คอเรนไดเทอร์พีน จากเปลือกต้นเปล้าใหญ่ *Croton oblongifolius* Roxb.
จากกุยบุรี จังหวัดประจวบคีรีขันธ์

นางสาวสุภาพร ศิริมงคล

KAURANE DITERPENES FROM STEM BARK OF *Croton oblongifolius* Roxb.
FROM KUI BURI, PRACHUAP KHIRI KHAN PROVINCE

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A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science in Chemistry
Department of Chemistry
Faculty of Science
Chulalongkorn University
Academic Year 2000

Thesis Title   KAURANE DITERPENES FROM STEM BARK OF *Croton oblongifolius* Roxb. FROM KUI BURI, PRACHUAP KHIRI KHAN PROVINCE

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สุภาพร ศิริมงคล : คอเรนไดเทอร์พืน จากเปลือกต้นเปล้าใหญ่ *Croton oblongifolius* Roxb. จากกุยบุรี จังหวัดประจวบคีรีขันธ์ (KAURANE DITERPENES FROM STEM BARK OF *Croton oblongifolius* Roxb. FROM KUI BURI PRACHUAP KHIRI KHAN PROVINCE) .

อาจารย์ที่ปรึกษา : รศ. ดร. โสภณ   เริงสำราญ, อาจารย์ที่ปรึกษาร่วม : อ.ดร. นาตยา งามโรจนวณิชย์ 110 หน้า. ISBN 974-346-996-6.

สกัดเปลือกต้นเปล้าใหญ่ *Croton oblongifolius* Roxb. แห้งและบดละเอียด ด้วยตัวทำละลายสารอินทรีย์ ประกอบด้วย เฮกเซน เอธิลอะซิเตต และเมทานอล ระเหยแยกตัวทำละลายออกจากสารสกัดแต่ละชนิดโดยวิธีลดความดัน จะได้รับสารสกัดหยาบจาก สารสกัดเฮกเซน เอธิลอะซิเตต และเมทานอล ตามลำดับ นำสารสกัดหยาบแต่ละชนิด ไปทำการแยกด้วยเทคนิคทางคอลัมน์โครมาโตกราฟีแยกสารได้สามชนิดทำการหาสูตรโครงสร้างของสารที่แยกได้ทั้งสามชนิดโดยอาศัยสมบัติทางกายภาพ ทางเคมี และข้อมูลทางสเปกโตรสโคปี สามารถพิสูจน์โครงสร้างได้ คือ kaur-16-en-19-oic acid (1) hard wickiic acid (2) และ สารผสมของ β-Sitosterol กับ Stigmasterol (3) จากการวิจัยพบว่าคูเรนไดเทอร์พืนนี้พบเป็นครั้งแรกในต้นเปล้าใหญ่ และได้ทำการสังเคราะห์อนุพันธ์ของคูเรนไดเทอร์พืน kaur-16-en-19-oic acid (1) สี่ชนิดคือ methyl kaur-16-en-19-oate (1a), kaur-16-en-19-ol (1b), 16,17-epoxy-kauran-19-oic acid (1c) และ 17-hydroxykaur-15-en-19-oic-acid (1d) เมื่อได้นำสารประกอบไดเทอร์พืนที่แยกได้จากธรรมชาติ และอนุพันธ์ต่างๆของสารดังกล่าว ไปทดสอบฤทธิ์ยับยั้งเซลล์มะเร็ง พบว่าสารเหล่านี้มีฤทธิ์ปานกลางจนถึงมีฤทธิ์น้อย

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

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

ปีการศึกษา...........2543..................   ลายมือชื่ออาจารย์ที่ปรึกษาร่วม……………..…...

SUPAPORN SIRIMONGKHON: KAURANE DITERPENES FROM STEM BARK OF *Croton oblongifolius* Roxb. FROM KUIBURI PRACHUAP KHIRI KHAN PROVINCE. THESIS ADVISOR : ASSOC. PROF. SOPHON ROEGNSUMRAN, Ph.D. THESIS Co-ADVISOR : Dr. NATTAYA NGAMROJNAVANICH 110 pp. ISBN 974-346-996-6.

The grounded air-dried stem barks of *Croton oblongifolius* Roxb. was extracted subsequently with organic solvents including hexane, ethyl acetate and methanol. The solvents in each crude extract were evaporated by evaporation under reduce pressure to obtain hexane extract crude, ethyl acetate extract crude and methanol extract crude, respectively. Each extract crude was isolated and purified using the column chromatography technique to result in three compounds. The structure of these compounds were characterized using their physical and chemical properties and spectral data. The structure of compound (1), compound (2), and compound (3) were proved to be kaur-16-en-19-oic acid (1), hardwickiic acid (2) and mixture of β-sitosterol and stigmasterol, respectively. This research indicated that the kaurane diterpene compound was first observed from the plant of *C. oblongifolius*. The compound (1) derivatives, including methyl kaur-16-en-19-oate (1a), kaur-16-en-19-ol (1b), 16,17-epoxy-kauran-19-oic acid (1c), and 17-hydroxykaur-15-en-19-oic acid(1d) were synthesized. The cytotoxic activity of isolated natural diterpenoids and their derivatives were assayed against cancer cell lines. The result indicated that most of tested compounds show a weak to moderate activity.

Department……….Chemistry………..Student's signature. …………..…………….

Field of study……..Chemistry…..…….Advisor's signature. …………………………

Academic year………2000………..…..Co- advisor's signature………..…………….

ACKNOWLEDGEMENT

First of all, the author wishes to express the deepest gratitude to her advisor,: Dr. Sophon Roengsumran and co-advisor Dr. Nattaya Ngamrojnavanich for invaluable advice, encouragement, and above all them inspirational comments. Besides, them complete understanding and deep insight into the organic chemistry has not only made work on this study a rewarding experience, but also has given precious experiences which the author wishes to apply in her future research. In addition, she would like to give a deep thanks to Dr. Amorn Petsom, for his excellent instruction, guidance, encouragement and support throughout this dissertation.

Grateful acknowledgements are made to Dr. Udom Kokphon, Dr. Chaiyo Chaichantipyuth and Dr. Anongrat Karntaing for serving as Examination Committee Members. Innumerable thanks are extended to Dr. Tirayut Vilaivan for guiding the research work and Dr. Nongnuj Jaiboon and Dr. Narongsak Chaichit for helping the analysis and determined by x-ray diffraction analysis.

The author also wishes to extend her profound thanks to all her friends for their cooperation and mental supports during this work. The financial supports of the Graduate School, Chulalongkorn University is also gratefully acknowledged. Pursuing the master program at Chulalongkorn University would have been impossible without this financial support.

Last but not least the author would like to dedicate this master thesis with great respect and love to her parents for all things that they have endured and sacrificed for her success.

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จุฬาลงกรณ์มหาวิทยาลัย

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LIST OF ABBREVIATIONS

TMS	tetramethylsilane
Hz	Hertz
ppm	part per million
δ	Chemical shift
s	singlet (NMS)
d	doublet (MR)
t	triplet (NMR)
q	quartet (NMR)
dd	double doublet
ddd	double doublet doublet
dt	double triplet
cm^{-1}	unit of wave number
M^{+}	molecular ion
m/z	mass to charge ratio
M.W.	molecular weight
ν_{max}	the wavelength at maximum sbsorption
br	broad
s	strong
m	medium
w	weak
%	percent
m.p.	melting point
Fig.	Figure
$^{\circ}$C	degree celsius
ml	milliliter (s)
mg	miligram
g	gram (s)
TLC	Thin Layer Chromatography
wt	weight

DEPT	Distortionless Enhancement by Polarisation Transfer
HMQC	Heteronuclear Multiple Quantum Correlation
HMBC	Heteronuclear Multiple Bond Correlation
COSY	Correlated Spectroscopy
NOESY	Nuclear Overhauser Enhancement Spectroscopy

CHAPTER I

INTRODUCTION

Croton oblongifolius Roxb.(Plao Yai) [1] belongs to the Euphorbiaceae family. It is a local medicinal plant, which has therapeutic properties. In Thailand, most researchers have concentrated on chemical constituents of a variety of *Croton*, especially *Croton oblongifolius* Roxb.(Plao Yai), *Croton sublyratus* Kurz. (Plao Noi), *Croton cascarilloide* Raeusch.(Plao Ngoen) and *Croton hutchisonianus* Hoss. (Plao Pae).

According to the study of *Croton oblongifolius* Roxb.(Plao Yai) from different areas, it was found that the chemical constituents are differently affected according to biological activities [2-8]. *Croton robustus* Kurz.(Plao Lueat) can be used as an antianemic agent. *Croton sublyratus* Kurz. (Plao Noi), Plaunotol from Plao Noi is an effective antipeptic ulcer drug available commercially. The barks and leaves of Plao Noi can be used as an antiulceric agent [9-11]. Moreover, plenty of interesting chemical constituents are found in Plao Ngoen [12] and Plao Pae [13]. Plao Ngoen can also be used as an antifebrile.

Croton oblongifolius Roxb. is one very interesting Thai medicinal plant because it is believed that all parts of the plant can be used as drugs. The leaves can be used as a tonic, the flowers are used as a teniacide, the fruits are used to treat dysmenorrhea, the seeds are used as a purgative, the barks are used to treat dyspepsia, and the roots are used to treat dysentery [14]. Moreover, this plant is widely distributed throughout Thailand.

1.1 General Characteristic of *Croton oblongifolius* Roxb.

Croton oblongifolius Roxb. is a medium sized deciduous tree in the Euphorbiaceae family. There are about 700 species in this family. In Thailand, it is commonly called Plao Yai (central) or Plao Luang (Northern). It is distributed throughout forests or shrubs below 700 meters above sea level. Its calyx and ovary are clothed with minute orbicular silvery scales. Leaves are 5.6-12.0 by 13.0-24.0 cm in

size. The shape of leaf blade is oblong-lanceolate. Its flowers are pale yellowish green and solitary in the axials of minute bracts on long erect racemes. The male flowers are located in the upper part of the length of pedicels of 4.0 mm. The calyx is more than 6.0 mm. long and segments are woolly. The twelve stamens are inflexed in bud and the length of filaments is 3.0 mm. In female flowers, the pedicels are short and stout. Its sepals are more acute than in the male with densely cliated margins. The diameter of the fruit is less the 1.3 cm., slightly 3-lobed and clothed with small orbicular and quite smooth on the back [15,16,17,18]. The picture of *Croton oblongifolius* Roxb. is shown in Fig.1.

From previous studies, the difference of diterpenoid compound were found in *Croton oblongifolius* Roxb. and some compound have been shown to inhibit the growth of cancer cells[6]. Therefore, *Croton oblongifolius* Roxb. contains a variety of diterpenoid compounds. To continue the investigation of *Croton oblongifolius* Roxb. plants from Amphoe Kuiburi Prachuap khiri khan province were studied. The NMR-screening of hexane extract crude indicated that this crude extract contained different diterpenoids which have been found previously. Therefore, it is of interest to study to chemical components as well as the biological activities of *Croton oblongifolius* Roxb., which was collected from Amphoe Kui buri, Prachuap khiri khan province.

1.2 The objectives of this research

1. To extract, isolate and purify the organic constituents from the stem barks of *Croton oblongifolius* Roxb. amphoe Kui buri, Prachuap khiri khan province.
2. To identify the structure of the isolated compounds which were obtained from the stem barks of *Croton oblongifolius* Roxb.
3. To determine the bioactivities of isolated compounds which were obtained from the stem barks of *Croton oblongifolius* Roxb.

Figure 1 The picture of *Croton oblongifolius* Roxb.

CHAPTER II

LITERATURE REVIEWS

2.1 Previous Study of Diterpenoid Compounds from *Croton oblongifolius* Roxb.

From the literature surveys, *Croton oblongifolius* Roxb. has been widely studied and many diterpenoid compounds have been isolated and characterized. According to the observation of chemical constituents of *Croton oblongifolius* Roxb. from various locations in Thailand, it was found that the main components have different types of structure. The chemical constituents found in *C. oblongifolius.* could be categorized into seven groups including cembrane diterpenoid, clerodane, labdane, halimane, pimarane, isopimarane and cleistanthane diterpenoid compounds, which are shown in Table 1.

Table 1 Chemical Constituents of *Croton oblongifolius* Roxb.

Plant parts	Substances	References
Bark	**cembrane diterpenoid** crotocembraneic acid neocrotocembraneic acid	6,7
	Poilaneic acid neocrotocembranal	6,19
Bark and Wood	**clerodane diterpenoid** (-)-Hard wickiic acid 11-Dehydro(-)-hardwickiic acid	21

Table 1 Chemical Constituents of *Croton oblongifolius* Roxb.(continue)

Plant parts	Substances	References
Bark	**labdane diterpenoid** **R** = CH₃ labda-7,12(*E*),14-triene = CHO labda-7-12(*E*),14-triene-17-al = CH₂OH labda-7,12(*E*),14-triene-17-ol = COOH labda-7,12(*E*),14-triene-17-oic acid	22
Bark	**Halimane diterpenoid** Crotohalimoneic acid Crotohalimaneic acid Benzoyl crotohalimanolic acid	6
Bark and Wood	**Pimarane diterpenoid** 19-Deoxyoblongifoliol Oblongilic acid	8,23

Table 1 Chemical Constituents of *Croton oblongifolius* Roxb.(continue)

Plant parts	Substances	References
Bark and Wood	**Pimarane diterpenoid** (continue) Oblongifoliol	24
Bark and Wood	**Isopimarane diterpenoid** ent-Isopimara-7,15-diene ent-Isopimara-7,15-diene-19-aldehyde	20,25
Bark	**Cleistanthane diterpenoid** Cleistantha-4,13,15-triene-3-oic acid	26

2.2 Biological activity review of diterpene compounds from *C.oblongifolius*

Diterpenoid compounds from *C. oblongifolius.* show biological activity such as cAMP phosphodiesterase inhibition, antimicrobial, antiplatelet aggregation, cytoxicity etc.

For example, the cemberane diterpene compound, neocrotocembranal [6], has activity against human tumor cell lines (P 388 cell line and 6 tumor cell lines; S-102 (hepatoma), Hep-G2 (hepatoma), SW 620 (colon), Chago (lung), Kato-3 (gastric), BT 474 (breast)). Crotocembraneic acid and neocrotocembraneic acid [6] have cAMP phosphodiesterase inhibitory activity.

The labdane diterpene, compounds from Prachub Kirikhun [22] have activity against human tumor cell lines and also show the antiplatelet aggregation activity.

The clerodane diterpene compoundss , for example hardwickiic acid [27], show antimicrobial activity.

The cleistanthane diterpene, compounds from Loei [26], have activity against human tumor cell lines.

Moreover, other diterpenoid compounds had been isolated from *C. oblongifolius.* such as, pimarane diterpene, compounds. These compounds were isolated from the aerial part of *Momordica balsamian*, showing antiviral activity against HIV. [28]

The isopimarane diterpene, compounds isolated from leaves of *Orthosiphon aristatus.*, show inhibitory activity on smooth muscle contractions caused by several stimulants. [29]

2.3 The literature reviews on Kaurane compounds

Table 2 Kaurane compounds from previously reports.
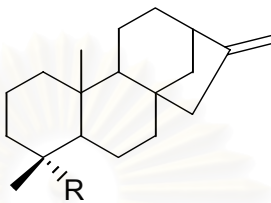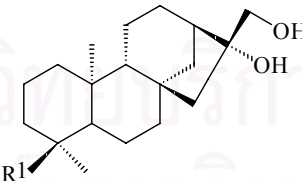
Botanical Names	Substances	References
Helianthus annus L.	R = COOH Kaur-16-en-19-oic acid = CH$_2$OH Kaur-16-en-19-ol	30
Iostephane madrensis	ent –kaur-16-en-19-oic acid 16α-hydroxy-ent-kaurane 15α-angeloyloxy-ent-kaur-16-en-19-oic acid 15α- hydroxy -ent-kaur-16-en-19-oic acid	31
Annona glabra	16α-methoxy-ent-kauran-19-oic acid 16α-hydro-ent-kauran-17-19-dimethyl ester 16α-acetoxy-ent-kauran-19-al-17-methyl ester 16α-hydro-19-ol-ent-kauran-17-oic acid	32
Croton hutchisonianus	R = H ent-kauran-16β,17-diol = CH$_2$OH ent-kauran-16β,17,18-triol	13
Mikania banisteriae	18,19-diacetoxy-ent-kaur-16-ene 17-oxo-ent-kaur-15(16)-en-18-oic acid ent-kaur-16-en-18-oic acid ent-kaur-16-en-18-ol	33

Table 2 Kaurane compounds from previously reports (continue)
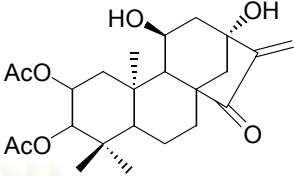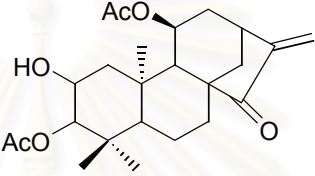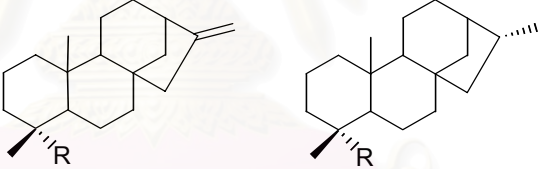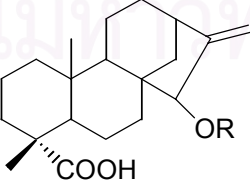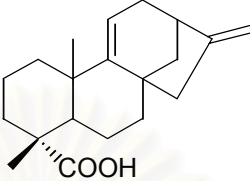
Botanical Names	Substances	References
Isodon rubescens	 2β-3β-diacetoxy-11β,13α-dihydroxy-ent-kaur-16-en-15-one 3β,11β-diacetoxy-2β,6α-dihydroxy-ent-kaur-16-en-15-one	34
Helianthus annuus	 Ia R= COOH IIa R= COOCH$_3$ Ib R= COOCH$_3$ IIb R= CH$_2$OH Ic R= CH$_2$OH IIc R= CH$_2$OOCCH$_3$ Id R= CH$_2$OOCCH$_3$	35
Mikania oblongifolia	 1a R= COCHCHPh 1b R= OH 1c R= Ac	36

Table 2 Kaurane compounds from previously reports (continue)

Botanical Names	Substances	References
Viguienra insignis	 ent-kaur-9(11)-16-dien-19-oic acid	37
Espolotiosis guacharaca	19-acetoxy-ent-kaurene 17-oxo-ent-kaur-15-en-19-oic acid ent-kaur-9(11),16(17)-dien-19-oic acid 12β-hydroxy-ent-kaur-9(11),16(17)-dien-19-oic acid 12-oxo-ent-kaur-9(11),16(17)-dien-19-oic acid 17-hydroxy-ent-kaur-15(16)en-19-oic acid 19-hydroxy-ent-kaurene 16α-hydroxy-ent-kaurene	38
Sideritis condicans	ent-16-kauren-7α-ol ent-16-kauren-18-ol ent-7α-acetoxy-16- kauren-18-ol ent-16-kauren-7α,18-diol ent-18-acetoxy-16-kauren-7α-ol ent-18-acetoxy-16-kauren-7-one	39
Mikania banisteriae	ent-kaur-16-en-18-al 18-acetoxy-ent-kaurene 18-hydroxy-16α,17-epoxy-ent-kaurene 4β-19-epoxy-18-nor-ent-kaurene	40

2.4 Biological activity review of kaurane compounds

In 1976, Elliger, C.G., *et. al*. reported that Kaurenoic acid from sunflower inhibited larval development in several *Lepidoptera* species.[41]

In 1995, Lajide, L., *et. al*. reported that kaur-16-en-19-oic acid (Compound 2) had the strongest antifeedant activity on termites among the *ent–*kauranes isolated from *Xylopia aethiopica*.[42]
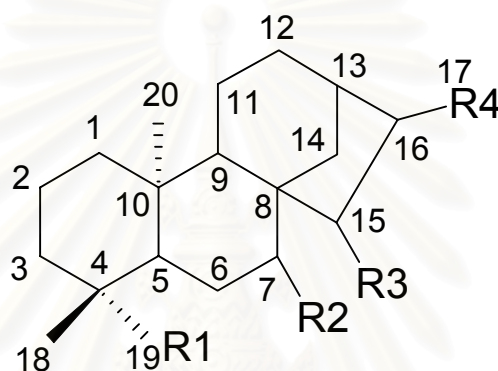
	R1	R2	R3	R4
Compound 1	COOH	β-OAc	H	H_2
Compound 2	COOH	H	H	H_2
Compound 3	CH_3	H	H	α-CH_3, β-OH
Compound 4	COOH	β-OAc	H	H_2
Compound 5	COOH	H	=O	H_2
Compound 6	CH_2OH	H	H	α-CH_3,β-OH

In 1997, Lobitz, G.O., *et.al*. found that *ent*-kaur-16-en-18-oic acid and *ent*-kaur-16-en-19-oic acid show antimicrobial and antiinflammatory properties and indicated that the reported compounds did not show acitivity on antifeedant.[33]

In 1999, Ohkoshi, E., *et. al.* reported that the cytotoxic activities of isolated compounds againt leukemia cell (L1210), among the isolated compounds 3 and 6, show relatively strong cytotoxicity.[43]

1	R= H	6	R1= CH$_2$,	R2= O
2	R= OH	7	R1= O,	R2= CH$_2$
3	R= OCOCH=CHPh			
4	R= OCOPh			
5	R= OCOC(CH$_3$)=CH$_2$			

CHAPTER III

EXPERIMENTAL

3.1 Plant Materials

The plant material of *Croton oblongifolius* Roxb. used in this study was collected from Amphoe Kui buri, Prachuap khiri khan Province, Thailand in August 1999. The plant specimen was compared against voucher specimen No. 084729 deposited in the herbarium of the Royal Forest Department, Bangkok, Thailand.

3.2 Extraction and Isolation

The air-dried stem bark of *Croton oblongifolius* Roxb. was milled to obtain the powdered plant material (6.5 kgs.). The plant material was subsequently extracted by solvents including hexane (13 liters×3), ethyl acetate (13 liters×3), and methanol (13 liters×2) respectively. After the solvent of hexane extract solution was evaporated, the results were hexane extract crude (100.04 g.), ethyl acetate extract crude (92.58 g.) and methanol extract crude (11.01 g.) respectively.

The result of extract crudes are presented in Table 3

Table 3 The various extract of the stem barks of *C. oblongifolius*.

Solvent extract	Appearance	Weight (g)	% wt. by wt. of the dried stem bark
Hexane	dark-yellowed oil	100.40	1.5446
ethyl acetate	dark-yellowed oil	92.58	1.4253
Methanol	dark-red gummy	11.01	0.1692

The procedure and results of extraction are shown in Scheme 1.

Ground air-dried barks of *Croton oblongifolius* Roxb. (6.5 Kg)

Hexane (13 liters×3)

marc

Hexane crude extract (100.04 g)

Ethyl acetate (13 liters×3)

Ethyl acetate crude extract (92.58 g)

marc

Methanol (13 liters×2)

residue

Methanol crude extract (11.01 g)
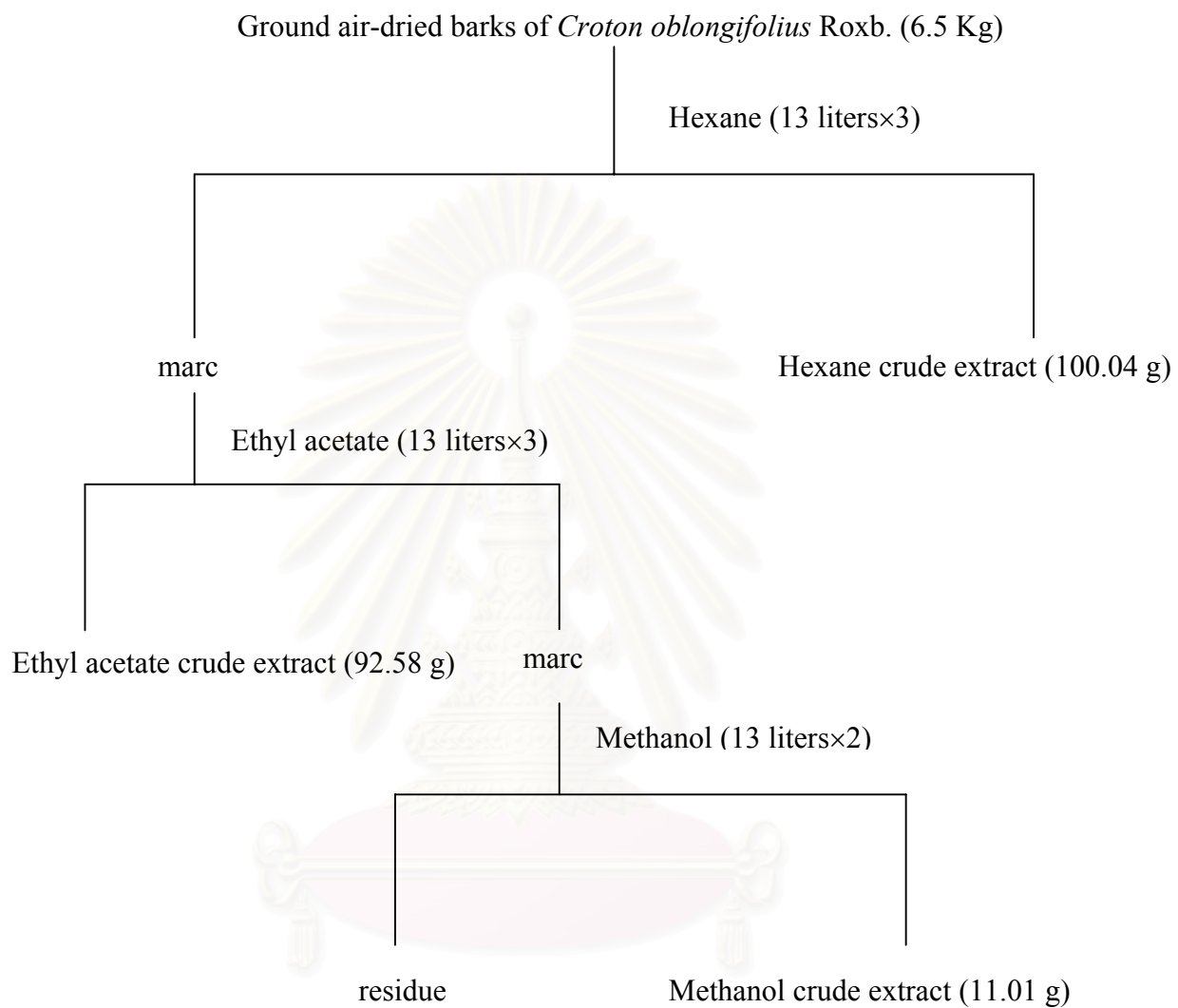
Scheme 1 Show the solvents subsequently extraction

3.3 Separation of Compound 1, 2, and Compound 3

The dark-yellow oil hexane crude (60 g) was fractionated by silica gel column chromatography using Merck's silica gel Art. 1.07734.1000 (70-230 mesh ASTM) as absorbent. The column was eluted with hexane-ethyl acetate gradient in a stepwise fashion. The similar fractions were combined and the solvent was removed by rotary evaporator to obtain compounds 1, 2 and 3 respectively. The ethyl acetate crude extract of croton oblongifolius Roxb gave similar compounds as in hexane crude extract.

Purification and properties of Compound 1

Compound 1 was obtained from the elution of siliga gel column chromatography with 5% ethyl acetate in hexane and was purified by re-crystallization with ethyl acetate and hexane to result a white solid crystal (32.86 g, 0.55% wt. by wt. of the dried stem bark). Compound 1 has mp. 171-172°C, $[\alpha]_D^{20}$ −109.6°(CHCl$_3$; c1.0), and show the Rf value 0.53 on TLC plate using 20% ethyl acetate in hexane as the mobile phase. Compound 1 is soluble in organic solvent such as hexane, ethyl acetate, chloroform and methanol.

The spectral data, UV (CHCl$_3$) λmax (logε): 242(2.90), FT-IR spectrum (Fig.18, Table 4), ^{1}H-NMR spectrum (CDCl$_3$, 500 MHz.)(Fig.19), ^{13}C-NMR spectrum (CDCl$_3$, 500 MHz.)(Fig.20, Table 7), m/z (EI) (Fig.22).

Purification and properties of Compound 2

Compound 2 was obtained from the elution of siliga gel column chromatography with 15% ethyl acetate in hexane and was purified by re-column chromatography using Merck's silica gel Art. 7734.1000 (70-230 mesh ASTM) as absorbent and eluting with 15% ethyl acetate in hexane to give a pure white solid crystal of Compound 2 (2.58 g, 0.04% wt. by wt. of the dried stem bark). Compound 2 has mp. 90-91°C, $[\alpha]_D^{20}$ −112.1°(CHCl$_3$; c1.0), and show the Rf value 0.40 on TLC

plate using 20% ethyl acetate in hexane as mobile phase. Compound 2 is soluble in organic solvent such as hexane, ethyl acetate, chloroform and methanol.

The spectral data, UV (CHCl$_3$) λmax (logε): 242(2.89), FT-IR spectrum (Fig.27, Table 12), ^{1}H-NMR spectrum (CDCl$_3$, 200 MHz.)(Fig.28), ^{13}C-NMR spectrum (CDCl$_3$, 200 MHz.)(Fig.29, Table 13), m/z (EI) (Fig.31).

Purification and properties of Compound 3

Compound 3 was obtained as a mixture from a silica gel column chromatography eluting the column with 50% ethyl acetate in hexane. Compound 3 was purified by re-crystallization with ethyl acetate and hexane to give a white needle crystal (0.26 g, 0.004% wt. by wt. of the dried stem bark). This mixture has mp. 142-145°C, show the Rf value 0.45 on TLC plate using 20% ethyl acetate in hexane as the mobile phase and soluble in organic solvent such as hexane, ethyl acetate, chloroform and methanol.

The spectral data, FT-IR spectrum (Fig.32, Table14), ^{1}H-NMR spectrum (CDCl$_3$, 200 MHz.)(Fig.33), ^{13}C-NMR spectrum (CDCl$_3$, 200 MHz.) (Fig.34, Table 15), m/z (EI) (Fig.35).

3.4 Purification and properties of modification

Methylation of Compound 1

Compound 1 (0.53 g, 1.76 mmol) was methylated with diazomethane in dichloromethane to give Compound 1a as a transparent oil (0.56 g, 1.76 mmol, quantitative yield). Compound 1a has   [α]$_D^{20}$ –91.9°(CHCl$_3$; c1.0) and show the Rf value 0.63 on TLC plate using 20% ethyl acetate in hexane as mobile phase and soluble in organic solvent such as hexane, ethyl acetate, chloroform and methanol.

The spectral data, UV(CHCl$_3$) λmax (logε): 242(3.60), FT-IR spectrum (Fig.36, Table 16) ^{1}H-NMR spectrum (CDCl$_3$, 200 MHz.)(Fig.37), ^{13}C-NMR spectrum (CDCl$_3$, 200 MHz.)(Fig.38, Table 17), m/z (EI) (Fig.40).

Reduction of Compound 1a

For the reduction of Compound 1a, methyl ester (0.10 g, 0.32 mmol) in 5 ml of anhydrous diethyl ether was added slowly from a dropping funnel into a stirred solution of lithium aluminum hydride (71 mg, 0.22 mmol) in 10 ml of anhydrous diethyl ether in a 25 ml round-bottom flask previously flushed with nitrogen. After the addition was completed, the reaction mixture was stirred for 20 hours at room temperature to give Compound 1b. The reaction was stopped and worked up in a usual manner. Compound 1b was a white solid (0.09 g, 0.30 mmol, 95.03%yield) after re-crystallization from ethyl acetate and hexane and was found to be soluble in hexane, chloroform, ethyl acetate and methanol, mp. 133-134°C, $[\alpha]_D^{20}$ −51.6°(CHCl$_3$; c1.0), Rf value 0.38 using 20% ethyl acetate in hexane as a mobile phase.

The spectral data, UV(CHCl$_3$) λmax (logε): 242(3.19), FT-IR spectrum (Fig.41, Table 18), ^{1}H-NMR spectrum (CDCl$_3$, 200 MHz.)(Fig.42), ^{13}C-NMR spectrum (CDCl$_3$, 200 MHz.)(Fig.43, Table 19), m/z (EI) (Fig.45).

Epoxidation of Compound 1

Compound 1 (0.50 g, 1.66 mmol) was epoxidized with m-CPBA (C$_7$H$_5$ClO$_3$, 314.2 mg, 1.82 mmol, 1.1eq) in 10 ml of dichloromethane and the reaction was performed in a 50 ml round-bottom flask. The reaction mixture was stirred for 15 hours at room temperature. The reaction was stopped and worked up in a usual manner. The mixture of Compound 1c and Compound 1d was obtained in this reaction. The mixture was separated by column chromatography on Merck's silica gel Art. 1.09385.1000 and eluting with 10% ethyl acetate in hexane. Similar fractions were combined and the solvent was removed by rotary evaporation. Compound 1c and Compound 1d were purified by re-crystallization with ethyl acetate and hexane.

