media was placed into each well of the 48-well plates, and 1 drop of the final solution of the cells listed in the previous section were be added to each well and allowed to grow to 75 % confluency (covering 75% of the bottom of the well). Next, our products were added to the wells at a concentration of 100 µM, except for four wells in which DMSO was added so that the final concentration was 0.01% (negative control). Once the PBD products were added in triplicate, the plates were incubated for 48 hours.

After 48 hours of incubation, 5-diphenyl-tetrazolium bromide (MTT) was added at 100 µg/well and will be incubated for 3-4 hours. After 3-4 hours of incubation, the supernatant fluid

was removed and 0.1 M HCl in isopropanol was added to each well to dissolve the resulting formazan crystals. The crystals were dissolved into solution well using a transfer pipette. The optical density of the resulting solution was measured at 570 nm. The optical density of the solution of the formazan crystals is directly correlated to the remaining number of viable cells in solution. Percent cell viability was calculated by comparing the concentration of formazan crystals formed in the negative control to the formazan crystals formed by the sample in which our PBD products are added. The cell viabilities of the negative controls were taken to be 100%.

NCI-60 Human Tumor Cell Lines Screen

NCI-60 Human Tumor Cell Lines Screen is a part of the National Cancer Institute's (NCI) Development Therapeutics Program (DTP)-Drug Synthesis and Chemistry Branch. The screening utilizes 60 different human tumor cell lines to identify and characterize novel compounds with growth inhibition or cell death. The 60 human tumor cell lines represent leukemia, melanoma, and cancers of the lung, colon, brain, ovary, breast, prostate, and kidneys. The NCI-60 screening is free of cost to those who submit compounds; however, compounds submitted are reviewed and only those that meet the guidelines set by the NCI are selected for screening.

Compounds that selected for the NCI-60 cell screening are initially dissolved in DMSO:glycerol 9:1 and tested at a single concentration of 10 μM. The One-dose data is reported in graphical form as a mean of the percent growth of treated cells (Figure 7). The value reported for the One-dose assay of the individual compounds is cell growth relative to the no-drug control in addition to the initial number of tumor cells present, which allows for the detection of both growth inhibition (values between 0 and 100) and tumor cell death (values less than 0).

Compounds that satisfy predetermined thresholds for the One-dose assay set by the NCI are tested by a Five-dose assay.

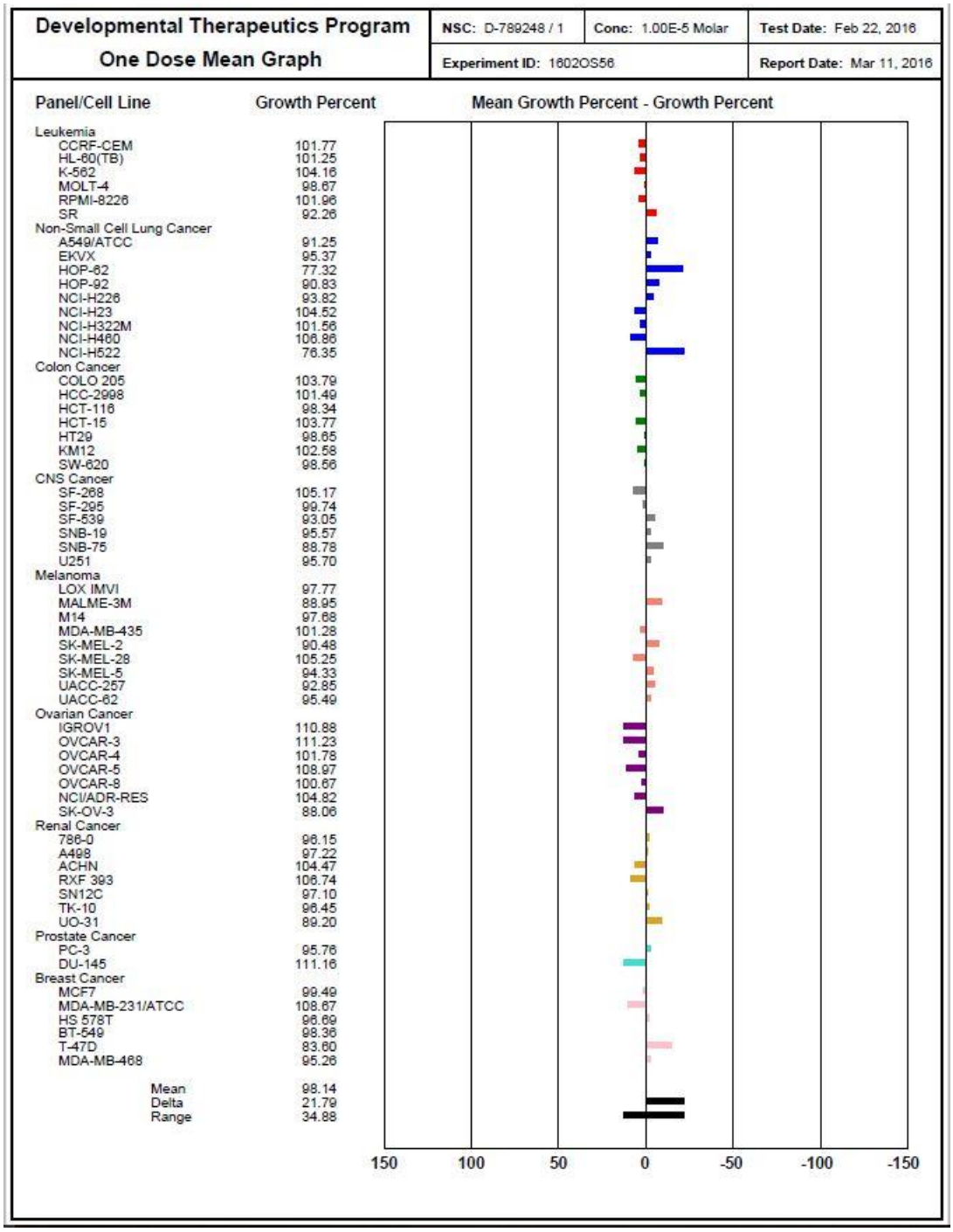


Figure 7: The NCI-60 Human Tumor Cell Lines Screen for **8**.

