องค์ประกอบทางเคมีและฤทธิ์ทางชีวภาพของเปลือกต้นเปล้าใหญ่ (*Croton oblongifolius* Roxb.)
จาก อำเภอเมือง จังหวัดอุดรธานี

นาย ศีลพงศ์ ใบเงิน

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาเคมี ภาควิชาเคมี
คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2542

ISBN 974-334-041-6

CHEMICAL CONSTITUENTS AND BIOLOGICAL ACTIVITY FROM THE STEM BARKS OF *Croton oblongifolius* Roxb. FROM AMPHOE MUANG, UDON THANI PROVINCE

Mr. Silapong Baiagern

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 1999

ISBN 974-334-041-6

Thesis Title	CHEMICAL CONSTITUENTS AND BIOLOGICAL ACTIVITY FROM THE STEM BARKS OF *Croton oblongifolius* Roxb. FROM AMPHOE MUANG, UDON THANI PROVINCE
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ศิลพงศ์ ใบเงิน : องค์ประกอบทางเคมีและฤทธิ์ทางชีวภาพของเปลือกต้นเปล้าใหญ่ (*Croton Oblongifolius* Roxb.) จาก อำเภอเมือง จังหวัดอุดรธานี CHEMICAL CONSTITUENTS AND BIOLOGICAL ACTIVITY FROM THE STEM BARKS OF *Croton oblongifolius* Roxb. FROM AMPHOE MUANG, UDON THANI PROVINCE อาจาย์ที่ปรึกษา : รศ.ดร. อมร เพชรสม; 105 หน้า.
ISBN 974-334-041-6

ได้สกัดแยกสารประกอบแลบเดนไดเทอร์ปีนอยด์ใหม่สองชนิดคือ labda-7,13(*Z*)-diene-17,12-olide และ labda-7,13(*Z*)-diene-17,12-olide-15-ol สารประกอบเคลโรเดนไดเทอร์ปีนอยด์ใหม่อีกหนึ่งชนิดคือ (-)-20-benzyloxyhardwickiic acid และ hardwickiic acid ซึ่งเป็นสารประกอบหลักจากส่วนสกัดเฮกเซน จากเปลือกต้นเปล้าใหญ่ที่อำเภอเมือง จังหวัดอุดรธานี และได้ทำการพิสูจน์โครงสร้างของสารใหม่นี้โดยอาศัยข้อมูลทางสเปกโตรสโกปี ซึ่งได้แก่ IR, MS, X-ray diffraction, 1D และ 2D NMR เทคนิคคือ DEPT, COSY, NOESY, HMBC, HMQC และโดยการสังเคราะห์อนุพันธ์ทางเคมีของสารประกอบเหล่านี้ พร้อมกันนั้นได้มีการทดสอบฤทธิ์ทางชีวภาพของสารประกอบทั้งหมดทั้งจากธรรมชาติและจากการสังเคราะห์ โดยทดสอบกับเซลล์ 6 ชนิดได้แก่ HS 27 (ไฟโบรบลาสต์), KATO (มะเร็งกระเพาะอาหาร), BT 474 (มะเร็งเต้านม), CHAGO (มะเร็งปอด), SW 620 (มะเร็งลำไส้ใหญ่) and HEP-G2 (มะเร็งตับ) ซึ่ง labda-7,13(*Z*)-diene-17,12-olide-15-ol มีฤทธิ์ในการยับยั้งเซลล์มะเร็ง HEP-G2 (มะเร็งตับ), CHAGO (มะเร็งปอด), SW 620 (มะเร็งลำไส้ใหญ่) และ KATO (มะเร็งกระเพาะอาหาร) มีค่า IC_{50} เท่ากับ 5, 6.4, 6.5 และ 7.1 μg/ml ตามลำดับ

ภาควิชา......เคมี...... **ลายมือชื่อนิสิต**..................
สาขาวิชา......เคมี...... **ลายมือชื่ออาจารย์ที่ปรึกษา**..................
ปีการศึกษา......2542...... **ลายมือชื่ออาจารย์ที่ปรึกษาร่วม**..................

SILAPONG BAIAGERN : CHEMICAL CONSTITUENTS AND BIOLOGICAL ACTIVITY FROM THE STEM BARKS OF *Croton oblongifolius* Roxb. FROM AMPHOE MUANG, UDON THANI PROVINCE THESIS ADVISOR : ASSO.PROF. AMORN PETSOM, Ph.D. 105 pp. ISBN 974-334-041-6

Two new labdane diterpenoid compounds, labda-7,13(*Z*)-diene-17,12-olide and labda-7,13(*Z*)-diene-17,12-olide-15-ol, one new clerodane diterpenoid compound, (-)-20-benzyloxyhardwickiic acid, and hardwickiic acid, which was the main product of the crude hexane, were isolated from the stem barks of *Croton oblongifolius* Roxb., which were collected from Amphur Muang, Udonthani Province. The structures of the new compounds were established by spectroscopic data including IR, MS spectra, X-ray diffraction, 1D and 2D NMR techniques (DEPT, COSY, NOESY, HMBC and HMQC) and chemical transformation. All of the compounds, both natural and synthetic, were subjected to biological activity test against a panel of six cell lines including HS 27 (fibroblast), KATO (gastric), BT 474 (breast), CHAGO (lung), SW 620 (colon) and HEP-G2 (hepatoma). Labda-7,13(Z)-diene-17,12-olide-15-ol exhibited cytotoxic activity against the HEP-G2 (hepatoma) cell, CHAGO (lung) cell, SW 620 (colon) cell and KATO (gastric) cell, *in vitro*, with IC_{50} values of 5, 6.4, 6.5, and 7.1 μg/ml, respectively.

ภาควิชา.....เคมี..... ลายมือชื่อนิสิต..........
สาขาวิชา.....เคมี..... ลายมือชื่ออาจารย์ที่ปรึกษา..........
ปีการศึกษา.....2542..... ลายมือชื่ออาจารย์ที่ปรึกษาร่วม..........

ACKNOWLEDGMENT

The author wishes to express his deepest appreciation to his advisor, Associate Professor Amorn Petsom, Ph.D., for his encouraging guidance, supervision and beneficial suggestions throughout the course of this research. The author is grateful for the suggestions and guidance given by Associate Professor Sophon Roengsumran, Ph.D., and Dr. Tirayut Vilaivan during the research work. He would also like to thank Associate Professor Udom Kokpol, Ph.D. for serving as chairman of his thesis committee and for the valuable comments from Assistant Professor Somchai Pengprecha, Ph.D. Gratitude is also extended to the Department of Chemistry, Faculty of Science and the Graduate School, Chulalongkorn University for the financial support.

Finally, special thanks is given to Dr. Nongnuj Jaiboon and Dr. Narongsak Chaichit for aiding in the analysis and determination carried out on X-ray diffraction analysis. Furthermore, the author wishes to express sincere gratitude to his parents, friends and girlfriend for their encouragement throughout the entire course of this study.

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ABBREVIATIONS

b.p.	=	Boiling point
br s	=	Broad singlet (for NMR spectra)
c	=	Concentration
^{0}C	=	Degree Celcius
$CDCl_3$	=	Deuterated chloroform
$CHCl_3$	=	Chloroform
CH_2Cl_2	=	Dichloromethane
cm	=	Centimeter
^{13}C-NMR	=	Carbon-13 nuclear magnetic resonance
COSY	=	Correlated SpectroscopY
d	=	Doublet (for NMR spectra)
dd	=	Doublet of doublet (for NMR spectra)
ddd	=	Doublet of doublet of doublet (for NMR spectra)
DEPT	=	Distortionless Enhancement by Polarization Transfer
DMSO	=	Dimethyl sulfoxide
δ	=	Chemical Shift
EI MS	=	Electron Impact Mass Spectrum
EtOAc	=	Ethyl acetate
g	=	Gram
^{1}H-NMR	=	Proton nuclear magnetic resonance
Hz	=	Hertz
HMBC	=	Heteromolecular Multiple Bond Correlation
HMQC	=	Heteromolecular Multiple Quantum Correlation
IR	=	Infrared spectrum
J	=	Coupling constant
kg	=	Kilogram

L	=	Liter
M^{+}	=	Molecular ion
mg	=	Milligram
MHz	=	Megahertz
ml	=	Milliliter
mm	=	Millimeter
m.p.	=	Melting point
MeOH	=	Methanol
M	=	Molar
m/z	=	Mass to charge ratio
M.W.	=	Molecular weight
MS	=	Mass spectrometry
No.	=	Number
NMR	=	Nuclear Magnetic Resonance
NOESY	=	Nuclear Overhauser Enhancement SpectroscopY
ppm	=	Part per million
q	=	Quartet (for NMR spectra)
s	=	Singlet (for NMR spectra)
t	=	Triplet (for NMR spectra)
TLC	=	Thin layer Chromatography
wt	=	Weight
R_f	=	Retention factor in chromatography

CHAPTER I

INTRODUCTION

In an attempt to overcome physical suffering, along with the desire for eternal life, mankind has unveiled the use of medicinal plants. Many have come to believe that medicinal plants or herbal medicines, originated from natural products, can be used as therapeutic drugs that have less potential in bringing about harmful side effects than synthetic drugs. In addition, these plants are often cheap and easily obtainable. Thus, there has been a worldwide trend towards the use of such medicinal drugs. Furthermore, there have been continual efforts to develop and to attain medicinal plants that are safe, reliable and effective. For example, an anti-peptic ulcer drug was developed from Plao noi (*Croton sublyratus* Kurz.), a Thai medicinal plant.

Plao Yai belongs to the Euphorbiaceae family [1], which comprises 800 genera and 5000 species, is known by its scientific name as *Croton oblongifolius* Roxb. Plao Yai is distributed throughout evergreen forests, deciduous forests and in the groves of brushwood. In Thailand, this medicinal plant is commonly known as Plao Yai (in Central Thailand), Plao Luang (in Northern Thailand), Poh (in Kamphaeng Phet Province), Khwa-wuu (in Karen, Kanchanaburi Province), Saa-kuu-wa (in Karen, Mae Hong Son Province) and as Haa-yoeng (in Shan, Mae Hong Son Province).

According to numerous Thai pharmacopoiea [2], all parts of Plao Yai are useful in terms of herbal medicine. For example, the bark can be used to inhibit chronic enlargements of the liver, the leaves can cure scabies, the fruits and seeds can be used as laxatives and the flowers can be used to kill parasites. In addition, the heartwood can be used in remedying faint and the roots (when not taken in large doses, which can be poisonous and harmful) can also be used to treat dysentery or chronic rheumatism.

1.1 General characteristics of the plants in the Genus Croton[3]

The genus Croton consists of 700 species of trees and shrubs. The leaves of these plants are usually alternate and have 2-glandular stipules at the base. The flowers are either single or clustered in the rhachis of the terminal raceme and the bracts are small. The male flowers contain 5-petals and 5-calyxes. Each male flower consists of many stamen that are inserted on a hairy receptacle. On the other hand, the female flowers are usually more ovate than the male flower. Its petals are either smaller than the sepals or are missing. The disk annular contains 4-6 glands that are opposite the sepals. Each female flower consists of three ovaries with a singular ovule in each cell. The seeds of these plants are smooth and have abundant albumen and broad cotyledons.

1.2 Botanical characteristics of *Croton oblongifolius* Roxb.[4]

Croton oblongifolius Roxb. is a medium-sized medicinal plant. The leaves fall between 5.6-12.0 cm by 13.0-24.0 cm in size. The leaf blade is oblong-lanceolate shaped. The flowers are solitary and pale yellowish-green. The female flowers are located in the lower part of the raceme, while the male flowers are located in the upper part of the raceme. The male flowers are narrowly shaped with pedicels 4.0 mm long. The male calyx are more than 6.0 mm and have segments that are ovate, obtuse and more than 2.5 mm long. The six male petals have a wooly texture and are 3.0 mm long and elliptic-lanceolate. The twelve stamens are inflexed in the bud and have filaments 3.0 mm long. The female flowers, the pedicels are short and stout. Its sepals are more acute than the sepals in the male flower and have densely ciliated margins. The fruit of the plant has a diameter of less than 1.3 cm and is slightly 3-lobed and is clothed with small orbicular scales. In addition, within each fruit there are eight seeds, each seed approximately 6.0 mm long, rounded and quite smooth on the back.

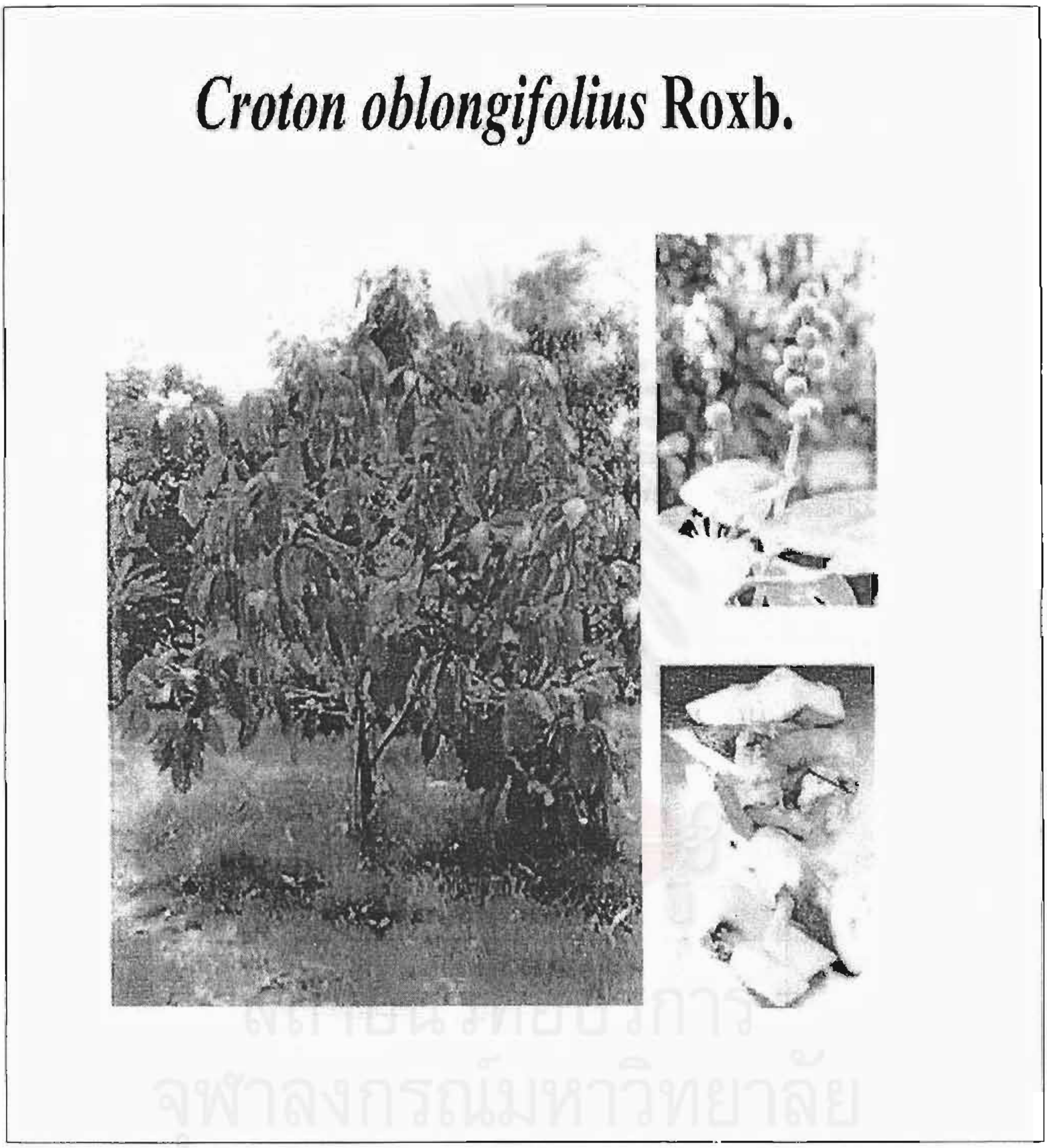


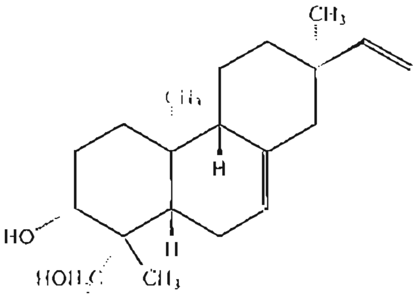
Figure 1. *Croton oblongifolius* Roxb.

The picture of the stem/ bark, leaves, flowers and fruits of *Croton oblongifolius* Roxb. are shown in Figure 1 [5].

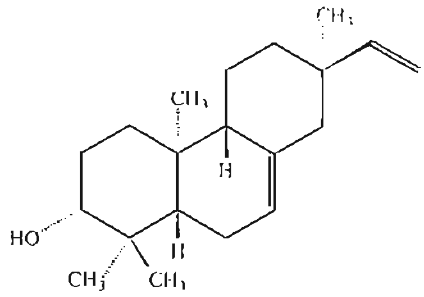
1.3 Previous research studies on diterpenoid compounds of *Croton oblongifolius* Roxb.

From numerous literature reviews, it is obvious that *Croton oblongifolius* Roxb. has been widely studied. As a result, many different organic compounds, mainly diterpenoid compounds, have been uncovered and are characterized in the following table.

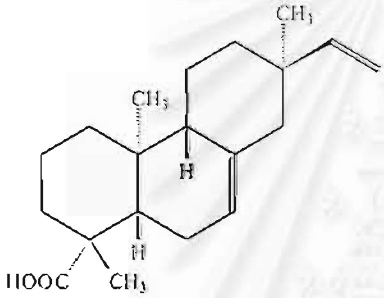
Plant Parts	Crude Extract	Organic Compounds	References
Stem barks	Hexane	Oblongifoliol	[6]
		19-Deoxyoblongifoliol	[7]
		Oblongifolic acid	[8]
		ent-Isopimara-7,15-diene	[9]
		ent-Isopimara-7,15-diene-19-aldehyde	[9]
		11-Dehydro(-)-hardwickiic acid	[10]
		(-)-Hardwickiic acid	[10]
		Crotocembraneic acid	[11]
		Neocrotocembraneic acid	[12]
		Neocrotocembranal	[13]
		Poilaneic acid	[13]
		Crovatin	[13]
		Isokolavenol	[13]
		Crotohalimaniec acid	[13]
		Benzoyl crotohalimanolic acid	[13]
		Crotohalimoneic acid	[13]
		Nidorellol	[13]



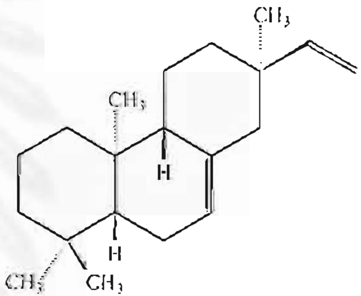
Oblongifoliol



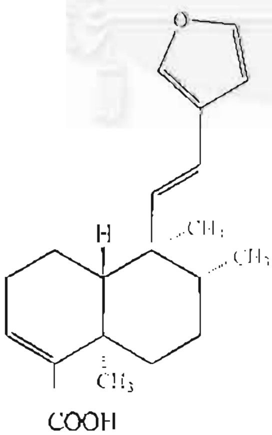
19-Deoxyoblongifoliol



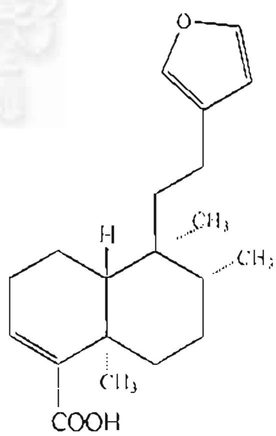
Oblongifolic acid



ent-Isopimara-7,15-diene

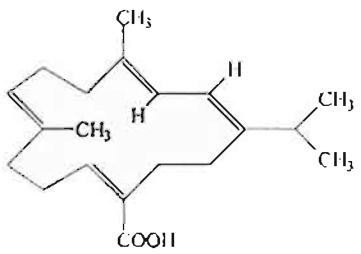


11-Dehydro-(-)-hardwickiic acid

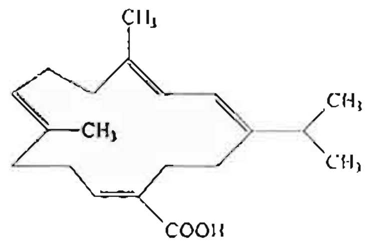


(-)-Hardwickiic acid

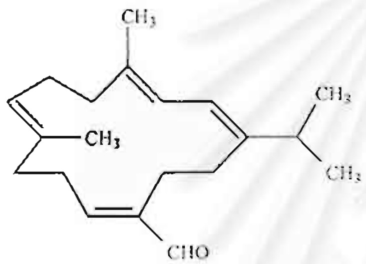
Figure 2. The structures of diterpenoid compounds from *Croton oblongifolius* Roxb.



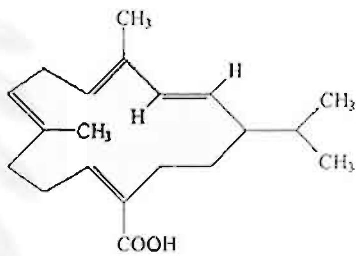
Crotocembraneic acid



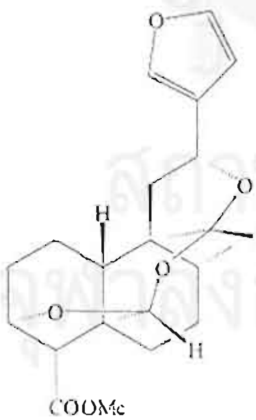
Neocrotocembraneic acid



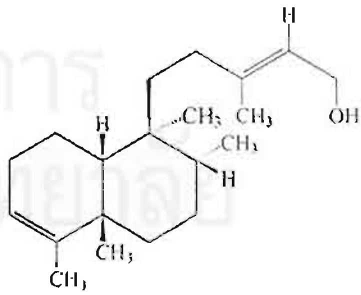
Neocrotocembranal



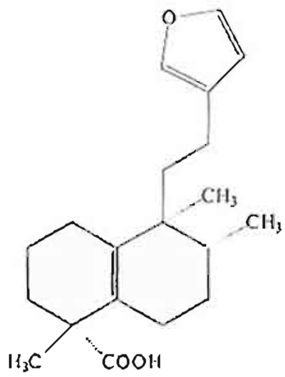
Poilaneic Acid



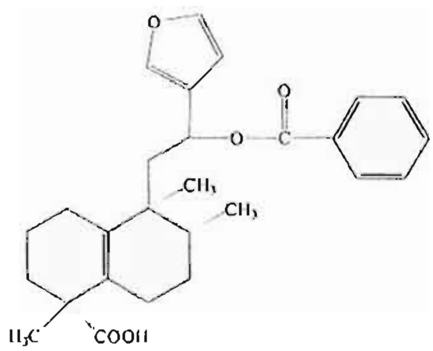
Crovatin



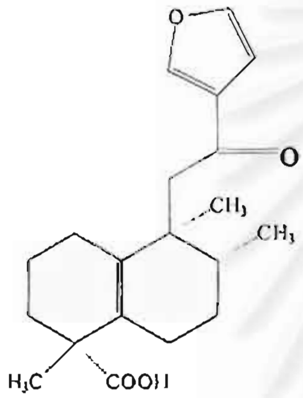
Isokolavenol



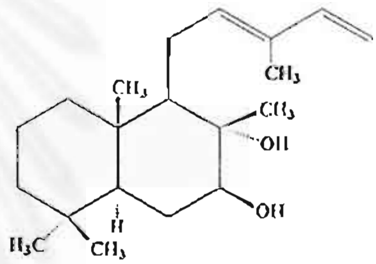
Crotohalimaneic acid



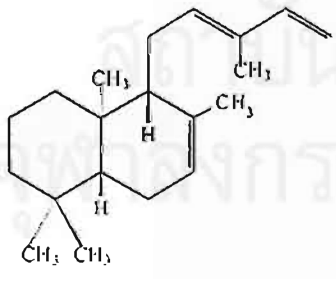
benzoyl crotohalimanolic acid



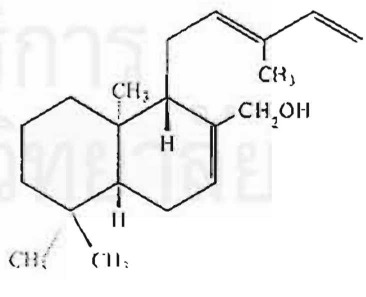
Crotohalimoneic acid



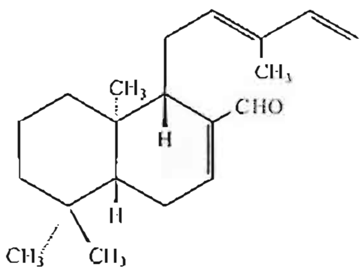
Nidorellol



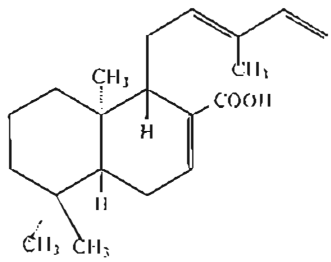
Labda-7,12(*E*),14-triene [14]



Labda-7,12(*E*),14-triene-17-ol [14]



Labda-7,12(*E*),14-triene-17-al [14]



Labda-7,12(*E*),14-triene-17-oic acid [14]

Figure 2. The structures of diterpenoid compounds from *Croton oblongifolius* Roxb. (continued)

From the literature review, the stem barks of *Croton oblongifolius* Roxb. can be used as therapeutic drugs [2]. Numerous compounds have been isolated from the stem barks of *Croton oblongifolius* Roxb. Some of these compounds have interesting biological activity. The diversity of chemical constituents found in the stem barks of *Croton oblongifolius* Roxb. from different places have led to continuous studying. Also, from primary ^{1}H-NMR scanning, it was found that the crude hexane spectrum of the stem barks of *Croton oblongifolius* Roxb., from Amphur Muang, Udonthani Province, was different from the spectrum of *Croton oblongifolius* Roxb. that were collected from other provinces. Therefore, it was intended to re-investigate the diterpenoid compounds of the stem barks of *Croton oblongifolius* Roxb.