Compound 1c is a white solid (0.21 g, 0.68 mmol, 40.72% yield), soluble in hexane, chloroform, ethyl acetate and methanol, mp. 191-192°C. $[\alpha]_D^{20}$ – 98.4°(CHCl$_3$; c1.0), Rf value 0.34 using 20% ethyl acetate in hexane as a mobile phase.

The spectral data, UV(CHCl$_3$) λmax (logε): 242(3.04), FT-IR spectrum (Fig.46, Table 20), ^{1}H-NMR spectrum (CDCl$_3$, 200 MHz.)(Fig.47), ^{13}C-NMR spectrum (CDCl$_3$, 200 MHz.)(Fig.48, Table 21), m/z (EI) (Fig.50).

Compound 1d is a white solid (0.29 g, 0.90 mmol, 54.48% yield) after re-crystallization from ethyl acetate and hexane, soluble in hexane, chloroform, ethyl acetate and methanol, mp. 192-193°C , $[\alpha]_D^{20}$ –101.6°(CHCl$_3$; c1.0), Rf value 0.10 using 20% ethyl acetate in hexane as a mobile phase.

The spectral data, UV(CHCl$_3$) λmax (logε): 242(3.15), FT-IR spectrum (Fig.51, Table 26), ^{1}H-NMR spectrum (CDCl$_3$, 200 MHz.)(Fig.52), ^{13}C-NMR spectrum (CDCl$_3$, 200 MHz.)(Fig.53, Table 27), m/z (EI) (Fig.55).

3.5 Instruments and Equipments

1. Nuclear Magnetic Resonance Spectrometer (NMR)

The ^{1}H and ^{13}C NMR spectra were recorded at 200.13 and 50.32 MHz. respectively, on a Bruker Model AC-F200 spectrometer, and at 500.00 and 125.65 MHz on a JEOL JNM-A500 spectrometer in CDCl$_3$. Chemical shifts are given in parts per million using residual protonated solvent as a reference. HMQC, HMBC, COSY and NOESY experiments were performed on the JEOL JNM-A500 spectrometer. .

2. X-ray Diffractrometer

Results for the x-ray diffractrometer were obtained on a SIEMEN SMART diffractrometer at Department of Physics, Faculty of Science, Thammasart University

3. Fourier Transform-Infrared spectrophotometer (FT-IR)

IR spectra were recorded on a Nicolet Impact 410 Spectrophotometer. Spectra of solid samples were recorded as KBr pellets. Liquid samples were recorded as thin films on NaCl cell.

4. Mass Spectrometer (MS)

Low resolution mass spectra were obtained with a Fison Instruments Mass Spectrometer model Trio 2000 at 70 eV.

5. Optical Rotation

The optical rotation spectra were recorded on a Perkin-Elmener 341 polarimeter.

3.6 Chemicals

3.6.1 Solvent used in this research such as hexane, dichloromathane, ethyl acetate and methanol were of commercial grade and were purified prior to use by distillation.

3.6.2 Other chemicals

1. Merck's silica gel 60 Art. 1.07734.1000 (70-230 mesh ATMS) was used as absorbent for column chromatography.

2. Merck's silica gel 60 Art 1.09385.1000 (230-400 mesh ATMS) was used as absorbent for column chromatography.

3. Merck's TLC aluminium sheet, silica gel 60F 254 precoated 25 sheets, 20×20 cm^2, layer 0.2 mm. was used to identical fraction.

3.7 Biological assay

Cytotoxicity Test [44,45]

Bioassay of cytotoxic activity against towards 6 tumor cell lines, which were Hs 27 (fibroblast), Kato-3 (gastric), BT 474 (breast), Chago (lung), SW 620 (colon) and HEP-G2 (hepatoma) culture *in vitro* was performed by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) colorimetric method [15-16]. In principle, the viable cell number / well is directly proportional to the production of formazan, which following solubilization, can be measured spectrophotometrically.

Samples were also tested for cytotoxic activity towards 6 tumor cell lines which were harvested from exponential-phase maintenance cultures (T-75 cm^2 flask), counted by trypan blue exclusion, and dispensed within replicate 96-well culture plates in 100-μl volumes using a repeating pipette. Following a 24-h incubation at 37^oC, 5% CO_2, 100% relative humidity, 100 μl of culture medium, culture medium containing sample was dispensed within appropriate wells (control group, N = 6; each sample treatment group, N = 3). Peripheral wells of each plate (lacking cells) were utilized for sample blank (N = 2) and medium / tetrazolium reagent blank (N = 6) "background" determinations. Culture plates were then incubated for 4 days prior to the addition of tetrazolium reagent. MTT stock solution was prepared as follows: 5 mg MTT / ml PBS was steriled and filtered with 0.45 -μm filtered units. MTT working solution was prepared just prior to culture application by diluting MTT stock solution 1 : 5 (v/v) in prewarmed standard culture medium. MTT working solution (50μl) was added to each culture well resulting in 50 μg MTT/ 250 μl total medium volume) and cultures were incubated at 37^oC for 4 to 24 h depending upon individual cell line requirements. Following incubation cell monolayers and formazan were inspected microscopically: Culture plates containing suspension lines or any detached cells were centrifuged at low speed for 5 min. All 10-20 μl of culture medium supernatant was removed from wells by slow aspiration through a blunt 18-guage

needle and replaced with 150 µl of DMSO using a pipette. Following through formazan solubilization, the absorbance of each well was measured using a microculture plate reader at 540 nm (single wavelength, calibration factor = 1.00).

Cell line growth and growth inhibition were expressed in terms of mean (± 1 SD) absorbance units and / or percentage of control absorbance (± 1 SD%) following subtraction of mean "background" absorbance.

The biological assay of Compound 1, 1a, 1b, 1c, 1d, 2, Compound 3 were performed by following the above mentioned procedure and the results of cytotoxicity testing against the 6 cancer cell lines are presented in Table 28.

CHAPTER IV

RESULTS AND DISCUSSION

The stem bark investigation of *C. oblongifolius.* from Amphoe Kui buri, Prachuap khiri khan Province indicates that compounds 1, 2, and 3 have been isolated and purified by using solvent extraction and chromatography techniques. The structural characterization of these compounds were proposed from spectral data including UV, IR, NMR, MS, and also x-rays crytallography. The cytotoxicity of these isolated compounds have been observed against cancer cell lines by following the standard procedure. The detail of this research will be described as in the following.

4.1 The Results of extraction process

Separation of hexane extract crude.

The hexane extract crude was obtained as a dark-yellowed oil (100.04 g) after evaporation. The hexane extract crude (60 g) was fractionated by silica gel column chromatography using Merck's silica gel Art. 1.07734.1000 (70-230 mesh ASTM) as absorbent. The column was eluted with hexane-ethyl-acetate gradient in a stepwise fashion to give compounds 1, 2 and 3 respectively.

Separation of ethyl acetate extract crude.

The ethyl acetate extract crude (80 g) was separated on Silica gel Art. 1.07734.1000 (70-230 mesh ASTM) using the column chromatography technique. The column was eluted with hexane, hexane-ethyl acetate, ethyl acetate, ethyl acetate-methanol, respectively. Comparison between hexane extract crude and ethyl acetate extract crude by ^{1}H, ^{13}C-NMR [Fig. 56, 57] and TLC was carried out. It was found that the ethyl acetate crude extract contained similar compounds as in the hexane crude extract.

Separation of methanol crude extract.

The methanol extract crude was obtained as a gummy residue (11.01g). It was dissolved in all solvent and this crude could not be purified by silica gal column chromatography.

4.2. Structure elucidation of Compound 1

Characterization of Compound 1

The IR spectrum of Compound 1 (Fig.18) revealed the presence of carboxylic group according to the broad absorption band between 3300 to 2400 cm^{-1} and the strong absorption band at 1695 cm^{-1}due to the carboxylic acid carbonyl stretching. The IR spectrum of Compound 1 is summarized in Table 4.

Table 4 The IR absorption bands assignment of Compound 1.

Wave number (cm^{-1})	Intensity	Vibration
3300-2400	Broad	O-H stretching vibration of acid
2950	Strong	C-H stretching vibration of -CH$_3$, -CH$_2$
1695	Strong	C=O stretching vibration of acid
1461	Medium	C=C stretching vibration of aliphatic
872	Medium	C-H out of plane bending vibration

The ^{1}H-NMR spectrum (Fig.19, Table 5) of Compound 1 indicate that it possesses two olefinic protons at 4.74, 4.80 ppm and two methyl group at 0.90 and 1.24 ppm

The ^{13}C-NMR spectrum (Fig.20, Table 5) showed 20 lines. One signal of carboxylic acid appeared at 185.1 ppm.

DEPT 90 experiments (Fig.21), indicated the presence of three saturated methines at 57.0, 55.1, and 43.8 ppm.

DEPT 135 spectrum (Fig.21) showed two methyl carbons at 28.9 and 15.5 ppm and ten methylene carbons at 103.1, 48.9, 41.3, 40.6, 39.6, 37.7, 33.1, 21.8, 19.1

and 18.4 ppm, which indicated that the carbon signals at 185.1, 155.6, 44.2, 43.7 and 39.7 ppm were quaternary.

The MS spectrum showed the fragmentation as follow, m/z (EIMS) (Fig.22): 302[M^+], 287(61), 259(52), 243(49), 241(45), 213(30), 187(19), 159(21), 148(25), 147(25), 131(75), 105(90), 91(100), 79(66).

The molecular formula of $C_{20}H_{30}O_2$ was proposed from the molecular ion at m/z 302(Fig. 22) and also from the elemental analysis of Compound 1 it was found that DBE was equal to 6 and the IR and NMR spectral show the Compound 1 have the unsaturated double bond and a carboxylic substituent group. THerefore Compound 1was shown to have a tetracyclic skeleton.

The information from 2D-NMR techniques; HMQC correlation (Fig.23, Table 5), HMBC correlation (Fig.24, Table 6), COSY correlation (Fig.25, Table 6) and NOESY correlation (Fig.26) were used to assist the interpretation of the Compound 1 structure.

Table 5 The HMQC spectral data of Compound 1.

^{13}C-NMR (ppm)	^{1}H-NMR (ppm), coupling constant (Hz)
15.5(q)	0.90s
18.4(t)	1.59br, 1.60br
19.1(t)	1.85br, 1.883br
21.8(t)	1.81d (J =3.50)
28.9(q)	1.24s
33.1(t)	1.47br, 1.61br
37.7(t)	2.16d (J=14.04), 1.12d (J=4.88)
39.6(t)	1.13dd(J=4.89, 11.29), 1.99dd(J=1.53, 11.30)
39.7(s)	-
40.6(t)	0.83br, 0.81d(J=4.27)
41.3(t)	1.15dt(J=3.36,3.05), 1.44br
43.7(s)	-
43.8(d)	2.63t(J= 4.89,9.46)
44.2(s)	-
48.9(t)	2.05br, 2.04d (J=1.52)
55.1(d)	1.05d (J=7.02)
57.0(d)	1.06d (J=7.34)
103.1(t)	4.80s, 4.74s
155.6(s)	-
185.1(s)	-

Table 6 The HMQC, HMBC and COSY spectral data of Compound 1.

Position	δ_C	δ_H	HMBC (H to C)	COSY
1	40.6(t)	0.83br 0.81d	C-3C-2,C-20	H-1(0.83), H-2(1.83,1.85) H-1(0.81), H-2(1.83,1.85)
2	19.1(t)	1.83br 1.85br	C-1	H-1(0.81,0.83), H-2(1.85), H-3(1.12, 2.16) H-1(0.81), H-3(2.16)
3	37.7(t)	4.88d 2.16d	C-18	H-2(1.83,1.85), H-3(2.16) H-2(1.83,1.85),H-3(1.12),H-5(1.06)
4	43.7(s)	-	-	-
5	57.0(d)	1.06d	C-1,C-6,C-7,C-18, C-20	H-6(1.81,1.91)
6	21.8(t)	1.81d 1.91dt	C-5,C-7	H-5(1.06), H-7(1.51,1.44) H-5(1.06), H-7(1.51,1.44)
7	41.3(t)	1.51dt 1.44br	C-14, C-6	H-6(1.91), H-7(1.44) H-6(1.81,1.91)
8	44.2(s)	-	-	-
9	55.1(d)	1.05d	C-7,C-10,C-12,C-15, C-20	H-11(1.60,1.59)
10	39.7(s)	-	-	-
11	18.4(t)	1.60br 1.59br	C-12	H-9(1.05), H-12(1.47,1.61) H-9(1.05), H-12(1.47,1.61)
12	33.1(t)	1.47br 1.61br	C-14	H-11(1.60,1.59), H-13(2.63) H-11(1.60,1.59), H-13(2.63)
13	43.8(d)	2.63t	C-12,C-14, C-11	H-12(1.47,1.61), H-14(1.13,1.99)
14	39.6(d)	1.13d 1.99d	C-7,C-12	H-13(2.63), H-14(1.99) H-13(2.63), H-14(1.13)
15	48.9(t)	2.05br 2.04d	C-13	H-14(1.13,1.99), H15(2.04) H-14(1.13), H15(2.05)
16	155.6(s)	-	-	-
17	103.1(t)	4.80s 4.74s	C-15	H-15(2.04,2.05), H-13(2.63) H-15(2.04,2.05), H-13(2.63)
18	28.9(q)	1.24s	C-3,C-5	-
19	185.1(s)	-	-	-
20	15.5(q)	0.90s	C-1,C-9	H-1(0.81)

Compound 1 showed spectral data identical to that of kaur-16-en-19-oic acid, which was reported in the literature [46]. The ^{13}C-NMR signal of Compound 1 and kaur-16-en-19-oic acid are presented in the Table 7 as follows.

Table 7 The ^{13}C-NMR spectra of Compound 1 with kaur-16-en-19-oic acid

Position	δ_C (ppm)	
	Compound 1	kaur-16-en-19-oic acid
1	40.6(t)	40.7(t)
2	19.1(t)	19.1(t)
3	37.7(t)	37.7(t)
4	43.7(s)	43.2(s)
5	57.0(d)	57.0(d)
6	21.8(t)	21.8(t)
7	41.3(t)	41.3(t)
8	44.2(s)	44.2(s)
9	55.1(d)	55.1(d)
10	39.7(s)	39.7(s)
11	18.4(t)	18.4(t)
12	33.1(t)	33.1(t)
13	43.8(d)	43.8(d)
14	39.6(t)	39.7(t)
15	48.9(t)	48.9(t)
16	155.6(s)	155.8(s)
17	103.1(t)	103.0(t)
18	28.9(q)	28.9(q)
19	185.1(s)	184.9(s)
20	15.5(q)	15.6(q)

The chemical shift on ^{13}C-NMR spectrum of Compound 1 and kaur-16-en-19-oic acid were compared signal by signal. This result indicated that the structure of Compound 1 is identical to kaur-16-en-19-oic acid. Thus, it can be concluded that Compound 1 is kaur-16-en-19-oic acid.
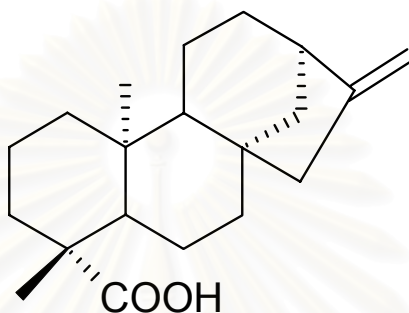

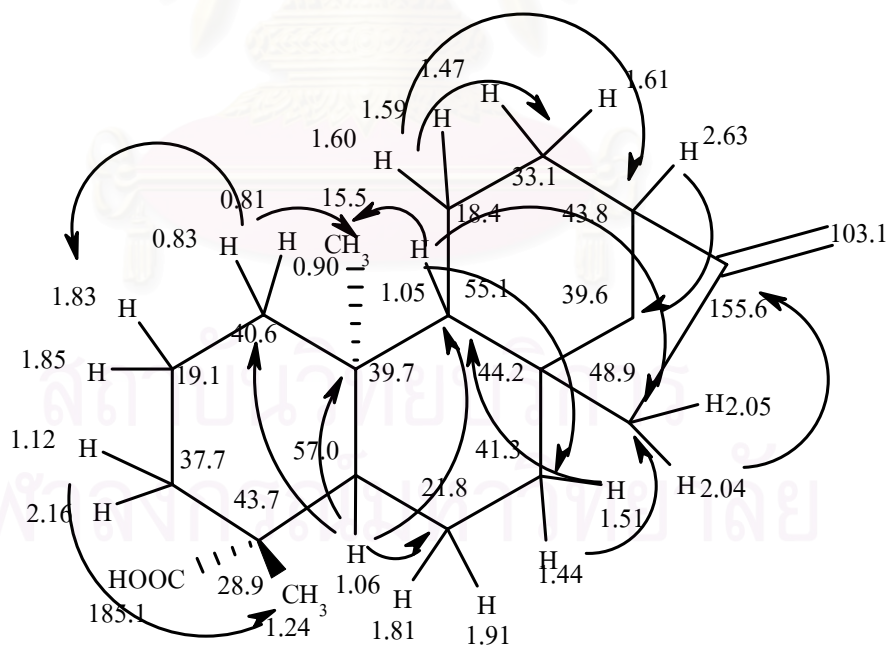**Figure 2** The structure of Compound 1

Figure 3 The HMBC correlation of Compound 1
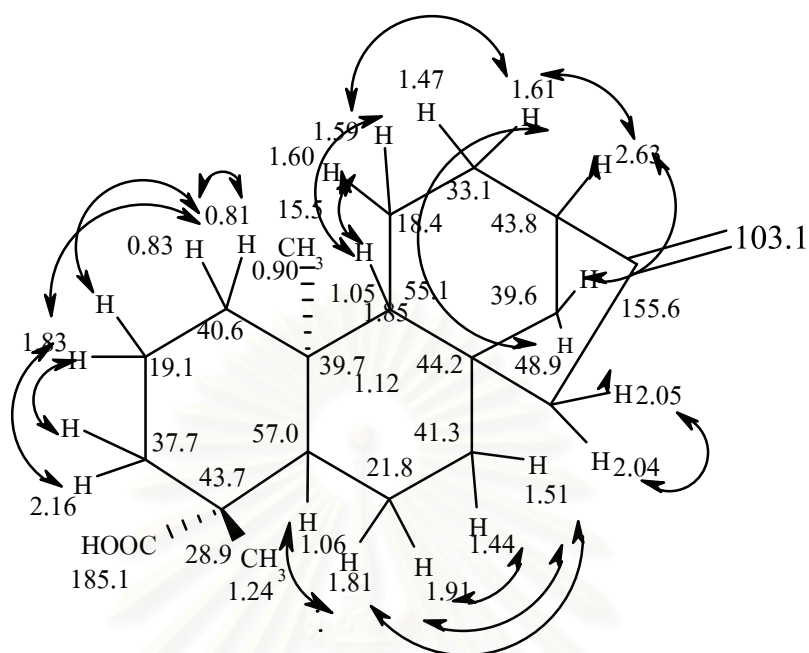
Figure 4 The COSY correlation of Compound 1

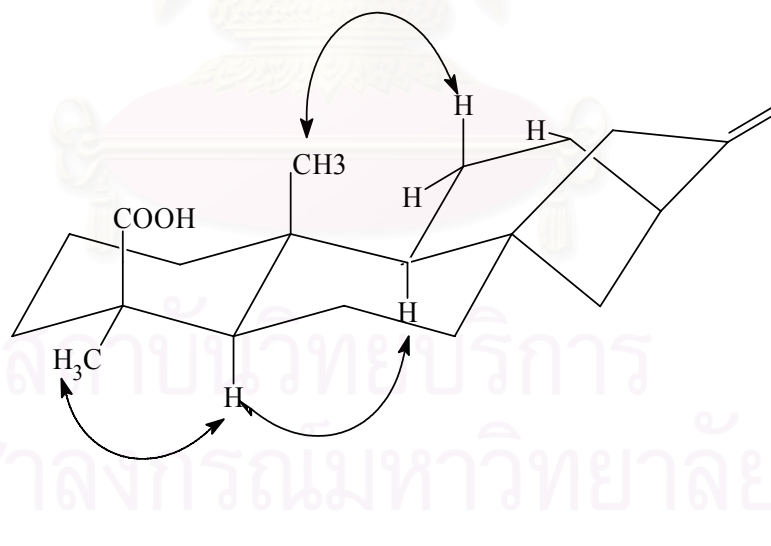**Figure 5** The NOESY correlation of Compound 1

Moreover, the structure of Compound 1 was also confirmed by x-ray diffraction analysis, which indicated that Compound 1 was identical to Kaur-16-en-19-oic acid. The ortep structure of Compound 1 is shown in Fig.6, and x-ray diffraction data are presented in Tables 8, 9, 10 and 11, respectively.
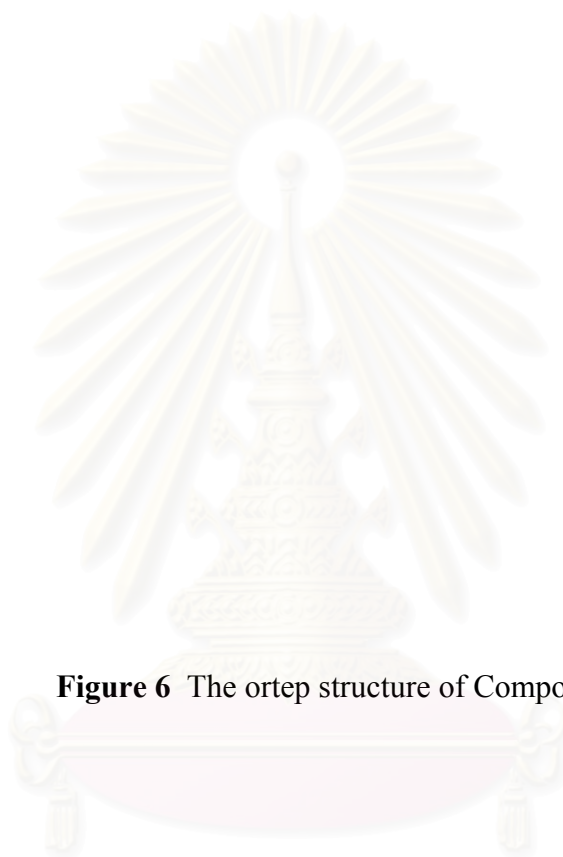

Figure 6 The ortep structure of Compound 1

Table 8 Crystal data and structure refinement for 1

Empirical formula	$C_{20}H_{30}O_2$
Formula weight	302.44
Temperature	293(2) K
Wavelength	0.71073 A$^°$
Crystal system, space group	Orthorhombic, P2(1)2(1)2(1)
Unit cell dimensions	a = 12.3870(2) A$^°$ alpha = 90 deg.
	b = 23.9113(4) A$^°$ beta = 90 deg.
	c = 24.2397(2) A$^°$ gamma = 90 deg.
Volume	7179.54(18) A$^{°3}$
Z, Calculated density	16, 1.119 Mg/m^3
Absorption coefficient	0.070 mm^{-1}
F(000)	2656
Theta range for data collection	1.20 to 30.42 deg.
Index ranges	$-17 \leq h \leq 15, -16 \leq k \leq 34, -33 \leq l \leq 32$
Reflections collected / unique	52074 / 20431 [R(int) = 0.0323]
Completeness to 2theta = 30.42	96.0%
Refinement method	Full-matrix least-squares on F^2
Data / restraints / parameters	20431/ 0 / 849
Goodness-of-fit on F^2	1.107
Final R indices [I > 2sigma(I)]	R1 = 0.0646, wR2 = 0.1371
R indices (all data)	R1 = 0.0575, wR2 = 0.1120
Absolute structure parameter	0.4(10)
Largest diff. peak and hole	0.159 and -0.182 e.A^{-3}

Table 9 Atomic coordinates (x 10^4) and equivalent isotropic displacement parameters (A^2 x 10^3) for 1

	X	Y	Z	U(eq)[*]
C(1)	2815(2)	3646(2)	1932(2)	82(1)
C(2)	2602(3)	4020(2)	1443(2)	91(1)
C(3)	2612(3)	3686(1)	898(2)	83(1)
C(4)	1820(2)	3186(1)	893(1)	54(1)
C(5)	2042(2)	2823(1)	1415(1)	52(1)
C(6)	2035(2)	3148(1)	1974(1)	59(1)
C(7)	2099(2)	2820(1)	372(1)	59(1)
C(8)	1603(2)	2223(1)	350(1)	61(1)
C(9)	1749(3)	1928(1)	901(1)	73(1)
C(10)	1395(3)	2278(1)	1396(1)	64(1)
C(11)	427(3)	2233(2)	137(2)	86(1)
C(12)	1557(5)	1888(1)	-642(2)	117(2)
C(13)	2154(3)	2027(2)	-126(1)	85(1)
C(14)	653(2)	3422(1)	883(1)	66(1)
C(15)	926(2)	3354(1)	2166(1)	569(1)
C(16)	2423(3)	2760(2)	2455(1)	903(1)
C(17)	1980(4)	3117(2)	-193(1)	86(1)
C(18)	908(5)	3004(2)	-492(2)	124(2)
C(19)	578(4)	2402(2)	-468(2)	117(2)
C(20)	1787(9)	1854(3)	-1149(2)	181(4)
O(2)	797(2)	3828(1)	2362(1)	81(1)
O(3)	154(2)	2989(1)	2147(1)	74(1)

[*]U(eq) is defined as one third of the trace of the orthogonalized

Table 10 Bond distances (A°) for 1.

Bond Distances	Distances (A°)	Bond Distances	Distances (A°)
O(2)-C(15)	1.241(3)	C(7)-C(8)	1.553(4)
O(3)-C(15)	1.294(3)	C(7)-C(17)	1.549(4)
C(1)-C(2)	1.510(5)	C(8)-C(9)	1.520(4)
C(1)-C(6)	1.534(4)	C(8)-C(11)	1.547(5)
C(2)-C(3)	1.543(5)	C(8)-C(13)	1.563(4)
C(3)-C(4)	1.547(4)	C(9)-C(10)	1.528(4)
C(4)-C(14)	1.553(4)	C(11)-C(19)	1.532(6)
C(4)-C(5)	1.559(3)	C(12)-C(20)	1.329(7)
C(4)-C(7)	1.574(4)	C(12)-C(13)	1.489(5)
C(5)-C(6)	1.531(4)	C(12)-C(19)	1.566(7)
C(5)-C(10)	1.562(4)	C(17)-C(18)	1.537(6)
C(6)-C(15)	1.532(4)	C(18)-C(19)	1.498(6)
C(6)-C(17)	1.566(4)		

Table 11 Bond angles (deg) for 1

Angles	(A°)	Angles	(A°)
C(2)-C(1)-C(6)	113.7(3)	C(9)-C(8)-C(11)	114.3(3)
C(1)-C(2)-C(3)	111.3(3)	C(9)-C(8)-C(7)	110.5(2)
C(2)-C(3)-C(4)	113.8(3)	C(11)-C(8)-C(7)	111.7(2)
C(3)-C(4)-C(14)	108.0(2)	C(9)-C(8)-C(13)	111.0(2)
C(3)-C(4)-C(5)	108.2(2)	C(11)-C(8)-C(13)	99.9(2)
C(14)-C(4)-C(5)	112.2(2)	C(7)-C(8)-C(13)	108.9(3)
C(3)-C(4)-C(7)	107.4(2)	C(8)-C(9)-C(10)	113.7(2)
C(14)-C(4)-C(7)	113.2(2)	C(9)-C(10)-C(5)	109.8(2)
C(5)-C(4)-C(7)	107.7(2)	C(19)-C(11)-C(8)	102.0(3)
C(10)-C(5)-C(4)	110.9(2)	C(20)-C(12)-C(13)	127.0(6)
C(10)-C(5)-C(6)	116.5(2)	C(20)-C(12)-C(19)	126.4(6)
C(4)-C(5)-C(6)	115.3(2)	C(13)-C(12)-C(19)	106.7(3)
C(15)-C(6)-C(1)	109.6.(2)	C(12)-C(13)-C(8)	106.8(3)
C(15)-C(6)-C(16)	103.8(2)	O(2)-C(15)-O(3)	122.3(2)
C(1)-C(6)-C(16)	108.4(2)	O(2)-C(15)-C(6)	121.7(2)
C(15)-C(6)-C(5)	115.3(2)	O(3)-C(15)-C(6)	115.8(2)
C(1)-C(6)-C(5)	109.2(2)	C(18)-C(17)-C(7)	114.8(3)
C(16)-C(6)-C(5)	110.4(2)	C(19)-C(18)-C(17)	112.7(3)
C(8)-C(7)-C(17)	110.8(3)	C(18)-C(19)-C(11)	109.0(3)
C(8)-C(7)-C(4)	116.8(2)	C(18)-C(19)-C(12)	109.2(5)
C(17)-C(7)-C(4)	115.6(2)	C(11)-C(19)-C(12)	101.6(3)

4.3 Structure elucidation of Compound 2

Characterization of Compound 2

The IR spectrum of Compound 2 (Fig.27) showed the presence of a carboxylic group with correspondence to the broad absorption band between 3500 to 2200 cm^{-1} and the strong absorption band at 1697 cm^{-1}due to the carboxylic acid carbonyl stretching. The IR spectrum of Compound 2 is summarized in Table 12.

Table 12 The IR absorption bands assignment of Compound 2.

Wave number (cm^{-1})	Intensity	Vibration
3400-2400	Broad	C-H stretching vibration of acid
2960, 2865	Strong	C-H stretching vibration of -CH$_3$, -CH$_2$
1672	Strong	C=O stretching vibration of acid
1629, 1410	Weak	C=C stretching vibration of olefin
1250	Strong	C-O stretching vibration

The ^{1}H-NMR spectrum (Fig.28) of Compound 2 possessed three methyl group at 0.75(3H), 0.81(3H) and 1.24 (3H) ppm, three olefinic protons of furanoid groups at 7.35, 7.20 and one vinylic proton at 6.87 ppm.

The ^{13}C-NMR spectrum (Fig.29, Table 13) showed 20 lines, which the carbonyl group of carboxylic acid corresponded to the signal at 172.7ppm.

DEPT 90 experiments (Fig.30) indicated the presence of four sp^2 methine carbons at 111.0, 138.4, 140.3 and 142.7 ppm and two saturated methines at 36.2 and 46.7 ppm.

DEPT 135 spectrum (Fig.30) showed six methylene carbons at 17.5, 18.2, 27.3, 27.5, 35.8 and 38.6 ppm and three methyl carbons at 15.9, 18.3, and 20.5 ppm, which indicated that the carbon signals at 37.6, 38.8, 125.5, 141.5 and 172.7 ppm were quaternary.

The MS spectrum showed the fragmentation as follows, m/z (EIMS) (Fig.31) : 316[M$^+$], 299(10), 283(6), 221(18), 203(23), 137(22), 125(100), 96(53), 95(40), 81(55).

Compound 2 showed a molecular ion with m/z = 316 ($C_{20}H_{28}O_3$)(Fig.31), which indicated a DBE of 7. Compound 2 must consist of one carbonyl group of carboxylic acid, one ring of furan (DBE=3) in addition to the one double bonds.

It could be concluded that Compound 2 exhibited the ^{13}C-NMR chemical shifts similar to hardwickiic acid. The ^{13}C-NMR chemical shift of Compound 2 and hardwickiic acid were compared as presented in Table 1. Therefore, Compound 2 was assigned as hardwickiic acid (Fig. 7), which was previously isolated from *Solidago rugosa* [47].

Table 13 The ^{13}C-NMR spectra of Compound 2 and hardwickiic acid .

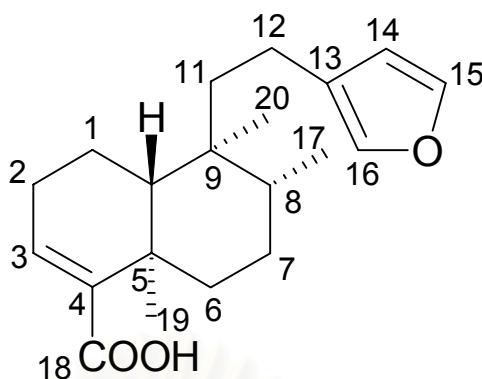| Position	δ_C (ppm)	
	Compound 2	hardwickiic acid
1	35.8 (t)	35.8 (t)
2	18.2 (t)	18.2 (t)
3	140.3 (d)	140.3 (d)
4	141.5 (s)	141.5 (s)
5	37.6 (s)	37.6 (s)
6	38.7 (t)	38.7 (t)
7	27.3 (t)	27.3 (t)
8	36.3 (d)	36.3 (d)
9	38.8 (s)	38.8 (s)
10	46.7 (s)	46.7 (s)
11	17.5 (t)	17.5 (t)
12	27.5 (t)	27.5 (t)
13	125.6 (s)	125.6 (s)
14	111.0 (d)	111.0 (d)
15	142.7 (d)	142.7 (d)
16	138.4 (d)	138.4 (d)
17	15.9 (q)	15.9 (q)
18	172.6 (s)	172.6 (s)
19	20.5 (q)	20.5 (q)
20	18.3 (q)	18.3 (q)

Figure 7 The structure of Compound 2

4.4 Structure and elucidation of Compound 3

Characterization of Compound 3

The IR spectrum of Compound 3 (Fig.32) showed the presence of a hydroxy group according to the broad absorption band at 3200 to 3700 cm^{-1}. The IR spectrum of Compound 3 is summarized in Table 14.

