Phytochemical and bioactivity investigations on *Aptosimum elongatum* Engl. extracts

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Phytochemical and bioactivity investigations on *Aptosimum elongatum* Engl. extracts

By

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Co-Supervisor: Dr. Anke. Wilhelm

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DECLARATION

I declare that the dissertation hereby submitted by me for the M.Sc. degree at the University of the Free State is my own independent work and has not previously been submitted by me at another University/Faculty. I further more cede copyright of the dissertation in favour of the University of the Free State.

Tsebo Sentle Molahloe

Date

This dissertation is dedicated to my son,

Akhumzi Tsentle Molahloe

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Tsebo Sentle Molahloe

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Abstract

In ancient times, the use of plants for traditional medicinal purposes has formed the foundation of what we call modern medicine today. Phytochemical investigations of plants used in traditional medicine have led to the discovery of many active compounds used in drugs today. *Aptosimum elongatum* (Scrophulariaceae) is an indigenous South African plant that has been used by the Khomani bushman for traditional remedies, both human and veterinary. However, to our knowledge a full phytochemical investigation has never been performed on this plant.

In the search for new bioactive constituents, the dried and ground aerial parts of *A. elongatum* were extracted consecutively with dichloromethane (DCM) and then methanol (MeOH). Five compounds were isolated from the two crude extracts via various chromatography techniques and were identified as glucosylated iridoids, a class of monoterpenoids. Several spectroscopic and spectrometry techniques were used for structure elucidation, including ^{1}H and ^{13}C 1D NMR, 2D COSY, HSQC, HMBC, and NOESY NMR experiments, high resolution electrospray ionization mass spectrometry, and infrared spectra.

The methanol crude extract yielded four compounds, three of which were identified as the known glucosylated iridoids angeloside (formerly isolated from *Angelonia integerrima* (Scrophulariaceae)), geniposidic acid, formerly isolated from *Gardenia jasminoides* Ellis (Rubiaceae)), and caryoptoside (formerly isolated from *Lamium album* (Lamiaceae)), and one novel glucosylated iridoid esterified at C-7 with a foliamenthoyl group, closely related to picconioside IV (formerly isolated from *Picconia excelsa* (Oleaceae), but with an extra hydroxy group. This compound was also isolated from the DCM crude extract, together with a second novel glucosylated iridoid, identical to the previous compound except for foliamenthoyl esterification at both C-7 and C-6″ (glucose moiety).

The crude MeOH and DCM extracts of *A. elongatum* showed moderate antioxidant activity, but only the DCM extract exhibited moderate inhibition of acetylcholinesterase, and GABAergic activity. Of the isolated compounds, caryoptoside and the mono foliamenthoate derivative showed antioxidant activity, and only the mono foliamenthoate derivative tested positive for AChE inhibition. Due to time constraints the isolated compounds have not been tested in the GABAergic assay.

Glucosylated iridoids have been isolated from a number of plants belonging to the Scrophulariaceae, and even from the Aptosimum genus, but this is the first report on a phytochemical study of *Aptosimum elongatum*.

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List of abbreviations

Ach	Acetylcholine
AChE	Acetylcholinesterase
AIDS	Acquired immunodeficiency syndrome
Al_2O_3	Alumina
AD	Alzheimer's disease
A. elongatum	Aptosimum elongatum
$CDCl_3$	Chloroform-d
^{13}C NMR	Carbon-13 nuclear magnetic resonance
CNS	Central nervous system
Col. nr.	Voucher specimen number
COSY	Homonuclear correlation spectroscopy
2D	Two-dimensional
DPPH	2,2-diphenyl-1-picrylhydrazyl
DCM	Dichloromethane
EtOAc	Ethyl acetate
EtOH	Ethanol
FTIR	Fourier transforms infrared spectrometry
GABA	γ-aminobutyric
GEA	Geniposidic acid
^{1}H NMR	Proton nuclear magnetic resonance
HCl	Hydrochloric acid

HMBC	Hetero-nuclear multiple bond correlation spectroscopy
HPLC	High-performance liquid chromatography
HR ESIMS	High-Resolution Electronspray Mass Spectrometry
HSQC	Hetero-nuclear single quantum correlation spectroscopy
HTS	High-throughput screening
Hx	Hexane
I	Nuclear spin
IPSPs	Mediate fast inhibitory postsynaptic potentials
IR	Infrared
LCMS	Liquid chromatography–mass detector
LCNMR	Liquid chromatography – nuclear magnetic resonance spectroscopy
LCPDA	Liquid chromatography–photodiode array detector
MeOD	Methanol-d4
MeOH	Methanol
MS	Mass spectrometry
MVA	Mevalonic acid
MW	Molecular Weight
NMB	National Museum Bloemfontein
NMR	Nuclear magnetic resonance
NOESY	Nuclear overhauser effect spectroscopy
ROS	Reactive oxygen species
SiO_2	Silica gel
SiOH gel ccc	Silica gel column chromatography column

TCM	Traditional Chinese Medicine
TLC	Thin-layer chromatography
TMS	Tetramethylsilane

Chapter 1: Introduction and Aims

Herbalism can be defined as the study or practice of medicinal and therapeutic uses of plants. Medicinal plants have been used traditionally over centuries for various ailments and therefore played a critical role in the development of human cultures around the world, especially in primary health systems. Scientists have drawn on traditional knowledge to identify extractable compounds from the plant organs for medicinal purposes or activities.[1]

Higher plants are a source of millions of natural products, with an immense variety of structural variations.[2,3] Only 15% of approximately 250 000 plant species worldwide have been phytochemically investigated, and the number of plants evaluated for the presence of biologically active compounds are even less.[4] A number of plant secondary metabolites have been used in conventional medicine, and as drug precursors, drug prototypes and pharmacological probes.[5,6] South Africa is one of the most bio-diverse areas in the world and has nearly 10% of the world's flora. The country has approximately 22 000 angiosperm species and an estimated 3000 plants are used as medicine on a regular basis.[7,8] The majority of medicinal plants are collected in the veld in South Africa, where in Europe plants used for medicine are cultivated on a large scale.[9] Surprisingly, only a few of the South African medicinal plants have been commercialized to some extent and are available as processed materials,[10] but some promising medicinal plants from South Africa have not yet been compiled in a reliable reference system.

Medicinal plants that are used by ancient cultures for a variety of lethal diseases are poorly understood and many diseases pose serious complications to the lives and health of humans. Thus, there is a need to identify and process naturally occurring compounds from medicinal plants. This may lead to novel drug discovery which may cure a variety of diseases.

The Bushmen are believed to be the first people of southern Africa, known to have practiced a hunter-gatherer mode of production.[11] Therefore, the Khomani Bushmen of the southern Kalahari are considered to be one of the most ancient cultures. They had an extensive knowledge of remedies extracted from various medicinal plants, which were passed on to new generations up to the present.[12] The emphasis of studies that have been done on the Bushman's

ecology, has been on plants used for food and water, while medicinal plants and their uses have remained poorly understood.[13]

The Karoo violets (Genus: Aptosimum), known locally as "magatho", meaning "wash-out", from the family Scrophulariaceae, are indigenous to southern Africa and has been used by the Khomani Bushmen as traditional medicine. Manetti (2011) reported that the root, stem and leaves of *Aptosimum albomarginatum* Marloth and Engl. and the stem, leaves and flowers of *Aptosimum elongatum* Engl. were used as medicine by the Khomani Bushmen.[13] Van Wyk et al. (2008)[14] reported that the powdered ash of *Aptosimum procumbens* (Lehm.) Steud. (Whole plant) was applied to burn wounds to dry it out. Therefore, we selected one of the lesser known Karoo violets, *Aptosimum elongatum*, to investigate its phytochemistry and bioactivity of crude extracts in medicinal applications, and to isolate new bioactive compounds as potential novel drugs.

The general objective of this study can be summarized as follows:

To establish information that can be used to standardize compound(s) of *Aptosimum elongatum* which are responsible for its medicinal properties.

The specific objectives of this study were the phytochemical and bioactivity investigation of *A. elongatum* via:

(i) The consecutive extraction of the aerial parts of *A. elongatum* with DCM and MeOH.

(ii) The evaluation of the phytochemical profile of the resultant DCM and MeOH crude extracts of *A. elongatum* via compound class screening.

(iii) The isolation and structure elucidation of the chemical structures of compounds isolated from *A. elongatum*.

(iv) The screening of the crude extracts and isolated compounds for antioxidant activity (DPPH) and inhibition of acetylcholinesterase activity.

(v) The screening of the crude extracts for GABAergic activity via a two micro-electrode system utilizing *Xenopus* oocytes.

We envisage that bioactive compounds from *A. elongatum* can present us with new herbal remedies and we aim to develop the isolated compounds as markers for the standardization of herbal formulations of *A. elongatum*. Secondly, this study may lead to the discovery of

privileged scaffolds for the development of novel drugs, which may help in the fight against certain diseases. The results of our bioactivity testing of isolated compounds may validate the use of this plant, thus providing preliminary scientific justification for the traditional medicinal uses of this ethno remedy, which is an important step towards its acceptance and development as an alternative therapeutic agent.

Chapter 2: Literature Review

2. History of phytomedicines and drug discovery

2.1. Medicinal plants

Traditional medical practice remains the largest healthcare system in the world. The first records of the use of plants for their therapeutic or medicinal properties were depicted on clay cuneiform scripts from Mesopotamia in 2600 BC, which reported an expanded medicinal system consisting of about 1000 plant derived medicines, including documented oils from *Cypressus sempervirens* (Cypress) and *Cedrus* species (cedar), *Glycyrrhiza glabra* (licorice), *Commiphora species* (myrrh), and *Papaver somniferum* (poppy juice), which are all still used to treat ailments ranging from cough and colds to parasitic infection and inflammation.[15]

In Egypt, medicinal knowledge was dated back to about 2900 BC and the information was recorded in the Ebers Papyrus in about 1550 BC, which contains over 700 plant-based drugs ranging from gargles, pills, infusions and ointments. The papyrus covers concepts like contraception, diagnosis of pregnancy and other gynaecological matters, intestinal disease and parasites, eye and skin problems, dentistry and the surgical treatment of abscesses and tumors, bone-setting and burns. [16]

Some of the largest traditional medicine systems are found in the Hindu Kush Himalayas, including Ayurvedic medicine and Chinese medicine.[17] Ayurveda is still widely practiced in India. It had its origins from the believe that the Hindu god Brahma sent his knowledge of healing to the sages to save mankind from disease.[18] These medicinal remedies (Veda) were recorded in four main knowledge bases, namely Yajur Veda, Rig Veda, Sam Veda and Atharva Veda. The writings of Agnivesha, one of the earliest scholars on Ayurveda, was lost, but live on in the Charaka Samhita and Sushrut Samhita (1000-500 BC), which are currently the main compilations of the Vedas. The Charaka Samhita and Sushrut Samhita give detailed descriptions of over 700 herbs. The Charaka Samhita describes all aspects of Ayurvedic

medicine and Sushruta Samhita describes the Science of Surgery.[19] Ayurveda is also called the "science of longevity" because it offers a complete system to live a long, healthy life.[20]

Traditional Chinese Medicine (TCM) has been extensively documented over thousands of years and is collected largely in the Chinese Materia Medica, dated from 1100 BC in the Wu She Er Bing Fang, containing 52 prescriptions. These manuscripts were only discovered in 1973 during the excavation of the Ma Wang Dui tomb at Changsha, Hunan.[21] Shen-Nong Ben-Cao-Jing, a Chinese book on agriculture and medicinal plants written between about 200 and 250 BC, contains 365 entries on medicaments and their descriptions. These include 252 plant parts, 67 animal parts and minerals.[22] The Chinese government is currently playing an active role in promoting the ancient remedies of traditional medicine, integrating it with western medicine practices.

The Western version of herbal medicine began in the cradle of Western civilisation in Ancient Greece around the fifth century BC. Greek philosophers like Thales, Anaximander, Anaximenes, and Empedocles believed that Air, Earth, Water and Fire were the four basic elements of all creation. Hippocrates, however, is widely acknowledged as the "father" of Western medicine and he developed a holistic approach to medicine based on the four humours Blood, Lymph, Gall and Mucus as the four major components of living.[23] Hippocrates developed a set of ethics for medical treatments that is still in use today. He proclaimed among others: "follow the medical rules, no other; act only for the Good, upon own critical knowledge and own responsibility; give neither drug nor advice for death; keep own life pure and saint; keep medical profession pure and saint; all seen or heard of people's life will remain unspoken; respect all this, as it is written and sworn, for maintaining life and (medical) profession honoured; by all mankind, for all times to come..."[23] During the first century AD, Dioscorides, a Greek physician, pharmacologist and botanist, wrote De Materia Medica, which described herbs and plant remedies he collected while travelling with the Roman army. It was used as reference book for physicians for centuries. In the period 150 – 200 AD Galen wrote approximately 30 books on pharmacology and medicine, describing complex prescriptions and formulas used in compounding drugs, sometimes containing dozens of ingredients ("galenicals").[24] These developments caused the slow disappearance of traditional herbalists, with their "simple" remedies, and traditional herbal medicine was only preserved by Catholic monks throughout the middle Ages. Herbal knowledge of Arab physicians was incorporated into Western herbal medicine in about 800 AD, and with the discovery of the Americas in 1500

AD, a variety of the New World medicinal plants became available to Europeans. Significant contributions to the European medicinal system came from the ancient New World, gaining knowledge of ancient herbal medicine systems from Mexican traditional medicine. Mexican traditional medicine included the knowledge of several indigenous groups, namely Nahua, Maya, Mixe and Zapotec, as well as the Inca civilisation.[25] Medicinal plants continued to be the main source of products used for the maintenance of health in Western conventional medicine until the nineteenth century, when urea was accidentally synthesized by Friedrich Wohler in 1828.[26] This first organic synthesis in human history ushered in the age of synthetic compounds. Over the next 100 years, synthetic drugs became the mainstay of Western conventional medicine, with phytomedicines playing a secondary role.

African traditional medicine has been practised since antiquity and was a holistic shaman-based system. Owing to the rich biological and cultural diversity in Africa, African traditional medicine has evolved in different societies with marked regional differences in healing.[27] Some practitioners use only herbal remedies (herbalists), some use spiritual healing (diviners), and some practice both.[28] Unfortunately, knowledge of plants and remedies used has not been documented, since this information has only been transferred by word of mouth from father to son, or mentor to student over the ages. In South Africa a large part of the population still uses traditional medicine, especially in isolated rural areas. The last two decades has seen an increase in research projects that document, and phytochemically investigate, plants and herbs purported to treat targeted illnesses via information gathered from traditional healers and/or communities.[29,30,31,32] Table 2.1 documents some of the South African medicinal plants that have been commercialized.

Table 2. 1: ***Some commercialized medicinal plants from South Africa***

Plant	Common name	Traditional uses	Commercialization	Source
Agathosma betulina (Rutaceae)	Buchu	Antipyretic, antispasmodic, cough, flu remedy, cold, diuretic, kidney and urinary tract, gout, infections, haematuria, prostatitis, cholera, stomach ailments, rheumatism, antiseptic bruises[33,34]	Extracted oil: 700 Euros/kg Buchu seed: R20 000/kg[35] Cultivated material: $56/kg.[36]	Veld and small cultivation farms [26]
Aloe ferox (Asphodelaceae)	Cape Aloe or bitter Aloe	Skin and hair treatments,[33] burns, insect bites, sores, and sunburn (leaves directly applied), and arthritis, conjunctivitis, toothaches, sinusitis, and stomach	Main wild-harvested commercially traded species[38] Dried leaf exudate: 400 tonnes per annum - worth	wild-harvested and small scale cultivation[31]

		pains,[33] leaf and stem decoction emetics, leaves and roots for hypertension and stress[37]	to rural harvesters (small-scale) R12–15 million/year[39]	
Aspalathin linearis (Fabaceae)	Rooibos or "long-life tea" (Africa)	For relieve of heartburn and nausea in pregnancy, babies: colic relieve and milk substitute[40]	Important commercial crop Cultivated since 1940's.[23]About 5000 tons/year - retail sales value of R429 million/year Exports exceed local use.[41]	Large scale cultivation and small percentage wild harvested[33]
Harpagophytum procumbens (Pedaliaceae)	Devil's claw, grapple plant, wood spider	rheumatism, arthritis, diabetes, gastrointestinal, disturbances, menstrual difficulties, neuralgia, headache, heartburn, gout[42]	Registered herbal medicine (Harpagophyti radix) in France and Germany, or food supplement in the UK, The Netherlands, the USA, Far East Harvest: 700 tonnes/year	Wild-harvested Government regulation needed to ensure continuous supply
Pelargonium sidoides (Geraniaceae)	Kalwerbossie (Afrikaans)	disorders of the gastrointestinal tract[42]	One of the most successful phytomedicines in the world EPs 7630 (Umckaloabo) - ethanolic extract, from the tuberous roots, (treat respiratory tract infections)[43] Eg. Germany: € 80 million/year[44] Umkalor - mother tincture marketed in Ukraine, Russia, and Latvia[45]	Mostly wild-harvested[46]

In addition to plant secondary metabolites, marine organisms, vertebrates, invertebrates and microorganisms are sources of natural products and have been used as naturally active pharmacophores through the ages.[47,48]

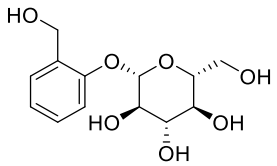
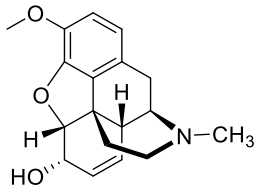
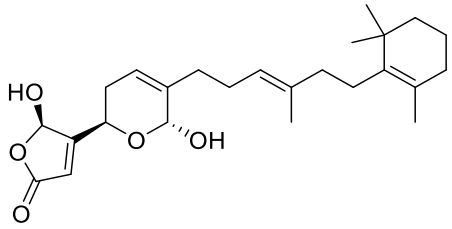
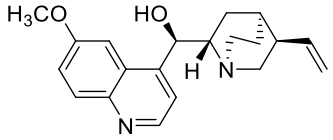
2.2 Significance of medicinal plants in drug discovery

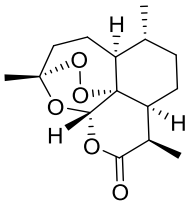
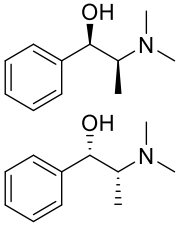
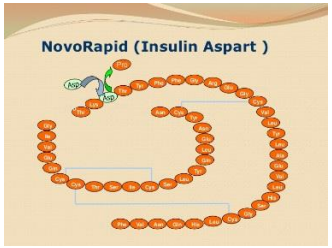
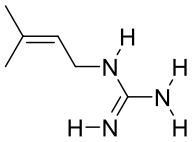
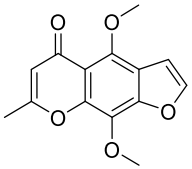
Studies indicate that worldwide approximately 250 000 to 350 000 plants species have been identified and of these, 35 000 plants species have been used for medicinal purposes.[49] Approximately 80% of 122 pure isolated compounds on the market derived from 94 plants species, were originally used for the same or related ethno medical purposes.[16]

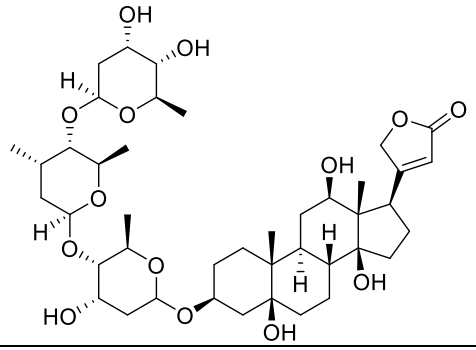
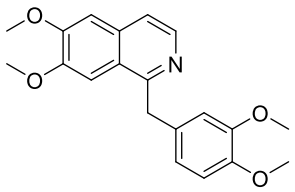
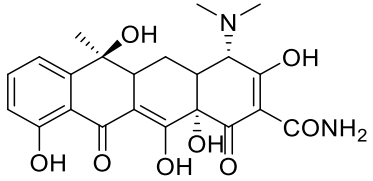
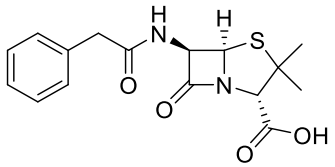
Over the last centuries, secondary metabolites have been the main source for drug discovery and development, particularly in the areas of cancer and infectious diseases.[50] About 100

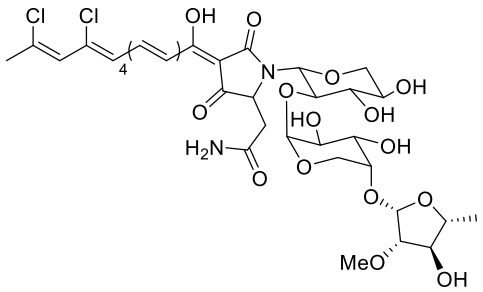
compounds derived from natural products, which are active against cancer and infectious diseases, are currently undergoing clinical trials and at least 100 more compound primaries from plants and microbial derivatives are presently in preclinical development.[51] Natural products and their derivatives signifies over 50% of all drugs clinically used in the world, and of these, isolated natural products contribute about 25%.[52] Almost every pharmacological class of drugs contains a natural product.[53]

Table 2. 2*: Examples of drugs derived from natural products that are presently used in clinical practise[40,41,42]*

Lead compound	Chemical structure	Developed drug	Source	Pharmacological use
Salicin		Aspirin	*Salix alba* (Plant)	Analgesic; anti- coagulant
Codeine		Merperidine, pentazocine, & propoxyphene	*Papaver sommiferum* (Plant)	Analgesic
Manoalide		Manoalide	*Luffariella variabilis* (Marine organism, sponge)	Analgesic; anti-inflammatory; antibiotic
Quinine		Chloroquine and mefloquine	*Cinchona officinalis* L. (Plant)	Anti-malarial

Artemisinin		OZ277 and the artemisinin dimeric analogue	*Artemisia annua* (Plant)	Anti-malarial
Ephedrine		Salbutamol & salmetrol	*Ephedra sinica* (Plant)	Anti-Asthmatic
Insulin		NovoRapid	Animal (Mammals)	Anti-diabetic
Galegine		Metformun	*Galega officinalis* (Plant)	Anti- diabetic
Khellin		Chromolyn	*Ammi visnaga* (Plant)	Bronchodilator

Digoxin		Lanoxin	*Digitalis purpurea* (Plant)	Congestive heart failure
Papaverine		Verapamil	*Papaver sommiferum* (Plant)	Anti- hypertensive
Tetracycline		Achromycin, doxycycline, etc.	*Streptomyces* (Micro-organism)	Antibiotic
Penicillin		Nafpenzal DC, Nafcillin (vet)	*Penicillium chrysogenum* (Micro-organism)	Antibiotic

Aurantoside A			*Melophlus sp.* (Marine organism, sponge)	Antifungal

These drugs are used for treatment of different diseases. Furthermore, the bioactive compounds derived from plants also serve as pharmacological tools, for example lysergic acid.[57]

Two examples of plant derived lead compounds are discussed below:

2.2.1 Analgesic and anticoagulants[58]

Codeine was isolated from *Papaver somniferum* and it plays a role as the model substrate for the development of analgesics like meperidine, pentazocine and propoxyphenene. Secondly, salicin is a lead compound isolated from the bark of *Salix alba* (willow tree), which led to potent pain killers and anticoagulant drugs like the universally known aspirin, diflusinil and mesalazine (Figure 2.1).

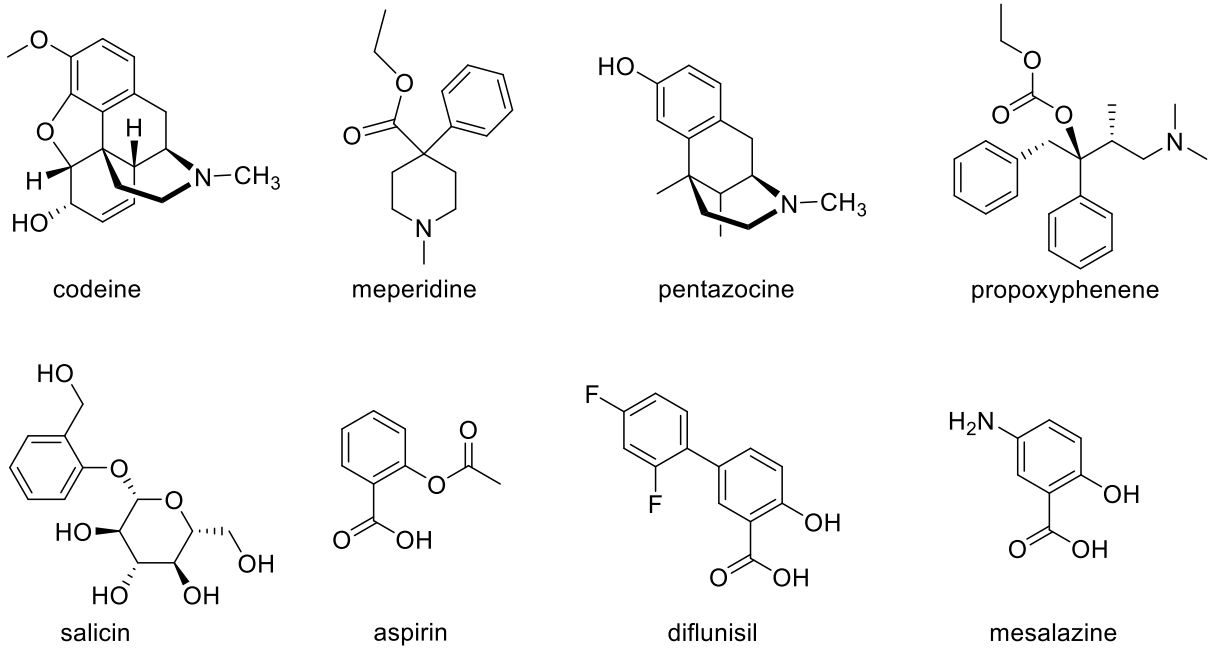


Figure 2.1*: Drug derivatives from codeine and salicin*

2.2.2 Anti-malarial

Quinine was isloted from the bark of *Cinchona officinalis*, and it formed the basis for the synthesis of anti-malarial drugs, chloroquine and mefloquine. However, many tropical regions have reported the development of malarial strains that are resistant to chloroquine and mefloquine. Isolation of a new anti-malarial lead compound named artemisinin from *Artemisia*

annua, which has been used for the treatment of fevers in traditional medicine for decades, addressed this issue.[16,59] Artemisinin is a sesquiterpene lactone containing an endoperoxide group, which shows high activity against resistant plasmodium strains. A series of derivatives including ethers and carbonates have been synthesized to overcome the lipophilic nature of artemisinin. Among them, artemether, arteether, and artesunate are being licensed as drugs in an increasing number of countries.[60,61,62,63] In addition, two promising analogues, OZ277 and an artemisinin dimeric analogue, have been synthesized and both these analogues are now used for the treatment of malaria in many countries.[16]

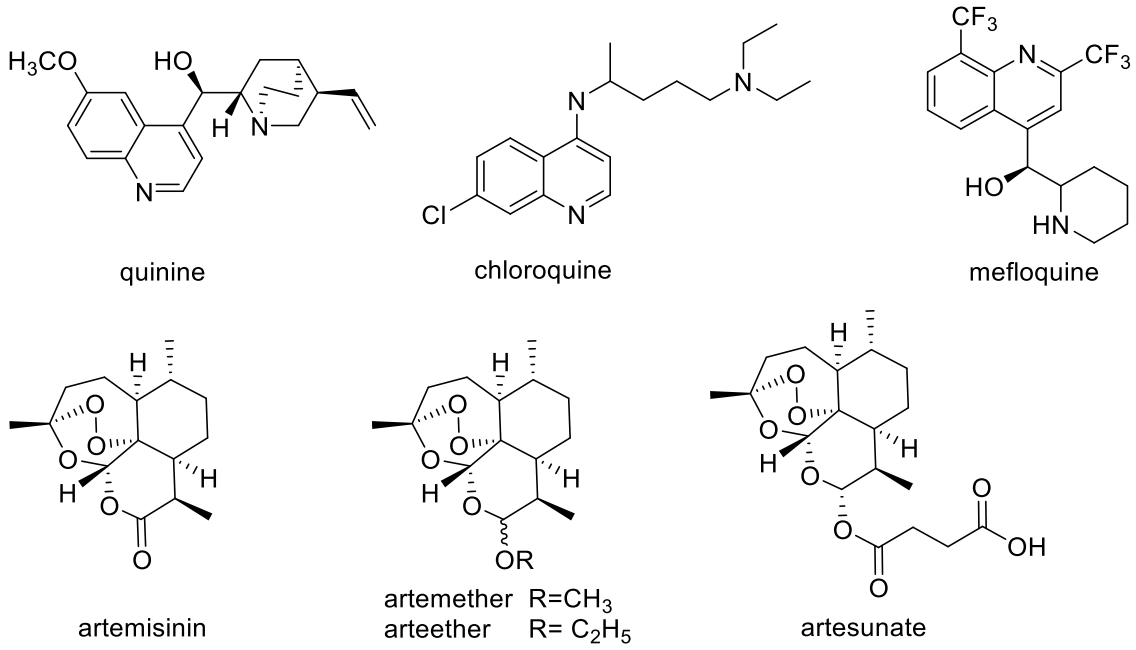


Figure 2.2*: Drugs developed from quinone and artemisinin* [64]

As mentioned above, natural products are the key to success for the development of new drugs and the identification of new compounds and it might be expected that the identification of new metabolites from natural products would be the core of pharmaceutical discovery efforts.[65] However, many pharmaceutical companies have eliminated their natural product research in the past 15 years.[66,67] The difficulties in dealing with natural products and the technical shortcomings in discovering new bioactive compounds in a complex extract, are probably the main reasons for the elimination of natural product research.[65] These limitations include lack of reproducible results, the incompatibility of the crude extract with high throughput assay procedures, the presence of artefacts in some extracts, the high cost of collection of natural

product samples, a long resupply time for active extracts, laborious isolation of active compounds from the extract, difficulty with large scale supply if a drug is developed from natural sources, slow growth and scarce distribution of the species.[68,69,70,71]

2.3 Approaches to Natural Products Drug Discovery

In order to discover drugs from natural products via new methods, a multidisciplinary approach is required to utilise innovative and available technologies to package natural product compounds for drug development and medical practice. The successful use of such an approach will lead to the development of next-generation drugs to combat the health challenges of today and the future.

There are two ways towards drug discovery

- Traditional methods
- Modern methods

2.3.1 Traditional methods

The extract is fractionated and the active compound is isolated and identified. Every step of fractionation and isolation is usually guided by bioassays, and the process is called bioactivity-guided isolation. However, the process can be slow, inefficient and labour intensive.[72] Bioactivity-guided isolation is a multidisciplinary approach to drug discovery. A positive result in the target assay for biological activity of a crude extract is followed by fractionation and subsequent activity testing of the individual fractions. The fractions with biological activity undergo further fractionation, until pure compounds are obtained. In the fractionation process, different techniques are used such as thin layer chromatography, column chromatography, high performance liquid chromatography, etc.[53]

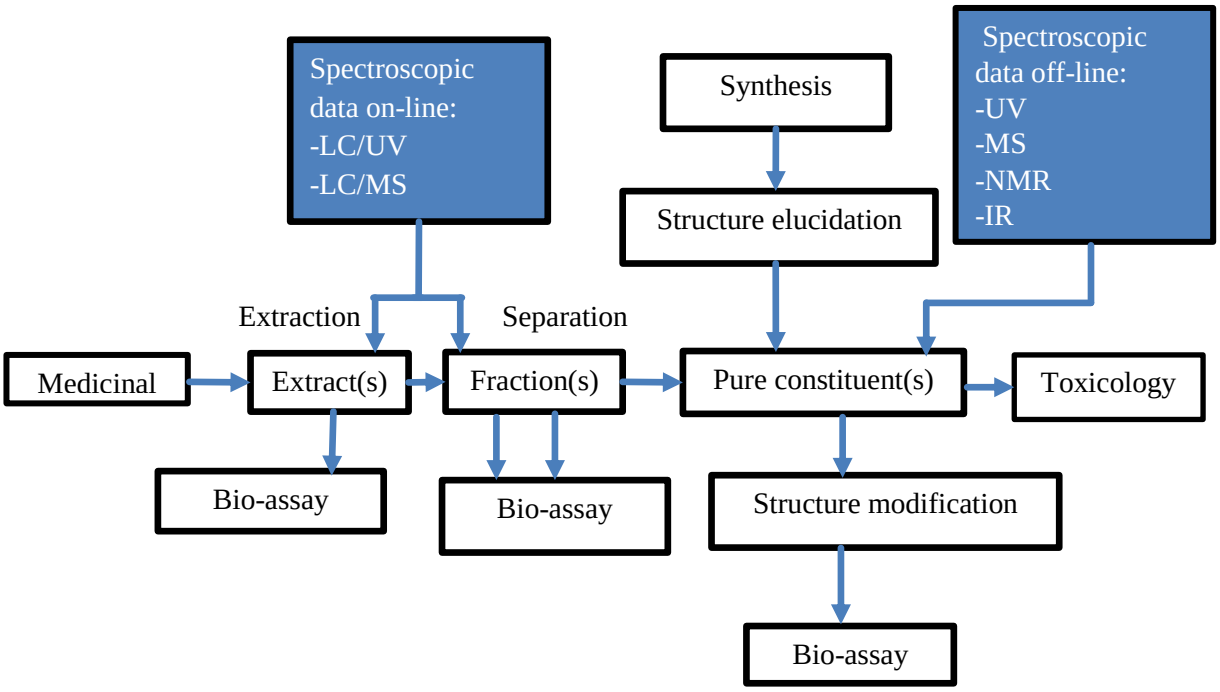


Figure 2.3*: Overview of bioactivity guided isolation*

Thus, isolation of pure compounds is an arduous and time consuming process and new strategies had to be developed to facilitate the process.

2.3.2 Modern methods

2.3.2.1 High-throughput screening (HTS)

HTS is a scientific experimentation method. It involves the screening of large compound libraries of thousands of molecules for activity against biological targets with the use of robotics, automation, several assays in a short time and large-scale data analysis. In order to incorporate natural products in the modern HTS programmes, a natural product library containing a collection of dereplicated natural products needs to be compiled. Dereplication eliminates re-isolation or recurrence of similar or same compounds from various extracts. A number of techniques are used for dereplication, eg. LC-MS (liquid chromatography–mass detector), LC-PDA (liquid chromatography–photo-diode-array detector), and LC-NMR (liquid chromatography-nuclear magnetic resonance spectroscopy). It is nowadays possible to build a 'chemically diverse' and 'high quality' natural product library that can be suitable for any modern HTS programmes. Natural product libraries that contain crude extracts,

chromatographic fractions or semi-purified compounds are also available. However, the best results can be obtained from a fully identified, pure natural product library as it presents scientists with the option to rapidly manage the 'lead' for further development.[72,73]

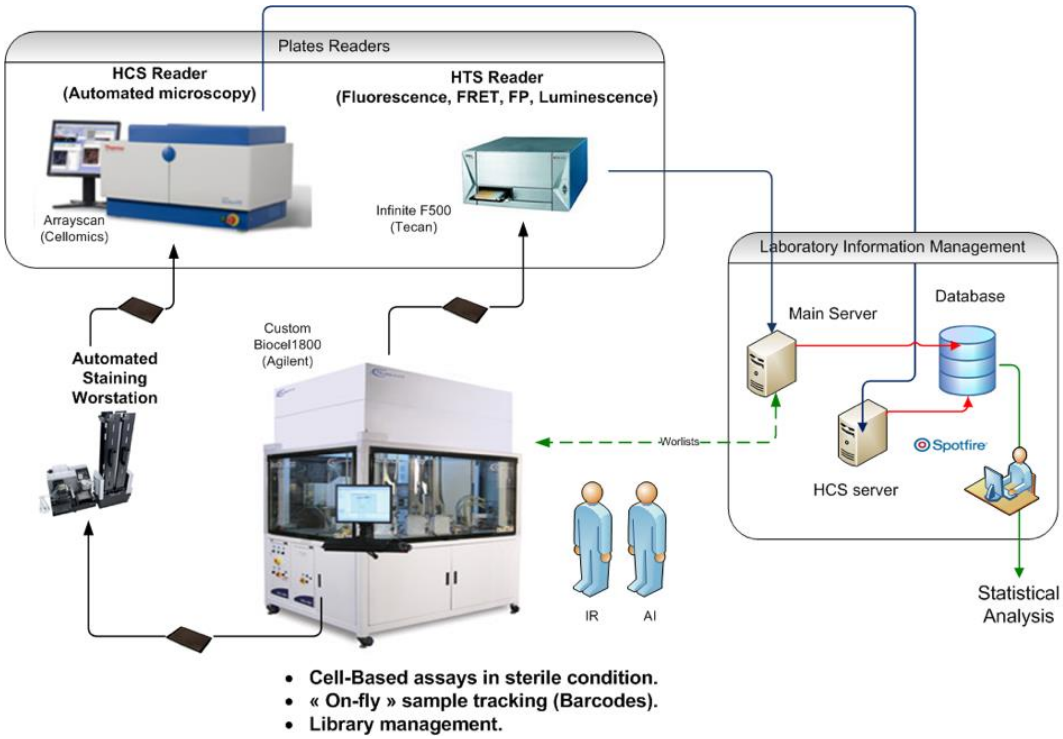


Figure 2.4*: **High Throughput Screening Core Facility (HTS Core Facility)[74]***

2.3.2.2 Innovative extraction methods

A multidisciplinary approach includes innovative extraction methods such as membrane separation technology, semi-bionic extraction, etc.[75]

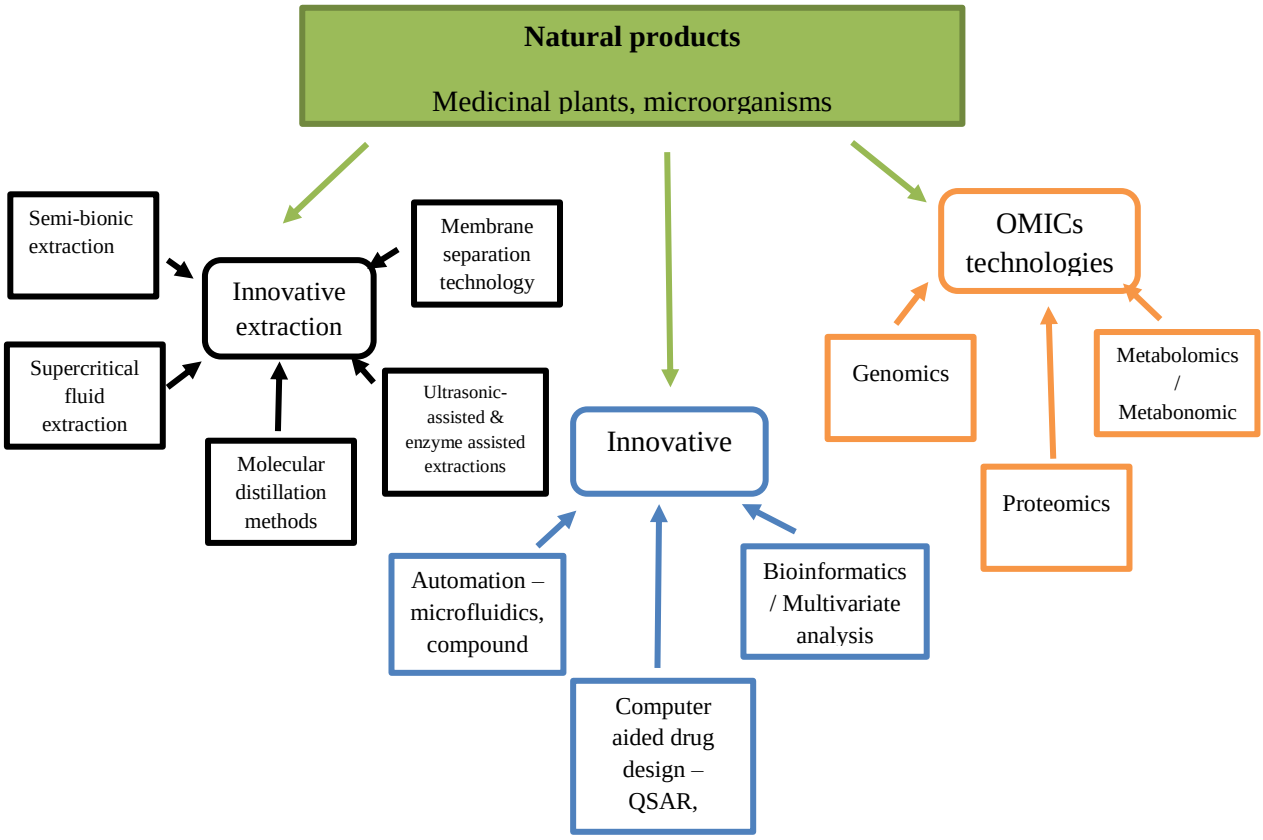


Figure 2.5*: Innovative technologies for natural product drug discovery. Application of these technologies can potentially lead to novel drug candidates from natural products.*

2.3.2.3 Systems biology approach

A systems biology approach together with the application of available technologies such as genomics, proteomics, transcriptomics, metabolomics/metabonomics, computational strategies and automation will potentially open an opportunity for innovative drug design resulting in better drug candidates. In the application of innovative technologies combined with systems biology, the focus should not be an analytic approach of trying to find a single active compound, but to take the synergistic effects of compounds into account.[75] Advances include: [76,77]

- The SepBox from Sepiatec Company for automatic isolation. This instrument can prepare pure compounds from a crude extract by preparative iterative HPLC.

- After HPLC separation, on-line, multi pharmacological detections are possible today, which permit parallel flow bioassay lines for biological activity, spectrometric data and selectivity analyses to obtain structural information.
- Progress in metabolomics will soon allow the prediction of the chemical composition of a plant extract through genome, proteome (enzymes) and transcriptome data.

2.3.3 Advantages and disadvantages of drug discovery from natural products

The advantages can be summarized as follows:[78]

- Large numbers of natural products with good chemical diversity are available.
- Natural products are "naturally bioactive". They have their origins in life organisms and have been designed to play a biological role.
- There is a long term history of usage, which gives evidence of toxicity.
- It enjoys a wider public acceptance.
- Limitations of the original molecules can be circumvented if the natural resources serve as starting point, since the original isolate can be delivered as a drug candidate or as a precursor for semi-synthetic drug development.

The disadvantages include:

- Selection of crude extracts, fractions or pure compounds for pharmacological screening is difficult.
- The concentration of active compounds in an extract or a fraction is unknown.
- Biological interferences occur between natural products and enzymatic-based screening tests.
- Medicinal chemists often find natural products chemically too complex, and access to biodiversity is considered to be difficult, too expensive, together with uncertain and difficult re-supply issues.
- The Convention on Biodiversity concedes access of biodiversity to everybody, but it is difficult to find the right administrative centre, which has the legal mandate to deal with these issues, in practice.
- The rights attached to natural products are not clear cut, and patenting a natural product is problematic.

- After purification of an active compound, semi-synthetic or synthetic derivatives of the compound must be synthesized to improve activity and to get quantitative structure activity-relationship information.
- The drug discovery and eventual commercialization would put substantial pressure on the resource and might lead to undesirable environmental problems.

2.4 Selection and collection of plants

2.4.1 Criteria for selection of plants of interest

Careful consideration is necessary when selecting a plant to investigate. Due to the large number of plants species that have been previously investigated, selecting new plants species is a difficult task. Medicinally useful plants are a good starting point in selecting a plant, and thus a thorough literature search followed by a survey among traditional health practitioners is important. Important information include chemotaxonomic criteria, traditional medicine information, like uses and preparation procedures, field observations and random collection.[79] Selection can thus also be based on phylogenetic and chemotaxonomic information of compounds from certain genera and families.[80,81]

2.4.2 Collection and identification of a plant specimen

In South Africa permits from government departments in charge of the environment or indigenous plant control must be obtained in advance for all plant collections, not only for threatened and protected taxa.[82] Identification of the desired species is done by a botanist in the field and the necessary plant samples are prepared to be saved in a herbarium, since plant classification is frequently changing due to shifts in species alignments and groupings that are made as new research is conducted.[80] During the collection and drying of plant specimens', precaution must be taken to avoid the formation of artefacts.

