Isolation and Synthesis of Bioactive Compounds from Plants

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ABSTRACT

As a part of a continuing search for bioactive compounds with the International Cooperative Biodiversity Group (ICBG), and in collaboration with the Natural Products Discovery Institute of the Institute for Hepatitis and Virus Research (IHVR), twelve plant extracts were investigated for their antiproliferative activity against the A2780 cell line, three plant extracts were investigated for their antimalarial activity against *Plasmodium falciparum*, and three plant extracts were investigated for their anti-inflammatory activity (PPAR-γ inhibition). Bioassay-guided fractionation of extracts led to the identification of four new antiproliferative compounds (**2.1**–**2.3**, **3.1**), five new anti-inflammatory compounds (**6.4a**, **6.5a–b**, **6.6a**, **6.6c**), and twenty-eight known compounds from eight of the extracts. In addition, mallotojaponin C, an antimalarial natural product, and derivatives were synthesized and investigated for their antimalarial activity.

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Chapter 1: Introduction

1.1 Importance of Natural Products

As of 2011, 25–50% of marketed drugs trace their origins to natural products.[1] From 1981 to 2010, the number of new small molecule chemical entities approved by the FDA has steadily decreased but natural products and natural product derivatives (% "N", Figure 1-1) continue to be a source for new chemical entities.[2]

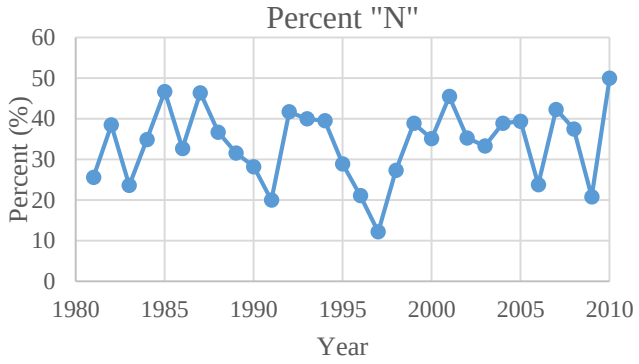
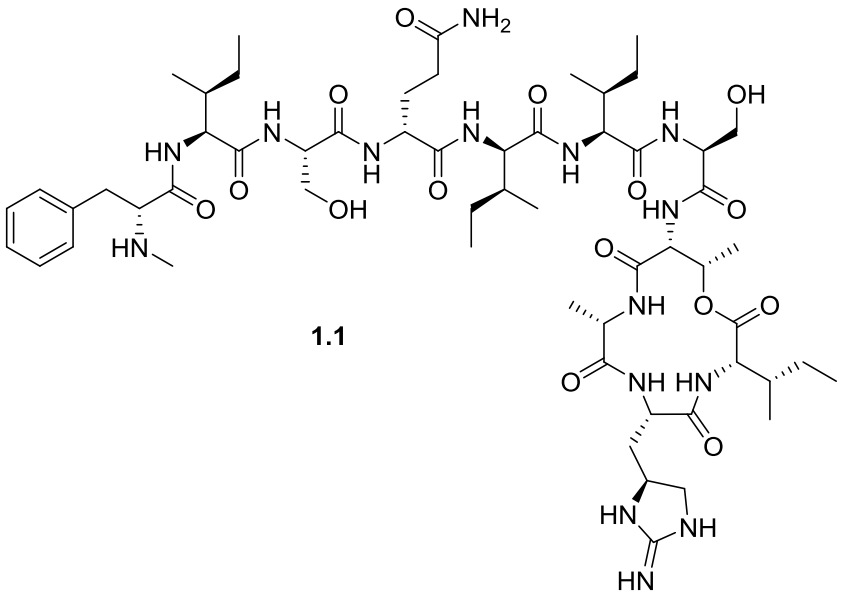


Figure 1-1. Percentage of new small molecule chemical entities approved by the FDA from natural sources (N). Adapted from Newman, D. J.; Cragg, G. M. Natural products as sources of new drugs over the 30 years from 1981 to 2010. *J. Nat. Prod.* **2012**, *75*, 311–35.

Natural products have continued to be a source of novel compounds, and as one recent example the complex peptide teixobactin (Figure 1-2), was reported to be a new antibiotic with activity against drug-resistant *Staphylococcus aureus* and *Mycobacterium tuberculosis*.[3] In spite of successes such as these, pharmaceutical companies have shifted their focus away from natural product isolation for a number of reasons, one of them being the repeated isolation of known compounds. If known compounds can be quickly identified, companies can make their isolations much more efficient and practical; hence, the need for dereplication techniques. Other modifications of the traditional bioassay-guided approach to natural products are emerging.[4]



1.1

Figure 1-2. Structure of teixobactin.

1.1.1 Anticancer Agents

Natural products have had a long history of being used for medicinal purposes and have played an important role in the development of anticancer drugs.[5] One example of a natural product success is the isolation and structure elucidation of paclitaxel (Figure 1-3).[6] More recently, romidespin[7] (2009) and omacetaxine mepesuccinate[8] (2012) are natural products that have been approved by the FDA for the treatment of cancer.[9] Since the 1940s, 49% of new small molecule chemical entities approved by the FDA for the treatment of cancer are either natural products or natural product derivatives.[2]

While progress has been made and cancer rates are declining, cancer continues to affect a large number of people.[10] Some types of cancer, while less common, are particularly difficult to treat. Over 14,000 of the 20,000 women diagnosed with ovarian cancer in the U.S. died in 2011.[11] Natural product research has provided complex structures, such as paclitaxel, that may not have

been discovered by other methods. The potential number of undiscovered natural products is seemingly limitless, leaving the possibility of many undiscovered novel chemical entities.

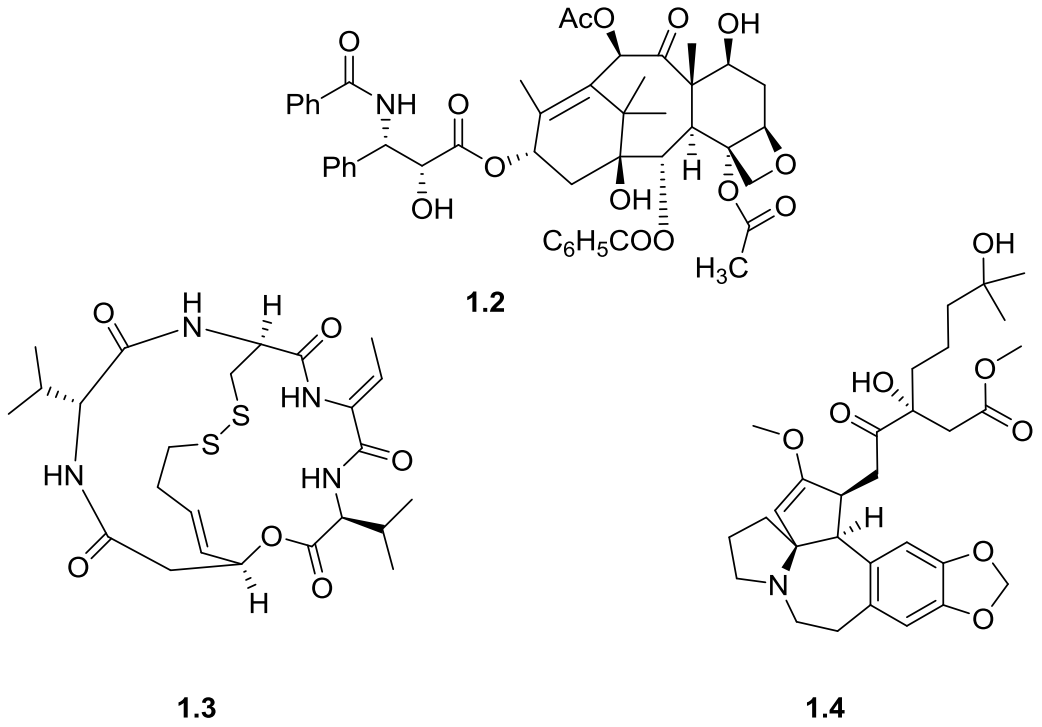


Figure 1-3. Structures of paclitaxel (**1.2**), romidespin (**1.3**), and omacetaxine mepesuccinate (**1.4**).

1.1.2 Antimalarial Agents

While malaria is not widespread in the U.S. (approx. 1,500 cases per year), there were an estimated 655,000 deaths and 216 million infections requiring clinical treatment worldwide in 2010. As of 2010, 3.3 billion people were considered to be at risk for malaria while 1.2 billion people in the African and Southeast Asia regions were considered to be at high risk (>1 case per 1,000 people). The African region contains the highest rate of transmission of malaria worldwide; 91% of deaths occurred in the African region. Children are particularly vulnerable to the disease—86% of global deaths involved children.[12,13] *Plasmodium falciparum* is responsible for approximately 90% of cases worldwide; furthermore, almost 2.5 billion people in South America and Asia are also at risk from *P. vivax.*[14]

Natural products have a long history of being a viable source of antimalarial compounds.[2] As early as the second century BCE, *Artemisia annua* was described as a medicinal treatment for malaria. In 340 CE, the plant's antifever properties were recorded, followed by the isolation of the active ingredient, artemisinin, in 1971.[12] Artemisinin derivatives continue to be prescribed today, although usually in combination with other drugs to avoid resistance. Combination treatments have proven to be highly effective; however, signs of resistance have developed in parts of the world such as Western Cambodia.[15] Quinine, whose origins trace to the 17th century, is still used for the treatment of malaria, although its use is now limited to cases of severe malaria. The quinine-inspired drug chloroquine was once widely used, but resistance to it is more widespread than artemisinin resistance.[16]

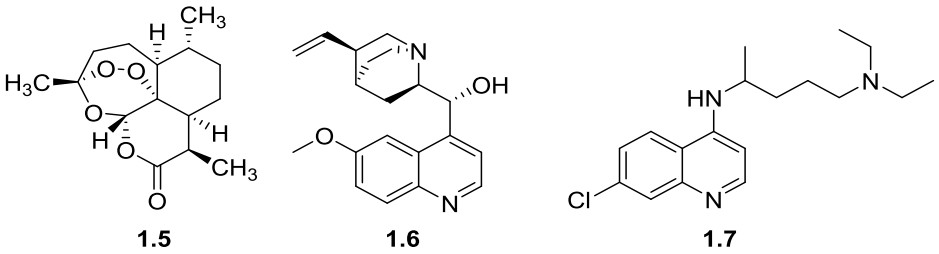


Figure 1-4. Structure of artemisinin (**1.5**), quinine (**1.6**), and chloroquine (**1.7**).

Due to the low profitability of developing antimalarial drugs, the pharmaceutical industry has not been a major player in the development of new antimalarial drugs. Orphan drug incentives have increased the number of orphan drugs approved by the FDA to an all-time high of 41% (17) of all drug approvals in 2014, a sign that neglected diseases are being increasingly targeted.[17] While no malaria drugs were approved in 2014, there are multiple drugs currently in clinical trials, some shown in Figure 1-5, that will hopefully prove to be effective.[14,18-30] Challenges include not only developing compounds to which the parasite does not develop resistance, but also developing a new class of drugs that stop the transmission of malaria.

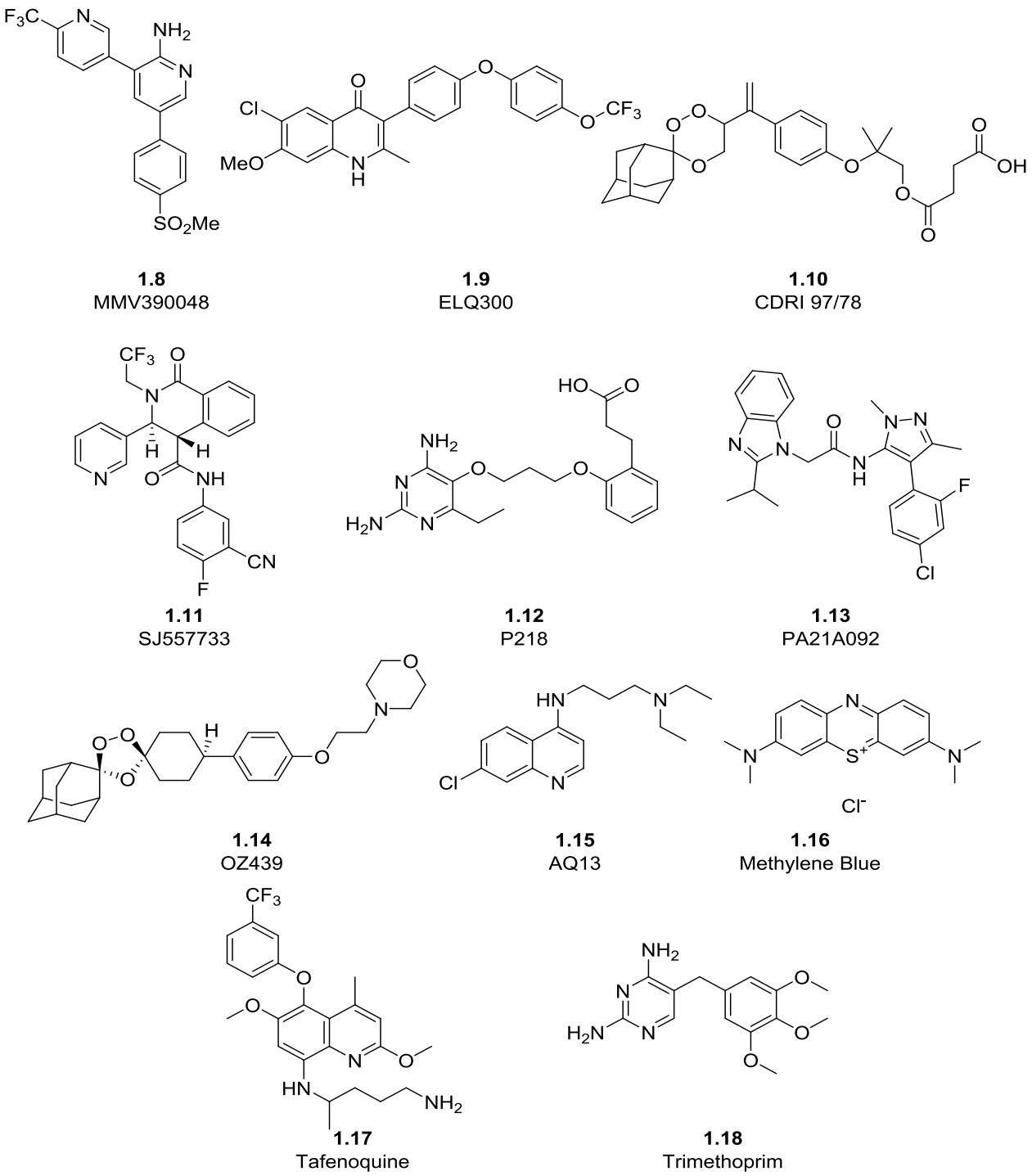
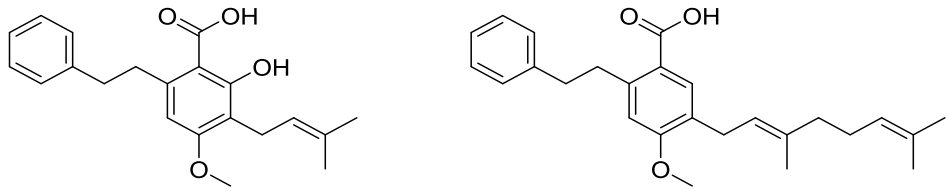


Figure 1-5. Structures of selected antimalarial compounds in clinical trials. Phase 1: MMV390048, ELQ300, CDRI 97/78, SJ557733, P218, PA21A092. Phase 2: OZ439, AQ13, methylene blue. Phase 3: tafenoquine, trimethoprim.

1.1.3 Anti-inflammatory Agents

Natural products and natural product derivatives have a long history of being used as anti-inflammatory agents. Aspirin is perhaps one of the most well-known anti-inflammatory natural product derivatives, while recent natural products with anti-inflammatory properties include aphanamenes C-P[31] and tricalysins A-H.[32] Anti-inflammatory agents not only reduce pain but also have been suspected to reduce the risk of cancer.[33] Activation of the peroxisome proliferator-activated receptor gamma (PPAR-γ) has been shown to reduce inflammation.[34] PPAR-γ agonists, such as the amorfrutins (Figure 1-6), have been shown to have anti-inflammatory properties, decreasing the inflammatory response of many cells and showing potential as a target for treatment of inflammatory diseases.[35,36]



1.19
Amorfrutin A

1.20
Amorfrutin B

Figure 1-6. Structure of amorfrutins A and B.

PPAR-γ agonists have also been shown to combat type 2 diabetes by improving sensitivity to insulin through increasing the levels of adiponectin.[37-39] Thiazolidindione, a PPAR-γ agonist, has been used for the treatment of type 2 diabetes but has unpleasant side effects. Natural products have been a source of PPAR-γ agonists in the past;[39] thus, plants are being searched for PPAR-γ agonists. Recent reports (Figure 1-7) include the isolation of homoisoflavonoids (**1.21, 1.22**),[40] hikiamides (**1.23–1.25**),[41] and isosilybin A (**1.26**).[38]

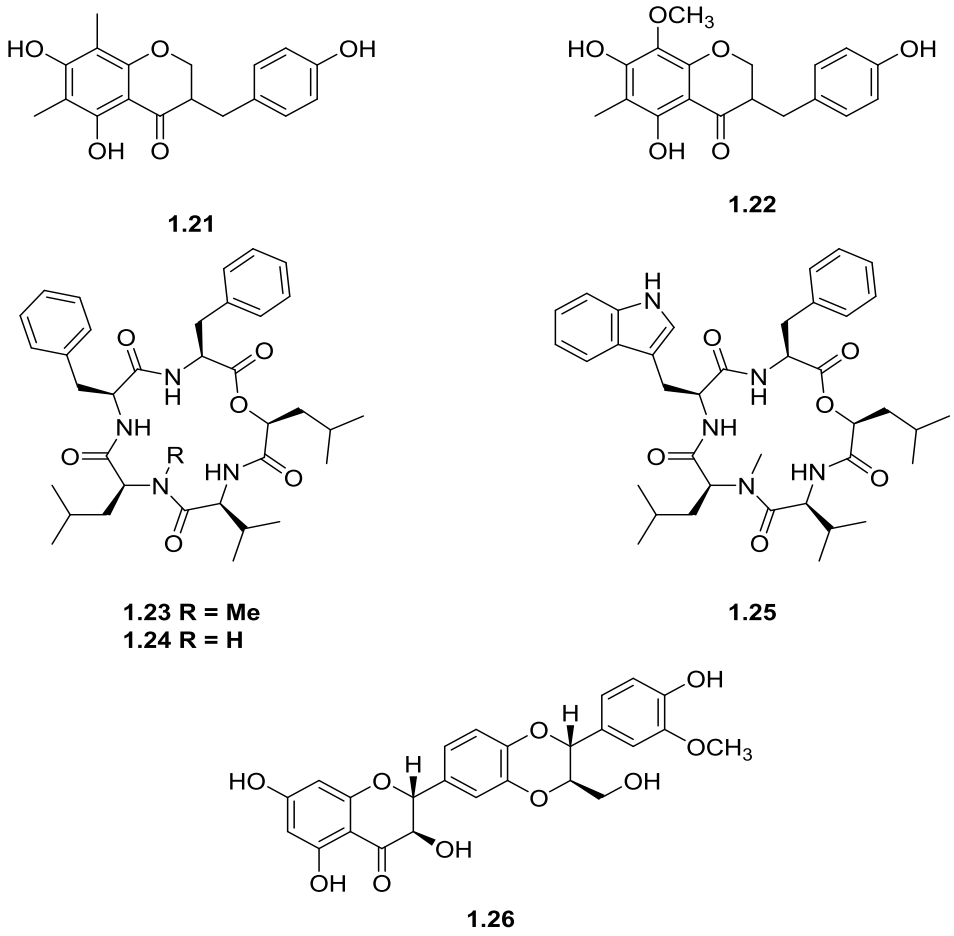


Figure 1-7. Structures of selected recently identified PPAR-γ agonists.

1.2 Sources of Natural Products

1.2.1 Madagascar ICBG Project

In 1993, a group from Virginia Tech, headed by Dr. Kingston, and in cooperation with Missouri Botanical Garden, Conservation International, Bristol-Myers Squibb, and Bedrijf Geneesmiddelen Voorziening Suriname (BGVS), was funded by the NIH to investigate natural products extracts from Suriname. This International Cooperative Biodiversity Group (ICBG) continued until 1998, when the project transitioned to Madagascar.[42] The plants are collected and identified by the Missouri Botanical Garden in collaboration with the Centre National d'Application de Recherche Pharmaceutique (CNARP). These extracts are collected with no regard to previous reports of medicinal activities. The extractions are performed by Dr. Vincent E. Rasamison (CNARP). These extracts are then made available to Virginia Tech where they are tested for the antiproliferative, antimalarial, and anti-inflammatory activities. If the extract shows interesting bioactivity, then it is fractionated in an attempt to identify the bioactive components.

1.2.2 Merck Collection at NPDI

In 2011, the entirety of Merck's natural products library was donated to the Institute for Hepatitis and Virus Research (IHVR).[43] This resulted in the founding of the Natural Product Discovery Institute (NPDI)[44] in order to facilitate the use of and caretaking of this collection. This library, one of the largest in the world, is available to researchers. The Kingston Group at Virginia Tech was among the first to gain access to this library.[45] Similar to the extracts from Madagascar, these extracts are tested for their antiproliferative and antimalarial activities and investigated further as merited.

1.3 Isolation of Bioactive Compounds

1.3.1 Bioassay-guided Fractionation

Bioassay-guided fractionation is used to prevent the isolation of inactive compounds. Without guidance from bioassays, extracts are fractionated and often only the major components are isolated and identified. After isolation, the compounds may then be analyzed for bioactivity. This leads to the isolation of many compounds that do not have significant bioactivity. As an alternative, bioassay-guided fractionation can be used to isolate bioactive compounds. When utilizing bioassay-guided fractionation, the crude extract and every fraction is tested for bioactivity. Ideally, after each fractionation step, bioactivity will have increased in some fractions, in comparison to the parent fraction, while decreasing in others (Figure 1-8). Only fractions that exhibit bioactivity are investigated further. This prevents the isolation of inactive compounds and promotes the isolation of compounds with desired bioactivities. Examples of bioassay-guided fractionation are present throughout this dissertation.

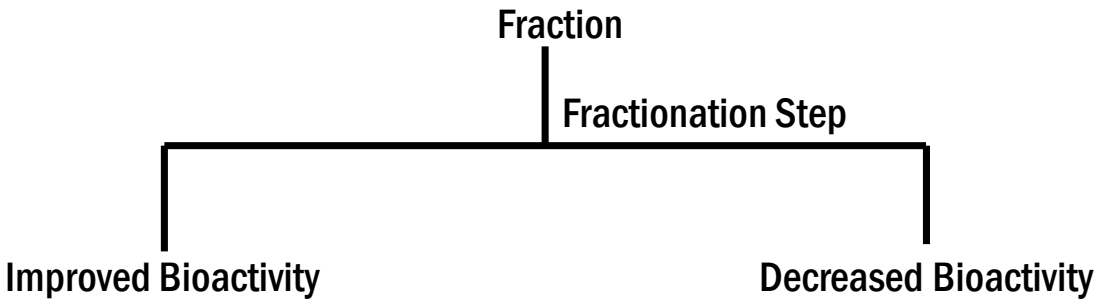
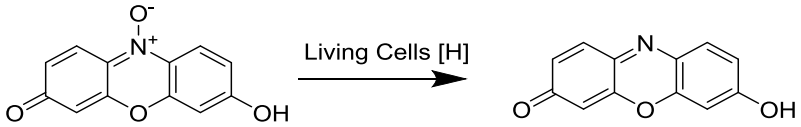


Figure 1-8. Bioassay-guided fractionation.

1.3.2 Antiproliferative Bioassay

In order to isolate antiproliferative compounds, the inhibition of the A2780 cell line is measured. The A2780 cell line is a drug-sensitive ovarian cancer cell line.[46] Cells are placed in a

96-well plate, treated with varying concentrations of fractions or compounds dissolved in DMSO, and incubated for 2 days. After 2 days, the cells are treated with resazurin. Resazurin is a weakly fluorescent compound that is reduced to resorufin, which is fluorescent, by living cells (Figure 1-9).[47] The fluorescence is measured and the inhibition is calculated. Inhibition can be measured at multiple concentrations in order to calculate an IC_{50} value. A detailed procedure has been published.[48,49]

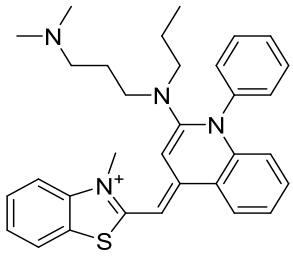


1.27 **1.28**

Figure 1-9. Reduction of resazurin (**1.27**) to resorufin (**1.28**).

1.3.3 Antimalarial Bioassay

Plasmodium falciparum (Dd2), is a chloroquine resistant strain. The parasite is grown in human blood. Ring stage parasites are treated with varying concentrations of either compounds, extracts, or fractions, and incubated for 3 days. Parasite growth is then determined by DNA quantitation using SYBR® Green I (Figure 1-10), which shows fluorescence enhancement when in contact with *P. falciparum*.[50] Detailed procedures have been published.[51]



1.29

Figure 1-10. Structure of SYBR® Green I.

1.3.4 Anti-inflammatory Bioassay

Inhibition of PPAR-γ activity was measured using a cell-based reporter assay. Cells were co-transfected with DNA plasmid and pRL reporter control. The transfected cells were treated and incubated. After incubation, the cells were harvested and the luciferase activity, which was normalized to pRL activity, was measured. The intensity of the luciferase fluorescence is directly related to the gene expression level of PPAR-γ. Detailed procedures have been published.[52]

1.3.5 Isolation Methods

In order to successfully isolate natural products, a researcher must have access to a large number of isolation methods due to the uniqueness of each extract. Liquid–liquid partitioning, column chromatography, and solid phase extraction (SPE) techniques are used extensively. High performance liquid chromatography (HPLC) is used for final purification.

1.3.6 Structure Elucidation

In contrast to synthetic chemistry, a natural products chemist must determine the structure of a compound without the benefit of knowing the reactants. Mass spectrometry (MS) is used to determine the molecular formula, which is then used to calculate the degree of unsaturation. 1D and 2D NMR spectrometry is used extensively to determine the 2D structure of the compound. Often, 2D NMR techniques, such as NOESY, can be used to determine the relative configuration of the unknown compound, either with or without chemical modification. In order to determine the absolute configuration of a compound, techniques other than NMR must be employed, such as ECD, polarimetry, or Mosher ester analysis.

1.3.7 Problems

A large problem with bioassay-guided fractionation is the investment of a large number of resources being committed to the investigation of an extract before the structures of the active components are identified. If the bioactive compounds are known and can be quickly identified, ideally early in the fractionation process, large amounts of time and other resources can be saved.

1.4 Dereplication Strategies

1.4.1 Development of Dereplication

In the field of natural product isolation, dereplication plays a large role in the efficiency of investigators. Dereplication is the process of quickly identifying that the active compound in an extract is or is not already known. By being able to quickly and effectively dereplicate extracts, researchers can save valuable time and money by not isolating compounds that are already known. Dereplication seems to naturally associate with high throughput screening where a large number of fractions are screened for activity and structural data – which can then be used to recognize known compounds.

1.4.2 Solid Phase Extraction

Dereplication is more successful when compounds can be effectively separated. While SPE does not provide any spectroscopic data, it can take advantage of the various functional groups that natural products possess to quickly obtain pure compounds. SPE takes advantage of these differences to effectively separate compounds; for example, strong anion and strong cation cartridges can be used to separate carboxylic acids and amines.[53] Mixed-mode polymeric reverse

phase (RP) anion exchange columns can retain carboxylates, phenyls, and enols and separate them based on relative polarity as well.[53] C_{18} and Sephadex cartridges have also been used to separate compounds based on polarity and size, respectively.[54] By using orthogonal separations, a pure compound can be obtained and further spectroscopic data can be gathered for a quick dereplication.

1.4.3 Mass Spectrometry

MS data is readily available and can be used to obtain a molecular formula which can be used to search many readily available databases. This technique is commonly coupled with liquid chromatography to obtain spectroscopic data for pure compounds. Traditional MS can sometimes give false positives so tandem MS/MS is utilized in order to reduce the risk of assigning an incorrect mass by investigating the fragment ions as well as the parent ion. The investigation of fragments can create characteristic "fingerprints" which can be used for a much more detailed library search.[55]

Forward analysis MS/MS is a technique in which a sample is fragmented and the resulting fragment masses are added up to determine the molecular formula of the parent sample. This is then checked through reverse analysis in which the resulting fragment masses are verified through differentiation.[56] This greatly reduces the risk of assigning an incorrect molecular formula while also giving more information about the fragments. However, MS/MS database data is limited: in 2009 MS/MS data were available for only approximately 14,000 spectra in the NIST/EPA/NIH Mass Spectral Database.[56] A least-squares data analysis can also be applied to match predictions of fragmentation with actual fragmentation to help verify the molecular weight.[56] Another method is MS/MS/MS that can be used to obtained detailed information about the fragmentation. However,

the spectra are quite complicated and have a low signal to noise ratio, making them inadequate for accurate mass determination.[55]

Jarussophon et al.[56] demonstrated that MS/MS has been very accurate when used to determine exact masses of compounds between 138–1569 Da. A total of 86 of 96 compounds (90%) were successfully assigned an accurate molecular weight; furthermore, after a least-squares analysis, the success rate was raised to 97% (93 of 96 compounds). MS/MS has also been used to successfully assign the molecular formula of paclitaxel, $C_{47}H_{51}NO_{14}$.[55] This demonstrates a potential to eliminate errors in molecular formula determination, reducing false positives and improving the accuracy of dereplication, and hence reducing the chances that a novel compound will be mistaken for an already known compound. The detailed information about fragmentation also eases structure determination.

1.4.4 Nuclear Magnetic Resonance Spectroscopy

NMR is commonly used in natural product dereplication due to the wealth of structural information it provides. However, it is relatively insensitive, so efforts must be taken to improve the signal-to-noise ratio (S/N) for small amounts of sample, a common occurrence in natural product isolation. An issue with the small amount of sample and the need for highly sensitive analysis is the appearance of contaminants which can arise from sources such as solvents, glassware, and columns.[57] This can be very difficult to avoid as one must go out of his or her way to prevent contamination and may have to use different protocols than are typically used in a synthetic laboratory. One commonly used method to improve the S/N ratio is to increase the number of scans; this improves the S/N ratio as a function of $n^{1/2}$ (n = number of scans).[57] While

effective, this is often impractical in shared NMR facilities as it ties up machines for long periods of time.

A large amount of background noise in NMR spectra is a result of the large number of electronics that are used for analysis. This noise results in many and various background signals at room temperature. To eliminate some of this background noise, and thus improve the S/N ratio, one can use a cryoprobe, an NMR spectrometer in which the rf coils and the preamplifier are cooled to approximately 20 K. Cooling improves the S/N ratio by approximately four-fold.[57]

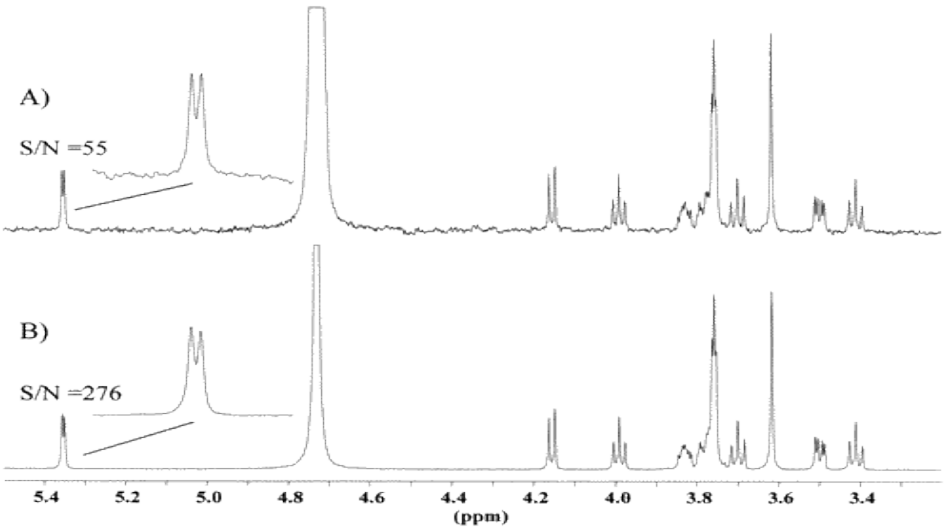


Figure 1-11. Comparison of ^{1}H NMR of sucrose in D_2O. (A) 172 µg in 550 µL in a 5-mm NMR tube. (B) 172 µg in 5 µL in a 1-mm probe tube. Reprinted with permission from *Anal. Chem*., **2002**, *74*, 4464–4471. Copyright 2002 American Chemical Society.

Another way to improve the S/N ratio is to decrease the volume of the tube. When a fixed amount of sample is available, a smaller tube size allows for preparation of a more concentrated sample due to the reduced amount of solvent need to fill the tube. This increases the effective mass contained within the tube, greatly increasing the S/N ratio. Molinski reports that a low volume capillary probe (5–7 µL) gives a five times greater mass sensitivity compared to a traditional 5 mm NMR probe (Figure 1-11).[57] Capillary NMR (1 mm probe) has been demonstrated to greatly

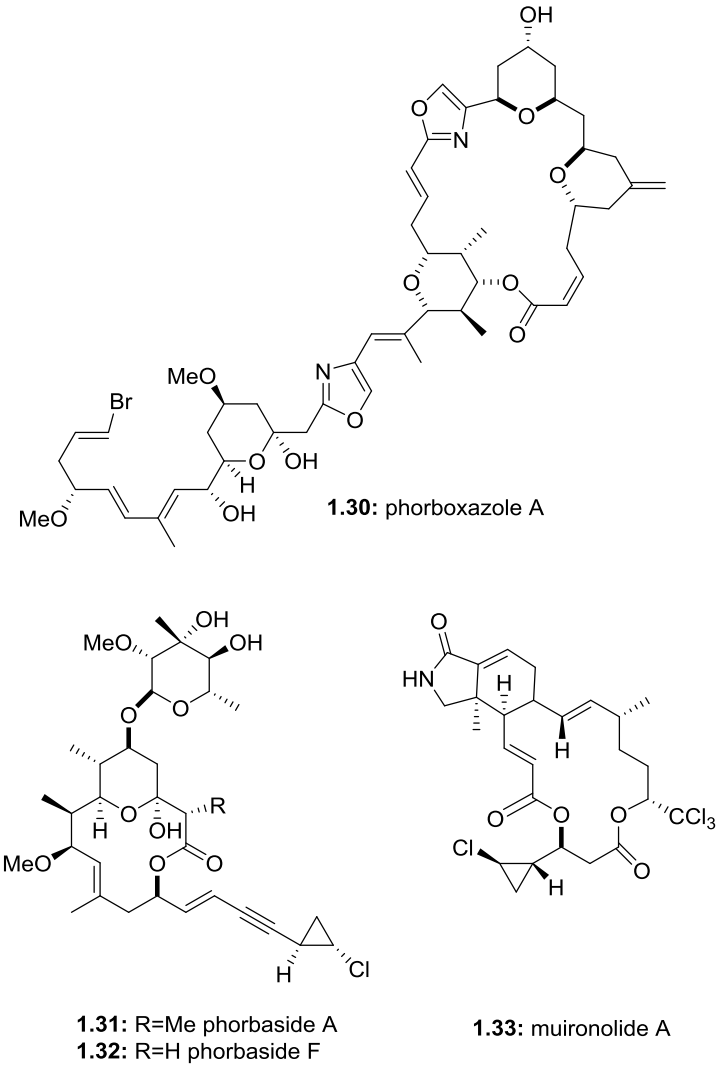


Figure 1-12. Compounds isolated from *Phobas sp*.: **1.30**: 186 mg (180 µmol) identified in 1995. **1.31**, **1.32**: ~1 mg and 0.007 mg, respectively (1.5 µmol combined), identified in 2003. **1.33**: ~0.09 mg (0.15 µmol), identified in 2009.

increase the S/N of sucrose by approximately 5-fold compared to a traditional 5 mm probe, as seen in Figure 1-11, due to a 110-fold increase in concentration.[58]

Compounds isolated from *Phobas sp.* are examples of a natural product extract story that evolved with technology (Figure 1-12). In 1995, compound **1.30** was isolated and its structure resolved with 180 µmol of compound on a 500 MHz NMR with a room temperature probe. Later, in 2003, the structures of **1.31** and **1.32** were determined with 1.5 µmol of compound on a 600 MHz NMR equipped with a 5 mm cryoprobe. Furthermore, in 2009, the structure of **1.33** was determined with 0.15 µmol of compound and a 600 MHz NMR equipped with a 1.7 mm microcryoprobe.[59]

1.4.5 High Performance Liquid Chromatography

As early as 1979, HPLC was recognized as a viable method for the separation of natural products; this acted as a replacement for complex separation solutions.[60] HPLC has advanced since then, increasing in speed, capacity, versatility, and resolution. In particular, the availability of various column types greatly improves the separation capabilities of HPLC.

HPLC profiling is the technique involving the coupling of HPLC analysis with activity testing and spectroscopy; this can be used to identify a compound through a database search

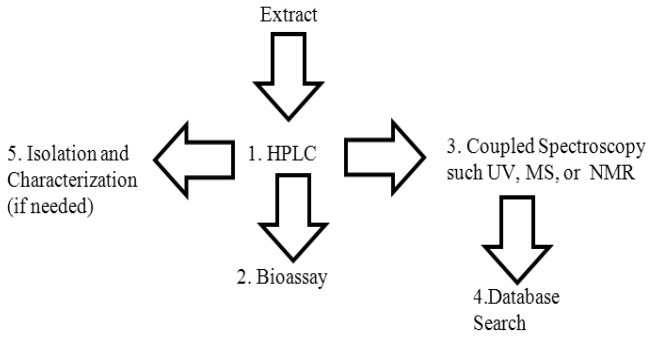


Figure 1-13. A schematic outlining HPLC profiling. Adapted from: Potterat, O.; Hamburger, M. *Curr. Org. Chem.* **2006**, *10*, 899.

without isolating the compound.[61] HPLC profiling (Figure 1-13) can also be used to guide isolations by prioritizing isolation of the active compounds within the sample instead of isolation of all compounds within the sample.

HPLC efficiency is limited by the back-pressure generated from the packing density of the column. While it is true that an increase in flow rate decreases time spent, it also decreases the resolution and generates a higher back-pressure that may be more than the HPLC pump can withstand. This often causes leaks and other damage to the equipment. One way to increase flow rate without generating high back pressures is to use a monolithic column—a column containing porous channels instead of particles; however, monolithic columns are not readily available or readily compatible when coupled with mass spectrometers.[62]

Another way to improve HPLC resolution is to decrease particle size; however, this increases back pressures creating the need for high pressure devices (>400 bar).[60] By decreasing the particle size, the number of theoretical plates are increased which increases resolution, although it is not always predictable to what extent the resolution will be increased.[63] This creates a demand for ultra performance liquid chromatography (UPLC) machines that can take advantage of smaller particle size and shorter column length; allowing hours to be saved on separations.

When choosing a UPLC method, the most important feature to examine is the maximum available pressure due to the continuing decrease in particle size and the ability of higher pressure systems to operate at higher flow rates.[60] UPLC users often have to choose between a shorter column or a slower flow rate, sacrificing either time or resolution; however, the resolution and time savings are still much improved compared to HPLC. In general, column length for UPLC is approximately one-third that of HPLC columns and the flow rate is approximately three times as fast, resulting in analysis times that are nine times faster than comparable HPLC runs.[60] UPLC is

not without its drawbacks. Pressures as high as 5000–7000 bar can cause solvent compression and generate heat from friction, causing difficulties in obtaining good separations.[64]

UPLC has been shown to decrease separation time when used in the pharmaceutical industry without sacrificing quality.[65] A success story of UPLC is the analysis of the medicinal plant *Gingko biloba*. Eugster successfully transferred HPLC methods to a UPLC machine, decreasing the particle size from 5 µm to 1.7 µm and shortening the column from 4.6 mm to 2.1 mm, thus reducing the analysis time nine-fold. Furthermore, if the column length is not shortened, far greater resolution is achieved and the analysis still takes significantly less time than the original analysis.[60]

1.4.6 HPLC Detection Methods

Wolfender offers a comprehensive study of HPLC detector methods, summarized in Table 1-1. UV is the most widely coupled detection method with HPLC. While it detects many compounds, it will miss non-UV absorbing compounds, making it an imperfect detector. Another detection method is fluorescence detection (FD), which is more sensitive than UV detection, but most natural products do not fluoresce, making FD a limited detection method. Chemiluminescence (CL) is another method, similar to FD, that detects the light emission of a molecule in its excited state; however, most natural products do not chemiluminesce.

Electrochemical detection (ECD) can also be coupled with HPLC and is more effective than FD and CL because many natural products have electrochemical activity. A drawback is that ECD is a destructive technique that causes reduction within the compound which can be an issue with natural products that are often in short supply.[66]

Table 1-1. Performance comparison of different HPLC detectors. Retyped from: J. L. Wolfender *Planta Med.***2009**, *75*,719.

	Type	Dynamic Range	Sensitivity	Specificity	Chromatographic Compatabilities	Structural Information	Ease of Use	Widespread use for NPs	Cost
UV	Spec.	***	***	**	***c	*	****	****	*
DAD	Spec.	***	***	**	***c	**	****	***	**
FD	Spec.	**	****	***	****	*	***	*	**
EC	Spec.	****	****	**	**	-	**	**	*
RID	Uni.	**	**	-	**d,e	-	****	*	*
ELSD	Uni.	**a	**	-	**e	-	****	*	*
CAD	Uni.	****a	***	-	**e	-	****	-g	**
MS	Uni./ Spec.	**	****	***	**e	***	**	***	***
MS-MS	Uni./ Spec.	**	****	****	**e	****	*	***	****
NMR.	Uni.	**b	*	*	*f	****	*	**	****

[a] Non-linear response. [b] NMR provides absolute quantification but is not used in this way in the on-flow mode. [c] Requires solvents and buffers with low UV cut-offs. [d] Not compatible with gradients. [e] Usually not compatible with non-volatile buffers. [f] Need deuterated water and deuterated modifiers in the on-flow mode. [g] Relatively new technique.

Refractive index detection (RID) was first used in 1942 but is very limited in its application. It works well for detecting carbohydrates and sugars but suffers from a lack of sensitivity and high susceptibility to background noise and baseline drift. Flame ionization detection is another seldom used method. While it is highly sensitive, it detects all carbon containing compounds, and thus requires a 100% H_2O mobile phase which greatly limits it utility.[66]

Originating in 1966, evaporative light scattering detection (ELSD) is a very effective detection method often coupled with HPLC. ELSD evaporates all volatile components and solvent and measures the amount to which light is scattered by the remaining particles; this technique has the advantage of detecting all components that are non-volatile or less volatile than the solvent. An attempt to replace ELSD was made in 2002 with the implantation of charged aerosol detection (CAD). CAD works similarly to ELSD but the particles remaining after evaporation are passed

through a charged corona discharge needles and the resulting charge is recorded by an electrometer.[66]

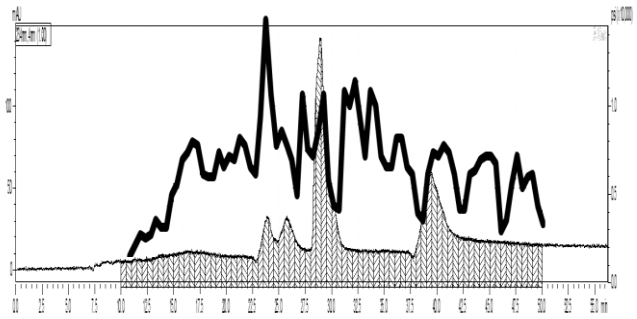


Figure 1-14. An example of coupling HPLC data with bioassay data. The shaded area of the chromatogram was collected into a 96-well plate which was then submitted for bioassay. The bold line represents relative inhibition.

MS and NMR can also be coupled with HPLC in order to provide structural data instead of just indicating the presence of a compound.[67] A typical setup includes separation into a 96-well plate for bioassay submission (Figure 1-14). By using a splitter, most of the sample can be separated into a container, such as a 96-well plate, which can be submitted for bioassay while a very small amount is sent to the mass spectrometer for analysis.[68] Cordell and Shin successfully demonstrated this principle in 1999 when they confirmed the activity of two active compounds in *Semecarpus anacardium* while gathering mass data at the same time.[68] Efficient gathering of data like this enables one to effectively dedicate his or her resources to isolating active compounds instead of blind chasing of compounds without knowing their activity. TOF MS has also been identified as an effective device to couple with UPLC, due to the quick mass analysis time; the entire separation and mass analysis takes approximately 10 minutes, compared to what would traditionally take 30 minutes by HPLC.[69] This demonstrates that MS analysis can keep up with the high speed of UPLC, preventing a bottleneck in the system.

Capillary NMR can also be a powerful tool to couple with HPLC. It has been shown to be a very effective resource to determine the structure of a compound in very small amounts. It was demonstrated by the isolation of chrysaibol from *Sepedonium chrysosperimum* which started with only 700 µg of crude extract, with only 33 µg being needed for the capillary NMR analysis.[70] In another example, Schroeder et al. isolated 13 different steroids in 11 different fractions when starting from only 50 fireflies, used capillary NMR to identify compounds from a very small sample.[71]

1.4.7 Database Searches

In 2001, LC-UV or LC-MS or a combination of the two methods was the preferred method for early dereplication of natural products; however, these methods do not allow for identification of structure,.[72] although they did allow for partial identification of the active components and the structure could later be determined. At this time, LC-NMR was becoming more widely used but the lack of published data in searchable libraries made dereplication difficult.[72] Furthermore, NMR techniques were not as developed as they currently are, so if only a very small amount of the compound could be isolated, obtaining useful NMR data may have been difficult or impossible. If spectroscopic data can be obtained, dereplication can be accomplished with the assistance of various databases. Three commonly used searchable databases are the Dictionary of Natural Products, MarinLit, and AntiBase.[73] SciFinder® can also be used when a possible structure is known to see if the structure is already published. These databases are only as effective as one makes them. One should note which structural features are searchable parameters within accessible databases to help guide one's gathering of spectral data.[74]

HPLC retention times have been demonstrated to be an effective tool in assisting dereplication. UV data coupled with retention times has been successfully used for database dereplication when using an in-house library.[73] However, great care has to be taken to ensure that HPLC conditions remain the same so that retention times do not vary. This makes retention time an inefficient parameter for dereplication because of the difficulties of ensuring that HPLC parameters remain the same at various facilities—limiting this technique to in-house libraries.

A dereplication method with much more transferability is the use of LC-NMR. While direct LC-NMR is the preferred approach, if one does not have access to an LC-NMR instrument one can first perform the LC separation and then obtain NMR spectra. NMR data is easily transferred between facilities. In order to successfully use NMR data to search databases, one must simply identify some easily recognizable features in the spectrum; in many cases it is not necessary to determine the entire structure once a database match has been made.

1.5 Synthetic Approaches

While natural products have been used for medicinal purposes, their effectiveness is enhanced by synthetic approaches. A synthetic route can be used to provide access to natural products that are in short supply. However, often the structure of the natural product is too complex to be synthesized so a semi-synthetic route may be used to modify a different naturally occurring compound (often a precursor in the biosynthetic pathway). For example, paclitaxel can be isolated from the bark of several yew species; however, these species are very slow growing and in order to meet supply demands without damaging the source, an alternative source of paclitaxel was sought.[75-77] It was found that paclitaxel can be efficiently synthesized in three steps from 10-

deacetylbaccatin, which can be isolated in high yield from the needles of *Taxus baccata* L (Figure 1-15).[78]

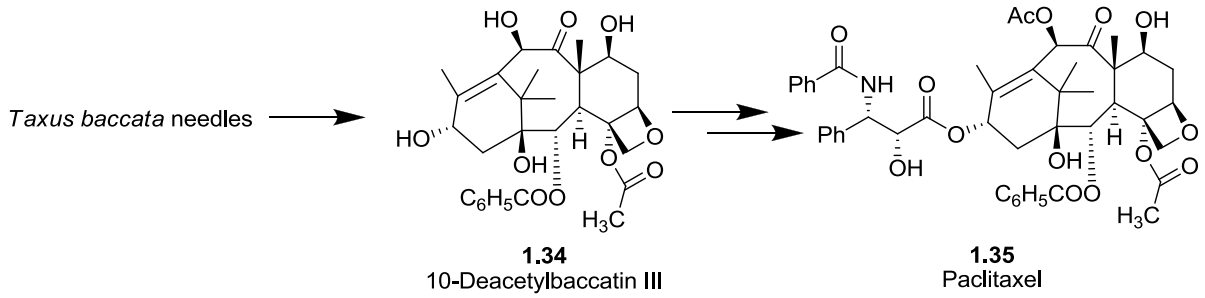


Figure 1-15. Semisynthetic route to obtain paclitaxel.

Bioactivities of natural products can often be improved by synthetic modification. If the natural product is not too complex, a study of the natural product and related compounds can be performed in an attempt to identify compounds with increased bioactivity (see Chapter 5). Some examples of natural product derivatives are shown in Figure 1-16.[79]

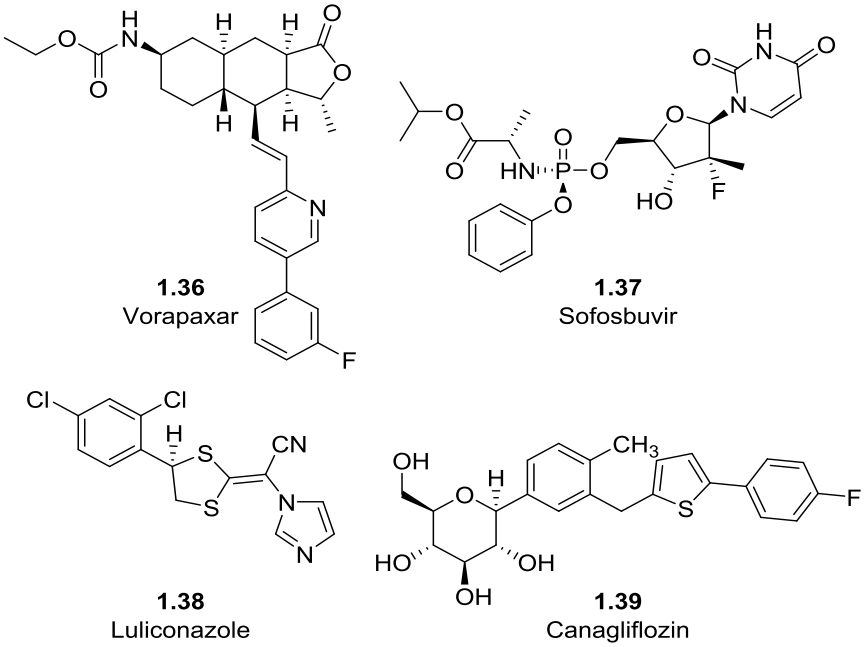


Figure 1-16. Natural product derivatives approved by the FDA from Janurary 2013–June 2014.

1.6 References

1. Kingston, D. G. I., Modern natural products drug discovery and its relevance to biodiversity conservation. *J. Nat. Prod.* **2011,** *74* (3), 496-511.

2. Newman, D. J.; Cragg, G. M., Natural products as sources of new drugs over the 30 years from 1981 to 2010. *J. Nat. Prod.* **2012,** *75* (3), 311-335.

3. Ling, L. L.; Schneider, T.; Peoples, A. J.; Spoering, A. L.; Engels, I.; Conlon, B. P.; Mueller, A.; Schaberle, T. F.; Hughes, D. E.; Epstein, S.; Jones, M.; Lazarides, L.; Steadman, V. A.; Cohen, D. R.; Felix, C. R.; Fetterman, K. A.; Millett, W. P.; Nitti, A. G.; Zullo, A. M.; Chen, C.; Lewis, K., A new antibiotic kills pathogens without detectable resistance. *Nature* **2015,** *517* (7535), 455-459.

4. Harvey, A. L.; Edrada-Ebel, R.; Quinn, R. J., The re-emergence of natural products for drug discovery in the genomics era. *Nat. Rev. Drug Discovery* **2015,** *14* (2), 111-129.

5. Cragg, G. M.; Grothaus, P. G.; Newman, D. J., Impact of natural products on developing new anti-cancer agents. *Chem. Rev.* **2009,** *109* (7), 3012-3043.

6. Wani, M. C.; Taylor, H. L.; Wall, M. E.; Coggon, P.; McPhail, A. T., Plant antitumor agents. VI. Isolation and structure of Taxol, a novel antileukemic and antitumor agent from *Taxus brevifolia*. *J. Am. Chem. Soc.* **1971,** *93* (9), 2325-2327.

7. Ueda, H.; Nakajima, H.; Hori, Y.; Fujita, T.; Nishimura, M.; Goto, T.; Okuhara, M., FR901228, a novel antitumor bicyclic depsipeptide produced by chromobacterium violaceum No. 968. I. Taxonomy, fermentation, isolation, physico-chemical and biological properties, and antitumor activity. *J. Antibiot.* **1994,** *47* (3), 301-310.

8. Powell, R. G.; Weisleder, D.; Smith, C. R.; Rohwedder, W. K., Structures of harringtonine, isoharringtonine, and homoharringtonine. *Tetrahedron Lett.* **1970,** *11* (11), 815-818.

9. Butler, M. S.; Robertson, A. A.; Cooper, M. A., Natural product and natural product derived drugs in clinical trials. *Nat. Prod. Rep.* **2014,** *31* (11), 1612-1661.

10. Edwards, B. K.; Noone, A. M.; Mariotto, A. B.; Simard, E. P.; Boscoe, F. P.; Henley, S. J.; Jemal, A.; Cho, H.; Anderson, R. N.; Kohler, B. A.; Eheman, C. R.; Ward, E. M., Annual report to the nation on the status of cancer, 1975-2010, featuring prevalence of comorbidity and impact on survival among persons with lung, colorectal, breast, or prostate cancer. *Cancer* **2014,** *120* (9), 1290-1314.

11. Center for Disease Control and Prevention - Ovarian Cancer Statistics. http://www.cdc.gov/cancer/ovarian/statistics/ (accessed November 30, 2014).

12. Center for Disease Control and Prevention - Malaria. http://www.cdc.gov/malaria/ (accessed November 30, 2014).

13. *World Malaria Report: 2011*. World Health Organization: Geneva, 2011.

14. Wells, T. N.; van Huijsduijnen, R. H.; Van Voorhis, W. C., Malaria medicines: a glass half full? *Nat. Rev. Drug Discovery* **2015,** *14* (6), 424-442.

15. Guerin, P. J.; Bates, S. J.; Sibley, C. H., Global resistance surveillance: ensuring antimalarial efficacy in the future. *Curr. Opin. Infect. Dis.* **2009,** *22* (6), 593-600.

16. Kappagoda, S.; Singh, U.; Blackburn, B. G., Antiparasitic therapy. *Mayo. Clin. Proc.* **2011,** *86* (6), 561-583.

17. Mullard, A., 2014 FDA drug approvals. *Nat. Rev. Drug Discovery* **2015,** *14* (2), 77-81.

18. Titus, E. O.; Craig, L. C.; Golumbic, C.; Mighton, H. R.; Wempen, I. M.; Elderfield, R. C., Identification by distribution studies. IX. Application to metabolic studies of 4-aminoquinoline antimalarials 1. *J. Org. Chem.* **1948,** *13* (1), 39-62.

19. Milhous, W. K.; Weatherly, N. F.; Bowdre, J. H.; Desjardins, R. E., *In vitro* activities of and mechanisms of resistance to antifol antimalarial drugs. *Antimicrob. Agents Chemother.* **1985,** *27* (4), 525-530.

20. Lamontagne, M. P.; Blumbergs, P.; Smith, D. C., Antimalarials .16. Synthesis of 2-substituted analogs of 8-[(4-amino-1-methylbutyl)amino]-6-methoxy-4-methyl-5-[3-(trifluoromethyl)phenoxy]quinoline as candidate antimalarials. *J. Med. Chem.* **1989,** *32* (8), 1728-1732.

21. Singh, C.; Puri, S. K. Preparation of 4-(1-trioxanylethenyl)phenoxyalkanols and esters as antimalarial agents. US6316493B1, 2001.

22. Mzayek, F.; Deng, H.; Mather, F. J.; Wasilevich, E. C.; Liu, H.; Hadi, C. M.; Chansolme, D. H.; Murphy, H. A.; Melek, B. H.; Tenaglia, A. N.; Mushatt, D. M.; Dreisbach, A. W.; Lertora, J. J.; Krogstad, D. J., Randomized dose-ranging controlled trial of AQ-13, a candidate antimalarial, and chloroquine in healthy volunteers. *PLoS Clin. Trials* **2007,** *2* (1), e6.

23. Zoungrana, A.; Coulibaly, B.; Sie, A.; Walter-Sack, I.; Mockenhaupt, F. P.; Kouyate, B.; Schirmer, R. H.; Klose, C.; Mansmann, U.; Melssner, P.; Muller, O., Safety and efficacy of methylene blue combined with artesunate or amodiaquine for uncomplicated falciparum malaria: a randomized controlled trial from Burkina Faso. *PLoS Clin. Trials* **2008,** *3* (2), e1630.

24. Charman, S. A.; Arbe-Barnes, S.; Bathurst, I. C.; Brun, R.; Campbell, M.; Charman, W. N.; Chiu, F. C.; Chollet, J.; Craft, J. C.; Creek, D. J.; Dong, Y.; Matile, H.; Maurer, M.; Morizzi, J.; Nguyen, T.; Papastogiannidis, P.; Scheurer, C.; Shackleford, D. M.; Sriraghavan, K.; Stingelin, L.; Tang, Y.; Urwyler, H.; Wang, X.; White, K. L.; Wittlin, S.; Zhou, L.; Vennerstrom, J. L., Synthetic ozonide drug candidate OZ439 offers new hope for a single-dose cure of uncomplicated malaria. *Proc. Natl. Acad. Sci. U. S. A.* **2011,** *108* (11), 4400-4405.

25. Deprez-Poulain, R.; Flipo, M.; Piveteau, C.; Leroux, F.; Dassonneville, S.; Florent, I.; Maes, L.; Cos, P.; Deprez, B., Structure-activity relationships and blood distribution of antiplasmodial aminopeptidase-1 inhibitors. *J. Med. Chem.* **2012,** *55* (24), 10909-10917.

26. Younis, Y.; Douelle, F.; Feng, T. S.; Gonzalez Cabrera, D.; Le Manach, C.; Nchinda, A. T.; Duffy, S.; White, K. L.; Shackleford, D. M.; Morizzi, J.; Mannila, J.; Katneni, K.; Bhamidipati, R.; Zabiulla, K. M.; Joseph, J. T.; Bashyam, S.; Waterson, D.; Witty, M. J.; Hardick, D.; Wittlin, S.; Avery, V.; Charman, S. A.; Chibale, K., 3,5-Diaryl-2-aminopyridines as a novel class of orally active antimalarials demonstrating single dose cure in mice and clinical candidate potential. *J. Med. Chem.* **2012,** *55* (7), 3479-3487.

27. Yuthavong, Y.; Tarnchompoo, B.; Vilaivan, T.; Chitnumsub, P.; Kamchonwongpaisan, S.; Charman, S. A.; McLennan, D. N.; White, K. L.; Vivas, L.; Bongard, E.; Thongphanchang, C.; Taweechai, S.; Vanichtanankul, J.; Rattanajak, R.; Arwon, U.; Fantauzzi, P.; Yuvaniyama, J.; Charman, W. N.; Matthews, D., Malarial dihydrofolate reductase as a paradigm for drug development against a resistance-compromised target. *Proc. Natl. Acad. Sci. U. S. A.* **2012,** *109* (42), 16823-16828.

28. Jimenez-Diaz, M. B.; Ebert, D.; Salinas, Y.; Pradhan, A.; Lehane, A. M.; Myrand-Lapierre, M. E.; O'Loughlin, K. G.; Shackleford, D. M.; Justino de Almeida, M.; Carrillo, A. K.; Clark, J. A.; Dennis, A. S.; Diep, J.; Deng, X.; Duffy, S.; Endsley, A. N.; Fedewa, G.; Guiguemde, W. A.; Gomez, M. G.; Holbrook, G.; Horst, J.; Kim, C. C.; Liu, J.; Lee, M. C.; Matheny, A.; Martinez, M. S.; Miller, G.; Rodriguez-Alejandre, A.; Sanz, L.; Sigal, M.; Spillman, N. J.; Stein, P. D.; Wang, Z.; Zhu, F.; Waterson, D.; Knapp, S.; Shelat, A.; Avery, V. M.; Fidock, D. A.; Gamo, F. J.; Charman, S. A.; Mirsalis, J. C.; Ma, H.; Ferrer, S.; Kirk, K.; Angulo-Barturen, I.; Kyle, D. E.; DeRisi, J. L.; Floyd, D. M.; Guy, R. K., (+)-SJ733, a clinical candidate for malaria that acts through

ATP4 to induce rapid host-mediated clearance of *Plasmodium*. *Proc. Natl. Acad. Sci. U. S. A.* **2014,** *111* (50), E5455-5462.

29. Nilsen, A.; Miley, G. P.; Forquer, I. P.; Mather, M. W.; Katneni, K.; Li, Y.; Pou, S.; Pershing, A. M.; Stickles, A. M.; Ryan, E.; Kelly, J. X.; Doggett, J. S.; White, K. L.; Hinrichs, D. J.; Winter, R. W.; Charman, S. A.; Zakharov, L. N.; Bathurst, I.; Burrows, J. N.; Vaidya, A. B.; Riscoe, M. K., Discovery, synthesis, and optimization of antimalarial 4(1H)-quinolone-3-diarylethers. *J. Med. Chem.* **2014,** *57* (9), 3818-3834.

30. Burrows, J.; Wyvratt, M.; Vaidya, A.; Kortagere, S.; Fan, E.; Chatterjee, A. K.; Nagle, A. S.; Kato, T. Preparation of 5-(acylamino)-4-(4-fluorophenyl)-1-methylpyrazole derivatives as antimalarial agents. WO2015008246A1, 2015.

31. Zhang, H. J.; Zhang, Y. M.; Luo, J. G.; Luo, J.; Kong, L. Y., Anti-inflammatory diterpene dimers from the root barks of *Aphanamixis grandifolia*. *Org. Biomol. Chem.* **2015,** *13* (27), 7452-7458.

32. Shen, C. P.; Luo, J. G.; Yang, M. H.; Kong, L. Y., Cafestol-type diterpenoids from the twigs of *Tricalysia fruticosa* with potential anti-inflammatory activity. *J. Nat. Prod.* **2015,** *78* (6), 1322-1329.

33. Rayburn, E. R.; Ezell, S. J.; Zhang, R., Anti-inflammatory agents for cancer therapy. *Mol. Cell. Pharmacol.* **2009,** *1* (1), 29-43.

34. Kapadia, R., Mechanisms of anti-inflammatory and neuroprotective actions of PPAR-gamma agonists. *Front. Biosci.* **2008,** *13* (13), 1813.

35. Dwivedi, A. K.; Gurjar, V.; Kumar, S.; Singh, N., Molecular basis for nonspecificity of nonsteroidal anti-inflammatory drugs (NSAIDs). *Drug Discovery Today* **2015,** *20* (7), 863-873.

36. Fuhr, L.; Rousseau, M.; Plauth, A.; Schroeder, F. C.; Sauer, S., Amorfrutins are natural PPARγ agonists with potent anti-inflammatory properties. *J. Nat. Prod.* **2015,** *78* (5), 1160-1164.

37. Matsuda, H.; Nakamura, S.; Yoshikawa, M., Search for new type of PPARγ agonist-like anti-diabetic compounds from medicinal plants. *Biol. Pharm. Bull.* **2014,** *37* (6), 884-891.

38. Pferschy-Wenzig, E. M.; Atanasov, A. G.; Malainer, C.; Noha, S. M.; Kunert, O.; Schuster, D.; Heiss, E. H.; Oberlies, N. H.; Wagner, H.; Bauer, R.; Dirsch, V. M., Identification of isosilybin a from milk thistle seeds as an agonist of peroxisome proliferator-activated receptor gamma. *J. Nat. Prod.* **2014,** *77* (4), 842-847.

39. Wang, L.; Waltenberger, B.; Pferschy-Wenzig, E. M.; Blunder, M.; Liu, X.; Malainer, C.; Blazevic, T.; Schwaiger, S.; Rollinger, J. M.; Heiss, E. H.; Schuster, D.; Kopp, B.; Bauer, R.; Stuppner, H.; Dirsch, V. M.; Atanasov, A. G., Natural product agonists of peroxisome proliferator-activated receptor gamma (PPARγ): a review. *Biochem. Pharmacol.* **2014,** *92* (1), 73-89.

40. Lin, H.-R., Two homoisoflavonoids act as peroxisome proliferator-activated receptor agonists. *Med. Chem. Res.* **2015**, *24*, 2898-2905.

41. Fukuda, T.; Sudoh, Y.; Tsuchiya, Y.; Okuda, T.; Matsuura, N.; Motojima, A.; Oikawa, T.; Igarashi, Y., Hikiamides A-C, cyclic pentadepsipeptides from *Fusarium sp*. TAMA 456. *J. Nat. Prod.* **2015,** *78* (4), 797-802.

42. Miller, J. S.; Birkinshaw, C.; Callmander, M., The Madagascar International Cooperative Biodiversity Group (ICBG): using natural products research to build science capacity. *Ethnobotany Research & Applications 3* **2005**, 283-286.

43. Hepatitis B Foundation: Press Room: Institute for hepatitis and virus research receives one of world's largest natural products library from Merck. http://www.hepb.org/news/release_6_06_2011.htm (accessed July 3, 2015).

44. About - Natural Products Discovery Institute. http://www.npdi-us.org/about/ (accessed July 2, 2015).

45. Natural Products Discovery Institute Makes its First Deal - Natural Products Discovery Institute. http://www.npdi-us.org/2011/11/npdi-makes-first-deal/#more-1 (accessed July 2, 2015).

46. Louie, K. G.; Behrens, B. C.; Kinsella, T. J.; Hamilton, T. C.; Grotzinger, K. R.; McKoy, W. M.; Winker, M. A.; Ozols, R. F., Radiation survival parameters of antineoplastic drug-sensitive and drug-resistant human ovarian-cancer cell-lines and their modification by buthionine sulfoximine. *Cancer Res.* **1985,** *45* (5), 2110-2115.

47. O'Brien, J.; Wilson, I.; Orton, T.; Pognan, F., Investigation of the Alamar Blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity. *Eur. J. Biochem.* **2000,** *267* (17), 5421-5426.

48. Cao, S.; Brodie, P. J.; Miller, J. S.; Randrianaivo, R.; Ratovoson, F.; Birkinshaw, C.; Andriantsiferana, R.; Rasamison, V. E.; Kingston, D. G. I., Antiproliferative xanthones of *Terminalia calcicola* from the Madagascar rain forest. *J. Nat. Prod.* **2007,** *70* (4), 679-681.

49. Pan, E.; Harinantenaina, L.; Brodie, P. J.; Miller, J. S.; Callmander, M. W.; Rakotonandrasana, S.; Rakotobe, E.; Rasamison, V. E.; Kingston, D. G. I., Four diphenylpropanes and a cycloheptadibenzofuran from *Bussea sakalava* from the Madagascar dry forest. *J. Nat. Prod.* **2010,** *73* (11), 1792-1795.

50. Smilkstein, M.; Sriwilaijaroen, N.; Kelly, J. X.; Wilairat, P.; Riscoe, M., Simple and inexpensive fluorescence-based technique for high-throughput antimalarial drug screening. *Antimicrob. Agents Chemother.* **2004,** *48* (5), 1803-1806.

51. Harinantenaina, L.; Bowman, J. D.; Brodie, P. J.; Slebodnick, C.; Callmander, M. W.; Rakotobe, E.; Randrianaivo, R.; Rasamison, V. E.; Gorka, A.; Roepe, P. D.; Cassera, M. B.;

Kingston, D. G. I., Antiproliferative and antiplasmodial dimeric phloroglucinols from *Mallotus oppositifolius* from the Madagascar dry forest. *J. Nat. Prod.* **2013,** *76* (3), 388-393.

52. Bassaganya-Riera, J.; Guri, A. J.; Lu, P.; Climent, M.; Carbo, A.; Sobral, B. W.; Horne, W. T.; Lewis, S. N.; Bevan, D. R.; Hontecillas, R., Abscisic acid regulates inflammation via ligand-binding domain-independent activation of peroxisome proliferator-activated receptor gamma. *J. Biol. Chem.* **2011,** *286* (4), 2504-2516.

53. Månsson, M.; Phipps, R. K.; Gram, L.; Munro, M. H. G.; Larsen, T. O.; Nielsen, K. F., Explorative solid-phase extraction (E-SPE) for accelerated microbial natural product discovery, dereplication, and purification. *J. Nat. Prod.* **2010,** *73*, 1126-1132.

54. Cardellina, J. H., II; Munro, M. H. G.; Fuller, R. W.; Manfredi, K. P.; McKee, T.; Tischler, M.; Bokesch, H. R.; Gustafson, K. R.; Beutler, J. A.; Boyd, M. R., A chemical screening strategy for the dereplication and prioritization of HIV-inhibitory aqueous natural products extracts. *J. Nat. Prod.* **1993,** *56*, 1123-1129.

55. Konishi, Y.; Kiyota, T.; Draghici, C.; Gao, J.-M.; Yeboah, F.; Acoca, S.; Jarussophon, S.; Purisima, E., Molecular formula analysis by an MS/MS/MS technique to expedite dereplication of natural products. *Anal. Chem.* **2007,** *79*, 1187-1197.

56. Jarussophon, S.; Acoca, S.; Gao, J.-M.; Deprez, C.; Kiyota, T.; Draghici, C.; Purisima, E.; Konishi, Y., Automated molecular formula determination by tandem mass spectrometry (MS/MS). *Analyst* **2009,** *134*, 690-700.

57. Molinski, T. F., NMR of natural products at the nanomole-scale. *Nat. Prod. Rep.* **2010,** *27*, 321-329.

58. Schlotterbeck, G.; Ross, A.; Hochstrasser, R.; Senn, H.; Kuehn, T.; Marek, D.; Schett, O., High-resolution capillary tube NMR. A miniaturized 5-µL high-sensitivity TXI probe for mass-limited samples, off-line LC NMR, and HT NMR. *Anal. Chem.* **2002,** *74*, 4464-4471.

59. Molinski, T. F., Microscale methodology for structure elucidation of natural products. *Curr. Opin. Biotechnol.* **2010,** *21*, 819-826.

60. Eugster, P. J.; Guillarme, D.; Rudaz, S.; Veuthey, J.-L.; Carrupt, P.-A.; Wolfender, J.-L., Ultra high pressure liquid chromatography for crude plant extract profiling. *J. AOAC Int.* **2011,** *94*, 51-70.

61. Potterat, O.; Hamburger, M., Natural products in drug discovery - concepts and approaches for tracking bioactivity. *Curr. Org. Chem.* **2006,** *10*, 899-920.

62. Nguyen, D. T. T.; Guillarme, D.; Rudaz, S.; Veuthey, J.-L., Chromatographic behaviour and comparison of column packed with sub-2µm stationary phases in liquid chromatography. *J. Chromatogr., A* **2006,** *1128*, 105-113.

63. Novakova, L.; Matysova, L.; Solich, P., Advantages of application of UPLC in pharmaceutical analysis. *Talanta* **2006,** *68*, 908-918.

64. Nguyen, D. T. T.; Guillarme, D.; Rudaz, S.; Veuthey, J.-L., Fast analysis in liquid chromatography using small particle size and high pressure. *J. Sep. Sci.* **2006,** *29*, 1836-1848.

65. Wren, S. A. C.; Tchelitcheff, P., Use of ultra-performance liquid chromatography in pharmaceutical development. *J. Chromatogr., A* **2006,** *1119*, 140-146.

66. Wolfender, J.-L., HPLC in natural product analysis: the detection issue. *Planta Med.* **2009,** *75*, 719-734.

67. Lin, Y.; Schiavo, S.; Orjala, J.; Vouros, P.; Kautz, R., Microscale LC-MS-NMR platform applied to the identification of active cyanobacterial metabolites. *Anal. Chem.* **2008,** *80*, 8045-8054.

68. Cordell, G. A.; Shin, Y. G., Finding the needle in the haystack. The dereplication of natural product extracts. *Pure Appl. Chem.* **1999,** *71*, 1089-1094.

69. Plumb, R. S.; Jones, M. D.; Rainville, P.; Castro-Perez, J. M., The rapid detection and identification of the impurities of simvastatin using high resolution sub 2 μm particle LC coupled to hybrid quadrupole time of flight MS operating with alternating high-low collision energy. *J. Sep. Sci.* **2007,** *30*, 2666-2675.

70. Mitova, M. I.; Murphy, A. C.; Lang, G.; Blunt, J. W.; Cole, A. L. J.; Ellis, G.; Munro, M. H. G., Evolving trends in the dereplication of natural product extracts. 2. The isolation of chrysaibol, an antibiotic peptaibol from a New Zealand sample of the mycoparasitic fungus sepedonium chrysospermum. *J. Nat. Prod.* **2008,** *71*, 1600-1603.

71. Gronquist, M.; Meinwald, J.; Eisner, T.; Schroeder, F. C., Exploring uncharted terrain in nature's structure space using capillary NMR spectroscopy: 13 steroids from 50 fireflies. *J. Am. Chem. Soc.* **2005,** *127*, 10810-10811.

72. Bradshaw, J.; Butina, D.; Dunn, A. J.; Green, R. H.; Hajek, M.; Jones, M. M.; Lindon, J. C.; Sidebottom, P. J., A rapid and facile method for the dereplication of purified natural products. *J. Nat. Prod.* **2001,** *64*, 1541-1544.

73. Lang, G.; Mayhudin, N. A.; Mitova, M. I.; Sun, L.; van, d. S. S.; Blunt, J. W.; Cole, A. L. J.; Ellis, G.; Laatsch, H.; Munro, M. H. G., Evolving trends in the dereplication of natural product extracts: new methodology for rapid, small-scale investigation of natural product extracts. *J. Nat. Prod.* **2008,** *71*, 1595-1599.

74. Corley, D. G.; Durley, R. C., Strategies for database dereplication of natural products. *J. Nat. Prod.* **1994,** *57*, 1484-1490.

75. Miller, R. W.; Powell, R. G.; Smith, C. R.; Arnold, E.; Clardy, J., Antileukemic alkaloids from *Taxus wallichiana* Zucc. *J. Org. Chem.* **1981,** *46* (7), 1469-1474.

76. Sénilh, V.; Blechert, S.; Colin, M.; Guénard, D.; Picot, F.; Potier, P.; Varenne, P., Mise en évidence de nouveaux analogues du Taxol extraits de *Taxus baccata*. *J. Nat. Prod.* **1984,** *47* (1), 131-137.

77. Magri, N. F.; Kingston, D. G. I., Modified Taxols. 2. Oxidation products of Taxol. *J. Org. Chem.* **1986,** *51* (6), 797-802.

78. Denis, J. N.; Greene, A. E.; Guenard, D.; Gueritte-Voegelein, F.; Mangatal, L.; Potier, P., Highly efficient, practical approach to natural Taxol. *J. Am. Chem. Soc.* **1988,** *110* (17), 5917-5919.

79. Tao, L.; Zhu, F.; Qin, C.; Zhang, C.; Chen, S.; Zhang, P.; Zhang, C.; Tan, C.; Gao, C.; Chen, Z.; Jiang, Y.; Chen, Y. Z., Clustered distribution of natural product leads of drugs in the chemical space as influenced by the privileged target-sites. *Sci. Rep.* **2015,** *5*, 9325.

Chapter 2: Antiproliferative Trihydroxyalkylcyclohexenones from *Pleiogynium timoriense*

2.1 Introduction

2.1.1 Abstract

Investigation of a dichloromethane (DCM) extract of the bark of *Pleiogynium timoriense* from the former Merck collection of natural product extracts for antiproliferative activity indicated that it was active with an IC_{50} value of 1.3 μg/mL against the A2780 ovarian cancer cell line. Bioassay-directed fractionation of this extract yielded the three new bioactive trihydroxyalkylcyclohexenones **2.1**–**2.3**. Their structures were determined by a combination of spectroscopic and chemical methods. Compounds **2.1**–**2.3** exhibited submicromolar antiproliferative activity against the A2780 human ovarian cancer cell line, with IC_{50} values of 0.8, 0.7, and 0.8 μM, respectively. This is a modified and expanded version of previously published work (Eaton, A. L.; et al. *J. Nat. Prod.*, **2015**, *78* (*7*), 1752–1755).[1]

Figure 2-1. *Pleiogynium timoriense.* Used under Creative Commons (CC BY-NC-ND 3.0) from <http://www.tropicos.org/Image/100118516>.

2.1.2 Author Contributions

The author of this dissertation (Alexander L. Eaton) completed the fractionation of the extract and the identification of the compounds described as well as the drafting of the manuscript. Ms. Peggy Brodie performed the antiproliferative bioassay (A2780) on all fractions and compounds. Dr. Michael Goetz provided the extract from the Natural Product Discovery Institute (NPDI). Dr. Liva Harinantenaina obtained the ECD spectra. Dr. David G. I. Kingston was a mentor for this work.

2.1.3 Previous Investigations of Pleiogynium timoriense

P. timoriense, also known as the Burdekin plum, is a tree found in northeast Australia and Malaysia as well as locations in the south-central Pacific and southwestern Pacific.[2] Its fruit is used to make jam,[3] and its leaves have been reported to be a source of antioxidants. Twelve compounds, including kaempferol, gallic acid, various kaempferol, quercitin, and myricetin glycosides, and three galloyl derivatives have been identified from the ethanolic extract of the leaves.[4] It has also been reported that cyanidin 3-glucoside can be found in the fruit of *P. timoriense*.[5,6] The DCM fraction was selected for fractionation based on its antiproliferative activity and the lack of reported antiproliferative compounds from the species.

2.1.4 Chemical Investigation of Pleiogynium timoriense

As part of an investigation of the former Merck natural products extract library for antiproliferative constituents, now maintained by the Natural Products Discovery Institute,[7] we identified a DCM fraction of the ethanol extract of the bark of *Pleiogynium timoriense* (Anacardiaceae) as a promising extract with an IC_{50} value of 1.3 μg/mL against the A2780 ovarian

cancer cell line. The DCM fraction (0.30 g) was fractionated using by Sephadex LH-20 column chromatography and two rounds of C_{18} HPLC to yield the three active compounds **2.1**–**2.3**.

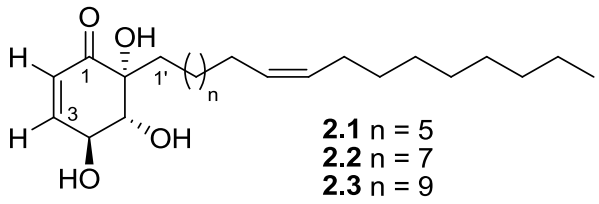
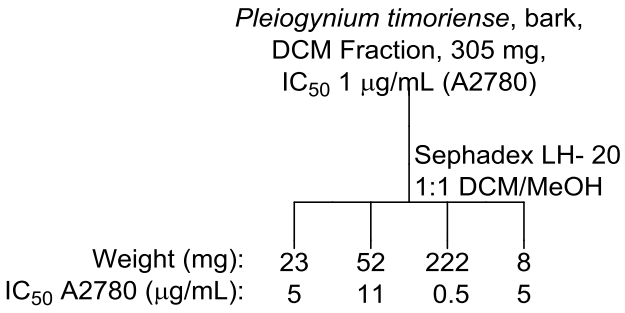


Figure 2-2. Compounds isolated from *Pleiogynium timoriense*.

2.2 Results and Discussion

2.2.1 Isolation of Compounds from Pleiogynium timoriense

The DCM fraction of the bark of *Pleiogynium timoriense* was partitioned by using Sephadex LH-20 chromatography (Scheme 2-1). This yielded one fraction with increased activity that was separated further by C_{18} HPLC to yield semipure **2.1-2.3**. Compounds **2.1-2.3** were purified by C_{18} HPLC. Compound **2.2** was obtained in the largest amount and was investigated first.



Scheme 2-1. Fractionation of *Pleiogynium timoriense.*

2.2.2 Structure Elucidation

^{13}C NMR and HRESIMS data indicated that compound **2.2** had the molecular formula $C_{25}H_{44}O_4$ ($[M+H]^+$ *m/z* 409.3291, calcd for $C_{25}H_{45}O_4^+$ 409.3312). Its ^{1}H NMR spectrum indicated the presence of an *α,β*-unsaturated carbonyl group (H-2, δ 6.1, 1H, dd, *J* = 10.2, 0.7 Hz; H-3 δ 6.8, 1H, ddd, *J* = 10.1, 3.9, 1.3 Hz), which was confirmed by its ^{13}C NMR spectrum (C-1, δ 200.2; C-2, δ 126.4; C-3, δ 145.9). A large peak for methylene protons in the ^{1}H NMR spectrum (δ 1.22-1.34) as well as a triplet at δ 0.88 (H-19′, 3H, t, *J* = 6.9 Hz) indicated the presence of a long alkyl chain in **2.2**. This was consistent with the ^{13}C NMR data, which showed 10 signals at approximately δ 29 as well as signals at δ 23.0 (C-2′), 22.7 (C-18′), and 14.7 (C-19′). The NMR spectroscopic data are comparable to those of other known trihydroxyalkylcyclohexenone derviatives.[8-11] The presence of a double bond within the alkyl chain was indicated by a signal at δ 5.34 (2H, m) in the ^{1}H NMR spectrum as well as by COSY correlations from δ 5.34 (H-10′, H-11′) to δ 2.00 (H-9′, H-12′).

COSY correlations between H-3 and H-4 as well as H-4 and H-5 were used to establish the positions of C-4 and C-5 (Figure 2-3). The lack of other correlations in the COSY spectra indicated that C-5 must be attached to an oxygenated tertiary carbon (C-6). HMBC correlations of H-2 to C-6, H-4 to C-6, and H-5 to C-1 (Figure 2-3) indicated that the structure contained a cyclic moiety, which is consistent with the calculated hydrogen deficiency index of four.

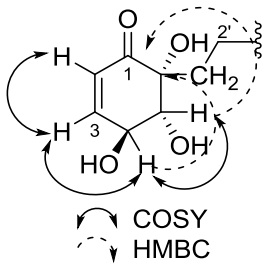


Figure 2-3. Selected 2D NMR correlations of **2.2**.

The configuration of the double bond in the alkyl chain was assigned as *Z* based on the shifts of the adjacent carbon atoms (δ 27.2 C-9′ and C-12′), which would have been more shielded in the case of an *E*-configuration (δ ~32).[12,13] The connectivity of the alkyl chain at C-6 was determined from the HMBC spectrum, which showed long-range correlations from H-1′ to C-6. The remaining ^{13}C NMR signals were assigned using HSQC and HMBC spectroscopy. Complete NMR assignments of all carbons and protons for **2.2** are reported in Table 2-1.

The location of the double bond in the alkenyl chain was determined through MS analysis of the products resulting from derivatization with dimethyl disulfide(), following the method of Mansour[14] and Roumy.[10] The LC-MS of the dimethyl disulfide derivative of **2.2** contained a strong fragment ion at *m/z* 329.19 (calcd $[C_{17}H_{29}O_4S]^+$ 329.18), indicating a $\Delta^{10',11'}$ double bond.

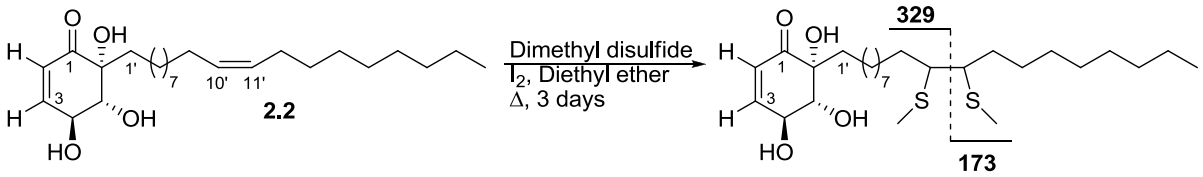


Figure 2-4. Dimethyl disulfide derivatization and fragmentation of **2.2.**

The ^{1}H NMR spectra of compounds **2.1** and **2.3** were similar to those of compound **2.2** (Table 2-1). The structures of **2.1** and **2.3** were assigned by comparison of NMR and MS data with those of **2.2**. ^{1}H and ^{13}C NMR spectroscopic data indicated that the structure of the cyclic moiety was identical based on chemical shifts and coupling constants. The only differences in the structures were due to the length of the alkyl chain and the location of the double bond within the chain. HRESIMS and ^{13}C NMR data were used to determine that **2.1** contained two fewer methylene groups than **2.2** ($[M+H]^+$ *m/z* 381.2972, calcd for $C_{23}H_{41}O_4^+$ 381.2999) and that **2.3** contained two additional methylene groups ($[M+H]^+$ *m/z* 437.3604, calcd for $C_{27}H_{49}O_4^+$

437.3625). LC-MS analysis of the dimethyl disulfide derivative of **2.1** showed a strong fragment ion at *m/z* 301.12 (calcd for $[C_{15}H_{25}O_4S]^+$ 301.15), and that of the same derivative of **2.3** showed a strong fragment ion at 357.20 (calcd for $[C_{19}H_{33}O_4S]^+$ 357.21). These results indicated $\Delta^{9',10'}$ and $\Delta^{12',13'}$ double bonds in **2.1** and **2.3**, respectively.

Table 2-1. NMR spectroscopic data of **2.1**–**2.3**.

	2.1[a]		**2.2**[b]		**2.3**[a]	
position	δ_C, type	δ_H, (*J* in Hz)	δ_C, type	δ_H, (*J* in Hz)	δ_C, type[j]	δ_H, (*J* in Hz)
1	200.2, C		200.2, C		201.6, C	
2	126.4, CH	6.10, dd (10.1, 0.8)	126.4, CH	6.10, dd (10.2, 0.7)	126.3, CH	6.10, dd (10.2, 0.8)
3	145.8, CH	6.80, ddd, (10.1, 3.9, 1.4)	145.9, CH	6.80, ddd, (10.1, 3.9, 1.3)	145.5, CH	6.80, ddd, (10.1, 4.0, 1.5)
4	68.6, CH	4.62, brs	68.5, CH	4.62, brs	68.5, CH	4.63, brs
5	75.4, CH	3.98, brs	75.4, CH	3.98, brs	75.3, CH	3.98, dd, (3.0, 1.5)
6	78.1, C		78.1, C		77.8, C	
1′	36.1, CH_2	1.83,m	36.1, CH_2	1.83,m	36.1, CH_2	1.83,m
2′	23.0, CH_2	1.13, m	23.0, CH_2	1.13, m	22.7, CH_2	1.13, m
3′	29.72, CH_2[c]	1.26, brs[g]	29.80, CH_2[c]	1.26, brs[g]	29.4, CH_2[c]	1.25, brs[g]
4′	29.78, CH_2[c]	1.26, brs[g]	29.78, CH_2[c]	1.26, brs[g]	29.4, CH_2[c]	1.25, brs[g]
5′	29.7, CH_2[c]	1.26, brs[g]	29.7, CH_2[c]	1.26, brs[g]	29.4, CH_2[c]	1.25, brs[g]
6′	29.5, CH_2[c]	1.26, brs[g]	29.53, CH_2[c]	1.26, brs[g]	29.4, CH_2[c]	1.25, brs[g]
7′	27.19, CH_2[d]	2.00, m[h]	29.53, CH_2[c]	1.26, brs[g]	29.4, CH_2[c]	1.25, brs[g]
8′	129.9, CH[e]	5.34, m[i]	29.51, CH_2[c]	1.26, brs[g]	29.4, CH_2[c]	1.25, brs[g]
9′	129.8, CH[e]	5.34, m[i]	27.22, CH_2[d]	2.00, m[h]	29.4, CH_2[c]	1.25, brs[g]
10′	27.23, CH_2 [d]	2.00, m[h]	129.86, CH[e]	5.34, m[i]	29.4, CH_2[c]	1.25, brs[g]
11′	29.32, CH_2[c]	1.26, brs[g]	129.93, CH[e]	5.34, m[i]	27.1, CH_2[d]	2.00, m[h]
12′	29.2, CH_2[c]	1.26, brs[g]	27.22, CH_2[d]	2.00, m[h]	129.8, CH[e]	5.35, m[i]
13′	29.34, CH_2[c]	1.26, brs[g]	29.4, CH_2[c]	1.26, brs[g]	129.8, CH[e]	5.35, m[i]
14′	29.34, CH_2[c]	1.26, brs[g]	29.33, CH_2[c]	1.26, brs[g]	27.1, CH_2[d]	2.00, m[h]
15′	31.9, CH_2	1.26, brs[g]	29.33, CH_2[c]	1.26, brs[g]	29.4, CH_2[c]	1.25, brs[g]
16′	22.7, CH_2	1.26, brs[g]	29.30, CH_2[c]	1.26, brs[g]	29.4, CH_2[c]	1.25, brs[g]
17′	14.1, CH_3	0.88, t (6.9)	31.9, CH_2	1.26, brs[g]	29.4, CH_2[c]	1.25, brs[g]
18′			22.7, CH_2	1.26, brs[g]	29.4, CH_2[c]	1.25, brs[g]
19′			14.2, CH_3	0.88, t (6.9)	31.8, CH_2	1.25, brs[g]
20′					22.6, CH_2	1.25, brs[g]
21′					14.0, CH_3	0.88, t (6.9)
OH		3.60[f]		3.59[f]		exchanges
OH		2.94[f]		2.90[f]		exchanges
OH		2.41[f]		2.37[f]		exchanges

[a]$CDCl_3$, 500 MHz, 150 MHz
[b]$CDCl_3$, 500 MHz, 125 MHz
[c, d, e, f]Interchangeable assignment within a column
[g, h, i]Overlapping signals
[j]Obtained from HMBC and HSQC spectra

Determination of the configurations of the stereogenic centers on the cyclohexenone ring of **2.2** proved to be a challenge. Comparison of ^{1}H NMR shifts and coupling constants with those of similar known trihydroxyalkylcyclohexenones was not definitive in determining the relative configuration.[9,11,15] However, the observed coupling constants of H-2 and H-3 in its ^{1}H NMR spectrum (H-2, dd, J = 1.1, 10.2 Hz; H-3, ddd, J = 1.1, 3.7, 10.2 Hz) are consistent with those of similar alkylcyclohexenones with the same relative configuration as proposed.[16] In order to further support our proposed relative configuration, the cyclic double bond of **2.2** was selectively reduced with diphenylsilane in the presence of $ZnCl_2$ and $Pd(PPh_3)_4$ to give the substituted cyclohexanone **2.4**. Irradiation of H-3 in a selective NOE experiment indicated correlations to H-1′ and H-4, but no correlation was observed to H-5b (δ 1.84), which indicated that H-3 was equatorial (Figure 2-5). The small coupling constants observed for H-3 in the ^{1}H NMR spectrum of **2.4** (1H, dd, J = 4.5, 1.1 Hz) allowed the assignment of an equatorial orientation for H-4 in **2.4** and, thus, the corresponding pseudoequatorial orientation of H-4 in **2.2**. A correlation was observed from H-1′ (δ 1.7) to H-6a (δ 2.7) in a 2D NOESY spectrum, indicating that the alkyl chain was in an axial

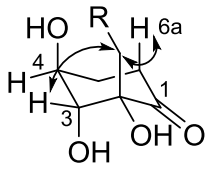


Figure 2-5. Selected NOE correlations of **2.4**.

orientation. The conformational preference for three axial substituents and only one equatorial substituent in **2.4** is presumably due to hydrogen bond formation between the carbonyl and the C-2 hydroxy groups.

The absolute configuration of **2.1** was determined by application of the dibenzoate chirality rule.[17,18] Surprisingly, acylation of **2.1** with *p*-bromobenzoyl chloride yielded the tri-*p*-

bromobenzoate **2.5**. The corresponding *J* couplings observed in the ^{1}H NMR spectrum of **2.5** (H-2, dd, *J* = 10.5, 2.2 Hz; H-3, dd, *J* = 10.5, 2.3 Hz; H-4, ddd, *J* = 7.5, 2.3, 2.2 Hz; H-5, d, *J* = *7.5*)

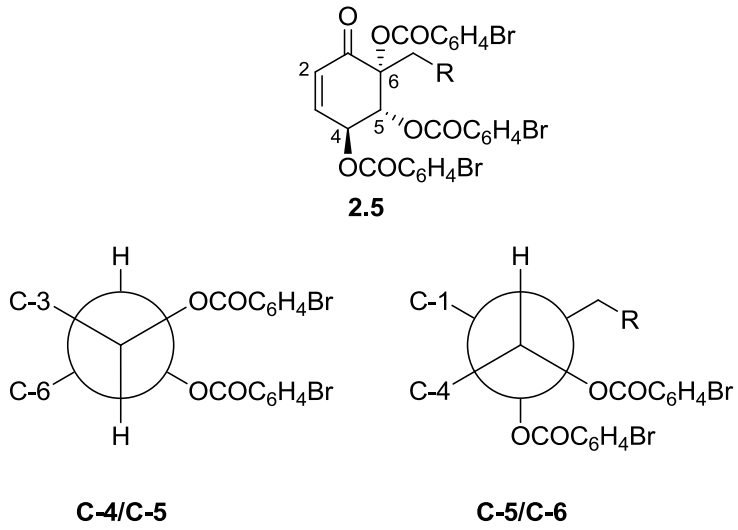


Figure 2-6. Structure of **2.5** and Newman projections of the C-4/C-5 and C-5/C-6 bonds of its major conformer.

are significantly different from those of **2.1**–**2.3**, indicating a change to a major conformation with the C-4 and C-5 *p*-bromobenzoate groups and the C-6 alkenyl side chain equatorial and only the C-6 *p*-bromobenzoate group axial (Figure 2-6). The resulting ECD spectrum (see Supporting Information 13.18) showed a positive Cotton effect at 253 nm, as predicted by the Newman projections of the C-4/C-5 and C-5/C-6 bonds. The expected weaker negative second Cotton effect is presumably buried in the strong positive background ellipticity. Thus, the absolute configuration of **2.1** is assigned as 4*S*,5*R*,6*R*. Additionally, since **2.1** ($[\alpha]^{22}_{D}$ +21) and **2.3** ($[\alpha]^{22}_{D}$ +19) have similar values of optical rotation to **2.1** ($[\alpha]^{22}_{D}$ +23), their absolute configurations must also be 4*S*,5*R*,6*R*.

The three isolated compounds are similar in structure to other known hydroxyalkylcyclohexenones that are found from *Tapirira obtusa*, *T. guianesnsis*, and *Lannea edulis* in the family Anacardiaceae.[10,12,19] Furthermore, they contain the same oxygenation pattern

as the zeylenones, many of which have been isolated from various members of the *Uvaria* genus (Annonaceae).[20-23]

2.2.3 Antiproliferative Activity

The antiproliferative activities of the three new compounds were determined against the A2780 ovarian cancer cell line. All three compounds exhibited moderate antiproliferative activity, with IC_{50} values of 0.8 ± 0.4, 0.7 ± 0.3, and 0.8 ± 0.5 μM, respectively.

2.3 Experimental Section

2.3.1 General Experimental Procedures

Optical rotations were recorded on a JASCO P-2000 polarimeter, and UV spectra were measured on a Shimadzu UV-1201 spectrophotometer. ECD analysis was performed on a JASCO J-810 spectropolarimeter with a 0.1 cm cell in MeOH at room temperature under the following conditions: speed 50 nm/min, time constant 1 s, bandwidth 2.0 nm. ^{1}H and ^{13}C NMR spectra were recorded using either Bruker Avance 500 or 600 spectrometers. ^{1}H-^{1}H three-bond *J*-coupling values were calculated from ^{1}H NMR spectra. MS were obtained on an Agilent 6220 LC-TOF-MS or a Thermo Electron TSQ LC-ESI-MS. Semipreparative HPLC was performed using Shimadzu LC-10AT pumps coupled with a Shimadzu SPD M10A diode array detector, a SCL-10A system controller, and a Cogent Bidentate C_{18} column (250 x 10 mm) or a Varian Lichrosorb 5 Diol column (250 x 10 mm). Bioactive compounds were checked for purity by analytical HPLC analysis using a Shimadzu SPD M10A diode array detector, a Sedex 75 ELSD, a SCL-10A system controller, and a Cogent Biodentate C_{18} column (75 x 4.6 mm).

2.3.2 Plant Material

Bark of *Pleiogynium timoriense* (DC) Leenh. was collected by Dr. Paul Cox under the auspices of the New York Botanical Garden (NYBG) from a seaward-facing forest on the island of Eua, Tonga in July 1987; a voucher specimen, PC01113 (ID number 40077), is on deposit at the NYBG.

2.3.3 Extraction and Isolation

An EtOH extract of the bark of *P. timoriense* was subjected to liquid−liquid partitioning to give active hexanes, DCM, and aqueous MeOH fractions with IC_{50} values against the A2780 ovarian cancer cell line of 3.0, 1.3, and 6.2 μg/mL, respectively. The active DCM fraction 23050-C6 (0.30 g) was fractionated using Sephadex LH-20 column chromatography (1:1 DCM/MeOH) to generate an active fraction (222 mg, IC_{50} 0.5 μg/mL). This fraction was further purified utilizing C_{18} HPLC (MeOH/H_2O gradient) to yield 3 semipure active fractions. These fractions were further purified using C_{18} HPLC (MeCN/H_2O gradient) to yield the active compounds **2.1** (5.4 mg), **2.2** (6.9 mg), and **2.3** (1.4 mg).