CHAPTER 3

RESULTS AND DISCUSSION

Chemistry

Syntheses of Pyrrolo[2,1-c][1,4]benzodiazepine (PBD) Derivatives

The synthesis of all PBD derivatives were accomplished with the readily available core structure of the PBDs (**1**) using L-proline and isatoic anhydride (from Isatis indigotica[20]) as the starting materials. As depicted in the synthesis plan (Scheme 3), the cyclocondensation of an equimolar mixture of L-proline and isatoic anhydride in DMF at 155 ^{o}C by a standard protocol[14,21,22] afforded Compound **1.** The product of this reaction was then recrystallized from a 10:1 v/v mixture of acetone and DMF to obtain an 82% yield of Compound **1** as off white crystals. Thionation of Compound **1** with 0.5 equiv Lawesson's reagent in THF at room temperature also afforded Compound **2** in 87% yield.[23]

Our attempts to synthesize the tetracyclic PBD analogs **3-13** from Compound **2** as shown in Scheme 3 was highly successful. Catalytic amination of Compound **2** using n-propylamine in the presence of mercury (II) chloride as catalyst afforded Compound **3** in an 88% yield after being recrystallized from nitromethane. The cyclization of Compound **3** with bis (2,4,6-trichlorophenyl)-2-phenylmalonate at 190 ^{o}C using a zincke apparatus to afford the pyrimidine-annulated Compound **4** in 75% yield by a neat reaction approach. Cyclization reactions of N,N'-disubstituted amidines with bis (2,4,6-trichlorophenyl)-2-phenylmalonates have generally been shown to result in the formation of pyridinium-4-olates. One possible mechanistic explanation for the synthesis of these compounds is the loss of two molecules of trichlorophenol through a ketene intermediate during ring closure.[14]

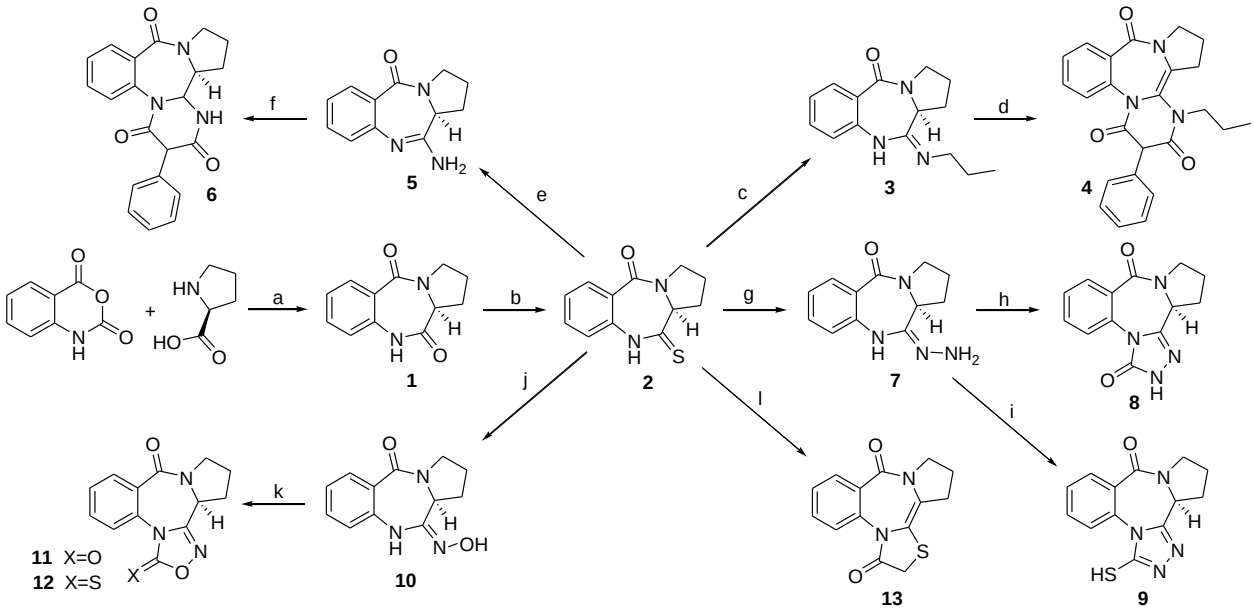
A standard protocol was followed for the treatment of Compound **2** with hydrazine monohydrate in ethanol at room temperature[14] to afford Compound **7** in a quantitative yield (i.e.

99%) which was further used as the main precursor for the syntheses of tetracyclic triazole PBD (**8**) and mercapto triazole PBD (**9**). Carbonylation of **7** with 1,1'-carbonyldiimidazole (CDI) in dioxane under reflux resulted in the formation of Compound **8** and upon further purification by flash gel column chromatography, pure **8** was afforded as white crystals in 99% yield. The loss of the two good leaving imidazole groups on the CDI during ring closure is a possible mechanistic explanation for this synthesis. Compound **8** was unambiguously confirmed by single crystal X-ray analysis.[24-27] Treatment of Compound **7** with thiocarbonyldiimidazole (TDI) in dioxane under reflux afforded Compound **9** and upon further purification by flash gel column chromatography resulted in yellow pure solid of **9**. Recrystallization from methanol afforded yellow crystals in 80% yield. Unlike the carbonylation of **7**, the thionylation of **7** with TDI resulted in the formation of a thiol compound instead of a thionyl compound. The delocalization of electrons onto the large-sized sulphur atom aided the transfer of proton from the nitrogen atom to the sulphur to form the thiol product.

A slight modification of the protocol used in Rekowski et al. and Bartsch et al.[28,29] for the synthesis optimization of Compound 10 was employed. Compound **2** was treated with hydroxylamine hydrochloride under basic conditions. The use of K_2CO_3 as a relatively milder base in place of triethylamine increased the % yield significantly to 94% as compared to 74% reported in the literature.[29] One possible explanation for this observation could be the ability of the K_2CO_3 to effectively neutralize the HCl making the hydroxylamine a very good nucleophile and readily available to attack the electrophilic thionyl carbon in **2**. Recrystallization from nitromethane afforded Compound **10** as yellow needle-like crystals.

Compound **10** was further used as the main precursor for the synthesis of oxadiazole (**11**) and thionyl oxadiazole (**12**) by respective carbonylation and thiocarbonylation reactions which

have been reported in literature.[28,29] A slight modification was made to the reported protocol to optimize the synthesis of **11** and **12** by treating **10** with carbonyldiimidazole and thiocarbonyldiimidazole under reflux in dioxane for 12 hours to afford **11** and **12** in 88% and 90% yield, respectively. According to the literature, both reactions under reflux in THF take 24 hours to form their respective products in 84% yields.



