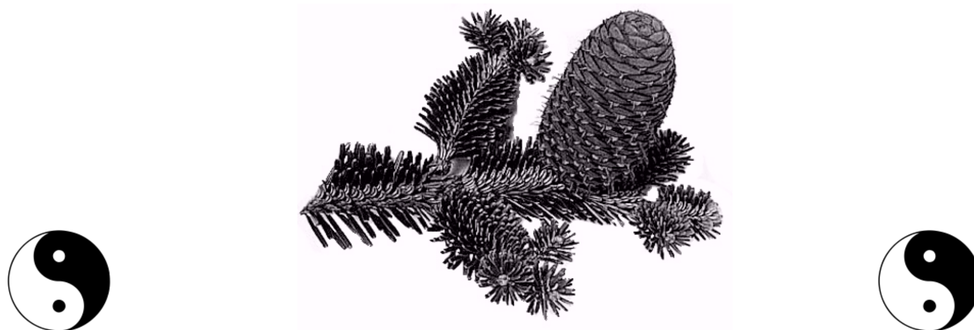
Herbal plants used in Traditional Chinese Medicine as a source of compounds with potential medical utilities – Identification, characterization and organization



by

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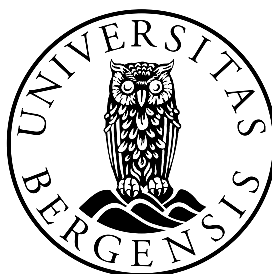
Thesis for the Master Degree in Pharmacy



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May 2010

Preface

This Master thesis has been written at the Gade Institute and the Center of Pharmacy, University of Bergen, Norway, with the laboratory part carried out at Modern Research Center for Traditional Chinese Medicine (MRCTCM), Second Military Medical University, Shanghai, P.R. China.

The study presented in this thesis was carried out as part of an international staff exchange programme titled “Traditional Chinese Medicine in the Post-genomic Era: Identifying Lead Therapeutic Compounds Against Cancer”. The European Council is acknowledged for the support of this program by FP7-PEOPLE-IRSES-2008, Marie Curies Actions – International Research Staff Exchange Scheme 2009 – 2013, project number 230232. The practical part of the thesis was carried out at the Modern Research Centre of Traditional Chinese Medicine at the Second Military Medical University in Shanghai from August 2009 to December 2009.

First I would like to thank Professor Wei-dong Zhang at the Modern Research Centre of Traditional Chinese Medicine and Professor Karl-Henning Kalland at the Gade Institute for supervision of the thesis work and the opportunity to do part of my master in Shanghai, a great experience, both academically and socially, that I will remember for a long time. I will also like to thank Professor Wei-Dong Zhang, leader at MRCTCM, for the opportunity to join them in their TCM research, and of course Dr. Lei Shan for his administration and help during our stay in Shanghai.

A special thanks to Yu Ping (Joe) for letting me join him and help him in his research, for his guidance in the laboratory, for all the interesting conversations and the chocolate at the lab. And a big thanks to all the staff at MRCTCM for being very helpful and making our four months stay in Shanghai a pleasant experience

I should thank my fantastic Granny, Rosalie Spencer Gloppestad, for proof reading my English.

At last, but not least, a big thank you to Nina Osmundsen, my fellow pharmacy student and partner in crime, for enduring four months in Shanghai with me.

Lars Kjetil Spencer Gloppestad, Bergen, May 2010

Abstract

Eight compounds were obtained from an ethanol extract from the aerial parts of *Abies forrestii,* by use of several different chromatographic techniques (size exclusion, TLC, normal and reverse phase). The chemical structure was determined for three of them by use of MS- and NMR-techniques (the known diterpenes abietic acid, dehydroabietic acid, 9,13(β)-epidioxy-8(14)-abieten-18-oic acid) and for one of them by comparing to a standard on TLC (β-sitosterol). The rest was either not pure enough or was obtained in a too low concentration. The pulverized plant material was extracted with ethanol, purified with ethylacetate and fractionated with gradient elution over a silica gel column and a MCI-column. The compounds were isolated and purified over silica gel-, Sephadex LH-20- and ODS-columns, and with P-TLC. The structures of the compounds were determined by a combination of LC-ESI MS, ^{1}H- and ^{13}C NMR data.

The extracted compounds are well known compounds in other *Abies* species, but no records have been found describing earlier extraction from *Abies forrestii.*

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1 Introduction

1.1 TCMCANCER project

This master thesis is part of the project *Traditional Chinese Medicine in the Post-Genomic Era: Identifying Lead Therapeutic Compounds Against Cancer (TCMCANCER)*. It is a collaboration project between *Centre de Recherche Public de la Santé* in Luxembourg, *Institute of Medicinal Plant Development* in Beijing, China, *Modern Research Center of Traditional Chinese Medicine, Second Military Medical University* in Shanghai, China and the *Gade Institute, University of Bergen* in Norway.

The TCMCANCER project objectives are to identify and characterize new lead compounds with anticancer activities from Chinese medicinal herbs in Traditional Chinese Medicine (TCM), and transfer of expertise between Chinese and European labs. The project also includes a staff exchange program funded by the EC Commission through the Marie Curie Actions (project number 230232). The laboratory part of this thesis was therefore carried out at the *Modern Research Center of Traditional Chinese Medicine* in Shanghai.

1.1.1 Modern Research Center of Traditional Chinese Medicine (MRCTCM), Second Military Medical University (SMMU)

MRCTCM at SMMU in Shanghai has eight major research teams working on bioactive constituents of TCM, quality control, metabolism, metabolism and pharmacokinetic research, metabonomics research, pharmacology model research, composite formula research, novel drug research and development (R&D) and natural products libraries. Their mission is a "Globalization of Traditional Chinese Medicine" and "Construction of a new research platform of TCM with approaches and methods in structural biology, chemical biology, medicinal chemistry, analytical chemistry, metabolism, pharmacokinetics, metabonomics, pharmacology and toxicology.

The staff at MRCTCM is skilled in isolation and characterization of chemical compounds from TCM, and over 70 species of medicinal plants have been investigated for the chemical compounds. Over 3000 compounds have been obtained, and about 400 of them were new.

MRCTCM has developed a Natural Products Library with 7100 standard TCM extracts and 3000 purified and chemically defined natural compounds derived from herbal and plant TCM extracts.

1.2 Traditional Chinese Medicine (TCM)

Here follows a short description of the theories in TCM as a background for how herbs and plants are used to treat illnesses in TCM.

TCM, Traditional Chinese Medicine, was developed in Asia over a period of several thousand years. The earliest written record of TCM is the Huangdi Internal Classic, and it is regarded as the first formulation of the theory in Chinese medicine. It is thought to be a text from before, or during the early period, of the Han-dynasty (206 BC-220 AD).[1]

TCM was, as Western medicine, influenced by the philosophical thoughts of its time. Western medicine has a logical background, where cause and effect are used to describe, not only the world we live in, but also humans. TCM was influenced by the ancient Chinese philosophy, which also affected the thoughts of Confucianism, Taoism/Daoism and Buddhism. The theories of *Yin/Yang*, *Qi* and the *Five Elements* are therefore an integral part of both TCM and also these thoughts and religions.[1]

1.2.1 Yin and Yang

In the west, *Yin and Yang* are often wrongly regarded as representations of good and evil, right and wrong, black and white. Although in some ways true, *Yin and Yang* are actually two complementing sides of an issue, like up and down, sun and shadow, male and female. The *Yin and Yang* theory is complex, and although it is integrated in TCM, it will not be explained thoroughly here. The basic idea is that everything is changing, balancing between two opposites, *Yin and Yang*. Everything is connected and nothing can be isolated from the whole. An illness has different elements of yin and yang. Because of the *Yin/Yang* theory, TCM does not have the same need to find a physiological cause of an illness as Western medicine does. The reason for the illness is the changes and the relationships in the body, the yin and yang. They only have to find the cause of the imbalance and help restore it.[2]

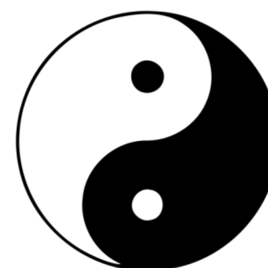


Figure 1.1: Ying/Yang

1.2.2 Qi

The notion of *Qi* is even more complex than the *Yin/Yang* theory, but it is an important part of Chinese medicine. *Qi* is generally explained as what connects everything, but also is everything and is in everything. It is the cause, the process and the outcome and it can take many forms. In TCM and in the body, *Qi* has been given a more specific meaning and role. It enters the body from parents, food and air, and five major functions of *Qi* in the body are often described (although every activity could be some sort of *Qi*).[2]

- *Qi* protects the body from external invasion and combats anything that manages to penetrate (like microorganisms). The cause of an illness due to penetration would therefore be deficient Qi, and a treatment would be to increase Qi, either by herbs or changes in the environment.
- *Qi* is the source and is all movement in the body, including walking, thinking, speaking, and aging. Qi is moving around in the body and normal movement is associated with normal physiological activity. However, movement can be disrupted or move wrongly, which will give rise to disharmony in the body.
- *Qi* is the source of transformation, like when food is transformed to blood, thought or Qi itself
- *Qi* warms the body, as a whole and in limbs and organs
- *Qi* also keeps everything in stability and in place. It keeps blood in the blood vessels, maintains the structure of the body and governs the loss of water.

There are many different types of *Qi*, some more important than others. Every organ has its own type of *Qi* with its own activity based on the specific organ. *Meridian Qi* is the *Qi* that flows in the meridians, which are the pathways where *Qi* flows between organs and body parts. This is the basic of the part of TCM known as acupuncture. *Protective Qi* has already been discussed as protecting the body from external invasions.[2]

Disharmonies of *Qi* give rise to illnesses. Deficient *Qi* has already been described for *Protective Qi*, but it applies to all types of *Qi* in the body. Stagnant *Qi* is a different disharmony, where normal movement of *Qi* is disrupted or flows the wrong way, like when *Stomach Qi* moves upward instead of the normal downward, and thereby causing nausea and vomiting.[2]

1.2.3 Fundamental Textures

Qi is one of *The Fundamental Textures* in the body. The others are *Blood* (not necessarily the same as Western blood), *Essence*, *Spirit* and *Fluids*. It can be explained as the basic textures and substances of humans.

The main activity of *Blood* is nourishing and moistening of the parts of the body by circulating. While *Essence* is what makes a living being unique from inorganic material, *Spirit* is what separates human life from animal life. *Essence* is responsible for development, birth, aging and decline. At last, *Fluids* are all body liquids other than *Blood*. Their function is, as *Blood,* to moisten and nourish.[2]

1.2.4 The Organs in TCM

Talking about organs in TCM it is not necessarily the same as describing organs in Western medicine. What the Chinese call an Organ is more the function and activities associated by the organ, than the physical form. An organ in Chinese medicine can therefore include more than a Western organ since it is defined by the function. Organs with no physical form can therefore also be described, which would not be possible in Western medicine. Eleven organs are usually recognized in TCM, five *Yin Organs* that produce, store, transform and regulate the fundamental textures, and six *Yang Organs* that receive, break down and absorb food, and excrete unused parts. The *Yin Organs* are *Heart*, *Lungs*, *Spleen*, *Liver* and *Kidneys*. The *Yang Organs* are *Gall Bladder*, *Stomach*, *Small Intestine*, *Large Intestine*, *Bladder* and *The Trippel Burner* (a non-physical organ).[2]

1.2.5 The cause of illness or the pattern of symptoms

The Chinese physician does not really look for the cause of the illness. He looks for *Patterns of Disharmony* by studying the symptoms of the patient. When trying to explain the reason for disharmony, three categories of factors are often described: environment, emotions and way of life. These factors are not only reasons for illness, but also symptoms and description of illness. There is a circular logic in Chinese medicine. In environmental factors, they talk about *Pernicious Influences*, which have some origin from the Theory of Five elements. They are expressed as climatic phenomena; *Wind*, *Cold*, *Heat/Fire*, *Dampness* and *Dryness*, and are usually external factors that invade the body, but they could also arise internally in the body.[2]

1.2.6 Chinese Herbal medicine

While Western pharmaceutical drugs are based on single compounds with specific physiological effect, Chinese herbal medicine are based on herbs, which give rise to wide arrays of compounds and effects. It is not only the usage of the one herb, but also the combination of different herbs that give these wide effects. Herbs are combined, based on their characters, functions and their ability to counteract unwanted effects of each other.[3] The Chinese herbs concept would also include non-botanical substances like insects, minerals and animal parts.

In later years, ready-made formulas for general conditions, called Patent medicine, have also been produced. Raw ingredients or extracts of herbs are formulated into factory made pills. The basic principle of combining different herbs is still used here, but the pills are not tailor made for a specific patient, but for easy use in general illnesses. The products are sold side by side of the natural herbs used for individualized treatment.[3]

Figure 1.2: Chinese Patent medicine (Wikimedia.org)

Nomenclature

The nomenclature of Chinese herbs (in Chinese) is based on several different systems, which are also combined to form names which describes the specific herb. The name may be based on the physical appearance of the herb, for example if it resembles an ear. Specific smell or taste of the herb may be included, and its geographical growth location in China. Plants not indigenous to China could have names indicating where they come from, as *Xi Yang Shen* (Panacis quinquefolii) translated as Western ginseng. Geographical names could often include an indication that the geographical climate has a special impact on the quality of the herb. Other things included in the name could be special properties of the plant's life cycle, the specific plant/animal part, the herbs therapeutic function and colour. The colour is often very important since it also provides information on the therapeutic attributes of the herb.[4]

Classification

With over 10 000 herbs to chose from, the Chinese physician/herbal practitioner needs a system to find the best herbs for his patient. There are, and have been, several ways to classify Chinese herbs over the years. Different ones are used in different texts and combinations are also very much used.[4]

Classification by therapeutic action and side effects

This is a very safe and efficient method of classification. Here the herbs are graded as *superior*, *medium* and *inferior*. Superior herbs have very good therapeutic actions; they have few or no side effects, and they can be used over longer periods. Medium graded herbs have therapeutic benefits and possible side effects, which should be balanced against each other. Inferior herbs usually have a greater amount of side effects and toxicities than benefits. They should only be used when the benefits outweigh the risks.[4]

Classification by source

Not the best system for inexperienced physician, but easy if you know what you are looking for. The herbs are categorized into their source of origin. That is: animal, insects, shellfish, fish, minerals and plants. The botanical herbs can be further classified by which part of the plant they come from.[4]

Classification by organs

Sometimes herbs are classified according to which organs they perform their main effects on.[4]

Classification by therapeutic function

This is the most usual way of classifying since it gives an easy way to find the herbs that matches the disharmony of the patient. The herbs are here classified by the effects they have on the *Fundamental textures (Qi, Blood, Essence, Spirit, Fluids), Yin/Yang and Pernicious Influences.[4]*

- Disperse
- Astringe
- Tonify
- Sedate
- Warm
- Cool
- Moisten
- Dry
- Eliminate toxin
- Move

Tonifying meaning to support and strengthen. Consolidating is like gathering together, and dispersing would mean to move and redistribute.[3]

Characteristics

The characteristics describe different properties of the herbs. They are used together with the therapeutic functions to select and combine appropriate herbs for the treatment, and all of them have to be considered when treating the patient.[3-4]

- ***Thermal property/nature of herbs****: Hot, Warm, Cool, Cold, Neutral*

  A herb can have a warming or cooling character. These are matched with the patient and his illness, where cool/cold herbs are used to treat illnesses that include *Heat,* like fever and sore throat, and vice versa. *Neutral* herbs can be used for both Cold and Hot illnesses.
- ***Taste****: Sour, Bitter, Sweet, Bland Acrid, Salty, Astringent*

  The different tastes correspond often with specific functions, and with organs and parts of the body where the effect is most dominant.
- ***Configuration****: Shape, Texture, Moisture*

  The configuration may mimic parts of the body, and these similarities have sometimes been used to describe functions of the herb.
- ***Colour***

  Colours work in much the same way as the configuration in directing the use of the herbs to specific organs.
- ***Direction****: Upwards, Downwards, Inward, Outward*

  Both herbs and diseases can have some kind of direction or movement, like cough and vomiting which moves upward. The movement of diseases and disharmonies can be matched with herbs that counteract the movement. A herb with specific direction could also be used to "guide" the effect of other herbs in the same direction.

We see that although a herb could purge *Heat* from the body, a more specific herb with *Heat* purging effect on the *Liver* could be a better choice for an illness affecting that organ. A cooling herb with the wrong direction will result in inappropriate treatment. Based on these characteristics, and which organs and textures that are influenced, combinations of Herbs are matched to the patient and his specific *Pattern of Disharmony*.[3-4]

1.2.7 Chinese Herbal Medicine as a source of compounds for Modern Medicine

The history and knowledge of Chinese herbal medicine is as old as the Chinese culture itself, over 3000 years old. The fact that it is still used and is an important part of the Chinese society is a good reason for Western medicine to show interest in its knowledge. The Chinese people themselves are also carrying out a lot of research on the constituents in Chinese herbs.

Western medicine's interest in Chinese herbs, and Chinese interest are not necessarily similar, but both could benefit from each other. Western medicine's main interest is to find new single compounds that could be used as lead compounds to developed new medicine in the west. Although the Chinese has seen the benefit in Western medicine, they still believe in a holistic approach to herbal treatment. Their research is carried out in a Western fashion, but their aim is to figure out how their herbal recipes are synergistically working together. Zaho et al. (2009) focused on this in their review paper on network-based TCM pharmacology study.[5] Chinese medicine is going through a modernization, where modern technology and academic thinking are used at the same time as the traditional theory of TCM is kept alive.

Although TCM is a large source for new lead compounds in drug research, Western medicine could also learn and benefit from the holistic way of thinking, and the Chinese research into the synergistically effects of herbs. Combination therapy has already become an important part of Western treatment of diseases like HIV and cancer.

