Important Differences and Potential Synergies between Traditional Chinese Medicine and Western Medicine, and the Isolation of Natural Products from *Bretschneidera Sinensis*

-Master thesis in Pharmacy-

By

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Summary

Traditional Chinese medicine (TCM) has been an important medical system in China for thousands of years. Lately, certain techniques like acupuncture and herbal medicines are also becoming increasingly popular in the West. Although the two medical systems are fundamentally different, both TCM and Western medicine are practiced alongside each other in China. Patients can choose between TCM, which offers a holistic approach to treatment and diagnosis, or Western medicine and its more mechanical view of disease.

In this paper the two medical systems are compared, and important differences and potential synergies are discussed. From this discussion it is evident that one of the most relevant ways the two can influence each other are to use TCM as a source of information in network medicine which is getting more and more popular and is proposed to be the future paradigm in drug discovery[1]. But for the time being, the study of constituents in herbal medicines from TCM as a possible source of novel lead compounds seems to be even more relevant. Natural products have proven a good source of complex and biologically active compounds that have even resulted in blockbuster drugs.

As a part of an international staff exchange programme titled "Traditional Chinese Medicine in the Post-genomic Era: Identifying Lead Therapeutic Compounds Against Cancer" this last potential for synergetic influence was investigated. Compounds from *Bretschneidera Sinensis hemsl.* a plant used in TCM was extracted, purified and structurally characterized at the Modern Research Centre of Traditional Chinese Medicine in Shanghai in hopes of finding novel compounds with possible anti-cancer properties.

Bretschneidera Sinensis hemsl. has never before been investigated, and eight compounds were isolated from the plant for the first time. 4 of these compounds were structurally characterized, and found to be previously known compounds. Although no new compounds were isolated, biologically active compounds were found, and this work adds to previous studies that indicate that TCM is indeed a valuable source of biologically active components.

Abbreviations

TCMCANCER project	(Acronym for:) Traditional Chinese Medicine in the Post-genomic Era: Identifying Lead Therapeutic Compounds Against Cancer
TCM	Traditional Chinese Medicine
CRP-Santé	Centre de Recherche Public de la Santé
MRCTCM	Modern Research Centre of Traditional Chinese Medicine
SMMU	Second Military Medical University
IMPLAD	Institute of Medicinal Plant Development
CC	Column chromatography
HPLC	High performance liquid chromatography
TLC	Thin liquid chromatography
pTLC	Preparative thin liquid chromatography
GC	Gas chromatography
H_2SO_4	Sulphuric Acid
Ac	Acetone
EtOAc	Ethyl acetate
$CHCl_3$	Trichloromethane
PE	Petroleum ether
MeOH	Methanol
RPM	Rotations per minute
MS	Mass spectrometry
NMR	Nuclear magnetic resonance spectroscopy
ESI	Electronspray ionization
FT	Fourier transformation
FID	Free Induction Decay

1 INTRODUCTION

1.1 TCMCANCER Project

This master thesis was performed as a part of the Marie Curie Actions – International Research Staff Exchange Scheme, FP7-People-IRSES-2008 supported by the European Comission in the project entitled "Traditional Chinese Medicine in the Post-genomic Era: Identifying Lead compounds Against Cancer" (TCMCANCER, project number 230232). The project aims to identify and characterize lead therapeutic compounds against cancer based on the knowledge of traditional Chinese medicine (TCM) and their subsequent testing in preclinical cancer models, genome analysis and finally screening in animal models. The international exchange program allows for specialized knowledge to be spread to different institutions. There are four separate partners participating; The Gade Institute at the University of Bergen in Norway, The Centre de Recherche Public de la Santé (CRP-Santé) in Luxembourg, the Modern Research Centre for Traditional Chinese Medicine (MRCTCM) at the Second Military Medical University (SMMU) in Shanghai and the Institute of Medicinal Plant Development (IMPD) in Beijing. At the MRCTCM European scientists are trained in purification and structure determination of natural products isolated from herbs used in TCM. The work at the MRCTCM is the first step of the ladder, and aims to select herbs and herbal extracts to examine; the purification of compounds in amounts that allow for *in vitro* and *in vivo* testing and thereafter the structural characterization of the compounds. The compounds are thereafter tested *in vitro* using high throughput screening technologies at the CRP-Santé. The Gade Institute in Norway is responsible for establishing phenotypic and genomic screening systems for TCM, while the IMPLAD will test the compounds *in vivo* and in animal models.

The staff exchange program is funded by the European Commission, and funds were received during my stay in Shanghai from August to December of 2009.

1.2 Aim of Study

The objective of this master thesis is based on the aims in the TCMCANCER project, where the intended work of the European scientists transferred to the MRCTCM are clearly stated in the "Description of Work" annex of the project. The aim of this master thesis is partly divided

in two, with a theoretical part to serve as background information for the practical part of the thesis. Both aims are connected to the TCMCANCER project.

The first aim of the thesis was therefore to get a general understanding of the concepts of traditional Chinese medicine (TCM), with emphasis on herbal medicine. Thereafter the aim was to compare the theory and beliefs of TCM to those of modern Western medicine, and look at important differences and potential synergies between the two and discuss if there are areas where the two can influence one another in a beneficial way.

The second aim of this paper is tightly connected to the aims of the TCMCANCER project. The project states that European scientists at the MRCTCM will take part in isolation, purification of compounds from a plant used in TCM to learn the basics of different extraction procedures and purification methods. The techniques of structural characterization will also be acquired through the stay at MRCTCM. The second aim of this master thesis was therefore to participate in the isolation and purification of compounds in *Bretschneidera Sinensis hemsl.* and the following structural characterization of the isolated compounds. The goal was to isolate novel compounds that could be put through further testing in the later stages of the TCMCANCER project and hopefully establish biological activity against cancer.

2 Theory

2.1 Western medicine

Western medicine has undergone drastic changes from ancient times when disease was attempted cured with magic and religious rituals until today's intricate knowledge about the different functions of the organs and even the genes in different cells. The origin of Western medicine is found in ancient Greece several hundred years before the birth of Christ [2-4]. Thales, "the father of rational thinking", one of the first philosophers who looked past the supernatural and tried to explain nature by logic and rational thinking, Hippocrates who is now widely known as the father of modern medicine and Galen are all important historical figures. Although their theories were not always correct, and sometimes even erroneous, they helped shape Western medicine towards the medical system that exists today. In order to understand modern Western medicine it is necessary to look back in history. The earliest Western medicine had several parallels to Chinese medicine, and a historical perspective will help us understand when and why the two developed in such different directions.

2.1.1 Medicine in ancient Greece

Modern Western medicine is based on the observation of the patient and the clinical symptoms he or she presents. This thought was first presented around year 400 B.C. by Hippocrates. The thoughts of Hippocrates contrasted the earlier views on medicine and disease which were then connected to magic and the ancient Greek Gods. The theory Hippocrates presented was that the body consisted of four humors (fluids) in the same way as nature consisted of four elements. The four humors were black bile, yellow bile, phlegm and blood, and healthy humans had a well balanced ratio of these four fluids. This meant that disease was seen as something originating inside the body, in contrast to today's knowledge of outside pathogens. In addition his theory was that there were four elemental conditions in which the human body could find itself; hot, cold, moist and dry. A disease was cured by utilizing the healing powers of nature after the likes of purgatives, emetics or sometimes even surgery (although only used as a last resort) [3-4] in order to reinstate the balance of the four humors. The Hippocratic method of practicing medicine was centred on the patient [5]. His thoughts were that the treatment of only one part of the body, or centred just on the disease would be an unsuccessful treatment. The physician evaluated the patient thoroughly by inspecting the symptoms, smelling the various liquids and listening to the body among other things. To keep the body balanced, the Greek emphasized the importance of a good diet,

exercise and cleanliness, which are all still highly relevant today. Although the four humor theory has been rejected and replaced with more accurate knowledge of anatomy and physiology, Hippocrates' theories represented a turning point towards modern medicine. He proposed the idea that disease resulted from natural causes, and eliminated magic and divine interference as causes and/or cure of disease. Instead he prompted that the curing of a disease should be based on observation of the patient, rational thinking and previous experiences [4-5]. The erroneous theories he proposed can be attributed to the lack of knowledge about the functions of the human body. This can in part be explained by the fact that science was in a very primitive state in this period of time and that dissection was not seen as an acceptable source of research rather that the lack of trying or the lack of will to understand medical phenomenon[5]. The Hippocratic Oath, an ethical code intended for all physicians, is also based on the ideas of Hippocrates and is still in use today. The thought and theories of Hippocrates are found in "the Hippocratic Corpus", a collection of 50-70 books assumed to be the works of Hippocrates[3]. In recent times, it has become clear that they are not the works of only one man. Historians have determined the works as being written and collected over a 50 year period, most likely by several authors. These might have pupils of Hippocrates or some of his followers and is likely to be a written version of his convictions. Although other physicians and philosophers proposed other theories and explanations, the four humors theory more or less became the prominent medical theory from the time of Hippocrates throughout the years of the Middle Ages[5].

Another philosopher whose influence on Western medicine has been enormous is Claudius Galen (129-199 AD). His theories, highly influenced by Hippocrates, persisted as the dominant medical system for over 1500 years [4, 6]. Galen continued to forward the theory of the four humors, and further associated them to the four conditions of the body; hot, cold, moist and dry. He developed an idea that the body contained three systems, and the presence of pneuma; pneuma physigon (associated with brain, nerves, liver and veins), and phneuma zoticon (associated with the heart and arteries). These two pneumas were substances essential to life. The three systems were the brain and nerves, the heart and arteries and the liver and veins. In contrast to Hippocrates, Galen was a firm believer in pharmaceuticals. He produced several mixtures consisting of a large amount of ingredients which were called galenicals. The galenicals were used to correct the balance of the four humors, and were employed after the principle of opposites. Fever (heat) was treated with a remedy believed to induce cold etc. Another important difference from Hippocratic thought was that Galen believed that the body

should be cured one organ at a time instead of being seen as a whole. This resulted in organ specific "medicines", which has continued to influence medical thought. Galen introduced dissections as an important tool for medical knowledge, and frequently performed dissections on living animals (vivisection). Galen promoted his thoughts and theories well, lecturing and performing public dissections who gathered crowds of people, and his reputation grew. His work was even translated to several languages which helped promote his thoughts widely. Although some of Galen's work contributed to medicine moving forward, his dominating position actually lead to a period of little medical progress. In addition to the stagnation of medical progress in the years following Galen, other important sanitary progress was lost. Year 476 has been marked as the year where the Roman Empire fell, and with it several sanitary systems were forgotten. The Roman Empire had developed systems for waste removal, sewer systems, aqueducts to obtain pure water and even an early version of the toilet, all of which were lost and forgotten for hundreds of years. For a 1500 year period his thoughts and teachings were the leading medical system, and not until the early stages of the scientific revolution in the 1500s did someone question Galen's theories.

2.1.2 The scientific revolution

William Harvey (born 1578) was the scientist who would finally replace the existing dogma that had been the leading medical view for a millennium. Galen's theories explaining the presence of blood was that it was constantly being created by the liver, being made from ingested food and drink received from the stomach and intestines[7]. The blood was thereafter carried to every part of the body by the systemic veins. The blood was consumed or turned into flesh in the various parts of the body, driven by the right ventricle of the heart. The left ventricle was responsible for creating a pulse for the movement of blood in the arteries that were responsible for absorbing pneuma from the lungs. The blood in the left ventricle was thought to come from the right ventricle through perforations or pores in the interventricular septum. Several physicians found this theory to contain flaws as the anatomy knowledge grew, but it was still prevailing until William Harvey eventually proposed his theory. The Renaissance (ranging from 1450 to 1700) created a new environment in society where religious beliefs and superstition were no longer accepted as the only answer. The power of the church was questioned which was followed by questioning of previous medical theories as the church had been the main source of healing and therapeutic agents[8]. Several previously known truths were challenged, and from this a method for exploring observations grew; the method which is now known as the Scientific Method. The main principle was to encourage

experimentation as a method for answering questions, and it led to several revolutionary discoveries which have resulted in the labelling of this period in time as "the scientific revolution". In the field of medicine, this period was revolutionized by the increased knowledge about anatomy. While dissection of human bodies was not allowed in the time of Galen, forcing him only to dissect animals, the Black Death (1347-1348) caused the church to allow dissection of dead humans to find the cause of the disease [8]. This was the first step in the direction of allowing dissection to be allowed as part of education, which was permitted in 1537.

The first attempt at dethroning Galen was made by Paracelcus (1493-1541). He rejected the four humor theory, and suggested that disease resulted from outside the body. In addition he used new materials as treatments, e.g. lead, arsenic and sulphur. But unlike Galen he was not able to convince people that his ideas were true, and they did not prevail. A second attempt was made by Vesaliaus (1514-64). As Paracelcus, he spoke openly about what he believed to be wrong in Galen's work. Vesalius studied anatomy thoroughly, and discovered that the works of Galen most likely were based on the sole dissection of animals, as he found several discrepancies. Another figure who made a substantial contribution towards a much more accurate anatomy knowledge was Leonardo da Vinci (1452-1519). But even if the anatomy knowledge grew and became more accurate, the knowledge of bodily functions was still almost nonexistent and Galen's blood system theory was still the only one. Although the names mentioned, and more after them noted that the flow of blood between the ventricles did not seem possible, it took another 100 years before the circulatory explanation took over as the leading theory.

William Harvey was convinced, as several other scientists been before him, that there were no perforations in the interventricular septum, and started his investigations. The age in which Harvey was born was one where the experimental culture grew, and experiments were seen as essential tools for new discoveries. This allowed him to perform experiments and dissections without being frowned upon. He performed several experiments whose results were consistent with his theories of a circulating blood system. He proved that the amount of blood moving through a single point in the body is far more than is ingested as nutrients. He also proved that the veins emptied in direction to the heart, and was refilled from the periphery. An experiment performed in public was cutting open a live snake and compressing the vein entering the heart, which resulted in a small and bloodless heart [7]. As some of the aspects of Galen's theory were questioned by different scientists, one might wonder why his dogma persisted as

long as it did. One reason may be his position, as mentioned earlier. Galen became an incredibly respected name, and he grew to become almost "more than a man". In addition, experimental procedures were not introduced as a popular scientific method until the millennium, making his theories difficult to disprove, with only rational and logical thinking as the available means. But maybe most important was the fact that there was no other plausible explanation available. Although some sceptics found flaws in Galen's theory, they did not understand enough of the human body to propose an alternative theory. The result was that Galen's theories prevailed until an alternative explanation was finally presented. Harvey did meet resistance when he presented his ideas, but as he presented his results he started gathering followers. One of these was the renowned philosopher and scientific thinker Descartes. Descartes was a firm believer that everything in nature can be explained by the use of science and mathematics. He broke with the ideas of the ancient Greeks that pneuma was present as a life giving force, and reduced the body to the likings of a machine that could be explained by science. This led to science being separated from religion, and eventually became the leading thought in modern Western society [8]. Harvey's discoveries were a starting point for research within physiology, and as followers continued his work using the experimental methods new discoveries continued to be made.

2.1.3 The "Germ Theory"

Despite the considerable progress made within the field of anatomy and eventually physiology, the cause of disease was still unclear. Treating disease by attempting to restore balance was still being used as late as the beginning of the 19th century, although physicians and scientists were not as convinced of its accuracy as they had been before. The scientific method of answering problems that developed during the Renaissance laid the ground for questioning "known" truths, and this would soon lead to the discovery of more accurate theories that would revolutionize the field of medicine. One of the leading causes of death was infections, and the *cause* of the disease became a focal point for scientists. Ignaz Semmelweis (1818–1865) was one of the physicians who started investigating the cause of infections in hospital wards [9]. In 1847 he told surgeons to wash their hands before seeing patients after performing autopsies, as he suspected the infections were related to the lack of cleanliness. Semmelweis' suspicions were confirmed as the mortality rate in his ward dropped significantly as a result of the increase in hygiene. Despite his impressive results, he was not able to convince his colleagues to embrace the theory, and it took another twenty years before the idea was rediscovered by Joseph Lister, and the importance of sterilization of instruments

and hands were acknowledged. The work of Joseph Lister was facilitated as a result of the discoveries of Louis Pasteur and Robert Koch. Louis Pasteur assumed that as disease is able to spread between people, something must be present to spread it. He proposed that there was a presence of invisible microorganisms in the air which he called germs, and presented the germ theory in 1870. The presence of bacteria was actually visualized almost 200 years earlier through the first light microscope, but its creator Anton van Leeuwenhoek (1632 – 1723) did not think of any connection between the newly discovered bacteria and disease and the significance of the discovery was not realized at the time. Pasteur also disproved the theory of spontaneous generation (vitalism), which had been a leading theory so far. Building on Pasteur's work, Koch presented his postulates, tying the presence of germs and the development of disease together. His postulates functioned as an explanation of disease, and he also developed culture medium in which he grew different bacteria. Together they developed vaccines and their work represents a turning point in medical history as more and more bacterial species, and thereby the causes of several diseases were discovered. Pasteur was a respected scientist, and his expertise was called in to consult by for instance Napoleon III, who wanted him to figure out why the wine went sour just after production. This lead to the development of pasteurization and also showed the possibility of using science and the scientific method in solving all sorts of problems. After the discovery of bacteria, there were still some diseases that were unexplainable, but the discovery of the tobacco mosaic virus (1890), as an infectious agent that remained in fluids that were filtered through membranes that removed bacteria, made it apparent that there were microorganisms that were even smaller than bacteria. In the following years scientists continued to make progress, and new advances in technology lead to multiple new discoveries such as x-rays for diagnostic purposes and a number of new medicines including the revolutionary discovery of penicillin finally making infections curable.

2.1.4 Evidence based medicine

In 1972 Archie Cochrane presented an idea based on the scientific method which he called "evidence based medicine" that became one of the foundations of modern Western medicine. The basis of his thoughts was that after an evaluation of the patient's condition the physician should investigate scientific papers and statistics and evaluate the different treatments to find the best possible solution for the patient. Rejecting the treatments that did not have an effect and continuing with the treatments that did have an effect was not a new idea. But the documentations and experiments that were being produced, resulted in a large amount of

available research material that had been tested and validated, and which was previously unavailable. Cochrane also focused on the importance of conducting clinical trials. The administration of different treatments for comparison had until that point not been considered, as the physicians previously decided the treatment dependent on the existing dogma of their time. This new thought was to look for scientific and statistic *evidence* that the treatments were effective, and discard any that was not able to show efficacy.

Employing the scientific method has resulted in a detailed knowledge of the body, down to the cells and genes. From the 1950s to 1960s it became possible to study the molecular components of cells, and the structural determination of DNA was achieved in 1953 [9]. It had already been determined that DNA contained genetic information, and with the human genome project (1999-2003) every single nucleotide of all the human genes has been determined. The scientific investigation of the body and its construction is constantly resulting in new discoveries, and opening the door to new possibilities. The mapping of the human genome makes it possible to perform genetic testing to look for genetic markers of diseases, utilizing gene therapy and individualizing pharmacotherapy. There have been several events contributing to shape the basis of the Western medical system into what it is today. Therapeutic agents in modern Western medicine have been through extensive testing for quality control and efficacy, and the lead compounds mechanism of action, side-effects profile and ADME (adsorption, distribution, metabolism and elimination) profile have been mapped. Thorough knowledge about the lead compound is required before the compound is considered to be approved for use and this is expected by the patient. This is the result of a medical tradition that has developed over several hundreds of years. The common denominator in the work of Descartes, Harvey, Cochrane, Pasteur and several others combined with the changes that occurred in the Renaissance have all contributed towards the scientific method, causal relationships and evidence based medicine as the basis of Western medicine.

2.2 Traditional Chinese medicine

As described in the previous paragraph Western medicine has developed into a system based on science and statistics, and the curing of a disease starts with identifying the *cause* of the disease. Western medicine is based on the theory of cause and effect, and the doctor will try to isolate the symptom down to a distinct, treatable cause separate from the patient. In order to

try and understand traditional Chinese medicine (TCM) we need to adapt a new way of thinking. We need to forget about causal pathways, and stop isolating illness as something that is separate from the person as a whole. In TCM nothing can really be understood if it is taken out of context. Everything is considered as having its place in a bigger picture, a way of thinking that can be partially explained by the Yin Yang theory. The result of this influence is that a TCM practitioner will never look for a cause for the disease, in fact the cause is considered irrelevant. The focus is not on the disease itself or its cause, but rather on the patient, and what the patient experiences.

2.1 Yin-Yang theory

Chinese medicine dates back thousands of years. Although the validity of the sources has been questioned in later years, the concept of Ying and Yang, acupuncture and herbal medicines are said to date almost 5000 years back [5]. The Chinese way of thinking has been greatly influenced by the Chinese traditional religion. This has been the leading religion in China for thousands of years, and helped shape the cultural tradition in the country. Chinese traditional religion, or Chinese folk tradition as it is also called, is a form of religion that developed from several other religions or ethical systems. Its inspirations have been drawn from Confucianism, Taoism, Buddhism and ancestor worship. The common denominator of these religions is the focus on humanity and ethical rules of conduct. Confucianism presented a version of the Golden rule, Taoism taught the importance of compassion, humility and moderation, and Buddhism taught (in very simple terms) that good actions are rewarded in your next life. From these religions (especially Taoism) the Chinese got the concept of Yin and Yang, which is a central element in understanding TCM. The Yin and Yang symbol represents that everything is part of a whole [10]. The symbol represents how opposites are defined by each other, and that nothing can be understood when taken out of context. Without dark, there is no light, without action there can be no inaction. For Yin to exist, Yang needs to be present, as the one cannot be defined without the presence of the other. The basic thought is that everything can be defined as having both a Yin and a Yang quality. The original character for Yin translates to the shady side of a slope [10], and typical Yin qualities are qualities such as rest, passivity, darkness, cold and calmness. The Yang character could originally be translated to the sunny side of a slope, and is characterized by qualities opposite to Yin qualities, namely things like activity, light, excitement and warmth. To underline the fact that everything is part of a continuous

The Yin Yang symbol

whole, Yin and Yang qualities can be divided further, into new Yin and Yang aspects within Yin and Yang, which can be further divided and so on. Yin and Yang balance each other, and a harmonious relationship is present when neither one is in excess, causing the other one to be weakened. To achieve this balance, Yin and Yang are constantly transforming, creating each other and supporting each other [10]. This theory is rooted in the Chinese culture causing a distinct difference between the Chinese and Western way of thinking. Because everything is part of a whole, and is constantly transforming as a result of naturally occurring changes, the relationship between cause and effect is largely absent in Chinese philosophy. Light does not cause darkness and action does not cause inaction, rather they are part of a relationship where the one transforms into the other as a process of natural change [11]. The concept of Yin and Yang, that harmony is dependent on balance and natural transformation, is transferred to the body in TCM. Health is believed to be a result of a well balanced relationship between Yin and Yang qualities, and a person's ability to adapt to change in order to maintain this balance is crucial for maintaining a good health. This results in the cause of disease being irrelevant when examining a patient, as changes occur in the absence of an external factor but rather as a result of natural transformations and cooperation between Yin and Yang qualities.
Modern biochemistry and detailed physiology is not really relevant for practitioners of TCM. As diagnosis and healing is based on observation of the patient's condition, and not what caused the condition, TCM practitioners never sought this kind of knowledge. Instead TCM has a way of seeing the body and its constituents that is very different from the Western view. The Yin-Yang theory is an essential concept in every aspect of traditional Chinese thought, also with regards to the body and its function. An equally important concept is the concept of Qi and the other essential textures. The thought is that everything, both living and nonliving matter contain Qi.

2.2.2 The fundamental textures

Qi and the Yin-Yang theory are dependent on each other; because Qi is present in everything, it is the substance that allows everything to change and transform into other shapes and forms. Although Qi is believed to be the factor that makes changes possible, the Qi does not cause change, but rather it is present throughout the transformation as well as before and after the change. The Qi found in humans originate from three separate sources; transferred from parent to child (Original Qi), acquired from nutrition (Grain Qi) and as a product of the air we breathe (Natural Qi). Together they make up the Qi of the person, which serves several important functions in the body. Qi is seen as a protector of the body from hurtful external

influences, it is required for transforming nutrients to new forms (Qi, blood, fluids) that is necessary for the body, it also supports the organs, the movement and the bodily fluids so that they stay in their proper place and it maintains the normal temperature. Although the Qi has many functions in the body, it is not seen as the cause of anything, as the Qi of nutrients or organs are not seen as an entity that is separate from where it belongs. As something changes, so does its Qi. A large number of different types of Qi have been identified, but in terms of TCM, there are five important ones; Organ Qi, Meridian Qi, Nutritive Qi, Protective Qi and Qi of the Chest [10]. Organ Qi is associated with every organ, and its action is dependent on the nature of the organ. Meridian Qi is associated with the meridians and flow through them ensuring and adjusting the internal harmony. Nutritive Qi is present in blood and helps transform food and other nutrients into blood, while protective Qi is the Qi responsible for protecting the body. The Qi of the chest is associated with the heart and lungs and their functions. In correlation with the Yin-Yang theory, an imbalance in the Qi results in disharmony and may result in illness. The two major disharmonies that can manifest are deficient Qi and stagnant Qi [10]. Deficient Qi may affect the entire body leading to a lack of energy, or it may be centred on a specific organ, resulting in a less than optimal function of this organ. Stagnant Qi is the term used to describe a Qi that is not flowing properly. This may result in pain or reduced function of organs. In addition to Qi, TCM describes four other textures of the body; Blood, Essence, Spirit and Fluids. The Blood, not to be mistaken for blood as it appears in Western medicine and physiology, is thought to be created from nutrients. The Blood circulates through the vessels and meridians of the body, and its task is to bring nourishment to the different areas. In relation to Qi, Blood is seen as a Yin aspect, while Qi is regarded as a Yang aspect. The two control each other as the Blood nourishes the organs and their Qi, while Qi helps create the Blood and keep it in its place. In this way they represent the Yin-Yang theory where everything is part of a whole and dependent upon each other. As with Qi, Blood can also be deficient or congealed/stagnant.

Essence is the substance that separates living organisms from the nonliving. Essence is thought to have a dual origin; prenatal Essence is inherited from the parents as the Original Qi, while the postnatal Essence is acquired from nutrients and the surrounding environment. A balanced and well functioning Essence is required for normal development. An Essence that is not balanced may lead to developmental problems such as retardation of growth and infertility. The Spirit is the substance that separates humans from animals. The Spirit it what makes humans able to form special connections to something or someone, and is the

substance that allows for relationships with ancestors, relationships that are not physically possible. In addition it is the Spirit that allows awareness and moral thoughts and personal convictions. It is Spirit that makes it possible for humans to shape their own life. The Fluids are the liquids which appear in the body other than Blood [10]. They have the same tasks as Blood, although they are not as potent and deficiencies of the Fluids mostly result in dryness.