Thus, the objectives of this research were as follows:

1. To extract and isolate the diterpenoid compounds of the stem barks of *Croton oblongifolius* Roxb., from Amphur Muang, Udonthani Province
2. To identify the structural formula of the isolated substances.
3. To investigate the biological activity of the compounds thus obtained.

CHAPTER II

EXPERIMENTS

2.1 General experimental procedures

All solvents were distilled prior to use. Melting points were determined on a Fischer-Johns melting point apparatus and are reported uncorrected. The optical rotation was determined on a Perkin-Elmer 341 polarimeter. UV-VIS spectra were recorded on a Milton-Roy Spectronic 3000 Array UV-VIS spectrophotometer. IR spectra were obtained on a Nicolet Impact 410 Spectrophotometer. Spectra of solid samples were recorded as KBr pellets and liquid samples were recorded as thin films (KBr cells). Low-resolution mass spectra were obtained with a Fisons Instruments Mass Spectrometer model Trio 2000 at 70 eV. ^1H 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 reference. COSY, NOESY, HMQC and HMBC experiments were performed on the JOEL JNM-A500 Spectrometer. X-ray diffraction experiments were carried out on SIEMEN SMART diffractrometer at Department of Physics, Faculty of Science, Thammasat University. Elemental analyses were measured on a Perkin Elmer PE2400 SERIES II (CHN/ O ANALYSER). Silica gel (Merck Kieselgel 60 and silica TLC plates (Si gel 60 F_{254}) were purchased from Merck Company.

Plant material

The stem bark sample of *C. oblongifolius* used in this study was collected from Amphur Muang, Udonthani Province, Thailand in June 1998. The botanical identification of the plant was determined by comparing with the voucher specimen No. 084729 in the herbarium collection of the Royal Forest Department of Thailand.

2.2 Extraction and Isolation

The powdered, sun-dried stem bark (6.0 kg) of *C. oblongifolius* was extracted with hexane. The hexane extract was filtered and evaporated in vacuo to obtain a yellowish-green oil (450 g). The remaining portion of the powdered stem bark was re-extracted with MeOH. The hexane extract (450 g) was repeatedly re-extracted with hexane, chloroform, ethyl acetate and methanol respectively.

Biological evaluation. Bioassay of cytotoxic activity against human tumor cell culture in vitro was performed by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] colorimetric method [14, 15, 16].

2.3 Single crystal x-ray diffraction experiment

A colourless needle-shaped crystal of Diol (Compound 4b) was obtained from chloroform. All data were collected at room temperature ($25 \pm 1\ ^{O}C$) using graphite monochromated MoK_{α} radiation (lamda=0.71073 A^{O}) on SIEMEN SMART Diffractometer. The data were corrected for Lorentz and polarization effects. The crystal experimental data of Compound 4b are given in Table 15.

The structure was solved by direct methods using SHELXS-97 and refined by full matrix least-square on F^2 using SHELXL-97 with anisotropic thermal parameters for all the non-hydrogen atoms. All the hydrogen atoms were found in difference Fourier maps and were included in refinement. The fraction coordinates of both non-hydrogen and hydrogen atoms and selected bond distances and angles of Compound 4b are listed in Tables 16, 17, 18, 19 and 20, respectively.

2.4 Purification and properties of the compounds eluted from column chromatography of hexane crude extract

2.4.1 Purification and properties of Compound 1

Compound 1 was eluted with pure hexane. The solvent was removed by rotary evaporation and the residue was purified by column chromatography (Merck's silica gel Art. 1.09385.1000). It is soluble in hexane, dichloromethane, chloroform, ethyl acetate, diethyl ether and methanol.

Compound 1 is a white crystalline solid (32 g, 7.11% yield from crude hexane and 0.53% yield from starting material), $[\alpha]^{25}_{D}$ -85.5° ($CHCl_3$, *c* 1.0). Found C 75.9; H 8.9% Calc. C 75.9, H 8.9%. The R_f value was 0.30 in 100% chloroform (SiO_2).

FT-IR spectrum (KBr), λ_{max} (cm^{-1}): 2400-3600 (br), 2971, 2930 and 2873 (m), 1685(s), 1634(m), and 1460 and 1396(m) and 1381(m) and 1280(m). (Fig.21)

^{1}H-NMR spectrum ($CDCl_3$, 200 MHz) δ (ppm) 7.33(1H, s), 7.19(1H, s), 6.85 (1H, s), 6.26(1H, s), 2.16-2.50(6H, m), 1.40-1.73(8H, m), 1.26(3H, s), 0.84(3H, d), 0.76(3H, s). (Fig.22)

^{13}C-NMR spectrum ($CDCl_3$, 50 MHz) δ (ppm) 172.8(s), 142.6(d), 141.5(s), 140.2(d), 138.3(d), 125.4(s), 110.9(d), 46.7(d), 38.8(s), 38.6(t), 37.6(s), 36.3(d), 35.8 (t), 27.5(t), 20.5(q), 18.4(q), 18.2(t), 17.5(t), 16.0(q). (Fig.22, 23)

EI MS spectrum *m/z*: 316 [M^+] 299, 283, 221, 203, 105 and 96 (Fig.24).

2.4.2 Purification and properties of Compound 2

Compound 2 was eluted with 10% chloroform in hexane. Similar fractions were combined and the solvents were removed by rotary evaporation. In order to remove the acid part, 30 ml diethyl ether was first added to the combined crude fraction in a separating funnel. Next 50 ml of 10% NaOH was added. After shaking, the organic layer of the crude fraction was separated from the acid part, which was then discarded. The solvent in the organic layer was then removed by rotary evaporation. The non-acidic crude was separated by preperative thin layer chromatography in 30%

ethyl acetate in hexane. From the resulting 3 bands, Compound 2 was the second band with a R_f = 0.48. This compound is soluble in dichloromethane, chloroform, ethyl acetate, diethyl ether and methanol.

Compound 2 is a white crystalline solid (1.2 g, 0.02%), $[\alpha]^{25}_{D}$ -0.8° ($CHCl_3$, *c*1.0), R_f; 0.48 (30% ethyl acetate in hexane), mp 82-85°C, UV (EtOH) λ_{max} 244 sh (log ε 3.56), Found C 79.4; H 9.9% Calc. C 79.4, H 9.9%.

FT-IR spectrum (KBr) (Fig.25) ν_{max} (cm^{-1}) : 3426(br), 2924(s), 1716(s), 1639 (s), 1454(m), 1367(m), 1250(s), 1122(w).

^{1}H-NMR spectrum ($CDCl_3$, 500 MHz) (Fig.26) δ (ppm) : 7.33(1H, dd), 5.59 (1H, s), 4.60(1H, dd), 2.37(1H, m), 2.25(1H, dd), 2.09(1H, m), 1.78(1H, m), 1.76(1H, m), 1.66(3H, s), 1.64(3H, s), 1.55(1H, m), 1.50(1H, m), 1.50(1H, m), 1.48(1H, m), 1.31(1H, dd), 1.20(1H, m), 1.06(1H, m), 0.92(3H, s), 0.90(3H, s), 0.76(3H, s).

^{13}C-NMR spectrum ($CDCl_3$, 125 MHz) (Fig.27) δ (ppm) : 166.1(s), 142.8(d), 134.0(s), 125.9(s), 123.3(d), 84.8(d), 49.3(d), 48.9(d), 41.9(t), 38.7(t), 34.7(s), 32.9(q), 32.8(s), 27.3(t), 25.1(t), 21.5(q), 18.6(t), 13.4(q), 13.1(q), 11.4(q).

m/z (EI) (rel int.) (Fig.29) : 302[M^+](70), 287(25), 217(50), 203(50), 179(50), 161(53), 133(40), 124(65), 109(100), 105(40).

2.4.3 Purification and Properties of Compound 3

Compound 3 was the first band of the preperative thin layer chromatography in 30% ethyl acetate in hexane with R_f = 0.24. This compound is soluble in dichloromethane, chloroform, ethyl acetate, diethyl ether and methanol.

Compound 3 is a transparent oil (0.12 g, 0.002%), $[\alpha]^{25}_{D}$ -1.5° ($CHCl_3$, *c* 1.0), R_f; 0.24 (30% ethyl acetate in hexane), UV (EtOH) λ_{max} 243 sh (log ε 3.38), Found C 75.4; H 9.4% Calc. C 75.4, H 9.5%.

FT-IR spectrum (neat) (Fig.34) ν_{max} (cm^{-1}) : 3395(br), 2919(s), 1700(s), 1634 (s). 1244(s).

^{1}H-NMR spectrum ($CDCl_3$, 500 MHz) (Fig.35) δ (ppm) : 7.30(1H, ddd), 5.70 (1H,m), 4.60(1H, dd),4.25(2H,ddd), 2.40(1H, m), 2.27(1H, m), 2.10(1H, m), 1.85(1H, ddd), 1.76(1H, m),1.72(3H,s), 1.53(1H, m), 1.50(1H, m), 1.50(1H, m), 1.49(1H, m), 1.32(1H, dd), 1.20(1H, m), 1.05(1H, m), 0.92(3H, s), 0.89(3H, s), 0.76(3H, s).

^{13}C-NMR spectrum ($CDCl_3$, 125 MHz) (Fig.36) δ (ppm) : 165.8(s), 143.3(d), 136.2(s), 127.1(d), 125.7(s), 83.7(d), 58.9(t), 49.1(d), 48.9(d), 41.9(t), 38.7(t), 34.7(s), 32.9(q), 32.8(s), 27.3(t), 25.2(t), 21.4(q), 18.5(t), 13.4(q), 12.2(q).

m/z (EI) (rel int.) (Fig.38) : 318[M^+](10), 175(55), 145(70), 105(90), 95(100), 81(85), 77(40).

2.4.4 Purification and properties of Compound 4

Compound 4 was obtained from 50% chloroform in hexane. Similar fractions were combined and the solvents were removed by rotary evaporation and further purified by column chromatography (Merck's silica gel Art. 1.09385.1000; the column was eluted with 30% ethyl acetate in hexane). This compound is soluble in chloroform, ethyl acetate, diethyl ether and methanol.

Compound 4 is a viscous transparent oil (3.20 g, 0.05%), $[\alpha]^{25}_{D}$ -46.79° ($CHCl_3$, *c* 1.0), R_f: 0.42 (30% ethyl acetate in hexane), UV (EtOH) λ_{max} 243 sh (log ε 3.59), Found C 74.3; H 7.4% Calc. C 74.3, H 7.4%.

FT-IR spectrum (neat) (Fig.43) ν_{max} (cm^{-1}) : 3500-3100 (br), 1716(s), 1675(s), 1250(s).

^{1}H-NMR spectrum ($CDCl_3$, 500 MHz) (Fig.44) δ (ppm) : 8.01(2H, d), 7.55(1H, dd), 7.45(2H, dd), 7.35(1H, d), 7.24(1H, s), 6.92(1H, dd), 6.28(1H, d), 4.50(1H, d), 4.30(1H, d), 2.53(1H, ddd), 2.40(1H, m), 2.35(1H, m), 2.25(1H, m), 2.20(1H, m), 2.08 (1H, m), 1.95(1H, m), 1.93(1H, m), 1.78(1H, m), 1.72(1H, m), 1.65(1H, m), 1.58(1H, d), 1.53(1H, m), 1.32(3H, s), 1.24(1H, m), 1.02(3H, d).

^{13}C-NMR spectrum ($CDCl_3$, 125 MHz) (Fig. 45) δ (ppm) : 172.0(s), 166.8(s), 142.9(d), 140.9(s), 140.5(d), 138.5(d), 132.9(d), 130.4(s), 129.5(d), 129.5(d), 128.5(d), 128.5(d), 125.1(s), 110.9(d), 67.7(t), 47.4(d), 42.3(s), 37.7(s), 36.4(d), 36.0(t), 32.4(t), 28.1(t), 27.2(t), 20.2(q), 19.2(t), 17.9(t), 16.9(q).

m/z (EI) (rel int.) (Fig. 47) : 436[M^+](18), 314(45), 125(50), 105(100).

2.5 Chemical transformation of Compound 4

2.5.1 Methylation of compound 4

Compound 4 (1 g, 2.293 mmol) was methylated with CH_2N_2 in Et_2O under the conditions described earlier [17]. The reaction product was purified with 10% ethyl acetate in hexane to give Compound 4a as a viscous oil (830 mg, 83% yield), $[\alpha]^{25}_D$ -90.9° ($CHCl_3$, *c* 1.0), R_f: 0.84 (10% ethyl acetate in hexane), UV (EtOH) λ_{max} 241 sh (log ε 3.85), Found C 74.6; H 7.6% Calc. C 74.5, H 7.6%.

FT-IR spectrum (neat) (Fig.52) ν_{max} (cm^{-1}) : 1726(s), 1613(s), 1280(s), 870-670 (w).

^{1}H-NMR spectrum ($CDCl_3$, 200 MHz) (Fig.53) δ (ppm) : 8.01(2H, d), 7.55(1H, d), 7.45(2H, d), 7.32(1H, s), 7.20(1H, s), 6.65(1H, s), 6.25(1H, s), 4.48(1H, d), 4.30 (1H, d), 3.67(3H, s), 2.55(1H, m), 2.42(1H, m), 2.35(1H, m), 2.23(1H, m), 2.18(1H, m), 2.05(1H, m), 1.95(1H, m), 1.91(1H, m), 1.78(1H, m), 1.73(1H, m), 1.62(1H, m), 1.58(1H, m), 1.52(1H, m), 1.32(3H, s), 1.24(1H, m), 1.00(3H, d).

^{13}C-NMR spectrum ($CDCl_3$, 50 MHz) (Fig.53) δ (ppm) : 167.6(s), 166.8(s), 142.9(d), 141.9(s), 138.5(d), 137.1(d), 132.9(d), 130.4(s), 129.5(d), 129.5(d), 128.5(d), 128.5(d), 125.2(s), 110.9(d), 67.7(t), 51.2(q), 47.3(d), 42.3(s), 37.7(s), 36.4(d), 36.1(t), 32.4(t), 27.8(t), 27.2(t), 20.3(q), 19.3(t), 17.9(t), 17.0(q).

m/z (EI) (rel int.) (Fig.55) : 450[M^+](20), 328(30), 139(45), 105(100), 94(50), 77(40).

2.5.2 Reduction of Compound 4a

The methyl ester (500 mg, 1.11 mmol) in 20 ml of anhydrous diethyl ether was added slowly from a dropping funnel into a stirred solution of lithium aluminum hydride (1.2 g, 31.57 mmol) in a 20 ml of anhydrous diethyl ether in a 50 ml round-bottom flask previously flushed with nitrogen. After the addition was completed, the reaction mixture was stirred for 5 hours at room temperature. The reaction was stopped and worked up in the usual manner. The organic layer was concentrated by rotary evaporation and purified by column chromatography (Merck's silica gel Art. 1.09385.1000) and eluted with 50% ethyl acetate in hexane to obtain Compound 4b (320 mg, 64%), $[\alpha]^{25}_{D}$ -32.4° ($CHCl_3$, *c* 1.0), R_f; 0.52 (50% ethyl acetate in hexane), UV (EtOH) λ_{max} 242.5 sh (log ε 2.55), EA; Found C 75.4; H 9.5% Calc. C 75.4, H 9.5%.

FT-IR spectrum (neat) (Fig.56) ν_{max} (cm^{-1}) : 3700-3050(br), 1644(s), 1019(s).

^{1}H-NMR spectrum ($CDCl_3$, 200 MHz) (Fig.57) δ (ppm) : 7.33(1H, s), 7.21(1H, s), 6.26(1H, s), 5.57 (1H, s), 4.07(2H, s), 3.70(2H, s), 2.54(1H, m), 2.40(1H, m), 2.37 (1H, m), 2.24(1H, m), 2.18(1H, m), 2.06(1H, m), 1.95(1H, m), 1.92(1H, m), 1.78(1H, m), 1.70(1H, m), 1.65(1H, m), 1.58(1H, m), 1.52(1H, m), 1.26(3H, m), 1.10(1H, s), 0.92(3H, d).

^{13}C-NMR spectrum ($CDCl_3$, 50 MHz) (Fig.57) δ (ppm) : 147.7(s), 142.7(d), 138.5(d), 125.6(s), 122.2(d), 111.0(d), 65.5(t), 62.8(t), 46.9(d), 43.1(s), 37.8(s), 36.6 (d), 36.2(t), 31.9(t), 27.2(t), 27.2(t), 20.9(q), 19.8(t), 17.8(t), 17.2(q).

m/z (EI) (rel int.) (Fig.59) : 318[M^+](10), 175(55), 145(70), 105(90), 95(100), 81(85), 77(40).

2.5.3 Hydrolysis of Compound 4

A solution of Compound 4 (500 mg, 1.146 mmol) in 30 ml of MeOH was refluxed with 20 ml of NaOH solution for 24 hours. The product was acidified with 20% HCl solution and purified by silica gel CC eluting with 50% ethyl acetate in hexane to give Compound 4c (412 mg, 84.2% yield), $[\alpha]^{25}_{D}$ -17.9° ($CHCl_3$, *c* 1.0), R_f; 0.86 (50% ethyl acetate in hexane), UV (EtOH) λ_{max} 242 sh (log ε 3.41), EA; Found C 72.3; H 8.5% Calc. C 72.2, H 8.6%.

FT-IR spectrum (neat) (Fig.60) ν_{max} (cm^{-1}) : 3600-3050(br), 1675(s), 1623(s).

^{1}H-NMR spectrum ($CDCl_3$, 200 MHz) (Fig.61) δ (ppm) : 7.35(1H, s), 7.20(1H, s), 6.90(1H, s), 6.25(1H, s), 3.70(2H, d), 2.55(1H, m), 2.41(1H, m), 2.35(1H, m), 2.24 (1H, m), 2.19(1H, m), 2.08(1H, m), 1.97(1H, m), 1.93(1H, m), 1.78(1H, m), 1.72(1H, m), 1.65(1H, m), 1.58(1H, m), 1.53(1H, m), 1.25(1H, m), 1.15(3H, s), 0.95(3H, d).

^{13}C-NMR spectrum ($CDCl_3$, 50 MHz) (Fig.61) δ (ppm) : 172.3(s), 142.8(d), 141.3(s), 140.0(d), 138.5(d), 125.5(s), 111.0(d), 65.5(t), 47.4(d), 43.2(s), 37.6(s), 36.2 (d), 36.1(t), 32.2(t), 28.1(t), 27.3(t), 20.1(q), 19.1(t), 17.8(t), 17.0(q).

m/z (EI) (rel int.) (Fig.63) : 332[M^+](90), 299(85), 219(75), 189(100), 173(80), 151(75), 157(50).

CHAPTER III

RESULTS AND DISCUSSION

3.1 Isolation of crude extract of *Croton oblongifolius* Roxb.

3.1.1 Separation of hexane crude extract

The hexane crude extract was obtained as a yellowish green oil (450 g) after evaporation. The crude extract (450 g) was fractionated by Silica gel column chromatography using Merck's silica gel Art. 7734.1000 (70-230 mesh ASTM) as adsorbent. The column was eluted with hexane-chloroform gradient in a stepwise fashion. The separation of hexane crude extract gave compounds 1-4 shown in Table 1.

3.1.2 Separation of chloroform crude extract

Concentrated chloroform crude extract (80 g) was separated on Silica gel 70-230 mesh ASTM using column chromatography technique. The column was eluted with hexane, hexane-chloroform, chloroform, and chloroform-methanol, respectively. About 125 ml of each eluted fraction was collected and was then evaporated to give about 30 ml of gummy residue.

3.1.3 Separation of methanol crude extract

The remaining extraction from chloroform resulted in the methanol crude extract (20 g). It was not possible to purify the methanol crude extract because the crude extract had a brown solid characteristic after partition with chloroform and could not dissolve in methanol.

Table 1. The results of separation of hexane crude extract by column chromatography.

Compounds	Physical appearance	% wt. by wt.
1	White solid	0.53
2	White solid	0.02
3	Transparent oil	0.002
4	Transparent oil	0.053

3.2 Structural elucidation of the isolated compounds from the stem barks of *Croton oblongifolius* Roxb.

3.2.1 Structure elucidation of Compound 1

The IR spectrum of Compound 1 is shown in Fig.21 and the absorption peaks were assigned as shown in Table 2. Its IR spectrum showed important absorption bands at 2400-3600 cm^{-1} (O-H stretching vibration of alcohol), 2971, 1930 and 1873 cm^{-1} (C-H stretching vibration), 1685 cm^{-1} (C=O stretching vibration of carbonyl group) and 1634 cm^{-1} (C-C stretching vibration of alkene).

Table 2. The IR absorption bands assignment of Compound 1

Wave number (cm^{-1})	Intensity	Tentative Assignment
2400-3600	Broad	O-H stretching vibration of acid
2971, 2930, 2873	Strong	C-H stretching vibration of -CH3, -CH2
1685	Strong	C=O stretching vibration of carbonyl group
1634	Medium	C=C stretching vibration of alkene

The ^{1}H-NMR spectrum (Fig.22) of Compound 1 indicated that it possesses three methyl groups (δ 0.76, 0.84 and 1.26 ppm), three olefinic protons of furanoid group (δ 7.33, 7.19 and 6.26 ppm) and one vinylic proton (δ 6.85 ppm).

The ^{13}C-NMR, DEPT-90, and DEPT-135 spectrum (Fig.23) showed 120 signals. Six signals of olefinic carbons appeared at δ 142.6, 141.5, 140.2, 138.3, 125.4 and 110.9 ppm. The signal at 172.8 ppm should be the carbonyl of carboxylic acid.

