องค์ประกอบทางเคมีของลำต้นปอทะเล <u>HIBISCUS</u> <u>TILIACEUS</u> LINN.
และฤทธิ์ทางชีวภาพ

นายก้องเกียรติ พวงรอด

CHEMICAL CONSTITUENTS OF THE STEMS OF HIBISCUS TILIACEUS LINN. AND THEIR BIOLOGICAL ACTIVITY
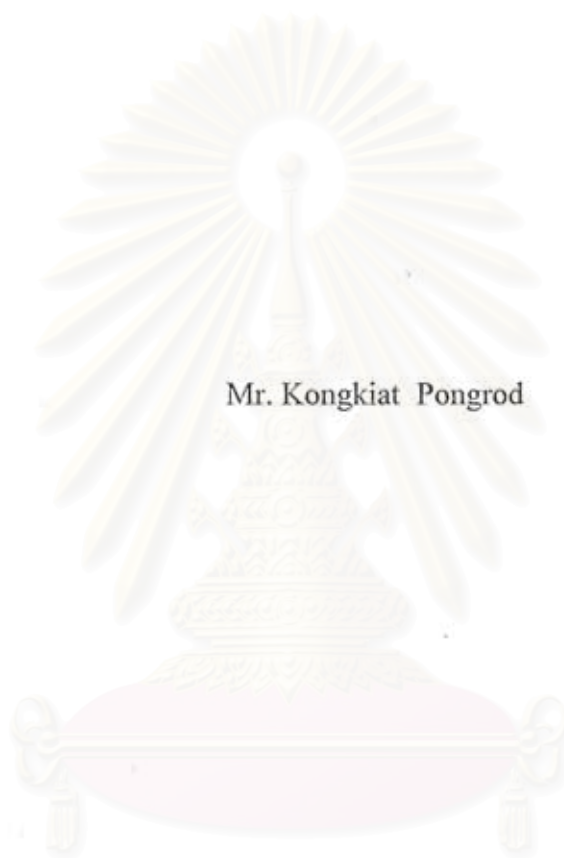
Mr. Kongkiat Pongrod

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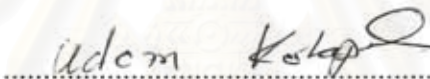Chulalongkorn University
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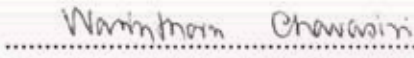|	
Thesis Title	Chemical Constituents of the Stems of *Hibiscus tiliaceus* Linn. And Their Biological Activity
By	Mr. Kongkiat Pongrod
Department	Chemistry
Thesis Advisor	Assistant Professor Warinthorn Chavasiri, Ph.D.

Accepted by the Faculty of Science, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree
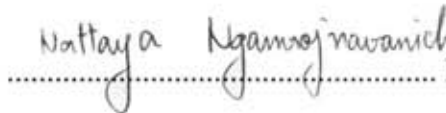
.... Dean of Faculty of Science

(Associate Professor Wanchai Phothiphichitr, Ph.D.)

Thesis Committee:

.... Chairman

(Associate Professor Udom Kokpol, Ph.D.)

.... Thesis Advisor

(Assistant Professor Warinthorn Chavasiri, Ph.D.)

.... Member

(Associate Professor Pipat Karntiang, Ph.D.)

.... Member

(Nattaya Ngamrojanavanich, Ph.D.)

ก้องเกียรติ   พวงรอด : องค์ประกอบทางเคมีของลำต้นปอทะเลและฤทธิ์ทางชีวภาพ
(CHEMICAL CONSTITUENTS OF THE STEMS OF <u>HIBISCUS</u> <u>TILIACEUS</u> LINN.
AND THEIR BIOLOGICAL ACTIVITY) อ.ที่ปรึกษา ผศ.ดร. วรินทร   ชวศิริ; 161 หน้า
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จากการศึกษาเบื้องต้นพบว่า สิ่งสกัดเมทานอลของลำต้นและใบปอทะเล แสดงความเป็นพิษ
ต่อไรสีน้ำตาลในระดับสูง สามารถแยกสารได้ 8 ชนิด จากสิ่งสกัดของลำต้นได้แก่ ของผสมเอสเทอร์
โซ่ตรง, ของผสมระหว่าง friedelin และ friedelan-3β-ol, ของผสมกรดคาร์บอกซิลิกโซ่ตรง, ของผสม
campesterol, β-sitosterol และ stigmasterol, 2,5-dimethoxy-1,4-benzoquinone, 5-hydroxy-7,8-
dimethoxycoumarin,   5-hydroxy-3,7-dimethoxyflavone   และ   5-hydroxy-3,3′,4′,5′,7-
pentamethoxyflavone จากสิ่งสกัดของใบสามารถแยกสารได้ 6 ชนิด คือ ของผสมระหว่าง friedelin
และ friedelan-3β-ol, β-amyrin, lupeol, p-methoxycinnamic acid, gossypol และ vanillic acid ผลการ
ทดสอบความเป็นพิษต่อไรสีน้ำตาลพบว่า สาร 2,5-dimethoxy-1,4-benzoquinone, 5-hydroxy-3,7-
dimethoxyflavone, 5-hydroxy-3,3′,4′,5′,7-pentamethoxyflavone, p-methoxycinnamic acid และ
gossypol แสดงความเป็นพิษต่อไรสีน้ำตาลในระดับสูง

ภาควิชา..................เคมี.................. ลายมือชื่อนิสิต.........ก้องเกียรติ พวงรอด.....................

สาขาวิชา..................เคมี.................. ลายมือชื่ออาจารย์ที่ปรึกษา..

ปีการศึกษา...............2542....................

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From the preliminary screening test, the methanolic crude extracts of stems and
leaves of *Hibiscus tiliaceus* Linn. exhibited the significant cytotoxic activity against brine
shrimp (*Artemia salina* Linn.). Eight substances were isolated from the crude extract and
were identified as a mixture of long chain esters, a mixture of friedelin and friedelan-3β-
ol, a mixture of long chain carboxylic acids, a mixture of campesterol, β-sitosterol and
stigmasterol, 2,5-dimethoxy-1,4-benzoquinone, 5-hydroxy-7,8-dimethoxy coumarin, 5-
hydroxy-3,7-dimethoxyflavone and 5-hydroxy-3,3',4',5',7-pentamethoxyflavone. The
separation of the crude extract of the leaves of *H. tiliaceus* afforded six substances. Their
structures were characterized as a mixture of friedelin and friedelan-3β-ol, β-amyrin,
lupeol, *p*-methoxycinnamic acid, gossypol and vanillic acid. The results of brine shrimp
cytotoxicity test indicated that 2,5-dimethoxy-1,4-benzoquinone, 5-hydroxy-3,7-
dimethoxyflavone, 5-hydroxy-3,3',4',5',7-pentamethoxyflavone, *p*-methoxycinnamic acid
and gossypol displayed high cytotoxic activity on brine shrimp lethality assay.

ภาควิชา.................เคมี................. ลายมือชื่อนิสิต..

สาขาวิชา.................เคมี................. ลายมือชื่ออาจารย์ที่ปรึกษา............................

ปีการศึกษา.............2542...................

ACKNOWLEDGEMENT

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br	broad	m.p.	melting point
°C	degree Celsius	MS	mass spectrometry
cm^{-1}	unit of wavenumber	MW	molecular weight
d	doublet (NMR)	*m/z*	mass per charge
dd	doublet of doublet (NMR)	NMR	nuclear magnetic resonance
dec	decomposed	ppm	part per million
DMSO	dimethylsulfoxide	q	quartet (NMR)
dt	doublet of triplet (NMR)	rel. int.	relative intensity
g	gram (s)	R_f	retardation factor
Hz	hertz	s	strong (IR)
IR	infrared	s	singlet (NMR)
J	coupling constant	t	triplet (NMR)
LC_{50}	concentration that caused	w	weak (IR)
	50% lethality	wt	weight
lit	literature	δ	chemical shift
m	multiplet (NMR)	%	percent
m	medium (IR)	μg	microgram (s)
mL	milliliter (s)	μL	microliter (s)

CHAPTER 1
INTRODUCTION

Located in the tropical hemisphere, Thailand exhibits plants biodiversity. Some 15,000 species of vascular plants have been recorded in Thailand. Flora experts reported that only 15% to 30% of the country's flora have been recorded. This means that there are some 40,000 - 80,000 species of plants that exist in the country.[1] Therefore, Thailand is a country endowed with a variety of plants which has strong potential for therapeutic applications and have been used extensively for treating disease from ancient time and are still the principle source of medicines today. In addition, plants are still a main source of bioactive compounds for the development of new therapeutic agents or as tools to examine biological process.

Currently in many industrialized countries there has been increased concern over rising medical costs, and the failure of modern medicine to treat efficiently those diseases that have become the leading causes of death in many countries, namely, heart disease and cancer. These trends have helped increasing worldwide interest in alternative medicine.[1]

Thailand, while still a developing country in many respects, has begun to suffer from many same problems as industrialized countries, including rising medical costs and increasing in the number of people who suffer from "modern disease" such as heart disease, high blood pressure, terminal stage cancer, and Acquire Immune Dificiency Syndrom (AIDS). In coping with these problems, Thailand is fortunate in that it has many indigenous resource available for confronting such trends, namely **herbal medicine**.

Traditionally, herbal medicines have been used as mixtures which involved balancing and enhancing certain chemical attributes. There are many examples of the usefulness of herbal medicine. Tuber has been used as a cream for relieving pain. The seed of custard apple has been used to heal lice and fungal infections. The herb curmin (*Curcuma Longa*), has been used to relive stomach flatulence[2]

Many natural products from medicinal plants have been extracted and studied scientifically, and incorporated into Western-style medical practices, especially in the development of drugs. Some kinds of modern drugs derived from medicinal plants. For instance, vinblastine and vincristine, dimeric indole alkaloid isolated from *Catharanthus roseus*, which were extremely important in the treatment of leukemia[3].
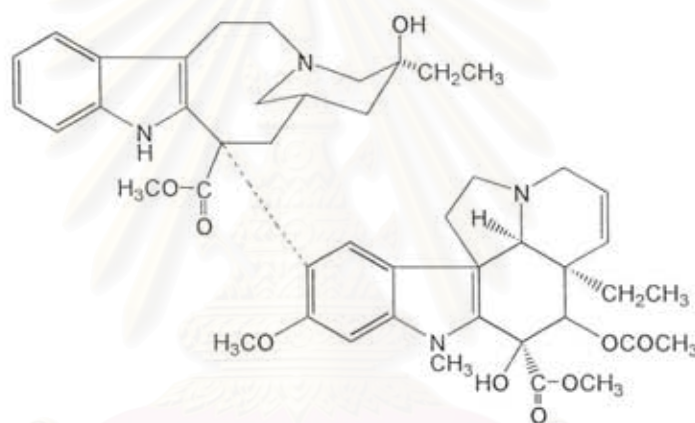
vinblastine
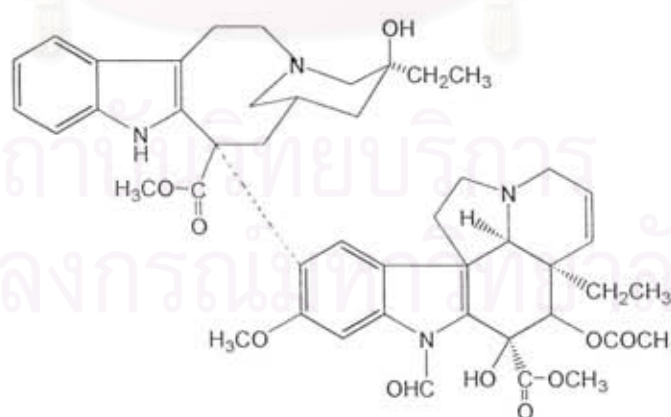
vincristine

Croton sublyratus (plau-noi), Thai medicinal plant, which is used extensively in local herbal medicine. Investigation of the chemical constituents of leaves by A. Ogiso and his coworkers led to the isolation of anti-peptic ulcer diterpene alcohol, plaunotol.[2]
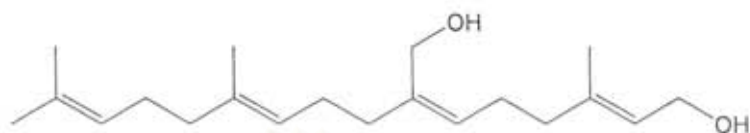
plaunotol

Calanolide **A** and inophyllums **P**, two coumarins with anti-HIV1 activity were reported to be the constituents of *Calophyllum* species.[4]
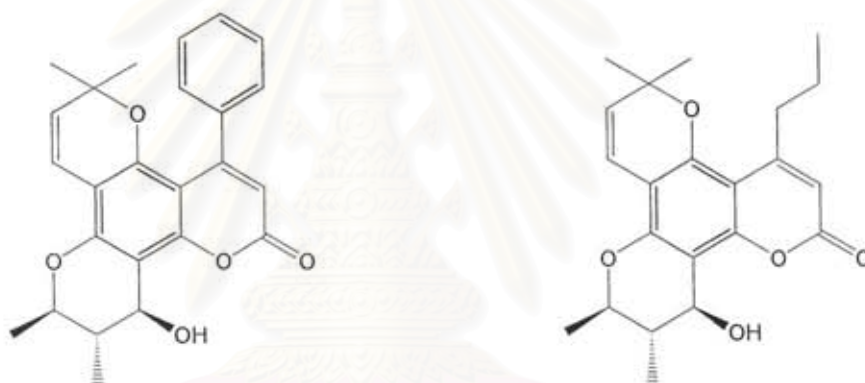
Calanolide **A**   Inophyllums **P**

Artemisia annua (Quinghaosu), a Chinese traditional herbal medicine used as a treatment for fever and malaria. This plant produced sesquiterpene lactone, artemisinin, which is responsible for their respective anti malarial properties.[5]
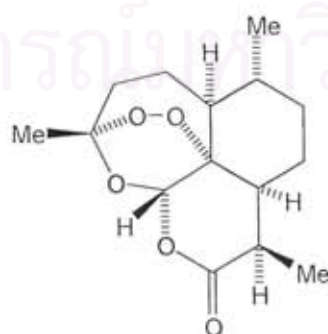
artemisinin

Among the medicinal plants available in Thailand, plants belonging to Malvaceae family are one of Thai medicinal plants, which widely used as traditional herbal medicine. For instance, *Sida rhombifolia* (kad morn): the root has been used for various medicinal purposes.[2] The examination of the root by J. Pyrex and M. Chari in 1983 led to the isolation of the alkaloid compounds namely cryptolepine, ephedrine, hypaphorine, phenylethylamine and vacicine.[6] The synthetic derivatives of vacicine, bromhexine, which could be presently used to treat chronic bronchitis and have been commercialized.[2]

Thespesia populnea, commonly known as the Portia tree, is widespread in Southeast asia. The heartwood contained thespesone, thespone and in addition with mansonones D, E and F.[8] These mansonones are known to induce contact dermatitis in man, to inhibit tumor formation and to have antifungal properties. They also affected lipid peroxidation and cytochrome P450 activity.[7] Recently, M Martina and co- workers isolated a new mansonone from the Hawaii heartwood and identified as 7-hydroxy-2,3,5,6-tetrahydro-3,6,9-trimethylnaphtho[1,8-b,c]pyran-4,8-dione.[8]
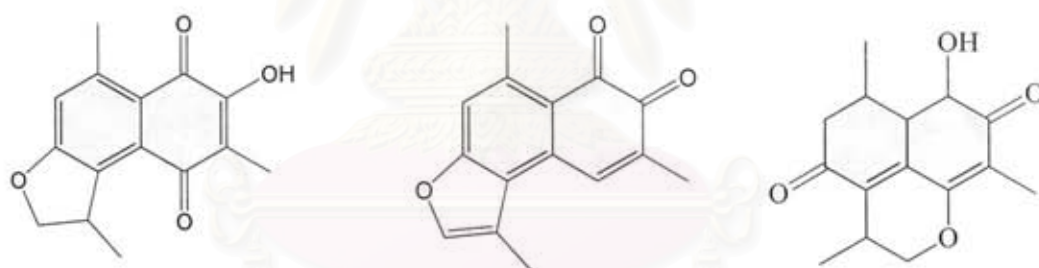
Thespesone   Thespone   new mansonone
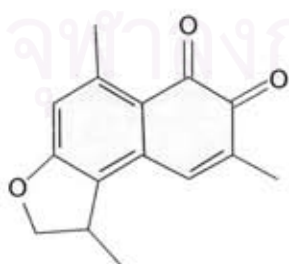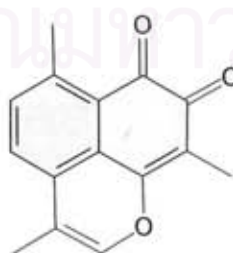
mansonone **D**   mansonone **E**   mansonone **F**
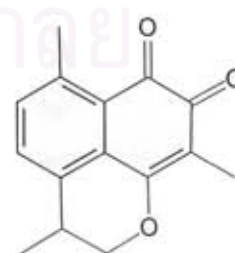
H. tilaceus is classified to be a mangrove plant belonging to family Malvaceae. This tree, coming from tropical orient and pacific region, bears little outward resemblance to the popular shurb *Hibiscus*, thought it is increasingly being used as an ornamental in seaside garden. It has large heart-shape leaves, downy underneath, and five-petaled. Hibiscus type yellows turn a darker hue before they fall. The tree trends to creep and twist along the ground rather than growing upright.[11]

H. tiliaceus is an indigenous plant to tropical countries which has a variety of medicinal application. Fresh leaves used to relieve sore throat and severe cough by squeezed into cold water and drunk. It was also taken as tea to treat stomach flatulence. The fresh bark is used to treat skin disorder.[12]

Fig 1.1 *Hibiscus tiliaceus* Linn.

Chemical Constituents Studies of *Hibiscus tiliaceus*

The wood and root of *Hibiscus tiliaceus* were first investigated by R.H. Thompson and coworker in 1980. The chloroform extract of Fijian wood yielded the series of hibiscones A -D (**1**)-(**4**) and also lapachol (**9**). The *H. tiliaceus* material from Sri Lanka yielded hibiscones A-D and hibiscoquinones A -D (**5**)-(**8**).[11]
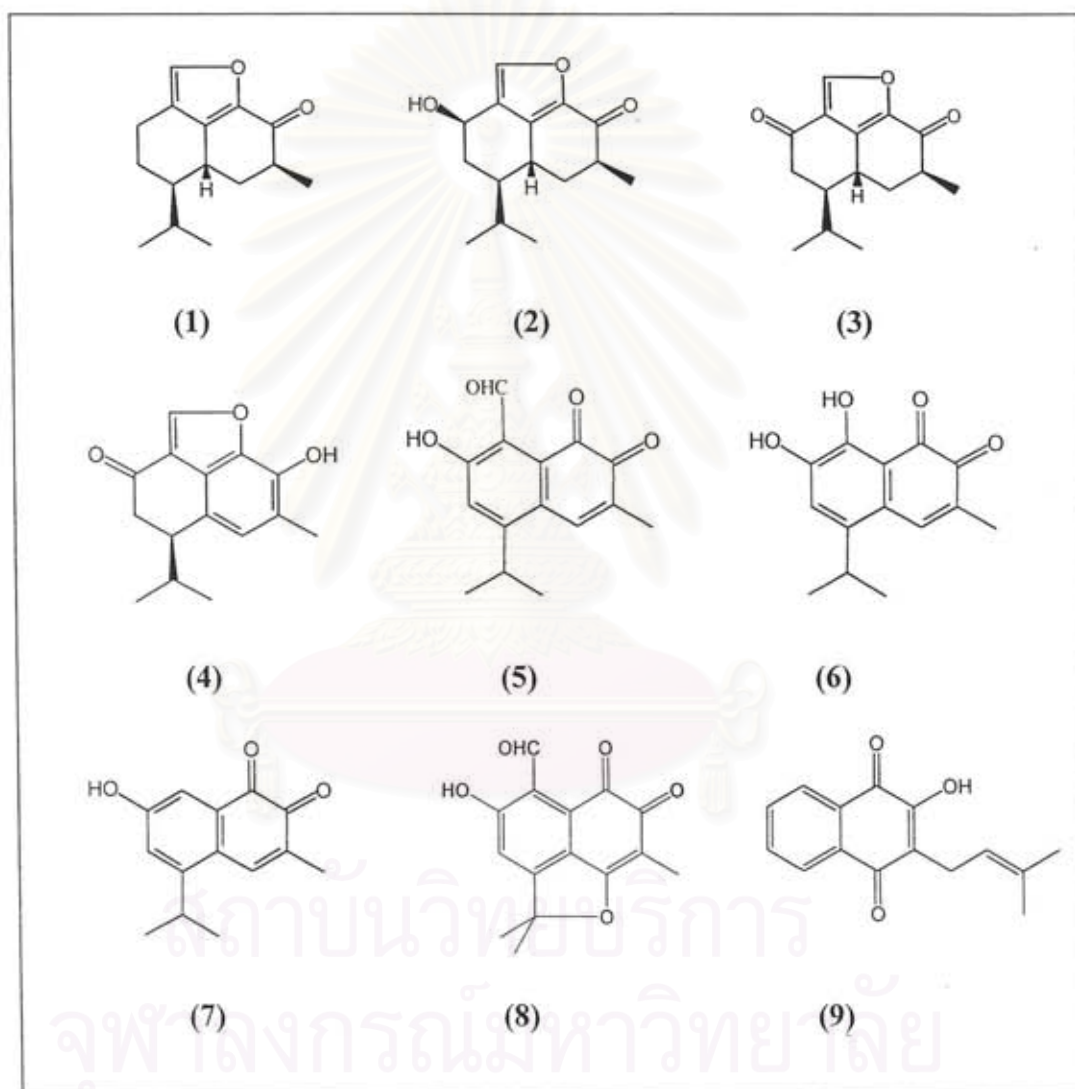
Fig 1.2 Chemical constituents of the wood and root of *H. tiliaceus*

The roots of *Hibiscus tiliaceus* from Brazil contained none of the foregoing compounds; however four related pigments were isolated. These were identified as mansonones D, E and F and gossypol (**10**).[11]

(**10**)
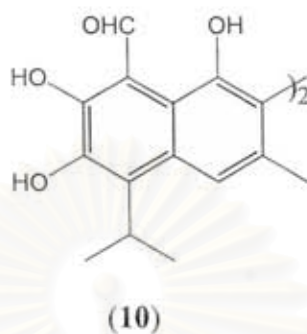
Investigation of the fruit of *H. tiliaceus* by S. Subramanian in 1973 led to the isolation of β-sitosterol, *para*-coumaric acid (**11**) and ferulic acid (**12**).[12]

(**11**)   (**12**)
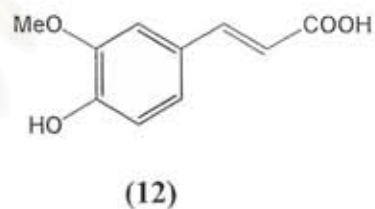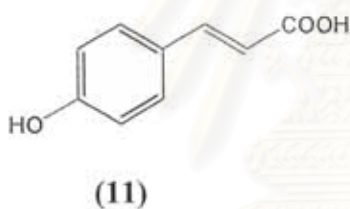
Chemical Constituents Studies of *Hibiscus* Genus

Plants in *Hibiscus* genus are known to be a rich source of steroids, triterpenoids, higly oxidized sesquiterpenoids, and flavonoids. Steroids and triterpenoids found in *Hibiscus* genus are reported in Table 1.1. The structures of these compounds are shown in Figs 1.3 and 1.4.

Table 1.1 Steroids and triterpenoids found in *Hibiscus* genus

Scientific name	Plant Part	Steroid and triterpenoid	Ref
H. abelmoschus	stem	campesterol, cholesterol, ergosterol β-sitosterol, stigmasterol	13
	leaf	campesterol, cholesterol, ergosterol β-sitosterol, stigmasterol	13
H. sabdariffa	seed	campesterol, cholesterol, ergosterol β-sitosterol, stigmasterol	14
	petal	campesterol, cholesterol, β-sitosterol stigmasterol	15
H. rosa-sinensis	leaf	campesterol, cholesterol, β-sitosterol stigmasterol, taraxerol	16
	flower	ergosterol, campesterol, β-sitosterol stigmasterol	15
H. cannabinus	stem	β-amyrin, campesterol, β-sitosterol stigmasterol	15
H. syriacus	root bark	betulin, campesterol, β-sitosterol stigmasterol	17
	leaf	campesterol, cholesterol, ergosterol β-sitosterol, stigmasterol	15
H. mutabilis	root bark	β-amyrin, betulinic cid, campesterol β-sitosterol, stigmasterol	15

R = H   : cholesterol

R = Me   : campesterol

R = Et   : β-sitosterol

stigmasterol

ergosterol
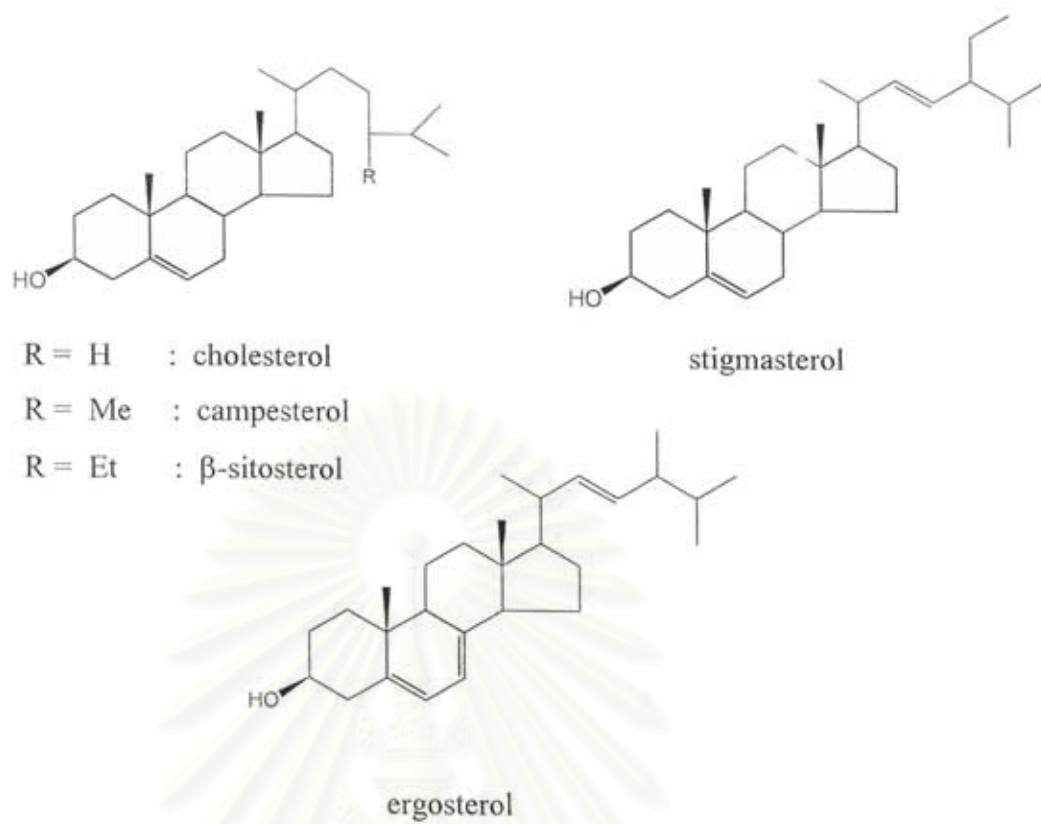
Fig 1.3 Steroids found in *Hibiscus* genus

β-amyrin

taraxerol

R = CH₂OH : betulin

R = CO₂H   : betulinic acid
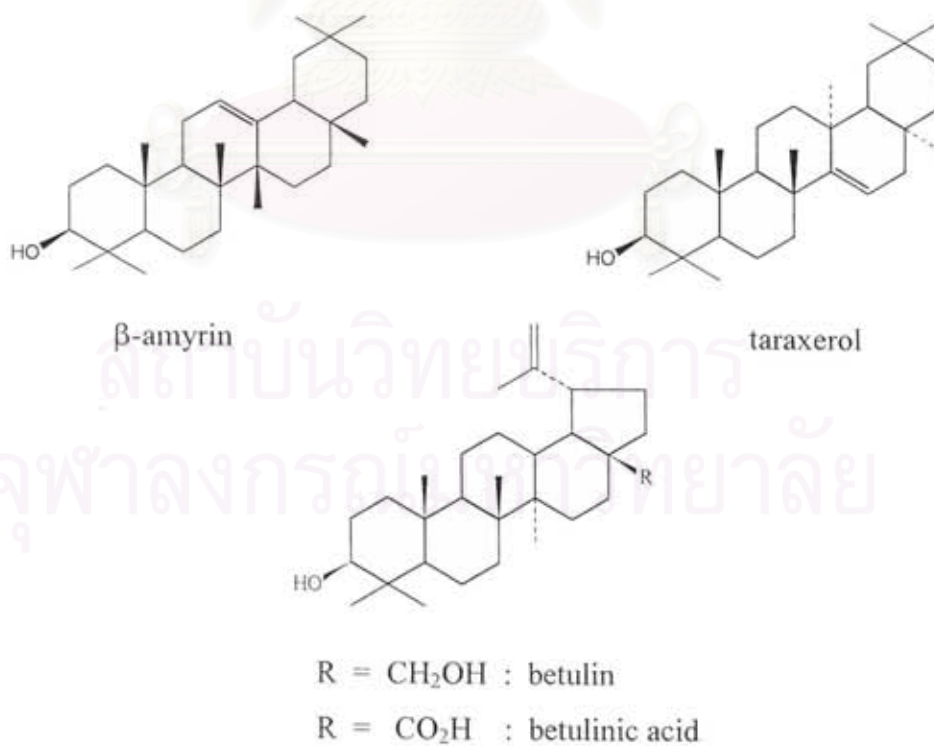
Fig 1.4 Triterpenoids found in *Hibiscus* genus

Owing to their attractive biological activities, plants in *Hibiscus* genus have been extensively investigated. Various organic compounds were isolated and found to exhibit biological activities. For instance, B. Maurer and A. Grieder isolated a new macrocyclic lactone from the seed of *Hibiscus abelmoschus* and identified as (Z)-5-tetradecen-14-olide (**13**) in 1977 in addition to ambreetolide (**14**), (Z)-5-dodecenyl acetate (**15**) and (Z)-tetradecenyl acetate (**16**).[18]

(13)

(14)

(15)

(16)
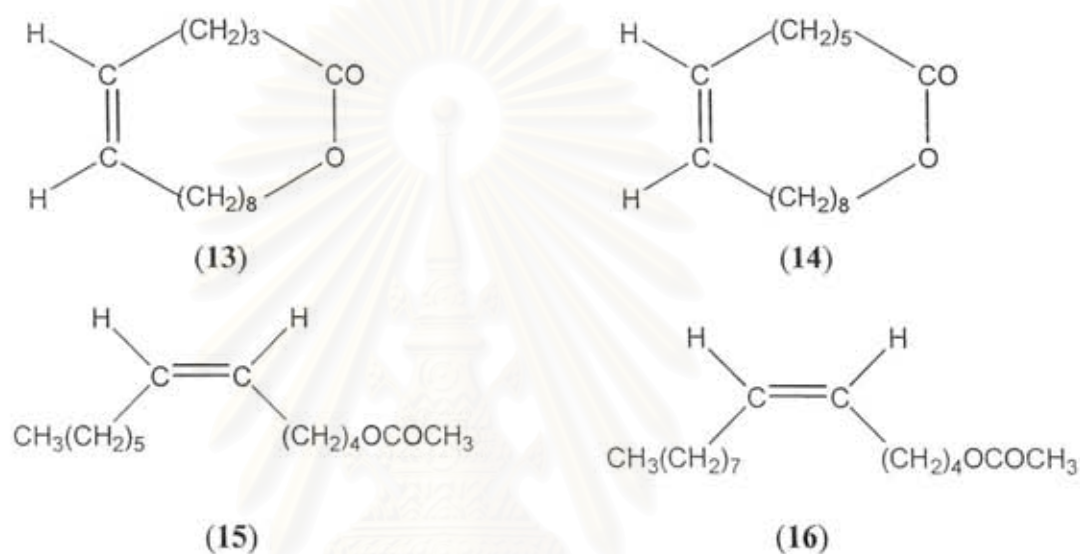
In 1981, F.V. Tkhin' et al isolated a new bioactive catechin from the root of *Hibiscus cannabinus* and characterized as 3,3',4',5',7-pentahydroxyflavan-(8→4)-3,3',4',5,7-pentahydroxyflavan (**17**).[19]
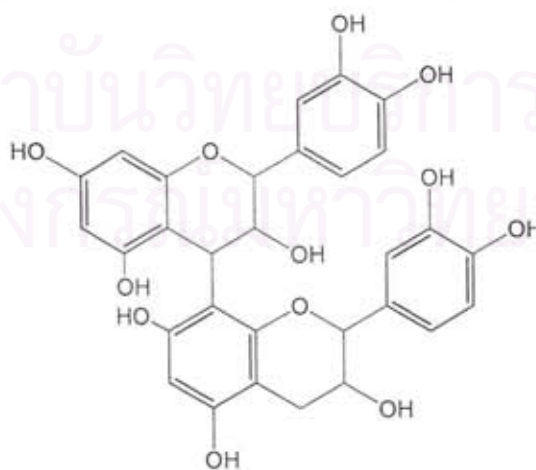
(17)

In 1995, S. Cafferty et al reported the isolation of mangiferin (**18**) and isomangiferin (**19**) from the leaves of *H. liliastrum*.[20]

(**18**)   (**19**)
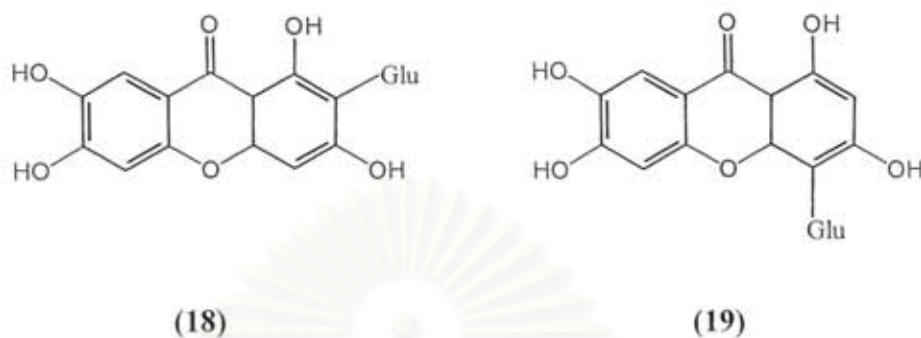
Many cyclic peptides with unique structures and biological activities have been isolated from microbial and marine origins. A few compounds including, lyciumins, citrusins, astins, yunnanins, curcacycline A, cleromycine I, segetalins and dichotomycins were isolated from higher plants. Including hibispeptin A (**20**), a novel cyclic peptide from the root bark of *H. syriacus*, a traditional Chinese herbal medicines which have been used as antipyritic, anthelmintic and antifungal agents in the Orient, has been isolated by Bong-Sik and co-workers in 1996.[21]

(**20**)
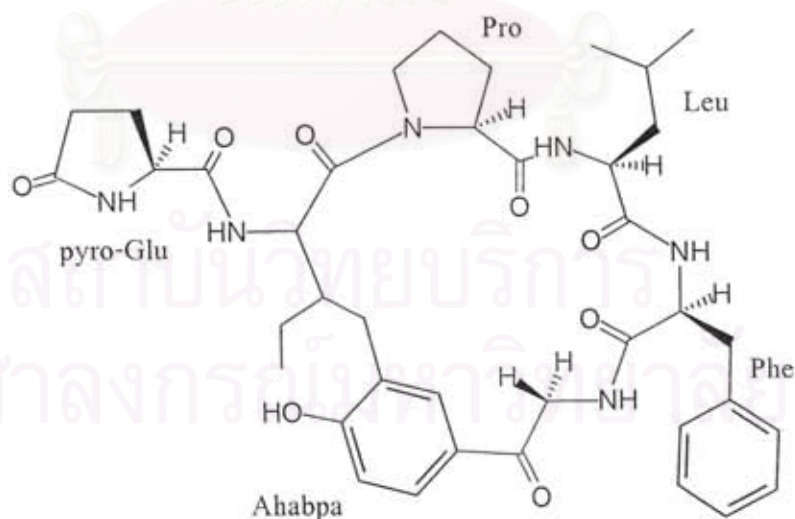
In 1997, three new naphthalene derivatives, designated as syriacusins A **(21)**, B **(22)** and C **(23)**, were isolated from the chloroform extract of the root bark of *H. syriacus* by Yoo et al. These compounds inhibited lipid peroxidation with IC_{50} of 0.54, 5.90 and 1.02 ppm, respectively. The first compound also showed cytotoxicity against some human cancer cell lines with an ED_{50} of 1.5 - 2.4 ppm. The structure of syriacusins A-C are shown below.[22]

(21)　　　　　　　　**(22)**　　　　　　　　**(23)**
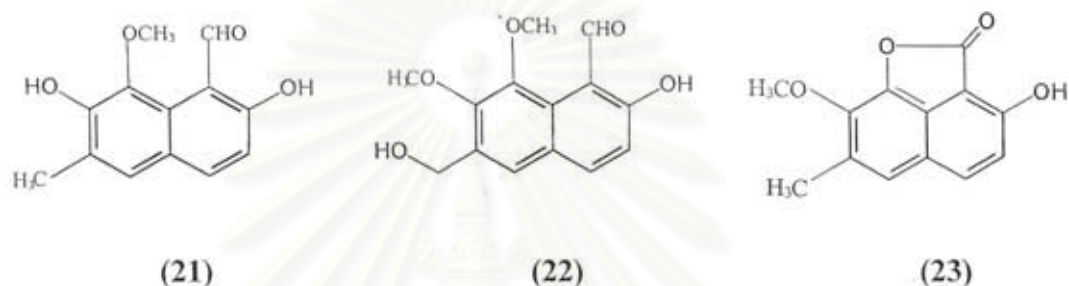
Two years later, B.S. Yunn and co-workers reported two new active triterpene caffeates as the constituents from the same part of this plant and identified as 3β,23,28-trihydroxy-12-oleanene-23-caffeate **(24)** and 3β,23,28-trihydroxy-12-oleanene-3-caffeate **(25)**. These compounds showed lipid peroxidation inhibitory activity and significant cytotoxicity against a panel of human cancer cell lines.[23]

(24)　　R^1 = OH,　R^2 =

(25)　　R^2 = OH,　R^1 =
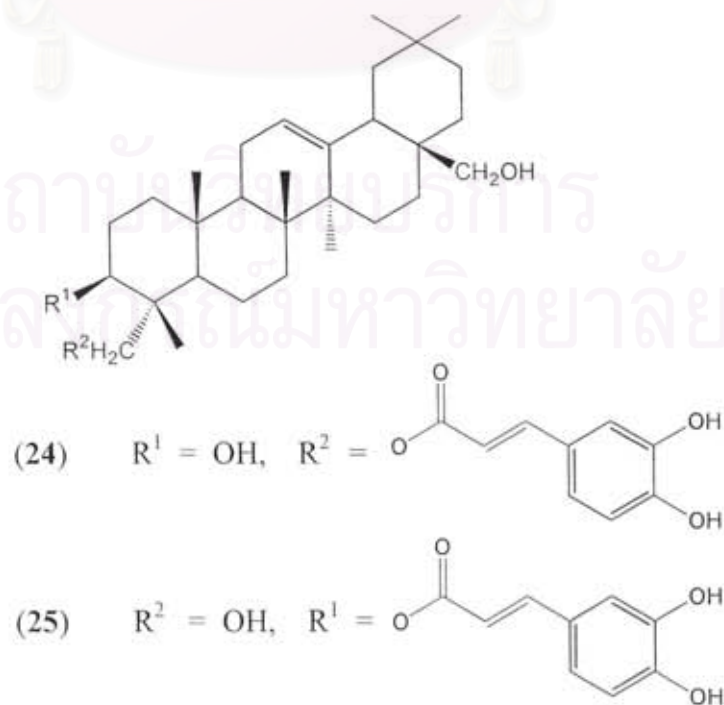
In 1972 J.B. Lowry investigated the chemical constituents of *Hibiscus* flowers and reported the floral anthocyanins for 11 *Hibiscus* species.[24] The results are shown in Table 1.2 and the structures are shown below.

Table 1.2 Floral anthocyanins of some plants in Malvaceae

Species	Compounds
H. archboldianus Borss	Cy 3-sambubioside (**26**) ; Cy 3-glucoside
H. macrophyllus Roxb.	Cy 3-glucoside (**27**)
H. tiliaceus L.	Cy 3-glucoside
H. cannabinus L.	Dp 3-sambubioside (**28**)
	Dp 3-glucoside (**29**)
H. sabdariffa L.	Dp and Cy 3-sambubioside
	Dp and Cy 3-glucoside
H. surattensis L.	Cy 3-sambubioside
H. rosa-sinensis L.	Cy 3-sophoroside
H. mutabilis L.	Cy 3-sambubioside ; Cy 3-glucoside
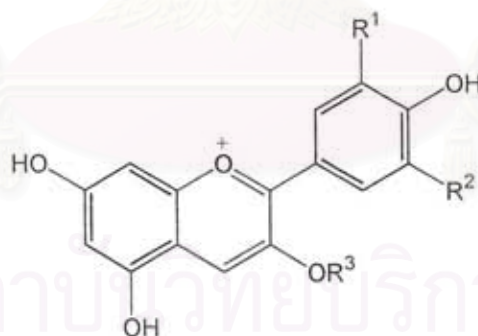
	R^1	R^2	R^3
26	OH	H	Glu
27	OH	H	Glu
28	OH	OH	Sam
29	OH	OH	Sam

Cy = cyanidin ; Dp = Delphinidin

Other isolated organic compounds from *Hibiscus* plants are displayed in Table 1.3 and the structures of some of these compounds are shown in Fig 1.5.

Table 1.3 Chemical constituents of plants in *Hibiscus* genus

Species	Plant Part	Organic compounds	Ref
H. abelmoschus	seed	**Fatty acid**: arachidic acid, behenic acid, linoleic acid, linolenic acid, myristic acid dodecyl acetate, dodecenyl acetate	18
H. cannabinus	leaf	car-3-ene (**30**), citral (**31**), phellandrene (**32**) limonene (**33**), α-terpeneol (**34**), piperitol (**35**)	13
	stem	3,8-dimethyl-1,2-naphthoquinone (**36**) 2,8-dihydroxy-4,7-dimethoxy-6-methyl-1-naphthaldehyde (**37**), *para*-tolualdehyde	25
H. sabdariffa	petal	**Fatty acid**: arachidic acid, behenic acid lauric acid, linoleic acid, linolenic acid, myristic acid, oleic acid, palmitic acid, palmitoleic acid anisaldehyde (**38**), benzoic acid (**39**), gallic acid (**40**), protocatechuic acid (**41**)	26 / 15
	flower	α-terpenyl acetate, quercitin (**42**) gossypetin (**43**), hibiscetin (**44**)	27
H. syriacus	flower	pelargonidin-3-O-malonylglucoside (**45**) peonidin-3-O-malonylglucoside (**46**) malvidin-3-O-malonylglucoside (**47**)	28
H. rosa-sinensis	leaf	**Fatty acid**: arachidic acid, behenic acid, lauric acid, myristic acid, oleic acid, palmitic acid stearic acid	29
	flower	quercitin, quercitin-3,7-di-O-β-D-glucoside	30
H. mutabilis	flower	nonacosane, tetratriacontanol, hexyl stearate quercitin, kaempferol	30

30 **31** **32** **33**

34 **35** **36** **37**

38 **39** **40** **41**

	R¹	R²	R³	R⁴	R⁵
42	OH	OH	H	OH	H
43	OH	OH	OH	OH	H
44	OH	OH	OH	OH	OH

	R¹	R²
45	H	H
46	OMe	H
47	OMe	OMe
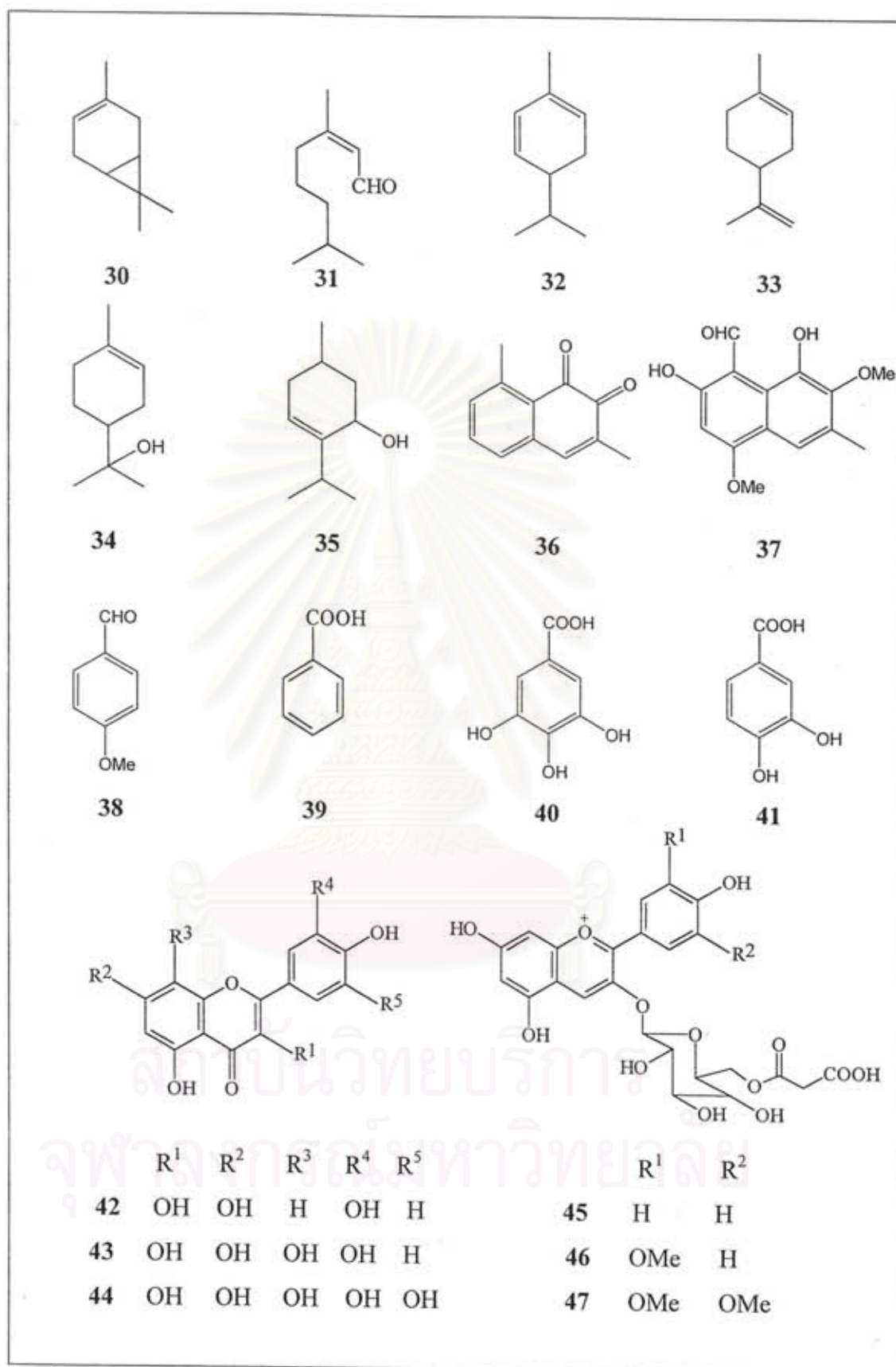
Fig 1.5 Other chemical constituents of plants in *Hibiscus* Genus

1.6 The Goal of This Research

Besides the preliminarily biological activities screening test, the methanolic crude extract of the stems of *H. tiliaceus* displayed moderate cytotoxicity against brine shrimp (*Artemia salina* Linn) with LC_{50} of 4.68 ppm. The methanolic extract crude extract of leaves exhibited the significant plant growth inhibition against *Mimosa pigra* Linn. Moreover, the methanolic extract of leaves also gave promising cytotoxic activity results; however, it was lack of information of chemical constituents and their biological activity. According to these preliminarily results, it is so attractive to investigate the chemical constituents which responsible for those biological activities. Therefore, the following goals can be summarized:

1. To isolate the bioactive substances from the leaves and stems of *H. tiliaceus*
2. To elucidate the chemical structures of isolated compounds
3. To study the biological activity of isolated compounds

CHAPTER 2
EXPERIMENTAL

2.1 Plant Material

Hibiscus tiliaceus Linn. was collected in April 1995 from Samutsongkram province. A voucher specimen is deposited in the herbarium of the Royal Foresty Department of Thailand (no. 117913).