Scheme 3: Reagents and conditions: Reagents and conditions: (a) DMF, 155 °C, 5h, 82.0%; (b) Lawesson's reagent, THF, rt, 15h, 87.0%; (c) n-propylamine, $HgCl_2$, rx, 1h, 88%; (d) bis(2,4,6-trichlorophenyl)-2-phenylmalonate, 190 ^oC, 10 min, 75%; (e) NH_3(g)(anhy.), $HgCl_2$, THF, rx, 1h, 88% (f) bis(2,4,6-trichlorophenyl)-2-phenylmalonate, 210-220 ^oC, 10 min, 63%; (g) $N_2H_4.H_2O$(98%), EtOH(abs.), rt, 15h, 99.0%; (h) CDI, dioxane (anhy.), reflux, 24h, 99.0%; (i) TDI, dioxane (anhy.), rx, 24h, 80.0%; (j) $NH_2OH.HCl$, K_2CO_3, ethanol, rt, 24h, 82%; (k) CDI, dioxane, rx, 12h, 88% / TDI, dioxane, rx, 12h, 90%; (l) α-bromoacetyl chloride, THF, rt, under N_2(g), 15h, 75%.
CDI: 1,1'-carbonyldiimidazole, TDI: 1,1'-thiocarbonyldiimidazole

X-ray Structural Analysis

Slow evaporation of a saturated solution of Compound **8** in ethyl acetate/hexane (4:1 v/v) afforded colorless single crystals with an orthorhombic unit cell. The ORTEP molecular structure and the crystallographic numbering are shown in Figure 8. The crystal strucutre in the

elemental cell features intermolecular hydrogen bonds to the three units of molecules at H3A···C1=O1, H3B···C10=O2, and O3···N4–H4A, respectively. A twisted helical conformation of the molecular structure can be visualized directly from the 6:7:5 pyrrolobenzodiazepine ring system while the 7-membered ring adopting a boat arrangement. This finding is in agreement with the dihedral angles of N(2)-C(8)-C(9)-N(1) and N(1)-C(1)-C(2)-C(7) which were determined to be 58.5(2)^{o} and 38.6(2)^{o}, respectively. The boat conformation was further confirmed by the torsion angles of -6.0(2)^{o} for C(9)-N(1)-C(1)-C(2) and -2.5(2)^{o} for C(1)-C(2)-C(7)-N(2). The bond distance of C(8)-N(3) was determined to be 128.8 pm and corresponds to the imino C(sp^2)=N(sp^2) double bond. In addition, the bond length N(2)-C(8) was measured to be a single bond with a distance of 136.9 pm which confirms the construction of 1,2,4-triazol-3(4H)-one annulated moiety. It's interesting to note that the presence of a distinct C(8)-C(9) single bond with a distance of 149.96 pm, unambiguously excludes any tautomerization and verifies its parent S-configuration at carbon C11a which gives the molecules a right-handed twist to interact with DNA double helix.

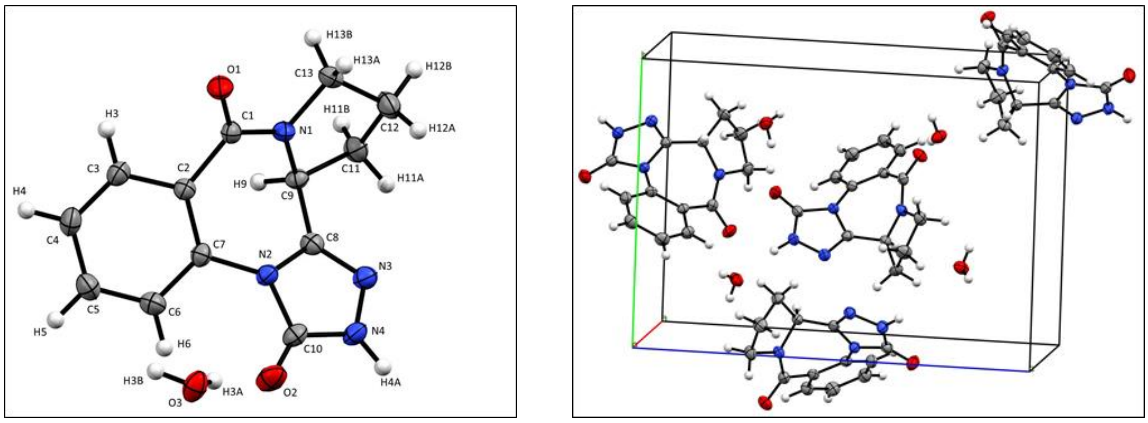


Figure 8: ORTEP projection of the X-ray crystal structure and Packing diagram of Compound **8**.

Biological Activity

MTT Cell Viability Test

To determine whether our PBD compounds were cytotoxic towards cancer cells, the MTT assay was initially used for selected cell lines. The MTT assay is a colorimetric assay that uses 3-(4,5-Dimethylthiazol-2-Yl)-2,5-diphenyltetrazolium bromide (MTT) to assess cell metabolic activity. Based on the previously reported works, the preferred cancer cell lines that were used for the MTT assay were breast (SK-BR-3, MCF-7), colon (CaCo-2, HCT 116), melanoma (SKMEL-2), and pancreatic (Mia Paca) cell lines. The PBD compounds were dosed at 100 µM, and allowed to incubate for 48 hours. The optical densities of the crystals were then measured at 590 nm using a BioTek Synergy HT spectrophotometer.

The results show that nearly all of the novel PBD compounds, except for Compound **13**, reduced the cell viability of MCF-7 cells by roughly up to 27% (Figure 9). In contrast, virtually none of the PBD analogs, with the exception of Compounds **1** and **10**, had an anti-proliferative effect on the SKMEL-2 cell line. Additionally, all compounds that were tested on HCT-116 and Mia Paca showed no cytotoxic effects. Compound **1** showed a decrease in cell viability for the SKBR-3, MCF-7, SKMEL-2, and CaCo 2 cell lines; however, the PBD dilactam showed no cytotoxicity towards HCT 116 or Mia Paca. The greatest amount of cytotoxicity for **1** was a reduction of cell viability by 20.3% and 20.1% for both MCF-7 and SKMEL-2 cell lines, respectively. Compound **2** showed a clear reduction of cell viability for MCF-7 and CaCo 2, while having the greatest amount of anti-proliferative effects on the MCF-7 cell line (17.7%). However, **2** did not noticeably decrease the cell viability of cell lines SKBR-3, SKMEL-2, HCT 116, or Mia Paca. Compound **3** had a relatively low effect on the cell viabilities of MCF-7 (10.4%) and CaCo 2 (7.5%) cells, while had no negative effect on the cell growth of SKBR-3,

Mia Paca, SKMEL 2, and HCT 116 cell lines. Compound **4** had a noticeable effect on the cell viabilities of SKBR-3 (17.3%) and MCF-7 (22.5%). However, **4** had no negative effects on the cell growth of SKMEL-2, CaCo 2, HCT 116, or Mia Paca cell lines. Compound **5** showed the highest growth inhibition against MCF-7 cell line (26.4%) when compared with other tested analogs. **5** had no negative effect on the cell viability of SKBR-3, SKMEL-2, and CaCo 2. Compounds **6** and **8** displayed some reduction in the cell viability of SKMEL-2 with values of 19.9% and 17.0%, respectively. Compounds **10** and **11** showed their highest inhibition activity versus SKMEL-2 breast cell line with 13.0% and 10.5%, respectively. Compound **13** had a selective anti-proliferative effect on CaCo 2 cell line (13.6%).