***Figure 1.3: Traditional Chinese medicine market** (Wikimedia.org)*

1.3 Abies (Fir)

At MRCTCM the genus *Abies* has been extensively investigated, the constituents of different species being thoroughly screened. Their focus has been on *Abies* species that are indigenous to China. Some of the earlier species investigated at MRCTCM include *Abies delavayi* [6], *Abies chensiensis* [7] and *Abies georgei [8]*

***Figure 1.4: Abies koreana** (Wikimedia.org - Jan Mehlich)*

Abies (Fir) is an important genus of the *Pinaceae* family, including about 50 different species, which occur in the highlands of Asia, Europe, North and Middle America and North Africa. 14 of these species are endemic to China.[9] It has earlier been reported that crude extracts and metabolites from *Abies* plants have showed a range of bioactivities. A review article from Yang et al. (2008) at MRCTCM, on studies of constituents in *Abies,* reports bioactivities like antitumor, antimicrobial, antiulcerogenic, anti-inflammatory, antihypertensive, antitussive and CNS activities.[10] The cytotoxic activities observed were from compounds isolated in *Abies sachalinensis* [11] and *Abies Koreana* [12-13].

Different *Abies* species have also been used in folk medicine against sicknesses. In Turkey *Abies cilicica* has been reported used against a wide range of conditions like wounds, vascular diseases, gastric ulcers, bronchitis, common cold and tuberculosis.[14] Usage of *Abies bommülleriana* to help wound healing and against conditions as stomach ache, indigestion, wound healing and common cold has also been reported in Turkey.[15]

Figure 1.5: Abies used in Norway

Yang et al. (2008) reports that the main compounds isolated from *Abies* species are terpenoids. Some flavonoids and lignans have been extracted, and a few other compounds like phenols, steroids, fatty acids and fatty alcohols have been found.[10]

1.3.1 Abies forrestii (Forrest fir)

Abies forrestii is a woody plant that can grow to 20 m. It grows mainly in Sichuan, Yunnan and Tibet provinces of the People's Republic of China and is named after the botanist George Forrest.[16]

Not much research has been done on the chemical constituents of *Abies forrestii.* A study of the essential oil from the needles showed the major components to be the monoterpenes α-pinen, β-pinen and limonene. Other compounds were detected in small amounts.[17]

1.4 Natural Products

The earliest records of plants used as drugs are from the Sumerians in Mesopotamia around 3000 BC, and approximately 40% of today's modern drugs are of natural origin[18]. At the start of the 19th century, morphine was isolated from opium. It was the first pure pharmacological active compound that was isolated from a crude drug, and it was the start of a new era in medicine with isolation and chemical identification of the active compounds in medicinal plants[18]. The idea of pure compounds is today one of the basic principles in Western medicine.

Many natural products, especially from plants, have been used as drugs or lead compounds for drugs. Paclitaxel (Taxol®), a diterpene first isolated from the yew *Taxus brevifolia* and now widely used in cancer treatment, is one of many examples of this.[18] Records of traditional use of plants as drugs have been, and are still an important factor in drug discovery and development. Research into this is called ethnopharmacology. Chinese medicine with records of more than 10 000 herbal remedies[4], is an enormous source for discovering new bioactive compounds. The antimalaria drug artemisinin was first isolated from the plant *Artemisia annua,* which in Chinese medicine are used to treat malaria.[18]

1.4.1 Terpenes

Terpenes are a class of hydrocarbons, with a wide variety of compounds produced by many sorts of plants. They are formed by branched C_5H_8 units called isoprene, or isopentenyl diphosphate (IPP) when activated (see Figure 1.6). Different types of terpenes are formed by combination of two or more isoprene units: monoterpenes (C_{10}), sesquiterpenes (C_{15}), diterpenes (C_{20}), sesterterpenes (C_{25}), triterpenes (C_{30}) and tetraterpenes (C_{35}). Plant steroids could be described as terpenes as they are regarded as derivatives of triterpenes.[18]

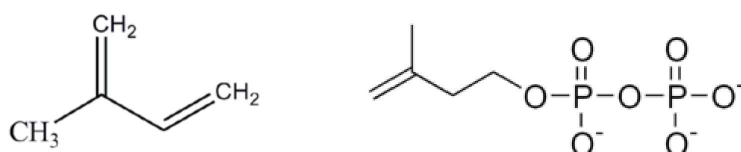


Figure 1.6: Isoprene and Isopentenyl diphosphate

Diterpenes

Diterpenes are one of many important natural products, found both as single compounds and as basic components of other larger important molecules, as chlorophyll. They exist both as straight carbon chains and as cyclic components. One good example of straight chain diterpenes is phytol, which is one component of vitamin E and vitamin K_1. Mono-, di-, tri-, and tetracyclic diterpenes can all arise from the same biosynthetic pathway. Diterpenes usually originate from the acyclic biosynthetic precursor geranylgeranyl diphosphate (GGPP), which is a combination of four IPPs.[18]

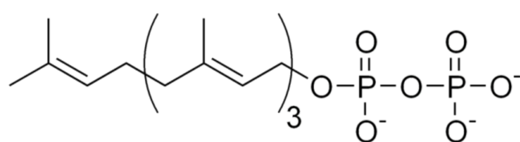


Figure 1.7: Geranylgeranyl diphosphate (GGPP)

There have been proposed cyclication reactions from GGPP to cyclic diterpenes and further on to corresponding alcohols, aldehydes and acids.[18-19] An example for tricyclic *Abietic acid* is shown in Figure 1.8

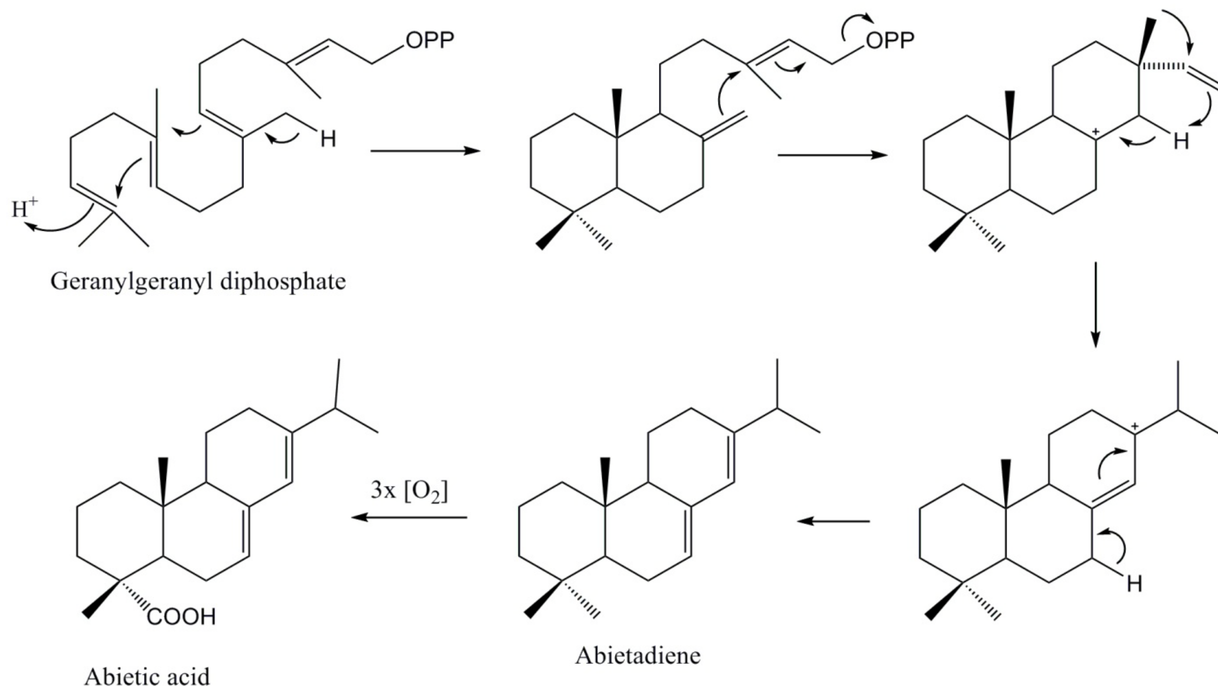


Figure 1.8: Proposed reaction mechanism for biosynthesis of abietic acid from geranylgeranyl diphosphate[18-19]

Triterpenes and steroids

Triterpenes are C_{30}-compounds formed from the precursor squalene (Figure 1.9), which is a combination of six IPPs. Squalene goes through different cyclication reaction usually forming pentacyclic and tetracyclic structures. The tetracyclic compound cycloartenol (Figure 1.10) is the precursor for further biosynthesis of steroids in plants.[18]

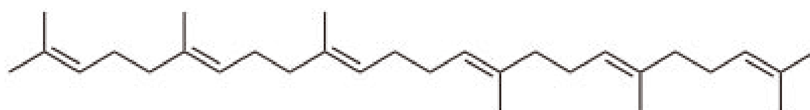


Figure 1.9: Squalene

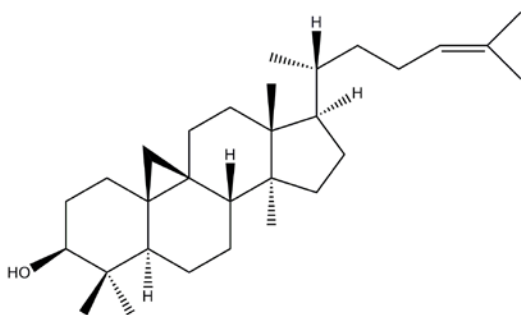


Figure 1.10: Cycloartenol

From triterpenes, compounds like the antibiotic fusidic acid, cardiac glycosides, used in the treatment of congestive heart failure, and vitamin D are formed. Fusidic acid is produced by the fungus *Acremonium fusidioides,* and cardiac glycosides can be found in Foxgloves (*Digitalis purpurea*).[18]

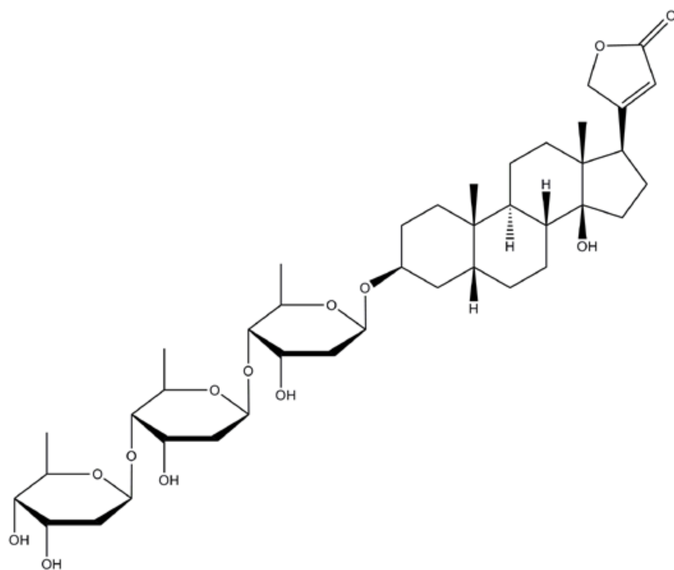


Figure 1.11: The cardiac glycoside digitoxin from Digitalis purpurea

2 Materials

2.1 Chemicals

All standard chemicals used in the experiments, were provided by one of these manufactures:

- Jiangsu Qiangsheng Chemical Co., Ltd, Changshu city, China
- Shanghai Zaituo Trade co., Ltd, Shanghai, China
- Changshu Yangyuan Chemical Co., Ltd, China
- Sinopharm Chemical Reagent Co., Ltd, Shanghai, China

NMR solvent

- Chloroform-D (D, 99.8%)+0.03% v/v TMS; *Tenglong Weibo Technology Co., Ltd, China*

Abbreviations used for some common chemicals

A - Acetone

C – Chloroform

D – Dichloromethane

E - Ethylacetate

EtOAc – Ethylacetate

EtOH – Ethanol

M - Methanol

MeOH – Methanol

P – Petroleum ether

2.2 Apparatuses and equipment

Rotary evaporators and water baths

- Heidolph Laborota 4000 and Water bath
- Eyela Rotary Evaporator N-1000 and Water bath SB-2000
- Senco *Rotary Evaporator and Water bath*

UV-lamp emitting light at 254 nm or 365 nm

- Shanghai Jingke Industrial Co. Ltd, WHF-203

Spectroscopic apperatures

- Bruker Avance 600 NMR spectrometer

- Agilent LC/MSD Trap XCT mass spectrometer

Stationary phases for column chromatography

- Silica gel, 100-200 μm and 10-40 μm; *Huiyou Silica Gel Development Co. Ltd., Yantai, PR China*
- YMC-GEL ODS-A, 50 μm; *YMC, MA, U.S.A*
- MCI-GEL CHP20P, 75-150 μm; *Mitsubishi Chemical Corporation*
- Sephadex LH-20, 40-70 μm; *Amersham Pharmacia Biotech AB, Uppsala, Sweden*

TLC-plates

- Baocengxiguijiaoban
  - Silica diameter: 10-40 μm
  - Layer thickness: 0.15-0.2 mm (A-TLC), 0.4-0.5 mm (P-TLC)

3 Methods and procedures

During the experimental procedure, different chromatography and spectroscopic methods were used. Here the methods and the basic procedures are described. The experimental work is described more thoroughly in chapter 4.

3.1 Chromatography

The basic principle of chromatography is the distribution of compounds between two phases, a mobile phase that passes over/through a stationary phase. This method can be used for separation of compounds with different characteristics that distribute differently between the phases.[20]

The stationary phase can be either a solid or a liquid, and the mobile phase may be a liquid or a gas. The different kinds of chromatographic techniques can be classified from the nature of the phases used. Here liquid chromatography has been used, that is chromatography with a liquid as the mobile phase. Liquid chromatography is well suited, and is a much used technique for preparative isolation of natural compounds.[20]

It can be useful to classify the chromatographic techniques based on the mode of separation. The molecules can be separated based on their differences in size, biological specificity, intermolecular bonding, molecular charges and solubility. The most relevant techniques are adsorption, partition, size-exclusion and ion-exchange. Techniques used in this experiment are described beneath.

3.1.1 Normal phase adsorption chromatography

In adsorption chromatography, there is a dynamic equilibrium of the solutes between the stationary phase and the mobile phase. It changes throughout the length of the column, so a stable equilibrium is never achieved anywhere in the column. Molecules are constantly adsorbed and desorbed, a process that is different for each type of solute depending on their physical properties and ability to form intermolecular bonds (hydrogen bonds, van der Waals forces, dipole-dipole interactions) with two phases.[20]

In normal phase adsorption chromatography, the stationary phase is more polar than the mobile phase and will interact more strongly with the most polar molecules than with molecules with lower polarity. A normal phase stationary phase will therefore retain polar compounds longer, and the less polar compounds will elute first.[20]

Silica-Gel

Silica gel is a polymer of silicon oxide ($SiO_2.H_2O$), forming a three dimensional structure. The silica gel structure has exposed silanol groups on its surface, giving it a polar nature and opportunity to form strong hydrogen bonds with compounds. Polar compounds that easily form hydrogen bonds, as carboxylic acids, amines and amides, will be more strongly retained on the silica gels than other less polar compounds. Water and methanol have the potential to dissolve silica-gel itself and it could contaminate the eluates. Silica gel used for open column chromatography is usually in the size range of 40-200 µm. Smaller particles can give a high backpressure, meaning that the flow of eluent is reduced because of the tight packing in the column, and the elution would take longer.
This can be overcome by using pressure, as in high pressure liquid chromatography (HPLC), or vacuum suction.[20]

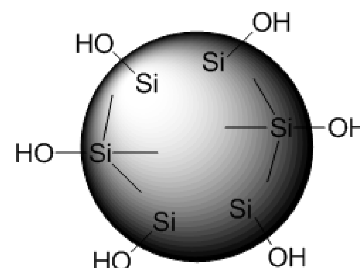


Figure 3.1: Illustration of Silica gel

Procedure

For CC over silica gel, a wide variation of mobile phase was used. By using analytical TLC, an appropriate mobile phase was selected with an Rf value of approximately 0.2 for the target compound. The sample was dissolved in a solvent and an appropriate amount of silica gel was added. The minimum amount of silica gel should be the same weight as the sample, and the maximum should not be more than twice the weight of the sample. By using a smaller amount of silica gel for the sample, there would be less to load in the column and it would be more time efficient. The mixture of sample and silica was dried using a rotavapor, fitted with cotton to avoid dry silica being sucked away. The sample would adhere to the silica.

A column of appropriate size was selected on basis of the amount of silica to be used. The amount of silica should be a minimum of 10 times, and maximum of 30 times the weight of the

silica-sample. A larger amount of silica-gel would give a better separation of compounds that are hard to separate, but the column would also take a longer time to complete.

The silica gel was added to the column and packed by using vacuum suction. The surface of the silica should be flat and smooth. Then the silica gel sample was loaded on to the top, and a small amount of silica was added on top of it for protection, before adding the mobile phase.

The column was eluted by use of vacuum suction, and fractions of appropriate size, determined by the size of the column, were collected. By using TLC, the endpoint of the column was detected when there were no more compounds emerging. Acetone was then used to wash out any remaining compounds.