Table 14 The IR absorption bands assignment of Compound 3.

Wave number (cm^{-1})	Intensity	Vibration
3200-3700	Broad	O-H stretching vibration of alcohol
2923,2858	Strong	C-H stretching vibration of -CH$_3$, -CH$_2$
1694	Strong	C=C stretching vibration of olefin
1461	Weak	O-H bending vibration of alcohol
1265	Medium	C-H bending vibration of-CH$_3$, -CH$_2$
1170	Weak	C-O stretching vibration
872	Weak	C-H out of plane bending vibration

^{1}H-NMR spectrum (Fig.33) of Compound 3 showed the proton of hydroxyl group at 3.53 ppm. The protons at 5.08 and 5.34 ppm were the signals of vinyl

protons. The signals at 0.66-2.27 ppm were the signals of angular methyl, methylene and methine groups of the steroids.

^{13}C-NMR spectrum (Fig.34, Table15) of Compound 3 showed the olefinic carbon signals at 121.71,129.26, 138.31 and 140.74 ppm. The carbon signal at 71.80 ppm exhibited the C-OH of the steroid.

The MS spectrum showed the fragmentation as follows, m/z (EIMS)(Fig.35): 414[M^+], 412[M^+], 396(12), 381(7), 329(12), 300(17), 271(36), 255(60), 213(45), 159(59), 145(71), 105(63), 95(69), 81(69), 55(100).

According to the information of a Compound 3, it was suggested that Compound 3 could be a mixture of steroid. To confirm the structure, ^{13}C-NMR spectrum of Compound 3 was compared to stigmasterol and β-sitosterol as shown in Table 15[48]. From all of the data, it could be concluded that Compound 3 was a mixture of stigmasterol assigned to be $C_{29}H_{50}O$ and EIMS [M^+] (m/z = 414)(Fig.35) which indicated 5 DBE and β-sitosterol assigned to be $C_{29}H_{48}O$ and EIMS [M^+] (m/z = 412)(Fig.35) which indicated 6 DBE. The structure of these compound in a mixture are presented in Fig.8.

Table 15 The ^{13}C-NMR spectra of Compound 3, Stigmasterol and β-Sitosterol.

Position	δ_C (ppm)		
	Compound 3	Stigmasterol	β-Sitosterol
1	37.3	37.4	37.1
2	31.7	31.7	31.8
3	71.8	71.8	71.9
4	42.3	42.4	42.4
5	140.7	140.0	140.9
6	121.7	121.7	121.8
7	31.9	31.9	32.0
8	31.9	31.9	32.0
9	50.2	50.3	50.3
10	36.5	36.6	36.6
11	21.1	21.1	21.1
12	39.7	39.8	39.9
13	42.3	42.4	42.4
14	56.8	57.0	56.8
15	24.3	24.4	24.3
16	28.9, 28.2	28.9	28.2
17	56.0	56.0	56.2
18	12.2, 11.8	12.2	11.9
19	19.4	19.4	19.4
20	40.5, 36.1	40.5	36.2
21	21.1, 19.1	21.1	19.1
22	138.3, 33.9	138.4	34.0
23	129.3, 29.2	129.4	29.3
24	51.2, 50.1	51.3	50.3
25	31.9, 26.1	31.9	26.2
26	19.0, 18.8	19.0	18.8
27	21.1, 19.8	21.1	19.8
28	25.4, 23.1	25.4	23.1
29	12.0, 11.9	12.0	11.9
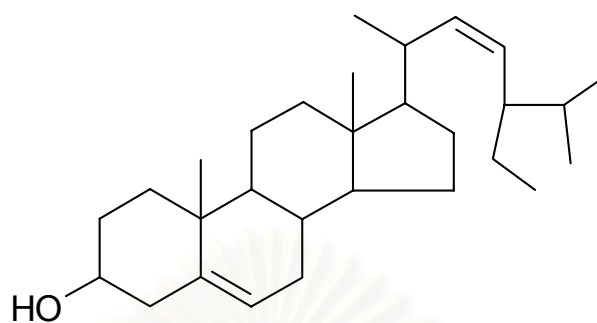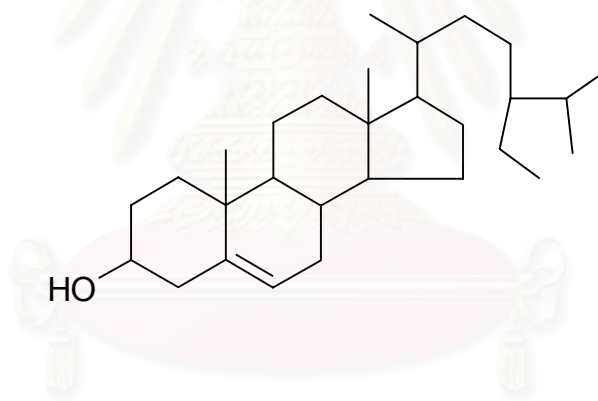
β-sitosterol

stigmasterol

Figure 8 The structure of stigmasterol and β-sitosterol

4.5 Purification and properties of modification of Compound 1

Methylation of Compound 1

Compound 1 (0.53 g, 1.76mmol) was methylated with diazomethane in dichloromethane to give Compound 1a as a transparent oil (0.56 g, 1.76 mmol, quantitative yield). The pathway for methylation of Compound 1 is shown in [Fig.9]
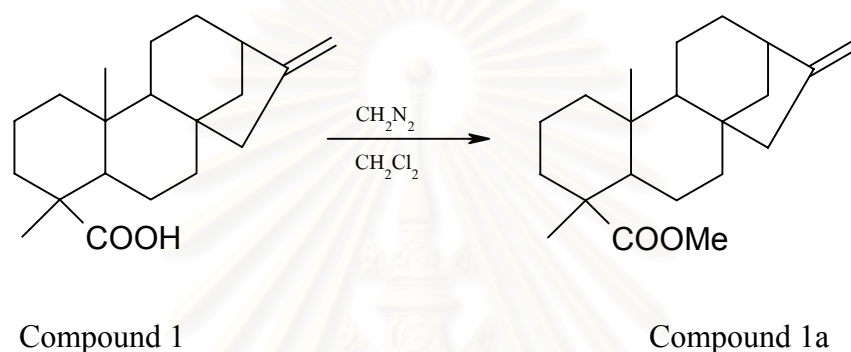

Figure 9 The methylation pathway of Compound 1

Characterization of Compound 1a

The IR spectrum of Compound 1a (Fig.36) showed important absorption bands at 2929 and 2824 cm^{-1}, and a strong absorption band at 1726 cm^{-1}due to the carboxylic acid carbonyl stretching. The IR spectrum of Compound 1a is summarized in Table 16.

Table 16   The IR absorption bands assignment of Compound 1a.

Wave number (cm^{-1})	Intensity	Vibration
2929,2824	Strong	C-H stretching vibration of -CH$_3$, -CH$_2$
1762	Strong	C=O stretching vibration of carbonyl group
1588	medium	C=C stretching vibration of olefin
1147	medium	C-O stretching vibration of ester

The ^{1}H-NMR spectrum (Fig.37) of Compound 1a indicated that it possesses two olefinic protons at 4.71 and 4.77 ppm, two methyl groups at 1.14 and 0.78 ppm, one methyl ester group at 3.61 ppm.

The ^{13}C-NMR spectrum (Fig.38, Table 17) showed 21 lines. Two signal of olefinic carbons appeared at 155.9and 103.0 ppm. The signal at 178.1 ppm should be the ester group and signal at 51.1 ppm should be the methyl ester.

DEPT 90 experiments (Fig.39) indicated the presence of three saturated methines at 57.1, 55.1 and 44.2 ppm.

DEPT 135 spectrum (Fig.39) showed three methyl carbons at 15.2, 28.7 and 51.1ppm, ten methylene carbons at 103.0, 49.0, 44.2, 43.8, 41.3, 39.7, 38.1, 21.9, 19.1 and 18.4 ppm, which indicated that the carbon signals at 178.1, 155.9, 44.2, 43.8 and 39.14 ppm were quaternary.

The MS spectrum showed the fragmentation as follows, m/z (EIMS) (Fig.40) : 316[M$^+$], 301(10), 273(19), 257(24), 241(19), 213(8), 159(17), 147(26), 131(74), 121(92), 107(72), 105(74), 91(100), 79(73).

The molecular formula of Compound 1a was indicated as $C_{21}H_{32}O_2$ (DBE = 6) and showed molecular ion at m/z = 316 (Fig.40). The ^{13}C-NMR spectrum was similar to that of Compound 1(Table 17), except for the moving upfield position of C-19 carboxylate ester (δ_C = 178.1) instead with of Compound 1 (δ_C = 185.1), and revealed the presence of a carbomethoxyl group [δ_H 3.61(3H, s, OMe); δ_C 51.1q, OMe] (Fig.38,39). These results indicated of that Compound 1a (Fig 10) was a methyl ester of Compound 1.

Table 17 The ^{13}C-NMR spectra of Compound 1a and methyl-Kaur-16-en-19-oate [49]

Position	δ$_C$ (ppm)	
	Compound 1a	methyl-Kaur-16-en-19-oate
1	40.8(t)	40.8(t)
2	19.1(t)	19.2(t)
3	38.1(t)	38.21(t)
4	43.8(s)	43.9(s)
5	57.1(d)	57.1(d)
6	22.0(t)	22.0(t)
7	41.3(t)	41.4(t)
8	44.2(s)	44.3(s)
9	55.1(d)	55.1(d)
10	39.7(s)	39.7(s)
11	18.5(t)	18.5(t)
12	33.1(t)	33.2(t)
13	43.8(d)	43.9(d)
14	39.4(t)	39.5(t)
15	49.0(t)	49.0(t)
16	155.9(s)	155.9(s)
17	103.0(t)	103.0(t)
18	28.7(q)	28.8(q)
19	178.1(s)	178.1(s)
20	15.4(q)	15.5(q)
21	51.1(q)	51.2(q)
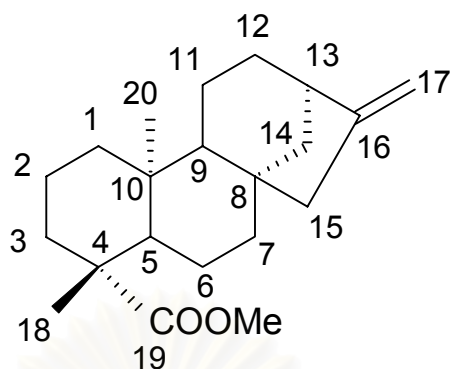
Figure 10 The structure of Compound 1a

Reduction of Compound 1a

Compound 1a(0.10 g. 0.32 mmol) was reduced with lithium aluminium hydride in diethyl ether to give Compound 1b(0.09 g. 0.30 mmol 95.03% yield).

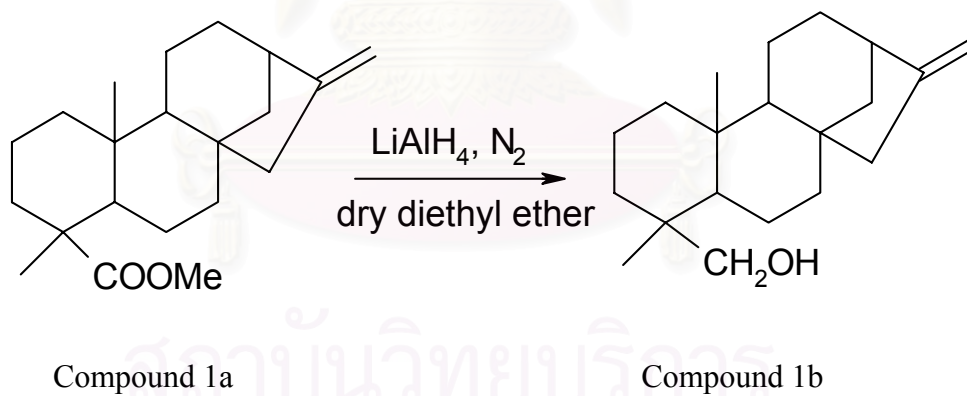The pathway for reduction of Compound 1a is shown in Fig.11.

Compound 1a   Compound 1b

Figure 11 The reduction pathway of Compound 1a

Characterization of Compound 1b

The IR spectrum of Compound 1b (Fig.41) showed the presence of a hydroxy group according to the broad and strong absorption band at 3100 to 3650 cm^{-1}. The IR spectrum of Compound 1b is summarized in Table 18.

Table 18 The IR absorption bands assignment of Compound 1b.

Wave number (cm^{-1})	Intensity	Vibration
3100-3650	Broad	O-H stretching vibration of alcohol
2923,2858	Strong	C-H stretching vibration of -CH$_3$, -CH$_2$
1658	Weak	C=C stretching vibration of olefin
1440	Weak	C-O stretching vibration of alcohol

The ^{1}H-NMR spectrum (Fig.42) of Compound 1b indicated that it possesses two olefinic protons at 4.77 and 4.71 ppm, two methyl groups at 0.99 and 0.94 ppm, and methylene alcohol at 3.42 and 3.73 ppm.

The ^{13}C-NMR spectrum (Fig.43, Table19) showed 20 lines. Two signal of olefinic carbons appeared at 155.9 and 103.0 ppm and signal at one methylene carbon of alcohol at 65.5 ppm.

DEPT 90 experiments (Fig.44) indicated the presence of three saturated methines at 56.8, 56.2 and 44.2 ppm.

DEPT 135 spectrum (Fig.44) showed two methyl carbons at 18.1 and 27.1 ppm, eleven methylene carbons at 103.0, 65.5, 49.1, 41.6, 40.5, 39.7, 35.7, 33.2, 20.5, 18.3 and 18.2 ppm, which indicated that the carbon signals at 155.9, 44.0, 39.2 and 38.7 ppm were quaternary.

The MS spectrum showed the fragmentation as follows, m/z (EIMS) (Fig.45) : 288[M$^+$], 273(11), 257(47), 229(7), 187(4), 175(1), 161(13), 147(23), 145(24), 131(70), 123(100), 109(70), 105(70), 91(92), 81(78), 61(67).

The molecular formula of Compound 1b was indicated to be C$_{20}$H$_{32}$O and showed molecular ion at m/z = 288 [Fig.45] (DBE =5). The ^{13}C-NMR spectrum was

similar to that of Compound 1a, except for the moving upfield position of C-19 of methylene carbon of alcohol (δ_C = 65.5 ppm) instead of δ_C = 178.1ppm carboxylate ester.

The comparison of spectral data for Compound 1b and Kaur-16-en-19-ol are presented in Table 19. Information on kaur-16-en-19-ol has already been published [50].

Table 19 The ^{13}C-NMR spectra of Compound 1b and Kaur-16-en-19-ol.

Position	δ_C (ppm)	
	Compound 1b	Kaur-16-en-19-ol
1	40.5(t)	40.5(t)
2	18.3(t)	18.3(t)
3	35.7(t)	35.7(t)
4	39.3(s)	39.3(s)
5	56.9(d)	56.9(d)
6	20.5(t)	20.5(t)
7	41.7(t)	41.7(t)
8	44.0(s)	.44.0(s)
9	56.2(d)	56.2(d)
10	38.7(s)	38.7(s)
11	18.2(t)	18.2(t)
12	33.2(t)	33.2(t)
13	44.2(d)	44.2(d)
14	39.7(t)	39.7(t)
15	49.1(t)	49.1(t)
16	155.9(s)	155.9(s)
17	103.0(t)	103.0(t)
18	27.1(q)	27.1(q)
19	65.5(t)	65.5(t)
20	18.1(q)	18.1(q)

The chemical shift of carbon in Compound 1b was compared with Kaur-16-en-19-ol, the spectra showed close similarity as in Table 19. Thus, Compound 1b and Kaur-16-en-19-ol had similar structures. The structure of Compound 1b is shown in Fig.12.
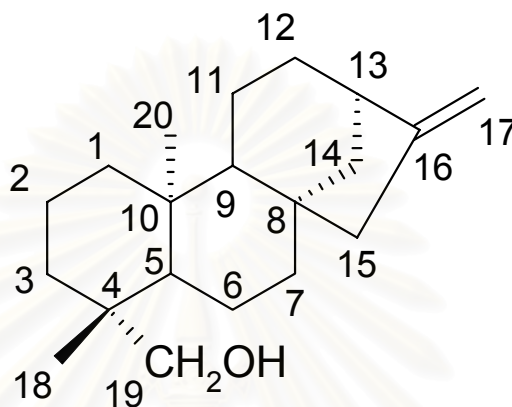
Figure 12 The structure of Compound 1b

Epoxidation of Compound 1

Compound 1 (0.50 mg, 1.66 mmol) was epoxidized with m-CPBA ($C_7H_5ClO_3$, 314.2 mg, 1.82 mmol, 1.1eq) in dichloromethane with nitrogen to give the mixture of Compound 1c(0.21 g, 0.68 mmol, 40.72%yield) and Compound 1d(0.29 g, 0.90 mmol, 54.48% yield).

The pathway for epoxidation of Compound 1 is shown in Fig.13.

Compound 1   Compound 1c   Compound 1d
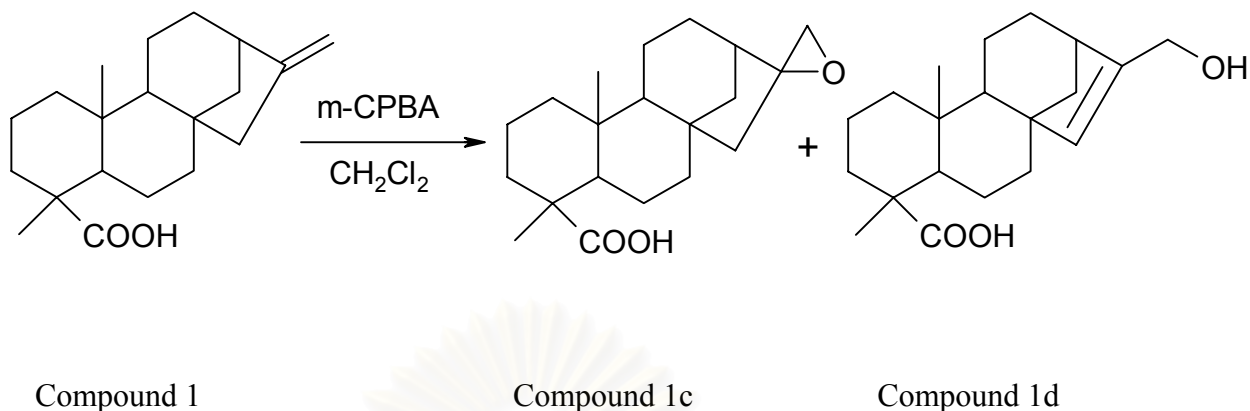
Figure 13 The epoxidation pathway of Compound 1

Characterization of Compound 1c

The IR spectrum of Compound 1c (Fig.46) showed the presence of a carboxylic group according to the broad absorption band at 3300 to 2400 cm^{-1} . The IR spectrum of Compound 1c is summarized in Table 20.

Table 20 The IR absorption bands assignment of Compound 1c

Wave number (cm^{-1})	Intensity	Vibration
3300-2400	Broad	O-H stretching vibration of acid
2930,2858	Strong	C-H stretching vibration of -CH$_3$, -CH$_2$
1694	Strong	C=O stretching vibration of acid
1440	Weak	C-O stretching vibration of acid
1250	Medium	C-O symmetric stretching of epoxide
952, 785	Weak	C-O asymmetric stretching of epoxide

The ^{1}H-NMR spectrum (Fig.47) of Compound 1c indicated that it possesses two methyl groups at 0.95 and 1.21 ppm, methylene epoxide at 2.85 ppm.

The ^{13}C-NMR spectrum (Fig.48, Table 21) showed 20 lines. One signal of carboxylic acid appeared at 184.2 ppm and two signals of methylene carbon of epoxide at 66.7 and 50.4 ppm.

DEPT 90 experiments (Fig.49) indicated the presence of three saturated methines at 56.9, 54.9 and 42.4 ppm.

DEPT 135 spectrum (Fig.49) showed two methyl carbons at 15.8 and 28.9 ppm, ten methylene carbons at 50.4, 48.6, 41.1, 40.7, 29.0, 38.4, 37.7, 21.7, 19.5 and 19.0 ppm, which indicated that the carbon signals at 184.2, 66.7, 45.4, 43.7 and 39.8 ppm were quaternary.

The MS spectrum showed the fragmentation as follows, m/z (EIMS) (Fig.50) : 318[M^+], 303(7), 285(7), 273(30), 272(21), 261(31), 246(21), 231(10), 215(8), 203(12), 185(9), 175(90), 166(19), 145(23), 135(32), 121(67).

The molecular formula of Compound 1c was indicated as $C_{20}H_{30}O_3$ (DBE=6) and showed molecular ion at m/z = 318 (Fig.50). The ^{13}C-NMR spectrum was similar to that of Compound 1 except for the moving upfield position of C-16 one quaternary carbon at δ_C = 66.7 ppm and C-17 methylene carbon of epoxide at δ_C = 50.4 ppm instead δ_C = 155.6 ppm and δ_C = 103.1ppm, reapectively. It could be concluded that Compound 1c was 16,17-epoxy-kauran-19-oic acid. (Fig.14). The ^{13}C-NMR chemical shift of Compound 1c and of 16,17-epoxy-kauran-19-oic acid are shown in Table 21.

Moreover, the structure of Compound 1c was also confirmed by X-ray diffraction analysis. The result indicated that Compound 1c had obsolete configuration identical to 16,17-epoxy-kauran-19-oic acid. The ortep structure of Compound 1c shown in Fig.15 and the x-ray diffraction data are presented in Tables 22, 23, 24 and 25.

Table 21 The ^{13}C-NMR spectra of Compound 1c and 16,17-epoxy-kauran-19-oic acid [51]

Position	δ_C (ppm)	
	Compound 1c	16,17-epoxy-kauran-19-oic acid
1	40.7(t)	40.7(t)
2	19.8(t)	19.6(t)
3	37.7(t)	37.7(t)
4	43.7(s)	43.7(s)
5	56.9(d)	56.9(d)
6	21.7(t)	21.7(t)
7	41.1(t)	41.1(t)
8	45.4(s)	45.4(s)
9	54.9(d)	55.0(d)
10	39.8(s)	39.7(s)
11	19.0(t)	19.0(t)
12	29.0(t)	29.0(t)
13	42.4(d)	42.5(d)
14	38.4(t)	38.4(t)
15	48.6(t)	48.7(t)
16	66.7(s)	66.4(s)
17	50.4(t)	50.4(t)
18	28.9(q)	28.9(q)
19	183.8(t)	183.8(t)
20	15.8(q)	15.8(q)
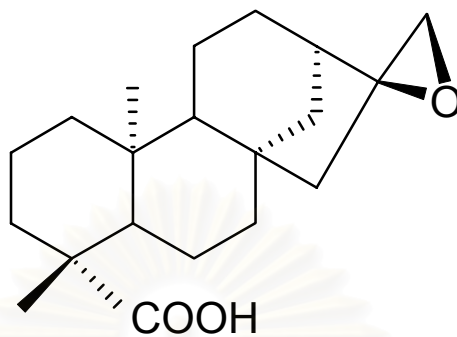
Figure 14 The structure of Compound 1c

Figure 15 The ortep structure of Compound 1c

Table 22 Crystal data and structure refinement for 1c

Empirical formula	$C_{20}H_{30}O_3$
Formula weight	318.44
Temperature	293(2) K
Wavelength	0.71073 A$^{\circ}$
Crystal system, space group	Orthorhombic, P2(1)2(1)2(1)
Unit cell dimensions	a = 12.3507(2) A$^{\circ}$ alpha = 90 deg.
	b = 23.9230(4) A$^{\circ}$ beta = 90 deg.
	c = 24.0752(2) A$^{\circ}$ gamma = 90 deg.
Volume	7113.40(18) A$^{\circ 3}$
Z, Calculated density	16, 1.189 Mg/m^3
Absorption coefficient	0.078 mm^{-1}
F(000)	2784
Theta range for data collection	1.69 to 30.51 deg.
Index ranges	-17 ≤ h ≤16, -33 ≤ k ≤33, -34 ≤ l ≤18
Reflections collected / unique	52897 / 20436 [R(int) = 0.0348]
Completeness to theta = 30.51	96.7%
Refinement method	Full-matrix least-squares on F^2
Data / restraints / parameters	20436/ 0 / 893
Goodness-of-fit on F^2	1.057
Final R indices [I > 2sigma(I)]	R1 = 0.0760, wR2 = 0.1920
R indices (all data)	R1 = 0.1155, wR2 = 0.2236
Absolute structure parameter	1.5(12)
Largest diff. peak and hole	0.477 and -0.420 e.A^{-3}

Table 23 Atomic coordinates (x 10^4) and equivalent isotropic displacement parameters (A^2 x 10^3) for 1c

	X	Y	Z	U(eq)[*]
C(1)	522(2)	4473(2)	4253(2)	57(1)
C(2)	-226(3)	4382(2)	3741(2)	82(1)
C(3)	39(4)	3857(2)	3417(2)	85(1)
C(4)	19(4)	3335(2)	3788(2)	76(1)
C(5)	770(3)	3380(2)	4301(1)	49(1)
C(6)	490(3)	2878(2)	4694(2)	54(1)
C(7)	690(5)	2282(2)	4454(2)	89(1)
C(8)	1801(6)	2020(2)	4623(3)	117(1)
C(9)	2054(5)	2009(2)	5218(3)	98(1)
C(10)	1110(5)	1930(2)	5589(2)	91(1)
C(11)	394(4)	2432(2)	5651(2)	75(1)
C(12)	928(3)	2906(2)	5296(2)	56(1)
C(13)	696(4)	3480(2)	5546(2)	65(1)
C(14)	1056(4)	3961(2)	5176(1)	60(1)
C(15)	428(3)	3932(2)	4619(1)	50(1)
C(16)	75(4)	4978(2)	4587(2)	83(1)
C(17)	1636(3)	4659(2)	4069(1)	53(1)
C(18)	1388(6)	1648(3)	6114(2)	96(2)
C(19)	2122(4)	2733(2)	5344(2)	79(1)
C(20)	1948(3)	3375(2)	4091(2)	62(1)
O(1)	1799(2)	4854(1)	3599(1)	74(1)
O(2)	2387(2)	4653(1)	4446(1)	69(1)
O(3)	789(9)	1356(3)	5693(4)	228(4)

[*]U(eq) is defined as one third of the trace of the orthogonalized

Table 24 Bond distances (A°) for 1c

Bond Distances	Distances (A$^\circ$)	Bond Distances	Distances (A$^\circ$)
C(1)-C(17)	1.512(5)	C(8)-C(9)	1.479(9)
C(1)-C(16)	1.554(6)	C(9)-C(10)	1.523(8)
C(1)-C(2)	1.555(6)	C(9)-C(19)	1.550(8)
C(1)-C(15)	1.567(5)	C(10)-O(3)	1.451(9)
C(2)-C(3)	1.514(8)	C(10)-C(18)	1.473(7)
C(3)-C(4)	1.536(7)	C(10)-C(11)	1.498(7)
C(4)-C(5)	1.549(5)	C(11)-C(12)	1.566(5)
C(5)-C(20)	1.541(5)	C(12)-C(13)	1.527(5)
C(5)-C(6)	1.574(5)	C(12)-C(19)	1.536(6)
C(5)-C(15)	1.567(5)	C(13)-C(14)	1.522(5)
C(6)-C(12)	1.547(5)	C(14)-C(15)	1.519(5)
C(6)-C(7)	1.559(6)	C(17)-O(1)	1.240(4)
C(7)-C(8)	1.563(9)	C(17)-O(2)	1.298(4)
		C(18)-O(3)	1.435(11)

Table 25 Bond angles (deg) for 1c

Angles	(A°)	Angles	(A°)
C(17)-C(1)-C(16)	104.3(3)	O(3)-C(10)-C(18)	58.8(2)
C(17)-C(1)-C(2)	110.5(3)	O(3)-C(10)-C(11)	125.5(2)
C(16)-C(1)-C(2)	108.0(3)	C(18)-C(10)-C(11)	114.8(2)
C(17)-C(1)-C(15)	115.9(2)	O(3)-C(10)-C(9)	124.1(3)
C(16)-C(1)-C(15)	109.8(2)	C(18)-C(10)-C(9)	116.5(2)
C(2)-C(1)-C(15)	108.1(2)	C(11)-C(10)-C(9)	107.4(2)
C(3)-C(2)-C(1)	113.3(2)	C(10)-C(11)-C(12)	106.1(3)
C(14)-C(4)-C(7)	111.8(2)	C(13)-C(12)-C(19)	113.1(6)
C(2)-C(3)-C(4)	113.4(2)	C(13)-C(12)-C(6)	110.0(6)
C(3)-C(4)-C(5)	107.6(2)	C(19)-C(12)-C(6)	113.2(3)
C(20)-C(5)-C(4)	113.6(2)	C(13)-C(12)-C(11)	110.9(3)
C(20)-C(5)-C(6)	107.2(2)	C(19)-C(12)-C(11)	99.6(2)
C(4)-C(5)-C(6)	112.4(2)	C(6)-C(12)-C(11)	109.5(2)
C(20)-C(5)-C(15)	108.2(2)	C(14)-C(15)-C(1)	116.4(2)
C(6)-C(5)-C(15)	107.5(2)	C(14)-C(15)-C(5)	111.3(3)
C(12)-C(6)-C(7)	109.3(2)	C(1)-C(15)-C(5)	114.4(3)
C(12)-C(6)-C(5)	117.1(2)	O(1)-C(17)-C(2)	121.8(3)
C(7)-C(6)-C(5)	116.3(2)	O(1)-C(17)-C(1)	121.7(5)
C(6)-C(7)-C(8)	114.2(3)	O(2)-C(17)-C(1)	116.2(3)
C(9)-C(8)-C(7)	112.7(2)	O(3)-C(18)-C(10)	59.9(4)
C(8)-C(9)-C(10)	111.9(2)	C(12)-C(19)-C(9)	101.4(4)
C(8)-C(9)-C(19)	109.0(3)	C(18)-O(3)-C(10)	61.4(5)
C(10)-C(9)-C(19)	100.7(2)		

Characterization of Compound 1d

   The IR spectrum of Compound 1d (Fig.51) showed the presence of a hydroxy group according to broad absorption band at 3277 to 3697 cm^{-1} and presence of a carboxylic group according to the broad absorption band at 3300 to 2400 cm^{-1} . IR spectrum of Compound 1d is summarized in Table 26.

Table 26 The IR absorption bands assignment of Compound 1d

Wave number (cm^{-1})	Intensity	Vibration
3277-3697	Broad	O-H stretching vibration of alcohol
3300-2400	Broad	O-H stretching vibration of acid
2986,2924	Strong	C-H stretching vibration of -CH$_3$, -CH$_2$
1695	Strong	C=O stretching vibration of acid
1465	Medium	C=C stretching vibration of olefin
1393	Medium	C-O stretching vibration of acid
1019	Medium	C-O symmetric stretching

   The ^{1}H-NMR spectrum (Fig.52) of Compound 1d indicated that it possesses two methyl groups at 0.96 and 1.22 ppm, one olefinic proton at 5.35 ppm, methylene alcohol, at 4.19 ppm.

   The ^{13}C-NMR spectrum (Fig.53, Table 27) showed 20 lines. Two signal of olefinic carbons appeared at 146.7and 135.9 ppm, signal of carboxylic acid at 178.0 ppm and signal of one methylene carbon of alcohol at 61.3 ppm.

   DEPT 90 experiments (Fig.54) indicated the presence of four methines at 135.9, 57.5, 50.5 and 41.8 ppm.

   DEPT 135 spectrum (Fig.54) showed two methyl carbons at 15.9 and 29.1 ppm, nine methylene carbons at 61.3, 44.0, 41.9, 40.5, 39.4, 26.1, 21.7, 19.9 and 20.1 ppm, which indicated that the carbon signals at 178.0, 146.7, 49.0, 43.9 and 40.1 ppm were quaternary.

The MS spectrum showed the fragmentation as follows, m/z (EIMS) (Fig.55) : 318[M$^+$], 300(35), 285(26), 260(21), 239(14), 213(6), 198(63), 185(14), 163(13), 159(18), 145(22), 131(28), 121(39), 117(40), 105(74), 91(100).

The molecular formula of Compound 1d was indicated to be C$_{20}$H$_{30}$O$_3$ (DBE= 6) and showed molecular ion at m/z = 318 [Fig.55]. The ^{13}C-NMR spectrum of Compound 1d was similar to that of Compound 1, except for the moving upfield position of C-15, one olefinic carbon at δ_C = 135.9 ppm, and C-16 low field unsaturated quaternary carbon at δ_C = 146.7 ppm, instead of Compound 1 at δ_C = 48.9 ppm, and δ_C = 155.6 ppm, respectively. It could be concluded that Compound 1d was of 17-hydroxykaur-15-en-19-oic acid. The 17-hydroxy-kaur-15-en-19-oic acid has been isolated from other *Espeletia* spiecies [52].

The ^{13}C-NMR chemical shifts of Compound 1d and of 17-hydroxy-kaur-15-en-19-oic acid are shown in Table 27.