2.2.3 TCM physiology; Organ networks and Meridians

The meridians are believed to be pathways in which Blood and Qi travels, but they are seen as something separate from Blood vessels. There are 12 meridians associated with the 12 Organs, and an additional extra 8 that results in a network of 20 meridians that connect every aspect of the body together. A harmonious body requires good flow in the meridians. If the flow in one of the meridians is disrupted, the connecting Organ may experience disharmony that results in illness. An Organ that is not well balanced may cause the flow in the connecting meridian to be disrupted, which may result in pain. The meridians are very important for the TCM practitioner, and have been used as an explanation of the concept of acupuncture. The meridians connect the inner parts of the body to the outer parts, which makes it possible to affect the inner organs by piercing the outer part of the body. The meridian concept is utilized in herbal medicine as well; the herbs are believed to enter the meridians and then reintroduce balance. The herbs a practitioner uses are dependent on the diagnosis. To make a diagnosis, knowledge about the Organs is vital. The Organs described in TCM are not the same as the organs described in Western physiology. In TCM the Organs are more like Organ networks that controls and influences both physical and psychological functions [11]. The Organs are defined on the basis of their function and what they do in the body, not as a single and permanent entity. As everything else, the different Organs have Yin and Yang properties. Yin Organs are considered to be positioned deeper in the body, and are responsible for transformation and storage, and are closely related to the fundamental textures. The Yin Organs are the Heart, Lung, Spleen, Liver and Kidney (and the Pericardium). Yang Organs are considered to be less connected to the fundamental textures, and thus less influential on psychological aspects. The Yang Organs are the Gallbladder, Stomach, Small intestine, Large intestine, Bladder (and Triple burner (a non-anatomical organ which is believed to control the body's Water)). The main tasks of the Yang organs are to adsorb useful nutrients and excrete the waste. Each Organ has an Organ in the other group to which it is tightly connected. The Spleen is connected to the Stomach. The Stomach processes the food and absorbs nutrients which it passes to the Spleen, and removes waste. The Spleen is fundamental in the digestion

process as it is the Organ that creates Blood and Qi from nutrients. A functioning Spleen is needed to maintain a functioning circulation. The Spleen is also important in psychological function. It is said to control one's ability to make decisions and to be a source of motivation. A person that either makes hasty decisions or is not able to make decisions at all might be influenced by a disharmony in the Spleen. The mouth and lips are said to be an image of the Spleens condition. The Liver is connected to the Gall bladder, which stores and excretes bile. Bile is in this context the result of excess Blood and Qi. The circulation of Blood is thought to be controlled by the Liver, which also regulates the Qi. Disharmonies of the Liver may therefore affect both the circulation of Blood and Qi. The eyes are said to be related to the Liver, and disorders in the eye are believed to originate in the Liver. The Kidneys are connected to the Bladder which eliminates urine. The Kidneys are supposed to store the Essence which regulates maturation as described above, and Kidneys that are not harmonious may result in lack of normal body development. The Kidneys are also believed to be related to the ear and diseases of the ear. The Heart is connected to the Small intestine which continues the work of the Stomach. The Heart stores the Spirit, and disharmony in the heart may reduce the person's awareness and moral. The Heart regulates the Blood flow, and a harmonious heart results in a good and even flow and pulse. The tongue is an image of the Heart's condition, and is an important diagnostic tool. The Lungs are connected to the Large intestine that continues the work of the Small intestine. The Lungs are responsible for the emotions, and balance of the Liver is required for the person not to become hysterical or lose all emotions. The Lungs combine the natural Qi with the internal Qi, and is also associated with the Qi of the Chest. A deficiency or imbalance in the Lungs may cause deficient Qi all over the body. Diseases of the nose and throat are believed to be connected to the Lungs.

2.2.4 Diagnosis and treatment

Factors that may result in disease if the body is not in harmony are environmental influences, emotions and the way of life. There are six pernicious influences that are named after meteorological conditions; wind, cold, fire, dampness, dryness and summer heat [10-11]. The presence of one or more of these influences results from an imbalance in the Yin-Yang relationship within the body. This weakens the protective Qi and allows external influences to affect the body's environment. A pernicious influence may be both internal and external, the external being more sudden in onset and more acute, while the internal ones are characterized by more chronic conditions. The signs and symptoms of the imbalance resulting from the various influences correspond to the name of the influence. Wind is a Yang condition, being

quick and dry. External Wind may be compared to the infection in Western medicine. Internal wind is characterized by excess activity, and Wind is usually followed by another pernicious influence. Cold is naturally characterized by the patient feeling cold. Cold in the body will manifest as it does in nature; by contracting and freezing. Cold is considered Yin, and may manifest when Yang is deficient as a result of reduced Kidney function. Fire is Yang, typically hot and active. As described with Cold, Fire manifest as it does in nature; fever is typical. A damp condition is recognized as being wet and heavy, and affecting the lower part of the body. It is classified as a Yin condition. Dryness and Summer Heat are not as important as the other four influences. They are usually not considered by practitioners. The common feature in all of these influences is that treatment is issued on the basis of the condition of the patient and the symptoms. The cause of the symptoms is always irrelevant. The way of life may also affect the internal balance. The spleen and stomach are influenced by the diet, as they are the organs most closely related to the food intake. A lifestyle of too much or too little activity at the wrong time may be the result of internal imbalance, or may generate imbalance over time. In a similar manner excessive emotions are signs of a person that is not harmonious. Although all of these are things that would be considered causes in Western medicine, they are not so in TCM. In TCM the cause becomes the effect. For instance, excess emotions may eventually lead to an imbalance in the body which again results in excess or an imbalance of emotions. In this line of thought, the principle of causal pathways is not relevant.

When a TCM physician is examining a patient, it is important to consider all symptoms and signs the patient presents. All aspects of the patient need to be considered, not just the presenting symptom that is bothering the patient. The presenting symptom will actually often be of less importance to the practitioner. The aim of the TCM doctor is to detect the patients "pattern of disharmony". According to the yin yang theory, a healthy person is a person in which everything is in harmony, or well balanced. The way of treating a patient in TCM is therefore to try and restore the imbalance, and the means of doing so are completely unrelated to what may have caused the imbalance. This is achieved by aiding the self-healing powers of the body by the use of herbs, acupuncture or other treatments, or by removing what stops the self-regulating ability from functioning [12]. A person's pattern of disharmony will depend on several aspects of his/her life, not just the physiological. Patients presenting a symptom that leads to the same diagnosis in Western medicine are likely to receive the same standardized treatment. In TCM, these people are likely to receive different treatments as they would show

different patterns of disharmony based on their personality as well as their physical appearance and characteristics. Together all these observations constitute a pattern of disharmony, and this disharmony can be treated. The first step to a mapping the disharmony is by looking at the eight principal patterns of disharmony: Yin/yang, interior/exterior, excess/deficiency and hot/cold [10]. The signs are interpreted to see if the disharmony is affecting internal or external parts of the body, correlating to the different pernicious influences. A person with deficient Qi, Blood or Organ activity will act slowly, have little energy and appear pale. The opposite would be the case if the disharmony is caused by a pattern of excess. If a person is cold it is indicative of a pattern of Cold, which could be result from insufficient yang or cold pernicious influences. After considering these basic patterns, the next step is to take a closer look and evaluate the status of the Blood, Qi, and Yin and Yang properties, followed by an evaluation of the Organs. One symptom can mean something completely different if accompanied by another symptom, according to the theory that nothing can be understood on its own. The examination of a patient is usually very thorough as there are a lot of patterns to consider. The way of diagnosing a patient is through "The Four Examinations" (which are actually five as two of the words are the same in Chinese); By looking at the patient, listening and smelling the patient, talking to the patient and touching the patient. By looking at the patient the physician notice the shape and apparent physical form and the way he behaves. For example a shy and small person may indicate a pattern of deficiency. But the most important factor in this group is the tongue. The tongue is studied thoroughly considering its size, shape, colour and movement. The physician listens to the patients voice and if present the type of cough. Different voices and cough are signs that point to various disharmonies. Questions about feeling hot or cold and personal background are considered most important, but also pain, urination and other bodily functions are investigated. Together with the tongue, the pulse is one of the most important factors in making a diagnosis. There are a vast number of different pulses, distinguished by their shape, strength, length, rhythm and so on. The different pulses correlate to different conditions or disharmonies in the body.

Although the Yin-Yang theory is dominant in TCM, there is another theory worth mentioning; the five phase theory. This theory introduces another five ways of categorization, and developed a while after the concept of Yin and Yang [10]. The Yin-Yang theory and the five phases are not separate theories, and are used together to achieve a more complex picture. Yin and Yang aspects can be classified further into one of the five phases. And the five phases

can be classified as either yin or yang. The five phases are fire, metal, wood, water and earth [11]. The phases are symbols of different qualities, and each of them represents activities and cycles of development. Fire is seen as an optimal state of activity, decline is expected. Metal represents the declining state while wood represents a state of increasing activity and growth. Water is a phase of calm about to change, while earth balances the other phases and is described as a phase of balance. The five phases are also used to describe the climatic changes that occur during the four seasons, and there are connections between the phases and the different organs. But because the physiology of the five phases theory does not always correlate with the eight principle patterns, the five phases theory has become very flexible and is sometimes disregarded by TCM physicians. Although not always utilized in clinical practice, the five phase theory remains an important theory in TCM.

2.2.5 Chinese history and the use of TCM

The end of Imperial China came in the beginning of the 1900s as a result of the increasing trade with European countries [13]. The difficulties with the British, the following opium wars and opening of more ports for trade eventually led to the fall of the empire. The creation of a new political system was problematic, understandable for a country with a history of thousands of years of imperial ruling. A divided country ruled by warlords was somewhat united by 1923 by the Nationalist party [13]. This was achieved by the help of the Soviet Union. China then sided with the allies during the First World War, and both events opened the door to Western influences. In 1949 the People's Republic of China was funded by Mao Ze Dong, and the communist rule led to a major change in Chinese society and the health care. As part of the new heath care regime, healthcare now became available to everyone, and TCM was integrated with Western medicine. With help from Russia hospitals and medical schools were introduced to China, and the Western influence grew. Although the Western influences were present, TCM enjoyed an increase in popularity during these years. The medical services were largely nonexistent, which encouraged the use of TCM. The knowledge were available, the treatments where cheap, and hospitals treated patients with herbal medicine and acupuncture. A major setback came during the Cultural Revolution (1966-1976). The communist rule was intensified, and symbols of the old society were destroyed and in some part prohibited, and education almost came to an end and doctors were in large part sent to work in rural areas [14]. Although this led to a situation where education almost came to a halt, the doctors in rural areas served as teachers for over a million "barefoot doctors". These were farmers that received a minimal of medical training making them able to

treat the most common diseases and to initiate preventive care. In retrospect, this may be seen as a positive development; growing knowledge of herbal medicine, an increase in barefoot doctors making health care available to rural areas at low cost. Mao Ze Dong died in 1976, and this ended the Cultural Revolution and this set the scene for another major change in Chinese health care and also an increasing interest in TCM.

TCM is deeply rooted in the Chinese culture. From my visit to China, it was evident that TCM is still very much in use. From the use of ingredients in food believed to be beneficial to health, modern pharmacies selling herbal remedies and dried natural products to the very visible purple marks of cupping on people in the streets, it was apparent that traditional treatment is still in use. The two main forms of TCM in use today are acupuncture and herbal medicine. Acupuncture has been adopted by the West and utilized for a while, and in later years herbal remedies and natural products are also constantly increasing in popularity [15-16]. In China, when a pattern of disharmony has been diagnosed, the practitioner chooses the appropriate treatment. Several texts are available as well as the traditional pharmacopoeias, and the physician has many basic prescriptions (herbal remedies) to choose from. Herbal medicine usually contains several ingredients. When the practitioner has chosen the appropriate prescription, the natural products are regulated by adding more of some and less of others dependent on the patient. Herbs are categorized on the basis of their qualities: Some herbs are classified as warm, some as cold. The shape of the herbs is considered when categorizing them as well as taste and their properties[11]; if they tonify, consolidate, disperse or have a purging effect. The principle of opposites is used when treating Heat and Cold disharmonies: Cold is attempted rebalanced by the use of warm herbs. "The Doctrine of Signatures" is another principle in use, and the theory is that similarities between plants and body parts indicate what the plant will be able to treat efficiently. An example is the Ginseng root which resembles a human body and thus is believed to strengthen the entire body. An herb's properties are based on the assumed effect of the herb. The ability to move stagnant Qi or Blood is the property of a dispersing herb. The opposite is found in consolidating herbs that treat patterns of deficiency. An herb with purging properties is able to rid the body of unwanted substances and can be used in conditions of stagnation that has become chronic. A tonifying herb is basically an herb that strengthens the body and is also able to rebalance patterns of deficiency.

The combined use of ingredients is thought to be beneficial by reducing side-effects and increasing the therapeutic effect. A typical mixture is composed of ingredients that serve

different purposes. The different ingredients are called the emperor drug (to which the main effect is attributed), minister drug (aid the emperor or target coexisting symptoms), assistant drug (aids the two above or treats minor symptoms) and servant drug (either target the treatment to a point in the body, or generally helps the other ingredients to act harmoniously) [15, 17]. The Chinese pharmacopeias, Chinese Materia Medica have developed throughout history. The Chinese history dates more than 4000 back to the first known dynasty, the Xia dynasty. The first known herbal writings dates back to the Han dynasty, who ruled from AD 25 – 220 [17]. Since these earliest texts, the number of herbs has continued to grow, from 365 remedies in the first Materia Medica to about 8000 in the Encyclopedia of Chinese Materia Medica [17]. In addition to the increase in number, there has also been an increase in knowledge, as the remedies have been used as treatments for thousands of years.

2.2.6 Health care in modern China

The communist rule from the 1950s to the 1970s led to an improvement in health care where basic care was available for everyone [18]. After the end of the Cultural Revolution and the death of Mao, a change was made to the health care market with the introduction of the market reform. This led to a health care system that was no longer universally available, and too expensive for some groups of society. The reduction of government funding resulted in heath care being a service where you now needed to pay for the services. As a consequence the best health care was found where people were able to pay; in the larger cities. The medical aid available in smaller towns and the country side was reduced and the cost was in some cases too high. Eventually the government increased funding to lower the prices on basic services [19], which led to hospitals making a profit where it was possible by increasing the costs of drugs and the use of technological equipment. In addition the overuse of drugs grew in order to improve profits.

The cost of Western medicine has caused the government to increase the encouragement of the use of TCM [20]. Today China has a medical system where TCM and modern Western medicine is practiced alongside each other [21-22]. TCM accounts for 40% of all health care in China, not counting self medication which is frequently used, especially as prophylactics or health promoting drugs. In rural areas the use of TCM is even more prominent. And the development of TCM continues; 95% of all Western hospitals have a TCM department [21], 60 000 new students enrol in TCM universities each year and patients in hospitals can choose which type of treatment they want [20]. The modern health care system in China has developed into a system where the two complement each other. Acute illnesses are usually

treated by Western medicine and technology, while chronic conditions and preventive measures are more commonly treated by the use of TCM. All Western doctors receive some TCM education, and all TCM practitioners receive some Western medicine education at university. From a Western perspective TCM has been acknowledged as a field of interest, and it appears that traditional treatments have an effect. But to be fully accepted among Western practitioners the biochemical components need to be investigated and RCT have to be conducted. Although research on TCM has increased recent years, there are still some issues that result from the different perspectives. TCM considers the withholding of treatment unethical, making RCT problematic. In addition the RCT model assumes that one disease can be treated with the same remedy, and that patients are a homogenous group, which is not consistent with the traditional Chinese thought. The use of several herbs and the thought that they complement each other also makes the testing of effects problematic as from a Western point of view it is difficult to locate the source (biochemical constituent) that causes the effect. Regardless of the problems, interest and research in TCM is increasing and its potential in drug discovery is recognized.

2.3 Comparing Western and traditional Chinese medicine

When comparing the theoretical framework of TCM to that of modern Western medicine, there are few, if any, similarities. The fundamental concept on which we base our thoughts of disease, pharmacotherapy and even view of the world appear to be completely different. And from a modern point of view it is. But historically it was not. Medical theory in the West, from ancient times throughout the medieval period show several similarities to TCM considering both how the concept of disease was defined and how the therapy was used. The period which appears to have separated the two is the Renaissance in Europe from 1450 - 1700. During this period changes in all aspects of the society including medicine were abundant, and in large part they shaped the Western medical theory. The contrast in the political environments in China and the West raises the question if it was the political changes during the Renaissance that opened the door to critical thinking that eventually developed into the scientific method and modern science. With China opening up to the rest of the world it was expected that Western medical thought would replace TCM, but this is not the case. Instead it is evident that TCM is becoming increasingly popular in the West, and what appears to be a harmonious relationship between the two has developed in China. The research in the field of TCM has increased and studies document its effect, and at the same time novel

chemical structures are being isolated from TCM plants. Can this ancient traditional knowledge introduce a new dimension to Western medicine and therapeutics?

2.3.1 Early medicine, culturally dependent

The theory behind TCM might seem complex and maybe even farfetched for someone from the West. The holistic world view, concept of Yin and Yang, Qi, fundamental textures and the Organ networks are hard to grasp. Especially the absence of causal relationships is very distant from the scientific perspective of the West. But if we look back in history, there are clearly similarities between the early medical tradition in ancient Greece and medieval ages to traditional medicine practiced in China. It was fairly revolutionary theories that were presented when medicine in the West abandoned the old ideas of divine influence, and turned towards nature. Although TCM appears to date further back than Western medicine, some of the central concepts and ideas in the two are very similar in a historic perspective. Both Chinese and the Western medicine introduced by Hippocrates embraced the role of nature. Chinese medicine regarded it as a part of a macroorganism containing everything, while the West considered it as a healing force. Both TCM and early Western medicine focused on the internal balance, and the development of disease as a result of imbalance. The West emphasized the importance of nature, and the composition of the body was also influenced by this. The four humors were based on the four elements, as were the four conditions in which the body could be. The composition of the body in TCM is based on the five fundamental textures, and there were six conditions the body could be in. The idea of the physician as a catalyst of rebalance was shared between the two. The West believed the nature would help the healing process over time following suitable treatment by the physician. TCM theory believes the body will rebalance itself with the aid of herbs or acupuncture prescribed by the physician. The use of herbal medication was one of Galen's important contributions to medicine, and the herbs he used were used to rebalance the humors. He also introduced the concept of pneuma, the substance essential to life, which shows some resemblance to the concept of Qi, Essence and Spirit. The early medicine was based on the existing view of the world and the culture of its time. Chinese medicine is highly influenced, and in some part based on especially Taoist and Confucian thoughts that were the dominant religion at the time. In the medieval years in the West natural product treatment was tolerated by the church, but it was tightly controlled and the development of new ideas was not encouraged as it was considered blasphemy and as criticism towards the Church. Although the view of disease had turned away from the superstitious, the Church was still the most powerful institution of the

time, and to turn against it was not an option (yet). Early medicine was clearly culture bound and based on the current beliefs and way of thought. There were limited scientific means available and really no reason to test the current hypotheses. TCM was deeply rooted in religious beliefs and with its holistic view there was no reason to look for the cause of anything. Western medicine was partly bound by the prominent church and Galen's ideas were so dominating no one could challenge them and society shaped the medical ideas of the time both in the East and the West.

2.3.2 Changes in the West, continuance in the East

The change that would separate TCM and Western medicine came during the Renaissance and would eventually create the fundamental difference between the two. During this period Western medicine, mathematics and logic were introduced as scientific tools which were culturally independent [23]. These new tools made it possible to test hypothesis and make new discoveries. The Renaissance was a period of new discoveries and revolutionizing inventions, but also a period of social and cultural change. The increasing discontent and open criticism of the church's position eventually led to an environment where it became acceptable to question previous truths and theories. All aspects of society and science were questioned, including medicine. The changes that occurred during this period transformed Western medicine into a science, where nothing is accepted unless it can be proven and tested. The previous theories about internal harmonies, balance and humors were abandoned and a new more detailed *mechanical* view was introduced. The mechanism behind the disease eventually developed; the idea that an effect/symptom is always preceded by a cause that can be isolated and cured. This period changed the foundations of Western medicine, and created a gap between Western and Chinese medicine. The entire view of the world changed, and eventually led to the thought that everything in nature can be explained by laws of physics and mechanics. The idea that disease has a distinct cause makes it possible to see the disease as separated from the body and treat it by standardized treatments.

This type of revolution did not happen in China, and has been proposed as a reason why the scientific method never really reached TCM [23-24]. It seems like a natural conclusion that the development of a society where free thinking and new ideas were subjected to open debate was a necessity for scientific progress. The Chinese were living in an imperial system at the time of the scientific revolution, and it has been proposed that this type of society did not allow for open debate required for progress. Until the Cultural Revolution in the 60s and 70s, the idea of challenging old theories and truths were almost nonexistent. But this might be an

answer that is a little too simple, as the Chinese had been the country of leading technology until this period. Several inventions were present in China long before they were found in the West, for instance gunpowder and paper, and also things as wheelbarrows and the mechanical clock [23]. The church's position has also been proposed as a reason for the oppression of new ideas and science in the West, because the same tendency is seen in other countries. But this can also be debated as the religious system in China, Taoism and Confucianism, can be described as more of an ethical rule of conduct than religion. New technology will usually develop when there is a need for it, as something that is given attention from many people will increase the likelihood that a solution is developed. The scientific method was embraced in all aspects of society as scientist understood that real-life problems could be solved with hypothesis and tests. The scientific revolution is a unique period in history and the consequences where the development of a new way of thought in the West. Although it is clear that this was the point where Western and Chinese medicine were separated, the reasons why it only happened in the West are hard to grasp. To look at the occurrences in the West and point out their absence in China has been proposed as being too simple [23]. It is cannot be assumed that the same events in China would have led to the scientific revolution originating in China instead. The answer to why science did not develop in the same manner in China will always result in speculation, as there are so many possible suggestions, and it is impossible to be certain of the consequences of certain events. But that does not stop people from trying.

In my opinion the absence of the scientific revolution and the continuation of TCM results from several factors, including the ones described above. I think the imperial system partly inhibited the concept of free thinking and challenging of old truths, and I think the oppression of the church in the West did hold back the scientific development. The way we behave and think are based on our experiences and how we perceive the world. Taoism and Confucianism greatly influenced the Chinese society over a long period of time. The Taoist view of the world probably became integrated in the Chinese culture and thereby shaping the perception of the world, and also of medicine. Although this holistic approach is difficult to apprehend from a Western perspective, it is easily understandable that it could become as rooted in a society with very little contact with the outside world as the scientific line of thought has become in the West. When the history and tradition of the Taoist thought and TCM is considered, developing over a 3000 year period it is even more understandable. The cultural influence of the West results in it being difficult to even grasp the theory of holistic views and

no causal relationships, because the scientific method is so deeply rooted in our society. But we must consider the possibility that there exists a system that is equally rooted in the Chinese society, and that this system does not consider the intricate knowledge of everything and the mechanical theories to be relevant. Maybe it is as simple as that the scientific revolution "was not needed" in China at this time. Their perception of the world did not require or encourage the chase for causal pathways, and their medical system had a 5000 years old history of experience and probably a noticeable effect as the system has lasted as long as it has. The difference between the two medical systems can probably be traced back to the cultural influences in the two countries that led to a fundamental difference in the perception of the world and everything in it.

2.3.3 Potential synergies – the best of two worlds

Despite the obvious differences between TCM and Western medicine, TCM is becoming increasingly popular in the West, and TCM is sustaining in China even though Western medicine was introduced several decades ago. The Chinese still believe in their old theories and treatments, and people in the West are turning towards natural products for a variety of illnesses. One reason is the beliefs that natural products are "safer" and results in fewer side-effects. The pharmaceutical industry has appreciated the possibilities that TCM could be a possible source of many new lead compounds, and the isolation of natural products has resulted in blockbuster drugs. But there are still obstacles. The lack of characterization of lead compounds and efficacy studies such as RCTs prevent TCM from being accepted by Western practitioners and scientists. Increased use of modern science to evaluate the TCM treatments seems to be a necessity if the use of TCM is going to be accepted in the West. But there have also been objections to some of the evaluation methods, for instance RCTs among practitioners of TCM. Through the attempt to perform quality controls and efficacy studies, it has become obvious that TCM may be a great source for novel chemical compounds. In recent years the interest in multicomponent therapeutics has increased, and pharmaceutical companies are starting to explore the use of combinations of drugs as opposed to the one drug, one target theory. TCM might serve as an inspiration in this field, and it appears that the ancient knowledge of TCM may complement the Western medicine in more than one way.

TCM is regarded as an important part of the medical system in China, but it is still regarded as an alternative form of medicine in the West. This does not keep traditional medicines like herbs and acupuncture from increasing in popularity in the West. Herbal medicines are gradually becoming more accepted by the general public, and the sales of medicinal herbs and

extracts are believed to increase by 4 billion dollars each year [16]. The “fascination” of synthetic drugs and their effects that revolutionized Western society by making it possible to cure disease is declining. The focus of consumers nowadays is often centred on the possible side-effects that may follow in addition to the therapeutic effect. Natural products and medicinal herbs appeal to consumers for several reasons. Herbal medicines may seem more natural and thus safer and may be considered less likely to cause side-effects. The overwhelming positive effects that are sometimes portrayed by the media may also be a factor. The thought of TCM that puts the patient in the centre may also be appealing. In short people are looking for alternative ways to maintain health, prevent disease and increase their quality of life. This has resulted in various forms of TCM being practiced in over 100 countries [22, 25]. In both Germany and Britain the use of acupuncture is increasing and accepted as a part of the health care covered by insurance and government [25], and TCM courses are available in Universities.

The health care in China consists of a combination of TCM and modern Western medicine. By teaching TCM in Western medical schools and Western medicine in TCM universities and offering both services in most hospitals the goal is to provide the best possible care for the patient by using the best practices from each system. In TCM hospitals, Western equipment and surgery are used. This is partly described by the financial situation, where Western tests are more profitable for the hospital, but it is also a consequence of the merging of the two systems. In most situations Western medicine, both treatment and diagnostic tools, is utilized in acute situations while TCM are more popularly used to maintain health and in chronic conditions. The treatment prescribed for various diseases are often a combination of Western drugs and TCM. The idea of alternative medication in chronic disease, especially idiopathic disease, might seem alluring. The effects of TCM have been observed and documented over thousands of years and the reduced side-effects compared to Western medication would appear to make it a good candidate for this kind of diseases. But I believe that the cultural difference is too big at the moment. The concept of evidence based medicine is too rooted in the Western medical society to approve of any TCM, and especially herbal medication unless the safety, efficacy and even mechanism of action have been investigated. In addition to the issues with efficacy, there is the problem of possible interaction. If an herbal medication does produce an effect, it may very well be able to interfere with any other medication the patient is using. The problem with the need for these studies originates in the theories of TCM. The herbal medicines consist of several herbs and active ingredients, and are often used for

chronic diseases [22]. The theory behind TCM is that the medicines help the body to rebalance and harmonize. This might lead to a time delay between the treatment and the effect, and in chronic conditions it is often more complicated to evaluate the effects resulting from the treatment. These are all issues that need to be handled when researching the effects of TCM. In addition there has been a debate about the use of RCTs in evaluation of TCM. Critics point out that RCTs are not suitable as the theory if the test is not compatible with that of TCM. TCM emphasizes the use of individualized treatment based on the patient, and the same disease (seen from a Western perspective) might not receive the same treatment. The treatments are very complex and are composed by several active components, and the standardized categorization of disease and treatment clashes with the fundamentals of TCM. It has been argued that the standard efficacy tests of TCM directed against a single target or single disease is unreasonable [12]. The effect of TCM is not believed to originate from a single target but is considered multi target treatments that help the body restore its balance. This means that efficacy studies of singe constituents of TCM is also considered unreasonable as the medicines are originally mixtures of ingredients and the effect of a complete remedy cannot be measured by the effects of its constituents. These are the beliefs of some TCM practitioners, but there are also those who believe that the future for TCM lies in the isolation and characterization of the active components of medicines [15, 26].

The idea of multicomponent therapeutics has increased in later years [27-30], and the idea behind the synergistic effects of the holistic TCM is being reintroduced [31]. The mapping of the human genome and intricate knowledge of gene sequences and proteins originally led to the development of the one-gene-one-drug theory, with the discovery of what seemed to be disease causing genes. But with continuous research it has become apparent that this theory may be too simple and that several genes and biological pathways contribute to the complexity of many diseases such as cancer, diabetes and arthritis. Two of the most common reasons for cancellations of potential new drugs during development are toxicity levels and the side-effects profile. These are some of the presumed advantages of multicomponent drugs. The idea is that a drug consisting of several active ingredients acting at several stages in a disease network can be delivered in lower doses with increased efficacy through synergy, and a reduced amount of side-effects consistent with the theory of TCM [27, 32]. Another advantage is that such therapy might reduce the incidence of drug resistance by acting at different sites in the pathway that eventually leads to the disease [29]. This makes some people question if the future of Western medicine might be found in combining already

existing drugs as well as isolating and developing new chemical entities [32]. Some challenges present themselves whenever different drugs are combined. The pharmacokinetic properties of combination of drugs need to be properly studied to look for any changes resulting from the addition of new drugs. Potential interactions between drugs also need to be accounted for. It has been suggested that TCM might be a source of inspiration to help reduce these problems. The formulas and therapeutics in TCM have been developed throughout thousands of years, and have been tested on an incredible number of people. Traditional medicines may thus serve as a starting point for multicomponent therapies. Perhaps the thousand years old traditions and theories of TCM might help Western medicine evolve further.

Although the multicomponent therapeutic model is increasing in popularity, the isolation of compounds is still highly relevant, and the isolation of natural products has resulted in several blockbuster drugs (*see chapter 2.4).* At the present time I think that the scientific model is too dominant in the Western medical community to approve of therapies where the effect cannot be quantified and thoroughly described. The use of TCM in the West is still considered as an alternative treatment, and the way I see it, the incorporation of Western science is needed if the ancient experiences and knowledge in TCM are going to impact Western medicine. The herbal medicine of TCM would probably not have persisted for thousands of years if it did not offer some effect. From a Western point of view, I am inclined to think of this effect as a result of active components in the different herbs. There are more than eleven thousand different plant species utilized as sources for TCM [26]. This is an enormous source of plant material that has been used as medicines and is believed to cause an effect on the human body. From a Western point of view, this is a large source of potentially new compounds with biological effects. Before a method of evaluating the effect of TCM that will satisfy both parts, I think the isolation of active compounds from TCM herbs and the utilization of the multicomponent therapeutic theory are the areas where the two disciplines can help each other the most. I do not doubt that medicinal herbs and alternatives will continue to be explored by the general public in the West. But from a scientific point of view I believe that isolation and characterization of active compounds as a tool in drug discovery and evaluation offers a significant potential in discovering new lead compounds and therefore is the area where the two can influence each other the most. In time the development of multicomponent therapeutics from isolated compounds might be a way of combining the best of the two disciplines in a synergistic way.

