# ANTI-TUMOR NATURAL PRODUCT RESEARCH FOCUSED ON PLANTS FOUND IN THE SOUTHERN APPALACHIAN REGION

By

Matthew John Flood II

A Thesis
Submitted to the
Faculty of the Graduate School
of
Western Carolina University
in Partial Fulfillment
of the Requirements for the Degree
of
Master of Science

Committee:

_____ Director

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_____

_____ Dean of the Graduate School

Date: _____

Summer 2010

Western Carolina University

Cullowhee, North Carolina

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A thesis presented to the faculty of the Graduate School of
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By

Matthew John Flood II

Director: Dr. Jason Clement
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Dr. Scott Huffman, Chemistry and Physics

August 2010

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And finally I would like to thank Dan White for helping to dial it in the past few years.

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# LIST OF ABBREVIATIONS

$CH_2Cl_2$ = dichloromethane
COSY = Correlation Spectroscopy
ESI-MS = Electrospray Ionization Mass Spectrometry
g = gram
HMBC = Heteronuclear Multiple Bond Correlation Spectroscopy
HPLC = High Performance Liquid Chromatography
HSQC = Heteronuclear Single Quantum Coherence
$IC_{50}$ = half maximal inhibitory concentration
*m/z* = mass-to-charge ratio
MeOH = Methanol
mg = milligrams
NA = No activity
nm = nanometer
NMR = Nuclear Magnetic Resonance
NT = Not Tested
$\delta_C$ = chemical shift of carbon NMR reported in parts per million
$\delta_H$ = chemical shift of proton NMR reported in parts per million
µM = micromolar
µg/mL = micrograms per milliliter

# ABSTRACT

Anti-Tumor Natural Products Research Focused on Plants Found in the Southern Appalachian Region

Matthew John Flood II

Western Carolina University (July 2010)

Director: Dr. Jason Clement

Natural products have been an integral part of society since the beginning of recorded history. Various types of herbs, plants, and even marine organisms have been used as both topical and ingestible remedies for a variety of health concerns and problems throughout the years. Our research efforts focus on the isolation and characterization of antitumor compounds from plant extracts from western North Carolina, a surprisingly diverse and under studied region of the country. Recent work has been done on the plant species *Aralia racemosa* and *Arnoglossum atriplicifolium*. Crude extracts were initially separated using either a modified Kupchan Partitioning or standard acid/base extraction, and then further purified using various chromatographic techniques. Two compounds resulted from this work on the two species and were characterized using various methods of mass spectrometry, and advanced NMR experiments including COSY, HMBC, and HSQC. An *ent*-kaurane diterpenoid was isolated from *Aralia racemosa*, and a highly oxygenated oplopane derivative from *Arnoglossum atriplicifolium.* The activity of the compounds was determined through a MCF-7 assay (breast cancer), with the control group being a FS-4 assay, both carried out by Wake Forest University. This is the first report of the antitumor activity of each of these compounds.

# 1. OVERVIEW OF PREVIOUS NATURAL PRODUCT STUDIES AND OVERVIEW OF METHODOLOGY

## 1.1 Introduction to Natural Products

Natural products have been an integral part of society since the beginning of recorded history. Various types of herbs, plants, and even marine organisms have been used as both topical and ingestible remedies for a variety of health concerns and problems throughout the years. However, around the turn of the nineteenth century, research started to focus on isolating and purifying compounds from natural sources as opposed to just accepting the medicinal powers of crude extracts. The methods and ideas of trying to isolate compounds that are already present in nature has continued on into areas of present day chemical research and development.[1] Natural products research has introduced many revolutionary products into modern medicine during its history. Natural products chemistry has brought forth compounds that have gone on to eventually lead to advanced cancer treatments, pain relievers, dietary supplements, and the treatment of a wide range of health issues.

Much of current natural product research is focused on finding effective cancer treatments. Cancer has become a main interest of natural products chemistry over the years due to its widespread diagnosis. In the United States alone one out of every four deaths can be attributed to cancer, making it second only to heart disease as the leading cause of death in America.[2]

The isolation and characterization of biologically active compounds from natural sources is called pharmacognosy. In pharmacognosy, there are three main areas of

inquiry: extraction, biological evaluation of extracts, and isolation/characterization of active principles of the extracts. Pharmacognosy differs from the more basic form of natural products chemistry, phytochemistry, which focuses solely on extraction, isolation, and characterization of compounds that may serve as taxonomic markers. Our research efforts will focus on the isolation and characterization of antitumor compounds from plant extracts from western North Carolina.

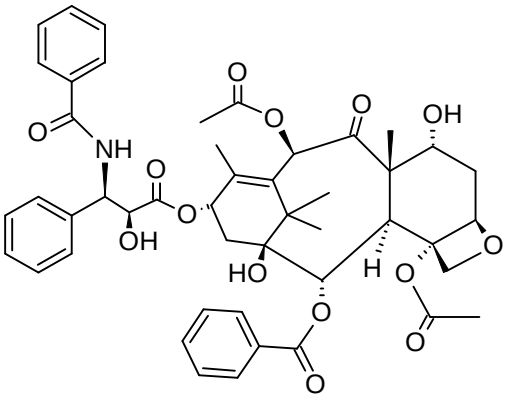
The isolation and characterization of a pure compound from a crude extract can be a fairly lengthy process. The main guiding factor in these isolations is the use of bioassays. A bioassay is a way to test biologically active compounds against a specific biological system. Initially a crude extract is prepared and tested against one or more bioassays to check for bioactivity. If the results are positive, further testing is required to find the responsible compound(s). In our case, if a crude sample is found to inhibit proliferation of MCF-7 cells, it is sent to our research labs to isolate the biologically active compounds. Once the crude sample is obtained, it needs to be fractionated, usually through multiple steps of liquid chromatography. Once the initial fractions are prepared they are run against the bioassays to test for activity. This is done to ensure that time is not wasted isolating inactive compounds. When active sub-fractions are obtained, they are tested in the assay to confirm the activity of the parent fractions. If the purified compounds show promise from the assays, they are characterized and their structure is determined using various spectroscopic methods. Once a biologically active natural product has been isolated and shows promising biological activity, it is often synthetically produced in larger quantities. This is necessary because in most cases there is simply not enough of the natural source material to supply the drug companies for

mass production. To try and replicate or improve upon the original molecule's success, synthetic derivatives may also be developed and tested. A synthetic approach that is affordable and more time efficient than isolation methods is desirable to pharmaceutical companies in order to make the drug(s) more marketable to the public.

The search for an effective cure for cancer has been a major goal in pharmaceutical research for quite some time and will most likely continue to be until a reliable and definite cure has been found or cancer has been made manageable. Many compounds that have already been isolated have shown great promise in helping the fight against cancer. The real test is to find compounds that are biologically selective to fight cancer, that is to say the compounds selectively fight the cancer cells as opposed to simply killing all cells. These types of compounds are desirable because if the isolated compounds destroyed all cells (both tumorous and healthy) they are of little use to the pharmaceutical industry. While many of the compounds that have gone on to clinical trials do not fully meet these standards, they do possess the ability to fight off cancerous cells while preserving the majority of healthy cells. Of these molecules which have been isolated, many have continued on to clinical trials and also into the market. Although the current drugs have shown promise and are somewhat effective, researchers are still looking for more selective drugs that prolong life and improve the quality of life for cancer patients. The most successful anti-tumor natural products are briefly reviewed in section 1.2.

## 1.2 Background

### 1.2.1 Taxol




**1.1**

Taxol (**1.1**) was first discovered and isolated in the late 1960's from the stem bark of *Taxus brevifolia* (Western yew). The compound was of great interest to scientists as it showed potent anti-leukemia and anti-tumor properties in cell assays.[3] Since the first isolation, taxol has also been isolated from other sources including the endophytic tree fungi *Taxomyces andreanae* and *Bartalinia robillardoides*.[4]

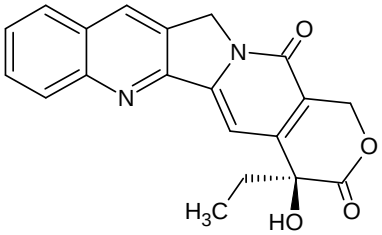
The mechanism used by taxol to inhibit cell growth was unique at the time of its discovery. The compound works to inhibit the depolymerization of microtubules into tubulin during cell division. Under normal circumstances microtubules will depolymerize back to tubulin upon command allowing the cell to further multiply. However, taxol works to inhibit the breaking down of microtubules inside the cell which prevents the cell from further dividing (mitosis).[4]

The major problem that held taxol back from becoming a very viable factor in fighting cancer was availability.[4] Natural taxol requires 60,000 pounds of bark from 12,000 trees which only produce 2.5 kilograms of taxol. Production levels like these

cannot be sustained because of the risk posed to the Western Yew tree. These shortcomings led to the development of syntheses of taxol, the most well known being a semisynthesis of taxol from 10-deacetylbaccatin III by Dr. Robert A. Holton's research group at Florida State University in the late eighties. This allowed taxol to be produced on a larger scale and at more reasonable prices to both consumers and producers.[5]

Currently, taxol is being marketed under the generic name paclitaxel, and is used mainly to treat ovarian, breast, and lung cancers. Due to taxol's poor water solubility, the drug has poor bioavailability in patients. This problem has led to the development of analogs like Docetaxel, a more potent and more water-soluble synthetic analog, which in essence uses the same mechanism to fight advanced breast cancers and also lung cancer.[6]

### 1.2.2 Camptothecin



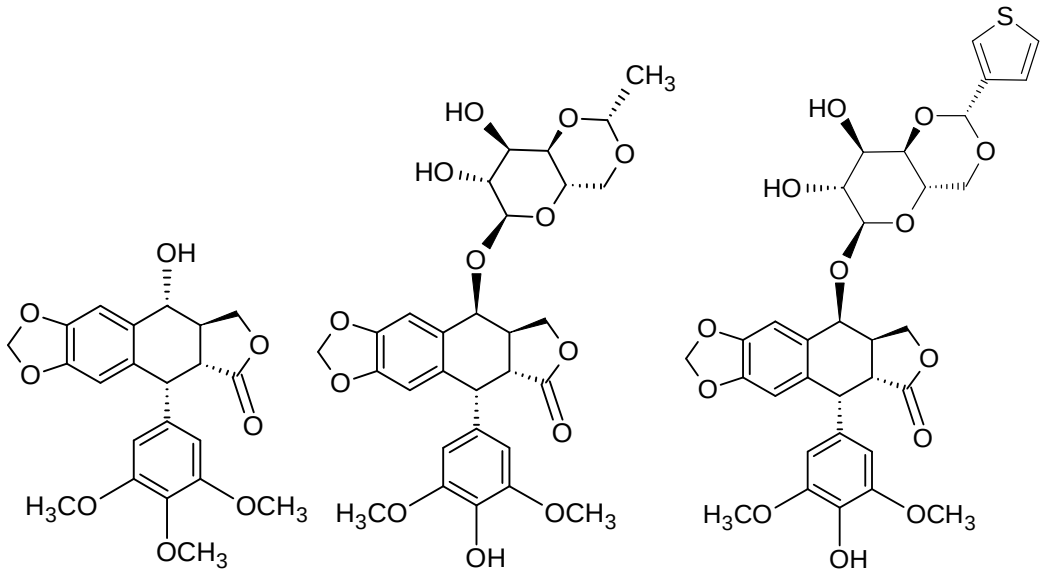

**1.2**

Camptothecin (**1.2**) was initially isolated from the wood and bark of *Camptotheca acuminata,* a tree found in China. The compound was found to have a very high activity against L1210 and P388 leukemia cell assays. These promising experimental results led to clinical trials involving camptothecin. [7]

The clinical trials of camptothecin showed promise in both phase 1 and 2 trials when paired with a campthothecin sodium salt (Phase 1 and 2 trials being the earliest tests of a drug's effects and efficacy). The compound was found to be effective against

gastric, intestinal, head, and neck tumors. The mechanism of action was later found to be inhibition of the enzyme topisomerase I, an enzyme that has been shown to play a role in DNA replication, transcription, and recombination, therefore inhibiting tumor growth. [7] Upon further study camptothecin was also found to be useful in the fight against otherwise resistant breast cancers. However, the drug never made it to clinical use due to more successful analogues being developed that were close to ten times as powerful.[8,9] First generation synthetic derivatives were eventually developed and marketed that included both Topotecan and Irinotecan, more water soluble versions of the natural product, making the drugs easier to administer. Both drugs are used for ovarian and colon cancer treatment. Camptothecin research has continued as scientists believe that the effects of the mechanism (specifically as a topisomerase I poison) have not yet been fully realized and second generation analogues are currently under development. [6]

### 1.2.3 Podophyllotoxin



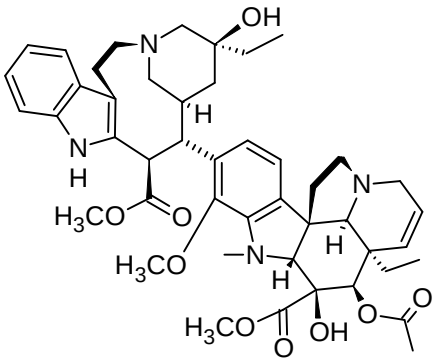

**1.3** **1.4** **1.5**

Podophyllotoxin (**1.3**), a lignan found in the resin of the dried roots of the plant *Podophyllum peltatum* (commonly known as mayapple), commonly found in the eastern United States, is another natural product that has come into focus in recent years.[10] The compound was first used during the nineteenth century to treat various diseases, venereal warts, and syphilis along with other ailments, which has led to more recent studies on the drug.[11]

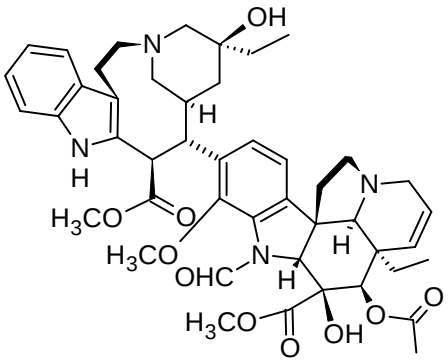
As its mechanism of action, podophyllotoxin works to inhibit the polymerization of tubulin. Unlike taxol, where the compound prevents the breaking down of the microtubules inside the cell, podphyllotoxin prevents microtubule formation from tubulin. If the microtubules (or spindles) are unable to form, then the cell is unable to divide the cell's chromosomes, resulting in sudden apoptosis of the cell in the metaphase.[9]

While podophyllotoxin was thought to be a promising and effective anti-tumor agent as clinical trials began, the compound was soon found to create severe gastrointestinal problems in patients. These disappointing results led scientists to try and develop synthetic derivatives that were more "patient-friendly." Etoposide (**1.4**) and teniposide (**1.5**) were eventually synthetically derived from the basic structure of podophyllotoxin, and were found to be more successful in clinical trials.[12]

### 1.2.4 Vinca Alkaloids



**1.6**



**1.7**

The vinca alkaloids, vinblastine (**1.6**) and vincristine (**1.7**), were both isolated from the leaves of the *Catharanthus roseus*, commonly known as the Madagascar periwinkle. Vincristine was found to be effective against non-Hodgkin's lymphoma in clinical trials, while vinblastine was also found to be effective against non-Hodgkin's lymphoma along with breast, testicular, bladder, and lung cancers.[13] The mechanism of action of the vinca alkaloids to inhibit these various diseases is the inhibition of tubulin polymerization in the cell. This in turn makes it impossible for the cell to divide, which then proceeds to apoptosis.[12,14]

The mass production of the vinca alkaloids has been difficult, as it takes a large amount of plant to obtain either of the vinca alkaloids (about 3 g are produced per metric ton of Madagascar Periwinkle). Scientists have dealt with this shortcoming by resorting to producing the compounds through mass cultivation, as the Periwinkle is a very easy and quick plant to grow.[12,13]

Natural products chemistry has become a mainstay in the pharmaceutical industry, and especially in anti-tumor research. Along with the previous compounds, natural products research has brought about many new and successful treatments into all

fields of medicine.[15] Our research group will continue to focus on the discovery and isolation of new compounds effective in the treatment of breast cancer, in hopes of finding a lead in the creation of a new anti-tumor drug/treatment.

### 1.3 Overview of Methods

The separation of natural products relies heavily on different forms of liquid chromatography. Methods that include open-column chromatography, high performance liquid chromatography (both normal and reverse phase), along with liquid partitioning are implemented in order to purify compounds of interest. Once a specific compound has been isolated, we are then able to use nuclear magnetic resonance (NMR) spectroscopy, mass spectrometry, and various other spectroscopic methods in order to characterize and identify the compound of interest.

#### 1.3.1 Liquid Chromatography

Chromatography is a method of separation used by scientists to obtain fractions and also pure compounds out of crude extracts. Separations are completed through the use of a mobile phase being passed through the stationary phase in a column. Stationary phases can be made up of a variety of substances including silica gels and synthetic polymers. [16]

Silica gels rely on polarity differences in order to separate substances eluting from the column. Silanol groups in the gel tend to have higher dipole moments allowing sample groups with complementary dipole moments to be attracted to these stationary phase groups. These polarity differences allow for the sample groups with higher polarities to remain bound to the stationary phase longer, and therefore elute slower.[13] One of our more frequently used techniques for separating extract fractions is a reverse-

phase $C_{18}$ silica column. Reverse-phase chromatography differs from normal-phase chromatography in the fact that it elutes compounds in the reverse order as normal phase under most circumstances. Generally, in reverse phase the more hydrophilic compounds are eluted first followed by the more hydrophobic compounds. Hydrophobic compounds tend to hang on to the long carbon chains of the $C_{18}$, due to London dispersion forces, longer than the hydrophilic compounds which elute fairly quickly.[16,17]

Some synthetic polymers can also work as chromatography stationary phases by a size exclusion mechanism. Size exclusion basically works around the idea that larger particles will pass through the column quicker that smaller ones. While not as efficient as other forms of liquid chromatography, these polymers allow for delicate samples to be separated with less fear of the sample decomposing as can sometimes happen with other forms of liquid chromatography.[16]

For the purposes of our research, high performance liquid chromatography will be the most useful tool. HPLC basically follows the same principles as normal liquid chromatography, except the technique usually involves the use of a smaller particle packing in the column at a higher pressure. HPLC results in much more efficient separations, which is very appealing to researchers with limited amounts of time, resources, and samples.[13] If sample size is not an issue, a preparative HPLC column can be used, which allows for a larger sample size to be used at a higher flow rate. This process allows for separations to be performed at a higher efficiency.[16]

### 1.3.2 Bioassay Guided Fractionation

Bioassay guided fractionation is a technique aimed at isolating bioactive compounds in a quick and effective manner. The basis behind the idea is that the

biologically active compounds are only present in active fractions as determined through the assays, so we focus on these select fractions to find the compounds of interest. The crude sample is fractionated and screened by the bioassay. Inactive fractions are then set aside. The process of removing the inactive fractions prevents the isolation of any non-active compounds and usually only active fractions are obtained. The idea is the non-active fractions are uninteresting and are subsequently removed from the process. The remaining known active fractions are then fractionated further and resulting sub-fractions are tested for activity. By this iterative process of fractionating only active fractions, the active compounds are isolated from the crude extract while less interesting compounds are eliminated. The isolations are tested along the way to see if they are selectively cytotoxic against both healthy and tumor cells.