2.3.4 Antiproliferative Bioassay

The assay was performed at Virginia Tech according to specifications previously described.[24,25] The A2780 cell line is a drug-sensitive ovarian cancer cell line.[26]

2.3.5 Spectroscopic Properties

Compound 2.1: $[\alpha]^{22}_{D}$ +23 (*c* 0.5 $CHCl_3$); UV (MeOH) λ_{max} (log ε) 215 (3.59) nm; ECD (MeOH) $[\Delta\varepsilon]_{330\ nm}$ -0.17, $[\Delta\varepsilon]_{242\ nm}$ +1.72, $[\Delta\varepsilon]_{209\ nm}$ -1.07; ^{1}H NMR ($CDCl_3$, 500 MHz) see Table 2-1; ^{13}C NMR ($CDCl_3$, 150 MHz) see Table 2-1; HRESIMS $[M+H]^+$ *m/z* 381.2972 (calcd for $C_{23}H_{41}O_4^+$ 381.2999), $[M+Na]^+$ *m/z* 403.2805 (calcd for $C_{23}H_{40}NaO_4^+$ 403.2819).

Compound 2.2: $[\alpha]^{22}_{D}$ +21 (*c* 0.7 $CHCl_3$); UV (MeOH) λ_{max} (log ε) 215 (3.44) nm; ^{1}H NMR ($CDCl_3$, 500 MHz) see Table 2-1; ^{13}C NMR ($CDCl_3$, 125 MHz) see Table 2-1; HRESIMS $[M+H]^+$ *m/z* 409.3291 (calcd for $C_{25}H_{45}O_4^+$ 409.3312), $[M+Na]^+$ *m/z* 431.3125 (calcd for $C_{25}H_{44}NaO_4^+$ 431.3132).

Compound 2.3: $[\alpha]^{22}_{D}$ +19 (*c* 0.1 $CHCl_3$); UV (MeOH) λ_{max} (log ε) 215 (3.25) nm; ^{1}H NMR ($CDCl_3$, 500 MHz) see Table 2-1; ^{13}C NMR ($CDCl_3$, 150 MHz) see Table 2-1; HRESIMS $[M+H]^+$ *m/z* 437.3604 (calcd for $C_{27}H_{49}O_4^+$ 437.3625), $[M+Na]^+$ *m/z* 459.3431 (calcd for $C_{27}H_{48}NaO_4^+$ 459.3445).

Reduction of Compound 2.2. Compound **2.2 (**5.8 mg, 0.014 mmol) was dissolved in $CHCl_3$ (3 mL) and Ph_2SiH_2 (5.3 μL, 5.3 mg, 0.028 mmol), $ZnCl_2$ (0.5 mg, 0.0037 mmol), and $Pd(PPh_3)_4$ (0.3 mg, 0.00026 mmol) were added. The reaction mixture was stirred for 4 h at rt. The solvent was removed under reduced pressure and the residue was purified by utilizing silica gel column open column chromatography (7:3 hexanes/EtOAc) to yield 2.3 mg of **2.4** (0.0056 mmol, 39%).

Compound 2.4: ^{1}H NMR (CD_3OD, 600 MHz) δ 5.38 (2H, m, H-10′, H-11′), 4.03 (1H, q, *J* = 4.3 Hz, H-4), 3.71 (1H, dd, *J* = 4.5, 1.1 Hz, H-5), 2.70 (1H, ddd, *J* = 13.4, 11.7, 5.6 Hz, H-2a), 2.37 (1H, dt, *J* = 13.4, 5.2 Hz, H-2b), 2.16 (2H, m, H-3a, H-1′b), 1.97 (4H, brs, H-9′, H-12′), 1.84 (1H, m, H-3b), 1.70 (1H, ddd, *J* = 13.9, 12.4, 4.3 Hz, H-1′a), 1.29 (24H, brs, H-3′, H-4′, H-5′, H-6′, H-7′, H-8′, H-13′, H-14′, H-15′, H-16′, H-17′, H-18′) 1.08 (2H, m, H-2′), 0.90 (3H, t, *J* = 7.0 Hz, H-19′); HRESIMS $[M+H]^+$ *m/z* 411.3472 (calcd for $C_{23}H_{43}O_4^+$ 411.3479), $[M+Na]^+$ *m/z* 433.3288 (calcd for $C_{23}H_{42}NaO_4^+$ 433.3288).

***p*-Bromobenzoylation of Compound 2.1.** Compound **2.1 (**0.4 mg, 0.001 mmol) was dissolved in DCM (2 mL), and 44.9 mg (0.37 mmol) of DMAP and 80.7 mg (0.37 mmol) of *p*-bromobenzoyl chloride were added. The reaction mixture was stirred for 1.5 h at rt. The solution was diluted with DCM (5 mL) and washed with H_2O (5 mL x 3), 3 M HCl (5 mL), and brine (5 mL). The organic layer was dried with anhydrous $MgSO_4$ and the solvent was removed under vacuum. The resulting residue was purified utilizing diol HPLC (hexanes/EtOAc gradient) to yield compound **2.5** (0.8 mg, 82%).

Compound 2.5: $[\alpha]^{22}_D$ +9 (*c* 0.07 MeOH); UV (MeOH) λ_{max} (log ε) 207 (4.12) nm, 248 (4.17); ECD (MeOH) $[\Delta\varepsilon]_{253\,nm}$ +23.84, $[\Delta\varepsilon]_{234\,nm}$ 0.00, $[\Delta\varepsilon]_{202\,nm}$ +7.94; ^{1}H NMR ($CDCl_3$, 500 MHz) 7.92 (2H, d, *J* = 8.6 Hz), 7.85 (2H, d, *J* = 8.6 Hz), 7.77 (2H, d, *J* = 8.6 Hz), 7.64 (2H, d, *J* = 8.6 Hz), 7.58 (2H, d, *J* = 8.6 Hz), 7.53 (2H, d, *J* = 8.6 Hz), 6.86 (1H, dd, *J* = 10.5, 2.3 Hz, H-3), 6.37 (1H, dd, *J* = 10.5, 2.2 Hz, H-2), 6.30 (1H, ddd, *J* = 7.5, 2.2, 2.2 Hz, H-4), 6.05 (1H, d, *J* = 7.5 Hz, H-5), 5.34 (2H, m, H-11′, H-12′), 2.35 (1H, m, H-1′a), 2.07 (1H, m, H-1′b), 1.98 (4H, m, H-10′, H-13′), 1.29 (20H, brs, H-3′, H-4′, H-5′, H-6′, H-7′, H-8′, H-9′, H-14′, H-15′, H-16′), 1.23 (2H, m, H-

2′), 0.88 (3H, t, *J* = 7.0 Hz); HRESIMS $[M+H]^+$ *m/z* 927.1055 (calcd for $C_{44}H_{50}Br_3O_7^+$ 927.1057), $[M+H]^+$ *m/z* 929.1048 (calcd for $C_{44}H_{50}Br_3O_7^+$ 929.1038), $[M+H]^+$ *m/z* 931.1082 (calcd for $C_{44}H_{50}Br_3O_7^+$ 931.1020), $[M+H]^+$ *m/z* 933.1018 (calcd for $C_{44}H_{50}Br_3O_7^+$ 933.1001).

2.4 **References**

1. Eaton, A. L.; Rakotondraibe, L. H.; Brodie, P. J.; Goetz, M.; Kingston, D. G. I., Antiproliferative trihydroxyalkylcyclohexenones from *Pleiogynium timoriense*. *J. Nat. Prod.* **2015,** *78* (7), 1752-1755.

2. Cavalcanti, S. B. T.; Teles, H. L.; Silva, D. H. S.; Furlan, M.; Young, M. C. M.; Bolzani, V. S., New tetra-acetylated oligosaccharide diterpene from *Cupania vernalis*. *J. Braz. Chem. Soc.* **2001,** *12* (3), 413-416.

3. Gachet, M. S.; Kunert, O.; Kaiser, M.; Brun, R.; Zehl, M.; Keller, W.; Munoz, R. A.; Bauer, R.; Schuehly, W., Antiparasitic compounds from *Cupania cinerea* with activities against *Plasmodium falciparum* and *Trypanosoma brucei rhodesiense*. *J. Nat. Prod.* **2011,** *74* (4), 559-566.

4. Al Sayed, E.; Martiskainen, O.; Sinkkonen, J.; Pihlaja, K.; Ayoub, N.; Singab, A.-E. N.; El-Azizi, M., Chemical composition and bioactivity of *Pleiogynium timoriense* (Anacardiaceae). *Nat. Prod. Commun.* **2010,** *5* (4), 545-550.

5. NetZel, M.; NetZel, G.; Tian, Q. G.; Schwartz, S.; Konczak, I., Native Australian fruits—a novel source of antioxidants for food. *Innov Food Sci Emerg* **2007,** *8* (3), 339-346.

6. Netzel, M.; Netzel, G.; Tian, Q.; Schwartz, S.; Konczak, I., Sources of antioxidant activity in Australian native fruits. Identification and quantification of anthocyanins. *J. Agric. Food. Chem.* **2006,** *54* (26), 9820-9826.

7. Dai, Y.; Harinantenaina, L.; Brodie, P. J.; Goetz, M.; Shen, Y.; TenDyke, K.; Kingston, D. G. I., Antiproliferative homoisoflavonoids and bufatrienolides from *Urginea depressa*. *J. Nat. Prod.* **2013,** *76* (5), 865-872.

8. Fraser, C. J.; Howell, G. P.; Harrity, J. P. A., A furan Diels–Alder cycloaddition approach to scyphostatin analogues. *Org. Biomol. Chem.* **2012,** *10* (45), 9058-9066.

9. Li, X.; Choi, H. D.; Kang, J. S.; Lee, C. O.; Son, B. W., New polyoxygenated farnesylcyclohexenones, deacetoxyyanuthone A and its hydro derivative from the marine-derived fungus *Penicillium sp*. *J. Nat. Prod.* **2003,** *66* (11), 1499-1500.

10. Roumy, V.; Fabre, N.; Portet, B.; Bourdy, G.; Acebey, L.; Vigor, C.; Valentin, A.; Moulis, C., Four anti-protozoal and anti-bacterial compounds from *Tapirira guianensis*. *Phytochemistry* **2009,** *70* (2), 305-311.

11. Schapiro, V.; Cavalli, G.; Seoane, G. A.; Faccio, R.; Mombru, A. W., Chemoenzymatic synthesis of chiral enones from aromatic compounds. *Tetrahedron: Asymmetry* **2002,** *13* (22), 2453-2459.

12. Correia, S. D.; David, J. M.; David, J. P.; Chai, H. B.; Pezzuto, J. M.; Cordell, G. A., Alkyl phenols and derivatives from *Tapirira obtusa*. *Phytochemistry* **2001,** *56* (7), 781-784.

13. Jie, M. S. F. L. K.; Pasha, M. K.; Alam, M. S., Synthesis and nuclear magnetic resonance properties of all geometrical isomers of conjugated linoleic acids. *Lipids* **1997,** *32*, 1041-1044.

14. Mansour, M. P.; Holdsworth, D. G.; Forbes, S. E.; Macleod, C. K.; Volkman, J. K., High contents of 24:6(n-3) and 20:1(n-13) fatty acids in the brittle star *Amphiura elandiformis* from Tasmanian coastal sediments. *Biochem. Syst. Ecol.* **2005,** *33* (7), 659-674.

15. Fraser, C. J.; Howell, G. P.; Harrity, J. P., A furan Diels-Alder cycloaddition approach to scyphostatin analogues. *Org. Biomol. Chem.* **2012,** *10* (45), 9058-9066.

16. Katoh, T.; Izuhara, T.; Yokota, W.; Inoue, M.; Watanabe, K.; Nobeyama, A.; Suzuki, T., Enantiocontrolled synthesis of the epoxycyclohexenone moieties of scyphostatin, a potent and specific inhibitor of neutral sphingomyelinase. *Tetrahedron* **2006,** *62* (7), 1590-1608.

17. Harada, N.; Nakanishi, K., Determining the chiralities of optically active glycols. *J. Am. Chem. Soc.* **1969,** *91* (14), 3989-3991.

18. Liu, H. W.; Nakanishi, K., Pyranose benzoates - an additivity relation in the amplitudes of exciton-split CD curves. *J. Am. Chem. Soc.* **1982,** *104* (5), 1178-1185.

19. Queiroz, E. F.; Kuhl, C.; Terreaux, C.; Mavi, S.; Hostettmann, K., New dihydroalkylhexenones from *Lannea edulis*. *J. Nat. Prod.* **2003,** *66* (4), 578-580.

20. Liao, Y.-H.; Xu, L.-Z.; Yang, S.-L.; Dai, J.; Zhen, Y.-S.; Zhu, M.; Sun, N.-J., Three cyclohexene oxides from *Uvaria grandiflora*. *Phytochemistry* **1997,** *45* (4), 729-732.

21. Palframan, M. J.; Kociok-Kohn, G.; Lewis, S. E., Photooxygenation of a microbial arene oxidation product and regioselective Kornblum-DeLaMare rearrangement: total synthesis of zeylenols and zeylenones. *Chemistry* **2012,** *18* (15), 4766-4774.

22. Takeuchi, Y.; Shi, Q. W.; Sugiyama, T.; Oritani, T., Polyoxygenated cyclohexenes from the Chinese tree, *Uvaria purpurea*. *Biosci. Biotechnol. Biochem.* **2002,** *66* (3), 537-542.

23. Ho, D. V.; Kodama, T.; Le, H. T.; Phan, K. V.; Do, T. T.; Bui, T. H.; Le, A. T.; Win, N. N.; Imagawa, H.; Ito, T.; Morita, H.; Nguyen, H. T., A new polyoxygenated cyclohexene and a new megastigmane glycoside from Uvaria grandiflora. *Bioorg. Med. Chem. Lett.* **2015**.

24. Cao, S.; Brodie, P. J.; Miller, J. S.; Randrianaivo, R.; Ratovoson, F.; Birkinshaw, C.; Andriantsiferana, R.; Rasamison, V. E.; Kingston, D. G. I., Antiproliferative xanthones of *Terminalia calcicola* from the Madagascar rain forest. *J. Nat. Prod.* **2007,** *70* (4), 679-681.

25. Pan, E.; Harinantenaina, L.; Brodie, P. J.; Miller, J. S.; Callmander, M. W.; Rakotonandrasana, S.; Rakotobe, E.; Rasamison, V. E.; Kingston, D. G. I., Four diphenylpropanes and a cycloheptadibenzofuran from *Bussea sakalava* from the Madagascar dry forest. *J. Nat. Prod.* **2010,** *73* (11), 1792-1795.

26. Louie, K. G.; Behrens, B. C.; Kinsella, T. J.; Hamilton, T. C.; Grotzinger, K. R.; McKoy, W. M.; Winker, M. A.; Ozols, R. F., Radiation survival parameters of antineoplastic drug-sensitive and -resistant human ovarian cancer cell lines and their modification by buthionine sulfoximine. *Cancer Res.* **1985,** *45* (5), 2110-2115.

Chapter 3: Bioactive Diterpene Glycosides from *Molinaea retusa* from the Madagascar Dry Forest

3.1 Introduction

3.1.1 Abstract

As a part of the ICBG collaboration, an ethanol extract of *Molinaea retusa* Radlk. (Sapindaceae) was investigated on the basis of it moderate antiproliferative activity against the A2780 human ovarian cancer cell line (IC_{50} 16 μg/mL). One new compound, 2″,3″,4″,6′-de-*O*-acetylcupacinoside (**3.1**, IC_{50} 15.4 μM) and two known compounds, cupacinoside (**3.2**, IC_{50} 9.5 μM) and 6′-de-*O*-acetylcupacinoside (**3.3**, IC_{50} 10.9 μM), were isolated by bioassay-directed fractionation using liquid–liquid partitioning, column chromatography, and HPLC. Cupacinoside (**3.2**) and 6-de-*O*-acetylcupacinoside (**3.3**) also had moderate antiplasmodial activities, with IC_{50} values of 4.0 and 6.4 μM, respectively, against *Plasmodium falciparum*, Dd2 strain. The structures were determined using spectroscopic methods. This is an expanded version of a previously published article on this species (Eaton, A. L.; et al. *Nat. Prod. Commun.* **2013,** *8* (9), 1201-1203).[1]

Figure 3-1. Flowers of *Molinaea retusa*. Used under Creative Commons (CC BY-NC-ND 3.0) from <http://www.tropicos.org/Image/100127490>.

3.1.2 Author Contributions

The author of this dissertation (Alexander L. Eaton) conducted the isolation and structure elucidation of the described compounds and drafted the manuscript. Dr. Liva Harinantenaina provided advice for the isolation and structure elucidation of the compounds described. Ms. Peggy Brodie performed the antiproliferative bioassay (A2780) on all fractions and compounds. Dr. Jessica D. Wiley and Dr. Maria B. Cassera performed the antimalarial bioassay (*Plasmodium falciparum*, Dd2 strain). Dr. Martin W. Callmander from the Missouri Botanical Garden supervised the collection of the plant. Richard Randrianaivo from the Missouri Botanical Garden assisted with collection of the plant. Roland Rakotondrajaona from the Centre National d'Application de Recherche Pharmaceutique (CNARP) collected and identified the plant. Dr. Vincent E. Rasamison (CNARP) carried out the plant extraction under the supervision of Dr. Etienne Rakotobe. Dr. David G. I. Kingston is the corresponding author for the published article and was a mentor for this work.

3.1.3 Previous Investigations of Molinaea retusa

Molinaea retusa is a member of the Sapindaceae family, which is made up of approximately 1900 species. *Molinaea retusa* is one of ten members of the *Molinaea* genus, all of which are endemic to Madagascar.[2] While plants of the Sapindaceae family have been the subject of multiple phytochemical studies, the genus *Molinaea* has not previously been investigated. Plants of the Sapindaceae family are known to contain terpenoids, flavonoids, and ceramides, with varying types of activity, including antiproliferative and antiplasmodial activities.[3-10]

3.1.4 Chemical Investigation of Molinaea retusa

As a part of our continuing search for bioactive natural products from Madagascar, an extract of *Molinaea retusa* was obtained. The crude ethanol extract of the roots were found to inhibit the grown of human ovarian cancer cells (A2780) with an IC50 value of 16 μg/mL. Bioassay-guided fractionation of the extract led to the identification of three diterpene glycosides, comprising the new compound 2″,3″,4″,6′-de-*O*-acetylcupacinoside and the two known compounds, cupacinoside and 6-de-*O*-acetylcupacinoside. All three compounds were found to have antiproliferative activity. Cupacinoside and 6-de-*O*-acetylcupacinoside also had antiplasmodial activity against *Plasmodium falciparum*, Dd2 strain. The isolation, structure elucidation, and bioactivity of the compounds will be discussed (Figure 3-2).

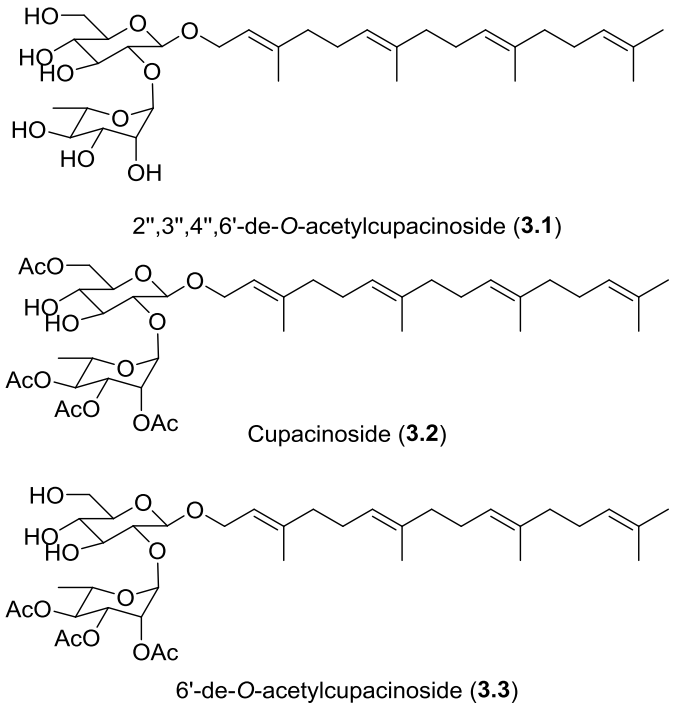
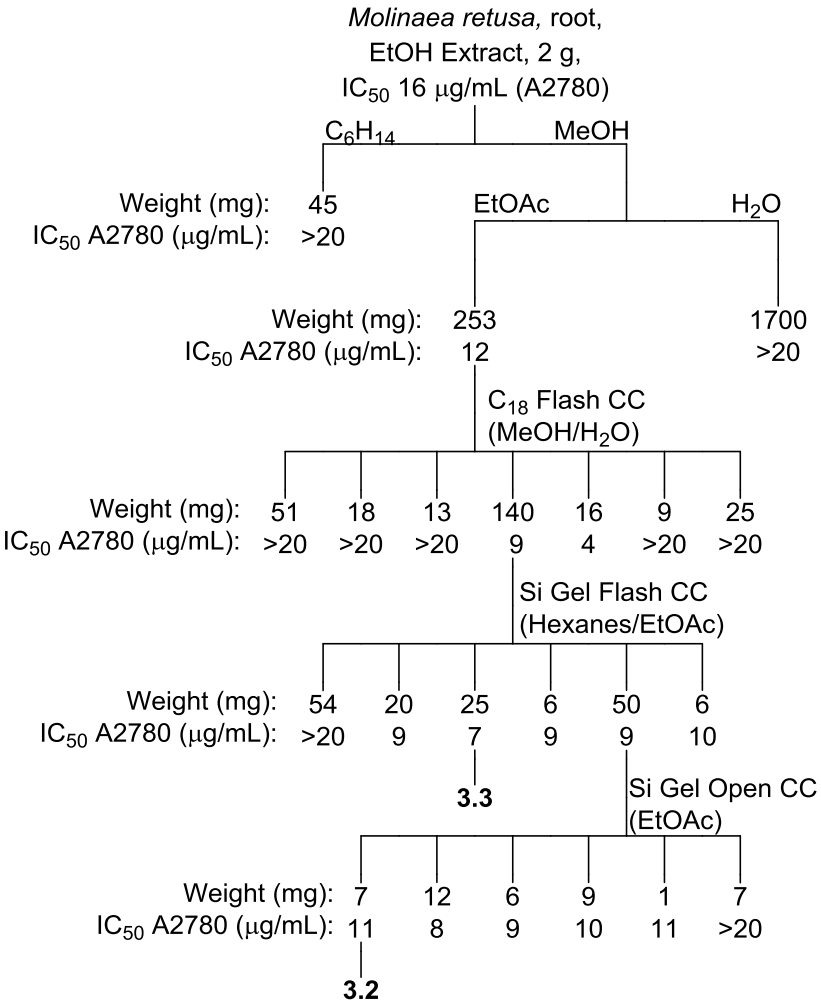


Figure 3-2. Compounds isolated from *Molinaea retusa*.

3.2 Results and Discussion

3.2.1 Isolation of Compounds from Molinaea retusa

Three diterpene glycosides were isolated from the roots of *Molinaea retusa* utilizing bioassay-guided fractionation (Scheme 3-1). The crude ethanol extract (1.99 g) was dissolved in methanol and extracted with hexane. The methanol fraction was dried under reduced pressure and dissolved in water. The aqueous solution was then extracted using ethyl acetate resulting in two fractions (three total, including the hexane fraction). The ethyl acetate fraction was found to have improved antiproliferative activity again the A2780 cell line, so it was fractionated further utilizing C_{18} flash chromatography and silica gel flash chromatography to yield cupacinoside (**3.2**) and 6-de-*O*-acetylcupacinoside (**3.3**).



Scheme 3-1. Separation of *Molinaea retusa* root extract.

During fractionation it was evident that another minor active compound was present, so an additional 2 g of crude ethanol extract was obtained (Scheme 3-2). This fractionation was completed with the goal of isolating as much as possible of the third compound. In the same manner as the first fractionation, liquid–liquid partitioning was performed, resulting in hexane, ethyl acetate, and water fractions. The ethyl acetate fraction was once again found to be active. The ethyl acetate fraction was fractionated utilizing C_{18} flash chromatograph, followed by open column chromatography with an amino solid phase and ethyl acetate/methanol mobile phase (85:15). This resulted in an active fraction which was further fractionated using silica gel column chromatography (EtOAc mobile phase), followed by purification utilizing high performance liquid chromatography (Figure 3-3) yielding 2″,3″,4″,6′-de-*O*-acetylcupacinoside (3.1). A detailed description of the isolation procedure can be found in the Experimental Section (3.3).

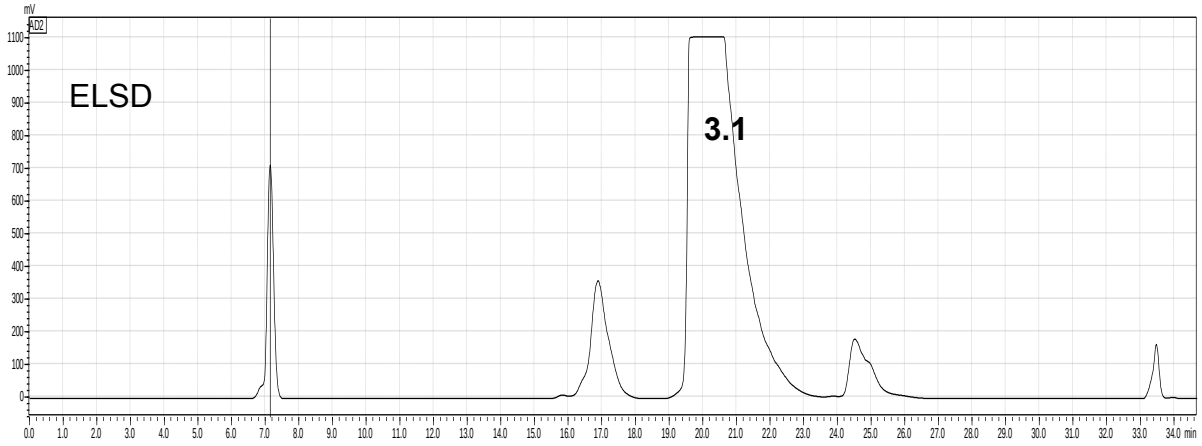
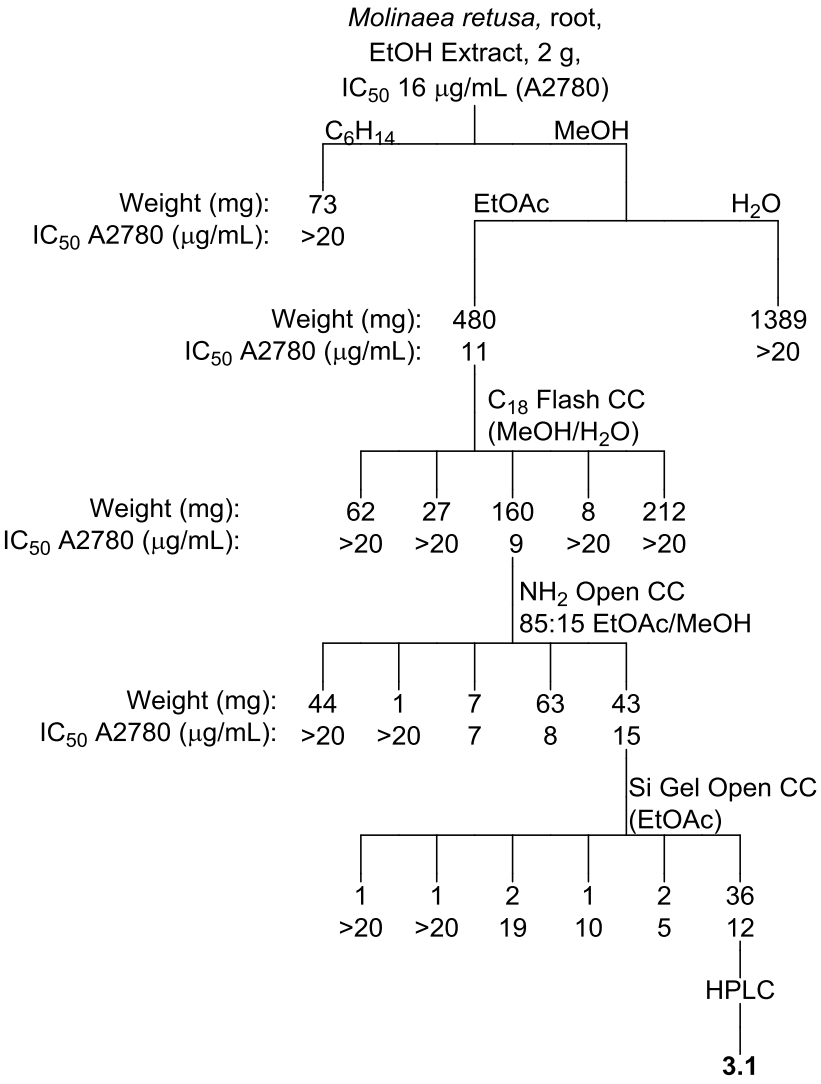


Figure 3-3. HPLC Chromatogram (ELSD) showing purification of 2″,3″,4″,6′-de-*O*-acetylcupacinoside (**3.1**).



Scheme 3-2. Fractionation of *Molinaea retusa* root extract to obtain compound **3.1.**

*3.2.2 Identification of Cupacinoside (**3.2**) and 6′-de-O-acetylcupacinoside (**3.3**)*

Two of the compounds, cupacinoside (**3.2**) and 6′-de-*O*-acetylcupacinoside (**3.3**) have previously been reported and were identified through comparison of ^{1}H NMR, ^{13}C NMR, and HRESIMS spectroscopic data with previously published data (Table 3-1).[3]

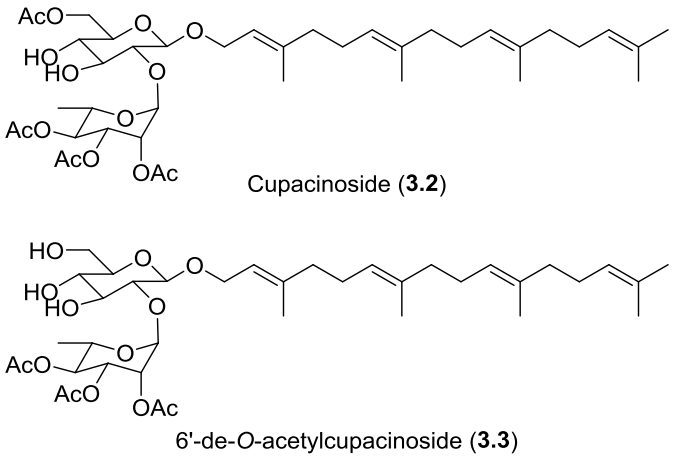


Figure 3-4. Structure of known compounds isolated from *Molinaea retusa*.

Table 3-1. ^{1}H and ^{13}C NMR spectroscopic data of cupacinoside (**3.2**) and 6′-de-*O*-acetylcupacinoside (**3.3**).

		cupacinoside (**3.2**)[a]		6′-de-*O*-acetylcupacinoside (**3.3**)[b]	
	position	δ_C, type	δ_H (*J* in Hz)	δ_C, type	δ_H (*J* in Hz)
isoprene chain	1	65.9, CH_2	4.37, dd (11.5, 6.6)	66.4, CH_2	4.39[i]
			4.16, dd (11.5, 7.6)		4.20, dd (11.7, 7.7)
	2	119.5, CH	5.36, t (6.7)	121.1, CH	5.39, brt
	3	141.4, C		142.3, C	
	4	39.7[c], CH_2	1.95-2.10[i]	40.7[c], CH_2	1.95-2.10[i]
	5	26.4[d], CH_2	2.01-2.17[i]	27.8[d], CH_2	2.01-2.17[i]
	6	123.6[e], CH	5.10[i]	125.1[e], CH	5.10[i]
	7	135.5[f], C		135.9[f], C	
	8	39.7[c], CH_2	1.95-2.10[i]	40.8[c], CH_2	1.95-2.10[i]
	9	26.8[d], CH_2	2.01-2.17[i]	27.6[d], CH_2	2.01-2.17[i]
	10	124.1[e], CH	5.10[i]	125.4[e], CH	5.10[i]
	11	135[f], C		136.4[f], C	
	12	39.7[c], CH_2	1.95-2.10[i]	40.9[c], CH_2	1.95-2.10[i]
	13	26.7[d], CH_2	2.01-2.17[i]	27.5[d], CH_2	2.01-2.17[i]
	14	124.4, CH	5.10[i]	125.5, CH	5.10[i]
	15	131.3, C		132.1, C	
	16	17.7, CH_3	1.60, s	17.6, CH_3	1.61, s
	17	25.7, CH_3	1.68, s	25.9, CH_3	1.67, s
	18	15.98[g], CH_3	1.59, s	16.1[g], CH_3	1.60, s
	19	15.99[g], CH_3	1.59, s	16.2[g], CH_3	1.61, s
	20	16.5, CH_3	1.69, s	16.7, CH_3	1.73, s
β-glucopyranosyl	1′	100.4, CH	4.40, d (7.8)	101.4, CH	4.44, d (7.8)
	2′	77.4, CH	3.51, dd (9.1, 7.9)	79.0, CH	3.38, dd (9.2, 7.9)
	3′	77.1, CH	3.65, t (9.0)	79.0, CH	3.53, t (9)
	4′	70.0, CH	3.32, t (9.3)	71.8, CH	3.26[i]
	5′	73.7, CH	3.39, ddd (9.7, 3.8, 2.2)	77.9, CH	3.26[i]
	6′	63.1, CH_2	4.54, dd (12.3, 3.9)	62.7, CH_2	3.87, dd (11.9, 1.5)
			4.25, dd (12.3, 2.4)		3.67, dd (11.9, 5.3)
	6′ -OAc	20.9, CH_3	2.12, s		
		172.2, C			
α-rhamnopyranosyl	1″	97.8, CH	5.19, d (1.4)	99.2, CH	5.22, d (1.5)
	2″	69.8, CH	5.31, dd (3.4, 1.7)	71.1, CH	5.34, dd (3.4, 1.7)
	2″ -OAc	21.0, CH_3	2.14, s	20.8[h], CH_3	2.03, s
		170.3, C		171.6, C	
	3″	69.4, CH	5.25, dd (10.1, 3.5)	71.0, CH	5.19, dd (10.2, 3.4)
	3″ -OAc	20.8, CH_3	1.99, s	20.7[h], CH_3	2.12, s
		170.3, C		171.6, C	
	4″	71.0, C	5.07[i]	72.3, CH	5.00, t (10.1)
	4″ -OAc	20.8, CH_3	2.03, s	20.6[h], CH_3	1.94, s
		169.9, C		171.6, C	
	5″	66.6, CH	4.26[i]	67.5, CH	4.39[i]
	6″	17.1, CH_3	1.16, d (6.2)	17.8, CH_3	1.11, d (6.2)

[a]Obtained in $CDCl_3$, 500 MHz (δ_H), 125 MHz (δ_C)
[b]Obtained in CD_3OD, 500 MHz (δ_H), 125 MHz (δ_C)
[c, d, e, f, g,h]Interchangeable signals
[i]Overlapping signals

*3.2.3 Identification of 2″,3″,4″,6′-de-O-acetylcupacinoside (**3.1**)*

The structure of compound **3.1** was found to be very similar to those of compounds **3.2** and **3.3** based on comparison of their ^{1}H NMR and ^{13}C NMR spectroscopic data. The ^{1}H NMR and ^{13}C NMR showed that five methyl groups (δ_H 1.60, 6H, s; δ_H 1.60, 3H, s; δ_H 1.61, 3H, s; δ_H 1.67, 3H, s; and δ_H 1.70, 3H, s) together with a broad triplet at δ_H 5.39 were present, consistent with the presence of a geranylgeraniol group as found in **3.2** and **3.3**.[3] The ^{1}H and ^{13}C NMR spectra exhibited two anomeric protons, suggesting the presence of two sugar units. The signal in the ^{1}H NMR spectrum at δ_H 4.35 (d, J = 7.7 Hz) is indicative of the anomeric proton on a β-glucopyranosyl unit. The remaining carbon and proton signals for the glucopyranosyl moiety were assigned through HSQC and HMBC correlations and have shifts corresponding to glucopyranosyl based on comparison with published literature data.[3] The doublet (J = 6.2 Hz) signal at δ_H 1.21 (H-6″) is characteristic of the methyl group present in a rhamnopyranosyl unit. The anomeric proton signal was assigned as δ_H 5.12 through use of HMBC and HMQC correlations. The coupling constant was not directly obtained, due to overlap of signals in the ^{1}H NMR spectrum, but could be inferred through the coupling constant present for the H-2″ signal (dd, J = 3.4, 1.7 Hz). Since 3.4 Hz represents the coupling of H-2″ to H-3″, the coupling of 1.7 Hz represents of coupling of H-1″ to H-2″. Furthermore, the signal of C-5″ was δ_C 69.8 which is consistent with α-rhamnopyranosyl.[11]

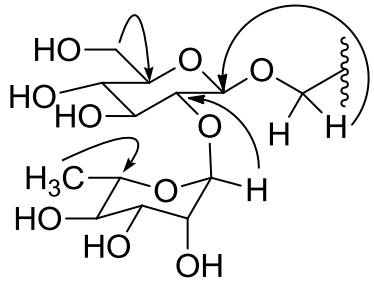
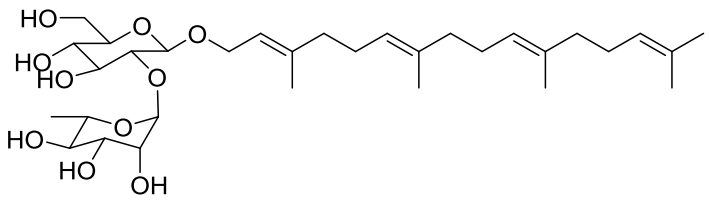


Figure 3-5. Key HMBC correlations of 2″,3″,4″,6′-de-*O*-acetylcupacinoside (**3.1**).

Assignments of protons and carbons for the α-rhamnopyranosyl unit were made based on HMBC and HSQC correlations and coupling constants present in the ^{1}H NMR spectrum. A correlation in the HMBC from H-1 to C-1′ indicated that the β-glucopyranosyl group is attached to the aglycone, and a correlation from H-1″ to C-2′ indicated that the α-rhamnopyranosyl group is connected to the β-glucopyranosyl unit at the 2′ position (Figure 3-5). The aglycone connectivity and sugar linkage were thus confirmed to be the same in **3.1** as in **3.2** and **3.3**, as expected due to the similarity of their spectroscopic data.

In comparison to **3.2** and **3.3**, H-2″, H-3″, and H-4″ of **1** were shifted upfield (*ca.* –1.6 ppm); conversely, C-2″, C-3″, and C- 4″ were shifted downfield (*ca*. +2.7 ppm) which is in agreement with the lack of acetylation. The ^{13}C NMR spectrum further indicated a lack of acetylation due to the absence of peaks downfield of δ_C 160. The molecular formula ($C_{32}H_{54}O_{10}$) was established using HRESIMS which yielded an ion at *m/z* 643.3727 corresponding to $[M+HCOO]^-$ (calcd 643.3694 for $C_{33}H_{55}O_{12}$). Compound **3.1** was thus determined to be 2″,3″,4″,6′-de-*O*-acetylcupacinoside. Complete NMR assignments can be seen in Table 3-2.



2",3",4",6'-de-*O*-acetylcupacinoside (**3.1**)

Figure 3-6. Structure of **3.1.**

Table 3-2. ^{1}H and ^{13}C NMR spectroscopic data of 2″,3″,4″,6′-de-*O*-acetylcupacinoside (**3.1**).

	position	δ_C, type	δ_H (*J* in Hz)
isoprene chain	1	66.4, CH_2	4.37, dd (11.7, 6.6)
			4.18, dd (11.7, 7.5)
	2	121.5, CH	5.39, bt
	3	141.8, C	
	4	40.7[a], CH_2	1.94-2.10[f]
	5	27.8[b], CH_2	2.01-2.17[f]
	6	125.3[c], CH	5.11f
	7	135.9[d], C	
	8	40.8[a], CH_2	1.94-2.10[f]
	9	27.6[b], CH_2	2.01-2.17[f]
	10	125.4[c], CH	5.11[f]
	11	136.2[d], C	
	12	40.9[a], CH_2	1.94-2.10[f]
	13	27.5[b], CH_2	2.01-2.17[f]
	14	125.5[c], CH	5.11[f]
	15	132, C	1.61
	16	17.5, CH_3	1.67
	17	25.9, CH_3	1.67
	18	16.14[e], CH_3	1.61
	19	16.12[e], CH_3	1.60
	20	16.6, CH_3	1.7
β-glucopyranosyl	1′	101.6, CH	4.35, d (7.7)
	2′	79.9, CH	3.35, t (8.5)
	3′	79.2, CH	3.44, t (9.0)
	4′	71.8, CH	3.28, t (8.8)
	5′	77.8, CH	3.21, ddd (9.7, 5.7, 2.3)
	6′	62.8, CH_2	3.86, dd (12.0, 2.3)
			3.67, dd (12.0, 5.8)
α-rhamnopyranosyl	1″	102.6, CH	5.12[f]
	2″	72.2, CH	3.92, dd (3.4, 1.7)
	3″	72.4, CH	3.64, dd (9.6, 3.4)
	4″	73.9, CH	3.37, t (9.6)
	5″	69.8, CH	4.01, dq (6.2, 9.6)
	6″	18.0, CH_3	1.21, d (6.2)

Obtained in CD_3OD, 500 MHz (δ_H), 125 MHz (δ_C)
[a, b, c, d, e]Interchangeable signals within a column
[f]Overlapping signals

3.2.4 Antiproliferative and Antiplasmodial Activity

Antiproliferative activities (Table 3-3) of compounds **3.1**–**3.3** against a human ovarian cancer cell line (A2780) were comparable to published activities against a rat skeletal myoblast cell line (L-6). Compounds **3.2** and **3.3** were previously isolated from *Cupania cinerea* (Sapindaceae) with activities of 1.3 μM (**3.2**) and 2.1 μM (**3.3**) against *P. falciparum* (K1 strain) and activities of 11.6 μM (**3.2**) and 8.7 μM (**3.3**) against rat skeletal myoblast cells (L-6 cell line).[3] Likewise antimalarial activities (Table 3-3) against *P. falciparum* (Dd2 strain) were comparable to previously reported activities against *P. falciparum* (K1 strain).[3] Compound **3.1** was not tested for antimalarial activity due to decomposition before testing could be completed.

Table 3-3. Activities of isolated compounds against the A2780 cell line and *Plasmodium falciparum.*

Compound	Antiproliferative activity against A2780, IC_{50}, μM	Antiplasmodial activity against *P. falciparum* Dd2 strain, IC_{50}, μM
2″,3″,4″,6′-de-*O*-Acetylcupacinoside (**3.1**)	15.4	Not determined
Cupacinocide (**3.2**)	9.5	4.0 ± 0.6
6′-de-*O*-Acetylcupacinoside (**3.3**)	10.9	6.4 ± 0.5

3.3 Experimental Section

3.3.1 General Experimental Procedures

Optical rotations were recorded on a JASCO P-2000 polarimeter. The UV spectrum was measured using a Shimadzu UV-1201 spectrometer. ^{1}H and ^{13}C NMR spectra were recorded with a Bruker Advance 500 spectrometer. ^{1}H-^{1}H three-bond *J*-coupling values were calculated from ^{1}H NMR spectra. Mass spectra were obtained with an Agilent 6220 LC-TOF-MS. Preparative HPLC was performed by using Shimadzu LC-10AT pumps coupled with a Sedex 75 Evaporative Light Scattering Detector (ELSD), a Shimadzu SPD M10A diode array detector, a SCL-10A system controller, and a Varian Dynamax NH_2 column (250 x 10 mm). Flash chromatography collection

was performed using a Biotage Horizon Pump coupled with a Biotage Horizon Flash Collector and a Biotage Horizon UV Detector.

3.3.2 Plant Material

Roots of *Molinaea retusa* Radlk. (Sapindaceae) (collection: Roland Rakotondrajaona et al. 366) were collected at an elevation of 275 m in November 2005, 2 km northwest of the village of Ankijabe in the Bemôsy forest, Vohemar, Daraina, Antsiranana, Sava region, 13°14′41" S 049°37′53" E, northern Madagascar. The sample was collected from a bush 4 m tall with orange fruits and black seeds.

3.3.3 Extraction and Isolation

Dried and powdered *M. retusa* roots (275 g) were extracted with EtOH for 24 h to yield 28.6 g of EtOH extract, of which 8.1 g was made available to Virginia Tech. Liquid–liquid partition of this extract (2 g) yielded an active EtOAc fraction (253 mg, IC_{50} 12 μg/mL). Purification of the EtOAc fraction was performed on a C_{18} column ($MeOH/H_2O$ gradient) using a flash chromatography fraction collector, and yielded one active sub-fraction (140 mg, IC_{50} 9 μg/mL). The sub-fraction was fractionated further on a flash chromatography fraction collector utilizing a silica gel column with a hexanes/EtOAc gradient yielding compound **3.3**(25 mg, IC_{50} 10.9 μM) and one other active fraction (55 mg, IC_{50} 9 μg/mL). The active fraction was fractionated further through the use of an open silica gel column (EtOAc mobile phase) yielding compound **3.2** (7 mg, IC_{50} 9.5 μM). A second fractionation was carried out to identify a minor active compound detected during the first fractionation. Liquid–liquid partitioning of 2 g of EtOH extract yielded an active EtOAc fraction (480 mg, IC_{50} 11 μg/mL) which was separated by a C_{18} column ($MeOH/H_2O$

gradient) to yield one active fraction (160 mg, IC_{50} 9 μg/mL). Compound **3.1** (17 mg, IC_{50} 15.4 μM) was obtained using an NH_2 open column (85:15 EtOAc/MeOH mobile phase) followed by fractionation using a silica gel open column (EtOAc mobile phase), followed by purification using high pressure liquid chromatography (HPLC) utilizing a silica gel column with a hexanes/EtOAc gradient.

3.3.4 Antiproliferative Bioassay

Assay was performed at Virginia Tech according to specifications previously described.[12,13] The A2780 cell line is a drug-sensitive ovarian cancer cell line.[14]

3.3.5 Antimalarial Bioassay

Assay was performed at Virginia Tech as previously described.[15]

3.3.6 Spectroscopic Properties

2′′,3′′,4′′,6′-de-*O*-acetylcupacinoside (3.1): $[\alpha]_D^{23}$ −22.0 (*c* 0.84, MeOH); UV/Vis λ_{max} (MeOH) nm (log ε): 203 (3.22); ^{1}H NMR (500 MHz, CD_3OD): See Table 3-2; ^{13}C NMR (125 MHz, CD_3OD): See Table 3-2; HRESIMS $[M+HCOO]^-$ *m/z* 643.3727 (calcd for $C_{33}H_{55}O_{12}^-$ 643.3699), $[M+Cl]^-$ *m/z* 633.3426 (calcd for $C_{32}H_{54}O_{10}Cl^-$ 633.3411), *m/z* $[M-H]^-$ 597.3652 (calcd for $C_{32}H_{53}O_{10}^-$ 597.3644).

Cupacinoside (3.2): ^{1}H NMR (500 MHz, $CDCl_3$): See Table 3-1; ^{13}C NMR (125 MHz, $CDCl_3$): See Table 3-1; HRESIMS $[M+NH_4]^+$ *m/z* 784.4438 (calcd for $C_{40}H_{66}NO_{14}^+$ 784.4478),

$[M+Na]^+$ *m/z* 789.3997 (calcd for $C_{40}H_{62}O_{14}Na^+$ 789.4032), *m/z* $[M+K]^+$ 805.3787 (calcd for $C_{40}H_{62}O_{14}^+$ 805.3771).

6′-de-*O*-acetylcupacinoside (3.3): ^{1}H NMR (500 MHz, CD_3OD): See Table 3-1; ^{13}C NMR (125 MHz, CD_3OD): See Table 3-1; HRESIMS $[M+NH_4]^+$ *m/z* 742.4357 (calcd for $C_{38}H_{64}NO_{13}^+$ 742.4372), $[M+Na]^+$ *m/z* 747.3910 (calcd for $C_{38}H_{60}O_{13}Na^+$ 747.3926).

3.4 References

1. Eaton, A. L.; Harinantenaina, L.; Brodie, P. J.; Cassera, M. B.; Bowman, J. D.; Callmander, M. W.; Randrianaivo, R.; Rakotondrajaona, R.; Rakotobe, E.; Rasamison, V. E.; Kingston, D. G. I., A new bioactive diterpene glycoside from *Molinaea retusa* from the Madagascar dry forest. *Nat. Prod. Commun.* **2013,** *8* (9), 1201-1203.

2. Mabberley, D. J., *The Plant-Book: A Portable Dictionary of the Vascular Plants*. 2nd ed.; University Press: Cambridge, 1997.

3. Gachet, M. S.; Kunert, O.; Kaiser, M.; Brun, R.; Zehl, M.; Keller, W.; Munoz, R. A.; Bauer, R.; Schuehly, W., Antiparasitic compounds from *Cupania cinerea* with activities against *Plasmodium falciparum* and *Trypanosoma brucei rhodesiense*. *J. Nat. Prod.* **2011,** *74* (4), 559-566.

4. Cao, S.; Brodie, P.; Callmander, M.; Randrianaivo, R.; Razafitsalama, J.; Rakotobe, E.; Rasamison, V. E.; TenDyke, K.; Shen, Y.; Suh, E. M.; Kingston, D. G. I., Antiproliferative triterpenoid saponins of *Dodonaea viscosa* from the Madagascar dry forest. *J. Nat. Prod.* **2009,** *72* (9), 1705-1707.

5. Cavalcanti, S. B. T.; Teles, H. L.; Silva, D. H. S.; Furlan, M.; Young, M. C. M.; Bolzani, V. S., New tetra-acetylated oligosaccharide diterpene from *Cupania vernalis*. *J. Braz. Chem. Soc.* **2001,** *12* (3), 413-416.

6. Fu, H.; Guo, Y.; Li, W.; Dou, D.; Kang, T.; Koike, K., A new angeloylated triterpenoid saponin from the husks of *Xanthoceras sorbifolia* Bunge. *J. Nat. Med.* **2010,** *64* (1), 80-84.

7. Ito, A.; Chai, H. B.; Kardono, L. B.; Setowati, F. M.; Afriastini, J. J.; Riswan, S.; Farnsworth, N. R.; Cordell, G. A.; Pezzuto, J. M.; Swanson, S. M.; Kinghorn, A. D., Saponins from the bark of *Nephelium maingayi*. *J. Nat. Prod.* **2004,** *67* (2), 201-205.

8. de Oliveira, S. Q.; de Almeida, M. T. R.; Maraslis, F.; Silva, I. T.; Sincero, T. C. M.; Palermo, J. A.; Cabrera, G. M.; Caro, M. S. B.; Simões, C. M. O.; Schenkel, E. P., Isolation of three new ent-labdane diterpenes from *Dodonaea viscosa* Jacquin (Sapindaceae): Preliminary evaluation of antiherpes activity. *Phytochem. Lett.* **2012,** *5* (3), 500-505.

9. Tantangmo, F.; Lenta, B. N.; Ngouela, S.; Kamdem, L. M.; Weniger, B.; Tsamo, E.; Lobstein, A.; Vonthron-Senecheau, C., New Sphingolipids and Other Constituents of *Pancovia laurentii*. *Helv. Chim. Acta* **2010,** *93* (11), 2210-2217.

10. Zhang, L. B.; Ji, J.; Lei, C.; Wang, H. Y.; Zhao, Q. S.; Hou, A. J., Isoprenylated flavonoid and adipogenesis-promoting constituents of *Dodonaea viscosa*. *J. Nat. Prod.* **2012,** *75* (4), 699-706.

11. Eberhard, B.; Voelter, W., *Carbon-13 NMR Spectroscopy: High Resolution Methods and Applications in Organic Chemistry and Biochemistry*. 3rd ed.; VCH: New York, 1989; p 513.

12. Cao, S.; Brodie, P. J.; Miller, J. S.; Randrianaivo, R.; Ratovoson, F.; Birkinshaw, C.; Andriantsiferana, R.; Rasamison, V. E.; Kingston, D. G. I., Antiproliferative xanthones of *Terminalia calcicola* from the Madagascar rain forest. *J. Nat. Prod.* **2007,** *70* (4), 679-681.

13. Pan, E.; Harinantenaina, L.; Brodie, P. J.; Miller, J. S.; Callmander, M. W.; Rakotonandrasana, S.; Rakotobe, E.; Rasamison, V. E.; Kingston, D. G. I., Four diphenylpropanes and a cycloheptadibenzofuran from *Bussea sakalava* from the Madagascar dry forest. *J. Nat. Prod.* **2010,** *73* (11), 1792-1795.

14. Louie, K. G.; Behrens, B. C.; Kinsella, T. J.; Hamilton, T. C.; Grotzinger, K. R.; McKoy, W. M.; Winker, M. A.; Ozols, R. F., Radiation survival parameters of antineoplastic drug-sensitive and drug-resistant human ovarian-cancer cell-lines and their modification by buthionine sulfoximine. *Cancer Res.* **1985,** *45* (5), 2110-2115.

15. Harinantenaina, L.; Bowman, J. D.; Brodie, P. J.; Slebodnick, C.; Callmander, M. W.; Rakotobe, E.; Randrianaivo, R.; Rasamison, V. E.; Gorka, A.; Roepe, P. D.; Cassera, M. B.; Kingston, D. G. I., Antiproliferative and antiplasmodial dimeric phloroglucinols from *Mallotus oppositifolius* from the Madagascar dry forest. *J. Nat. Prod.* **2013,** *76*, 388-393.

Chapter 4: Bioactive Oleanane Glycosides from *Polyscias duplicata* from the Madagascar Dry Forest

4.1 Introduction

4.1.1 Abstract

In a search for antiproliferative compounds as part of the International Cooperative Biodiversity Group (ICBG) program, an ethanol extract of *Polyscias duplicata* was investigated due to its antiproliferative activity against the A2780 human ovarian cell cancer line (IC_{50} 6 μg/mL). Seven known oleanane glycosides, 3β-[(α-L-arabinopyranosyl)oxy]-16α-hydroxyolean-12-en-28-oic acid (**4.1**, IC_{50} 8 μM), 3β-[(α-L-arabinopyranosyl)oxy]-16α,23-dihydroxyolean-12-en-18-oic acid (**4.2**, IC_{50} 13 μM), 3β-[(*O*-β-D-glucopyranosyl-(1→3)-α-L-arabinopyranosyl)oxy]-16α-hydroxyolean-12-en-28-oic acid (**4.3**, IC_{50} 7 μM), 3β-[(*O*-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranosyl)oxy]-16α-hydroxyolean-12-en-28-oic acid (**4.4**, IC_{50} 2.8 μM), 3β-[(*O*-β-D-glucopyranosyl-(1→3)-α-L-arabinopyranosyl)oxy]-23-hydroxyolean-12-en-28-oic acid (**4.5**, IC_{50} 10 μM), 3β-[(*O*-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranosyl)oxy]-23-hydroxyolean-12-en-28-oic acid (**4.6**, IC_{50} 3.4 μM), and 3β-[(α-L-arabinopyranosyl)oxy]-23-hydroxyolean-12-en-28-oic acid (**4.7**, IC_{50} 3.4 μM) were isolated by bioassay-guided fractionation, using liquid–liquid partitioning, column chromatography, and HPLC. The structures were determined by using spectroscopic methods. This is a slightly modified and expanded version of previously published work (Eaton, A.L.; et al. *Nat. Prod. Commun.* **2015,** *10* (4), 567-570).[1]

Figure 4-1. *Polyscias duplicata.* Used under Creative Commons (CC BY-NC-ND 3.0) from <http://www.tropicos.org/Image/76737>.

4.1.2 Author Contributions

The author of this dissertation (Alexander L. Eaton) completed the fractionation, isolation, and identification of the described compounds as well as the drafting of the manuscripts. Ms. Peggy Brodie performed the antiproliferative bioassay (A2780) on all fractions and compounds. Martin W. Callmander from the Missouri Botanical Garden supervised the collection of the plant. Roland Rakotondrajaona from the Centre National d'Application de Recherche Pharmaceutique (CNARP) collected and identified the plant. Etienne Rakotobe (CNARP) supervised the work at CNARP, and Vincent E. Rasamison (CNARP) carried out the plant extraction. Dr. David G. I. Kingston is the corresponding author for the published article and was a mentor for this work.

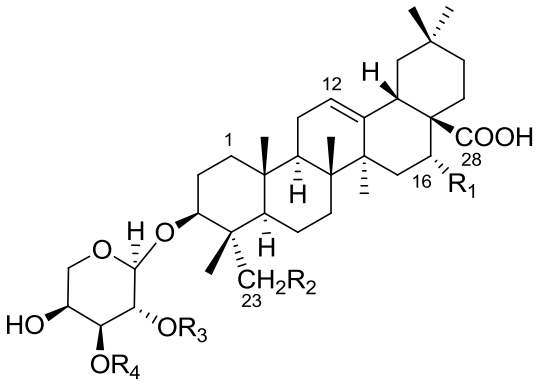
4.1.3 Previous Investigations of Polyscias duplicata

Work was initiated on this extract in part because there had been no previous phytochemical investigations of plants from the genus *Gastonia.* Subsequent to the initiation of our work the plant was reidentified as *Polyscias duplicata* (Thouars ex Baill.) Lowry & G. M. Plunkett, also a member of the Araliaceae family, which contains approximately 1,300 species.[2] Although there has been no published phytochemical work on *P. duplicata*, the phytochemistry of the genus *Polyscias* has been well investigated, and members of this genus are known to contain oleanane glycosides.[3-8] Although all of the oleanane glycosides obtained in this work have been isolated previously, this is the first report of the isolation of compounds **4.1**, **4.3**, and **4.5** from the genus *Polyscias.* Compounds **4.2** and **4.4** have been isolated from *P. fulva*,[9] and **4.6** and **4.7** have been isolated from both *P. fulva* and *P. dichroostachya.*[9,10]

4.1.4 Chemical Investigation of Polyscias duplicata

The antiproliferative ethanol extract of *Polyscias duplicata* was subjected to bioassay-directed fractionation and isolation of the major bioactive constituents. This led to the identification of seven known oleanane glycosides, which were found to be weakly cytotoxic (IC_{50} 3-13 μM) against the A2780 human ovarian cancer cell line. Comparison of ^{13}C NMR data and HRESIMS data with literature values led to their identification as seven oleanane glycosides (Figure 4-2), 3β-[(α-L-arabinopyranosyl)-oxy]-16α-hydroxyolean-12-en-28-oic acid (**4.1**, IC_{50} 8 μM),[11] 3β-[(α-L-arabinopyranosyl)oxy]-16α,23-dihydroxy-olean-12-en-18-oic acid (**4.2**, IC_{50} 13 μM),[12] 3β-[(*O*-β-D-glucopyranosyl-(1→3)-α-L-arabinopyranosyl)oxy]-16α-hydroxyolean-12-en-28-oic acid (**4.3**, IC_{50} 7 μM),[13] 3β-[(*O*-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranosyl)oxy]-16α-hydroxyolean-12-en-28-oic acid (**4.4**, IC_{50} 2.8 μM),[11] 3β-[(*O*-β-D-glucopyranosyl-(1→3)-α-

L-arabinopyranosyl) oxy]-23-hydroxyolean-12-en-28-oic acid (**4.5**, IC_{50} 10 μM),[12] 3β-[(*O*-α-L-rhamnopyranosyl-(1→2)-α-L-arabino-pyranosyl)oxy]-23-hydroxyolean-12-en-28-oic acid (**4.6**, IC_{50} 3.4 μM),[14] and 3β-[(α-L-arabinopyranosyl)oxy]-23-hydroxyolean-12-en-28-oic acid (**4.7**, IC_{50} 3.4 μM).[15]



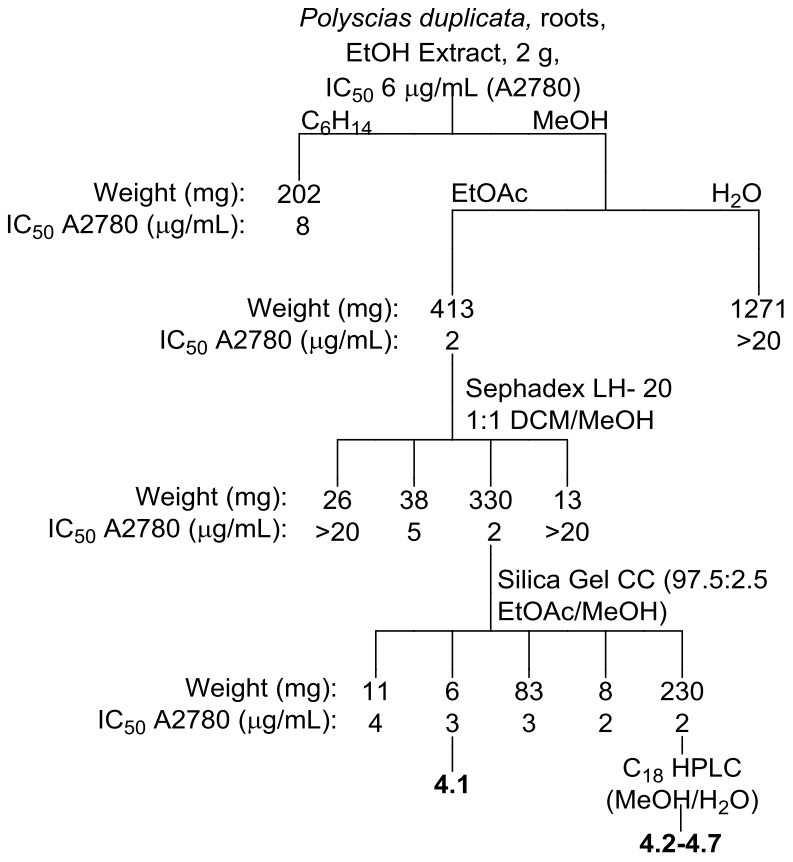
	R_1	R_2	R_3	R_4
4.1	OH	H	H	H
4.2	OH	OH	H	H
4.3	OH	H	H	Glc
4.4	OH	H	Rha	H
4.5	H	OH	H	Glc
4.6	H	OH	Rha	H
4.7	H	OH	H	H

Figure 4-2. Compounds isolated from *Polyscias duplicata*.

4.2 Results and Discussion

*4.2.1 Isolation of Compounds **4.1**–**4.7** from Polyscias duplicata*

The crude ethanol extract of *Polyscias duplicata* (IC_{50} 6 μg/mL) was fractionated with liquid–liquid partitioning yielding an active ethyl acetate fraction. The ethyl acetate fraction was further partitioned with Sephadex LH-20 column chromatography followed by silica gel column chromatography to yield **4.1** and an active fraction. A portion of the active fraction was separated by C_{18} HPLC to yield seven antiproliferative compounds (**4.1**–**4.7**). An overview of this extraction can be seen in Scheme 4-1, and details can be found in the Experimental Section of this chapter (4.3).



Scheme 4-1. Isolation of **4.1**–**4.7** from *Polyscias duplicata*.

*4.2.2 Identification of Compounds **4.1**–**4.7***

Initial inspection of the ^{1}H NMR spectra indicated that **4.1–4.7** were very similar in structure. HRESIMS was used to determine the molecular formulas of **4.1–4.7**. A database search in the Dictionary of Natural Products was performed to determine possible structures. Due to the scarcity of published ^{1}H NMR data, ^{13}C NMR experimental data were compared to published values to confirm the structure of **4.1–4.7**. Partial ^{1}H NMR assignments are presented at the end of this chapter and complete ^{1}H NMR spectra can be found in the supporting information.

4.2.3 Antiproliferative Activity

The isolated compounds were subjected to antiproliferative bioassay against the A2780 ovarian cancer cell line. The results are shown in Table 3-3, and indicate that two of the most active compounds (**4.4** and **4.6**) had a rhamnose at the C2' position. A hydroxyl group at C16 appeared to reduce activity, as indicated by a comparison of **4.2** and **4.7**.

Table 4-1. Activities of isolated compounds against the A2780 cell line.

Compound	Antiproliferative activity against A2780, IC_{50} (μM)
3β-[(α-L-arabinopyranosyl)oxy]-16α-hydroxyolean-12-en-28-oic acid (**4.1**)	6 ± 3
3β-[(α-L-arabinopyranosyl)oxy]-16α,23-dihydroxy-olean-12-en-18-oic acid (**4.2**)	16 ± 4
3β-[(*O*-β-D-glucopyranosyl-(1→3)-α-L-arabino-pyranosyl)oxy]-16α-hydroxyolean-12-en-28-oic acid (**4.3**)	5 ± 4
3β-[(*O*-α-L-rhamnopyranosyl-(1→2)-α-L-arabino-pyranosyl)oxy]-16α-hydroxyolean-12-en-28-oic acid (**4.4**)	2.8 ± 0.2
3β-[(*O*-β-D-glucopyranosyl-(1→3)-α-L-arabino-pyranosyl)oxy]-23-hydroxyolean-12-en-28-oic acid (**4.5**)	13 ± 2
3β-[(*O*-α-L-rhamnopyranosyl-(1→2)-α-L-arabino-pyranosyl)oxy]-23-hydroxyolean-12-en-28-oic acid (**4.6**)	3 ± 0.5
3β-[(α-L-arabinopyranosyl)oxy]-23-hydroxyolean-12-en-28-oic acid (**4.7**)	3.3 ± 0.1
Paclitaxel	0.07 ± 0.02

4.3 Experimental Section

4.3.1 General Experimental Procedures

^{1}H and ^{13}C NMR spectra were recorded with a Bruker Advance 500 spectrometer. ^{1}H-^{1}H three-bond *J*-coupling values were calculated from ^{1}H NMR spectra. Mass spectra were obtained with an Agilent 6220 LC-TOF-MS. Preparative HPLC was performed using Shimadzu LC-10AT pumps coupled with a Sedex 75 Evaporative Light Scattering Detector (ELSD), a Shimadzu SPD M10A diode array detector, a SCL-10A system controller, and a Cogent Biadentate C_{18} column (250 x 10 mm).

4.3.2 Plant Material

Roots of *Polyscias duplicata* (Thouars ex Baill.) Lowry & G. M. Plunkett (Araliaceae) were collected at an elevation of 189 m on October 5, 2006, 15 km from the village of Saharenana in the Sahafary forest, Sadjoavato, Antsiranana II, 12°34'48" S 049°26'31" E (*R. Rakotondrajaona 396*). The sample was collected from a tree 7 m tall with a thorny trunk, with green spherical fruit that turn purple upon reaching maturity.

4.3.3 Extraction and Isolation

Polyscias duplicata roots (250 g) were ground and extracted with ethanol at room temperature to yield 16.2 g of extract, of which 5.3 g were made available to Virginia Tech. Liquid–liquid partitioning of a 2 g portion of this extract yielded an active EtOAc fraction (413 mg, IC_{50} 2 μg/mL). Purification of the EtOAc fraction was performed on a Sephadex LH-20 column (1:1 dichloromethane/methanol) which yielded an active sub-fraction (330 mg, IC_{50} 2 μg/mL). The sub-fraction was fractionated further utilizing a silica gel column (97.5:2.5 EtOAc/MeOH) yielding one active compound (**4.1**, 6 mg) and an active sub-fraction (230 mg, IC_{50} 2 μg/mL). A portion of this sub-fraction (88 mg) was further fractionated using high pressure liquid chromatography (HPLC) utilizing a C_{18} column with a methanol/water gradient yielding six bioactive compounds (**4.2**, 10 mg; **4.3**, 7 mg; **4.4**, 4 mg; **4.5**, 4 mg; **4.6**, 5 mg; **4.7**, 32 mg).

4.3.4 Antiproliferative Bioassay

Assay was performed at Virginia Tech according to specifications previously described.[16] The A2780 cell line is a drug-sensitive ovarian cancer cell line.[17]

4.3.5 Spectroscopic Properties

3β-[(α-L-arabinopyranosyl)oxy]-16α-hydroxyolean-12-en-28-oic acid (4.1): $[\alpha]^{22}_{D}$ +9.3 (*c* 0.1 MeOH); ^{1}H NMR (500 MHz, C_5D_5N, Partial Assignment): 0.91 (CH_3, s), 0.98 (CH_3, s), 1.06 (CH_3, s), 1.09 (CH_3, s), 1.21 (CH_3, s), 1.29 (CH_3, s), 1.88 (CH_3, s), 4.8 (H-1′, d, *J* = 7.1 Hz), 5.28 (H-16, br s), 5.67, (H-12, t, *J* = 3.1 Hz); ^{13}C NMR (150 MHz, C_5D_5N): 16.1 (C-25), 17.4 (C-24), 17.9 (C-26), 19.0 (C-6), 24.3 (C-11), 25.2 (C-30), 27.1 (C-2), 27.7 (C-27), 28.7 (C-23), 31.5 (C-20), 33.2 (C-22), 33.8 (C-29), 34.0 (C-7), 36.6 (C-15), 36.7 (C-21), 37.5 (C-10), 39.3 (C-1), 40.0 (C-4), 40.4 (C-8), 41.9 (C-18), 42.6 (C-14), 47.7 (C-9), 47.7 (C-19), 49.4 (C-17), 56.4 (C-5), 67.2 (C-5′), 70.0 (C-4′), 73.4 (C-2′), 75.1 (C-16), 75.2 (C-3′), 89.1 (C-3), 107.9 (C-1′), 122.8 (C-12), 145.6 (C-13), 180.3 (C-28, from HMBC); HRESIMS $[M+Na]^+$ *m/z* 627.3804, (calcd for $C_{35}H_{56}NaO_8^+$ 627.3867).

3β-[(α-L-arabinopyranosyl)oxy]-16α,23-dihydroxy-olean-12-en-18-oic acid (4.2): $[\alpha]^{22}_{D}$ +20.3 (*c* 0.1 MeOH); ^{1}H NMR (500 MHz, C_5D_5N, Partial Assignment): 0.95 (CH_3, s), 0.99 (CH_3, s), 1.05 (CH_3, s), 1.08 (CH_3, s), 1.20 (CH_3, s), 1.80 (CH_3, s), 5.01 (H-1′, d, *J* = 7.2 Hz), 5.25 (H-16, br s), 5.67 (H-12, t, *J* = 3.2 Hz); ^{13}C NMR (150 MHz, C_5D_5N): 14.0 (C-24), 16.7 (C-25), 18.0 (C-26), 18.6 (C-6), 24.3 (C-11), 25.3 (C-30), 26.5 (C-2), 27.6 (C-27), 31.4 (C-20), 33.0 (C-22), 33.6 (C-7), 33.7 (C-29), 36.6 (C-15), 36.6 (C-21), 37.4 (C-10), 39.3 (C-1), 40.4 (C-8), 41.9 (C-14), 42.5 (C-18), 43.9 (C-4), 47.6 (C-19), 47.8 (C-5), 48.2 (C-9), 49.4 (C-17), 65.0 (C-5′), 67.4 (C-23), 70.0 (C-4′), 73.5 (C-2′), 75.1 (C-16), 75.2 (C-3′), 82.4 (C-3), 107.0 (C-1′), 122.7 (C-12), 145.6 (C-13), 180.4 (C-28, from HMBC); HRESIMS $[M+H]^+$ *m/z* 621.4037 (calcd for $C_{35}H_{57}O_9^+$ 621.3997), $[M+NH_4]^+$ *m/z* 638.4270 (calcd for $C_{35}H_{60}NO_9^+$ 638.4263),v$[M+Na]^+$ *m/z* 643.3789 (calcd for $C_{35}H_{56}NaO_9^+$ 643.3817), $[M+K]^+$ *m/z* 659.3563(calcd for $C_{35}H_{56}KO_9^+$ 659.3556),

$[2M+H]^+$ *m/z* 1241.7838 (calcd for $C_{70}H_{113}O_{18}^+$ 1241.7921), $[2M+Na]^+$ *m/z* 1263.7660 (calcd for $C_{70}H_{112}NaO_{18}^+$ 1263.7741).

3β-[(*O*-β-D-glucopyranosyl-(1→3)-α-L-arabino-pyranosyl)oxy]-16α-hydroxyolean-12-en-28-oic acid (4.3): $[\alpha]^{22}_D$ +15 (*c* 0.1 MeOH); ^{1}H NMR (500 MHz, C_5D_5N, Partial Assignment): 0.89 (CH_3, s), 1.05 (CH_3, s), 1.06 (CH_3, s), 1.08 (CH_3, s), 1.21 (CH_3, s), 1.23 (CH_3, s), 1.86 (CH_3, s), 4.98 (H-1′, d, *J* = 5.8 Hz), 5.21 (H-1″, d, *J* = 7.7 Hz), 5.27 (H-16, br s), 5.67 (H-12, t, *J* = 3.3 Hz); ^{13}C NMR (150 MHz, C_5D_5N): 16.2 (C-25), 17.4 (C-24), 18.1 (C-26), 19.1 (C-6), 24.4 (C-11), 25.5 (C-30), 27.1 (C-2), 27.8 (C-27), 28.8 (C-27), 31.6 (C-20), 33.2 (C-22), 33.9 (C-29), 34.1 (C-7), 36.7 (C-21), 36.8 (C-15), 37.6 (C-10), 39.4 (C-1), 40.1 (C-4), 40.5 (C-8), 42.1 (C-18), 42.7 (C-14), 47.8 (C-19), 47.9 (C-9), 49.6 (C-17), 56.5 (C-5), 63.1 (C-6″), 65.5 (C-5′), 68.8 (C-4′), 72.1 (C-4″), 74.0 (C-2′), 75.3 (C-16), 77.0 (C-2″), 78.7 (C-3″), 78.7 (C-5″), 81.6 (C-3′), 89.4 (C-3), 105.4 (C-1′), 106.6 (C-1″), 122.8 (C-12), 145.8 (C-13), 180.0 (C-28, from HMBC); HRESIMS $[M+NH_4]^+$ *m/z* 784.4763 (calcd for $C_{41}H_{70}NO_{13}^+$ 784.4842), $[M+Na]^+$ *m/z* 789.4332 (calcd for $C_{41}H_{66}NaO_{13}^+$ 789.4396), $[M+K]^+$ *m/z* 805.4099 (calcd for $C_{41}H_{66}KO_{13}^+$ 805.4135).

3β-[(*O*-α-L-rhamnopyranosyl-(1→2)-α-L-arabino-pyranosyl)oxy]-16α-hydroxyolean-12-en-28-oic acid (4.4): $[\alpha]^{22}_D$ –20 (*c* 0.1 MeOH); ^{1}H NMR (500 MHz, C_5D_5N, Partial Assignment): 0.89 (CH_3, s), 1.04 (CH_3, s), 1.08 (CH_3, s), 1.10 (CH_3, s), 1.18 (CH_3, s), 1.21 (CH_3, s), 1.65 (H-6″, d, *J* = 6.2 Hz), 1.87 (CH_3, s), 4.93 (H-1′, d, *J* = 5.5 Hz), 5.26 (H-16, br s), 5.66 (H-12, t, *J* = 3.3 Hz), 5.26 (H-16, br s); ^{13}C NMR (150 MHz, C_5D_5N): 16.1 (C-25), 17.4 (C-24), 17.9 (C-26), 18.9 (C-6), 18.9 (C-6′), 24.2 (C-11), 25.5 (C-30), 27.0 (C-2), 27.7 (C-27), 28.5 (C-23), 31.4 (C-20), 32.8 (C-22), 33.8 (C-29), 33.9 (C-7), 36.6 (C-15), 36.6 (C-21), 37.5 (C-10), 39.4 (C-1), 39.9 (C-

4), 40.3 (C-8), 42.0 (C-18), 42.6 (C-14), 47.7 (C-9), 47.7 (C-19), 49.5 (C-17), 56.4 (C-5), 65.1(C-5′), 69.1 (C-4′), 70.3 (C-5″), 72.8 (C-2″), 73.0 (C-3″), 74.2 (C-3′), 74.5 (C-4″), 75.2 (C-16), 76.3 (C-2′), 89.2 (C-3), 102.1 (C-1″), 105.2 (C-1′), 122.6 (C-12), 145.7 (C-13), 180.6 (C-28, from HMBC); HRESIMS $[M+NH_4]^+$ *m/z* 768.4916 (calcd for $C_{41}H_{70}NO_{12}^+$ 768.4893), $[M+Na]^+$ *m/z* 773.4409 (calcd for $C_{41}H_{66}NaO_{12}$ 773.4446).

3β-[(*O*-β-D-glucopyranosyl-(1→3)-α-L-arabino-pyranosyl)oxy]-23-hydroxyolean-12-en-28-oic acid (4.5): $[\alpha]^{22}_D$ +34.7 (*c* 0.1 MeOH); ^{1}H NMR (500 MHz, C_5D_5N, Partial Assignment): 0.94 (CH_3, s), 0.94 (CH_3, s), 1.01 (CH_3, s), 1.03 (CH_3, s), 1.04 (CH_3, s), 1.23 (CH_3, s), 5.20 (H-1′, d, *J* = 5.9 Hz), 5.21 (H-1″, d, *J* = 7.7 Hz), 5.49 (H-12, t, *J* = 3.4 Hz); ^{13}C NMR (150 MHz, C_5D_5N): 13.4 (C-24), 16.5 (C-25), 17.9 (C-26), 18.6 (C-6), 24.1 (C-11), 24.2 (C-30), 24.3 (C-16), 26.3 (C-2), 26.5 (C-27), 28.8 (C-15), 31.4 (C-20), 33.3 (C-7), 33.7 (C-22), 33.7 (C-29), 34.7 (C-21), 37.3 (C-10), 39.1 (C-1), 40.1 (C-8), 42.4 (C-14), 42.5 (C-18), 43.9 (C-4), 46.9 (C-19), 47.1 (C-17), 48.3 (C-5), 48.5 (C-9), 62.9 (C-6″), 65.3 (C-23), 65.4 (C-5′), 68.7 (C-4′), 71.8 (C-4″), 74.1 (C-2′), 76.7 (C-2″), 78.6 (C-3″), 78.7 (C-5″), 81.8 (C-3), 82.6 (C-3′), 104.3 (C-1″), 106.4 (C-1′), 123.5 (C-12), 145.3 (C-13), 180.4 (C-28, from HMBC); HRESIMS $[M+H]^+$ *m/z* 767.4568 (calcd for $C_{41}H_{67}O_{13}^+$ 767.4576), *m/z* $[M+NH_4]^+$ 784.4849 (calcd for $C_{41}H_{70}NO_{13}^+$ 784.4842), *m/z* $[M+Na]^+$ 789.4396 (calcd for $C_{41}H_{66}NaO_{13}^+$ 789.4396), *m/z* $[M+K]^+$ 805.4202 (calcd for $C_{41}H_{66}KO_{13}^+$ 805.4135), *m/z* $[2M+H]^+$ 1533.9075 (calcd for $C_{82}H_{133}O_{26}^+$ 1533.9080), *m/z* $[2M+Na]^+$ 1555.8879 (calcd for $C_{82}H_{132}NaO_{26}^+$ 1555.8899), *m/z* $[2M+K]^+$ 1571.8557 (calcd for $C_{82}H_{132}KO_{26}^+$ 1571.8638).

3β-[(*O*-α-L-rhamnopyranosyl-(1→2)-α-L-arabino-pyranosyl)oxy]-23-hydroxyolean-12-en-28-oic acid (4.6): $[\alpha]^{22}_D$ +18.7 (*c* 0.1 MeOH); ^{1}H NMR (500 MHz, C_5D_5N, Partial Assignment):

0.94 (CH_3, s), 0.95 (CH_3, s), 1.01 (CH_3, s), 1.03 (CH_3, s), 1.09 (CH_3, s), 1.24 (CH_3, s), 1.66 (H-6″, d, *J* = 6.2 Hz), 5.13 (H-1′, d, *J* = 6.3 Hz), 5.48 (H-12, t, *J* = 3.5 Hz), 6.31 (H-1″, br s); ^{13}C NMR (150 MHz, C_5D_5N): 14.4 (C-24), 16.5 (C-25), 17.9 (C-26), 18.5 (C-6″), 18.9 (C-6), 24.1 (C-30), 24.2 (C-16), 24.2 (C-11), 26.5 (C-2), 26.6 (C-27), 28.8 (C-15), 31.3 (C-20), 33.3 (C-22), 33.7 (C-7), 33.7 (C-29), 34.7 (C-21), 37.3 (C-10), 39.4 (C-1), 40.1 (C-8), 42.4 (C-18), 42.5 (C-14), 44.0 (C-5), 46.9 (C-19), 47.1 (C-17), 48.1 (C-4), 48.6 (C-9), 64.4 (C-23), 66.1 (C-5′), 69.8 (C-5″), 70.1 (C-4′), 72.8 (C-2″), 72.9 (C-3″), 74.5 (C-4″), 75.1 (C-3′), 76.2 (C-2′), 81.4 (C-3), 102.1 (C-1″), 104.8 (C-1′), 122.9 (C-12), 145.3 (C-13), 180.1 (C-28, from HMBC); HRESIMS $[M+H]^+$ 751.4586 *m/z* (calcd for $C_{41}H_{67}O_{12}^+$ 751.4627), $[M+NH_4]^+$ *m/z* 768.4873 (calcd for $C_{41}H_{70}NO_{12}^+$ 768.4893), $[M+Na]^+$ *m/z* 773.4405 (calcd for $C_{41}H_{66}NaO_{12}^+$ 773.4446), $[M+K]^+$ *m/z* 789.4262 (calcd for $C_{41}H_{66}KO_{12}^+$ 789.4186).

3β-[(α-L-arabinopyranosyl)oxy]-23-hydroxyolean-12-en-28-oic acid (4.7): $[\alpha]^{22}_D$ +49.7 (*c* 0.1 MeOH); ^{1}H NMR (500 MHz, C_5D_5N, Partial Assignment): 0.93 (CH_3, s), 0.94 (CH_3, s), 0.95 (CH_3, s), 1.01 (CH_3, s), 1.03 (CH_3, s), 1.24 (CH_3, s), 4.99 (H-1′, d, *J* = 7.2 Hz), 5.48 (H-12, t, *J* = 3.1 Hz); ^{13}C NMR (150 MHz, C_5D_5N): 14.0 (C-24), 16.5 (C-25), 17.8 (C-26), 18.5 (C-6), 24.0 (C-30), 24.1 (C-16), 24.2 (C-11), 26.5 (C-2), 26.5 (C-27), 28.7 (C-15), 31.3 (C-20), 33.3 (C-7), 33.6 (C-29), 33.6 (C-22), 34.6 (C-21), 37.3 (C-10), 37.1 (C-1), 40.1 (C-8), 42.3 (C-18), 42.5 (C-14), 43.9 (C-4), 46.8 (C-19), 47.0 (C-17), 48.0 (C-9), 48.5 (C-5), 64.9 (C-23), 67.3 (C-5′), 70.0 (C-4′), 73.5 (C-2′), 75.1 (C-3′), 82.3 (C-3), 107.0 (C-1′), 122.9 (C-12), 145.2 (C-13), 180.6 (C-28); HRESIMS $[M+NH_4]^+$ *m/z* 622.4254 (calcd for $C_{35}H_{60}NO_8^+$ 622.4313), $[M+Na]^+$ *m/z* 627.3826 (calcd for $C_{35}H_{56}NaO_8^+$ 627.3867), $[M+K]^+$ *m/z* 643.3664 (calcd for $C_{35}H_{56}KO_8^+$ 643.3607), $[2M+H]^+$ *m/z*

1209.8025 (calcd for $C_{70}H_{113}O_{16}^{+}$ 1209.8023), $[2M+Na]^{+}$ *m/z* 1231.7725 (calcd for $C_{70}H_{112}NaO_{16}^{+}$ 1231.7843), $[2M+K]^{+}$ *m/z* 1247.7511 (calcd. for $C_{70}H_{112}KO_{16}^{+}$ 1247.7582).

4.4 References

1. Eaton, A. L.; Brodie, P. J.; Callmander, M. W.; Rakotondrajaona, R.; Rakotobe, E.; Rasamison, V. E.; Kingston, D. G. I., Bioactive oleanane glycosides from *Polyscias duplicata* from the Madagascar dry forest. *Nat. Prod. Commun.* **2015,** *10* (4), 567-570.

2. Mabberley, D. J., *The Plant-Book: A Portable Dictionary of the Vascular Plants*. 2nd ed.; University Press: Cambridge, 1997.

3. Bedir, E.; Toyang, N. J.; Khan, I. A.; Walker, L. A.; Clark, A. M., A new dammarane-type triterpene glycoside from *Polyscias fulva*. *J. Nat. Prod.* **2001,** *64* (1), 95-97.

4. Chaturvedula, V. S.; Schilling, J. K.; Miller, J. S.; Andriantsiferana, R.; Rasamison, V. E.; Kingston, D. G. I., New cytotoxic oleanane saponins from the infructescences of *Polyscias amplifolia* from the Madagascar rainforest. *Planta Med.* **2003,** *69* (5), 440-444.

5. Cioffi, G.; Vassallo, A.; Lepore, L.; Venturella, F.; Dal Piaz, F.; Tommasi, N., Antiproliferative oleanane saponins from *Polyscias guilfoylei*. *Nat. Prod. Commun.* **2008,** *3* (10), 1667-1670.

6. Paphassarang, S.; Raynaud, J.; Lussignol, M.; Becchi, M., Triterpenic glycosides from *Polyscias scutellaria*. *Phytochemistry* **1989,** *28* (5), 1539-1541.

7. Paphassarang, S.; Raynaud, J.; Lussignol, M.; Cabalion, P., A New Oleanolic Glycoside from *Polyscias scutellaria*. *J. Nat. Prod.* **1990,** *53* (1), 163-166.

8. Vo, D. H.; Yamamura, S.; Ohtani, K.; Kasai, R.; Yamasaki, K.; Nguyen, T. N.; Hoang, M. C., Oleanane saponins from *Polyscias fruticosa*. *Phytochemistry* **1998,** *47* (3), 451-457.

9. Mitaine-Offer, A. C.; Tapondjou, L. A.; Lontsi, D.; Sondengam, B. L.; Choudhary, M. I.; Atta ur, R.; Lacaille-Dubois, M. A., Constituents isolated from *Polyscias fulva*. *Biochem. Syst. Ecol.* **2004,** *32* (6), 607-610.

10. Gopalsamy, N.; Gueho, J.; Julien, H. R.; Owadally, A. W.; Hostettmann, K., Molluscicidal saponins of *Polyscias dichroostachya*. *Phytochemistry* **1990,** *29* (3), 793-795.

11. Grishkovets, V. I.; Sidorov, D. Y.; Yakovishin, L. A.; Arnautov, N. N.; Shashkov, A. S.; Chirva, V. Y., Triterpene glycosides of *Hedera canariensis*. I. Structures of glycosides L-A, L-B_1, L-B_2, L-C, L-D, L-E_1, L-G_1, L-G_2, L-G_3, L-G_4, L-H_1, L-H_2, and L-I_1 from the leaves of *Hedera canariensis*. *Chem. Nat. Compd.* **1996,** *32* (3), 360-365.

12. Joshi, B. S.; Moore, K. M.; Pelletier, S. W.; Puar, M. S.; Pramanik, B. N., Saponins from *Collinsonia canadensis*. *J. Nat. Prod.* **1992,** *55* (10), 1468-1476.

13. Strigunov, V. S.; Grishkovets, V. I.; Shashkov, A. S.; Chirva, V. Y., Triterpene glycosides from *Tetrapanax papyriferum*. III. Minor monodesmoside glycosides from stem bark. *Chem. Nat. Compd.* **2002,** *37* (5), 462-465.

14. Shao, C. J.; Kasai, R.; Xu, J. D.; Tanaka, O., Saponins from roots of *Kalopanax septemlobus* (Thunb.) Koidz., Ciqiu: structures of kalopanax-saponins C, D, E and F. *Chem. Pharm. Bull.* **1989,** *37* (2), 311-314.

15. Mshvildadze, V.; Elias, R.; Faure, R.; Debrauwer, L.; Dekanosidze, G.; Kemertelidze, E.; Balansard, G., Triterpenoid saponins from berries of *Hedera colchica*. *Chem. Pharm. Bull.* **2001,** *49* (6), 752-754.

16. Cao, S.; Brodie, P. J.; Miller, J. S.; Randrianaivo, R.; Ratovoson, F.; Birkinshaw, C.; Andriantsiferana, R.; Rasamison, V. E.; Kingston, D. G. I., Antiproliferative xanthones of *Terminalia calcicola* from the Madagascar rain forest. *J. Nat. Prod.* **2007,** *70* (4), 679-681.

17. Louie, K. G.; Behrens, B. C.; Kinsella, T. J.; Hamilton, T. C.; Grotzinger, K. R.; McKoy, W. M.; Winker, M. A.; Ozols, R. F., Radiation survival parameters of antineoplastic drug-sensitive

and drug-resistant human ovarian-cancer cell-lines and their modification by buthionine sulfoximine. *Cancer Res.* **1985,** *45* (5), 2110-2115.

Chapter 5: Synthesis of Mallotojaponin C and Derivatives

5.1 Introduction

5.1.1 Abstract

The phloroglucinol mallotojaponin C was isolated recently from *Mallotus oppositifolius* and was shown to have both antiplasmodial and cytocidal activities against *Plasmodium falciparum.* The natural product, mallotojaponin C, was synthesized in two steps from 2′,4′,6′-trihydroxyacetophenone. Derivatives of mallotojaponin C were synthesized in an attempt to improve the bioactivity. Two derivatives, **5.12** and **5.13**, were found to have similar antiplasmodial activity to that of mallotojaponin C.

5.1.2 Author Contributions

The author (Alexander L. Eaton) of this dissertation performed the syntheses described. Ms. Shuqi Zhao assisted with the synthesis of compounds **5.5** and **5.16**. Antimalarial assays (*Plasmodium falciparum,* Dd2) were performed by Dr. Seema Dalal under the supervision of Prof. Maria Belen Cassera. Dr. David G. I. Kingston was a mentor for this work.

5.1.3 Previous Investigations and Project Goals

The phloroglucinol mallotojaponin C (**5.1**) was isolated in 2013 by Liva Harinantenaina from *Mallotus oppositifolius*, a member of the Euphorbiaceae family,[1] and was shown to have both cytostatic and cytocidal activity against chloroquine/mefloquine-resistant *Plasmodium falciparum* (Dd2 strain). Related compounds previously isolated or prepared and tested for their antimalarial activity include methylated mallotojaponin C (**5.2**), mallotophenone (**5.3**), and mallotojaponin B (**5.4**). This testing of **5.1**–**5.4** indicated the importance of the alkenyl side chain and the phenolic

hydroxy substituents at C-2 and C-6 for activity. Many monomeric phloroglucinols have been investigated for their antimalarial activity, although few have given positive results.[2-4] Herein is presented a synthesis of mallotojaponin C and an investigation of the activities of mallotojaponin C derivatives.

Table 5-1. Antimalarial activities of mallotojaponin derivatives previously investigated.[1]

Compound	IC_{50} (μM)
5.1	0.14 ± 0.04
5.2	2.5 ± 0.5
5.3	>10
5.4	0.75 ± 0.3

5.2 Results and Discussion

5.2.1 Synthesis

Compounds **5.1** and **5.5–5.18** (Figure 5-1) were synthesized during the course of this work. Mallotojaponin C (**5.1**) was synthesized in two steps starting from 2′,6′-dihydroxy-4′-methoxyacetophenone. The first step involved the straightforward synthesis of **5.11** by the prenylation of the commercially available compound **5.10**. The synthesis of **5.1** from **5.11** proved to be more challenging. The synthesis of **5.1** requires the coupling of two molecules of **5.11** by formaldehyde. The initial coupling conditions used were based upon previous work with similar substrates,[5-7] but in the case of **5.1** it was apparent that the alkene moiety was readily hydrated under the standard acidic conditions. Most of the previously published work was on derivatives not containing an alkene moiety, so this problem did not arise in these cases. This presented a number of difficulties. In order to improve the yield of **5.1**, the synthesis of the model compound **5.8** was investigated (Figure 5-2) because of the reduced cost of **5.15** in comparison to **5.10**. Various solvents, catalysts, and reaction durations were used in attempts to improve the yield of

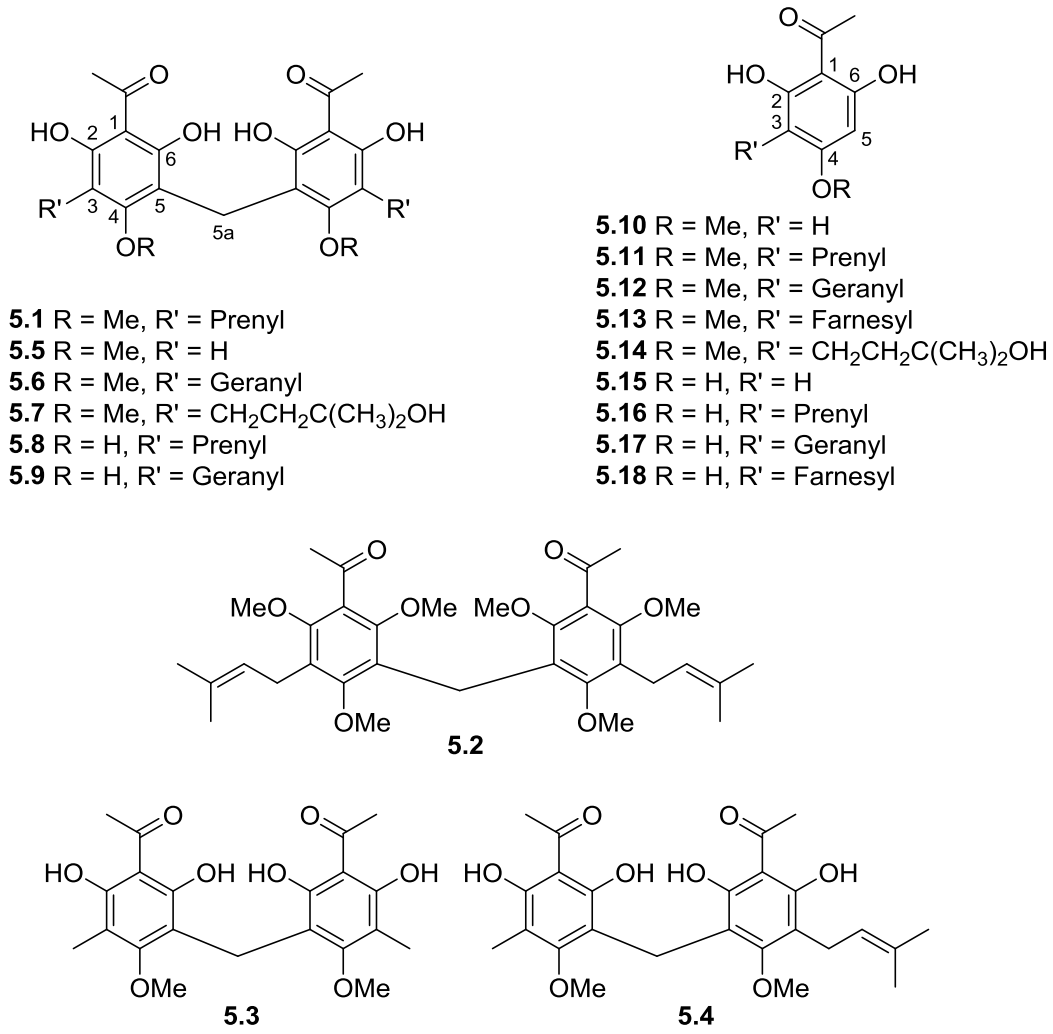


Figure 5-1. Compounds investigated for antimalarial activity.

5.8 and to prevent hydration and other undesired reactions (Table 5-2). The best solvent was found to be acetonitrile, and the use of concentrated sulfuric acid as catalyst reduced hydration of the alkene. These conditions allowed the reaction to be completed in less than 30 minutes, and this

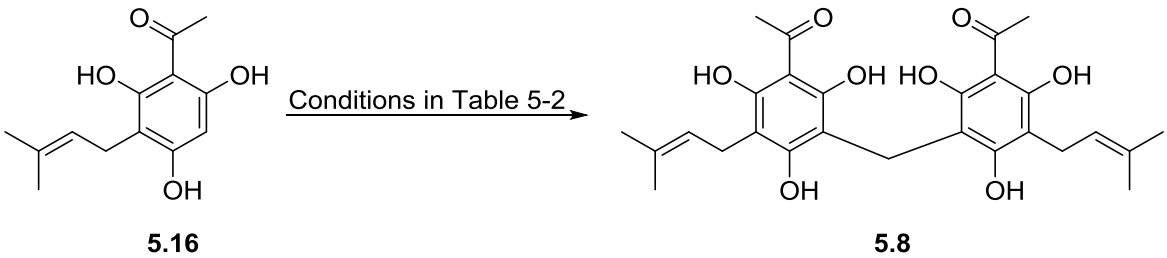


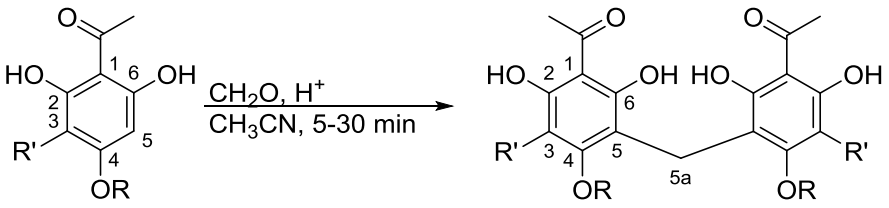
Figure 5-2. Synthesis of **5.8**.

short reaction time reduced the amount of hydration. Although the yield of the coupling reaction was only 15%, the desired product could be purified. The structure was confirmed by MS and comparison of ^{1}H and ^{13}C NMR spectra to previously published spectra.[1]

Table 5-2. Some conditions attempted for synthesis of **5.8**.