2.4 Natural products in drug discovery

Natural products have had an enormous influence on the history of medicine. From the isolation of morphine, the first pure natural compound to be isolated from a plant and the discovery of penicillin to the large contribution to cancer therapy in recent years, where over 60% of the available therapy (or therapy currently in testing) are natural products or based on a natural product [33], these compounds have continued to be an important contribution to the discovery of new drugs. The use of natural products is not a new invention; on the contrary, it may even go further back in time than modern man. Findings in grave sites being over 60000 years old suggest that even Neanderthals might have been aware of medicinal effects of some plants [34]. For a long period of time, natural products were the only medicinal compounds available, which is understandable as some of the oldest medicinal text dates back to 2600 BC. Ancient texts have been recovered from all over the world, the oldest ones mainly focused on medicines of plant origin, some of which are still in use today [34-35]. Natural products have been used as medicines throughout the world for thousands of years. Despite the (probable) lack of contact between the continents at this time, the earliest texts found in the Occident and the Orient show similar diseases were treated with the same medicinal plants [34]. This suggests that the earliest developed medical systems were quite similar. One of the cross roads for the Western and oriental medical systems may have been the isolation of the first pure compound from a medicinal plant. Morphine was extracted from opium in the earliest years of the 1800s by Friedrich Wilhelm Sertürner, a German pharmacist. Until this time, the medical theory in the Occident had been an empirical science based solely on practical theory and observations [35]. The isolation of morphine was part of a revolution in Western drug discovery and development starting in the beginning of the 1800s, and a shift from the use of crude extracts ("polypharmacy") towards pharmacology and the use of pure compounds was initiated. The isolation of morphine was quickly followed by the purification of natural products by the Western pharmaceutical companies who developed pure compounds instead of producing crude extracts which had been the case before.

In the beginning of the 21 century, it was argued that one third to one quarter of all top selling drugs worldwide were natural products or derived thereof [36-37]. The main therapeutic areas where new and approved drugs from natural origin are most prominent are within infectious diseases, cancer and drugs treating hypertension and inflammation [38]. The works of Newman, Cragg and Snader [38-41] have provided a solid proof of the influence natural products has on the number of new chemical entities. Their latest article covers a 25 year long

period (1981-2006), and the role of natural products in drug discovery is clearly evident; A total of 1184 new chemical entities (not including combinations of older drugs or drugs with new indications) were approved in this 25 year period and over half of these (52%) were natural products, derived there of or natural product mimics [38]. In comparison only 30% of these new chemical entities were completely synthetic. Within the area of cancer therapy, the importance of natural products is even more evident. Cancer is one of the therapeutic areas where drugs derived from natural products have been (and still are) a large part of the available therapy. In fact, the largest group of the new chemical entities approved during the years of 1891 – 2006 is the group that contains the "naturally derived" drugs [38]. The same article shows that the group that contains natural products that has not been modified in any way is the third largest group. Combining these two groups with the other groups with natural product affiliation (synthetic analogues and natural mimics), the total is that 65% of all new chemical entities approved for cancer treatment in this 25 year period have some connection to natural products [38]. Counting all available cancer therapy, over 70% of the therapeutics has a connection to natural products [33]. This shows that natural products have the possibility to be a significant source of therapeutic agents, and from experience, cancer is definitely one of the therapeutic areas where natural products appear to be an indispensable source of agents.

Although natural products have been such an important source of new chemical entities, the emerging trend the last decade has been to significantly reduce the natural product research within the pharmaceutical companies [40, 42-43]. Combinatorial chemistry combined with high throughput screening seemed to be a more ideal way to search for new lead compounds as this could be done faster and thus at a lesser cost. The pharmaceutical industry is first and foremost a business, and the technological and chemical advances the last decades led to a large economical growth that continued to grow year by year. This has resulted in a great pressure enforced by the shareholders to continually produce new blockbuster drugs [42]. To meet these demands, the companies are always looking to make the research and development of new drugs more efficient; the process needs to be quick and profitable. There are several reasons why natural products were deemed less suitable as drug candidates. Natural products usually exist in a crude extract, which makes the screening process more difficult [42, 44]. In addition, if there are any hits in the screening, the isolation of the active compound may be very time consuming compared to the screening of synthetic library where the pure compounds are present in known amounts. Another issue is the availability of natural

products. Seasonal variation and environmental changes may affect both the presence and the availability of the active components of the natural products. In addition to this, the natural product resources are not everlasting, and they are sometimes protected by local and global regulations. These potential problems made the combinatorial chemistry seem very promising. But the results of this new method of choice are yet to come, as only one de novo drug developed by combinatorial chemistry has been approved for use since 1981 [38, 40]. The usefulness of the combinatorial chemistry method as a tool for drug *discovery* has therefore been questioned, as the libraries that were made showed very little diversity and the compounds were all too similar. As a consequence the recent development has been to focus more on the diversity of the libraries often using nature as an inspiration; producing molecules that are similar to natural products in terms of complexity [40].

Technological advances has continued to reduce the time needed to isolate, purify and characterize natural products, making them more able to compete with the synthetics as a source of potential lead compounds [36, 42, 45]. Prefractionation of extracts aid the initial screening, advances in fractionation techniques and spectroscopic methods all contribute to making the road from plant or fermentation broth to pure compound shorter. Creating natural product libraries and utilizing the combinatorial chemistry to optimize lead compounds, create analogues or making synthetic or semi-synthetic routes of production will be a way of getting the best of two worlds. Natural products are unique with their complex structures and are the one class of molecules said to be an exception of Lipinski's rules [46]. A drug development program including a natural product program offers an extraordinary chemical diversity that cannot be matched by synthesis. The most diverse chemical collection will always be the best starting point for discovering new lead compounds, and natural products can be an important contribution to this diversity.

2.5 Modern Research Centre for Traditional Chinese Medicine

The Modern Research Centre for Traditional Chinese Medicine (MRCT) is a research department belonging to the Second Military Medical University (SMMU) in Shanghai working towards a globalization of TCM and to construct a new research platform of TCM. The MRCT has systematically investigated over 70 species of medicinal plants, and 30 new species are under investigation each year. Through their work, over 3000 single compounds have been indentified, and about 400 of these are novel compounds. Researchers at MRCTCM are continuously publishing results of new chemical entities and are thereby contributing to the modernization of TCM. In addition to publishing new knowledge of TCM,

the MRCTCM has also built its own Natural Products Library. This library consists of 7100 standard TCM extracts and 3000 purified and structurally characterized natural compounds derived from herbal extracts used in TCM. Compounds are being added to the library each year by researches, making it possible to conduct comprehensive bio-activity screenings. The library has grown to become the largest library of natural products in China. The compounds are organized in a MDL database which allows for easy access to properties such as bio-activity and chemical structure of natural compounds.

The practical part of this master thesis was performed in coalition with a research group of the MRCTCM, who focuses on medicinal plants which are endemic but rare in China, and it is a criterion that the plants have been used in TCM. The aim of the MRCTCM is to obtain novel natural compounds from these plants and increase the existing library of natural compounds. The compounds are later sequentially studied at molecule, cell and animal levels.

2.5.1 Bretschneidera Sinensis hemsl.

Bretschneidera Sinensis hemsl. is an endemic and endangered plant in China. The genus Bretschneideraceae consists of only one species, *Bretschneidera sinensis*, which is a large and evergreen tree attaining 20 meters in height. The bark of *Bretschneidera sinensis* is said to be effective in relieving stomachache, alleviating pain and harmonizing menstruation. During the fall of 2009 the bark of the tree was under investigation at the MRCTCM at the SMMU in Shanghai. The compounds present in the bark of *Bretschneidera Sinensis* have never before been characterized as this is the first time the plant is being investigated with respect to potential therapeutic compounds. In correlation to the aims of the TCMCANCER project the second aim of this master thesis was the isolation and purification of compounds from the petroleum ether (PE) fraction of the bark of *Bretschneidera Sinensis*.

Illustration 2.1 – Bretschneidera Sinensis hemsl.

3 Materials

Table 3.1 – Solvents used in fractionation, isolation and for NMR analysis

Solvent	Supplier
Acetone	Sinopharm Chemical Reagent Co., Ltd
Trichloromethane	Sinopharm Chemical Reagent Co., Ltd
Petroleum ether	Changshu Yangyuan Chemical Co., Ltd
Ethyl acetate	Shanghai Zaituo Trade Co., Ltd
Methanol	Jiangsu Qiangsheng Chemical Co., Ltd
Sulphuric acid	Sinopharm Chemical Reagent Co., Ltd
Ethanol	Changshu Yangyuan Chemical Co., Ltd
Vanillin powder	Sinopharm Chemical Reagent Co., Ltd
For NMR:	
Solvent	Supplier
Acetone, D6, 0.5mL	Cambridge Isotope Laboratories Inc.
Trichloromethane, CDCl3, 0.6mL	J&K Chemical Ltd.
Methanol D4 0,6 ml	Cambridge Isotope Laboratories Inc.

Table 3.2 - Stationary phases used in column chromatography, TLC and pTLC

Material	Thickness (mm)	Particle size (um)	Size	Supplier
Silica gel	-	100-200	-	Huiyou Silica gel Development Co., Ltd
Silica gel	-	10-40	-	Huiyou Silica gel Development Co., Ltd
TLC plate, Silica GF254	0.15-0.2	10-40	10x2.5, 20x5	Baocengceng Xiguijiaoban
pTLC plate, Silica	0.4-0.5	10-40	20x20	Baocengceng Xiguijiaoban
Sephadex LH-20	-	40-70	-	GE Healthcare Bio-Sciences AB
Capillary tube, 0.5mm diam	-	-	-	Shanghai Sunlex Merchandising Co.,Ltd
Cabillary tube, 0.3 mm diam	-	-	-	Shanghai Sunlex Merchandising Co.,Ltd

Table 3.3 - Technical equipment

Equipment	Supplier
Rotary evaporator W201	Shanghai SENCO Technology Co. Ltd
Pump used in CC	RiSheng: RS-88
	Shengzhe: BS 310
Latex gloves	Unigloves
UV-machine	WFH 203 Shanghai Jingke Industrial Co. Ltd
NMR tubes	Norell Inc.
Automatic fraction collector	Shanghai Qingpu-Huxi Instruments Factory: BSZ-100
Syringe 200-1000ul	Dragon Laboratory Instruments Ltd
NMR Spectrometer	Bruker Avance 600
Mass spectrometer	Agilent LC/MSD Trap XCT

4 Methods

4.1 Chromatographic methods

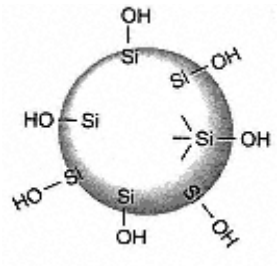
For the isolation and purification of compounds present in *Bretschneidera sinensis*, different chromatographic methods were employed. Chromatography has been used as a method to separate the different compounds in a mixture since the beginning of the 20th century [47]. By letting a mobile phase flow over a densely packed stationary phase the different compounds in a mixture will be distributed between the stationary phase and mobile phase with regards to their chemical properties. Throughout the last decade, there have been extreme advances within the field of chromatography, with the result that modern day technology is able to separate compounds from complicated mixtures [47]. In this master thesis, the chromatographic methods used were open column chromatography and preparative and analytical thin liquid chromatography. Although newer and in many cases more effective methods are available, low pressure column chromatography is still employed for the basic isolation of natural products at MRCTCM due to its easy operation and its low cost.

4.1.1 Column chromatography

Silica gel

Silica gel is a stationary phase commonly used to separate natural compounds, with the structure being tetrahedral units of silicone oxide formed into polymers. Silica gel is a porous material with a large surface area and thus offers a multitude of possible interaction sites to the solutes. Smaller particles offer a larger surface area, and thus they offer better separation. An average particle size used in open column chromatography (CC) is 40-200 µm. Smaller particles will pack more densely and result in back pressures when the column is eluted, which increases the time needed to elute the column. Particles smaller than 40 µm are normally used in high performance liquid chromatography (HPLC).

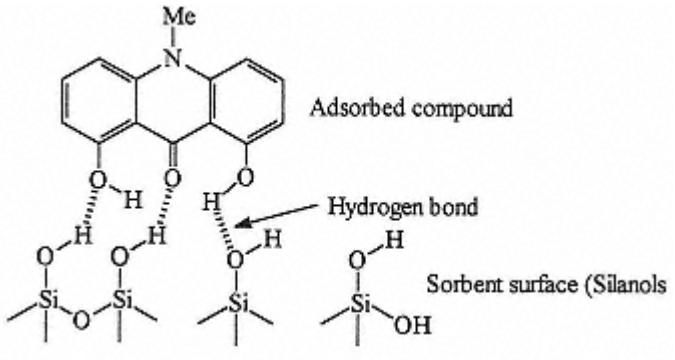
Figure 4.1 Silica gel structure



Silica gel generally retains solutes based on their hydrogen-bonding potential. Exposed silanol groups on the surface of the Silica gel structure are able to form strong hydrogen bonds with the solutes, *see fig 4.1 and 4.2 [48]*. This means that polar compounds will form stronger bonds to the Silica gel and will thus be eluted slower than non polar compounds. Strongly bond (polar) compounds require polar solvents used as the mobile phase to be eluted.

In complex mixtures a gradient system is commonly employed where the polarity of the mobile phase is gradually increased, to elute more polar compounds. The silanol groups on Silica gel can be chemically modified to produce a non-polar stationary phase. In this case the mobile phase will be more polar than the stationary phase, a situation which is called reversed phase chromatography. The different Silica gels used in this master thesis are given in table 3.2. Silica gel was used in both analytical thin liquid chromatography (TLC), preparative thin liquid chromatography (pTLC) and column chromatography (CC).

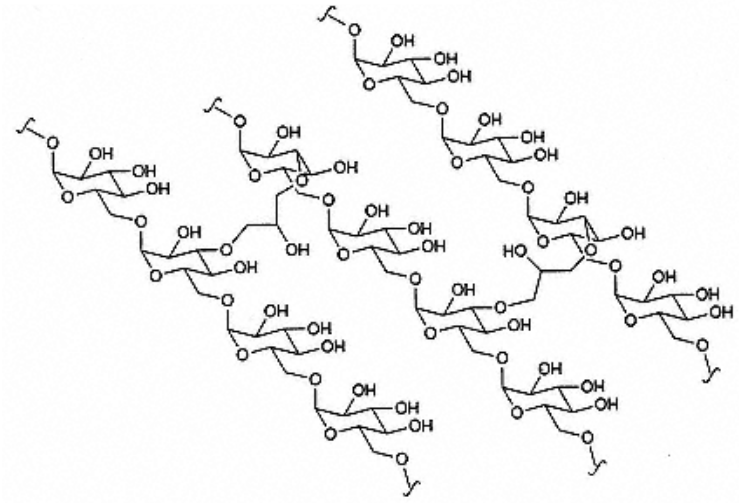
Figure 4.2 – Silica gel bonding, normal phase



Sephadex

Sephadex is a stationary phase that results from dextran polymers being cross-linked by epichlorohydrin, which results in a three dimensional network, *see fig. 4.3 [48]*. Although dextran is a water soluble molecule, the cross linking of the polymer makes the gel insoluble in water. The gel swells in polar liquids due to the hydrophilic nature of dextran, a property which can be used to chromatograph natural compounds based on their size. The swelling of the gel determines the range of molecular weights in which the gel is useful, and a denser gel will be able to separate compounds of lower molecular weights. In addition to the size-exclusion mechanism, adsorption, partition and possibly ion-exchange mechanisms also play a part in separation, which means that larger particles may sometimes be eluted earlier than expected [48]. Sephadex gel can be used with most solvents (with the exception of strong acids that would hydrolyze the glycosidic linkages) and the materials are rather inert, which means that irreversible adsorption rarely occurs, and the entire sample is recovered from the gel. Another advantage is that the gel can be used several times without regeneration.

Figure 4.3 – Dextran crosslinking



Sephadex LH-20 was used in this master thesis, and is a hydroxypropylated Sephadex G-25, which is able to separate compounds with molecular weights in the range of 100-5000 Da. The hydroxypropylation in the LH series adds lipophilicity to the gel, and the gel swells to about 4 times its dry volume when in contact with polar organic solvents, which makes this gel a good choice for the separation of natural products soluble in organic solvents. Sephadex gel can separate molecules by two different mechanisms. When a single solvent is used, the gel swells and the compounds are separated mainly by size exclusion, although adsorption may also play a part. The other method is to use a mixture of solvents. This results in the most polar of the solvents being picked up by the gel, and leads to the formation of a two-phase system where the stationary and the mobile phase are of different composition. The mode of separation now becomes that of partition. In this case the best results are obtained when a mixture of solvents with different polarity is used. Both separation methods were utilized in this master thesis.

4.1.2 Planar chromatography

Separation of compounds on thin layers of stationary phase coated on a glass, plastic or aluminium plates is called planar chromatography. The most common form of planar chromatography in the field of natural products isolation is thin liquid chromatography (TLC), as it is an easy and cheap method for isolating and purifying compounds. Analytical TLC is generally used to monitor and detect compounds throughout the isolation process, while preparative TLC (pTLC) is used to isolate compounds and as a final purifying step. Newer preparative methods are available, but pTLC is still very much in use as it is a simple, not very time consuming process that can isolate compounds at a very low cost. Compounds in the range of 1 mg to 1 g can be isolated by pTLC.

A spot of the sample is plotted onto the TLC plate, which is then placed in a tank containing sufficient solvent. The solvent is "pulled" up the plate through capillary action, and the compounds are separated based on the chemical properties of both the compounds and the stationary phase. When Silica gel is used, the mechanism of separation is adsorption, as mentioned in earlier paragraphs. In normal phase chromatography, the most polar will be retained more than the less polar compounds. The *Rf*-value quantifies the migration, and makes it possible to compare different compounds developed in the same solvent.

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

From the formula it is apparent that the *Rf*-value is a ratio, and will always be in the range of 0 (no migration) – 1 (migration together with solvent). The R_f value for any single compound is constant when the same solvent system in the same ratio is used.

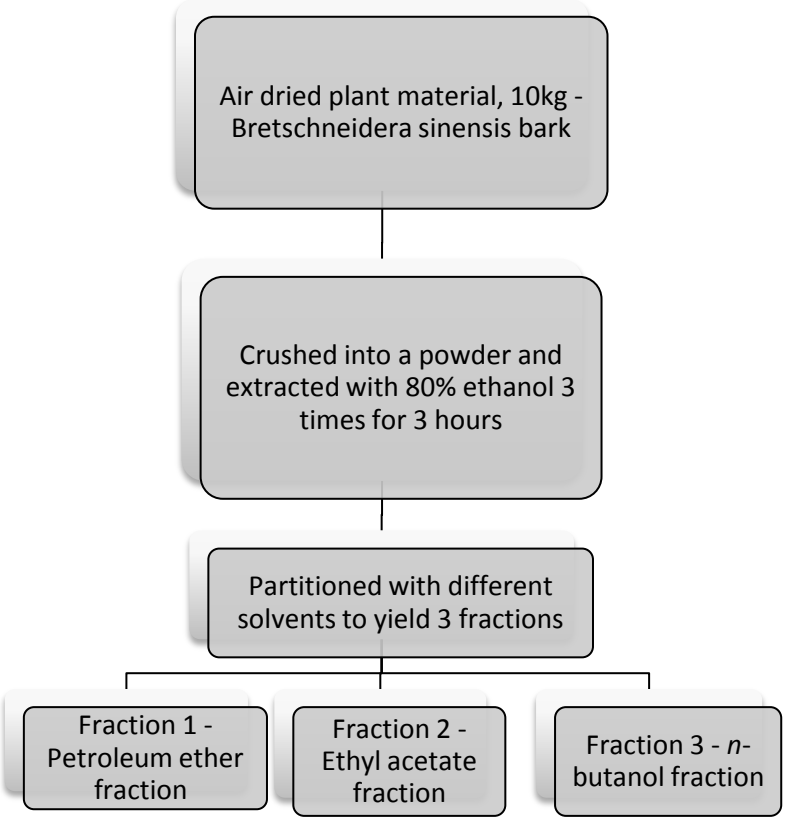
For the detection of compounds on TLC and pTLC plates, both destructive and non-destructive detection agents are available. Investigation under UV-light is a non-destructive way of detecting compounds, and is possible as UV-active compounds are incorporated into the stationary phase by the manufacturer. The presence of compounds will therefore appear as a dark spot on a light surface under UV-light at 254 nm or 365 nm. Many compounds will emit a distinctive fluorescent light under 365 nm. An example would be chlorophyll, which can be recognized by its red fluorescent light. Spray detection is a destructive method for compound detection, as the detection is based on a chemical reaction between the compound and reagent. Compounds that are not visible in UV-light will require spray detection to be visualized. In the case of preparative TLC it is important that any spray detection reagent used is limited to a small portion of the plate, as the reaction will destroy the compound(s) and prevent them from being investigated further. TLC and pTLC plates used in this master thesis were always investigated in UV light followed by spray detection.

4.2 General procedures

4.2.1 Preparation of plant material

As the extraction and isolation of natural products are a time consuming process, it was not possible to start the extraction from the plant itself given the time limit of four months. It was therefore decided that with the time available, it would be best to start the extraction from a fraction of the plant. The fraction that was investigated was prepared from *Bretschneidera sinensis*, a plant already under investigation at the MRCTCM at the SMMU in Shanghai. *Figure 4.4* gives an overview over the work that had already been done to prepare the petroleum ether fraction, which was the fraction studied in this thesis.

Figure 4.4 – Preparation of the petroleum ether fraction



The aim of the laboratory work was to contribute to the already ongoing work of characterizing the chemical constituents of the *Bretschneidera Sinensis* bark and during this work collect compounds for further testing in correlation to the aims of the TCMCANCER project. There were no known compounds that were targeted from the start in the isolation process; the aim was rather to achieve a thorough overview of the compounds present in the plant, and by doing so contribute to the compound library of the MRCTCM and potentially discover new compounds that may be of therapeutic value. The work was therefore conducted in a manner that step by step divided the petroleum ether (PE) fraction into smaller and smaller fractions that contained fewer compounds. Target compounds were selected as the amount of compounds in the fractions were reduced, and were based on the apparent amount of compound and the difficulty in isolating them. The techniques used throughout the isolation process were open column chromatography (CC) and thin liquid chromatography (TLC), the latter in both analytical and preparative scale.

4.2.2 Preparation of Silica gel columns

In the open column chromatographic separations the main stationary phase employed was Silica gel. The particle size of the Silica gel used in the columns throughout the thesis was 10-

40µm unless otherwise stated. All the Silica columns were packed by dry packing, and the sample was without exception loaded by dry loading. In the cases where the weight of Silica packed in the column was based on the weight of sample, this is stated specifically in the relevant sections. Regardless, the procedure employed for packing the columns were the same: The desired amount of Silica was put in a column of appropriate size. The exit valve of the column was let open, and the column was tapped repeatedly to allow the air to escape the column. The packing was tested by turning the column to a horizontal level, and giving it a shake. The column was deemed sufficiently packed when the Silica gel stayed in place during this test.

The preparation of the sample for dry loading did also follow the same procedure in all Silica column separations. The sample was concentrated by removing most of the solvent under reduced pressure prior to being transferred from its container to a porcelain bowl of appropriate size. The desired amount of Silica gel, with particle size corresponding to the size of the particles already packed in the column, was added to the porcelain bowl and the content mixed thoroughly by the use of a spatula. The content was left to dry until the mixture started to thicken and eventually turned into a dry lumpy mass. Throughout the drying process the mixture was stirred to ensure a uniform thickening. The lumpy mass was crushed into a fine powder using the spatula. When the powder was completely dry and free from solvent, the powder was added to the column and the column was tapped a few timed to pack the sample.

4.2.3 Preparation of Sephadex LH-20 columns

The Sephadex used in the columns was Sephadex LH-20. Sephadex is a stationary phase that can be reused, and the columns used in this thesis were columns that had been used before, and they were subsequently pre-packed. There were several columns with Sephadex LH-20 stationary phases available; each one to be used with only one mobile phase to ensure the best possible results each time the column was used. Columns for partition chromatography and size exclusion were both available. In this thesis, Sephadex-LH20 was used as the stationary phase on two occasions, and both columns had been used before. On one occasion the column was eluted with a solvent system consisting of three solvents, petroleum ether, trichloromethane and methanol (5:5:1 respectively) and the other occasion size-exclusion chromatography was employed by the use of a single solvent; acetone (Ac).

The sample was loaded by wet loading when using Sephadex LH-20 columns. Any solvents present in the fraction to be separated were evaporated by the use of a rotary evaporator,

before the compounds were dissolved in the mobile phase. The sample was thereafter carefully loaded onto the column by a pipette. The sample container was washed three times with the mobile phase and was added to the column in the same manner as the sample. The exit valve was then opened to allow the sample to be adsorbed onto the stationary phase, and afterwards the mobile phase was added to the column, and fractions were collected by an automatic fraction collector.

4.2.4 Compound detection and isolation by TLC and pTLC

During the collection of fractions from CC, the fractions were continuously tested by TLC. TLC was used in analytical for compound detection and preparative scale for isolation of compounds. The only plates used in this thesis were glass plates pre-coated with Silica gel. The specifications of the plates are given in the Materials section in *table 3.2*.

4.2.4.1 Procedure for development of TLC plates

A TLC plate of appropriate size was chosen (see Materials section for specifications) or alternatively a TLC plate was cut into smaller plates to obtain a plate of appropriate size. A line was drawn 0.7mm-10mm above the end of the plate, and depending on the amount of samples under investigation, a number of marks were made onto this line, and numbered according to the number of the samples. The samples were then plotted onto their numbered spot by the use of a capillary tube with a diameter of 0.3mm and allowed to dry. The mobile phase was freshly made for each test, and the solvents chosen for the mobile phase were measured by the use of graded pipettes, and put directly into the TLC tank. After the solvents had been added, the tank was shaken vigorously to mix the solvents. The TLC plate was put into the tank, and was not removed until the solvent font reached the top of the plate. The plate was removed from the tank and was either left to dry naturally in a fume cabinet, or was dried by the use of a hand held dryer (hair dryer).

4.2.4.2 Procedure for development of pTLC plates

Two lines were drawn 3 cm above the end of the pTLC plate, with an approximately 2mm wide gap between them. A capillary tube with the diameter of 0.5mm was used to plot a line of dots in between the two lines. This was repeated until the plotted line was completely adsorbed on the Silica gel which was controlled by turning the plate over, and visibly confirming that a consistent line could also be seen on the back of the plate. To obtain a consistent line it was almost always necessary to repeat the plotting of the line about three times. The number of pTLC plates used was dependent on the amount of sample available for pTLC analysis. Once the plate showed a clear visible line on both sides of the plate, a new

plate was prepared to be plotted. One pTLC tank had the capacity of developing two pTLC plates at once. If there was only one tank to be used in the development of the plates, the solvents of the mobile phase were measured by the use of a measuring cylinder of an appropriate size, and put directly into the tank and then mixed as described in the former paragraph. If more than one tank would be used, the mobile phase was made by measuring the solvents by a measuring cylinder and transferring them to an empty solvent bottle. The solvents were mixed within this bottle, and thereafter equally distributed in the desired amount of pTLC tanks. The pTLC plates were put in the tanks, and the mobile phase was allowed to travel to the top of the plate before they were removed from the tank. The pTLC plates were allowed to dry naturally in a fume cabinet before further investigations.

4.2.4.3 UV-detection and colour developing agents

Both the TLC and pTLC plates were investigated under UV-light with wavelength of mainly 254nm (although 365nm was sometimes applied). In both cases, the compounds absorbing UV-light was marked by the use of a pencil. The analytical plates were always investigated in a colour developing agent as well as UV-light. The two colour developing agents used during this thesis were a 10% sulphuric acid in ethanol solution and a solution made from 1 g vanillin dissolved in a mixture of 25 ml sulphuric acid and 150 ml ethanol, the two are hereafter named sulphuric acid (H_2SO_4) colour developing agent and vanillin colour developing agent respectively for convenience. For the analytical TLC plates, the whole plate was covered in the chosen colour developing agent by the use of a pipette. Thereafter, the plate was placed on a heating plate, and heated until colouration appeared. In the case of pTLC, an approximately 1.5 cm wide piece of the plate was cut off, and this piece was tested in the same manner as the analytical plate.

