Isolation and Structure Elucidation of Anticancer and Antimalarial Natural Products

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ABSTRACT

As part of an International Cooperative Biodiversity Group (ICBG) program and a collaborative research project with the Natural Products Discovery Institute, twenty plant extracts were investigated for their antiproliferative and antimalarial activities. Bioassay guided fractionation of thirteen extracts led to the identification of three new antiproliferative compounds, ethyl leptaulosides A−C (**5.1**−**5.3**), six new antiplasmodial compounds, apoplanesiacarpan A and B (**2.4**−**2.5)**, (±)-rhodomyrtosone F (**3.1**), (±)-calliviminone C (**3.2**), 3α-angeloyloxy-15-hydroxylabda-7,13-dien-16,15-olid-18-oic acid (**4.1**), 3α-angeloyloxy-15-methoxylabda-7,13-dien-16,15-olid-18-oic acid (**4.2**), and twenty-six known compounds. The structures of these compounds were elucidated by using a combination of 1D (^{1}H and ^{13}C) and 2D NMR spectroscopy, mass spectrometry, UV, IR, CD, optical rotation, and chemical modifications. Compounds **5.1** and **5.2** showed moderate antiproliferative activity against the A2780 human ovarian cancer cell line assay with IC_{50} values of 3 µM and 10 µM, respectively. Compound **3.1** showed potent antiplasmodial activity with an IC_{50} value of 100 nM, while compounds **3.2** and **4.1** showed moderate antiplasmodial activity with IC_{50} values of 4 µM and 10 µM, respectively. The other compounds had IC_{50} values larger than 20 µg/mL, and were thus either inactive or only weakly active.

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GENERAL AUDIENCE ABSTRACT

Plant based natural products have a long history of being used for medicinal purposes and have played an important role in the modern drug discovery program, with the best known examples being paclitaxel as an anticancer drug, and quinine and artemisinin as antimalarial drugs. Despite great progress in fighting malarial and cancer, both diseases remain difficult to combat due to emergence of drug resistance in malarial parasites and hardness to treat various types of cancer. Therefore, it is urgent to discover new antimalarial and anticancer agents to treat these deadly diseases. This research focuses on identifying new antimalarial and anticancer agents from plant extracts. Investigation of twenty plant extracts led to the isolation of three new anticancer, six new antimalarial and twenty-six known compounds. The isolation and structure elucidation of these new bioactive compounds will be discussed in this dissertation.

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Table of Contents

Chapter 1: Introduction

1.1 Natural products and drug discovery

Natural products (secondary metabolites) provide attractive sources for drug discovery with enormous structural and chemical diversity. Natural products have a rich history in drug discovery programs. From 1959 to 1973, over 25% of US prescriptions contained active ingredients derived from plants while 13.3% and 2.7% were derived from microbial and animal sources.[1] A detailed analysis revealed that 37% of small molecule drugs approved by the US Food and Drug Administration (FDA) between 1981 and 2014 were related to natural products.[2] Natural product-related drugs have been used against a wide range of disease types, including in particular cancer and infectious disease.[3] It is estimated less than 10% of the world's biodiversity has been studied for potential biological activities with only 5 to 15% of the approximately 250 000 species of higher plants systematically investigated, less than 1% of bacterial and 5% of fungal species known, and the potential of the marine environment barely tapped.[4] Therefore, natural products discovery will continue to be an interesting topic.

1.1.1 Antimalarial natural products

Malaria is one of the world's most devastating diseases. It is caused by parasites of the genus *Plasmodium* (*P. falciparum*, *P. malariae, P. ovale,* and *P. vivax*).[5] *P. falciparum* is the most causative species, and causes about 80% of all malaria infections and about 90% of deaths from malaria. [6] About 3.2 billion people are at risk for contracting malaria, leading to an estimated 198 million malaria cases and nearly 600,000 deaths in 2013.[7]

Plant based natural products have played an important role in malaria treatment with the best known examples being quinine (**1.1**) and artemisinin (**1.2**). Quinine, also known as "the drug

to have relieved more human suffering than any other in history",[8] was isolated form the bark of the cinchona tree, *Cinchona officinalis* and other *Cinchona* species.[6] It was considered as a miracle drug in combating malaria until the parasite developed resistance.[9] Artemisinin, also known as Qinghaosu, was isolated from *Artemisia annua* by Chinese scientists.[10] Artemisinin-based combination therapies (ACTs) are currently the preferred first-line antimalarials used against *P. falciparum* (the deadliest of the five species that infect humans), and ACTs have been adopted for first-line treatment in most of the countries where *P. falciparum* is endemic. [7] The ACTs combine an artemisinin (ART) group of drugs (artesunate, artemether, dihydroartemisinin, artemisinin and arteether) with another antimalarial compound (for example, mefloquine, amodiaquine, and piperaquine).[11] However, the rapid development of resistance to the partner drug and recent concerns about artemisinin resistance[12] underline the need for the development of new drugs to expand our repertoire of antimalarial agents to use in combination therapies.[11]

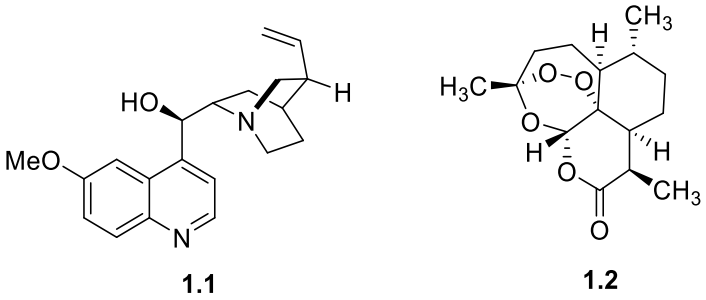


Figure 1-1. Structures of quinine (**1.1**) and artemisinin (**1.2**)

To date, compounds isolated from plants still play a major role in the discovery and development of antimalarial drugs, as evidenced by the fact that many compounds with antimalarial activity continue to be found in plants. [6, 13-14] The Kingston group's ongoing screening of plant extracts for antiplasmodial compounds has resulted in the isolation of a variety of interesting natural products with potent activity. These compounds include the dimeric

phloroglucinols mallotojaponins B and C (**1.3-1.4**, IC_{50} = 0.75 ± 0.30 and 0.14 ± 0.04 μM respectively) isolated from a tropical plant of the genus *Mallotus*,[15] ococymosin from *Ocotea cymosa* (**1.5**, IC_{50} = 0.45 ± 0.02 μM),[16] and the anthraquinone scutianthraquinone B (**1.6**, IC_{50} = 1.14 μM) from *Scutia myrtina*.[17] The discovery of novel naturally occurring antimalarial agents from plants is thus an important research objective.

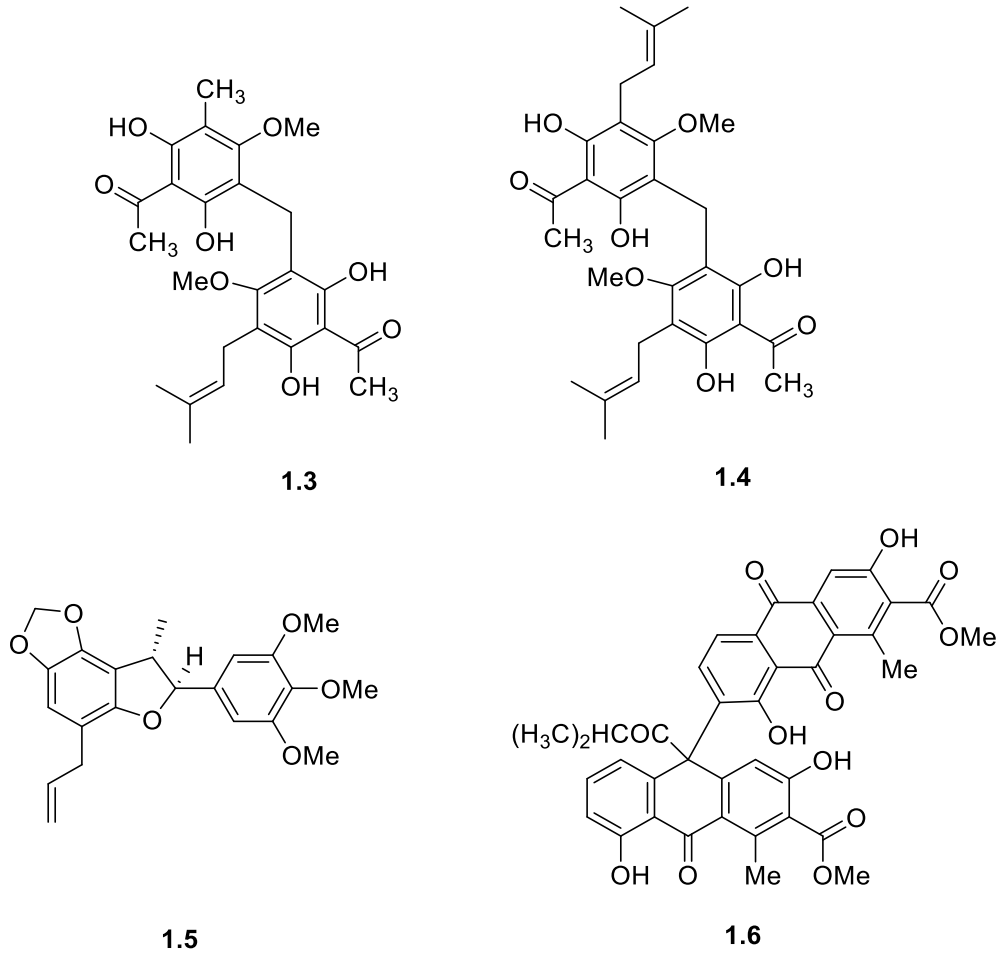


Figure 1-2. Antiplasmodial agents isolated by Kingston research group

1.1.2 Anticancer natural products

The use of natural products as cancer remedies has a long history.[18] Plant-derived compounds that are used in cancer treatment include vincristine (**1.7**), irinotecan (**1.8**), etoposide

(**1.9**) and paclitaxel (**1.10**).[19] Despite this successful history, there is still a need for better anticancer drugs. So the search for anticancer agents derived from plants is still of great interest. One of the most exciting plant-based anticancer agents in development is flavopiridol (**1.11**), representing the first cyclin-dependent kinase inhibitor.[19] Flavopiridol is a synthetic derivative of rohitukine, isolated from the plant *Amoora rohituka* (Maliaceae).[19-20] Mechanistic study suggested that flavopiridol can interfere with the phosphorylation of cyclin-dependent kinases, therefore impeding their activation and blocking cell-cycle progression at growth phase 1 (G_1) or G_2.[21-22]

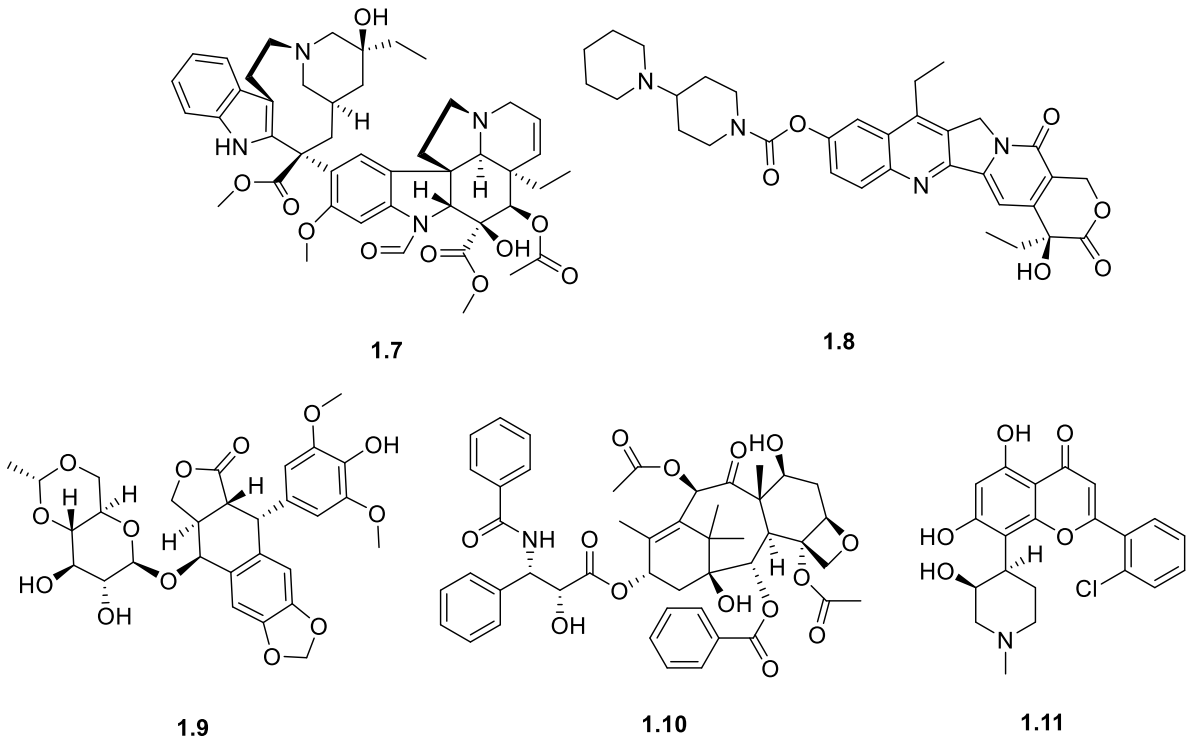


Figure 1-3. Structures of vincristine (**1.7**), irinotecan (**1.8**), etoposide (**1.9**), paclitaxel (**1.10**) and flavopiridol (**1.11**)

From 1998 to 2013, the Kingston research group isolated a variety of classes of antiproliferative natural products from plant materials collected in Madagascar. One of the most

exciting examples was 3α-*O*-(β-D-glucopyranosyl)desoxypodophyllotoxin (**1.12**), a lignan obtained from *Cleistanthus boivinianus*, which exhibited potent antiproliferative activity with an IC_{50} value of 33.0 ± 3.6 nM.[23]

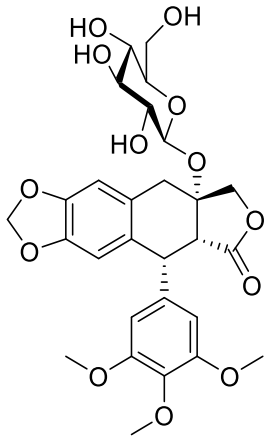


Figure 1-4. Structure of 3α-*O*-(β-D-glucopyranosyl)desoxypodophyllotoxin (**1.12**)

1.2 Discovery of new plant-based bioactive natural products

1.2.1 Plant materials

1.2.1.1 The Madagascar ICBG program

In 1993, Dr. Kingston's research group at Virginia Tech, in cooperation with the Missouri Botanical Garden, Conservation International, Bristol-Myers Squibb, and Bedrijf Geneesmiddelen Voorziening Suriname (BGVS) started to investigate natural product extracts from Suriname, as part of an International Cooperative Biodiversity Group (ICBG), funded by the NIH.[24-25] Although Dr Kingston's first phase of ICBG funding began in Suriname, the project transitioned to Madagascar from 1998 to 2013.[25] Madagascar is home to around 13,000 plant species, and probably more than 90% of them are endemic.[24] The plant materials of the ICBG Madagascar program were collected and identified by the Missouri Botanical Garden in collaboration with the

Centre National d'Application de Recherches Pharmaceutique (CNARP). These extracts were collected with no regard to previous reports of medicinal activities. The extractions were performed by Dr. Vincent E. Rasamison at CNARP. These extracts were then made available to Virginia Tech where they were tested for antiproliferative and antimalarial activities. Extracts showing interesting bioactivity were fractionated to identify the bioactive components.

1.2.1.2 Merck Collection at NPDI

In June 2011, the Natural Products Discovery Institute (NPDI) was established as a result of an unprecedented gift by Merck and Company of its entire U.S. Natural Products Library and the Schering-Plough Legacy Culture Collection to the Hepatitis B Foundation's research affiliate, the Institute for Hepatitis and Virus Research.[26] The NPDI's collection was comprised of more than 22,000 plant, 35,000 actinomycetous and 45,000 fungal extracts, which is one of the largest natural product extract collections in the world.[27-28] NPDI collaborated with the Kingston group at Virginia Tech to search for novel anti-cancer and anti-malarial agents from these extracts.[26]

1.3 Isolation of bioactive natural products

1.3.1 Bioassay-guided fractionation

Bioassay-guided fractionation is a popular technique used in natural product chemistry. It is particularly useful when the active component is not known.[29] In bioassay-guided fractionation, the crude extract is separated into several fractions using different separation techniques. Each fraction is then evaluated by bioassay, and only fractions with the desired biological activity in the bioassay are selected for further fractionation until pure compounds with the biological activity are isolated.[30] Bioassay-guided fractionation greatly reduces the chance of isolating inactive compounds.

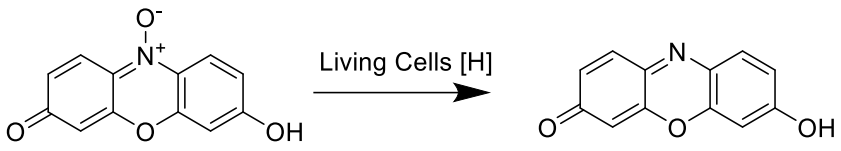
1.3.2 Isolation techniques

Isolation and purification of bioactive natural products from crude plant extracts can be described as finding a needle in a hay stack.[31] In order to obtain the pure active compound, a variety of isolation techniques are needed. The fastest and simplest technique used is liquid-liquid partition, which separates fractions based on their relative solubility in different solvents. Liquid Chromatography (LC), including open column chromatography and High Performance Liquid Chromatography (HPLC) are probably the most widely used isolation techniques. Relatively non-specific detectors are suitable for HPLC, such detectors include UV, refractive index (RI), evaporative light scattering (ELSD) or charged aerosol detectors (CAD).[30] HPLC separation is often detected by UV and ELSD in this dissertation. The purpose of combining these detectors is to show the presence of all compounds, and sometimes to use the UV information to facilitate a tentative identification of an unknown compound. The commonly used stationary phases include size-exclusion Sephadex LH-20, normal phase silica gel, reverse phase C18, and diol-modified silica gel among other options. Thin-layer chromatography (TLC) and solid phase extraction (SPE) are also popular separation techniques due to their ease of application. Other separation techniques include gas chromatography (GC), capillary electrophoresis (CE) and Ultra High Performance Liquid Chromatography (uHPLC), but they were not used in this work and thus will not be discussed in this dissertation.

1.3.3 Antiproliferative bioassay

The antiproliferative activities of all fractions and compounds were evaluated by the drug-sensitive A2780 human ovarian cancer cell line assay using Alamar Blue reagent.[32] The AlamarBlue assay is designed to measure quantitatively the proliferation of various human and animal cell lines. The assay incorporates an oxidation-reduction (REDOX) indicator that both

fluoresces and changes color in response to chemical reduction resulting from cell growth.[33] Continued cell growth maintains a reduced environment to reduce AlamarBlue (resazurin **1.8**) into resorufin (**1.9**).[33] The percentage of inhibition can then be calculated by analyzing the level of AlamarBlue (**1.8**) converted into the fluorescent resorufin (**1.9**).

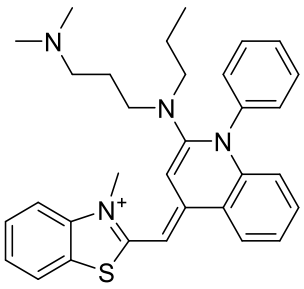


1.8 **1.9**

Figure 1-3. Resazurin Reduced to Resorufin by Living Cells

1.3.4 Antimalarial bioassay

The antimalarial activities of all fractions and compounds were evaluated by growth inhibition of the chloroquine resistant *Plasmodium falciparum* (Dd2) strain in a 72 h growth assay with minor modifications from a previously reported method.[34-35] Ring stage parasite cultures (100 µL per well, with 1% hematocrit and 1% parasitaemia) were grown for 72 h in the presence of increasing concentrations of the inhibitor in a 5.05% CO_2, 4.93% O_2 and 90.2% N_2 gas mixture at 37 ºC. After 72 h in culture, parasite viability was determined by DNA quantitation using SYBR Green I (SG) as described previously.[35] Upon binding to double-stranded DNA (dsDNA), the fluorescence of the SG/dsDNA complex is enhanced > 1000-fold.[36] The half-maximum inhibitory concentration (IC_{50}) values are the average of three independent determinations with each determination in duplicate, and are expressed ± S.E.M.



1.10

Figure 1-4. Structue of SYBR Green I

1.4 Dereplication

1.4.1 Introduction

There is no doubt that natural products provide a diverse and promising source for drug discovery to combat various diseases. However, one of the greatest challenges facing natural product chemists is wasting time on the isolation, purification and characterization of known compounds.[37] The traditional approach to the isolation and identification of natural products depends on extensive chromatography, including HPLC, followed by mass spectrometry and NMR spectroscopic analysis, which can be a time consuming process. It could be extremely disappointing to isolate known compounds with biological activities that are previously reported. Therefore, dereplication (*i.e*., the identification and discarding of known compound at the earliest possible stage) has become a matter of great interest, and numerous dereplication methods have been developed.

1.4.2 Dereplication Methods

Dereplication methods rely mainly on analytical techniques and database searching (e.g., from NIST).[38] Currently, dereplication is much easier due to the rapid development of modern

hyphenated techniques such as liquid chromatography–mass spectrometry (LC-MS), liquid chromatography-nuclear magnetic resonance (LC-NMR), gas chromatography-mass spectrometry (GC-MS), liquid chromatography-nuclear magnetic resonance-mass spectrometry (LC-NMR-MS) and other methods.[37]

1.4.2.1 LC- MS and LC-MS/MS

LC-MS provides important information including retention time, molecular mass, molecular formular, and sometimes fragmentation patterns. LC-MS techniques accompanied by MS libraries and databases, allow hit searching to some extent.[38] Accuracy is fundamental for successful dereplication, and accurate masses need to be determined within ± 5 ppm. The instrument accuracy performance is usually validated by analyzing a known compound in one of the first analytical runs and in each analytical run if a known compound was detected.[39] LC-MS had been successfully applied in the rapid dereplication of quinolines from an extract of *Choisya ternate*,[40] taxoids from the bark of *Taxus wallichiana,*[41] cucurbitacins from stem bark of *Elaeocarpus chinensis,*[42] among other examples. Comparing with LC-MS, LC-MS/MS provides characteristic fragmentation details which can be helpful to identify structures. For example, loss of CO_2 is favored in acids, and phenolic compounds frequently give fragments corresponding to a tropylium-like ion. In a work by Spanjer and *et al.*, more than 20 mycotoxins were successfully identified in a single 30-min run by LC-MS/MS.[43]

1.4.2.2 LC-NMR

LC-NMR, combining the separation power of HPLC with the superior structural information of nuclear magnetic resonance (NMR),[44] provides a reliable technique in the analysis of phytochemicals in plant mixtures,[37] especially when LC-MS does not allow confident identification of the compounds. LC-NMR has been applied to characterize a wide range of plant-

based natural products, including flavanones,[45] napthoquinones,[46] alkaloids,[47] sesquiterpene lactones[48] and many more. The NMR spectrum can be obscured in part by solvent protons. To minimize such interference, solvent signal suppression is mandatory, and can be achieved by presaturation, soft-pulse multiple irradiation or water suppression enhancement.[49-50] Introduction of a solid-phase extraction (SPE) to the LC-NMR system has also been applied, which allowed recovery of analytes from the HPLC mobile phase for NMR analysis.[50] Compounds trapped by SPE cartridges can then be eluted into NMR tubes with minimized interference from HPLC mobile phase components.[50] Unfortunately, lack of sensitivity and automation of interpretation is still a challenge of LC-NMR. Also, the lack of searchable NMR databases is preventing LC-NMR from being used as a front line dereplication technique.[44]

1.4.2.3 LC-NMR-MS

Compared with LC-MS and LC-NMR, LC-NMR-MS is the most powerful tool to provide comprehensive structural data for non-volatile compounds. LC-NMR-MS makes it possible for parallel on-line NMR and MS analysis, therefore providing complementary data and minimizing ambiguities between LC–MS and LC–NMR systems.[51] In the parallel analysis, eluent is split to two flows, and the balance between the two flows can be adjusted by a splitter. The typical split ratio is 95:5 for NMR vs. MS, since NMR is less sensitive.[37] The method has been applied in identification and confirmation several plant metabolites.[52-53] With the latest shielded magnets, a benchtop ion trap MS can now be placed less than 1 m from the center of a 500 MHz magnet without adverse performance. This short distance also minimized LC peak broadening, and enhanced NMR sensitivity.[51] In stopped-flow and loop storage mode, the limits of detection at the ^{1}H observation frequency of 600 MHz for 500 MW analytes are around 100 ng. While in continuous flow or on-flow modes, using NMR as the real-time detector, sensitivity and resolution

are limited by the short residence time of analytes, and typically more than 10 μg per analyte is needed for quality results at the ^{1}H observation frequency of 500 MHz.[49]

1.4.2.4 Database Search

Database searches offer an alternative dereplication approach, if spectroscopic data of the desired compound can be obtained. The most used databases in this dissertation are the Dictionary of Natural Products (DNP) and SciFinder. The DNP is the only comprehensive and fully-edited database documenting virtually all known natural products.[54] An effective DNP database dereplication should combine parameters like molecular formula, molecular weight and ^{1}H NMR key signals. If the structure of a compound is elucidated, SciFinder can then be used to confirm if the structure has been published. It is also possible to search SciFinder through molecular formula. However, it usually generates too many hits, and NMR interpretation must be used to eliminate "undesired" structures.

1.5 Structure elucidation of bioactive natural products

Conventional structure elucidation of bioactive natural products mainly depends on spectroscopic analysis, including NMR, IR, UV spectroscopy and mass spectrometry. Determination of stereochemistry usually depends on interpretation of NMR spectra, including 2D spectra, circular dicroism spectroscopy, and optical rotation values. Dereplication of known compounds relies mainly on mass spectrometry, 1D NMR spectroscopic analysis, and database searches. Structure elucidation of new compounds requires more in-depth spectroscopic analysis. Chemical modifications may be needed when spectroscopic analysis does not allow confident structure elucidation. Modification methods applied in this dissertation include methylation, acid hydrolysis, and Mosher's ester analysis. Specific examples of employing these methods will be provided in chapter 2, chapter 5 and chapter 6.

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Chapter 2: Antiplasmodial Isoflavanes and Pterocarpans from *Apoplanesia paniculata*

This chapter is a modified and expanded version of a previously published work (Su, Q.; Krai, P.; Goetz, M.; Cassera, M. B.; Kingston, D. G. I. Antiplasmodial isoflavanes and pterocarpans from *Apoplanesia paniculata*. *Planta Med.* **2015**, *81*, 1128−1132.). Contributions of co-authors of the articles are described as follows in the order of the names listed. The author of this dissertation (Qingxi Su) completed the fractionation of the extract and the structural elucidation of the compounds described and performed the antiproliferative bioassay (A2780), as well as preparation of the manuscript. Dr. Priscilla Krai performed the antimalarial Dd2 bioassay on all fractions and compounds under the guidance of Dr. Maria B. Cassera. Dr. Michael Goetz provided the extract from the Natural Product Discovery Institute (NPDI). Dr. David G. I. Kingston was a mentor for this work and is the corresponding author for the published article.

Figure 2-1. Image of *Apoplanesia paniculata.* Used under Creative Commons (CC BY-NC-ND 3.0) from < http://tropicos.org/Image/100282291>.

2.1 Introduction

As part of the Kingston group's continuing search for bioactive natural products, an EtOH extract from the root of *Apoplanesia paniculata* (Fabaceae) was selected for investigation for the presence of antiplasmodial agents. The crude extract displayed weak activity against the *Plasmodium falciparum* Dd2 strain (IC_{50} > 20 μg/mL). Several antibacterial and antifungal isoflavones had been isolated from the related specie *Flemingia paniculata*.[1] No phytochemistry work had been performed on *Apoplanesia paniculata.*

Bioassay-guided fractionation of an EtOH extract of the roots of the plant *Apoplanesia paniculata* (Fabaceae) led to the isolation of the three known compounds amorphaquinone (**2.1**), pendulone (**2.2**), melilotocarpan C (**2.3**) and the two new pterocarpans, apoplanesiacarpan A and B (**2.4** and **2.5**). The structures of the known compounds **2.1**–**2.3** were determined by comparison of their ^{1}H NMR, mass spectrometry and circular dichroism spectra with the data reported in the literature. The structures of **2.4** and **2.5** were determined based on 1D and 2D NMR experiments, mass spectrometry, circular dichroism spectra, and chemical modification. Compounds **2.1** and **2.2** exhibited good antiplasmodial activity with IC_{50} values of 6 ± 2 and 7 ± 1 μM, respectively. Compound **2.3** exhibited weak antiplasmodial activity (42 ± 5 μM), while compounds **2.4** and **2.5** were inactive. Compound **2.6** was synthesized to confirm the structure of **2.5**, and it showed enhanced antiplasmodial activity (16 ± 1 μM) compared to its analogues **2.3**–**2.5**.

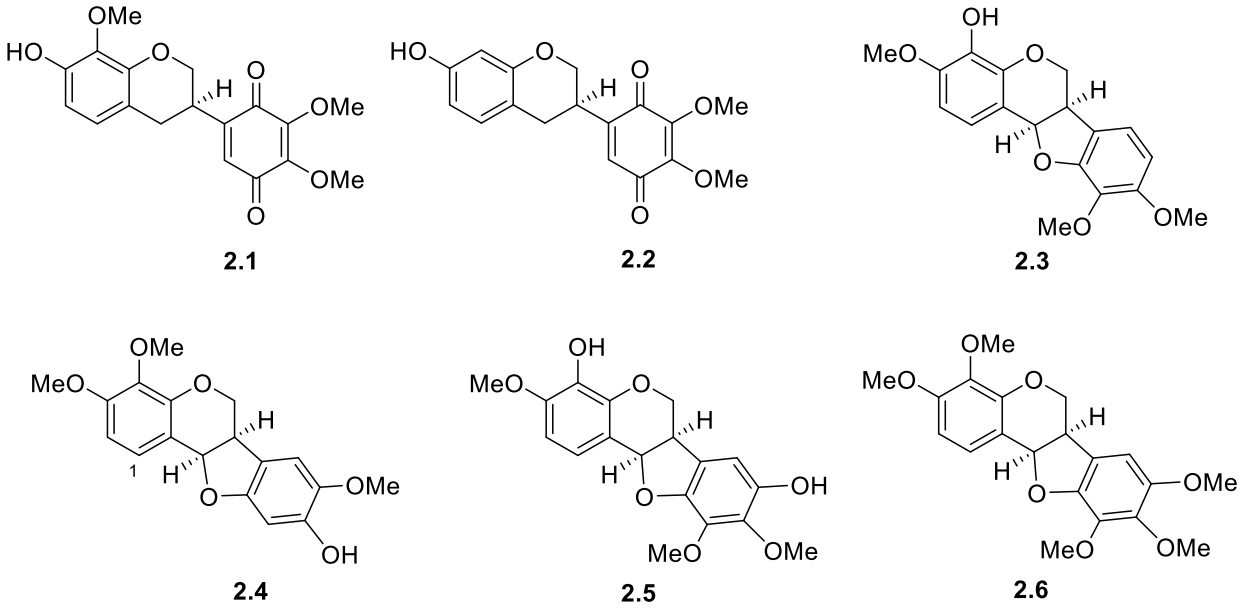
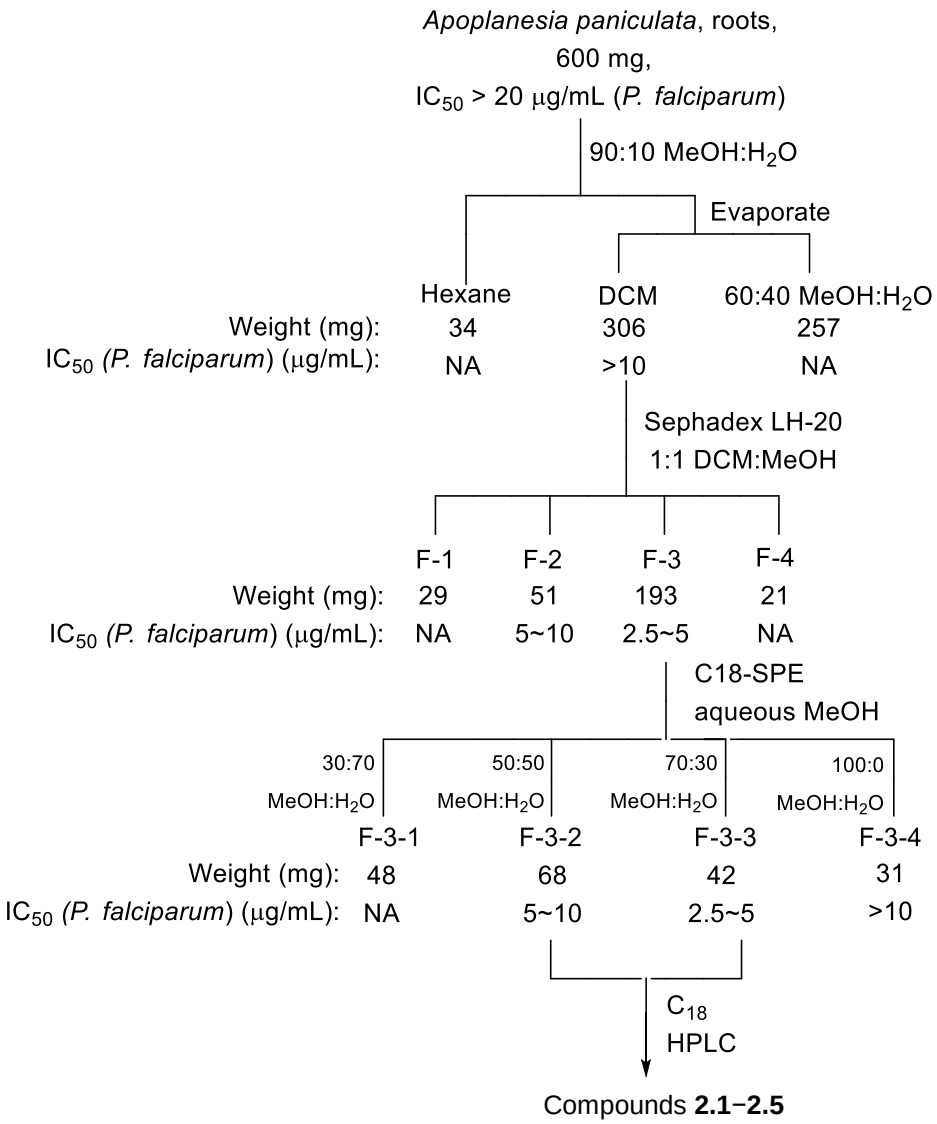


Figure 2-2. Structures of compounds **2.1**–**2.6**

2.2 Results and Discussion

2.2.1 Isolation of active compounds

The dichloromethane-soluble fraction obtained from a liquid-liquid partition of the ethanol extract of *Apoplanesia paniculata* (600 mg) showed weak antiplasmodial activity (IC_{50} value of >10 µg/mL). Since the plant was never studied for antiplasmodial activity, the crude extract was selected for fractionation in order to identify its bioactive compounds. Fractionation of the dichloromethane-soluble materials by chromatography on Sephadex LH-20, reverse phase SPE, and C_{18} HPLC yielded three known compounds amorphaquinone (**2.1**), pendulone (**2.2**), melilotocarpan C (**2.3**) and the two new pterocarpans, apoplanesiacarpan A and B (**2.4** and **2.5**). (Scheme 2-1)



Scheme 2-1 Bioassay-guided separation of *Apoplanesia paniculata*

2.2.2 Structure elucidation of compounds **2.1**–**2.3**

The known compounds amorphaquinone (**2.1**),[2] pendulone (**2.2**),[3] and melilotocarpan C (**2.3**)[4] were identified by comparison of their spectroscopic and physical data with literature data. The *3R* configuration of compounds **2.1** and **2.2** were confirmed by comparison of their circular dichroism spectra with those of previously reported data.[5-6]

2.2.3 Structure elucidation of compound **2.4**

Compound **2.4** was obtained as a white powder. The quasi-molecular ion peak at *m/z* 331.1168 $[M+H]^+$ (calcd for $C_{18}H_{19}O_6^+$, 331.1176) corresponded to a molecular formula of $C_{18}H_{18}O_6$. Its pterocarpan skeleton was confirmed by comparison of its ^{1}H-NMR data with that of compound **2.3** (melilotocarpan C). The ^{1}H and ^{13}C-NMR spectra of **2.4** (Table 2-1) showed signals for three methoxy groups (δ_H 3.83, 3.84 and 3.87, each 3H, s), an AB aromatic spin system [δ_H 7.21 (1H, d, J = 8.6 Hz) and 6.67 (1H, d, J = 8.6 Hz)] and two aromatic proton singlets (δ_H 6.83 and 6.47, each 1H, s). The positions of the sp^2 protons and methoxy groups were confirmed by HSQC, HMBC and NOE (Figure 2-3). The HMBC correlations between H-11a (δ_H 5.46, d, J = 6.8 Hz) and C-11b (δ_C 114.6) and between H-1 (δ_H 7.21, d, J = 6.8 Hz) and C-11b indicated that the AB spin system belongs to the A-ring. HMBC correlations between H-2 (δ_H 6.67, d, J = 8.6 Hz) and C-3 (δ_C 153.9) and C-4 (δ_C 137.8), between 3-OMe (δ_H 3.87, s) and C-3, and between 4-OMe (δ_H 3.84, s) and C-4 confirmed the placement of two methoxy groups in the A ring. The position of MeO-8 was confirmed by the NOE correlation between 8-OMe (δ_H 3.83, s) and H-7 (δ_H 6.47, s). Other key HMBC and NOE correlations are shown in Figure 2-3. A coupling constant of 6.8 Hz between H-6a and H-11a suggested these two protons were *cis*-diaxial; a larger coupling constant of 13-14 Hz would have been observed for the *trans* configuration.[7] The assigned relative configuration was in agreement with the fact that all natural pterocarpans found so far have the 6a,11a-*cis* configuration.[8] The absolute configurations of 6a and 11a were determined as (*R*,*R*) by comparison of the circular dichroism spectrum of **2.4** with that of known compounds.[9-10]

Table 2-1. ^{1}H NMR (500 MHz, $CDCl_3$) and ^{13}C NMR (125 MHz, $CDCl_3$) Chemical Shift Data (δ, ppm) for Compounds **2.4**–**2.6**

Position	2.4		2.5		2.6
	δ_H, (J in Hz)[a]	δ_C,type[b]	δ_H, (J in Hz)[a]	δ_C, type[b]	δ_H, (J in Hz)[a]
1	7.21 d (8.6)	125.9 CH	7.08 d (8.6)	121.4 CH	7.29 d (8.6)
2	6.67 d (8.6)	106.3 CH	6.67 d (8.6)	105.4 CH	6.68 d (8.6)
3	-	153.9 C	-	147.5 C	
4	-	137.8 C	-	134.1 C	-
5	-	149.8 C	-	143.7 C	-
6	4.34 dd (11.0, 5.1) 3.63 dd (11.0, 11.0)	67.1 CH_2	4.36 dd (11.0, 5.1) 3.73 dd (11.0, 11.0)	66.7 CH_2	4.38 dd (11.0, 5.1) 3.72 dd (11.0, 11.0)
6a	3.52 m	40.5 CH	3.53 m	40.7 CH	3.53 m
6b	-	118.2 C	-	122.9 C	-
7	6.47 s	110.7 CH	6.60 s	104.7 CH	6.59 s
8	-	140.2 C	-	140.0 C	-
9	-	147.3 C	-	138.9 C	-
10	6.83 s	95.1 CH	-	137.8 C	-
11	-	153.1 C	-	143.4 C	-
11a	5.46 d (6.8)	78.4 CH	5.50 d (6.8)	78.3 CH	5.51 d (6.8)
11b	-	114.6 C	-	114.1 C	-
3-OMe	3.87 s	56.5 CH_3	3.91 s	56.5 CH_3	3.89 s
4-OMe	3.84 s	61.4 CH_3	-	-	3.86 s
8-OMe	3.83 s	56.5 CH_3	-	-	3.84 s
9-OMe	-	-	3.91 s	60.5 CH_3	3.86 s
10-OMe	-	-	3.98 s	61.5 CH_3	3.98 s

[a] Data (δ) measured at 500 MHz; s = singlet, d = doublet, dd = doublet of doublets, m = multiplet. *J* values are in Hz and are omitted if the signals overlapped as multiplets. The overlapped signals were assigned from HSQC and HMBC spectra without designating multiplicity.

[b] Data (δ) measured at 125 MHz; CH_3, CH_2, CH, and C multiplicities were determined by HSQC experiments.

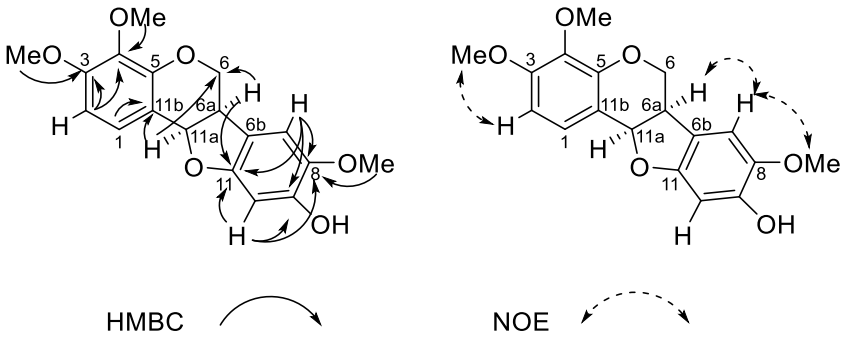


Figure 2-3. Key HMBC and NOESY correlations of compound **2.4**

2.2.4 Structure elucidation of compound **2.5**

Compound **2.5** was obtained as a white powder. The quasi-molecular ion peak at *m/z* 347.1127 $[M+H]^+$ (calcd for $C_{18}H_{19}O_7^+$, 347.1125) indicated a molecular formula of $C_{18}H_{18}O_7$. Characteristic signals for H-6 (δ_H 4.36, dd, J = 11.0, 5.1 Hz and 3.73, dd, J = 11.0, 11.0 Hz), H-6a (δ_H 3.53, m) and H-11a (δ_H 5.50, d, J = 6.8 Hz) in a pterocarpan skeleton were observed in its ^{1}H-NMR spectrum (Table 2-1). The ^{1}H NMR spectrum also showed the presence of three methoxy groups (δ_H 3.91, 3.91 and 3.98, each 3H, s), an AB aromatic proton spin system (δ_H 7.08, d, J = 8.6 Hz and 6.67, d, J = 8.6 Hz), and a single aromatic proton (δ_H 6.60, s). The HMBC correlation between H-11a and C-1 indicated that the AB spin system belonged to the A-ring. The NOE correlation between H-2 (δ_H 6.67, d, J = 6.8 Hz) and MeO-3 (δ_H 3.91, s) and between H-2 and C-4 (δ_C 134.1) confirmed the placement of the methoxy and hydroxyl groups in the A ring. The remaining assignment to be made was the position of the hydroxyl group and methoxy groups in ring D. NOE correlations between H-6a and H-7, and HMBC correlations between H-7 and C-8,

C-9 were observed, indicating that carbons 8, 9, and 10 were oxygenated. No NOE correlation of H-7 with any methoxy group was observed, suggesting the placements of hydroxyl and methoxyl groups on the D-ring for compound **2.5** might be as shown in Figure. 2-4.

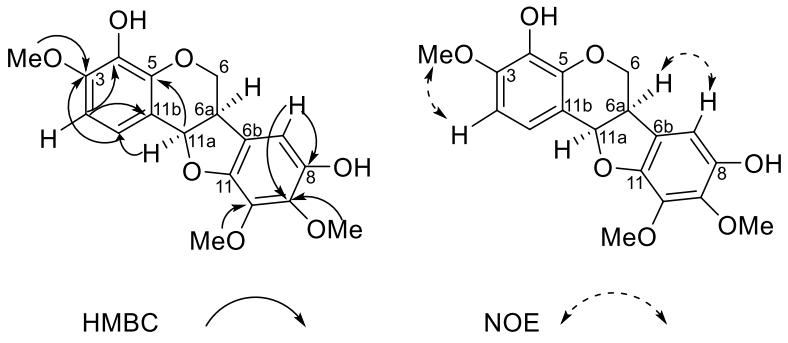
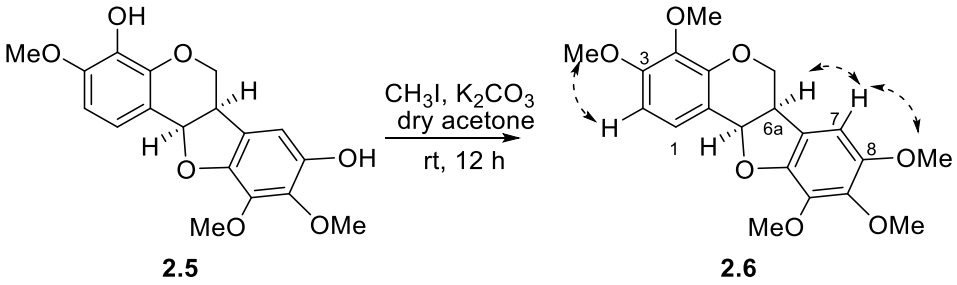


Figure 2-4. Key HMBC and NOESY correlations of compound **2.5**

To confirm this proposed structure, compound **2.5** was methylated to give **2.6** (Figure. 2-5). A key NOE correlation between H-7 (δ_H 6.59, s) and MeO-8 (δ_H 3.84, s) was observed for **2.6**, indicating that the HO-8 group in **2.5** was methylated, and thus confirming structure **2.5**. The use of methylation reaction to assist structure elucidation was also performed to confirm the structure of mallotojaponin B.[11]



2.5 **2.6**

Figure 2-5 Methylation of **2.5** to **2.6** and key NOESY correlations observed for **2.6**

2.2.5 Bioactivities

Compounds **2.1**–**2.6** were evaluated for their antimalarial activity against the drug-resistant Dd2 strain of *P. falciparum*, and their antiproliferative activity against the A2780 human ovarian cancer cell line. Quinones **2.1** and **2.2** showed moderate inhibition of the growth of the drug-resistant Dd2 strain of *P. falciparum*, with IC_{50} values of 6 ± 2 and 7 ± 1 μM, respectively, and also displayed moderate antiproliferative activity against the A2780 human ovarian cancer cell line, with IC_{50} values of 7 ± 1 and 21 ± 4 μM. Quinones **2.1** and **2.2** have been previously reported to exhibit antileishmanial,[12] antitumor,[13] antiparasitic and antimicrobial activities.[6] Melilotocarpan C (**2.3**) exhibited weak antiplasmodial activity (42 ± 5 μM), while apoplanesiacarpan A (**2.4**) and apoplanesiacarpan B (**2.5**) were inactive (IC_{50} > 20 μg/mL). The methylated derivative **2.6** was however moderately active, with an IC_{50} value of 16 ± 1 μM (Table 2-2).

Table 2-2. Antiplasmodial and antiproliferative activity data of compounds **2.1**–**2.6**.

Compound	*P. falciparum* Dd2 strain IC_{50} (μM)	A2780 ovarian cancer cells IC_{50} (μM)
Amorphaquinone (**2.1**)	6 ± 2	7 ± 1
Pendulone (**2.2**)	7 ± 1	21 ± 4
Melilotocarpan C (**2.3**)	42 ± 5	> 60
Apoplanesiacarpan A (**2.4**)	> 60	> 60
Apoplanesiacarpan B (**2.5**)	> 58	> 58
Methylapoplanesiacarpan B **(2.6**)	16 ± 1	> 56
Artemisinin (positive control)	6 ± 1	Not tested

Pterocarpans are a large group of compounds derived from isoflavanoids and have been reported to display cytotoxic,[14] antifungal,[15] and antimycobacterial,[16] activities. They might be interesting antimalarial agents as some pterocarpan derivatives have been reported to exhibit leishmanicidal activity.[12] The reduced activity of **2.3**–**2.6** compared with the activity of **2.1** and **2.2** suggested the quinone fraction of the molecules might be important for increased activity.

Comparison of the IC_{50} values of **2.3**–**2.6** suggests the number and placement of the methoxy and hydroxyl groups on the pterocarpan backbone can also affect the biological activity.

2.3 Experimental Section

2.3.1 General experimental procedures

Optical rotations were recorded on a JASCO P-2000 polarimeter. UV spectra were measured on a Shimadzu UV-1201 spectrophotometer. IR spectra were measured on a MIDAC M-series FTIR spectrometer. NMR spectra were recorded in $CDCl_3$ on a Bruker Avance 500 spectrometer. Chemical shifts are given in δ (ppm), and coupling constants are reported in Hz. Mass spectra were obtained on an Agilent 6220 LC-TOF-MS in the positive ion mode. Open column chromatographies were performed using Sephadex LH-20 (I.D.×L 3 × 50 cm) and C_{18} (I.D.×L 2.5 × 10 cm, 40−63 μm). Semi-preparative HPLC was performed on a Shimadzu LC-10AT instrument with a semipreparative C18 Varian Dynamax column (5 μm, 250 × 10 mm). All isolated compounds were purified to 95% purity or better, as judged by HPLC (both UV and ELSD detection) and ^{1}H NMR spectroscopies before determining bioactivity.

2.3.2 Plant material

Roots of *A. paniculata* C. Presl. were collected from plants along a river edge in Guatemala, close to Zapaca, Teculutan by Juan Jose Castillo under the auspices of the New York Botanical Garden. Voucher specimens of the plant are on deposit under the accession number JJC02914a, b and c (specimen ID 41281) at the NYBG.

2.3.3 Extraction and isolation

Dried, powdered plant material was exhaustively extracted with EtOH at room temperature to give an extract designated 60029-6C; a total of 600 mg of this extract was made available to Virginia Tech. Extract 60029-6C (600 mg, IC_{50} >20 µg/mL in the *P. falciparum* Dd2 antimalarial assay) was suspended in aqueous MeOH (MeOH/H_2O 9:1, 100 mL) and extracted with hexane (5 × 100 mL portions) to give 34 mg of hexane-soluble material. The aqueous fraction was then diluted to MeOH/H_2O 6:4 (150 mL) and further extracted with CH_2Cl_2 (5×100 mL portions) to give a CH_2Cl_2-soluble fraction (306 mg) with an IC_{50} value of >10 µg/mL; the residual MeOH-H_2O soluble fraction (257 mg) was inactive in the *P. falciparum* Dd2 antimalarial assay. The CH_2Cl_2 fraction was subjected to size exclusion open column chromatography on a Sephadex LH-20 column (I.D.×L 3×50 cm) eluted with CH_2Cl_2/MeOH 1:1. Four fractions were obtained on the basis of TLC profile. The four factions were denoted as F1 (29 mg), F2 (51 mg), F3 (193 mg) and F4 (21 mg). The most active fraction F3 exhibited an IC_{50} between 2.5 and 5 µg/mL, and was then subjected to column chromatography on C_{18} silica gel (I.D.×L 2.5×10 cm, 40-63 µm). Elution with aqueous MeOH with the MeOH/H_2O ratios of 30:70, 50:50, 70:30 and 100:0 (200 mL each) gave four subfractions indexed F3-1 (48 mg), F3-2 (68 mg), F3-3 (42 mg) and F3-4 (31 mg). Fractions F3-2 (68 mg, IC_{50} between 5 and 10 µg/mL) and F3-3 (42 mg, IC_{50} between 2.5 and 5 µg/mL) were combined. Further separation of the combined fractions was carried out by HPLC on a semipreparative C_{18} Varian Dynamax column (5 µm, 10 × 250 mm) eluted with a solvent gradient from CH_3CN/H_2O, 30:70 to 70:30 from 0 to 30 min, to 100:0 from 30 to 40 min, ending with 100% CH_3CN from 40 to 50 min at a flow rate of 2.5 mL/min. This process gave **2.1** (2.9 mg, t_R 35 min), **2.2** (2.8 mg, t_R 36 min,), **2.3** (3.0 mg, t_R 42 min), **2.4** (4.2 mg, t_R 39 min), and **2.5** (6.2 mg, t_R 25 min).

2.3.4 Antiproliferative bioassays

The antiproliferative activities of all fractions and compounds were evaluated by the drug-sensitive A2780 human ovarian cancer cell line assay. [17] In brief, A2780 cells grown to 90-95% confluency were collected and resuspended in growth medium (RPMI1640 supplemented with 10% fetal bovine serum and 2 mM L-glutamine). Cells were counted using a hemacytometer, and a solution containing 2.5×10^5 cells per mL was prepared in growth media. Eleven columns of a 96-well microtiter plate were seeded with 199 μL of cell suspension per well, and the remaining column contained 200 μL media only (no cells). The plate was incubated for 3 h at 37 °C/5%CO_2 to allow the cells to adhere to the wells. Following this incubation, potential antiproliferative agents, prepared in H_2O/DMSO 50:50, were added to the wells to make a series of concentrations at 20 μg/mL, 4 μg/mL, 0.8 μg/mL and 0.16 μg/mL. One column of wells was left with no inhibitor (negative control), and four dilutions of a known compound (paclitaxel or actinomycin D) were included as a positive control. The plate was incubated for 2 days at 37 °C/5% CO_2, then the medium gently shaken from the wells and replaced with reaction medium (supplemented growth medium containing 1% AlamarBlue) and incubated for another 3 h. The level of AlamarBlue converted into a fluorescent compound by living cells was then analyzed using a Cytofluor series 4000 plate reader (Perseptive Biosystems) with an excitation wavelength of 530 nm, an emission wavelength of 590 nm, and gain of 45. The percent inhibition of cell growth was calculated using the 0% and 100% controls present on the plate, and an IC_{50} value (concentration of agent required to inhibit 50% cell growth) was calculated using a linear extrapolation of the data that lay on either side of the 50% inhibition level. Samples were analyzed in triplicate on at least two separate experiments to produce a reliable IC_{50} value.

2.3.5 Antimalarial bioassays

The effect of each fraction and pure compounds on *in vitro* parasite growth of Dd2 strain was measured in a 72 h growth assay in the presence of inhibitor as described previously with minor modifications.[18-19] Ring stage parasite cultures (100 µL per well, with 1% hematocrit and 1% parasitaemia) were grown for 72 h in the presence of increasing concentrations of the inhibitor in a 5.05% CO_2, 4.93% O_2 and 90.2% N_2 gas mixture at 37 ºC. After 72 h in culture, parasite viability was determined by DNA quantitation using SYBR Green I as described previously.[19] The IC_{50} values are the average of three independent determinations with each determination in duplicate, and are expressed ± S.E.M.

2.3.6 Spectroscopic properties

Apoplanesiacarpan A (**2.4**)**:** white powder; $[\alpha]_D^{23}$ −54.8 (c 0.023, MeOH); UV (MeOH) λ_{max} (log ε) 208 (4.7), 234 (4.1), 297 (3.9) nm; CD (MeOH) $[\Delta\varepsilon]_{297\ nm}$ +1.9, $[\Delta\varepsilon]_{234\ nm}$ −14.1; IR (film) ν_{max} 1773, 1523, 1216 and 1080 cm^{-1}; ^1H and ^{13}C NMR data, see Table 2-1; HRESIMS (pos.): *m/z* 331.1188 $[M+H]^+$ (calcd. for $C_{18}H_{19}O_6^+$, 331.1177), 353.0990 $[M+Na]^+$ (calcd. for $C_{18}H_{18}\ NaO_6^+$, 353.0996).

Apoplanesiacarpan B (**2.5**)**:** white powder; $[\alpha]_D^{23}$ −80.2 (c 0.12, MeOH); UV (MeOH) λ_{max} (log ε) 229 (5.1), 238 (5.0), 297 (4.6) nm; CD (MeOH) $[\Delta\varepsilon]_{297\ nm}$ +3.9, $[\Delta\varepsilon]_{234\ nm}$ −65.5; IR (film) ν_{max} 1775, 1613, 1523, 1218 and 1080 cm^{-1}; ^1H and ^{13}C NMR data, see Table 2-1; HRESIMS (pos.): *m/z* 347.1127 $[M+H]^+$ (calcd for $C_{18}H_{19}O_7^+$, 347.1125), 369.0948 $[M+Na]^+$ (calcd for $C_{18}H_{18}NaO_7^+$, 369.0945).