3.1.2 Reverse phase adsorption chromatography

Reverse phase chromatography utilizes the same principles as normal phase, but the phases are the other way around. The mobile phase is the more polar phase and the stationary phase is the less polar one, retaining the compounds with the lowest polarity.[20]

Octadecasilyl (ODS)

Octadecasilyl is a bonded phase silica, which means that the physical properties of the silica have been altered by reaction and bonding of the silanol groups. Here the silanol groups have been octadesilylated, which means that 18 carbon alkyl chains have replaced the silanol groups on the surface, giving the silica a high degree of hydrophobicity. A potential problem with bonded phase silica is non-reacted silanol groups that would reduce the hydrophobicity. These groups can to some degree be deactivated by end-capping, meaning reacting them with a small silating agent such as trimethylchlorosilane (see Figure 3.2).[20]

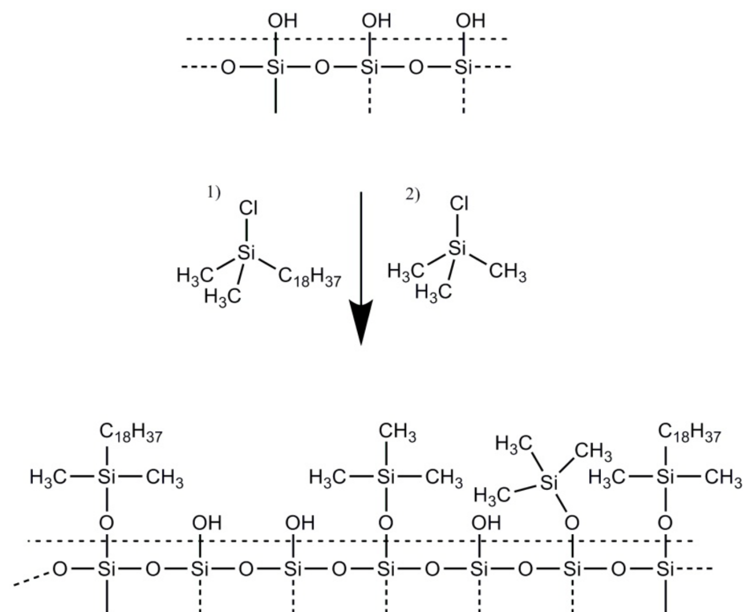


Figure 3.2: Preparation of end-capped octadecasilyl (ODS) in two steps

Procedure

For CC over ODS, a graduated system of methanol and water was used. A 1000 ml column with 800 ml ODS was prepared and saturated with the first mobile phase. The liquid front was lowered to the ODS-surface before loading the sample dissolved in 100% methanol. The liquid front was again lowered to the ODS surface, letting the sample settle on the ODS. Then more mobile phase was carefully added, and the column was eluted.

MCI Gel polystyrene

The polystyrene used here is a styrene-divinylbenzene copolymer with reverse phase capabilities (see Figure 3.3). Compared to silica-based reverse phase materials, polystyrene-based materials are not as expensive and the problem of exposed silanol groups is avoided. [20]

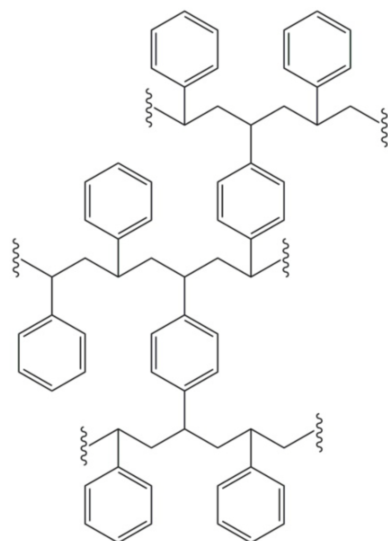


Figure 3.3: Styrene-divinylbenzene copolymer

Procedure

For CC over MCI, a graduated system of methanol and water was used. A 1500 ml column with 1300 ml MCI-gel was prepared and saturated with the first mobile phase. The liquid front was lowered to the MCI-surface before loading the sample. The sample is not always soluble in the first mobile phase, and a large sample then has to be loaded in small steps to avoid the top of the column getting to a high methanol concentration, washing and adding the correct mobile phase between each step. After loading the sample, more mobile phase was carefully added and the column was eluted using vacuum suction.

3.1.3 Size exclusion chromatography

In size exclusion chromatography, compounds are separated based on their differences in size. The chromatography particles have surface-pores, which molecules can penetrate, and can thereby be retained. Small molecules will penetrate the pores more easily than large molecules, and they will therefore have a longer way to travel and be more retained than the larger molecules.[20]

Sephadex LH-20

Sephadex is a water-insoluble polymer of carbohydrates, formed by crosslinking of water-soluble dextran with epichlorohydrin. Since it is very hydrophilic, it swells in water and this swelling has an influence on the size exclusion properties of the gel.[20]

Sephadex LH-20 is a hydroxypropylated Sephadex G-25. The number in the Sephadex G-series is the amount of water picked up when the Sephadex is swelling. Sephadex G-25 picks up 2.5 ml/g of dry beads. A lower number therefore indicates a higher density and is suitable for smaller compounds. The hydroxypropylation gives Sephadex LH-20 a more lipophilic character, but retaining the hydrophilicity. The added lipophilicity gives the gel the property of adequate swelling in organic solvents.[20]

When using one single solvent as mobile phase, the separation is based on size exclusion. When using a solvent mixture, the most polar solvent will be taken up by the gel, forming a two phased system. Chromatography with a solvent mixture will therefore not only give separation based on size, but also by partition in the different phases.[20]

Procedure

For CC over Sephadex LH-20, a mixture of dichloromethane and methanol in a ratio of 1:1 and a column of 600 ml was used. A maximum of 2 g sample in a mixture of dichloromethane and methanol in a ratio of 1:1 was applied to the top. Approximately 200 ml were collected before starting to collect fractions of appropriate sizes. This first eluted 200 ml was tested with TLC to confirm that there were no compounds in it. By using TLC, the endpoint of the column was detected when there were no more compounds emerging.

3.1.4 Thin-Layer Chromatography (TLC)

Thin-layer chromatography (TLC) is a form of what we call planar liquid chromatography, which is the separation of a mixture on a thin layer of adsorbent percoated on a plate; glass, aluminium or plastic. TLC is a very cheap and quick method to separate a mixture, and since it only needs a small amount of sample and not much equipment; it is widely used in natural product isolation, both analytical and preparative.[20]

In the experiments here, the TLC-plates were percoated with silica and therefore utilizing the earlier described principles of adsorption chromatography (see chapter 3.1.1). A sample is applied to the plate with the stationary phase, and the plate is placed in a small bath of mobile phase just covering, and wetting the edge of it. The mobile phase migrates up the plate by capillary forces, developing the plate by moving the compounds of the sample. On silica-coated plates, polar compounds will be more retained than nonpolar compounds and the different compounds can be separated.[20]

TLC can be used preparative to separate the compounds; it can be used analytically to decide which mobile phase would be suitable for column chromatography of the sample, to monitor the separation process, and to compare different samples/compounds.

For a specific system of sorbent and solvent, an Rf-value can be used to describe the migration of compounds.[20]

$$R_f = \frac{Compound\ distance\ from\ origin}{Solvent\ front\ distance\ from\ origin}$$

Analytical TLC

As mentioned, analytical TLC can be used to decide on a suitable mobile phase for CC, compare different samples and monitor the separation process. When deciding on a mobile phase for CC, TLC can give an idea on how the compounds will separate in the column. For silica columns for example, a Rf-value for the corresponding TLC between 0.3 and 0.7 would give a good separation. When comparing samples and assessing the purity of a compound it is always a good rule to test a compound with two different solvent systems, since an apparently pure spot could be a mix of two compounds with identical Rf-value in that specific solvent system.

There are many different methods to detect compounds on a TLC-plate after it has been developed, both nondestructive, where it is possible to recover the compound afterwards, and destructive methods. UV-detection, which is nondestructive, is one common method to visualize compounds. An UV-active compound, which has been mixed into the adsorbent on the TLC-plate, will emit a pale coloured light when exposed to UV-light of the right wavelength (usually 254 and 365 µm). Compounds that absorb light at the specific wavelength appear as darks spots against the light background. Longwave UV-light, as 365 µm, will also detect compounds that fluoresce, for example chlorophyll.

Iodine adsorbs as a yellow/brown colour to most compounds, so by placing a developed TLC plate in an iodine atmosphere, an enclosed jar with iodine crystals, visualizing is possible. In the majority of cases iodine is reversible bound to the compound and will rapidly evaporate when removed from the jar.

Spray detection is a destructive method where a reagent, which is sprayed on to the plate, reacts with the developed compounds. There are different sprays that react with specific types

of compounds and other ones that react with a wide variety of classes visualizing most of the compounds on the plate. Here a 10% sulphuric acid ethanol solution was used as a wide variety-visualizing reagent.

Silica gel has some disadvantages when analyzing acidic and basic compounds. Especially acidic compounds have a tendency to “tail” on silica, and they will therefore not give clear spots. This is because of interactions between the acidic groups and the silanol on the silica. It can be resolved by adding a small amount of acid (e.g. acetic acid) to the mobile phase, which will keep the acidic groups in their nonionized form. A weak base would do the same for a problematic basic compound.

Preparative TLC (P-TLC)

Preparative TLC could be described as a large scale TLC. It has to some degree been replaced by more advanced techniques such as HPLC (high-pressure liquid chromatography), but because of the low cost, speed and simplicity of the method, it is still in common use. The possibility to separate compounds in small amounts in the range from 1 mg to 1 g is very useful. The normal use of P-TLC would be for the final purification and separation of a mixture with few compounds.[20]

In comparison to analytical TLC, which is carried out on plates with 0.1-0.2 mm sorbent thickness, P-TLC plates have 0.5-4 mm sorbent thickness. A scale up from analytical TLC to P-TLC is not straightforward, and often includes some trial and error. In many cases, the polarity of the mobile phase has to be reduced to achieve the same Rf-values as in analytical TLC.[20]

Procedure

By using analytical TLC, an appropriate mobile phase was selected with an Rf value of approximately 0.5. The sample was dissolved in the minimum amount of solvent and applied in a straight line approximately 1.5 cm above the bottom of a silica coated PTLC glass plate. The amount of sample applied on one plate should be between 50-100mg.

20-30 ml of the mobile phase was added to one chamber in a development tank. The plate was placed in a second chamber for 20 min, to let the evaporating mobile phase saturate the atmosphere and the plate, before moving it to the chamber with mobile phase for development.

UV-lamp detection was used to detect UV-light absorbing compounds. By removing a small part of the edge of the plate and using H_2SO_4 spray reagents on this, it is possible to get an idea of how the compounds have migrated.

The plate was divided into different fractions, based on the results from the UV- and spray reagent detection, and the silica gel was scraped off. The fractions were ground to a fine powder of silica gel and mixed with 10 ml of silica gel (100-200 mesh) to make the sample more permeable for solvent in the column. A column was packed with 20 ml silica gel (100-200 mesh) together with the fraction, and eluted five times with 70 ml acetone.

3.2 Analytical spectroscopic methods

NMR and MS were performed on the isolated compounds by the analytical department at SMMU. The samples were dissolved in chloroform-D.

3.2.1 Nuclear Magnetic Resonance (NMR) spectroscopy

NMR is a technique used to study the number of specific magnetic atoms in a compound, in this case hydrogen-1 and carbon-13 nuclei. It can also provide information of the immediate environment of the distinct nucleus.[21]

Every nucleus has a charge and some have the property of spin, they behave as they are spinning. The property of spin and charge together, generates a magnetic field, giving the nucleus what we call a magnetic moment. The nucleus's magnetic field can be affected by an applied magnetic field. This will make the spinning nucleus precess about its own axis with an angular frequency ω, the Larmor frequency, which is proportional to the strength of the applied magnetic field. The precession generates an oscillating electric field with the same frequency. The electrical field will absorb energy from an electromagnetic wave with identical frequency, giving a spin change. We say it resonates with the electromagnetic radiation.[21]

In NMR, the sample is placed in an applied magnetic- and electromagnetic field and thereby generating the resonance. The electrons around a nucleus generate a magnetic field that shields the nucleus from the applied magnetic field. Similar nuclei will have slightly different electronic environments dependent on their chemical environment, and will resonate at slightly

different frequency. By measuring these differences, we can learn something about chemical environments of the nuclei and the structure of the compound.[21]

It is difficult to measure exact resonance frequencies. Instead, the resonance is measured relative to a reference compound. The resonance of a nucleus in a compound is therefore reported as how far it is shifted from the reference compound. The universally used standard reference compound tetramethylsilane (TSM), $(CH_3)_4Si$, is added to the sample. The shift is dependent on the applied magnetic field, and a field-independent measure, chemical shift (δ), is therefore obtained by dividing the shift (Hz) with the spectrometer frequency (MHz).[21]

$$\delta = \frac{shift\ in\ HZ}{spectrometer\ \ frequency\ \ in\ MHz}$$

^{1}H

^{1}H has two spin states and is the most common nucleus studied in NMR.

In a NMR-spectrum, the area under a peak is proportional to the number of nuclei generating the peak. Integrals of the peaks are therefore measured to give an indication of how many nuclei the molecules contain of each type. This is widely used for ^{1}H-NMR spectrums since it gives easy and reliable results. As for ^{13}C NMR spectrums, integrals are not that commonly used because they are less reliable.[21]

A signal can be split into multiplets of peaks, spin-spin splitting. This arises because neighbouring nuclei with spin have different spin states in the molecules of the solution. These small differences give different magnetic environments in the molecules and will therefore give rise to small differences in the chemical shift. In ^{1}H NMR spectroscopy, spin-spin splitting is common between hydrogen on neighbouring carbons (three-bond distance). The protons are coupled to each other and the signals are split into $(n+1)$ signals, where n is the number of protons on neighbouring carbons. The splitting, the distance between the peaks, are described by the coupling constant, J, expressed in HZ. Two hydrogen signals splitting each other will have the same coupling constant and it can therefore be used to deduce the structure of the molecule.[21]

^{13}C

The dominating carbon-isotope in nature is ^{12}C, but it is NMR inactive since it does not have any spin or magnetic moment. The ^{13}C-isotope has, however, a magnetic moment, but since the

natural occurrence is very low and the magnetogyric ratio of ^{13}C also is low, the resonance is weaker compared to hydrogen and more difficult to detect.[21]

Since the probability of two ^{13}C atoms being adjacent to each other in the same molecule is very slim, we rarely see homonuclear (carbon-carbon) splitting. Instead, we can observe heteronuclear (carbon-hydrogen) splitting. Since the coupling constants for this splitting are relatively large, in the range of 100-250 Hz, the multiplets often overlap with one another. Therefore, most carbon spectrums are obtained as proton-decoupled spectrums where only singlets are observed. The decoupling could also intensify some signals and thereby improve the signal-to-noise ratio.[21]

The proton-decoupled spectrums lose information on numbers of hydrogens attached to the carbons. A technique called DEPT (Distortionless Enhancement by Polarization Transfer) can be used to obtain this information. The DEPT experiment used here are DEPT-135, which generates spectrums of single peaks, which are either positive or negative. Carbon atoms with odd numbers of hydrogen attached to them, methines and methyls, appear as negative peaks, whereas carbons that bear even numbers of hydrogen, methylenes, appear as positive peaks. Quaternary carbons, unprotonated ones, give no signal in the DEPT-135 specter.[21]

3.2.2 MS (Mass Spectrometry)

Mass spectrometry is a way to determine the molecular mass of a compound. We can also compare fragmentation patterns of the compound.

The basic principles in mass spectrometry involve converting molecules to ions and then accelerating them into an analyzer, which separates them according to their mass-to-charge ratio (m/z). Since the charge on most of the formed ions is one, m/z describes the mass of the ions.[21]

There are different ways to introduce compounds to the mass spectrometer and different ways to ionize and detect them. Here we have used a liquid chromatography-mass spectrometer (LC/MS) with electrospray ionization (ESI) and ion trap. The LC separates compounds in the sample before introducing it to the mass spectrometer, thereby achieving a purer sample. ESI is often used for natural products since it generates less fragmentation and gives better results for large compounds.[22] Since we here are most interested in the mass of the compound, the molecular ion (M^+), less fragmentation is preferable. Both positive and negative ions can be

measured from the ESI. Positive ions are often a complex of one or more sample molecules and ions from the solution, as sodium and hydrogen ($M+H^+$, $M+Na^+$), and negative ions are often the sample molecules without one hydrogen ($M-H^-$).[22]

3.3 Natural Products Library at MRCTCM

As mentioned in the introduction (chapter 1.1.1), MRCTCM has developed their own Natural Products Library with more than 3000 purified single compounds and 7100 standard TCM extracts. Every compound purified from a new plant will be included in the library.

All information of the compounds stored in the library is organized in a MDL ISIS Database, which is a searchable system for managing chemical and biological information. With this database, it is possible to do quick and flexible queries according to specialised terms for natural products like phenotype, chemical structure, bio-activities, plant source and TCM usage.

Figure 3.4: Natural Products Library at MRCTCM

4 Results and discussion

At the Modern Research Center of Traditional Chinese Medicine, chemical compounds are extracted from herbs and plants. Biologically active compounds are targeted and the goal is to map the different potential active compounds in the herbal drug. This is very time-consuming, and when I arrived at the laboratory, the group was already working on the plant *Abies forrestii*. I joined them in their work and was given a fraction (**E_2a**), derived from the laboratory's earlier work, and compounds were purified and isolated from this starting material.

4.1 Plant material, extraction and separation

The aerial parts of *Abies forrestii* were collected from Bayi, Linzhi City, Tibet, in August 2008 and authenticated by Prof. Han-Ming Zhang in the Department of Pharmacognosy, Second Military Medical University. A voucher specimen was deposited at the herbarium of School of Pharmacy, Second Military Medical University, Shanghai, People's Republic of China.

The plant material (7.7 kg) was dried and then pulverized. The pulverized material was extracted three times with 80% ethanol (EtOH) under reflux, each for 3 hours. The extracts were combined and concentrated to a smaller volume. It was performed liquid-liquid extraction with ethylacetate (EtOAc) of the extract. The EtOAc extract was separated into seven subfractions (E_{1}-$_{7}$) by column chromatography (CC) over silica gel (100-200 mesh) with gradient elution (P:C, C, C:M=95:5, C:M=9:1, C:M=8:2, C:M=7:3, C:M=5:5). Fraction E_2 was filtrated, and the filtrate was subjected to CC over MCI gel with 90% methanol (MeOH), 100% MeOH and Acetone, giving three subfractions, respectively E_2a-b.
A flowchart of the extraction and separation is shown in Figure 4.1.