While we usually assume that only one compound is responsible for the activity in these assays, this is not always the case. It has been theorized that multiple compounds can combine to have a greater biological activity, a process known as synergism. This suggests that it is also necessary to examine multiple compound combinations in assays to see how compounds work with each other. Our collaborators at the Bent Creek Institute are interested in the study of synergistic mixtures as a part of plant-based medicines, so we will therefore be including them as part of our research plan.[18] For the purpose of our research our fractions and pure compounds will be tested in the FS-4 and MCF-7 cell assays. FS-4 cells are wild-type, non-tumorous human foreskin fibroblast cells, while MCF-7 is an estrogen receptor-positive breast tumor cell line.[19,20]

The MCF-7 assay is an MTS assay that works off the reduction of a tetrazolium salt to a colored (purple) formazan product. The reduction is driven by a reductase

enzyme found only in viable cells. Cells that do not reduce the salt are not considered viable, and therefore the inhibition of cell proliferation by the fraction being tested can be measured.

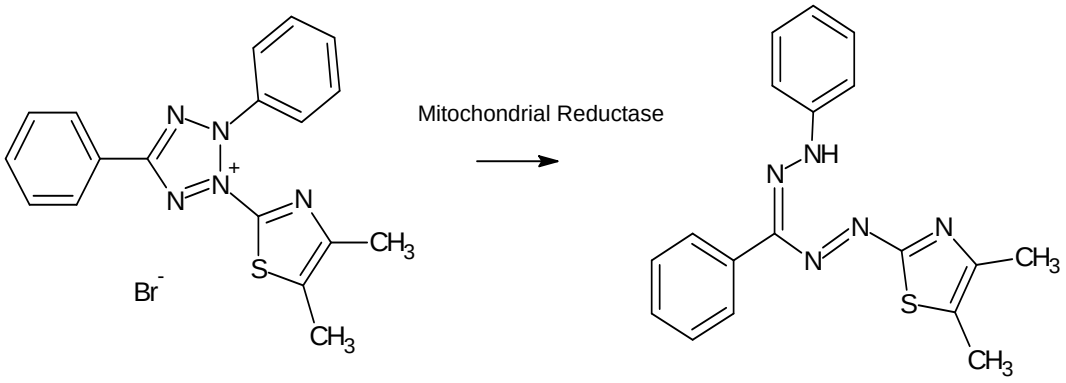



**Figure 1.1**: Reduction of Tetrazolium Salt to Formazan Product

### 1.3.3 Mass Spectrometry

Mass spectrometry is a technique used to identify the molecular formula of analyte molecules. The instrument we will be using for the mass spectrometry employs the electrospray ionization technique to produce ions for analysis. Electrospray ionization works to turn a sample into an aerosol form and then produces ions by inducing a large charge into the sample droplets. The droplet is then subjected to a drying gas (usually $N_2$) in order to remove any excess solvent. As evaporation of the remaining solvent continues on the droplets, eventually the electrostatic repulsions between the concentrated droplets become too great and cause the droplet to break apart into ions. The ions are then accelerated to the quadrupole ion-trap where they are trapped and separated by their mass-to-charge ratio (*m/z*), by a mass filter.[21] The ions are then filtered, detected, and reported by their abundances when they impact the detector and

electron multiplier and the mass-to-charge ratio is determined. Although mass spectrometry is a very useful and efficient tool for molecule analysis, it usually cannot be used to determine the complete structure of organic molecules because it provides no information about the actual absolute configuration of the molecule. Due to this restriction mass spectrometry is primarily a confirmatory tool in our lab. [16]

### 1.3.4 NMR

Nuclear magnetic resonance is our primary tool in determining the structure of our unknown compounds. The basis of NMR deals with the total spin and magnetic dipoles of atomic nuclei. NMR spectroscopy deals with the study of nuclear spin of atoms when exposed to a magnetic field. This phenomenon can be applied to help decipher atomic structures through the orientations of their respective magnetic moments. NMR is very helpful in determining the structures of molecules, as it measures the respective resonances of different nuclei in different positions and analyzes their interactions. The study of these resonances helps us deduce the locations of different atoms in the molecule.[22] Our research will mainly use $^{1}$H NMR along with $^{13}$C NMR, which measure the effects produced by hydrogen nuclei and carbon nuclei respectively. While these 1D NMR experiments provide much information about the structure of a molecule, they oftentimes do not supply enough information to obtain a complete structure. To obtain complete molecular structure 2D NMR spectroscopy is used, which correlates data from $^{13}$C and $^{1}$H NMR spectra. These spectra provide us with information about C-H connectivity and interactions, which allows us to assign a structure to our isolated compound in three dimensions. [23]

There are four main types of advanced NMR experiments used in our research: DEPT-135, COSY, HSQC, and HMBC. DEPT-135 is a carbon experiment that is used to distinguish between methines, methylenes, and methyls while quaternary carbons do not appear in the spectrum. Carbons are differentiated by their orientation relative to the baseline. Methines and methyls are shown in an "up" orientation or "positive," while methylenes are shown as "down" or negative. The COSY experiment allows for proton to proton spin-spin coupling to be detected. Signals in COSY tend to arise from neighboring protons that are two or three bonds apart, however any system with spin-spin coupled protons can give a COSY correlation. The HSQC experiment allows for the determination of one bond couplings between hydrogens and carbons. The experiment is useful in assigning protons to their respective carbons and vice versa. The HMBC experiment measures long-range couplings between carbons and protons, while suppressing the one bond HSQC correlations. HMBC allows for two to three bond correlations to be observed in the molecule of interest. In certain, more rare conditions it is possible to observe four and five bond correlations using the HMBC experiment.

# 2. ISOLATION AND CHARACTERIZATION OF 17-HYDROXY-16α-ENT-KAURAN-19-AL, A DITERPENOID FROM THE PLANT *ARALIA RACEMOSA*

## 2.1 Introduction

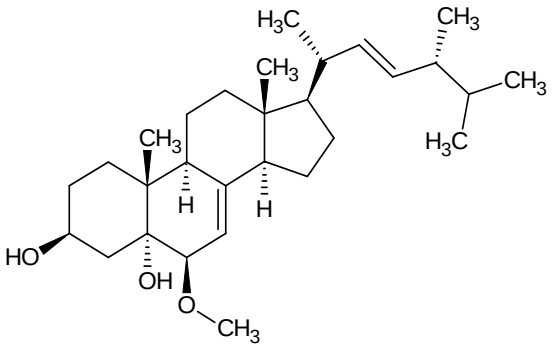
Aside from a few phytochemical studies, the chemistry of the plant *Aralia racemosa* has not been fully characterized up to this point. Using an assay for the breast cancer cell line MCF-7, the *ent*-kaurane diterpenoid 17-hydroxy-16α-*ent*-kauran-19-al was isolated from an extract of the plant *Aralia racemosa* by bioassay-guided fractionation. Characterization was completed using various 1D and 2D NMR experiments, along with mass spectrometry.

### 2.1.1 Previous Investigation into *Aralia* species

There are many plant species in the southern appalachian region that have not been fully characterized chemically. Previously, the genus *Aralia* has yielded promising results in a variety of assays, with activities ranging from anti-inflammatory properties to hepatitis and diabetes treatments. The species *A. racemosa* has been studied very little up to this point, with research mainly focusing on phytochemical studies.[24] The previous history of the genus and biological activity found in these types of plants, along with the lack of study of the plant *Aralia racemosa* specifically, make it a prime candidate for our research.

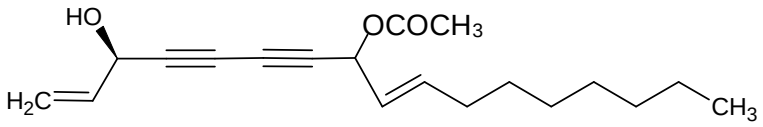
Many different types of compounds have been isolated from the *Aralia* species including sterols,[26] falcarindiols,[27] saponins, and terpenes, all of which have shown some kind of biological activity *in vitro.*[28] One important set of compounds are the sterols

found in *Aralia cordata*, a commonly studied species. Ik Soo Lee *et al.* isolated and characterized four sterols from the plant including 3β, 5α-dihydroxy-6β-methoxyergosta-7,22-diene (**2.1**), which showed significant cytotoxicity against murine L1210 leukemia cells, human K562 cells, and mouse LLC Lewis lung carcinoma cells with $IC_{50}$ (μM) values of 11.7, 11.9, and 15.1 respectively. Other sterols isolated from the *A. cordata* yielded similar structures and showed less activity.[25] Also according to older results compound **2.1** exhibited cytotoxicity against small cell lung, ovarian, skin, and colon cancers.[26]

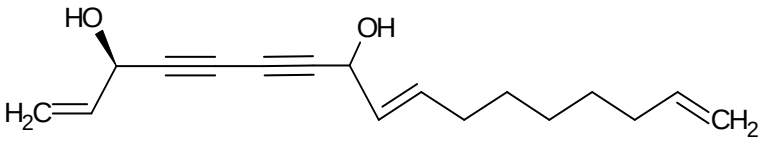



**2.1**

The falcarindiol (**2.2** and **2.3**) derivatives found in *Aralia cordata* have shown to be quite effective in inhibition of the COX-1 enzyme *in vitro*, with $IC_{50}$ ranging from 11.7 to 50.4 μM. COX-1 and COX-2 are well known for their role in the biosynthesis of prostaglandins and thromboaxanes from arachidonic acid, which if inhibited can have analgesic and antipyretic effects on the target. The absolute configuration of these falcarindiol derivatives was not reported by Dang *et al.*[27]

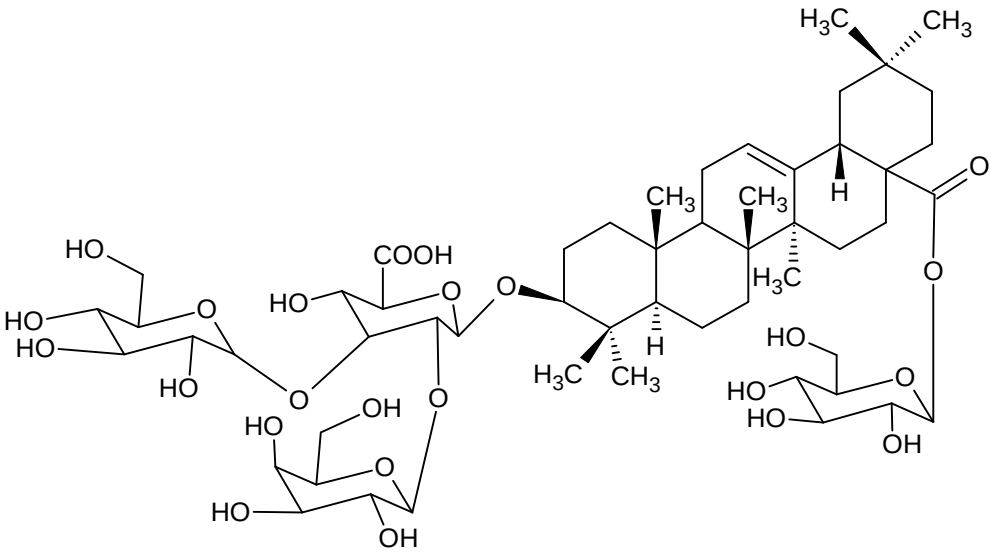



**2.2**




**2.3**

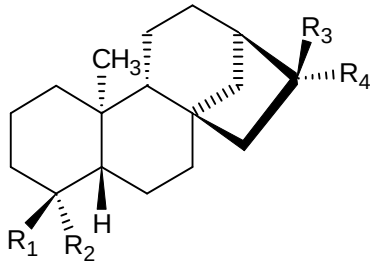
The triterpene saponin **2.4**, isolated from the root bark of *Aralia dasyphylla,* is a oleanic based saponin isolated by Kai Xiao *et al.* This triterpene saponin isolated showed significant activity against two separate cultured human cancer cell lines. The compound exhibited very good potency with a $IC_{50}$ values of 1.2 µM and 0.02 µM for the KB mouth cancer) and Hela-$S_3$ (cervical cancer) respectively.[28]




**2.4**

The *Aralia* species also synthesizes many different derivatives from the *ent*-kaurane diterpenoid skeletal structure. Many of the derivatives are active against the

COX-1 and COX-2 enzymes previously mentioned, making them possible analgesic and antipyretic (fever-reducing) agents.




| | $R_1$ | $R_2$ | $R_3$ | $R_4$ |
|---|---|---|---|---|
| **2.5** | COOH | $CH_3$ | $CH_3$ | OH |
| **2.6** | COOH | $CH_3$ | = | |
| **2.7** | COOH | $CH_3$ | OH | $CH_2OH$ |
| **2.8** | CHO | $CH_3$ | COOH | H |

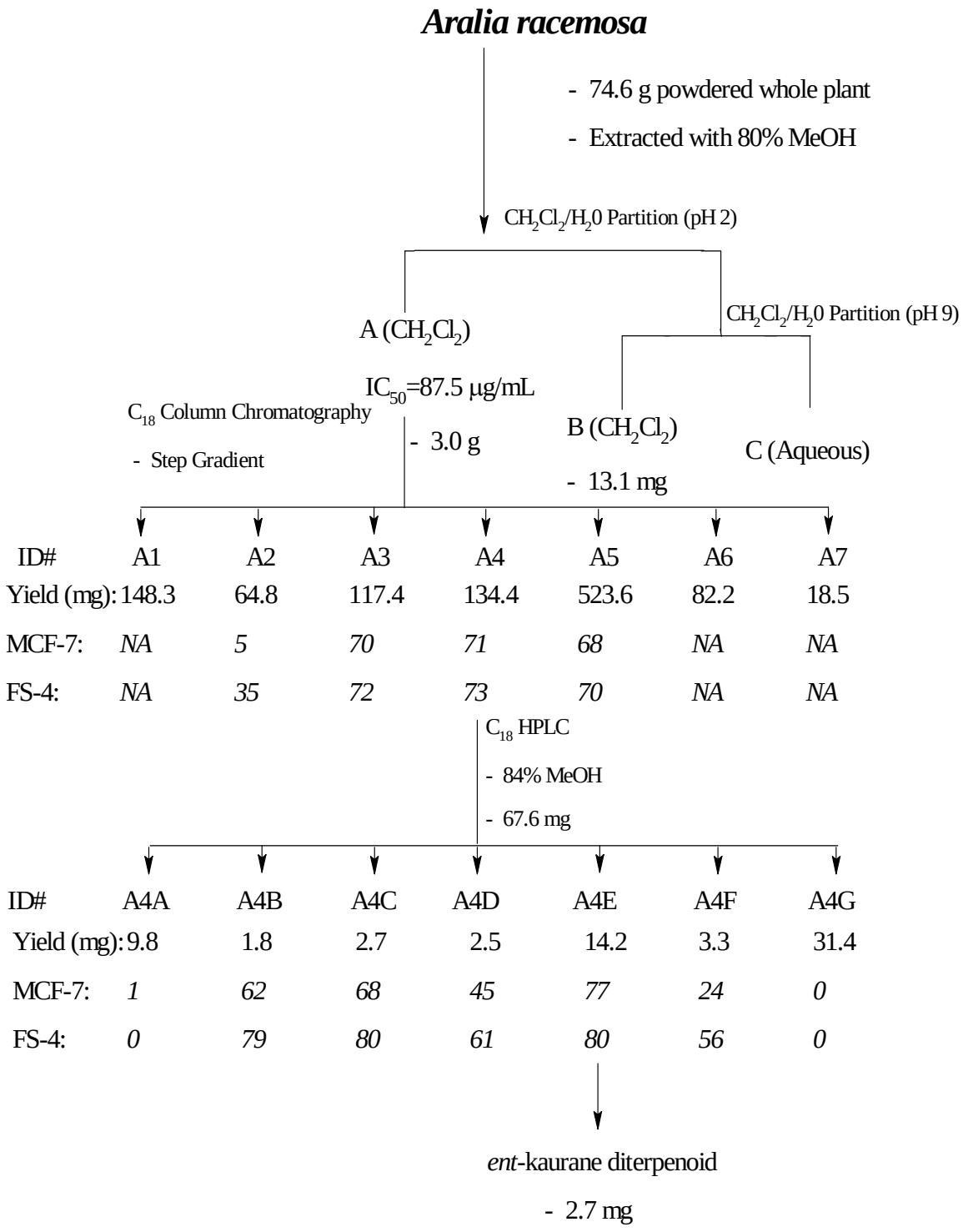
Specifically Ik Soo Lee *et al.* isolated compound **2.5** from the aerial parts of *Aralia cordata*, which was found to be somewhat active inhibiting the COX-1 enzyme with an $IC_{50}$ of 227 µM.[25] Another derivative of the above skeleton was isolated and found to inhibit the COX-1 and COX-2 enzymes with $IC_{50}$ values of 121.6 and 127.6 µM respectively.[27]

## 2.2 Results and Discussion

### 2.2.1 Isolation and Characterization of 2.9

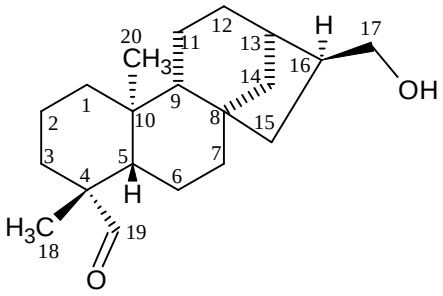
The isolation of **2.9** is depicted in Figure **2.1**. The plant material and crude extract were collected and prepared by the Bent Creek Institute. The crude extract was subjected to a standard acid/base extraction, resulting in three fractions. The *Aralia racemosa* acidic fraction showed promising activity in the MCF-7 cell assay, with an $IC_{50}$ = 87.5 µg/mL, while the other two fractions were set aside for later study. Preliminary fractionation was completed by Tim Willis and Sara Garrett in the Clement Lab using reverse phase $C_{18}$ open column chromatography with a step-gradient solvent system. The 80% MeOH/$H_2O$ fraction was chosen for further study due to increased activity when compared to other fractions and amount available for study. The 80% MeOH/$H_2O$ fraction was then subjected to reverse phase $C_{18}$ HPLC using an 84% MeOH/$H_2O$ method, and compound **2.9** was isolated from the fraction.