Table 27 The ^{13}C-NMR spectra of Compound 1d and 17-hydroxykaur-15-en-19-oic acid.[53]

Position	δ_H (ppm)	
	Compound 1d	17-hydroxykaur-15-en-19-oic acid
1	41.9(t)	42.3(t)
2	19.9(t)	19.8(t)
3	39.4(t)	39.8(t)
4	43.9(s)	44.0(s)
5	57.5(d)	57.2(d)
6	21.7(t)	22.1(t)
7	44.0(t)	44.4(t)
8	49.0(s)	49.0(s)
9	50.5(d)	50.0(d)
10	40.1(s)	40.7(s)
11	20.1(t)	20.5(t)
12	26.1(t)	26.4(t)
13	41.8(d)	42.2(s)
14	40.5(t)	40.9(t)
15	135.9(d)	136.0(d)
16	146.7(s)	147.1(s)
17	61.3(t)	61.1(t)
18	29.1(q)	29.9(q)
19	178.0(s)	178.0(s)
20	15.9(q)	16.2(q)

The spectral data of Compound 1d was similar to that of 17-hydroxykaur-15-en-19-oic acid, which has been published in previously [54].
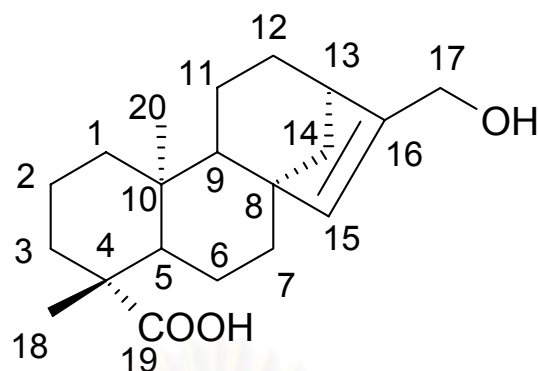
Figure 16 The structure of Compound 1d

4.6 Result of biological activity test

The *in vitro* activity of some compounds (10 μg/ml) from *Croton oblongifolius* **Roxb**. exhibited against 5 cell lines, which composed of Kato-3 (gastric), BT 474 (breast), Chago (lung), SW 620 (colon) and Hep-G2 (hepatoma) cancer are reported in Table 28.

Table 28 Cytotoxic activity against tumor cell lines of some compounds from *C.oblongifolius*.

Compound* 10 μg/ml	% survival					
	Hs 27 (fibroblast)	Kato-3(gastric)	BT474 (breast)	Chago (lung)	SW 620 (colon)	Hep-G2 (hepatoma)
1	108	73	80	52	46	77
2	108	79	124	107	110	103
1a	123	69	51	87	56	74
1b	83	40	74	94	60	67
1c	100	90	120	101	104	104

* dissolved in ethanol

All of compounds showed weak cytotoxic activity against Hs 27 (fibroblast), Kato-3 (gastric), BT 474 (breast), Chago (lung), SW 620 (colon) and Hep-G2 (hepatoma) cancer. The Compound 1 (kaur-16-en-19-oic acid) showed no antifeedant, antimicrobial and antiinflammable properties, which have been reported previously

[34]. Moreover, Compound 1 was known to have a plant growth stimulating activity [49]. The cytotoxicity of Compounds 1, 1a, 1b, 1c, and 1d against Hs 27, Kato-3, BT 474, Chago, SW 620 and Hep-G2 tumor cells were the first reported in this thesis. In addition, bioassay against P-388 has been reported previously for kaur-16-en-19-oic acid (Compound 1)[55] and hardwickiic acid (Compound 2) [6].

CHAPTER V

CONCLUSION

In this research, the chemical constituents of the stem bark of *Croton oblongifolius* Roxb. from Amphoe Kui buri, Changwad Prachuap khiri khan was investigated. The chromatographic separation of hexane and ethyl acetate crude extract gave kaur-16-en-19-oic acid (Compound 1, 32.68g, 0.55% of dry plant), hardwickiic acid(Compound 2, 2.58g, 0.04% of dry plant) and a mixture of stigmaterol and β-sitosterol(Mixture 3, 0.26g, 0.004% of dry plant), are presented in Table 29. The structure were determined from spectral data, including UV, IR, MS, and NMR and also by comparison with the spectral data previously reported.

Kaur-16-en-19-oic acid was found as a major constituent. Even though Kaur-16-en-19-oic acid was first isolated from *Helianthus annus* L. [30] but the presence of Kaur-16-en-19-oic acid had never been reported in *Croton* species except kaur-16,17-diol and kaur-16-17-18-triol in *C. hutchisonianus*. Therefore, this research work represented the first report of Kaur-16-en-19-oic acid in *Croton oblongifolius* Roxb.

The derivatives of Compound 1, such as methyl kaur-16-en-19-oic acid (1a), Kaur-16-en-19-ol (1b), 16,17-epoxy-kauran-19-oic acid (1c), and 17-hydroxykaur-15-en-19-oic acid (1d), were synthesized by known methods. The crystal structure of kaur-16-en-19-oic acid (1) and 16,17-epoxy-kauran-19-oic acid (1c) were determined by x-ray diffraction analysis and their ortep structure were shown in Fig. 6 and 15, respectively. It is the first report of crystal structure of both compounds.

Table 29 Isolated substances from *C. oblongifolius* in this research.

Compound	Name of compound	Weight (g)	% wt. by wt. of the starting material
1	Kaur-16-en-19-oic acid	32.86	0.55
2	Hardwickiic acid	2.58	0.04
3	Stigmasterol, β-Sitosterol	0.26	0.004

Table 30 The synthesis of Kaur-16-en-19-oic acid derivative

Compound	Name of compound	Weight (g)	% wt. by wt. of the starting material
1a	Methyl kaur-16-en-19-oate	0.56	Quantitative yield
1b	Kaur-16-en-19-ol	0.09	95.03
1c	16,17-epoxy-kauran-19-oic acid	0.21	40.72
1d	17-hydroxykaur-15-en-19-oic acid	0.29	54.48

Each compound in this research work was subjected to the cytotoxic activity test against a panel of human cancer cell lines, including Hs-27 (human cell line), Chago (lung), SW620 (colon), BT474 (breast), Kato-3 (gastric), and Hep-G2 (hepatoma). The result indicated that these compound have weak to moderate cytotoxicity against the tested cell lines (Table28). Nonetheless, there are a number of reports on other activities of these compounds, for example, antimicrobial, anti-inflammatory properties of Compound 1.

Suggestion for the future work

1. **The investigation of chemical constituents of *Croton oblongifolius* Roxb. should be continued in order to find new sources of diterpenoids and better understanding of the biodiversity of this species.**

2. **The chemistry of kaur-16-en-19-oic acid should be explored farther in order to find the possible application of this compound and it is derivative.**

R

1   COOH
1a COOMe
1b CH $_2$OH

1c

1d

2

β-sitosterol   stigmaterol
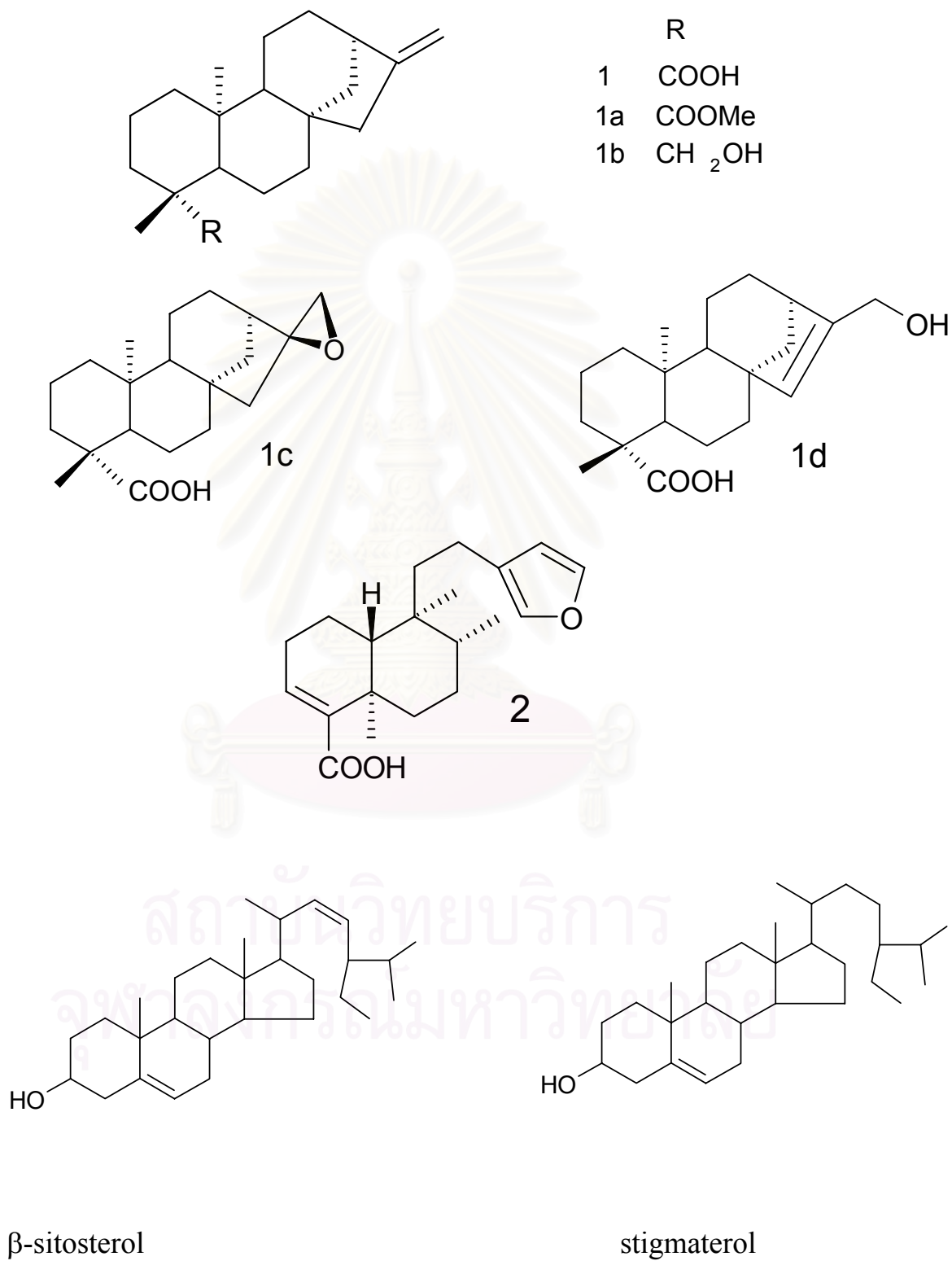
Figure17 The structure of compounds obtained from *C. oblongifolius* in this research.