2.4.3 Preparation of a plant specimen as a herbarium voucher

A herbarium voucher can be defined as a pressed plant specimen deposited in a herbarium for future reference and it provides large amounts of information on plant taxa and vegetation regions. A herbarium voucher supports research work and is used to identify the plant species

which are investigated. Herbarium voucher specimens are the key in cross-referencing if any name changes occurs.[82] The plant specimens are prepared by pressing and drying in a plant press. A plant press is an instrument which consists of a wooden frame which keeps cardboard and blotter paper together with straps. The cardboard helps with air flow and the blotter paper absorbs the moisture from the plants, leaving the plants to dry, thus preserving the morphological integrity of the plants. After drying, plant specimens are mounted on herbarium paper for long term storage.[80] The herbarium vouchers have information labels with the specimen's name and family, collector's name and number, the locality, collection date and descriptive notes.[82]

2.5 General review of the Scrophulariaceae family

2.5.1 The Scrophulariaceae family

2.5.1.1 The scientific classification of Scrophulariacease family[83]

Kingdom:	Plantae
Suphylum:	Euphyllophytina
Infraphylum:	Radiatopses
Subclass:	Magnoliidae
Superorder:	Asteranae
Order:	Lamiales
Family:	Scrophulariaceae

2.5.1.2 Distribution of the Scrophulariaceae family

The Scrophulariaceae family is one of the major plant families consisting of herbs, shrubs or vines with 220 genera and about 3000 species distributed worldwide. The name Scrophulariaceae is derived from the European species of Scrophularia, common figwort, which is used to treat haemorrhoids.[84] The majority of members of the Scropulariaceae family are hemi-parasites, meaning part of their nutrition is derived by parasitizing other plants. However, they are photosynthetic plants.[85]

In Africa, the Scrophulariaceae family is commonly known as snapdragon from the foxglove family with more or less 65 genera and 1700 species. Forty-seven genera and 825 species are native to southern Africa, with two genera and three species naturalized, and three genera and 23 species cultivated in southern Africa. In recent studies of the Scrophulariaceae and related families, a large number of genera have been moved to other families in the Lamiales (mainly Plantaginaceae, Orobanchaceae and to a lesser extent Stilbaceae).[86]

2.5.1.3 Morphology of the Scrophulariaceae family

This family of flowering plants includes herbs, which are annual or perennial herbs, shrubs with bilateral or rarely radical symmetry, a few trees and semi-parasites.[85] The leaves may be opposite or alternate, simple or pinnately lobed and with or without stipulate depending on the global basis.[84] The family is characterized by its bisexual flower with tubular corollas, which is bilaterally symmetrical and a varying number of stamens (four stamens are the most common, presenting as two long and two short stamens).[84,85] The gynoecium consists of a single bicarpellate pistil, with a larger ovary that consists of two chambers containing many ovules.[84]

2.5.2 The genus *Aptosimum*

2.5.2.1 The scientific classification of the *Aptosimum* genus[83]

Kingdom:	Plantae
Suphylum:	Euphyllophytina
Infraphylum:	Radiatopses
Subclass:	Magnoliidae
Superorder:	Asteranae
Order:	Lamiales
Family:	Scrophulariaceae
Tribe:	Aptosimeae
Genus:	*Aptosimum*

2.5.2.2 Distribution of the *Aptosimum* genus

Aptosimum is a genus within the kingdom of Plantae, order of Lamiales and family of Scrophulariaceae, with about 40 species native to Africa, and with 20 species occurring in Southern Africa.[83,86] The highest species diversity is found in Namibia, with the majority

occurring in the western, drier parts of Namibia.[87] Some species of *Aptosimum* prefer certain environmental conditions to grow, eg. *A. decumbens* and *A. elongatum* prefer sandy soil like Kalahari sand, *A. albomarginatum* and *A. spinescens* prefer calcareous soil and *A. suberosum* prefers to grow in the saline calcrete of the Etosha Pans.[87] *Aptosimum* can be described as a woody herb or sub-shrub, which has alternating one veined leaves. The flowers of the genus are axillary, solitary or in short cymes with a five lobed calyx which have a campanulate tube.[87] The corolla has five rounded lobes and there are four stamens, two long and two short.[87] The 20 species found in South Africa have similar flowers, which makes it difficult to distinguish between them, but their leaves are different and thus enables identification of the species.[87]

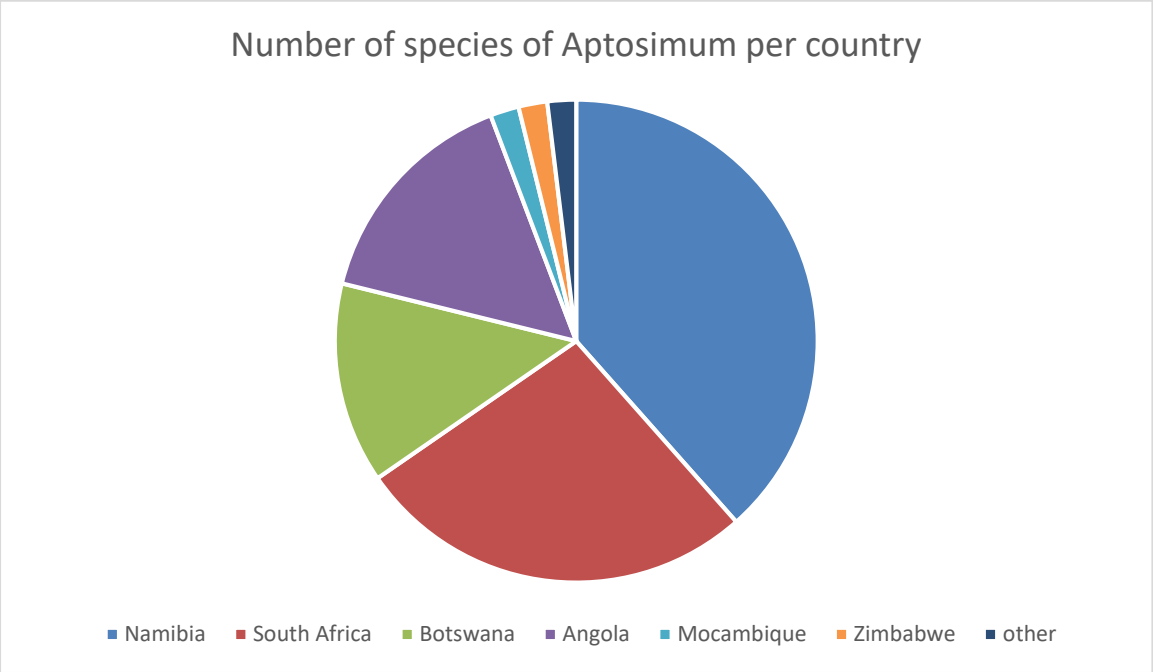


Figure 2.6*: Relative number of species of Aptosimum found in different countries in southern Africa* [87]

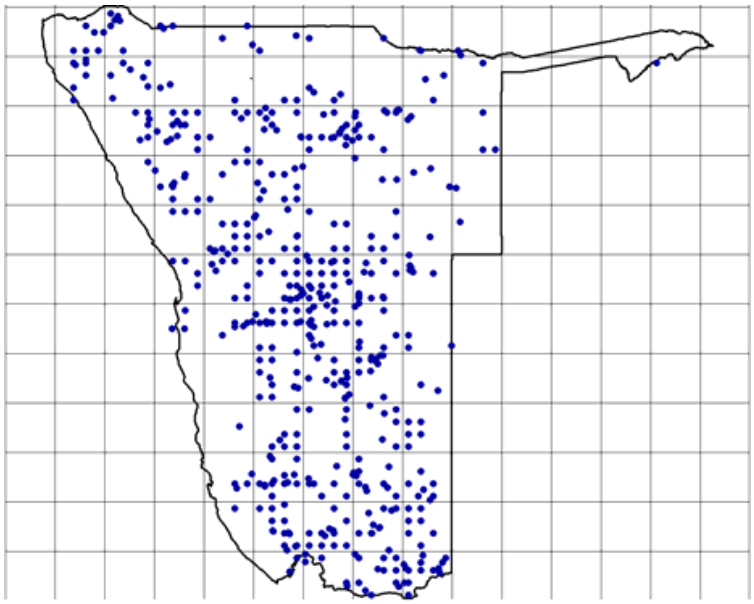


Figure 2.7*: Distribution of Aptosimum in Namibia* [87]

2.5.2.3 Morphology of *Aptosimum* genus[88]

Aptosimum genus can be described as perennial undershrubs but sometimes flowering in the herbaceous state, low, with or without elongated and often procumbent branches, sometimes cushion-forming or tufted, but mostly woody at base. The leaves alternate and are usually densely crowded on long or short shoots. They are linear, lanceolate, elongated or spathulate, entire, 1-nerved, midrib sometimes persistent, and spinescent. Flowers are solitary in leaf axils or in short axillary cymes, sessile or subsessile, bibracteolate, and dark blue to violet. The calyx is tubular, 5-lobed to various depths; tube campanulate; lobes linear to deltoid or ovate, ± valvate in bud. The corolla is tubular, slightly irregular, and 5-lobed. The tube is elongated, much longer than calyx, widening suddenly above the short, narrow base into a long throat, and wide mouth. The limb patent is much shorter than the tube, and oblique. The lobes are free, rounded, ± equal, and two 2 posteriors outside in the bud. There are four stamens, didynamous, included, and arising in the lower part of the corolla tube. The filaments are filiform, the anthers of a shorter, posterior pair smaller than those of the longer, anterior pair and sometimes sterile. The anthers are bithecate, transverse and ciliatehispid. The thecae are confluent so dehiscing along a single transverse line. The nectary is usually saucer-shaped. The ovary is bilocular with many ovules. The style is filiform with exceeding stamens. The stigma is small and obscurely bidentate, emarginate or subcapitate. The fruit is a thick-walled capsule, obovoid, ± globose,

broadly ovoid or ovoid. The upper part is compressed at right angles to the septum, emarginated and septicidal, but opening only at the top and often persistent. The valves are usually bifid. The many seeds are obovoid or flattened-globose and testa adpressedly reticulate.

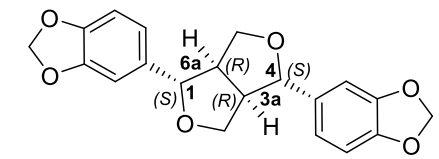
The capsules of *Aptosimum* are thick-walled and woody, splitting open only at the truncate to a rounded apex, which is compressed at right angles to the septum and dehiscing only at the apex.[89]

Figure 2.8*: Aptosimum genus flowering plant*[87]

Figure 2.9*: Aptosimum capsule*[87]

2.5.2.4 Phytochemistry of *Aptosimum*

The *Aptosimum* genus has been used as a traditional medicine in South Africa for decades.[90] However, only three species of *Aptosimum* have been phytochemically investigated namely, *A. spinescens*, *A. indivisum*, and *A. procumbens*. The medicinal applications of *A. spinescens* include headache, backache and stomach ache, and eight lignans have been isolated: sesamin **(2.1)**, spinescin **(2.2)**, piperitol **(2.3)**, pinoresinol **(2.4)**, pinoresinol dimethyl ether **(2.5)**, pinoresinol monomethyl ether **(2.6)**, Aptosimone **(2.7)**, and Aptosimol **(2.8).**[91,92] *A. indivisum* is used for the treatment of stomach ache/upset stomach and three compounds were isolated, namely the iridoid shanzhiside methyl ester **(2.9**), verbascoside **(2.10)** and the flavanone pinocembrin 7-*O*-β-neohesperidoside **(2.11)**.[92,93] *A. procumbens* was used to treat 'krimpsiekte' in sheep, an illness that affects the joints, muscles and stomach, often resulting in starvation and death.[94] It afforded iridoid glycosides angeloside **(2.12)**, shanzhiside methyl ester **(2.13)**, barlerin **(2.14)**, foliamethoylshanzhiside methyl ester **(2.15)** and the pinocembrin 7-*O*-β-neohesperidoside **(2.11)**.[93]



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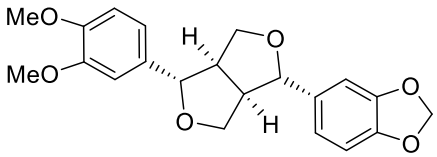

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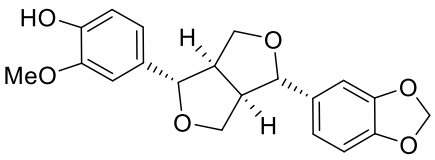

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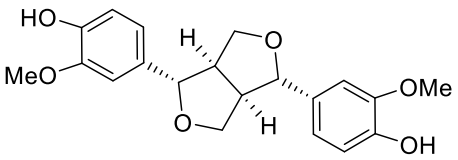

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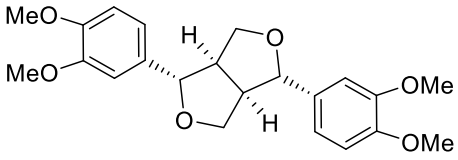

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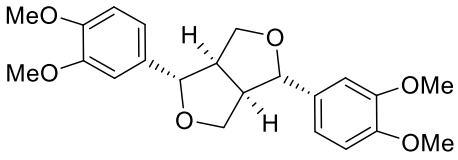

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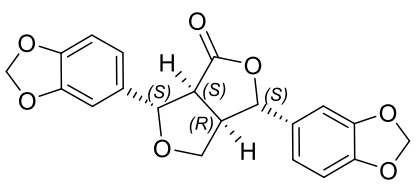

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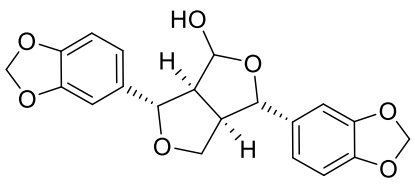

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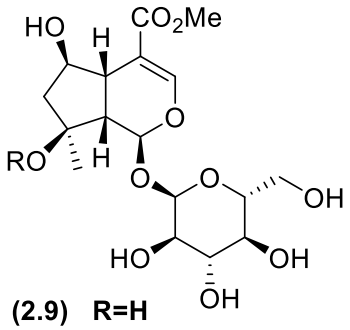

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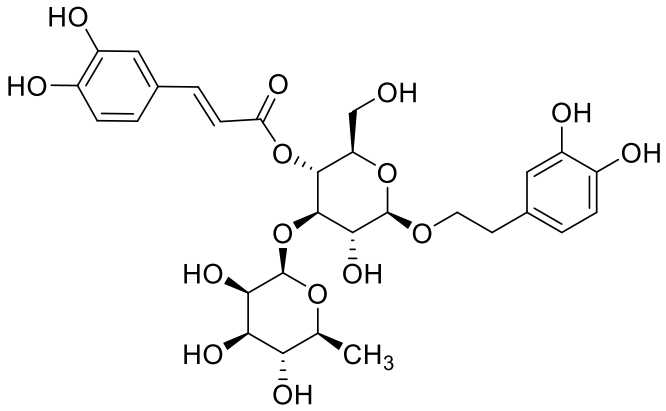

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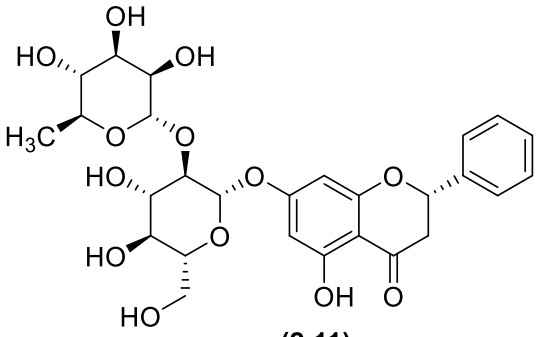

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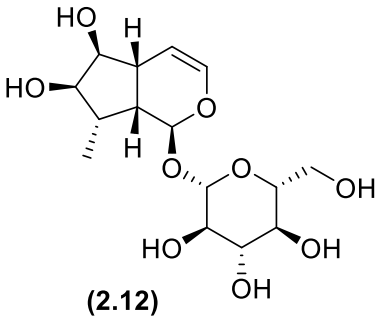

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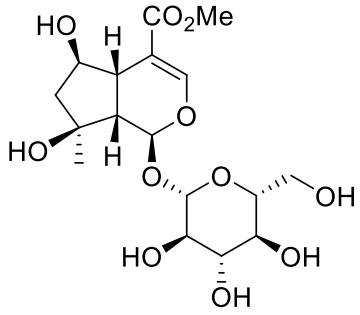

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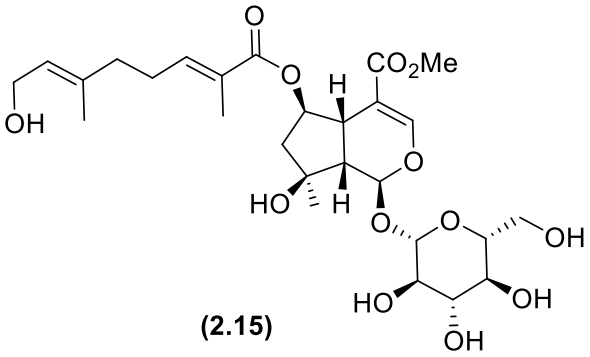

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2.6 A review of analytical methods used in natural product research

The qualitative and quantitative studies of bioactive compounds from plant materials mostly rely on the selection of proper methods. In this section, some of the commonly used methods in natural products research are discussed

2.6.1 Phytochemistry screening

Phytochemicals are chemicals resulting from plants and the term is used to describe the large number of secondary metabolic compounds found in plants[95]. A phytochemical screening assay is a simple, quick and inexpensive procedure that gives the researcher a quick answer to the various types of phytochemicals in a mixture and it is an important tool in bioactive compound analyses.[95] Some of the bioactive substances that can be derived from plants are flavonoids, alkaloids, carotenoids, tannin, antioxidants, proteins, amino acids and phenolic compounds.[95]

Table 2.3: ***Structural features and activities of various phytochemicals from plants[96]***

Phytochemicals	Structural features	Example(s)	Activities
Phenols and Polyphenols	C3 side chain, -OH groups, phenol ring	Catechol, Epicatechin, Cinnamic acid	Antimicrobial, Anthelmintic, Antidiarrhoeal
Flavonoids	Phenolic structure, one carbonyl group Hydroxylated phenols, C6-C3 unit linked to an aromatic ring Flavones + 3-hydroxyl group	Chrysin, Quercetin, Rutin	Antimicrobial Antidiarrhoeal
Alkaloids	Heterocyclic nitrogen compounds	Berberine, Piperine, Palmatine, Tetrahydropalmatine	Antimicrobial, Anthelmintic, Antidiarrhoeal
Glycosides	Sugar + non carbohydrate moiety	Amygdalin	Antidiarrhoeal
Terpenoids and essential oils	Acetate units + fatty acids, extensive branching and cyclized	Capsaicin	Antidiarrhoeal
Lectins and Polypeptides	Proteins	Mannose-specific agglutinin, Fabatin	Antiviral
Coumarins	Phenols made of fused benzene and α- pyrone rings	Warfarin	Antiviral

Table 2.4: ***Mechanism of action of some phytochemicals[96]***

Phytochemicals	Activity	Mechanism of action
Polyphenols and Tannins	Antimicrobial	Binds to adhesins, enzyme inhibition, substrate deprivation, complex with cell wall, membrane disruption, metal ion complexation
	Antidiarrhoeal	Makes intestinal mucosa more resistant and reduces secretion, stimulates normalization of deranged water transport across the mucosal cells and reduction of the intestinal transit, blocks the binding of B subunit of heat-labile enterotoxin to GM1, resulting in the suppression of heat-labile enterotoxin-induced diarrhea, astringent action
	Anthelmintic	Increases supply of digestible proteins by animals by forming protein complexes in rumen, interferes with energy generation by uncoupling oxidative phosphorylation, causes a decrease in G.I. metabolism
Flavonoids	Antimicrobial	Complex with cell wall, binds to adhesins.
	Antidiarrhoeal	Inhibits release of autocoids and prostaglandins, Inhibits contractions caused by spasmogens, Stimulates normalization of the deranged water transport across the mucosal cells, Inhibits GI release of acetylcholine
Alkaloids	Antimicrobial	Intercalates into cell wall and DNA of parasites.
	Antidiarrhoeal	Inhibits release of autocoids and prostaglandins.
	Anthelmintic	Possess anti-oxidating effects, thus reduces nitrate generation which is useful for protein synthesis, suppresses transfer of sucrose from stomach to small intestine, diminishing the support of glucose to the helminthes, acts on CNS causing paralysis
Glycosides	Antidiarrhoeal	Inhibits release of autocoids and prostaglandins
Terpenoids and essential oils	Antimicrobial	Membrane disruption
	Antidiarrhoeal	Inhibits release of autocoids and prostaglandins
Lectins and Polypeptides	Antiviral	Blocks viral fusion or adsorption, forms disulfide bridges
Coumarins	Antiviral	Interaction with eucaryotic DNA

2.6.2 Preparation and extraction of plant material

Plant material must be dried and powdered and to avoid degradation of compounds the grinding machine used on the plant material must not become too hot.[97] Various techniques can be used for the extraction of compounds.[98] Extraction is the first step, and the selection of estraction

solvent(s) is crucial. Low polarity solvents will yield lipophilic components and alcoholic solvents will give a large spectrum of polar and non-polar compounds.[97]

2.6.3 Removal of tannins

Tannins are a group of secondary metabolites which are commonly distributed in the plant kingdom. Tannins are used in the preparation of leather,[99] cold setting adhesives, mud drilling, etc. Removal of tannins from plant extracts or fractions is essential before sending the samples for biological testing, since tannins may lead to false positives during biological screenings.[97]

2.6.4 Techniques

2.6.4.1 Thin-layer chromatography

Thin-layer chromatography (TLC) is a chromatography technique used to separate non-volatile mixtures.[100] It is often used to analyse the fractions collected from column chromatography to determine if the fraction contains more than one component and if fractions can be combined without affecting their purity.[101] Separation depends on the relative affinity of compounds towards stationary and mobile phases on the TLC plate. The compounds are driven by capillary action to travel over the surface of the stationary phase with the mobile phase.[102] While the compounds travel over the surface of the stationary phase, the compounds with higher affinity to the stationary phase travel slowly, while those with less affinity to the stationary phase travel faster.[102] Thus, separation of components in the mixture is achieved. Once separation occurs, the individual components are visualized as spots on the plate after staining or under UV light.[102]

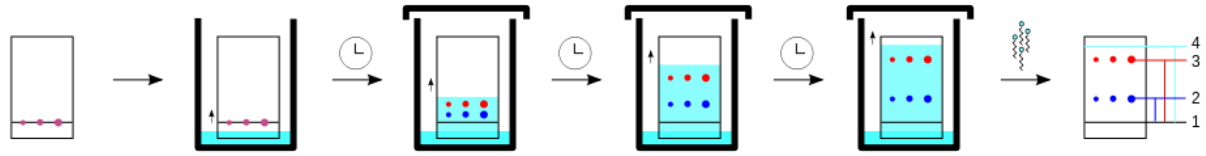


Figure 2.10: ***Development of a TLC plat; a purple spot separates into a red and blue spot***[103]

2.6.4.2 Column chromatography

Column chromatography is a purification technique used for isolation of desired compounds from a mixture.[101] In column chromatography, the stationary phase, which is a solid adsorbent,

is placed in a vertical glass column. The mobile phase (a liquid) is added to the top surface of the adsorbent and flows through the column by either gravity or external pressure.[101]

To purify a crude extract using column chromatography, the crude extract is applied on the surface of the solid phase and passes through the column with the eluent (liquid phase) by gravity or by the application of air pressure.[102] Equilibrium is reached between the solute adsorbed on the adsorbent and the eluting solvent flowing through the column.[102] Owing to the different interactions of the components in the extract with the stationary and mobile phases, the speed of movement down the column will vary and separation will be achieved.[102] The individual components, or elutants, are collected in fractions as the eluant moves out of the column.[102]

Silica gel (SiO_2) and alumina (Al_2O_3) are the two adsorbents commonly used for column chromatography. The polarity of the solvent which is passed through the column affects the relative rates at which compounds move through the column.[102] Polar solvents can compete better with polar molecules for the polar sites on the adsorbent surface and will also solvate the polar constituents better. Consequently, a highly polar solvent will move highly polar molecules rapidly through the column.[101] If a solvent is too polar, movement becomes too rapid, and little or no separation of the components of a mixture will result.[101] If a solvent is not polar enough, no compounds will elute from the column.[101] Proper choice of an eluting solvent is thus vital for the successful application of column chromatography as a separation technique. Often a series of increasingly polar solvent systems are used to elute a column. A non-polar solvent is used first to elute the less-polar compounds. Once the less polar compounds have been collected, a more polar solvent is added to elute the more polar compounds.[101]

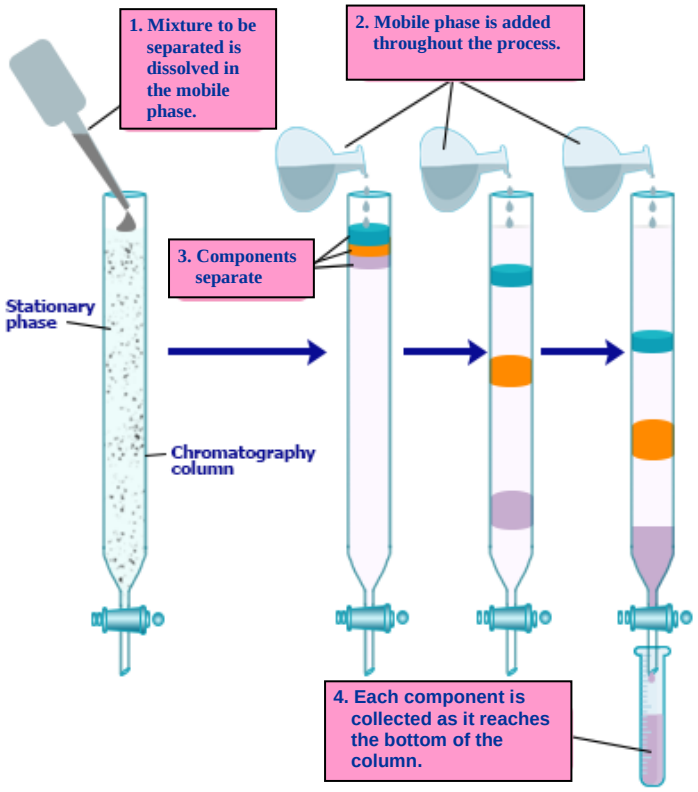


Figure 2.11: ***Separation of components during column chromatography***[104]

2.6.4.3 High-performance liquid chromatography

High-performance liquid chromatography (HPLC), also known as high-pressure liquid chromatography, is a chromatographic technique firstly used to separate a mixture of compounds and, secondly, it is used to identify, quantify and purify the individual components of the mixture.[105]

2.6.4.4 Mass spectrometry

Mass spectrometry (MS) is a technique used to allow the determination of the molecular mass and the molecular formula of a compound, as well as certain structural features via fragmentation patterns.[106] A small sample of the compound is vaporized and then ionized as a result of an electron being removed (positive mode) or added (negative mode) from each molecule, producing a molecular ion.[106] The majority of the molecular ions break apart into cations, radicals, neutral molecules, and other radical cations.[106] The bonds that are most likely to break are the bonds that are the weakest and they result in the formation of the most stable products or fragments.[107] These fragments are detected individually on the basis of their mass-

to-charge ratios.[107] The information that is acquired and displayed by the mass spectrum allows the analyst to reconstruct the original molecule and identify it. Besides the significant applicability to molecular compound identification, mass spectrometry also finds application in elemental analysis, such as to determine which isotopes of an element might be present in a sample.[101]

2.6.4.5 NMR spectroscopy[108]

Nuclear magnetic resonance (NMR) spectroscopy is the method of choice to elucidate the structures of organic compounds. Although a bigger sample of material is needed to obtain useful spectra relative to mass spectrometry, the method is non-destructive and modern instruments can yield useful data up to about 1 mg of sample. Organic chemists use nuclear magnetic resonance (NMR) spectroscopy to determine a molecule's carbon-hydrogen framework and are used in conjunction with other techniques like mass spectrometry and infrared spectroscopy.

Isotopes of particular interest and use to organic chemists are ^{1}H, ^{13}C, ^{19}F and ^{31}P, all of which have a nuclear spin (I) of ½. A spinning charge generates a magnetic field, and the resulting spin-magnet has a magnetic moment (**μ**) proportional to the spin. In the presence of an external magnetic field ($\mathbf{B_0}$), two spin states exist, **+½** and **-½**. The magnetic moment of the lower energy **+½** state is aligned with the external field, and that of the higher energy **-½** spin state is opposed to the external field. Electromagnetic irradiation of a correct frequency leads to absorption in energy and "flips" the lower energy state to a higher state. The frequency at which a certain proton or carbon nucleus absorbs are thus characterictic of that nucleus and depends on the surrounding electron environment. A proton nuclear magnetic resonance (^{1}H NMR) spectrum gives information about the environments of the various hydrogen atoms in a molecule and a carbon-13 nuclear magnetic resonance (^{13}C NMR) spectrum does the same for the carbon atoms. Two-dimensional (2D) NMR experiments give information defined by two frequency axes instead of one. The most commonly used 2D experiments include homonuclear correlation spectroscopy (COSY), which indicates direct proton-proton correlations, heteronuclear single-quantum correlation spectroscopy (HSQC), indicating direct carbon-proton correlations, heteronuclear multiple-bond correlation spectroscopy (HMBC), indicating carbon-proton correlations over two to four bonds (HMBC) and nuclear Overhauser effect spectroscopy (NOESY), that gives information about correlations between nuclei in space.

2.6.4.6 Infrared (IR) spectroscopy[109]

Infrared (IR) spectroscopy is an instrument used to determine functional groups such as the CO, CN, COR (where R is an alkyl group), COOR, and so forth present in a molecule. This instrument uses the similarity between the energies in infra-red radiation and energies involved in bond vibrations to analyse chemical compounds.

2.7 Biological Assays

Bioassay screening or pharmacological evaluations are used to guide the isolation process towards the pure bioactive component. Bioassays can be divided into two categories: primary and secondary bioassay screens. Primary bioassays are assays that are efficiently applied to a large number of samples (crude extracts) to determine if any bioactivity of the desired type is present. Secondary bioassays are more detailed screening of the lead compounds in different model systems in order to select compounds for clinical trials.[110]

Three primary assays, (i) antioxidant, (ii) acetylcholine esterase inhibition and (iii) GABAergic acitivity tests were used for the purpose of this study.

2.7.1 Antioxidant activity (Radical scavenging)

Empiriological and experimental evidence suggest that free radicals and reactive oxygen species (ROS) are implicated in more than 100 diseases, including malaria, acquired immunodeficiency syndrome (AIDS), heart disease, stroke, arteriosclerosis, diabetes, cancer and gastric ulcers.[111,112] Free radicals are defined as molecules containing one or more unpaired electrons in a molecular orbital.[113] In the human body they are generated via various endogenous systems, exposure to different physiochemical conditions or pathological states. Figure 2.12 depicts the formation reaction of reactive oxygen species (ROS), also known as free radicals, in the human body.

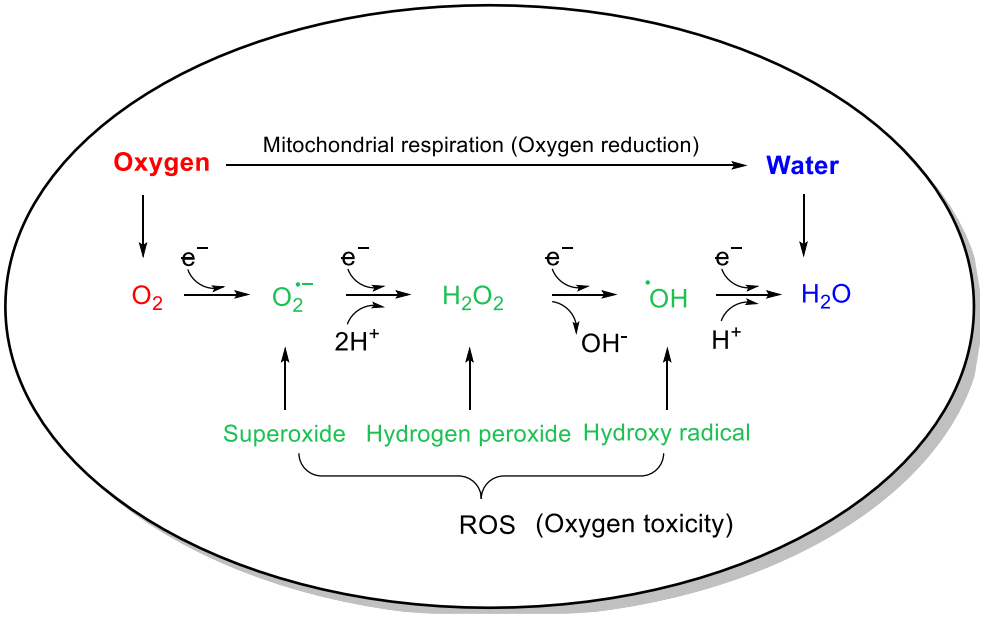


Figure 2.12: ***The formation of reactive oxygen species (ROS)***[114]

Free radical scavenging via antioxidants can protect the human body from ROS effects and may retard progression of many chronic diseases, maintain nutritional quality, minimize lipid oxidative rancidity in foods and increase shelf life.[111,115] Antioxidants relieve oxidative stress, i.e. preventing free radicals from damaging biomolecules such as proteins, DNA and lipids.[116] Antioxidants are molecules which can donate an electron to an ROS, yielding an electron paired molecule and a resulting, more stable, antioxidant radical. Ascorbic acid (Vitamin C) is a good example of a natural antioxidant. The mechanism of action can be explained as follows (Figure 2.13):

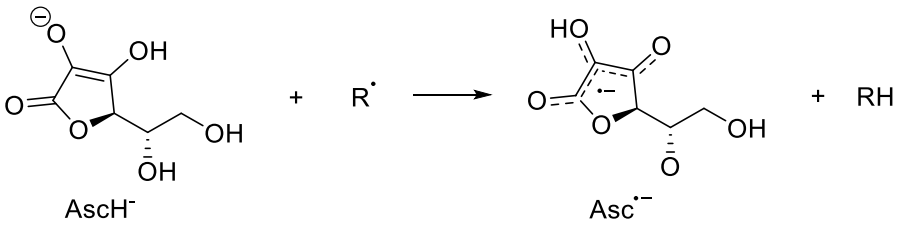


Figure 2.13: ***Mechanism of action when ascorbic acid scavenges a radical***

The ascorbic acid radical is much more stable due to resonance.

2.7.1.1 Qualitative testing via thin layer chromatography (TLC) plates

In the radical scavenging assay, a TLC plate is loaded with the targeted extract or compound and developed in the solvent system of choice. After drying of the TLC, it is sprayed with a solution of 2,2-diphenyl-1-picrylhydrazyl (DPPH), which is a violet coloured radical. Reaction with any antioxidant species present on the plate, will show as yellow spots on a violet background. The active zone is exhibited as pale yellow spots against a violet background.[117]

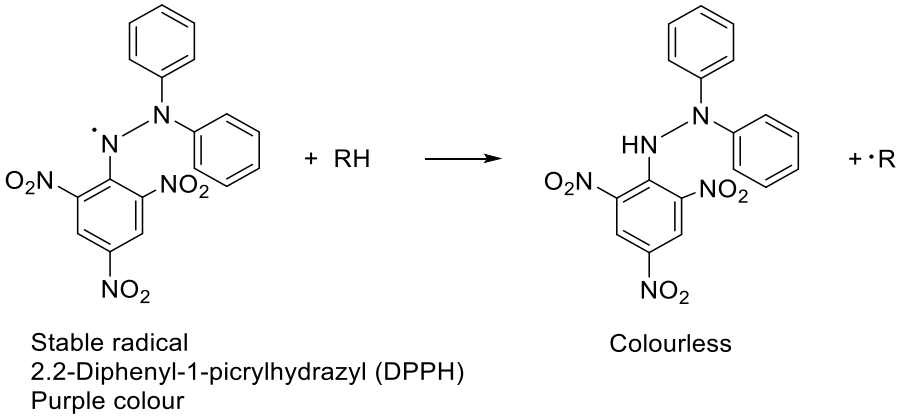


Figure 2.14: ***TLC assay for the detection of radical scavengers. The TLC plate is sprayed with a 0.3% solution of DPPH in methanol and radical scavengers appear as yellow-white spots on a purple background.***

2.7.1.2 Quantitative antioxidant testing[113]

Quantitative antioxidant testing, or the spectroscopic dilution test, is used to evaluate the amount of free radical scavenging activity. During quantitative testing for radical scavenging activities the absorbance of a standard methanolic DPPH solution is determined at 517 nm. A dilution series of the sample being tested is prepared, each sample in the concentration series diluted with DMSO, and subsequently added to the prepared DPPH solution. The absorbance of each sample is measured over a period of 5 min, and the respective radical scavenging is calculated from the decrease in absorbance at 517 nm using the following equation,

$$100 \text{ X } [\ \frac{\left(A0\ X\ \frac{2.95}{3.00}\right) - At}{\left(A0\ X\ \frac{2.95}{3.00}\right) + Ap}\ ]$$

A_0 = absorbance before addition of the test solution
A_t = absorbance after 5 min reaction time
Ap = absorbance after 5 min reaction time if all DPPH has been scavenged

2.7.2 Acetylcholinesterase (AChE) inhibition

Acetylcholine (ACh) is one of the key neurotransmitters in the brain that influences memory and cognitive functions. Low concentrations of ACh in certain individuals can lead to Alzheimer's disease (AD), ataxia and myasthenia gravis.[118] AD is a neurodegenerative disorder, which is characterized by loss of memory, cognitive decline, severe behavioural abnormalities and ultimately death, and is a common cause of senile dementia in elderly humans.[119] The key enzyme playing a role in the break-down of ACh is acetylcholinesterase (AChE). The principal role of AChE is the termination of nerve impulse transmission at the cholinergic synapses by rapid hydrolysis of ACh.[120,121] Inhibition of this enzyme can therefore serve as a promising strategy for the treatment of these neurological disorders. Presently the newest drugs (e.g. galantamine, rivastigmine and donepezil), available for the treatment of AD and dementia is based on the inhibition of acetylcholinesterase function by correcting a deficiency of the key enzyme in the breaking down of acetylcholine.[113]

2.7.2.1 TLC bioautographic assay test[122]

A TLC bioautographic assay was introduced for the screening of plant extracts for inhibition of acetylcholinesterase activity to search for new potential drugs. The TLC bioautographic assay method relies on the cleavage by acetylcholinesterase of 1-naphthyl acetate to form 1-naphthol, which then reacts with Fast Blue salt B to give a purple coloured diazonium dye (Figure 2.15). Regions containing acetylcholinesterase inhibitors appear as white spots on a purple background.

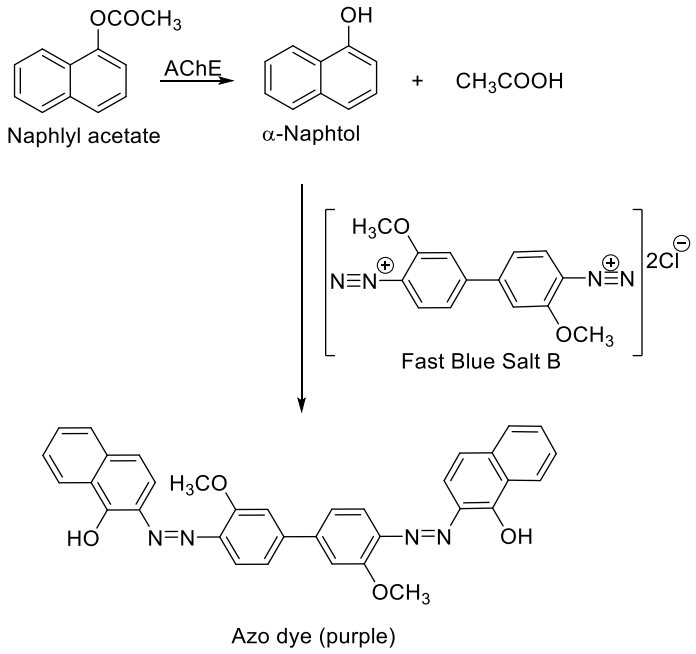


Figure 2.15: ***Reaction of AChE with naphthyl acetate and the substrate formation of the purple dye in the TLC assay.***[122]

2.7.3 Gamma-aminobutyric acid (GABA)

Gamma-amino butyric acid (GABA) is a naturally occurring major inhibitory neurotransmitter in the mammalian central nervous system. It conveys chemical messages through the brain and the nervous system, and is playing a role regulating communication between brain cells. GABA plays a key role in the regulation of synaptic transmission throughout the brain, affecting numerous psychological and physiological processes by inhibiting or reducing the activity of the nerve cells or neurons.[123]

As the chief mediator of inhibitory synaptic transmission in the mammalian central nervous system, GABA influences behaviour using three types of GABA receptors: $GABA_A$, $GABA_B$ and $GABA_C$ receptors. $GABA_A$ and $GABA_C$ receptors are fast ionotropic receptors belonging to a family of ligand gated ion channels, while $GABA_B$ receptors are metabotropic receptors with seven transmembrane domains coupled to G-proteins. Activation of GABA receptors by GABA induces membrane hyperpolarisation, reduces the frequency of the generation of action potentials and results in inhibition.[124]

2.7.3.1 $GABA_A$ receptors

$GABA_A$ receptors are the major and fastest inhibitory ligand gated neurotransmitter receptors found in the central nervous system (CNS). They play a major role in almost all brain

physiological functions.[125] $GABA_A$ receptors are heteropentameric proteins, consisting of a central chloride ion-selective channel gated by GABA.[125] The central channel is surrounded by five homologous or heterogonous subunit isoforms. There are a total of 19 different subunit isoforms: six α (α1-6), three β (β1-3), three γ (γ1-3), δ, ε, θ, π and three ρ (ρ1-3) subunits that have been identified in the human genome that can form $GABA_A$ receptors in many combinations.[126,127] The most common is a combination of 2α, 2β and 1γ subunits (Figure 2.16).[128]

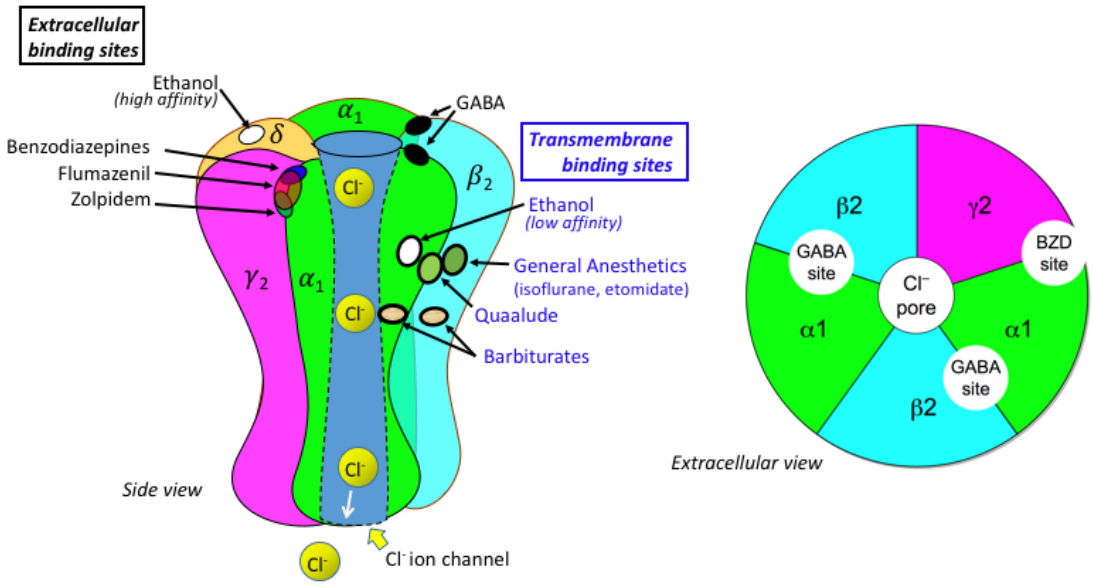


Figure 2.16: ***Structure of the frequent combination of two α1, two β2 and one γ2 subunits of the $GABA_A$ receptor[129]***

GABA exerts most of its effects via $GABA_A$ receptors. $GABA_A$ receptors may be embedded in the post-synaptic membrane where they mediate transient and fast synaptic inhibition that occurs in milliseconds, or they may be located at the extra-synaptic positions where they respond to ambient concentrations of GABA and mediate long-term inhibition. GABA released from the presynaptic membrane terminals rapidly diffuses across the synaptic cleft and binds to binding sites at post-synaptic $GABA_A$ receptors during fast synaptic inhibition. These receptors undergo a rapid conformational change after binding, that opens the integral chloride channel and allow the flow of chloride ions down the chemical gradient, across the post-synaptic membrane. This ensures propagation of neurotransmission and represents the basis of neural communication.[130]

$GABA_A$ receptor-mediated events have two effects on the post-synaptic membrane: a change in the membrane potential due to the movement of Cl^--ions through the membrane (hyperpolarizing inhibition) and an increase of the post-synaptic membrane conductance (shunting inhibition). Synaptic receptors detect millimolar concentrations of GABA and mediate fast inhibitory post-synaptic potentials (IPSPs), while extra-synaptic receptors detect micromolar concentrations of GABA and thus mediate slower IPSPs and also tonic conductances. Tonic and phasic conductances is the basis of different physiological and behavioral processes.

2.7.3.2 Modulation of $GABA_A$ receptors by pharmacological agents

$GABA_A$ receptors have binding sites for a variety of clinically important substances, such as barbiturates, benzodiazepines and benzodiazepine-site ligands, intravenous and volatile anaesthetics, neuroactive steroids, and ethanol all of which reach at least some of their pharmacological effects after binding to the $GABA_A$ receptor complex.[131,132,133] Increased activity of the $GABA_A$ receptor causes ataxia, anxiolysis, myorelaxation, hypnosis, sedation, anaesthesia and anterograde amnesia, while a decrease in $GABA_A$ receptor activity leads to increased vigilance, memory enhancement, anxiety, and seizures.[131,132,134] $GABA_A$ receptor modulators are thus widely used in the treatment of insomnia, anxiety disorders, restlessness, aggressive behaviours and epilepsy.[132,135] Thus, the $GABA_A$ receptor is a highly-relevant drug target and due to severe side effects of commonly available drugs there is a pressing need for the development of novel $GABA_A$ receptor modulators. Plant extracts contain a high amount of $GABA_A$ receptor ligands and a variety of natural products modulating $GABA_A$ receptors have been identified. Examples of these natural product modulators are flavonoids, monoterpenes, diterpenes, neolignans and β-carbolines.[136,137] Herring and co-workers developed an automated two-microelectrode functional assay, using *X. laevis* (African clawed frog) oocytes expressing human $GABA_A$ receptors of the desired subtype composition, to analyse plant extracts for GABAergic acitivity (Figure 2.17).

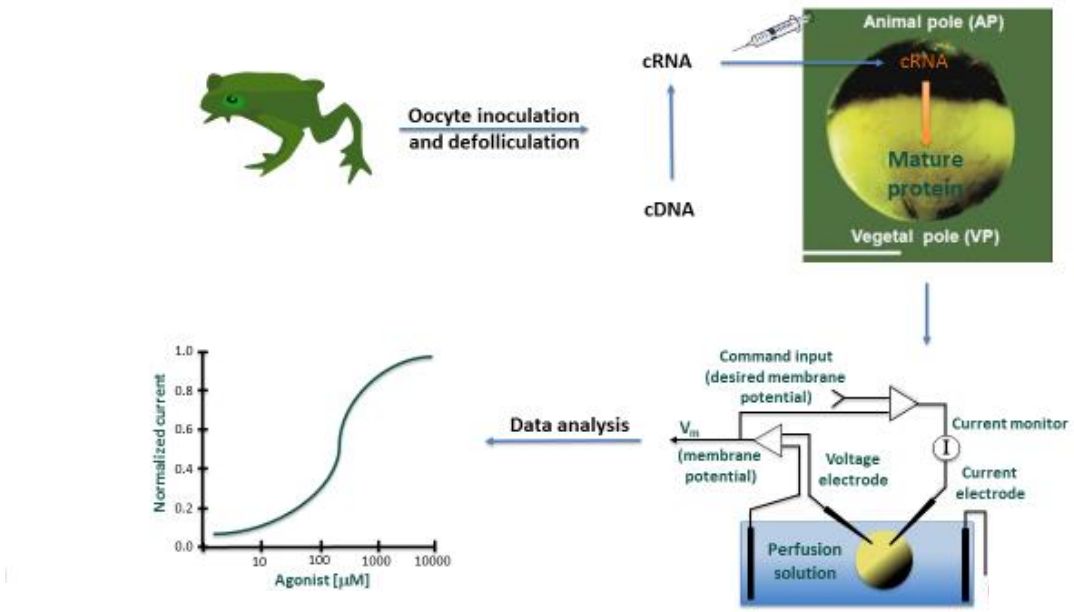


Figure 2.17: ***Automated two-microelectrode functional assay, using X. laevis oocytes expressing human GABA$_A$ receptors of the desired subtype composition***

Oocytes from *Xenopus laevis* are used as a heterologous expression system for the expression of the recombinant $GABA_A$ receptor. Since the $GABA_A$ receptor is a chloride-conducting channel, chloride-currents are measured. Compounds which enhance activity of the channel, becomes evident by enlarged chloride-currents. Figure 2.18 shows that for example, compound **12** (an example from a previous plant extract) at a concentration of 30 µM enhanced the chloride-current about 5-fold (500%), and at 100 µM more than 6-fold (~600%). This is referred to as potentiation of GABA-induced currents (I_{GABA} potential). In general, a +30% I_{GABA} potentiation enhancement was denoted as an internal limit. Fractions with negative values decrease the GABA-induced chloride currents (limit: -30%). Those fractions might contain inhibitors, which are relevant from the toxicological point of view, as they might induce convulsions or epilepsy (Figure 2.18).