**Figure 2.1:** Isolation of **2.9**




* Assay data shown is percent inhibition as determined at a sample concentration of 100 µg/mL unless otherwise noted.

Compound **2.9** was found to give a pseudomolecular ion of *m/z* 305.1 ([M+H]) when examined by low resolution ESI-MS. $^{1}H$ and $^{13}C$ NMR data were collected in *d*-chloroform ($CDCl_3$) and reported in Table 2.1. The $^{1}H$ NMR spectrum of **2.9** showed several key proton signals, including two methyl singlets ($\delta_H$ 0.86 and 0.99), an oxygenated proton doublet signal ($\delta_H$ 3.39), and an aldehyde proton singlet ($\delta_H$ 9.76). The $^{13}C$ NMR experiment yielded twenty signals. These signals suggested the presence of both an oxygenated carbon and an aldehyde carbon ($\delta_C$ 67.61 and 206.08 respectively). A DEPT-135 experiment helped in the determination of the presence of four methines, one oxygenated methine, nine methylenes, one oxygenated methylene, two methyls, and by comparison with $^{13}C$ NMR data three quaternary carbons. The DEPT-135 experiment and MS ion helped to drastically reduce the number of chemical formula possibilities. Also, IR analysis provided evidence of an O-H stretch at 3378 $cm^{-1}$. Along with the $^{1}H$ NMR data, $^{13}C$ NMR data, IR, and MS analysis, the compound was determined to have a molecular formula of $C_{20}H_{32}O_2$ with a mass of 304.2 g/mol. Using the molecular formula, the degree of unsaturation was determined to be five for compound **2.9**.




**2.9**

**Table 2.1:** $^{13}C$ and $^{1}H$ NMR Data for **2.9**

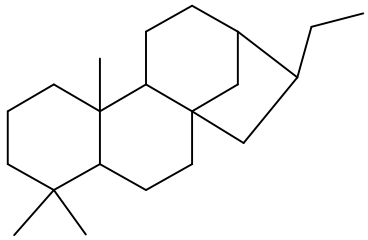
| Position | $\delta_C$[a] | DEPT-135 | $\delta_H$[a] | Multiplicity |
|---|---|---|---|---|
| 1 | 39.98 | $CH_2$ | 0.80 | m |
| | | | 1.85 | m |
| 2 | 18.83 | $CH_2$ | 1.58 | m |
| 3 | 34.32 | $CH_2$ | 1.00 | m |
| | | | 2.14 | m |
| 4 | 48.53 | C | - | - |
| 5 | 56.77 | CH | 1.14 | dd, $J_1$=12.3, $J_2$=2.2 |
| 6 | 20.48 | $CH_2$ | 1.65 | m |
| | | | 1.86 | m |
| 7 | 41.65 | $CH_2$ | 1.52 | m |
| 8 | 44.70 | C | - | - |
| 9 | 54.88 | CH | 1.04 | m |
| 10 | 39.43 | C | - | - |
| 11 | 18.49 | $CH_2$ | 1.46 | m |
| 12 | 31.31 | $CH_2$ | 1.45 | m |
| | | | 1.59 | m |
| 13 | 38.27 | CH | 2.10 | m |
| 14 | 37.56 | $CH_2$ | 1.00 | m |
| | | | 1.84 | m |
| 15 | 45.16 | $CH_2$ | 0.94 | m |
| | | | 1.60 | m |
| 16 | 43.40 | CH | 1.97 | m |
| 17 | 67.61 | $CH_2$ | 3.39 | m |
| | | | 3.42 | m |
| 18 | 24.39 | $CH_3$ | 0.99 | s |
| 19 | 206.08 | CH | 9.76 | s |
| 20 | 16.40 | $CH_3$ | 0.86 | s |

*All units in ppm

[a]Spectra were acquired using $CDCl_3$ (*d*-chloroform)

With the presence of twenty carbons confirmed, a $C_{20}$ skeletal structure was proposed. The skeleton along with the presence of two methyls, an aldehyde, a hydroxyl group, and a high degree of unsaturation with no double bonds, the structure was

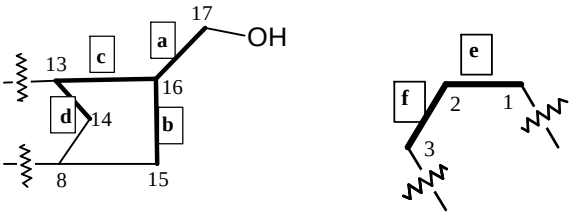
hypothesized to be an *ent*-kaurane derivative (**2.10**), a class of compounds previously found in the *Aralia* genus.



**2.10**

A HSQC analysis was performed on compound **2.9** showing carbon-proton one bond coupling. These results are depicted in Table **2.1**. These HSQC correlations allowed for the interpretation of the COSY and HMBC spectra.

COSY analysis of compound **2.9** suggested the presence of several key structural segments that are shown in Figure **2.2.** The proton signal at $\delta_H$ 3.39 ppm was coupled to a signal at $\delta_H$ 1.97 ppm. Due to the multiplicity of the signal at $\delta_H$ 3.39 ppm, it was determined to be the methylene attached to the hydroxyl group. The methine signal at $\delta_H$ 1.97 ppm showed a correlation to a methylene signal at $\delta_H$ 0.94 ppm. A crosspeak was also observed between the methine and methylene at $\delta_H$ 1.97 and $\delta_H$ 2.10 ppm. Also a crosspeak was observed between the methine and methylene at 1.84 and $\delta_H$ 2.10 ppm. This series of correlations established segment A (Figure **2.2**). A coupling was also observed between the methylene protons at $\delta_H$ 0.80 ppm and 1.58 ppm, and also the methylene protons at $\delta_H$ 1.58 ppm and 2.10 ppm. These correlations helped to determine segment B in Figure **2.2**. COSY correlations can be seen in Figure **2.3**. Other COSY correlations were present in the COSY experiment but are also shown in HMBC correlations so they are not specifically reported.




**Segment A** **Segment B**

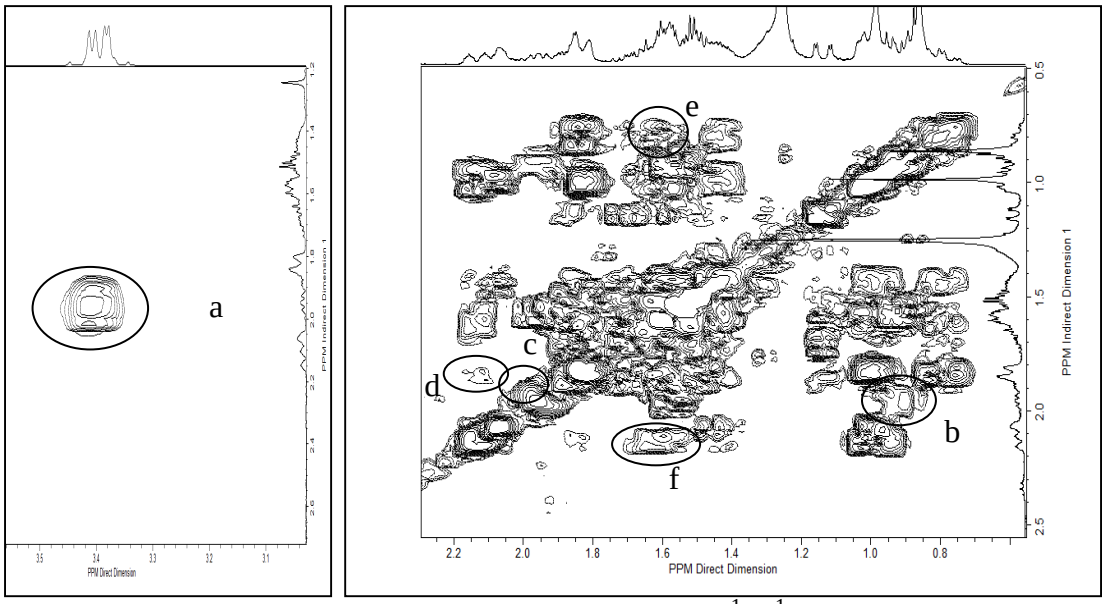



**Figure 2.2:** Structural Segments for **2.9** from the $^{1}H$-$^{1}H$ COSY Experiment

*a = a, b = b, c = c, …

The HMBC spectrum allowed for the determination of the connectivity of the previously mentioned structural segments. Correlations between the methyl signal at $\delta_H$ 0.99 ppm and the carbon signals at $\delta_C$ 48.53 and 34.32 ppm suggested the connectivity of segment B to the methyl through a quarternary carbon at $\delta_C$ 48.53 ppm. The HMBC also allowed for a correlation to be made between the methyl protons at $\delta_H$ 0.99 ppm and the carbon at $\delta_C$ 56.77 ppm. This carbon showed a correlation to the proton signal at $\delta_H$ 0.86 ppm, a methyl proton attached to the methyl carbon at $\delta_C$ 16.40 ppm in segment B. The

aldehyde proton at $\delta_H$ 9.76 ppm also showed a correlation to the methylene at $\delta_C$ 34.32 ppm and the methine at $\delta_C$ 48.53 ppm. A correlation was also observed between the methine proton at $\delta_H$ 1.14 ppm and the aldehyde carbon at $\delta_C$ 206.08 ppm. These correlations provided evidence of segment C in Figure **2.3**.

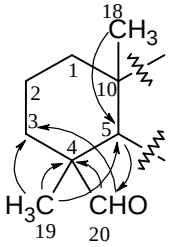



**Figure 2.3**: Segment C of 2.9

The HMBC spectrum also provided correlations to the hydroxylated carbon (segment A). The proton signal of the $CH_2$ group at $\delta_H$ 3.39 ppm shows a correlation to the carbon at $\delta_C$ 43.40 ppm, and also one to the methylene at $\delta_C$ 45.16 ppm. The proton at $\delta_H$ 1.97 ppm attached to the carbon at $\delta_C$ 43.40 ppm was shown to have a correlation to the carbon at $\delta_C$ 38.27 ppm, which also showed a correlation to the proton at $\delta_H$ 1.58 ppm, which is in fact part of segment A. The methylene proton at $\delta_H$ 0.94 ppm showed a correlation to the methine at $\delta_C$ 54.88 ppm. The proton at $\delta_H$ 0.94 ppm also showed a correlation to the quaternary carbon at $\delta_C$ 44.70 ppm. These correlations provided evidence of segment D in Figure **2.4**. Additional key 2D information and correlations are shown in Figures **2.6** and **2.7.**

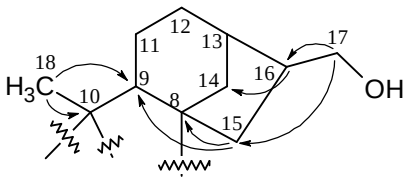



**Figure 2.4:** Segment D of **2.9**

In order to see if compound **2.9** was a known compound, a literature search was performed for compounds with a molecular formula of $C_{20}H_{32}O_2$ and the presence of a substructure similar to segment D. The search yielded several results, but after refining the results to include the presence of an aldehyde and four rings, and taking into consideration the isolation of an *ent*-kaurane derivative in previous work completed on the plant in our lab,[29] two possibilities were found. The preliminary data, along with the unsaturation number matched well with that of 17-hydroxy-16β-*ent*-kauran-19-al (**2.9**), a known diterpenoid that has been isolated from similar plant species. Additional key COSY and HMBC correlations are shown in Figures **2.6** and **2.7**.

A discrepancy was found, though, as the orientation of the hydroxyl group on C-17 in the proposed structure did not match correctly with the published data.[31] Further investigation of literature related to the *Aralia* genus and *ent*-kaurane diterpenoids revealed a great bit of confusion in the labeling of similar compounds. The main discrepancies occurred at the location of the hydroxyl group at C-17 and the surrounding carbons and hydrogens, due to either incorrect assigning of the carbons in previously published work or citations of these publications in subsequent studies. The discrepancy revolves around the chemical shift of C-17 and its orientation in relation to C-11. Dräger *et al.* summarized the older incorrect assignments and incorrect publications and

presented their own case for the correct chemical shifts of the carbons in a supplemental paper dealing with diterpenes from the plant *Stillingia sanguinolenta*.[30] The Dräger group observed and reported that a structure having the H-16 oriented towards the C-11 resonates at $\delta_H$ 3.3 ppm, while the C-17 resonates at a frequency of $\delta_C$ 67 ppm. While the epimer with H-16 oriented away from the C-11 resonates at $\delta_H$ 3.7 ppm, and the C-17 is shifted upfield around $\delta_C$ 64.0 ppm. The data in Table **2.1** reports these chemical shifts to be $\delta_H$ 3.39 ppm and $\delta_C$ 67.61 ppm respectively. This data led to our assignment of the H-16 proton as being oriented towards the C-11 carbon (depicted in Figure **2.5**).

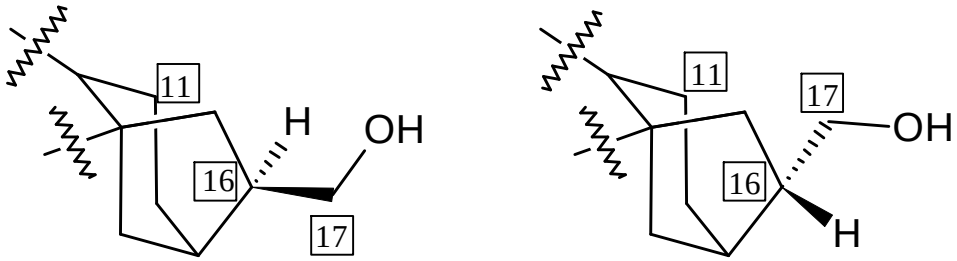
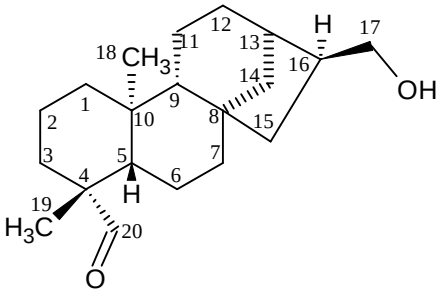



**Figure 2.5:** Possible Orientations of the Hydroxyl Group in Compound **2.9**

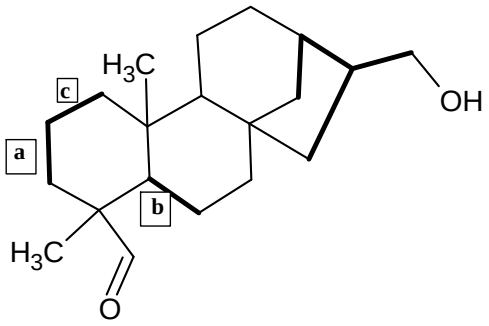



**2.9**

Due to budget constraints at the Bent Creek Institute (BCI), compound **2.9** has not yet been submitted for assay testing as a pure compound and therefore exact assay data cannot be reported. However, a previous fraction containing compound **2.9** was tested.

The results were positive with the semi-pure compound showing 75.7% inhibition of MCF-7 cells at 100 µg/mL, and 70.3% inhibition of FS-4 cells at 100 µg/mL. The fraction contained some minor impurities of a falcarindiol derivative with known activity. Therefore the activity of compound **2.9** was not able to be confirmed due to the ending of our antitumor natural products drug discovery collaboration with BCI.

It should be noted that several problems arose from the particular MCF-7 assay. Several instances of disappearing activity, inconsistent results, and varied protocol were observed. While some of the activity inconsistencies could possibly be due to the synergistic effects described in chapter 1, it was hypothesized that the assay itself was subject to significant variability. It would be possible to rule sample degradation out by testing for synergism. An experiment could be performed by initially testing a parent fraction and subsequent daughter fractions to see if activity was lost. If in fact activity was lost in the experiment, daughter fractions could be recombined to test if the initial parent fraction's activity can be recovered. If in fact it is recovered, then synergistic effects are most likely the cause for activity loss. This particular experiment was unable to be performed due to monetary and time constraints.