There were thirteen sp^3 carbon signals at δ 46.7(d), 38.8(s), 38.6(t), 37.6(s), 36.3(d), 35.8(t), 27.5(t), 25.3(t), 20.5(q), 18.48(q), 18.2(t), 17.5(t) and 16.0(q) ppm.

Its molecular formula was established as $C_{20}H_{28}O_3$, which was confirmed by observing the molecular ion at *m/z* 316 (Fig.24). The molecular formula, $C_{20}H_{28}O_3$, of Compound 1 defined a degree of unsaturation of seven, therefore, Compound 1 must consist of one ring of furan (DBE = 3) in addition to one double bond, two rings and one carbonyl group of carboxylic acid.

Compound 1 exhibited the ^{13}C-NMR chemical shifts identical to hardwickiic acid [18]. A comparison of the ^{13}C-NMR chemical shifts of Compound 1 and hardwickiic acid is shown in Table 3. These data indicated that Compound 1 was hardwickiic acid.

Figure 3. The structure of Compound 1

Table 3. ^{13}C-NMR chemical shifts of Compound 1 and Hardwickiic acid

Carbon	Chemical shifts (ppm)	
	Compound 1	Hardwickiic acid
1	17.5t	17.5t
2	27.5t	27.5t
3	140.2d	140.2d
4	141.5s	141.5s
5	37.6s	37.6s
6	35.8t	35.8t
7	27.5t	27.3t
8	36.3d	36.3d
9	38.8s	38.8s
10	46.7d	46.7d
11	38.6t	38.6t
12	18.2t	18.2t
13	125.4s	125.5s
14	110.9d	110.9d
15	142.6d	142.7d
16	138.3d	138.3d
17	16.0q	16.0q
18	172.8s	172.8s
19	20.5q	20.5q
20	18.2q	18.3q

3.2.2 Structure Elucidation of Compound 2

The IR spectrum of Compound 2 (Fig.25) is summarized in Table 4.

Table 4. The IR absorption bands assignment of Compound 2

Wave number (cm^{-1})	Intensity	Tentative Assignment
2924	Strong	C-H stretching vibration of $-CH_2$, $-CH_3$
1726	Strong	C=O stretching vibration
1639	Strong	C=C stretching vibration
1454, 1367	Medium	$-CH_2$, $-CH_3$ bending
1250	Strong	C-O stretching vibration

The ^{1}H-NMR spectrum (Fig.26, Table 5) of Compound 2 showed three methyl groups attaching to quaternary carbons (0.76, 0.90 and 0.92 ppm) and two olefinic methyl groups (1.64 and 1.66 ppm) and two olefinic protons (5.59 and 7.33 ppm).

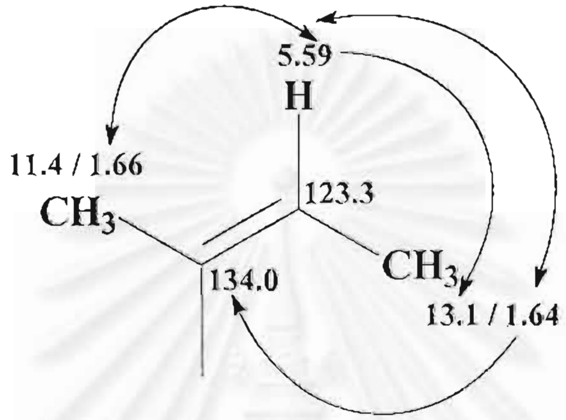
The ^{13}C-NMR spectrum (Fig.27, Table 5) showed 20 lines. Four signals of olefinic carbons appeared at 142.8, 134.0, 125.9 and 123.3 ppm.

DEPT 90 Experiments (Fig.28), indicated the presence of two sp^2 methine carbons at 123.3 and 142.8 ppm and three saturated methines at 48.9, 49.3 and 84.8 ppm. The DEPT-135 spectrum (Fig.28) showed five methylene carbons at 41.9, 38.7, 27.3, 25.1 and 18.6 ppm and five methyl carbons at 32.9, 21.5, 13.4, 13.1 and 11.4 ppm (Table 5), which indicated that the carbon signals at 166.1, 134.0, 125.9, 34.7 and 32.8 were quaternary.

Compound 2 showed a molecular ion with *m/z* 302 ($C_{20}H_{30}O_2$), which indicated DBE of 6. The information from 2D-NMR techniques, COSY correlations (Fig.32, Table 6), HMQC correlations (Fig.30. Table 6), HMBC correlations (Fig.31, Table 6) were used to assist in the interpretation of the structure of Compound 2.

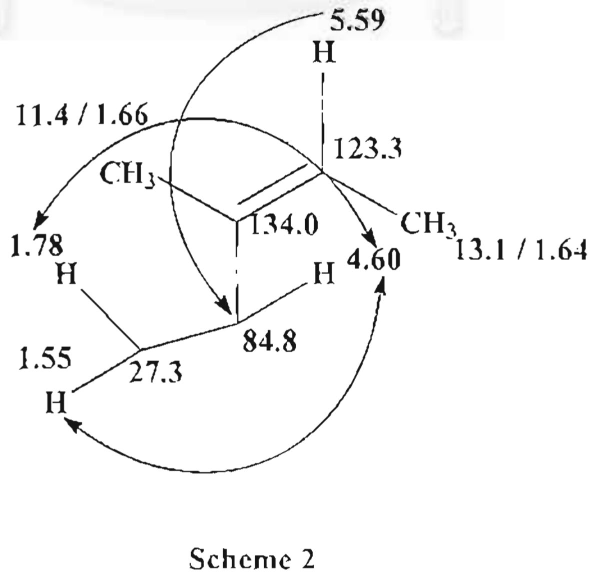
Two-dimensional NMR techniques were used for assisting the structure assignment. The protons directly attached to the carbons in Compound 2 were assigned by HMBC spectra (Fig.31, Table 6).

Crucial long-range ^{1}H-^{13}C correlations were obtained by HMBC correlations (Fig.31), the proton at 5.59 was coupled with methyl carbon at 13.1 ppm and the proton of methyl (1.64 ppm) was coupled with carbon at 134.0 ppm. The COSY spectrum (Fig.32) showed that the proton at 5.59 ppm was coupled with the proton of methyl at 1.64 and 1.66 ppm (see Scheme 1).



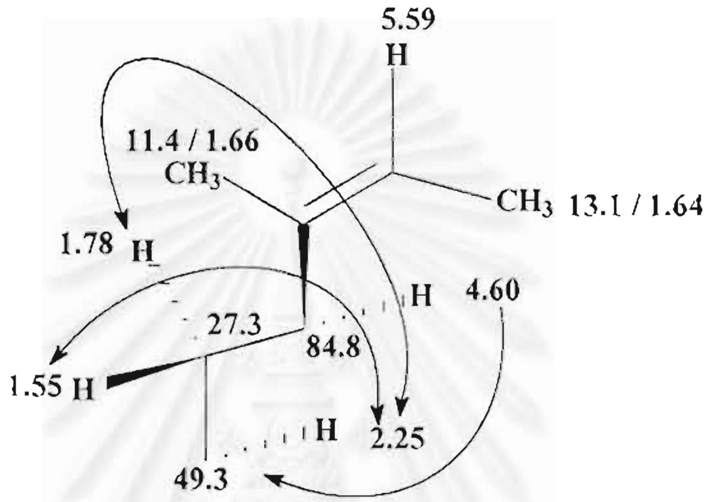
Scheme 1

The HMBC spectrum showed that the proton at 5.59 ppm was coupled with the carbon at 84.8 ppm. And according to COSY spectrum, the proton at 4.60 ppm was coupled with the proton at 1.78 and 1.55 ppm (see Scheme 2).



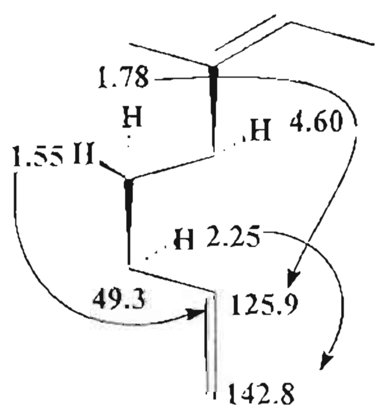
Scheme 2

HMBC spectrum showed that the proton at 4.60 ppm was coupled with the carbon at 49.28 ppm. According to COSY spectrum, the proton at 2.25 ppm was coupled with the proton at 1.78 and 1.55 ppm. Moreover, cis-conformation, which was assigned by NOESY spectrum (Fig.33), indicated the appearance of coupling between the proton at 4.60 ppm and the protons at 1.78 and 2.25 ppm (see Scheme 3).



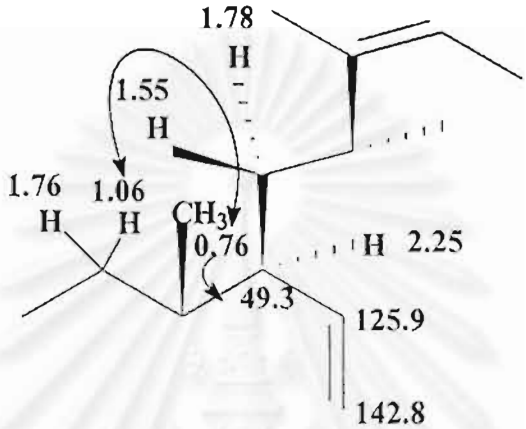
Scheme 3

HMBC spectrum showed that the proton at 1.78 ppm and 1.55 ppm were coupled with the carbon at 125.9 ppm and the proton at 2.25 ppm was coupled with the carbon at 142.8 ppm (see Scheme 4).



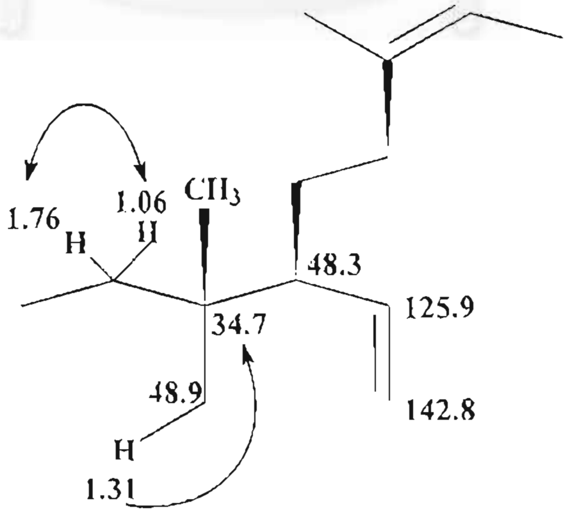
Scheme 4

According to HMBC spectrum and COSY spectrum, the proton of methyl at 0.76 ppm was coupled with the carbon at 49.3 ppm and the proton at 1.06 ppm, respectively. Moreover, cis-conformation, which was assigned by NOESY spectrum (Fig.33), indicated the appearance of coupling between the proton of methyl at 0.76 ppm and the proton at 1.55 ppm (see Scheme 5).



Scheme 5

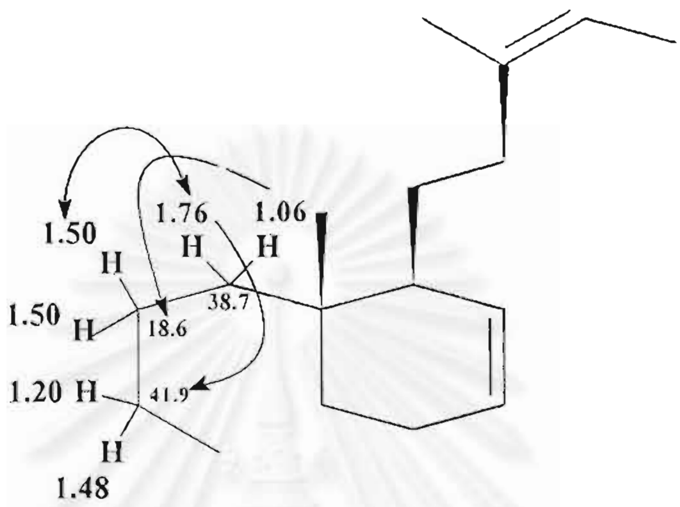
In accordance to COSY spectrum, the proton at 1.06 ppm was coupled with the proton at 1.76 ppm. According to HMBC spectrum, the proton at 1.31 ppm was coupled with the carbon at 34.7 ppm (see Scheme 6).



Scheme 6

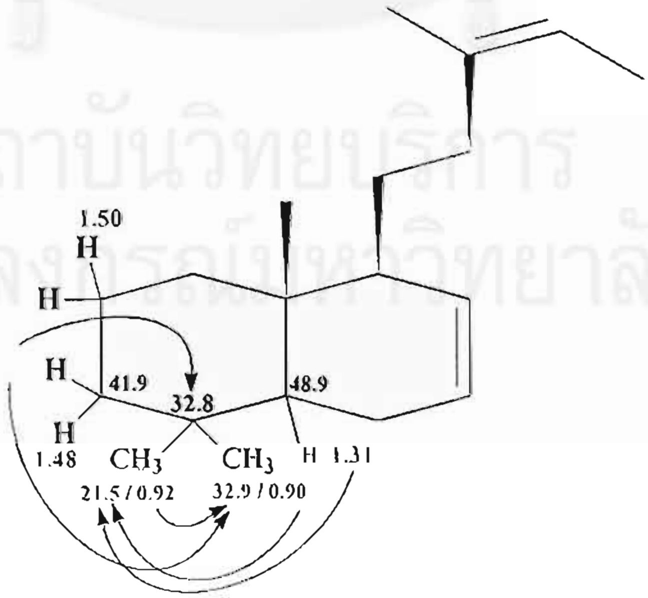
หน้านี้หายไป ไม่มีในต้นฉบับที่นำมาสแกน

According to HMBC spectrum, the proton at 1.06 ppm was coupled with the carbon at 18.6 ppm and the proton at 1.76 ppm was coupled with the carbon at 41.9 ppm. Moreover, according to COSY spectrum, the proton at 1.50 ppm was coupled with the proton at 1.76 ppm and also with the proton at 1.20 ppm (see Scheme 9).



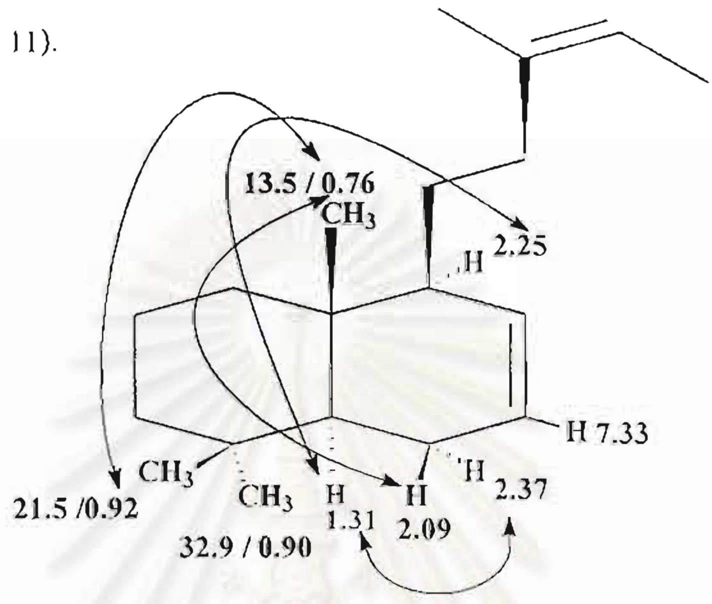
Scheme 9

The proton at 1.20 ppm was coupled with the carbon at 32.8 ppm and the methyl carbon at 32.9 ppm. Moreover, the proton at 1.31 ppm was coupled with the carbon methyl at 21.5 ppm. HMBC spectrum showed that the methyl protons at 0.90 ppm were coupled with the methyl carbon at 21.5 ppm and the methyl protons at 0.92 ppm were coupled with the methyl carbon at 32.9 ppm (see Scheme 10).



Scheme 10

Cis-conformation, which was assigned by NOESY spectrum (Fig.33), indicated the appearance of coupling between methyl protons at 0.76 ppm and the methyl proton at 0.92 and the proton at 2.09 ppm. Moreover, according to NOESY spectrum, the proton at 1.31 ppm was coupled with the proton at 2.25 ppm and the proton at 2.37 ppm (see Scheme 11).



Scheme 11

From connecting the carbon at 125.9 ppm with the carbonyl carbon at 166.0 ppm and closed lactone ring between the carbon at 84.8 ppm with the oxygen atom of lactone, a lactone ring was formed. Thus, the structure of Compound 2 was obtained as shown in Figure 4. The COSY correlations and long-range C-H correlations by HMBC spectrum are summarized in Figure 5 and 6 respectively.

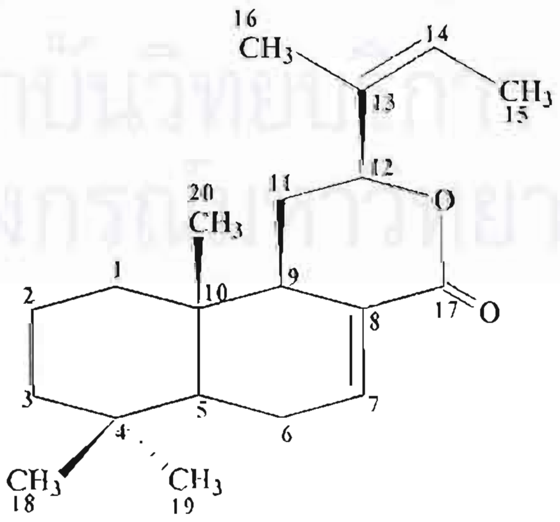


Figure 4. The structure of Compound 2

Table 5. The HMQC spectral data of Compound 2

^{13}C-NMR (ppm)	^{1}H-NMR (ppm), coupling constant (Hz)
11.4q	1.66s
13.1q	1.64s
13.4q	0.76s
18.6t	1.50m, 1.50m
21.5q	0.92s
25.1t	2.09ddd(*J*=2.7, 4.6, 12.2), 2.37m
27.3t	1.55m, 1.78m
32.8s	-
32.9q	0.90s
34.7s	-
38.7t	1.06ddd(*J*=3.7, 12.8, 16.2), 1.76m
41.9t	1.20m, 1.48m
48.9d	1.31dd(*J*=5.2, 12.2)
49.3d	2.25m
84.8d	4.60dd(*J*=1.5, 11.3)
123.3d	5.59s
125.9s	-
134.0s	-
142.8d	7.33ddd(*J*=2.7, 2.7, 5.5)
166.1s	-

Table 6. The HMQC, HMBC and COSY spectral data of Compound 2

Position	δ_C[a]	δ_H	HMBC (H to C)	COSY
1	38.7(t)	1.06	C-2, C-3, C-9, C-10	H-1(1.76), H-20(0.76)
		1.76	C-3, C-20	H-1(1.06), H-2(1.50)
2	18.6(t)	1.50	C-3, C-4	H-2(1.50), H-3(1.20, 1.48)
		1.50	C-3, C-4	H-2(1.50), H-3(1.20, 1.48)
3	41.9(t)	1.20	C-2, C-4, C-19	H-2(1.50), H-3(1.48), H-18(0.92)
		1.48	C-1, C-5	H-3(1.20)
4	32.8(s)	-	-	-
5	48.9(d)	1.31	C-4, C-6, C-9, C-10, C-18, C-19, C-20	H-6(2.09), H-6(2.37)
6	25.1(t)	2.09	C-7	H-5(1.31), H-6(2.37)
		2.37	C-7	H-5(1.31), H-6(2.37), H-7(7.33)
7	142.8(d)	7.33	C-6	H-6(2.37, 2.09), H-9(2.25)
8	125.9(s)	-	-	-
9	49.3(d)	2.25	C-7	H-7(7.33), H-11(1.55, 1.78)
10	34.7(s)	-	-	-
11	27.8(t)	1.55	C-8, C-12	H-9(2.25), H-11(1.78), H-12(4.60)
		1.78	C-8, C-16	H-9(2.25), H-11(1.55), H-12(4.60)
12	84.8(d)	4.60	C-9, C-13, C-14, C-16	H-11(1.55, 1.78)
13	134.0(s)	-	-	-
14	123.3(d)	5.59	C-12, C-15, C-16	H-15(1.64), H-16(1.66)
15	13.1(q)	1.64	C-13, C-14	H-14(5.59)
16	11.4(q)	1.66	C-12, C-13, C-14	H-14(5.59)
17	166.1(s)	-	-	-
18	21.5(q)	0.92	C-3, C-4, C-5, C-19	H-3(1.20), H-19(0.90)
19	32.9(q)	0.90	C-3, C-4, C-5, C-18	H-18(0.92)
20	13.4(q)	0.76	C-1, C-5, C-9, C-10	H-1(1.06)

[a]Carbon type as determined by DEPT experiments spectra : s = singlet, d = doublet, t = triplet, q = quartet.