2.3.7 Methylation of *Apoplanesiacarpan* B (**2.5**)

Apoplanesiacarpan B (**2.5**, 2.5 mg) was dissolved in anhydrous acetone (2 mL) and treated with K_2CO_3 (120 mg) and methyl iodide (200 µL). The mixture was stirred at room temperature

for 24 h. The reaction mixture was evaporated and dissolved in water. The aqueous solution was then extracted with EtOAc to yield the methylated product **2.6**, which was further purified on C18 HPLC eluted by 80-100% CH_3CN in H_2O to give 2.0 mg of pure **2.6**. Structure elucidation of compound **2.6** was based on its ^1H NMR data (Table 2-1); HRESIMS (pos.): *m/z* 375.1421 $[M+H]^+$ (calcd for $C_{20}H_{23}O_7^+$, 375.1439), 397.1237 $[M+Na]^+$ (calcd for $C_{20}H_{22}NaO_7^+$, 397.1258).

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Chapter 3: Antiplasmodial phloroglucinol derivative isolated from *Syncarpia glomulifera* (Myrtaceae)

This chapter is a modified and expanded version of a previously published work (Su, Q.; Dalal, S.; Goetz, M.; Cassera, M. B.; Kingston, D. G. I., Antiplasmodial phloroglucinol derivatives from *Syncarpia glomulifera*. *Bioorg. Med. Chem.*, **2016**, *24*, 2544−2548). Contributions of co-authors of the articles are described as follows in the order of the names listed. The author of this dissertation (Qingxi Su) completed the fractionation of the extract and the structural elucidation of the compounds described as well as preparation of the manuscript. Dr. Seema Dalal performed the antimalarial Dd2 bioassay on all fractions and compounds under the guidance of Dr. Maria B. Cassera. Dr. Michael Goetz provided the extract from the Natural Product Discovery Institute (NPDI). Dr. David G. I. Kingston was a mentor for this work and is the corresponding author for the published article.

Figure 3-1. *Syncarpia glomulifera.* Flowers and leaves at Kahakapao Reservoir Haleakala Ranch, Maui - Credit: Forest and Kim Starr - Plants of Hawaii - Image licensed under a Creative Commons Attribution 3.0 License, permitting sharing and adaptation with attribution

3.1 Introduction

As part of the Kingston group's continuing search for bioactive natural products, a MeOH extract from the stem bark of a Hawaiian specimen of *Syncarpia glomulifera* was selected for investigation for potential antiplasmodial natural products, as the crude extract displayed moderate activity against the *Plasmodium falciparum* Dd2 strain (IC_{50} around 1.25 µg/mL). *Syncarpia glomulifera* is a member of the Myrtaceae family, which is known for its high foliar terpene concentrations.[1] Previous studies have reported the isolation of antibacterial and cytotoxic triterpenoids from the bark extract of *S. glomulifera* from Paluma, North Queensland, Australia.[2] However, no work has been reported on the isolation of any antimalarial agent from this species.

Bioassay guided fractionation of a MeOH extract of the stem bark of *Syncarpia glomulifera* (Myrtaceae) led to the isolation of the two new phloroglucinol derivatives (±)-rhodomyrtosone F (**3.1**) and (±)-calliviminone C (**3.2**), the three known triterpenes, betulinic acid (**3.3**), ursolic acid-3-acetate (**3.4**), and ursolic acid (**3.5**), and 1-(2,4,6-trihydroxyphenyl)-1-hexanone (**3.6**) (see Figure 3-2). Compound **3.1** exhibited strong antiplasmodial activity (IC_{50} = 100 ± 20 nM), while compounds **3.2**–**3.4** were moderately active and **3.5** and **3.6** were inactive in this assay. The structures of **3.1** and **3.2** were elucidated based on analyses of their MS data, 1D and 2D NMR spectra, and comparison with related compounds.

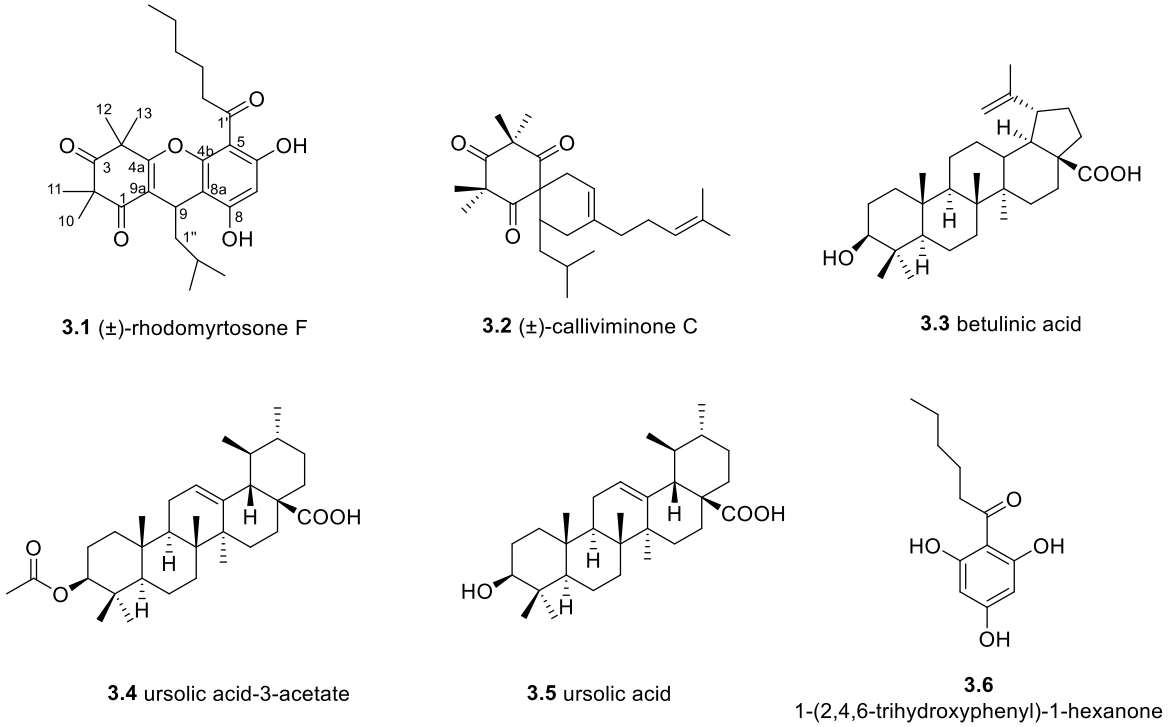
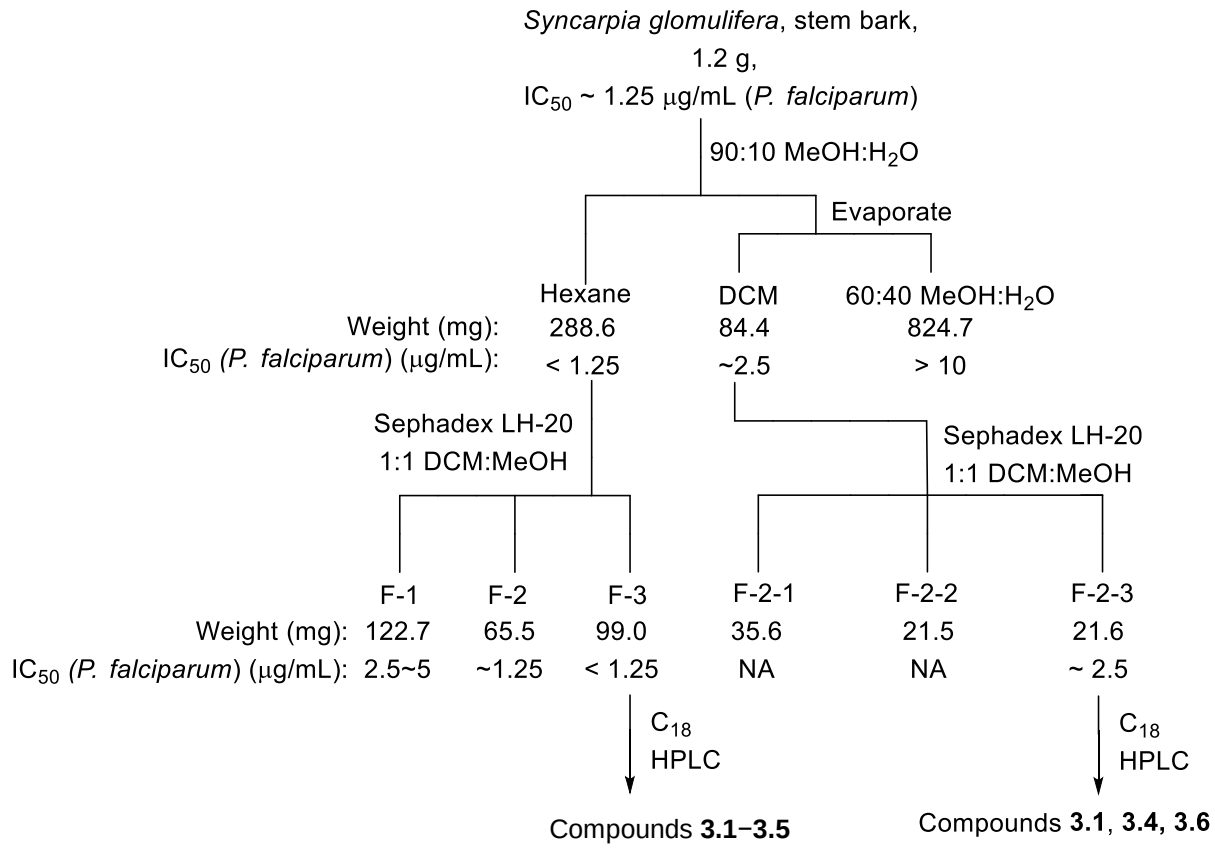


Figure 3-2. Structures of compounds **3.1**–**3.6**

3.2 Results and Discussion

3.2.1 Isolation of active compounds

The methanol extract from the stem bark of *Syncarpia glomulifera* (1.2 g) was selected for investigation based on its moderate antiplasmodial activity (IC_{50} value around 1.25 µg/mL) against the Dd2 drug-resistant strain of *P. falciparum*. Fractionation of the methanol-soluble materials by liquid-liquid partition, open column chromatography on Sephadex LH-20, followed by C18 HPLC yielded two new phloroglucinols, named as (±)-rhodomyrtosone F (**3.1**) and (±)-calliviminone C (**3.2**), along with four known compounds (**3.3**–**3.6**) (Scheme. 3.1).



Scheme 3.1 Bioassay-guided fractionation of *Syncarpia glomulifera*

3.2.2 Structure elucidation of compounds **3.1**

Compound **3.1** was isolated as a yellowish gum. The protonated molecule peak at *m/z* 457.2591 $[M+H]^+$ (calcd for $C_{27}H_{37}O_6^+$, 457.2585) suggested a molecular formula of $C_{27}H_{36}O_6$ with 10 degrees of unsaturation. The ^1H NMR, ^{13}C NMR and HSQC spectra (Table 3-1) indicated the presence of 3 carbonyl, 9 quaternary, 3 methine, 5 methylene and 7 methyl carbons in the structure. The cyclohexene-1,3-dionyl moiety of compound **3.1** was suggested by HMBC correlations of 10- and 11-Me (δ_H 1.36 and 1.39, each 3H, s) to C-1, C2 and C-3 (δ_C 197.6, 56.2 and 212.3), and of 12- and 13-Me (δ_H 1.43 and 1.54, each 3H, s) to C-3, C-4 (δ_C 47.3) and C-4a (δ_C 166.9). In addition H-9 (δ_H 4.24, d, J = 5.6 Hz) correlated to C-1, C-9a (δ_C 114.3) and C-4a (δ_C 166.9). An aromatic ring

was assigned to connect to C-9 due to the deshielded chemical shift of H-9 along with HMBC correlations of H-9 to C-4b, C-8a and C-8 (δ_C 162.6, 106.9 and 155.8). The placement of the aromatic proton H-7 was determined based on its HMBC correlations with C-8a, C-8 and C-6 (δ_C 158.5). C-4a, C-4b, C-6, C-8 were oxygenated due to their low field signals (δ_C 155.8~166.9). The identifications and placements of the acyl and isobutyl groups were confirmed by COSY and HMBC correlations (Figure. 3-2) along with comparison of NMR data with those of related compounds.[3-4] An oxygen atom was assigned to connect C-4a and C-4b to give a tricyclic skeleton, based on the ^{13}C NMR signals of C-4a and C-4b, and on the degree of unsaturation.

Compound **3.1** had an optical rotation of zero, indicating that it was isolated as a racemate. It was thus assigned the structure (±)-4,9-dihydro-6,8-dihydroxy-2,2,4,4-tetramethyl-5-hexanoyl-9-isobutyl-1*H*-xanthene-1,3(2*H*)-dione and named as (±)-rhodomyrtosone F based on its similarity to rhodomyrtone[5] and rhodomyrtosones B[4] and E.[6]

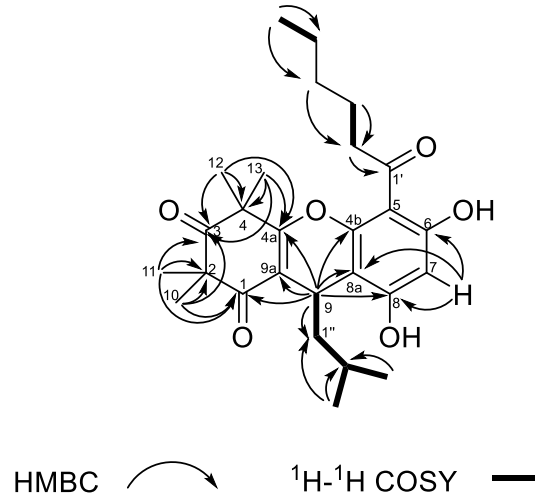


Figure 3-2. Key HMBC and COSY correlations of compound **3.1**.

3.2.3 Structure elucidation of compound **3.2**

Compound **3.2** was isolated as light yellow gum. The protonated molecule peak at *m/z* 387.2896 $[M+H]^+$ (calcd for $C_{25}H_{39}O_3^+$, 387.2894) suggested a molecular formula of $C_{25}H_{38}O_3$ with 7 degrees of unsaturation. The ^1H NMR, ^{13}C NMR and HSQC spectra of **3.2** showed 3 carbonyl, 5 quaternary, 4 methine, 5 methylene and 8 methyl carbons in the structure. The structure of compound **3.2** was elucidated to contain a 1,3,5-cyclohexanetrione moiety based on HMBC correlations of 14- and 15-Me (δ_H 1.33 and 1.39, each 3H, s) to C-1, C-2 and C-3 (δ_C 208.3, 56.3 and 213.1), of 12- and 13-Me (δ_H 1.37 and 1.39, each 3H, s) to C-3, C-4 and C-5 (δ_C 213.1, 56.9, 208.3), and of 13- and 15-Me to C-6 (δ_C 67.7). Additional HMBC correlations of H-7a (δ_H 2.17, 1H, m) to C-6, C-8 and C-9 (δ_C 67.7, 116.1 and 136.7); H-7b (δ_H 2.49, 1H, brd, J = 17.2 Hz) to C-1, of H-10a (δ_H 2.13, 1H, m) to C-6, C-8 and C-9, and of H-11 (δ_H 2.31, 1H, m) to C-5 and C-6 indicated a spiro-[5,5]-undec-8-ene skeleton. The presence of an isobutyl group was determined by the cross peaks between H-1″ (δ_H 0.78 and 1.39, each 1H, m) to H-2″ (δ_H 1.63, 1H, m), and of H-2″ to 3″-Me (δ_H 0.87, 3H, d, J = 7.0 Hz) and 4″-Me (δ_H 0.87, d, J = 7.0 Hz) in the ^1H-^1H COSY spectra. The placement of the isobutyl group was confirmed by a ^1H-^1H COSY correlation of H-1″a (δ_H 0.78, 1H, m) to H-11. The presence of the 4-methylpentene unit was established by HMBC data. Key HMBC correlations include H-1′ (δ_H 1.93, 2H, m) to C-2′ (δ_C 26.1); H-2′ (δ_H 2.03, 2H, m) to C-3′ (δ_C 124.1); 5′-Me (δ_H 1.67, 3H, d, J = 1.4 Hz), 6′-Me (δ_H 1.59, 3H, s) to C-3′ and C-4′ (δ_C 131.7). The 4-methyl-pentene unit was placed at C-9 on the basis of ^3J HMBC coupling between H-1' and C-10 (δ_C 30.7). Compound **3.2** was a racemate at C-11 based on its zero optical rotation, and its structure was thus elucidated as (±)-2,2,4,4-tetramethyl-11-isobutyl-9-(4-methyl-3-penten-1-yl)- spiro[5.5]undec-8-ene -1,3,5-trione, and named as (±)-calliviminone C, based on its similar structure to (±)-calliviminones A and B.[7]

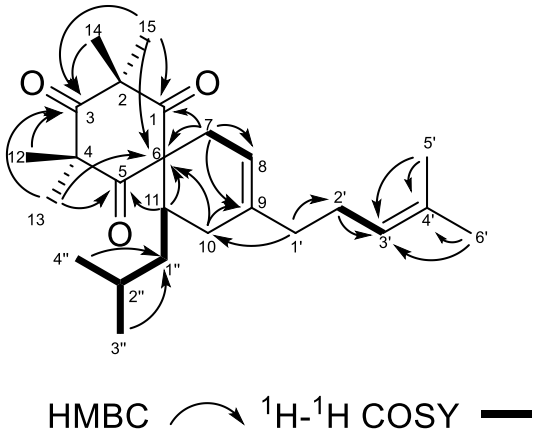


Figure 3-3. Key HMBC and COSY correlations of compound **3.2.**

3.2.4 Structure elucidation of compounds **3.3**−**3.6**

Structures of compounds **3.3**−**3.6** were elucidated as the four known compounds betulinic acid (**3.3**),[8] ursolic acid-3-acetate (**3.4**),[9] ursolic acid (**3.5**),[9] and 1-(2,4,6-trihydroxyphenyl)-1-hexanone (**6**),[10] by comparison of their ^{1}H NMR and mass spectrometric data with literature data.[8-10]

Table 3-1. ^{1}H and ^{13}C NMR Data (δ, ppm) for compounds **3.1**- **3.2** (500 and 125 MHz) in $CDCl_3$

	3.1			**3.2**	
Position	δ_H, (*J* in Hz)[a]	δ_C, type[b]	Position	δ_H, (*J* in Hz)[a]	δ_C, type[b]
1		197.6, C	1		208.3, C
2		56.2, C	2		56.3, C
3		212.3, C	3		213.1, C
4		47.3, C	4		56.9, C
4a		166.9, C			
4b		162.6, C			
5		107.5, C	5		208.3, C
6		158.5, C	6		67.7, C
7	6.06 s	95.0, CH	7	2.49 brd (17.2), 2.17 m	29.5, CH_2
8		155.8, C	8	5.31 m	116.1, CH
8a		106.9, C			
9	4.24, t (5.6)	25.3, CH	9		136.7, C
9a		114.3, C			
10	1.36 s	24.3, CH_3	10	2.13 m, 1.98 m	30.7, CH_2
11	1.39 s	24.7, CH_3	11	2.31 m	34.1, CH
12	1.43 s	24.7, CH_3	12	1.37 s	26.5, CH_3
13	1.54 s	24.9, CH_3	13	1.39 s	26.3, CH_3
			14	1.33 s	24.5, CH_3
			15	1.39 s	25.8, CH_3
1′		206.9, C	1′	1.93 m	37.3, CH_2
2′	3.09 t (7.2)	44.6, CH_2	2′	2.03 m	26.1, CH_2
3′	1.71 m	24.4, CH_2	3′	5.03 tq (7.0, 1.4)	124.1,CH
4′	1.36 m	31.8, CH_2	4′		131.7, C
5′	1.36 m	22.7, CH_2	5′	1.67 d (1.4)	25.8, CH_3
6′	0.91 t (7.2)	14.2, CH_3	6′	1.59 s	17.9, CH_3
1″	1.43 m	46.0, CH_2	1″	1.39 m, 0.78 m	39.2, CH_2
2″	1.35 m	25.3, CH	2″	1.63 m	25.6, CH
3″	0.84 d (6.0)	23.3, CH_3	3″	0.87 d (7.0)	24.4, CH_3
4″	0.87 d (6.0)	23.6, CH_3	4″	0.85 d (7.0)	21.1, CH_3

[a] Data (δ) measured at 500 MHz; s = singlet, br s = broad singlet, d = doublet, dd = doublet of doublets, ddd = doublet of doublets of doublets, dt = doublet of triplets, m = multiplet. *J* values are in Hz and are omitted if the signals overlapped as multiplets. The overlapped signals were assigned from HSQC and HMBC spectra without designating multiplicity.
[b] Data (δ) measured at 125 MHz; CH_3, CH_2, CH, and C multiplicities were determined by HSQC experiments.

3.2.5 Bioactivities

Compounds **3.1**−**3.6** were evaluated for their antimalarial activity against the drug-resistant Dd2 strain of *P. falciparum* (Table 3-2). Compound **3.1** exhibited the strongest activity with an IC_{50} of 100 ± 20 nM. Acylphloroglucinols with similar structures to compound **3.1** have been reported to show antibacterial activity,[11-12] and examples of phloroglucinol derivatives as antimalarial agents can be found in the literature,[13-14] but compound **3.1** is an order of magnitude more potent than tomentosone A,[14] the closest analog with reported antimalarial activity. Compound **3.1** was also tested for cytotoxicity toward human embryonic kidney cells (HEK) and no toxicity was detected up to 3.125 μM while 58% inhibition was observed at the highest dose tested (50 μM). Compound **3.2** contains an unusual spiro-[5,5]-undec-8-ene skeleton. The first examples of such carbon Diels-Alder adducts between a phloroglucinol and a terpenoid were (±)-calliviminones A and B, isolated from *Callistemon viminalis*.[7] Compound **3.2** exhibited moderate antiplasmodial activity (IC_{50} 4 ± 1 μM), which is the first reported antiplasmodial activity of a phloroglucinol with the spiro-[5,5]-undec-8-ene skeleton. Compound **3.2** did not show toxicity up to the highest dose tested (100 μM). Betulinic acid (**3.3**) showed moderate inhibitory effect on the Dd2 strain of *P. falciparum*, and it has previously been reported to inhibit the 3D7 stain of *P. falciparum*, a chloroquine-sensitive strain.[15] Ursolic acid (**3.5**) and its 3-acetate (**3.4**) have been reported to be antibacterial and antioxidant agents,[16] but these compounds were inactive against the Dd2 strain of *P. falciparum*, as previously observed.[17] Compound **3.6**, 1-(2,4,6-trihydroxyphenyl)-1-hexanone, is a possible biosynthetic precursor of **3.1**; it was inactive against *P. falciparum* at the highest dose tested (90 μM).

Table 3-2. Antiplasmodial activity data of compounds **3.1**−**3.6**

Compound	*P. falciparum* Dd2 strain IC_{50} (μM)
(±)-Rhodomyrtosone F (**3.1**)	0.10 ± 0.02
(±)-calliviminone C (**3.2**)	4 ± 1
Betulinic acid (**3.3**)	3.0 ± 0.4
Ursolic acid-3-acetate (**3.4**)	12.0 ± 0.1
Ursolic acid (**3.5**)	Not active (> 40)
1-(2,4,6-trihydroxyphenyl)-1-hexanone (**3.6**))	Not active (> 90)
Artemisinin (positive control)	6 ± 1

3.3 Experimental Section

3.3.1 General experimental procedures

UV spectra were measured on a Shimadzu UV-1201 spectrophotometer. IR spectra were measured on a MIDAC M-series FTIR spectrometer. NMR spectra were recorded in $CDCl_3$ on a Bruker Avance 500 spectrometer in $CDCl_3$; chemical shifts are given in δ (ppm), and coupling constants are reported in Hz. Mass spectra were obtained on an Agilent 6220 LC-TOF-MS in the positive ion mode. Optical rotations were recorded on a JASCO P-2000 polarimeter. Open column chromatography was performed using Sephadex LH-20 (I.D.×L 3×50 cm). Semi-preparative HPLC was performed on a Shimadzu LC-10AT instrument with a semipreparative C_{18} Phenomenex Luna column (5 μm, 250×10 mm). All isolated compounds were evaluated for purity by HPLC (both UV and ELSD detection) and by NMR before bioactivity assay. Purity of each compound was checked on a Phenomenex Luna C_{18} column (5 μm, 250×10 mm) and a Cogent Bidentate C_{18} column (4 μm, 76×4.6 mm). All isolated compounds were purified to 95% purity or better, as judged by HPLC (both UV and ELSD detection) and ^{1}H NMR spectroscopies before determining bioactivity.

3.3.2 Plant material

Stem bark from *S. glomulifera* (Sm.) Nied was collected by Gary J. Ray in Hawaii in June 1998 near Pupukea Road, Pupukea (21.38.39 N, 158.00.54 W). A specimen is on deposit at the New York Botanical Garden under the accession number GR01020c (ID 89558).

3.3.3 Extraction and isolation

Dried, powdered plant material was exhaustively extracted with MeOH at room temperature to give an extract designated 0040244-10F; a total of 3 g of this extract was made available to Virginia Tech and 1.2 g of the material was used in the bioassay guided fractionation. Extract 0040244-10F (1200 mg, IC_{50} around 1.25 µg/mL, tested in antimalarial assay) was suspended in aqueous MeOH (MeOH/H_2O 9:1, 100 mL) and extracted with hexane (5×100 mL portions) to give an active hexane-soluble fraction (288.6 mg, IC_{50} < 1.25 µg/mL). The aqueous fraction was then diluted to MeOH/H_2O 6:4 (150 mL) and further extracted with CH_2Cl_2 (5×100 mL portions) to yield the CH_2Cl_2-soluble fraction (84.4 mg, IC_{50} approximately 2.5 µg/mL) and the aqueous fraction (824.7 mg, IC_{50} > 10 µg/mL). The active hexane fraction was then subjected to a size exclusion open column chromatography on Sephadex LH-20 (I.D×L 3×50 cm) eluted with CH_2Cl_2/MeOH 1:1. Three fractions (F1-1: 122.7 mg, F1-2: 65.5mg, F1-3: 99.0 mg) were collected based on TLC analyses. The active fraction F1-3 (IC_{50} < 1.25 µg/mL) was further separated by HPLC on a semipreparative C_{18} column (Phenomenex Luna column, 5 µm, 250 X10 mm) eluted with a solvent gradient from CH_3OH/H_2O, 75:25 to 85:15 from 0 to 10 min, to 95:5 from 10 to 20 min, ending with 100% CH_3OH from 20 to 40 min at a flow rate of 2.5 mL/min. This process gave compounds **3.3** (4.8 mg, t_R 23.5 min), **3.4** (4.2 mg, t_R 31 min), and crude mixtures containing compound **3.1** (t_R 26 min) and **3.2** (t_R 27.5 min). The collected mixture containing compound **3.1** (5.1 mg) was then further purified by HPLC on the same column eluted

with a solvent gradient from CH_3CN/H_2O, 85:15 to 90:10 from 0 to 15 min, to 100:0 from 15 to 25 min, ending with 100% CH_3CN from 25 to 40 min at a flow rate of 2.5 mL/min. This process gave compounds **3.1** (3.6 mg, t_R 28 min) and **3.5** (1.3 mg, t_R 26 min). Mixture containing compound **3.2** (2.0 mg) was also purified by HPLC on the semipreparative C_{18} column with aqueous acetonitrile as above. This process gave purified compound **3.2** (1.4 mg, t_R 36 min).

The moderately active dichloromethane fraction was also subjected to the size exclusion open column using the method described above, to yield three sub-fractions (F2-1: 35.6 mg, F2-2: 21.5 mg, F2-3: 21.6 mg). Fraction F2-3 (IC_{50} around 2.5 µg/mL) was separated by HPLC on the Luna C_{18} column, eluted with a solvent gradient from CH_3OH/H_2O, 60:40 to 70:30 from 0 to 10 min, to 90:10 from 10 to 30 min, ending with 100% CH_3OH from 30 to 40 min at a flow rate of 2.5 mL/min. This process yielded compound **3.6** (2.1 mg, t_R 24.5 min) and small amount of compound **3.1** (0.2 mg, t_R 33.0 min) and compound **3.4** (0.4 mg, t_R 41.5 min).

3.3.4 Antimalarial bioassays

The effect of each fraction and pure compounds on in vitro parasite growth of Dd2 strain was measured in a 72 h growth assay in the presence of inhibitor as described previously with minor modifications.[18-19] Ring stage parasite cultures (100 µL per well, 1% hematocrit and 1% parasitaemia) were grown for 72 h in the presence of increasing concentrations of the inhibitor in a humidified chamber at 37 °C and low oxygen conditions (5.06% CO_2, 4.99% O_2, and 89.95% N_2). After 72 h in culture, parasite viability was determined by DNA quantitation using SYBR Green I as described previously.[19] The IC_{50} values were calculated with KaleidaGraph software using a nonlinear regression curve fitting. IC_{50} values are the average of three independent determinations with each determination in duplicate, and are expressed ± S.E.M. Artemisinin was used as the positive control with an IC_{50} of 6 ± 1 nM.

3.3.5 In vitro cytotoxicity against HEK293 cells

Compounds were evaluated for their cytotoxicity against normal cell line HEK293 (Human Embryonic Kidney). Briefly, 10,000 HEK cells per well were plated in a clear bottom 96 well plate. After allowing the cells to adhere, the media was replaced with 100 µL of media containing varying amounts of the test compound and incubated for 24 hours. Later, 10 µL of resazurin sodium salt (Sigma) at 0.125 mg/mL was added to each well and incubated for 2 h. Cell viability was determined by measuring the fluorescence at 585 nm after excitation at 540 nm.

3.3.6 Spectroscopic properties

(±)-Rhodomyrtosone F (**3.1**): yellowish gum; $[\alpha]_D^{23}$ 0 (*c* = 0.22, MeOH); UV (MeOH) λ_{max} (log ε) 223 (3.31), 261 (3.01), 292 (2.51), 333 (1.40) nm; IR (film) ν_{max} 2935, 2872, 1721, 1629, 1430, 1389, 1108 and 1091 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 3-1; HRESIMS (pos.): *m/z* 457.2591 $[M+H]^+$ (calculated for $C_{27}H_{37}O_6^+$, 457.2585); 474.2831 $[M+NH_4]^+$ (calcd for $C_{27}H_{40}NO_6^+$, 474.2851).

(±)-Calliviminone C (**3.2**): yellowish gum; $[\alpha]_D^{23}$ 0 (*c* = 0.15, MeOH); UV (MeOH) λ_{max} (log ε) 214 (3.53), 297 (2.67); IR (film) ν_{max} 2357, 2335, 2146 and 1699 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 3-1; HRESIMS (pos.): *m/z* 387.2896 $[M+H]^+$ (calcd for $C_{25}H_{39}O_3^+$, 387.2894).

3.4 References

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Chapter 4: New Antiplasmodial Diterpenes from *Gutierrezia sarothrae*

This chapter is a modified and expanded version of a previously published work (Su, Q.; Dalal, S.; Goetz, M.; Cassera, M. B.; Kingston, D. G. I., New Antiplasmodial diterpenes from *Gutierrezia sarothrae. Nat. Prod. Commun.* **2016**, *11*, 719−721). Contributions of co-authors of the articles are described as follows in the order of the names listed. The author of this dissertation (Qingxi Su) completed the fractionation of the extract and the structural elucidation of the compounds described as well as preparation of the manuscript. Dr. Seema Dalal performed the antimalarial Dd2 bioassay on all fractions and compounds under the guidance of Dr. Maria B. Cassera. Dr. Michael Goetz provided the extract from the Natural Product Discovery Institute (NPDI). Dr. David G. I. Kingston was a mentor for this work and is the corresponding author for the published article.

Figure 4-1. *Gutierrezia sarothrae.* Used with permission from David Stang (zipecodezoo.com). Retrieved from < http://tropicos.org/Image/58007>.

4.1 Introduction

In continuation of our investigation of antimalarial agents from plants,[1-2] a MeOH extract from the plant *Gutierrezia sarothrae* (Asteraceae) was evaluated for antiproliferative activity against drug-resistant Dd2 strain of *Plasmodium falciparum*. The extract was selected for bioassay−guided fractionation based on its initial weak activity against this assay (IC_{50} between 5 and 10 μg/mL). The family Asteraceae (or Compositae) contains many perennial herbs as well as subshrubs that are important weeds on rangelands.[3] *Gutierrezia sarothrae*, a member of the Asteraceae family, is widely distributed in rangelands of western North America.[4] The plant has been used externally to treat bee stings, snake bites, flesh wounds,[5] and rheumatism.[6] Previous studies have suggested the plant could contain monoterpenes,[7] volatile components such as cryptone and *β*-eudesmol,[4] flavonoids,[8] and labdane derivatives.[9-10] An antitumor proteinaceous substance has also been reported to exist in the plant.[11] However, the plant has not previously been studied for potential antimalarial constituents.

Bioassay-guided fractionation afforded the two new labdane derivatives 3α-angeloyloxy-15-hydroxylabda-7,13-dien-16,15-olid-18-oic acid (**4.1**) and 3α-angeloyloxy-15-methoxylabda-7,13-dien-16,15-olid-18-oic acid (**4.2**). The structures of **4.1** and **4.2** were elucidated by interpretation of 1D and 2D NMR spectroscopic data, mass spectrometry, and comparison with the data of related compounds reported in the literature. We report herein the bioassay-guided isolation and structure elucidation of the two new compounds, as well as their antimalarial activity.

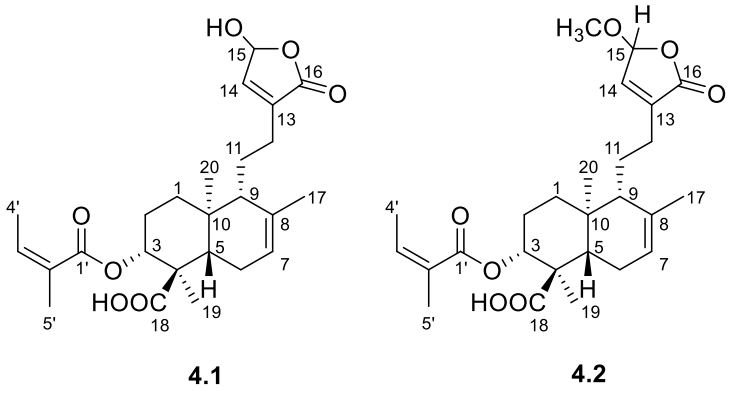
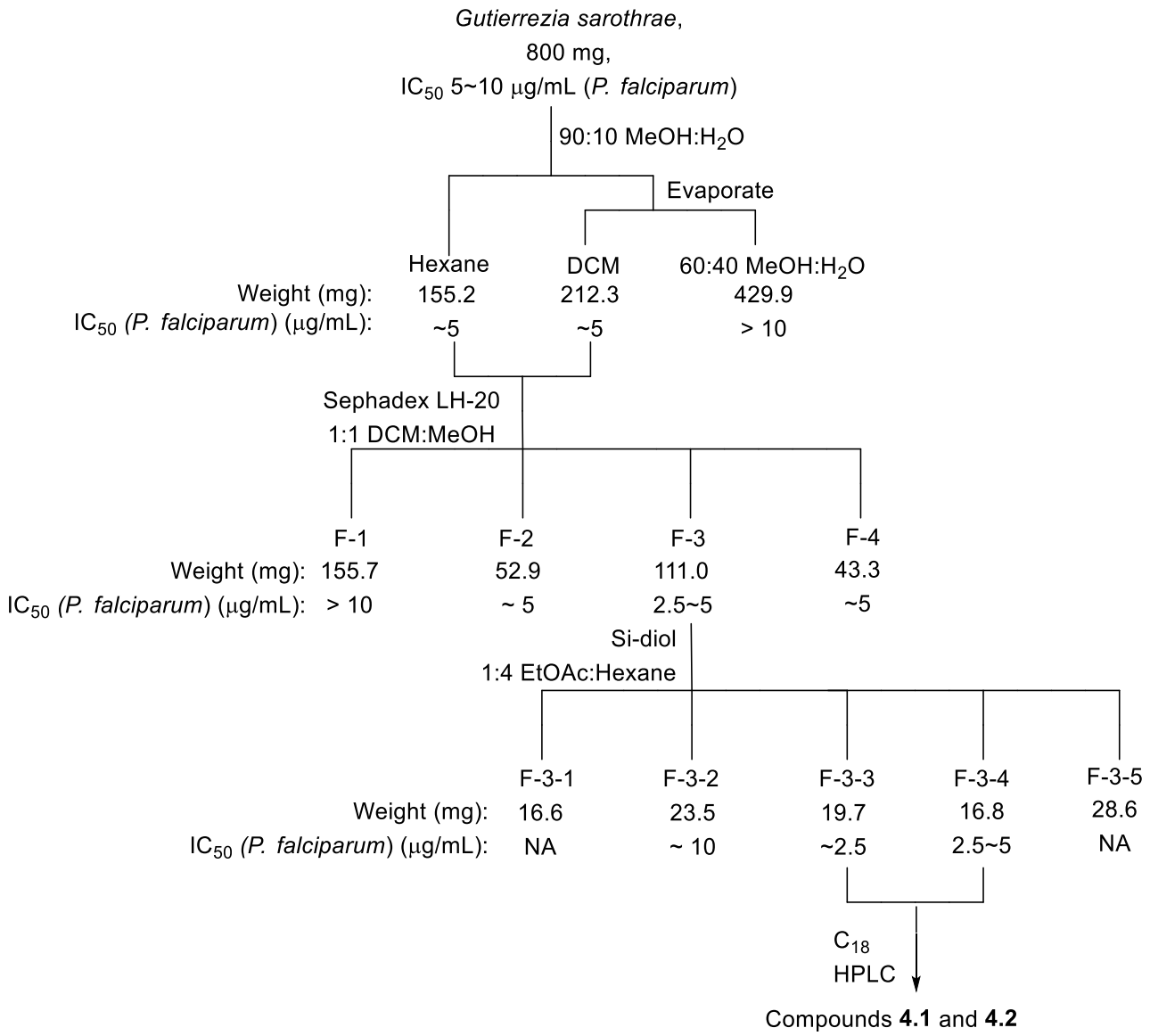


Figure 4-2. Structures of compounds **4.1** and **4.2** isolated from *G. sarothrae*

4.2 Results and Discussion

4.2.1 Isolation of active compounds

The methanol extract from the plant of *Gutierrezia sarothrae* (800 g) was selected for investigation based on its moderate antiplasmodial activity (IC_{50} between 5 to 10 µg/mL) against the Dd2 drug-resistant strain of *P. falciparum*. The methanol-soluble extract of *G. sarothrae* was suspended in MeOH/H_2O 9:1 and extracted with hexane. The aqueous methanol was then diluted to MeOH/H_2O 6:4 and extracted by CH_2Cl_2. The active hexane and CH_2Cl_2 fractions were combined with IC_{50} around 5 µg/mL. The combined fraction was then subjected to open column chromatography on Sephadex LH-20 followed by normal phase diol open column chromatography. The most active fraction from the diol column was subjected to semi-preparative C_{18} HPLC to yield two new diterpenes (**4.1** and **4.2**). The fractionation tree is shown in Scheme 4-1 and a detailed description of the isolation procedure is given in the Experimental Section.



Scheme 4-1 Bioassay-guided separation of *Gutierrezia sarothrae*

4.2.2 Structure elucidation of compound **4.1**

Compound **4.1** was obtained as colorless oil and had the molecular formula $C_{25}H_{34}O_7$ as indicated by HRESIMS analysis (*m/z* 469.2218 $[M+Na]^+$, calcd. for $C_{25}H_{34}O_7Na^+$, 469.2197;

915.4543 $[2M+Na]^+$, calcd. for $C_{50}H_{68}O_{14}Na^+$, 915.4501). Its ^1H NMR spectrum (Table 4-1) indicated the presence of five methyl groups including three singlets (δ_H 0.82, 1.30 and 1.72, each 3H) and two multiplets (δ_H 1.93, dq, *J* = 7.0, 1.5 Hz, 3H and 1.83, m, 3H); five downfield protons between δ 5.23 and 6.88; and a number of multiplets between δ 1.30 and 2.55. The ^{13}C NMR (Table 4-1) and HSQC spectra of **4.1** indicated the presence of 3 carbonyl groups, 5 quaternary, 7 methine, 5 methylene and 5 methyl carbons. The labdane diterpene skeleton was established based on HMBC correlations of Me-17 (δ_H 1.72, s, 3H) to C-7, C-8 and C-9 (δ_C 121.9, 134.7 and 54.0); Me-19 (δ_H 1.30, s, 3H) to C-3, C-4 and C-5 (δ_C 77.7, 51.2 and 46.1); Me-20 (δ_H 0.82, s, 3H) to C-1, C-5 and C-9 (δ_C 36.2, 46.1 and 54.0) (Figure 4-2). A carboxylic acid group was assigned to the C-3 position due to the HMBC correlation between Me-19 and C-18 (δ_C 179.2). Further examination of its ^1H NMR spectrum revealed characteristic signals of an angeloyl group. An olefinic proton (δ_H 6.03, qq, *J* = 7.0, 1.5 Hz, 1H), two methyl groups (δ_H 1.93, dq, *J* = 7.0, 1.5 Hz, 3H) and 1.83, m, 3H), and the corresponding carbons (δ_C 167.3, 128.1, 138.3, 15.9 and 20.7) were assigned unambiguously to C-1' – C-5' based on HSQC and HMBC correlations. The HMBC spectrum also established correlations between the C-12 methylene protons (δ_H 2.55, m and 2.30, m, each 1H) to C-13, C-14 and C-16 (δ_C 138.4, 143.6 and 171.3), and from the oxygenated H-15 (δ_H 6.12, s, 1H) to C-13 and H-14 (δ_H 6.88, s, 1H), to C-15 (δ_C 96.7). These correlations suggested the presence of a hydroxyl-furanone moiety, as shown in Figure 4-2. The relative configuration of compound **4.1** was confirmed by NOESY correlations between 3-H to 5-H, Me-19 to Me-20, and Me-20 to 11-H (Figure 4-2). The spectroscopic data showed satisfactory agreement with those recorded for related labdane derivatives by Bohlmann *et al.* [10]

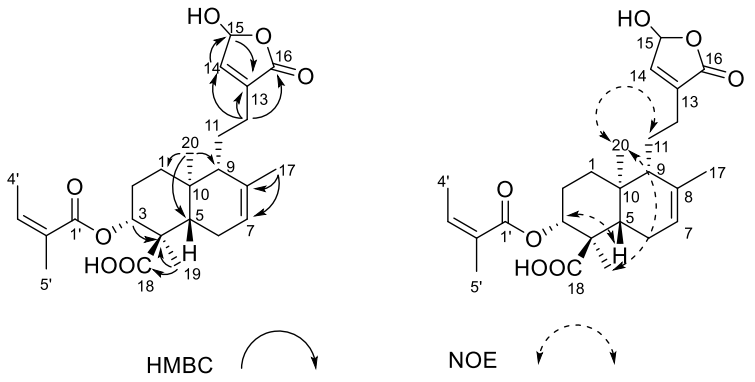


Figure 4-2. HMBC (left) and NOESY (right) correlations of compound **4.1**

4.2.3 Structure elucidation of compound **4.2**

Compound **4.2** was obtained as a colorless oil and had the molecular formula $C_{26}H_{36}O_7$ as indicated by HRESIMS analysis (*m/z* 461.2560 $[M+H]^+$, calcd. for $C_{26}H_{37}O_7^+$, 461.2534; 483.2361 $[M+Na]^+$, calcd. for $C_{26}H_{36}NaO_7^+$, 483.2354). Examination of its ^1H NMR spectroscopic data indicated that compound **4.2** was similar to compound **4.1**, except for the presence of a methoxy group in place of a hydroxyl group. Placement of the methoxy group was determined by an HMBC correlation between the methoxy group and C-15. Examination of the NOESY correlations of compound **4.2** suggested its relative configuration to be identical to that of compound **4.1**. Compound **4.2** was obtained as a mixture of C-15 epimers that were not separable by HPLC, resulting in two sets of signals for C-13, C-14, C-15 and -OMe.[12] Compound **4.2** might possibly be an artefact formed by reaction of **4.1** with MeOH during the extraction of the plant material. However, we were unable to collect fresh plant material and extract it with a different solvent to confirm this hypothesis.

Table 1. ^{1}H and ^{13}C NMR Data (δ, ppm) for compounds **4.1**−**4.2** in $CDCl_3$

	4.1		**4.2**	
Position	δ_H[a]	δ_C[b]	δ_H[a]	δ_C[b]
1	1.97 m, 1.36 m	36.7, CH_2	1.93 m, 1.31m	36.7, CH_2
2	1.93 m, 1.68 m	25.1, CH_2	2.05 m, 1.65 m	25.2, CH_2
3	5,23 dd (11.9, 4.0)	77.7, CH	5.27 dd (12.0, 3.7)	77.7, CH
4	-	51.2, C	-	51.1, C
5	2.05 m	46.1, CH	2.02 m	46.1, CH
6	1.95 m, 2.07 m	23.6, CH_2	1.84 m, 1.68 m	23.8, CH_2
7	5.36 brs	121.9, CH	5.35 brs	122.1, CH
8	-	134.7, C	-	134.6, C
9	1.79 m	54.0, CH	1.75 m	54.2, CH
10	-	36.2, C	-	36.2, C
11	1.74 m, 1.49 m	25.0, CH_2	1.69 m, 1.48 m	25.0, CH_2
12	2.55 m, 2.30 m	27.4, CH_2	2.54 m, 2.27 m	27.5, CH_2
13	-	138.4, C	-	138.7/ 138.8, C
14	6.88 s	143.6, CH	6.80 s	142.1/ 142.2, CH
15	6.12 s	96.7, CH	5.75 d (1.3)	102.6/ 102.7, CH
16	-	171.3, C	-	171.4, C
17	1.72 s	22.1, CH_3	1.71 s	22.1, CH_3
18	-	179.2, C	-	180.2, C
19	1.30 s	12.0, CH_3	1.27 s	12.1, CH_3
20	0.82 s	14.1, CH_3	0.81 s	14.0, CH_3
1'	-	167.3, C	-	167.5, C
2'	-	128.1, C	-	128.1, C
3'	6.03 qq (7.0, 1.5)	138.3, CH	6.02 qq (7.0, 1.5)	138.3, CH
4'	1.93 dq (7.0, 1.5)	15.9, CH_3	1.93 brd (7.5)	15.9, CH_3
5'	1.83 m	20.7, CH_3	1.82 brs	20.8, CH_3
-OMe			3.59 s	57.35/57.32, CH_3

[a] Data (δ) measured at 500 MHz; s = singlet, br s = broad singlet, d = doublet, dd = doublet of doublets, ddd = doublet of doublets of doublets, dt = doublet of triplets, m = multiplet. *J* values are in Hz and are omitted if the signals overlapped as multiplets. The overlapped signals were assigned from HSQC and HMBC spectra without designating multiplicity.
[b] Data (δ) measured at 125 MHz; CH_3, CH_2, CH, and C multiplicities were determined by HSQC experiments.

4.2.4 Bioactivities

Compounds **4.1** and **4.2** were evaluated for their antimalarial activity against the chloroquine/mefloquine-resistant Dd2 strain of *P. falciparum*. Compound **4.1** exhibited moderate antiplasmodial activity with an IC_{50} value of 10 ± 4 μM in this assay, while compound **4.2** was inactive at doses below 20 μg/mL. The activity of compound **4.1** might thus be associated with its ring-opened α, β-unsaturated aldehyde form, since **4.2** is incapable of ring opening under mild

conditions. Unfortunately, some biological activities were lost after fractionation by Si-diol open column chromatography (see scheme 4-1).

4.3 Experimental Section

4.3.1 General experimental procedures

Optical rotations were recorded on a JASCO P-2000 polarimeter. UV spectra were measured on a Shimadzu UV-1201 spectrophotometer. IR spectra were measured on a MIDAC M-series FTIR spectrometer. NMR spectra were recorded in $CDCl_3$ on a Bruker Avance 500 spectrometer. Chemical shifts are given in δ (ppm), and coupling constants are reported in Hz. Mass spectra were obtained on an Agilent 6220 LC-TOF-MS in the positive ion mode. Open column chromatographies were performed using Sephadex LH-20 (I.D.× L 3×50 cm) and Si-diol (I.D.×L 2.5×35 cm, 40-63 μm). HPLC separation was performed on a Shimadzu LC-10AT instrument with a semipreparative C18 Phenomenex Luna column (5 μm, 250 ×10 mm).

4.3.2 Plant material

Leaves and stems of *G. sarothrae* Kuntze were collected on the edge of a lake near Saskatoon, Canada (52.10N, 106.40W) by Cori M. Morenberg under the auspices of the New York Botanical Garden. A voucher specimen is on deposit under accession number CM00068a.

4.3.3 Extraction and isolation

Dried, powdered plant material was exhaustively extracted with MeOH to give a MeOH-soluble extract designated X-5689; a total of 800 mg of this extract was made available to Virginia Tech. This extract (IC_{50} between 5 and 10 μg/mL against *P. falciparum* Dd2 strain) was suspended in aqueous MeOH (MeOH/H_2O 9:1, 100 mL) and extracted with hexane (5×100 mL portions).

Evaporation of the solvent afforded 155.2 mg of hexane soluble materials. The aqueous fraction was then diluted to MeOH/H_2O 6:4 (150 mL) and further extracted with CH_2Cl_2 (5×100 mL portions) to yield 212.3 mg of CH_2Cl_2 fraction and 429.9 mg of aqueous fraction. The active hexane and CH_2Cl_2 fractions (IC_{50} around 5 µg/mL) were combined and subjected to size exclusion open column chromatography (I.D.×L 3×50 cm) on Sephadex LH-20 eluted with CH_2Cl_2/MeOH 1:1. Four fractions (F1: 155.7 mg, F2: 52.9 mg, F3: 111.0 mg, F4: 43.3 mg) were collected based on TLC profile. The most active fraction F3 (IC_{50} between 2.5~5 µg/mL) was then subjected to Si-diol open column chromatography (I.D.×L 2.5×35 cm, 40−63 µm) eluted with EtOAc/Hexane 1:4 to give five subfractions indexed F3-1 (16.6 mg), F3-2 (23.5 mg), F3-3 (19.7 mg), F3-4 (16.8 mg) and F3-5 (28.6 mg). Fractions F3-3 (IC_{50} around 2.5 µg/mL) and F3-4 (IC_{50} between 2.5~5 µg/mL) were combined and further separated by HPLC on a semipreparative C_{18} column (Phenomenex Luna column, 5 µm, 250×10 mm) eluted with a solvent gradient from CH_3CN/H_2O, 50:50 to 60:40 from 0 to 10 min, to 100:0 from 10 to 30 min, ending with 100% CH_3CN from 30 to 40 min at a flow rate of 2.5 mL/min. This process gave compounds **4.1** (2.3 mg, t_R 12.5 min) and **4.2** (1.2 mg, t_R 22.0 min).

4.3.4 Antimalarial bioassays

The effect of each fraction and pure compounds on *in vitro* parasite growth of Dd2 strain was measured in a 72 h growth assay in the presence of inhibitor as described previously with minor modifications.[13-14] Ring stage parasite cultures (100 µL per well, with 1% hematocrit and 1% parasitaemia) were grown for 72 h in the presence of increasing concentrations of the inhibitor in a 5.05% CO_2, 4.93% O_2 and 90.2% N_2 gas mixture at 37 ^oC. After 72 h in culture, parasite viability was determined by DNA quantitation using SYBR Green I as described previously.[14] The half-maximum inhibitory concentration (IC_{50}) values were calculated with KaleidaGraph software

using a nonlinear regression curve fitting. IC_{50} values are the average of three independent determinations with each determination in duplicate, and are expressed ± S.E.M. Artemisinin was used as the positive control with an IC_{50} of 6 ±1 nM.

4.3.5 Spectroscopic properties

3α-Angeloyloxy-15-hydroxylabda-7,13-dien-16,15-olid-18-oic acid (**4.1**): Colorless oil, $[\alpha]_D^{23}$: +21.7 (c 0.037, MeOH); UV (MeOH) λ_{max} (log ε) 221 (4.83); IR (film) ν_{max} 3332, 1657, 1451, 1024 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): Table 4-1. ^{13}C NMR (125 MHz, $CDCl_3$): Table 4-1. HRESIMS (pos.): *m/z* $[M+Na]^+$, calcd. for $C_{25}H_{34}O_7Na^+$: 469.2197, found: 469.2218; $[2M+Na]^+$, calcd. for $C_{50}H_{68}O_{14}Na^+$, 915.4501, found: 915.4543.

3α-Angeloyloxy-15-methoxylabda-7,13-dien-16,15-olid-18-oic acid (**4.2**): Colorless oil. $[\alpha]_D^{23}$: +16.5(c 0.030, MeOH); UV (MeOH) λ_{max} (log ε) 218 (4.89); IR (film) ν_{max} 3324, 1641, 1452, 1017 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): Table 4-1. ^{13}C NMR (125 MHz, $CDCl_3$): Table 4-1. HRESIMS (pos.): *m/z* $[M+H]^+$, calcd. for $C_{26}H_{37}O_7^+$, 461.2534, found: 461.2560; $[M+Na]^+$, calcd. for $C_{26}H_{36}NaO_7^+$, 483.2354, found: 483.2361.

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Chapter 5: Antiproliferative triterpenoid saponins from *Leptaulus citroides* Baill. from the Madagascar rain forest

This chapter is a modified and expanded version of a previously published work (Su, Q., Brodie, P. J., Liu, Y., Miller, J. S., Andrianjafy, N. M., Antsiferana, R., Rasamison, V. E. and Kingston, D. G. I., Antiproliferative triterpenoid saponins from *Leptaulus citroides* Baill. from the Madagascar rain forest. *Nat. Prod. Bioprospect*., **2016**, *6*, 31−39). Contributions of co-authors of the articles are described as follows in the order of the names listed. The author of this dissertation (Qingxi Su) completed the fractionation of the extract and the structural elucidation of the compounds described, and the drafting of the manuscript. Ms. Peggy Brodie and Dr. Yixi Liu performed the antiproliferative bioassay (A2780) on all fractions and compounds. Dr. James S. Miller and Dr. Naina M. Andrianjafy from Missouri Botanical Garden collected and identified the plant. Dr. Vincent E. Rasamison from Madagascar carried out the initial plant extraction, supervised by Dr. Rabodo Antsiferana. Dr. David G. I. Kingston was a mentor for this work and is the corresponding author for the published article.

Figure 5-1. *Leptaulus citroides Baill.* Used under Creative Commons (CC BY-NC-ND 3.0) from <http://tropicos.org/Image/100125562>.

5.1 Introduction

As part of the Kingston group's continuing ICBG Madagascar project, the EtOH extracts from the roots and wood of the Madagascan plant *Leptaulus citroides* were selected for investigation on the basis of their weak antiproliferative activity against the A2780 human ovarian cancer cell line (IC_{50} = 20 µg/mL).

Bioassay-guided fractionation of the roots and wood extract of the plant led to the isolation of ethyl esters of three new triterpenoid saponins (**5.1**–**5.3**) and the known sesquiterpenoid cinnamosmolide (**5.4**). The structures of **5.1**–**5.3** were elucidated by extensive 1D and 2D NMR experiments, mass spectrometry and chemical modification. Compounds **5.1**, **5.2**, and **5.4** showed moderate cytotoxicity against the A2780 human ovarian cancer cell line with IC_{50} values of 3.0 ± 0.3, 10 ± 1 and 2.0 ± 0.2 µM, respectively.