4.2.4.4 Compound recovery from pTLC plates

After detecting compounds by investigation under UV-light and by the use of a colour developing agent, the desired compounds were recovered from the plate. The area (uncontaminated by colour developing agent) containing the desired compound was removed from the plate by the use of a spatula and put on a piece of paper. If the sample had been plotted on more than one plate, the compound from the different plates were scraped off and put on the same paper. After the compound had been completely removed from the plate, the Silica gel pieces on the paper were crushed into a fine powder by using the bottom of a round bottom flask. The powder was then transferred to small columns (1 cm diameter, 20 cm in length), which had been clogged at the end by a small amount of cotton which acted as a

filter. The column was tapped repeatedly to allow the Silica gel to be evenly packed. The compounds were recovered by washing the Silica gel three times with acetone by filling the column and collecting the eluted solvent.

4.2.5 Selection of mobile phases

The selection of mobile phases used in Silica gel columns or for pTLC was without exception based on TLC results. A mobile phase used to separate or purify a fraction in either a Silica column or on a pTLC plate was selected by testing the fraction in question in various solvent systems, and investigating the degree of compound separation, as well as the *Rf*-values of the compounds. Throughout the thesis the following solvents were combined in various combinations to obtain a mobile phase of advantageous properties: Methanol (MeOH), acetone (Ac), ethyl acetate (EtOAc), trichloromethane ($CHCl_3$) and petroleum ether (PE). When a fraction was to be separated by column chromatography, the favourable mobile phase would put the *Rf*-values of the desired compounds in the area of 0.2. In the case of separation or isolation by pTLC, the mobile phase was deemed suitable if the TLC results showed the compounds to be separated well as well as the compounds having *Rf*-values in the area of 0.4-0.7.

4.3 Generation of subfractions from PE fraction

The PE fraction was separated into crude fractions by column chromatography. 284.5 g of Silica gel with particle size 100-200 µm was dry packed in a column, and 33 g of the same Silica gel was used to prepare the sample for dry loading. The column was eluted with a gradient system of PE and EtOAc. The step gradient consisted of six steps; 30:1, 15:1, 8:1, 4:1, 2:1 and 1:1 PE to EtOAc respectively. 150-200 ml fractions were collected from the column and the solvent was evaporated on a rotary evaporator at 59˚C with 80rpm. The resulting residue from each fraction was transferred to a small sample glass, and numbered according to the order they were collected in. The residues from the first five fractions were combined in a 100 ml Erlenmeyer flask. The compounds collected in these fractions were multiple and of very low polarity, and would be very difficult to separate by standard methods with the time available. This fraction was therefore removed from the experimental part of this master thesis and not studied any further. The fraction collected after these fractions was named fraction number one.

Every other fraction collected from the column was tested on TLC to determine when the polarity of the mobile phase needed to be changed, and to combine collected fractions into subfractions that appeared to contain the same compounds. *Table 5.1* gives an overview of the subfractions created by combining fractions, and this table also provides an overview over the subfractions investigated further in this thesis. The subfractions obtained from the Silica column are hereby referred to as S_x, where the S refers to the primary Silica column, and the x represent the number of the fraction in question. E.g. the subfraction created by combining fractions 32 and 33 obtained from the Silica column is referred to as $S_{32\text{-}33.}$ Subfractions for further separations were chosen dependent on the apparent number of compounds present, and by an equally important factor; the apparent amount of compounds present in the subfraction.

Any crystals precipitating in the fractions were carefully washed with petroleum ether and the excess solvent was removed and transferred to another sample glass. The crystals were tested by TLC.

4.4 Isolation and purification of compounds from a selection of subfractions

4.4.1 Subfraction 32-33

The subfraction $S_{32\text{-}33}$ was separated into smaller fractions by the use of a Silica gel column, eluted with an 8:1 ratio of PE and Ac, respectively. A pressure pump was used to increase the eluting speed, and fractions of approximately 8 ml volume were collected. Subfraction 4-7 from this column was attempted separated by pTLC in a mobile phase consisting of a 3.5:1 ratio of PE to EtOAc, before the compounds were recovered and combined with S_{31}. Compounds in subfraction 8-11 from this column were purified by the use of pTLC developed in a 10:1 $CHCl_3$ and EtOAc mobile phase.

4.4.2 Subfraction 61-82

Subfraction $S_{61\text{-}82}$ was separated further by CC with Silica gel as the stationary phase, and the mobile phase being a two step gradient system consisting of PE and EtOAc. 2 g of Silica gel was used to prepare the sample for loading. The first step in the gradient system was a ratio of 5:1 of the two solvents, and the second step a 3:1 ratio. Fractions of approximately 8 ml were collected in test tubes. Three subfractions (B, C and D) obtained from this column were separated further by column chromatography using Sephadex LH-20 as the stationary phase,

and a mobile phase consisting of PE, $CHCl_3$ and MeOH in a 5:5:1 ratio respectively. Subfraction 15-18 from the Sephadex LH-20 column separation was purified by pTLC in a 5:1 ratio of $CHCl_3$ to EtOAc.

Subfraction 39-57 obtained from the same Sephadex LH-20 separation was attempted separated by size-exclusion chromatography with Sephadex LH-20 as the stationary phase and Ac as a single solvent mobile phase. Compounds in fraction 16-17 obtained from the size-exclusion separation were purified by two subsequent pTLC separations. The first one in a mobile phase consisting of a 2:1 ratio of petroleum ether to ethyl acetate, and the second one purified a group of compounds recovered from the first pTLC in a 7:1 ratio of $CHCl_3$ and Ac.

4.4.3 Subfraction 56-61

The $S_{56\text{-}61}$ fraction was separated by the use of column chromatography, with Silica gel as the stationary phase and PE and EtOAc in a 4:1 ratio as the mobile phase. Fraction 6-20 from this column was separated further by the use of pTLC, with $CHCl_3$and EtOAc, 5:1 as the mobile phase. 6 pTLC plates were used for the separation. Two fractions were recovered from the plates for further separation, and they were purified separately by the use of pTLC, both fractions developed in a 2:1 ratio of PE and EtOAc as the mobile phase.

4.5 Spectroscopic methods

For the structural elucidation of the isolated compounds, mass spectrometry (MS) and nuclear magnetic resonance (NMR) were used. Mass spectrometry was used to find the molecular mass of the unknown compounds, and in combination with the NMR results to calculate a base formula and the molecular formula. 1D nuclear magnetic resonance was used as a "start off point" to determine the structure of the unknowns.

4.5.1 Mass spectrometry

Mass spectrometry (MS) has progressed to become a very important tool in the discovery of natural products, and the amount of information that can now be obtained from MS analysis is substantial [35, 49]. But at the same time it still is, as it traditionally was, a very useful method for finding structural information (molecular weight) of unknown compounds. Early mass spectrometry was limited to analyzing only small volatile substances, but modern instruments have the capacity to investigate a broad range of molecules: from small organic molecules to large biological macromolecules [49]. This is mainly due to the development of soft ionization techniques such as the electron spray ionization (ESI) which was the ionization

method employed in this thesis. ESI is called a soft ionization technique because the ionization of the sample is caused by the addition or removal of a proton, not leaving much energy for the fragmentation of the ions. ESI has become one of two preferable methods used to ionizing samples in MS [50].

Two possible ionization methods are available, namely positive and negative ionization. The appropriate method is chosen on the basis of the compound: A compound containing functional groups that easily accept protons, e.g. amines, are ionized by positive ionization. Compounds containing functional groups that easily donate protons are ionized by negative ionization. In the spectroscopic analysis in this master thesis the compounds were unknown and both methods were employed. The charged ions are usually singly charged molecular ions which means that they are protonated $(M+H)^+$ or deprotonated $(M-H)^-$ molecular ions. But there is another observable way of ionizing the molecules, namely by adduct ionization. A Na^+ ion added to the molecule instead of a proton results in an adduct molecule and will show up in the spectra as a peak corresponding to M+23. Other common adduct ions appearing in the positive ionization are K^+ and NH_4^+ (+ 39 and +18 respectively) [51]. In negative ionization, Cl^- is a common adduct ion appearing at M+35.

The rule of 13 and the unsaturation index

When the molecular mass has been found, the rule of 13 can be used to calculate a base formula, which is the number of carbon and hydrogen atoms corresponding with the molecular weight. The rule of 13 is calculated as shown below.

$$\frac{\boldsymbol{M}}{\boldsymbol{13}} = \boldsymbol{n} + \frac{\boldsymbol{r}}{\boldsymbol{13}}$$

The base formula: C_nH_{n+r}. By combining this with the information obtained from the NMR spectra, any other atoms presumably present in the compound (e.g. oxygen or nitrogen) can be added to the base formula by removing the corresponding number of carbon and hydrogen atoms. The number of carbon peaks in the NMR spectra must also be taken into account in evaluating the probability of the calculated molecular formula.

The index of hydrogen deficiency/unsaturation index is symbolized by the letter U and is, as the name implies, the number of unsaturated carbons present in the compound, i.e. ring structures and double bonds. The unsaturation index is found by the formula below:

$$U = \frac{(n - r + 2)}{2}$$

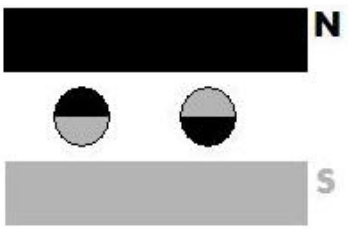
The unsaturation index combined with the NMR spectra is a good indicator to if the calculated formula is the correct one: If the NMR spectra reveal an aromatic structure, the unsaturation index needs to be at least 4. On the other hand, if the unsaturation index seems unreasonably high, it might be an indication that one or more carbon atoms should be removed and replaced by the corresponding amount of hydrogen atoms. Used together with MS and NMR, the rule of thirteen and the unsaturation index are therefore very helpful tools in determining the molecular formula and getting more information about the structure in the form of double bonds and ring structures.

4.5.2 NMR Spectroscopy

Nuclear magnetic resonance spectroscopy is routinely used to study the structure of simple chemical compounds in one dimensional experiments [52]. For more complex compounds, two dimensional techniques are more appropriate and has also become commonplace in the laboratory. NMR is a relatively insensitive method of analysis where even the most advanced instruments require samples in the µmol range (at least) [53]. Still, NMR is widely used, as the amount of information possible to obtain from the spectra outweighs/compensates for the insensitivity issue. The NMR experiments give information about the different magnetic environments present in the molecules, and serve as an important source of information for structural elucidation. In this master thesis only 1D NMR spectroscopy was used.

When the nuclei of certain atoms present in a magnetic field are influenced by another oscillating magnetic field, the resulting phenomenon is called nuclear magnetic resonance (NMR). The NMR phenomenon is dependent upon a property called spin, which can be found in several isotopes. Almost every element has an isotope with a spin $\neq 0$, and the spin property is a requirement for the element to produce a signal. Another requirement is that the isotope is present in a large enough amount to be detected by the apparatus, which limits the types of atoms that can be studied, and also explains the sensitivities of different atoms [52]. Under the influence of a magnetic field, a proton will align itself in one of two possible orientations corresponding to a high energy level or a low energy level *fig. 4.5 [52]*. The NMR signal is detected as the nuclei transitions from one energy level to the other, high to low or low to high. For this transition to be detected there needs to be an excess population in one of the energy levels. If the number of nuclei in the two energy levels were equal, there would be no net signal as the transitions occurred as they would cancel each other out. The

Fig 4.5 Energy levels in NMR



excess population is therefore what makes it possible to observe signals in a NMR experiment, and this excess is found in the lower energy level. By increasing the operating frequency of the NMR instrument, the number of nuclei in the lower level is increased as the difference between the two energy levels increases.

Pulsed Fourier Transform (FT) instruments are preferred as it is faster and more sensitive than the Continuous Wave instrument which is the option. In the FT instrument all the different protons are excited at the same time by a very short pulse/burst of energy that contains a large range of frequencies. The range of frequencies is big enough to excite all the protons which will emit electromagnetic radiation when they after a period of time return to their original energy state. The different nuclei will emit different electromagnetic radiation, which is called the free induction decay (FID).The FID decrease with time as the nuclei's energy gradually decrease. The different electromagnetic radiation can be extracted from the FID by the use of a computer, and the Fourier transform analysis, and converted into a frequency domain signal which can then be interpreted. Information of the NMR machine used to characterize the compounds is given in *Table 3.3* in the Methods section.

4.6 Preparation of compounds for NMR and MS analysis

The pure compounds were dissolved in either chloroform-d, D_6 acetone or MeOD depending on their chemical properties. N_1 was dissolved in MeOD, N_2 and N_7 in $CDCl_3$ and N_3 in D6 acetone. N_4 was dissolved in a mixture of a mixture of D_6 Ac and $CDCl_3$, N_6 and N_8 were dissolved in $CDCl_3$. Before the preparation of samples for NMR and MS analysis, the small glasses containing the compounds were weighed. The NMR solvent was then added to the compound, and washed over the compound by the use of a Finn pipette. A small amount (approximately 0.5 ml) of the dissolved compound was transferred to a NMR tube, which was labelled with its containing compound's name and solvent used to dissolve it. Another small amount of the already dissolved compound was transferred to a small centrifugation tube labelled with the compound's name for the MS analysis. The compounds were allowed to dry completely, and then weighed again to determine the amount of compound removed for the analysis. The finished samples were delivered to the analytical department who carried out the actual analysis.

5 Results and discussion

5.1 Generation of subfractions through column chromatography

106 test tubes containing approximately 8 ml each were collected from the column chromatography of the PE fraction. These were combined to yield 20 subfractions, listed in *table 5.1*. The combinations of fractions were based on TLC results: Examination of colour after testing the TLC plates in a sulphuric acid colour developing agent and the *Rf*-values were considered. This was a crude separation of compounds, and the fractions were combined first and foremost to enrich the amount of compounds that already appeared to be of substantial mass. *Table 5.1* also gives an overview of the results obtained from the TLC test performed which was used as the basis of combining the fractions into these subfractions.

Table 5. 1 - TLC results from initial column chromatography

Subfraction	Basis for combining the fractions, colour	Basis for combining, Rf-values
S1-4	Orange/brown and pink	0.62 and 0.4
S5	No defined spot	-
S6-8	Brown	0.44
S9	Difficult to differentiate a spot	-
S10-13	Purple and brown spots	0.4 and 0.47
S14-16[1]	Difficult to differentiate a spot	-
S17-20[1]	Pink	0.22
S21-23	Pink and purple	0.2 and 0.24
S25-26[1]	Yellow/brown spots	0.5 and 0.7
S27-30	brown and blue spots	0.5 and 0.6
S31	No defined spot	-
S32-33[2]	Dark puple	0.5
S34-39	Green and yellow spots	0.6 and 0.4
S40-45	Brown	0.3
S46-48	Green	0.9
S49-55	Green	0.8
S56-61[2]	Light green and dark blue	0.6 and 0.4
S62-71[2]	Dark blue and purple	0.4 and 0.35
S72-81[2]	Brown, dark blue and purple	0.5, 0.4 and 0.35
S82-89	Dark	0.25

[1] Pure compound collected from one or more of the original fractions before combination into subfraction
[2] Subfractions chosen for further separation

In fractions 16, 17, 18 and 26 white crystals precipitated. The crystals were tested on TLC in 8:1 petroleum ether and ethyl acetate, to determine if the crystals were the same compound

and the results of this test is given in *table 5.2*. The crystals in fractions 16, 17 and 18 were determined to be the same compounds based on their *Rf*-values which were very similar. These crystals were therefore combined in a sample glass and named N3 awaiting NMR and MS analysis. The crystal in fraction 26 had a significantly different *Rf*-value, and it was therefore concluded that this was another pure compound. This compound was labelled N2 and set aside with N3 for future NMR and MS analysis.

Table 5.2 - TLC results obtained when testing precipitated crystals

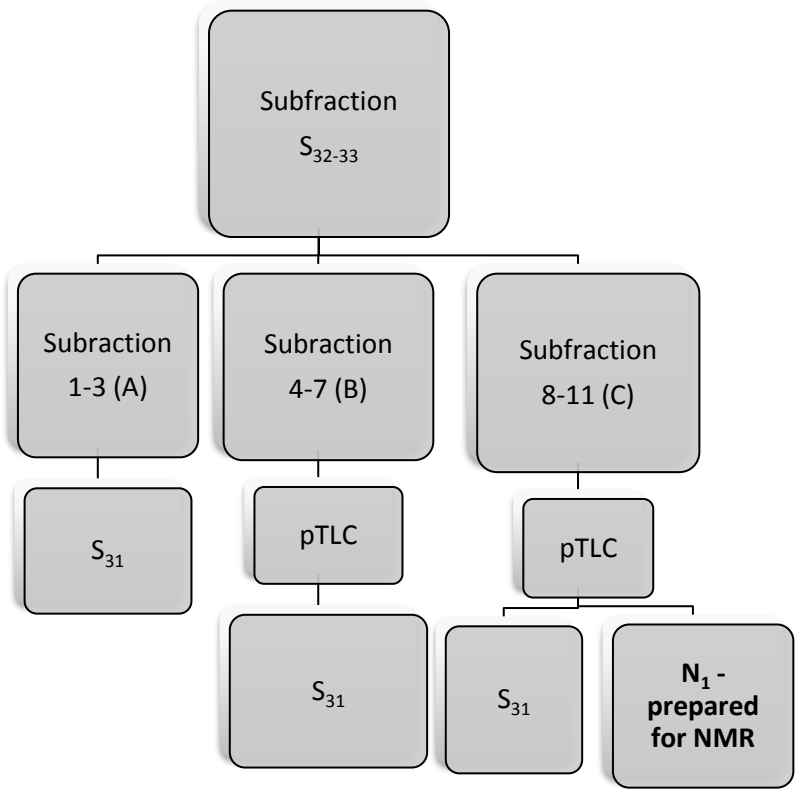
Fraction number	Rf-value
16	0.32
17	0.33
18	0.34
26	0.24

5.2 Isolation and purification of compounds

5.2.1 Subfraction S_{32-33}

Figure 5.1 gives a brief overview of the work done to isolate and purify compounds in subfraction S_{32-33}, which eventually led to the discovery of N_1.

Figure 5.1 – Separation of compounds in S_{32-33}



18 fractions were collected from the Silica column, and by testing these fractions on TLC in the mobile phase 4:1, petroleum ether and acetone, 3 subfractions were created; A, B and C. The combination of fractions into these subfractions was based on colour (after the addition of H_2SO_4 colour developing agent) and the apparent *Rf*-values. Subfraction A did not appear to contain any compounds present in an amount that would be sufficient for isolation and this subfraction was added to S_{31} to potentially enrich these compounds.

A purple spot in fraction B was chosen as the target compound and attempted purified by pTLC in a 3.5:1 PE:EtOAc mobile phase but this was unsuccessful, as the UV-investigation and colour development in H_2SO_4 revealed what appeared to be several compounds that were not separated at all. A TLC test in a new mobile phase: $CHCl_3$ and EtOAc (10:1) revealed that the target compound was actually a spot composed of several compounds present in small amounts. The subfraction was therefore combined with S_{31} to enrich these compounds.

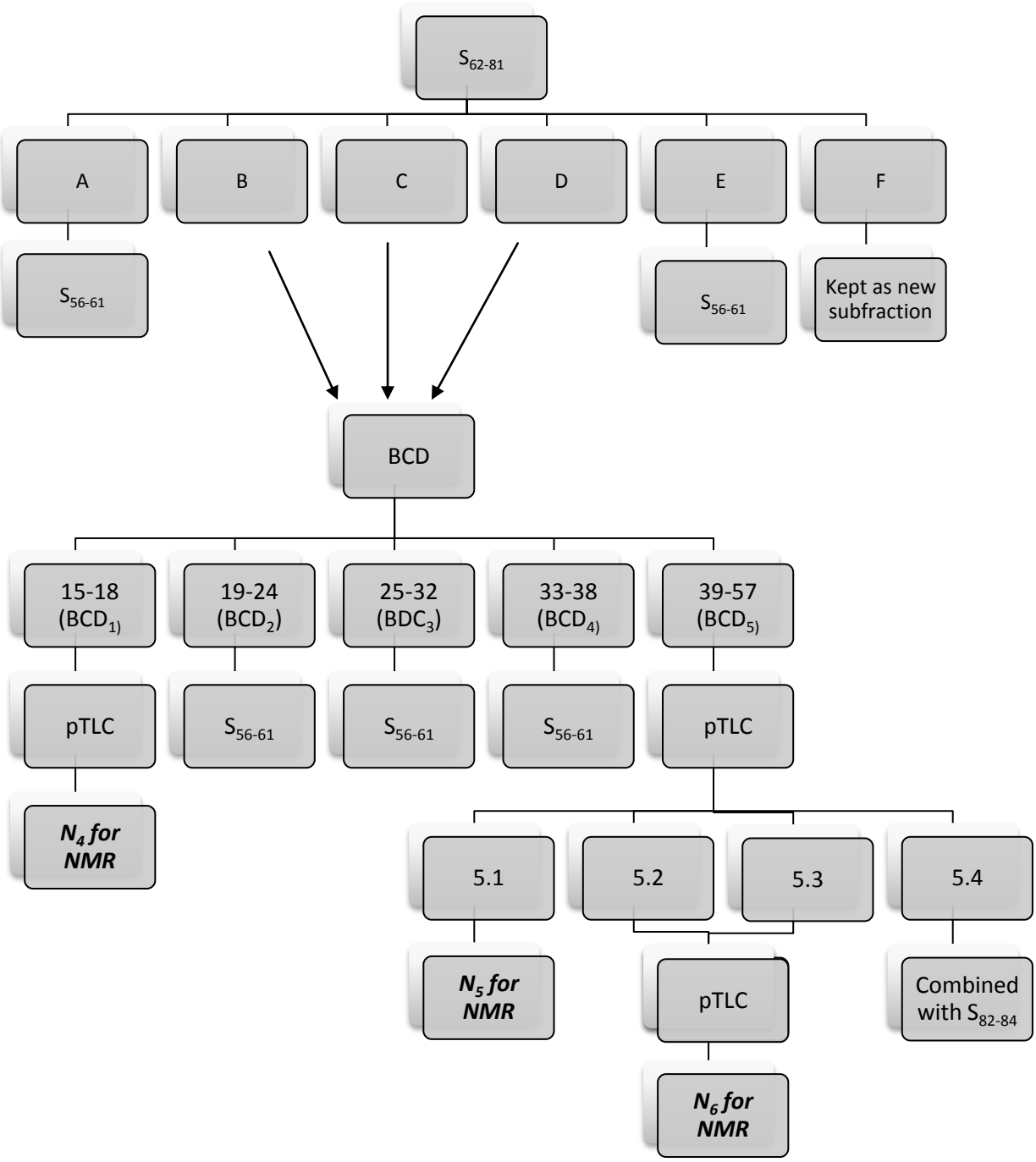
N_1 was obtained from purification of subfraction C. The subfraction was tested on TLC in 10:1 $CHCl_3$ and EtOAc and a clear pink spot was chosen as the target compound. The target compound was purified by pTLC, which indicated the compound being present in substantial mass and clearly separated from the other compounds. The compound was named N1 and set aside for preparation for NM and NMR analysis, the results of which are given in *section 5.3.2*. The remaining compounds were combined with S_{31}.

Originally it was believed that fraction B contained the main compound of subfraction $S_{32\text{-}33}$. The lack of proper evaluation of available mobile phases resulted in time being spent on a useless pTLC test which could have been avoided if the preparations had been done more thoroughly. Even small subfractions contain a vast number of natural products that are not necessarily easily visibly separated by TLC. A thorough preparation and evaluation of mobile phases will often prevent unnecessary work and thereby result in a more efficient use of time, as seen in this case.

5.2.2 Subfractions $S_{62\text{-}71}$ and $S_{72\text{-}81}$

Subfractions $S_{62\text{-}71}$ and $S_{72\text{-}81}$ combined to form subfraction $S_{62\text{-}81}$. *Figure 5.2* gives a summation of the work done to isolate compounds from subfraction $S_{62\text{-}81}$, which resulted in the isolation and purification of N_4, N_5, and N_6.

Figure 5.2 – Separation of compounds in S_{62-81}



S_{82-81} was put in a Silica gel column and eluted with a two step gradient system, PE:EtOAc (5:1 followed by 3:1). But the separation was not particularly good. As shown in *figure 5.2* the Silica gel column chromatography resulted in 5 fractions, namely A-F. The fractions B, C and D contained a compound that was coloured orange in sulphuric acid colour developing agent with the same *Rf*-value, and this compound was chosen as the target compound. The

TLC results also showed the presence of other compounds in addition to the target compound that had to be separated. The separation of the compounds in the BCD fraction by column chromatography using Sephadex gel, PE:$CHCl_3$:MeOH (5:5:1) resulted in 5 subfractions as noted in *figure 5.2*, based on *Rf*-value and colour when developed on TLC and tested in sulphuric acid and vanillin colour developing agent. These results are given in *table 5.3*.

Table 5.3 – Fractions obtained from Sephadex column

Fraction	Appearance of defined spots after H2SO4 addition	Rf-value	Appearance of defined spots, vanillin	Rf-values
BCD1	Orange spot	0.5	One clear spot	0.5
BCD2	No defined spot	-	No clear spot	-
BCD3	Brown	0.4	Three clear spots	0.45, 0.4 and 0.2
BCD4	No defined spot	-		-
BCD5	2 brown spots, highly stained	0.2 and 0.3	One long spot	0.25

Based on the results in *table 5.3*, fraction BCD1 was determined to contain the target compound. This fraction was purified by pTLC. When investigated under UV-light, the plate only showed 2 compounds with very weak UV-absorption at 254 nm. From previous TLC tests, it was clear that the target compound did absorb UV-light but it was visualized by staining with both vanillin and H_2SO_4 and a wide band with Rf-value 0.5 clearly separated from the other compounds. The compound was recovered, tested on TLC with four different solvent systems (PE:EtOAc 3:1, PE:Ac 4:1, $CHCl_3$:Ac 6:1, $ChCl_3$:EtOAc 5:1) all of which gave results indicating a pure compound as they each resulted in a single orange spot. The compound was labelled N_4 and set aside for structural determination.

Based on the results form *table 5.3* the fraction BCD5 was also chosen for further investigation as the spots showed significant colour which suggested compounds of substantial mass. Size exclusion separation was attempted by a Sephadex column with acetone as the mobile phase, with poor result. The size-exclusion separation did not separate the compounds further, and all fractions obtained from the Sephadex column were combined again and attempted separated by pTLC in PE:EtOAc (2:1).

The pTLC results from the separation of BCD_5 showed the following: Under UV-light, three bands were visible. One glowed neon blue under wavelength 365 and was easily separated. This compound was recovered and labelled N_5. The second band was very wide and appeared to be a combination of more than one compound. The third compound was not of interest at the present time as it did not seem to be either pure or present in significant amounts. The

wide band was recovered and attempted purified by pTLC in a new mobile phase (7:1 ratio of $CHCl_3$:Ac). This resulted in two separate bands, one of which appeared to be a pure compound with *Rf*-value 0.2 that was recovered from the plate and labelled N_6.