In vitro Cytotoxicity

In order to validate our preliminary in-house MTT cell viability results and further expand the number of cancer cell lines, nine PBD analogs were selected for the single-dose *in vitro* cytotoxicity using a panel of NCI-60 cell lines as part of Developmental Therapeutics Program (DTP) at the National Cancer Institute. Similar results were obtained in agreement with the MTT data (Table 1). All the compounds were shown to have some non-selective growth inhibition on T-47D breast cell line ranging from 6.7 to 12.7%. Interestingly, Compounds **3** and **4** displayed their highest inhibition on SNB-75 (CNS) (12.3%) and NCI-H522 (NSCL) (12.2%), respectively. The best inhibition activity was exhibited by **6** which exclusively acts on the renal UO-31 cell line with 28.5% growth inhibition.

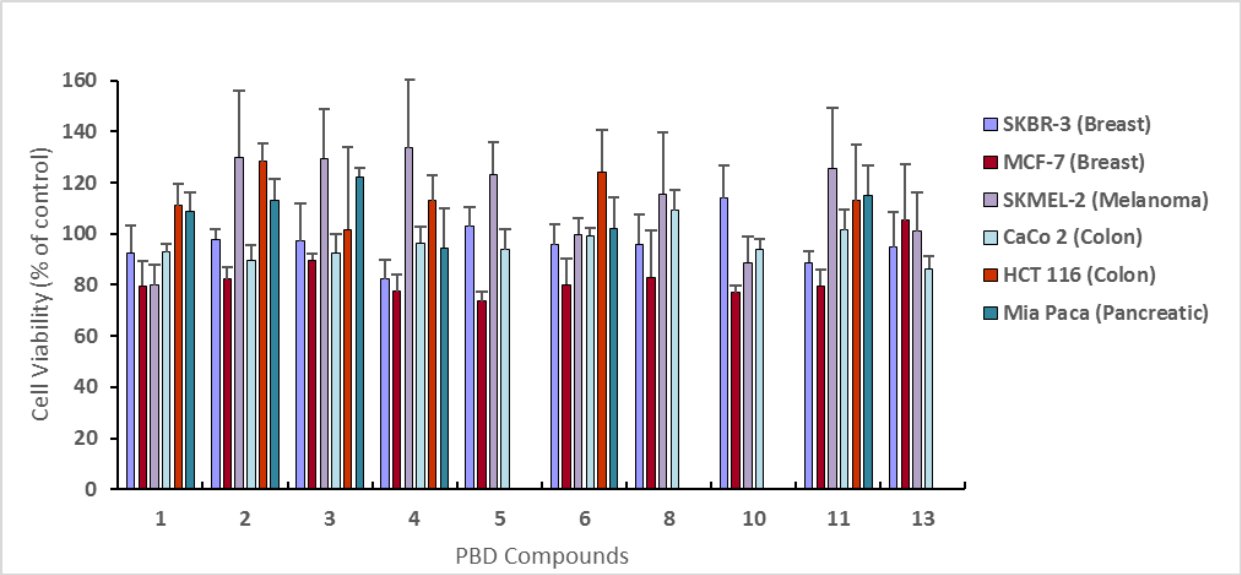


Figure 9: *In vitro* cytotoxicity assay of PBD analogues using the MTT Assay.

Cancer	Cell line	PBD Compounds-Growth Percent								
		3	**4**	**5**	**6**	**8**	**10**	**11**	**12**	**13**
Leukemia	HL-60(TB)	101.38	103.22	103.05	101.26	101.25	90.92	103	94.58	87.83
	SR	102.79	103.91	95.32	103.31	92.26	99.34	103.8	91.96	101.39
NSCL	HOP-62	101.62	89.44	84.60	90.25	77.32	91.39	80.34	92.91	87.54
	NCI-H522	84.29	77.82	83.64	78.63	76.35	79.92	90.49	76.82	81.50
Colon	HCT-116	100.33	96.01	96.12	99.44	98.34	98.34	101.09	95.49	88.64
	HT29	97.57	99.46	101.60	95.26	98.65	94.64	95.04	99.63	91.31
CNS	SNB-75	77.68	87.15	83.05	84.45	88.78	93.52	89.29	90.43	85.08
	U251	98.25	99.23	91.64	98.40	95.70	96.42	96.22	95.79	96.22
Melanoma	SKMEL-2	95.73	91.26	96.42	96.80	90.48	91.71	97.71	82.08	94.75
	MALME-3M	92.15	95.68	97.19	94.85	88.95	95.21	83.85	98.70	100.22
Ovarian	NCI/ADR-RES	97.24	95.76	102.94	95.62	104.82	100.80	99.31	104.39	100.73
	SK-OV-3	103.74	98.55	95.20	106.55	88.06	101.62	88.46	92.95	90.84
Renal	UO-31	85.88	83.90	78.66	71.55	89.20	86.24	88.38	87.04	80.50
	TK-10	92.55	94.15	94.62	90.33	96.45	92.33	95.19	96.05	98.39
Prostate	PC-3	98.28	95.79	89.08	90.37	95.76	95.37	97.71	90.70	88.45
Breast	T-47D	88.30	88.54	89.48	91.74	83.60	92.91	93.26	91.42	87.23
	HS 578T	94.34	98.16	92.43	94.98	96.69	99.98	98.31	96.79	94.58

Table 1: *In vitro* cytotoxicity data of PBD derivatives against a number of NCI-60 cell lines.

CONCLUSIONS

In conclusion, PBD dilactam (**1**) (formed from the cyclocondensation of commercially available isatoic anhydride with L-proline in DMF for 5 hours) was synthesized and used as the core structure for all of the PBD analogous in this project. Compound **2**, which was synthesized via the thionation of dilactam **1**, was used as the starting material for the syntheses of PBDs **3-13**. In order to test the anti-proliferative activity of the PBD products, the MTT assay was first implemented. For the MTT assay, the PBD products were the most effective against the MCF-7; all of the PBDs, except for Compound **13**, reduced the cell viability of MCF-7 cells to roughly 27%. In contrast to MCF-7, virtually none of the PBD analogs, with the exception of compounds **1** and **10**, had an anti-proliferative effect on the SKMEL-2 cell line. Additionally, all compounds that were tested on HCT-116 and Mia Paca showed no cytotoxic effects.