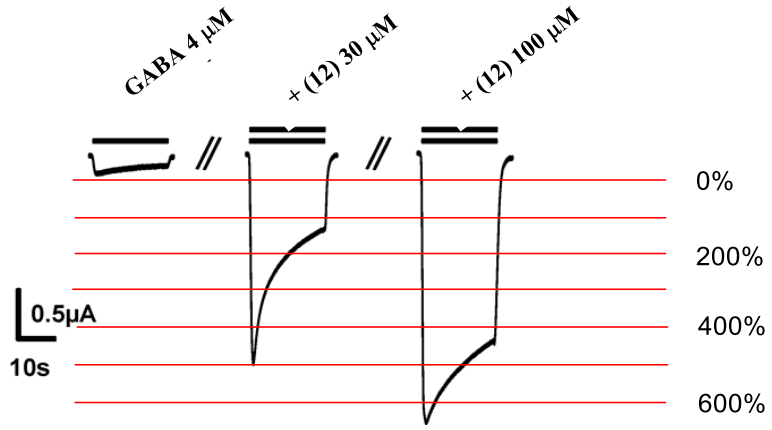


Figure 2.18: ***Current trace that is evoked by 4 µM of GABA (left) and two other traces which are the result of a co-application of GABA plus a compound.***

2.8 Conclusion

In this Chapter, some general aspects of phytomedicines were discussed in terms of the history of phytomedicines and drug discovery, and approaches to natural products drug discovery, which include traditional methods and modern methods. Collection and selections of plants as well as the general review of the *A. elongatum* family and genus. The analytical methods used in natural product research were also illustrated along with the main biological assays used in this study.

Chapter 3: Results and discussion

3.1 Introduction

Phytochemical investigations of plants used in traditional medicine have led to the discovery of many active compounds used in drugs today. In South Africa, with its large diversity of plants, only a small percentage of traditional medicinal plants has been investigated, and to our knowledge only four reports on the herbal remedies used by the indigenous people of the semi-arid Karoo region have been published.[138] Species from the *Aptosimum* genus of the family Scrophulariaceae is virtually unknown with only *Aptosimum procumbens*, *Aptosimum albomarginatum*, and *Aptosimum elongatum* mentioned as herbal remedies used by the Bushmen in South Africa.[139] The aim of this investigation was to explore the phytochemistry and biological activities of *A. elongatum* to broaden our knowledge on possible biological applications of this medicinal plant.

3.2 General review of *Aptosimum elongatum*

3.2.1 Scientific classification of *A. elongatum*[140]

Kingdom:	Plantae
Suphylum:	Euphyllophytina
Infraphylum:	Radiatopses
Subclass:	Magnoliidae
Superorder:	Asteranae
Order:	Lamiales
Family:	Scrophulariaceae
Tribe:	Aptosimeae
Genus:	*Aptosimum*
Species:	*A. elongatum*
Binomial name:	*Aptosimum elongatum* Engl.

3.2.2 Distribution of the Scrophulariaceae family

A. elongatum is distributed in Namibia and parts of southern Africa in the Free State, Gauteng, Limpopo, Mpumalanga, Northern Cape, and North West.[140,141] The plant is found on scrub and open wooded grassland mainly on the deep Kalahari sand and sometimes along the road, sand dunes and sand soil.[141] This species has been reported to be used for both human and veterinary medicines,[142,143] mostly for burn wounds. The Khomani Bushmen from the Kalahari Desert calls it *magatho* or "washout" and the aerial parts (stem, leaf and flowers) are used.[144]

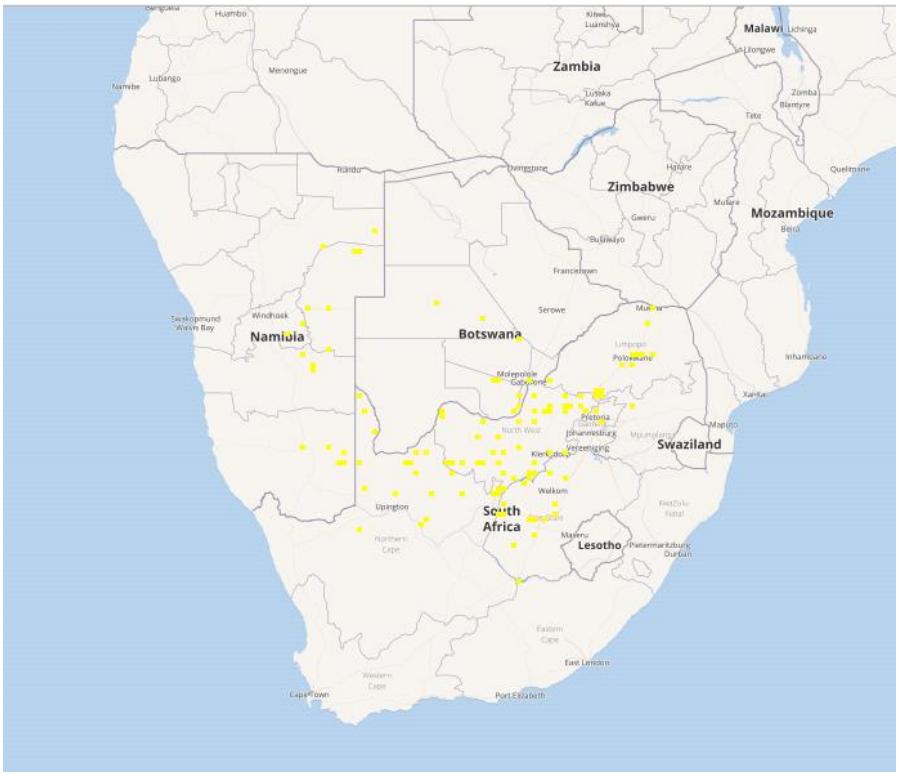


Figure 3.1*: Distribution of A. elongatum*[140]

3.2.3 Morphology of *A. elongatum*[141]

A. elongatum can be described as a small procumbent shrub, widely spread into slender branches that are up to 60 cm long and covered with white dense hair.[141] The leaves are covered with hair and are 10 - 18 x 3.5 - 5 mm in size. The flowers are deep blue to purple.[141] It is easily recognized by its dense, prostrate growth form and the dense indumentum that makes the plant

appear grey, especially during low rainfall periods or in the dry winter months.[145] It is similar to *A. eriocephalum*, but does not have the woolly calyxes and glabrous, clear petiole leaves.[145]

Figure 3.2*: Flowering A. elongatum in the dry season when pubescence is more pronounced. PHOTO: H. KOLBERG[145]*

3.3 Phytochemical investigation of *A. elongatum*

3.3.1 Plant Collection

The aerial parts (stems, leaves and flowers) of *A. elongatum* were collected and identified by Dr P. Zietsman (National Museum, Bloemfontein) at Bethulie, coordinates -30.503046, 25.986662, a Karoo town in the southern Free State, South Africa. A plant sample was stored in the herbarium of the National Museum, Bloemfontein.

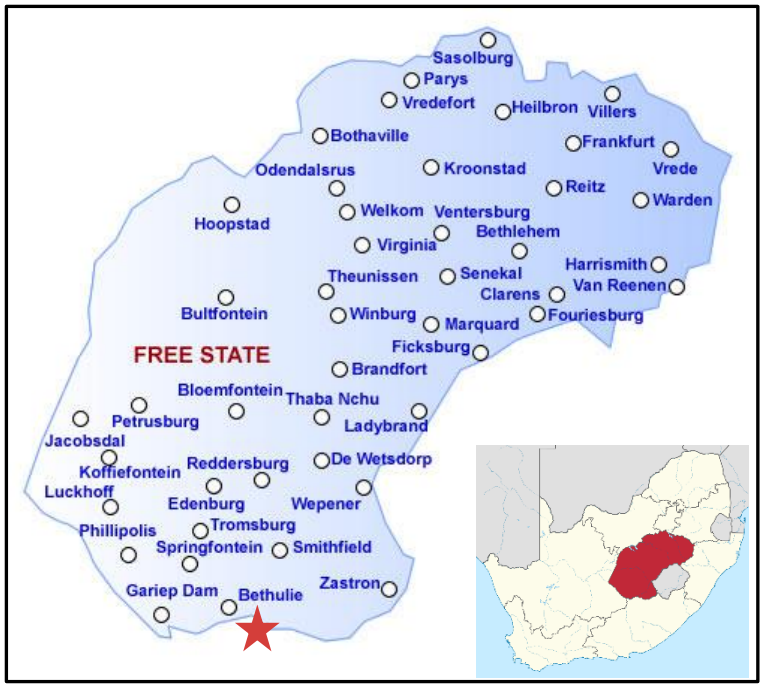


Figure 3.3*: Geographic location of Bethulie in the Free State, South Africa*

3.3.2 Extraction and structural elucidation

The aerial parts of *A. elongatum* was dried and ground to a fine powder. The dried powder (137.6 g) was extracted consecutively with DCM and MeOH to yield two main crude extracts (3.6 g and 17.7 g, respectively). The two crude extracts were subjected to general phytochemical screening and the results are summarized in Table 3.1.

The DCM and MeOH crude extracts were further fractionated as indicated in Figure 3.4 and 3.5, respectively, leading to the isolation of one compound from the DCM crude extract, one compound found in both the DCM and MeOH crudes and three compounds from the MeOH crude extract.

Table 3.1*: Results of phytochemical screening of the DCM and MeOH crude extracts*

Detection	Type of test	Observation
Carbohydrates	Benedict's Test	Orange precipitate indicated the presence of carbohydrates.
	Fehling's Test	Red precipitate indicating the presence of carbohydrates.
Flavonoids	Alkaline Reagent Test	Intense yellow colour indicated the presence of flavonoids in the MeOH crude extract. Addition of dil. acid gave no change in the yellow colour.
Phenols	Ferric Chloride Test	A black colour was observed indicating the presences of phenols
Saponins	Froth Test	No foam observed indicating the absence of saponins in the MeOH crude extract
Terpenoids	Salkowski's Test	A golden yellow colour was observed, indicating the presence of terpenoids

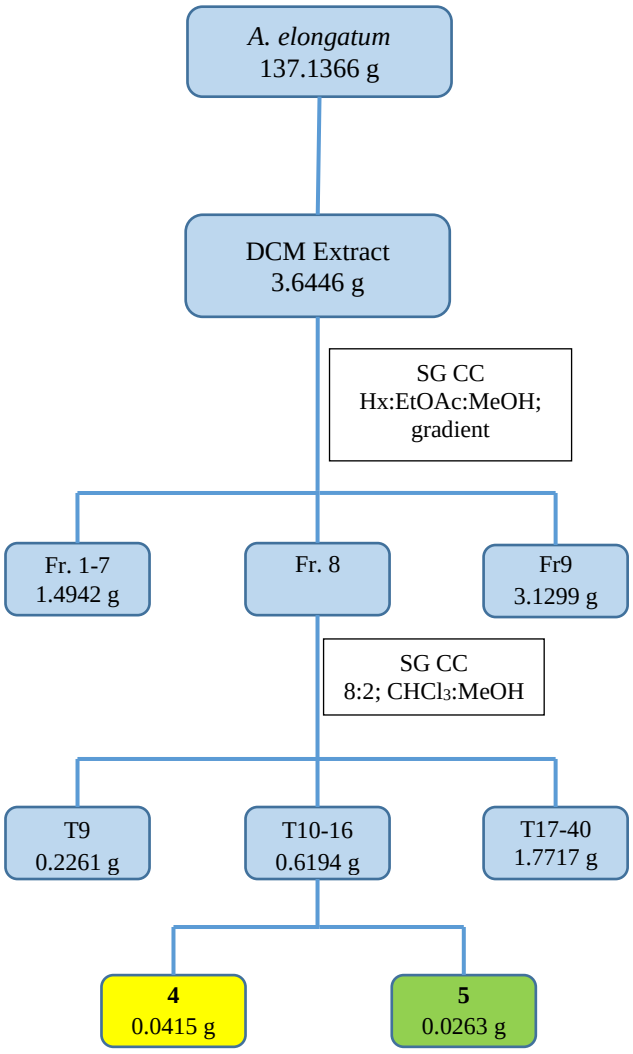


Figure 3.4*: Schematic representation of the isolation of compounds 4 and 5 from the DCM extract of A. elongatum*

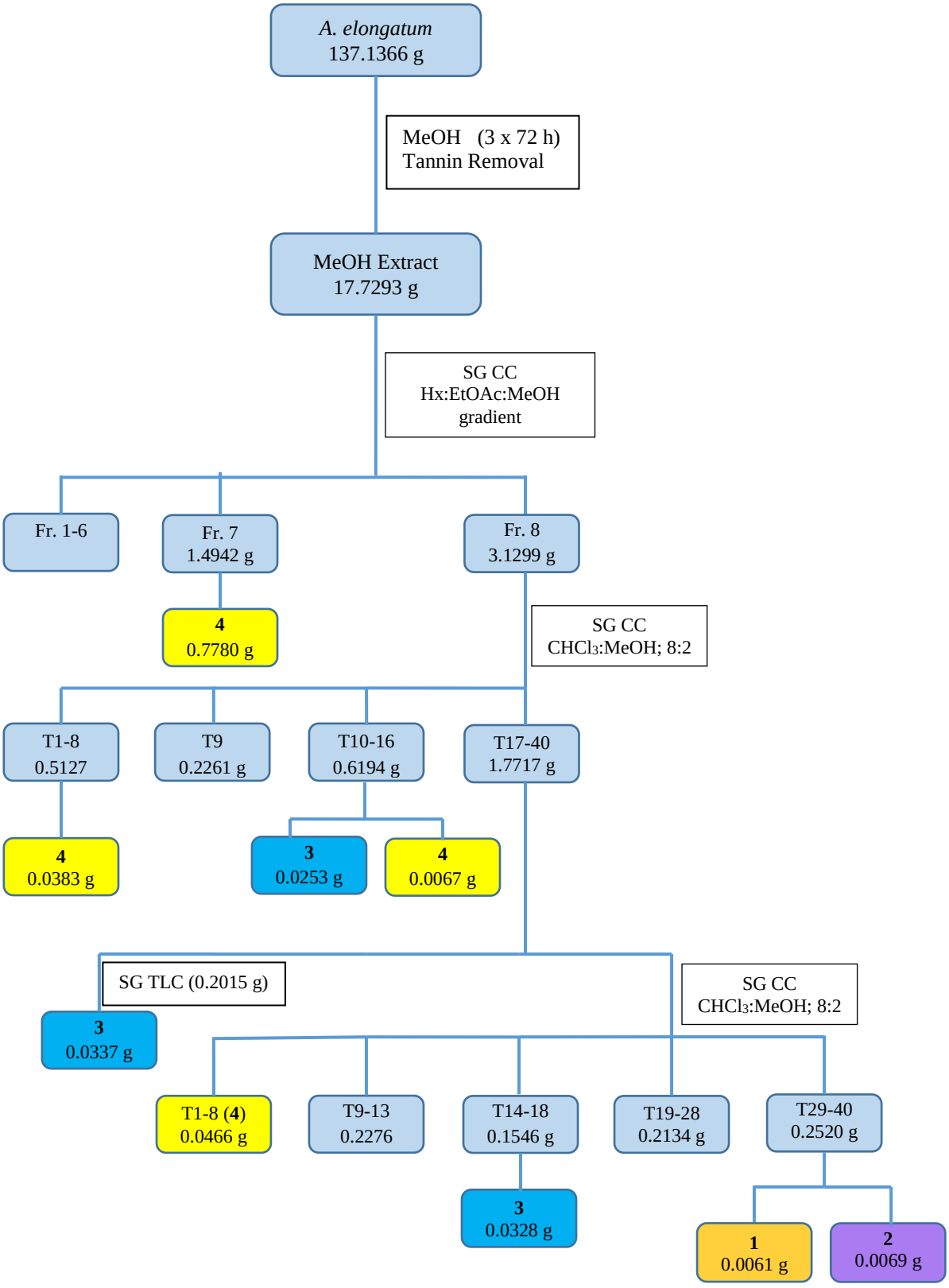
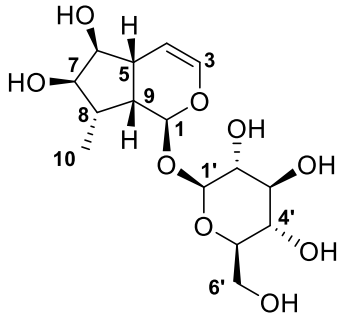


Figure 3.5: ***Schematic representation of the isolation of compounds 1, 2, 3 and 4 from the MeOH extract of A. elongatum***

3.3.2.1 Structural elucidation of Compound **1**



Compound **1** was obtained as a clear yellow oil. The HR ESI-MS of compound **1** gave an $[M+H]^+$ quasi molecular ion peak at *m/z* 349.1444, which is consistent with the molecular formula $C_{15}H_{24}O_9$ (calc. 349.1499), indicating a degree of unsaturation (or index of hydrogen deficiency) of four (Figure 3.6). IR spectroscopy is an important technique for structure elucidation to provide information on the functional groups in the molecule. A broad peak centred at 3343 cm^{-1} was attributed to the OH bond stretching vibration. The C-H symmetrical and asymmetrical stretching vibrations were observed as strong, sharp peaks at 2850 and 2918 cm^{-1}, respectively. Absorption at 1657 cm^{-1} is characteristic of double bonds and the peaks centred at 1040 cm^{-1} correspond to C–O stretching vibrations (Figure 3.7).

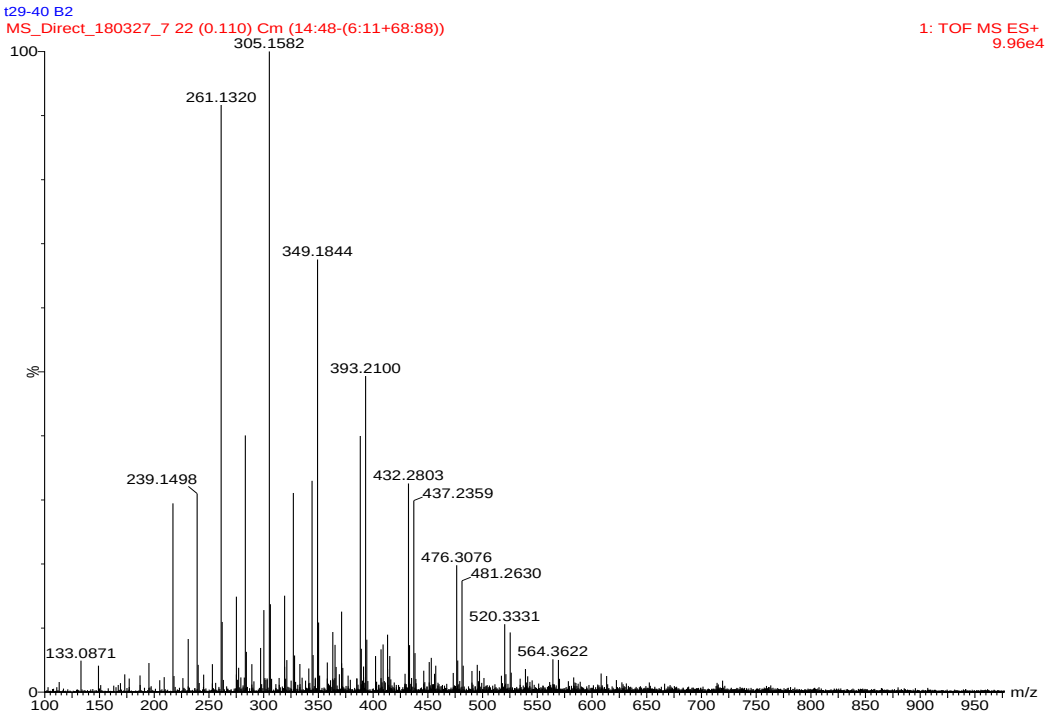


Figure 3.6*: HRESI-MS spectrum of 1*

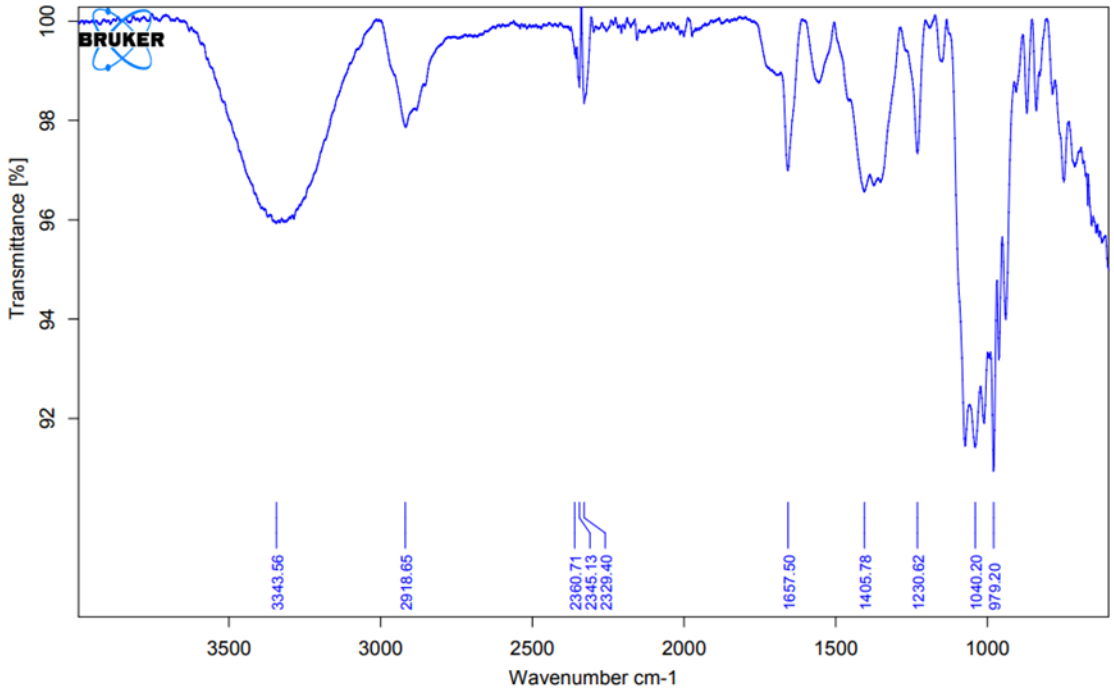


Figure 3.7*: IR spectrum of 1*

The ^{1}H NMR spectrum of compound **1** (Plate 1, Table 3.2) showed the following characteristics: an anomeric proton at δ_H 4.63 (J = 7.9 Hz), which is consistent with the larger axial-axial coupling expected for a β-D-glucoside moiety, a distinct doublet of doublets integrating for one proton in the aromatic region at δ_H 6.20 (H-3, J = 2.1, 6.4 Hz), which correlates with a proton at δ_H 4.79 (H-4, J = 2.8, 6.5 Hz) in the 2D COSY experiment (Plate 4), indicating a double bond connected to two methine carbons, a one proton doublet at δ_H 5.41 (H-1, J = 2.2 Hz) and an aliphatic three-proton doublet at δ_H 1.14, indicating a methyl group. Thus, if the degree of saturation is four, this indicates a bicyclic structure (one double bond and one cyclic sugar leaving two cyclic moieties). In the 2D COSY experiment (Plate 4), correlations between the proton at δ_H 2.65 (H-5) with three protons at δ_H 4.79 (H-4, double bond), 3.77 (H-6, oxygenated) and 2.71 (H-9), respectively, and between the protons at δ_H 2.71 (H-9) and 5.41 (H-1, acetal), 2.65 (H-5) and 2.21 (H-8), respectively, clearly confirmed the bicyclic nature of compound **1**, the two rings joined through the sharing of the mutual protons at δ_H 2.71 (H-9) and 2.65 (H-5). The low field chemical shift of the second double bond hydrogen at δ_H 6.20 (H-3) indicates that it is connected to an oxygen, suggesting an heterocyclic six-membered ring connected to an oxygenated pentacyclic ring. This arrangement of atoms is characteristic of the iridane skeleton found in iridoids and the compound was therefore identified as an iridoid glycoside (Compound **1**).

The ^{13}C NMR and ^{13}C APT spectra (Plate 2 and 3) showed 15 resonances (Table 3.2): five methine and one methylene oxygenated carbons corresponding to a glucose moiety, two oxygenated carbons, most likely hydroxymethines, resonating at δ_C 77.0 and 79.6 (C-6 and C-7), respectively, two sp^2 methine carbons with chemical shifts at δ_C 139.9 and 104.2 (C-3 and C-4), respectively, three aliphatic methine carbons at δ_C 36.8, 37.5 and 38.5 (C-5, C-8 and C-9), respectively, and one methyl carbon at δ_C 14.3. The important correlations observed in the 2D HMBC experiment (Plate 6) are depicted in Figure 3.8. These correlations enabled us to confirm the positions of the β-*O*-glucoside moiety, the double bond and the two hydroxy groups at C-6 and C-7, respectively.

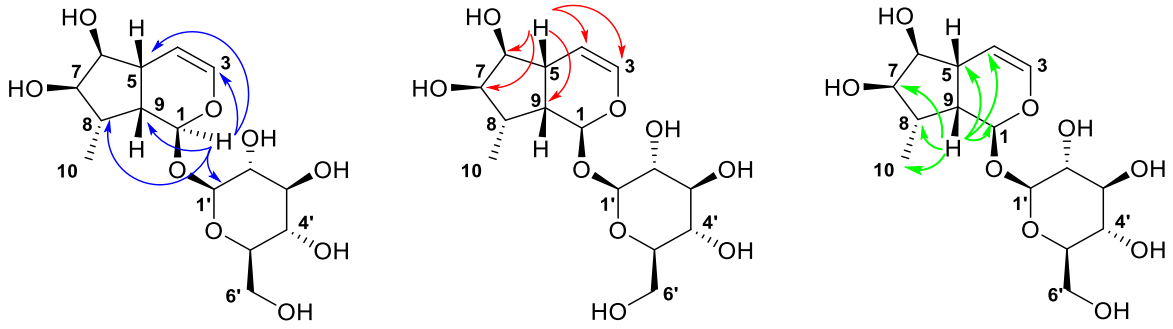


Figure 3.8*: HMBC correlations of H-1, H-5 and H-9*

The relative configuration were confirmed via coupling constants and long distance 2D NOESY experiments (Figure 3.9, Plate 7). NOE correlations between H-1 and H-7 and H-10, respectively, confirmed the β orientation of the hydroxy group at C-7, and the α orientation of the methyl group, respectively. Correlations of H-8 and H-9 indicates that they are syn relative to each other.

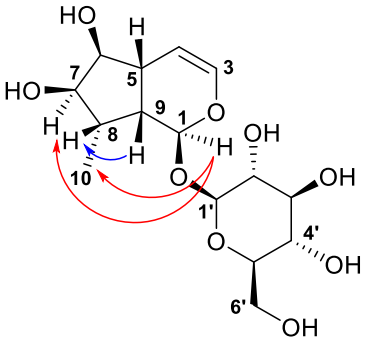


Figure 3.9*: 2D NOESY correlations confirming the absolute configuration of 1*

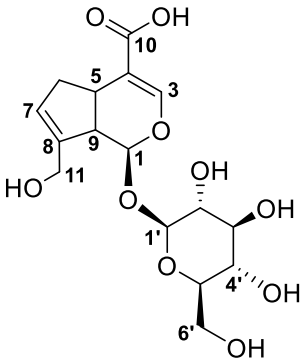
Compound **1** was determined to be (2*S*,3*R*,4*S*,5*S*,6*R*)-2-(((1*S*,4a*R*,5*S*,6*R*,7*S*,7a*R*)-5,6-dihydroxy-7-methyl-1,4a,5,6,7,7a-hexahydrocyclopenta[c]pyran-1-yl)oxy)-6-(hydroxy-

methyl)tetrahydro-2*H*-pyran-3,4,5-triol. The carbon and proton shifts compare favourably to the known compound, angeloside, first isolated from *Angelonia integerrima* (Scrophulariaceae).[146]

Table 3.2*: ^{1}H and ^{13}C NMR data of angeloside 1 [600 MHz, CD_3OD, δ (ppm), J (Hz)]*

	δ ^{1}H Experimental	δ ^{13}C Experimental	δ ^{1}H Published	δ ^{13}C Published
1	5.41 (1H, d, *J* = 2.2)	93.4	5.39 (1H, d, J = 2.3)	95.0
3	6.20 (1H, dd, *J* = 2.1, 6.4)	139.9	6.18 (1H, dd, J = 6.2, 1.9)	140.3
4	4.79 (1H, dd, *J* = 2.8, 6.5)	104.3	4.77 (1H, dd, J = 6.2 and 2.9)	105.7
5	2.65 (1H, br. dd, *J* = 2.4, 8.7)	36.8	2.62 (1H, dddd, J = 8.5, 2.9, 1.9,1.9)	36.7
6	3.77 (1H, dd, *J* = 1.8, 3.8)	77.0	3.75(1H, dd, J = 3.9, 1.9)	80.0
7	3.73 (1H, dd, *J* = 3.9, 8.4)	79.6	3.71 (1H, dd, J = 8.4, 3.9)	77.4
8	2.21 (1H, br. dt, *J* = 7.7, 11.3)	37.5	2.19 (1H, ddq, J = 11.1, 8.4, 7.3)	37.3
9	2.71 (1H, m)	38.5	2.69 (1H, ddd, J = 11.1, 8.5, 2.3)	38.7
10	1.14 (3H, d, *J* = 7.3)	13.0	1.12 (3H, d, J = 7.3)	13.9
1′	4.63 (1H, d, *J* = 7.9)	97.9	4.61 (1H, d, J = 8.1)	98.8
2′	3.25 (1H, dd, *J* = 7.9, 9.4)	73.4	3.19 (dd. J=9.2, 8.1)	73.5
3′	3.41 (1H, dd, *J* = 8.8, 8.8)	76.6	3.37 (1H, dd, J = 9.2, 9.2)	76.4
4′	3.32 (1H, obs.)	70.3	3.30 (1H, obs.)	70.4
5′	3.33 (1H, obs.)	76.8	3.29 (1H, obs.)	77.0
6′eq	3.87 (1H, dd, *J* = 1.9, 12.1)	61.4	3.88 (1H, dd, J = 11.9 and 1.6)	61.5
6′ax	3.68 (1H, dd**,** *J* = 5.3, 12.0)		3.67 (1H, ddd, J= 11.9, 4.1, 1.6)	

3.3.2.2 Structural elucidation of Compound **2**



Compound **2** was obtained as a clear yellow oil and the purity can be observed on the HPLC chromatogram (Figure 3.10).

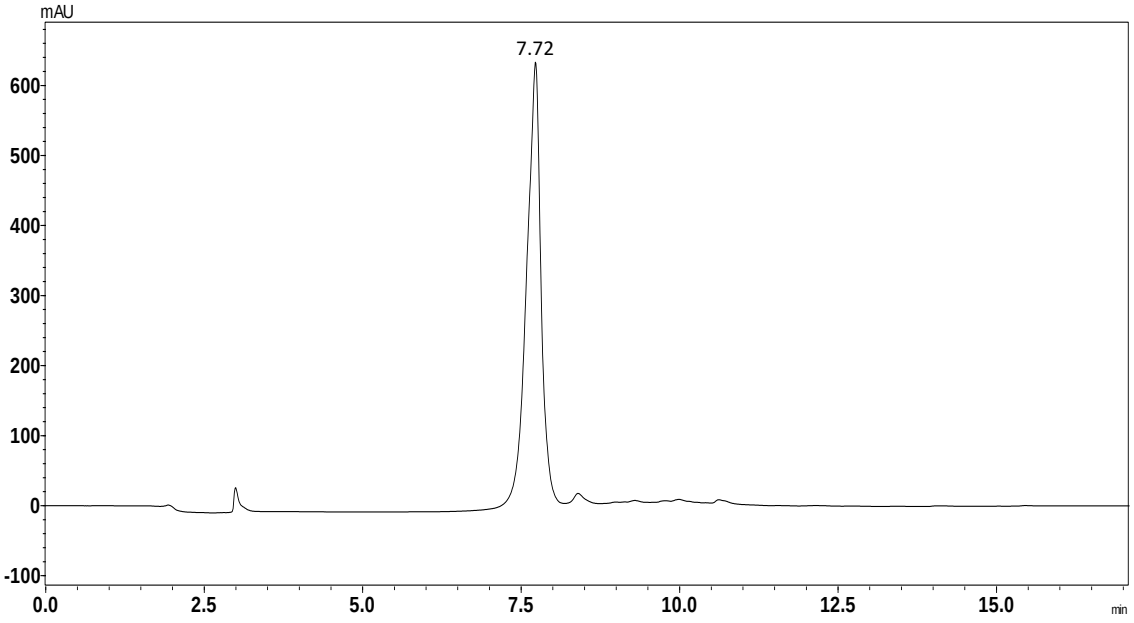


Figure 3.10*: HPLC chromatogram of compound 2*

The ESI-MS of compound **2** gave an $[M+H+Na]^+$ quasi molecular ion peak at *m/z* 397.1107, which is consistent with the molecular formula $C_{16}H_{23}O_{10}Na$ (calc. 397.1111) (Figure 3.11). The IR spectrum of **2** closely resembled the IR spectrum of **1**, except for an absorption peak at 1729 cm^{-1}, indicating the presence of a carbonyl group (Figure 3.12). Further peaks correlate with OH absorbances at 3321 cm^{-1}, double bonds at 1638 cm^{-1} and C-O vibrations at 1039 cm^{-1}.

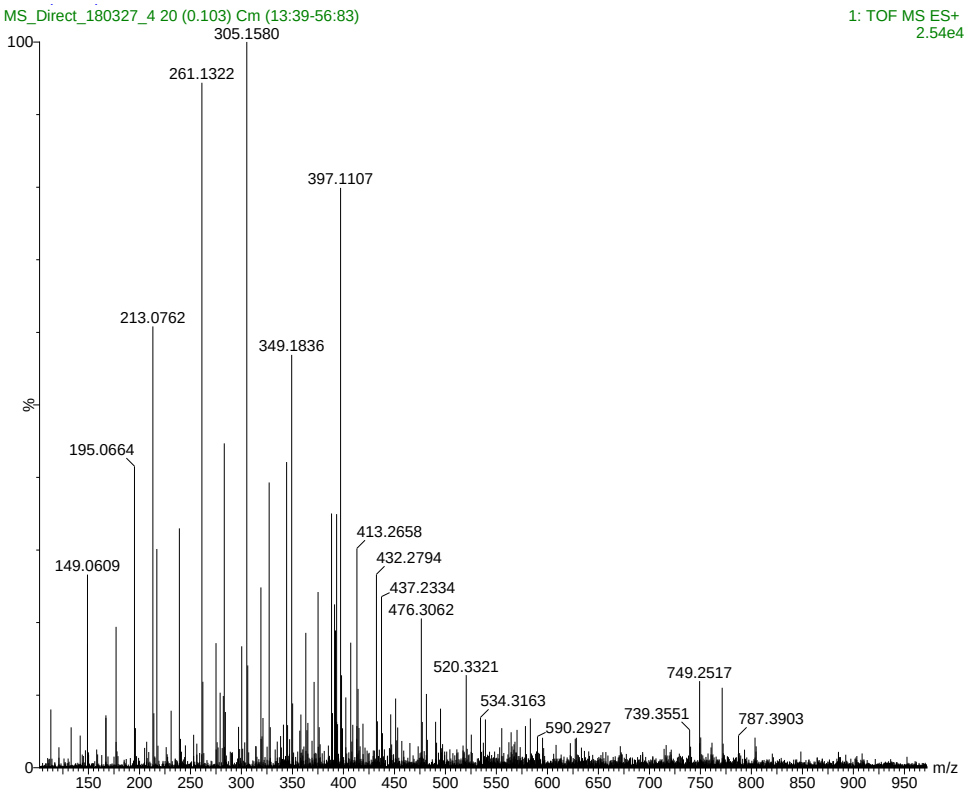


Figure 3.11*: ESI-MS spectrum of 2*

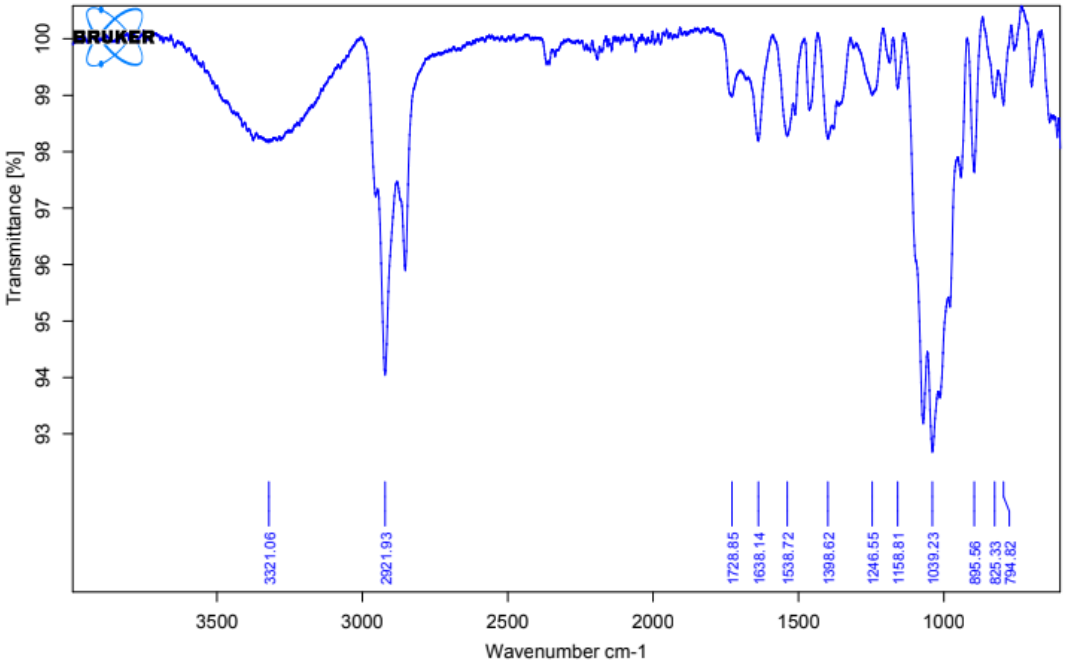


Figure 3.12*: IR spectrum of 2*

The formula $C_{16}H_{22}O_{10}$ implies a degree of unsaturation of six. The IR spectrum indicated the presence of a carbonyl carbon, which was confirmed by the 2D HMBC spectrum (Plate 13) with a resonance at δ_C 173.5 (the carbonyl carbon resonance did not show in the ^{13}C NMR

spectrum). Inspection of the ^{1}H NMR of **2** (Table 3.3, Plate 8) showed an anomeric proton at δ_H 4.74, an acetal proton doublet at δ_H 5.13 and two one proton broadened singlets at δ_H 5.81 (H-7) and 7.36 (H-3), respectively, indicating two double bonds. Thus, we once again have a bicyclic system in order to satisfy the degree of unsaturation (one carbonyl double bond, two double bonds, one cyclic glucoside and the bicyclic system). Comparison of the general 2D HMBC correlations of **2** with those of compound **1**, indicated that **2** also has a glucosylated iridoid skeleton. The positions of the carbonyl moiety and the double bonds were confirmed via 2D HMBC experiments (Figure 3.13).

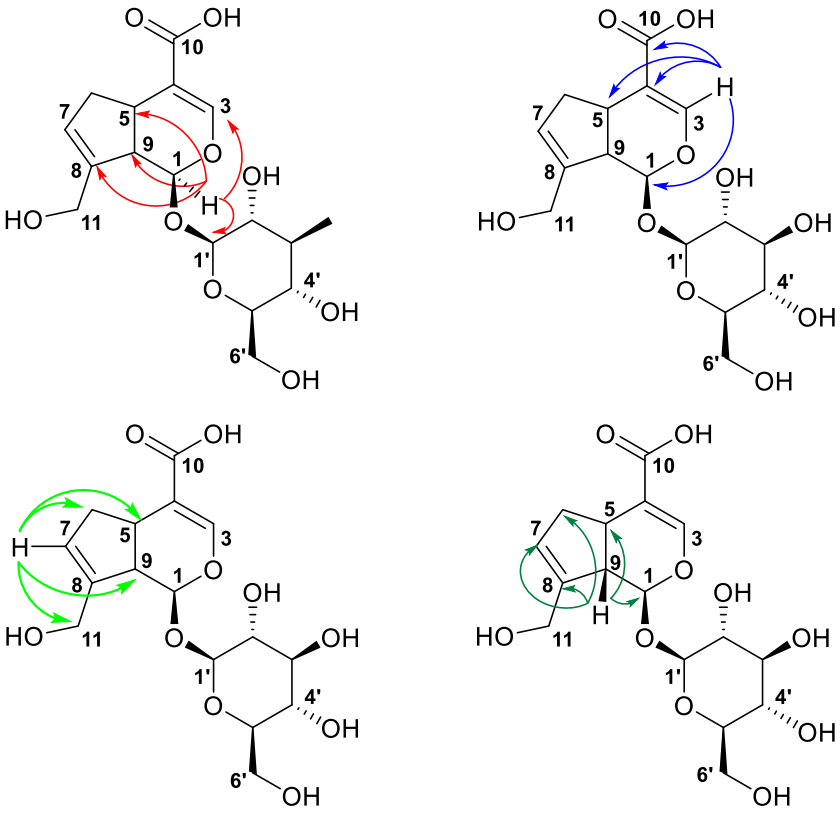


Figure 3.13*: HMBC correlations of H-1, H-3, H-7 and H-9*

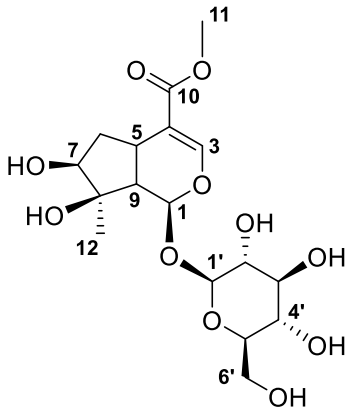
Compound **2** was characterized as (1*S*)-7-(hydroxymethyl)-1-(((2*S*,3*R*,4*S*,5*S*,6*R*)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2*H*-pyran-2-yl)oxy)-1,4a,5,7a-tetrahydrocyclo-penta-[c]pyran-4-carboxylic acid. The carbon and proton shifts compare favourably to the known compound, geniposidic acid, isolated from *Gardenia jasminoides* Ellis (Rubiaceae).[147]

Table 3.3: ***^{1}H (Plate 8) and ^{13}C NMR (Plate 9) data of geniposidic acid 2 [600 MHz, CD_3OD, δ (ppm), J (Hz)]***

	δ ^{1}H Experimental	δ ^{13}C Experimental	δ ^{1}H Published	δ ^{13}C Published
1	5.13 (1H, d, *J* = 7.7)	96.6	5.13 (1H, d, J=5.7))	98.09
3	7.36 (1H, s)	149.1	7.42 (1H, s)	152.65
4		115.9 (HMBC)		113.44
5	3.25 (1H, obsc.)	36.0	3.08 (1H, m)	36.87
6eq	2.87 (1H, br. dd, *J* = 8.0, 16.4)	38.5	2.92 (1H, m)	40.07
6ax	2.12 (1H, ddd, *J* = 2.4, 8.1, 16.6)		2.14 (1H, m)	40.07
7	5.81 (1H, br. s)	127.1	5.84 (1H, s)	128.52
8		143.4		144.96
9	2.71 (1H, dd, *J* = 7.7, 7.7)	45.9	2.92 (1H, m)	47.29
10		173.5 (HMBC)		171.95
11a	4.33 (1H, d, *J* = 14.3)	60.2	4.37 (1H, d, J=14.4)	61.62
11b	4.21 (1H, d, *J* = 14.3)		4.24 (1H, d, J=14.0)	
1′	4.74 (1H, d, *J* = 7.9)	99.0	4.77 (1H, d, J=7.2)	100.30
2′	3.25 (1H, dd, *J* = 7.9, 9.4)	73.5	3.28 (1H, m)	74.95
3′	3.41 (1H, dd, *J* = 8.8, 8.8)	76.5	3.46 (1H, m)	77.86
4′	3.32 (1H, obs.)	70.2	3.29 (1H, m)	71.56
5′	3.33 (1H, obs.)	76.9	3.29 (1H, m)	78.29
6′eq	3.87 (1H, dd, *J* = 1.9, 12.1)	61.2	3.89 (1H, m)	62.65
6′ax	3.68 (1H, dd, *J* = 5.3, 12.0)		3.63 (1H, m)	

Geniposidic acid (GEA), is a natural occurring chemical compound, classified as an iridoid glucoside, found in a variety of plants. GEA is known to be present in a number of folk medicines used as bitter tonics, sedatives, febrifuges, and cough medicines, remedies for wounds and skin disorders and as hypotensives.[148] In several medicinal herbs, GEA is an active ingredient and it has pharmacological effects on hypertension, inflammation, diabetes, atherosclerosis, and cancer.[149] It furthermore displays antihypertensive, anti-obesity, anti-inflammatory, antioxidative and anticancer chemotherapeutic properties, and it was found to be effective against jaundice and hepatic disorders.[150,151] In a study of the bioactive compounds present in *Eremophila longifolia* (Scrophulariaceae), a medicinal plant used by the Australian Aboriginal people, it was found that the main contributor to the cardiovascular activity exhibited by the crude extract of the plant was geniposidic acid.[152]

3.3.2.3 Structural elucidation of Compound **3**



Compound **3** was obtained as a clear yellow oil and the purity can be observed on the HPLC chromatogram (Figure 3.14). The HR ESI-MS of **3** gave an $[M+H+Na]^+$ quasi molecular ion peak at *m/z* 429.1370, which is consistent with the molecular formula $C_{17}H_{27}O_{11}Na$ (calc. 429.1373) (degree of unsaturation of 5) (Figure 3.15). Salient in the IR spectrum is OH-stretching vibrations at 3324 cm^{-1}, C-H stretching vibrations centred at 2917 cm^{-1}, a conjugated carbonyl absorption at 1691 cm^{-1}, double bonds at 1640 cm^{-1} and C-O absorbances at 1071 cm^{-1} (Figure 3.16).