COSY

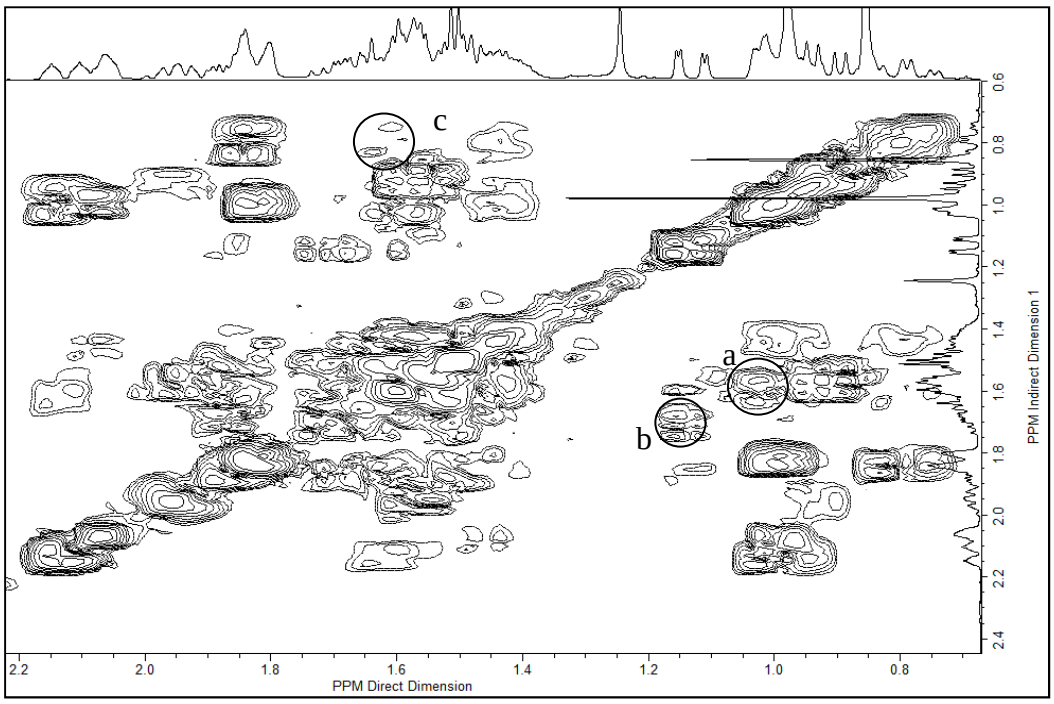



**Figure 2.6:** Additional COSY Correlations for Compound **2.9**

*a = a, b = b, c = c, …

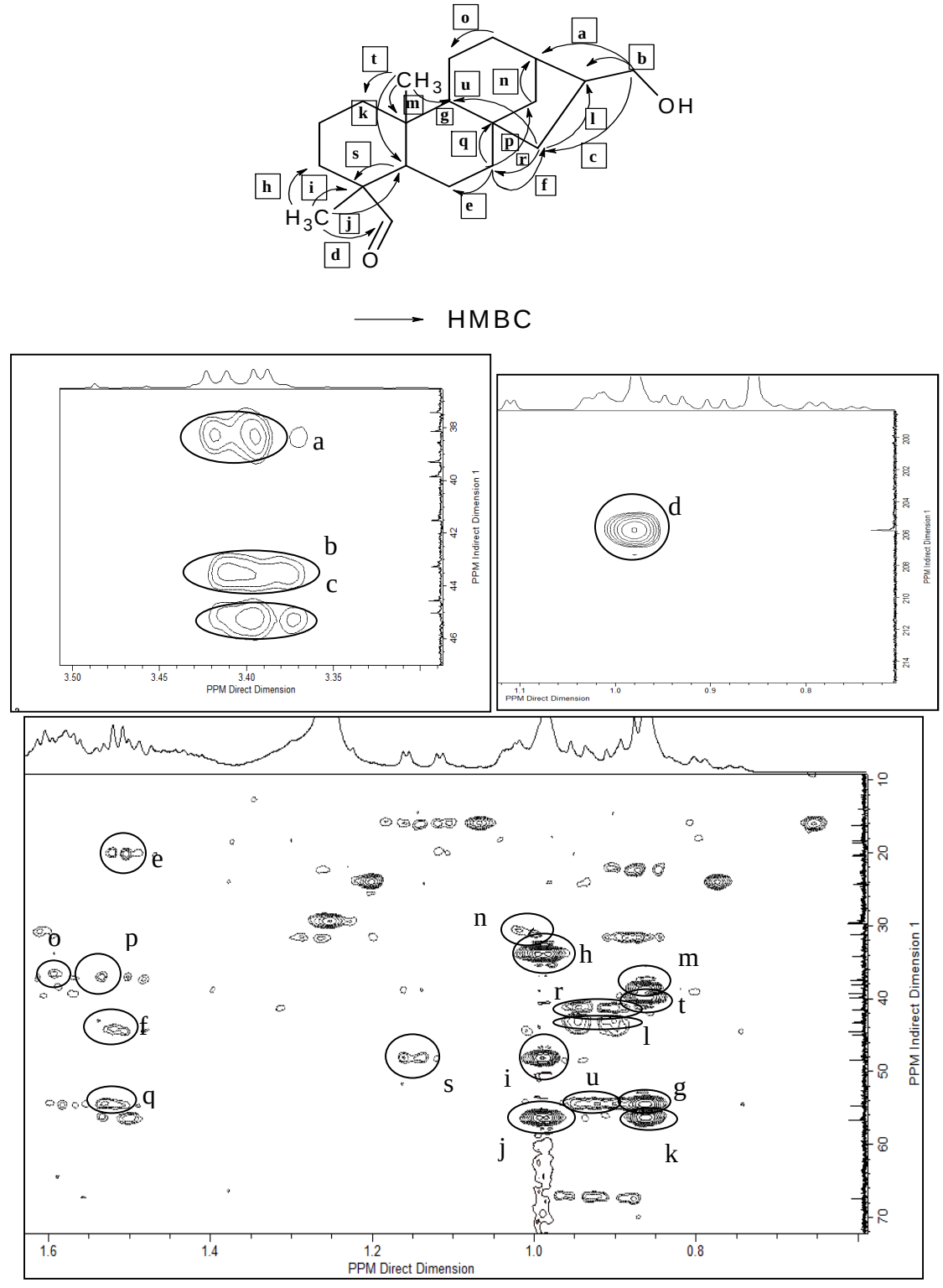


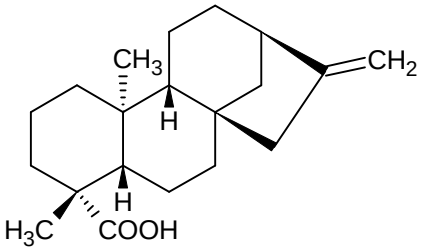

**Figure 2.7:** HMBC Correlations of Compound **2.9**

*a = a, b = b, c = c, …

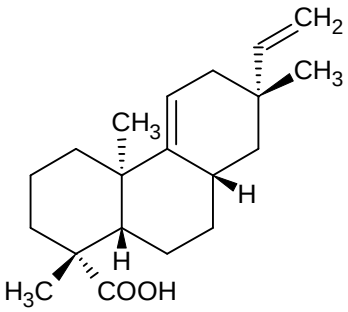
### 2.2.2 Previous Investigation of *ent*-Kaurane Diterpenoids

The *Aralia* genus is not the only type of plant to produce these *ent*-kaurane of diterpenoids. The plant *Annona squamosa* is also documented as producing these types of compounds. Yang-Chang Wu *et al.* isolated a number of *ent*-kauranoic diterpenoids from the fruits of *Annona squamosa*. Aside from the compound *ent*-16β,17-dihydroxykauran-19-oic acid (**2.7**), the majority of the compounds did not show any significant activity in the HIV assay. *ent*-16β,17-Dihydroxykauran-19-oic acid was identified as showing activity against HIV replication in H9 lymphocyte cells. The compound exhibited an $IC_{50}$ of 0.263 μM in the assay referenced.[31] *Annona squamosa* has also proved to be moderately effective in anti-platelet aggregation through the studies of Yu-Liang Yang *et al.* Yang *et al.* isolated and characterized twenty new and known *ent*-kaurane diterpenoids, two of which were found to inhibit platelet aggregation, the previously discussed *ent*-kaur-16-en-19-oic acid (**2.6**) and 16α-hydro-19-al-*ent*-kauran-17-oic acid (**2.8**). The compound **2.8** completely inhibited platelet aggregation induced by arachidonic acid and collagen at 200 μM, and compound **2.6** completely inhibited platelet aggregation induced by collagen at 200 μM.[32]

*ent*-Kaurenoic acid (**2.11**) was also previously isolated by the Clement group.[29] The pure compound was isolated from the same acidic extract as compound **2.9**, following the bioassay-guided fractionation scheme previously reported. Compound **2.11** was isolated along with a cytotoxic diterpenoid (**2.12**). Compound **2.11** was determined to be inactive after bioassay results were received, while **2.12** was reported as inhibiting 73% of MCF-7 cells at a concentration of 100 μg/mL and inhibiting 73% of FS-4 cells at a concentration of 100 μg/mL.[29]




**2.11**




**2.12**

## 2.3 Experimental

**General Experimental Procedures**. HPLC separations were performed on a Perkin Elmer Series 200 Pump and UV/Vis Detector using either a Varian Dynamax 250*10.0 mm (L*ID) Microsorb 100 Å-5mm C18 with a Varian Dynamax Guard ½" Microsorb 100-5 C18 guard column or a Varian Dynamax 250*21.4 mm (L*ID) Microsorb 100-5 C18 with a Varian Dynamax Guard 1" Microsorb 100-5 C18 guard column. The IR spectrum was recorded with a Perkin Elmer Spectrum One FT-IR spectrophotometer. NMR spectra were recorded on a JEOL 300 MHz Eclipse + FT NMR with 5mm FG/TH Tunable Probe. Mass spectral data were obtained on a Finnigan LTQ MS-Ion Mass API Source operating with a LC Packings Ultimate 3000 using a $C_{18}$ PepMag 100 3µm 100Å 15 cm column.

**Plant Material and Sample Preperation.** Whole plants were harvested by Josh Kelly in rich oak-hickory cove forests in Buncombe County, N.C. Plant material was ground and initially extracted by Ryan M. Kelly of the Bent Creek Institute, at the North Carolina Arboretum. Plants were lyophilized and ground to 250 nm particle size. Voucher samples were collected and stored at the Bent Creek Institute's Germplasm Repository at the North Carolina Arboretum. The powdered plant material (74.6 g) was extracted with

80% MeOH:$H_2O$, filtered, then extracted again with 2:1 $CH_2Cl_2$:MeOH. The 80% MeOH:$H_2O$ extract was concentrated by rotary evaporation to remove the MeOH, and the remaining aqueous material was partitioned by an acid-base extraction. All plant material collection and extract preparation was completed by Bent Creek Institute.

**Isolation and Characterization of 2.9.** The crude extract was subjected to an acid/base extraction, and the extract was initially partitioned between $CH_2Cl_2$ and $H_2O$ (pH 2). The aqueous layer was basified (pH 9), and further partitioned with $CH_2Cl_2$. The remaining $H_2O$ fraction was set aside as the aqueous fraction. The $CH_2Cl_2$ fraction following acidification of this extract was received from Bent Creek Institute under the sample number BCI00041B. Both the acidic fraction and basic fraction showed positive results in the MCF-7 assay. The fraction was selected for further investigation due to the partitioning resulting in a greater mass than all other fractions. The acidic fraction was fractionated further by the use of a $C_{18}$ open column, using various concentrations of aqueous methanol as a solvent system. Four active fractions resulted from the separation. The 80% MeOH fraction was selected to continue fractionation based on improved assay data. The 80% MeOH fraction was subjected to repeated $C_{18}$ reverse-phase HPLC that yielded seven fractions. $^1$H NMR analysis of one of the fractions showed compound **2.9** present in pure form. More extract was obtained and fractioned from Bent Creek Institute in order to recover enough of compound **2.9** for analysis following the same protocols and fractionations.

**Assay Preparation**. High-throughput 96-well format viability assays were used to determine the anti-tumor effects of natural product chromatographic fractions. Breast (MCF-7) tumor cells were the representative tumor cell line, while human foreskin

fibroblasts (FS4) were the representative non-tumor tissue. MCF-7 and FS4 cells were maintained in Dulbecco's Modified Eagle Medium and 10% Fetal Bovine Serum. Each type of cell was seeded at ~5,000 cells/well in 96-well plates. After seeding for 24-36 hours, fresh medium was added to the cells in triplicate. Each sample had a three-point dilution yielding concentrations of 100 µg/ml, 50 µg/ml, and 10 µg/ml. After 36 hours the viability of each treated group was assessed relative to the control group by the MTS assay (Promega®). The assay measured the cell's viability through its capability to reduce the tetrazolium salt, MTS, into a soluble formazan product that can be spectrophotometrically detected at 492 nm. Extracts/Fractions/Compounds that elicit cell death or reduced proliferation were detected by a loss of absorbance when compared to untreated cells.

**17-Hydroxy-16α-ent-kauran-19-al** (**2.9**): white amorphous powder; IR (neat film) 3378, 2925, 2852, 1718, 1463, 1369, 1248, 1060, 1019, 970, 906, 890, 763, 738 $cm^{-1}$; $^{1}H$ NMR ($CDCl_3$) see Table 2.1; $^{13}C$ NMR ($CDCl_3$) see table 2.1; LRESI-MS (positive ion) 305.1 ($[M+H]^+$, calc. for $C_{20}H_{32}O_2$: 305.2)

# 3. ISOLATION AND CHARACTERIZATION OF 1β-ACETOXY-6α, 7α - DIANGELOYLOXY-2-OXO-OPLOPA-3, 14Z, 11, 12-DIENE-13-AL, AN OPLOPANE DERIVATIVE FROM THE PLANT *ARNOGLOSSUM ATRIPLICIFOLIUM* (*CACALIA ATRIPLICIFOLIA*)

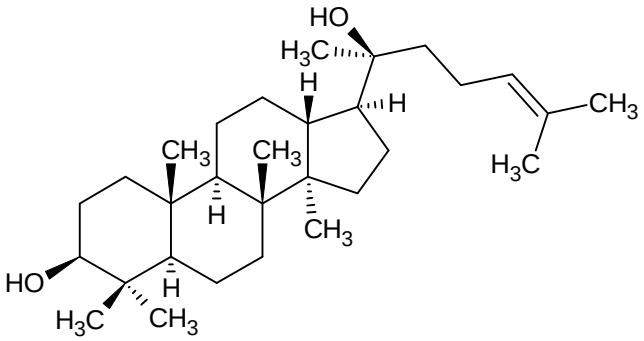
## 3.1 Introduction

The plant *Arnoglossum atriplicifolium* has been studied chemically very little throughout history. Using the breast cancer cell line assay MCF-7, 1β-Acetoxy-6α, 7α-diangeloyloxy-2-oxo-oplopa-3, 14Z, 11, 12-diene-13-al, an oplopane derivative was isolated from an extract of the plant, *Arnoglossum atriplicifolium (Cacalia atriplicifolia)* by bioassay-guided fractionation. The results for the isolation and characterization of the natural product 1β-Acetoxy-6α, 7α-diangeloyloxy-2-oxo-oplopa-3, 14Z, 11, 12-diene-13-al, are provided below. The biological activity of the oplopane derivative from *Arnoglossum atriplicifolium* is reported below.

### 3.1.1 Previous Investigation into the *Arnoglossum (Cacalia)* Species

Previously, the genus *Arnoglossum* (previously known as *Cacalia*) has shown some promising results in a variety of assays, with activities ranging from anti-fungal and anti-malarial properties all the way to anti-tumor activities against certain tumor cell types. The species *A. atriplicifolium* has been studied very little up to this point from a bioactivity standpoint, with research mainly focusing on phytochemical and oil studies. The previous history of the genus and compounds found in these types of plants, along with the lack of study of the plant *Arnoglossum atriplicifolium* and its bioactivity specifically, make it a prime candidate for our research.

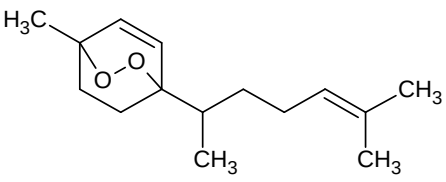
The species *Arnoglossum (Cacalia)* has yielded many different kinds of compounds in previous studies, including oplopanes, furans, coumarins, triterpenoids, and kaurenoids. Most of these studies were purely phytochemical while others were based on earlier studies in search of natural sources of both oil and rubber for industrial uses. In a study aimed at isolating oil and rubber compounds out of *Cacalia atriplicifolia* as potential oil and rubber sources, Spencer isolated the compound dammarenediol II (**3.1)**. Around a quarter of the seed oil from *Cacalia atriplicifolia* is composed of the long-chain fatty esters of **3.1.** Also, a small amount of the seed oil was reported to be composed of long-chain fatty esters from oleanolic acid.[33]



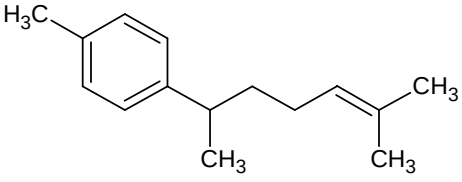

**3.1**

The genus *Cacalia* has also yielded some positive results when tested against various bioassays *in vitro.* Nishikawa *et al.* isolated the bisabolane sesquiterpenoid endoperoxide, 3,6-Epidoxy-1,10-bisaboladiene (**3.2**) from the edible wild-plant *Cacalia delphiniifolia*, a plant found commonly in the northern part of Japan. The group isolated **3.2** and tested it against both the K562 cell assay (human chronic myelogenous leukemia) and the LNCaP cell assay (human prostate carcinoma). The results were positive showing $IC_{50}$ values to be 9.1 µM and 23.4 µM respectively. For comparison the group ran α-Curcumene (**3.3)**, a known anti-tumor agent commonly found in a large number of

plants and essential oils. Compound **3.3** presents an interesting comparison as it lacks an endoperoxide moiety, but aside from the endoperoxide the structure is basically the same. When **3.3** was exposed to the same assays, it exhibited over ten times less potency (256.5 µM and 277.5 µM), suggesting the endoperoxide moiety is indeed a major part of the activity of **3.2**.[34]

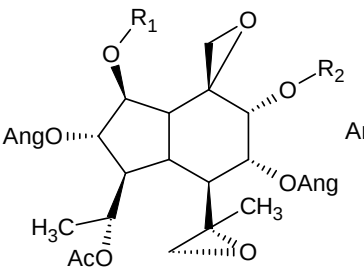


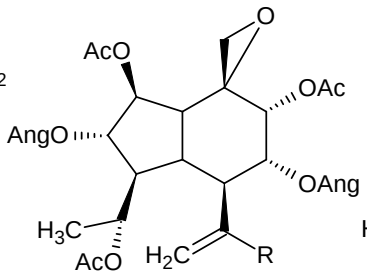

**3.2**

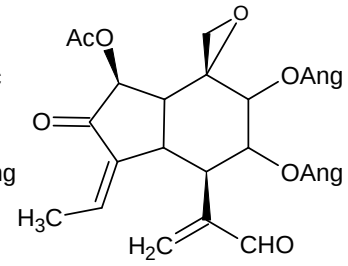


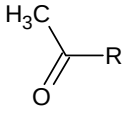
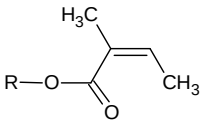

**3.3**

The species *C. atriplicifolia (A. atriplicifolium)* has also been studied from a phytochemical standpoint. Bohlmann *et al.* isolated a series of highly oxygenated oplopane derivatives. Bohlmann's group was in an ongoing investigation into the tribe *Senecionea*, which led to their study of the aerial parts of *Arnoglossum atriplicifolium*. The oplopane derivatives (**3.3, 3.4, 3.5, 3.6, 3.7)** were reported with their respective $^{1}$H NMR data.[35]




| | $R_1$ | $R_2$ | | R | | 3.7 |
|---|---|---|---|---|---|---|
| **3.3** | **H** | **Ang** | **3.5** | **CHO** | **Ac =** |  |
| **3.4** | **Ac** | **Ac** | **3.6** | **$CH_2OH$** | **OAng =** |  |

## 3.2 Results and Discussion

### 3.2.1 Isolation and Characterization of 3.8

Isolation of 1β-Acetoxy-6α, 7α-diangeloyloxy-2-oxo-oplopa-3, 14*Z*, 11, 12-diene-13-al (**3.8**) is given in Figure **3.1**. An acidic fraction of *Arnoglossum atriplicifolium* (see section 2.3 for the protocol for sample preparation) was initially found to be active in BCI's antitumor assay screening, with 89% inhibition of MCF-7 cell proliferation at 125 μg/mL. The *Arnoglossum atriplicifolium* crude extract subsequently generated by organic solvent extraction showed promising activity in the MCF-7 cell assay, with an $IC_{50}$ = 19.0 μg/mL. The crude extract was then subjected to a modified Kupchan partitioning, resulting in four fractions, two of which were active. After bioassay guided fractionation by reverse phase $C_{18}$ open column chromatography, reverse phase $C_{18}$ HPLC, and normal phase $SiO_2$ HPLC on active fractions, **3.8** was isolated as the most active fraction in the crude sample. Due to the small sample size of **3.8** that prevented in depth analysis, more of the compound needed to be isolated. In a simultaneous bioassay guided experiment conducted in the Clement Lab, two undergraduate students isolated compound **3.8** from the hexane fraction in greater yield from a fraction generated at BCI. After comparison of the samples by $^{1}H$ NMR analysis it was determined that the compounds were the same and analysis was continued with the sample of greater mass.