2.2 General Procedure

NMR spectra were recorded in $CDCl_3$ on a Bruker ACF 200 and a Jeol JNM-A500 spectrometer using tetramethylsilane (TMS) as an internal reference in ^1H and ^{13}C measurement and standard Bruker pulse sequence for two - dimensional experiments. Assignment of ^{13}C-NMR chemical shifts was made with the aids of DEPT spectra. The FT - IR spectra were recorded on a Fourier Transform Infrared Spectrophotometer model Impact 410 (KBr pellets). The mass spectra were obtained on Fission Instrument model Trio 2000 operating at 70 eV ionization voltage.

Melting points were determined with a Fisher - Johns melting point apparatus and are uncorrected. Gas chromatography GC -7AG instrument equipped with a flame ionization detector with N_2 as a carrier gar. Medium Pressure Liquid Chromatography was performed on Buchi, model B-680 A. Silica gel Merck Kieselgel 60, no. 7734, 7749, 7731 were used for column chromatography, preparative thin layer chromatography (prep TLC), and quick column chromatography respectively. TLC analyses were performed with Whatman precoated silica gel (Merck Kieselgel 60 PF_{254}) and spots on the plate were observed under UV light and visualized by spraying with 10% H_2SO_4 in ethanol followed by heating.

2.3 Chemicals

All solvents used in this research were purified prior to use by standard protocol except for those which were reagent grades.

2.4 Chemical Tests

2.4.1. Liebermann-Burchard Reaction

This is a general test for steroid or triterpenoid compound. To a solution of the sample to be tested (2-3 mg) in dry chloroform (0.5 mL) was added a few drops of acetic anhydride with shaking, followed by one drop of concentrated sulfuric acid and the color change was observed after a few minutes. The deep green colour suggested the presence of steroidal ring system and the purple colour indicated a triterpenoid skeleton.

2.4.2 Cyanidin test

This is a test for flavonoid compounds. To an alcoholic solution (1.0 mL) of the sample (2-3 mg) was added pieces of magnesium and 1-3 drops of concentrated hydrochloric acid. Any color developed within a few minutes was observed. The shade of color is suggestive of the class of flavonoid nucleus.

2.4.3 Alkaloid test

Dragendroff's reagent

Add a few drops of Dragendroff's reagent into the sample solution. The orange precipitate suggested the presence of alkaloid nucleus.

Kraut's reagent

The sample solution was added a few drops of Kraut's reagent yielded brown precipitate. This positive test exhibited the presence of alkaloid nucleus.

Marme's reagent

To a methanol solution of the sample to be tested was added a few drop of Marme's reagent. The brown precipitate indicated the occurrence of alkaloid compounds.

2.5 Chemical Reactions

2.5.1 Synthesis of 4-methoxycinnamic acid

Malonic acid (1.64 g, 15.8 mmol) is dissolved in 10 mL of anhydrous pyridine, *p*-methoxybenzaldehyde (3.18 g, 13.1 mmol) and piperidine (0.11 g, 0.13 mL) are added, and the solution is refluxed for 1.5 h. Carbon dioxide evolution has ended by this time. The solution is cooled to RT, poured into a mixture of 10 g of ice, 5 mL of conc. HCl and 13 mL of H_2O, precipitating the acid as colorless solid. The solid is collected by filtration, washed with ice water, crystallized from EtOH and dried in *vacuo* to give colorless needles. The yield is 2.60 g (70%), m.p. 185-186°C. TLC revealed the R_f value of 0.77 (solvent: 20% EtOAc-MeOH).

2.6 Extraction Procedure

Ground sun-dried leaves (13 kg) and stems (18 kg) were separately successively percolated with *n*-hexane, dichloromethane, ethyl acetate and methanol for three days at room temperature. The residue was repeated for several times until the solution was colorless and then the filtered solution was removed under vacuum giving crude extracts. The extraction procedure is shown in Scheme 2.1.

Ground sun-dried samples of

Hibiscus tiliaceus

| hexane

hexane extract   residue

| dichloromethane

dichloromethane extract   residue

| ethyl acetate

ethyl acetate extract   residue

| methanol

methanol extract   marc

(discarded)
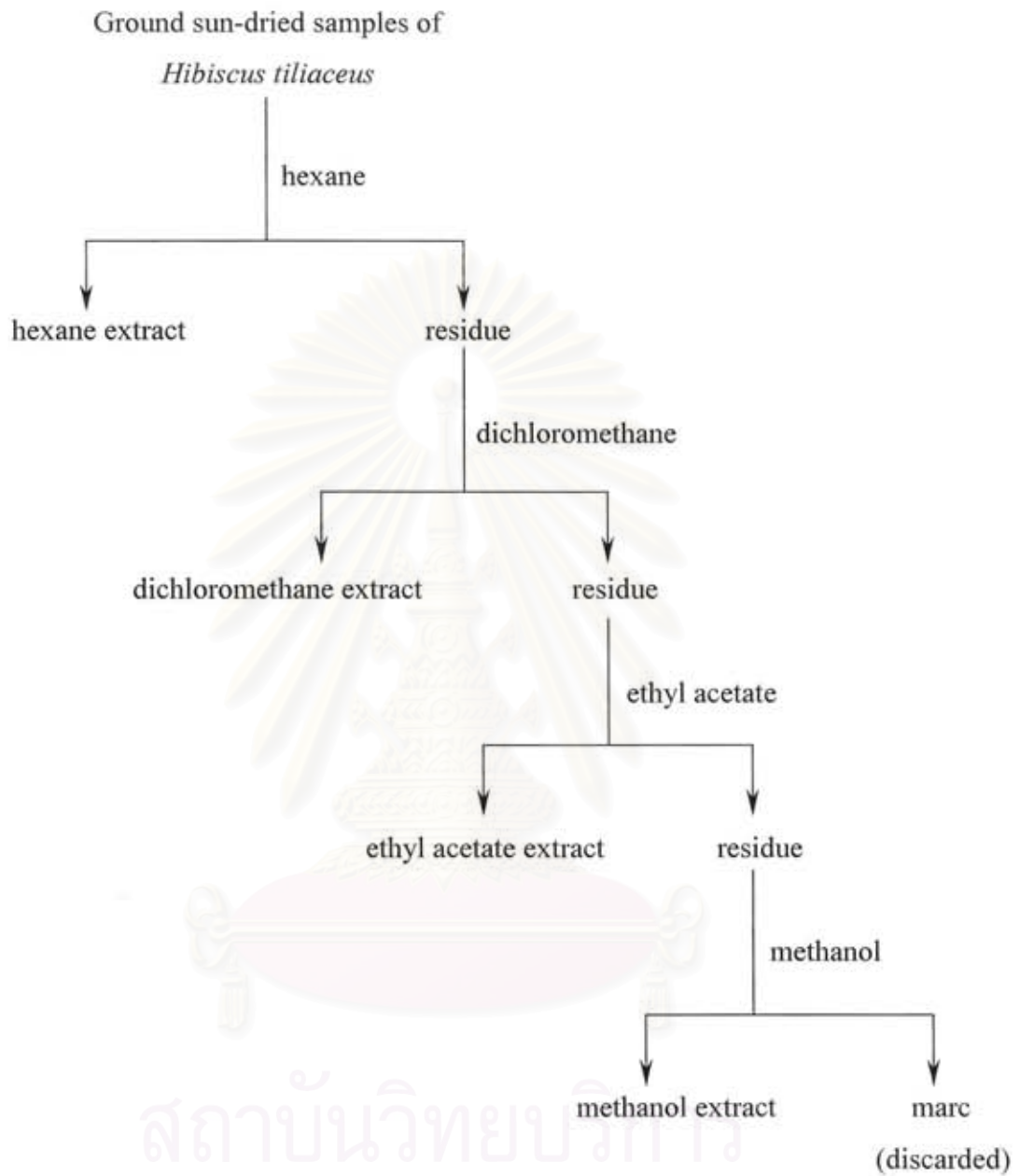
Scheme 2.1 The general extraction procedure

2.7 Bioassay Experiments

In the search for bioactive substances from *Hibiscus tiliaceus*, the bioactivities of crude extracts, fractions, and pure compounds were routinely assayed using the following bioassay experiments.

2.7.1 Brine Shrimp Cytotoxicity Lethality Test (BSCLT)[31]

The samples were assayed at 10, 100, and 1,000 ppm using 10 second-star larvae of the brine shrimp (*Artemia salina*, Leach) in triplicate. The brine shrimp were observed periodically over 24 - h period. After 24 h contact, the number of surviving organisms was recorded and the LC_{50} was evaluated by the calculation using the probit statistical analysis program.

2.7.2 Anti Cell Lines Cytotoxicity Test[32]

Human tumor cell lines were kindly performed in Beijing Medical School, Beijing, Republic of China.

Cytotoxicity assays of the extracts and fractions were carried out by modification of the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] colorimetric method. All assays were performed in triplicate. The results were expressed as a percentage, relative to control incubation, and the effective dose required to inhibit cell growth by 50 % (ED_{50}) was determined.

2.7.3 Antioxidant Test[32]

There were two methods employed for anti-oxidative test.

a) Reduction of 2,2-diphenyl-1-pidrylhydrazyl [=2,2-Diphenyl-1-(2,4,6-trinitrophenyl)hydrazyl; DPPH] radical. TLC autographs assay: after developing and drying TLC plates were sprayed with a 0.2% DPPH solution in MeOH. The plates were examined at 30 min after spraying. Active compounds appeared as yellow spots against a purple background.

b) Bleaching of a β-carotene. TLC autographic assay: after developing and drying, TLC plates were sprayed with a β-carotene solution in dichloromethane (0.2 mg/mL). The plates were exposed to 254 nm UV light for 20 min before examination. β-carotene undergoes bleaching except in places where antioxidative substances prevented the degradation. Active compounds appeared as orange spots against a white background.

CHAPTER 3
RESULTS AND DISCUSSION

In the course of screening for biologically active constituents from Thai mangrove plants, the methanolic extract of the stems of *H. tiliaceus* exhibited significant cytotoxic activity against brine shrimp (*Artemia salina* Linn). Previously, a series of sesquiterpenoid quinones has been isolated from the stems; nevertheless, the biological activities of these compounds have not been reported. Thus, in this investigation, the biological activity study was conducted using brine shrimp cytotoxic lethality assay as a guide. Moreover, the methanolic extract of leaves also gave promising cytotoxic activity results; however, it was lack of information of chemical constituents and their biological activity. Thereby, searching for biologically active constituents from the leaves of *H. tiliaceus* should be rationalized.

3.1 The results of extraction

The stems and leaves of *Hibiscus tiliaceus* Linn. were extracted following the procedures described in Chapter 2. The results of extraction are summarized as shown in Table 3.1.

Table 3.1 The results of extraction of stems and leaves of *H.tiliaceus*

Plant part	Solvent	Fraction no.	Weight (g)	%w/w
Stem (18.0 kg)	hexane	I	40.2	0.22
	dichloromethane	II	35.0	0.20
	ethyl acetate	III	48.5	0.27
	methanol	IV	60.3	0.34
Leaf (13.0 kg)	hexane	V	207.4	1.60
	dichloromethane	VI	225.8	1.74
	ethyl acetate	VII	186.5	1.43
	methanol	VIII	121.3	0.93

From the results of extraction, it was observed that the yields of extraction derived from the leaves were higher than those obtained from the stems in every solvent extract.

3.2 The results of biological activity screening tests

3.2.1 Brine shrimp cytotoxicity test[31]

Various crude extracts of *H. tiliaceus* were preliminarily screened for cytotoxicity against brine shrimp (*Artemia salina* Linn.) according to the procedures described in Chapter 2. The results are displayed in Table 3.2.

Table 3.2 The results of brine shrimp cytotoxicity test

Plant part	Solvent	Fraction	LC$_{50}$ (ppm)	Activity
stem	hexane	I	33.04	medium activity
	dichloromethane	II	23.67	medium activity
	ethyl acetate	III	11.72	medium activity
	methanol	IV	60.05	medium activity
leaf	hexane	V	41.32	medium activity
	dichloromethane	VI	15.64	medium activity
	ethyl acetate	VII	17.56	medium activity
	methanol	VIII	122.24	low activity

According to the above results, Fractions I, II, III, V, VI and VII displayed interesting cytotoxic activity against brine shrimp. These crude extracts were thus selected for further investigation of the chemical constituents.

3.2.2 Anti cell line cytotoxicity test[32]

In vitro cytotoxicity test against four human tumor cell lines were kindly carried out by the staff of Beijing Medical School, Beijing, Replublic of China employing standard seven - day MTT assays for HL - 60 (*Leukemia carcinoma*),

Bel-7402 (*Hepatocellular carcinoma*), HCT-8 (*Colon carcinoma*) and BGC-823 (*Gastric carcinoma*). The results are shown in Tables 3.3 and 3.4.

Table 3.3 The results of cytotoxicity test against HL-60 and Bel-7402 cell lines

Fraction	Concentration (µg/mL)	Inhibition (%)	
		HL-60	Bel-7402
I	1	18.70	1.86
	10	21.29	9.21
	100	71.68	73.89
II	1	3.37	-1.64
	10	17.31	-1.80
	100	83.72	88.29
III	1	-39.66	2.82
	10	-24.65	10.18
	100	57.40	72.85
IV	1	-22.61	3.90
	10	-12.86	3.67
	100	-53.89	21.32

Table 3.4 The results of cytotoxicity test against HCT-8 and BGC-823 cell lines

Fraction	Concentration	Inhibition (%)	
	(µg/mL)	HCT-8	BGC-823
I	1	28.22	19.78
	10	40.65	16.30
	100	67.09	50.00
II	1	37.47	46.55
	10	40.00	40.63
	100	87.57	91.68
III	1	28.78	17.20
	10	29.25	18.26
	100	55.42	68.06
IV	1	11.86	25.62
	10	11.21	29.82
	100	2.42	44.74

In addition to the results derived from the brine shrimp cytotoxicity test, Fractions **I**, **II** and **III** also exhibited moderate cytotoxicity against some human tumor cell lines.

3.2.3 Antioxidant test[32]

The methanolic extracts of leaves, stems and roots of *H. tiliaceus* were tested for anti-oxidant activity according to the procedure described in Chapter 2.   The results are reported in Table 3.5.

Table 3.5 The results of antioxidant test

Plant part	Activity	
	β-carotene	DPPH
Leaf	-	+
Stem	-	+
Root	-	+

+ : active,   - : inactive

According to the results of brine shrimp cytotoxic lethality assay and anti-oxidant test, the crude extracts of stems and leaves of this plant gave attractive bioassay results. The trends outline from preliminary screening test pointed toward the presence of bioactive substances in stems and leaves of *H. tiliaceus*. Thus, chemical investigation of these two parts were conducted.

3.3 Chemical constituents studies of the stems of *H. tiliaceus*

3.3.1 Separation of hexane crude extract (Fraction I)

The hexane crude extract (Fraction I) as yellow-brown solid, 38.0 g was separated by silica gel column chromatography. The column was initially eluted by 10% CH_2Cl_2-hexane, followed by increasing polarity of solvent. Eluting solvent was collected for each fraction approximately 500 mL and then concentrated under vacuum to a small volume. Each fraction was checked by TLC and the equivalent fractions were combined. The results of the separation of Fraction I are shown in Table 3.6.

Table 3.6 The results of the separation of Fraction I

Eluents (%volume by volume)	Fraction No. (500 mL)	Remarks	Weight (g)
10% CH$_2$Cl$_2$-hexane	1-8	white amorphous solid (Mixture 1)	0.17
20% CH$_2$Cl$_2$-hexane	9-24	pale yellow oil	trace
30% CH$_2$Cl$_2$-hexane	25-45	white crystal + yellow oil (Mixture 2)	3.07
40% CH$_2$Cl$_2$-hexane	46-54	yellow oil	trace
50% CH$_2$Cl$_2$-hexane	55-64	white ppt in pale yellow oil (Mixture 3)	1.43
	65-76	white crystal + yellow oil (Mixture 4)	3.47
	77-80	pale yellow oil	0.50
	81-86	yellow brown oil	1.75
80% CH$_2$Cl$_2$-hexane	87-101	brown oil	0.37
90% CH$_2$Cl$_2$-hexane	102-104	brown oil	0.69
100% CH$_2$Cl$_2$	105-115	dark brown viscous liquid	2.28
5% MeOH- CH$_2$Cl$_2$	116-130	dark brown viscous liquid	1.46

3.3.2 Separation of dichloromethane crude extract (Fraction II)

Fraction II as dark green viscous solid, 40 g was separated by open column chromatography using silica gel as an adsorbent. The separation procedure employed was the same as that used for the separation of Fraction II. The results are indicated in Table 3.7.

Table 3.7 The results of the separation of Fraction II

Eluent (%volume/volume)	Fraction No.	Remarks	weight (g)
10% CH₂Cl₂-hexane	1-20	white amorphous solid	1.00
20% CH₂Cl₂-hexane	21-30	pale yellow oil	trace
40% CH₂Cl₂-hexane	31-40	white needle + dark oil	3.02
50% CH₂Cl₂-hexane	41-50	dark brown solid	trace
60% CH₂Cl₂-hexane	51-58	dark green brown solid	0.27
80% CH₂Cl₂-hexane	59-70	dark brown oil	0.19
100% CH₂Cl₂	71-92	red needle (**Compound 1**) + red brown oil	0.85
	93-95	yellow solid (**Compound 2**) + yellow oil	1.02
10% EtOAc-CH₂Cl₂	96-100	dark green oil	1.95
20% EtOAc- CH₂Cl₂	101-114	dark green oil	1.08
30% EtOAc- CH₂Cl₂	115-125	dark green oil	2.16
50% EtOAc- CH₂Cl₂	126-136	dark brown semi solid	1.47
80% EtOAc- CH₂Cl₂	137-150	dark brown semi solid	0.23
100% EtOAc	151-160	black viscous solid	1.88
5% MeOH-EtOAc	161-170	black solid	trace
10% MeOH-EtOAc	171-181	black solid	trace
50% MeOH-EtOAc	182-190	black solid	trace

3.3.3 Separation of ethyl acetate crude extract (Fraction III)

The ethyl acetate extract, Fraction III (45 g) was subjected to column chromatography over silica gel which initially eluted with *n*-hexane, followed by increasing amount of dichloromethane in hexane. The results of the separation of Fraction III are shown below.

Table 3.8 The results of the separation of Fraction III

Eluent (% volume by volume)	Fraction No.	Remarks	Weight (g)
10% CH$_2$Cl$_2$-hexane	1-10	yellow oil + white amorphous solid	1.56
20% CH$_2$Cl$_2$-hexane	11-25	pale yellow oil + white needle	1.87
30% CH$_2$Cl$_2$-hexane	26-40	yellow oil + white amorphous solid	2.04
40% CH$_2$Cl$_2$-hexane	41-58	orange oil	0.98
60% CH$_2$Cl$_2$-hexane	59-73	greenish oil	0.86
80% CH$_2$Cl$_2$-hexane	74-85	yellow solid + greenish oil (**Compound 3**)	1.24
100% CH$_2$Cl$_2$	86-100	pale yellow solid (**Compound 4**)	0.40
10% EtOAc-CH$_2$Cl$_2$	101-120	yellow-green oil + yellow solid	2.03
30% EtOAc-CH$_2$Cl$_2$	121-135	yellow solid in yellow oil	0.50
50% EtOAc-CH$_2$Cl$_2$	136-144	brown oil	0.74
70% EtOAc-CH$_2$Cl$_2$	145-160	brown solid in dark brown oil	1.12
80% EtOAc-CH$_2$Cl$_2$	161-170	dark brown oil	2.30
100% EtOAc	171-190	dark brown oil	1.72
10% MeOH-EtOAc	191-200	brown residue	trace
50% MeOH-EtOAc	201-215	brown residue	trace

3.4 Purification, properties and structural elucidation of organic compounds isolated from the stems of *H. tiliaceus*

3.4.1 Purification, properties and structural elucidation of Mixture 1

Mixture **1** was obtained as white precipitate in yellow oil from the separation of Fraction I by eluting with 10% CH_2Cl_2 in hexane. The white precipitate was crystallized by a mixture of acetone and chloroform. Mixture **1** as white solid, m.p.70-72 °C, 20 mg (0.05% w/w of hexane extract) was obtained. The TLC (silica gel) exhibited only one spot at R_f 0.85 (solvent system : chloroform).

The IR spectrum (Fig 1) of Mixture **1** showed the absorption band belonging to C=O stretching vibration of an ester at $1731 cm^{-1}$ (s) and C-O stretching vibration at $1178 cm^{-1}$ (s). The C-H stretching vibration and C-H bending vibration of $-CH_2-$ and CH_3- were observed at 2847 and $1465 cm^{-1}$, respectively. The additional absorption peak at $717 cm^{-1}$ was due to $-CH_2-$ (for chain, 4 carbons). From this spectroscopic data, Mixture **1** was identified as a mixture of saturated long chain aliphatic esters.[33]

3.4.2 Purification, properties and structural elucidation of Mixture 2

As a white precipitate in pale yellow oil, Mixture **2** was obtained after it was eluted with 30 % CH_2Cl_2 in hexane. The oil was removed by washing with methanol for several times. White needle (30 mg, 0.075% w/w of extract), m.p. 140-145°C, was formed after recrystallization from hot hexane. This mixture showed two spots with R_f value of 0.82 and 0.68 (silica gel/dichloromethane).

After monitoring by colour tests, this substance gave a purple colour with Liebermann-Burchard's reagent. It also showed the positive result to 2,4-DNP reagent. This indicated the presence of triterpenoid nucleus containing carbonyl functional group of either aldehyde or ketone in this molecule.

The IR spectrum (Fig 2) revealed the characteristic absorption peak of O-H stretching vibration of hydroxy group at $3400-3500 cm^{-1}$, C-H stretching vibration of CH_2, CH_3 at 2950 and $2850 cm^{-1}$ and C=O stretching vibration of aldehyde or ketone at $1708 cm^{-1}$.

The ^{1}H NMR spectrum of Mixture **2** (Fig 3) exhibited the signals in an aliphatic region at δ 0.50-2.50 ppm which were corresponded to the proton signals of methyl, methylene and methine protons. Other signals were observed at δ 3.40 ppm as multiplet with 1H integration consistent with the signal of proton on a carbon attached to an oxygen atom.

The ^{13}C NMR spectrum (Fig 4) gave good agreement with IR and ^{1}H NMR spectra. It exhibited a total of 44 signals of carbon which suggested that this compound be a mixture. The spectrum revealed the chemical shifts of carbon very close to those of friedelin-type triterpene. From the literature survey, the generally found triterpenoid belonging to friedelan group which widely distributed in plants is fridelin and friedelan-3β-ol. The ^{13}C NMR chemical shift of each carbon was assigned by comparison with those of literature data.[34] The carbonyl carbon of friedelin was observed at δ 214.3 ppm whereas the signal at δ 72.7 ppm should be the carbon bearing hydroxy group belonging to friedelan-3β-ol. Other signals around 61.3 to 6.80 ppm were compatible with methyl, methylene, methine and quarternary carbons. The ^{13}C NMR chemical assignment of Mixture **2** is shown in Table 3.9.

Thus, the structures of Mixture **2** could be deduced as a mixture of fridelin and friedelan-3β-ol. This deduction was also reinforced by direct comparison of R_f value with that of the authentic sample. The result of TLC comparison showed that the R_f value of Mixture **2** is identical with that of friedelin and friedelan-3β-ol.

Table 3.9 Comparison of carbon chemical shift of component 1 with fridelan 3β-ol and component 2 with fridelin.

Carbon position	Fridelin	Component 2	Fridelan-3β-ol	Component 1
1	22.4	20.3	16.4	16.4
2	41.5	41.5	35.3	35.3
3	213.1	213.1	72.8	72.8
4	58.2	58.2	49.3	49.2
5	42.1	42.1	37.9	37.8
6	41.3	41.3	47.8	49.0
7	18.2	18.2	17.6	17.5
8	53.1	53.1	53.2	53.1
9	37.4	37.4	37.2	37.1
10	59.5	59.5	61.4	61.3
11	35.6	35.6	35.6	35.6
12	30.5	30.5	30.7	30.5
13	38.3	38.3	38.4	38.3
14	39.7	39.7	39.7	39.7
15	32.4	32.4	32.4	32.4
16	36.0	35.6	36.2	36.0
17	30.0	30.0	30.0	30.0
18	42.8	42.8	42.9	42.8
19	35.3	35.5	35.4	35.5
20	28.1	28.2	28.2	28.2
21	32.8	32.8	32.9	32.8
22	39.2	39.2	39.3	39.2
23	6.8	6.8	11.6	11.6
24	14.6	14.6	15.8	15.8
25	17.9	17.9	18.3	18.2
26	18.6	18.6	18.7	18.6
27	20.2	20.2	20.1	20.2
28	32.1	32.1	32.1	32.1
29	35.0	35.0	35.0	35.0
30	31.6	31.7	31.8	31.8

The mass spectrum (Fig 5) also supported the above evidence, it gave expected molecular ion at both m/z 426 and 428 which corresponded to the ^{1}H and ^{13}C NMR spectra. Other fragmentation series belonging to friedelan-3β-ol were observed at m/z 413, 395, 304, 275, 249, 207, 205, 195, 193 and 165 together with significant fragmentation ion peaks of friedelin at m/z 411, 341, 302, 274, 273, 247, 218, 205, 193 and 191. The possible mass fragmentation pattern of Mixture **2** is shown in Scheme 3.1.

By means of TLC and spectral comparison, Mixture **2** was obviously concluded to be a mixture of friedelin and friedelan-3β-ol. Their structures are shown below.

friedelin   friedelan-3β-ol
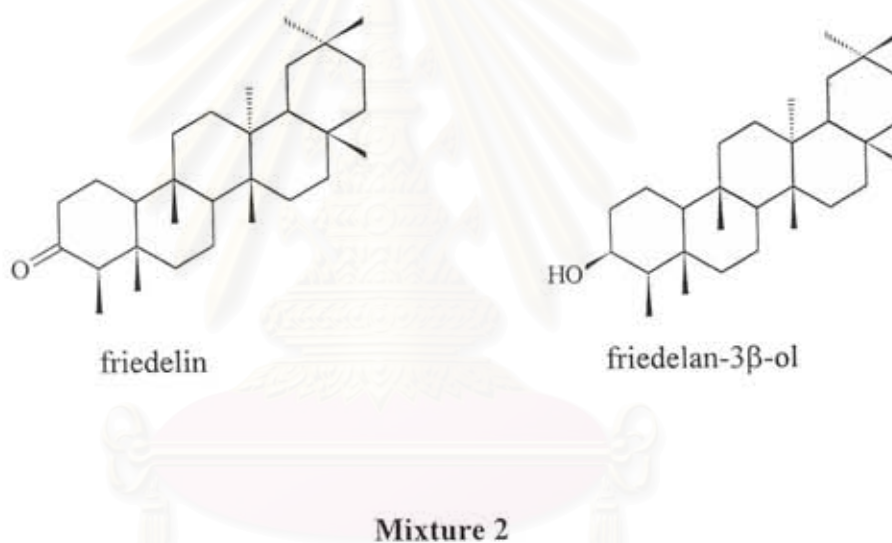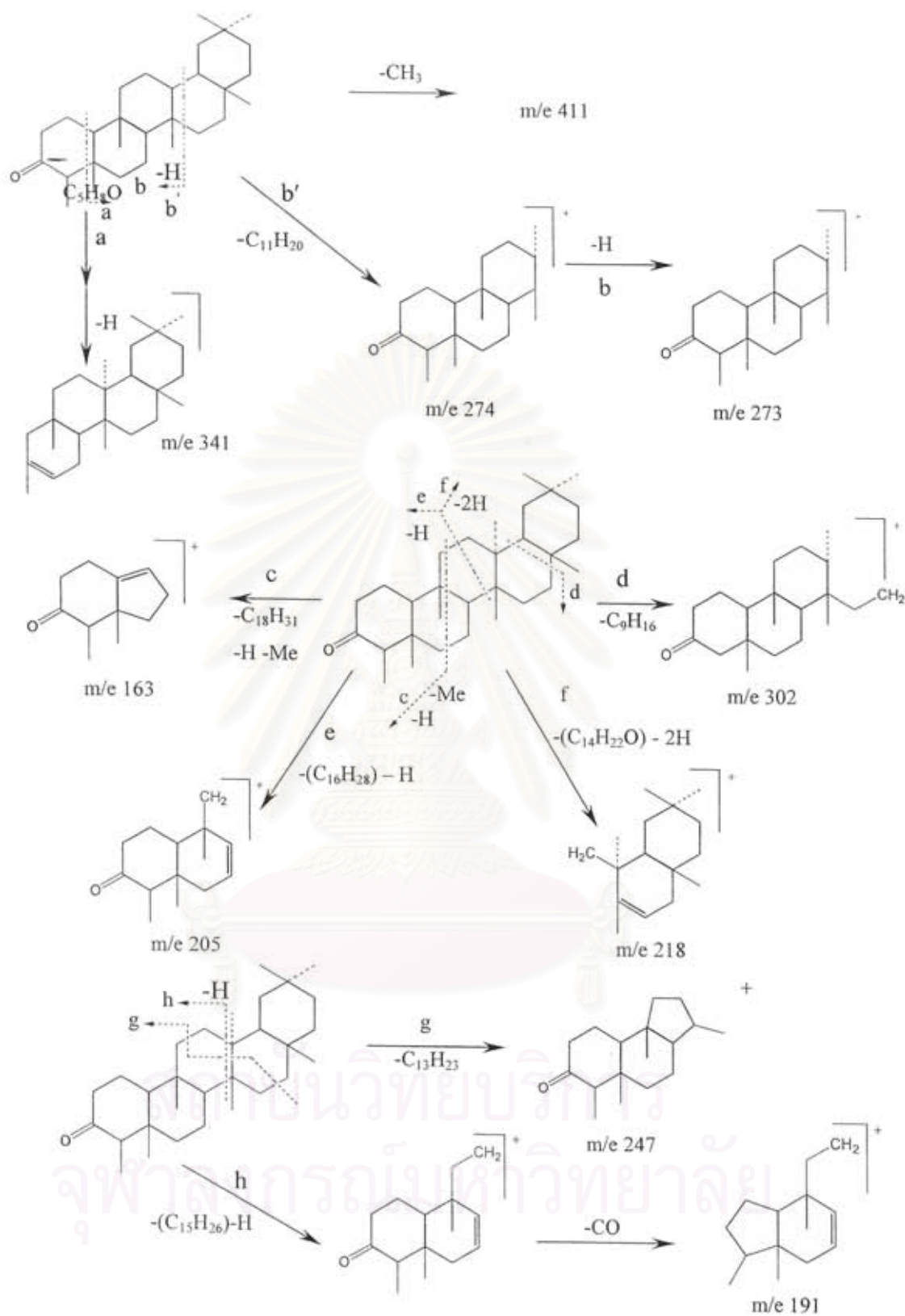
Mixture 2

Scheme 3.1 The possible mass fragmentation pattern of Mixture **2**
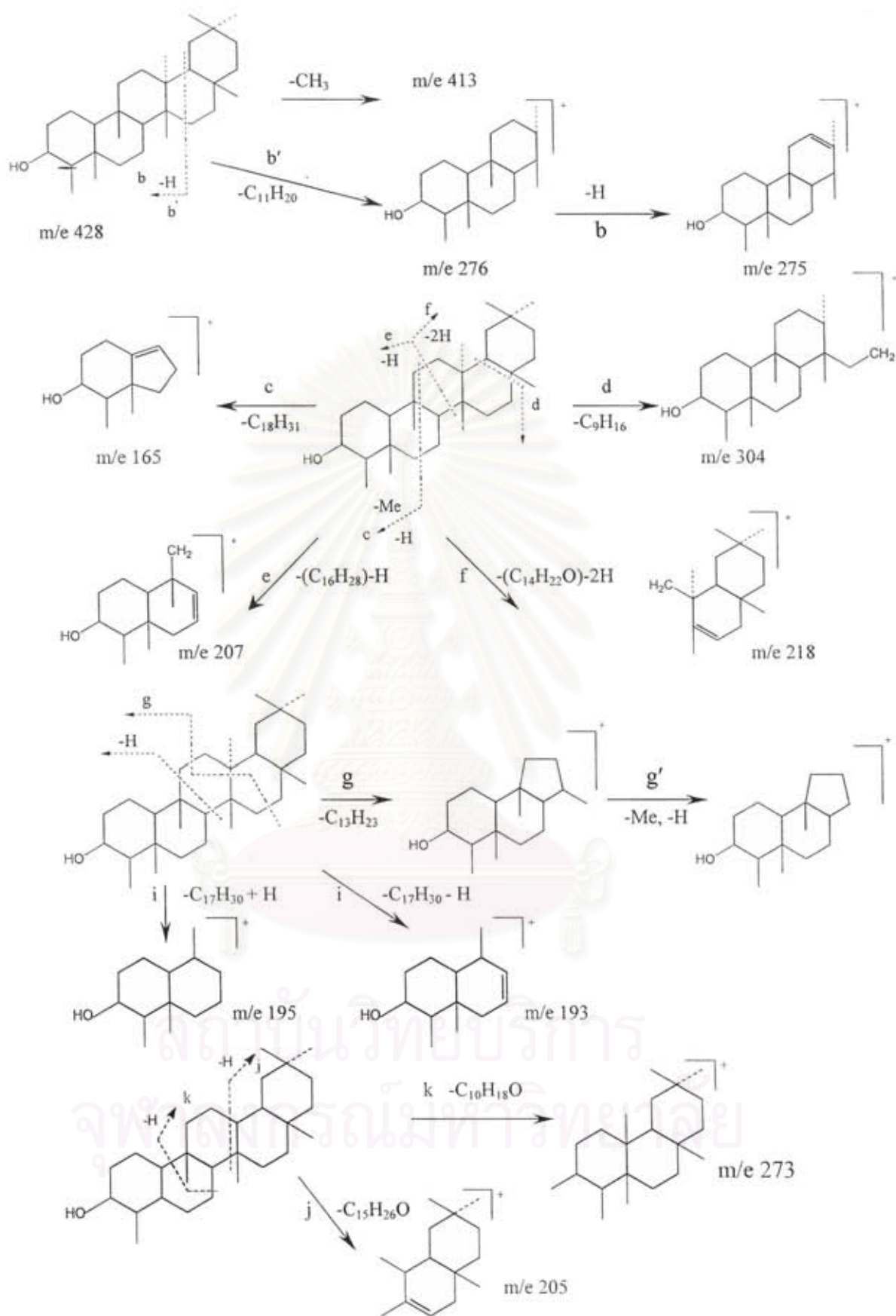
Scheme 3.1 (cont.)

3.4.3 Purification, properties and structural elucidation of Mixture 3

Mixture 3 was obtained from Fraction nos. 55-64 of hexane crude extract (Fraction I) as white precipitate. The white precipitate was recrystallized by acetone : chloroform (1:1) yielding white precipitate (32 mg, 0.08% w/w of hexane extract), m.p. 70-73°C. It showed a single spot on TLC with R_f value of 0.85 (silica gel/dichloromethane).

The IR spectrum (Fig 6) showed an absorption band of O-H stretching vibration of carboxylic acid at 3000-3400 cm^{-1} (very broad), C=O stretching vibration of carboxylic acid at 1705 cm^{-1}(s). The absorption band at 2919 and 1465 cm^{-1} revealed the presence of methylene and methyl groups. In addition, the absorption bands due to -CH$_2$- at 717 cm^{-1} was observed.

With the aids of IR spectroscopic evidence, it could be concluded that Mixture 3 is a mixture of long chain carboxylic acids.[33]

3.4.4 Purification, properties and structural elucidation of Mixture 4

The white solid in yellow oil was obtained from Fraction I. The yellow oil was removed with methanol. The remained solid was recrystallized from chloroform : hexane (1:1) for several times to afford white needle designated as Mixture 4, 1.64 g (0.004% w/w of hexane extract), m.p. 140-143°C. The TLC plate (silica gel) displayed R_f value of 0.45 (solvent : dichloromethane). Mixture 4 was soluble in dichloromethane, but slightly soluble in hexane. This substance gave a deep green colour with Liebermann-Burchard's reagent which suggested the presence of a steroid nucleus.

The IR spectrum (Fig 7) exhibited the absorption band of O-H stretching vibration at 3500-3430 cm^{-1} (s, b) and C-O stretching vibration at 1050 and 1020 cm^{-1}. In addition, the band at 1650 cm^{-1} revealed the presence of C=C functional group; the stretching and bending vibration of -CH$_3$ and -CH$_2$ were detected at 2960-2840 cm^{-1} and 1460 cm^{-1}, respectively.

According to the above data, this substance should be a steroidal compound. To analyze the composition of this mixture, it was subjected to gas chromatography and the chromatogram of the mixture was compared with that of three standard steroids: campesterol, stigmasterol and β-sitosterol (Fig 8). The result of GC analysis is shown in Table 3.10.

Table 3.10 The composition of steroids in Mixture **4**

Name	Retention time (min)	% composition
campesterol	17.28	9.03
stigmasterol	18.43	35.20
β-sitosterol	20.96	55.77

Thus, it could be concluded that Mixture **4** was a mixture of campesterol, β-sitosterol and stigmasterol.

β-sitosterol

campesterol

stigmasterol
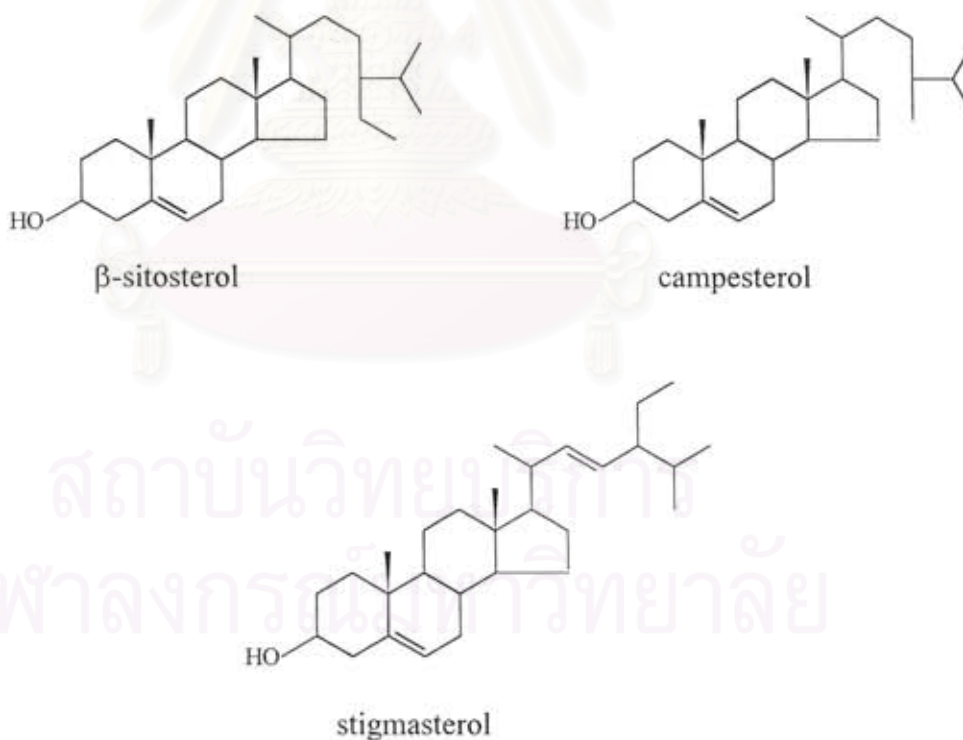
Mixture **4** mixture of steroid

3.4.5 Purification, properties and structural elucidation of Compound 1

After elution with 80% CH_2Cl_2-hexane, Compound **1** was gained as orange solid in brown oil. The brown oil was removed by washing with methanol for several times. The orange needle (m.p. 250°C, 8 mg, 0.02 % w/w of dichloromethane extract) was obtained after recrystallization with a mixture of chloroform - methanol. It showed a single spot with R_f value of 0.74 in dichloromethane solvent system.

Its IR spectrum (Fig 9) showed absorption bands of aromatic moiety at 2991 and 1593 cm^{-1} and of two conjugated carbonyl groups at 1696 and 1644 cm^{-1}, which are characteristics of a quinonoid nucleus. The molecular ion peak $[M]^+$ in EI mass spectrum (Fig 10) appeared at m/z 168 which was corresponded to a molecular formular $C_8H_8O_4$.

The ^1H NMR spectrum (Fig 11) showed an important signal at δ 5.70 ppm (2H, s) compatible with olefinic protons. Methoxy protons were detected at δ 3.70 ppm (6H, s). In addition, the ^{13}C NMR spectrum (Fig 12) exhibited total four carbon signals: at δ 188.0 ppm ascribing for the carbonyl carbons of quinonoid skeleton, the signal at δ 107.5 ppm consistent with olefinic carbons and the signal at δ 56.0 ppm coinciding with methoxy carbons.

All above data suggested that Compound **1** be a 1,4-benzoquinone containing two methoxy groups. Therefore, three possible structures of Compound **1** could be depicted as shown below.

A   B   C
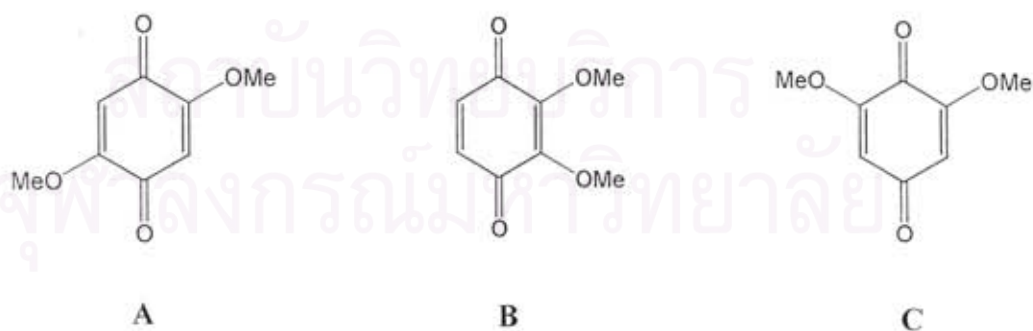
The alternative structure B can be discarded on the fact that the olefinic proton signals were observed as 2H singlet. The proton signal of structure B should appear as a doublet due to the coupling of proton at *ortho* position to each other. However, the ^{13}C NMR spectrum indicated that structure A should be corresponded to Compound **1** rather than structure C. This was because the spectrum gave only four carbon signals due to carbonyl carbons were suited in the same environment. This observation was confirmed by comparison of ^{1}H and ^{13}C NMR spectra of Compound **1** with those of 2,6-dimethoxy-1,4-benzoquinone (structure C), which was isolated from the heartwoods of *Rhizophora apiculata* in 1988.[35] The results are reported in Table 3.11.

Table 3.11 Comparison of chemical shift of structure A and 2,6-dimethoxy-1,4-benzoquinone (structure C)

Carbon No.	Chemical shift (ppm)			
	Structure A		Structure C	
	δ_C	δ_H	δ_C	δ_C
1	188.0	-	186.8	-
2	158.0	-	157.3	-
3	107.5	5.70	107.4	5.80
4	188.0	-	176.7	-
5	158.0	5.70	107.4	5.80
6	107.5	-	157.3	-
OMe	56.0	3.80	56.5	3.80

The mass spectrum (Fig 10) also supported structure A. It gave the parent ion peak at m/z 168.0 together with other fragmentation ion peaks at 153.0, 140.0, 138.0, 125.0, 97.0 and 69.0. The possible mass fragmentation pattern is given in Scheme 3.2.
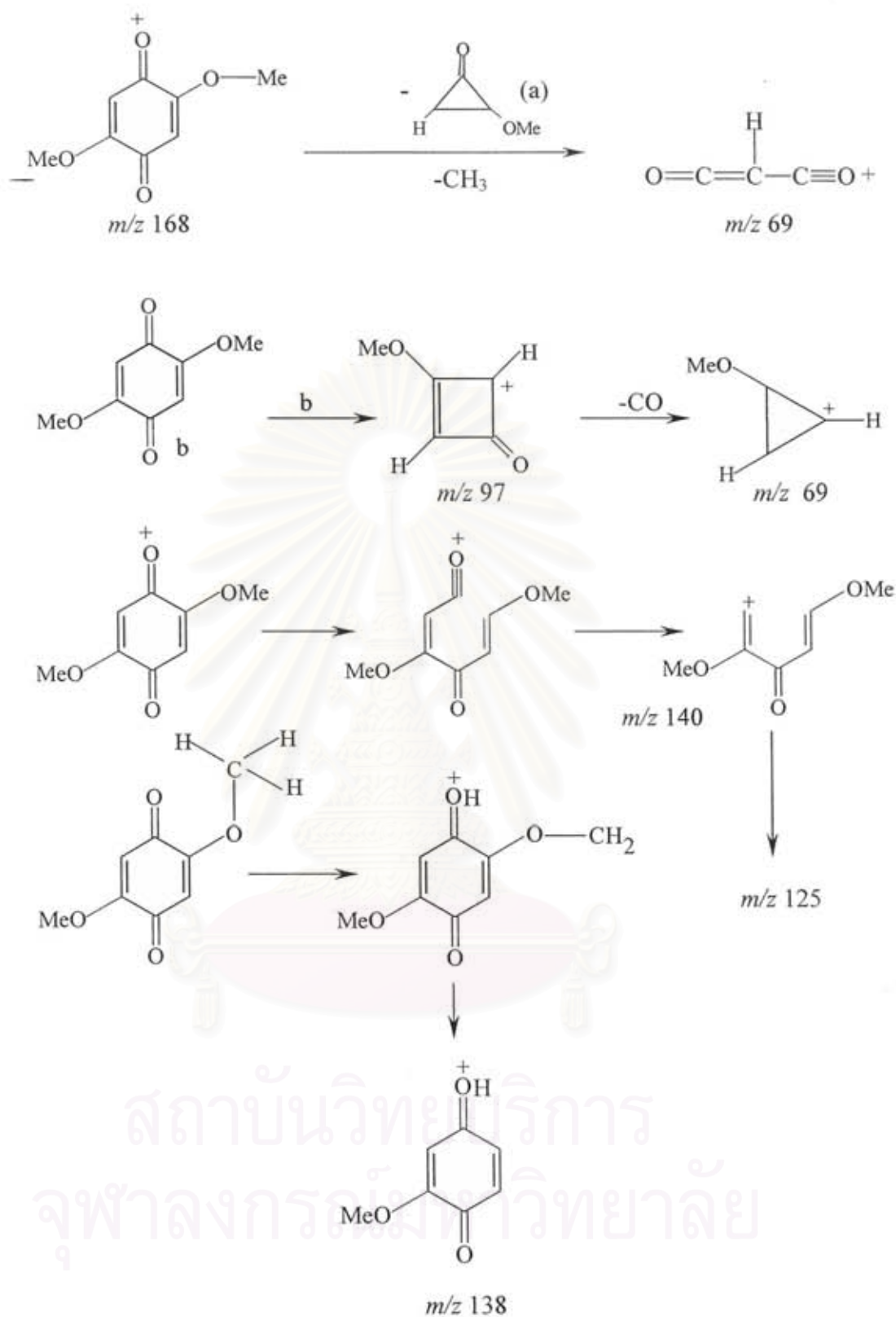
Scheme 3.2 The possible mass fragmentation pattern of Compound **1**

According to the physical properties and spectral evidences, it could be concluded that Compound **1** was 2,5-dimethoxy-1,4-benzoquinone. The structure of this compound is shown below.
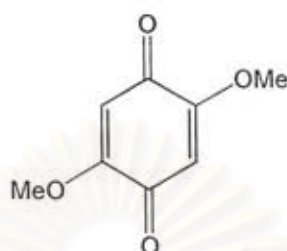
Compound 1

2,5-dimethoxy1,4-Benzoquinone

3.4.6 Purification, properties and structural elucidation of Compound 2

Compound **2** was obtained as yellow solid in brown oil. The oil was removed by washing with methanol and the remaining solid was recrystallized from hexane-ethyl acetate several times. White needle, m.p. 178-180 °C, (8 mg, 0.02% w/w of CH_2Cl_2 extract) was obtained. It showed one spot on TLC with R_f value of 0.65 in CH_2Cl_2 as a solvent system. Compound **2** is soluble in chloroform, dichloromethane and ethyl acetate.