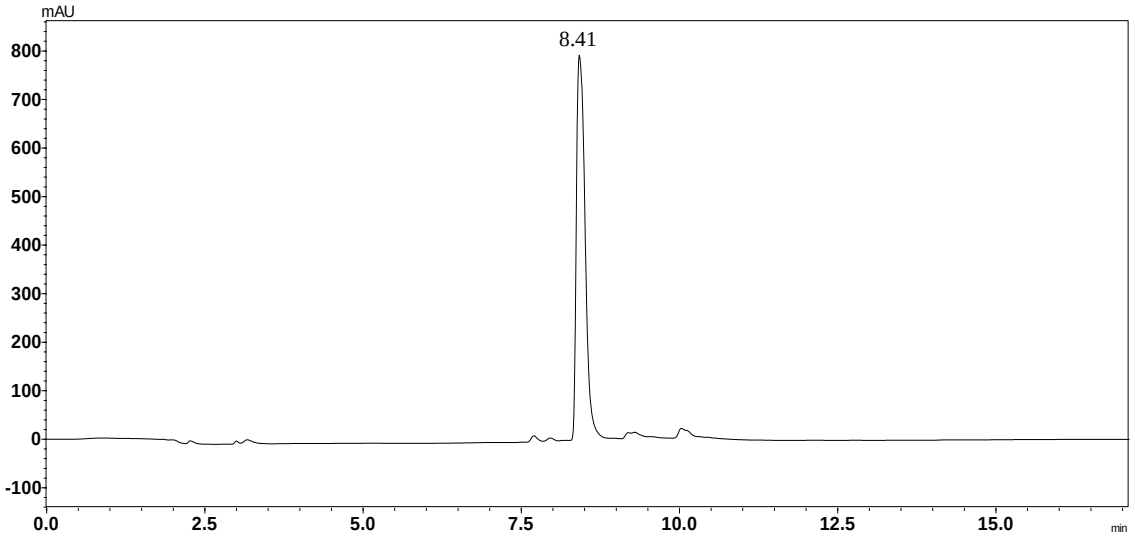


Figure 3.14*: HPLC chromatogram of 3*

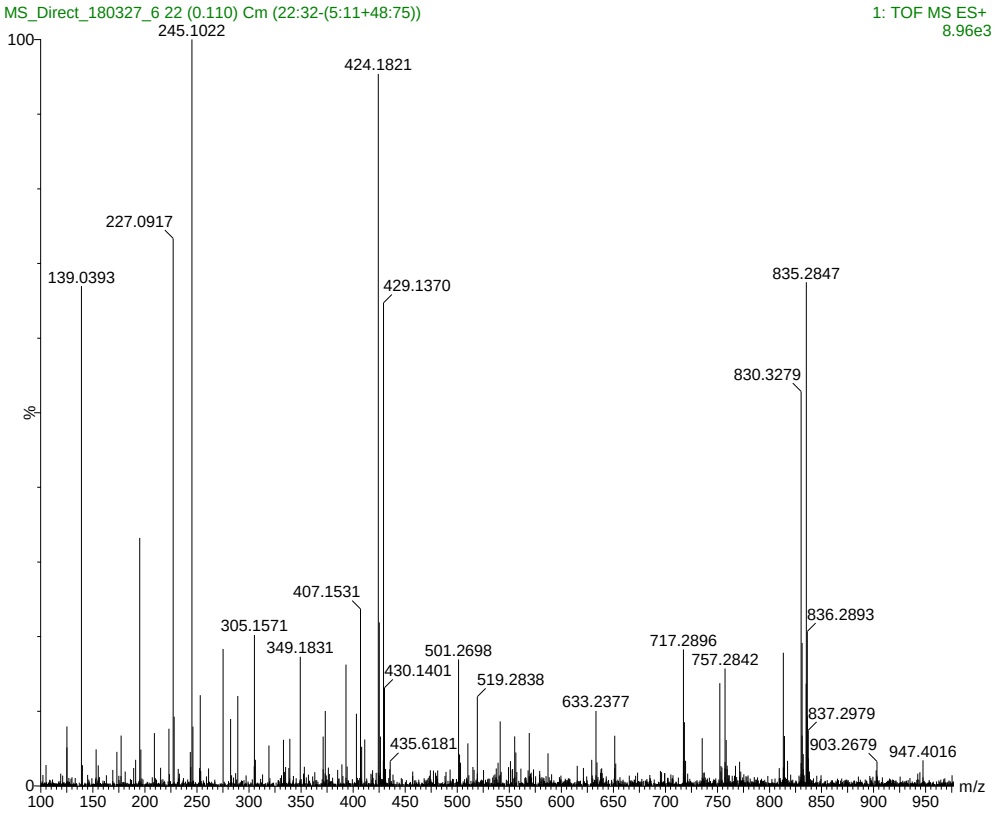


Figure 3.15*: ESI-MS spectrum of 3*

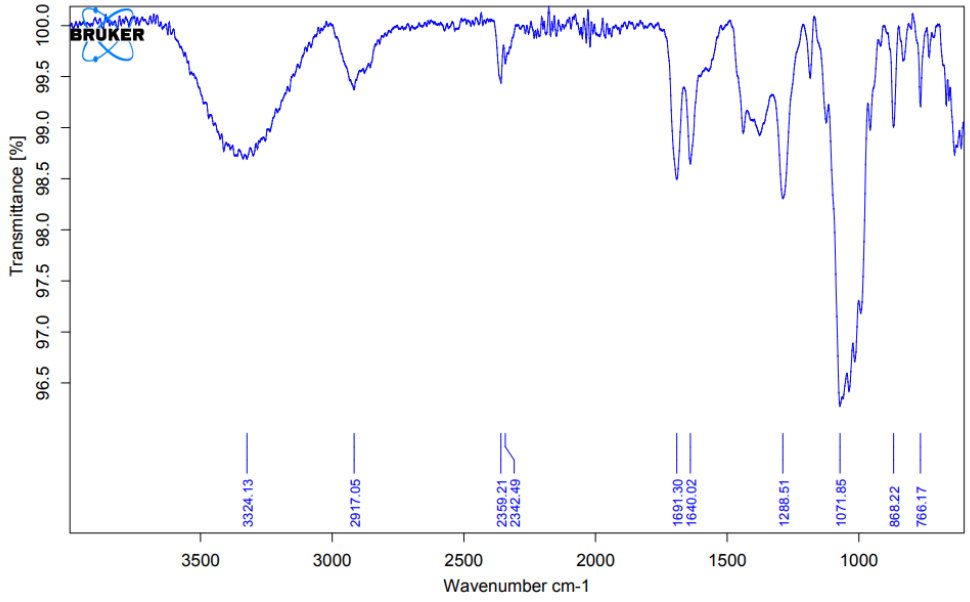


Figure 3.16*: IR spectrum of 3*

The ^{1}H NMR data (Table 3.4, plate 15) suggests a glucosylated iridoid skeleton as for the previous compounds with H-1 at δ_H 5.60 (d, J = 2.1 Hz), H-3 as a singlet at δ_H 7.39 and H-1′

at δ_H 4.66 (d, *J* = 8.0 Hz). The small *J*-value (2.1 Hz) of H-1 indicates an α-orientation, while the large coupling constant (8.0 Hz) observed for H-1′ indicates a β-anomer for the glucoside. Characteristic in the ^{1}H NMR spectrum is the presence of two methyl groups, one resonating at δ_H 1.22 (H-12) and an oxygenated methyl at δ_H 3.72 (H-11), respectively. The ^{13}C APT experiment (plate 17) showed 17 carbon atoms with a carbonyl resonance at δ_C 167.8 indicating an ester, a conjugated oxygenated methine carbon at δ_C 150.2 (C-3), two methyl carbons at δ_C 48.1 (C-11) and 20.5 (C-12), respectively, and two acetal methine carbons at δ_C 93.8 (C-1) and 98.4 (C-1′), respectively. Seven oxygenated methine carbons between δ_C 93.8 and 61.5 correspond to five glucoside carbons and C-7 and C-8, respectively. The positions of the various functional groups were determined via 2D HMBC correlations (Figure 3.17).

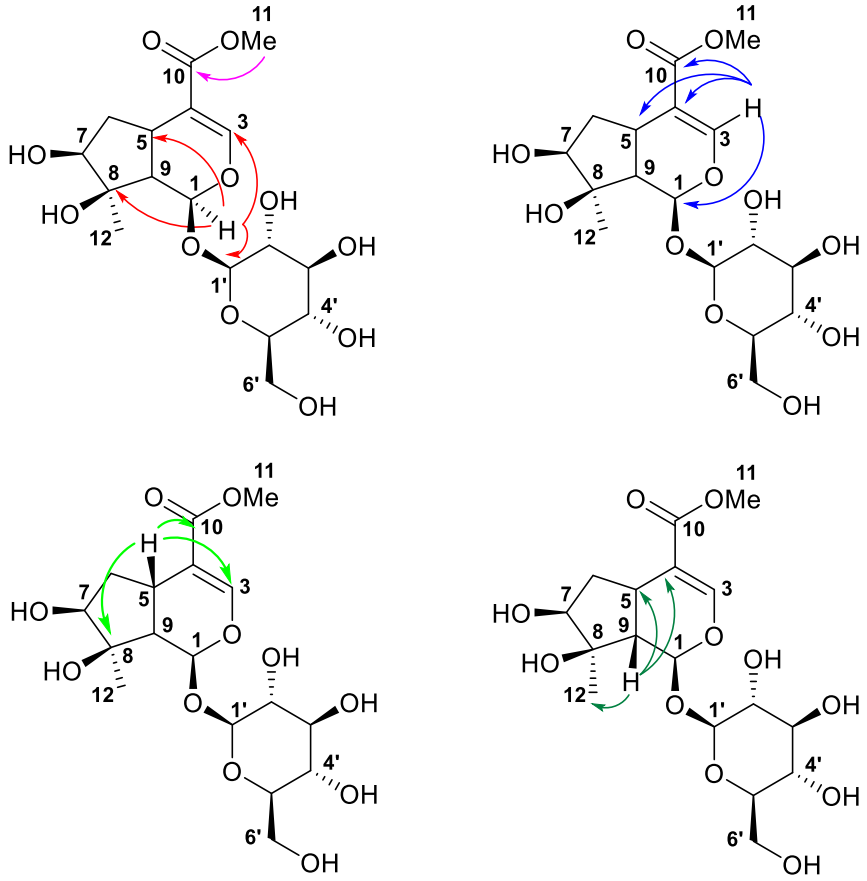


Figure 3.17*: HMBC correlations of H-1, H-3, H-5, H-9 and OMe*

The relative configuration of **3** was determined via 2D NOESY experiments (plate 21). Correlations between H-12 and H-7 indicate that these protons are cis (α-orientation), and correlation between H-1 and H-1′ and H-6′$_{ax}$, respectively, also suggests an α-orientation for these protons. Correlations between H-5 and H-9 and H-6$_{eq}$, respectively, show that these protons have a cis, β-orientation as indicated.

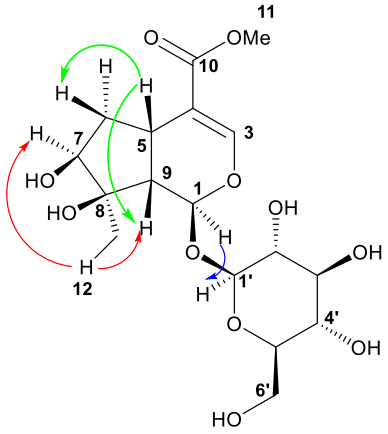


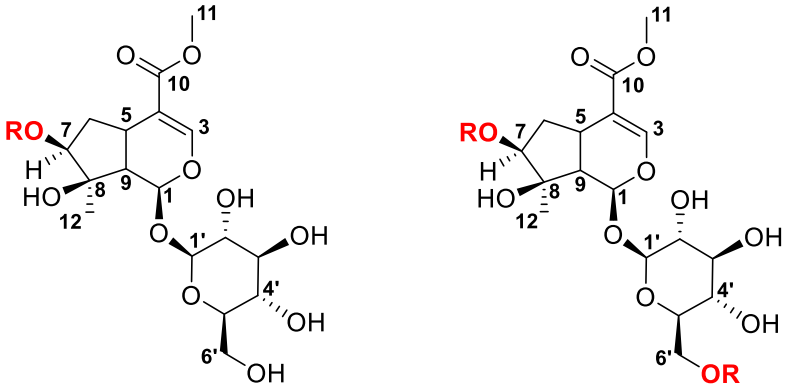
Figure 3.18*: NOE correlations confirming the relative configuration of 3*

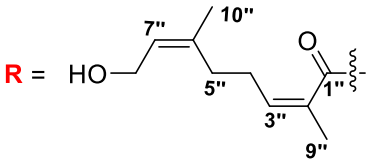
Compound **3** was identified as methyl (1*S*,6*S*,7*R*)-6,7-dihydroxy-7-methyl-1-(((2*S*,3*R*,4*S*,5*S*,6*R*)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2*H*-pyran-2-yl)oxy)-1,4a,5,6,7,7a-hexahydro-cyclopenta[c]pyran-4-carboxylate. The NMR data of **3** show a good correlation with those of the known iridoid, caryoptoside.[153]

Table 3.4*: ^{1}H and ^{13}C NMR data of caryoptoside 3 [600 MHz, CD_3OD, δ (ppm), J (Hz)]*

	δ ^{1}H Experimental	δ ^{13}C Experimental	δ ^{1}H Published	δ ^{13}C Published
1	5.60 (1H, d, *J* = 2.1)	93.8	5.58 (1H, d, *J* = 2.0)	95.1
3	7.39 (1H, s)	150.2	7.37 (1H,s)	151.5
4		112.8	-	114.2
5	3.14 (1H, br. dd, *J* = 6.1, 9.7)	26.1	n/a	27.6
6eq	2.24 (1H, ddd, *J* = 3.1, 9.7, 14.6)	37.4	2.22 (1H, m, *J* = 3.0, 9.4, 14.4)	38.8
6ax	1.68 (1H, ddd, *J* = 5.4, 6.3, 14.6)		1.65 (1H, dt, *J* = 5.7, 14.4)	
7	3.66 (1H, dd, *J* = 3.1, 5.4)	76.9	n/a	79.1
8		78.6	-	79.9
9	2.60 (1H, dd, *J* = 2.0, 10.5)	48.1	2.58 (1H, d, *J* = 10.5)	51.6
10		167.8	-	169.2
11	3.72 (3H, s)	50.3	n\a	51.6
12	1.22 (3H, s)	20.5	1.19 (3H, s)	21.9
1′	4.66 (1H, d, *J* = 8.0)	98.4	4.63 (1H, d, *J* = 7.9)	99.8
2′	3.20 (1H, dd, *J* = 8.0, 9.1)	73.3	3.18 (1H, dd, *J* = 9.0, 8.0)	74.7
3′	3.39 (1H, dd, *J* = 9.0, 10.0)	77.7	3.36 (1H, t)	78.0
4′	3.29 (1H, dd, *J* = 8.9, 9.2)	70.2	3.25 (1H, m)	71.6
5′	3.35 (1H, m)	76.6	3.27 (1H, d, *J* = 8.5)	78.3
6′eq	3.92 (1H, dd, *J* = 2.2, 12.0)	61.5	3.89 (1H, dd, *J* = 1.7, 11.9)	62.9
6′ax	3.68 (1H, dd, *J* = 6.1, 12.0)	61.5	3.66 (1H, m)	

3.3.2.4 Structural elucidation of Compounds **4** and **5**





Compounds **4** and **5** were both obtained as pale yellow oils. The HR ESI-MS of compound **4** gave an $[M+H+Na]^+$ molecular ion peak at *m/z* 595.2380, which is consistent with the molecular formula $C_{27}H_{41}O_{13}Na$ (calc. 595.2367) (Figure 3.21), and compound **5** gave an $[M+H+Na]^+$ molecular ion peak at *m/z* 761.3330, which is consistent with the molecular formula $C_{37}H_{55}O_{15}Na$ (calc. 761.3360) (Figure 3.22). Salient in the IR spectra of **4** and **5** are the pronounced conjugated carbonyl absorbances observed at 1692.9 cm^{-1} and 1697.5 cm^{-1}, respectively (Figures 3.23 and 3.24, respectively). Additional absorbances were almost identical to those observed for compounds **1** – **3**.

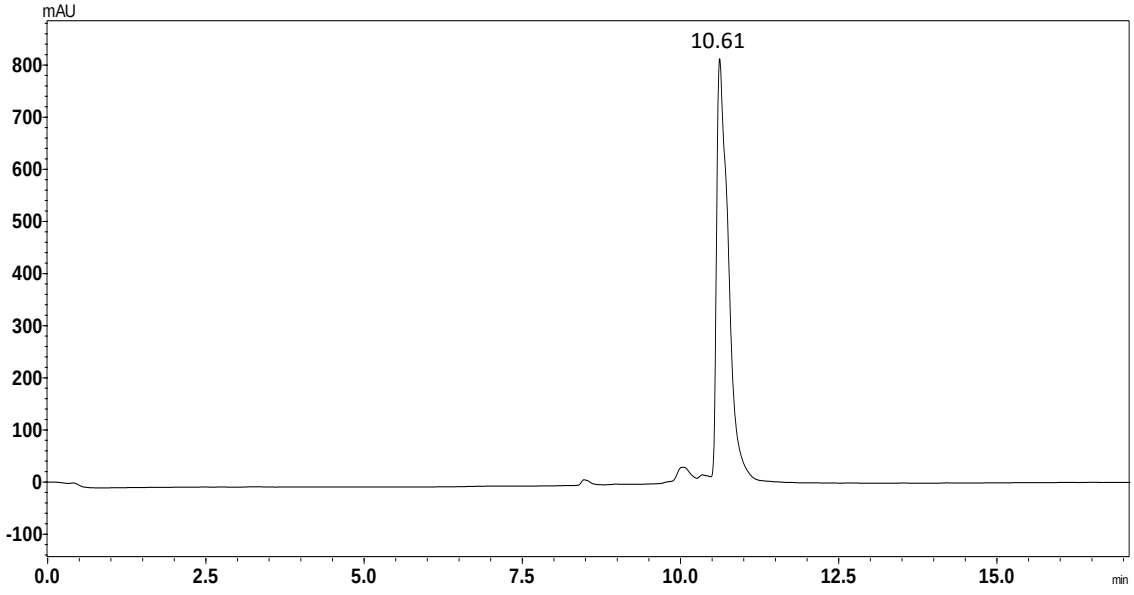


Figure 3.19*: HPLC chromatogram of 4*

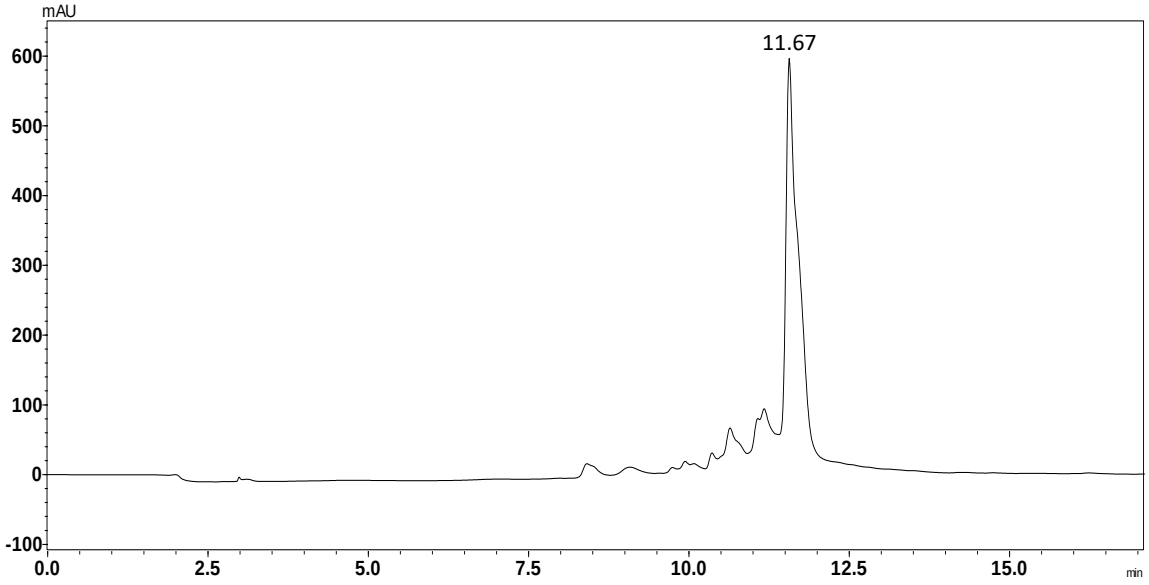


Figure 3.20*: HPLC chromatogram of 5*

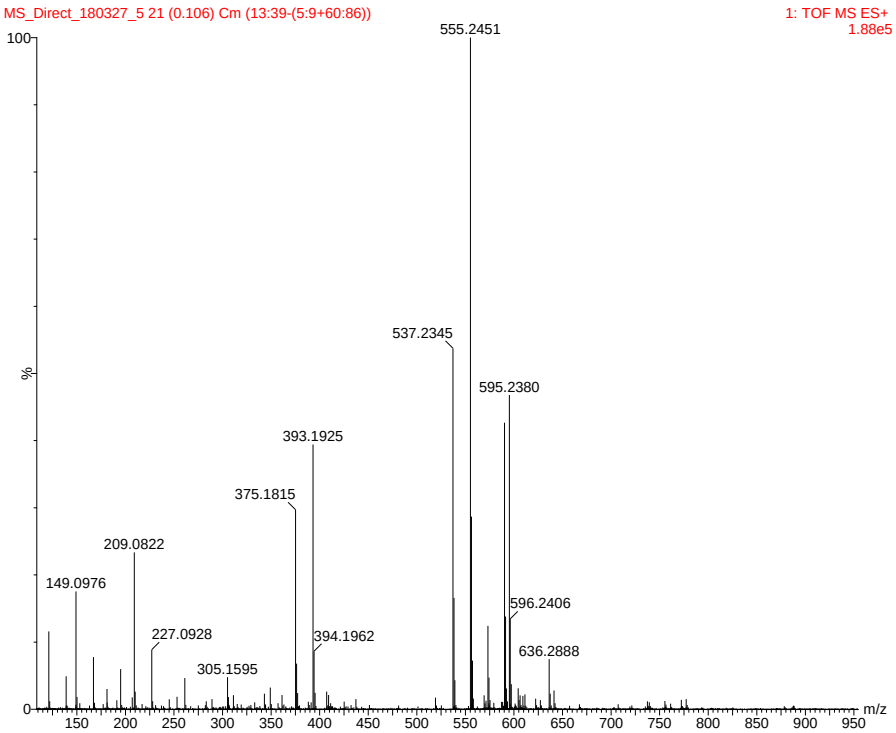


Figure 3.21*: ESI-MS spectrum of 4*

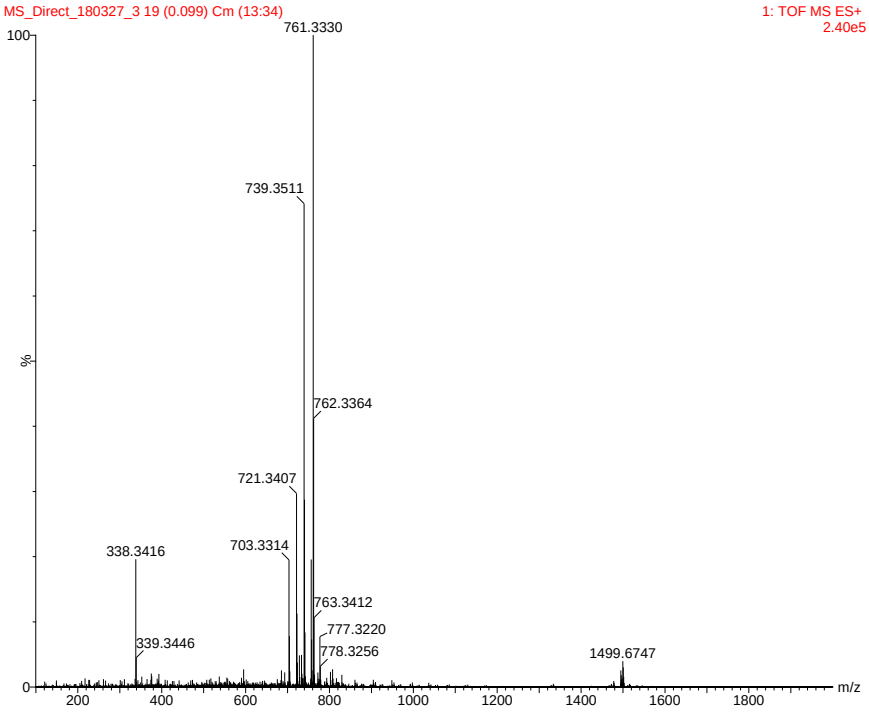


Figure 3.22*: ESI-MS spectrum of 5*

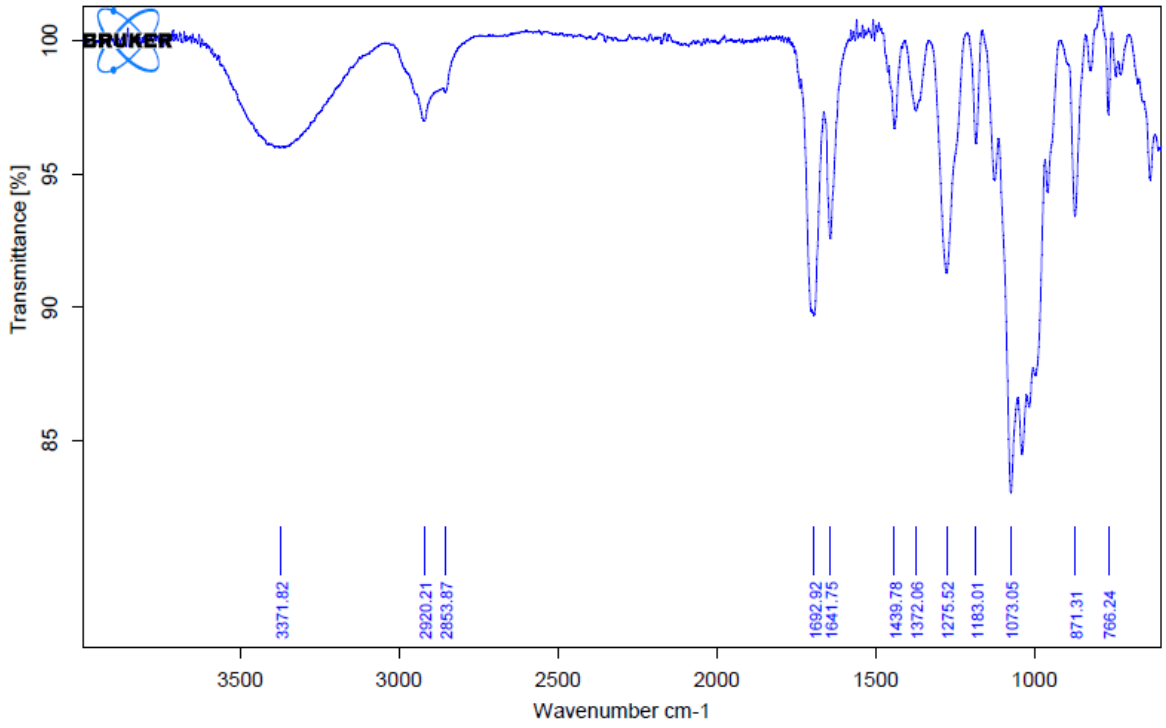


Figure 3.23*: IR spectrum of 4*

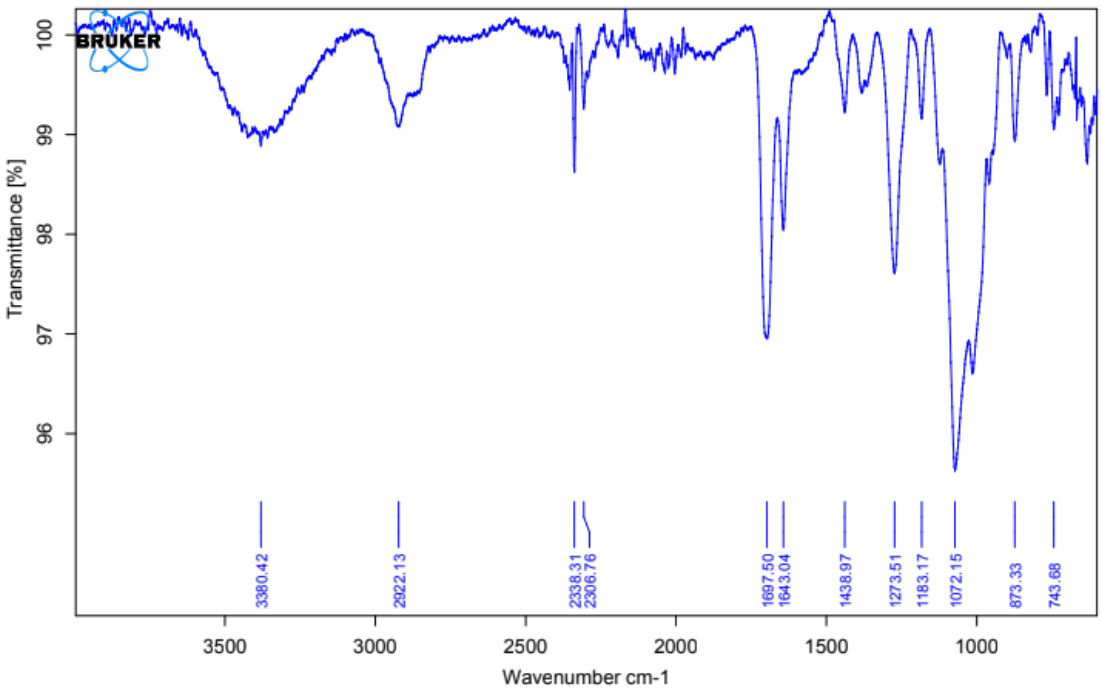


Figure 3.24*: IR spectrum of 5*

The ^{1}H NMR spectra of **4** and **5** (Table 3.5, plates 22 and 29) displayed the characteristic resonances of the glucosylated iridoids described previously, with H-1 resonating at δ_H 5.61 and 5.47 for **4** and **5**, respectively, H-3 at δ_H 7.41 and 7.42, respectively, and the anomeric proton H-1′ at δ_H 4.66 and 4.69, respectively. Comparison of the ^{1}H and ^{13}C NMR spectra of **4** and **5** with those of **1** – **3**, showed a close correlation between the iridoid glucoside skeleton of caryoptoside **3** and that of **4** and **5**, except for the downfield shift of H-7 from 3.73 ppm in **3** to 4.87 and 4.82 ppm for **4** and **5**, respectively, indicating that **4** and **5** may be 7-*O*-derivatives of **3**. The ^{13}C NMR spectra of **4** showed 10 more carbon resonances (relative to **3**), and from the ^{13}C APT experiment these were identified as one ester carbonyl resonance at δ_C 167.7, two quaternary and two primary olefinic carbons, four methylene carbons of which one were oxygenated, and two methyl carbons. The ^{1}H NMR spectrum of **4** displayed two olefinic protons at δ_H 6.86 as a tq and 5.42 as a tq, respectively, indicating their positions between a methylene and methyl group. Long distance 2D HMBC correlations between the vinylic proton at δ_H 6.86 (H-3″) and the methyl group at δ_C 11.2 (C-9″), the methylene carbon at δ_C 37.7 (H-5″), the vinylic carbon at δ_C 127.6 and the ester carbonyl at δ_C 167.7, and between the vinylic proton at δ_H 5.42 (H-7″) and the methyl group at δ_C 14.8 (C-10″), the oxygenated methylene carbon at δ_C 58.0 (H-8″), and the methylene carbon at δ_C 37.7 (H-5″) indicated that the ester moiety of the ten additional carbon resonances was a linear monoterpenoid, determined as an

isomer of foliamenthoate (Figure 3.25).[154] The 2D HMBC correlation between H-7 and H-1″ confirmed the position of the ester chain.

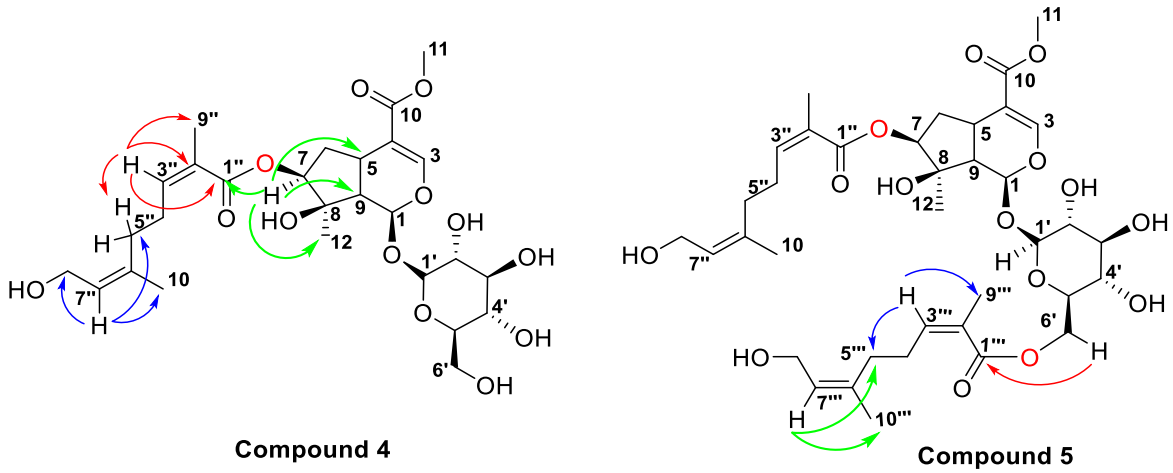


Figure 3.25*: 2D HMBC correlations observed for 4 and 5*

Comparison of the NMR data of **4** with that of the known compound picconioside IV[154] showed a close resemblance, except that compound **4** has a hydroxy group at C-8. Compound **4** is thus a new 7-*O*-foliamenthoate derivative of caryoptoside. An 1D NOE experiment showed correlation between H-3″ and H-9″ and correlation between H-7″ and H-10″, indicating, together with the small allylic coupling constants of ^{3}J = 1.8 and 1.3 Hz, respectively, *cis* configurations for the double bonds of the side-chain (Figure 3.26), while 2D NOESY correlations between H-1 and H-1′, H-12 and H-7 confirmed the relative configuration of the iridoid skeleton (Figure 3.26).

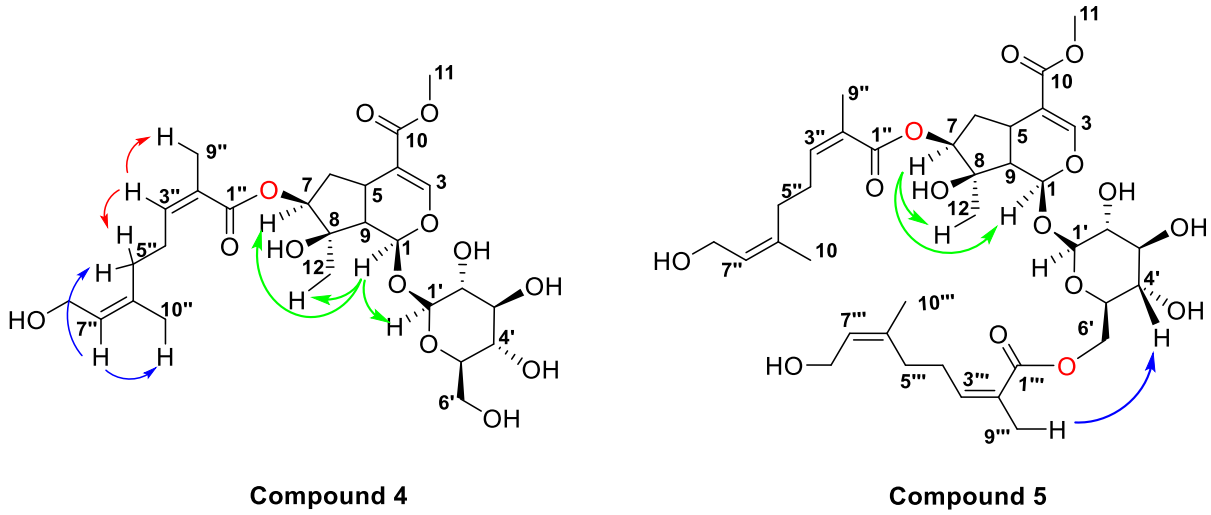


Figure 3.26: ***1D and 2D NOE correlations confirming the absolute configuration of 4 and 5***

The ^{13}C and ^{1}H NMR spectra of compound **5** are almost identical to those of **4**, except for the presence of a second foliamenthoate moeity, and the downfield shifting of $6'_{eq}$ and $6'_{ax}$, from 3.90 and 3.68 ppm, respectively for **4**, to 4.49 and 4.27 ppm, respectively, for **5**, suggesting that the second esterification occurred on *O*-6′. The position of the second foliamenthoate moeity was confirmed via a 2D HMBC experiment with correlation between H-$6'_{eq}$ and H-$6'_{ax}$ and C-1‴ (Figure 3.25). Owing to lack of material, 1D and 2D NOESY experiments performed on compound **5** were mostly inconclusive, and the few correlations observed are depicted in Figure 3.26. The stereochemistry of the geometric isomers of the foliamenthoate moieties were deduced from those of compound **4**, since all coupling constants were identical to those observed for **4**.

Table 3.5*: ^{1}H and ^{13}C NMR data of compounds 4 and 5 [600 MHz, CD_3OD, δ (ppm), J (Hz)]*

	4 δ ^{1}H (*J*-values in Hz)	**4 δ ^{13}C**	**5 δ ^{1}H (*J*-values in Hz)**	**5 δ ^{13}C**
1	5.61 (1H, d, *J* = 2.5)	93.4	5.47 (1H, d, *J* = 2.9)	93.3
3	7.41 (1H, d, *J* = 1.6)	150.2	7.42 (1H, s)	150.3
4		112.6		112.6
5	3.18 (1H, m)	26.1	3.22 (1H, m)	26.9
6a	2.30 (1H, ddd, *J* = 2.3, 9.8, 15.2)	35.8	2.27 (1H, ddd, *J* = 2.5, 10.0, 15.1)	35.9
6b	1.80 (1H, ddd, *J* = 5.8, 6.6, 15.3)		1.81 (1H, dt, *J* = 6.5, 15.0)	
7	4.87 (1H, dd, *J* = 2.4, 5.4)	80.3	4.82 (1H, dd, *J* = 3.0, 5.8)	80.1
8		78.2		78.3
9	2.66 (1H, dd, *J* = 2.0, 10.8)	47.7	2.60 (1H, dd, *J* = 2.8, 10.3)	48.2
10		167.6		167.6
11	3.72 (3H, s)	50.3	3.71 (3H, s)	50.3
12	1.28 (3H, s)	20.8	1.27 (3H, s)	21.0
1′	4.66 (1H, d, *J* = 7.9)	98.5	4.69 (1H, d, *J* = 7.9)	98.2
2′	3.21 (1H, dd, *J* = 7.9, 9.2)	73.2	3.23 (1H, dd, *J* = 8.6, 8.9)	73.3
3′	3.39 (1H, dd, *J* = 9.0, 9.0)	76.6	3.40 (1H, obsc.)	76.5
4′	3.29 (1H, dd, *J* = 8.7, 9.8)	70.2	3.38 (1H, obsc.)	70.2
5′	3.34 (1H, m)	76.9	3.56 (1H, ddd, *J* = 2.3, 5.1, 9.7)	74.4
$6'_{eq}$	3.90 (1H, dd, *J* = 2.3, 12.0)	61.4	4.49 (1H, dd, *J* = 2.2, 12.0)	63.1
$6'_{ax}$	3.68 (1H, dd, *J* = 6.1, 12.0)		4.27 (1H, dd, *J* = 5.3, 11.9)	
1″		167.7		167.7
2″		127.6		127.6
3″	6.86 (1H, tq, *J* = 1.2, 6.8)	142.1	6.86 (1H, tq, *J* = 1.6, 7.4)	142.2
4″	2.37 (2H, m)	26.6	2.38 (2H, m)	26.6
5″	2.19 (2H, t, *J* = 7.6)	37.7	2.19 (2H, t, *J* = 8.0)	37.7
6″		137.1		137.1
7″	5.42 (1H, tq, *J* = 1.4, 7.3)	124.4	5.41 (1H, m)	124.3
8″	4.11 (2H, d, *J* = 6.7)	58.0	4.10 (2H, d, *J* = 6.6)	58.0
9″	1.87 (3H, s)	11.2	1.86 (3H, s)	11.8
10″	1.71 (3H, s)	14.8	1.71 (3H, s)	14.8
1‴				168.0
2‴				127.4
3‴			6.81 (1H, tq, *J* = 1.6, 7.4)	142.2
4‴			2.35 (2H, m)	26.6
5‴			2.17 (2H, t, *J* = 8.0)	37.8
6‴				136.9

7‴			5.41 (1H, m)	124.4
8‴			4.10 (2H, d, *J* = 6.6)	58.0
9‴			1.85 (3H, s)	11.8
10‴			1.70 (3H, s)	14.8

Compound **4** was identified as methyl (1*S*)-7-hydroxy-6-(((2*Z*,6*Z*)-8-hydroxy-2,6-dimethyl-octa-2,6-dienoyl)oxy)-7-methyl-1-(((2*S*,3*R*,4*S*,5*S*,6*R*)-3,4,5-trihydroxy-6-(hydroxymethyl)-tetra-hydro-2*H*-pyran-2-yl)oxy)-1,4a,5,6,7,7a-hexahydrocyclopenta[c]pyran-4-carboxylate (trivial name: 7-*O*-((2*Z*,6*Z*)-foliamenthoate)caryoptoside) and compound **5** as methyl (1*S*)-7-hydroxy-6-(((2*Z*,6*Z*)-8-hydroxy-2,6-dimethylocta-2,6-dienoyl)oxy)-7-methyl-1-((2*S*,3*R*,4*S*,5*S*,6*R*)-3,4,5-trihydroxy-6-((((2*Z*,6*Z*)-8-hydroxy-2,6-dimethylocta-2,6-dienoyl)-oxy)methyl)tetrahydro-2*H*-pyran-2-yl)oxy)-1,4a,5,6,7,7a-hexahydrocyclopenta[c]-pyran-4-carboxylate (trivial name: 7-*O*-((2*Z*,6*Z*)-foliamenthoate)-6″-O-((2Z,6Z)-foliamenthoate) cary-optoside.

3.4 Glucosylated Iridoids

3.4.1 Biosynthesis

Iridoids are monoterpenes, which are found in a large number of plant families as natural constituents. Iridoids are typically found in plants as glycosides, most often bound to glucose. There are four main classes of iridiods namely, aglycone iridoids, secoiridoids, bisiridoids and iridoid glycosides (the most abundant).These compounds contain a cyclopentane ring which is usually fused to a six-membered oxygen heterocycle, forming the cyclopenta[c]pyranoid skeleton known as an iridane skeleton (Figure 3.27).[155]

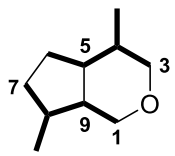
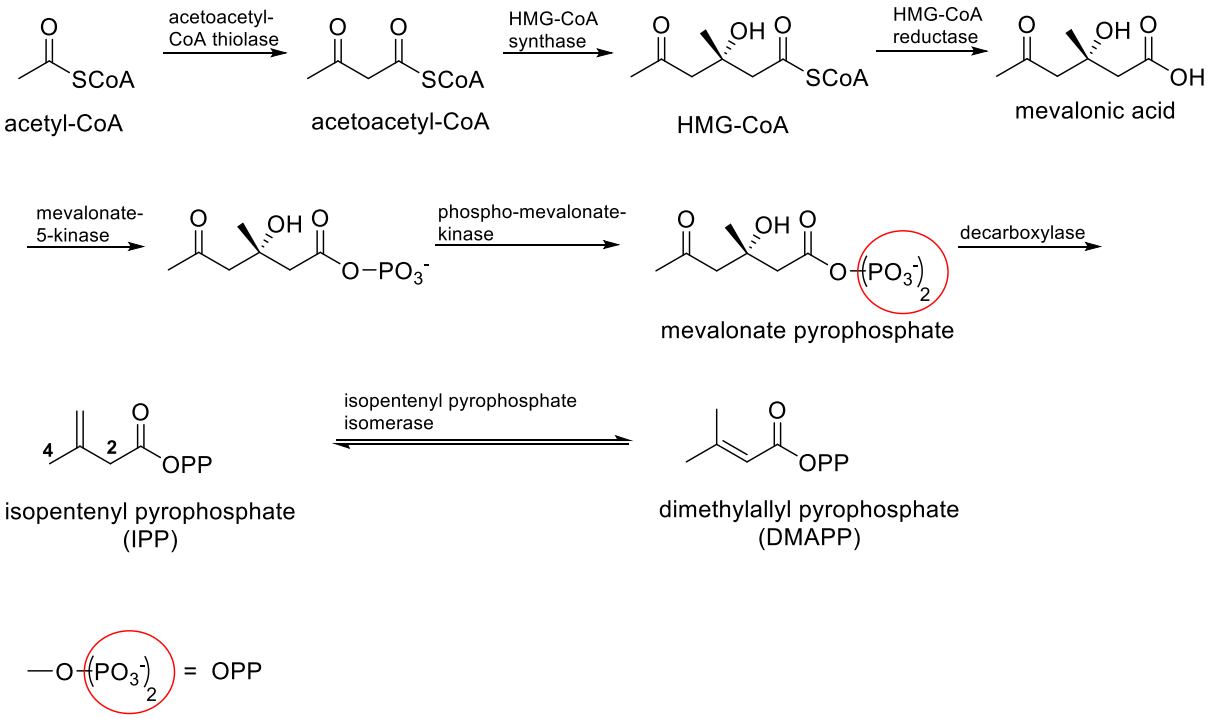


Figure 3.27: ***General chemical structure of the iridane skeleton***

Iridoids generally have nine carbons with a tenth carbon bonded to C-4. This carbon may be a methyl group or it may form part of a carbonyl or secondary alcohol functional group. Structural variations and diversification of iridoid types are achieved by the introduction of

additional carbons, functional groups and double bonds into the skeleton.[156] They are synthesized via the mevalonate pathway (Scheme 3.1).[157]



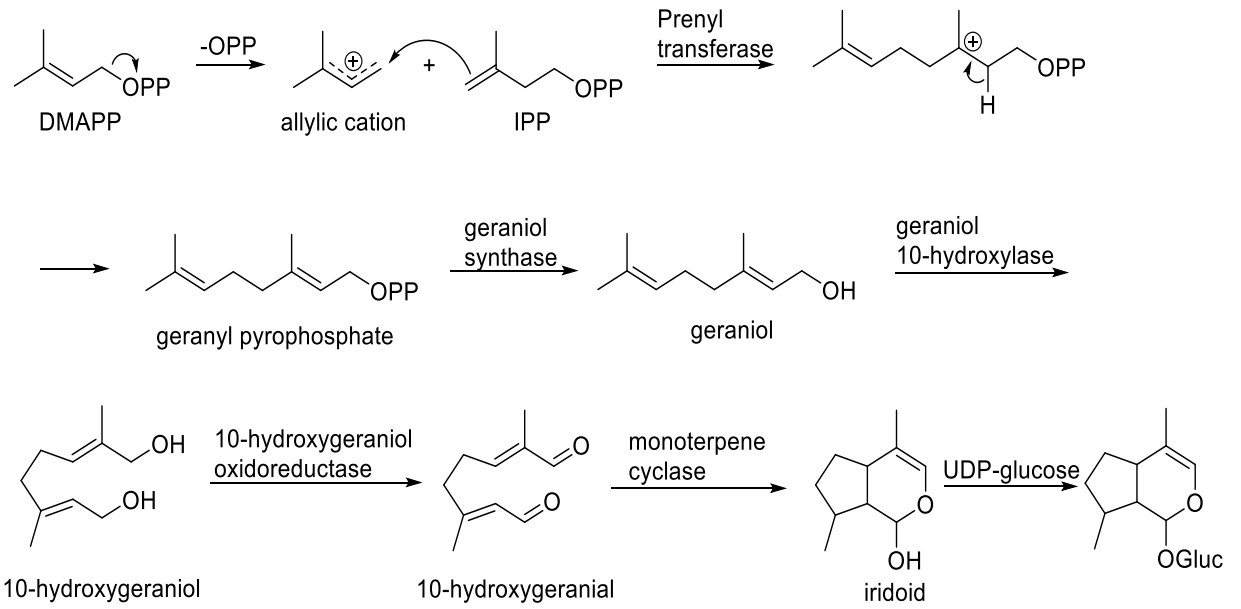
Scheme 3.1: *The mevalonate pathway to form DMAPP*

Plants have the ablity to utilize the mevalonate pathway, because the mevalonate pathway enzymes are found in the cytosol.[155] Mevalonic acid (MVA) forms the key intermediate isopentenyl pyrophosphate, which is converted to dimethylallyl pyrophosphate by means of allylic isomerization. The reaction is catalyzed by the isopentenyl pyrophosphate isomerase enzyme. The mechanism involves the removal of the proton at C-2 followed by addition of a proton from water at C-4 and is stereospecific.[158]

IPP and DMAPP reacts to form the key intermediate geranyl pyrophosphate, followed by hydroxylation and oxidation to yield the glucosylated iridoid (Scheme 3.2).[155]

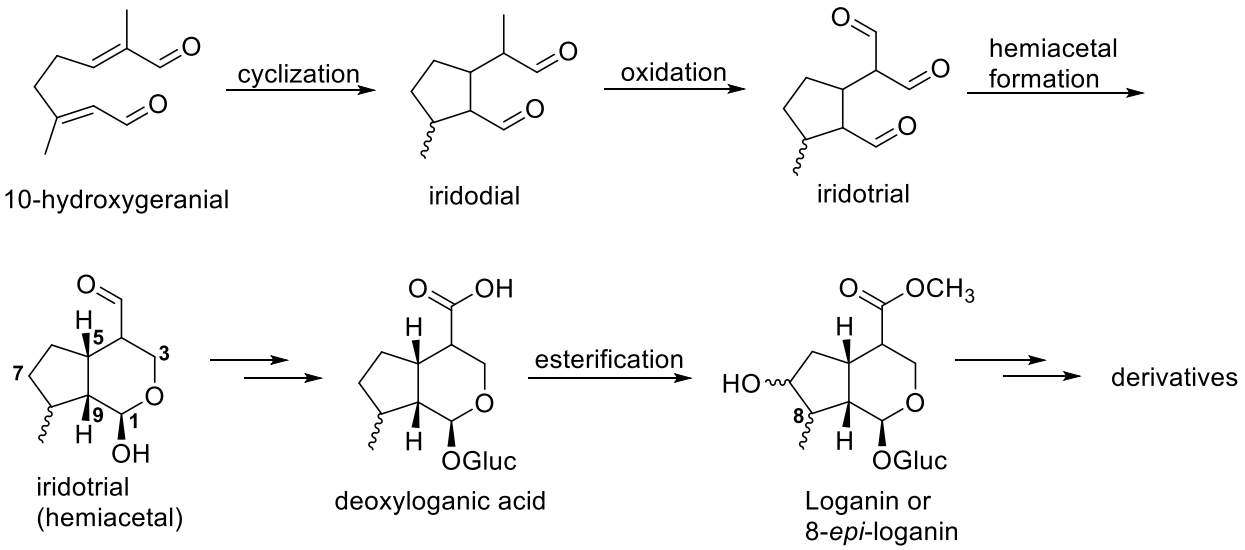
The head-to-tail addition of dimethylallyl pyrophosphate to isopentenyl pyrophosphate forms geranyl pyrophosphate, which is catalysed by an enzyme called prenyl transferase. This reaction involves the ionization of DMAPP to the matching allylic cation, which causes improvement of the electrophilicity of the substrate and aids in the alkylation of IPP. The formation of a tertiary carbocation intermediate is accomplished by electrophilic addition to the double bond of IPP. Loss of the C-2 proton generates the monoterpenoid geranyl pyrophosphate. The reaction is stereospecific and the *E*-geometrical isomer is formed

exclusively. Geraniol synthase followed by hydroxylase and then oxidoreductase yields 10-hydroxygeranial, which cyclizes to the 1-hydroxy iridoid structure.