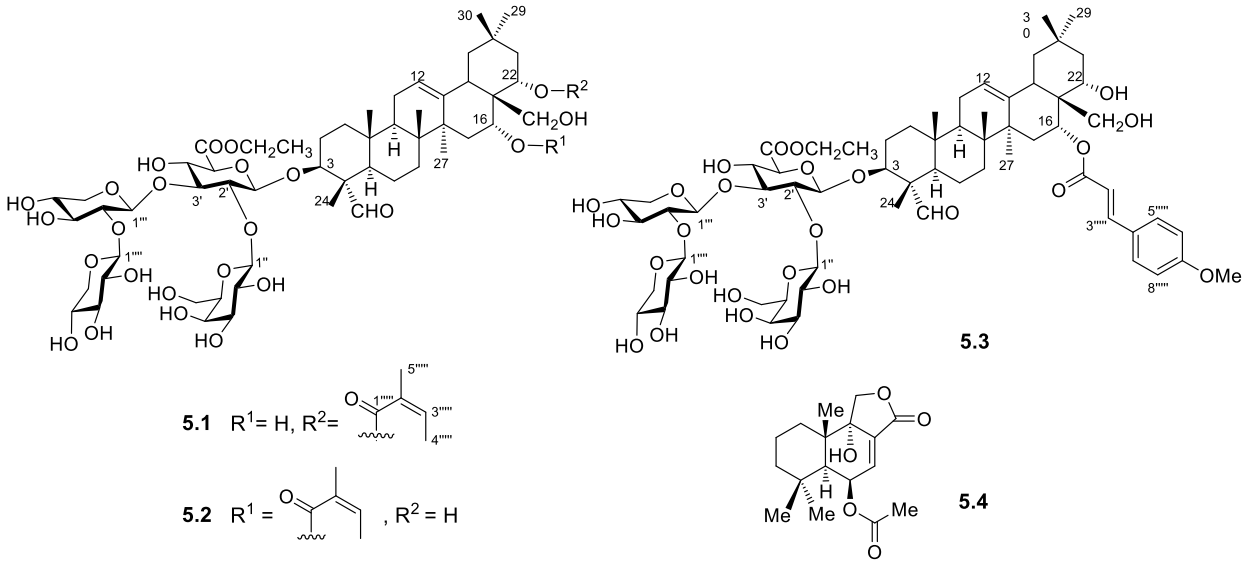
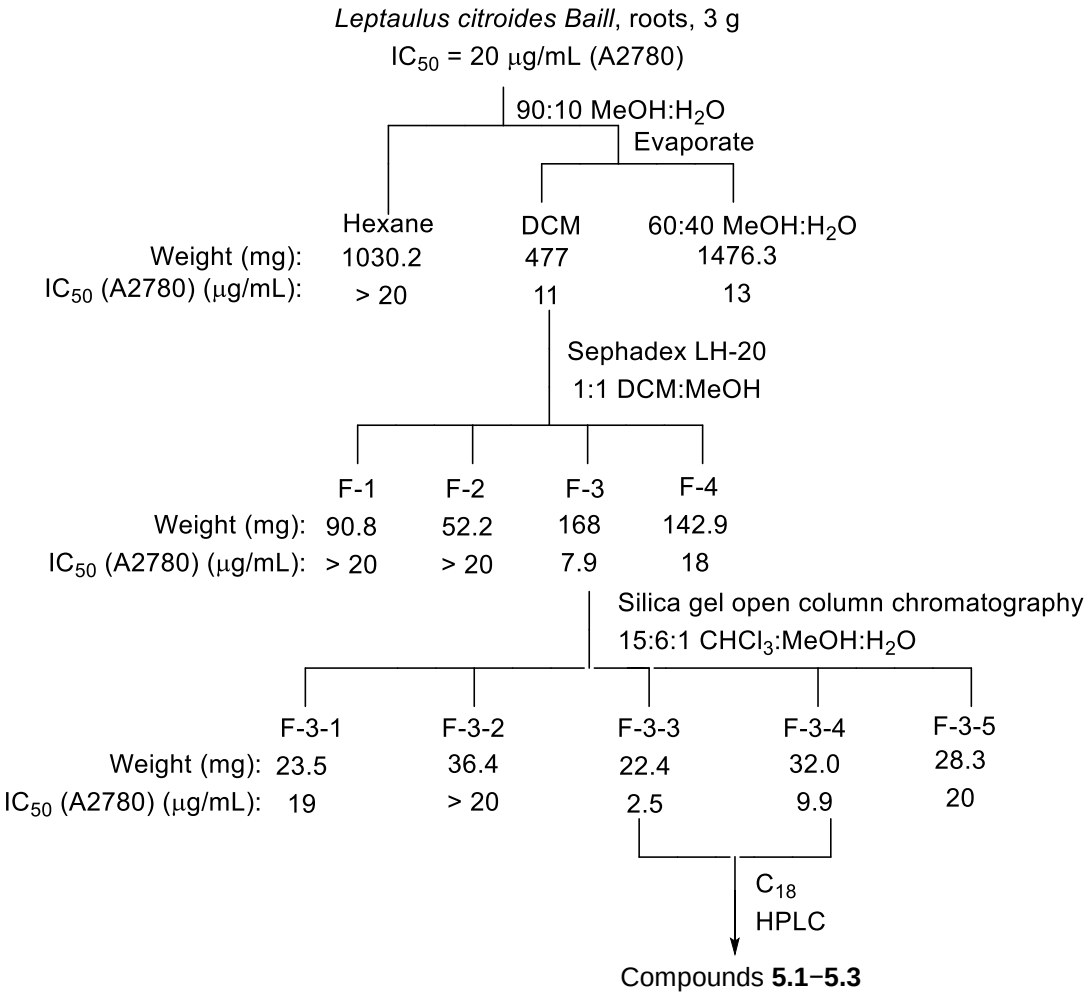


Figure 5-2. Structures of compounds **5.1**–**5.4**

5.2 Results and Discussion

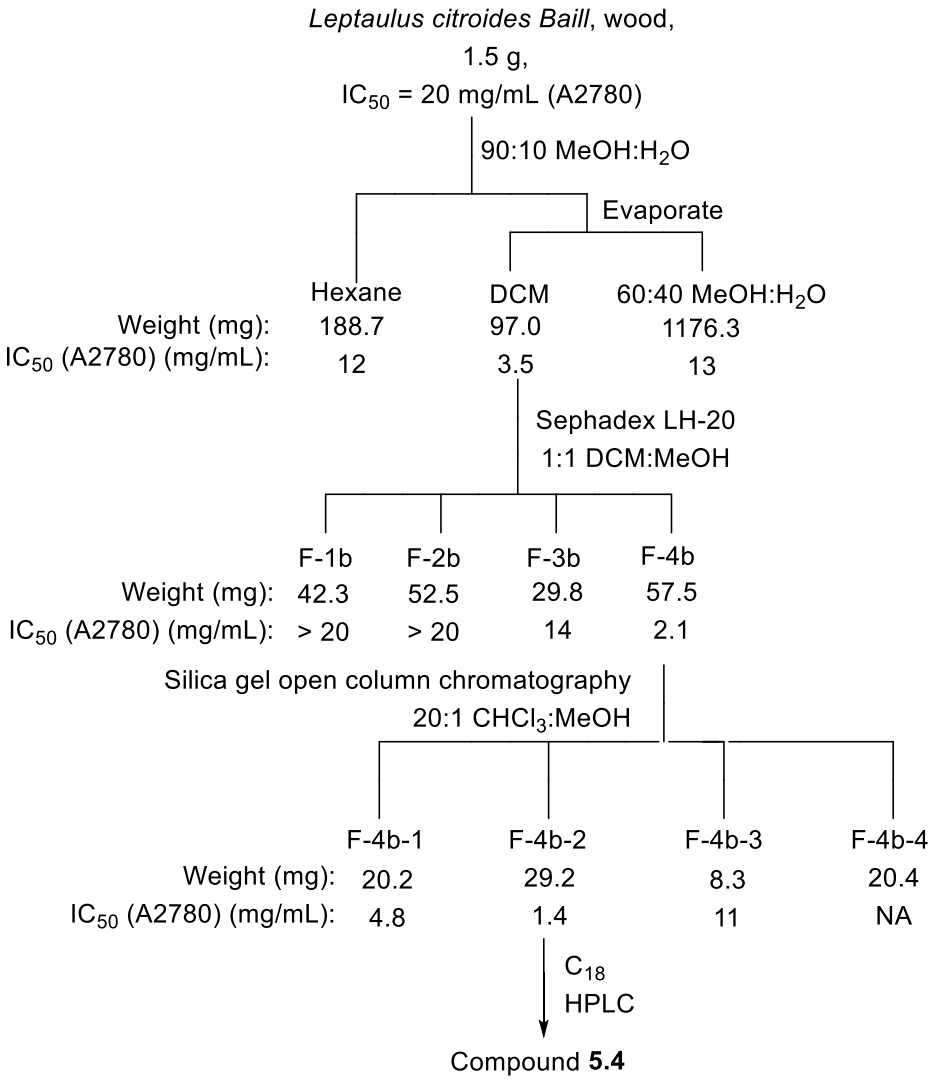
5.2.1 Isolation of active compounds

The crude EtOH extract of the roots of *L. citroides* (MG1619, 3g) exhibited weak antiplasmodial activity (IC_{50} = 20 µg/mL). Since the plant was never investigated for potential bioactive, especially antiproliferative compounds, the crude extract was selected for fractionation in order to identify its bioactive compounds. Fractionation of the EtOH-soluble materials by liquid-liquid partition, open column chromatography on Sephadex LH-20, silica-gel, followed by C_{18} HPLC yielded three three new triterpenoid saponins (**5.1**–**5.3**) (Scheme 5.1).



Scheme 5.1 Bioassay-guided separation of root extract of *Leptaulus citroides*

The EtOH extract of the wood of *L. citroides* (MG1620, 1.5g) with weak antiplasmodial activity (IC_{50} = 20 µg/mL), was also fractionated in pursuit of new antiproliferative agents. Fractionation of the extract MG1620 by liquid-liquid partition, open column chromatography on Sephadex LH-20, silica-gel, followed by C_{18} HPLC yielded the known sesquiterpenoid cinnamosmolide (**5.4**) (Scheme 5.2).



Scheme 5.2 Bioassay-guided separation of wood extract of *Leptaulus citroides*

5.2.2 Structure elucidation of compound **5.1**

Ethyl leptauloside A (**5.1**) was obtained as an amorphous white powder. Its HRESIMS revealed a sodiated quasi-molecular ion peak at *m/z* 1223.5806 $[M+Na]^+$, corresponding to the molecular formula $C_{59}H_{92}O_{25}$. Compound **5.1** was assigned as an olean-12-ene triterpene derivative based on its 1D and 2D NMR spectra. The ^1H NMR spectrum of the aglycone part of compound **5.1** displayed characteristic signals of six singlet methyl groups [δ_H 0.91, 0.95, 1.03, 1.05, 1.16 and 1.50, (each 3H, all s, 29, 26, 25, 30, 24, 27-H_3)], an olefin group (δ_H 5.35, t, *J* = 3.5 Hz, H-12) and an aldehyde group (δ_H 9.44, s). The position of the olefin group was confirmed by HMBC correlations of H_3-27 (δ_H 1.50, s) to C-13 (δ_C 144.1), and H-12 to C-11 (δ_C 24.6).[1] The aglycone moiety was oxygenated at C-3, 16, 22, and 28, based on the HMBC correlations between H_3-24 (δ_H 1.16, s) and C-3 (δ_C 86.3); H-22/H_3-29/H_3-30(δ_H 5.44, dd, *J* = 12.1, 5.6 Hz/ 0.91 s/ 1.05 s) and C-21 (δ_C 42.1); between H-16/ H-22 (δ_H 4.11, brs/ δ_H 5.44, dd, *J* = 12.1, 5.6 Hz) and C-17 (δ_C 45.3); and between H_2-28 (δ_H 3.05, d, *J* = 10.9 Hz/ 3.25m) and C-22 (δ_C 73.8). The aldehyde group [(δ_H /δ_C): 9.44, s/210.6] was located at C-4 based on the HMBC correlation between the aldehyde proton and C-24 (δ_C 10.8), in addition to comparison of 1D NMR data of **5.1** with previously reported data of similar compounds [2]. The 1D NMR and HSQC spectra of compound **5.1** showed the characteristic chemical shifts and coupling patterns of an angeloyl group, with one olefinic proton [δ_H 6.07 (1H, qq, *J* = 7.3, 1.5 Hz, H-3'''')], two methyl groups [δ_H 1.98 (3H, dq, *J* = 7.3, 1.5 Hz, H_3-4'''') and 1.90 (3H, m, H_3-5'''')], a carboxyl carbon (δ_C 168.8, C-1'''') and two sp^2 carbons (δ_C 138.1 and 130.0, C-3'''' and C-2''''), in agreement with the NMR data of related compounds.[1-3] The angeloyl group was assigned to C-22 based on the HMBC correlation between H-22 and the angeloyl carbonyl carbon. The spectroscopic data of the aglycone of **5.1** showed good agreement with the data of similar compounds reported previously.[2-6] The relative

configurations of the aglycone and tetrasaccharide moiety were determined from coupling constants and ROESY correlations. The aldehyde group at C-4 was assigned the α-equatorial orientation on the basis of ROESY correlations of H_3-24 (δ_H 1.16, s) to the *β*-axially oriented H_3-25 (δ_H 1.03, s) and of H-23 (δ_H 9.44, s) to H-3 (δ_H 3.87, m) and H-5 (δ_H 1.35, m), both of which were *β*-axial (Figure 1). H-16 (δ_H 4.11, brs) was assigned a *β*-equatorial orientation based on its appearance as a broad singlet, indicating small coupling constants, while H-22 (δ_H 5.44, dd, *J* = 12.3, 5.6 Hz) had the *β*-axial orientation based on its coupling constants; these assignments were confirmed by comparison with the chemical shifts and coupling constants of related protons of apodytine A-C.[4] The relative configurations of the aglycone were supported by comparison of its ^{1}H and ^{13}C NMR data with those of assamsaponin A and camelliasaponin B1.[6]

The structure of the sugar moiety of **5.1** was elucidated on the bases of ^{1}H-^{1}H COSY, TOCSY, ROESY, HSQC, and HMBC spectra. Four anomeric protons [δ_H 4.97 (1H, d, *J* = 7.2 Hz), 4.92 (1H, d, *J* = 7.1 Hz), 4.51 (1H, d, *J* = 7.6 Hz) and 4.45 (1H, d, *J* = 7.5 Hz)] correlated with carbons at δ_C 102.7, 102.0, 107.5 and 104.8 were observed in the HSQC spectrum of **5.1**, indicating the presence of four sugar units. The four sugar units were identified as *β*-glucuronopyranosyl (GlcA-1′-6′), *β*-galactopyranosyl (Gal-1″-6″), *β*-xylopyranosyl (Xyl-1‴-5‴), and *β*-xylopyranosyl (Xyl-1⁗-5⁗), by comparison of its ^{13}C NMR data with those of apodytine B,[4] isotheasaponin B_1,[7] and assamsaponin A.[6] HMBC correlations of H-1″ (δ_H 4.97, d) to C-2′ (δ_C 77.8), H-1‴ (δ_H 4.92, d) to C-3′ (δ_C 83.6), and H-1⁗ (δ_H 4.51, d) to C-2‴ (δ_C 85.1) indicated the connectivity of the four sugar units (Figure 5-3). The HMBC correlation of H-1' (δ_H 4.45, d, *J* = 7.5 Hz) to C-3 (δ_C 86.3), indicated that the tetrasaccharide moiety was connected to the aglycone at C-3. An ethyl ester group was present at C-5′ (δ_C 76.5) based on the COSY correlations of H_2-7′ (δ_H 4.22, q, *J* = 7.1 Hz) to H_3-8′ (δ_H 1.28, t, *J* = 7.1 Hz) and HMBC correlations of H_2-7′ (δ_H 4.22, q, *J* = 7.1 Hz)

and H-5′ (δ_H 3.84, d, J = 9.2 Hz) to a carbonyl carbon C-6′ (δ_C 170.3). These facts led to the assignment of ethyl leptauloside A (**5.1**) as 3-*O*-{ethyl[*β*-D-galactopyranosyl-(1→2)][*β*-D-xylopyranosyl-(1→2)-*β*-D-xylopyranosyl-(1→3)]-*β*-D-glucopyranosiduronate}-(3*β*, 4*α*, 16*α*, 22*α*)-16,28-dihydroxy-22-{[(2*Z*)-2-methyl-1-oxo-2-buten-1-yl]oxy}-23-oxoolean-12-en-3-yl.

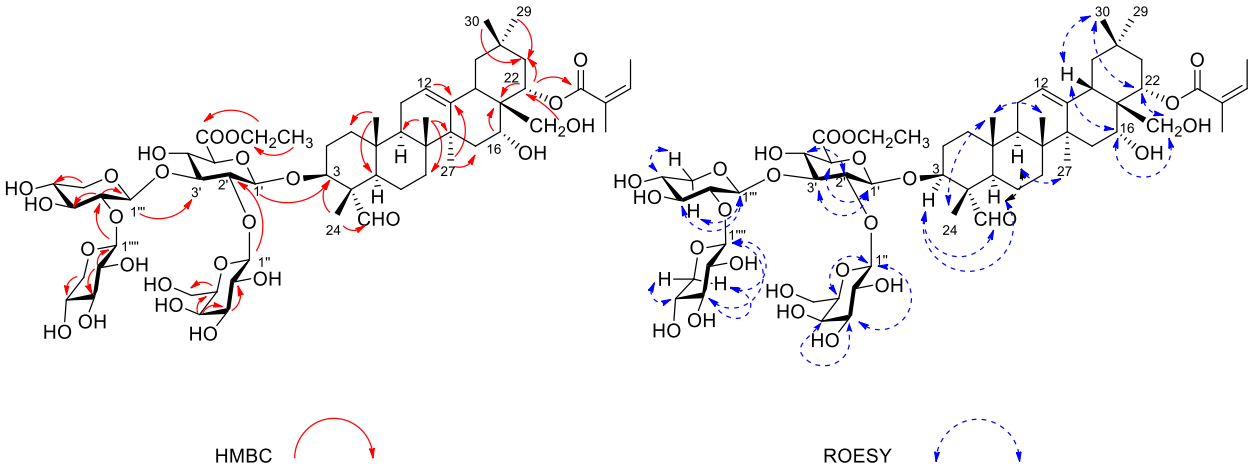


Fig. 5-3. Key HMBC (left) and ROESY (right) correlations of compound **5.1**

The structures and absolute configurations of the sugar moieties of **5.1** were confirmed by the method described by Tanaka and Kouno.[8] Compound **5.1** was subjected to acid hydrolysis and the resulting mixture was derivatized by treatment with L-cysteine methyl ester and *o*-tolylisothiocyanate (Fig. 5-4). Comparison of the HPLC retention times of the resulting *o*-tolythiocarbamoyl-thiazolidine derivatives of the sugar units with those of standards prepared from L-cysteine methyl ester and D and L-glucose, D and L-galactose, D and L-xylose, D and L-arabinose and D-glucuronic acid, and from D-cysteine methyl ester and D-glucuronic acid, confirmed the structures of the carbohydrate units as D-galactose, two D-xyloses, and D-glucuronic acid.

Ethyl esters of glucuronopyranosyl derivatives are unlikely to be natural products, so the ethyl ester was probably formed as the plant material was extracted with EtOH and the solvent

was evaporated. It was regrettably not feasible to collect fresh plant material and extract it with a different solvent to confirm this hypothesis.

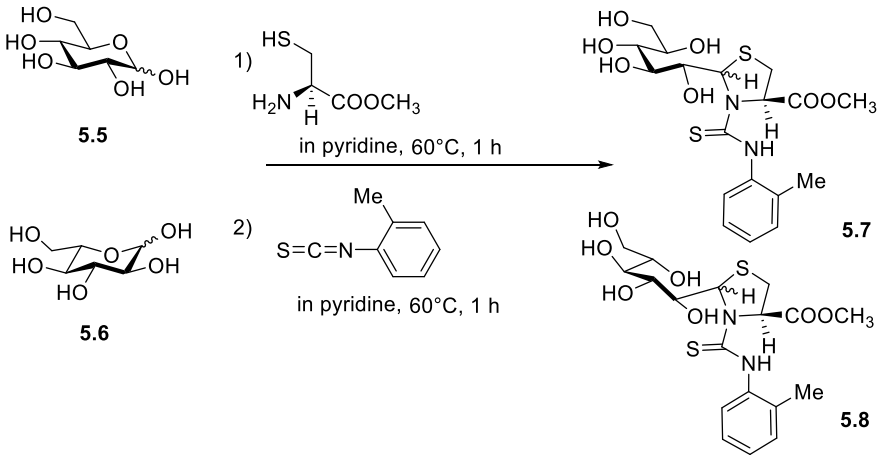


Fig. 5-4. Reactions of D- and L-glucose (**5.5** and **5.6**) with L-cysteine methyl ester and *o*-tolylisothiocyanate

5.2.3 Structure elucidation of compound **5.2**

Ethyl leptauloside B (**5.2**) was obtained as an amorphous white powder. The quasi-molecular ion peak at *m/z* 1223.5781 $[M+Na]^+$ corresponding to a molecular formula of $C_{59}H_{92}O_{25}$. Further comparison of 1D NMR data of **5.2** suggested it is an isomer of **5.1** with the same sugar moiety and a similar aglycone to that of **5.1**. The only difference between **5.1** and **5.2** was the position of the angeloyl and hydroxyl groups. The angeloyl group was assigned to C-16 in **5.2**, as indicated by the deshielded signal of H-16 (δ_H 5.63, brs), and the hydroxyl group was assigned to C-22 due to the more shielded signal of H-22 (δ_H 4.06, dd, J = 12.3, 5.6 Hz) compared to that of compound **5.1**.[4, 7] The aglycone of **5.2** was therefore elucidated as identical to that of theasaponin G_2.[9] Compound **5.2** contained same the sugar moieties as **5.1** as shown by the essentially identical 1D NMR data of the two compounds. The structure of **5.2** was further confirmed by comparison of HSQC, HMBC and ROESY data with those of **5.1**. Thus, the structure of ethyl leptauloside B

(**5.2**) was determined as 3-*O*-{ethyl[*β*-D-galactopyranosyl-(1→2)][*β*-D-xylopyranosyl-(1→2)- *β*-D-xylopyranosyl-(1→3)]-*β*-D-glucopyranosiduronate}-(3*β*,4*α*,16*α*,22*α*)-22,28-dihydroxy-16-{[(2*Z*)-2-methyl-1-oxo-2-buten-1-yl]oxy}-23-oxoolean-12-en-3-yl (Figure. 5-2).

5.2.4 Structure elucidation of compound **5.3**

Ethyl leptauloside C (**5.3**) was OBTAINED as an amorphous white powder. The quasi-molecular ion peak at *m/z* 1301.5982 $[M+Na]^+$ corresponded to a molecular formula of $C_{64}H_{94}O_{26}$. Comparison of ^1H and ^{13}C NMR data with those of **5.2** indicated that they were closely related except that signals of the angeloyl group were replaced by those of a *p*-methoxycinnamoyl group in the spectra of **5.3**. The ^1H NMR spectrum of **3** showed signals of a *para*-substituted benzene ring at δ_H 7.54 (2H, d, J = 8.8 Hz) and 6.98 (2H, d, J = 8.8 Hz), two olefinic protons with *E*-configuration δ_H 7.75 (1H, d, J = 16.0 Hz) and δ_H 6.35 (1H, d, J = 16.0 Hz) and a methoxy group δ_H 3.84 (3H, s). These signals suggested the occurrence of a 4-methoxycinnamoyl group, and this was confirmed by HMBC correlations between the methoxy group (δ_H 3.84, s, 3H) with C7, and $H_{\text{-acyl-3}}$ (δ_H 7.75, d, J = 16.0 Hz) with $C_{\text{-acyl-4}}$ (δ_C 128.2). Comparisons of chemical shifts and coupling constants of H-16 and H-22 in **5.2** and **5.3** suggested the 4-methoxycinnamate group was attached to C-16 [4,7]. Thus, the structure of **5.3** was determined as 3-*O*-{ethyl[*β*-D-galactopyranosyl-(1→2)][*β*-D-xylopyranosyl-(1→2)-*β*-D-xylopyranosyl-(1→3)]-*β*-D-glucopyranosiduronate}- (3*β*, 4*α*,16*α*, 22*α*) -22,28-dihydroxy-16-[((*E*)-4-methoxycinnamoyl)oxy]-23-oxoolean-12-en-3-yl (Figure. 5-2).

5.2.5 Structure elucidation of compound **5.4**

Compound **5.4** was isolated as a white solid. Its structure was assigned as shown based on comparison of its spectroscopic data with those reported in the literature.[10] Cinnamosmolide (**5.4**) has also been reported to display antifungal and α-glucosidase inhibitory activities.[11-12]

Table 5-1. ^{1}H NMR (500 MHz, CD_3OD) and ^{13}C NMR (125 MHz, CD_3OD) Chemical Shift Data (δ, ppm) for Compounds of **5.1-5.3**

Position	1		2		3		Position	1		2		3	
	$^{1}H^{a}$	$^{13}C^{b}$	$^{1}H^{a}$	$^{13}C^{b}$	$^{1}H^{a}$	$^{13}C^{b}$		$^{1}H^{a}$	$^{13}C^{b}$	$^{1}H^{a}$	$^{13}C^{b}$	$^{1}H^{a}$	$^{13}C^{b}$
1	1.12 m, 1.73 m	39.3 CH_2	1.12 m, 1.71 m	39.3 CH_2	1.12 m, 1.71m	39.3 CH_2	2'- β- Gal						
2	1.92 m 1.77 m	25.7 CH_2	1.95 m, 1.77 m	25.7 CH_2	1.95m, 1.77 m	25.7 CH_2	1"	4.97 d (7.2)	102.7 CH	4.97 d (7.2)	102.8 CH	4.97 d (7.2)	102.8 CH
3	3.87 m	86.3 CH	3.86 m	86.2 CH	3,86 m	86.2 CH	2"	3.47 m	73.4 CH	3.46 m	73.4 CH	3.46 m	73.4 CH
4	/	56.2 C	/	56.2 C	/	56.2 C	3"	3.49 m	75.1 CH	3.49 m	75.1 CH	3.49 m	75.1 CH
5	1.35 m	48.8 CH	1.33 m	49.0 CH	1.33 m	48.9 CH	4"	3.80 m	71.0 CH	3.80 m	71.0 CH	3.80 m	71.0 CH
6	0.95 m, 1.54 m	21.2 CH_2	0.95 m, 1.54 m	21.1 CH_2	0.95 m, 1.54 m	21.1 CH_2	5"	3.62 m	76.6 CH	3.62 m	76.6 CH	3.62 m	76.6 CH
7	1.26 m, 1.63 m	32.5 CH_2	1.25 m, 1.54 m	33.1 CH_2	1.28 m, 1.56 m	33.1 CH_2	6"	3.71 m 3.78 m	62.5 CH_2	3.71 m 3.78 m	62.5 CH_2	3.71 m 3.78 m	62.5 CH_2
8	/	42.6 C	/	41.4 C	/	41.4 C	3'- β- Xyl						
9	1.79 m	48.0 CH	1.76 m	48.0 CH	1.79 m	47.9 CH	1'''	4.92 d (7.1)	102.0 CH	4.92 d (7.1)	102.0 CH	4.92 d (7.1)	102.0 CH
10	/	37.0 C	/	37.0 C	/	37.0 C	2'''	3.39 m	85.1 CH	3.39 m	85.1 CH	3.39 m	85.1 CH
11	1.96 m, 1.80 m	24.6 CH_2	1.95 m, 1.76 m	24.5 CH_2	1.93 m, 1.78 m	25.4 CH_2	3'''	3.58 m	77.5 CH	3.58 m	77.5 CH	3.58 m	77.5 CH
12	5.35 t (3.5)	124.2 CH	5.37 t (3.7)	124.9 CH	5.39 t (3.6)	125.0 CH	4'''	3.56 m	71.0 CH	3.57 m	71.0 CH	3.57 m	70.9 CH
13	/	144.1 C	/	142.7 C	/	142.8 C	5'''	3.23 m (ax) 3.90 dd (11.4, 5.4) (eq)	66.7 CH_2	3.23 m (ax) 3.90 dd (11.4, 5.4) (eq)	66.7 CH_2	3.23 m (ax) 3.90 dd (11.4, 5.4) (eq)	66.7 CH_2
14	/	41.8 C	/	42.5 C	/	42.6 C	2'''- β- Xyl						
15	1.31 m, 1.73 m	35.2 CH_2	1.42 m, 1.99 m	32.3 CH_2	1.50 m, 1.99 m	32.3 CH_2	1''''	4.51 d (7.6)	107.5 CH	4.51 d (7.6)	107.5 CH	4.50 d (7.6)	107.5 CH
16	4.11 brs	70.9 CH	5.63 brs	71.7 CH	5.64 brs	72.4 CH	2''''	3.27 m	76.2 CH	3.27 m	76.2 CH	3.27 m	76.2 CH
17	/	45.3 C	/	44.7 C	/	44.9 C	3''''	3.31 m	77.8 CH	3.32 m	77.8 CH	3.32 m	77.9 CH
18	2.52 brd (12.0)	41.3 CH	2.22 brd (12.0)	42.6 CH	2.26 brd (12.0)	42.6 CH	4''''	3.51 m	71.0 CH	3.51 m	71.0 CH	3.51 m	71.0 CH
19	1.06 m, 2.49 m	47.9 CH_2	1.12m, 2.24 dd	48.0 CH_2	1.21 m 2.40 t (13.5)	48.1 CH_2	5''''	3.20m (ax) 3.96 dd (11.4, 5.4) (eq)	67.3 CH_2	3.20m 3.96 dd (11.4, 5.4) (eq)	67.3 CH_2	3.20m 3.96 dd (11.4, 5.4) (eq)	67.3 CH
20	/	33.2 C	/	32.1 C	/	31.8 C	Acyl						
21	1.56 m, 2.27 m	42.1 CH_2	1.39 m, 1.67 m	44.7 CH_2	1.46 m 1.76 m	44.6 CH_2	1''''	/	168.8 C	/	168.8 C	/	167.9 C

22	5.44 dd (12.1, 5.6)	73.8 CH	4.06 dd (12.1, 5.6)	73.8 CH	4.08 dd (12.1, 5.6)	73.8 CH	2''''	/	130.0 C	/	129.3 C	6.35 d (16.0)	117.2 CH
23	9.44 s	210.6 CH	9.43 s	210.5 CH	9.41 s	210.4 CH	3''''	6.07 qq (7.3, 1.5)	138.1 CH	6.08 qq (7.3, 1.5)	139.0 CH	7.75 d (16.0)	145.8 CH
24	1.16 s	10.8 CH_3	1.16 s	10.8 CH_3	1.15 s	10.8 CH_3	4''''	1.98 dq (7.3, 1.5)	15.9 CH_3	1.96 dq (7.3, 1.5)	15.9 CH_2	/	128.2 C
25	1.03 s	16.4 CH_3	1.03 s	16.4 CH_3	1.03 s	16.4 CH_3	5''''	1.90 m	20.9 CH_3	1.99 m	21.3 CH_3	7.54 d (8.8)	130.8 CH
26	0.95 s	17.3 CH_3	0.98 s	17.2 CH_3	0.99 s	17.2 CH_3	6''''					6.98 d (8.8)	115.6 CH
27	1.50 s	27.7 CH_3	1.36 s	27.5 CH_3	1.38 s	27.7 CH_3	7''''					/	163.3 C
28	3.05 d (10.9) 3.25 m	64.8 CH_2	3.30 m, 3.62 m	70.1 CH_2	3.23 m, 3.64 m	69.9 CH_2	8''''					6.98 d (8.8)	115.6 CH
29	0.91 s	33.6 CH_3	0.94 s	33.4 CH_3	1.02 s	34.0 CH_3	9''''					7.54 d (8.8)	130.8 CH
30	1.05 s	25.2 CH_3	0.99 s	25.3 CH_3	1.04 s	25.5 CH_3	-OMe					3.84 s	55.9 CH_3
3-β-GlcA													
1'	4.45 d (7.5)	104.8 CH	4.45 d (7.5)	104.8 CH	4.45 d (7.5)	104.8 CH							
2'	3.76 m	77.8 CH	3.76 m	77.9 CH	3.76 m	77.8 CH							
3'	3.74 m	83.6 CH	3.74 m	83.6 CH	3.74 m	83.6 CH							
4'	3.58 m	70.9 CH	3.58 m	70.9 CH	3.58 m	70.9 CH							
5'	3.84 d (8.2)	76.5 CH	3.84 d (8.2)	76.5 CH	3.84 d (8.2)	76.5 CH							
6'	/	170.3 C	/	170.3 C	/	170.3 C							
7'	4.22 q (7.1)	62.5 CH_2	4.22 q (7.1)	62.5 CH_2	4.22 q (7.1)	62.5 CH_2							
8'	1.28 t (7.1)	14.4 CH_3	1.28 t (7.1)	14.4 CH_3	1.28 t (7.1)	14.4 CH_3							

[a] Assignments based on analysis of 2D NMR spectra. Data (δ) measured at 500 MHz; s = singlet, br s = broad singlet, d = doublet, dd = doublet of doublets, ddd = doublet of doublets of doublets, dt = doublet of triplets, m = multiplet. *J* values are in Hz and are omitted if the signals overlapped as multiplets. The overlapped signals were assigned from HSQC and HMBC spectra without designating multiplicity.

[b] Data (δ) measured at 125 MHz; CH_3, CH_2, CH, and C multiplicities were determined by HSQC experiments.

5.2.6 Bioactivities

Compounds **5.1-5.4** were evaluated for antiproliferative activity against the A2780 human ovarian cancer cell line (Table 5-2). Compounds **5.1**, **5.2** and **5.4** showed IC_{50} values of 3.0, 10, and 2.0 μM respectively, while compound **3** was inactive (IC_{50} > 20 μg/mL, inhibiting 14% of cells growth at a concentration of 20 μg/mL) in this assay. Previous studies suggested that acylation with angeloyl groups at C-21 and C-22 can affect the biological activities of oleanane triterpenoid saponins.[1, 3, 13-14] These results support the importance of an angeloylated 22-hydroxyl group for antiproliferative activity. The reduced activity of compound **5.3** comparing to **5.2** is possibly due to the bulkiness of the 4-methoxycinnamate group that acylates the C-16 hydroxyl group.

Table 5-2. Antiproliferative activity data of compounds **5.1**–**5.4**.

Compound	A2780 ovarian cancer cells IC_{50} (μM)
Ethyl Leptauloside A (**5.1**)	3.0 ± 0.3
Ethyl Leptauloside B (**5.2**)	10 ± 1
Ethyl Leptauloside C (**5.3**)	NA*
Cinnamosmolide (**5.4**)	2.0 ± 0.2

* IC_{50} > 20 μg/mL, the exact IC_{50} value was not determined.

5.3 Experimental Section

5.3.1 General experimental procedures

IR and UV spectra were measured on MIDAC M-series FTIR and Shimadzu UV-1201 spectrophotometers, respectively. 1D and 2D NMR spectra were recorded on a Bruker Avance 500 spectrometer in CD_3OD; chemical shifts are given in δ (ppm), and coupling constants are reported in Hz. Mass spectra were obtained on an Agilent 6220 LC-TOF-MS in the positive ion

mode. Optical rotations were recorded on a JASCO P-2000 polarimeter. Open column chromatography was performed using Sephadex LH-20 and silica gel (40−63 μm, Silicycle Co. USA). HPLC was performed on a Shimadzu LC-10AT instrument with a semipreparative C18 (Phenomenex Luna column, 5 μm, 250×10 mm), a Shimadzu SPD M10A diode array detector, and a SCL-10A system controller. All isolated compounds were purified to 95% purity or better, as judged by HPLC (both UV and ELSD detection) before determining bioactivity.

5.3.2 Plant material

Leptaulus citroides Baill. (Cardiopteridaceae) (vernacular name Tabonaka) were collected by N. M. Andrianjafy and coworkers at an elevation of about 600 m from a 10 m tall tree. Collection was made on a slope near the town of Ambodimangavalo in the district of Vavatenina, on the d'Ihofika river near the Andranofantsona camp, at coordinates 17°39'07"S 048°58'14"E (-17.6519400, 48.9705500). Duplicate voucher specimens (Andrianjafy 323) were deposited at the Centre National d'Application des Recherches Pharmaceutiques (CNARP), the Herbarium of the Department of Forestry and Fishery Research (TEF), and the Missouri Botanical Garden, St. Louis, Missouri (MO).

5.3.3 Extraction and isolation

The EtOH extract of the root of *L. citroides* (MG 1619, 3 g, IC_{50} = 20 μg/mL) was suspended in aqueous MeOH (MeOH/H_2O 9:1, 100 mL) and extracted with hexane (5 × 100 mL portions). The aqueous fraction was then diluted to 60% MeOH and further extracted with CH_2Cl_2 (5 × 100 mL portions) to give a CH_2Cl_2 fraction (477 mg) with an IC_{50} value of 11 μg/mL. This fraction was further subjected to size exclusion open column chromatography on Sephadex LH-20 (I.D. × L 3 × 50 cm) eluted with CH_2Cl_2/MeOH 1:1 to yield four fractions, of which the most active fraction F3 (168 mg) exhibited an IC_{50} of 7.9 μg/mL. Fraction F3 was applied to a silica gel column (I.D.

× L 3 × 50 cm, 40−63 μm) and eluted with $CHCl_3$/MeOH/H_2O 15:6:1 to give five fractions based on TLC profile. Fractions F3-3 (22.4 mg, IC_{50} = 2.5 μg/mL) and F3-4 (32.0 mg, IC_{50} = 9.9 μg/mL) were combined and further separated by HPLC on a semipreparative C_{18} column (Phenomenex Luna column, 5 μm, 25 × 1 cm) with elution by a solvent gradient from CH_3OH/H_2O 50:50 to 60:40 from 0 to 10 min, to 70:30 from 20 to 30 min, to 100:0 from 30 to 35 min, ending with 100% CH_3OH from 35 to 45 min. This process gave crude compounds **5.1** (3.4 mg, t_R 22 min) and **5.2** (3.0 mg, t_R 23 min), and compound **5.3** (3.0 mg, t_R 26 min). Compounds **5.1** and **5.2** were each purified by HPLC on a semipreparative C_{18} column (Phenomenex Luna column, 5 μm, 250 × 10 mm) eluted with a same solvent gradient from CH_3CN/H_2O 30:70 to 40:60 from 0 to 10 min, to 50:50 from 10 to 40 min, ending with 100% CH_3CN from 40 to 45 min to give purified compounds **5.1** (3.0 mg, t_R 27 min) and **5.2** (2.8 mg, t_R 40 min).

The EtOH extract of the wood of *L. citroides* (MG 1620, 1.5 g, IC_{50} = 20 μg/mL) was subjected to liquid-liquid partition using same procedures described above. The active dichloromethane fraction (97 mg, IC_{50} = 3.5 μg/mL) was subjected to Sephadex LH-20 (I.D. × L 3 × 50 cm) chromatography to give four fractions. The active fraction F-4b (57.5 mg, IC_{50} = 2.1 μg/mL) was then subjected to open silica gel column (I.D. × L 3 × 50 cm, 40−63 μm) eluted with $CHCl_3$-MeOH, 20:1 to give four fractions. Fraction F-4b-2 (29.2 mg, IC_{50} = 1.4 μg/mL) was further separated by HPLC on a semipreparative C_{18} column (Phenomenex Luna column, 5 μm, 250 ×10 mm) eluted by a solvent gradient from CH_3CN/H_2O, 70:30 to 90:10 from 0 to 30 min, to 100:0 from 30 to 40 min, ending with 100% CH_3CN from 40 to 45 min. This process gave compound **5.4** (7.6 mg, t_R 32 min).

5.3.4 Antiproliferative bioassays

Antiproliferative activities were determined at Virginia Tech against the drug-sensitive A2780 human ovarian cancer cell line as previously described.[15]

5.3.5 Spectroscopic properties

Ethyl leptauluside A (**5.1**): White powder; $[\alpha]_D^{23}$ +3.0 (*c* = 0.19, MeOH); UV (MeOH); λ_{max} (log ε) 207 (6.11) nm; IR (film) ν_{max} 3350, 1773, 1613, 1521, 1158, 1078 cm^{-1}. ^1H- and ^{13}C-NMR: see Table 5-1. HRESIMS *m/z* 1223.5806 $[M+Na]^+$; $C_{59}H_{92}O_{25}Na^+$, (calc. 1223.5820).

Ethyl leptauluside B (**5.2**): White powder; $[\alpha]_D^{23}$ −7.7 (*c* = 0.18, MeOH); UV (MeOH) λ_{max} (log ε) 209 (6.11) nm; IR (film) ν_{max} 3378, 1773, 1613, 1521, 1078 cm^{-1}. ^1H- and ^{13}C-NMR: see Table 5-1. HRESIMS *m/z* 1223.5781 $[M+Na]^+$, $C_{59}H_{92}O_{25}Na^+$; (calc. 1223.5820).

Ethyl leptauluside C (**5.3**): White Powder; $[\alpha]_D^{23}$ +9.6 (*c* = 0.12, MeOH); UV (MeOH) λ_{max} (log ε) 330 (3.79), 282 (3.98), 225 (4.05), 209 (4.09) nm; IR (film) ν_{max} 3386, 1773, 1609, 1518, 1175, 1078, 706 cm^{-1}. ^1H- and ^{13}C-NMR: see Table 5-1. HRESIMS *m/z* 1301.5982 $[M+Na]^+$, $C_{64}H_{94}O_{26}Na^+$; (calc. 1301.5926).

5.3.6 Hydrolysis of ethyl leptauloside A (**5.1**) and absolute configuration of its carbohydrate moieties.

Authentic methyl 2-(polyhydroxyalkyl)-3-(*o*-tolylthiocarbamoyl)-thiazolidine-4(*R*)-carboxylates were prepared from D- and L-galactose, D- and L-glucose, D- and L-xylose, D- and L-arabinose, and D-glucuronic acid by reaction with L-cysteine methyl ester and *o*-tolylisothiocyanate as described.[8] Since L-glucuronic acid was not available, the enantiomeric (o-tolylthiocarbamoyl)-thiazolidine-4(*S*)-carboxylate of D-glucuronic acid was prepared by reaction with D-cysteine methyl ester and *o*-tolylisothiocyanate. Compound **5.1** (2.0 mg) was treated with

3 M HCl for 4 h at 100 °C, and the solution was then neutralized with sodium bicarbonate and extracted thrice with EtOAc. The aqueous fraction was evaporated to dryness under reduced pressure. The resulting mixture of carbohydrates (0.7 mg) was then dissolved in 0.5 mL pyridine, 0.9 mg of L-cysteine methyl ester was added, and the mixture was heated at 60 °C for 1 h. *o*-tolylisothiocyanate (0.9 mg) was then added to the mixture, which was again heated at 60 °C for 1 h. The reaction mixture was directly analyzed by reverse-phase HPLC on a Phenomenex Luna column (5 µm, 250×10 mm), eluted with isocratic 0.1 % formic acid in CH_3CN/H_2O 15:85 at a flow rate of 2.5 mL/min for 5 min, followed by 0.1 % formic acid in CH_3CN/H_2O 25:75 for 30 min, and a wash with 100 % CH_3CN for 10 min. The resulting chromatogram contained three major peaks with retention times of 25.29, 29.12, and 31.25 min, identical to those of the derivatives of D-xylose, D-galactose and D-glucuronic acid (Table 5-3). Co-injection of each of the derivatives obtained by hydrolysis of compound **5.1** with its corresponding synthetic counterpart confirmed the identity of the compounds. The peak corresponding to the D-xylose derivative was approximately twice as large as that for the D-galactose derivative, consistent with the presence of two D-xylose units in compound **5.1**.

Table 5-3. Retention times of the thiocarbamoyl-thiazolidine

Aldose	**Absolute configuration**	**t_R (min)**
Galactose	D	25.35
	L	26.08
Glucose	D	28.04
	L	26.76
Glucuronic acid	D	29.03
	L [a]	28.26
Xylose	D	31.32
	L	30.02
Arabinose	D	31.71
	L	30.28

[a] The t_R was obtained by reacting D-glucuronic acid with D-cysteine methyl ester

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Chapter 6: Antiproliferative and Antiplasmodial Chromene Derivative and a Plastoquinone from *Huberantha perrieri.*

6.1 Introduction

As part of the ICBG Madagascar project, the EtOH extracts from the leaf of the plant *Huberantha perrieri* (Annonaceae) was selected for investigation due to its activity against A2780 human ovarian cancer cell lines with IC_{50} value of 18 μg/mL. The Annonaceae family contains about 135 genera and 2500 species, and most of them are distributed in the tropical zones.[1] The Annonaceae family represent an important source for drug discovery. Different categories of bioactive compounds such as alkaloids[2-3] flavonoids[4-5] and acetogenins[6-7] with diverse bioactivities have been identified from plants of the Annonaceae family. The genus *Huberantha* comprises 27 species,[8] and none of them have been investigated for potential antiproliferative or antiplasmodial activities.

Figure 6-1.Leaf of *Huberantha perrieri*. Used under Creative Commons (CC BY-NC-ND 3.0) from <http://tropicos.org/Image/100127805>

Bioassay-guided fractionation of the extract led to the isolation of two known compounds, **6.1** and **6.2**. The structures of **6.1** and **6.2** were confirmed by NMR experiments, mass spectrometry and Mosher's analysis. Compounds **6.1** and **6.2** were evaluated for their antiproliferative and antiplasmodial activities, and showed moderate cytotoxic activity against the A2780 human ovarian cancer cell line assay with IC_{50} values of 9 ± 1 µM and 17 ± 2 µM respectively. Their antiplasmodial activities were also evaluated, both compounds exhibited moderate antimalarial activity with IC_{50} values of 5 ± 1 µM and 2.0 ± 0.4 µM respectively.

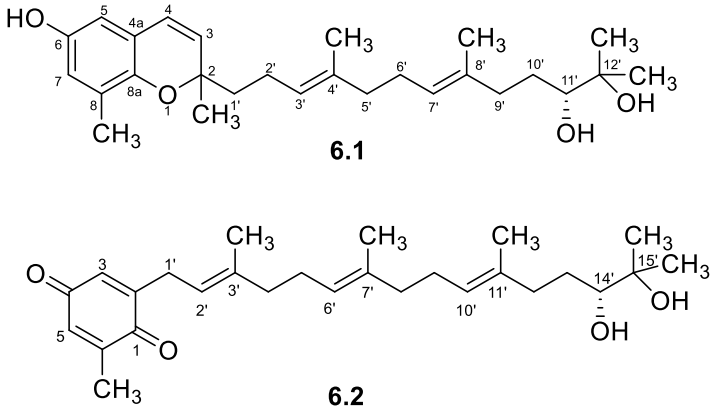


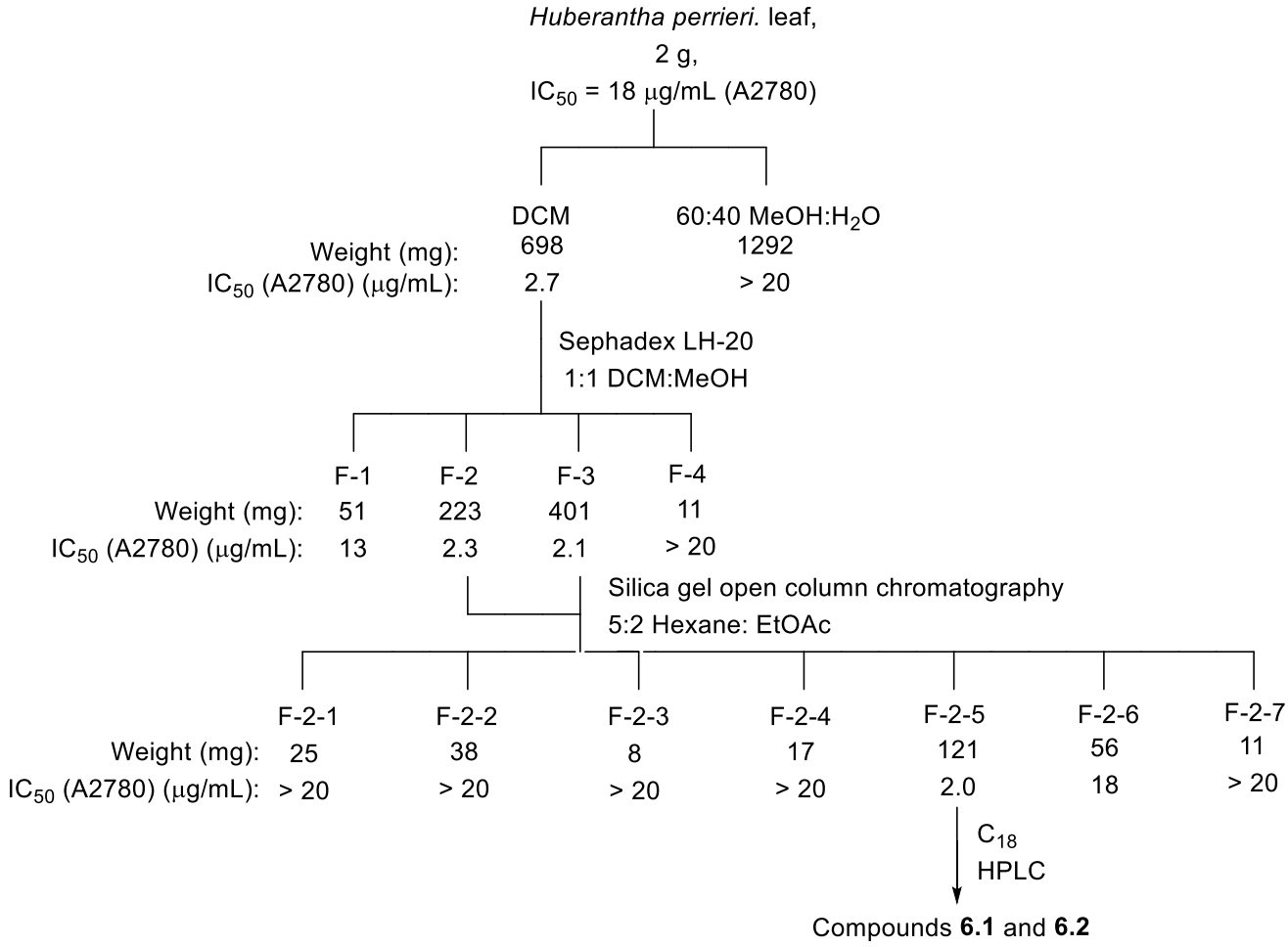
Figure 6-2. Structures of compounds **6.1** and **6.2**

6.2 Results and Discussion

6.2.1 Isolation of active compounds

The crude EtOH extract of the leaf of *Huberantha perrieri* (MG 3588, 2.0 g) exhibited weak antiproliferative activity (IC_{50} = 18 µg/mL) against the A2780 cell line assay. Since the plant was never investigated before, the crude extract was fractionated in order to identify its bioactive compounds. The crude extract was subjected to liquid-liquid partition, column chromatography on

Sephadex LH-20, Si-gel column chromatography and C18 HPLC. These process led to the isolation of two known compounds (**6.1** and **6.2**) (Scheme 6-1).



Scheme 6-1 Bioassay-guided separation of leaf extract of *Huberantha perrieri*

6.2.2 Identification of compound **6.1**

Compound **6.1** was isolated as a pale yellowish oil. Its molecular formula was determined to be $C_{27}H_{40}O_4$ from its HRESIMS ($[M+H]^+$ ion peak at *m/z* = 429.3026, calcd for $C_{27}H_{41}O_4^+$ 429.2999). Six singlet methyl groups were observed in the ^1H NMR (δ_H 1.15, 1.19, 1.36, 1.57, 1.59 and 2.14, each 3H, all s, 12'-Me, 12'-Me, 2-Me, 4'-Me, 8'-Me and 8-Me). It also had signals at δ_H 5.15 (H-3', t, *J* = 7.4 Hz), δ_H 5.10 (H-7', t, *J* = 7.4 Hz), δ_H 5.56 (H-4, d, *J* = 9.9 Hz), δ_H 6.23

(H-3, d, J = 9.9 Hz), δ_H 6.32 (H-5, d, J = 2.9 Hz), δ_H 6.47 (H-7, d, J = 2.9 Hz), and δ_H .3.35 (H-11', dd, J = 10.3, 1.5 Hz). The ^{1}H NMR data of compound **6.1** were consistent with the known compound reported by M. Iwashima and *et al.*[9] However, although the ^{13}C NMR spectrum of the currently isolated compound **6.1** showed almost identical signals to the literature,[9] twin signals (1:1) were observed that corresponded to C-3', C-4', C-8', 4'-Me and 8'-Me (Table 6-1). The twinned signals could be due to the presence of stereoisomers at C-2' or C-11', or to the presence of both *E* and *Z* isomers at C3', C-7'.

To confirm the stereochemistry at the double bonds of **6.1**, its NOESY correlations were analyzed. Key NOE correlations (Figure 6-3) between 2'-H to 4'-Me, 6'-H to 8'-Me, 3'-H to 5'-H, and 7'-H to 9'-H were observed, and no correlations between 3'-H to 4'-Me, or 7'-H to 8'-Me were observed, which confirmed the *E* configuration of both double bonds. Furthermore, the C-2' and C-6' allylic carbons would each be expected to have two very different ^{13}C NMR chemical shifts, if both *E* and *Z* isomers of the double bonds were present,[10-11] but only one signal was in fact observed for these carbons. This observation indicated the isomers must differ in configuration at C-2 or C-11'. Unfortunately, they could not be separated by various column chromatographic methods, so an indirect method was used to establish the point of difference.

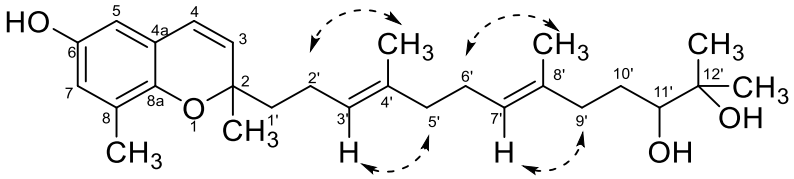


Figure 6-3. Key NOE correlations of compounds **6.1**

Table 6-1. ^{1}H and ^{13}C NMR Data (δ, ppm) for compound **6.1** (500 and 125 MHz) in $CDCl_3$

Position	^{1}H (J in Hz)[a]	^{13}C, type[b]
2		77.8, C
3	6.23 d (9.9)	130.7, CH
4	5.56 d (9.9)	122.9, CH
4a		121.4, C
5	6.32 d (2.9)	110.3, CH
6		148.8, C
7	6.47 d (2.9)	117.1, CH
8		126.3, C
8a		144.8, C
1'	1.66 m	40.7, CH_2
2'	2.06 m	22.6, CH_2
3'	5.15 br t (7.4)	124.32/ 124.34, CH
4'		134.97/ 135.00, C
5'	1.96 m	39.5, CH_2
6'	2.11 m	26.5, CH_2
7'	5.10 br t (7.4)	125.0, CH
8'		134.86/ 134.89, C
9'	1.97 m	36.8, CH_2
10'	1.41 m, 1.57 m	29.6, CH_2
11'	3.35 dd (10.4, 1.5)	78.3, C
12'		73.1, C
2-Me	1.36 s	25.9, CH_3
8-Me	2.14 s	15.4, CH_3
4'-Me	1.57 s	15.86/ 15.87, CH_3
8'-Me	1.59 s	15.87/ 15.89, CH_3
12'-Me	1.15 s 1.19 s	23.3, CH_3 26.4, CH_3

[a] Assignments based on analysis of 2D NMR spectra.
[b] Data (δ) measured at 500 MHz and 125 MHz; s = singlet, br s = broad singlet, d = doublet, dd = doublet of doublets, t = triplet, br t = broad triplet, m = multiplet. *J* values are in Hz and are omitted if the signals overlapped as multiplets. The overlapped signals were assigned from HSQC and HMBC spectra without designating multiplicity.
[c] Data (δ) measured at 125 MHz; CH_3, CH_2, CH, and C multiplicities were determined by HSQC experiments.

To confirm the absolute configuration at C-12' of **6.1**, Mosher's analysis was performed. Compound **6.1** was reacted with (*S*)-MPA-Cl and (*R*)-MPA-Cl respectively in NMR tubes to form the enantiomeric pair of MPA diesters.[12-13] The reaction mixtures were directly analyzed by ^{1}H NMR, and the absolute configuration of C-12 was determined as *R* based on the $\Delta\delta_H$ ($\delta_S - \delta_R$) values shown in Figure 6-4. Mosher's analysis also suggested that only the *R* isomer at C-11' was present, since only one set of shifts was observed. These findings indicate that the presence of two isomers, as evidenced by the twinned ^{13}C NMR signals, can only be due to the presence of two epimers at the C-2 position.