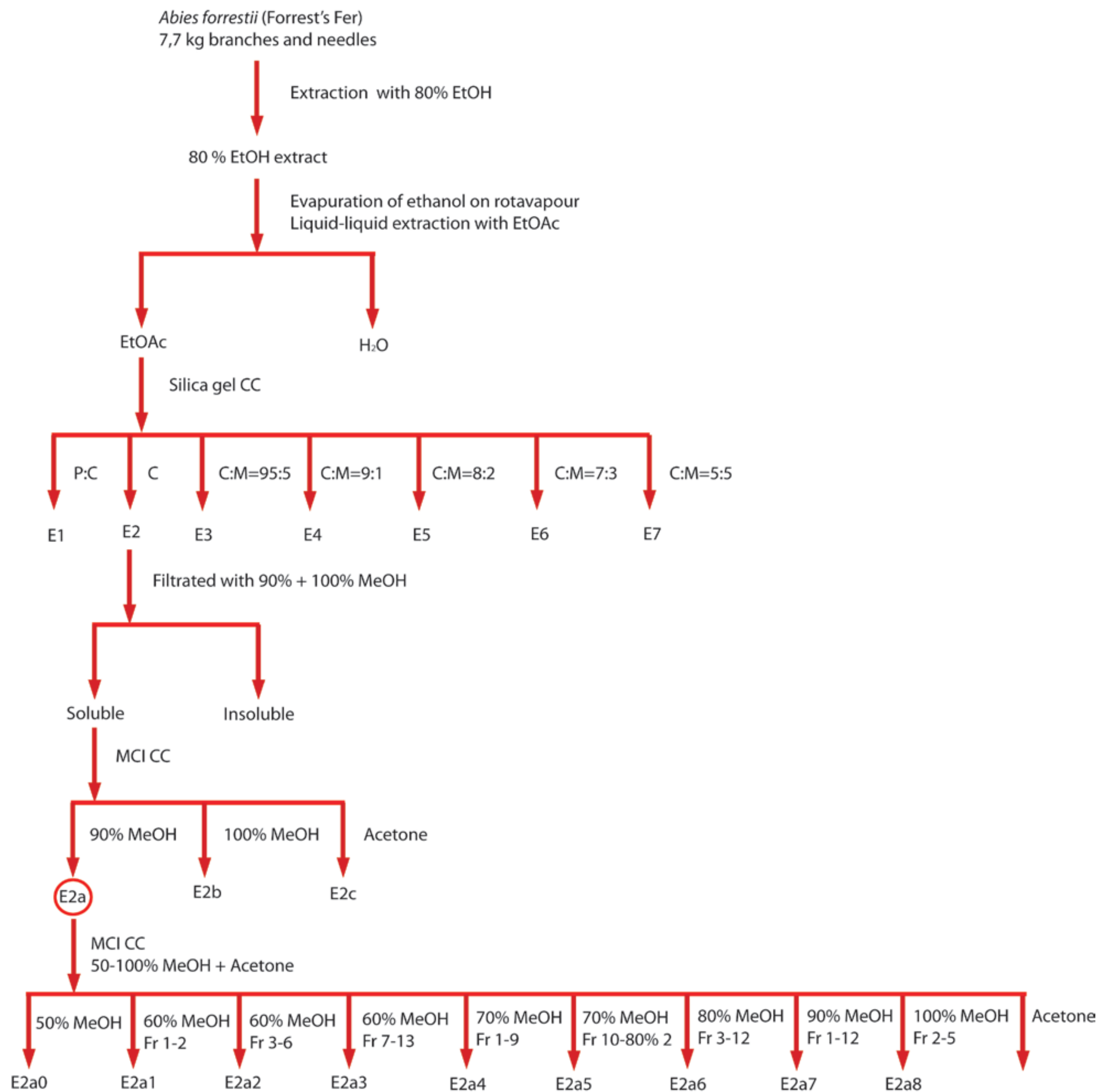


Figure 4.1: Extraction and separation for Abies forrestii

4.2 Isolation and purification

Figure 4.2 shows a flowchart of the isolation and purification from E2a.

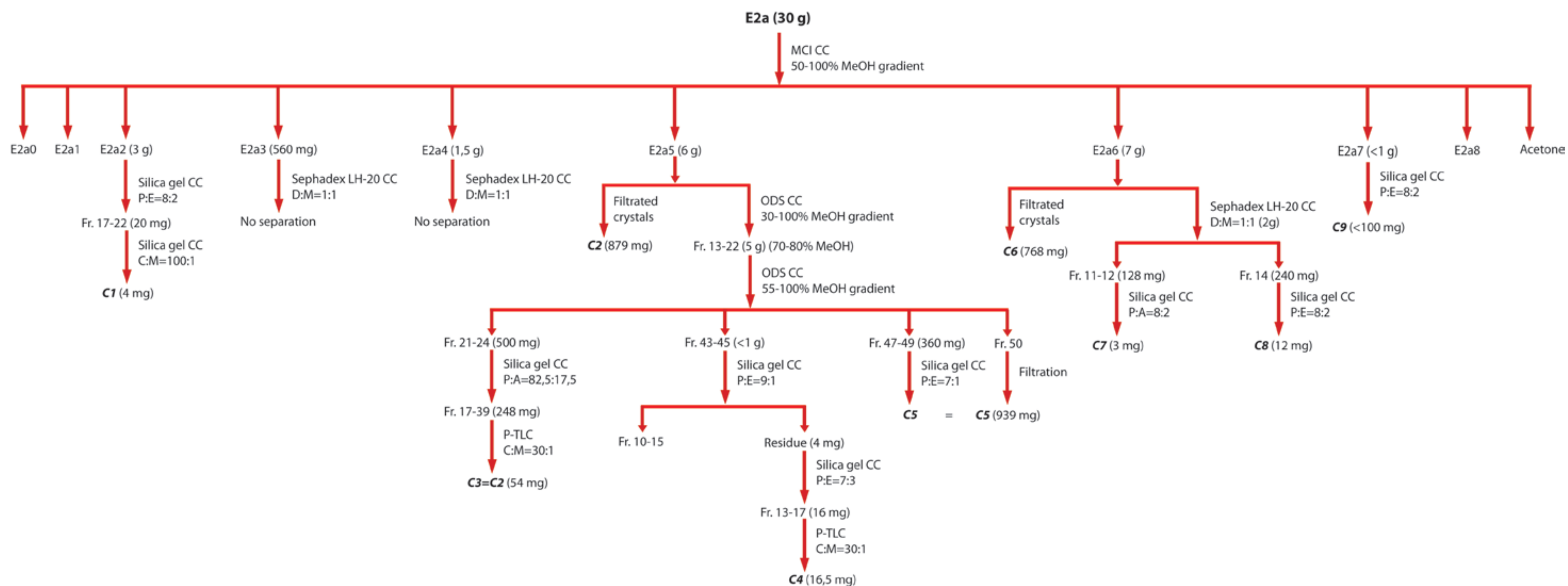


Figure 4.2: Isolation and purification of compounds in fraction E2a from Abies forrestii

4.2.1 E2a - MCI

This fraction was subjected to size exclusion CC over MCI-gel. The column was prepared and saturated with a 50% solution of methanol to water.

Fraction **E2a** (30 g) was dissolved in 100% methanol (it could not dissolve in 50% methanol) and loaded to the column in small steps. The column was eluted with 12x500 ml for each mobile phase, except the last one, which was eluted with only 5x500 ml. The fractions were dried on a rotavapor (50 ^{o}C, 90 rpm) and then dissolved in 100% methanol. It was used a graduated mobile phase system of methanol and water v/v: 50% methanol, 60% methanol, 70% methanol , 80% methanol, 90% methanol, 100% methanol, 100% acetone.

TLC of the fractions and combining of similar fractions gave 9 subfractions, $E2a_{0\text{-}8}$.

4.2.2 E2a2

4.2.2.1 E2a2 - Silica

For this fraction, **E2a2** (3 g), normal phase CC over silica-gel with dry loading was used obtain one of the compounds of the fraction.

TLC with P:E=8:2 showed the target spot with Rf=0.21. A 300 ml column packed with 200 ml silica gel (10-40 µm) was used. The sample silica was prepared with 2X10 ml silica gel (100-200 mesh). 55 fractions were collected, where fraction number 1 was 100 ml, number 2-50 was 20 ml and number 51-55 was 50 ml. Acetone was used to wash out the remaining compounds in the column.

TLC of the fractions with P:E=7:3, showed a main single spot in fractions 17-27, and two spots from fraction 28. Fractions 17-22 were thought to be the most pure and were combined to one, but TLC with D:M=50:1 showed an extra spot in addition to the target spot.

4.2.2.2 E2a2 – Silica (17-22) – Silica

For this fraction, **E2a2** $_{\mathbf{(17\text{-}22)}}$ (20 mg), it was again used normal phase CC over silica-gel with dry loading to purify the compound.

TLC with C:M=100:1 showed the target spot with Rf=0.5. A 300 ml column packed with 200 ml silica gel (10-40 µm) was used. The sample silica was prepared with 2X1 ml silica gel (100-200

mesh). 25 fractions of 20 ml were collected and acetone was used to wash out the remaining compounds in the column.

TLC of the fractions with C:M=100:1, showed a main single spot in fractions 9-19, strongest from 8-11. Fractions 9-15 were thought to be the most pure and were combined to one. The combined fraction was tested on TLC with C:M=100:1, which confirmed it as one purified compound with UV-absorption at 254 nm, and iodine adsorption. The weight was measured to be 4 mg, and the compound, named **C1,** was prepared for NMR- and MS-detection.

4.2.3 E2a3

4.2.3.1 E2a3 – Sephadex LH-20

For this fraction, **E2a3** (560 mg), size exclusion CC over Sephadex LH-20 was used to separate the compounds. 34 fractions of 10 ml each were collected.

TLC of the fractions with C:M=20:1 showed compounds from fraction 15-21, but these were not very well separated. All the fractions were combined again.

4.2.4 E2a4

4.2.4.1 E2a4 – Sephadex LH-20

For this fraction, **E2a4** (1.5 g), size exclusion CC over Sephadex LH-20 was used to separate the compounds. 40 fractions of 10 ml each were collected.

TLC of the fractions with C:M=20:1 did not show any good separation of the compounds and all the fractions were combined again.

4.2.5 E2a5

In fraction **E2a5,** some crystals had crystallized. The fraction was filtrated by use of vacuum suction and filter paper, and the crystals were washed with the filtrate. The crystals were obtained as a yellow amorphous powder. It was tested on TLC and detected with H_2SO_4 spray reagent, showing one single spot. The weight of the crystals was 879 mg and the compound

was given the name **C2.** It was prepared for MS- and NMR-detection dissolving it in chloroform-D.

4.2.5.1 E2a5 - ODS

This fraction, **E2a5** (6 g) was subjected to reverse phase CC over ODS to separate the compounds. The column was eluted with a graduated mobile phase system of methanol and water v/v: 30%, 50%, 70%, 80%, 90% and 100%, collecting 8 fractions of 250 ml for each mobile phase.

TLC with C:M=20:1 showed that all the compounds had eluted in the fractions 13-22 (70%-80% methanol), but they were not very well separated.

4.2.5.2 E2a5 – ODS (13-22) – ODS

For the fraction, $\mathbf{E2a5_{(13\text{-}22)}}$ (5 g), reverse phase CC over ODS was used to separate the compounds of the fraction. The column was eluted with a graduated mobile phase system of methanol and water v/v: 55%, 60%, 65%, 70%, 75%, 80% and 100%, collecting 10 fractions of 250 ml for each mobile phase.

TLC with C:M=20:1 was used to determine which fractions should be combined with each other, and which fractions were suitable for further separation and purification. Fraction 21-24 looked promising for further purification. In addition, fractions 43-45, and 47-49 showed compounds that could be easily separated.

4.2.5.3 E2a5 – ODS (13-22) – ODS (21-24) – Silica gel

The fraction $\mathbf{E2a5_{(13\text{-}22)\text{-}(21\text{-}24)}}$ (500 mg) was subjected to normal phase CC over silica gel to obtain one of the compounds of the fraction.

TLC with P:E=82.5:17.5 showed the target spot with Rf=0.21. The target spot was brown when sprayed with H_2SO_4. A 300 ml column packed with 200 ml silica gel (10-40 µm) was used. The sample silica was prepared with 2X4 ml silica gel (100-200 mesh). 46 fractions, where fraction number 1 was 100 ml, number 2-35 was 20 ml and number 36-46 was 40 ml, were collected. Acetone was used to wash out the remaining compounds in the column.

TLC of the fractions with C:M=20:1, showed a main spot in fractions 13-45, getting weaker from fraction 35, and two spots in fractions 19-39. The spots did not show any UV-absorption at this point. Fractions 17-39 were combined and the solvent evaporated, giving the mass of the fractions to 248 mg.

The combined fractions were plotted on two P-TLC plates, 80 mg on each, which was developed with C:M=30:1. UV-detection showed more than the two compounds seen earlier. The plate was divided into three fractions based on the result of the H_sSO_4 spray reagent and UV-detection. After extraction and filtrating, the fractions were tested on TLC with C:M=20:1 and P:E=7:3. One of them seemed to be a pure compound. It was a yellow amorphous powder, which adsorbed iodine, but not UV light. The weight was determined to be 54.4 mg, and it was named **C3.** Later comparison to **C2**, using TLC, showed them to be the same compound**.**

4.2.5.4 E2a5 – ODS (13-22) – ODS (43-45) – Silica gel

This fraction, **E2a5**$_{\mathbf{(13\text{-}22)(43\text{-}45)}}$ (<1 g), was subjected to normal phase CC over silica gel to obtain one of the compounds of the fraction.

From TLC it was found that P:E=9:1 gave Rf=0.25 for the target spot, the spot with the largest Rf value of two main spots. A 300 ml column packed with 160 ml silica-gel (10-40 µm) was used. The sample silica was prepared with 2X3 ml silica gel (100-200 mesh). 24 fractions, where fraction number 1 was 40 ml, number 2-16 was 20 ml and number 17-24 was 30 ml, were collected. Acetone was used to wash out the remaining compounds in the column.

TLC of the fractions with P:E=8:2 showed a single spot in fractions 7-16, and two spots in fractions 18-24. Fractions 10-15 were believed to be the most purified fractions, and these were combined and then tested on TLC with P:E=8:2 and P:A=8:2. P:A separated the spot and showed several compounds in the fraction.

4.2.5.5 E2a5 – ODS (13-22) – ODS (43-45) – Silica gel 2

The acetone residue from **E2a5**$_{\mathbf{(13\text{-}22)(43\text{-}45)}}$ (4 mg) was prepared for a second normal phase CC over silica gel to separate the second main spot.

P:E=7:3 gave a Rf-value of 0.20 for the target spot. A 300 ml column packed with 160 ml silica-gel (10-40 µm) was used. The sample silica was prepared with 2X3 ml silica gel (100-200 mesh). 37 fractions; where fraction number 1 was 60 ml, number 2-30 was 20 ml, and number 31-37 was 40 ml, were collected. Acetone was used to wash out the remaining compounds in the column.

TLC of the fractions with P:A=7:3 showed a single spot in fractions 7-31, with UV absorption at 235 nm and a red colour when sprayed with H_2SO_4. Fractions 13-17 were believed to be the most purified, and these were combined and then tested on TLC with C:M=30:1. The TLC showed some other small spots.

The combined fractions were plotted on two P-TLC plates, which were developed with C:M=30:1. UV-detection showed the target compound separated from the rest, and it was marked and collected based on the H_sSO_4 spray reagent and UV-detection. After extraction and filtrating, the compound was tested on TLC, where a pure compound was found. The compound was a brown/yellow amorphous powder with a weight of 16.5 mg. It was named **C4** and prepared for MS- and NMR-detection dissolving it in chloroform-D.

4.2.5.6 E2a5 – ODS (13-22) – ODS (50)

In fraction $\mathbf{E2a5_{(13\text{-}22)(50)}}$, some crystals had crystallized. The fraction was filtrated by use of vacuum suction and filter paper, and the crystals were washed with the filtrate. The crystals were tested on TLC D:M=50:1 and detected with H_2SO_4 spray reagent, showing one single pink spot. The spot also absorbed UV at 254 nm. The weight of the crystals was 939 mg and the compound was given the name **C5.** It was prepared for MS- and NMR-detection dissolving it in chloroform-D.

4.2.5.7 E2a5 – ODS (13-22) – ODS (47-49) – Silica gel

For this fraction, $\mathbf{E2a5_{(13\text{-}22)(47\text{-}49)}}$ (360 mg), normal phase CC over silica-gel was used to obtain one of the compounds of the fraction.

From TLC it was found that P:E=7:1 gave Rf=0.24 for the target spot. A 300 ml column packed with 200 ml silica gel (10-40 µm) was used. The sample silica was prepared with 10X10 ml silica gel (100-200 mesh). 44 fractions, where fraction number 1 was 100 ml, number 2-41 was

20 ml and number 41-44 was 60 ml, were collected. Acetone was used to wash out the remaining compounds in the column.

TLC of the fractions with C:M=30:1 and detection with H_2SO_4 spray reagent showed a single spot in fractions 18-38, getting weaker from fraction 34. Fractions 24-33 were believed to be the most purified, and these were combined and concentrated. The concentrated fraction was tested on TLC D:M=50:1 together with **C5** from fraction 50. Detection with H_2SO_4 spray reagent showed that the target compound was the same as **C5** from fraction 50.