Scheme 3.2: ***Biosynthetic formation of a glucosylated iridoid***

Carboxylated (C-11) glycoside iridoids are formed from the iridodial 10-hydroxygeranial to yield loganin or 8-*epi*-loganin (Scheme 3.3).[159]



Scheme 3.3: ***Biosynthesis of 11-carboxylated glucosylated iridoids***

10-hydroxygeranial is cyclized to an iridodial, followed by oxidation to form an iridotrial that exists in a keto and hemiacetal form.[155] Hemiacetal formation of the iridotrial (keto form) leads to cyclization, thus forming the heterocyclic ring of the iridoid skeleton. Oxidation of the

remaining aldehyde functional group yields deoxyloganic acid. Most iridoids are glycosides (almost exclusively glucose) and glycosylation transforms the hemiacetal into an acetal. Methylation of the carboxylic acid results in esterification and in the formation of deoxyloganin. Hydroxylation at C-7 of deoxyloganin yields loganin (Scheme 3.3).[160]

3.5 Biological screening

Both the DCM and MeOH extracts were subjected to biological tests in order to evaluate their biological activities. The tests were performed according to strict protocols (see Experimental part) so that results from different extracts, fractions and compounds were clear and comparable.

The tests used in this study were as follows:
- Radical scavenging test with DPPH
- Acetylcholinesterase inhibition test
- GABAergic activity

In the radical scavenging test with DPPH, the methanol extract of *A. elongatum* showed activity, and gave the most intensive active zones on TLC. In the acetylcholinesterase inhibition test, the dichloromethane extract of *A. elongatum* showed activity, and gave the most intensive active zones on TLC. In the GABAergic activity test, the dichloromethane extract of *A. elongatum* showed activity.

3.5.1 Radical scavenging assay

Qualitative TLC assay

Radical scavenging activity was measured in-house as TLC bioassays, in which a known amount of crude extract of *A. elongatum* and fractions were deposited on a thin-layer chromatography (TLC) plate and developed in a suitable solvent system prior to the respective assays, and after drying of the TCL it was sprayed with a solution of 2,2-diphenyl-1-picrylhydrazyl (DPPH), which is a violet coloured radical. This allowed separation of the compounds in the extract or fraction, leading to easy localization of active zones and tracing of

active compounds in a complex matrix. The method can thus be employed for the target-directed isolation of these constituents. The number of active zones, seen as white spots on TLC, together with the intensities of the active zones gave a measure of how active the extract was. In the radical scavenging test with DPPH, the methanol extract of *A. elongatum* was the most active, and gave the most intensive active zones on TLC, whereas the DCM extract showed no activity. Figure 3.28 shows the methanol crude extract of *A. elongatum* with the reference.

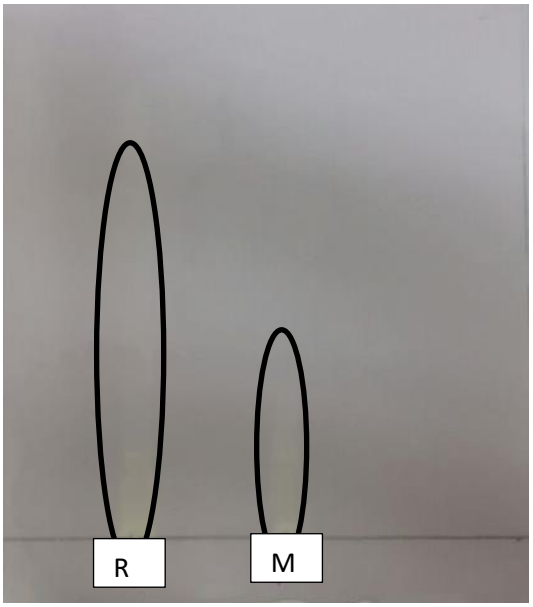


Figure 3.28*: MeOH extract and reference*

From the MeOH crude extract of *A. elongatum*, a gradient column was performed and nine fractions were afforded. Fraction 8 and 9 showed activity (Figure 3.29). Fraction 8 was further isolated into fractions namely F8t1-8, F8t9, F8t10-16, F8t17-40 from fraction 8. The fractions were tested for antioxidant activity and all four new fractions from fraction 8 gave intensive active zones on TLC showing high activity. Figure 3.29 shows the antioxidant activity of the fractions.

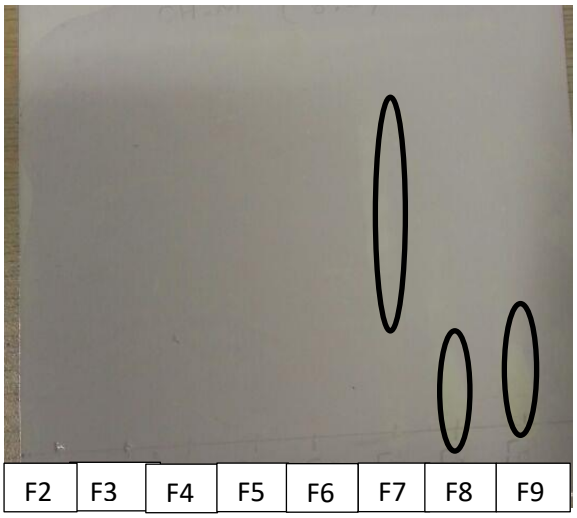


Figure 3.29*: All fractions from MeOH crude extract.*

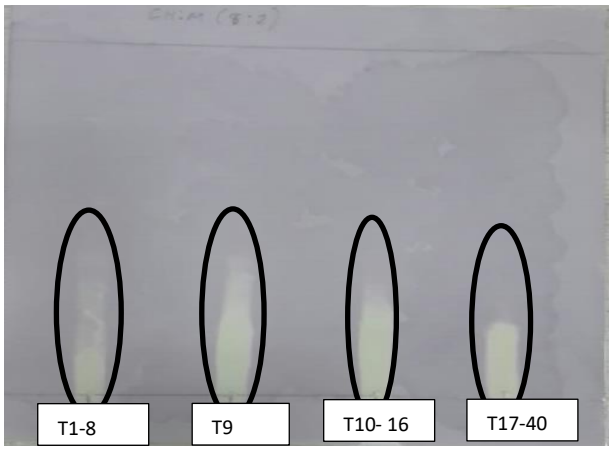


Figure 3.30: *The four fractions from fraction 8 (MeOH extract).*

Compound **5**, isolated from the DCM extract of *A. elongatum*, displayed no activity, as can be expected since the DCM crude extract was also inactive. However, compounds **3** and **4**, isolated from the MeOH extract, showed significant anti-oxidant acitivity, while compound **2** and **1**, from the MeOH extract, showed no activity. Figure 3.31 shows the anti-oxidant results of compounds **1 – 4**.

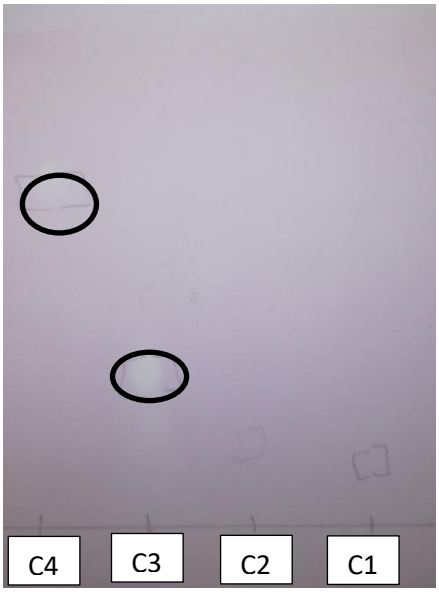


Figure 3.31: ***Anti-oxidant results of compounds 1 – 4.***

3.5.2 Acetylcholinesterase inhibition test

Acetylcholinesterase inhibition activity was measured in-house as TLC bioassays, in which a known amount of crude extract of *A. elongatum* and fractions were deposited on a TLC plate and developed in a suitable solvent system prior to the respective assays, again allowing separation of the compounds in the extract or fraction, leading to easy localization of active zones and tracing of active compounds. The bioautographic assay method relies on cleavage by acetylcholinesterase of 1-naphthyl acetate to form 1-naphthol, which then reacts with Fast Blue salt B to give a purple coloured diazonium dye, which turn to white after reaction with a AChE inhibitor. In the acetylcholinesterase inhibition test, the DCM extract of *A. elongatum* was the most active, and gave the most intensive active zones on TLC, whereas the MeOH extract showed no activity. Figure 3.32 shows the MeOH crude extract and DCM crude extract of *A. elongatum* with the reference compound.

Figure 3.32: ***Results of the AChE inhibition test of the crude extracts***

Compounds **4** and **5**, were isolated from the DCM extract. Only compound **4** showed significant AChE inhibition activity.

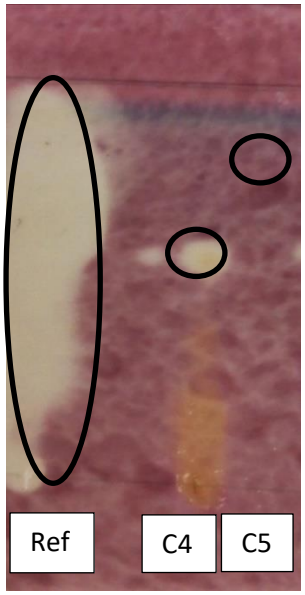


Figure 3. 33*: AChE inhibition by compounds isolated from the DCM extract*

3.5.3 GABAergic activity

As stated in Chapter 2.7.3.2 the $GABA_A$ receptor is a chloride-conducting channel and thus chloride-currents are measured. Compounds which enhance activity of the channel, becomes evident by enlarged chloride-currents. The results are reported as a percentage of potentiation of GABA-induced currents (I_{GABA} potential). In general, a ±30% I_{GABA} potentiation enhancement was denoted as an internal limit. Fractions with negative values decrease the GABA-induced chloride currents. Those fractions might contain inhibitors, which are relevant from the toxicological point of view, as they might induce convulsions or epilepsy. Fractions with values of +30% or more, act as activators and thus may exert a calming influence, indicating possible positive effects in disorders like anxiety, sleeping disorders and epilepsy. The results are summarized in Table 3.6.

Table 3.6: ***I_{GABAA} potentiation of the chloride channel by fractions from the MeOH and DCM extracts of A. elongatum. (MF = MeOH fraction and DF = DCM fraction).***

Code	I_{GABA} pot.; %	SEM
MF81	-0,7	2,4
MF82	-13,8	2,5
MF83	-10,5	4,3
DF8b	30,6	0,1
DF8c	60,5	23,3

3.6 Conclusion

Five compounds were isolated from the DCM and MeOH crude extracts via various chromatography techniques and were identified as glucosylated iridoids, a class of monoterpenoids. Several spectroscopic and spectrometry techniques were used for structure elucidation. The carbon-hydrogen backbone and connectivities and relative configuration were determined via ^{1}H and ^{13}C 1D NMR, 2D COSY, HSQC, HMBC, and NOESY NMR experiments. IR data confirmed the functional groups present in each compound, and high resolution electrospray ionization mass spectrometry confirmed the formula of each compound. HPLC data demonstrated the purity of the isolated terpenoids. The DCM extract

yielded two novel iridoid glycosides, characterized by the presence of one foliamenthoyl group at *O*-C-7 for Compound **4** and two foliamenthoyl groups at *O*-C-7 and *O*-C-6′, respectively, for Compound **5**. The MeOH extract afforded three known iridoid glycosides, angeloside, geniposidic acid and caryoptoside.

In the radical scavenging test with DPPH, the methanol extract of *A. elongatum* showed good anti-oxidant activity, and gave the most intensive active zones on TLC. Compounds isolated from the methanol extract that showed antioxidant activity were **3** and **4**, while the DCM extract exhibited activity in the acetylcholinesterase inhibition test, and gave the most intensive active zones on TLC. GABAergic activity showed moderate activity from the dichloromethane extract of *A. elongatum.*

Glucosylated iridoids have been isolated from a number of plants belonging to the Scrophulariaceae, and even from the Aptosimum genus, but this is the first report on a phytochemical study of *Aptosimum elongatum*.

Chapter 4: Experimental

4.1 Introduction

In phytochemistry, consecutive extraction, isolation and structure elucidation of chemical structures of compounds are vital objectives. Chromatographic methods such as TLC and open column chromatography assist with the isolation of pure compounds and physicochemical techniques such as NMR, MS, IR and HPLC for identifying and determining the chemical structures of isolated compounds. The five pure isolated compounds in this study were fully characterized by various spectroscopic techniques and the procedures followed using these techniques are discussed in this section. These compounds play a key role in this study and are discussed in **Chapter 3**.

4.2 Plant collection

The *A. elongatum* plant was collected in March 2016. The plant was collected and identified by Dr. Pieter Zietsman, National Museum, Bloemfontein, South Africa. Voucher specimens are deposited in the National Museum, Bloemfontein, South Africa. The collection date, location and voucher specimen number (Col. nr.) are shown in Table 4.1. The delivery date is the date the sample was received in the Chemistry Department, UFS

Table 4.1: ***Plant collection***

Abbreviation	Voucher nr.	Plant name	Plant parts	Dry mass	Location	Delivery date
A. elongatum	6440	*Aptosimum elongatum*	Aerial parts	137.64 g	Bethulie	08/04/2016

4.3 Phytochemical screening

Phytochemical examinations were carried out for all the extracts as per the standard methods.

4.3.1 Detection of carbohydrates

The DCM and MeOH extracts (3 mL) were dissolved individually in distilled water (5 mL) and filtered. The filtrates were used to test for the presence of carbohydrates

Benedict's Test:

Benedict's reagent: Sodium citrate (17 g) and anhydrous sodium carbonate (10 g) were dissolved in slightly warm distilled water (80 mL). Copper(II)sulphate (17 g) was dissolved in distilled water (10 mL), separately. The copper sulphate solution (10 mL) was slowly added to the sodium carbonate-citrate solution (80 mL) with constant stirring. The final volume was made up with distilled water (100 mL).

Each of the DCM and MeOH filtrates (2 mL) were treated with Benedict's reagent (1 mL), and heated gently. An orange red precipitate indicated the presence of reducing sugars in both extracts.

Fehling's Test:

Fehling's reagent A: Copper(II)sulfate (7 g) and concentrated sulfuric acid (0.1 mL) were dissolved in water (20 mL), and the reagent subsequently made up to 100 mL with distilled water. **Fehling's reagent B**: Sodium potassium tartrate (35 g) and sodium hydroxide (15 g) were dissolved in water (20 mL), and the volume made up to 100 mL with distilled water.

Each of the DCM and MeOH filtrates (2 mL) were hydrolyzed with dil. HCl, neutralized with an alkali and heated with equal amounts of Fehling's A & B solution (1 mL). Formation of a red precipitate indicates the presence of reducing sugars.

4.3.2 Detection of saponins

Froth Test: The DCM and MeOH extracts (2 mg) were diluted with distilled water to 20 mL, and shaken in a graduated cylinder for 15 minutes. Formation of a layer of foam (1 cm) indicated the presence of saponins.

Foam Test: The DCM and MeOH extracts (5 mg) were shaken with 2 mL of water. Foam formed during agitation persisted for ten minutes indicating the presence of saponins.

4.3.3 Detection of terpenoids

Salkowski's Test: Portions of the DCM and MeOH extracts (2 mg each) were treated with chloroform (5 mL) and filtered. The filtrates (2 mL) were treated with a few drops of concentrated sulphuric acid, shaken and allowed to stand. The appearance of a golden yellow colour indicated the presence of triterpenes.

4.3.4 Detection of phenols

Ferric Chloride Test: Portions of the DCM and MeOH extracts (0.5 mg) were treated with 3-4 drops of a dilute ferric chloride solution. Formation of blueish-black colour indicated the presence of phenols.

4.3.5 Detection of flavonoids

Alkaline Reagent Test: Portions of the DCM and MeOH extracts (0.5 mg) were treated with a few drops of a diluted sodium hydroxide solution. Formation of an intense yellow colour, which became colourless after addition of dilute acid, indicated the presence of flavonoids.

4.4 Extraction of *A. elongatum*

Powdered aerial plant material of *A. elongtum* (137.6 g) was extracted consecutively with DCM (500 mL, overnight, x 3) and MeOH (500 mL, overnight, x 3), with stirring. The respective extracts was filtered and concentrated under vacuum. This afforded the DCM extract (3.6 g)

and the MeOH extract (17.7 g), respectively. Both the DCM and MeOH extracts were selected for further studies. The yield obtained from the extracts is given in Table 4.2:

Table 4.2*: Yield of A. elongatum plant extracts*

	Aerial plant material (g)
Mass of dry plant material	137.6
Mass of dry extract	21.4
% yield	15.53%

4.5 Removal of tannins

To remove the tannins the dried MeOH extract (21.4g) was dissolved in MeOH (100 mL). The extract was loaded onto a Solid-Phase Extraction (SPE, Phenomenex) and polyamide gel (CC-6; 50 g) column. The solid phase gel column was washed with MeOH (2 x 250 mL). The volume of the combined MeOH fractions was evaporated under reduced pressure to yield the tannin free extract (17.7 g).

4.6 Chromatographic methods

4.6.1 Thin-layer chromatography (TLC)

TLC was carried out on silica gel 60 F254 precoated aluminium sheets (Merck). The solvent systems used were Hx-EtOAc, MeOH-EtOAc, and $CHCl_3$-MeOH in different ratios. After development, TLC plates were first observed under UV light (254 nm and 366 nm) for locating chromophoric compounds. Further detection was achieved by spraying with a *p*-anisaldehyde/CH_3COOH/conc. H_2SO_4 (v/v/v, 0.5/50/1 mL) solution. The sprayed plates were heated to about 100 ℃, and spots of different colours developed according to the nature of the compounds.

4.6.2 Open gradient column chromatography on silica gel

Open gradient column chromatography was employed for the first stage of fractionation. Silica gel 60 (0.040-0.063 mm Merck) was used. The eluent systems used was from non-polar to polar with each fraction (Table 4.3).

Table 4.3: ***Non-polar to polar eluent system applied for each fraction***

Fraction number	Eluent system	Ratio
1	Hx	10
2	Hx: EtOAc	8:2
3	Hx: EtOAc	6:4
4	Hx: EtOAc	4:6
5	Hx: EtOAc	2:8
6	Hx: EtOAc	1:9
7	EtOAc	10
8	MeOH-EtOAc	2:8
9	MeOH-EtOAc	3:7

4.6.3 Open column chromatography

Open column chromatography was performed using Merck Silica gel 600 (0.040-0.063 mm) at the final stages of purification. TLC analysis was used to determine the eluent systems. Different column sizes and flow rates were used and fractions were collected in test tubes.

4.6.4 Preparative Thin-Layer Chromatography

Preparative thin-layer chromatography was carried out on 20 x 20 cm glass-backed, 0.25 mm silica plates for the isolation of the compounds. TLC analysis was used to determine the eluent systems. The UV- active compounds were scraped off and washed with methanol and acetone by filtration.

4.7 Isolation strategy

In this work, gradient elution in column chromatography was chosen as the major separation technique. This technique is based on the significant difference in polarity of two or more solvents systems, for instance polar and non-polar solvents are employed. During the separation, the ratio of polar to non-polar solvents is varied, either continuously or in a series of steps. Separation efficiency is greatly enhanced by gradient elution.

4.8 Fractionation of the MeOH extract and DCM extract

The MeOH and DCM extracts of the whole parts of *A. elongatum* were fractionated via open gradient column chromatography. The solvent system used was Hx/EtOAc/MeOH for both extracts. Each extract was eluted in nine fractions (Tables 4.4 and 4.5).

Table 4.4: *DCM extract fractionated*

Fraction number	Eluent system	Ratio	Mass (g)
1	Hx	10	0.1326
2	Hx: EtOAc	8:2	0.1893
3	Hx: EtOAc	6:4	0.1811
4	Hx: EtOAc	4:6	0.1888
5	Hx: EtOAc	2:8	0.1458
6	EtOAc	10	0.5781
7	MeOH:EtOAc	1:9	2.9942
8	MeOH:EtOAc	2:8	5.1299
9	MeOH:EtOAc	3:7	1.5088

Table 4.5: *MeOH extract fractionated*

Fraction number	Eluent system	Ratio	Mass (g)
1	Hx	10	0.0973
2	Hx: EtOAc	8:2	0.0701
3	Hx: EtOAc	6:4	0.2840
4	Hx: EtOAc	4:6	0.5984

5	Hx: EtOAc	2:8	0.0891
6	EtOAc	10	0.0904
7	MeOH:EtOAc	1:9	0.1369
8	MeOH:EtOAc	2:8	0.9569
9	MeOH:EtOAc	3:7	0.5088

From the MeOH extract, Fractions 7 and 8 were further purified using EtOAc/MeOH as solvent system in different ratios. Four sub-fractions were collected from fraction 7 (Table 4.6) and four sub-fractions from Fraction 8 (Table 4.7).

Table 4.6: ***Fraction 7 of MeOH extract***

Test number	**Mass (g)**
T1-24	0.1302
T25-28	0.2217
T29-32	0.3643
T36-40	0.7788

Table 4.7*: Fraction 8 of MeOH extract*

Test number	**Mass (g)**
T1-8	0.5127
T9	0.2261
T10-16	0.6194
T17-40	1.7798

4.9 Isolation of pure compounds

An isolation scheme for *A. elongatum* is presented below (Figure 4.1 and 4.2) to show the five different compounds collected from the whole dry plant material of *A. elongatum*. The different compounds are indicated by different colours due to the fact that some were collected from more than one fraction. Compounds **1** (tan), compound **2** (purple), and compound **3** (royal blue) were isolated from the MeOH crude. Compound **4** (yellow) was isolated from both the DCM and MeOH crudes, while compound **5** (green) were isolated from the DCM crude.

Table 4.8*: indicates the mass of each compound*

Compound	Name of the compound	colour	Mass(g)
1	Angeloside	tan	0.0069
2	Geniposidic acid	purple	0.0061
3	Caryoptoside	blue	0.0918
4	7-*O*-foliamenthoate caryoptoside	yellow	0.9111
5	6″,7-*O*-difoliamenthoate caryoptoside	green	0.0263

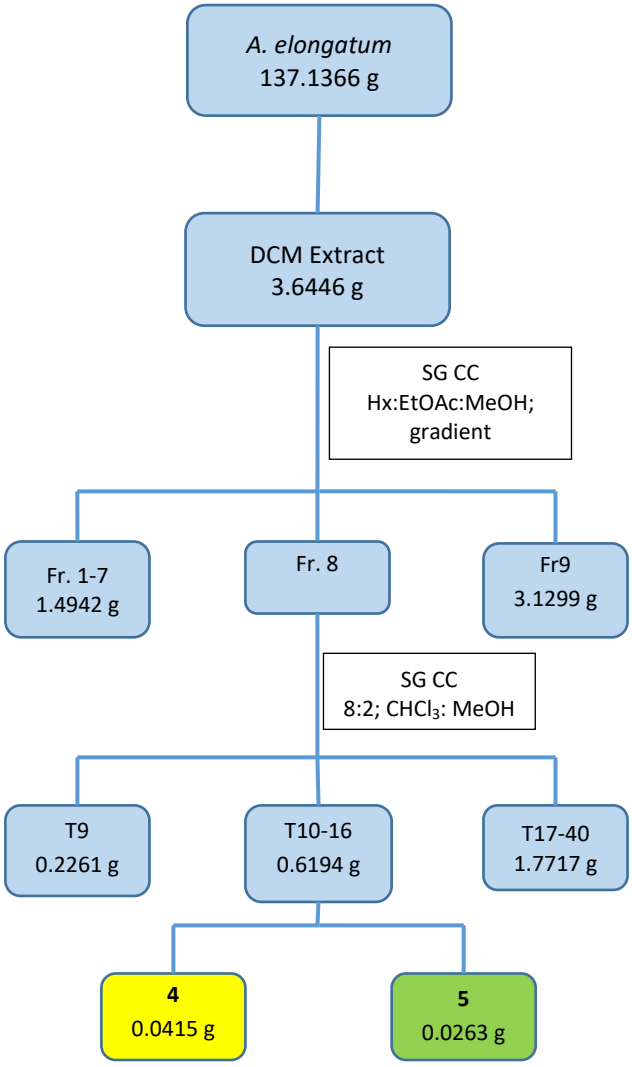


Figure 4.1: ***Schematic representation of the isolation of compounds 4 and 5 from the DCM extract of A. elongatum***

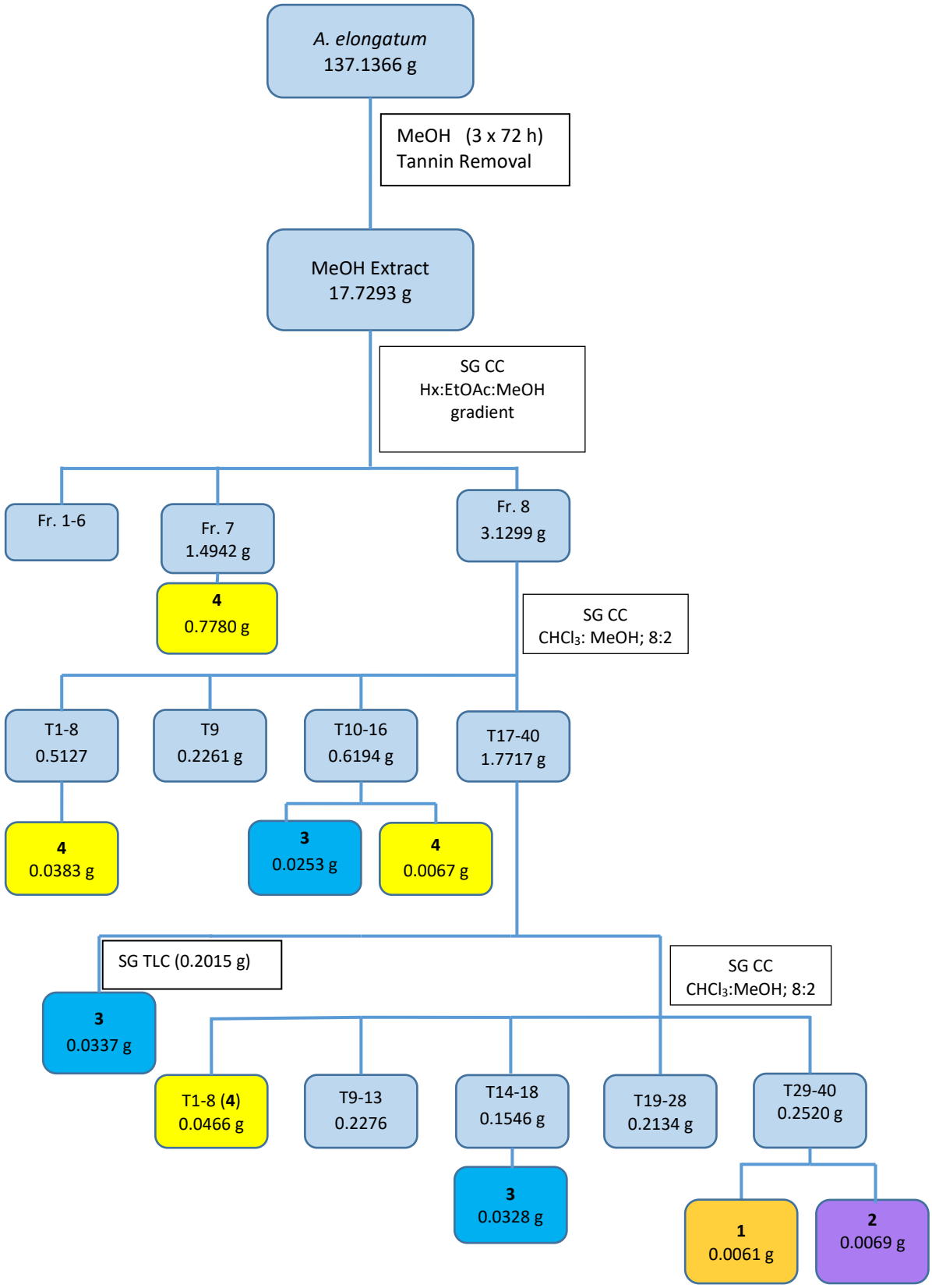


Figure 4.2: ***Schematic representation of the isolation of compounds 1, 2, 3 and 4 from the MeOH extract of A. elongatum***

4.9.1 Physical data for (2*S*,3*R*,4*S*,5*S*,6*R*)-2-(((1*S*,4a*R*,5*S*,6*R*,7*S*,7a*R*)-5,6-dihydroxy-7-methyl-1,4a,5,6,7,7a-hexahydrocyclopenta[c]pyran-1-yl)oxy)-6-(hydroxy-methyl)tetrahydro-2*H*-pyran-3,4,5-triol (1) (Angeloside)

Description	Transparent yellow oil
Empirical Formula	$C_{15}H_{24}O_9$
Observed Molecular weight	349.1844
Theoretical Molecular weight	349.1499
1H NMR (MeOH)	Plate 1, Table 3.2
13C NMR	Plate 2, Table 3.2
13C APT	Plate 3
COSY	Plate 4
HSQC	Plate 5
HMBC	Plate 6
NOESY	Plate 7

4.9.2 Physical data for (1*S*)-7-(hydroxymethyl)-1-(((2*S*,3*R*,4*S*,5*S*,6*R*)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2*H*-pyran-2-yl)oxy)-1,4a,5,7a-tetrahydrocyclo-penta-[c]pyran-4-carboxylic acid (2) (Geniposidic acid)

Description	Transparent yellow oil
Empirical Formula	$C_{16}H_{22}O_{10}$
Observed Molecular weight	397.1107 (Na-adduct)
Theoretical Molecular weight	397.1111 (Na-adduct)
1H NMR (MeOH)	Plate 8, Table 3.3
13C NMR	Plate 9, Table 3.3
13C APT	Plate 10
COSY	Plate 11
HSQC	Plate 12
HMBC	Plate 13

4.9.3 Physical data for methyl (1*S*,6*S*,7*R*)-6,7-dihydroxy-7-methyl-1-(((2*S*,3*R*,4*S*,5*S*,6*R*)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2*H*-pyran-2-yl)oxy)-1,4a,5,6,7,7a-hexahydrocyclopenta[c]pyran-4-carboxylate (3) (Caryoptoside)

Description	pure yellow oil
Empirical Formula	$C_{17}H_{26}O_{11}$
Observed Molecular weight	429.1370 (Na-adduct)
Theoretical Molecular weight	429.1373 (Na-adduct)
1H NMR (MeOH)	Plate 15, Table 3.4
13C NMR	Plate 16, Table 3.4
13C APT	Plate 17
COSY	Plate 18
HSQC	Plate 19
HMBC	Plate 20
NOESY	Plate 21

4.9.4 Physical data for methyl (1*S*)-7-hydroxy-6-(((2*Z*,6*Z*)-8-hydroxy-2,6-dimethylocta-2,6-dienoyl)oxy)-7-methyl-1-(((2*S*,3*R*,4*S*,5*S*,6*R*)-3,4,5-trihydroxy-6-(hydroxymethyl)-tetra-hydro-2*H*-pyran-2-yl)oxy)-1,4a,5,6,7,7a-hexahydrocyclopenta[c]pyran-4-carboxylate (4)

Description	pale yellow oil
Empirical Formula	$C_{27}H_{41}O_{13}$
Observed Molecular weight	595.2380 (Na-adduct)
Theoretical Molecular weight	595.2367 (Na-adduct)
1H NMR (MeOH)	Plate 22, Table 3.5
13C NMR	Plate 23, Table 3.5
13C APT	Plate 24
COSY	Plate 25
HSQC	Plate 26
HMBC	Plate 27
NOSEY	Plate 28

4.9.5 Physical data for methyl (1*S*)-7-hydroxy-6-(((2*Z*,6*Z*)-8-hydroxy-2,6-dimethylocta-2,6-dienoyl)oxy)-7-methyl-1-(((2*S*,3*R*,4*S*,5*S*,6*R*)-3,4,5-tri-hydroxy-6-((((2*Z*,6*Z*)-8-hydroxy-2,6-dimethylocta-2,6-dienoyl)oxy)-methyl)-tetrahydro-2*H*-pyran-2-yl)oxy)-1,4a,5,6,7,7a-hexahydrocyclopenta[c]pyran-4-carboxylate (5)

Description	pale yellow oil
Empirical Formula	$C_{37}H_{55}O_{15}$
Observed Molecular weight	761.3330 (Na-adduct)
Theoretical Molecular weight	761.3360 (Na-adduct)
1H NMR (MeOH)	Plate 29, Table 3.5
13C NMR	Plate 30, Table 3.5
DEPT	Plate 31
COSY	Plate 32
HSQC	Plate 33
HMBC	Plate 34
NOSEY	Plate 35

4.10 Physicochemical methods

4.10.1 Nuclear magnetic resonance spectra (NMR)

^{1}H and ^{13}C NMR spectra were obtained on a Bruker Avance 600 MHz spectrometer (600 MHz for ^{1}H NMR and 150 MHz for ^{13}C NMR). Different deuterated solvents were used for various samples and the operating temperature was set to 25 ^{o}C. Tetramethylsilane (TMS) was used as internal standard for ^{1}H resonances (0 ppm).

For advanced and two-dimensional spectra including ^{13}C APT/DEPT, and 2D COSY, NOESY, HSQC, HMBC, the standard pulse sequences and processing macros were employed as provided in the original software.

4.10.2 Mass spectrometry (MS)

Low-resolution mass spectrometry of the compounds was done by Dr G.Kemp of the Department of Microbial, Biochemical, and Food Biotechnology, University of the Free State.

High-resolution mass spectrometry of the compounds was done by Mr E.van Schalkwyk of the Central Analytical Facilities, Stellenbosch University.

4.10.2.1 Spectrometry

MS was performed on a MD Sciex 3200 QTrap fitted with an electrospray (Turbo-ion spray) ionization source. Data acquisition was in the positive mode and to produce mass spectra of the preparations, the first quadrupole was scanned through m/z = 100 – 675 at unit resolution. The declustering potential was set at 35 V and all other settings were adjusted for optimal sensitivity of the base peak observed in the spectra.

4.10.2.2 Analysis

Samples were introduced into the electrospray source by manually injecting each sample into a constantly flowing stream of injection solvent [acetonitrile: 0.1% formic acid (50:50) (v/v)] at a flow rate of 50 µL/min. Mass spectra were acquired by combining all spectra across the injection peak and subtracting the background.

4.10.2.3 Sample preparation

Each sample was dissolved in 1 mL acetonitrile and 50 µL of each was diluted with the addition of 950 µL injection solution. Of this dilution, 10 µL volumes were then injected for analysis as described above.

4.10.2.4 Interpretation of mass spectra

The base peak in each mass spectrum represents the intact protonated ion of the main component in each preparation (positive mode). The *m/z* value is therefore that of the ion expressed as [M+H]+. The monoisotopic mass of the uncharged molecule would thus be one mass unit higher than the *m/z* value displayed on the mass spectra.

4.10.3 High-performance liquid chromatography (HPLC)

The methanol extracts were analysed by high-performance liquid chromatography (HPLC) on a Shimadzu instrument (Tokyo, Japan; CBM 20A-communication bus module, DGU 20AS-degasser, LC 20-AD-PUMP A&B, CTO 10AS UP-column oven) with a photo diode array detector (PDA).

A reverse phase column and guard phase column (Phenomenex $C_{18,}$ 250 mm x 4.6 mm with a diameter of 5 µm) were used. Solution A was water +0.1% FA and solution B contained methanol +0.1% FA. Initially 0 – 5 min, 10-50% solution B; 5.1 – 10 min, 80-90% solution B; 10.1 – 15 min, 90% solution B; 15.1 – 17min, 10% solution B at a flow rate of 1 mL/min and

a constant temperature of 25ºC. The eluent was detected by measuring the absorbance between 200 and 400 nm.

4.10.4 Infrared spectroscopy (IR)

Solid state Fourier transform infrared spectrometry (FTIR) spectra were recorded as a neat compound on a Bruker Tensor 27 spectrometer in the range of 4000 – 400 cm^{-1}

4.11 Biological methods

4.11.1 Radical scavenging assays

4.11.1.1 Qualitative TLC assay

A TLC plate was spotted with the respective test samples, developed (EtOAc:MeOH 8:2) and left to dry. After spraying with a methanolic DPPH solution (3 mg/mL), the active zone was observed as yellow spots on a violet background.

4.11.2 Acetylcholinesterase

A solution of each sample from each crude extract and isolated compounds (10 mg/mL) in MeOH was prepared and 10 µL of these test solutions were loaded on a TLC plate (10 cm x 10 cm) and migrated (*A. elongatum*, DCM and MeOH extracts; MeOH:$CHCl_3$; 8:2). The TLC plates were dried and sprayed evenly with an AChE solution (5 mL) (from solution of 440 U ACHE in 70 mL, pH 7.8 Tris) (Sigma C3389). The plates were left for 5 min. A solution of α-naphthyl acetate (6 mg) in ethanol (2.5 mL) was added to a solution of Fast Blue Salts B (25 mg) (Sigma D9805) in H_2O (10 mL). The mixture was sprayed onto the TLC plates and after 5 min. the active extracts appeared as white spots on a purple background. Huperzine (1 mg/100mL in MeOH) was used as reference.

4.11.3 GABA screening to identify possible $GABA_A$ receptor activity

A. elongatum extracts and fractions were screened for $GABA_A$ receptor activity making use of a two-microelectrode voltage clamp assay on *X. laevis* oocytes. This assay is based on the same technique as the above described hERG screening, except for different cRNA. Synthesis of capped runoff poly (A+) cRNA transcripts was obtained from linearized cDNA templates (pCMV vector). After enzymatic isolation, the oocytes were injected with 10-50 mL of DEPC-treated water (Sigma) containing the different cRNAs of α1, β2 and γ2 subunits.

The $GABA_A$ receptor activity screening was done by the Department of Pharmacology and Toxicology, University of Vienna, Austria

4.12 Conclusion

In conclusion, a detailed procedure of the analytical methods used in natural product research was used to reach the objectives of this project and was presented in this chapter. 600 MHz NMR, IR and MS was used to elucidate the chemical structures of the isolated compounds. The results obtained from these tools are described in detail in **Chapter 3**.

Chapter 5: Evaluation and future research

5.1 Introduction

In this chapter, the results obtained during the course of this study, are evaluated compared to the objectives indicated in Chapter 1 on the basis of their scientific relevance and success. Secondly, to identify possible future research based on the results obtained in this study.

5.2 Evaluation of the study

The aim of the study was to investigate *A. elongatum*, the phytochemistry and bioactivity of its crude extracts in medicinal applications, and to isolate new bioactive compounds as potential novel drugs.

The objectives of this study as outlined at the beginning of this study were as follows:

- The consecutive extraction of the aerial parts (whole plant) of *A. elongatum* with DCM and MeOH.
- The evaluation of the phytochemical profile of the resultant DCM and MeOH crude extracts of *A. elongatum* via compound class screening.
- The isolation and structure elucidation of the chemical structures of compounds isolated from *A. elongatum*.
- The screening of the crude extracts and isolated compounds for antioxidant activity (DPPH) and inhibition of acetylcholinesterase activity.
- The screening of the crude extracts for GABAergic activity via a two micro-electrode system utilizing *Xenopus* oocytes.

All the given aims and objectives outlined in Chapter 1 were successfully reached as reflected by the results discussed in Chapter 3. MeOH and DCM extracts were obtained from the aerial parts of *A. elongatum.* Both extracts were screened for activity as radical scavengers, inhibitors of acetylcholinesterase and GABAergic activity. The crude MeOH and DCM extracts of *A.*

elongatum showed moderate antioxidant activity, but only the DCM extract exhibited moderate inhibition of acetylcholinesterase and GABAergic activity.

Five compounds have been isolated and characterized. Two of the isolated iridoid glucosides were identified as new compounds, and the remaining three were previously isolated and are known as angeloside, geniposidic acid and caryoptoside, but it is the first time these compounds are reported in this plant. All the compounds have been characterized by analytical methods used in natural product research such as NMR, IR, HPLC and MS. This, coupled with the findings from the bioassay studies on the crude extract, justify the use of *A. elongatum* in traditional medicine. Furthermore, standardization of herbal formulations from *A. elongatum* can be possible by using the characterized compounds as markers in the improved traditional medicine.

5.3 Future research

The following set of objectives can be attempted during future research:

- Further studies on the two novel compounds isolated from *A. elongatum* to understand the phytochemistry and bioactivity function.
- Isolation and identification of more compounds from the plant.
- Toxicity studies on all five characterized compounds are recommended, so as to establish their safety to human beings.
- GABAergic bioassay tests on the compounds isolated from the active DCM extract.
- Phytochemical studies on the roots of *A. elongatum* are recommended in order to establish if they share the same phytochemical and bioactivity profile as the aerial parts; this will solve the problem of harvesting the whole plant for the treatment of disease.