$^{1}H$ and $^{13}C$ NMR data were collected in *d*-chloroform ($CDCl_3$) and also *d*-benzene ($C_6H_6$) and reported in Table **3.1**. The $^{1}H$ NMR spectrum of **3.8** showed several key proton signals. These included an acetate methyl singlet ($\delta_H$ 2.09), two epoxide doublets ($\delta_H$ 2.84 and 3.06), an aldehyde singlet ($\delta_H$ 9.64), and several doublets of quartets ($\delta_H$ 1.89 and 2.01) and quartets of quartets ($\delta_H$ 6.03 and 6.17). The $^{13}C$ NMR experiment

yielded twenty-three signals in $CDCl_3$. Key signals observed included two carbonyls at $\delta_C$ 193.26 and 199.07 ppm, several $sp^2$ alkene carbon signals, and several oxygenated carbon signals. Two of the oxygenated carbons appeared to be part of an epoxide as the chemical shifts of the signals, were shifted downfield to $\delta_C$ 50.97 and 56.98 ppm. The $^1H$ spectra also showed two doublets at $\delta_H$ 2.84 and 3.06 ppm, which confirmed the presence of an epoxide in compound **3.7**. A DEPT-135 experiment helped in the determination of the presence of *at least* eight methines, one methylene, and six methyls. A HSQC analysis was also performed on compound **3.8** showing carbon-proton one bond coupling. These results are depicted in Table **3.1**. These HSQC correlations allowed for the interpretation of the HMBC spectra. Further investigation of the $^{13}C$ spectrum revealed that several carbon signals were not appearing in the spectra due to conformational issues, and possible overlapping of signals. A prime example of these discrepancies were the presence of three esterified methine protons in the 1D spectrum, while only two oxygenated carbons were observable in the $^{13}C$ spectrum. Several signals were observed in the HSQC and HMBC spectra that were not clearly observable in the $^1H$ spectrum. The conformational issues will be addressed later in this section. These issues were handled in order to confirm the structure.

**Figure 3.1:** Isolation of Compound **3.8**

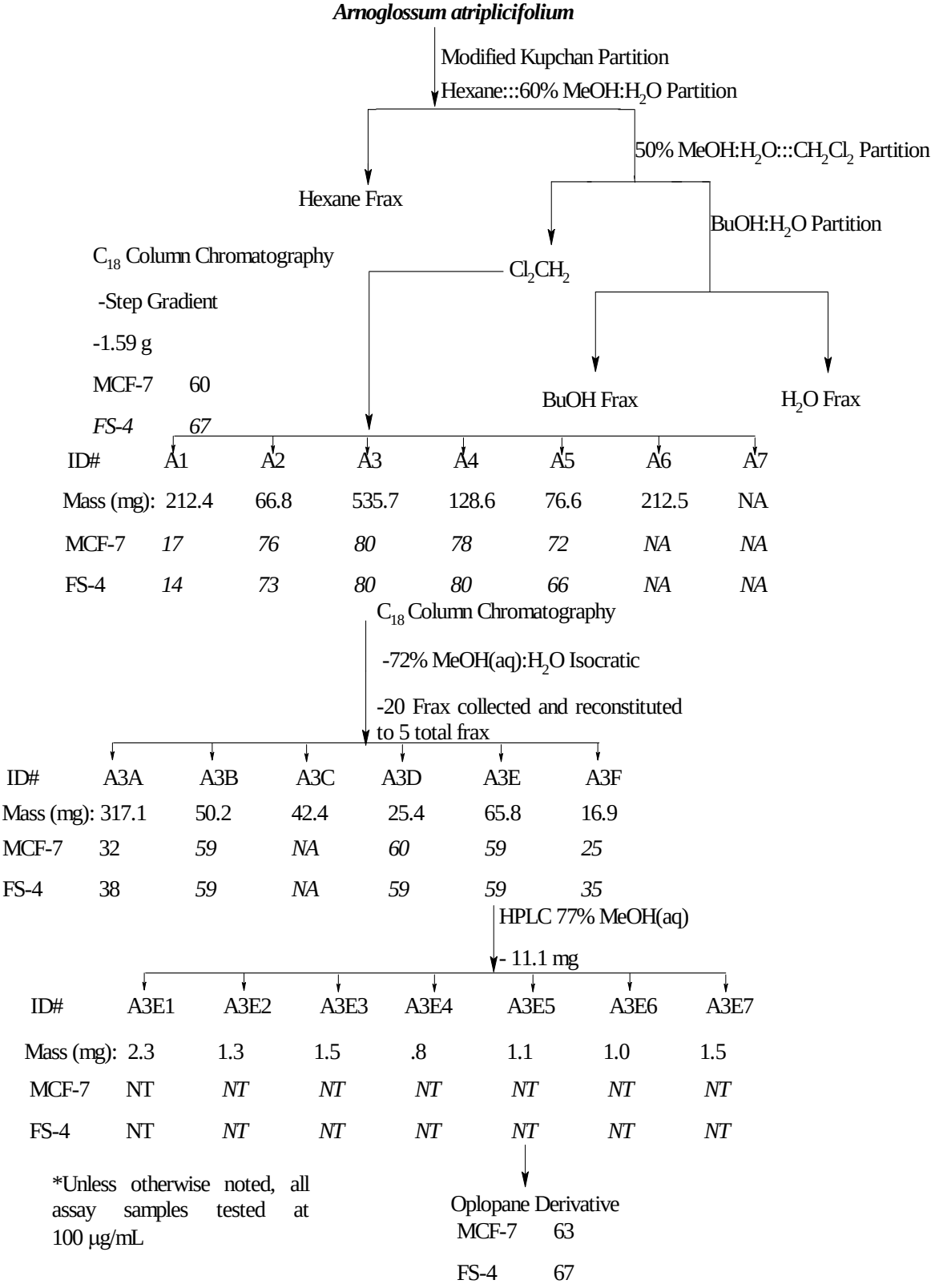


**Table 3.1:** $^{13}C$ and $^{1}H$ NMR Data for **3.8**

| | $\delta_C$[a] | DEPT-135 | $\delta_H$[a] | | $\delta_H$[b] | |
|---|---|---|---|---|---|---|
| 1 | 70.99 | CH | 5.13 | d, $J_1$ = 3.1 | 5.07 | d, $J_1$ = 4.8 |
| 2 | 199.07 | C | - | - | - | - |
| 3 | 136.23 | C | - | - | - | - |
| 4 | + | CH | + | - | + | - |
| 5 | + | CH | + | - | 3.71 | m |
| 6 | + | CH | 5.30 | bs | 5.47 | dd, $J_1$ = 11.0, $J_2$ =3.0 |
| 7 | 74.02 | CH | 4.86 | d | 5.17 | d, $J_1$ = 3.1 |
| 8 | 56.98 | C | - | - | - | - |
| 9 | 42.20 | CH | 2.47 | dd, $J_1$ = 5.2, $J_2$ = 2.0 | 2.34 | dd, $J_1$ = 12.9, $J_2$ =4.8 |
| 10 | 50.97 | $CH_2$ | 2.84 | d, J = 4.5 | 2.93 | d, $J_1$ = 4.8 |
| | | | 3.06 | d, J = 4.5 | 2.62 | d, $J_1$ = 4.6 |
| 11 | + | C | - | - | - | - |
| 12 | 136.23 | $CH_2$ | 6.29 | s | 5.75 | s |
| | | | 6.63 | - | 5.38 | s |
| 13 | 193.26 | CH | 9.64 | s | 9.20 | s |
| 14 | 138.32 | CH | 5.90 | bs | 5.79 | m |
| 15 | 15.10 | $CH_3$ | 2.08 | dd, $J_1$ = 5.2, $J_2$ = 2.0 | 1.92 | m |
| Ang1 1 | 166.04 | C | - | - | - | - |
| Ang2 1 | 166.01 | C | - | - | - | - |
| Ac 1 | 170.01 | C | - | - | - | - |
| Ang1 2 | 127.16 | C | - | - | - | - |
| Ang2 2 | 126.81 | C | - | - | - | - |
| Ac 2 | 21.02 | $CH_3$ | 2.09 | s | 1.74 | s |
| Ang1 3 | 140.13 | CH | 6.17 | qq, $J_1$ = 7.2, $J_2$ = 1.3 | 5.77 | m |
| Ang2 3 | 140.39 | CH | 6.03 | qq, $J_1$ = 7.2, $J_2$ = 1.4 | 5.68 | dq, $J_1$ = 7.0, $J_2$ = 1.5 |
| Ang1 4 | 20.42 | $CH_3$ | 1.97 | m | 1.95 | m |
| Ang2 4 | 20.88 | $CH_3$ | 1.67 | m | 1.72 | m |
| Ang1 5 | 16.20 | $CH_3$ | 2.01 | dq, $J_1$ = 7.3, $J_2$ = 1.5 | 1.97 | dq, $J_1$ = 7.7, $J_2$ = 1.5 |
| Ang2 5 | 15.90 | $CH_3$ | 1.89 | dq, $J_1$ = 7.4, $J_2$ = 1.5 | 1.87 | dq, $J_1$ = 7.0, $J_2$ = 1.6 |

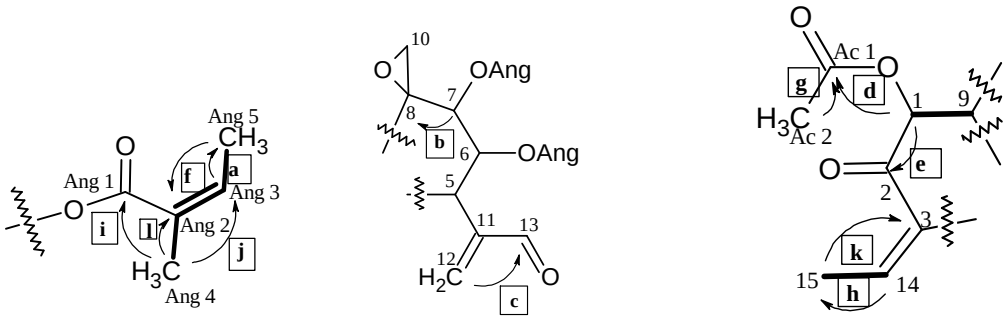
*All units in ppm

[a]Spectra were acquired using $CDCl_3$ (*d*-chloroform)

[b]Spectra were acquired using $C_6D_6$ (*d*-benzene)

+ = signal unobservable in spectrum

Low resolution positive ion ESI-MS of compound **3.8** gave a pseudomolecular ion of *m/z* 501.4 ([M+1]) when examined. Upon analysis of $^{1}H$, $^{13}C$, DEPT-135, HSQC NMR data, and MS data for **3.8** it was determined that compound **3.8** was very similar to compound **3.7** reported by the Bohlmann group as mentioned in section 3.1. Compound **3.7** was also reported with a molecular ion of 500.2 *m/z* ($[M^{+}]$) with molecular formula of $C_{27}H_{32}O_9$, further providing clues that there were missing carbons in the $^{13}C$ NMR data for compound **3.7**. The molecular formula of compound **3.8** could not be $C_{27}H_{32}O_9$ with the nnumber of carbons observed in the $^{13}C$ spectra. Due to chemical shift similarities at the protons on C-5, C-10, C-13, C-15, C-21, along with several others between compound **3.8** and the Bohlmann compound **3.7**, it was hypothesized that compound **3.8** had a chemical formula of $C_{27}H_{32}O_9$ with a mass of 500.4 g/mol, and was most likely the same as compound **3.7**. From this information it was also deduced that compound **3.8** had a degree of unsaturation of eleven rings plus double bonds. The remaining step was to use 2D NMR data to confirm whether compound **3.8** was the same as **3.7**. Working under the hypothesis that compound **3.8** was the same as the published Bohlmann compound **3.7**, $^{1}H$-$^{1}H$ COSY correlations and HMBC correlations were examined to see if the compounds were indeed the same to test the hypothesis.




A B C

= COSY = HMBC

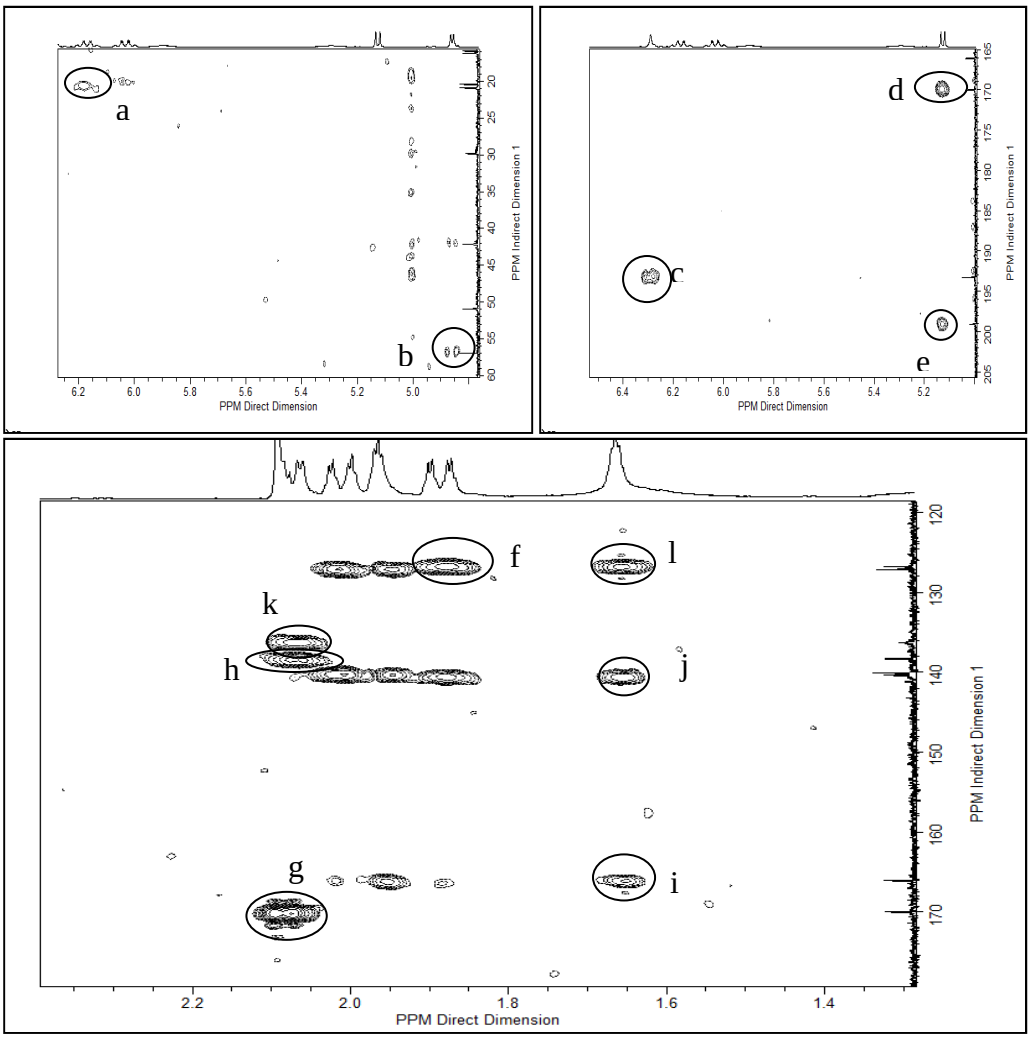



**Figure 3.2:** HMBC Correlations of Segments A, B, and C

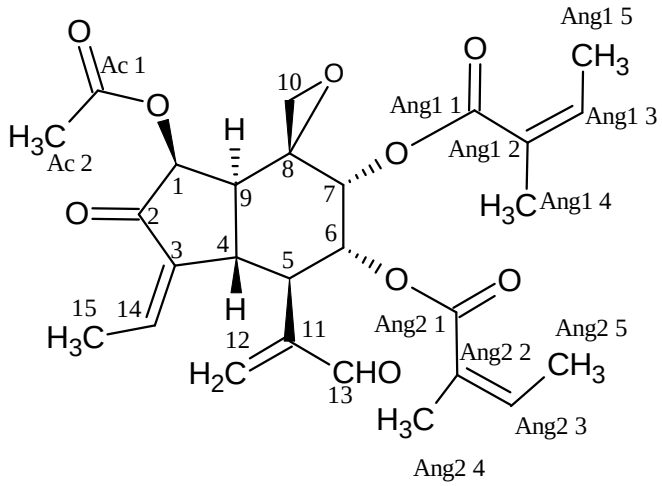
*a = a, b = b, c = c, …

Several COSY correlations were found that pieced together each of the angeloxy groups as seen in Segment A. Coupling was observed between the methine group at $\delta_H$ 6.03 and the methyl group at $\delta_H$ 1.89, in the angeloxy group. A coupling was also observed between these two methyl groups at $\delta_H$ 1.67 and 1.89. HMBC correlations were also observed in the angeloxy groups, specifically between the quaternary carbon at $\delta_C$ 126.81 and the methyl protons at $\delta_H$ 1.67 and 1.89. A correlation was also observed between the quaternary carbon at $\delta_C$ 126.81 and the methine at $\delta_H$ 6.03. Also, a second quaternary carbon at $\delta_C$ 166.01 was observed as correlating to the methyl proton at $\delta_H$ 1.67. A second angeloxy group was also observed that showed similar correlations, in both the HMBC and COSY at similar chemical shifts. These correlations, seen in Figure **3.3**, helped establish the presence of two angeloxy groups in compound **3.8**.

The conformational issues previously mentioned were resolved by running addition NMR experiments in *d*-benzene at 70° C. These parameters allowed for compound **3.8** to achieve a single energy state as opposed to the many different energy states that were present under normal conditions. The presence of a single energy state allowed for the resolution of several signals in compound **3.8** that are depicted in Table **3.1**.