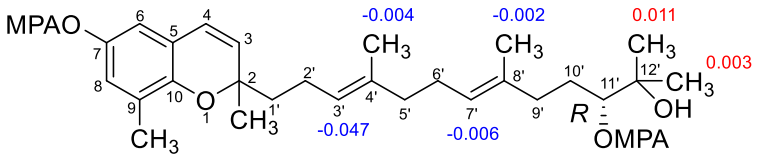


Figure 6-4. Absolute configuration of the secondary hydroxyl group of **6.1**

The previous observation of slight differences in the ^{13}C NMR shifts of 2',3',5',7',8'-C and 4',8'-Me of *2R,4'R,8'R-α*-tocopherol as compared with the same carbons in its *2S,4'R,8'R-α*-tocopherol disasteromer support the contention that the presence of both epimers at C-2 in compound **6.1** can account for the observed twinning of selected ^{13}C NMR signals.[14]

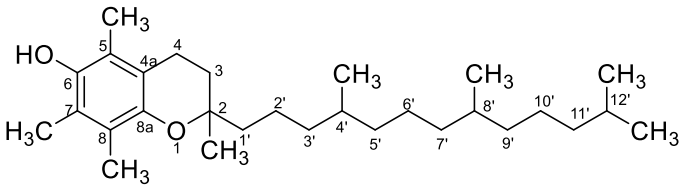


Figure 6-5. Structure of *α*-tocopherol

Compound **6.1** was thus dereplicated as the known (3*R*,6*E*,10*E*)-13-(2,8-dimethyl-6-hydroxy-2*H*-chromen-2-yl)-2,6,10-trimethyltrideca-6,10-diene-2,3-diol isolated from brown alga *Sargassum micracanthum*.[9] The NMR data reported in this reference did not mention the presence of twinned resonances, however.

6.2.3 Identification of compound **6.2**

Compound **6.2** was obtained as yellowish oil. Its structure was dereplicated as the known plastoquinone (Figure 6-2) by comparison of its mass spectrometry and NMR data with the literature data.[9]

6.2.4 Bioactivities

Compounds **6.1** and **6.2** were evaluated for antiproliferative and antiplasmodial activities (Table 6-2). Compounds **6.1** and **6.2** have been reported to exhibit antioxidant and antiviral activities,[9] but their antiproliferative and antiplasmodial activities are being reported for the first time. Interestingly, compound **6.2** had better antiplasmodial activity than **6.1**, probably due to its quinone moiety.

Table 6-2. Antiproliferative activity data of compounds **6.1** and **6.2**.

Compound	A2780 ovarian cancer cells IC_{50} (µM)	*P. falciparum* Dd2 strain IC_{50} (µM)
6.1	9 ± 1	5 ± 1
6.2	17 ± 2	2.0 ± 0.4

6.3 Experimental Section

6.3.1 General experimental procedures

UV spectra were measured on a Shimadzu UV-1201 spectrophotometer. IR spectra were measured on a MIDAC M-series FTIR spectrometer. NMR spectra were recorded in $CDCl_3$ on a

Bruker Avance 500 spectrometer in $CDCl_3$; chemical shifts are given in δ (ppm), and coupling constants are reported in Hz. Mass spectra were obtained on an Agilent 6220 LC-TOF-MS in the positive ion mode. Optical rotations were recorded on a JASCO P-2000 polarimeter. Open column chromatography was performed using Sephadex LH-20 (I.D.×L 3×50 cm). Semi-preparative HPLC was performed on a Shimadzu LC-10AT instrument with a semipreparative C_{18} Phenomenex Luna column (5 µm, 250×10 mm). All isolated compounds were evaluated for purity by HPLC (both UV and ELSD detection) and by NMR before bioactivity assay.

6.3.2 Plant material

The extracts of *Huberantha perrieri* (Cardiopteridaceae) was collected by Richard Randrianaivo and coworkers at an elevation of about 200 m from a 8 m tall tree with a DBH (diameter at breast height) of 15 cm. Collection was made from the rainforest 2 km to the west of Ankijabe, at coordinates 13°14'17"S 049°37'50"E (-13.2380555, 49.6305556). Duplicate voucher specimens (Randrianaivo 1279) were deposited at the Centre National d'Application des Recherches Pharmaceutiques (CNARP), the Herbarium of the Department of Forestry and Fishery Research (TEF), and the Missouri Botanical Garden, St. Louis, Missouri (MO).

6.3.3 Extraction and isolation

The EtOH extract of the leaf of *H. perrieri* (MG 3588, 2 g, IC_{50} = 12 µg/mL) was suspended in aqueous MeOH (MeOH/H_2O 9:1, 100 mL) and extracted with CH_2Cl_2 (5 × 100 mL portions). The aqueous fraction was then diluted to 60% MeOH and further extracted with CH_2Cl_2 (5 × 100 mL portions) to give 698 mg of CH_2Cl_2-soluble materials with an IC_{50} value of 2.7 µg/mL. This fraction was further subjected to size exclusion open column chromatography on Sephadex LH-20 (I.D. × L 3 × 50 cm) eluted with CH_2Cl_2/MeOH 1:1 to yield four fractions, of which the most active fractions F2 (223 mg, IC_{50} = 2.3 µg/mL) and F3 (168 mg, IC_{50} = 2.1 µg/mL) were combined

and applied to a silica gel column (I.D. × L 3 × 50 cm, 40−63 μm) eluted with hexane/EtOAc 5:2 to give seven fractions based on TLC profile. Fraction F-2-5 (121 mg, IC_{50} = 2.0 μg/mL) was further separated by HPLC on a semipreparative C_{18} column (Phenomenex Luna column, 5 μm, 250 × 10 mm) with elution by a solvent gradient from CH_3OH/H_2O, 60:40 to 70:40 from 0 to 10 min, to 100:0 from 10 to 40 min, ending with 100% CH_3OH from 40 to 55 min. This process gave compounds **6.1** (12 mg, t_R 22 min) and **6.2** (3.0 mg, t_R 31 min).

6.3.4 Antiproliferative bioassays

Antiproliferative activities were determined at Virginia Tech against the drug-sensitive A2780 human ovarian cancer cell line as previously described.[15]

6.3.5 Antimalarial bioassays

The effect of each fraction and pure compounds on in vitro parasite growth of Dd2 strain was measured in a 72 h growth assay in the presence of inhibitor as described previously with minor modifications.[16-17] Ring stage parasite cultures (100 μL per well, 1% hematocrit and 1% parasitaemia) were grown for 72 h in the presence of increasing concentrations of the inhibitor in a humidified chamber at 37 °C and low oxygen conditions (5.06% CO_2, 4.99% O_2, and 89.95% N_2). After 72 h in culture, parasite viability was determined by DNA quantitation using SYBR Green I as described previously.[17] The IC_{50} values were calculated with KaleidaGraph software using a nonlinear regression curve fitting. IC_{50} values are the average of three independent determinations with each determination in duplicate, and are expressed ± S.E.M. Artemisinin was used as the positive control with an IC_{50} of 6 ±1 nM.

6.3.6 Spectroscopic properties

Compound **6.1**: Light yellowish oil. $[\alpha]_D^{23}$ +11.0 (*c* = 0.2, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$): See Table 6-1; ^{13}C NMR (125 MHz, $CDCl_3$): See Table 6-2; HRESIMS $[M+H]^+$ *m/z* = 429.3026 (calcd for $C_{27}H_{41}O_4^+$ 429.2999).

Compound **6.2**: Light yellowish oil. $[\alpha]_D^{23}$ +10.4 (*c* = 0.50, $CHCl_3$); ^{1}H-NMR (500 MHz, $CDCl_3$) δ ppm: 1.16 (3H, s, one of 15-Me), 1.20 (3H, s, one of 15-Me), 1.42 (1H, m, one of H-13), 1.58 (1H, m, one of H-13), 1.59 (3H, s, 7-Me), 1.60 (3H, s, 11-Me), 1.62 (3H, s, 3-Me), 1.99 (1H, m, one of H-4'), 1.99 (1H, m, one of H-8'), 2.00 (1H, m, one of H-9'), 2.06 (3H, s, 6-Me), 2,06(1H, m, one of H-4), 2.06(1H, m, one of H-8), 2.06 (1H, m, one of H-5'), 2.07 (1H, m, one of H-9'), 2.08 (1H, m, one of H-12'), 2.09 (1H, m, one of H-5'), 2.20 (1H, m, one of H-12'), 3.13 (2H, br d, *J* = 7.3 Hz, H-1'), 3.36 (1H, dd, *J* = 1.5, 9.8 Hz, H-14'), 5.10 (1H, dt, *J* = 0.9,6.8 Hz, H-6'), 5.15 (1H, dt, *J* = 0.8, 6.8 Hz, H-10'), 5.19 (1H,dt, *J* = 0.9, 7.3 Hz, H-2'), 6.47 (1H, d, *J* = 3.0 Hz, H-3), 6.55(1H, d, *J* = 3.0 Hz, H-5). ^{13}C-NMR (125 MHz, $CDCl_3$) δ ppm: 16.0 (q, 3'-Me), 16.1 (q, 7'-Me), 16.1 (q, 6-Me),16.2 (q, 11'-Me), 23.2 (q, 15'-Me), 26.5(t, C-5'), 26.5 (q,15'-Me), 26.7 (t, C-9'), 27.7 (t, C-1'), 29.7 (t, C-13'), 37.0 (t,C-12'), 39.8 (t, C-8'), 39.8 (t, C-4'), 73.1(s, C-15'), 78.3(s, C-14'), 118.1 (d, C-2'), 124.0 (d, C-6'), 125.2(d, C-10'),132.4 (s, C-3), 133.3 (d, C-5), 135.0 (s, C-7'), 135.4 (s, C-11'), 140.0 (s, C-3'), 146.1 (s, C-6), 148.7 (s, C-2), 188.1(s, C-4), 188.3 (s, C-1). HRESIMS $[M+Na]^+$ *m/z* = 451.2807 (calcd for $C_{27}H_{40}O_4Na^+$ 451.2824).

6.3.7 Conversion of **6.1** to an Enantiomeric Pair of MPA Diesters

In a 25 mL round-bottom flask, 5.00 mg of (*R*)-(−)-α-methoxyphenylacetic acid ((*R*)-MPA) was dissolved in 5 mL of CH_2Cl_2 with 15 mg of oxalyl chloride. A catalytic amount of dimethylformamide was then added to the reaction. The reactants were mixed with a magnetic stir bar in an ice bath for 1 h to obtain 4.98 mg of (*R*)-(−)-α-methoxyphenylacetyl chloride (91% yield).

The in-NMR-tube reaction was carried out to prepare the MPA ester derivatives. Compound **6.1** (2 mg) dissolved in $CDCl_3$ was transferred to a clean NMR tube,[12] and the solvent was completely evaporated under an N_2 gas flow. (*R*)-(−)-α-Methoxyphenylacetyl chloride (4.98 mg) was dissolved in $CDCl_3$ (0.5 mL) and added to the NMR tube immediately under an N_2 gas flow, and the NMR tube was shaken carefully to mix **6.1** and (*R*)-MPA chloride. The reaction NMR tube was kept at 0 °C for 24 h to form the (*R*)-MPA diester and was directly analyzed by ^{1}H NMR spectroscopy. The (*S*)-MPA derivative was prepared from (*S*)-MPA by the same method.

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Chapter 7: Investigation of Other Antimalarial and Antiproliferative Plant Extracts

7.1 Introduction

7.1.1 Abstract

In the continuing search for novel antimalarial and antiproliferative agents, some extracts were investigated without any novel bioactive compounds being identified. Bioassay guided fractionation of these extracts yielded only known compounds with/without biological activities, or no compounds due to various reasons. This chapter summarizes this work.

7.1.2 Author Contributions

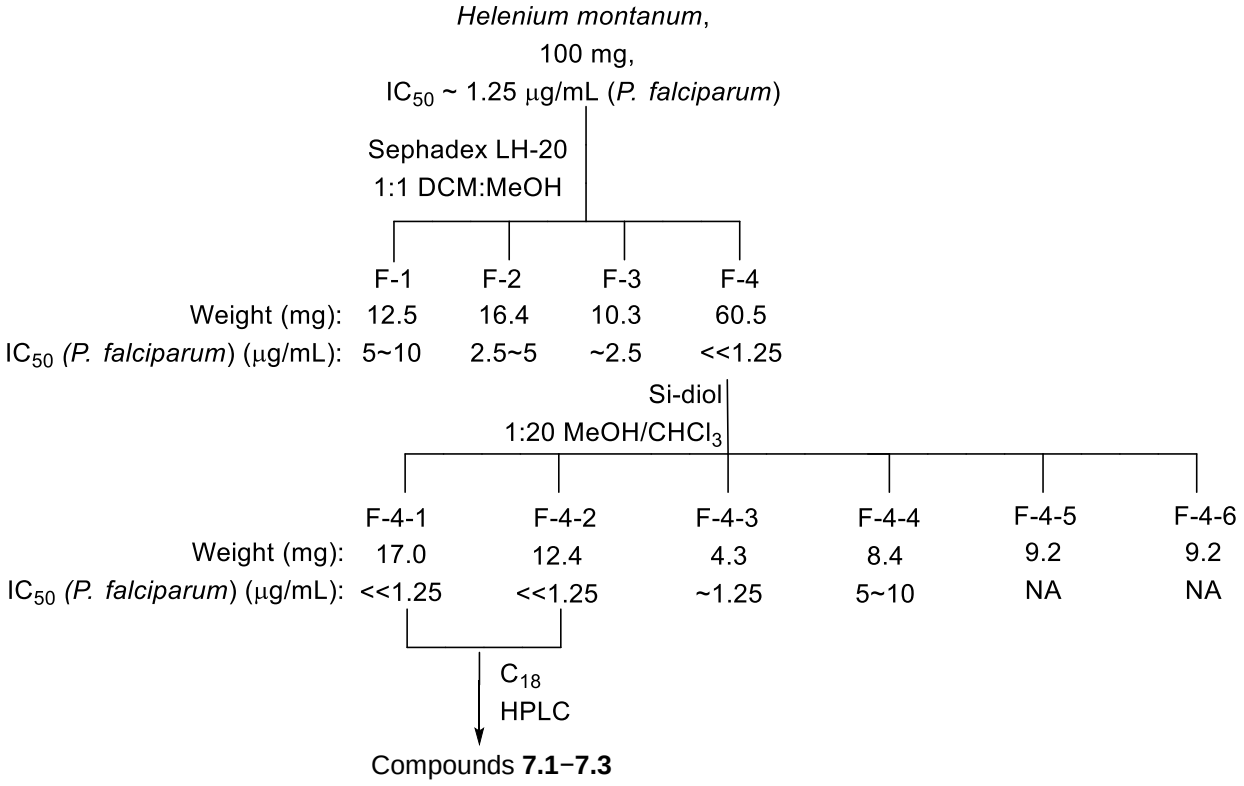
The author (Qingxi Su) of this dissertation performed the fractionation of all of these extracts and dereplicated the structures of compounds shown in this chapter, except for the extract of *Antiaris madagascariensis* (Moracreae) MG3973 (section 7.3.2), which was performed by Mr. Nick Cordova under the guidance of the author. Dr. Jessica D. Wiley, Dr. Seema Dalal, Dr. Priscilla Krai and Dr. Maria B. Cassera performed the antimalarial bioassays (*Plasmodium falciparum*, Dd2 strain). Ms. Peggy Brodie, Ms. Ming Wang, Dr. Yixi Liu and the author (Qingxi Su) performed the A2780 antiproliferative bioassays on all fractions and compounds. Dr. David G. I. Kingston was a mentor for this work.

7.2 Investigation of Other Antimalarial Extracts

7.2.1 *Helenium montanum* (Asteraceae), 40265-04B

Open column chromatography of the dichloromethane extract of *Helenium montanum* (Asteraceae) (100 mg, IC_{50} around 1.25 μg/mL) on Sephadex LH-20 furnished an active fraction

with an IC_{50} value less than 1.25 μg/mL. Successive Si-diol open column chromatography followed by C_{18} HPLC of the active fraction yielded three known sesquiterpene lactones (**7.1**−**7.3**) (Figure 7-2-1) with potent to moderate antimalarial activities. The detailed fractionation procedures are described in Scheme 7-2-1.



Scheme 7-2-1 Bioassay-guided separation of *Helenium montanum* extract

The structures of compounds **7.1**−**7.3** were assigned by comparison of their spectroscopic data with those in the literature.[1-3] Compounds **7.1**−**7.3** exhibited potent to moderate antiplasmodial activity against the Dd-2 strain of P. *falciparum* with IC_{50} values of 2.0 ± 0.2 μM, 6 ± 1 μM, and 16 ± 1 μM respectively. *Helenium* is an intensively studied genus, which is reported to contain a variety of sesquiterpene lactones.[2-3] Antiplasmodial, antiproliferative and

antimicrobial activities of related compounds have been well investigated.[4-7] Since the active components isolated from the plant were sesquiterpene lactones, the minor moderately active components are very likely to fall into the same category. Therefore the extract was not investigated further.

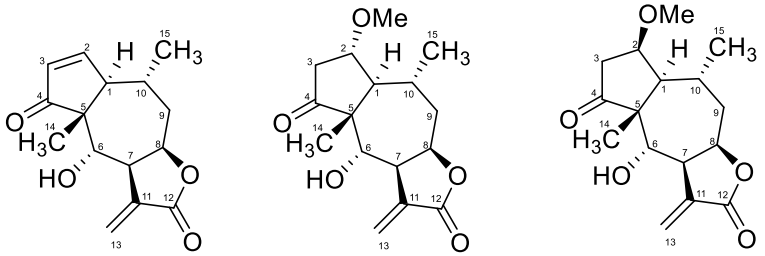
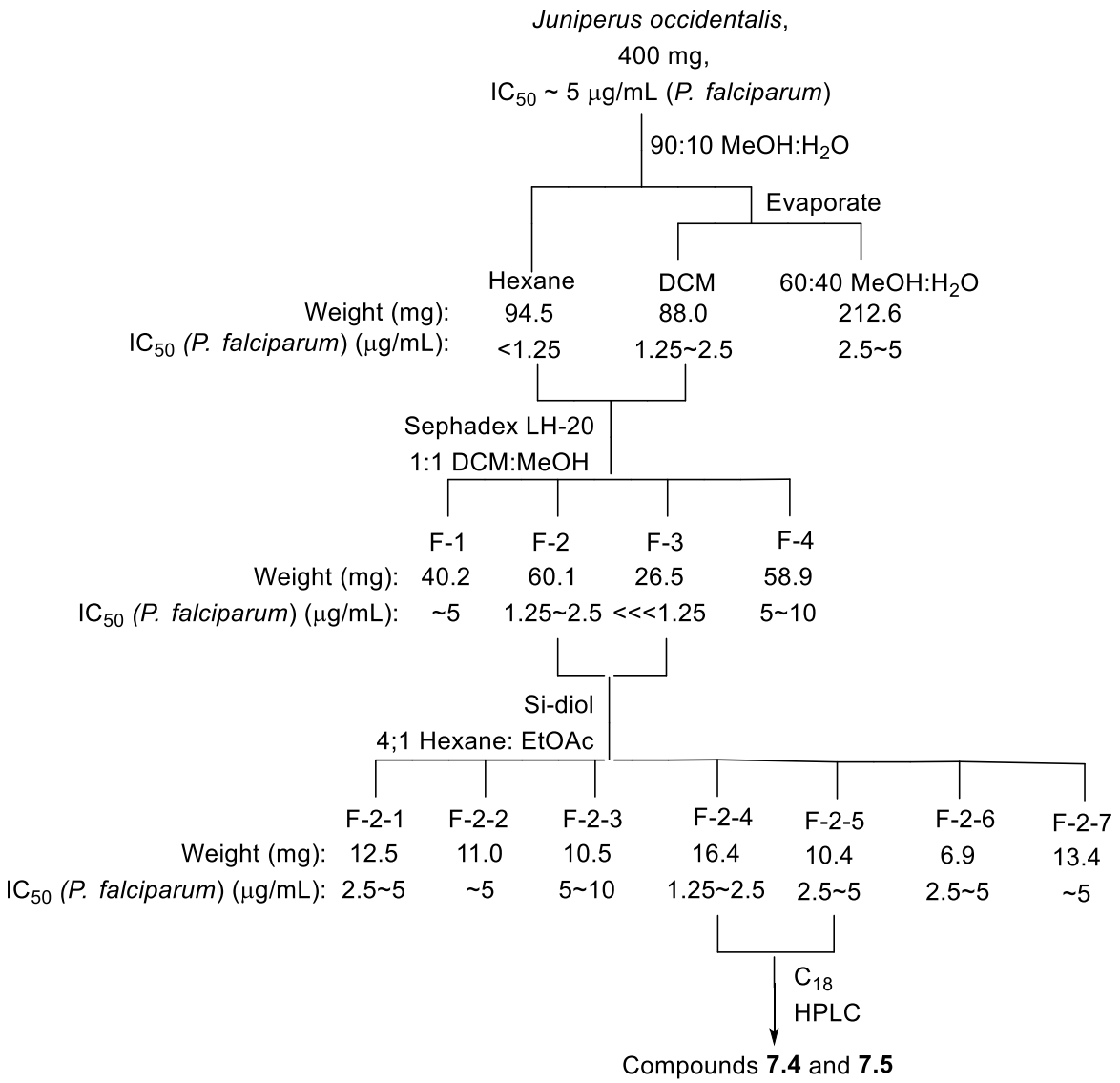


Figure 7-2-1. Structures of compounds **7.1**−**7.3**

7.2.2 *Juniperus occidentalis* (Cupressaceae)

The methanol extract of *Juniperus occidentalis* was selected to investigate its potential antiplasmodial agents, due to its moderate inhibitory activity against the Dd2 strain of *P. falciparum* with an IC_{50} value of 5 μg/mL. The methanol extract of *J. occidentalis* (400 mg, IC_{50} around 5 μg/mL) was fractionated by liquid-liquid partition, open column chromatography on sephadex LH-20 and Si-diol, followed by C_{18} HPLC. These procedures yielded two known diterpenes (**7.4** and **7.5**) (Figure 7-2-2) which were inactive in the malarial assay. Biologiccal activities were partially lost in the Si-diol open column chromatography and we were unable to identify the active components by C_{18} HPLC due to the limited amount of extract. The detailed fractionation procedures are described in Scheme 7-2-2.



Scheme 7-2-2 Bioassay-guided separation of *Juniperus occidentalis* extract

Juniperus is a well investigated genus, and compounds isolated from related plants included phenol glucosides, biflavones, and labdane-type diterpenes.[8-10] Structures of **7.4** and **7.5** were confirmed by comparison of their spectroscopic data with literature data.[11-14]

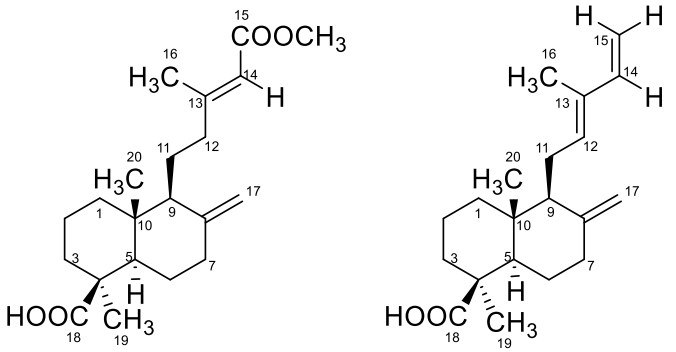
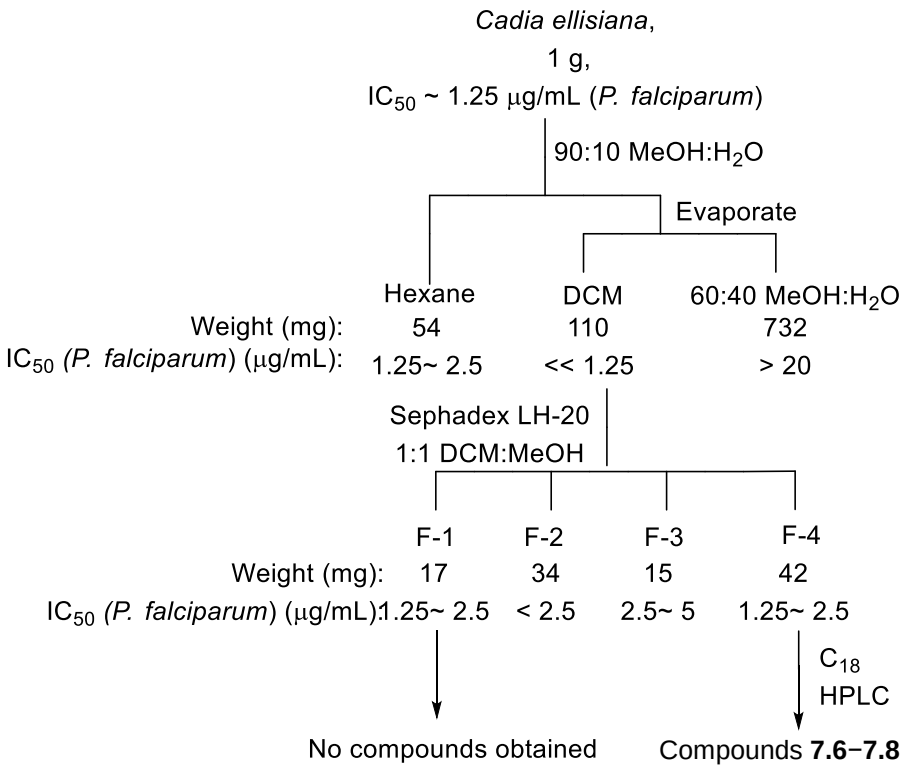


Figure 7-2-2. Structures of compounds **7.4** and **7.5**

7.2.3 *Cadia ellisiana* (Fabaceae) MG0390

An ethanol extract of the bark of *Cadia ellisiana* was selected for bioassay guided fractionation, due to its good inhibitory activity against the Dd2 strain of *P. falciparum* with an IC_{50} value around 1.25 μg/mL. The crude extract (1 g, IC_{50} around 1.25 μg/mL) was fractionated by liquid-liquid partition, open column chromatography on Sephadex LH-20, and followed by C_{18} HPLC. These procedures gave three known compounds (**7.6**−**7.8**) (Figure 7-2-3) which were weakly active in the malarial assay (IC_{50} = 65 ± 20 μM, 43 ± 13 μM, and 23 ± 2 μM respectively). The structures of **7.6**−**7.8** were dereplicated by comparison of their spectroscopic data with the literature.[15-17]



Scheme 7-2-3 Bioassay-guided separation of *Cadia ellisiana* extract

The first fraction (F-1) obtained from Sephadex LH-20 open column exhibited the best biological activity. Unfortunately, no compounds were obtained from this fraction, even though a variety of column chromatographies were tried. The last fraction (F-4) collected from Sephadex LH-20 was further examined by C_{18} HPLC, however, the major compounds (**7.6−7.8**), which were obtained from this fraction, showed only weak activity in the bioassay. The detailed fractionation procedures are described in Scheme 7-2-3.

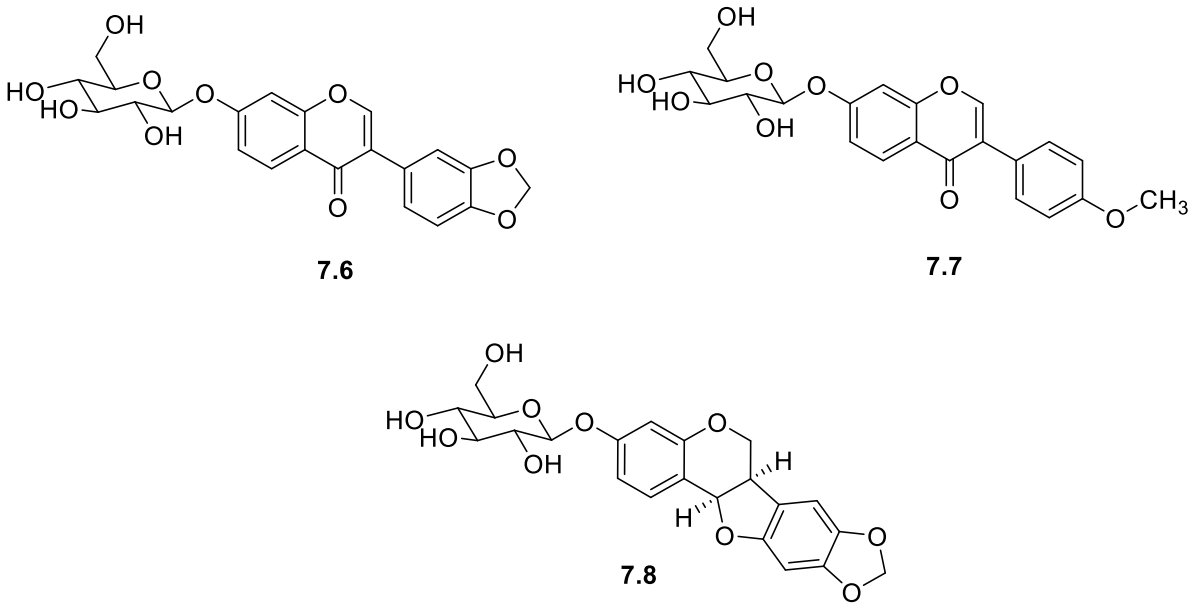


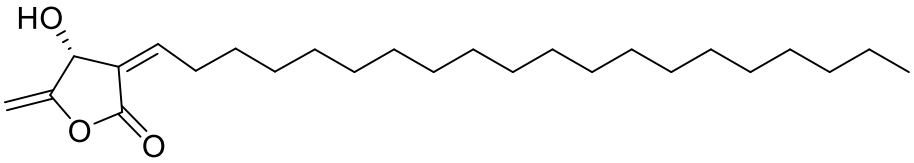
Figure 7-2-3. Structures of Compounds **7.6−7.8**

7.2.4 *Tambourissa thouvenotii* (Monimiaceae) MG 0346

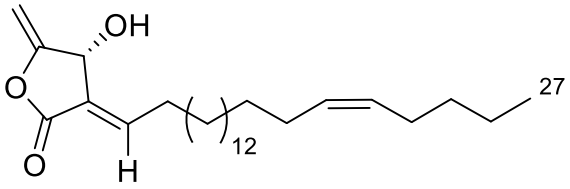
The ethanol extract of the wood part of *Tambourissa thouvenotii* was selected to investigate antiplasmodial activity, due to its weak inhibitory activity against the Dd2 strain of *P. falciparum* (IC_{50} between 5 and 10 μg/mL). Phytochemical investigations on the genus of *Tambourissa* resulted in the identification of a number of volatile terpenes and curcumene from *T. leptophylla*.[18] A hydroxybutanolide named as tambouranolide with moderate cytotoxic activity had also been identified from the root parts of this specie.[19] However, the genus was not previously investigated for antiplasmodial agents.

The crude extract was partitioned by liquid-liquid partition, open column chromatography on Sephadex LH-20, Diaion, C_{18} SPE and C_{18} HPLC (Scheme 7-2-4). Two antiplasmodial agents, compounds **7.9** and **7.10**, were obtained from the hexane-soluble material with IC_{50} values of 4.0 ± 0.4 μM and 14 ± 3 μM. Compound **7.9** was dereplicated as the known compound miaolinolide,

which had been previously reported to display strong inhibitory activity on LPS-induced TNF-α production.[20] Compound **7.10** was identical to the previously isolated compound from the roots of the same plant.[19] Therefore, the extract was not investigated further.

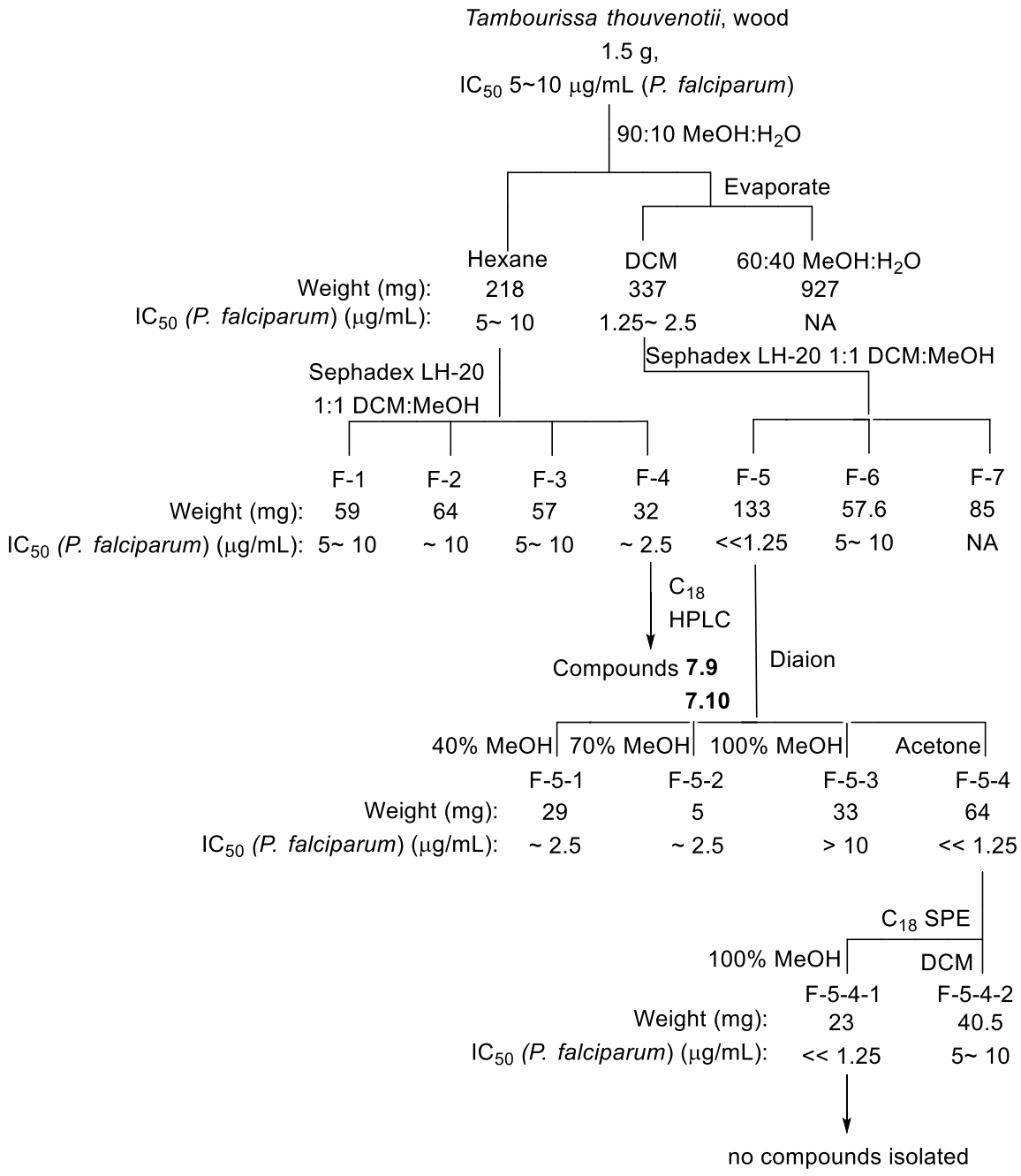


7.9



7.10

Figure 7-2-4. Structures of Compounds **7.9** and **7.10**

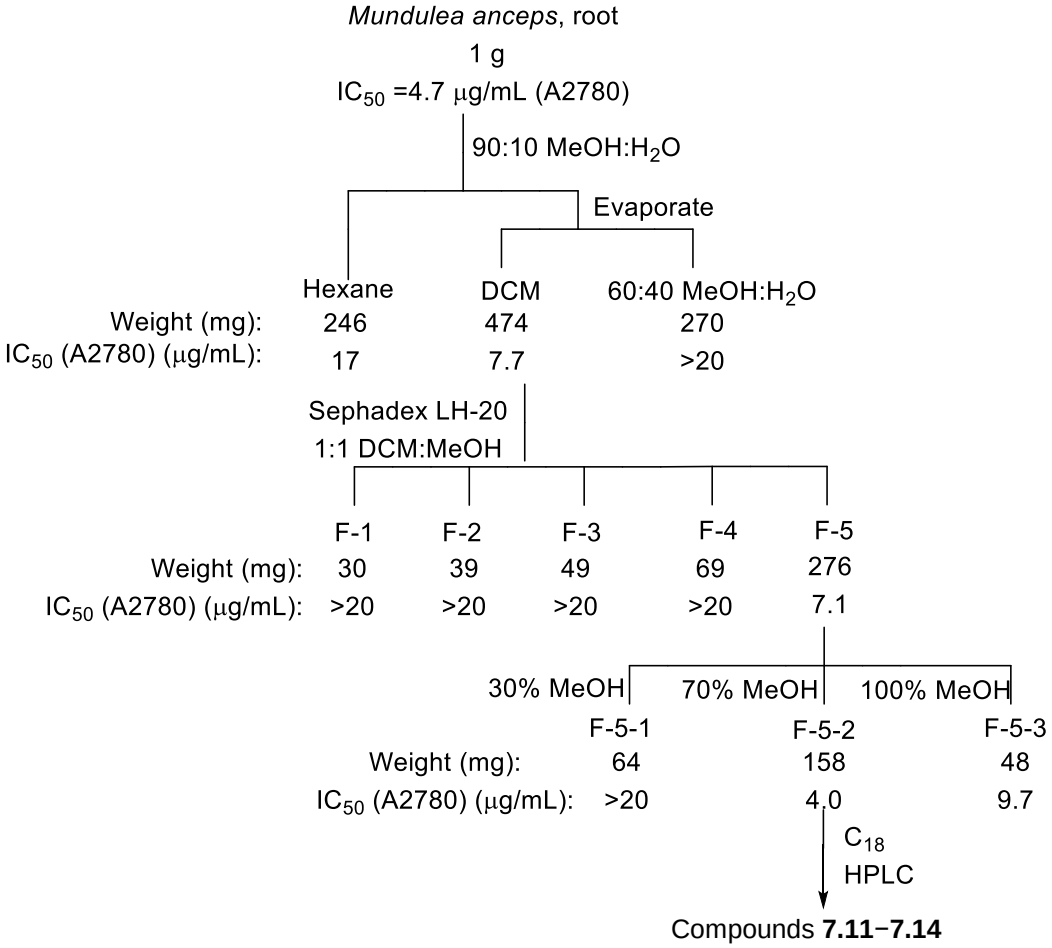


Scheme 7-2-4 Bioassay-guided separation of *Tambourissa thouvenotii* extract

7.3 Investigation of Other Anticancer Extracts

7.3.1 *Mundulea anceps* (Fabaceae) MG4344

As part of an International Cooperative Biodiversity Group (ICBG) program, the ethanol extract of the leaf of *Mundulea anceps* was investigated due to its moderate antiproliferative activity against the A2780 human ovarian cancer cell line assay with an IC_{50} value of 4.7 μg/mL. The crude extract (1 g, IC_{50} = 4.7 μg/mL) was fractionated by liquid-liquid partition, open column chromatography on Sephadex LH-20, C_{18} SPE and followed by C_{18} HPLC. These procedures yielded four known flavanones (**7.11**–**7.14**) (Figure 7-3-1). The detailed fractionation procedures are shown in scheme 7-3-1.



Scheme 7-3-1 Bioassay-guided separation of *Mundulea anceps* extract

The structures of 6,8-diprenylaromadendrin (**7.11**),[21] lonchocarpol A (**7.12**)[22] and mundulea-flavanone B (**7.13**)[23] were elucidated by mass spectrometry, NMR spectroscopies, optical rotation, and circular dichroism data in comparison with the literature. Compound **7.14** was identified as the previously isolated chlorospermin from a *Glycosmis sp.* (Rutaceae).[24] However its optical rotation was opposite to that reported.[24] The absolute configuration of **7.14** was confirmed by analysis of its circular dichroism (CD) spectroscopic data.

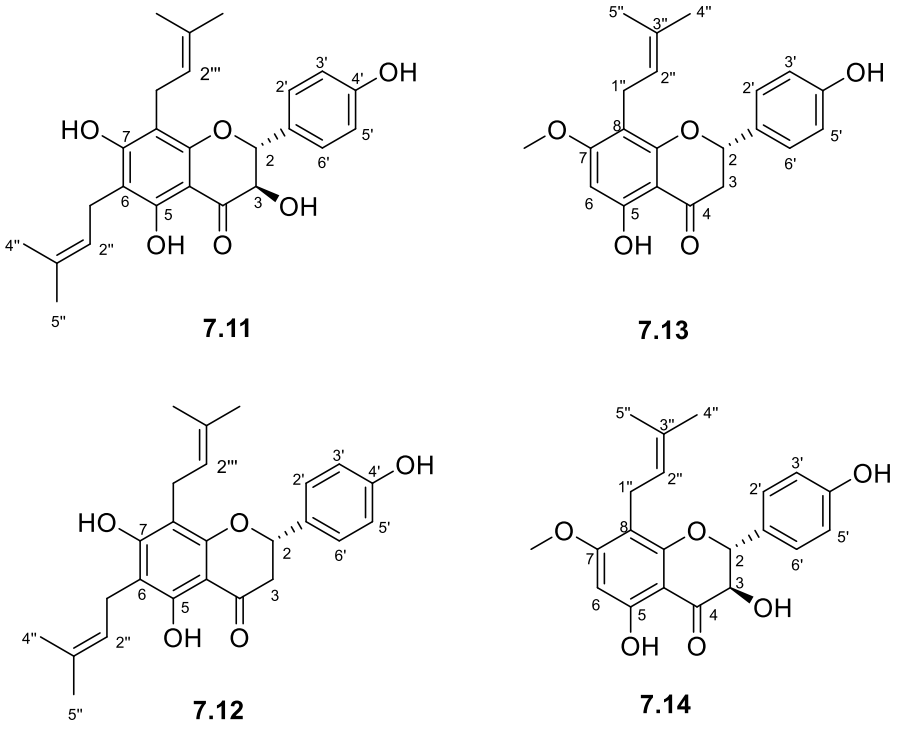


Figure 7-3-1. Structures of Compounds **7.11**−**7.14**

Compound **7.14** was obtained as a light yellow amorphous solid. The positive HRESIMS gave a peak at *m/z* 371.1462 $[M+H]^+$ (calcd for $C_{21}H_{23}O_6^+$, 371.1489) indicating the formula of $C_{21}H_{22}O_6$. Compound **7.14** showed identical ^1H and ^{13}C NMR spectroscopic data as chlorospermin. However, an opposite optical rotation value, $[\alpha]_D^{23}$ = +14 (c = 0.0675, $CHCl_3$), was obtained, in

comparison with the literature data $[\alpha]_D^{20} = -18$ (c = 0.4, $CHCl_3$).[24] A large coupling constant (*J* = 12 Hz) between 2-H and 3-H were observed, suggesting compound **7.14** was a 2,3-*trans*-dihydroflavonol derivative.[25] Considering the optical rotation value, compound **7.14** might be an enantiomer of chlorospermin. In order to confirm the absolute configuration of **7.14**, its CD spectroscopy was analyzed. The CD spectrum gave a positive Cotton effect in the 320 nm region due to n→π* transition, and a negative Cotton effect at 291 nm due to π→π* transition.[25-26] Similar Cotton effects were also observed from related 3-hydroxyflavanones with (*2R,3R*) stereochemistry,[25-27] therefore the stereochemistry of **7.14** was determined as (*2R,3R*). The literature reported the opposite optical rotation, most probably because the literature compound was the enantiomer of **7.14**.

Compounds **7.11−7.14** were evaluated for their antiproliferative activity with the A2780 human ovarian cancer cell line assay. Compounds **7.11−7.13** exhibited weak to moderate activity with IC_{50} values of 24 ± 2 μM, 14 ± 1 μM and 29 ± 3 μM. Compound **7.14** was inactive in the ±assay (IC_{50} > 20 μg/mL). The assay result suggested that the presence of two isoprene units on the A-ring, and a hydroxyl group at C-3 is preferred for better biological activity.

7.3.2 *Antiaris madagascariensis* (Moracreae) MG3973

In a continuing research to identify novel antiproliferative agents, an ethanol extract of the leaves of *Antiaris madagascariensis* was obtained. The genus *Antiaris* belongs to the Moraceae family, and has been well investigated. One of the most intensively investigated species is *Antiaris toxicaria*, which was previously found to contain a variety of cardiac glycosides with potent cardiotonic and antiproliferative activities.[28-29] Compounds isolated from this related species

include cardenolides,[30-31] prenylflavanones,[32] phenylpropanoid and lignan derivatives,[33] and coumarin derivatives.[34] Since the species of *Antiaris madagascariensis* was not previously investigated, and the crude extract displayed strong inhibitory activity against the A2780 human ovarian cancer cell line assay with an IC_{50} value of 3.9 μg/mL, the extract was selected for further investigation. The crude extract (2 g, IC_{50} = 3.9 μg/mL) was fractionated by liquid-liquid partition, open column chromatography on Sephadex LH-20, C_{18} SPE and by C_{18} HPLC. Bioassay-guided fractionation led to the isolation of the known malayoside [35] (**7.15**) (Figure 7-3-2) with potent antiproliferative activity with an IC_{50} value of 0.22 ± 0.02 μM. The detailed fractionation procedures are shown in scheme 7-3-2.

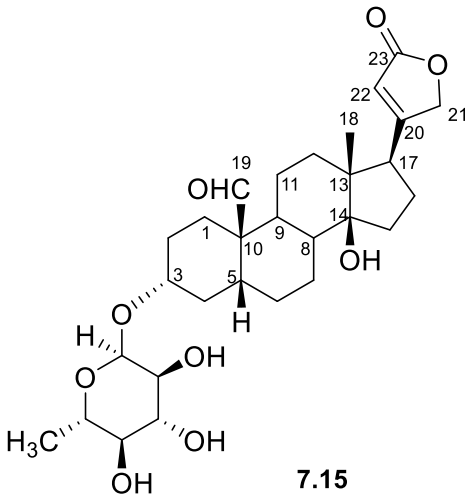
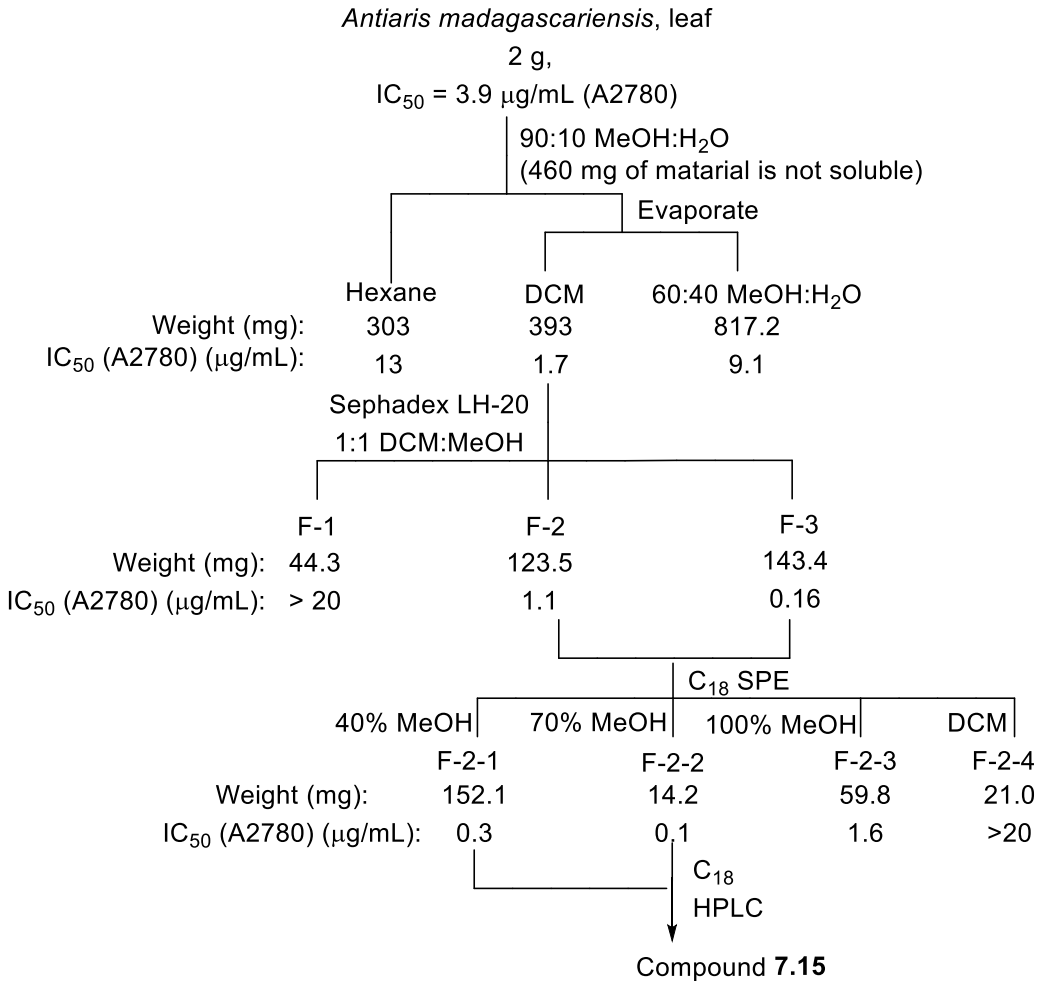


Figure 7-3-2. Structure of compound **7.15**

The structure of **7.15** was dereplicated by its HRESIMS and ^{1}H NMR spectroscopic data.[28, 36] Throughout the partitioning of this extract, several fractions also showed moderate to weak bioactivity. The most active compound in the extract was identified as the well investigated malayoside.[28-29] Therefore, those fractions with moderate to weak activities were not investigated since they were likely to contain known compounds.

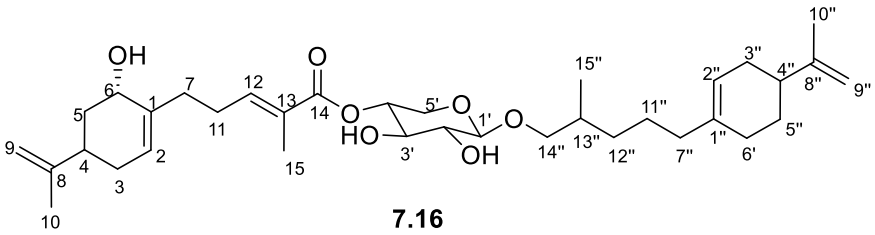


Scheme 7-3-2 Bioassay-guided separation of *Antiaris madagascariensis* extract

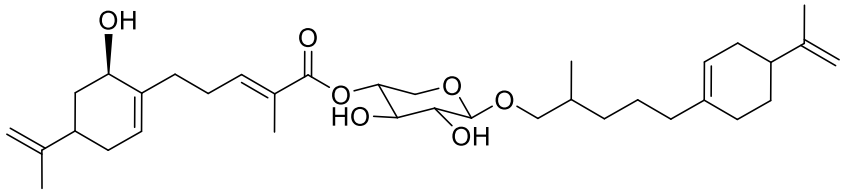
7.3.3 *Nidorella resedifolia* (Asteraceae) 60073-11G

The methanol extract of *Nidorella resedifolia* was selected for investigation since its hexane soluble materials displayed weak antiproliferative activity against the A2780 human ovarian cell line assay. Previous research on several species belonging to the *Nidorella* genus indicated these species contained diterpenes and sesquiterpene derivatives.[37-38] However the species of *Nidorella resedifolia* had never previously been studied for potential antiproliferative

agents. Bioassay-guided fractionation (Scheme 7-3-2) of the crude extract (300 mg, IC_{50} > 20 μg/mL) by liquid-liquid partition, open column chromatography on Sephadex LH-20, and C_{18} HPLC led to the isolation of two known sesquiterpene xylosides (**7.16**-**7.17**) (Figure 7-3-3) with moderate antiproliferative activities with IC_{50} values of 11 ± 1 μM and 12 ± 1 μM respectively. This is the first report of the antiproliferative activity of these sesquiterpene xylosides.



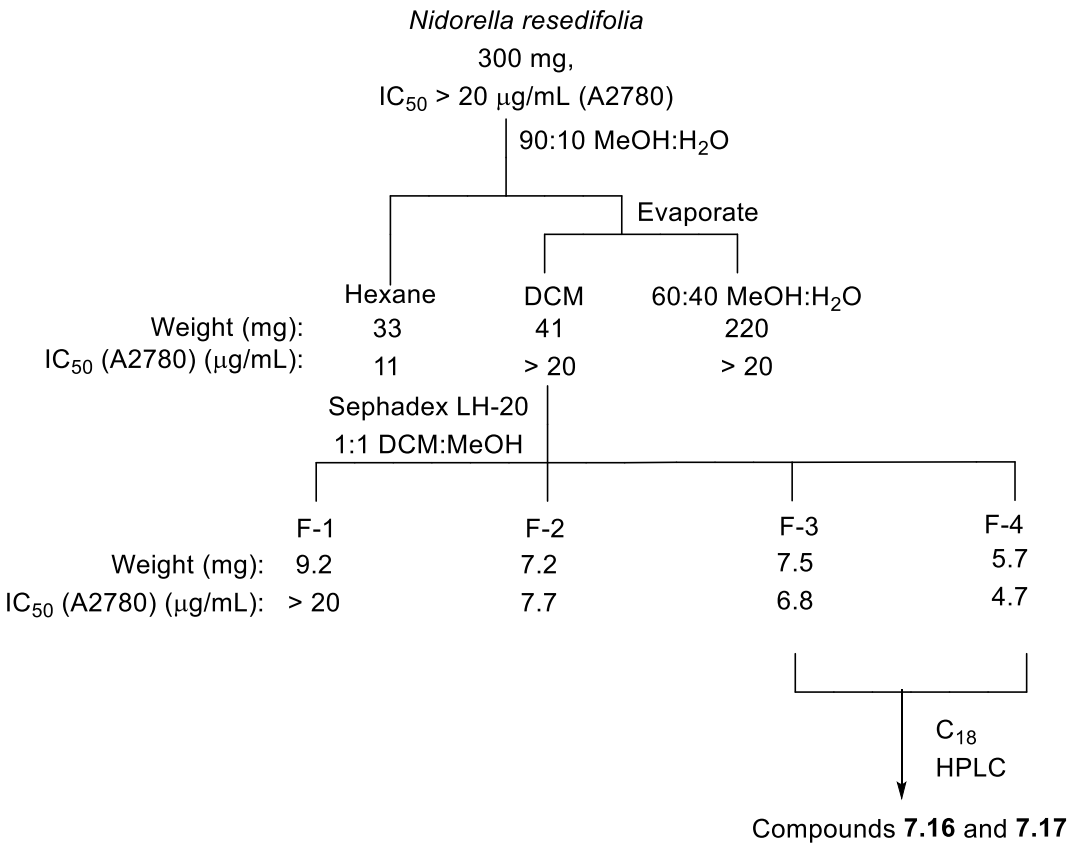
7.16



7.17

Figure 7-3-3. Structures of Compounds **7.16** and **7.17**

Structures of the two compounds were elucidated by comparison of their HRESIMS and ^{1}H NMR data with literature data.[38]



Scheme 7-3-3 Bioassay-guided separation of *Nidorella resedifolia* extract

7.4 Suspended Extracts

The extracts listed in this part were selected for investigation due to their bioactivity during initial screening. However, no compounds were identified in the bioassay guided fractionation. Fractionation procedures resulted in either inactive fractions against the bioassay or inadequate materials for further investigation.

7.4.1 *Asteropeia rhopaloides* (Asteropiaceae) MG 0277-0276

Ethanol extracts of the bark and stem of *Asteropeia rhopaloides* (Asteropiaceae) were investigated due to their antiplasmodial activity (IC_{50} ~ 1 μg/mL, IC_{50} ~ 2 μg/mL). The extracts were subjected to liquid-liquid partition, Sephadex LH-20 open column chromatography and preparative C_{18} HPLC. However, the active compounds were not identified, due to the complexity and limited amount of the active fraction.

7.4.2 *Eustachys paspaloides* (Poaceae) 71089-2H

The methanol extract of *Eustachys paspaloides* was selected for investigation due to its antiproliferative activity (IC_{50} = 2 μg/mL). The crude extract (600 mg) was subjected to liquid-liquid partition, open column chromatography on Sephadex LH-20, C_{18} SPE and C_{18} HPLC. Unfortunately, the only active component that could be identified was the phthalate ester, bis (2-ethylhexyl) 1,2-benzenedicarboxylate (**7.18**).This compound was most probably a contaminant arising from the use of Tygon tubing to transfer solvent during extract preparation. Other minor active compounds could not be identified due to the small amount present. The extract was not investigated further.

