การพิสูจน์โครงสร้างของสารที่ออกฤทธิ์ทางชีวภาพจากใบพญายอ

นายเกษทัย ปัญญาคม

STRUCTURAL ELUCIDATION OF BIOACTIVE

COMPOUNDS OF *CLINACANTHUS NUTANS*

(BURM. F.) LINDAU LEAVES

Ketthai Panyakom

A Thesis Submitted in Partial Fulfillment of the Requirements for the

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OF *CLINACANTHUS NUTANS* (BURM. F.) LINDAU LEAVES

Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for a Master's Degree.

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พญายอเป็นพืชในวงศ์ Acanthaceae จัดเป็นยาพื้นบ้านของไทยที่รู้จักและใช้กันมายาวนาน มีสรรพคุณรักษาอาการอักเสบ ถอนพิษแมลงสัตว์กัดต่อย รักษาโรคเริม งูสวัด และอาการแพ้ต่างๆ เมื่อนำส่วนสกัดด้วยคลอโรฟอร์มของใบพญายอ มาแยกด้วยเทคนิคทางโครมาโทกราฟี สามารถ แยกสารบริสุทธิ์ได้ 6 ชนิด ใช้ข้อมูลทางสเปกโทรสโกปี (DEPT, COSY, NOESY, HSQC และ HMBC) พิสูจน์โครงสร้างของสารบริสุทธิ์ทั้งหมดที่แยกได้ พบสารที่มีสูตรโครงสร้างคล้ายคลึงกับ คลอโรฟิลล์ เอ และคลอโรฟิลล์ บี 5 ชนิด คือ 13^2-hydroxy-$(13^2$-$S)$-chlorophyll b, 13^2-hydroxy-$(13^2$-$R)$-chlorophyll b, 13^2-hydroxy-$(13^2$-$R)$-phaeophytin b, 13^2-hydroxy-$(13^2$-$S)$-phaeophytin a และ 13^2-hydroxy-$(13^2$-$R)$-phaeophytin a รวมทั้งพบสารในกลุ่มกลูโคไซด์คือ stigmasteryl-3-O-β-D-glucopyranoside ซึ่งสารเหล่านี้ยังไม่มีรายงานว่าพบในพืชชนิดนี้มาก่อน

สาขาวิชาเคมี   ลายมือชื่อนักศึกษา_____

ปีการศึกษา 2549   ลายมือชื่ออาจารย์ที่ปรึกษา_____

KETTHAI PANYAKOM : STRUCTURAL ELUCIDATION OF

BIOACTIVE COMPOUNDS OF *CLINACANTHUS NUTANS* (BURM. F.)

LINDAU LEAVES. THESIS ADVISOR : ASST. PROF. SANTI

SAKDARAT, Ph.D. 140 PP.

CHLOROPHYLL A AND CHLOROPHYLL B RELATED COMPOUNDS/

CLINACANTHUS NUTANS LINDAU

Clinacanthus nutans (Burm. f.) Lindau, a Thai medicinal plant of family Acanthaceae, is well-known plant used in Thai traditional medicine as an anti-inflammatory drug for the treatment of insect bites, herpes infection and allergic responses. The chloroform crude extract was purified by column chromatography and preparative thin layer chromatography to give six pure compounds. Structural elucidation of the isolated compounds was carried out on the basis of spectral analyses, including DEPT, COSY, NOESY, HSQC and HMBC. Five of these were known compounds with structures related to chlorophyll a and chlorophyll b namely 13^2-hydroxy-(13^2-*S*)-chlorophyll b, 13^2-hydroxy-(13^2-*R*)-chlorophyll b, 13^2-hydroxy-(13^2-*R*)-phaeophytin b, 13^2-hydroxy-(13^2-*S*)-phaeophytin a and 13^2-hydroxy-(13^2-*R*)-phaeophytin a. The other was stigmasteryl-3-*O*-β-D-glucopyranoside, which was not previously reported in this species.

School of Chemistry   Student's Signature_____

Academic Year 2006   Advisor's Signature_____

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Finally, I dedicate this work to my parents, my sister and the rest of my family. They provided me with every opportunity to succeed in life and the encouragement I needed to succeed.

Ketthai Panyakom

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LIST OF SCHEMES

LIST OF ABBREVIATIONS

A. D.	anno domini
br	broad
$^{\circ}$C	degree Celcius
CDCl$_3$	chloroform-d_1
COSY	correlation spectroscopy
d	doublet
dd	doublet of doublets
DEPT	distortionless enhancement by polarization transfer
dq	doublet of quartets
g	gram
HMBC	heteronuclear multiple-bond correlation
HSQC	heteronuclear single quantum coherence
Hz	Hertz
IR	infrared spectroscopy
J	coupling constant in Hertz
m	multiplet
mL	milliliter
nm	nanometer
NMR	nuclear magnetic resonance spectroscopy
NOESY	nuclear overhauser effect spectroscopy

LIST OF ABBREVIATIONS (Continued)

OPD	out patient department
ppm	parts per million
q	quartet
s	singlet
t	triplet
TLC	thin-layer chromatography
UV	ultraviolet radiation
UV-Vis	ultraviolet-visible radiation
δ	chemical shift in ppm
λ_{max}	maximum absorption wavelength
ν_{max}	maximum absorption wavenumber

CHAPTER I

INTRODUCTION

1.1   General Introduction

By definition, natural is an adjective referring to something that is present in or produced by nature and not artificial or man-made (Gad, 2005). The term natural products is applied to materials derived from plants, microorganisms, invertebrates and vertebrates, which are fine biochemical factories for the biosynthesis of both primary and secondary metabolites (Figure 1.1). Secondary metabolites play ecologically significant roles in how the living organisms deal with their surroundings and therefore are important for their ultimate survival (Bhat, Nagasampagi and Sivakumar, 2005). Secondary metabolites include products of overflow metabolism as a result of nutrient limitation, or shunt metabolites produced during idiophase, by defense mechanisms, as regulator molecules, and so on (Cannell, 1998).

Natural product research remains one of the main means of discovering bioactive compounds. Since little is known about the etiology of many human, animal and plant diseases, it is difficult to design potentially active molecules for their treatment and therefore leads from natural sources will continue to be sought (Block, 1989). Until recently, most natural product chemists have been more concerned with the isolation and structural elucidation of secondary metabolites than with their biological activity. In the past, it was left largely to the biologists to alert chemists to interesting interactions that might be mediated by metabolites, but there was little

CO_2 + Light

Calvin Cycle

CARBOHYDRATE METABOLISM

Amino acids

Glycosides

Oligosaccharides

Polysaccharides

Inositols

Antibiotics

Pyruvate

PYRIMIDINE PURINE METABOLISM

Nucleic acids

Shikkimate

Flavonoids

AMINO ACID METABOLISM

Peptides

ACETYL-CoA

Fatty acids

Acetogenins

Terpenoids

Steroids
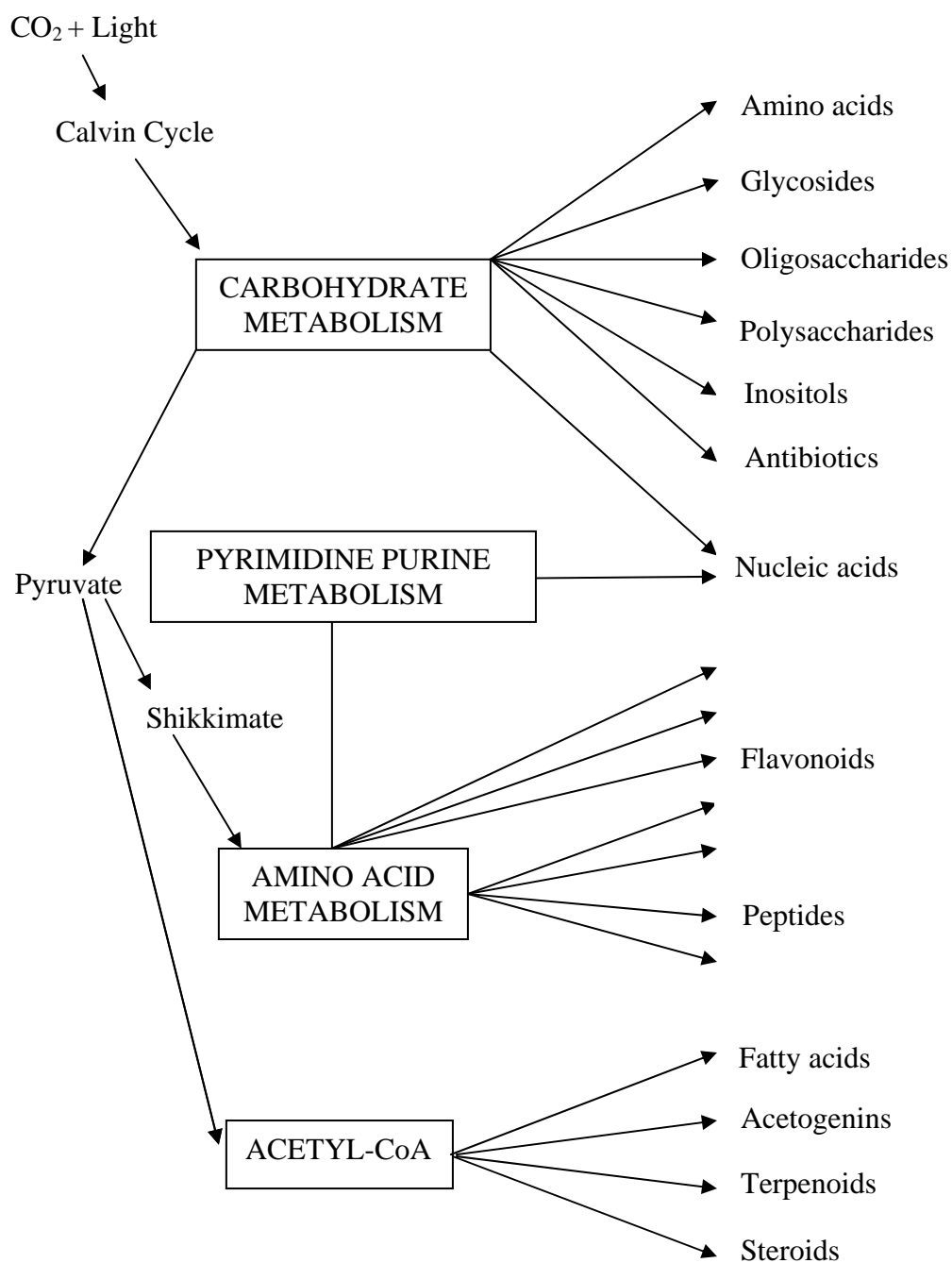
Figure 1.1 Outline of biogenesis of natural products.

Adapted from Bhat, Nagasampagi and Sivakumar (2005).

dialog. The modern trend toward multidisciplinary research is a recognition of the necessity of collaboration if more complex problems in the life sciences are to be solved. As a consequence, the traditional separation between various disciplines is becoming less distinct. Increasingly, natural product chemists are realizing that the detection, isolation, and structural determination of a metabolite are only the first steps toward answering much broader questions (Colegate and Molyneux, 1993).

1.2   Natural Products Research and Development

The World Health Organization (WHO) estimates that 80% of the world's inhabitants mainly depend on traditional medicines as sources for their health care (Farnsworth, Akerele, Bingel, Soejarto and Guo, 1985). Over 100 chemical substances that are considered to be important drugs that are either currently in use or have been widely used in one or more countries in the world have been derived from a little under 100 different plants. Approximately 75% of these substances were discovered as a direct result of chemical studies focusing on the isolation of active substances from plants used in folk medicine (Gad, 2005).

Starting from the first records, plants have been the roots of folk medicine (Cragg, Newman and Snader, 1997). Some of these plants are still in use as drugs today for the treatment of ailments ranging from coughs and colds to parasitic infections and inflammation (Newman, Cragg and Snader, 2000). Several "modern" drugs were first isolated from plants. For example, morphine (1.1) was first discovered from the opium poppy (*Papaver somniferum*) in 1816 (Cragg and Newman, 2001), quinine (1.2) was isolated in 1820 from *Cinchona officinalis* (Clark, 1996; Phillipson, 2001), aspirin (1.3) was isolated in 1829 from willow (*Salix* spp.)

(Mann, 1992; Butler, 2004) and pilocarpine (1.4) was isolated in 1876 from jaborandi

(*Pilocarpus mycrophyllus*) (Cragg, 2002).
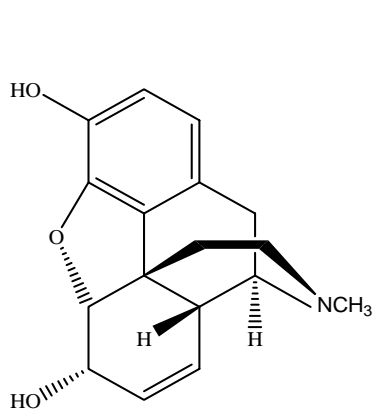
(1.1) Morphine
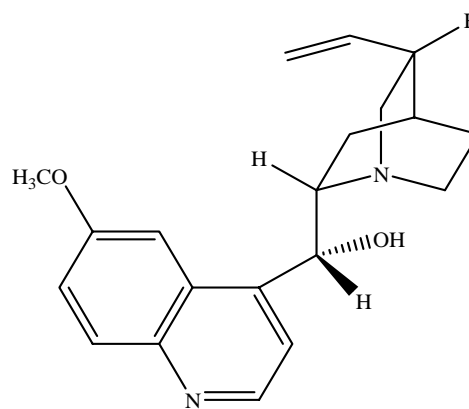
(1.2) Quinine
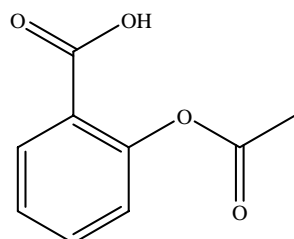
(1.3) Aspirin
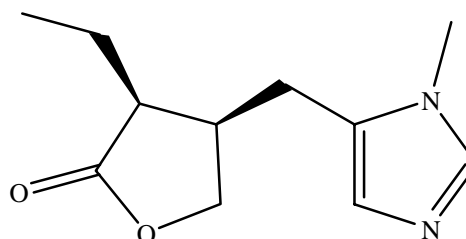
(1.4) Pilocarpine

Moreover, in the research for natural products, a common tendency is to use

screening techniques that monitor bioactivity toward a problem of current interest

(Colegate *et al*., 1993). The discovery of antibacterial filtrate (penicillin, 1.5) by

Fleming in 1928 from *Penicillium notatum* provided researchers a new method for

identifying potentially useful drugs. Between 1942 and 1944, much effort was

concentrated on discovering further antibiotics using that method. The output of this

antibiotic research was prolific and included examples such as chloramphenicol (1.6),

chlortetracycline (1.7) and erythromycin (1.8). All of these compounds are still in use as drugs today (Gad, 2005).
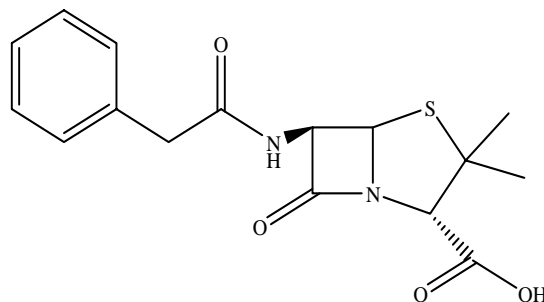
(1.5) Penicillin
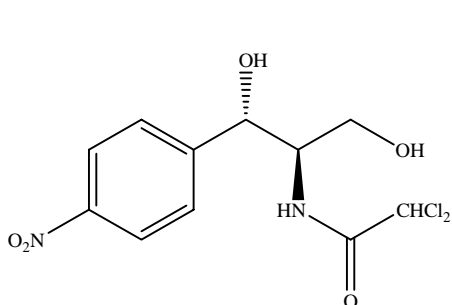
(1.6) Chloramphenicol
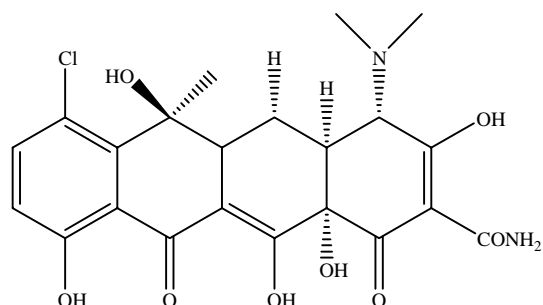
(1.7) Chlortetracycline
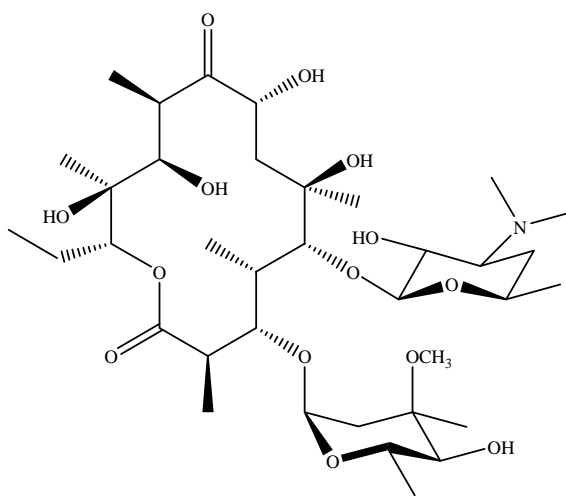
(1.8) Erythromycin

1.3   Natural Products in Pharmaceuticals

As the former examples show, natural products are important pharmaceuticals. In a study of the pharmaceuticals on the market from 1981-2002, only 43% of the drugs were totally synthetic, while the remaining 57% were obtained from natural sources. The data shown in Figure 1.2 categorizes natural sources in the following way: biological – usually a large peptide or protein either isolated from an organism or cell line or produced by biotechnological means in a surrogate host; natural product; natural product derivative – derived from a natural product and is usually a semi-synthetic modification; synthetic drug – totally synthetic drug, often found by random screening and modification of an existing agent; synthetic drug with a natural product pharmacophore – made by total synthesis, but the pharmacophore is from natural product and vaccine (Newman, Cragg and Snader, 2003).
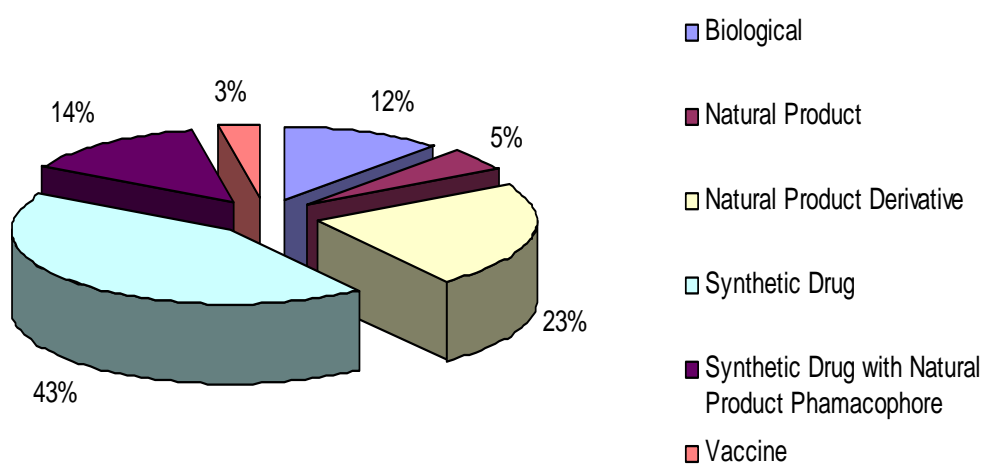
Figure 1.2 Portions of pharmaceuticals containing natural products (1981-2002).

Adapted from (Newman, Cragg and Snader, 2003).

1.4   Importance of Standardization and Quality Control

Despite the availability of a number of modern efficacious drugs, herbal medicines are still widely used and their importance is increasing because of the presence of certain incurable diseases such as AIDS and some chronic diseases. It is well known that any agent intended to be used as a drug should be uniform in quality regarding origin, the content of therapeutically active constituents and cleanliness. Many herbal medicines being sold in the market are fake products. Some medicinal plant materials are sold under the same local name but derived from different origins; hence, their curative effects are different and some may even lead to toxicity. The natural variations of active constituents in herbal materials are also vital to the efficacy of herbal medicines. Besides, these drugs are often contaminated with microorganisms, which may cause various infections to consumers. To overcome the risk of the possible health hazards mentioned above, standard specifications and quality control procedures for these medicines are required by manufacturers as well as by regulatory authorities to improve the image of herbal medicinal products (Dechatiwongse na Ayudhaya, 1997).

There are several important factors affecting the efficacy and safety of medicinal plant materials, namely: (Dechatiwongse na Ayudhaya, 1997 and Chavalittumrong, 2000).

1.4.1 Plant Name There are some difficulties with the plant names because some plants have the same local name but belong to different botanical origins or vice versa. If wrong medicinal plant materials are used, they will possess not only undesirable activities, but also unexpected toxicities.

1.4.2 Part Used The right part of a plant should be used. Different parts generally contain different substances.

1.4.3 Age The proper harvesting time for medicinal plants should be known to obtain high content of therapeutic principles.

1.4.4 Preparation and Storage Suitable processes of cleaning, cutting, drying and preservation should be followed to prevent the contamination or deterioration of crude plant materials.

1.4.5 Quality Though we know the correct plant name, the right part used, the proper harvesting time, suitable process of preparation and storage, we still cannot assume that raw materials of high quality can be obtained. Chemical constituents of plants are usually affected by external factors; *e.g.* climate, the physical features of the land, irrigation, fertilizer, *etc.*

Hence, it is essential to set up scientific criteria for standardization and quality control of herbal medicines to ensure that the consumers receive drugs of high quality.

1.5 General Control Methods

The standard control of herbal remedies is performed by two aspects (Dechatiwongse na Ayudhaya, 1997 and Chavalittumrong, 2000, 2001 and 2002).

1.5.1 Establishment of Standard Specifications of Herbal Raw Materials

According to World Health Organization (WHO) documents and other useful information, the specifications for medicinal plant materials should include the following:

(a) Name (Scientific Name, English Name, Local Name)

(b) Synonyms

(c)   Definition

(d)   Constituents

(e)   Plant Description

(f)   Description of Plant Material

  - General Appearance

  - Organoleptic Properties

  - Microscopic Characteristics

(g)   Packaging and Storage

(h)   Identification

  - Chemical Reaction

  - Thin-Layer Chromatographic Analysis

(i)   Purity Tests

  - Microbiological

  - Foreign Matter

  - Total Ash

  - Acid-Insoluble Ash

  - Water-Soluble Extract

  - Alcohol-Soluble Extract

  - Moisture

  - Pesticide Residues

  - Arsenic and Heavy Metals

  - Radioactive Residues

(j)   Determination of Major/Active Chemical Constituents

(k)   Medical Uses and Doses

1.5.2 Development of Good Manufacturing Practice (GMP) Guidelines for the Manufacture of Herbal Medicinal Products

The purpose of these guidelines is to outline steps which should be taken, as necessary and appropriate, by manufacturers of herbal medicinal products with the objective of ensuring that their products are of the nature and quality intended. Successful and concurrent implementation of these GMP guidelines will not only improve the image of the herbal medicinal products industry, but also promote international recognition and acceptance and consequently, escalate the export potential of these products. The Good Manufacturing Practices for Pharmaceutical Products, Supplementary guidelines for the manufacture of herbal medicinal products; WHO/Pharm/93.561 (Lou, 1993) should be followed to develop the above mentioned guidelines.

1.6 Quality Assurance and Regulatory Control of Herbal Medicines in Thailand

Medicinal plants have been used in Thailand for a long period of time. Documentarily, the first record appeared during the Sukhothai period (14th century A. D.) and the use of traditional drug formulas began during the Ayudhya period. Traditional drugs have been popular in the Kingdom throughout the Ayudhya and Rattanokosin periods. King Rama III (about 200 years ago) ordered the collection of traditional drug formulas including the diagnosis of diseases, traditional massage, literature and poetry, and their subsequent inscription on stone plates installed on the walls of two temples (Wat Po and Wat Raja Orasa Ram). Again during the years 1895-1900 King Rama V ordered the collection of all knowledge of traditional

medicines from the noble sand doctors, and the printing and distribution for the first time in the two volumes of the official pharmacopoeia called "Tamrapaettayasartsongkroh". Since the use of herbal medicines is quite often derived from empirical experience, the standardization and quality control of these preparations are usually lacking, which hampers an increased utilization of such medicines. Thai pharmacists and scientists have therefore realized that this problem should be solved to upgrade the quality of herbal raw materials and their finished products (Dechatiwongse na Ayudhaya, 1997).

Generally, the term "herbal medicines" applies to medicinal products whose active ingredients consist exclusively of plant materials or vegetable drug preparations. This notion of herbal remedies has broad international recognition since WHO has defined the term in the same way.

The chemistry of medicinal herbs is very complex. Not all the constituents present in the plant have therapeutic activity, some are poisonous *e.g.* pyrrolizidine and tropane alkaloids. Phytochemistry deals with the study of the chemical composition of plant material. Plants are used in various forms varying from powders to extracts. Powder represents the drug in ground form and these types of preparations are considered to be crude. The Pharmacopoeia mentions standardized vegetable powders for therapeutic application (Sawasdimongkol, 1995).

Herbal systems of medicine have become increasingly popular in recent years. A recent study from the US demonstrated that about 34% of the general population used one or the other system at least once a year. In India 76% of patients visiting the general medicine OPD of a tertiary care hospital use alternative therapies. In light of growing demand for herbal drugs, the quality control and assurance is of primary

importance. The standardized herbal extracts are considered to be more scientific than crude drugs. The commonly employed technique for removal of active substances from the crude drug is called extraction. Selection of the solvent is very critical in preparing extracts, because the active constituents of the plants have affinity for solvents.

Extracts are prepared by separating the soluble matter from vegetable tissues by application of a suitable solvent like alcohol, water or ether. The resultant liquid is concentrated by evaporation to obtain liquid extract or concentrated nearly to dryness to obtain solid extract. Depending on the solvent used, the extracts are classified as alcoholic, ethereal or aqueous (the solid and liquid extract classification is based on method of preparation, the alcoholic, aqueous and ethereal extract classification is based on type of solvent used) (Pothisiri, 1996).

The standardized herbal extract is a preparation, which contains a certain fixed proportion of the active constituent. For example, a standardized extract of *Papaver somniferum* contains not less than 9.5% of morphine. The concept of standardization has great impact on the quality of herbal products. Standardization helps in adjusting the herbal drug formulation to a defined content of a constituent or constituents with therapeutic activity. The latest method of preparing herbal extracts is by successive macerating of the powdered drug in order of increasing polarity. This process is known as successive solvent extraction and is carried out in a special assembly known as a soxhlet apparatus. The biological source of the drug has great impact on the finished product in herbal drug preparation. Proper identification of the drug is significant for phytochemical screening, which further exerts importance on therapeutic activity of the medicinal herb. Thus, presence of an identification standard

is a must in the finished product of herbal drug preparation. A constituent of a medicinal herb, which is used for quality control and assurance of a herbal product, is known as a marker compound. A marker compound may or may not have therapeutic activity (Sawasdimongkol, 1995; Malhotra and Pal Singh, 2002).

Nowadays, we talk about active constituents of drugs. An active constituent is truly responsible for therapeutic activity of a medicinal plant. The extracts are further subjected to chemical tests for identification of the plant constituents. The isolated constituents are of further importance to the pharmaceutical industry for applied research.

1.7   Research Objectives

1.7.1   To extract, purify and isolate chemical constituents from the leaves of *Clinacanthus nutans* (Burm. f.) Lindau.

1.7.2   To identify chemical constituents from the leaves of *Clinacanthus nutans* (Burm. f.) Lindau.

CHAPTER II

HISTORICAL

2.1 *Clinacanthus nutans* (Burm. f.) Lindau

Clinacanthus nutans (Burm. f.) Lindau belongs to the family of Acanthaceae. This family includes many species, which are known to have medical properties. In Thailand, many Thai medicinal plants have been collected, including medicinal plants of the family *Acanthaceae* which are *Andrographis paniculata* (Burm. f.) Nees, *Acanthus ebrateatus* Vahl, *Barleria lupulina* Lidl, *Clinacanthus nutans* (Burm. f.) Lindau, *Justicia betonica* Linn, *Rhinacanthus nasutus* Linn, *Ruellia tuberosa* Linn and *Thunbergia laurifolia* Linn (Hancharnlerd, Babprasert and Phisuksanthiwattana, 1994) (Table 2.1).

Table 2.1 Medicinal Plants of the Family Acanthaceae and Their Traditional Use in Thailand.

Plant Species	Characteristic	Seasonal Blooming	Traditional Use
1. *Acanthusebra teatus* Vahl	Shrub	Rainy season	All stem and seeds for healing of wounds and worm protection. Fresh leaves for healthy hair

Table 2.1 (Continued).

Plant Species	Characteristic	Seasonal Blooming	Traditional Use
2. *Andrographis paniculata* (Burm. f.) Nees	Perennial shrub	All year	Leaves and stems for healing sore throat and diarrhea
3. *Barleria lupulina* Lindl.	Shrub	Rainy season	Blended leaves for healing inflammation after insect bites
4. *Clinacanthus nutans* (Burm. f.) Lindau	Shrub	In January	Fresh leaves for healing burns and scalds
5. *Justicia betonica* Linn.	Shrub	All year	No information
6. *Rhinacanthus nasutus* Linn.	Small Shrub	In January	Leaves and roots for healing skin diseases Roots are antiseptic
7. *Ruellia tuberosa* Linn.	Perennial shrub (long life)	Rainy season	Roots for healing urinary tract inflammation and reducing toxicity
8. *Thunbergia laurifolia* Linn.	Shrub	Rainy season	Leaves for healing stomachache

In Thailand, herpes simplex virus (HSV) infection is still considered one of our national health problems. The disease is highly infectious and the prevalence of the antibodies to herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2) in the normal populace was shown to be as high as 60% and 55%, respectively. Acyclovir, the antiviral drug of choice for the treatment of HSV infection, is quite expensive and the consumption of this medicine in our country is increasing at approximately 20% annually (Jayavasu, 1998).

C. nutans has been traditionally used in Thailand for the treatment of herpes infections. Laboratory investigations in Thailand have indicated that the extract of this herb exhibits anti-viral properties against the herpes simplex virus (HSV) (Thawaranantha, Balachandra, Jongtrakulsiri, Chavalittumrong, Bhumiswasdi and Jayavasu, 1992) and varicella zoster virus (VZV) (Jayavasu, Chavaltittumrong, Polachandara, Dechatiwongse na Ayudhaya and Jongtrakulsiri, 1992). *C. nutans* cream was later clinically shown to be as efficacious as acyclovir in relieving pain and healing herpes simplex and herpes zoster without causing a burning sensation, the side effect experienced by some patients using acyclovir (Sangkitpporn, Polchanandara, Thawatsupa, Bunchob and Chavaltittumrong, 1993; Jayavasu, 1998).

Typical *C. nutans* products are currently being used in many Thai hospitals to replace topical acyclovir in treating herpes simplex and herpes zoster (Sangkitpporn, Polchanandara, Balachandra, Dechatiwongse na Ayudhaya, Bunchob and Jayavasu, 1995). In addition, this herb exhibits an excellent and rapid acting, anti-inflammatory property (Chuakul, 1986; Tanasomwong, 1986), making a *C. nutans* topical cream or lotion a good natural product for the relief of minor skin inflammation and insect bites (Satayavivad, Bunyapraphatsara, Kittisiripornkul and Tanasomwong, 1996).

C. nutans has been considered as one of five medicinal plants in the National List of Essential Drugs A. D. 1999. It has been promoted for treatment of herpes simplex, herpes zoster and skin pruritis in the Primary Health Care Programme (Ministry of Public Health, 1999).

Because of its interesting biological activities and the fact that very little is known in the literature, it was desirable to phytochemically investigate this plant in details.

2.2 Specification of *Clinacanthus nutans*

Botanical Name: *Clinacanthus nutans* (Burm. f.) Lindau.

Family: Acanthaceae.

Common Name: Slaed Pang Pon (Tua mia), Phayaa Yo, Phak Man Kai, Phak Lin Khiat (Chiang Mai), Phayaa Plong Thong, Phayaa Plong Kham (Lampang), Pho-so-chaang (Karen-Mae Hong Sorn) (เต็ม สมิตินันทน์, 2544).

Botanical Description: This plant is a shrub 1-3 m high with pubescent branches. Leaves are simple, opposite, narrowly elliptic oblong or lanceolate, 2.5-13 cm long and 0.5-1.5 cm wide. The leaves have apex acute or acuminate; margin exsculptate-dentate or subentire; base cuneate, obtuse, rounded or truncate often oblique; pubescence on the nerves; petiole 3-15 mm long. Flowers are in dense cymes at the top of the branches and their branchlets; cymes 5-α flowered, often terminating drooping horizontal branches but themselves erect, subsecund and combined into a large lax, leafy panicle. Each flower has calyx densely patently glandular-pubescent, about 1 cm long; corolla glandular-pubescent, about 3.5 cm, dull red with green base; lower lip (turned upwards) with yellow streaks, apically sordidly yellow or greenish

yellow; stamens 2, inserted in the throat, more or less appressed against the upper lip. Ovary is compressed, 2-celled, 2 ovules in each cell; having style filiform, shortly bidentate. Capsule is oblong, basally contracted into a short, solid stalk 4-seeded (Backer and Bakhuizen, 1965; Perry, 1986). Its morphological features are illustrated in Figure 2.1

Figure 2.1 Morphological illustration of *Clinacanthus nutans* (Burm. f.) Lindau.

Ecology and Distribution: cultivated and found in deciduous forests.

Propagation: normally propagated by cuttings.

Loss on Drying: not more than 14%.

Total Ash: not more than 21%.

Acid-Insoluble Ash: not more than 4%.

Alcohol-Soluble Extract: not less than 27%.

Water-Soluble Extract: not less than 26%.

Ether-Soluble Extract: not less than 1%.

Ethnomedical Use: for the treatment of burn, herpes zoster and insect bite; antipyretic (Kharnngan, 1991).

2.3   Pharmacological Study

C. nutans has several pharmacological studies as exemplified below:

2.3.1   Activity Against Snake Venom

A water extract of leaves reduced mortality rate in snake venom treated mice from 100% to 63 ± 3.34%, while a 95% alcohol extract (2.0 g/kg) was ineffective when injected intravenously, intraperitoneally or given orally (Thongharb and Tejasen, 1977). A water extract was also effective in counteracting snake venom when tested on rat phrenic-nerve diaphragm preparations (Cherdchu, Poopyruchpong, Adchariya, Patanaba Nangkost and Ratanabangkoon, 1977).

2.3.2   Antiinflammatory Activity

Leaf extracts, especially a butanol extract, reduced inflammation induced by carrageenin injection in rats. The active fraction was reported to be unstable. One of the active ingredients in *C. nutans* was identified as a flavonoid compound (Satayavivad *et al.,* 1996).

2.3.3   Activity Against Herpes Simplex Virus (HSV)

In 1992 Jayavasu and Thawaranantha groups reported the ability of the ethanol extract of *C. nutans* leaves to inactivate HSV-2 compared with that of acyclovir. The activity was measured *in vitro* using a plaque reduction assay of HSV-2 in baby hamster kidney cell line. Based on 50 percent inhibited dose, the results showed the ethanol extract of *C. nutans* leaves inhibited plaque formation by HSV-2.

Jayavasu's group further reported the clinical trial in the treatment of genital herpes patients with *C. nutans* extract, it showed that patients who used *C. nutans* as well as those who used acyclovir, could develop crust lesion within 3 days and healing within 7 days while the placebo group had crusting time in 4-7 days and healing time in 7-14 days or more. This meant that *C. nutans* and acyclovir have good efficacy in shortening the duration of infection and reducing severity. Besides, *C. nutans* has no sticky, burning, stinging pain and side effects. In conclusion, it is believed that *C. nutans* is suitable for the treatment of genital herpes patients.

In 1993 Sangkitporn, Polchanandara, Thawatsupa, Bunchob and Chavaltittumrong reported the results of treatment of recurrent Genital Herpes Simplex virus infection with *C. nutans* extract, the numbers of patients with lesion crusting within three days and with lesion crusting within seven days were significantly increased in the *C. nutans* extract-treated group and the acyclovir-treated group compared with the placebo-treated group. There was no side effect of *C. nutans* during treatment. Mild, transient burning or pains associated with application of acyclovir were found in 21 male patients (24.7%) and in 18 female patients (23.1%).

2.3.4 Activity Against Varicella Zoster Virus (VZV)

In 1992 Jayavasu, Chavaltittumrong, Polachandara, Dechatiwongse na Ayudhaya and Jongtrakulsiri reported the ability of the extract of *C. nutans* leaves to inactivate VZV. The activity was measured *in vitro* using a plaque reduction assay and DNA hybridization. Based on 50 percent inhibited dose, the results showed the extract of *C. nutans* leaves inhibited plaque formation by VZV. From the present findings, based on the result of inactivation assay, it was recognized that the *in vitro*

antiviral activity of *C. nutans* might be a direct interaction of the extract with the virus.

In 1993 Sangkitporn, Polchanandara, Thawatsupa, Bunchob and Chavaltittumrong reported the results of treatment of herpes zoster with a topical formulation of 5 percent *C. nutans* extract. There was no difference of patient characteristics in the *C. nutans* extract-treated group and in the placebo group, but the numbers of patients with lesion crusting within three days and healing within seven days and ten days were significantly greater in the *C. nutans* extract-treated group than in the placebo group. Furthermore, the pain score was reduced more rapidly in the *C. nutans* extract treated group and there were no side effects from the study medication. *C. nutans* cream is more effective than placebo in the treatment of herpes zoster and the patients healed faster than patients in the placebo group (Charuwichitratana, Wongrattanapasson, Timpatanapong and Bunjob, 1996).

2.3.5 Toxicological Study

Acute toxicity studies of ethanolic extract of *C. nutans* leaves given orally, subcutaneously or intraperitoneally, did not produce any side effects of toxicity in the animal. A subchronic toxicity study was performed by daily oral administration of the extract to rats for 90 days. Histopathalogical examination of internal organs did not show any abnormalities that could be due to the effect of the extract (Chavalittumrong, Attawish, Rugsamon and Chuntapet, 1995). In addition, ethanol extract of *C. nutans* was shown to inhibit growth of yellow head baculoving and also to induce phagocytosis activity in tiger prawn (Chavalittumrong *et al.*, 1995). The results of these studies show that *C. nutans* has strong potential to be developed as a novel source of medicine to treat herpes infections.