In order to verify our preliminary in-house MTT cell viability results, and to further expand the number of cancer cell lines used to test the PBD compounds, nine PBD analogs were selected for the single-dose *in vitro* cytotoxicity using a panel of NCI-60 cell lines as part of Developmental Therapeutics Program (DTP) at the National Cancer Institu4te. The PBDs produced similar results in the NCI-60 cell lines as compared to the cell lines used for the MTT Assay. The best anti-proliferative activity against the NCI-60 cell lines was exhibited by PBD **6** which exclusively acts on the renal UO-31 cell line with 28.5% growth inhibition.

FUTURE WORK

In order to increase cytotoxicity, unsaturation of the PBDs at the C2 position of the core structure may be included in future projects. Additionally, tetracyclic PBDs may have steric hindrance when entering the minor groove, which would prevent the alkylation of DNA; successful compounds such as anthramycin could be modified to contain a tetracyclic structure in order to determine whether steric hindrance should be considered.

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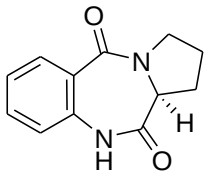
APPENDICES

Appendix A1: ^{1}H-NMR Spectrum for Compound **1** in DMSO-d6



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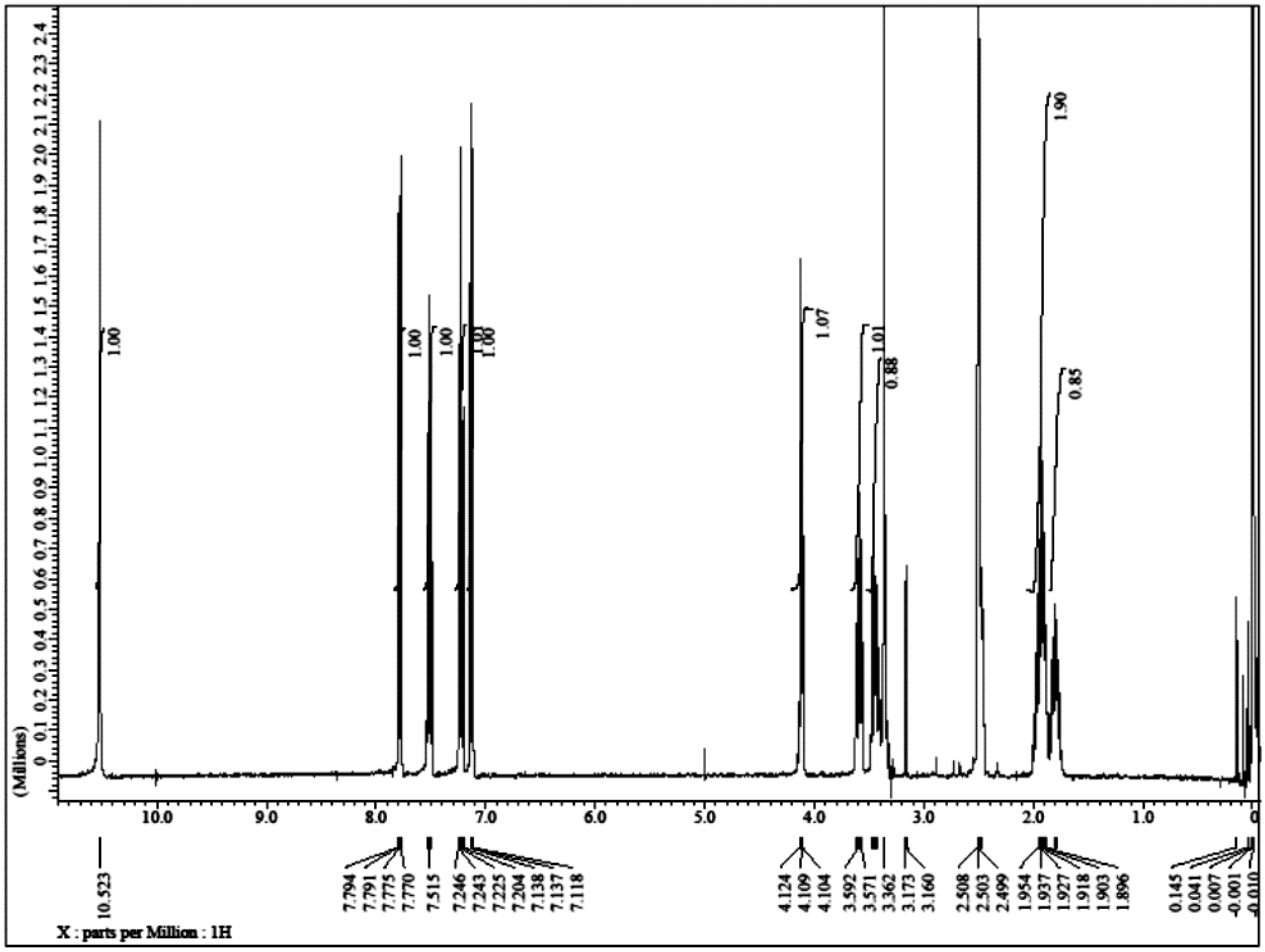
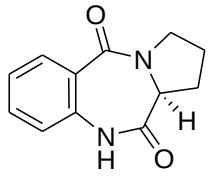