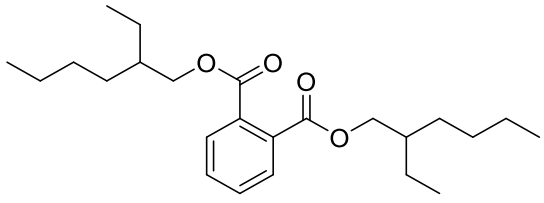


Figure 7-4-1. Structure of compound **7.18**

7.4.3 *Myrsiphyllum asparagoides* (Asparagaceae) 71091-11E

The methanol extract of *Myrsiphyllum asparagoides* was investigated due to its reported antiproliferative activity (IC_{50} = 2.9 μg/mL, A2780). The extract was subjected to liquid-liquid partitioning. After liquid-liquid partition, all fractions showed decreased bioactivity (IC_{50} > 20 μg/mL); also, retesting of the original extract showed a loss of bioactivity (IC_{50} > 20 μg/mL). This extract was not investigated further.

7.4.4 *Rhopalocarpus triplinervis* MG 4242

The methanol extract of the bark of *Rhopalocarpus triplinervis* was investigated due to its reported antiproliferative activity (IC_{50} = 12 μg/mL, A2780). The extract was subjected to liquid-liquid partitioning. After liquid-liquid partition, all fractions showed decreased bioactivity (IC_{50} > 20 μg/mL); also, retesting of the original extract showed a loss of bioactivity (IC_{50} > 20 μg/mL). This extract was not investigated further.

7.4.5 *Acoelorrhaphe wrightii* (Arecaceae) 0039966-02H

The methanol extract of *Acoelorrhaphe wrightii* was investigated due to its initial antiplasmodial activity (IC_{50} = 2 μg/mL). The extracts were subjected to liquid-liquid partition and Sephadex LH-20 column chromatography. Unfortunately, all of the fractions showed decreased bioactivity (IC_{50} > 20 μg/mL). Furthermore, retesting of the original extract showed a loss of bioactivity (IC_{50} > 20 μg/mL). These extracts were not investigated further.

7.4.6 *Cnestis polyphylla* (Connaraceae) MG 0256-0257

Ethanol extracts of the stem and leaf of *Cnestis polyphylla* were investigated due to their initial antiplasmodial activity (IC_{50} around 1.25 to 2.5 μg/mL). The extracts were subjected to liquid-liquid partition, Sephadex LH-20 column chromatography and Si-gel column chromatography. After successive column chromatography, the fractions showed decreased

bioactivity (IC_{50} > 20 μg/mL). Furthermore, recombining the fractions showed a loss of bioactivity (IC_{50} > 20 μg/mL). These extracts were not investigated further.

7.4.7 *Mitriostigma axillare* (Rubiaceae) 115275-3D

The ethanol extract of *Mitriostigma axillare* was selected for investigation due to the antiproliferative activity of its dichloromethane-soluble materials (IC_{50} = 3.1 μg/mL). The crude extract (800 mg) was subjected to liquid-liquid partition, open column chromatography on Sephadex LH-20 and Si-gel, followed by C_{18} HPLC. Unfortunately, the minor active compounds could not be identified due to the small amount present.

7.5 References

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Chapter 8: Summary and General Conclusions

In a continuing search for bioactive natural products, more than twenty plant extracts were selected for bioassay guided inspection. Thirteen of them were fractionated to yield nine new and twenty-seven known compounds. The research work indicated that plant materials continue to be an important source for natural products drug discovery.

Even though many known compounds were isolated, some of them showed new biological activities that were not previously reported. The structures of the known compounds were confirmed by comparison of spectroscopic properties with published data. The research challenge was to purify and determine the structures of the new bioactive compounds, from limited amounts of plant material. Extensive purification procedures were performed to ensure clean NMR spectroscopic data could be obtained, and the bioassay results of each compound were reliable. The structure elucidation of some bioactive compounds relied not only on extensive NMR experiments, but also to ECD experiments and chemical modifications.

Table 8-1 Summary of Antimalarial Natural Products Isolated

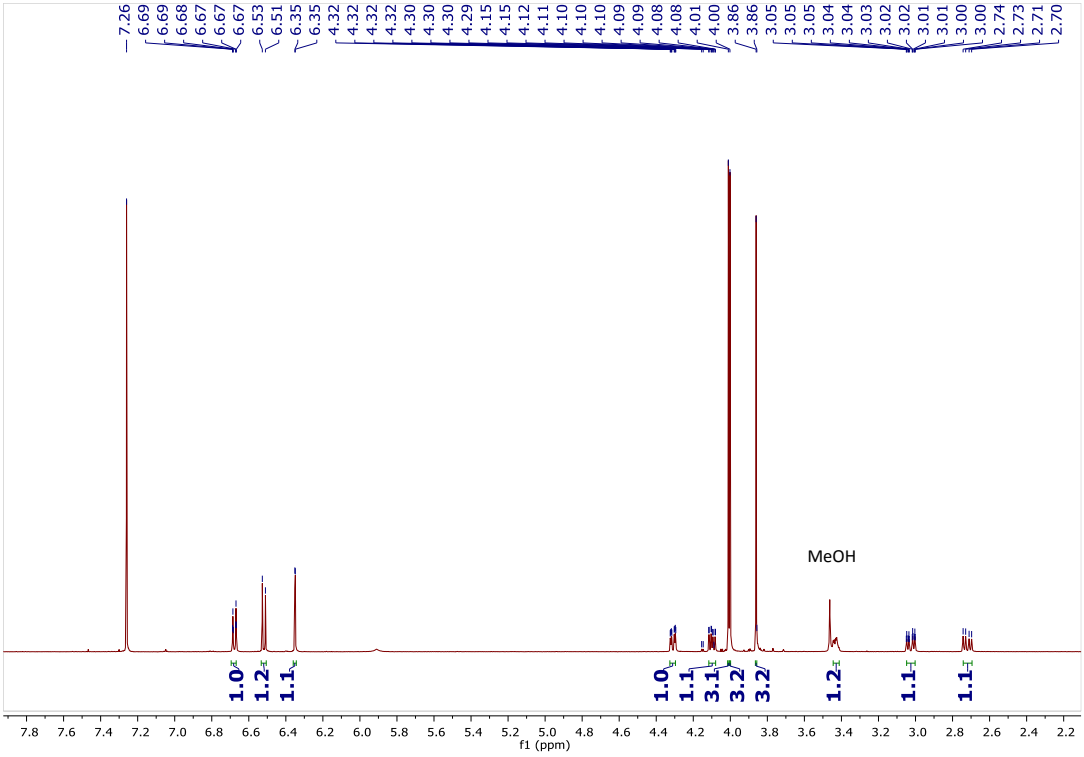
Compound	Plant	IC_{50} (μM)	New/Known
2.1	*Apoplanesia paniculata*	6 ± 2	Known
2.2	*Apoplanesia paniculata*	7 ± 1	Known
2.3	*Apoplanesia paniculata*	42 ± 5	Known
2.4	*Apoplanesia paniculata*	> 60	New
2.5	*Apoplanesia paniculata*	> 58	New
3.1	*Syncarpia glomulifera*	0.10 ± 0.02	New
3.2	*Syncarpia glomulifera*	4 ± 1	New
3.3	*Syncarpia glomulifera*	3.0 ± 0.4	Known
3.4	*Syncarpia glomulifera*	12 ± 0.1	Known
3.5	*Syncarpia glomulifera*	> 40	Known
3.6	*Syncarpia glomulifera*	> 90	Known
4.1	*Gutierrezia sarothrae*	10 ± 4	New
4.2	*Gutierrezia sarothrae*	> 44	New
6.1	*Polyalthia blume.*	5 ± 1	Known
6.2	*Polyalthia blume*	2.0 ± 0.4	Known
7.1	*Helenium montanum*	2.0 ± 0.2	Known
7.2	*Helenium montanum*	6 ± 1	Known
7.3	*Helenium montanum*	16 ± 1	Known
7.4	*Juniperus occidentalis*	> 57	Known
7.5	*Juniperus occidentalis*	> 60	Known
7.6	*Cadia ellisiana*	> 45	Known
7.7	*Cadia ellisiana*	43 ± 10	Known
7.8	*Cadia ellisiana*	23 ± 2	Known
7.9	*Tambourissa thouvenotii*	4.0 ± 0.4	Known
7.10	*Tambourissa thouvenotii*	14 ± 3	Known

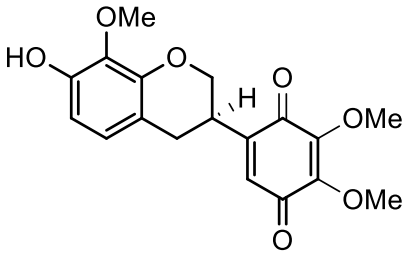
Table 8-2 Summary of Antiproliferative Natural Products Isolated

Compound	Plant	IC_{50} (μM)	New/Known
2.1	*Apoplanesia paniculata*	7 ± 1	Known
2.2	*Apoplanesia paniculata*	21 ± 4	Known
2.3	*Apoplanesia paniculata*	> 60	Known
2.4	*Apoplanesia paniculata*	> 60	New
2.5	*Apoplanesia paniculata*	> 58	New
5.1	*Leptaulus citroides*	3.0 ± 0.3	New
5.2	*Leptaulus citroides*	10 ± 1	New
5.3	*Leptaulus citroides*	> 16	New
5.4	*Leptaulus citroides*	2.0 ± 0.2	Known
6.1	*Polyalthia blume*	9 ± 1	Known
6.2	*Polyalthia blume*	17 ± 2	Known
7.11	*Mundulea anceps*	24 ± 2	Known
7.12	*Mundulea anceps*	14 ± 1	Known
7.13	*Mundulea anceps*	29 ± 3	Known
7.14	*Mundulea anceps*	> 54	Known
7.15	*Antiaris madagascariensis*	0.22 ± 0.02	Known
7.16	*Nidorella resedifolia*	11 ± 1	Known
7.17	*Nidorella resedifolia*	12 ± 1	Known

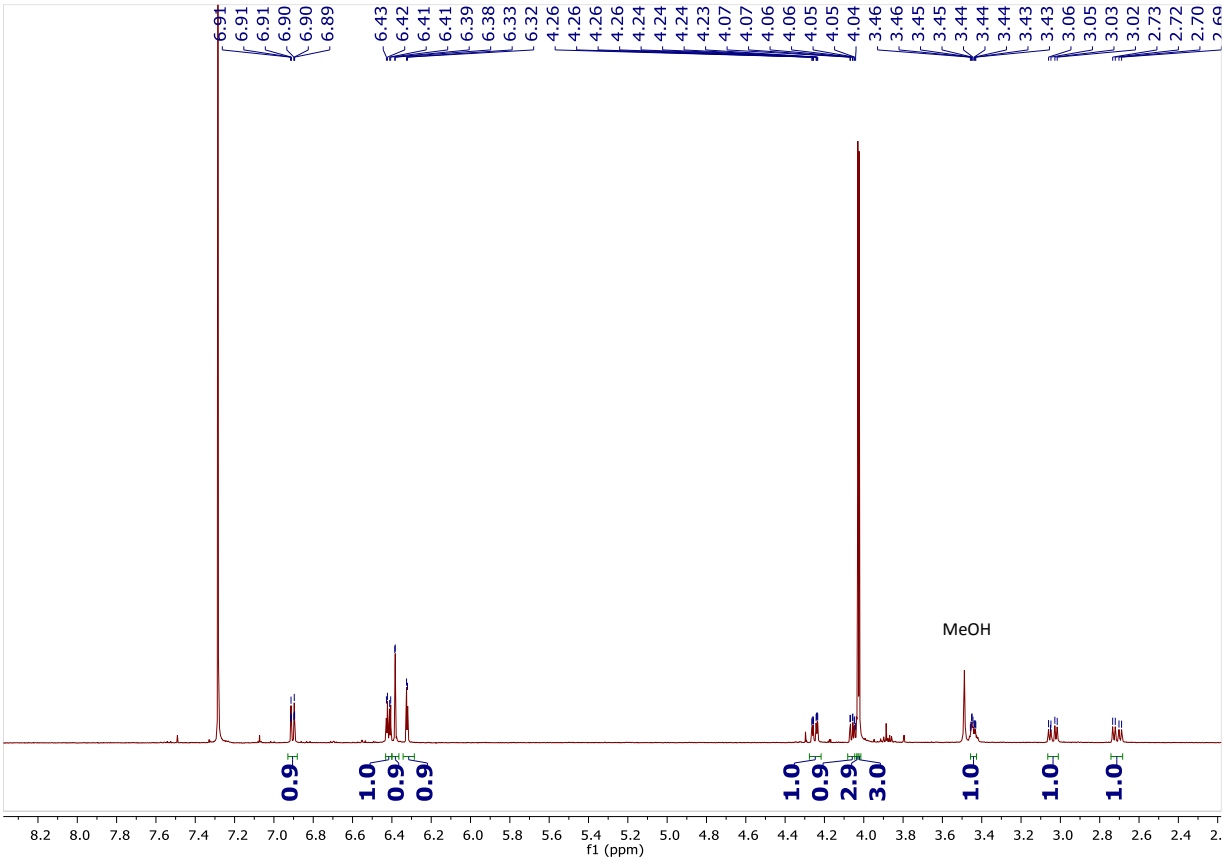
Chapter 9: Supporting Information

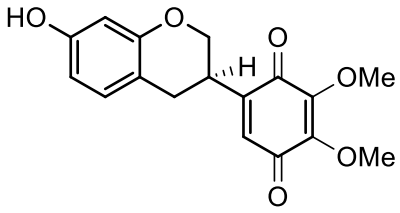
9.1 ^{1}H NMR spectrum of compound **2.1** ($CDCl_3$, 500 MHz)



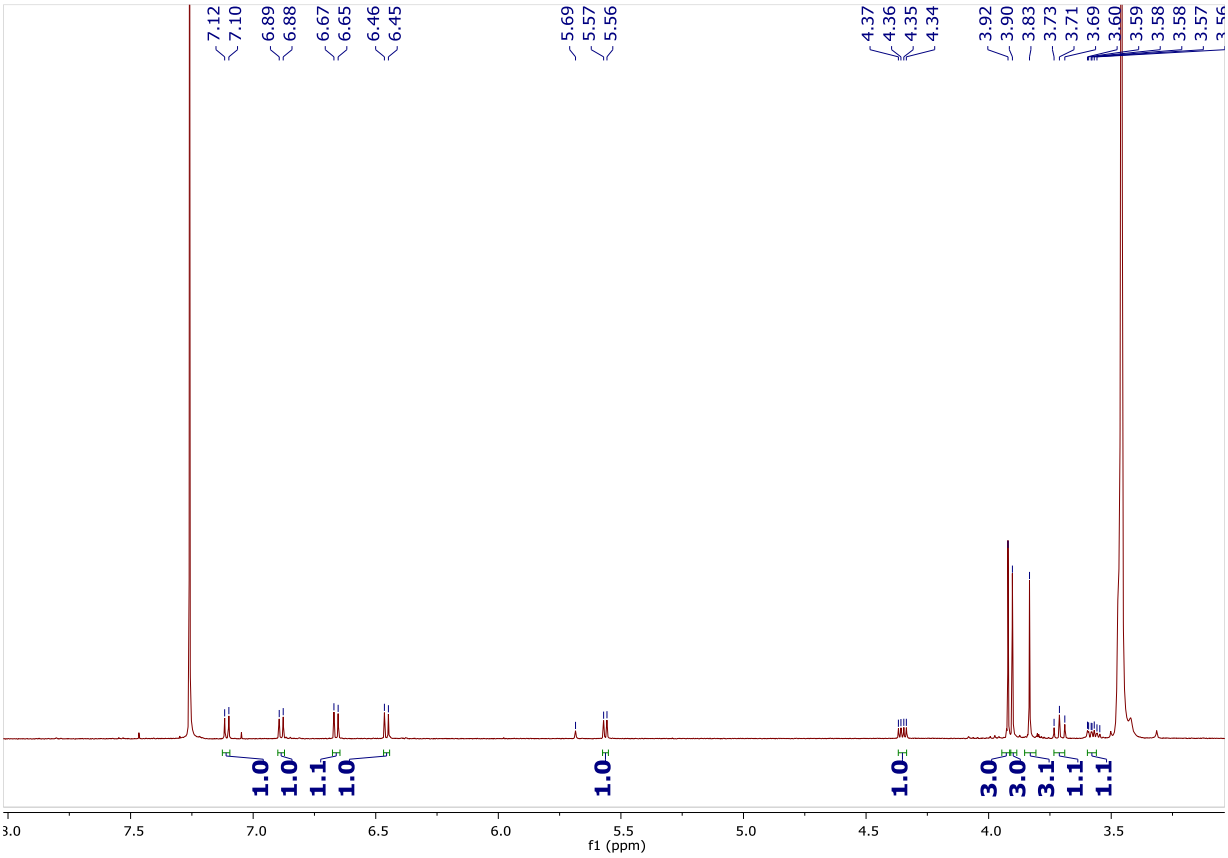


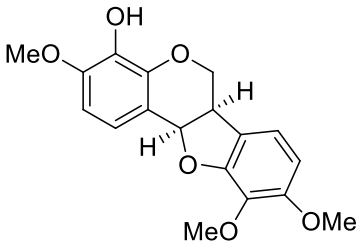
9.2 ^{1}H NMR spectrum of compound **2.2** ($CDCl_3$, 500 MHz)





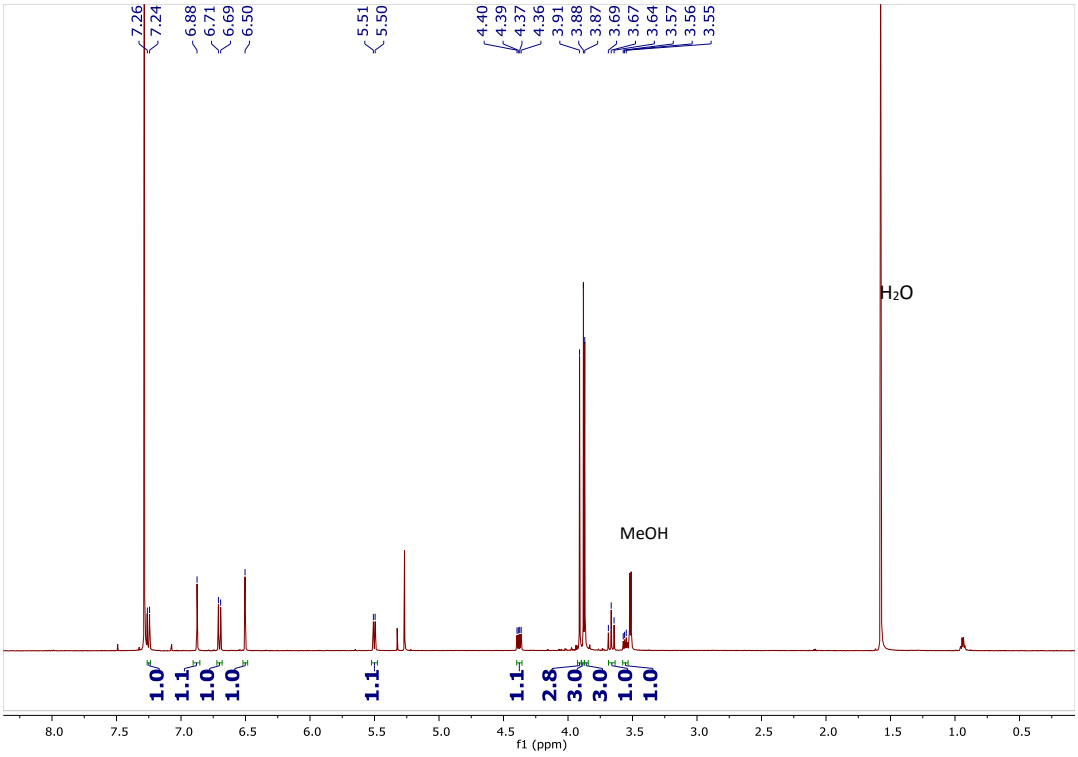
9.3 ^{1}H NMR spectrum of compound **2.3** ($CDCl_3$, 500 MHz)

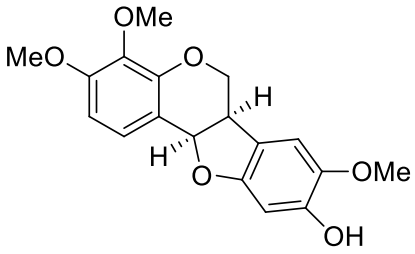




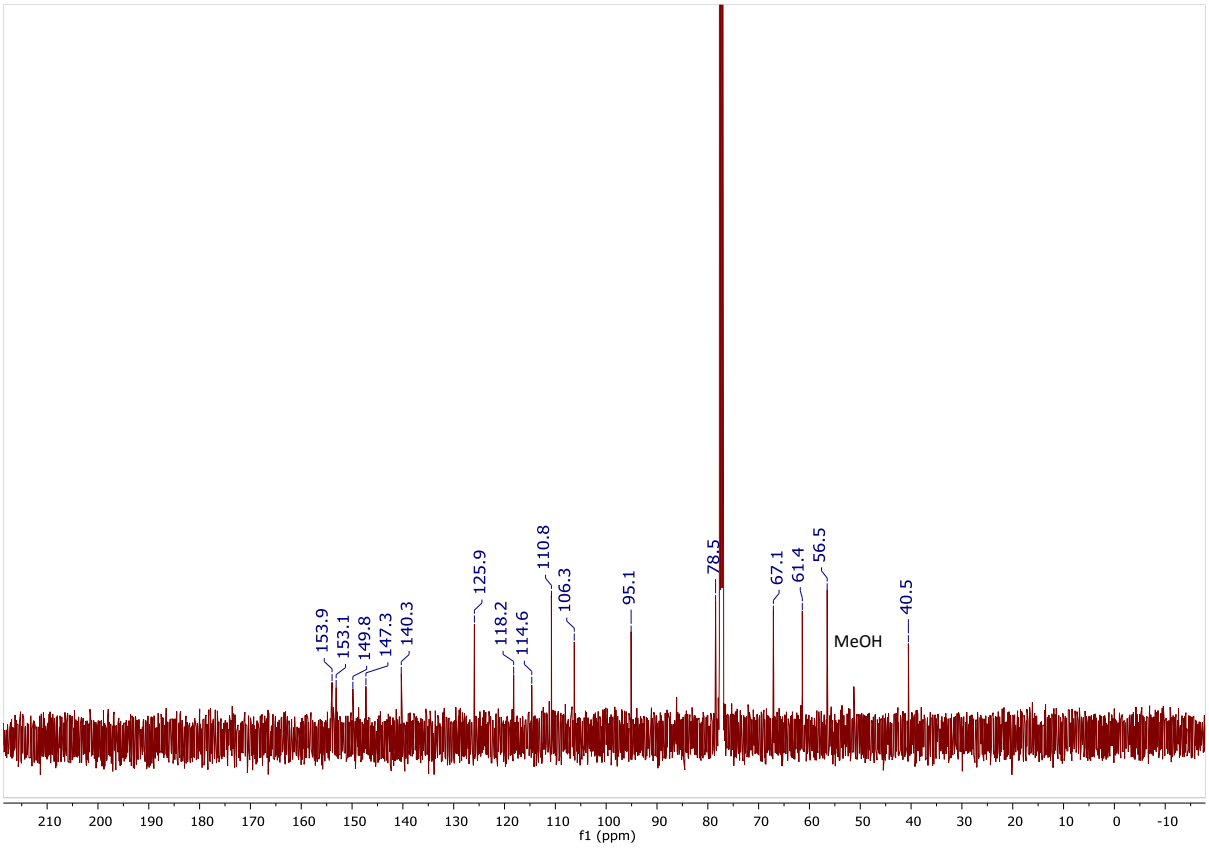
9.4 ^{1}H and ^{13}C NMR spectra of **2.4**

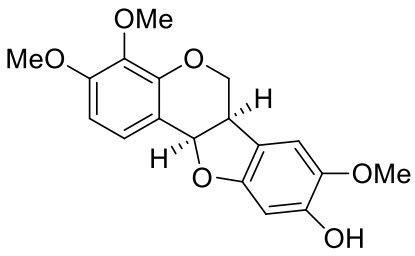
9.4.1 ^{1}H NMR spectrum of compound **2.4** ($CDCl_3$, 500 MHz)





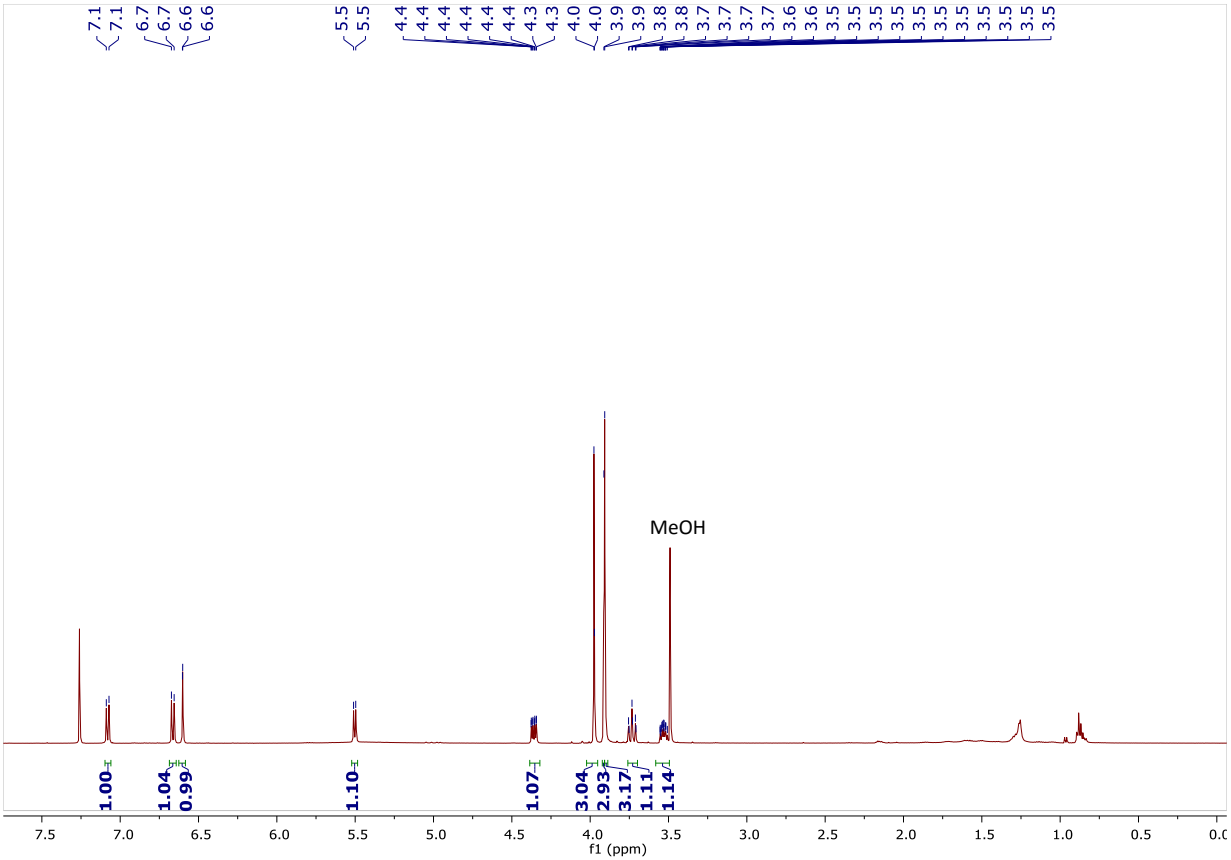
9.4.2 ^{13}C NMR spectrum of compound **2.4** ($CDCl_3$, 125 MHz)

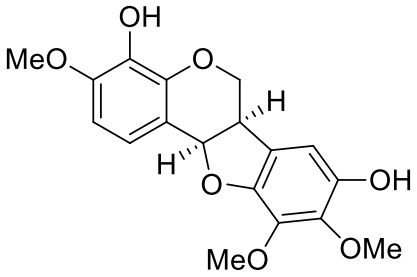




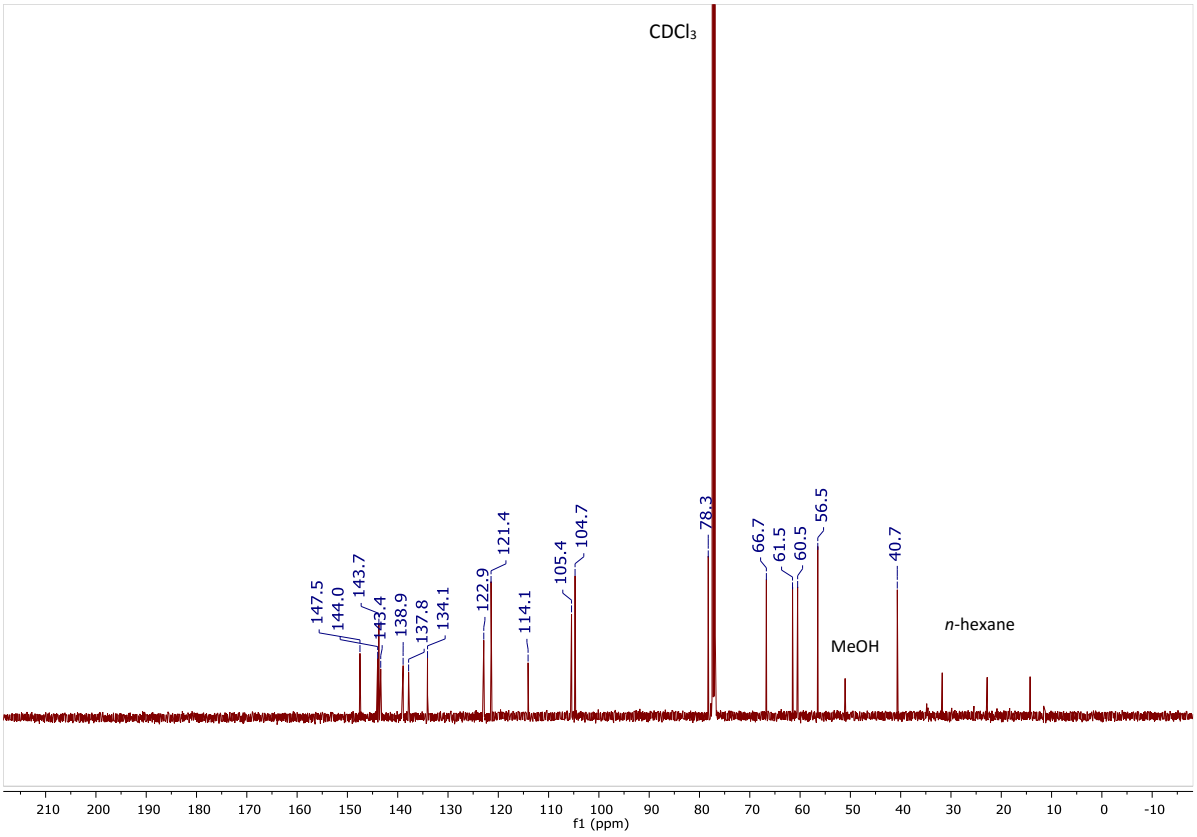
9.5 ^{1}H and ^{13}C NMR spectra of **2.5**

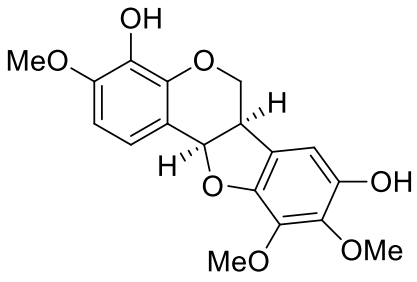
9.5.1 ^{1}H NMR spectrum of compound **2.5** ($CDCl_3$, 500 MHz)



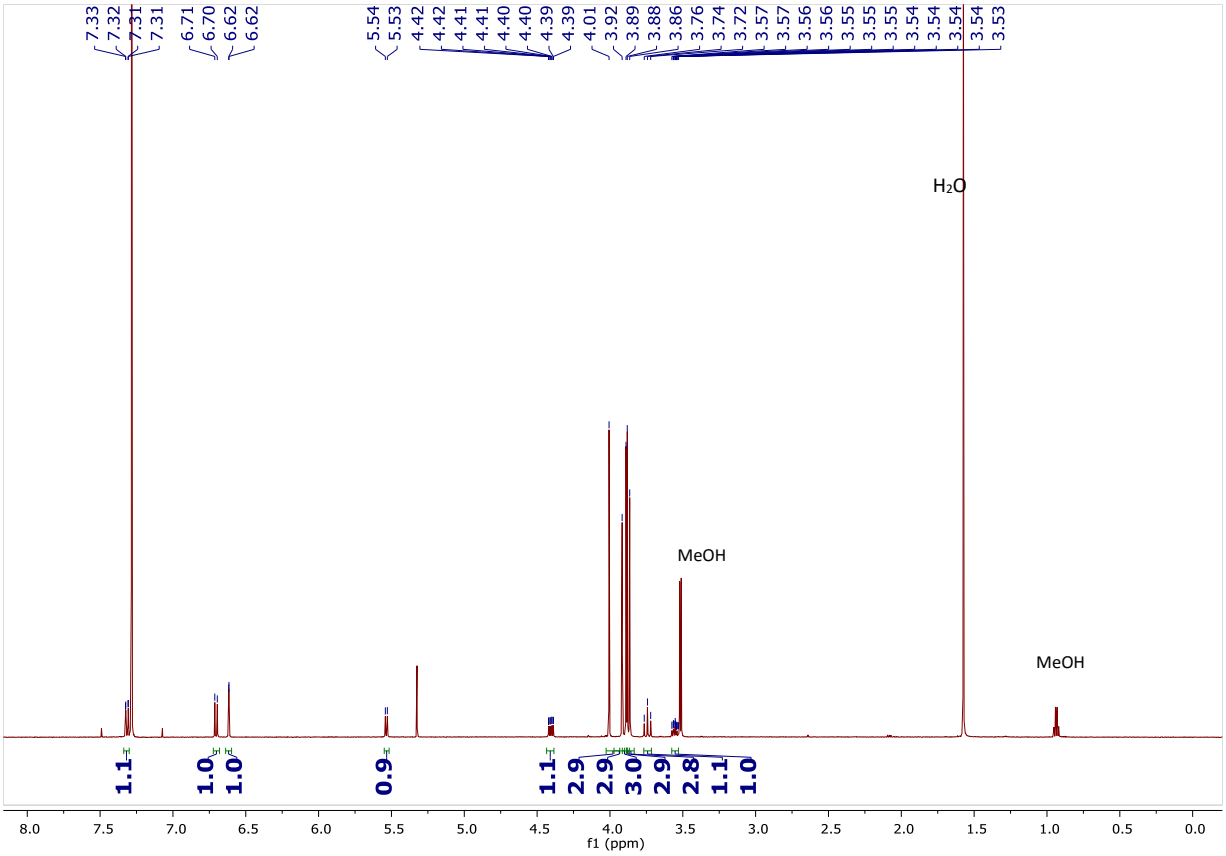


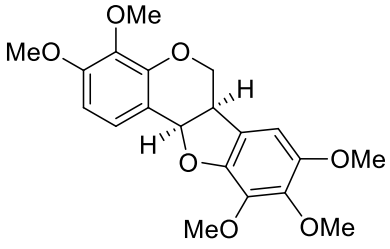
9.5.2 ^{13}C NMR spectrum of compound **2.5** ($CDCl_3$, 125 MHz)





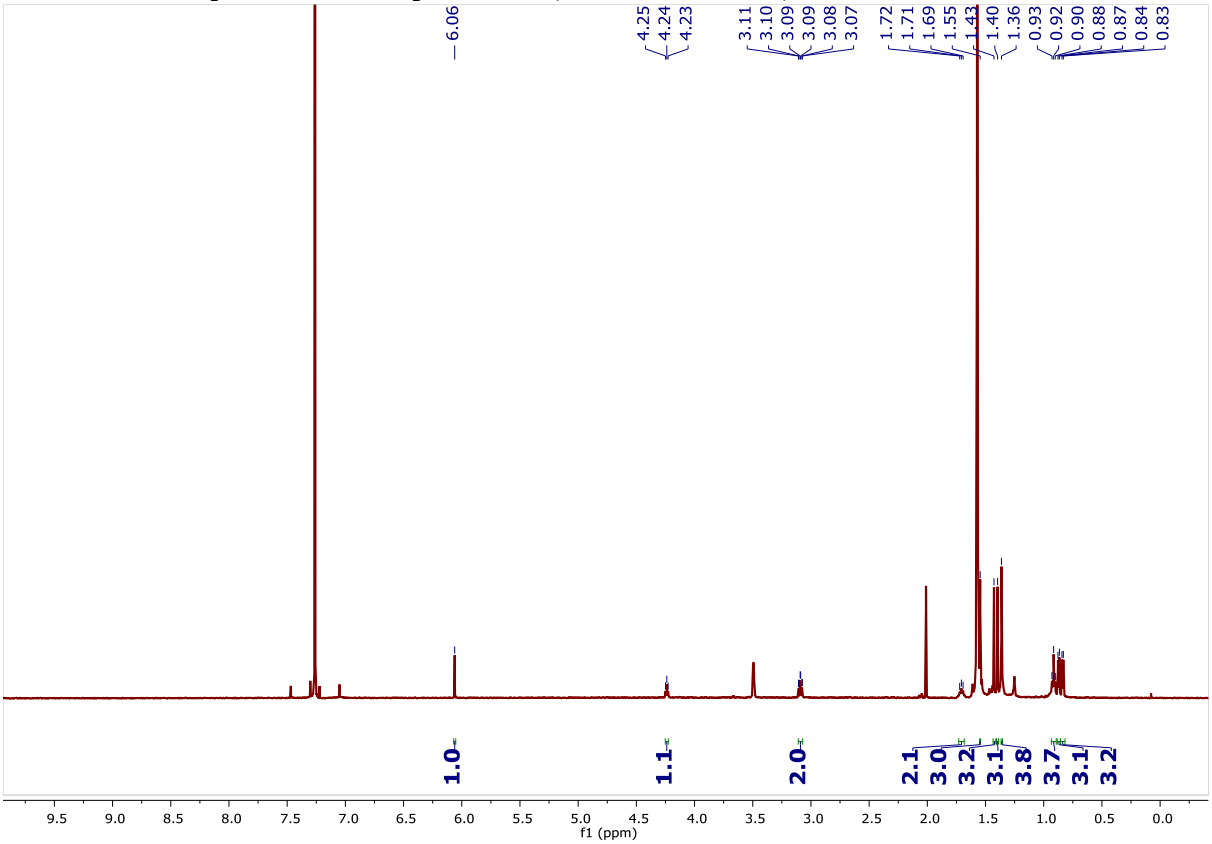
9.6 ^{1}H NMR spectrum of compound **2.6** ($CDCl_3$, 500 MHz)

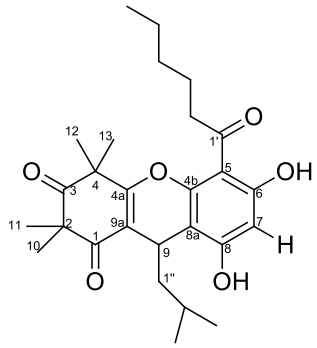




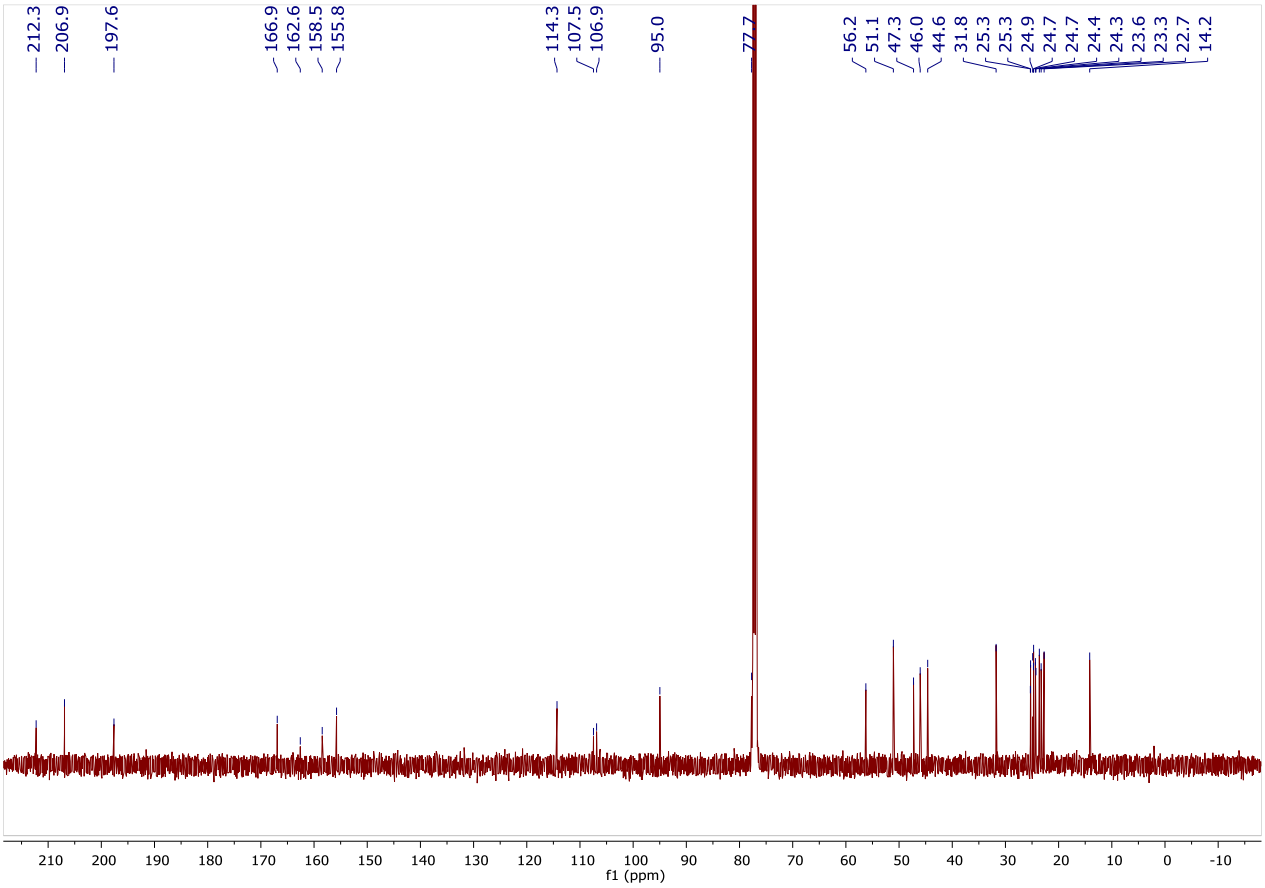
9.7 ^{1}H and ^{13}C NMR spectra of compound **3.1**

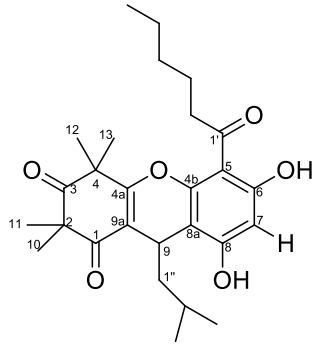
9.7.1 ^{1}H NMR spectrum of compound **3.1** ($CDCl_3$, 500 MHz)





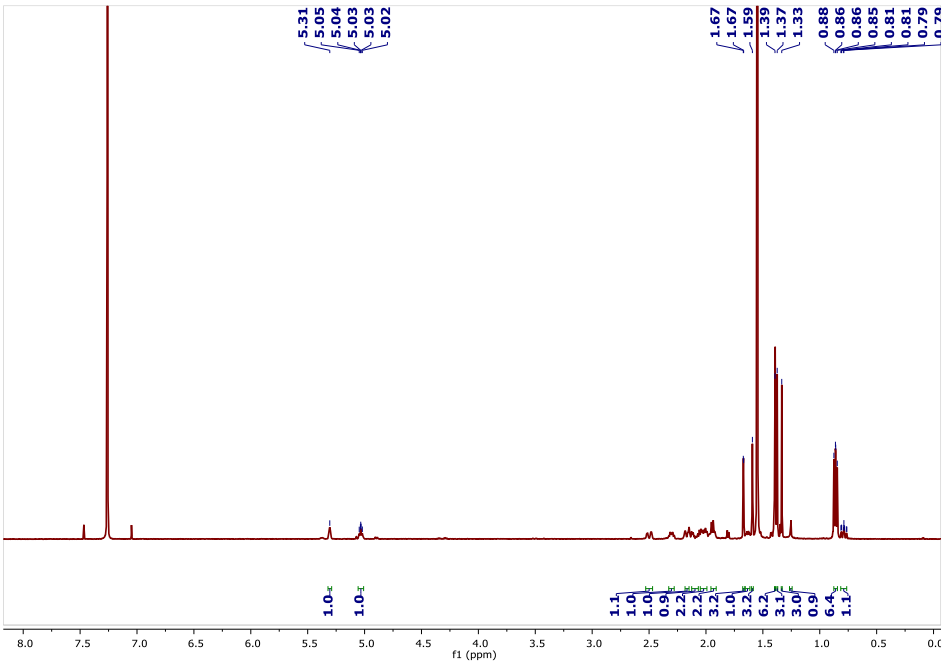
9.7.2 ^{13}C NMR spectrum of compound **3.1** ($CDCl_3$, 125 MHz)

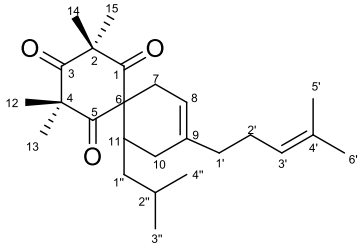




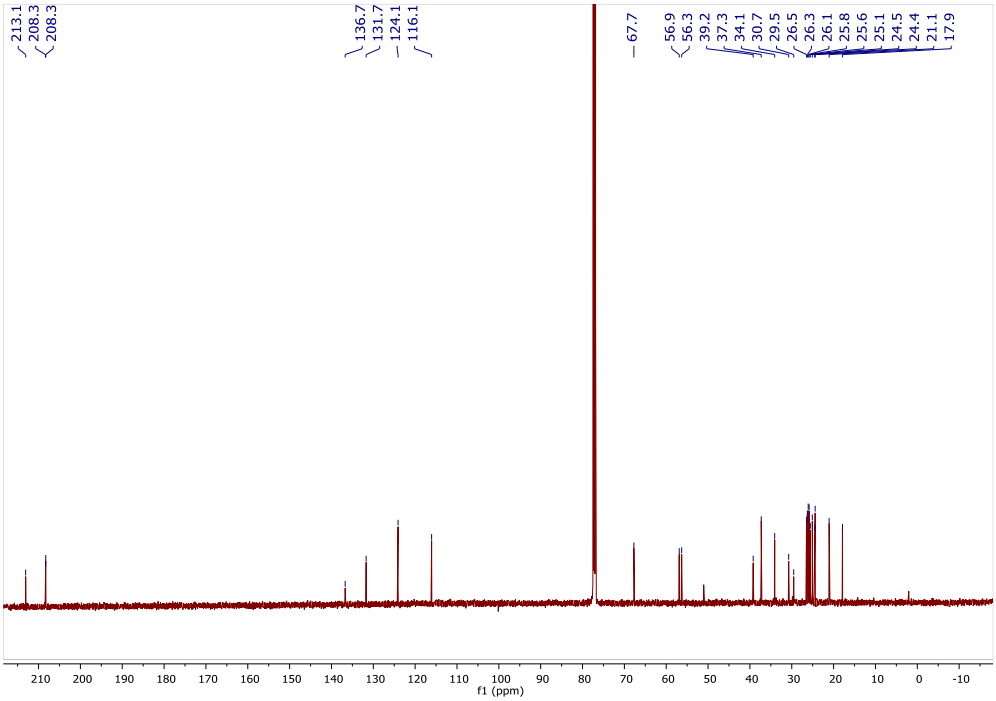
9.8 ^{1}H and ^{13}C NMR spectra of **3.2**

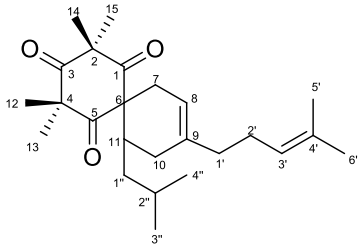
9.8.1 ^{1}H NMR spectrum of compound **3.2** ($CDCl_3$, 500 MHz)



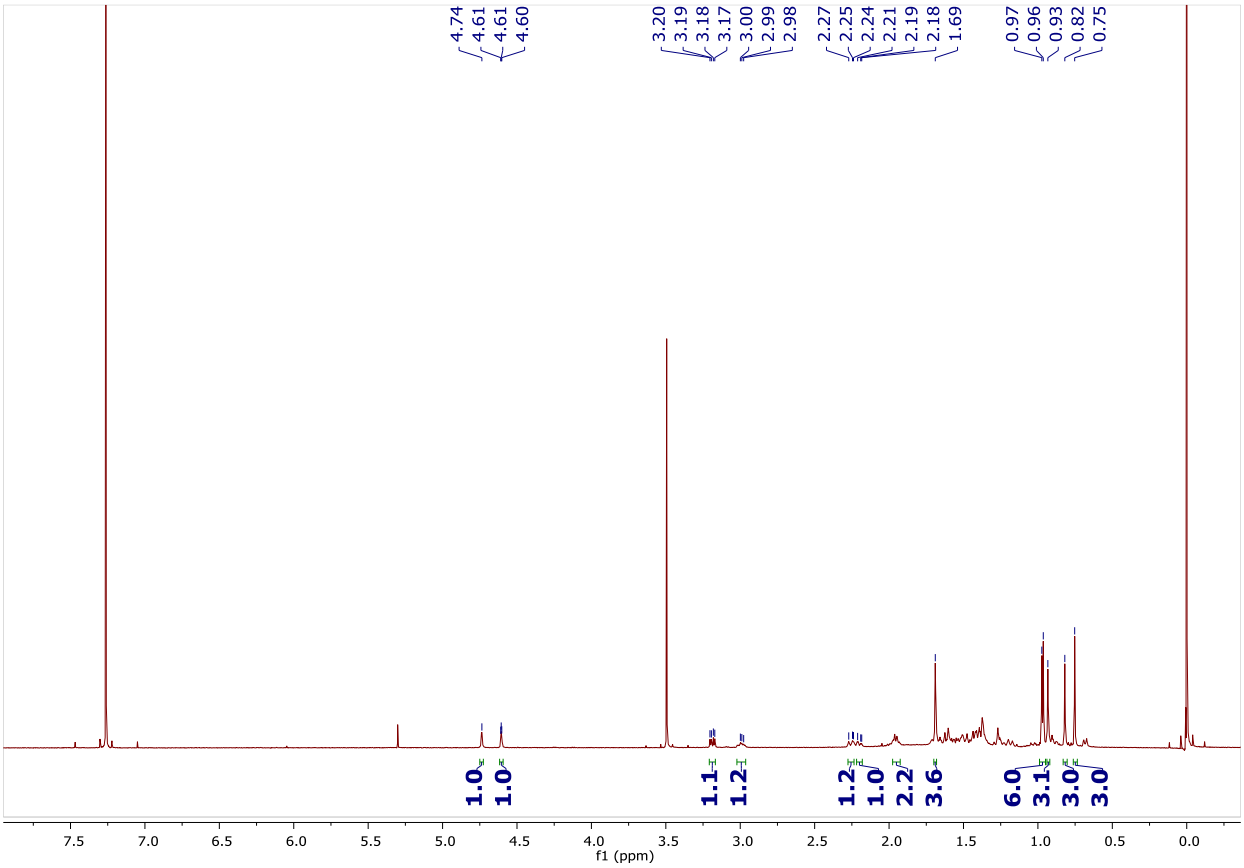


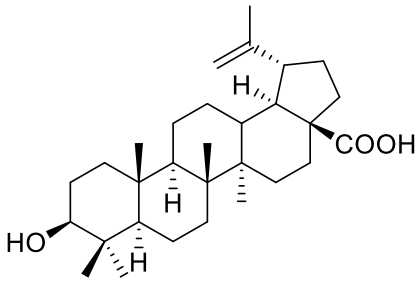
9.8.2 ^{13}C NMR spectrum of compound **3.2** ($CDCl_3$, 125 MHz)



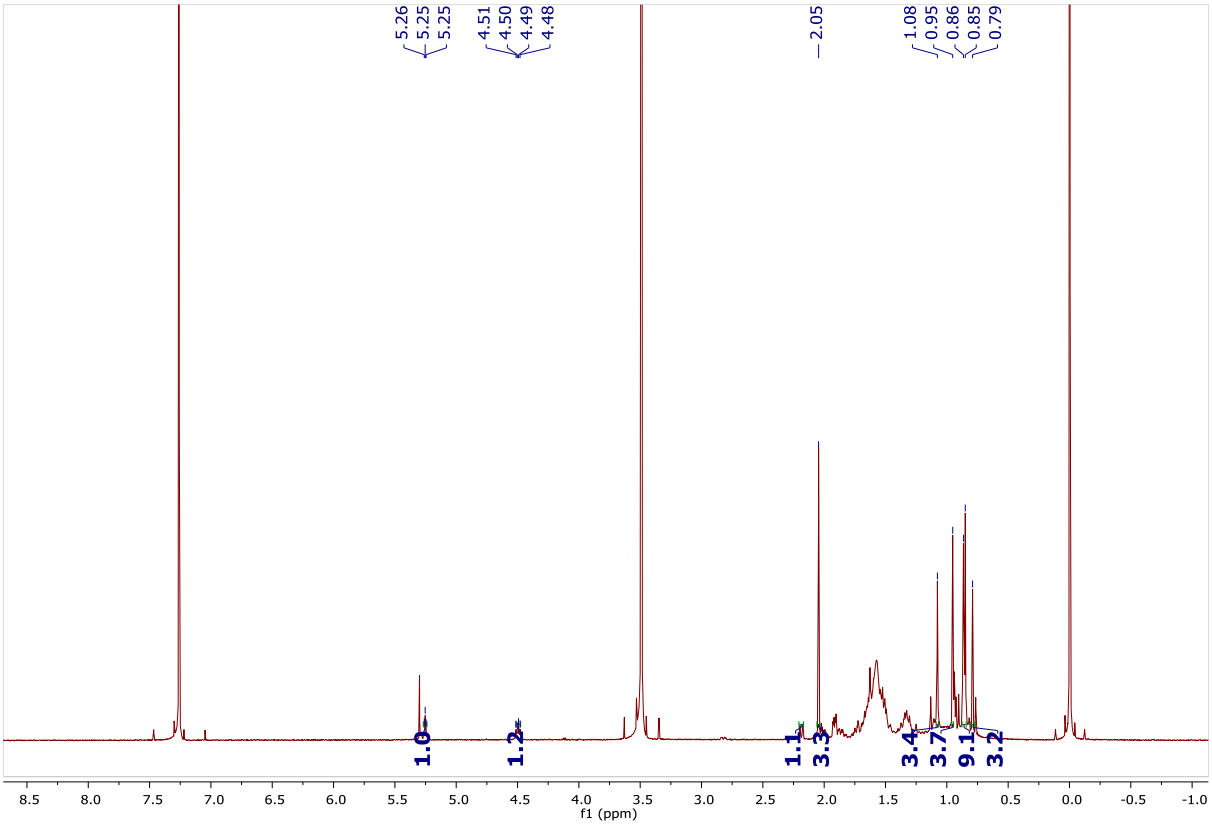


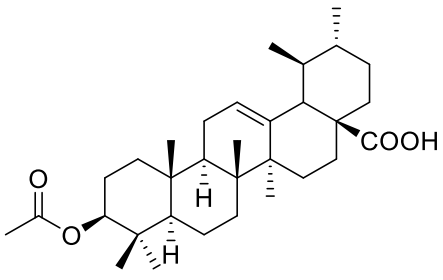
9.9 ^{1}H NMR spectrum of compound **3.3** ($CDCl_3$, 500 MHz)