The Department of Medical Sciences, Ministry of Public Health transferred the technology of *C. nutans* antiviral cream, including extraction and the formulation of *C. nutans* cream, to the Government Pharmaceutical Organization to produce this cream for the market (Jayavasu, 1998). This case illustrates that traditional medicine can be very valuable and useful for modern scientific studies.

Consequently, a systematic procedure for the quality control of the crude drug and raw material has to be established. Currently, the control of *C. nutans* to produce antiviral cream uses a bioactivity test (plaque reduction assay) which requires considerable time and cost. As it is important to reduce the cost of such control, chemical study of active components should be conducted to obtain the quality control of crude extract of *C. nutans* by developing chemical reference standards for the active components isolated from *C. nutans*.

2.4   Chemical Constituents

C. nutans has been chemically investigated previously as below:

Dampawan (1976) and Dampawan, Huntrakul and Reutrakul (1977) reported the isolation of stigmasterol (2.1), lupeol (2.2), and β-sitosterol (2.3) from a light petroleum extract of the leaves, stems and roots of *C. nutans*.

(2.1) Stigmasterol   **(2.2) Lupeol**
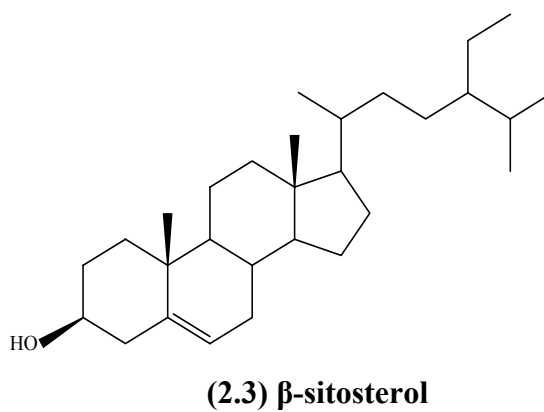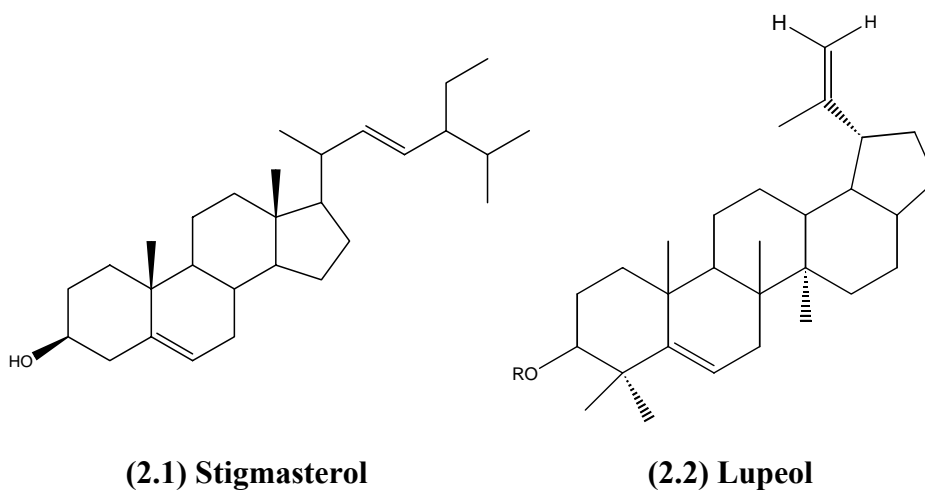
(2.3) β-sitosterol

In 1983 Lin, Li and Yu reported the isolation of betulin (2.4), lupeol (2.2) and β-sitosterol (2.3) from the roots of *C. nutans*.

(2.4) Betulin
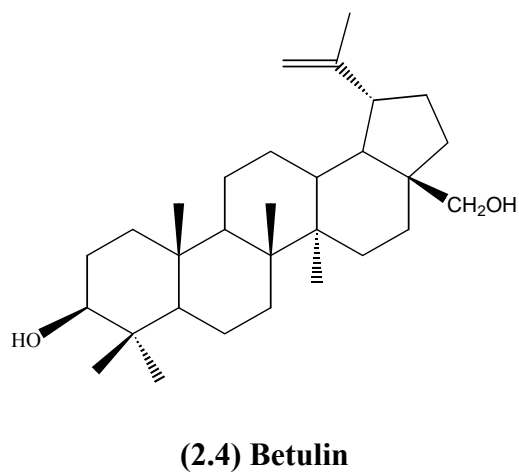
Although chemical studies were started in 1976, very little advance was made at that time, because of unsystematic approaches in the isolation and lack of suitable techniques for the characterization of the components. However, as a result of the development in instrumental and chemical techniques, in 1997 Teshima, Kaneto, Ohtani, Kasai, Lhieochaiphant and Picheasoonthon isolated six known C-glucosyl flavones [vitexin (2.5), isovitexin (2.6), shaftoside (2.7), isomollupentin-7-*O*-β-glucopyranoside (2.8), orientin (2.9) and isoorientin (2.10)] from the methanol extract of the stems and leaves of *C. nutans*.
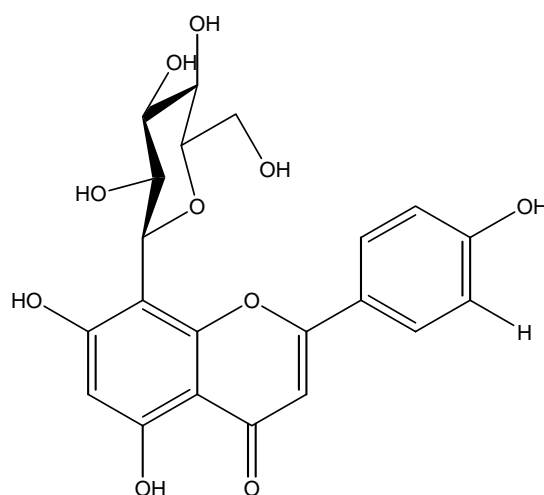
(2.5) Vitexin
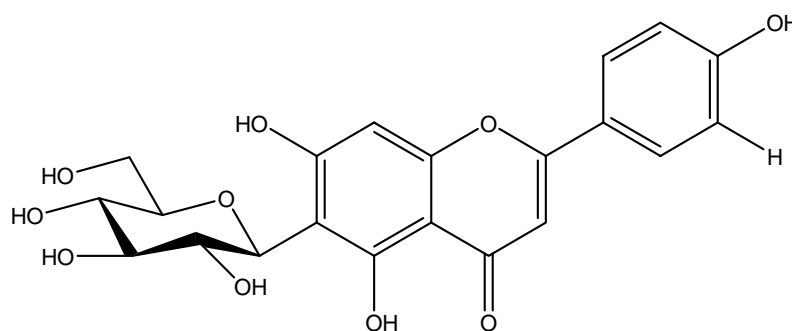
(2.6) Isovitexin
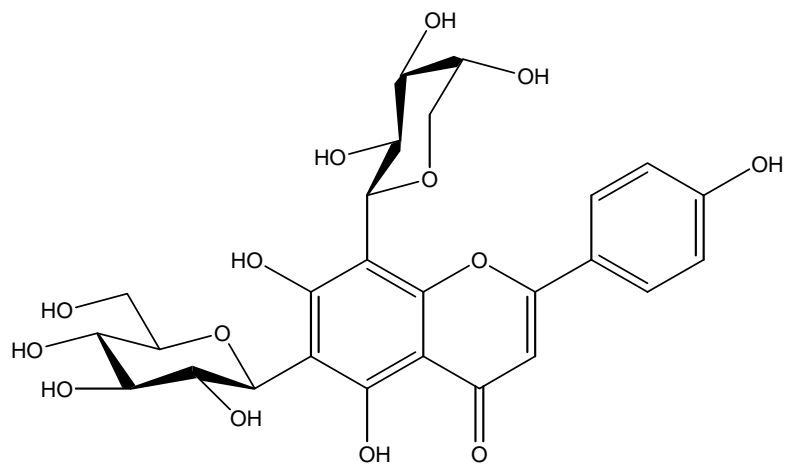
(2.7) Shaftoside
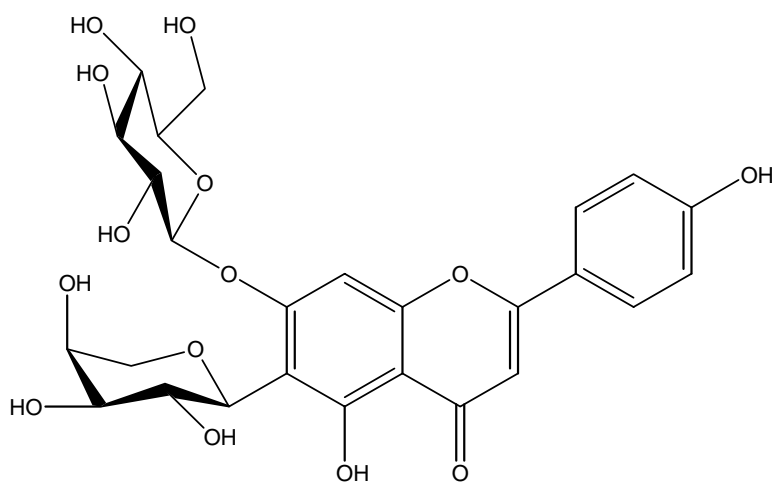
(2.8) Isomollupentin-7-*O*-β-glucopyranoside
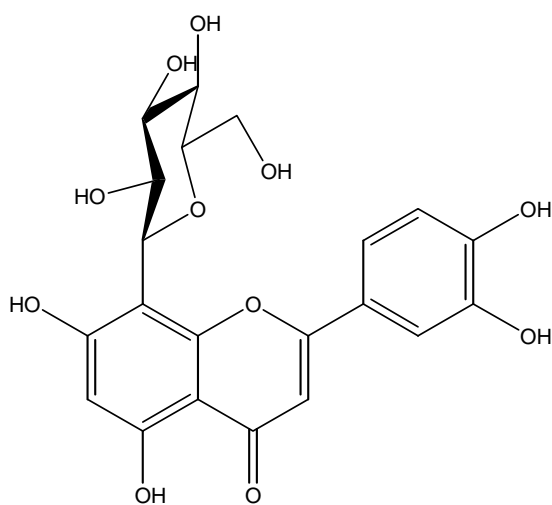
(2.9) Orientin
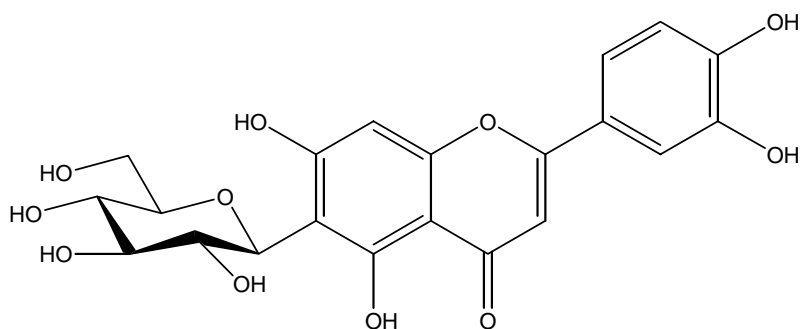
(2.10) Isoorientin

In 1998 Teshima, Kaneto, Ohtani, Kasai, Lhieochaiphant and Picheasoonthon isolated five sulfur-containing glucosides [clinacoside A (2.11), clinacoside B (2.12), clinacoside C (2.13), cycloclinacoside A1 (2.14) and cycloclinacoside A2 (2.15)] from the butanol and water soluble portions of the methanol extract of the stems and leaves of *C. nutans*.
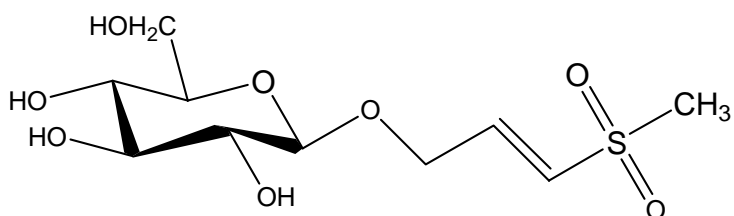
(2.11) Clinacoside A
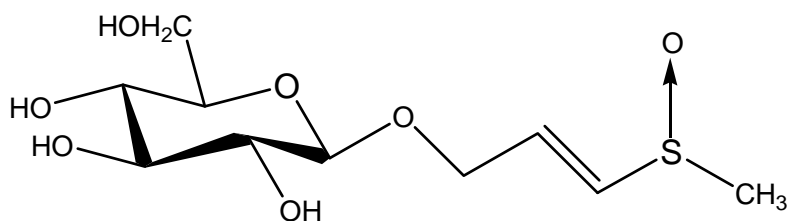
(2.12) Clinacoside B
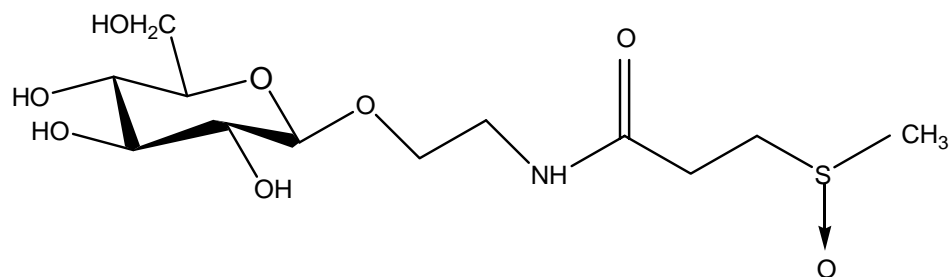
(2.13) Clinacoside C
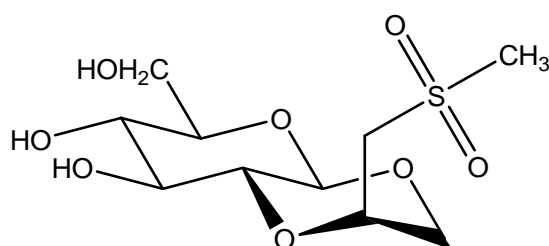
(2.14) Cycloclinacoside A1
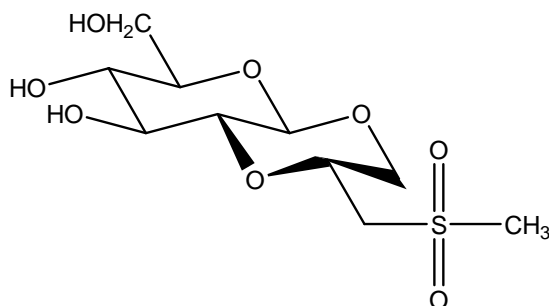
(2.15) Cycloclinacoside A2

In 2001 Satakhum reported the isolation of two glycoglycerolipids (1,2-*O*-dilinolenoyl-3-*O*-β-D-galactopyranosylglycerol (2.16) and 1-*O*-palmitoyl-2-*O*-linolenoyl-3-*O*-[α-D-galactopyranosyl-(1" ⟶ 6')-*O*-β-D-galacctopyranosyl] glycerol (2.17)) from the leaves of *C. nutans*. Further investigation by Suwanborirux's group in 2003 found anti-HSV activity of both compounds.

| | R₁ | R₂ |
| R₁ | R₂ |

Major Hexadecanoyl (16:0)   Hexadecatrienoyl (16:3)

Octadecanoyl (18:0)   Hexadecatrienoyl (16:3)

Minor Hexadecanoyl (16:0)   Octadecanoyl (18:0)

Octadecanoyl (18:0)   Hexadecanoyl (16:0)

(2.16) 1,2-*O*-dilinolenoyl-3-*O*-β-D-galactopyranosylglycerol

R₁   R₂

Major Octadecanoyl (18:0)   Octadecatetraenoyl (18:4)

Octadecatetraenoyl (18: 4)   Octaadecanoyl (18: 0)

Minor Hexadecanoyl (16:0)   Octadecatrienoyl (18:3)
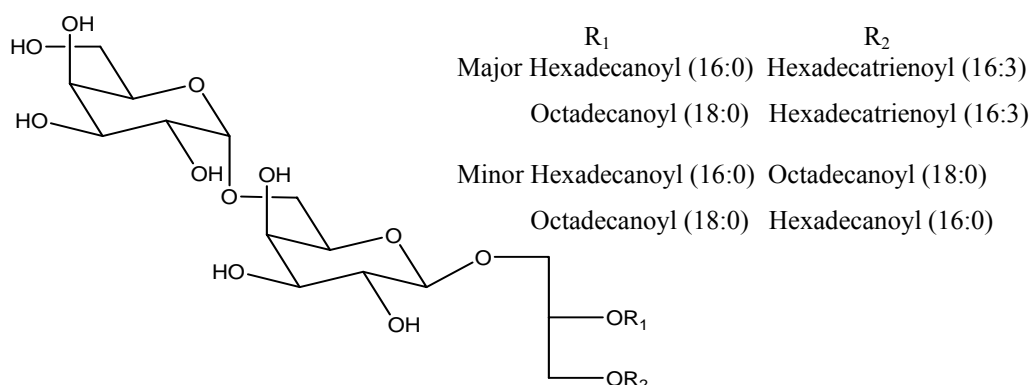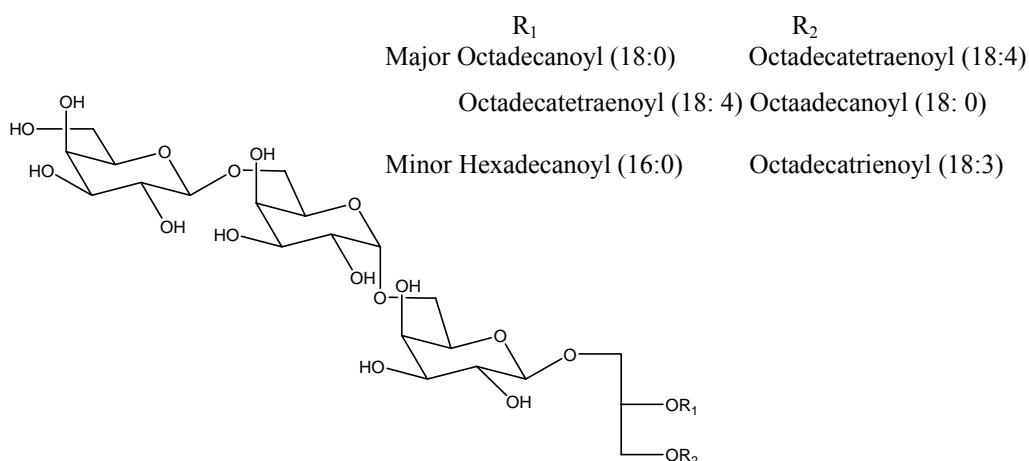
(2.17) 1-*O*-palmitoyl-2-*O*-linolenoyl-3-*O*-[α-D-galactopyranosyl-

(1"⟶ 6')-*O*-β-D-galacctopyranosyl] glycerol

In 2001 Dechatiwongse na Ayudhya, Sakdarat, Shuyprom, Pattamadilok, Bansiddhi, Waterman, P. G. and Karagianis, G. reported the isolation of 13^2-hydroxy-$(13^2$-*S*)-phaeophytin b (2.18), purpurin 18 phytylester (2.19) and phaeophorbide a (2.20) from the chloroform extract of the leaves of *C. nutans*.

(2.18)   **(2.19)**   **(2.20)**
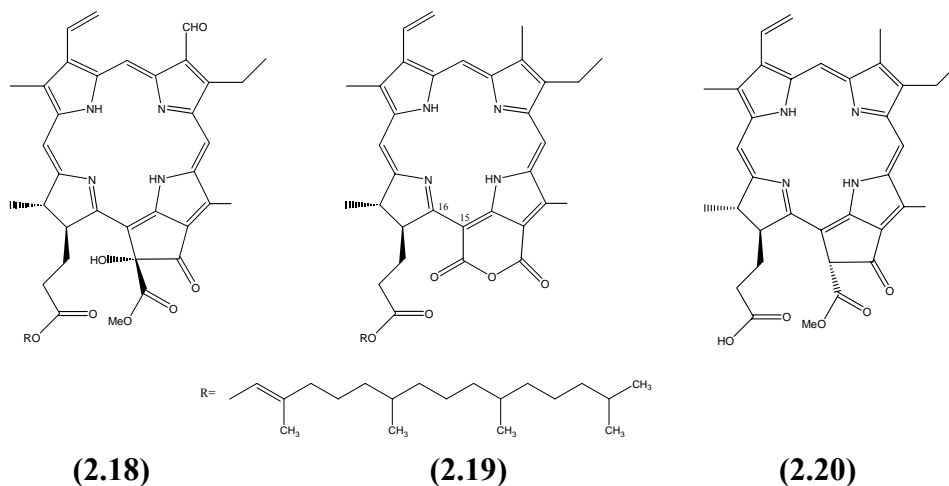
(2.18) 13^2-hydroxy-(13^2-*S*)-phaeophytin b   **(2.19) Purpurin 18 phytylester**

(2.20) Phaeophorbide a

In 2004 Tuntiwachwuttikul, Pootaeng-on, Phansa and Taylor reported the isolation of a mixture of nine cerebrosides (2.21) and a monoacylmonogalactosylglycerol (2.22) from the leaves of *C. nutans*. The structures of the cerebrosides were characterized as 1-*O*-β-D-glucosides of phytosphingosines, which comprised a common long-chain base, (2*S*,3*S*,4*R*,8*Z*)-2-amino-8(*Z*)-octadecene-1,3,4-triol with nine 2-hydroxy fatty acids of varying chain lengths (C16, C18, C20-26) linked to the amino group. The glycosylglyceride was characterized as (2*S*)-1-*O*-linolenoyl-3-*O*-β-D-galactopyranosylglycerol.
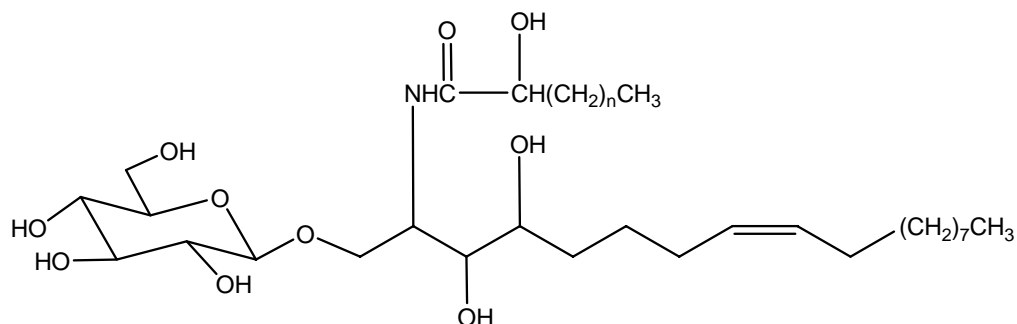
n= 13, 15, 17-23

(2.21) Cerebrosides
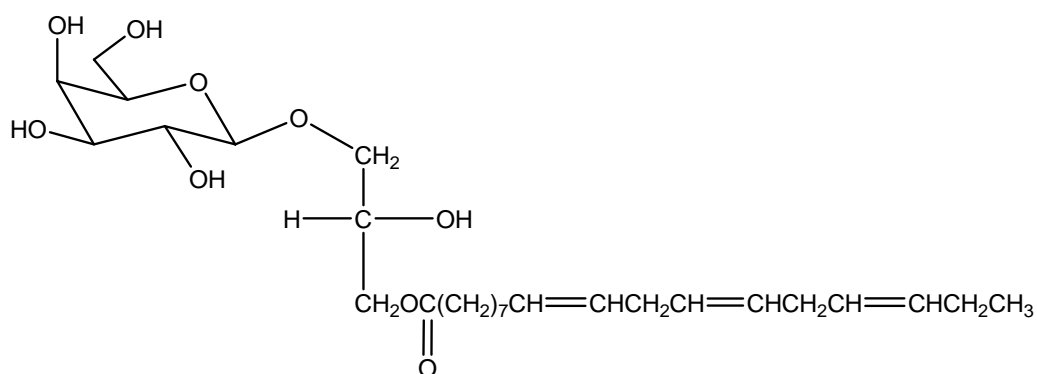
(2.22) (2S,3S,4R,8Z)-2-amino-8(Z)-octadecene-1,3,4-triol

Although the chemical constituents have been reported, the active components have not been identified. This project attempts to isolate and characterize such chemical principles of *C. nutans*.

Our research group has been interested in this plant due to wide use in traditional medicine. Because of its interesting biological activities and the fact that very little is known about it in the literature, it is desirable to phytochemically investigate this plant in details.

In this project, six compounds were separated from the leaves of *C. nutans*. The isolated compounds could be identified as chlorophyll a and chlorophyll b related compounds, together with the stigmasterol glucoside. Structures of isolated compounds were characterized on the basis of NMR spectra by comparison of their NMR spectral data with literature values.

Details on isolation and structural determination of chemical constituents will be discussed in the next section of this thesis.

CHAPTER III

EXPERIMENTAL

All experiments have been performed at Suranaree University of Technology, but some instruments were not included which indicated in each item.

3.1   Source of Plant Materials

Fresh aerial parts of *C. nutans* were collected from the Faculty of Pharmaceutical Sciences, Khon Kaen University, Khon Kaen, Thailand. The specimens were authenticated by the Botanical section, Medicinal Plant Research Institute, Department of Medical Sciences, Ministry of Public Health. The leaves were separated from the stems, washed thoroughly and dried in an oven at 50 $^{\circ}$C. The dried samples were ground to powder.

3.2   General Techniques

3.2.1 Solvents

The organic solvents were commercial grade and distilled prior to use for extraction and as eluents for thin-layer and column chromatography. Reagent grade solvents were used for recrystallization.

3.2.2 Other Chemicals

3.2.2.1 Merck's silica gel 60 Art. 7734 (70-230 mesh ASTM) was used as adsorbent for normal column chromatography and 9385 (230-400 mesh ASTM) for

flash chromatography.

3.2.2.2 Merck's silica gel 60 G Art. 7731 and 60 GF$_{254}$ Art. 7730 were applied as adsorbent for preparative TLC.

3.2.2.3 Merck's TLC aluminum sheet, silica gel 60 F$_{254}$ precoated 20x20 cm^2 in size with layer thickness of 0.2 mm was used to identify the identical fractions.

3.2.3 **Chromatography Techiques**

3.2.3.1 Thin-Layer Chromatography (TLC)

Techniques: One way, ascending.

Adsorbent: Silica gel 60 F$_{254}$ pre-coated on aluminum 2x5 cm^2 plate (Merck).

Visualization: 1. Under daylight.

2. Under ultraviolet light at 254 and 365 nm.

3. By staining TLC plates with anisaldehyde-sulfuric acid reagent.

3.2.3.2 Column Chromatography

Adsorbent: Unless indicated otherwise, silica gel with particle size of less than 0.63 mm (Merck) was used throughout the experiments.

Packing method: Slurry packing.

Sample loading: The sample was dissolved in a small amount of a suitable organic solvent, mixed with a small quantity of silica gel 60 with particle sizes of 0.063-0.200 mm, air dried and added gently onto the top of column.

Elution: After loading of the sample, the column was eluted with a suitable solvent system using the gradient technique.

3.2.3.3 Preparative Chromatography

Preparative layer chromatography plates were prepared as follows. A mixture of Merck's silica gel 60 GF$_{254}$ for preparative layer chromatography (200 g) in water (480 mL) was shaken thoroughly until the suspension was homogeneous. It was allowed to stand in a stoppered flask for 1-2 hours before being spreaded onto eight 20x20 cm^2 plates. The plates were left to dry in still air for 24 hours and then in an oven at 100 ^{o}C for 1 hour. Bands were detected by viewing under UV lamp at 254 and 365 nm.

3.3   Instrumentation

3.3.1   Rotatory evaporator (*Buchi R-114* Vacuum System)

The Buchi rotatory evaporator was used to evaporate the large amount of volatile solvents.

3.3.2   UV-Visible Spectrometer

UV-visible absorbance was measured on a Hewlett Packard 8452A diode array UV-Vis spectrophotometer at Medicinal Plant Research Institute, Department of Medical Sciences.

3.3.3   FT-IR Spectrophotometer

IR spectra were recorded on a Model Spectrum GX, Perkin-Elmer spectrophotometer at Medicinal Plant Research Institute, Department of Medical Sciences. Spectra of solid samples were recorded as potassium bromide (KBr) pellets

3.3.4   NMR Spectrometer

The ^{1}H-NMR and DEPT ^{13}C-NMR spectra were recorded at 500 MHz and 125 MHz, respectively on a *Bruker DRX-500* NMR spectrophotometer at

Department of Chemistry, Indiana University in deuterated pyridine (C_6D_5N). The chemical shifts were recorded in parts per million with reference to the solvent signals.

The numbers of hydrogen atoms attached to each carbon atom were obtained from *J*-modulated spin-echo experiments. 2-D NMR spectroscopy was performed with standard COSY (^1H-^1H correlation), NOESY, HSQC (^{13}C-^1H correlation) and HMBC (long range carbon-proton correlation) pulse sequences.

3.4 Extraction

The two and a half kilograms of dried powder of leaves of *C. nutans* were extracted consecutively with hexane and chloroform in a soxhlet extraction apparatus. The extracted solutions were then filtered through filter paper. The filtrates were concentrated to remove solvent by evaporation under reduced pressure on a rotary evaporator giving 72.64 g of hexane crude extract as dark brown gum and 59.53 g of chloroform crude extract as dark green slush. The extraction sequence is shown in Scheme 3.1.

```
┌─────────────────────────────────────────────────────┐
│      Dried powder of leaves of C. nutans (2500 g)     │
└─────────────────────────────────────────────────────┘
                         │
                         │  -soxhlet extracted with hexane
                         │  -filtered, evaporated
          ┌──────────────┴──────────────┐
    ┌───────────┐              ┌──────────────────────┐
    │   Marc    │              │  Hexane crude extract │
    └───────────┘              └──────────────────────┘
         │
         │           (dark brown gum, 72.64 g, 2.91% w/w)
         │
   ┌─────┴──────────────────┐
┌────────┐        ┌────────────────────────┐
│  Marc  │        │  Chloroform crude extract │
└────────┘        └────────────────────────┘
```

(dark green slush, 59.53 g, 2.38% w/w)
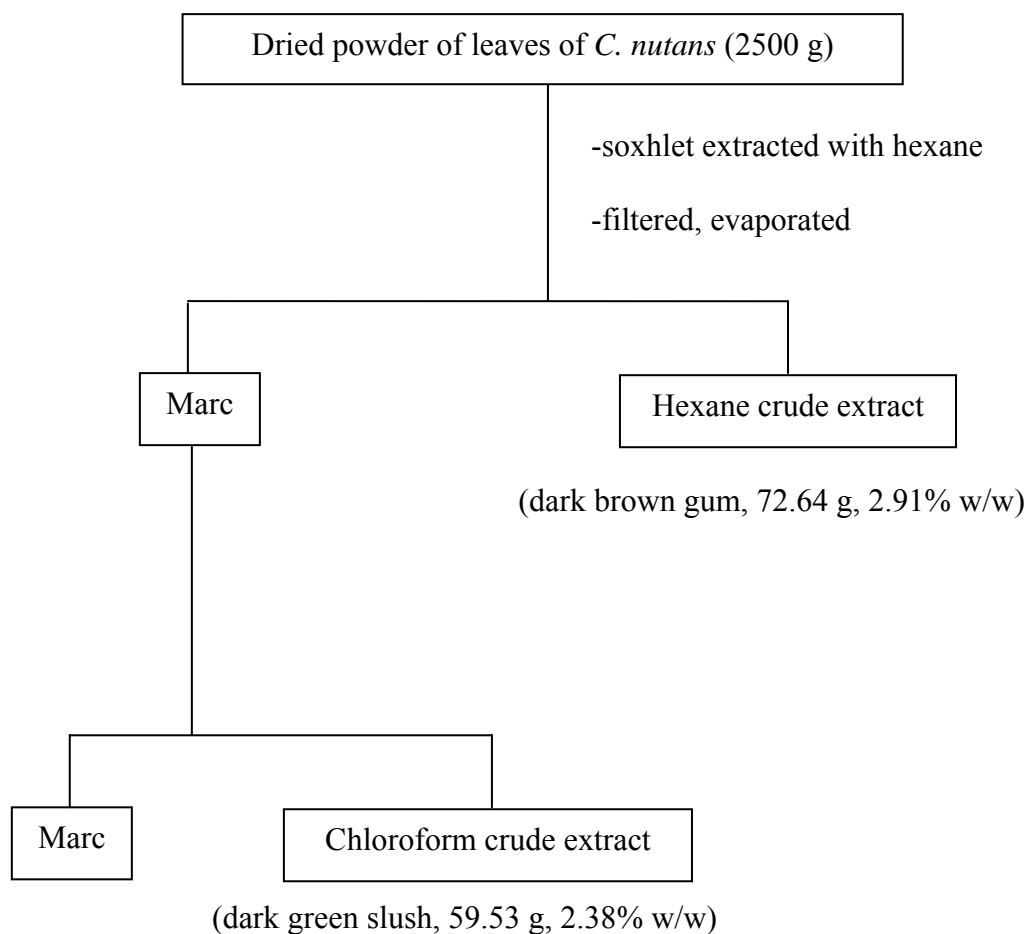
Scheme 3.1 Extraction of the leaves of *C. nutans*.

3.5   Isolation

The chloroform crude extract was concentrated in vacuum to give a residue (59.53 g) which was chromatographed on a silica gel 60 column. The column was eluted successively with hexane-ethyl acetate (1:1), ethyl acetate, chloroform-ethanol (1:1), and ethanol. Every fraction of 2000 mL was collected and concentrated to a small volume and four major fractions (I 19.48 g, II 4.67 g, III 15.65 g and IV 3.70 g) were separated by monitoring with TLC in order to combine the fractions which had the same compounds. The isolation sequence is shown in Scheme 3.2.

```
                    ┌─────────────────────┐
                    │  Chloroform Extract  │
                    │     (59.53 g)        │
                    └─────────────────────┘
                              │
                              │   column chromatography, silica gel
                              │   -hexane: ethyl acetate 1:1
                              │   -ethyl acetate
                              │   -chloroform: ethanol 1:1
                              │   -ethanol
```

Fraction I	Fraction II	Fraction III	Fraction IV
(19.48 g)	(4.67 g)	(15.65 g)	(3.70 g)
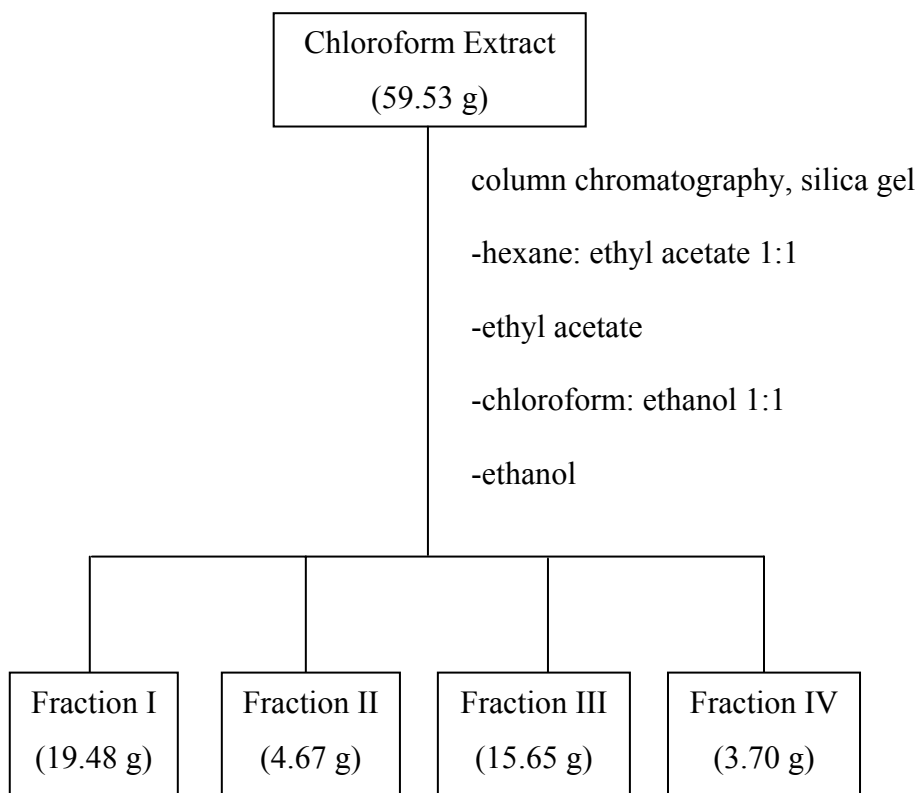
Scheme 3.2 Isolation of chloroform crude extract.

A portion of fraction I (1.0045 g) was further separated by preparative thin-layer chromatography (hexane-ethyl acetate 7:3) to afford five fractions (A 0.0367 g, B 0.0341 g, C 0.0693 g, D 0.0498 g and E 0.0539 g). Fraction A (0.0367 g) was further purified by preparative thin-layer chromatography using the same developing solvent to give crude compound 1 (0.0105 g) and 2 (0.0098 g), which were recrystallized from methanol to obtain pure compound 1 as bright green powder (0.0052 g) and compound 2 as bright green powder (0.0048 g). The isolation sequence of compounds 1 and 2 is shown in Scheme 3.3.
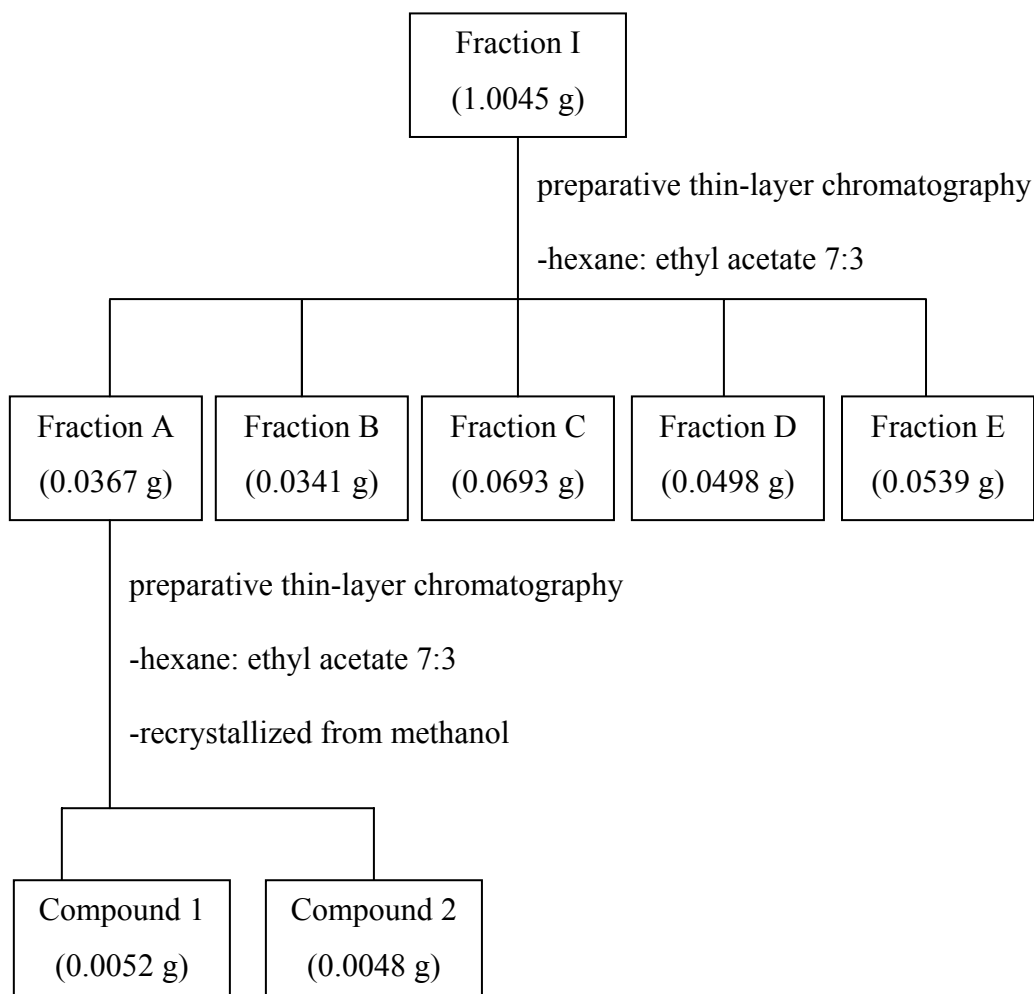
Scheme 3.3 Isolation of compounds 1 and 2 from chloroform crude extract.