Chapter 6: References

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Plate 1 1H NMR (600 MHz) spectrum of Compound 1 MeOD

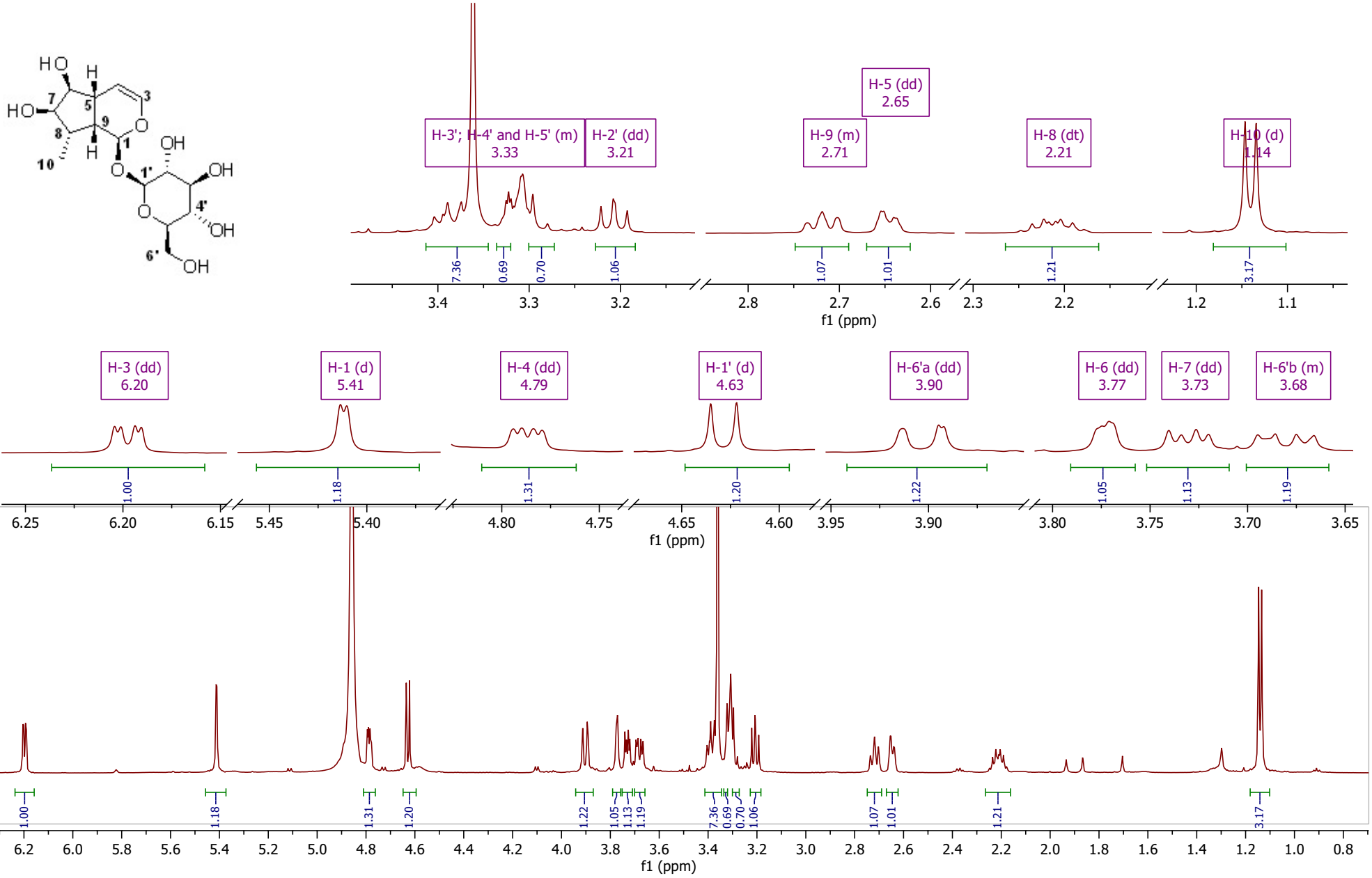


Plate 2 13C NMR (150 MHz) spectrum of Compound 1 MeOD

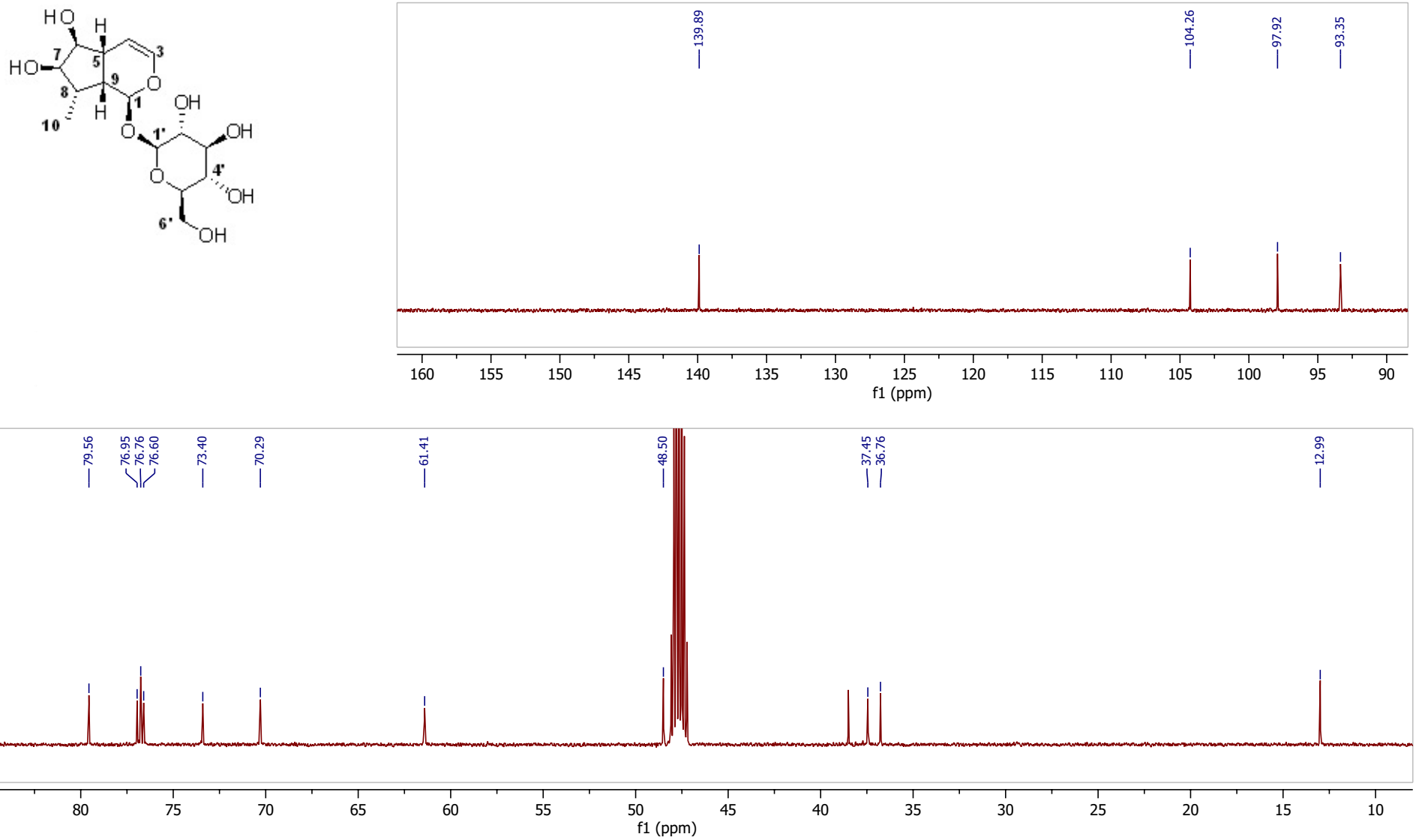
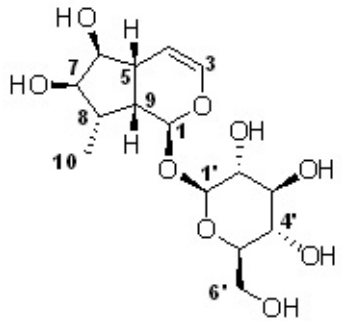
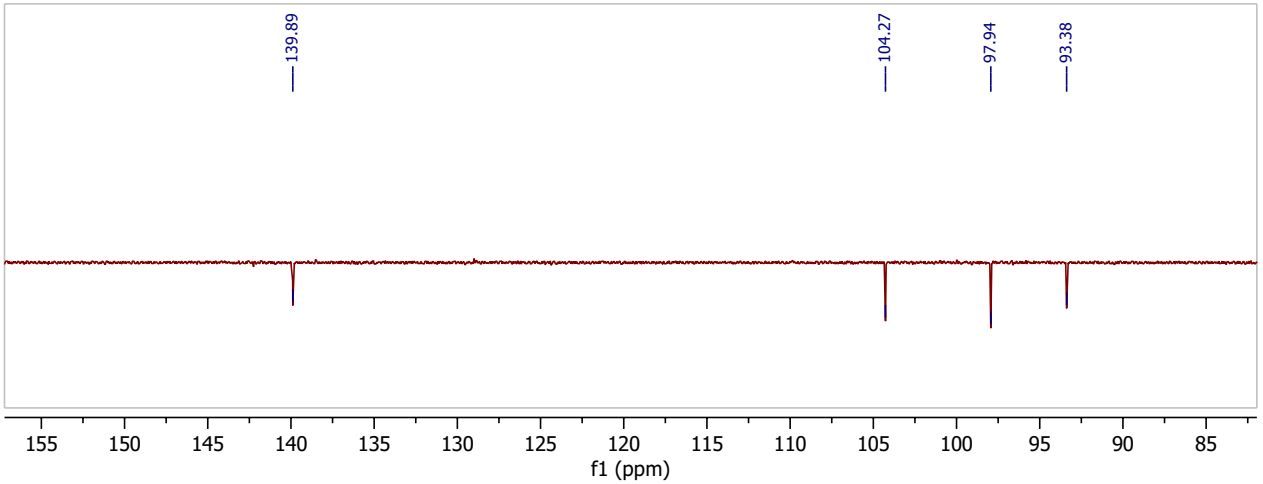


Plate 3 13C APT NMR (150 MHz) spectrum of Compound 1 MeOD





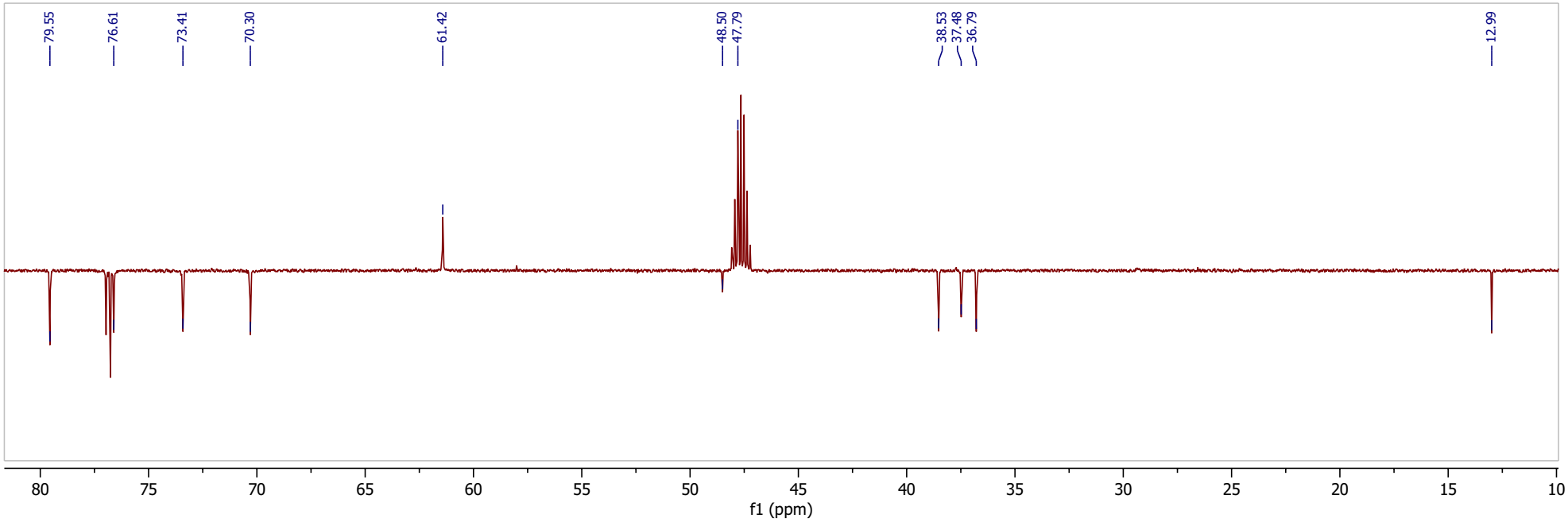


Plate 4 2D COSY NMR spectrum of Compound 1 MeOD

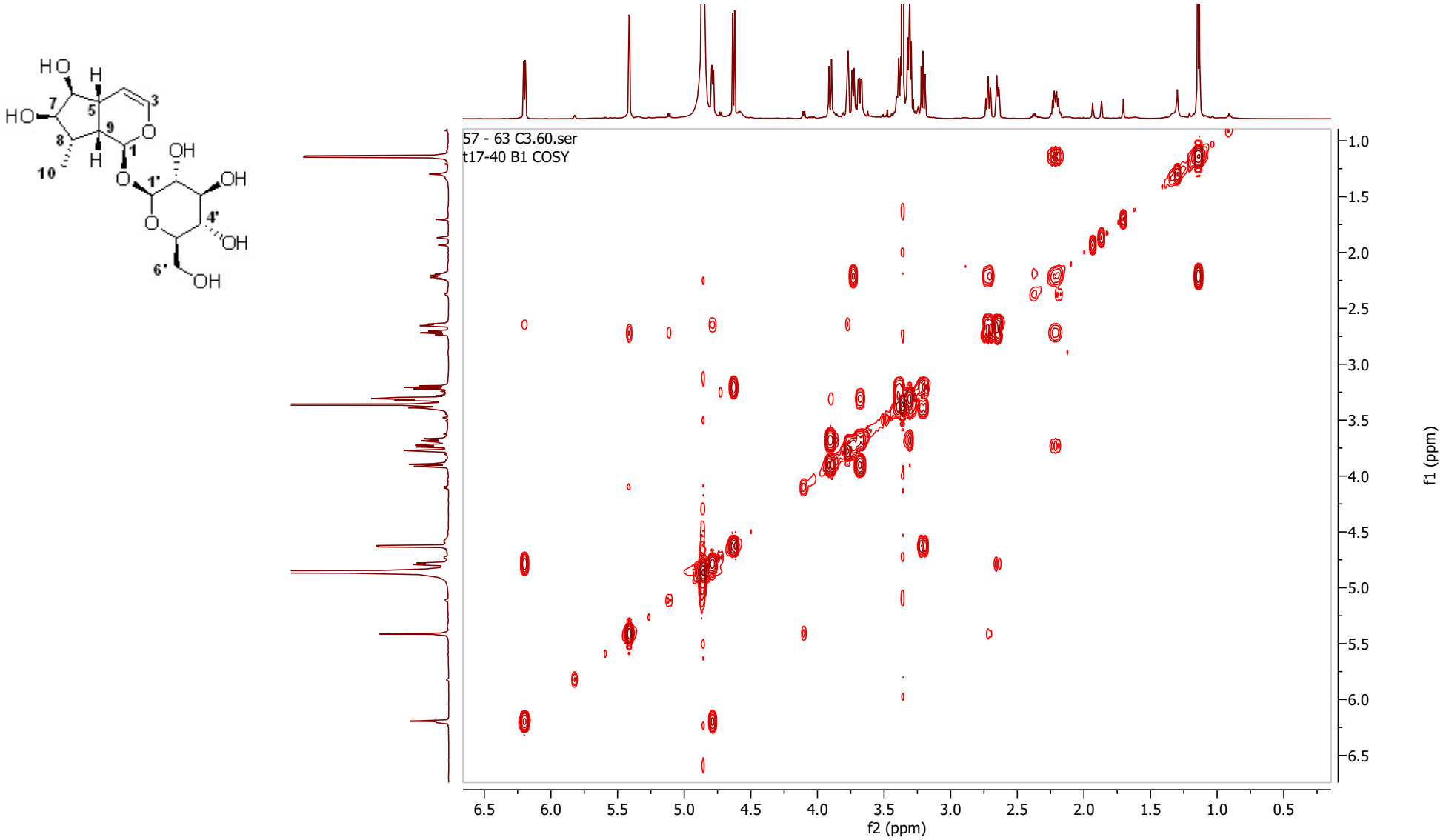


Plate 5 2D HSQC spectrum of Compound 1 MeOD

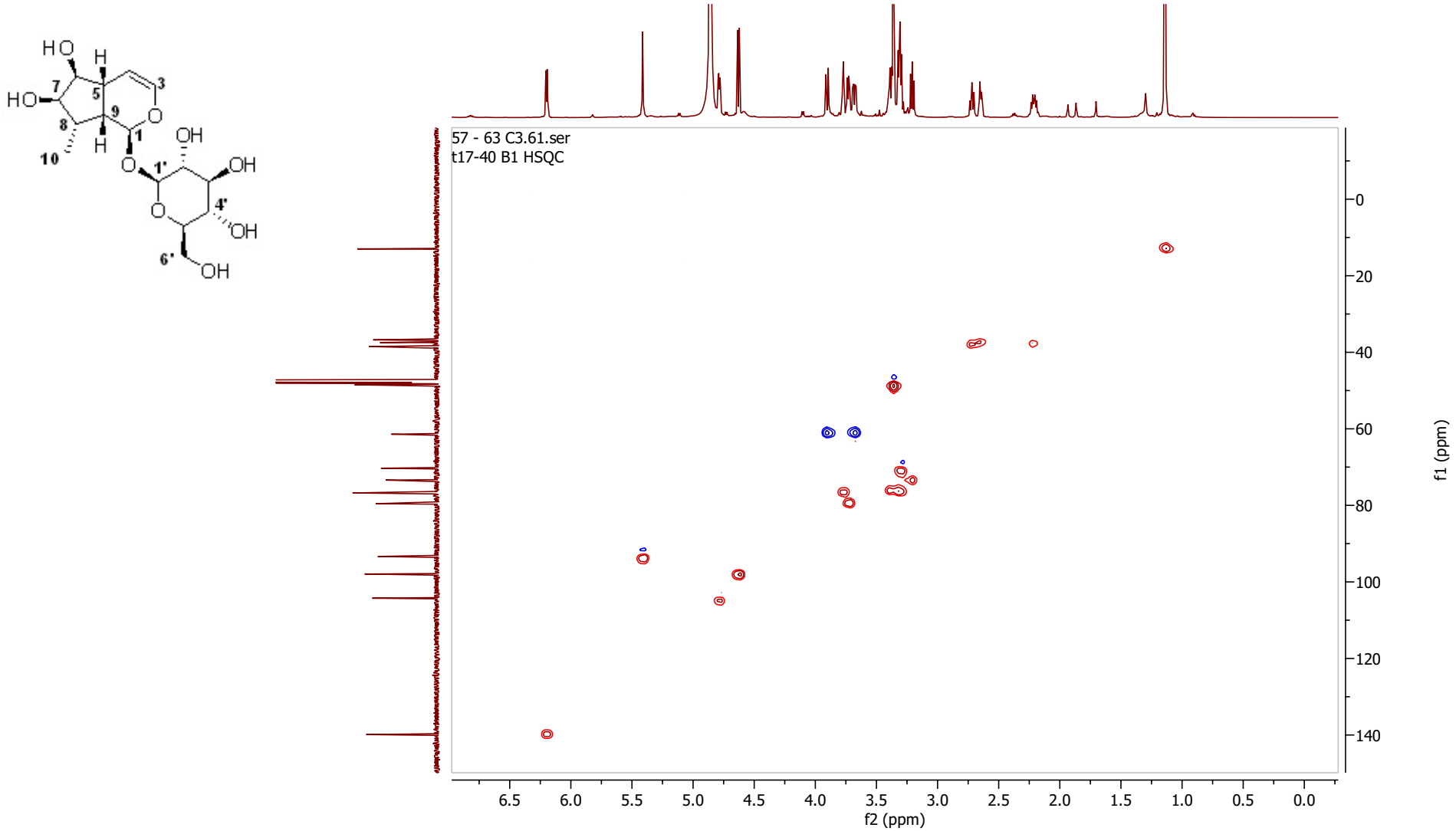


Plate 6 2D HMBC spectrum of Compound 1 MeOD

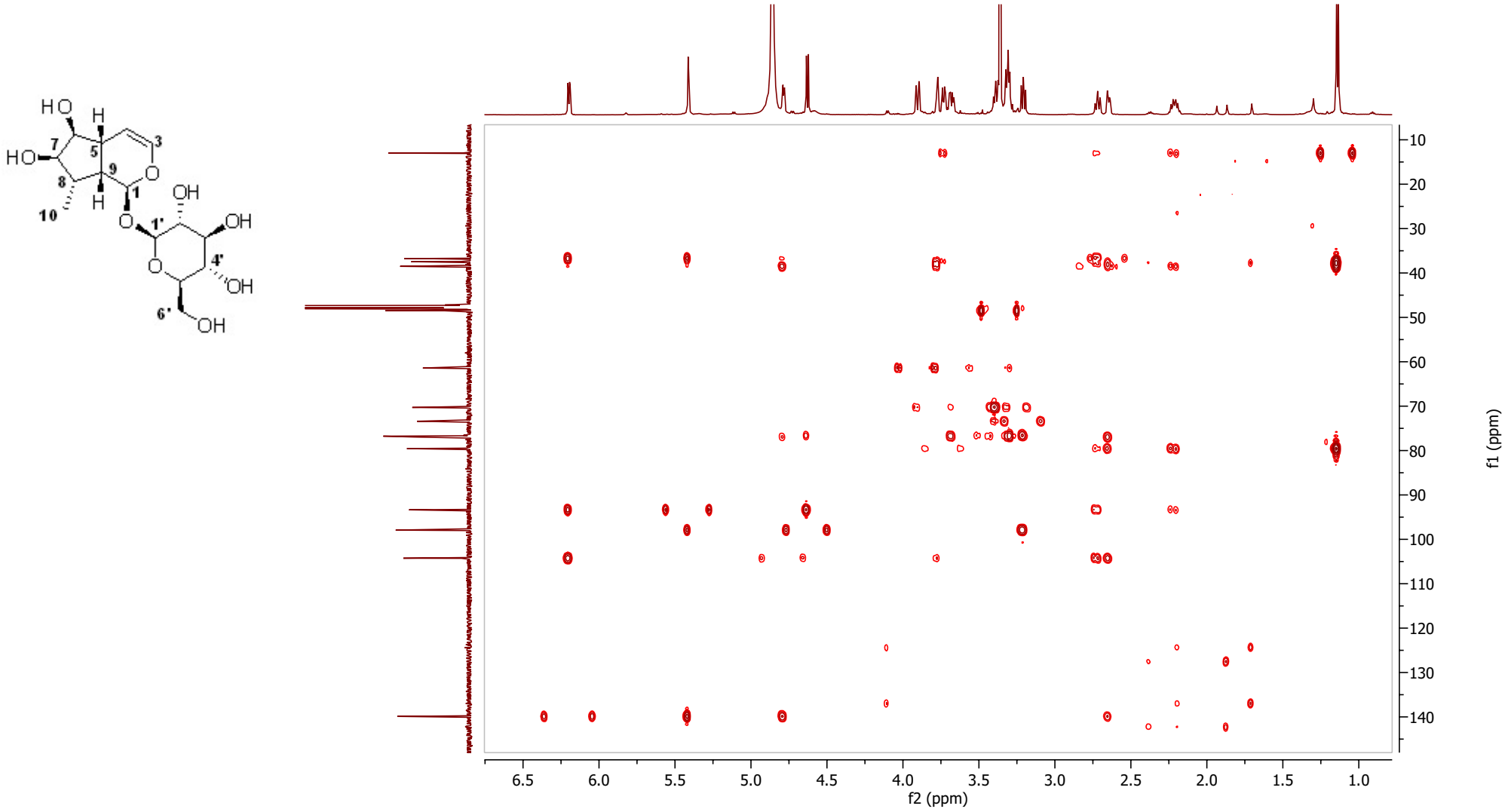
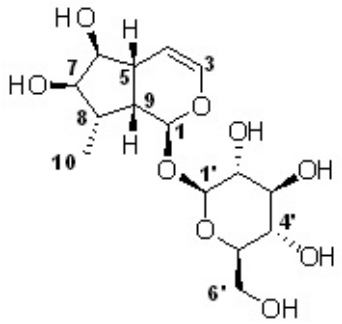


Plate 7 2D NOESY spectrum of Compound 1 MeOD



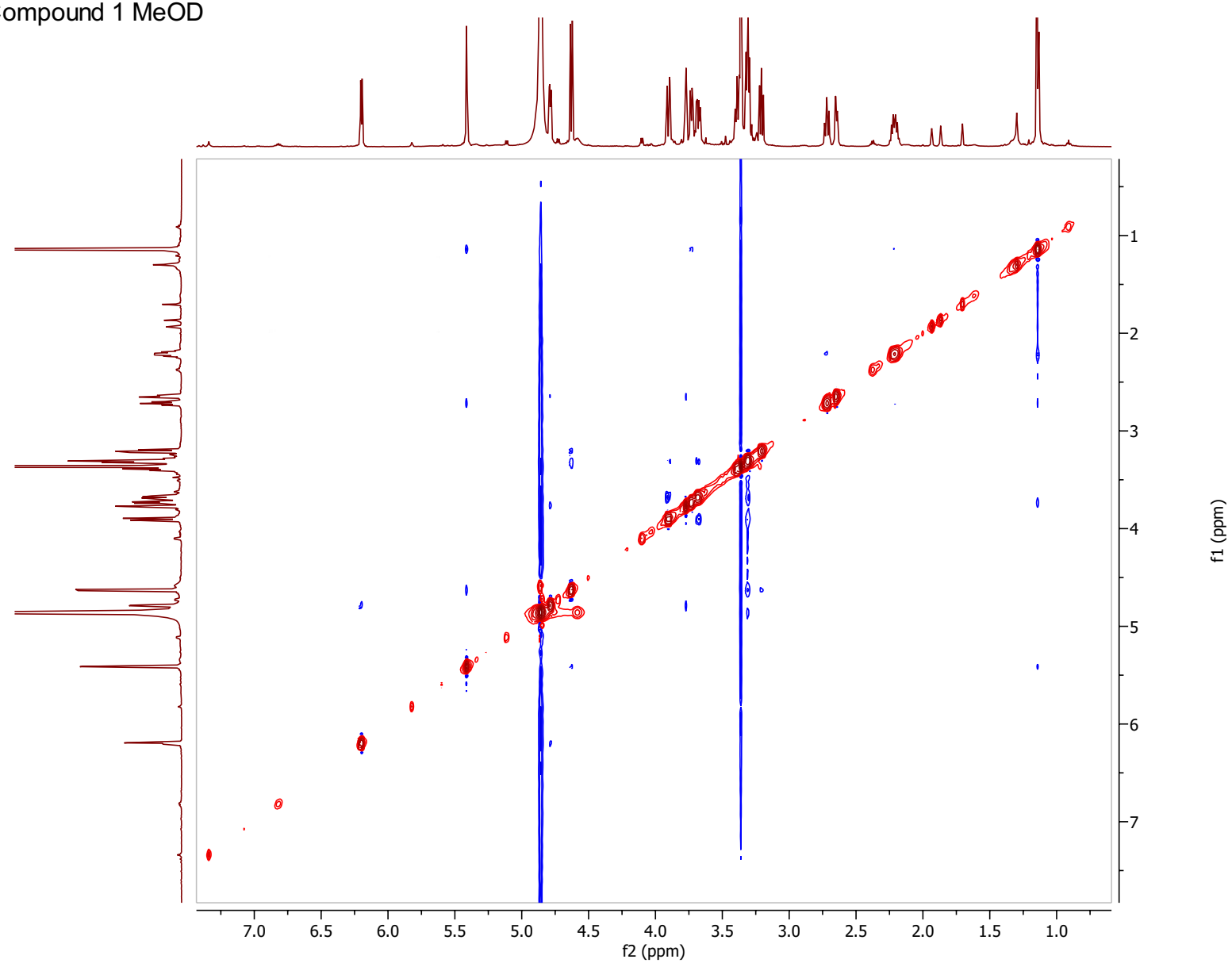
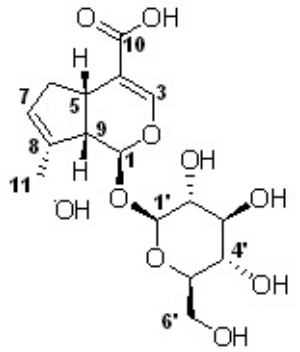
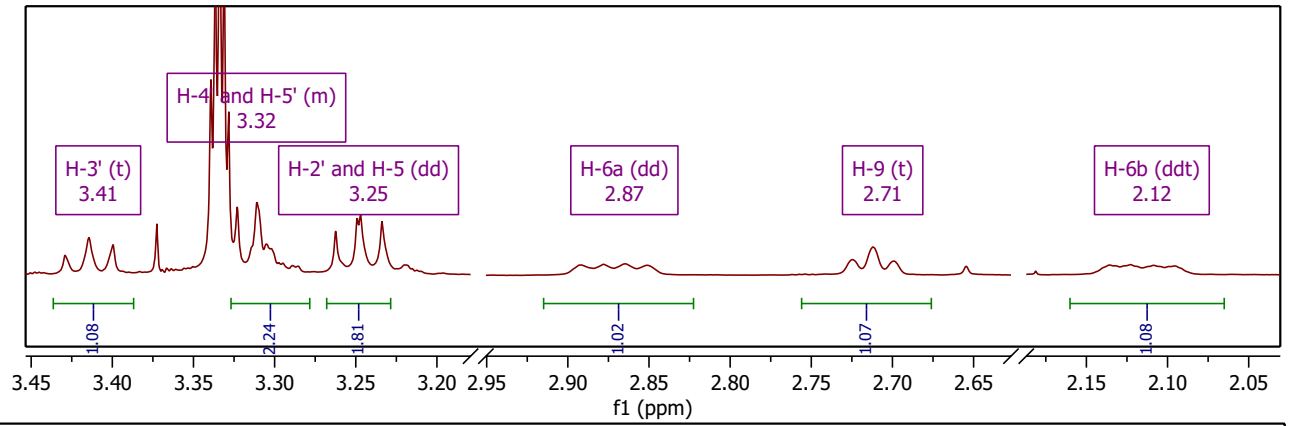
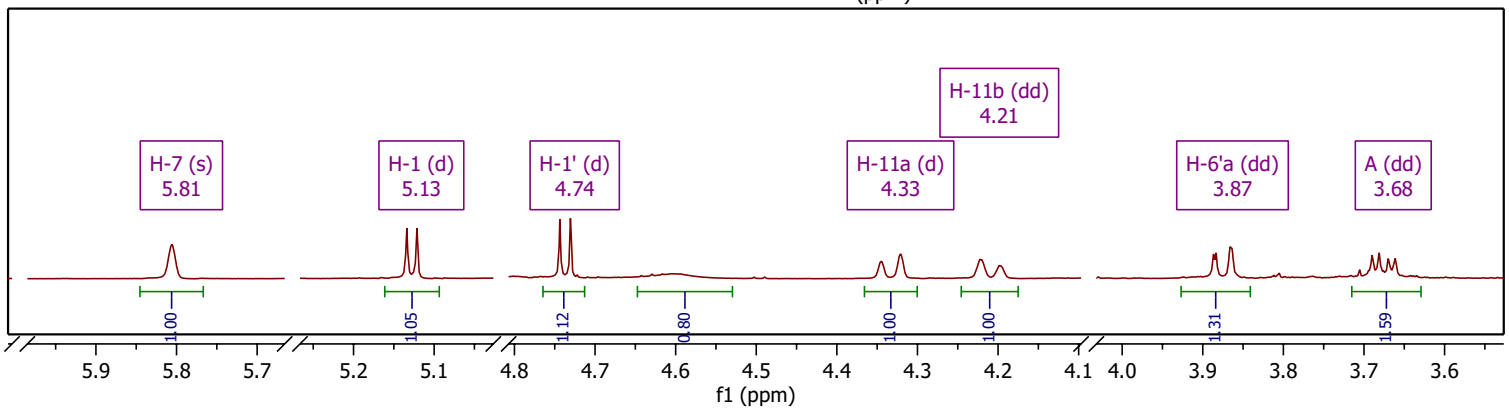


Plate 8 1H NMR (600 MHz) spectrum of Compound 2 MeOD







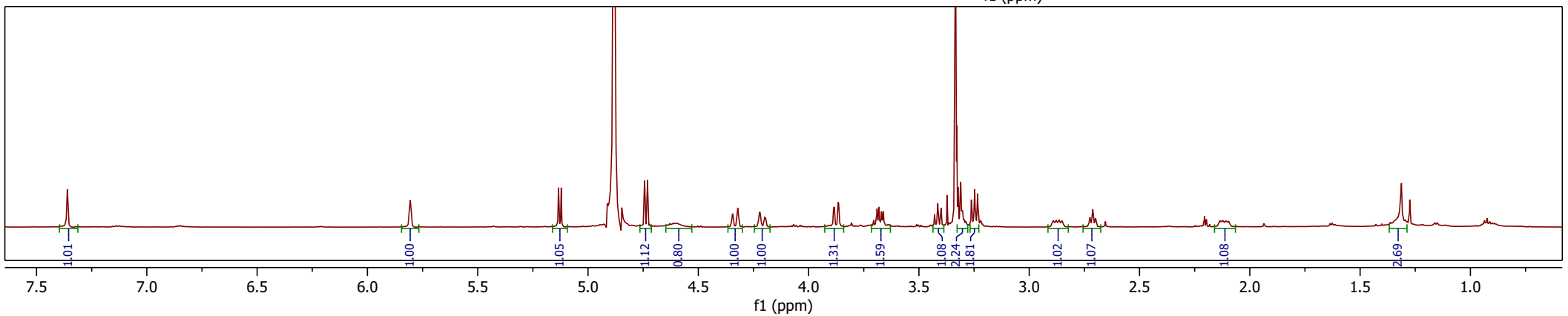
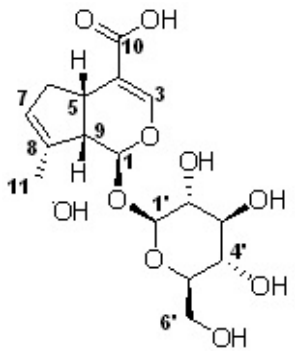
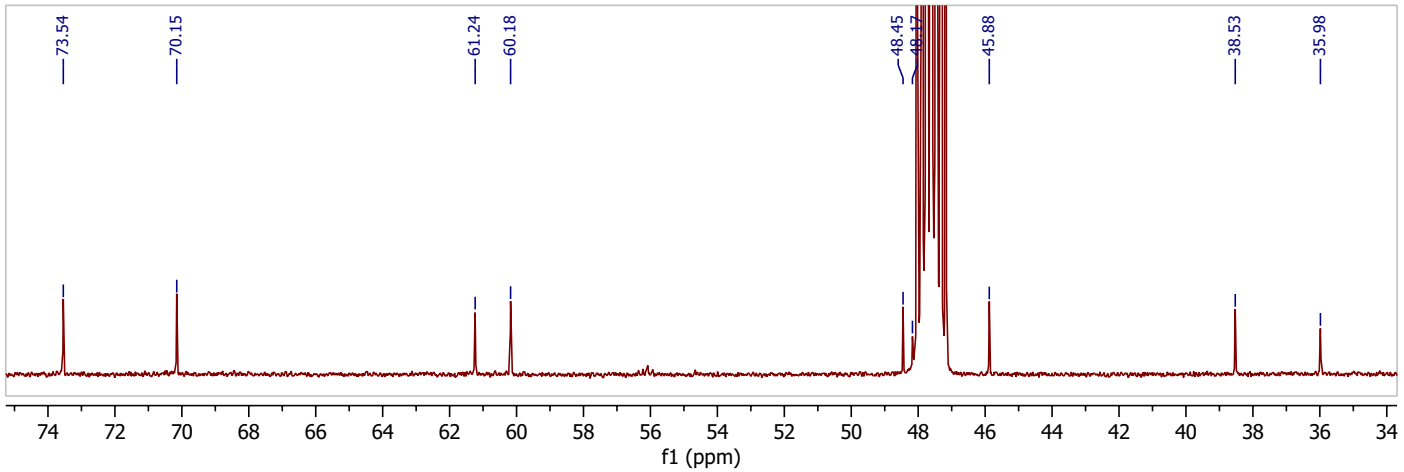


Plate 9 13C NMR (150 MHz) spectrum of Compound 2 MeOD





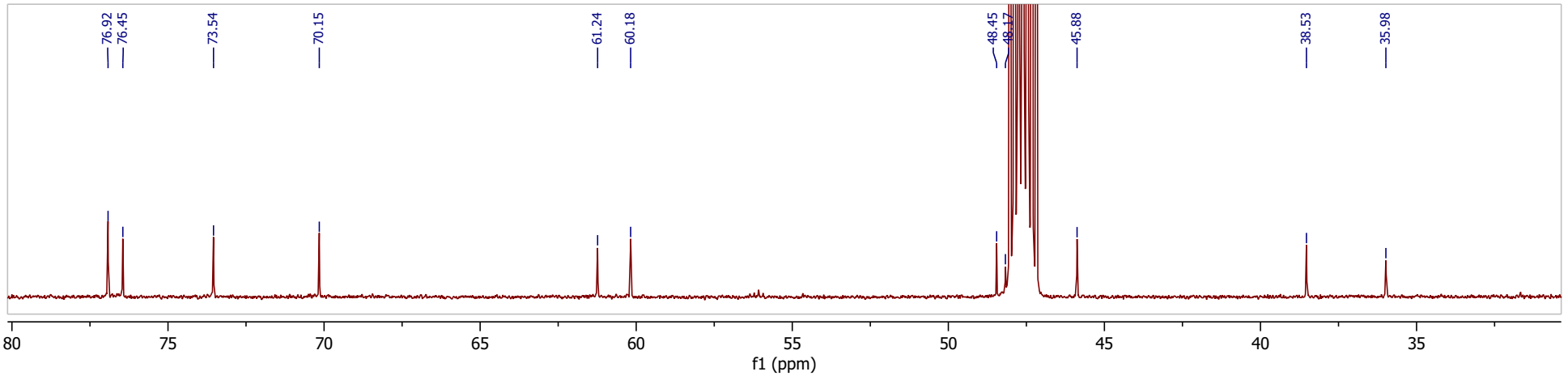
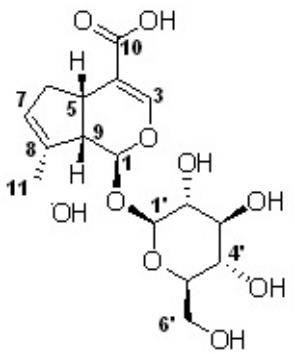
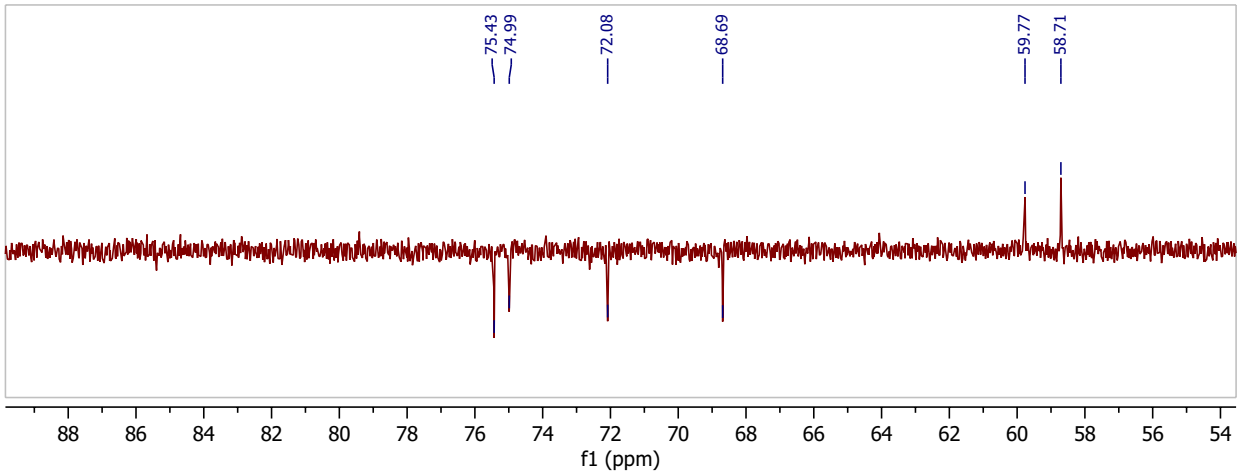


Plate 10 13C APT NMR spectrum of Compound 2 MeOD





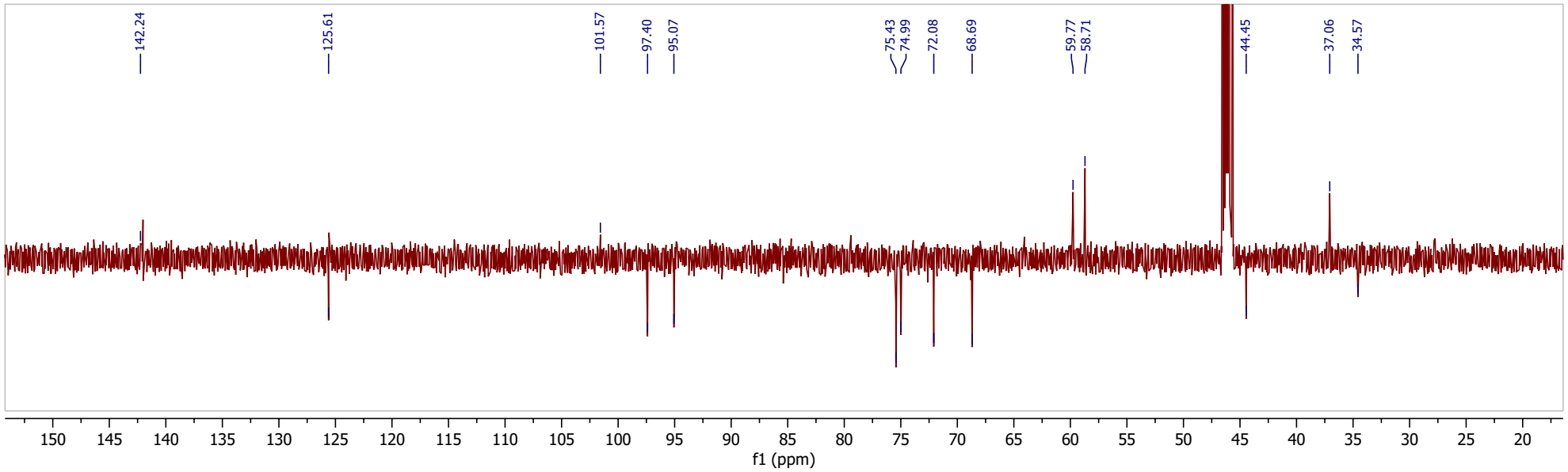
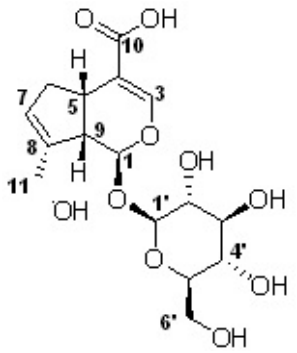


Plate 11 2D COSY spectrum of Compound 2 MeOD



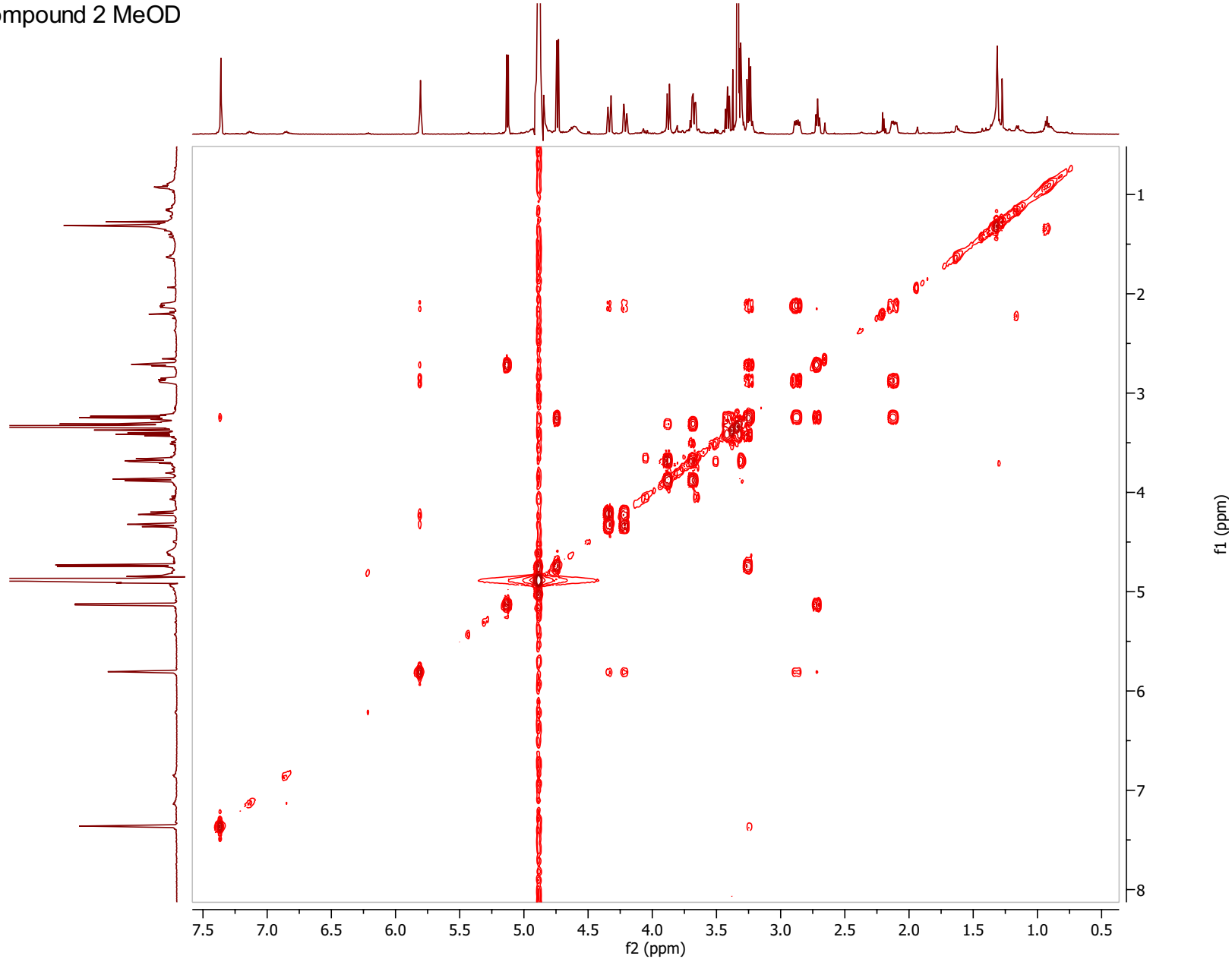
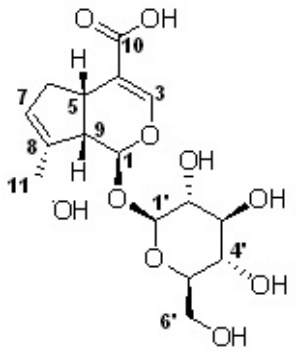