The IR spectrum (Fig 13) clearly revealed the presence of phenolic hydroxy group at 3324 cm^{-1}, α,β-unsaturated carbonyl of lactone at 1710 cm^{-1}. The characteristic absorption peak due to an aromatic moiety was observed at 1624 cm^{-1}. This data suggested that Compound **2** possesses a conjugated system, such as carbonyl moiety. Moreover, its IR spectrum pattern was found to be similar to those of reported coumarins.[36] Therefore, Compound **2** may be classified as coumarin.
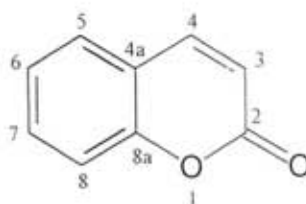
The ^{1}H NMR spectrum (Fig 14) exhibited the charateristic signals due to H-3 and H-4 of a coumarin skeleton at δ 6.22 and 7.96 ppm (1H each, d, J = 10.0 Hz) respectively. The isolated aromatic proton was observed at δ 6.44 (1H, s) in addition to two methoxy groups which were detected at δ 3.90 and 3.92 (3H each, s).

The ^{13}C NMR spectrum (Fig 15) displayed the resonance signals for all carbons and the multiplicity of each carbon could be assigned by DEPT spectra (Fig 16). The carbonyl carbon appeared at 161.4 ppm that was found to be similar to those common coumarins.[37] Furthermore, five quarternary carbons at δ 155.6, 151.8, 145.6, 131.5 and 102.5 ppm, three methine carbons at δ 138.5, 111.8 and 92.4 ppm and the presence of two methoxy groups at δ 61.4 and 56.2 ppm were also observed.

The ^{1}H-^{1}H NOESY spectrum (Fig 17) showed that H-4 proton had no coupling interaction with the other carbons except for H-3 proton. This result indicated the absence of proton attached to C-5 position. Consequently, the substituent located at C-5 position should be a hydroxy group.

The spectroscopic evidence suggested that Compound 2 be a coumarin containing one hydroxy group and two methoxy groups.

From the HMBC spectrum (Fig 18), the carbonyl carbon of coumarin detected at δ 161.4 ppm was coupled with olefinic protons at δ 6.22 and 7.96 ppm of H-3 and H-4, respectively. This clearly pointed out that the C-3 and C-4 positions in the coumarin ring should be unsubstituted. The proton at C-4 position was correlated to the aromatic carbon at C-3 position at 111.8 ppm, C-5 position at 145.6 ppm and also the signal at δ 151.8 ppm. The latter signal could be assigned for C-8a of the coumarin moiety.
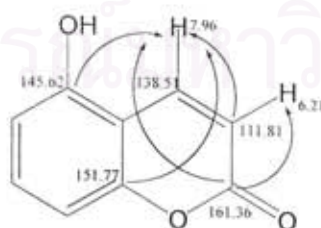
In addition, the C-H long range correlation in HMBC spectrum provided further information. The singlet aromatic proton at C-6 position at δ 6.45 ppm was coupled with the carbon of C-4a position at δ 102.5 ppm, C-7 at δ 155.6 and C-8 at δ 131.5 ppm. The location of hydroxy and methoxy groups were also able to be determined by HMBC spectrum as follows: the HMBC cross peaks were observed between methoxy protons at δ 3.90 ppm and an aromatic carbon at C-7 position at δ 155.6 ppm and also between the other methoxy protons at δ 3.90 and the carbon at C-8 position at δ 131.5 ppm.
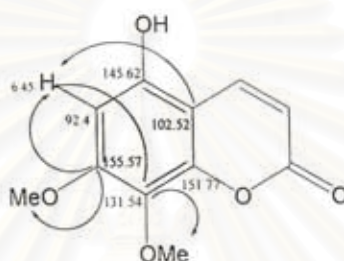
These locations were supported by the ^{1}H-^{1}H through space coupling in NOESY spectrum (Fig 17). This spectrum showed cross peak between the singlet aromatic proton of the C-6 position at δ 6.45 ppm and methoxy group at δ 3.92 ppm.

Supported by the above spectroscopic data, it could be concluded that Compound 2 was 5-hydroxy-7,8-dimethoxycoumarin. The structure is shown below.
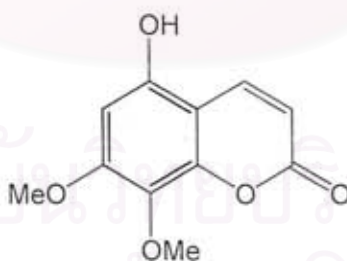
Compound 2

3.4.7 Purification, properties and structural elucidation of Compound 3

After recrystallization pale yellow solid derived from Fraction Nos. 74-85 of ethyl acetate extract with hexane-ethyl acetate, pale yellow needle (m.p. 140-142°C, 18 mg, 0.045% w/w of extract) was obtained. This compound revealed one spot on TLC with R_f value of 0.56 in dichloromethane solvent system. Compound **3** is soluble in various solvents such as dichloromethane, chloroform, ethyl acetate and slightly soluble in methanol. This compound gave a deep red colour with cyanidin test which is the characteristic of flavonol compound.

The IR spectrum (Fig 19) of Compound **3** showed the absorption band of a hydroxy group at 3400-3300 cm^{-1}. The strong absorption band at 1664 cm^{-1} revealed the presence of α,β-unsaturated carbonyl and the characteristic absorption peak due to an aromatic moiety was observed at 1550 cm^{-1}.

The EI mass spectrum (Fig 20) of this compound gave a molecular ion at *m/z* 298 in accordance with a flavonoid containing one hydroxy and two methoxy groups which was corresponding to the molecular formular $C_{17}H_{14}O_5$.

The ^{1}H NMR spectrum (Fig 21) exhibited two multiplet signals at δ 7.52 and 8.05 ppm, corresponding to 2H and 3H protons, respectively. This observation implied the characteristics of unsubstituted benzene ring. The spectrum also showed two doublet signals at δ 6.38 and 6.46 ppm of one proton each for *meta*-positioned aromatic ring proton. The presence of two methoxy groups was indicated by two singlet signals at δ 3.87 and 3.88 ppm. The other signal at δ 12.58 ppm was clearly indicated the presence of the chelated hydroxy group.

The ^{13}C NMR (Fig 22) and DEPT spectra (Fig 23) displayed 15 signals of carbon. The signals of quarternary carbons at δ 179.0, 165.6, 162.1, 156.9, 155.9, 139.7 and 130.5 ppm, five methine carbons at δ 130.9, 128.6, 128.4, 98.0 and 92.2 ppm and the methoxy carbons at 60.4 and 55.8 ppm were visualized. The chemical shift of the carbonyl carbon at δ 179.0 ppm revealed the characteristic feature of a flavonol compound (5-hydroxyflavone).

From the above spectroscopic data, Compound **3** was suggested to be methoxygenated flavonol compound bearing two methoxy groups.

The positions which were substituted by two methoxy groups could be assigned by ^{1}H NMR spectrum. This could also be confirmed through long-range [1]H-^{13}C coupling deduced from the HMBC spectrum (Fig 24). Since the absence of signal for H-3 at δ 6.30 ppm, the methoxy group should therefore be located at C-3. This fact was collaborated, on the HMBC spectrum, by the correlation of methoxy protons at δ 3.87 ppm and δ C-3 at δ 139.7 ppm. The ^{1}H NMR spectrum further exhibited the two doublet 1H proton signal at 6.38 and 6.46 ppm with coupling constant 8 Hz. The splitting pattern and the coupling constant were indicative of the assignment for *meta*-disubstitution. Thus, this methoxy group could be placed at C-7. This assignment was also supported by the correlation of the proton signal at δ 3.87 ppm and the carbon signal at δ 165.6 ppm in the HMBC spectrum.
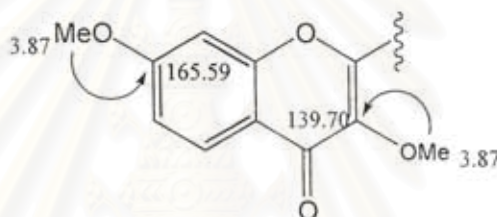
The aromatic singlet signal at δ 6.46 ppm could be assigned for H-8, which correlated with C-7 and C-9 (at δ 156.9 ppm). The remaining aromatic proton at δ 6.38 ppm (H-6) was also correlated to C-7 and C-5 (δ 162.1 ppm). In addition, the chelated hydroxy proton (δ 12.58 ppm) was found to have a close relationship with three aromatic carbons at δ 97.7, 106.2 and 162.1 ppm.
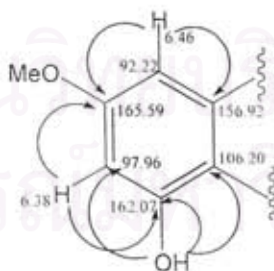
The complete assignment of ^{1}H and ^{13}C NMR chemical shift of Compound **3** is displayed in Table 3.12.

Table 3.12 The ^{1}H and ^{13}C chemical shift assignment of Compound **3**

Position	Chemical shift (ppm)	
	^{1}H NMR	^{13}C NMR
2		155.9
3		139.7
4		179.0
5		162.0
6	6.38 (2H, d, J = 2.14 Hz)	98.0
7		165.6
8	6.46 (2H, d, J = 2.13 Hz)	92.2
9		156.2
10		106.2
1'		139.7
2'	8.06 (2H, m)	128.4
3'	7.52 (3H, m)	128.6
4'	7.52 (3H, m)	130.5
5'	7.52 (3H, m)	128.6
6'	8.06 (2H, m)	128.4
C$_3$-OCH$_3$	3.87 (3H, s)	55.8
C$_7$-OCH$_3$	3.88 (3H, s)	60.4
C$_5$-OH	12.58 (1H, s)	-

In addition, the observed NOE correlations in the NOESY spectrum (Fig 25) between the aromatic proton signal at 6.46 and 3.87 ppm, 6.37 and 3.87 ppm and 8.07 and 3.88 ppm suggested the complete structure of Compound **3**.
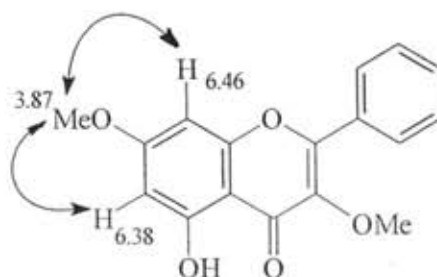
According to the above spectroscopic data, Compound **3** can be deduced as 5-hydroxy-3,7-dimethoxyflavone. Its structure is shown below.
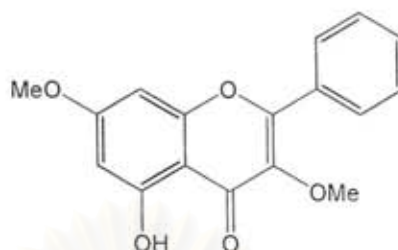
Compound 3

5-hydroxy-3,7-dimethoxyflavone

3.4.8 Purification, properties and structural elucidation of Compound 4

Compound **4** was obtained as yellow solid in greenish oil from Fraction Nos. 86-100 of Fraction **III** (see Table 3.8). The greenish oil was removed by washing with methanol. The remaining yellow solid was recrystallized from chloroform-methanol to yield yellow needle, m.p. 144-145°C (12 mg, 0.03% w/w of ethyl acetate extract). Compoud **4** was soluble in dichloromethane, chloroform, ethyl acetate but slightly soluble in hexane and methanol. This compound displayed a single spot with R_f value of 0.6 (solvent system : 100% ethyl acetate).

Compound **4** gave a deep red colour with cyanidin test, which is the characteristic of flavonol. In the EIMS mass spectrum (Fig 26), the $[M]^+$ at m/z 388 was attributed for the molecular formular $C_{20}H_{20}O_8$.

The IR spectrum (Fig 27) exhibited an absorption band due to O-H stretching vibration of hydroxy group at 3300-3400 cm^{-1}. The absorption band at 1670 cm^{-1} which corresponded to the C=O stretching vibration of, possibly, an α,β-unsaturated ester were observed. In addition, the absorption peak at 1500 cm^{-1} suggested the presence of aromatic moiety.

The ^{1}H NMR spectrum of Compound **4** (Fig 28) exhibited the singlet aromatic proton signal at δ 6.36, 6.44 (1H each, d, J = 2.1 Hz) and a singlet signal with 2H integration at δ 7.36 ppm. The signal of methoxy protons appeared at δ 3.88 (6H, s) and 3.94 ppm (9H, s). The signal for chelated hydroxy group at δ 12.58 ppm (1H, s) was also detected.

The ^{13}C NMR and DEPT 90, 135 spectra (Fig 29 and 30) displayed the signals of carbons as follows: a carbonyl carbon at δ 178.8 ppm, eight quarternary carbons at δ 165.6, 162.0, 156.7, 155.6, 153.1, 140.6, 139.4 and 125.4 ppm, three methine carbons at δ 106.1, 97.9 and 92.2 and four methoxy carbons at 61.0, 60.3, 56.3 and 55.8 ppm.

According to spectroscopic evidence and colour test, it could be concluded that Compound 4 was a flavonol compound bearing five methoxy groups.

The ^{1}H NMR spectrum of Compound 4 was found to be very close to that of Compound 3. To illustrated this, in Compound 3 the aromatic signal belonging to an unsubstituted B ring in the range of 7.30-8.00 ppm (5H, m) could not be detected in Compound 4. The notified signal in that region was the aromatic singlet signal at δ 7.25 ppm (2H). This observation firmly indicated the substitution on B ring. The singlet signals at δ 3.82 ppm with 6H integration and at δ 3.94 ppm with 9H integration were attributed to two methoxy groups and three methoxy groups, respectively. The comparison of ^{1}H NMR chemical shift of Compounds 3 and 4 is recorded in Table 3.13.

Table 3.13 Comparison of ^{1}H NMR chemical shift of Compounds **3** and **4**

Position	^{1}H NMR chemical shift (ppm)	
	Compound **3**	Compound **4**
6	6.38 (2H, d, J = 2.14 Hz)	6.36 (1H, d, J = 2.13 Hz)
8	6.46 (2H, d, J = 2.13 Hz)	6.45 (1H, d, J = 2.44 Hz)
2′	8.06 (2H, m)	7.36 (2H, s)
3′	7.52 (3H, m)	-
4′	7.52 (3H, m)	-
5′	7.52 (3H, m)	-
6′	8.06 (2H, m)	7.36 (2H, s)
5-OH	12.58 (1H, s)	12.58 (1H, s)
3-OMe	3.87 (3H, s)	3.88 (6H, s)
7-OMe	3.88 (3H, s)	3.94 (9H, s)
3′-OMe	-	3.94 (9H, s)
4′-OMe	-	3.88 (6H, s)
5′-OMe	-	3.94 (9H, s)

The ^{1}H NMR manifestly pointed out that Compound **4** must be a pentamethoxyflavonol compound. Its structure could be deduced as 5-hydroxy-3,3′,4′,5′,7-pentamethoxyflavone. This deduction was established by comparison comparing the ^{1}H NMR spectrum of this compound with those of the known flavonoid, myricetin-3,3′,4′,5′,7-pentamethyl ether.[38] The result is displayed in Table 3.14.

Table 3.14 Comparison of ^{1}H NMR chemical shift of Compound **4** with myricetin-
3,3′,4′,5′,7-pentamethyl ether

Carbon	^{1}H NMR chemical shift (ppm)	
no.	myricetin-3,3′,4′,5′,7-pentamethyl ether	Compound **4**
6	6.30 (2H, d, J = 2.14 Hz)	6.36 (1H, d, J = 2.13 Hz)
8	6.43 (2H, d, J = 2.13 Hz)	6.45 (1H, d, J = 2.44 Hz)
2′	7.36 (2H, s)	7.36 (2H, s)
6′	7.36 (2H, s)	7.36 (2H, s)
5-OH	12.55 (1H, s)	12.58 (1H, s)
2(OMe)	3.86 (6H, s)	3.88 (6H, s)
3(OMe)	3.90 (9H, s)	3.94 (9H, s)

The EI mass spectrum (Fig 26) also supported the proposed structure of Compound **4**. It revealed significant mass fragmentation ion, besides the molecular ion, at m/z 373 [M-Me]⁺, 357 [M-OMe]⁺, 345 [M-MeCO]⁺. Other fragmentation ion patterns were proposed as shown in Scheme 3.2.
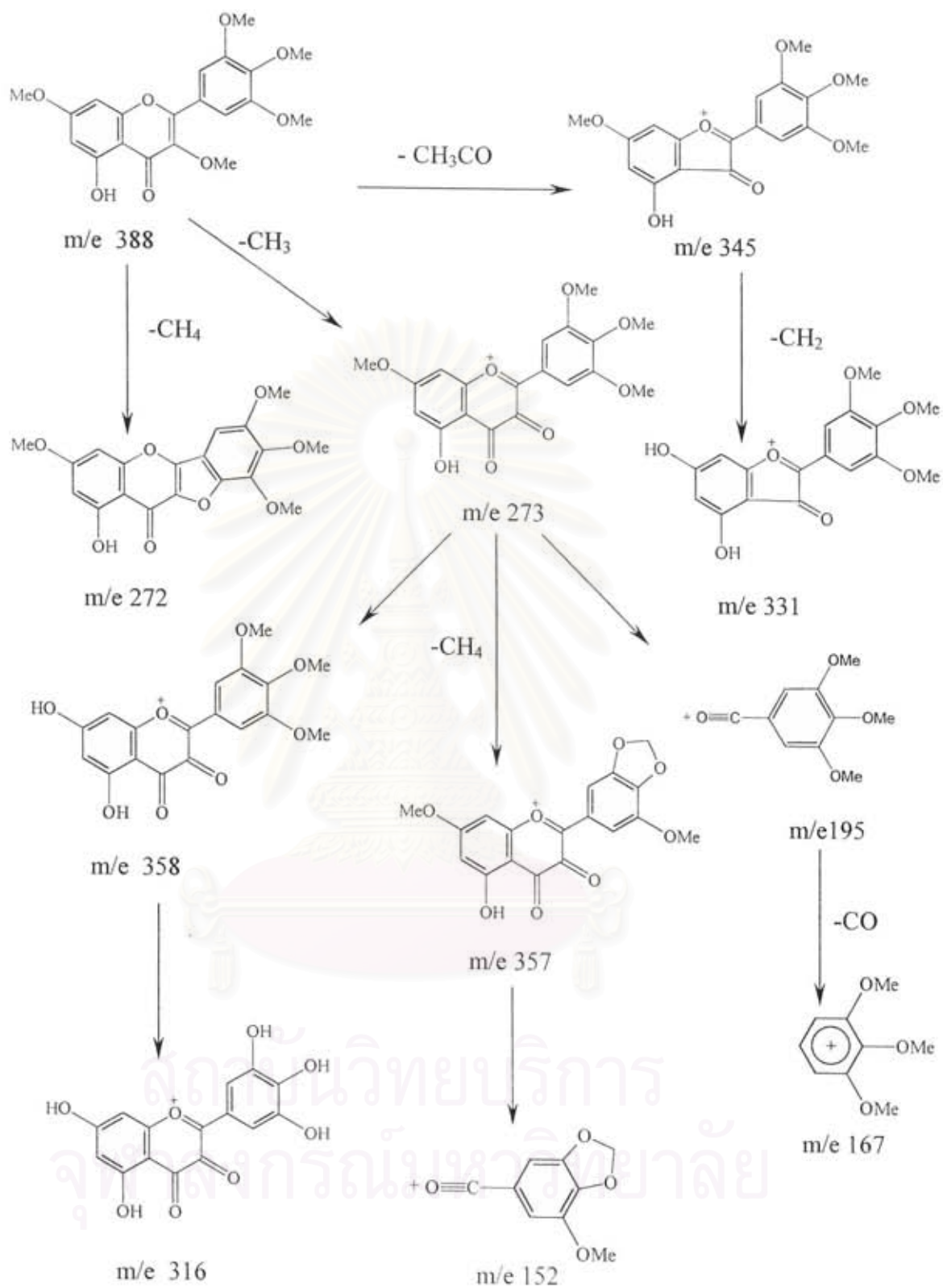
Scheme 3.3 The possible mass fragmentation of Compound **4**

Through the search from chemical literatures, the ^{13}C chemical assignment of this compound has not been reported. Thus, the tentative assignment for carbon signals was tabulated in Table 3.15.

Table 3.15 Tentative ^{13}C chemical shift assignment of Compound **4**

Position	^{13}C NMR chemical shift (ppm)	
	Compound 3	Compound 4
2	155.9	156.7
3	139.7	139.4
4	179.0	178.8
5	162.0	162.0
6	98.0	97.9
7	165.6	165.7
8	92.2	92.2
9	156.2	155.6
10	106.2	106.1
1′	139.7	125.4
2′	128.4	106.1
3′	128.6	153.1
4′	130.5	140.6
5′	128.6	153.1
6′	128.4	106.1
3-OCH$_3$	55.8	55.82
7-OCH$_3$	60.4	61.0
3′- OCH$_3$	-	56.3
4′- OCH$_3$	-	60.3
5′- OCH$_3$	-	56.3

From the above data, it could be concluded that Compound **4** is a flavonoid compound namely 5-hydroxy-3,3′,4′,5′,7-petamethxoyflavone[38] and its structure is shown below.
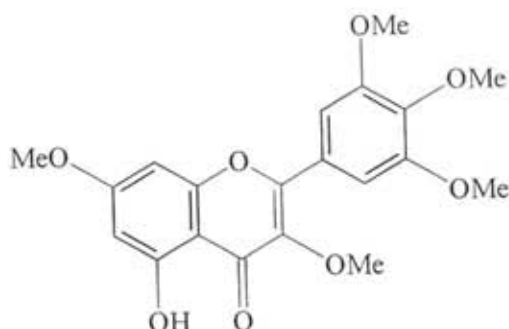
Compound 4

5-hydroxy-3,3',4',5',7-petamethxoyflavone

3.5 Chemical constiuents of the leaves of *H. tiliaceus*

3.5.1 Separation of dichloromethnae extract of leaves (Fraction VI)

The dichloromethane extract of leaves (Fraction VI) was further separated into small fractions by quick column chromatography using silica gel 60G Art. 7731 as adsorbent. Portion (200 g) of this crude extract was adsorbed on sillica gel 60G Art. 7734 and subjected to column chromatography. The column was initially eluted with hexane and gradually changed to a mixture of dichloromethane and hexane, dichloromethane and a mixture of dichloromethane and methanol. Eluting solvent approximately 8 L for each fraction was collected and then concentrated to a small volume. The results of separation are shown in Table 3.16.

Table 3.16 The results of the separation of Fraction VI by silica gel quick column chromatography

Eluent (%vol/vol)	Fraction	Remarks	Weight (g)
100% hexane	VIA	red solid + red oil	33.61
10% CH_2Cl_2- hexane	VIB	yellow solid mass	14.56
30% CH_2Cl_2- hexane	VIC	white solid + yellow oil	28.77
50% CH_2Cl_2- hexane	VID	white solid + greenish oil	20.82
80% CH_2Cl_2- hexane	VIE	dark green solid	16.45
100% CH_2Cl_2	VIF	dark green gum	22.38
10% MeOH-CH_2Cl_2	VIG	dark green viscous solid	17.86

3.5.1.1 Brine shrimp cytotoxicity test of fractions derived from Fraction VI

Each fraction derived from the separation of dichloromethane extract of leaves (Fraction VI) by quick column chromatography was further screened for cytotoxicity against brine shrimp (*Artemia salina* Linn.) according to the procedure described in Chapter 2. The results are shown in Table 3.17.

Table 3.17 The results of brine shrimp cytotoxicity test of various fractions derived from the separation of Fraction VI

Fraction No.	LC_{50}	Activity
VIA	70.64	medium activity
VIB	66.94	medium activity
VIC	32.68	medium activity
VID	24.39	medium activity
VIE	18.86	medium activity
VIF	23.06	medium activity
VIG	44.87	medium activity

3.5.2 Fractionation of Fraction VII by quick column chromatography

The ethyl acetate extract of leaves (Fraction VII) was evaporated to dryness under vacuum to yield dark green residue. The residue was fractioned using a silica gel 60G Art..7731 column eluting with hexane, and a gradient introduction of dichloromethane was added up to 100 %, followed by a mixture of methanol and dichloromethane. The results of separation are shown in Table 3.18.

Table 3.18 The results of the separation of Fraction VII by quick column chromatography

Eluent (%vol/vol)	Fraction	Remarks	Weights (g)
50% CH$_2$Cl$_2$-hexane	**VIIA**	white solid + yellow oil	18.82
80%CH$_2$Cl$_2$-hexane	**VIIB**	yellow-green syrupy mass	12.68
100% CH$_2$Cl$_2$-hexane	**VIIC**	dark brown syrupy mass	20.81
5% MeOH-CH$_2$Cl$_2$	**VIID**	dark brown viscous solid	15.34
10% MeOH-CH$_2$Cl$_2$	**VIIE**	dark brown viscous solid	7.56
50% MeOH-CH$_2$Cl$_2$	**VIIF**	brown solid	11.64

3.5.2.1 Brine shrimp lethality test for ethyl acetate extract of leaves (Fraction VII)

Each small fraction derived from the separation of ethyl acetate extract by quick column chromatography was subjected to brine shrimp cytotoxicity test. The results of brine shrimp cytotoxicity test are displayed in Table 3.19.

Table 3.19 The results of brine shrimp cytotoxicity test of various fractions derived from the separation of Fraction VII

Fraction No.	LC$_{50}$	Activity
VIIA	40.82	medium activity
VIIB	30.68	medium activity
VIIC	56.75	medium activity
VIID	18.97	medium activity
VIIE	12.43	medium activity
VIIF	28.52	medium activity

3.6 Separation of fractions derived from Fractions VI and VII

3.6.1 Separation of Fraction VIA

Fraction VIA was subjected to silica gel column chromatography using a mixture of dichloromethane and hexane as eluents. The results of separation are shown in Table 3.20.

Table 3.20 The results of the separation of Fraction VIA

Eluent % (vol/vol)	Fraction No.	Remarks	Weight (g)
100% hexane	1-5	white amorphous solid	5.21
10%CH_2Cl_2-hexane	6-10	pale yellow oil + white precipitate	2.58
20%CH_2Cl_2-hexane	11-25	orange viscous liquid	1.37
30%CH_2Cl_2-hexane	26-35	pale yellow semisolid	0.65
40%CH_2Cl_2-hexane	36-43	pale yellow oil	1.46
60%CH_2Cl_2-hexane	44-57	orange viscous liquid	1.17
80%CH_2Cl_2-hexane	58-74	orange oil	0.92
100%CH_2Cl_2	75-80	pale yellow oil	0.68

3.6.2 Separation of Fraction VIB

After elution with 10% CH_2Cl_2-hexane, yellow solid mass was gained. Thin layer chromatography indicated there were at least two components in this fraction. This fraction was then separated by rechromatographed over silica gel using hexane and a mixture of hexane containing increasing proportions of dichloromethane as eluents. The results of separation are shown in Table 3.21.

Table 3.21 The results of the separation of Fraction VIB

Eluent % (vol/vol)	Fraction No.	Remarks	Weight (g)
100% hexane	1-3	white amorphous solid	1.25
10%CH_2Cl_2-hexane	4-8	white needle + yellow oil	4.05
20%CH_2Cl_2-hexane	9-15	white needle (Compound **5**)	2.86
30%CH_2Cl_2-hexane	16-20	orange semi solid	0.44
40%CH_2Cl_2-hexane	21-30	white precipitate + pale yellow oil	1.82
60%CH_2Cl_2-hexane	31-37	white amorphous solid	0.53
80%CH_2Cl_2-hexane	38-46	yellow viscous solid	0.27
100% CH_2Cl_2	47-51	yellow viscous solid	0.16
10%EtOAc- CH_2Cl_2	52-60	pale yellow oil	0.10
30%EtOAc- CH_2Cl_2	61-70	pale yellow oil	0.37
50%EtOAc- CH_2Cl_2	71-78	pale yellow oil	0.62

3.6.3 Separation of Fraction VIC

Fraction VIC contained white needle in pale yellow oil (26.8 g, 13.4% wt/wt of dichloromethane extract). This fraction was first examined by TLC. The results revealed that there were at least two components in this fraction (silica gel, solvent : dichloromethane). It showed the same R_f value as that for a Mixture **2** (a mixture of friedelin and friedelan-3β-ol). Thus, a portion (10 g) of this fraction was decided to reseparate by silica gel column chromatography. The results of separation are shown in Table 3.22.

Tabe 3.22 The results of the separation of Fraction VIC

Eluent	Fraction No.	Remark	Weight (g)
100% hexane	1-7	white amorphous solid	0.52
10% CH_2Cl_2-hexane	8-15	deep red oil	0.61
20% CH_2Cl_2-hexane	16-22	orange semi solid	0.38
	23-28	white needle + bright yellow oil (**Mixture 2**)	2.46
30% CH_2Cl_2-hexane	29-35	white solid + pale yellow oil	1.29
50% CH_2Cl_2-hexane	36-40	pale yellow oil	0.26
60% CH_2Cl_2-hexane	41-45	pale yellow oil	0.44
	46-52	white precipitate + yellow oil	0.18
80% CH_2Cl_2-hexane	53-55	pale yellow oil	0.63
100% CH_2Cl_2	56-60	pale yellow oil	0.24
10% EtOAc-CH_2Cl_2	61-68	colourless oil	0.37
20% EtOAc-CH_2Cl_2	69-77	colourless oil	0.14

3.6.4 Separation of Fraction VID

The TLC of Fraction VID presented at least two spots (silica gel, solvent: dichloromethane). This fraction was reseparated by column chromatography using silica gel as an adsorbent. The results of separation are shown in Table 3.23.

Table 3.23 The results of the separation of Fraction VID

Eluent % (vol/vol)	Fraction No.	Remarks	Weight (g)
10%CH$_2$Cl$_2$-hexane	1-8	yellow viscous solid	1.06
	9-13	white needle + yellow oil	3.48
20%CH$_2$Cl$_2$-hexane	14-20	pale yellow oil	2.46
30%CH$_2$Cl$_2$-hexane	21-25	white precipitate	1.50
40%CH$_2$Cl$_2$-hexane	26-32	white precipitate pale yellow oil (Compound **6**)	5.21
	33-38	white needle	1.27
60%CH$_2$Cl$_2$-hexane	39-45	yellow viscous solid	0.84
80%CH$_2$Cl$_2$-hexane	46-50	yellow viscous solid	0.63
100% CH$_2$Cl$_2$	51-55	pale yellow solid	0.12
5%MeOH- CH$_2$Cl$_2$	56-60	pale yellow solid	0.35

3.6.5 Separation of Fraction VIE

Fraction VIE was obtained as yellow gum after eluted with 80% CH$_2$Cl$_2$-hexane. This fraction was further purified by silica gel column chromatography. The results of separation are shown in Table 3.24.

Table 3.24 The results of the separation of Fraction VIE

Eluent % (vol/vol)	Fraction No.	Remarks	Weight (g)
50% CH$_2$Cl$_2$-hexane	1-5	white needle + pale yellow oil	0.63
80% CH$_2$Cl$_2$-hexane	6-15	white precipitate + greenish oil	1.47
100% CH$_2$Cl$_2$	16-20	dark green viscous solid	2.03
5% MeOH-CH$_2$Cl$_2$	21-30	dark green solid	2.64
10% MeOH-CH$_2$Cl$_2$	31-35	dark brown gum	0.81
20% MeOH-CH$_2$Cl$_2$	36-42	brown oil	0.95
50% MeOH-CH$_2$Cl$_2$	43-52	brown oil	0.35

3.6.6 Separation of Fraction VIF

Fraction VIF as dark green gum, 22.3 g (11.15% wt/wt of dichloromethane extract of leaves) was further purified by subjecting to column chromatography over silica gel. The results of separation are shown in Table 3.25.

Table 3.25 The results of the separation of Fraction VIF

Eluent % (vol/vol)	Fraction No.	Remarks	Weight (g)
50%CH$_2$Cl$_2$-hexane	1-3	white needle + yellow oil	2.73
80%CH$_2$Cl$_2$-hexane	4-10	dark green solid	0.86
100% CH$_2$Cl$_2$	11-18	greenish oil	0.53
10%EtOAc-CH$_2$Cl$_2$	19-24	pale green oil	1.34
20%EtOAc-CH$_2$Cl$_2$	25-30	greenish-brown residue	1.82
30%EtOAc-CH$_2$Cl$_2$	31-40	dark brown viscous solid	0.52
50%EtOAc-CH$_2$Cl$_2$	41-48	white powder + pale green oil	2.74
60% EtOAc-CH$_2$Cl$_2$	49-55	white needle + yellow oil	0.24
80% EtOAc-CH$_2$Cl$_2$	56-60	dark green solid	0.86
100%EtOAc	61-70	greenish-brown oil	0.17
10%EtOAc-MeOH	71-75	brown residue	trace
30%EtOAc-MeOH	76-80	brown residue	trace
50%EtOAc-MeOH	81-88	dark brown residue	trace

3.6.7 Separation of Fraction VIG

Fraction VIG, 5% MeOH-CH$_2$Cl$_2$, was first examined by TLC using CH$_2$Cl$_2$ as a developing solvent. The results indicated that there were two major components in this fraction. Thus, the concentrated extract was further separated by silica gel column chromatography and initially eluted with 50%CH$_2$Cl$_2$-hexane. The results of separation are tabulated in Table 3.26.

Table 3.26 The results of the separation of Fraction VIG

Eluent % (vol/vol)	Fraction No.	Remarks	Weight (g)
50% CH_2Cl_2-hexane	1-8	white neddle + yellow oil	2.73
80% CH_2Cl_2-hexane	9-15	pale yellow oil	0.86
100% CH_2Cl_2	16-20	yellow oil	0.18
10% EtOAc-CH_2Cl_2	21-30	orange semisolid	1.34
20% EtOAc-CH_2Cl_2	31-35	white precipitate pale yellow oil	1.82
30% EtOAc-CH_2Cl_2	36-42	yellow viscous solid	0.52
40% EtOAc-CH_2Cl_2	43-50	white powder + pale green oil	2.74
60% EtOAc-CH_2Cl_2	51-56	dark green solid	0.18
80% EtOAc-CH_2Cl_2	57-65	dark green solid	0.18
100% EtOAc-CH_2Cl_2	66-74	dark green viscous solid	1.42
5% MeOH-EtOAc	75-80	pale green oil	trace
10% MeOH-EtOAc	81-85	greenish brown residue	trace
50% MeOH-EtOAc	86-95	greenish brown residue	trace

3.6.8 Separation of Fraction VIIA

As yellow green gum, Fraction VIIA was gained after elution with 50% CH_2Cl_2-hexane. TLC monitoring revealed the existence of a mixture of steroid and Compound **6**. Portion (10 g) of this fraction was then separated by silica gel column chromatography. The results of separation are dsiplayed in Table 3.27.

Table 3.27 The results of the separation of Fraction VIIA

Eluent (%vol/vol)	Fraction No.	Remarks	Weight (g)
10% CH$_2$Cl$_2$-hexane	1-5	white amorphous solid	1.25
30% CH$_2$Cl$_2$-hexane	6-10	yellow viscous solid	0.28
40% CH$_2$Cl$_2$-hexane	11-18	pale yellow solid	trace
50% CH$_2$Cl$_2$-hexane	19-25	white crystal + greenish oil	2.74
80% CH$_2$Cl$_2$-hexane	26-31	greenish solid	0.63
100%CH$_2$Cl$_2$	32-36	greenish solid	3.76
10% EtOAc-CH$_2$Cl$_2$	37-40	yellow semi solid	1.97
30% EtOAc-CH$_2$Cl$_2$	41-46	greenish oil	0.43
50% EtOAc-CH$_2$Cl$_2$	47-53	pale green oil	0.58
80% EtOAc-CH$_2$Cl$_2$	54-60	yellow brown oil	0.16
100% EtOAc	61-65	dark brown oil	0.85
10% MeOH-EtOAc	66-70	dark brown oil	1.13

3.6.9 Separation of Fraction VIIB

The brownish-yellow viscous solid which was obtained by elution with 80% CH$_2$Cl$_2$-hexane was rechromatograped by column chromatography using silica gel as an adsorbent. The results of separation are shown in Table 3.28.

Table 3.28 The results of the separation of Fraction VIIB

Eluent (%vol/vol)	Fraction No.	Remarks	Weight (g)
5% EtOAc-hexane	1-6	white solid + yellow oil	0.16
8% EtOAc-hexane	7-12	pale yellow oil	0.62
10% EtOAc-hexane	13-20	white amorphous solid + pale yellow oil	2.85
15% EtOAc-hexane	21-27	white solid + orange oil	1.67
20% EtOAc-hexane	28-33	white powder + yellow oil	0.76
40% EtOAc-hexane	34-42	pale brown oil	1.81
60% EtOAc-hexane	43-50	greenish oil	0.38
80% EtOAc-hexane	51-55	dark green solid	0.61

3.6.10 Separation of Fraction VIIC

Fraction VIIC was obtained as yellow brown viscous solid by elution with 100% CH_2Cl_2. These fractions were then subjected to silica gel column chromatography using a mixture of hexane and ethyl acetate as eluents. The results of separation are exhibited in Table 3.29.

Table 3.29 The results of the separation of Fraction VIIC

Eluent (%vol/vol)	Fraction No.	Remarks	Weight (g)
5% EtOAc-hexane	1-10	white solid + yellow oil	0.75
	11-20	yellow syrupy mass	0.97
8% EtOAc-hexane	21-28	white crystal + pale yellow oil	1.82
10% EtOAc-hexane	29-35	white crystal + yellow oil	1.04
	36-43	yellow precipitate	0.37
15% EtOAc-hexane	44-52	orange oil + white solid	1.48
20% EtOAc-hexane	53-60	yellow oil	0.18
	61-65	yellow oil + white precipitate	0.25
40% EtOAc-hexane	66-70	yellow brown solid	0.95
60% EtOAc-hexane	71-75	brownish-green oil	0.21
80% EtOAc-hexane	76-83	brown oil	0.62

3.6.11 Separation of Fraction VIID

Fraction VIID was obtained as dark brown solid after it was eluted by 5% methanol in dichloromethane. This fraction was then separated by column chromatography using silica gel as an adsorbent. The results of separation are shown in Table 3.30.

Table 3.30 The results of the separation of Fraction VIID

Eluent (%vol/vol)	Fraction No.	Remarks	Weight (g)
5% EtOAc-hexane	1-4	yellow viscous solid	1.58
8% EtOAc-hexane	5-10	pale yellow oil	0.84
10% EtOAc-hexane	11-15	white crystal + pale yellow oil (Compound 7)	1.32
15% EtOAc-hexane	16-22	dark brown solid	0.27
20% EtOAc-hexane	23-34	dark brown solid	4.64
40% EtOAc-hexane	35-40	yellow brown solid	2.31
60% EtOAc-hexane	41-48	dark green residue	0.84
80% EtOAc-hexane	49-57	dark brown viscous solid	1.08
100% EtOAc	58-62	pale brown residue	0.24
20% MeOH-EtOAc	63-68	red brown viscous solid	0.55

3.6.12 Separation of Fraction VIIE

Thin layer chromatography indicated at least two components were existed in this fraction. Further purification was performed by column chromatography. The results of separation are displayed in Table 3.31.

Table 3.31 The results of the separation of Fraction VIIE

Eluent (%vol/vol)	Fraction No.	Remarks	weight (g)
20% EtOAc-hexane	1-7	yellow brown solid (Compound 8)	0.64
40% EtOAc-hexane	8-13	yellow brown solid	2.31
60% EtOAc-hexane	14-20	dark green residue	0.84
80% EtOAc-hexane	21-28	dark brown viscous solid	1.08
100% EtOAc	29-35	pale brown residue	0.24
20% MeOH-EtOAc	36-40	red brown viscous solid	0.55
40% MeOH-EtOAc	41-45	dark brown viscous solid	0.28

3.6.13 Separation of Fraction VIIF

Fraction VIIF was obtained by elution with 50% methanol in dichloromethane. Evaporation under reduced pressure left a dark green sticky mass which was chromatographed by column chromatography using silica gel as an adsorbent. The results of separation are displayed in Table 3.32.

Table 3.32 The results of the separation of Fraction VIIF

Eluent (%vol/vol)	Fraction No.	Remarks	Weight (g)
10% EtOAc-hexane	1-5	yellow oil	0.14
	6-12	yellow solid + yellow oil	0.52
20% EtOAc-hexane	13-20	dark brown solid	4.64
40% EtOAc-hexane	21-24	white needle + greenish oil	2.31
60% EtOAc-hexane	25-32	brown oil	0.84
	33-38	dark green solid	1.76
80% EtOAc-hexane	39-47	reddish-brown residue	1.08
100% EtOAc	48-53	dark brown viscous solid	0.24
	54-60	pale green oil	0.13
20% MeOH-EtOAc	61-65	dark green oil	0.55
40% MeOH-EtOAc	66-70	dark brown viscous solid	0.28
60% MeOH-EtOAc	71-75	dark green viscous solid	0.08
80% MeOH-EtOAc	76-80	dark green viscous solid	0.17
100% MeOH	81-90	dark green viscous solid	0.62

3.7 Purification, properties and structural elucidation of isolated compounds isolated from the leaves of *H. tiliaceus*

3.7.1 Purification, properties and structural elucidation of Compound 5

After elution with 10% CH_2Cl_2-hexane of Fraction VID, white crystal in yellow oil was obtained. The yellow oil was removed with methanol and the remaining solid was recrystallization by a mixture of chloroform and methanol to yield needle with melting point 196-198°C, 100 mg, (0.05% wt/wt of dichloromethane extract). This compound gave a violet colour with Liebermann-Burchard's reagent which suggested the presence of triterpenoid skeleton.

The IR spectrum (Fig 31) displayed broad band in the range of 3400-3500 cm^{-1} belonging to O-H stretching and the absorption peak of C-O stretching vibration at 1040 cm^{-1}. The additional bands of trisubstituted olefinic moiety were also observed at 1634 and 815 cm^{-1}.

The information gained from ^1H NMR spectrum (Fig 32) was in good agreement with that from IR spectrum *i.e.*, a significant signal at δ 5.12 ppm (1H, m) was attributed to an olefinic proton. Another signal at δ 3.18 ppm (H, t, J = 6.93 *Hz*) was consistent with the signal of a proton on a carbon bearing hydroxy group.

The molecular formular of this compound was established as $C_{30}H_{50}O$ by mass spectrum (Fig 33). It displayed the molecular ion peak at 426. The important fragmentation ions were observed at m/z 218, 207 and 189. This suggested that Compound 5 should be a member of α-amyrin or β-amyrin series. The possible mass fragmentation pattern of Compound 5 is illustrated in Scheme 3.4.
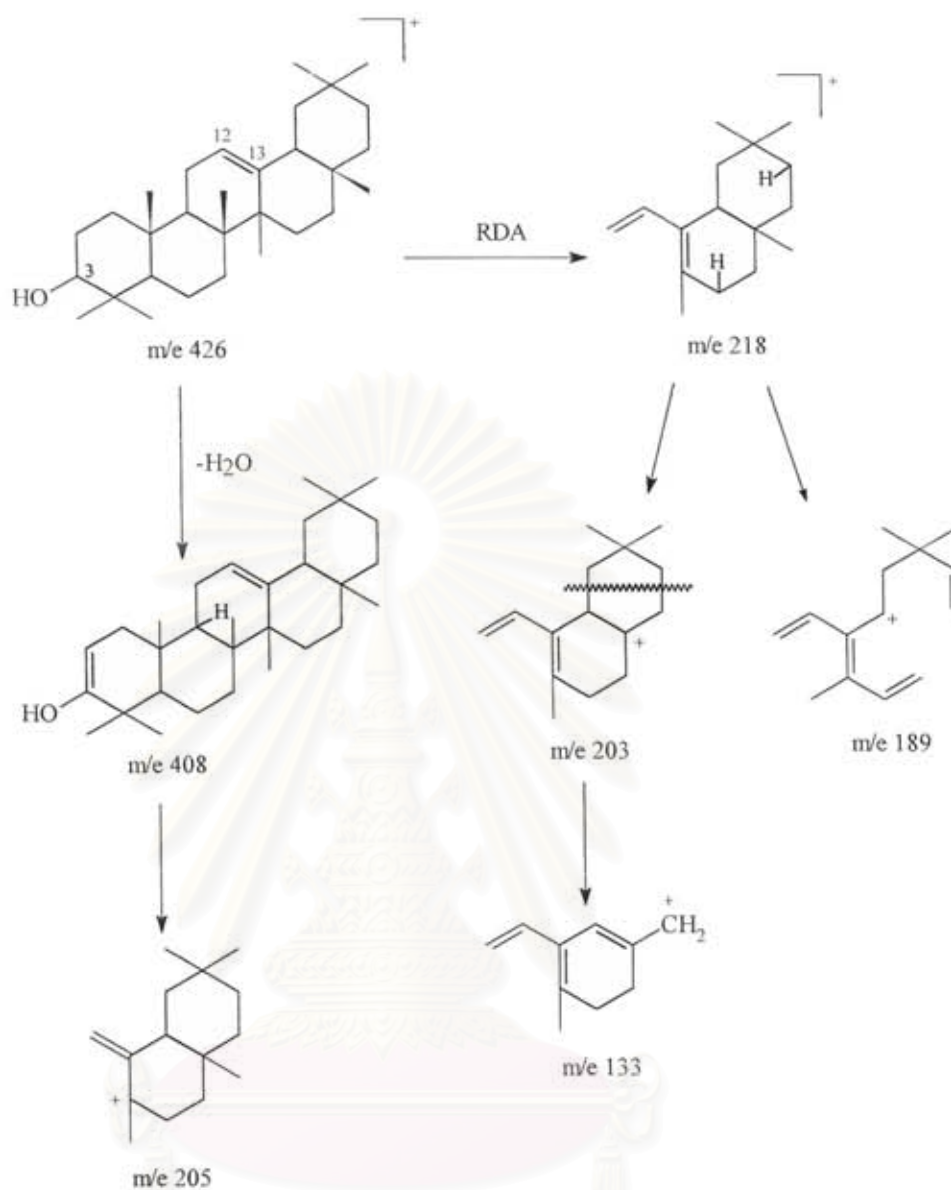
Scheme 3.4 The possible mass fragmentation pattern of Compound **5**

The ^{13}C NMR spectrum (Fig 34) displayed two olefinic carbons at δ 145.1 and 121.7 ppm. The signal at 79.0 ppm could be assigned for the carbon signal adjacent to oxygen atom. The other signals around 55.14 to 15.45 ppm were compatible with methyl, methylene, methine and quarternary carbons. The comparison ^{13}C NMR chemical shifts of Compound **5** and those of β-amyrin is summarized as in Table 3.33.