Fraction B (0.0341 g) was further purified by preparative thin-layer chromatography using the same developing solvent to give crude compound 3 (0.0141 g), which was recrystallized from methanol to obtain pure compound 3 as dark green powder (0.0044 g). The isolation sequence of compound 3 is shown in Scheme 3.4.

```
┌─────────────────┐
│ Fraction B      │
│ (0.0314 g)      │
└─────────────────┘
        │
        │   preparative thin-layer chromatography
        │
        │   -hexane: ethyl acetate 7:3
        │
        │   -recrystallized from methanol
┌─────────────────┐
│ Compound 3      │
│ (0.0044 g)      │
└─────────────────┘
```
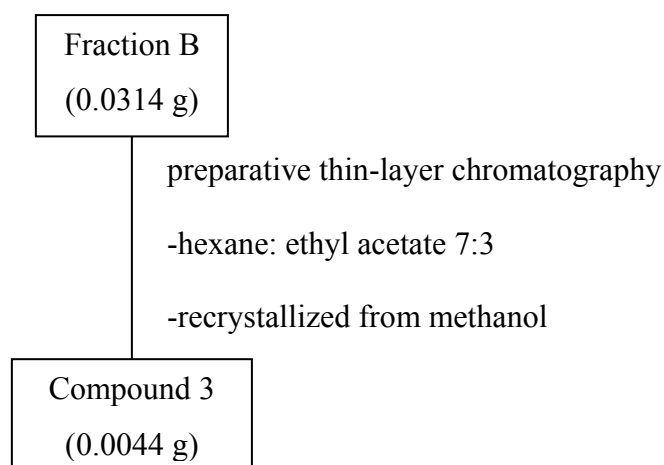
Scheme 3.4 Isolation of compound 3 from chloroform crude extract.

Fraction D (0.0498 g) was further purified by preparative thin-layer chromatography using the same developing solvent to give crude compound 4 (0.0117 g) and 5 (0.0113 g), which were recrystallized from chloroform-methanol mixed solvent to obtain pure compound 4 as green powder (0.0061 g) and compound 5 as green powder (0.0057 g). The isolation sequence of compounds 4 and 5 were as shown in Scheme 3.5.

```
            ┌─────────────────┐
            │ Fraction D      │
            │ (0.0498 g)      │
            └─────────────────┘
                    │
                    │   preparative thin-layer chromatography
                    │
                    │   -hexane: ethyl acetate 7:3
                    │
                    │   -recrystallized from chloroform-methanol mixed solvent
            ┌───────┴────────────────────┐
   ┌─────────────────┐          ┌─────────────────┐
   │ Compound 4      │          │ Compound 5      │
   │ (0.0061 g)      │          │ (0.0057 g)      │
   └─────────────────┘          └─────────────────┘
```
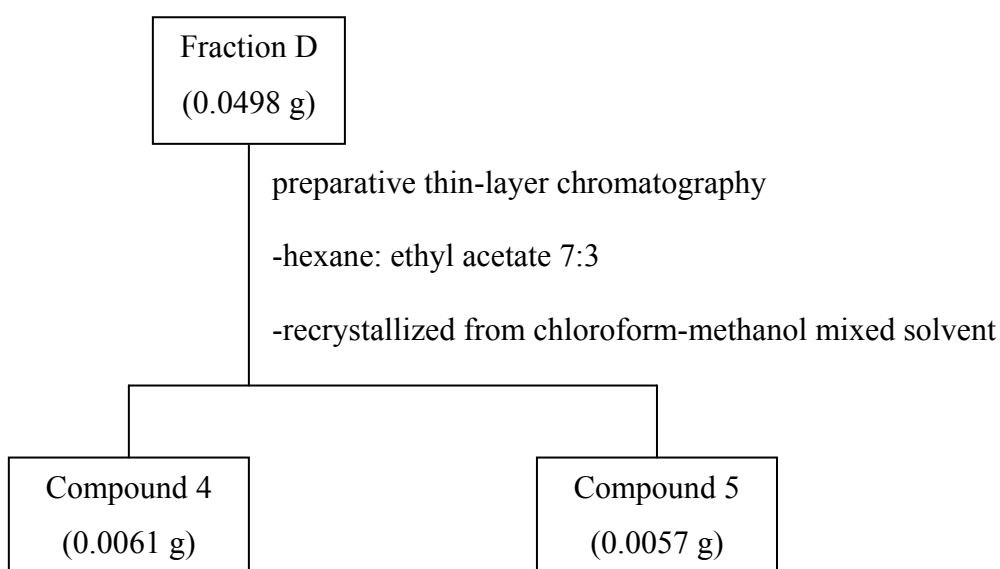
Scheme 3.5 Isolation of compounds 4 and 5 from chloroform crude extract.

Fraction IV (3.70 g) was chromatographed on a silica gel 60 column, eluting with chloroform, followed by chloroform-methanol gradient to provide pure compound 6 as white powder (1.04 g). The isolation sequence of compound 6 is shown in Scheme 3.6.

```
┌─────────────────┐
│   Fraction IV    │
│    (3.70 g)      │
└────────┬─────────┘
         │   column chromatography, siliga gel 60
         │   -chloroform: methanol 10:0 - 0:10
┌────────┴─────────┐
│   Compound 6     │
│    (1.04 g)      │
└─────────────────┘
```
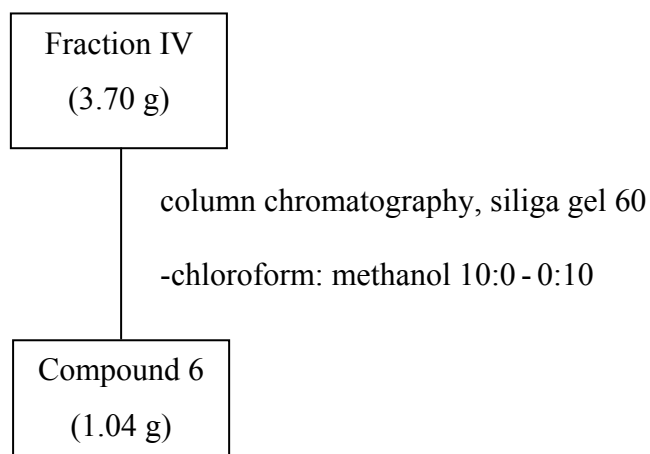
Scheme 3.6 Isolation of compound 6 from chloroform crude extract.

3.6   Purification and Characterization of the Isolated Compounds

3.6.1 Purification and Characterization of Compound 1

Compound 1 was obtained from 1.0045 g of fraction I of the first column chromatography separation of chloroform crude extract. Repetitive silica gel column chromatography separation is indicated in Scheme 3.3. The solvent was removed by rotary evaporator and the compound was recrystallized from methanol several times to provide 0.0052 g of pure compound. This compound is soluble in chloroform.

UV (CHCl$_3$) λ_{max} values are 408, 506, 535, 612 and 668 nm (Figure A.1).

FT-IR spectrum ν_{max} (KBr) values are 3028, 1730, 1638 and 1310 cm^{-1} (Table 4.1 p48 and Figure A.2).

^{1}H NMR δ (ppm) values, in pyridine-d_5, are 1.56 (3H, d, $J = 7.25$ Hz, 18^1-H), 1.75 (3H, t, $J = 7.55$ Hz, 8^2-H), 3.27 (3H, s, 2^1-H), 3.68 (3H, s, 12^1-H), 3.74 (3H, s, 13^4-H), 4.18 (2H, q, $J = 7.65$ Hz, 8^1-H), 4.49 (1H, dq, $J = 7.30$ Hz, 18-H), 5.14 (1H, m, 17-H), 6.06 (1H, dd, $J = 2.28$, 11.39 Hz, 3^2-H), 6.49 (1H, dd, $J = 2.28$, 15.19 Hz, 3^2-H), 8.55 (1H, s, 20-H), 10.06 (1H, s, 10-H), 10.91 (1H, s, 5-H) and 11.57 (1H, s, 7^1-H) (Table 4.2 p49 and Figure A.3).

DEPT ^{13}C NMR δ (ppm) values, in pyridine-d_5, are 195.4 (13^1-C), 188.8 (7^1-C), 170.4 (19-C), 92.2 (13^2-C), 173.5 (17^3-C), 156.6 (1-C), 140.9 (3-C), 139.4 (11-C), 149.5 (2-C), 130.9 (3^1-C), 120.8 (3^2-C), 110.5 (10-C), 104.5 (5-C), 94.1 (20-C), 53.3 (13^4-C), 50.8 (17-C), 50.6 (18-C), 31.6 (17^2-C), 31.1 (17^1-C), 23.3 (18^1-C), 19.9 (8^2-C), 19.9 (8^1-C), 13.3 (12^1-C) and 12.8 (2^1-C) (Table 4.3 p50 and Figure A.4).

3.6.2 Purification and Characterization of Compound 2

Compound 2 was obtained from 1.0045 g of fraction I of the first column chromatography separation of chloroform crude extract. Repetitive silica gel column chromatography separation is indicated in Scheme 3.3. The solvent was removed by rotary evaporator and the compound was recrystallized from methanol several times to provide 0.0048 g of pure compound. This compound is soluble in chloroform.

UV (CHCl$_3$) λ_{max} values are 410, 507, 536, 612 and 666 nm (Figure A.9).

FT-IR spectrum ν_{max} (KBr) values are 3030, 1728, 1630 and 1306 cm^{-1} (Table 4.8 p58 and Figure A.10).

^{1}H NMR δ (ppm) values, in pyridine-d_5, are 1.56 (3H, d, J = 7.25 Hz, 18^1-H),1.75 (3H, t, J = 7.55 Hz, 8^2-H), 3.27 (3H, s, 2^1-H), 3.68 (3H, s, 12^1-H), 3.74 (3H, s, 13^4-H), 4.18 (2H, q, J = 7.65 Hz, 8^1-H), 4.50 (1H, dq, J = 7.30 Hz, 18-H), 5.54 (1H, m, 17-H), 6.06 (1H, dd, J = 2.28, 11.39 Hz, 3^2-H), 6.49 (1H, dd, J = 2.28, 15.19 Hz, 3^2-H), 8.57 (1H, s, 20-H), 10.06 (1H, s, 10-H), 10.91 (1H, s, 5-H) and 11.57 (1H, s, 7^1-H) (Table 4.9 p60 and Figure A.11).

DEPT ^{13}C NMR δ (ppm) values, in pyridine-d_5, are 195.3 (13^1-C), 188.7 (7^1-C), 171.7 (19-C), 92.4 (13^2-C), 173.3 (17^3-C), 156.4 (1-C), 140.8 (3-C), 139.3 (11-C), 149.5 (2-C), 130.9 (3^1-C), 120.7 (3^2-C), 109.9 (10-C), 104.2 (5-C), 94.2 (20-C), 53.4 (13^4-C), 50.4 (17-C), 50.3 (18-C), 31.0 (17^2-C), 31.0 (17^1-C), 23.3 (18^1-C), 19.9 (8^2-C), 19.6 (8^1-C), 12.9 (12^1-C) and 12.8 (2^1-C) (Table 4.10 p61 and Figure A.12).

3.6.3   Purification and Characterization of Compound 3

Compound 3 was obtained from 1.0045 g of fraction I of the first column chromatography separation of chloroform crude extract. Repetitive silica gel column chromatography separation is indicated in Scheme 3.4. The solvent was removed by rotary evaporator and the compound was recrystallized from methanol several times to provide 0.0044 g of pure compound. This compound is soluble in chloroform.

UV (CHCl$_3$) λ_{max} values are 412, 505, 526, 603 and 656 nm (Figure A.17).

FT-IR spectrum ν_{max} (KBr) values are 3429, 2940, 1720, 1630 and 1310 cm^{-1} (Table 4.15 p69 and Figure A.18).

^{1}H NMR δ (ppm) values, in pyridine-d_5, 1.71 (3H, d, J = 7.25 Hz, 18^1-H),1.83 (3H, t, J = 7.55 Hz, 8^2-H), 3.35 (3H, s, 2^1-H), 3.70 (3H, s, 12^1-H), 3.85 (3H, s, 13^4-H), 4.22 (2H, q, J = 7.65 Hz, 8^1-H), 4.60 (1H, dq, J = 7.30 Hz, 18-H), 5.41 (1H, m, 17-H), 6.21 (1H, dd, J = 2.28, 11.39 Hz, 3^2-H), 6.54 (1H, dd, J = 2.28, 15.19 Hz, 3^2-H), 8.89 (1H, s, 20-H), 10.16 (1H, s, 10-H), 11.00 (1H, s, 5-H) and 11.50 (1H, s, 7^1-H) (Table 4.16 p71 and Figure A.19).

DEPT ^{13}C NMR δ (ppm) values, in pyridine-d_5, are 196.5 (13^1-C), 191.7 (7^1-C), 178.7 (19-C), 92.1 (13^2-C), 173.6 (17^3-C), 150.0 (1-C), 135.5 (3-C), 135.9 (11-C), 135.5 (2-C), 124.3 (3^1-C), 121.5 (3^2-C), 114.6 (10-C), 104.9 (5-C), 95.1 (20-C), 54.9 (13^4-C), 51.8 (17-C), 50.3 (18-C), 31.6 (17^2-C), 31.1 (17^1-C), 22.7 (18^1-C), 18.7 (8^2-C), 18.7 (8^1-C), 12.3 (12^1-C) and 12.1 (2^1-C) (Table 4.17 p72 and Figure A.20).

3.6.4 Purification and Characterization of Compound 4

Compound 4 was obtained from 1.0045 g of fraction I of the first column chromatography separation of chloroform crude extract. Repetitive silica gel column chromatography separation is indicated in Scheme 3.5. The solvent was removed by rotary evaporator and the compound was recrystallized from chloroform-methanol mixed solvent several times to provide 0.0061 g of pure compound. This compound is soluble in chloroform.

UV (CHCl$_3$) λ_{max} values are 408, 506, 536, 613 and 670 nm (Figure A.25).

FT-IR spectrum ν_{max} (KBr) values are 3430, 2924, 1741, 1620 and 1460 cm^{-1} (Table 4.22 p80 and Figure A.26).

^{1}H NMR δ (ppm), in pyridine-d_5, are 1.61 (3H, d, J = 7.25 Hz, 18^1-H),1.76 (3H, t, J = 7.55 Hz, 8^2-H), 3.43 (3H, s, 2^1-H), 4.02 (3H, s, 12^1-H), 3.75 (3H, s, 13^4-H), 3.83 (2H, q, J = 7.65 Hz, 8^1-H), 4.64 (1H, dq, J = 7.30 Hz, 18-H), 5.24 (1H, m, 17-H), 6.19 (1H, dd, J = 2.28, 11.39 Hz, 3^2-H), 6.45 (1H, dd, J = 2.28, 15.19 Hz, 3^2-H), 9.05 (1H, s, 20-H), 10.07 (1H, s, 10-H), 9.91 (1H, s, 5-H) and 3.29 (1H, s, 7^1-H) (Table 4.23 p81 and Figure A.27).

DEPT ^{13}C NMR δ (ppm) values, in pyridine-d_5, 194.6 (13^1-C), 11.6 (7^1-C), 172.5 (19-C), 91.1 (13^2-C), 173.6 (17^3-C), 141.4 (1-C), 136.3 (3-C), 139.3 (11-C), 132.0 (2-C), 129.9 (3^1-C), 123.0 (3^2-C), 104.7 (10-C), 100.5 (5-C), 95.0 (20-C), 53.6 (13^4-C), 54.4 (17-C), 50.7 (18-C), 33.1 (17^2-C), 33.5 (17^1-C), 23.1 (18^1-C), 18.2 (8^2-C), 20.1 (8^1-C), 12.9 (12^1-C) and 12.5 (2^1-C) (Table 4.24 p83 and Figure A.28).

3.6.5 Purification and Characterization of Compound 5

Compound 5 was obtained from 1.0045 g of fraction I of the first column chromatography separation of chloroform crude extract. Repetitive silica gel column chromatography separation is indicated in Scheme 3.5. The solvent was removed by rotary evaporator and the compound was recrystallized from chloroform-methanol mixed solvent several times to provide 0.0057 g of pure compound. This compound is soluble in chloroform.

UV (CHCl$_3$) λ_{max} values are 412, 507, 537, 612 and 668 nm (Figure A.33).

FT-IR spectrum ν_{max} (KBr) values are 3429, 2995, 1740, 1617 and 1455 cm^{-1} (Table 4.29 p91 and Figure A.34).

^{1}H NMR δ (ppm), in pyridine-d_5, are 1.74 (3H, d, J = 7.25 Hz, 18^1-H),1.68 (3H, t, J = 7.55 Hz, 8^2-H), 3.40 (3H, s, 2^1-H), 3.73 (3H, s, 12^1-H), 3.71 (3H, s, 13^4-H), 3.66 (2H, q, J = 7.65 Hz, 8^1-H), 4.60 (1H, dq, J = 7.30 Hz, 18-H), 5.77 (1H, m, 17-H), 6.18 (1H, dd, J = 2.28, 11.39 Hz, 3^2-H), 6.37 (1H, dd, J = 2.28, 15.19 Hz, 3^2-H), 8.98 (1H, s, 20-H), 9.88 (1H, s, 10-H), 9.70 (1H, s, 5-H) and 3.19 (1H, s, 7^1-H) (Table 4.30 p92 and Figure A.35).

DEPT ^{13}C NMR δ ppm, in *pyridine-d_5*, 194.7 (13^1-C), 11.5 (7^1-C), 173.0 (19-C), 91.2 (13^2-C), 173.9 (17^3-C), 142.6 (1-C), 136.7 (3-C), 138.6 (11-C), 132.8 (2-C), 129.8 (3^1-C), 123.3 (3^2-C), 105.1 (10-C), 98.6 (5-C), 91.8 (20-C), 53.1 (13^4-C), 51.3 (17-C), 51.1 (18-C), 32.7 (17^2-C), 32.4 (17^1-C), 23.1 (18^1-C), 18.0 (8^2-C), 19.3 (8^1-C), 12.4 (12^1-C) and 12.5 (2^1-C) (Table 4.31 p93 and Figure A.36).

3.6.6 Purification and Characterization of Compound 6

Compound 6 was obtained from 3.70 g of fraction IV of the first column chromatography separation of chloroform crude extract. Repetitive silica gel column chromatography separation is indicated in Scheme 3.6. To provide pure compound 6 as white powder (1.04 g).

FT-IR spectrum ν$_{max}$ (KBr) values are 3500-3200, 2390, 1640, 1470, 1383, 1250, 1160, 1075 and 1019 cm^{-1} (Table 4.36 p101 and Figure A.41).

^{1}H NMR δ (ppm) values, in pyridine-d_5, 0.95 (1H, m, 9-H), 2.00 (2H, m, 12-H), 5.07 (1H, d, J = 7.7 Hz, 1$^'$-H), 4.08 (1H, m, 2$^'$-H), 7.07 (1H, d, J = 4.0 Hz, 2$^'$-OH), 6.40 (1H, t, J = 6.2 Hz, 6$^'$-OH), 4.30 (1H, m, 3$^'$-H), 1.60 (1H, m, 24-H), 4.00 (1H, m, 3-H), 0.95 (1H, m, 14-H), 0.77 (3H, s, 18-H), 0.95 (3H, m, 21-H), 1.45 (1H, m, 20-H), 1.40 (2H, m, 11-H), 1.40 (1H, m, 8-H) and 1.25 (2H, m, 15-H) (Table 4.37 p102 and Figure A.42).
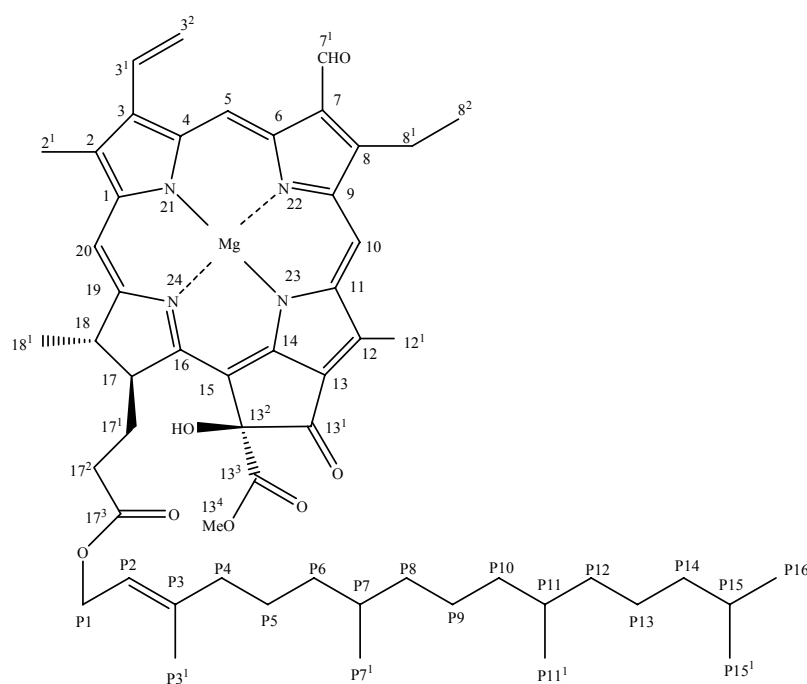
DEPT ^{13}C NMR δ (ppm) values, in pyridine-d_5, 141.4 (5-C), 122.4 (6-C), 139.3 (22-C), 130.0 (23-C), 122.4 (3-C), 37.4 (1-C), 57.3 (14-C), 30.0 (5-C), 40.5 (20-C), 40.5 (12-C), 56.8 (17-C), 13.0 (29-C), 103.1 (1´-C), 75.8 (2´-C), 79.1 (3´-C), 72.2 (4´-C), 78.9 (5´-C) and 63.4 (6´-C) (Table 4.38 p105 and Figure A.43).

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Structural Elucidation of 13^2-hydroxy-(13^2-S)-chlorophyll b

Figure 4.1 Structure of Compound 1.

The characteristic bright green color and the UV spectrum (Figure A.1) of compound 1 suggested that compound 1 is a chlorophyll derivative compound (Watanabe, Yamamoto, Ihshkawa, Yagi, Sakata, Brinen and Clardy, 1993).

The IR spectrum of compound 1 is shown in Figure A.2 and the absorption peaks were assigned as shown in Table 4.1.

Table 4.1 IR Absorption Band Assignment of Compound 1.

Wave number (cm^{-1})	Intensity	Assignment
3028	m	C-H Stretch
1730, 1638	m	C=O Stretch
1310	m	C-H Bend

Information from ^{1}H-NMR (Figure A.3) and DEPT ^{13}C-NMR (Figure A.4) spectra showed the necessary diagnostic peaks to be identified as a chlorophyll b derivative with a hydroxyl group on C-13^2. The 3H-2^1 and 3H-12^1 resonances occurred as sharp singlets at δ 3.27 and 3.68 ppm, respectively. H-5, H-10, and H-20 were found as sharp singlets at δ 10.91, 10.06 and 8.55 ppm, respectively. A vinyl group was present at C-3 as indicated by the H-3^1 doublet of doublets at δ 8.26 ($J =$ 11.39, 15.19 Hz) ppm and the H-3^2 (E) and the H-3^2 (Z) doublet of doublets at δ 6.49 ($J =$ 2.28, 15.19 Hz) ppm and 6.06 ($J =$ 2.28, 11.39) ppm, respectively. An ethyl group was found to occur at C-8. The 2H-8^1 resonances appeared as a quartet at δ 4.18 ($J =$ 7.65 Hz) ppm and 3H-8^2 occurred as a triplet at δ 1.75 ($J =$ 7.55 Hz) ppm. Similarly, as with chlorophyll b, a five membered carbocyclic ring was present at position 13. A keto group was found at C-13^1 as indicated by the fully substituted carbon resonance at δ 195.4 ppm. The C-13^2 resonances occurred at δ 92.2 ppm as with chlorophyll b and a methyl ester was also present at C-13^3. This was indicated by C-13^3 occurring as a fully substituted carbon resonance at δ 175.0 ppm and the C-13^4 methyl carbon resonance being present at δ 53.3 ppm. The H-13^4 resonance was found as a sharp singlet at δ 3.74 ppm. The characteristic signals of phytyl ester appeared at δ 5.38 (H-P2), 4.66 (H-P1), 61.6 (C-P1) and 120.8 (C-P2) ppm.

Compound 1 exhibited the ^{1}H-NMR and ^{13}C-NMR chemical shifts identical to 13^2-hydroxy-(13^2-S)-chlorophyll b (Hynninen, Leppakases and Mesilaakso, 2006). A comparison of the ^{1}H-NMR and ^{13}C-NMR chemical shifts of compound 1 and 13^2-hydroxy-(13^2-S)-chlorophyll b (Hynninen *et al.*, 2006) are shown in Tables 4.2 and 4.3. These data indicated that compound 1 is 13^2-hydroxy-(13^2-S)-chlorophyll b (Figure 4.1).

Table 4.2 The ^{1}H-NMR Chemical Shifts of Compound 1$^{\#}$ and 13^2-hydroxy-(13^2-S)-chlorophyll b*.

Proton Position	Chemical Shifts in ppm (Coupling Constant in Hz)	
	Compound 1	13^2-hydroxy-(13^2-S)-chlorophyll b
2^1	3.27 (*s*)	3.30 (*s*)
3^1	8.26 (*dd*, J = 11.39, 15.19)	8.30 (*dd*, J = 11.60, 17.80)
3^2 (*E*)	6.49 (*dd*, J = 2.28, 15.19)	6.28 (*dd*, J = 1.50, 17.80)
3^2 (*Z*)	6.06 (*dd*, J = 2.28, 11.39)	6.04 (*dd*, J = 1.50, 11.60)
5	10.91 (*s*)	10.17 (*s*)
7^1	11.57 (*s*)	11.20 (*s*)
8^1	4.18 (*q*, J = 7.65)	4.19 (*q*, J = 7.60)
8^2	1.75 (*t*, J = 7.55)	1.79 (*t*, J = 7.60)
10	10.06 (*s*)	9.92 (*s*)
12^1	3.68 (*s*)	3.62 (*s*)
13^2-OH	6.68 (*s*)	6.16 (*s*)

Table 4.2 (Continued).

Proton Position	Chemical Shifts in ppm (Coupling Constant in Hz)	
	Compound 1	13^2-hydroxy-(13^2-S)-chlorophyll b
13^4-OMe	3.74 (s)	3.60 (s)
17	5.14 (m)	4.08 (m)
18	4.49 (dq, J = 7.30)	4.49 (dq, J = 7.30)
18^1	1.56 (d, J = 7.25)	1.64 (d, J = 7.30)
20	8.55 (s)	8.49 (s)

[#]500 MHz ^{1}H, pyridine d_5
[*]500 MHz ^{1}H, acetone d_6

Table 4.3 The ^{13}C-NMR Chemical Shifts of Compound 1[#] and 13^2-hydroxy-(13^2-S)-

chlorophyll b[*].

Carbon Position	Chemical Shifts in ppm	
	Compound 1	13^2-hydroxy-(13^2-S)-chlorophyll b
1	156.6	157.5
2	149.5	136.8
2^1	12.8	12.4
3	140.9	141.1
3^1	130.9	130.9
3^2	120.8	120.8
4	137.3	149.8

Table 4.3 (Continued).

Carbon Position	Chemical Shifts in ppm	
	Compound 1	13^2-hydroxy-(13^2-*S*)-chlorophyll b
5	104.5	104.2
6	148.7	149.4
7	132.2	131.6
7^1	188.8	188.5
8	156.4	155.6
8^1	19.9	19.6
8^2	19.9	19.4
9	143.3	143.6
10	110.5	111.1
11	139.4	149.3
12	131.4	138.9
12^1	13.3	12.8
13	149.0	130.5
13^1	195.4	193.5
13^2	92.2	90.5
13^3	175.0	173.8
13^4	53.3	53.2
14	163.2	163.9
15	110.7	108.9

Table 4.3 (Continued).

Carbon Position	Chemical Shifts in ppm	
	Compound 1	13^2-hydroxy-(13^2-*S*)-chlorophyll b
16	160.1	161.4
17	50.8	52.1
17^1	31.1	32.2
17^2	31.6	31.6
17^3	173.5	173.8
18	50.6	49.7
18^1	23.3	23.3
19	170.4	171.6
20	94.1	94.4

[#]125 MHz ^{13}C, pyridine d_5
[*]125 MHz ^{13}C, acetone d_6

Further studies of the structure of compound 1 were done by analysis of the COSY, HSQC, HMBC and NOESY correlations (see also Tables 4.4-4.7).

The COSY spectrum (Figure A.5) indicated that one proton doublet of doublets at δ 8.26 (H-3^1) ppm coupled to one proton doublet of doublets at δ 6.49 (H-3^2 *E*) ppm and one proton doublet of doublets at 6.06 (H-3^2 *Z*) ppm. The signal at δ 5.38 (H-P2) ppm correlated only with the signal at δ 4.66 (H-P1) ppm. The signal of one proton at 4.49 (H-18) ppm showed a cross peak with a signal at δ 1.56 (H-18^1) ppm. Study of the COSY (H-H) spectrum of compound 1 led to the complete assignment of ^{1}H and ^{13}C shift signals as shown in Table 4.4.

Table 4.4 Observed Correlations in the COSY (H-H) Spectrum of Compound 1.

δ ^{1}H-NMR in ppm (Position)	COSY in ppm (Coupled Hydrogen)
8.26 (H-3^1)	6.49 (H-3^2 *E*), 6.06 (H-3^2 *Z*)
4.18 (H-8^1)	1.75 (H-8^2)
5.14 (H-17)	2.56, 2.47 (H-17^1)
2.56, 2.47 (H-17^1)	2.58, 2.49 (H-17^2)
4.49 (H-18)	1.56 (H-18^1)
5.38 (H-P2)	4.66 (H-P1)

One bond correlation (HSQC) data revealed that the proton at δ 10.06 (H-10) ppm was attached to the carbon at δ 110.5 (C-10) ppm, the proton at δ 10.91 (H-5) ppm was attached to the carbon at δ 104.5 (C-5) ppm, the proton at δ 8.55 (H-20) ppm was attached to the carbon at δ 94.1 (C-20) ppm, the proton at δ 8.26 (H-3^1) ppm was attached to the carbon at δ 130.9 (C-3^1) ppm, the protons at δ 6.49 (H-3^2 *E*) and 6.06 (H-3^2 *Z*) ppm were attached to the carbon at δ 120.8 (C-3^2) ppm, the proton at δ 5.14 (H-17) ppm was joined with the carbon at δ 50.8 (C-17) ppm, the proton at δ 4.49 (H-18) ppm was joined with the carbon at δ 50.6 (C-18) ppm, the proton at δ 3.68 (H-12^1) ppm was joined with the carbon at δ 13.3 (C-12^1) ppm, the proton at δ 4.18 (H-8^1) ppm was joined with the carbon at δ 19.9 (C-8^1) ppm, and the proton at δ 3.27 (H-2^1) ppm was joined with the carbon at δ 12.8 (C-2^1) ppm. The protons attached to the carbons in compound 1 were assigned by the HSQC spectrum as shown in Figure A.6 and Table 4.5.

**Table 4.5** Observed Correlations in HSQC Vicinal (C-H) Spectrum of Compound 1.

δ ^{1}H-NMR in ppm (Position)	HSQC in ppm (Attached Carbon)
3.68 (H-12^1)	13.3 (C-12^1)
8.26 (H-3^1)	130.9 (C-3^1)
6.49 (H-3^2 *E*)	120.8 (C-3^2)
6.06 (H-3^2 *Z*)	120.8 (C-3^2)
10.91 (H-5)	104.5 (C-5)
11.57 (H-7^1)	188.8 (C-7^1)
4.18 (H-8^1)	19.9 (C-8^1)
1.75 (H-8^2)	19.9 (C-8^2)
10.06 (H-10)	110.5 (C-10)
5.14 (H-17)	50.8 (C-17)
4.49 (H-18)	50.6 (C-18)
1.56 (H-18^1)	23.3 (C-18^1)
8.55 (H-20)	94.1 (C-20)

In the H-C long range coupling spectrum obtained by HMBC, the proton at δ 6.49 (H-3^2 *E*) ppm showed cross peaks with the carbons at 130.9 (C-3^1) and 140.9 (C-3) ppm, the proton at δ 6.06 (H-3^2 *Z*) ppm showed a cross peak with the carbon at 140.9 (C-3) ppm, the proton at δ 1.56 (H-18^1) ppm showed cross peaks with the carbons at 170.4 (C-19) ppm, 50.6 (C-18) and 50.8 (C-17) ppm, the proton at δ 5.14 (H-17) ppm showed a cross peak with the carbon at 23.3 (C-18^1) ppm, the proton at δ 3.27 (H-2^1) ppm showed cross peaks with the carbons at 140.9 (C-3) and 156.6 (C-1)

ppm and the proton at δ 8.55 (H-20) ppm showed a cross peak with the carbon at

149.5 (C-2) ppm (Figure A.7, Figure 4.2 and Table 4.6).
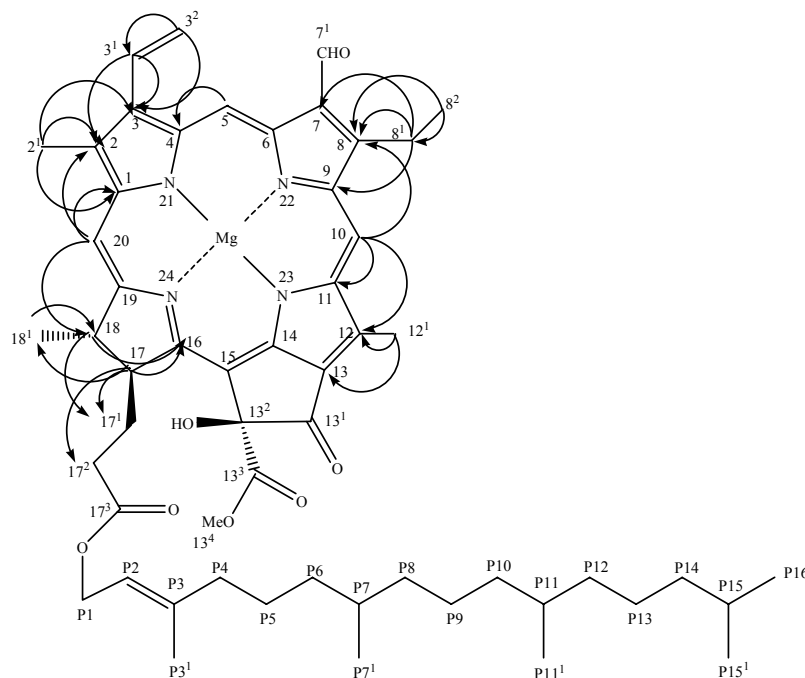
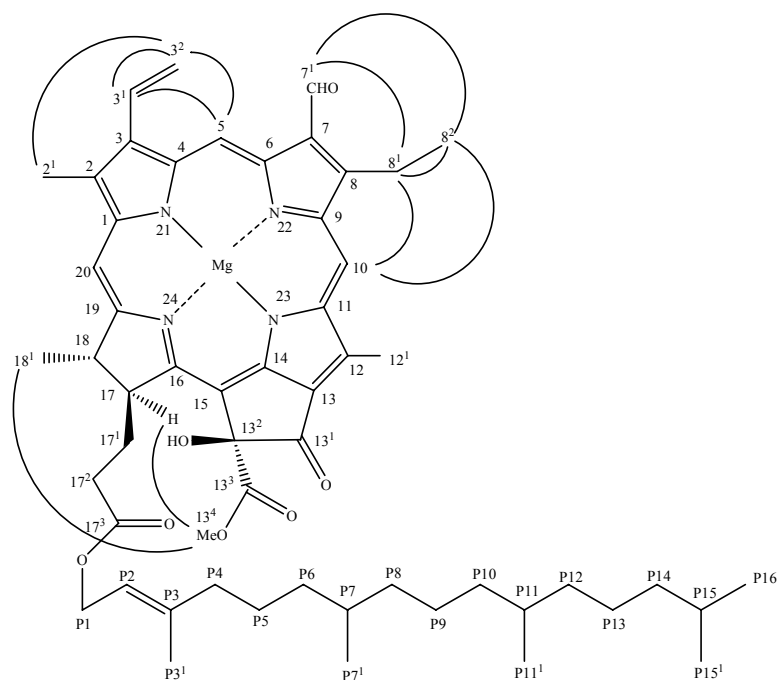**Figure 4.2** The HMBC correlations of compound 1.

Table 4.6 Observed Long Range C-H Correlations in the HMBC of Compound 1.

δ ^{1}H-NMR in ppm (Position)	HMBC in ppm (Correlation Carbon)
3.27 (H-2^1)	156.6 (C-1), 149.5 (C-2), 140.8 (C-3)
6.49 (H-3^2 E)	130.9 (C-3^1), 140.9 (C-3)
6.06 (H-3^2 Z)	140.9 (C-3)
10.91 (H-5)	140.9 (C-3)
4.18 (H-8^1)	19.9 (C-8^2)
1.75 (H-8^2)	19.9 (C-8^1), 156.4 (C-8)
10.06 (H-10)	139.4 (C-11), 156.4 (C-8)

Table 4.6 (Continued).