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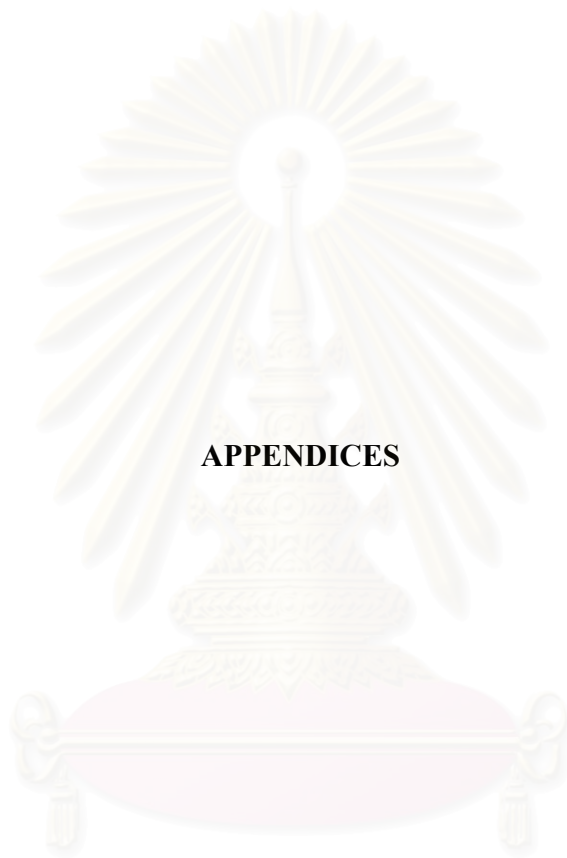
APPENDICES
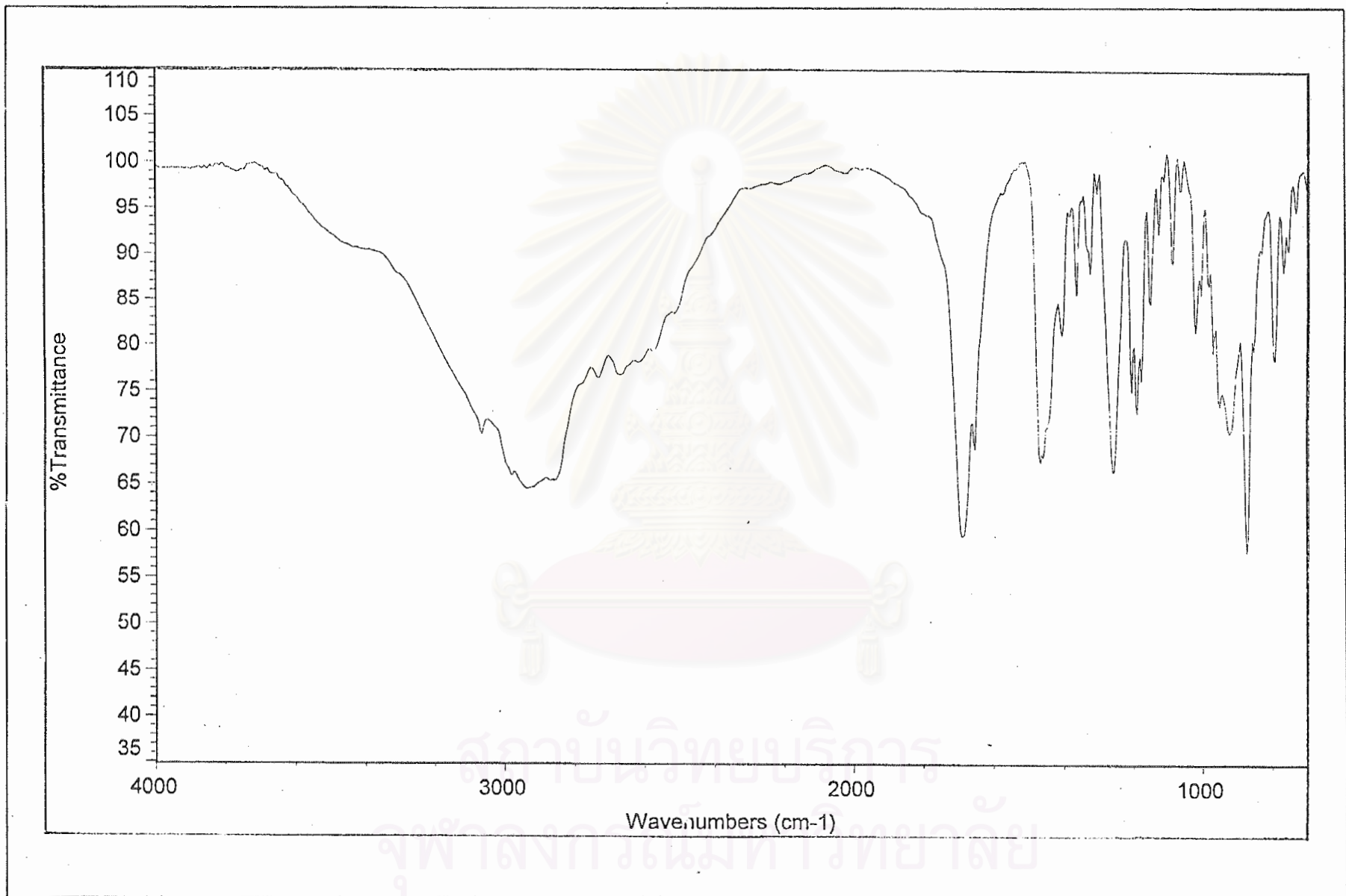
Figure 18   The IR spectrum of compound 1
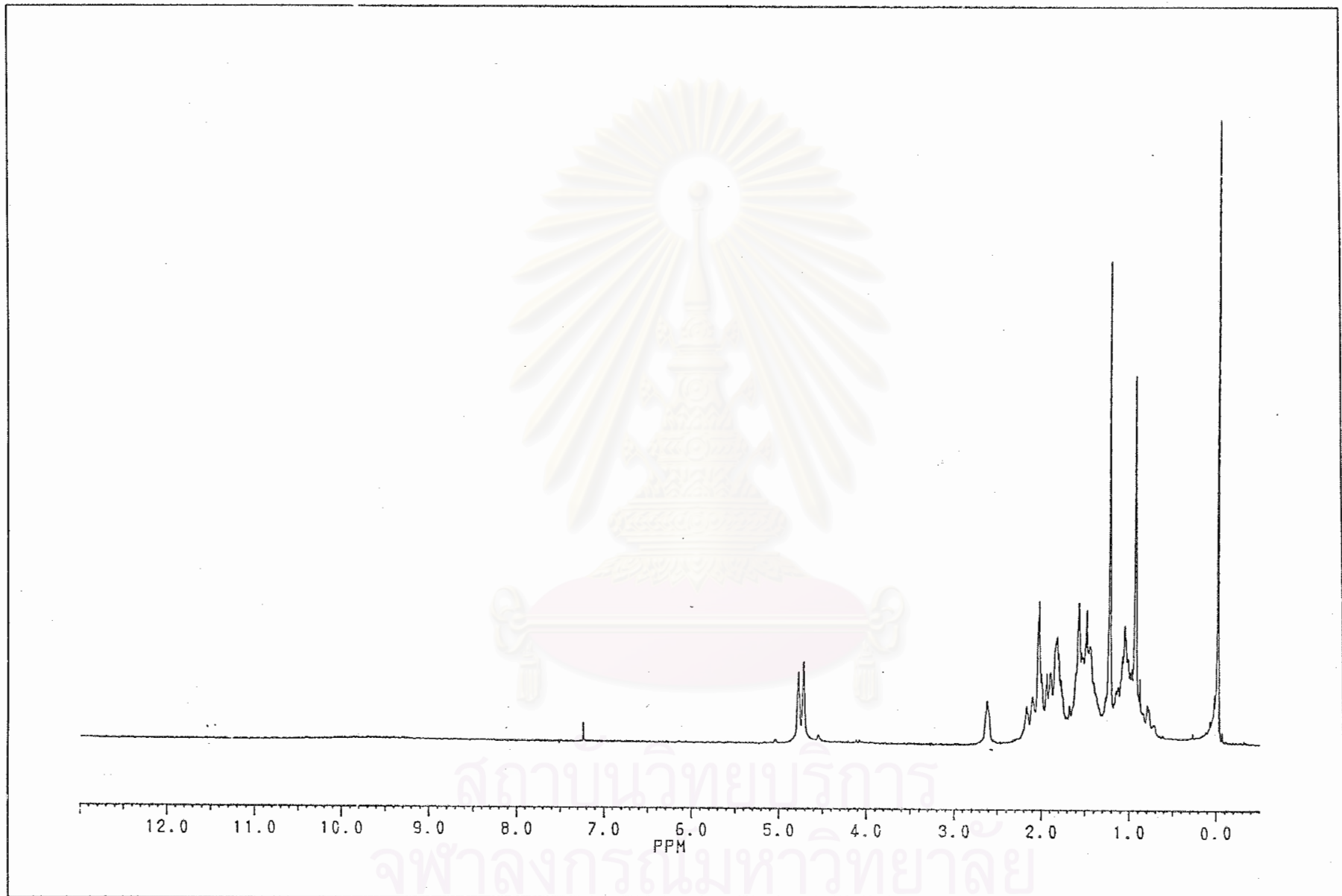
Figure 19 The ^{1}H-NMR spectrum of compound 1
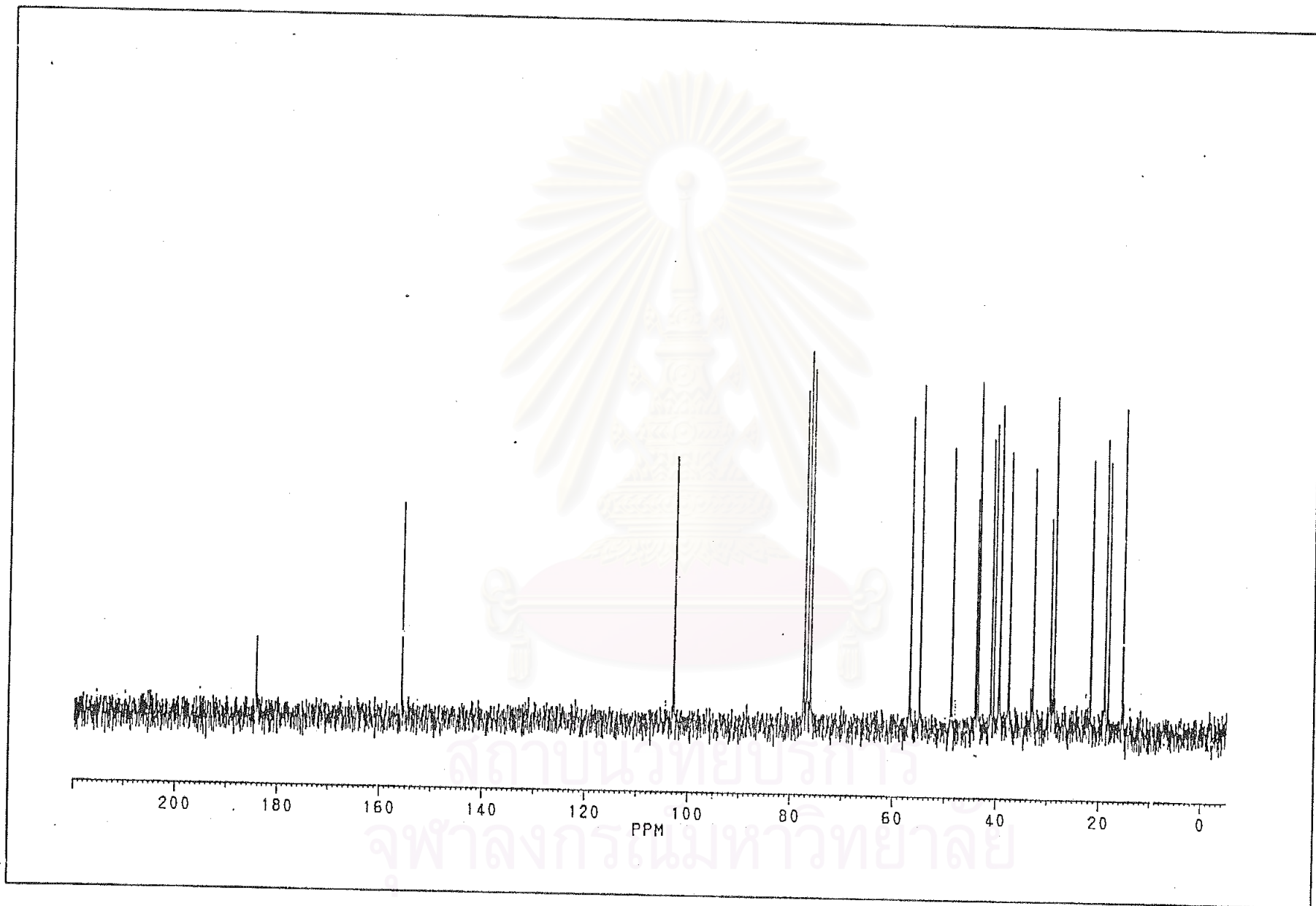
Figure 20 The ^{13}C-NMR spectrum of compound 1
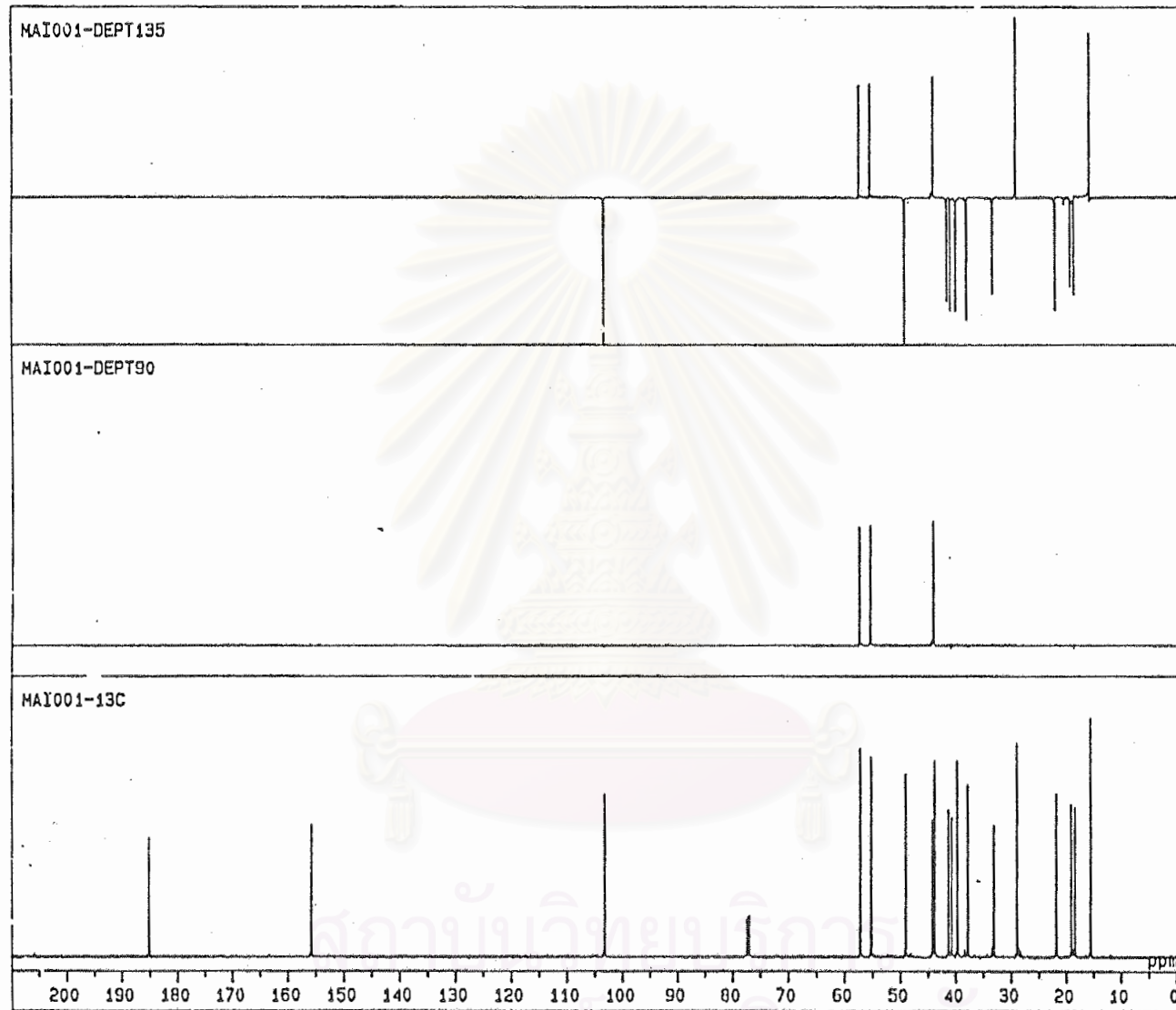
Figure 21   DEPT 90, 135 and ^{13}C-NMR spectrum of compound 1
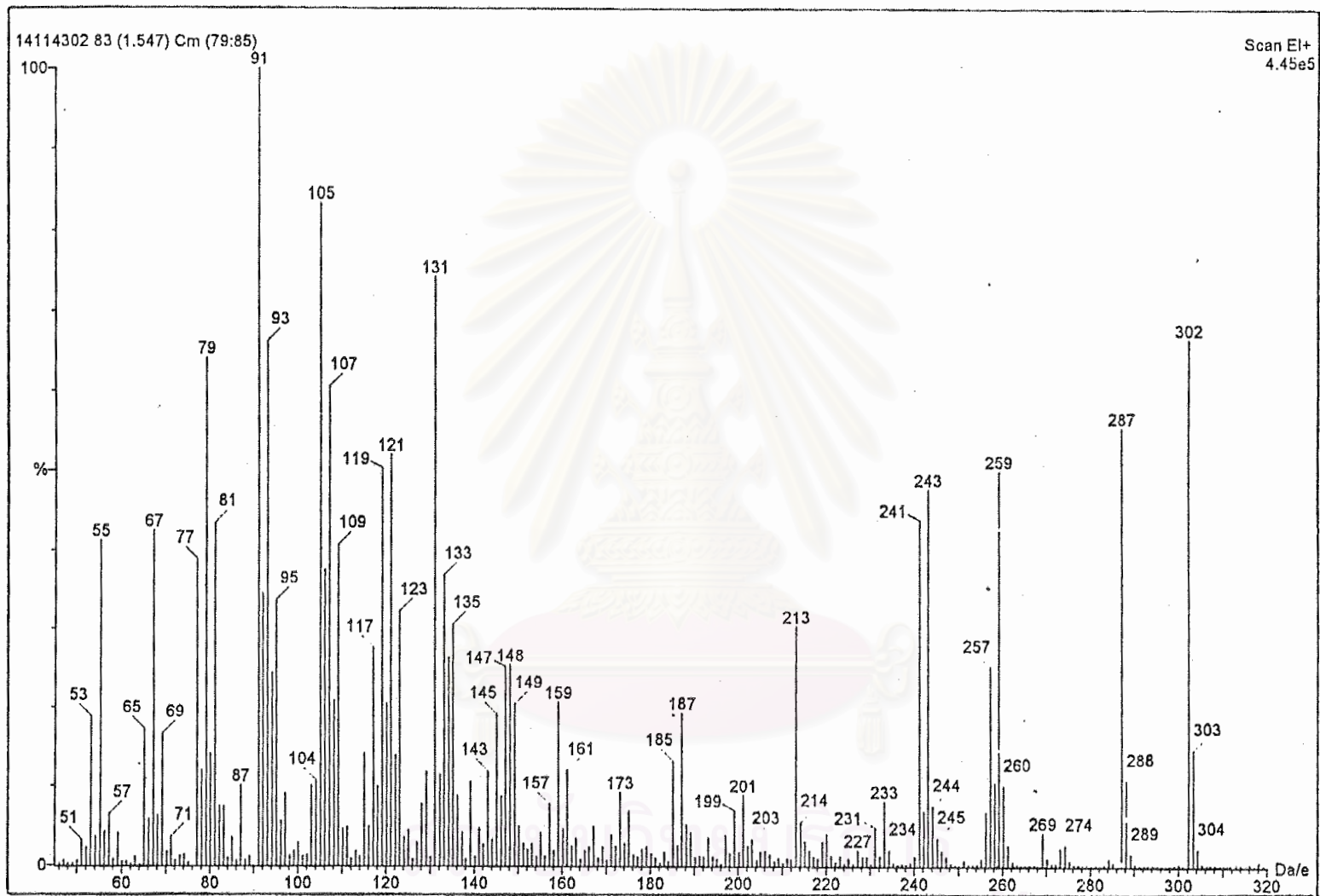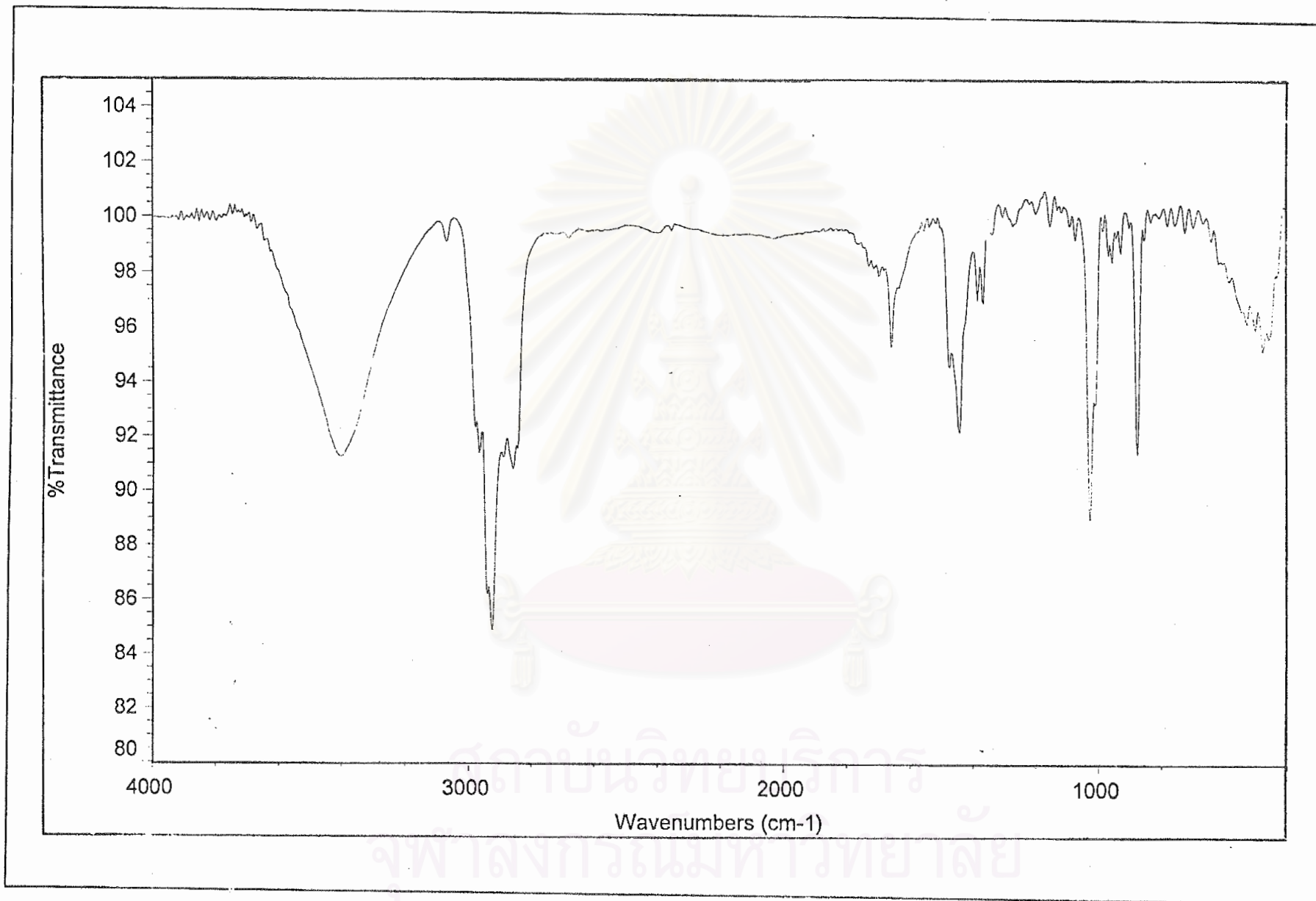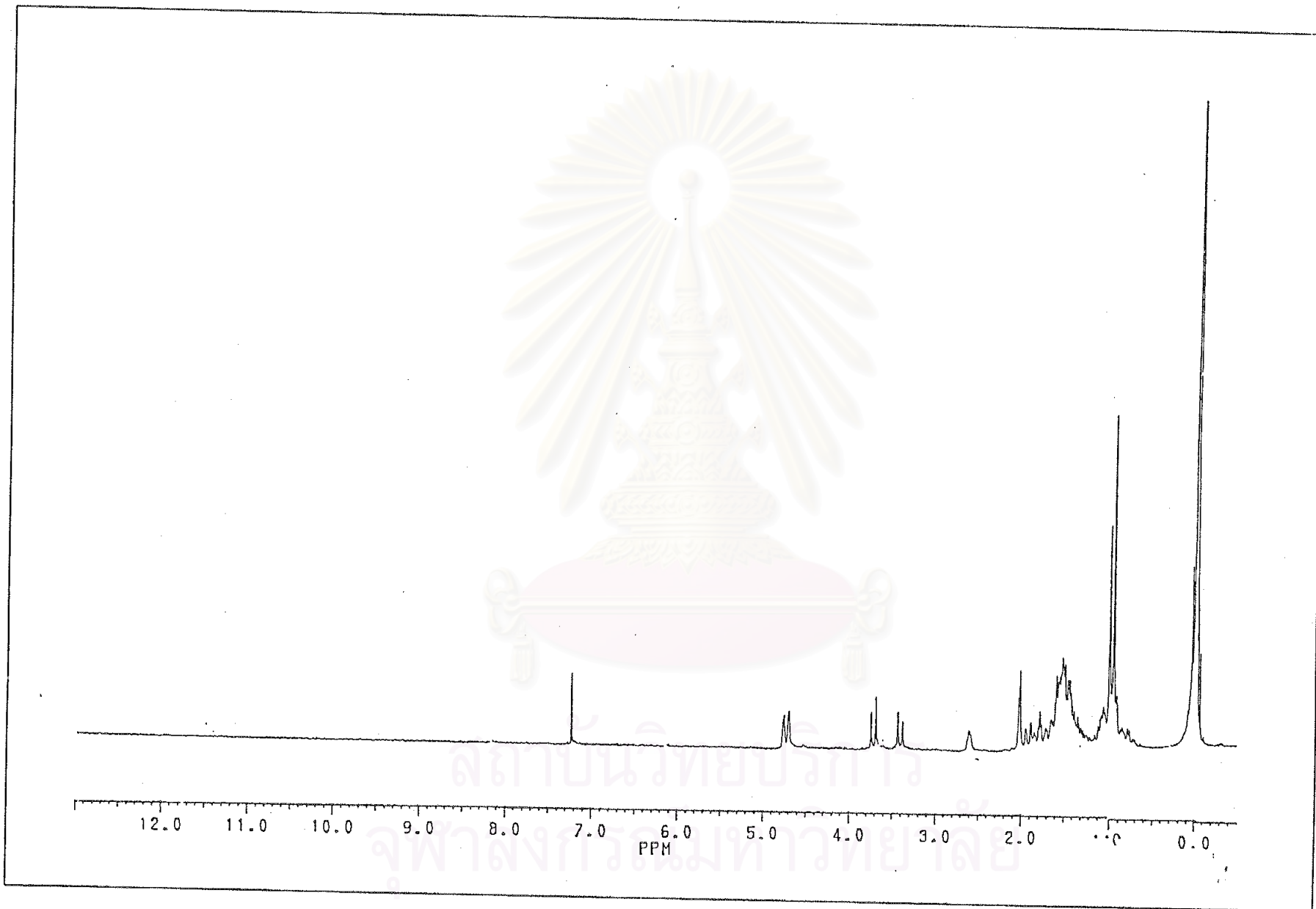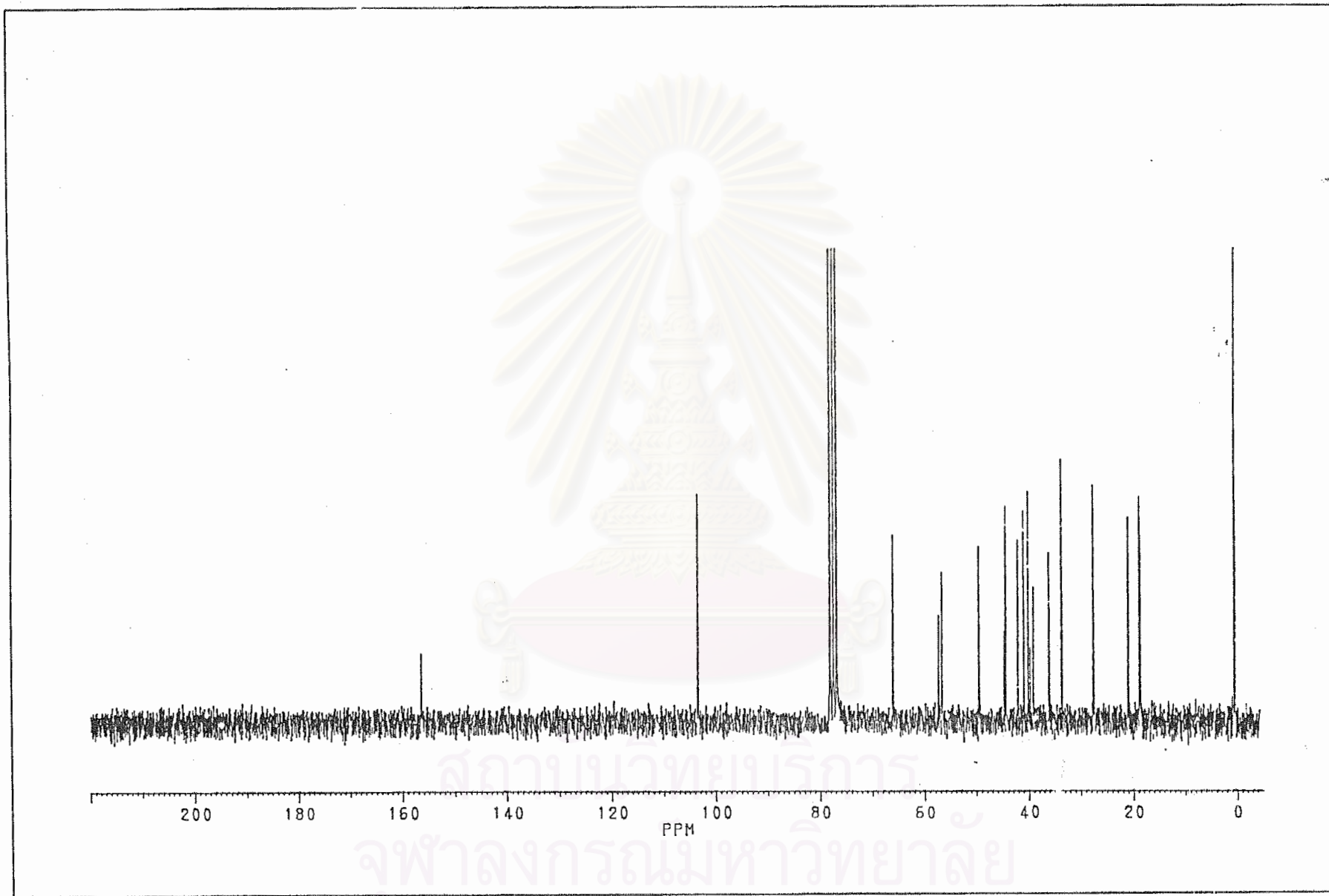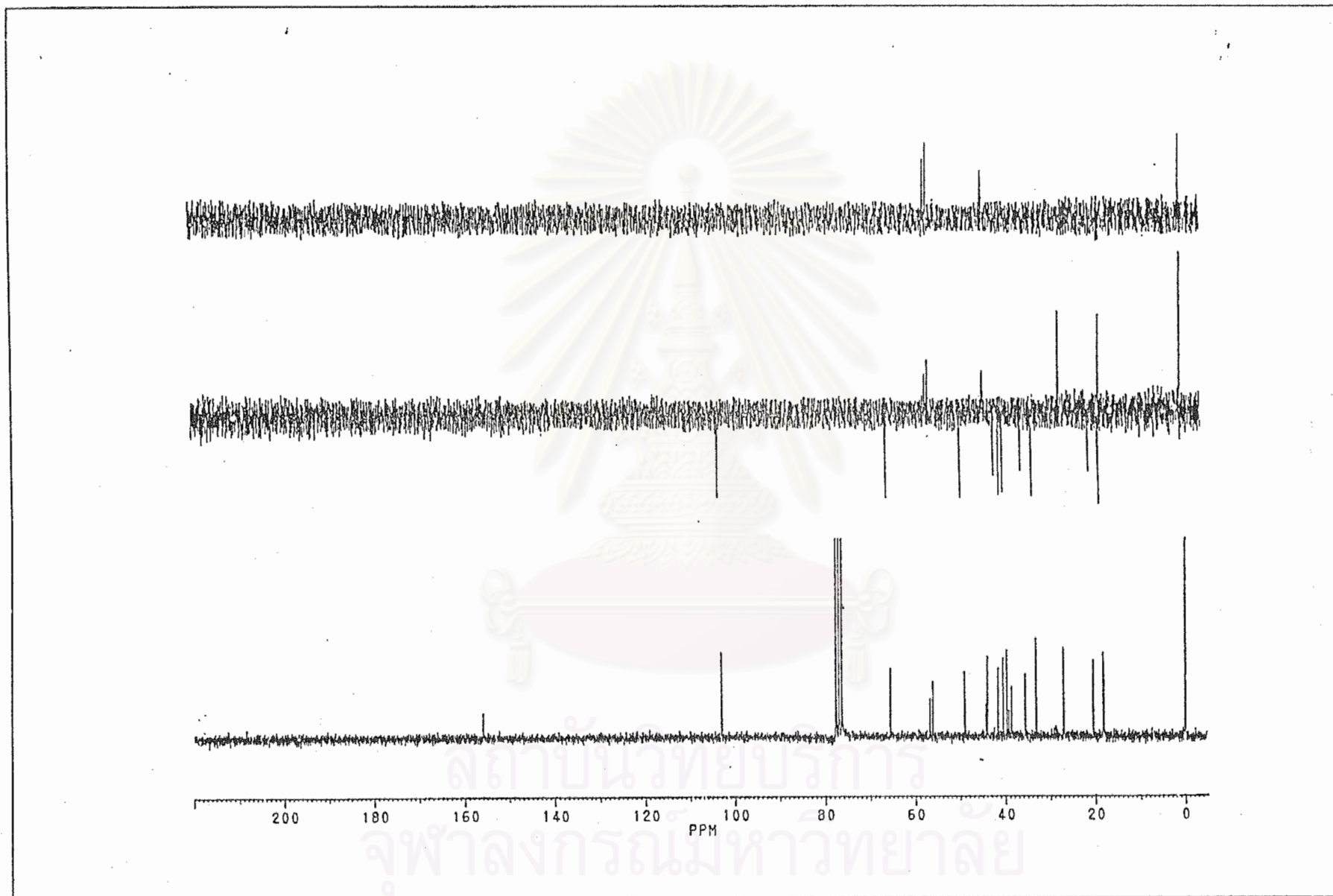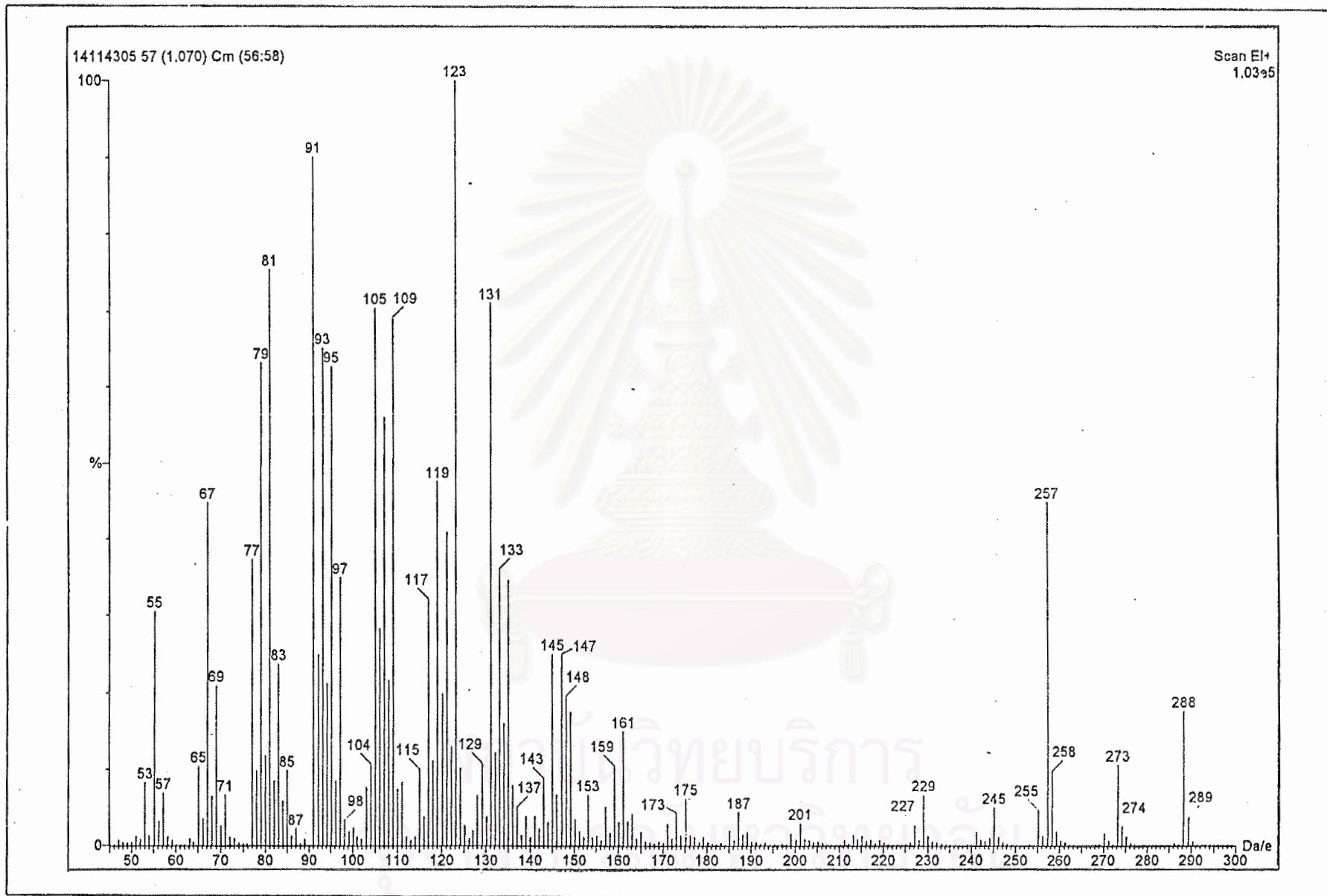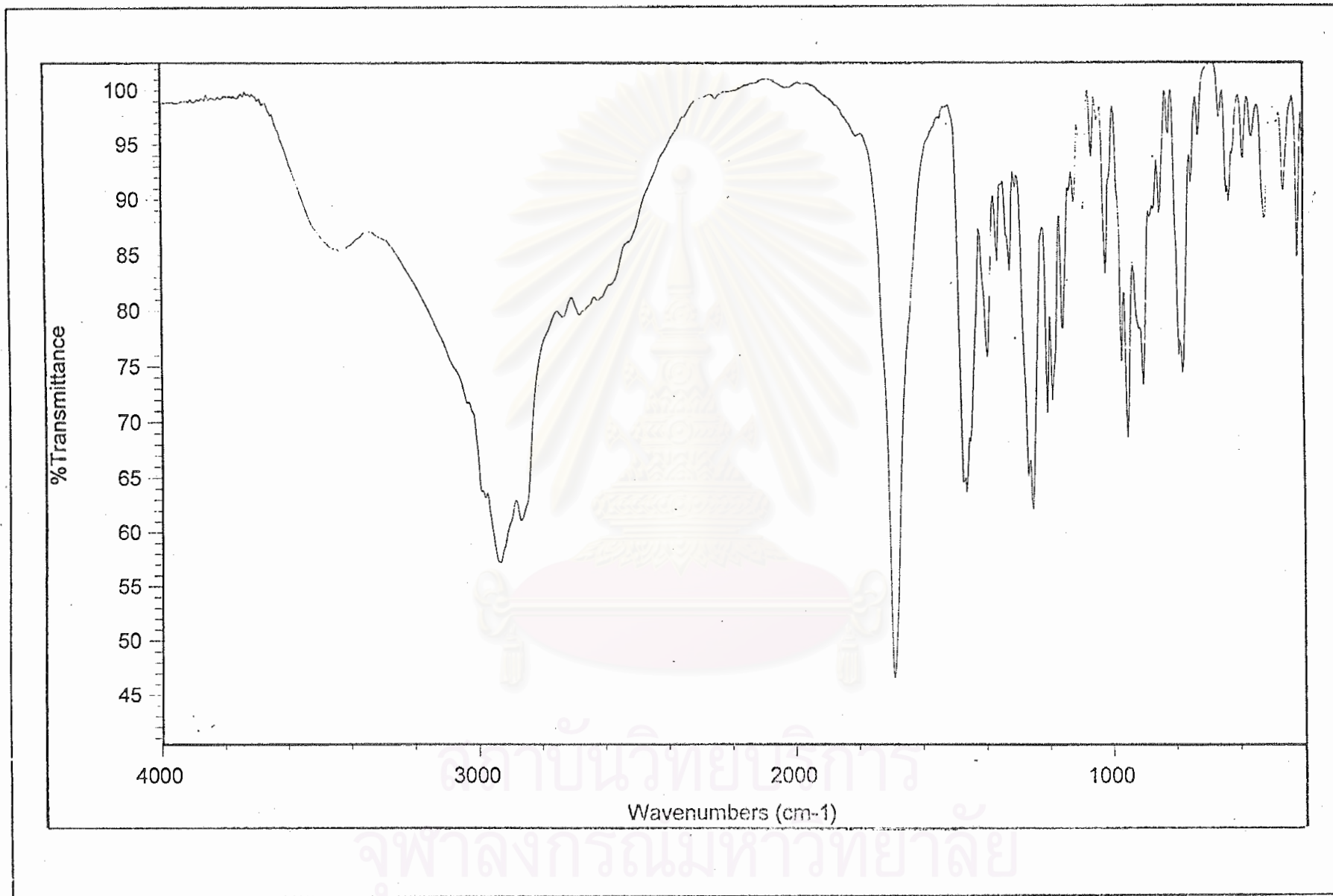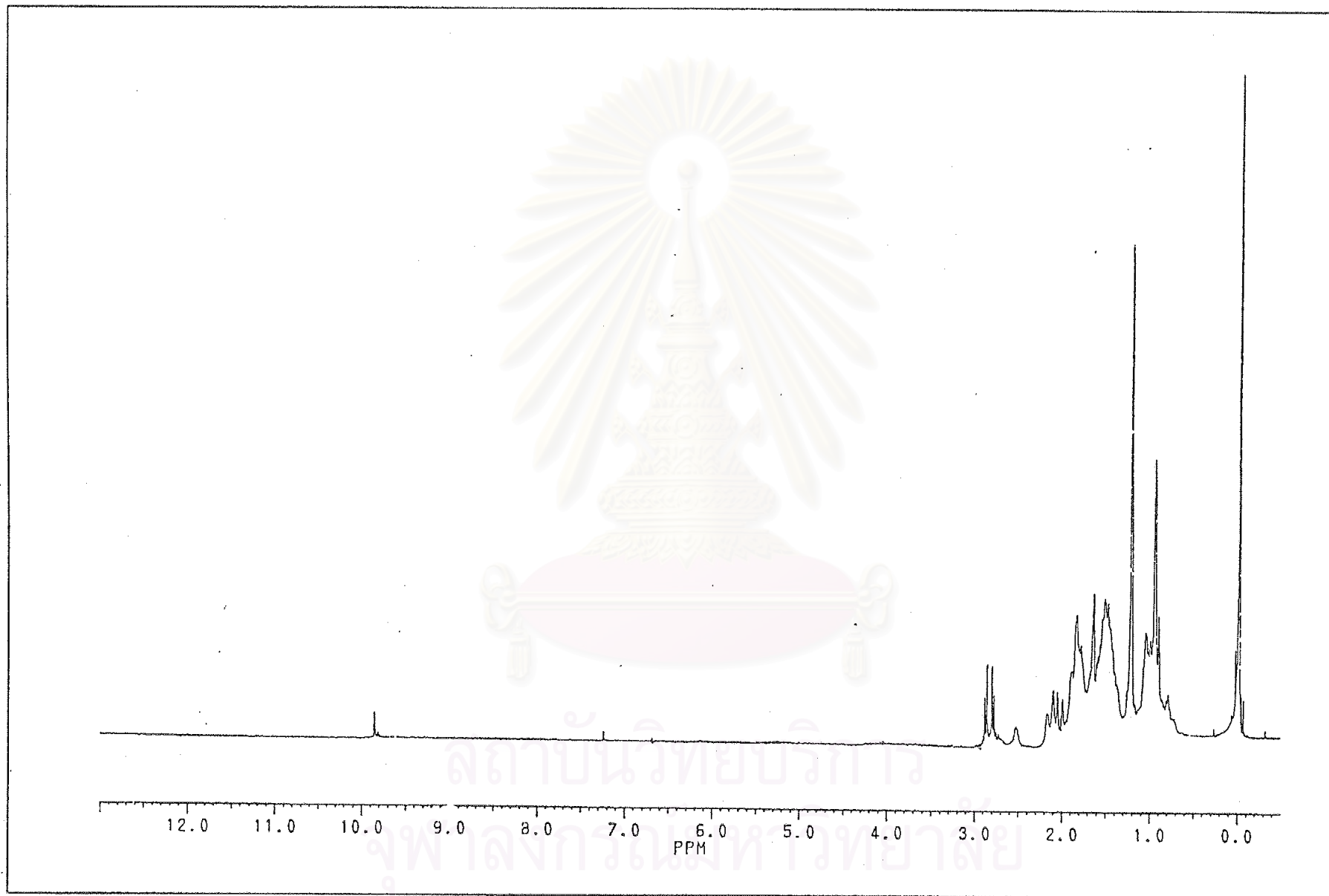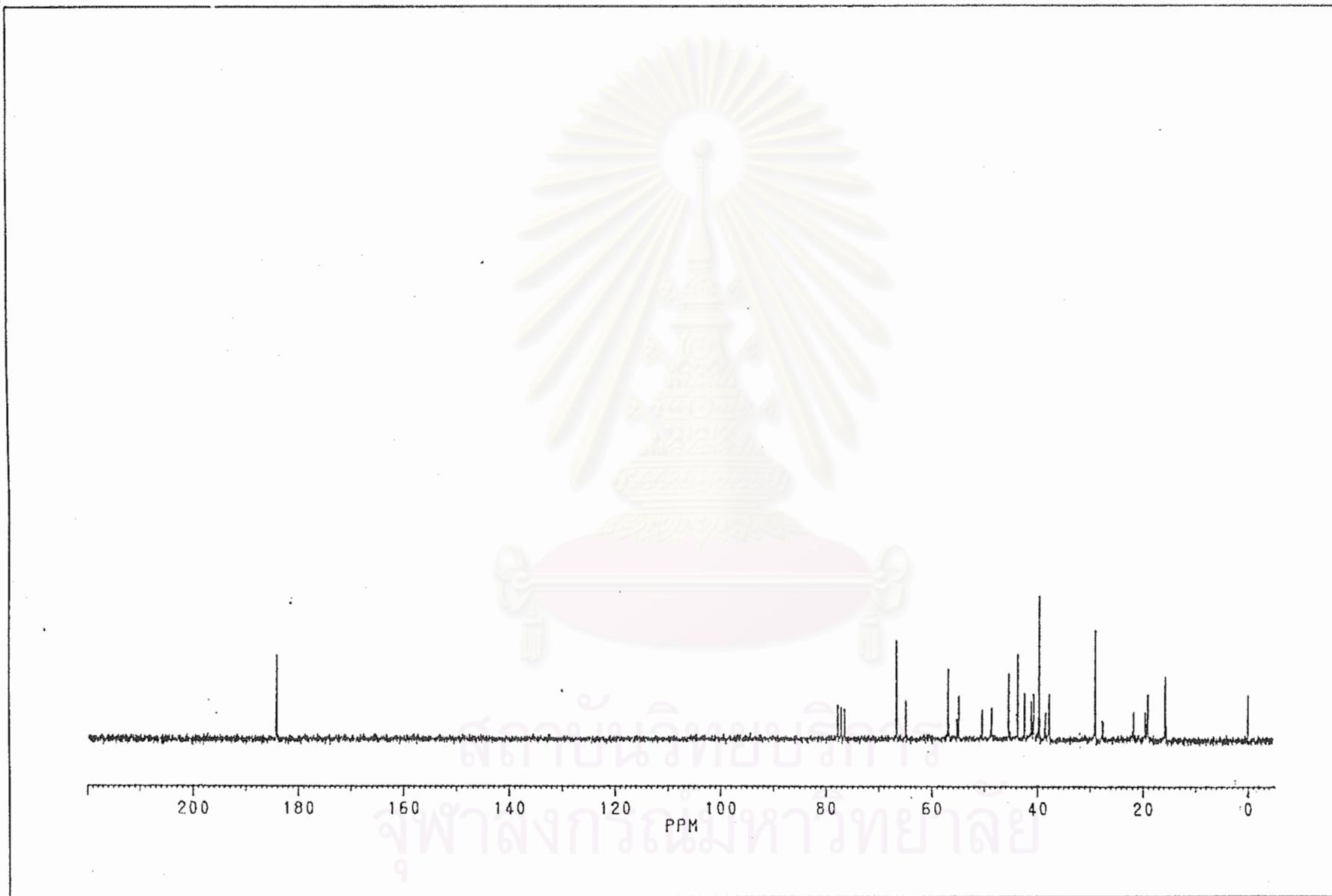
Figure 22 The EI MS spectrum of compound 1
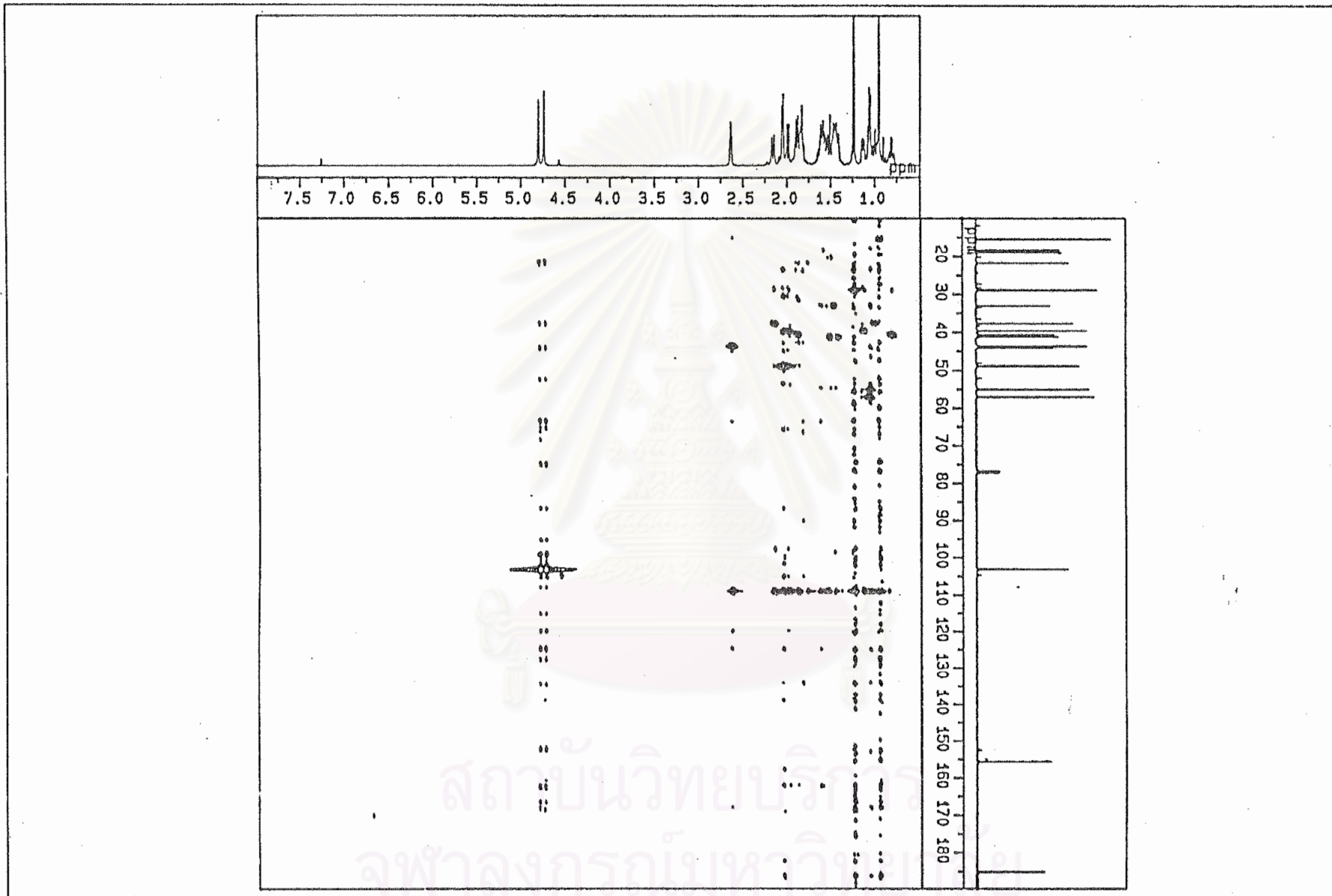