Table 3.33 The comparison of ^{13}C chemical shifts of β-amyrin with Compound **5**[35]

Carbon No.	Chemical shift (ppm)	
	β-amyrin	Compound **5**
1	38.5	38.6
2	27.0	26.9
3	78.9	79.0
4	38.7	38.8
5	55.1	55.2
6	18.3	18.4
7	32.6	32.7
8	39.7	39.8
9	47.6	47.6
10	37.0	36.9
11	23.4	23.5
12	121.7	121.7
13	145.0	145.1
14	41.7	41.7
15	28.3	28.4
16	26.2	26.2
17	32.5	32.5
18	47.2	47.2
19	46.8	16.8
20	31.1	31.1
21	34.8	34.7
22	37.2	37.4
23	28.1	28.1
24	15.5	15.5
25	15.5	15.6
26	16.8	16.8
27	26.0	26.0
28	27.3	27.4
29	33.2	33.3
30	23.6	23.7

Based on the ^{1}H NMR, ^{13}C NMR and MS spectral data together with their physical properties, it could be concluded that Compound **5** was β-amyrin. Its structure is shown below.
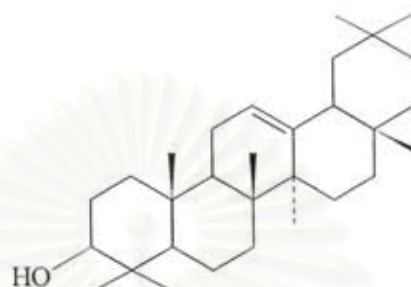
Compound 5: β-amyrin

3.7.2 Purification, properties and structural elucidation of Mixture 5

Mixture **5** was gained as white needle in pale yellow oil from the separation of Fractions VI and VII. The pale yellow oil was get rid of by washing with methanol and the remaining white needle was recrystallized with a mixture of hexane and chloroform for several times to yield bright white needle, m.p. 143-145°C, 75 mg (0.04% wt/wt of dicloromethane extract). TLC displayed only one spot with R_f value of 0.38 in dichloromethane solvent system. Mixture **5** was soluble in dichloromethane but slightly soluble in hexane and methanol. This substance gave a deep green colour with liebermann-Burchard's reagent which suggested the presence of steroidal moeity.

The IR spectrum (Fig 35) exhibited an absorption band belonging to a hydroxy group at 3400-3500 cm^{-1} and an absorption of disubstituted alkene at 969 cm^{-1}. The band at 1050 and 1020 cm^{-1} was corresponded to C-O stretching vibration and the band at 1650 cm^{-1} revealed the presence of C=C stretching vibration. The C-H stretching and bending vibrations of –CH$_3$- and –CH$_2$- were also observed at 2960-2840 cm^{-1} and 1460 cm^{-1}, respectively.

In addition to a colour test, IR spectrum and its physical properties, this substance was found to possess very close to those of Mixture **3** (a mixture of campesterol, stigmasterol and β-sitosterol) which was obtained previously from the stems. Thus, this mixture was analyzed by GLC method. When compared the analyzed gas chromatogram (Fig 36) of this mixture with that of a mixture of standard steroids (a mixture of campesterol, β-sitosterol and stigmasterol). It was found that Mixture **5** was infact a mixture of stigmasterol and β-sitosterol. The composition of this mixture is reported in Table 3.34.

Table 3.34 The composition of steroids in Mixture **5**

Standard Steroid	Retention time of standard steroid (min)	Retention time of Mixture (min)	Composition (%)
campesterol	-	-	-
stigmersterol	18.61	18.65	44.72
β-sitosterol	21.17	21.18	55.28

3.7.3 Purification, properties and structural elucidation of Compound 6

Compound **6** was obtained as white precipitate in yellow oil from Fraction VI. After removing the oil by washing with methanol, the remaining white precipitate was recrystallized with a mixture of chloroform and methanol yielding white needle, m.p. 214-216°C, 40 mg (0.02% wt/wt of dichloromethane extract). Compound **6** gave a purple colour with Liebermann-Burchard's reagent which was indicative of a triterpenoid skeleton. This compound was soluble in hexane and dichloromethane but slightly soluble in ethyl acteate and methanol.

The IR spectrum (Fig 37) displayed the absorption band due to O-H stretching vibration at 3400-3500 cm^{-1}. The additional band of vinylidine group (CH$_3$-C=CH$_2$) at 3070, 1645 and 889 cm^{-1} together with gem dimethyl group at 1385 and 1180 cm^{-1} were also observed.

The ^{1}H NMR spectrum (Fig 38) exhibited the important signal at δ 4.65 and 4.55 ppm (2H each) which compattible with a terminal methylene proton ($CH_2=CH-$). The multiplet signal of methine proton on a carbon attached to an oxygen atom with one proton integration was detected at δ 3.15 ppm. Other signals in the region of 0.60-1.70 were attributed to methyl, methylene and methine protons.

The ^{13}C NMR spectrum (Fig 39) displayed a total of 30 signals of carbons. It showed the olefinic carbon signals at δ 158.0 and 116.7 ppm and the signal at δ 79.0 ppm was corresponded to the carbon bearing hydroxy group. The signals of methyl, methylene, methine and quarternary carbons were also evidence at δ around 55.37 to 14.57 ppm. The ^{13}C NMR chemical shifts of Compound **5** were found to be very close to those of the known triterpenoidal compound namely lupeol. The ^{13}C NMR chemical shifts assignment of Compound **6** is shown in Table 3.35.

Table 3.35 The ^{13}C NMR chemical assignment of Compound **6**[35]

Carbon	Chemical shift (ppm)	
No.	Lupeol	Compound 6
1	38.8	38.8
2	27.5	27.5
3	79.0	79.0
4	38.9	38.9
5	55.4	55.3
6	18.4	18.4
7	34.4	34.3
8	40.9	40.9
9	50.5	50.4
10	37.2	37.2
11	21.0	21.0
12	25.2	25.2
13	38.1	38.1
14	42.9	42.8
15	27.5	27.5
16	35.7	35.6
17	43.1	43.0
18	48.4	43.3
19	48.0	48.0
20	150.9	150.9
21	29.9	29.9
22	40.0	40.0
23	28.0	27.9
24	15.4	15.4
25	16.1	16.1
26	16.0	16.0
27	14.6	14.6
28	18.0	17.9
29	109.3	109.3
30	19.3	19.3

The information obtained from mass spectrum (Fig 40) gave good agreement with those from IR, ^{1}H NMR and ^{13}C NMR spectra. It gave the expected molecular ion at m/z 426 which was corresponded to the molecular formular $C_{30}H_{50}O$ together with the series of fragmentation at m/z 383, 207, 189 and 95 which suggested that this compound should be a member of lupane type triterpenoid compound. The possible mass fragmentation pattern of Compound 6 is presented in Scheme 3.5.
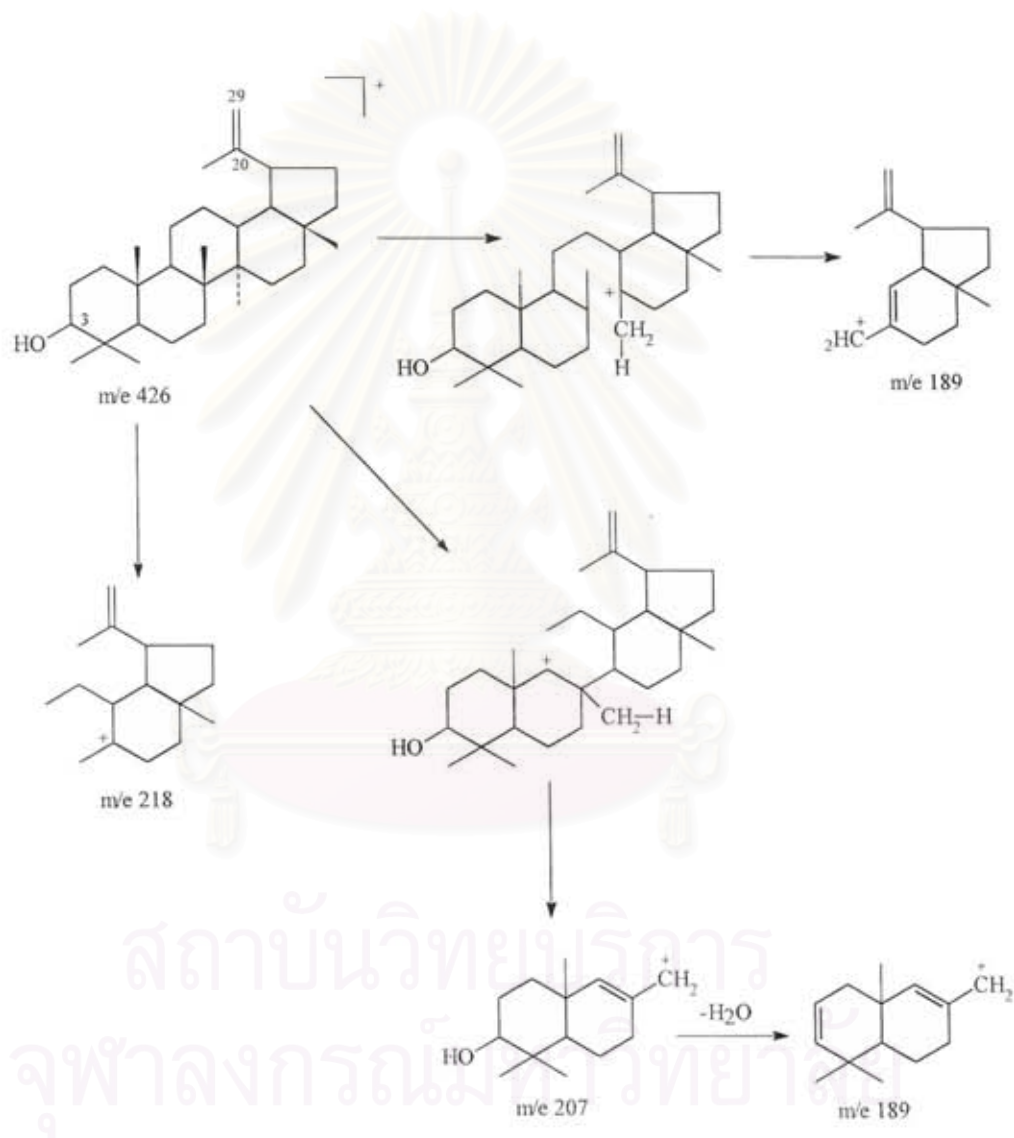
Scheme 3.5 The possible mass fragmentation pattern of Compound 6

According to the above spectroscopic data, it was obviously to be concluded that Compound **6** was lupeol and the structure is shown below.
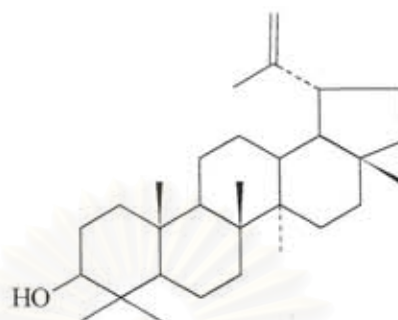
Compound 6, lupeol

3.7.4 Purification, properties and structural elucidation of Compound 7

Compound 7 was isolated from the silica gel column chromatography of Fractions VIIE and VIIF by elution with 10% ethyl acetate in hexane. After recrystallization with a mixture of chloroform and methanol, white crystal product 46 mg (0.03% wt/wt of ethyl acetate extract), m.p. 170-172°C and R_f value of 0.77 (20% EtOAc-MeOH) was obtained . This compound was easily soluble in ethyl acetate and dimethyl sulfoxide, but slightly soluble in dichloromethane. It gave nagative results to the Liebermann-Burchard's and cyanidin reagents.

The IR spectrum (Fig 41) exhibited broad bands due to O-H stretching vibration of carboxylic acid at 3500-3400 cm^{-1} and a conjugated carbonyl group at 1685 cm^{-1}. The absorption peaks belonging to C-H stretching vibration of aromatic moiety were detected at 1659 and 1512 cm^{-1}. The additional absorption band at 828 cm^{-1} indicated the C-H out of plane deformation of benzene ring (*p*-substitution, 2H adjacent).

The ^{1}H NMR spectrum (Fig 42) showed the doublet signal at δ 6.38 and 7.55 ppm (1H each, $J = 16.18$ *Hz*) which was compatible with *trans* olefinic proton. A pair of 2H doublet signal centered at 7.55 and 7.63 ppm ($J = 8.85$ *Hz*) were attributed to the two *ortho* and the two *meta* hydrogens, respectively. The sharp singlet with the integration equal to three protons detected at 3.80 ppm could be assigned for methoxy protons.

The ^{13}C NMR spectrum (Fig 43), DEPT 90 and DEPT 135 spectra (Fig 44) displayed signals of eight carbon signals which could be assigned as follows: the carbonyl carbon at 167.8 ppm, four methine aromatic carbons at 114.3 and 129.9 (2C each). The signal at 114.3 and 129.9 ppm could be fixed for olefinic carbons. The signal at 55.3 ppm consistent with methoxy carbons and two additional quarternary carbons were observed at 126.8 and 160.9 ppm.

From the above spectroscopic information, it could be deduced that this compound would be *trans*-cinnamic acid derivative bearing one methoxy group. Considering the ^{1}H NMR spectrum, the splitting pattern and its coupling constant revealed an *para* disubstituted benzene ring. There were two alternatives to place the methoxy group in this structure, one was the methoxy group on benzene ring and the other was methoxy group as methyl ester. The two possible structures are illustrated below.
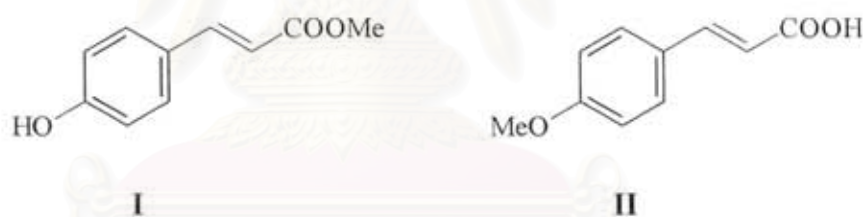
The best information that could be used for distinguishing these two structures was the information obtained from IR spectrum. Its IR spectrum exhibited the broad peak belonging O-H stretching vibration of carboxylic group at 3500-3150 cm^{-1}. This observation suggested that Compoud 7 be corresponded to structure II rather than structure I. This was because the absorption band due to O-H stretching vibration of phenolic group appeared as a sharp peak In addition, the mass fragmentation pattern was also supported to structure II. The mass spectrum (Fig 45) exhibited the molecular ion peak at m/z 178 and the prominent ion peak at m/z 161 and 133 which were formed by loss of OH and CO$_2$H, respectively.[39] The possible mass fragmentation of this compound is shown in Scheme 3.7.

MeO—⟨C₆H₄⟩—CH=CH-C(=O⁺)-OH

m/z 178

MeO—⟨C₆H₄⟩—CH=CH-C≡O⁺

m/z 161

MeO—⟨C₆H₄⟩—CH=CH⁺

m/z 133
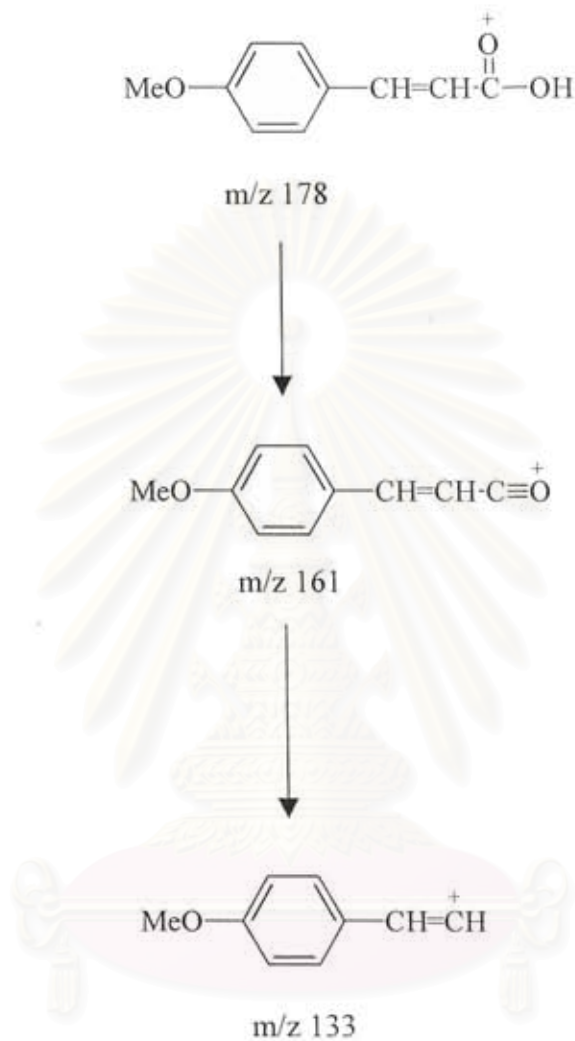
Scheme 3.6 The possible mass fragmentation pattern of Compound 7

According to the mass fragmentation pattern, the structure (**I**) was found to be corresponded to Compound 7 rather than structure (**II**). In addition, the physical properties and spectral data of Compound 7 are identical with the synthetic *trans-p-*methoxycinnamic acid. The ^{1}H and ^{13}C NMR chemical shift assignments of this compound is illustrated in Table 3.36.

Table 3.36 The ^{1}H and ^{13}C NMR chemical shift assignment of Compound 7

Position	Chemical shift (ppm)	
	δ_H	δ_C
1	-	126.8
2	6.97(d, J = 6.72 Hz)	114.3
3	7.63 (d, J = 6.72 Hz)	130.0
4	-	160.1
5	6.97(d, J = 6.72 Hz)	130.0
6	7.63 (d, J = 6.72 Hz)	114.3
7	7.55 (d, J = 16.18 Hz)	143.7
8	6.38 (d, J = 16.17 Hz)	116.5
9	-	167.8
OMe	3.80 (3H, s)	55.3

Supported by spectroscopic data and the comparison with the synthetic sample, Compound 7 was no doubt to be concluded to be *trans-p*-methoxycinnamic acid. The structure of this compound is shown below.
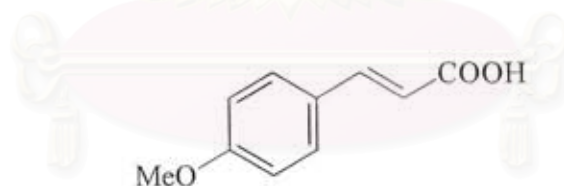
Compound 7: *Trans-p*-methoxycinnamic acid

3.8.5 Purification, properties and structural elucidation of Compound 8

Compound **8**, as bright yellow needle with m.p. 200-202°C, 120 mg (0.08% wt/wt of crude extract) was isolated from the separation of ethyl acetate extract of leaves. Compound **8** showed a single spot on TLC with R_f value of 0.76 (silica gel, ethyl acetate). This compound gave negative results to Liebermann-Burchard's and Br_2 in CCl_4 reagents, but showed a positive test to 2,4-DNP reagent.

The IR spectrum (Fig 46) exhibited a strong absorption peak at 1600 cm^{-1} which suggested the presence of internally hydrogen-bonded carbonyl moiety. The characteristic absorption peak due to the O-H stretching vibration of hydroxy group and aromatic moiety were observed at 3500 and 1600 cm^{-1}, respectively.

The ^{1}H NMR spectrum (Fig 47) of Compound **8** displayed a signal of low field aldehyde proton at δ 11.6 ppm (1H, s), a chelated hydroxy proton appeared at 14.80 ppm (1H, s), and an isolated aromatic proton signal was observed at δ 7.75 ppm. Two singlet signals were detected at δ 6.29 and 6.32 ppm (1H each) which may be assigned as protons of hydroxy group. The spectrum also showed the aromatic methyl protons at δ 2.14 ppm and further exhibited the presence of isopropyl group with six-proton integration as a doublet signal at δ 1.52 ppm (J = 7.63 *Hz*). The methine proton of this moiety was detected at δ 3.86 ppm. (1H, m)

The ^{13}C NMR spectrum (Fig 48) showed a total of fourteen carbon atoms. From DEPT 90 and DEPT 135 spectra (Fig 49) suggested the presence of aldehyde carbonyl at δ 199.3 ppm, two methine carbons at δ 118.1 and 27.9 ppm, nine quarternary carbons at δ 156.0, 150.5, 143.4, 134.1, 133.7, 129.7, 115.9, 114.7 and 111.8 ppm and three methyl carbons at δ 20.3 (2C) and 20.2 ppm.

The above spectroscopic data and the information obtained from colour test implied that this compound was not an unsaturated steroid, triterpenoid or flavonoid compound, but this compound should be a phenolic compound containing aldehyde functional group.

The overall ^{1}H and ^{13}C NMR spectra are generally in good agreement with that of gossypol, a sesquiterpene aldehyde, which widely distributed in Malvaceae family and previously isolated from the roots of *H. tiliaceus*.[11]

Moreover, the structure of Compound **8** was confirmed by comparison its ^{1}H NMR spectrum with the literature data of gossypol.[40] The results are recorded in Table 3.37.

Table 3.37 The comparison of the ^{1}H NMR chemical shifts of Compound **8** and gossypol

Position	Chemical shift (ppm)	
	Compound **8**	Gossypol
4-ArH	7.74 (1H, s)	7.45 (1H, s)
-CHO	11.06 (1H, s)	11.11 (1H, s)
3-CH$_3$	2.14 (3H, s)	2.39 (3H, s)
(CH$_3$)$_2$CH	3.86 (1H, m)	3.81 (1H, m)
(**CH$_3$**)$_2$CH	1.52 (6H ,d, $J = 7.0$ Hz)	1.48 (6H, d, $J = 7.0$ Hz)
1-OH	14.80 (1H, s)	14.75 (1H, s)
6-OH	6.30 (1H, s)	6.00 (broad)
7-OH	6.32 (1H, s)	6.00 (broad)

The EI mass spectrum (Fig 50) was also supported the structure of Compound **8**, it exhibited the expected molecular ion peak at m/z 518 (5%). Other significant fragmentation ions which formed by loss of 1 and 2 mol of H$_2$O were detected at m/z 500 (85%) and 482 (100%). These fragmentations suggested the presence of an aldehyde groups at C-1and hydroxy group at C-2 and C-8. Furthermore, the spectrum displayed the ion peaks due to elimination of one and two moles of H$_2$O and a methyl group at m/z 485 (12%) [M$^+$-H$_2$O-Me] and 467 (93%) [M$^+$-2H$_2$O-Me]. The possible mass fragmentation pattern of Compound **8** is illustrated in Scheme 3.8.[40]
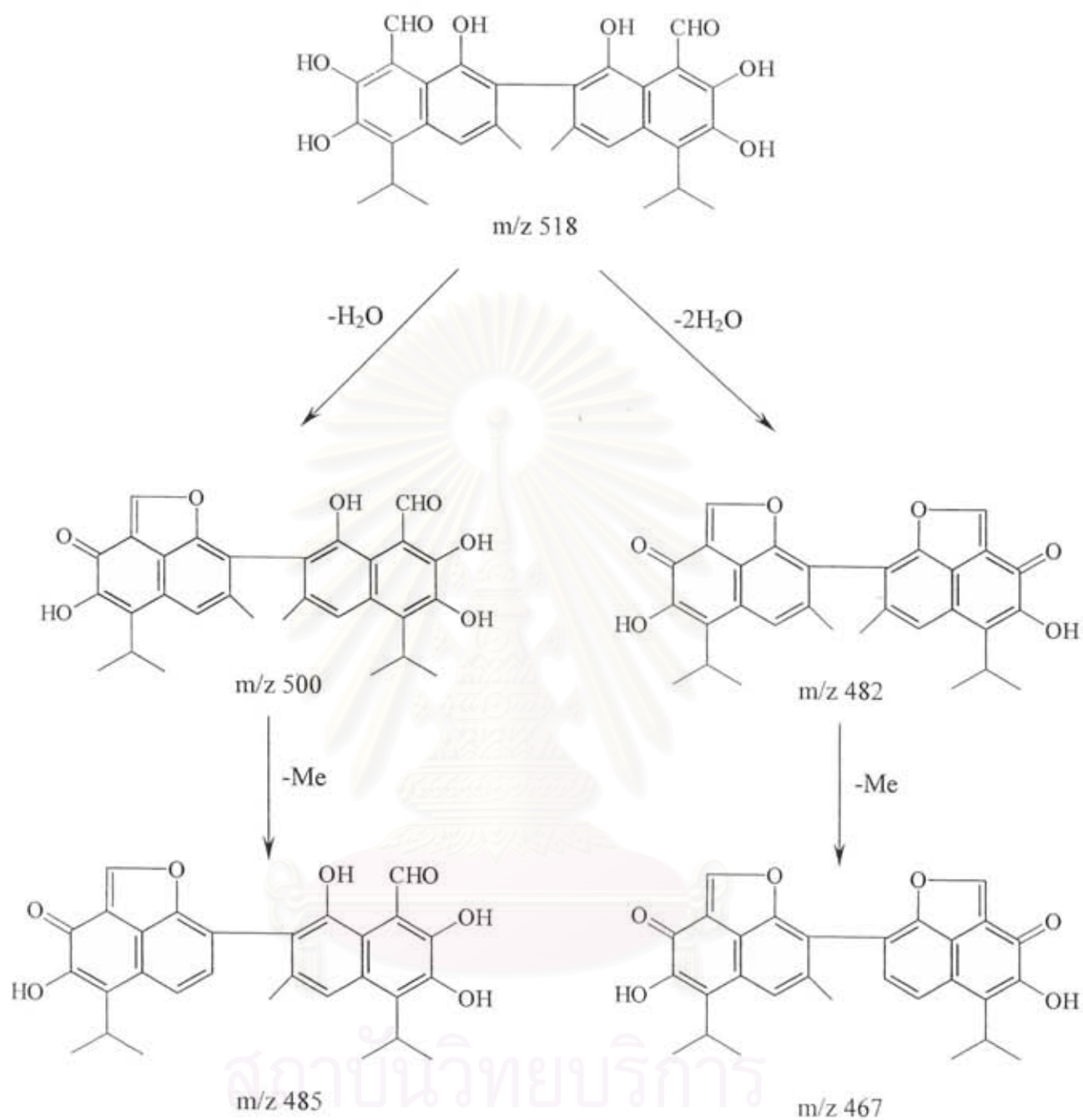
Scheme 3.7 Possible mass fragmentation pattern of Compound **8**

All above spectroscopic information including colour test, it was clear to conclude that Compound **8** was gossypol.[40] The structure is shown below.
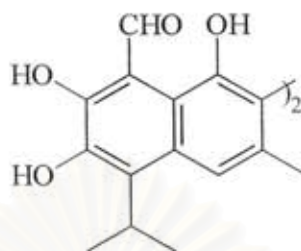
Compound 8: gossypol

3.7.6 Purification, properties and structural elucidation of Compound 9

White solid and greenish oil was obtained from the combination of Fraction Nos. 21-24 of Fraction VIIF. After monitoring by TLC, Fraction **a** was found to contain a mixture of Compounds **8** and **9** (a minor constituent of this fraction). Hence, it was decided to separate by rechromatography over silica gel using 60% ethyl acetate in hexane as eluent to yield Compound **9** as white needle-like crystal with melting point 208-210° C, 45 mg (0.024 % wt/wt of dichloromethane extract), R_f 0.82 (silica gel, 40% MeOH:CH$_2$Cl$_2$). This compound was soluble in ethyl acetate and methanol, but slightly soluble in dichloromethane. It gave negative results to Liebermann-Burchard's, 2,4-DNP and cyanidin reagents.

The IR spectrum (Fig 51) of Compound **9** exhibited a broad absorption band due to O-H stretching vibration of carboxylic acid at 3400-3500 cm^{-1}, C=O stretching vibration of conjugated carbonyl moiety at 1651 cm^{-1} and C=C stretching vibration of aromatic functional group at 1615 and 1542 cm^{-1}.

The ^{1}H NMR spectrum (Fig 52) displayed the doublet signal at δ 6.85 ppm with 1H integration which could be assigned for an aromatic proton. Another aromatic signal was observed at δ 7.48 ppm (2H) and the three-proton singlet signal at δ 3.85 ppm was campatible to a methoxy group.

The ^{13}C NMR spectrum (Fig 53) exhibited a total of 8 signals. The DEPT 90 and DEPT 135 spectra (Fig 54) displayed one methoxy carbon at δ 55.6 ppm, three methine carbons at δ 112.2, 115.1 and 123.6 ppm, four quarternary carbons at δ 121.7, 147.3, 151.5 and 167.3 ppm, respectively. The last signal was corresponded to the carbonyl carbon of carboxylic acid.

According to the above spectroscopic evidence, it can be proposed that this compound would be benzoic acid derivative, vanillic acid. The physical properties and spectral data are additionally identical with those of authentic sample. Its structure is shown below.
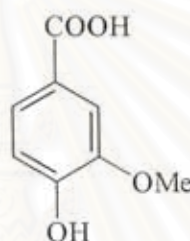
Compound 9: vanillic acid

3.8 The results of biological activities test of isolated compounds

All of isolated organic substances from both the stems and the leaves of from *H. tiliaceus* were evaluated for biological activity using brine shrimp cytotoxicity test and antioxidative activity as bioassay. The results of biological activity study of these compounds are displayed in Table 3.38.

Table 3.38 The results of brine shrimp cytotoxicity test and antioxidative test of
isolated substances

substances	Brine shrimp cytotoxicity test		antioxidative test	
	LC$_{50}$ (ppm)	Activity	DPPH	β-carotene
Mixture 1	15430.25	no activity	-	-
Mixture 2	3005.42	no activity	-	-
Mixture 3	970.63	no activity	-	-
Mixture 4	387.74	low activity	-	-
Compound 1	8.52	high activity	+	-
Compound 2	not tested	not tested	not tested	not tested
Compound 3	0.001	high activity	+	-
Compound 4	0.08	high activity	+	-
Compound 5	21992.65	no activity	-	-
Compound 6	181.16	low activity	-	-
Compound 7	9.96	high activity	+	-
Compound 8	2.42	high activity	+	-
Compound 9	22.68	medium activity	-	-

From the above results, a mixture of long chain esters (Mixture **1**), a mixture
of friedelin and friedelan-3β-ol (Mixture **2**), a mixture of long chain carboxylic acids
(Mixture **3**) and a mixture of steroids (Mixture **4**) did not show significant cytotoxic
activity against brine shrimp and also did not exhibit antioxidative activity. The two
flavonoids compounds: 5-hydroxy-3,7-dimethoxyflavone (Compound **3**) and 5-
hydroxy-3,3',4',5',7-pentamethoxyflavone (Compound **4**) displayed the highest
cytotoxicity activity. 2,5-Dimethoxy-1,4-benzoquinone (Compound **1**), *p*-
methoxycinnamic acid (Compound **7**), gossypol (Compound **8**) and vanillic acid
(Compound **9**) showed the significant cytotoxic activity on brine shrimp lethality test.
In addition, these compounds gave positive results to antioxidant test.

3.9 Biological activites of isolated substances from literature survey

Mixture **1** was isolated from both hexane and dichloromethnae extracts of stems of *H. tiliaceus* and was characterized as a mixture of long chain ester. This substance is widely distributed in the lipophilic fraction of various plants. Bioassay results indicated that this compound did not exhibit cytotoxicity against brine shrimp and also gave the negative result to the antioxidative test.

Mixture **2**, a mixture of friedelin and friedelan-3β-ol was separated from the hexane and dichloromethane extract of both stems and leaves. This substance occurrs in a number of plants used in traditional herbal medicine and have been utilized as anti-inflamatory and anticonvulsant agent.[41] Nevertheless, they did not show the significant cytotoxicity activity test against brine shrimp and had no effect on anti oxidant assay.

Mixture **3** was isolated from the stems and was elucidated its structure as a mixture of long chain carboxylic acids. The bioassay results of this compound did not show significant biological activity tests to both brine shrimp cytotoxicity and antioxidative activity test. From the literature search, a mixture of long chain carboxylic acids with carbon number 21, 22, 24,25, 27, 28, 30, 31, 33, 34 and 36 exhibited high feeding inhibition against insect boll weevil.[35]

Mixture **4** was isolated from the stems and was identified as a mixture campesterol β-sitosterol and stigmasterol. They did not exhibit significant brine shrimp cytotoxic lethality assay and gave negative results to antioxidant activity test.

Compound **1** was isolated from the dichloromethane extract of stems and its structure was established as 2,5-dimethoxy-1,4-benzoquinone. From the literature survey, it was found that this compound was isolated only from higher fungi (polyporus fumosus) but the isolation from plants has not previously reported.[42] The bioassay results revealed that Compound **1** showed the high activity on brine shrimp cytotoxic lethality assay and also gave a positive result to the antioxidative test.

Compoumd **2** was separated from dichloromethane extract of stems and was characterized as 5-hydroxy-7,8-dimethoxycoumarin. Compound **2** was first reported to be a constituent of the arial part of *Artemisia lanciniata* (Asteraceae)[43] and there is

no reported on this compound furthermore. Unfortunately, this compound was obtained in so such small amount that the biological activity could not be evaluated.

Compound **3** was isolated from the ethyl acetate extract of stems and was characterized as 5-hydroxy-3,7-dimethoxyflavone. This compound is a rare methoxyflavone which was first isolated from *Cheilanthes kaulfussi* (Polypodaceae).[45] Compound **3** exhibited high cytotoxicity against brine shrimp and showed positive results to free radical scavenging activity.

Compound **4** was separated from the ethyl acetate extract of stems. It structure was established as 5-hydroxy-3,3',4',5',7-pentamethoxyflavone. From the literature survey, Compound **4** has been isolated from the bud of *Betula nigra* (Betulaceae) in 1975 by E. Wollenweber.[44] Recently, this compound has been isolated from the leaves of *Bosisto floydii* (Rutaceae)[45]; however, the biological activity has not been carried out and reported. In our bioassay experiments, Compound **4** displayed the significant cytotoxicity activity against brine shrimp and also gave positive results to antioxidative test.

Compound **5** was separated from dichloromethane extract of leaves and was identified its structure as β-amyrin. This compound is a member of an oleanane triterpene which occurrs in several plants, especially mangrove plants. From the bioassay results, Compound **5** did not display the significant biological activity on both brine shrimp cytotoxicity and antioxidant activity tests.

Compound **6** was isolated from dichloromethane extract and was identified its structure as lupeol. Lupeol is a lupane-type pentacyclic triterpene which is widely distributed in nature and has been shown to exhibit a variety of biological activities for instance, anti-inflamatory activity, cytotoxic activity against Hep-GZ, A-431, and H-411E tumor cell lines.[46] The biological activity test indicated that Compound **6** exhibited low cytotoxicity activity against brine shrimp and did not show the antioxidant activity.

Compound **7** was isolated from the ethyl acetate extract and its structure was characterized as *p*-methoxycinnamic acid. From the chemical literatures, the biological activities of this compound has not been reported. *In vitro* cytotoxicity, against brine shrimp, exhibited the promising results and gave the positive test to antioxidant activity.

Compound **8** was separated from the ethyl acetate extract of leaves and was identified as gossypol. This compound is a highly-oxidized sesquiterpenoid with the cadinene skeleton which occurrs in wild species of *Gossypium* and other Malvaceae genera. Therefore, its occurrence in *Hibiscus tiliaceus* is not surprising. The naturally occurring compound, gossypol, was found to possess an insecticidal activity.[47]

Compound **9** was isolated from the leaves and was identified as vanillic acid. Generally, benzoic acid derivatives possessed an antimicrobial activity and plant growth inhibition. The results of biological activity test showed the medium activity on brine shrimp lethality assay and also exhibited the positive result to antioxidant activity test.

3.10 Structure activity relationship of cinnamic acid derivatives and benzoic acid derivatives.

3.10.1 Structure activity relationship of cinnamic acid derivatives

Compound 7, *p*-methoxycinnamic acid was isolated from the leaves of *H. tiliaceus* and exhibited high acitvity on brine shrimp cytotoxicity test. Since the structure of this compound was not complicated and the structure activity relationship (SAR) study of these compounds has not been reported, a series of nine related *p*-methoxycinnamic acid was collected and performed brine shrimp cytotoxicity test. This is therefore the first report to demonstrate the structure activity relationship of cinnamic acids with the brine shrimp lethality test. The LC_{50} of those mentioned compounds are reported as shown in Table 3.39.
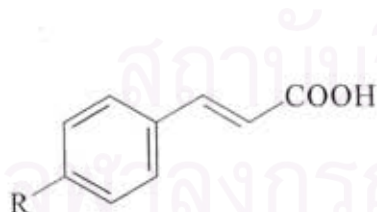
C1 :	R = H
C2 :	R = OH
C3 :	R = OMe
C4 :	R = $O(CH_2)_3CH_3$
C5:	R = $O(CH_2)_5CH_3$
C6:	R = $O(CH_2)_7CH_3$
C7:	R = $O(CH_2)_{11}CH_3$
C8:	R = CH_3
C9:	R = CF_3
C10:	R = $C(CH_3)_3$

Table 3.39 The results of brine shrimp cytotoxicity test of cinnamic acid derivatives

Compound	R	LC_{50} (ppm)	activity
C1	H	140.66	low activity
C2	OH	25.21	medium activity
C3	OMe	9.96	high activity
C4	butuyloxy	23.55	medium activity
C5	hexyloxy	0.035	high activity
C6	octyloxy	0.035	high activity
C7	dodecyloxy	421.99	low activity
C8	Me	34.71	medium activity
C9	CF_3	40.92	medium activity
C10	t-Bu	163.14	low activity

Table 3.39 shows the *in vitro* cytotoxicity activity against brine shrimp of cinnamic acid derivatives with substituent located at C-4 position. Using cinnamic acid (**C1**) as a reference compound, it can be seen that the substituent located at *para* position to a carboxylic group had a significant effect on cytotoxic activity against brine shrimp. To illustrated this, *p*-coumaric acid (*p*-hydroxycinnamic acid) (**C2**) displayed higher cytotoxicity than those of cinnamic acids.

Among selected cinnamic acids, it could be classified into two main groups according to their substituent attached to C-4 position. The first group was compound in the series of cinnamic acids bearing alkyloxy group located at C-4 position and the other was those in the series of cinnamic acids containing alkyl group located at C-4 position.

Considering the first series of compounds (**C3-C7**), the results of brine shrimp cytotoxic lethality test implied that the length of side chain in alkoxy group was greatly affected the activity. The most appropriate ones were observed when **R** group was hexyl and octyl groups. The longer chain length such as in Compound **C7** or the shorter chain length as in Compound **C4** revealed less activity.

In the series of cinnamic acid derivatives with the variation of alkyl group located at C-4 position (Compounds **C8**, **C9** and **C10**), all compounds displayed less potent activity than *p*-methoxycinnamic acid. It was also important to note that the cytotoxicity was decreased by large substituent.

From the above result, it could obviously be concluded that a number of carbon atom on side chain of *p*-alkoxycinnamic acid had a great effect on brine shrimp lethality activity. The highest activity was observed when the carbon number of alkoxy chain at C4-position was six or eight.

3.10.2 Structure activity relationship of benzoic acid acid derivatives

From the brine shrimp lethality assay, vanillic acid (Compound **9**) possessed medium activity. To compare the structure activity relationship of this compound and its analogues, eight commercially available benzoic acid derivatives were chosen for SAR study against brine shrimp. The results are shown in Table 3.40.

	R^1	R^2	R^3	R^4	R^5
B1	COOH	OH	H	OH	H
B2	COOH	H	OH	OH	H
B3	COOH	H	OH	H	OH
B4	COOH	H	OMe	OH	H
B5	COOH	H	OMe	OMe	H
B6	COOH	H	OH	OH	OH
B7	COOH	H	H	OH	H
B8	OH	H	OH	H	OH
B9	CHO	H	OMe	OH	H
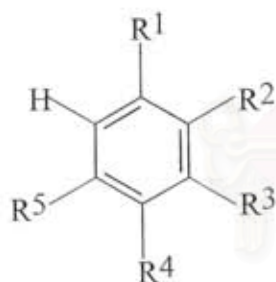
Table 3.40 The results of brine shrimp cytotoxicity test of vanillic acid and related compounds

Cpd	R					LC_{50}	activity
	R^1	R^2	R^3	R^4	R^5	(ppm)	
B1	COOH	OH	H	OH	H	10.02	medium
B2	COOH	H	OH	OH	H	15.50	medium
B3	COOH	H	OH	H	OH	0.10	high
B4	COOH	H	OMe	OH	H	29.20	medium
B5	COOH	H	OMe	OMe	H	43.35	medium
B6	COOH	H	OH	OH	OH	22.45	medium
B7	COOH	H	H	OH	H	12.97	medium
B8	OH	H	OH	H	OH	26.41	medium
B9	CHO	H	OMe	OH	H	103.42	low

Comparing the results of brine shrimp lethality test of vanillic acid (Compounds **B1-B7**) with those of 3,4-dimethoxybenzoic acid (**B5**) and 3,4-dihydroxybenzoic acid (**B2**), the latter compound displayed the higer activity than those of vanillic acid and 3,4-dimethoxybenzoic acid.

This result suggested that the methoxy group on benzene ring has a significant effect on cytotoxic activity of benzoic acid. The cytotoxicity is greatly reduced by a methoxy group. This same trend was also observed in 2,4-dihydroxybenzoic acid (**B1**) and 3,5-dimethoxybenzoic acid (**B3**). The LC_{50} of these two compounds were almost the same.

The number of hydroxy groups also had a significant effect on the cytotoxicity. To illustrate this, the LC_{50} of gallic acid (**B6**) and p-hydroxybenzoic acid (**B7**) were compared. From the above results, *p*-hydroxybenzoic acid (**B7**) displayed more potent activity than gallic acid (**B6**) approximately twice. Thus, it disclosed that the cytotoxicity of benzoic acid was perhaps decreased by increasing the number of hydroxy group on benzene ring.

The above trend pointed toward that dihydroxy benzoic acid showed more cytotoxic than those of trihydroxy benzoic acid and monohydroxy benzoic acid. Further SAR study on the effect of substitutent position was carried out by comparison of the LC_{50} of 2,4-dihydroxybenzoic acid (**B1**), 3,4-dihydroxybenzoic acid (**B2**) and 3,5-dihydroxybenzoic acid (**B3**).

The results manifestly showed that Compound **B1** with hydroxy groups located at *ortho* and *para* positions to carboxylic group showed the LC_{50} value almost the same as that for Compound **B2** where hydroxy group located at *meta* and *para* positions to carboxylic group. This observation implied that higher cytotoxic activity was observed when both hydroxy groups were located at *meta* positions to carboxylic group.

CHAPTER 4
CONCLUSION

In our search for biologically active constituents from Thai mangrove plants, the methanolic extract of stems and leaves of *Hibiscus tiliaceus* Linn. exhibited the significant cytotoxic activity against brine shrimp (*Artemia salina* Linn.) with LC_{50} values of 1.25 and 1.00 ppm, respectively. Thus, these two parts were selected for investigated the chemical constituents and their biological activity. Eight substances were isolated from the stems using traditional chromatographic techniques. By means of physical properties and various spectroscopic methods including 2D-NMR techniques, their structures were established as: a mixture of long chain aliphatic esters, a mixture of friedelin and friedelan-3β-ol, a mixture of long chain carboxylic acids, a mixture of campesterol, β-sitosterol and stigmasterol, 2,5-dimethoxy-1,4-benzoquinone, 5-hydroxy-7,8-dimethoxycoumarin, 5-hydroxy-3,7-dimethoxyflavone and 5-hydroxy-3,3',4',5',7-pentamethoxyflavone. In addition, chemical examination of the leaves led to the isolation of six substances and their structures were characterized as a mixtue of fridelin and friedelan-3β-ol, β-amyrin, a mixture of β-sitosterol and stigmasterol, lupeol, *p*-methoxycinnamic acid, gossypol and vanillic acid. From the bioassay results, 2,5-dimethoxy-1,4-benzoquinone, 5-hydroxy-7,8-dimethoxycoumarin, 5-hydroxy-3,7-dimethoxyflavone, 5-hydroxy-3,3',4',5',7-pentamethoxyflavone, *p*-methoxycinnamic acid and gossypol displayed high activity on brine shrimp cytotoxic lethality test and gave a positive result to the antioxidative test.

All of these isolated substances have not been reported to be the constituents of leaves and stems of this plant. This investigation can be summarized as shown in Table 4.1. The structures of isolated substances are illustrated in Fig 4.1.

$CH_3(CH_2)_nCOOR$

Mixture 1

Mixture 2

(Mix of R = OH, R′ = H and R, R′ = O)

$CH_3(CH_2)_nCOOH$

Mixture 3

Mixture 4

(Mix of R = $C_{20}H_{23}$, $C_{10}H_{21}$ and C_9H_{21})

Mixture 5

(Mix of R = $C_{10}H_{23}$ and C_9H_{21})

Compound 1

Compound 2

Compound 3

Compound 4

Compound 5

Compound 6

Compound 7

Compound 8

Compound 9

Fig 4.1 Isolated organic substances from the stems and leaves of *H. tiliaceus*

In addition, it was found that by employing bioassay guided methodology, five active compounds, 2,5-dimethoxy-1,4-benzoquinone (Compound **1**), 5-hydroxy-3,7-dimethoxyflavone (Compound **3**), 5-hydroxy-3,3',4',5',7-pentamethoxyflavone (Compound **4**), *p*-methoxycinnamic acid (Compound **7**) and gossypol (Compound **8**) could be isolated from both parts of this plant. The study of SAR also provided informative results to find out that some derivatives of isolated compounds showed more potent activity than the original compounds. For instance, *p*-hexyloxy and *p*-octyloxycinnamic acids (Compounds **C5** and **C6**) exhibited more potent activity than a parent compound (*p*-methoxycinnamic acid, Compound **7**).

Proposal for Future Work

It could be clearly seen that various biologically active compounds could be isolated from the active fractions of both stems and leaves of *H. tiliaceus*. Therefore, the bioassay directed fractionation is quite vital for disclosing biologically active compounds. Eventhough, the use of brine shrimp cytotoxic lethality assay is the first preliminary indication among well known bioassays, its results firmly were reliable and showed the good tendency towards studying on more sophisicated bioassays such as anticancer, antitumor in pharmaceutical aspects or insecticidal activity in agricultural aspects. Therefore, further studies on those mentioned bioassays should be conducted.