δ ^{1}H-NMR in ppm (Position)	HMBC in ppm (Correlation Carbon)
3.68 (H-12^1)	131.4 (C-12), 139.4 (C-11)
3.74 (H-13^4)	175.0 (C-13^3)
5.14 (H-17)	23.3 (C-18^1)
4.49 (H-18)	23.3 (C-18^1), 31.1 (C-17^1), 31.6 (C-17^2)
1.56 (H-18^1)	170.4 (C-19), 50.6 (C-18), 50.8 (C-17), 53.3 (C-13^4)
8.55 (H-20)	149.5 (C-2), 156.6 (C-1), 50.8 (C-17), 50.6 (C-18)

The NOESY correlation spectrum showed the appearance of coupling between the proton at δ 1.75 (H-8^2) ppm and the proton at 4.18 (H-8^1) ppm, coupling between the proton at δ 5.14 (H-17) ppm and the proton at δ 3.74 (H-13^4) ppm, coupling between the proton at δ 11.57 (H-7^1) ppm and the proton at δ 1.75 (H-8^2) and 4.18 (H-8^1) ppm, coupling between the proton at δ 10.91 (H-5) ppm and the protons at δ 6.49 (H-3^2 *E*) and 8.26 (H-3^1) ppm. The 3D structure of compound 1 could be drawn as shown in Figure 4.3. The NOE effects obtained from the NOESY spectra could be seen as indicated in Figure A.8, Figure 4.3 and Table 4.7 which are in good agreement with the proposed structure.

Figure 4.3 The NOESY correlations of compound 1.

Table 4.7 Observed NOE Correlations of Compound 1.

δ ^{1}H-NMR in ppm (Position)	NOESY in ppm (Cross Peaks)
8.26 (H-3^1)	6.49 (H-3^2 *E*), 6.06 (H-3^2 *Z*)
6.49 (H-3^2 *E*)	3.27 (H-2^1), 11.57 (H-7^1)
10.91 (H-5)	6.49 (H-3^2 *E*), 8.36 (H-3^1)
11.57 (H-7^1)	1.75 (H-8^2), 4.18 (H-8^1)
1.75 (H-8^2)	4.18 (H-8^1)
10.06 (H-10)	1.75 (H-8^2), 4.18 (H-8^1)
5.14 (H-17)	3.74 (H-13^4)
1.56 (H-18^1)	3.74 (H-13^4)
8.55 (H-20)	3.27 (H-2^1), 3.74 (H-13^4)

4.2 Structural Elucidation of 13^2-hydroxy-(13^2-R)-chlorophyll b
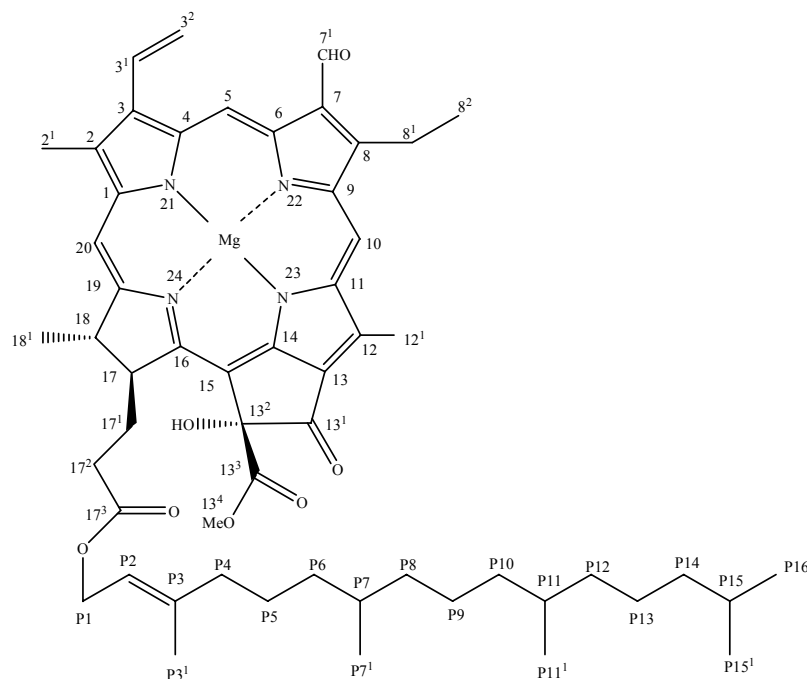
Figure 4.4 Structure of Compound 2.

Compound 2 was obtained as bright green powder. The UV spectrum (Figure A.9) showed absorptions of a chlorophyll derivative compound (Watanabe *et al.*, 1993). The IR spectrum of compound 2 is shown in Figure A.10 and the absorption peaks were assigned as shown in Table 4.8.

Table 4.8 IR Absorption Band Assignment of Compound 2.

Wave number (cm^{-1})	Intensity	Assignment
3030	m	C-H Stretch
1728, 1630	m	C=O Stretch
1306	m	C-H Bend

Information from ^{1}H-NMR (Figure A.11) and DEPT ^{13}C-NMR (Figure A.12) spectra showed the necessary diagnostic peaks to be identified as a chlorophyll b derivative with a hydroxyl group on C-13^2. The 3H-2^1 and 3H-12^1 resonances occurred as sharp singlets at δ 3.27 and 3.68 ppm, respectively. H-5, H-10, and H-20 were found as sharp singlets at δ 10.91, 10.06 and 8.57 ppm, respectively. A vinyl group was present at C-3 as indicated by the H-3^1 doublet of doublets at δ 8.26 (J = 11.39, 15.19 Hz) ppm and the H-3^2 (E) and the H-3^2 (Z) doublet of doublets at δ 6.49 (J = 2.28, 15.19 Hz) and 6.06 (J = 2.28, 11.39) ppm, respectively. An ethyl group was found to occur at C-8. The 2H-8^1 resonances appeared as a quartet at δ 4.18 (J = 7.65 Hz) ppm and 3H-8^2 occurred as a triplet at δ 1.75 (J = 7.55 Hz) ppm. Similarly, as with chlorophyll b, a five membered carbocyclic ring was present at position 13. A keto group was found at C-13^1 as indicated by the fully substituted carbon resonance at δ 195.3 ppm. The C-13^2 resonances occurred at δ 92.4 as with chlorophyll b and a methyl ester was also present at C-13^3. This was indicated by C-13^3 occurring as a fully substituted carbon resonance at δ 174.4 ppm and the C-13^4 methyl carbon resonance being present at δ 53.3 ppm. The H-13^4 resonance was found as a sharp singlet at δ 3.74 ppm. The characteristic signals of phytyl ester appeared at δ 5.13 (H-P2), 4.42 (H-P1), 61.4 (C-P1) and 119.3 (C-P2) ppm.

Compound 2 exhibited the ^{1}H-NMR and ^{13}C-NMR chemical shifts identical to 13^2-hydroxy-(13^2-R)-chlorophyll b (Hynninen et $al.,$ 2006). A comparison of the ^{1}H-NMR and ^{13}C-NMR chemical shifts of compound 1 and 13^2-hydroxy-(13^2-R)-chlorophyll b (Hynninen et $al.$, 2006) are shown in Tables 4.9 and 4.10. These data indicated that compound 2 is 13^2-hydroxy-(13^2-R)-chlorophyll b (Figure 4.4).

Table 4.9 The ^{1}H-NMR Chemical Shifts of Compound 2$^\#$ and 13^2-hydroxy-(13^2-R)-

chlorophyll b*.

Proton Position	Chemical Shifts in ppm (Coupling Constant in Hz)	
	Compound 2	13^2-hydroxy-(13^2-R)-chlorophyll b
2^1	3.27 (*s*)	3.30 (*s*)
3^1	8.26 (*dd*, *J* = 11.39, 15.19)	8.30 (*dd*, *J* = 11.60, 17.80)
3^2 (*E*)	6.49 (*dd*, *J* = 2.28, 15.19)	6.28 (*dd*, *J* = 1.50, 17.80)
3^2 (*Z*)	6.06 (*dd*, *J* = 2.28, 11.39)	6.04 (*dd*, *J* = 1.50, 11.60)
5	10.91 (*s*)	10.17 (*s*)
7^1	11.57 (*s*)	11.20 (*s*)
8^1	4.18 (*q*, *J* = 7.65)	4.19 (*q*, *J* = 7.60)
8^2	1.75 (*t*, *J* = 7.55)	1.79 (*t*, *J* = 7.60)
10	10.06 (*s*)	9.92 (*s*)
12^1	3.68 (*s*)	3.62 (*s*)
13^2-OH	6.46 (*s*)	6.12 (*s*)
13^4-OMe	3.74 (*s*)	3.60 (*s*)
17	5.54 (*m*)	4.63 (*m*)
18	4.50 (*dq*, *J* = 7.30)	4.50 (*dq*, *J* = 7.30)
18^1	1.56 (*d*, *J* = 7.25)	1.64 (*d*, *J* = 6.95)
20	8.57 (*s*)	8.52 (*s*)

$^\#$500 MHz ^{1}H, pyridine d_5
*500 MHz ^{1}H, acetone d_6

Table 4.10 The ^{13}C-NMR Chemical Shifts of Compound 2$^{\#}$ and 13^2-hydroxy-(13^2-R)-chlorophyll b*.

Carbon Position	Chemical Shifts in ppm	
	Compound 2	13^2-hydroxy-(13^2-R)-chlorophyll b
1	156.4	157.4
2	149.5	136.9
2^1	12.8	12.4
3	140.8	141.1
3^1	130.9	130.9
3^2	120.7	120.9
4	137.1	149.7
5	104.2	104.2
6	148.6	149.2
7	131.9	131.6
7^1	188.7	188.5
8	156.2	155.8
8^1	19.8	19.8
8^2	19.6	19.6
9	143.5	143.5
10	109.9	111.0
11	139.3	149.1
12	131.0	139.3

Table 4.10 (Continued).

Carbon Position	Chemical Shifts in ppm	
	Compound 2	13^2-hydroxy-(13^2-R)-chlorophyll b
12^1	12.9	12.8
13	149.1	130.5
13^1	195.3	193.4
13^2	92.4	90.7
13^3	174.4	174.4
13^4	53.4	53.4
14	163.2	163.8
15	109.2	109.2
16	160.4	160.4
17	50.4	50.4
17^1	31.0	31.0
17^2	31.0	31.0
17^3	173.3	173.3
18	50.3	50.3
18^1	23.3	23.3
19	171.7	171.7
20	94.2	94.2

[#]125 MHz ^{13}C, pyridine d_5
[*]125 MHz ^{13}C, acetone d_6

Further studies of the structure of compound 2 were done by analysis of the COSY, HSQC, HMBC and NOESY correlations (see also Tables 4.11- 4.14).

The COSY spectrum (Figure A.13) indicated that one proton doublet of doublets at δ 8.26 (H-3^1) ppm coupled to one proton doublet of doublets at δ 6.49 (H-3^2 *E*) ppm and one proton doublet of doublets at 6.06 (H-3^2 *Z*) ppm. The signal at δ 5.13 (H-P2) ppm correlated only with the signal at δ 4.42 (H-P1) ppm. The signal of one proton at 4.50 (H-18) ppm showed a cross peak with a signal at δ 1.56 (H-18^1) ppm. Study of the COSY (H-H) spectrum of compound 1 led to the complete assignment of ^{1}H and ^{13}C shift signals as shown in Table 4.11.

Table 4.11 Observed Correlations in the COSY (H-H) Spectrum of Compound 2.

| δ ^{1}H-NMR in ppm (Position)	COSY in ppm (Coupled Hydrogen)
8.26 (H-3^1)	6.49 (H-3^2 *E*), 6.06 (H-3^2 *Z*)
4.18 (H-8^1)	1.75 (H-8^2)
5.54 (H-17)	2.34, 2.30 (H-17^1)
2.34, 2.30 (H-17^1)	2.21, 1.95 (H-17^2)
4.49 (H-18)	1.56 (H-18^1)
4.42 (H-P2)	4.42 (H-P1)

One bond correlation (HSQC) data revealed that the proton at δ 10.06 (H-10) ppm was attached to the carbon at δ 109.9 (C-10) ppm, the proton at δ 10.91 (H-5) ppm was attached to the carbon at δ 104.2 (C-5) ppm, the proton at δ 8.57 (H-20) ppm was attached to the carbon at δ 94.2 (C-20) ppm, the proton at δ 8.26 (H-3^1)ppm was attached to the carbon at δ 130.9 (C-3^1) ppm, the protons at δ 6.49 (H-3^2 *E*) and 6.06

(H-3^2 Z) ppm were attached to the carbon at δ 120.7 (C-3^2) ppm, the proton at δ 5.54 (H-17) ppm was joined with the carbon at δ 50.8 (C-17) ppm, the proton at δ 4.50 (H-18) ppm was joined with the carbon at δ 50.3 (C-18) ppm, the proton at δ 3.68 (H-12^1) ppm was joined with the carbon at δ 12.9 (C-12^1) ppm, the proton at δ 4.18 (H-8^1) ppm was joined with the carbon at δ 19.8 (C-8^1) ppm, and the proton at δ 3.27 (H-2^1) ppm was joined with the carbon at δ 12.8 (C-2^1) ppm. The protons attached to the carbons in compound 2 were assigned by the HSQC spectrum as shown in Figure A.14 and Table 4.12.

Table 4.12 Observed Correlations in HSQC Vicinal (C-H) Spectrum of Compound 2.

δ ^{1}H-NMR in ppm (Position)	HSQC in ppm (Attached Carbon)
3.68 (H-12^1)	12.9 (C-12^1)
8.26 (H-3^1)	130.9 (C-3^1)
6.49 (H-3^2 E), 6.06 (H-3^2 Z)	120.7 (C-3^2)
10.91 (H-5)	104.2 (C-5)
11.57 (H-7^1)	188.7 (C-7^1)
4.18 (H-8^1)	19.8 (C-8^1)
1.75 (H-8^2)	19.6 (C-8^2)
10.06 (H-10)	109.9 (C-10)
5.54 (H-17)	50.4 (C-17)
4.50 (H-18)	50.3 (C-18)
1.56 (H-18^1)	23.3 (C-18^1)
8.57 (H-20)	94.2 (C-20)

In the H-C long range coupling spectrum obtained by HMBC, the proton at δ 6.49 (H-3^2 E) ppm showed cross peaks with the carbons at 130.9 (C-3^1) and 140.9 (C-3) ppm, the proton at δ 6.06 (H-3^2 Z) ppm showed a cross peak with the carbon at 140.8 (C-3) ppm, the proton at δ 1.56 (H-18^1) ppm showed cross peaks with the carbons at 171.7 (C-19), 50.3 (C-18) and 50.4 (C-17) ppm, the proton at δ 5.54 (H-17) ppm showed a cross peak with the carbon at 23.3 (C-18^1) ppm, the proton at δ 3.27 (H-2^1) ppm showed cross peaks with the carbons at 140.8 (C-3) and 156.4 (C-1) ppm and the proton at δ 8.57 (H-20) ppm showed a cross peak with the carbon at 149.5 (C-2) ppm (Figure A.15, Figure 4.5 and Table 4.13).
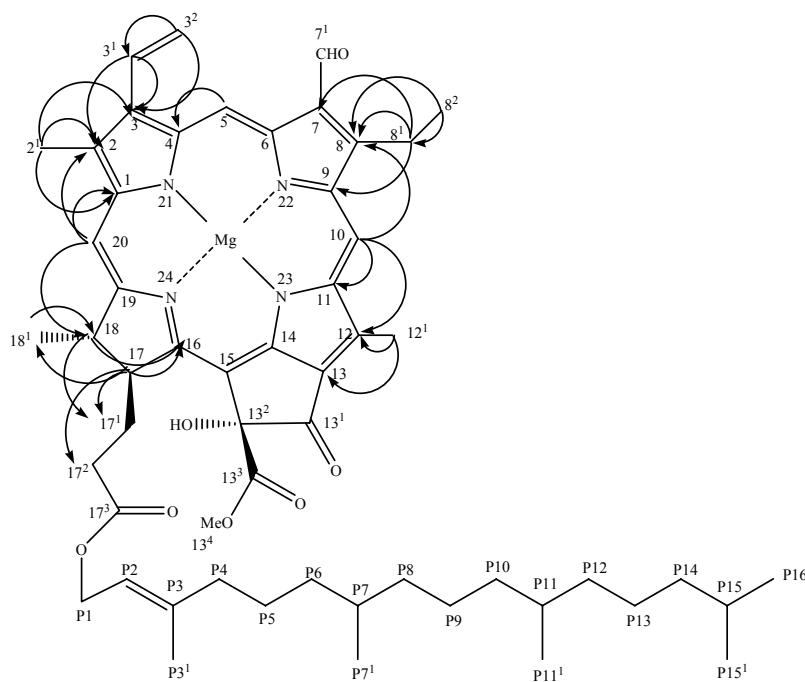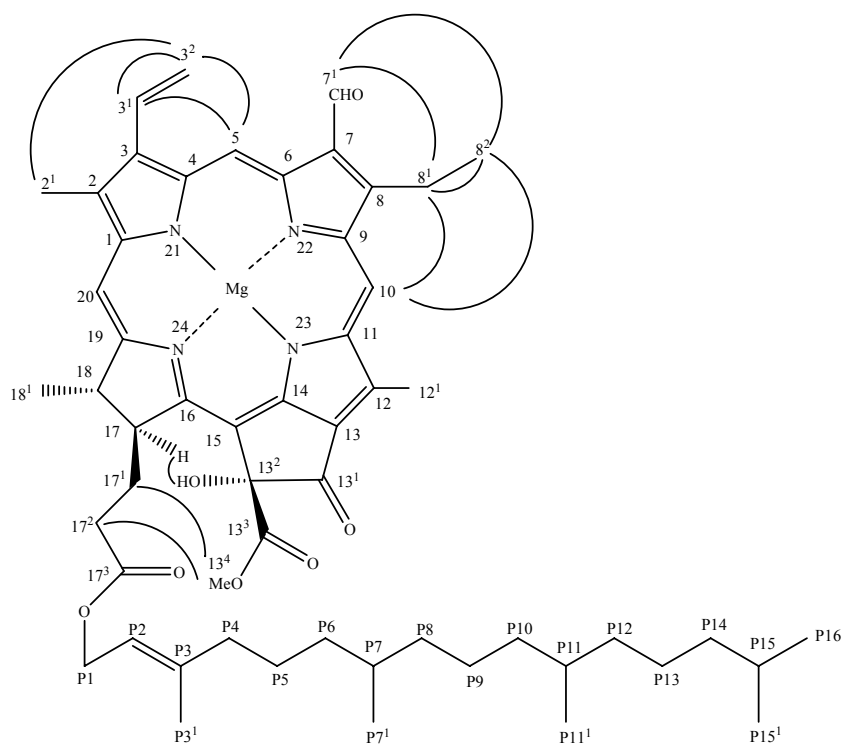

Figure 4.5 The HMBC correlations of compound 2.

Table 4.13 Observed Long Range C-H Correlations in the HMBC of Compound 2.

δ ^{1}H-NMR in ppm (Position)	HMBC in ppm (Correlation Carbon)
3.27 (H-2^1)	156.4 (C-1), 149.5 (C-2), 140.8 (C-3)
6.49 (H-3^2 E)	130.9 (C-3^1), 140.8 (C-3)
6.06 (H-3^2 Z)	140.8 (C-3)
10.91 (H-5)	140.8 (C-3)
4.18 (H-8^1)	19.9 (C-8^2)
1.75 (H-8^2)	19.9 (C-8^1)
10.06 (H-10)	139.3 (C-11)
3.68 (H-12^1)	131.0 (C-12), 139.3 (C-11)
3.74 (H-13^4)	174.4 (C-13^3)
5.54 (H-17)	23.3 (C-18^1)
4.50 (H-18)	23.3 (C-18^1), 31.0 (C-17^1), 31.0 (C-17^2)
1.56 (H-18^1)	171.7 (C-19), 50.3 (C-18), 50.4 (C-17), 53.4 (C-13^4)
8.57 (H-20)	149.5 (C-2), 156.4 (C-1), 50.4 (C-17), 50.3 (C-18)

The NOESY correlation spectrum showed the appearance of coupling between the proton at δ 1.75 (H-8^2) ppm and the proton at 4.18 (H-8^1) ppm, coupling between the proton at δ 2.34 (H-17^1) ppm and the proton at δ 3.74 (H-13^4) ppm, coupling between the proton at δ 11.57 (H-7^1) ppm and the proton at δ 1.75 (H-8^2) and 4.18 (H-8^1) ppm, coupling between the proton at δ 10.91 (H-5) ppm and the protons at δ 6.49 (H-3^2 E) and 8.26 (H-3^1) ppm. The 3D structure of compound 2 could be drawn as

shown in Figure 4.6. The NOE method was used to determine the stereochemical configuration at C-13^2 between compounds 1 and 2. The NOE effects obtained from the NOESY spectra could be seen as indicated in Figure A.16, Figure 4.6 and Table 4.14.

**Figure 4.6** The NOESY correlations of compound 2.

Table 4.14 Observed NOE Correlations of Compound 2.

δ ^{1}H-NMR in ppm (Position)	NOESY in ppm (Cross Peaks)
8.26 (H-3^1)	6.49 (H-3^2 E), 6.06 (H-3^2 Z)
6.49 (H-3^2 E)	3.27 (H-2^1), 11.57 (H-7^1)
10.91 (H-5)	6.49 (H-3^2 E), 8.26 (H-3^1)
11.57 (H-7^1)	1.75 (H-8^2), 4.18 (H-8^1)

Table 4.14 (Continued).

δ ^{1}H-NMR in ppm (Position)	NOESY in ppm (Cross Peaks)
1.75 (H-8^2)	4.18 (H-8^1)
10.06 (H-10)	1.75 (H-8^2), 4.18 (H-8^1)
2.34 (H-17^1)	3.74 (H-13^4)
2.21 (H-17^2)	3.74 (H-13^4)
5.54 (H-17)	6.46 (H-13^2)

4.3   Structural Elucidation of 13^2-hydroxy-(13^2-*R*)-phaeophytin b
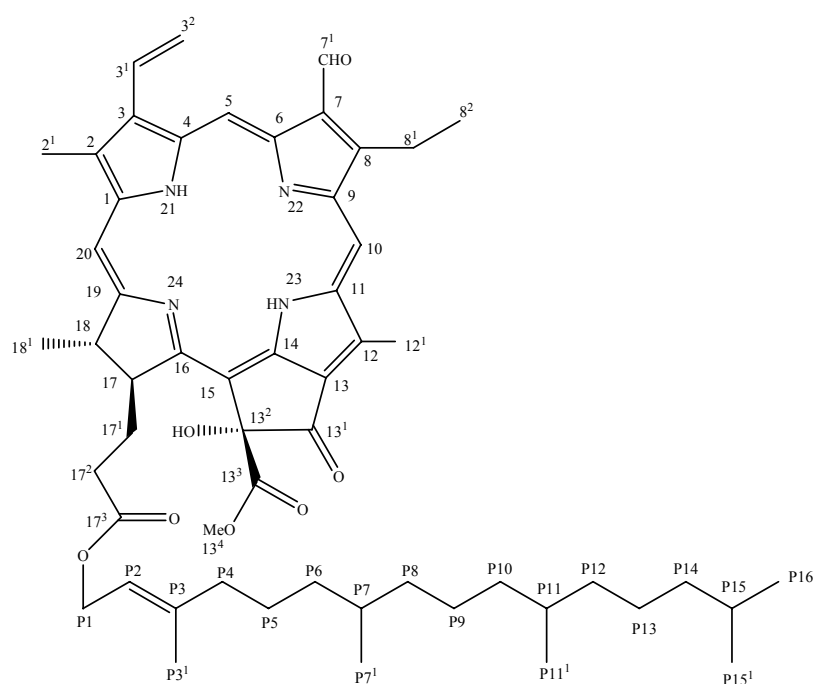
Figure 4.7 Structure of Compound 3.

Compound 3 was obtained as dark green powder. The UV spectrum (Figure A.17) showed absorptions of a chlorophyll derivative compound (Watanabe *et al*.,

1993). The IR spectrum of compound 3 is shown in Figure A.18 and the absorption peaks were assigned as shown in Table 4.15.

Table 4.15 IR Absorption Band Assignment of Compound 3.

Wave number (cm^{-1})	Intensity	Assignment
3429	s	N-H Stretch
2940	m	C-H Stretch
1720, 1630	m	C=O Stretch
1310	m	C-H Bend

Information from ^{1}H-NMR (Figure A.19) and DEPT ^{13}C-NMR (Figure A.20) spectra showed the necessary diagnostic peaks to be identified as a phaeophytin b derivative with a hydroxyl group on C-13^2. The 3H-2^1 and 3H-12^1 resonances occurred as sharp singlets at δ 3.35 and 3.70 ppm , respectively. H-5, H-10, and H-20 were found as sharp singlets at δ 11.01, 10.16 and 8.89 ppm, respectively. A vinyl group was present at C-3 as indicated by the H-3^1 doublet of doublets at δ 8.19 ($J =$ 11.39, 15.19 Hz) ppm and the H-3^2 (E) and the H-3^2 (Z) doublet of doublets at δ 6.54 ($J =$ 2.28, 15.19 Hz) and 6.06 ($J =$ 2.28, 11.39) ppm, respectively. An ethyl group was found to occur at C-8. The 2H-8^1 resonances appeared as a quartet at δ 4.22 ($J =$ 7.65 Hz) ppm and 3H-8^2 occurred as a triplet at δ 1.83 ($J =$ 7.55 Hz) ppm. Similarly, as with phaeophytin b, a five membered carbocyclic ring was present at position 13. A keto group was found at C-13^1 as indicated by the fully substituted carbon resonance at δ 196.5 ppm. The C-13^2 resonances occurred at δ 92.1 ppm as with phaeophytin b and a methyl ester was also present at C-13^3. This was indicated by C-13^3 occurring as

a fully substituted carbon resonance at δ 172.8 ppm and the C-13^4 methyl carbon resonance being present at δ 54.9 ppm. The H-13^4 resonance was found as a sharp singlet at δ 3.85 ppm. The characteristic signals of phytyl ester appeared at δ 5.40 (H-P2), 4.72 (H-P1), 61.9 (C-P1) and 119.0 (C-P2) ppm.

Compound 3 exhibited the ^{1}H-NMR and ^{13}C-NMR chemical shifts identical to 13^2-hydroxy-(13^2-R)-phaeophytin b (Buchanan, Hashimoto and Asakawa, 1996). A comparison of the ^{1}H-NMR and ^{13}C-NMR chemical shifts of compound 3 and 13^2-hydroxy-(13^2-S)-phaeophytin b (Shuyprom, 2005) are shown in Tables 4.16 and 4.17. These data indicated that compound 3 is 13^2-hydroxy-(13^2-R)-phaeophytin b (Figure 4.7).

Table 4.16 The ^{1}H-NMR Chemical Shifts of Compound 3$^{\#}$ and 13^2-hydroxy-(13^2-S)-phaeophytin b$^{\#}$.

Proton Position	Chemical Shifts in ppm (Coupling Constant in Hz)	
	Compound 3	13^2-hydroxy-(13^2-S)-phaeophytin b
2^1	3.35 (*s*)	3.45 (*s*)
3^1	8.19 (*dd*, *J* = 11.39, 15.19)	8.18 (*dd*, *J* = 10.90, 11.65)
3^2 (*E*)	6.54 (*dd*, *J* = 2.28, 15.19)	6.54 (*dd*, *J* = 2.50, 17.80)
3^2 (*Z*)	6.21 (*dd*, *J* = 2.28, 11.39)	6.21 (*dd*, *J* = 2.50, 12.60)
5	11.00 (*s*)	11.01 (*s*)
7^1	11.50 (*s*)	11.48 (*s*)
8^1	4.22 (*q*, *J* = 7.65)	4.19 (*q*, *J* = 7.65)

Table 4.16 (Continued).

Proton Position	Chemical Shifts in ppm (Coupling Constant in Hz)	
	Compound 3	13^2-hydroxy-(13^2-*S*)-phaeophytin b
8^2	1.83 (*t*, *J* = 7.55)	1.81 (*t*, *J* = 7.60)
10	10.16 (*s*)	10.16 (*s*)
12^1	3.70 (*s*)	3.72 (*s*)
13^2-OH	6.20 (*s*)	6.52 (*s*)
13^4-OMe	3.85 (*s*)	3.85 (*s*)
17	5.41 (*m*)	5.23 (*m*)
18	4.60 (*dq*, *J* = 7.30)	4.67 (*dq*, *J* = 7.30)
18^1	1.71 (*d*, *J* = 7.25)	1.74 (*d*, *J* = 7.30)
20	8.89 (*s*)	8.92 (*s*)
21-NH	-1.32 (*br, s*)	-1.33 (*br, s*)
23-NH	0.68 (*br, s*)	0.66 (*br, s*)

[#]500 MHz ^{1}H, pyridine d_5

Table 4.17 The ^{13}C-NMR Chemical Shifts of Compound $3^{\#}$ and 13^2-hydroxy-$(13^2$-$S)$-phaeophytin b$^{\#}$.

Carbon Position	Chemical Shifts in ppm	
	Compound 3	13^2-hydroxy-$(13^2$-$S)$-phaeophytin b
1	150.0	144.0
2	135.5	133.5
2^1	12.1	12.4
3	135.4	137.5
3^1	124.3	129.5
3^2	121.5	123.0
4	136.0	137.4
5	104.9	102.6
6	152.0	160.2
7	134.3	138.9
7^1	191.7	188.0
8	148.3	148.2
8^1	18.7	19.6
8^2	18.7	19.7
9	150.2	151.8
10	108.2	114.6
11	135.9	138.1
12	135.7	138.8

Table 4.17 (Continued).

Carbon Position	Chemical Shifts in ppm	
	Compound 3	13^2-hydroxy-(13^2-S)-phaeophytin b
12^1	12.3	12.5
13	135.8	128.9
13^1	196.5	194.9
13^2	92.1	91.3
13^3	172.8	174.0
13^4	54.9	53.6
14	150.4	151.1
15	106.0	111.0
16	162.5	166.8
17	51.8	51.5
17^1	31.1	32.4
17^2	31.6	31.7
17^3	173.6	173.5
18	50.3	51.1
18^1	22.7	23.3
19	178.7	175.6
20	95.1	95.1

[#]125 MHz ^{13}C, pyridine d_5

Further studies of the structure of compound 3 were done by analysis of the COSY, HSQC, HMBC and NOESY correlations (see also Tables 4.18-4.21).

The COSY spectrum (Figure A.21) indicated that one proton doublet of doublets at δ 8.19 (H-3^1) ppm coupled to one proton doublet of doublets at δ 6.54 (H-3^2 *E*) ppm and one proton doublet of doublets at 6.21 (H-3^2 *Z*) ppm. The signal at δ 5.40 (H-P2) ppm correlated only with the signal at δ 4.72 (H-P1) ppm. The signal of one proton at 4.60 (H-18) ppm showed a cross peak with a signal at δ 1.71 (H-18^1) ppm. Study of the COSY (H-H) spectrum of compound 3 led to the complete assignment of ^{1}H and ^{13}C shift signals as shown in Table 4.18.

Table 4.18 Observed Correlations in the COSY (H-H) Spectrum of Compound 3.

δ ^{1}H-NMR in ppm (Position)	COSY in ppm (Coupled Hydrogen)
8.19 (H-3^1)	6.54 (H-3^2 *E*), 6.21 (H-3^2 *Z*)
4.22 (H-8^1)	1.83 (H-8^2)
5.41 (H-17)	2.81, 2.67 (H-17^1)
2.81, 2.67 (H-17^1)	2.79, 2.46 (H-17^2)
4.60 (H-18)	1.71 (H-18^1)
5.40 (H-P2)	4.72 (H-P1)

One bond correlation (HSQC) data revealed that the proton at δ 10.16 (H-10) ppm was attached to the carbon at δ 114.6 (C-10) ppm, the proton at δ 11.00 (H-5) ppm was attached to the carbon at δ 102.6 (C-5) ppm, the proton at δ 8.89 (H-20) ppm was attached to the carbon at δ 95.1 (C-20) ppm, the proton at δ 8.19 (H-3^1) ppm was attached to the carbon at δ 124.3 (C-3^1) ppm, the protons at δ 6.54 (H-3^2 *E*) and 6.21

(H-3^2 Z) ppm were attached to the carbon at δ 121.5 (C-3^2) ppm, the proton at δ 5.41 (H-17) ppm was joined with the carbon at δ 51.8 (C-17) ppm, the proton at δ 4.60 (H-18) ppm was joined with the carbon at δ 50.3 (C-18) ppm, the proton at δ 3.70 (H-12^1) ppm was joined with the carbon at δ 12.3 (C-12^1) ppm, the proton at δ 4.22 (H-8^1) ppm was joined with the carbon at δ 18.7 (C-8^1) ppm, and the proton at δ 3.35 (H-2^1) ppm was joined with the carbon at δ 12.1 (C-2^1) ppm. The protons attached to the carbons in compound 3 were assigned by the HSQC spectrum as shown in Figure A.22 and Table 4.19.

Table 4.19 Observed Correlations in HSQC Vicinal (C-H) Spectrum of Compound 3.

δ ^{1}H-NMR in ppm (Position)	HSQC in ppm (Attached Carbon)
3.70 (H-12^1)	12.3 (C-12^1)
8.19 (H-3^1)	124.3 (C-3^1)
6.54 (H-3^2 E), 6.21 (H-3^2 Z)	121.5 (C-3^2)
11.00 (H-5)	104.9 (C-5)
11.50 (H-7^1)	191.7 (C-7^1)
4.22 (H-8^1)	18.7 (C-8^1)
1.83 (H-8^2)	18.7 (C-8^2)
10.16 (H-10)	114.6 (C-10)
5.41 (H-17)	51.8 (C-17)
4.60 (H-18)	50.3 (C-18)
1.71 (H-18^1)	22.7 (C-18^1)
8.89 (H-20)	95.1 (C-20)

In the H-C long range coupling spectrum obtained by HMBC, the proton at δ 6.54 (H-3^2 *E*) ppm showed cross peaks with the carbons at 124.3 (C-3^1) and 135.4 (C-3) ppm, the proton at δ 6.21 (H-3^2 *Z*) ppm showed a cross peak with the carbon at 135.4 (C-3) ppm, the proton at δ 1.71 (H-18^1) ppm showed cross peaks with the carbons at 178.7 (C-19), 50.3 (C-18) and 51.8 (C-17) ppm, the proton at δ 5.41 (H-17) ppm showed a cross peak with the carbon at 22.7 (C-18^1) ppm, the proton at δ 3.35 (H-2^1) ppm showed cross peaks with the carbons at 135.4 (C-3) and 150.0 (C-1) ppm and the proton at δ 8.89 (H-20) ppm showed a cross peak with the carbon at 133.5 (C-2) ppm. (Figure A.23, Figure 4.8 and Table 4.20).
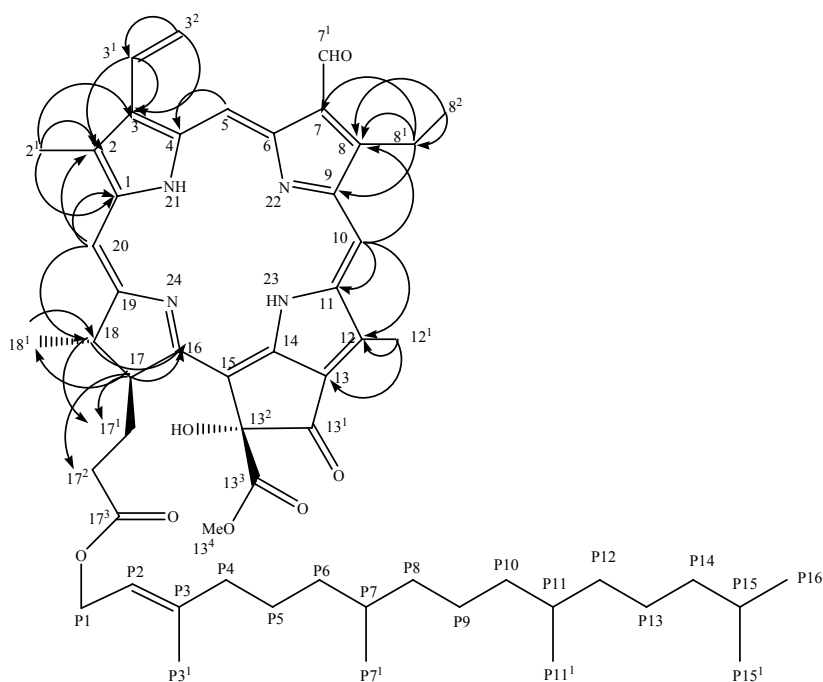
Figure 4.8 The HMBC correlations of compound 3.

Table 4.20 Observed Long Range C-H Correlations in the HMBC of Compound 3.