Plate 12 HSQC NMR spectrum of Compound 2 MeOD



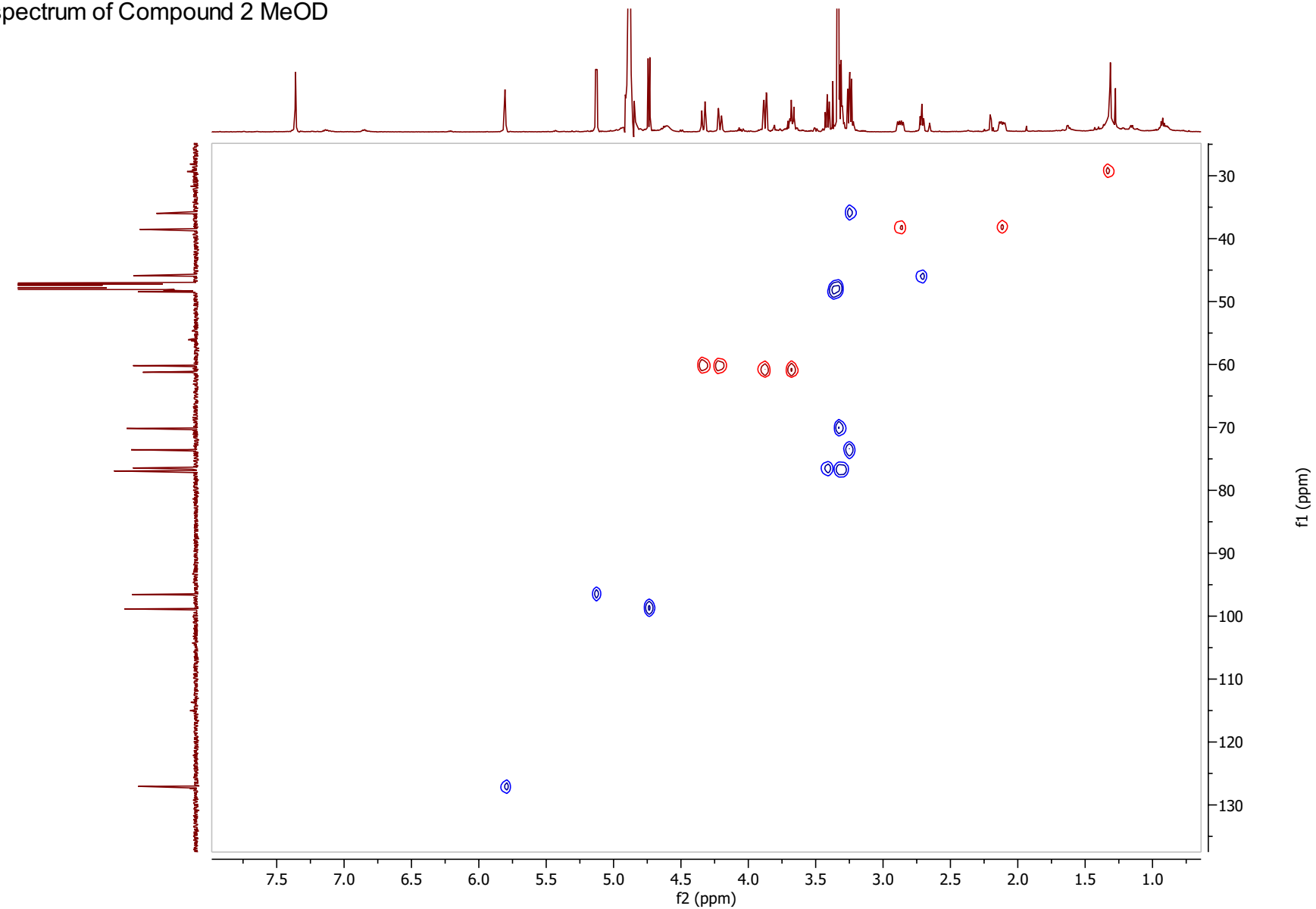


Plate 13 2D HMBC spectrum of Compound 2 MeOD

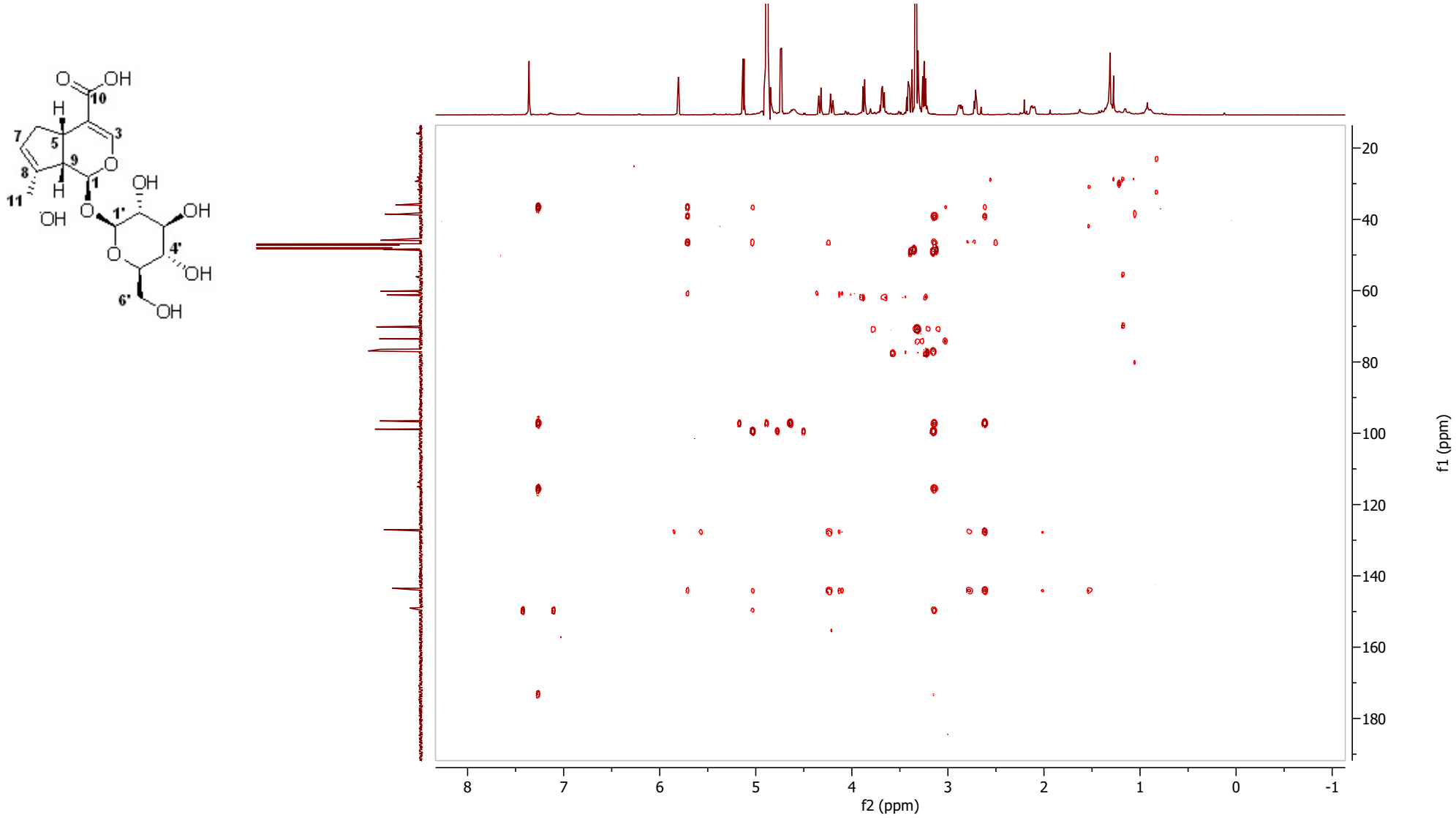


Plate 15 1H NMR (600 MHz) spectrum of Compound 3 MeOD

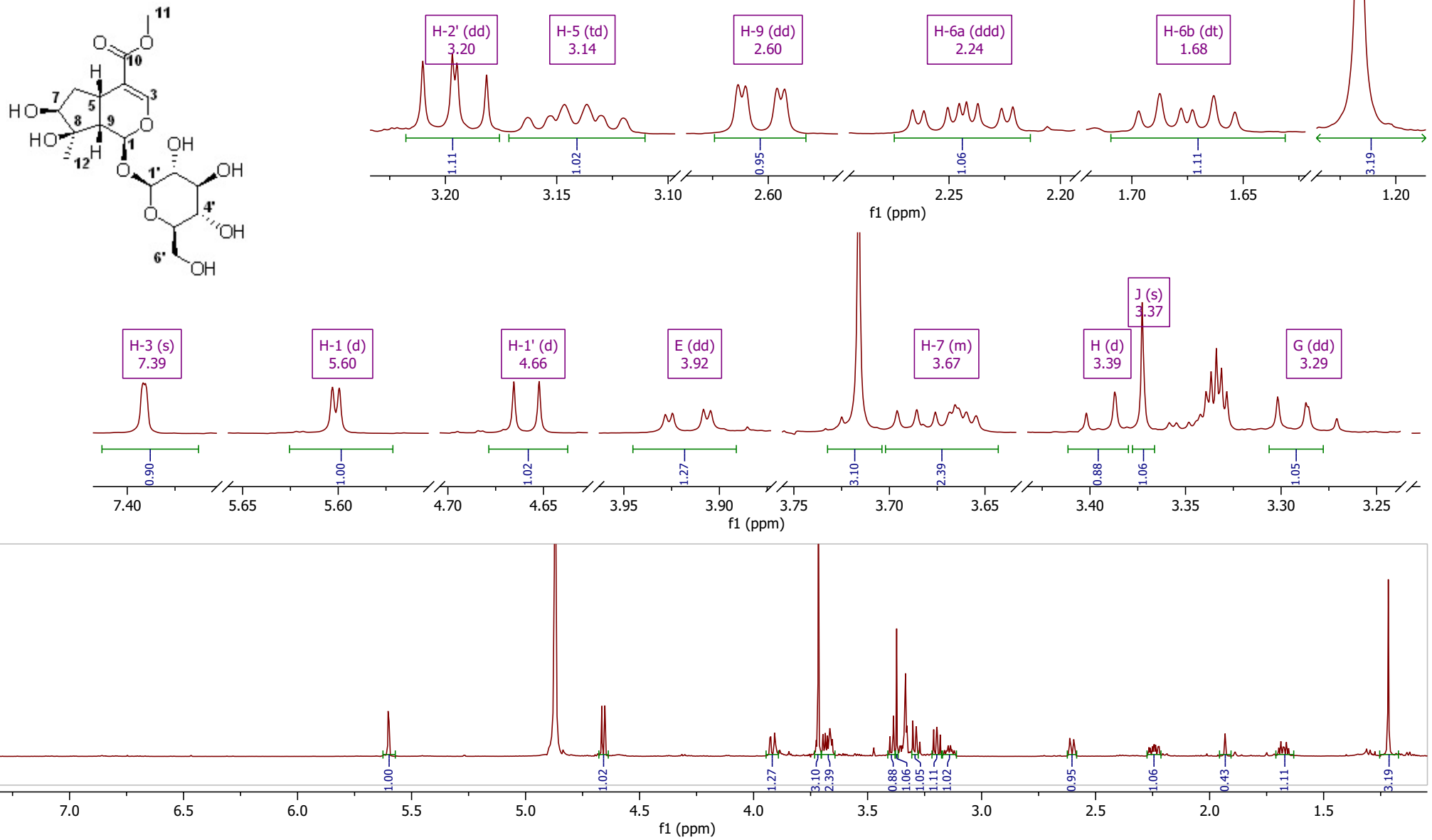
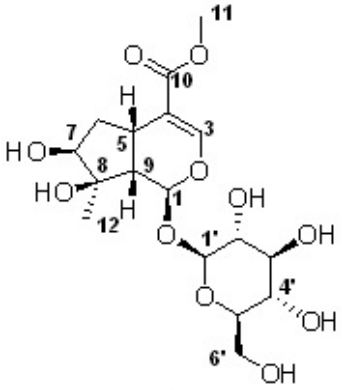
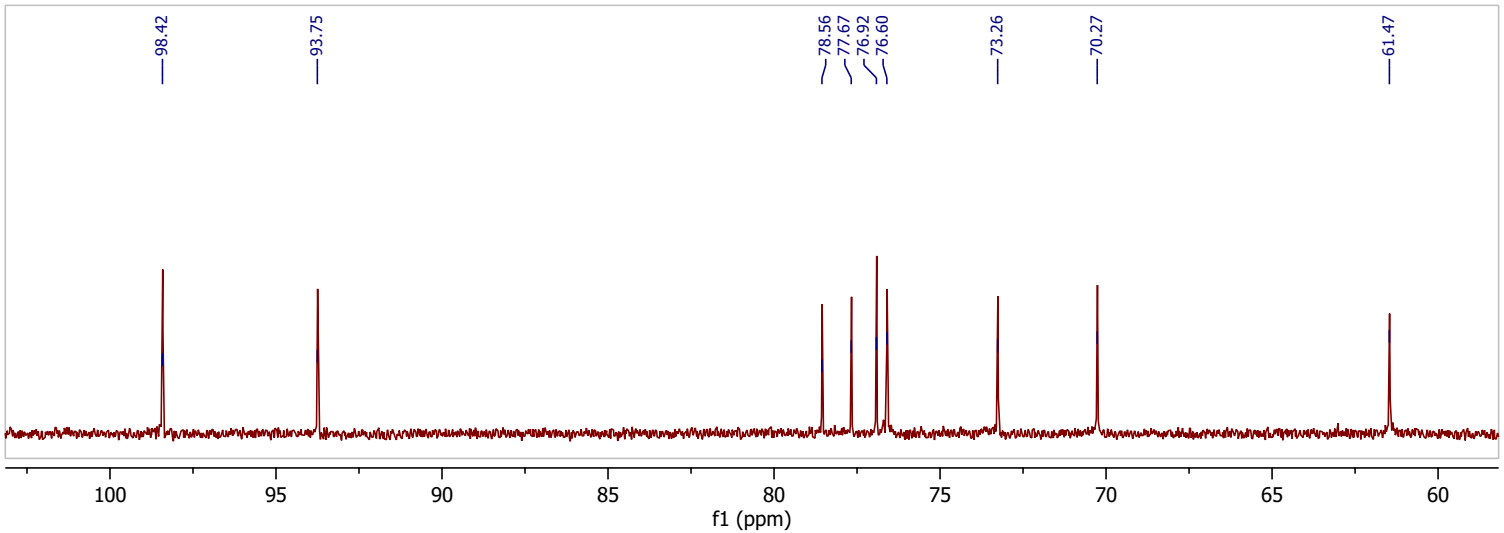


Plate 16 13C NMR (150MHz) spectrum of Compound 3 MeOD





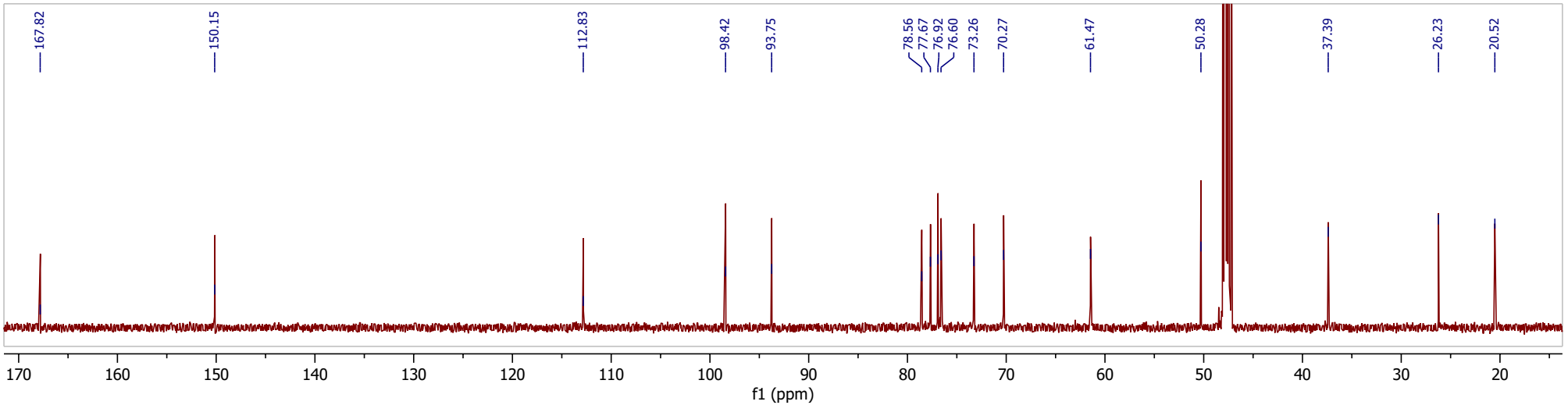
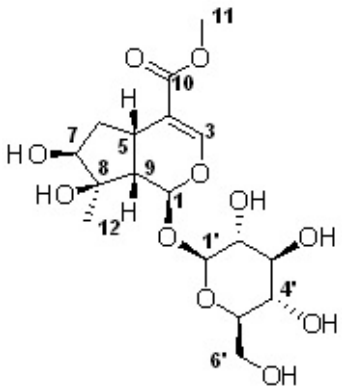
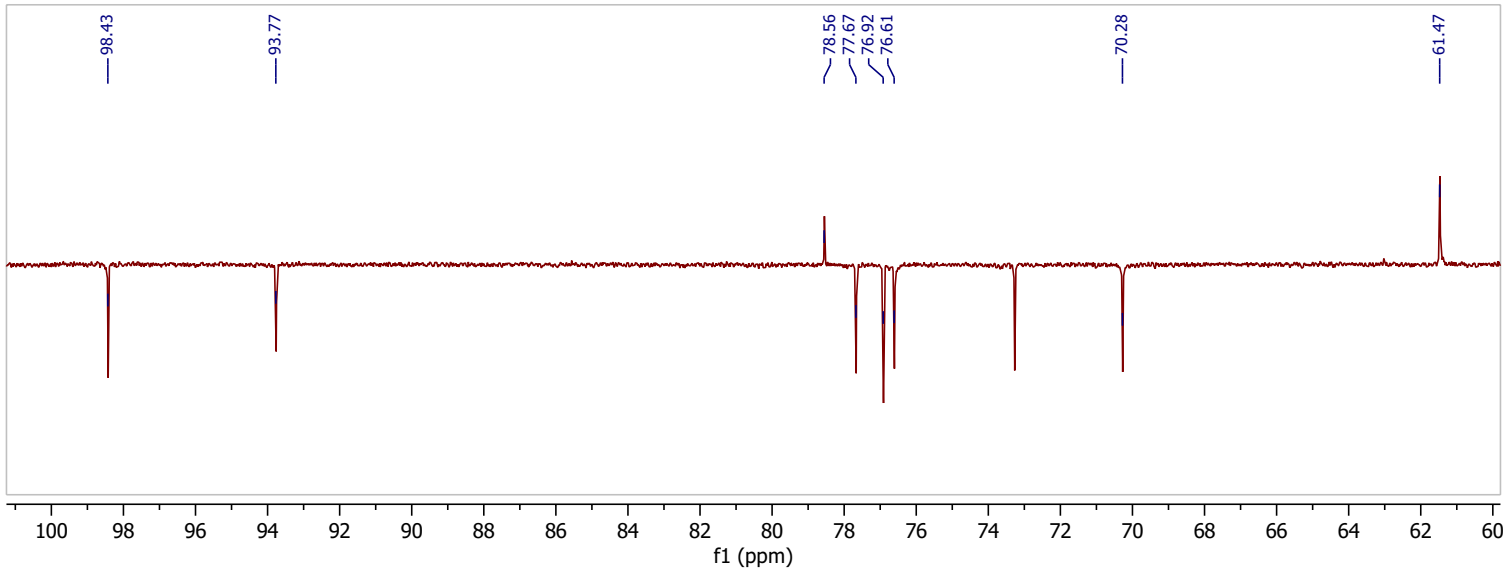


Plate 17 13C APT NMR (150MHz) spectrum of Compound 3 MeOD





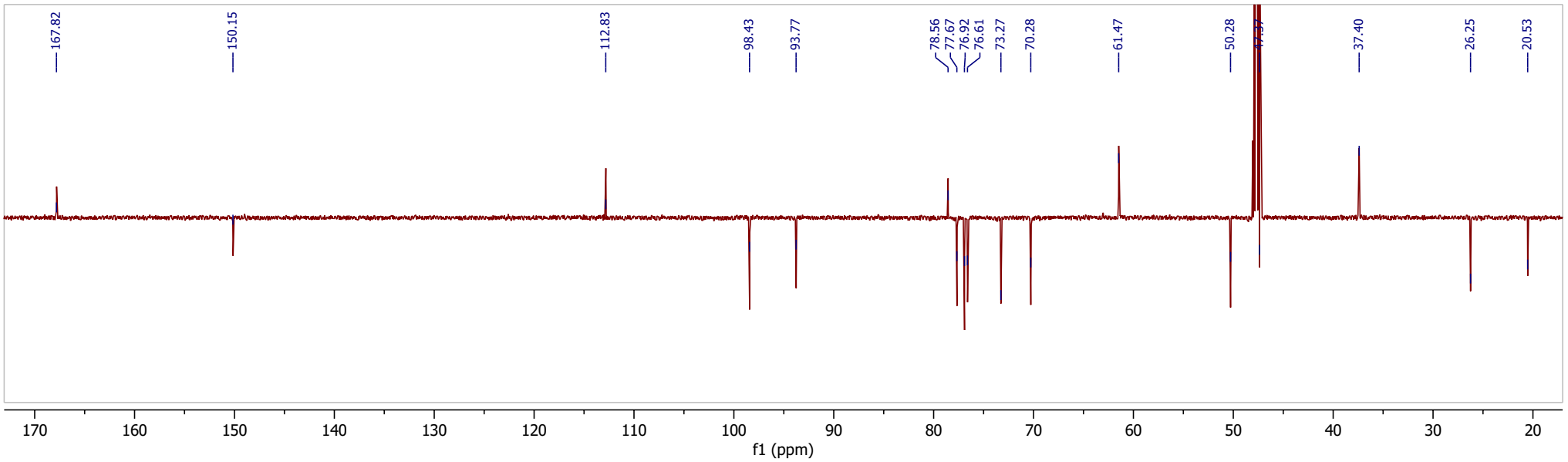
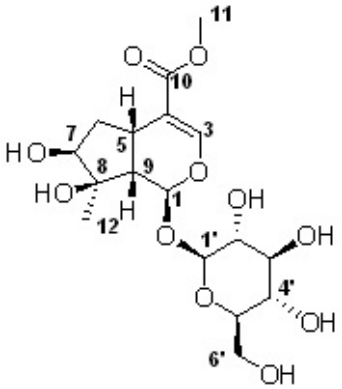


Plate 18 2D COSY spectrum of Compound 3 MeOD



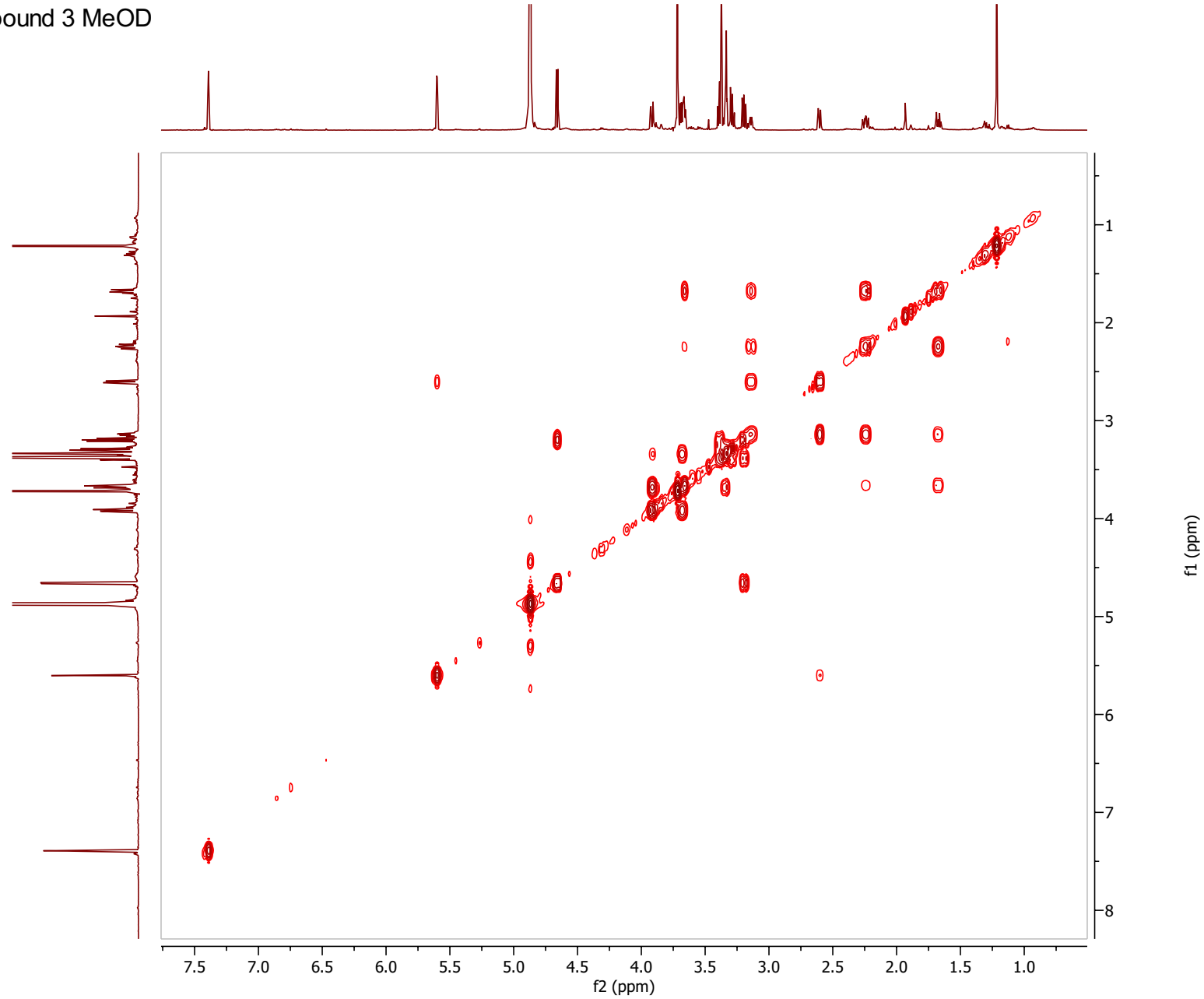


Plate 19 2D HSQC NMR spectrum of Compound 3 MeOD

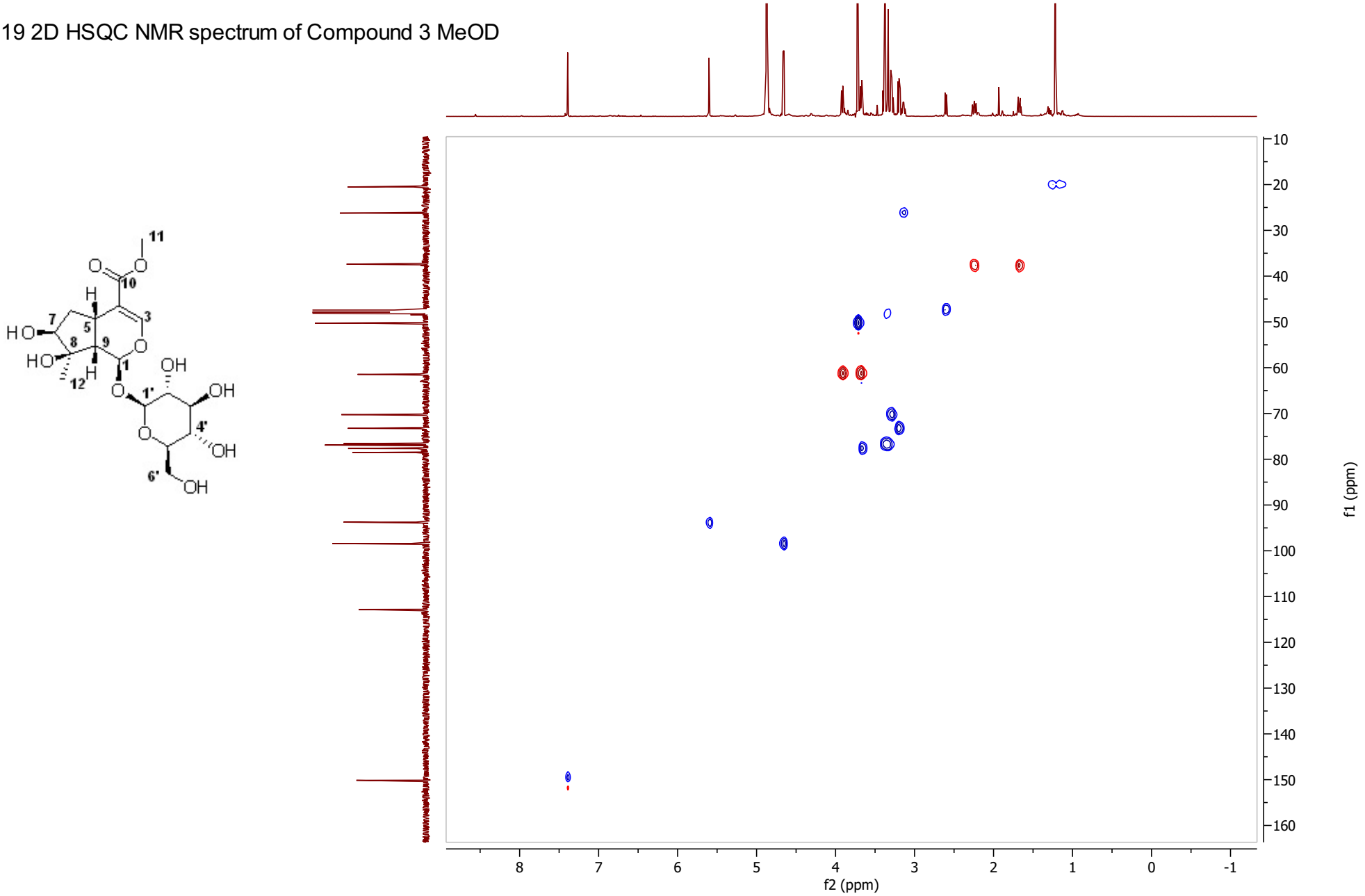
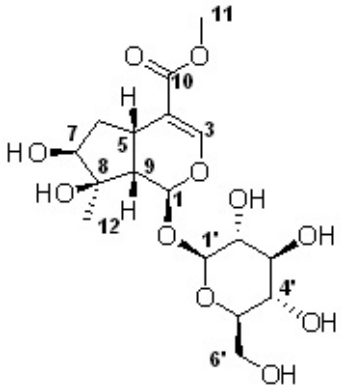


Plate 20 2D HMBC NMR spectrum of Compound 3 MeOD



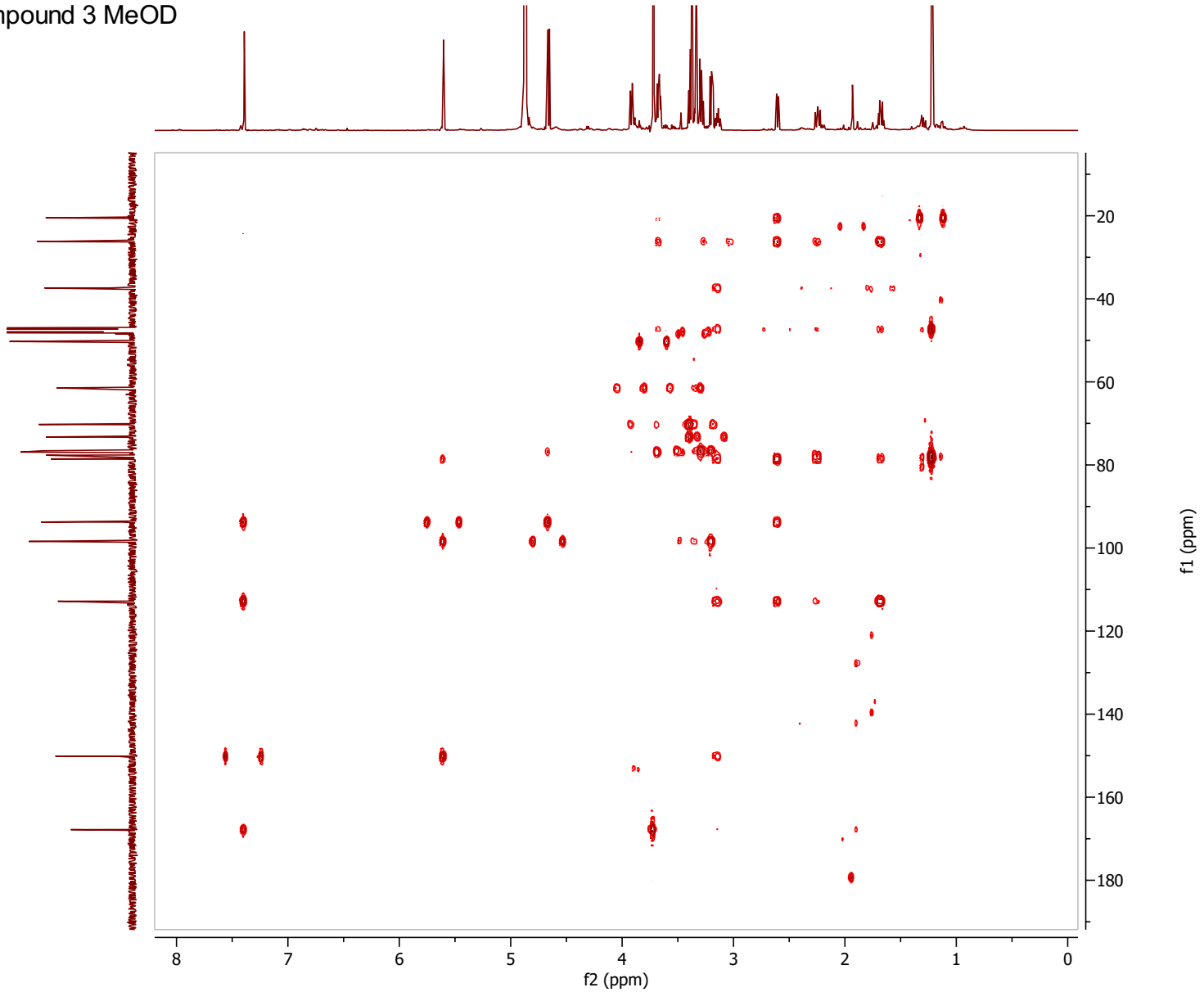
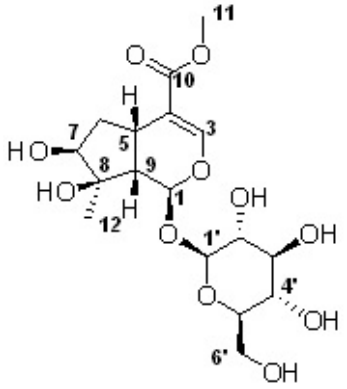


Plate 21 2D NOESY NMR spectrum of Compound 3 MeOD



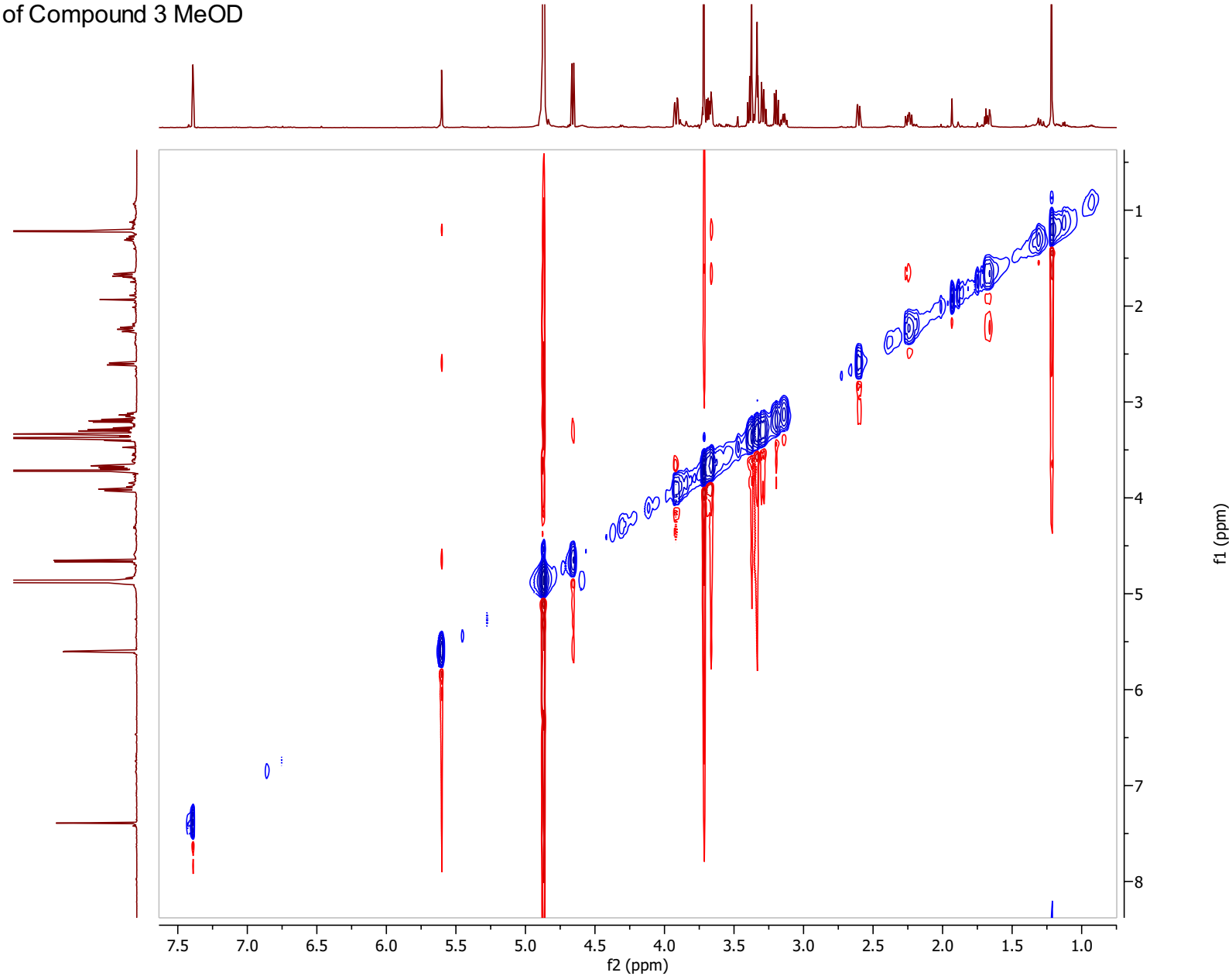
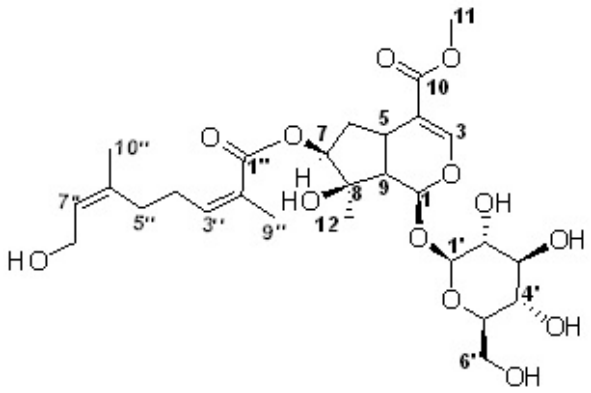
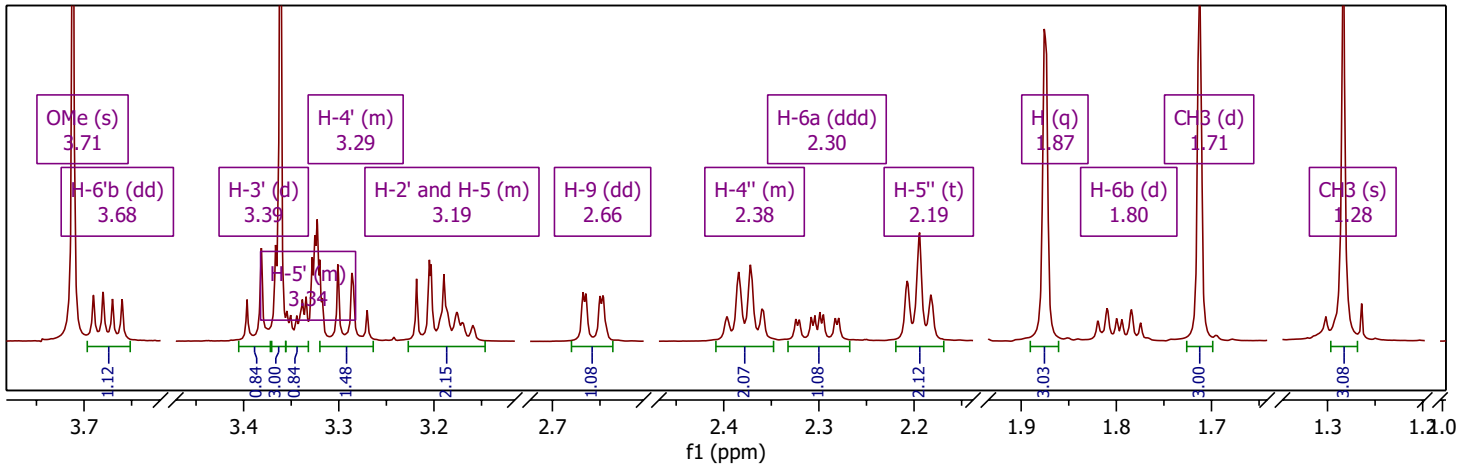
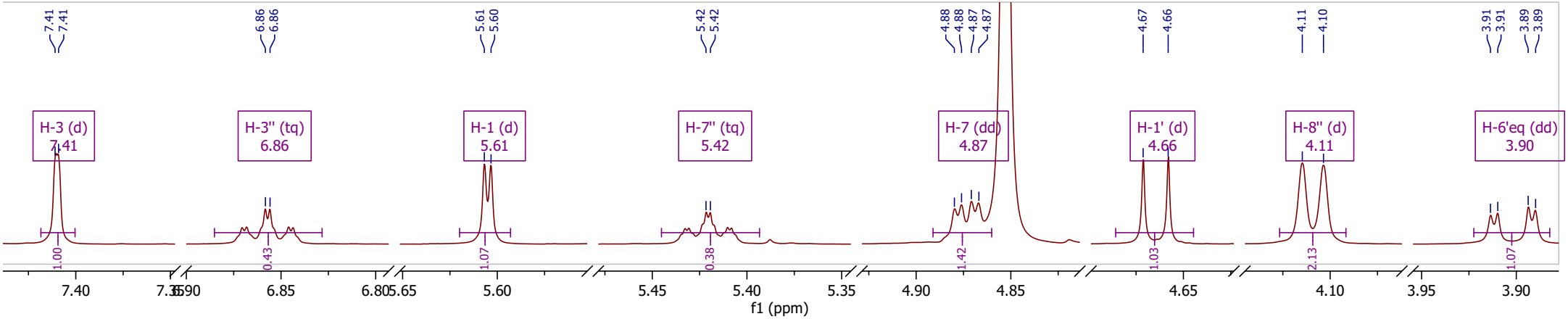


Plate 22 1H NMR (600 MHz) spectrum of Compound 4 (CD3OH)







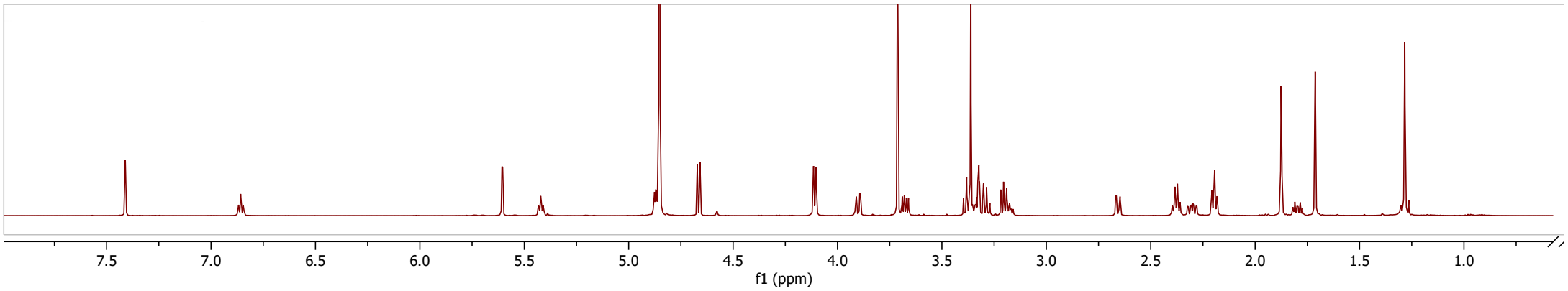
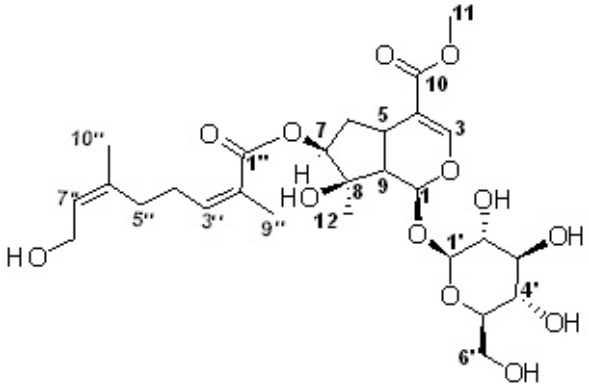
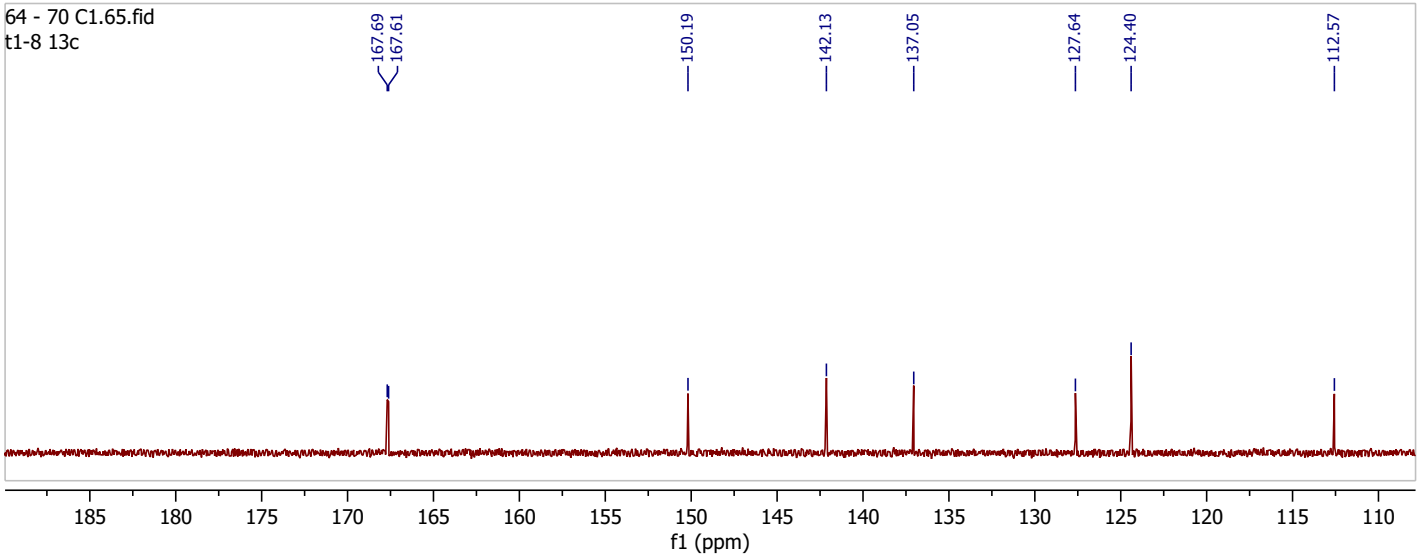