4.2.6 E2a6

In fraction **E2a6,** some crystals had crystallized. The fraction was filtrated by use of vacuum suction and filter paper, and the crystals were washed with the filtrate. The crystals were tested on TLC D:M=50:1 and detected with H_2SO_4 spray reagent, showing one single spot. The weight of the crystals was 768 mg and the compound was given the name **C6.** It was prepared for MS- and NMR-detection dissolving it in chloroform-D.

4.2.6.1 E2a6 – Sephadex LH-20

Fraction **E2a6** (6 g) was subjected to size exclusion CC over Sephadex LH-20 to separate the compounds. Part of the sample, 2 g, was applied to the column and 27 fractions of 15 ml each were collected.

TLC of the fractions with C:M=50:1 showed three main spots suitable for further separation and purification. Comparison of the fractions, by use of TLC, with earlier purified compounds **C6** and **C2**, showed one of the main spots to be the same as **C6** .

4.2.6.2 E2a6 – Sephadex LH-20 (11-12) – Silica gel

For fraction **E2a6$_{(11-12)}$** (128 mg), normal phase CC over silica gel was used to obtain one of the compounds of the fraction.

From TLC it was found that P:A=8:2 gave an Rf=0.30 for the target spot. A 150 ml column packed with 100 ml silica-gel (10-40 µm) was used. The sample silica was prepared with 2X2 ml silica gel (100-200 mesh). 30 fractions, where fraction number 1 was 40 ml and number 2-30

was 20 ml, were collected. Acetone was used to wash out the remaining compounds in the column.

TLC of the fractions with P:A=75:25 and detection with H_2SO_4 spray reagent showed a single spot in fractions 9-19. Fractions 11-17 were believed to be the most purified, and these were combined and concentrated. The combined fraction was tested on TLC with D:M=50:1, which confirmed it as one purified compound with UV-absorption at 254 nm, and iodine adsorption. The weight was measured to be 3 mg, and the compound was named **C7** and prepared for MS and NMR-detection dissolving it in chloroform-D.

4.2.6.3 E2a6 – Sephadex LH-20 (14) – Silica gel

This fraction, **$E2a6_{(14)}$** (240 mg) was subjected to normal phase CC over silica-gel to obtain one of the compounds of the fraction.

From TLC it was found that P:E=8:2 gave an Rf=0.31 for the target spot. A 150 ml column packed with 100 ml silica-gel (10-40 µm) was used. The sample silica was prepared with 2X3 ml silica gel (100-200 mesh). 30 fractions, where fraction number 1 was 40 ml and number 2-30 was 20 ml, were collected. Acetone was used to wash out the remaining compounds in the column.

TLC of the fractions with P:A=75:25 and detection with H_2SO_4 spray reagent showed a single spot in fractions 10-30. Fractions 18-22 were believed to be the most purified, and these were combined and concentrated. The combined fractions were tested on TLC with D:M=50:1, which confirmed that it was one purified compound with no UV-absorption at 254 nm, but iodine adsorption. The weight was measured to be 12 mg, and the compound was named **C8** and prepared for MS- and NMR-detection dissolving it in chloroform-D.

4.2.7 E2a7

This fraction, **E2a7** (<1 g), was subjected to normal phase CC over silica gel to obtain one of the compounds of the fraction.

From TLC it was found that P:E=8:2 gave Rf=0.22 for the target spot. A 300 ml column packed with 160 ml silica-gel (10-40 µm) was used. The sample silica was prepared with 2X3 ml silica

gel (100-200 mesh). 30 fractions, where fraction number 1 was 40 ml, number 2-30 was 20 ml, were collected. Acetone was used to wash out the remaining compounds in the column.

TLC of the fractions with P:A=7:3 showed the pure target spot in fractions 7-9. Fraction 7 was dried and the weight of the compound, named **C9**, was measured to be <100 mg. The compound was, by comparison to a standard on TLC, determined to be β-sitosterol

4.3 Structural determination by MS and NMR

The isolated compounds were subjected to LC-ESIMS, and both positive and negative spectrums were recorded. ^{1}H NMR spectrums of the compound were recorded at 600 MHz (25 ^{o}C). Proton-decoupled ^{13}C NMR spectrums, together with DEPT-135 spectrums, were recorded at 150 MHZ (25 ^{o}C)

4.3.1 C6

The positive ESIMS spectrum (Figure 4.3) shows a main ion peak at *m/z* 325, which was believed to be $[M+Na]^+$. Less intense peaks can be observed at *m/z* 303 and *m/z* 627, and believed to be $[M+H]^+$ and $[2M+Na]^+$, respectively. The negative spectrum shows a main peak at *m/z* 301 and a medium peak at *m/z* 603, thought to be $[M-H]^-$ and $[2M-H]^-$, respectively. The molecule mass of **C6** was therefore believed to be 302 amu.

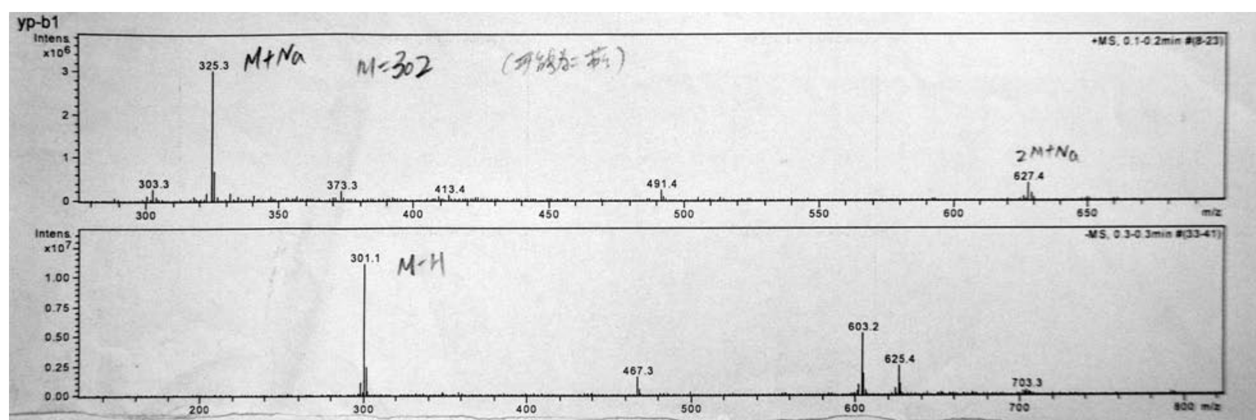


Figure 4.3: LC-ESIMS spectrum of C6, positive at the top, negative at the bottom

The ^{13}C NMR spectrum (Figure 4.4) shows 20 carbon signals (Table 4.4), including 4 carbons in the vinylic region [δ_C 120.7, 122.5, 135.7, 145.4], and on carbonyl [δ_C 185.1]. Two of the vinylic carbons (120.7 ppm and 122.5 ppm) have a positive signal in the DEPT-135 spectrum, indicating one hydrogen attached to each. Since the carbonyl has no signal in the DEPT-135 spectrum, we can rule out the possibility of an aldehyde group. It is most likely an acid, ketone, amid or ester. The ^{1}H NMR spectrum (Figure 4.5) does not show any signals (11.0-12.0 ppm) indicating carboxylic acid. Since the compound was dissolved in chloroform-D, there is a possibility that the carboxylic hydrogen has been exchanged by deuterium and therefore has a weak or no signal.

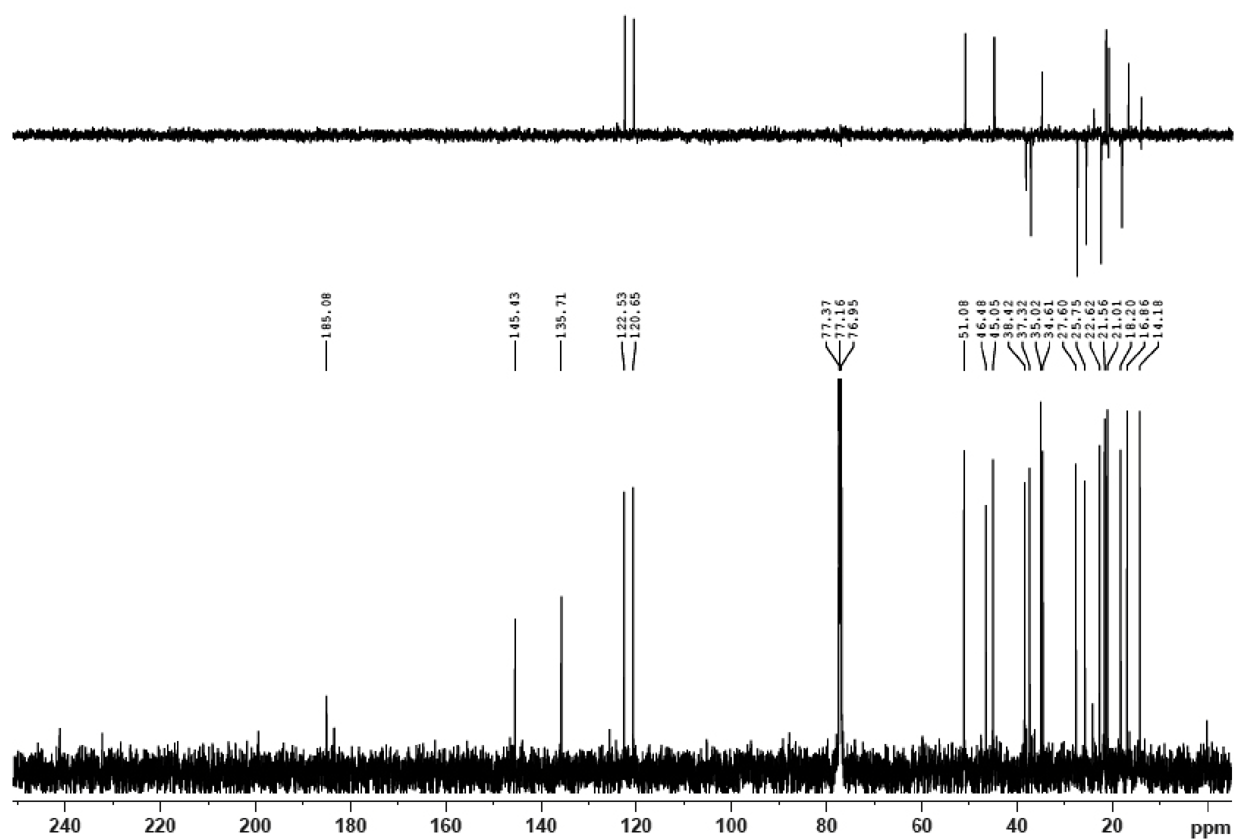


Figure 4.4: Proton-decoupled ^{13}C NMR and DEPT-135 spectra ($CDCl_3$, 150 MHz) of C6 recorded at 25°C

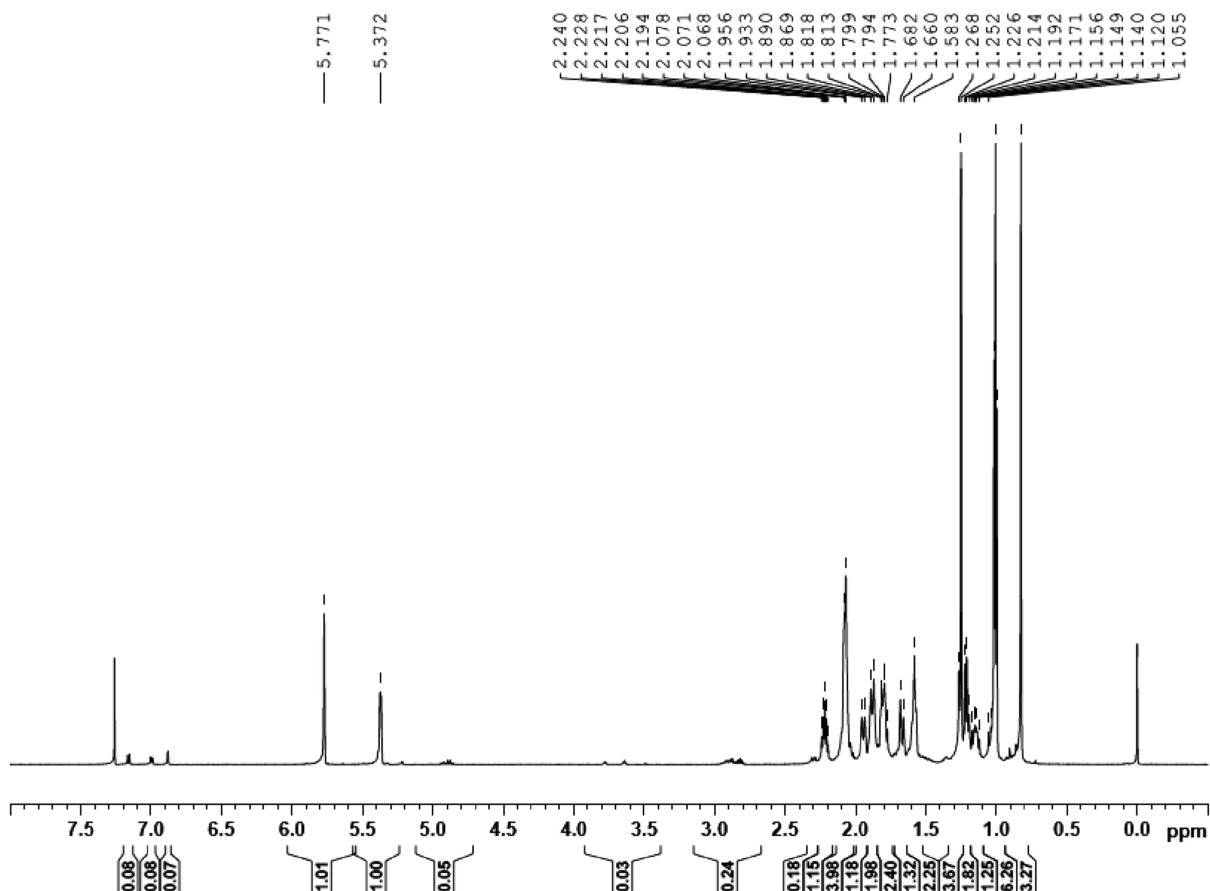


Figure 4.5: ^{1}H NMR spectrum ($CDCl_3$, 600 MHz) of C6 recorded at 25°C

In the vinylic region of the ^{1}H NMR spectrum we can see two signals, [δ_H 5.77, 5.37], both with an integral of 1. These correspond to the two vinylic carbons with positive DEPT-135 signal. They both seem like unsplit singlets, but the signal at 5.37 ppm, which has half the intensity compared to the signal at 5.77 ppm, could be a multiplet. There are no signals indicating the presence of an amide (5.0-9.0 ppm). Besides the two vinylic signals, the^{1}H NMR spectrum has three other well distinguish peaks in the region from 0.75-1.35 ppm (methyls) (Table 4.1 and Figure 4.6). The signals at δ_H 1.25 and 0.83 are two singlets, each with an integral of 3, representing two methyl proton signals. At δ_H 1.00 there is a multiplet with an integral of 6. This is most likely two duplets, at 1.01 ppm and 1.00 ppm, which are not well separated. The coupling constant for both of them is 6 Hz, maybe being split by the same hydrogen. They most likely represent two isopropyl methyl signals.

Table 4.1: ¹H NMR Spectroscopic data of compound C6

¹H δ (ppm)	Integral	Multiplicity	J (Hz)	
5,77	1	S	-	H-14
5,37	1	M	-	H-7
1,25	3	s	-	Me-18
1,01	3	m*	6	Me-16
1,00	3	m*	6	Me-17
0,83	3	S	-	Me-20

* *Assumed to be two duplets overlapping*

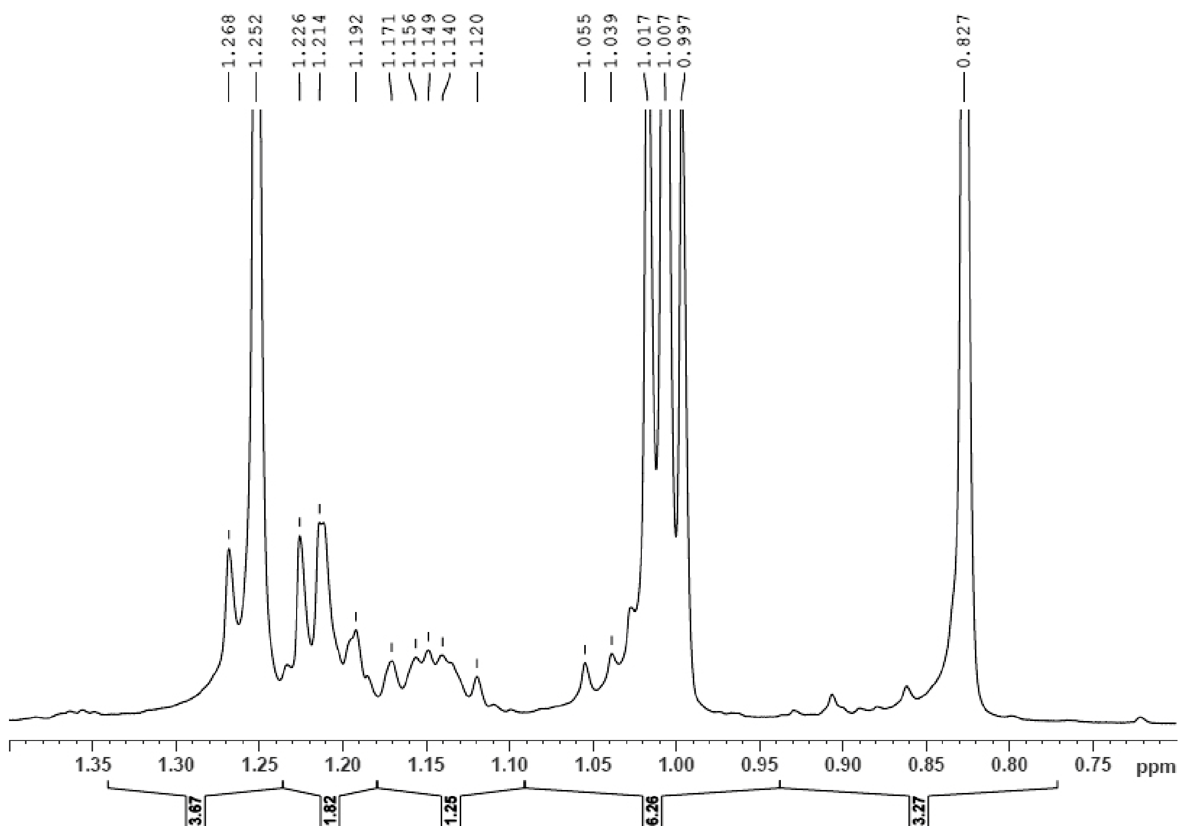


Figure 4.6: ¹H NMR spectrum ($CDCl_3$, 600 MHz), 0.75-1.35 ppm, of C6 recorded at 25°C

With a molecular weight of 302 amu, and by assuming the presence of 20 carbons and an acid group, we get the molecular formula of $C_{20}H_{30}O_2$, which has 6 degrees of unsaturation. Since three of these are attributed to the one carbonyl and the two vinylic groups, the molecule is

believed to contain a tricyclic nucleus, which will make up for the three last unsaturations. Tricyclic C_{20} natural products are typical diterpenes. By comparing with earlier isolated tricyclic diterpenes from other *Abies* species reported in Yang et al's. (2008) review article[10], the compound was thought to be abieta-7-13-diene-18-oic acid (abietic acid). This was then confirmed by comparing the NMR-data with data from earlier work[23-24]. The carbon signals were assigned according to the data from these earlier works.