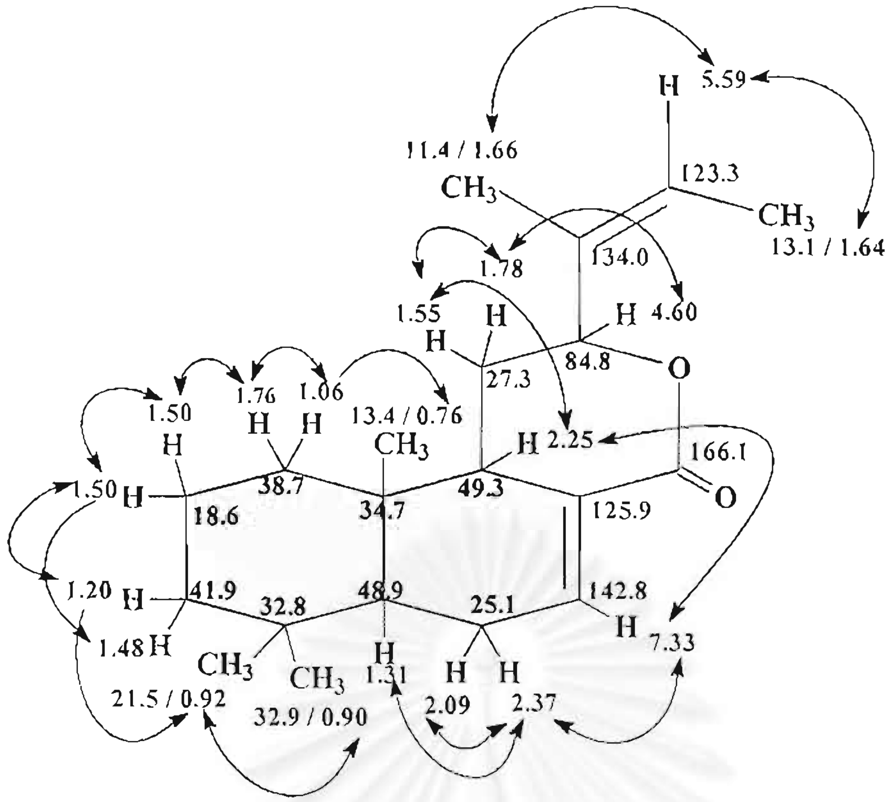


Figure 5. The COSY correlations of Compound 2

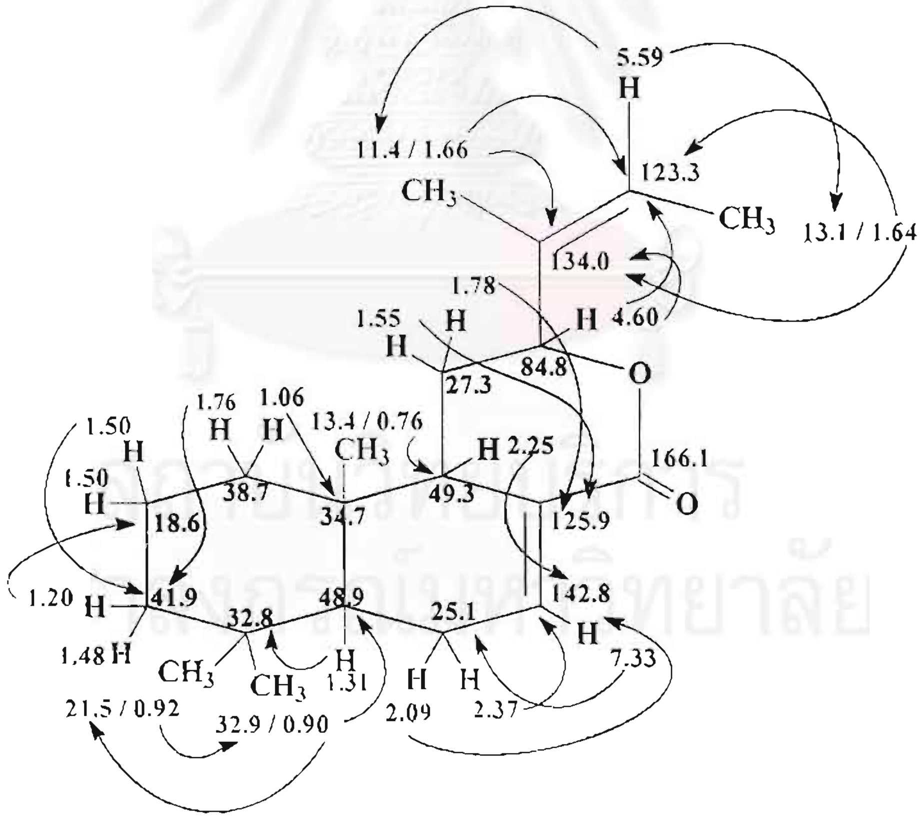


Figure 6. The HMBC correlations of Compound 2

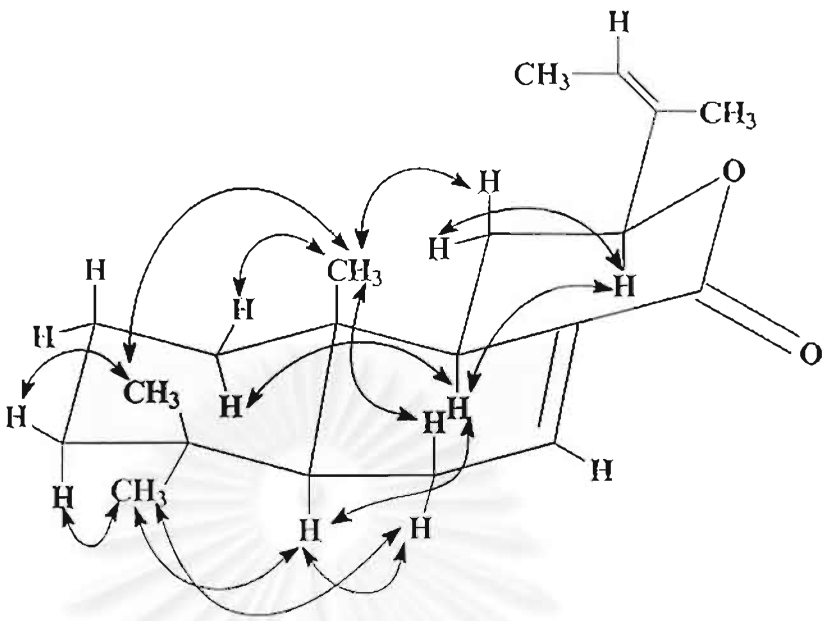


Figure 7. The NOESY correlations of Compound 2

3.2.3 Structure elucidation of Compound 3

The IR spectrum of Compound 3 (Fig.34) showed the presence of a hydroxy group according to the broad absorption band between 3700 to 3050 cm^{-1}.

Table 7. The IR absorption bands assignment of Compound 3

Wave number (cm^{-1})	Intensity	Tentative assignment
3700-3050	Broad	O-H stretching vibration of alcohol
2919	Strong	C-H stretching vibration of $-CH_2$, $-CH_3$
1700	Strong	C=O stretching vibration
1634	Medium	C=C stretching vibration
1244	Medium	C-O stretching vibration

The ^1H-NMR spectrum (Fig.35) indicated that Compound 3 possessed three methyl groups attaching to quaternary carbons (0.76, 0.89 and 0.91 ppm) and one olefinic group (1.72 ppm) and two olefinic protons (5.70 and 7.30 ppm) and two protons for the alcohol functional group (4.25 ppm).

The ^{13}C-NMR spectrum (Fig.36, Table 8) showed the carbonyl group of lactone corresponding to the signal at 165.7 ppm. The signals of olefinic carbons appeared at 143.3, 136.2, 127.1 and 125.7 ppm respectively.

DEPT 90 Experiments (Fig.37), indicated the presence of two sp^2 methine carbons at 127.1 and 143.3 ppm and three saturated methines at 48.9, 49.1 and 83.7 ppm. The DEPT-135 spectrum (Fig.37) showed six methylene carbons at 58.9, 41.9, 38.7, 27.3, 25.2 and 18.5 ppm and four methyl carbons at 32.8, 21.4, 13.4 and 12.2 ppm (Table 8), which indicated that the carbon signals at 165.8, 136.2, 125.7, 34.7 and 32.8 were quaternary.

The molecular formula of Compound 3 was assigned to be $C_{20}H_{30}O_3$ according to microanalysis and EI MS [M^+] (*m/z* 318), which indicated a DBE of 6. The ^{13}C-NMR spectrum (Fig.38, Table 8) of Compound 3 was similar to that of Compound 2 except for the downfield positions of C-16 (58.9 ppm) when compared to that of Compound 2 (11.4 ppm). Its ^{1}H-NMR spectrum (Fig.35) showed doublet of doublet of doublet signals (δ_H 4.25, *J*=6.7, 12.8, 18.9) of 2H-16. Comparison of spectral data including ^{1}H-NMR and ^{13}C-NMR including DEPT analysis, NOESY correlations, COSY correlations, HMQC correlations and HMBC correlations of this compound with that of Compound 2 demonstrated that Compound 3 differed from 2 only in having a hydroxy group attached to C-16. Based on the spectral data discussed above and shown below, the structure of Compound 3 was assigned to be labda-7,13(*Z*)-diene-17,12-olide-15-ol (Fig.8).

Table 8. The HMQC spectral data of Compound 3

^{13}C-NMR (ppm)	^{1}H-NMR (ppm), coupling constant (Hz)
12.2q	1.72s
13.4q	0.76s
18.5t	1.50m, 1.50m
21.4q	0.92s
25.2t	2.10m, 2.40m
27.3t	1.53m, 1.85ddd(*J*=2.1, 3.9, 13.1)
32.8s	-
32.9q	0.89s
34.7s	-
38.7t	1.05m, 1.76m
41.9t	1.20m, 1.49m
48.9d	1.32dd(*J* = 4.9, 11.9)
49.1d	2.27m
58.9t	4.25ddd(*J*=6.7, 12.8, 18.9)
83.7d	4.60dd(*J* = 1.5, 11.6)
125.7s	-
127.1d	5.70m
136.2s	-
143.3d	7.30ddd(*J* = 2.7, 2.7, 5.2)
165.8s	-

Table 9. The HMQC, HMBC and COSY spectral data of Compound 3

Position	δ_C [a]	δ_H	HMBC (H to C)	COSY
1	38.7(t)	1.05	-	H-1(1.76), H-20(0.76)
		1.76	C-10	H-1(1.06)
2	18.5(t)	1.50	-	H-2(1.50), H-3(1.20)
		1.50	C-3	H-2(1.50), H-3(1.49)
3	41.7(t)	1.20	-	H-2(1.50), H-18(0.92)
		1.49	-	H-2(1.50)
4	32.8(s)	-	-	-
5	48.9(d)	1.32	C-4, C-9, C-10, C-18, C-19, C-20	H-6(2.10), H-6(2.40)
6	25.2(t)	2.10	-	H-5(1.32), H-6(2.40)
		2.40	C-7, C-8	H-5(1.32), H-6(2.10), H-7(7.30)
7	143.3(d)	7.30	-	H-6(2.40, 2.10)
8	125.7(s)	-	-	-
9	49.1(d)	2.27	-	H-11(1.85)
10	34.7(s)	-	-	-
11	27.3(t)	1.53	C-12	H-11(1.85)
		1.85	-	H-11(1.53)
12	83.7(d)	4.60	C-9, C-11, C-13, C-14, C-15	H-11(1.85)
13	136.2(s)	-	-	-
14	127.1(d)	5.70	C-15	H-15(1.72), H-16(4.25)
15	12.2(q)	1.72	C-13, C-14	H-14(5.70)
16	58.9(t)	4.25	C-11, C-13, C-14, C-15	H-14(5.70)
		4.25	C-11, C-13, C-14, C-15	H-14(5.70)
17	165.8(s)	-	-	-
18	21.4(q)	0.92	C-2, C-19	H-3(1.20), H-19(0.89)
19	32.9(q)	0.89	C-18	H-6(1.32), H-18(0.92)
20	13.4(q)	0.76	-	H-1(1.05)

[a]Carbon type as determined by DEPT experiments spectra : s = singlet, d = doublet, t = triplet, q = quartet.

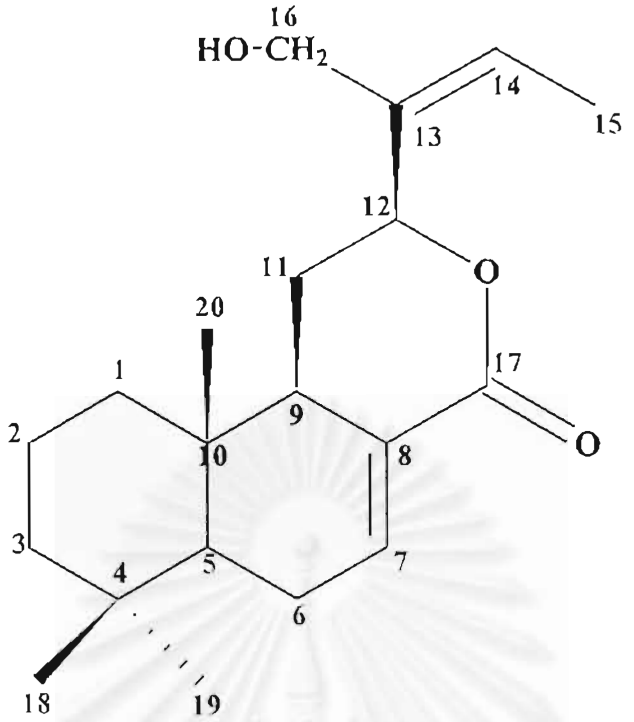


Figure 8. The structure of Compound 3

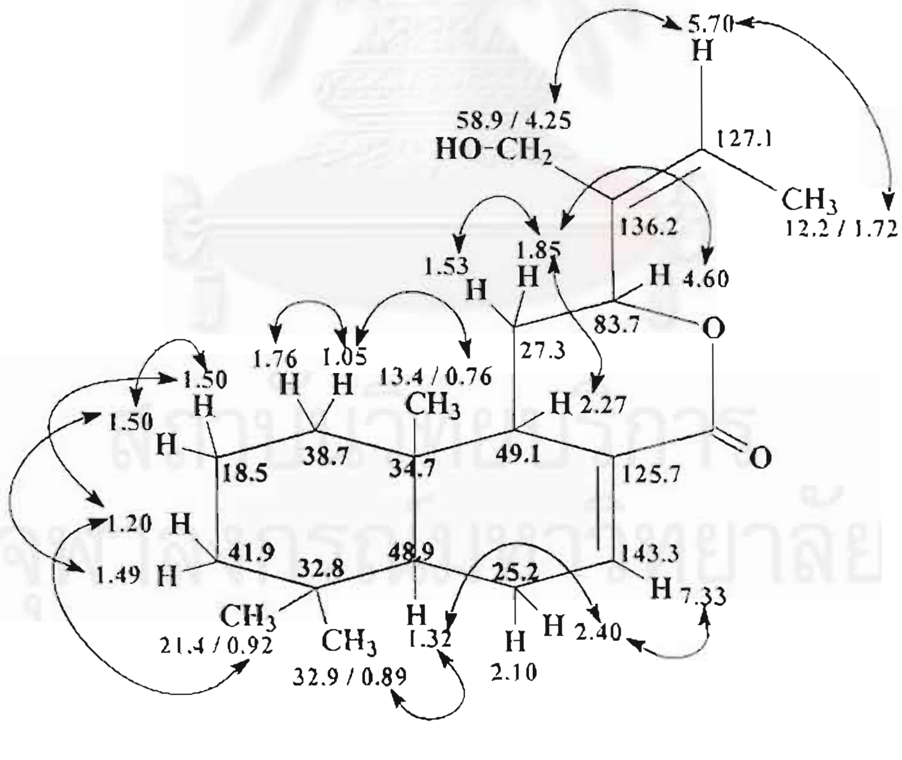


Figure 9. The COSY correlations of Compound 3

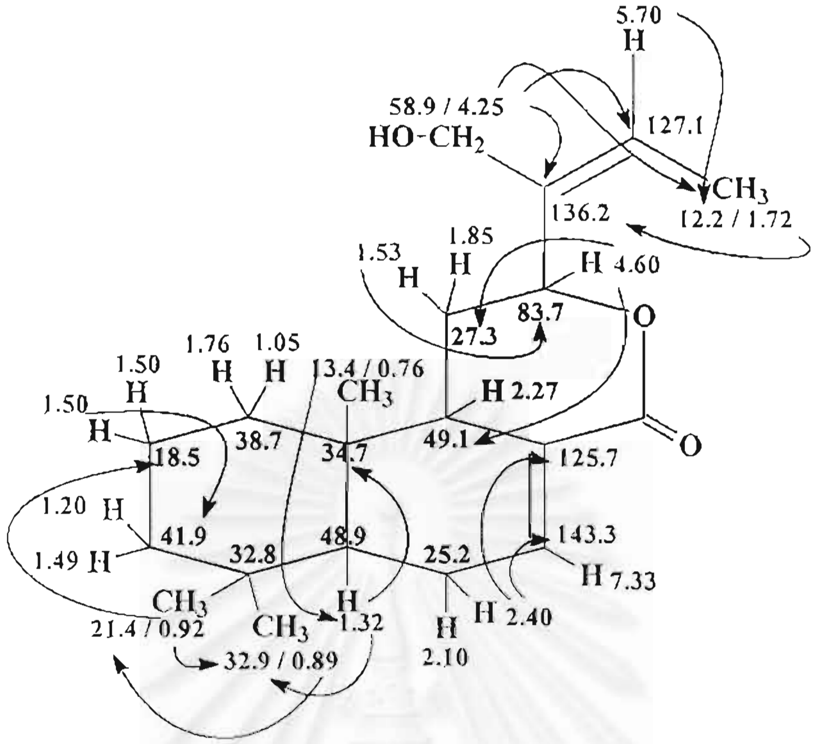


Figure 10. The HMBC correlations of Compound 3

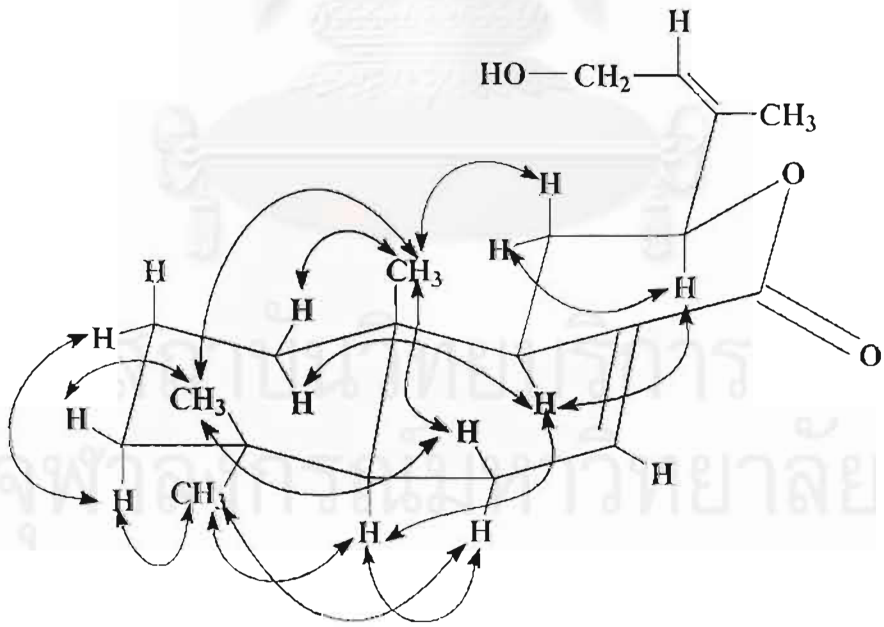


Figure 11. The NOESY correlations of Compound 3

3.2.4 Structure elucidation of Compound 4

The IR spectrum of Compound 4 (Fig.43) showed the presence of a carboxylic group according to the broad absorption band between 3500 to 3100 cm^{-1} and the strong absorption band at 1716 cm^{-1} due to the carboxylic acid carbonyl stretching.

Table 10. The IR absorption bands assignment of Compound 4

Wave number (cm^{-1})	Intensity	Tentative assignment
3500-3100	Broad	O-H stretching vibration of alcohol
2955, 2924	Strong	C-H stretching vibration of $-CH_2$, $-CH_3$
1716, 1675	Strong	C=O stretching vibration
1629	Medium	C=C stretching vibration
1250	Medium	C-O stretching vibration

The molecular formula of Compound 4 was assigned to be $C_{27}H_{32}O_5$ according to microanalysis and EI MS [M^+] (*m/z* 436), which indicated a DBE of 12. The ^{13}C-NMR spectrum (Fig. 45, Table 11) of Compound 4 was similar to that of Compound 1 except for the downfield positions of C-20 (67.73 ppm) when compared to that of Compound 1 (18.02 ppm). Its ^1H-NMR spectrum (Fig.44) showed two doublet signals (δ_H 4.30, J = 11.9 and δ_H 4.50, J = 11.9) of 2H-20. Comparison of spectral data including ^1H-NMR and ^{13}C-NMR including DEPT analysis, NOESY correlations, COSY correlations, HMQC correlations and HMBC correlations of this compound with that of Compound 1 demonstrated that Compound 4 differed from Compound 1 only in having a benzoyl ester group attached C-20. Based on the spectral data discussed above and shown below, the structure of Compound 4 was assigned to be (-)-20-benzyloxyhardwickiic acid (Fig. 12).

Table 11. The HMQC spectral data of Compound 4

^{13}C-NMR (ppm)	^{1}H-NMR (ppm), coupling constant (Hz)
16.9q	1.02d(J = 6.7)
17.9t	2.25m, 2.40m
19.2t	1.72m, 1.95m
20.2q	1.32s
27.2t	1.53m, 1.65m
28.1t	2.20m, 2.35m
32.4t	1.93m, 2.08m
36.0t	1.24m, 2.53ddd(J = 3.0, 3.0, 12.8)
36.4d	1.78m
37.7s	-
42.3s	-
47.4d	1.58d(J = 12.5)
67.7t	4.30d(J = 11.9), 4.50d(J = 11.9)
110.9d	6.28d(J = 1.5)
125.1s	-
128.5d	7.45dd(J = 7.6, 7.6)
128.5d	7.45dd(J = 7.6, 7.6)
129.5d	8.01d(J = 1.2)
129.5d	8.01d(J = 1.2)
130.4s	-
132.9d	7.55dd(J = 7.6, 7.6)
138.5d	7.24s
140.5d	6.92dd(J = 2.5, 4.6)
140.9s	-
142.9d	7.35d(J = 1.5)
166.8s	-
172.0s	-

Table 12. The HMQC, HMBC and COSY spectral data of Compound 4

Position	δ_C[a]	δ_H	HMBC (H to C)	COSY
1	19.2(t)	1.72m	C-5	H-2(2.35)
		1.95m	C-2, C-3, C-5	H-2(2.20)
2	28.1(t)	2.20m	C-3	H-1(1.95), H-2(2.35), H-3(6.92)
		2.35m	C-1, C-3, C-10	H-1(1.72), H-2(2.20), H-3(6.92)
3	140.5(d)	6.92dd(*J*=2.45,4.58)	C-1, C-5, C-18	H-2(2.20), H-2(2.35)
4	140.9(s)	-	-	-
5	37.7(s)	-	-	-
6	36.0(t)	1.24m	C-19	H-6(2.53), H-7(1.65)
		2.53ddd(*J*=3.05,3.05,12.82)	C-8, C-10	H-6(1.24), H-7(1.65), H-7(1.53)
7	27.2(t)	1.53m	C-6, C-9	H-6(2.53), H-8(1.78)
		1.65m	-	H-6(1.24)
8	36.3(d)	1.78m	-	H-17(1.02)
9	42.3(s)	-	-	-
10	47.4(d)	1.58d(*J*=12.51)	C-2, C-4, C-5, C-9, C-19	H-1(1.72)
11	32.4(t)	1.93m	C-10, C-12	H-11(2.08), H-12(2.40)
		2.08m	C-10, C-12	H-11(1.93), H-12(2.40), H-12(2.25)
12	17.9(t)	2.25m	C-11, C-13, C-14, C-16	H-11(2.08), H-12(2.40)
		2.40m	C-13, C-16	H-11(1.93), H-11(2.08), H-12(2.25)
13	125.1(s)	-	-	-
14	110.9(d)	6.28d(*J*=1.53)	C-13, C-16	H-15(7.35)
15	142.9(d)	7.35d(*J*=1.53)	C-13, C-14, C-16	H-14(6.28)
16	138.5(d)	7.24s	C-13, C-14, C-15	-
17	16.9(q)	1.02d(*J*=6.71)	C-7, C-8, C-9	H-8(1.78)
18	172.0(s)	-	-	-
19	20.2(q)	1.32s	C-4, C-5, C-6, C-10	-
20	67.7(t)	4.30d(*J*=11.9)	C-9, C-10, C-11, C-21	H-20(4.50)
		4.50d(*J*=11.9	C-8, C-9, C-11, C-21	H-20(4.30)
21	166.8(s)	-	-	-
22	130.4(s)	-	-	-
23	129.5(d)	8.01d(*J*=1.22)	C-21, C-23, C-25	H-24(7.45)
24	128.5(d)	7.45dd(*J*=7.63,7.63)	C-22, C-24	H-23(8.01), H-25(7.55)
25	132.9(d)	7.55dd(*J*=7.63,7.63)	C-23	H-24(7.45)
26	128.5(d)	7.45dd(*J*=7.63,7.63)	C-22, C-26	H-25(7.55), H-27(8.01)
27	129.5(d)	8.01d(*J*=1.22)	C-21, C-25, C-27	H-26(7.45)

[a]Carbon type as determined by DEPT experiments spectra : s = singlet, d = doublet, t = triplet, q = quartet.