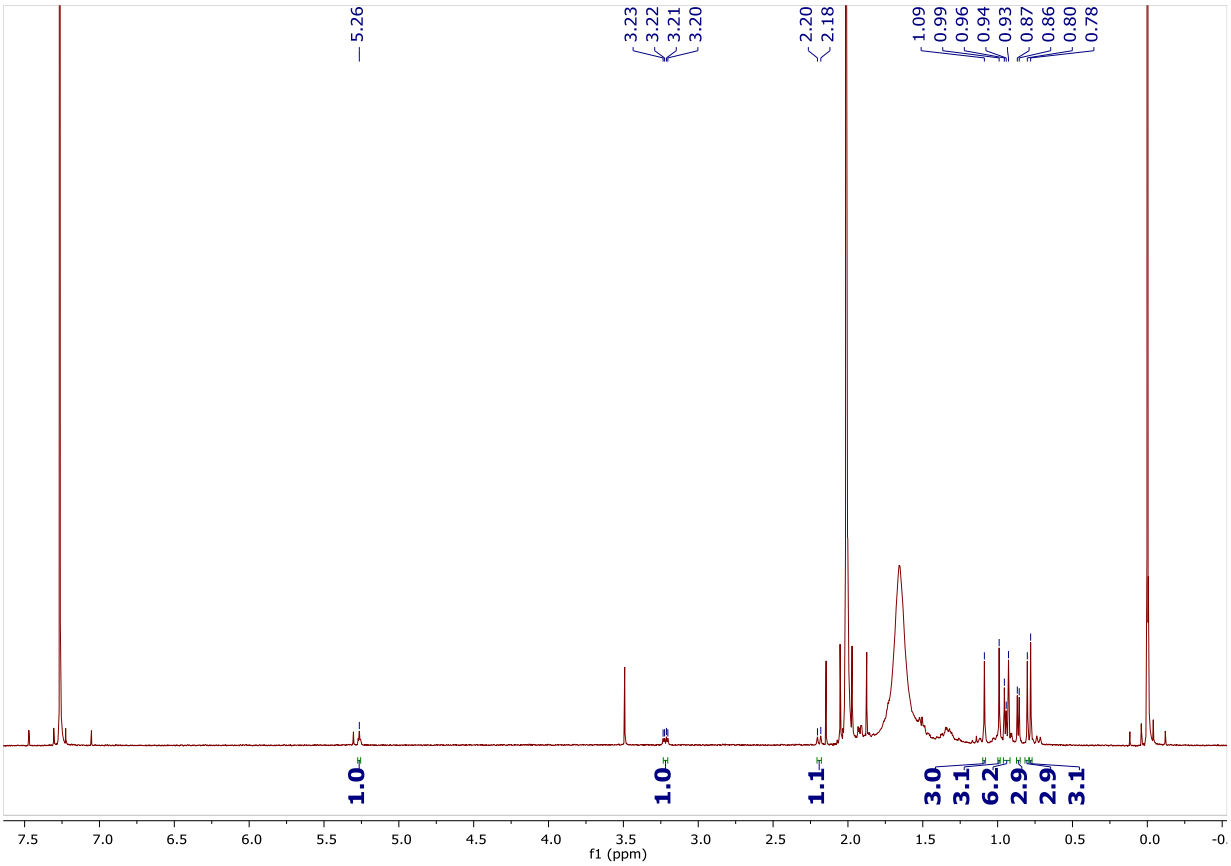


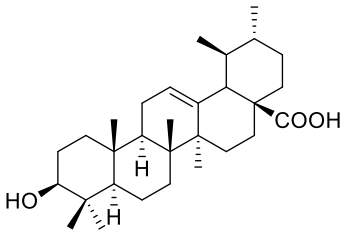
9.10 ^{1}H NMR spectrum of compound **3.4** ($CDCl_3$, 500 MHz)



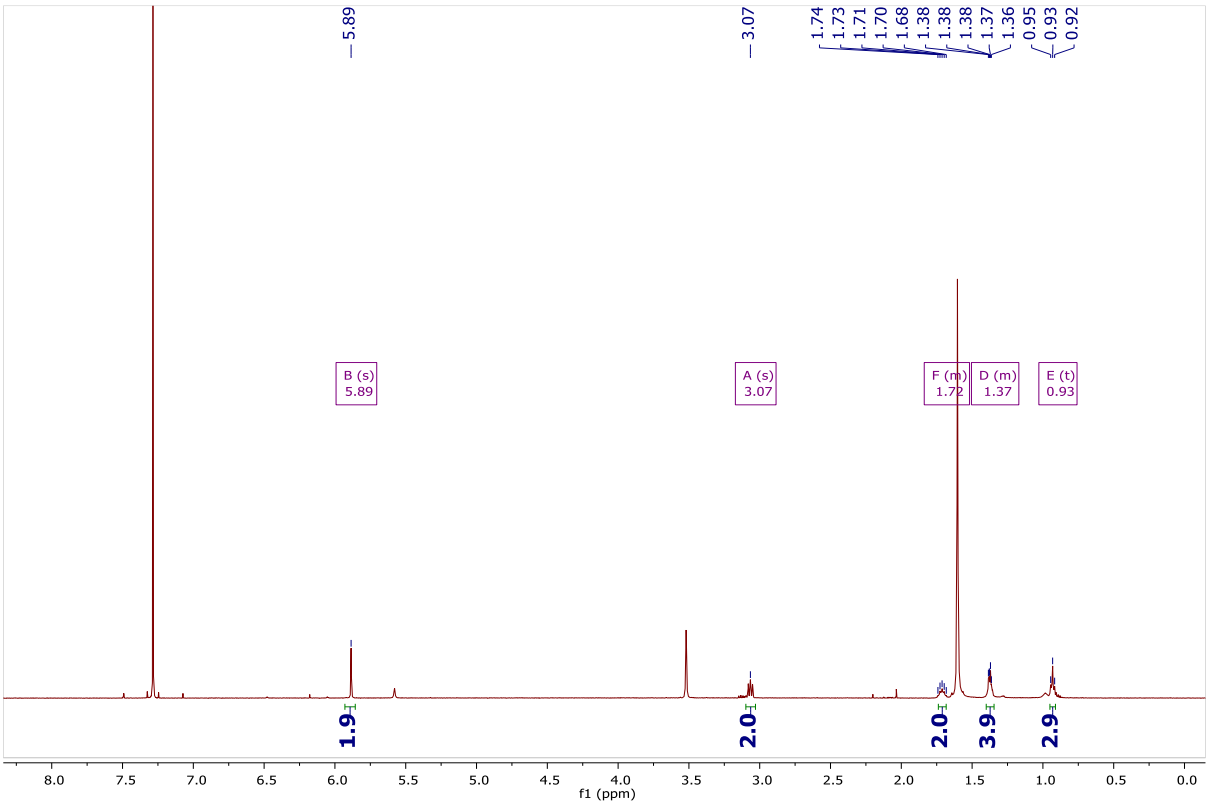


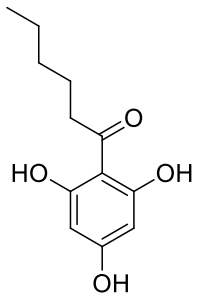
9.11 ^{1}H NMR spectrum of compound **3.5** ($CDCl_3$, 500 MHz)





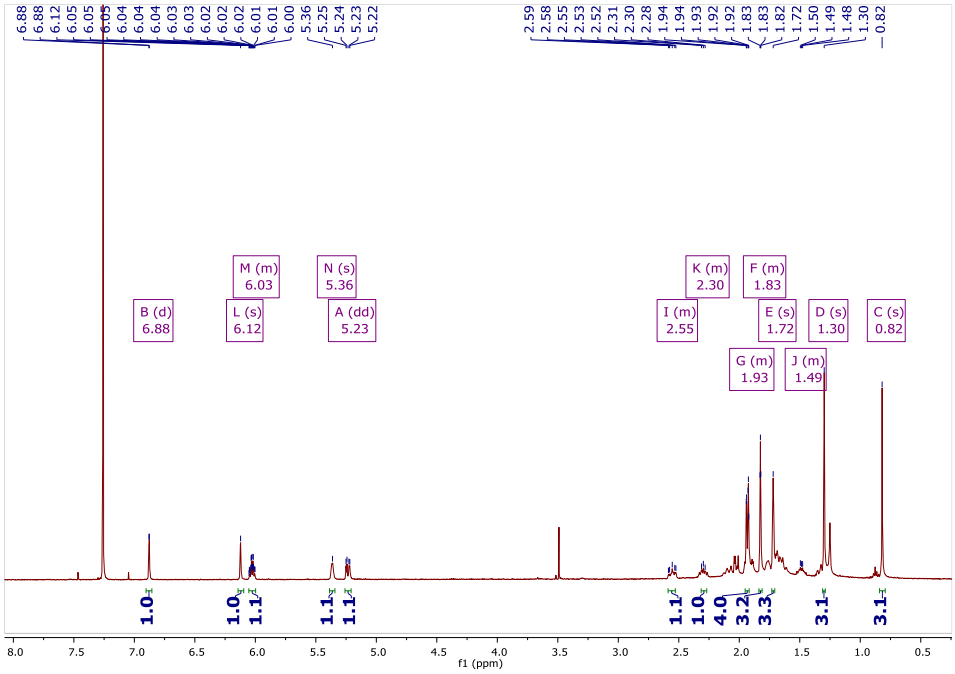
9.12 ^{1}H NMR spectrum of compound **3.6** ($CDCl_3$, 500 MHz)

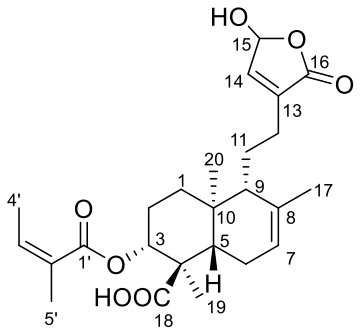




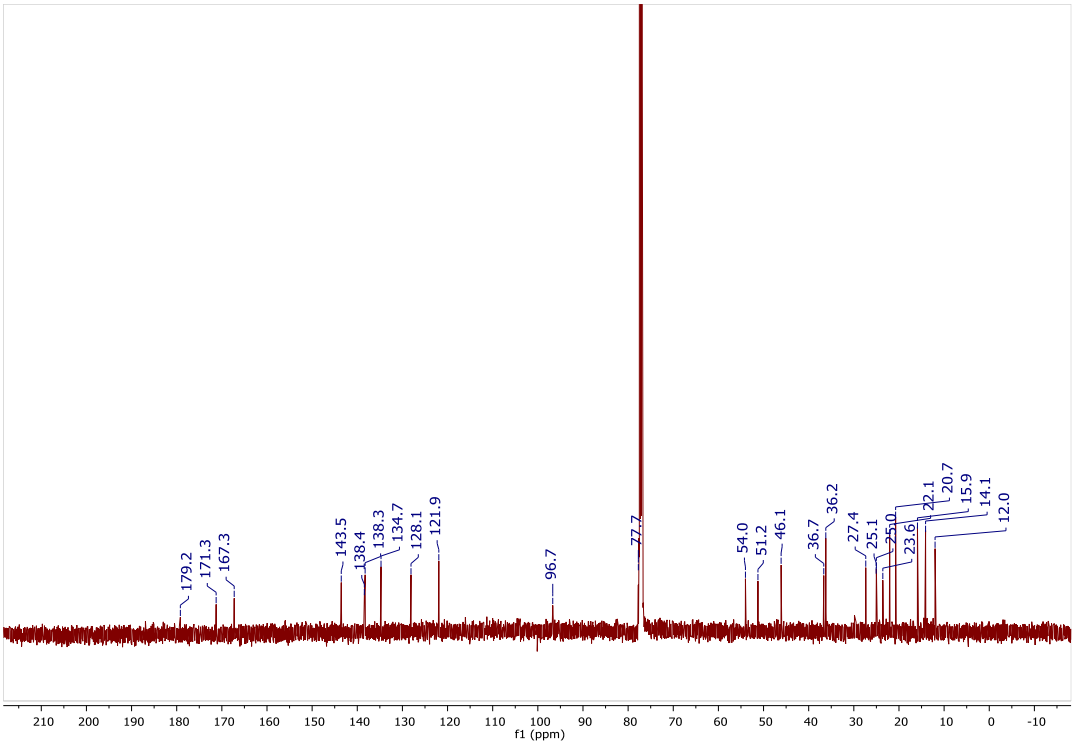
9.13. ^{1}H and ^{13}C NMR spectra of compound **4.1**

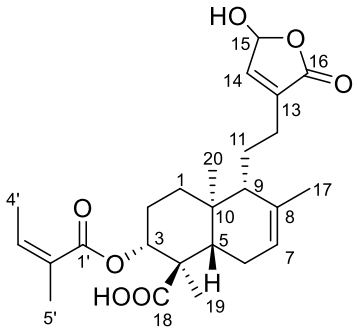
9.13.1 ^{1}H NMR spectrum of compound **4.1** ($CDCl_3$, 500 MHz)





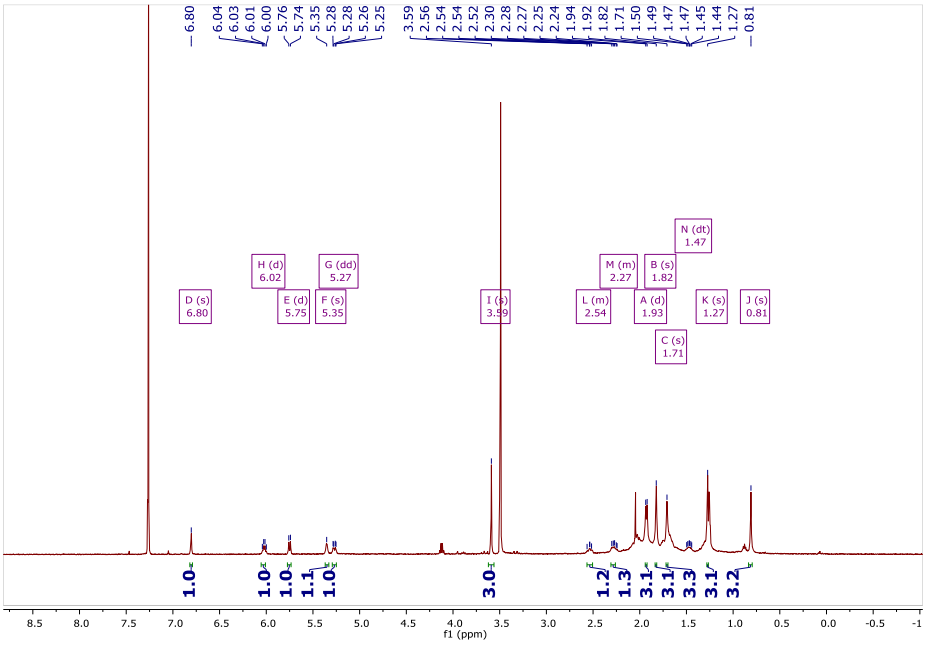
9.13.2 ^{13}C NMR spectrum of compound **4.1** ($CDCl_3$, 125 MHz)

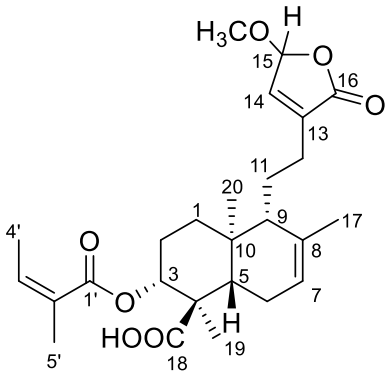




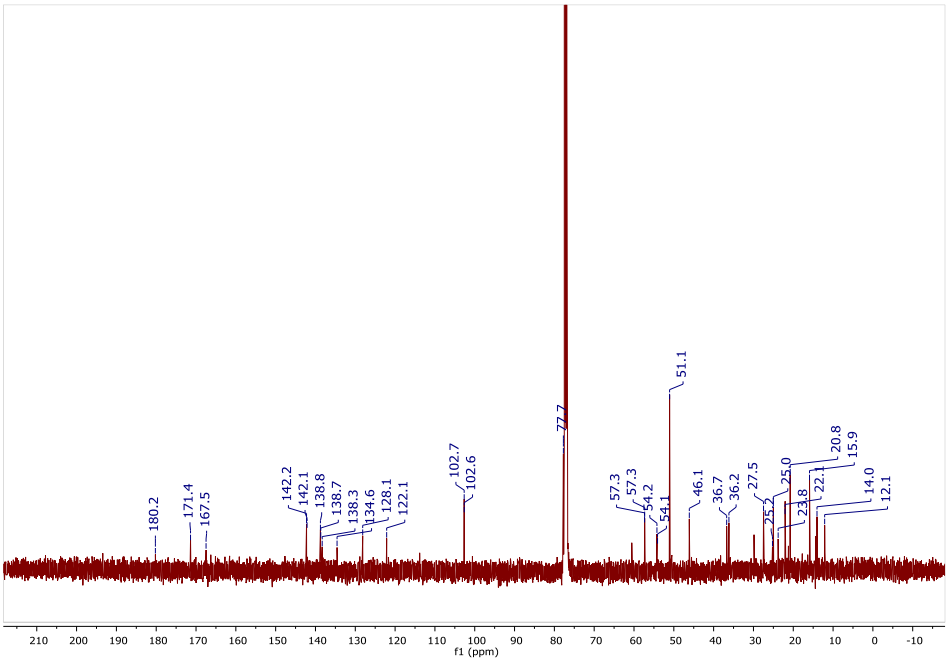
9.14. ^{1}H and ^{13}C NMR spectra of compound **4.2**

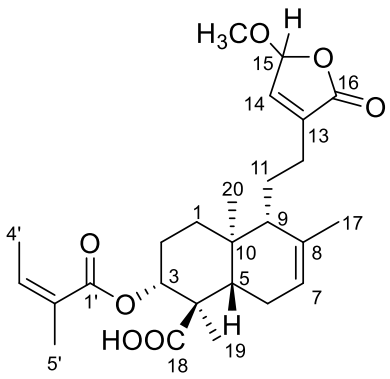
9.14.1 ^{1}H NMR spectrum of compound **4.2** ($CDCl_3$, 500 MHz)





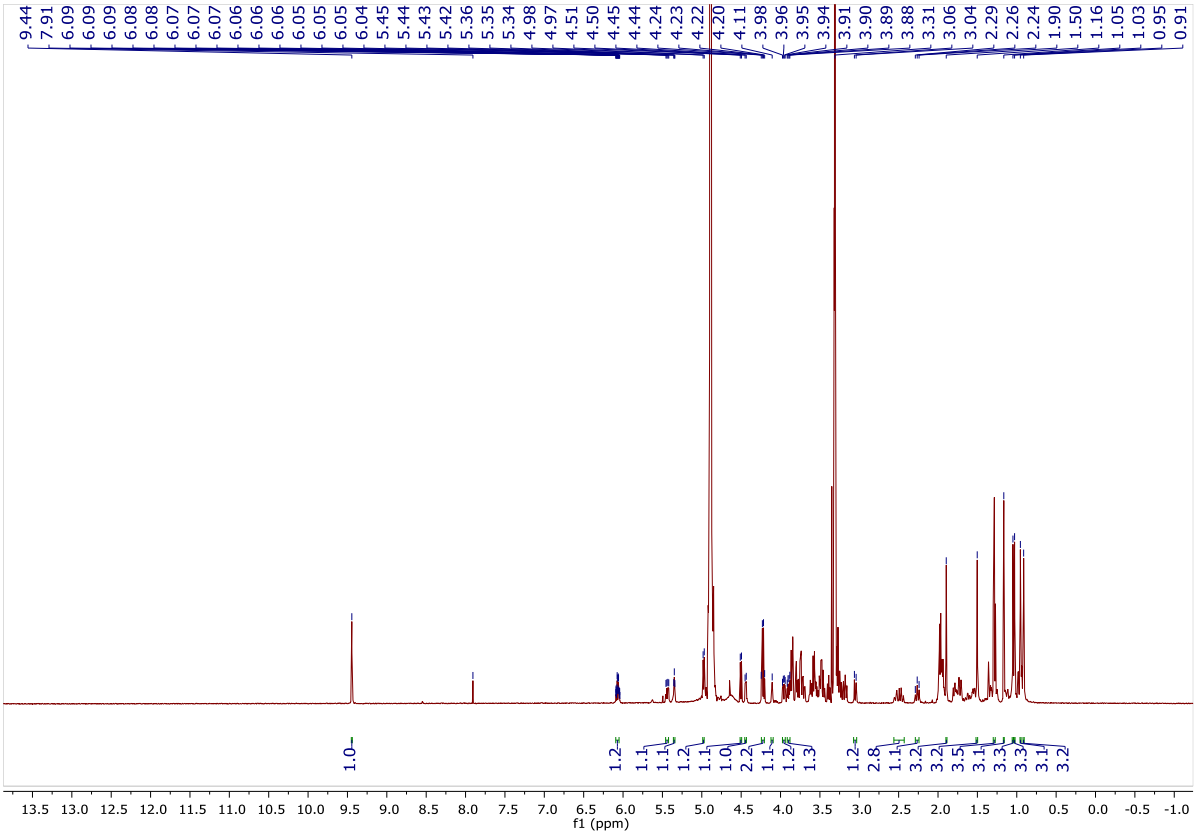
9.14.2 ^{13}C NMR spectrum of compound **4.2** ($CDCl_3$, 125 MHz)

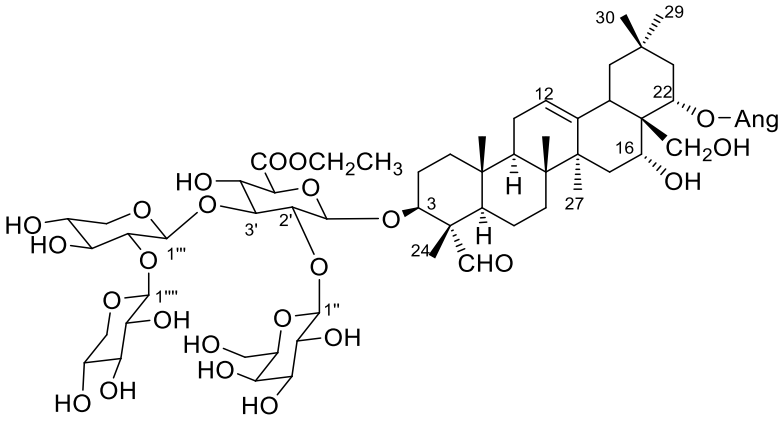




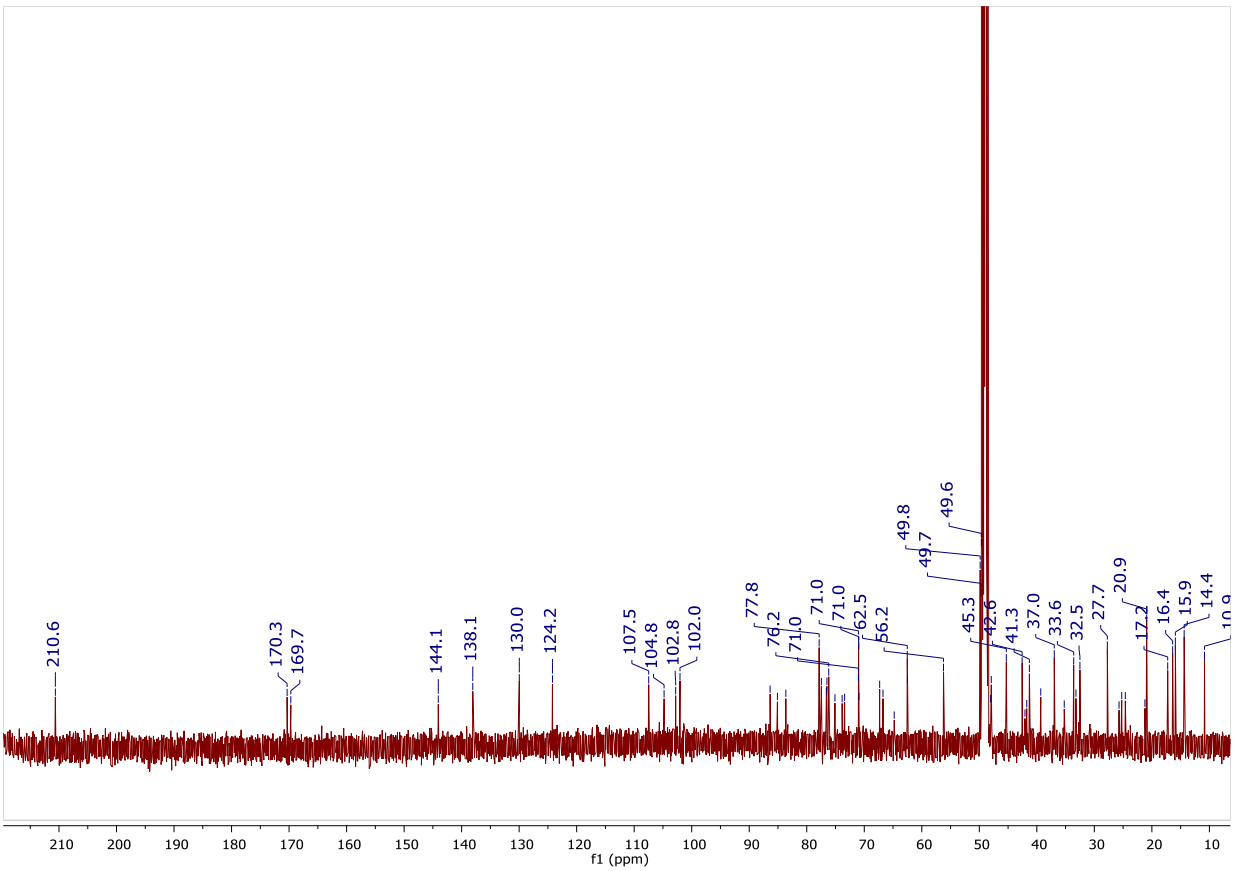
9.15. ^{1}H and ^{13}C NMR spectra of compound **5.1**

9.15.1 ^{1}H NMR spectrum of compound **5.1** (CD_3OD, 500 MHz)



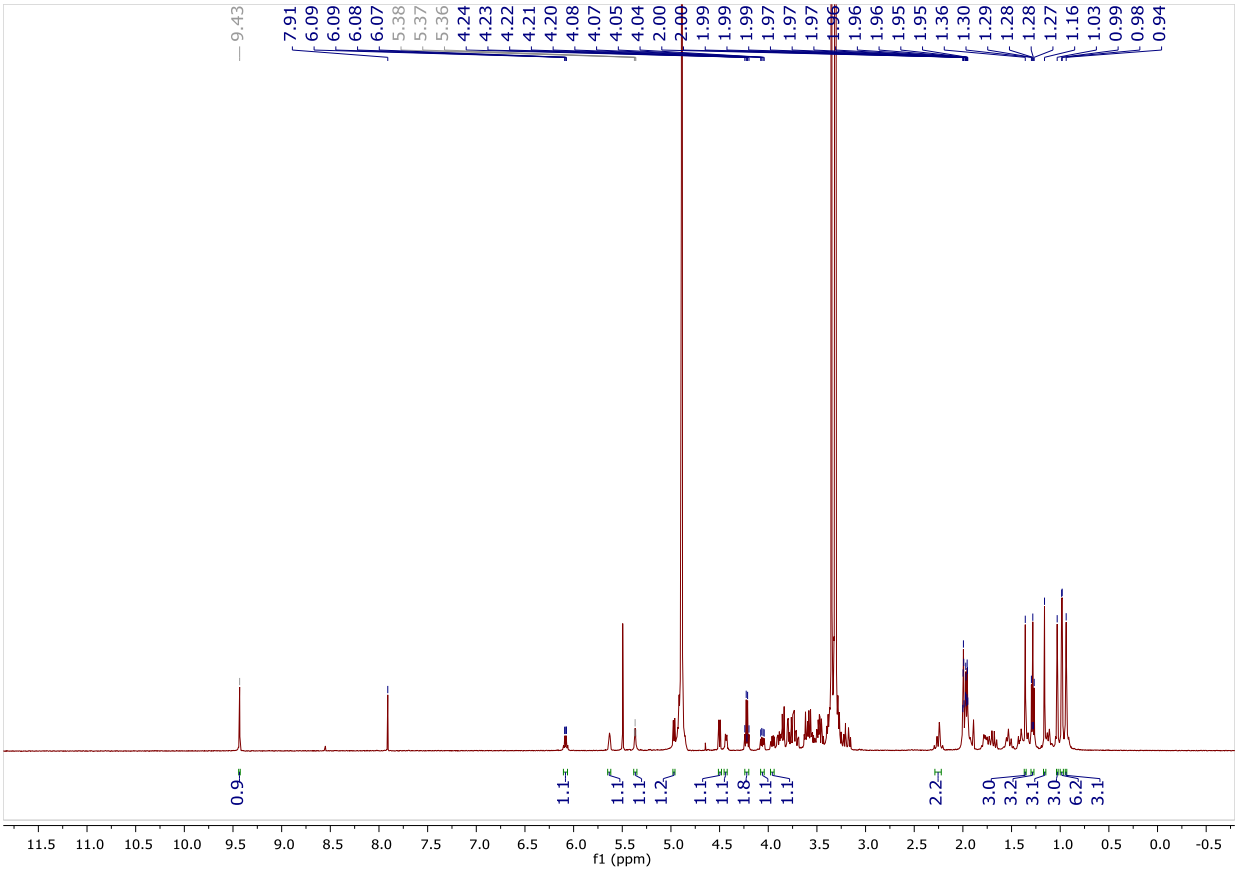


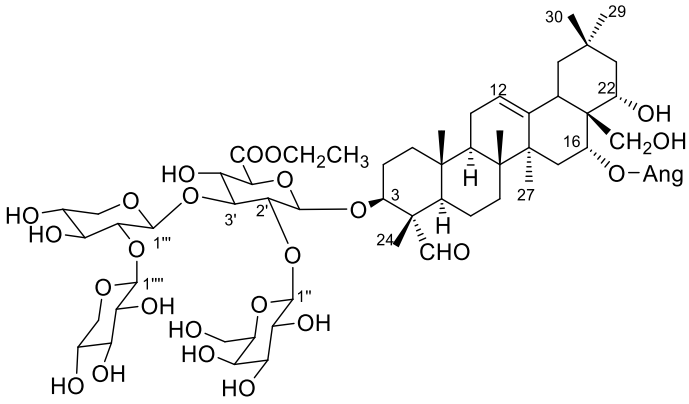
9.15.2 ^{13}C NMR spectrum of compound **5.1** (CD_3OD, 125 MHz)



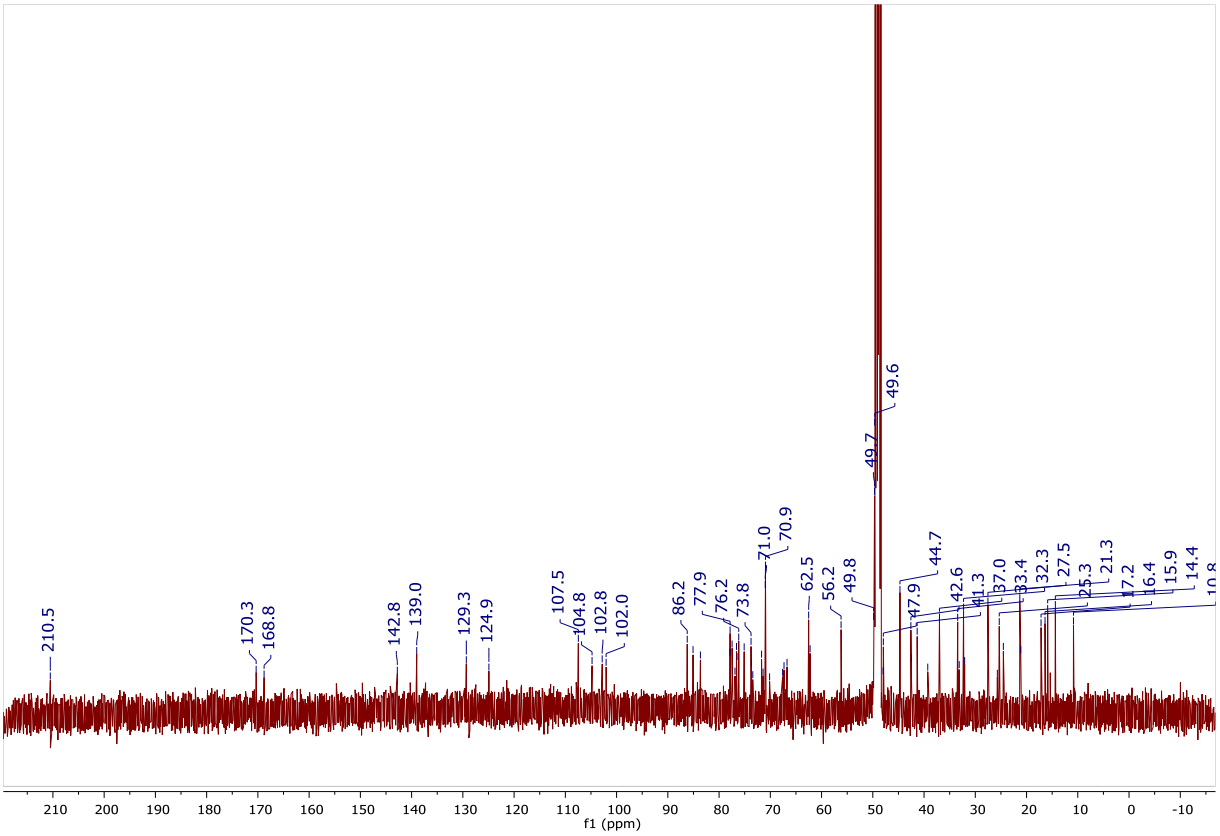
9.16. ^{1}H and ^{13}C NMR spectra of compound **5.2**

9.16.1 ^{1}H NMR spectrum of compound **5.2** (CD_3OD, 500 MHz)



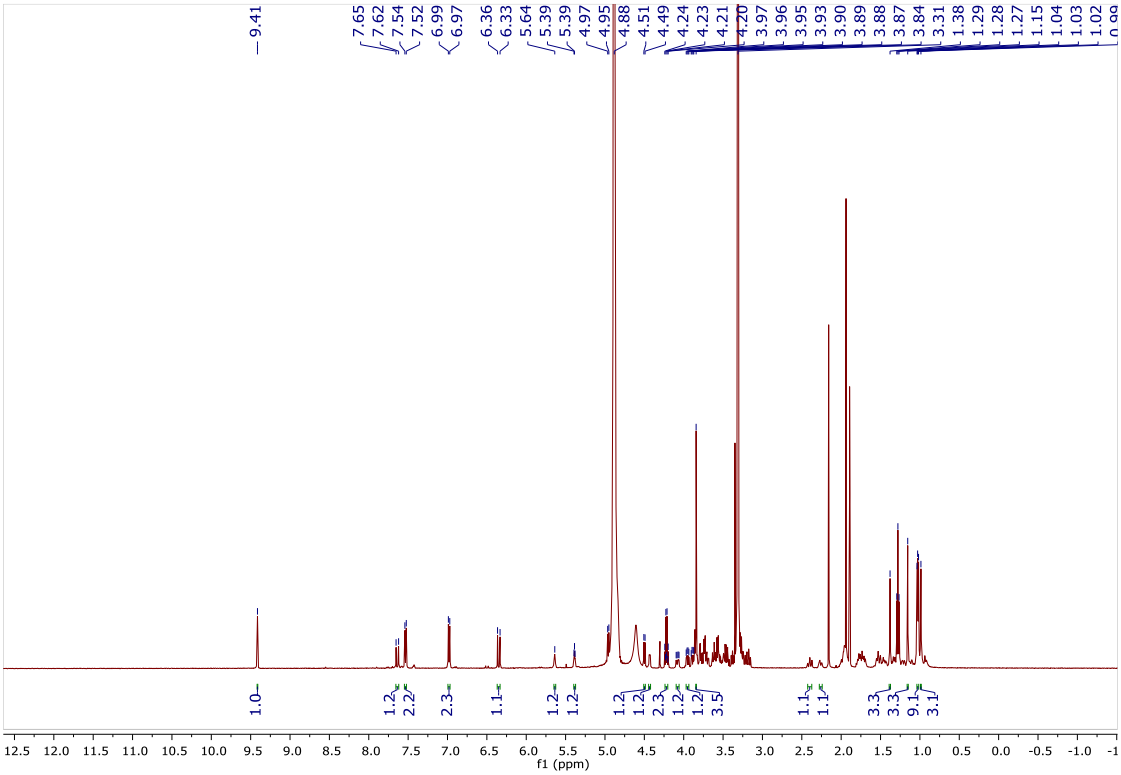


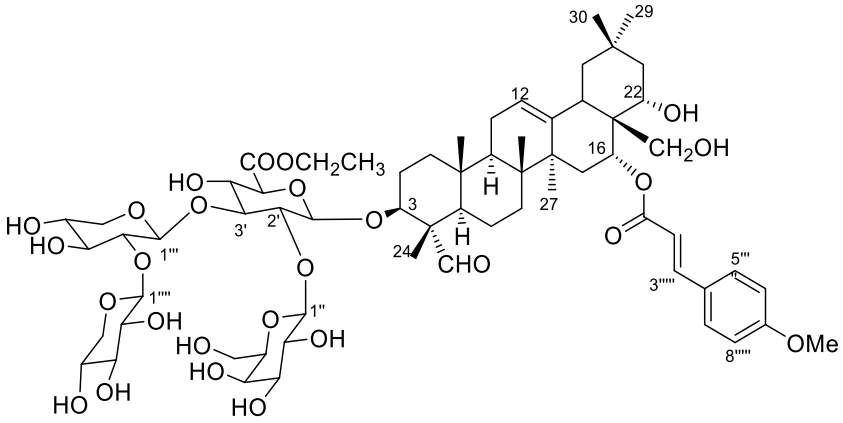
9.16.2 ^{13}C NMR spectrum of compound **5.2** (CD_3OD, 500 MHz)



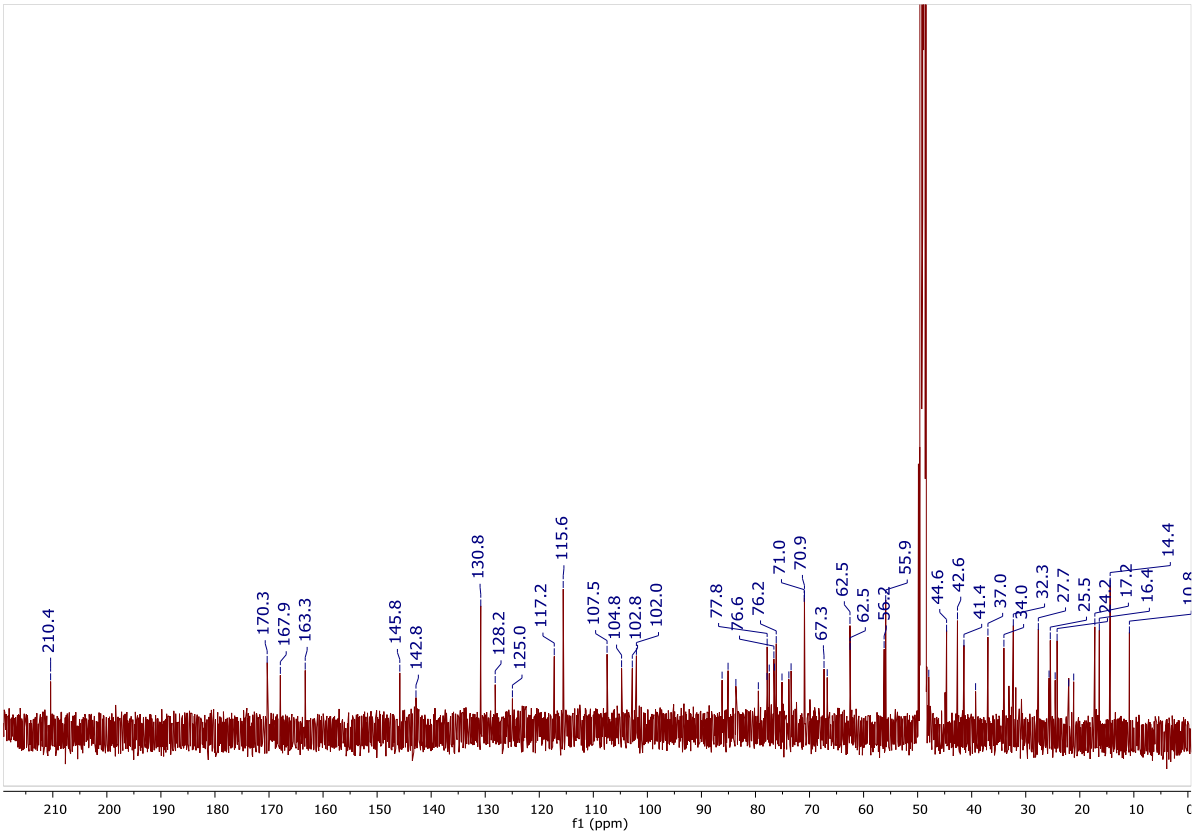
9.17. ^{1}H and ^{13}C NMR spectra of compound **5.3**

9.17.1 ^{1}H NMR spectrum of compound **5.3** (CD_3OD, 500 MHz)

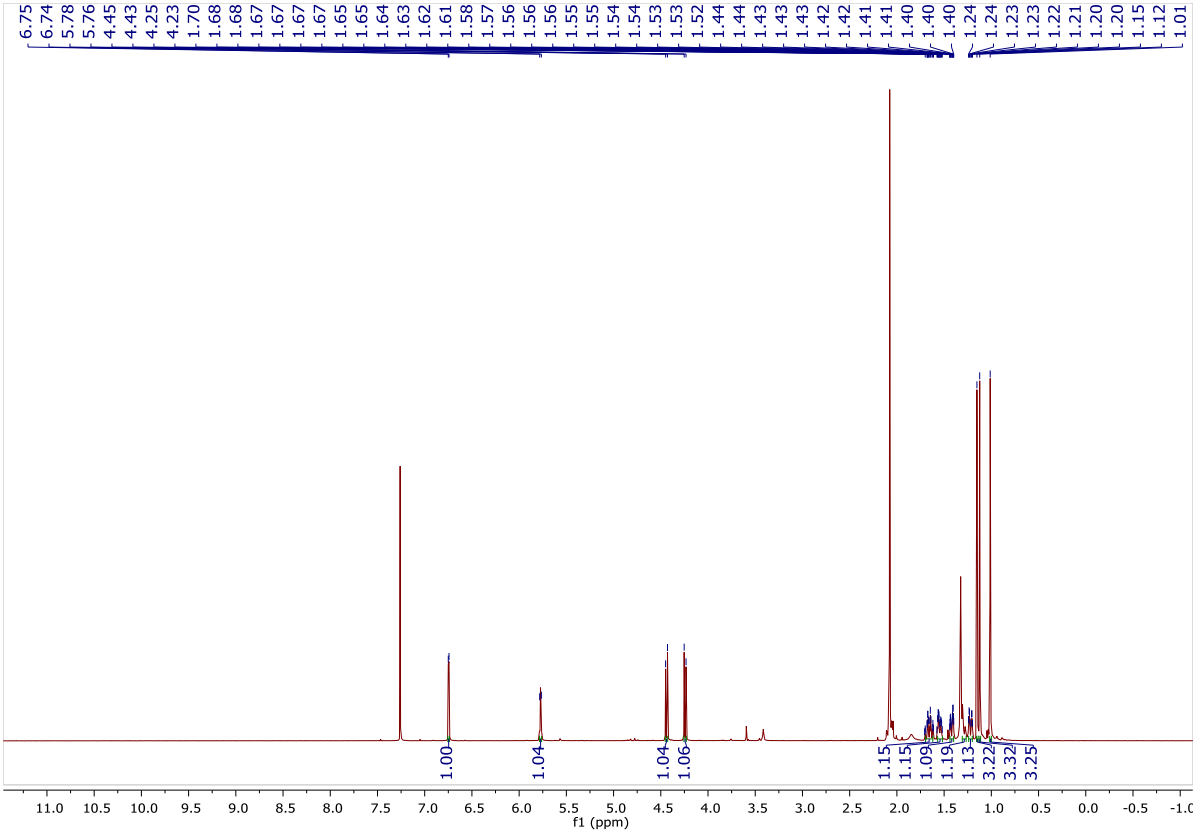


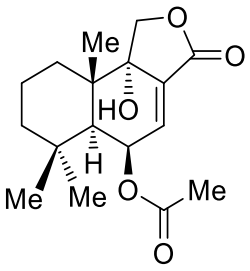


9.17.2 ^{13}C NMR spectrum of compound **5.3** (CD_3OD, 500 MHz)



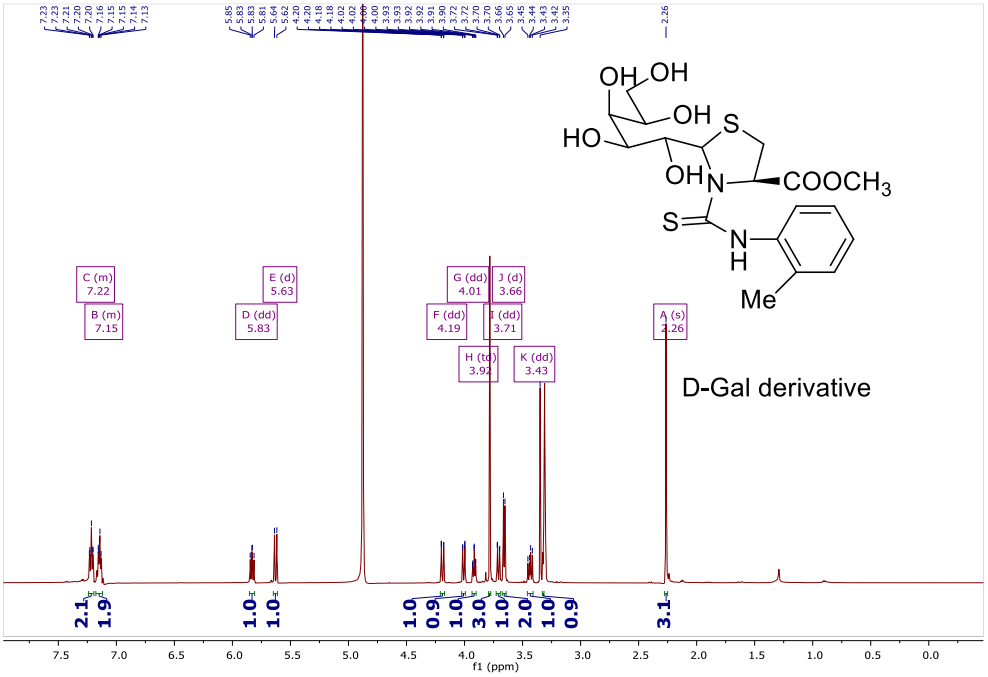
9.18. ^{1}H NMR spectrum of compound **5.4** ($CDCl_3$, 500 MHz)

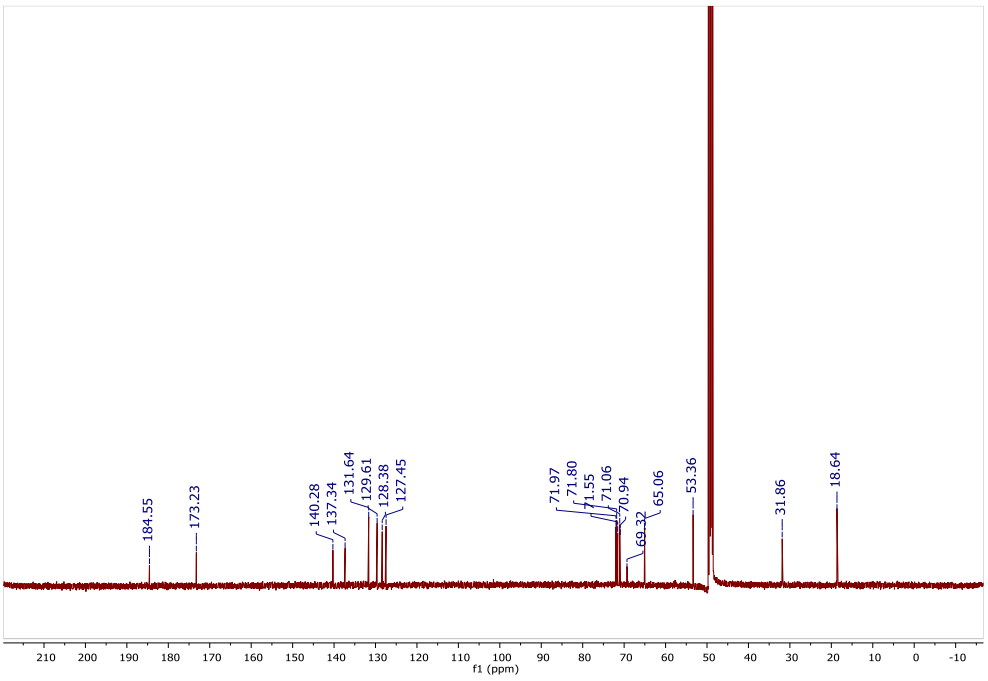




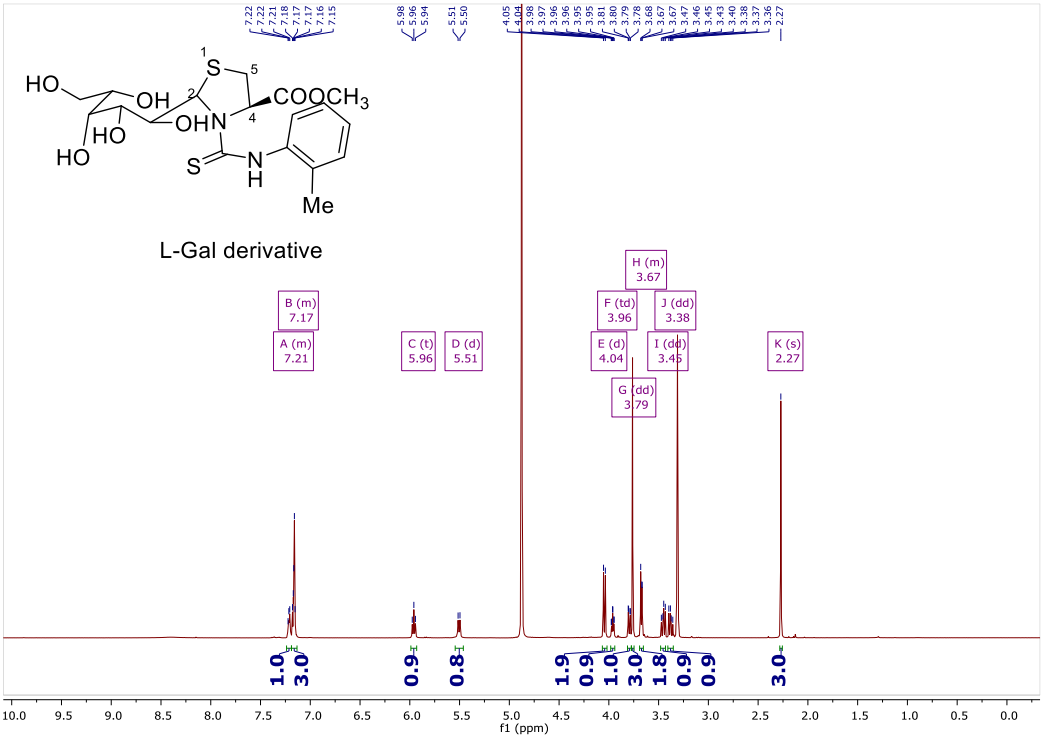
9.19. ^{1}H and ^{13}C NMR spectra of standard D/L-sugar derivatives

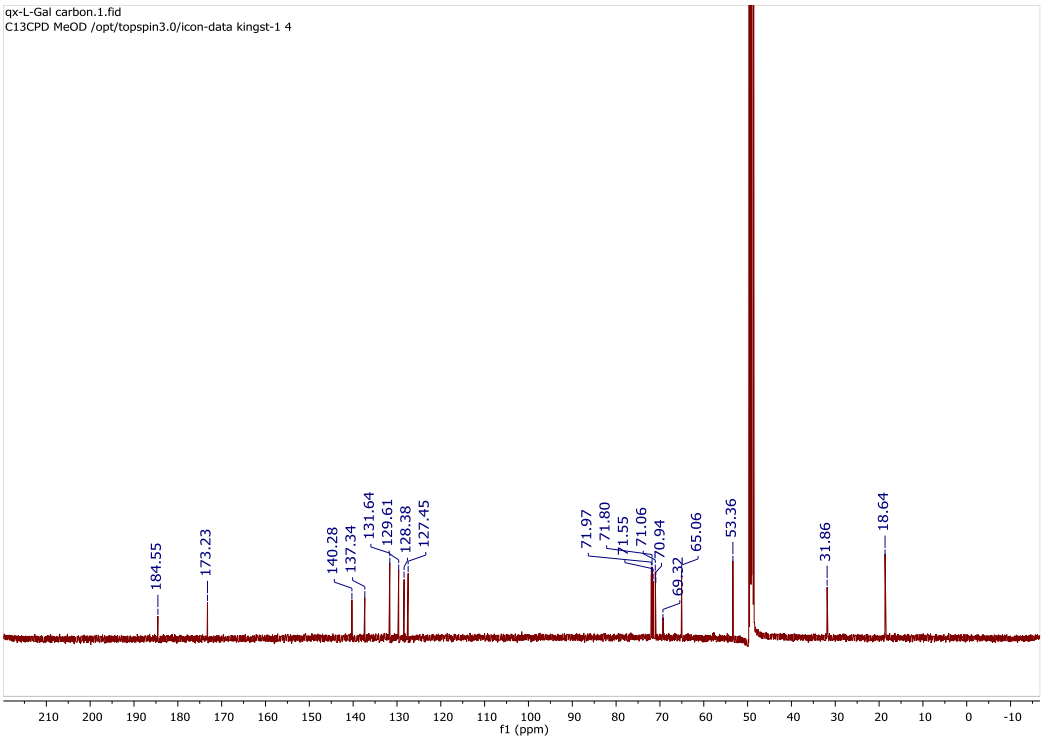
9.19.1 ^{1}H and ^{13}C NMR spectra of standard D-galactose derivative (CD_3OD, 500 and 125 MHz)



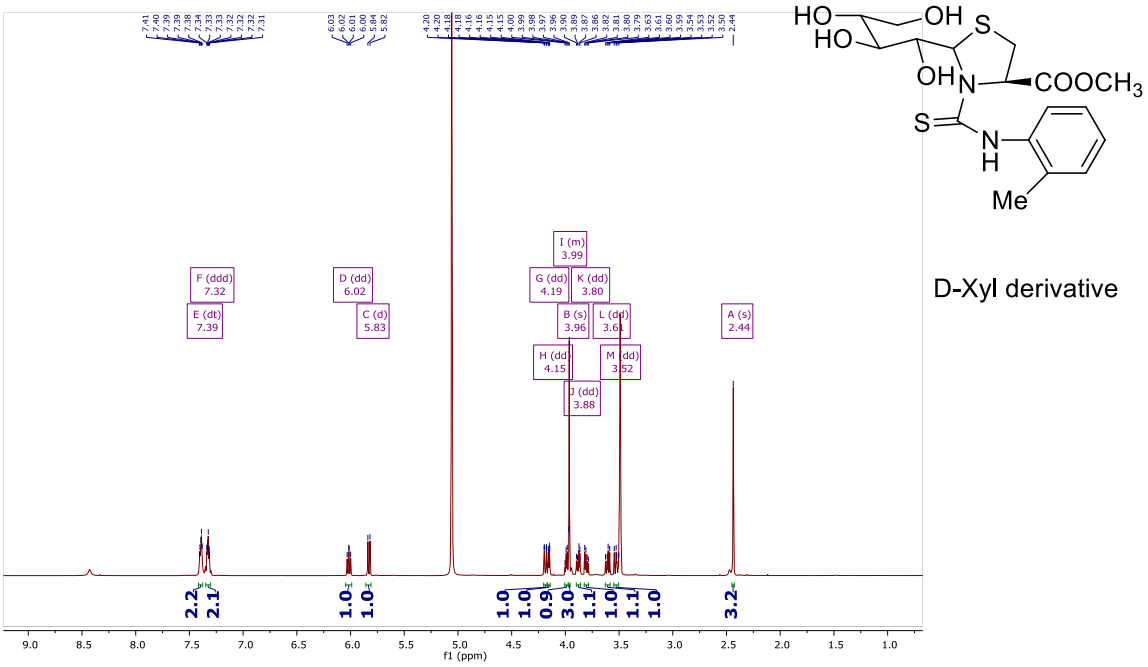


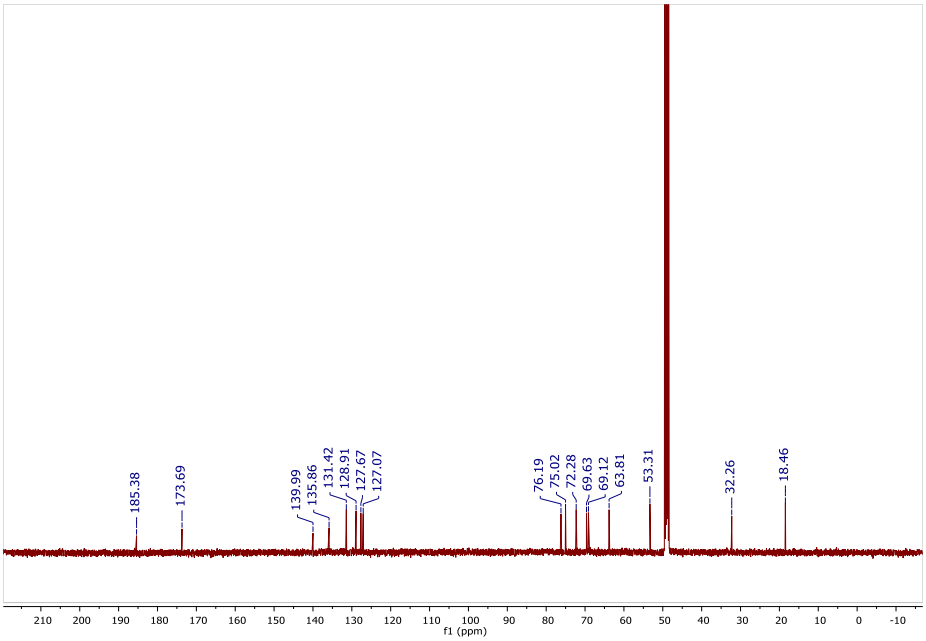
9.19.2 ^{1}H and ^{13}C NMR spectra of standard L-galactose derivative (CD_3OD, 500 and 125 MHz)