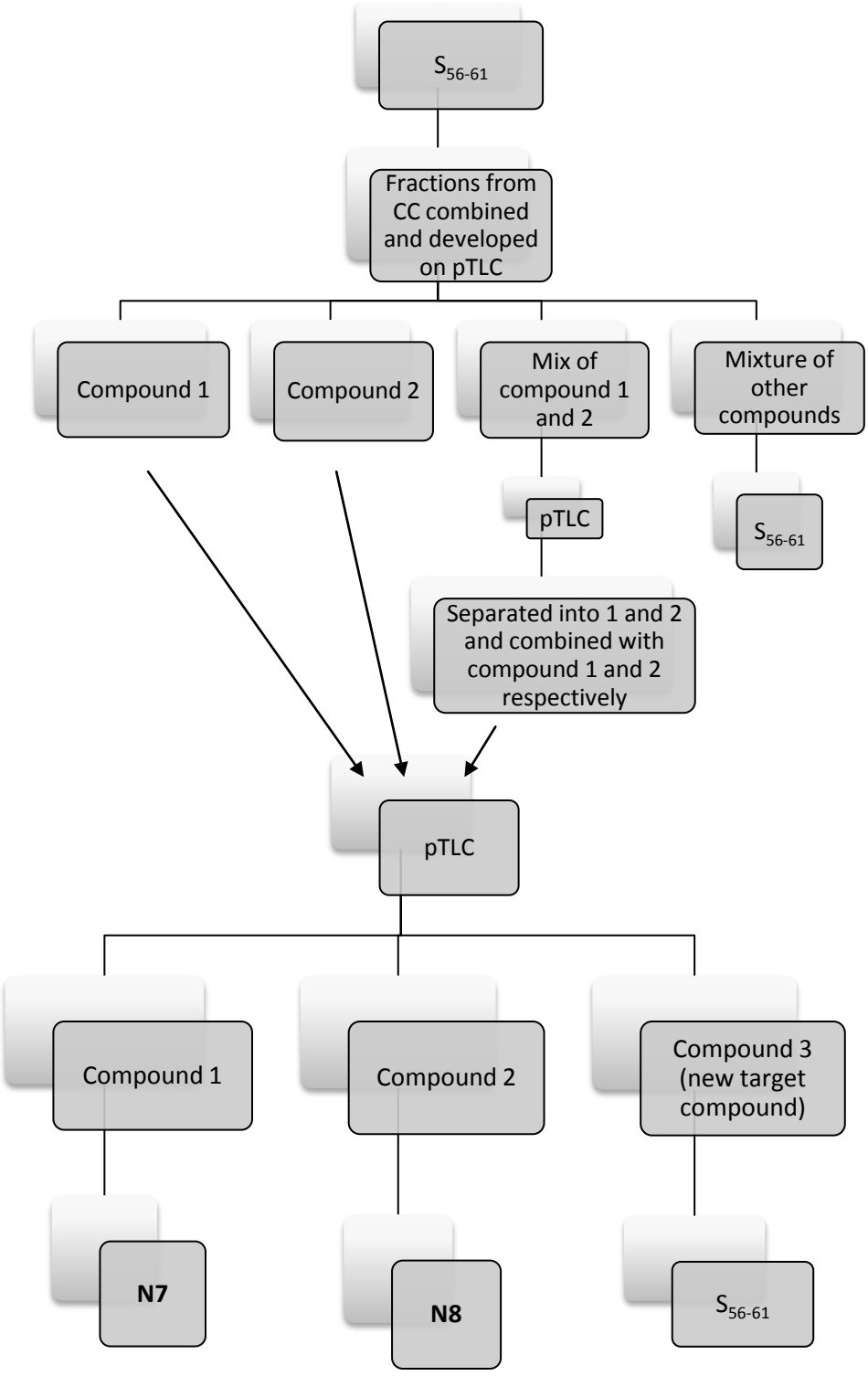
The compounds were tested on TLC to confirm that they were pure compounds. N_5 was tested in two different solvent systems (PE:EtOAc 2:1 and $CHCl_3$:Ac 7:1) and displayed a very weak colouration barely visible separate from the target compound that might indicate a second compound. As the possible second compound seemed to be present only in minimal amounts and it had already been attempted separated by pTLC, the compound was set aside and left to dry before the evaluation if it would be delivered for structural determination later on. N_6 was tested in four different solvent systems (PE:EtOAc 3:1, PE:Ac 5:1, $CHCl_3$:Ac 7:1, $ChCl_3$:EtOAc 5:1) and developed in vanillin as a colour developing agent. Every plate indicated that the compound was pure, as the only visible spot was a clear pink spot.

The compounds in fraction 62-81 were very difficult to separate. After attempting to separate the compounds by both adsorption and partition chromatography without any of them being a success, size exclusion separation was attempted. Several mobile phases had already been tested on TLC to achieve a better separation by the use of Silica gel columns, but without result. Size-exclusion separation in the Sephadex column was an attempt to separate the compounds which seemed to be of very low polarity, and of very similar polarity. The lack of separation achieved by size-exclusion chromatography indicated that the compounds in this fraction were also similar in size, which added to the difficulty in separating them. A different chromatographic method might have improved the results, and given a better yield as less material might have been lost as the transfer and pTLC recovery steps might not have been needed, see *section 5.3*.

5.2.3 Subfraction 56-61

The separation of fraction 56-61 by column chromatography using Silica gel and eluting the column with a 4:1 ratio of PE and EtOAc resulted in 25 fractions of approximately 8 ml each. TLC results showed the separation attempt to be unsuccessful, as almost all fractions appeared to contain more or less the same compounds. The only achievement obtained by running this column was the removal of small amounts of chlorophyll and other unwanted pigments. The work done to separate compounds N7 and N8 from $S_{56\text{-}61}$ are schematically given in *figure 5.3*.

Figure 5.3 – Separation of compounds from S_{56-61}



Before the CC several mobile phases were tested but none of them appeared to be optimal. A clear blue spot was initially chosen as the target compound, and the aim was to isolate this compound by the use of CC. The solvent systems either did not separate the different compounds well enough, or the *Rf*-values of the target compound were too high, that would

have led to all the compound(s) being eluted right away. The combination of $CHCl_3$ and EtOAc showed significantly better separation on TLC that PE:EtOAc. The problem with using the first combination was that it was too polar, and the compounds were not likely to be retained by the Silica gel in the column.

After testing several ratios of $CHCl_3$ and EtOAc, it was decided that the combination was too polar to put the target at a *Rf*-value of about 0.2 and the mobile phase of PE:EtOAc was chosen instead, despite the fact that the separation of compounds were not as good.

After CC the fraction was attempted separated by pTLC ($CHCl_3$:EtOAc, 5:1) to remove the vast amount of pigment still present in the fraction. The column chromatography was not successful, and it was difficult to find a mobile phase that gave good separation other than the $CHCl_3$:EtOAc which gave good results on silica. pTLC was therefore chosen as the separation method to achieve some separation of compounds and to remove pigments.

The pTLC plates showed at least 11 compounds absorbing UV-light at 254 nm. 6 plates were used for the separation. 3 of which appeared to display 2 compounds of substantial mass which were clearly separated from other compounds which were chosen as the target compounds. Pieces of the pTLC plates were tested in both sulphuric acid and vanillin colour developing agents. The sulphuric acid coloured the compounds blue and pink, while both compounds were coloured deep purple by vanillin. The two compounds were recovered and labelled 1 and 2. The remaining 3 plates showed compound 1 and 2 not to be separated from other compounds, and they were removed from the plate together, and plotted on a new pTLC and developed in the same mobile phase. This resulted in two prominent bands on the plate, one developed both pink and blue colour in sulphuric acid, while the other one was coloured brown/red. Both were removed from the plate, labelled 1* and 2*, and tested on TLC (PE:EtOAc 2:1) to compare them to the previously removed fractions labelled 1 and 2.

Table 5.4 – TLC results following pTLC of $S_{56\text{-}61}$

Fraction	Number of visible spots	Colour in sulphuric acid	*Rf*-values
1	2	One clear blue spot, one pink/brown	0.63 and 0.79
1*	2	One blue spot, one pink/brown	0.61 and 0.76
2	2	One brown spot + one weakly coloured	0.75 and 0.81
2*	1	One weak brown spot	0.75

Based on the results in table 5.4, compounds 1* and 2* were added to the previously recovered compounds 1 and 2. These were tested on TLC, in a 2:1 ratio of PE and EtOAc, and two target compounds were selected as the target compounds, the blue spot in fraction number 1 and the brown spot in fraction number 2.

The target compounds in each of the two fractions 1 and 2 were purified by pTLC, both by the use of 2:1 ratio of PE and EtOAc as the mobile phase. Both target compounds were well separated, and recovered from its plates. The target compound from fraction 1 was named N7, and the target compound obtained from fraction 2 was named N8. Both compounds were tested in two different solvent systems (PE:EtOAc and $CHCl_3$:EtOAc), and appeared to be pure compounds as only one spot developed in both cases.

Another compound was removed from the pTLC plate where fraction 1 had been plotted. This appeared to be a third compound of substantial mass and was labelled N9. The TLC performed to confirm the purity revealed that the compound was actually a mixture of two compounds, as two spots were visualized after developing the TLC in a mobile phase of $CHCl_3$ and EtOAc and staining with vanillin. These compounds were added to the rest of the compounds removed and set aside from the first pTLC plate to enrich these compounds.

5.3 Evaluation of the isolation process

The open column chromatography of the different subfractions was very time consuming. Silica gel with an average particle size of 10-40µg was used to fractionate the subfractions. An advantage of a tightly packed column is that it will result in a better separation, but the downside is that it will also increase the time needed for the column to be eluted. An attempt to speed up the eluting process was made by using a pump whenever silica gel columns were employed. Without the pump the speed was too slow and the process too time consuming. When looking at the results and the isolation process described above, it is obvious that some of the columns did not have the desired effect and did not separate the compounds very well at all. This means that some of the most time consuming work performed during the thesis sometimes led to very little progress. The compounds present in the PE fraction were compounds of very low polarity which might have contributed to the difficulty in separating them. A more efficient technique might have been the use of preparative HPLC which is faster and the densely packed columns offer a very good separation, or GC that is a very useful tool in the isolation of compounds from herbal medicines.

HPLC is becoming increasingly popular, and is used in both analytical and preparative scale. But HPLC is still significantly more expensive than open CC, which is inexpensive and very easily performed. HPLC experiments are dependent upon expensive equipment, and in a laboratory where there are many people the accessibility might be reduced, as the equipment is shared. These are some of the reasons why CC is still commonly used in isolation of natural products, and was also the case at MRCTCM. CC is a very simple and inexpensive method of separating natural products, and can provide very good results if the preparations are done correctly. This was one of the main techniques employed at the MRCTCM for the basic isolation of unknown compounds. One of the aims of the TCMCANCER relevant for this master thesis was to spread basic knowledge of natural products isolation from TCM. CC is a common and traditional way of isolation natural products, and combined with the expenses and availability issues of the HPLC equipment, it was the natural choice of technique.

In retrospect it is apparent to think that some of the results might have been better and the work might have been less tedious if HPLC had been employed. But some of the time spent on columns and pTLC could probably have been avoided if the preparation had been better. In subfraction S_{32-33} the importance of testing several solvent systems became apparent as the compound initially chosen as the target compound turned out to be a combination of more than one compound after developing pTLC plates. The lack of preparation led to the use of equipment and time that could have been saved. Although pTLC does separate better than a normal TLC plate, it might have been discovered if the initial TLC test had been performed more thoroughly. In subfraction S_{62-81} there were used more than one pTLC for the final purifying step. This is also something that might have been avoided if the preparation and choice of mobile phase had been more thorough. Subfraction S_{56-61} was separated and the compound isolated entirely by the use of pTLC. In this case many solvent systems had been explored, but none of them exhibited promising properties. The plotting of pTLC was tedious as the subfraction could not be evaporated too much as there was a lot of chlorophyll present and the mixture turned thick and difficult to work with. In this case HPLC or GC might have been a easier and fast alternative to the time consuming process of developing a lot of pTLC plates and trying to recover as lot of the compounds as possible.

It is obvious that isolation of natural products is a time consuming process, but a lot of time can actually be saved by using a little more time in the preparations. In addition to saving time, the separation and the purity of isolated compounds might improve.

5.4 Characterization of compounds

Eight compounds were isolated from the PE fraction. The characteristics of these compounds are given below in *table 5.5*.

Table 5.5 – Characteristics of the isolated compounds

Compound	Visual characteristics	Weight (mg)
N1	White/yellowish amorphous powder	9,3
N2	White crystals/powder	208
N3	White crystals	19,5
N4	Pale amorphous powder	8,0
N5	Brownish/white amorphous powder	5,4
N6	White amorphous powder	2,0
N7	Pale amorphous powder	17,7
N8	White amorphous powder	15,2

5.4.1 N5 – excluded due to impurities

The TLC tests of compound N5 showed a very weak spot that might be an additional compound, but it was decided to let the powder dry and evaluate later. When the crystals dried the result was a white powder with brownish discolouration, and it did not look like a pure compound at all. As the previous TLC results had shown that N5 might be a mixture of two compounds, the discolouration was interpreted as proof of this and N5 was excluded from the samples delivered to the analysis lab for NMR testing and it was decided not to study this compound(s) further.

5.4.2 Structural determination of N1

The ESI-MS results of N1 are given as attachment 1.1. The attachment shows peaks at the following mass to charge (*m/z)* ratios: 168.0 [M+H], 190.0 [M+Na], 357.0 [2M+Na], 165.9 [M-H] and 332.8 [2M-1]. On the basis of these peaks, the molecular weight of N1 was determined to be 167 Da. The ^{13}C-NMR and ^{1}H-NMR spectra are given as attachment 1.2 and 1.3. The interpretation of the peaks in the ^{13}C-NMR and ^{1}H-NMR spectra are given in *table 5.6 and 5.7*.

Table 5.6 Interpretation of 13C-NMR spectrum

Carbon no.*	Ppm	Assignment
1	128.1	Aromatic carbon
2	116.5	Aromatic carbon
3	146.1	Aromatic carbon
4	145.1	Aromatic carbon
5	117.3	Aromatic carbon
6	121.7	Aromatic carbon
1'	177.8	Carbonyl group
2'	42.7	Aliphatic carbon

* See figure 5.4

Table 5.7 Interpretation of 1H-NMR spectrum

Ppm	Shape	Integral	Coupling constant	Assignment
3.30	Singlet	2	-	Aliphatic CH2
6.60	Doublet of doublets	1	7.4 Hz and 1.8 Hz	Aromatic CH
6.73	Doublet	1	7.4 Hz	Aromatic CH
6.75	Doublet	1	1.8 Hz	Aromatic CH

The sources available for structural characterization were a ^{13}C-NMR and a ^{1}H-NMR spectrum in addition to the ESI-MS. The ^{13}C-NMR and ^{1}H-NMR spectra show signal patterns consistent with an aromatic ring (carbon peaks in the area of 110-174 ppm and proton peaks in the area of 6.5-8 ppm). In the carbon spectra there are 8 signals which indicate a C8-sceleton. The peak at 177.8 in the ^{13}C-NMR spectrum is suggestive of a carbonyl; possibly an acid group. The information from the MS spectrum gave a molecular weight of 167. An odd numbered molecular mass is the result of an odd number of nitrogen atoms in the structure. Based on the low molecular mass, the evidence of an aromatic ring, a possible acid group and an aliphatic CH_2 group, *one* nitrogen atom is most likely. A structure containing an aromatic ring and a carbonyl indicate 5 degrees of unsaturation. By adding the information gathered so far, the molecular formula $C_8H_9NO_3$ is found by the rule of thirteen.

The three proton signals in the aromatic region with an integral of three (combined) indicate that only three protons are present on the aromatic ring, resulting in 3 substitutes attached to the aromatic ring. ^{13}C-NMR signals at 146.1 ppm and 145.1 ppm indicate two electronegative groups attached to the ring. The signal at 42.7 ppm in the carbon-13 spectrum and the singlet at 3.3 ppm in the proton spectrum indicate a CH_2 group attached to the ring. The available groups deduced so far are an amino group, a CH_2 group, an acid group and from the

molecular formula the remaining group is a hydroxyl group. The carbon-13 signal at 42.7 ppm and proton signal at 3.3 ppm indicate that the CH_2 group is not directly linked to one of the more electronegative atoms N and O as the peaks would probably be found further downfield if this was the case. This suggests that an acetic acid is the third substituent on the aromatic ring in addition to an amino and a hydroxyl group. The substitution pattern is found by examining the aromatic region of the proton spectrum. The ^{1}H-NMR spectrum results are given in *table 5.7*. The doublet of doublets at 6.60 ppm has one coupling constant 7.4 Hz (^{3}J) and one 1.8 Hz (^{4}J). The doublet at 6.73 ppm has one coupling constant being 7.4 Hz indicating a three bond coupling to the first proton. The last peak in the spectra at 6.75 ppm is a doublet with coupling constant 1.8 Hz indicating a four bond coupling to the first proton. Combining the information deduced from the spectra, N1 was assumed to be one of the two known compounds 2-(4-amino-3-hydroxyphenyl) acetic acid (structure 1) or 2-(3-amino-4-hydroxyphenyl) acetic acid.

To confirm the structure as one of these compounds, the NMR values were compared to published literature [54-56]. The comparison is given in *tables 5.8* and *5.9*.

Table 5.8
Comparison of 1H-NMR results of N1 to 2-(4-amino-3-hydroxyphenyl) acetic acid

1H- NMR, N1 (ppm)	2-(4-amino-3-hydroxyphenyl)acetic acid (ppm)[55]
3.59	3.30
6.86	6.60
6.96	6.72
7.22	6.76

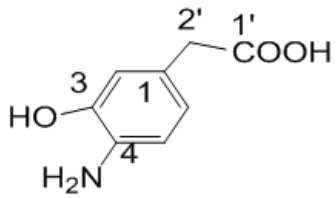
Table 5.9
Comparison of 13C-NMR results of N1 to 2-(3-amino-4-hydroxyphenyl)acetic acid

13C-NMR , N1 (ppm)	2-(3-amino-4-hydroxyphenyl)acetic acid (ppm) [54]
43.7	40.5
116.5	114.1
117.3	115.4
121.1	117.3
128.1	125.7
145.1	136.1
146.1	142.8
177.8	173.2

Table 5.8 compares the ^{1}H-NMR results of compound N1 and 2-(4-amino-3-hydroxyphenyl) acetic acid. The values are similar, with the results of N1 being consistently slightly higher

(≈0.3 ppm) than 2-(4-amino-3-hydroxyphenyl) acetic acid. *Table 5.9* compares the ^{13}C-NMR peaks of N1 compared to those of 2-(3-amino-4-hydroxyphenyl) acetic acid. Most values are similar with compound N1 being consistently higher than 2-(3-amino-4-hydroxyphenyl) acetic acid (≈2 -3 ppm). But one value does stand out with a 10 ppm difference to the reference compound. This indicates that the structure of N1 is not identical to 2-(3-amino-4-hydroxyphenyl) acetic acid. Based on the results above, compound N1 was determined to be the known compound 2-(4-amino-3-hydroxyphenyl) acetic acid.

Figure 5.4 – Structure of N1



N_1

5.4.3 Characterization of compound N2

The ESI-MS results of N2 are given as attachment 2.1. The attachment shows peaks at the following mass to charge (*m/z)*ratios: 437.2 [M+Na], 413.2 [M-H], 449.2 [M+Cl^-] and 827.3 [2M-1]. On the basis of these peaks, the molecular weight of N2 was determined to be 414 Da. The ^{13}C-NMR and ^{1}H-NMR spectra are given as attachment 2.2 and 2.3. The interpretation of the peaks in the ^{13}C-NMR spectrum is given in *table 5.10* where the question marks represent peaks that were difficult to identify as positive or negative in the DEPT-135.

From the molecular weight of 414, the rule of thirteen gives a base formula of $C_{31}H_{42}$. This equals an unsaturation index of 11. The peak at 71.7 ppm indicates an oxygenated carbon. When adding one oxygen atom to the molecular formula, the result is $C_{30}H_{38}O$ which has an unsaturation index of 12. The peaks at 121.6 and 140.7 ppm in the ^{13}C-NMR indicate olefinic carbon atoms, but other than this all peaks indicate aliphatic carbons. This is also indicated by the ^{1}H-NMR spectrum of N2 (see *table 5.12*), which show a single signal at 5.34 suggestive of a proton connected to an olefinic carbon atom, while the rest of the proton signals are upfield in the spectrum, not indicating any more double bonds. As the structure appears to contain only one double bond, an unsaturation index of 12 seems unlikely with the number of carbon atoms. The removal of one carbon and addition of twelve hydrogen atoms results in the molecular formula $C_{29}H_{50}O$ which has a hydrogen deficiency of 5, indicating a structure with a 4 ring skeleton and one double bond (corresponding to the two carbon signals at 121.6 and 140.7 ppm).

The DEPT-135 spectrum was difficult to interpret, especially in the upfield region. It would

Table 5.10 – Interpretation of ^{13}C-NMR spectra of N2

N2 (ppm)	DEPT-135	Assignment
11.8	?	?
11.9	Positive (?)	Aliphatic CH_3 or CH
18.8	?	?
19.0	?	?
19.4	Positive (?)	Aliphatic CH_3 or CH
19.8	Negative (?)	Aliphatic CH_2
21.0	Negative (?)	Aliphatic CH_2
23.0	Negative (?)	Aliphatic CH_2
24.3	Negative	Aliphatic CH_2
26.0	Positive	Aliphatic CH_3 or CH
28.2	Negative	Aliphatic CH_2
29.1	Positive	Aliphatic CH_3 or CH
31.6	Negative	Aliphatic CH_2
31.9	Positive	Aliphatic CH_3 or CH
31.9	?	?
33.9	Negative	Aliphatic CH_2
36.1	Positive (?)	Aliphatic CH_3 or CH
36.5	No peak	Aliphatic (quaternary)
37.2	Negative	Aliphatic CH_2
39.7	Negative	Aliphatic CH_2
42.2	Negative	Aliphatic CH_2
42.3	No peak	Aliphatic (quaternary)
45.8	Positive	Aliphatic CH_3 or CH
50.1	Positive	Aliphatic CH_3 or CH
56.0	Positive	Aliphatic CH_3 or CH
56.7	Positive	Aliphatic CH_3 or CH
71.7	Positive	Oxygenated (CH-O)
121.6	Positive	Olefinic (C=CH)
140.7	Positive	Olefinic (quaternary)

be preferable with an enlargement of the different regions of the carbon spectrum and its corresponding DEPT-135 spectrum to make a complete assignment of carbon peaks which was not possible with the current magnification of the spectrum (as demonstrated in *table 5.10*). In the ^{13}C-NMR spectrum of N2 only 28 carbon atoms can be counted. This might be due to overlapping signals. Although this is uncommon in carbon spectra, it might be the case in these spectra as almost all signals are grouped relatively closely together in the upfield part of the spectrum. The upfield region has been enlarged to make an assignment easier. This enlargement shows that the peak at 31.9 is slightly taller than all the other peaks in this area, possibly due to two carbon atoms overlapping.

A search in the "Spectral Database for Organic Compounds", SDBS [57], gave a single hit when a search for the molecular formula $C_{29}H_{50}O$ was entered. The resulting structure was β-sitosterol, but no NMR spectra were available for this compound. A literature search was conducted, and the ^{13}C-NMR spectrum of N2 were compared to that of β-sitosterol from literature [58] which also allowed the different carbon peaks in the spectra to be assigned to the different carbons. The results from this comparison are given in *table 5.11*. This also gave an indication that the assumption that the peak at 31.9 was actually two different protons was correct, as there are two very similar signals in β-sitosterol than corresponds well to these values.

Table 5.11 Comparison of 13C-NMR spectra of N2 to that of β-Sitosterol

Carbon no.*	β-Sitosterol (ppm) [58]	N2
1	37.7	37.2
2	32.3	31.6
3	72.2	71.7
4	42.8	42.2
5	141.2	140.7
6	122.1	121.6
7	32.2	31.9
8	32.3	31.9
9	50.6	50.1
10	36.9	36.5
11	21.5	21.0
12	40.2	39.7
13	42.8	42.3
14	57.2	56.7
15	24.7	24.3
16	28.7	28.2
17	56.5	56.0
18	12.4	11.8
19	19.8	19.4
20	36.6	36.1
21	19.2	18.8
22	34.4	33.9
23	26.5	26.0
24	46.2	45.8
25	29.6	29.1
26	20.2	19.8
27	19.5	19.0
28	23.5	23.0
29	12.3	11.9

*See figure 5.5

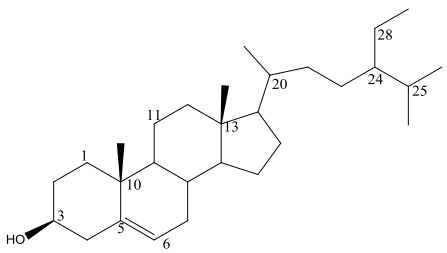
The values of N2 are consistent with the values of β-sitosterol, with the values of N2 being approximately 0.5 ppm lower than those of β-sitosterol. The corresponding values made it possible to assign values to every carbon atom in the structure which was not possible based on the available material. The solvents used to dissolve the compounds for analysis were the same, and the results should in theory be exactly the same. The small difference in the results may be accounted for by the fact that the compounds were analyzed by different kinds of NMR machines.

The ^{1}H-NMR spectrum was not easily interpreted. Most signals were located upfield in the spectra causing most of the signals to overlap, resulting in peaks that are difficult to separate and integrals that seem unlikely (a total of 69 protons). When the molecular weight gave the most likely molecular formula $C_{29}H_{50}O$, and the carbon spectrum is consistent with that of β-sitosterol, it is improbable that the molecule actually contains 69 protons. Because only 1D NMR analysis were available, and the results did overlap the way they did, the ^{1}H-NMR spectrum was not completely interpreted, but compared to previously interpreted spectra found in literature [58]. To identify a known structure, it is rarely necessary to identify every proton signal to confirm the structure. The most easily identifiable peaks are given in *table 5.12* and compared to those of β-sitosterol found in literature [58].

Table 5.12 1H-NMR spectra of N2 and comparison to β-sitosterol

ppm (β-sitosterol) [58]	ppm (N3)	Shape (N3)	Integral (N3)	Coupling constant (Hz) (N3)	Assignment (N3)
5.39	5.34	Brs	1	-	olefinic proton
3.56	3.52	m	1	-	CH-OH
1.05	1.01	s	-	-	aliphatic protons
0.96	0.97	d	-	6.6	Aliphatic protons
0.89	0.84	t	-	7.8	Aliphatic protons
0.87	0.83	d	-	6.6	Aliphatic protons
0.85	0.81	d	-	6.6	Aliphatic protons
0.72	0.67	s	3	-	Aliphatic protons, CH_3

Figure 5.5 Structure of compound N2, β-sitosterol



5.4.4 Structural determination of N3

The ESI-MS results of N1 are given as attachment 3.1. The attachment shows several peaks, but none of them are useful in determining the molecular weight, as it is not possible to link the different peaks to a common value. This suggests that compound N3 is a highly non-polar compound. ESI/MS has poor sensitivity for detection of non-polar compounds, which may result in inconclusive results as seen in attachment 3.1. [49-50]. The ^{13}C-NMR and ^{1}H-NMR spectra are given as attachment 3.2 and 3.3. The interpretation of the peaks in the ^{13}C-NMR spectrum is given in *table 5.13*, and the most easily identifiable peaks of the proton spectrum are given in *table 5.14*. The proton spectrum of N3 was similar to N2 with regards to the compounds being non polar resulting in proton peaks overlapping upfield in the spectrum making the interpretation of every peak very difficult.

The ^{13}C-NMR spectrum indicates a 30-carbon skeleton. A peak at 205 ppm in the ^{13}C-NMR spectrum (positive in DEPT-135) indicates an aldehyde group, consistent with the peak at 9.70 in the ^{1}H-NMR spectrum. The two peaks at 149.1 and 108.7 are suggestive of two carbon atoms bound together by a double bond. The two protons bound to the carbon atom giving rise to the peak at 108.7 ppm are found at 4.78 ppm and 4.77 ppm indicating that the protons are not chemically equivalent. A carbon peak at 76.7 indicates an oxygenated carbon with one proton attached (positive peak in DEPT-135). The absence of other carbon peaks in this area of the spectrum suggests a hydroxyl group. The rest of the spectrum show aliphatic carbons and protons, including 6 methyl groups identified by the proton spectrum which is suggestive of a compound containing a multiple ring structure as seen in N2. The MS spectrum of N3 is also similar to that of N2. As the molecular mass was not found by the ESI/MS, a definitive molecular formula is not possible to obtain. From the information found in the NMR spectrum, it is suggested that N3 has a C_{30} skeleton, at least 41 hydrogen atoms (found by summarizing the integrals) and probably 2 oxygen atoms. But without the molecular mass and only 1D NMR, the structural characterization was very difficult.