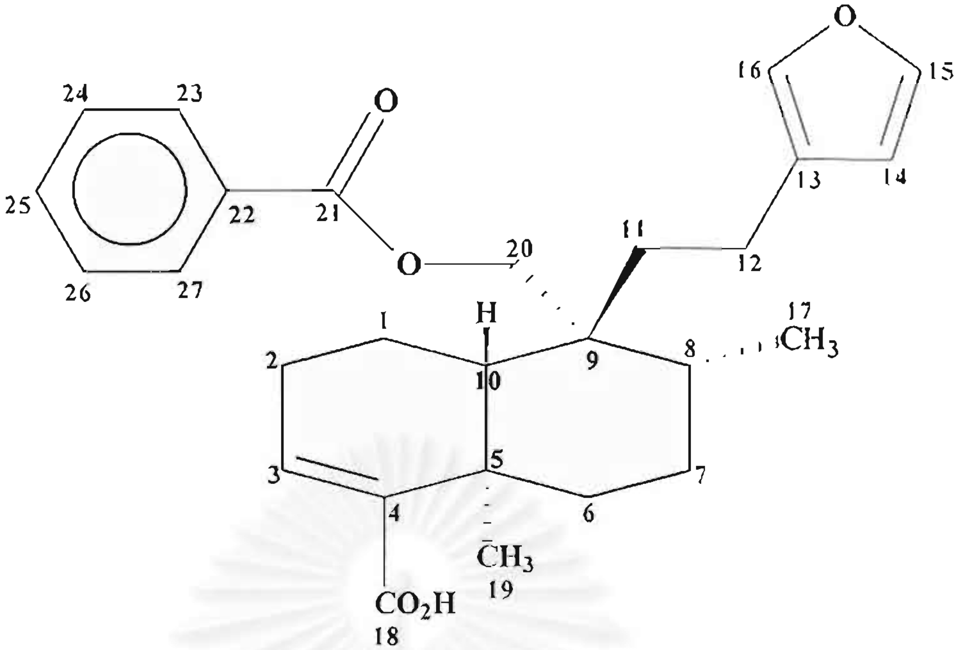


Figure 12. Structure of Compound 4

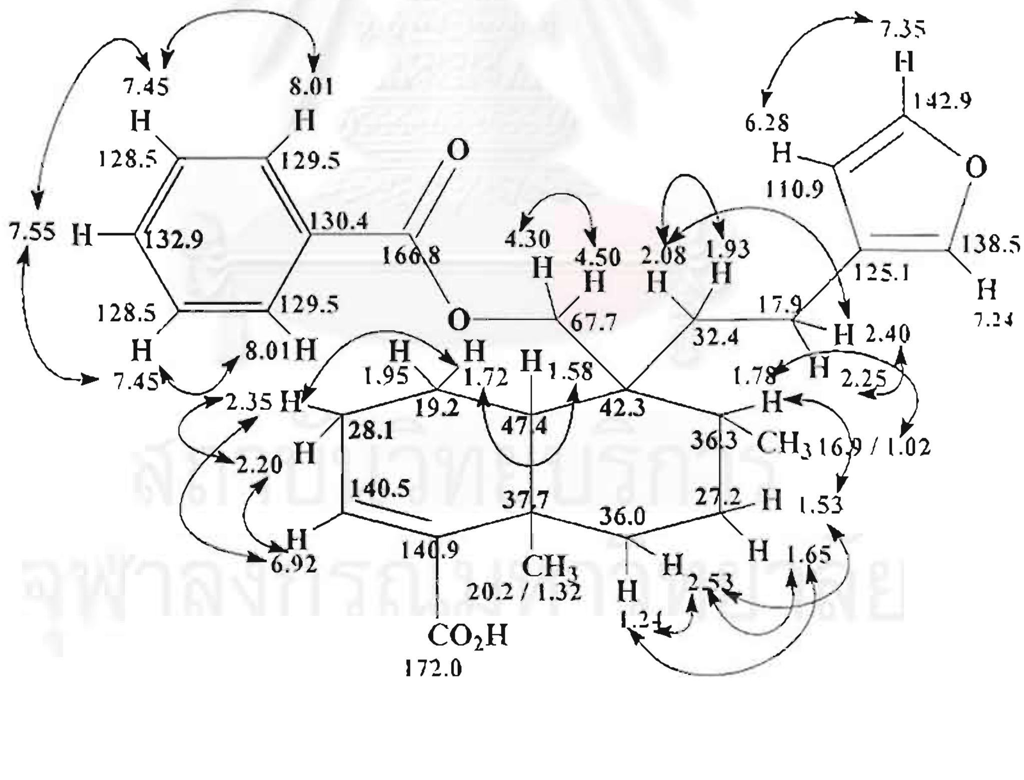


Figure 13. The COSY correlations of Compound 4

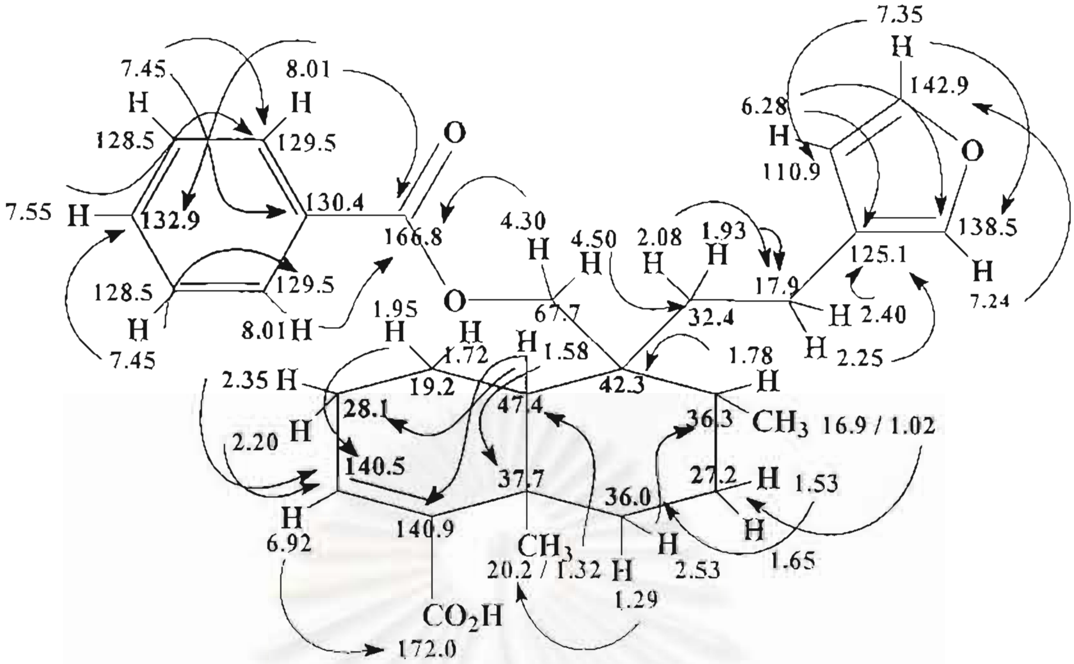


Figure 14. The HMBC correlations of Compound 4

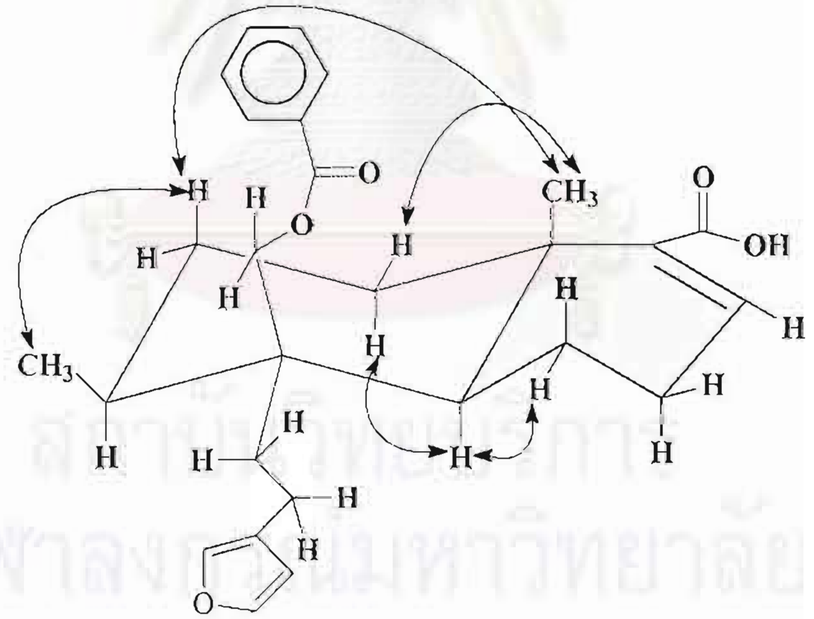
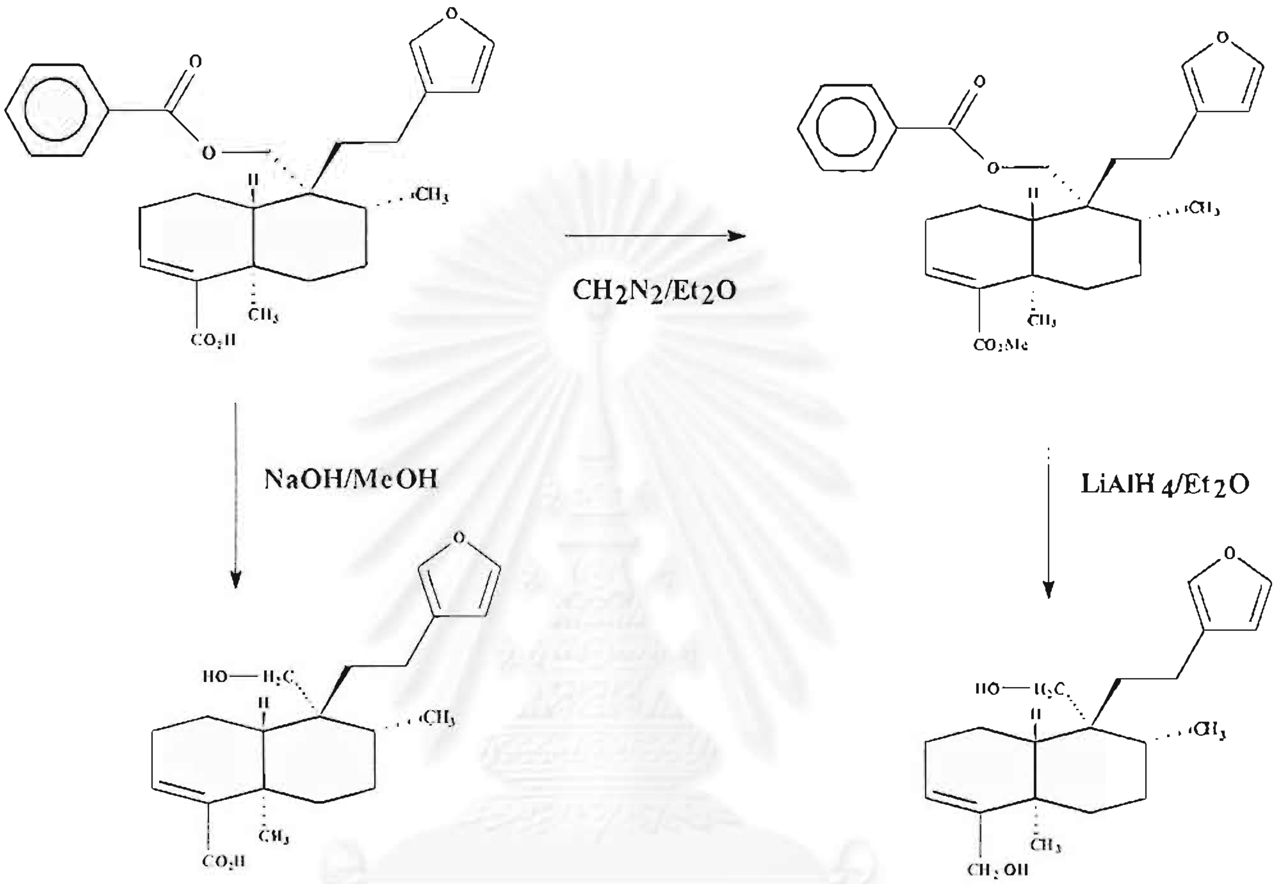


Figure 15. The NOESY correlations of Compound 4

3.3 Modification of compounds from *Croton oblongifolius* Roxb.

3.3.1 Modification of Compound 4

The pathway of modification of Compound 4 is shown in Scheme 12.



Scheme 12. Modification pathway of Compound 4

3.3.1.1 Methylation of Compound 4

The Compound 4 was methylated with diazomethane in diethyl ether to give methyl ester, Compound 4a, as a viscous transparent oil. The IR spectrum of Compound 4a is shown in Fig. 52 and the absorption peaks were assigned as shown in Table 13. Its IR spectrum showed important absorption bands at 1726, 1613(C=O stretching vibration), 1280(C-O stretching vibration of ester) and 870-670 cm^{-1} (Aromatic).

Table 13. The IR absorption bands assignment of Compound 4a

Wave number (cm^{-1})	Intensity	Tentative assignment
2590, 2868	Strong	C-H stretching vibration of $-CH_2$, $-CH_3$
1726, 1613	Strong	C=O stretching vibration
1629	Medium	C=C stretching vibration
1280	Medium	C-O stretching vibration

The ^{1}H-NMR spectrum (Fig.53) of Compound 4a showed that it possesses two methyl groups of protons (δ_H 1.32 and 1.00 ppm), one methylene ester protons (δ_H 4.48 and 4.30 ppm), eight olefinic protons (δ_H 8.01, 8.01, 7.55, 7.45, 7.45, 7.32, 7.20, 6.65 and 6.25ppm) and one methyl ester group (δ_H 3.67 ppm).

The ^{13}C-NMR, DEPT-90 and DEPT-135 spectrum (Fig.54) showed 28 signals. Twelve signals of olefinic carbons appeared at δ 142.9, 141.9, 137.1, 138.5, 132.9, 130.4, 129.5, 129.5, 128.5, 128.5, 125.2 and 110.9 ppm. The signals at 167.6 and 166.8 ppm should be the carboxyl groups of ester. There were thirteen sp^3 signals at δ 19.3(t), 27.8(t), 37.7(s), 36.1(t), 27.2(t), 36.4(d), 42.3(s), 47.3(d), 32.4(t), 17.9(t), 17.0 (q), 20.3(q) and one methyl ester at 51.2 ppm.

Its molecular formula was established as $C_{28}H_{34}O_5$, which was confirmed by observing molecular ion at *m/z* 450 (Fig. 55).

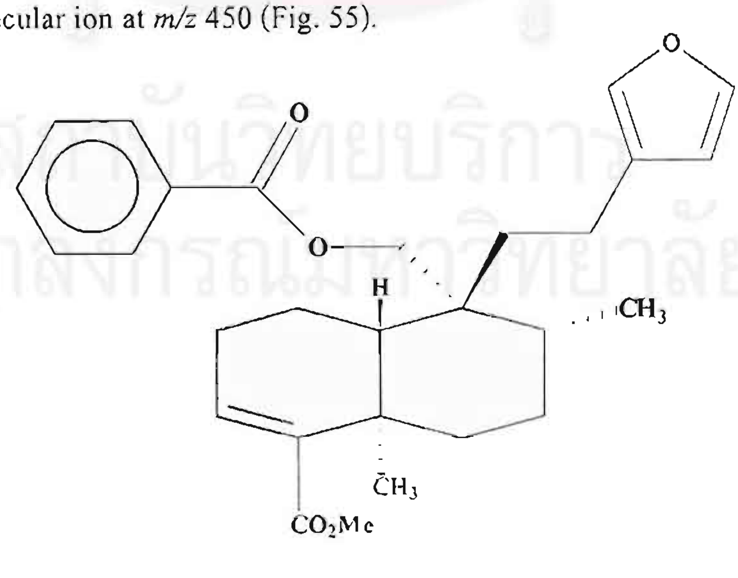


Figure 16. The structure of Compound 4a

3.3.1.2 Reduction of Compound 4a

The Compound 4a was reduced with lithium aluminum hydride in diethyl ether to give a diol compound, Compound 4b, as a white crystalline solid. The IR spectrum of Compound 4b is shown in Fig.56 and the absorption peaks were assigned as shown in Table 14. Its IR spectrum showed important absorption bands at 3700-3050 cm^{-1} (O-H stretching of alcohol), 2929 cm^{-1} (C-H stretching vibration), 1644 cm^{-1}(C=C stretching vibration of olefin) and 1019 cm^{-1} (C-O stretching vibration of primary alcohol).

Table 14. The IR absorption bands assignment of Compound 4b

Wave number (cm^{-1})	Intensity	Tentative assignment
3700-3050	Broad	O-H stretching vibration of alcohol
2929	Medium	C-H stretching vibration of -CH3, -CH2
1644	Weak	C=C stretching vibration of olefin
1019	Medium	C-O stretching vibration of primary alcohol

The ^1H-NMR spectrum (Fig.57) of Compound 4b showed that it possesses two methyl groups of protons (δ_H 1.10 and 0.92 ppm), two methylene alcohol protons (δ_H 4.07 and 3.70 ppm) and four olefinic protons (δ_H 7.33, 7.21, 6.26 and 5.57 ppm).

The ^{13}C-NMR, DEPT-90 and DEPT-135 spectrum (Fig.58) showed 20 signals. Six signals of olefinic carbons appeared at δ 147.7, 142.7, 122.2, 138.5, 125.6 and 111.0 ppm. There were fourteen sp^3 signals at δ 19.8(t), 27.2(t), 37.8(s), 36.2(t), 27.2 (t), 36.6(d), 43.2(s), 46.9(d), 31.9(t), 17.8(t), 17.2(q), 20.9(q), and two methylene carbons of alcohol at δ 65.5 and 62.8 ppm.

Its molecular formula was established as $C_{20}H_{30}O_3$, which was confirmed by observing molecular ion at *m/z* 318 (Fig. 59).

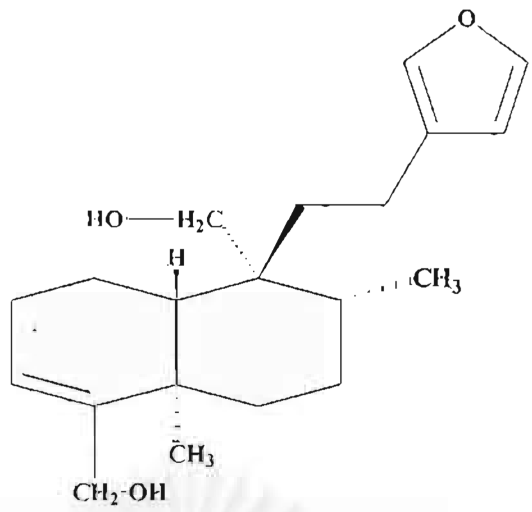


Figure 17. The structure of Compound 4b

Slow evaporation of a chloroform solution of the diol 4b gave crystals suitable for X-ray crystallographic analysis. A computer generated drawing of the final X-ray model of 4b is given in **Figure 18**. The packing diagram (**Figure 19**) revealed that each hydroxyl hydrogen atom is participating in a strong intermolecular hydrogen bond; O3-H3...O2(i) and O2-H2...O3(ii). These results in infinite hydrogen bonded chains along *a*-direction. The furan ring O(1) atom is not involved in hydrogen bonding. The resulting X-ray structure of (4b) are in agreement with the expected structure of the reduction product of (4) therefore the structure and relative stereochemistry of (4) previously deduced from NMR experiments is confirmed.

Table 15. Crystal data and structure refinement for Compound 4b

Empirical formula	$C_{20}H_{30}O_3$
Formula weight	318.44
Temperature	293(2) K
Wavelength	0.71073 A
Crystal system, space group	Orthorhombic, P_2 (1)2(1)2(1)
Unit cell dimensions	a = 7.94010(10) A alpha = 90 deg.
	b = 12.4435(2) A beta = 90 deg.
	c = 18.3666(2) A gamma = 90 deg.
Volume	1814.67(4) A^3
Z, Calculated density	4, 1.166 mg/m^3
Absorption coefficient	0.076 mm^{-1}
F(000)	696
Theta range for data collection	1.98 to 30.47 deg.
Index ranges	-10<=h<=5, -17<=k<=17, -25<= l<=26
Reflection collected / unique	13290 / 5225 [R(int) = 0.0181]
Completeness to 2theta = 30.34	96.6 %
Refinement method	Full-matrix least-squares on F^2
Data / restraints / parameters	5225 / 0 / 328
Goodness-of-fit on F^2	1.014
Final R indices [I > 2 sigma(I)]	R1 = 0.0378, wR2 = 0.1055
R indices (all data)	R1 = 0.0448, wR2 = 0.1127
Absolute structure parameter	-0.7 (8)
Largest diff. peak and hole	0.205 and –0.130 e. A^{-3}

Table 16. Atomic coordinates (x 10^4) and equivalent isotropic displacement parameters (A^2 X 10^3) for diol. (Compound 4b)

	x	y	z	U_{eq}
C (10)	2420 (1)	5292 (1)	3023 (1)	32 (1)
O (1)	-3290 (4)	6373 (2)	5348 (1)	138 (1)
O (2)	3692 (1)	3299 (1)	4520 (1)	46 (1)
C (1)	2962 (2)	6335 (1)	3412 (1)	42 (1)
O (3)	3413 (1)	7075 (1)	665 (1)	45 (1)
C (2)	2480 (2)	7300 (1)	2948 (1)	46 (1)
C (3)	2808 (2)	7124 (1)	2151 (1)	39 (1)
C (4)	3200 (2)	6183 (1)	1852 (1)	35 (1)
C (5)	3464 (1)	5155 (1)	2305 (1)	33 (1)
C (6)	2753 (2)	4170 (1)	1896 (1)	44 (1)
C (7)	2646 (2)	3166 (1)	2378 (1)	45 (1)
C (8)	1556 (2)	3333 (1)	3057 (1)	39 (1)
C (9)	2202 (1)	4288 (1)	3532 (1)	34 (1)
C (11)	891 (2)	4569 (1)	4137 (1)	39 (1)
C (12)	-777 (2)	5089 (2)	3897 (1)	57 (1)
C (13)	-1844 (2)	5414 (1)	4534 (1)	50 (1)
C (16)	-2331 (6)	6395 (2)	4725 (2)	119 (1)
C (15)	-3436 (3)	5323 (2)	5532 (1)	79 (1)
C (14)	-2587 (2)	4738 (2)	5057 (1)	61 (1)
C (18)	3471 (2)	6073 (1)	1039 (1)	45 (1)
C (19)	5387 (2)	5026 (1)	2416 (1)	45 (1)
C (17)	1364 (2)	2256 (1)	3452 (1)	52 (1)
C (20)	3881 (2)	4030 (1)	3919 (1)	41 (1)

Table 17. Bond lengths (A°) and angles (deg) for Diol. (Compound 4b)

C(10)	- C(1)	1.5429 (16)	C(6)	- C(7)	1.5325 (17)
C(10)	- C(5)	1.5667 (14)	C(7)	- C(8)	1.5323 (18)
C(10)	- C(9)	1.5707 (14)	C(8)	- C(17)	1.5318 (17)
O(1)	- C(15)	1.354 (3)	C(8)	- C(9)	1.5605 (16)
O(1)	- C(16)	1.375 (4)	C(9)	- C(20)	1.5446 (15)
O(2)	- C(20)	1.4380 (13)	C(9)	- C(11)	1.5622 (15)
C(1)	- C(2)	1.5227 (17)	C(11)	- C(12)	1.5388 (18)
O(3)	- C(18)	1.4245 (15)	C(12)	- C(13)	1.5009 (19)
C(2)	- C(3)	1.5023 (16)	C(13)	- C(16)	1.329 (3)
C(3)	- C(4)	1.3304 (16)	C(13)	- C(14)	1.406 (2)
C(4)	- C(18)	1.5140 (15)	C(15)	- C(14)	1.322 (3)
C(4)	- C(5)	1.5405 (15)			
C(5)	- C(6)	1.5443 (16)			
C(5)	- C(19)	1.5485 (17)			

Table 18. Bond lengths (A^{O}) and angles (deg) for Diol. (Compound 4b)