Solvent	Linker	Equivalents	Catalyst	Cat. Amt.	Heat (50° C)	Duration	Yield
$CHCl_3$	Trioxane	0.3	p-TsCl	Cat.	Y	2 h	Fail
$CHCl_3$	Trioxane	0.3	H_2SO_4	1 drop	Y	2 h	Fail
MeOH	Formaldehyde	0.5	H_2SO_4	1 drop	N	Overnight	Trace
MeOH	Formaldehyde	1	H_2SO_4	5 drops	N	Overnight	Trace
MeOH	Formaldehyde	3	H_2SO_4	5 drops	N	Overnight	Trace
MeOH	Formaldehyde	10	H_2SO_4	5 drops	N	Overnight	Trace
MeOH	Formaldehyde	100	H_2SO_4	5 drops	N	Overnight	Trace
MeOH	Formaldehyde	300	H_2SO_4	5 drops	N	Overnight	Trace
MeOH	Formaldehyde	1000	H_2SO_4	5 drops	N	Overnight	Trace
MeOH	Formaldehyde	1	H_2SO_4	1 drop	N	Overnight	Trace
MeOH	Formaldehyde	1	H_2SO_4	10 drops	N	Overnight	Trace
MeOH	Formaldehyde	1	HCl	1 drop	N	Overnight	Trace
MeOH	Formaldehyde	1	HCl	10 drops	N	Overnight	Trace
MeOH	Formaldehyde	1	H_2SO_4	1 drop	Y	Overnight	Fail
MeOH	Formaldehyde	1	H_2SO_4	10 drops	Y	Overnight	Fail
MeOH	Formaldehyde	1	HCl	1 drop	Y	Overnight	Fail
MeOH	Formaldehyde	1	HCl	10 drops	Y	Overnight	Fail
THF	Formaldehyde	1	H_2SO_4	5 drops	N	Overnight	Fail
MeCN	Formaldehyde	1	H_2SO_4	5 drops	N	Overnight	<10%
MeCN	Formaldehyde	2	H_2SO_4	5 drops	N	Overnight	~10%
MeCN	Formaldehyde	10	H_2SO_4	5 drops	N	30 min	~15%

In an attempt to improve upon the antiparasitic activity of the coupled compounds, compounds **5.5**–**5.9** were synthesized to investigate the effect of the length of the alkenyl chain and the substituent at C-5 on the bioactivity of the resulting compounds. Compound **5.8** has been previously synthesized, and its structure was confirmed by MS and comparison of ^{1}H and ^{13}C NMR spectra to previously published spectra.[7] The structures of **5.5**–**5.7** and **5.9** were confirmed by MS and comparison of ^{1}H and ^{13}C NMR spectra.



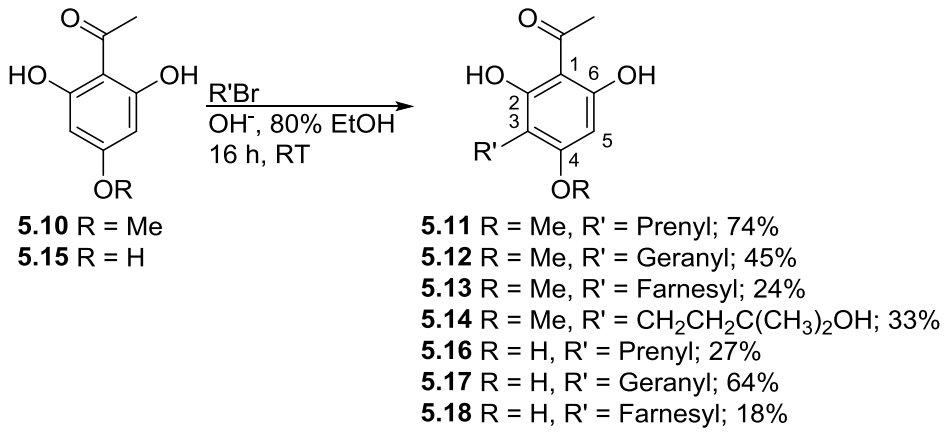
5.10 R = Me, R' = H
5.11 R = Me, R' = Prenyl
5.12 R = Me, R' = Geranyl
5.14 R = Me, R' = $CH_2CH_2C(CH_3)_2OH$
5.16 R = H, R' = Prenyl
5.17 R = H, R' = Geranyl

5.1 R = Me, R' = Prenyl; 32%
5.5 R = Me, R' = H; 38%
5.6 R = Me, R' = Geranyl; 4%
5.7 R = Me, R' = $CH_2CH_2C(CH_3)_2OH$; 50%
5.8 R = H, R' = Prenyl; 20%
5.9 R = H, R' = Geranyl; 27%

Figure 5-3. Synthesis of **5.1**, **5.5**–**5.9**

The synthesis of compounds **5.5**–**5.9** required the preparation of compounds **5.10**, **5.12**, **5.14**, **5.16**, and **5.17** as reactants. Alkylation of the precursors **5.10** and **5.15** was achieved by reaction of the phenol with either sodium hydroxide or lithium hydroxide and the appropriate alkenyl halide in ethanol at room temperature. The use of sodium hydroxide led to higher yields of **5.11**, **5.12**, **5.16**, and **5.17** than the use of lithium hydroxide. Compounds **5.11**,[8-11] **5.16**,[11-14] **5.17**,[15,16] and **5.18**[14,17] have previously been synthesized, and their structures were confirmed by MS and comparison of ^{1}H and ^{13}C NMR spectra to previously published spectra. The ethanol solvent was chosen based on a previous study of the effect of solvent on the C-alkylation of phloroglucinols and trials of various solvents to synthesize **5.11**.[18,19]

During the course of this work, the synthesis of other derivatives was attempted by replacing formaldehyde with various aldehydes (Figure 5-5). The synthesis of **5.19** initially appeared to be successful, as judged by its ^{1}H NMR spectrum, and the compound showed

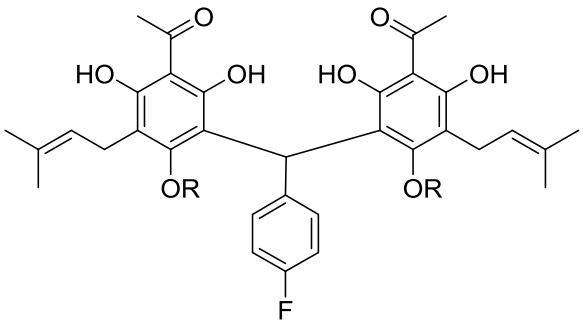


5.10 R = Me
5.15 R = H

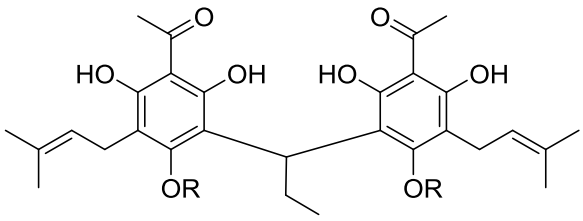
5.11 R = Me, R' = Prenyl; 74%
5.12 R = Me, R' = Geranyl; 45%
5.13 R = Me, R' = Farnesyl; 24%
5.14 R = Me, R' = $CH_2CH_2C(CH_3)_2OH$; 33%
5.16 R = H, R' = Prenyl; 27%
5.17 R = H, R' = Geranyl; 64%
5.18 R = H, R' = Farnesyl; 18%

Figure 5-4. C-alkylation of **5.10** and **5.15**.

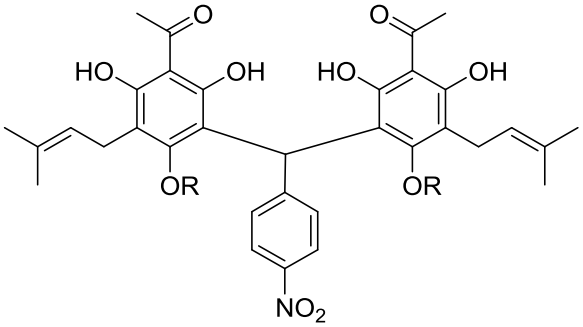
promising bioactivity, with an IC_{50} value in the 2–4 μM range. Unfortunately the compound proved to be unstable when stored in the freezer and decomposed before MS data could be obtained and an accurate IC_{50} value determined. Attempts to synthesize the other proposed derivatives **5.20 – 5.28** were unsuccessful.



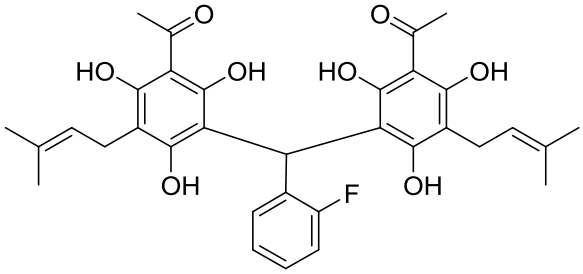
5.19 R = H
5.20 R = CH_3



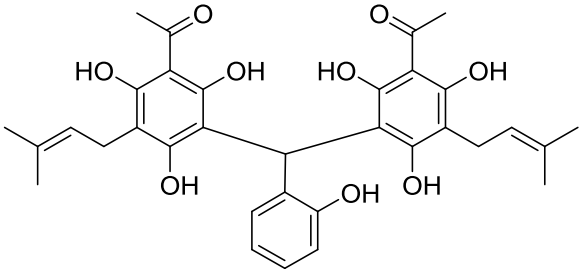
5.21 R = H
5.22 R = CH_3



5.24 R = H
5.25 R = CH_3



5.26



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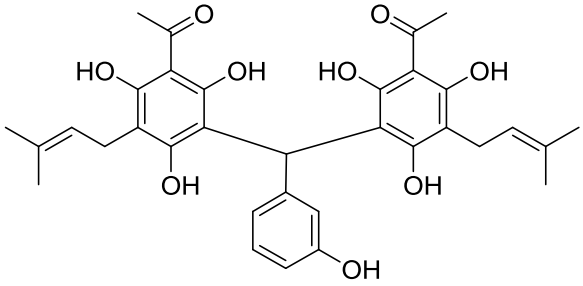


5.28

Figure 5-5. Attempted mallotojaponin C derivatives.

5.2.2 Antiplasmodial Activity

The synthetic compounds **5.1**–**5.19** were evaluated by Dr. Seema Dalal in Prof. Maria Belen Cassera's laboratory for their antiparasitic activities against the Dd2 strain of *P. falciparum.*

For the dimeric phloroglucinols (**5.1**–**5.9**), it was found that the prenyl side chain maximized the bioactivity, with the bis-prenylated natural product **5.1** having the best potency.

Table 5-3: Activities of compounds against *Plasmodium falciparum* Dd2.

Compound	IC_{50} (μM)
5.1	0.14 ± 0.04[1]
5.2	2.5 ± 0.5[1]
5.3	>10[1]
5.4	0.75 ± 0.3[1]
5.5	4.5 ± 2.5
5.6	1.7 ± 0.8
5.7	>10
5.8	>20
5.9	>10
5.10	>20
5.11	1.7 ±0.4
5.12	0.6 ± 0.08
5.13	0.4 ± 0.05
5.14	>20
5.15	>20
5.16	>20
5.17	6.2 ± 2.3
5.18	1.3 ± 0.8
5.19	>20

As previously noted, the replacement of hydroxy groups with methoxy groups reduced activity. A different trend was observed among the precursor compounds (**5.10**–**5.18**), where an increased alkenyl chain length led to an increase in antiplasmodial activity. Thus in the 4-*O*-methylated series **5.10**–**5.13**, the unalkylated compound **5.10** was essentially inactive, while the prenylated compound **5.11** was six-fold less potent than the corresponding geranylated compound **5.12**; the farnesylated compound **5.13** had a similar activity to **5.12**. Similar trends were noted with the unmethylated series **5.15**–**5.18**, with **5.15** and **5.16** being essentially inactive, the geranylated

compound **5.17** having an IC_{50} of 6.4 μM, and the farnesylated compound **5.18** having an IC_{50} of about 3 μM. Interestingly, this effect was not observed in dimeric phloroglucinol derivatives, since as noted above the geranylated compound 5.6 is less active than its prenylated counterpart **5.1**. Hydration of the alkene, as in **5.7** and **5.14**, also led to a decrease in bioactivity. Additionally, it was also found that the presence of a methoxy group at C-4 increased the bioactivity.

The reason for the increase in activity with an increase in alkenyl chain length is not clear at this time, but one possibility is that it is the result of a detergent-like effect. If this is the case, then the appeal of this class of compounds as antimalarial leads would be much reduced. It is hoped that ongoing studies in Prof. Maria Belen Cassera's laboratory will shed light on this and other mechanistic aspects of these interesting compounds.

5.3 Experimental Section

5.3.1 General Experimental Procedures

NMR spectra were recorded on a Varian 400 or Bruker Advance 500 MHz spectrometer. ^{1}H-^{1}H three-bond *J*-coupling values were calculated from ^{1}H NMR spectra. UV-Vis spectra were recorded on a Shimadzu UV-1201 spectrophotometer. Mass spectra were obtained with an Agilent 6220 LC-TOF-MS. Compounds **5.10** and **5.15** were purchased from Indofine Chemical Company and tested for bioactivity without any further purification. Compounds **5.2–5.3** were isolated from *Mallotus oppositifolius* and compound **5.4** was previously synthesized from **5.1**.[1] Preparatory HPLC separations were performed using Shimadzu LC-8A pumps coupled with a Shimadzu SPD-M10A diode array detector, a SCL-10A controller, and a Varian Dynamax C_{18} column (250 x 21.4 mm). Semipreparative HPLC separation were performed using Shimadzu LC-10AT pumps coupled with a Shimadzu SPD-M10A diode array detector, a SCL-10A system controller, and a Phenomenex Luna C_{18} column (250 x 10 mm). Bioactive compounds were checked for purity by

analytical HPLC analysis using Shimadzu LC-10AT pumps coupled with a Shimadzu SPD-M10A diode array detector, a Sedex 75 Evaporative Light Scattering Detector (ELSD), a SCL-10A system controller, and a Phenomenex Luna C_{18} column (250 x 4.6 mm).

5.3.2 Antimalarial Bioassay

Assay was performed at Virginia Tech as previously described.[1]

*5.3.3 Synthesis of Mallotojaponin C (**5.1**)*

Compound **5.11** (26.2 mg, 0.1 mmol) was dissolved in acetonitrile (~3 mL). Formaldehyde (37% aq., 76 μL, 1 mmol) was added, followed by the addition of conc. HCl (1 drop). The reaction mixture was stirred for 5 min at rt. The reaction mixture was diluted with water (10 mL) and extracted with EtOAc (3 x 10 mL). The organic solution was washed with sat. NaCl (10 mL), dried with $MgSO_4$, and the solvent was removed under reduced pressure. The residue was purified utilizing C_{18} HPLC (MeOH/H_2O gradient w/ 0.1% formic acid) to yield **5.1** (6.1 mg, 0.012 mmol, 32%). Reaction yield is based on unrecovered starting material.

Mallotojaponin C (5.1): light yellow powder; ^{1}H NMR ($CDCl_3$, 400 MHz): 1.68 (H-5′, 3H, s), 1.77 (H-4′, 3H, s) 2.70 (–OAc, 6H, s), 3.31 (H-1′, 4H, d, J = 6.5 Hz, 1H), 3.68 (H-5a, 2H, s), 3.98 (–OCH_3, 6H, s), 5.21 (H-2′, H, m), 9.06 (–OH, 2H, s), 13.49 (–OH, 2H, s); HRESIMS $[M+H]^+$ *m/z* 513.2469 (calcd for $C_{29}H_{37}O_8^+$ 513.2483), $[M+Na]^+$ *m/z* 535.2297 (calcd for $C_{25}H_{44}NaO_4^+$ 535.2302).

*5.3.4 Synthesis of **5.5***

Compound **5.10** (100 mg, 0.5 mmol) was dissolved in methanol (~20 mL). Formaldehyde (37% aq., 2 mL, 27 mmol) was added, followed by the addition of conc. H_2SO_4 (5 drops). The reaction mixture was stirred for 16 h at rt. The reaction mixture was diluted with water (20 mL) and extracted with EtOAc. The organic solution was washed with sat. aqueous NaCl, dried with $MgSO_4$, and the solvent was removed under reduced pressure. The residue was purified utilizing silica gel CC (1:1 hexanes/EtOAc) to yield **5.5** (53.7 mg, 0.012 mmol, 38%).

Compound 5.5: light yellow powder; ^{1}H NMR ($(CD_3)_2SO$, 400 MHz): 2.56 (–OAc, 6H, s), 3.61 (H-5a, 2H, s), 3.69 (–OCH_3, 6H, s), 6.00 (H-3, 2H, s), 10.87 (–OH, 2H, s), 13.48 (–OH, 2H, s); ^{13}C NMR ($(CD_3)_2SO$, 100 MHz) 15.2, 30.7, 32.7, 39.5, 55.4, 90.2, 104.4, 160.8, 162.3, 163.9, 203.1; HRESIMS $[M+H]^+$ *m/z* 377.1197 (calcd for $C_{19}H_{27}O_8^+$ 377.1231), $[M+Na]^+$ *m/z* 399.1020 (calcd for $C_{19}H_{20}NaO_8^+$ 399.1050).

*5.3.5 Synthesis of **5.6***

Compound **5.12** (49.9 mg, 0.16 mmol) was dissolved in acetonitrile (~3 mL). Formaldehyde (37% aq., 117 μL, 1.6 mmol) was added, followed by the addition of conc. H_2SO_4 (1 drop). The reaction mixture was stirred for 5 min at rt. The reaction mixture was diluted with water (10 mL) and extracted with EtOAc (3 x 10 mL). The organic solution was washed with sat. aqueous NaCl (10 mL), dried with $MgSO_4$, and the solvent was removed under reduced pressure. The residue was purified utilizing C_{18} HPLC (MeOH/H_2O gradient w/ 0.1% formic acid) to yield **5.6** (2.2 mg, 0.003 mmol, 4%).

Compound 5.6: light yellow powder; UV (MeOH) λ_{max} (log ε): 204 (4.32) nm, 289 (3.95); ^{1}H NMR ($CDCl_3$, 400 MHz): 1.56 (H-10′, 6H, s), 1.63 (H-9′, 6H, s), 1.77 (H-8′, 6H, s), 1.98 (H-5′, 4H, m), 2.05 (H-4′, 4H, bt, *J* = 7.4 Hz), 2.70 (–OAc, 6H, s), 3.31 (H-1′, 4H, d, *J* = 6.9 Hz), 3.68 (H-5a, 2H, s), 3.97 (–OCH_3, 6H, s), 5.04 (H-6′, 2H, m), 5.22 (H-2′, 2H, m), 9.09 (–OH, 2H, s), 13.47 (–OH, 2H, s); ^{13}C NMR ($CDCl_3$, 100 MHz): 16.2, 17.7, 22.7, 25.7, 26.6, 33.7, 39.6, 62.8, 108.4, 109.0, 114.1, 122.6, 124.2, 131.3, 135.7, 157.4, 159.6, 162.7, 205.3; HRESIMS $[M-H]^-$ *m/z* 647.3663 (calcd for $C_{19}H_{27}O_8^+$ 347.3589).

*5.3.6 Synthesis of **5.7***

Compound **5.12** (12.1 mg, 0.045 mmol) was dissolved in acetonitrile (~3 mL). Formaldehyde (37% aq., 34 μL, 0.46 mmol) was added, followed by the addition of conc. H_2SO_4 (1 drop). The reaction mixture was stirred for 5 min at rt. The reaction mixture was diluted with water (10 mL) and extracted with EtOAc (3 x 10 mL). The organic solution was washed with sat. aqueous NaCl (10 mL), dried with $MgSO_4$, and the solvent was removed under reduced pressure. The residue was purified utilizing C_{18} HPLC (MeOH/H_2O gradient w/ 0.1% formic acid) to yield **5.7** (6.2 mg, 0.011 mmol, 50%).

Compound 5.7: light yellow powder; UV (MeOH) λ_{max} (log ε) 213 (4.33) nm, 287 (4.25) nm, 354 (3.70) nm; ^{1}H NMR ($CDCl_3$, 400 MHz): 1.37 (H-4′, H-5′, 12H, s), 1.74 (H-2′, 4H, t, *J* = 6.7 Hz), 2.63 (–OAc, 6H, s), 2.66 (H-1′, 4H, t, *J* = 6.8 Hz), 3.65 (–OCH_3, 6H, s), 3.94 (H-5a, 2H, s), 13.80 (–OH, 2H, s); ^{13}C NMR ($CDCl_3$, 100 MHz): 17.1, 27.0, 31.9, 33.8, 60.3, 75.6, 104.8, 108.2, 114.4, 155.2, 162.7, 163.4, 204.4.

*5.3.7 Synthesis of **5.8***

Compound **5.16** (14.3 mg, 0.061 mmol) was dissolved in acetonitrile (~3 mL). Formaldehyde (37% aq., 45 μL, 0.60 mmol) was added, followed by the addition of conc. H_2SO_4 (1 drop). The reaction mixture was stirred for 30 m at rt. The precipitate was filtered and rinsed with MeOH to yield **5.8** (3 mg, 0.0062 mmol, 20%). The compound appeared to be > 90% pure by ^{1}H NMR spectroscopy.

Compound 5.8: light yellow powder; ^{1}H NMR ($(CD_3)_2SO$, 400 MHz): 1.59 (H-5′, 6H, s), 1.68 (H-4′, 6H, s), 2.63 (–OAc, 6H, s), 3.23 (H-1′, 4H, d, *J* = 6.7 Hz), 3.68 (H-5a, 2H, s) 5.04 (H-2′, 2H, m); ^{13}C NMR ($(CD_3)_2SO$, 100 MHz): 17.0, 17.8, 21.5, 25.5, 32.6, 48.6, 105.8, 106.4, 108.1, 123.0, 130.4, 159.5, 203.7; HRESIMS $[M+H]^+$ *m/z* 485.2140 (calcd for $C_{27}H_{33}O_8^+$ 485.2170), $[M+Na]^+$ *m/z* 507.1946 (calcd for $C_{27}H_{32}NaO_8^+$ 507.1989).

*5.3.8 Synthesis of **5.9***

Compound **5.17** (49.3 mg, 0.16 mmol) was dissolved in acetonitrile (~3 mL). 37% aq. formaldehyde (121 μL, 1.6 mmol) was added, followed by the addition of conc. H_2SO_4 (1 drop). The reaction mixture was stirred for 5 m at rt. The reaction mixture was diluted with water (10 mL) and extracted with EtOAc (3 x 10 mL). The organic solution was washed with sat. aqueous NaCl (10 mL), dried with $MgSO_4$, and the solvent was removed under reduced pressure. The residue was purified utilizing C_{18} HPLC (MeOH/H_2O gradient w/ 0.1% formic acid) to yield **5.9** (10.5 mg, 0.017 mmol, 27 %). Reaction yield is based on recovered unreacted starting material. Attempts at recrystallizing the compound in MeOH/H_2O were unsuccessful and resulted in decomposition before MS data could be obtained.

Compound 5.9: light yellow powder; UV (MeOH) λ_{max} (log ε) 208 (4.27) nm, 230 (4.17) nm, 292 (4.11) nm; ^{1}H NMR ($CDCl_3$, 400 MHz): 1.60 (H-10′, 6H, s), 1.68 (H-9′, 6H, s), 1.83 (H-8′, 6H, s), 2.11 (H-4′, H-5′, 8H, m), 2.67 (–OAc, 6H, s), 3.41 (H-1′, 4H, d *J* = 6.7 Hz), 3.79 (H-5a, 2H, s), 5.05 (H-6′, 2H, m), 5.21 (H-2′, 2H, m).

*5.3.9 Synthesis of **5.11***

Compound **5.10** (495 mg, 2.7 mmol) was dissolved in a solution of 80% ethanol (40 mL) containing NaOH (120 mg, 3 mmol). Prenyl bromide (346 μL, 3 mmol) was added and the reaction mixture was stirred for 16 h at rt. The reaction mixture was acidified with 3N HCl and extracted with DCM (3 x 50 mL). The organic solution was washed with sat. aqueous NaCl (50 mL), dried with $MgSO_4$, and the solvent was removed under reduced pressure. The residue was purified utilizing C_{18} HPLC to yield **5.11** (228 mg, 0.017 mmol, 74 %). Reaction yield is based on unrecovered starting material.

Compound 5.11: light yellow needles; UV (MeOH) λ_{max} (log ε) 215 (4.06) nm, 288 (4.09) nm, 333 (3.36); ^{1}H NMR (CD_3OD, 400 MHz): 1.60 (H-5′, 3H, s), 1.71 (H-4′, 3H, s), 2.60 (–OAc, 3H, s), 3.15 (H-1′, 2H, d, *J* = 7.2 Hz), 3.78 (–OCH_3, 3H, s), 5.10 (H-2′, m), 5.96 (H-5, 1H, s); ^{13}C NMR (CD_3OD, 100 MHz): 17.8, 22.0, 25.9, 33.1, 55.8, 90.9, 106.0, 108.9, 124.3, 131.1, 162.3, 163.5, 165.0, 205.0; HRESIMS $[M+H]^+$ *m/z* 251.1274 (calcd for $C_{14}H_{19}O_4^+$ 251.1278), $[M+Na]^+$ *m/z* 273.1114 (calcd for $C_{14}H_{18}NaO_4^+$ 273.1097).

*5.3.10 Synthesis of **5.12***

Compound **5.10** (150 mg, 0.82 mmol) was dissolved in a solution of 80% ethanol (40 mL) containing LiOH (40 mg, 1.7 mmol). Geranyl bromide (180 μL, 0.91 mmol) was added and the reaction mixture was stirred for 16 h at rt. The reaction mixture was acidified with 3N HCl and extracted with DCM (3 x 50 mL). The organic solution was washed with sat. aqueous NaCl (50 mL), dried with $MgSO_4$, and the solvent was removed under reduced pressure. The residue was purified utilizing C_{18} HPLC to yield **5.12** (78 mg, 0.12 mmol, 43 %). Reaction yield is based on recovered unreacted starting material.

B. NaOH method. Compound **5.10** (100 mg, 0.55 mmol) was dissolved in a solution of 80% ethanol (40 mL) containing NaOH (50 mg, 1.25 mmol). Geranyl bromide (120 μL, 0.60 mmol) was added and the reaction mixture was stirred for 16 h at rt. The reaction mixture was acidified with 3N HCl and extracted with DCM (3 x 50 mL). The organic solution was washed with sat. aqueous NaCl (50 mL), dried with $MgSO_4$, and the solvent was removed under reduced pressure. The residue was purified utilizing C_{18} HPLC to yield **5.12** (37 mg, 0.12 mmol, 45 %). Reaction yield is based on unrecovered starting material.

Compound 5.12: light yellow powder; UV (MeOH) λ_{max} (log ε) 216 (4.16) nm, 289 (4.14) nm, 333 (3.38) nm; ^{1}H NMR (CD_3OD, 400 MHz): 1.55 (H-10′, 3H, s), 1.60 (H-9′, 3H, s), 1.72 (H-8′, 3H, s), 2.00 (H-4′, H-5′, 4H, m), 2.63 (–OAc, 3H, s), 3.19 (H-1′, 2H, d, *J* = 7.0 Hz), 3.81 (–OCH_3, 3H, s), 5.04 (H-6′, m), 5.11 (H-2′, m), 6.01 (H-5, 1H, s); ^{13}C NMR (CD_3OD, 100 MHz): 16.1, 17.7, 21.9, 25.8, 26.3, 33.1, 40.9, 55.9, 90.8, 106.0, 109.0, 124.4, 125.5, 131.9, 134.8, 162.4, 163.6, 165.0, 205.1; HRESIMS $[M+H]^+$ *m/z* 319.1896 (calcd for $C_{19}H_{27}O_4^+$ 319.1904), $[M+Na]^+$ *m/z* 341.1707 (calcd for $C_{19}H_{26}NaO_4^+$ 341.1723).

*5.3.11 Synthesis of **5.13***

Compound **5.10** (100 mg, 0.55 mmol) was dissolved in a solution of 80% ethanol (40 mL) containing LiOH (26.4 mg, 1.1 mmol). Farnesyl bromide (164 μL, 0.61 mmol) was added and the reaction mixture was stirred for 16 h at rt. The reaction mixture was acidified with 3N HCl and extracted with DCM (3 x 50 mL). The organic solution was washed with sat. aqueous NaCl (50 mL), dried with $MgSO_4$, and the solvent was removed under reduced pressure. The residue was purified utilizing C_{18} HPLC to yield **5.13** (61 mg, 0.12 mmol, 24 %). Reaction yield is based on unrecovered starting material.

Compound 5.13: light yellow powder; UV (MeOH) λ_{max} (log ε) 213 (4.21) nm, 288 (4.14) nm, 333 (3.38) nm; ^{1}H NMR ($CDCl_3$, 400 MHz): 1.58 (H-15′, 3H, s), 1.59 (H-14′, 3H, s), 1.67 (H-13′, 3H, s), 1.82 (H-12′, 3H, s), 2.07 (H-4′, H-5′, H-8′, H-9′, 8H, m), 2.65 (–OAc, 3H, s), 3.34 (H-1′, 2H, d, *J* = 7.1 Hz), 3.82 (–OCH_3, 3H, s), 5.07 (H-6′, H-10′,2H, m), 5.19 (H-2′, 1H, m), 6.02 (H-5, 1H, s); ^{13}C NMR (CD_3OD, 100 MHz): 16.1, 17.7, 21.9, 25.9, 27.1, 27.6, 33.0, 40.7, 40.7, 56.0, 91.1, 106.0, 108.9, 124.4, 125.2, 125.4, 132.0 ,134.6, 135.7, 162.5, 163.5, 165.1, 205.4; HRESIMS $[M+H]^+$ *m/z* 387.2535 (calcd for $C_{24}H_{35}O_4^+$ 387.2530), $[M+Na]^+$ *m/z* 409.2346 (calcd for $C_{24}H_{34}NaO_4^+$ 409.2349).

*5.3.12 Synthesis of **5.14***

Compound **5.10** (49.6 mg, 0.27 mmol) was dissolved in an 80% ethanol solution (5 mL) containing NaOH (12 mg, 0.3 mmol). Prenyl bromide (35 μL, 0.3 mmol) was added and the reaction mixture was stirred for 16 h at rt. The reaction mixture was acidified with 3N HCl and

extracted with DCM (3 x 50 mL). The organic solution was washed with sat. aqueous NaCl (50 mL), dried with $MgSO_4$, and the solvent was removed under reduced pressure. The residue was purified utilizing silica gel CC (9:1 Hexanes/EtOAc w/ 0.1% Formic Acid) to yield **5.14** (24 mg, 0.089 mmol, 33 %).

Compound 5.14: light yellow powder; UV (MeOH) λ_{max} (log ε) 216 (4.03) nm, 290 (4.07) nm, 331 (3.39) nm; ^{1}H NMR (CD_3OD, 400 MHz): 1.37 (H-4′, H-5′, 6H, s), 1.77 (H-3′, 2H, t, *J* = 6.9 Hz), 2.53 (H-2′, 2H, t, *J* = 6.9 Hz), 2.58 (–OAc, 3H, s), 3.83 (–OCH_3, 3H, s), 6.01 (H-5, 1H, s); ^{13}C NMR (CD_3OD, 100 MHz):17.4, 26.9, 32.5, 33.6, 56.2, 77.1, 92.3, 102.1, 107.0, 157.7, 165.2, 166.5, 204.7; HRESIMS [M-OH]$^{\bullet}$ *m/z* 251.1278 (calcd for $C_{14}H_{19}O_4^{\bullet}$ 251.1283), [M-C_4H_9O]$^{\bullet}$ *m/z* 195.0646 (calcd for $C_{10}H_{11}O_4^{\bullet}$ 195.0657).

*5.3.13 Synthesis of **5.16***

Compound **5.15** (980 mg, 5.8 mmol) was dissolved in a solution of 80% ethanol (40 mL) containing LiOH (280 mg, 11.7 mmol). Prenyl bromide (776 μL, 6.7 mmol) was added and the reaction mixture was stirred for 16 h at rt. The reaction mixture was acidified with 3N HCl and extracted with DCM (3 x 50 mL). The organic solution was washed with sat. aqueous NaCl (50 mL), dried with $MgSO_4$, and the solvent was removed under reduced pressure. The residue was purified utilizing C_{18} HPLC to yield **5.16** (365 mg, 1.5 mmol, 27 %).

Compound 5.16: light yellow powder; ^{1}H NMR (CD_3OD, 400 MHz): 1.63 (H-5′, 3H, s), 1.73 (H-4′, 3H, s), 2.60 (–OAc, 3H, s), 3.17 (H-1′, 2H, d, *J* = 7.2 Hz), , 5.16 (H-2′, m), 5.89 (H-5, 1H, s); ^{13}C NMR (CD_3OD, 100 MHz): 17.8, 22.1, 26.0, 32.8, 94.7, 105.5, 107.9, 124.5, 131.1, 161.8,

163.9, 164.8, 204.6; HRESIMS $[M+H]^+$ *m/z* 237.1119 (calcd for $C_{13}H_{17}O_4^+$ 237.1121), $[2M+NH_4]^+$ *m/z* 490.2405 (calcd for $C_{26}H_{36}NO_8^+$ 399.1050).

*5.3.14 Synthesis of **5.17***

A. LiOH method. Compound **5.15** (253 mg, 1.5 mmol) was dissolved in a solution of 80% ethanol (40 mL) containing LiOH (65 mg, 2.7 mmol). Geranyl bromide (297 μL, 1.5 mmol) was added and the reaction mixture was stirred for 16 h at rt. The reaction mixture was acidified with 3N HCl and extracted with DCM (3 x 50 mL). The organic solution was washed with sat. aqueous NaCl (50 mL), dried with $MgSO_4$, and the solvent was removed under reduced pressure. The residue was purified utilizing C_{18} HPLC to yield **5.17** (147 mg, 0.48 mmol, 41 %). Reaction yield is based on unrecovered starting material.

B. NaOH method. Compound **5.15** (100 mg, 0.60 mmol) was dissolved in a solution of 80% ethanol (40 mL) containing NaOH (50 mg, 1.25 mmol). Geranyl bromide (130 μL, 0.65 mmol) was added and the reaction mixture was stirred for 16 h at rt. The reaction mixture was acidified with 3N HCl and extracted with DCM (3 x 50 mL). The organic solution was washed with sat. aqueous NaCl (50 mL), dried with $MgSO_4$, and the solvent was removed under reduced pressure. The residue was purified utilizing C_{18} HPLC to yield **5.17** (65 mg, 0.48 mmol, 64 %). Reaction yield is based on unrecovered starting material.

Compound 5.17: light yellow powder; ^{1}H NMR ($CDCl_3$, 400 MHz): 1.60 (H-10′, 3H, s), 1.68 (H-9′, 3H, s), 1.82(H-8′, 3H, s), 2.10 (H-4′, H-5′, 4H, m), 2.67 (–OAc, 3H, s), 3.37 (H-1′, 2H, d, *J* = 7.1 Hz), 5.05 (H-6′, m), 5.25 (H-2′, m), 5.85 (H-5, 1H, s); ^{13}C NMR (CD_3OD, 100 MHz): 16.2, 17.7, 22.0, 25.8, 27.7, 32.8, 40.9, 94.7, 105.5, 107.9, 124.6, 125.5, 131.9, 134.7, 161.8, 163.9,

164.8, 204.5; HRESIMS $[M+H]^+$ *m/z* 305.1744 (calcd for $C_{18}H_{25}O_4^+$ 305.1747), $[M+Na]^+$ *m/z* 327.1539 (calcd for $C_{18}H_{24}NaO_4^+$ 327.1567).

*5.3.15 Synthesis of **5.18***

Compound **5.15** (101 mg, 0.60 mmol) was dissolved in a solution of 80% ethanol (40 mL) containing LiOH (29 mg, 1.2 mmol). Farnesyl bromide (180 μL, 0.66 mmol) was added and the reaction mixture was stirred for 16 h at rt. The reaction mixture was acidified with 3N HCl and extracted with DCM (3 x 50 mL). The organic solution was washed with sat. aqueous NaCl (50 mL), dried with $MgSO_4$, and the solvent was removed under reduced pressure. The residue was purified utilizing C_{18} HPLC to yield **5.18** (61 mg, 0.081 mmol, 18 %). Reaction yield is based on unrecovered starting material.

Compound 5.18: light yellow powder; UV (MeOH) λ_{max} (log ε) 214 (4.21) nm, 291 (4.19) nm; ^{1}H NMR (CD_3OD, 400 MHz): 1.54 (H-15′, 3H, s), 1.56 (H-14′, 3H, s), 1.65 (H-13′, 3H, s), 1.79 (H-12′, 3H, s), 1.97 (H-4′, H-5′, H-8′, H-9′, 8H, m), 2.59 (–OAc, 3H, s), 3.18 (H-1′, 2H, d, *J* = 7.1 Hz), 5.05 (H-6′, H-10′,2H, m), 5.17 (H-2′, 1H, m), 5.89 (H-5, 1H, s); ^{13}C NMR (CD_3OD, 100 MHz): 14.7, 14.7, 16.3, 20.6, 24.5, 26.0, 26.3, 31.4, 39.4, 39.4, 93.3, 104.1, 106.5, 123.3, 123.9, 124.1, 130.4, 133.0, 134.3, 160.4, 162.5, 163.4, 203.1; HRESIMS $[M+H]^+$ *m/z* 373.2366 (calcd for $C_{23}H_{33}O_4^+$ 373.2373), $[M+Na]^+$ *m/z* 395.2169 (calcd for $C_{23}H_{32}NaO_4^+$ 395.2193).

5.4 **References**

1. Harinantenaina, L.; Bowman, J. D.; Brodie, P. J.; Slebodnick, C.; Callmander, M. W.; Rakotobe, E.; Randrianaivo, R.; Rasamison, V. E.; Gorka, A.; Roepe, P. D.; Cassera, M. B.; Kingston, D. G. I., Antiproliferative and antiplasmodial dimeric phloroglucinols from *Mallotus oppositifolius* from the Madagascar dry forest. *J. Nat. Prod.* **2013,** *76* (3), 388-393.

2. Bharate, S. B.; Bhutani, K. K.; Khan, S. I.; Tekwani, B. L.; Jacob, M. R.; Khan, I. A.; Singh, I. P., Biomimetic synthesis, antimicrobial, antileishmanial and antimalarial activities of euglobals and their analogues. *Bioorg. Med. Chem.* **2006,** *14* (6), 1750-1760.

3. Bharate, S. B.; Khan, S. I.; Yunus, N. A.; Chauthe, S. K.; Jacob, M. R.; Tekwani, B. L.; Khan, I. A.; Singh, I. P., Antiprotozoal and antimicrobial activities of O-alkylated and formylated acylphloroglucinols. *Bioorg. Med. Chem.* **2007,** *15* (1), 87-96.

4. Verotta, L.; Appendino, G.; Bombardelli, E.; Brun, R., In vitro antimalarial activity of hyperforin, a prenylated acylphloroglucinol. A structure-activity study. *Bioorg. Med. Chem. Lett.* **2007,** *17* (6), 1544-1548.

5. McGookin, A.; Robertson, A.; Simpson, T. H., 449. Rottlerin. Part VIII. The rottlerone change. *J. Chem. Soc.* **1951**, 2021.

6. Chauthe, S. K.; Bharate, S. B.; Sabde, S.; Mitra, D.; Bhutani, K. K.; Singh, I. P., Biomimetic synthesis and anti-HIV activity of dimeric phloroglucinols. *Bioorg. Med. Chem.* **2010,** *18* (5), 2029-2036.

7. Minassi, A.; Cicione, L.; Koeberle, A.; Bauer, J.; Laufer, S.; Werz, O.; Appendino, G., A multicomponent carba-betti strategy to alkylidene heterodimers - total synthesis and structure-activity relationships of arzanol. *Eur. J. Org. Chem.* **2012,** *2012* (4), 772-779.

8. Balani, S. K.; Gupta, R. K.; Krishnamurti, M., Synthesis of 5-hydroxy-3', 4', 5', 7-tetramethoxy-8-prenylflavanone. *J. Indian Chem. Soc.* **1980,** *57* (12), 1238-1239.

9. Sharma, V. K.; Gupta, S. R., Synthesis of helichromanochalcone and isoxanthiohumol. *Acta Chim. Hung.* **1983,** *113* (1), 93-95.

10. Hossain, M. A.; Salehuddin, S. M., Synthesis of amoradicin. *Indian J. Chem., Sect. B: Org. Chem. Incl. Med. Chem.* **2002,** *41* (11), 2399-2401.

11. Vogel, S.; Heilmann, J., Synthesis, cytotoxicity, and antioxidative activity of minor prenylated chalcones from *Humulus lupulus*. *J. Nat. Prod.* **2008,** *71* (7), 1237-1241.

12. Diller, R. A.; Riepl, H. M.; Rose, O.; Frias, C.; Henze, G.; Prokop, A., Synthesis of demethylxanthohumol, a new potent apoptosis-inducing agent from hops. *Chem. Biodiversity* **2005,** *2* (10), 1331-1337.

13. Lee, Y. R.; Li, X.; Kim, J. H., Concise total synthesis of biologically interesting mallotophilippens C and E. *J. Org. Chem.* **2008,** *73* (11), 4313-4316.

14. Basabe, P.; de Roman, M.; Marcos, I. S.; Diez, D.; Blanco, A.; Bodero, O.; Mollinedo, F.; Sierra, B. G.; Urones, J. G., Prenylflavonoids and prenyl/alkyl-phloroacetophenones: synthesis and antitumour biological evaluation. *Eur. J. Med. Chem.* **2010,** *45* (9), 4258-4269.

15. Sun, Q.; Schmidt, S.; Tremmel, M.; Heilmann, J.; Konig, B., Synthesis of natural-like acylphloroglucinols with anti-proliferative, anti-oxidative and tube-formation inhibitory activity. *Eur. J. Med. Chem.* **2014,** *85*, 621-628.

16. Huang, C.; Zhang, Z.; Li, Y., Total synthesis of (*R,S*)-sophoraflavanone C. *J. Nat. Prod.* **1998,** *61* (10), 1283-1285.

17. Wang, X.; Lee, Y. R., Efficient synthesis of polycycles bearing prenylated, geranylated, and farnesylated citrans: application to 3'-prenylrubranine and petiolin D regioisomer. *Tetrahedron* **2011,** *67* (47), 9179-9184.

18. Gissot, A.; Wagner, A.; Mioskowski, C., Buffer-induced, selective mono-C-alkylation of phloroglucinol: application to the synthesis of an advanced intermediate of catechin. *Tetrahedron* **2004,** *60* (32), 6807-6812.

19. Raikar, S. B.; Nuhant, P.; Delpech, B.; Marazano, C., Synthesis of polyprenylated benzoylphloroglucinols by regioselective prenylation of phloroglucinol in an aqueous medium. *Eur. J. Org. Chem.* **2008,** *2008* (8), 1358-1369.

Chapter 6: Identification of Anti-inflammatory Compounds from *Oncostemum bojerianum*

6.1 Introduction

6.1.1 Abstract

An ethanol extract of *Oncostemum bojerianum* A. DC. (Primulaceae) was investigated on the basis of its anti-inflammatory activity. Bioassay-guided fractionation of the extract utilizing liquid–liquid partitioning, column chromatography, and HPLC led to the identification of five known 5-alkyl-bis-resorcinol derivatives and the five previously unreported 5-alkyl-bis-resorcinol derivatives, (6′*Z*)-1,3-dihydroxy-5-[16′-(3″,5″-dihydroxyphenyl)hexadec-6′-enyl]benzene (**6.4a**), (6′*Z*)-1-acetoxy-3-hydroxy-5-[14′-(3″,5″-dihydroxyphenyl)tetradec-6′-enyl]benzene (**6.5a**), and (8′*Z*)-1-acetoxy-3-hydroxy-5-[14′-(3″,5″-dihydroxyphenyl)tetradec-8′-enyl]benzene (**6.5b**), (6′*Z*)-1-acetoxy-3-hydroxy-5-[14′-(3″,5″-dihydroxyphenyl)hexadec-6′-enyl]benzene (**6.6a**), and (10′*Z*)-1-acetoxy-3-hydroxy-5-[14′-(3″,5″-dihydroxyphenyl)hexadec-10′-enyl]benzene (**6.6c**). There structures were determined utilizing spectroscopic and chemical methods. This work has not been published elsewhere.

Figure 6-1. *Oncostemum bojerianum.* Used under Creative Commons (CC BY-NC-ND 3.0) from <http://www.tropicos.org/Image/100225463>.

6.1.2 Author Contributions

The author (Alexander L. Eaton) of this dissertation completed the fractionation of the extract, the identification of the described compounds, and the drafting of this manuscript. Stéphan Rakotonandrasana led the team that collected and identified the plant. Xiaoying Zhang and Dr. Josep Bassaganya-Riera provided testing for anti-inflammatory activity. Dr. David G. I. Kingston was a mentor for this work.

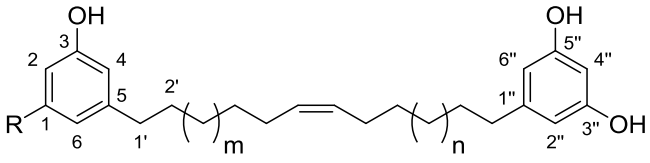
6.1.3 Previous Investigations of Oncostemum bojerianum

Oncostemum bojerianum A. DC., a member of the Primulaceae Batsch ex Borkh. Family is endemic to Madagascar. The genus, *Oncostemum,* contains approximately 100 species.[1] *O. bojerianum* was previously investigated at Virginia Tech and found to contain cytotoxic compounds.[2] Other members of the genus have not undergone phytochemical investigation.

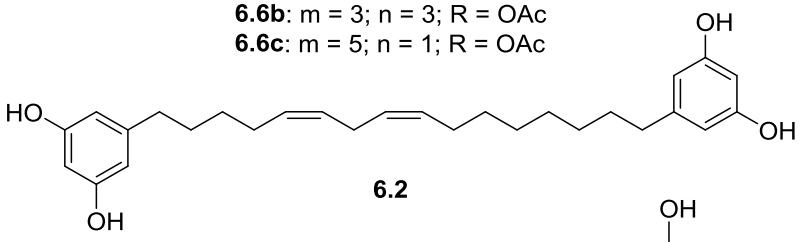
6.1.4 Chemical Investigation of Oncostemum bojerianum

The ethanol extract of the leaves of *Oncostemum bojerianum* A. DC. (Primulaceae) was selected for investigation due to its anti-inflammatory activity. The five known 5-alkyl-bis-resorcinol derivatives, (6′*Z*)-1,3-dihydroxy-5-[14′-(3″,5″-dihydroxyphenyl)tetradec-6′-enyl]benzene (**6.1**),[2-5] (5′*Z*, 8′*Z*)-1,3-dihydroxy-5-[16′-(3″,5″-dihydroxyphenyl)hexadeca-5′,8′-dienyl]benzene (**6.2**),[6] 1,3-dihydroxy-5-[14′-(3″,5″-dihydroxyphenyl)tetradecanyl]benzene (**6.3**),[5,7,8] (8′*Z*,)-1,3-dihydroxy-5-[16′-(3″,5″-dihydroxyphenyl)hexadec-8′-enyl]benzene (**6.4b**),[2,4] and (8′*Z*,)-1-acetoxy-3-hydroxy-5-[14′-(3″,5″-dihydroxyphenyl)hexadec-8′-enyl]benzene (**6.6b**)[2] and the five previously unreported 5-alkyl-bis-resorcinol derivatives, (6′*Z*)-1,3-dihydroxy-5-[16′-(3″,5″-dihydroxyphenyl)hexadec-6′-enyl]benzene (**6.4a**), (6′*Z*)-1-acetoxy-3-hydroxy-5-[14′-

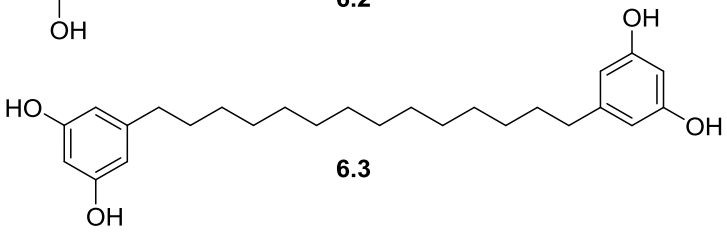
(3″,5″-dihydroxyphenyl)tetradec-6′-enyl]benzene (**6.5a**), and (8′*Z*)-1-acetoxy-3-hydroxy-5-[14′-(3″,5″-dihydroxyphenyl)tetradec-8′-enyl]benzene (**6.5b**), (6′*Z*)-1-acetoxy-3-hydroxy-5-[14′-(3″,5″-dihydroxyphenyl)hexadec-6′-enyl]benzene (**6.6a**), and (10′*Z*)-1-acetoxy-3-hydroxy-5-[14′-(3″,5″-dihydroxyphenyl)hexadec-10′-enyl]benzene (**6.6c**) were purified using liquid–liquid partitioning, Sephadex LH-20 size exclusion chromatography, and C_{18} HPLC. Their structures were identified by ^{1}H NMR, ^{13}C NMR, MS, and chemical derivation.



6.1: m = 1; n = 3; R = OH
6.4a: m = 1; n = 5; R = OH
6.4b: m = 3; n= 3; R = OH
6.5a: m = 1; n = 3; R= OAc
6.5b: m = 3; n = 1; R = OAc
6.6a: m = 1; n = 5; R = OAc
6.6b: m = 3; n = 3; R = OAc
6.6c: m = 5; n = 1; R = OAc



6.2



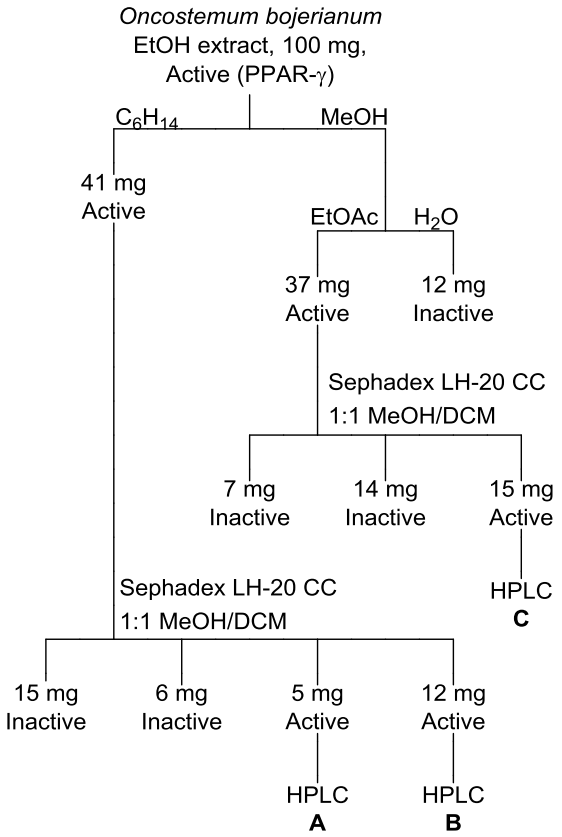
6.3

Figure 6-2. Compounds identified from *Oncostemum bojerianum.*

6.2 Results and Discussion

6.2.1 Fractionation of Oncostemum bojerianum

Bioassay guided fractionation was first performed on 100 mg of *Oncostemum bojerianum* extract (Scheme 6-1). Liquid–liquid partitioning yielded an active hexane fraction and an active ethyl acetate fraction. Sephadex open column chromatography of the hexane fraction yielded two active fractions which were analyzed further by C_{18} HPLC (**A** and **B**). Sephadex open column chromatography of the ethyl acetate fraction yielded one active fraction, which was analyzed further by C_{18} HPLC (**C**).



Scheme 6-1. Small scale bioassay-guided fractionation of *Oncostemum bojerianum*.

As seen by HPLC (Figure 6-3), these three fractions contained similar components. Small amounts were isolated and analyzed by ^{1}H NMR spectroscopy. The compounds present appear to be similar to cytotoxic bis 5-alkylresorcinol derivatives previously isolated from this extract.[2]

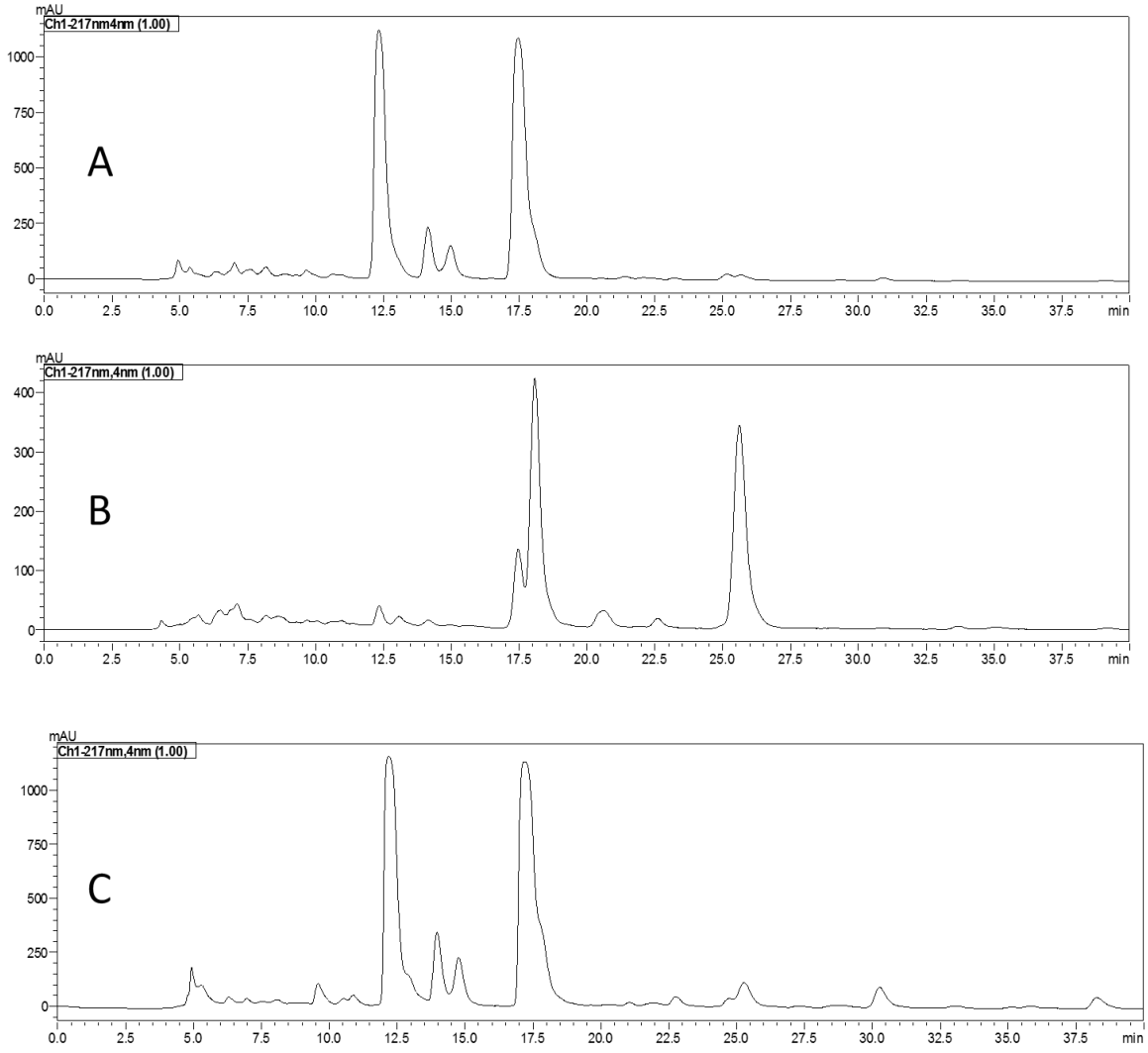
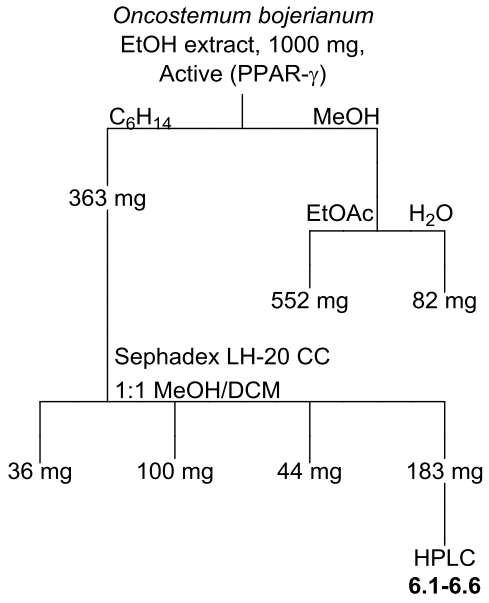


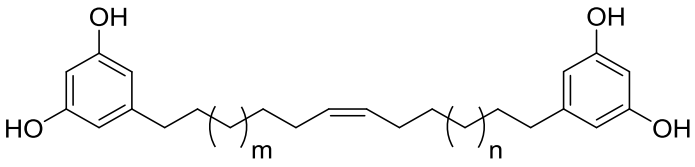
Figure 6-3. HPLC comparison of fractions A, B, and C. PDA Detector: 217 nm.

Due to the small amount of material obtained and the larger amount needed for bioassay, an additional 1 g of extract was worked up. In order to preserve material, the second extraction (Scheme 6-2) was guided by the first and by TLC instead of by the bioassay. The second extract underwent liquid–liquid partitioning in the same manner as the first. The hexane fraction was subjected to Sephadex LH-20 open column chromatography, yielding a fraction which appeared to have the six major components identified in the first fractionation. This fraction was subjected to C_{18} HPLC. The major components were collected and their structures and bioactivities were determined. Full details of these extractions can be found in the Experimental Section of this chapter (6.3).



Scheme 6-2. Fractionation of *Oncostemum bojerianum* to obtain compounds **6.1–6.6.**

*6.2.2 Identification of (6'Z)-1,3-dihydroxy-5-[14'-(3'',5''-dihydroxyphenyl)tetradec-6'-enyl]benzene (**6.1**)*



6.1: m = 1; n = 3

Figure 6-4. Structure of (6′Z)-1,3-dihydroxy-5-[14′-(3′′,5′′-dihydroxyphenyl)tetradec-6′-enyl]benzene.

Oncostemonols A-H have previously been isolated from this extract.[2] Comparison of ^{1}H NMR spectra (Table 6-1) indicated that **6.1** is also a 5-alkylresorcinol derivative. HRESIMS suggested a molecular formula of $C_{26}H_{37}O_4$ ($[M+H]^+$ *m/z* 413.2705, calcd for $C_{26}H_{37}O_4^+$ 413.2686). The method of Mansour[9] and Roumy[10] using MS analysis of the products (**6.8**)

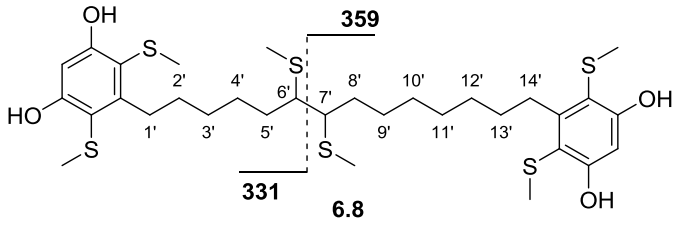


Figure 6-5. MS fragmentation of compound **6.8**.

resulting from derivatization with dimethyl disulfide was used to determine the location of the double bond in the alkenyl chain. The LC-MS of the dimethyl disulfide derivative (Figure 6-5) of **6.1** contained fragment ions at *m/z* 331.17 (calcd for $[C_{15}H_{23}O_2S_3]^{\bullet}$ 331.09) and *m/z* 359.17 (calcd for $[C_{17}H_{27}O_2S_3]^{\bullet}$ 359.12) indicating a $\Delta^{8',9'}$ double bond. Thus, the structure was determined to be **6.1**. Its NMR and MS data are in agreement with previous published data.[5] Complete ^{1}H NMR data can be found in Table 6-1.

Table 6-1. ^{1}H NMR data of compounds **6.1–6.4**.

	6.1	**6.2**	**6.3**	**6.4a**	**6.4b**
position	δ_H (J *in Hz)*	δ_H (J *in Hz)*	δ_H (J *in Hz)*	δ_H (J *in Hz)*	δ_H (J *in Hz)*
2	6.08, d (2.2)[a]	6.10, m[b]	6.07, t (2.2)[b]	6.08, d (1.8)[a]	6.08, d (1.8)[a]
4	6.12, d (2.2)[b]	6.14, t (2.4)[c]	6.12, d (2.2)[c]	6.12, bd (1.8)[b]	6.12, bd (1.8)[b]
6	6.12, d (2.2)[b]	6.14, t (2.4)[c]	6.12, d (2.2)[c]	6.12, bd (1.8)[b]	6.12, bd (1.8)[b]
1′	2.43, t (7.7)[c]	2.46, bt (7.7)[d]	2.43, bt (7.7)[d]	2.43, bt (7.6)[c]	2.43, bt (7.6)[c]
2′	1.57, m[d]	1.58, m[e]	1.55, m[e]	1.56, m[d]	1.56, m[d]
3′	1.32, m[e]	1.32, m[f]	1.30, m[f]	1.32, m[e]	1.32, m[e]
4′	1.32, m[e]	2.07, m[g]	1.30, m[f]	1.32, m[e]	1.32, m[e]
5′	2.02, m[f]	5.35, m[h]	1.30, m[f]	2.02, m[g]	1.32, m[e]
6′	5.34, bt (4.6)[g]	5.35, m[h]	1.30, m[f]	5.34, t (4.8)[h]	1.32, m[e]
7′	5.34, bt (4.6)[g]	2.79, t (6.6)	1.30, m[f]	5.34, t (4.8)[h]	2.02, m[g]
8′	2.02, m[f]	5.35, m[h]	1.30, m[f]	2.02, m[g]	5.34, t (4.8)[h]
9′	1.32, m[e]	5.35, m[h]	1.30, m[f]	1.32, m[e]	5.34, t (4.8)[h]
10′	1.32, m[e]	2.07, m[g]	1.30, m[f]	1.32, m[e]	2.02, m[g]
11′	1.32, m[e]	1.32, m[f]	1.30, m[f]	1.32, m[e]	1.32, m[e]
12′	1.32, m[e]	1.32, m[f]	1.30, m[f]	1.32, m[e]	1.32, m[e]
13′	1.57, m[d]	1.32, m[f]	1.55, m[e]	1.32, m[e]	1.32, m[e]
14′	2.43, t (7.7)[c]	1.32, m[f]	2.43, bt (7.7)[d]	1.32, m[e]	1.32, m[e]
15′		1.58, m[e]		1.56, m[d]	1.56, m[d]
16′		2.46, bt (7.7)[d]		2.43, bt (7.6)[c]	2.43, bt (7.6)[c]
2″	6.12, d (2.2)[b]	6.14, t (2.4)[c]	6.12, d (2.2)[c]	6.12, bd (1.8)[b]	6.12, bd (1.8)[b]
4″	6.07, d (2.2)[a]	6.10, m[b]	6.07, t (2.2)[b]	6.07, d (1.8)[a]	6.07, d (1.8)[a]
6″	6.12, d (2.2)[b]	6.14, t (2.4)[c]	6.12, d (2.2)[c]	6.12, bd (1.8)[b]	6.12, bd (1.8)[b]

Obtained in CD_3OD, 500 MHz (δ_H)
[a]Interchangeable Assignments within a Column
[b,c,d,e,f,g,h]Overlapping Signals

6.2.3 *Identification of (5′Z,8′Z)-1,3-dihydroxy-5-[16′-(3″,5″-dihydroxyphenyl)hexadeca-5′,8′-dienyl]benzene (**6.2**)*

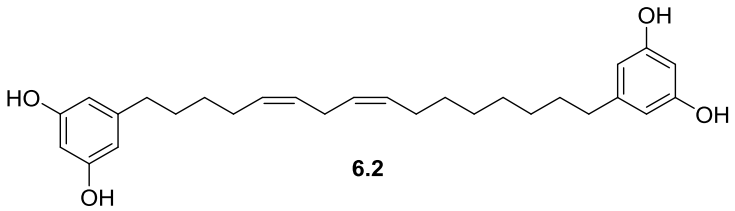


Figure 6-6. Structure of (5′Z,8′Z)-1,3-dihydroxy-5-[16′-(3″,5″-dihydroxyphenyl)hexadeca-5′,8′-dienyl]benzene.

Analysis of the ^{1}H NMR spectrum (Table 6-1) indicated that the structure of **6.2** was very similar to that of **6.1** and is also likely a bis-5-alkylresorcinol derivative. However, inspection of the ^{1}H NMR spectrum indicated that integration of the signal at δ_H 5.35 (4H, m) was consistent with the presence of two double bonds in the alkenyl chain. This was also consistent with the molecular formula, $C_{28}H_{38}O_4$, obtained from MS analysis ($[M+H]^+$ *m/z* 439.2853, calcd for $C_{28}H_{39}O_4^+$ 439.2843) and the calculated hydrogen deficiency index of ten. The double bonds were determined to be conjugated based on the ^{1}H NMR spectrum, which showed a signal at δ_H 2.79 (H-7′, 2H, t, *J* = 6.6 Hz). Fragment ions were observed in the MS at *m/z* 191.06 (calcd for $[C_{12}H_{15}O_2]^{\bullet}$ 191.10), *m/z* 245.16 (calcd for $[C_{16}H_{21}O_2]^{\bullet}$ 245.15) and *m/z* 247.10 (calcd for $[C_{16}H_{23}O_2]^{\bullet}$ 247.17 indicating $\Delta^{5',6'}$ and $\Delta^{8',9'}$ double bonds (Figure 6-7). ^{1}H NMR and MS data are consistent with published values.[6]

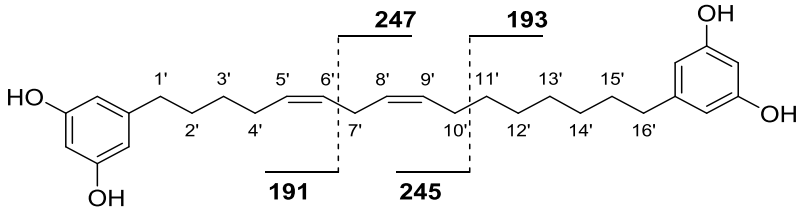


Figure 6-7. MS fragmentation of compound **6.2.**

*6.2.4 Identification of 1,3-dihydroxy-5-[14′-(3″,5″-dihydroxyphenyl)tetradecanyl]benzene (**6.3**)*

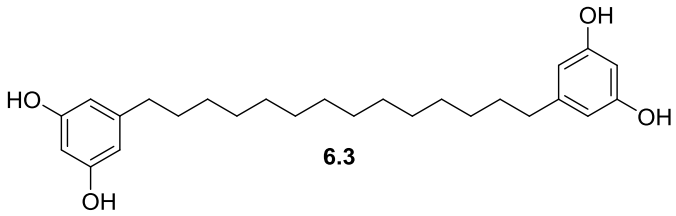
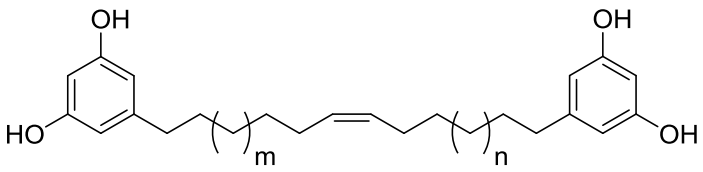


Figure 6-8. Structure of compound **6.3**.

Comparison of the ^{1}H NMR data (Table 6-1) indicated that the structure of **6.3** was similar to that of **6.1** and **6.2**. However, the ^{1}H NMR spectrum of **6.2** did not have a signal at δ_H ~5.3, indicating that an alkene moiety was no longer present. This was consistent with the molecular formula of $C_{26}H_{38}O_4$ ($[M+H]^+$ *m/z* 415.2848, calcd for $C_{26}H_{39}O_4^+$ 415.2843) and the calculated hydrogen deficiency index of eight. Thus, the structure of **6.3** was determined to be 1,3-dihydroxy-5-[14′-(3″,5″-dihydroxyphenyl)tetradecyl]benzene (**6.3**). ^{1}H NMR and MS data are consistent with published values.[7]

*6.2.5 Identification of (6'Z)-1,3-dihydroxy-5-[16'-(3'',5''-dihydroxyphenyl)hexadec-6'-enyl]benzene (**6.4a**) and (8'Z)-1,3-dihydroxy-5-[16'-(3'',5''-dihydroxyphenyl)hexadec-8'-enyl]benzene (**6.4b**)*



6.4a: m = 1; n = 5
6.4b: m = 3; n= 3

Figure 6-9. Structure of compounds **6.4a** and **6.4b**.

Upon initial inspection of the ^{1}H NMR spectrum (Table 6-1) of the substance identified as **6.4**, it appeared that its structure was very similar to that of **6.1.** The molecular formula was determined to be $C_{28}H_{40}O_4$ by HRESIMS ($[M+H]^+$ *m/z* 441.3041, calcd for $C_{28}H_{41}O_4^+$ 441.2999). This is consistent with the presence of a C_{16} alkenyl chain. However, inspection of the ^{13}C NMR spectrum indicated the presence of at least three sp^2 carbon atoms (δ_C 130.7, 130.8, 130.9) in the alkenyl chain. These data are not consistent with the calculated hydrogen deficiency index of nine.

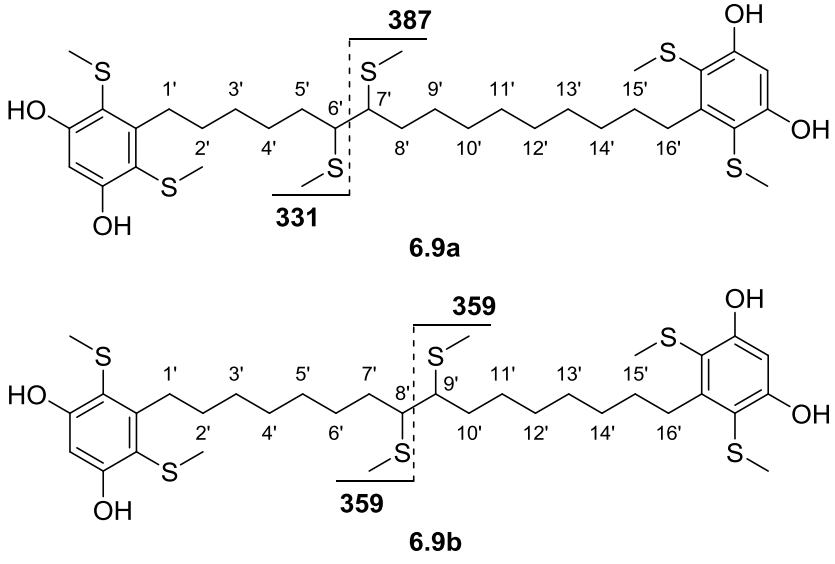
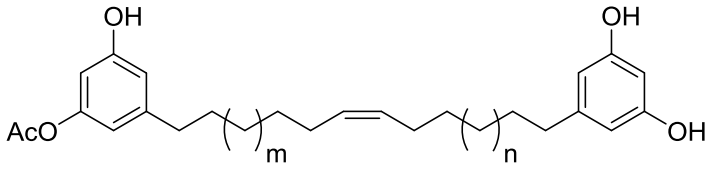


Figure 6-10. MS fragmentation of compounds **6.9a–b**.

Thus, it was proposed that **6.4** was not a single compound but rather two inseparable isomers, varying by the position of the double bond in the alkenyl chain. The same method[9,10] that was employed to determine the double bond location in **6.1** was used to determine the location of the double bonds in the alkenyl chain in **6.4**. The LC-MS of the dimethyl disulfide derivatives (**6.9a–b**) of **6.4** contained fragment ions at *m/z* 331.07 (calcd for $[C_{15}H_{23}O_2S_3]^{\bullet}$ 331.09), *m/z* 359.15 (calcd for $[C_{17}H_{27}O_2S_3]^{\bullet}$ 359.12), and *m/z* 387.18 (calcd for $C_{19}H_{31}O_2S_3]^{\bullet}$ 387.15) indicating $\Delta^{6',7'}$ and $\Delta^{8',9'}$ double bonds in the alkenyl chain (Figure 6-10). Thus, the structure of **6.4** was determined to be a mixture of **6.4a** and **6.4b.** Compound **6.4a** has not been previously reported, but compound **6.4b** has been reported, and its ^{1}H and ^{13}C NMR and MS data are in agreement with published values.[7]

6.2.6 *Identification of (6′Z)-1-acetoxy-3-hydroxy-5-[14′-(3″,5″-dihydroxyphenyl)tetradec-6′-enyl]benzene (**6.5a**) and (8′Z)-1-acetoxy-3-hydroxy-5-[14′-(3″,5″-dihydroxyphenyl)tetradec-8′-enyl]benzene (**6.5b**)*



6.5a: m = 1; n = 3
6.5b: m = 3; n = 1

Figure 6-11. Structures of compounds **6.5a** and **6.5b**.

Inspection of its ^{1}H NMR spectrum indicated that the structure of **6.5** was related to the structures of **6.1**–**6.4**. There was an additional signal at δ_H 2.25 (3H) suggesting the presence of an acetyl group. This was consistent with the molecular formula, $C_{28}H_{38}O_5$, obtained from HRESIMS ($[M+H]^+$ *m/z* 455.2810, calcd for $C_{28}H_{39}O_5^+$ 455.2792) and the calculated hydrogen deficiency index of ten. It was determined that the acetyl group was attached to one of the resorcinol rings by the presence of additional signals in the ^{1}H NMR spectrum at δ_H 6.09, 6.35, 6.40, and 6.52 which were not present in the ^{1}H NMR spectrum of **6.1**–**6.4**. The only other difference in the ^{1}H NMR spectrum was the additional signal at δ_H 2.54, which was assigned as H-1. This is consistent with

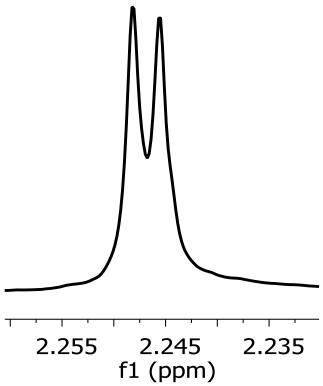


Figure 6-12. Signal in ^{1}H NMR spectrum of compound **6.5** at δ_H 2.25.

presence of an acetyl group since the benzene ring moieties will no longer exhibit identical signals in the aromatic region of the ^{1}H NMR spectrum.

Table 6-2. ^{1}H NMR spectra of **6.5a–6.6c**.

	6.5a	**6.5b**	**6.6a**	**6.6b**	**6.6c**
position	δ_H (J *in Hz)*	δ_H (J *in Hz)*	δ_H (J *in Hz)*	δ_H (J *in Hz)*	δ_H (J *in Hz)*
2	6.35, m	6.35, m	6.35, q (2.1)	6.35, q (2.1)	6.35, q (2.1)
4	6.40, m[a]	6.40, m[a]	6.40, m[a]	6.40, m[a]	6.40, m[a]
6	6.52, m[a]	6.52, m[a]	6.52, bs[a]	6.52, bs[a]	6.52, bs[a]
1′	2.54, t (7.5)	2.54, t (7.5)	2.54, m	2.54, m	2.54, m
2′	1.59, m[b]	1.59, m[b]	1.59, m[b]	1.59, m[b]	1.59, m[b]
3′	1.36, m[c]	1.36, m[c]	1.32, m[c]	1.32, m[c]	1.32, m[c]
4′	1.36, m[c]	1.36, m[c]	1.32, m[c]	1.32, m[c]	1.32, m[c]
5′	2.04, m[d]	1.36, m[c]	2.05, m[d]	1.32, m[c]	1.32, m[c]
6′	5.36, t (4.6)[e]	1.36, m[c]	5.36, bt (5.1)[e]	1.32, m[c]	1.32, m[c]
7′	5.36, t (4.6)[e]	2.04, m[d]	5.36, bt (5.1)[e]	2.05, m[d]	1.32, m[c]
8′	2.04, m[d]	5.36, t (4.6)[e]	2.05, m[d]	5.36, bt (5.1)[e]	1.32, m[c]
9′	1.36, m[c]	5.36, t (4.6)[e]	1.32, m[c]	5.36, bt (5.1)[e]	2.05, m[d]
10′	1.36, m[c]	2.04, m[d]	1.32, m[c]	2.05, m[d]	5.36, bt (5.1)[e]
11′	1.36, m[c]	1.36, m[c]	1.32, m[c]	1.32, m[c]	5.36, bt (5.1)[e]
12′	1.36, m[c]	1.36, m[c]	1.32, m[c]	1.32, m[c]	2.05, m[d]
13′	1.59, m[b]	1.59, m[b]	1.32, m[c]	1.32, m[c]	1.32, m[c]
14′	2.45, t (7.7)	2.45, t (7.7)	1.32, m[c]	1.32, m[c]	1.32, m[c]
15′			1.59, m[b]	1.59, m[b]	1.59, m[b]
16′			2.45, m	2.45, m	2.45, m
2″	6.14, d (2.2)[f]	6.14, d (2.2)[f]	6.14, d (2.1)[f]	6.14, d (2.1)[f]	6.14, d (2.1)[f]
4″	6.09, m	6.09, m	6.09, q (2.0)	6.09, q (2.0)	6.09, q (2.0)
6″	6.14, d (2.2)[f]	6.14, d (2.2)[f]	6.14, d (2.1)[f]	6.14, d (2.1)[f]	6.14, d (2.1)[f]
-OAc	2.25	2.25	2.25	2.25	2.25

Obtained in CD_3OD, 500 MHz (δ_H)
[a]Interchangeable assignments within a column
[b,c,d,e,f]Overlapping signals

Inspection of the expanded signal at 2.25 ppm for the methyl protons of the acetyl group revealed that it consisted of two signals of approximately equal magnitude separated by 1 Hz (Figure 6-12). LC-MS of the dimethyl disulfide derivatives of **6.5** (**6.10a–b**) showed fragment ions at *m/z* 327.05 (calcd for $[C_{16}H_{23}O_3S_2]^{\bullet}$ 327.11), *m/z* 331.01 (calcd for $[C_{15}H_{23}O_2S_3]^{\bullet}$ 331.09), *m/z* 355.24 (calcd for $[C_{18}H_{27}O_3S_2]^{\bullet}$ 355.14), and *m/z* 359.13 (calcd for $[C_{17}H_{27}O_2S_3]^{\bullet}$ 359.12) indicating the presence of $\Delta^{6',7'}$ and $\Delta^{8',9'}$ double bonds in the alkenyl chain (Figure 6-13).

Compounds **6.5a** and **6.5b** are thus monoacetylated derivatives of **6.1**. Due to compound **6.1**'s unsymmetrical nature, monoacetylation of one of the four hydroxy groups leads to two possible compounds, **6.5a** and **6.5b**, which are both observed. This is the first report of compounds **6.5a** and **6.5b**.

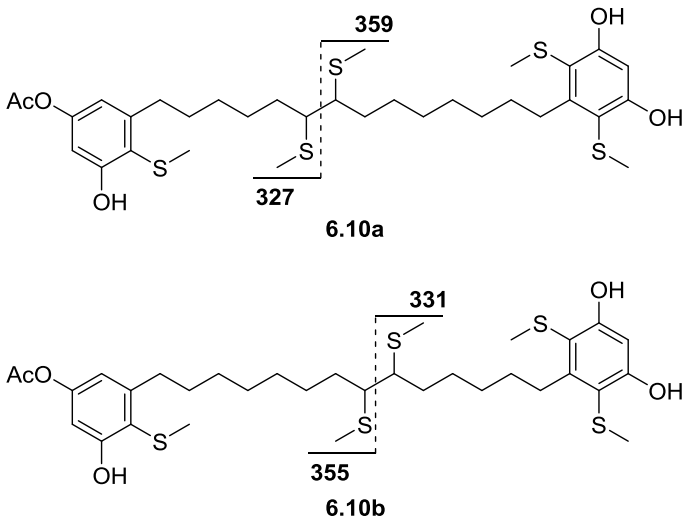
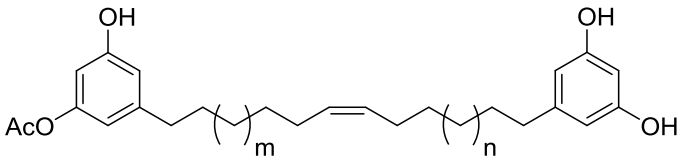


Figure 6-13. MS fragmentation of **6.10a–b**.

6.2.7 *Identification of (6′Z)-1-acetoxy-3-hydroxy-5-[14′-(3″,5″-dihydroxyphenyl)hexadec-6′-enyl]benzene (**6.6a**), (8′Z)-1-acetoxy-3-hydroxy-5-[14′-(3″,5″-dihydroxyphenyl)hexadec-8′-enyl]benzene (**6.6b**), and (10′Z)-1-acetoxy-3-hydroxy-5-[14′-(3″,5″-dihydroxyphenyl)hexadec-10′-enyl]benzene (**6.6c**)*



6.6a: m = 1; n = 5
6.6b: m = 3; n = 3
6.6c: m = 5; n = 1

Figure 6-14. Structures of compounds **6.6a**, **6.6b**, and **6.6c**.

Inspection of the ^{1}H NMR spectrum of compound 6.6 indicated that its structure was closely related to that of **6.5**. Similarly to compound **6.5**, a signal for an acetyl group at δ_H 2.25 consisted of three signals of similar intensity (Figure 6-15). From HRESIMS, the molecular formula was determined to be $C_{30}H_{42}O_5$ ($[M+H]^+$ m/z 483.3119, calcd for $C_{30}H_{43}O_5^+$ 483.3105). Due to the signals in the ^{1}H NMR spectrum at δ_H 2.25 and the previously identified isomeric mixtures, it was deduced that 6.6 was also a mix of inseparable isomers. LC-MS of the dimethyl disulfide derivative (**6.11a–c**) showed fragment ions at m/z 312.12 (calcd for $[C_{16}H_{24}O_2S_2]^{\bullet}$

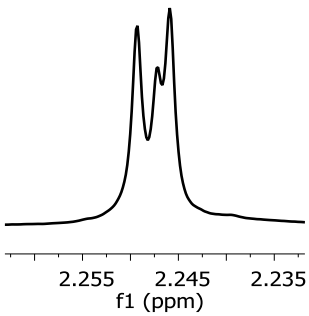


Figure 6-15. Signal in ^{1}H NMR spectrum of compound **6.6** at δ_H 2.25.

312.12), m/z 331.08 (calcd for $[C_{15}H_{23}O_2S_3]^{\bullet}$ 331.09), m/z 359.14 (calcd for $[C_{17}H_{27}O_2S_3]^{\bullet}$ 359.12), and m/z 387.11 (calcd for $[C_{19}H_{31}O_2S_3]^{\bullet}$ 387.15) indicating the presence of Δ6′,7′, Δ8′,9′,

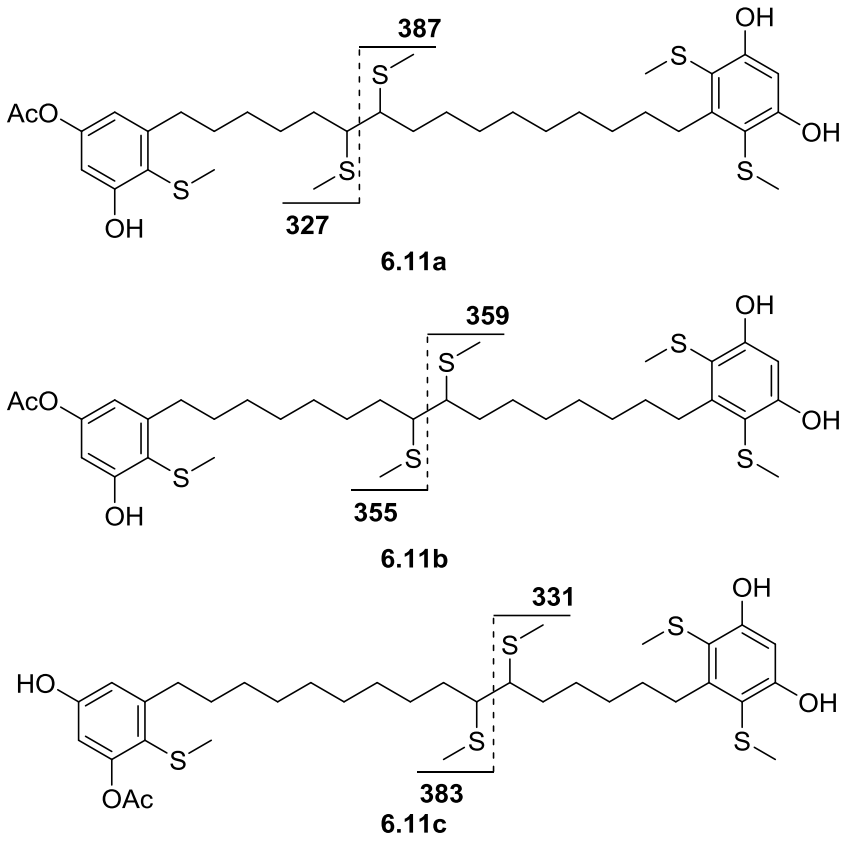


Figure 6-16. MS fragmentation of compounds **6.11a–c**.

and Δ10′,11′ double bonds in the alkenyl chain (Figure 6-14). Thus, the structures of **6.6a**, **6.6b**, and **6.6c** were proposed. Compounds **6.6a** and **6.6c** have not been previously reported. The related symmetrical isomer, **6.6b**, has previously been isolated from *Oncostemum bojerianum*.[2] Similar to what was observed in the case of **6.5a** and **6.5b,** compounds **6.6a–c** are monoacetylated derivatives of 6.4a and 6.4b. ^{1}H and ^{13}C NMR data are comparable to the published values of **6.6b**.[2]

6.2.8 Anti-inflammatory Activity

Table 6-3. PPAR-γ agonist activity.

Compound	EC_{50} μM
6.1	9
6.2	47
6.3	51
6.4a–b	24
6.5a–b	12
6.6a–c	45

Due to the inseparable nature of **6.4**–**6.6**, the bioactivities of the individual compounds could not be confirmed. 5-Alkylresorcinols have been reported to be cytotoxic[2,11] and to have DNA-cleaving properties.[5,7]

6.3 Experimental Section

6.3.1 General Experimental Procedures

^{1}H and ^{13}C NMR spectra were recorded using either a Bruker Advance 500 or 600 spectrometer. ^{1}H-^{1}H three-bond *J*-coupling values were calculated from ^{1}H NMR spectra. Mass spectra were obtained on an Agilent 6220 LC-TOF-MS or a Thermo Electron TSQ LC-ESI-MS. Semi-preparative HPLC was performed using Shimadzu LC-10AT pumps coupled with a Shimadzu SPD M10A diode array detector, a SCL-10A system controller, and a Cogent Bidentate C_{18} column (250 x 10 mm).

6.3.2 Plant Material

Leaves of *Oncostemum bojerianum* A. DC. (Primulaceae) (collection: Stéphan Rakotonandrasana et al.) were collected at an elevation of 1000 m in December 1999, 2 km north of the village of Ankosy near the boundary of the Zahamena National Park, 17°28′45" S 048°44′09" E. The sample was collected from a 4 m tall shrub with red petiole.

6.3.3 Extraction and Isolation

The ethanol extract of the leaves *Oncostemum bojerianum* (100 mg) was dissolved in methanol and extracted with hexanes. The methanol fraction was evaporated to dryness and dissolved in water. The water fraction was extracted with ethyl acetate. Both the hexanes (41 mg) and ethyl acetate (37 mg) fractions showed bioactivity. The hexanes fraction was separated further by Sephadex LH-20 open CC (1:1 MeOH/DCM) yielding two active fractions (5 mg, 12 mg) which were analyzed by C_{18} HPLC (**A** and **B**, respectively). Likewise, the ethyl acetate fraction was separated by Sephadex LH-20 CC (1:1 MeOH/DCM), yielding one active fraction (15 mg) which was analyzed by C_{18} HPLC (**C**). No compounds were identified from this fractionation.

A larger fractionation was undertaken in which 1 g of the ethanol extract of the leaves of *O. bojerianum* was fractionated. TLC was used to guide the second fractionation in an attempt to isolate the compounds detected in the first fractionation. This extract underwent liquid–liquid partitioning in the same manner as the first yielding a hexanes fraction (362 mg). This fraction was further partitioned using Sephadex LH-20 CC (1:1 MeOH/DCM). A fraction (183 mg) was then separated by C_{18} HPLC to yield **6.1–6.6** (8 mg, 3 mg, 3 mg, 12 mg, 4 mg, 6 mg, respectively).

6.3.4 Anti-inflammatory Assay

Anti-inflammatory activity was determined using a cell-based PPAR γ reporter assay as previously described.[12]

6.3.5 Spectroscopic Properties

(6′*Z*)-1,3-dihydroxy-5-[14′-(3′′,5′′-dihydroxyphenyl)tetradec-6′-enyl]benzene (6.1): ^{1}H NMR (500 MHz, CD_3OD): See Table 6-1; ^{13}C NMR (150 MHz, CD_3OD): 28.09, 28.12, 29.9, 30.29, 30.33, 30.5, 30.7, 30.8, 32.3, 32.4, 37.0, 101.0, 107.9, 130.8, 130.9, 146.3, 146.4, 159.3; HRESIMS $[M+H]^+$ *m/z* 413.2705(calcd for $C_{26}H_{37}O_4^+$ 413.2686), $[M+Na]^+$ *m/z* 435.2521 (calcd for $C_{26}H_{36}NaO_4^+$ 435.2506), $[2M+Na]^+$ *m/z* 847.5127 (calcd for $C_{52}H_{72}NaO_8^+$ 847.5119), $[2M+K]^+$ *m/z* 863.4844 (calcd for $C_{52}H_{72}KO_8^+$ 863.4859).

(5′*Z*, 8′*Z*)-1,3-dihydroxy-5-[16′-(3′′,5′′-dihydroxyphenyl)hexadeca-5′,8′-dienyl]benzene (6.2): ^{1}H NMR (500 MHz, CD_3OD): See Table 6-1; ^{13}C NMR (150 MHz, CD_3OD):26.1, 26.5, 29.0, 30.3, 99.6, 106.7, 106.9, 128.2, 129.3, 145.3, 158.3; HRESIMS $[M+H]^+$ *m/z* 439.2853 (calcd for $C_{28}H_{39}O_4^+$ 439.2843), $[2M+K]^+$ *m/z* 915.5250 (calcd for $C_{56}H_{76}KO_4^+$ 915.5172).

1,3-dihydroxy-5-[14′-(3′′,5′′-dihydroxyphenyl)tetradecanyl]benzene (6.3): ^{1}H NMR (500 MHz, CD_3OD): See Table 6-1; HRESIMS $[M+H]^+$ *m/z* 415.2848 (calcd for $C_{26}H_{39}O_4^+$ 415.2843), $[2M+Na]^+$ *m/z* 851.5443 (calcd for $C_{52}H_{76}NaO_8^+$ 851.5432).

(6′*Z*,)-1,3-dihydroxy-5-[16′-(3′′,5′′-dihydroxyphenyl)hexadec-6′-enyl]benzene (6.4a) and (8′*Z*,)-1,3-dihydroxy-5-[16′-(3′′,5′′-dihydroxyphenyl)hexadec-8′-enyl]benzene (6.4b): ^{1}H NMR (500 MHz, CD_3OD):See Table 6-1; ^{13}C NMR (150 MHz, CD_3OD): 28.09, 28.12, 29.9, 30.32, 30.32, 30.34, 30.4, 30.5, 30.6, 30.70, 30.71, 30.83, 30.84, 32.3, 32.4, 36.9, 37.0, 100.9, 101.0, 107.9, 130.7, 130.8, 130.9, 146.28, 146.34, 146.4, 159.25, 159.26; HRESIMS $[M+H]^+$ *m/z* 441.3041 (calcd for $C_{28}H_{41}O_4^+$ 441.2999), $[M+Na]^+$ *m/z* 463.2834 (calcd for $C_{28}H_{40}NaO_4^+$

463.2819), $[2M+Na]^+$ *m/z* 903.5759 (calcd for $C_{56}H_{80}NaO_8^+$ 903.5745), $[2M+K]^+$ *m/z* 919.5465 (calcd for $C_{56}H_{80}KO_8^+$ 919.5485).

(6′*Z*,)-1-acetoxy-3-hydroxy-5-[14′-(3′′,5′′-dihydroxyphenyl)tetradec-6′-enyl]benzene (6.5a) and (8′*Z*,)-1-acetoxy-3-hydroxy-5-[14′-(3′′,5′′-dihydroxyphenyl)tetradec-8′-enyl]benzene (6.5b): ^{1}H NMR (500 MHz, CD_3OD): See Table 6-2; ^{13}C NMR (150MHz, CD_3OD, From HMBC): 19.6, 26.7, 28.9, 29.1, 29.2, 29.4, 30.7, 31.2, 35.5, 35.7, 99.7, 106.1, 106.4, 112.2, 112.5, 129.4, 144.8, 145.0, 151.6, 157.8, 169.8; HRESIMS $[M+H]^+$ *m/z* 455.2810 (calcd for $C_{28}H_{39}O_5^+$ 455.2792), $[M+NH_4]^+$ *m/z* 472.3071 (calcd for $C_{28}H_{42}NO_5^+$ 472.3057), $[M+Na]^+$ *m/z* 477.2628 (calcd for $C_{28}H_{38}NaO_5^+$ 477.2611), $[M+K]^+$ *m/z* 499.2398 (calcd for $C_{28}H_{38}KO_5^+$ 493.2351), $[2M+Na]^+$ *m/z* 931.5346 (calcd for $C_{56}H_{76}NaO_{10}^+$ 931.5331), $[2M+K]^+$ *m/z* 947.5011 (calcd for $C_{56}H_{76}KO_{10}^+$ 947.5070).

(6′*Z*,)-1-acetoxy-3-hydroxy-5-[14′-(3′′,5′′-dihydroxyphenyl)hexadec-6′-enyl]benzene (6.6a), (8′*Z*,)-1-acetoxy-3-hydroxy-5-[14′-(3′′,5′′-dihydroxyphenyl)hexadec-8′-enyl]benzene (6.6b), and (10′*Z*,)-1-acetoxy-3-hydroxy-5-[14′-(3′′,5′′-dihydroxyphenyl)hexadec-10′-enyl]benzene (6.6c): ^{1}H NMR (500 MHz, CD_3OD): See Table 6-2; ^{13}C NMR (151 MHz, CD_3OD): 21.0, 28.05, 28.06, 28.09, 29.8, 30.0, 30.24, 30.25, 30.26, 30.27, 30.28, 30.30, 30.31, 30.33, 30.4 30.47, 30.54, 30.56, 30.60, 30.61, 30.63, 30.64, 30.65, 30.66, 30.68, 30.70, 30.71, 30.79, 30.80, 30.81, 30.82, 32.28, 32.29, 32.30, 32.4, 36.73, 36.74, 36.98, 36.99, 100.96, 100.98, 107.28, 107.31, 107.9, 113.7, 113.9, 130.7, 130.8, 130.85, 130.92, 131.0, 146.3, 146.35, 146.44, 146.5, 153.0, 159.28, 159.29, 170.6; HRESIMS $[M+H]^+$ *m/z* 483.3119 (calcd for $C_{30}H_{43}O_5^+$ 483.3119), $[M+NH_4]^+$ *m/z* 500.3383 (calcd for $C_{30}H_{46}NO_5^+$ 500.3371), $[M+Na]^+$ *m/z* 505.2937 (calcd for $C_{30}H_{42}NaO_5^+$ 505.2924),

$[M+K]^+$ *m/z* 521.2673 (calcd for $C_{30}H_{42}KO_5^+$ 521.2664), $[2M+Na]^+$ *m/z* 987.5960 (calcd for $C_{60}H_{84}NaO_{10}^+$ 987.5957), $[2M+K]^+$ *m/z* 1003.5600 (calcd for $C_{60}H_{84}KO_{10}^+$ 1003.5696).

6.4 References

1. Mabberley, D. J., *The Plant-Book: A Portable Dictionary of the Vascular Plants*. 2nd ed.; University Press: Cambridge, 1997.

2. Chaturvedula, V. S.; Schilling, J. K.; Miller, J. S.; Andriantsiferana, R.; Rasamison, V. E.; Kingston, D. G. I., New cytotoxic bis 5-alkylresorcinol derivatives from the leaves of *Oncostemon bojerianum* from the Madagascar rainforest. *J. Nat. Prod.* **2002,** *65* (11), 1627-1632.

3. Barr, J. R.; Scannell, R. T.; Yamaguchi, K., Structure elucidation of naturally occurring long-chain mono- and dienes. *J. Org. Chem.* **1989,** *54* (2), 494-496.

4. Cirigottis, K.; Cleaver, L. E. A., Chemical studies of the Proteaceae. Vii. An examination of the woods of 17 species for resorcinol derivatives. *Aust. J. Chem.* **1974,** *27* (2), 345.

5. Lytollis, W.; Scannell, R. T.; An, H. Y.; Murty, V. S.; Reddy, K. S.; Barr, J. R.; Hecht, S. M., 5-alkylresorcinols from *Hakea trifurcata* that cleave DNA. *J. Am. Chem. Soc.* **1995,** *117* (51), 12683-12690.

6. Suzuki, Y.; Esumi, Y.; Yamaguchi, I., Structures of 5-alkylresorcinol-related analogues in rye. *Phytochemistry* **1999,** *52* (2), 281-289.

7. Furstner, A.; Seidel, G., Shortcut syntheses of naturally occurring 5-alkylresorcinols with DNA-cleaving properties. *J. Org. Chem.* **1997,** *62* (8), 2332-2336.

8. Varma, R. S.; Manju, M.; Parthasarathy, M. R., New phenolic constituents of *Grevillea robusta* wood. *Phytochemistry* **1976,** *15* (9), 1418-1419.

9. Mansour, M. P.; Holdsworth, D. G.; Forbes, S. E.; Macleod, C. K.; Volkman, J. K., High contents of 24:6(n-3) and 20:1(n-13) fatty acids in the brittle star *Amphiura elandiformis* from Tasmanian coastal sediments. *Biochem. Syst. Ecol.* **2005,** *33* (7), 659-674.

10. Roumy, V.; Fabre, N.; Portet, B.; Bourdy, G.; Acebey, L.; Vigor, C.; Valentin, A.; Moulis, C., Four anti-protozoal and anti-bacterial compounds from *Tapirira guianensis*. *Phytochemistry* **2009,** *70* (2), 305-311.

11. Chuang, T. H.; Wu, P. L., Cytotoxic 5-alkylresorcinol metabolites from the leaves of *Grevillea robusta*. *J. Nat. Prod.* **2007,** *70* (2), 319-323.