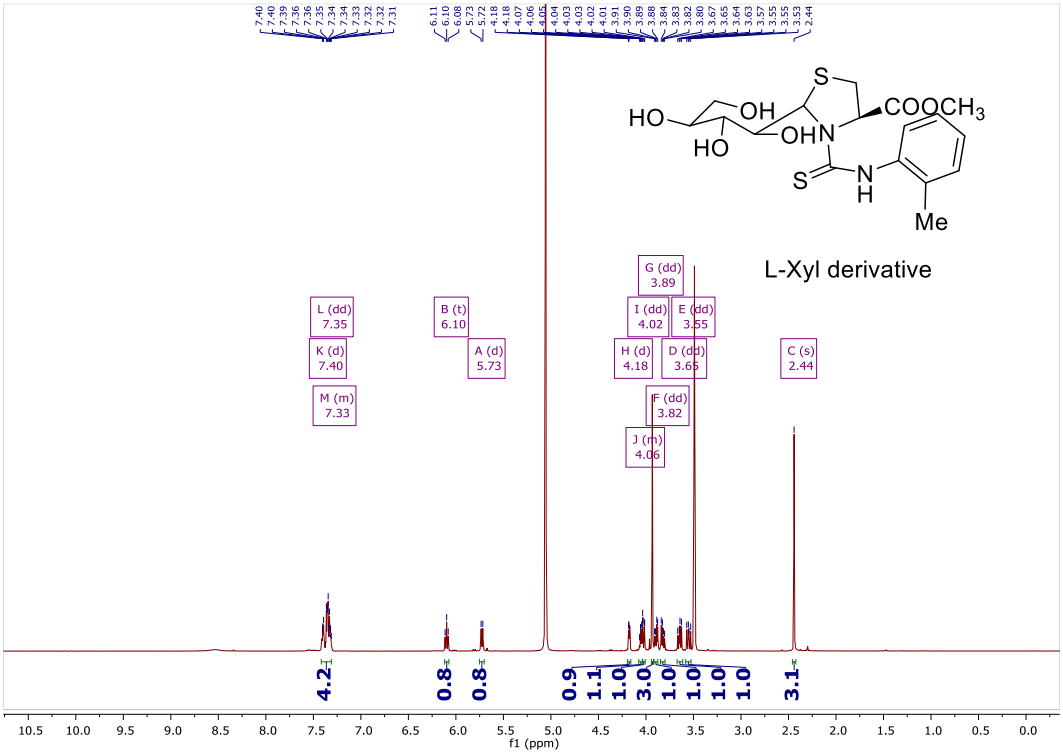


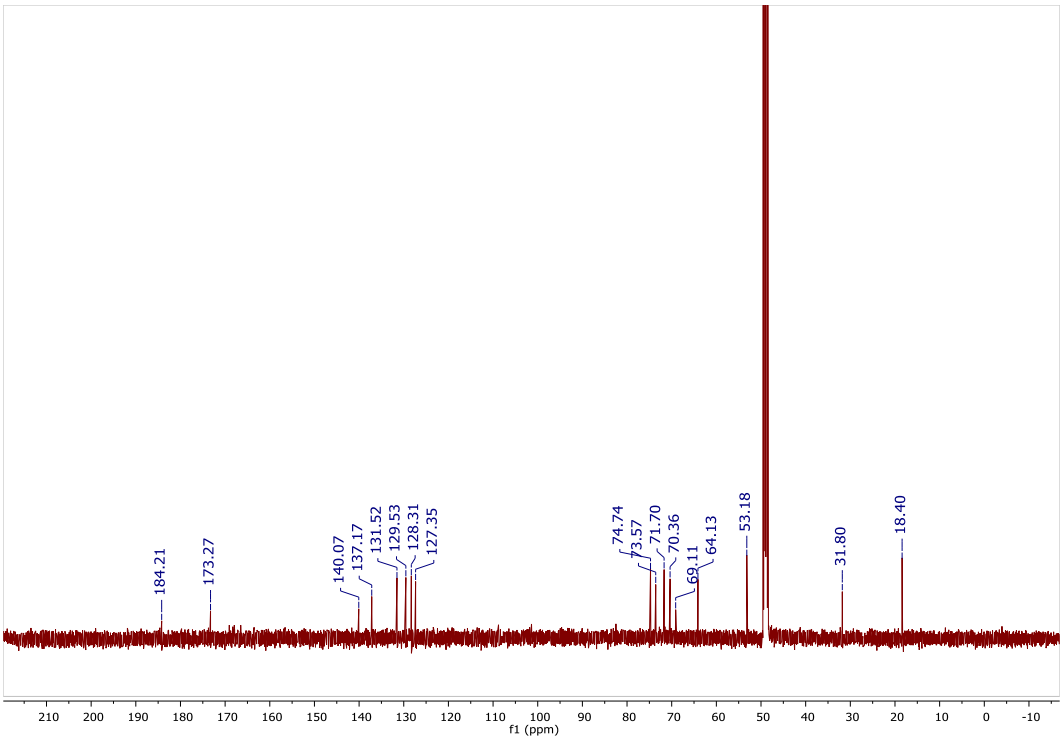
9.19.3 ^{1}H and ^{13}C NMR spectra of standard D-xylose derivative (CD_3OD, 500 and 125 MHz)



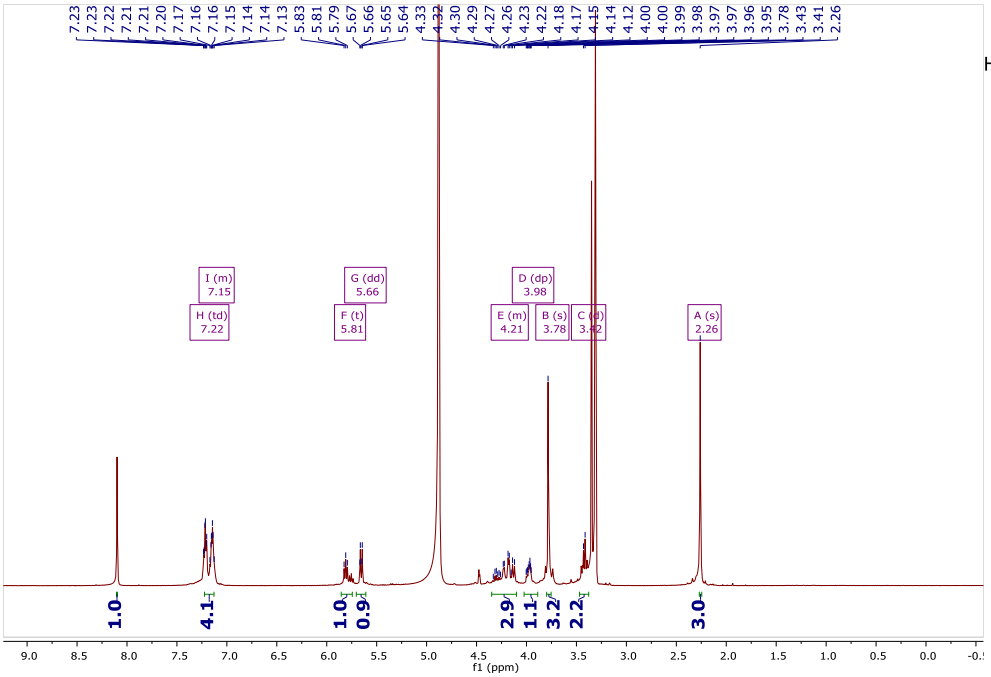


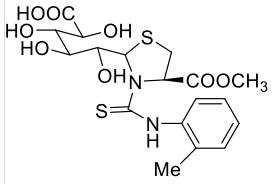
9.19.4 ^{1}H and ^{13}C NMR spectra of standard L-xylose derivative (CD_3OD, 500 and 125 MHz)



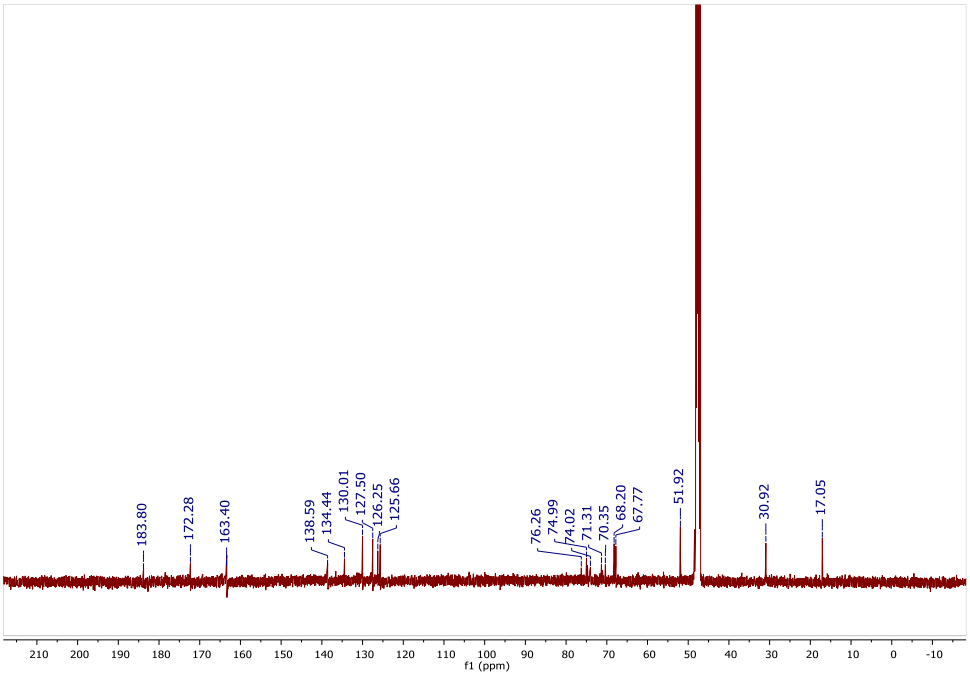


9.19.5 ^{1}H and ^{13}C NMR spectra of standard D-glucuronic acid derivative (CD_3OD, 500 and 125 MHz)

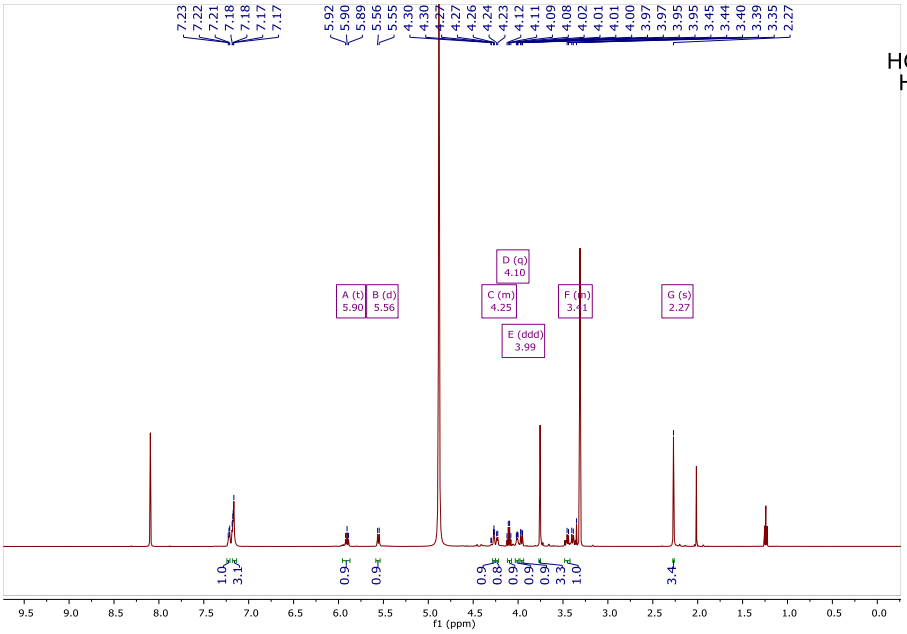


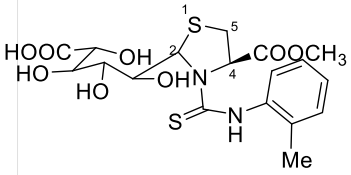


D-GlcA derivative

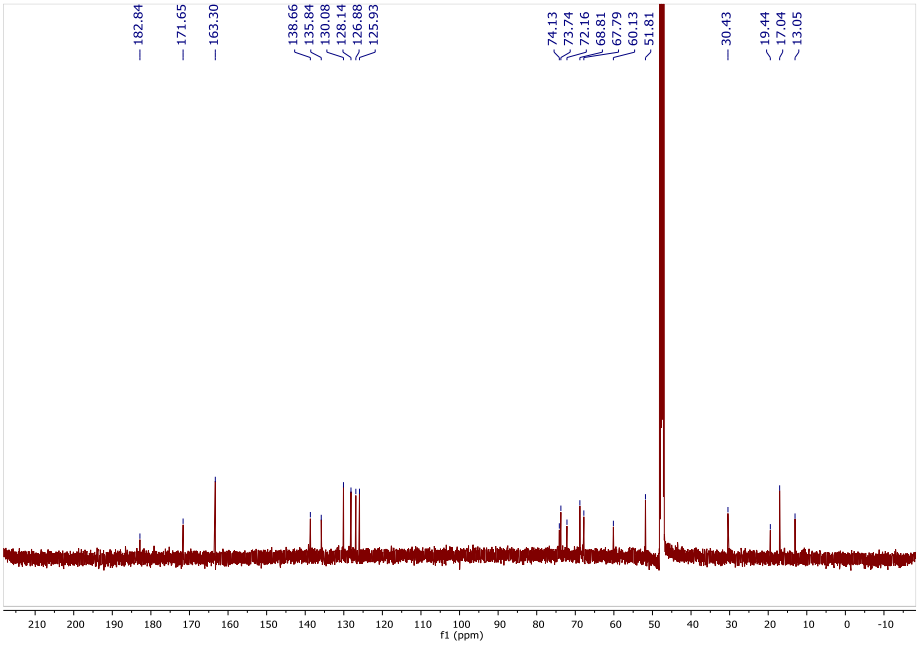


9.19.6 ^{1}H and ^{13}C NMR spectra of standard L-glucuronic acid derivative (CD_3OD, 500 and 125 MHz)

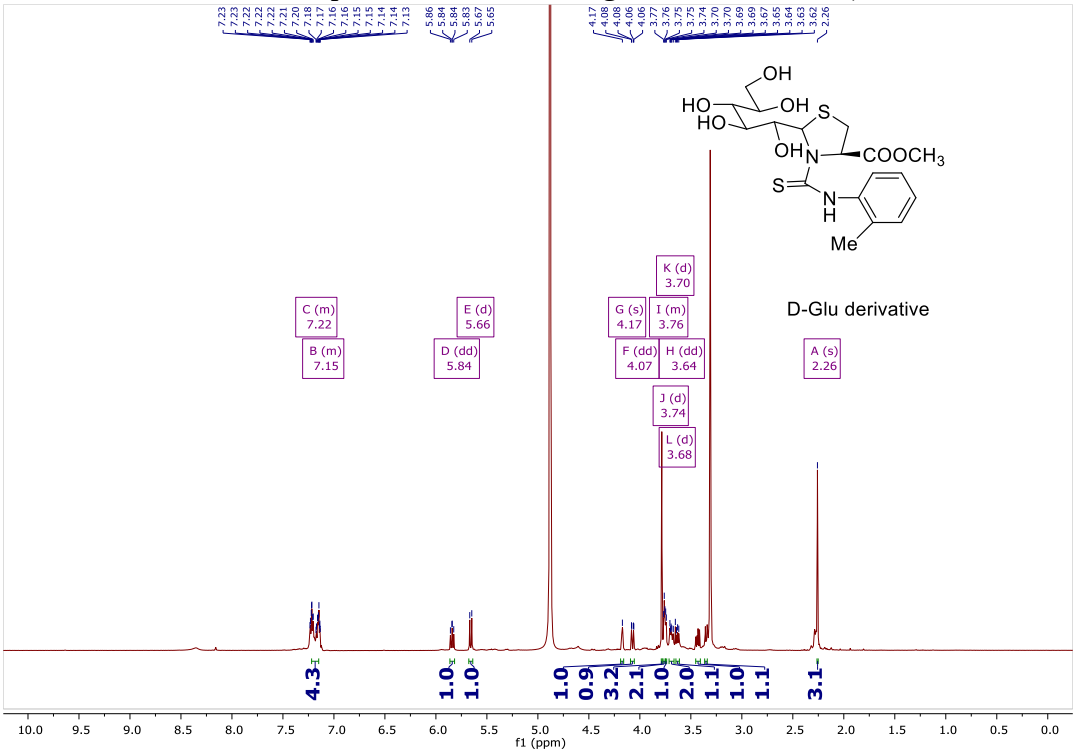


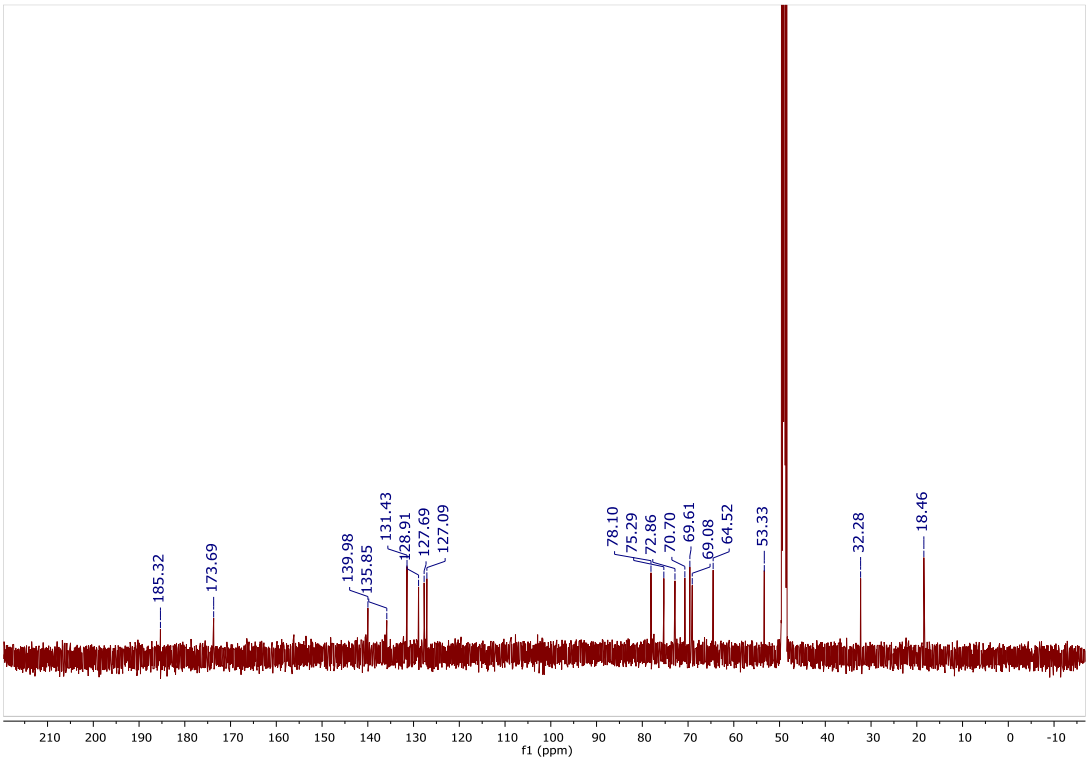


L-GlcA derivative

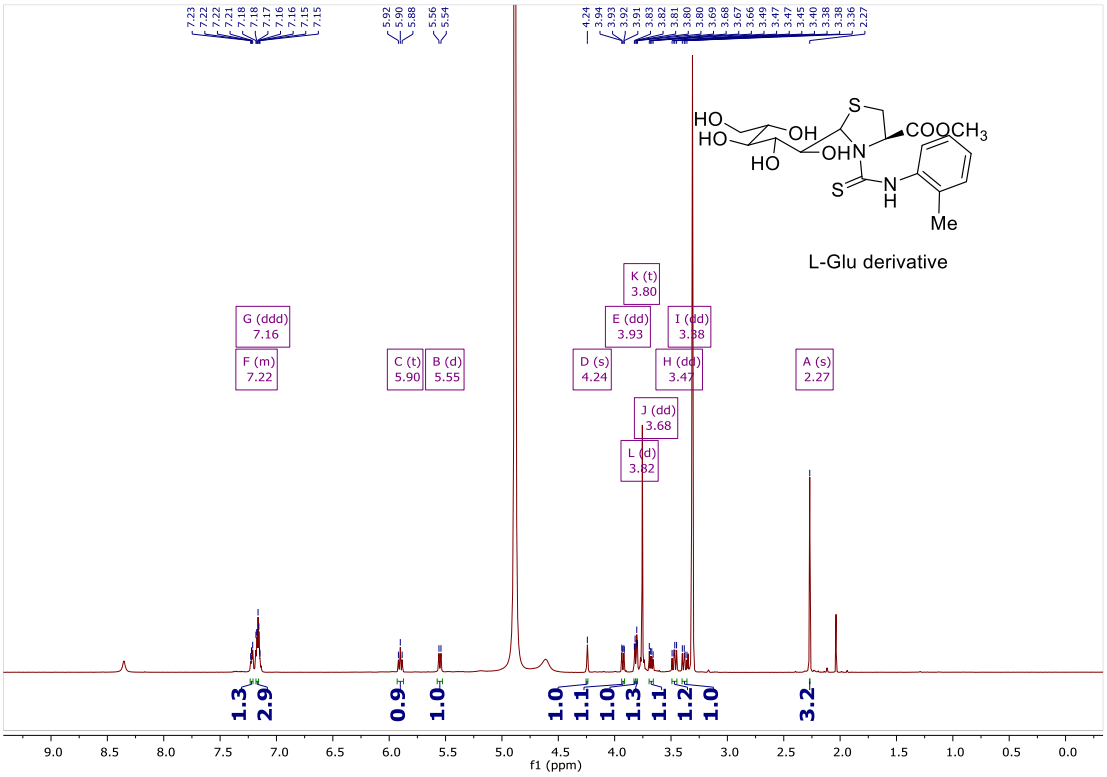


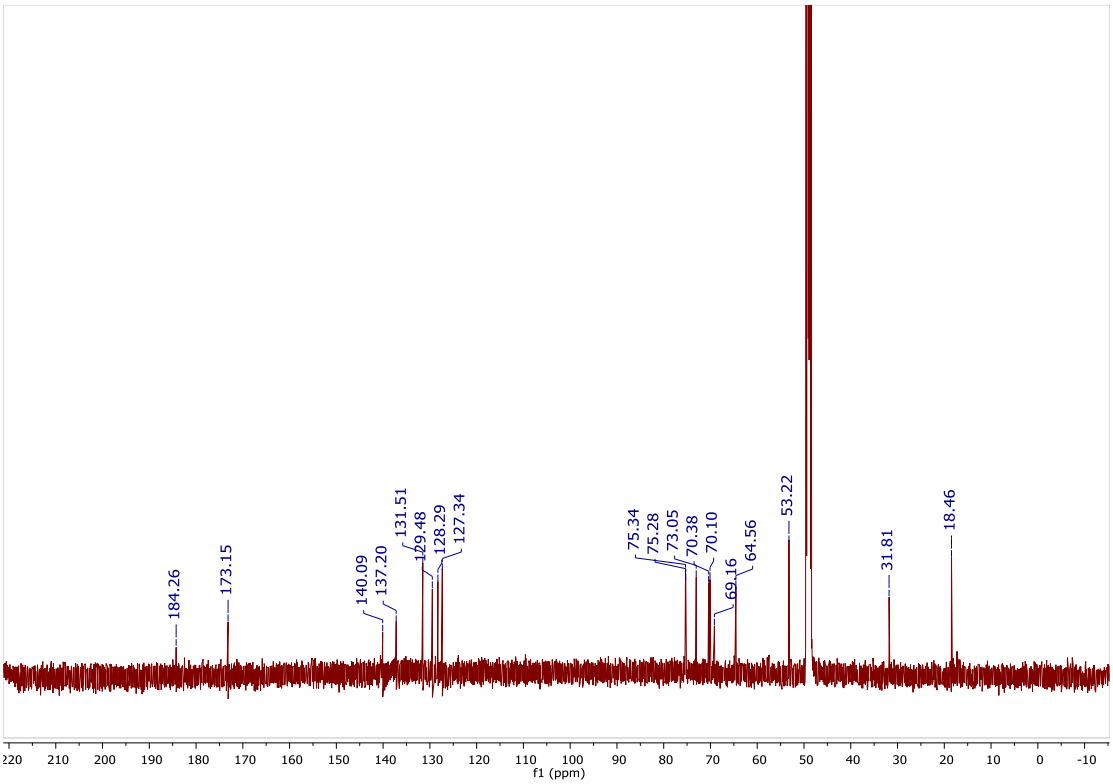
9.19.7 ^{1}H and ^{13}C NMR spectra of standard D-glucose derivative (CD_3OD, 500 and 125 MHz)



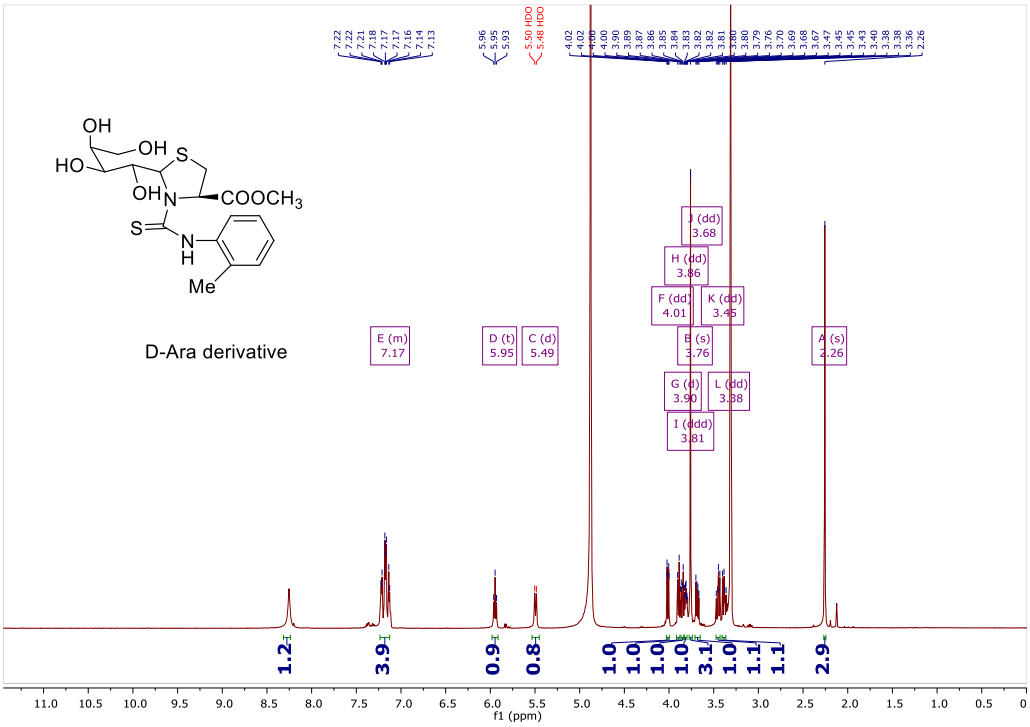


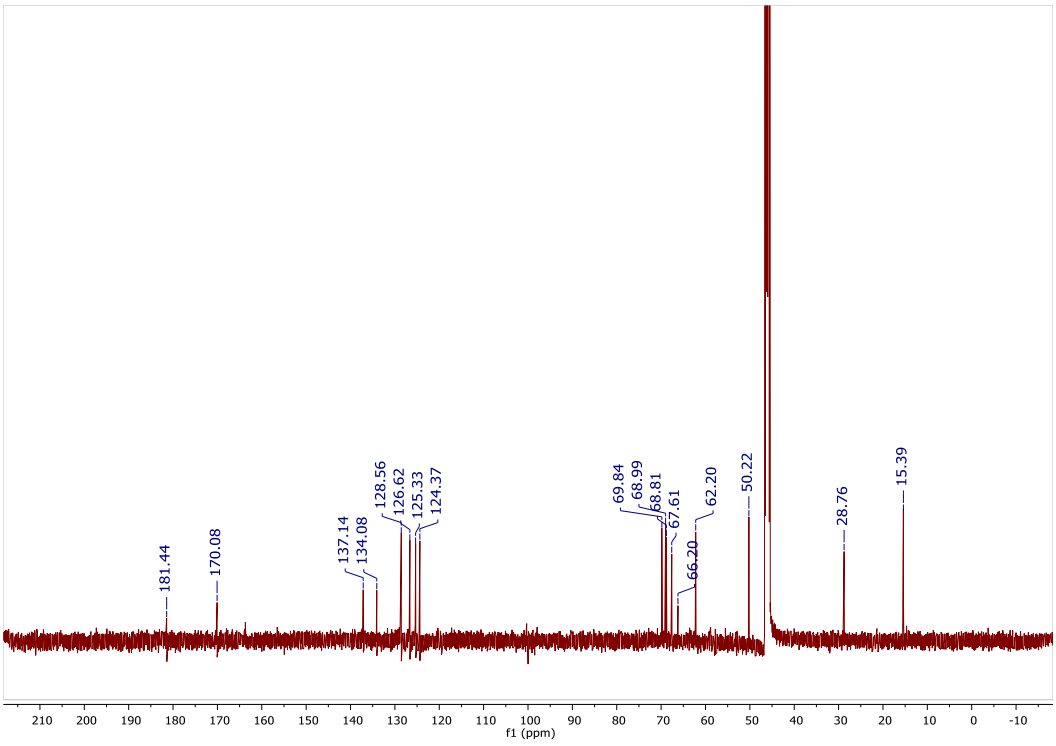
9.19.8 ^{1}H and ^{13}C NMR spectra of standard L-glucose derivative (CD_3OD, 500 and 125 MHz)



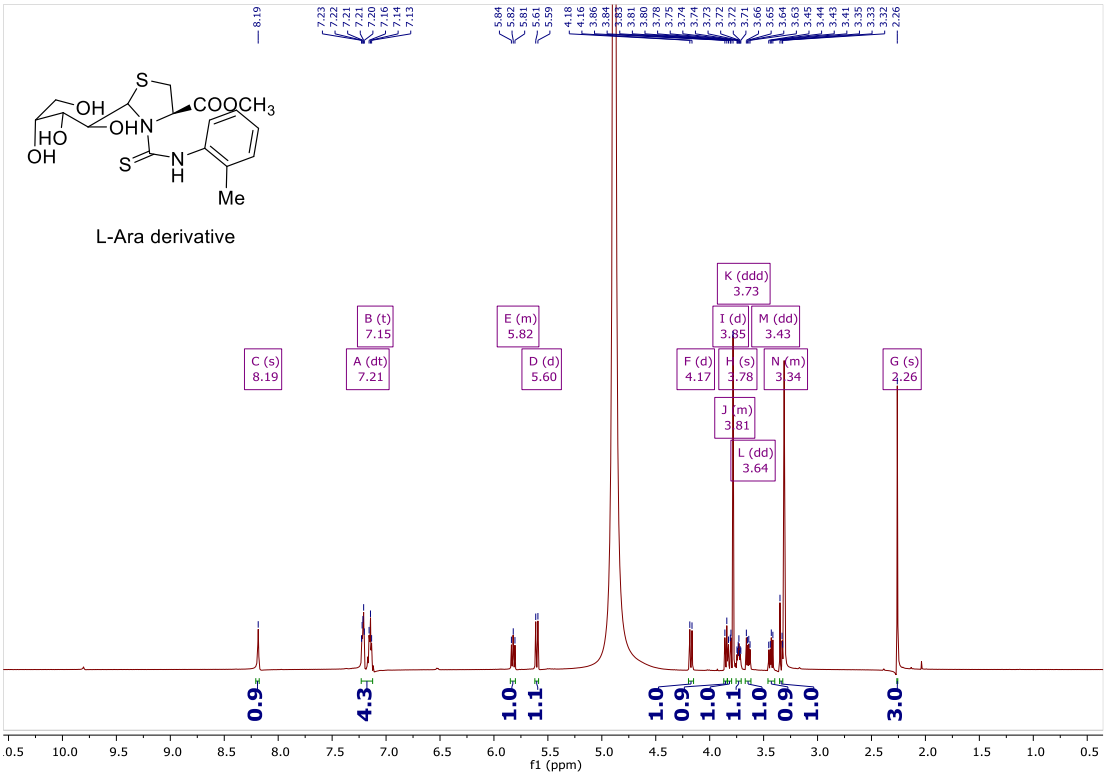


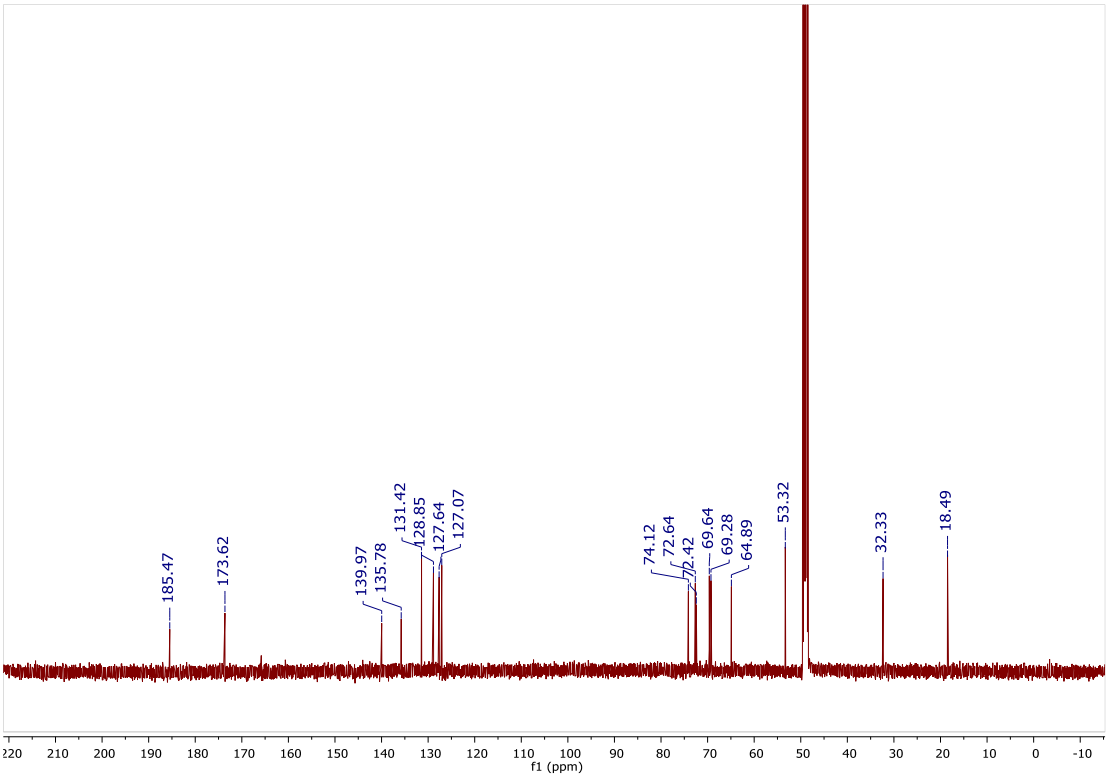
9.19.9 ^{1}H and ^{13}C NMR spectra of standard D-arabinose derivative (CD_3OD, 500 and 125 MHz)





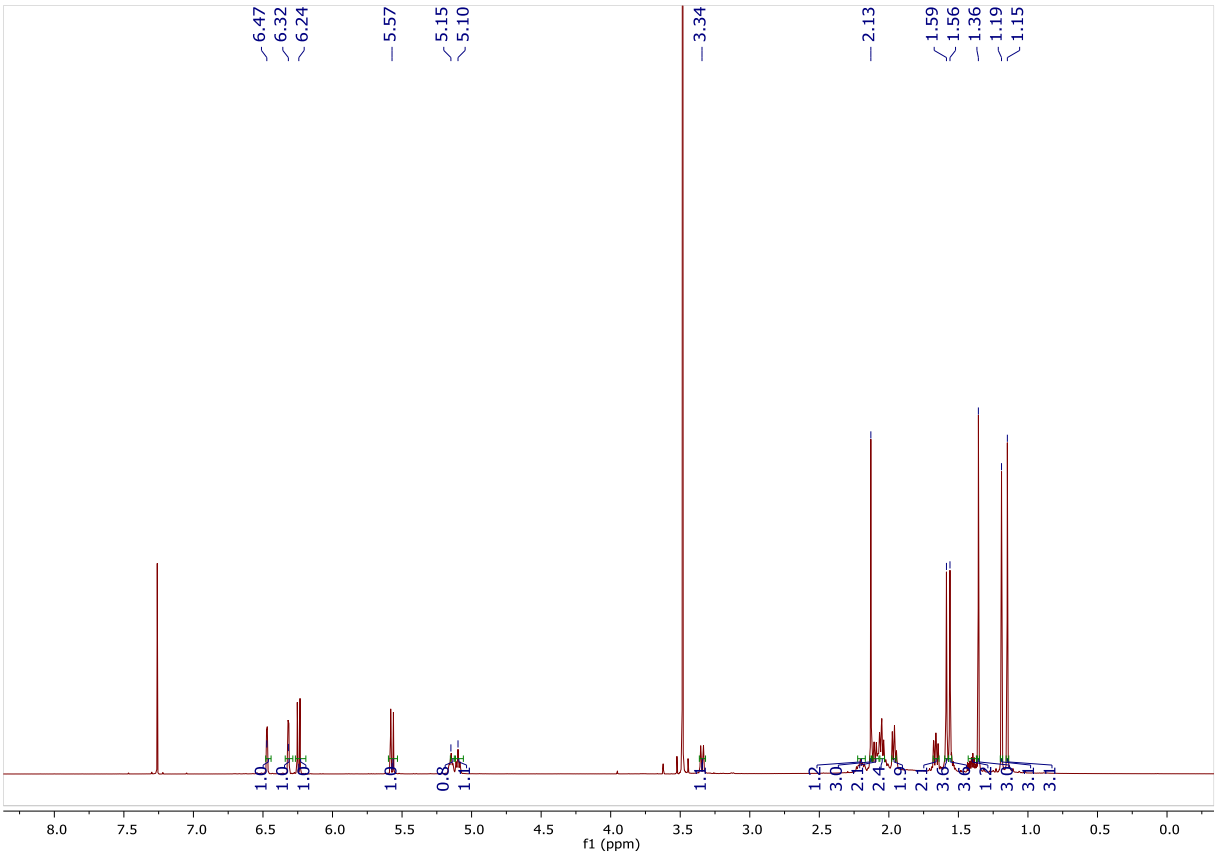
9.19.10 ^{1}H and ^{13}C NMR spectra of standard L-arabinose derivative (CD_3OD, 500 and 125 MHz)

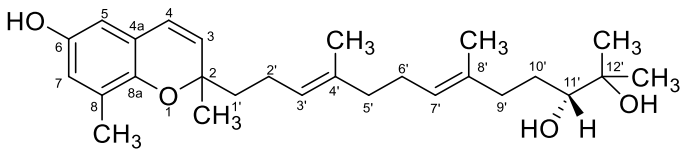




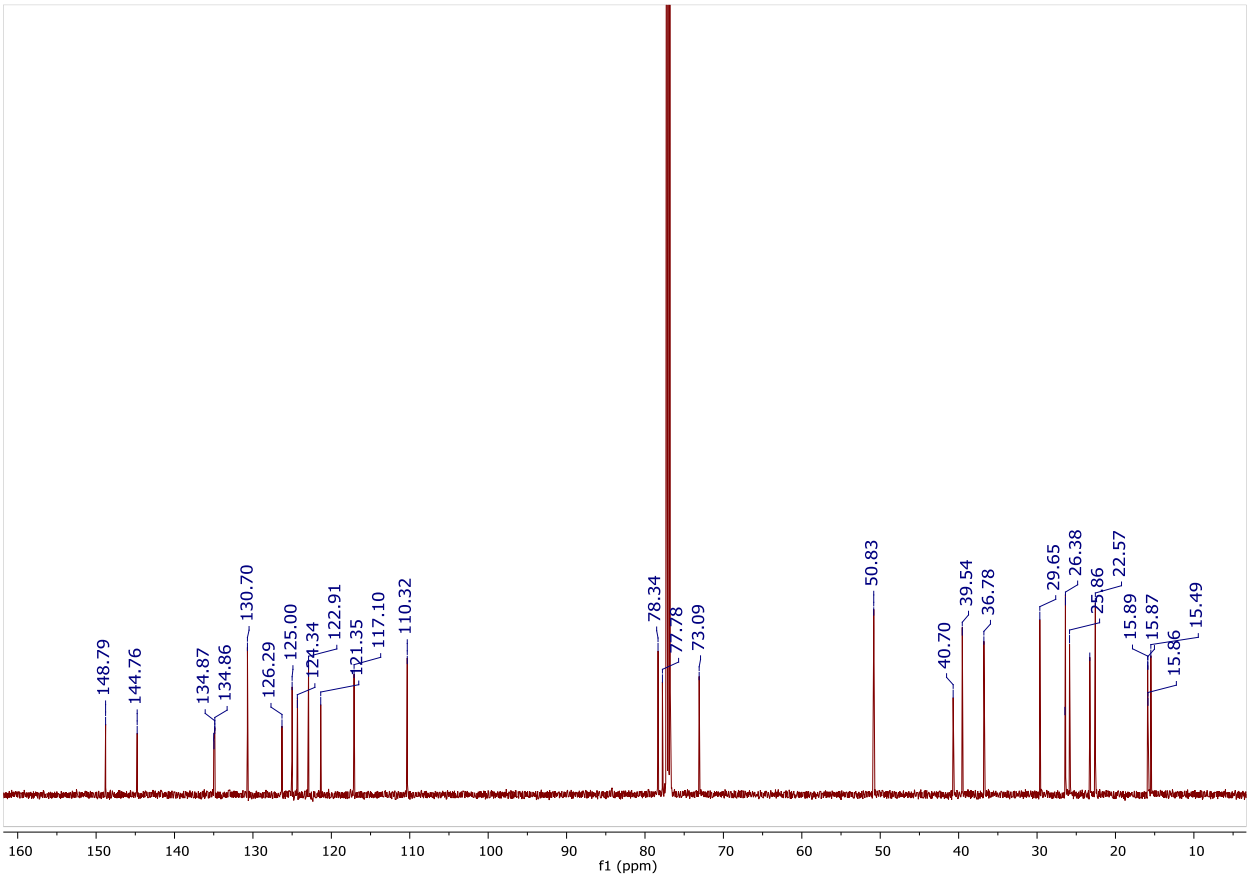
9.20 ^{1}H and ^{13}C NMR spectra of compound **6.1**

9.20.1 ^{1}H NMR spectrum of compound **6.1** ($CDCl_3$, 500 MHz)

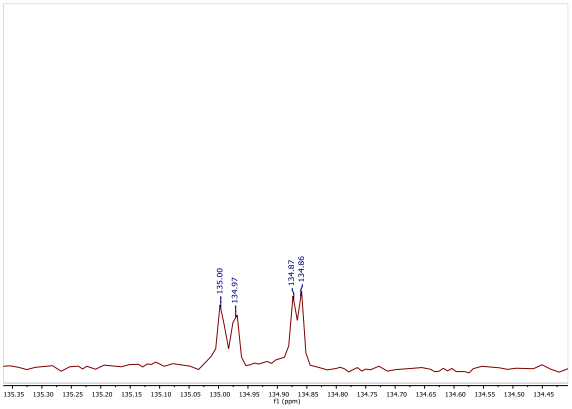


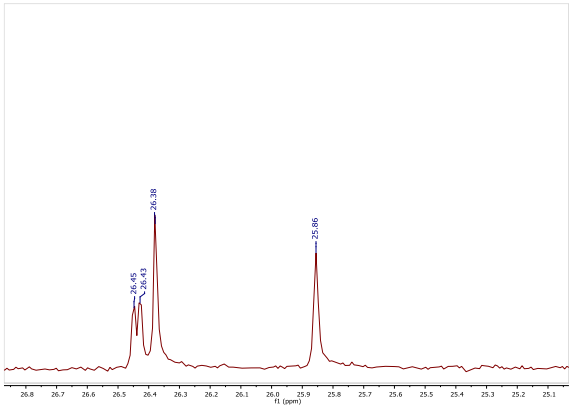


9.20.2 ^{13}C NMR spectrum of compound **6.1** ($CDCl_3$, 125 MHz)



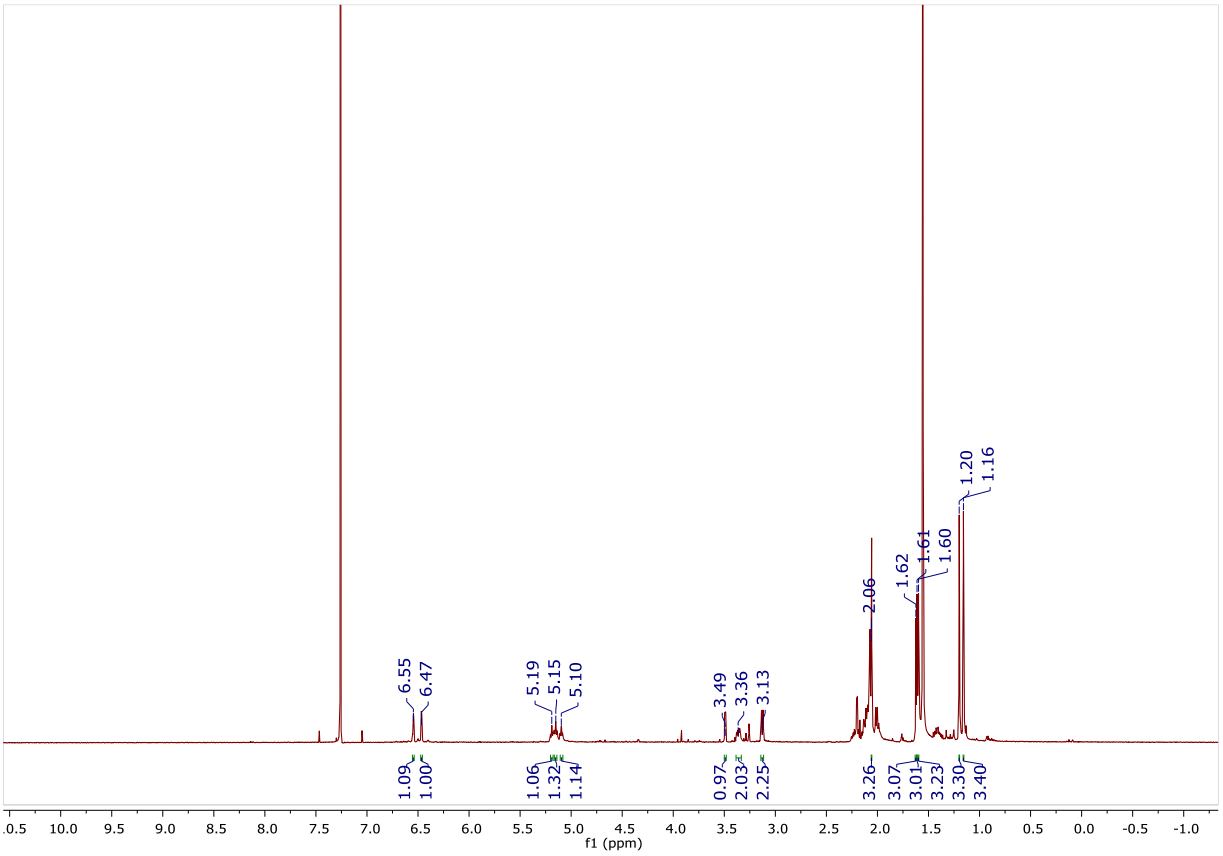
9.20.3 Twin signals observed in ^{13}C NMR spectrum of compound **6.1** ($CDCl_3$, 125 MHz)

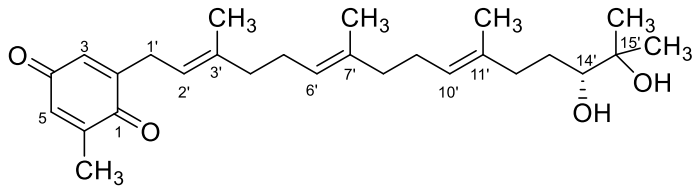




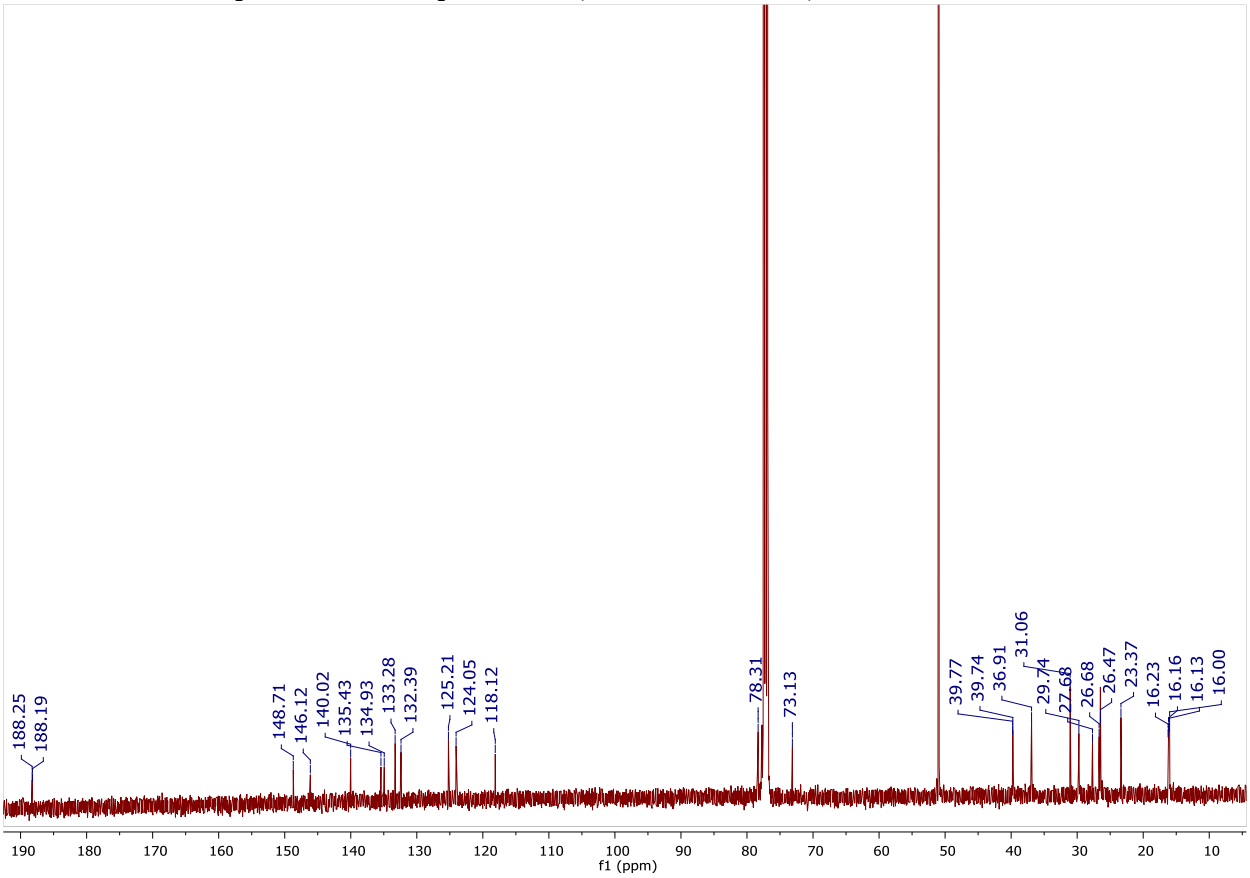
9.21 ^{1}H and ^{13}C NMR spectra of compound **6.2**

9.21.1 ^{1}H NMR spectrum of compound **6.2** ($CDCl_3$, 500 MHz)

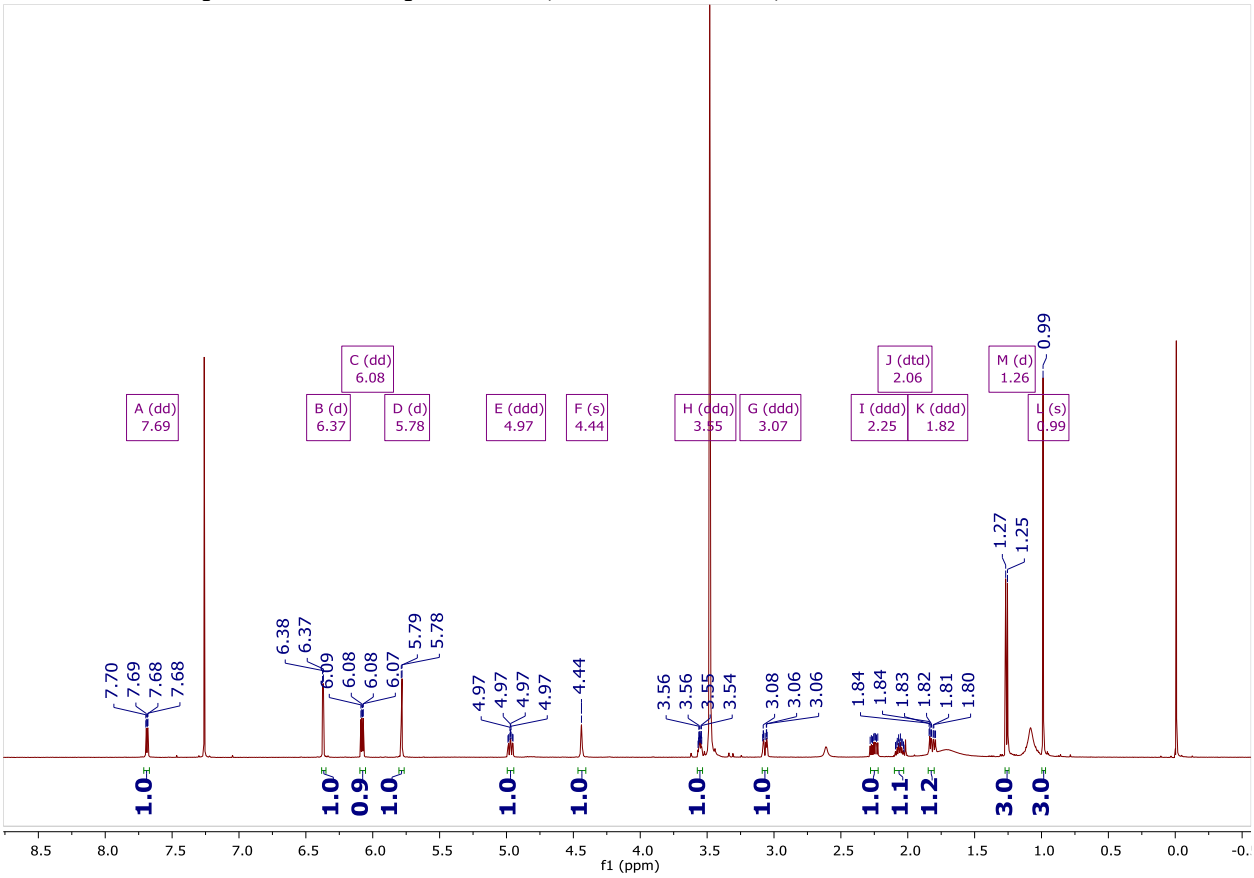


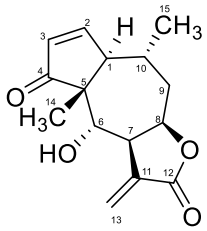


9.21.2 ^{13}C NMR spectrum of compound **6.2** ($CDCl_3$, 125 MHz)