Table 5.13 – Interpretation of ^{13}C-NMR spectra of N3

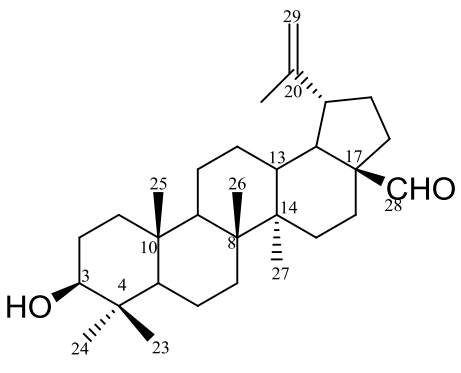
N3 (PPM)	DEPT-135	Assignment
12.8	Positive	Aliphatic (CH_3 or CH)
14.3	Positive	Aliphatic (CH_3 or CH)
14.5	Positive	Aliphatic (CH_3 or CH)
14.8	Positive	Aliphatic (CH_3 or CH)
17.2	Negative	Aliphatic (CH_2)
17.3	Positive	Aliphatic (CH_3 or CH)
19.7	Negative	Aliphatic (CH_2)
24.5	Negative	Aliphatic (CH_2)
26.5	Negative	Aliphatic (CH_2)
26.7	Positive	Aliphatic (CH_3 or CH)
27.7	Negative	Aliphatic (CH_2)
28.4	No peak ?	Aliphatic (quaternary)
28.8	Negative	Aliphatic (CH_2)
31.8	Negative ?	Aliphatic (CH_2)
33.3	Negative	Aliphatic (CH_2)
36.1	No peak	Aliphatic (quaternary)
37.7	Positive	Aliphatic (CH_3 or CH)
37.7	Negative	Aliphatic (CH_2)
37.8	No peak ?	Aliphatic (quaternary)
39.8	No peak	Aliphatic (quaternary)
41.5	No peak	Aliphatic (quaternary)
46.6	Positive	Aliphatic (CH_3 or CH)
46.9	Positive	Aliphatic (CH_3 or CH)
49.5	Positive	Aliphatic (CH_3 or CH)
54.4	Positive	Aliphatic (CH_3 or CH)
58.1	No peak	Aliphatic (quaternary)
76.7	Positive	Oxygenated carbon
108.7	Negative	Olefinic (CH_2)
149.1	No peak	Olefinic quaternary
205	Positive	Carbonyl, (CHO)

Table 5.14 – Most easily identifiable peaks in the 1H-NMR spectra of N3

Ppm	Shape	Integral	Assignment
0.76	S	3	Methyl group
0.84	S	-	Aliphatic protons
0.94	S	3	Methyl group
0.96	S	-	Aliphatic protons
1.03	S	3	Methyl group
1.72	S	3	Methyl group
4.62	Brs	1	Olefinic proton
4.77	Brs	1	Olefinic proton
9.70	S	-	Aldehyde

The determination of this structure was aided by the structural determination of N7. N3 and N7 showed very similar ppm values (with N7 having slightly higher values) in the ^{13}C-NMR spectra, suggesting similar structures. After finding the structure of compound N7, it was apparent that the difference between the two structures was the aromatic ring as there were no signals in the aromatic region of the ^{13}C-NMR spectrum of N3. An additional difference was that the carbonyl peak at 205 ppm in N3 suggesting that the aldehyde group was replaced by a peak at 183 ppm in the spectrum of N7, corresponding with an acid group. After the additional information obtained by comparison to N7, N3 was identified as betulinic aldehyde by comparing the results obtained from the spectrum to the literature [56, 59] as shown in *table 5.15*. The data from *table 5.15* identified compound N3 as betulinic aldehyde, and made it possible to assign the different peaks in the spectra to the different carbon atoms. The values from the spectra of N3 compared to the values form the literature, there is a good match between the peak values, with the values recorded in the spectrum of N3 being consistently 1-2ppm lower than those found in literature [59]. N3 was dissolved in acetone prior to the analysis, while the betulinic aldehyde was dissolved in $CDCl_3$ according to the literature [59]. This small difference in ppm values can be explained by the fact that the compounds were analyzed by different kinds of NMR machines in different solvents.

Figure 5.6 Structure of compound N3, betulinic aldehyde



N_3

Table 5.15 Comparison of 13C-NMR spectra of N3 to betulinic aldehyde

Carbon no*	N3 (ppm)	Betulinic Aldehyde [59]
1	37.7	38.7
2	26.5	27.3
3	76.7	78.9
4	37.8	38.8
5	54.4	55.5
6	17.2	18.2
7	33.3	34.3
8	39.8	40.8
9	49.5	50.4
10	36.1	37.1
11	19.7	20.7
12	24.5	25.5
13	37.7	38.7
14	41.5	42.5
15	28.4	29.2
16	27.7	28.8
17	58.1	59.3
18	46.9	48.0
19	46.6	47.5
20	149.1	149.7
21	28.8	29.8
22	31.8	33.2
23	27.6	27.9
24	14.3	15.4
25	14.5	15.9
26	14.8	16.1
27	12.8	14.2
28	205.0	205.6
29	108.7	110.1
30	17.3	19.0

* See figure 5.6

5.4.5 Structural determination of N4

The ESI-MS results of N1 are given as attachment 4.1. The attachment shows peaks at the following mass to charge (*m/z)* ratios: 449.1 [M-H] and 899.5 [2M-H] On the basis of these peaks, the molecular weight of N4 appears to be 450 Da. The ^{1}H-NMR and ^{13}C-NMR spectra are given as attachment 4.2 and 4.3. The attempt to interpret the peaks of the ^{13}C-NMR spectra is given in *table 5.16.*

Table 5.16 Interpretation of 13C-NMR spectra of N4

N4	DEPT-135	Assignment
13.5	Positive	Probably methyl group
22.1	Negative	CH_2
24.2	Negative	CH_2
24.3	Negative	CH_2
26.6	Negative	CH_2
28.4	Negative	CH_2
28.5	Negative	CH_2
28.7	Negative	CH_2
31.3	Negative	CH_2
33.3	Negative	CH_2
33.3	Negative	CH_2
61.5	Negative	Oxygenated, CH_2
68.4	Positive	Oxygenated, one proton
129.1	No peak	Olefinic, quaternary
129.4	Positive	Olefinic, one proton
172.5	No peak	Carbonyl group, ester (?)

From *table 5.16* it is obvious that the number of carbon atoms is far less that what would be expected from the molecular weight of 450 Da. A molecular weight of 450 results in the base formula $C_{34}H_{42}$, but the carbon spectrum only shows 16 peaks. When adding the 3 oxygen atoms a possible formula is $C_{30}H_{42}O_3$, which has an unsaturation index of 10. With the number of peaks in the ^{13}C-NMR spectrum, this seems unlikely, as does the molecular weight.

When looking at the ^{1}H-NMR spectrum it looks like the compound is not completely pure. There are some strong signals that are not in proportion to the weaker signals that are more abundant which is suggestive of an impure compound. The compound was dissolved in a mixture of Ac and $CHCl_3$, which are both easily visible as relatively peaks in the ^{1}H-NMR spectrum, but another set of peaks appear to be present in the spectrum which cannot be

explained by the solvent mixture. The impurities of the ^{1}H-NMR spectrum combined with a less than optimal signal to noise ratio in the ^{13}C-NMR spectrum prevented this compound from being structurally elucidated with the available material. From the similarities of the MS spectra to those of N2, N3 and N7, the compound may have some kind of multiple ring structure. Without a molecular weight, the structure cannot be elucidated, and the fact that the molecular weight does not match the number of peaks in the spectrum suggests that the assumed weight of 450 might be wrong, or that there are peaks that are not visible in the spectrum. Added to the impurities in the ^{1}H-NMR spectrum this led to compound N4 not being structurally characterized.

5.4.6 Structural determination of N6

No NMR results were obtained as the quantity of sample delivered for analysis was too small. Only 2 mg of compound 6 was isolated and purified from the petroleum ether fraction, and although the entire sample was dissolved and delivered to MS and NMR analysis no results were obtained from the NMR analysis. The ESI/MS spectra is given as attachment 6.1 and suggest a compound with molecular weight of 350 Da (Peaks at the following mass to charge (*m/z)*ratios: 349.2 [M-H] and 699.3 [2M-1]). But without any NMR results, the molecular formula is impossible to deduce, and the compound was not characterized.

5.3.7 Structural determination of N7

The ESI-MS results of N7 are given as attachment 7.1. The attachment shows peaks at the following mass to charge (*m/z)* ratios relating to the same molecular weight: 601.5 [M-H] and 637.4 [M+Cl^{-}]. On the basis of these peaks, the molecular weight of N7 was determined to be 602 Da. The ^{13}C-NMR and ^{1}H-NMR spectra are given as attachments 7.2 and 7.3. The interpretation of the peaks in the ^{13}C-NMR spectra is given in *table 5.17*, and the peaks of the ^{1}H-NMR spectra are given in *table 5.18*.

The ^{13}C-NMR spectrum show a poor signal to noise ratio, with very weak peaks, some of them were not possible to definitely identify as peaks when looking at the spectrum for the first time. But the molecular weight of 602 Da indicates a substantial amount of carbon atoms, and a closer look was necessary. Signals in the aromatic region of the ^{1}H-NMR spectrum were another reason to take a closer look at this part of the carbon spectrum.

Table 5.17 –Interpretation of ^{13}C-NMR spectra of N7

N7 (PPM)	DEPT-135	Assignment
14.7	Positive	Aliphatic (CH or CH_3)
16.0	Positive	Aliphatic (CH or CH_3)
16.2	Positive	Aliphatic (CH or CH_3)
16.6	Positive	Aliphatic (CH or CH_3)
18.1	Negative	Aliphatic (CH_2)
19.3	Positive	Aliphatic (CH or CH_3)
20.8	Negative	Aliphatic (CH_2)
23.8	Negative	Aliphatic (CH_2)
25.4	Negative	Aliphatic (CH_2)
28.0	Positive	Aliphatic (CH or CH_3)
29.7	Negative	Aliphatic (CH_2)
30.5	Negative	Aliphatic (CH_2)
32.1	Negative	Aliphatic (CH_2)
34.2	Negative	Aliphatic (CH_2)
37.1	Negative	Aliphatic (CH_2)
37.1	No peak	Aliphatic (quaternary)
38.0	No peak	Aliphatic (quaternary)
38.4	Positive	Aliphatic (CH or CH_3)
40.7	No peak	Aliphatic (quaternary)
42.4	No peak	Aliphatic (quaternary)
46.9	Positive	Aliphatic (CH or CH_3)
49.2	Positive	Aliphatic (CH or CH_3)
50.4	Positive	Aliphatic (CH or CH_3)
55.4	Positive	Aliphatic (CH or CH_3)
56.4	No peak	Aliphatic (quaternary)
80.9	Positive	Oxygenated
110 ?	Negative	Olefinic (CH_2)
115.8	Positive, high peak	Olefinic (CH)
116.2	Positive	Olefinic (CH)
127.3	No peak	Olefinic (quaternary)
129.9	Positive, high peak	Olefinic (CH)
144.1	Positive	Olefinic (CH)
150 ?	Positive?	Olefinic (CH)
157.7	Positive?	Olefinic (CH)
167 ?	No peak?	Carbonyl group, acid or ester
182 ?	No peak?	Carbonyl group, possibly acid

Table 5.18 – Interpretation of 1H-NMR spectra of N7

Ppm	Shape	Integral	Coupling constant	Assignment
0.91	S	3	-	Methyl group?
0.94	S	-	-	Methyl group?
0.98	S	-	-	Methyl group?
1.70	S	-	-	Methyl group?
3.02	Brs	1	-	Aliphatic proton
4.60	Brs	1	-	Oxygenated
4.61	Brs	1	-	Olefinic proton
4.74	Brs	1	-	Olefinic proton
6.29	D	1	15.6	Olefinic (conjugated) proton, trans
6.84	D	2	8.4	Aromatic protons
7.43	D	2	8.4	Aromatic protons
7.60	D	1	15.6	Olefinic (conjugated), trans

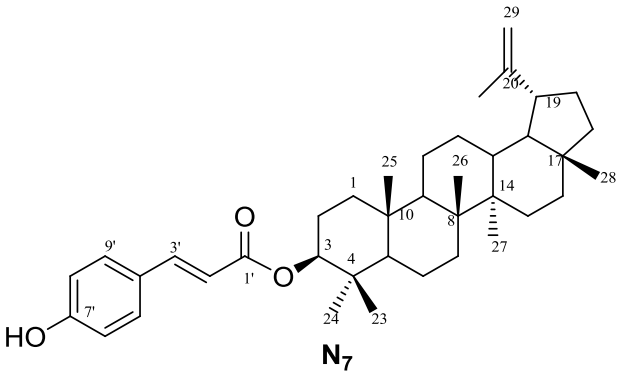
A large magnification of the spectrum was necessary to try to identify peaks in the aromatic region of the carbon spectra, where signals were particularly low. When studying the spectrum and its magnifications very closely, allowing even very small peaks to be counted, the number of peaks counted was 36. Two peaks, at 129.9 and 115.8 stand out because they are taller than the rest, possibly due to two chemically equivalent carbon atoms giving rise to peaks at the exact same value, indicating that the compound has a C_{38} skeleton. By summarizing the integrals in the ^{1}H-NMR spectrum, a total of approximately 60 protons are found. The peaks of the spectrum are like the proton spectra of the other compounds; a lot of peaks upfield in the spectrum causing the peaks to overlap, which in turn may cause the amount of protons to appear higher than what is actually the case. The molecular weight of 602 Da gives a base formula of $C_{46}H_{50}$. The peaks from the carbon and proton NMR spectra indicate the presence of only one aromatic ring, two carbonyl groups (182 ppm and 166 ppm) and two double bonds, indicating a degree of unsaturation of at least 8. The assumptions made when interpreting the spectra indicates the presence of 5 oxygen atoms. A formula of 38 carbon atoms and 5 oxygen atoms results in the molecular formula $C_{38}H_{66}O_5$, which is very unlikely as it results in an unsaturation index of only 7. When adding a carbon atom, the molecular formula becomes $C_{39}C_{54}O_5$, with an unsaturation index of 13 which is more likely. This indicates a structure containing a 5 ring skeleton as well as the two carbonyl groups, the aromatic ring and the two double bonds. The structure was confirmed by a literature search after finding the assumed structure in available literature at the MRCTCM [56]. The coupling

constants found in the ^{1}H-NMR spectrum made it possible to identify the structure as 3β-o-*trans*-coumaroylbetulinic acid, as the coupling constant found at the doublets at 7.60 and 6.29 ppm was 15.6 Hz which is correspondent with a trans bond.

From the comparison in *table 5.19* it is obvious that one of the signals overlapped, and was not possible to visualize when looking at the spectra, resulting in the assumed C_{38} skeleton. The peak at 38.37 appears to be a bit broad in the first magnification of the upfield region of the N7 spectrum, and when magnifying it excessively it is barely possible to see what appear to be two very poorly separated peaks.

The values of the ^{13}C-NMR spectrum of 3β-o-*trans*-coumaroylbetulinic acid are a good match with the values of N7, with N7 being slightly lower than those of 3β-o-trans-coumaroylbetulinic acid (approximately 0.3-0.7 ppm). As the compounds were analysed in different solvents, and by different kinds of NMR machines, the results will not be exactly the same, and the small differences in ppm values are expected.

Figure 5.7 Structure of compound N7, 3β-o-trans-coumaroylbetulinic acid



*Numbered carbons corresponding to carbons in table 5.19

Table 5.19 Comparison of 13C-NMR spectra to that of 3β-o-trans-coumaroylbetulinic acid

Carbon no*	N7	3β-o-trans-coumaroylbetulinic acid [60]
1	38.4	38.63
2	23.8	24.14
3	80.9	80.67
4	38.0	38.12
5	55.4	55.71
6	18.2	18.48
7	34.2	34.62
8	40.7	41.07
9	50.4	50.71
10	37.1	37.33
11	20.8	21.18
12	25.4	26.01
13	38.4	38.55
14	42.4	42.84
15	29.7	30.24
16	32.1	32.85
17	56.4	56.61
18	49.2	49.75
19	46.9	47.76
20	150.0	151.27
21	30.5	31.21
22	37.1	37.57
23	28.0	28.13
24	16.6	16.86
25	16.0	16.27
26	16.2	16.35
27	14.7	14.88
28	180.1	178.81
29	109.5	109.96
30	19.3	19.46
1'	167.0	166.65
2'	116.2	116.87
3'	144.1	143.80
4'	127.2	126.70
5'	129.9	133.64
6'	115.8	115.97
7'	157.7	160.55
8'	115.8	115.97
9'	129.9	133.64

5.4.8 Structural determination of N8

The ESI-MS results of N8 are given as attachment 8.1. The attachment shows peaks at the following mass to charge (*m/z)* ratios: 587.5 [M-H] and 623.4 [M+Cl^-]. On the basis of these peaks, the molecular weight of N8 was determined to be 588 Da. The only other available source of information is the ^{1}H-NMR spectrum, which is given as attachment 8.2. The ^{1}H-NMR spectrum show several strong peaks not in proportion to weaker peaks indicating an impure compound. As the results indicated an impure compound, this compound was not analyzed further by the NMR department, and there are no more available results to give an indication of what this compound with a molecular weight of 588 might be.

5.5 Evaluation of the structural determination process

Of the 8 compounds that were isolated from the plant, only 4 were pure enough or present in large enough amount to be structurally characterized, which was a rather disappointing result. All compounds had been tested rather thoroughly by the use of TLC and appeared to be pure compounds (except N5 as mentioned earlier). The isolated compounds were set aside as they were purified, and the collection of compounds was delivered to the NMR department at the end of my stay in Shanghai. This meant that when I received the results there were no more time to attempt an extra purifying step to remove the impurities. Maybe the compounds could have been purified if the compounds had been delivered as soon as they were isolated, and the results would have been received earlier. But from the results of the pTLC and TLC tests I did, all compounds appeared pure, and the impurities might have been too difficult to remove with the methods used.

Again it is relevant to think that more modern separation techniques, such as GC or HPLC might have resulted in a better separation and pure compounds that could have been characterized.

For the NMR analysis, only 1D NMR was used. If the compounds had been novel compounds it would have been necessary to use 2D NMR to be able to structurally characterize the compounds. But the 1D NMR was enough to identify the most characteristic groups and carbon skeletons of these compounds which allowed the data to be compared to literature available at the MRCTCM and to the data obtained from literature searches.

No new compounds were discovered, but the compounds were isolated from *Bretschneidera Sinensis* for the first time, as this plant has not previously been examined for biologically active compounds. Although the compounds were not new, they are known biologically active compounds. β-sitosterol is known to lower cholesterol levels [61], is used as treatment against benign prostate enlargement [62] and have even displayed an effect in breast cancer [63]. Betulinic acid has been found to induce apoptosis in human melanoma cells [36, 64], and betulinic acid derivates appear to be a new class of HIV-1 inhibitors [65]. Although only four compounds were structurally characterized from *Bretschneidera sinensis* some of the compounds are biologically active with a significant effect on various diseases. This makes me even more curious of what the other compounds might have been, and I wish the evaluation of the purity of the compounds would have been even more thorough which would possibly have resulted in the impurities being removed to yield pure compounds that could have been characterized.

6 Conclusion

The theoretical framework behind TCM and Western medicine has developed into two completely different medical systems. Although the initial thoughts in Western medicine resembled those of TCM, modern Western medicine has few, if any, similar traits to those in TCM. TCM is based on a holistic approach where nothing can be understood on its own when it is removed from the "the big picture". In comparison, Western medicine has a much more mechanical view, where the disease and symptoms are isolated into a single entity that can be cured. In TCM the herbal treatments consist of several components that work together to reduce side-effects and enhance the therapeutic effects. The goal of the treatments is to help the body to restore its internal balance and thereby being healthy. The most prominent idea in Western medicine has been to isolate smaller and more defined drug targets, and synthetically produce lead compounds to fit the target. After working with this thesis, my conclusion is that there are two areas in particular where TCM and Western medicine can influence each other.

The first area is in the field of network medicine. In later years network medicine has become increasingly popular in the West, and the use of "polypharmacuticals" opposed to the one-gene-one-drug theory is gaining attention. The combination of drugs means that the dose of each drug can be reduced, resulting in less side-effect. But at the same time the effect of the drugs acting at more than one step in the disease mechanism at once is believe to increase the effect compared to one drug therapy, similar to TCM theory. TCM is being proposed as a model of this type of medication, as it has been used for thousands of years and adverse effects are well documented. The transfer of TCM knowledge into the modern Western medicine may for instance serve as a kind of rough interaction studies indicating which drugs are worth combining. This is possible as more and more TCM are being studied and their active components are being isolated and characterized.

Although TCM has prevailed in China despite the introduction of modern Western medicine, a large part of Chinese scientists think that TCM needs to be modernized if its use is to be continued in China, and spread to other countries. One of the areas where research has been requested is within the field of quality control and *identification of active components* of TCM. This is the second area where I see a great potential for cooperation. Natural products have historically been an important source of novel compounds, and have resulted in blockbuster drugs (that is, a few drugs that in a large part make up the main part of the

industry's income). The TCMCANCER project is attempting to use this method and utilize the knowledge in TCM to obtain new compounds that exhibit anti-cancer properties.

From the work at the MRCTCM in Shanghai 4 of 8 compounds isolated from *Bretschneidera Sinensis* was structurally characterized. In correlation with the aims of the TCMCANCER project basic techniques in natural products extraction, purification and structural characterization was used for the isolation process. Although more advanced techniques and instrumentation was available at the MRCTCM, basic techniques such as the ones used in this thesis were commonly employed at the research centre. In retrospect it might seem as though more modern separation techniques such as GC or HPLC might have given a better separation and thereby the opportunity to characterize more than four compounds, especially as the fraction contained compounds of very low polarity and were hard to separate.

Although no novel compounds were isolated and characterized, three of the compounds and their derivates are biologically active, and knowledge about natural products isolation techniques has been acquired. Based on the findings, a more thorough investigation of *Bretschneidera Sinensis* would probably result in more biologically active compounds. The results add to the many other research papers that report the findings of bioactive compounds from plants. History shows that natural products are a good source of new chemical entities and TCM might provide an especially good source: The herbal remedies found in TCM have been used and tested for thousands of years and would probably not have lasted if they did not exhibit biological effect indicating the presence of active constituents.

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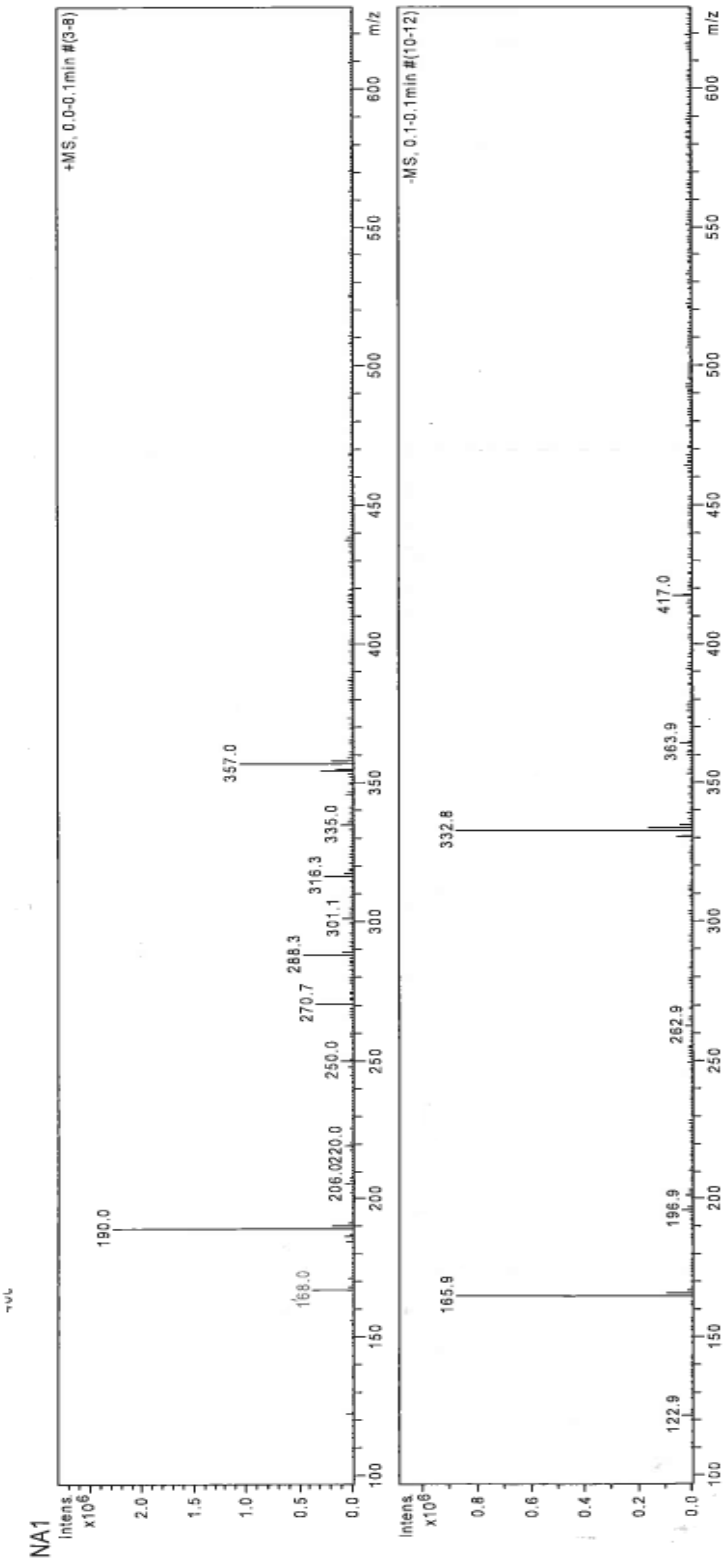
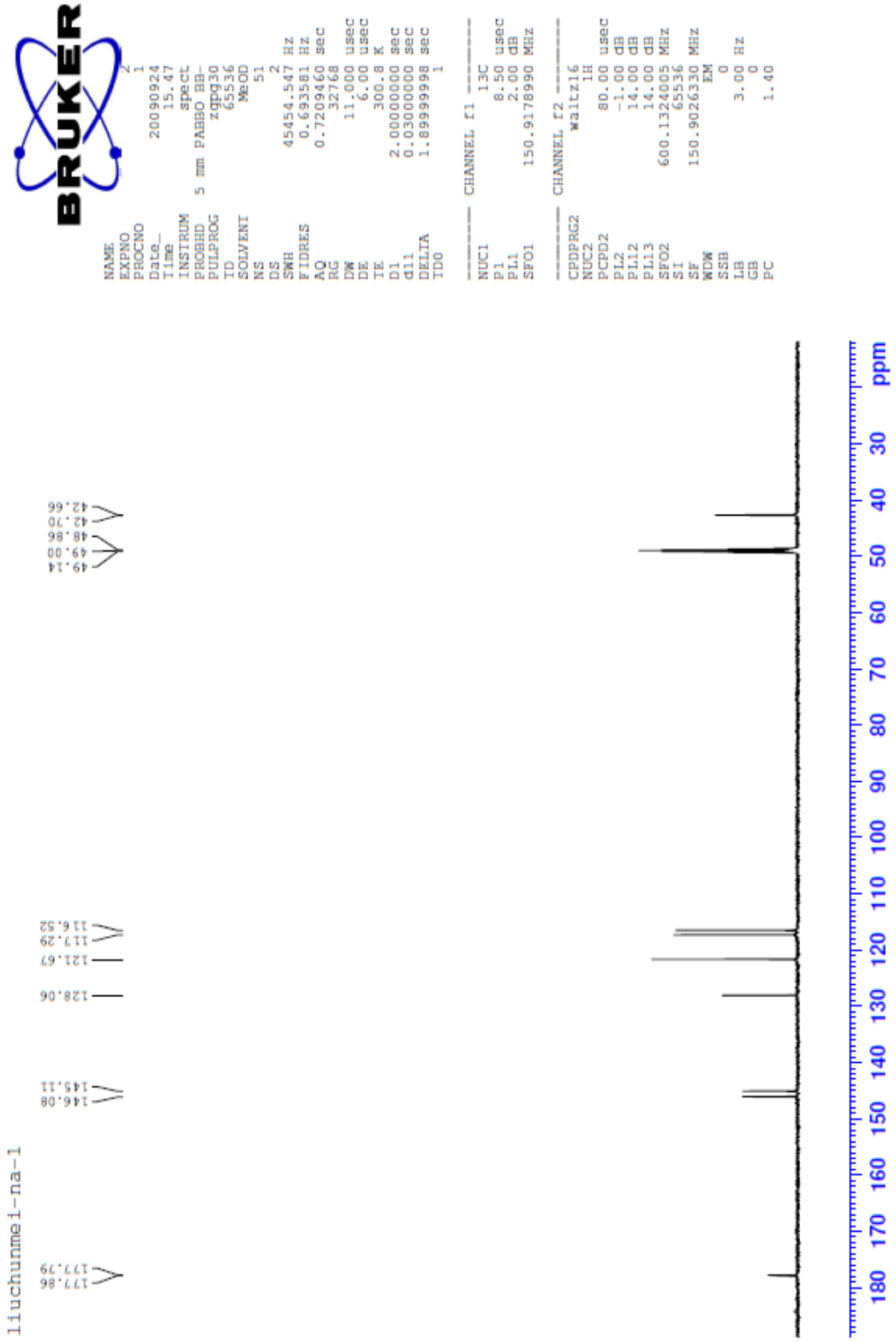
Attachment 1.1 – ESI-MS of N1