12. Bassaganya-Riera, J.; Guri, A. J.; Lu, P.; Climent, M.; Carbo, A.; Sobral, B. W.; Horne, W. T.; Lewis, S. N.; Bevan, D. R.; Hontecillas, R., Abscisic acid regulates inflammation via ligand-binding domain-independent activation of peroxisome proliferator-activated receptor gamma. *J. Biol. Chem.* **2011,** *286* (4), 2504-2516.

Chapter 7: Isolation of Antiproliferative Compounds from *Schismatoclada farahimpensis* Homolle

7.1 Introduction

7.1.1 Abstract

As a part of our continued search for antiproliferative compounds from plants endemic to Madagascar, an ethanol extract of *Schimatoclada farhimpensis* Homolle (Rubiaceae) (Figure 7-1) was investigated due to its antiproliferative activity (IC_{50} 13 µg/mL). Four known compounds, oleanolic acid 28-*O*-(*β*-D-glucopyranosyl) ester (**7.1**),[1] 3-*O*-(*β*-D-glucuronopyranosyl) oleanoic acid (**7.2**),[2] 3-*O*-(*β*-D-glucuronopyranosyl) oleanoic acid 28-*O*-(*β*-D-glucopyranosyl) ester (**7.3**),[3] and 3-*O*-(*β*-D-glucopyranosyl-(1→2)-*β*-D-glucuronopyranosyl) oleanoic acid (**7.4**),[4] were isolated using liquid–liquid partitioning, column chromatography, and HPLC. This is the first report of compounds isolated from the genus *Schismatoclada*. This work has not been published elsewhere.

Figure 7-1. Flower of *Schismatoclada farahimpensis*. Used under Creative Commons (CC BY-NC-ND 3.0) from <http://www.tropicos.org/Image/100225175>.

7.1.2 Author Contributions

Janssen Claudio completed the isolation of **7.1** and carried out all other fractionation steps except the final HPLC separation leading to the isolation of **7.2–7.4**. The author of this dissertation completed the isolation of **7.2**–**7.4**, the structure elucidation of compounds **7.1–7.4**, and the drafting of this manuscript. Plant collection in Madagascar and initial identification as *Schismatoclada sp.* was carried out by N. M. Andrianjafy under the oversight of Dr. James S. Miller. The plant taxonomy was updated to *S. farahimpensis* Homolle by Sally E. Dawson of the Royal Botanical Gardens, Kew, England. Dr. Vincent Rasamison prepared the bark extract of *S. farahimpensis* under the oversight of Dr. Etienne Rakotobe. Dr. David G. I. Kingston was a mentor for this work.

7.1.3 Previous Investigations of Schimatoclada farhimpensis

S. farahimpensis Homolle is endemic to Madagascar.[5] The genus *Schismatoclada* contains twenty species, all of which are found in Madagascar.[6] There have been no other phytochemical studies of any members of this genus.

7.1.4 Chemical Investigation of Schimatoclada farhimpensis

Bioassay of ethanol extracts of various parts of *S. farahimpensis* indicated that the bark extract had antiproliferative activity with an IC_{50} value of 13 µg/mL. This extract was selected for investigation due to its activity and the lack of prior chemical studies of any members of this genus. Four known oleanic acid derivatives, oleanolic acid 28-*O*-(β-D-glucopyranosyl) ester (**7.1**),[1] 3-*O*-(β-D-glucuronopyranosyl) oleanoic acid (**7.2**),[2] 3-*O*-(β-D-glucuronopyranosyl) oleanoic acid 28-*O*-(β-D-glucopyranosyl) ester (**7.3**),[3] and 3-*O*-(β-D-glucopyranosyl-(1→2)-β-D-glucuronopyranosyl)

oleanoic acid (**7.4**),[4] were isolated using liquid–liquid partitioning, Sephadex LH-20 size exclusion chromatography, C_{18} solid phase extraction, diol HPLC, and silica gel open column chromatography. Their structures were identified by comparison of ^{1}H NMR and MS data with the data of known compounds. The isolation, structure elucidation, and bioactivity of the compounds is discussed.

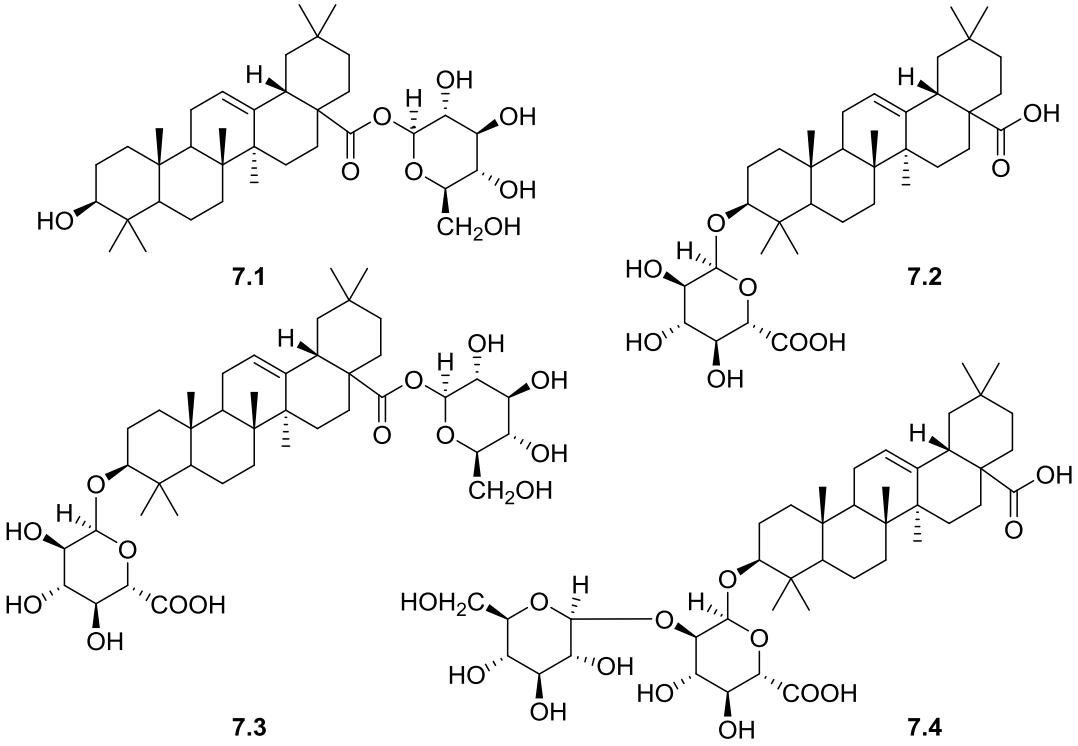
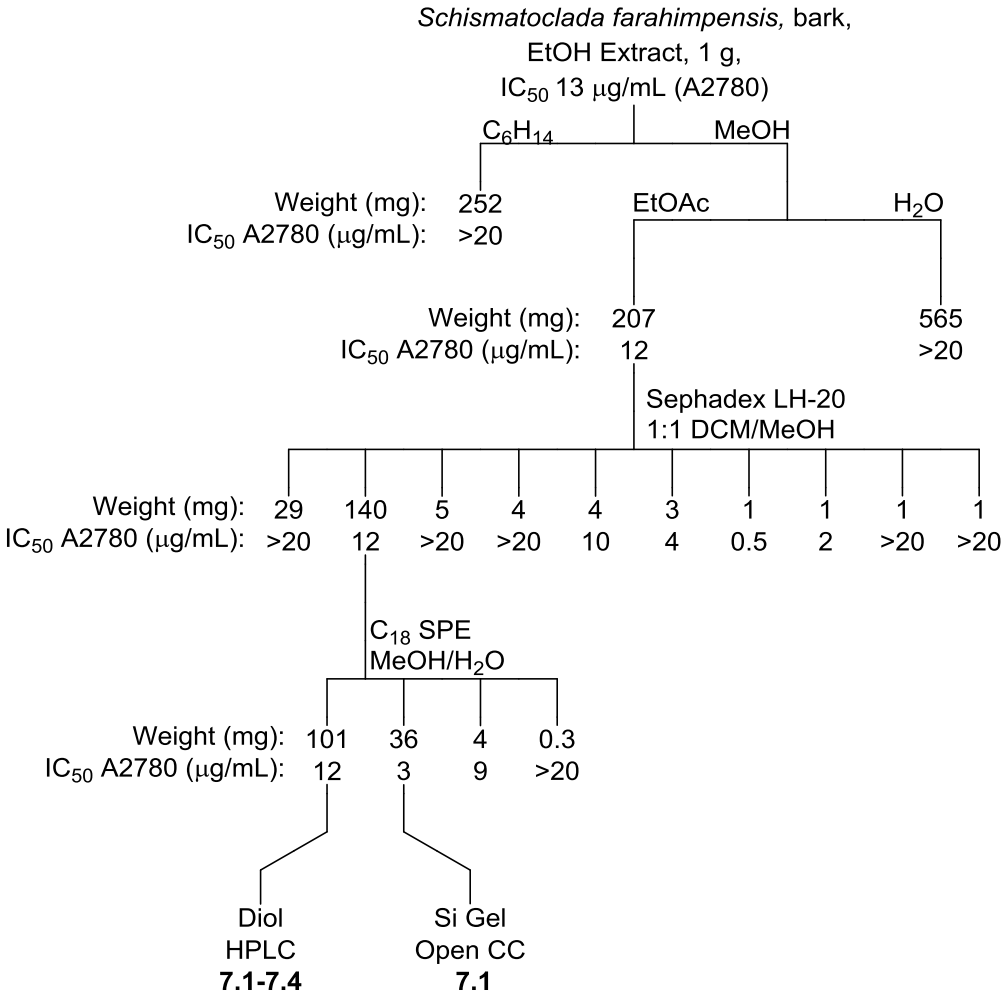


Figure 7-2. Structure of compounds isolated from *Schismatoclada farahimpensis.*

7.2 Results and Discussion

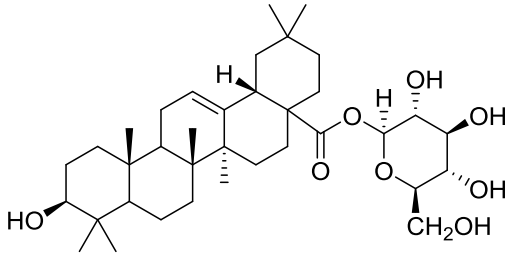
7.2.1 Isolation of Compounds from S. farahimpensis.

Liquid–liquid partitioning of the ethanol extract of *S. farahimpensis* bark led to a bioactive ethyl acetate fraction. This fraction was partitioned further utilizing Sephadex LH-20 open column chromatography, silica gel open column chromatography, C18 SPE, and diol HPLC to yield the four compounds **7.1–7.4**. This procedure is outlined in Scheme 7-1, and full details can be found in the Experimental Section of this chapter (7.3).



Scheme 7-1. Separation of the ethanol extract of *S. farahimpensis.*

7.2.2 Identification of oleanolic acid 28-O-(β-D-glucopyranosyl) ester

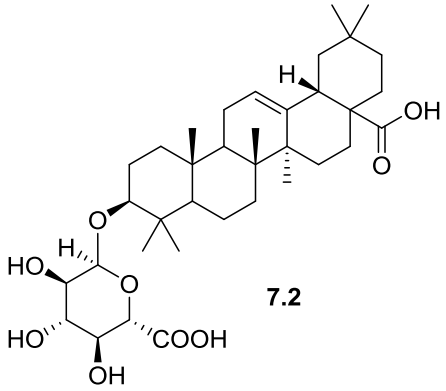


7.1

Figure 7-3. Structure of oleanolic acid 28-*O*-(β-D-glucopyranosyl) ester.

The molecular formula of **7.1** was determined to be $C_{36}H_{58}O_8$ from HRESIMS ($[M+Na]^+$ *m/z* 641.4031, calcd for $C_{36}H_{58}NaO_8^+$ 641.4024). There were seven singlet signals (δ_H 0.89, δ_H 0.91, δ_H 0.92, δ_H 1.04, δ_H 1.14, δ_H 1.24, δ_H 1.25) observed in the ^{1}H NMR spectrum corresponding to nine methyl groups. There was also a broad singlet signal downfield (δ_H 5.47), suggesting that **7.1** was an oleanolic acid derivative. In addition, there was a signal observed (δ_H 6.35, dd, J = 8.2, 1.4 Hz) representing the anomeric proton of a sugar. Furthermore, fragmentation observed in the mass spectrum ($[M-C_6H_{11}O_6]^+$ *m/z* 439.3569, calcd for $C_{30}H_{47}O_2^+$ 439.3571) suggested the molecule was an oleanolic acid glycoside. A literature search was performed and the structure was identified by comparison of HRESIMS and ^{1}H NMR data (C_5D_5N) to published values.[1] Partial ^{1}H NMR data can be found in Table 7-1.

7.2.3 *Identification of 3-O-(β-D-glucuronopyranosyl) oleanoic acid*



7.2

Figure 7-4. Structure of 3-*O*-(β-D-glucuronopyranosyl) oleanoic acid.

The molecular formula of **7.2** was determined to be $C_{36}H_{56}O_9$ from HRESIMS ($[M+Na]^+$ *m/z* 655.3814, calcd for $C_{36}H_{56}NaO_9^+$ 655.3817). Since the signal from the anomeric proton in its ^1H NMR spectrum was less downfield (δ_H 5.03) than that of **7.1**, it was speculated that the sugar moiety was connected at a different location to the oleanolic acid moiety. A fragmentation pattern similar to **7.1** was observed in the mass spectrum ($[M-C_6H_9O_7]^+$ *m/z* 439.3568, calcd for $C_{30}H_{47}O_2^+$ 439.3571) which suggested that the aglycone was identical in both compounds. A literature search was performed and the structure was identified by comparison of HRESIMS and ^1H NMR data (C_5D_5N) to published values.[2] Partial ^1H NMR data can be found in Table 7-1.

7.2.4 *Identification of 3-O-(β-D-glucuronopyranosyl) oleanoic acid 28-O-(β-D-glucopyranosyl) ester*

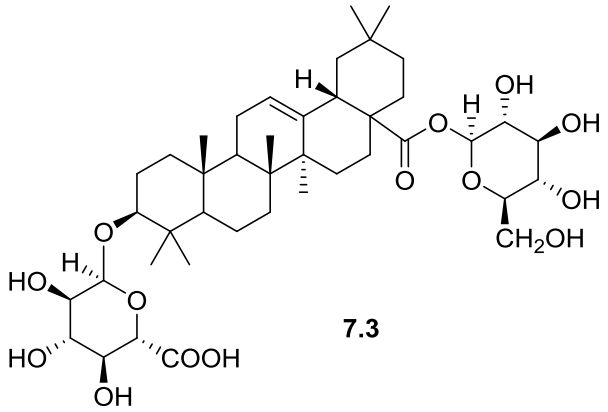
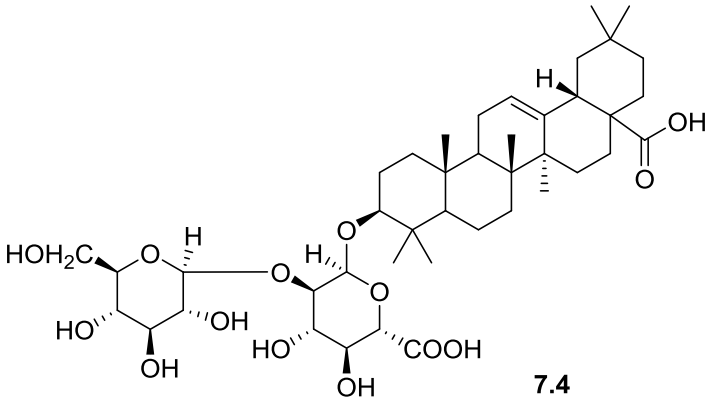


Figure 7-5. Structure of 3-*O*-(β-D-glucuronopyranosyl) oleanoic acid 28-*O*-(β-D-glucopyranosyl) ester.

The molecular formula of **7.3** was determined to be $C_{42}H_{66}O_{14}$ from HRESIMS ($[M+Na]^+$ *m/z* 817.4349, calcd for $C_{42}H_{66}NaO_{14}^+$ 817.4345) The ^1H NMR spectrum was very similar to **7.1** and **7.2** however, there were signals corresponding to two anomeric protons (δ_H 5.05, δ_H 6.35) instead of only one. The fragmentation pattern in the mass spectrum was consistent with the presence of a disubstituted oleanolic acid glycoside ($[M-C_{12}H_{19}O_{12}]^+$ *m/z* 439.3577, calcd for $C_{30}H_{47}O_2^+$ 439.3571). A literature search was performed and the structure was identified by comparison of HRESIMS and ^1H NMR data (C_5D_5N) to published values.[3] Partial ^1H NMR data can be found in Table 7-1.

7.2.5 *Identification of 3-O-(β-D-glucopyranosyl-(1→2)-β-D-glucuronopyranosyl) oleanoic acid*



7.4

Figure 7-6. Structure of 3-*O*-(β-D-glucopyranosyl-(1→2)-β-D-glucuronopyranosyl) oleanoic acid.

The molecular formula of **7.4** was determined to be $C_{42}H_{66}O_{14}$ from HRESIMS ($[M+Na]^+$ *m/z* 817.4355, calcd for $C_{42}H_{66}NaO_{14}^+$ 817.4345) The ^1H NMR spectrum was very similar to **7.3** however, the shift of the anomeric proton of the glucopyranosyl moiety (δ_H 5.42) was more upfield suggesting a different connectivity. The fragmentation pattern in the mass spectrum was consistent with the presence of a disubstituted oleanolic acid glycoside ($[M-C_6H_{11}O_6]^+$ *m/z* 439.3569, calcd for $C_{30}H_{47}O_2^+$ 439.3571). A literature search was performed and the structure was identified by comparison of HRESIMS and ^1H NMR data ($(CD_3)_2SO$) to published values.[4] Partial ^1H NMR data can be found in Table 7-1.

7.2.6 ^{1}H NMR Data

^{13}C NMR data for **7.1–7.4** is published in a number of solvents; however, ^{1}H NMR data is much less accessible. This published data is scattered, sometimes outdated, sometimes incomplete, and available in only a limited number of solvents. For convenience, partial ^{1}H NMR data recorded in both deuterated methanol and deuterated pyridine is reported to aid with future identifications of these compounds (Table 7-1).

Table 7-1. ^{1}H NMR data of **7.1**–**7.4**.

	7.1		**7.2**	
position	δ_H (*J* in Hz), C_5D_5N	δ_H (*J* in Hz), CD_3OD	δ_H (*J* in Hz), C_5D_5N	δ_H (*J* in Hz), CD_3OD
-CH_3	0.89	0.77	0.80	0.81
-CH_3	0.91	0.80	0.97	0.85
-CH_3	0.92	0.91	0.99	0.91
-CH_3	1.04	0.93	0.99	0.94
-CH_3	1.14	0.95	1.02	0.95
-CH_3	1.24	0.97	1.32	1.05
-CH_3	1.25	1.16	1.34	1.16
β-glucuronopyranosyl			5.03[a]	4.37, d (7.8)
β-glucopyranosyl	6.35, dd (8.2, 1.4)	5.38, d (8.1)		
olefinic	5.47, brs	5.25, t (3.6)	5.48, brs	5.24, t (3.6)
	7.3		**7.4**	
	δ_H (*J* in Hz), C_5D_5N	δ_H (*J* in Hz), CD_3OD	δ_H (*J* in Hz), C_5D_5N	δ_H (*J* in Hz), CD_3OD
-CH_3	0.83	0.80	0.81	0.81
-CH_3	0.89	0.85	0.96	0.86
-CH_3	0.92	0.91	0.99	0.91
-CH_3	1.00	0.93	1.02	0.94
-CH_3	1.10	0.95	1.11	0.95
-CH_3	1.29	1.05	1.30	1.08
-CH_3	1.32	1.16	1.32	1.16
β-glucuronopyranosyl	5.04, d (7.8)	4.38, d (7.8)	5.01, d (7.5)	4.50, d (7.5)
β-glucopyranosyl	6.35, d (8.1)	5.38, d (8.1)	5.42, d (7.5)	4.68, d (7.7)
olefinic	5.42, brs	5.25, t (3.8)	5.47, brs	5.24, t (3.7)

[a]Overlapping with signal from H_2O

7.2.7 Bioactivity

The isolated compounds, **7.1–7.4**, were not tested for their antiproliferative properties against the A2780 cell line due to a lack of access to bioassay testing. Oleanolic acid 28-*O*-(β-D-glucopyranosyl) ester (**1**) is known to inhibit elastase release by human neutrophils,[7] to exhibit antimicrobial bioactivity,[8] to inhibit gastric emptying and gastrointestinal transit in mice,[9] to be toxic to the Mexican bean beetle,[10] and to inhibit glycogen phosphorylase.[11] 3-*O*-(β-D-Glucuronopyranosyl) oleanoic acid (**2**) has been shown to exibit hepatotoxicity,[12] to inhibit pancreatic lipase,[13] to prevent the inhibition of gastric emptying and gastrointestinal transit in mice,[9] to exhibit molluscicidal activity,[14] to exhibit hypoglycemic activity in rats,[15] and to inhibit ethanol absorption in rats.[16] 3-*O*-(β-D-Glucuronopyranosyl) oleanoic acid 28-*O*-(β-D-glucopyranosyl) ester (**3**) has been shown to inhibit superoxide anion generation and elastase release by human neutrophils,[17] to exhibit hepatoprotective and hepatotoxic activities,[12] to have moderate cytotoxicity,[18] to inhibit cyclic AMP phosphodiesterase,[19] and to inhibit ethanol absorption in rats.[20] 3-*O*-(β-D-Glucopyranosyl-(1→2)-β-D-glucuronopyranosyl) oleanoic acid (**4**) has been shown to inhibit ethanol absorption in rats.[20]

7.3 Experimental Section

7.3.1 General Experimental Procedures

^{1}H and ^{13}C NMR spectra were recorded using a Bruker Advance 500 spectrometer and a Varian 400 spectrometer. ^{1}H-^{1}H three-bond *J*-coupling values were calculated from ^{1}H NMR spectra. Mass spectra were obtained with an Agilent 6220 LC-TOF-MS. Semi-preparative HPLC was performed using Shimadzu LC-10AT pumps coupled with a Shimadzu SPD M10A diode array detector, a SCL-10A system controller, and a Kromasil 60-5 Diol column (250 x 10 mm).

7.3.2 Plant Material

Bark of *S. farahimpensis* Homolle (Rubiaceae) was collected at an elevation of 560-630 m in June 2003 at 17°39′46" S 048°59′05" E. The plant had a shaft of 12 m, yellow wood, striated bark, green leaves, and green fruit with persistent sepals.

7.3.3 Extraction and Isolation

A ground dried sample of *S. farahimpensis* bark was extracted with EtOH at room temperature to yield crude EtOH extract designated MG 1964. A total of 0.98 g of this extract was made available to Virginia Tech. The dried ethanol extract (0.98 g, IC_{50} 13 µg/mL) was dissolved in methanol and extracted with hexanes. The methanol partition was dried, dissolved in water, and extracted with ethyl acetate. Of the resulting hexane, ethyl acetate, and aqueous fractions, only the ethyl acetate fraction showed improved activity (207 mg, IC_{50} 12 µg/mL). This fraction was further partitioned using Sephadex LH-20 column chromatography (1:1 DCM/MeOH) yielding ten fractions of which the major one had most of the activity (140 mg, IC_{50} 12 µg/mL). This fraction was subjected to C_{18} SPE with elution with MeOH/H_2O, which yielded four fractions, one of which exhibited greatly improved activity (36 mg, IC_{50} 3 µg/mL) and one of which retained most of the mass (101 mg, IC_{50} 12 µg/mL). The fraction with improved activity was fractionated by silica gel open column chromatography to yield **7.1** (6 mg, IC_{50} 2 µg/mL). A portion of the other fraction (40 mg) was separated using Diol HPLC to yield compounds **7.2** (1 mg), **7.3** (10 mg) and **7.4** (4 mg) as well as the previously isolated **7.1** (1 mg,).

7.3.4 Antiproliferative Bioassay

Antiprolierative bioassays were performed at Virginia Tech according to the procedures previously described.[21] The A2780 cell line is a drug-sensitive ovarian cancer cell line.[22]

7.3.5 Spectroscopic Properties

Oleanolic acid 28-*O*-(β-D-glucopyranosyl) ester (7.1): ^{1}H NMR (500 MHz, CD_3OD): See Table 3-2; ^{1}H NMR (500 MHz, C_5D_5N): See Table 3-2; HRESIMS $[M+NH_4]^+$ *m/z* 636.4533 (calcd for $C_{36}H_{62}NO_8^+$ 636.4470), $[M+Na]^+$ *m/z* 641.4031 (calcd for $C_{36}H_{58}NaO_8^+$ 641.4024), *m/z* $[M+K]^+$ 657.3799 (calcd for $C_{36}H_{58}KO_8^+$ 657.3763), $[M-C_6H_{11}O_6]^+$ *m/z* 439.3569 (calcd for $C_{30}H_{47}O_2^+$ 439.3571).

3-*O*-(β-D-glucuronopyranosyl) oleanoic acid (7.2): ^{1}H NMR (500 MHz, CD_3OD): See Table 3-2; ^{1}H NMR (500 MHz, C_5D_5N): See Table 3-2; HRESIMS $[M+Na]^+$ *m/z* 655.3814 (calcd for $C_{36}H_{56}NaO_9^+$ 655.3817), $[M-C_6H_9O_7]^+$ *m/z* 439.3568 (calcd for $C_{30}H_{47}O_2^+$ 439.3571).

3-*O*-(β-D-glucuronopyranosyl) oleanoic acid 28-*O*-(β-D-glucopyranosyl) ester (7.3): ^{1}H NMR (500 MHz, CD_3OD): See Table 3-2; ^{1}H NMR (500 MHz, C_5D_5N): See Table 3-2; HRESIMS $[M+NH_4]^+$ *m/z* 812.4832 (calcd for $C_{42}H_{70}NO_{14}^+$ 812.4791), $[M+Na]^+$ *m/z* 817.4349 (calcd for $C_{42}H_{66}NaO_{14}^+$ 817.4345), $[M-C_{12}H_{19}O_{12}]^+$ *m/z* 439.3577 (calcd for $C_{30}H_{47}O_2^+$ 439.3571).

3-*O*-(β-D-glucopyranosyl-(1→2)-β-D-glucuronopyranosyl) oleanoic acid (7.4): ^{1}H NMR (500 MHz, CD_3OD): See Table 3-2; ^{1}H NMR (500 MHz, C_5D_5N): See Table 3-2; ; ^{1}H NMR (400 MHz, $((CD_3)_2SO)$: 0.71 (3H, s), 0.74 (3H, s), 0.86 (3H, s), 0.87 (6H, s), 0.98 (3H, s), 1.09 (3H, s), 4.29

(1H, d, J = 6.8 Hz), 4.41 (1H, d, J = 7.7 Hz) 5.16 (1H, t, J = 3.8 Hz); HRESIMS $[M+Na]^+$ *m/z* 817.4355 (calcd for $C_{42}H_{66}NaO_{14}^+$ 817.4345), $[M-C_{12}H_{19}O_{12}]^+$ *m/z* 439.3580 (calcd for $C_{30}H_{47}O_2^+$ 439.3571).

7.4 References

1. Zhu, Y. Y.; Qian, L. W.; Zhang, J.; Liu, J. H.; Yu, B. Y., New approaches to the structural modification of olean-type pentacylic triterpenes via microbial oxidation and glycosylation. *Tetrahedron* **2011,** *67* (23), 4206-4211.

2. Kizu, H.; Kitayama, S.; Nakatani, F.; Tomimori, T.; Namba, T., Studies on Nepalese crude drugs. III. On the saponins of *Hedera nepalensis* K. Koch. *Chem. Pharm. Bull.* **1985,** *33* (8), 3324-3329.

3. Kawamura, N.; Watanabe, H.; Oshio, H., Saponins from roots of *Momordica cochinchinensis*. *Phytochemistry* **1988,** *27* (11), 3585-3591.

4. Study on hydrolysis kinetics of ginsenoside-Ro in alkaline medium and structural analysis of its hydrolyzate. *Zhongguo Zhongyao Zazhi* **2014,** *39* (5), 867-872.

5. Tropicos.org. Missouri Botanical Garden. http://www.tropicos.org/Name/50099148 (accessed June 08, 2015).

6. Mabberley, D. J., *The Plant-Book: A Portable Dictionary of the Vascular Plants*. 2nd ed.; University Press: Cambridge, 1997.

7. Chan, H. H.; Hwang, T. L.; Reddy, M. V.; Li, D. T.; Qian, K.; Bastow, K. F.; Lee, K. H.; Wu, T. S., Bioactive constituents from the roots of *Panax japonicus* var. *major* and development of a LC-MS/MS method for distinguishing between natural and artifactual compounds. *J. Nat. Prod.* **2011,** *74* (4), 796-802.

8. Chiozem, D. D.; Trinh-Van-Dufat, H.; Wansi, J. D.; Mbazoa Djama, C.; Fannang, V. S.; Seguin, E.; Tillequin, F.; Wandji, J., New friedelane triterpenoids with antimicrobial activity from the stems of *Drypetes paxii*. *Chem. Pharm. Bull.* **2009,** *57* (10), 1119-1122.

9. Li, Y.; Matsuda, H.; Yoshikawa, M., Effects of oleanolic acid glycosides on gastrointestinal transit and ileus in mice. *Bioorg. Med. Chem.* **1999,** *7* (6), 1201-1205.

10. Marquina, S.; Maldonado, N.; Garduno-Ramirez, M. L.; Aranda, E.; Villarreal, M. L.; Navarro, V.; Bye, R.; Delgado, G.; Alvarez, L., Bioactive oleanolic acid saponins and other constituents from the roots of *Viguiera decurrens*. *Phytochemistry* **2001,** *56* (1), 93-97.

11. Wen, X.; Sun, H.; Liu, J.; Cheng, K.; Zhang, P.; Zhang, L.; Hao, J.; Zhang, L.; Ni, P.; Zographos, S. E.; Leonidas, D. D.; Alexacou, K. M.; Gimisis, T.; Hayes, J. M.; Oikonomakos, N. G., Naturally occurring pentacyclic triterpenes as inhibitors of glycogen phosphorylase: synthesis, structure-activity relationships, and X-ray crystallographic studies. *J. Med. Chem.* **2008,** *51* (12), 3540-3554.

12. Kinjo, J.; Okawa, M.; Udayama, M.; Shono, Y.; Hirakawa, T.; Shii, Y.; Nohara, T., Hepatoprotective and hepatotoxic actions of oleanolic acid-type triterpenoidal glucuronides on rat primary hepatocyte cultures. *Chem. Pharm. Bull.* **1999,** *47* (2), 290-292.

13. Li, F.; Li, W.; Fu, H.; Zhang, Q.; Koike, K., Pancreatic lipase-inhibiting triterpenoid saponins from fruits of *Acanthopanax senticosus*. *Chem. Pharm. Bull.* **2007,** *55* (7), 1087-1089.

14. Odukoya, O. A.; Houghton, P. J.; Adelusi, A.; Omogbai, E. K. I.; Sanderson, L.; Whitfield, P. J., Molluscicidal triterpenoid glycosides of *Dialium guineense*. *J. Nat. Prod.* **1996,** *59* (6), 632-634.

15. Yoshikawa, M.; Murakami, T.; Harada, E.; Murakami, N.; Yamahara, J.; Matsuda, H., Bioactive saponins and glycosides. VII. On the hypoglycemic principles from the root cortex of *Aralia elata* SEEM.: structure related hypoglycemic activity of oleanolic acid oligoglycoside. *Chem. Pharm. Bull.* **1996,** *44* (10), 1923-1927.

16. Yoshikawa, M.; Murakami, T.; Harada, E.; Murakami, N.; Yamahara, J.; Matsuda, H., Bioactive saponins and glycosides. VI. Elatosides A and B, potent inhibitors of ethanol absorption, from the bark of *Aralia elata* SEEM. (Araliaceae): the structure-requirement in oleanolic acid glucuronide-saponins for the inhibitory activity. *Chem. Pharm. Bull.* **1996,** *44* (10), 1915-1922.

17. Huang, Q.; Qiao, X.; Xu, X., Potential synergism and inhibitors to multiple target enzymes of Xuefu Zhuyu Decoction in cardiac disease therapeutics: a computational approach. *Bioorg. Med. Chem. Lett.* **2007,** *17* (6), 1779-1783.

18. Liang, C.; Ding, Y.; Nguyen, H. T.; Kim, J. A.; Boo, H. J.; Kang, H. K.; Nguyen, M. C.; Kim, Y. H., Oleanane-type triterpenoids from *Panax stipuleanatus* and their anticancer activities. *Bioorg. Med. Chem. Lett.* **2010,** *20* (23), 7110-7115.

19. Nikaido, T.; Ohmoto, T.; Sankawa, U.; Tanaka, O.; Kasai, R.; Shoji, J.; Sanada, S.; Hiai, S.; Yokoyama, H.; Oura, H.; Kawashima, Y., Inhibitors of cyclic AMP phosphodiesterase in *Panax ginseng* C.A. Meyer and *Panax japonicus* C.A. Meyer. *Chem. Pharm. Bull.* **1984,** *32* (4), 1477-1483.

20. Yoshikawa, M.; Harada, E.; Matsuda, H.; Murakami, T.; Yamahara, J.; Murakami, N., Elatosides A and B, potent inhibitors of ethanol absorption in rats from the bark of *Aralia elata* SEEM.: the structure-activity relationships of oleanolic acid oligoglycosides. *Chem. Pharm. Bull.* **1993,** *41* (11), 2069-2071.

21. Cao, S.; Brodie, P. J.; Miller, J. S.; Randrianaivo, R.; Ratovoson, F.; Birkinshaw, C.; Andriantsiferana, R.; Rasamison, V. E.; Kingston, D. G. I., Antiproliferative xanthones of *Terminalia calcicola* from the Madagascar rain forest. *J. Nat. Prod.* **2007,** *70* (4), 679-681.

22. Louie, K. G.; Behrens, B. C.; Kinsella, T. J.; Hamilton, T. C.; Grotzinger, K. R.; McKoy, W. M.; Winker, M. A.; Ozols, R. F., Radiation survival parameters of antineoplastic drug-sensitive

and drug-resistant human ovarian-cancer cell-lines and their modification by buthionine sulfoximine. *Cancer Res.* **1985,** *45* (5), 2110-2115.

Chapter 8: Antimalarial Compounds from *Rhodospaera rhodanthema*

8.1 Introduction

8.1.1 Abstract

As a part of our continued search for bioactive compounds from plants, an ethanol extract of *Rhodospaera rhodanthema* (Anacardiaceae) was investigated due to its antiplasmodial activity (IC_{50} <6 µg/mL). Three known compounds, tetrahydroamentoflavone (**8.1**), pentagalloylglucopyranose (**8.2**), and 2,2′-methylenebis(6-*tert*-butyl-4-methylphenol) (**8.3**) were isolated using liquid–liquid partitioning, column chromatography, solid phase extraction, and high pressure liquid chromatography. The three compounds showed antiplasmodial activity against *Plasmodium falciparum*, Dd2 line (IC_{50} 2.3<x<4.6 µM, IC_{50} 2.7<x<5.3 µM, IC_{50} 6.8 ± 1.6 µM, respectively). This is the first report of the presence of these compounds in *Rhodospaera rhodanthema*. This work has not been published elsewhere.

8.1.2 Author Contributions

The author of this dissertation (Alexander L. Eaton) conducted the isolation and structure elucidation of the described compounds and drafted the manuscript. Dr. Liva Harinantenaina was a mentor for this work and provided advice for the isolation and structure elucidation of the compounds described. Dr. Jessica D. Wiley and Dr. Maria B. Cassera performed the antimalarial bioassay (*Plasmodium falciparum*, Dd2 strain), and Dr. Michael Goetz (Natural Product Discovery Institute) provided the plant extract. Dr. David G. I. Kingston served as a mentor for the work.

8.1.3 Previous Investigations of Rhodospaera rhodanthema

Rhodospaera rhodanthema (Anacardiaceae), the only member of the *Rhodospaera* genus, is endemic to northeast Australia.[1] It is a tree, up to 20 m high, and is commonly known as the deep yellow wood, yellow cedar or tulip satinwood.[2] It is known to contain gallic acid,[3] fisetin,[3] a fisetin glucoside[4], and quercetin.[3] *Rhodospaera rhodanthema* may also be referred to as *Rhus rhodanthema* but does not belong to the genus *Rhus* in a strict sense because it is not a true sumac.[5]

8.1.4 Chemical Investigation of Rhodospaera rhodanthema

As a part of our ongoing investigation of the former Merck natural products library, available through a collaboration with the Natural Products Discovery Institute, an extract of fronds from *Rhodospaera rhodanthema* was selected for investigation due to its activity against *Plasmodium falciparum* (IC_{50} <6 μg/mL). Three bioactive compounds were isolated (Figure 8-1), tetrahydroamentoflavone (**8.1**, IC_{50} 2.3<x<4.6 μM), pentagalloylglucopyranose (**8.2**, IC_{50} 2.7<x<5.3 μM), and 2,2′-methylenebis(6-*tert*-butyl-4-methylphenol) (**8.3**, IC_{50} 6.8 ± 1.6 μM),

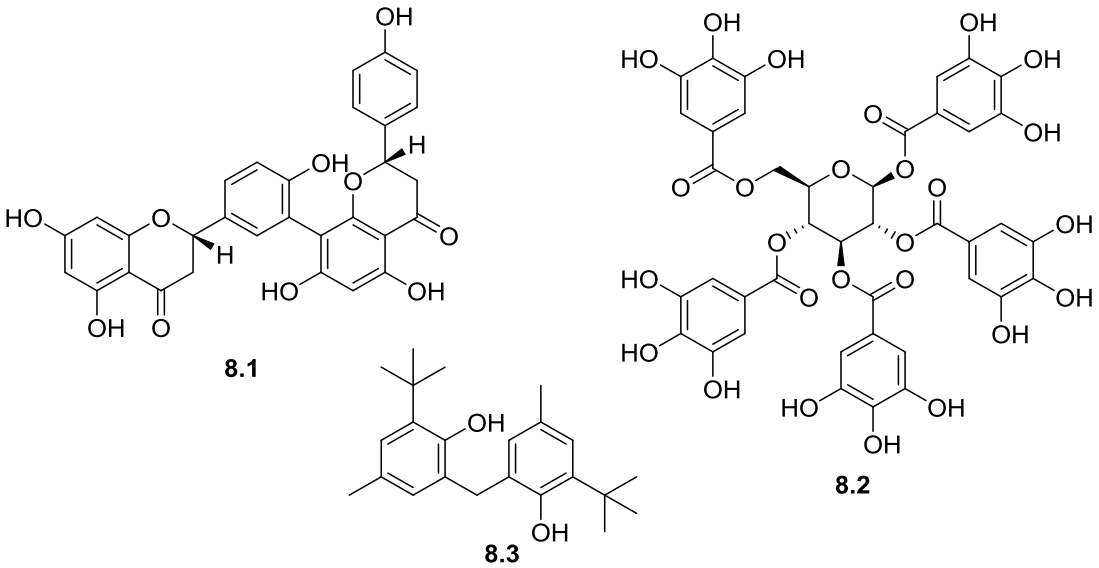


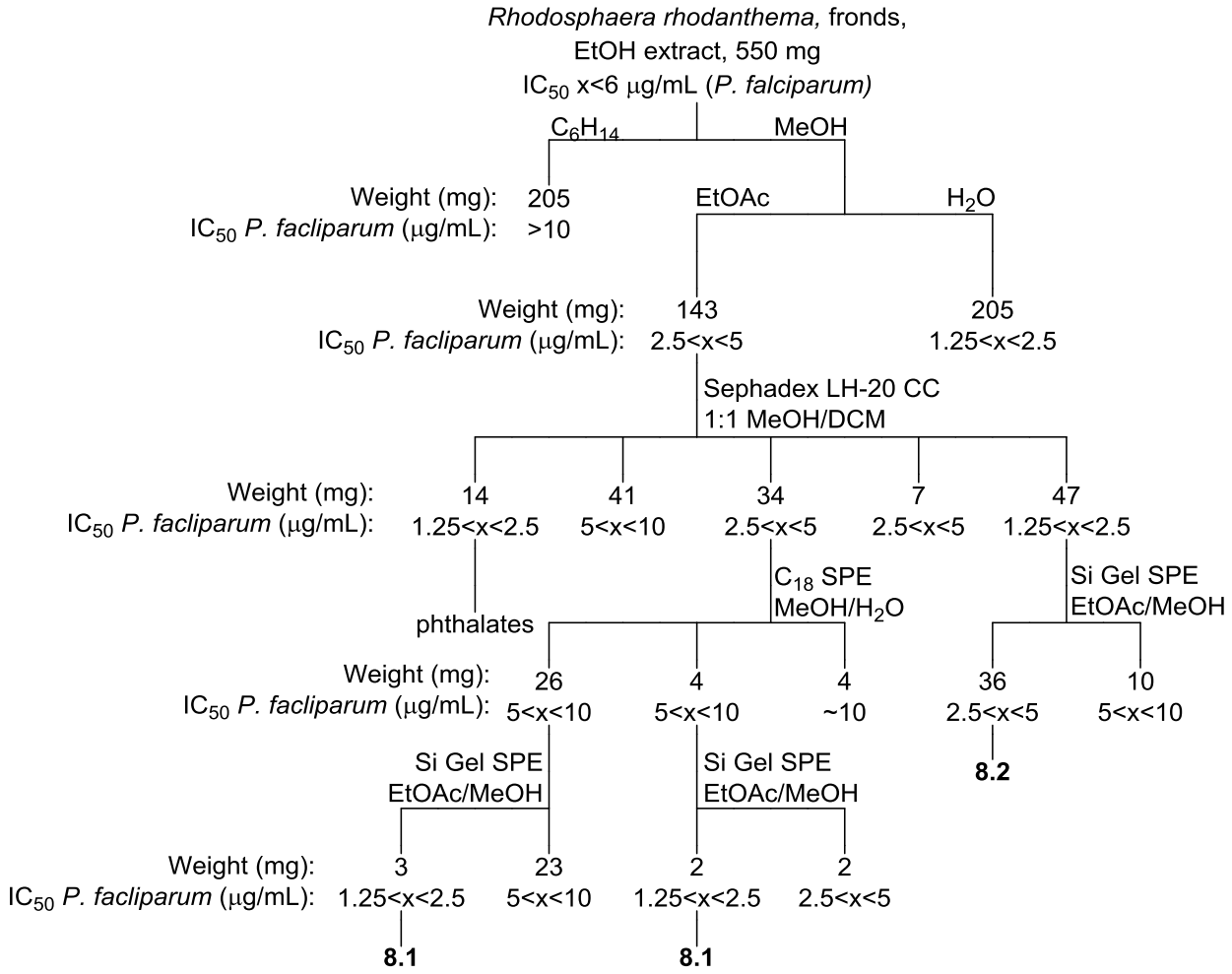
Figure 8-1. Compounds isolated from *Rhodosphaera rhodenthemia.*

through bioassay guided fractionation. The isolation, structure elucidation, and bioactivity of the compounds will be discussed.

8.2 Results and Discussion

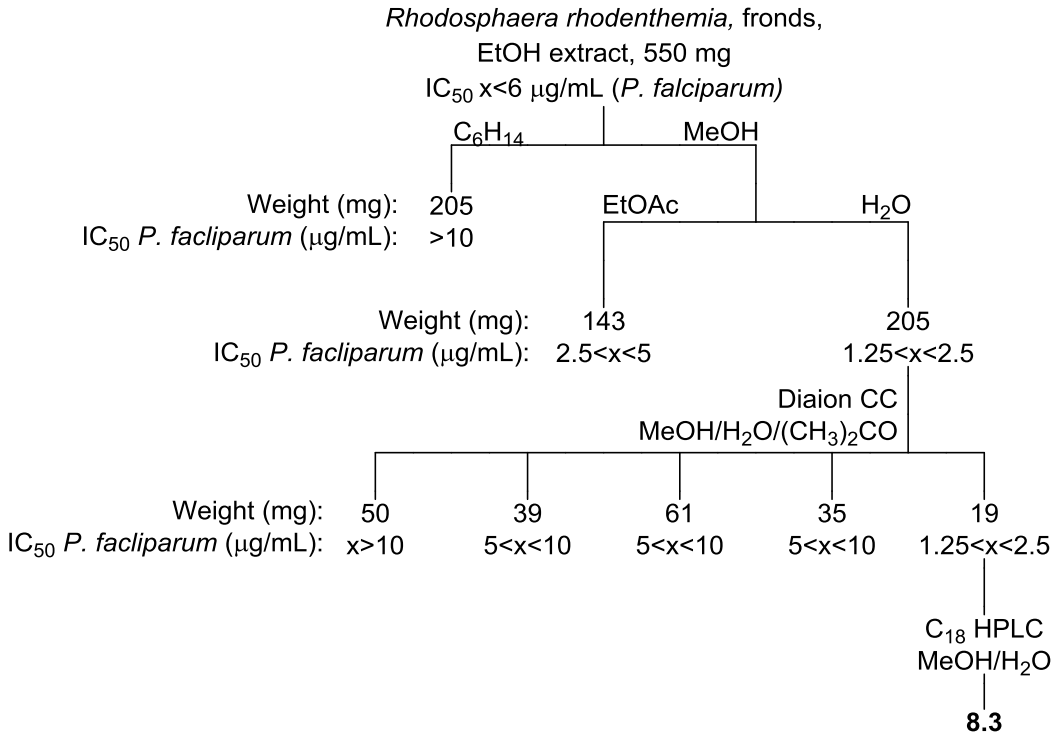
8.2.1 Isolation of Compounds from Rhodospaera rhodanthema

Liquid–liquid partitioning of the ethanol extract of the the fronds of *Rhodosphaera rhodanthema* yielded an active ethyl acetate fraction and an active water fraction. The active ethyl acetate fraction was further partitioned using chromatography on Sephadex LH-20, solid phase extraction, and high pressure liquid chromatography to afford compounds **8.1** and **8.2 (**Scheme 8-1**)**.



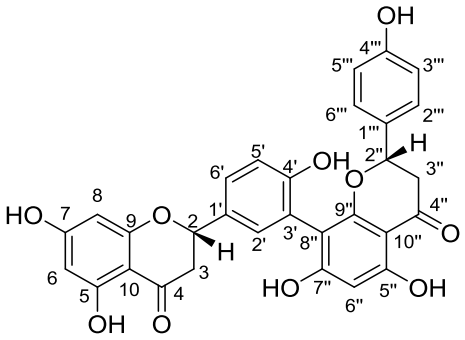
Scheme 8-1. Separation of the ethyl acetate fraction of *Rhodosphaera rhodanthema.*

The active water fraction was further subjected to chromatography on a Diaion column and high pressure liquid chromatography to give 2,2′-methylenebis(6-*tert*-butyl-4-methylphenol) (**8.3**) (Scheme 8-2). Full details can be found in the Experimental Section of this chapter (8.3). Although 2,2′-methylenebis(6-*tert*-butyl-4-methylphenol) has reportedly been isolated from *Aspergillus fumigatus*,[6] it is likely a contaminant since it does not fit into any known biosynthetic pathway and it is a widely used plasticizer. The ethyl acetate fraction was also found to be contaminated with phthalate, so further fractionation was stopped.



Scheme 8-2. Separation of water fraction of *Rhodospaera rhodanthema.*

8.2.2 Identification of Tetrahydroamentoflavanone



8.1

Figure 8-2. Structure of tetrahydroamentoflavanone.

Tetrahydroamentoflavone (**8.1**) was analyzed using ^{1}H NMR and HRESIMS. There were eight signals (δ_H 7.29, 1H, d, J = 2.3 Hz; δ_H 7.25, 1H, bd; δ_H 7.20, 2H, d, J = 8.6 Hz; δ_H 6.89, 1H, d, J = 8.3 Hz; δ_H 6.72, 2H, d, J = 8.6 Hz; δ 6.02, 1H, s; δ 5.90, 1H, d, J = 2.2 Hz; δ_H 5.88, 1H, d, J = 2.2 Hz) present in the aromatic region of the ^{1}H-NMR. The signals at δ_H 7.20 and δ_H 6.72 represent an AA'BB' ring spin system. The signal at δ_H 6.89 represents ortho-coupled protons on an aromatic ring and the signals at δ_H 5.90 and δ_H 5.88 represent meta-coupling protons. This data suggests the presence of four aromatic rings. There were also signals at δ_H 5.37 (1H, dd, J = 12.6, 3.1 Hz) and δ_H 5.32 (1H, d, J = 14.2 Hz) that suggested the presence of two oxygen bearing methine protons. The remaining signals (δ_H 3.05, 1H, dd, J = 17.1, 12.6 Hz; δ_H 3.04, 1H; δ_H 2.78, 1H, dd, J = 17.0, 3.1 Hz; δ_H 2.63, 1H, bd, J = 16.8 Hz) were recognized as likely being coupled to the peaks at δ_H 5.37 and δ_H 5.32. The molecular formula of the compound was determined to be $C_{30}H_{22}O_{10}$ from its mass spectrum ([M-H]$^-$ *m/z* 541.1242, calcd for $C_{30}H_{21}O_{10}^-$ 541.1140; [2M-H]$^-$ *m/z* 1083.2479, calcd for $C_{60}H_{43}O_{20}^-$ 1083.2353). A database search (Dictionary of Natural Products) was performed with the search parameters of the molecular weight (542 g/mol) and the presence of four aromatic rings with one being specified as a *p*-disubstituted benzene ring and one specified as a pentasubstituted benzene ring. This search yielded three results that were quickly

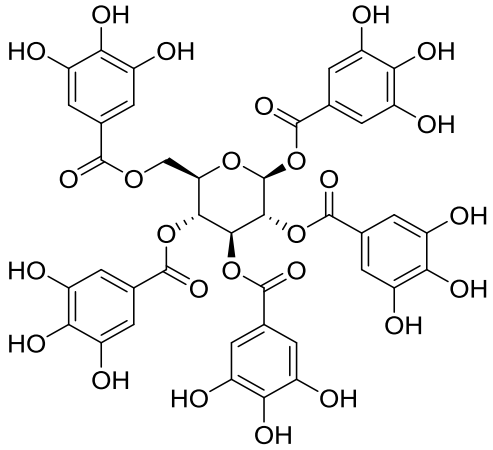
screened to find the structure of tetrahydroamentoflavanone. Experimental NMR data (Table 8-1) was compared to published ^{1}H-NMR data.[7,8] Absolute configuration was determined by comparison of published ECD data to experimental ECD data.[8,9]

Table 8-1. ^{1}H NMR spectroscopic data of tetrahydroamentoflavone (**8.1**).

position	δ_H (*J* in Hz)
2	5.32, bd (14.2)
3	2.98, bs[a]
	2.63, bd (16.8)[b]
6	5.90, d (2.2)[c]
8	5.88, d (2.2)[c]
2′	7.29, d (2.3)
5′	6.89, d (8.3)
6′	7.25, bd (8.2)
2″	5.37, dd (12.6, 3.1)
3″	3.05, dd, (17.1, 12.6)[a]
	2.78, dd(17.0, 3.1)[b]
6″	6.02, s
2‴, 6‴	7.20, d (8.6)
3‴, 5‴	6.72, d (8.6)

500 MHz, CD_3OD
[a,b,c]Interchangeable Assignment

8.2.3 Identification of Pentagalloylglucopyranose



8.2

Figure 8-3. Structure of pentagalloylglucopyranose.

Pentagalloylglucopyranose (**8.2**), was analyzed by using ^{1}H NMR, ^{13}C NMR, HSQC, and LC-MS. There are peaks appearing in five clusters (δ_C ~167, δ_C ~146, δ_C ~140, δ_C ~120, δ_C ~110) each containing five signals indicating the presence of a repeating unit. The signals at δ_C ~146 and δ_C ~110 are approximately twice as strong as the signals at δ_C ~140 and δ_C ~120 suggesting that they might represent more than one carbon. The five most downfield signals present in the ^{13}C-NMR (δ_C 167.9, δ_C 167.3, δ_C 167.0, δ_C 166.9, δ_C 166.2) suggested ester-linkages. The ^{13}C NMR showed a signal at δ_C 93.8 which was coupled with the proton at δ_H 6.24 (d, J = 8.3 Hz) (as seen by HSQC). These shifts are characteristic of an ester-linked β-hexopyranosyl. The other remaining proton signals (H-2: δ_H 5.59, dd, J = 9.8, 8.3 Hz; H-3: δ_H 5.91, t, J = 9.7 Hz; H-4: δ_H 5.62, t, J = 9.6 Hz; H-5: δ_H 4.40, m; H-6a: δ_H 4.52, bd, J = 10.5 Hz; H-6b: δ 4.39, m,) and carbon signals (C-2: δ_C 72.2, C-3: δ_C 74.1, C-4: δ_C 69.8; C-5: δ_C 74.4; C-6: δ_C 63.1 for the β-hexopyranosyl unit suggested the β-hexopyranosyl group to be a β-glucopyranosyl group. A structure with five galloyl groups attached to β-glucopyranosyl was proposed as **8.2**. Published ^{1}H and ^{13}C NMR spectra were

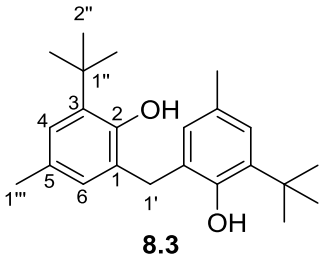
compared to experimental ^{1}H and ^{13}C NMR spectra (Table 8-2).[10,11] The molecular formula, $C_{41}H_{32}O_{26}$, was consistent with LC-MS data ($[M+H]^+$ *m/z* 941.29 calcd for $C_{41}H_{33}O_{26}^+$ 941.13). There was also a strong signal present in the LC-MS spectrum consistent with the loss of a galloyl group ($[M-C_7H_5O_5]^+$ *m/z* 771.21 calcd for $C_{34}H_{27}O_{21}^+$ 771.10).

Table 8-2. ^{1}H and ^{13}C NMR spectroscopic data of pentagalloylglucopyranose (**8.2**).

	position	δ_C, type[a]	δ_H (*J* in Hz)[b]
β-glucopyranosyl	1	93.8, CH	6.24, d, (8.3)
	2	72.2, CH	5.59, dd (9.8, 8.3)
	3	74.1, CH	5.91, t (9.7)
	4	69.8, CH	5.62, t (9.6)
	5	74.4, CH	4.40, m
	6	63.1, CH_2	4.52, bd (10.5)
			4.39, m
C=O		167.9, C	
		167.3, C	
		167.0, C	
		166.9, C	
		166.2, C	
galloyl 1	1	121.0, C[c]	
	2,6	110.6, CH[d]	6.90, s[g]
	3, 5	146.5, C[e]	
	4	140.8, C[f]	
galloyl 2	1	120.3, C[c]	
	2,6	110.5, CH[d]	6.95, s[g]
	3, 5	146.4, C[e]	
	4	140.3, C[f]	
galloyl 3	1	120.2, C[c]	
	2,6	110.4, CH[d]	6.98, s[g]
	3, 5	146.4, C[e]	
	4	140.3, C[f]	
galloyl 4	1	120.2, C[c]	
	2,6	110.4, CH[d]	7.06, s[g]
	3, 5	146.3, C[e]	
	4	140.1, C[f]	
galloyl 5	1	119.7, C[c]	
	2,6	110.3, CH[d]	7.12, s[g]
	3, 5	146.2, C[e]	
	4	140.0, C[f]	

Assignments made using HSQC and HMBC
[a]100 MHz, CD_3OD
[b]500 MHz, CD_3OD
[c,d,e,f,g]Interchangeable assignement

8.2.4 Identification of 2,2′-methylenebis(6-tert-butyl-4-methylphenol)



8.3

Figure 8-4. Structure of 2,2′-methylenebis(6-*tert*-butyl-4-methylphenol).

2,2′-Methylenebis(6-*tert*-butyl-4-methylphenol) (**8.3**) was analyzed using ^{1}H NMR, ^{13}C NMR, and HRESIMS. The proton NMR showed the presence of meta-coupled aromatic protons at δ_H 6.90 (1H, d, J = 1.9 Hz) and δ_H 6.79 (1H, d, J = 1.9 Hz). These two doublets, along with six aromatic signals (δ_C 151.3, δ_C 139.3, δ_C 130.6, δ_C 130.5, δ_C 129.4, δ_C 126.5) in the ^{13}C NMR suggested the presence of a tetrasubstituted benzene ring. The signal at δ_C 151.3 suggests that one of the substituents is a hydroxyl group. The signals at δ_H 2.19 (3H, s) in the ^{1}H NMR and δ_H 21.01 in the ^{13}C NMR indicate a methyl group attached to the ring. The singlet corresponding to nine protons in the ^{1}H NMR signal (δ_H 1.38, 9H), the strong signal in the ^{13}C NMR at δ_C 30.4, and the signal at δ_C 35.6 are consistent with the presence of a *tert*-butyl group in the molecule. The HRESIMS spectrum suggested that the molecular formula was $C_{23}H_{32}O_2$ ([M-H]$^-$ *m/z* 339.2346 calcd for $C_{23}H_{31}O_2^-$ 339.2330) . Considering only the number of carbon, hydrogen, and oxygen atoms accounted for thus far as well as the remaining ^{1}H NMR signal (δ_H 3.82, 1H, s) and ^{13}C NMR signal, the compound must be symmetrical. A dimeric structure was proposed as **8.3**. The published ^{1}H NMR spectrum is comparable to the experimental ^{1}H NMR spectrum.[12] This compound is a well-known synthetic antioxidant, and is almost certainly a contaminant that somehow ended up in the crude extract, and not a natural product.

Table 8-3. ^{1}H and ^{13}C NMR spectroscopic data of of 2,2′-methylenebis(6-*tert*-butyl-4-methylphenol) (**8.3**).

	position	δ_C, type	δ_H (*J* in Hz)
isoprene chain	1	129.4, C	
	2	151.3, C	
	3	139.3, C	
	4	126.5, CH	6.79, d (1.8)
	5	130.6, C	
	6	130.5, CH	6.90, d (2.0)
	1′	32.3, CH_2	3.82, s
	1″	35.6, C	
	2″	30.4, CH_3	1.38, s
	1‴	21.0, CH_3	2.19, s

Obtained in CD_3OD, 600 MHz (δ_H), 150 MHz (δ_C)

8.2.5 Antiplasmodial Activity

Compounds **8.1–8.3** were tested for their antiplasmodial activity against *Plasmodium falciparum*, Dd2 strain (Table 3-3). Tetrahydroamentoflavone has previously been tested for antiplasmodial activity against *P. falciparum*, K1 strain but its IC_{50} was found to be greater than 9.3 μM.[13] Tetrahydroamentoflavone has been shown to exhibit antileishmanicidal activity,[13] to inhibit cyclooxygenase[14], to inhibit xanthine oxidase,[8] to exhibit a protective effect against anoxia,[15] and to exhibit antioxidant properties.[16]

Table 8-4. Activities of isolated compounds against *Plasmodium falciparum.*

Compound	Antiplasmodial activity against *P. falciparum* Dd2 strain, IC_{50}, μM
Tetrahydroamentoflavone (**8.1**)	2.3<x<4.6
Pentagalloylglucopyranose (**8.2**)	2.7<x<5.3
2,2′-Methylenebis(6-*tert*-butyl-4-methylphenol) (**8.3**)	6.8 ± 1.6

8.3 Experimental Section

8.3.1 General Experimental Procedures

Optical rotations were recorded on a JASCO P-2000 polarimeter. ECD analysis was performed on a JASCO J-810 spectropolarimeter with a 0.1 cm cell in MeOH at room temperature under the following conditions: speed 50 nm/min, time constant 1 s, band width 2.0 nm. ^{1}H and ^{13}C NMR spectra were recorded using either a Bruker Advance 500 spectrometer or a Bruker Advance 600 spectrometer. ^{1}H-^{1}H three-bond *J*-coupling values were calculated from ^{1}H NMR spectra. Mass spectra were obtained on an Agilent 6220 LC-TOF-MS or a Thermo Electron TSQ LC-ESI-MS. Semi-preparative HPLC was performed by using Shimadzu LC-10AT pumps coupled with a Shimadzu SPD M10A diode array detector, a SCL-10A system controller, and a Cogent Bidentate C_{18} column (250 x 10 mm).

8.3.2 Plant Material

The ethanol extract of fronds from *Rhodospaera rhodanthema* was supplied by Dr. Michael Goetz from the Natural Products Discovery Institute (NPDI) collection.

8.3.3 Extraction and Isolation

The ethanol extract of the fronds of *Rhodospaera rhodanthema* (550 mg, IC_{50} x<6 μg/mL) was dissolved in methanol and extracted with hexanes. The methanol was removed and the residue was dissolved in water and extracted with ethyl acetate. This liquid–liquid partitioning created three fractions (F1: hexanes, F2: ethyl acetate, and F3: water) of which two showed improved bioactivity: the ethyl acetate fraction (143 mg, IC_{50} 2.5<x<5 μg/mL) and the water fraction (205 mg, IC_{50} 1.25<x<2.5 μg/mL).

The ethyl acetate fraction (F2) was further partitioned using Sephadex LH-20 open column chromatography to produce five fractions (F2-(1-5)) of which three showed activity (F2-1: 14 mg, IC_{50} 1.25<x<2.5 μg/mL; F2-3: 34 mg, IC_{50} 2.5<x<5 μg/mL; F2-5: 47 mg, IC_{50} 1.25<x<2.5 μg/mL). The first fraction, F2-1,was separated further using silica gel solid phase extraction (EtOH/MeOH) to yield two fractions, one of which had improved activity (8 mg IC_{50} x<1.25 μg/mL). The major component of this fraction was found to be phthalates by ^{1}H NMR spectroscopy. The third fraction, F2-3, was further separated by C_{18} SPE (MeOH/H_2O) in three fractions. Curiously, these fractions all showed reduced activity (F2-3a: 26 mg, IC_{50} 5<x<10 μg/mL; F2-3b: 4 mg, IC_{50} 5<x<10 μg/mL; F2-3c: 4 mg, IC_{50} ~10 μg/mL) so the two most active fractions (F2-3a, F2-3b) were chosen for further separation. Both of these fractions were separately purified with silica gel SPE (EtOAc/MeOH) and tetrahydroamentoflavone (**8.1**, IC_{50} 1.25<x<5 μg/mL) was isolated from each (3 mg, 2 mg, respectively). The other active fraction (F2-5) was separated using silica gel SPE (EtOAc/MeOH) to yield pentagalloylglucopyranose (**8.2**, 4 mg, IC_{50} 2.5<x<5 μg/mL).

The water fraction (F3) was partitioned using a Diaion column to yield five fraction, one of which showed moderate activity (20 mg, IC_{50} 2.5<x<5 μg/mL). This fraction was purified using C_{18} HPLC (MeOH/H_2O) to yield 2,2′-methylenebis(6-*tert*-butyl-4-methylphenol) (**8.3,** 20 mg, IC_{50} 2.5<x<5 μg/mL). Further fractionation was stopped due to the large amount of contaminants already isolated and the likelihood of isolating more contaminants.

8.3.4 Antimalarial Bioassay

Assay was performed at Virginia Tech as previously described.[17]

8.3.5 Spectroscopic Properties

Tetrahydroamentoflavone (8.1): $[\alpha]^{22}_{D}$ –22.4 (*c* 0.24 MeOH); CD (MeOH) $[\Delta\varepsilon]_{325\ nm}$ 2.2, $[\Delta\varepsilon]_{289\ nm}$ –9.4; ^{1}H NMR (500 MHz, CD_3OD): See Table 8-1; HRESIMS $[M-H]^-$ *m/z* 541.1242 (calcd for $C_{30}H_{21}O_{10}^-$ 541.1140), $[2M-H]^-$ *m/z* 1083.2479 (calcd for $C_{60}H_{43}O_{20}^-$ 1083.2353 $[M+HCOO]^-$).

Pentagalloylglucopyranose (8.2): ^{1}H NMR (500 MHz, CD_3OD): See Table 8-2; ^{13}C NMR (100 MHz, CD_3OD): See Table 8-2; MS $[M+H]^+$ *m/z* 941.29 (calcd for $C_{41}H_{33}O_{26}^+$ 941.13), $[M-C_7H_5O_5]^+$ *m/z* 771.21 (calcd for $C_{34}H_{27}O_{21}^+$ 771.10).

2,2′-methylenebis(6-*tert*-butyl-4-methylphenol) (8.3): ^{1}H NMR (600 MHz, CD_3OD): See Table 8-3; ^{13}C NMR (150 MHz, CD_3OD): See Table 8-3; HRESIMS $[M–H]^-$ *m/z* 339.2346 (calcd for $C_{23}H_{31}O_2^-$ 339.2330), $[M+Cl]^-$ *m/z* 375.2119 (calcd for $C_{23}H_{32}O_2Cl^-$ 375.2096).

8.4 **References**

1. Mabberley, D. J., *The Plant-Book: A Portable Dictionary of the Vascular Plants*. 2nd ed.; University Press: Cambridge, 1997.

2. PlantNET - FloraOnline. http://plantnet.rbgsyd.nsw.gov.au/cgi-bin/NSWfl.pl?page=nswfl&lvl=sp&name=Rhodosphaera~rhodanthema (accessed June 10, 2015).

3. Perkin, A. G., Yellow colouring principles contained in various tannin matters. Part VI. *Rhus cotinus* and *Rhus rhodanthema*. *J. Chem. Soc., Trans.* **1898,** *73*, 1016-1019.

4. Perkin, A. G., Yellow colouring matters obtained from *Rhus rhodanthema*, *Berberis oetnensis*, and *Rumex obtusifolius*. *J. Chem. Soc., Trans.* **1897,** *71*, 1194-1200.

5. Sando, C. E.; Bartlett, H. H., The flavones of *Rhus*. *Am. J. Bot.* **1918,** *5* (3), 112-119.

6. Kim, Y.-K.; Lee, H.-W.; Son, K.-H.; Kwon, B.-M.; Jeong, T.-S.; Lee, D.-H.; Shin, J.; Seo, Y.; Kim, S.-U.; Bok, S.-H., GERI-BP002-A, novel inhibitor of acyl-CoA : cholesterol acyltransferase produced by *Aspergillus fumigatus* F93. *J. Antibiot.* **1996,** *49* (1), 31-36.

7. Elsohly, M. A.; Craig, J. C.; Waller, C. W.; Turner, C. E., Biflavonoids from fruits of poison ivy *Toxicodendron radicans*. *Phytochemistry* **1978,** *17* (12), 2140-2141.

8. Arimboor, R.; Rangan, M.; Aravind, S. G.; Arumughan, C., Tetrahydroamentoflavone (THA) from *Semecarpus anacardium* as a potent inhibitor of xanthine oxidase. *J. Ethnopharmacol.* **2011,** *133* (3), 1117-1120.

9. Swamy, R. C.; Kunert, O.; Schuhly, W.; Bucar, F.; Ferreira, D.; Rani, V. S.; Kumar, B. R.; Appa Rao, A. V., Structurally unique biflavonoids from *Selaginella chrysocaulos* and *Selaginella bryopteris*. *Chem. Biodiversity* **2006,** *3* (4), 405-413.

10. Cui, C. B.; Zhao, Q. C.; Cai, B.; Yao, X. S.; Osadsa, H., Two new and four known polyphenolics obtained as new cell-cycle inhibitors from *Rubus aleaefolius* Poir. *J. Asian Nat. Prod. Res.* **2002,** *4* (4), 243-252.

11. Zhang, Y.; DeWitt, D. L.; Murugesan, S.; Nair, M. G., Novel lipid-peroxidation- and cyclooxygenase-inhibitory tannins from *Picrorhiza kurroa* seeds. *Chem. Biodiversity* **2004,** *1* (3), 426-441.

12. Casiraghi, G.; Casnati, G.; Pochini, A.; Puglia, G.; Ungaro, R.; Sartori, G., Uncatalyzed phenol-formaldehyde reactions. A convenient synthesis of substituted 2,2′-dihydroxydiphenylmethanes. *Synthesis* **1981,** *1981* (02), 143-146.

13. Kunert, O.; Swamy, R. C.; Kaiser, M.; Presser, A.; Buzzi, S.; Rao, A. V. N. A.; Schuhly, W., Antiplasmodial and leishmanicidal activity of biflavonoids from Indian *Selaginella bryopteris*. *Phytochem. Lett.* **2008,** *1* (4), 171-174.

14. Selvam, C.; Jachak, S. M., A cyclooxygenase (COX) inhibitory biflavonoid from the seeds of *Semecarpus anacardium*. *J. Ethnopharmacol.* **2004,** *95* (2-3), 209-212.

15. Zheng, J. X.; Zheng, Y.; Zhi, H.; Dai, Y.; Wang, N. L.; Fang, Y. X.; Du, Z. Y.; Zhang, K.; Li, M. M.; Wu, L. Y.; Fan, M., New 3',8''-linked biflavonoids from *Selaginella uncinata* displaying protective effect against anoxia. *Molecules* **2011,** *16* (8), 6206-6214.

16. Li, X. C.; Han, W. J.; Mai, W. Q.; Wang, L., Antioxidant activity and mechanism of tetrahydroamentoflavone in vitro. *Nat. Prod. Commun.* **2013,** *8* (6), 787-789.

17. Harinantenaina, L.; Bowman, J. D.; Brodie, P. J.; Slebodnick, C.; Callmander, M. W.; Rakotobe, E.; Randrianaivo, R.; Rasamison, V. E.; Gorka, A.; Roepe, P. D.; Cassera, M. B.; Kingston, D. G. I., Antiproliferative and antiplasmodial dimeric phloroglucinols from *Mallotus oppositifolius* from the Madagascar dry forest. *J. Nat. Prod.* **2013,** *76*, 388-393.

Chapter 9: Identification of Bioactive Compounds from *Curroria sp.*

9.1 Introduction

9.1.1 Abstract

In a continuing search for antiproliferative compounds from plants, an extract of *Curroria sp.* (Asclepiadaceae) was investigated due to its antiproliferative activity (IC_{50} 10 μg/mL) and lack of phytochemical studies of the genus. Five known compounds, isofraxetin 6-*O*-β-D-glucopyranoside (IC_{50} >54 μM), sarmentogenin α-L-diginoside (IC_{50} 0.3 μM), sarmentogenin α-L-diginosyl-β-D-glucopyranoside (IC_{50} 1 μM), pinoresinol (IC_{50} >56 μM), and apigenin (IC_{50} >74 μM) were isolated using liquid–liquid partitioning, column chromatography, solid phase extraction, and HPLC. The five compounds were quickly identified through utilizing of ^{1}H NMR, HRESIMS, and the Dictionary of Natural Products database. This work has not been published elsewhere.

9.1.2 Author Contributions

The author of this dissertation (Alexander L. Eaton) completed the fractionation of the extract and the identification of the compounds described. Ms. Peggy Brodie performed the antiproliferative bioassay (A2780) on all fractions and compounds. Dr. Michael Goetz provided the extract from the Natural Product Discovery Institute (NPDI). Dr. David G. I. Kingston was a mentor for this work.

9.1.3 Previous Investigations of Curroria sp.

The genus *Curroria* belongs to the Asclepiadaceae family and contains only four members. Plants from the Asclepiadaceae family have been reported to contain bioactive cardenolides, flavonoids, and triterpenes.[1] There have been no previous investigations of the genus *Curroria*.

9.1.4 Chemical Investigation of Curroria sp.

The above ground portion of *Curroria sp.* (Asclepiadaceae) was selected for investigation based on the lack of studies of the genus and the antiproliferative activity of its ethanol extract (IC_{50} 10 μg/mL, A2780). The extract was partitioned using liquid–liquid partitioning, Sephadex LH-20 CC, Diaion CC, C_{18} SPE, HPLC, and silica gel CC. The five known compounds isofraxetin 6-*O*-β-D-glucopyranoside (**9.1**), sarmentogenin α-L-diginoside (**9.2**), sarmentogenin α-L-diginosyl-β-D-glucopyranoside (**9.3**), pinoresinol (**9.4**), and apigenin (**9.5**) (**Error! Reference ource not found.**), were identified using HRESIMS and ^{1}H NMR spectroscopy.

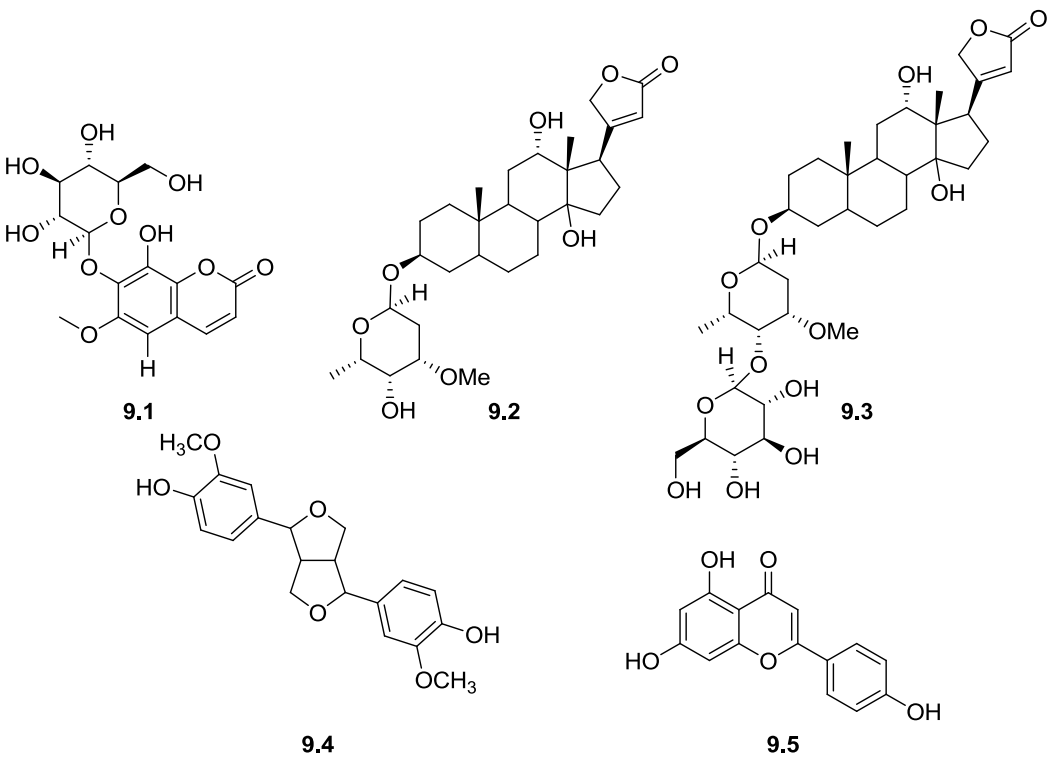
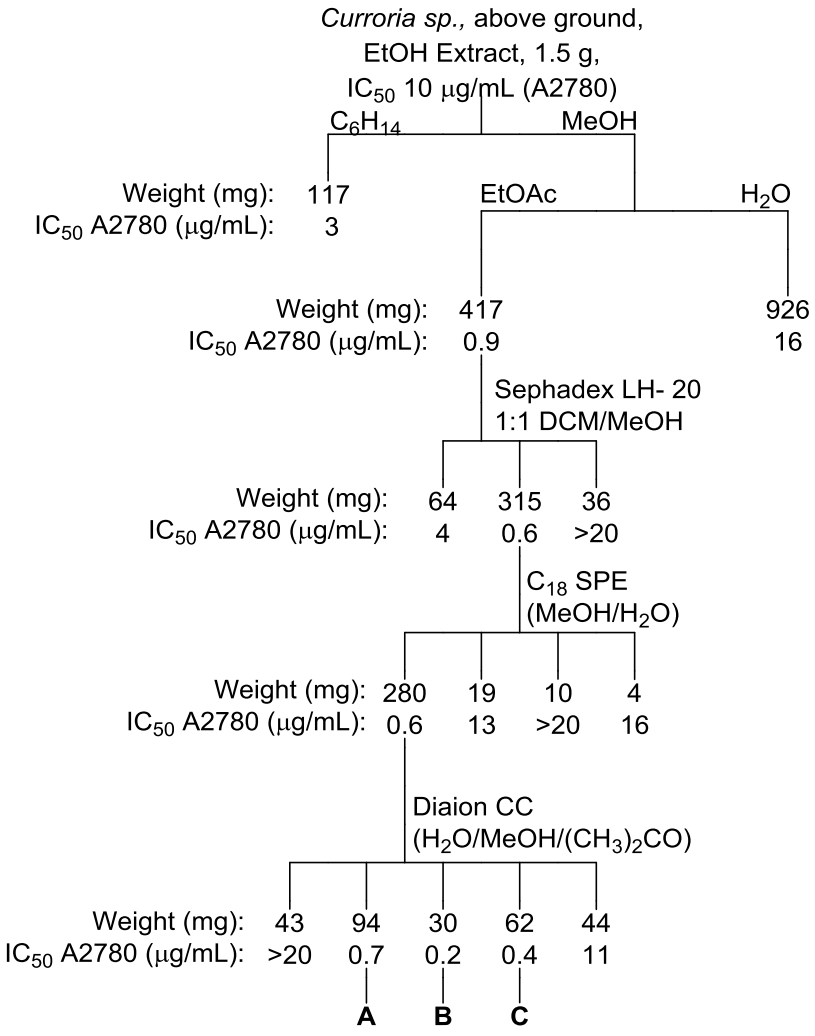


Figure 9-1: Compounds identified from *Curroria sp.*

9.2 Results and Discussion

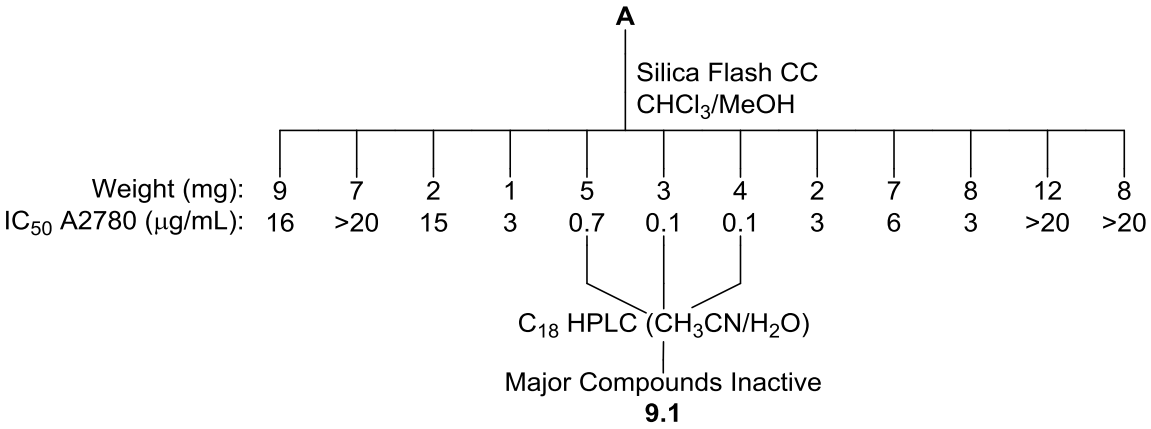
9.2.1 Isolation of Compounds from Curroria sp.

An ethanol extract of the above ground portion of *Curroria sp.* was subjected to liquid–liquid partitioning, yielding an active ethyl acetate fraction. The ethyl acetate fraction was further partitioned using Sephadex LH-20 column chromatography, yielding increased activity in one fraction. This fraction was further partition by C_{18} SPE and Diaion CC to yield three active fractions (**A, B, C,** Scheme 9-1).



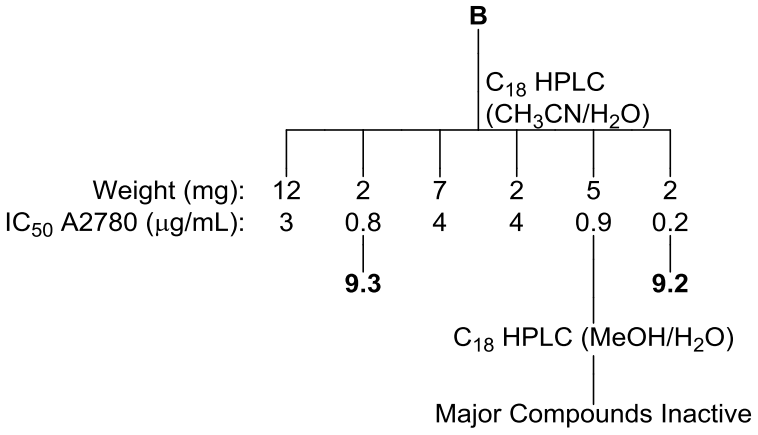
Scheme 9-1. Fractionation of *Curroria sp.*

Fraction **A** was partitioned further utilizing silica gel flash column chromatography and C_{18} HPLC to yield **9.1** (Scheme 9-2).



Scheme 9-2. Fractionation of A.

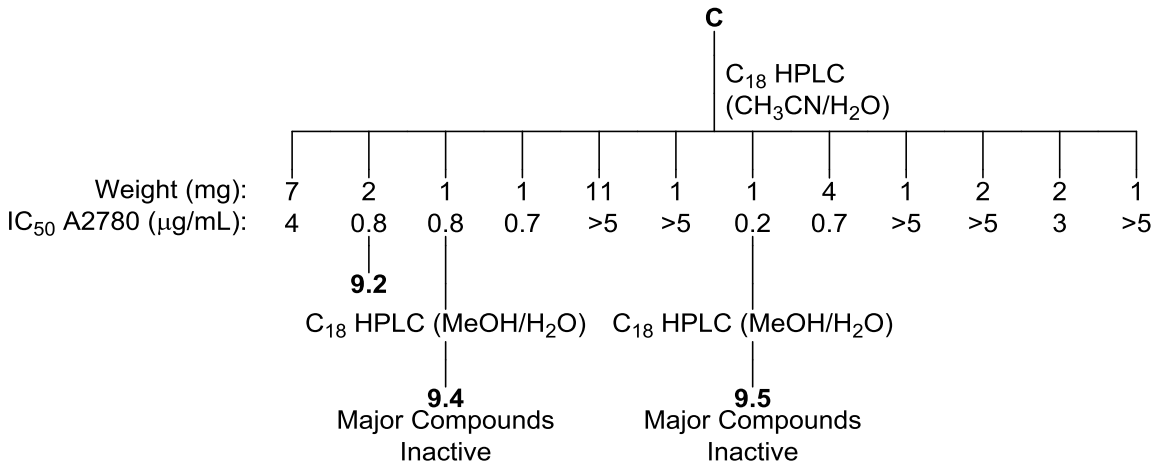
Fraction **B** was partitioned further by C_{18} HPLC to yield compounds **9.2** and **9.3** (Scheme 9-3).



Scheme 9-3. Fractionation of B.

Fraction **C** was partitioned further by C_{18} HPLC to yield compounds **9.2**, **9.4**, and **9.5** (Scheme 9-4). Throughout the partitioning of this extract, there were many fractions that exhibited strong bioactivity. However, the major constituents of these fractions were often inactive. The activity of the parent fraction was likely due to the small amount of cardenolides present, such as

9.2 and **9.3**. Unfortunately, these minor constituents could not be completely identified and were not investigated further since cardenolides have been studied extensively.[2,3]

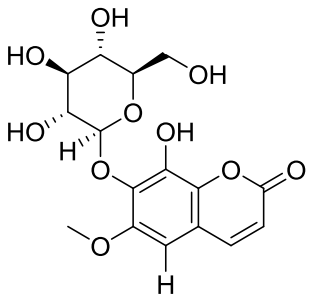


Scheme 9-4. Fractionation of C.

9.2.2 Dereplication of Known Compounds

In order to quickly identify the structures of compounds **9.1–9.5,** database searches utilizing the Dictionary of Natural Products were performed. This resulted in significantly fewer resources being invested in the structure elucidation of the compounds, saving both time and money.

9.2.2.1 Identification of Isofraxetin 6-O-β-D-glucopyranoside



9.1

Figure 9-2. Structure of isofraxetin 6-*O*-β-D-glucopyranoside.

The molecular formula of **9.1** was assigned as $C_{16}H_{18}O_{10}$ using HRESIMS ($[M+Na]^+$ *m/z* 393.0787, calcd for $C_{16}H_{18}NaO_{10}^+$ 393.0792). A quick analysis of the ^1H NMR spectrum showed some easily assignable signals. With only this information, a search was performed in the Dictionary of Natural Products Database, resulting in the identification of nine possible structures (Figure 9-3). The ^1H NMR data for each structure were compared to the experimental data, and the structure of **9.1** was assigned as isofraxetin 6-*O*-β-D-glucopyranoside.[4]

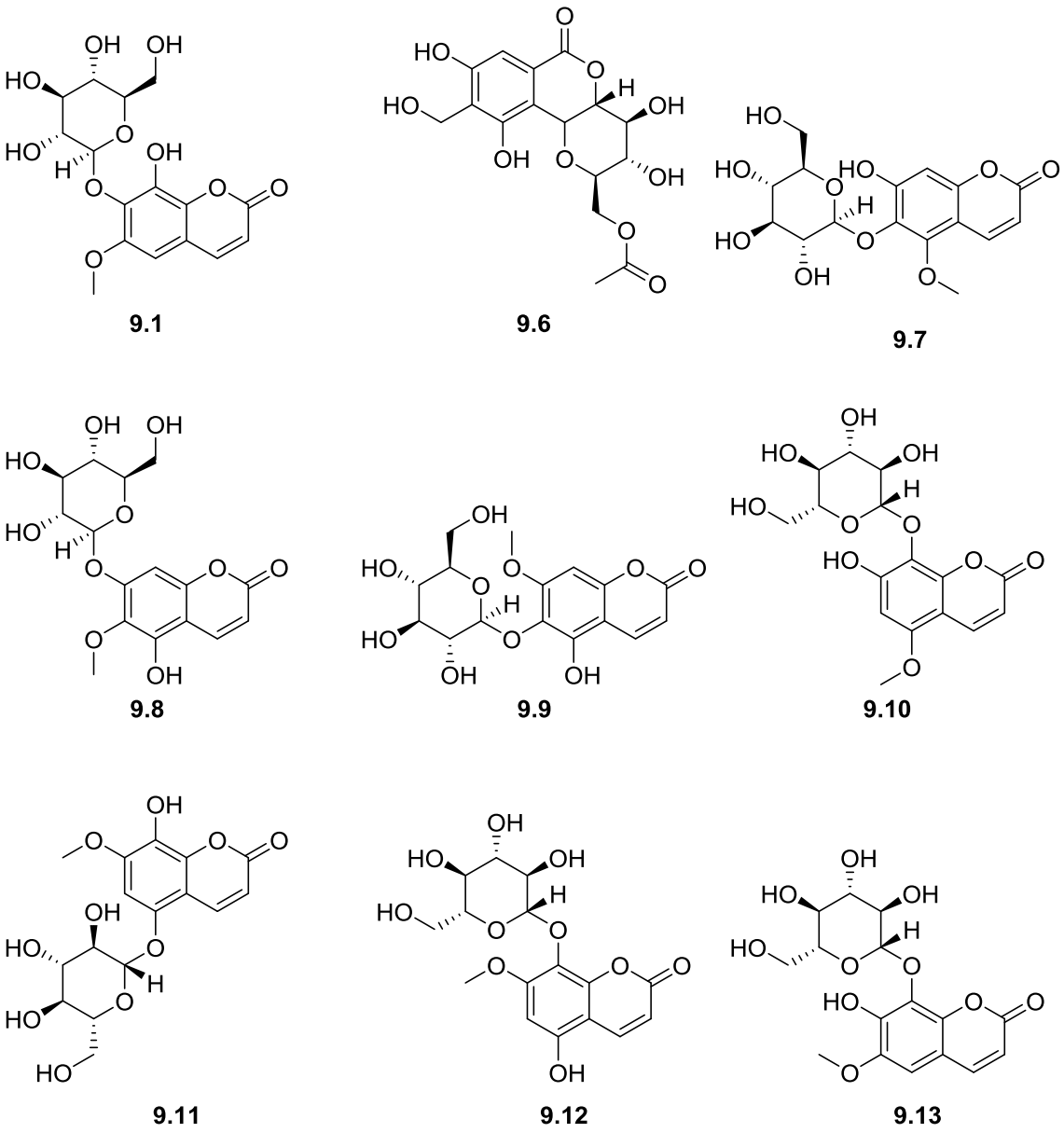
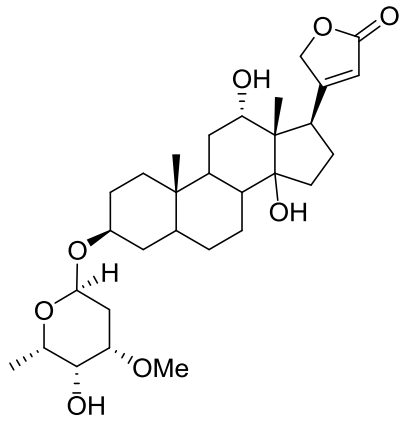


Figure 9-3: Possible structures of **9.1** from DNP search.

9.2.2.2 Identification of Sarmentogenin α-L-diginoside



9.2

Figure 9-4. Structure of sarmentogenin α-L-diginoside.

The same strategy employed to determine the structure of **9.1** was utilized to determine the structure of **9.2**. The molecular formula of **9.2** was found to be $C_{30}H_{46}O_8$ by HRESIMS ($[M+H]^+$ *m/z* 535.3248, calcd for $C_{30}H_{47}O_8^+$ 535.3265). In the ^1H NMR spectrum, there were three signals corresponding to $-CH_3$ groups (δ_H 0.90, s; δ_H 1.08, s; δ_H 1.21, d). Using only these easily identifiable features, a search was performed which resulted in 21 possible structures, containing four aglycones (Figure 9-5). ^1H NMR data were compared with those of the known compounds, and the structure was determined to be sarmentogenin α-L-diginoside.[5]

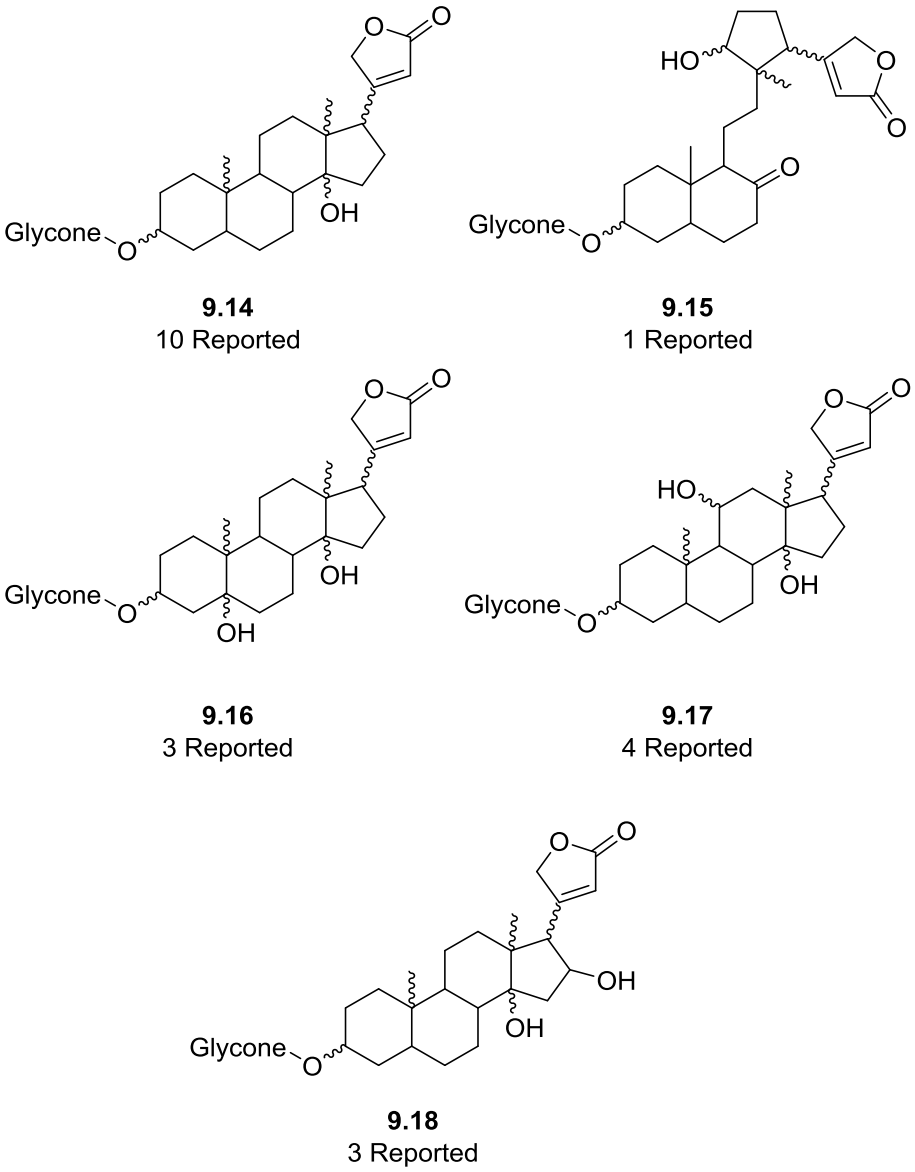


Figure 9-5. Possible aglycones of **9.2**.

9.2.3 Identification of Sarmentogenin α-L-diginosyl-β-D-glucopyranoside

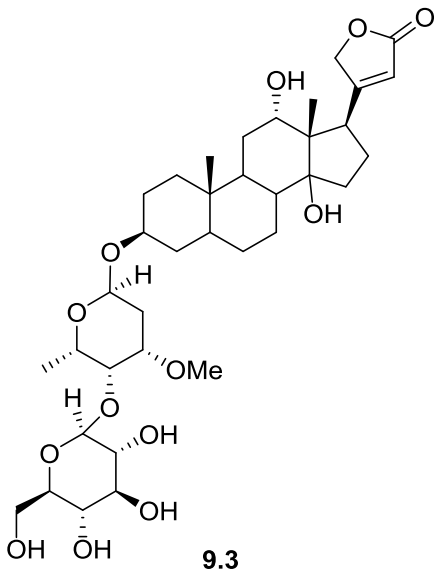
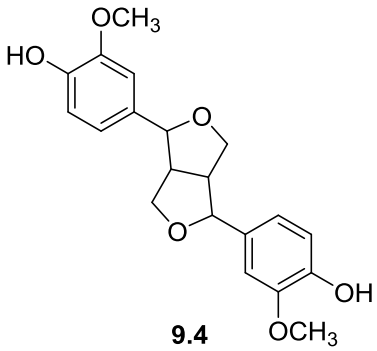


Figure 9-6. Structure of sarmentogenin α-L-diginosyl-β-D-glucopyranoside.

Unlike the structure determinations of **9.1** and **9.2**, the structure of **9.3** was not identified through a database search. The molecular formula was determined to be $C_{36}H_{56}O_{13}$ by HRESIMS ($[M+Na]^+$ *m/z* 719.3652, calcd for $C_{36}H_{56}NaO_{13}^+$ 719.3613). Based upon similarities in the ^1H NMR spectra of **9.3** and **9.2** (including peaks at δ_H 0.90, s; δ_H 1.07, δ_H 5.91) the aglycone was determined to be identical. The L-diginosyl moiety was also determined to be present, based upon comparison of ^1H NMR data with those of **9.2**. In addition, there was a glucopyranose moiety present based on the molecular formula and the appearance of additional signals in the ^1H NMR spectrum, including the signal for the anomeric proton at δ_H 4.62 (d, J = 7.8 Hz). The signals in the ^1H NMR spectrum for the aglycone and the sugar moieties are consistent with published values for sarmentogenin α-L-diginosyl-β-D-glucopyranoside.[5]

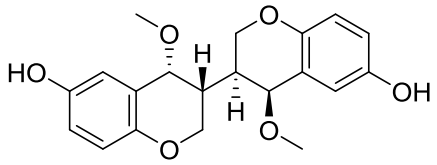
9.2.3.1 Identification of Pinoresinol



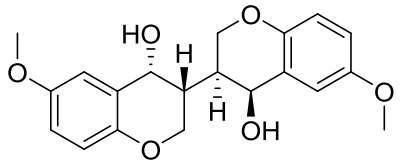
9.4

Figure 9-7. Structure of pinoresinol.

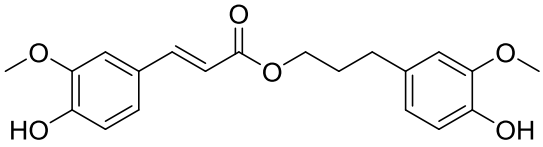
In order to determine the structure of **9.4**, the same strategy used for **9.1** and **9.2** was employed. The molecular formula was determined to be $C_{20}H_{22}O_6$ based on HRESIMS data ($[M+H]^+$ *m/z* 359.1470, calcd for $C_{20}H_{23}O_6^+$ 359.1489). The ^{1}H NMR spectrum suggested the presence of a 1,2,4-trisubstituted benzene moiety (B124; δ_H 6.95, d, J = 1.9 Hz; δ_H 6.82, ddd, J = 8.1, 1.9, 0.6 Hz; δ_H 6.71, d, J = 8.1 Hz) and a methoxy moiety (δ_H 3.86, s). However, when a search was performed based on the molecular formula, the presence of one benzene ring moiety, and the presence of one methoxy moiety, the search engine returned no results. Hence, it was postulated that the structure could be symmetrical based on the relatively small number of signals present in the ^{1}H NMR spectrum for the calculated molecular formula. Another search was performed assuming the compound was symmetrical (2 – B124 and 2 – methoxy moieties), which resulted in 16 possible structures (Figure 9-8). The published ^{1}H NMR data was compared with experimental data and the structure was assigned as pinoresinol.[6] Since the compound did not possess antiproliferative properties, the absolute configuration was not determined.