In aspect of structure and activity relationship study, this research obviously reinforces the necessity for this kind of work as the complementary part for natural product chemists to think over. The gained results, for example from this work provided better opportunity to disclose better biologically active compounds such as those belonging to cinnamic acid and benzoic acid derivatives.

Moreover, the chemical constituents and biological activity study of other parts of *H. tiliaceus* should be investigated. The results from that study should reveal the simililarity or difference and might lead to understanding of biosynthesis pathway of some major components.

Last but confidently not least, the results obtained from this work supported a promising concept of the fully utilization and biologically active compound searching from ideal and potential natural resources of Thai mangrove plants.

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APPENDICES
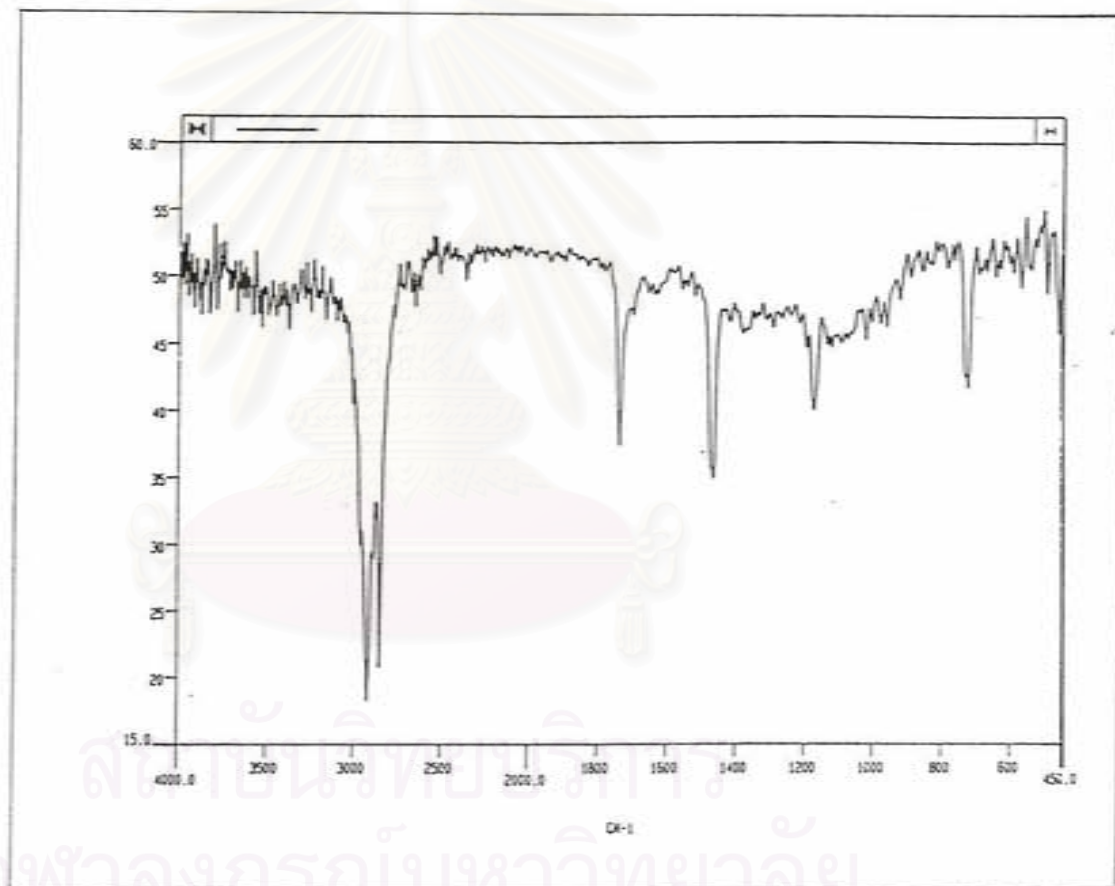
Fig 1 The IR spectrum of Mixture **1**
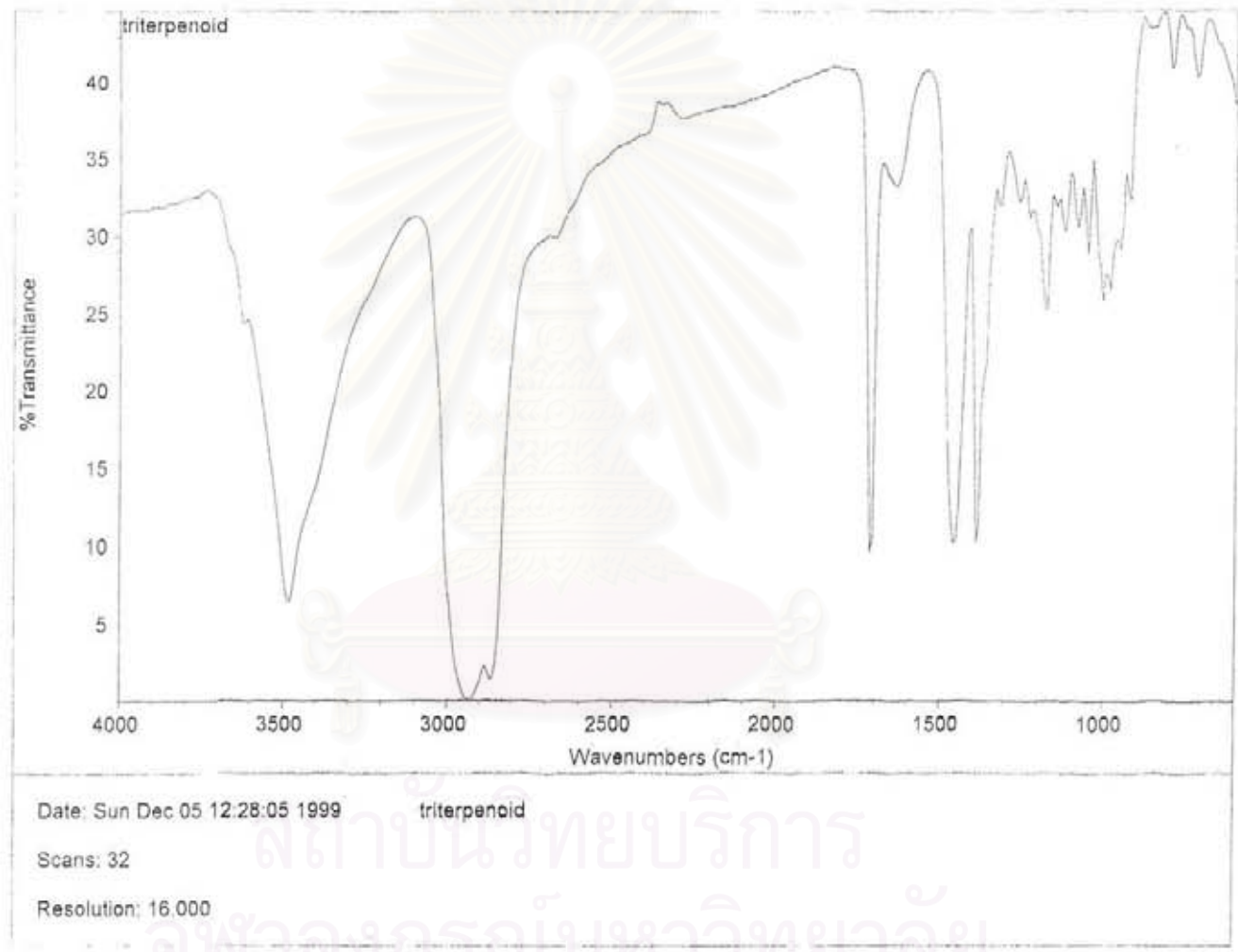
Fig 2 The IR spectrum of Mixture 2
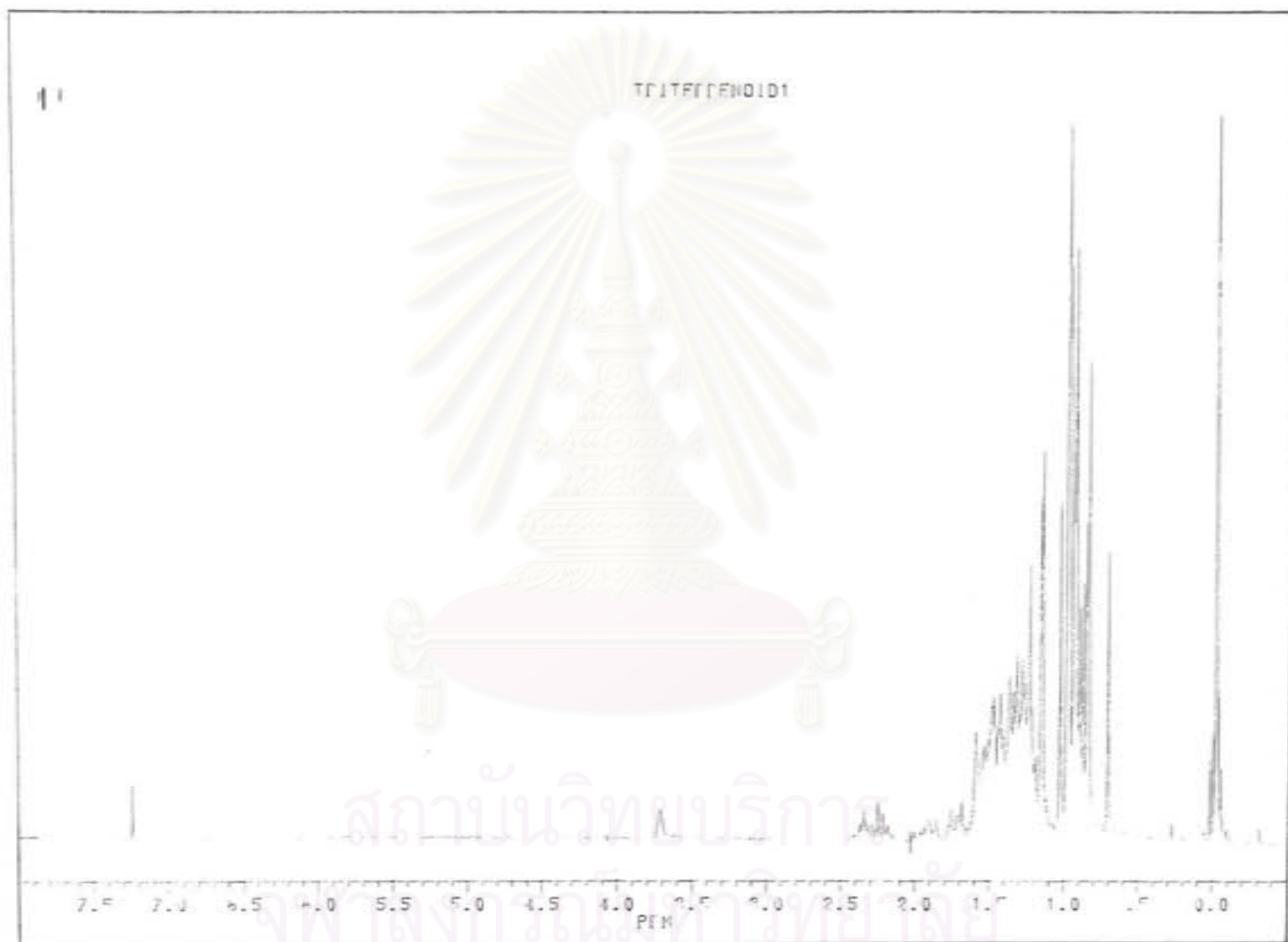
Fig 3 The ^{1}H NMR spectrum of Mixture 2
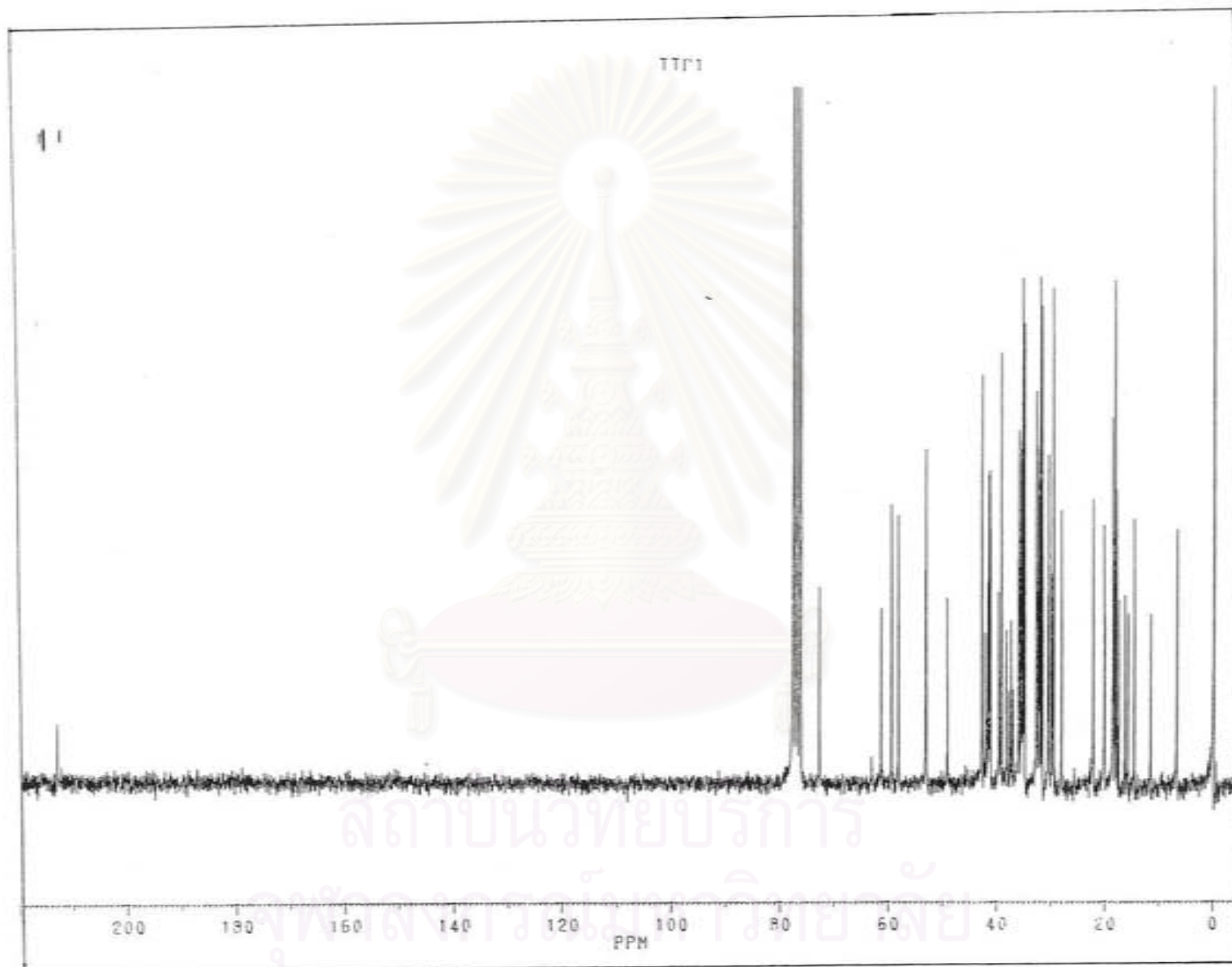
Fig 4 The ^{13}C NMR spectrum of Mixture 2
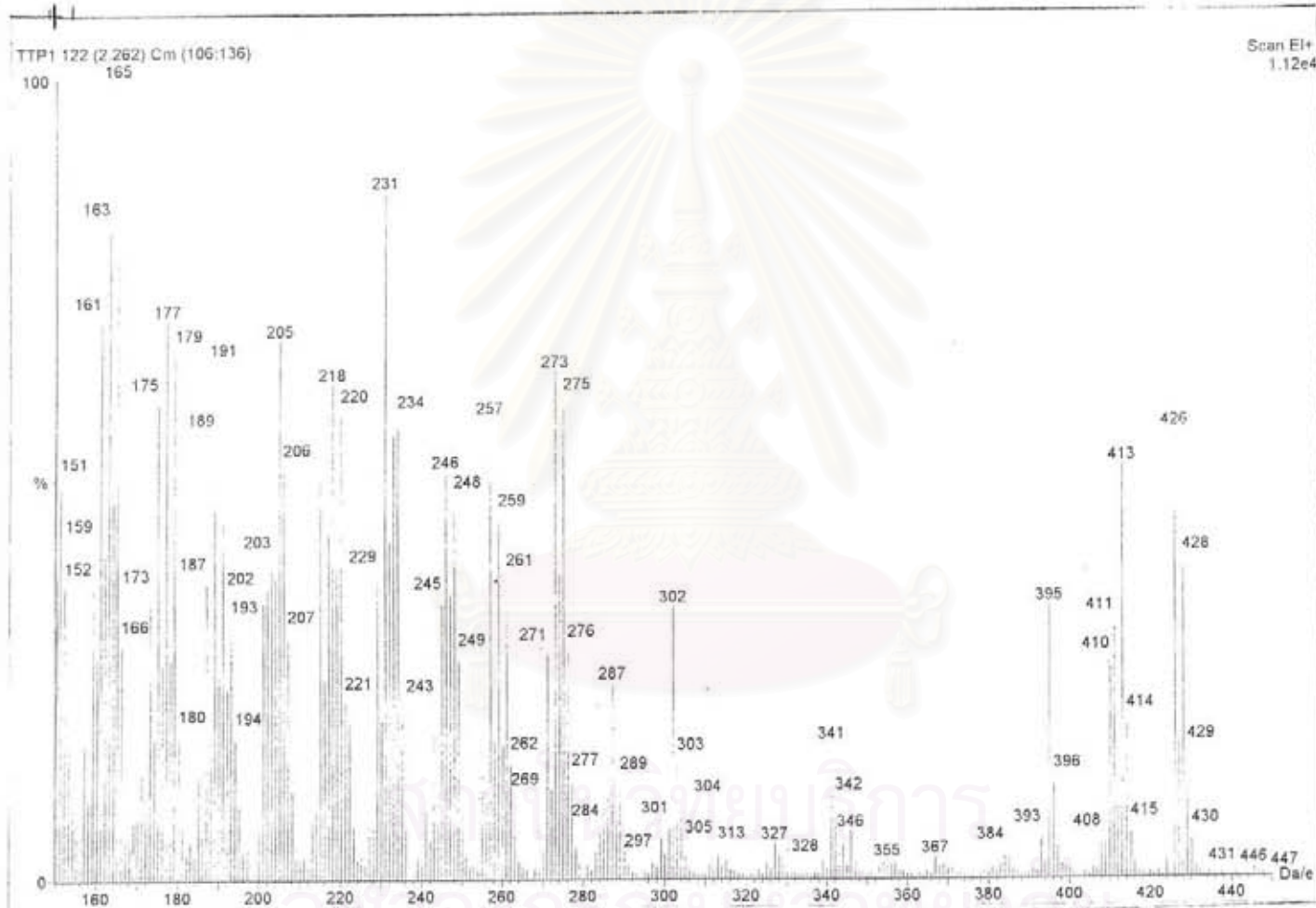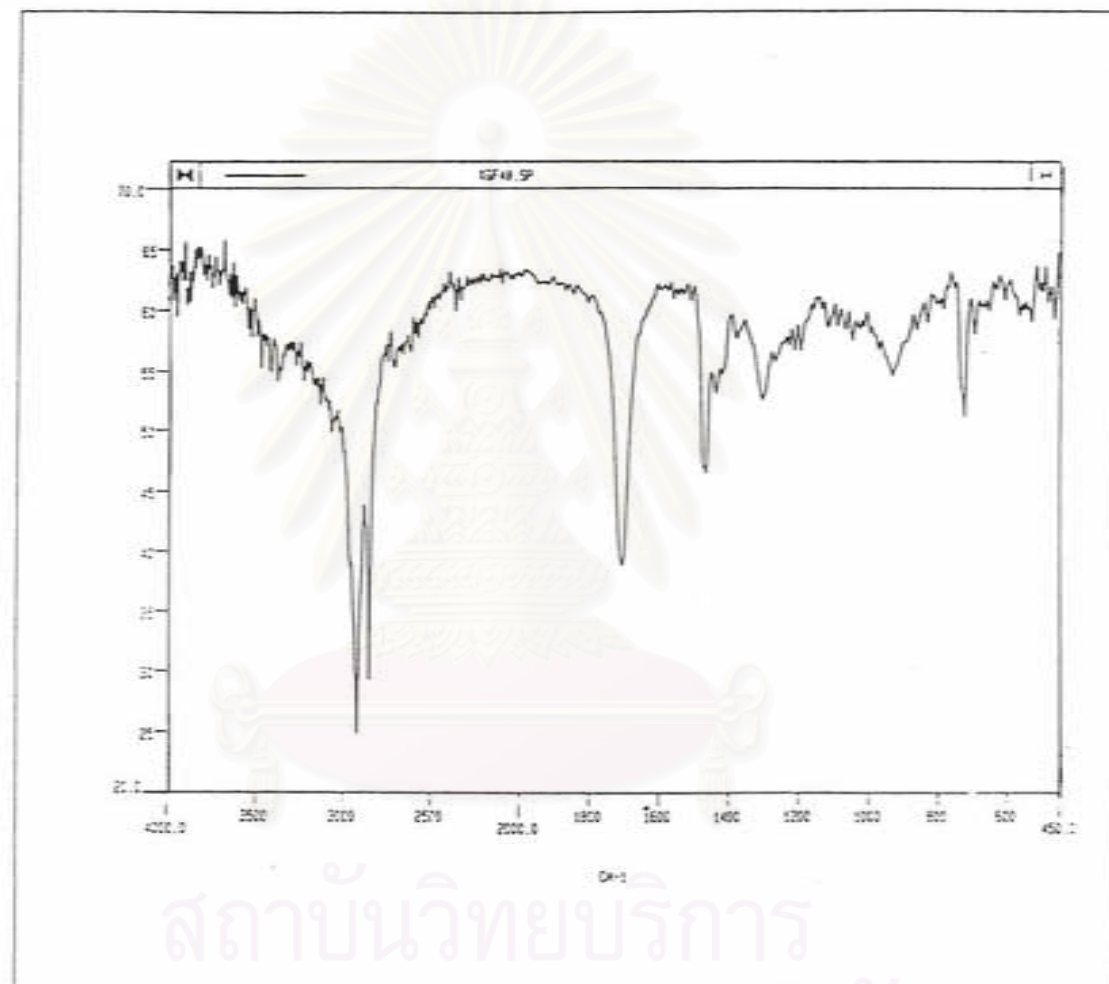
Fig 6 The IR spectrum of Mixture 3
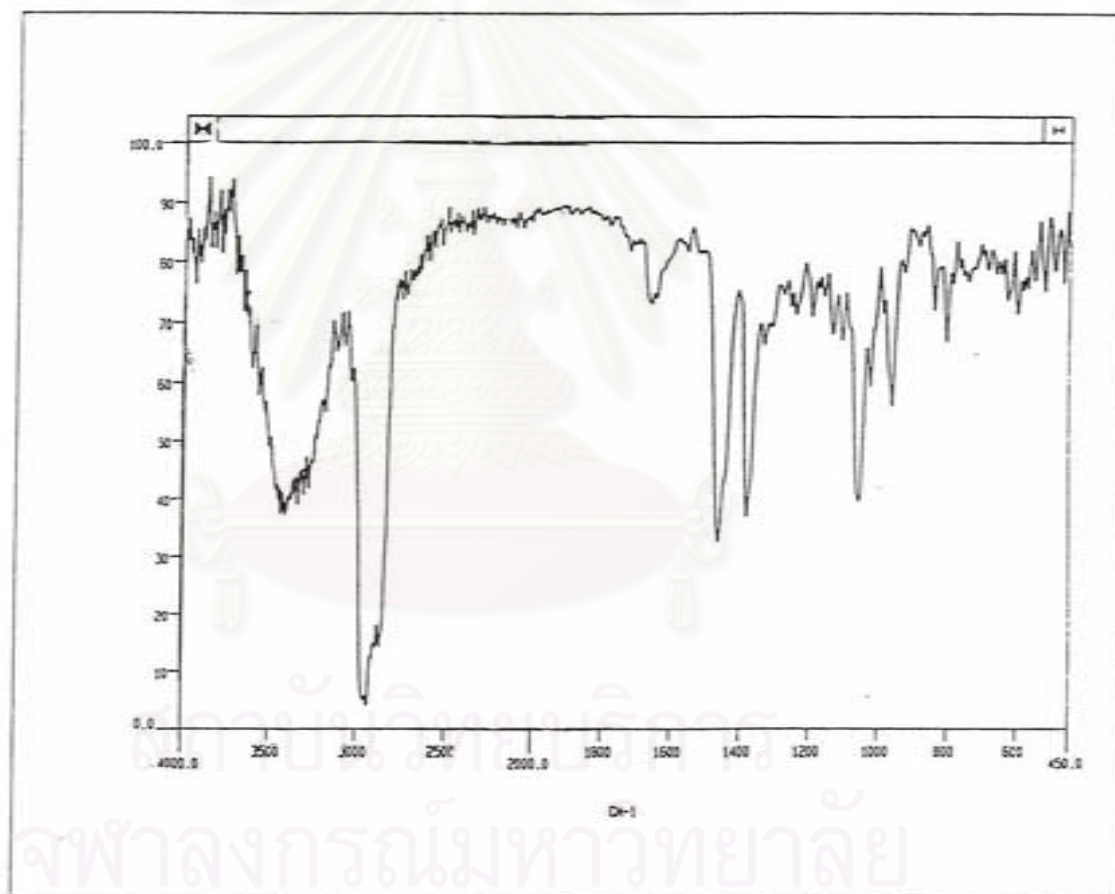
Fig 7 The IR spectrum of Mixture **4**

สเตอรอยด์มาตรฐาน
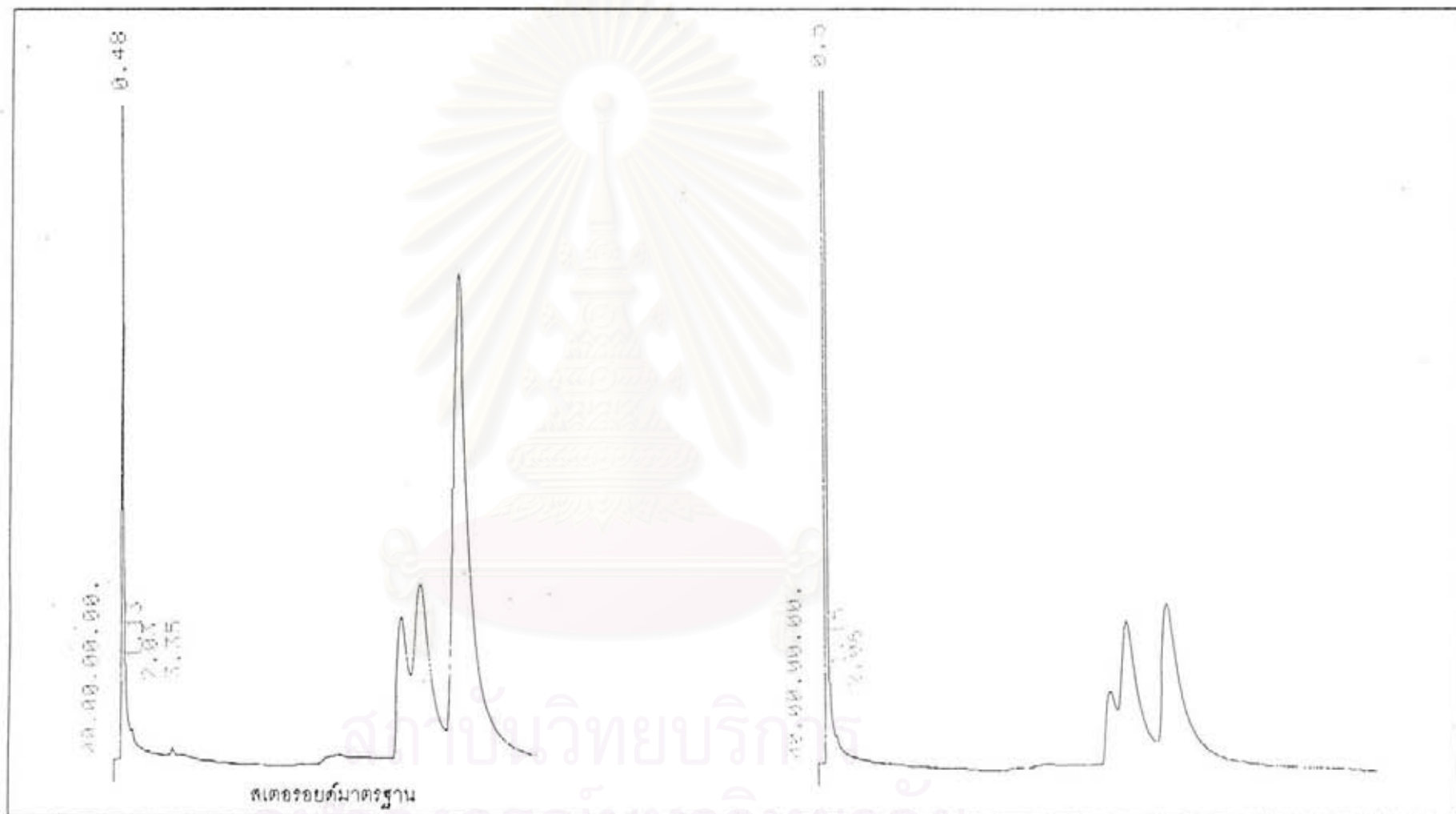
Fig 8 The GLC analysis of standard steroids and Mixture **4**
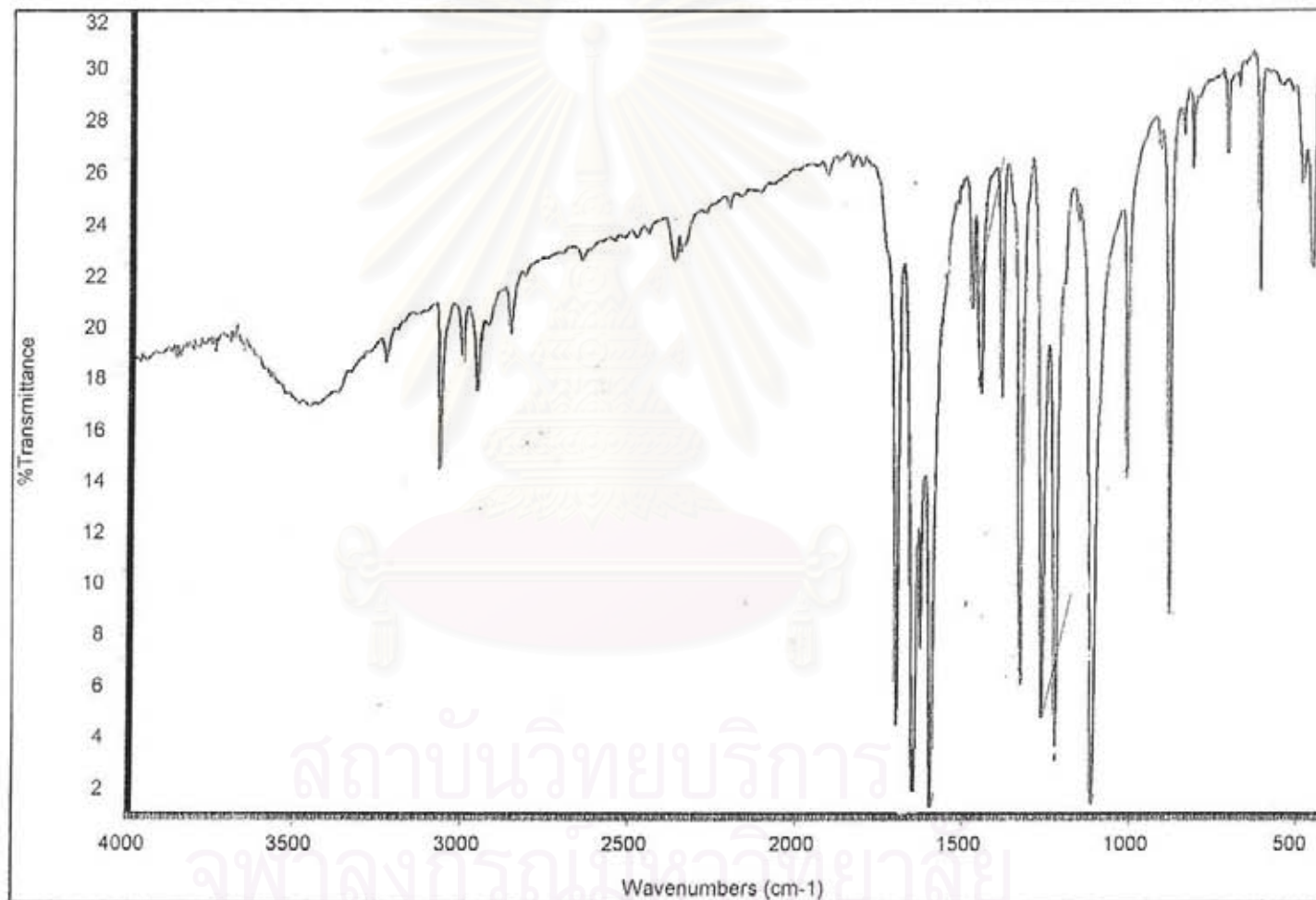
Fig 9 The IR spectrum of Compound 1
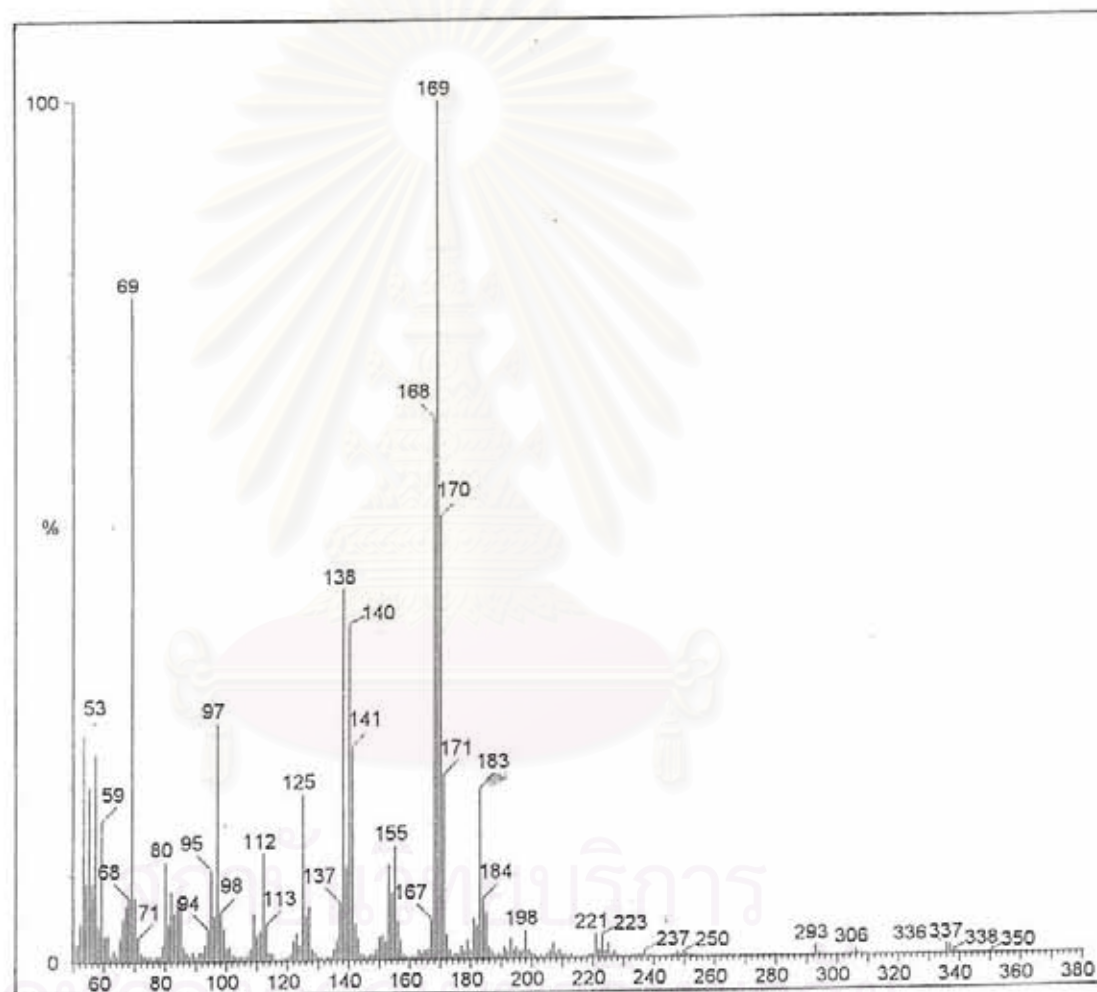
Fig 10 The mass spectrum of Compound 1
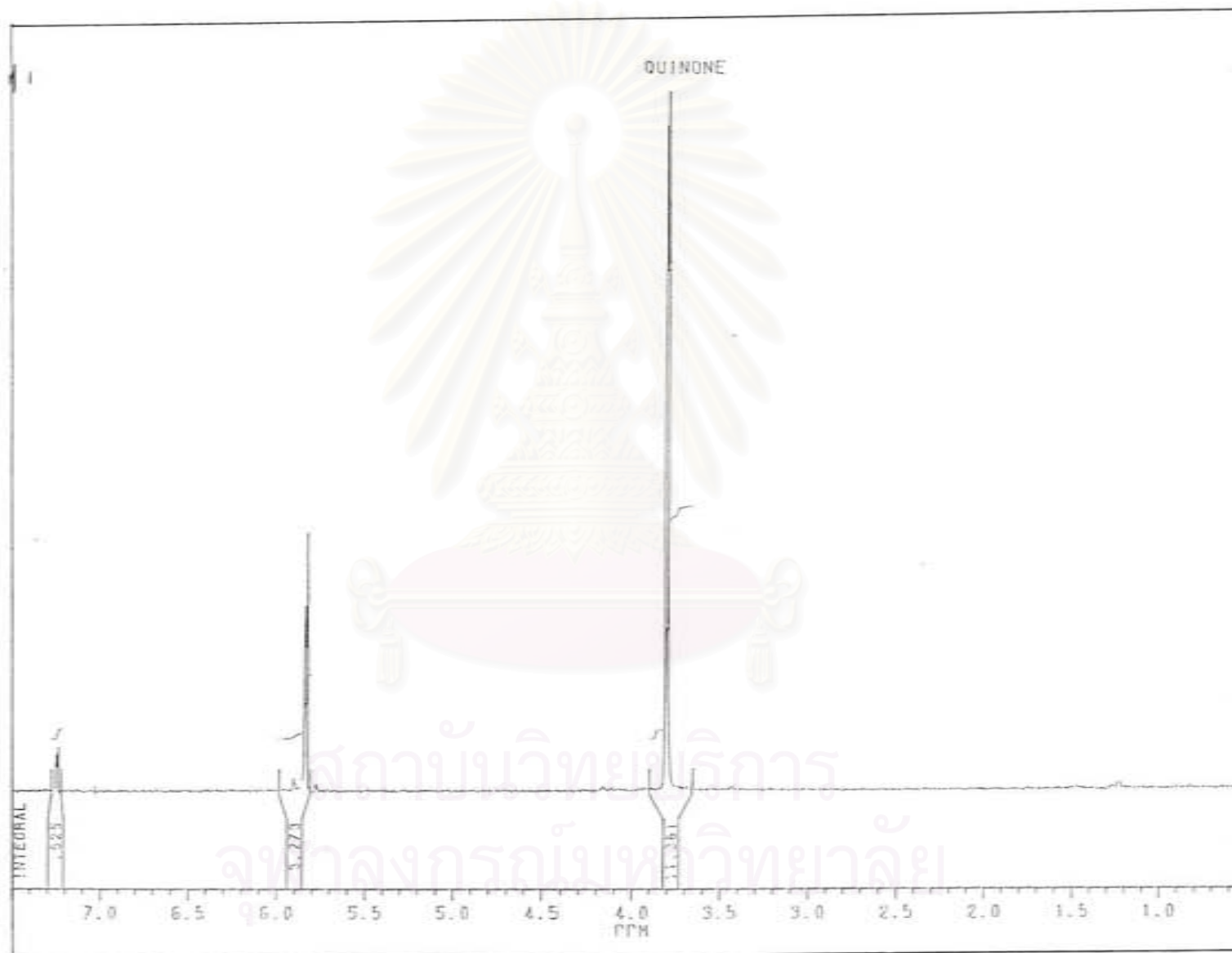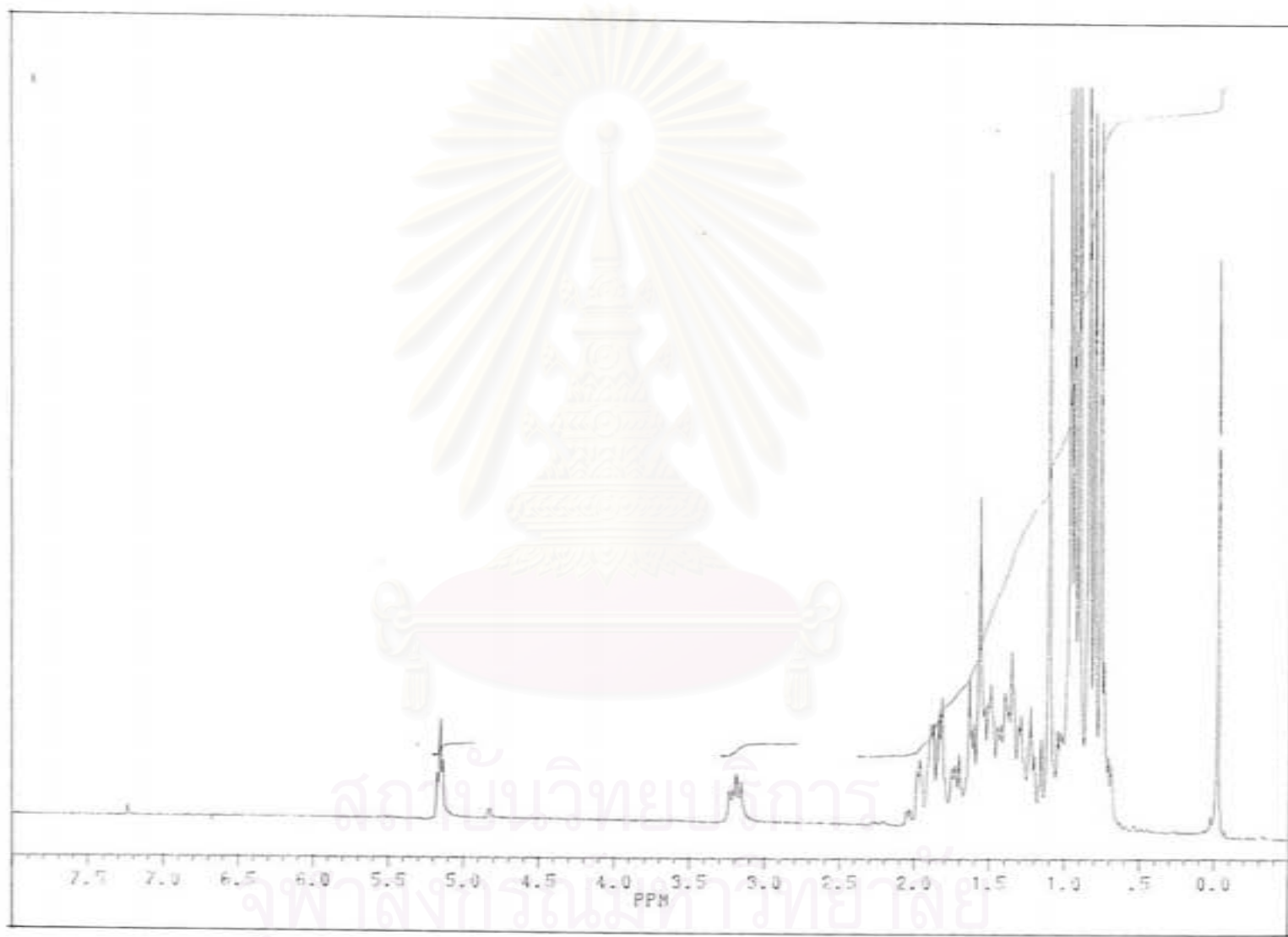
Fig 11 The ^{1}H NMR spectrum of Compound **1**
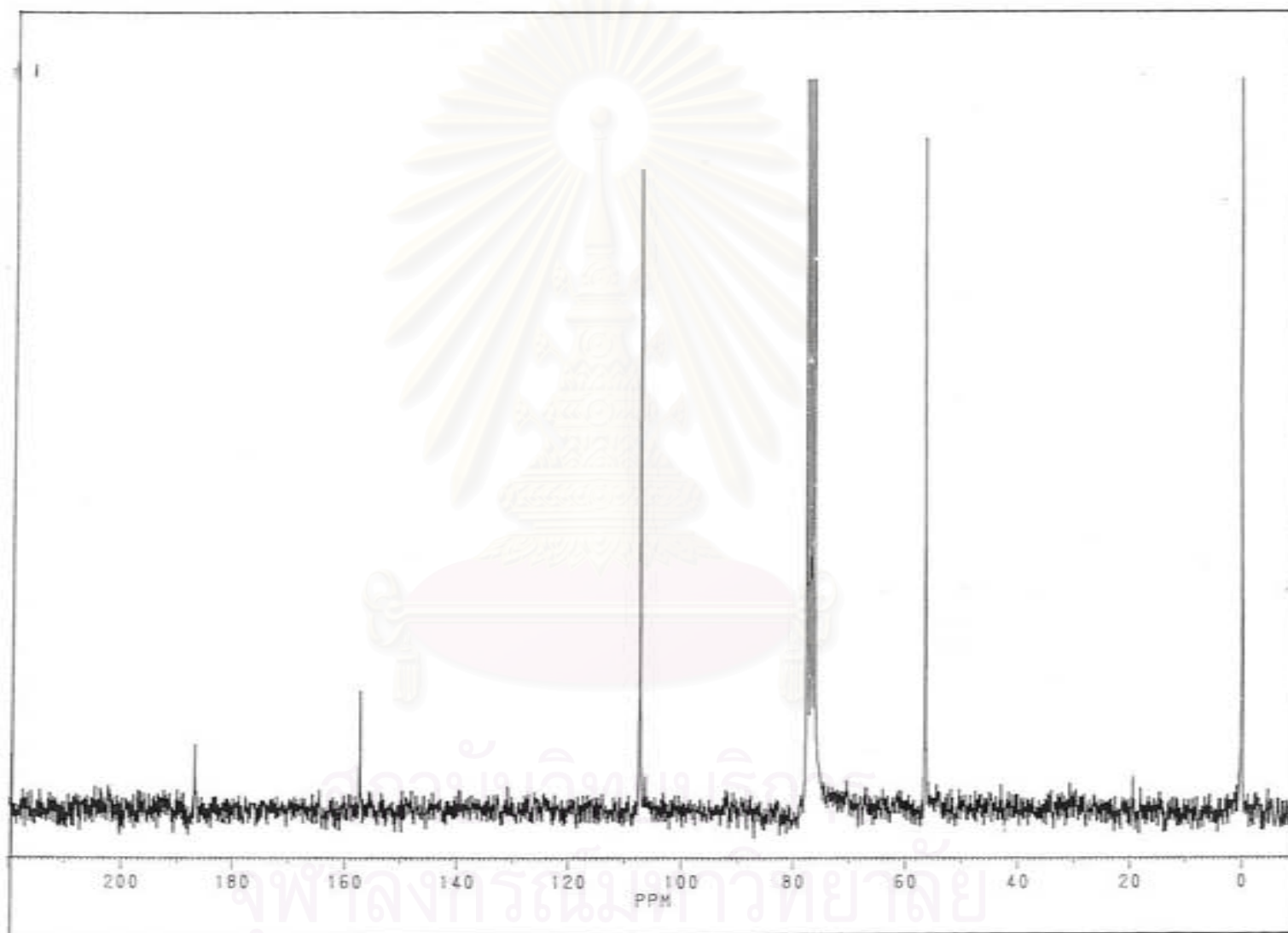
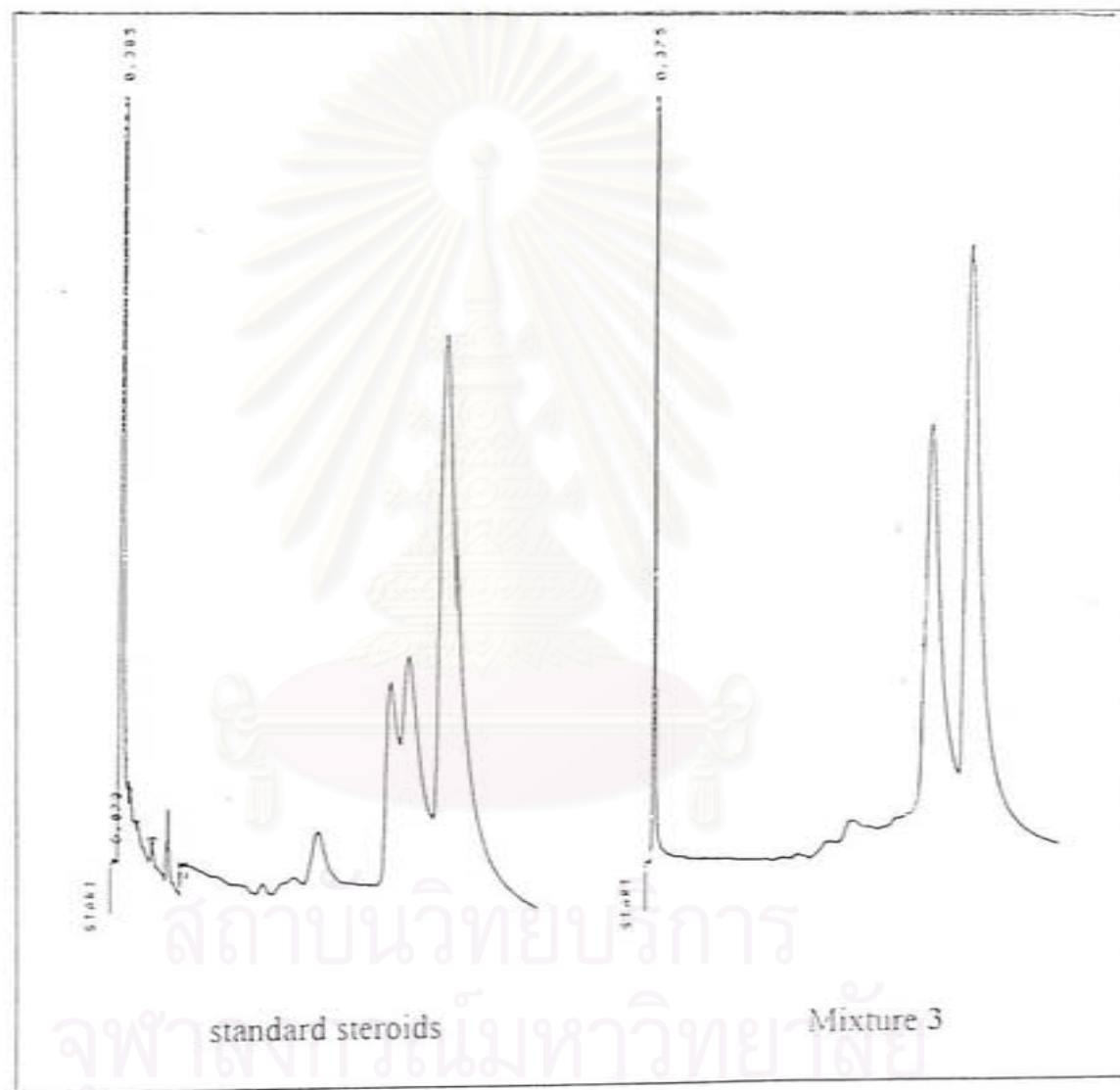**Fig 12** The ^{13}C NMR spectrum of Compound **1**
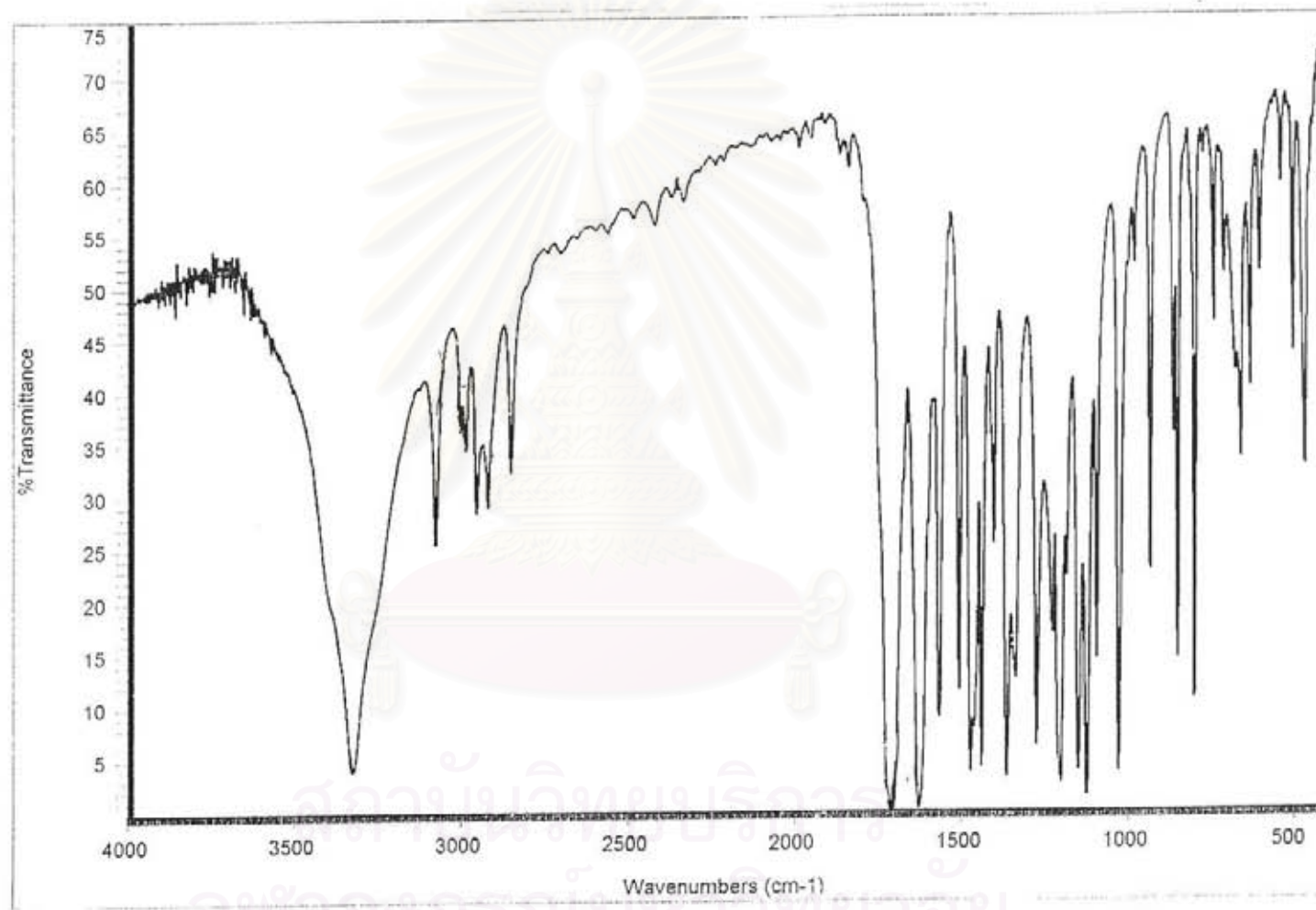
Fig 13 The IR spectrum of Compound **2**