δ ^{1}H-NMR in ppm (Position)	HMBC in ppm (Correlation Carbon)
3.35 (H-2^1)	150.0 (C-1), 136.5 (C-2), 135.4 (C-3), 135.9
6.54 (H-3^2 E)	124.3 (C-3^1), 135.4 (C-3)
6.21 (H-3^2 Z)	135.4 (C-3)
11.00 (H-5)	135.4 (C-3)
4.22 (H-8^1)	18.7 (C-8^2)
1.83 (H-8^2)	18.7 (C-8^1), 135.9 (C-11)
10.16 (H-10)	135.9 (C-11)
3.70 (H-12^1)	135.7 (C-12), 135.9 (C-11)
3.85 (H-13^4)	172.8 (C-13^3)
5.41 (H-17)	22.7 (C-18^1)
4.60 (H-18)	22.7 (C-18^1), 31.1 (C-17^1), 31.6 (C-17^2)
1.71 (H-18^1)	178.7 (C-19), 50.3 (C-18), 51.8 (C-17), 54.9 (C-13^4)
8.89 (H-20)	135.5 (C-2), 150.0 (C-1), 51.8 (C-17), 50.3 (C-18)

The NOESY correlation spectrum showed the appearance of coupling between the proton at δ 1.83 (H-8^2) ppm and the proton at 4.22 (H-8^1) ppm, coupling between the proton at δ 2.81 (H-17^1) ppm and the proton at δ 3.85 (H-13^4) ppm, coupling between the proton at δ 11.50 (H-7^1) ppm and the proton at δ 1.83 (H-8^2) and 4.22 (H-8^1) ppm, coupling between the proton at δ 11.00 (H-5) ppm and the protons at δ 6.54 (H-3^2 E) and 8.19 (H-3^1) ppm. The 3D structure of compound 3 could be drawn as

shown in Figure 4.9. The NOE method was used to determine the stereochemical configuration at C-13^2 between compound 3 and 13^2-hydroxy-(13^2-*S*)-phaeophytin b (Shuyprom, 2005). The NOE effects obtained from the NOESY spectra could be seen as indicated in Figure A.24, Figure 4.9 and Table 4.21.
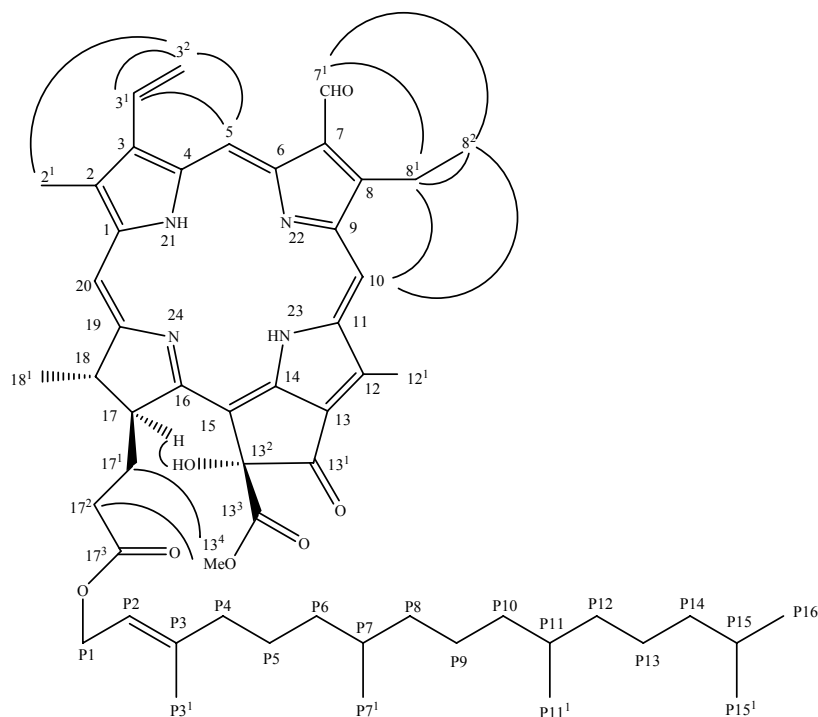
**Figure 4.9** The NOESY correlations of compound 3.

Table 4.21 Observed NOE Correlations of Compound 3.

δ ^{1}H-NMR in ppm (Position)	NOESY in ppm (Cross Peaks)
8.19 (H-3^1)	6.54 (H-3^2 *E*), 6.21 (H-3^2 *Z*)
6.54 (H-3^2 *E*)	3.35 (H-2^1), 11.50 (H-7^1)
11.00 (H-5)	6.54 (H-3^2 *E*), 8.19 (H-3^1)
11.50 (H-7^1)	1.83 (H-8^2), 4.22 (H-8^1)
1.83 (H-8^2)	4.22 (H-8^1)

Table 4.21 (Continued).

δ ^{1}H-NMR in ppm (Position)	NOESY in ppm (Cross Peaks)
10.16 (H-10)	1.83 (H-8^2), 4.22 (H-8^1)
2.81 (H-17^1)	3.85 (H-13^4)
2.79 (H-17^2)	3.85 (H-13^4)
5.41 (H-17)	6.20 (H-13^2)

4.4   Structural Elucidation of 13^2-hydroxy-(13^2-*S*)-phaeophytin a
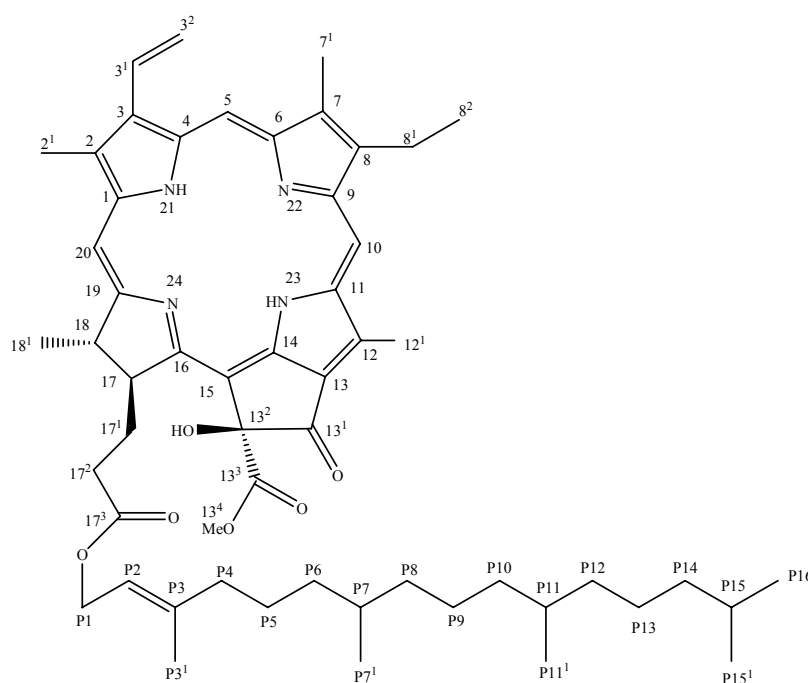
Figure 4.10 Structure of Compound 4.

Compound 4 was obtained as green powder. The UV spectrum (Figure A.25) showed absorptions of a chlorophyll derivative compound (Watanabe *et al*., 1993). The IR spectrum of compound 4 is shown in Figure A.26 and the absorption peaks were assigned as shown in Table 4.22.

Table 4.22 IR Absorption Band Assignment of Compound 4.

Wave number (cm^{-1})	Intensity	Assignment
3430	s	N-H Stretch
2924	m	C-H Stretch
1741, 1620	m	C=O Stretch
1460	m	C-H Bend

Information from ^{1}H-NMR (Figure A.27) and DEPT ^{13}C-NMR (Figure A.28) spectra showed the necessary diagnostic peaks to be identified as a phaeophytin a derivative with a hydroxyl group on C-13^2. The 3H-2^1 and 3H-12^1 resonances occurred as sharp singlets at δ 3.43 and 4.02 ppm, respectively. H-5, H-10, and H-20 were found as sharp singlets at δ 9.91, 10.07 and 9.05 ppm, respectively. A vinyl group was present at C-3 as indicated by the H-3^1 doublet of doublets at δ 8.26 (J = 11.39, 15.19 Hz) ppm and the H-3^2 (E) and the H-3^2 (Z) doublet of doublets at δ 6.45 (J = 2.28, 15.19 Hz) and 6.19 (J = 2.28, 11.39) ppm, respectively. An ethyl group was found to occur at C-8. The 2H-8^1 resonances appeared as a quartet at δ 3.83 (J = 7.65 Hz) ppm and 3H-8^2 occurred as a triplet at δ 1.76 (J = 7.55 Hz) ppm. A methyl group was present at C-7^1 as indicated by H-7^1 sharp singlet at δ 3.29 ppm. Similarly, as with phaeophytin a, a five membered carbocyclic ring was present at position 13. A keto group was found at C-13^1 as indicated by the fully substituted carbon resonance at δ 194.6. The C-13^2 resonances occurred at δ 91.1 ppm as with phaeophytin a and a methyl ester was also present at C-13^3. This was indicated by C-13^3 occurring as a fully substituted carbon resonance at δ 174.2 ppm and the C-13^4 methyl carbon resonance being present at δ 53.1 ppm. The H-13^4 resonance was found as a sharp

singlet at δ 3.69 ppm. The characteristic signals of phytyl ester appeared at δ 5.43 (H-P2), 4.69 (H-P1), 61.8 (C-P1) and 119.2 (C-P2) ppm.

Compound 4 exhibited the ^{1}H-NMR and ^{13}C-NMR chemical shifts identical to 13^2-hydroxy-(13^2-S)-phaeophytin a (Matsuo, Hamasaki and Nozaki, 1996). A comparison of the ^{1}H-NMR and ^{13}C-NMR chemical shifts of compound 4 and 13^2-hydroxy-(13^2-S)-phaeophytin a (Matsuo *et al*., 1996) are shown in Tables 4.23 and 4.24. These data indicated that compound 4 is 13^2-hydroxy-(13^2-S)-phaeophytin a (Figure 4.10).

Table 4.23 The ^{1}H-NMR Chemical Shifts of Compound 4[#] and 13^2-hydroxy-(13^2-S)-phaeophytin a[*].

Proton Position	Chemical Shifts in ppm (Coupling Constant in Hz)	
	Compound 4	13^2-hydroxy-(13^2-S)-phaeophytin a
2^1	3.43 (*s*)	3.43 (*s*)
3^1	8.26 (*dd*, J = 11.39, 15.19)	8.01 (*dd*)
3^2 (E)	6.45 (*dd*, J = 2.28, 15.19)	6.30 (*dd*)
3^2 (Z)	6.19 (*dd*, J = 2.28, 11.39)	6.20 (*dd*)
5	9.91 (*s*)	9.47 (*s*)
7^1	3.29 (*s*)	3.25 (*s*)
8^1	3.83 (*q*, J = 7.65)	3.70 (*q*)
8^2	1.76 (*t*, J = 7.55)	1.70 (*t*)
10	10.07 (*s*)	9.62 (*s*)

Table 4.23 (Continued).

Proton Position	Chemical Shifts in ppm (Coupling Constant in Hz)	
	Compound 4	13^2-hydroxy-(13^2-*S*)-phaeophytin a
12^1	4.02 (*s*)	3.74 (*s*)
13^2-OH	5.53 (*s*)	5.53 (*s*)
13^4-OMe	3.75 (*s*)	3.62 (*s*)
17	5.24 (*m*)	4.17 (*m*)
18	4.64 (*dq*, *J* = 7.30)	4.50 (*dq*)
18^1	1.80 (*d*, *J* = 7.25)	1.61 (*d*)
20	9.05 (*s*)	8.65 (*s*)
21-NH	-0.89 (*br, s*)	
23-NH	0.78 (*br, s*)	

[#]500 MHz ^{1}H, pyridine d_5
[*]400 MHz ^{1}H, CDCl$_3$

Table 4.24 The ^{13}C-NMR Chemical Shifts of Compound 4[#] and 13^2-hydroxy-(13^2-S)-phaeophytin a[*].

Carbon Position	Chemical Shifts in ppm	
	Compound 4	13^2-hydroxy-(13^2-S)-phaeophytin a
1	141.4	142.8
2	132.0	131.8
2^1	12.5	12.1
3	136.3	136.6
3^1	129.9	129.1
3^2	123.0	122.9
4	137.3	136.3
5	100.5	98.0
6	156.1	155.4
7	137.3	136.2
7^1	11.6	11.3
8	146.2	145.8
8^1	20.1	19.5
8^2	18.2	17.4
9	150.7	151.1
10	104.7	104.3
11	139.3	137.8
12	132.5	129.4

Table 4.24 (Continued).

Carbon Position	Chemical Shifts in ppm	
	Compound 4	13^2-hydroxy-(13^2-S)-phaeophytin a
12^1	12.9	12.3
13	136.5	127.0
13^1	194.6	192.0
13^2	91.1	89.0
13^3	174.2	172.8
13^4	53.6	53.4
14	150.7	149.8
15	111.7	107.7
16	168.3	162.5
17	54.4	51.8
17^1	33.5	31.1
17^2	33.1	31.6
17^3	173.6	173.6
18	50.7	50.3
18^1	23.1	22.7
19	172.5	172.4
20	95.0	93.6

[#] 125 MHz ^{13}C, pyridine d_5
[*] 100 MHz ^{13}C, CDCl$_3$

Further studies of the structure of compound 4 were done by analysis of the COSY, HSQC, HMBC and NOESY correlations (see also Tables 4.25-4.28).

The COSY spectrum (Figure A.29) indicated that one proton doublet of doublets at δ 8.26 (H-3^1) ppm coupled to one proton doublet of doublets at δ 6.45 (H-3^2 E) ppm and one proton doublet of doublets at 6.19 (H-3^2 Z) ppm. The signal at δ 5.43 (H-P2) ppm correlated only with the signal at δ 4.69 (H-P1) ppm. The signal of one proton at 4.64 (H-18) ppm showed a cross peak with a signal at δ 1.80 (H-18^1). Study of the COSY (H-H) spectrum of compound 4 led to the complete assignment of ^{1}H and ^{13}C shift signals as shown in Table 4.25.

Table 4.25 Observed Correlations in the COSY (H-H) Spectrum of Compound 4.

δ ^{1}H-NMR in ppm (Position)	COSY in ppm (Coupled Hydrogen)
8.26 (H-3^1)	6.45 (H-3^2 E), 6.19 (H-3^2 Z)
3.83 (H-8^1)	1.76 (H-8^2)
5.24 (H-17)	2.90, 2.69 (H-17^1)
2.90, 2.69 (H-17^1)	2.82, 2.45 (H-17^2)
4.64 (H-18)	1.80 (H-18^1)
5.43 (H-P2)	4.69 (H-P1)

One bond correlation (HSQC) data revealed that the proton at δ 10.07 (H-10) ppm was attached to the carbon at δ 104.7 (C-10) ppm, the proton at δ 9.91 (H-5) ppm was attached to the carbon at δ 100.5 (C-5) ppm, the proton at δ 9.05 (H-20) ppm was attached to the carbon at δ 95.0 (C-20) ppm, the proton at δ 8.26 (H-3^1) ppm was attached to the carbon at δ 129.9 (C-3^1) ppm, the protons at δ 6.45 (H-3^2 E) and 6.19

(H-3^2 Z) ppm were attached to the carbon at δ 123.0 (C-3^2) ppm, the proton at δ 5.24 (H-17) ppm was joined with the carbon at δ 54.4 (C-17) ppm, the proton at δ 4.64 (H-18) ppm was joined with the carbon at δ 50.7 (C-18) ppm, the proton at δ 4.02 (H-12^1) ppm was joined with the carbon at δ 12.9 (C-12^1) ppm, the proton at δ 3.76 (H-8^1) was joined with the carbon at δ 20.1 (C-8^1) ppm and the proton at δ 3.43 (H-2^1) ppm was joined with the carbon at δ 12.5 (C-2^1) ppm. The protons attached to the carbons in compound 4 were assigned by the HSQC spectrum as shown in Figure A.30 and Table 4.26.

Table 4.26 Observed Correlations in HSQC Vicinal (C-H) Spectrum of Compound 4.

δ ^{1}H-NMR in ppm (Position)	HSQC in ppm (Attached Carbon)
4.02 (H-12^1)	12.9 (C-12^1)
8.26 (H-3^1)	129.9 (C-3^1)
6.45 (H-3^2 E), 6.19 (H-3^2 Z)	123.4 (C-3^2)
9.91 (H-5)	100.5 (C-5)
3.29 (H-7^1)	11.6 (C-7^1)
3.83 (H-8^1)	20.1 (C-8^1)
1.76 (H-8^2)	18.2 (C-8^2)
10.07 (H-10)	104.7 (C-10)
5.24 (H-17)	54.4 (C-17)
4.64 (H-18)	50.7 (C-18)
1.80 (H-18^1)	23.1 (C-18^1)
9.05 (H-20)	95.0 (C-20)

In the H-C long range coupling spectrum obtained by HMBC, the proton at δ 6.45 (H-3^2 E) ppm showed cross peaks with the carbons at 129.9 (C-3^1) and 136.8 (C-3) ppm, the proton at δ 6.19 (H-3^2 Z) ppm showed a cross peak with the carbon at 136.3 (C-3) ppm, the proton at δ 1.80 (H-18^1) ppm showed cross peaks with the carbons at 172.5 (C-19), 51.1 (C-18) and 54.4 (C-17) ppm, the proton at δ 5.24 (H-17) ppm showed a cross peak with the carbon at 23.1 (C-18^1) ppm, the proton at δ 3.43 (H-2^1) ppm showed cross peaks with the carbons at 136.3 (C-3) and 141.4 (C-1)ppm and the proton at δ 9.05 (H-20) ppm showed a cross peak with the carbon at 132.0 (C-2) ppm (Figure A.31, Figure 4.11 and Table 4.27).
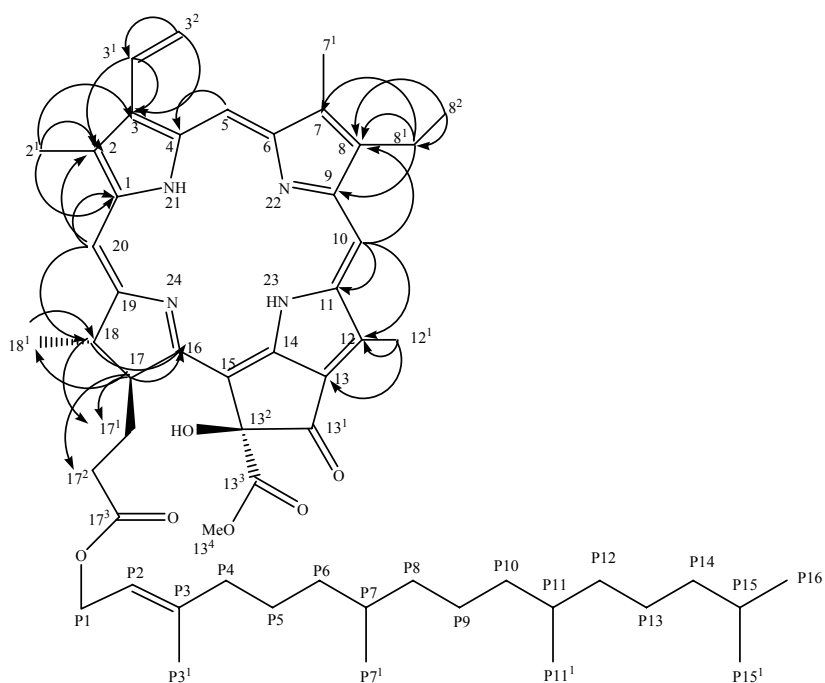
Figure 4.11 The HMBC correlations of compound 4.

Table 4.27 Observed Long Range C-H Correlations in the HMBC of Compound 4.

δ ^{1}H-NMR in ppm (Position)	HMBC in ppm (Correlation Carbon)
3.43 (H-2^1)	141.4 (C-1), 132.0 (C-2), 136.3 (C-3)
6.45 (H-3^2 E)	129.9 (C-3^1), 136.3 (C-3)
6.19 (H-3^2 Z)	136.3 (C-3)
9.91 (H-5)	136.3 (C-3)
3.83 (H-8^1)	18.2 (C-8^2)
1.76 (H-8^2)	20.1 (C-8^1)
10.07 (H-10)	139.3 (C-11)
4.02 (H-12^1)	132.5 (C-12), 139.3 (C-11)
3.75 (H-13^4)	174.2 (C-13^3)
5.24 (H-17)	23.1 (C-18^1)
4.64 (H-18)	23.1 (C-18^1), 33.5 (C-17^1), 33.1 (C-17^2)
1.80 (H-18^1)	172.5 (C-19), 50.7 (C-18), 54.4 (C-17), 53.6 (C-13^4)
9.05 (H-20)	132.0 (C-2), 141.4 (C-1), 54.4 (C-17), 50.7 (C-18)

The NOESY correlation spectrum showed the appearance of coupling between the proton at δ 1.76 (H-8^2) ppm and the proton at 3.83 (H-8^1) ppm, coupling between the proton at δ 5.24 (H-17) ppm and the proton at δ 3.75 (H-13^4) ppm, coupling between the proton at δ 3.29 (H-7^1) ppm and the proton at δ 1.76 (H-8^2) and 3.83 (H-8^1) ppm, coupling between the proton at δ 9.91 (H-5) ppm and the protons at δ 6.45 (H-3^2 E) and 8.26 (H-3^1). The 3D structure of compound 4 could be drawn as shown in Figure 4.12. The NOE effects obtained from the NOESY spectra could be seen as

indicated in Figure A.32, Figure 4.12 and Table 4.28 which are in good agreement with the proposed structure.
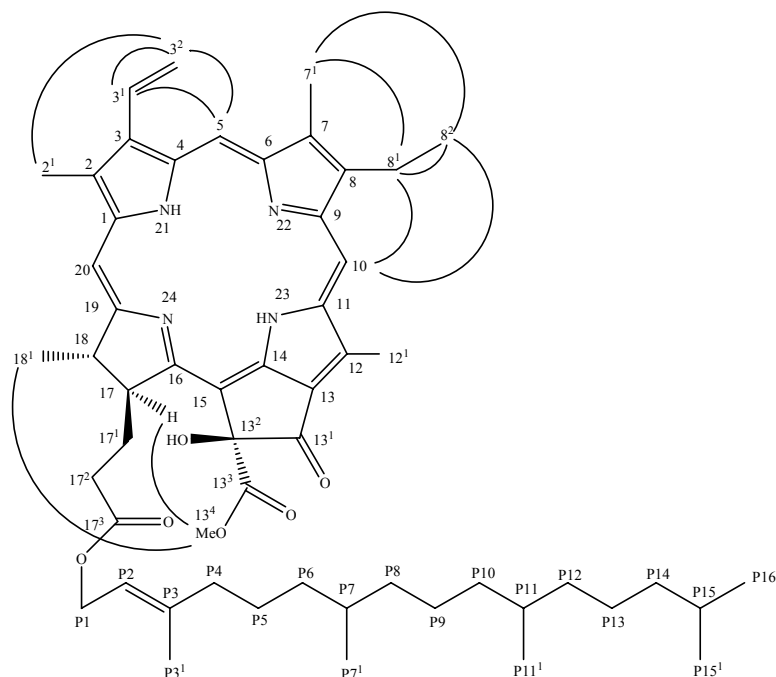
Figure 4.12 The NOESY correlations of compound 4.

Table 4.28 Observed NOE Correlations of Compound 4.

δ ^{1}H-NMR in ppm (Position)	NOESY in ppm (Cross Peaks)
8.26 (H-3^1)	6.45 (H-3^2 *E*), 6.19 (H-3^2 *Z*)
6.45 (H-3^2 *E*)	3.43 (H-2^1), 3.29 (H-7^1)
9.91 (H-5)	6.45 (H-3^2 *E*), 8.26 (H-3^1)
3.29 (H-7^1)	1.76 (H-8^2), 3.83 (H-8^1)
1.76 (H-8^2)	3.83 (H-8^1)
10.07 (H-10)	1.76 (H-8^2), 3.83 (H-8^1)

Table 4.28 (Continued).

δ ^{1}H-NMR in ppm (Position)	NOESY in ppm (Cross Peaks)
5.24 (H-17)	3.75 (H-13^4)
1.80 (H-18^1)	3.75 (H-13^4)
9.05 (H-20)	3.43 (H-2^1), 3.75 (H-13^4)

4.5 Structural Elucidation of 13^2-hydroxy-(13^2-*R*)-phaeophytin a
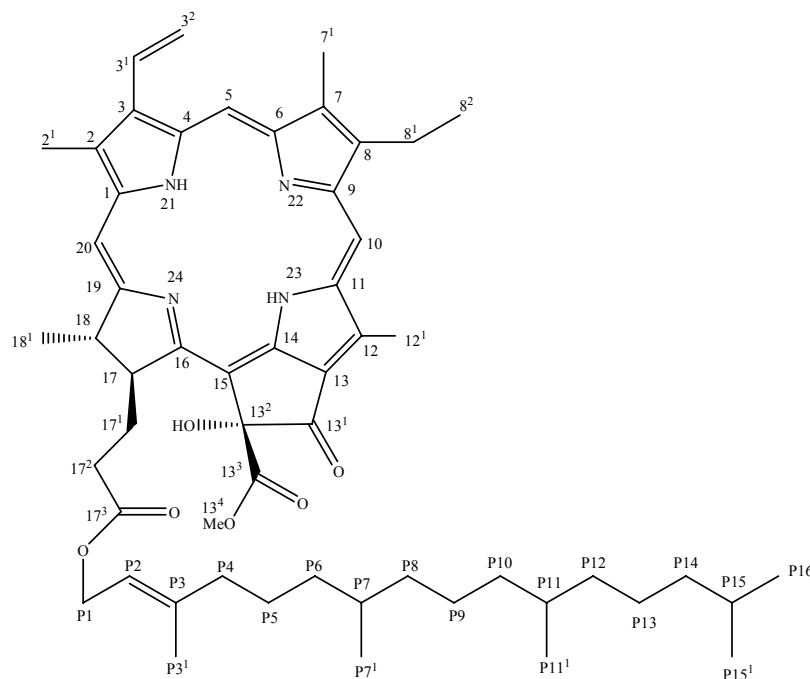
Figure 4.13 Structure of Compound 5.

Compound 5 was obtained as green powder. The UV spectrum (Figure A.33) showed absorptions of a chlorophyll derivative compound (Watanabe *et al*., 1993). The IR spectrum of compound 5 is shown in Figure A.34 and the absorption peaks were assigned as shown in Table 4.29.

Table 4.29 IR Absorption Band Assignment of Compound 5.

Wave number (cm^{-1})	Intensity	Assignment
3429	s	N-H Stretch
2995	m	C-H Stretch
1740, 1617	m	C=O Stretch
1455	m	C-H Bend

Information from ^{1}H-NMR (Figure A.35) and DEPT ^{13}C-NMR (Figure A.36) spectra showed the necessary diagnostic peaks to be identified as a phaeophytin a derivative with a hydroxyl group on C-13^2. The 3H-2^1 and 3H-12^1 resonances occurred as sharp singlets at δ 3.40 and 3.73 ppm, respectively. H-5, H-10, and H-20 were found as sharp singlets at δ 9.70, 9.88 and 8.93 ppm, respectively. A vinyl group was present at C-3 as indicated by the H-3^1 doublet of doublets at δ 8.10 (J = 11.39, 15.19 Hz) ppm and the H-3^2 (E) and the H-3^2 (Z) doublet of doublets at δ 6.37 (J = 2.28, 15.19 Hz) and 6.18 (J = 2.28, 11.39) ppm, respectively. An ethyl group was found to occur at C-8. The 2H-8^1 resonances appeared as a quartet at δ 3.66 (J = 7.65 Hz) ppm and 3H-8^2 occurred as a triplet at δ 1.86 (J = 7.55 Hz) ppm. A methyl group was present at C-7^1 as indicated by H-7^1 sharp singlet at δ 3.19 ppm. Similarly, as with phaeophytin a, a five membered carbocyclic ring was present at position 13. A keto group was found at C-13^1 as indicated by the fully substituted carbon resonance at δ 194.7 ppm. The C-13^2 resonances occurred at δ 91.2 ppm as with phaeophytin a and a methyl ester was also present at C-13^3. This was indicated by C-13^3 occurring as a fully substituted carbon resonance at δ 173.5 and the C-13^4 methyl carbon resonance being present at δ 53.1. The H-13^4 resonance was found as a sharp singlet at δ 3.71

ppm. The characteristic signal of phytyl ester appeared at δ 5.37 (H-P2), 4.68 (H-P1), 61.8 (C-P1) and 119.4 (C-P2) ppm.

Compound 5 exhibited the ^{1}H-NMR and ^{13}C-NMR chemical shifts identical to 13^2-hydroxy-(13^2-R)-phaeophytin a (Matsuo et $al.$, 1996). A comparison of the ^{1}H-NMR and ^{13}C-NMR chemical shifts of compound 5 and 13^2-hydroxy-(13^2-R)-phaeophytin a (Matsuo et $al.$, 1996) are shown in Tables 4.30 and 4.31. These data indicated that compound 5 is 13^2-hydroxy-(13^2-R)-phaeophytin a (Figure 4.13).

Table 4.30 The ^{1}H-NMR Chemical Shifts of Compound 5[#] and 13^2-hydroxy-(13^2-R)-phaeophytin a[*].

Proton Position	Chemical Shifts in ppm (Coupling Constant in Hz)	
	Compound 5	13^2-hydroxy-(13^2-R)-phaeophytin a
2^1	3.40 (s)	3.42 (s)
3^1	8.10 (dd, J = 11.39, 15.19)	8.01 (dd)
3^2 (E)	6.37 (dd, J = 2.28, 15.19)	6.30 (dd)
3^2 (Z)	6.18 (dd, J = 2.28, 11.39)	6.20 (dd)
5	9.70 (s)	9.46 (s)
7^1	3.19 (s)	3.25 (s)
8^1	3.66 (q, J = 7.65)	3.72 (q)
8^2	1.68 (t, J = 7.55)	1.70 (t)
10	9.88 (s)	9.61 (s)
12^1	3.73 (s)	3.72 (s)

Table 4.30 (Continued).

Proton Position	Chemical Shifts in ppm (Coupling Constant in Hz)	
	Compound 5	13^2-hydroxy-(13^2-R)-phaeophytin a
13^2-OH	5.35 (s)	5.35 (s)
13^4-OMe	3.71 (s)	3.64 (s)
17	5.77 (m)	4.48 (m)
18	4.60 (dq, $J = 7.30$)	4.69 (dq)
18^1	1.74 (d, $J = 7.25$)	1.68 (d)
20	8.93 (s)	8.63 (s)
21-NH	-1.48 (br, s)	
23-NH	0.67 (br, s)	

[#]500 MHz ^{1}H, pyridine d_5
[*]400 MHz ^{1}H, CDCl$_3$

Table 4.31 The ^{13}C-NMR Chemical Shifts of Compound 5[#] and 13^2-hydroxy-(13^2-R)-phaeophytin a[*].

Carbon Position	Chemical Shifts in ppm	
	Compound 5	13^2-hydroxy-(13^2-R)-phaeophytin a
1	142.6	142.9
2	132.8	131.9
2^1	12.5	12.1
3	136.7	136.5
3^1	129.8	129.1

Table 4.31 (Continued).

Carbon Position	Chemical Shifts in ppm	
	Compound 5	13^2-hydroxy-(13^2-R)-phaeophytin a
3^2	123.3	122.9
4	136.8	136.4
5	98.6	97.9
6	156.1	155.6
7	137.4	136.3
7^1	11.5	11.3
8	146.0	145.3
8^1	19.3	19.5
8^2	18.0	17.5
9	151.9	151.0
10	105.1	104.2
11	138.6	137.8
12	129.9	129.3
12^1	12.4	12.2
13	128.1	126.3
13^1	194.7	192.0
13^2	91.2	89.1
13^3	173.5	173.0
13^4	53.1	53.8

Table 4.31 (Continued).

Carbon Position	Chemical Shifts in ppm	
	Compound 5	13^2-hydroxy-(13^2-R)-phaeophytin a
14	150.7	150.2
15	111.2	107.6
16	163.5	161.9
17	51.3	50.8
171	32.4	31.2
172	32.7	31.9
173	173.9	173.5
18	51.1	50.2
181	23.1	22.7
19	173.0	172.8
20	91.8	93.4

[#] 125 MHz ^{13}C, pyridine d_5
[*] 100 MHz ^{13}C, CDCl$_3$

Further studies of the structure of compound 5 were done by analysis of the COSY, HSQC, HMBC and NOESY correlations (see also Tables 4.32-4.35).

The COSY spectrum (Figure A.37) indicated that one proton doublet of doublets at δ 8.10 (H-3^1) ppm coupled to one proton doublet of doublets at δ 6.37 (H-3^2 E) ppm and one proton doublet of doublets at 6.18 (H-3^2 Z) ppm. The signal at δ 5.37 (H-P2) ppm correlated only with the signal at δ 4.68 (H-P1) ppm. The signal of

one proton at 4.60 (H-18) ppm showed a cross peak with a signal at δ 1.74 (H-18^1) ppm. Study of the COSY (H-H) spectrum of compound 5 led to the complete assignment of ^{1}H and ^{13}C shift signals as shown in Table 4.32.

Table 4.32 Observed Correlations in the COSY (H-H) Spectrum of Compound 5.

δ ^{1}H-NMR in ppm (Position)	COSY in ppm (Coupled Hydrogen)
8.10 (H-3^1)	6.41 (H-3^2 E), 6.25 (H-3^2 Z)
3.66 (H-8^1)	1.73 (H-8^2)
5.77 (H-17)	2.94, 2.33 (H-17^1)
2.94, 2.33 (H-17^1)	2.83, 2.58 (H-17^2)
4.60 (H-18)	1.74 (H-18^1)

One bond correlation (HSQC) data revealed that the proton at δ 9.88 (H-10) ppm was attached to the carbon at δ 105.1 (C-10) ppm, the proton at δ 9.70 (H-5) ppm was attached to the carbon at δ 98.6 (C-5) ppm, the proton at δ 8.93 (H-20) ppm was attached to the carbon at δ 91.8 (C-20) ppm, the proton at δ 8.10 (H-3^1) ppm was attached to the carbon at δ 129.8 (C-3^1) ppm, the protons at δ 6.37 (H-3^2 E) and 6.18 (H-3^2 Z) ppm were attached to the carbon at δ 123.3 (C-3^2) ppm, the proton at δ 5.77 (H-17) ppm was joined with the carbon at δ 51.3 (C-17) ppm, the proton at δ 4.60 (H-18) ppm was joined with the carbon at δ 51.1 (C-18) ppm, the proton at δ 3.73 (H-12^1) ppm was joined with the carbon at δ 12.4 (C-12^1) ppm, the proton at δ 3.66 (H-8^1) ppm was joined with the carbon at δ 19.3 (C-8^1) ppm and the proton at δ 3.40 (H-2^1) ppm was joined with the carbon at δ 12.5 (C-2^1) ppm. The protons attached to the

carbons in compound 5 were assigned by the HSQC spectrum as shown in Figure A.38 and Table 4.33.

Table 4.33 Observed Correlations in HSQC Vicinal (C-H) Spectrum of Compound 5.

δ ^{1}H-NMR in ppm (Position)	HSQC in ppm (Attached Carbon)
3.73 (H-12^1)	12.4 (C-12^1)
8.10 (H-3^1)	129.8 (C-3^1)
6.37 (H-3^2 E)	123.3 (C-3^2)
6.18 (H-3^2 Z)	123.3 (C-3^2)
9.70 (H-5)	98.7 (C-5)
3.19 (H-7^1)	11.5 (C-7^1)
3.66 (H-8^1)	19.3 (C-8^1)
1.68 (H-8^2)	18.0 (C-8^2)
9.88 (H-10)	105.1 (C-10)
5.77 (H-17)	51.3 (C-17)
4.60 (H-18)	51.1 (C-18)
1.74 (H-18^1)	23.1 (C-18^1)
8.93 (H-20)	91.8 (C-20)

In the H-C long range coupling spectrum obtained by HMBC, the proton at δ 6.37 (H-3^2 E) ppm showed cross peaks with the carbons at 129.8 (C-3^1) and 136.7 (C-3) ppm, the proton at δ 6.18 (H-3^2 Z) ppm showed a cross peak with the carbon at 136.7 (C-3) ppm, the proton at δ 1.74 (H-18^1) ppm showed cross peaks with the carbons at 173.0 (C-19), 51.1 (C-18) and 51.3 (C-17) ppm, the proton at δ 5.77 (H-17)

ppm showed a cross peak with the carbon at 23.1 (C-18^1) ppm, the proton at δ 3.40
(H-2^1) ppm showed cross peaks with the carbons at 136.7 (C-3) and 142.6 (C-1) ppm
and the proton at δ 8.93 (H-20) ppm showed a cross peak with the carbon at 132.8 (C-
2) ppm (Figure A.39, Figure 4.14 and Table 4.34).
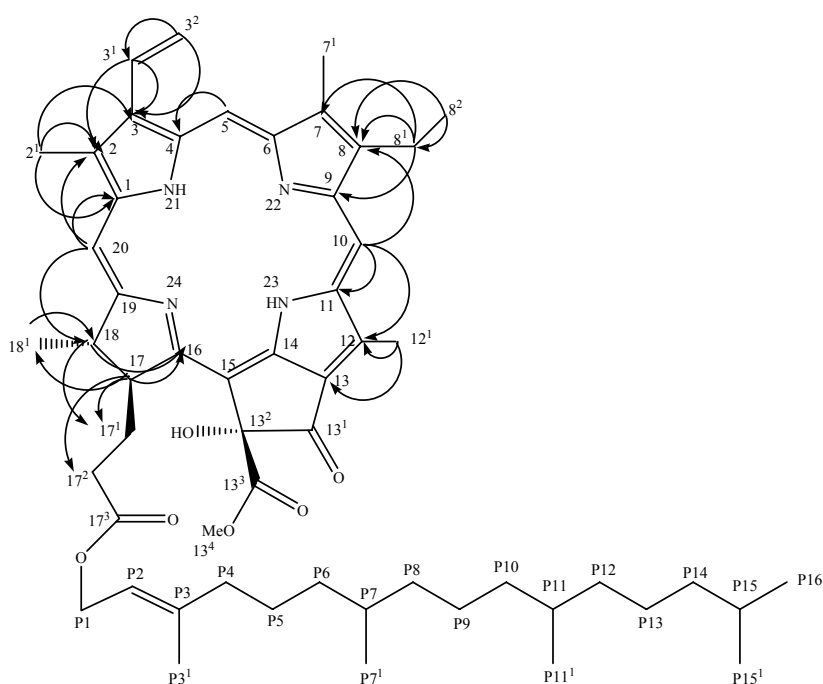
Figure 4.14 The HMBC correlations of compound 5.

Table 4.34 Observed Long Range C-H Correlations in the HMBC of Compound 5.

δ ^{1}H-NMR in ppm (Position)	HMBC in ppm (Correlation Carbon)
3.40 (H-2^1)	142.6 (C-1), 132.8 (C-2), 136.7 (C-3)
6.37 (H-3^2 E)	129.8 (C-3^1), 136.7 (C-3)
6.18 (H-3^2 Z)	136.7 (C-3)
9.70 (H-5)	136.7 (C-3)
3.66 (H-8^1)	18.0 (C-8^2)

Table 4.34 (Continued).