Attachment 1.2 – 13C NMR of N1



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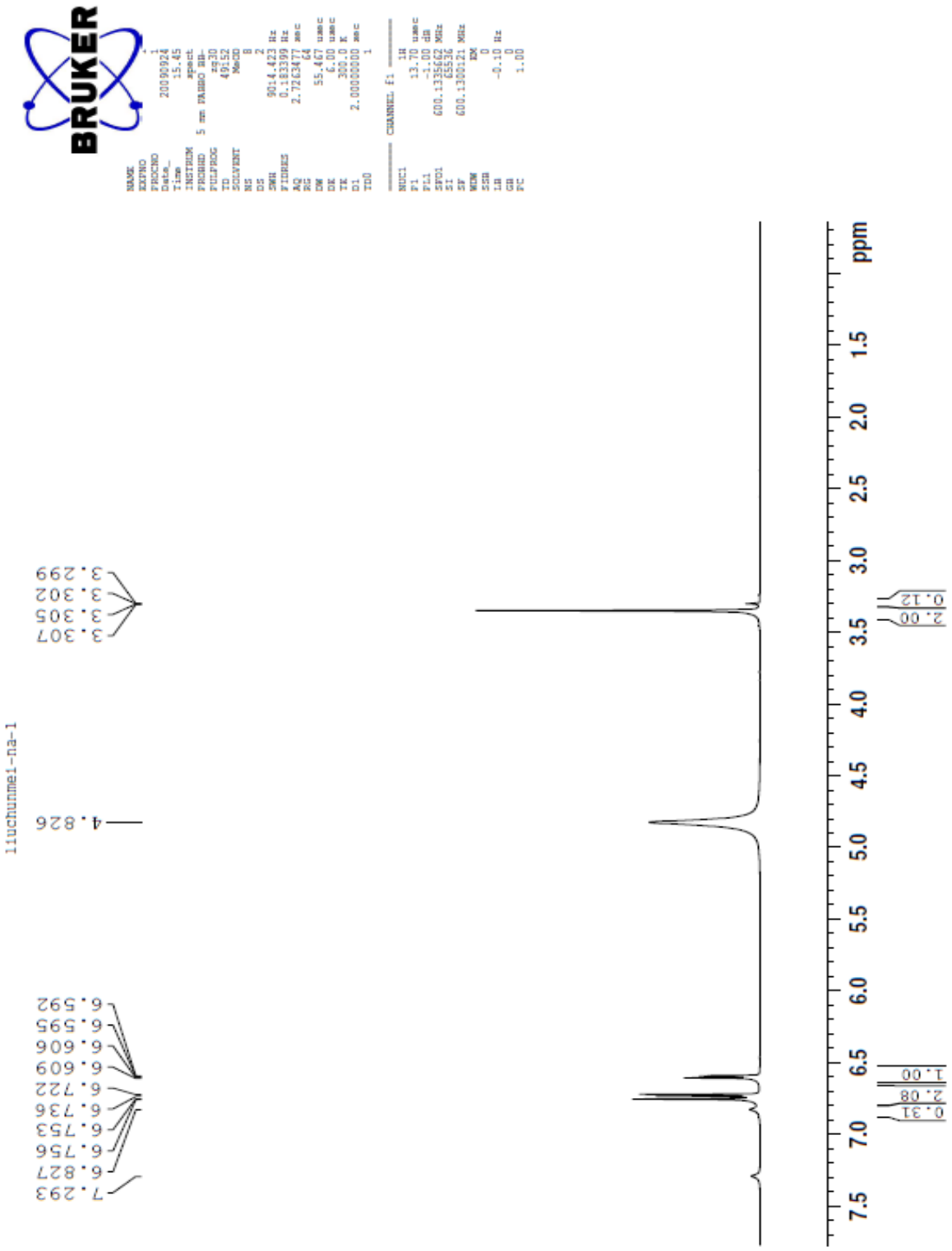
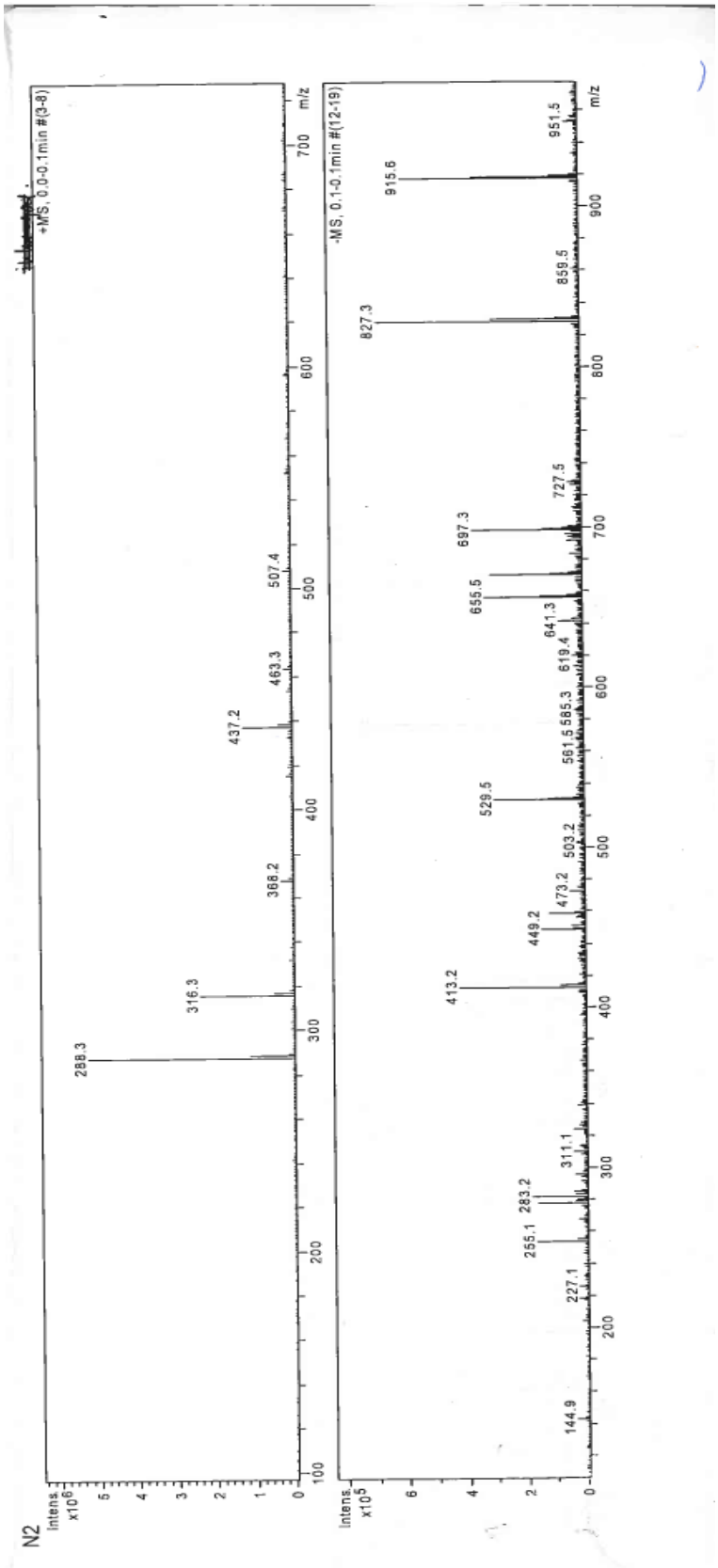
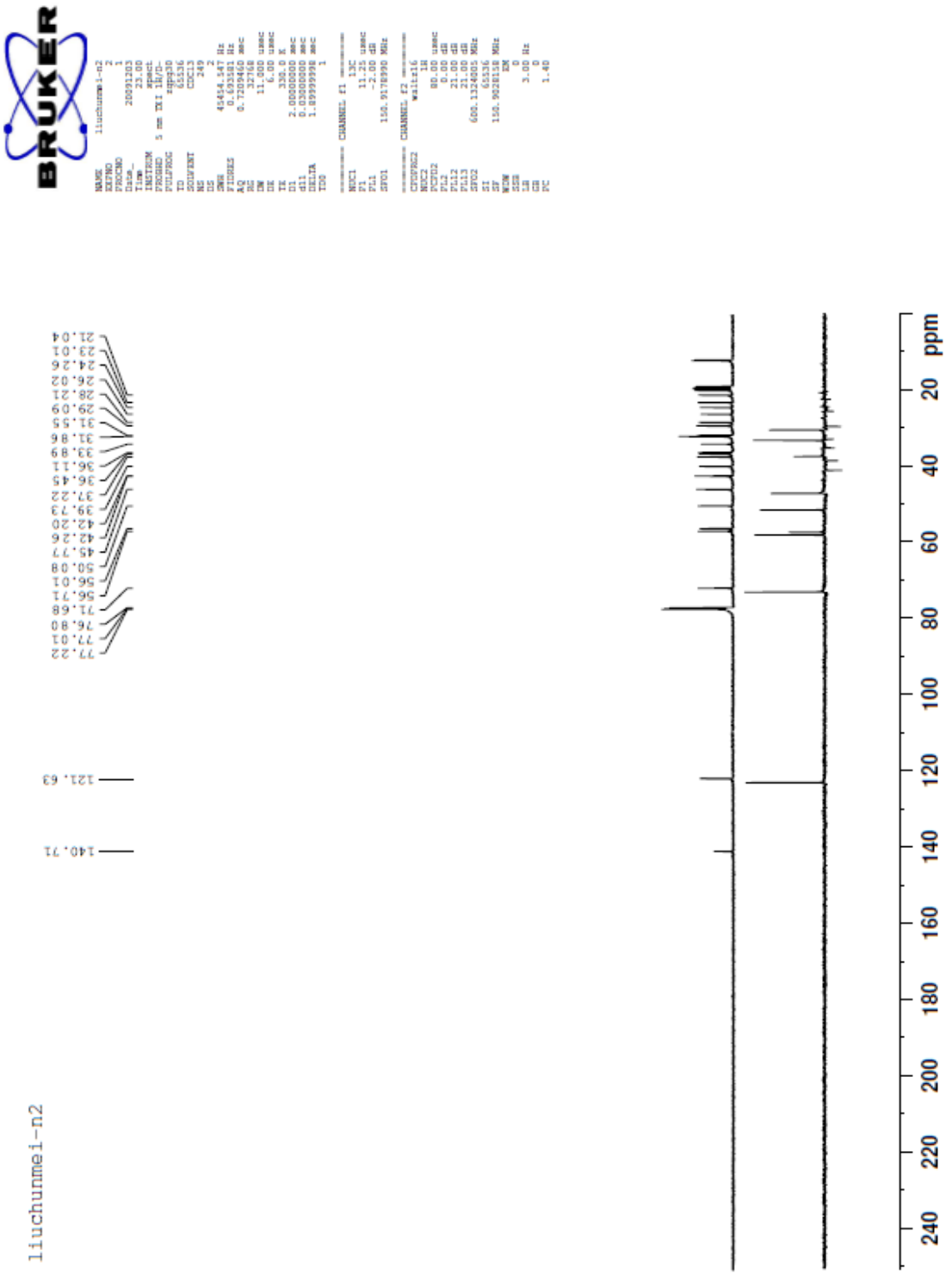