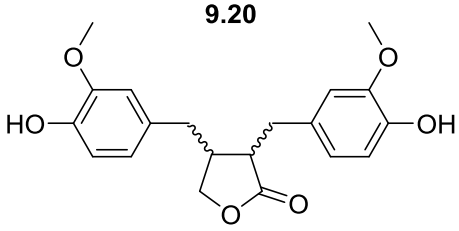
9.19



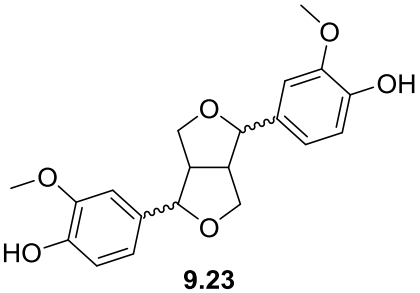
9.20



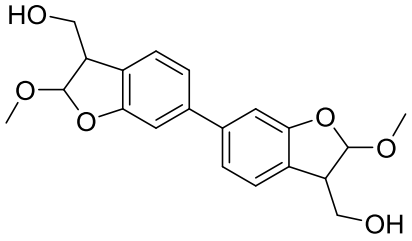
9.21



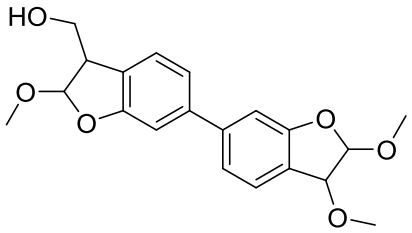
9.22
3 Reported Stereoisomers



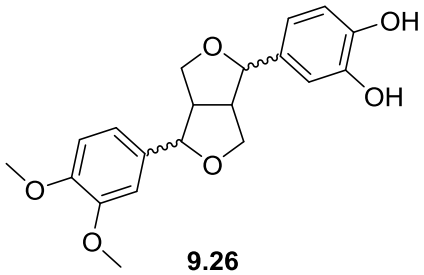
9.23
4 Reported Stereoisomers



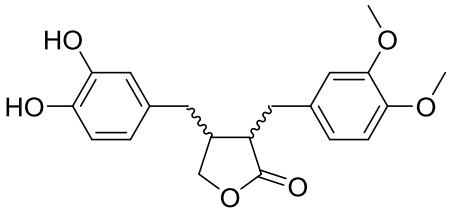
9.24



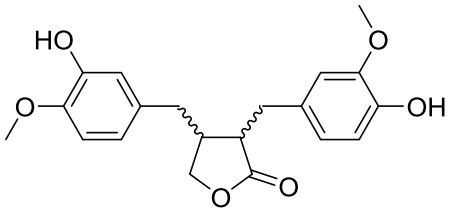
9.25



9.26
2 Reported Stereoisomers



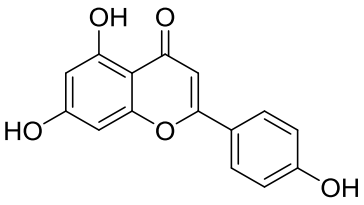
9.27



9.28

Figure 9-8. Possible structures of **9.4**.

9.2.3.2 Identification of Apigenin



9.5

Figure 9-9. Structure of apigenin.

The molecular formula of **9.5** was found to be $C_{15}H_{10}O_5$ by HRESIMS ($[M+H]^+$ *m/z* 271.5093, calcd for $C_{15}H_{11}O_5^+$ 271.0593). The ^1H NMR spectrum showed characteristic signals of AA′BB′ and ABX spin systems suggesting a 1,4-disubstituted benzene moiety (B14) and a 1,2,3,5-tetrasubstituted benzene moiety (B1235; δ_H 7.85, 2H, d, J = 8.8 Hz; δ_H 6.93, 2H, d, J = 8.8 Hz). A search was performed, resulting in eight possible structures (Figure 9-10). The experimental ^1H NMR data were compared with published values and the structure was assigned as apigenin.

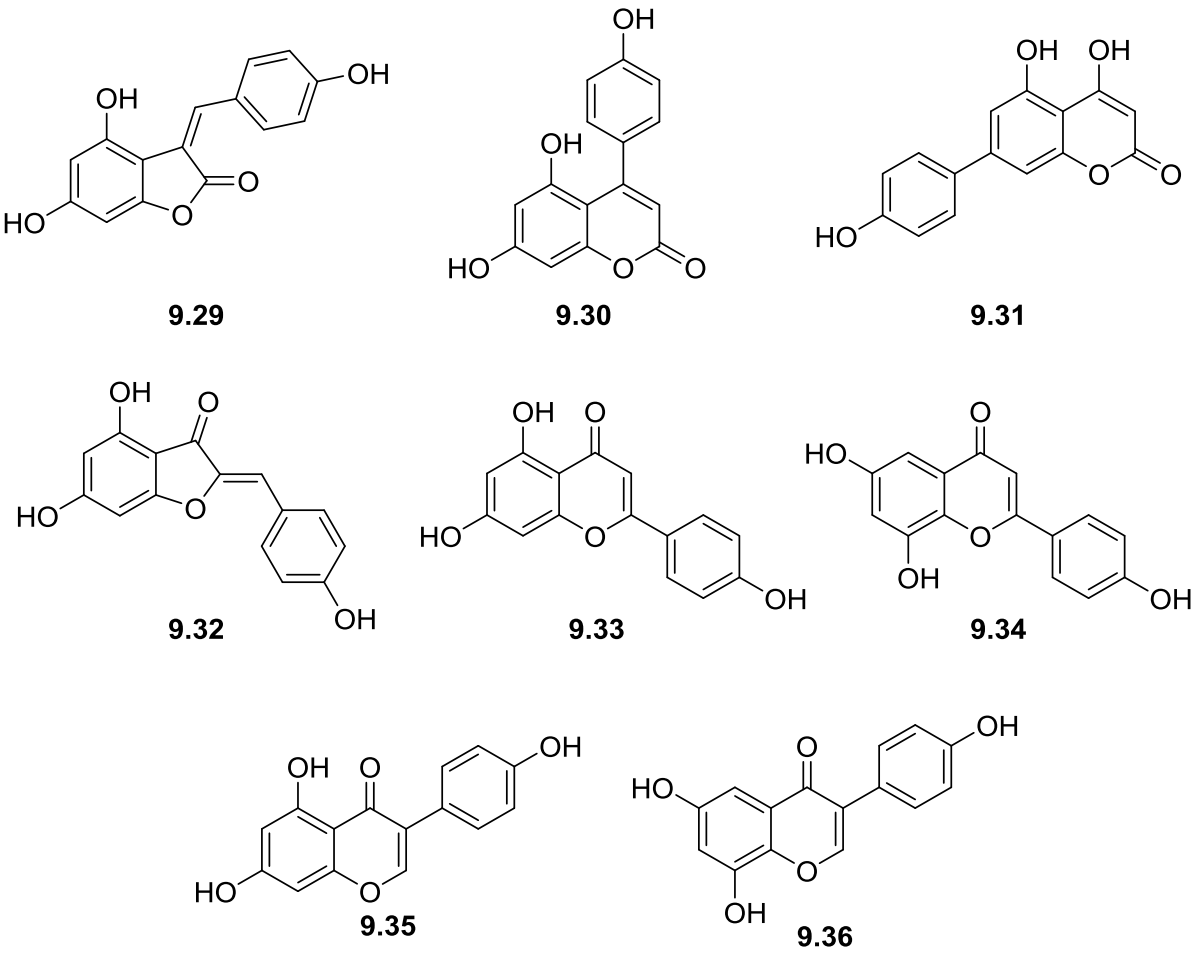


Figure 9-10. Possible structures of **9.5**.

9.2.4 Antiproliferative Activity

Compounds **9.1–9.5** were tested for their antiproliferative activity against the A2780 ovarian cancer cell line.. The active sarmentogenins have previously been reported to be cytotoxic.[7] Pinoresinol has been reported to have antioxidant properties.[8,9] Apigenin has previously been reported to have antiproliferative[10] and antimutagenic properties. [11]

Table 9-1: Activities of isolated compounds against the A2780 cell line and *Plasmodium falciparum.*

Compound	Antiproliferative activity against A2780, IC_{50} (μM)
Isofraxetin 6-*O*-β-D-glucopyranoside (**9.1**)	>54
Sarmentogenin α-L-diginoside (**9.2**)	0.3
Sarmentogenin α-L-diginosyl-β-D-glucopyranoside (**9.3**)	1
Pinoresinol (**9.4**)	>56
Apigenin (**9.5**)	>74

9.3 Experimental Section

9.3.1 General Experimental Procedures

^{1}H and ^{13}C NMR spectra were recorded using either a Bruker Advance 500 spectrometer. ^{1}H-^{1}H three-bond *J*-coupling values were calculated from ^{1}H NMR spectra. Mass spectra were obtained on an Agilent 6220 LC-TOF-MS. Semi-preparative HPLC was performed using Shimadzu LC-10AT pumps coupled with a Shimadzu SPD M10A diode array detector, a SCL-10A system controller, and a Cogent Bidentate C_{18} column (250 x 10 mm).

9.3.2 Plant Material

Dr. Michael Goetz provided the ethanol extract of the above ground portions of *Curroria sp.* from the Natural Product Discovery Institute (NPDI) repository.

9.3.3 Extraction and Isolation

The ethanol extract (1.5 g, IC_{50} 10 μg/mL) of the above ground portion of *Curroria sp.* (Asclepiadaceae) was dissolved in methanol and extracted with hexanes. The solvent was evaporated from the methanol partition, dissolved in water, and extracted with ethyl acetate. The ethyl acetate fraction (417 mg, IC_{50} 1 μg/mL) was partitioned using Sephadex LH-20 CC (1:1 DCM/MeOH) to yield a fraction with increased activity (315 mg, IC_{50} 0.6 μg/mL). This fraction was partitioned further using C_{18} solid phase extraction (MeOH/H_2O) yielding a fraction with increased activity that was partitioned with Diaion CC (H_2O/MeOH/$(CH_3)_2CO$) to yield **A** (94 mg, IC_{50} 0.7 μg/mL), **B** (30 mg, IC_{50} 0.2 μg/mL), and **C** (62 mg, IC_{50} 0.4 μg/mL). Fraction **A** was partitioned using silica flash CC ($CHCl_3$/MeOH) to yield three fractions with increased activity (5 mg, IC_{50} 0.8 μg/mL; 3 mg, IC_{50} 0.1 μg/mL; 4 mg, IC_{50} 0.1 μg/mL). These three fractions were partitioned using C_{18} HPLC (CH_3CN/H_2O) and compound **9.1** was identified (4 mg, IC_{50} >54 μM). Fraction **B** was partitioned using C_{18} HPLC (CH_3CN/H_2O) to yield **9.2** (2 mg, IC_{50} 0.3 μM) and **9.3** (2 mg, IC_{50} >1 μM). Fraction **C** was partitioned using C_{18} HPLC (CH_3CN/H_2O) to yield **9.2** (2 mg, IC_{50} 0.3 μM), **9.4** (1 mg, IC_{50} >56 μM), and **9.5** (1 mg, IC_{50} >74 μM)

9.3.4 Antiproliferative Bioassay

Assay was performed at Virginia Tech according to specifications previously described.[12] The A2780 cell line is a drug-sensitive ovarian cancer cell line.[13]

9.3.5 Spectroscopic Properties

Isofraxetin 6-*O*-β-D-glucopyranoside (9.1): Partial ^{1}H NMR spectrum (500 MHz, CD_3OD): 3.63 (3H, s), 4.44 (1H, d *J* = 7.4 Hz), 5.56 (1H, d, *J* = 9.0 Hz), 6.55 (1H, s) 7.59 (1H, d, *J* = 9.0 Hz), Table 3-2; HRESIMS $[M+Na]^+$ *m/z* 393.0787 (calcd for $C_{16}H_{18}Na_{10}^+$ 393.0792), $[M+K]^+$ *m/z* 409.0527 (calcd for $C_{16}H_{18}KO_{10}^+$ 409.0532), *m/z* $[2M+Na]^+$ 763.1657 (calcd for $C_{32}H_{36}NaO_{20}^+$ 763.1692).

Sarmentogenin α-L-diginoside (9.2): Partial ^{1}H NMR spectrum (500 MHz, CD_3OD): 0.90 (3H, s), 1.08 (3H, s), 1.21 (3H, d, *J* = 6.3 Hz), 3.43 (3H, s), 4.96 (1H, bs), 5.92 (1H, bs); HRESIMS $[M+H]^+$ *m/z* 535.3248 (calcd for $C_{30}H_{47}O_8^+$ 535.3265), $[M+ NH_4]^+$ *m/z* 552.3519 (calcd for $C_{30}H_{50}NO_8^+$ 552.3531), $[M+Na]^+$ *m/z* 557.3079 (calcd for $C_{30}H_{46}NaO_8^+$ 557.3085), $[M+K]^+$ *m/z* 573.2875 (calcd for $C_{30}H_{46}KO_8^+$ 573.2824), $[2M+Na]^+$ *m/z* 1091.6269 (calcd for $C_{60}H_{92}NaO_{16}^+$ 1091.6278).

Sarmentogenin α-L-diginosyl-β-D-glucopyranoside (9.3): Partial ^{1}H NMR spectrum (500 MHz, CD_3OD): 0.90 (3H, s), 1.07 (3H, s), 1.27 (3H, d, *J* = 6.3 Hz), 3.41 (3H, s), 4.62 (1H, d *J* = 7.8 Hz), 4.97 (1H, bs), 5.91 (1H, bs); HRESIMS $[M+Na]^+$ *m/z* 719.3652 (calcd for $C_{36}H_{56}NaO_{13}^+$ 719.3613).

Pinoresinol (9.4): ^{1}H NMR spectrum (500 MHz, CD_3OD): 3.16 (2H, m), 3.84 (2H, dd, *J* = 9.1, 3.5 Hz), 3.86 (6H, s), 4.24 (2H, dd, *J* = 9.0, 6.8 Hz), 4.71 (2H, d, *J* = 4.2 Hz), 6.77 (2H, d, *J* = 8.1 Hz), 6.82 (2H, ddd, *J* = 8.1, 1.9, 0.6), 6.95 (1H, d, *J* = 1.9 Hz); HRESIMS $[M+H]^+$ *m/z* 359.1470 (calcd for $C_{20}H_{23}O_6^+$ 359.1489).

Apigenin (9.5): ^{1}H NMR (500 MHz, CD_3OD): 6.18 (1H, d, *J* = 2.1 Hz), 6.42 (1H, d, *J* = 2.1 Hz), 6.57 (1H, s), 6.93 (2H, d, *J* = 8.8 Hz), 7.85 (2H, d, *J* = 8.8 Hz); HRESIMS $[M+H]^+$ *m/z* 271.0593 (calcd for $C_{15}H_{11}O_5^+$ 271.0601).

9.4 References

1. Ntie-Kang, F.; Yong, J. N., The chemistry and biological activities of natural products from Northern African plant families: from Aloaceae to Cupressaceae. *Rsc Adv* **2014,** *4* (106), 61975-61991.

2. Slingerland, M.; Cerella, C.; Guchelaar, H. J.; Diederich, M.; Gelderblom, H., Cardiac glycosides in cancer therapy: from preclinical investigations towards clinical trials. *Invest. New Drugs* **2013,** *31* (4), 1087-1094.

3. Steyn, P. S.; van Heerden, F. R., Bufadienolides of plant and animal origin. *Nat. Prod. Rep.* **1998,** *15* (4), 397-413.

4. Li, Z.; Li, X.; Li, D.; Gao, L.; Xu, J.; Wang, Y., A new coumarin glycoside from the husks of *Xanthoceras sorbifolia*. *Fitoterapia* **2007,** *78* (7-8), 605-606.

5. Chen, R.-F.; Abe, F.; Yamauchi, T.; Taki, M., Cardenolide glycosides of *Strophanthus divaricatus*. *Phytochemistry* **1987,** *26* (8), 2351-2355.

6. Xie, L.-H.; Akao, T.; Hamasaki, K.; Deyama, T.; Hattori, M., Biotransformation of pinoresinol diglucoside to mammalian lignans by human intestinal microflora, and isolation of *Enterococcus faecalis* strain PDG-1 responsible for the transformation of (+)-pinoresinol to (+)-lariciresinol. *Chem. Pharm. Bull.* **2003,** *51* (5), 508-515.

7. Ankli, A.; Heilmann, J.; Heinrich, M.; Sticher, O., Cytotoxic cardenolides and antibacterial terpenoids from *Crossopetalum gaumeri*. *Phytochemistry* **2000,** *54* (5), 531-537.

8. Yi, B.; Hu, L.; Mei, W.; Zhou, K.; Wang, H.; Luo, Y.; Wei, X.; Dai, H., Antioxidant phenolic compounds of cassava (*Manihot esculenta*) from Hainan. *Molecules* **2011,** *16* (12), 10157-10167.

9. Li, W. J.; Zhang, Z. H.; Cheng, X. L.; Liu, J.; He, Y.; Zhou, C.; Guo, Y.; Lin, R. C.; Wang, G. L., Two new compounds isolated from *Liriope muscari*. *Molecules* **2012,** *17* (8), 8773-8781.

10. Csupor-Löffler, B.; Hajdú, Z.; Zupkó, I.; Molnár, J.; Forgo, P.; Vasas, A.; Kele, Z.; Hohmann, J., Antiproliferative constituents of the roots of *Conyza canadensis*. *Planta Med.* **2011,** *77* (11), 1183-1188.

11. Miyazawa, M.; Hisama, M., Antimutagenic activity of flavonoids from *Chrysanthemum morifolium*. *Biosci., Biotechnol., Biochem.* **2003,** *67* (10), 2091-2099.

12. Cao, S.; Brodie, P. J.; Miller, J. S.; Randrianaivo, R.; Ratovoson, F.; Birkinshaw, C.; Andriantsiferana, R.; Rasamison, V. E.; Kingston, D. G. I., Antiproliferative xanthones of *Terminalia calcicola* from the Madagascar rain forest. *J. Nat. Prod.* **2007,** *70* (4), 679-681.

13. Louie, K. G.; Behrens, B. C.; Kinsella, T. J.; Hamilton, T. C.; Grotzinger, K. R.; McKoy, W. M.; Winker, M. A.; Ozols, R. F., Radiation survival parameters of antineoplastic drug-sensitive and drug-resistant human ovarian-cancer cell-lines and their modification by buthionine sulfoximine. *Cancer Res.* **1985,** *45* (5), 2110-2115.

Chapter 10: Identification of Potentially Anti-inflammatory Compounds from *Streblus dimepate*

10.1 Introduction

10.1.1 Abstract

An ethanol extract of *Streblus dimepate* (Bureau) C. C. Berg (Moriaceae) was investigated on the basis of its anti-inflammatory activity. Bioassay-guided fractionation of the extract utilizing liquid–liquid partitioning, column chromatography, and HPLC led to the identification of two known compounds, (2*Z*,3*S*,4*S*)-2-(11-dodecenylidene)-3-hydroxy-4-methylbutanolide and (2*Z*)-2-(dodecylidene)-3-hydroxy-4-methylbutanolide. The structures were determined by using spectroscopic methods. This work has not been published elsewhere.

Figure 10-1. *Streblus dimepate*. Used under Creative Commons (CC BY-NC-ND 3.0) from <http://www.tropicos.org/Image/100292631>.

10.1.2 Author Contributions

The author (Alexander L. Eaton) of this dissertation completed the fractionation of the extract, identification the described compounds, and drafting of this manuscript. Chris Birkinshaw led the team that collected and identified the plant. Xiaoying Zhang and Dr. Josep Bassaganya-Riera provided testing for anti-inflammatory activity. Dr. David G. I. Kingston was a mentor for this work.

10.1.3 Previous Investigations of Streblus dimepate

Streblus dimepate (Bureau) C.C. Berg, a member of the Moraceae family, is endemic to Madagascar. The genus contains approximately 25 species that have a variety of uses including paper making and medicinal purposes.[1] *S. asper* has been shown to have anti-inflammatory properties.[2,3]

10.1.4 Chemical Investigation of Streblus dimepate

As a part of our ongoing search for bioactive compounds from Madagascan plants, an extract of the leaves and fruits of *Streblus dimepate* was selected for investigation due to its activity as an agonist of PPAR-γ. Two compounds were identified (Figure 10-2), (2*Z*,3*S*,4*S*)-2-(11-dodecenylidene)-3-hydroxy-4-methylbutanolide (**10.1**) and (2*Z*)-2-(dodecylidene)-3-hydroxy-4-

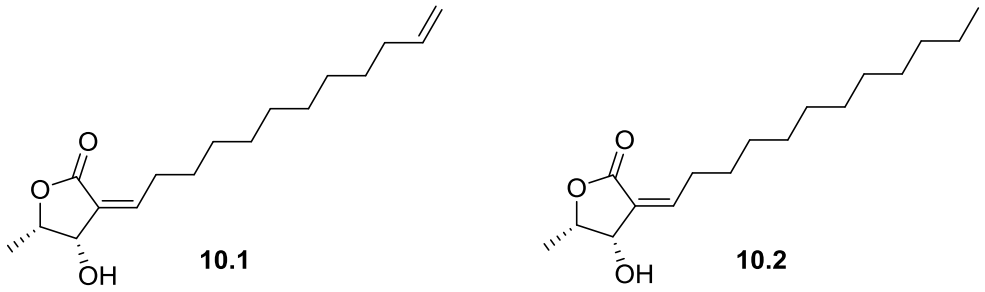


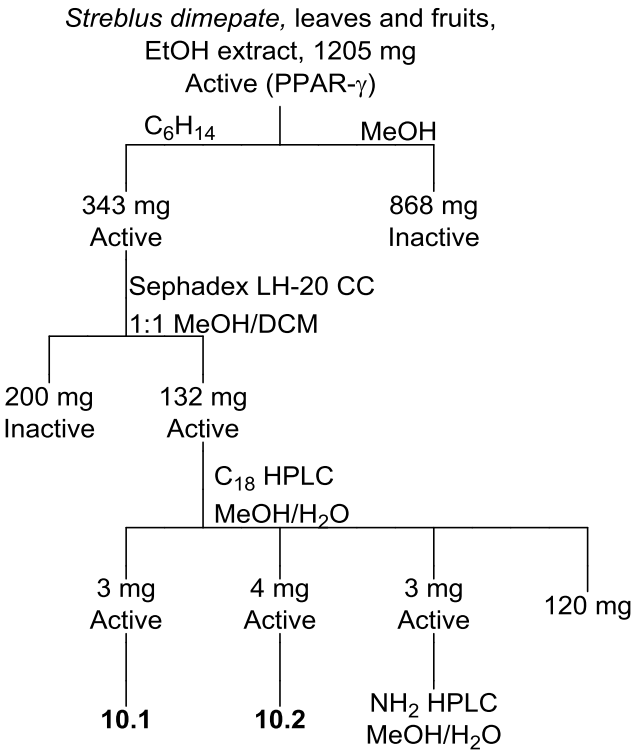
Figure 10-2. Structures of compounds identified from *Streblus dimepate*.

methylbutanolide (**10.2**), through bioassay guided fractionation. The isolation, structure elucidation, and the bioactivity of these compounds will be discussed.

10.2 Results and Discussion

10.2.1 Fractionation of Streblus dimepate

Liquid–liquid partitioning of the ethanol extract of the fruits and leaves of *Streblus dimepate*, yielded an active hexane fraction. This fraction was partitioned further by Sephadex LC-20 column chromatography to yield one active fraction. The active fraction was further purified using C_{18} HPLC to yield three semi-pure components. Two components (**10.1–10.2**) were identified by ^{1}H NMR spectroscopy, ^{13}C NMR spectroscopy, and MS. Purification of the third component was attempted on NH_2 HPLC, but the activity was lost and ^{1}H NMR spectroscopy indicated that the major component appeared to be degraded, so its structure was not determined. Other small amounts of structurally related compounds appeared to be present based on the UV spectra of HPLC peaks. However, isolation was discontinued for two reasons. Firstly, the bioassay required larger amounts of compound (~4 mg) to test than could be isolated from the available material. Secondly, it was requested that another plant extract (*Oncostemum bojerianum*) be prioritized to produce initial results for a grant application. The extraction is outlined in Scheme 10-1 and a detailed procedure can be found in the Experimental Section (10.3).



Scheme 10-1. Fractionation of *Streblus dimepate.*

10.2.2 Identification of (2Z,3S,4S)-2-(11-dodecenylidene)-3-hydroxy-4-methylbutanolide

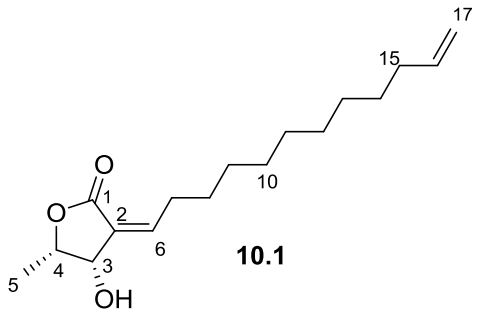


Figure 10-3. Structure of (2*Z*,3*S*,4*S*)-2-(11-dodecenylidene)-3-hydroxy-4-methylbutanolide (**10.1**).

The structure of (2*Z*,3*S*,4*S*)-2-(11-dodecenylidene)-3-hydroxy-4-methylbutanolide (**10.1**) was determined using ^{1}H NMR spectrometry, ^{13}C NMR spectrometry, and HRESIMS. From HRESIMS, the molecular formula was determined to be $C_{17}H_{28}O_3$ ($[M+H]^+$ *m/z* 281.2117, calcd for $C_{17}H_{29}O_3^+$ 281.2111) which indicates that the structure has four degrees of unsaturation. The ^{1}H NMR spectrum had signals at δ_H 2.03 (H-15, bq, *J* = 6.8 Hz), δ_H 5.81 (H-16, ddt, *J* = 16.9, 10.2, 6.8 Hz), δ_H 4.99 (H-17a, ddt, *J* = 17.1, 2.3, 1.6 Hz), and δ_H 4.93 (H-17b, ddt, *J* = 10.1, 2.3, 1.2 Hz) suggesting the presence of a terminal alkene. This was consistent with the ^{13}C NMR spectrum, which had signals at δ_C 33.8 (C-15), δ_C 139.3 (C-16), and δ_C 114.1 (C-17). The ^{1}H and ^{13}C NMR spectra also indicated the presence of a long saturated hydrocarbon chain (δ_H 1.27, m, H-9 – H-13; δ_C 29.5, 29.4, 29.4, 29.3, 29.1, 28.9, 28.8, C-8 – C-14). Furthermore, the ^{13}C NMR spectrum suggested the presence of an ester moiety (δ_C 168.5, C-1). Using this data, a search was performed using the Dictionary of Natural Products, and the structure was proposed to be 2-(11-dodecenylidene)-3-hydroxy-4-methylbutanolide. The configuration of the alkene moiety at C-2 was determined to be *Z* by comparison of ^{1}H NMR data with both *E* (δ_H ~6.95) and *Z* (δ_H ~6.55) isomers of similar compounds.[4-6] The relative configuration of the groups at C-3 and C-4 was determined to be *cis* based on comparison of observed coupling constants and ^{1}H NMR shifts with

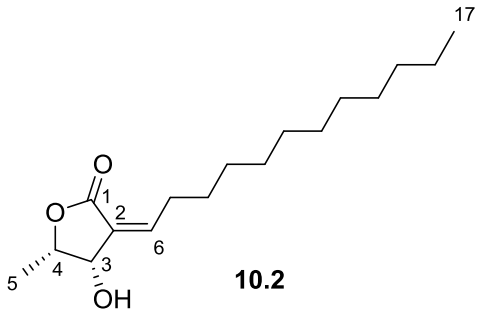
reference compounds.[4-6] Finally, the absolute configuration was determined to be (2*Z*,3*S*,4*S*) based on comparison of optical rotation data with data of the known compound.[5] Thus, the structure of (**10.1**) is (2*Z*,3*S*,4*S*)-2-(11-dodecenylidene)-3-hydroxy-4-methylbutanolide. Complete ^{13}C and ^{1}H NMR spectroscopy assignments can be seen in Table 10-1.

Table 10-1. NMR data of (2*Z*,3*S*,4*S*)-2-(11-dodecenylidene)-3-hydroxy-4-methylbutanolide (**10.1**).

position	δ_C, type	δ_H (*J* in Hz)
1	168.5, C	
2	129.3, C	
3	71.4, CH	4.66, dd (5.4, 1.1)
4	77.7, CH	4.56, qd (6.5, 5.3)
5	14.11, CH_3	1.40, d (6.5)
6	149.7, CH	6.56, td (7.7, 1.3)
7	27.9, CH_2	2.75, m
8	28.8[a], CH_2	1.47, m
9	29.5[a], CH_2	1.27, m[b]
10	29.4[a], CH_2	1.27, m[b]
11	29.4[a], CH_2	1.27, m[b]
12	29.3[a], CH_2	1.27, m[b]
13	29.1[a], CH_2	1.27, m[b]
14	28.9[a], CH_2	1.37, m
15	33.8, CH_2	2.03, bq (6.8)
16	139.2, CH	5.81, ddt (16.9, 10.2, 6.7)
17	114.1, CH_2	4.99, ddt (17.1, 2.3, 1.6)
		4.93, ddt (10.2, 2.3, 1.2)

Obtained in $CDCl_3$, 500 MHz (δ_H), 125 MHz (δ_C)
[a]Interchangeable Assignments
[b]Overlapping signals

10.2.3 Identification of (2Z)-2-(dodecylidene)-3-hydroxy-4-methylbutanolide



10.2

Figure 10-5. Structure of (2*Z*)-2-(dodecylidene)-3-hydroxy-4-methylbutanolide (**10.2**).

The structure of (2*Z*)-2-(dodecylidene)-3-hydroxy-4-methylbutanolide (**10.2**) was determined using ^{1}H NMR spectroscopy, ^{13}C NMR spectroscopy, and HRESIMS. The determination of **10.2** proved to be significantly more challenging that that of **10.1** due to the fact that it was not completely pure, as determined by LC-MS. A significant difference in the structure of **10.2** is the presence of an additional signal in the ^{1}H NMR (H-17, δ_H 0.89, t, J = 6.7 Hz) and ^{13}C NMR (δ_C 14.1) spectra, suggesting the presence of a terminal methyl group. While there were still signals present in the ^{1}H NMR spectrum (δ_H 2.03, δ_H 5.81, δ_H 4.99, δ_H 4.93) suggesting the presence of a terminal alkene, they were reduced in intensity; furthermore, the corresponding signals in the ^{13}C NMR spectrum were either absent (δ_C ~139.5) or significantly reduced in intensity (δ_C 114.1, δ_C 33.8). From HRESIMS and LCMS data the molecular formula was determined to be $C_{17}H_{30}O_3$ ($[M+H]^+$ *m/z* 283.2280, calcd for $C_{17}H_{31}O_3^+$ 283.2268). Thus, the structure was proposed as (2*Z*)-2-(dodecylidene)-3-hydroxy-4-methylbutanolide (**10.2**). Since the compound was identified from a mixture, the absolute configuration could not be confirmed; however, based on the ^{1}H NMR data, its relative stereochemistry is identical to that of (2*Z*,3*S*,4*S*)-2-(11-dodecenylidene)-3-hydroxy-4-methylbutanolide (**10.1**). Complete assignment of ^{1}H and ^{13}C

NMR spectroscopdata can be found in Table 10-2. NMR spectroscopic data is in agreement with published values for (2Z,3*R*,4*R*)-2-(dodecylidene)-3-hydroxy-4-methylbutanolide.[7]

Table 10-2. ^{1}H and ^{13}C NMR Data of (2*Z*)-2-(dodecylidene)-3-hydroxy-4-methylbutanolide (**10.2**).

position	δ_C, type	δ_H (*J* in Hz)
1	168.5, C	
2	129.3, C	
3	71.4, CH	4.66, dd (5.4, 1.2)
4	77.7, CH	4.56, qd (6.5, 5.3)
5	14.1, CH_3	1.40, d (6.6)
6	149.7, CH	6.56, td (7.7, 1.3)
7	27.9, CH_2	2.75, m
8	28.8[a], CH_2	1.47, m
9	29.6[a], CH_2	1.27, m[b]
10	29.6[a], CH_2	1.27, m[b]
11	29.5[a], CH_2	1.27, m[b]
12	29.4[a], CH_2	1.27, m[b]
13	29.3[a], CH_2	1.27, m[b]
14	29.5[a], CH_2	1.27, m
15	31.9, CH_2	2.03, bq (6.8)
16	22.7, CH_2	1.31, m
17	14.1, CH_3	4.99, ddt (17.1, 2.3, 1.6)

Obtained in $CDCl_3$, 500 MHz (δ_H), 125 MHz (δ_C)
[a]Interchangeable Assignments
[b]Overlapping signals

10.2.4 Anti-inflammatory Activity

Due to the large amount of pure compound (~3 mg) required to obtain an accurate EC_{50} value, the bioactivities of **10.1** and **10.2** were not determined. Butenolides, including **10.1**, have been reported to be cytotoxic.[5,8,9]

10.3 Experimental Section

10.3.1 General Experimental Procedures

Optical rotations were recorded on a JASCO P-2000 polarimeter. ^{13}C NMR spectra were recorded using a Bruker Advance 500 spectrometer. ^{1}H-^{1}H three-bond *J*-coupling values were calculated from ^{1}H NMR spectra. Mass spectra were obtained on an Agilent 6220 LC-TOF-MS or a Thermo Electron TSQ LC-ESI-MS. Semi-preparative HPLC was performed using Shimadzu LC-10AT pumps coupled with a Shimadzu SPD M10A diode array detector, a SCL-10A system controller, and either a Cogent Bidentate C_{18} column (250 x 10 mm) or a Cogent Amino HPS column (250 x 10 mm).

10.3.2 Plant Material

Leaves and fruits of *Streblus dimepate* (Bureau) C. C. Berg (Moriaceae) (collection: Chris Birkinshaw et al. 604) were collected at an elevation of 1200 m in December 1999 from a community forest outside Zahamena National Park, 3 km east of Andranomalaza Atsimo (17°39′12" S 048°38′39" E). The sample was collected from a 6 m tall tree with white bark and immature green fruit.

10.3.3 Extraction and Isolation

The ethanol extract (1205 mg) of the leaves and fruit of *Streblus dimepate* was dissolved in methanol and extract with hexanes. The active hexanes fraction (343 mg) was partitioned using Sephadex LH-20 open column chromatography (1:1 MeOH/DCM). This yielded on active fraction (132 mg) which was further purified by C_{18} HPLC (MeOH/H_2O gradient) to yield three active fractions (3 mg, 4 mg, 3 mg, respectively). The first fraction was identified as (2*Z*)-2-(dodecylidene)-3-hydroxy-4-methylbutanolide (**10.1**). The major component of the second fraction was identified as (2*Z*,3*S*,4*S*)-2-(11-dodecenylidene)-3-hydroxy-4-methylbutanolide (**10.2**). Purification of the third fraction was attempted using NH_2 HPLC (MeOH/H_2O gradient); however, the compound decomposed.

10.3.4 Anti-inflammatory Assay

Anti-inflammatory activity was determined using a cell-based PPAR γ reporter assay as previously described.[10]

10.3.5 Spectroscopic Properties

(2*Z*,3*S*,4*S*)-2-(11-dodecenylidene)-3-hydroxy-4-methylbutanolide (10.1): $[\alpha]_D^{23}$ –35 (*c* 0.046, $CHCl_3$); ^1H NMR (500 MHz, $CDCl_3$): See Table 3-2; ^{13}C NMR (125 MHz, $CDCl_3$): See Table 3-2; HRESIMS $[M+H]^+$ *m/z* 281.2117 (calcd for $C_{17}H_{29}O_3^+$ 281.2111), $[M+NH_4]^+$ *m/z* 298.2390 (calcd for $C_{17}H_{32}NO_3^+$ 298.2377), $[M+Na]^+$ *m/z* 303.1946 (calcd for $C_{17}H_{28}NaO_3^+$ 303.1931), $[2M+NH_4]^+$ *m/z* 578.4502 (calcd for $C_{34}H_{60}NO_6^+$ 578.4415), $[2M+Na]^+$ *m/z* 583.3906 (calcd for $C_{34}H_{56}NaO_6^+$ 583.6969), $[2M+K]^+$ *m/z* 599.3813 (calcd for $C_{34}H_{56}KO_6^+$ 599.3708).

(2*Z*)-2-(dodecylidene)-3-hydroxy-4-methylbutanolide (10.2): 1H NMR (500 MHz, CDCl3): See Table 3-1; ^{13}C NMR (125 MHz, $CDCl_3$): See Table 3-1; HRESIMS $[M+H]^+$ *m/z* 283.2280 (calcd for $C_{17}H_{31}O_3^+$ 183.2268), $[M+NH_4]^+$ *m/z* 300.2548 (calcd for $C_{17}H_{34}NO_3^+$ 300.2533), $[M+Na]^+$ *m/z* 305.2110 (calcd for $C_{17}H_{30}NaO_3^+$ 305.2087), *m/z* $[2M+K]^+$ 603.3944 (calcd for $C_{34}H_{60}KO_6^+$ 603.4021).

10.4 References

1. Mabberley, D. J., *The Plant-Book: A Portable Dictionary of the Vascular Plants*. 2nd ed.; University Press: Cambridge, 1997.

2. Agrawal, V. K.; Gambhir, S. S.; Wahi, A. K., A preliminary study on anti-inflammatory activity of some medicinal plants. *Indian J. Nat. Prod.* **1994,** *10* (1), 14-15.

3. Sripanidkulchai, B.; Junlatat, J.; Wara-aswapati, N.; Hormdee, D., Anti-inflammatory effect of *Streblus asper* leaf extract in rats and its modulation on inflammation-associated genes expression in RAW 264.7 macrophage cells. *J. Ethnopharmacol.* **2009,** *124* (3), 566-570.

4. Cheng, W.; Zhu, C.; Xu, W.; Fan, X.; Yang, Y.; Li, Y.; Chen, X.; Wang, W.; Shi, J., Chemical constituents of the bark of *Machilus wangchiana* and their biological activities. *J. Nat. Prod.* **2009,** *72* (12), 2145-2152.

5. Kwon, H. C.; Baek, N. I.; Choi, S. U.; Lee, K. R., New cytotoxic butanolides from *Lindera obtusiloba* BLUME. *Chem. Pharm. Bull.* **2000,** *48* (5), 614-616.

6. Takeda, K.; Sakurawi, K.; Ishii, H., Components of the Lauraceae family—I. *Tetrahedron* **1972,** *28* (14), 3757-3766.

7. Lee, S.-S.; Chang, S.-M.; Chen, C.-H., Chemical cconstituents from *Alseodaphne andersonii*. *J. Nat. Prod.* **2001,** *64* (12), 1548-1551.

8. Mansoor, T. A.; Hong, J.; Lee, C. O.; Sim, C. J.; Im, K. S.; Lee, D. S.; Jung, J. H., New cytotoxic metabolites from a marine sponge *Homaxinella sp*. *J. Nat. Prod.* **2004,** *67* (4), 721-724.

9. Tuchinda, P.; Udchachon, J.; Reutrakul, V.; Santisuk, T.; Taylor, W. C.; Farnsworth, N. R.; Pezzuto, J. M.; Kinghorn, A. D., Bioactive butenolides from *Melodorum fruticosum*. *Phytochemistry* **1991,** *30* (8), 2685-2689.

10. Bassaganya-Riera, J.; Guri, A. J.; Lu, P.; Climent, M.; Carbo, A.; Sobral, B. W.; Horne, W. T.; Lewis, S. N.; Bevan, D. R.; Hontecillas, R., Abscisic acid regulates inflammation via ligand-binding domain-independent activation of peroxisome proliferator-activated receptor gamma. *J. Biol. Chem.* **2011,** *286* (4), 2504-2516.

Chapter 11: Investigations of Other Extracts

11.1 Introduction

11.1.1 Abstract

The extracts discussed in this chapter were selected for investigation because they showed bioactivity during initial screening. However, due to various reasons discussed within, there were no compounds identified from the investigations of these extracts.

11.1.2 Author Contributions

The author (Alexander L. Eaton) of this dissertation performed the fractionation of all of these extracts except that of *Barringtonia racemosa* (Section 11.2.7) which was performed by Ms. Emily Berckman under the supervision of the author. Ms. Peggy Brodie performed the A2780 antiproliferative bioassays on all fractions and compounds. Dr. Jessica D. Wiley and Dr. Maria B. Cassera performed the antimalarial bioassays (*Plasmodium falciparum*, Dd2 strain). Xiaoying Zhang and Dr. Josep Bassaganya-Riera provided testing for anti-inflammatory activity. Dr. David G. I. Kingston was a mentor for this work.

11.2 Antiproliferative Extracts

11.2.1 Viguieranthus pervillei – MG 1401

An ethanol extract of the roots of *Viguieranthus pervillei* (Drake) Villiers (Fabaceae) was investigated on the basis of previously reported antiproliferative activity (IC_{50} 9 μg/mL, A2780). However, after liquid–liquid partitioning, it was found that the original ethanol extract (IC_{50} >20 μg/mL) had lost activity but the water fraction showed antiproliferative activity (IC_{50} 14 μg/mL). The water fraction was partitioned using Diaion column chromatography, C_{18} SPE, and C_{18} HPLC.

Unfortunately, the only active components that could be identified were phthalates and grease. Other minor active constituents were not identified due to the small amount present. The extract was not investigated further.

11.2.2 Viguieranthus sp. – MG 1386

An ethanol extract of the leaves of *Viguieranthus* Villers (Fabaceae) was investigated due to its reported antiproliferative activity (IC_{50} 9 μg/mL, A2780). This extract was subjected to liquid–liquid partitioning. After liquid–liquid partitioning, the fractions showed decreased bioactivity (IC_{50} >20 μg/mL); furthermore, retesting of the parent extract showed a loss of bioactivity (IC_{50} >20 μg/mL). This extract was not investigated further.

11.2.3 Petalodiscus platyrhachis – MG 3644

An ethanol extract of the leaves and inflorescences of *Petalodiscus* cf. *platyrachis* Baill. (Phyllanthaceae) was investigated due to its reported antiproliferative activity (IC_{50} 15 μg/mL, A2780). This extract was subjected to liquid–liquid partitioning. After liquid–liquid partitioning, the fractions showed decreased bioactivity (IC_{50} >20 μg/mL); furthermore, retesting of the parent extract showed a loss of bioactivity (IC_{50} >20 μg/mL). This extract was not investigated further.

11.2.4 Pandanus ankaranensis – MG 4521

An ethanol extract of the leaves of *Pandanus ankaranensis* Callm. & Laivao (Pandanaceae) was investigated due to its reported antiproliferative activity (IC_{50} 19 μg/mL, A2780). Retesting of the extract showed the bioactivity had decreased (IC_{50} > 20 μg/mL). The extract was

fractionated using liquid–liquid partitioning, Sephadex LH-20 column chromatography, C18 SPE, and HPLC. The major bioactive component was found to be phthalates, a contaminant.

11.2.5 Brexia madagascariens – MG 3934

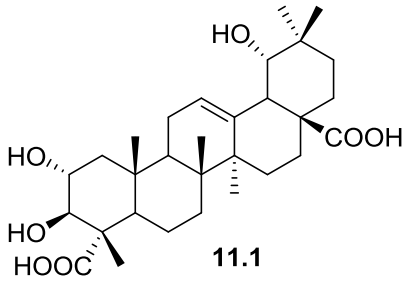
An ethanol extract of the roots of *Brexia madagascariensis* (Lam.) Ker Gawl. (Celastraceae) was investigated due to its reported antiproliferative activity (IC_{50} 14 μg/mL, A2780). Retesting of the extract showed activity had decreased (IC_{50} > 20 μg/mL). After liquid–liquid partitioning, the water fraction showed improved bioactivity (IC_{50} > 10 μg/mL). This fraction was partitioned further using Diaion column chromatography, Sephadex LH-20 column chromatography, and HPLC. This led to multiple fractions that appeared to contain pentacyclic triterpenes as judged by ^{1}H NMR spectroscopy. However, due to the small amount present in each fraction, the relative impurity of the fractions, the low bioactivity, and the lack of interest in the structures, the active components were not isolated. No further work has been completed on this extract.

11.2.6 Pandanus analamerensis – MG 3479

An ethanol extract of the leaves of *Pandanus analamerensis* Huynh (Pandanaceae) was investigated due to its reported antiproliferative activity (IC_{50} 17 μg/mL, A2780). This extract was subjected to liquid–liquid partitioning. After liquid–liquid partitioning, the fractions showed decreased bioactivity (IC_{50} >20 μg/mL); furthermore, retesting of the parent extract showed a loss of bioactivity (IC_{50} >20 μg/mL). This extract was not investigated further.

11.2.7 Barringtonia racemosa – MG 3283

An ethanol extract of the bark of *Barringtonia racemosa* (L.) Spreng. (Lecythidaceae) was investigated by Emily Berckman, under the supervision of the author of this dissertation, due to its reported antiproliferative activity (IC_{50} 10 μg/mL, A2780). While the active component was not identified fully, it was shown to be a derivative of bartogenic acid. This extract was not investigated further.



11.1

Figure 11-1. Structure of bartogenic acid.

11.3 Antimalarial Extracts

11.3.1 Tolmiea menziesii – 109993-4A

An extract of *Tolmiea menziesii* (Saxifragaceae) was selected for investigation due to its antiplasmodial activity (IC_{50} <6 μg/mL, *P. falciparum Dd2*). After fractionation utilizing a polyamide column, bioactivity was decreased (IC_{50} > 10 μg/mL). Since the original bioactivity was likely due to tannins, this extract was not investigated further.

11.3.2 Melanophylla aucubifolia – MG 238 – MG 239

Ethanol extracts of the bark and stems of *Melanophylla aucubifolia* Baker (Torricelliaceae) were investigated due to their antiplasmodial activity (IC_{50} ~1.25 μg/mL, IC_{50} < 1.25 μg/mL).

Extensive fractionation using liquid–liquid partitioning, Diaion column chromatography, Sephadex LH-20 column chromatography, SPE, and HPLC led to the identification of the active components as phthalates. Due to the high levels of contamination, these extracts were not investigated further.

11.4 Anti-inflammatory Extract

11.4.1 Trichilia sp. – MG 0072

An ethanol extract of the bark of *Trichilia sp.* P. Browne was investigated due to its potential anti-inflammatory activity (PPAR-γ agonist). However, after liquid–liquid partitioning, the fractions did not show greatly improved bioactivity. Due to a lack of interest from collaborators, the extract was not investigated further.

Chapter 12: General Conclusions

In a five year research period, 34 compounds were identified from eight different plants (**Error! Reference source not found.**). The various compounds identified represent a small ortion of the spectrum of unique and complex compounds that are produced by plants. The three previously unreported antiproliferative trihydroxyalkylcyclohexenones, **2.1**-**2.3**, isolated from *Pleiogynium timoriense* were perhaps the most interesting compounds isolated. This is not so much for their novelty, as similar compounds have been isolated previously, but for the challenge of determining their structures. The determination of their relative configurations required chemical modification followed by 2D NMR, while benzoylation in order to synthesize a derivative for ECD analysis gave an unexpected product. Fortunately, the results were interpretable and the absolute configuration was able to be determined. Overall, the structure elucidation of **2.1-2.3** proved to be very intellectually stimulating due to the multistep approaches needed to determine the absolute and relative configurations. This was not typically the case with other compounds which were isolated; the various configurations were solved by comparison of experimental NMR and optical rotation data to published values.

Identification of the compounds in this dissertation was greatly aided by access to large amounts of published data. Resources such of the Dictionary of Natural Products, SciFinder, and the large amount of scientific literature from the Virginia Tech library proved to be invaluable. These resources allowed known compounds to be quickly identified and provided direction for the identification of new compounds. Without these resources, hours of additional spectroscopic and chemical analysis would have been needed. As demonstrated by the identification of many known compounds within this work, dereplication techniques need to be employed to rapidly identify known compounds

The synthesis of mallotojaponin C and derivatives proved to be more challenging than initially suspected. While the synthetic route is short (2 steps), the low yields and instability of many attempted derivatives proved to be quite frustrating. It will be interested to see the results of continued mechanistic studies, particularly for compounds **5.1**, **5.12**, and **5.13**.

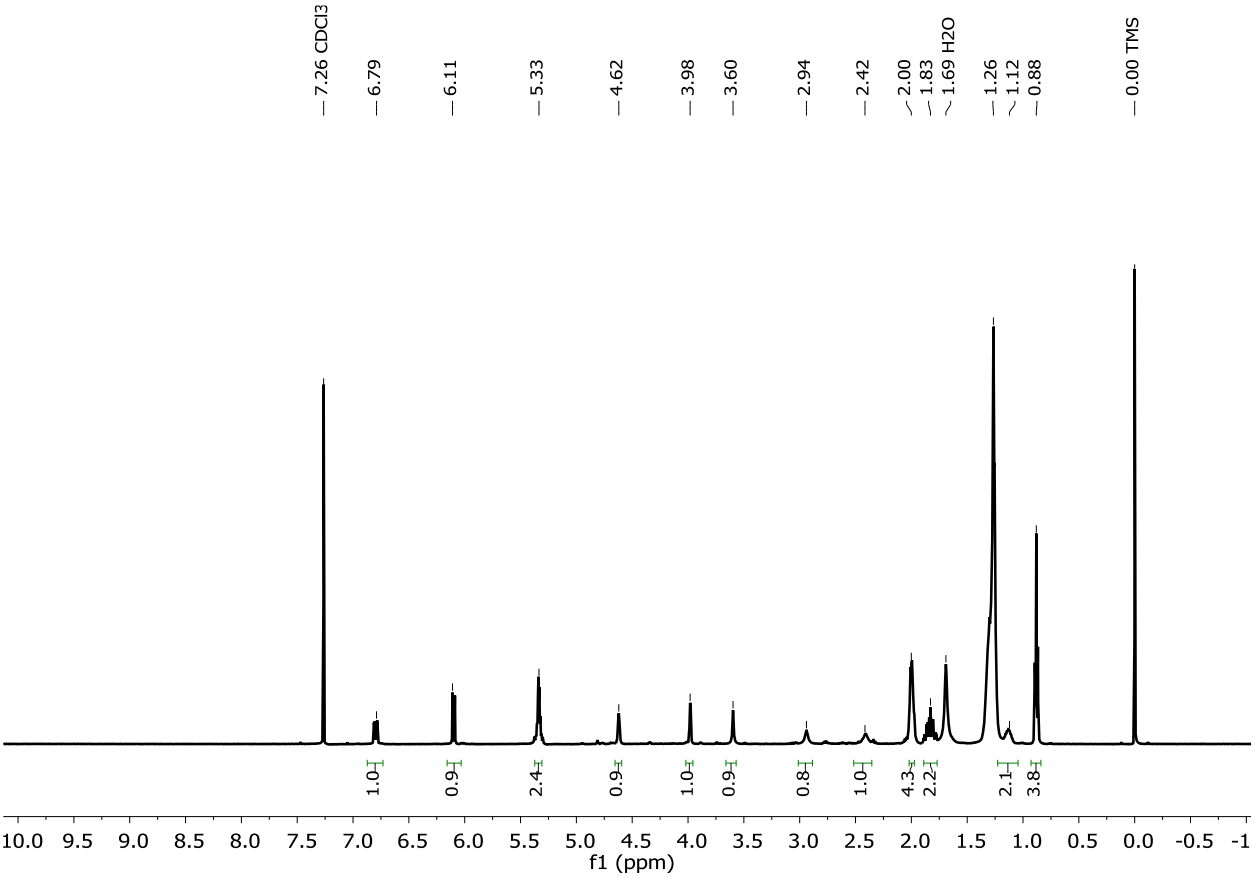
Table 12-1. Compounds identified from plants.

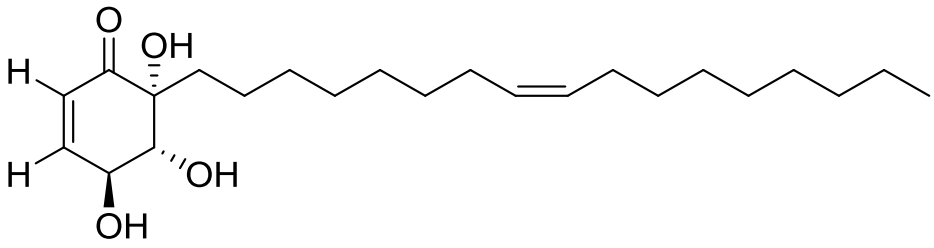
Compound	Plant	New/Known
2.1	*Pleiogynium timoriense*	New
2.2	*Pleiogynium timoriense*	New
2.3	*Pleiogynium timoriense*	New
3.1	*Molinaea retusa*	New
3.2	*Molinaea retusa*	Known
3.3	*Molinaea retusa*	Known
4.1	*Polyscias duplicata*	Known
4.2	*Polyscias duplicata*	Known
4.3	*Polyscias duplicata*	Known
4.4	*Polyscias duplicata*	Known
4.5	*Polyscias duplicata*	Known
4.6	*Polyscias duplicata*	Known
4.7	*Polyscias duplicata*	Known
6.1	*Oncostemum bojerianum*	Known
6.2	*Oncostemum bojerianum*	Known
6.3	*Oncostemum bojerianum*	Known
6.4a	*Oncostemum bojerianum*	New
6.4b	*Oncostemum bojerianum*	Known
6.5a	*Oncostemum bojerianum*	New
6.5b	*Oncostemum bojerianum*	New
6.6a	*Oncostemum bojerianum*	New
6.6b	*Oncostemum bojerianum*	Known
6.6c	*Oncostemum bojerianum*	New
7.1	*Schimatoclada farhimpensis*	Known
7.2	*Schimatoclada farhimpensis*	Known
7.3	*Schimatoclada farhimpensis*	Known
7.4	*Schimatoclada farhimpensis*	Known
8.1	*Rhodospaera rhodanthema*	Known
8.2	*Rhodospaera rhodanthema*	Known
8.3	*Rhodospaera rhodanthema*	Known
9.1	*Curroria sp.*	Known
9.2	*Curroria sp.*	Known
9.3	*Curroria sp.*	Known
9.4	*Curroria sp.*	Known
9.5	*Curroria sp.*	Known
10.1	*Streblus dimepate*	Known
10.2	*Streblus dimepate*	Known

In summary, the work described in this dissertation is indicative of the strength and challenge of plant-based natural products research at the present time. Its strength lies in its ability to isolate and identify bioactive compounds from complex plant extracts, while its major challenge is its repeated discovery of known compounds. The dereplication work described in the previous chapters is an example of a fruitful approach to this challenge.

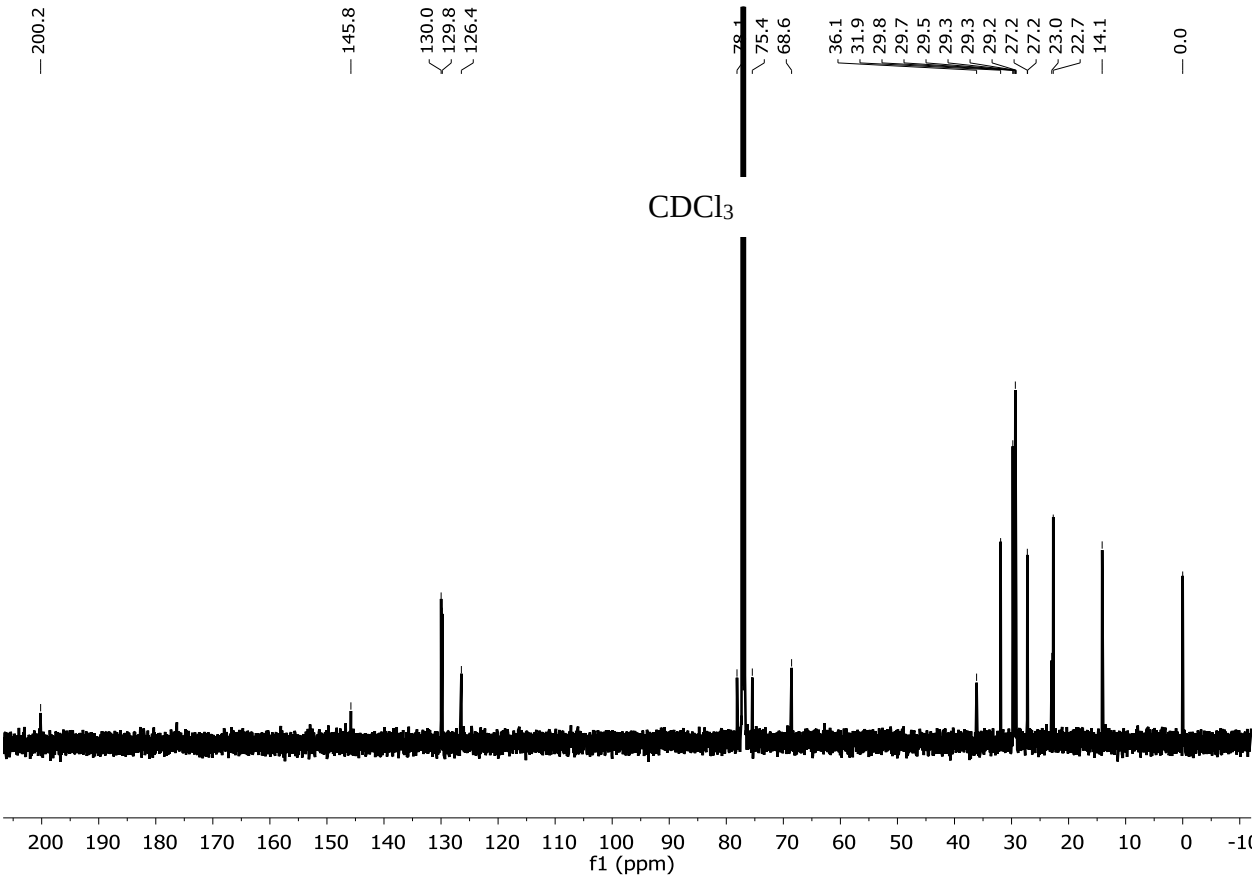
Chapter 13: Supporting Information

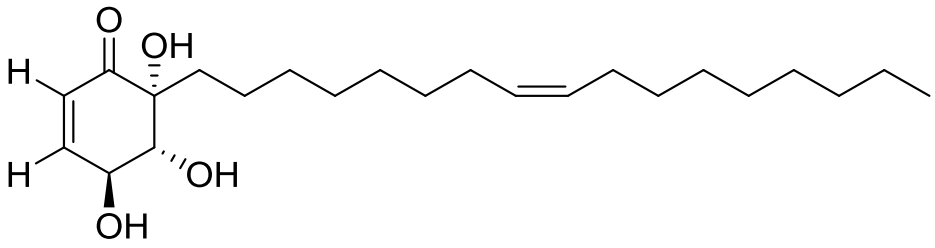
13.1 ^{1}H NMR of 2.1 ($CDCl_3$, 500 MHz)



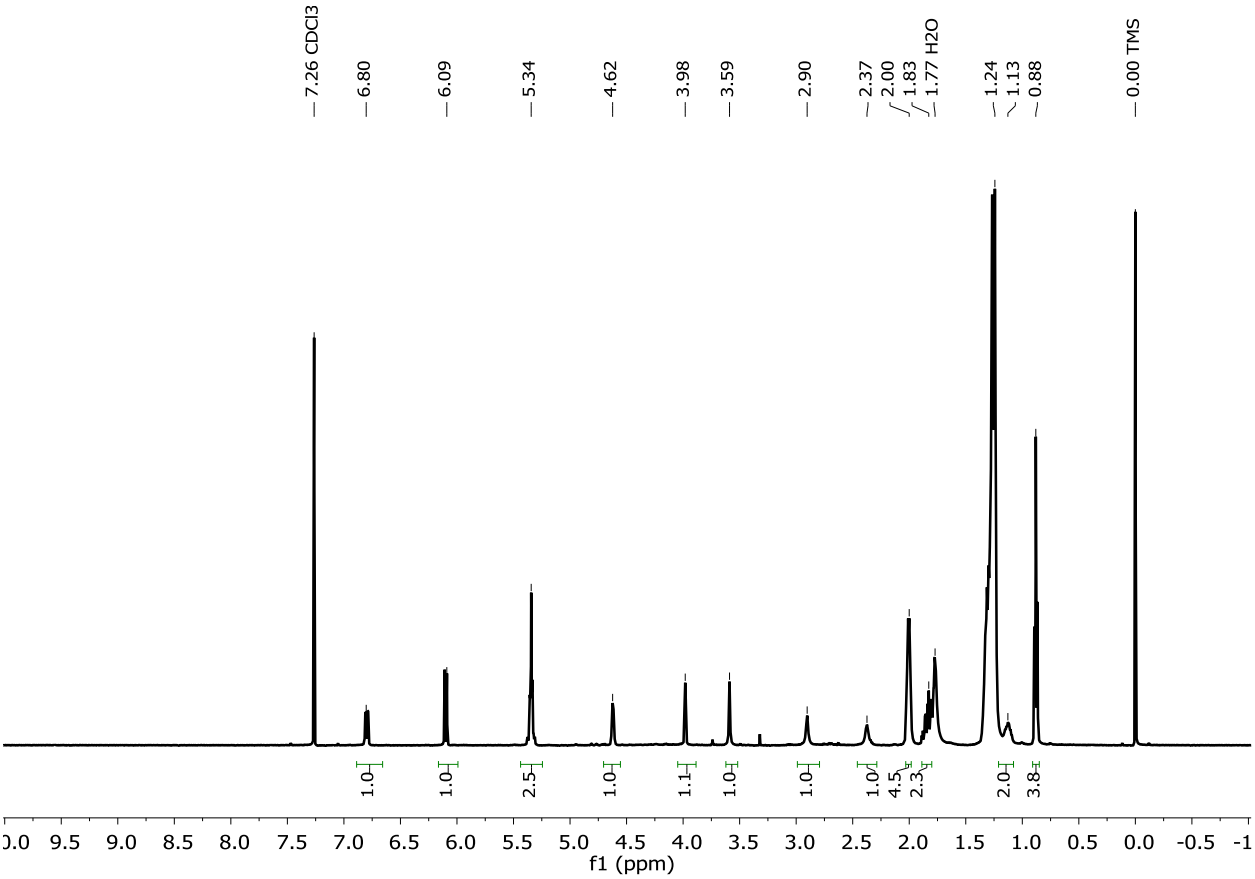


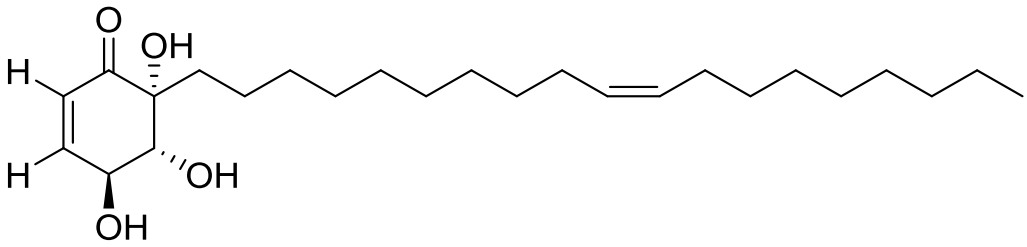
13.2 ^{13}C NMR of 2.1 ($CDCl_3$, 150 MHz)



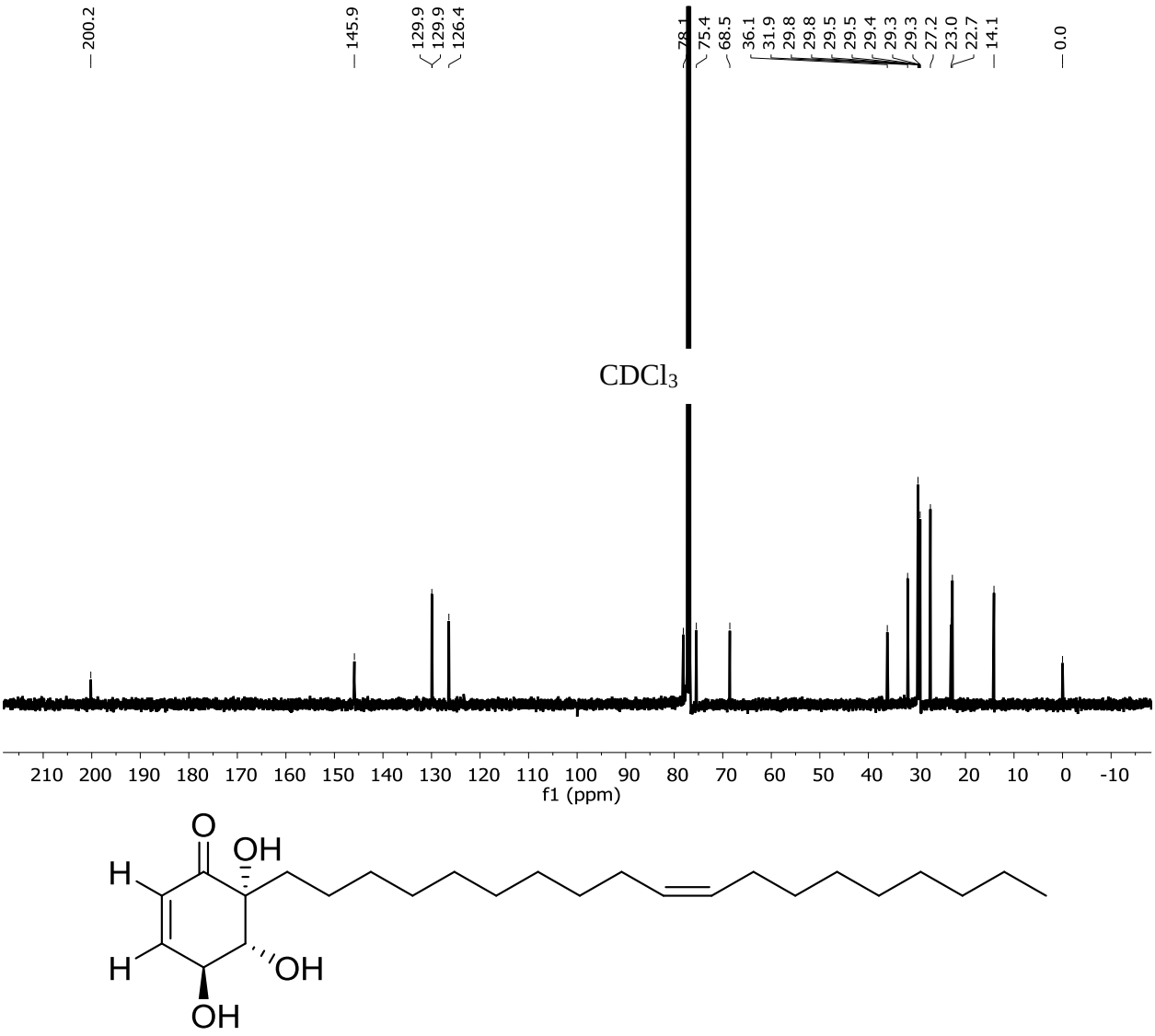


13.3 ^{1}H NMR of 2.2 ($CDCl_3$, 500 MHz)

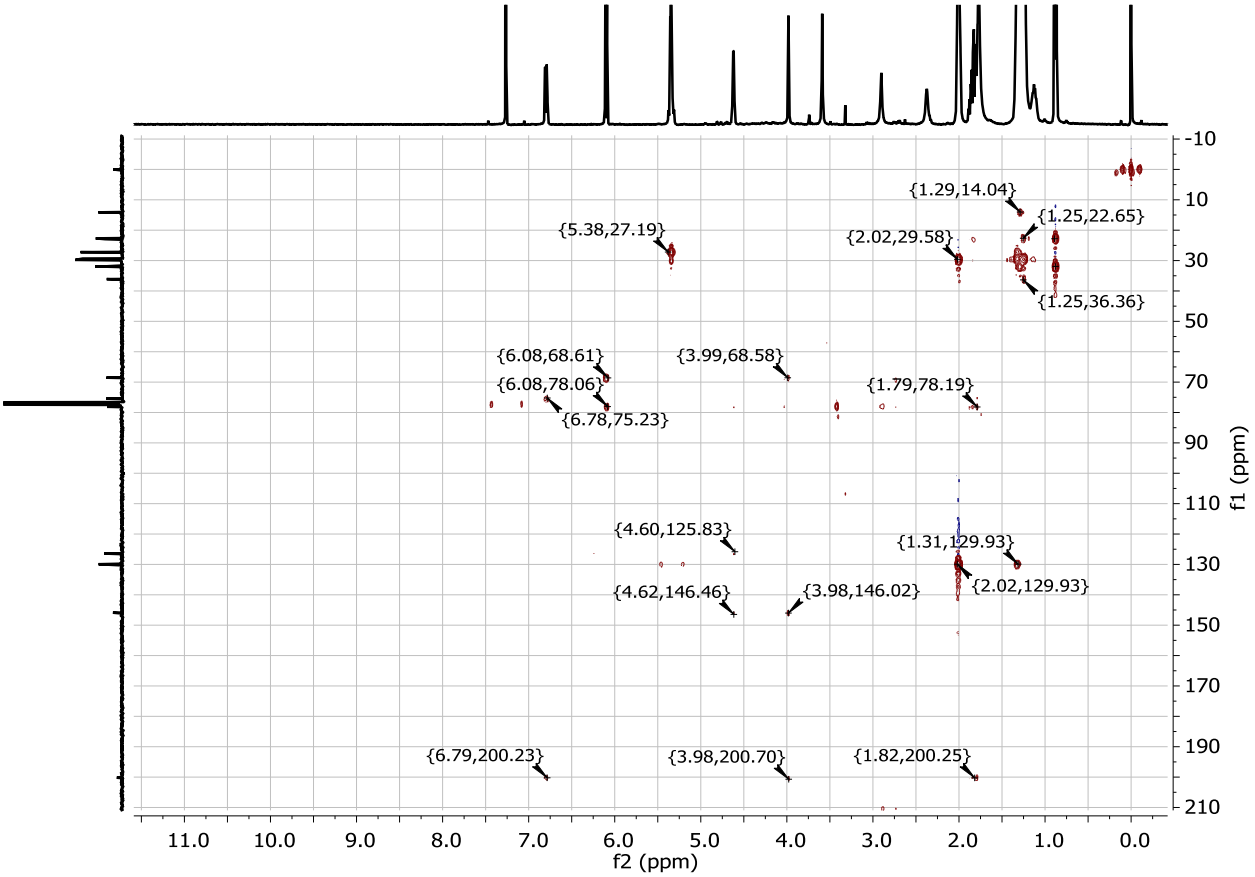


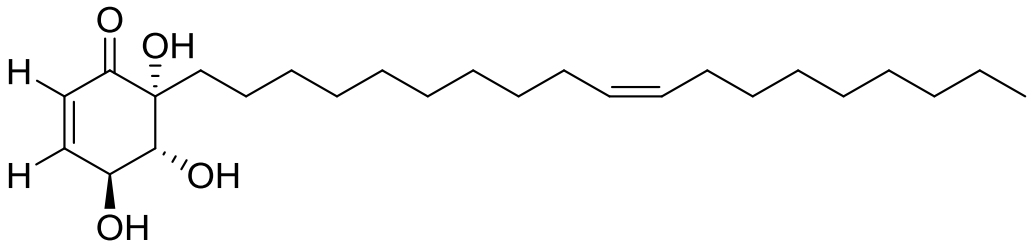


13.4 ^{13}C NMR of 2.2 ($CDCl_3$, 125 MHz)

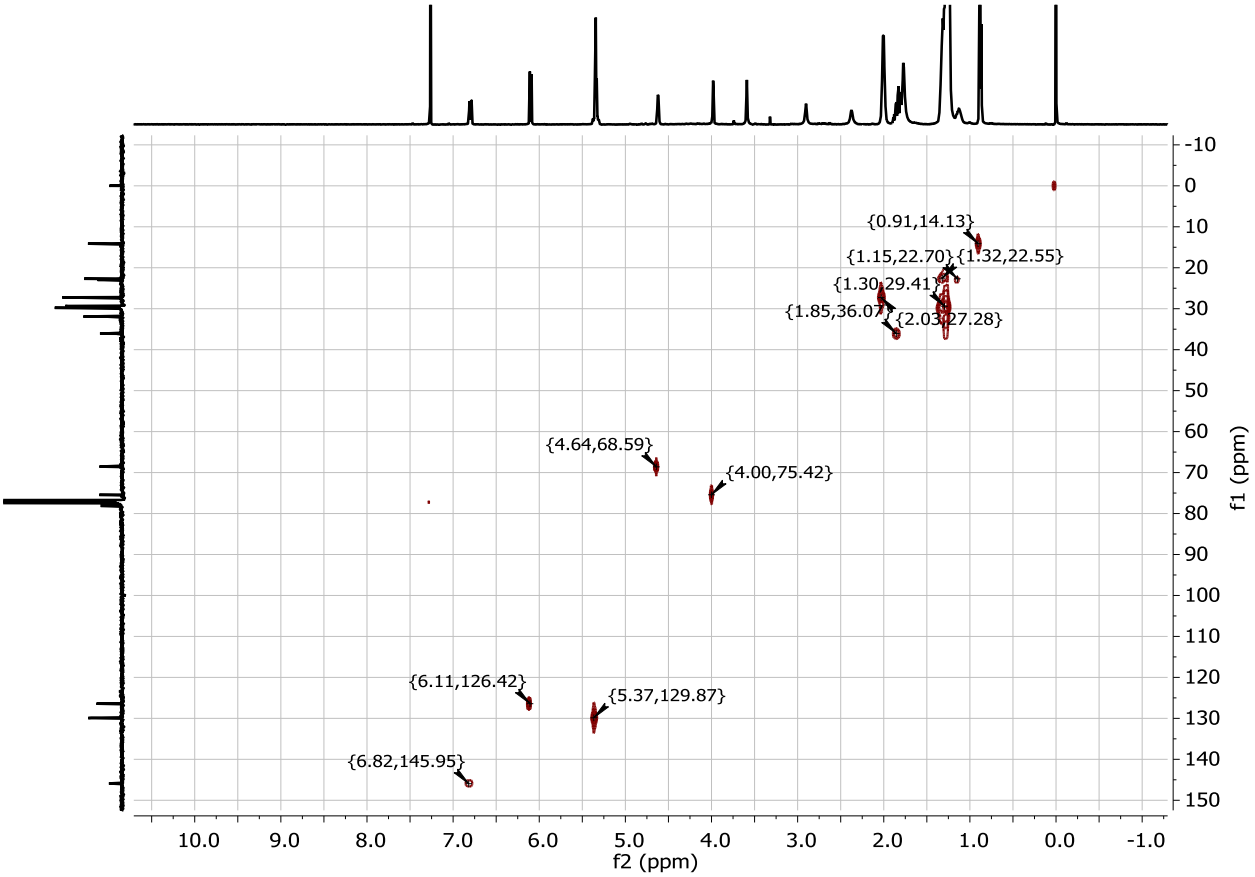


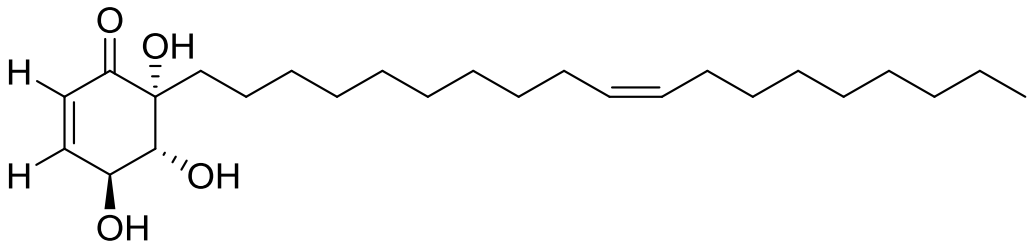
13.5 HMBC of 2.2 ($CDCl_3$, 600 MHz, 150 MHz)



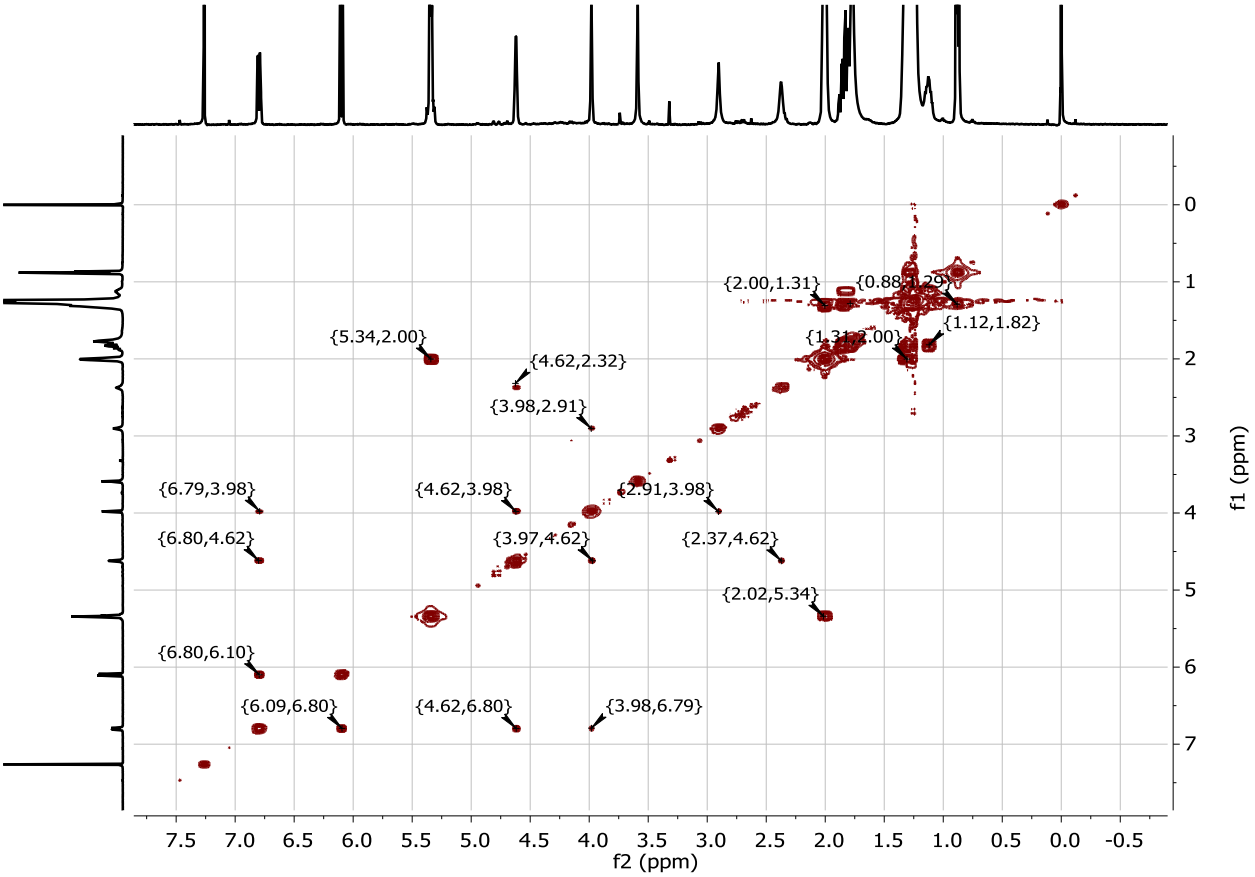


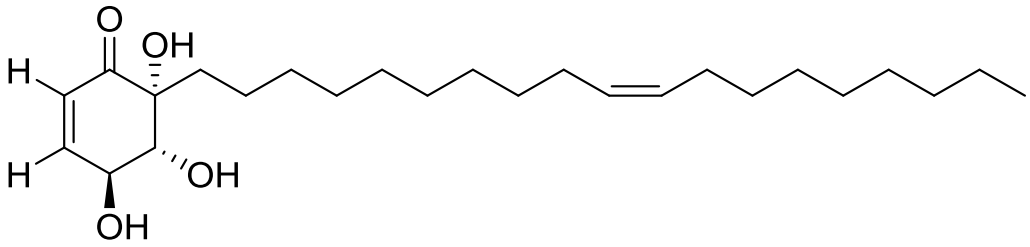
13.6 HSQC of 2.2 ($CDCl_3$, 500 MHz, 125 MHz)



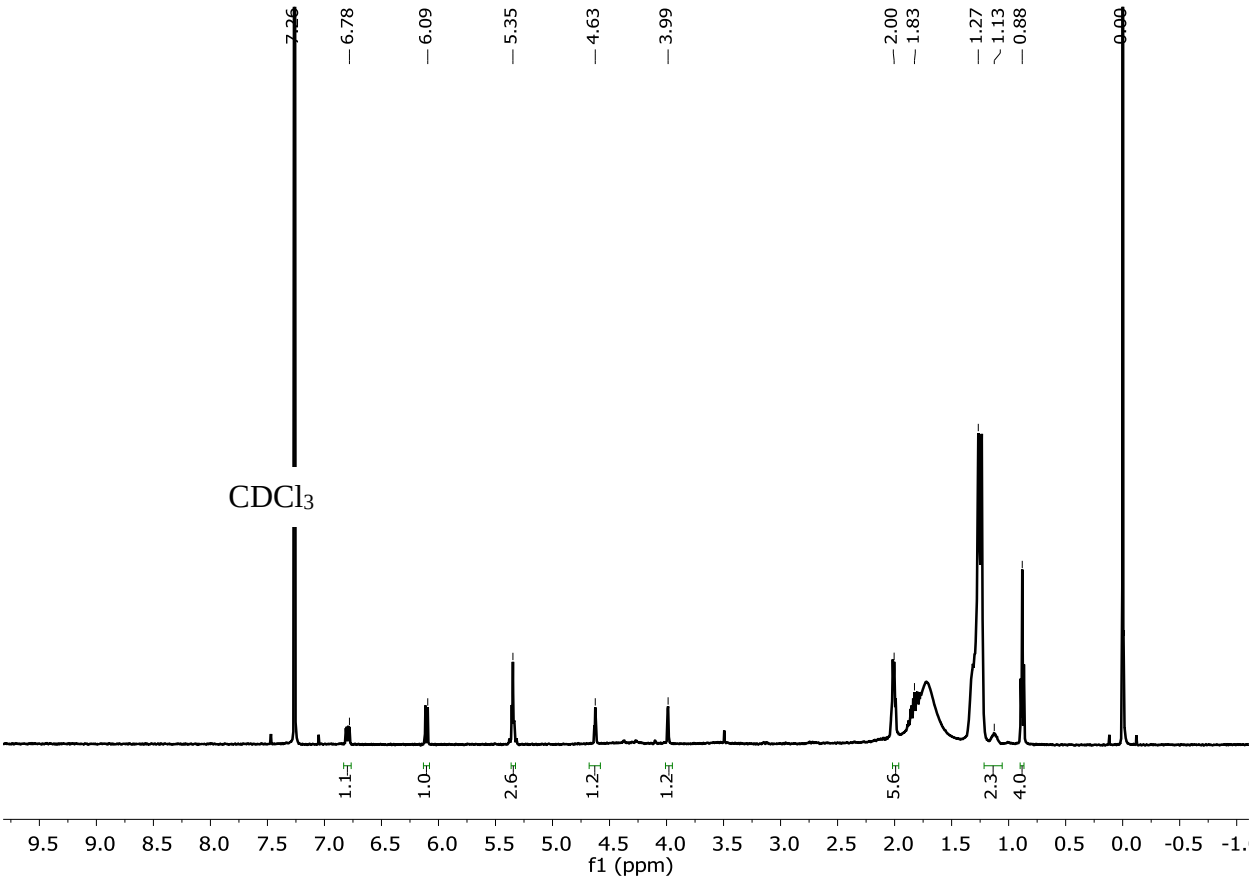


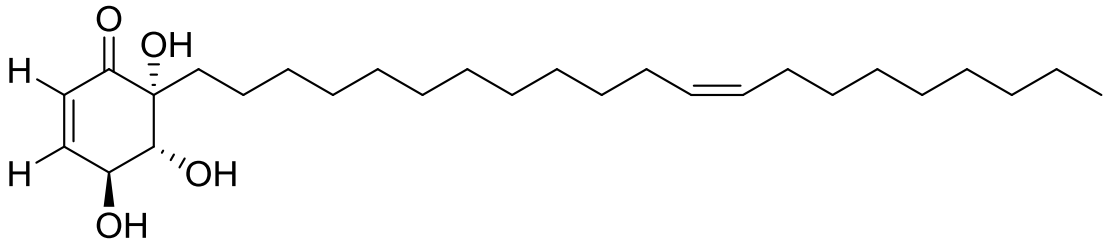
13.7 COSY of 2.2 ($CDCl_3$, 500 MHz)



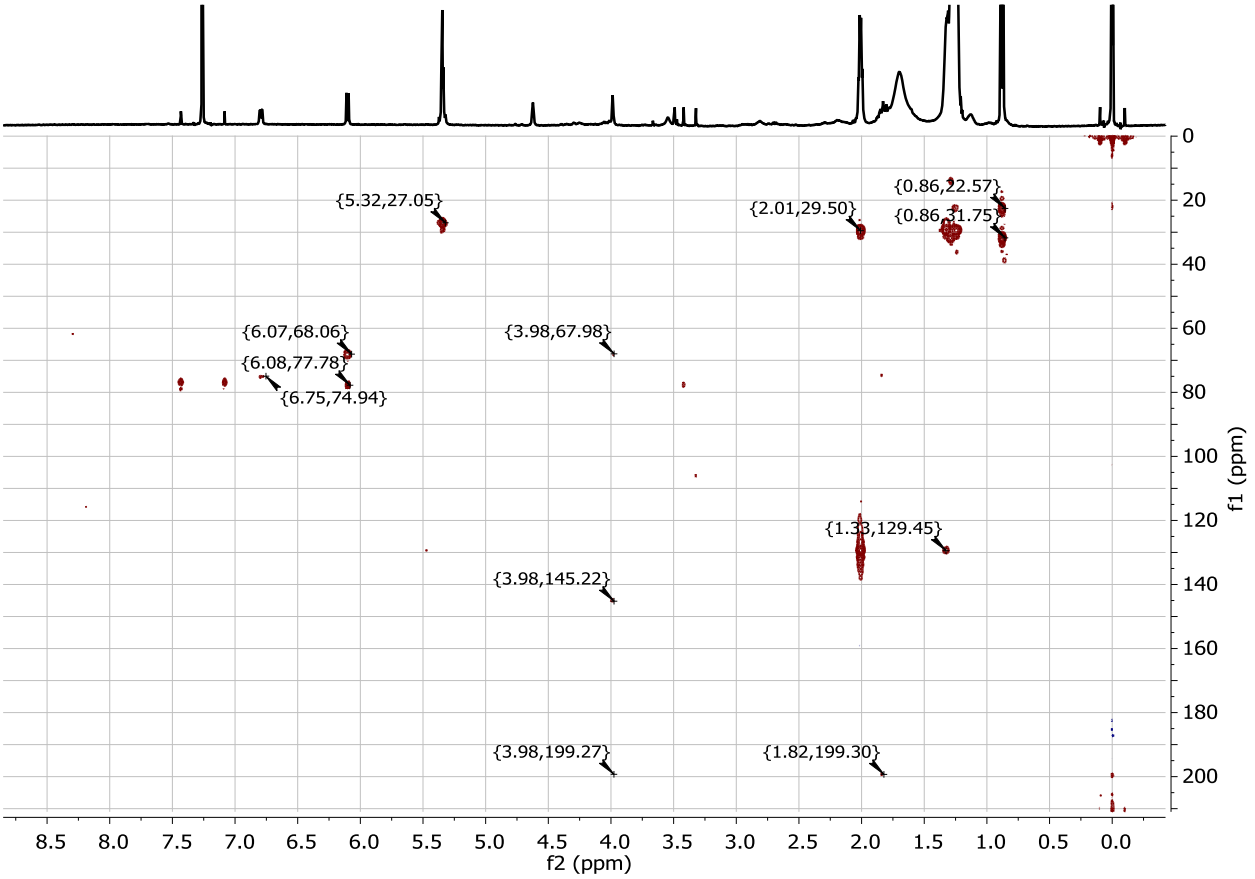


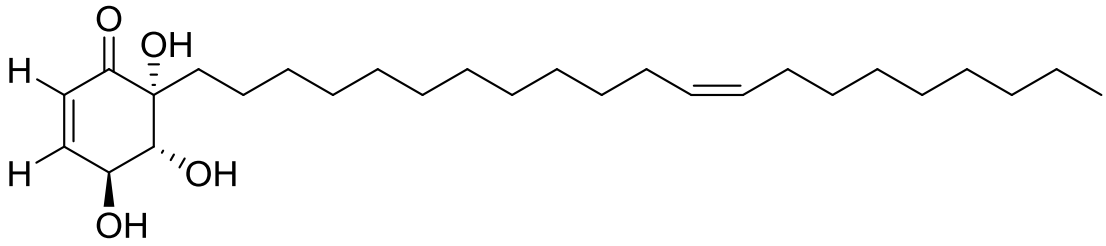
13.8 ^{1}H NMR of 2.3 ($CDCl_3$, 500 MHz)



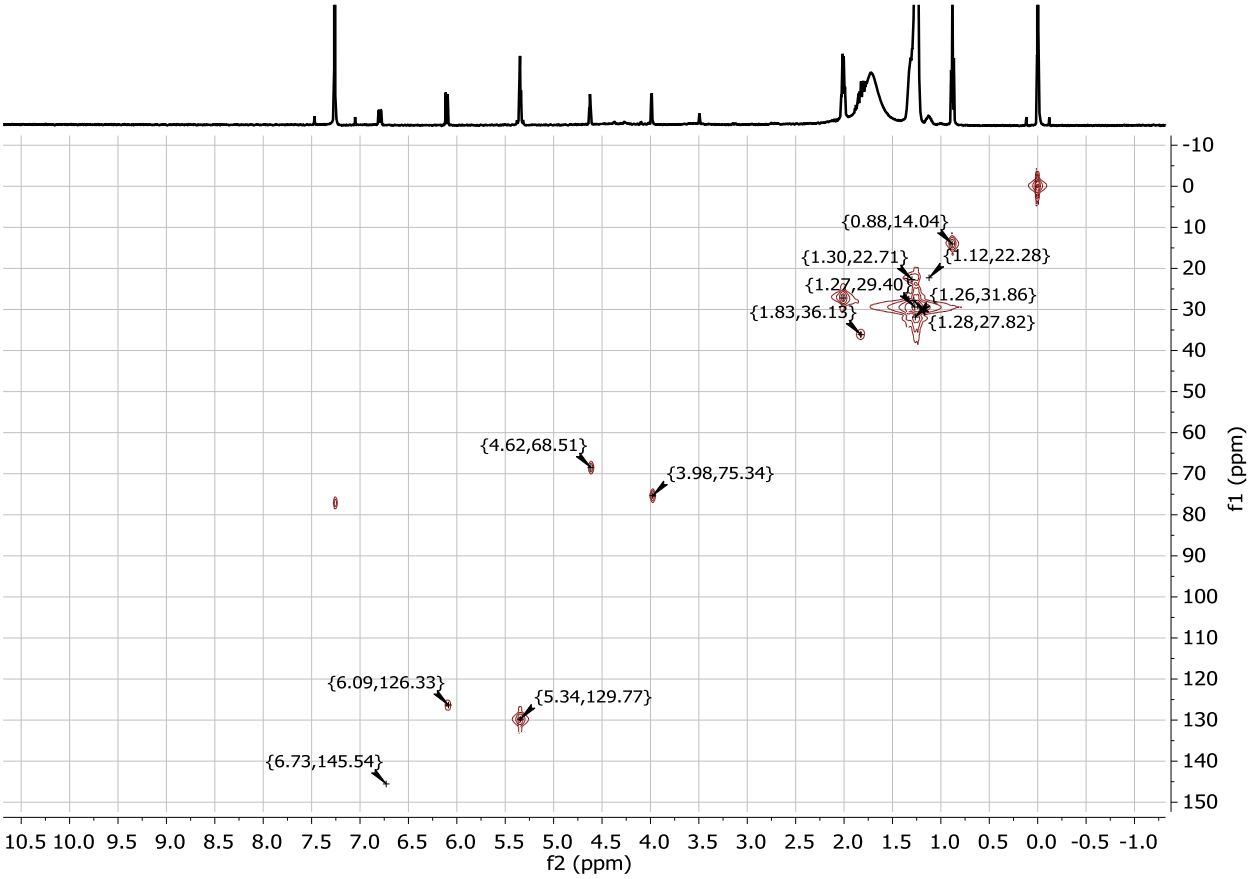


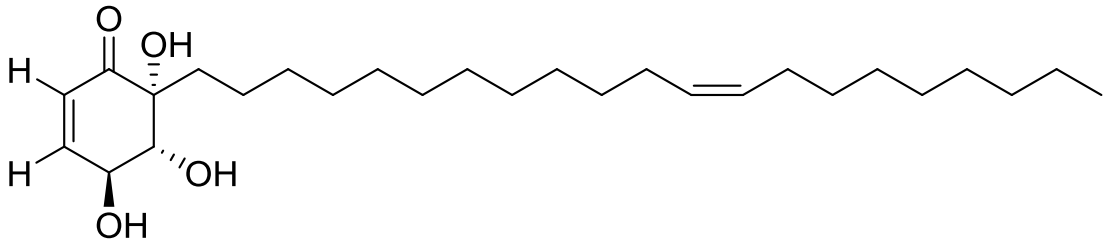
13.9 HMBC of 2.3 ($CDCl_3$, 600 MHz, 150 MHz)



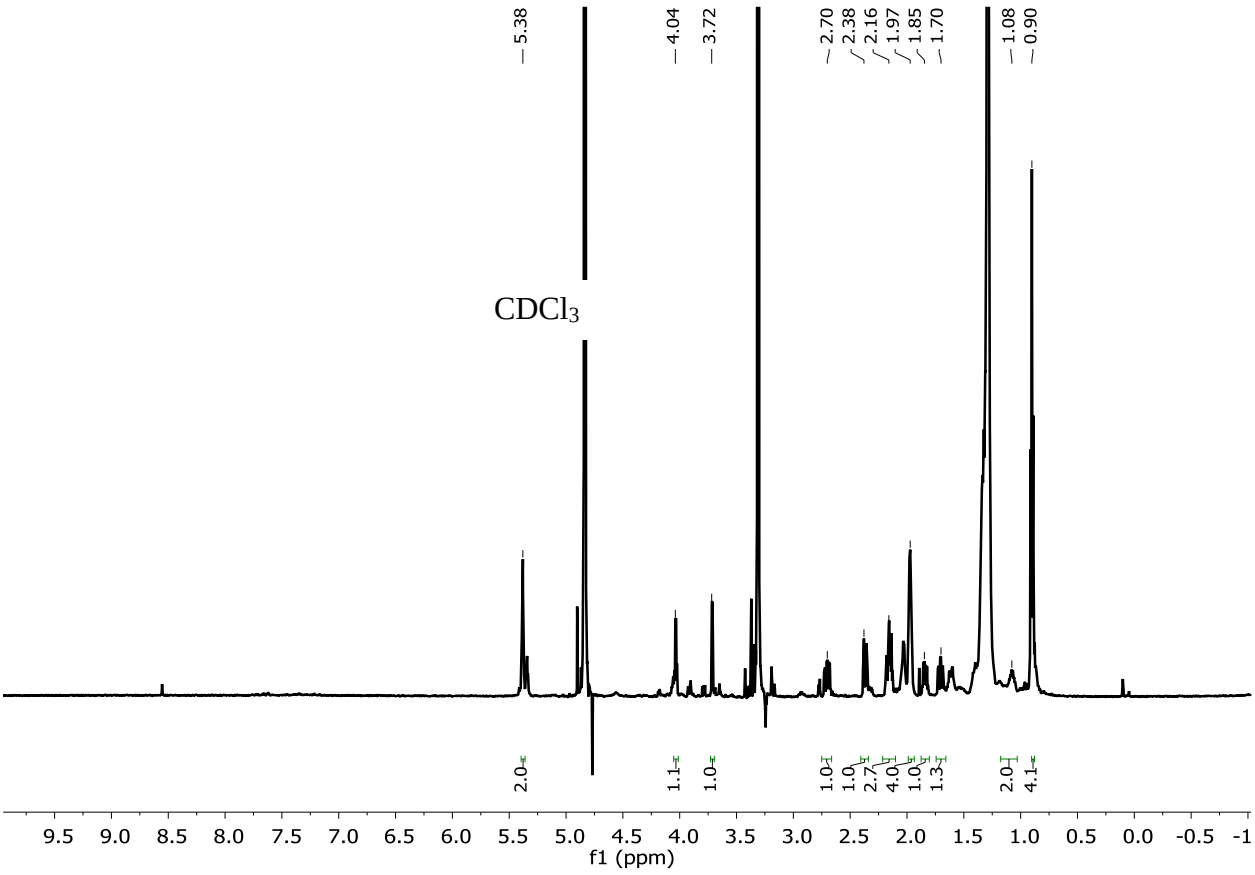


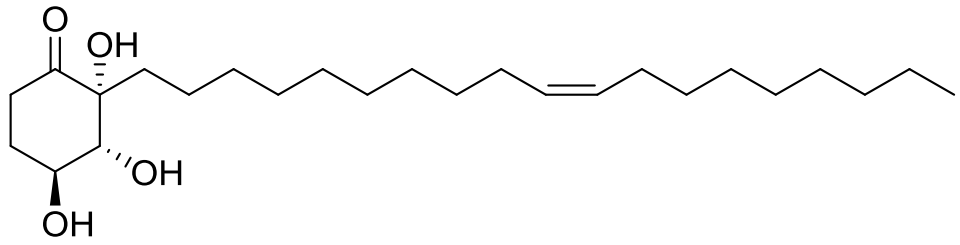
13.10 HSQC of 3.3 ($CDCl_3$, 600 MHz, 150 MHz)