Fig 14 The ^{1}H NMR spectrum of Compound **2**
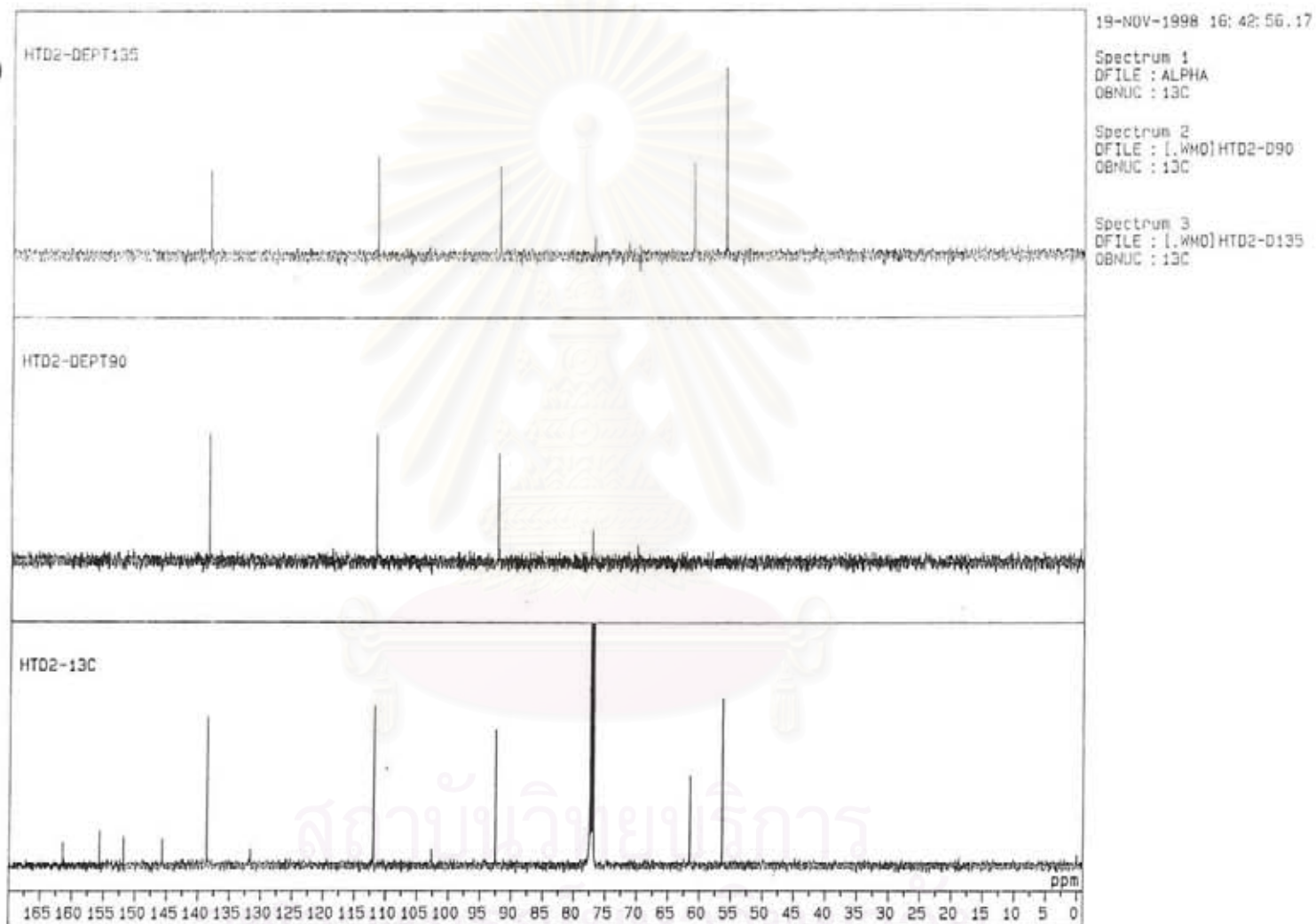
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* CHULALONGKORN UNIVERSITY *
*        JNM-A500           *
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COMNT : HTD2-1H H5X

EXMOD : SINGL
IRMOD : NON
POINT :     32768
FREQU : 10000.00 Hz
SCANS :         4
DUMMY :         4
ACQTM :     3.2768 sec
PD    :     3.0000 sec
RGAIN :        21
PW1   :     3.40 usec
OBNUC : 1H
OBFRQ :  500.00 MHz
OBSET : 162410.00 Hz

IRNUC : 1H
IRFRQ :  500.00 MHz
IRSET : 162410.00 Hz
IRATN :       120
IRRPW :      30.0 usec
IRBP1 :        25
IRBP2 :         2
IRANS :         0

ADBIT :        16
CTEMP :      29.1 c
CSPED :        11 Hz
SLVNT : CDCL3
RESOL :      0.31 Hz
BF    :      0.31 Hz
REFVL :      0.00 ppm
XE    :   4551.09 Hz
XS    :    265.96 Hz

OPERATOR :
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113

Fig 15 The ^{13}C NMR spectrum of Compound **2**

Fig 16 The DEPT 90 and DEPT 135 spectrum of Compound 2

Fig 17 The NOESY spectrum of Compound 2
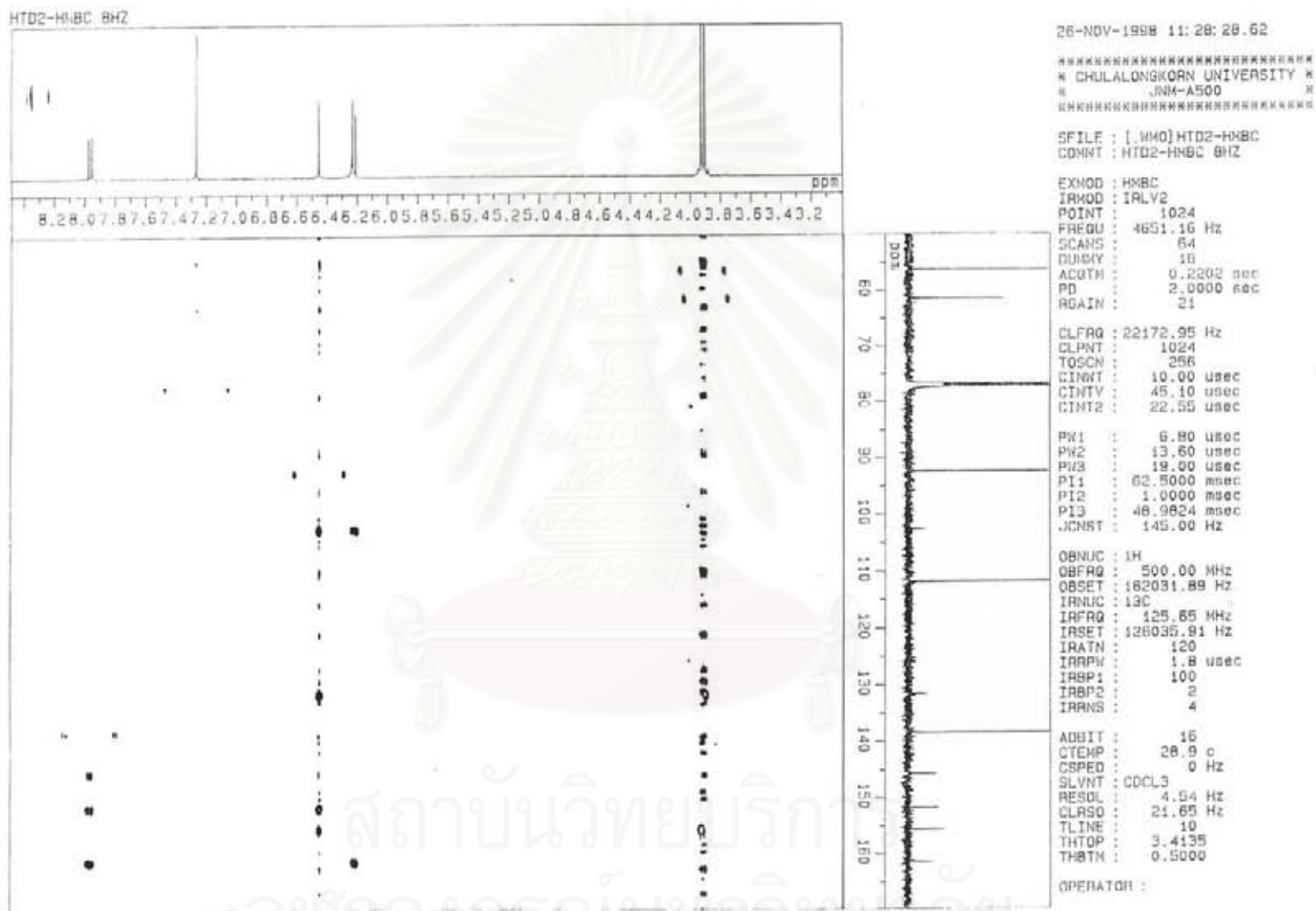
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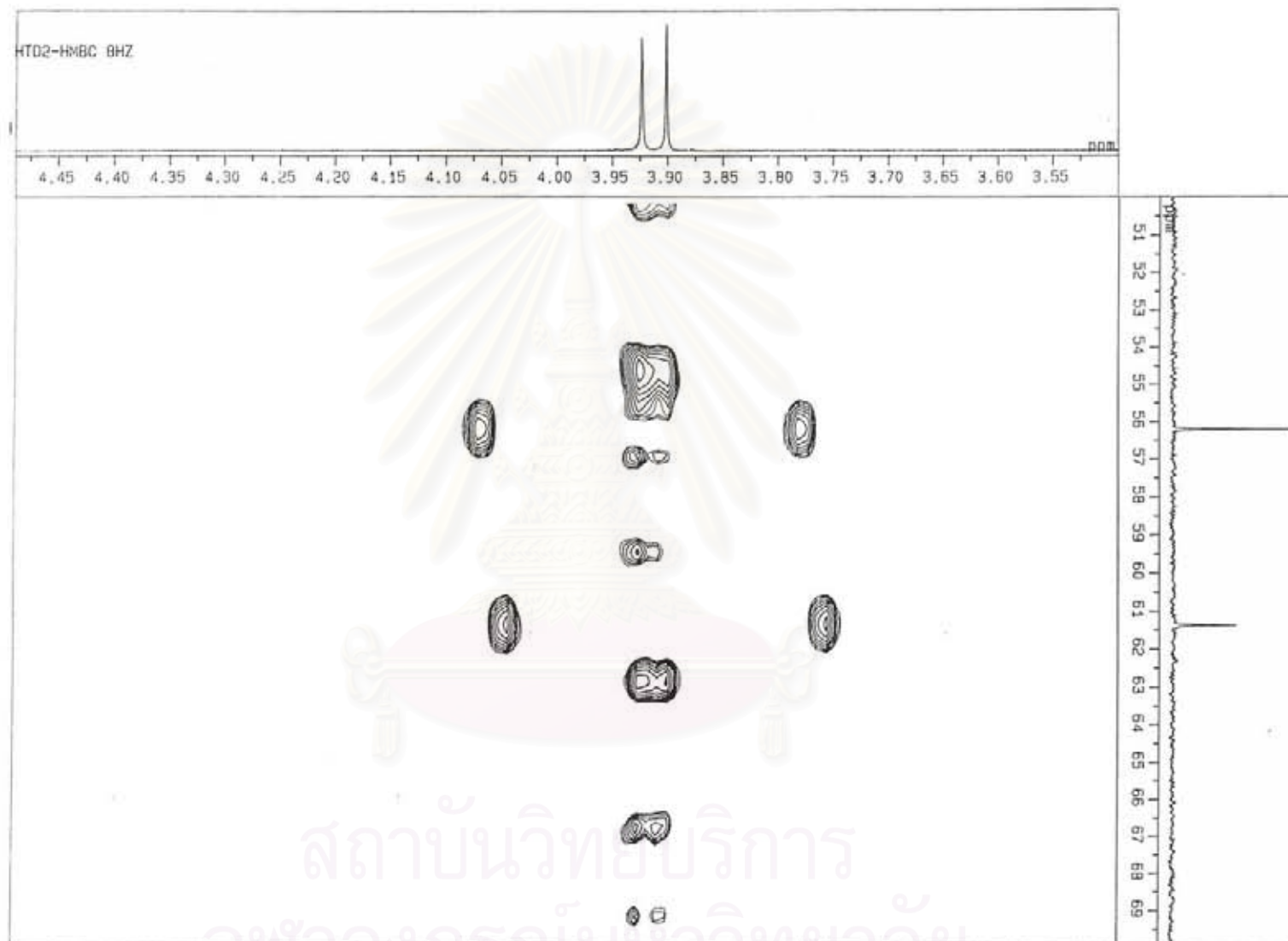
Fig 18A The HMBC spectrum of Compound **2**

Fig 18B The HMBC spectrum of Compound **2**

Fig 18C The HMBC spectrum of Compound **2**

Fig 18D The HMBC spectrum of Compound **2**
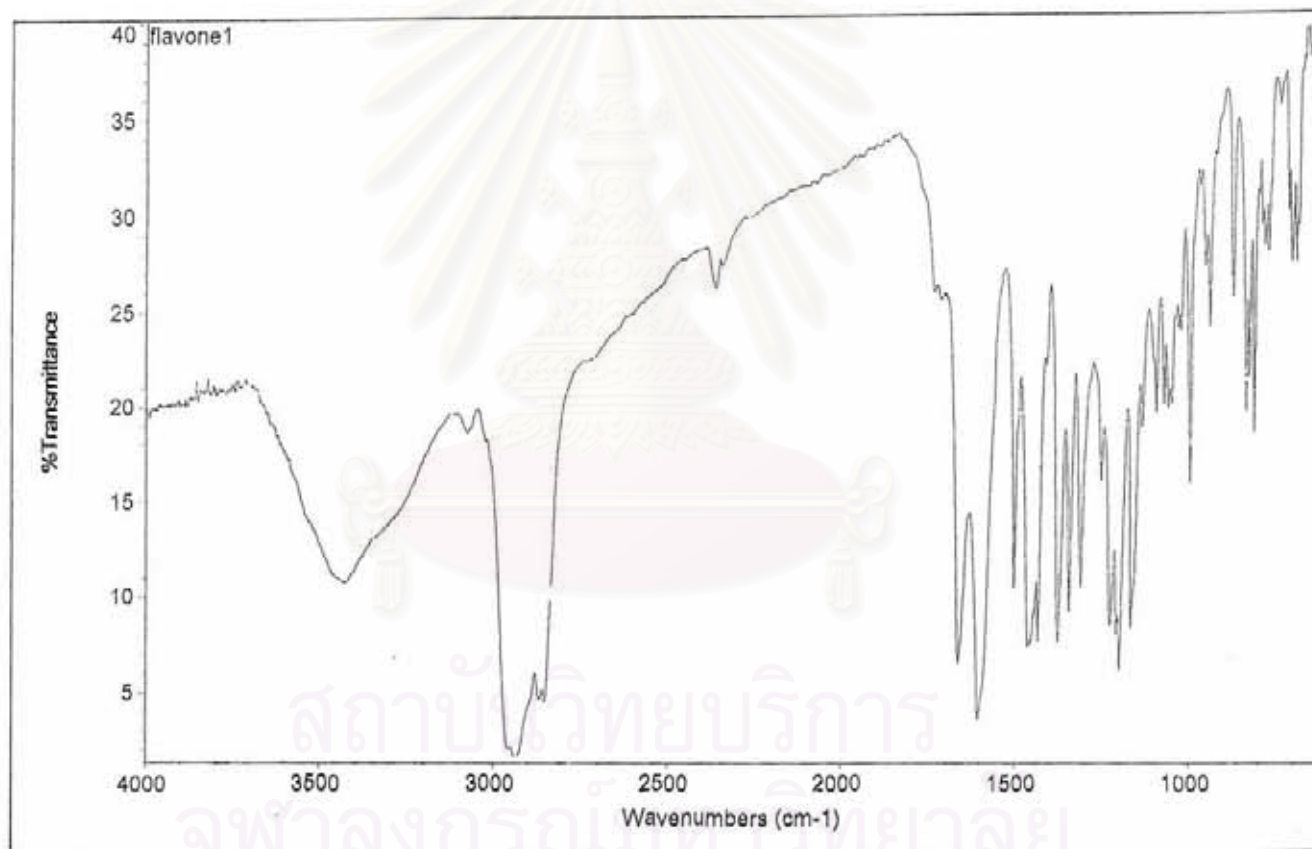
Fig 19 The IR spectrum of Compound 3
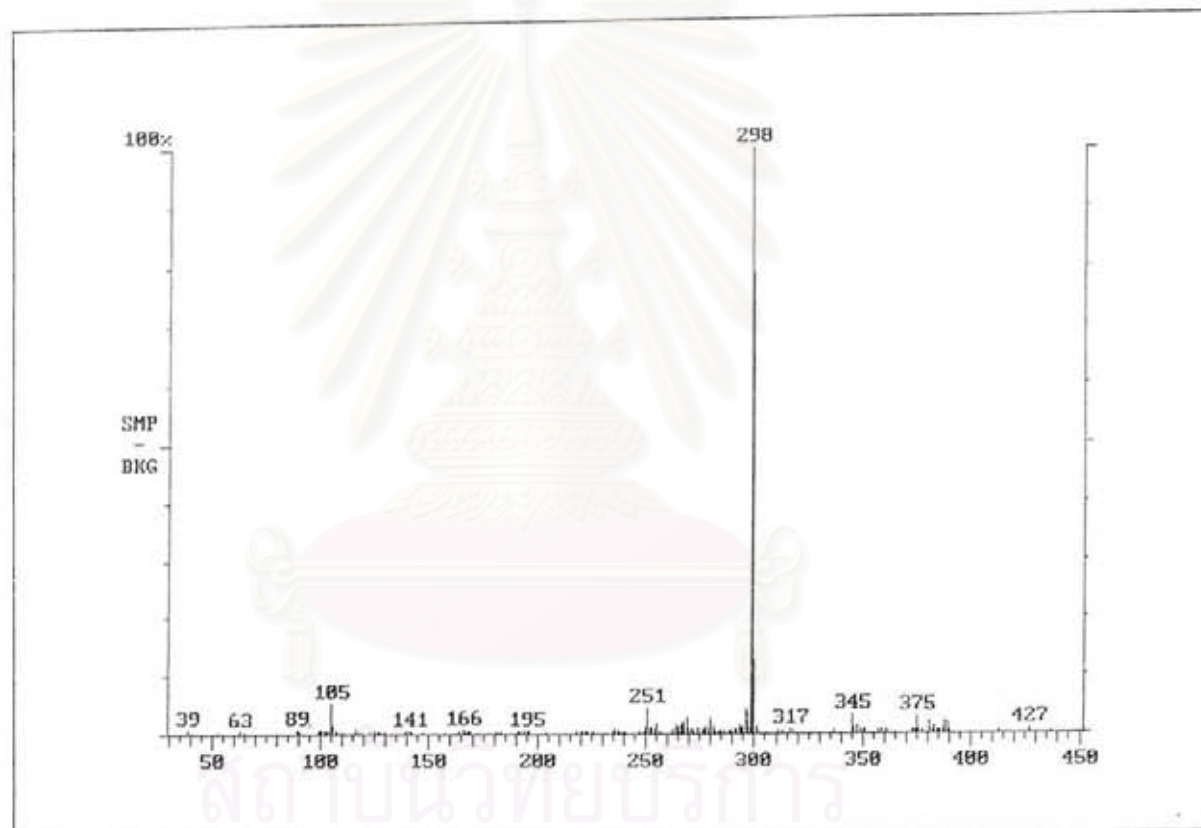
Fig 20 The mass spectrum of Compound **3**
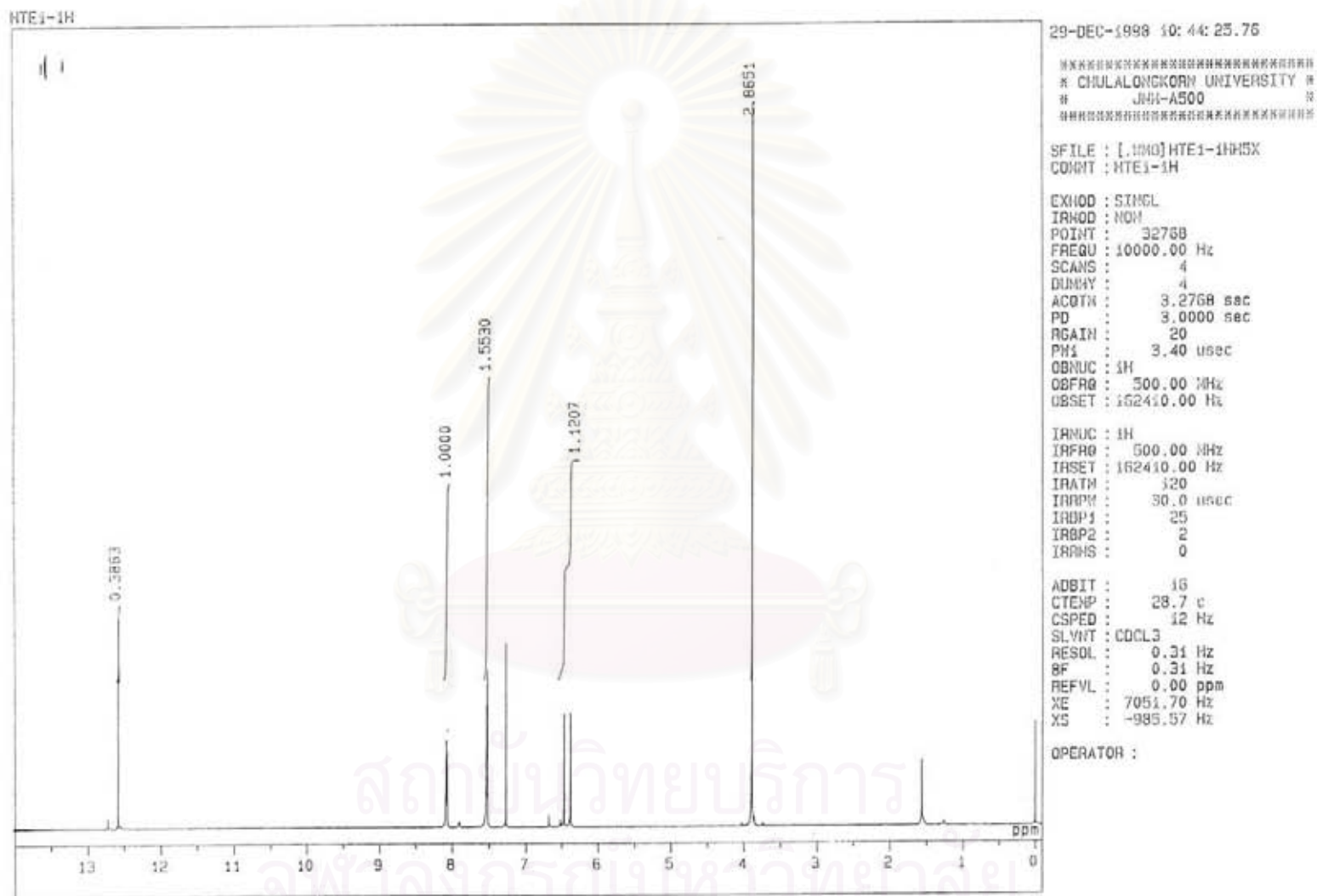
Fig 21 The ^{1}H NMR spectrum of Compound **3**
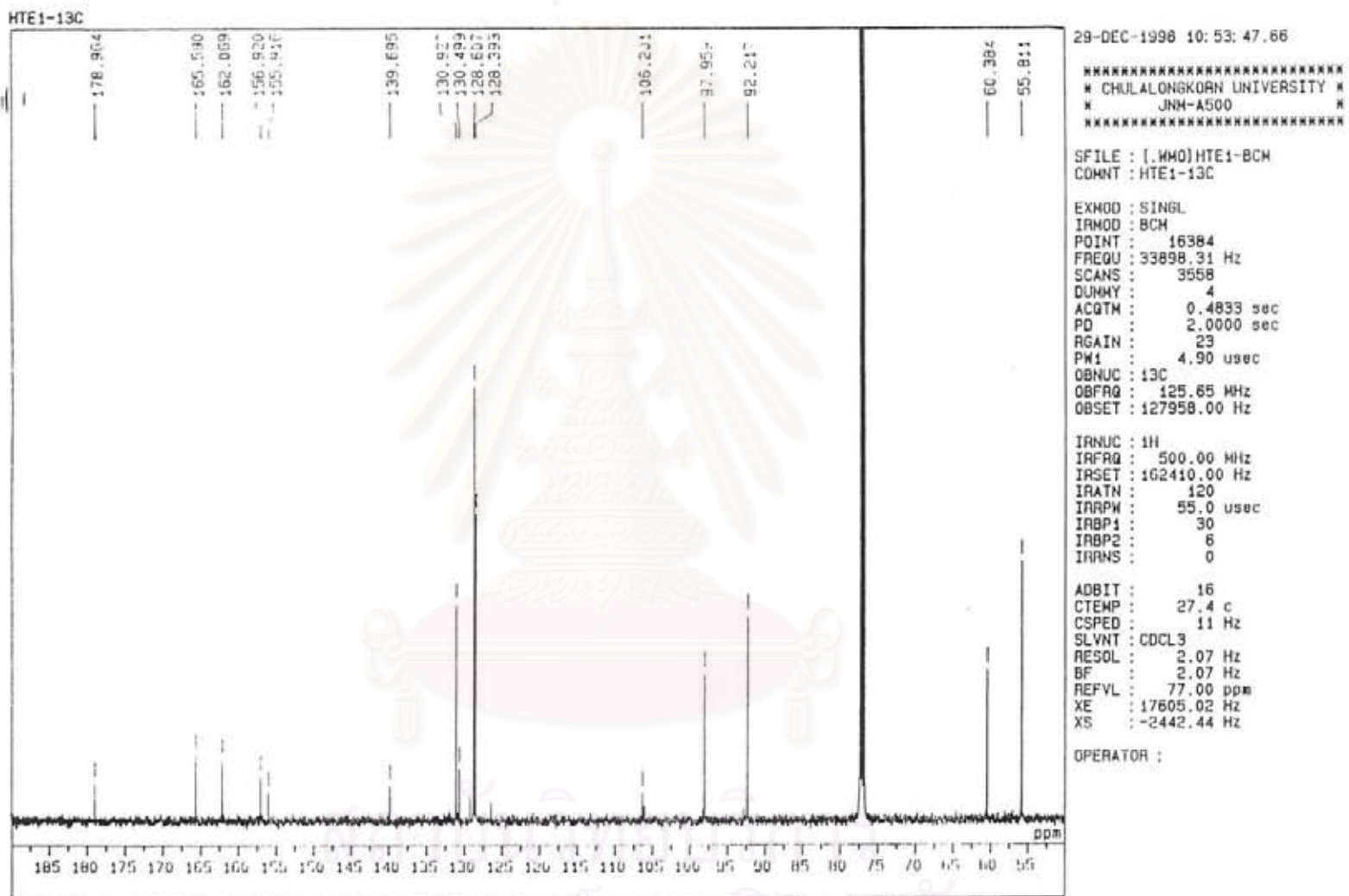
Fig 22 The ^{13}C NMR spectrum of Compound **3**
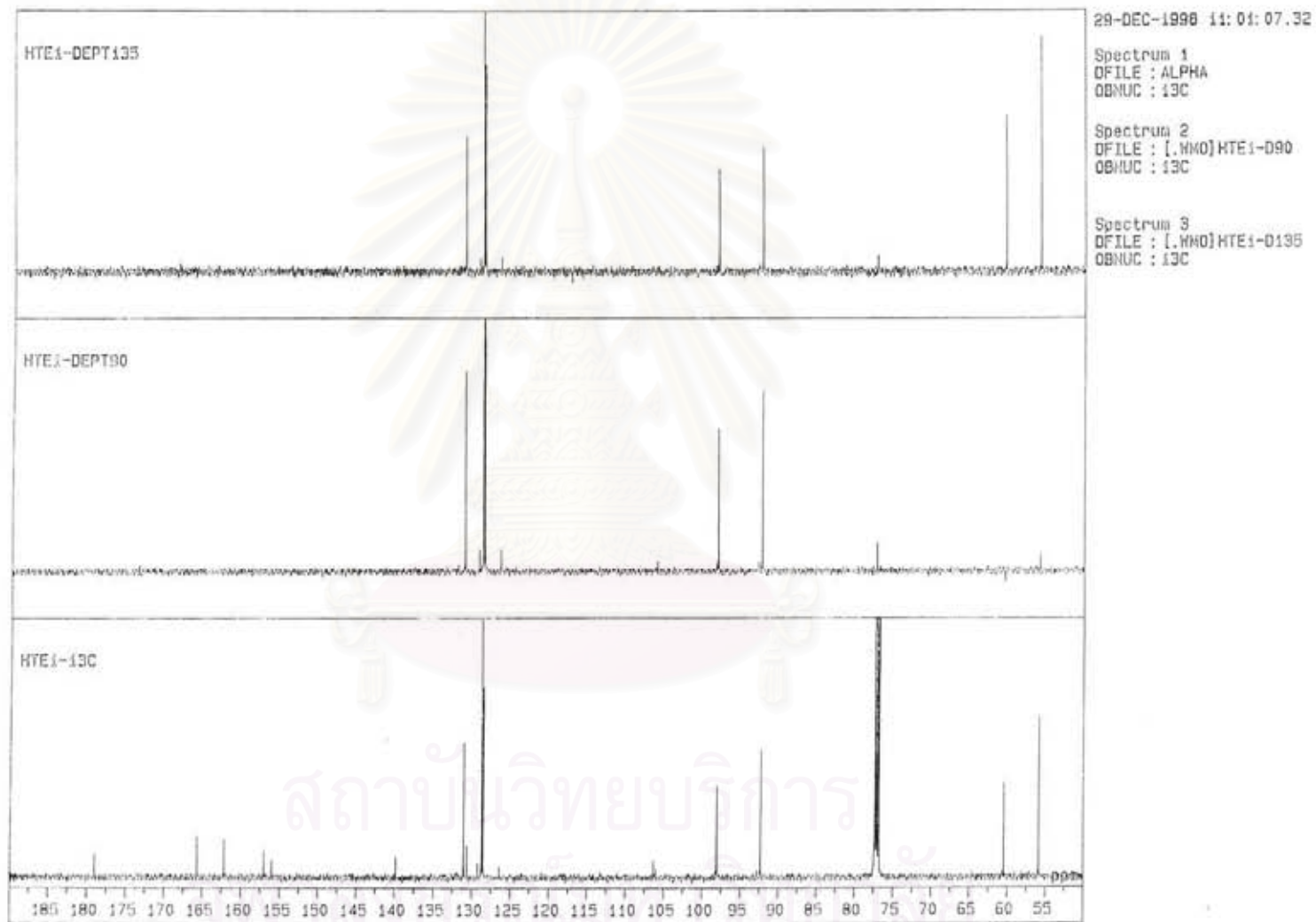
Fig 23 The DEPT 90 and DEPT 135 spectrum of Compound 3
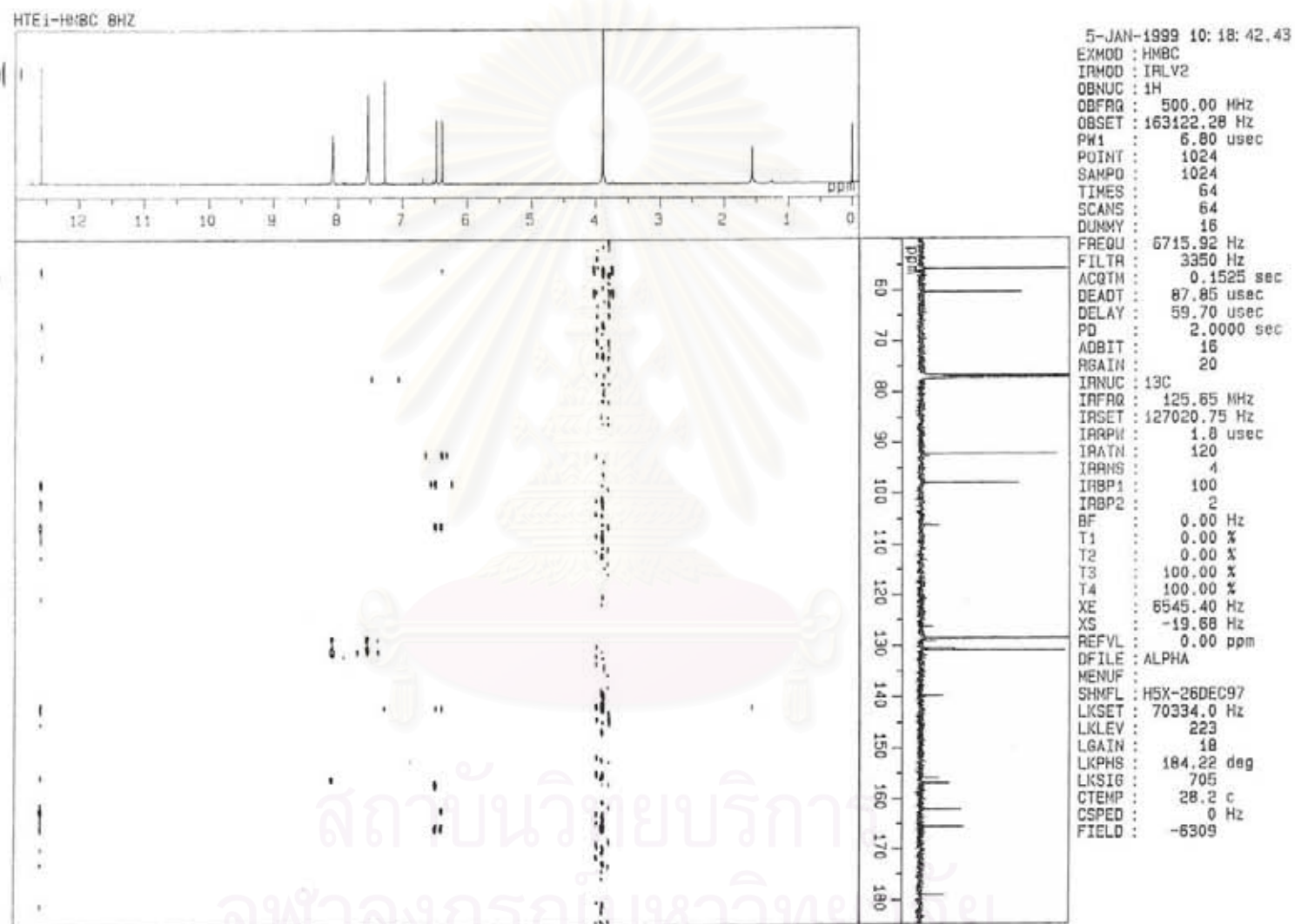
Fig 24 The HMBC spectrum of Compound 3

Fig 24A The HMBC spectrum of Compound **3**
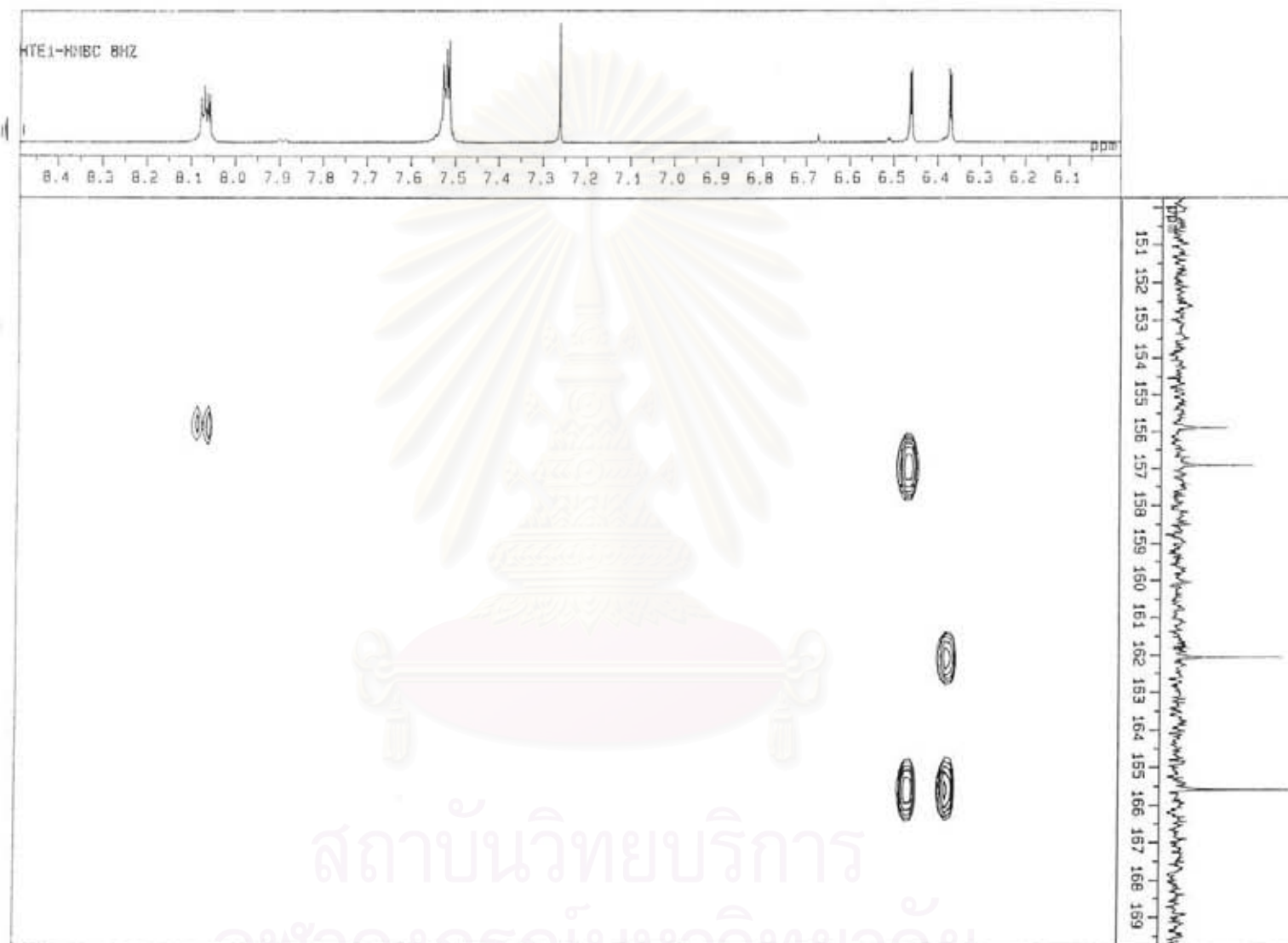
Fig 24B The HMBC spectrum of Compound **3**