A signal for the methine where the angeloxy group was connected to the rest of the skeleton of compound **3.8** also provided a clue as to the orientation of compound **3.8**. The methine proton at $\delta_H$ 4.86 showed a HMBC correlation to the quaternary carbon at $\delta_C$ 56.98, suggesting the epoxide was located near the angeloxy group. These correlations, seen in Figure **3.2**, along with the detected epoxide and angeloxy groups suggested a fragment such as segment B was present in compound **3.8**. The segment also suggests

that, if the Bohlmann structure is correct, C-5, C-6, and C-11 were not showing up in the spectra as they were not present in the $^{13}C$ spectrum nor showing any correlations in the 2D spectra.




**3.8**

Finally the assignments of the ketone and acetyl groups needed to be addressed. The acetoxylated methine group at $\delta_H$ 5.13 was found to be coupled with the methine at $\delta_H$ 2.47 through a COSY correlations, showing that the two methine groups are next to each other. The methyl proton signal at $\delta_H$ 2.08 was found to be coupled with the methine proton at $\delta_H$ 5.90 in COSY, indicating the position of one of the carbon-carbon double bonds in the group. HMBC analysis, shown in Figure **3.4**, showed correlations between the acetyl methyl proton at $\delta_H$ 2.09 and the quaternary carbon at $\delta_C$ 170.01, suggesting the presence of an acetate group in compound **3.8**. The quaternary carbon at $\delta_C$ 170.01 also showed a correlation to the methine proton at $\delta_H$ 5.13, strongly suggesting the assignment of the acetyl group. A correlation was also found between the quaternary

carbon at $\delta_C$ 136.23 and the methyl proton at $\delta_H$ 2.08, suggesting the methyl group is connected to the methine carbon of the C-C double bond. Also a key correlation could be observed to the ketone at $\delta_C$ 199.07 to the acetoxylated methine group at $\delta_H$ 5.13, confirming the location of the ketone and assignment of segment C.

In order to piece together segments B and C, a correlation was observed between the methine proton at $\delta_H$ 5.13 and the methine carbon at $\delta_C$ 74.02. This correlation provided a direct correlation between segment B and C, strongly suggesting that segments B and C were connected. These correlations along with the COSY data suggested the epoxide was located between the acetate and angeloxy substituents. These connections showed evidence of **3.8**, which was in accordance with the published Bohlmann *et al*. compound. Complete COSY and HMBC data are provided in Figures **3.2** and **3.3**.

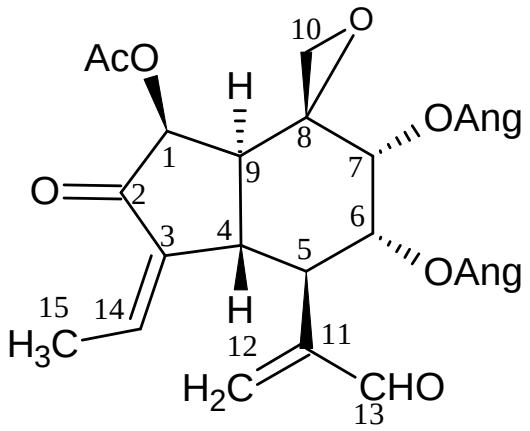



**Figure 3.3:** Proposed Structure of Compound **3.8**

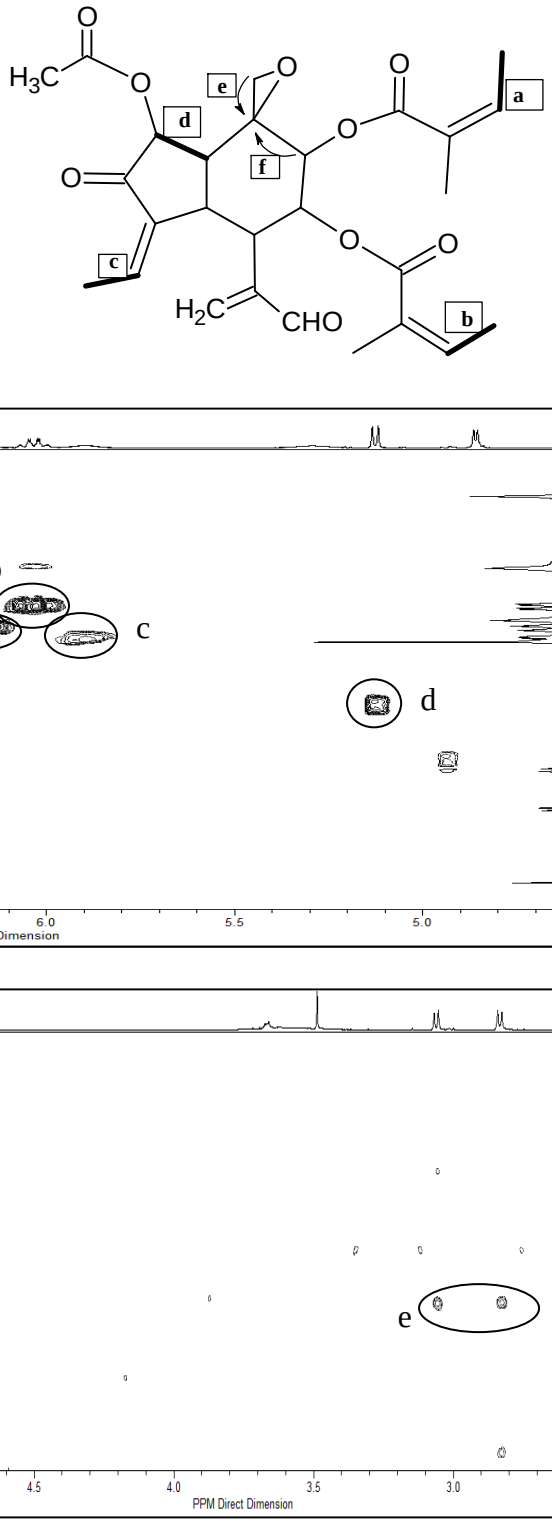



**Figure 3.4:** HMBC and COSY correlations of compound **3.8**

*a = a, b = b, c = c, …

Upon deeper investigation of the proton spectrum an important element was absent. The doublet of doublets that would be expected to be present representing the C-6 angeloxy group being split by both the C-5 and C-7 methine protons connecting the was not observed in the $^{1}H$ spectum. Several broad one-proton signals were observed in the region where this signal would be expected to appear. Upon further study of the Bohlmann *et al.* paper it was discovered that running the sample in *d*-benzene ($C_6D_6$) at a temperature of 70° C can result in improved spectrum and resolved peaks. This was reported in the Bohlmann *et al.* paper, as they were experiencing the same difficulties with broad singlets and unresolved peaks. After running the sample under these conditions a doublet of doublets was observed at $\delta_H$ 5.62. These peaks along with the subsequent comparison of all proton data, in both $C_6D_6$ and $CDCl_3$, further validated the proposed structure of the compound isolated (**3.8**). The absolute configuration for compound **3.8** was not determined, nor was it reported by Bohlmann *et al.*

Several attempts were made to improve upon the data set using the 300 MHz instrument with a direct probe. The direct probe (proton observe coil on outside of probe) causes an increase in proton signal to noise. This signal to noise increase on proton spectra proved to be problem in our work as many spectra were not as clear. Initially compound **3.8** was run at increased scans in order to improve signal to noise strength, to no avail. Several alternate 2D experiments including a NOE experiment, which measures proton coupling through space, to see if any additional cross peaks could be observed that would provide any information as to the orientation of compound **3.8.** The NOE also failed to provide any relevant information or peaks that would be expected from the spectrum. Also implemented was the use of a Shigemi® tube, a piece of

equipment that allows for a smaller solvent volume to be used, therefore increasing sample concentration. After implementation of a Shigemi® tube run at increased scans (experiments ranged from 12 to 36 hrs), some improvement was observed, but peaks were obviously still not resolved or showing up. In an attempt to acquire an improved data set for compound **3.8**, the compound was sent to a collaborator at Youngstown State in Ohio, with access to a 400 MHz NMR with an indirect probe. The higher field NMR should, in theory, improve upon the data set and possibly resolve peaks that the 300 MHz was unable to improve upon, while the indirect probe (proton observe coil on inside of probe) should allow for better resolution of the proton spectra. Upon receiving the outsourced spectra from the higher field NMR, no real improvement was observed and no further key correlations could be found. These failed attempts at trying to acquire better data for compound **3.8** led the group to believe that the best resource in deciphering the structure of compound **3.8** the previously used Bohlmann *et al.* paper, specifically using compound **3.7**, the 1β-Acetoxy-6α, 7α-diangeloyloxy-2-oxo-oplopa-3, 14Z, 11, 12-diene-13-al.

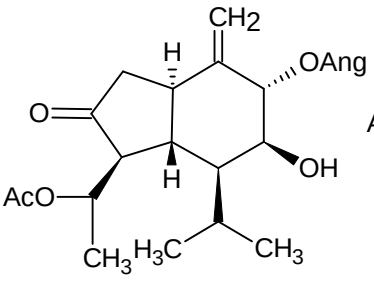
In order to obtain an improved data set, simply more of compound **3.8** would need to be isolated from the $CH_2Cl_2$ and hexane fractions of *Arnoglossum atriplicifolium.* Due to insufficient material mass provided from Bent Creek Institute and time and monetary constraints, this task was unable to be performed. Future plans include the isolation of more of the compound and determine the absolute configuration of compound **3.8**.

Compound **3.8** was determined to be the most active compound in the organic extract of *Arnoglossum atriplicifolium.* Compound **3.8** was found to inhibit 62.6% of MCF-7 cells at 100 µg/mL and 66.6% of FS-4 cells at the same concentration. The $IC_{50}$

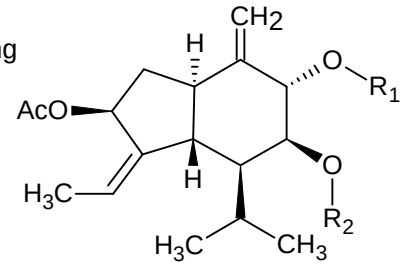
of compound **3.8** was determined to be 19.02 µg/mL. This is the first report of antitumor activity of a purified compound from the plant *Arnoglossum atriplicifolium*.

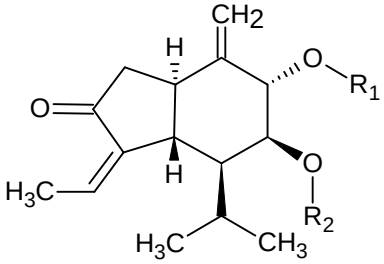
### 3.2.2 Previous Investigation of Oplopane Derivatives

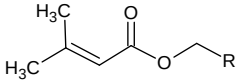
These types of oplopane derivatives have also been found in other plant species, such as *Acrisione denticulata.* Abdel Aal *et al.* isolated a total of eleven new oplopane derivatives and a new furolabdane from an extract of the aerial parts of *Acrisione denticulate* (*Senecio yegua*). The genus *Acrisione* is believed to be part of the same subtribe (Tussilagininae) as *Arnoglossum* (*Cacalia*), which the chemistry supports. The oplopanes reported had three main structures with several derivatives, **3.9 - 3.20**.[36]

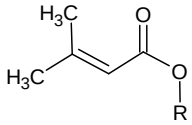



**3.9**




Mesen = 


Sen = 


| | $R_1$ | $R_2$ | | $R_1$ | $R_2$ |
|---|---|---|---|---|---|
| **3.10** | Ang | H | **3.14** | Mesen | H |
| **3.11** | H | Ang | **3.15** | Mesen | Ac |
| **3.12** | Mesen | H | **3.16** | Sen | H |
| **3.13** | H | H | **3.17** | Ang | H |
| | | | **3.18** | H | Ang |
| | | | **3.19** | Ang | H |
| | | | **3.20** | H | Ang |

### 3.3 Experimental

**General Experimental Procedures**. HPLC seperations were performed on a Perkin Elmer Series 200 Pump and UV/Vis Detector using either a Varian Dynamax 250*10.0 mm (L*ID) Microsorb 100-5 C18 with a Varian Dynamax Guard ½" Microsorb 100-5 C18 guard column or a Varian Dynamax 250*21.4 mm (L*ID) Microsorb 100-5 C18 with a Varian Dynamax Guard 1" Microsorb 100-5 C18 guard column. IR spectra were recorded with a Perkin Elmer Spectrum One FT-IR spectrophotometer. NMR spectra were recorded on a Jeol 300 MHz Eclipse + FT NMR with 5mm FG/TH Tunable Probe. Mass spectral data were obtained on a Finnigan LTQ MS-Ion Mass API Source operating with a LC Packings Ultimate 3000 using a $C_{18}$ PepMag 100 3µm 100Å 15 cm column.

**Plant Material and Sample Preperation**. Whole plants of *Arnoglossum atriplicifolium* were wild-harvested by botanist Josh Kelly, in rich cove forests of 3000-4000 ft elevation throughout Buncombe County, N.C. Voucher samples were collected and stored at the Bent Creek Institute's Germplasm Repository at the North Carolina Arboretum. Plant material was ground and extracted by Ryan M. Kelly of the Bent Creek Institute, either at the N.C. Arboretum or at Western Carolina University. Finely ground plant material was suspended in $CH_2Cl_2$:MeOH (1:1) overnight, at a ratio of 1 g plant material per 50 ml solvent. The resulting solution was then filtered, and the filtercake was resuspended in 100% methanol and left for 30 minutes. The MeOH extraction was filtered and combined with the $CH_2Cl_2$:MeOH solution and dried to produce the crude extract. Using a separate approach, additional plant material was extracted using pure $CH_2Cl_2$, followed by pure MeOH. The MeOH and $CH_2Cl_2$ extractions were then combined to produce

another crude extract. Extraction of approximately 240 g of powdered root material by these two methods yielded approximately 20 g of crude organic extract.

**Isolation and Characterization of 3.8.** The crude extract was subjected to a modified Kupchan Partitioning, where the crude extract was partitioned between hexane and 60% MeOH/$H_2O$ solution with the hexane fraction being set aside. Water was added to the remaining 60% MeOH (aq) mixture to make it 50% MeOH (aq), which was then subjected to a liquid partition with $CH_2Cl_2$, with the $CH_2Cl_2$ fraction set aside. The remaining MeOH was then rotovapped away to leave an aqueous solution. The aqueous solution was then washed with three aliquots of BuOH and separated out, to leave a BuOH fraction and aqueous fraction for a total of four fractions. The crude extract of the plant was received from Bent Creek Institute under the sample number BCI00041 II and BCI00041IV. Both the hexane fraction and dichloromethane fractions showed positive results in the MCF-7 assay. The $CH_2Cl_2$ fraction was further fractionated by the use of a $C_{18}$ open column, using various concentrations of MeOH/ $H_2O$ as a solvent system. Four active fractions resulted from the separation. The most active fraction, the 70% MeOH wash, was further fractionated through $C_{18}$ open column chromatography using C18 TLC of 72% MeOH/$H_2O$ to give a good overall separation of sample components, resulting in twenty fractions which were reconstituted into five total fractions. Three active fractions resulted from the separation. The 100% MeOH wash was selected for further fractionation based on total mass and activity. The 100% MeOH wash was then subjected to a $C_{18}$ reverse-phase HPLC method of 77% MeOH/$H_2O$ that yielded eight fractions, two of which were active. After a “clean-up” step of $SiO_2$ HPLC at 90%

Hexane/IPA, $^{1}$H NMR analysis of one of the fractions showed compound **3.8** present in pure form.

**Assay Preparation.** See section **2.3**.

**1β-Acetoxy-6*α*, 7*α*-diangeloyloxy-2-oxo-oplopa-3, 14Z, 11, 12-diene-13-al** (**3.8**): white amorphous powder; IR (neat film) 2927, 1725, 1645, 1457, 1371, 1225, 1154, 1044, 969, 849 $cm^{-1}$; $^{1}$H NMR ($CDCl_3$) see Table 3.1; $^{13}$C NMR ($CDCl_3$) see table 3.1; LRESI-MS (positive ion) 501.4 ($[M+H]^+$, calc. for $C_{27}H_{32}O_9$: 501.2)