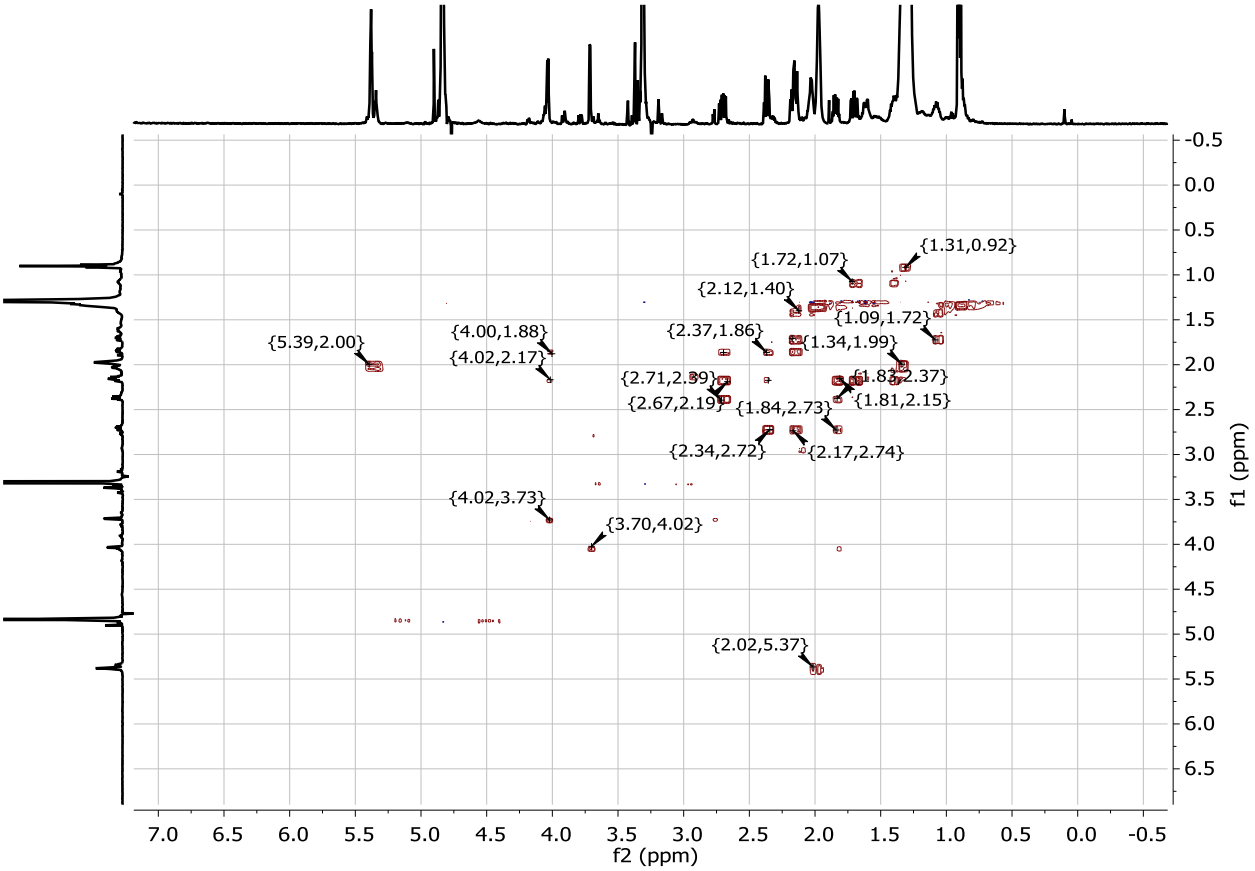


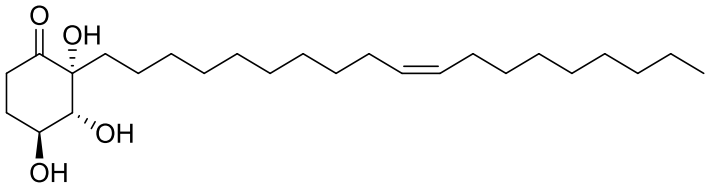
13.11 ^{1}H NMR of 2.4 (CD_3OD, 600 MHz)



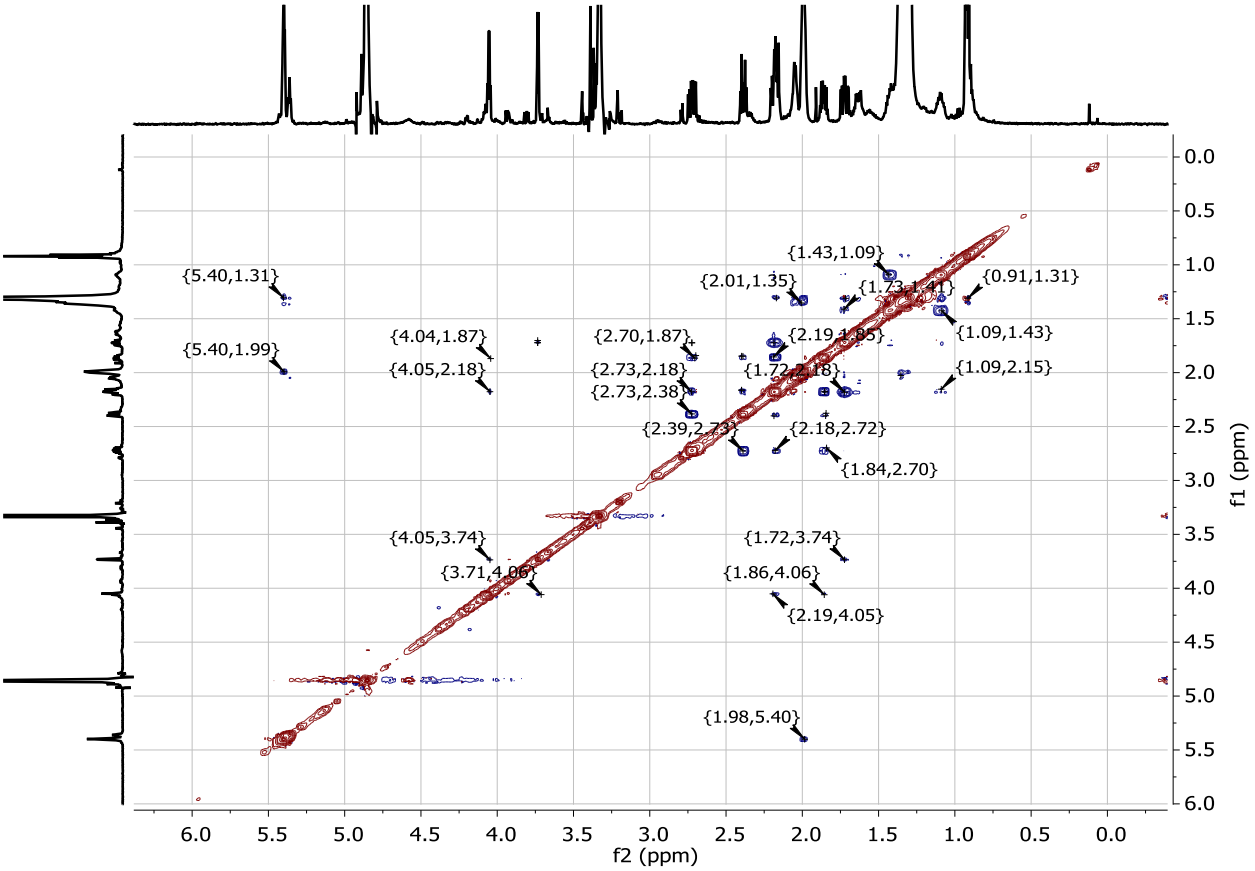


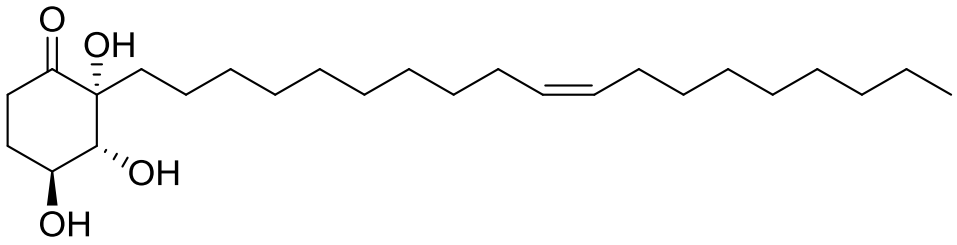
13.12 COSY of 2.4 (CD_3OD, 600 MHz)



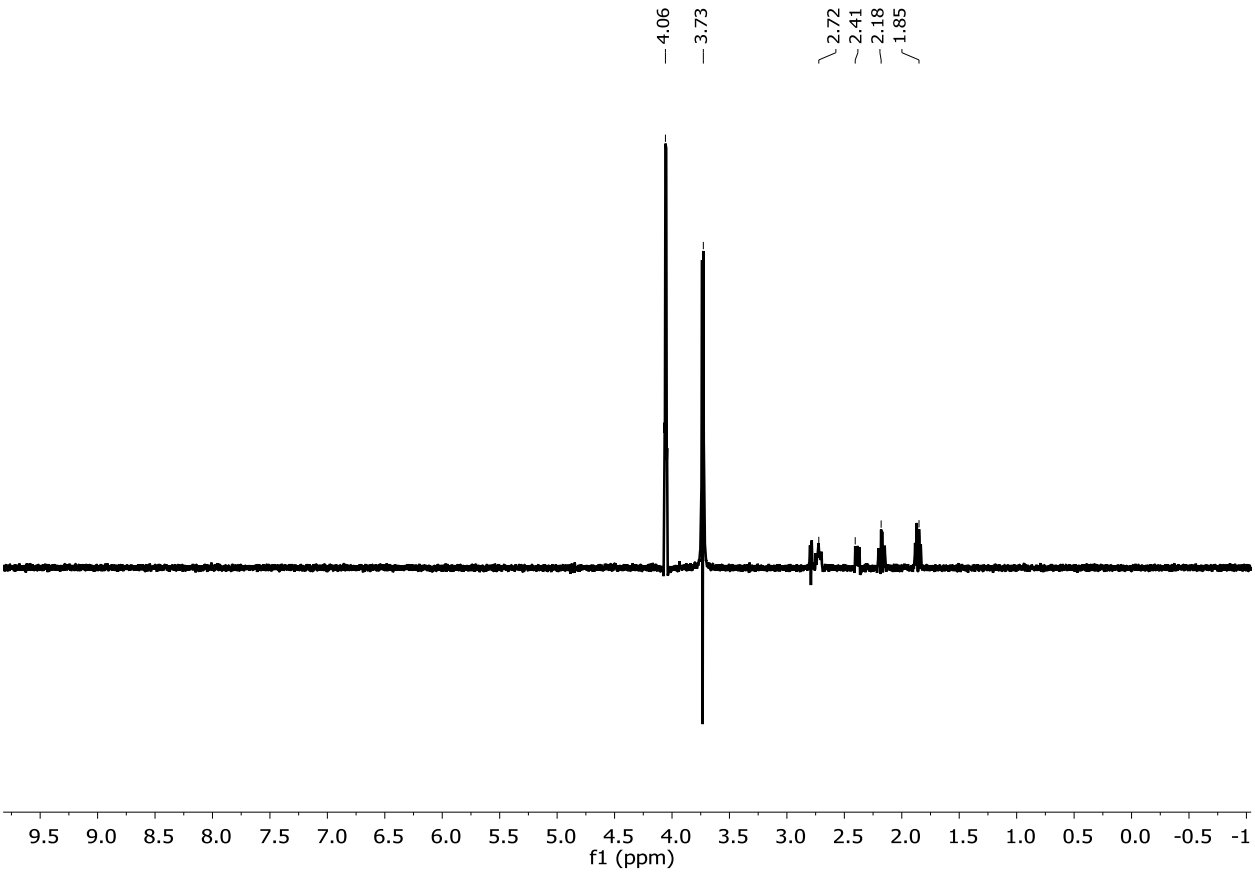


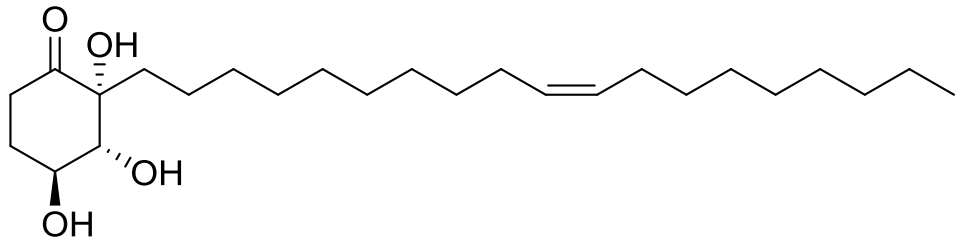
13.13 NOESY of 2.4 (CD_3OD, 600 MHz)



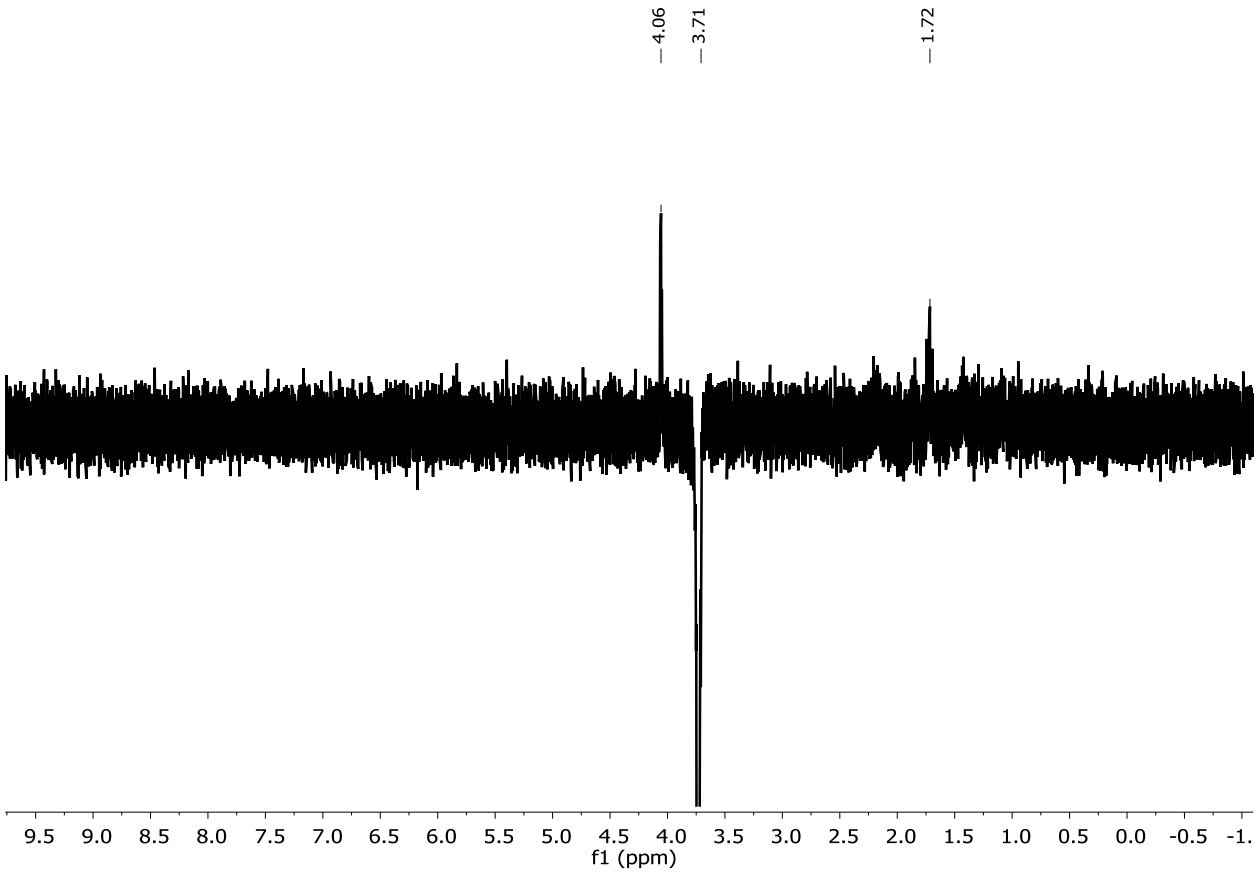


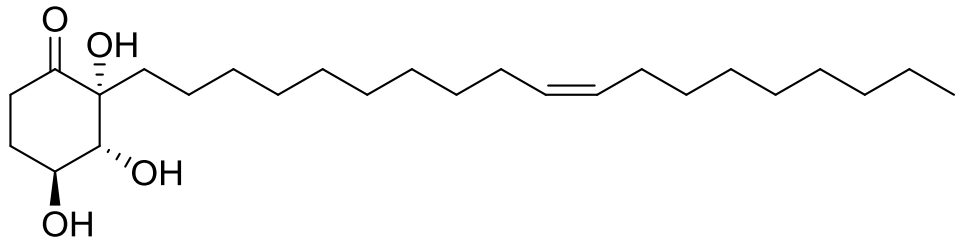
13.14 SEL. TOCSY of 2.4 (CD_3OD, 600 MHz, Irradiated at 3.7 ppm)



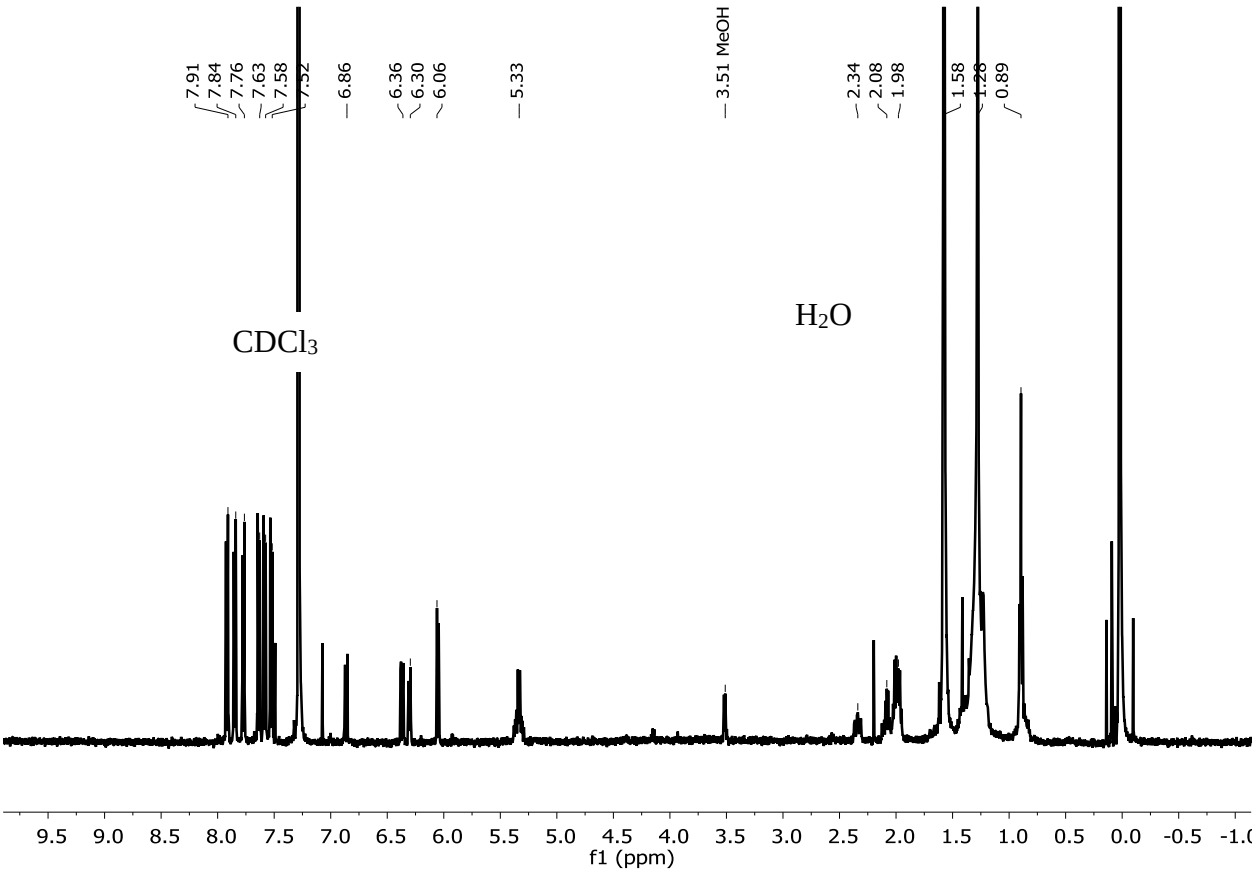


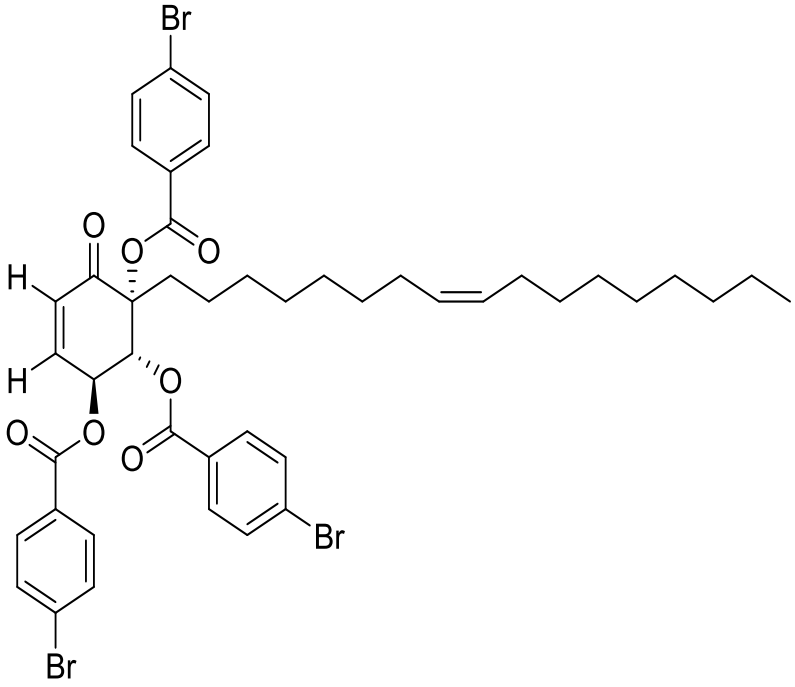
13.15 SEL. NOE of 2.4 (CD_3OD, 600 MHz, Irradiated at 3.7 ppm)



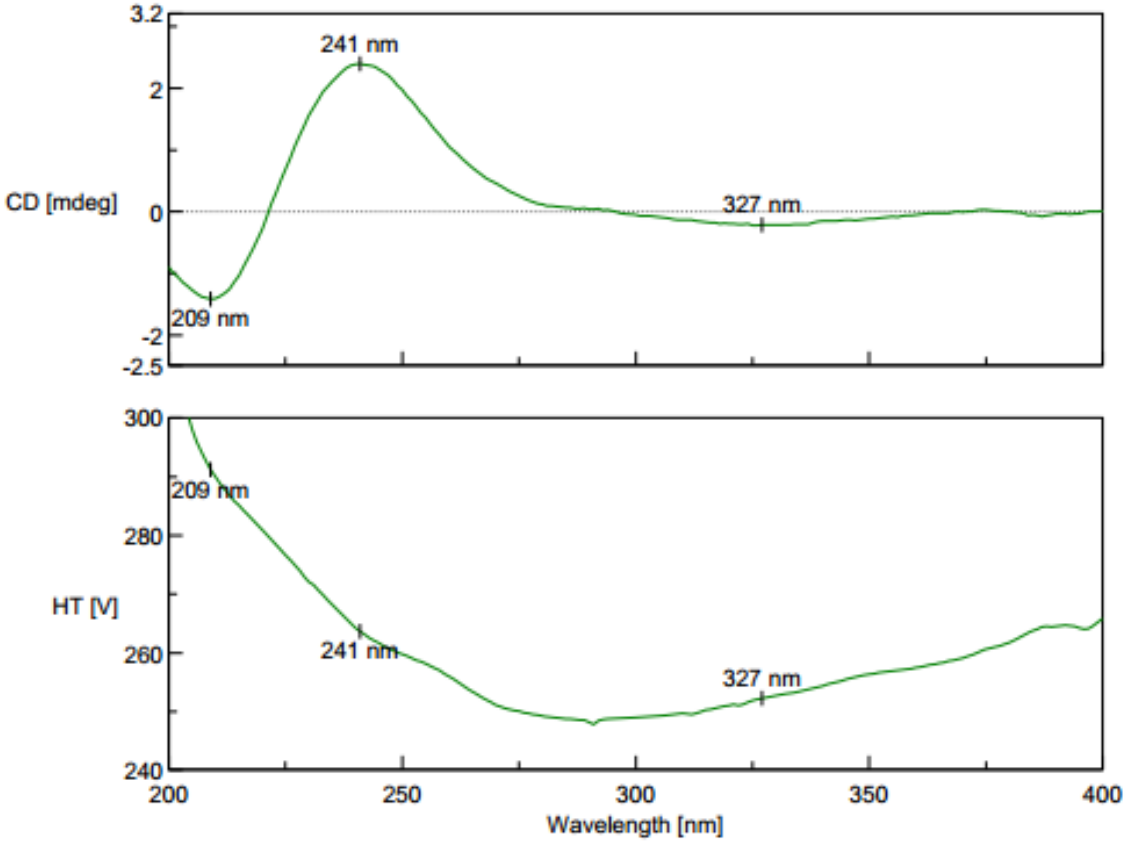


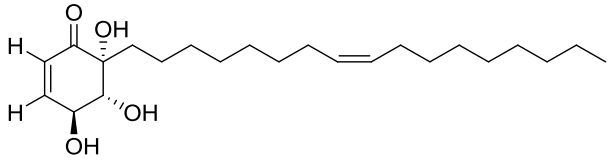
13.16 ^{1}H NMR of 2.5 (CD_3OD, 600 MHz)



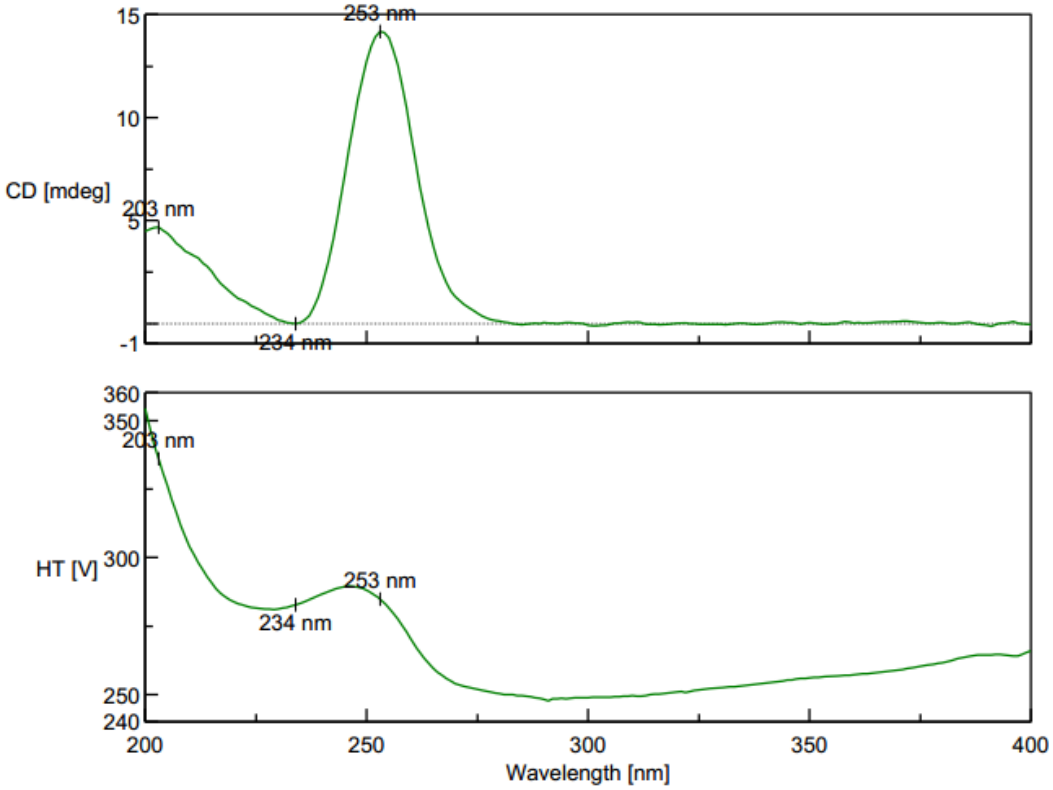


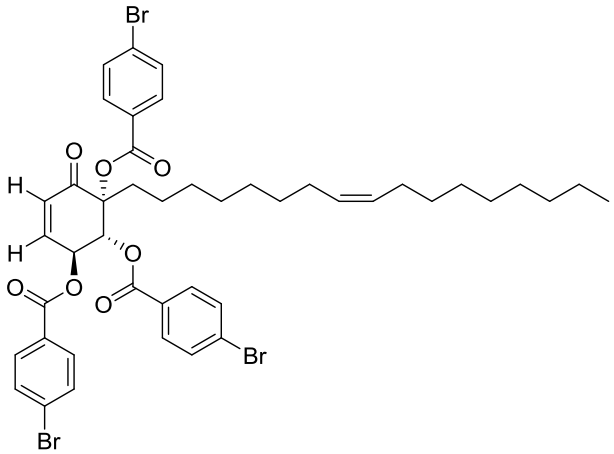
13.17 ECD Spectrum of 2.1 (MeOH)





13.18 ECD Spectrum of 2.5 (MeOH)

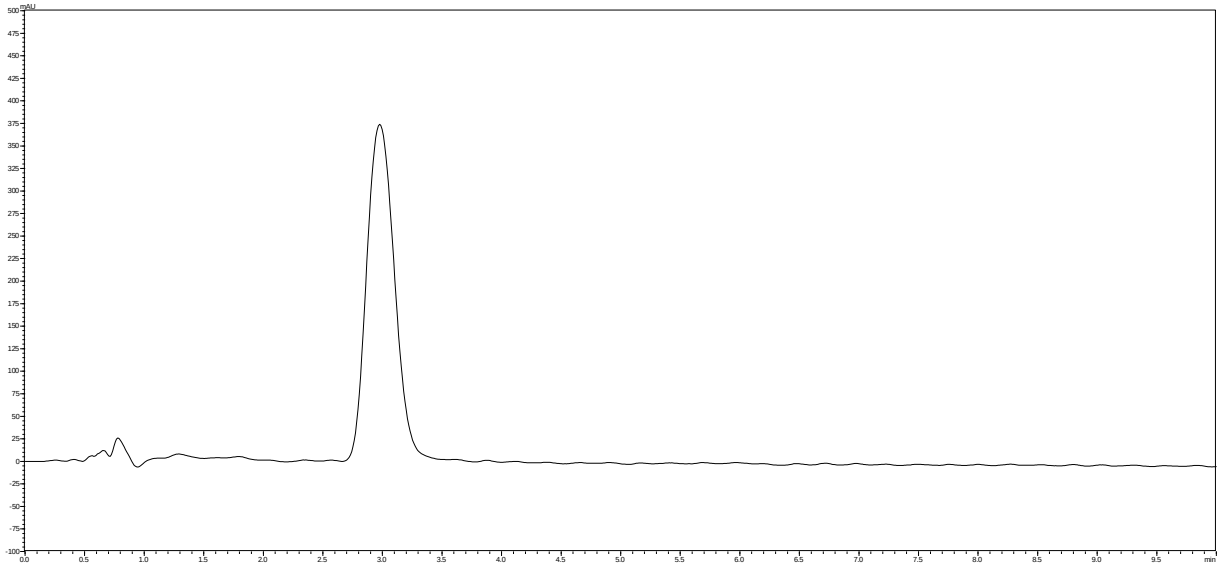




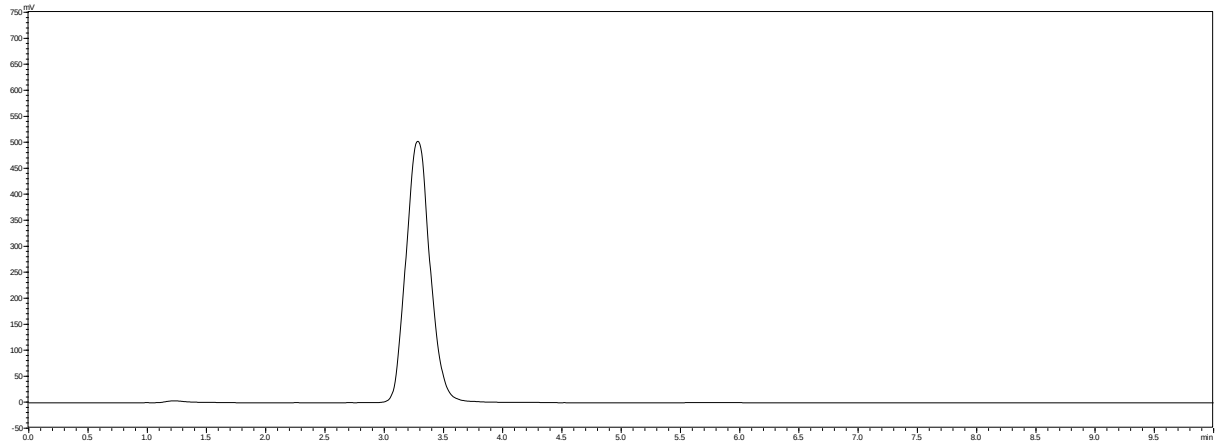
13.19 HPLC of 2.1

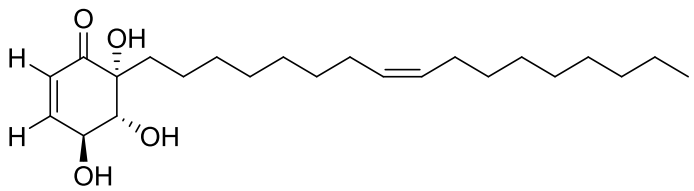
Column: Cogent Bidentate C18 100 A, 4 μm, 7.5 x 4.6 mm
Concentration: 1 mg/mL
Injection: 25 μL injection
Mobile Phase: 88% MeOH (aq.) with 0.01% Formic Acid

Detector: PDA (217 nm)



Detector: ELSD

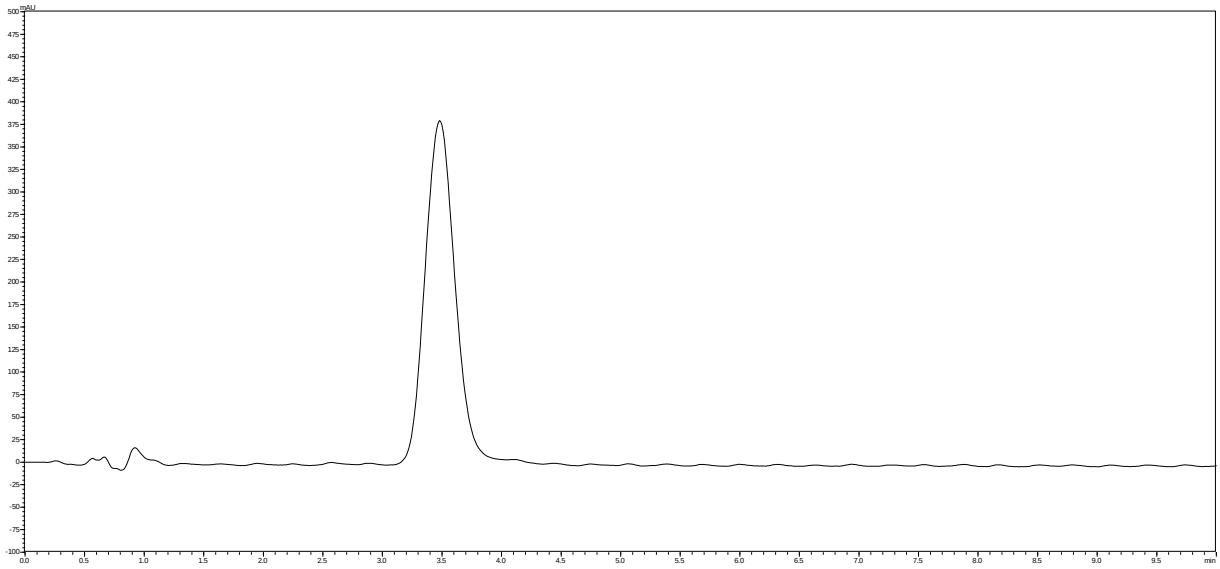




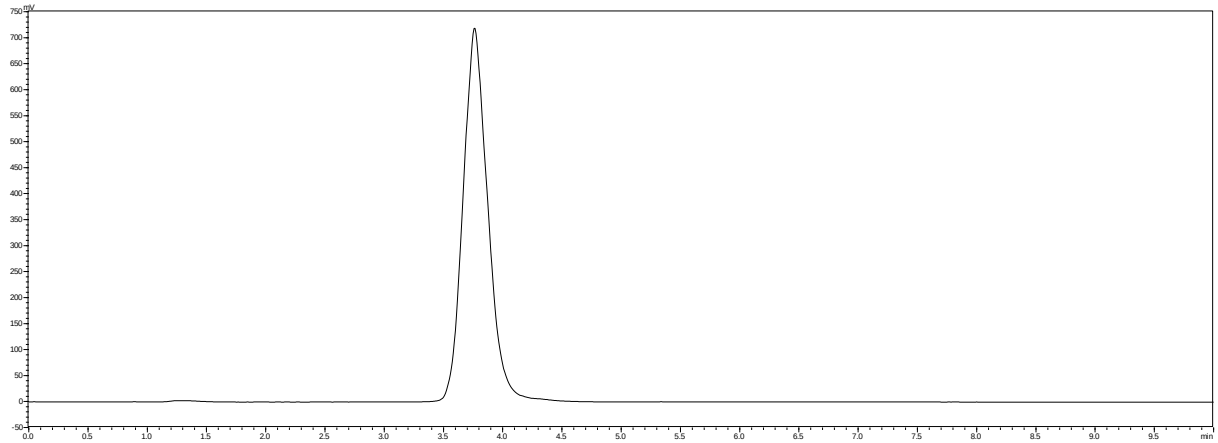
13.20 HPLC of 2.2

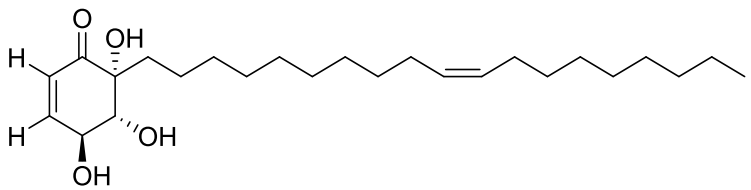
Column: Cogent Bidentate C18 100 A, 4 μm, 7.5 x 4.6 mm
Concentration: 1 mg/mL
Injection: 25 μL injection
Mobile Phase: 90% MeOH (aq.) with 0.01% Formic Acid

Detector: PDA (217 nm)



Detector: ELSD

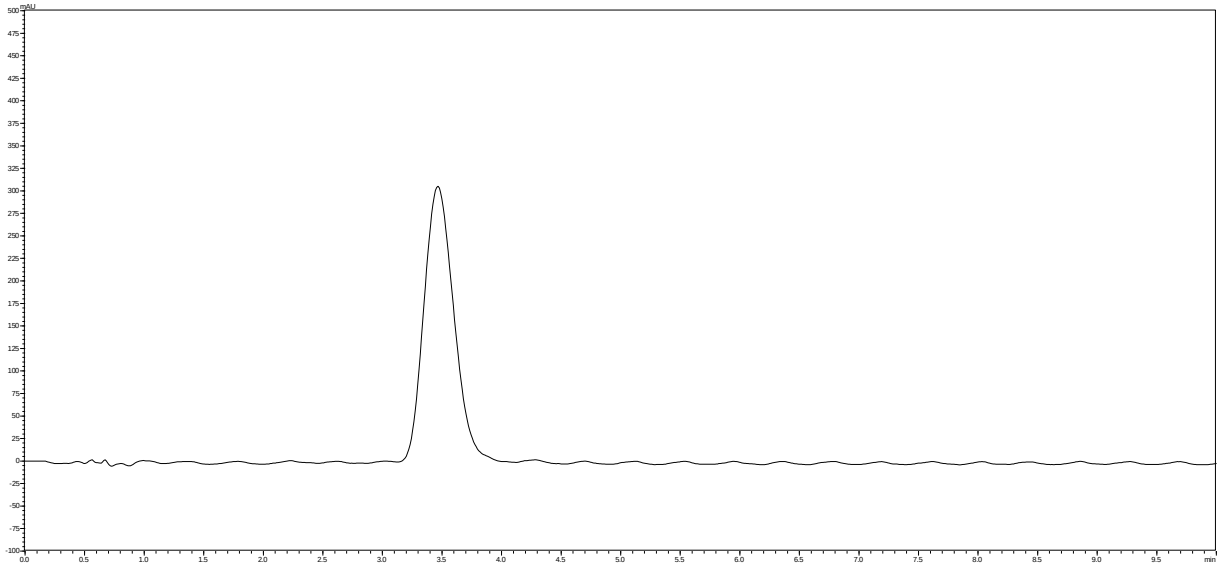




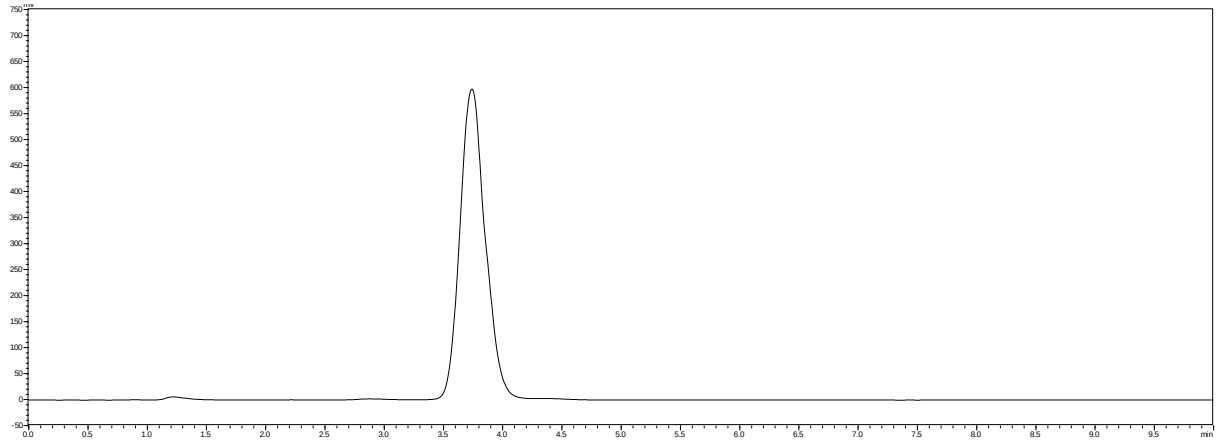
13.21 HPLC of 2.3

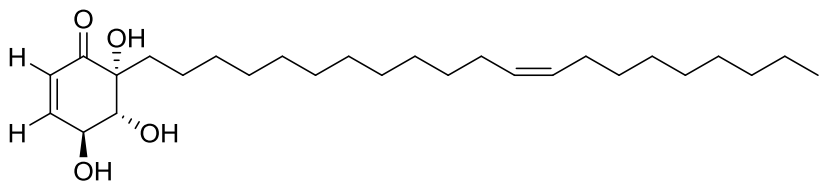
Column: Cogent Bidentate C18 100 A, 4 μm, 7.5 x 4.6 mm
Concentration: 1 mg/mL
Injection: 25 μL injection
Mobile Phase: 92.5% MeOH (aq.) with 0.01% Formic Acid

Detector: PDA (217 nm)

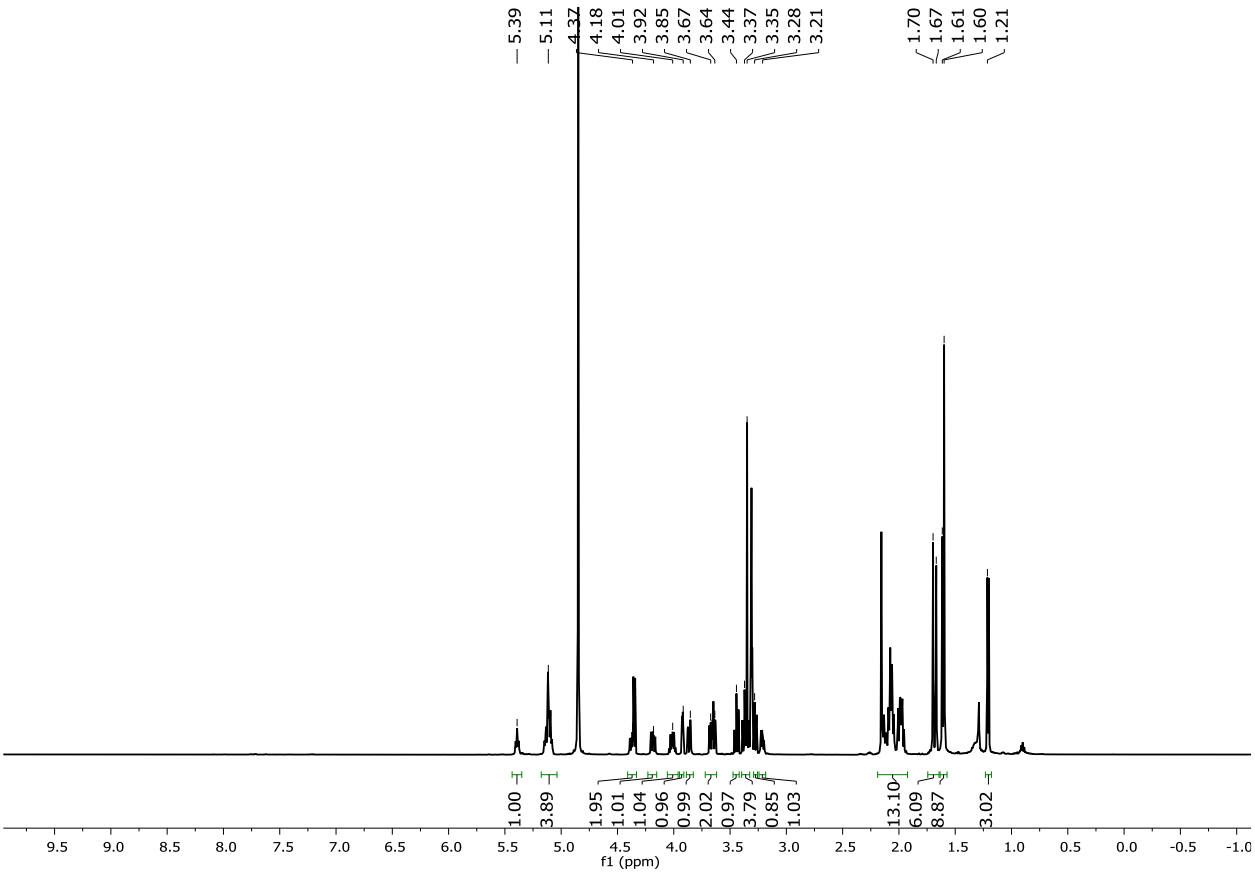


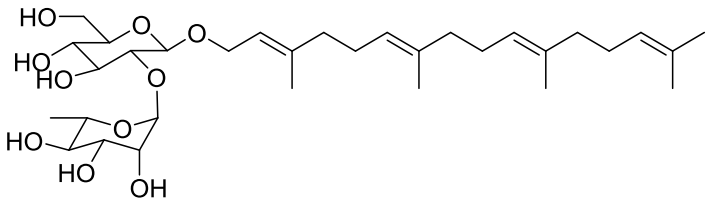
Detector: ELSD





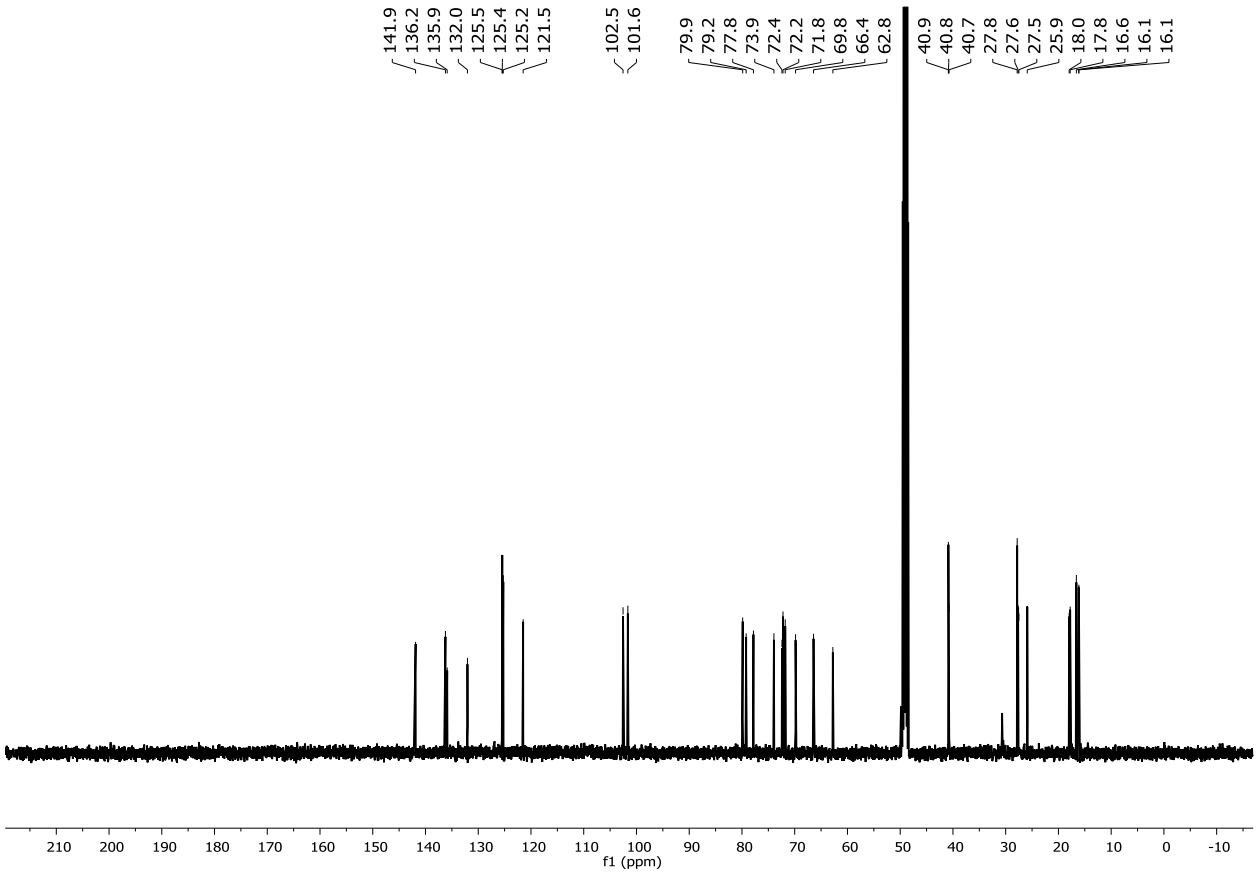
13.22 ^{1}H NMR of 3.1 (CD_3OD, 500 MHz)

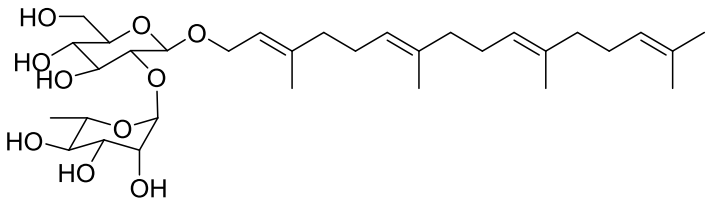




2'',3'',4'',6'-de-*O*-acetylcupacinoside (**3.1**)

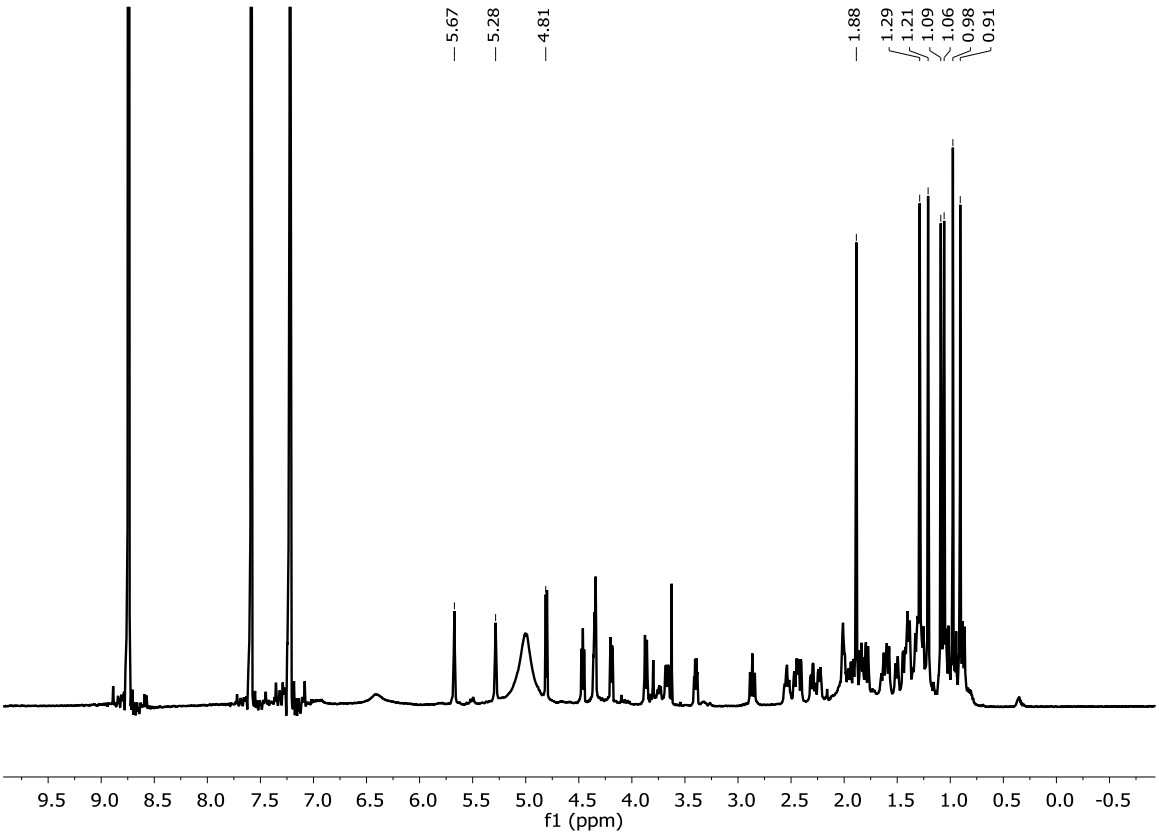
13.23 ^{13}C NMR of 3.1 (CD_3OD, 125 MHz)

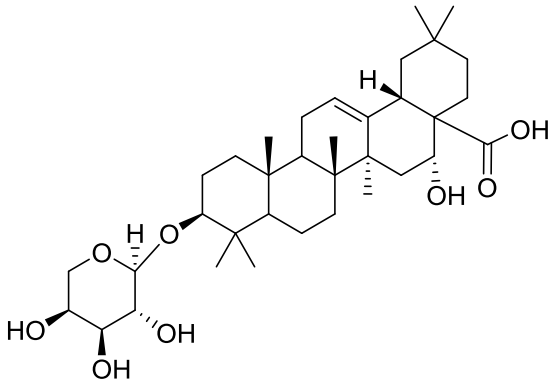




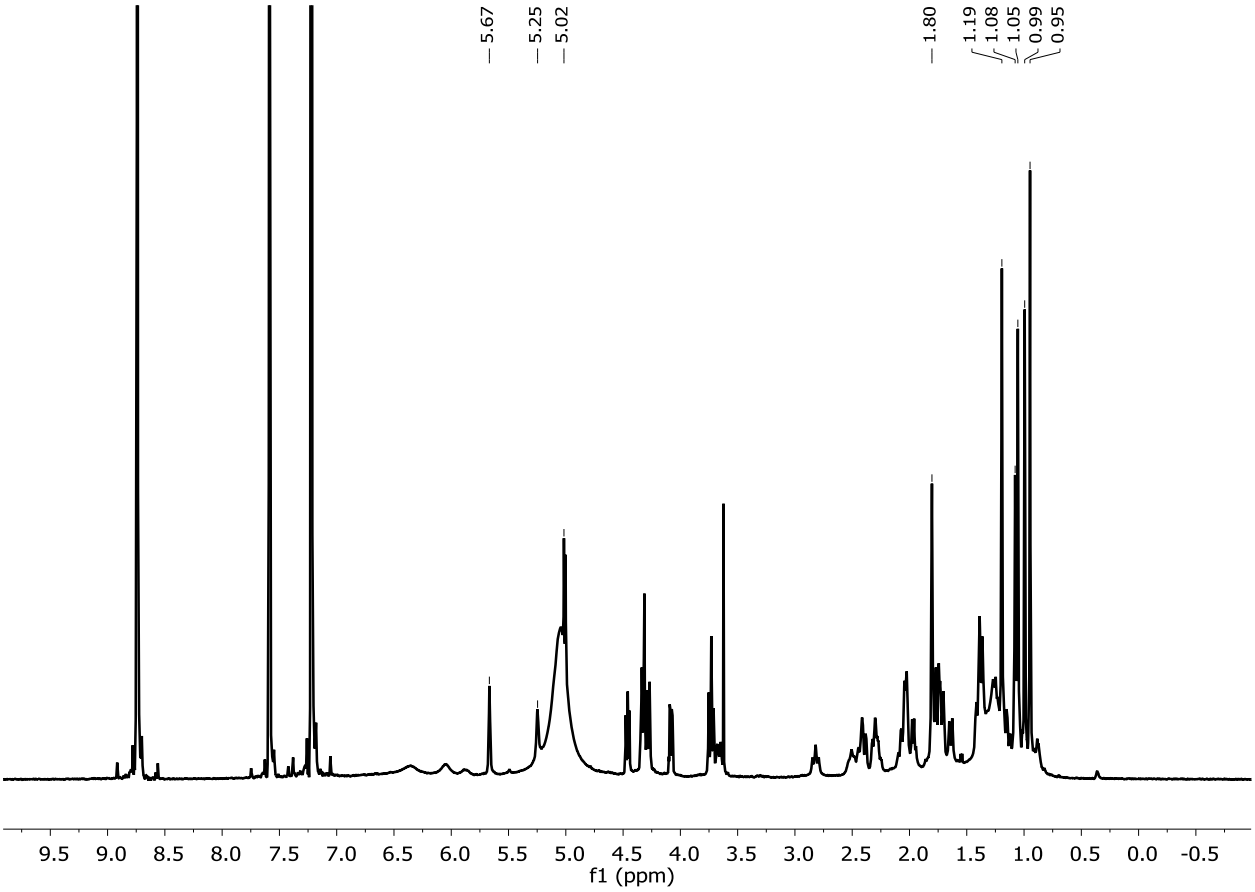
2'',3'',4'',6'-de-*O*-acetylcupacinoside (**3.1**)

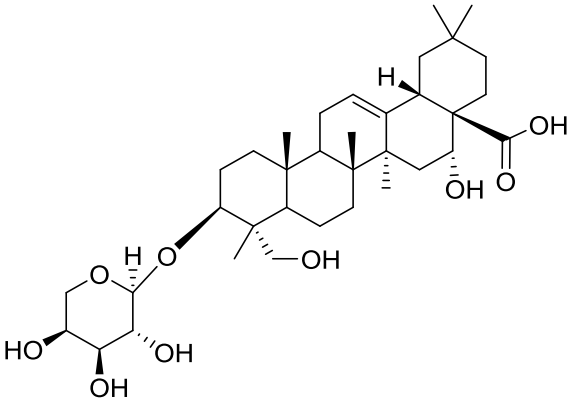
13.24 ^{1}H NMR of 4.1 (C_5D_5N, 600 MHz)



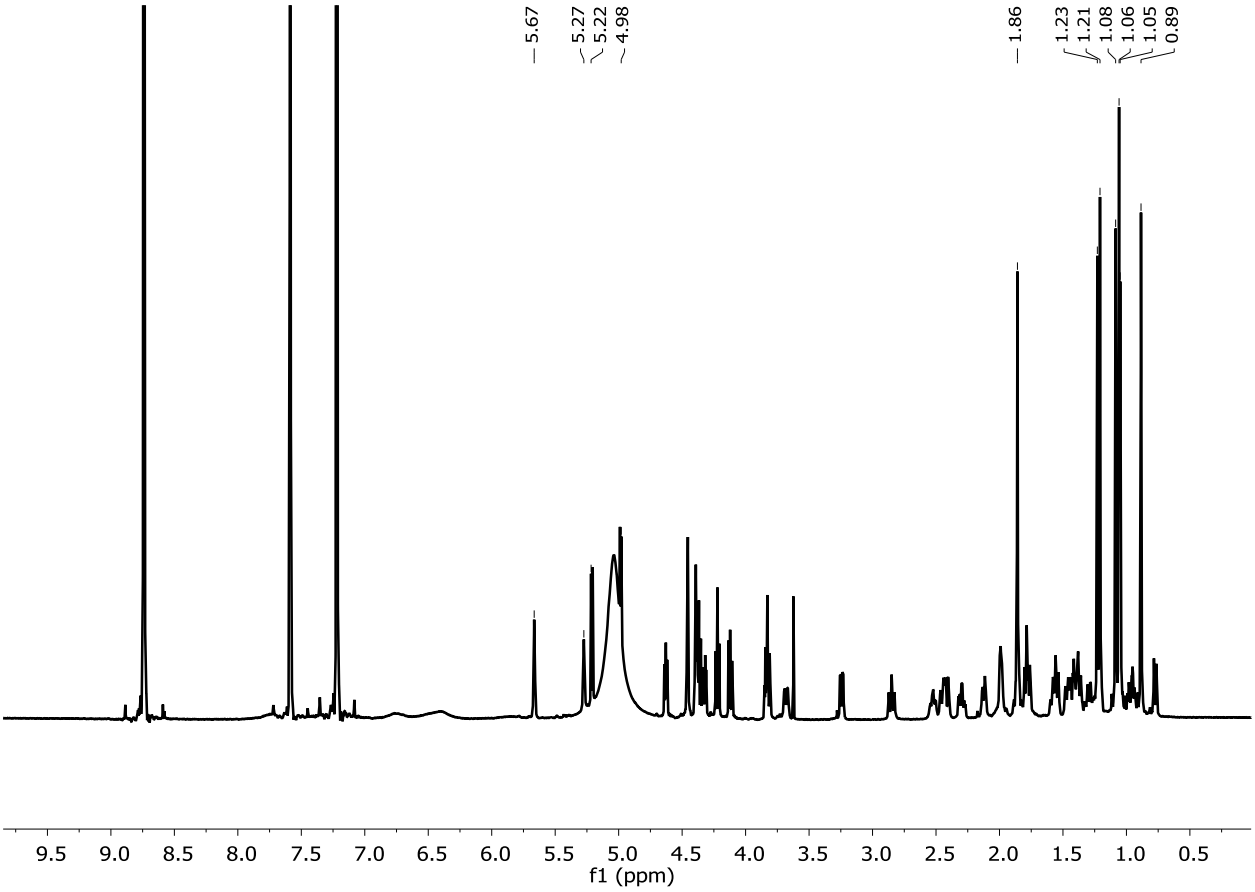


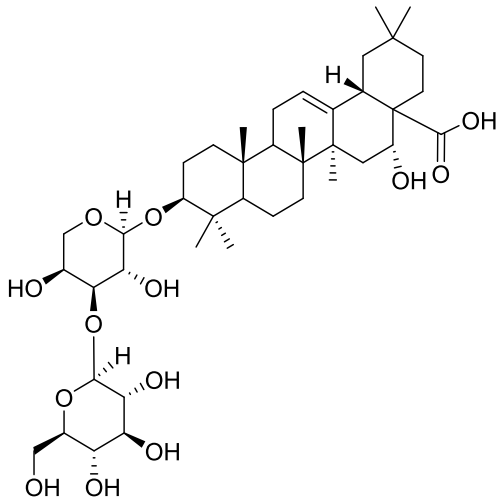
13.25 ^{1}H NMR of 4.2 (C_5D_5N, 600 MHz)



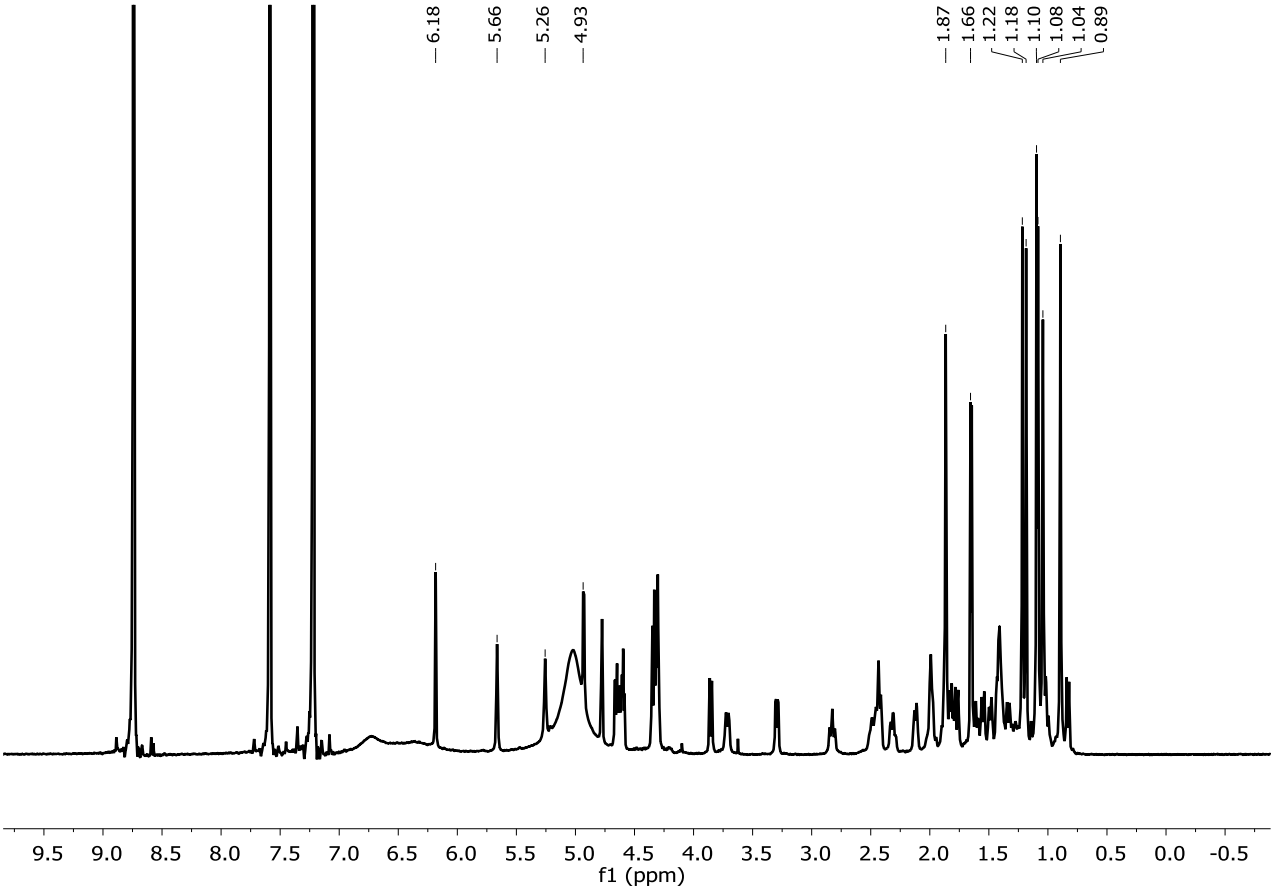


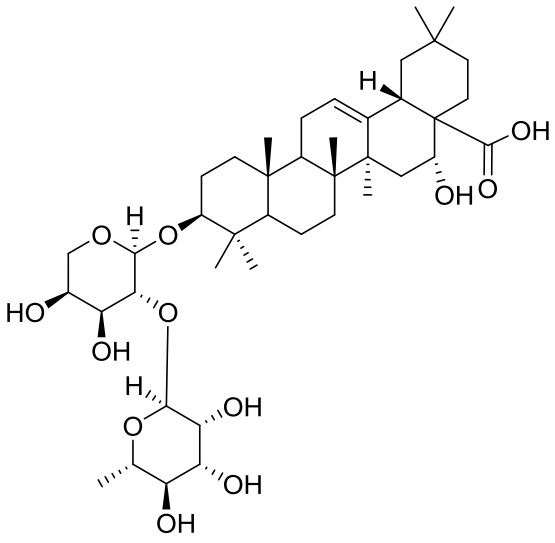
13.26 ^{1}H NMR of 4.3 (C_5D_5N, 600 MHz)



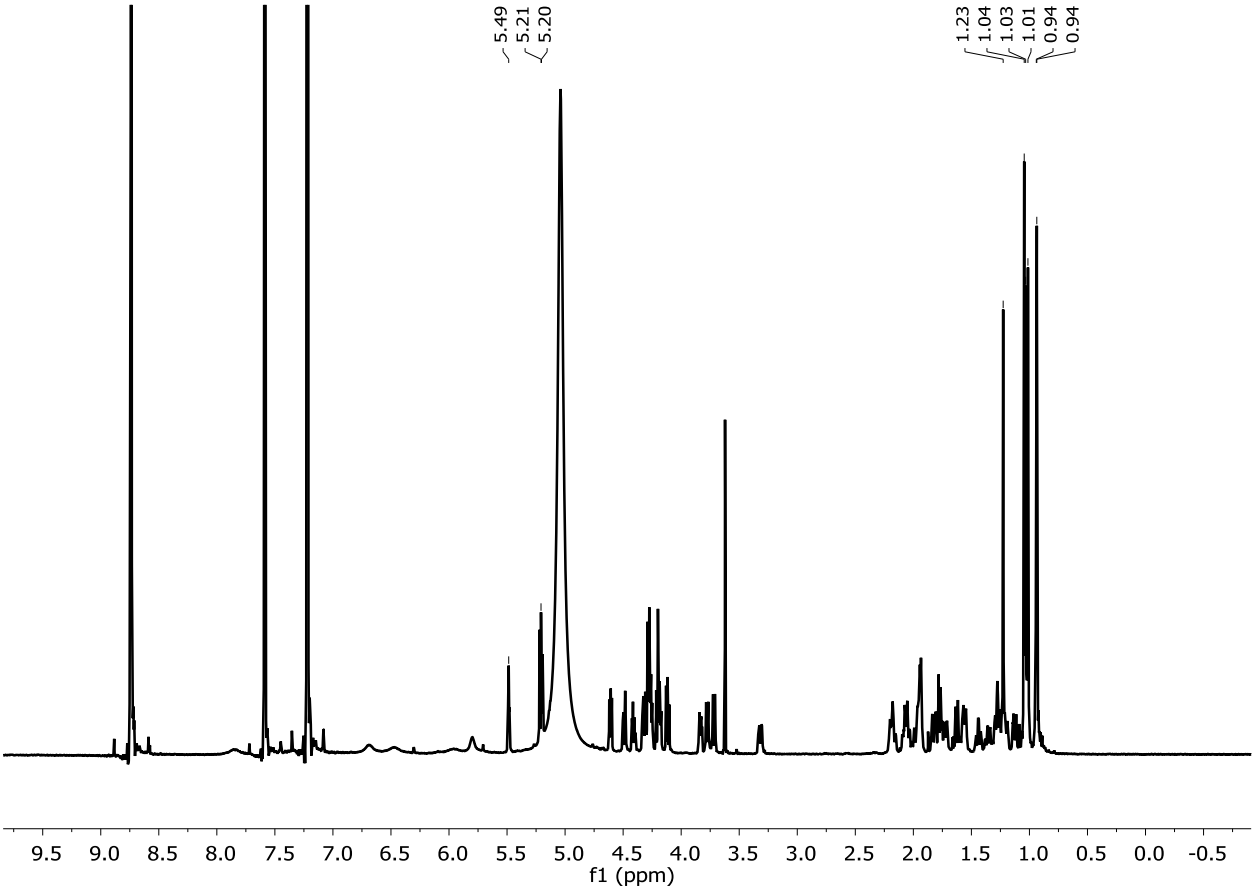


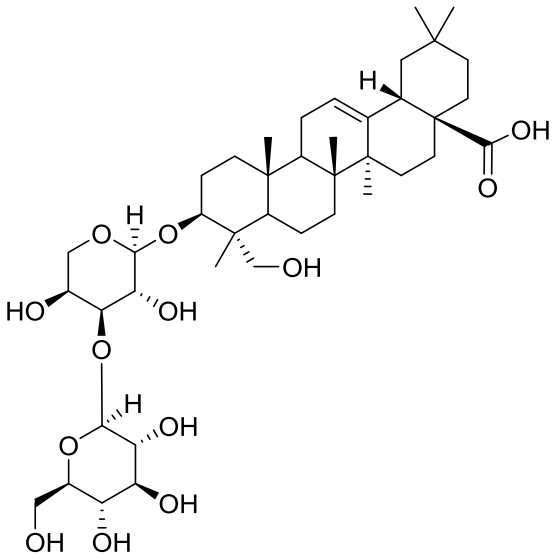
13.27 ^{1}H NMR of 4.4 (C_5D_5N, 600 MHz)



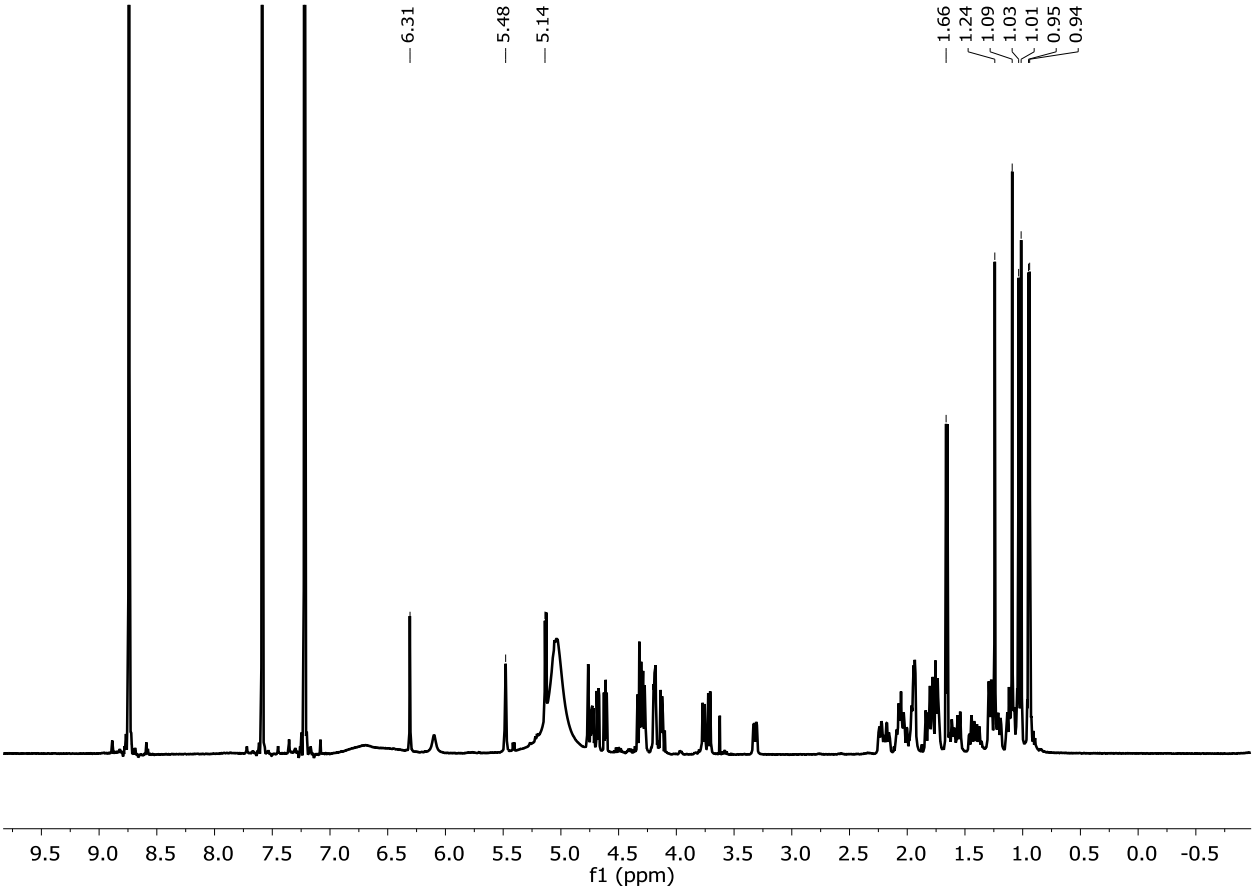


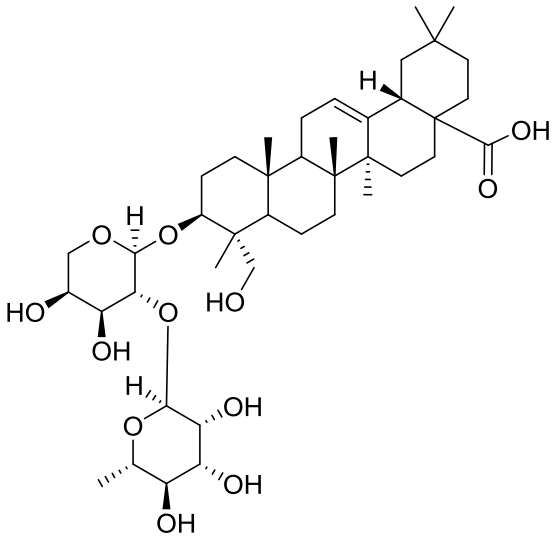
13.28 ^{1}H NMR of 4.5 (C_5D_5N, 600 MHz)



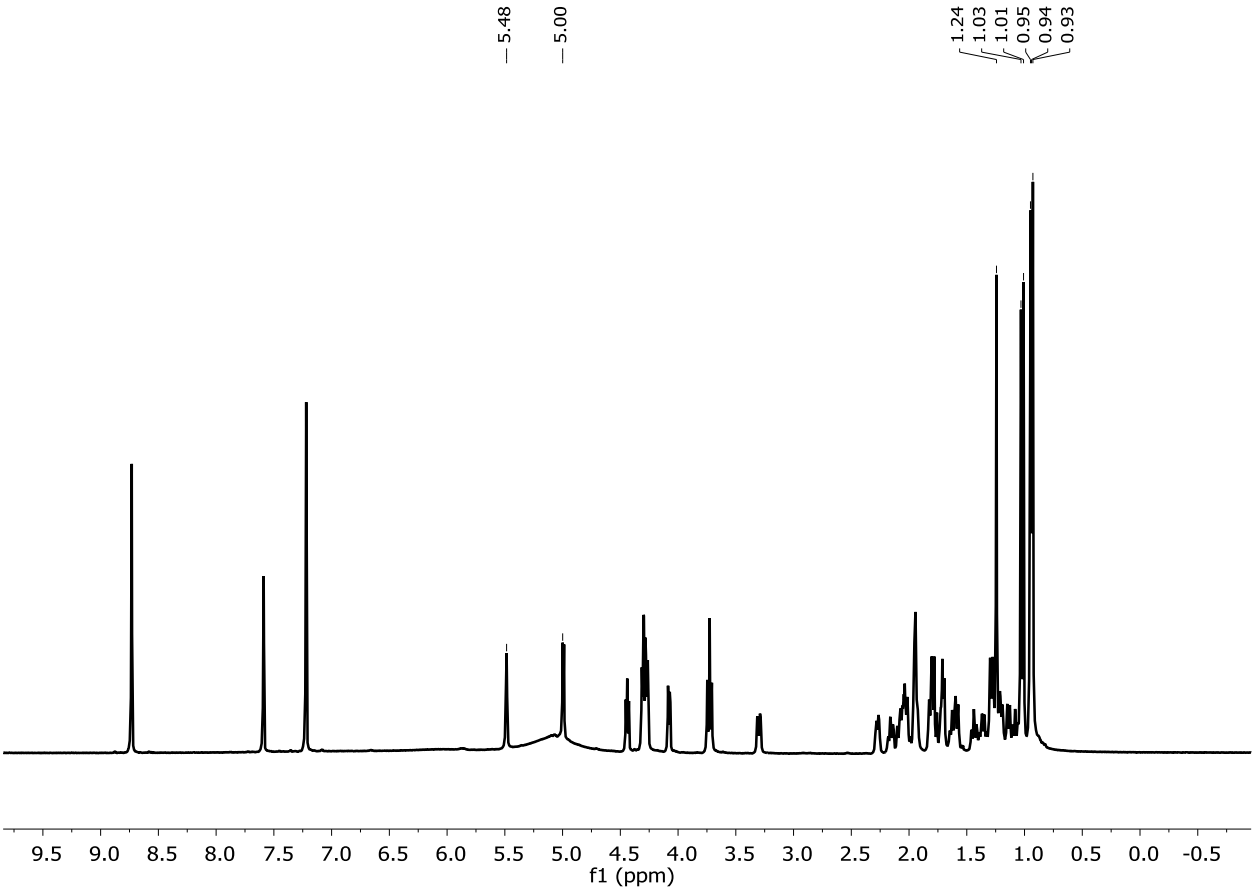


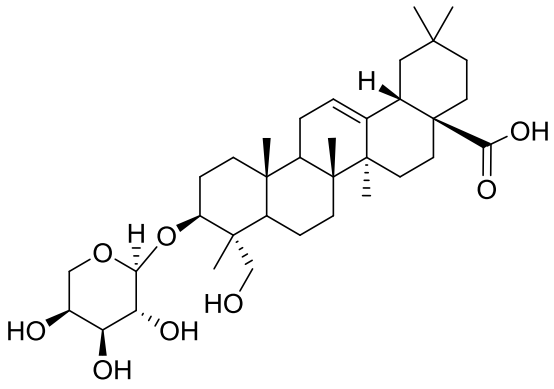
13.29 ^{1}H NMR of 4.6 (C_5D_5N, 600 MHz)





13.30 ^{1}H NMR of 4.7 (C_5D_5N, 600 MHz)

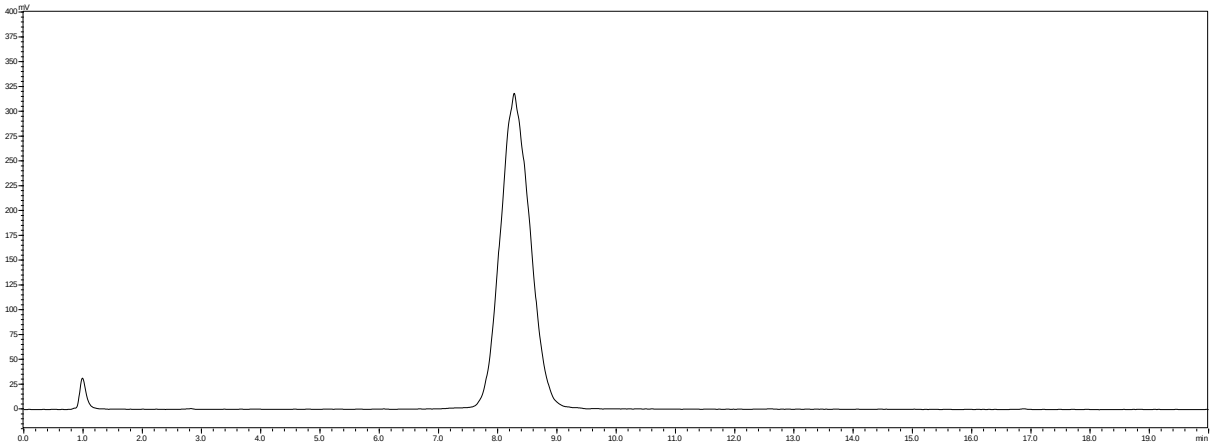


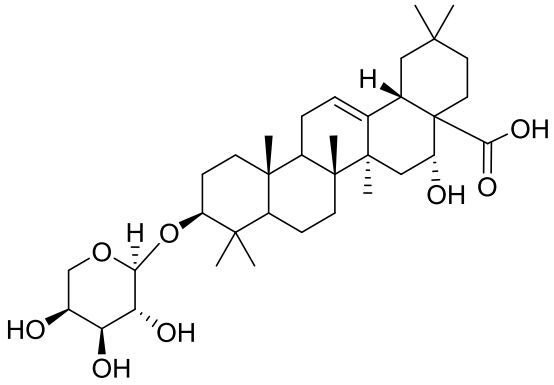


13.31 HPLC of 4.1

Concentration: 1 mg/mL in MeOH
Injection Volume: 25 μL
Column: Cogent C_{18}Analytical (75 x 4.6 mm)
Solvent: 69% MeOH/H_2O w/ 0.01% Formic Acid
Flow Rate: 1.5 mL/min

Detector: ELSD

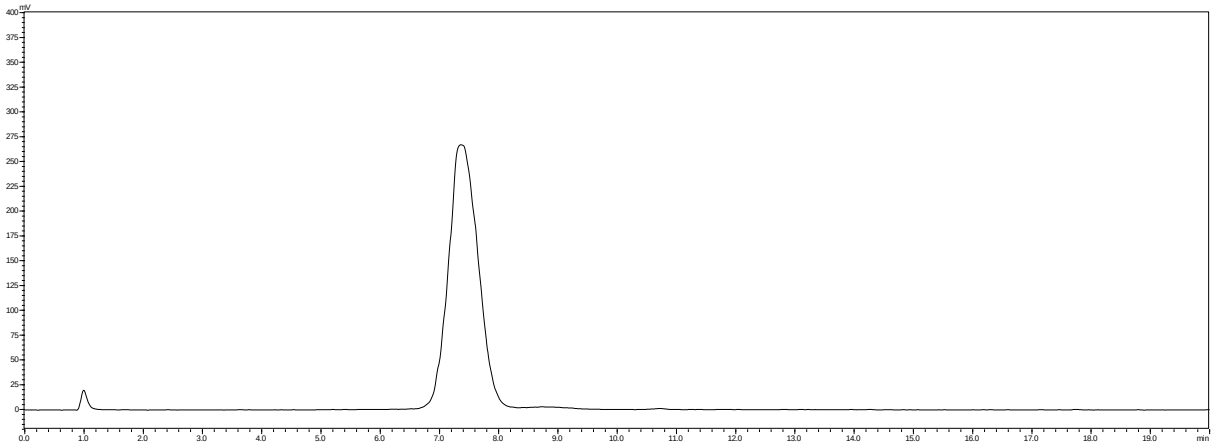


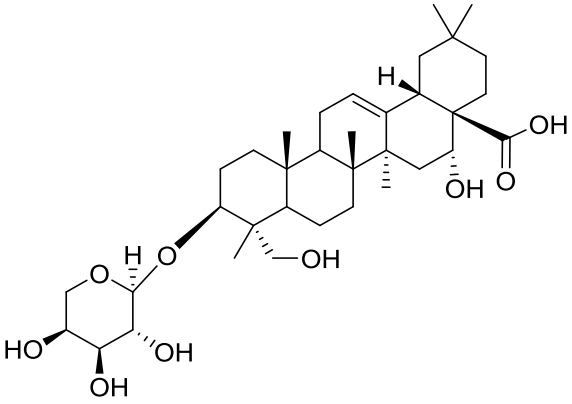


13.32 HPLC of 4.2

Concentration: 1 mg/mL in MeOH
Injection Volume: 25 μL
Column: Cogent C_{18}Analytical (75 x 4.6 mm)
Solvent: 66% MeOH/H_2O w/ 0.01% Formic Acid
Flow Rate: 1.5 mL/min

Detector: ELSD

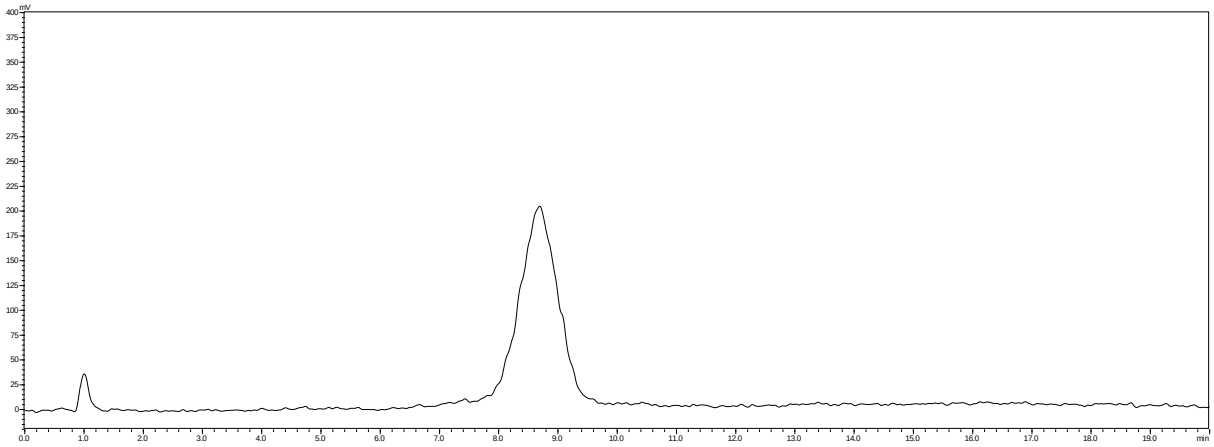


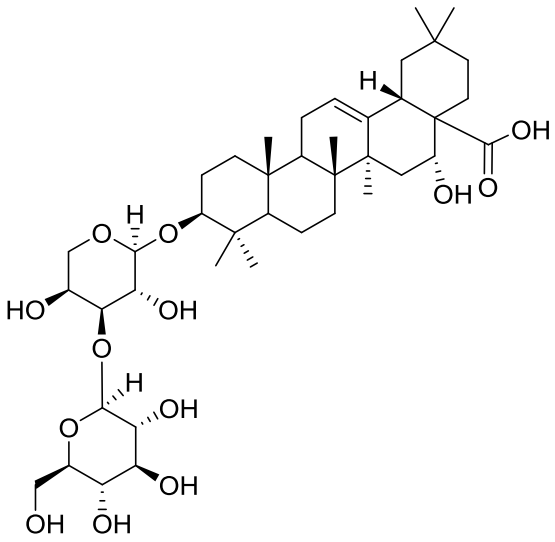


13.33 HPLC of 4.3

Concentration: 1 mg/mL in MeOH
Injection Volume: 25 μL
Column: Cogent C_{18}Analytical (75 x 4.6 mm)
Solvent: 66% MeOH/H_2O w/ 0.01% Formic Acid
Flow Rate: 1.5 mL/min

Detector: ELSD

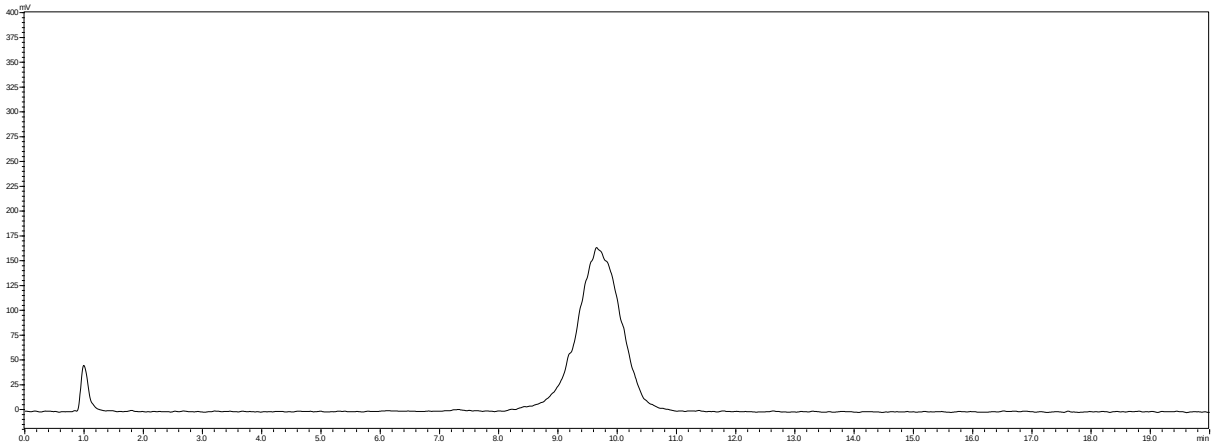


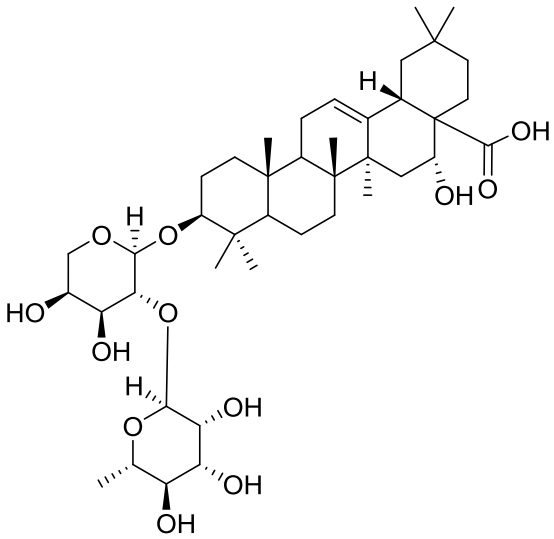


13.34 HPLC of 4.4

Concentration: 1 mg/mL in MeOH
Injection Volume: 25 μL
Column: Cogent C_{18}Analytical (75 x 4.6 mm)
Solvent: 67% MeOH/H_2O w/ 0.01% Formic Acid
Flow Rate: 1.5 mL/min

Detector: ELSD

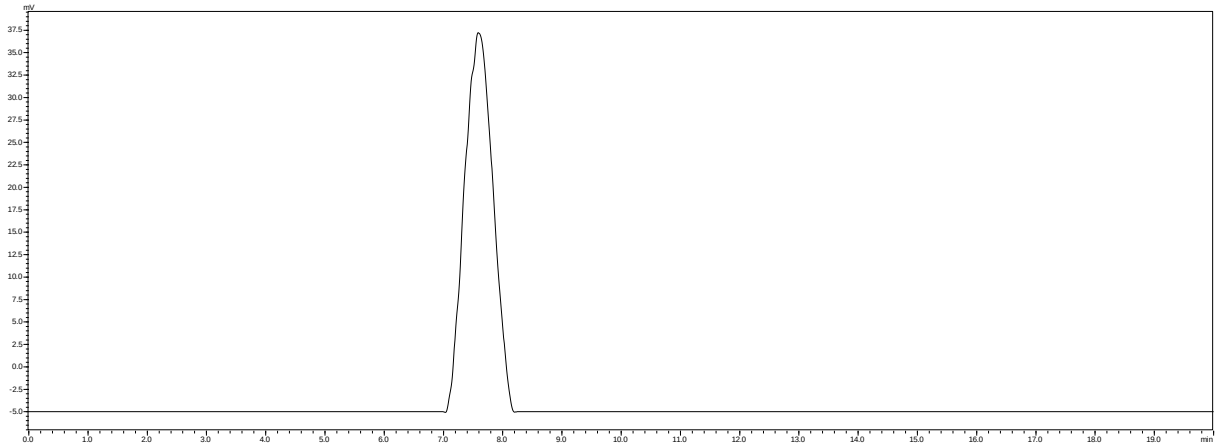


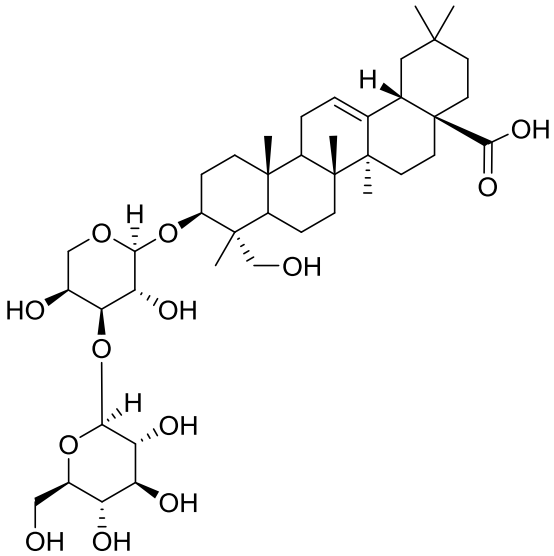


13.35 HPLC of 4.5

Concentration: 0.15 mg/mL in MeOH
Injection Volume: 25 μL
Column: Cogent C_{18}Analytical (75 x 4.6 mm)
Solvent: 70% MeOH/H_2O w/ 0.01% Formic Acid
Flow Rate: 1.5 mL/min

Detector: ELSD

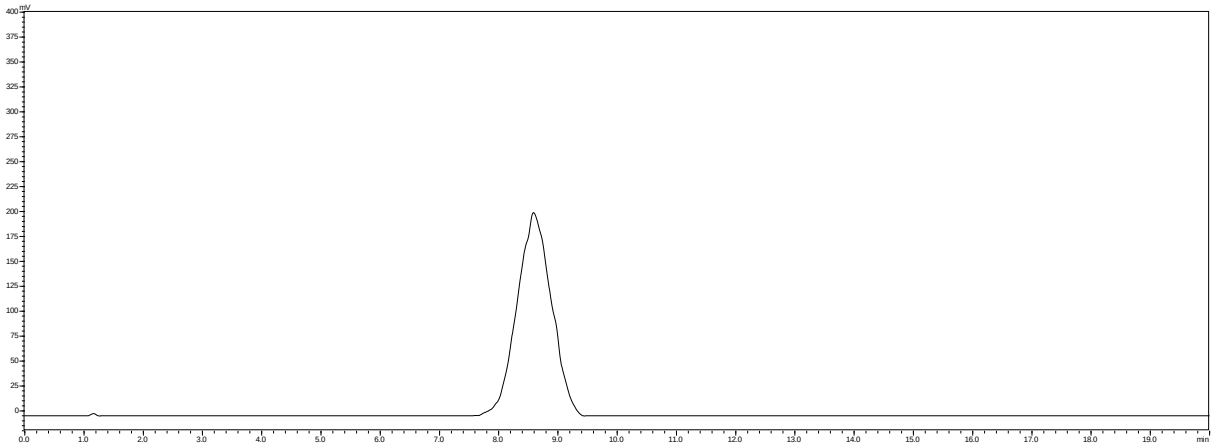


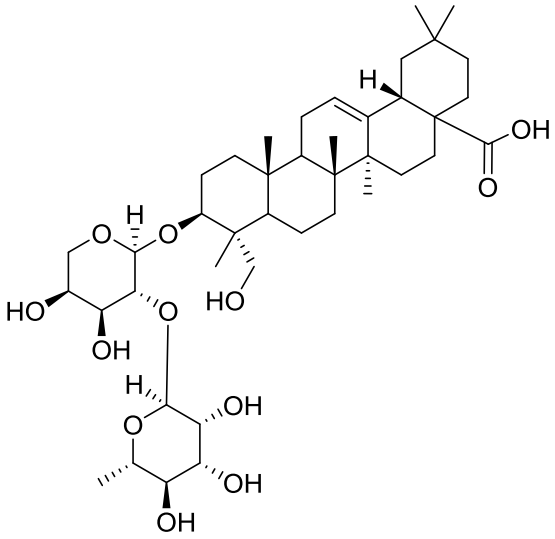


13.36 HPLC of 4.6

Concentration: 1 mg/mL in MeOH
Injection Volume: 25 μL
Column: Cogent C_{18}Analytical (75 x 4.6 mm)
Solvent: 70% MeOH/H_2O w/ 0.01% Formic Acid
Flow Rate: 1.5 mL/min