Fig 24C The HMBC spectrum of Compound 3

Fig 25 The NOESY spectrum of Compound **3**

Fig 26 The mass spectrum of Compound 4
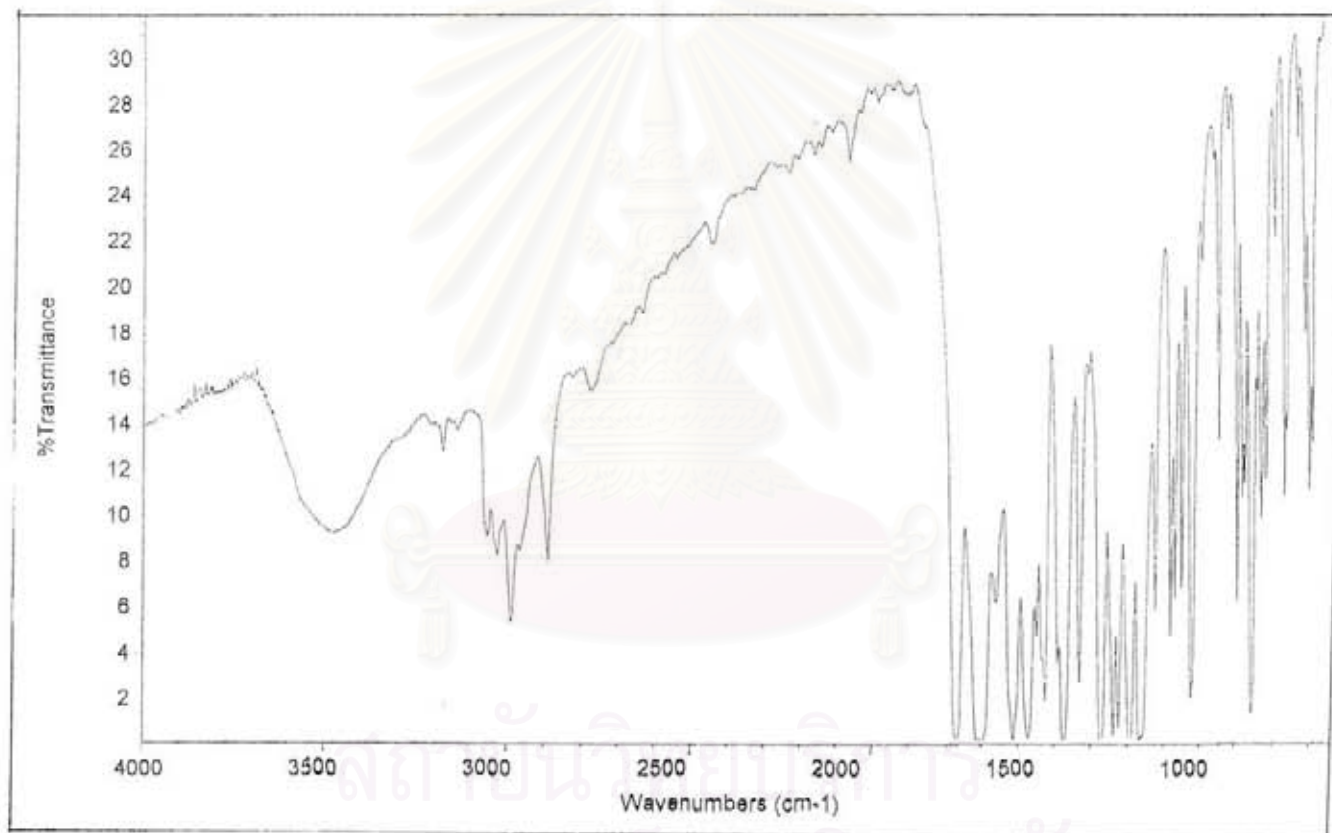
Fig 27 The IR spectrum of Compound **4**

Fig 28 The ^{1}H NMR spectrum of Compound **4**
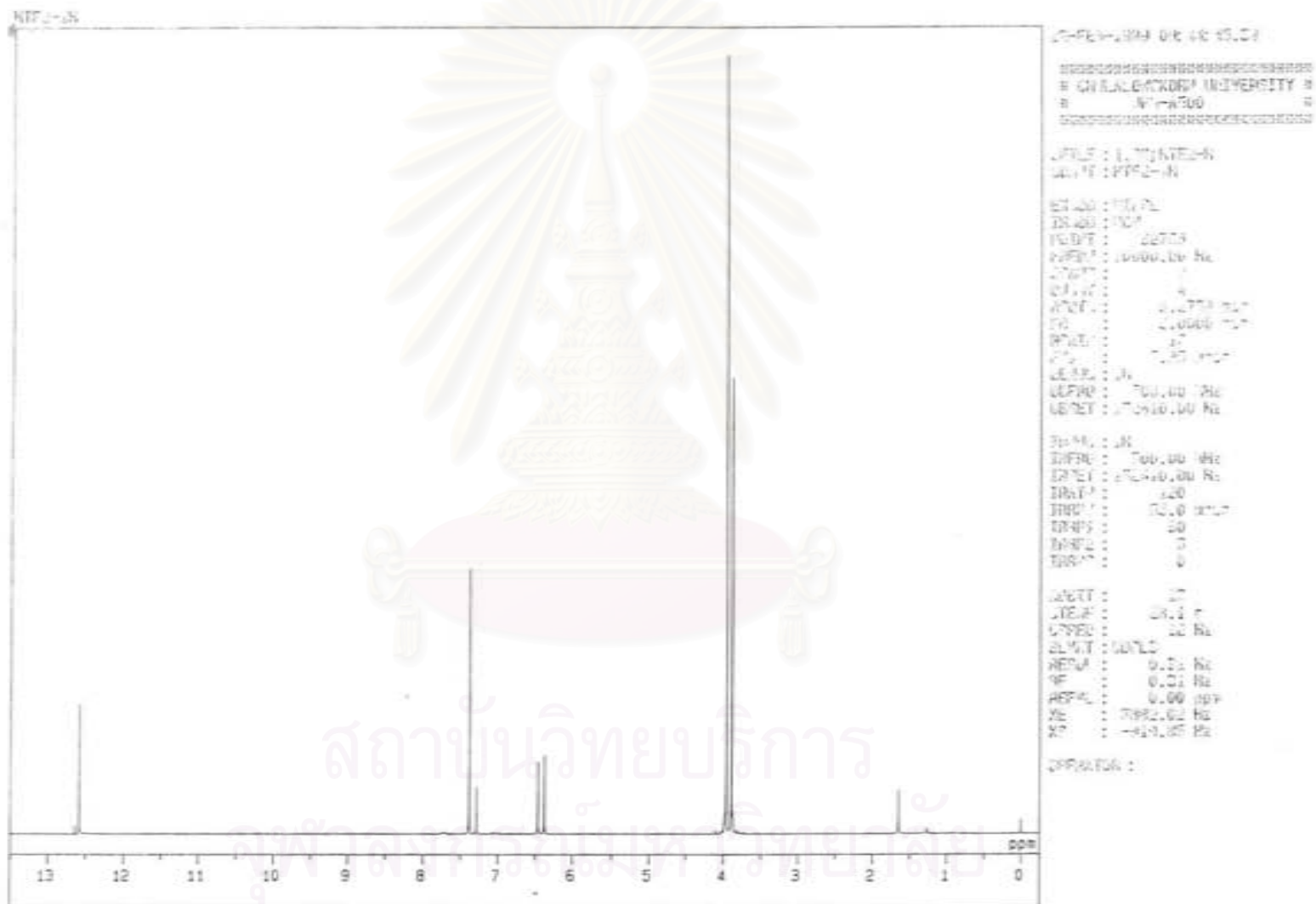
Fig 29 The ^{13}C NMR spectrum of Compound **4**
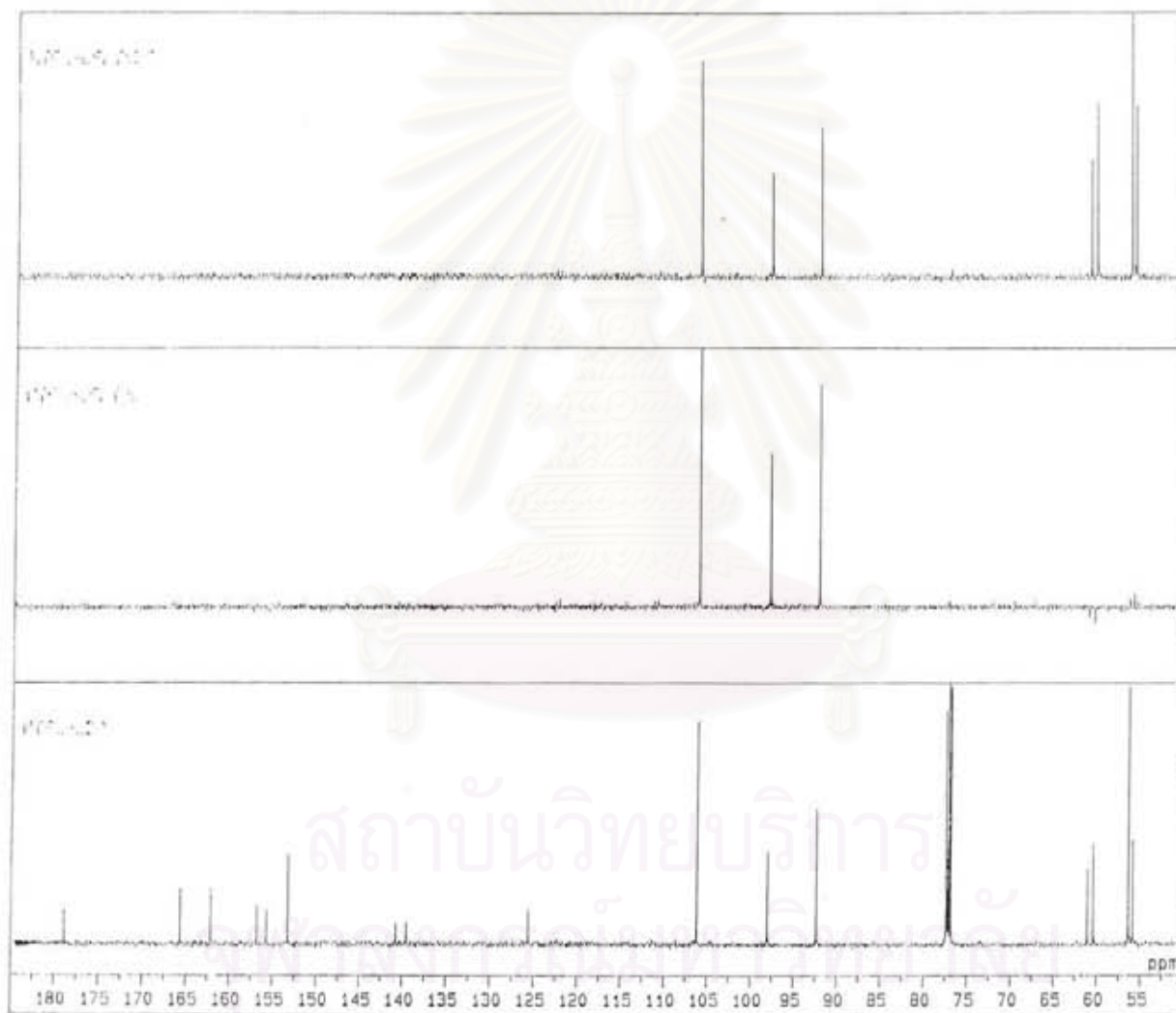
Fig 30 The DEPT 90 and 135 spectrum of Compound **4**

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Resolution: 16.000
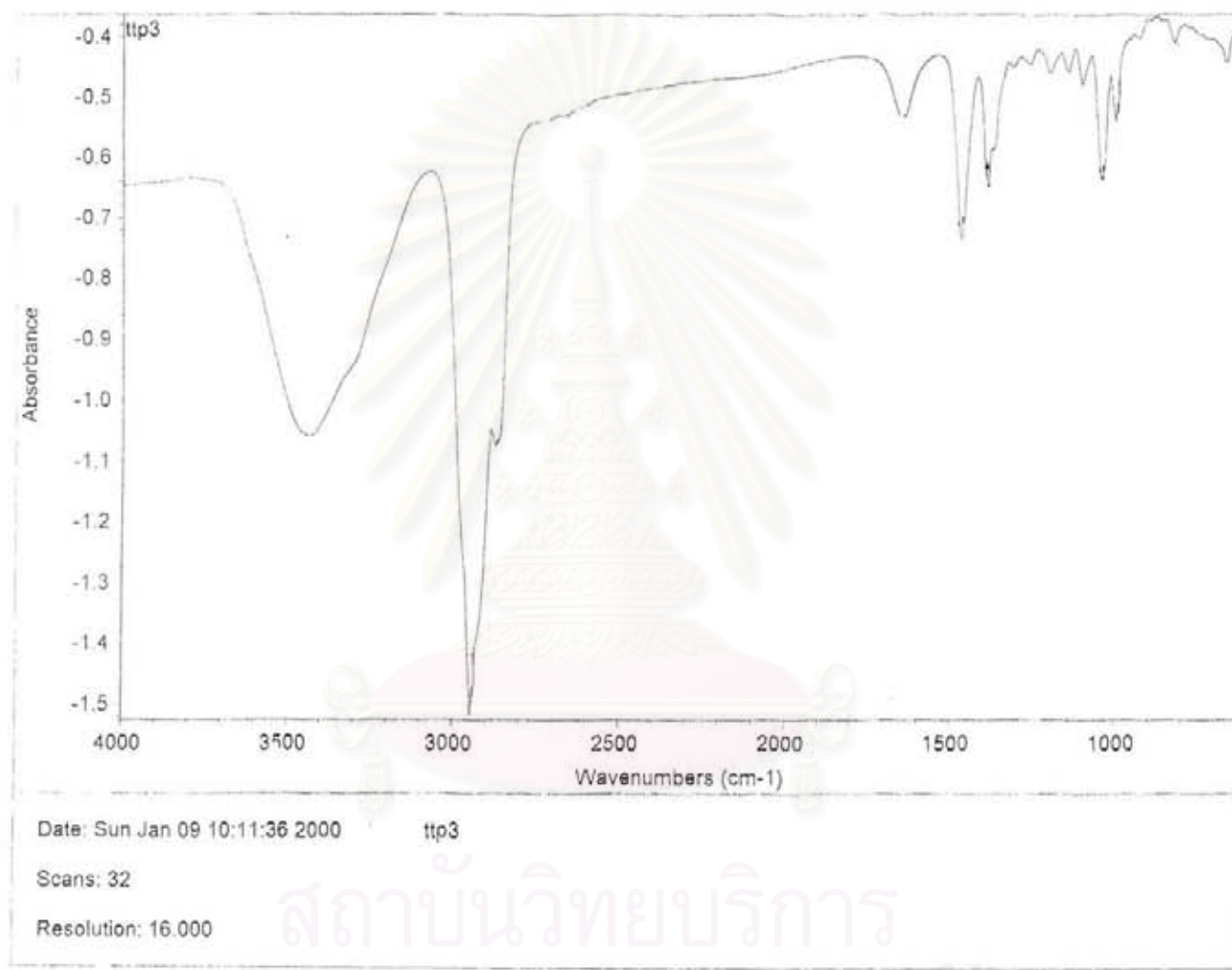
Fig 31 The IR spectrum of Compound **5**

Fig 32 The ^{1}H NMR spectrum of Compound **5**
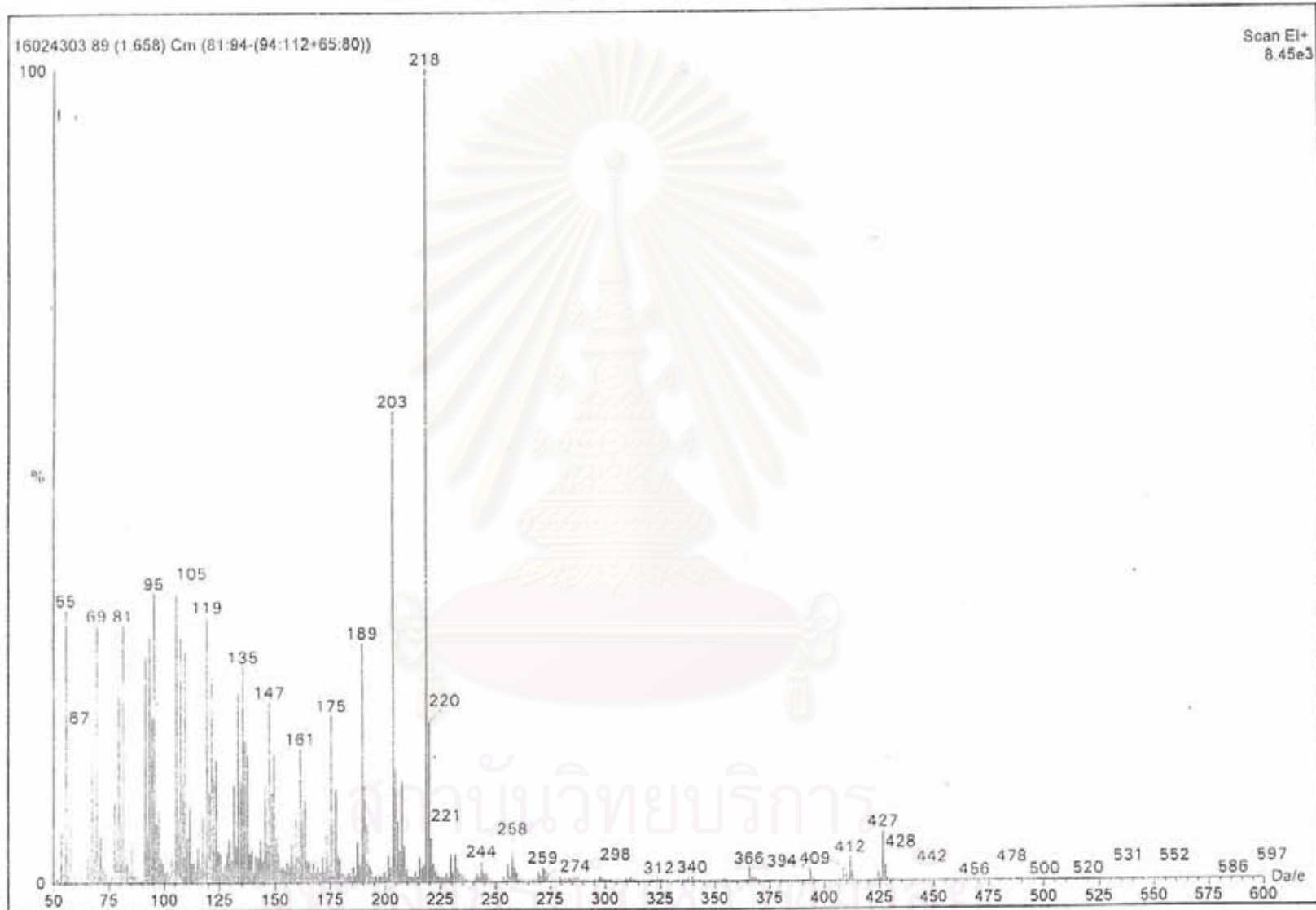
Fig 33 The mass spectrum of Compound **5**
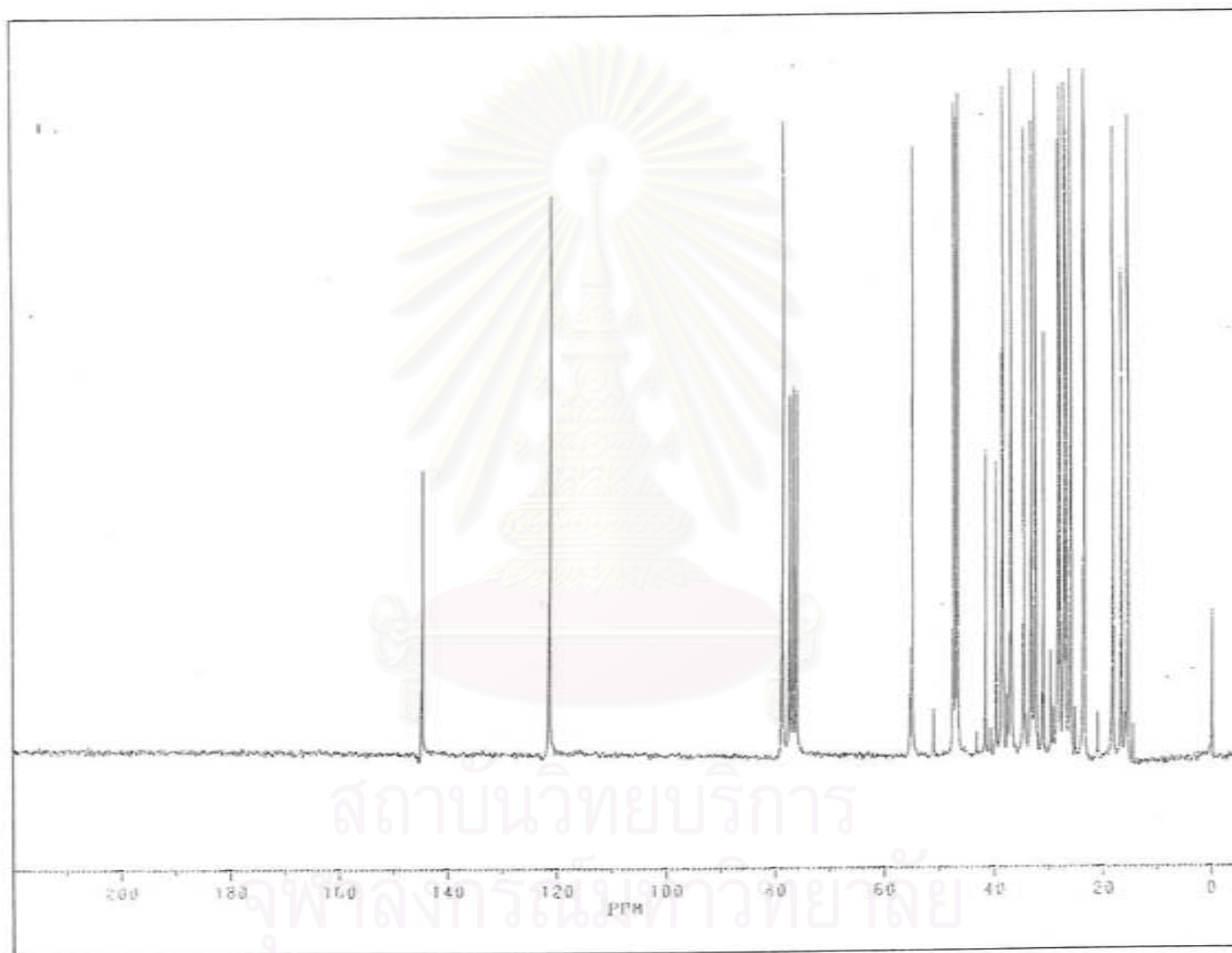
Fig 34 The ^{13}C NMR spectrum of Compound 5
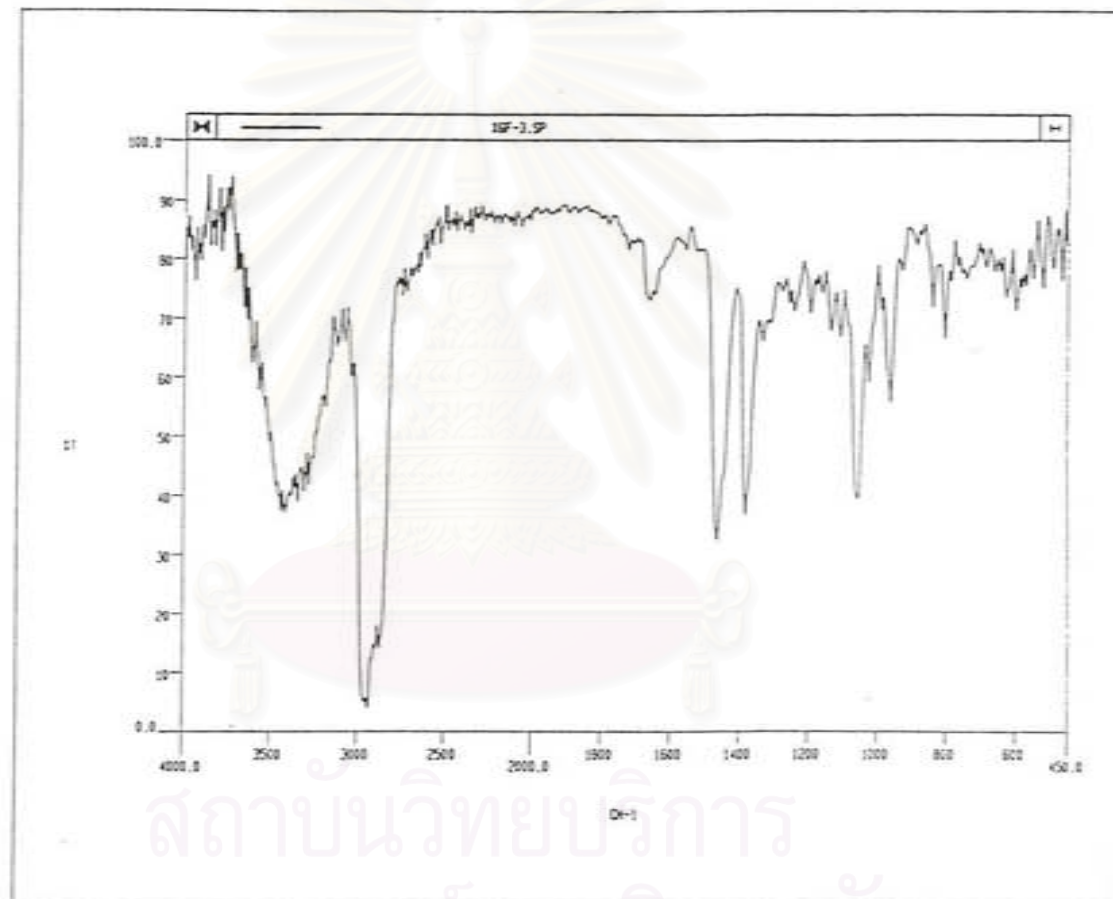
Fig 35 The IR spectrum of Mixture 5

Fig 36 The GLC analysis of standard steroids and Mixture **5**

-0.3 ttp4

Absorbance

Wavenumbers (cm-1)

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Scans: 32

Resolution: 16.000

143
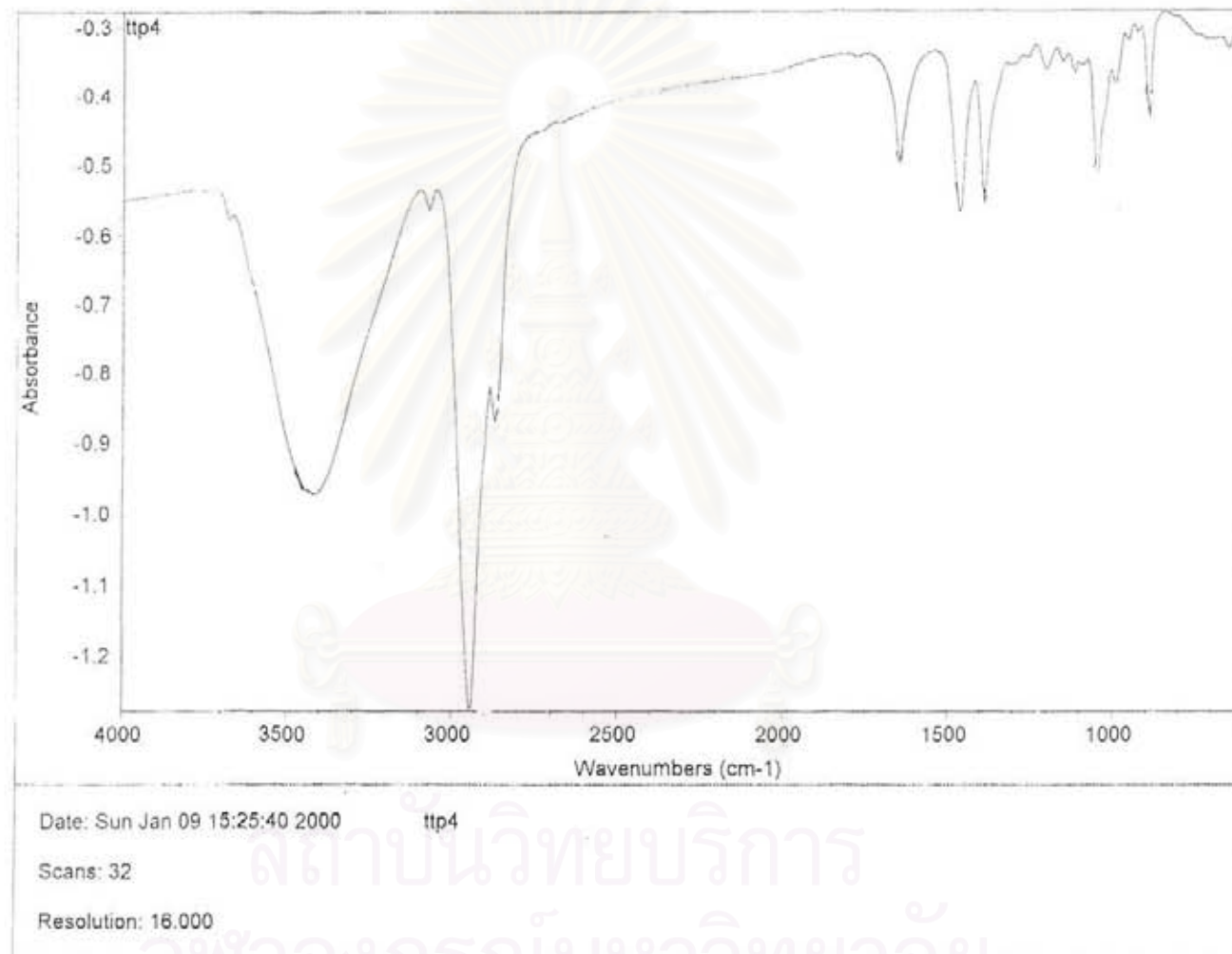
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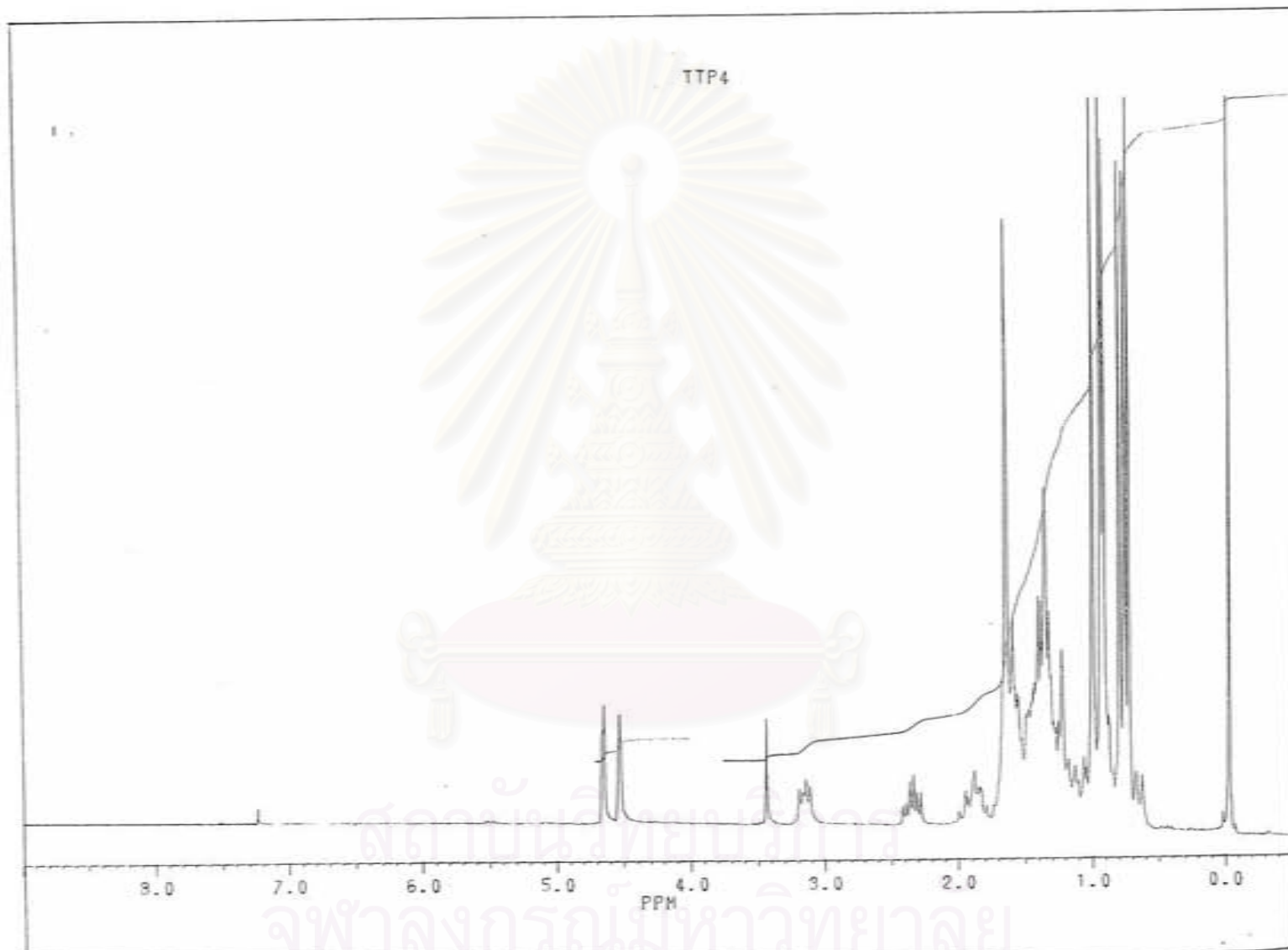
Fig 38 The ^{1}H NMR spectrum of Compound **6**
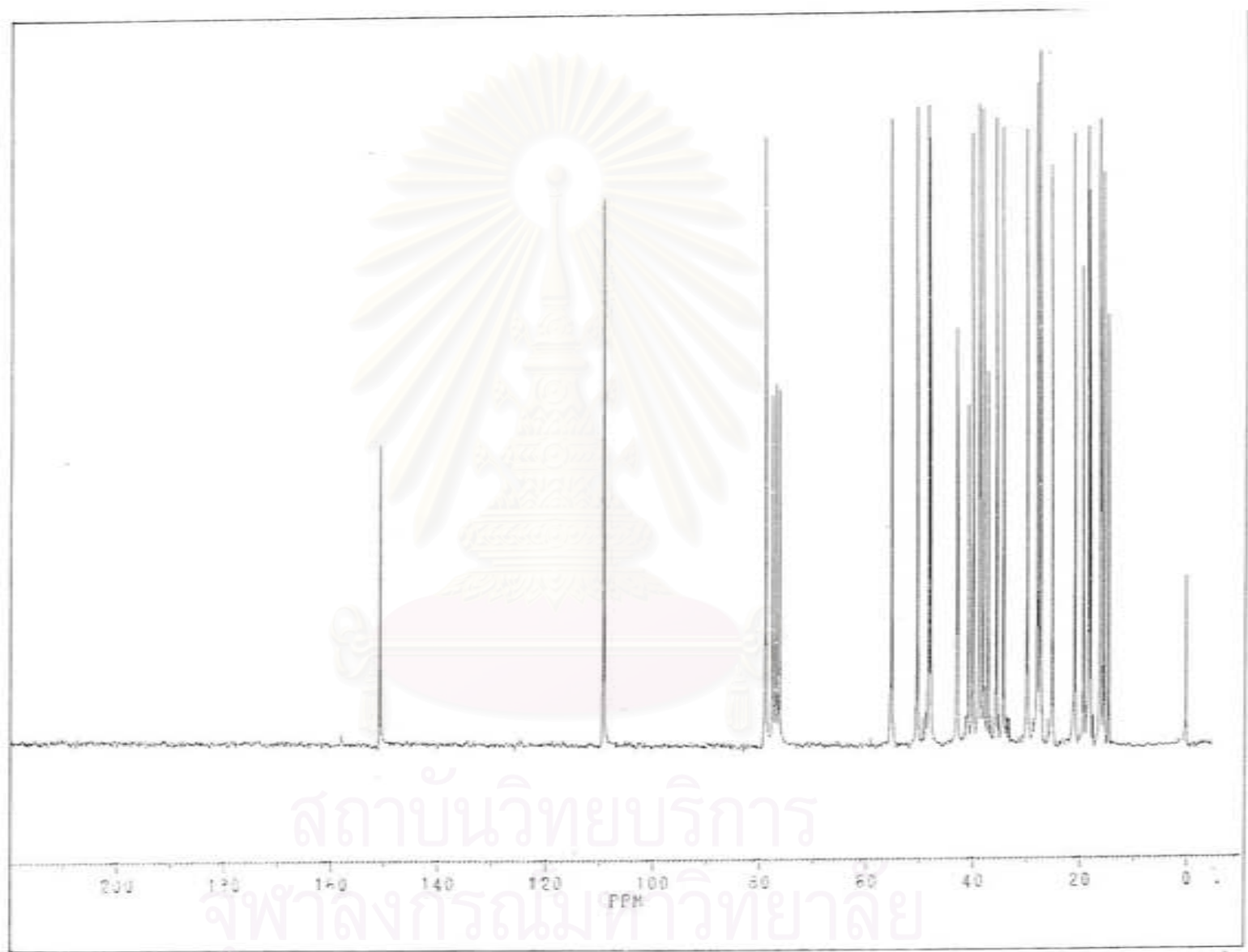
Fig 39 The ^{13}C NMR spectrum of Compound **6**