4.3.2 C2

The positive ESIMS spectrum (Figure 4.7) shows a main ion peak at *m/z* 357, which was believed to be $[M+Na]^+$. The less intense peak observed at *m/z* 691 was believed to be $[2M+Na]^+$. The negative spectrum shows a main peak at *m/z* 333 and medium peak at *m/z* 667, thought to be $[M-H]^-$ and $[2M-H]^-$, respectively. The molecule mass of **C2** was therefore believed to be 334 amu.

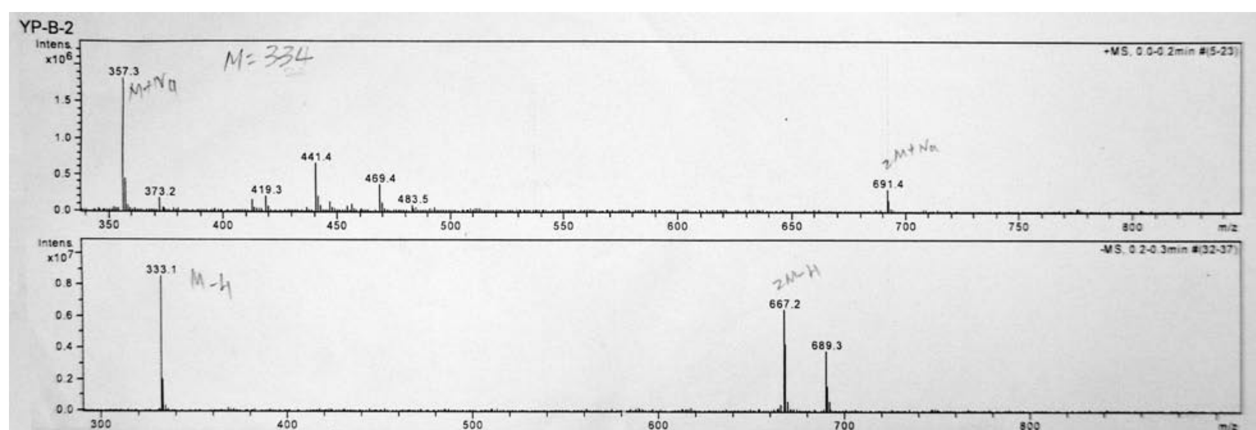


Figure 4.7: LC-ESIMS spectrum of C2, positive at the top, negative at the bottom

The ^{13}C NMR spectrum (Figure 4.8) shows 20 carbon signals (Table 4.4), including two carbons in the vinylic region and one carbonyl. The shifts of the two vinylic carbons are 143.9 ppm and 127.2 ppm, respectively, with no DEPT-135 signal and with positive signal. The carbonyl with shift 185.1 ppm has no signal in the DEPT-135 spectrum, indicating no aldehyde. Like for C6, the ^{1}H NMR spectrum (Figure 4.9) did not show any signal indicating a carboxylic acid, but also here the possibility of exchange with chloroform-D is likely. In the ^{13}C NMR spectrum, there are

also two signals at 82.2 ppm and 79.8 ppm with no DEPT-135 signal. These are in the region that could indicate either oxygen-bond carbons (C-O) (40-80 ppm) or alkyne carbons (65-90 ppm). They are very far shifted downfield for oxygen bond carbons, and there are no indications of ethers (3.2-3.8 ppm) or hydroxyl protons in the ^{1}H NMR spectrum. Hydroxyl protons are always very difficult to detect and may in this case be exchanged with chloroform-D.[21]

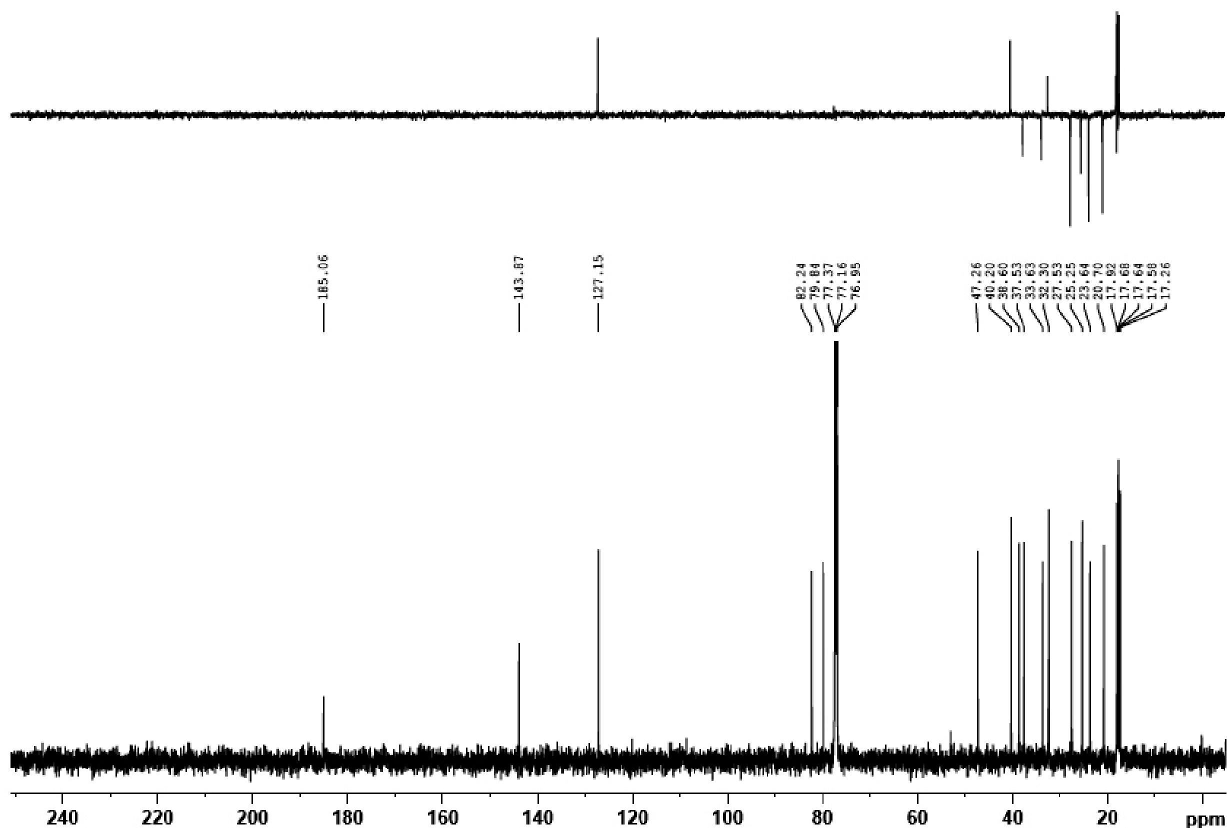


Figure 4.8: Proton-decoupled ^{13}C NMR and DEPT-135 spectra ($CDCl_3$, 150 MHz) of C2 recorded at 25°C

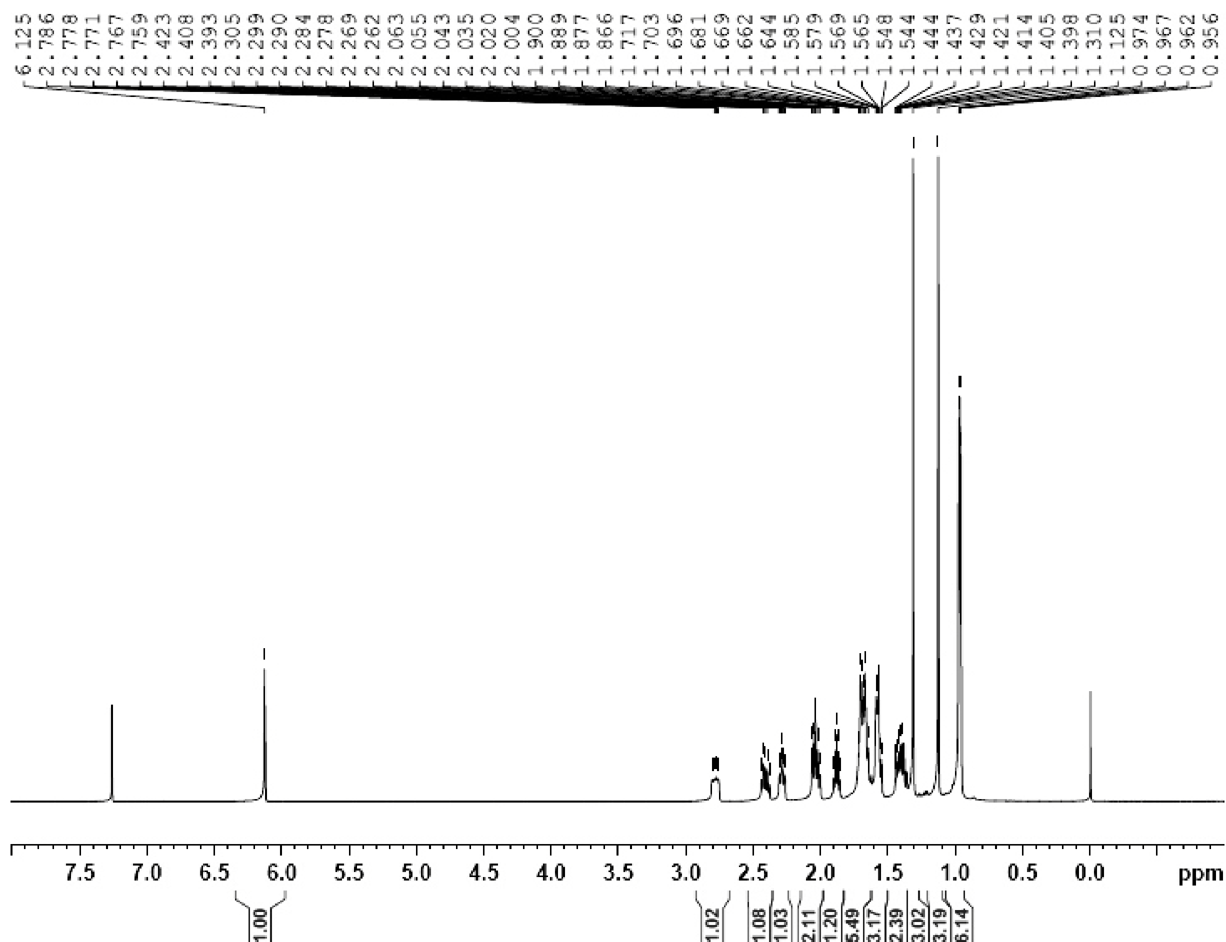


Figure 4.9: ^{1}H NMR spectrum ($CDCl_3$, 600 MHz) of C2 recorded at 25°C

In the vinylic region of the ^{1}H NMR spectrum, we can see one single peak at 6.13 ppm, with an integral of 1. This corresponds to the vinylic carbon with positive DEPT-135 signal. There are no signals indicating the presence of an amide (5.0-9.0 ppm). Besides the vinylic signal, the ^{1}H NMR spectrum has three other well distinguish peaks in the upfield region, from 0.75-1.35 ppm (methyls) (Figure 4.10 and Table 4.2). The peaks at δ_H 1.31 and 1.13 are two singlets, each with an integral of 3, representing two methyl proton signals. The other signal, at 0.97 ppm, looks like a quartet, but has an integral of 6 and is therefore probably two duplets overlapping, δ_H 0.97 and 0.96. The coupling constants for them are 4.2 Hz and 3.6 Hz, respectively. As in C6, they most likely represent two isopropyl methyl signals. Four methyls is consistent with the results from the ^{13}C NMR (Table 4.4)

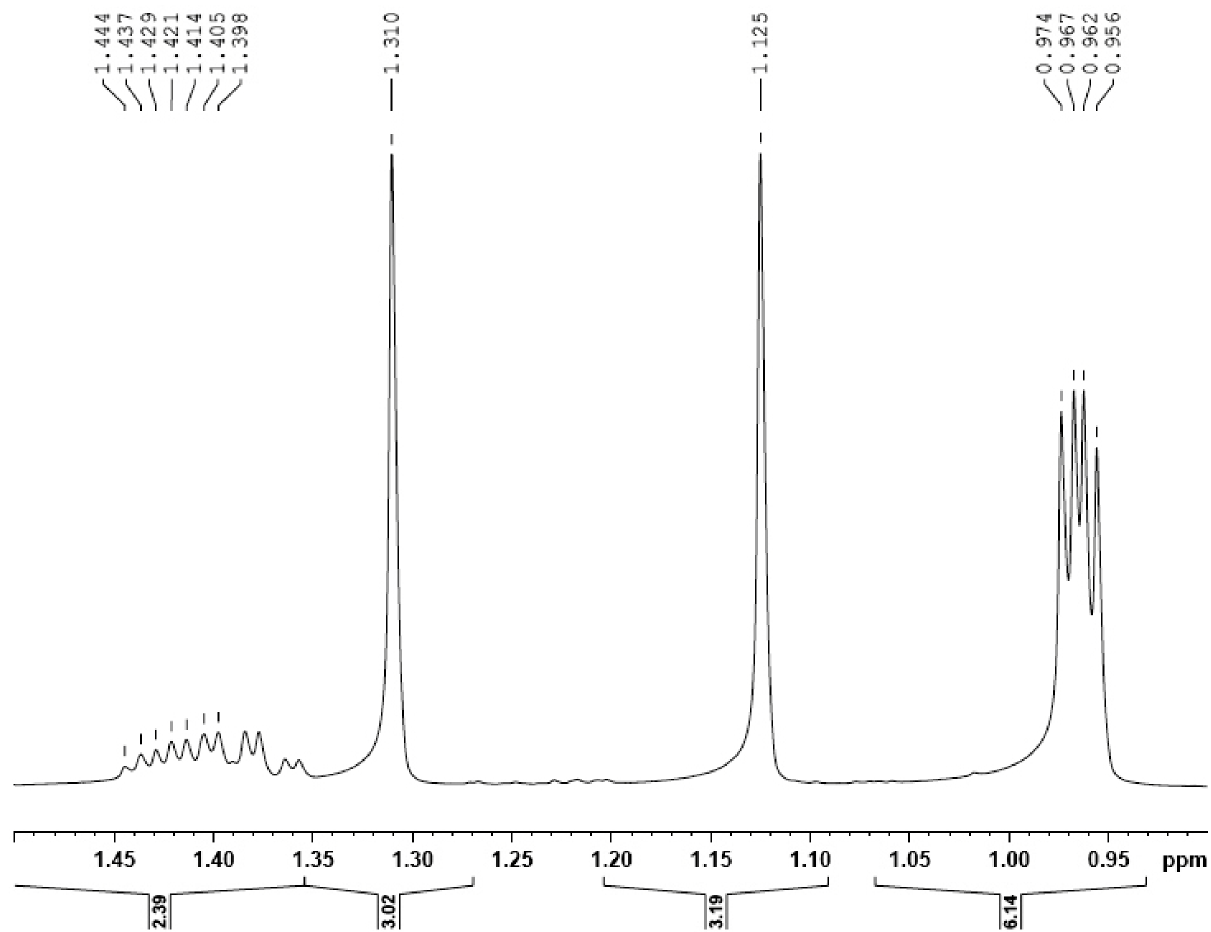


***Figure 4.10:** ^1H NMR spectrum ($CDCl_3$, 600 MHz), 0.9-1.5 ppm, of C2 recorded at 25°C*

***Table 4.2:** ^1H NMR Spectroscopic data of compound C2*

^1H δ (ppm)	Integral	Multiplicity	J (Hz)	
6,13	1	s	-	H-14
1,31	3	s	-	Me-18
1,13	3	s	-	Me-20
0,97	3	m*	4,2	Me-16
0,96	3	m*	3,6	Me-17

* *Assumed to be two duplets overlapping*

With the exception of five carbons, the vinylic plus one other, the carbon shifts for compound C2 are very similar to the shifts seen for C6 (see Table 4.4). It is therefore reasonable to expect the two compounds to have some similarities. Since the carbonyls in the two compounds have identical shift values, it is assumed that C2 also includes an acid. The difference in molecular mass of C6 and C2 is 32 amu, which corresponds to two oxygens. This is consistent with the two carbon signals at 82.2 ppm and 79.8 ppm. These could arise from two carbons bound to one oxygen each. These assumptions would give a molecular formula of $C_{20}H_{30}O_4$, which has 6 degrees of unsaturation. The carbonyl and vinylic group uses two unsaturations. The similarities of the carbon shift between C6 and C2 would indicate a similar tricyclic nucleus, which then leaves one unsaturation. If one double bond in C6 has been replaced by two oxygens and the only difference in the molecular formula is the two oxygens and no hydrogens, the two oxygens have to bind with each other, forming an epidioxy bridge. This adds up the last unsaturation.