Appendix A2: ^{1}H-NMR Spectrum for Compound **1** in DMSO-d6



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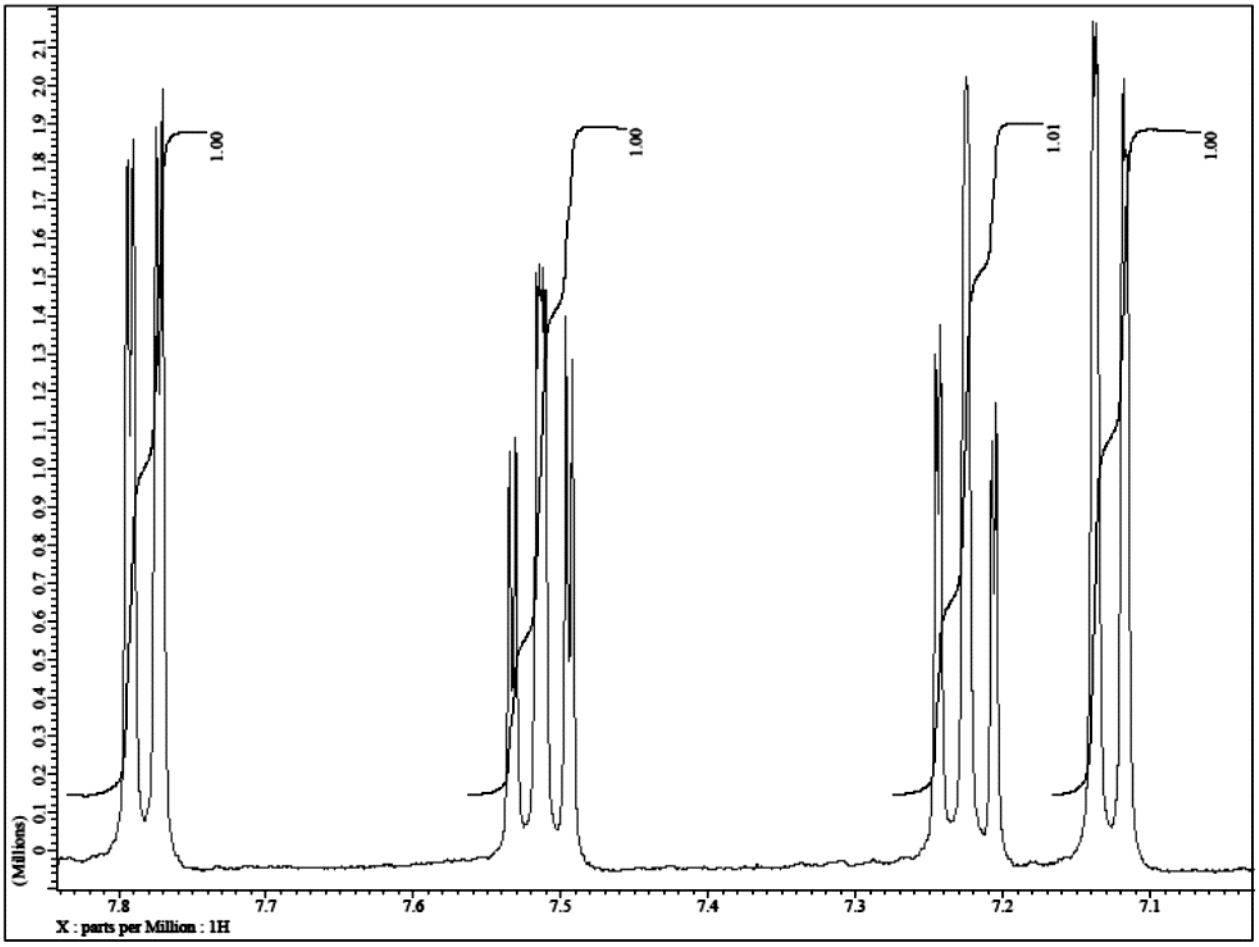
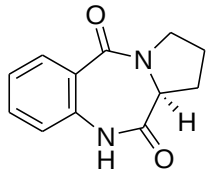


Appendix A3: ^{1}H-NMR Spectrum for Compound **1** in DMSO-d6



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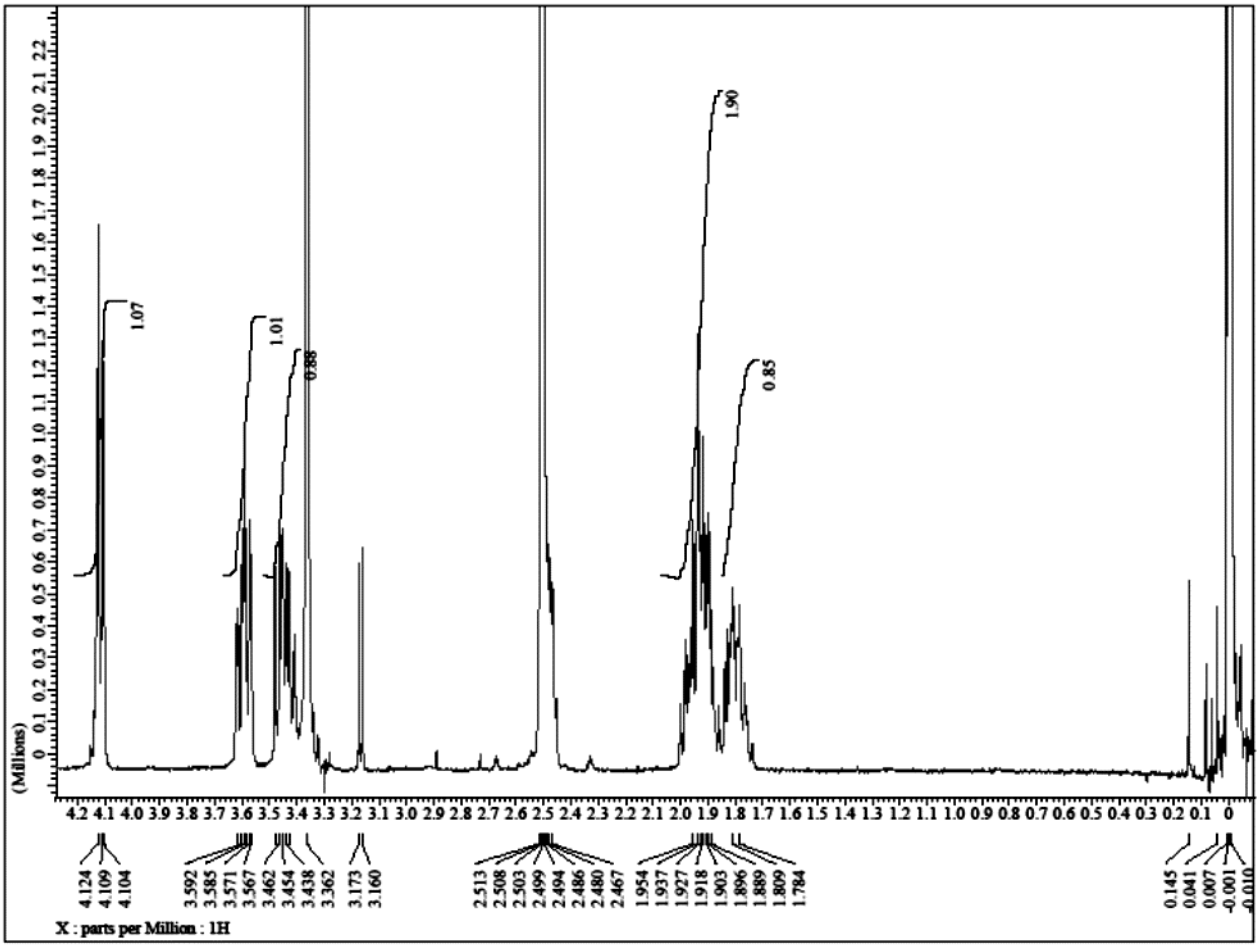
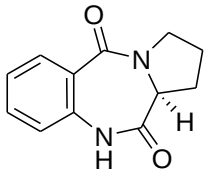