Figure 23   The HMQC-NMR spectrum of compound 1
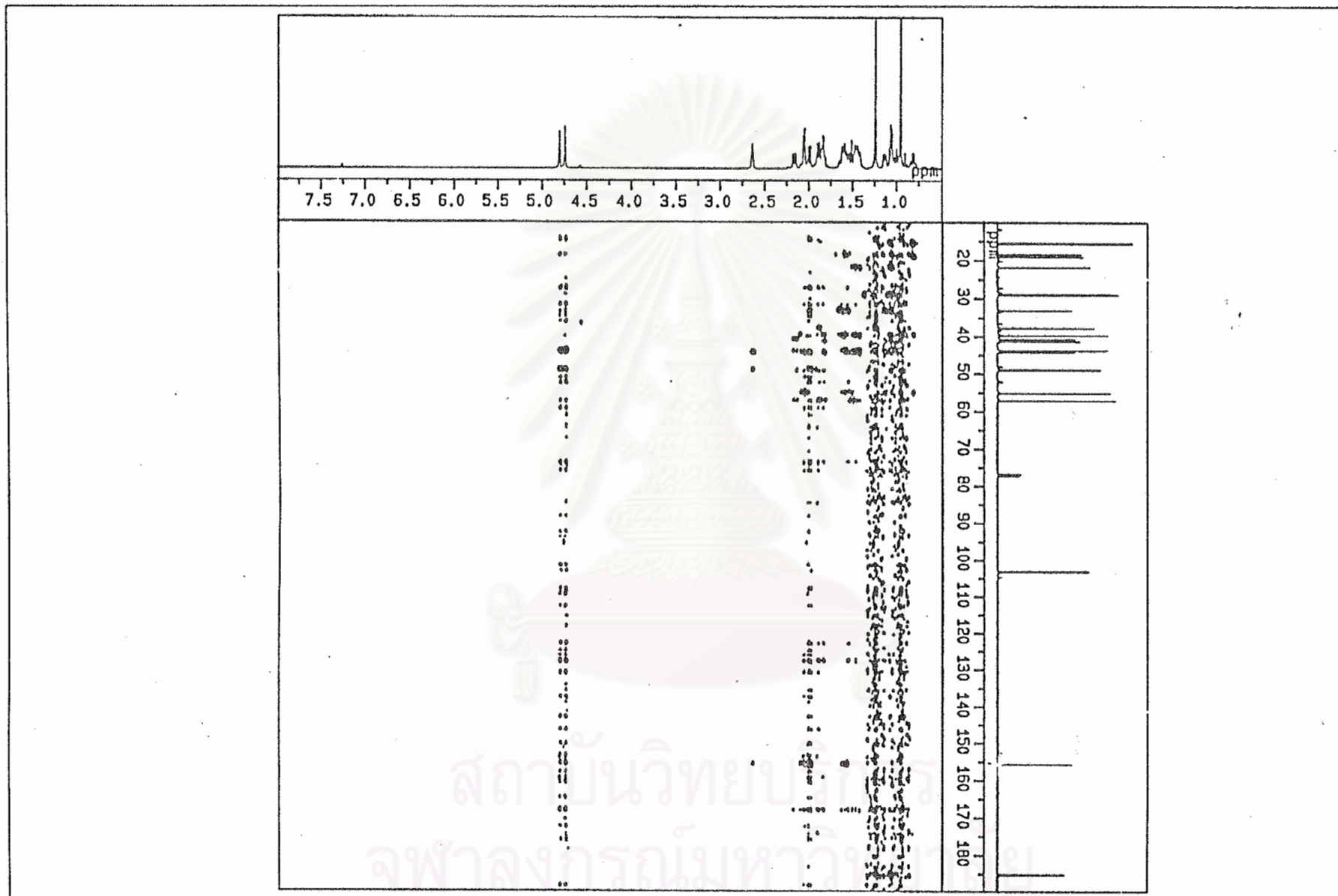
Figure 24 The HMBC-NMR spectrum of compound 1
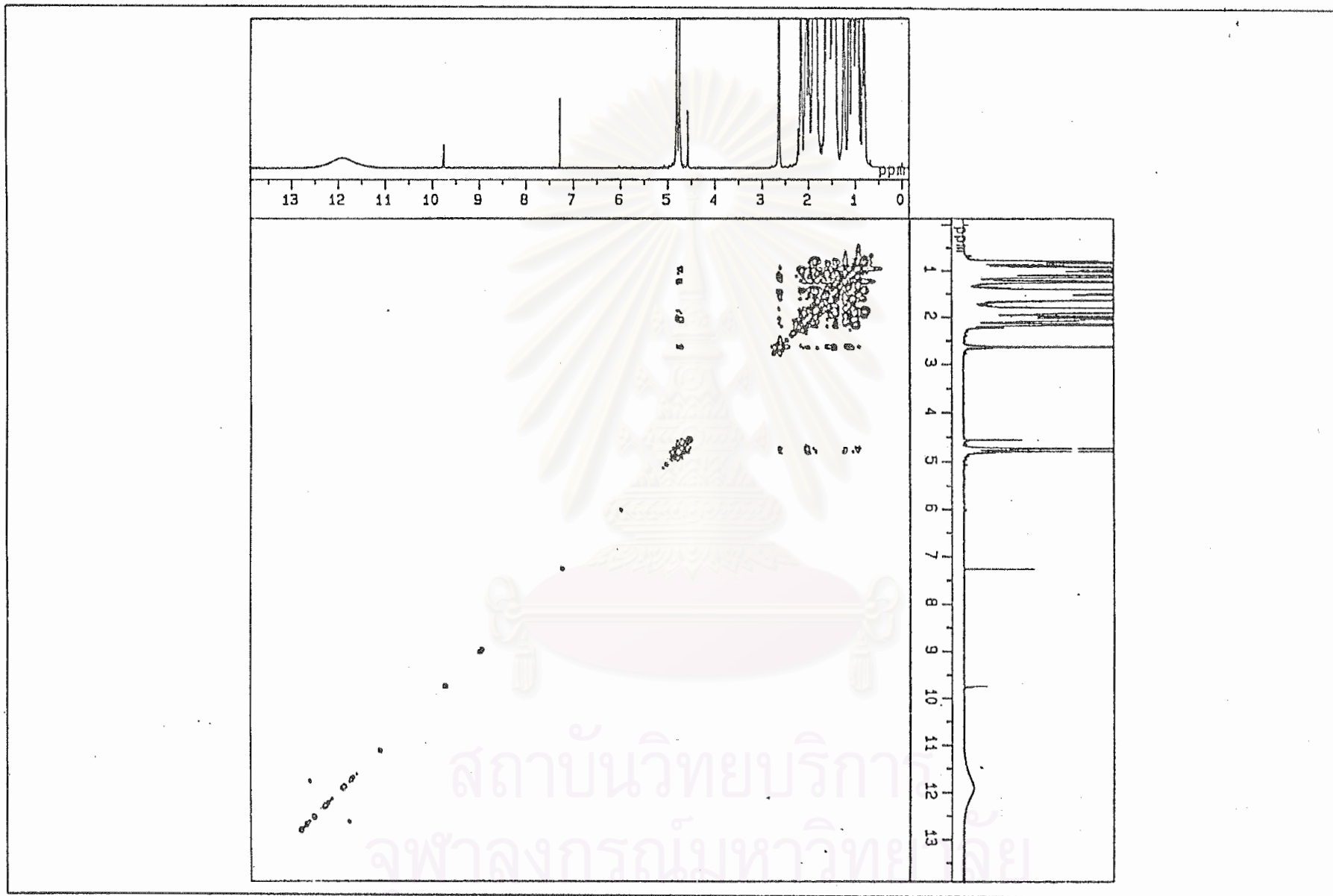
Figure 25 The COSY-NMR spectrum of compound 1
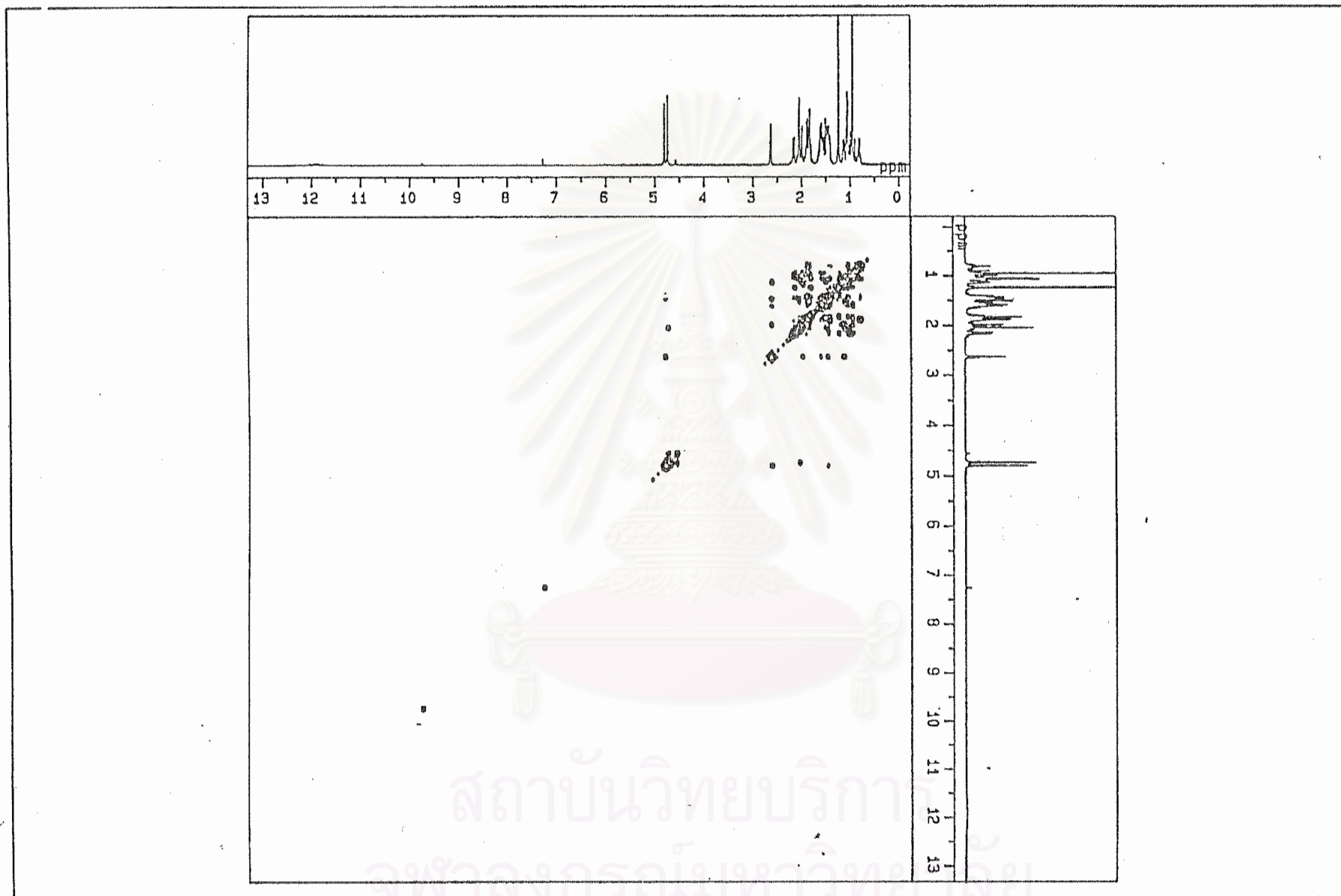
Figure 26   The NOESY-NMR spectrum of compound 1
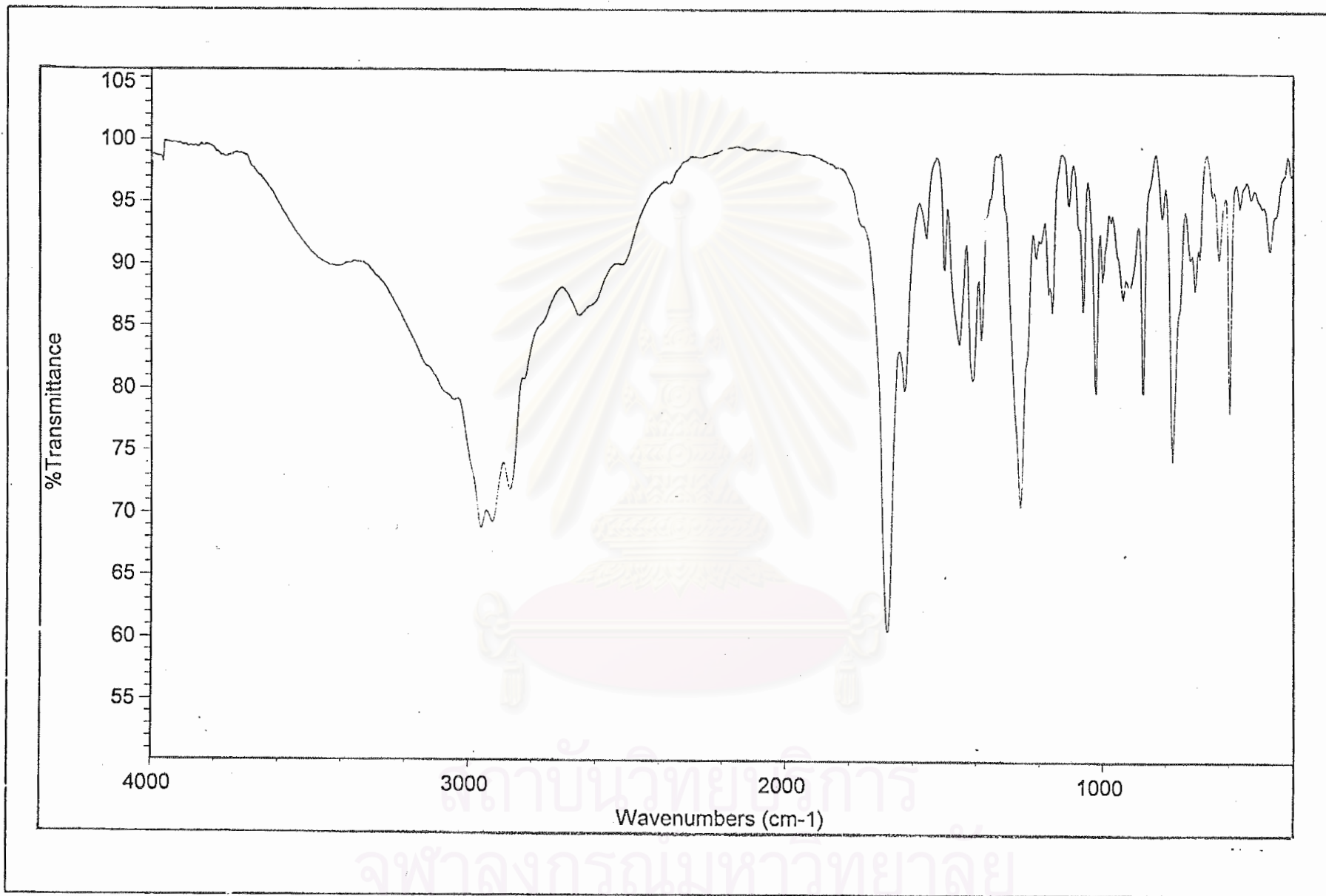
Figure 27 The IR spectrum of compound 2
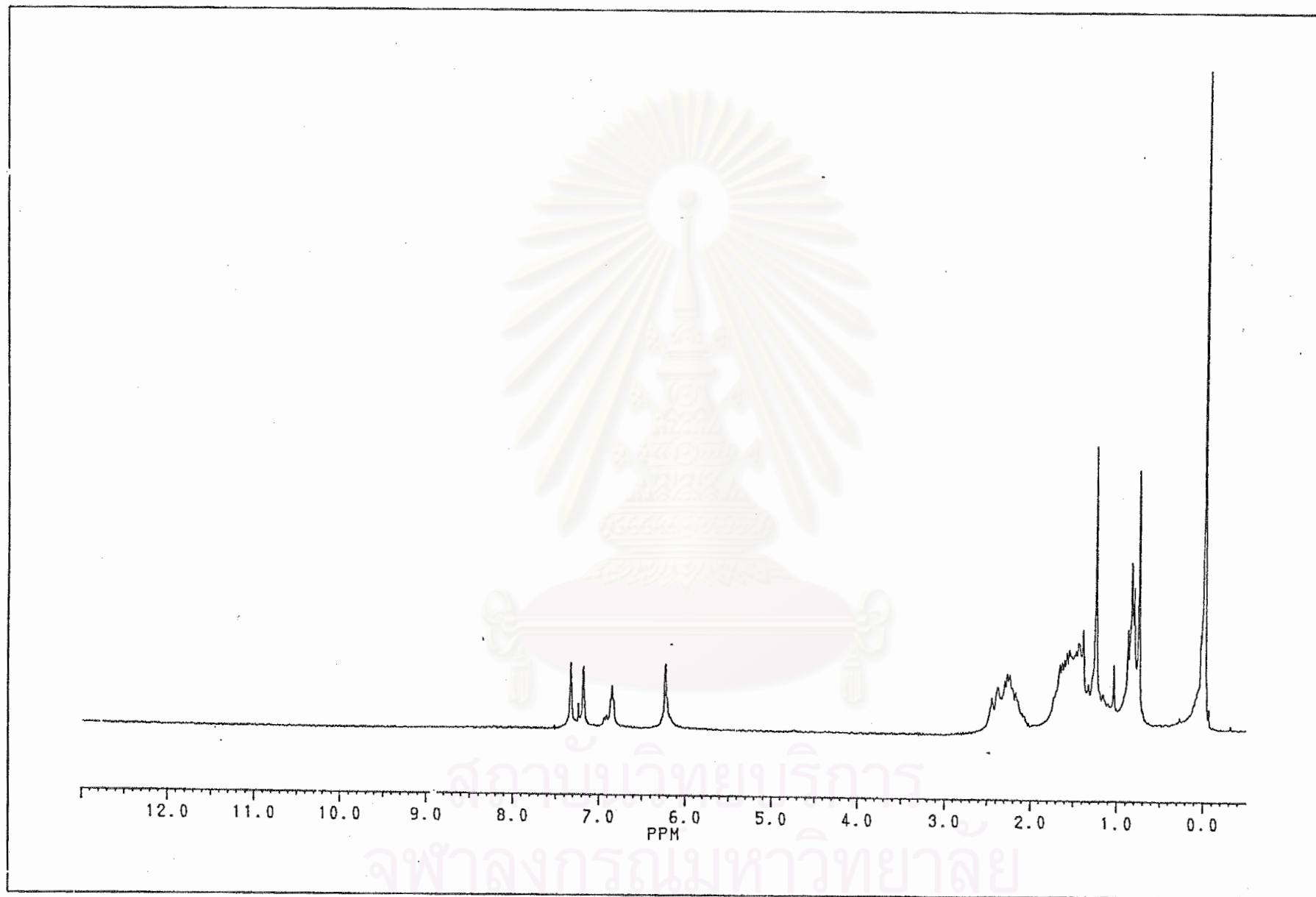
Figure 28 The ^{1}H-NMR spectrum of compound 2
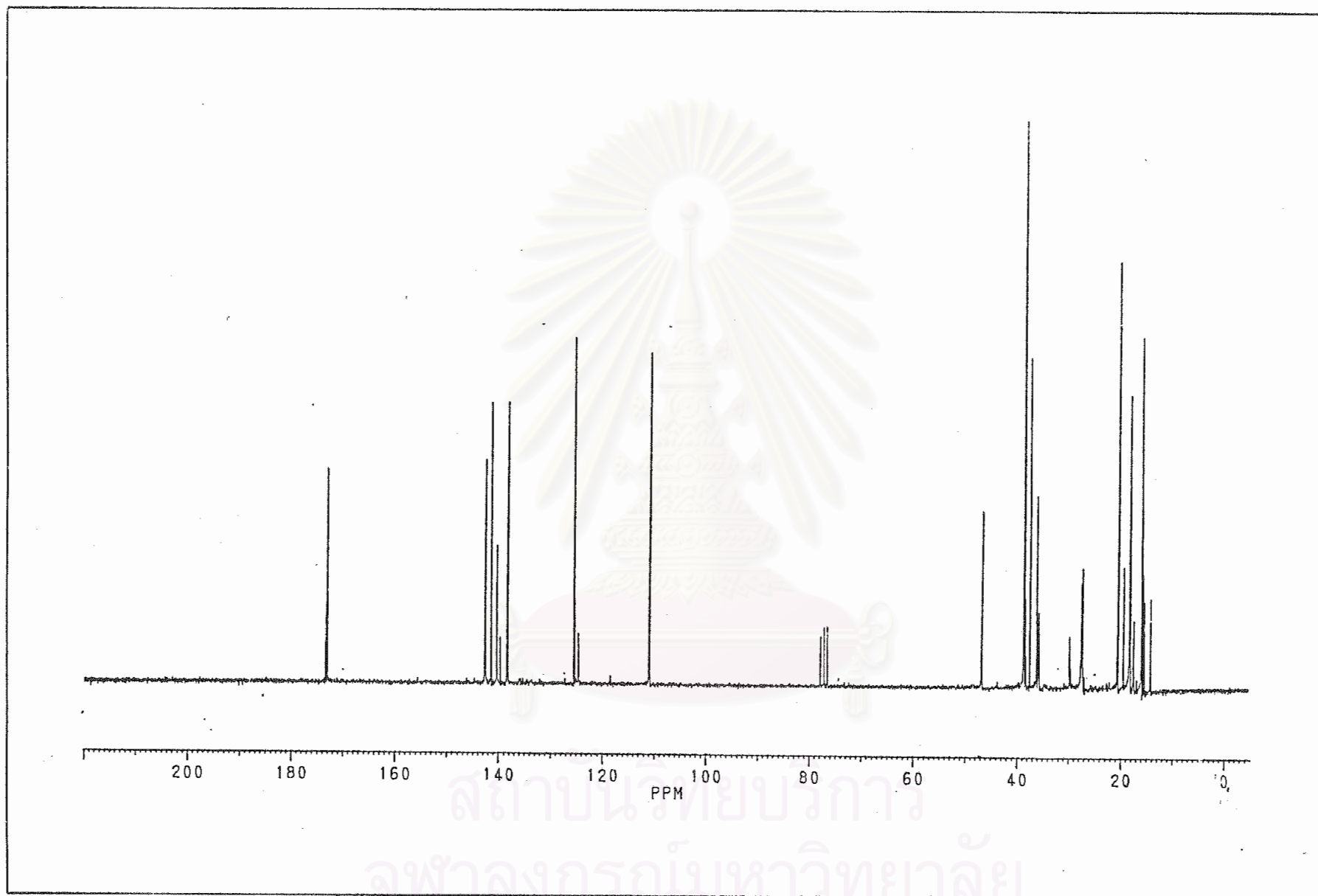
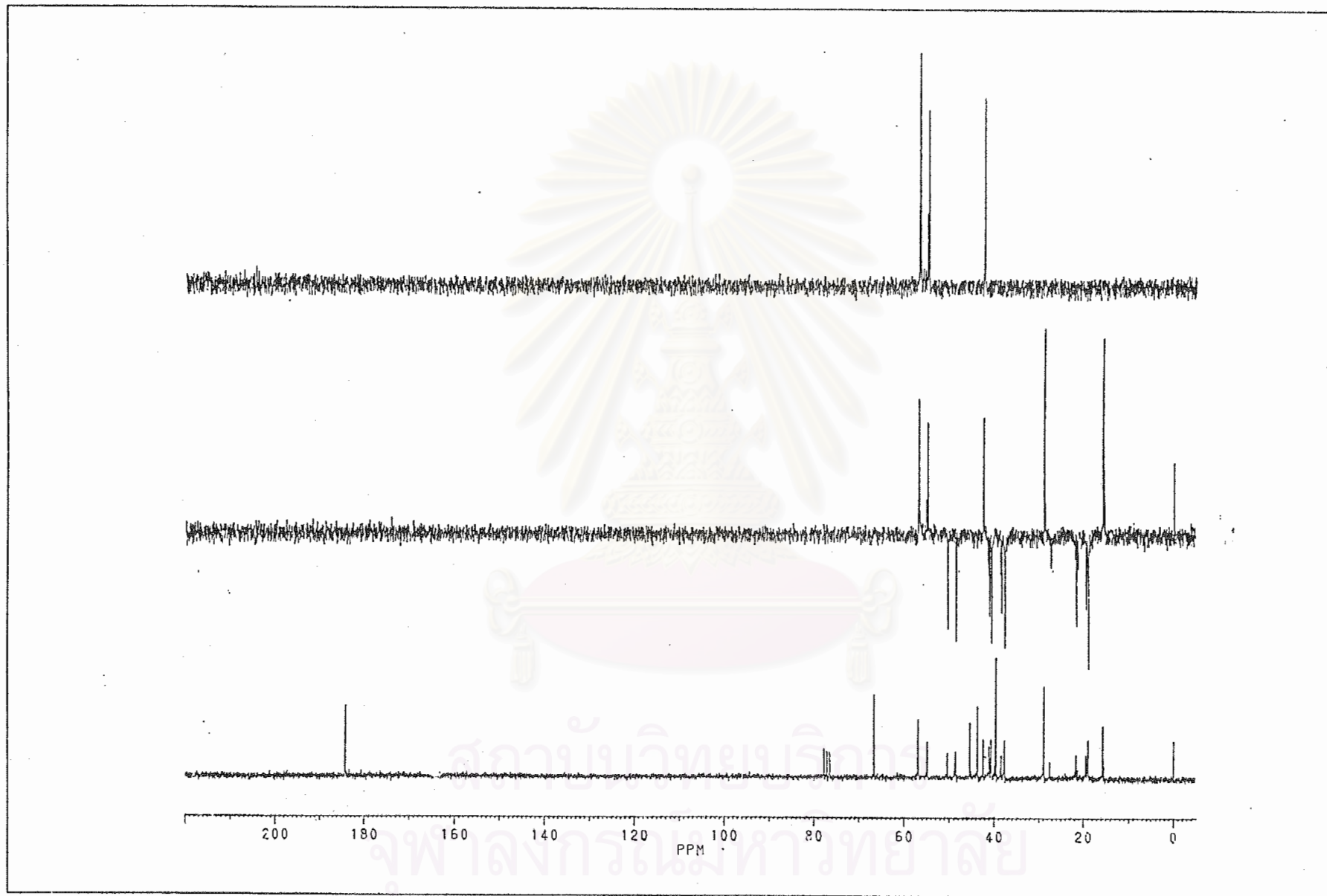Figure 29 The ^{13}C-NMR spectrum of compound 2
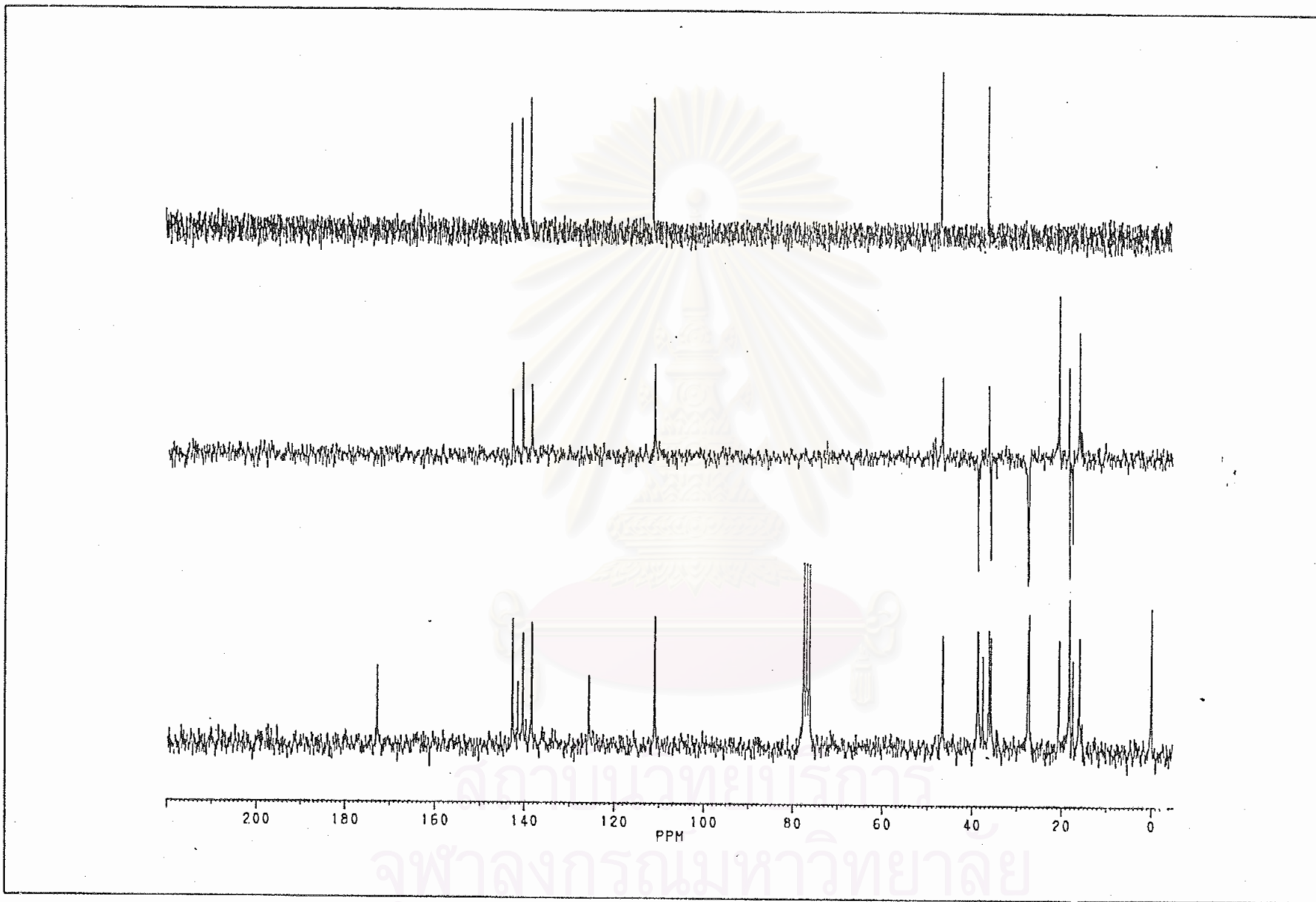
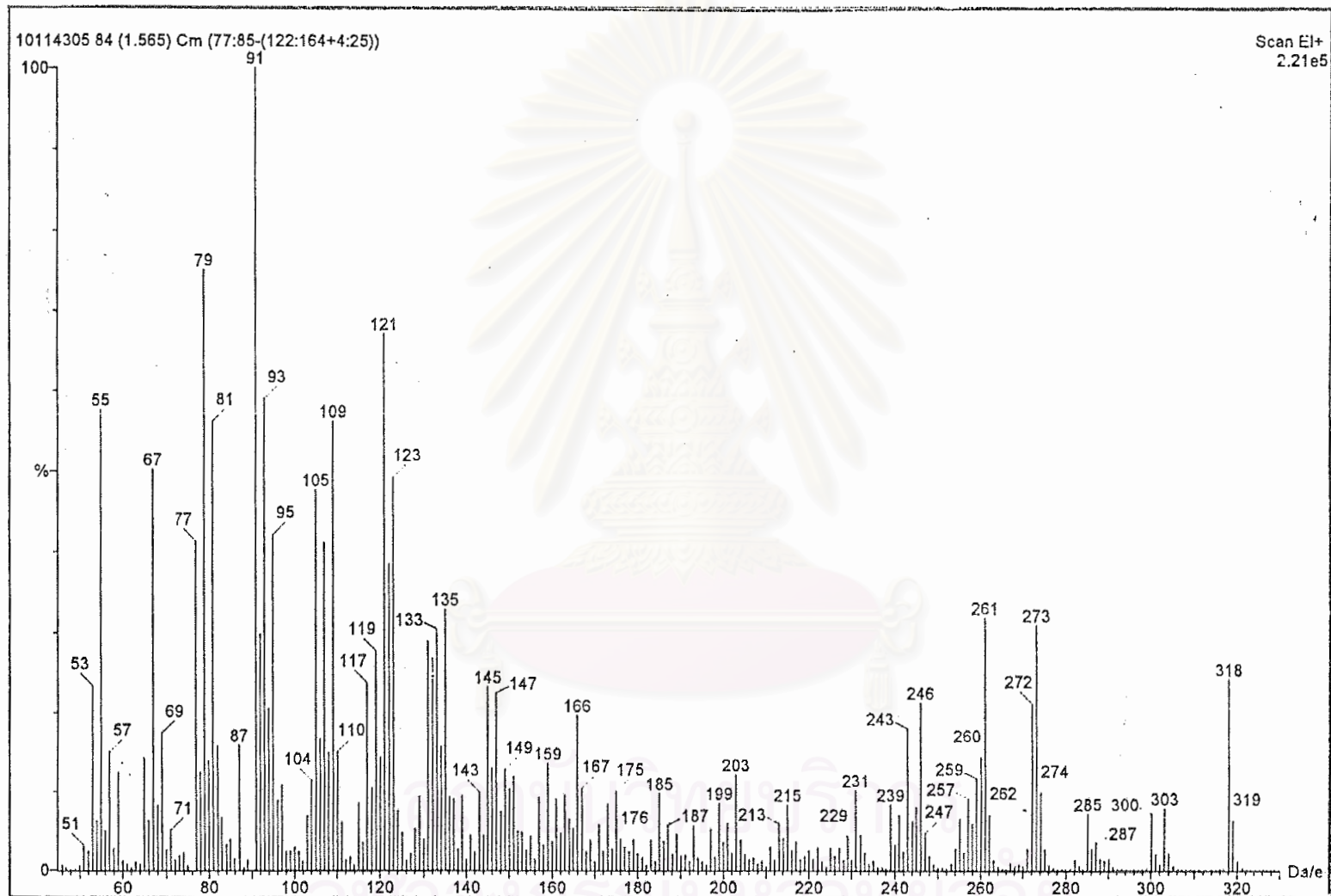**Figure 30** DEPT 90, 135 and ^{13}C-NMR spectrum of compound 2
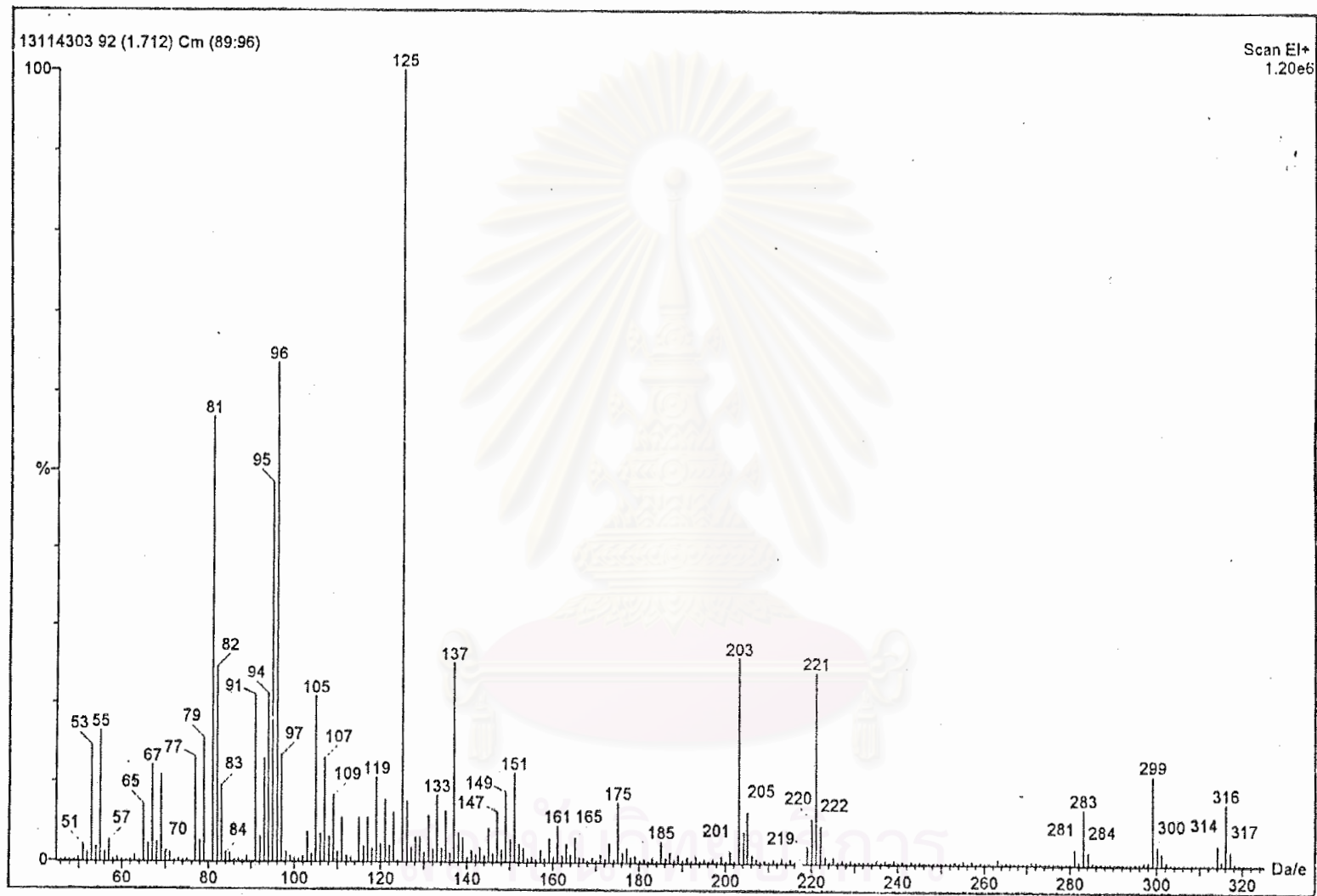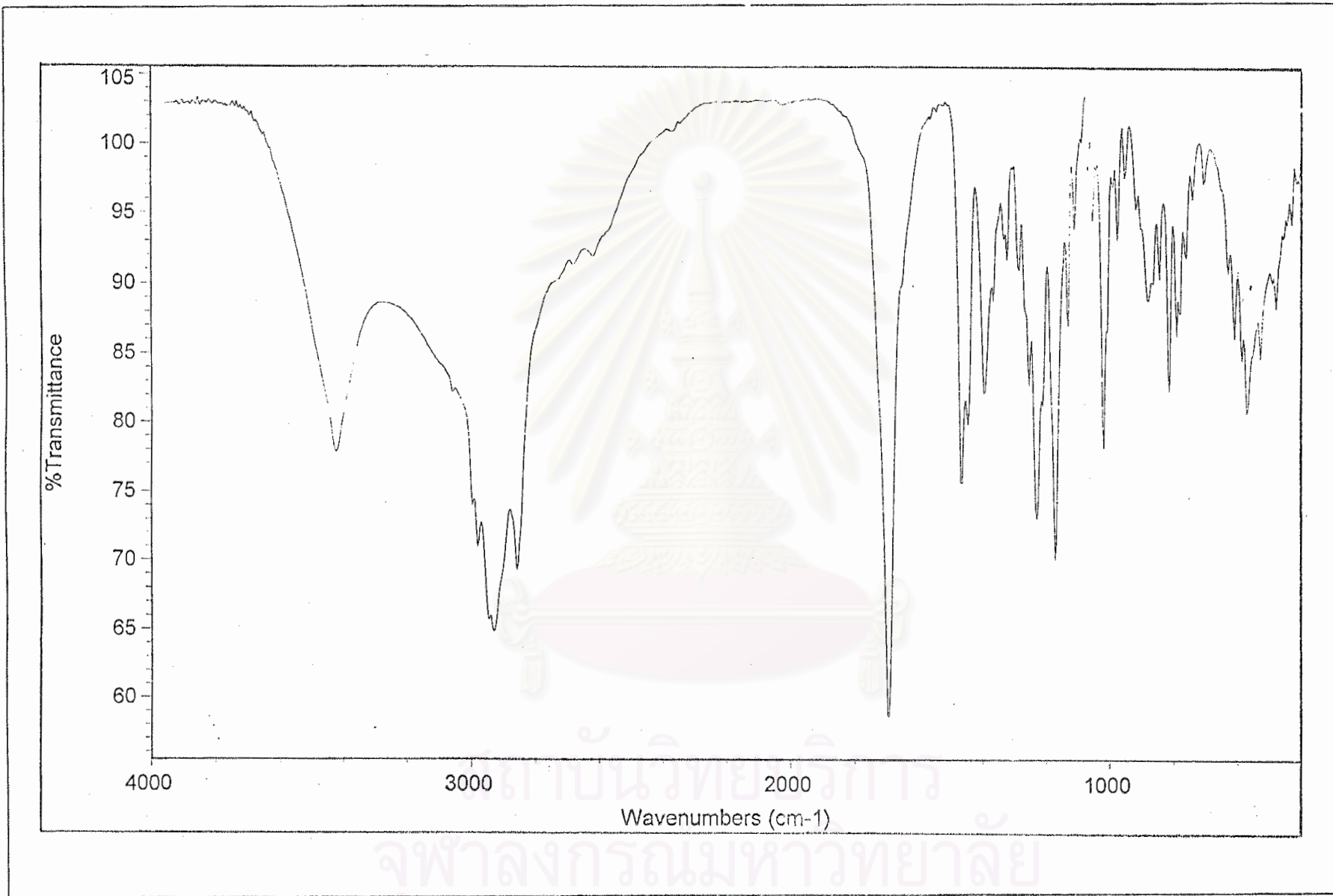
Figure 31 The EI MS spectrum of compound 2
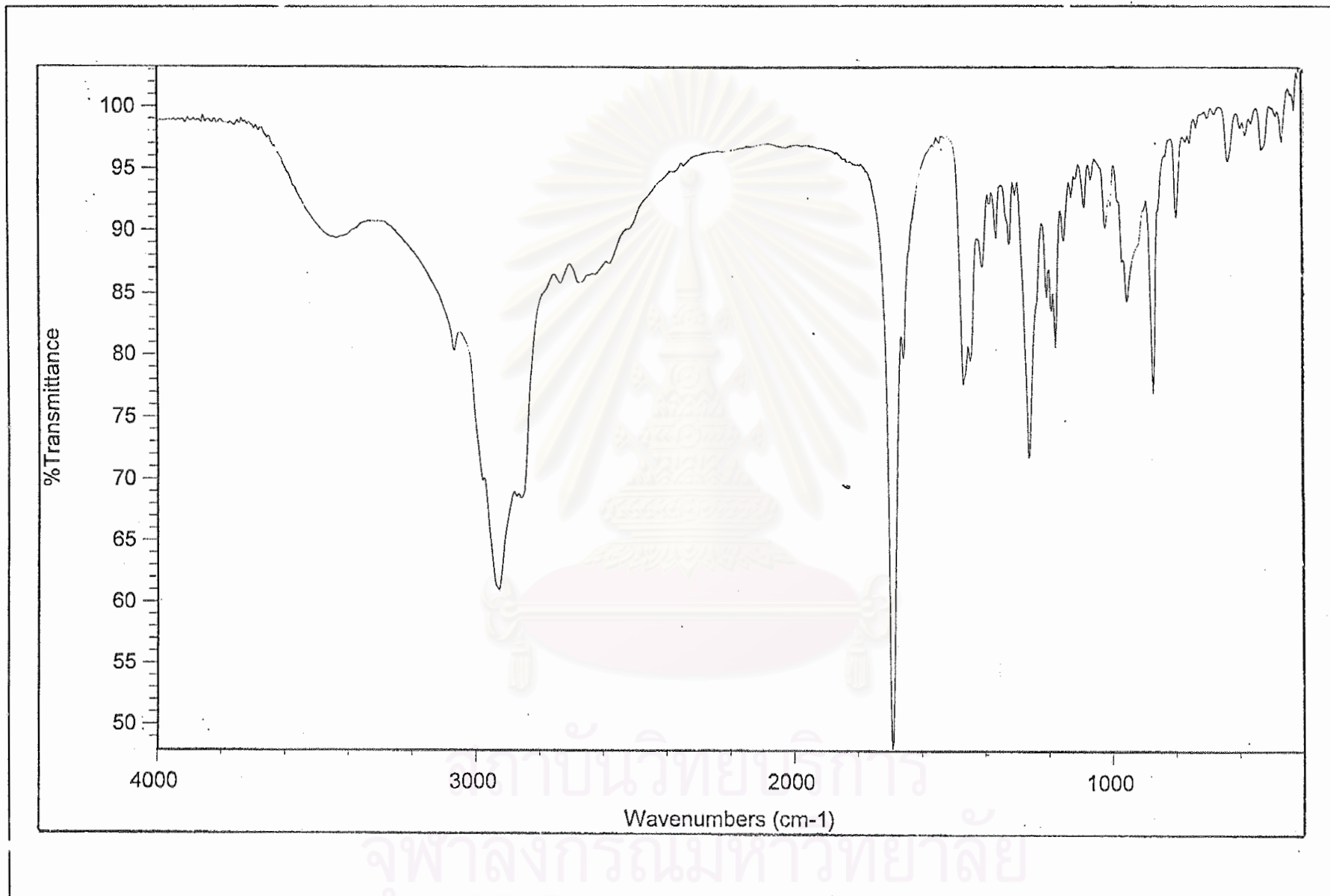