Fig 40 The mass spectrum of Compound **6**

Date: Sun Oct 31 12:50:09 1999   cinnamic acid

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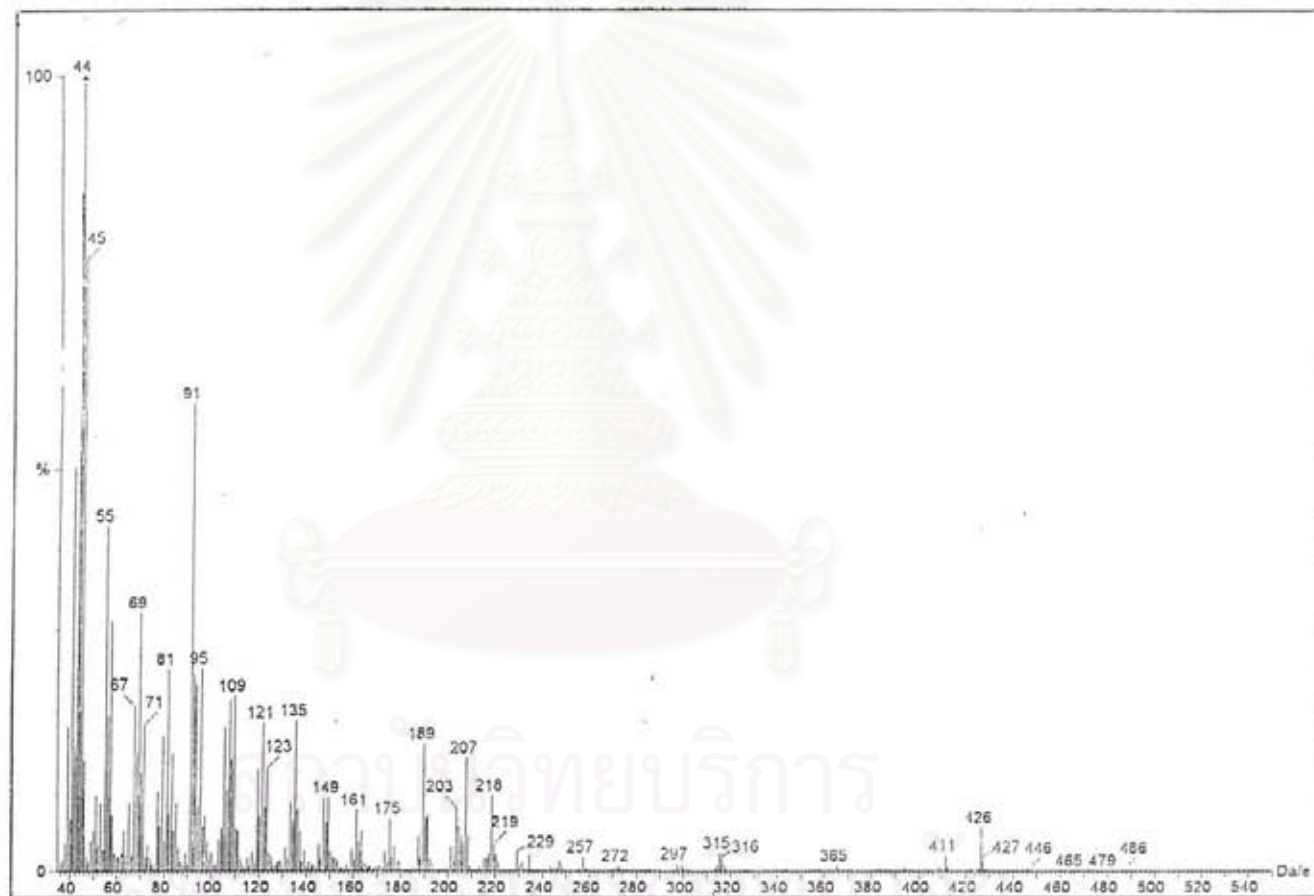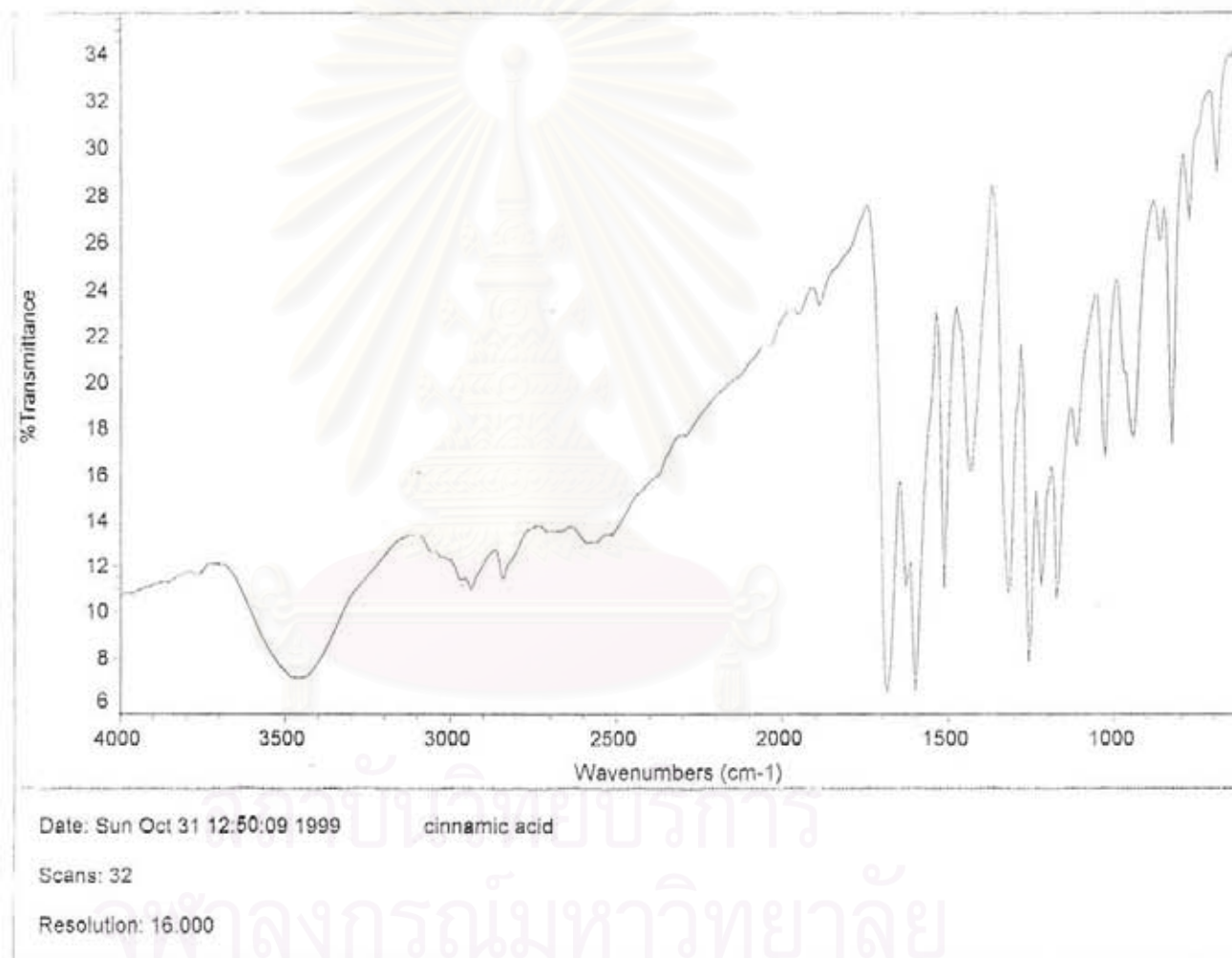
Fig 41 The IR spectrum of Compound 7
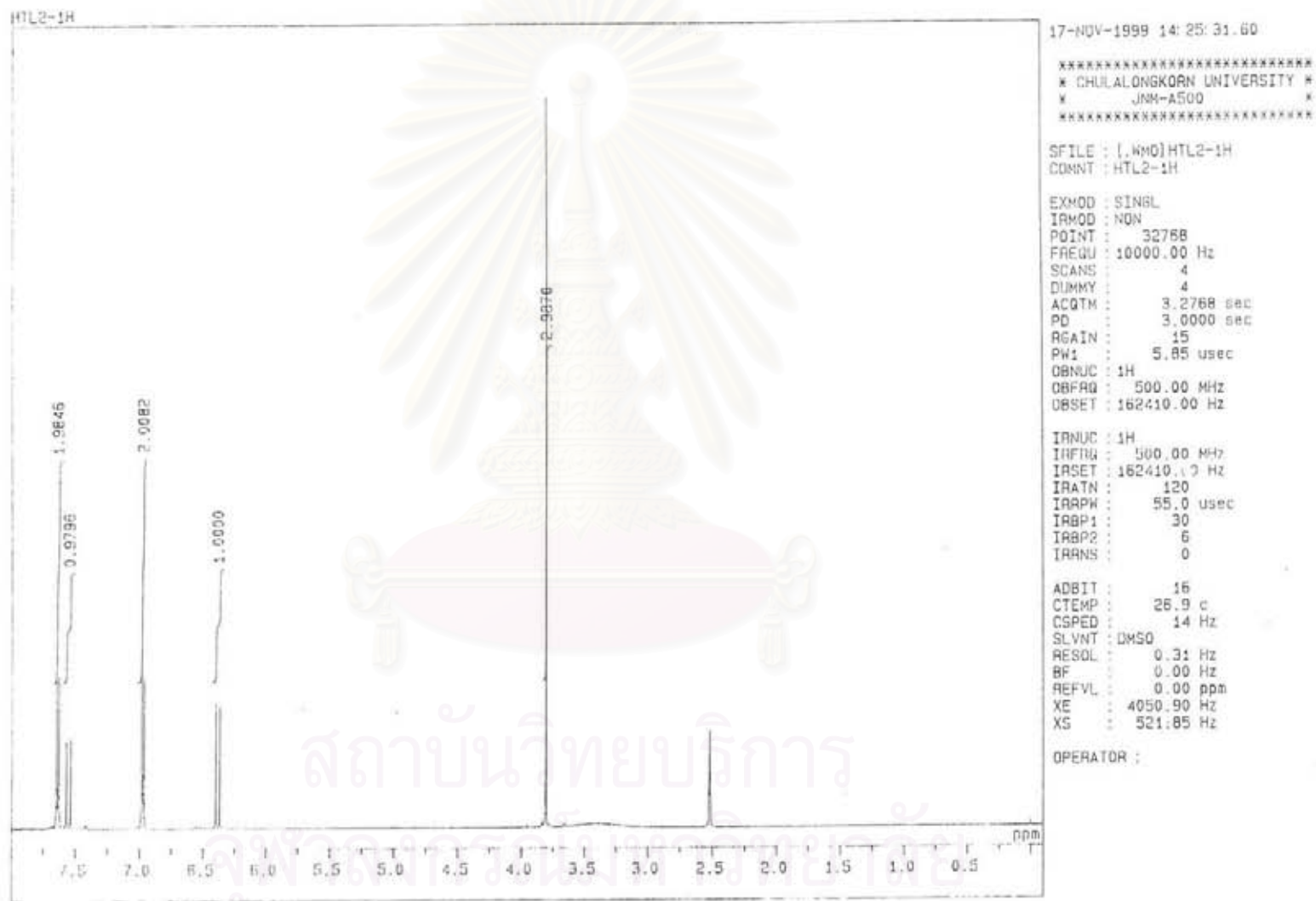
Fig 42 The ^{1}H NMR spectrum of Compound **7**
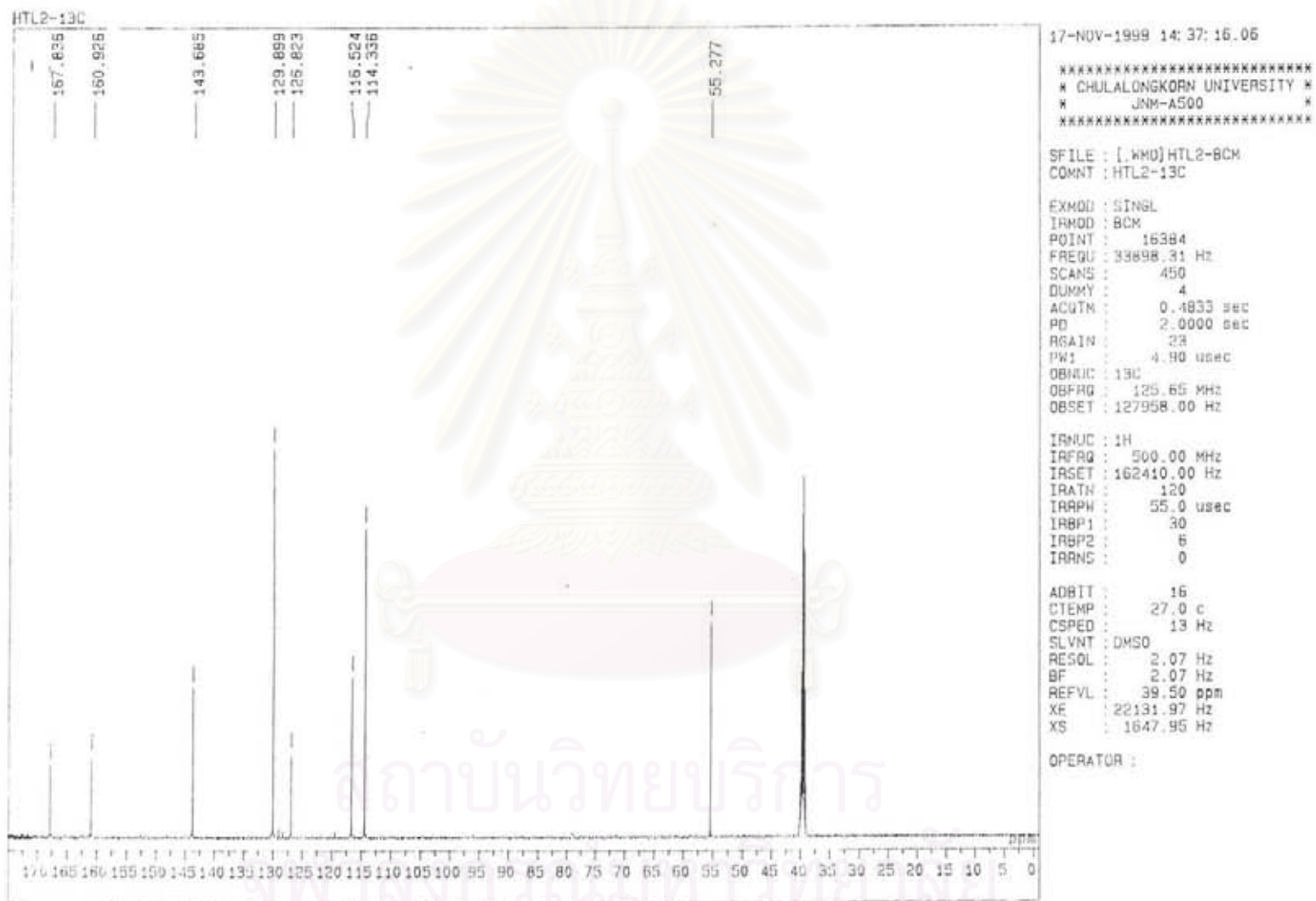
Fig 43 The ^{13}C NMR spectrum of Compound 7
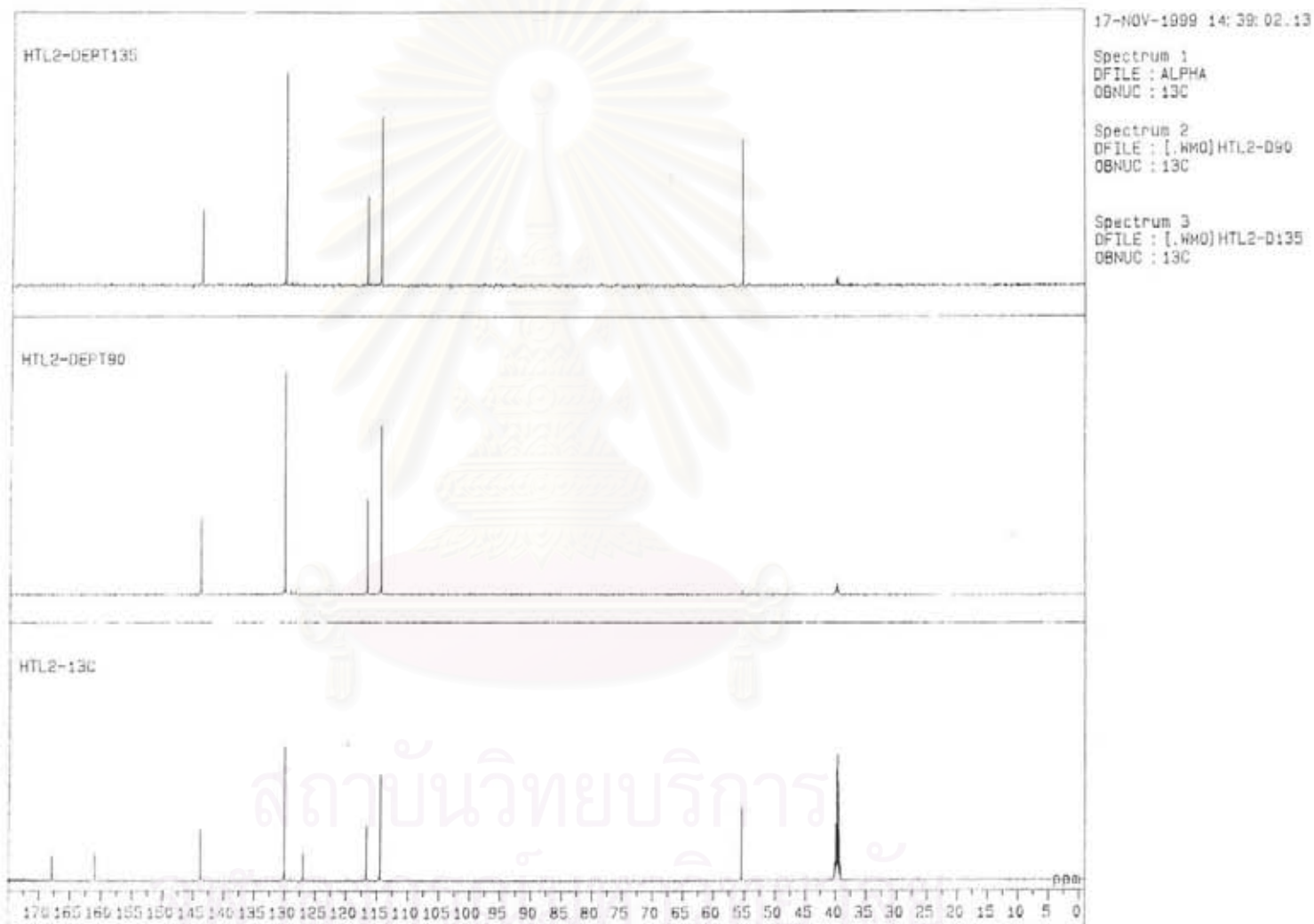
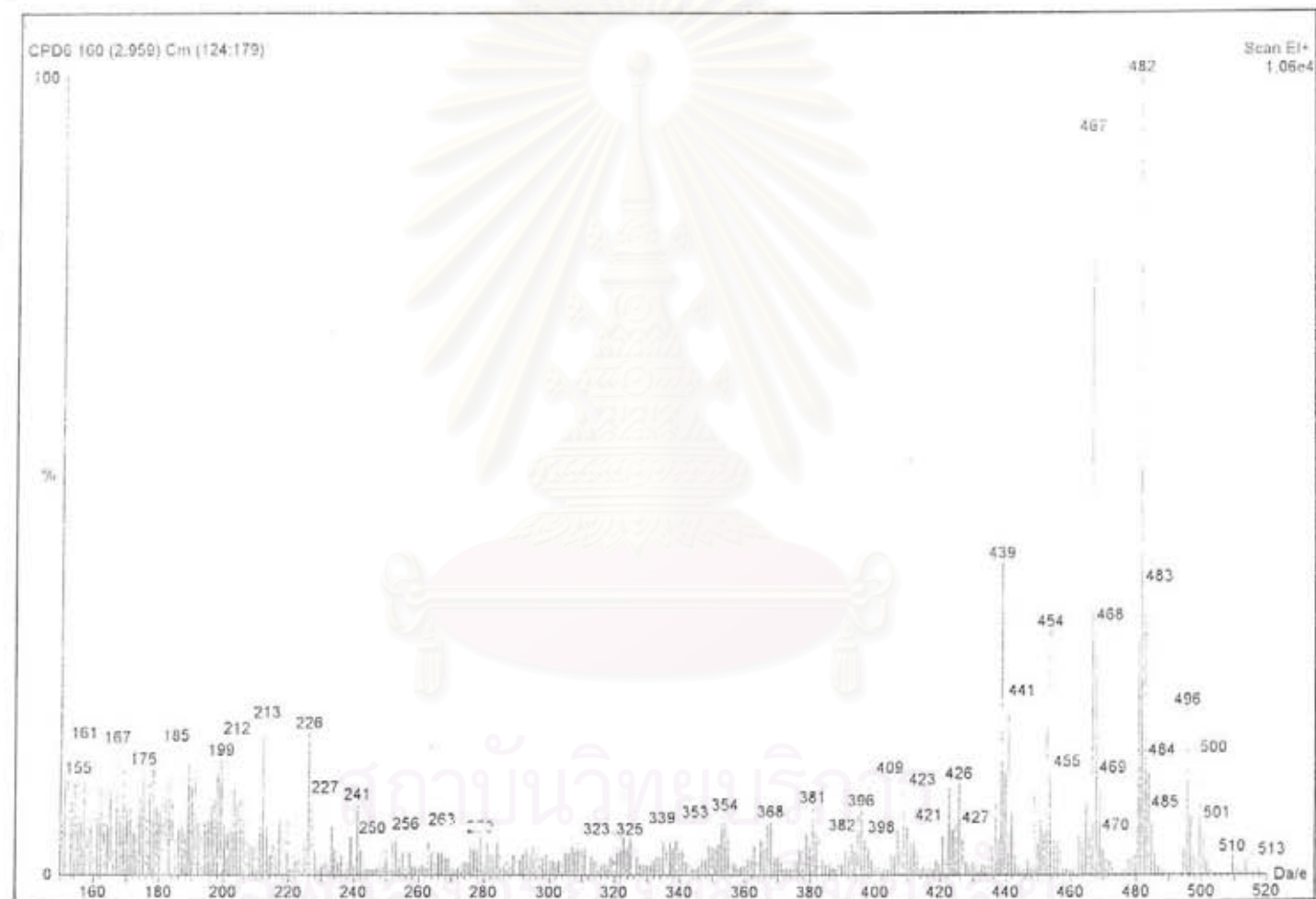**Fig 44** The DEPT 90 and 135 spectrum of Compound **7**
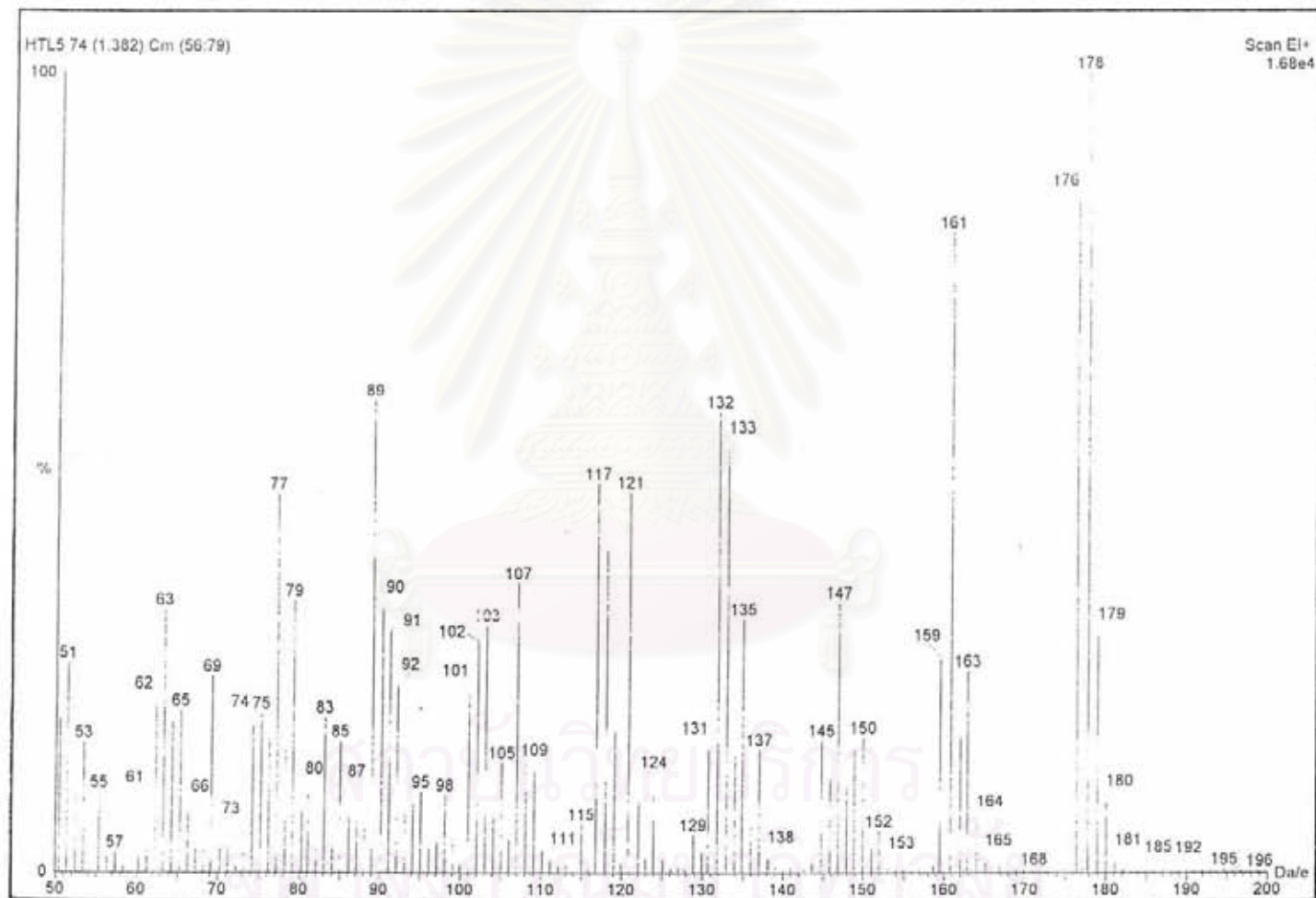
Fig 45 The mass spectrum of Compound 7
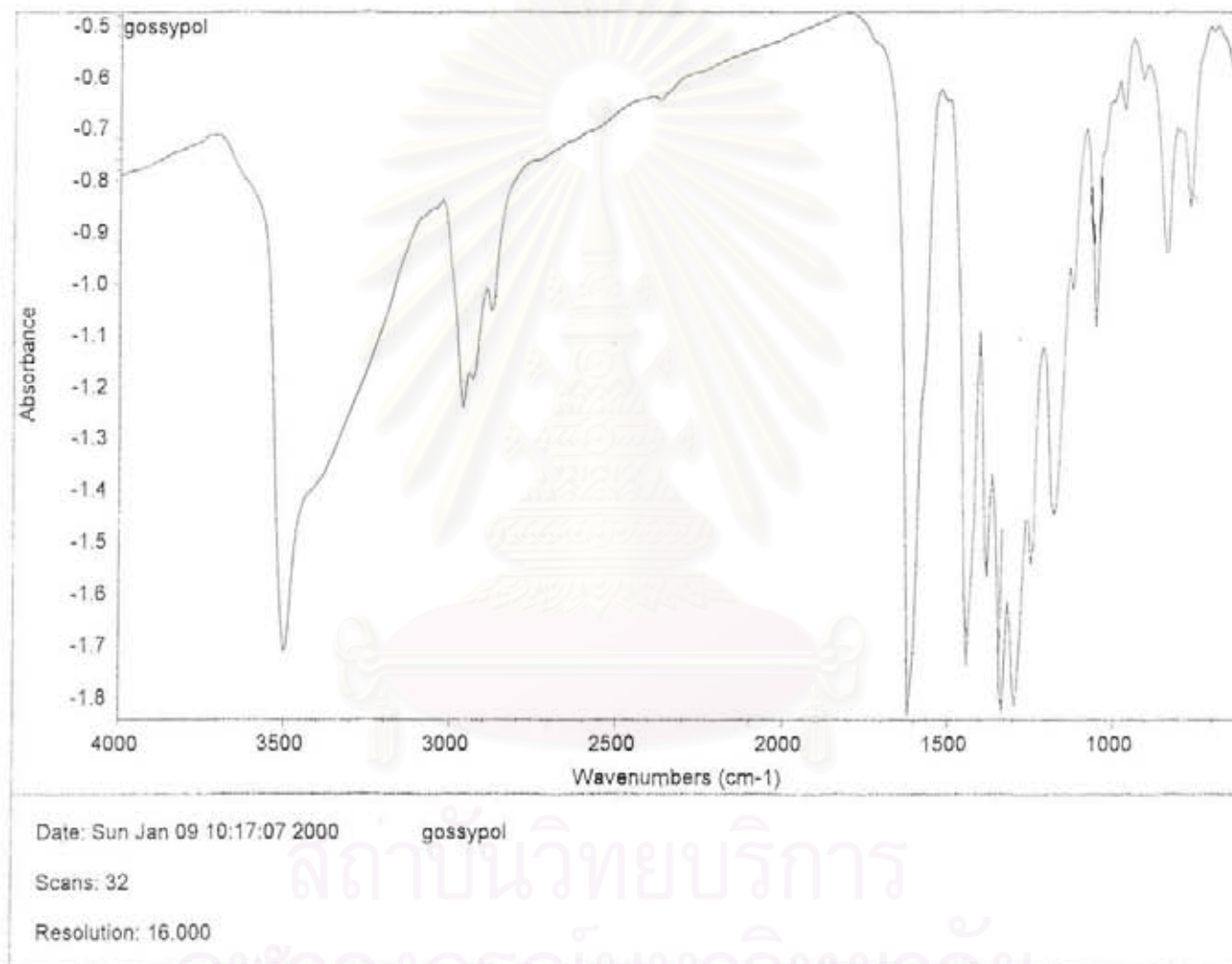
Fig 46 The IR spectrum of Compound 8
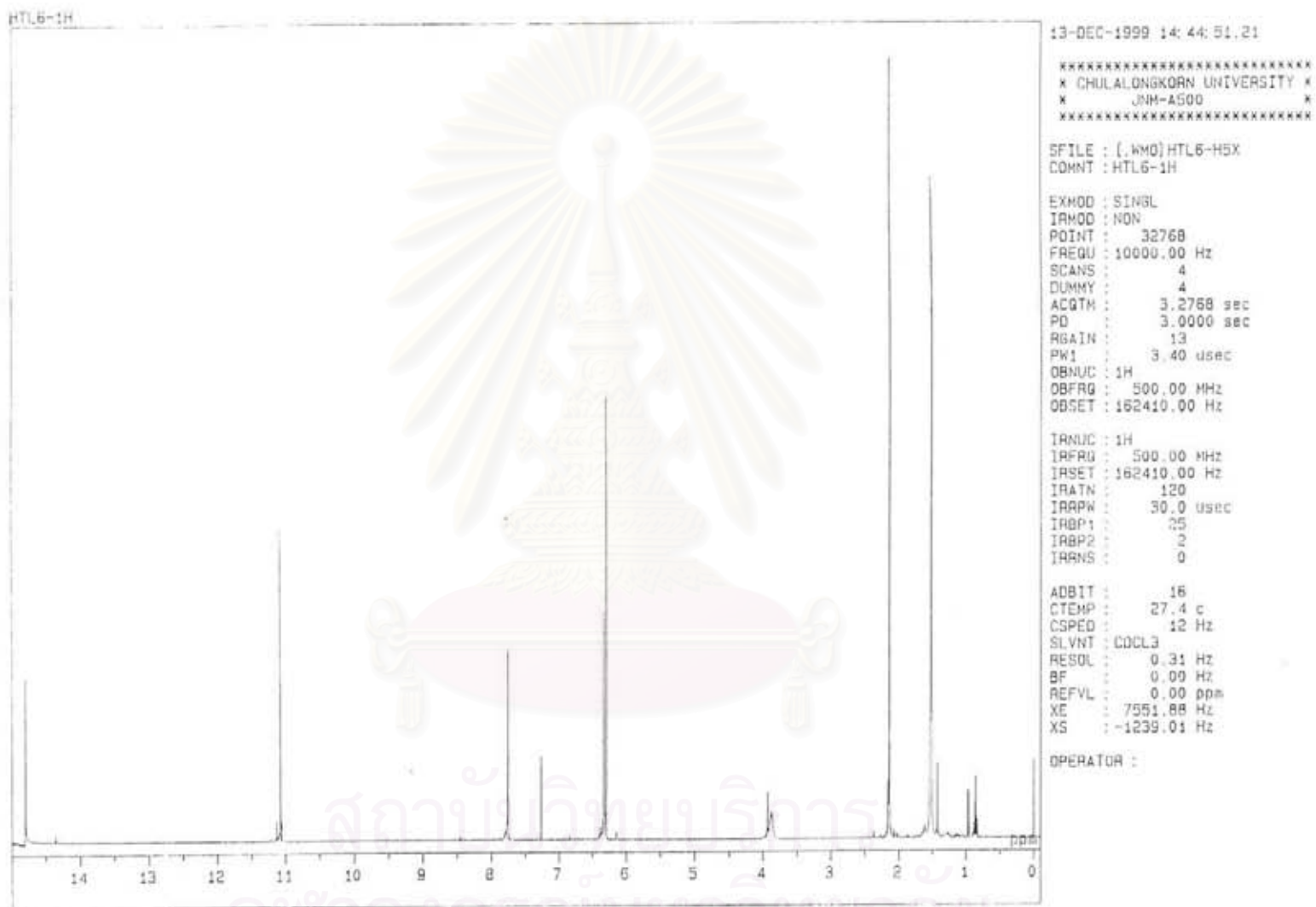
Fig 47 The ^{1}H NMR spectrum of Compound **8**
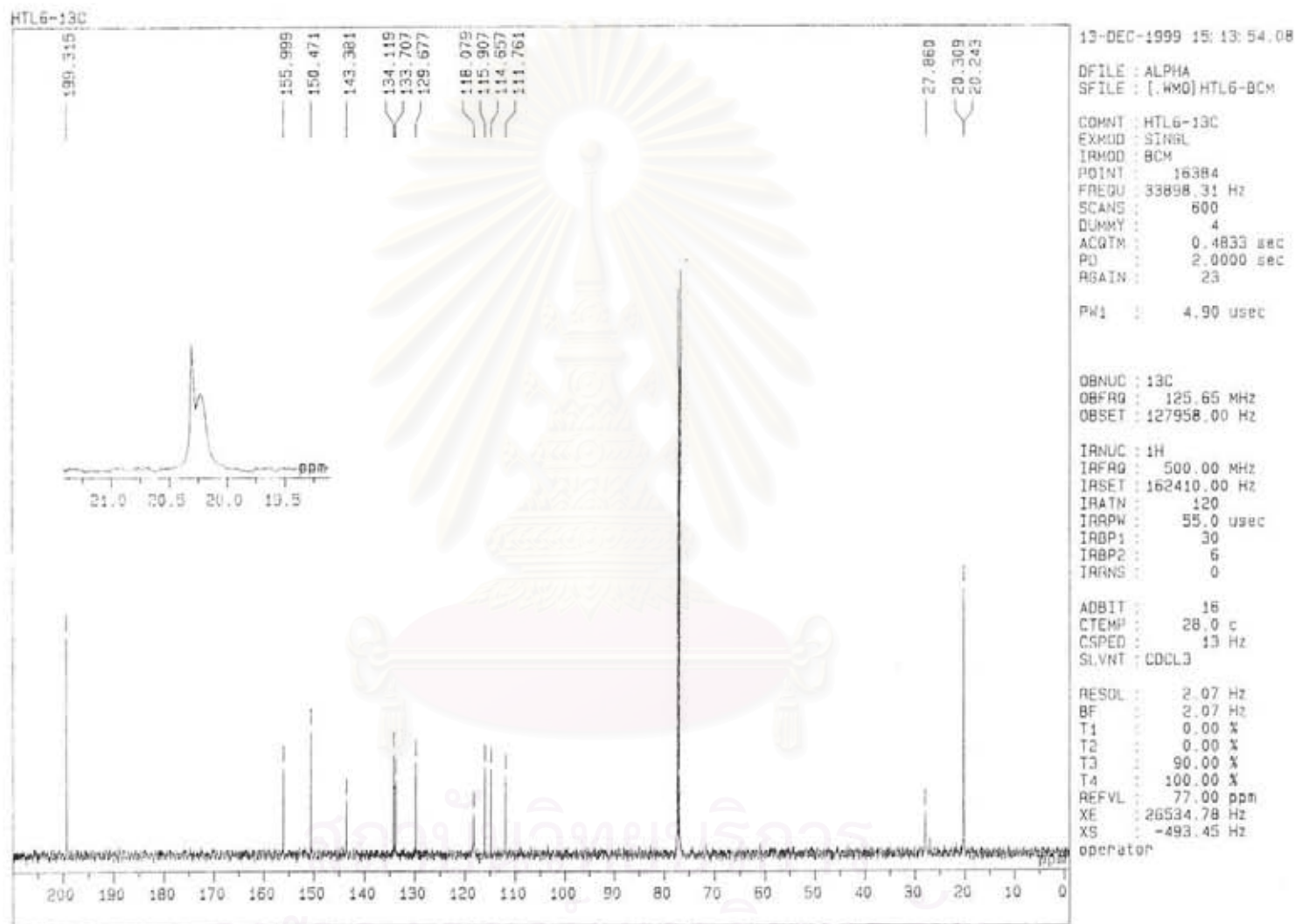
Fig 48 The ^{13}C NMR spectrum of Compound **8**
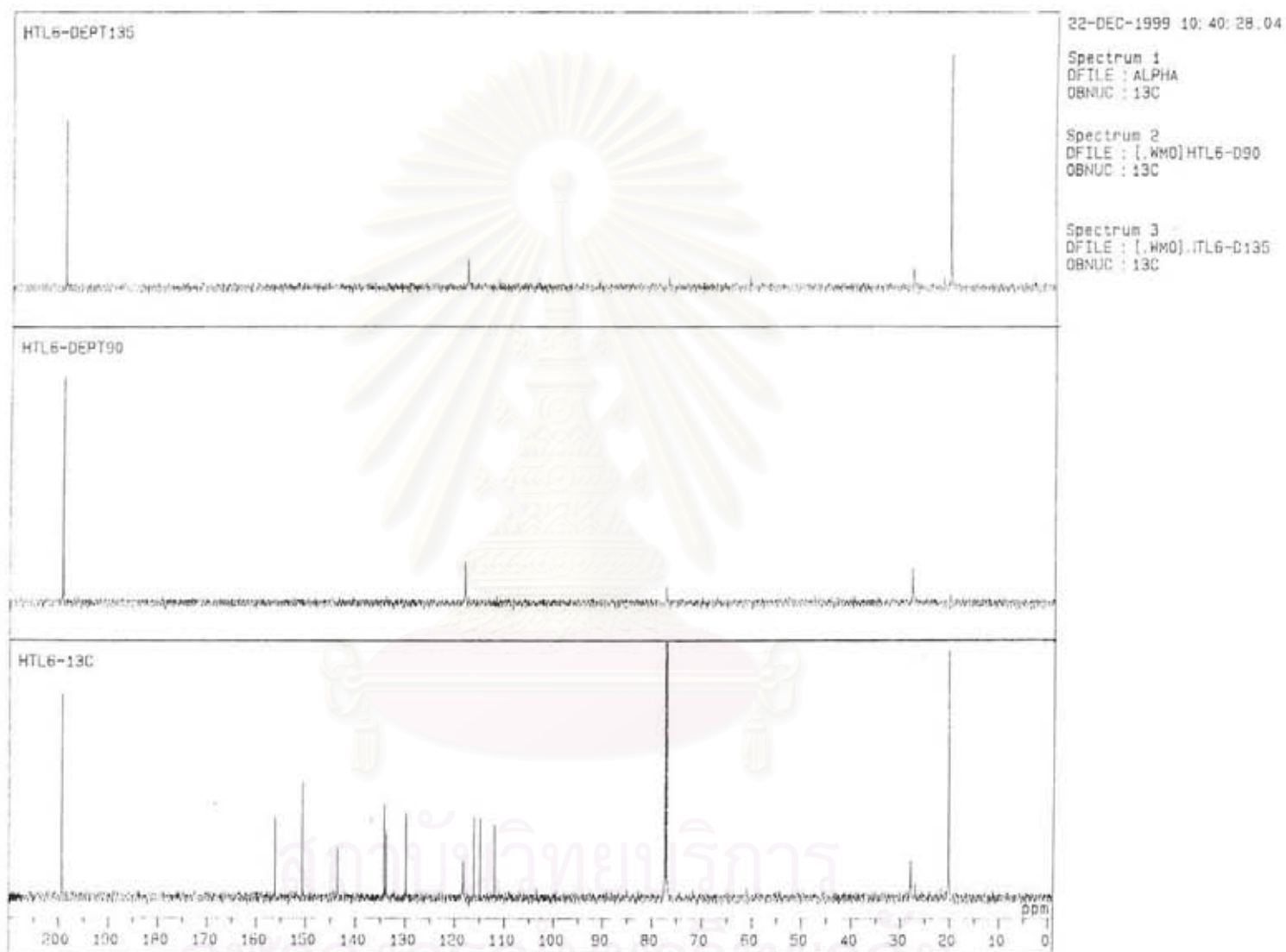
Fig 49 The DEPT 90 and 135 spectrum of Compound **8**

Fig 50 The mass spectrum of Compound **8**
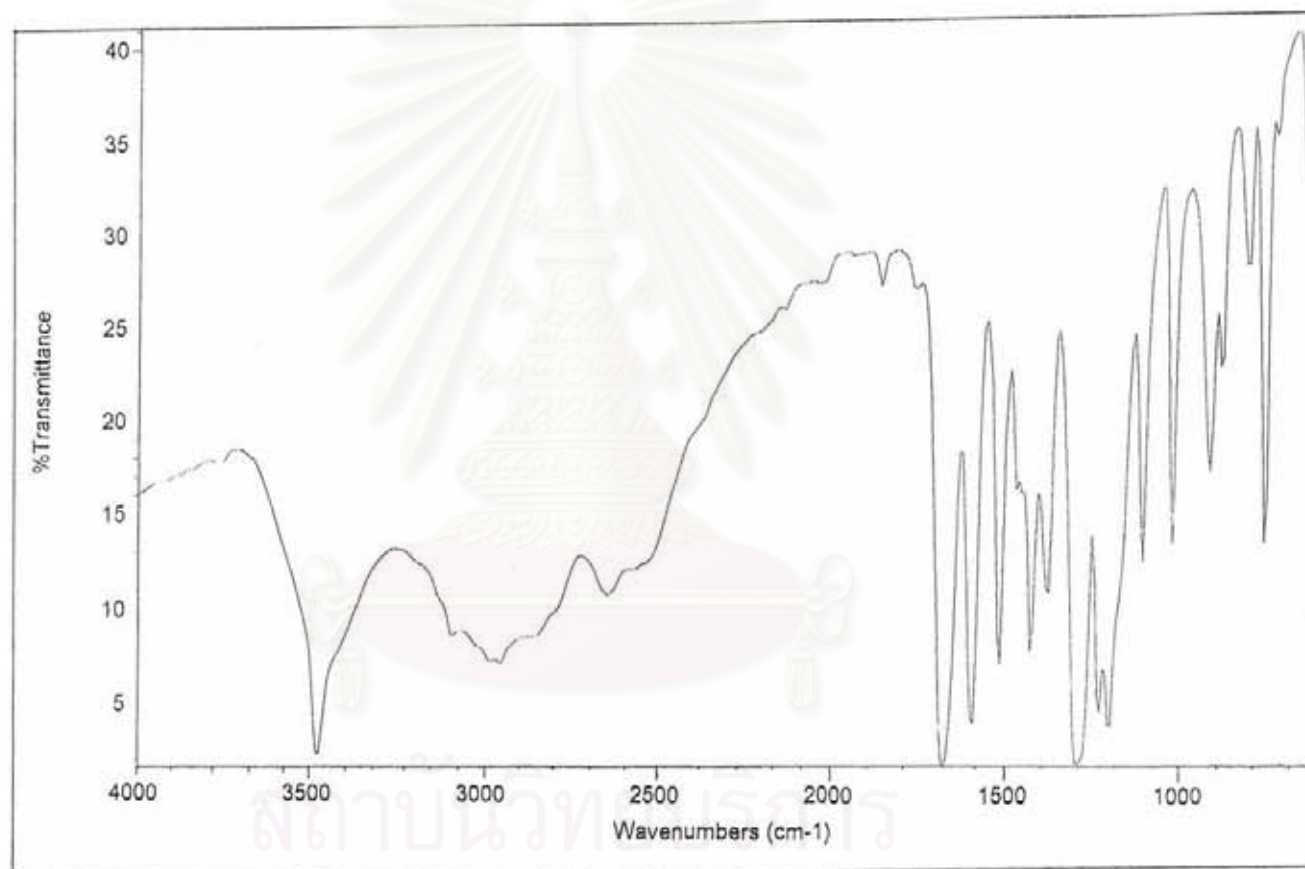
Fig 51 The IR spectrum of Compound **9**

HTL3-1H
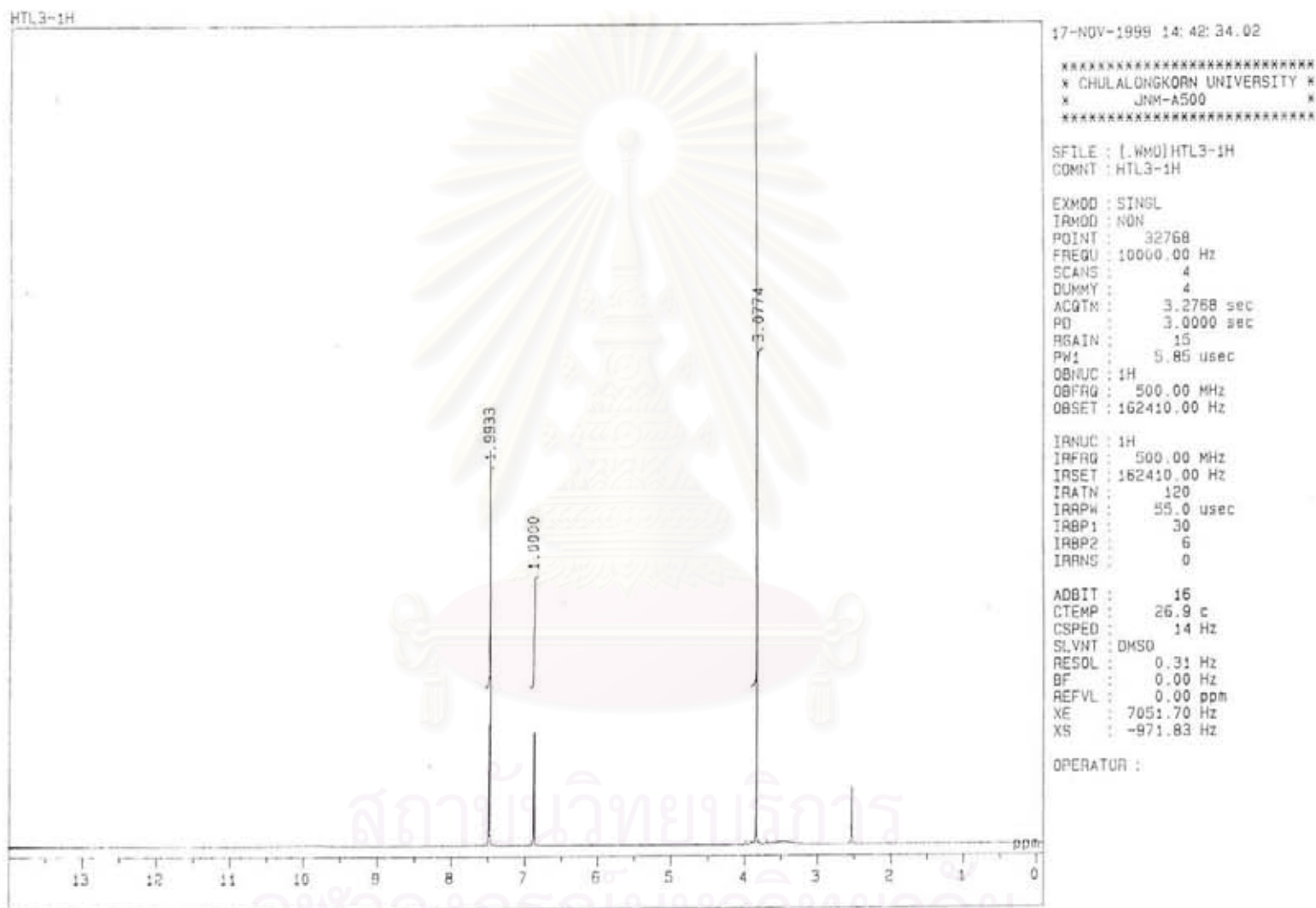
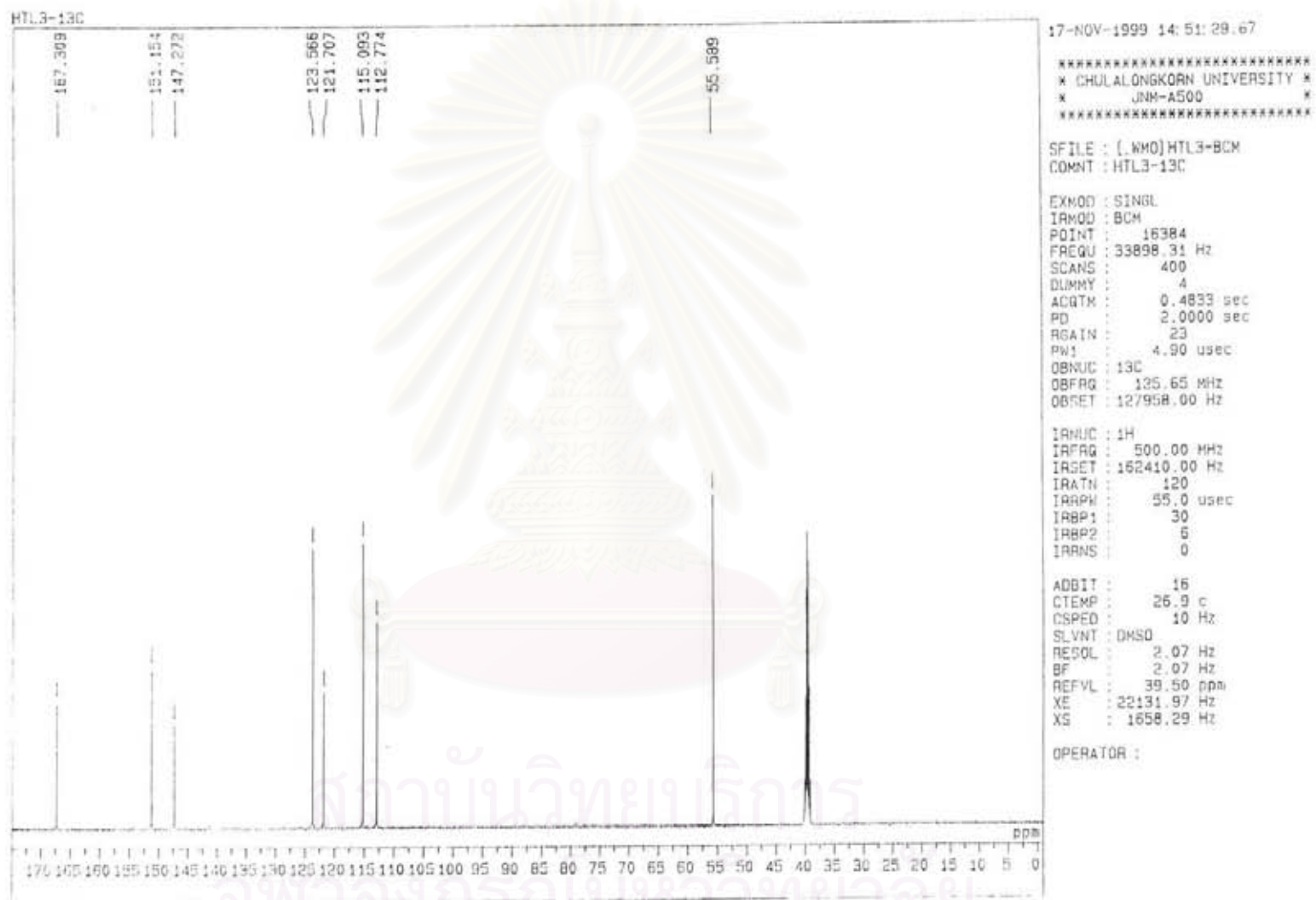Fig 52 The ^{1}H NMR spectrum of Compound 9

158

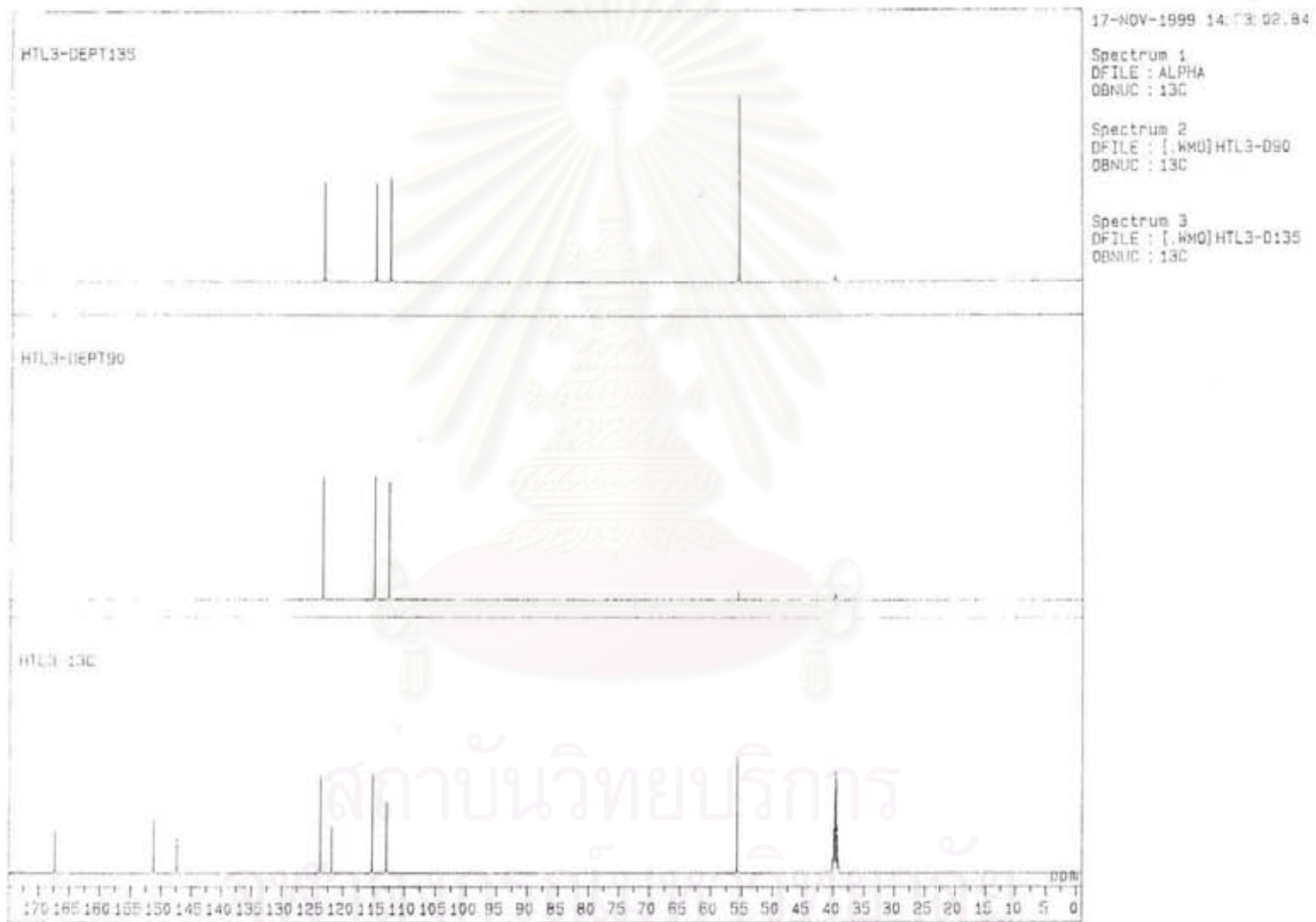**Fig 53** The ^{13}C NMR spectrum of Compound **9**

Fig 54 The DEPT 90 and 135 spectrum of Compound 9

VITA

Mr. Konkiat Pongrod was born on February 27, 1975 in Bangkok, Thailand. He received a Bachelor Degree of Science, majoring in Chemistry from Mahidol University. In 1997, he has been a graduate studying Organic Chemistry at Chulalongkorn University. He was supported by a research grant for his master degree's thesis from the graduate school, Chulalongkorn University and from Natural Products Research Unit of Department of Chemistry, Chulalongkorn University. His present address is 20/1 Soi. Jomthong 13, Jomthong Rd., Jomthong, Bangkok 10150.