Appendix A4: ^{13}C-NMR Spectrum for Compound **1** in DMSO-d6



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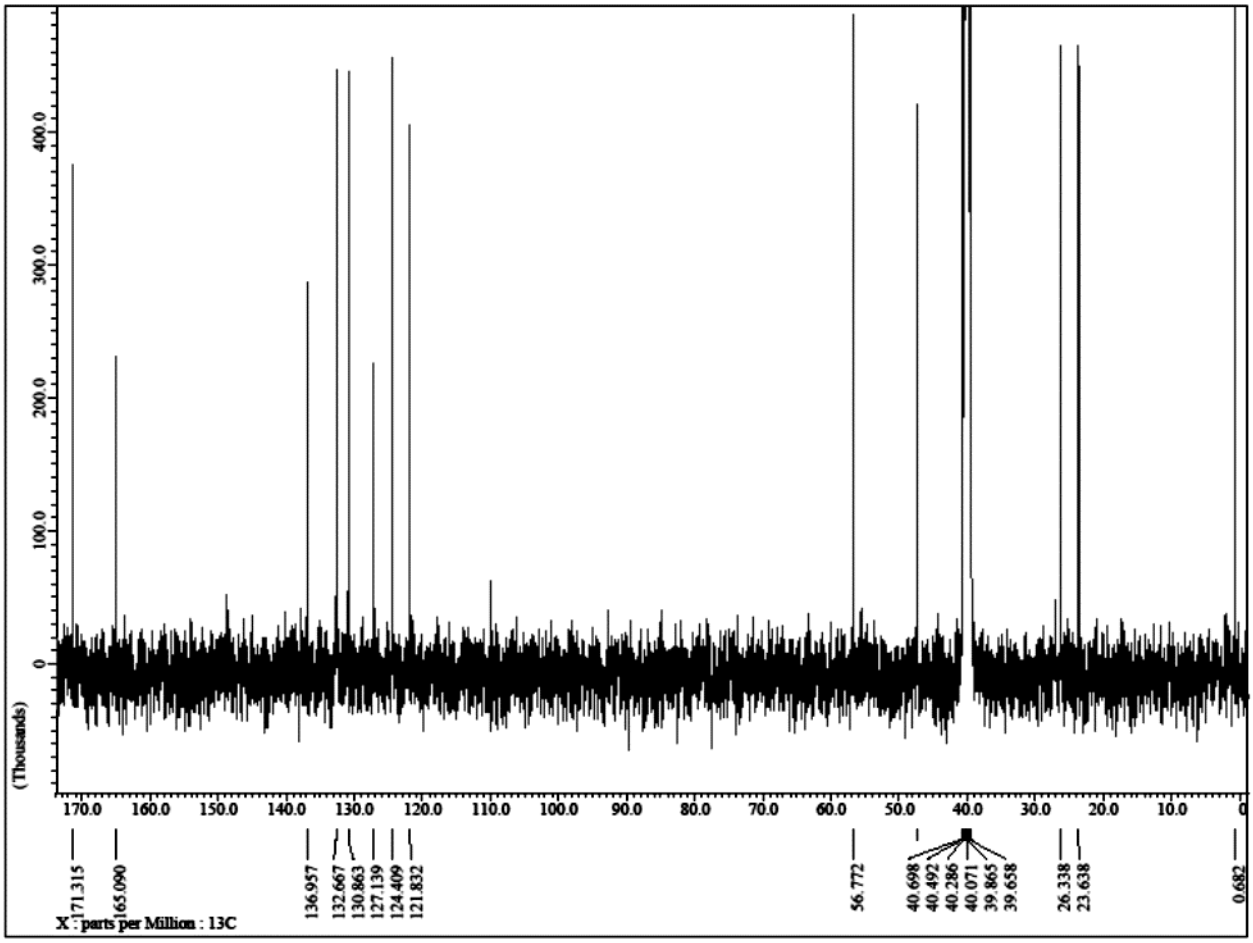
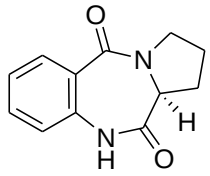


Appendix A5: C DEPT-135 NMR Spectrum for Compound **1** in DMSO-d6



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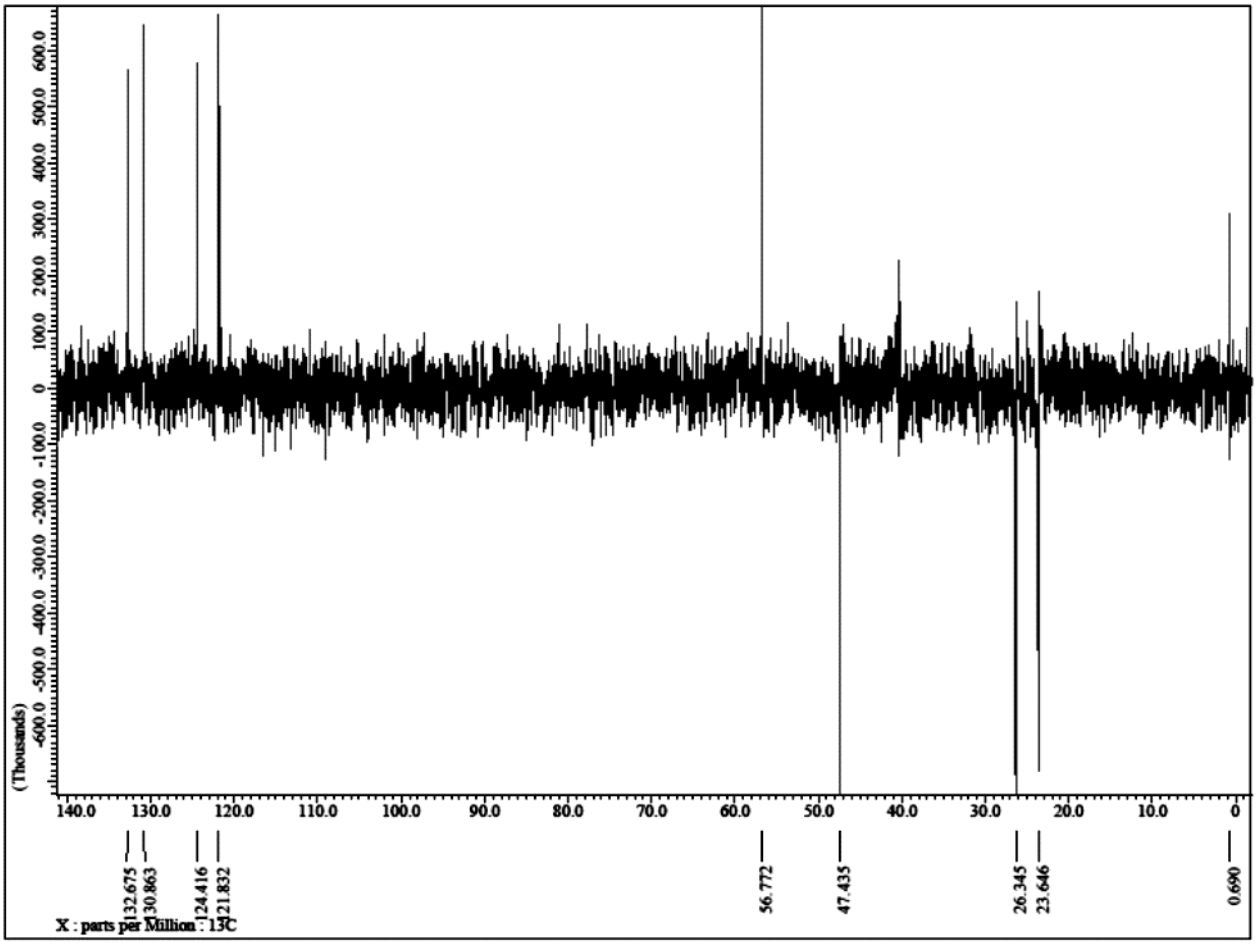
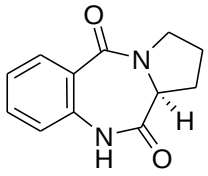