Figure 32 The IR spectrum of compound 3
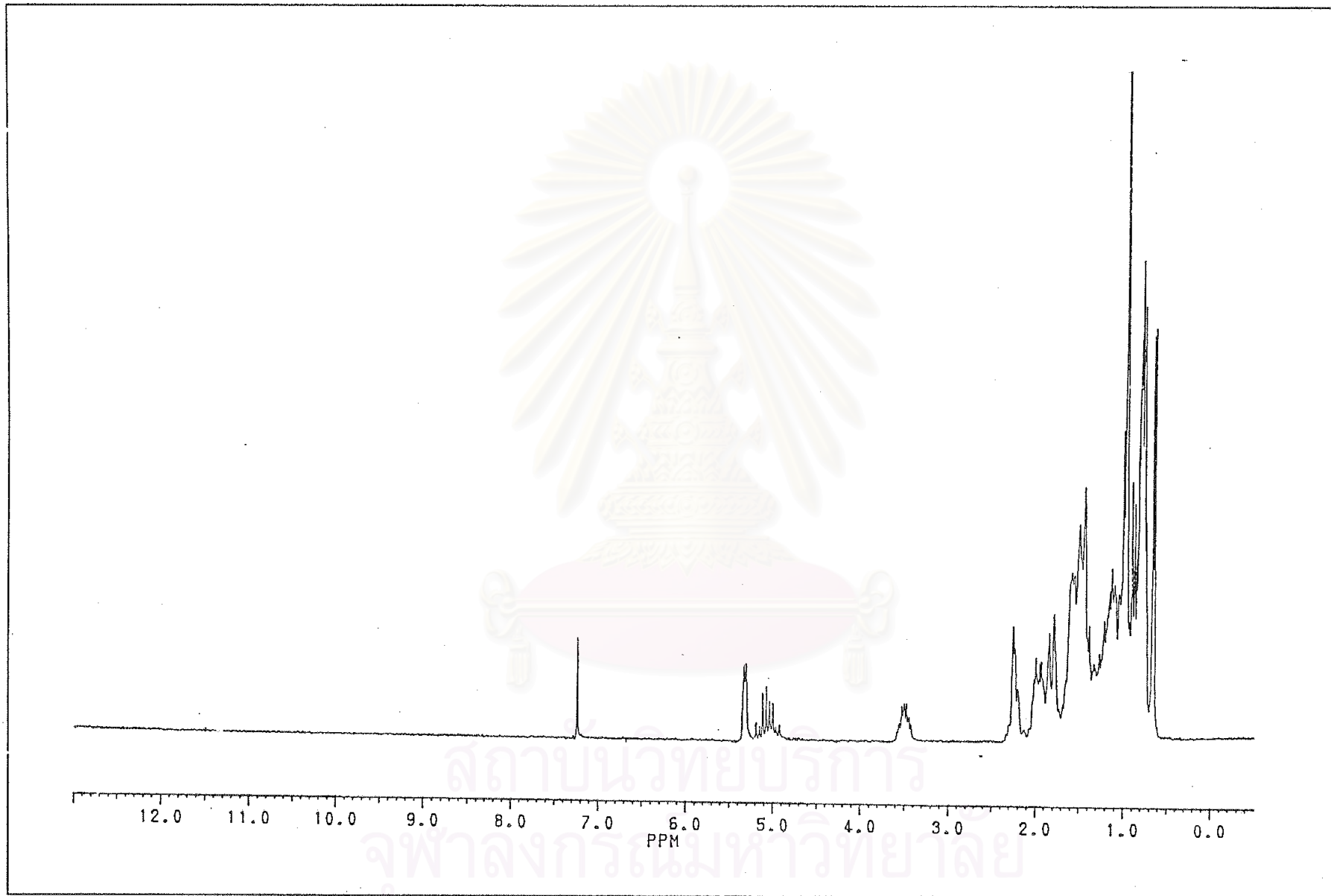
Figure 33 The ^{1}H-NMR spectrum of compound 3
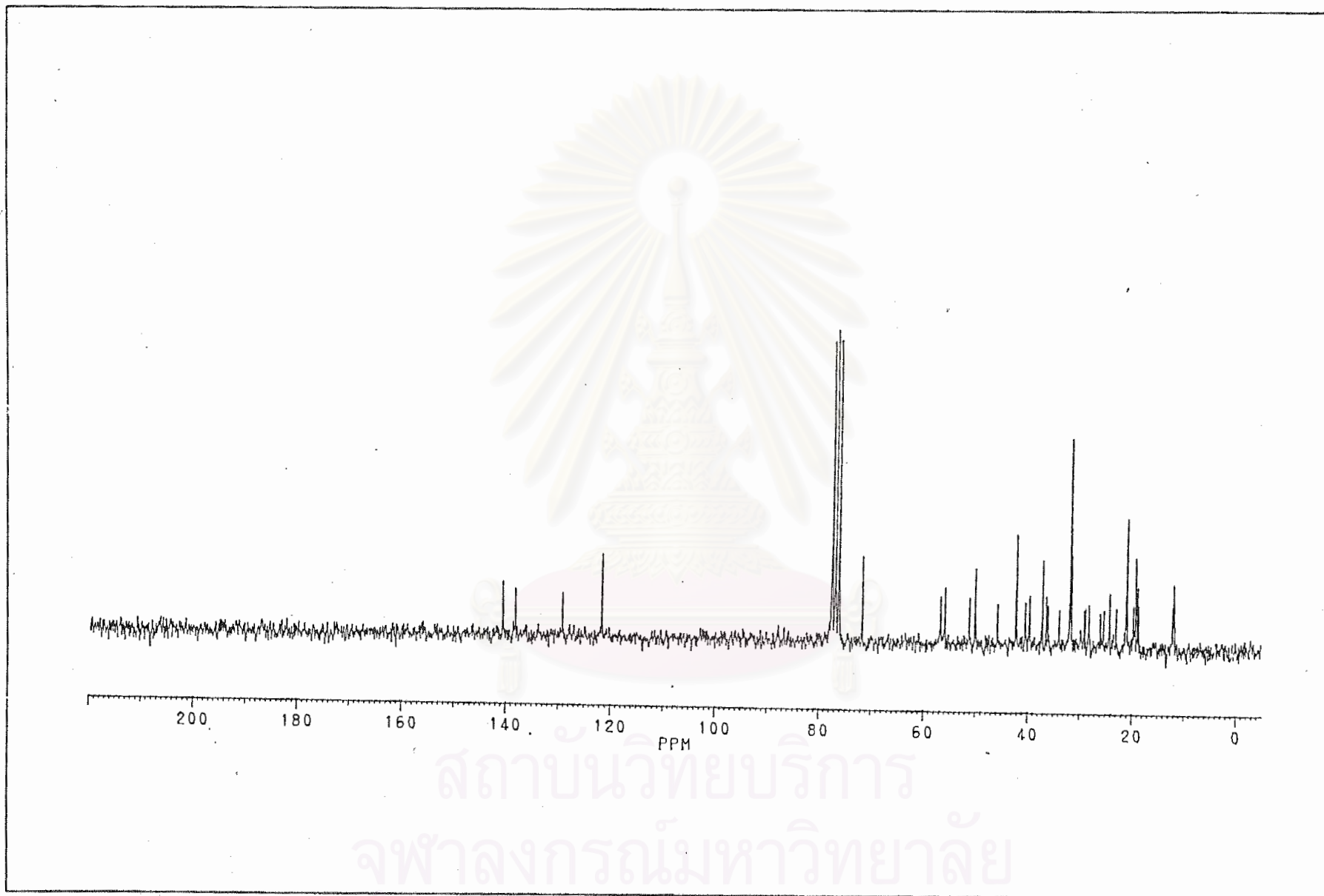
Figure 34 The ^{13}C-NMR spectrum of compound 3
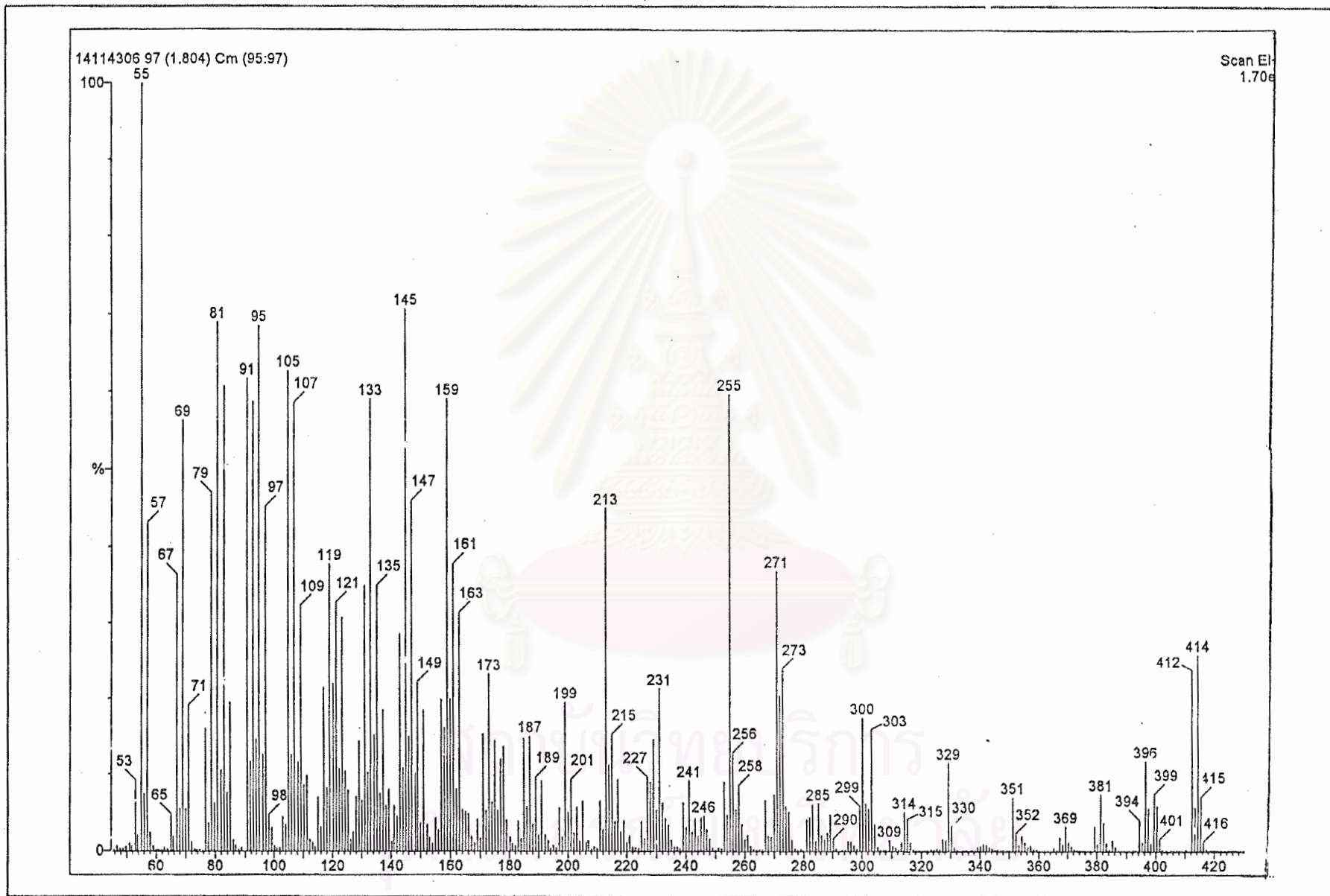
Figure 35 The EI MS spectrum of compound 3
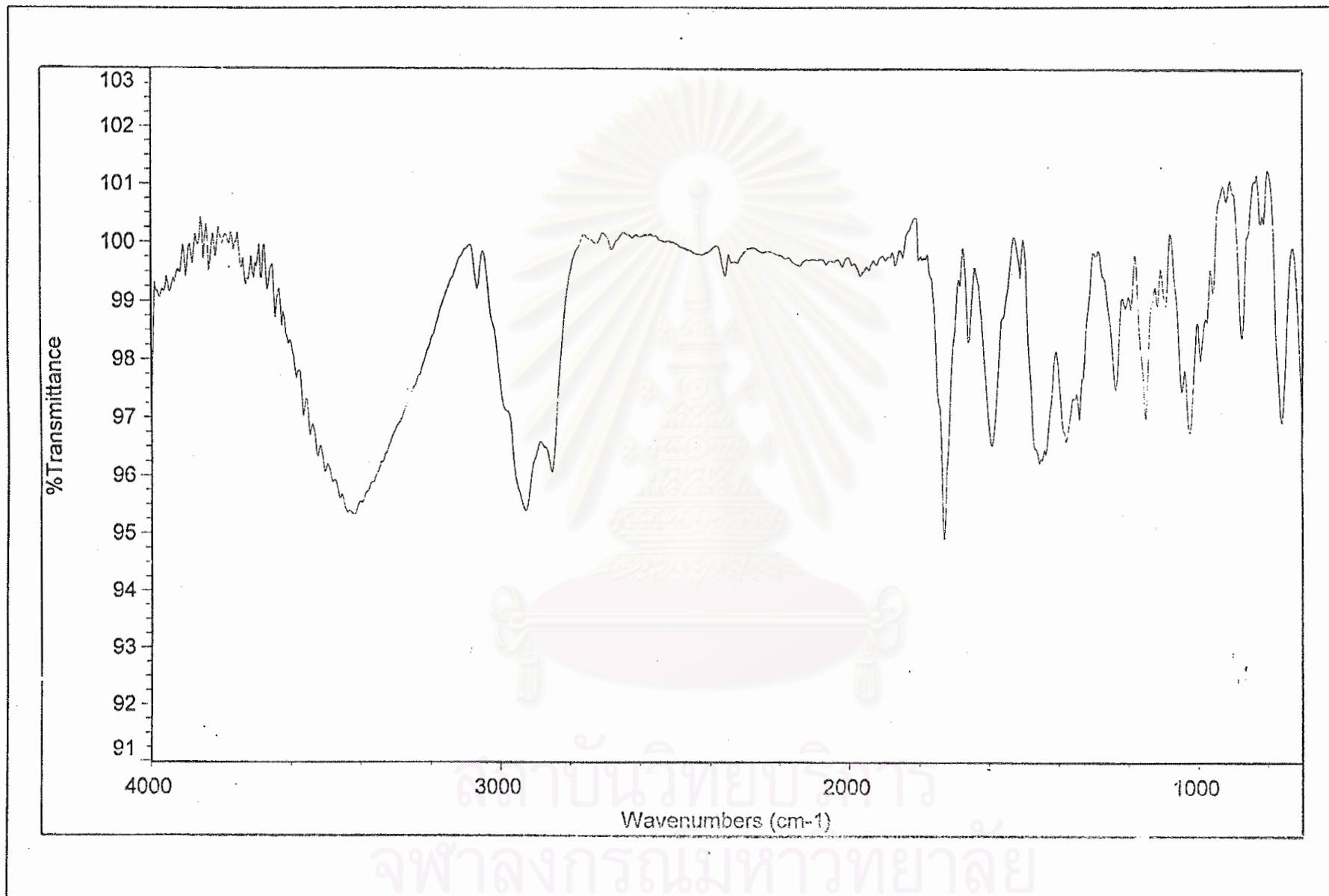
Figure 36 The IR spectrum of compound 1a
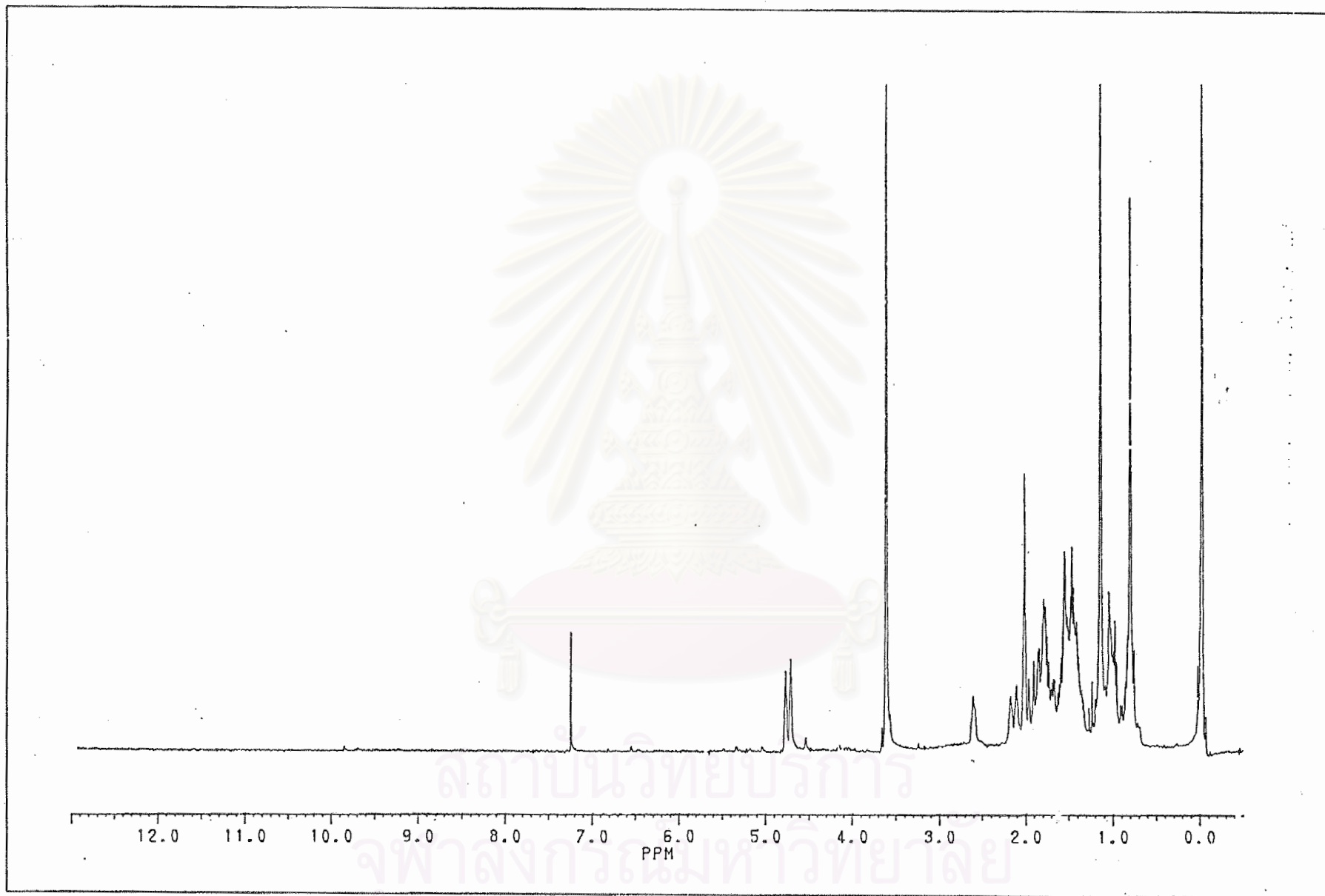
Figure 37 The ^{1}H-NMR spectrum of compound 1a
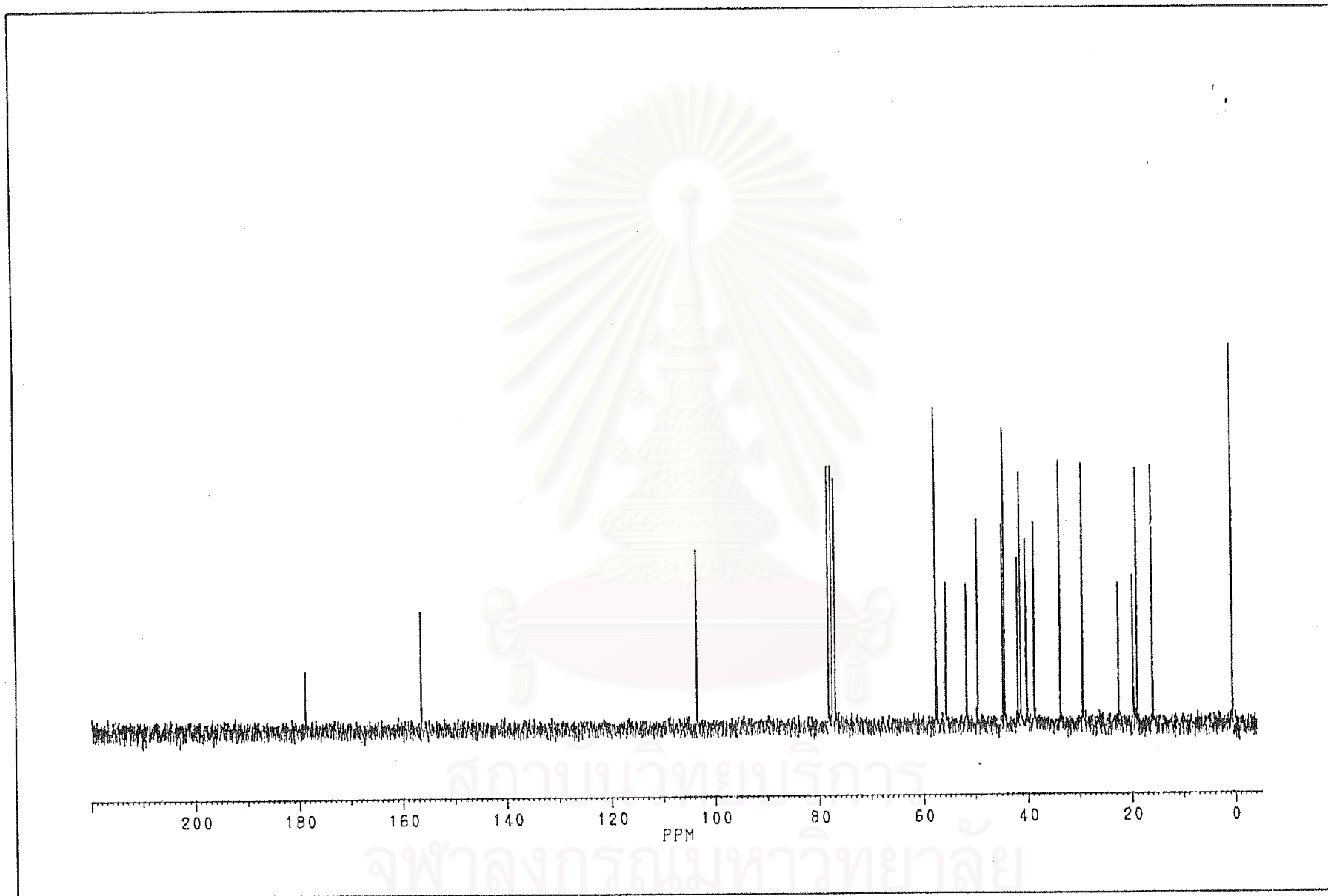
Figure 38 The ^{13}C-NMR spectrum of compound 1a
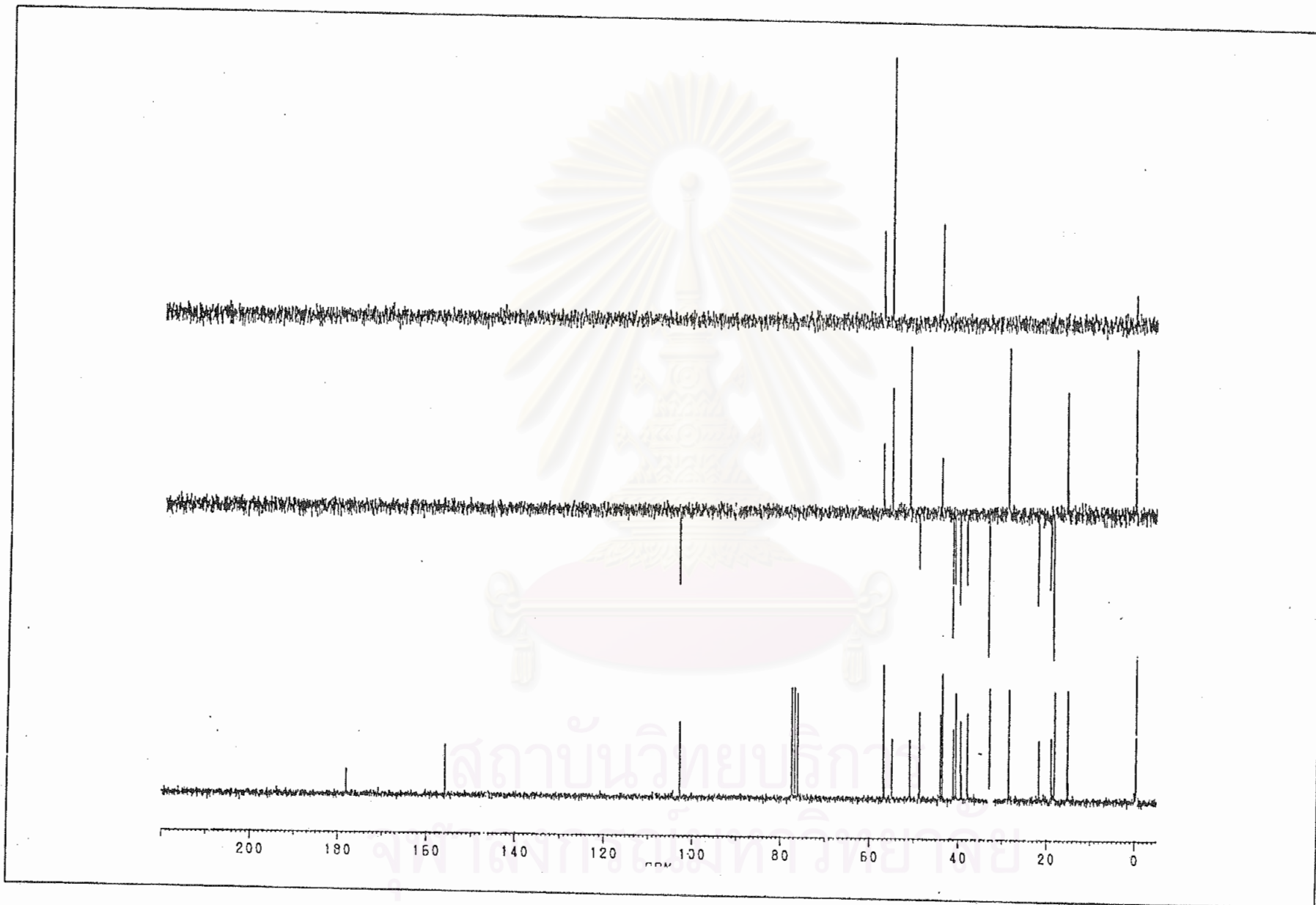
Figure 39 DEPT 90, 135 and ^{13}C-NMR spectrum of compound 1a
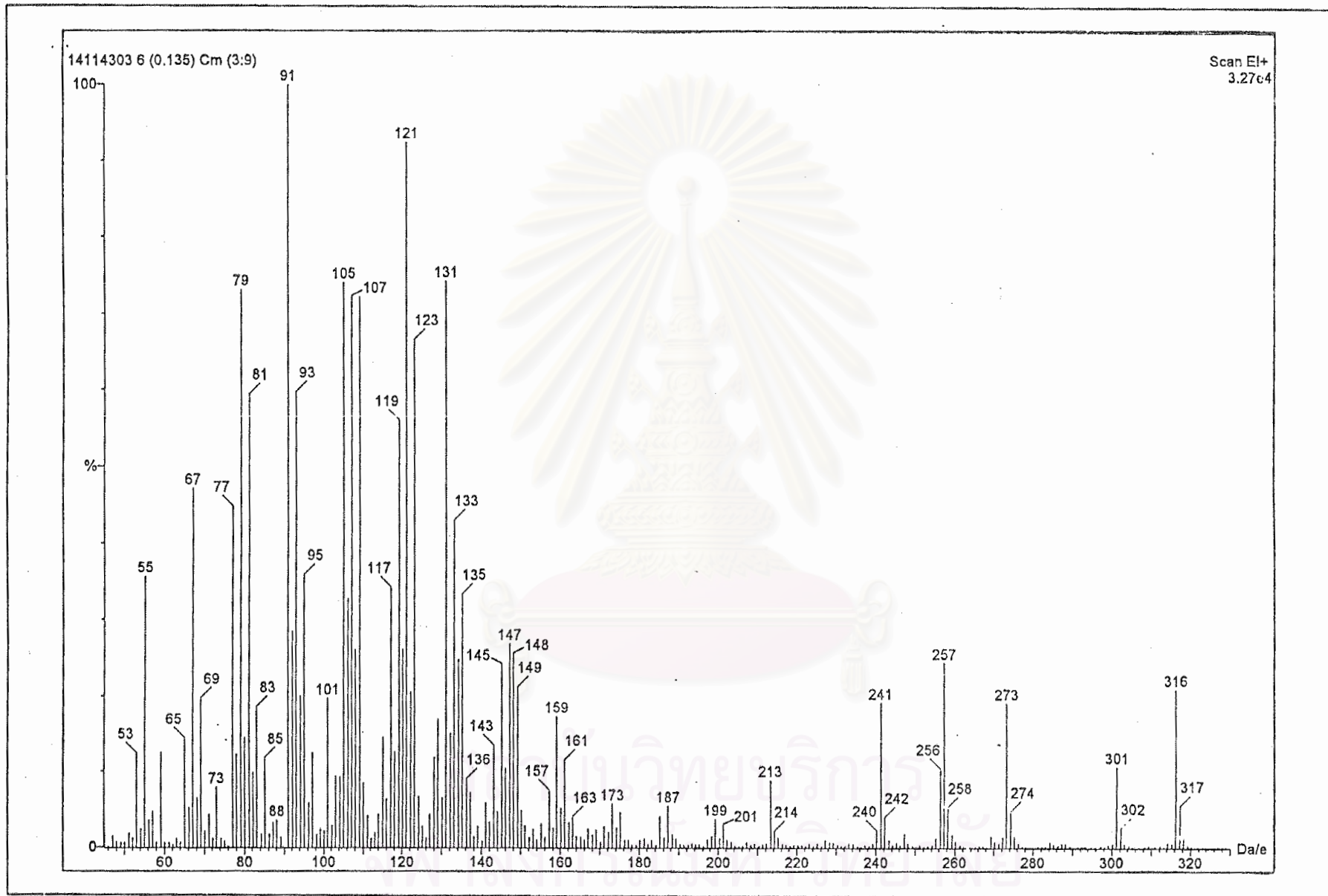
Figure 40 The EI MS spectrum of compound 1a
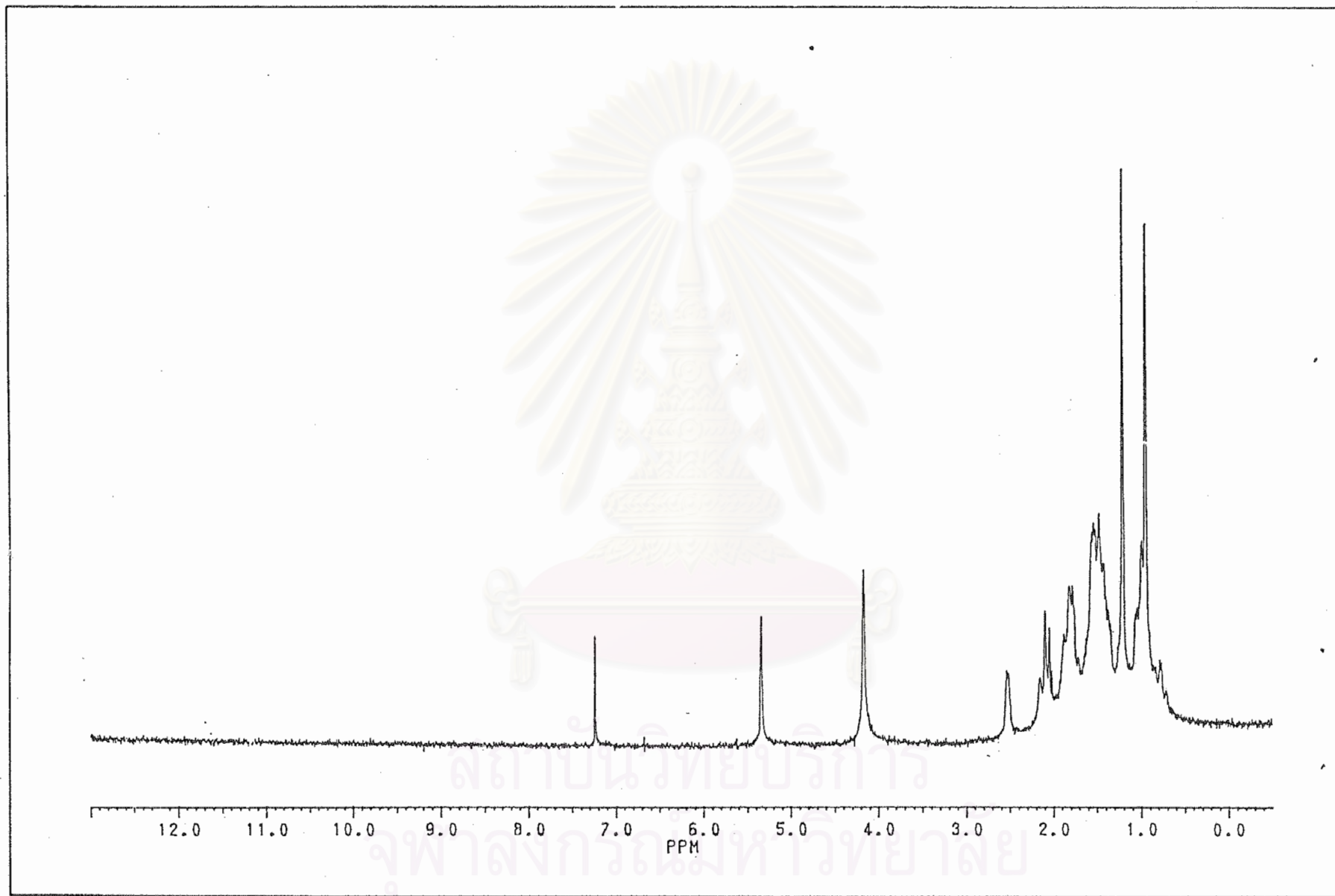
Figure 41 The IR spectrum of compound 1b
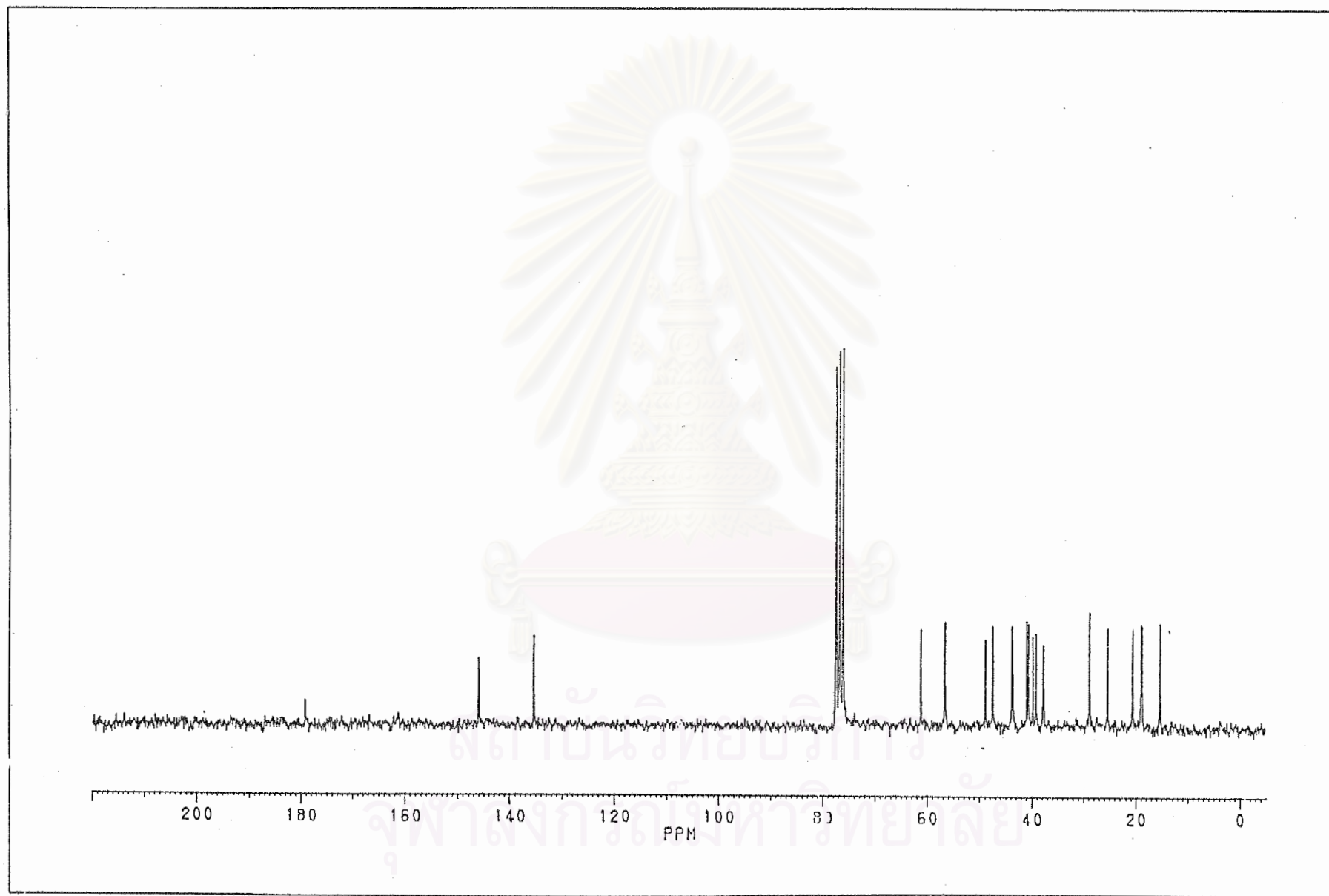
Figure 42 The ^{1}H-NMR spectrum of compound 1b