Detector: ELSD

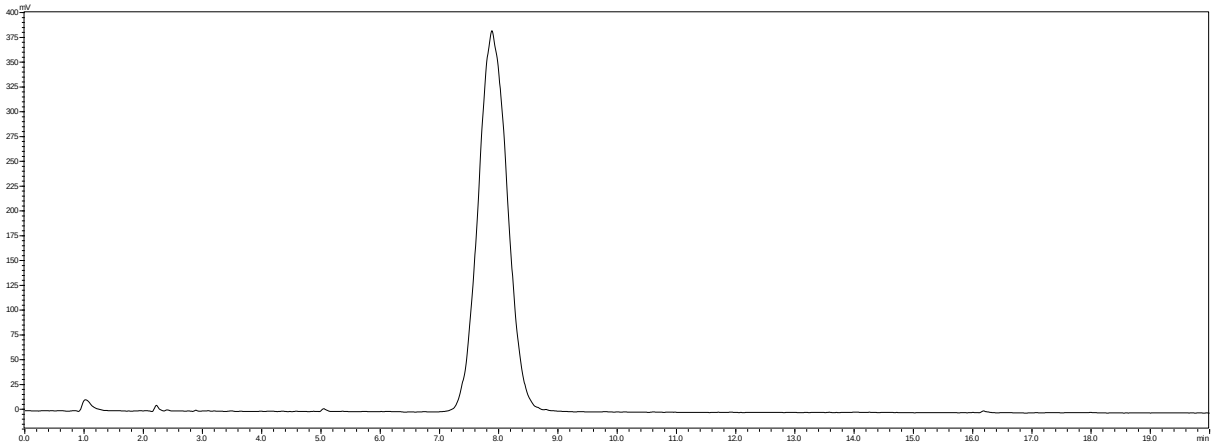


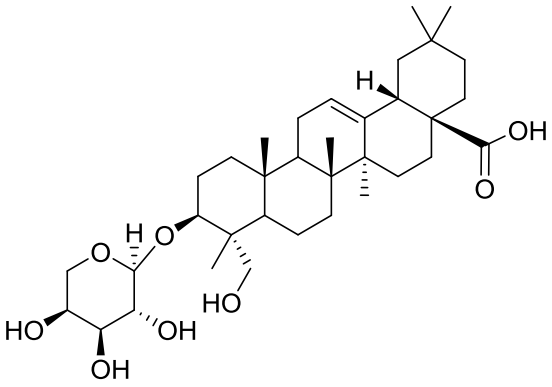


13.37 HPLC of 4.7

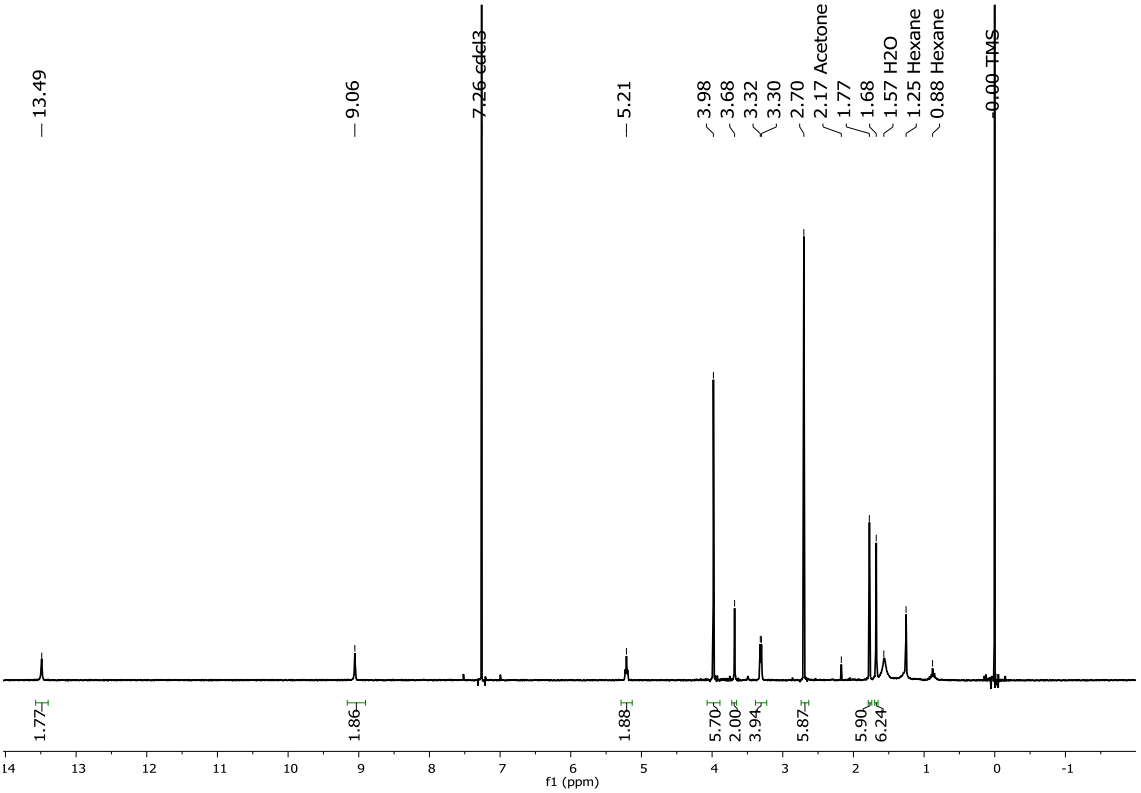
Concentration: 1 mg/mL in MeOH
Injection Volume: 25 μL
Column: Cogent C_{18}Analytical (75 x 4.6 mm)
Solvent: 72% MeOH/H_2O w/ 0.01% Formic Acid
Flow Rate: 1.5 mL/min

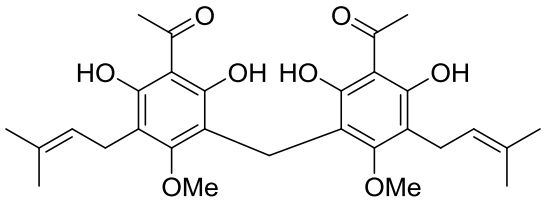
Detector: ELSD



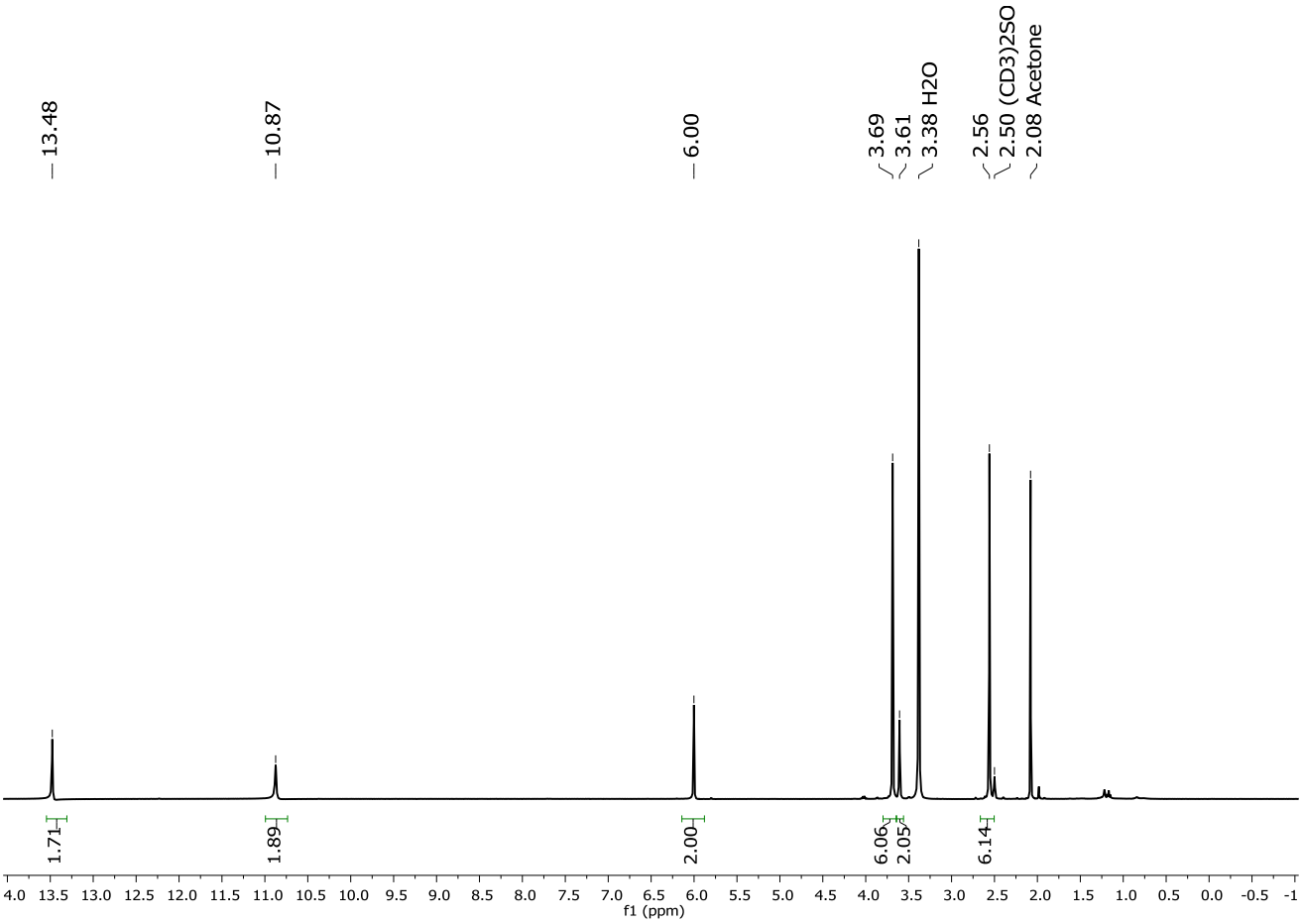


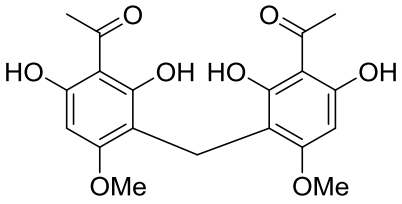
13.38 ^{1}H NMR of 5.1 ($CDCl_3$, 400 MHz)



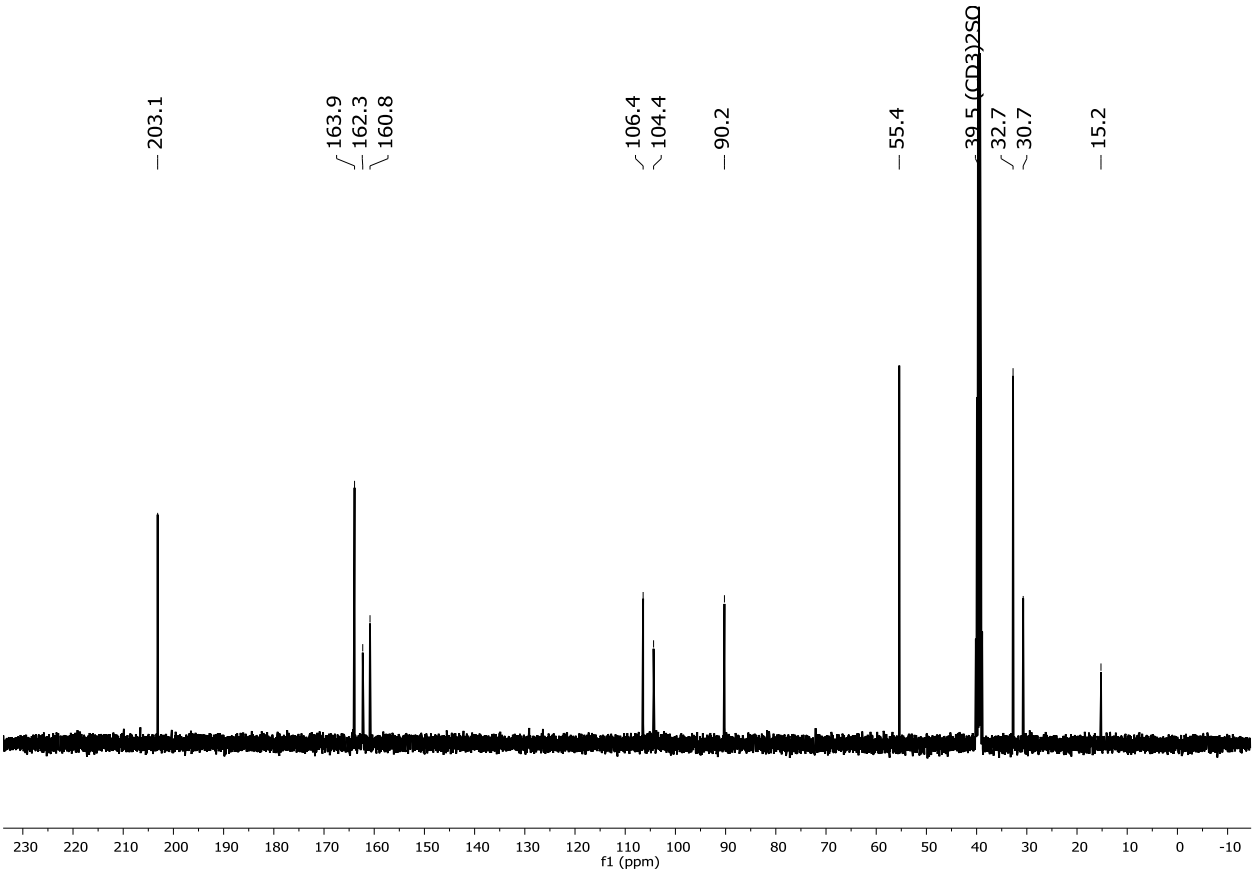


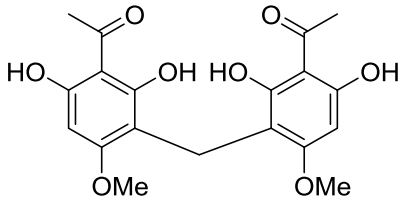
13.39 ^{1}H NMR of 5.5 ($(CD_3)_2SO$, 400 MHz)



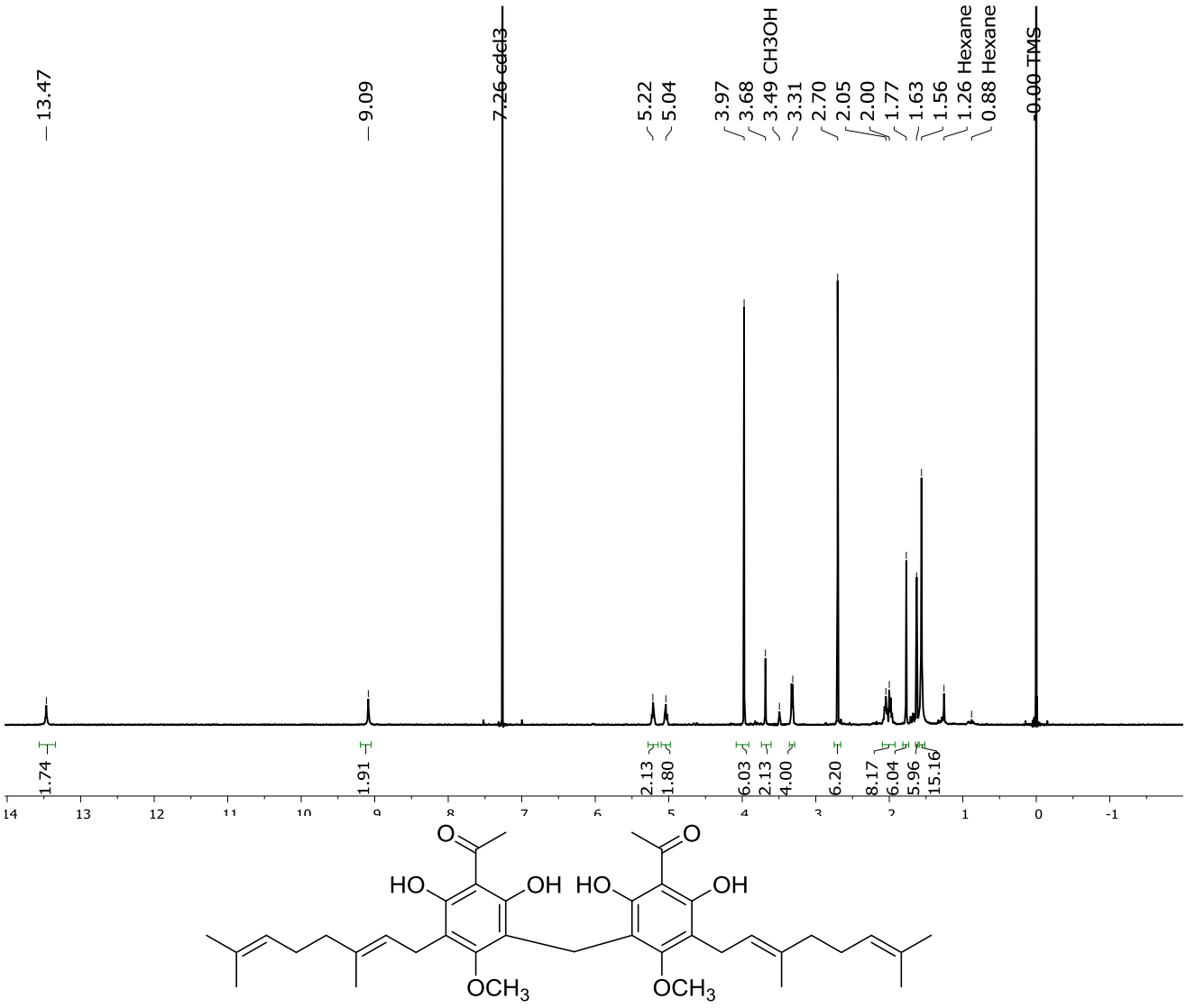


13.40 ^{13}C NMR of 5.5 ($(CD_3)_2SO$, 100 MHz)

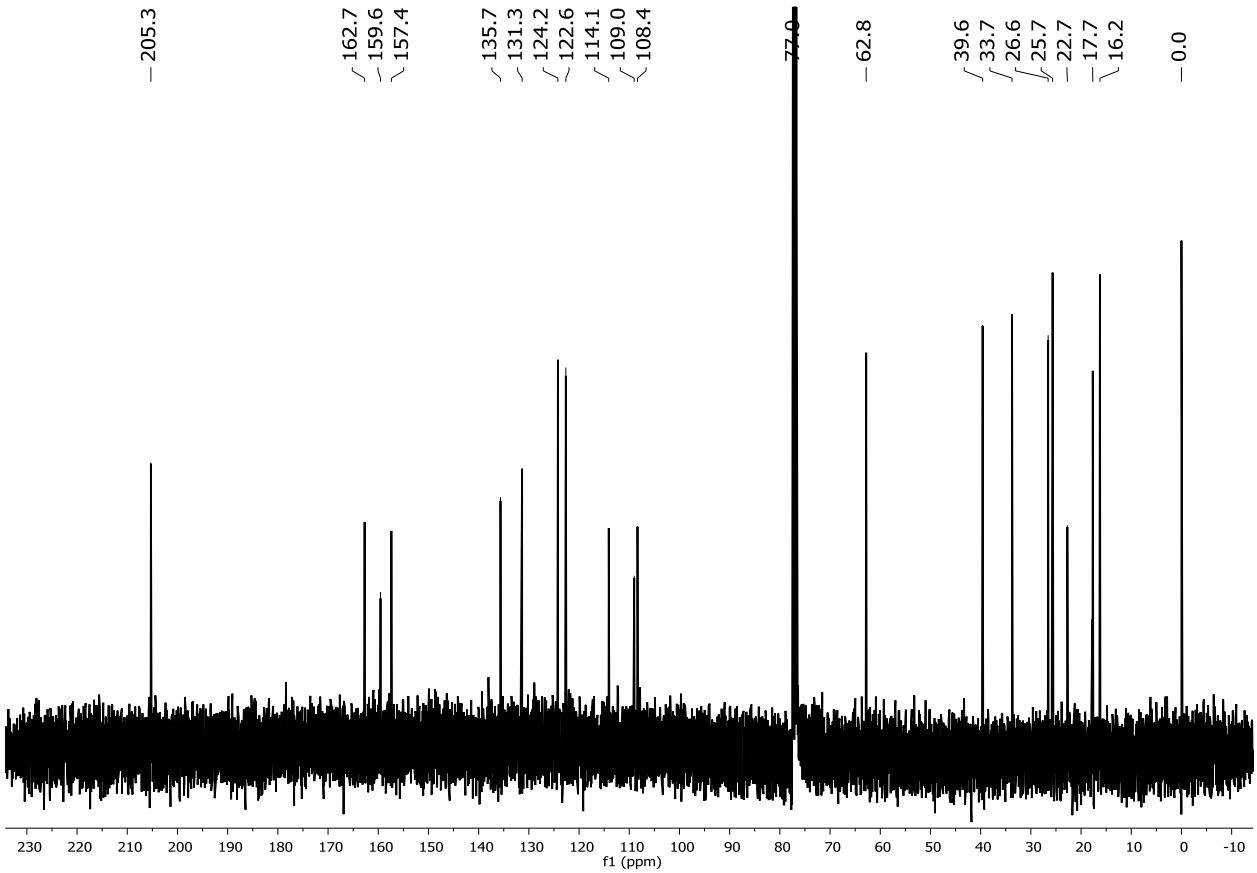


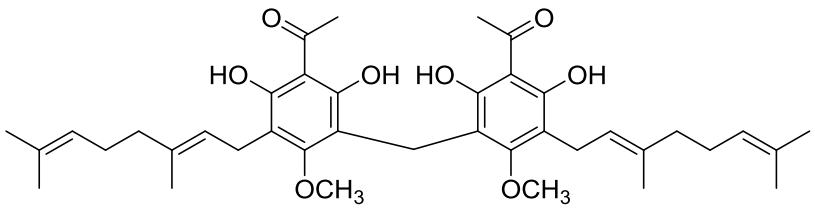


13.41 ^{1}H NMR of 5.6 ($CDCl_3$, 400 MHz)

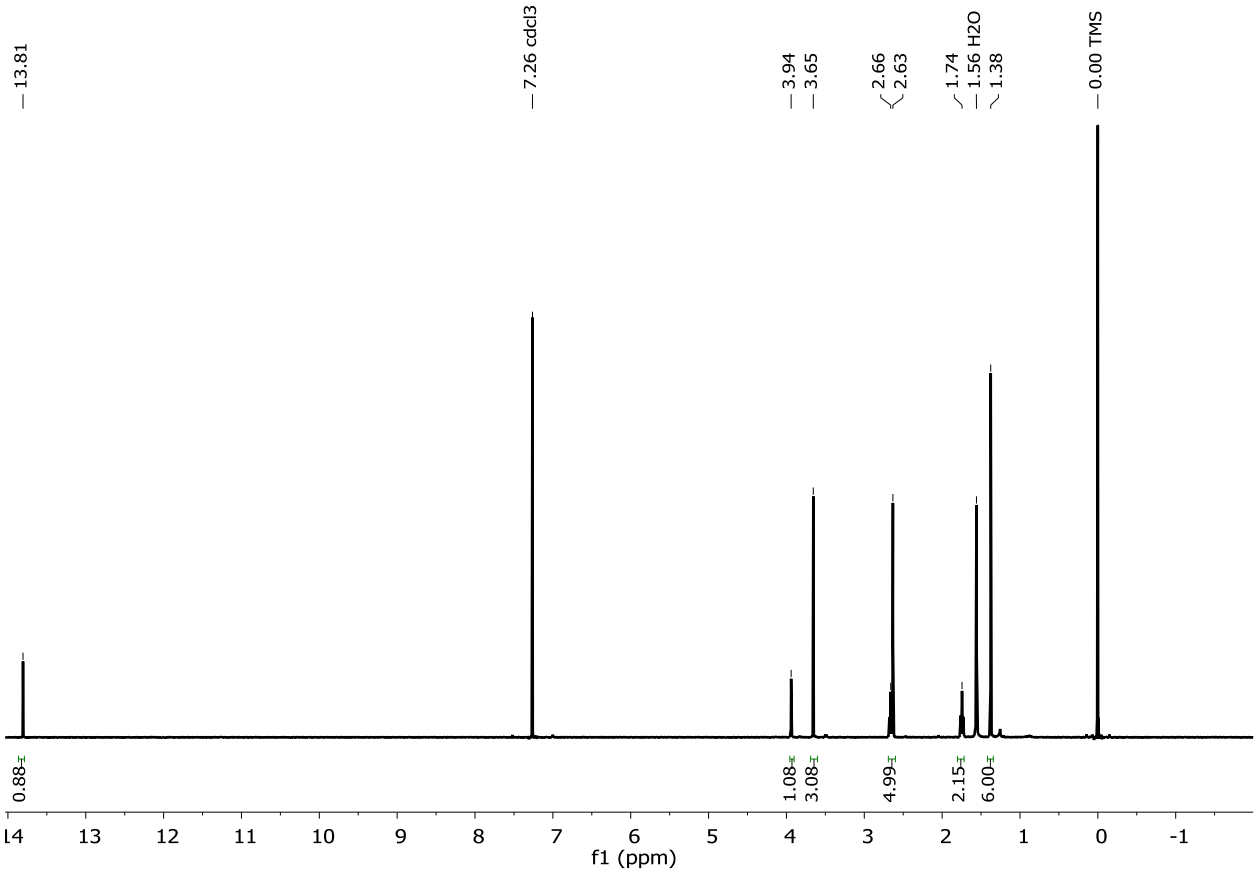


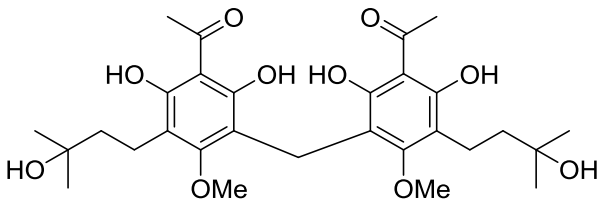
13.42 ^{13}C NMR of 5.6 ($CDCl_3$, 100 MHz)



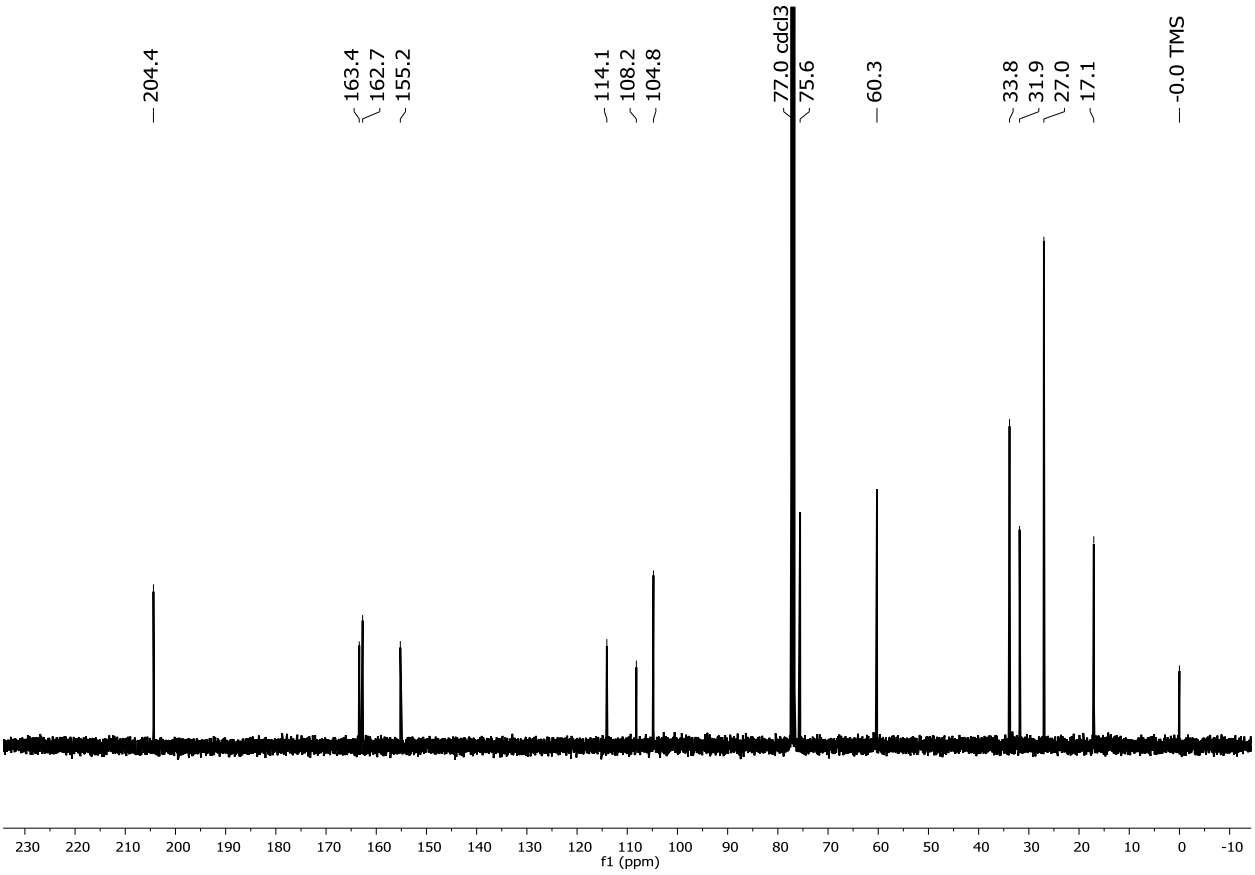


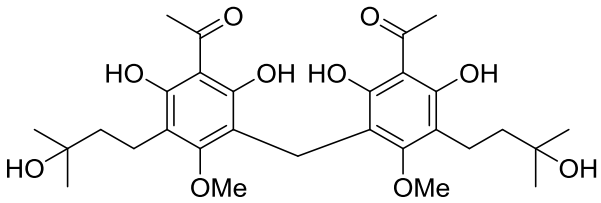
13.43 ^{1}H NMR of 5.7 ($CDCl_3$, 400 MHz)



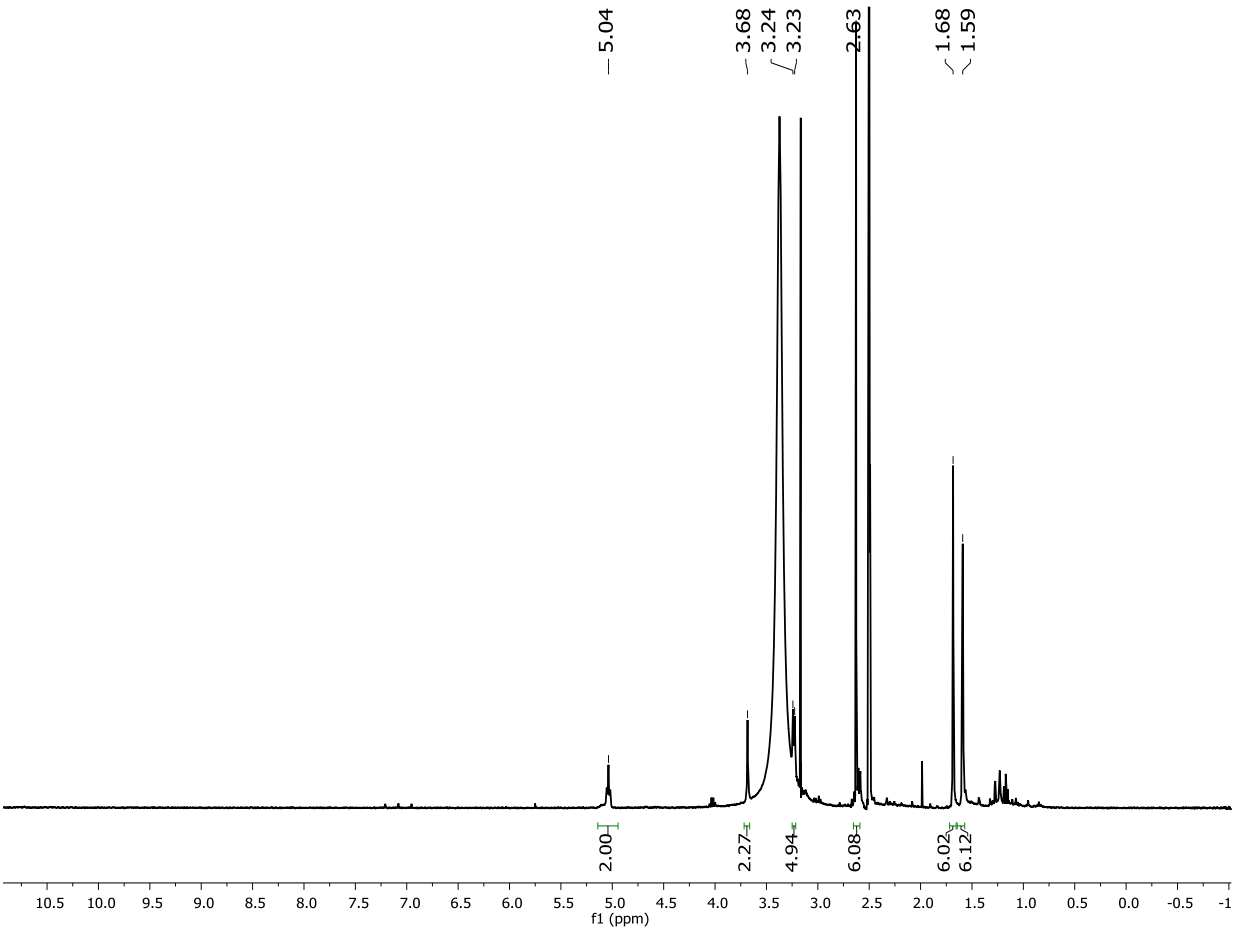


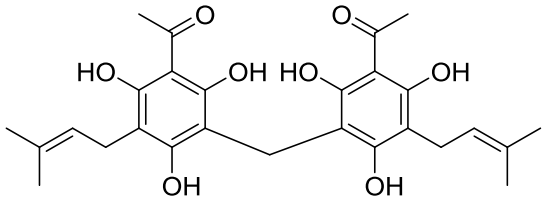
13.44 ^{13}C NMR of 5.7 ($CDCl_3$, 100 MHz)



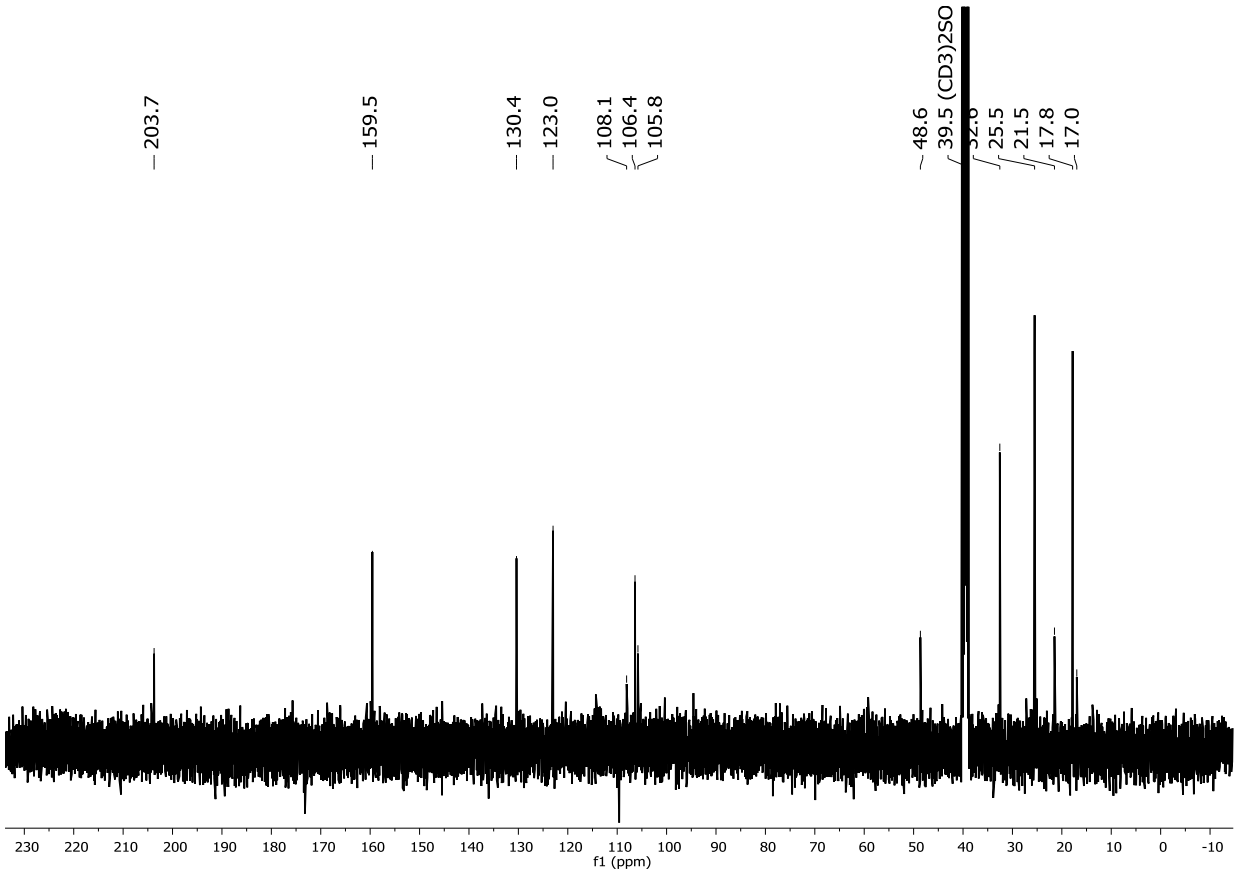


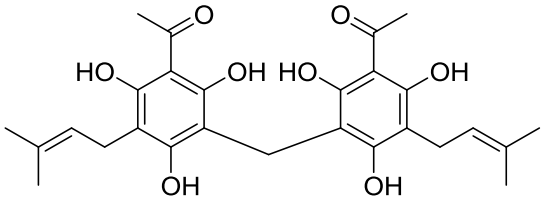
13.45 ^{1}H NMR of 5.8 ($(CD_3)_2SO$, 400 MHz)



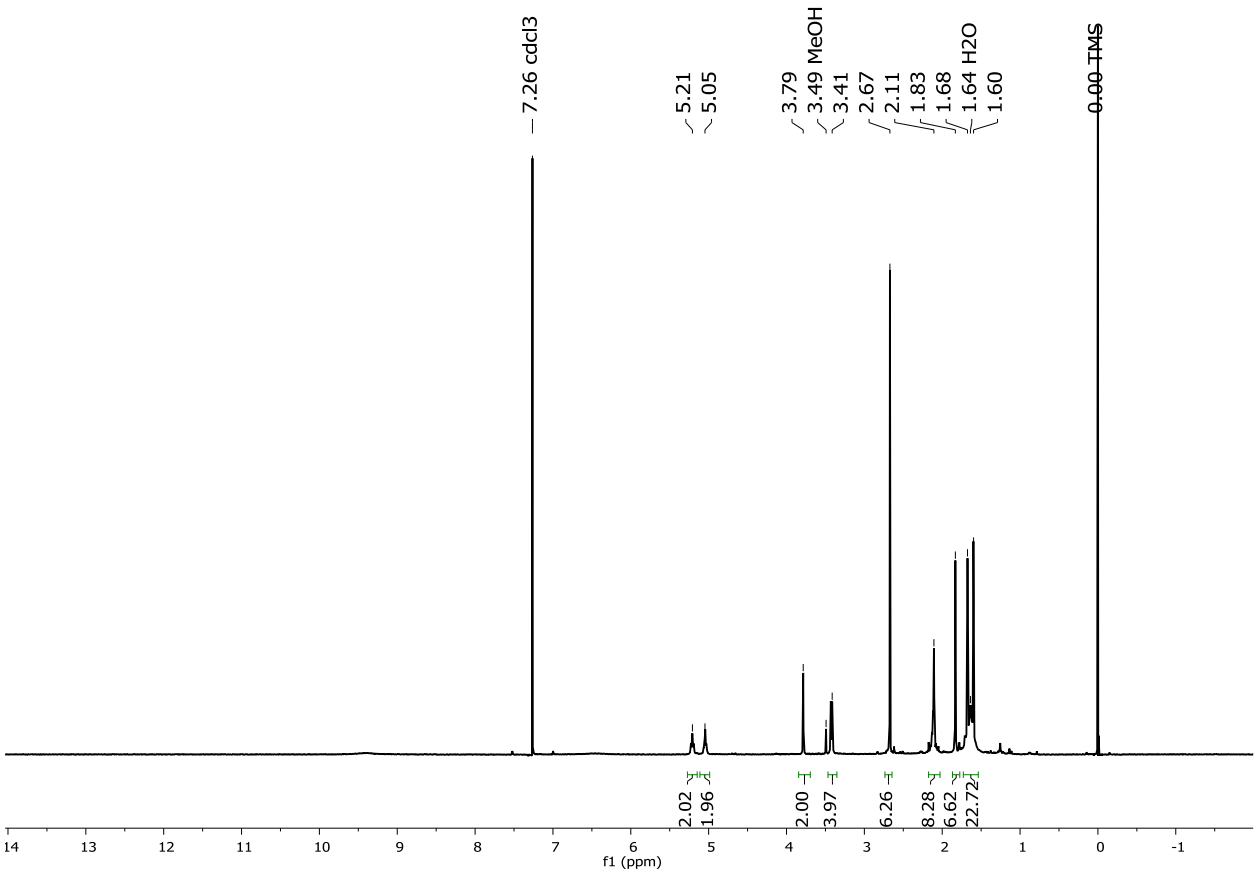


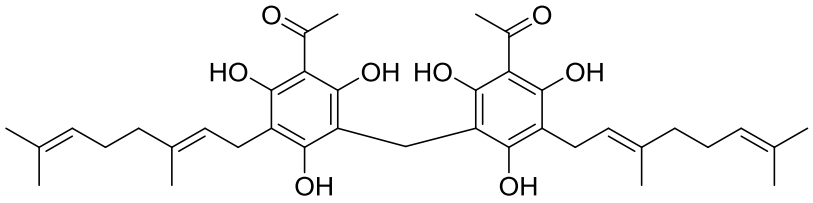
13.46 ^{13}C NMR of 5.8 ($(CD_3)_2SO$, 100 MHz)



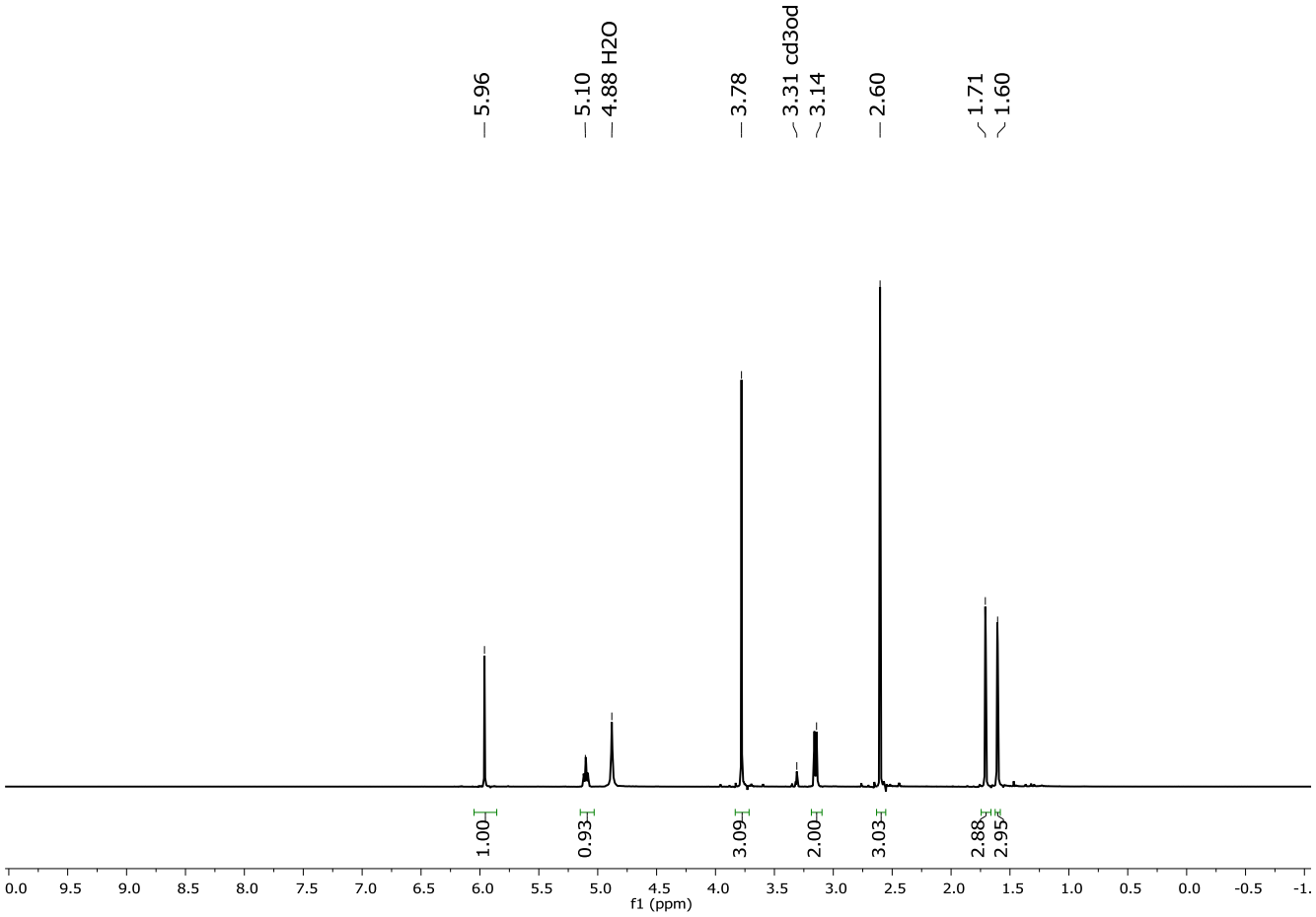


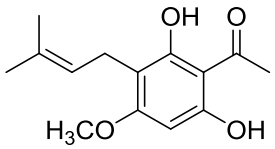
13.47 ^{1}H NMR of 5.9 ($CDCl_3$, 400 MHz)



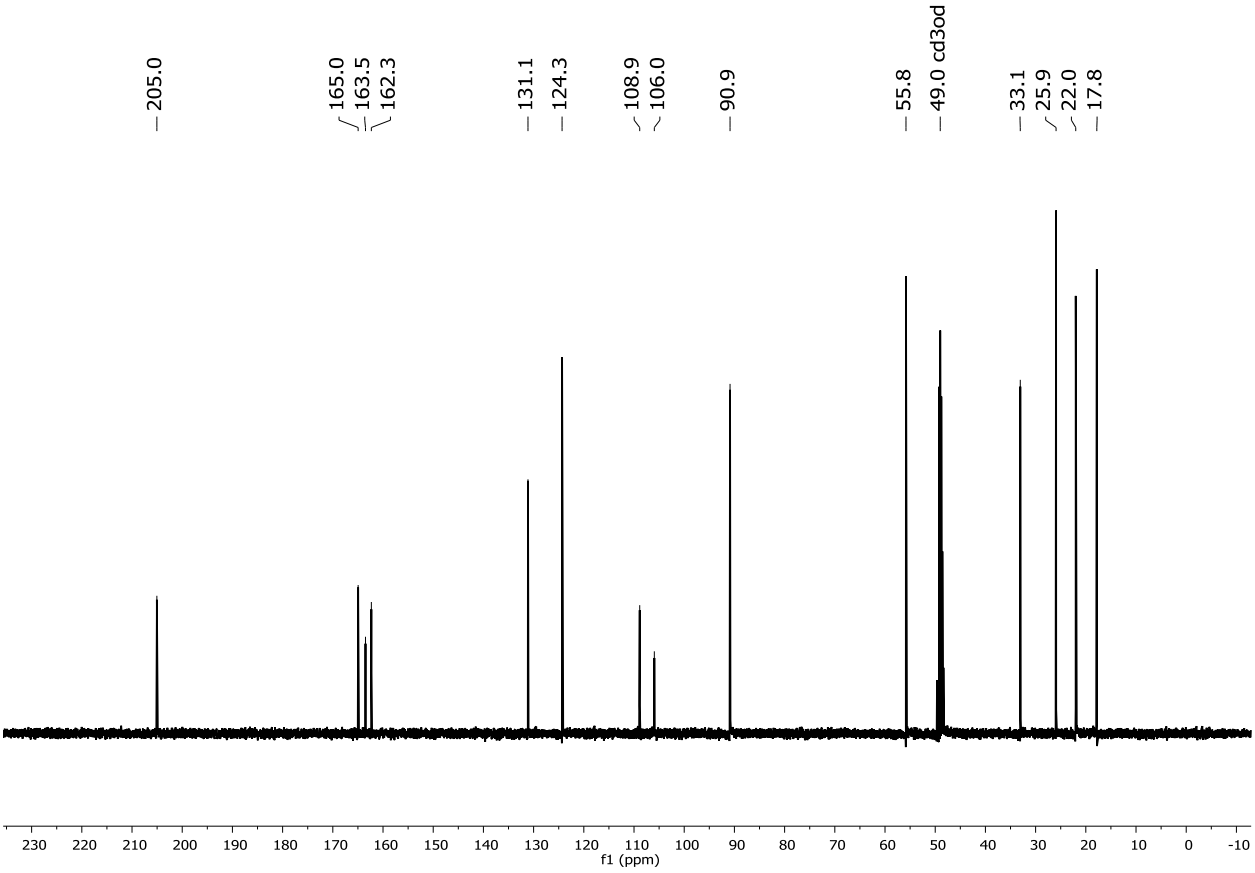


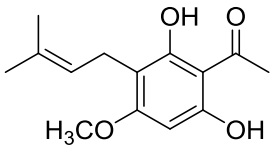
13.48 ^{1}H NMR of 5.11 (CD_3OD, 400 MHz)



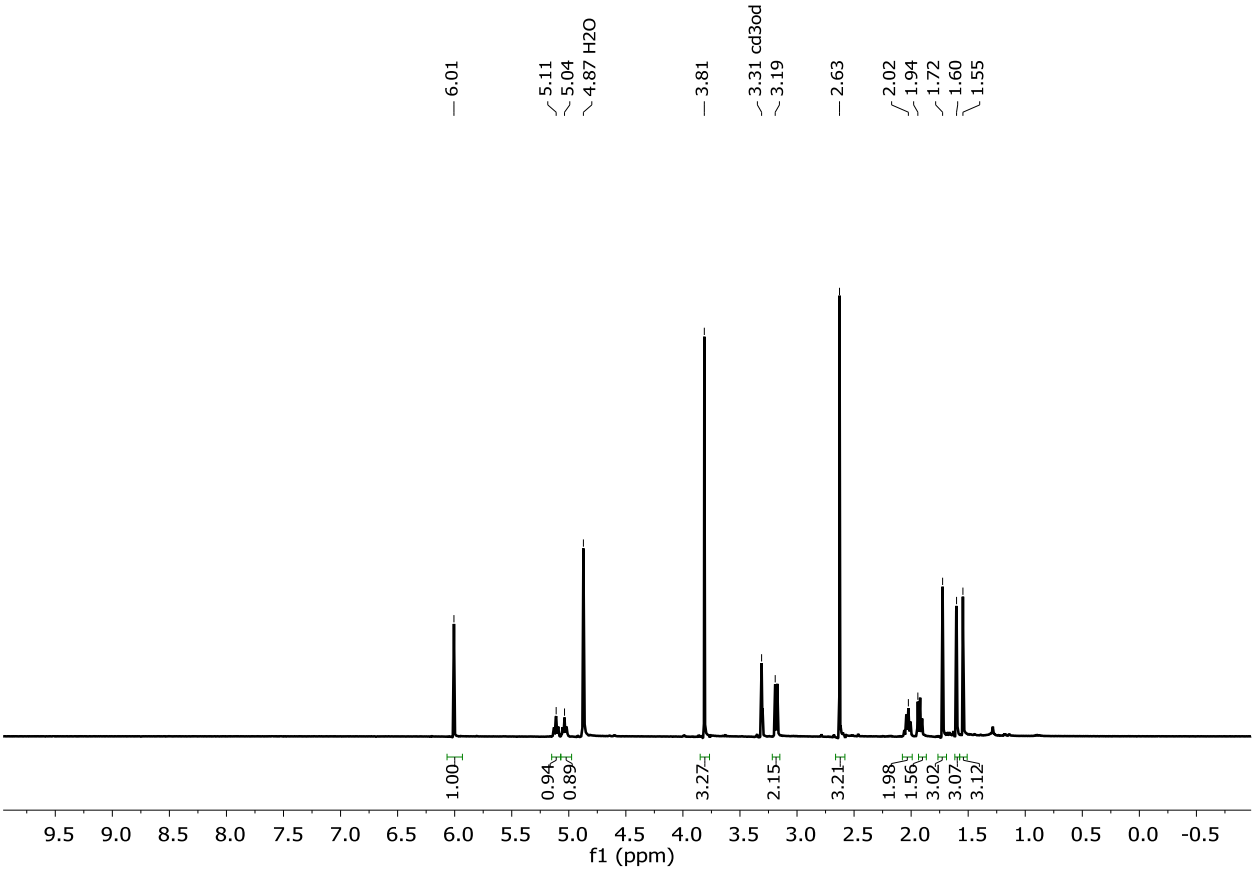


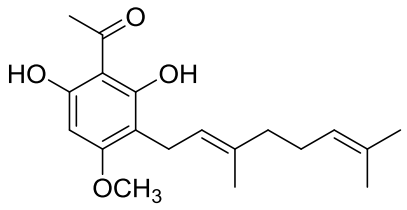
13.49 ^{13}C NMR of 5.11 (CD_3OD, 100 MHz)



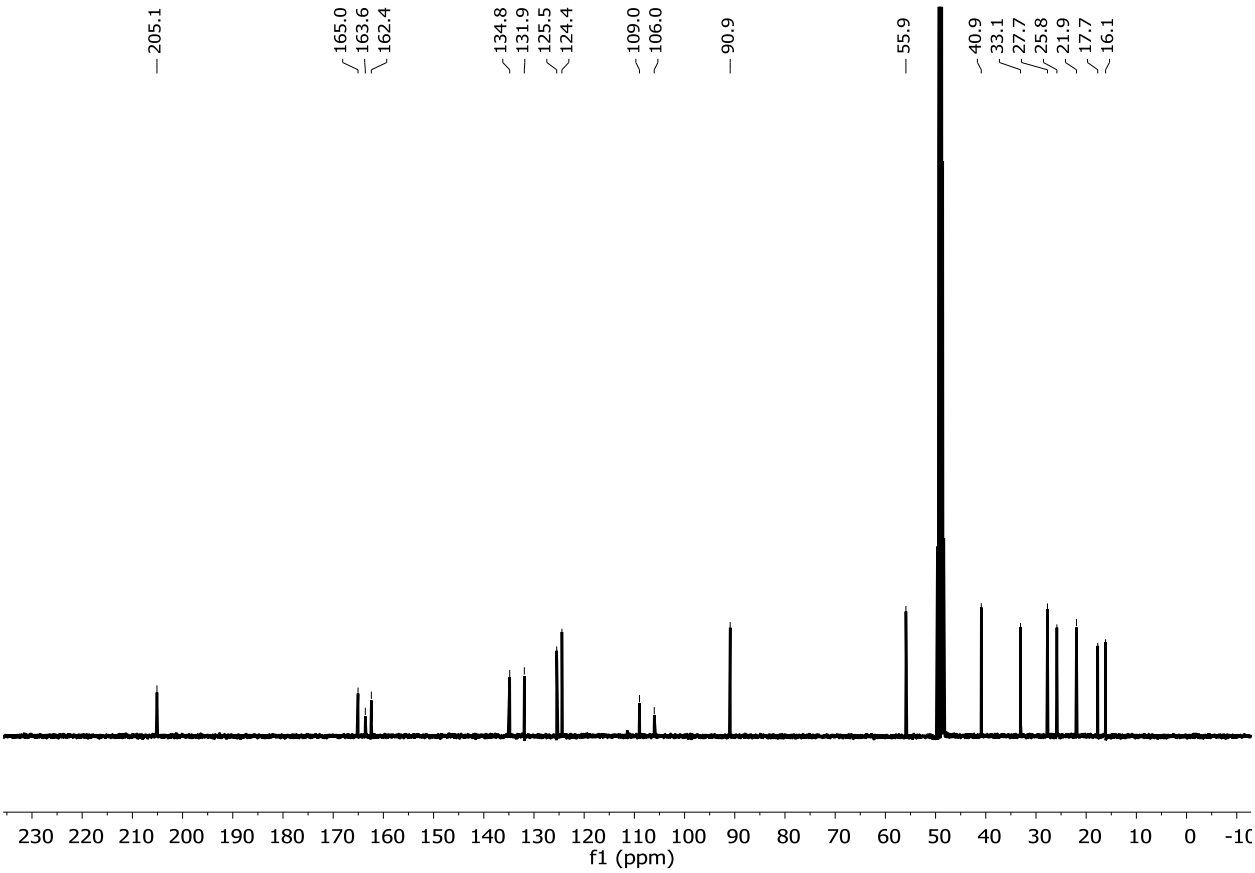


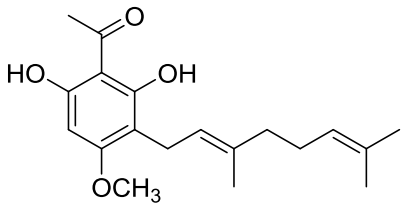
13.50 ^{1}H NMR of 5.12 ($CDCl_3$, 400 MHz)



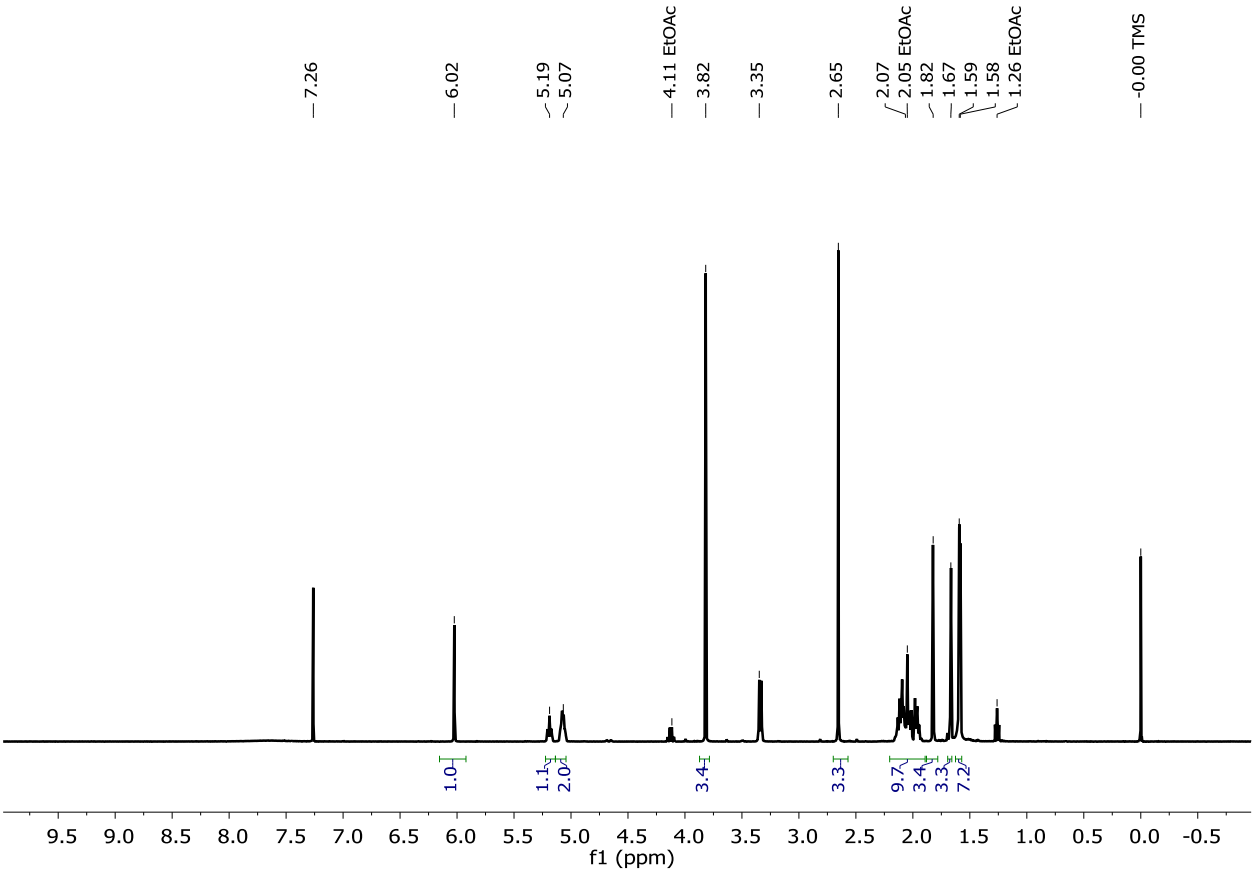


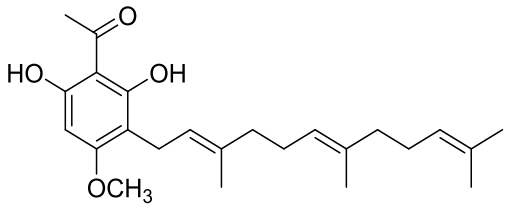
13.51 ^{13}C NMR of 5.12 ($CDCl_3$, 100 MHz)



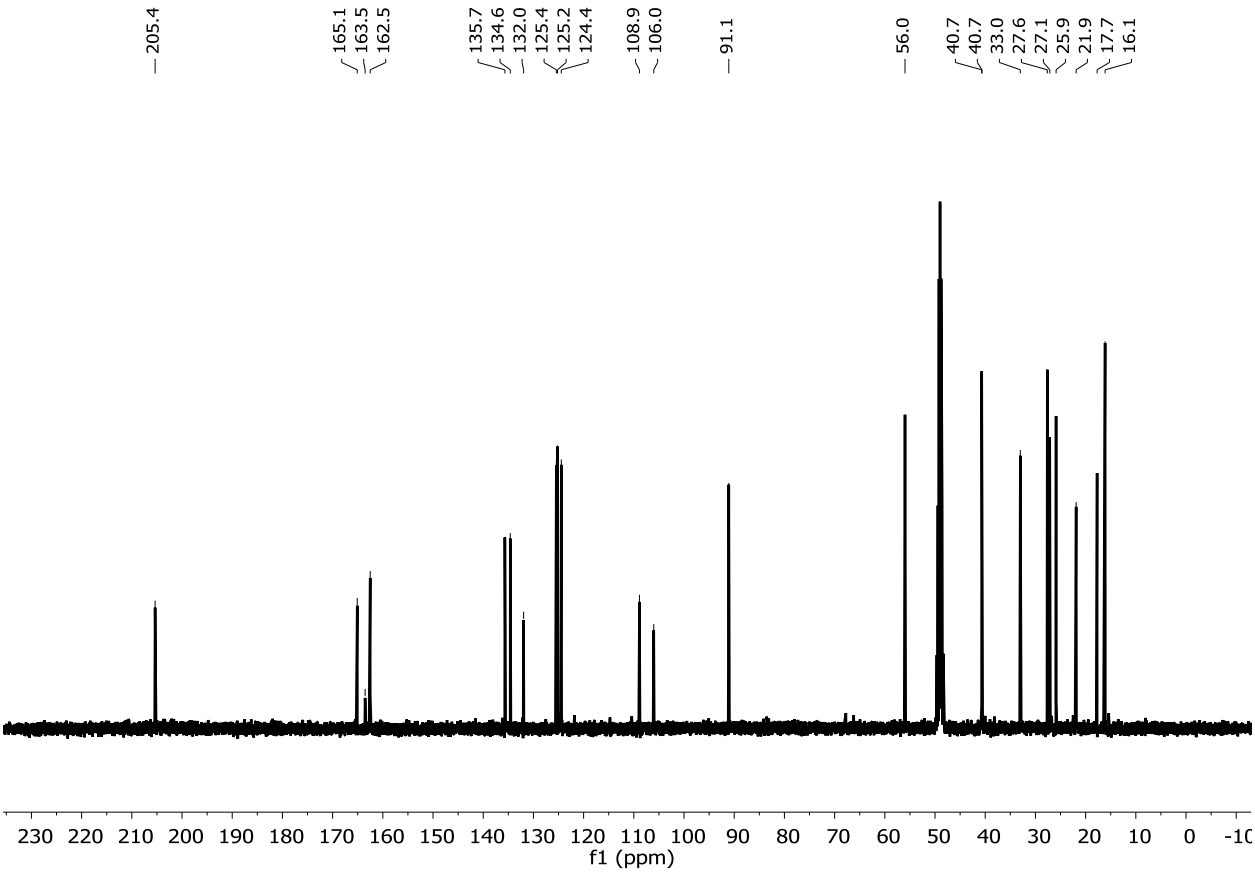


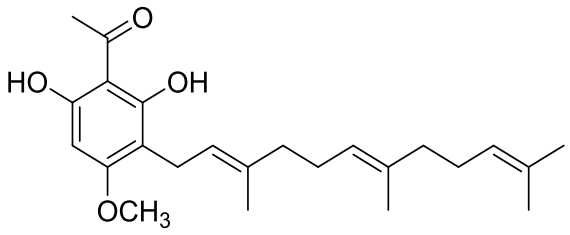
13.52 ^{1}H NMR of 5.13 ($CDCl_3$, 400 MHz)



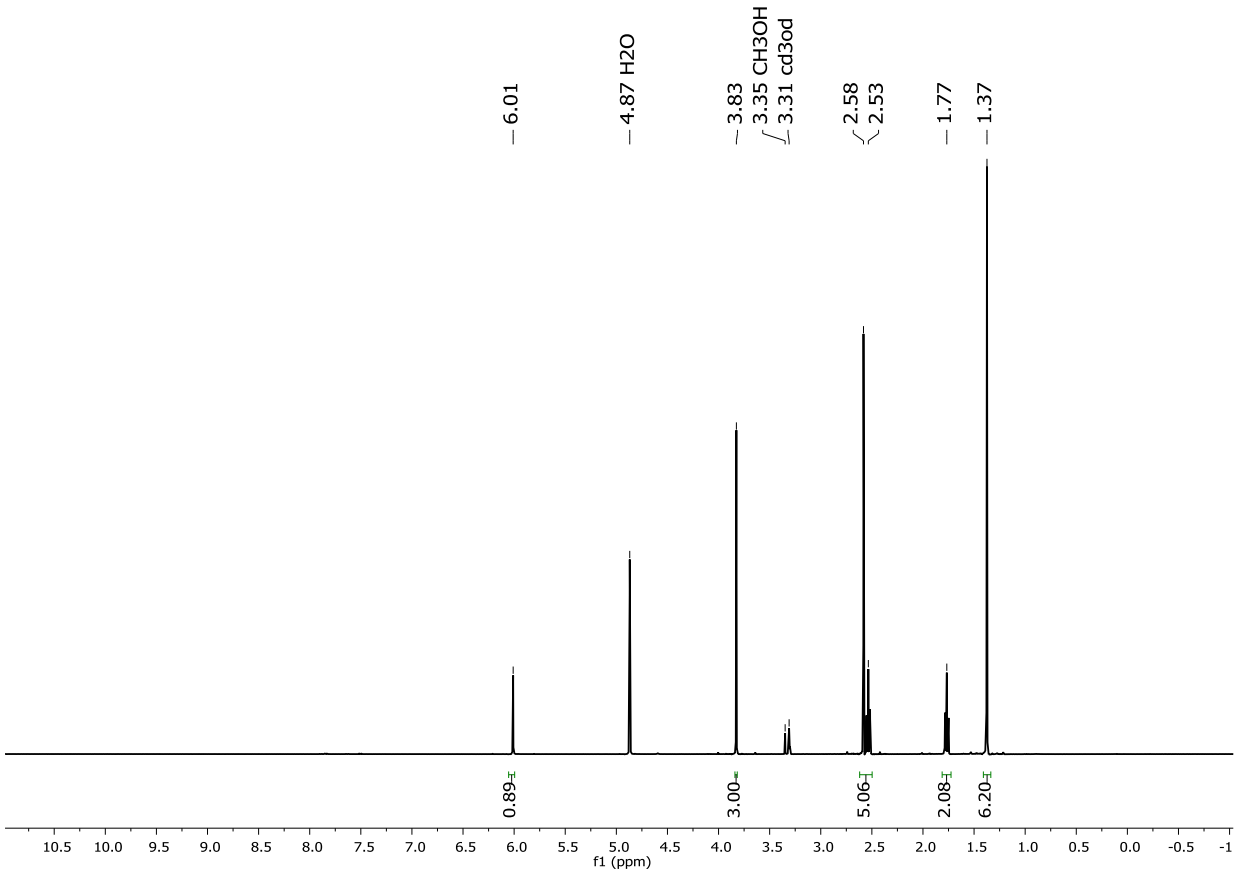


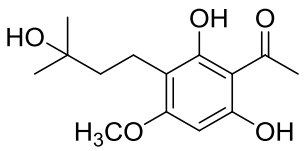
13.53 ^{13}C NMR of 5.13 ($CDCl_3$, 100 MHz)



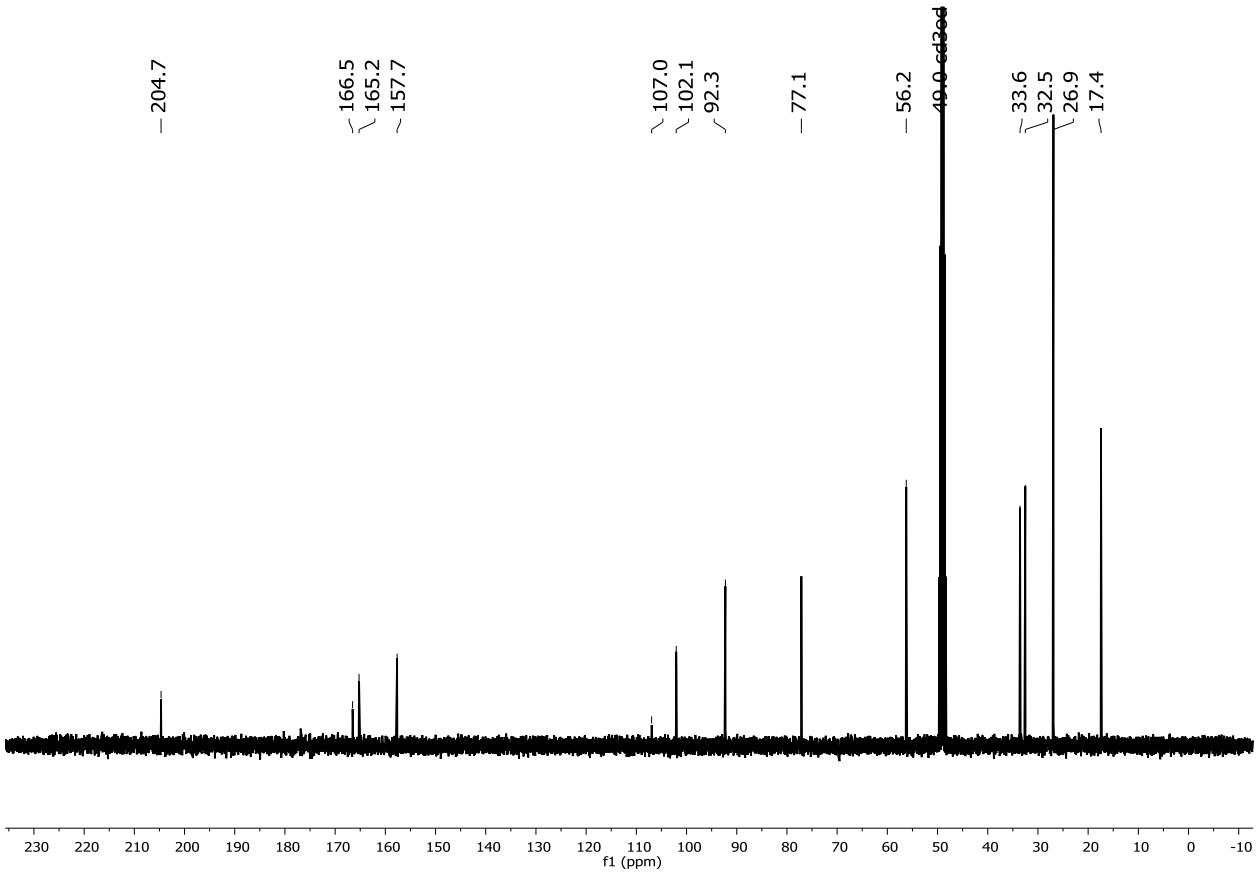


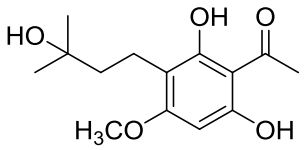
13.54 ^{1}H NMR of 5.14 (CD_3OD, 400 MHz)



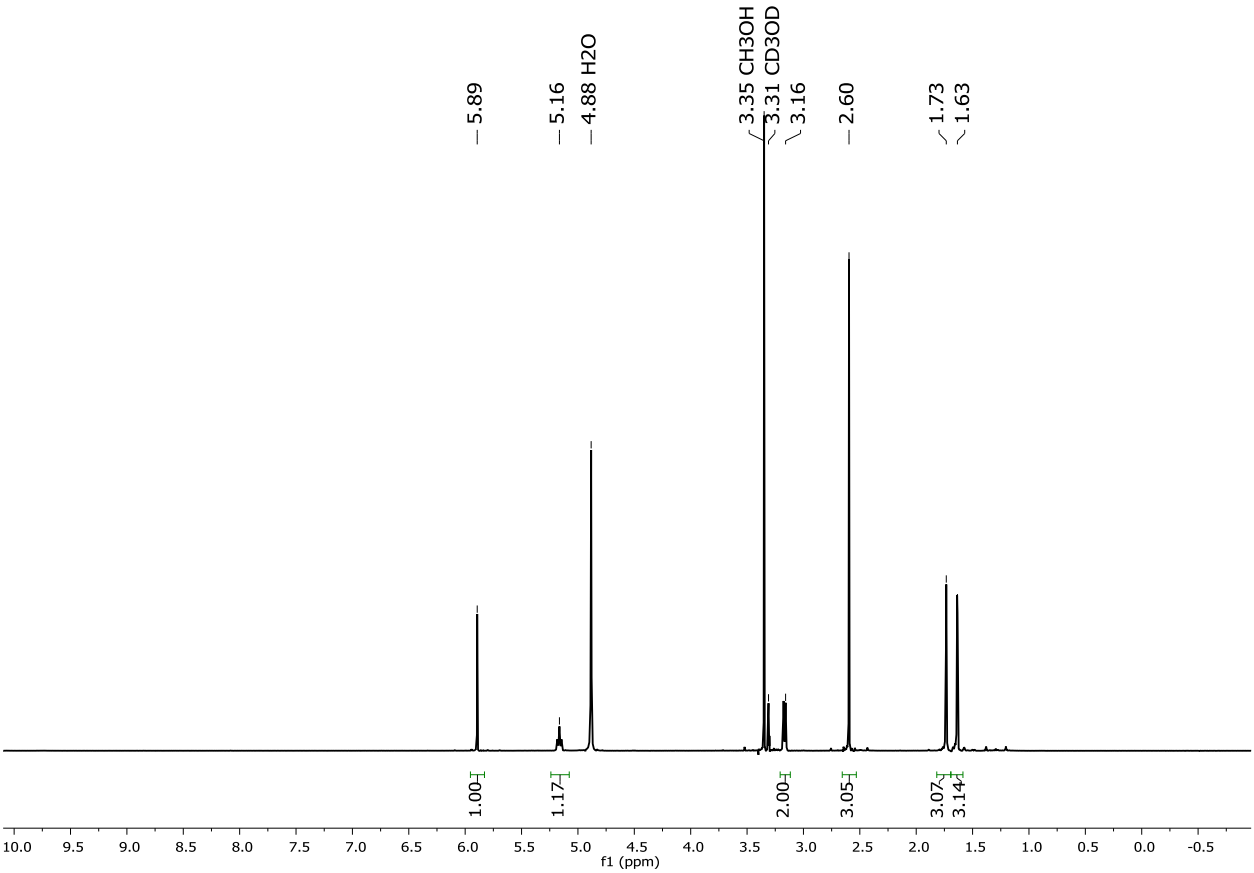


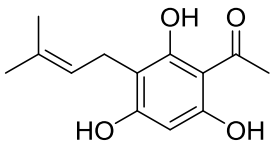
13.55 ^{13}C NMR of 5.14 (CD_3OD, 100 MHz)



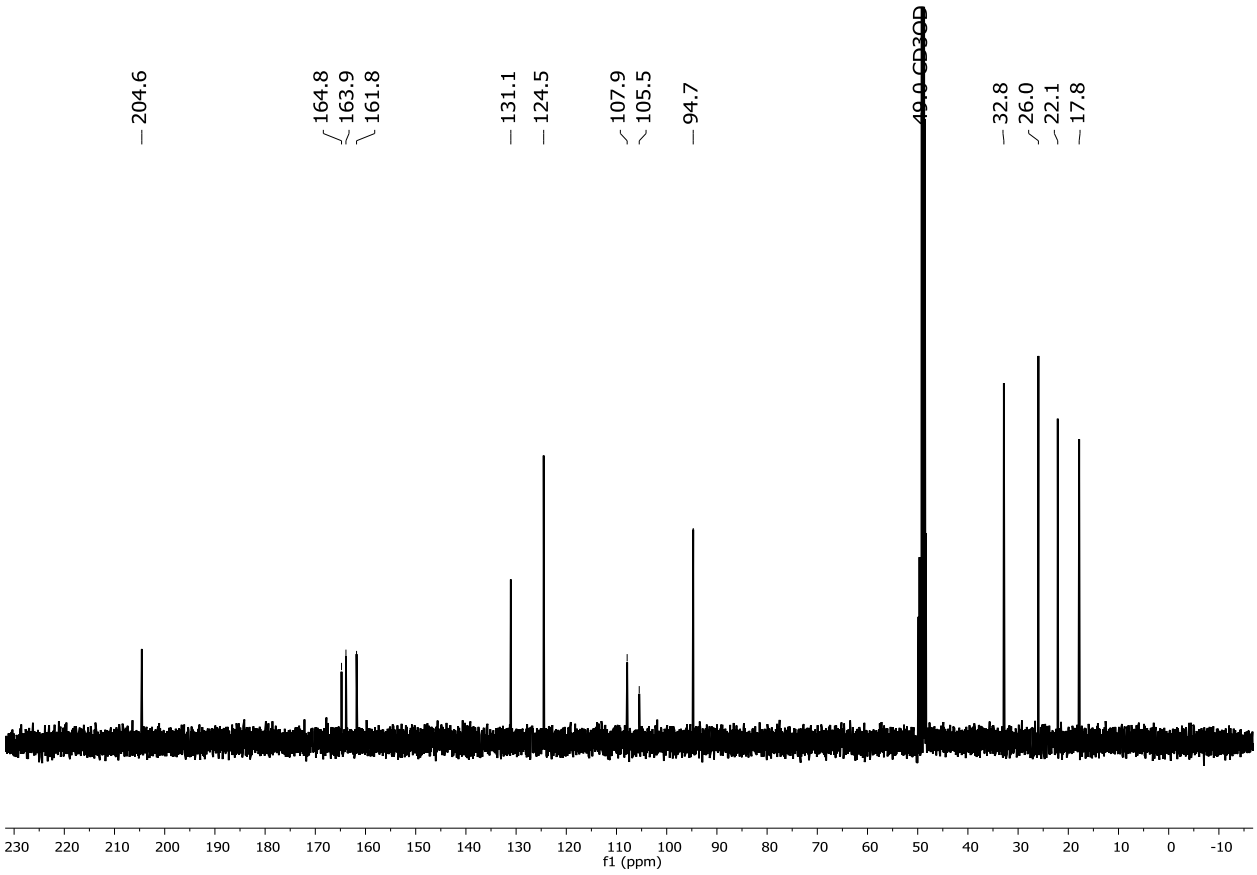


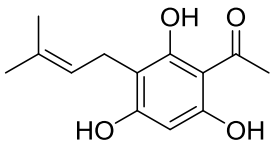
13.56 ^{1}H NMR of 5.16 (CD_3OD, 400 MHz)



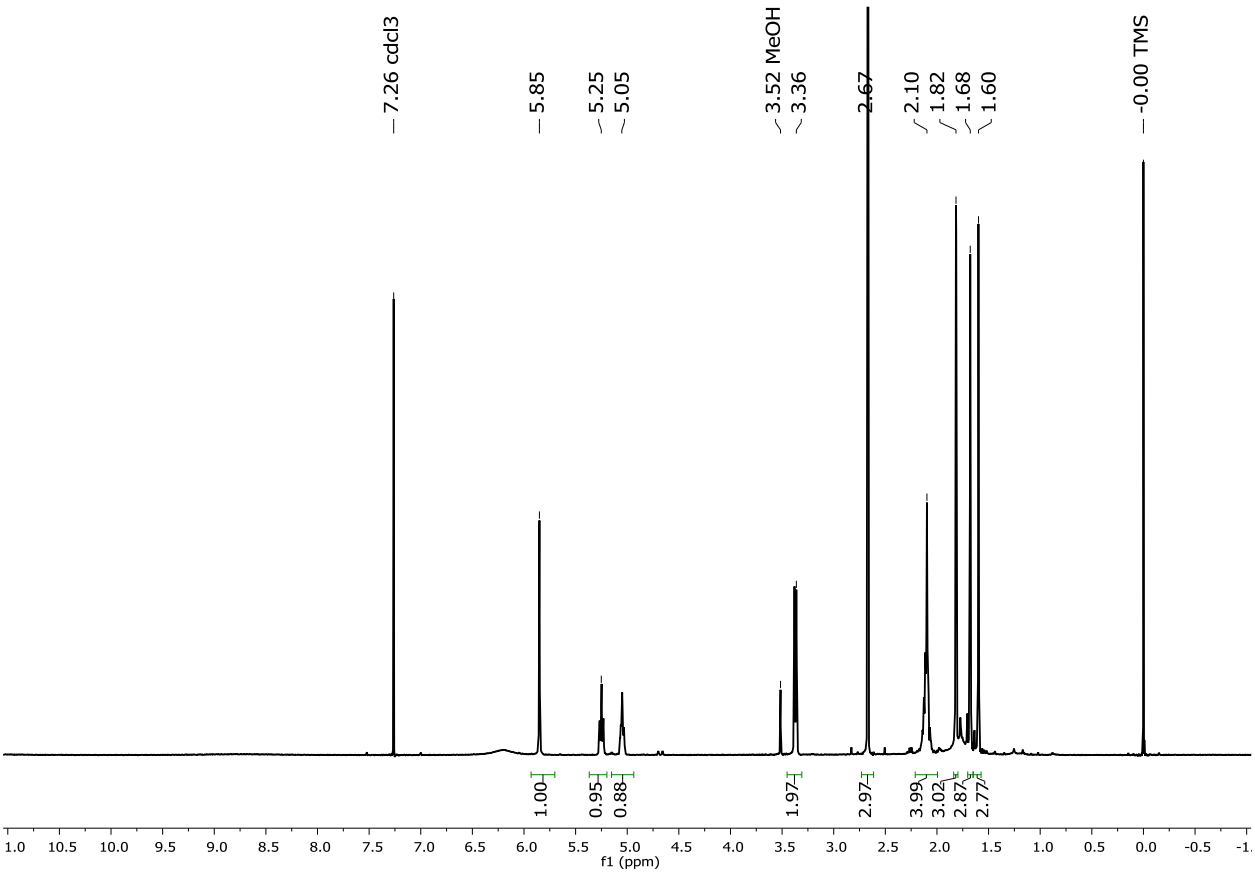


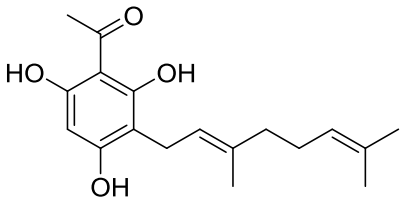
13.57 ^{13}C NMR of 5.16 (CD_3OD, 100 MHz)



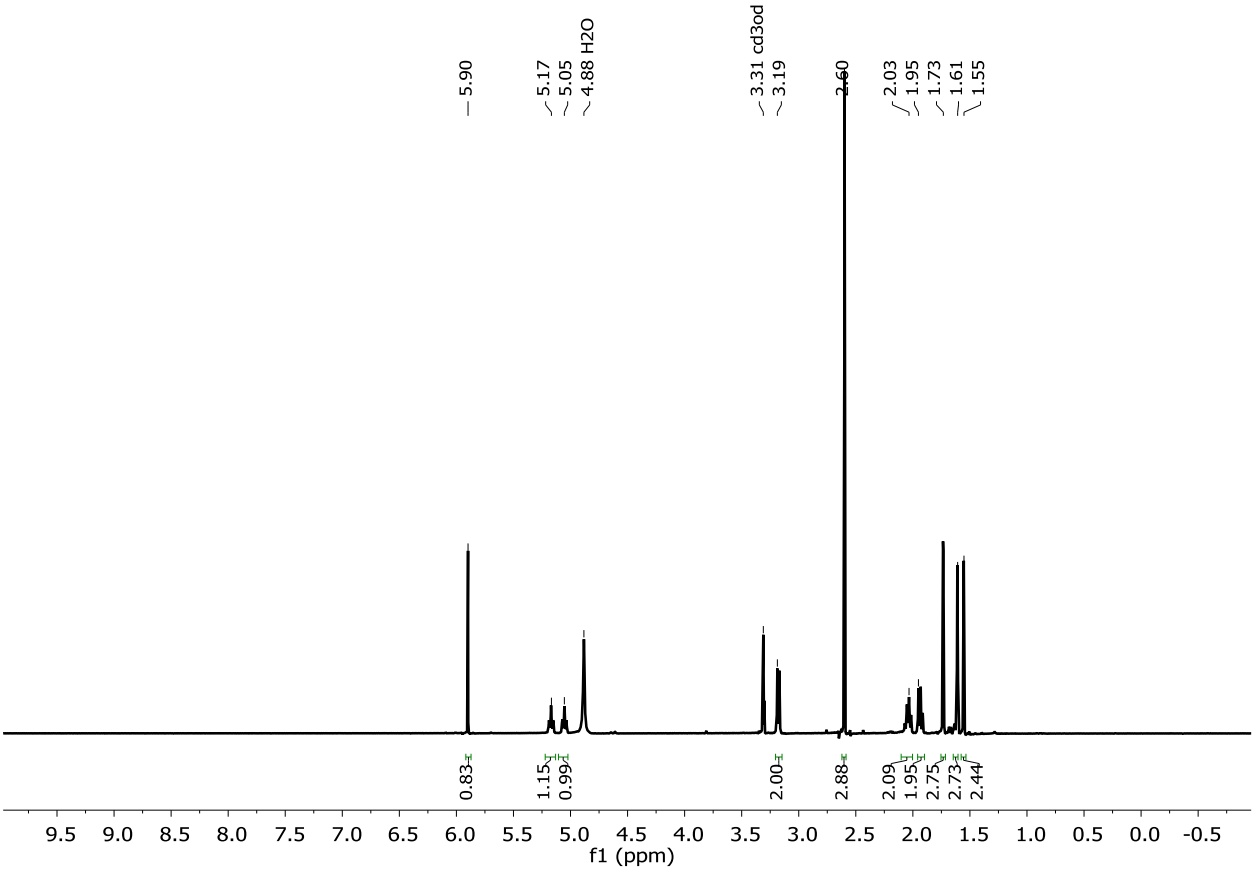


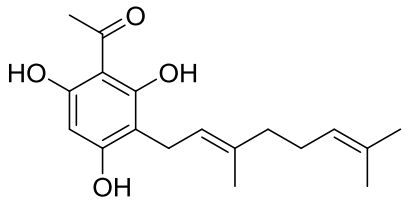
13.58 ^{1}H NMR of 5.17 ($CDCl_3$, 400 MHz)



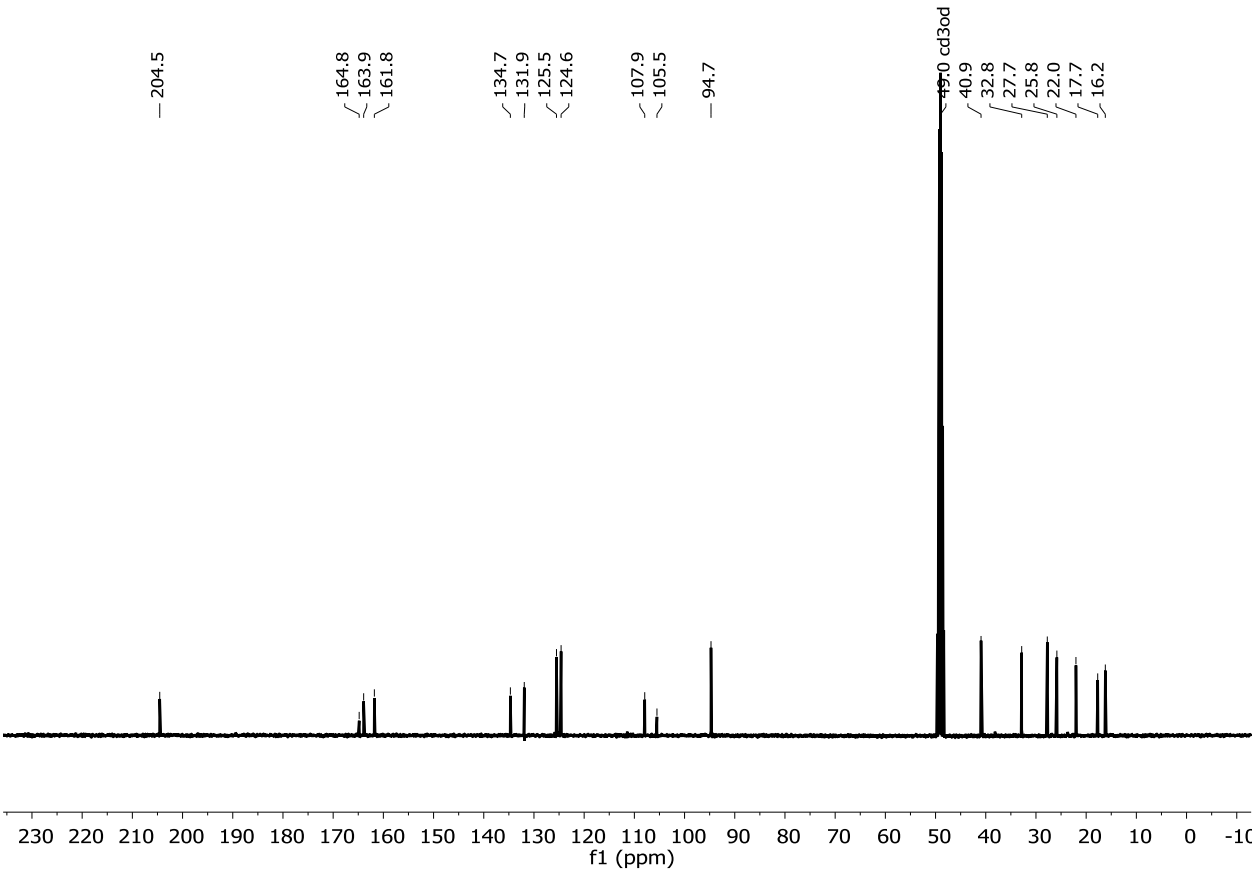


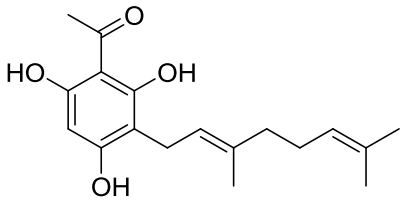
13.59 ^{1}H NMR of 5.17 (CD_3OD, 400 MHz)



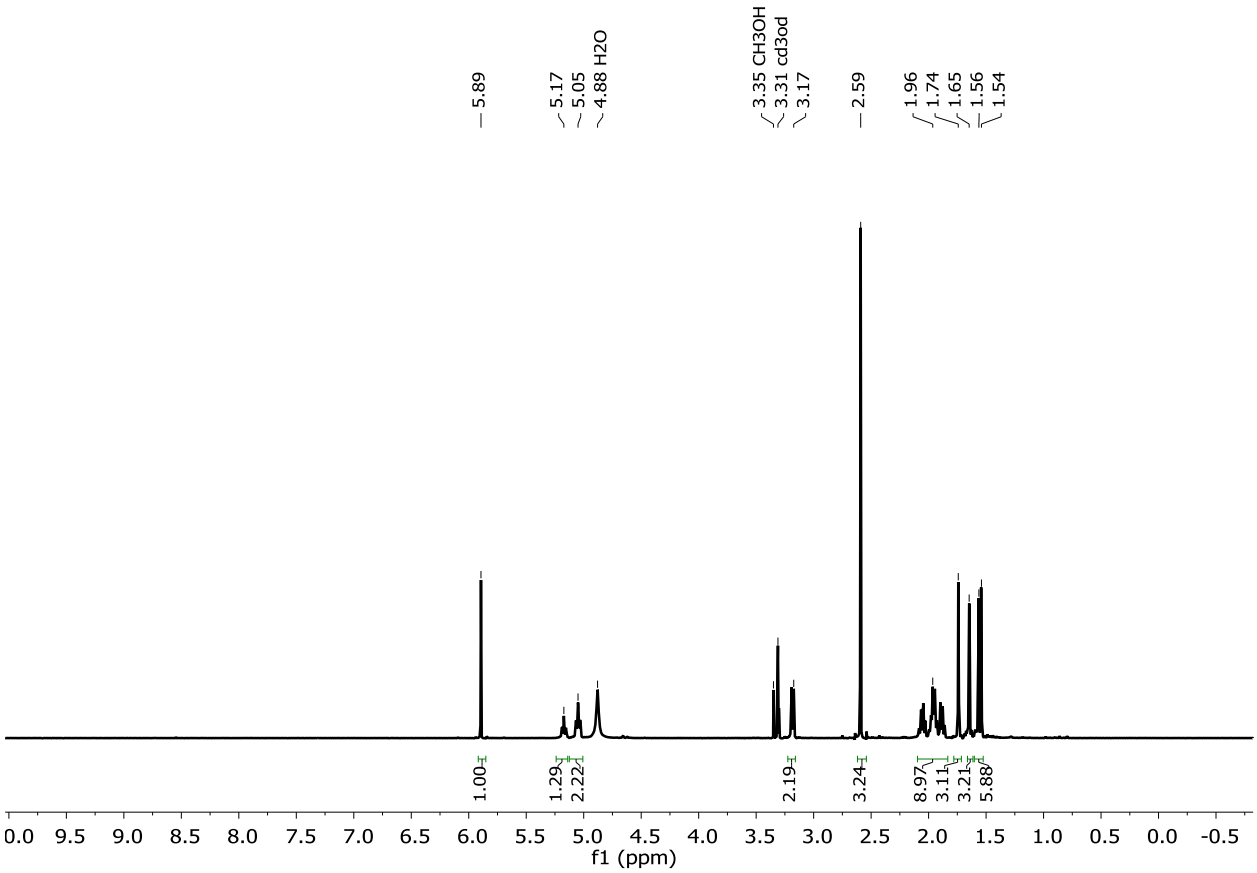


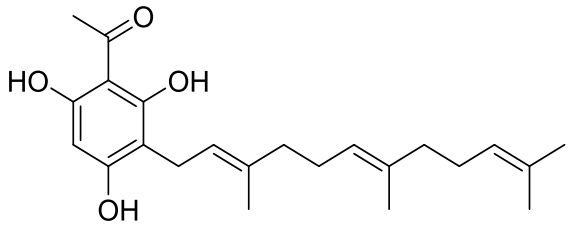
13.60 ^{13}C NMR of 5.17 (CD_3OD, 100 MHz)



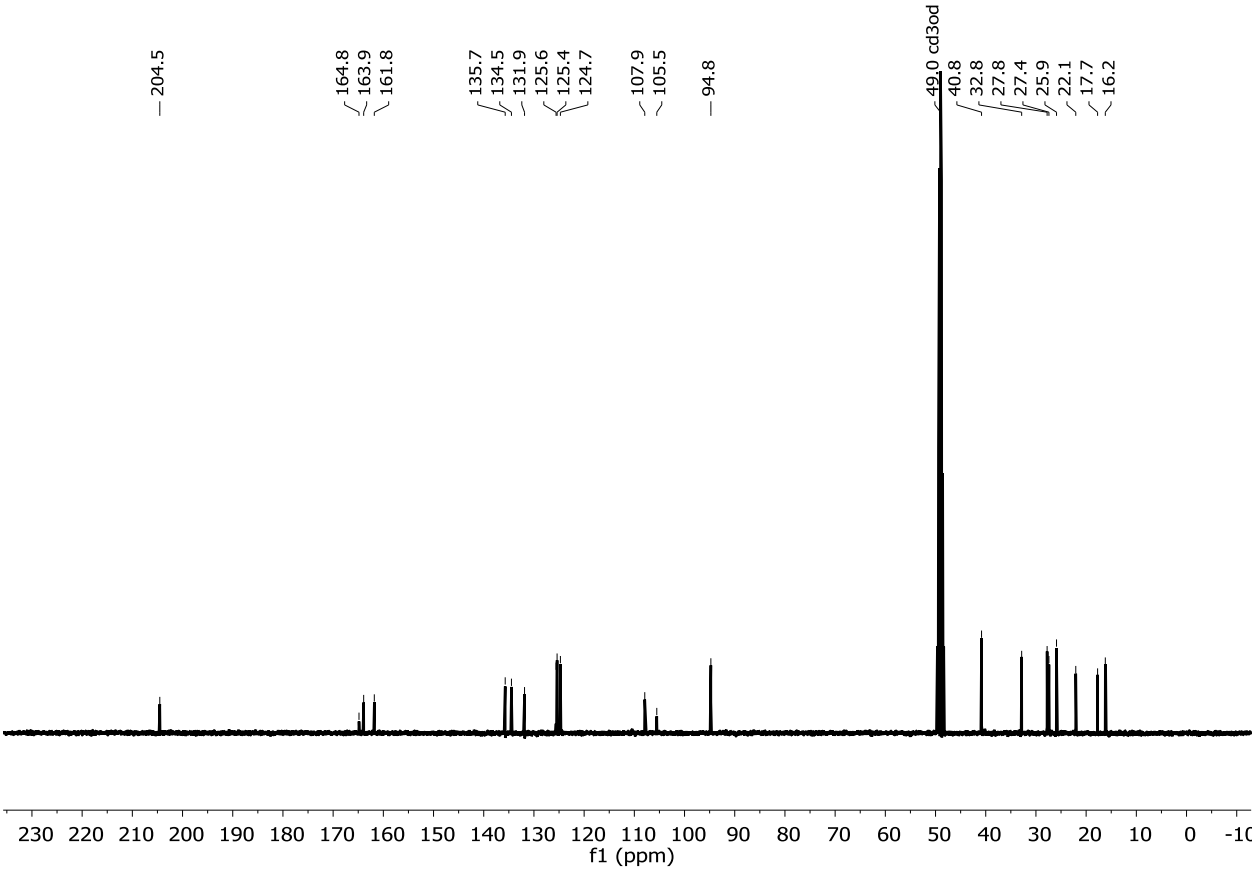


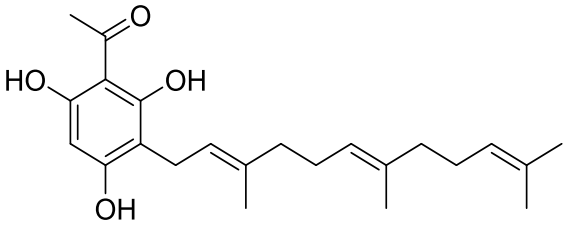
13.61 ^{1}H NMR of 5.18 (CD_3OD, 400 MHz)



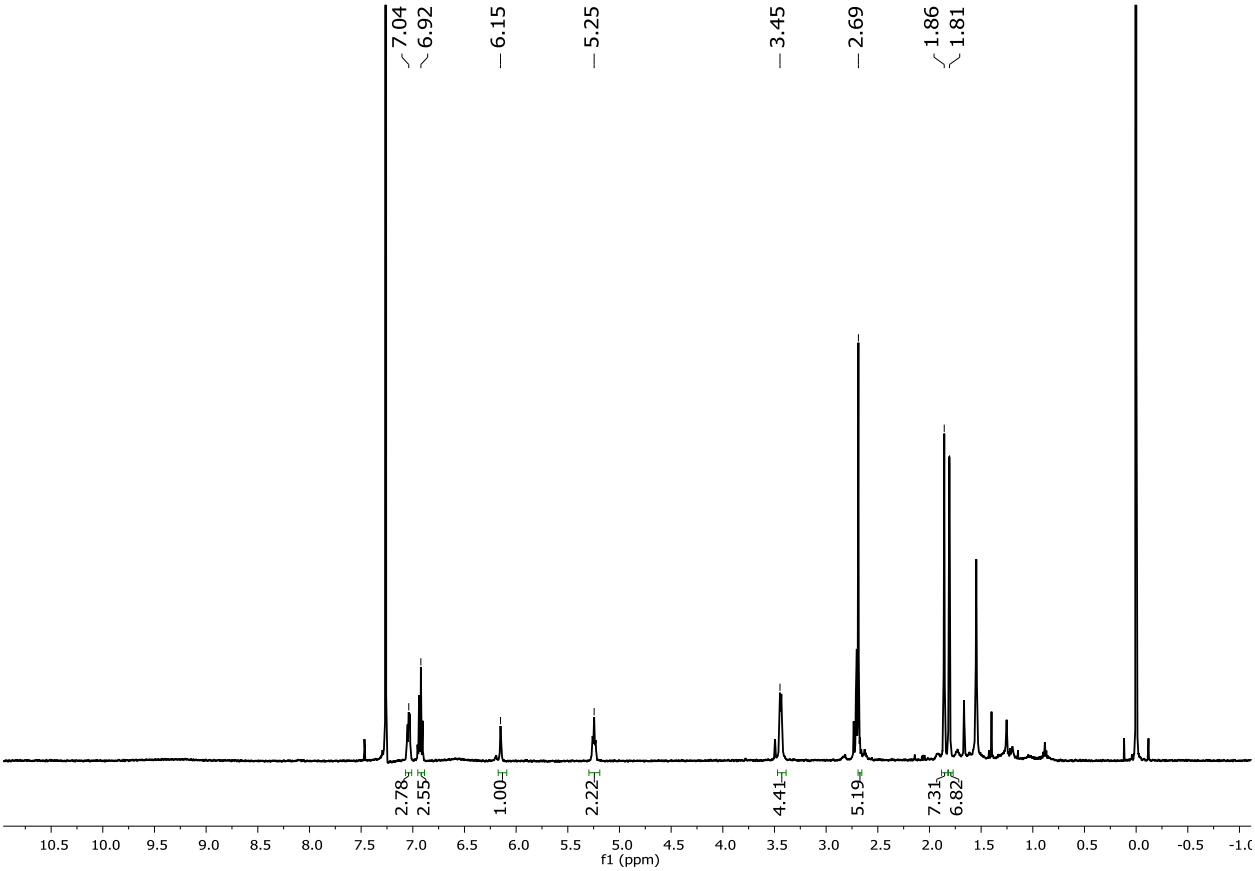


13.62 ^{13}C NMR of 5.18 (CD_3OD, 100 MHz)

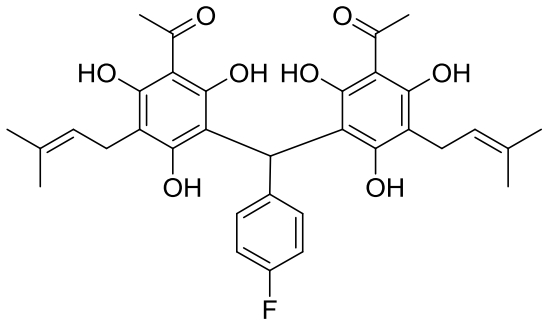




13.63 ^{1}H NMR of 5.19 ($CDCl_3$, 500 MHz)



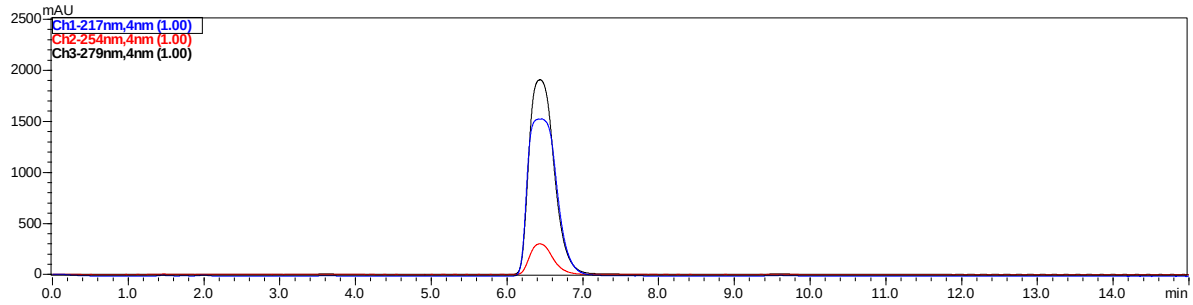
13.64



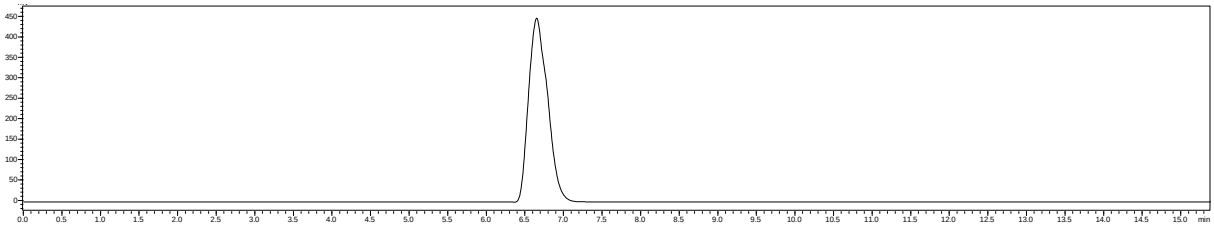
HPLC of 5.11

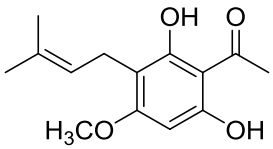
Column: Phenomenex Luna C_{18}, 250 x 4.6 mm
Concentration: 2.5 mg/mL
Injection: 10 µL injection
Mobile Phase: 80% MeOH (aq.)