Plate 23 13C NMR (150 MHz) spectrum of Compound 4 (CD3OH)





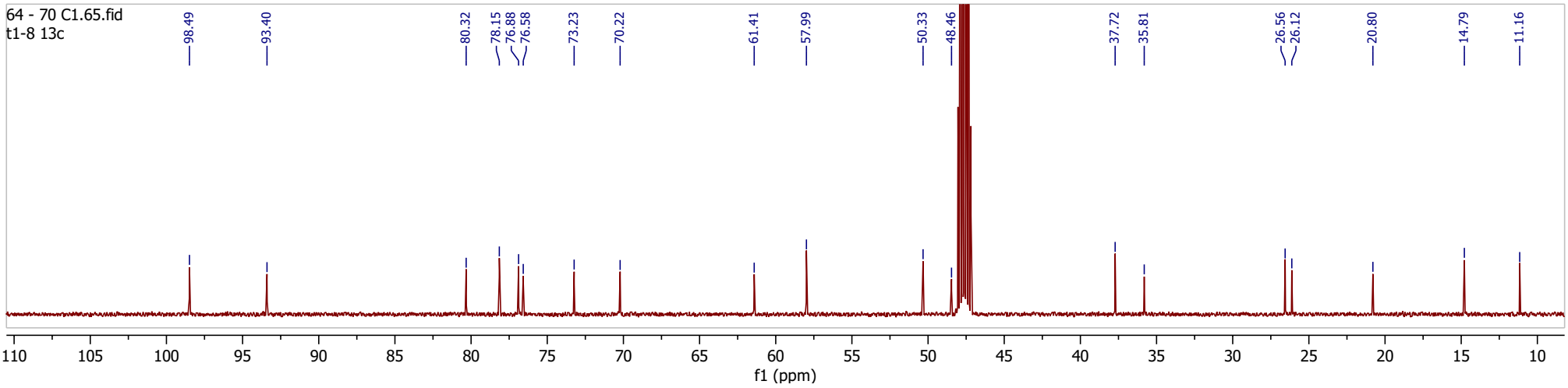


Plate 24 13C APT NMR (150 MHz) spectrum of Compound 4 (CD3OH)

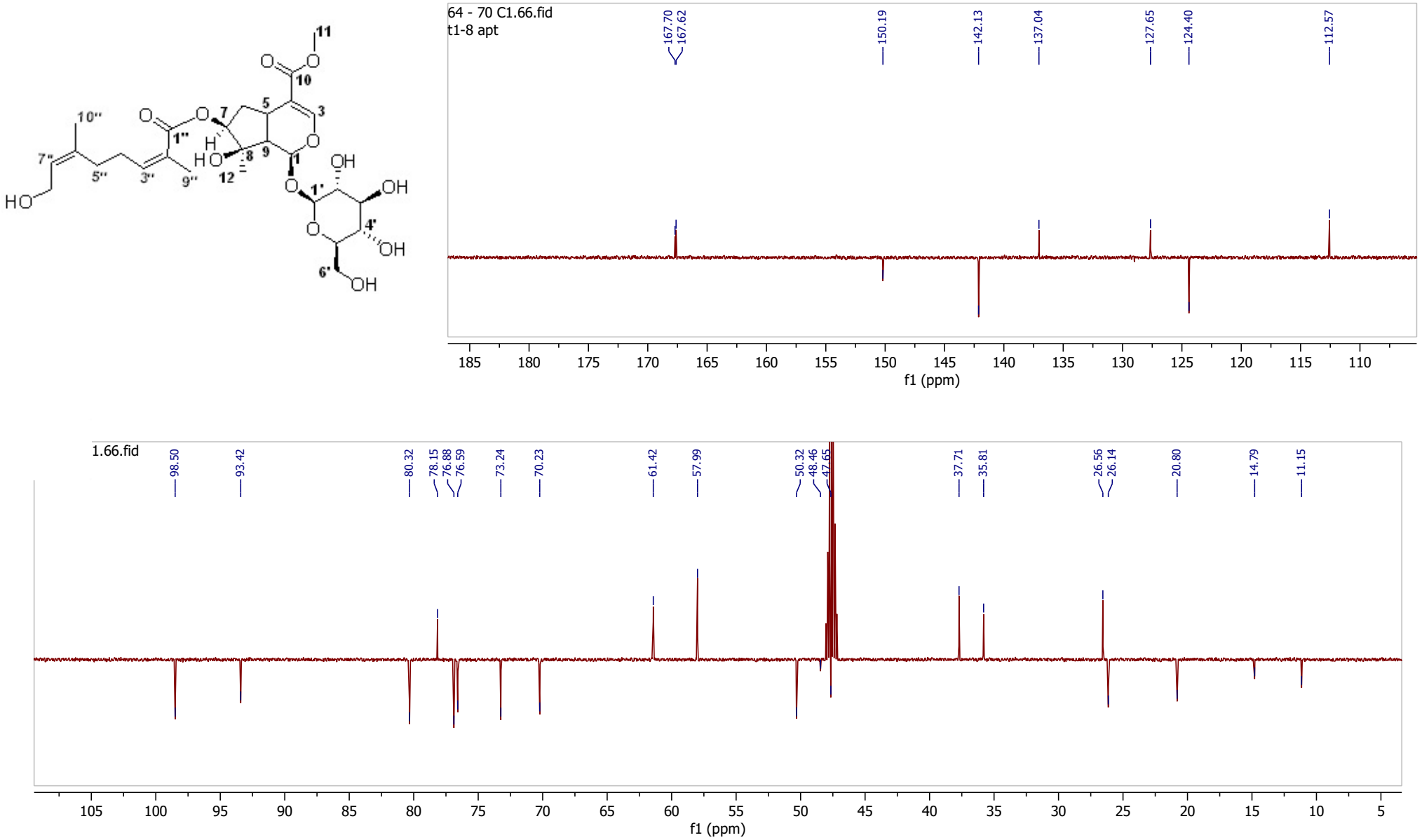


Plate 25 2D COSY NMR spectrum of Compound 4 (CD3OH)

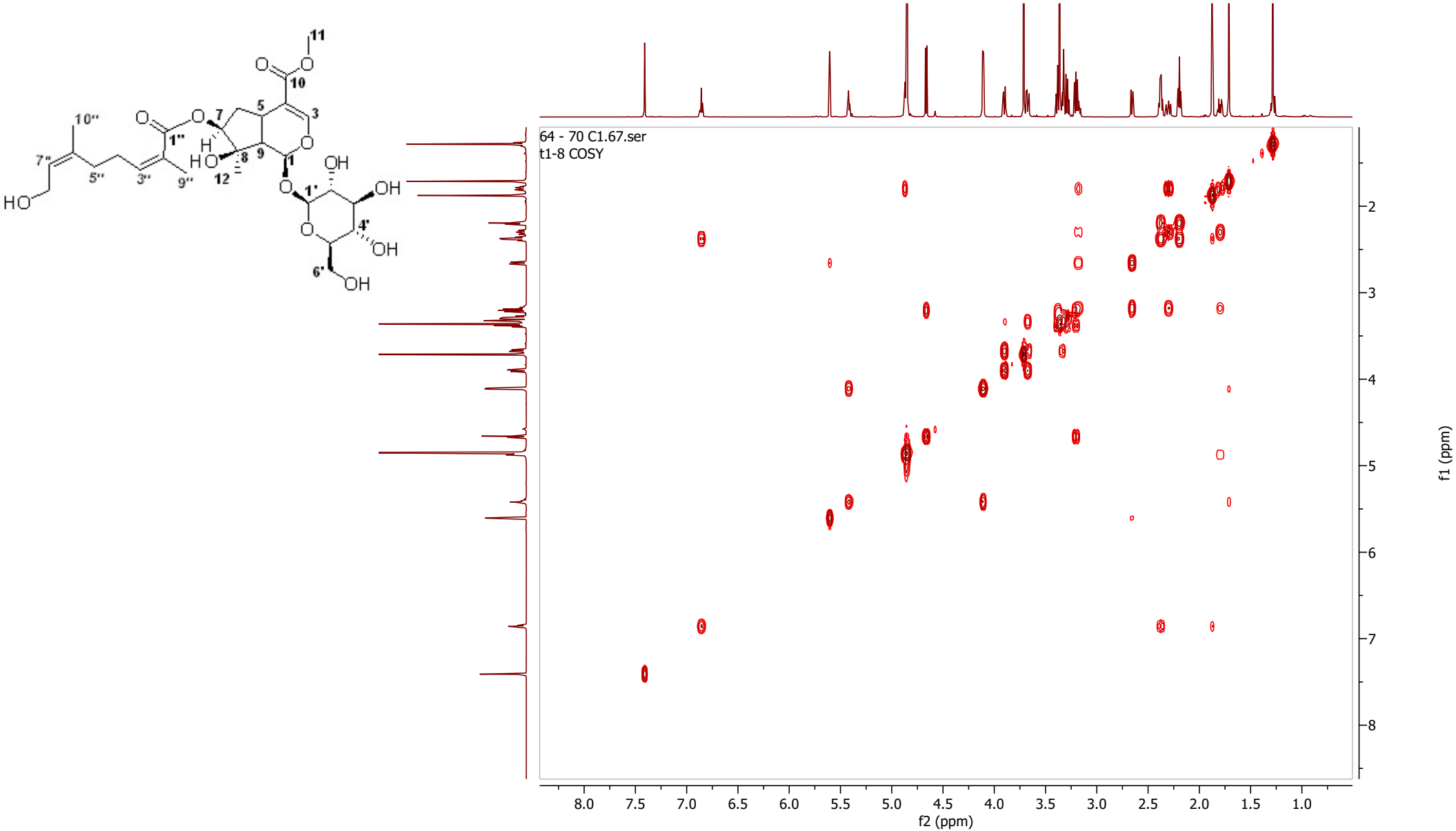


Plate 26 2D HSQC NMR spectrum of Compound 4 (CD3OH)

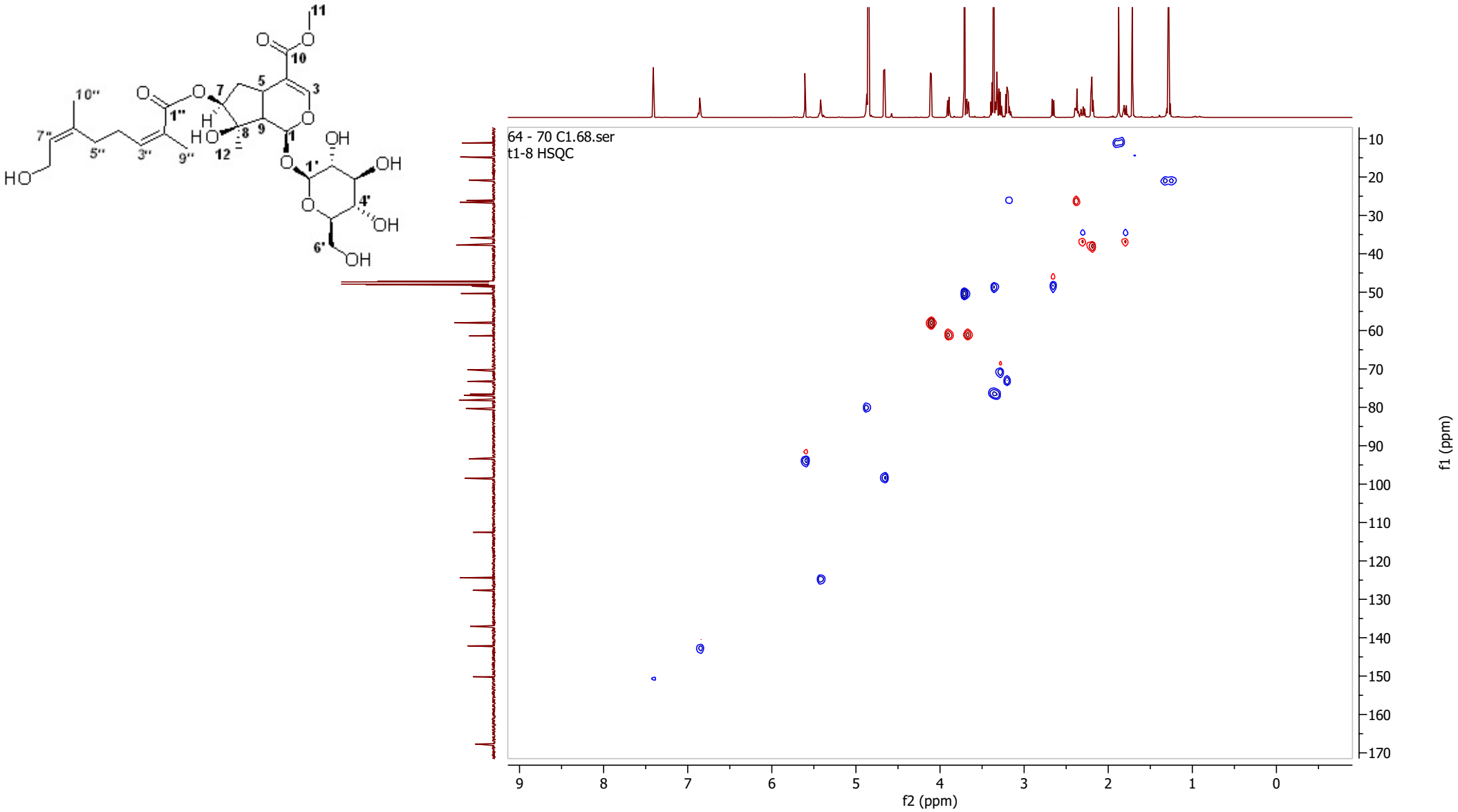


Plate 27 2D HMBC NMR spectrum of Compound 4 (CD3OH)

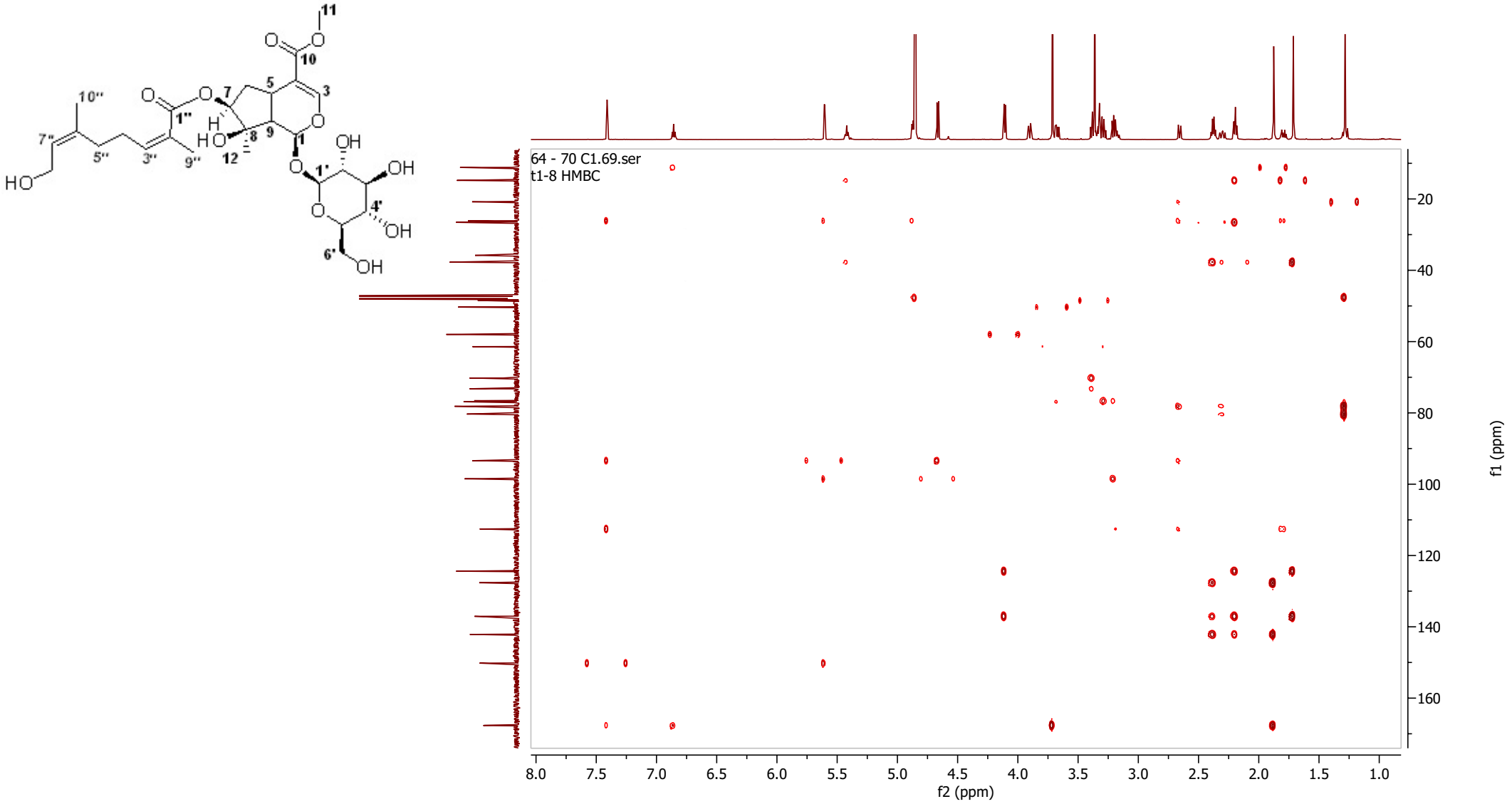


Plate 28 2D NOESY NMR spectrum of Compound 4 (CD3OH)

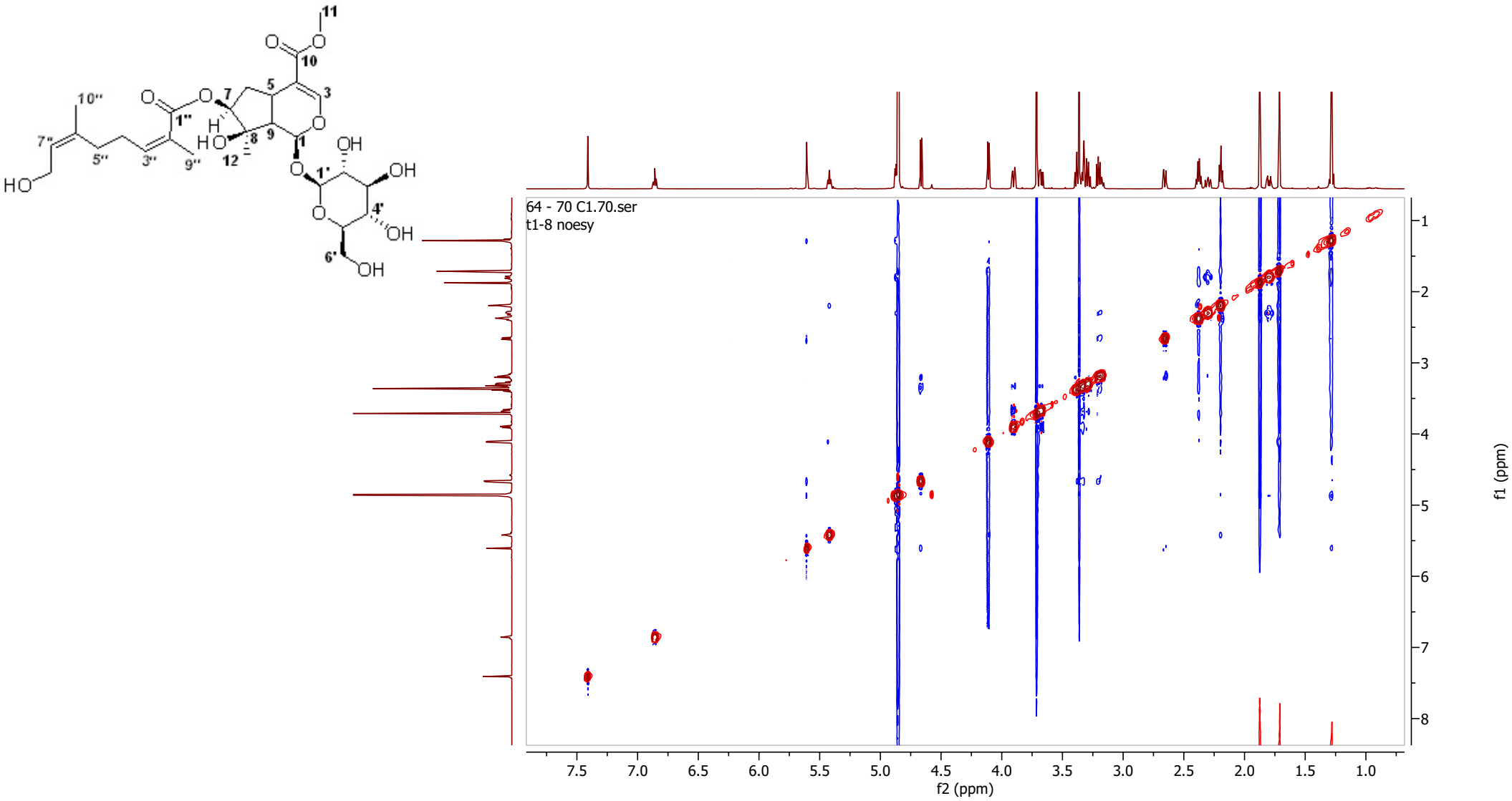
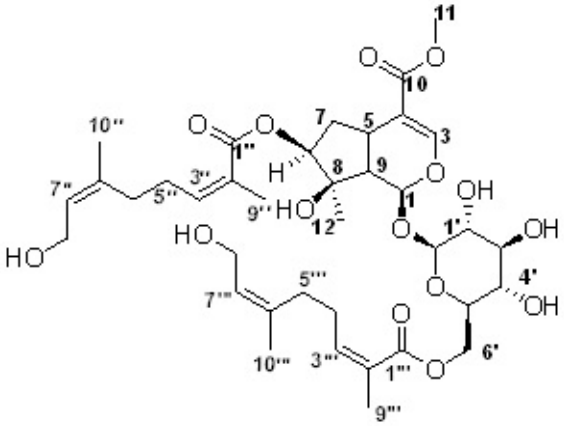
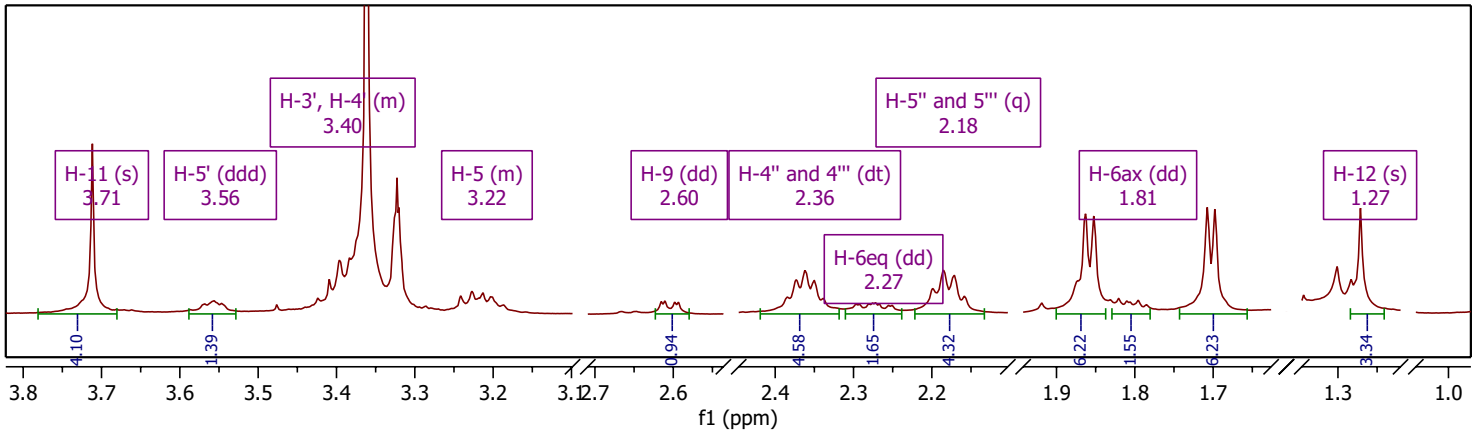
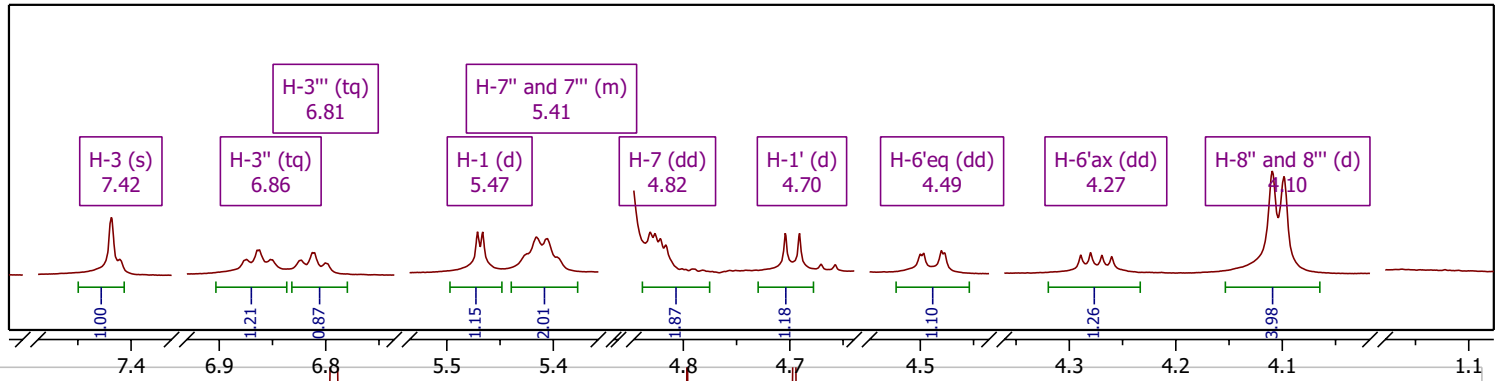


PLATE 29 1H NMR (600MHz) spectrum of Compound 5 MeOD







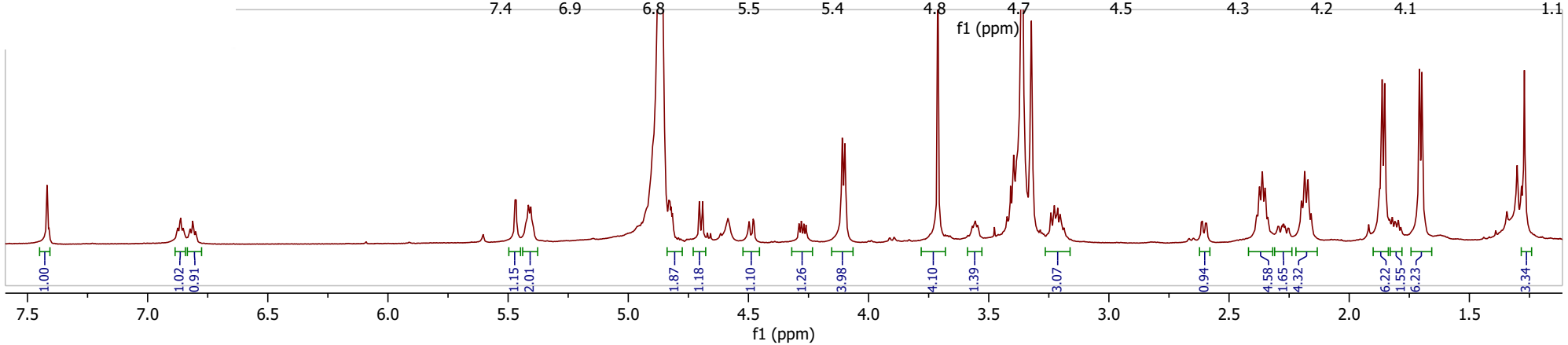
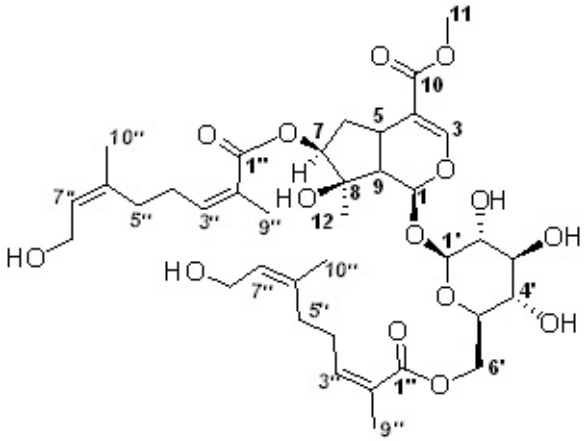
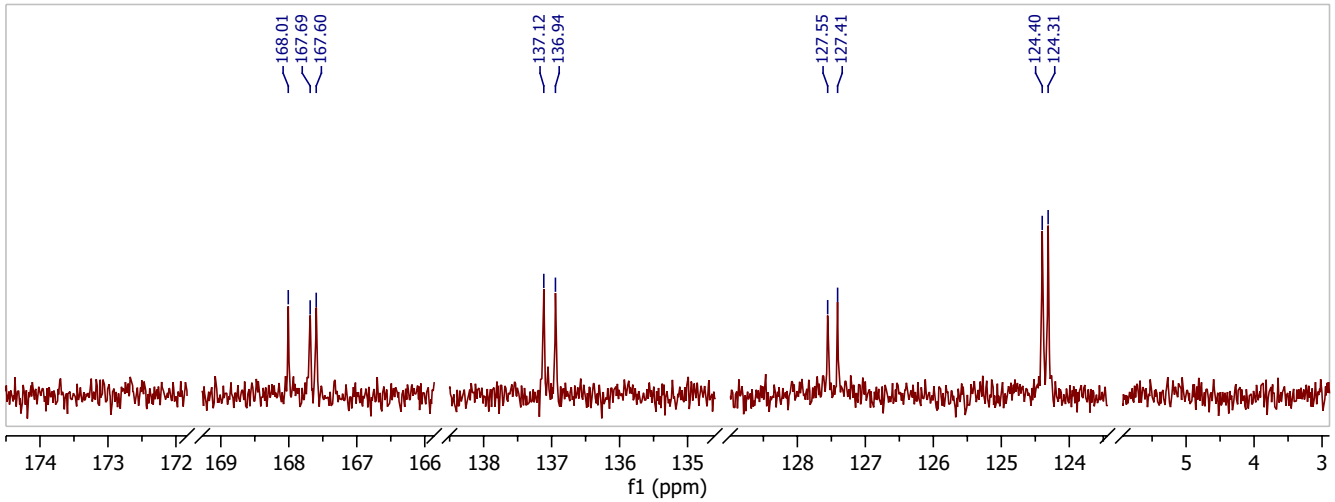


PLATE 30 13C NMR (150 MHz) spectrum of Compound 5 MeOD





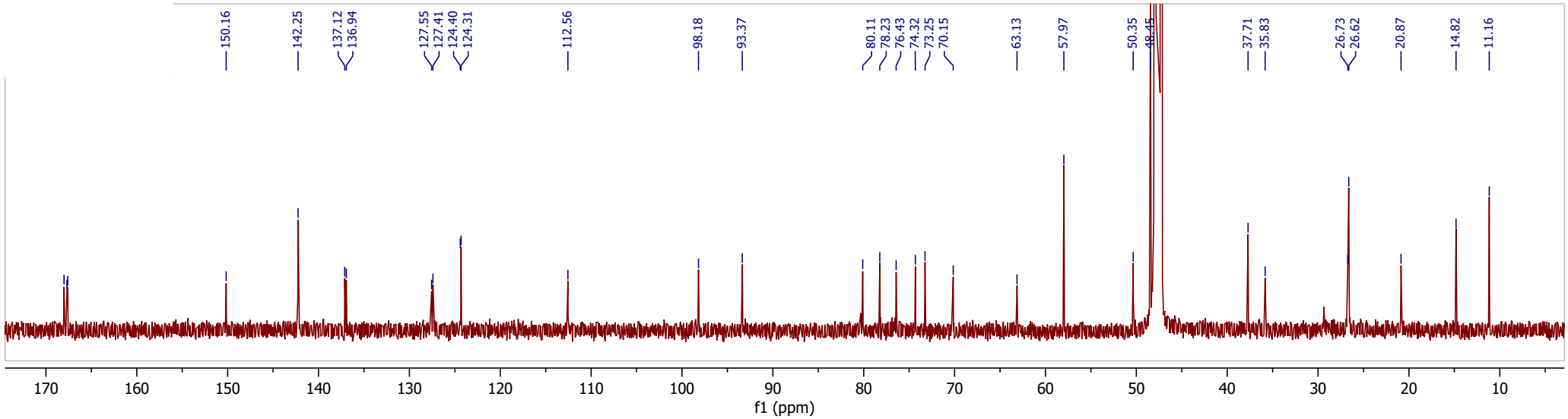
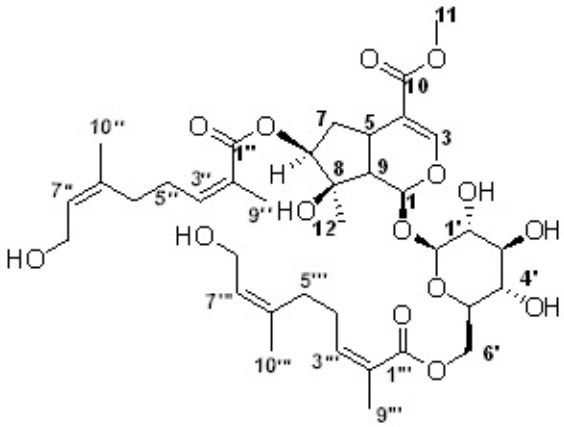
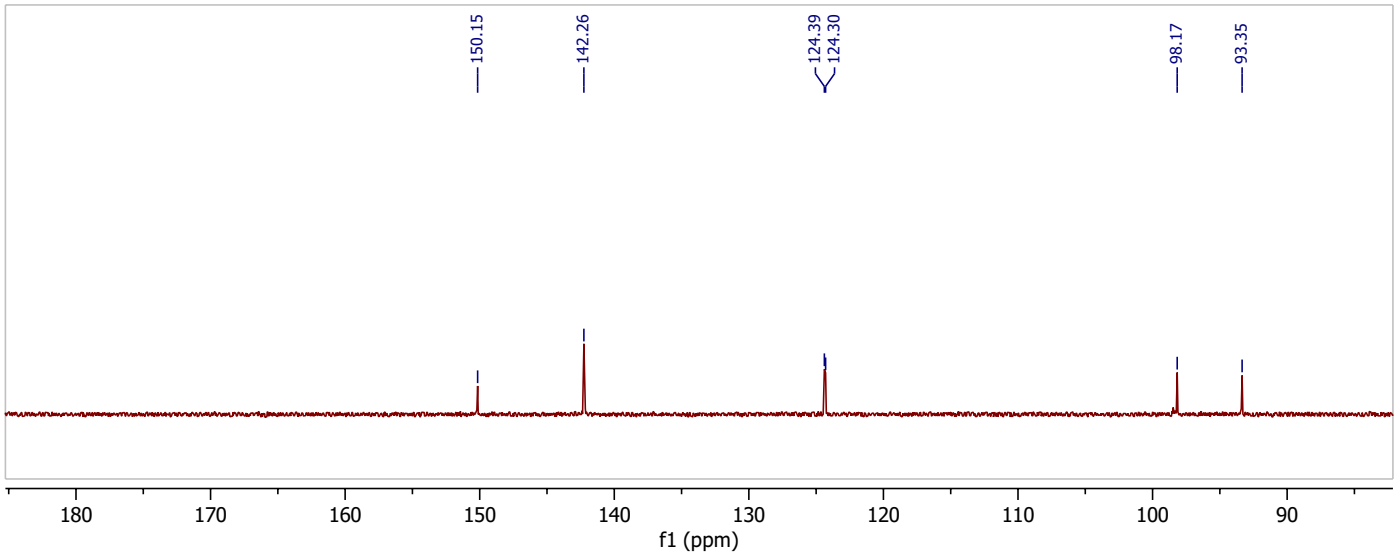


PLATE 31 13C DEPT (150 MHz) spectrum of Compound 5 MeOD





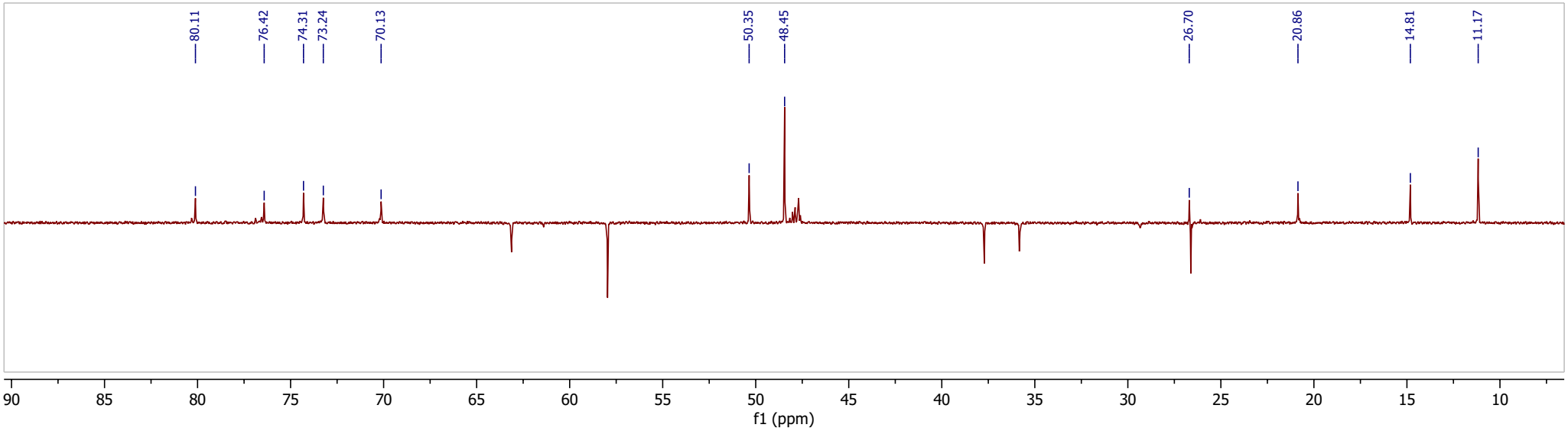


PLATE 32 2D COSY NMR spectrum of Compound 5 MeOD

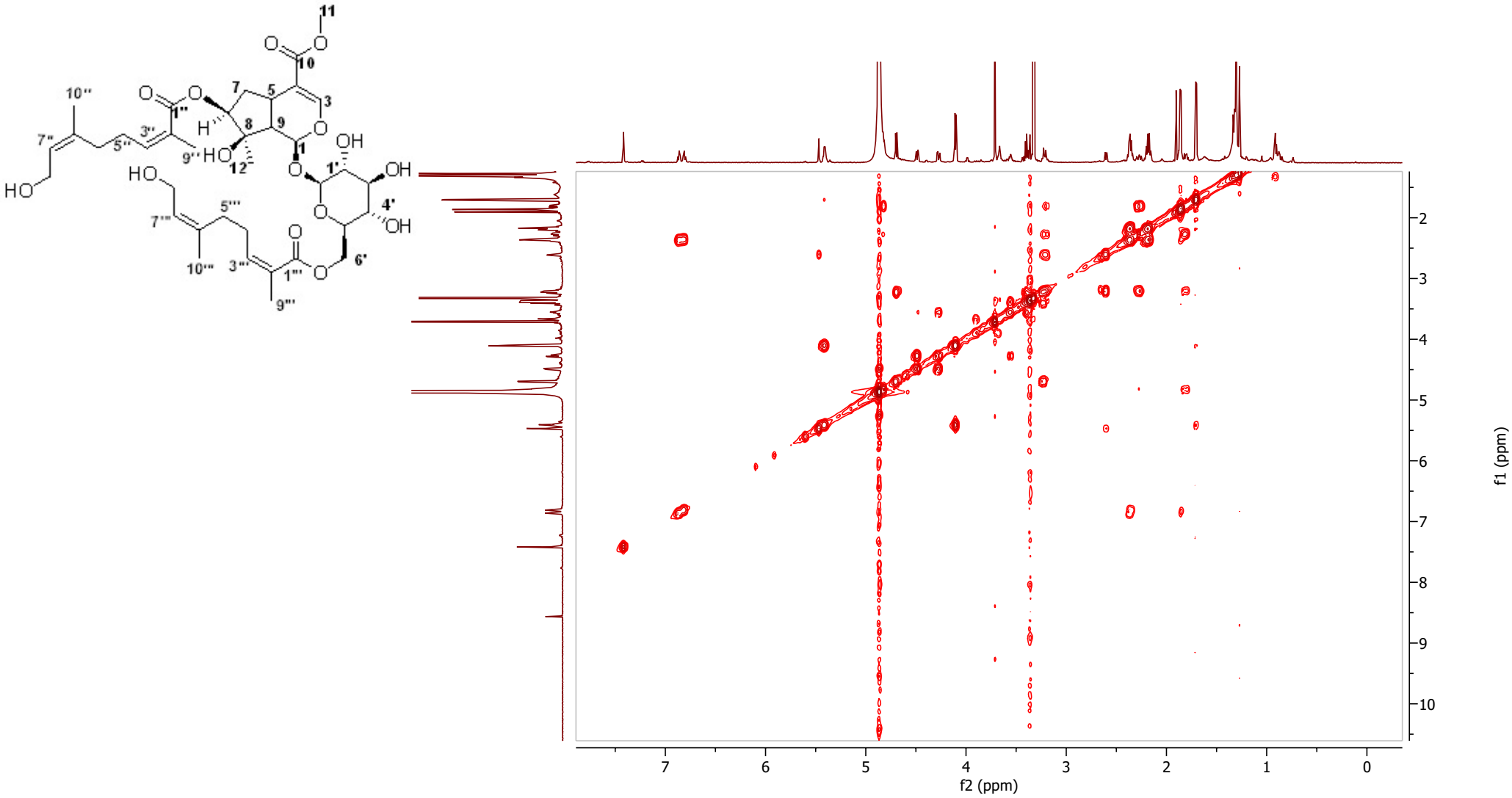
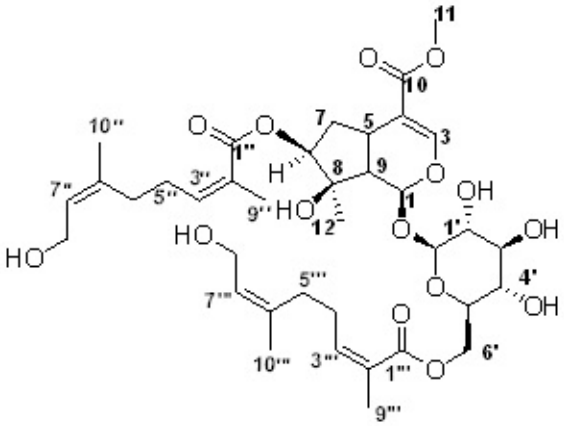


PLATE 33 2D HSQC NMR spectrum of Compound 5 MeOD



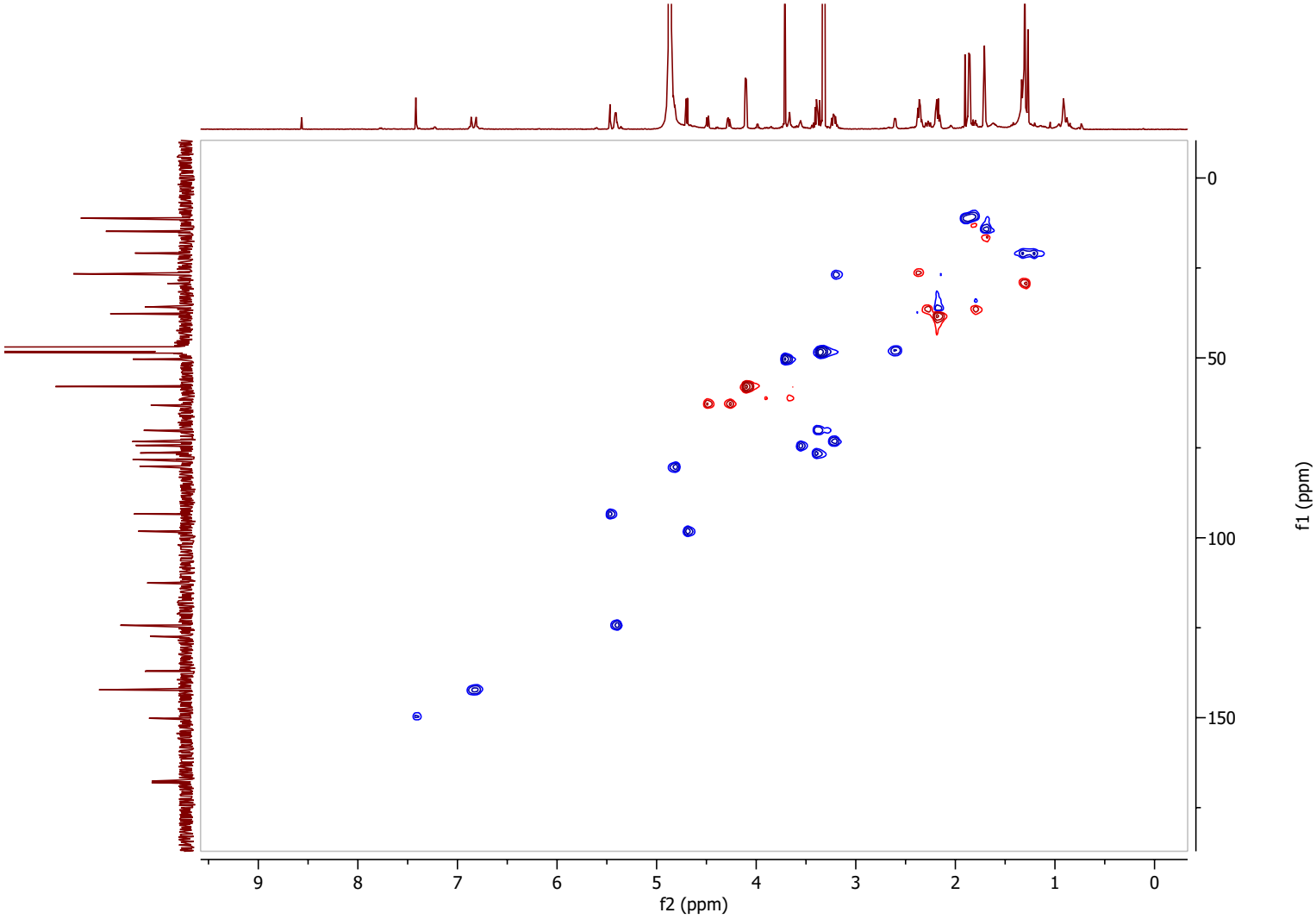


PLATE 34 2D HMBC NMR spectrum of Compound 5 MeOD