Yang et al's. (2008) review article on diterpenes from *Abies species [10]* shows a compound that match the description, a 9,13 -epidioxy-8(14)-abieten-18-oic acid. The NMR- data for C2 was compared to data from earlier work on 9,13-epidioxy-8(14)-abieten-18-oic acid, and confirmed to be 9,13(β)-epidioxy-8(14)-abieten-18-oic acid and the peaks were assigned according to this.[25]

4.3.3 C5

The positive ESIMS spectrum (Figure 4.11) showed a main ion peak at *m/z* 323, which was believed to be $[M+Na]^+$. The small peak observed at *m/z* 623 was believed to be $[2M+Na]^+$. The negative spectrum shows a main peak at *m/z* 299 and medium peak at *m/z* 599, thought to be $[M-H]^-$ and $[2M-H]^-$, respectively. The molecule mass of **C5** was therefore believed to be 300 amu.

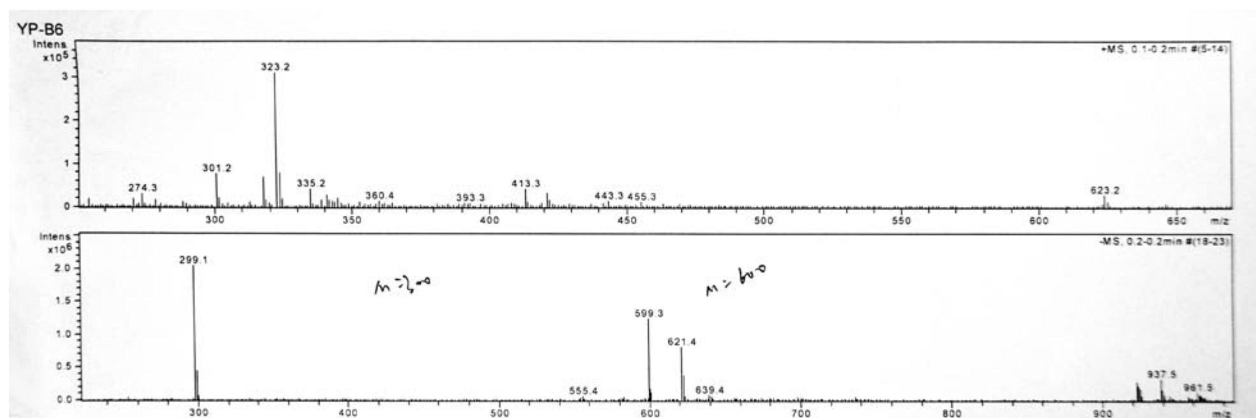


Figure 4.11: LC-ESIMS spectrum of C5, positive at the top, negative at the bottom

The ^{13}C NMR spectrum (Figure 4.12) shows 19 carbon signals (Table 4.4), including 6 carbons in the aromatic and vinylic region and one carbonyl. With six carbons in the aromatic region, it is reasonable to assume the presence of a benzene ring. The shifts for the 6 aromatic carbons are 146.9 ppm, 145.9, 134.8, 127.1, 124.3, 124.0, the three last ones with positive DEPT-135 signal and the others with no signal. The carbonyl with shift 185.2 ppm has no signal in the DEPT-135 spectrum, indicating no aldehyde. Like for C2, the ^{1}H NMR spectrum (Figure 4.14) did not show any signal indicating a carboxylic acid, but also here the possibility of exchange with chloroform-D is likely.

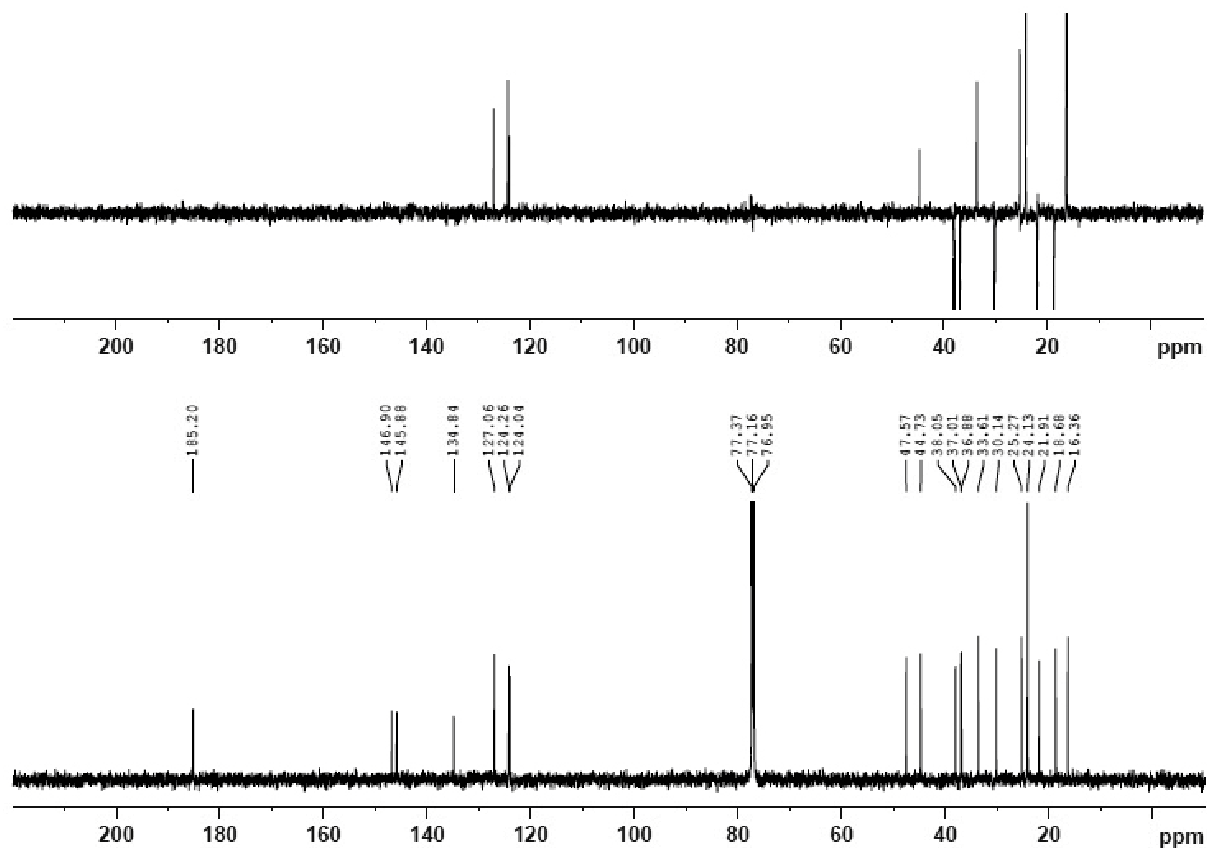


Figure 4.12: Proton-decoupled ^{13}C NMR and DEPT-135 spectra ($CDCl_3$, 150 MHz) of C5 recorded at 25°C

In the aromatic region of the ^{1}H NMR spectrum (Figure 4.13), there are three peaks with an integral of one, two doublets at 7.17 ppm and 7.01, and a singlet at 6.89 ppm. This corresponds to the three DEPT-135 positive aromatic carbons. The coupling constant for both the doublets is 8.4 Hz (see Table 4.3). They are probably in an ortho-position and splitting each other.

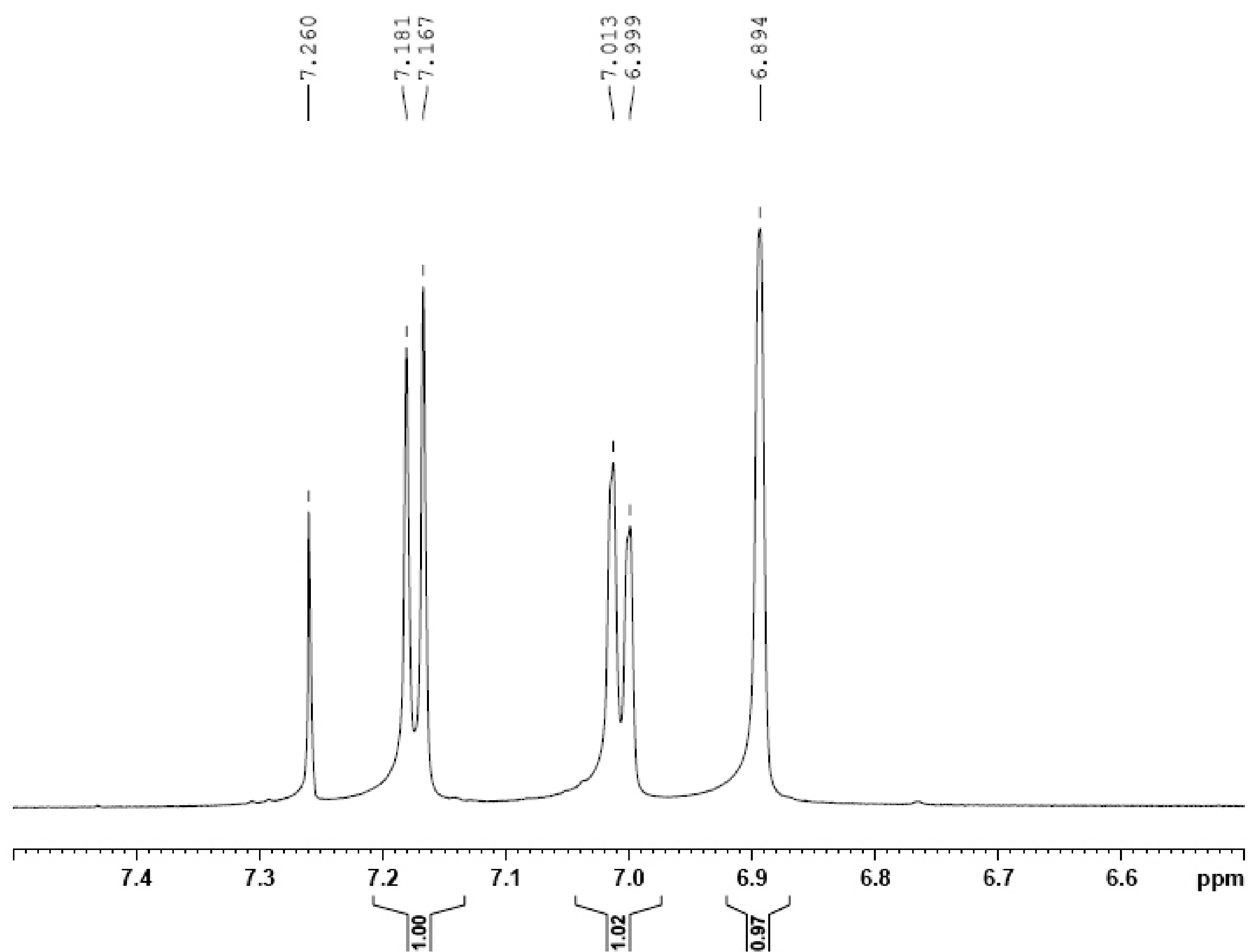


Figure 4.13: ^{1}H NMR spectrum ($CDCl_3$, 600 MHz), 6.5-7.5 ppm (aromat region), of C5 recorded at 25°C

Table 4.3: ^{1}H NMR Spectroscopic data of compound C5

^{1}H δ (ppm)	Integral	Multiplicity	J (Hz)	
7,17	1	d	8,4	H-11
7,01	1	d	8,4	H-12
6,89	1	s	-	H-14
1,29	3	s	-	Me-18
1,24	8	bd	-	Me-20
1,23			-	Me-16,17

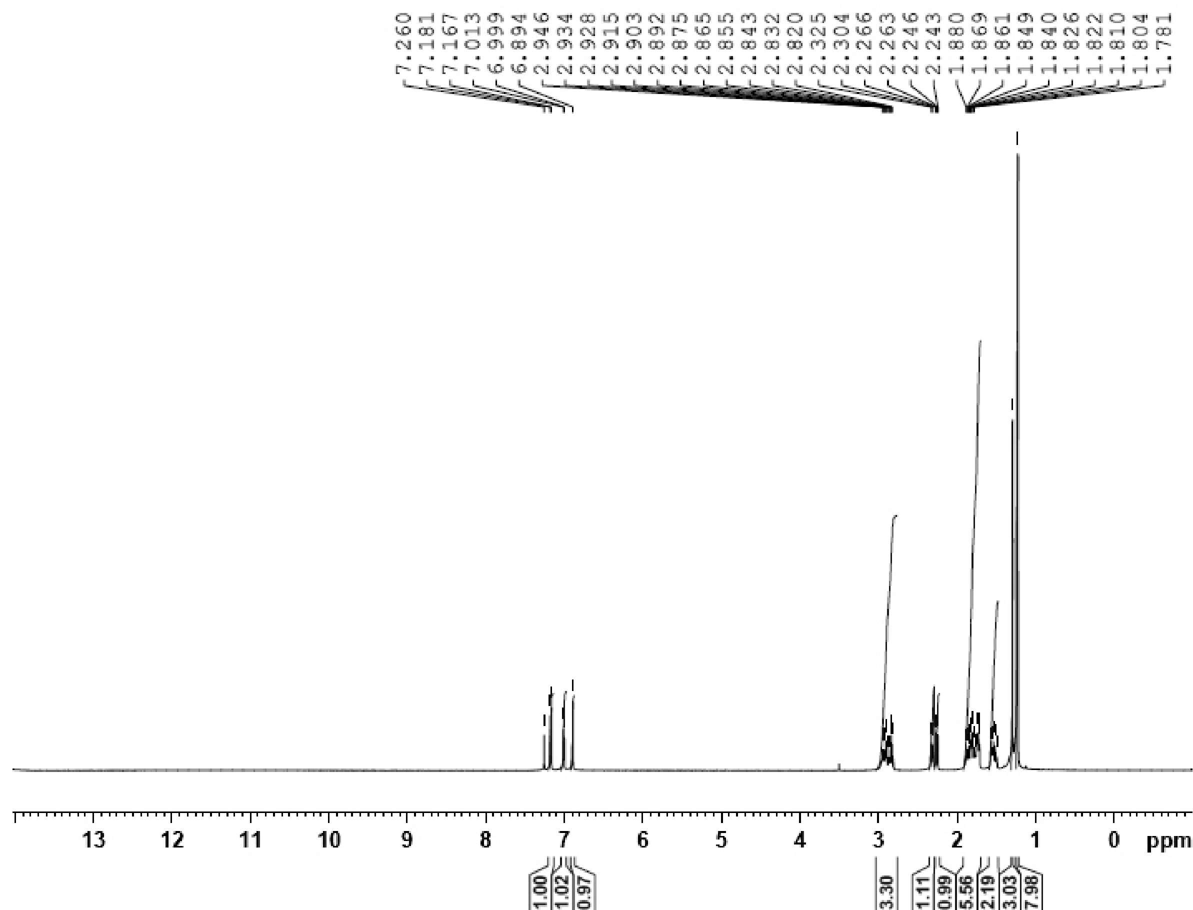


Figure 4.14: ***^{1}H NMR spectrum ($CDCl_3$, 600 MHz) of C5 recorded at 25°C***

In the ^{13}C NMR spectrum (Figure 4.12) a methyl peak can be seen at 24.1 ppm that is twice as intense as the other methyl peaks. Although integral information in ^{13}C spectra normally would be unreliable, it is reasonable to assume that this peak arises from two identical methyl groups and that it there are 20 carbons in the molecule.

The ^{1}H NMR spectrum shows three peaks in the methyl region (Figure 4.15), 1.29 ppm, 1.24 ppm and 1.23 ppm. None of these are well separated (see Figure 4.16), especially the two last ones, which could well be a doublet. These two have an integral of 8 together (Table 4.3). Since the ^{13}C spectrum showed three methyl groups with two them having the same shift, it is reasonable to believe that the two peaks with the integral of 8 actually represent three methyls where the hydrogen from two of them have the same shift.