C(1) – C(10) – C(5)	109.55(9)	C(17) – C(8) – C(9)	115.70(10)
C(1) – C(10) – C(9)	115.06(8)	C(7) – C(8) – C(9)	111.91(10)
C(5) – C(10) – C(9)	118.20(9)	C(20) – C(9) – C(8)	112.51(10)
C(15) – O(1) – C(16)	105.93(19)	C(20) – C(9) – C(11)	107.11(9)
C(2) – C(1) – C(10)	109.47(9)	C(8) – C(9) – C(11)	110.45(9)
C(3) – C(2) – C(1)	112.76(10)	C(20) – C(9) – C(10)	110.14(9)
C(4) – C(3) – C(2)	124.87(10)	C(8) – C(9) – C(10)	107.98(8)
C(3) – C(4) – C(18)	121.31(10)	C(11) – C(9) – C(10)	108.60(9)
C(3) – C(4) – C(5)	122.65(9)	C(12) – C(11) – C(9)	117.60(9)
C(18) – C(4) – C(5)	116.00(9)	C(13) – C(12) – C(11)	112.02(11)
C(4) – C(5) – C(6)	110.26(9)	C(16) – C(13) – C(14)	104.32(18)
C(4) – C(5) – C(19)	106.93(9)	C(16) – C(13) – C(12)	128.16(18)
C(6) – C(5) – C(19)	110.05(10)	C(14) – C(13) – C(12)	127.52(15)
C(4) – C(5) – C(10)	106.96(8)	C(13) – C(16) – O(1)	111.3(2)
C(6) – C(5) – C(10)	107.60(9)	C(14) – C(15) – O(1)	108.83(19)
C(19) – C(5) – C(10)	114.98(9)	C(15) – C(14) – C(13)	109.63(19)
C(7) – C(6) – C(5)	112.71(10)	O(3) – C(18) – C(4)	113.13(10)
C(8) – C(7) – C(6)	112.98(10)	O(2) – C(20) – C(9)	113.24(9)
C(17) – C(8) – C(7)	108.84(11)		

Table 19. Hydrogen coordinates (x 10^4) and isotropic displacement parameters (A^2 X 10^3) for Diol. (Compound 4b)

	x	y	z	U_{eq}
H (6A)	1580 (2)	4365 (12)	1727 (8)	39 (4)
H (10)	1233 (19)	5409 (11)	2873 (8)	32 (3)
H (2A)	1280 (3)	7433 (13)	3035 (10)	53 (5)
H (7A)	3790 (3)	2921 (15)	2526 (11)	59 (5)
H (1A)	2430 (3)	6434 (15)	3904 (10)	59 (5)
H (1B)	4220 (2)	6365 (14)	3497 (9)	51 (4)
H (8)	440 (2)	3524 (13)	2910 (9)	42 (4)
H (11A)	1410 (2)	5026 (13)	4478 (9)	42 (4)
H (7B)	2190 (3)	2567 (16)	2071 (11)	63 (5)
H (11B)	600 (2)	3921 (13)	4384 (9)	43 (4)
H (2B)	3100 (3)	7973 (18)	3112 (11)	71 (6)
H (18B)	4600 (3)	5739 (16)	953 (12)	66 (5)
H (6B)	3290 (3)	4055 (17)	1424 (11)	66 (5)
H (12A)	-500 (3)	5720 (2)	3605 (15)	93 (8)
H (19C)	5930 (2)	5623 (16)	2725 (11)	59 (5)
H (17B)	2470 (3)	1968 (16)	3597 (11)	67 (5)
H (18A)	2560 (3)	5545 (15)	833 (11)	62 (5)
H (3O)	2820 (3)	6962 (16)	275 (12)	63 (5)
H (2O)	4540 (3)	2948 (15)	4505 (10)	49 (4)
H (17A)	690 (3)	2323 (14)	3903 (11)	57 (5)
H (19B)	5680 (3)	4332 (17)	2613 (12)	67 (5)
H (20B)	4350 (2)	4723 (14)	4100 (9)	45 (4)
H (20A)	4690 (3)	3693 (15)	3579 (10)	55 (5)
H (17C)	830 (3)	1663 (18)	3143 (12)	75 (6)
H (12B)	-1450 (4)	4480 (2)	3539 (15)	102 (8)
H (19A)	5880 (3)	5051 (17)	1962 (13)	72 (6)
H (14)	-2640 (4)	3890 (3)	5053 (14)	116 (10)
H (15)	-4090 (5)	5200 (3)	5900 (2)	140 (13)
H (16)	-2280 (6)	6930 (3)	4500 (2)	153 (15)
H (3)	2640 (2)	7716 (13)	1856 (9)	44 (4)

Table 20. Hydrogen–bond geometry of Diol (Compound 4b)

D-H...A	d(D..A)	d(D-H)	d(H..A)	< D-H...A	A
O(3)-H(3)...O(2)	2.726	0.868	1.863	172.61	O2[-x+1/2, -y+1, z-1]
O(2)-H(2)...O(2)	2.778	0.800	1.983	172.89	O3[-x+1, y-1/2, -z+1]

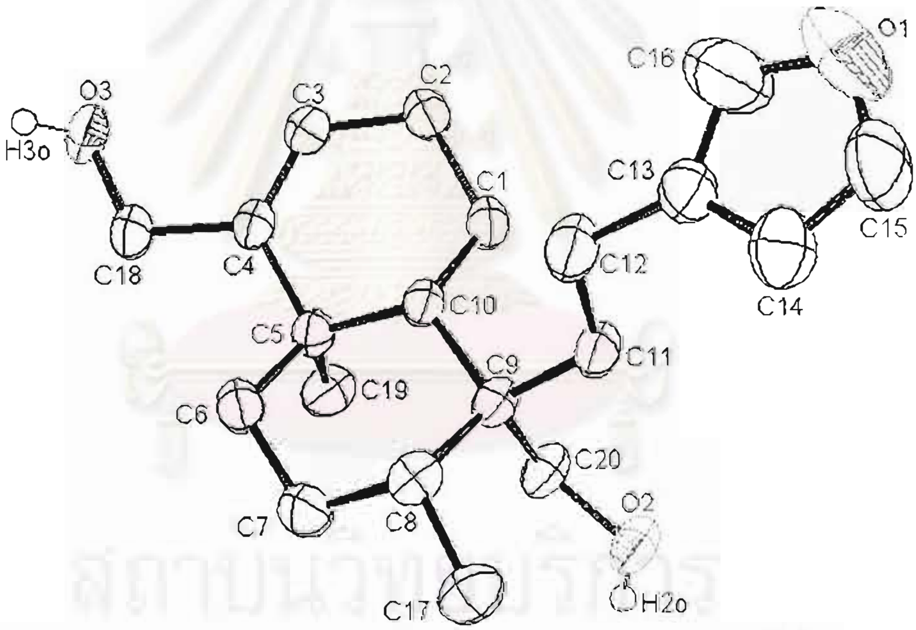


Figure 18. ORTEP drawing of Compound 4b

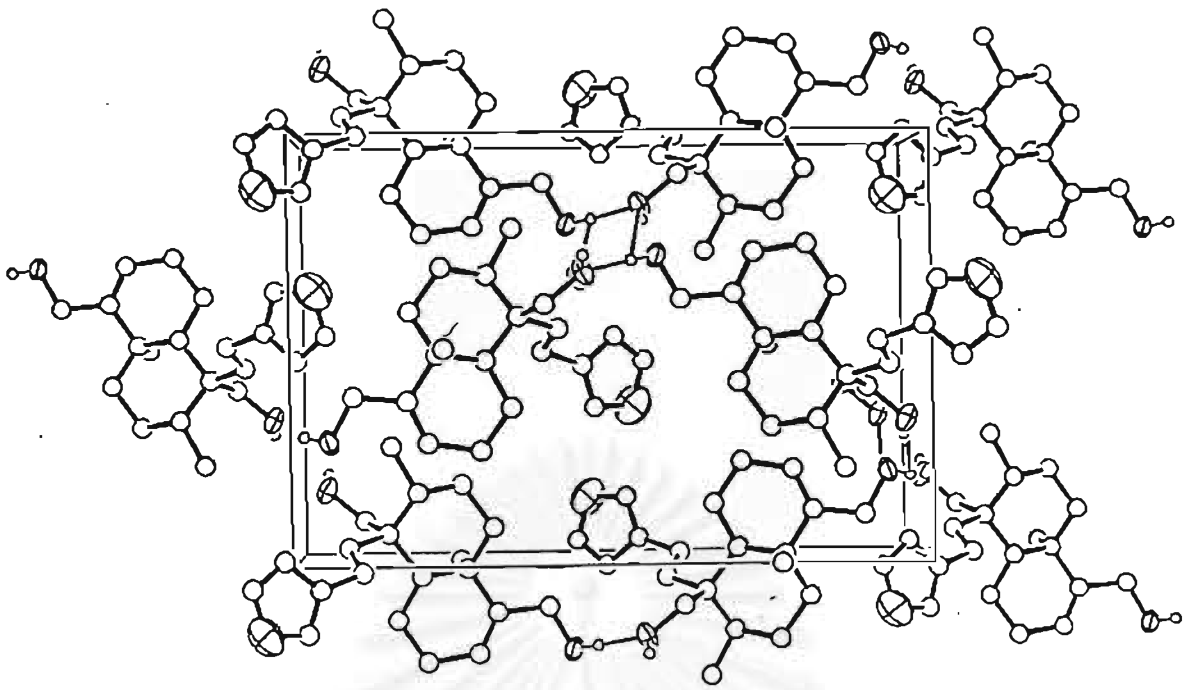


Figure 19. The packing *diagram* of Compound 4b

3.3.1.3 **Hydrolysis of Compound 4**

Compound 4 was hydrolyzed with NaOH in methanol to give alcohol of Compound 4, Compound 4c, as a viscous transparent oil. The IR spectrum of Compound 4c is shown in Fig.60 and the absorption peaks were assigned as shown in Table 21. Its IR spectrum showed important absorption bands at 3405 cm^{-1} (O-H stretching of alcohol), 2970 cm^{-1} (C-H stretching vibration), 1675 cm^{-1}(C=O stretching vibration of carbonyl of carboxylic acid) and 1623 cm^{-1}(C=C stretching vibration of olefin).

Table 21. The IR absorption band assignment of Compound 4c

Wave number (cm^{-1})	Intensity	Tentative assignment
3405	Broad	O-H stretching vibration of alcohol
2970	Strong	C-H stretching vibration of -CH3, -CH2
1675	Strong	C=O stretching vibration of carbonyl
1623	Medium	C=C stretching vibration of olefin

The ^{1}H-NMR spectrum (Fig.61) of Compound 4c showed that it possesses two methyl groups of protons (δ_H 1.15 and 0.95 ppm), one methylene alcohol proton (δ_H 3.70 ppm) and four olefinic protons (δ_H 7.35, 7.20, 6.90 and 6.25 ppm).

The ^{13}C-NMR, DEPT-90 and DEPT-135 spectrum (Fig.62) showed 20 signals. Six signals of olefinic carbons appeared at δ 142.8, 141.3, 140.0, 138.5, 125.5 and 111.0 ppm. There were thirteen sp^3 signals at δ 19.1(t), 28.1(t), 37.6(s), 36.2(t), 27.3(t), 36.1(d), 43.2(s), 47.4(d), 32.2(t), 17.8(t), 17.1(q), 20.1(q), and one methylene carbon of alcohol at δ 65.5 ppm.

Its molecular formula was established as $C_{20}H_{28}O_4$, which was confirmed by observing molecular ion at *m/z* 332 (Fig.63).

Figure 20. The structure of Compound 4c

Table 22. ^{13}C-NMR spectral data of Compounds 4 and derivatives (4a, 4b and 4c)

Position	Compound 4	Compound 4a	Compound 4b	Compound 4c
C-1	19.2(t)	19.3(t)	19.8(t)	19.1(t)
C-2	28.1(t)	27.8(t)	27.2(t)	28.1(t)
C-3	140.5(d)	137.1(d)	122.2(d)	140.0(d)
C-4	140.9(s)	141.9(s)	147.7(s)	141.3(s)
C-5	37.7(s)	37.7(s)	37.8(s)	37.6(s)
C-6	36.0(t)	36.1(t)	36.2(t)	36.2(t)
C-7	27.2(t)	27.2(t)	27.2(t)	27.3(t)
C-8	36.3(d)	36.4(d)	36.6(d)	36.1(d)
C-9	42.3(s)	42.3(s)	43.2(s)	43.2(s)
C-10	47.4(d)	47.3(d)	46.9(d)	47.4(d)
C-11	32.4(t)	32.4(t)	31.9(t)	32.2(t)
C-12	17.9(t)	17.9(t)	17.8(t)	17.8(t)
C-13	125.1(s)	125.2(s)	125.6(s)	125.5(s)
C-14	110.9(d)	110.9(d)	111.0(d)	111.0(d)
C-15	142.9(d)	142.9(d)	142.7(d)	142.8(d)
C-16	138.5(d)	138.5(d)	138.5(d)	138.5(d)
C-17	16.9(q)	17.0(q)	17.2(q)	17.1(q)
C-18 (C=O)	172.0(s)	167.6(s)	62.8(t)	172.3(s)
C-19	20.2(q)	20.3(q)	20.9(q)	20.1(q)
C-20	67.7(t)	67.7(t)	65.5(t)	65.5(t)
C-21	166.8(s)	166.8(s)	-	-
C-22	130.4(s)	130.4(s)	-	-
C-23	129.5(d)	129.5(d)	-	-
C-24	128.5(d)	128.5(d)	-	-
C-25	132.9(d)	132.9(d)	-	-
C-26	128.5(d)	128.5(d)	-	-
C-27	129.5(d)	129.5(d)	-	-
CH_3O-	-	51.2(q)	-	-

CHAPTER IV

BIOLOGICAL ACTIVITY

4.1 Biological assay

4.1.1 Cytotoxicity test

Bioassay of cytotoxic activity against 6 cell lines, including HS 27 (fibroblast), Kato (gastric), BT 474 (breast), Chago (lung), SW 620 (colon) and Hep-G2 (hepatoma) cancer, *in vitro* was performed by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] colorimetric method. [14-16] In principle, the viable cell number / well is directly proportional to the production of formazan, which following solubilization, can be measured spectrophotometrically.

The 6 cell lines 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°C, 5% CO_2, 100% relative humidity, 100 μl of culture medium, the culture medium, containing the 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 additions of tetrazolium reagent. MTT stock solution was prepared as follows: 5 mg MTT / ml PBS was sterile 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 pre-warmed standard culture medium. MTT working solution (50 μl) was added to each culture well resulting in 50 μg MTT/ 250 μl total medium volume) and cultures were incubated at 37°C for 4 to 24 h depending upon individual cell line requirements. Incubation cell monolayers and formazan were then 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 the 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.

4.2 Results of biological activity test

The *in vitro* activity of some compounds (10 μg/ml) from *Croton oblongifolius* against the 6 cell lines, for example, HS 27 (fibroblast), Hep-G2 (hepatoma), SW 620 (colon), Chago (lung), Kato (gastric), BT 474 (breast) cancer are reported in Table 23.

Table 23. Cytotoxic activity against 6 cell lines of some compounds from *C. oblongifolius*.

Compound	% Survival					
	HS 27 (fibroblast)	Hep-G2 (hepatoma)	SW 620 (colon)	Chago (lung)	Kato (gastric)	BT474 (breast)
2	157	61	73	72	47	75
3	93	56	67	44	50	70
4	130	74	84	111	64	109
4a	111	74	58	114	65	82
4b	107	53	97	113	70	91
4c	105	79	98	108	86	96

All compounds showed cytotoxic activity against 6 cell lines. Moreover, compound 3, which consisted of an alcohol group, showed remarkable cytotoxic activity against all cell lines tested.

Table 24. Cytotoxicity data of natural compounds and synthesized compounds

Compound	IC_{50} (μg/mL) for cell lines					
	HS 27 (fibroblast)	Hep-G2 (hepatoma)	SW 620 (colon)	Chago (lung)	Kato (gastric)	BT474 (breast)
2	>10	>10	8.1	8.8	6.8	>10
3	>10	5	6.5	6.4	7.1	>10
4c	>10	>10	>10	>10	>10	>10

Furthermore, hardwickiic acid, which was the main product of the crude hexane, and its derivatives have been tested for antimicrobial activity. It was found that no biological properties have been specifically related to hardwickiic acid. (-)-Hardwickiic acid showed a significant qualitative antibacterial activity against the Gram-positive bacteria (*B. subtilis*, *St. aureus*) and *M. smegmatis* [19].

CHAPTER V

CONCLUSION

From the study of chemical constituents found in the stem bark of *Croton oblongifolius* Roxb. from Amphur Muang, Udonthani Province, it was found that the main components were different from those obtained from other places. The chemical constituents found in *Croton oblongifolius* Roxb. could be categorized into two groups including clerodane diterpenoid and labdane diterpenoid compounds.

In this research, concerning the chemical constituents found in the stem bark of *Croton oblongifolius* Roxb. from Amphur Muang, Udonthani Province, two new labdane compounds were discovered including labda-7,13(*Z*)-diene-17,12-olide (1) and labda-7,13(*Z*)-diene-17,12-olide-15-ol (2). One new clerodane compound, (-)-20-benzyloxyhardwickiic acid, was also found. Moreover, hardwickiic acid was found to be the main constituent in this plant.

From the modification of (-)-20-benzyloxyhardwickiic acid, the carboxylic acid group was changed into methyl ester and reduce with $LiAlH_4$ to the diol. The single crystal x-ray crystallographic study of the diol confirmed the relative stereochemistry of the parent compound. The ester group was hydrolyzed into an alcohol. The derivatives of (-)-20-benzyloxyhardwickiic acid including methyl ester, diol and alcohol were new compounds.

The isolated compounds and their derivatives showed cytotoxic activity against 6 cell lines. Moreover, compound 3, (labda-7,13(*Z*)-diene-17,12-olide-15-ol), which consisted of an alcohol group, showed significant cytotoxic activity against 6 cell lines. Compound 3 exhibited cytotoxic activity against the HEP-G2 (hepatoma) cell, CHAGO (lung) cell, SW 620 (colon) cell and KATO (gastric) cell, *in vitro*, with IC_{50} values of 5, 6.4, 6.5 and 7.1 μg/ml, respectively.