δ ^{1}H-NMR in ppm (Position)	HMBC in ppm (Correlation Carbon)
1.68 (H-8^2)	19.3 (C-8^1), 138.6 (C-11)
9.88 (H-10)	138.6 (C-11)
3.73 (H-12^1)	129.9 (C-12), 138.6 (C-11)
3.71 (H-13^4)	173.5 (C-13^3)
5.77 (H-17)	23.1 (C-18^1)
4.60 (H-18)	23.1 (C-18^1), 32.4 (C-17^1), 32.7 (C-17^2)
1.74 (H-18^1)	173.0 (C-19), 51.1 (C-18), 51.3 (C-17),
	53.1 (C-13^4)
8.93 (H-20)	132.8 (C-2), 142.6 (C-1), 51.3 (C-17), 51.1 (C-18)

The NOESY correlation spectrum showed the appearance of coupling between the proton at δ 1.68 (H-8^2) ppm and the proton at 3.66 (H-8^1) ppm, coupling between the proton at δ 2.94 (H-17^1) ppm and the proton at δ 3.71 (H-13^4) ppm, coupling between the proton at δ 3.19 (H-7^1) ppm and the proton at δ 1.68 (H-8^2) and 3.66 (H-8^1) ppm, coupling between the proton at δ 9.70 (H-5) ppm and the protons at δ 6.37 (H-3^2 *E*) and 8.10 (H-3^1) ppm. The 3D structure of compound 5 could be drawn as shown in Figure 4.15. The NOE method was used to determine the stereochemical configuration at C-13^2 between compounds 4 and 5. The NOE effects obtained from the NOESY spectra could be seen as indicated in Figure A.40, Figure 4.15 and Table 4.35.
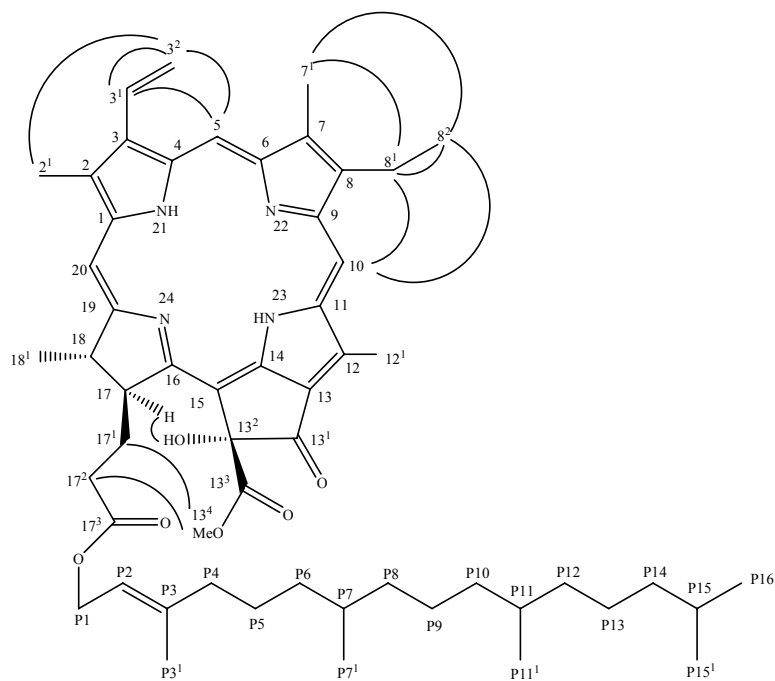
Figure 4.15 The NOESY correlations of compound 5.

Table 4.35 Observed NOE Correlation of Compound 5.

δ ^{1}H-NMR in ppm (Position)	NOESY in ppm (Cross Peaks)
8.10 (H-3^1)	6.37 (H-3^2 *E*), 6.18 (H-3^2 *Z*)
6.37 (H-3^2 *E*)	3.40 (H-2^1), 3.19 (H-7^1)
9.70 (H-5)	6.37 (H-3^2 *E*), 8.10 (H-3^1)
3.19 (H-7^1)	1.68 (H-8^2), 3.66 (H-8^1)
1.68 (H-8^2)	3.66 (H-8^1)
9.88 (H-10)	1.68 (H-8^2), 3.66 (H-8^1)
2.90 (H-17^1)	3.71 (H-13^4)
2.82 (H-17^2)	3.71 (H-13^4)
5.77 (H-17)	5.35 (H-13^2)

4.6   Structural Elucidation of Stigmasteryl-3-*O*-β-D-glucopyranoside
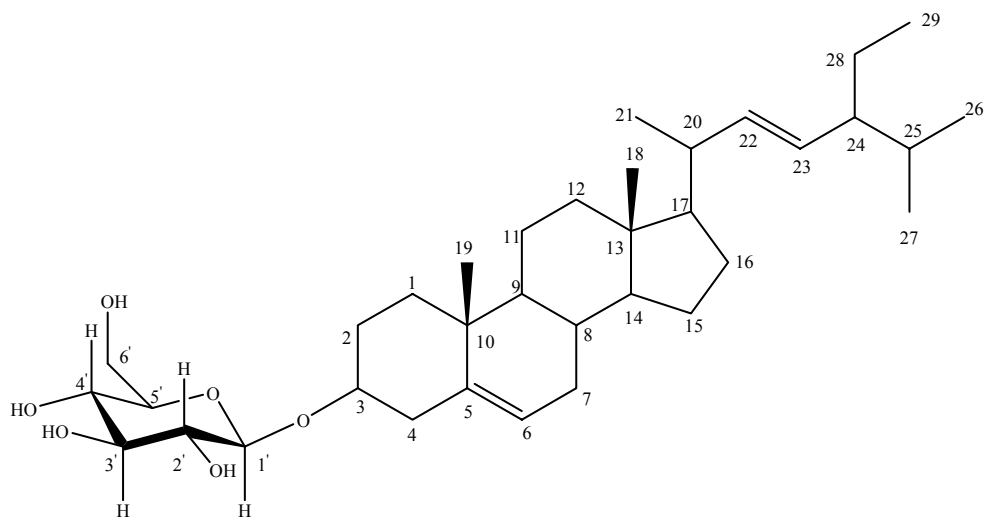
Figure 4.16 Structure of Compound 6.

Compound 6 was obtained as white powder. The IR spectrum of compound 6 is shown in Figure A.41 and the absorption peaks were assigned as shown in Table 4.36.

Table 4.36 IR Absorption Band Assignment of Compound 6.

Wave number (cm^{-1})	Intensity	Assignment
3500-3200, 2390	s	O-H Stretch
1640	w	C=C Stretch
1470, 1383	m	C-H Bend
1250	w	C-O Stretch
1160	m	C-O Stretch
1075, 1019	s	C-O Stretch

The ^{1}H-NMR (Figure A.42) and DEPT ^{13}C-NMR (Figure A.43) spectra of this compound showed the presence of three olefinic protons at δ 5.37 (*m*, br, H-6) ppm, 5.15 (*dd*, *J* = 15.20, 8.80) ppm, 5.08 (*dd*, *J* = 15.30, 8.80) ppm, respectively (Boonyaratavej and Petsom, 1991); six methyl groups in the region δ 0.70 to δ 0.97 [δ 0.70 (*s*, Me-18); δ 0.97 (*m*, Me-19); δ 0.95 (*m*, Me-21); 0.95 (*m*, Me-26, Me-27); 0.90 (*m*, Me-29)] ppm. These were found to be the signal of β-anomeric proton of glucopyranosyl a group of multiplet signals at δ 4.08 (*m*, H-2$^{'}$), δ 4.30 (*m*, H-3$^{'}$), δ 4.30 (*m*, H-4$^{'}$), δ 4.00 (*m*, H-5$^{'}$) and δ 4.43 (*m*, H-6$^{'}$) ppm with corresponding carbons at δ 103.1, 75.8, 79.1, 72.2, 79.0 and 63.4 (C-1$^{'}$- C-6$^{'}$) ppm. A comparison of the ^{1}H-NMR and ^{13}C-NMR chemical shifts of compound 6 and stigmasteryl-3-*O*-β-D-glucopyranoside (Alam, Chopra, Ali and Niwa, 1996) are shown in Tables 4.37 and 4.38.

Table 4.37 The ^{1}H-NMR Chemical Shifts of compound 6[#] and stigmasteryl-3-*O*-β-D-glucopyranoside[*].

Proton Position	Chemical Shifts in ppm (Coupling Constant in Hz)	
	Compound 6	stimasteryl-3-*O*-β-D-glucopyranoside
1α	1.00 (*m*)	0.99
1β	1.75 (*m*)	1.81
1$^{'}$	5.07 (*d*, *J* = 7.70)	4.21
2α	1.75 (*m*)	0.90
2β	2.15 (*m*)	1.27

Table 4.37 (Continued).

Proton Position	Chemical Shifts in ppm (Coupling Constant in Hz)	
	Compound 6	stigmasteryl-3-O-β-D-glucopyranoside
2$^{'}$	4.08 (*m*)	3.46
2$^{'}$-OH	7.07 (*br, d, J* = 4.00)	
3	4.00 (*m*)	3.47
3$^{'}$	4.30 (*m*)	3.40
3$^{'}$-OH	7.13 (*br*)	
4α	2.50 (*m*)	1.14
4β	2.75 (*m*)	1.78
4$^{'}$	4.30 (*m*)	3.11
4$^{'}$-OH	7.13 (*br*)	
5$^{'}$	4.00 (*m*)	3.62
6	5.37 (*br, m*)	5.32
6$^{'}$α	4.43 (*m*)	4.21
6$^{'}$β	4.58 (*m*)	4.41
6$^{'}$-OH	6.40 (*br, t, J* = 6.20)	
7α	1.60 (*m*)	1.48
7β	1.90 (*m*)	
8	1.40 (*m*)	1.49
9	0.95 (*m*)	0.91
11	1.40 (*m*)	1.18

Table 4.37 (Continued).

Proton Position	Chemical Shifts in ppm (Coupling Constant in Hz)	
	Compound 6	stigmasteryl-3-O-β-D-glucopyranoside
12β	2.00 (*m*)	1.96
12α	1.15 (*m*)	1.14
14	0.95 (*m*)	1.07
15	1.25 (*m*)	1.03
16α	1.30 (*m*)	1.64
16β	1.85 (*m*)	1.79
17	1.15 (*m*)	1.04
18	0.70 (*s*)	0.64
19	0.97 (*m*)	1.00
20	1.45(*m*)	1.31
21	0.95 (*m*)	0.98
22	5.10 (*dd, J* = 15.20, 8.80)	5.15
23	5.08 (*dd, J* = 15.30, 8.80)	5.01
24	1.60 (*m*)	0.92
25	1.75 (*m*)	1.44
26	0.95 (*m*)	0.89
27	0.95 (*m*)	0.81
28	1.30 (*m*)	1.05
29	0.90 (*m*)	0.82

[#]500 MHz ^{1}H, pyridine d_5; [*]600 MHz ^{1}H, DMSO d_6 with TMS

Table 4.38 The ^{13}C-NMR Chemical Shifts of Compound 6 and stigmasteryl-3-*O*-β-D-glucopyranoside.

Carbon Position	Chemical Shifts in ppm	
	Compound 6	stigmasteryl-3-*O*-β-D-glucopyranoside
1	37.4	38.3
1'	103.1	100.8
2	30.8	33.3
2'	75.8	70.1
3	78.6	76.9
3'	79.1	76.7
4	39.9	46.8
4'	72.2	73.4
5	141.4	140.4
5'	78.9	76.7
6	122.4	121.1
6'	63.4	61.0
7	32.7	31.3
8	32.6	31.4
9	50.9	49.6
10	37.4	36.2
11	21.8	22.6
12	40.5	39.6

Table 4.38 (Continued).

Carbon Position	Chemical Shifts in ppm	
	Compound 6	stigmasteryl-3-*O*-β-D-glucopyranoside
13	42.9	40.0
14	57.3	56.2
15	26.9	24.8
16	29.3	29.2
17	56.8	56.1
18	12.5	11.8
19	19.9	19.0
20	40.5	35.4
21	21.8	18.8
22	139.3	138.0
23	130.3	128.8
24	51.9	45.1
25	30.0	31.3
26	19.7	19.6
27	19.5	18.9
28	23.9	23.8
29	13.0	11.6

[#]125 MHz ^{1}H, pyridine d_5; [*]150 MHz ^{13}C, DMSO d_6 with TMS

The close agreement between our data and the literature values indicate that compound 6 is stigmasteryl-3-*O*-β-D-glucopyranoside (Figure 4.16).

CHAPTER V

CONCLUSION

The dried powder of the leaves of *C. nutans* was extracted with hexane and chloroform, respectively. The chloroform crude extract was isolated by column chromatography and preparative thin-layer chromatography to give six pure compounds. The chemical structures were characterized on the basis of NMR spectral analysis, including DEPT, COSY, HSQC, HMBC and NOESY in comparison with literature values. The structures of six isolated compounds are summarized as follows.

Compounds 1 and 2 were identified as 13^2-hydroxy-(13^2-*S*)-chlorophyll b and 13^2-hydroxy-(13^2-*R*)-chlorophyll b, respectively. The ^{1}H and ^{13}C-NMR spectroscopic evidence revealed the presence of the characteristic vinyl protons (H-5, H-10, and H-20), methyls attached to C-2, C-12 and C-18, an ethyl at C-8 and an aldehyde at C-7^1. The NOESY method was used to determine the stereochemical configuration at C-13^2. For the 13^2(*R*)-diastereomer, the NOESY experiment showed correlations between the 13^4-CH$_3$ protons and the 17^1-CH$_2$/17^2-CH$_2$ protons, as well as between the 13^2-COH proton and the 17-CH proton, implying that these protons were spatially close to one another. In the case of the 13^2(*S*)-diastereomer, the NOESY experiment showed correlations between the 13^4-CH$_3$ proton and the 17-CH proton, as well as between the 13^4-CH$_3$ protons and the 18^1-CH$_3$ protons, implying that these protons were spatially close to one another.

Compound 3 was identified as 13^2-hydroxy-(13^2-R)-phaeophytin b. The ^{1}H and ^{13}C-NMR spectra of compound 3 were similar to those of compounds 1 and 2, but with extra two NH protons for the chlorin ring. The NOESY method was also used to determine the configuration of this compound which was similar to that of compound 2.

Compounds 4 and 5 were identified as 13^2-hydroxy-(13^2-S)-phaeophytin a and 13^2-hydroxy-(13^2-R)-phaeophytin a, respectively. The ^{1}H and ^{13}C-NMR spectra of compounds 4 and 5 were similar to those of compound 3 with a slight difference in the position of the methyl proton at C-7^1. The NOESY method was also used to determine the configuration of compounds 4 and 5 which were similar to those of compounds 1 and 2, respectively.

Compound 6 was identified as stigmasteryl-3-O-β-D-glucopyranoside. The ^{1}H and ^{13}C-NMR spectra of compound 6 were in agreement with those obtained from the literature.

In terms of biological activities, chlorophyll related compounds isolated from plants and marine organisms have been shown to possess strong antioxidant activity (Watanabe et $al.,$ 1993). Compounds 3, 4 and 5 showed in $vitro$ cytostatic activity against hepatoma tissue culture at 33 μg/mL (Nakatani, Ourisson and Bech, 1981) and Compound 6 has been shown to have growth promoting activity and to be active against leukemia (Boonyaratavej and Petsom, 1991).

REFERENCES

REFERENCES

เต็ม สมิตินันทน์. (2544). **ชื่อพรรณไม้แห่งประเทศไทย**. ส่วนพฤกษศาสตร์ป่าไม้ สำนักวิชาการ
    ป่าไม้: กรมป่าไม้.

Alam, M. S., Chopra, N., Ali, M. and Niwa, M. (1996). Oleanen and stigmasterol
    derivatives from *Ambroma austa*. **Phytochemistry** 41: 1197-1200.

Backer, C. A. and Bakhuizen van den brink, R. C. (1965). **Flora of Java** Netherland:
    N.V.P. Noordhoff Groningen.

Bhat, S. V., Nagasampagi, B. A. and Sivakumar, M. (2005). **Chemistry of natural
    products.** New Delhi: Narosa Publishing House.

Block, J. H. (1989). **Progress in the design of bioactive molecules.** Washington
    D.C.: American Chemical Society.

Boonyaratavej, S. and Petsom, A. (1991). Chemical constituents of the roots of
    Bridelia tomentosa bl. **Journal of the Science Society of Thailand** 17: 61-
    69.

Butler, M. S. (2004). The role of natural product chemistry in drug discovery. **Journal
    of Natural Product** 67: 2141-2153.

Cannell, R. J. C. (1998). **Natural products isolation.** New Jersey: Humana Press.

Cherdchu, C., Poopyruchpong, N., Adchariya, R., Patanaba Nangkost, S. and
    Ratanabangkoon, K. (1977). The absence of antagonism between extract of
    Clinacanthus nutans and *Naja naja siamensis.* **The Journal of the Tropical
    Medicine and Public Health** 8: 249-254.

Chavalittumrong, P. (2000). **Standard of Thai herbal medicine:** *Andrographis paniculata* **(Burm. f.) Nees.** Bangkok: E.T.O. Press.

Chavalittumrong, P. (2001). **Standard of Thai herbal medicine:** *Curcuma longa* **Linn.** Bangkok: E.T.O. Press.

Chavalittumrong, P. (2002). **Standard of Thai herbal medicine:** *Cassia alata* **Linn.** Bangkok: E.T.O. Press.

Chavalittumrong, P., Attawish, A., Rugsamon, P. and Chuntapet, P. (1995). Toxicological study of *Clinacanthus nutans* (Burm. f.) Lindau. **Bulletin of the Department of Medical Science** 37: 323-337.

Charuwichitratana, S., Wongrattanapasson, N., Timpatanapong, P. and Bunjob, M. (1996). Herpes zoster: Treatment with *Clinacanthus nutans* cream. **International Journal of Dermatology** 35: 665-666.

Chuakul, W. (1986). **Chemical study of the anti-inflammatory agents from the leaves of Phayaa Plong Thong,** *Clinacanthus nutans* **(Burm. f.) Lindau.** M.Sc. Thesis, Faculty of Pharmacy, Mahidol University.

Clark, A. M. (1996). Natural products as a resource for new drugs. **Pharmaceutical Research** 13: 1133-1141.

Colegate, S. M. and Molyneux, R. J. (1993). **Bioactive natural products.** Florida: CRC Press.

Cragg, G. M. (2002). Natural product drug discovery and development. **Puerto Rico Health Sciences Journal** 21: 97-111.

Cragg, G. M. and Newman, D. J. (2001). Natural product drug discovery in the next millennium. **Pharmaceutical Biology** 39: 8-17.

Cragg, G. M., Newman, D. J. and Snader, K.M. (1997). Natural products in drug discovery and development. **Journal of Natural Product** 60: 52-60.

Dampawan, P. (1976). **Studies of the chemical constituents of the *Clinacanthus nutans* (Acanthaceae) and *Zingiber Cassumunar* Roxb.** M.Sc. Thesis, Mahidol University.

Dampawan, P., Huntrakul, C. and Reutrakul, V. (1977). Constituents of *Clinacanthus nutans* and the Crystal Structure of LUP-20 (29)-ene-3-one. **Journal of the Science Society of Thailand** 3: 14-26.

Dechatiwongse na Ayudhaya, T. (1997). Standardization and quality control of herbal medicines. **In International Symposium on the Standardization and Practice of Herbal Medicine** (pp 1-16). Korean: Seoul National University.

Dechatiwongse na Ayudhya, T., Sakdarat, S., Shuyprom, A., Pattamadilok, D., Bansiddhi, J., Waterman, P. G. and Karagianis, G. (2001). Chemical constituents of the leaves of *Clinacanthus nutans* Lindau. **Thai Journal of Phytopharmacy** 8: 1-8.

Farnsworth, N. R., Akerele, O., Bingel, A. S., Soejarto, D. D. and Guo, Z. (1985). Medicinal plants in therapy. **Bulletin of the World Health Organization** 63: 965-981.

Gad, S. C. (2005). **Drug discovery handbook.** New York: John Wiley and Sons.

Hancharnlerd, O., Babprasert, C. and Phisuksanthiwattana, Y. (1994). **Medicinal plants in Pakchong research station garden.** Faculty of Agriculture: Kasetsart University.

Hynninen, P. H., Leppakases T. S. and Mesilaakso, M. (2006). The enolate anions of chlorophylls a and b as ambident nucleophiles in oxidations with (L)- or (D)-(10-camphorsulfonyl)oxaziridine. Synthesis of $13^2(S/R)$hydroxychlorophylls a and b. **Tetrahedron** 62: 3412-3422.

Jayavasu, C., Chavaltitumrong, P., Polachandara, K., Dechatiwongse na Ayudhaya, T. and Jongtrakulsiri, S. (1992). The virucidal activity of *Clinacanthus nutants* Lindau extract against herpes simplex virus type 2: An *in vitro* study. **Bulletin of the Department of Medical Science** 34: 153-158.

Jayavasu, C. (1998). Clinical trial in the treatment of genital herpes patients with *Clinacanthus nutants* extract. In **The 9th Ministry of Public Health Symposium (57).** Bangkok: E.T.O. Press.

Kharnngan, D. (1991). **Specification of Thai medicinal plants.** Bangkok: E.T.O. Press.

Lin, J., Li, H. and Yu, J. (1983). Studies on the chemical constituents of Nui Xu Hua (*Clinacanthus nutans*). **Zhongcaoyao** 14: 337-338.

Lou, Z.C. (1993). Good manufacturing practices for pharmaceutical products, supplementary guidelines for the manufacture of herbal medicinal products. **WHO/Pharm/93.561/rev.1.** (pp 5-118). Geneva.

Malhotra, S. and Pal Singh, A. (2002). Hepatoprotective potential of phytomedicnes. **Gastroenterology** 6: 76-77.

Mann, J. (1992). **Murder, Magic, and Medicine.** Oxford: Oxford University Press.

Matsuo, A., Ono, K., Hamasaki, K. and Nozaki, H. (1996). Phaeophytins from cell suspension culture of the Liverwort *Plagiochila ovalifolia*. **Phytochemistry** 42: 427-430.

Ministry of Public Health, National Drug Committee. (1999). **National list of essential drugs A. D. 1999 (List of herbal medicinal products).** Bangkok: E.T.O. Press.

Nakatani, Y., Ourisson, G. and Bech, J.P. (1981). Chemistry and biochemistry of Chinese drugs VII. cytostatic pheophytins from Silkworm excreta and derived photocytotoxic pheophorbides. **Chemical and Pharmaceutical Bulletin** 29: 2261-2269.

Newman, D. J., Cragg, G. M. and Snader, K. M. (2000). The Influence of natural products upon drug discovery. **Natural Products Reports** 17: 215-234.

Newman, D. J., Cragg, G. M. and Snader, K.M. (2003). Natural products as sources of new drugs over the period 1981-2002. **Journal of Natural Product** 66: 1022-1037.

Perry, L. M. (1986). **Medicinal plants East and Southeast Asia.** Cambridge: The MIT Press.

Phillipson, J. D. (2001). Phytochemistry and medicinal plants. **Phytochemistry** 56: 237-243.

Pothisiri, P. (1996). **Medicinal use of botanicals an overview in Thailand food and drug administration.** Bangkok: E.T.O. Press.

Sangkitpporn, S., Polchanandara, K., Thawatsupa, P., Bunchob, M. and Chawalitumrong, P. (1993). Treatment of recurrent genital herpes simplex virus infection with *Clinacanthus nutans* extract. **Bulletin of the Department of Medical Service** 18: 226-231.

Sangkitpporn, S., Polchanandara, K., Balachandra, K., Dechatiwongse na Ayudhaya, T., Bunchob, M. and Jayavasu, C. (1995). Treatment of herpes zoster patients with *Clinacanthus nutans* (bi phaya yaw) extract. **Journal of the Medical Association of Thailand** 78: 624-627.

Satakhun, S. (2001). **Chemical constituents of *Clinacanthus nutans* leaves.** M.Sc. Thesis, Chulalongkron University.

Satayavivad, J., Bunyapraphatsara, N., Kittisiripornkul, S. and Tanasomwong, W. (1996). Analgsic and anti-inflammatory activities of extract of *Clinacanthus nutans* (Burm. f.) Lindau. **Thai Journal of Phytopharmceutical Sciences** 3: 7-17.

Sawasdimongkol, K. (1995). **Medicinal plants and Thai traditional medicine.** Department of Medical Sciences: Ministry of Public Health, Thailand.

Shuyprom, A. (2005). **Chemical composition investigation of the *Clinacanthus nutans* (Burm. f.) Lindau leaves.** M.Sc. Thesis, Suranaree University of Technology.

Suwanborirux, K., Satakhun, S., Janwitayanuchit, W., Songsaksakul, S., Patarapananich, C., Lipipun, V., Vayamhasuwan, P., Suttisiri, R., Pengsuparp, T., De-eknamkul, W., Vilaivan, T. and Pummangura, S. (2003). The secret of *Clinacanthus nutans*. **The Sixth JSPS-NRCT Joint Seminar: Recent Advances in Natural Medicine Research**: 1-6.

Tanasomwong, W. (1986). **The Screening of anti-inflammatory action of *Clinacanthus nutans* (Burm. f.): A critical evaluation of carrageenan induced hind paw edema model.** M.Sc. Thesis, Mahidol University.

Teshima, K., Kaneto, T., Ohtani, K., Kasai, R., Lhieochaiphant, S., Picheasoonthon, C. and Yomasaki, K. (1997). C-glycosyl flavones from *Clinacanthus nutans*. **Natural Medicines** 51: 557.

Teshima, K., Kaneto, T., Ohtani, K., Kasai, R., Lhieochaiphant, S., Picheasoonthon, C. and Yomasaki, K. (1998). Sulfur-containing glucosides from *Clinacanthus nutans*. **Phytochemistry** 48: 831-835.

Thawaranantha, D., Balachandra, K., Jongtrakulsiri, S., Chavalitturong, P., Bhumiswasdi, J. and Jayavasu, C. (1992). *In vitro* antiviral activity of *Clinacanthus nutans* on varicella-zoster virus. **Siriraj Hospital Gazette** 44: 285-291.

Thongharb, C. and Tejasen, P. (1977). The effect of Slaed Pang Pon (*Clinacanthus nutans*) on Thai Cobra Venom (*Naja naja siamensis*). **Thai Journal of Phytopharmaceutical Sciences** 2: 1057-1063.

Tuntiwachwuttikul, P., Pootaeng-on, Y., Phansa, P. and Taylor, W. C. (2004). Cerebrosides and a monoacylmonogalactosylglycerol from *Clinacanthus nutans*. **Chemical and Pharmaceutical Bulletin** 52: 27-32.

Watanabe, N., Yamamoto, K., Ihshkawa, H., Yagi, A., Sakata, K., Brinen, L. S. and Clardy, J. (1993). New chlorophyll a related compounds isolated as antioxidants from marine bivalves. **Journal of Natural Products** 56: 305-317.