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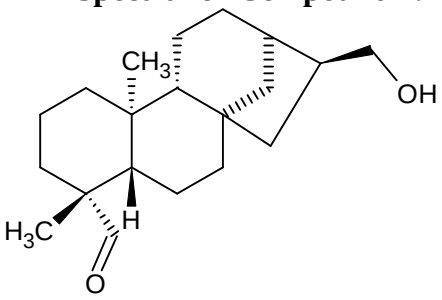
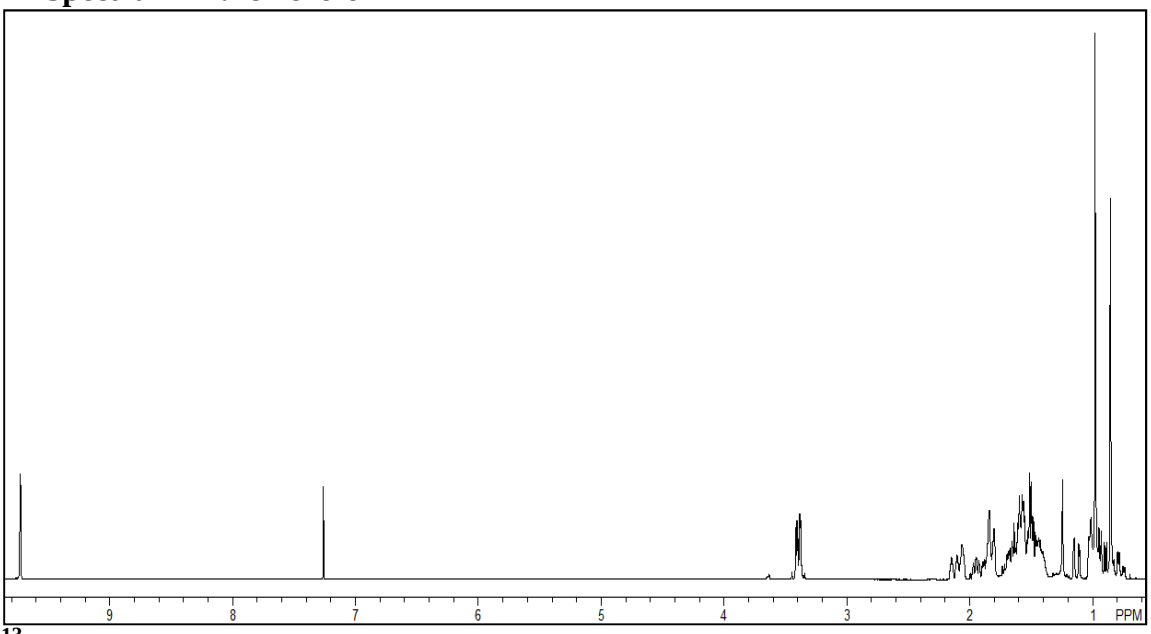
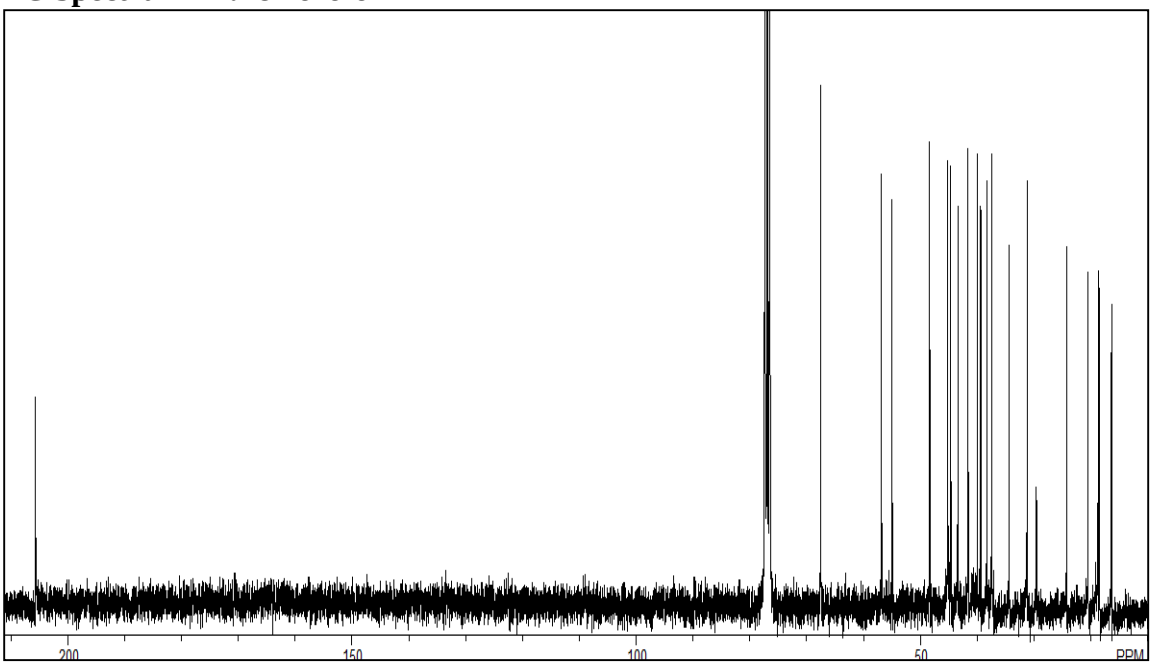
**Appendix A: $^{1}$H and $^{13}$C NMR Spectra for Compound 2.10**