Appendix A6: GC-MS Spectrum for Compound **1**



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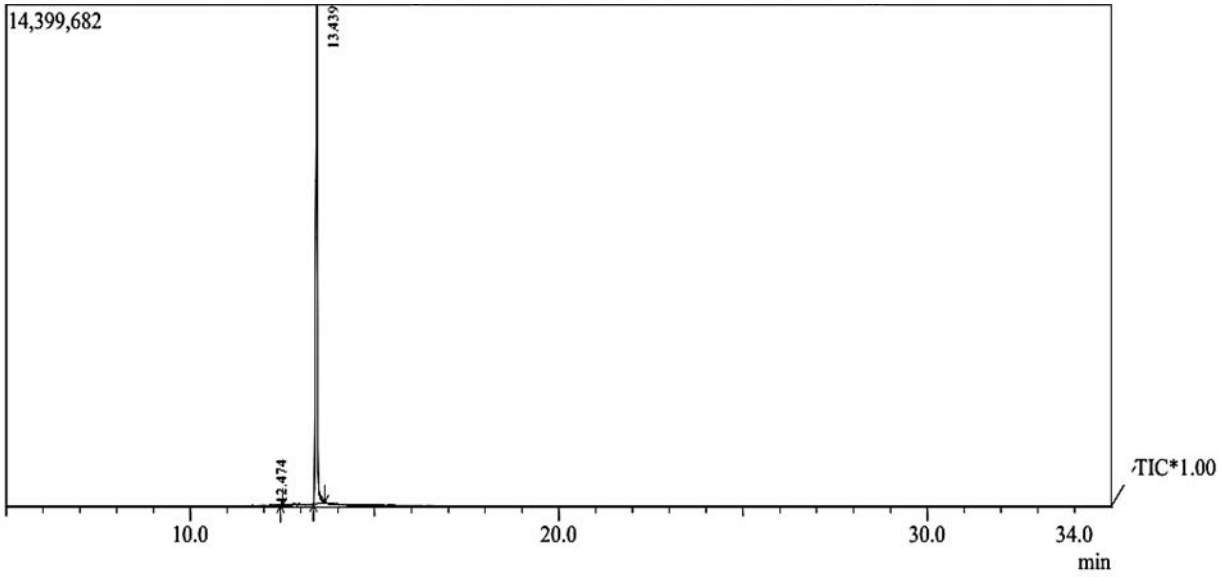

Peak Report TIC

Peak#	R.Time	I.Time	F.Time	Area	Area%	Height	Height%	A/H	Mark	Name
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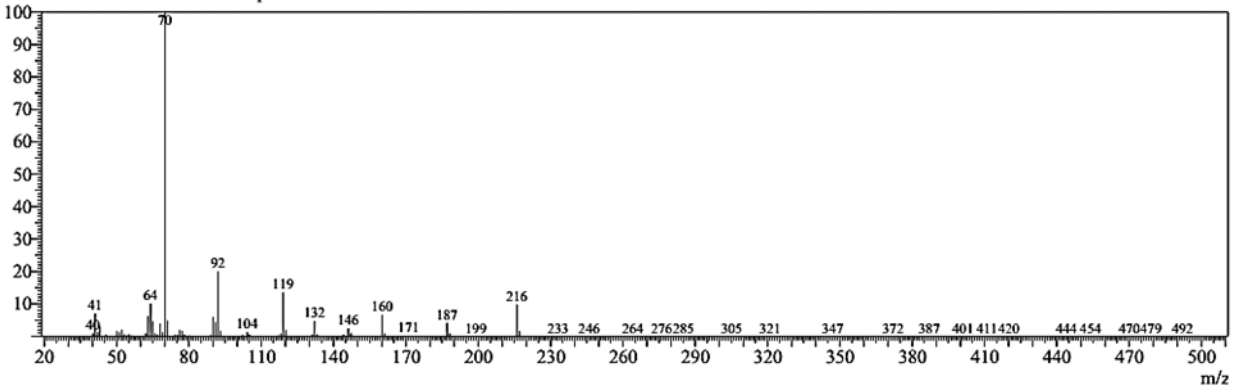
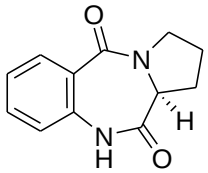
2	13.439	13.337	13.645	49809273	99.87	14299056	99.69	3.48		Pyrrolidine[2,1-c]-2H,5H-1,4-be
				49876327	100.00	14342993	100.00			

Line#:2 R.Time:13.4(Scan#:1206)
MassPeaks:251
RawMode:Averaged 13.4-13.4(1205-1207) BasePeak:70(5604477)
BG Mode:Calc. from Peak Group 1 - Event 1



Appendix A7: IR Spectrum for Compound **1**



1