Figure 43 The ^{13}C-NMR spectrum of compound 1b

Figure 44 DEPT 90, 135 and ^{13}C-NMR spectrum of compound 1b

Figure 45 The EI MS spectrum of compound 1b

Figure 46 The IR spectrum of compound 1c

Figure 47 The ^{1}H-NMR spectrum of compound 1c
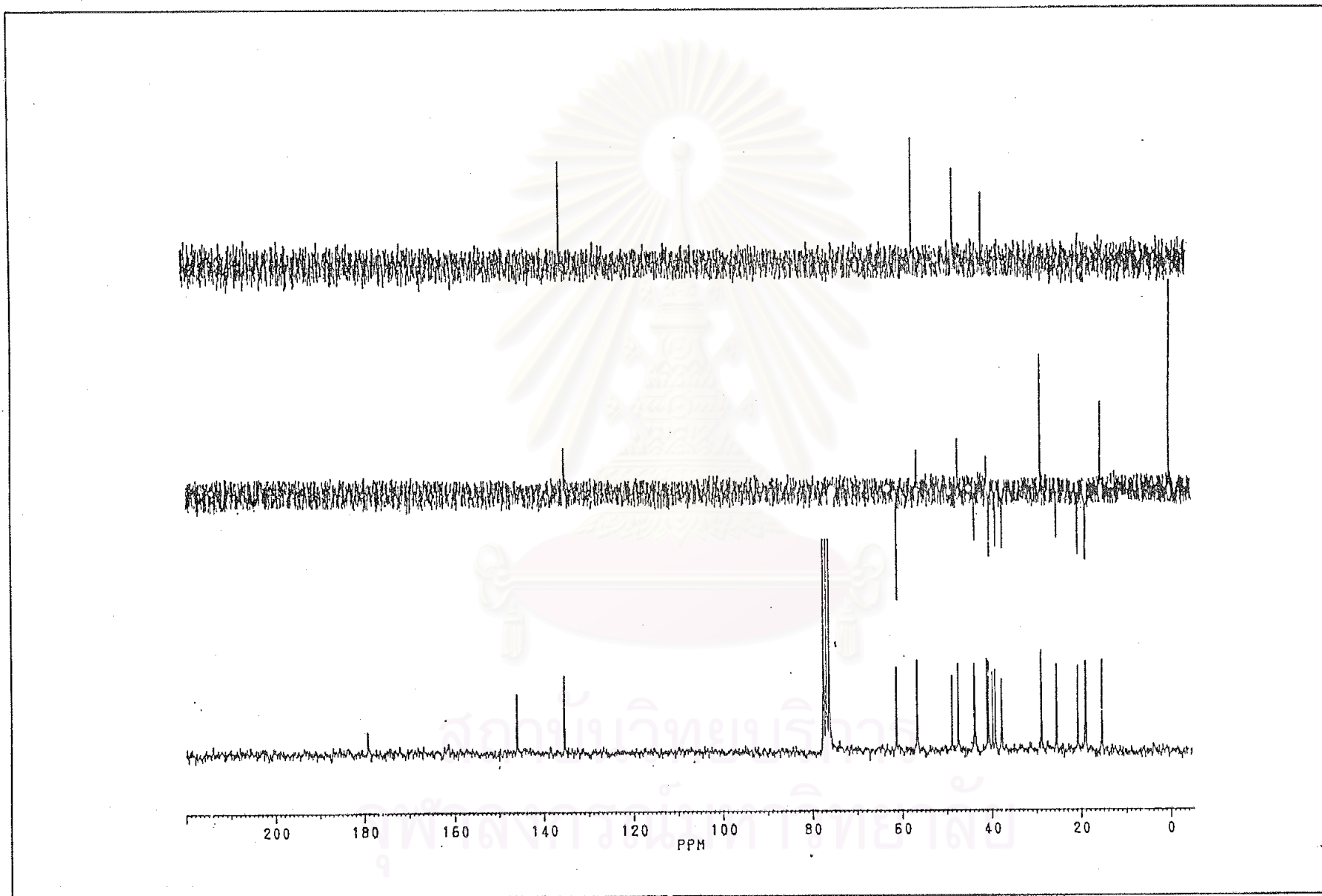
Figure 48 The ^{13}C-NMR spectrum of compound 1c

Figure 49 DEPT 90, 135 and ^{13}C-NMR spectrum of compound 1c

Figure 50 The EI MS spectrum of compound 1c

Figure 51 The IR spectrum of compound 1d

Figure 52 The ^{1}H-NMR spectrum of compound 1d

Figure 53 The ^{13}C-NMR spectrum of compound 1d

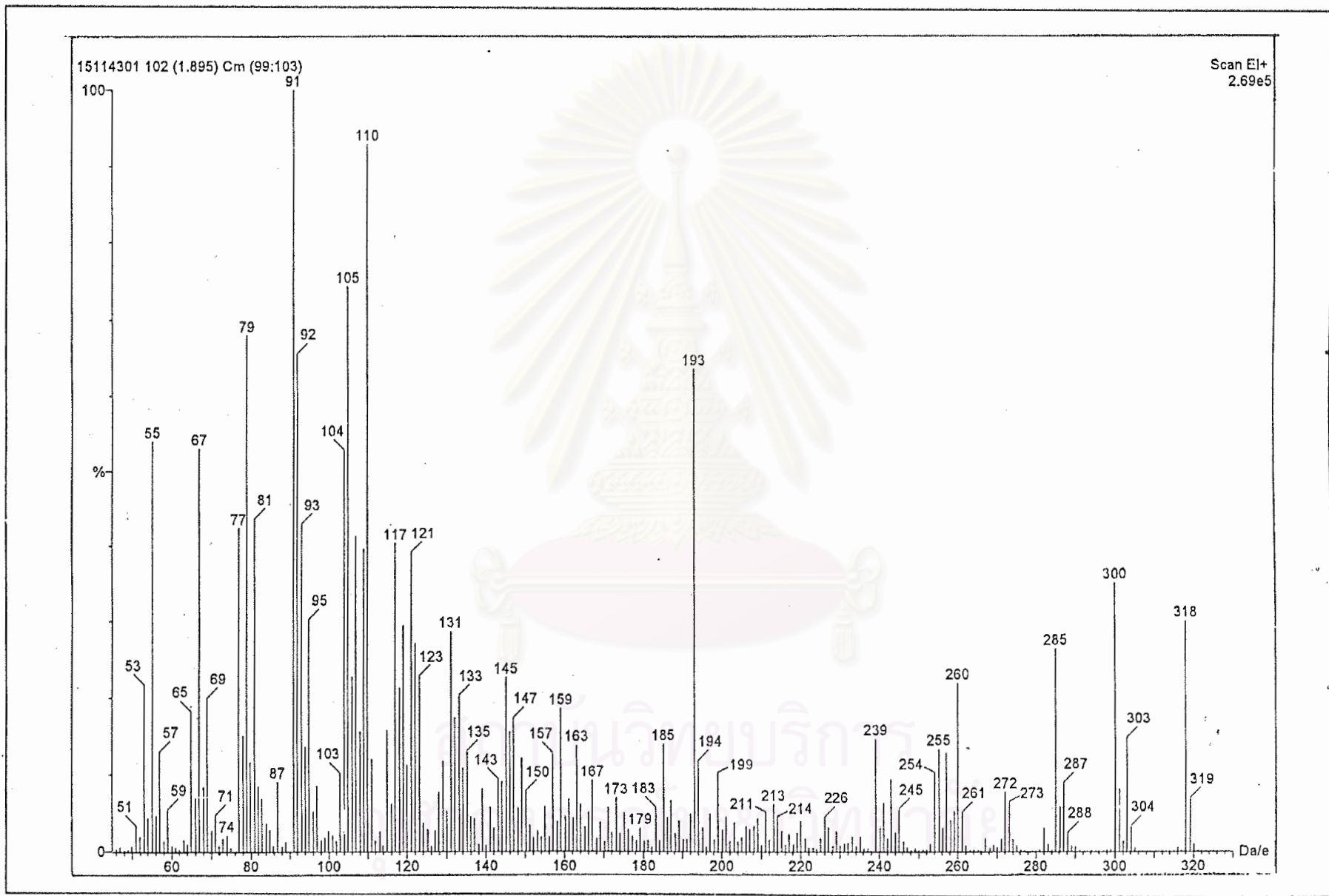Figure 54 DEPT 90, 135 and ^{13}C-NMR spectrum of compound 1d
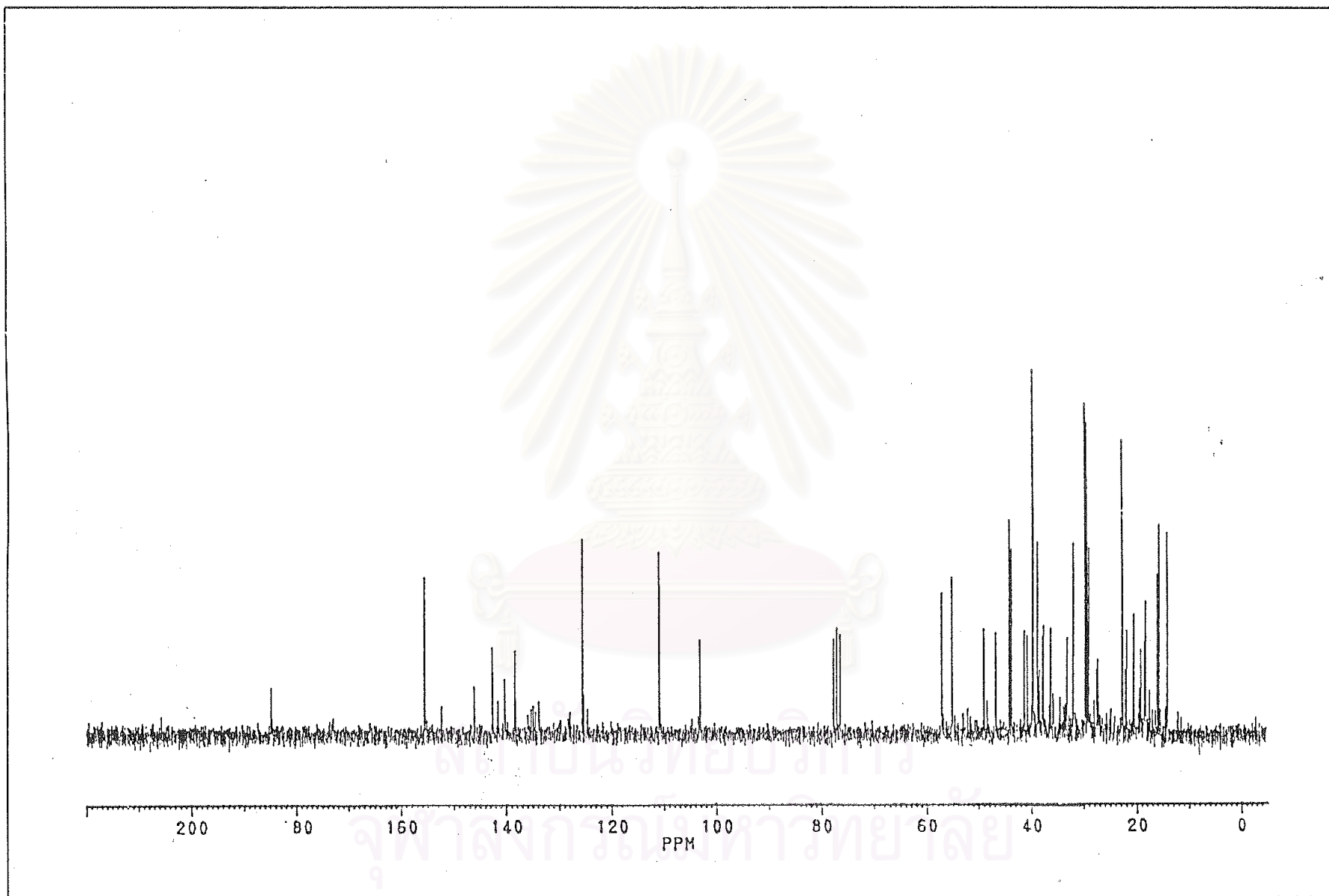
Figure 55 The EI MS spectrum of compound 1d

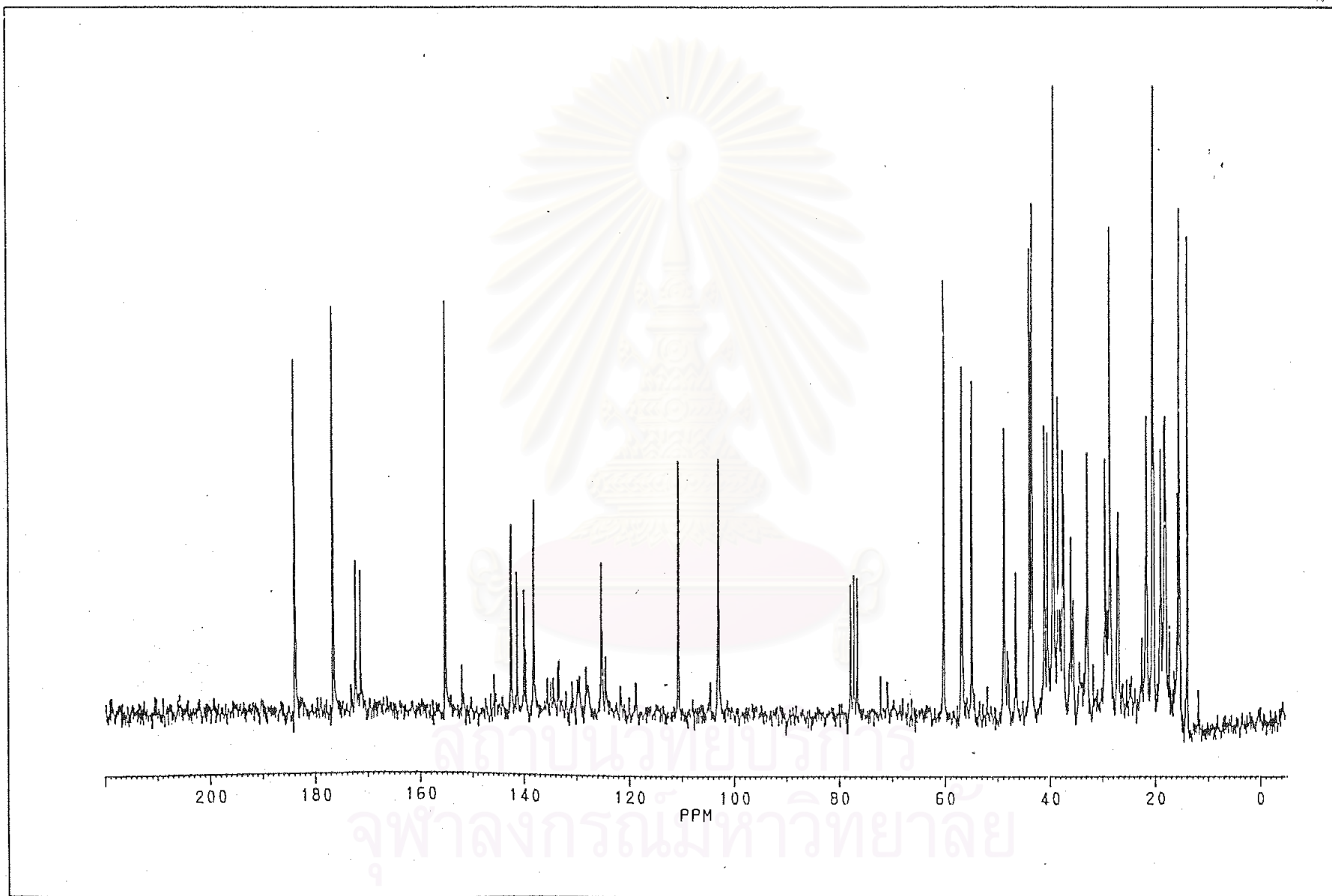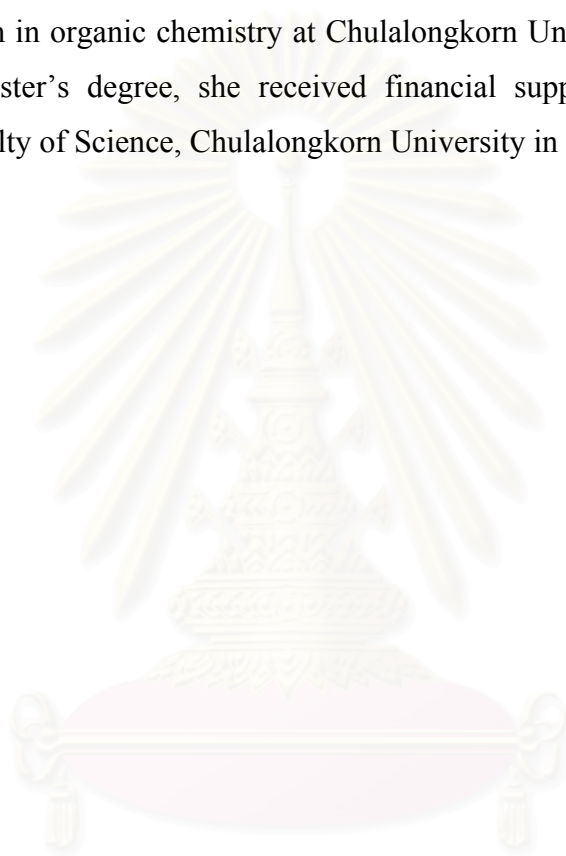Figure 56 The ^{13}C-NMR spectrum of crude hexane extraction

Figure 57 The ^{13}C-NMR spectrum of crude ethyl acetate extraction

VITA

Miss Supaporn Sirimongkhon was born on june 23, 1975 in Ratchaburi, Thailand. She graduated with a Bachelor Degree of science in Chemistry from Chulalongkorn University in 1997. In the same year, she was admitted into a Master Degree program in organic chemistry at Chulalongkorn University. During her study toward the Master's degree, she received financial support from Department of Chemistry Faculty of Science, Chulalongkorn University in 1999-2000.