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APPENDIX

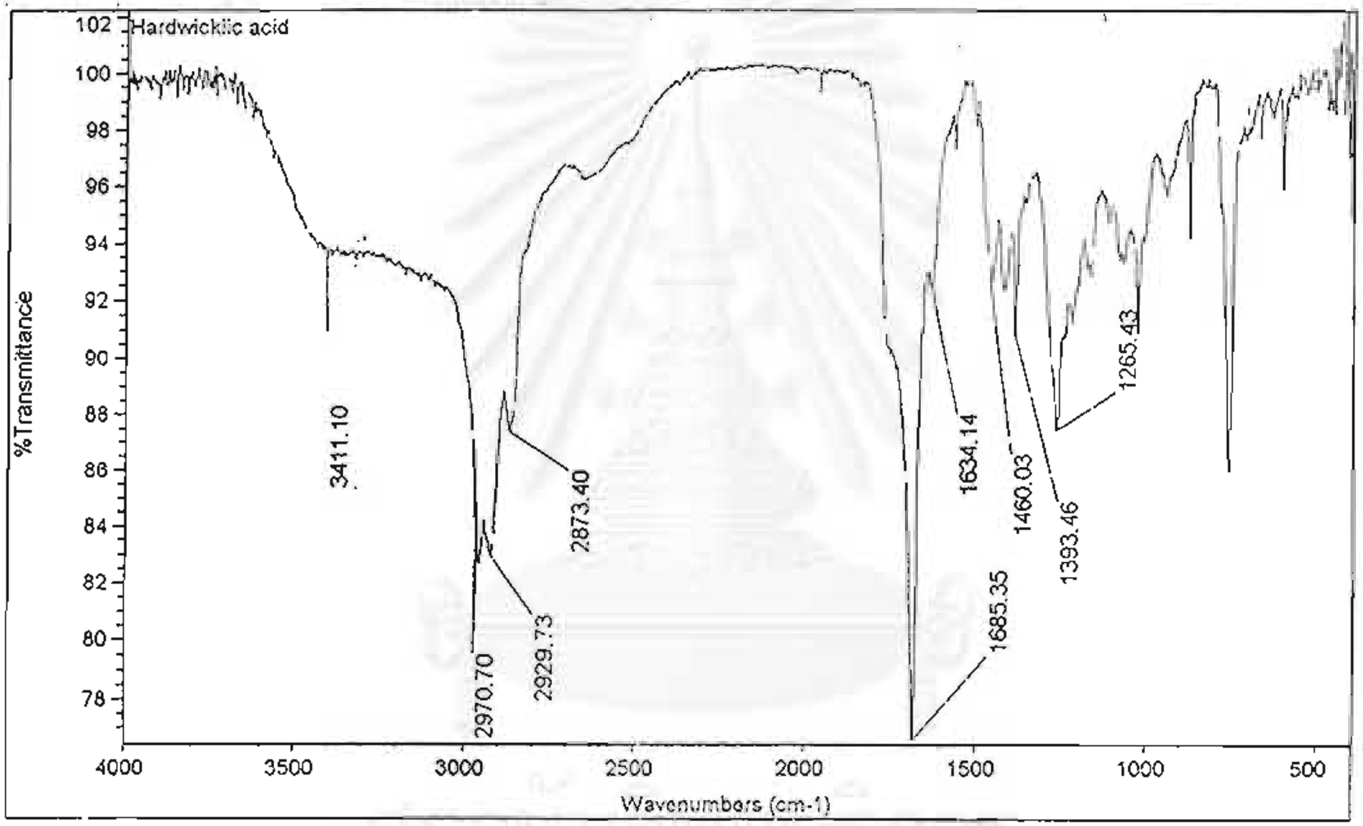

Figure 21. The IR spectrum of Compound 1

62

63

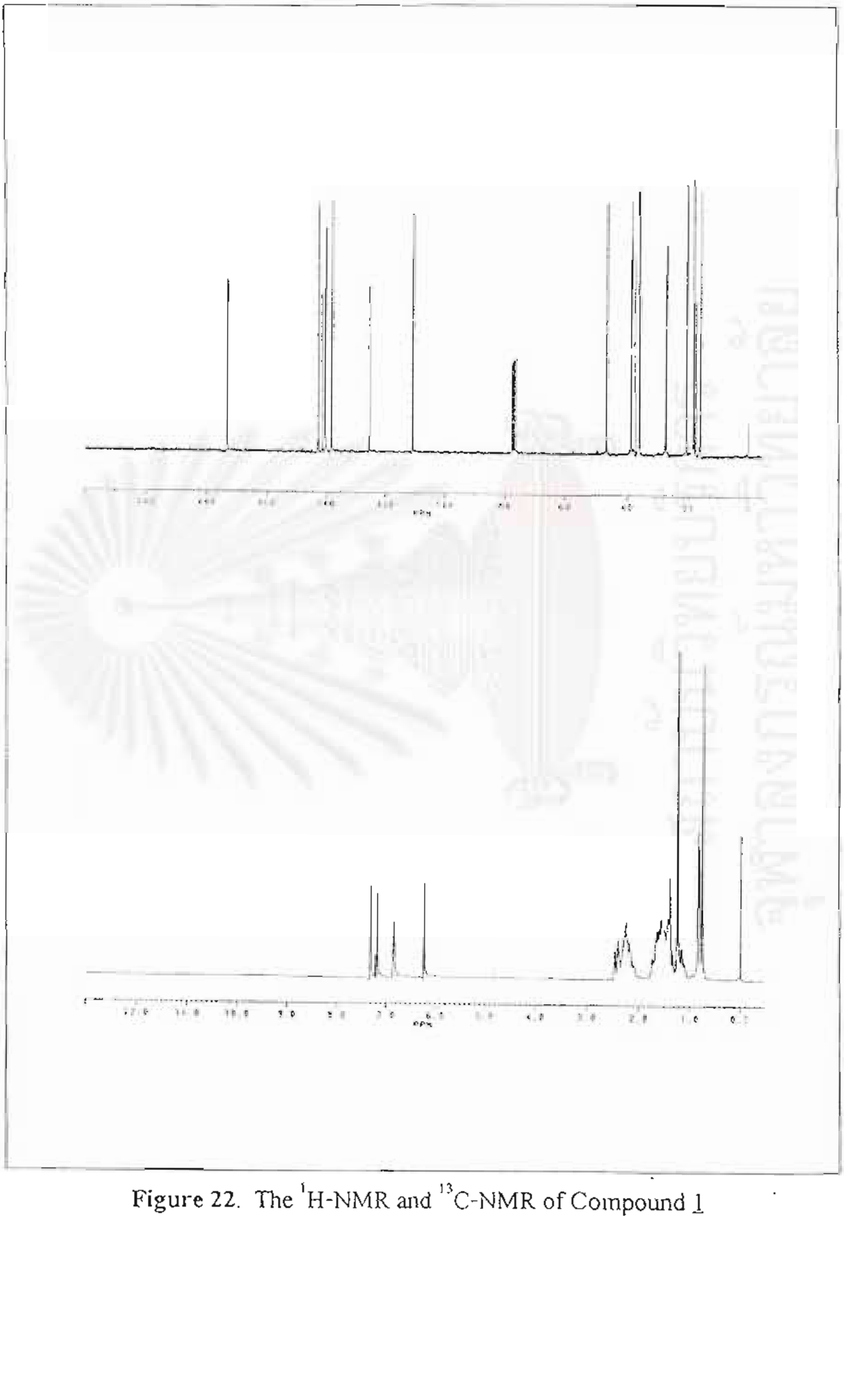

Figure 22. The ^{1}H-NMR and ^{13}C-NMR of Compound 1

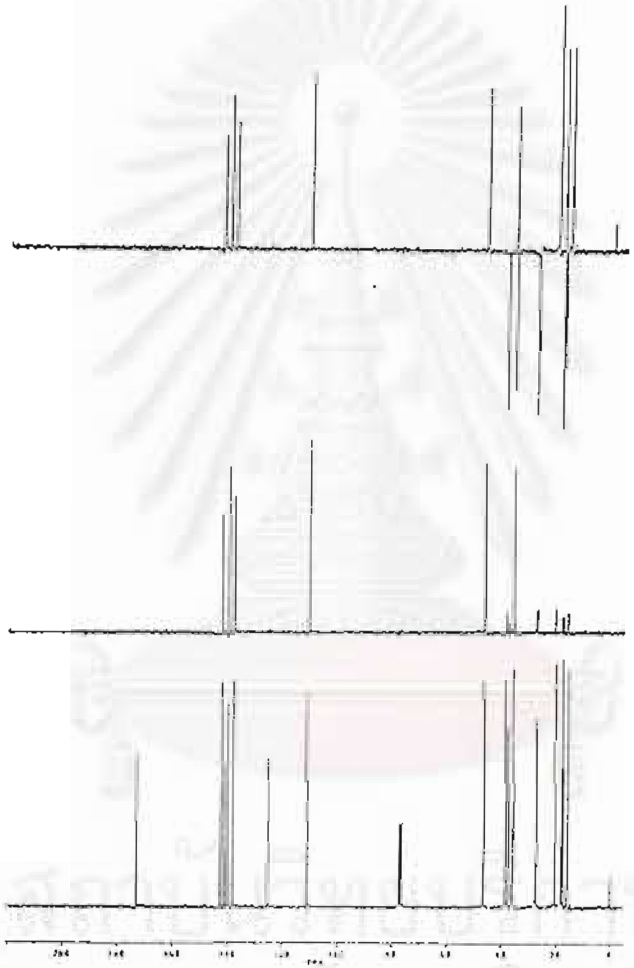

Figure 23. DEPT-135, 90 ^{13}C-NMR of Compound 1

64

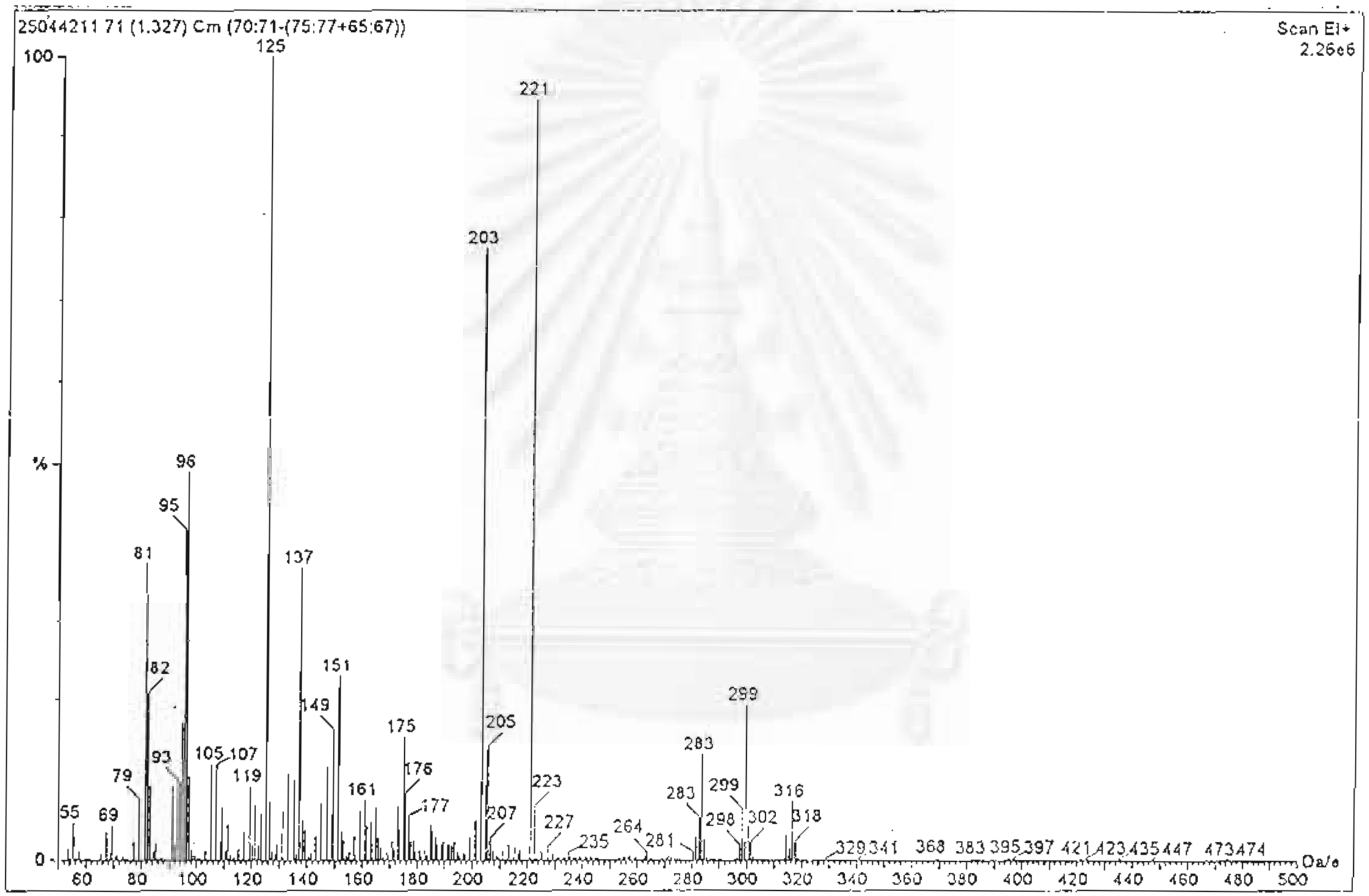

Figure 24. The EI MS spectrum of Compound 1

65

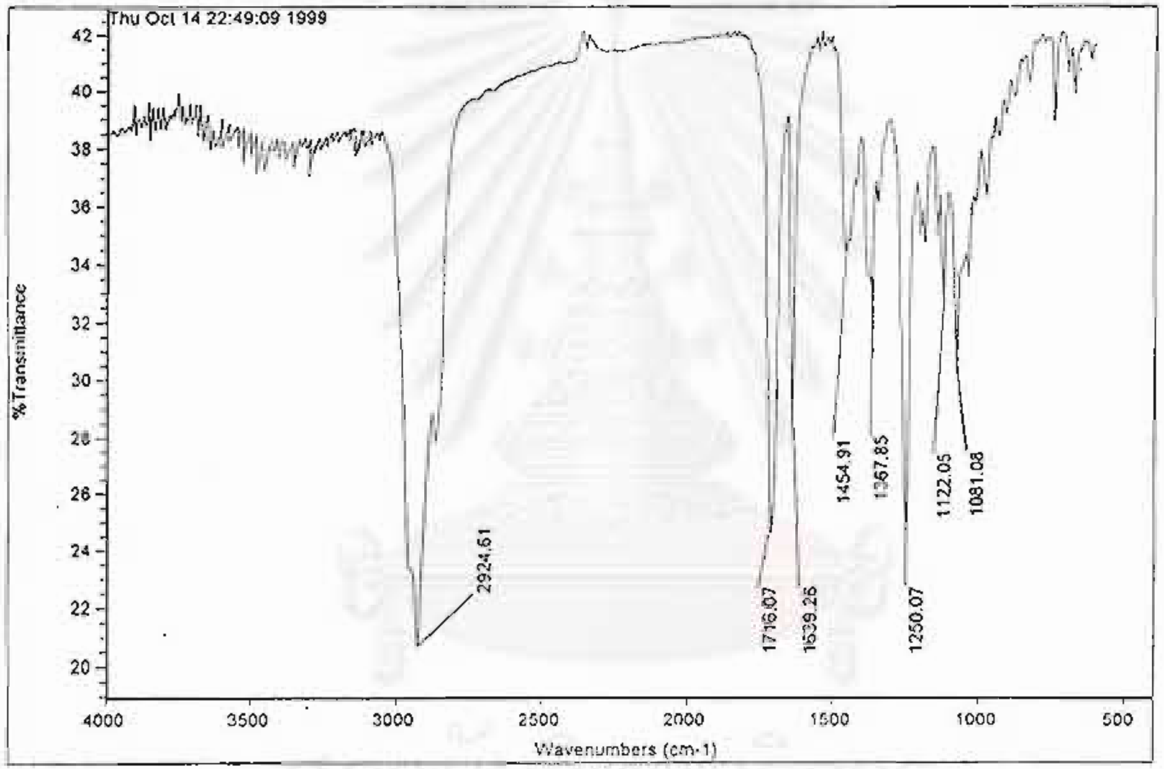

Figure 25. The IR spectrum of Compound 2

66

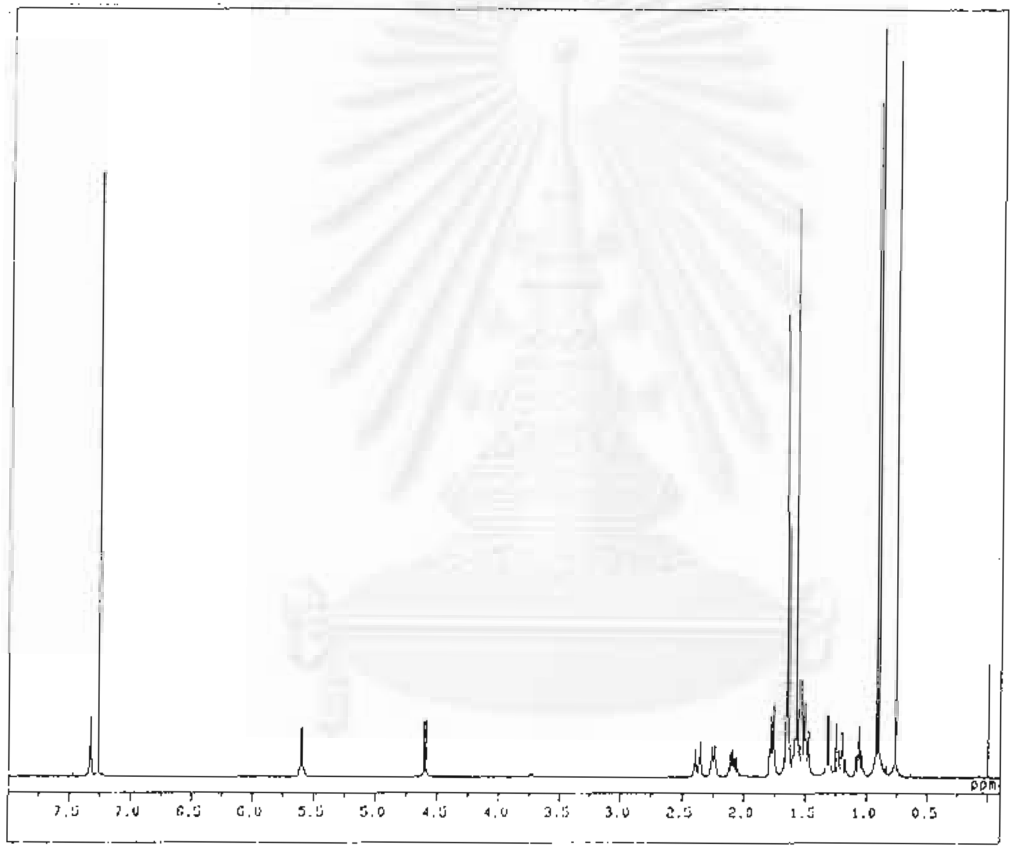

Figure 26. The ^{1}H-NMR spectrum of Compound 2

67

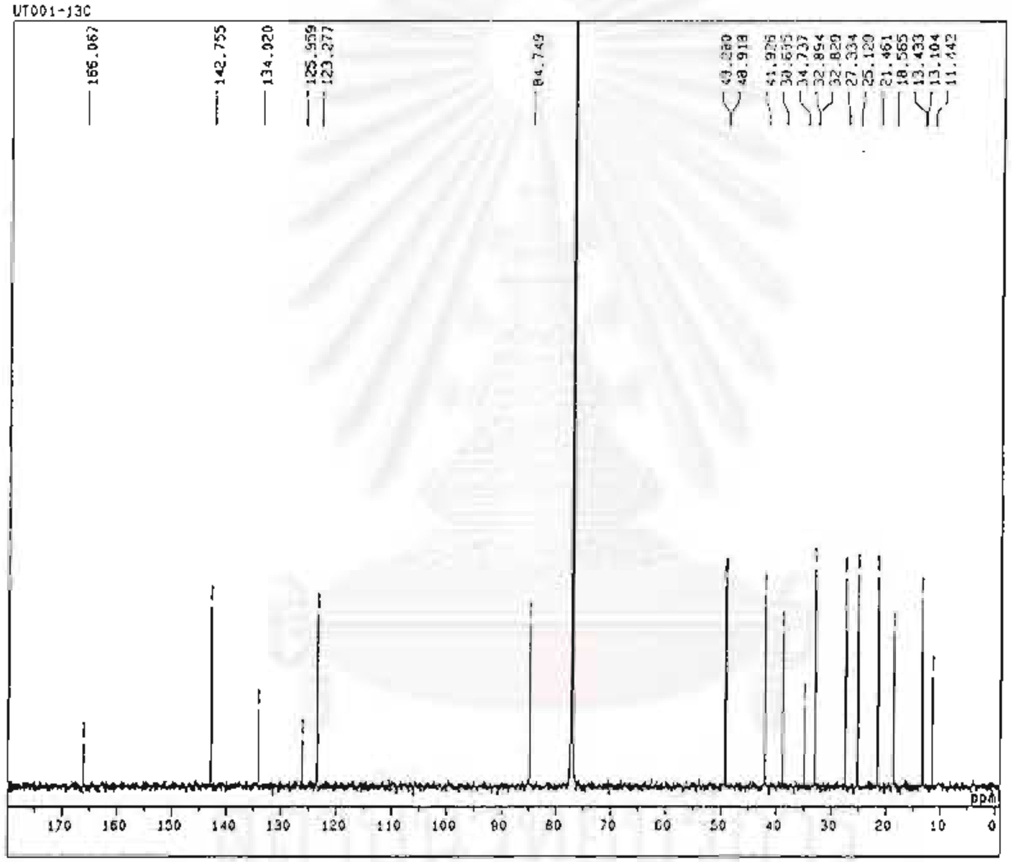

Figure 27. The ^{13}C-NMR spectrum of Compound 2

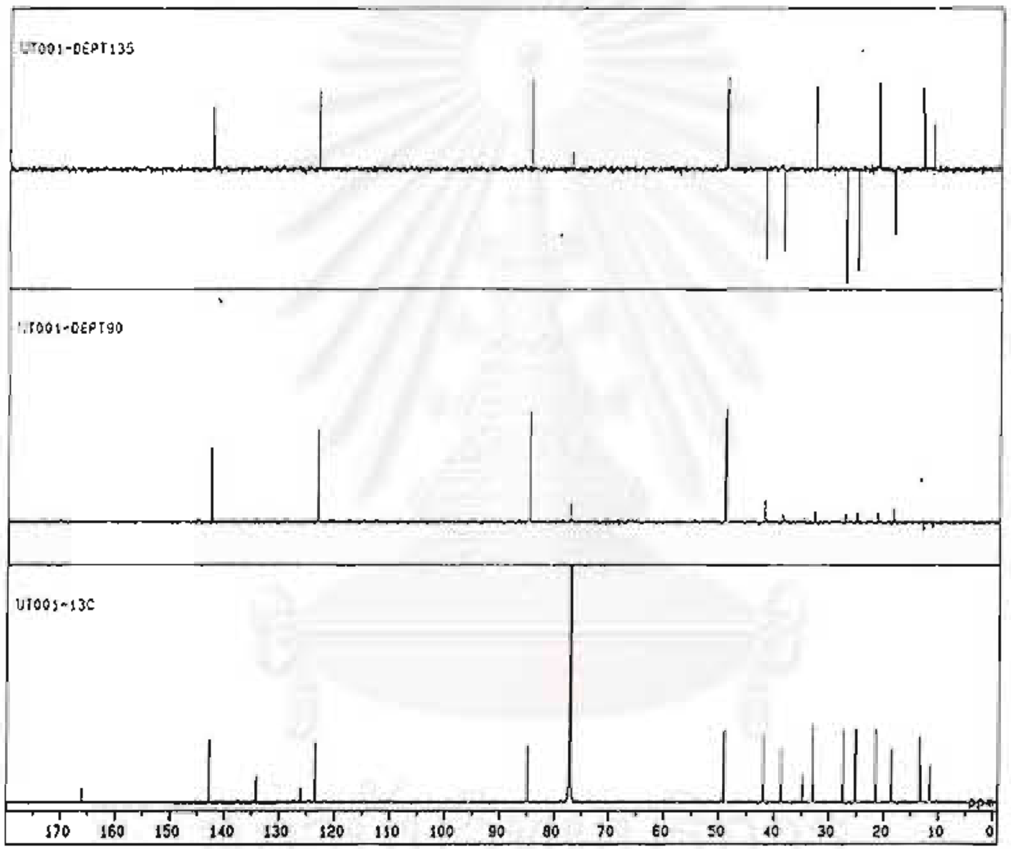

Figure 28. DEPT-135, 90 ^{13}C-NMR of Compound 2

69

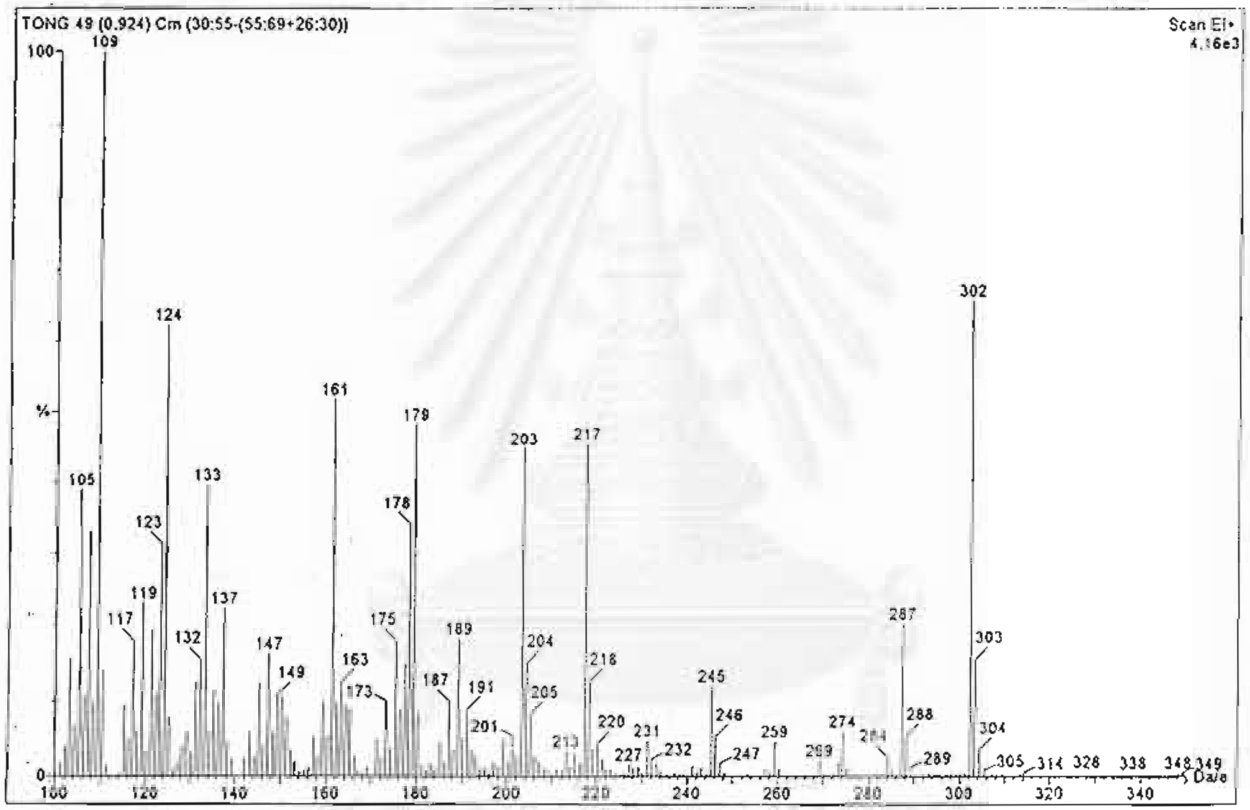