Detector: PDA



Detector: ELSD

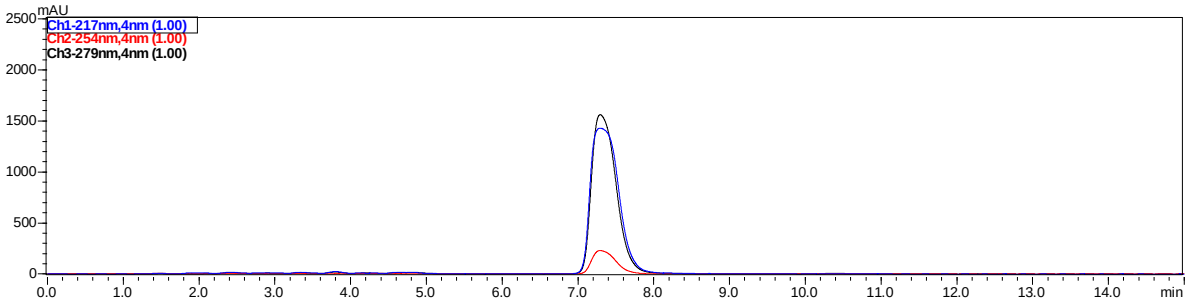




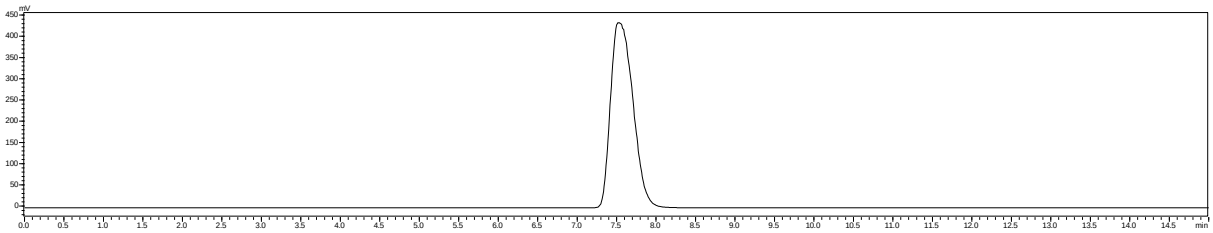
13.65 HPLC of 5.12

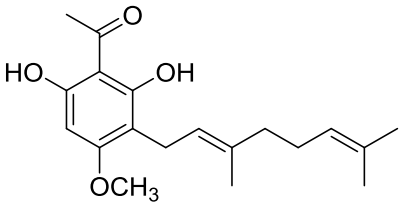
Column: Phenomenex Luna C_{18}, 250 x 4.6 mm
Concentration: 2.5 mg/mL
Injection: 10 µL injection
Mobile Phase: 88% MeOH (aq.)

Detector: PDA



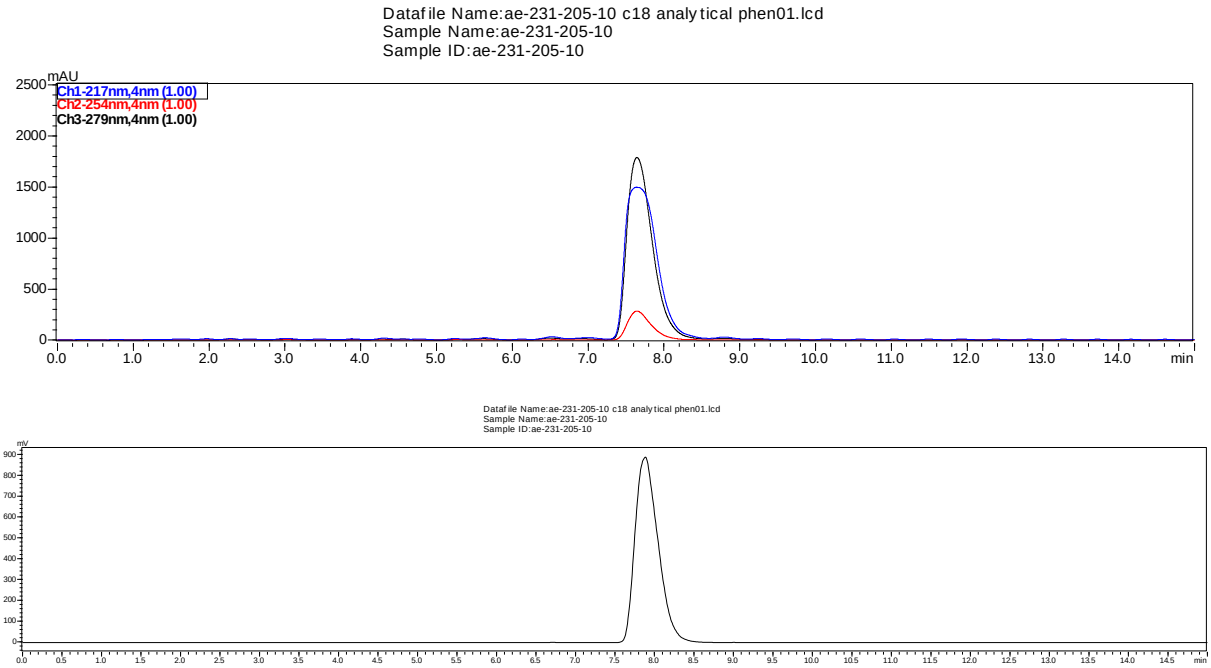
Detector: ELSD

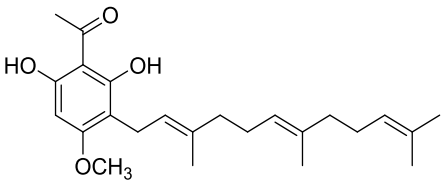




13.66 HPLC of 5.13

Column: Phenomenex Luna C_{18}, 250 x 4.6 mm
Concentration: 2.5 mg/mL
Injection: 10 μL injection
Mobile Phase: 93% MeOH (aq.)

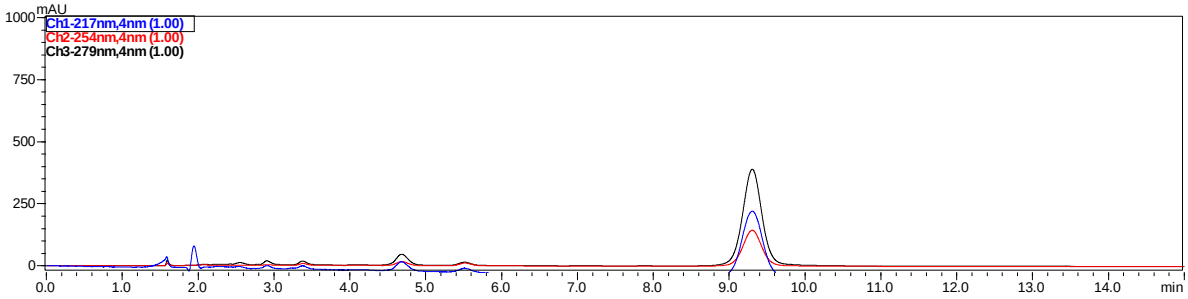




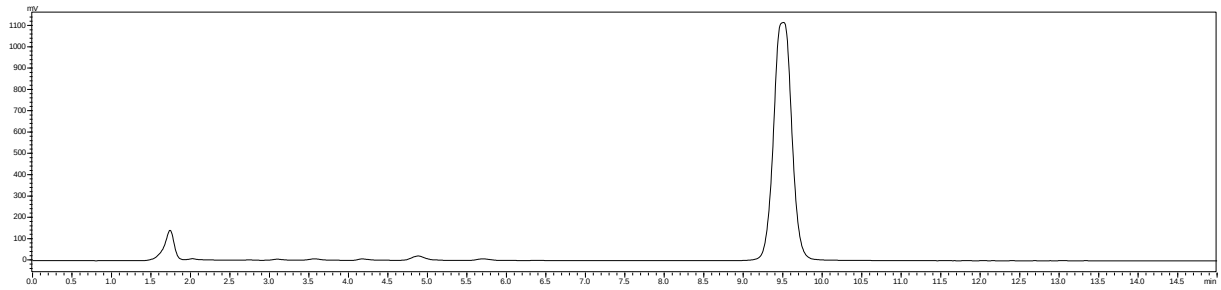
13.67 HPLC of 5.16

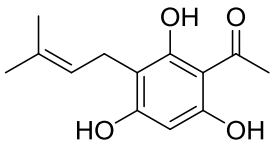
Column: Phenomenex Luna C_{18}, 250 x 4.6 mm
Concentration: 1.25 mg/mL
Injection: 10 µL injection
Mobile Phase: 100% MeOH (aq.)

Detector: PDA



Detector: ELSD

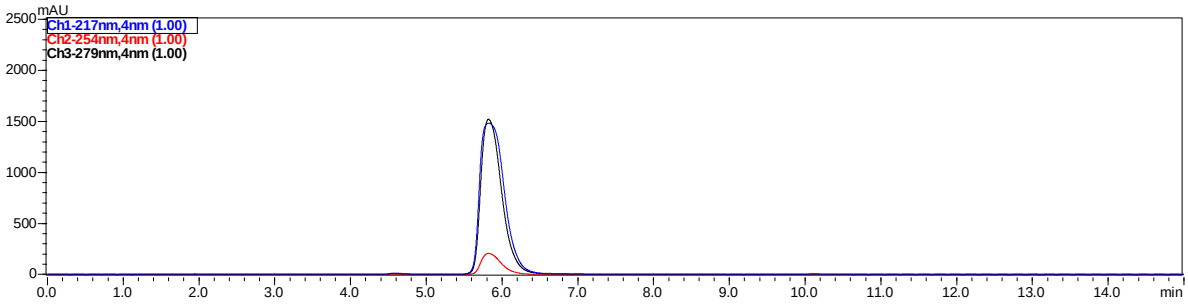




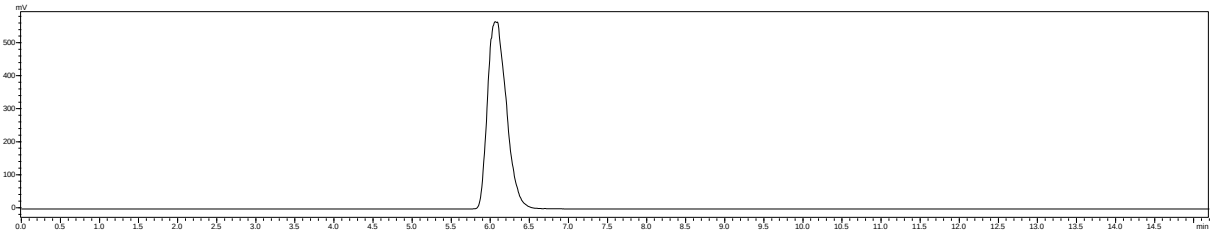
13.68 HPLC of 5.17

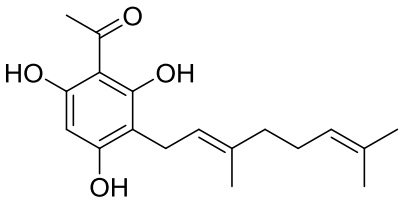
Column: Phenomenex Luna C_{18}, 250 x 4.6 mm
Concentration: 5 mg/mL
Injection: 5 µL injection
Mobile Phase: 85% MeOH (aq.)

Detector: PDA



Detector: ELSD

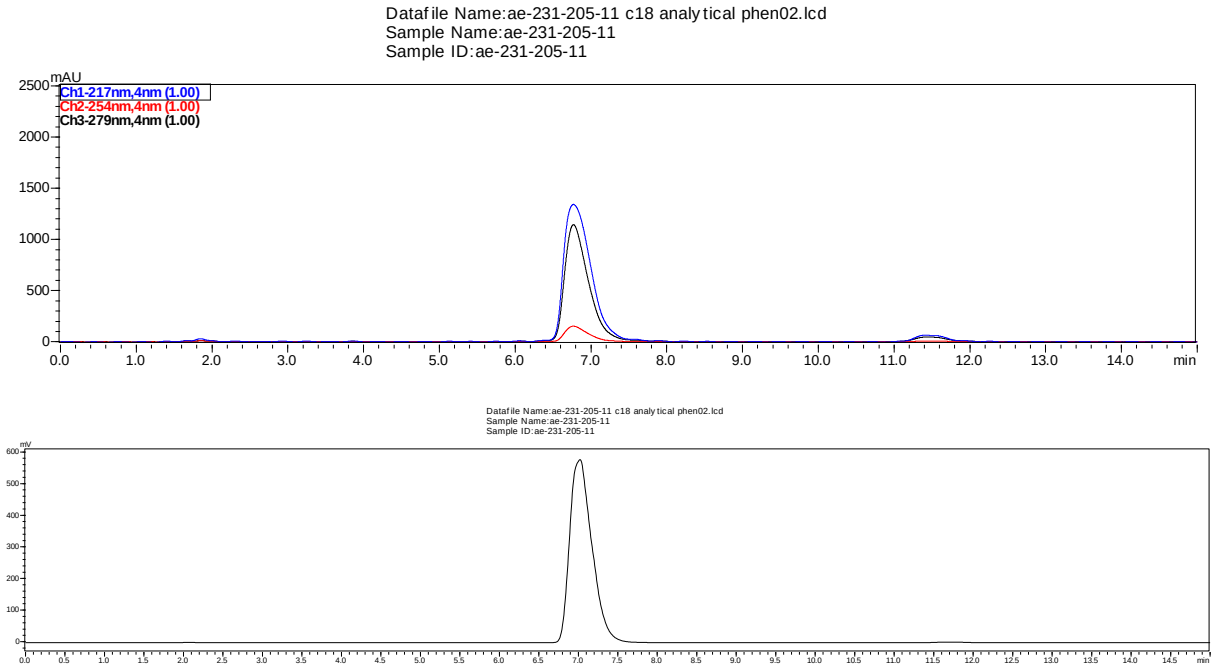


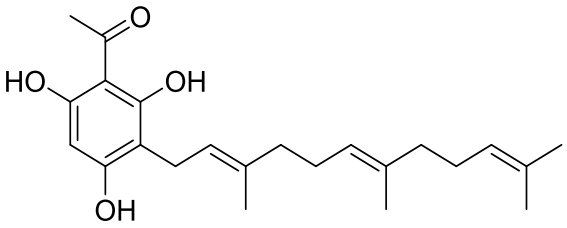


13.69 HPLC of 5.18

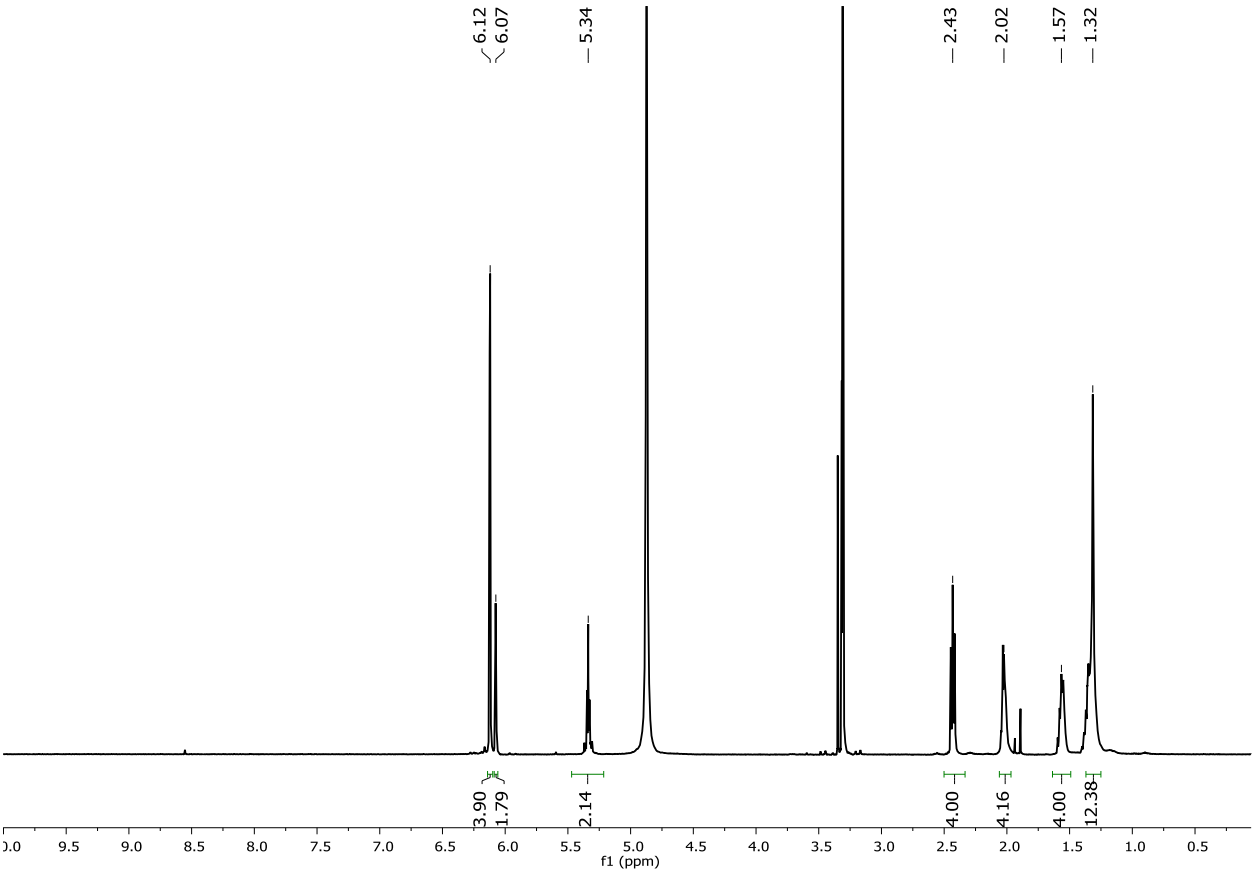
2.5 mg/mL; 10 uL; 90% MeOH Isocratic 250 x 4.6 Phenomenex Luna C18

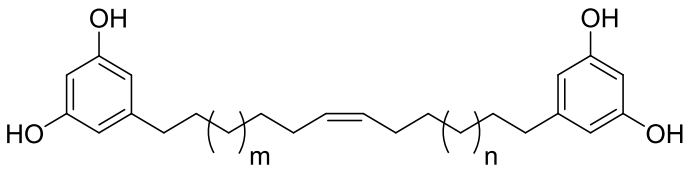
Column: Phenomenex Luna C_{18}, 250 x 4.6 mm
Concentration: 2.5 mg/mL
Injection: 10 μL injection
Mobile Phase: 90% MeOH (aq.)





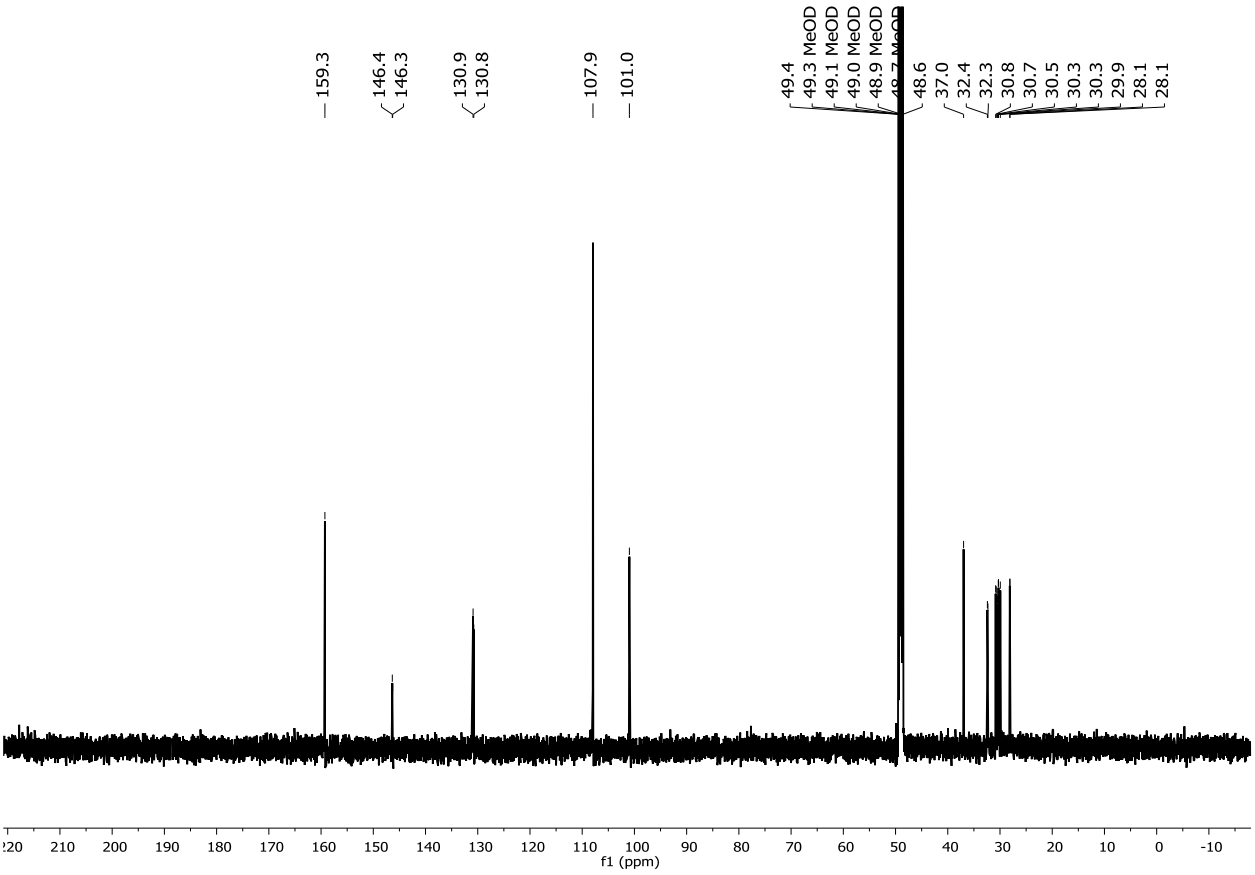
13.70 ^{1}H NMR of 6.1 (CD_3OD, 500 MHz)

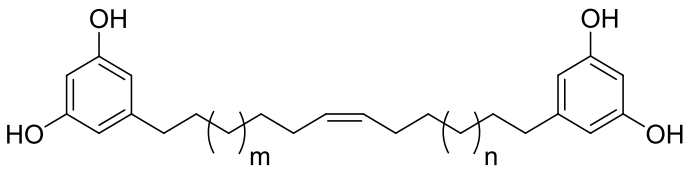




6.1: m = 1; n = 3

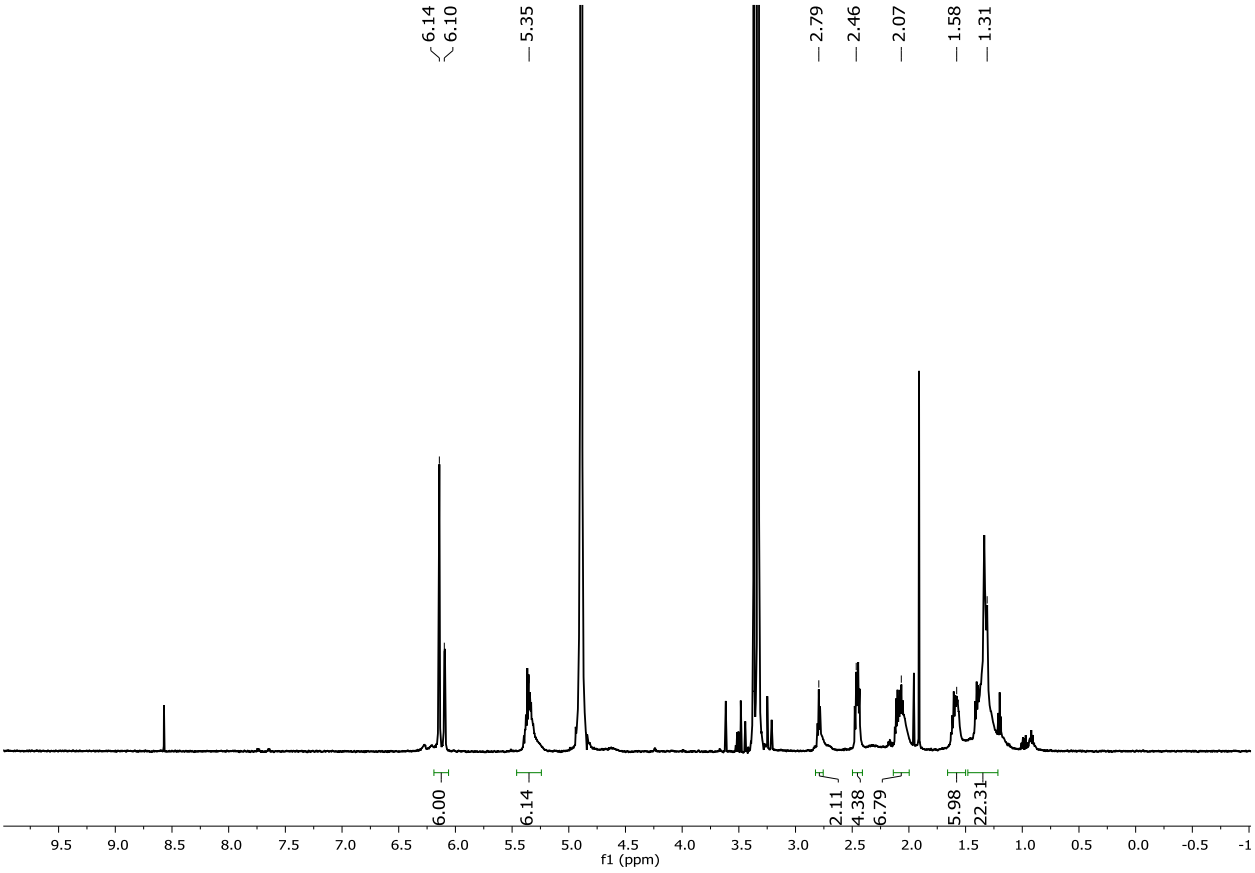
13.71 ^{13}C NMR of 6.1 (CD_3OD, 150 MHz)

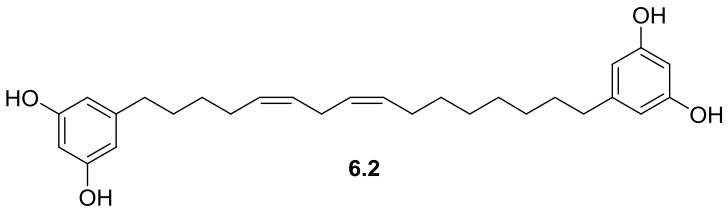




6.1: m = 1; n = 3

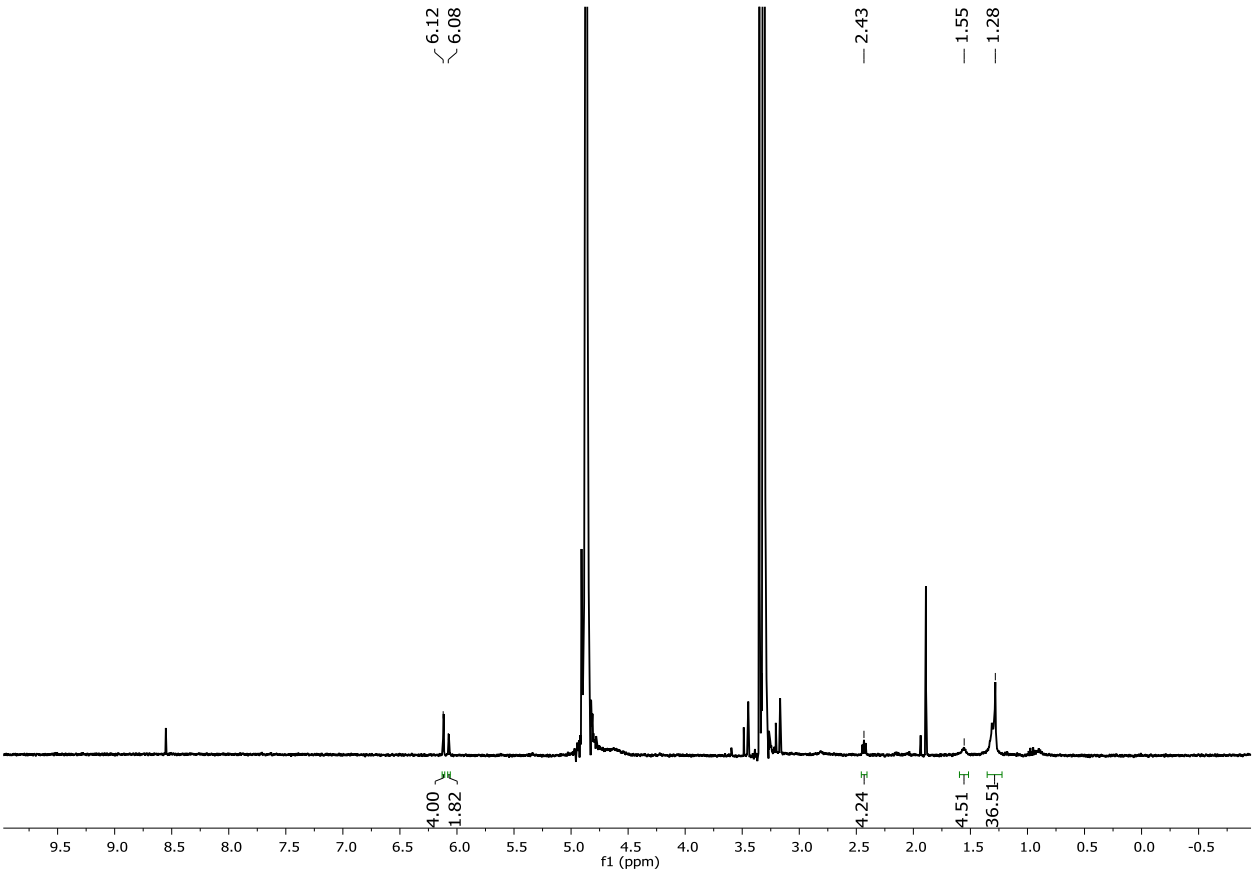
13.72 ^{1}H NMR of 6.2 (CD_3OD, 500 MHz)

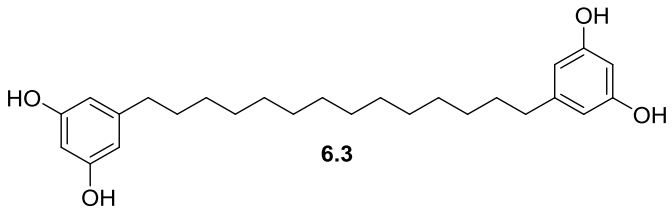




6.2

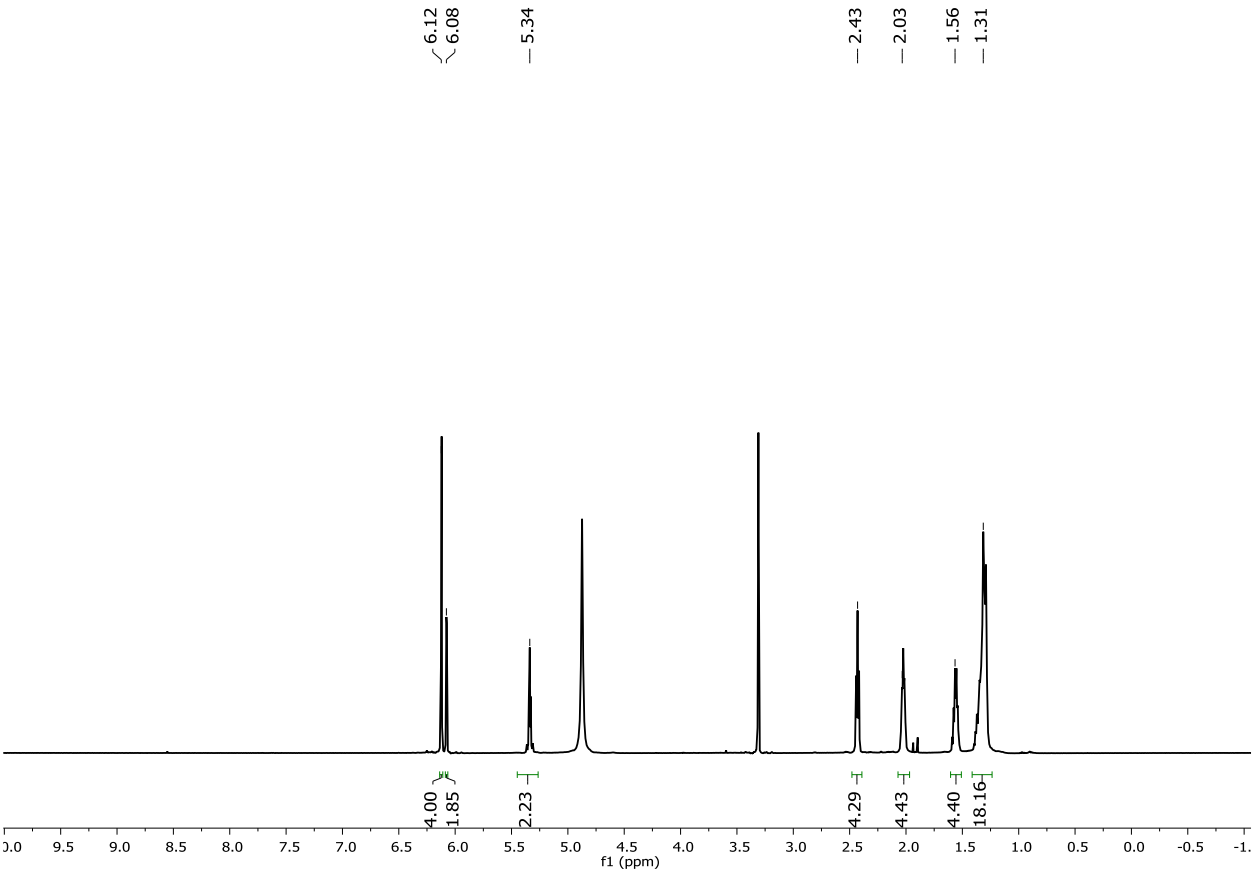
13.73 ^{1}H NMR of 6.3 (CD_3OD, 500 MHz)

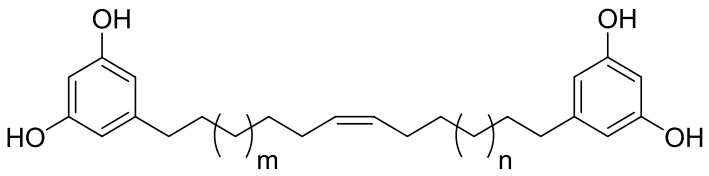




6.3

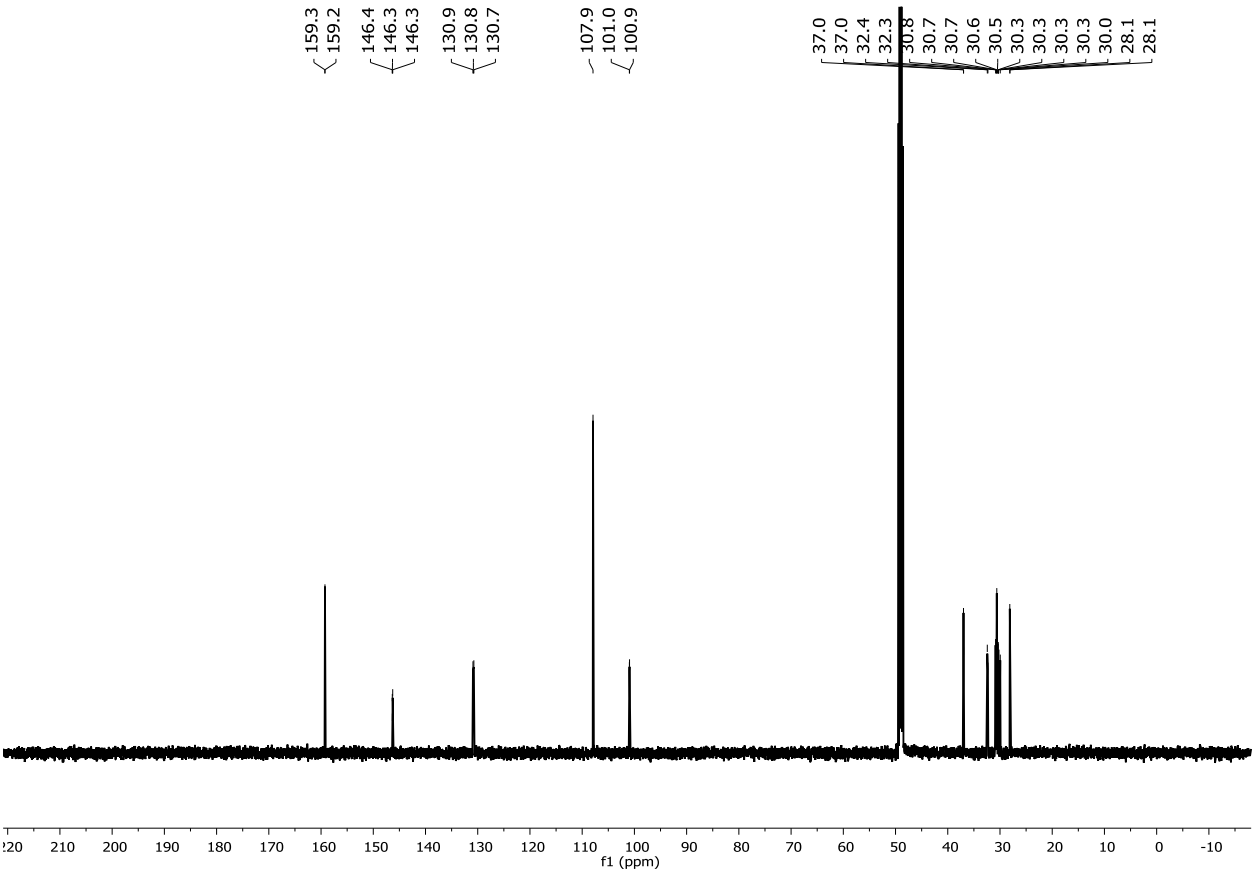
13.74 ^{1}H NMR of 6.4 (CD_3OD, 500 MHz)

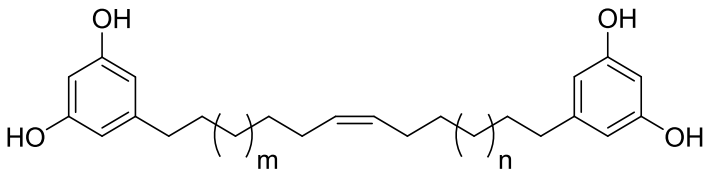




6.4a: m = 1; n = 5
6.4b: m = 3; n= 3

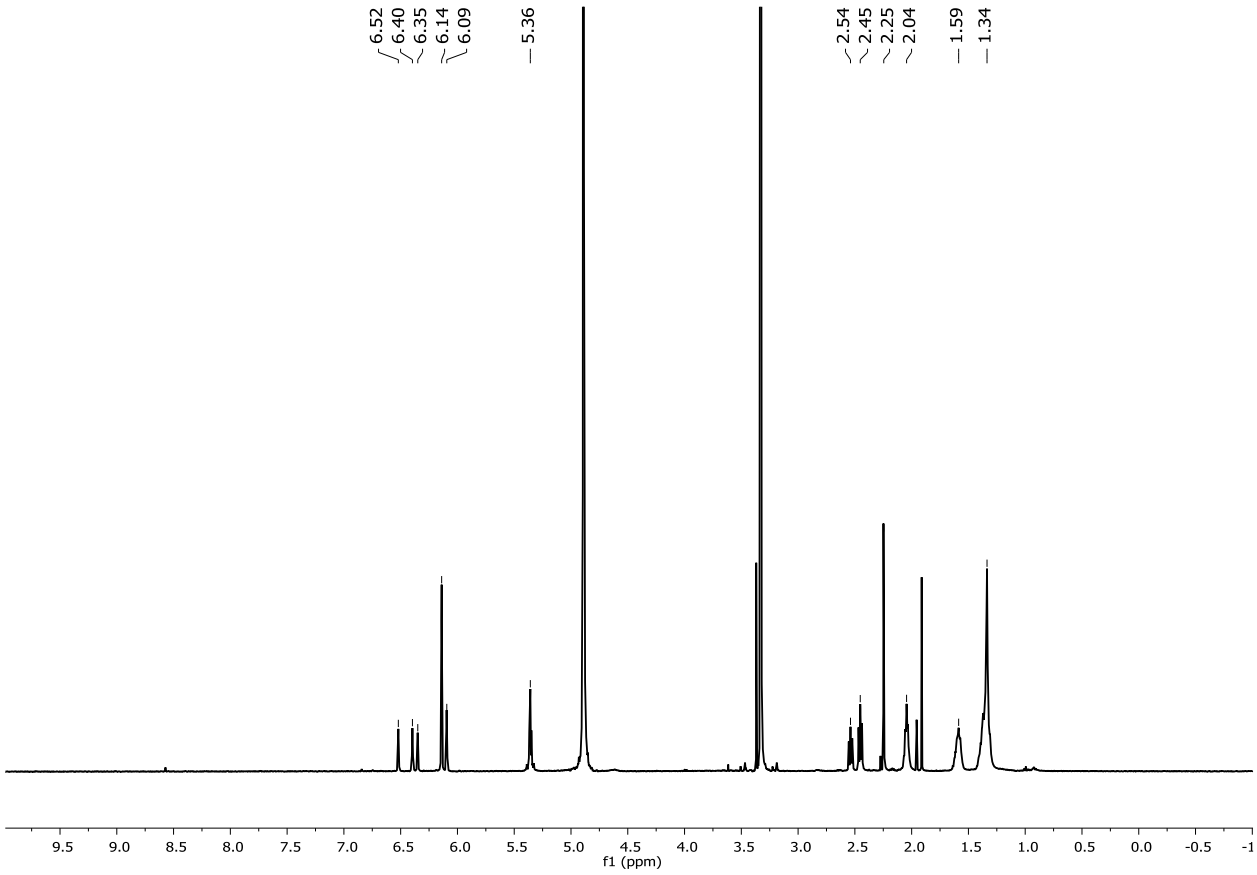
13.75 ^{13}C NMR of 6.4 (CD_3OD, 150 MHz)

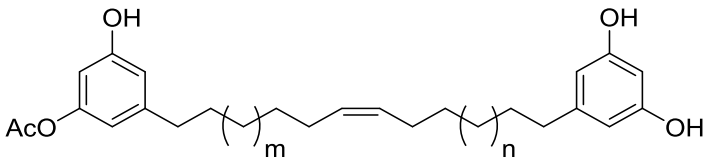




6.4a: m = 1; n = 5
6.4b: m = 3; n= 3

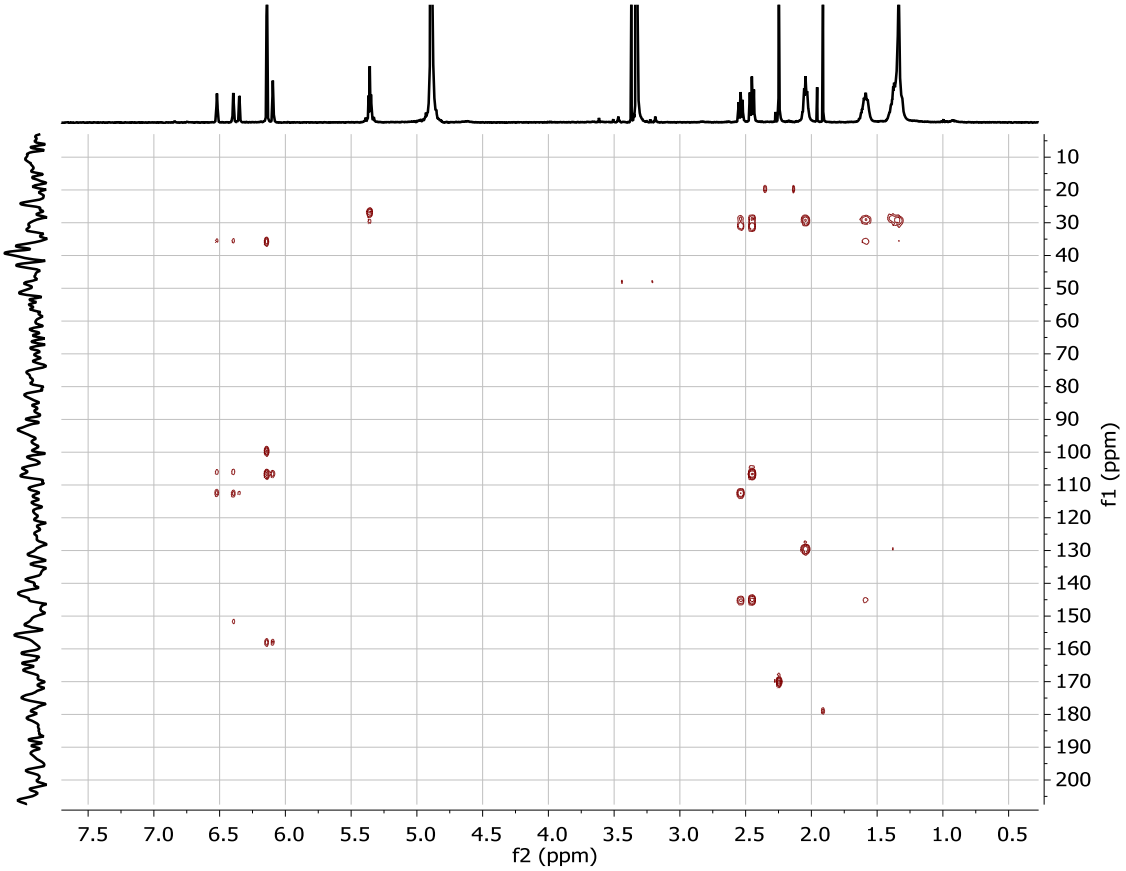
13.76 ^{1}H NMR of 6.5 (CD_3OD, 500 MHz)

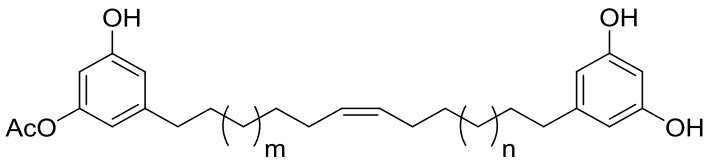




6.5a: m = 1; n = 3
6.5b: m = 3; n = 1

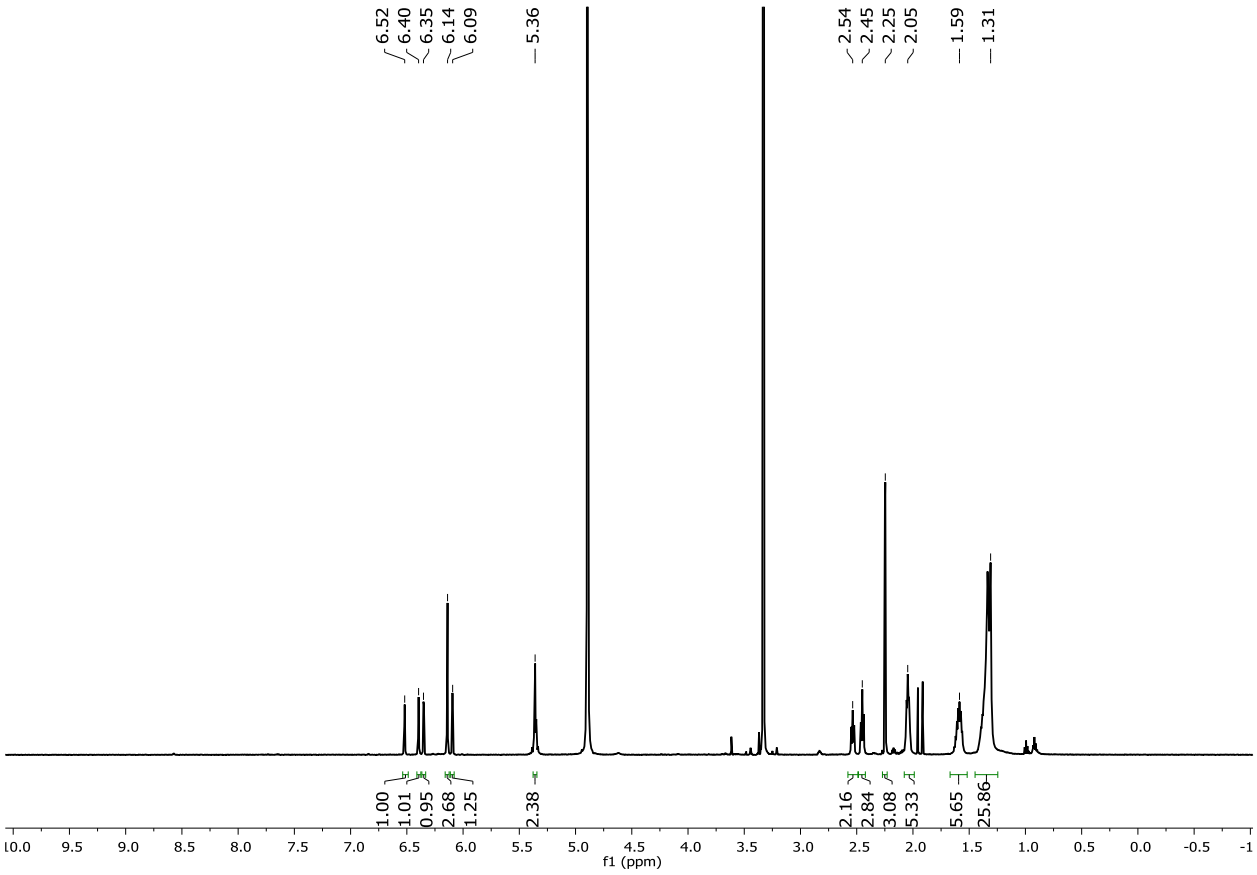
13.77 HMBC of 6.5 (CD_3OD; 600 MHz, 150 MHz)

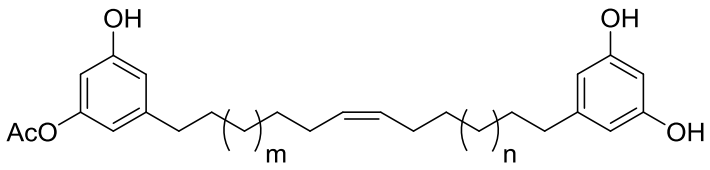




6.5a: m = 1; n = 3
6.5b: m = 3; n = 1

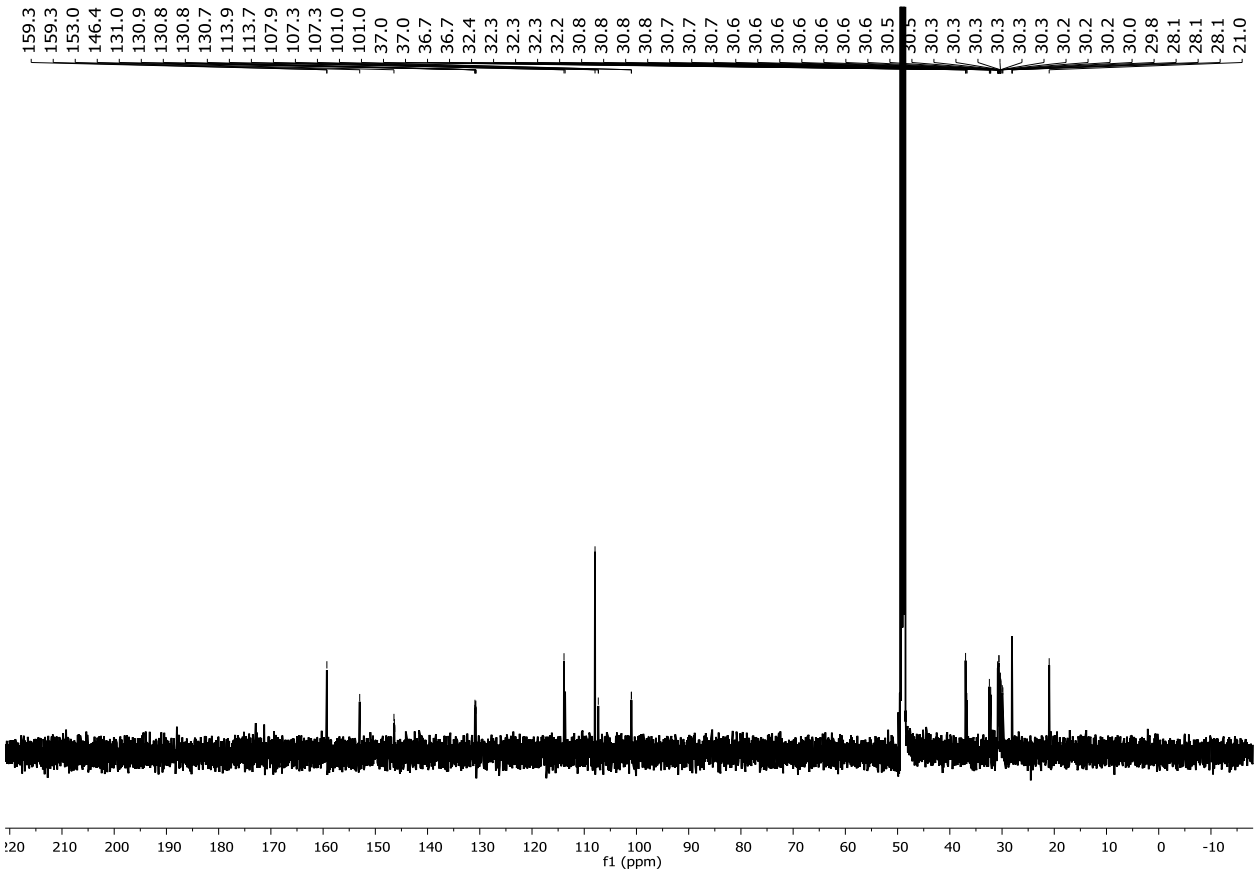
13.78 ^{1}H NMR of 6.6 (CD_3OD, 500 MHz)

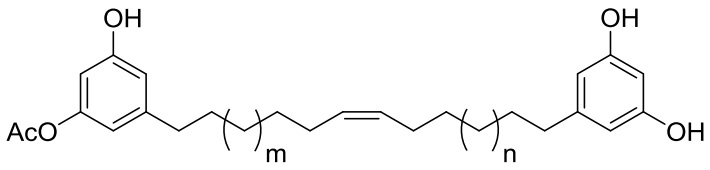




6.6a: m = 1; n = 5
6.6b: m = 3; n = 3
6.6c: m = 5; n = 1

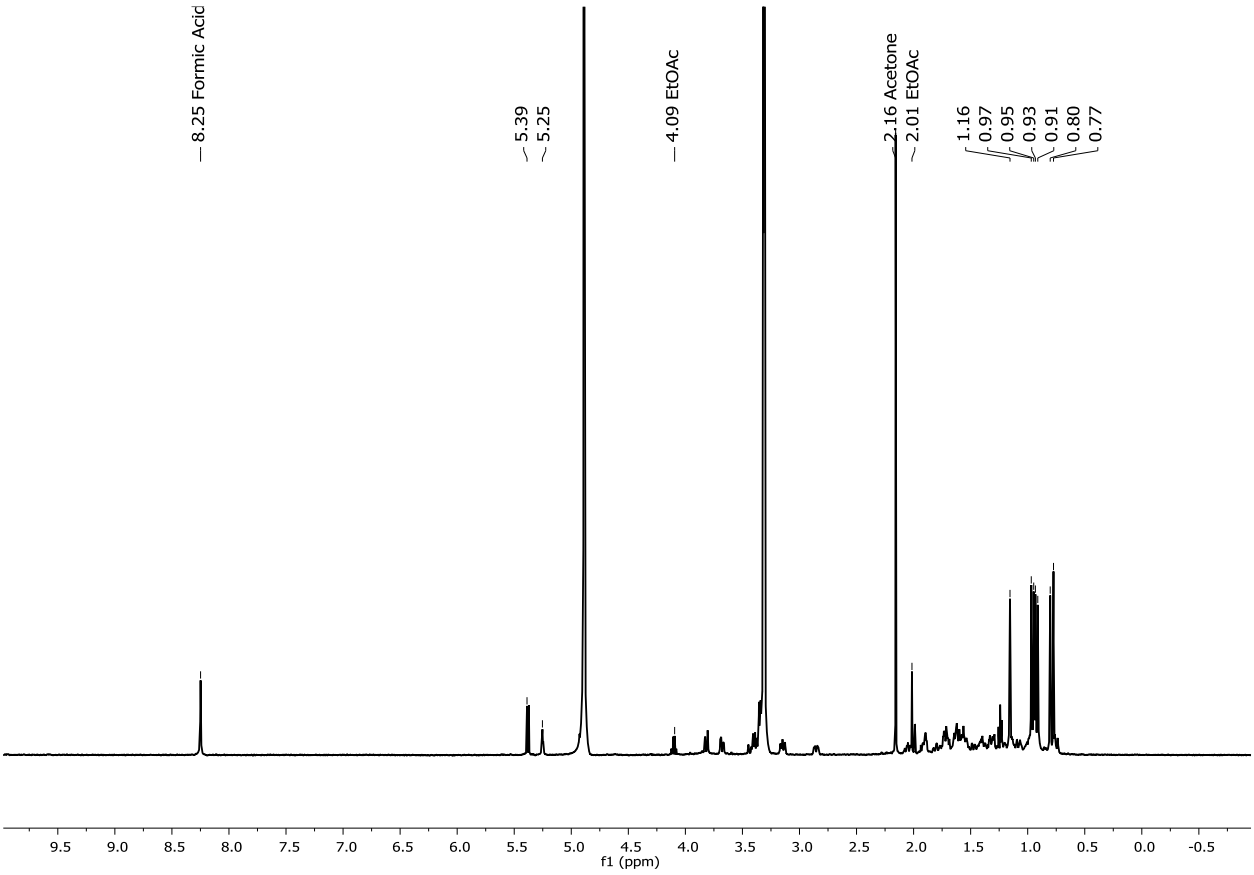
13.79 ^{13}C NMR of 6.6 (CD_3OD, 150 MHz)

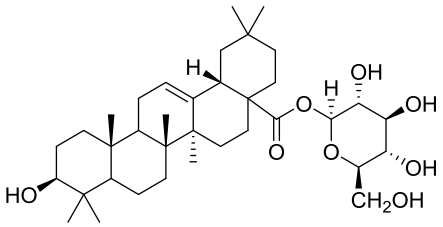




6.6a: m = 1; n = 5
6.6b: m = 3; n = 3
6.6c: m = 5; n = 1

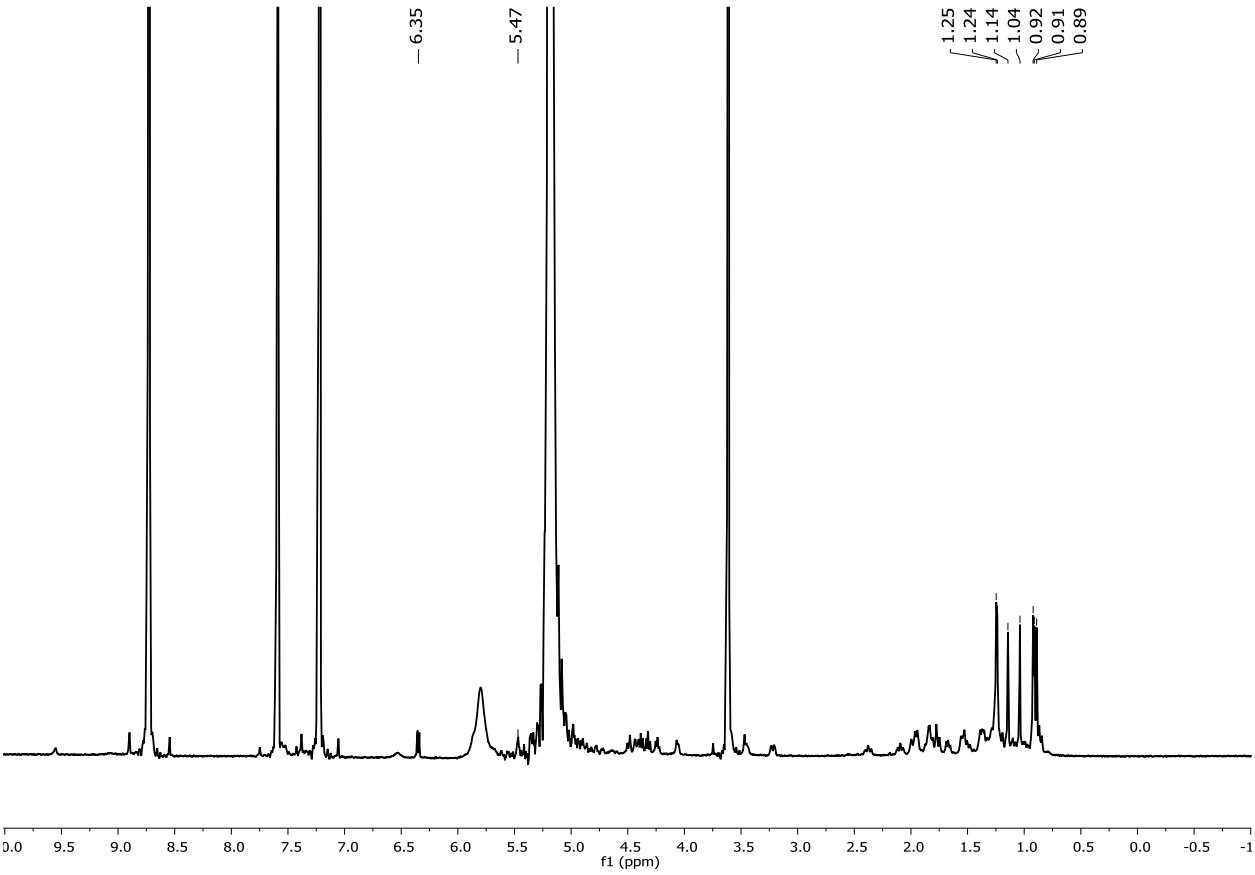
13.80 ^{1}H NMR of 7.1 (CD_3OD, 500 MHz)

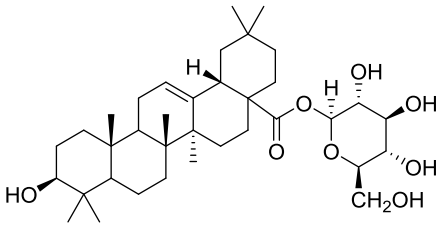




7.1

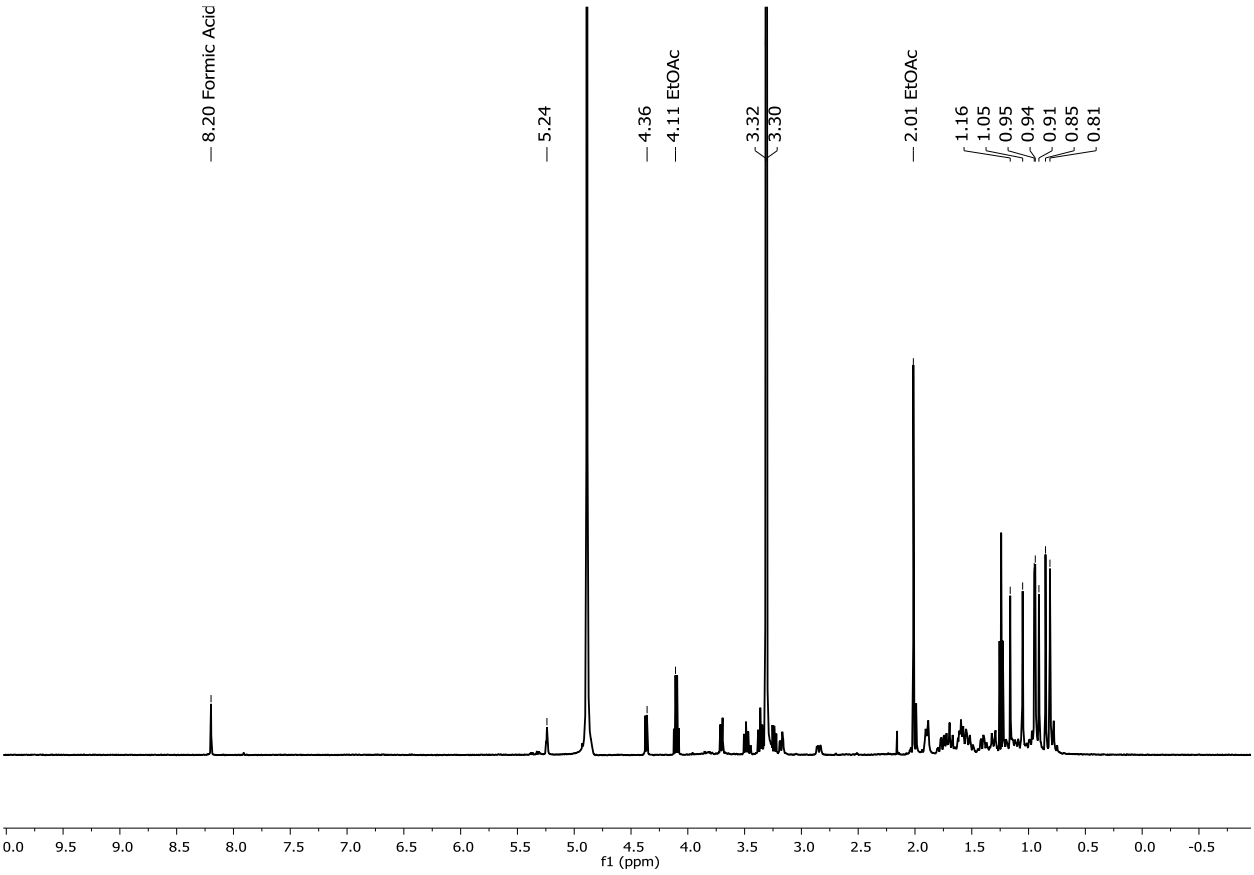
13.81 ^{1}H NMR of 7.1 (C_5D_5N, 500 MHz)

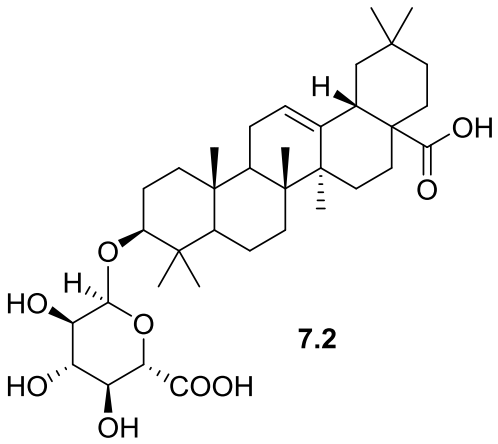




7.1

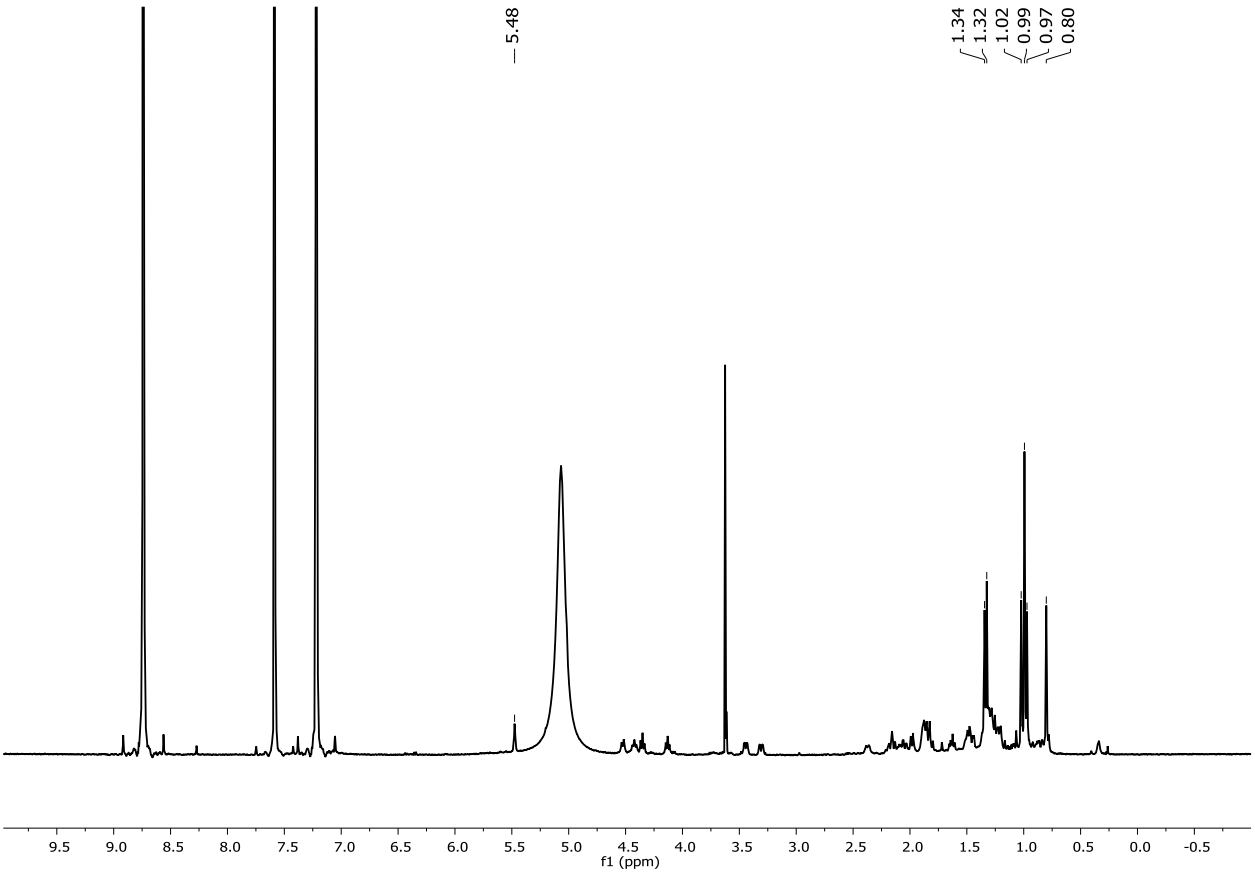
13.82 ^{1}H NMR of 7.2 (CD_3OD, 500 MHz)

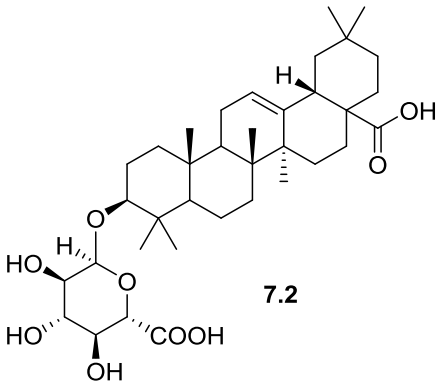




7.2

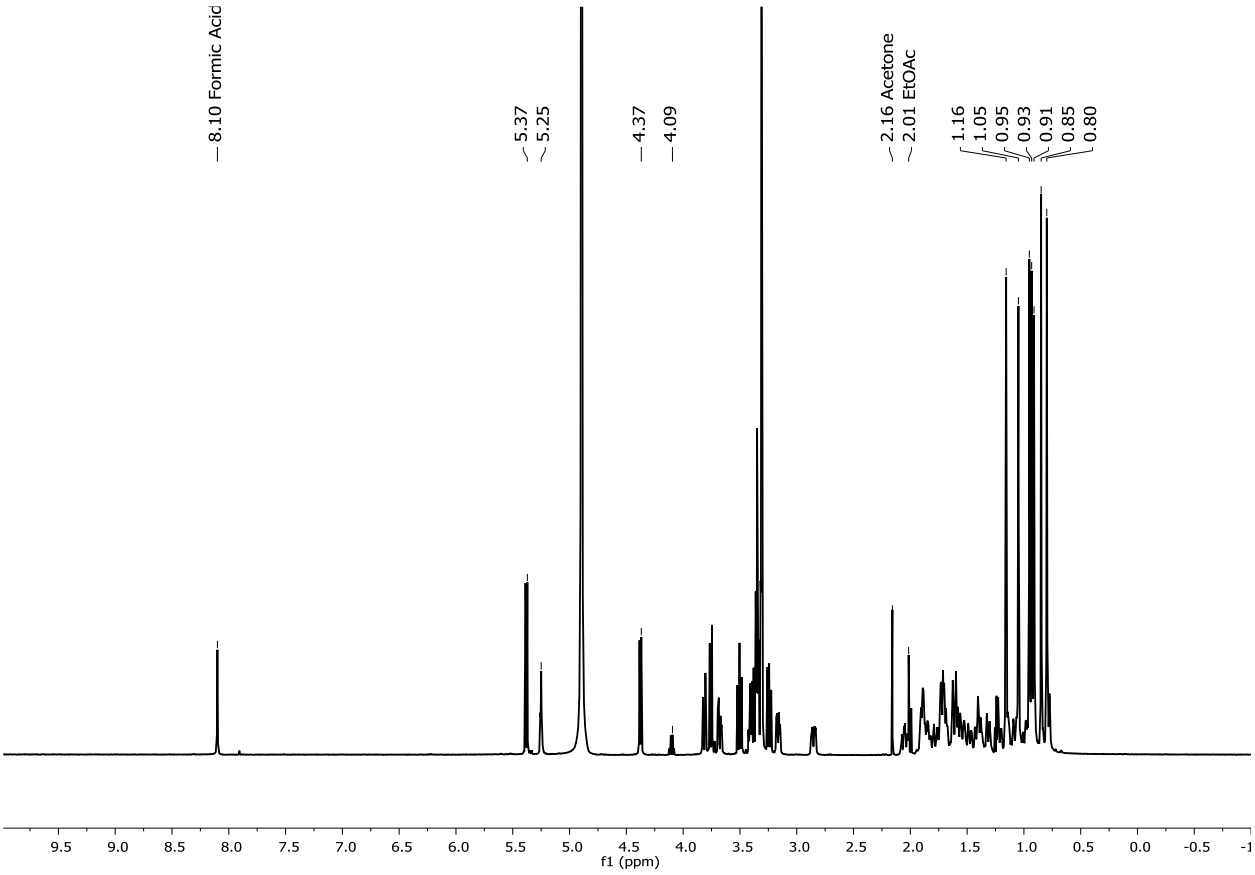
13.83 ^{1}H NMR of 7.2 (C_5D_5N, 500 MHz)

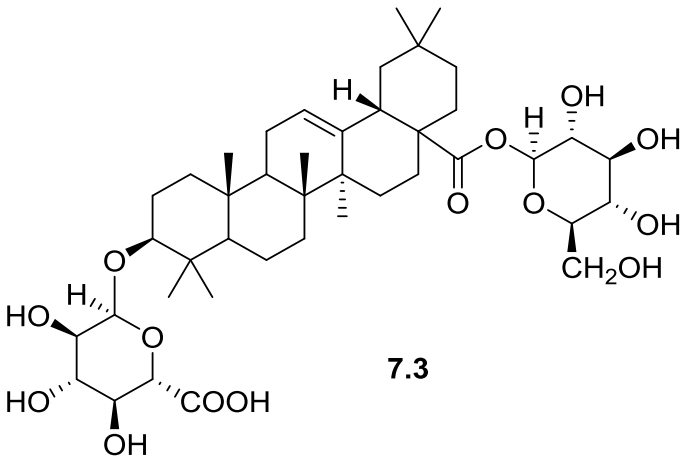




7.2

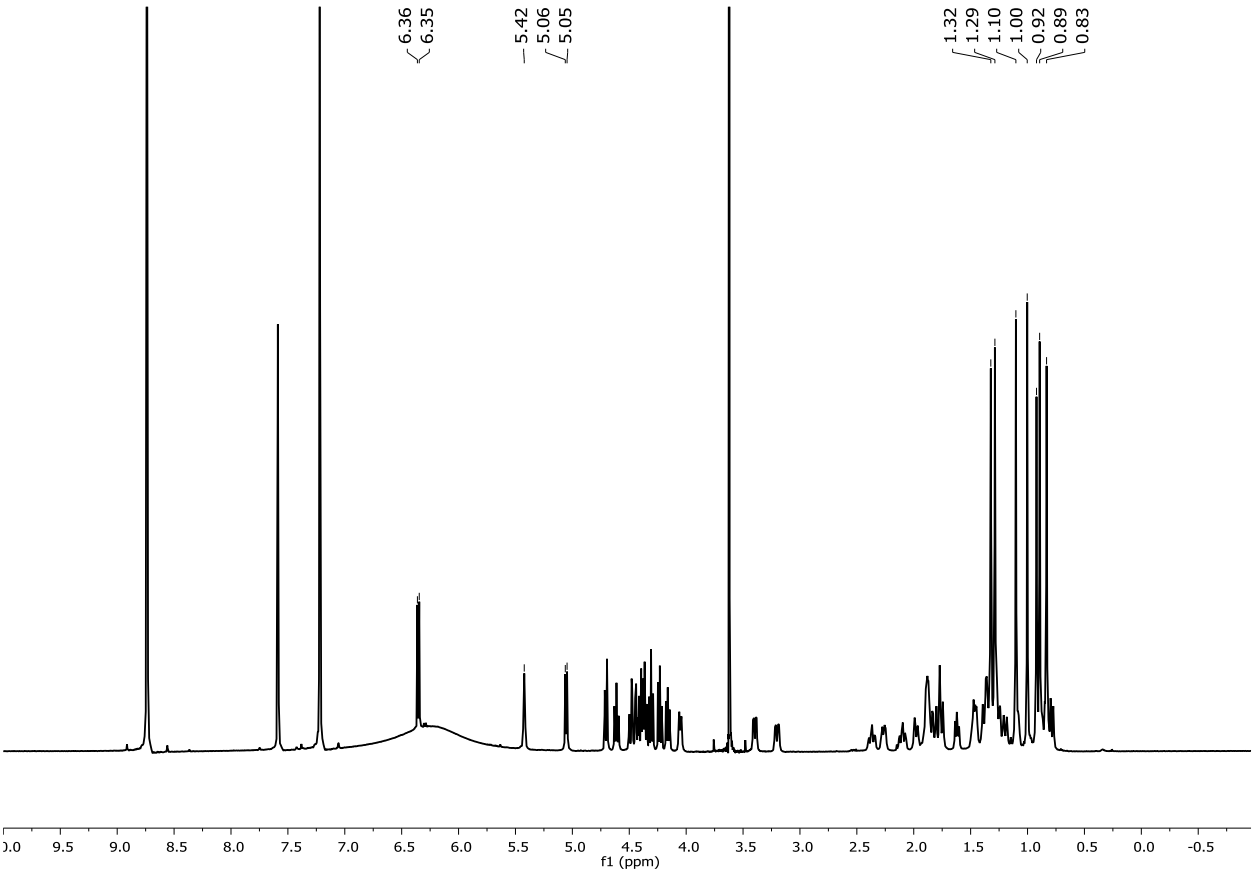
13.84 ^{1}H NMR of 7.3 (CD_3OD, 500 MHz)

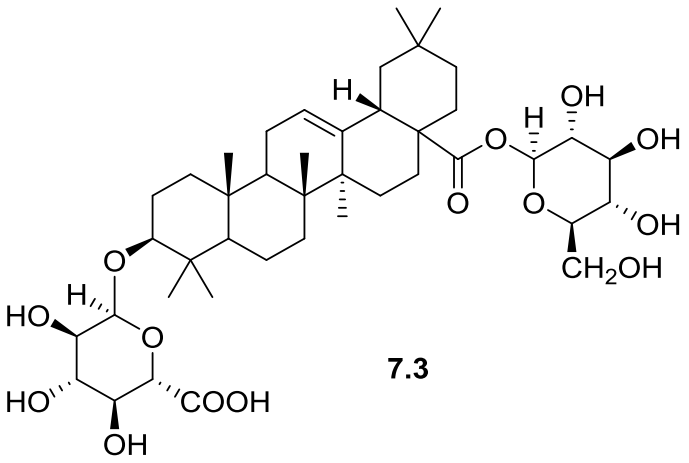




7.3

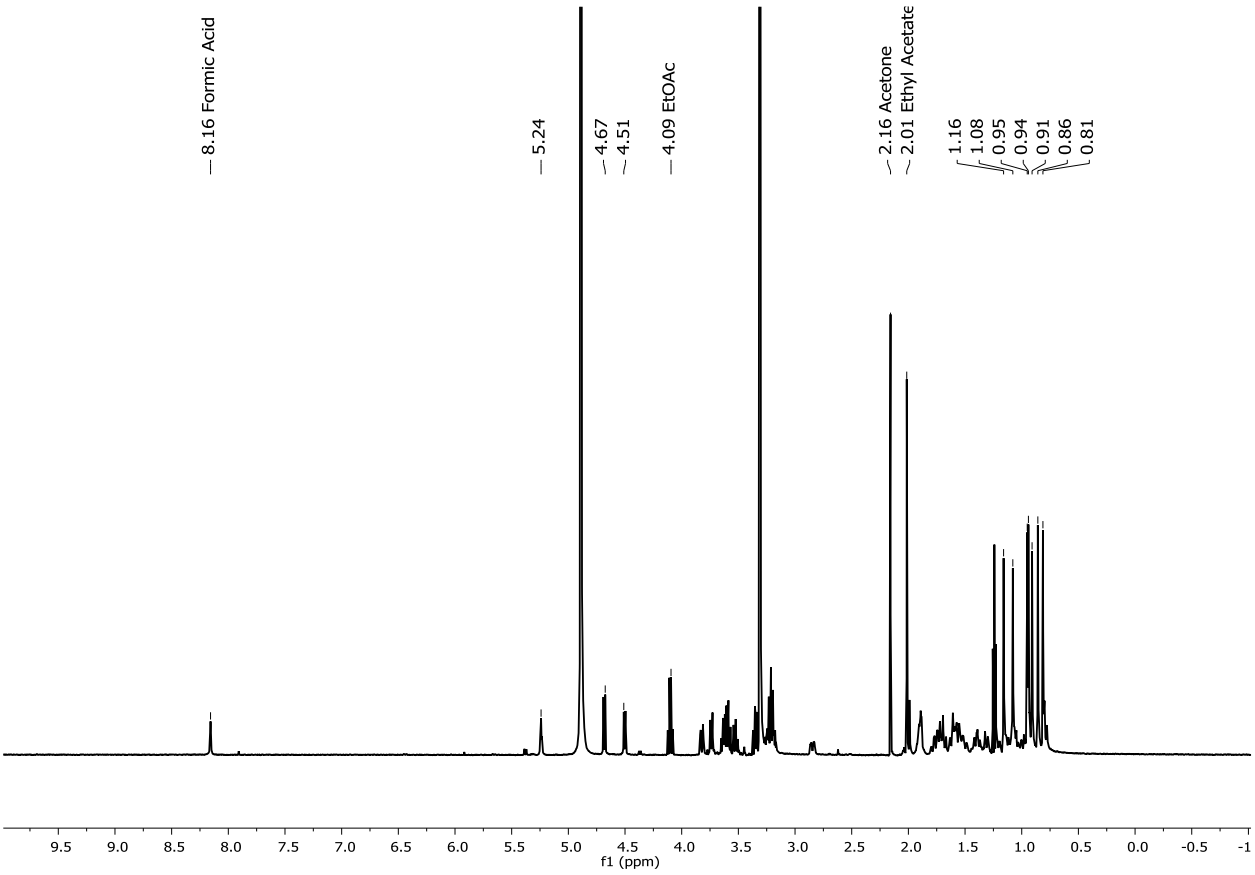
13.85 ^{1}H NMR of 7.3 (C_5D_5N, 500 MHz)

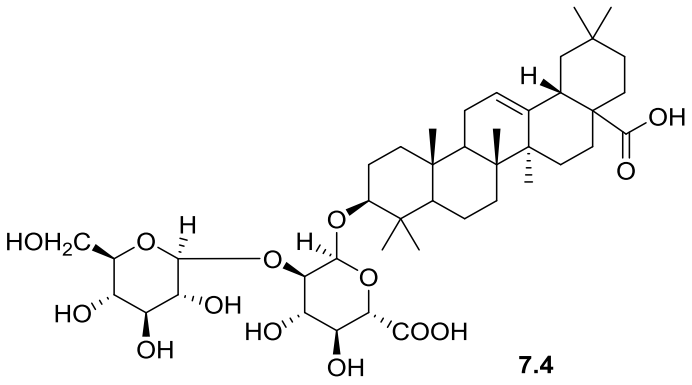




7.3

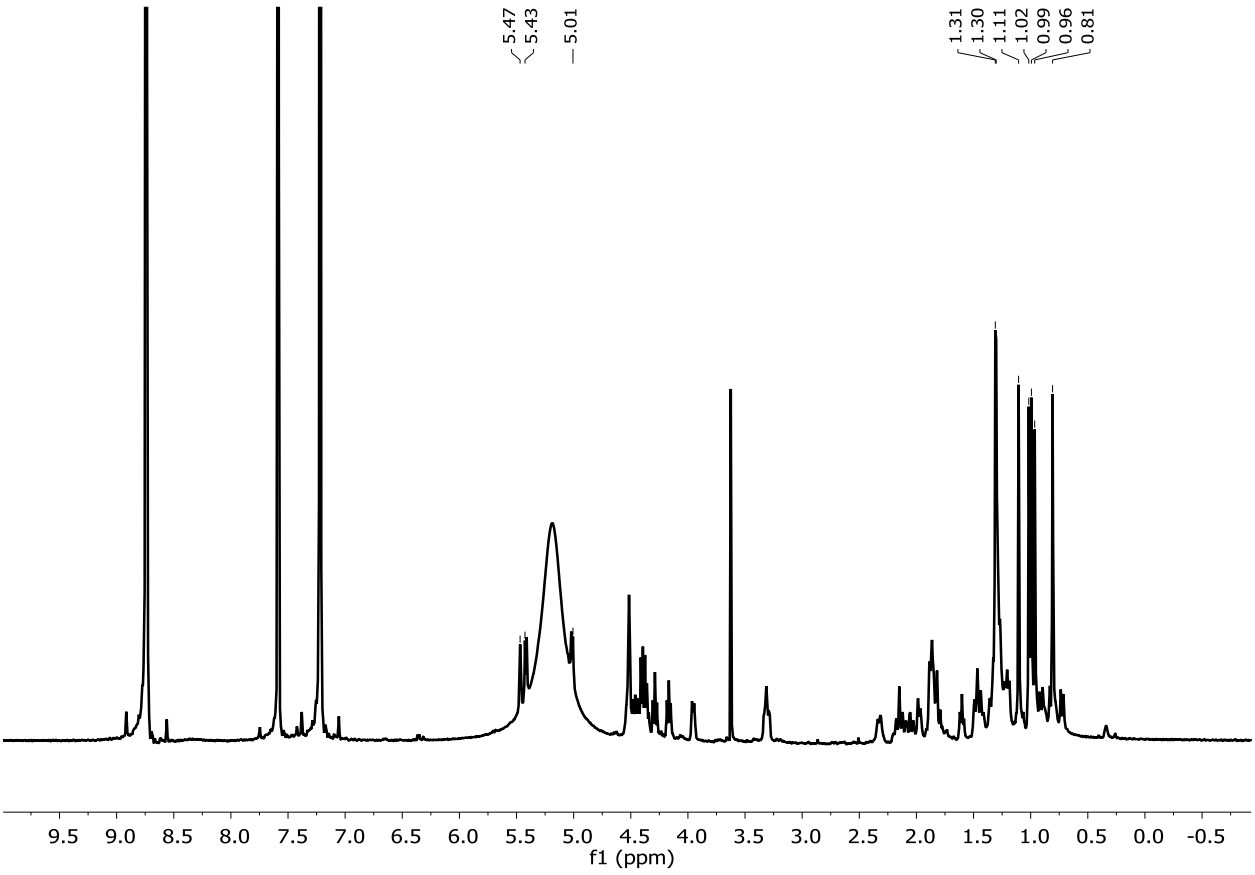
13.86 ^{1}H NMR of 7.4 (CD_3OD, 500 MHz)

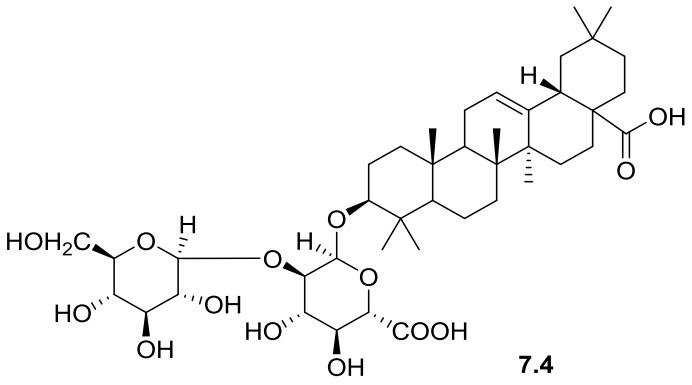




7.4

13.87 ^{1}H NMR of 7.4 (C_5D_5N, 500 MHz)





7.4