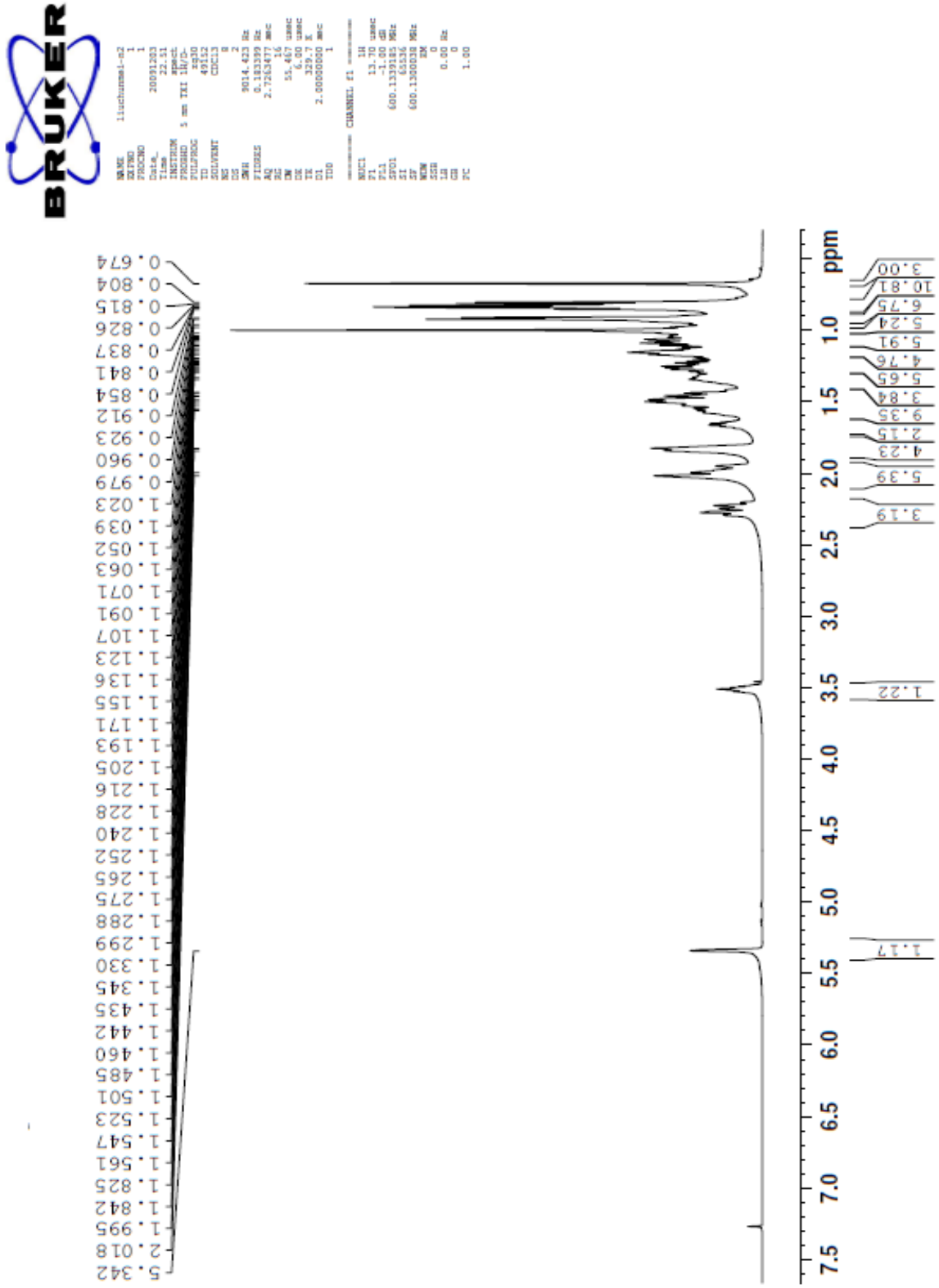
Attachment 2.1 – ESI-MS of N2



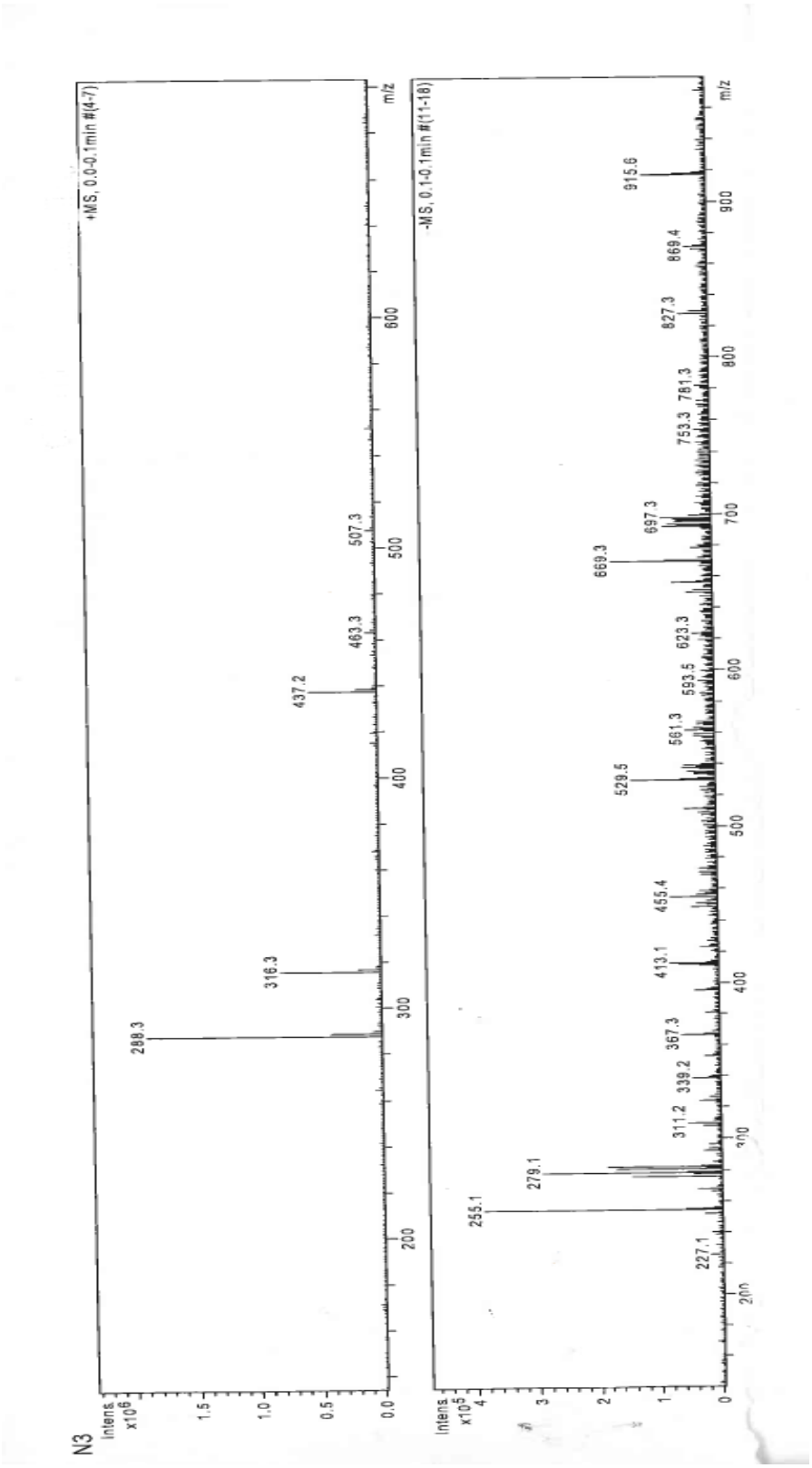
Attachment 2.2 – 13C NMR of N2



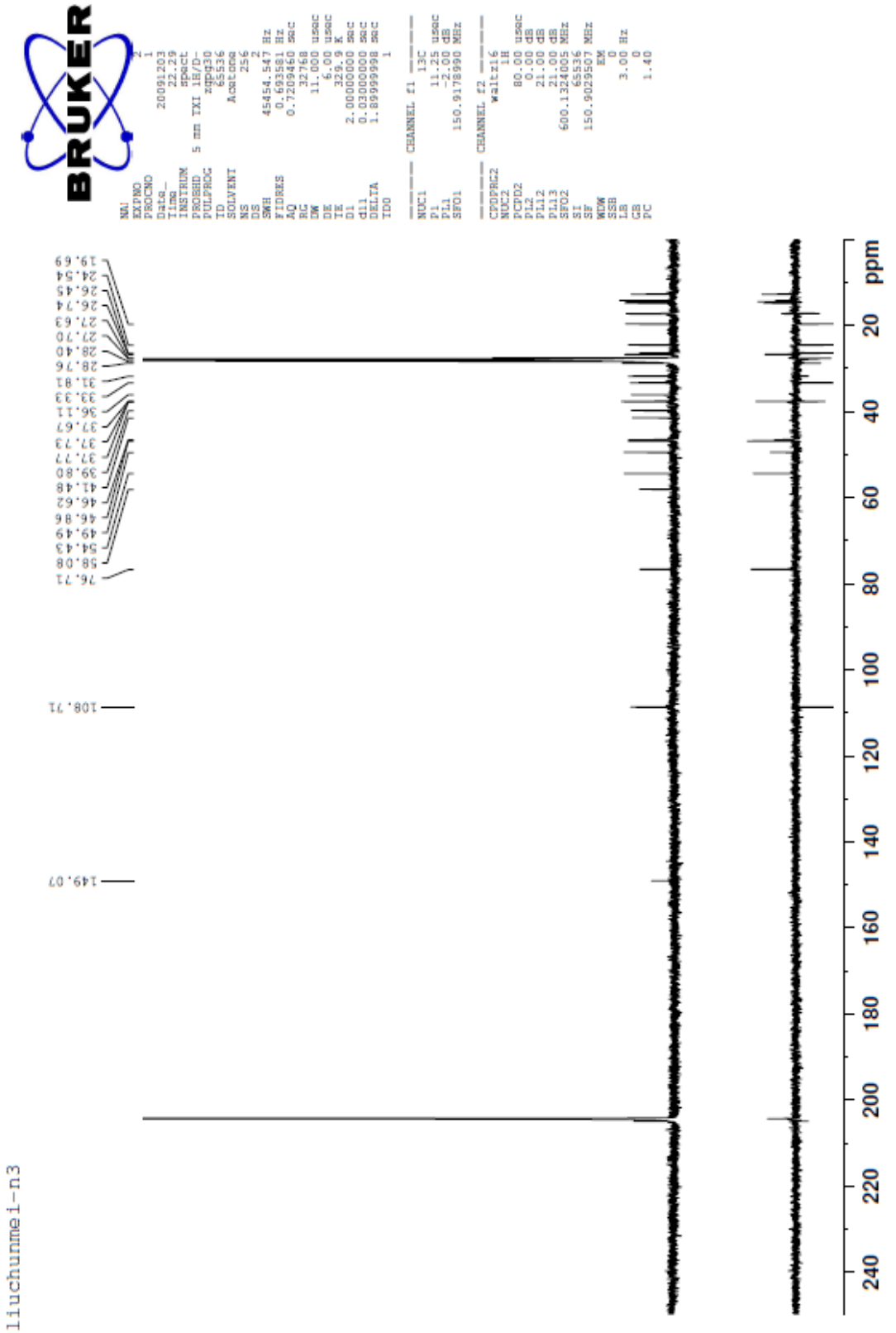
Attachment 2.3 – 1H NMR of N2



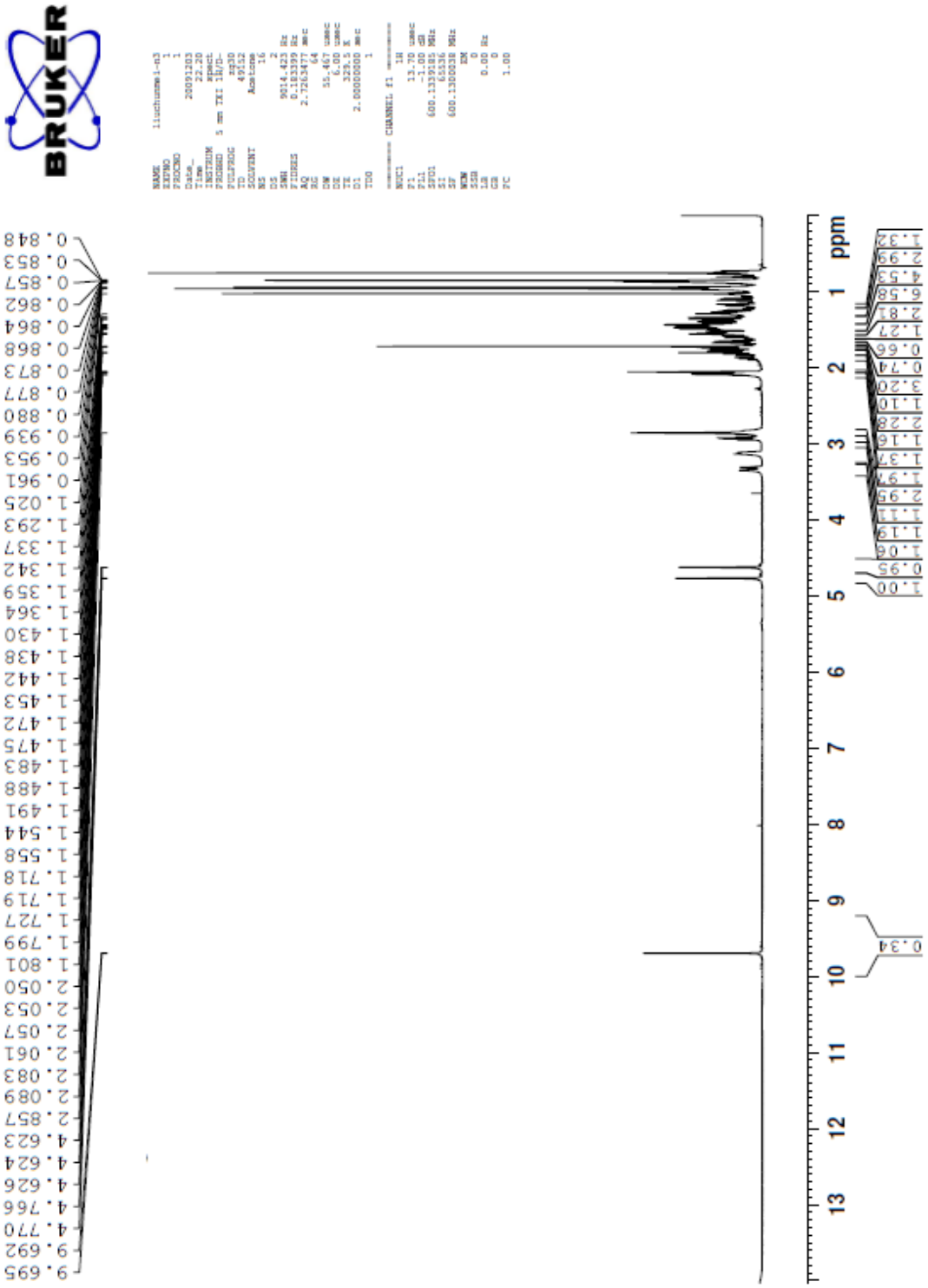
Attachment 3.1 – ESI-MS of N3



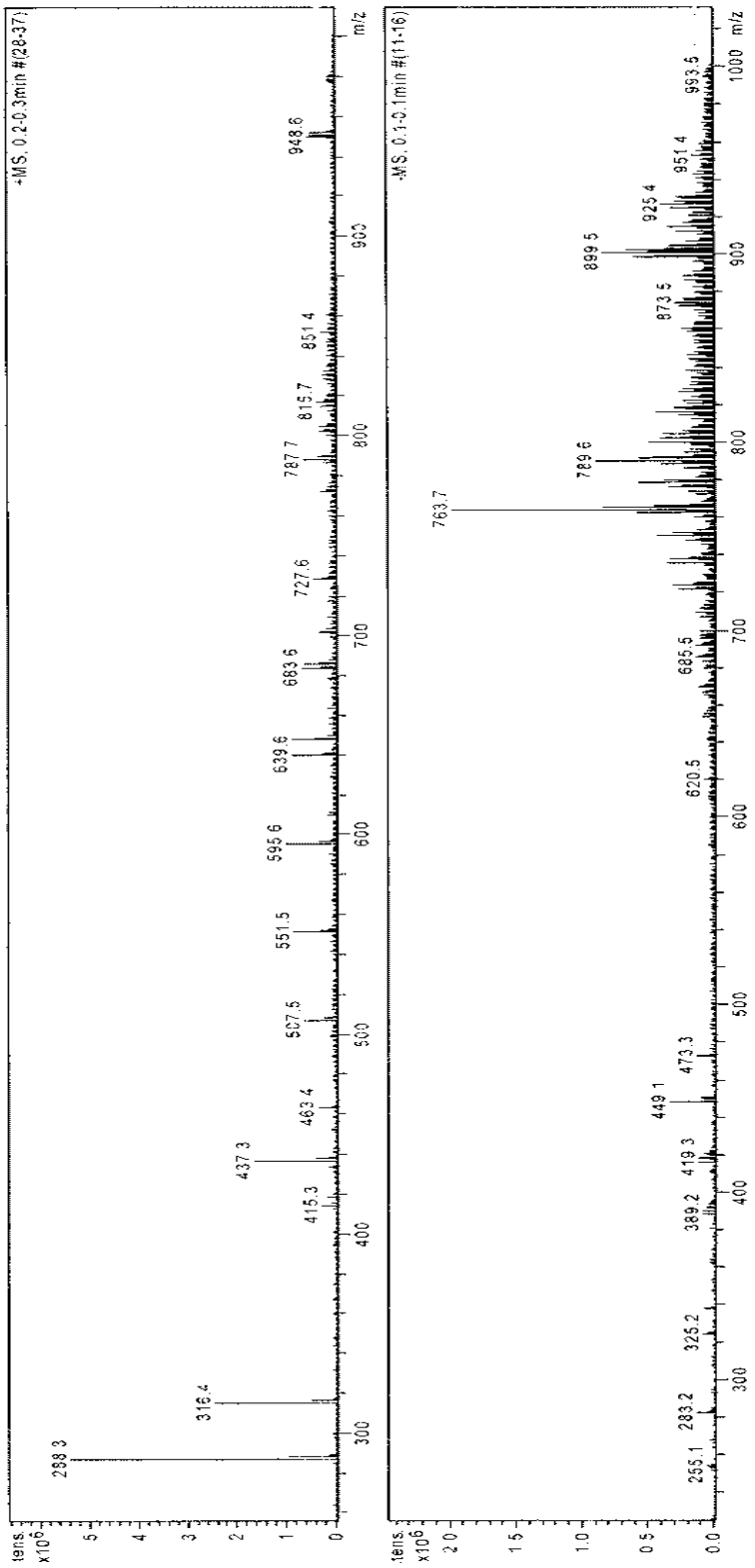
Attachment 3.2 – 13C NMR of N3



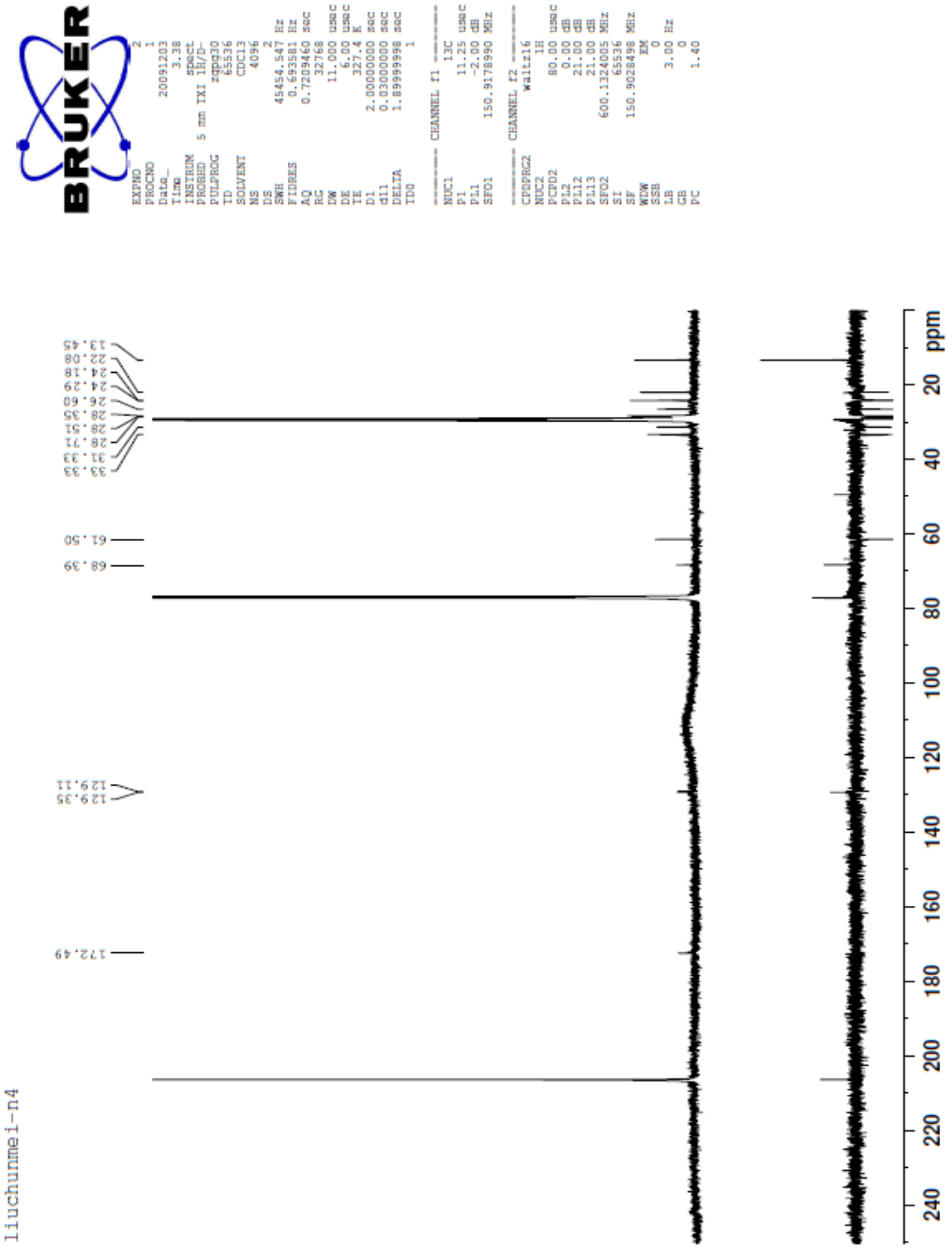
Attachment 3.3 – 1H NMR of N3



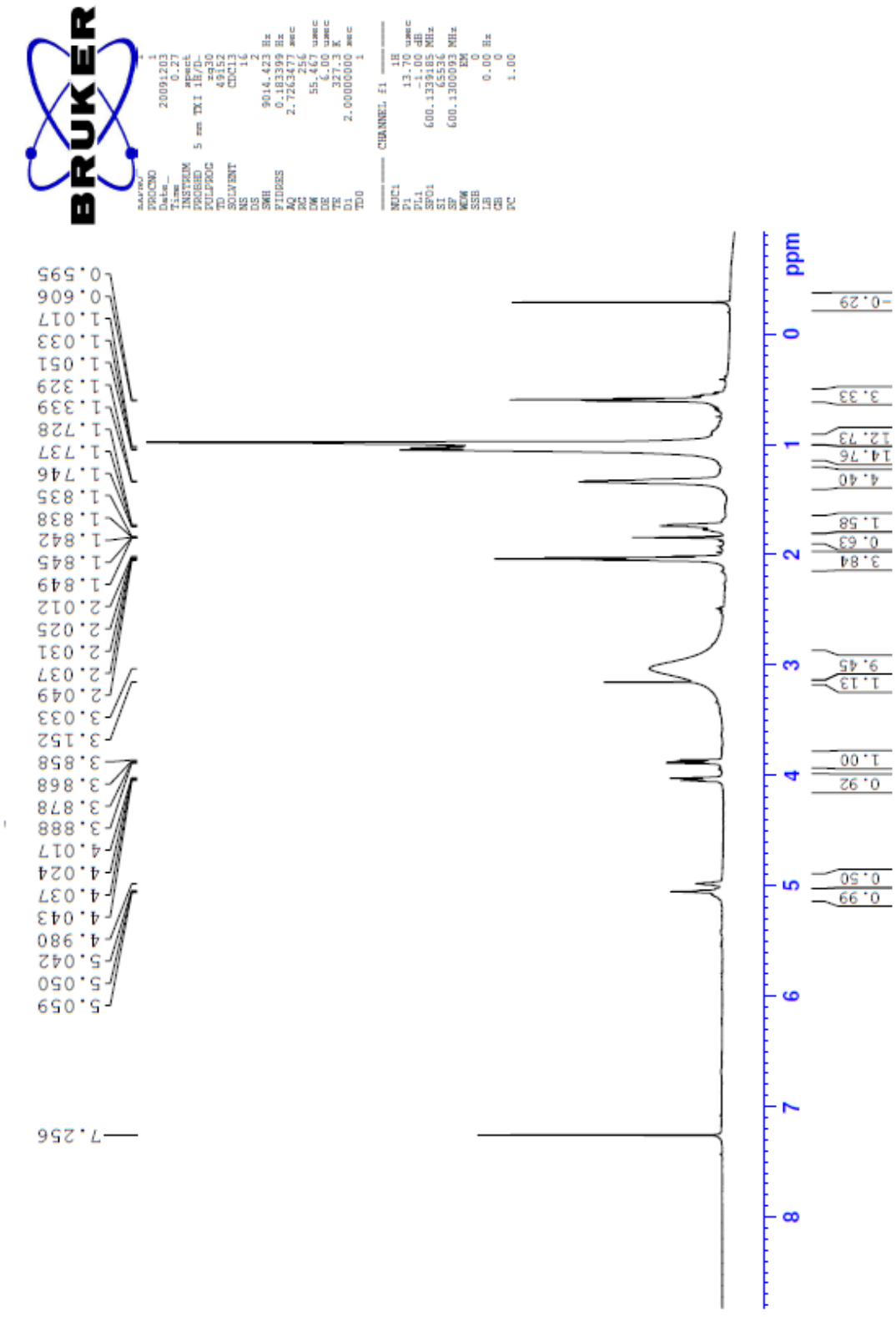
Attachment 4.1 – ESI-MS of N4



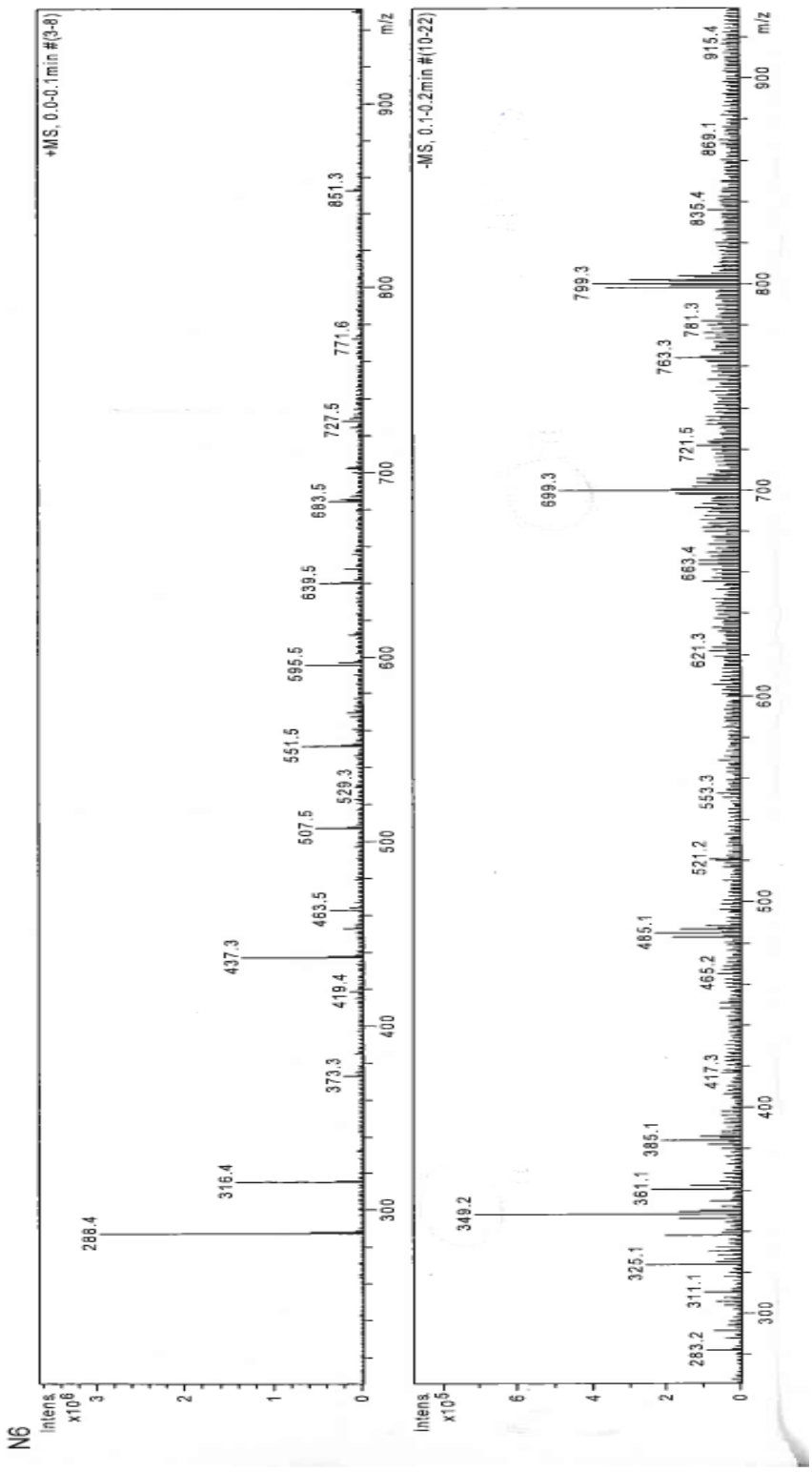
Attachment 4.2 – 13C NMR of N4



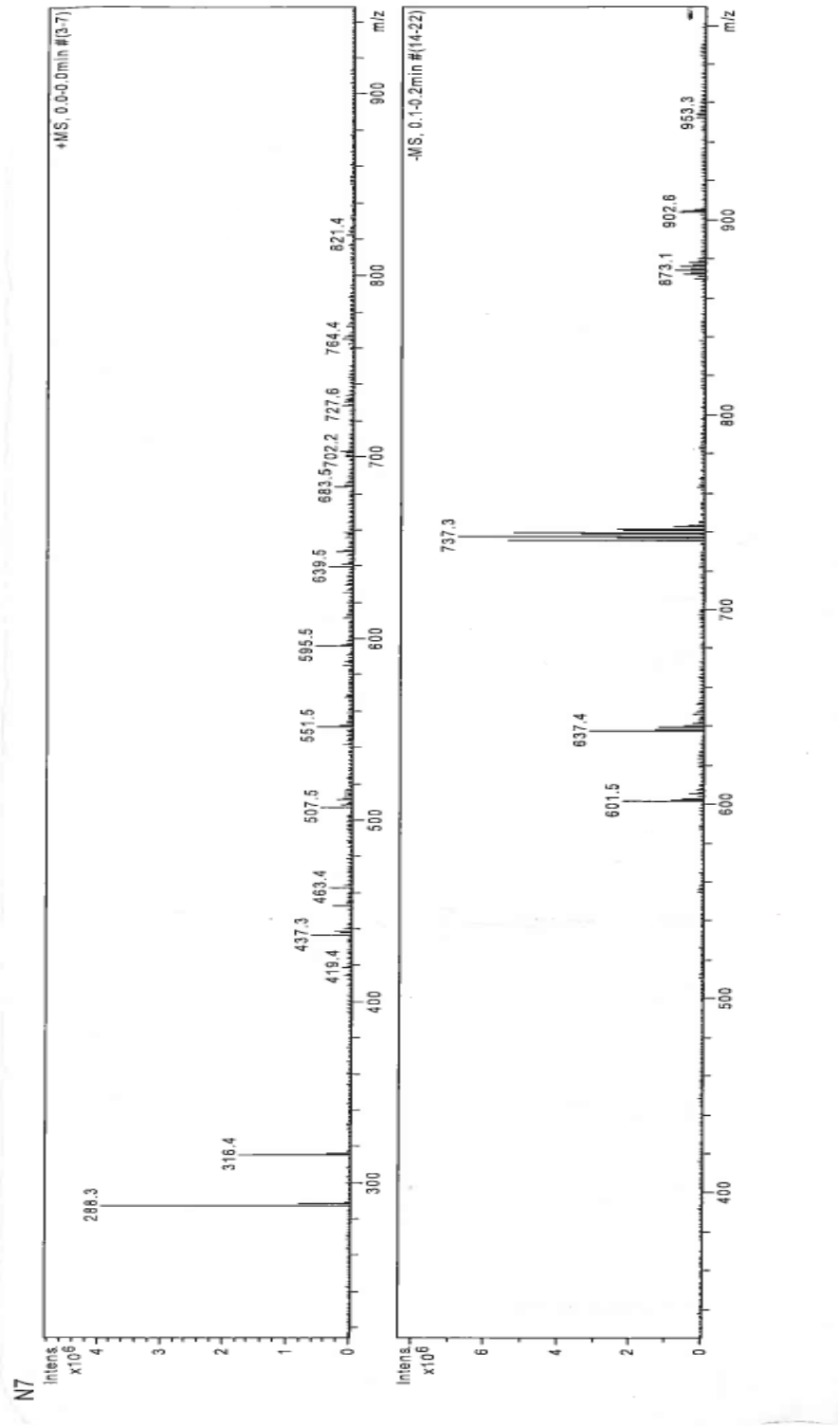
Attachment 4.3 – 1H NMR of N4



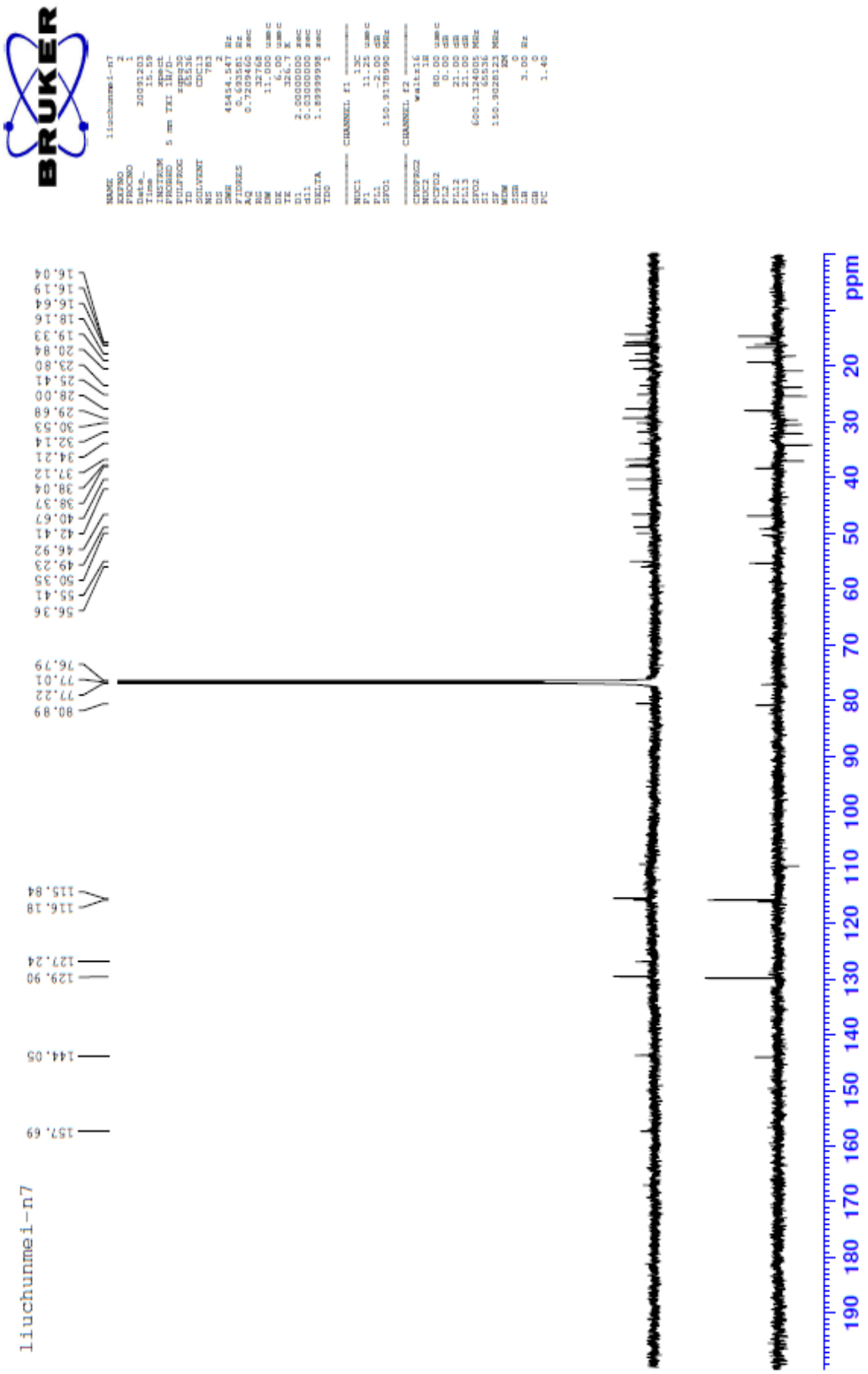
Attachment 6.1 – ESI-MS of N6



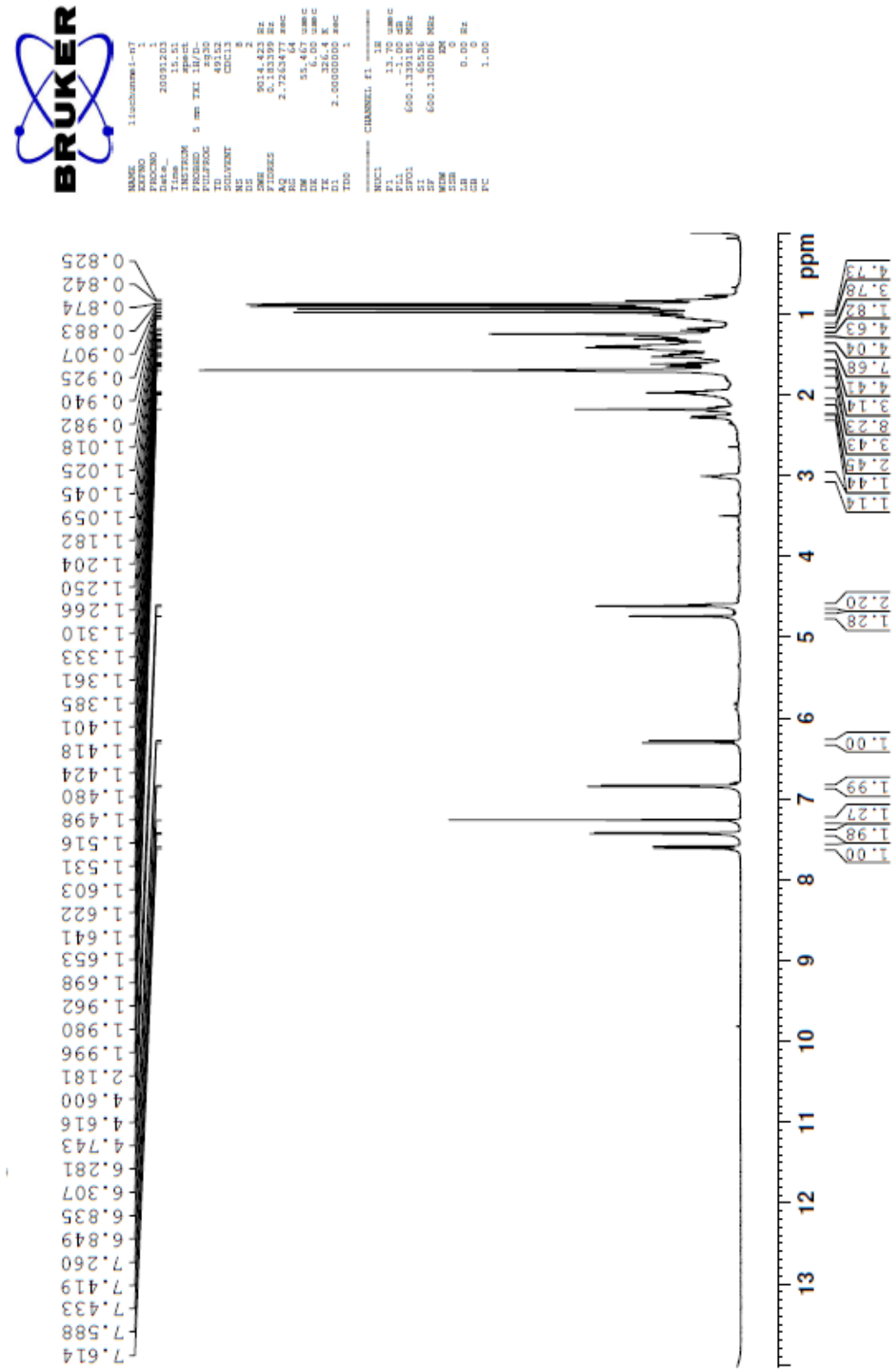
Attachment 7.1 – ESI-MS of N7



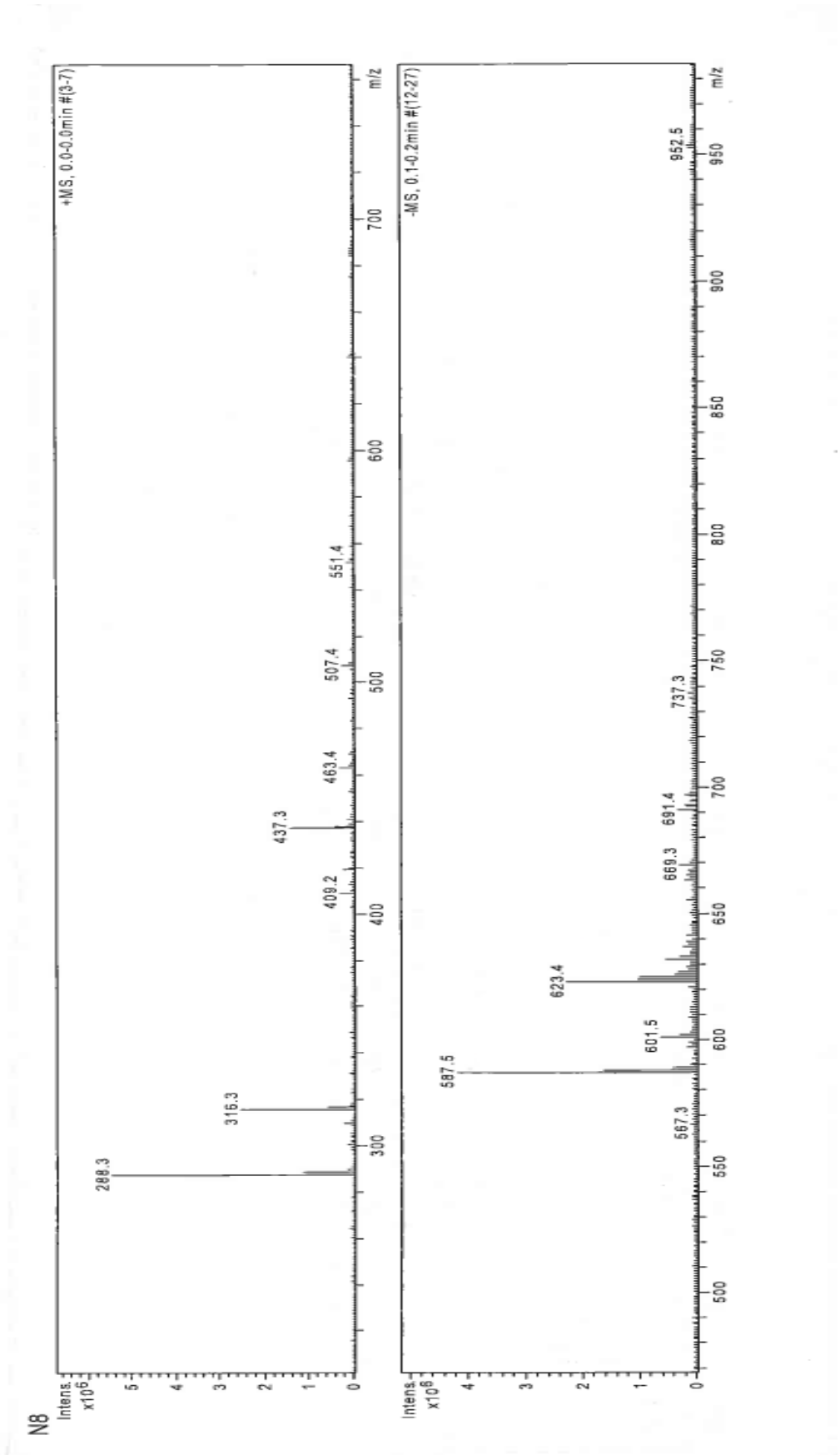
Attachment 7.2 – 13C NMR of N7



Attachment 7.3 – 1H NMR of N7



Attachment 8.1 – ESI-MS of N8



Attachment 8.2 – 1H NMR of N8