APPENDIX

APPENDIX
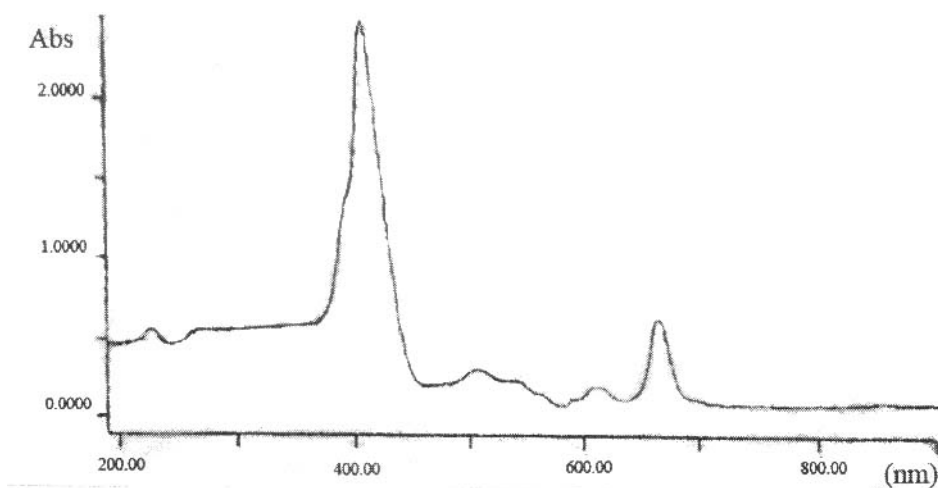
Figure A.1 UV-Vis spectrum of compound 1
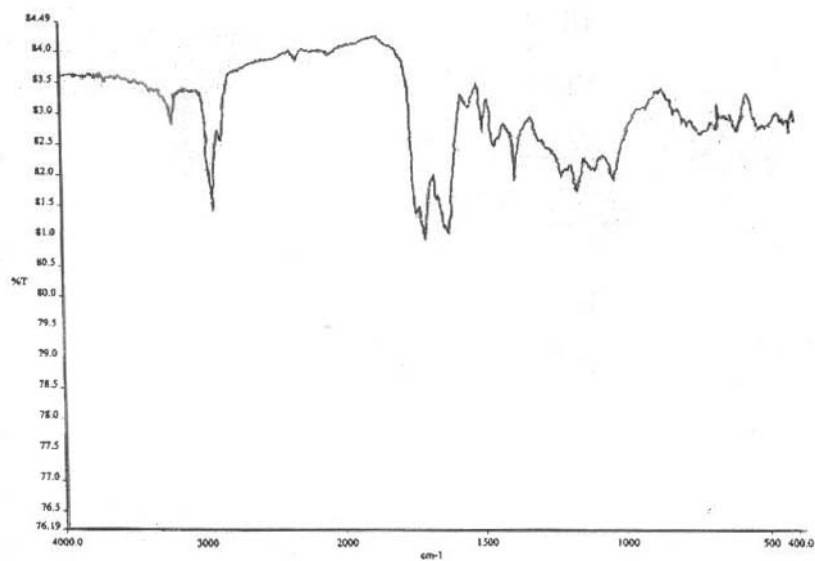
Figure A.2 IR spectrum of compound 1
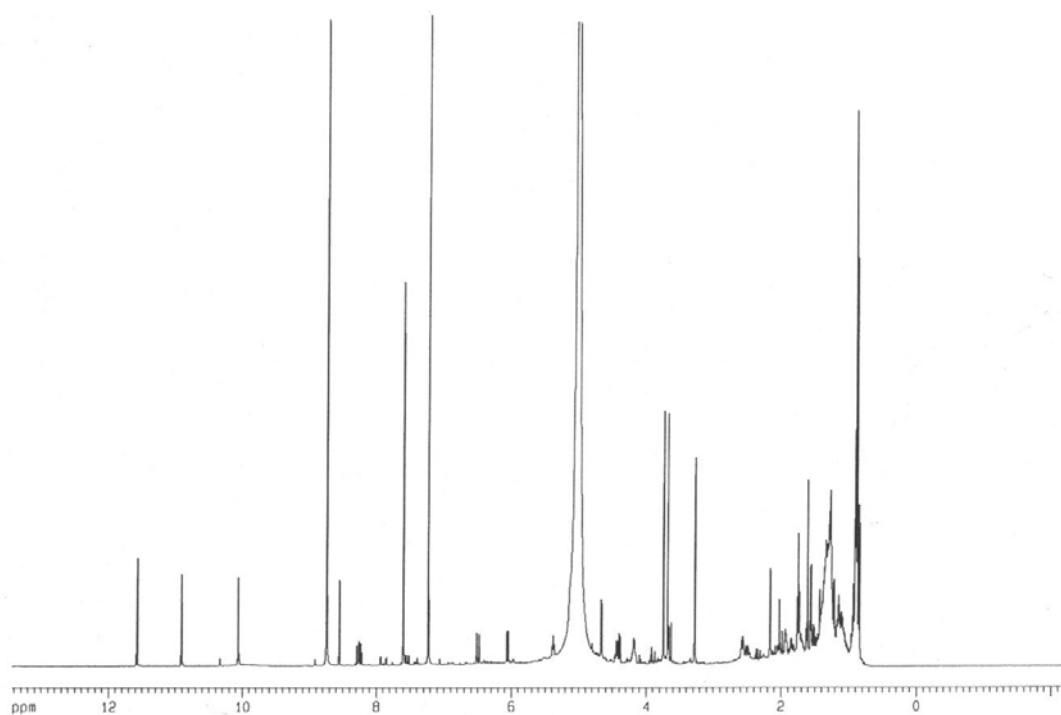
Figure A.3 ^{1}H-NMR spectrum of compound 1
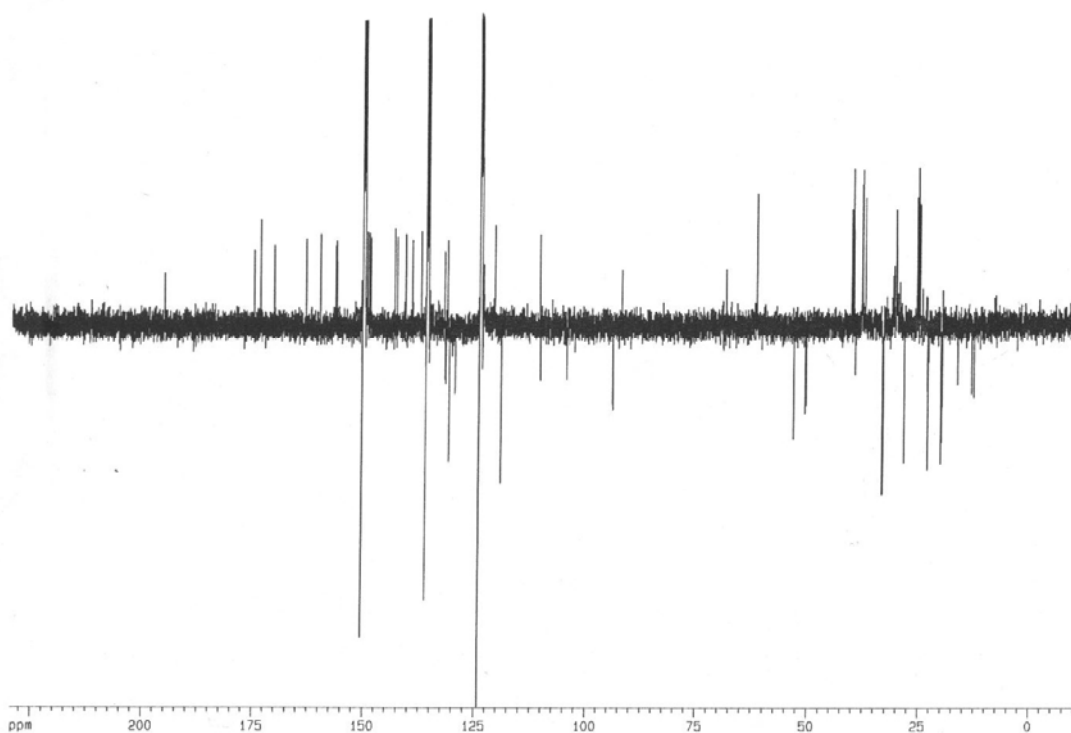
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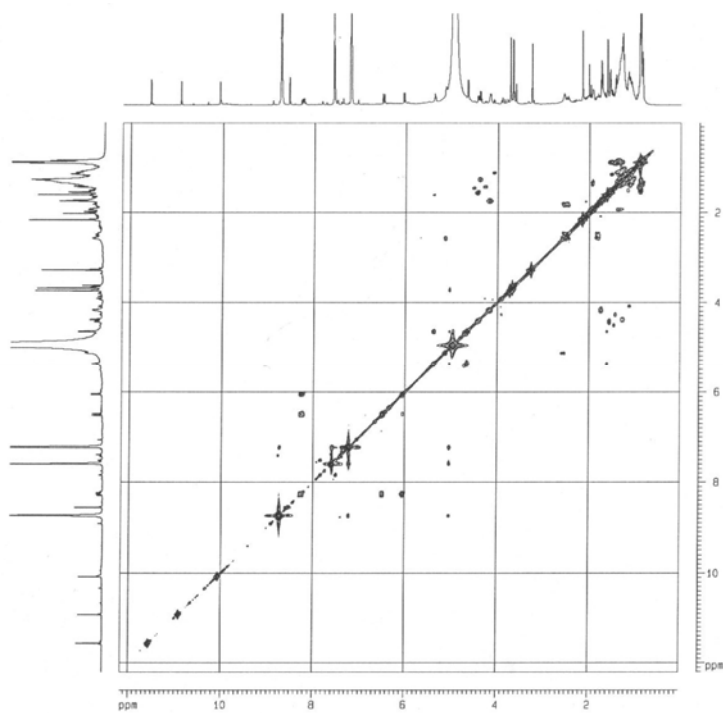
Figure A.5 ^{1}H,^{1}H-COSY spectrum of compound 1
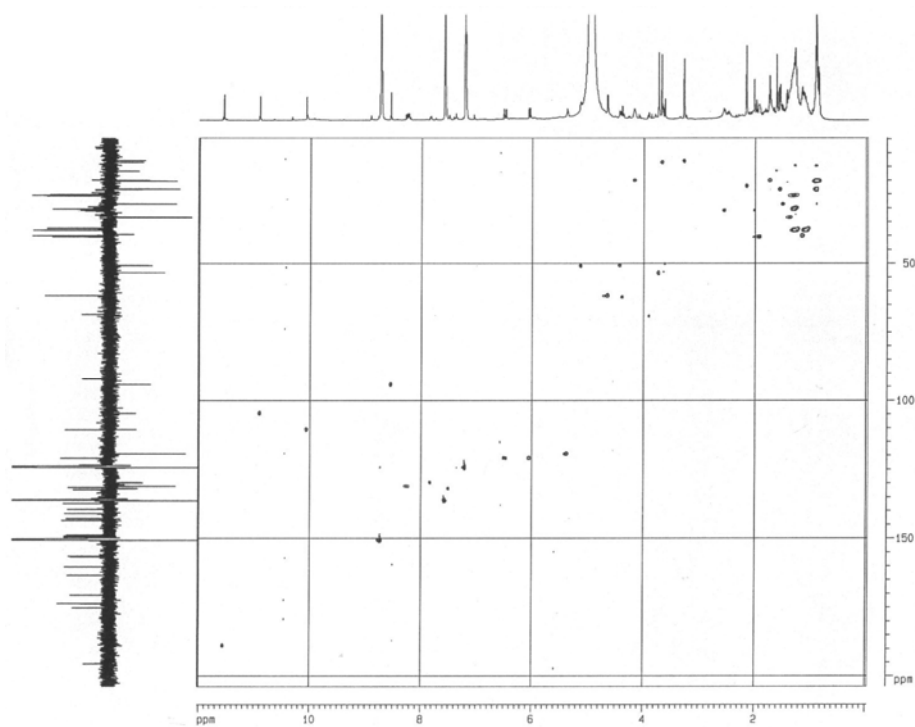
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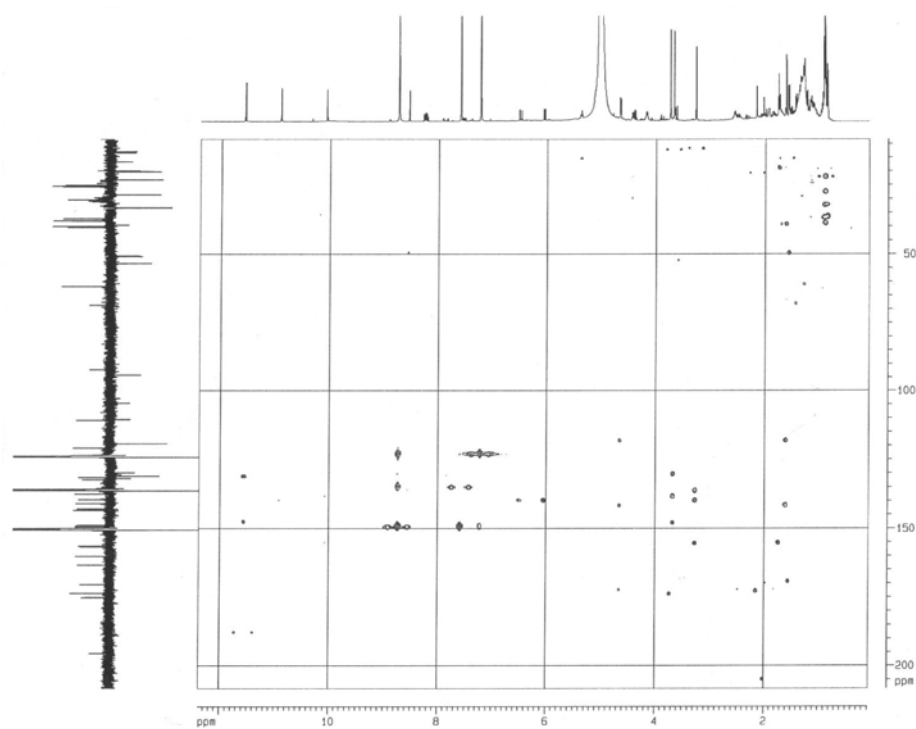
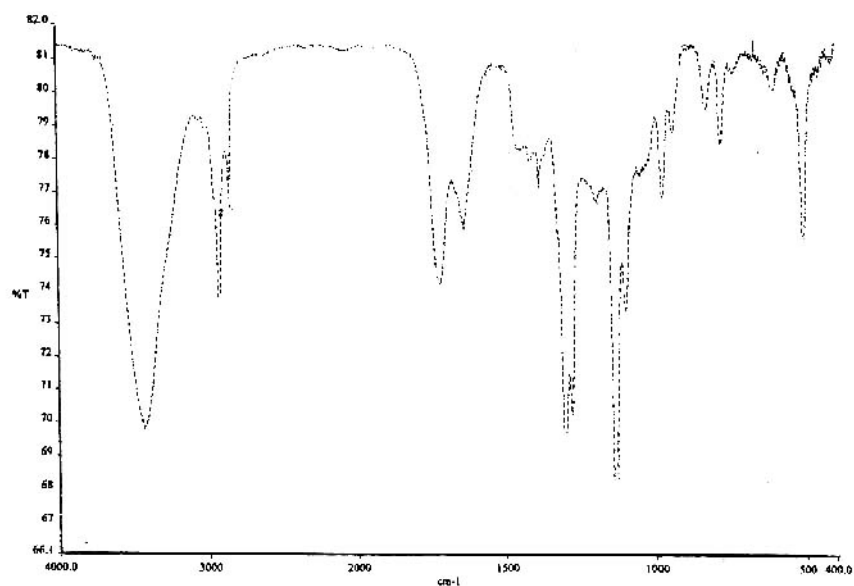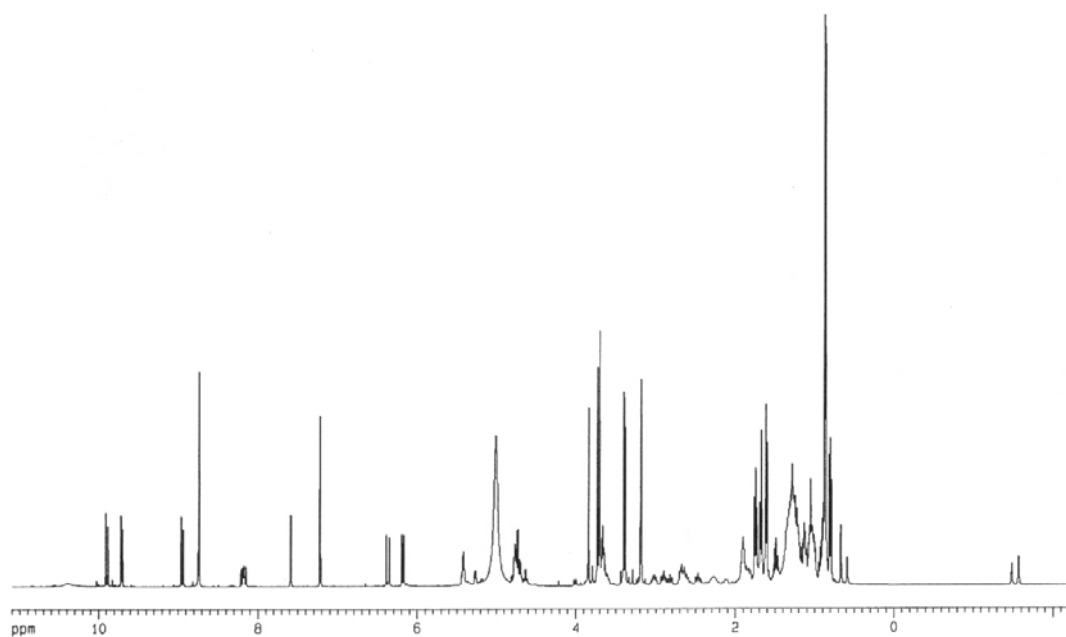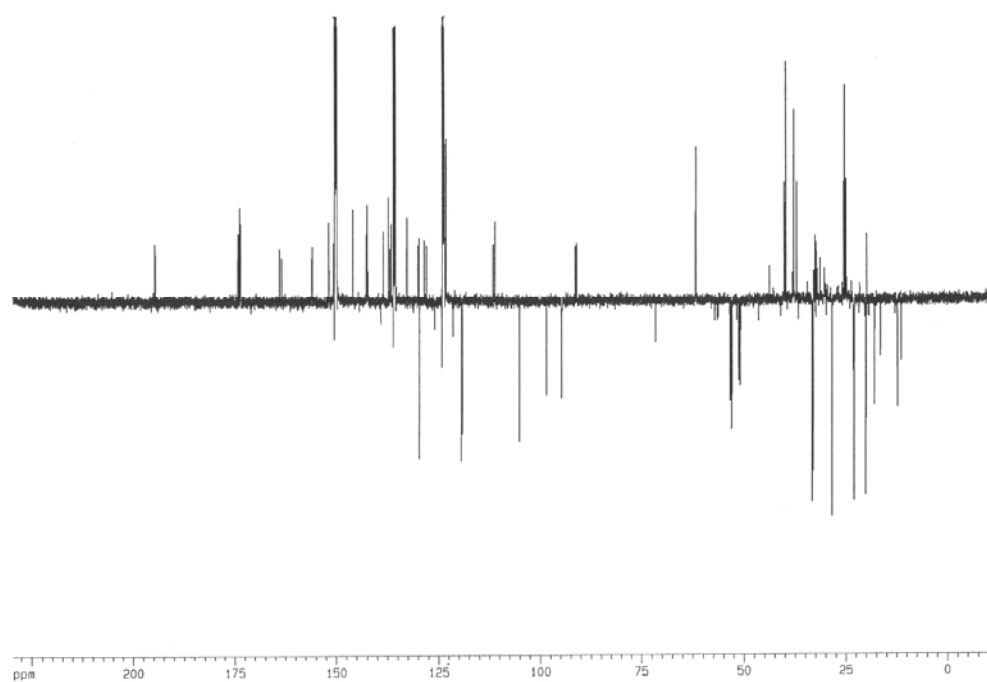**Figure A.7** HMBC spectrum of compound 1
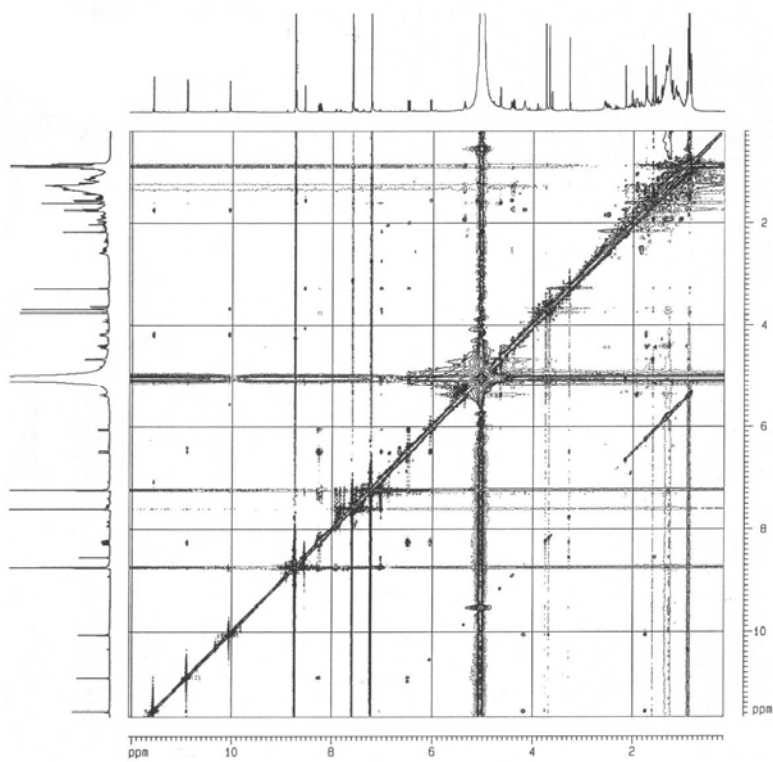
Figure A.8 NOESY spectrum of compound 1
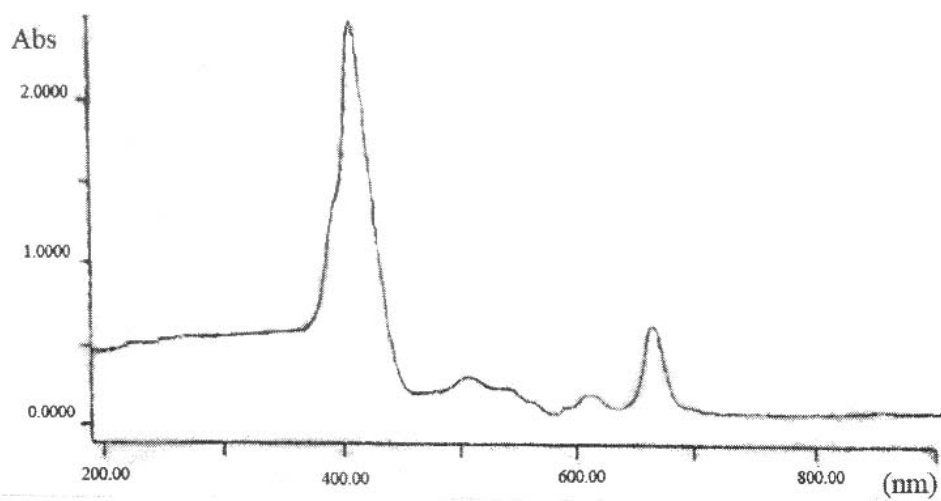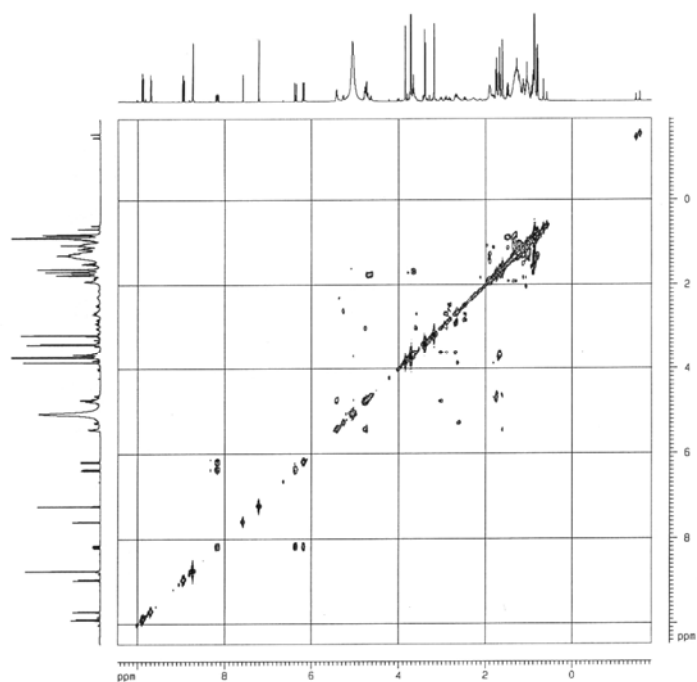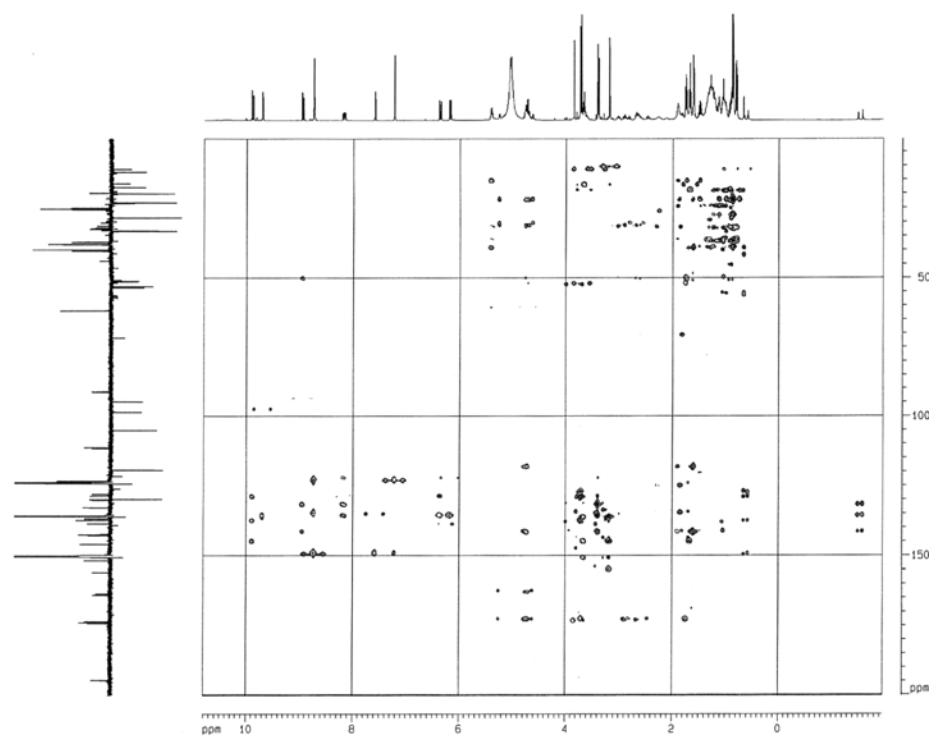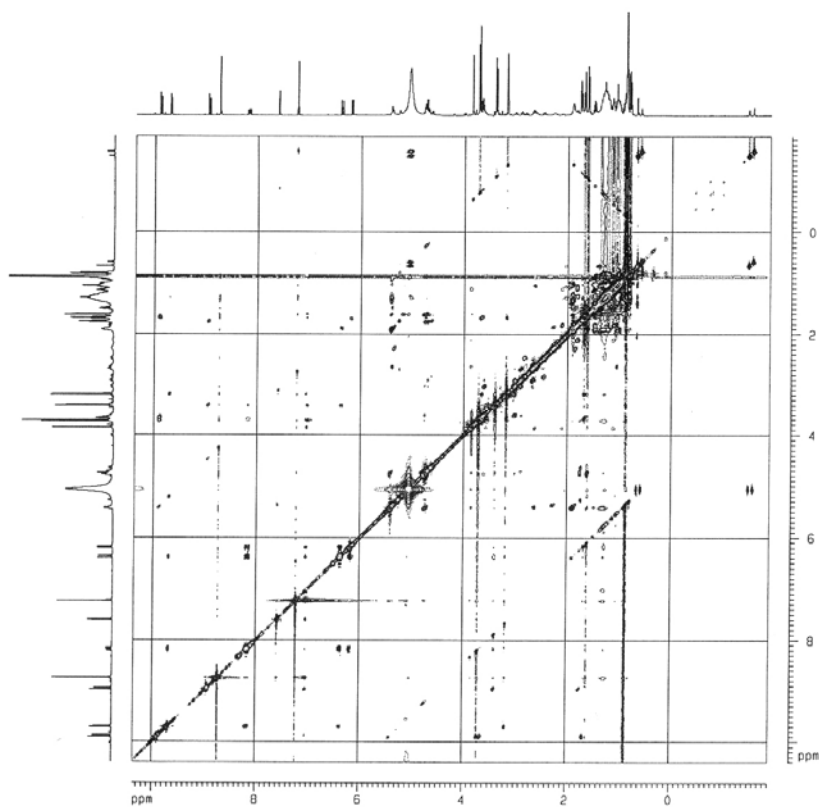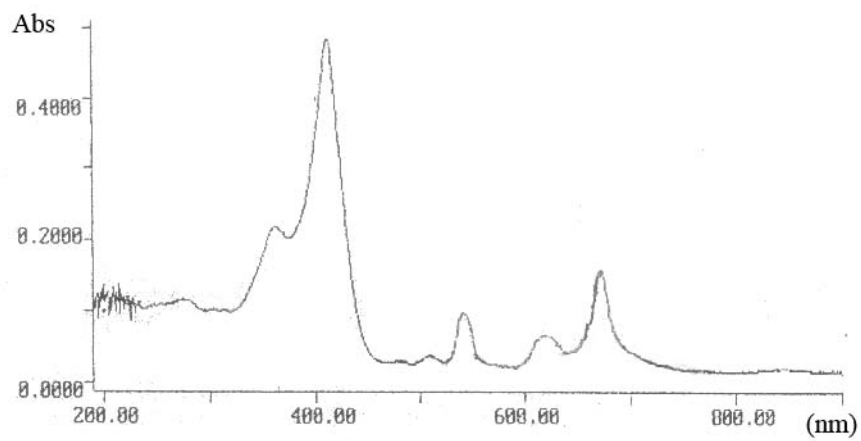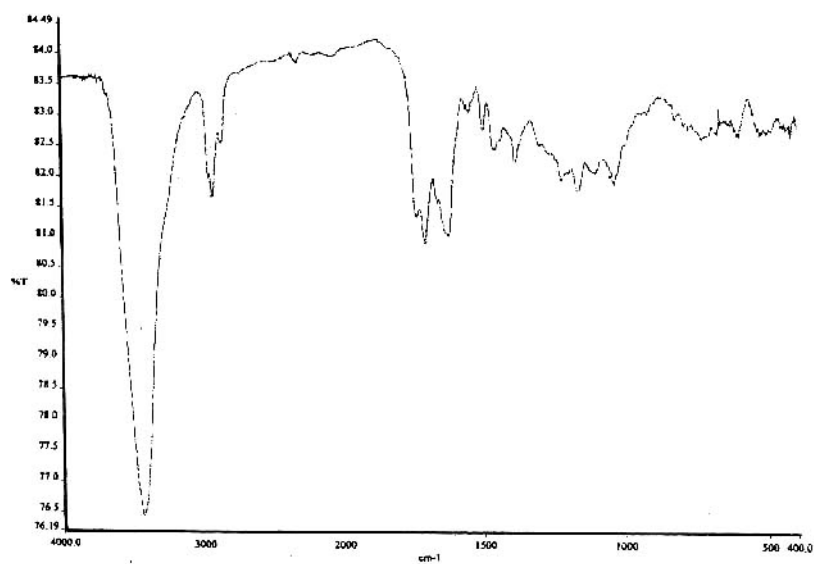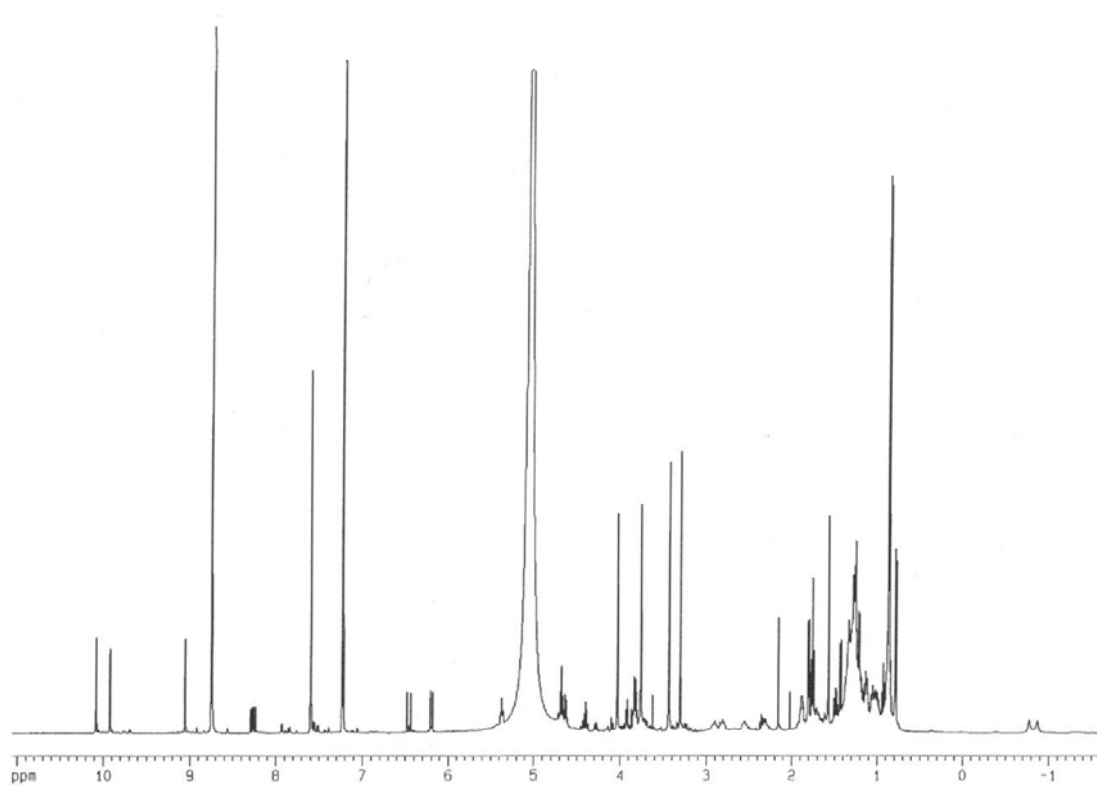
Figure A.9 UV-Vis spectrum of compound 2
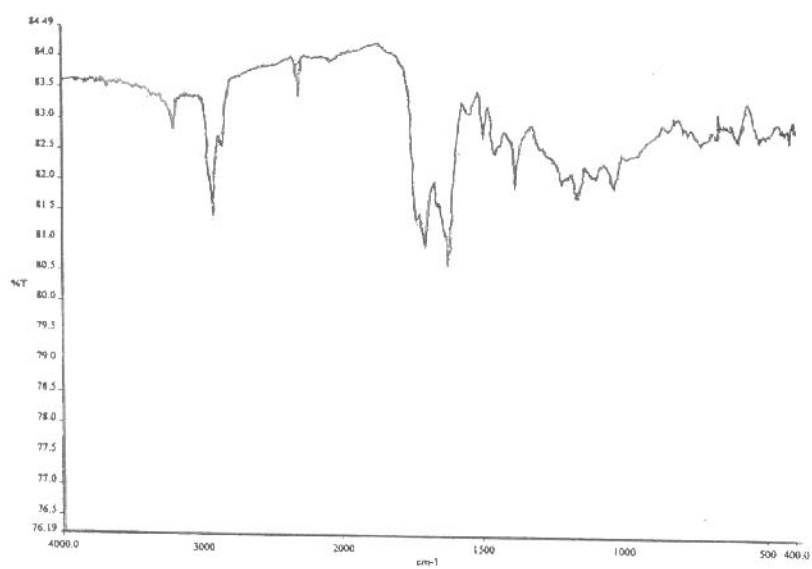
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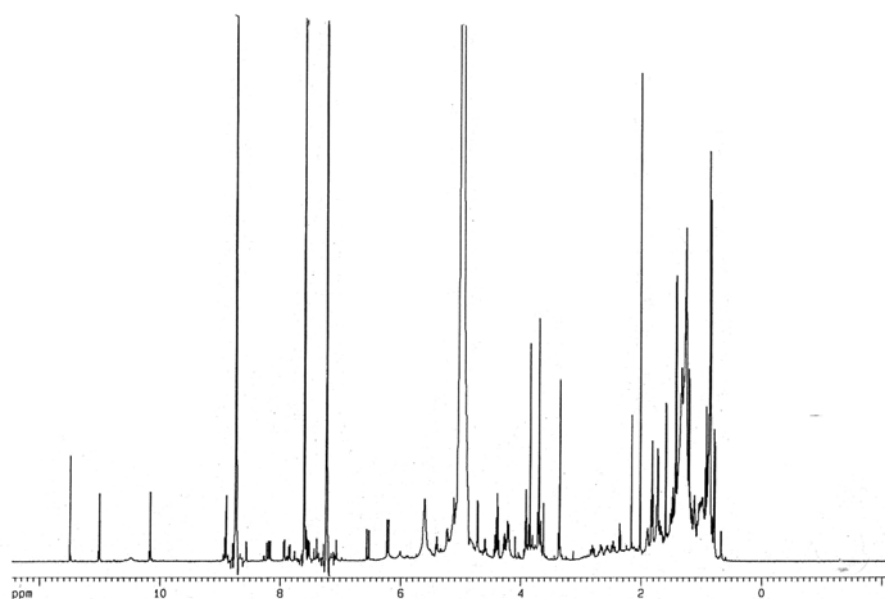
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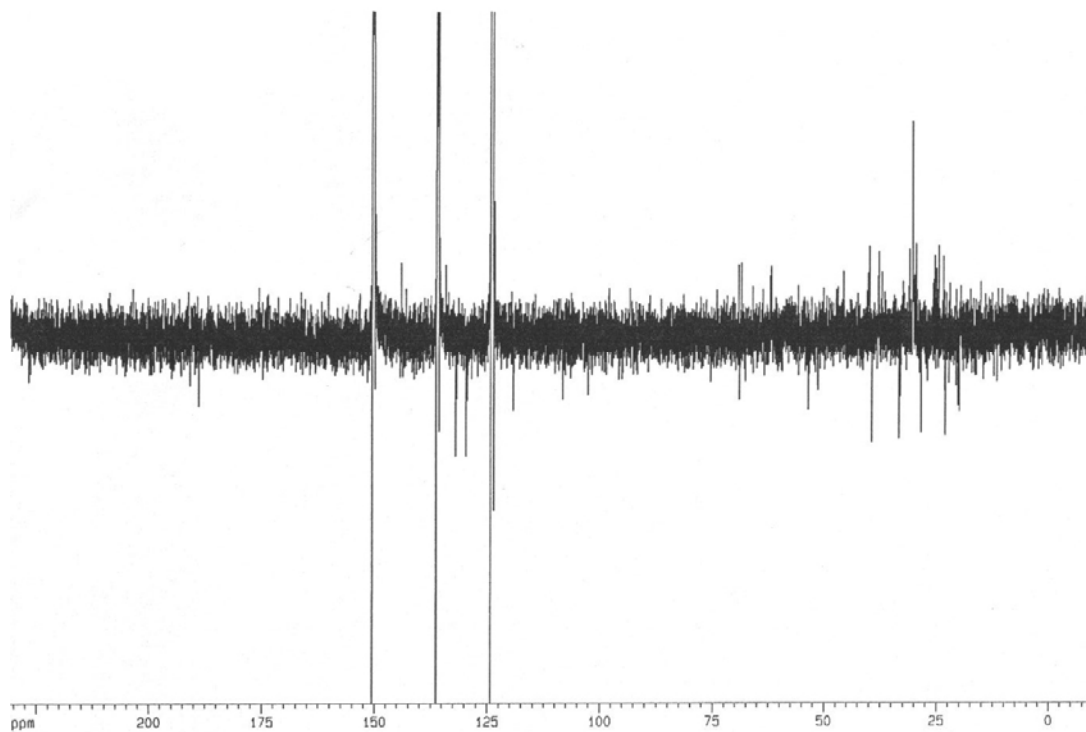
Figure A.12 DEPT ^{13}C-NMR spectrum of compound 2
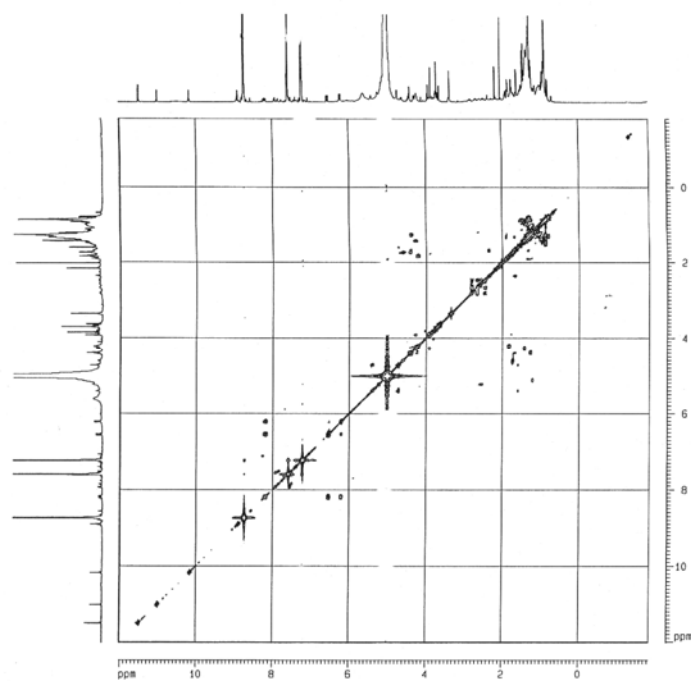
Figure A.13 ^{1}H,^{1}H-COSY spectrum of compound 2
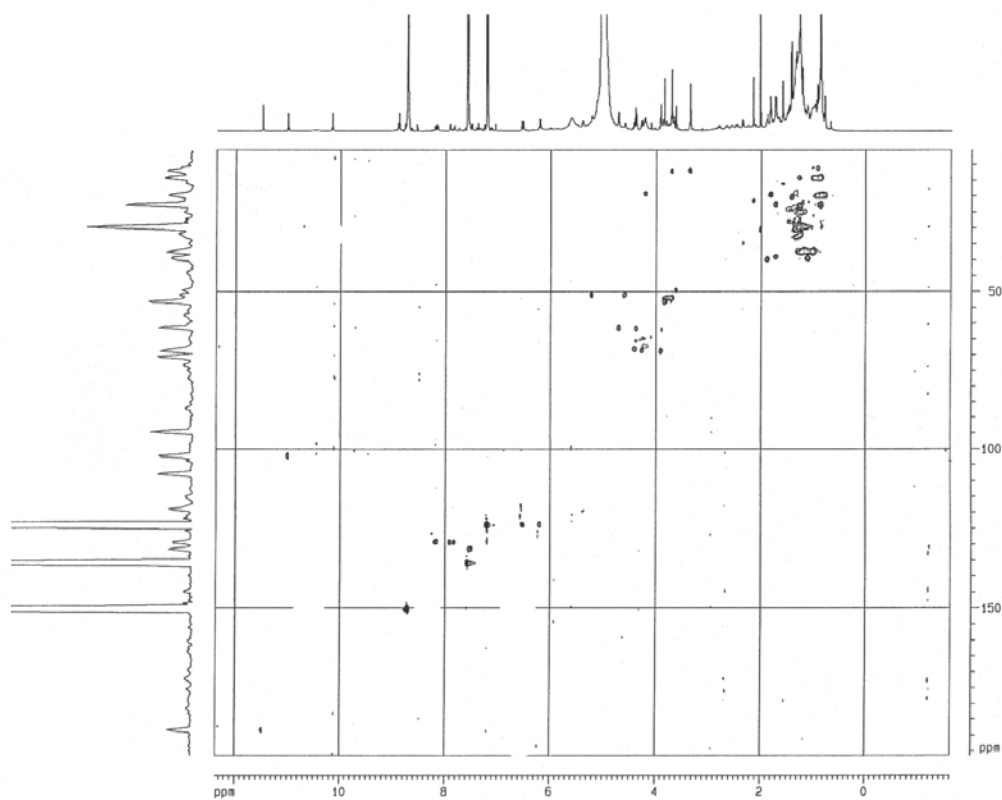
Figure A.14 HSQC spectrum of compound 2
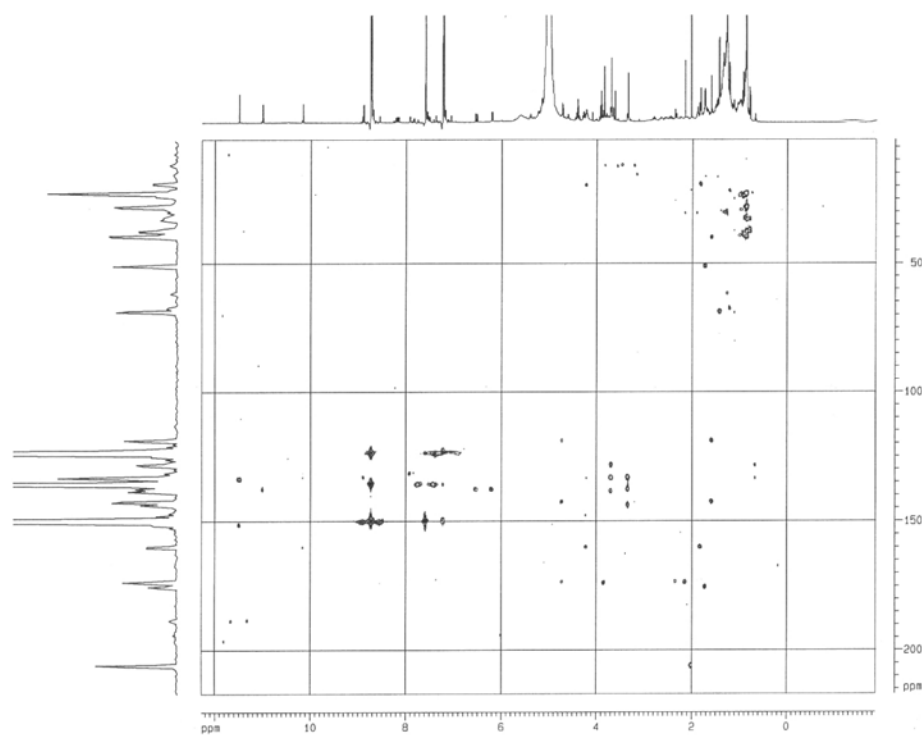
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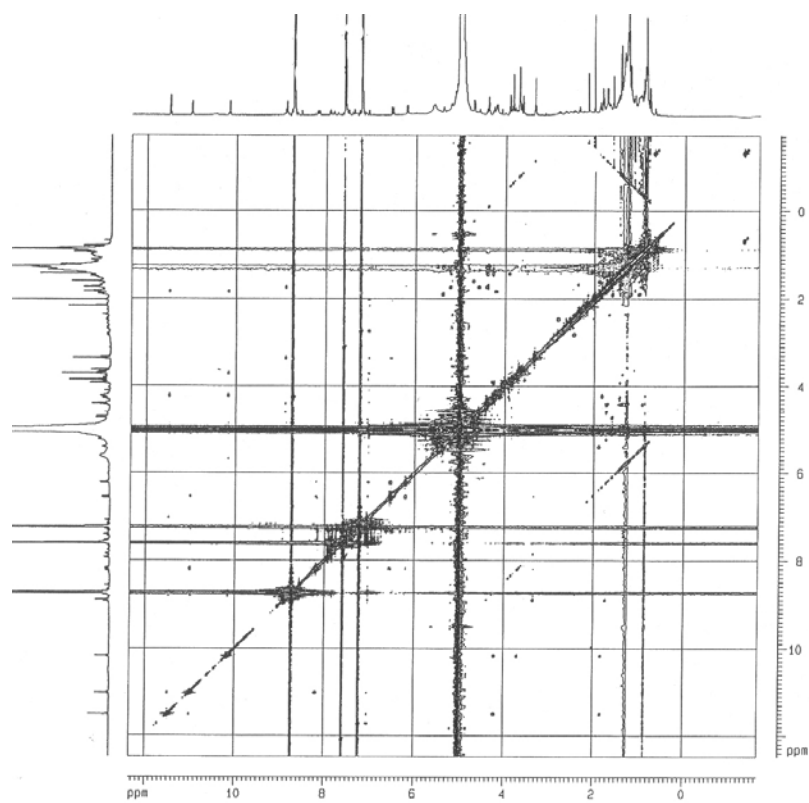
Figure A.16 NOESY spectrum of compound 2
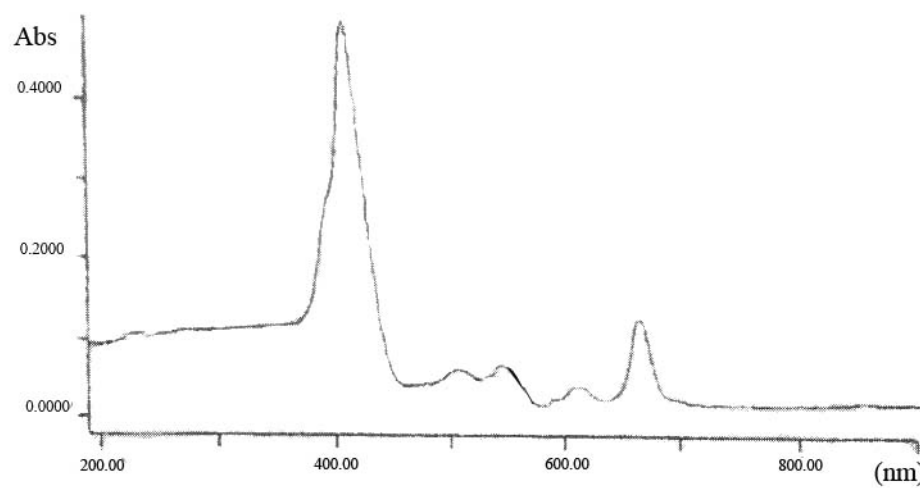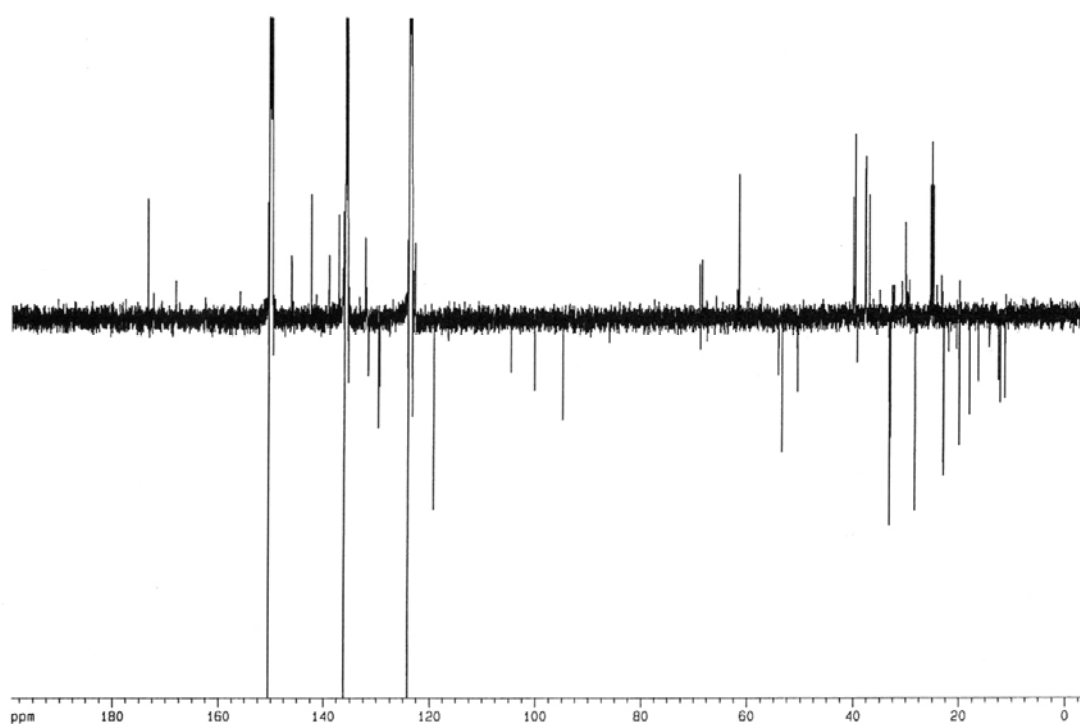
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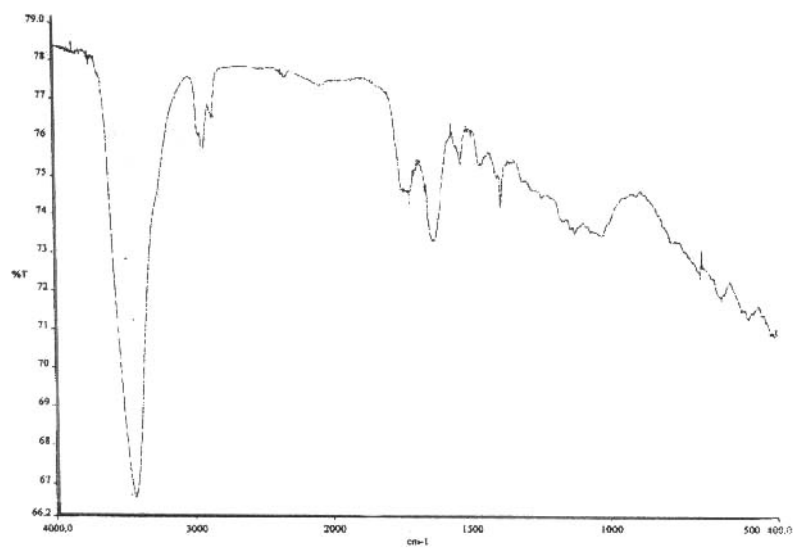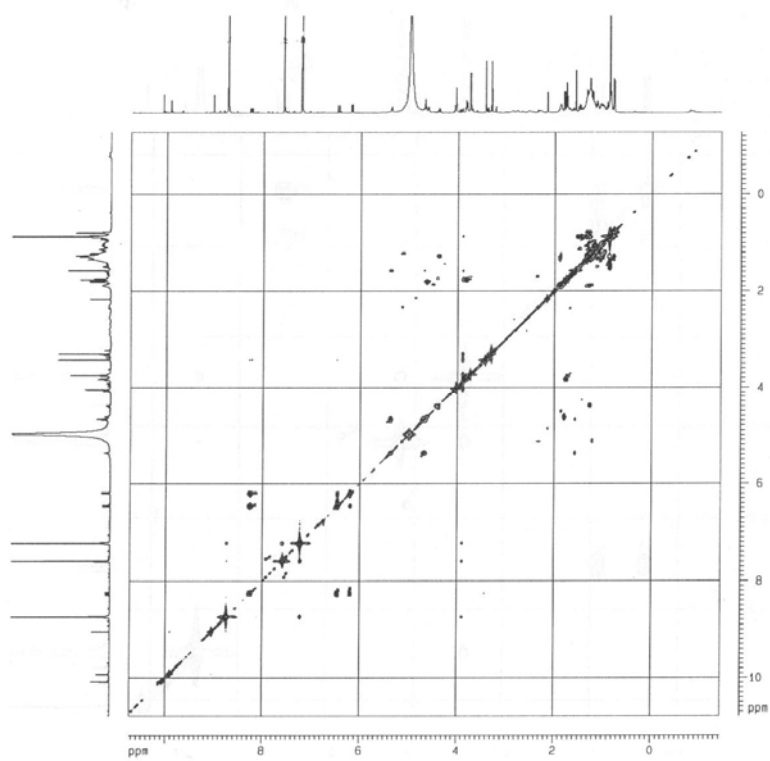