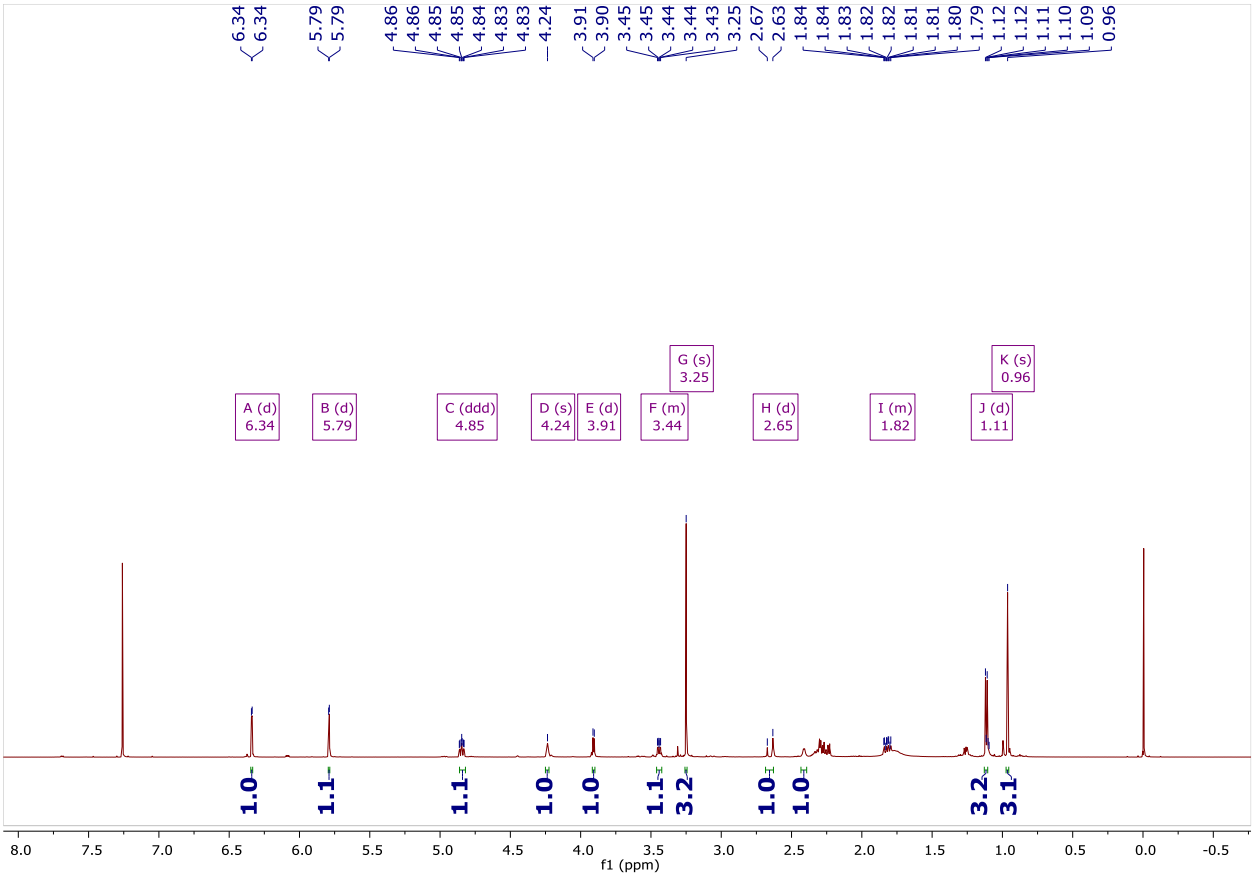


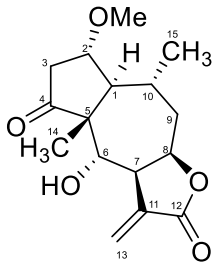
9.22 ^{1}H NMR spectrum of compound **7.1** ($CDCl_3$, 500 MHz)



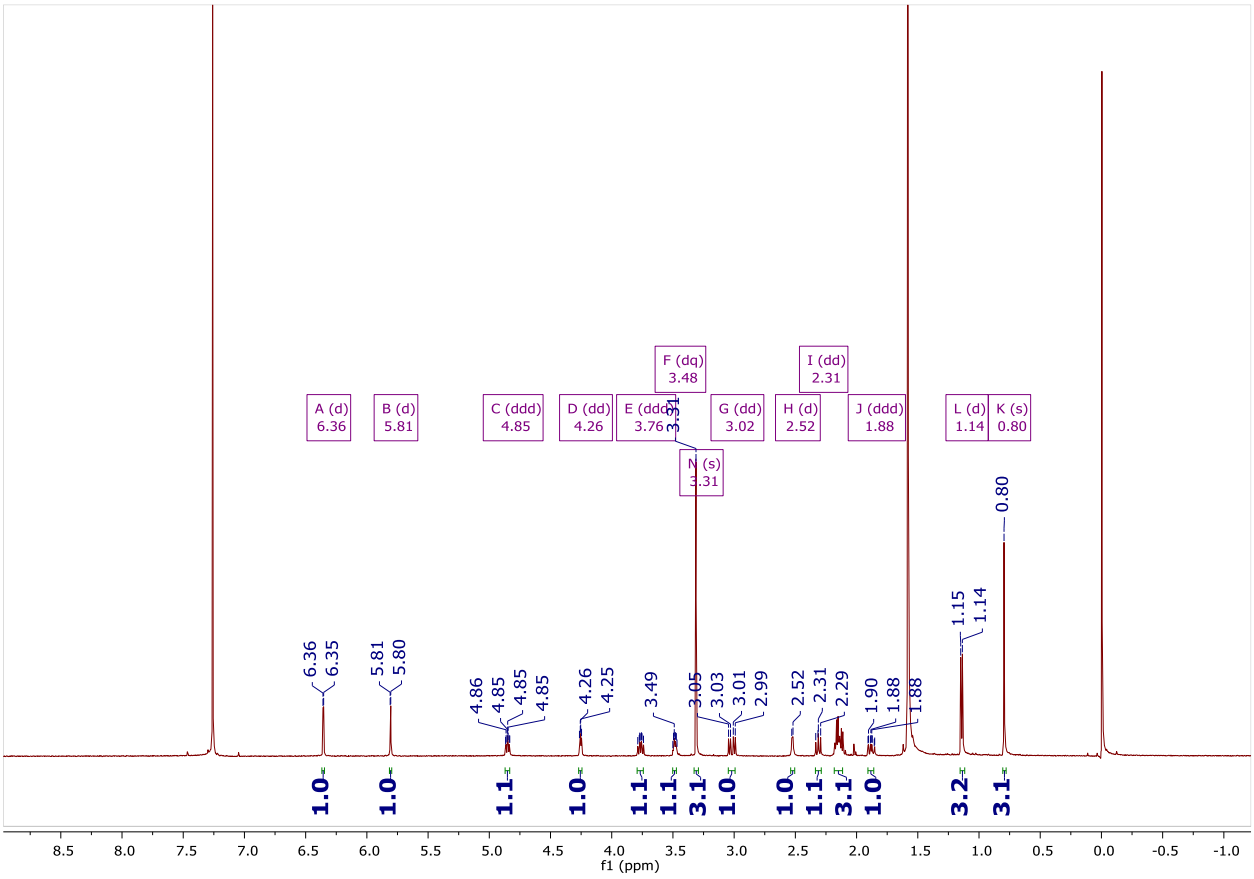


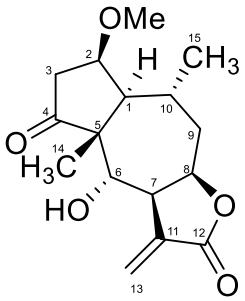
9.23 ^{1}H NMR spectrum of compound **7.2** ($CDCl_3$, 500 MHz)



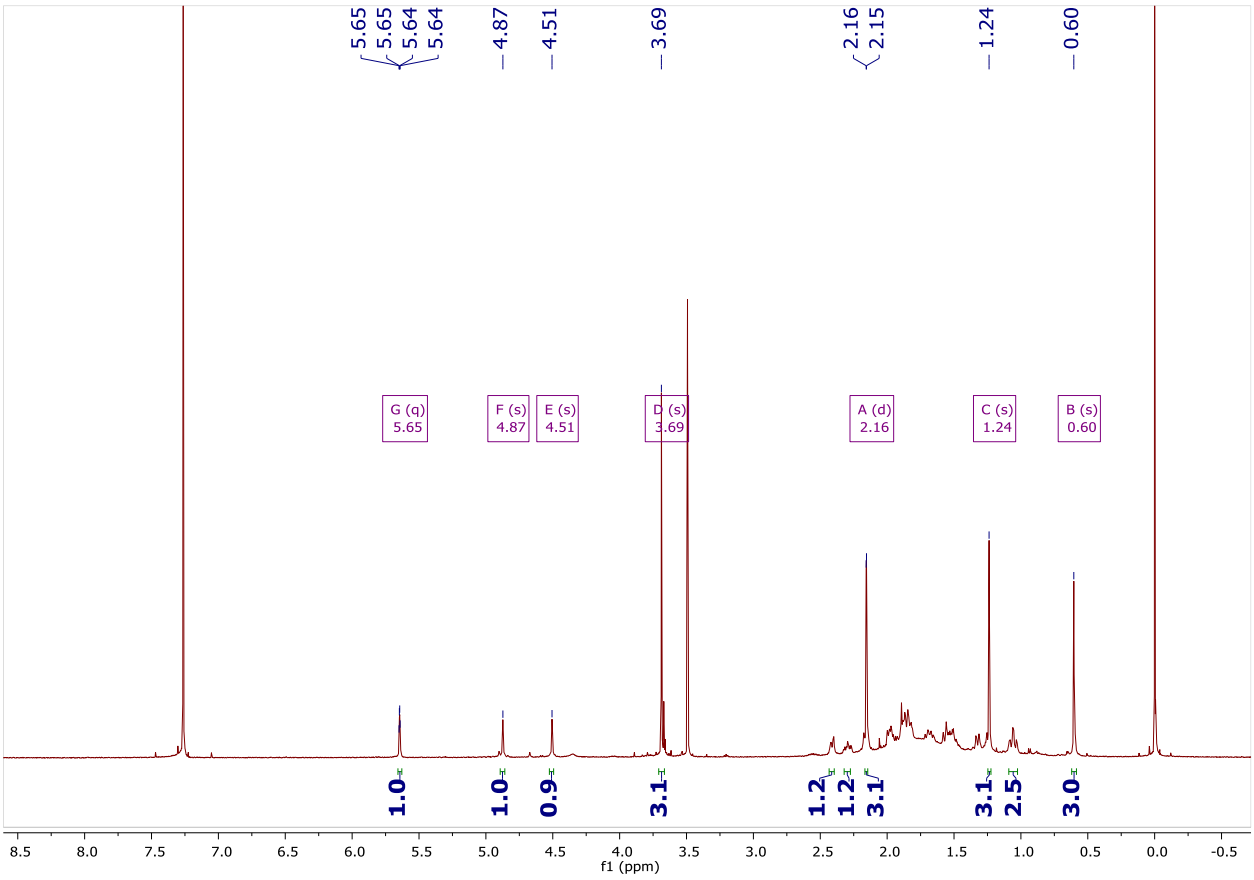


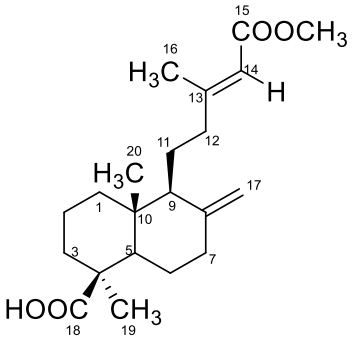
9.24 ^{1}H NMR spectrum of compound **7.3** ($CDCl_3$, 500 MHz)



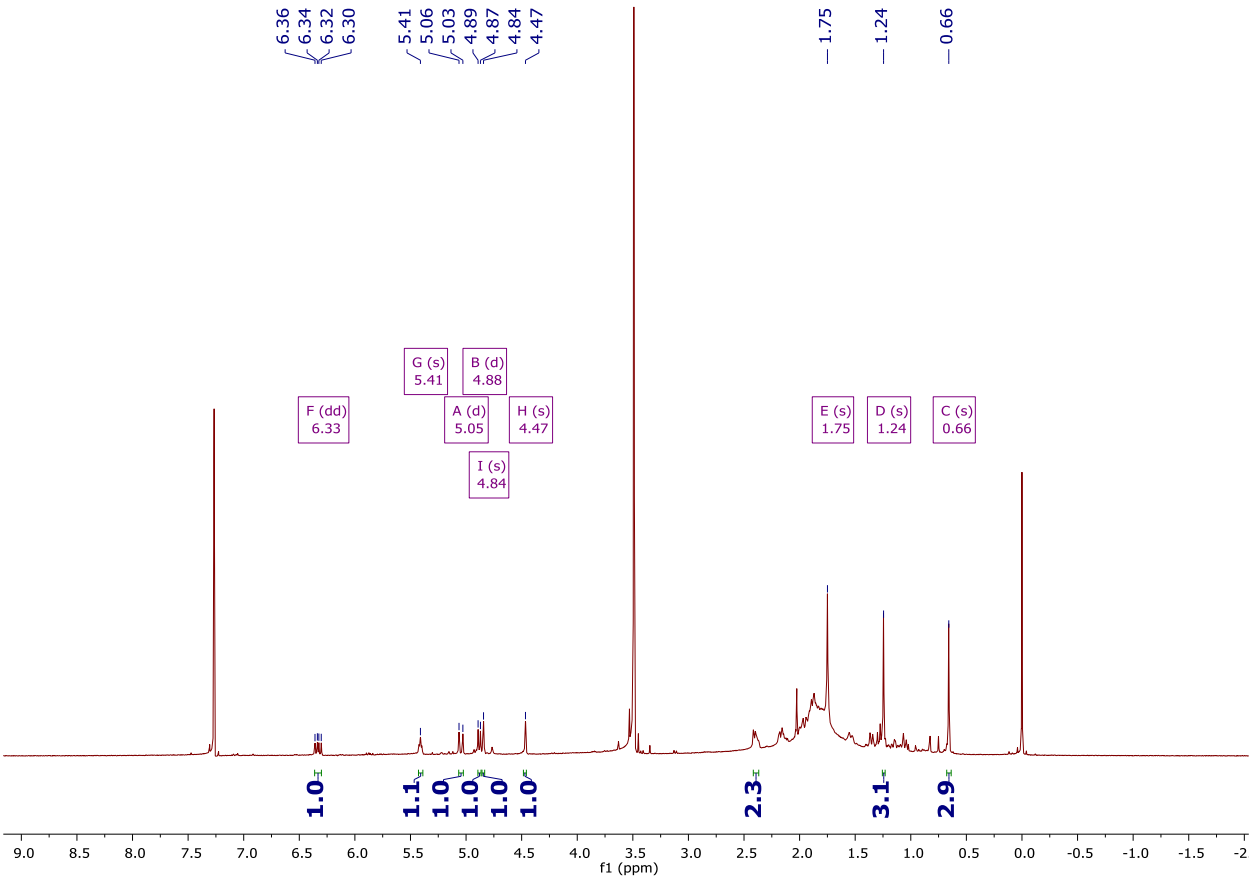


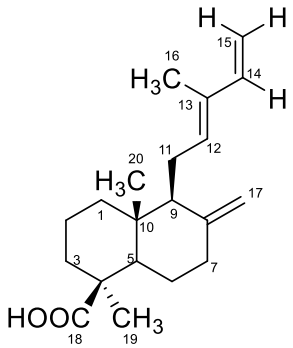
9.25 ^{1}H NMR spectrum of compound **7.4** ($CDCl_3$, 500 MHz)



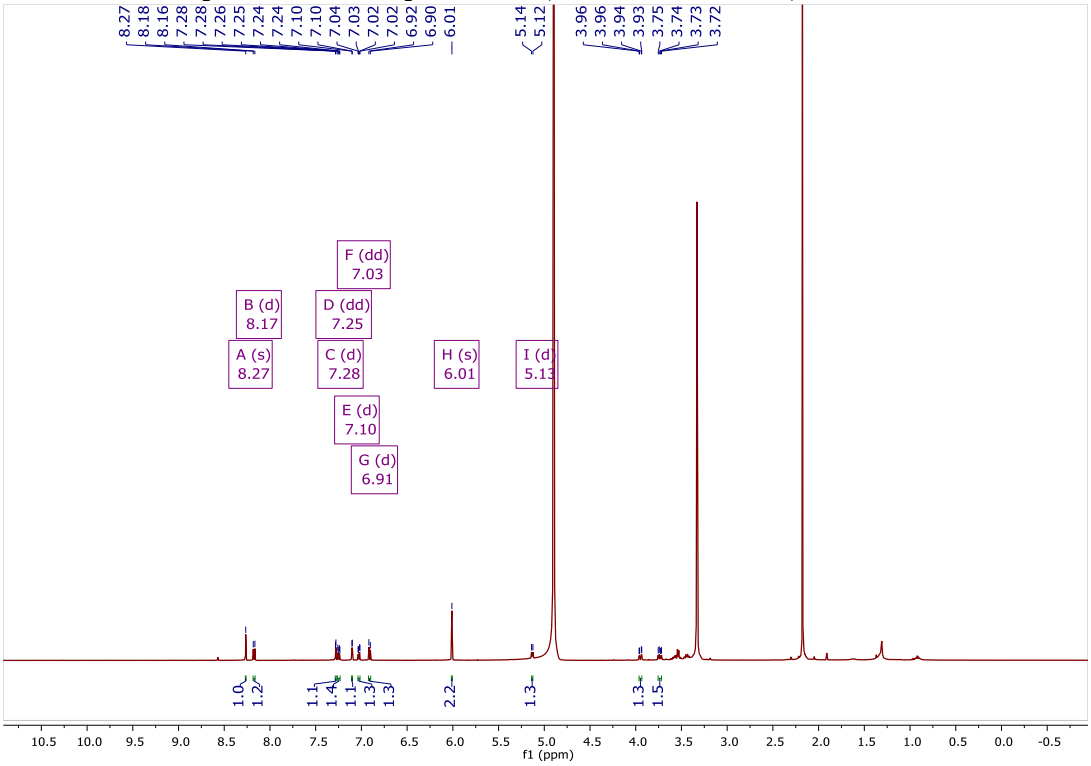


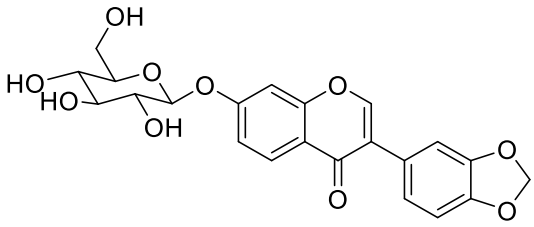
9.26 ^{1}H NMR spectrum of compound **7.5** ($CDCl_3$, 500 MHz)



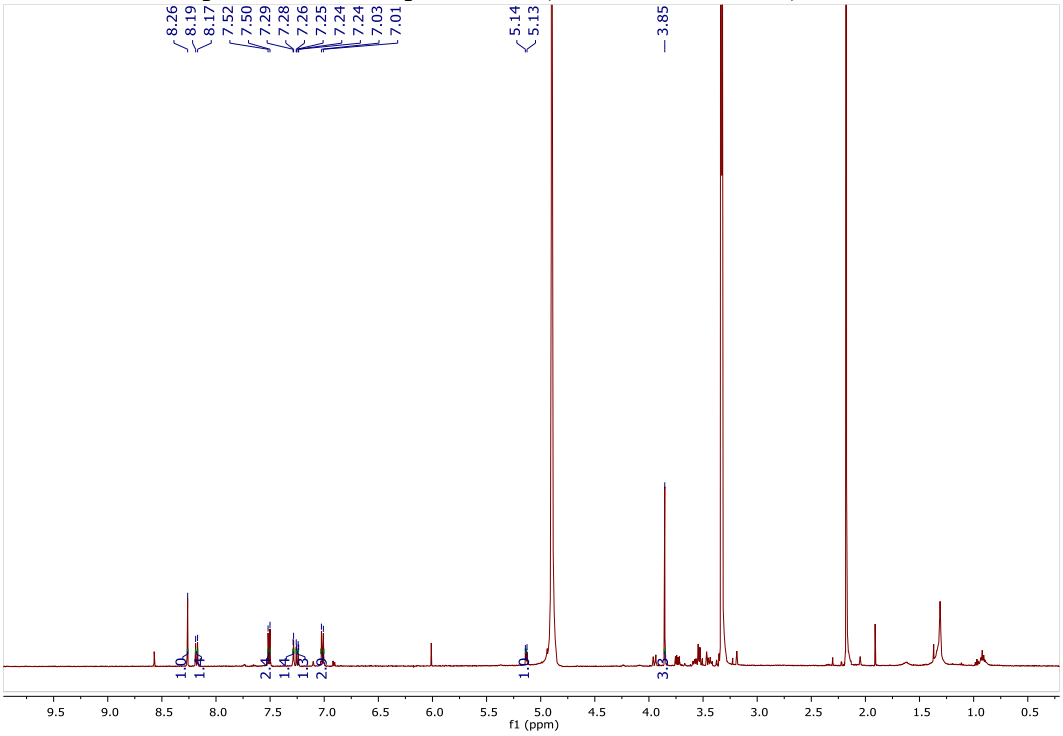


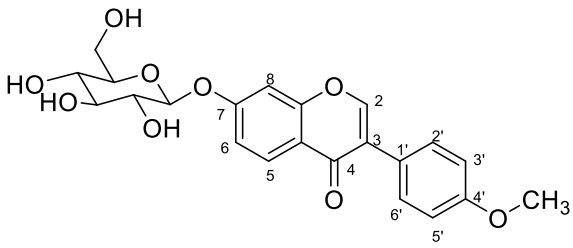
9.27 ^{1}H NMR spectrum of compound **7.6** (CD_3OD, 500 MHz)



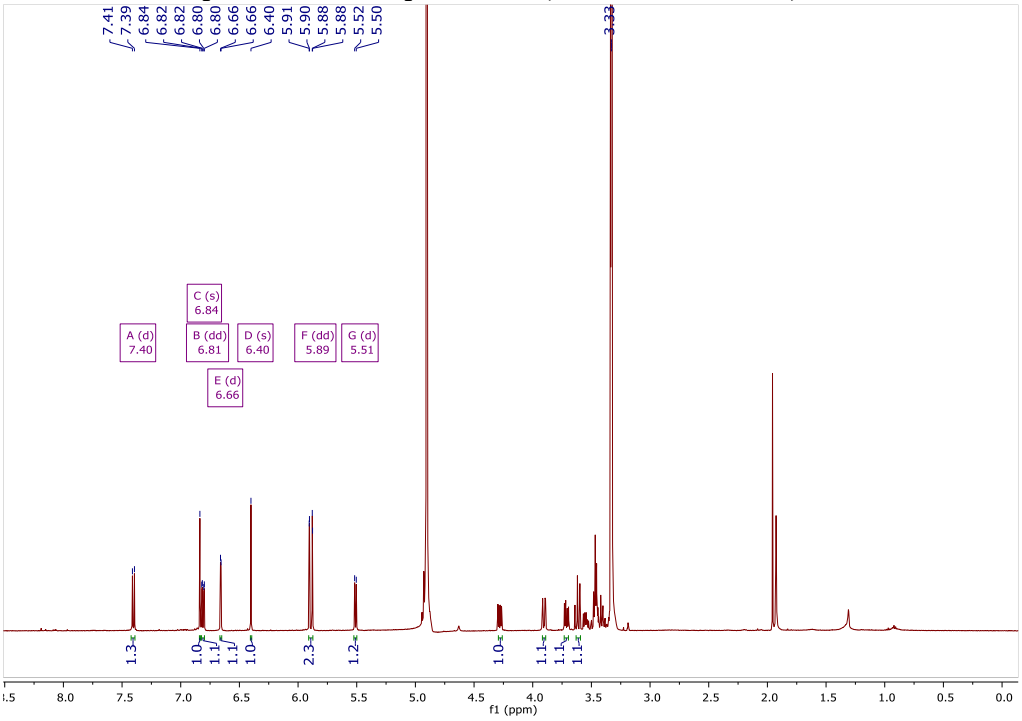


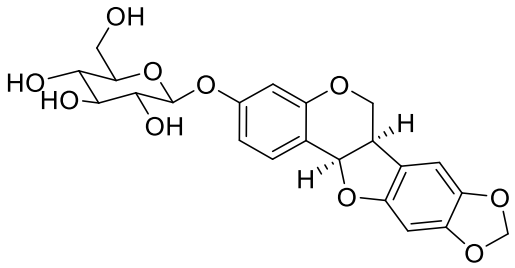
9.28 ^{1}H NMR spectrum of compound **7.7** (CD_3OD, 500 MHz)



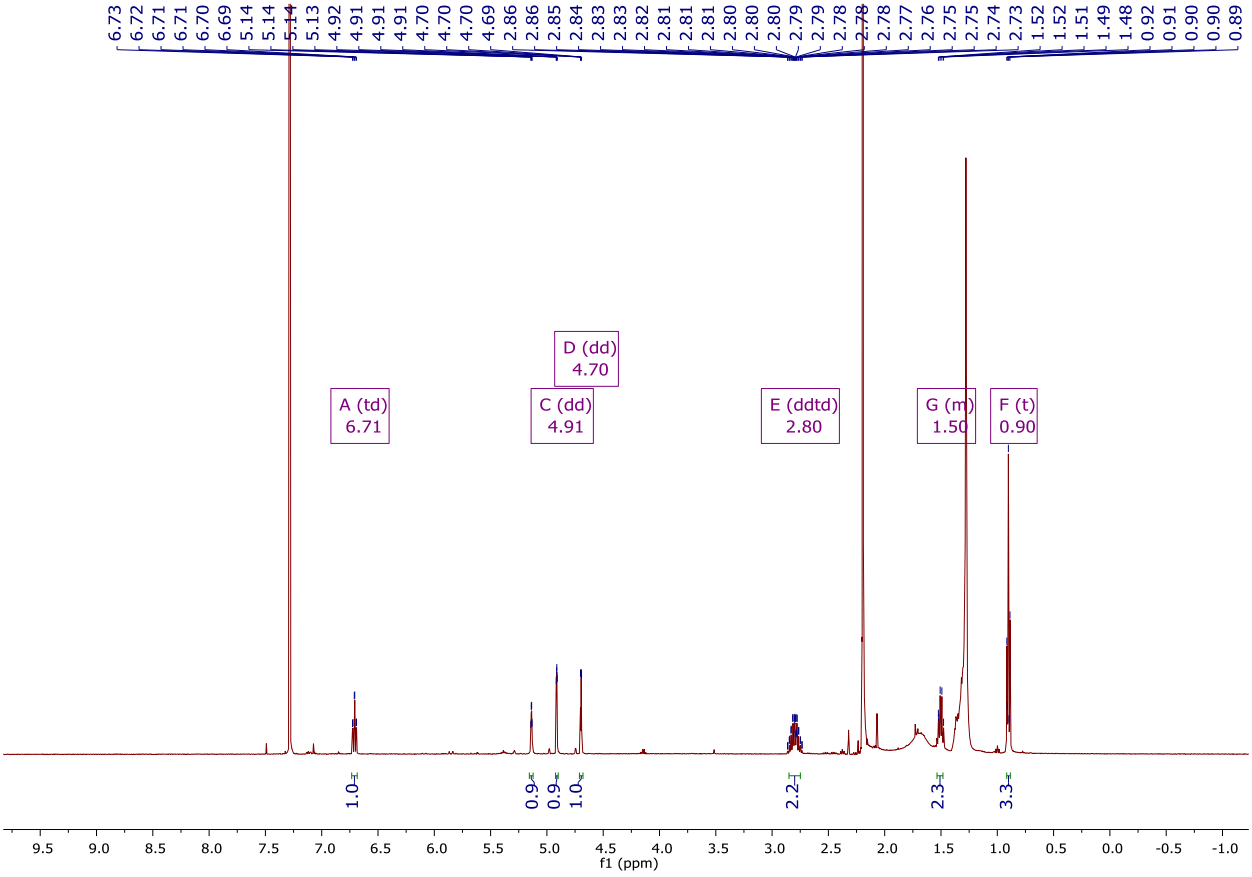


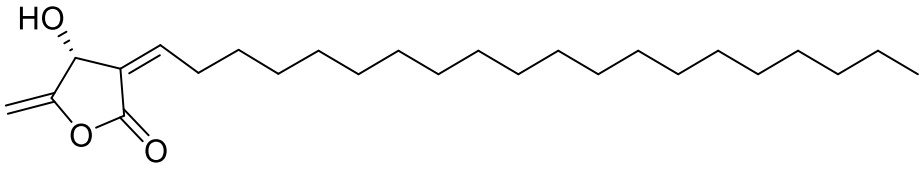
9.29 ^{1}H NMR spectrum of compound **7.8** (CD_3OD, 500 MHz)



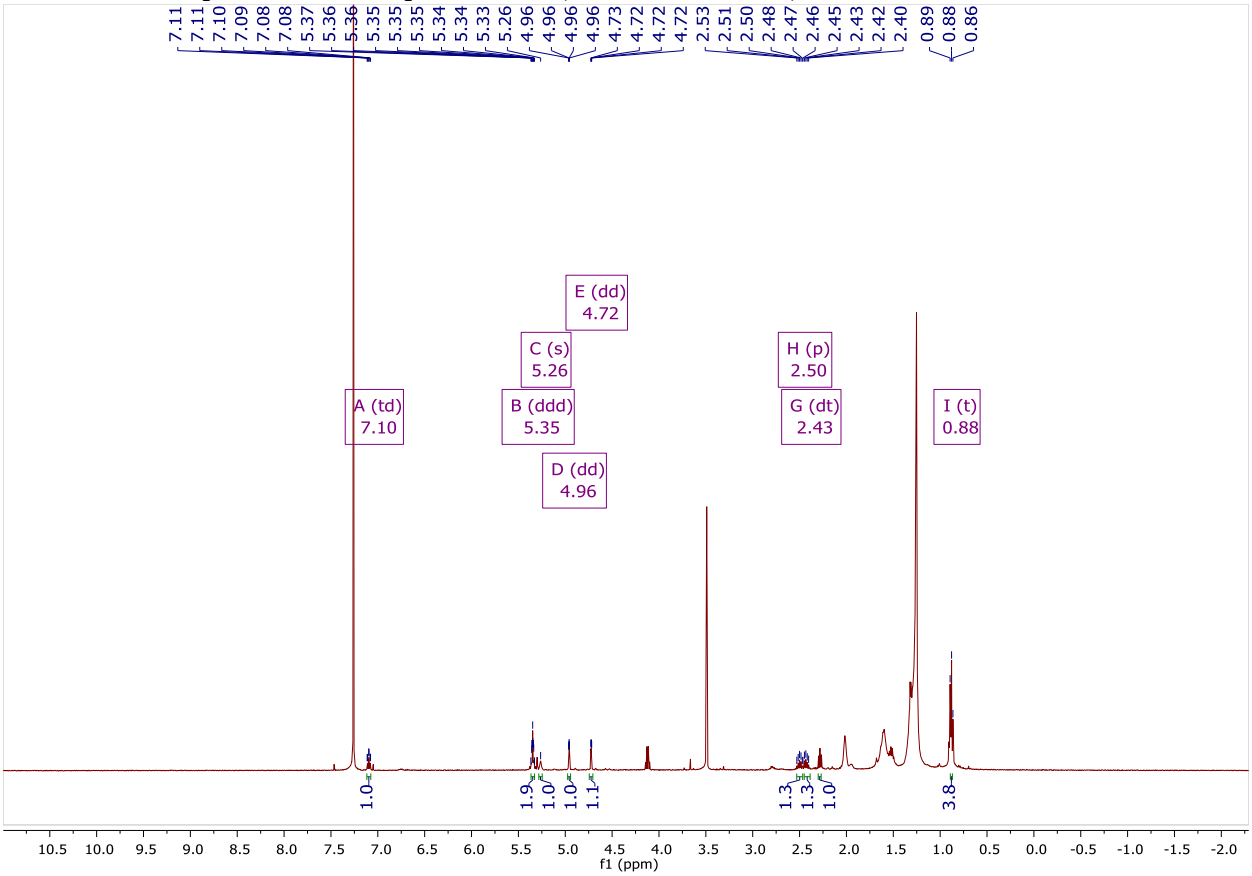


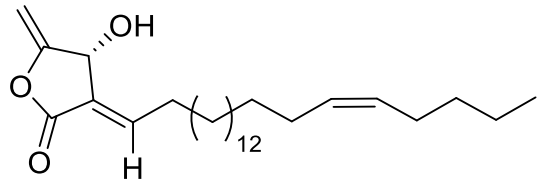
9.30 ^{1}H NMR spectrum of compound **7.9** ($CDCl_3$, 500 MHz)



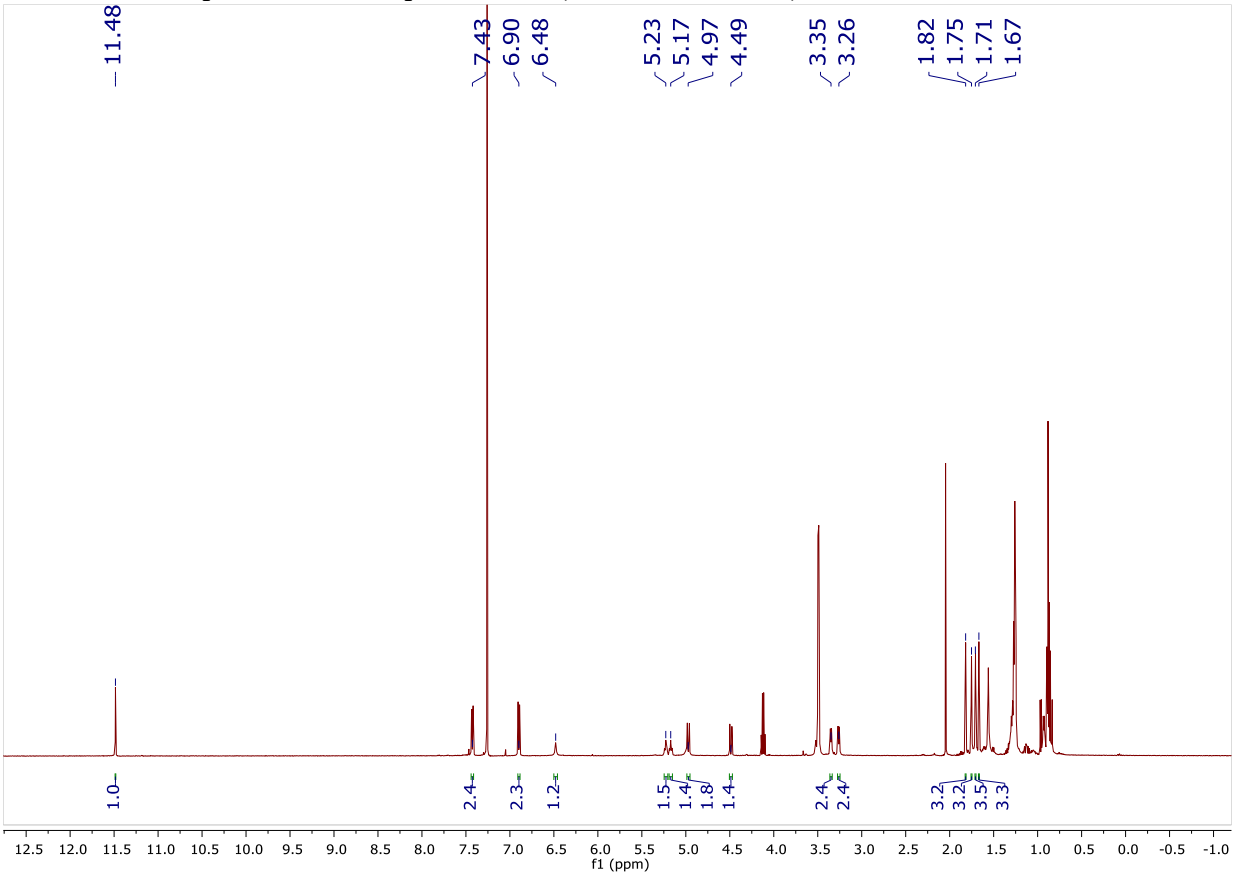


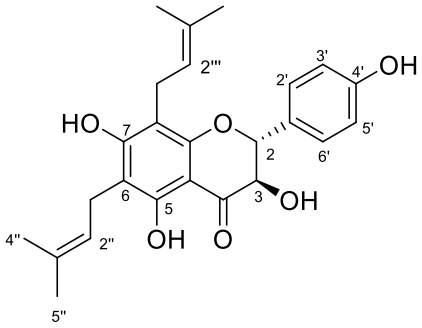
9.31 ^{1}H NMR spectrum of compound **7.10** ($CDCl_3$, 500 MHz)



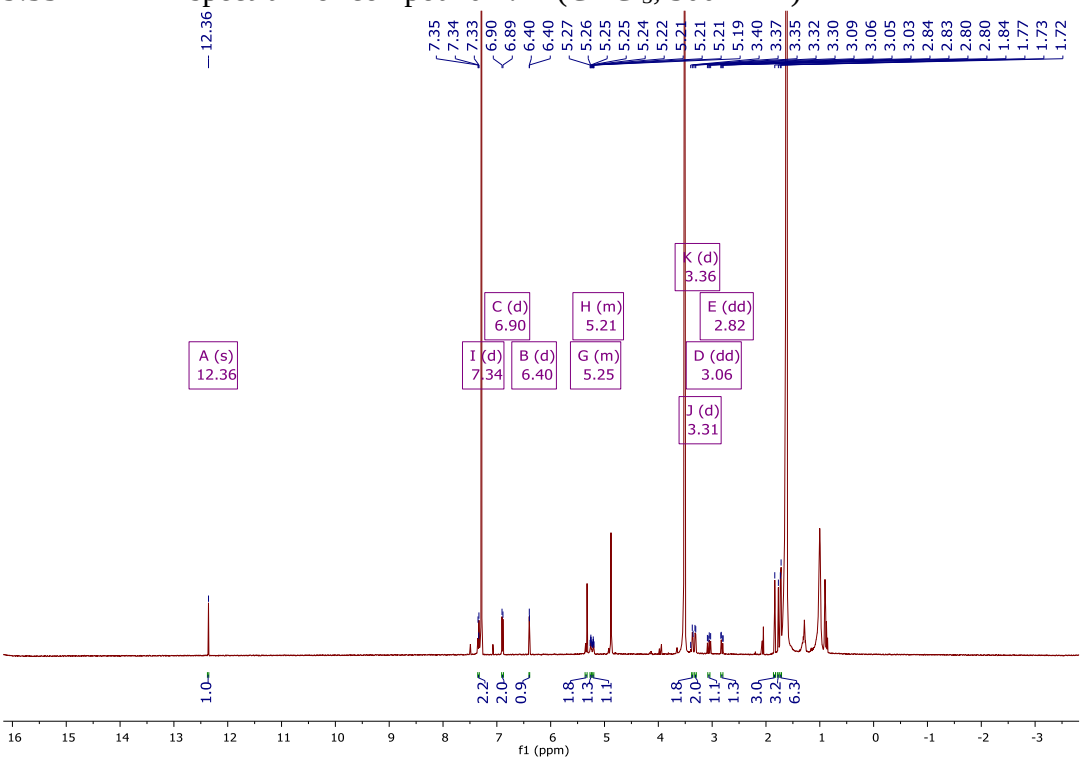


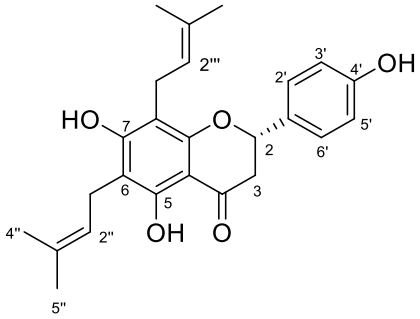
9.32 ^{1}H NMR spectrum of compound **7.11** ($CDCl_3$, 500 MHz)



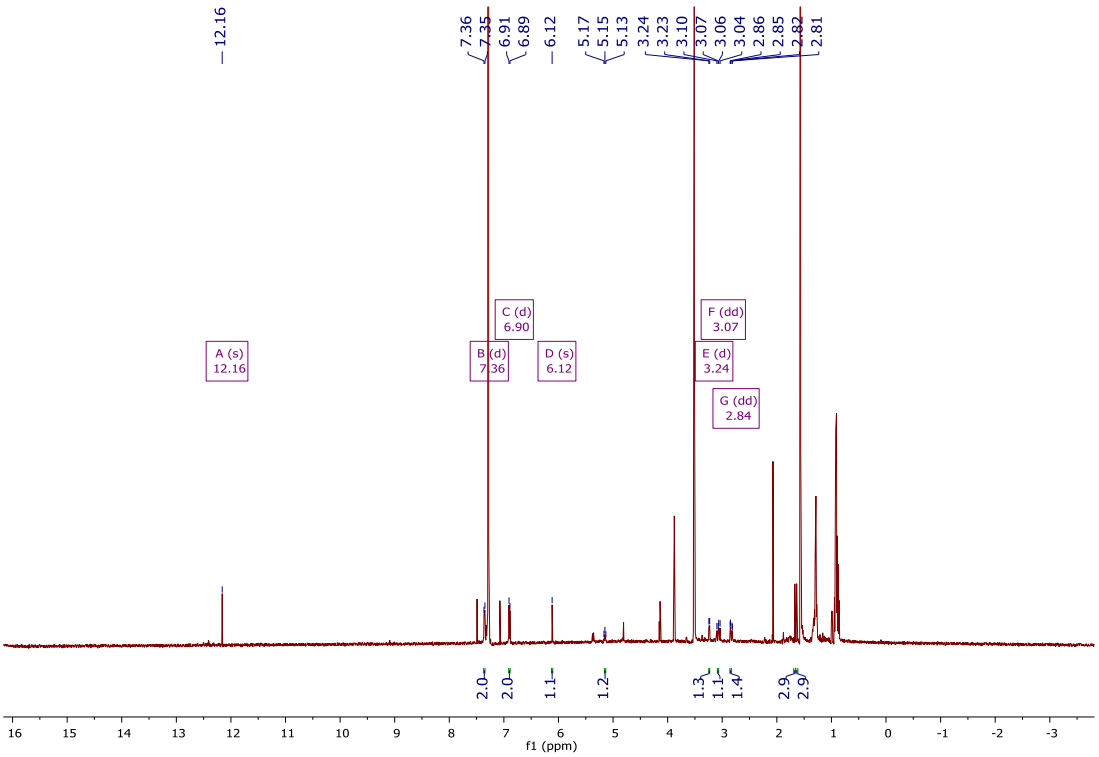


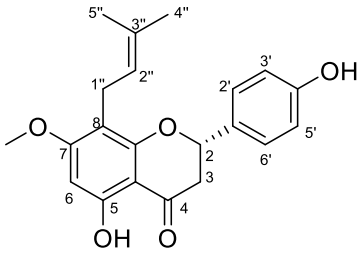
9.33 ^{1}H NMR spectrum of compound **7.12** ($CDCl_3$, 500 MHz)





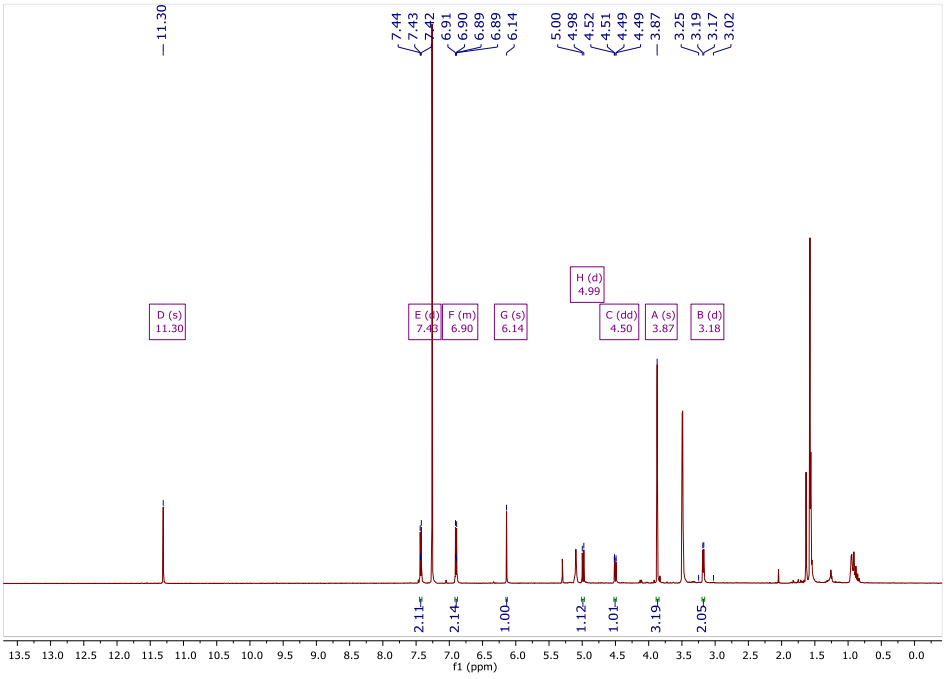
9.34 ^{1}H NMR spectrum of compound **7.13** ($CDCl_3$, 500 MHz)

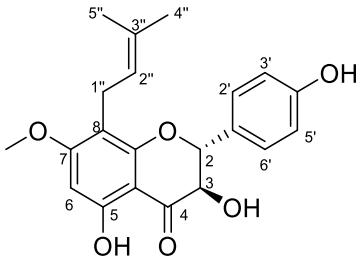




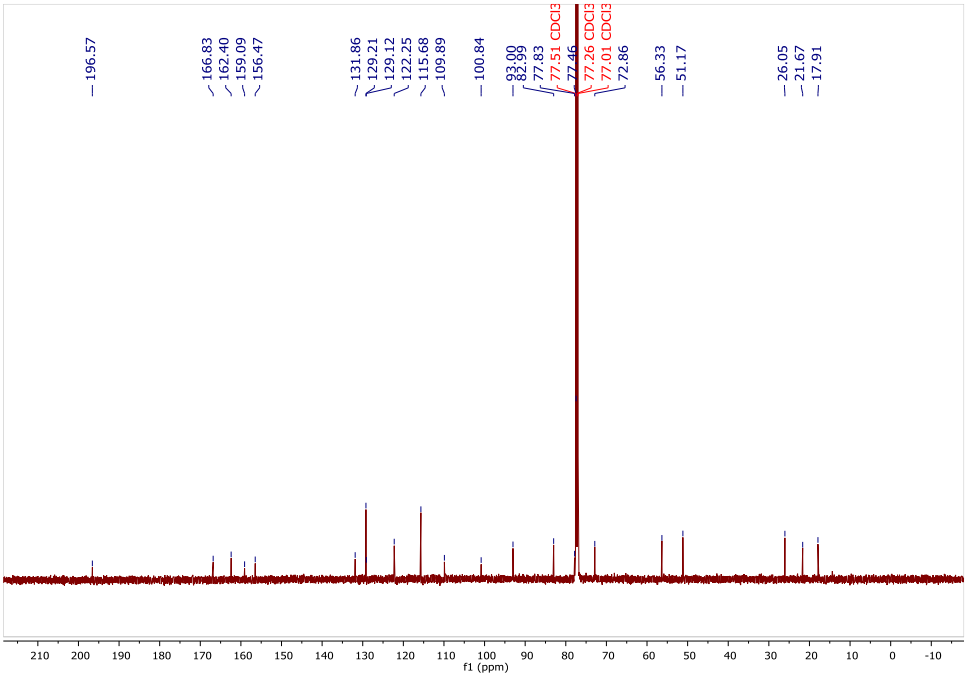
9.35 ^{1}H and ^{13}C NMR spectra of compound **7.14**

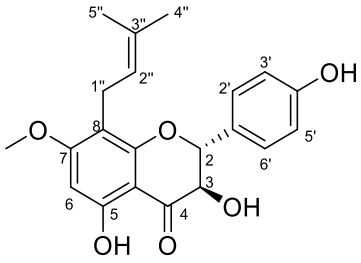
9.35.1 ^{1}H NMR spectrum of compound **7.14** ($CDCl_3$, 500 MHz)



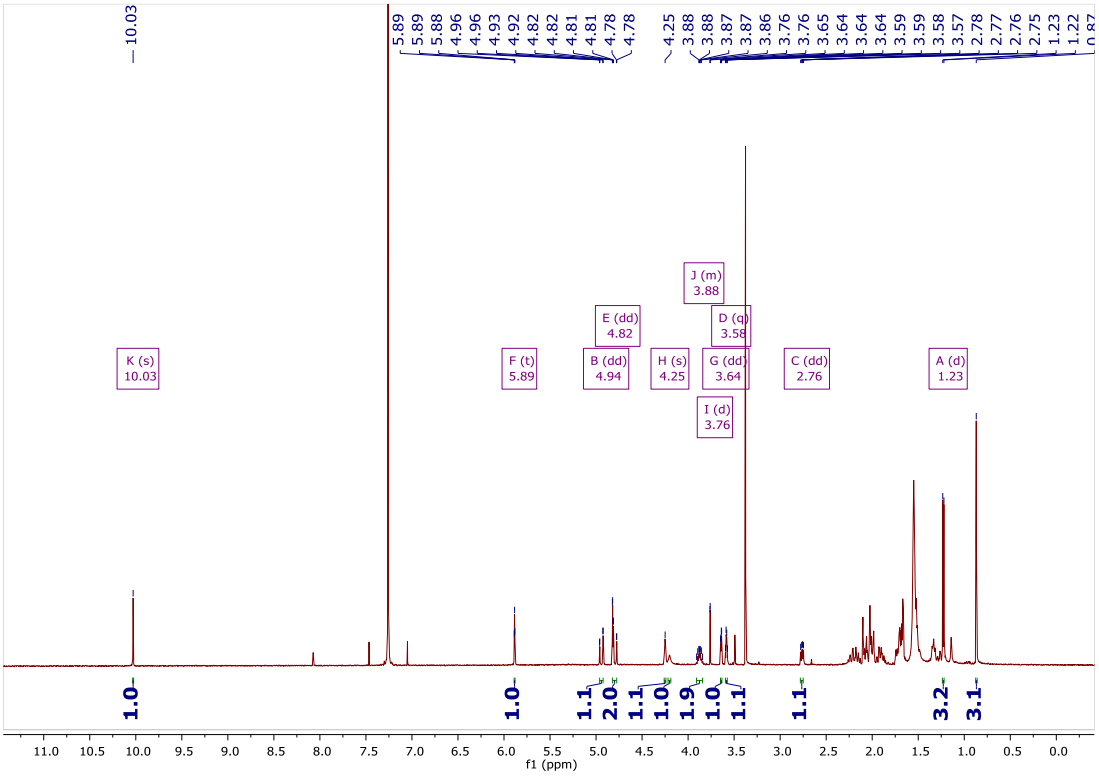


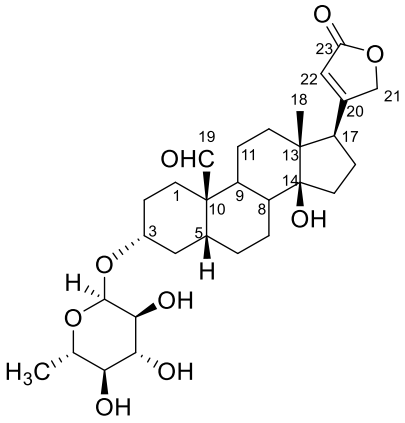
9.35.2 ^{13}C NMR spectrum of compound **7.14** ($CDCl_3$, 125 MHz)



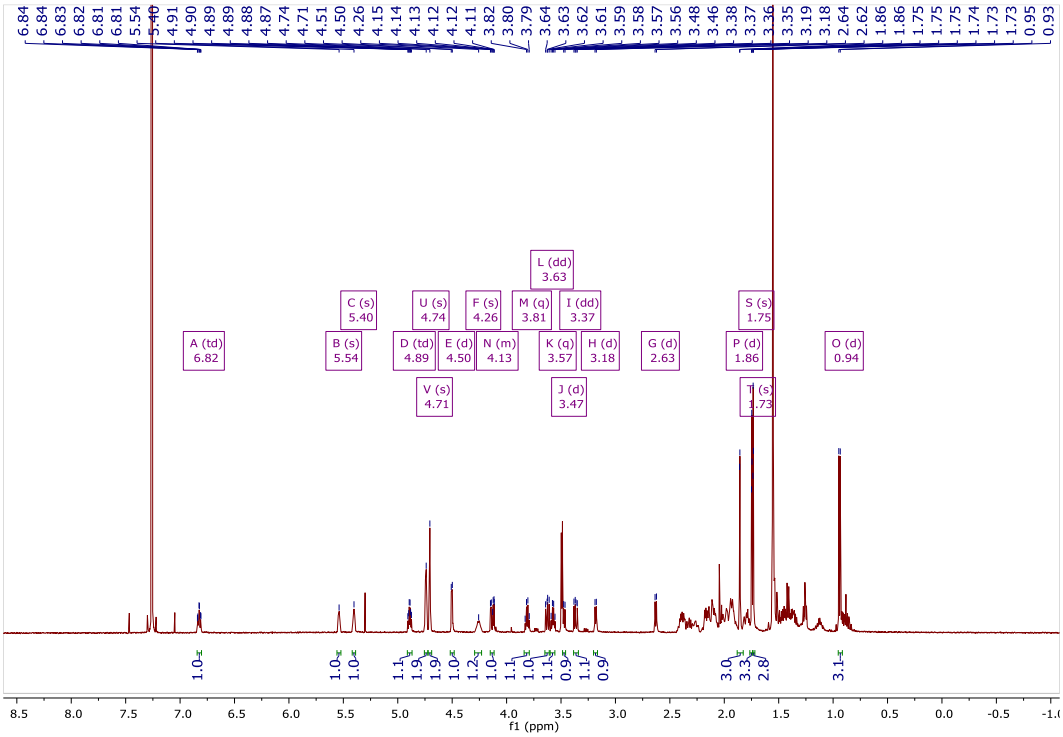


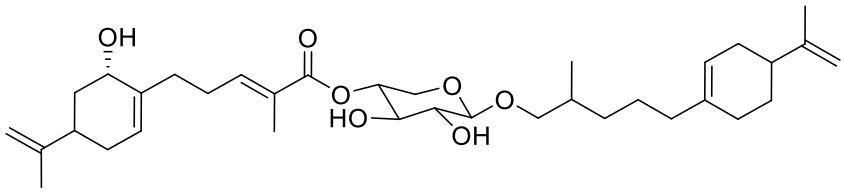
9.36 ^{1}H NMR spectrum of compound **7.15** ($CDCl_3$, 500 MHz)





9.37 ^{1}H NMR spectrum of compound **7.16** ($CDCl_3$, 500 MHz)





9.38 ^{1}H NMR spectrum of compound **7.17** ($CDCl_3$, 500 MHz)