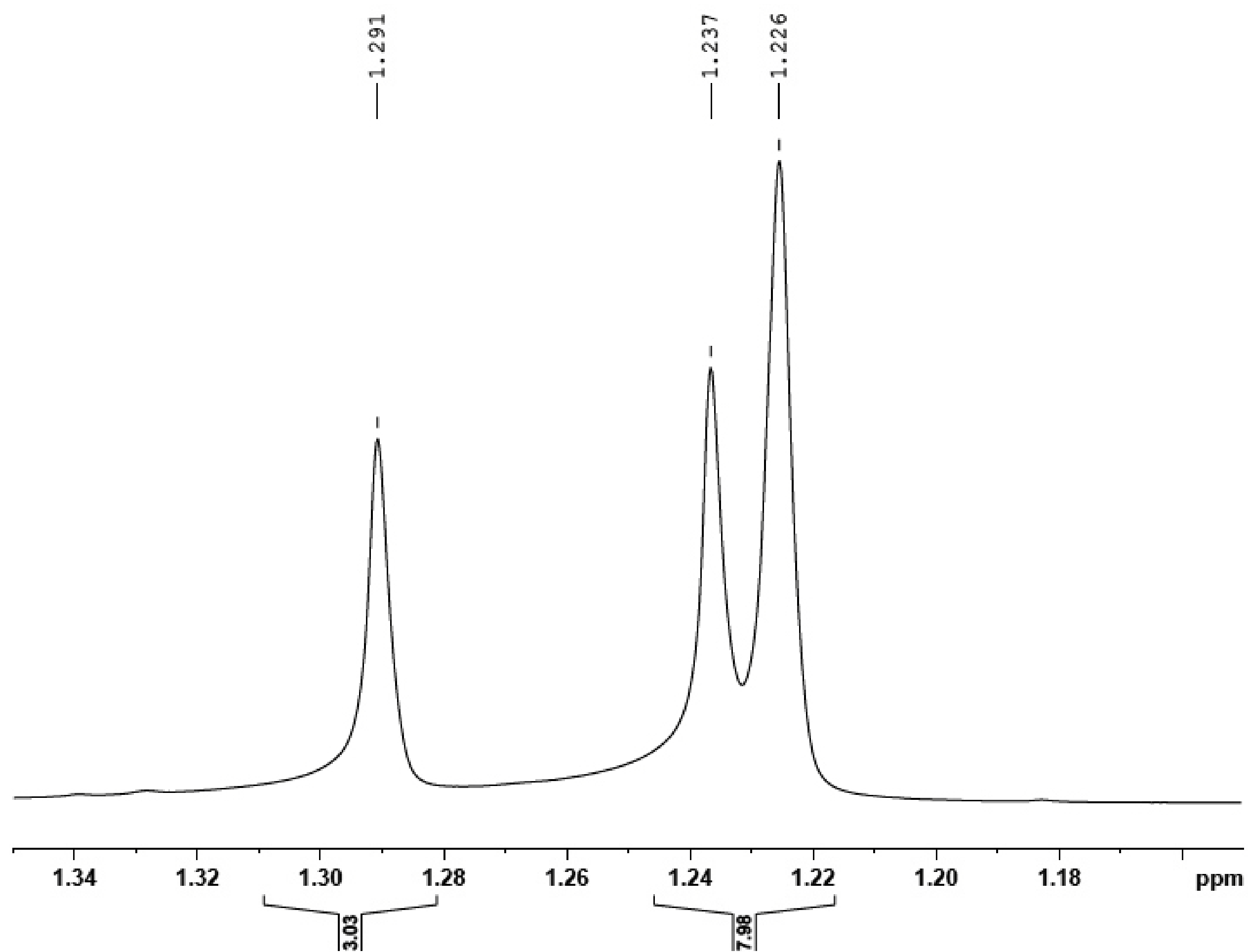


Figure 4.15: ^{1}H NMR spectrum ($CDCl_3$, 600 MHz), 1.16-1.35 ppm (methyl region), of C5 recorded at 25°C

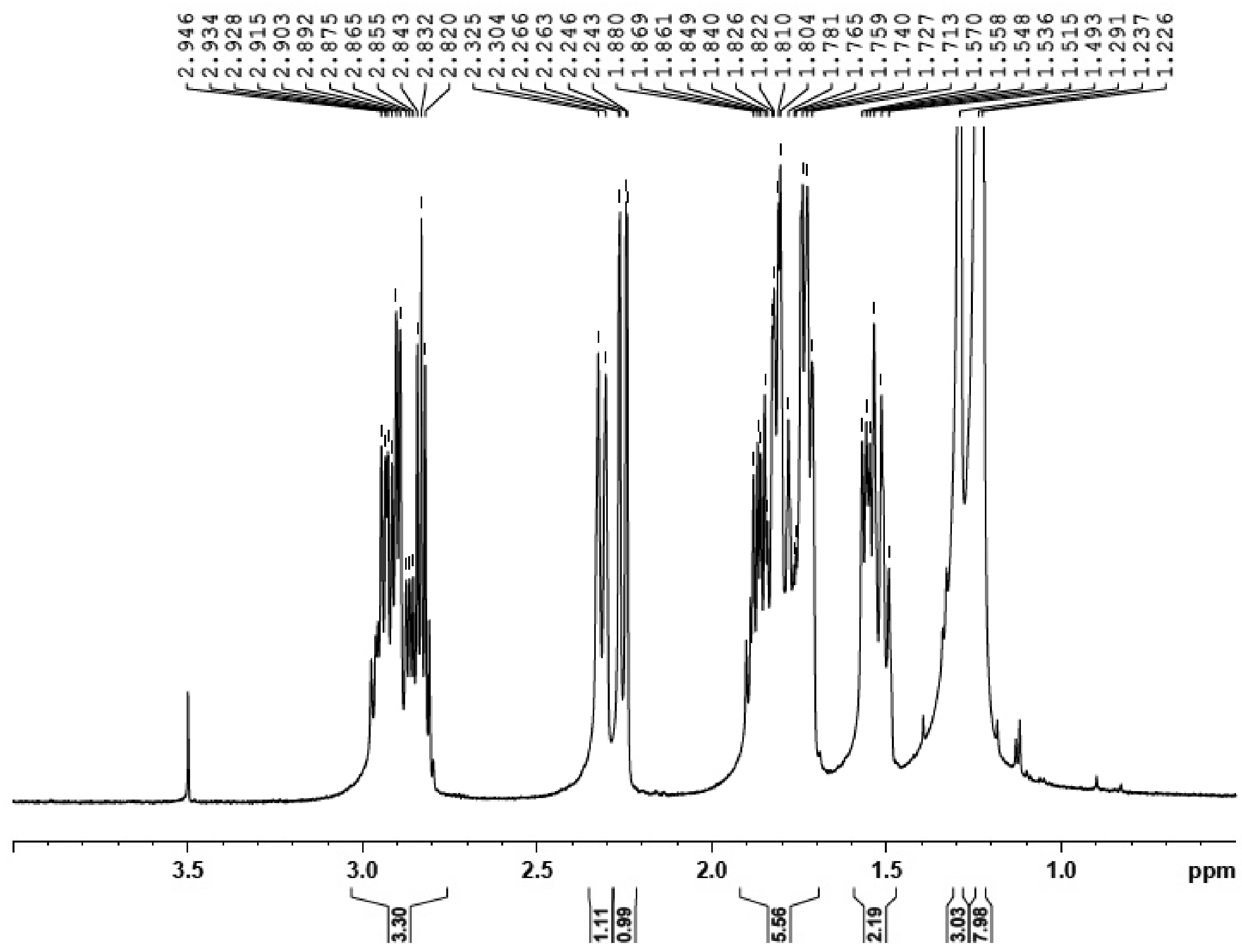


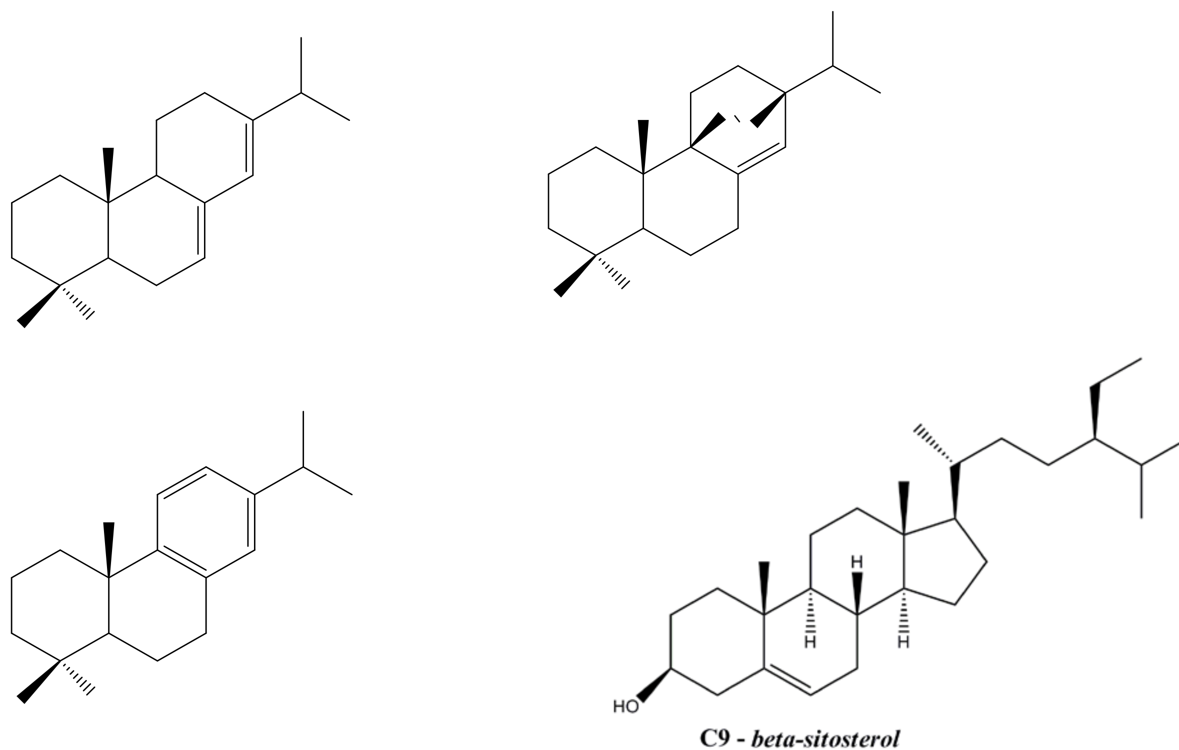
Figure 4.16: ^{1}H NMR spectrum ($CDCl_3$, 600 MHz), 0-4 ppm, of C5 recorded at 25°C

With a molecular weight of 300 amu, and by assuming the presence of 20 carbons and an acid group, we get the molecular formula of $C_{20}H_{28}O_2$, which has 7 degrees of unsaturation. Since five of these are attributed to the one carbonyl and the benzene ring, the molecule is believed to contain two more ring structures forming a tricyclic nucleus with the benzene ring. Like C2 and C6, it is probably a tricyclic C_{20}-diterpene. By comparing with earlier isolated tricyclic diterpenes from other *Abies* species reported in Yang et al's. (2008) review article[10], the compound was thought to be dehydroabietic acid. This was then confirmed by comparing the NMR-data with data from earlier work.[26] The carbon signals were assigned according to the data from this earlier work.

Table 4.4: ^{13}C NMR Spectroscopic data of compound C2, C5, C6

	C2		C5		C6	
C	**^{13}C δ (ppm)**	**Multip.**	**^{13}C δ (ppm)**	**Multip.**	**^{13}C δ (ppm)**	**Multip.**
1	33,6	t	38,1	t	38,4	t
2	17,7	t	18,7	t	18,2	t
3	37,5	t	36,9	t	37,3	t
4	47,3	s	47,6	s	46,5	s
5	40,2	d	44,7	d	45,1	d
6	20,7	t	21,9	t	25,8	t
7	27,5	t	30,1	t	120,7	d
8	143,9	s	134,8	s	133,7	s
9	82,2	s	146,9	s	51,1	d
10	38,6	s	37,0	s	34,6	s
11	23,6	t	124,3	d	22,6	t
12	25,3	t	124,0	d	27,6	t
13	79,8	s	145,9	s	145,4	s
14	127,2	d	127,1	d	122,5	d
15	32,3	d	33,6	d	35,0	d
16	17,3	q	24,1*	q	21,6	q
17	17,6	q	24,1*	q	21,0	q
18	17,6	q	25,3	q	16,9	q
19	185,1	s	185,2	s	185,1	s
20	17,9	q	16,4	q	14,2	q

* *One peak assumed to represent two carbons*



C9 - *beta-sitosterol*

4.3.4 C1, C4, C7, C8

C4, C7 and C8 unfortunately did not give any results. The analytical department at SMMU reported that the samples of C4 and C7 had too low a concentration, and that C8 was not pure enough to give any usable results.

C1 was reported to be a fatty acid, and unfortunately, no results were returned.

4.3.5 C9

C9 was determined to be the well-known steroid β-sitosterol by comparison to a standard on TLC.

4.4 Isolated compounds

The four isolated compounds are well known compounds, which all have previously been seen in other *Abies* species. No literature describing the extraction of these compounds form *Abies forrestii* has been found. It was still reasonable to assume the presence of them.

Three of the compounds are 20-carbon tricyclic diterpenes acids: abietic acid, 9,13(β)-epidioxy-8(14)-abieten-18-oic acid and dehydroabietic acid. These are also called diterpene resin acids. Conifers use diterpene resin acids as constituents in their secretion product oleoresin. The oleoresin is secreted when the conifer is wounded, as if by an insect. It can trap the insect and it is also believed that it may have a direct effect on some insects.[19]

4.4.1 Abietic acid (C6) and Dehydroabietic acid (C5)

Abietic and dehydroabietic acid are the main resin acids in colophony (rosin), which is the solid leftover when distilling oleoresin.[18] Since colophony is widely used in different products, we are exposed to colophony in many areas of daily life. It is therefore reported many clinical cases involving colophony. Most of these cases are bronchial asthma and contact dermatitis.[27] It is reasonable to assume that these resin acids or derivatives of them could cause some of these effects.

4.4.2 9,13(β)-epidioxy-8(14)-abieten-18-oic acid (C2)

9,13(β)-epidioxy-8(14)-abieten-18-oic acid are also a resin acid. It has been shown to be an oxidation product of palustric acid (Figure 4.17).[28] In earlier work on *Abies* species by Barrero et al. (1991), it has therefore been proposed that 9,13(β)-epidioxy-8(14)-abieten-18-oic acid is an oxidation product of palustric acid produced during the extraction and isolation procedure.[25]

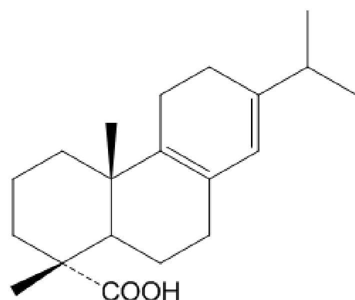


Figure 4.17: Palustric acid

4.4.3 β-sitosterol (C9)

β-sitosterol, a phytosterol, is structurally similar to cholesterol (Figure 4.18) and is one of the most common plant sterols [29]. In the 1950s and on, sitosterol was investigated as a potential means of lowering serum cholesterol levels by inhibiting absorption of cholesterol from the gut, absorbing sitosterol instead. But later in the 1980s, when the very efficient statins were introduced, the interest in sitosterol as a cholesterol lowering drug dropped.[29-30] In later years interest in phytosterols have been revoked, and potential effects in cancer and immune modulation have been investigated [31].

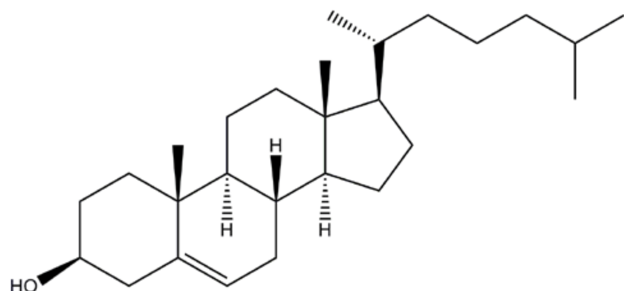


Figure 4.18: Cholesterol

5 Conclusion

The history and knowledge of Chinese herbal medicine is over 3000 years old, and over 10 000 herbs are recorded and are in use for different conditions. This is an enormous amount of knowledge that can be used to find new lead compounds for Western medicine.

TCM herbal medicine has been based on the old knowledge and developed over thousands of years, and the holistic way of thinking has been important. In this new era of medicine and research, modern TCM are still developing and acquiring new knowledge. By the use of Western research techniques Chinese medicine is going through a modernization where the synergistically and holistic thinking is still strong. Modern technology and academic thinking are used to find out more about the active compounds in the herbal recipes and how they may work together with each other. In this way the traditional theory of TCM is kept alive.

The genus *Abies,* of the *Pinaceae* family, has been extensively investigated at MRCTCM. Different *Abies* species have been used in folk medicine, and it has been reported bioactivities from crude extracts of several species. *Abies forrestii* is one of 14 species that are native to P.R. China, and little research has been done on this specific plant.

In this master thesis, eight compounds were obtained from an ethanol extract from the aerial parts of *Abies forrestii,* by use of several different chromatographic techniques (size exclusion, TLC, normal and reverse phase). The chemical structure was determined for three of them by use of MS- and NMR-techniques (the known diterpenes abietic acid, dehydroabietic acid, 9,13(β)-epidioxy-8(14)-abieten-18-oic acid) and for one of them by comparing to a standard on TLC (β-sitosterol). The rest was either not pure enough or was obtained in a too low concentration. The pulverized plant material was extracted with ethanol, purified with ethylacetate and fractionated with gradient elution over a silica gel column and a MCI-column. The compounds were isolated and purified over silica gel-, Sephadex LH-20- and ODS-columns, and with P-TLC. The structures of the compounds were determined by a combination of LC-ESI MS, ^{1}H- and ^{13}C NMR data.

The extracted compounds in this thesis will be included in MRCTCM's Natural Products Library and registered in their MDL ISIS Database, together with all other compounds that have been, and will be, extracted from *Abies forrestii* at the laboratory at MRCTCM. They are well known compounds in other *Abies* species, but no records have been found describing earlier extraction

from *Abies forrestii.* These studies provide further in-depth understanding of the chemical composition of *Abies*.

Abies and *Abies forrestii* is of continuing interest at MRCTCM and further research will continue. The Natural Products Library and the MDL ISIS Database are, and will become an even larger resource for discovering new medicines and for understanding how Chinese herbal treatment work. Discovering new medicines with lower toxicity for treatment of cancer, and finding new antibiotics is important for our future. With the growing bacterial resistance to common antibiotics, new antibiotics are especially important, and maybe new ways of thinking would also be beneficial. I believe that combination therapy will become more and more important when treating multiresistant bacteria, virus infections, and cancer. Chinese medicine could be a very good resource for learning more about combination treatment and how different constituents affect each other and the body.

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