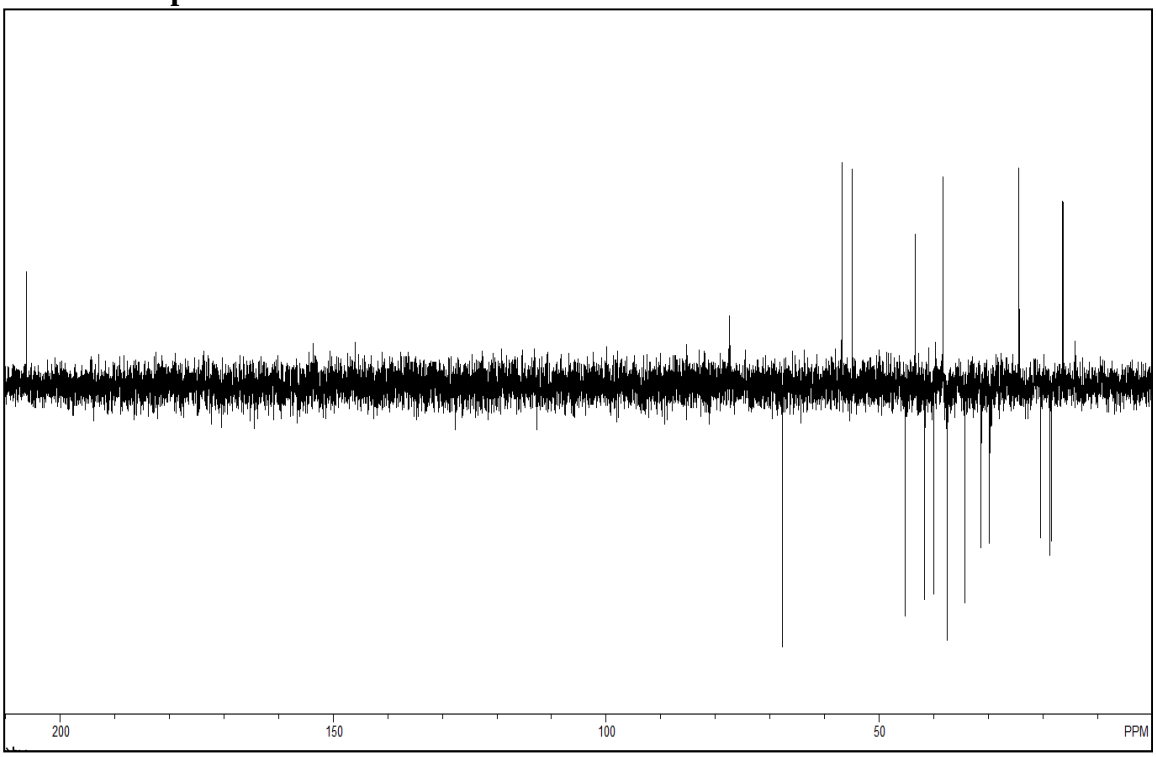

**$^{1}$H Spectrum in *d*-chloroform**



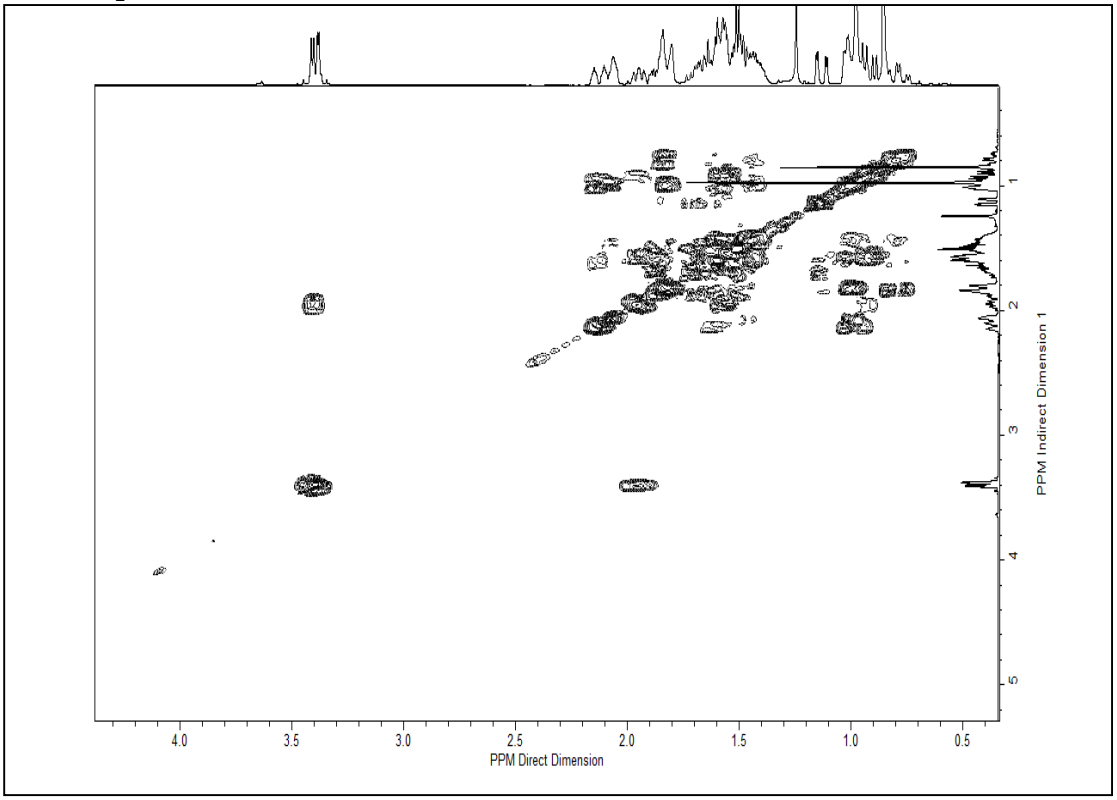

**$^{13}$C Spectrum in *d*-chloroform**



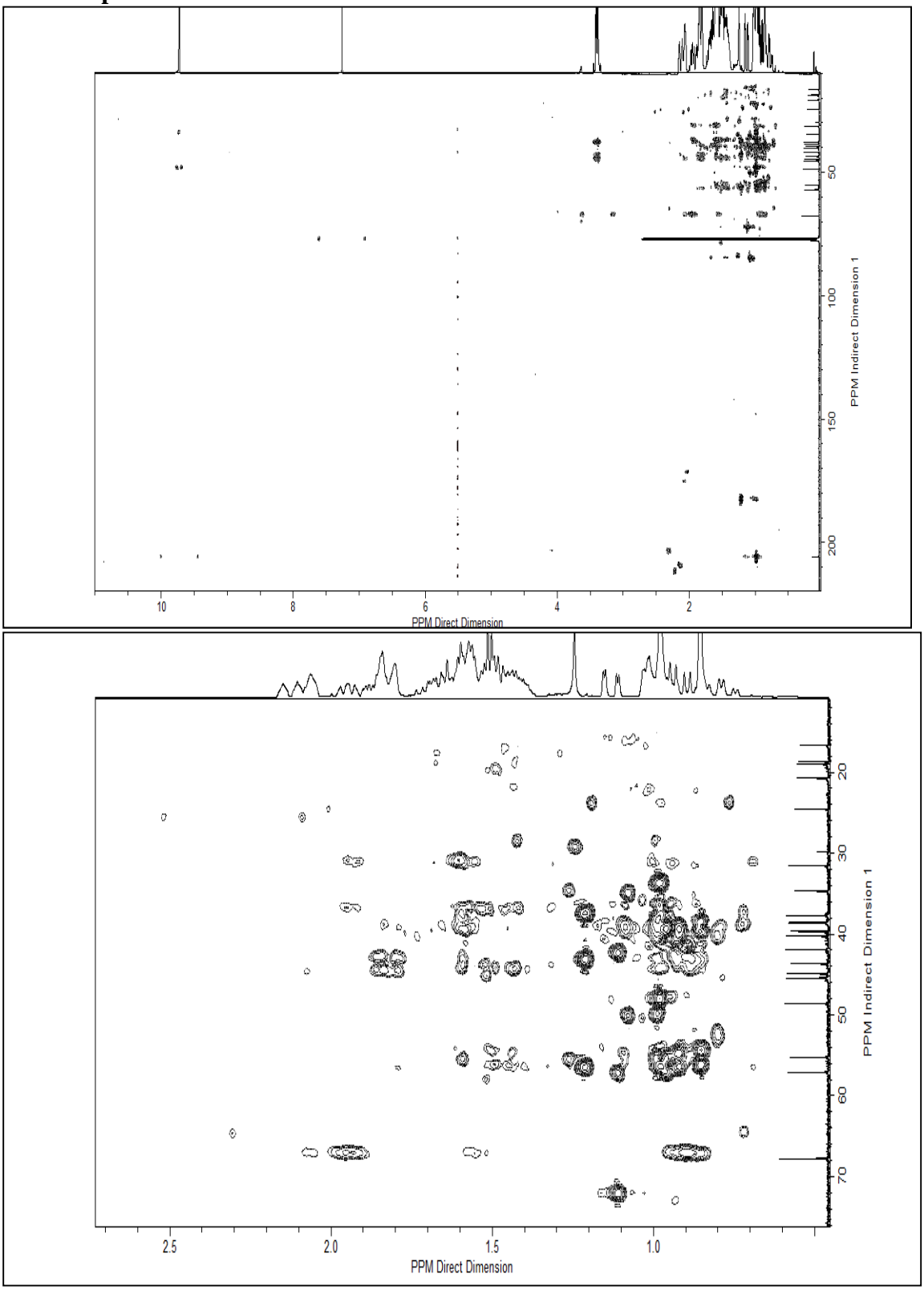
**DEPT-135 Spectrum in *d*-chloroform**



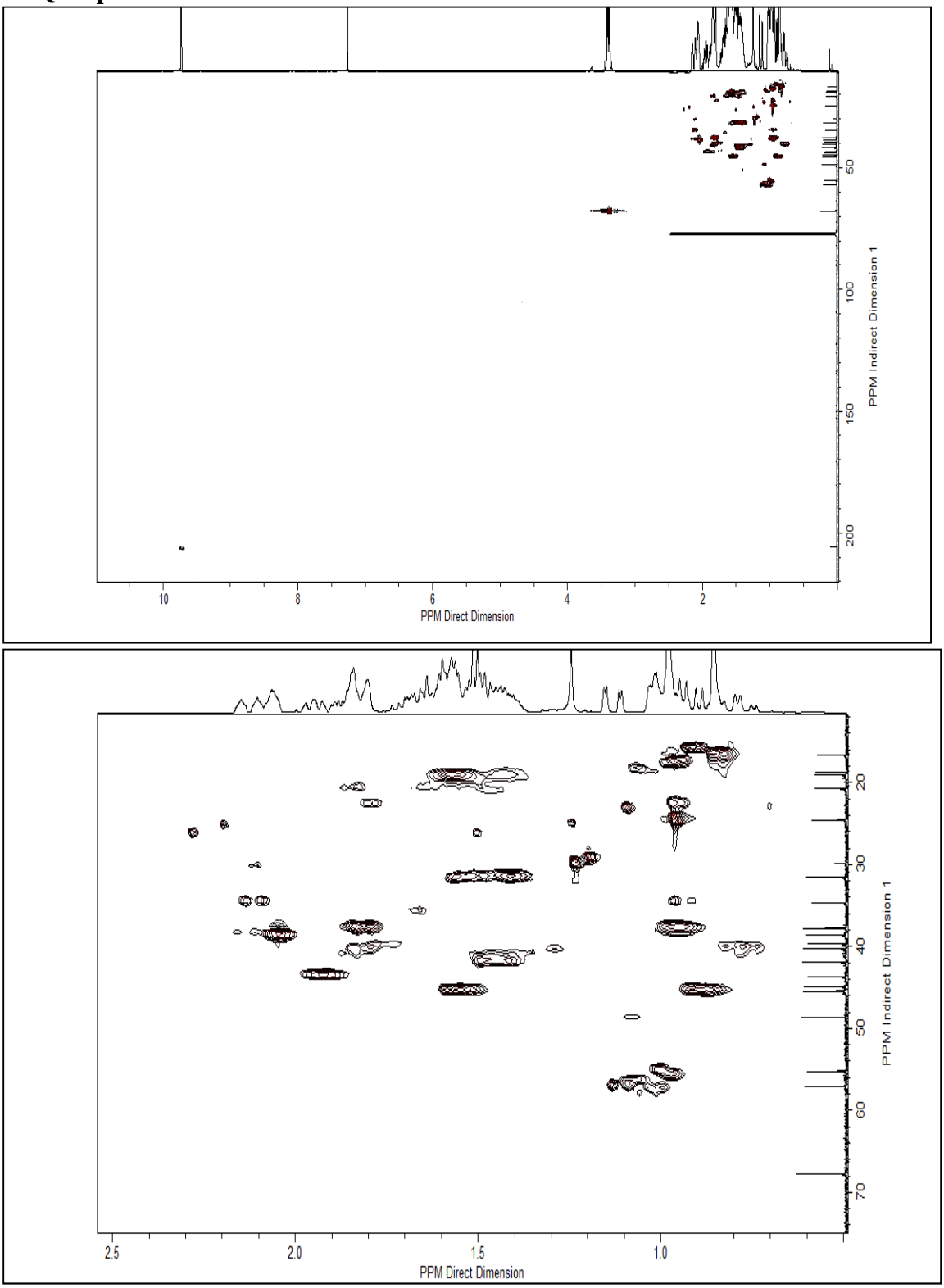

**COSY Spectrum in *d*-chloroform**



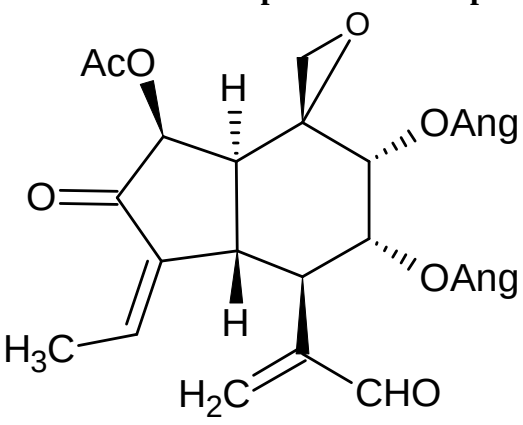
**HMBC Spectrum in *d*-chloroform**



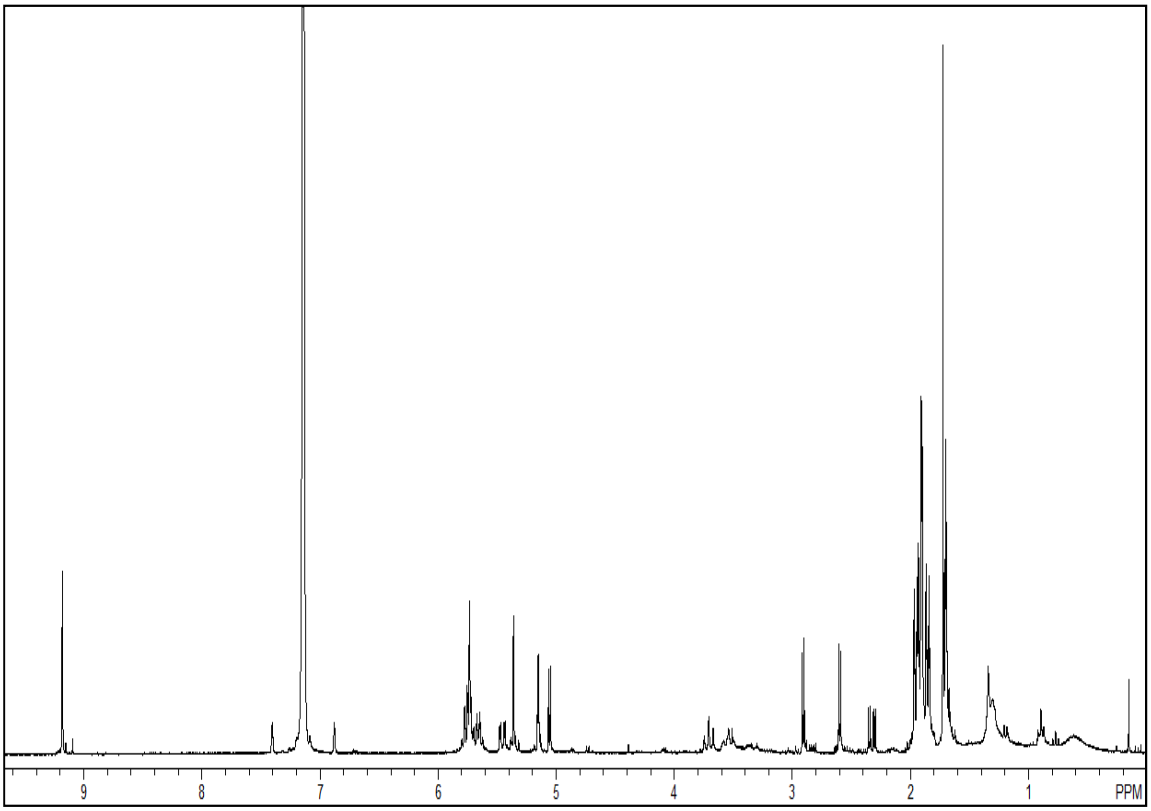
**HSQC Spectrum in *d*-chloroform**



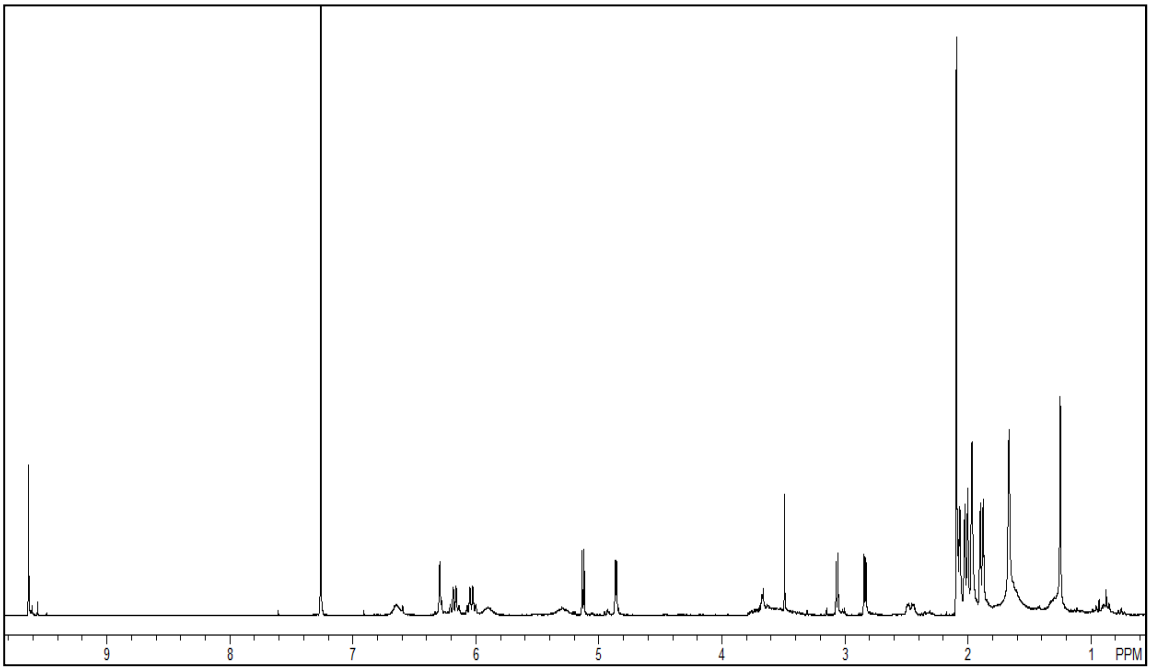
**$^{1}$H and $^{13}$C NMR Spectra for Compound 3.8**



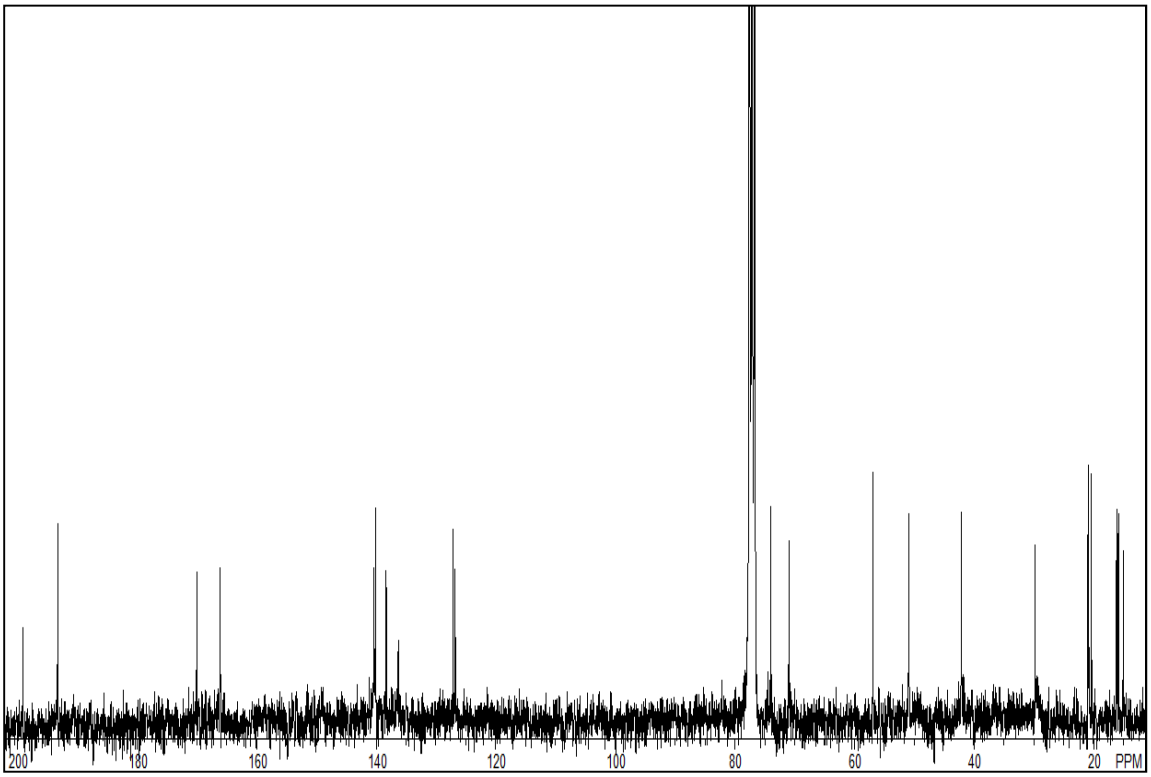

**$^{1}$H Spectrum in *d*-benzene**



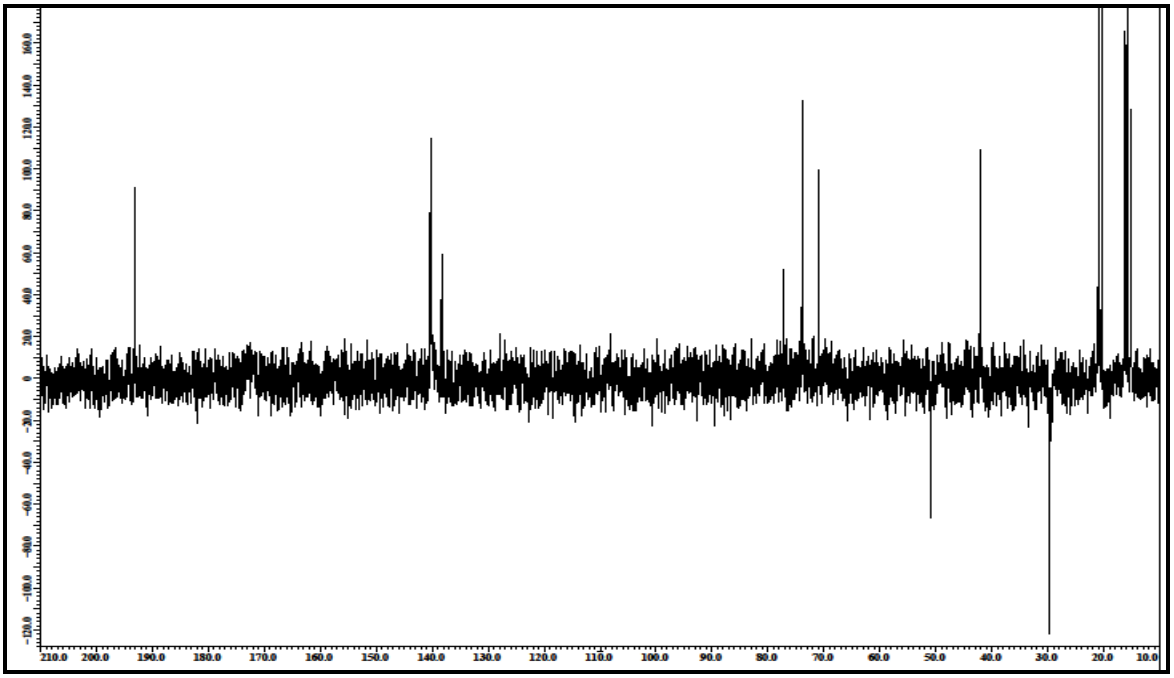
**$^{1}$H Spectrum in *d*-chloroform**



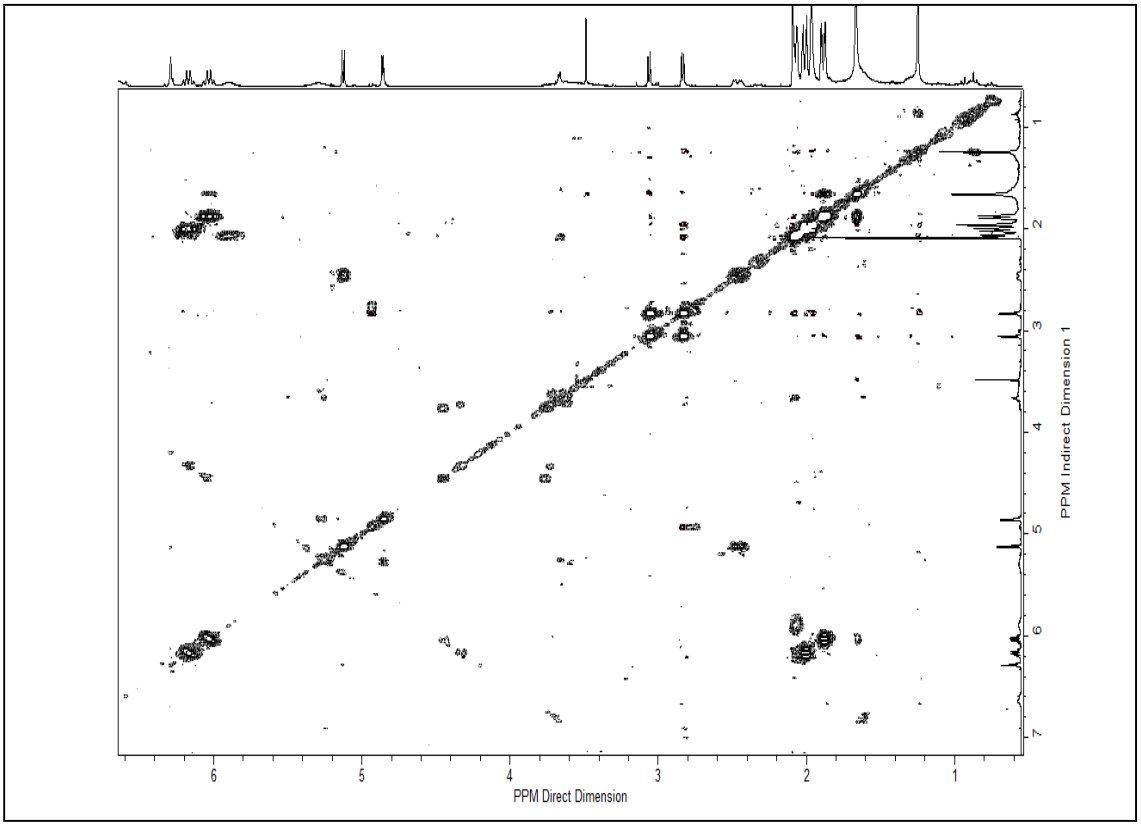

**$^{13}$C Spectrum in *d*-chloroform**



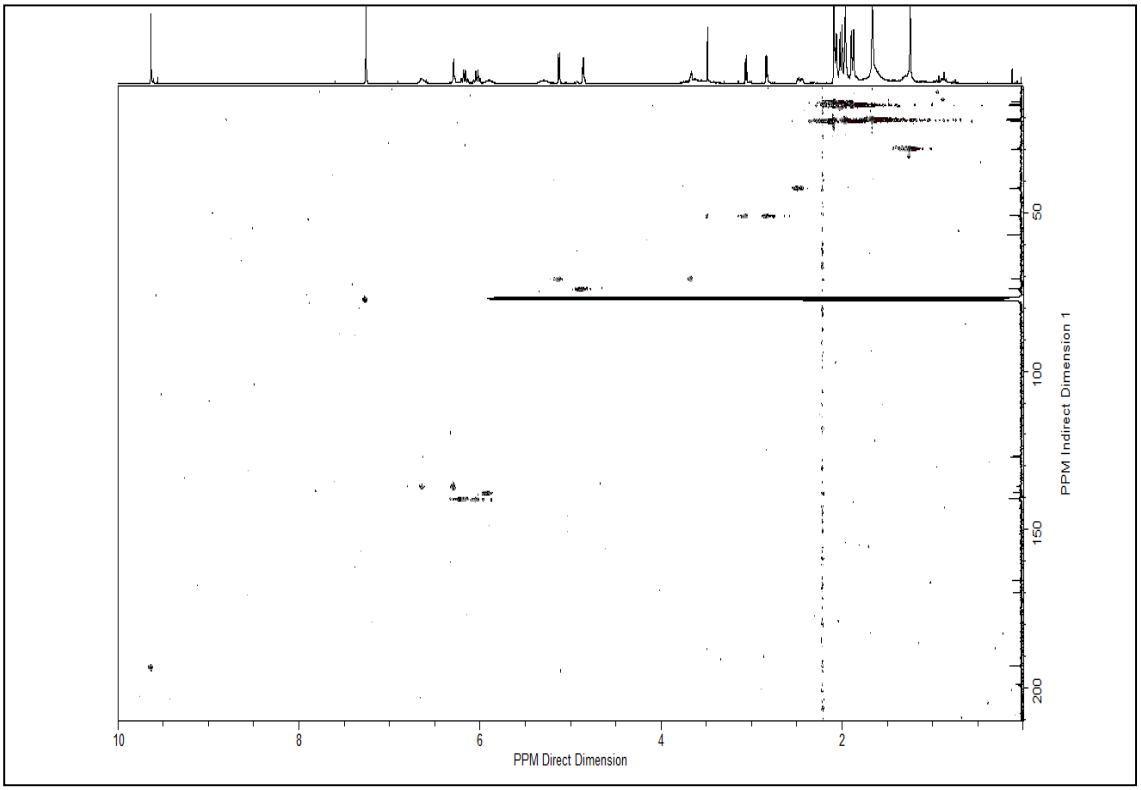
**DEPT-135 Spectrum in *d*-chloroform**

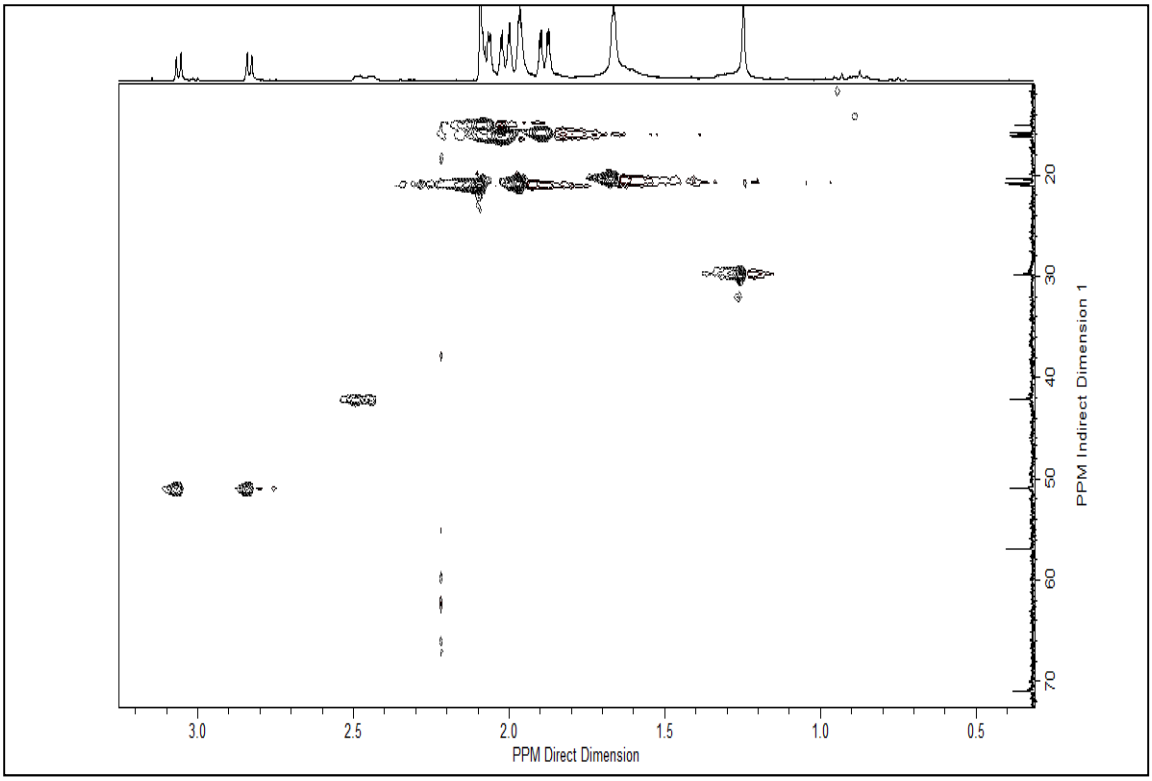


**COSY Spectrum in *d*-chloroform**



**HSQC Spectrum in *d*-chloroform**



**HMBC Spectrum in *d*-chloroform**