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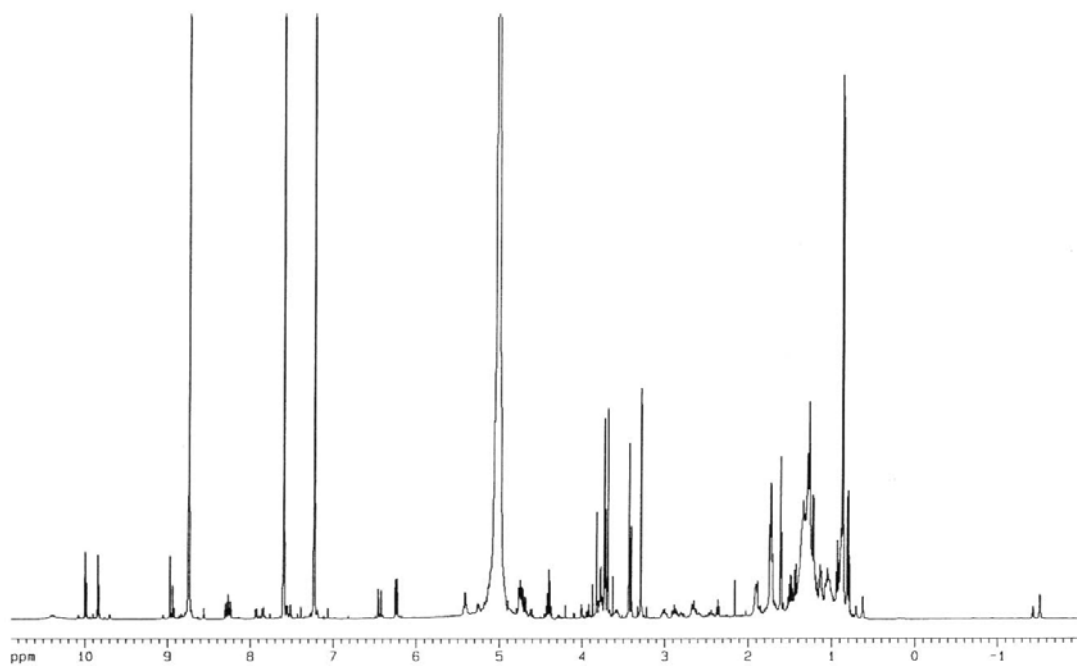
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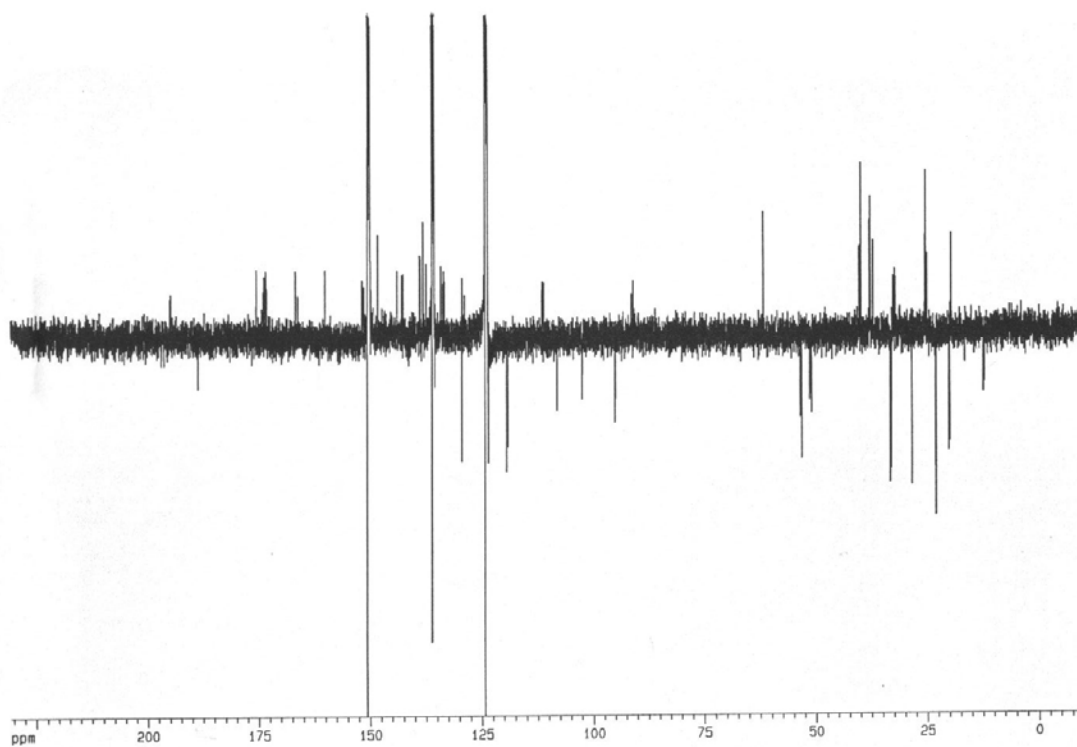
Figure A.20 DEPT ^{13}C-NMR spectrum of compound 3
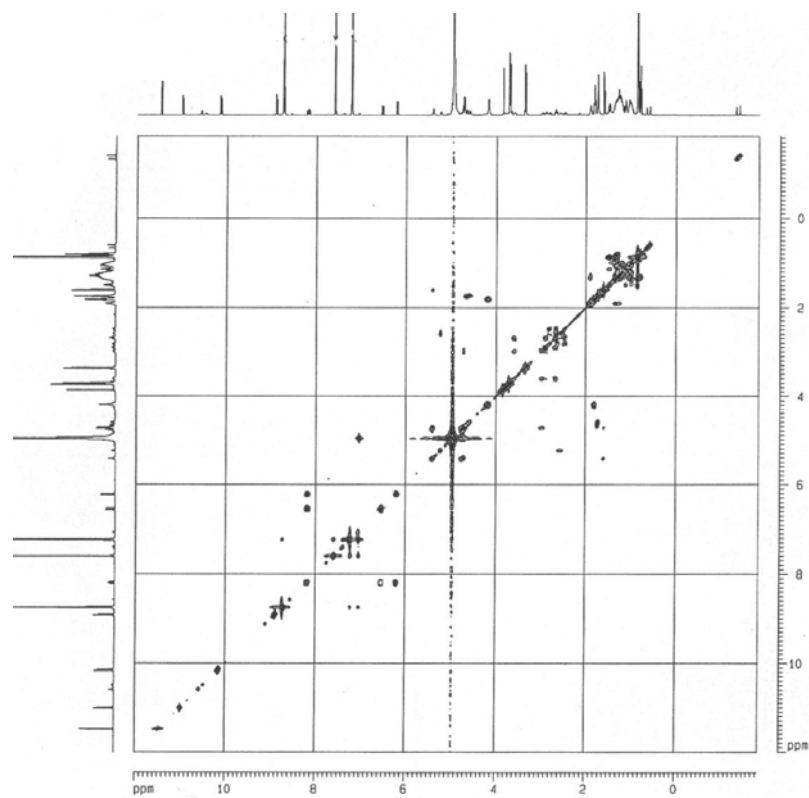
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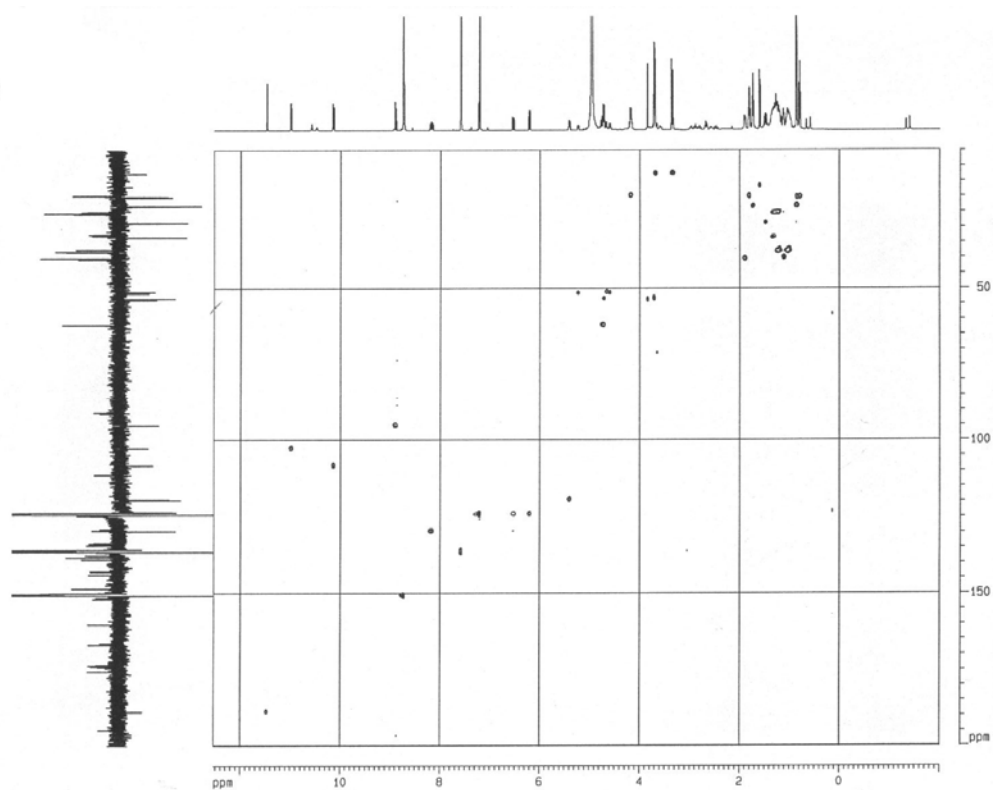
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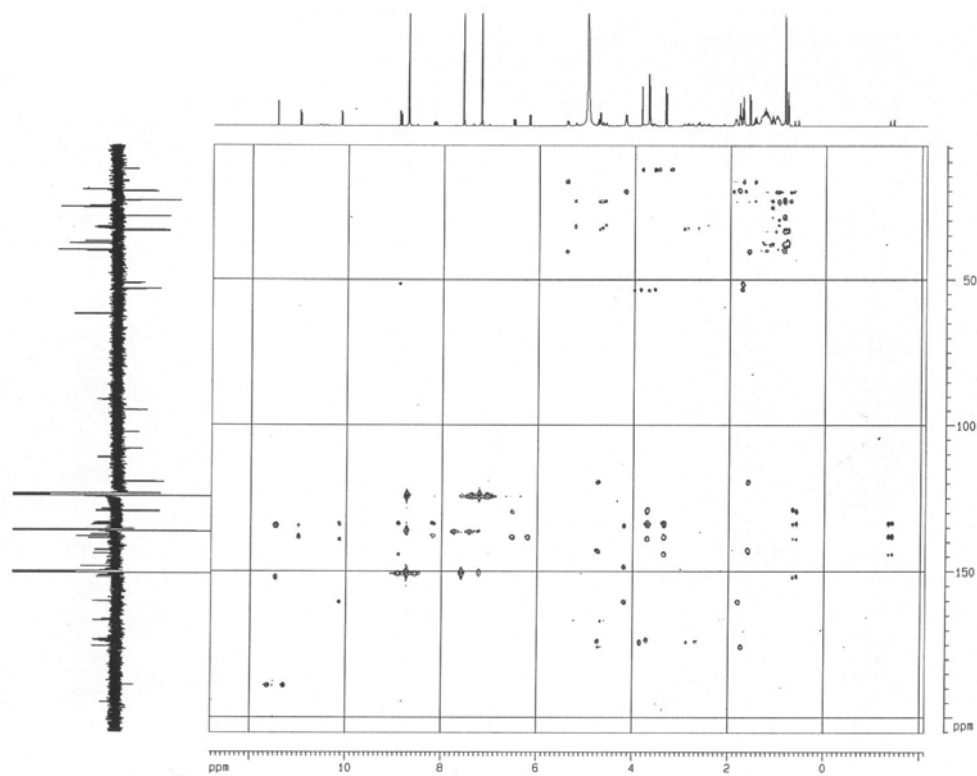
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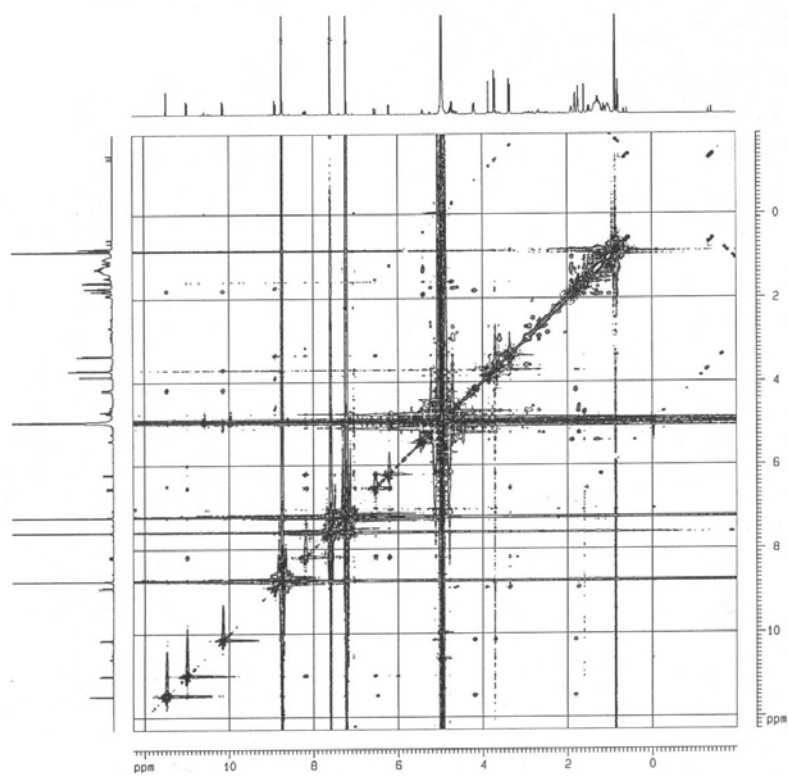
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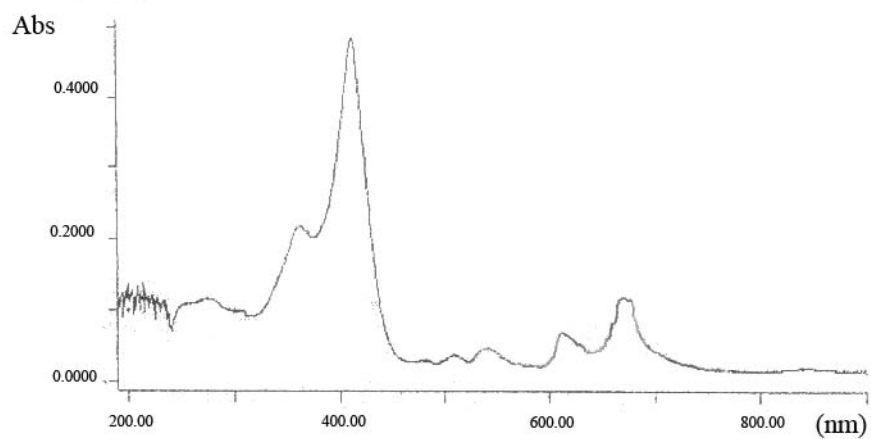
Figure A.25 UV-Vis spectrum of compound 4

Figure A.26 IR spectrum of compound 4

Figure A.27 ^{1}H-NMR spectrum of compound 4

Figure A.28 DEPT ^{13}C-NMR spectrum of compound 4

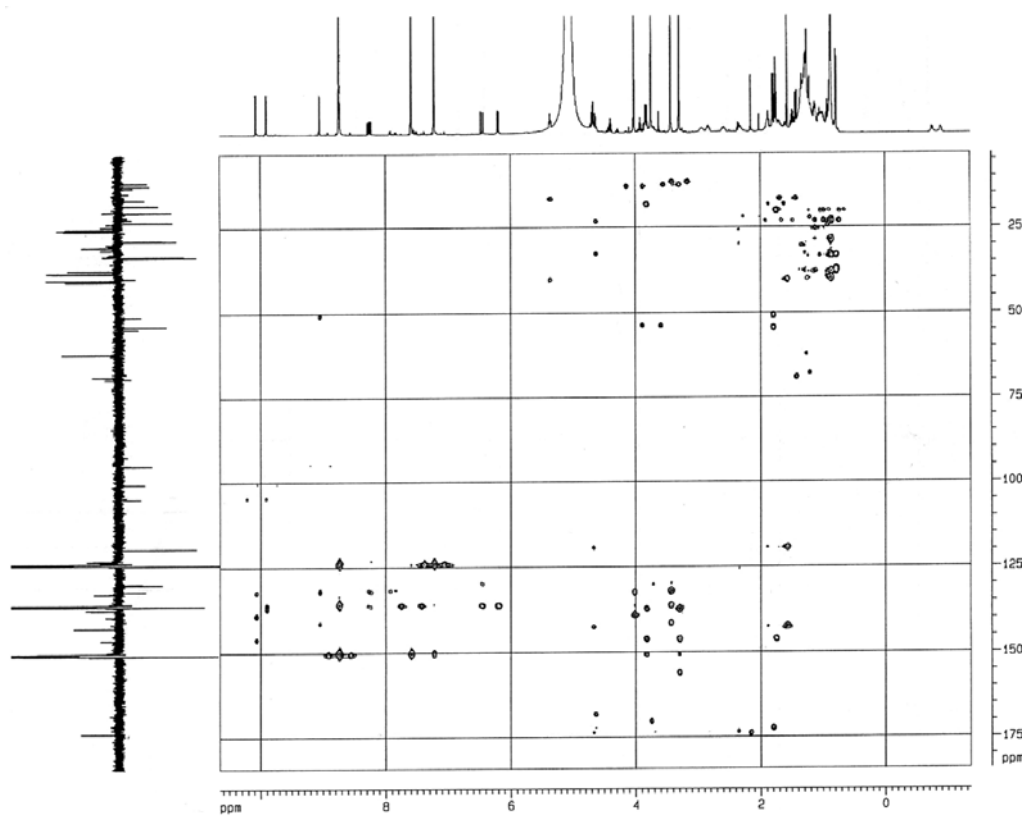**Figure A.29** ^{1}H,^{1}H-COSY spectrum of compound 4
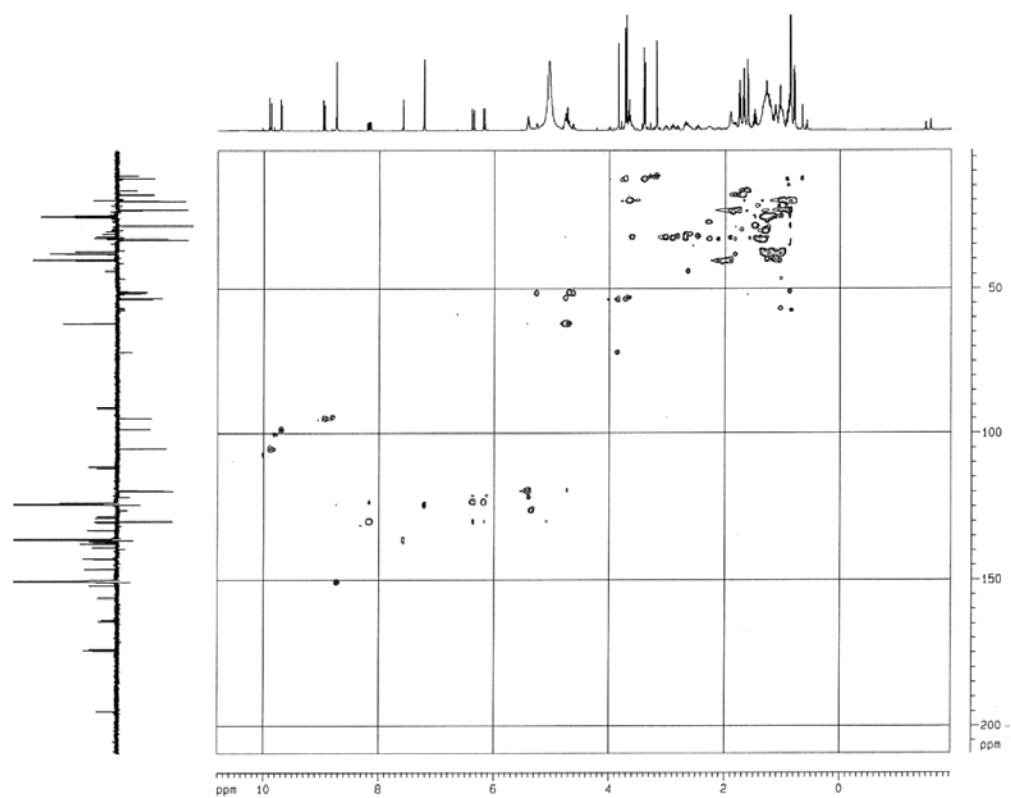
Figure A.30 HSQC spectrum of compound 4

Figure A.31 HMBC spectrum of compound 4

Figure A.32 NOESY spectrum of compound 4

Figure A.33 UV-Vis spectrum of compound 5

Figure A.34 IR spectrum of compound 5

Figure A.35 ^{1}H-NMR spectrum of compound 5

Figure A.36 DEPT ^{13}C-NMR spectrum of compound 5

Figure A.37 ^{1}H,^{1}H-COSY spectrum of compound 5
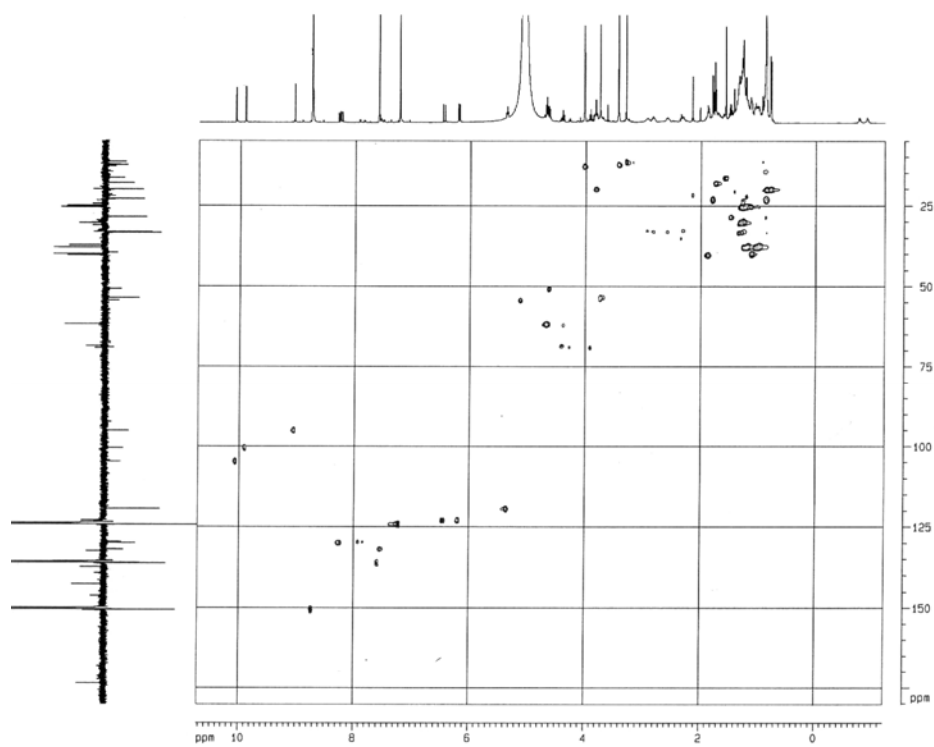
Figure A.38 HSQC spectrum of compound 5
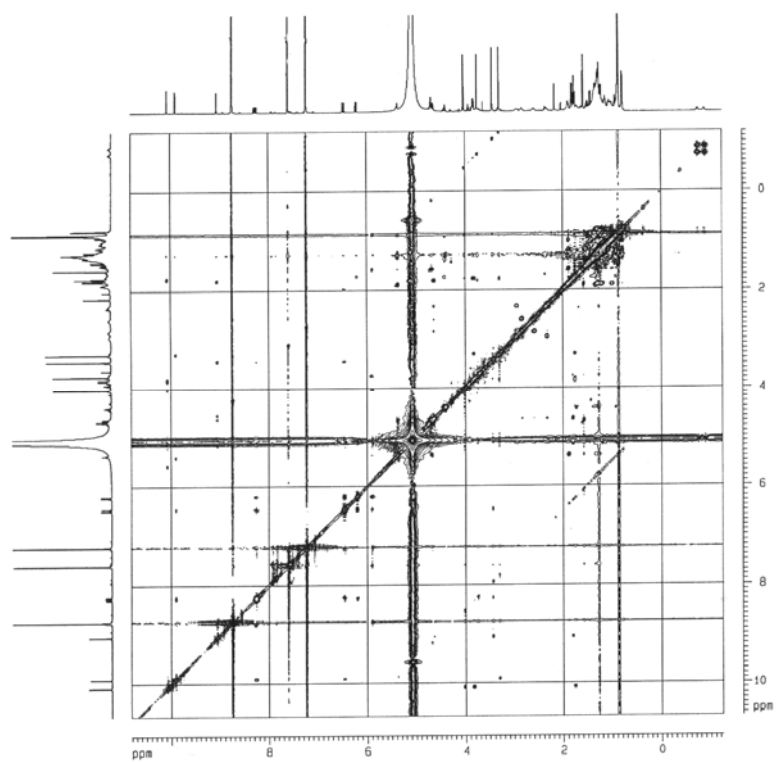
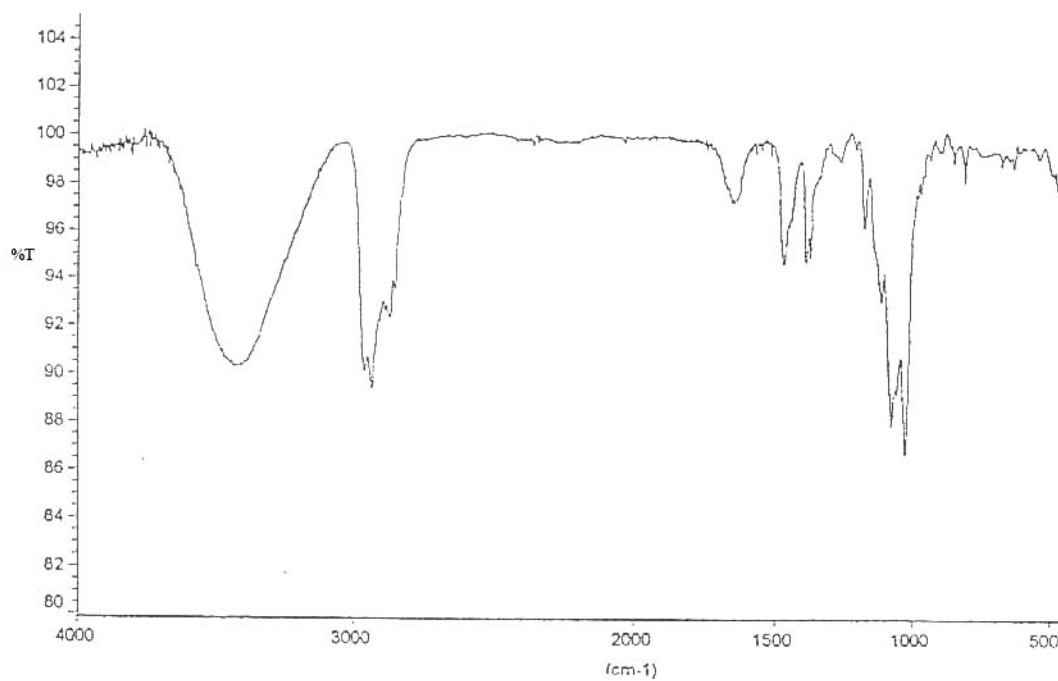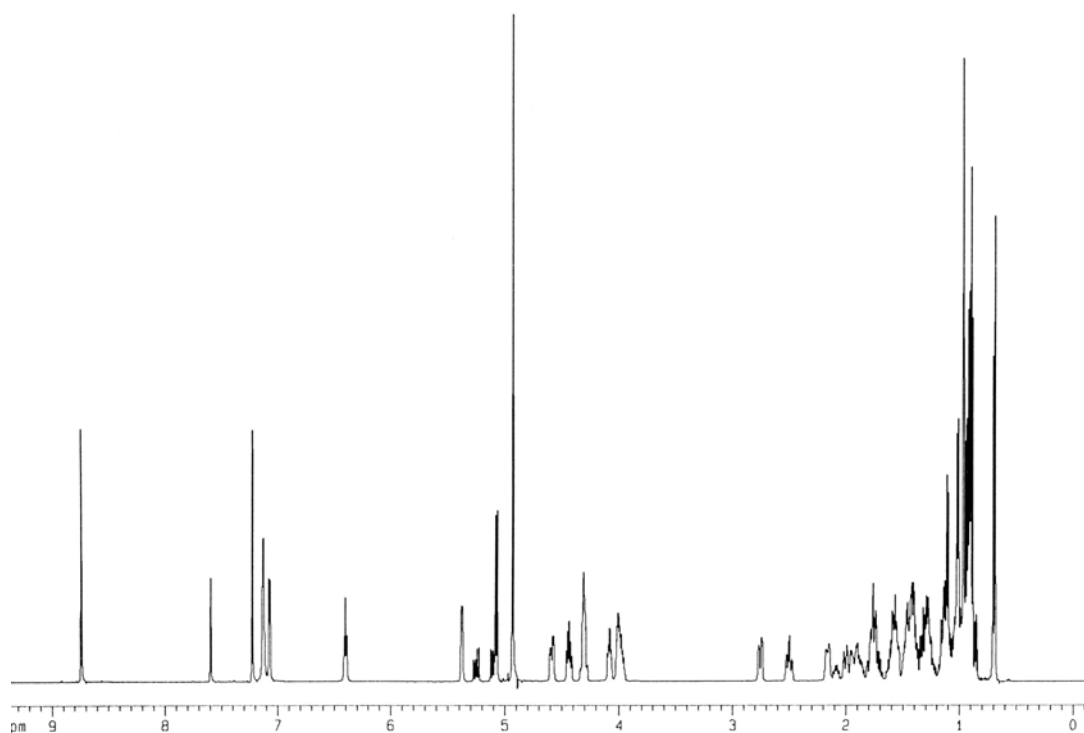**Figure A.39** HMBC spectrum of compound 5
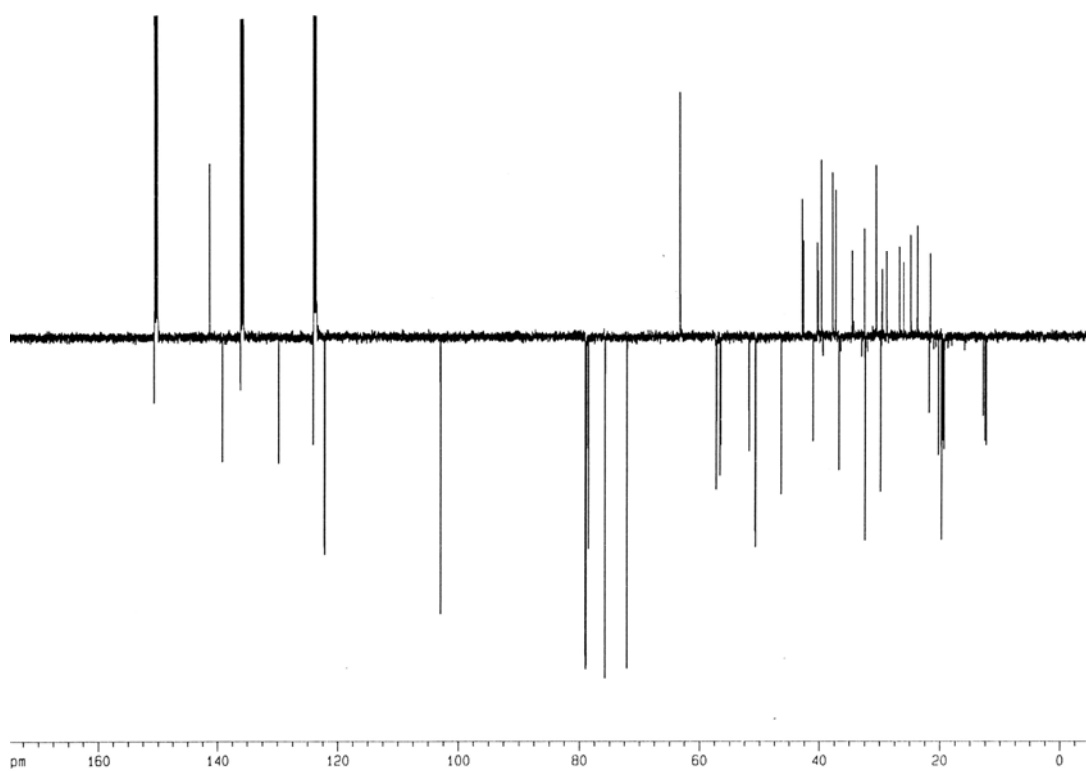

Figure A.40 NOESY spectrum of compound 5

Figure A.41 IR spectrum of compound 6

Figure A.42 ^{1}H-NMR spectrum of compound 6

Figure A.43 DEPT ^{13}C-NMR spectrum of compound 6

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the leaves of *Clinacanthus nutans* Lindau. **The 5th National Symposium on**

Graduate Research, at Bangkok, Thailand.

Sakdarat, S., Panyakom, K., Shuyprom, A. and Samart, N. (2006). Chemical

composition investigation of the *Clinacanthus nutans* Lindau leaves. **The**

10th International Kyoto Conference on New Aspects of Organic

Chemistry, at Kyoto, Japan.