Figure 29. The EI MS spectrum of Compound $\underline{2}$

70

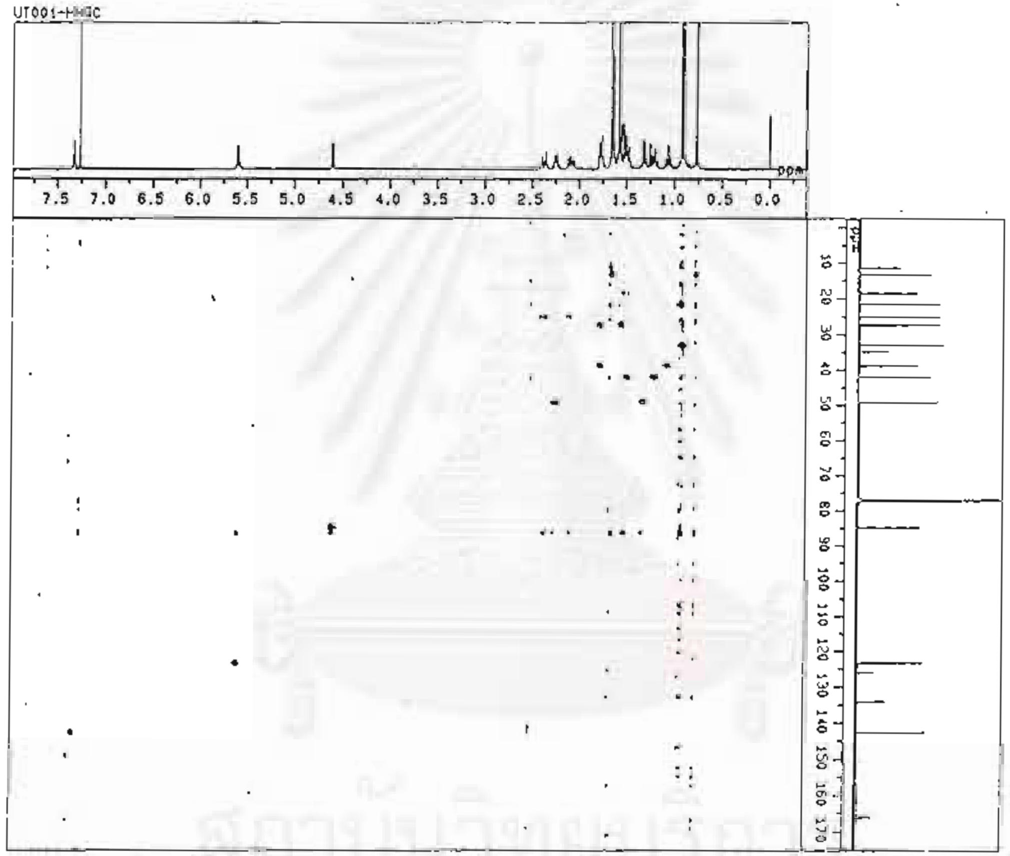

Figure 30. The HMQC-NMR spectrum of Compound 2

71

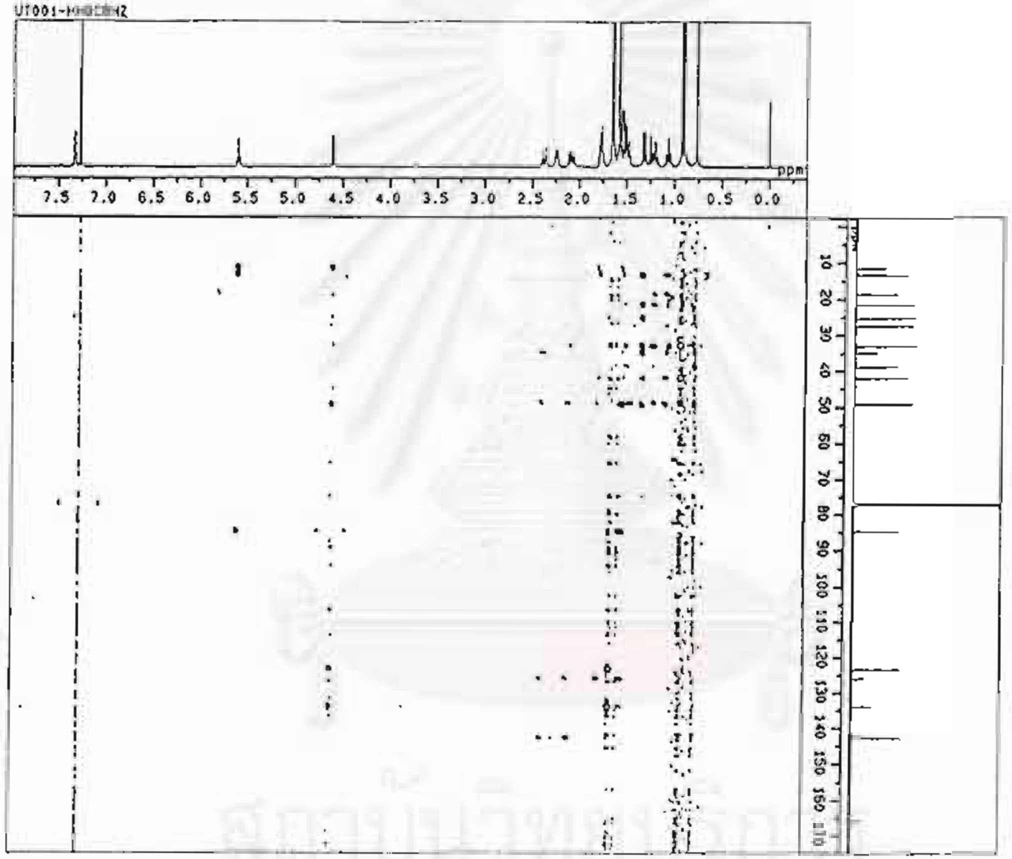

Figure 31. The HMBC-NMR spectrum of Compound 2

72

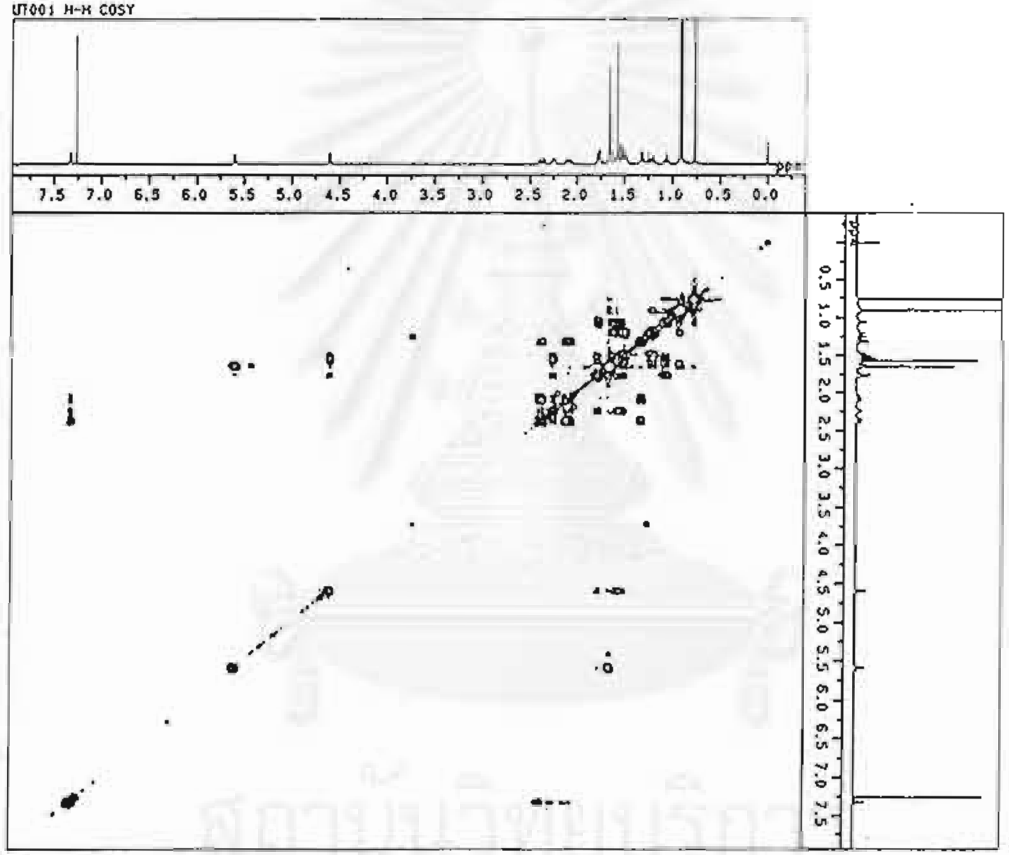

Figure 32. The COSY-NMR spectrum of Compound 2

73

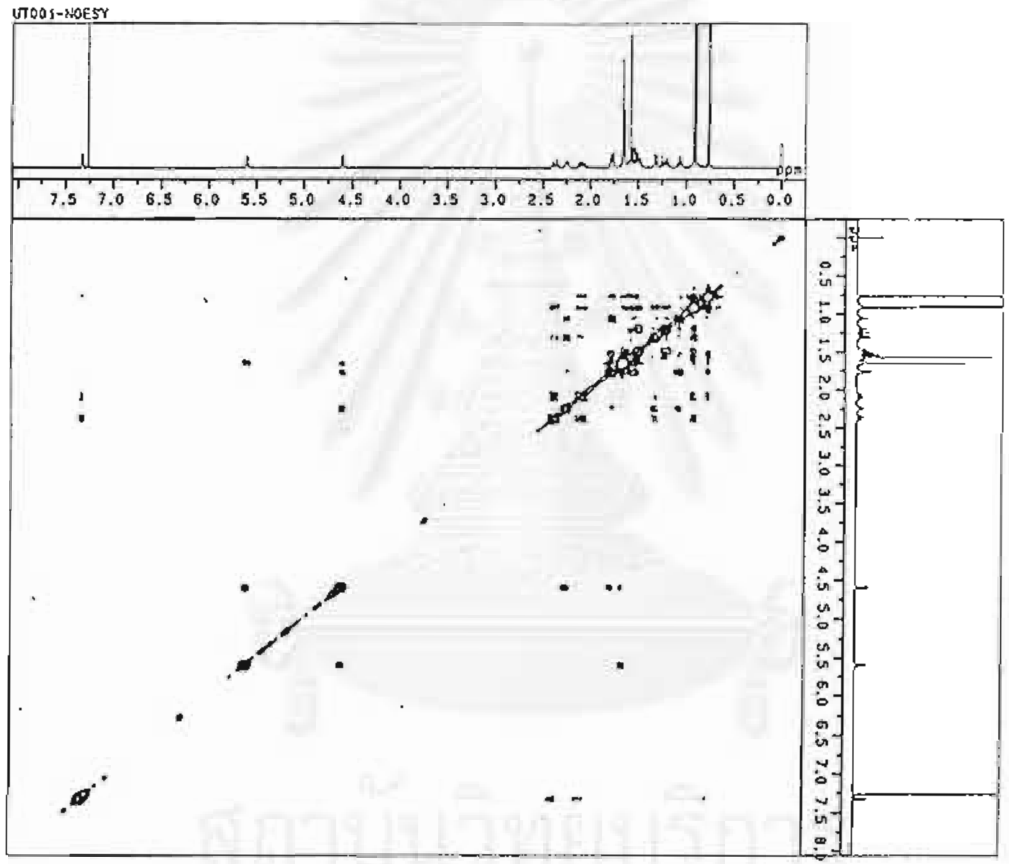

Figure 33. The NOESY-NMR spectrum of Compound 2

74

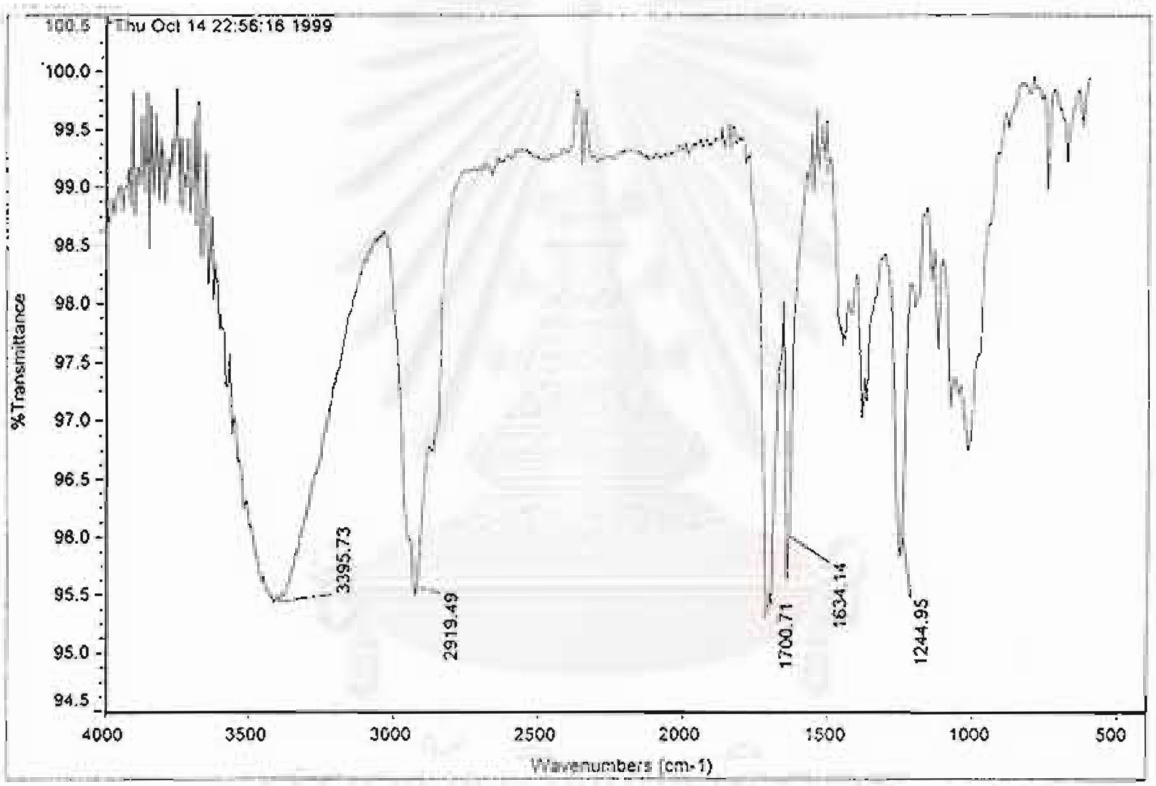

Figure 34. The IR spectrum of Compound 3

75

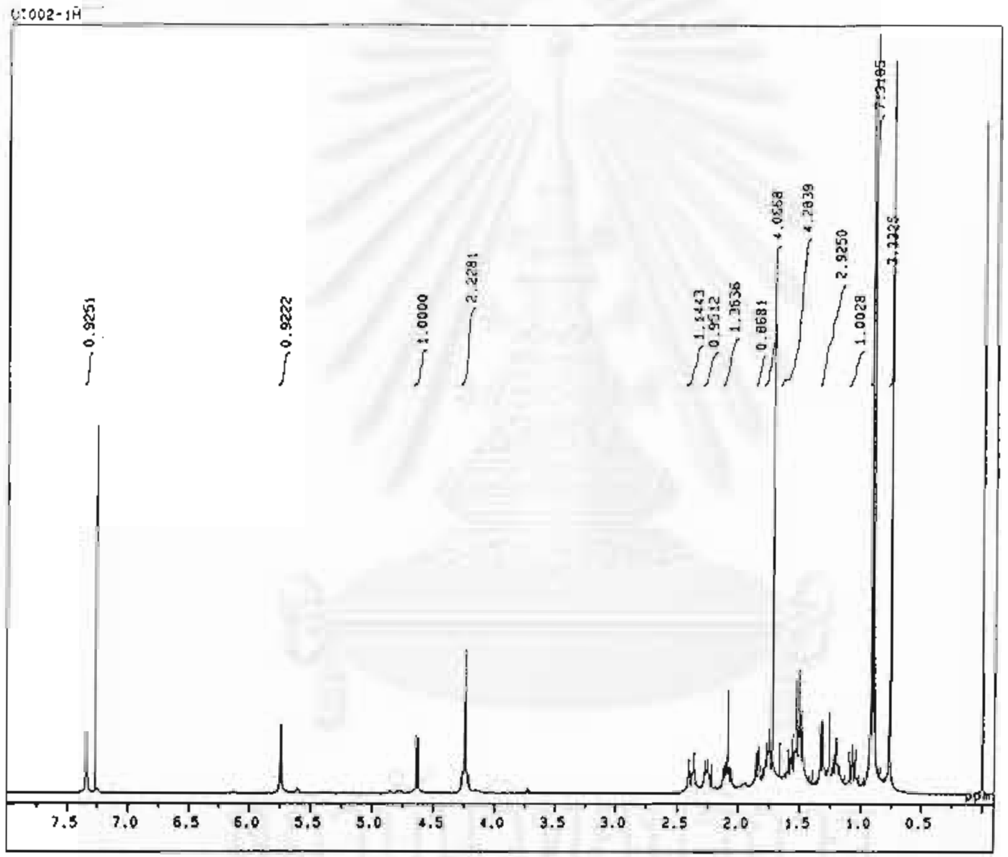

Figure 35. The [1]H-NMR spectrum of Compound 3

76

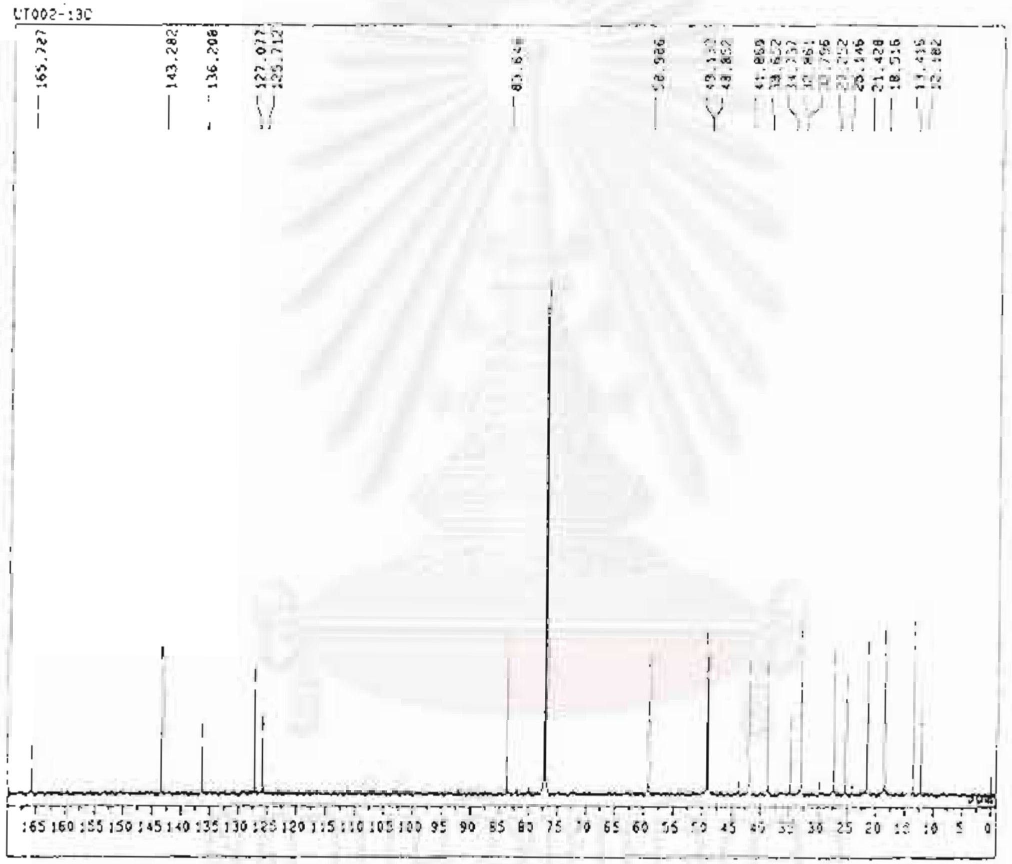

Figure 36. The ^{13}C-NMR spectrum of Compound 3

77

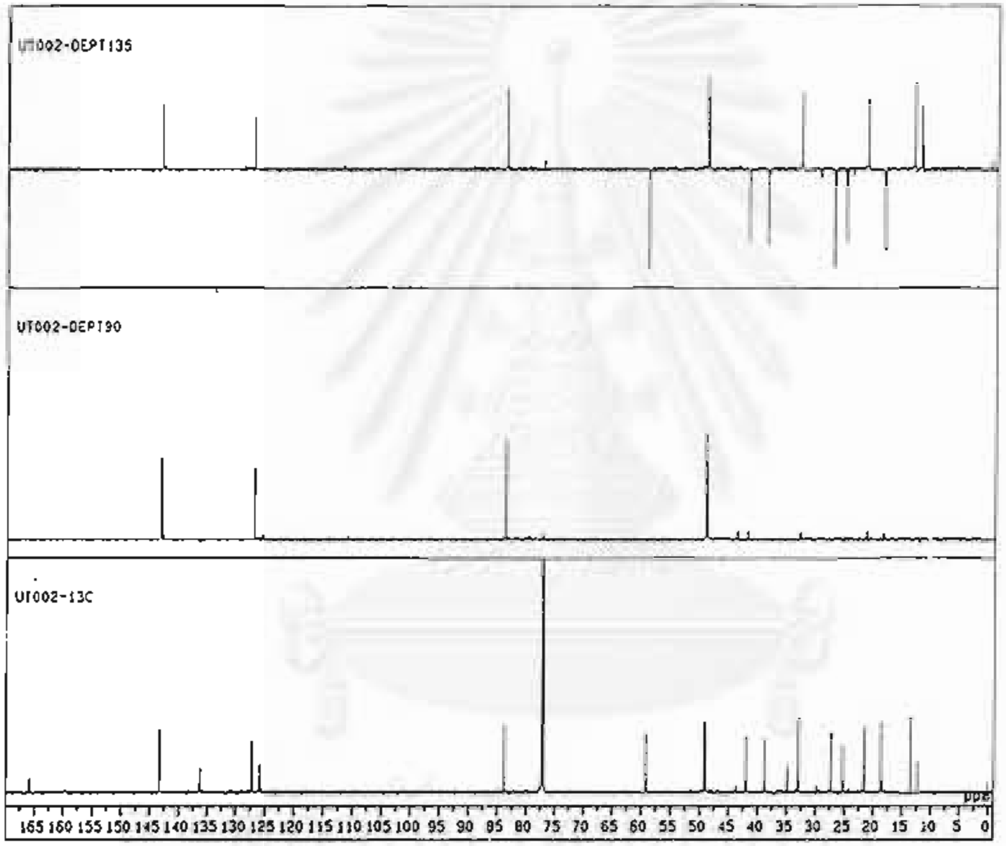

Figure 37. DEPT-135, 90 ^{13}C-NMR of Compound 3

78

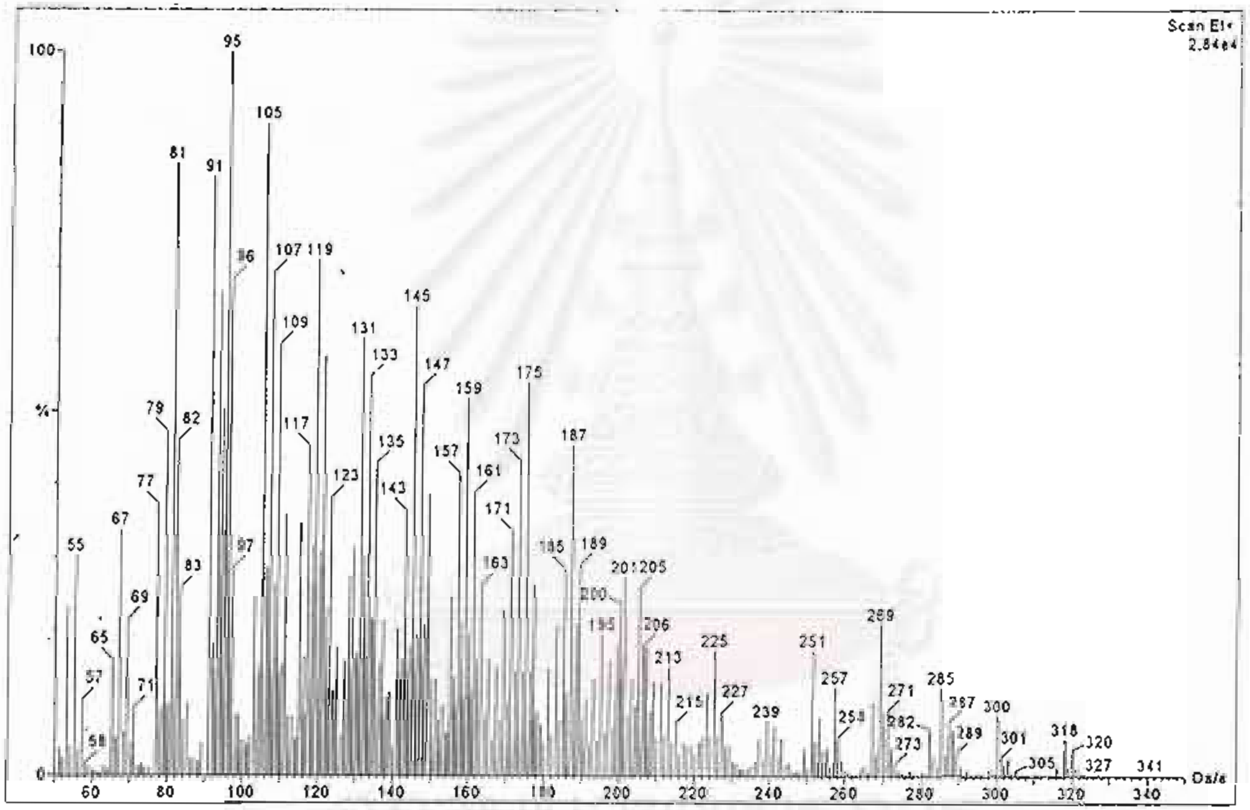

Figure 38. The EI MS spectrum of Compound 3

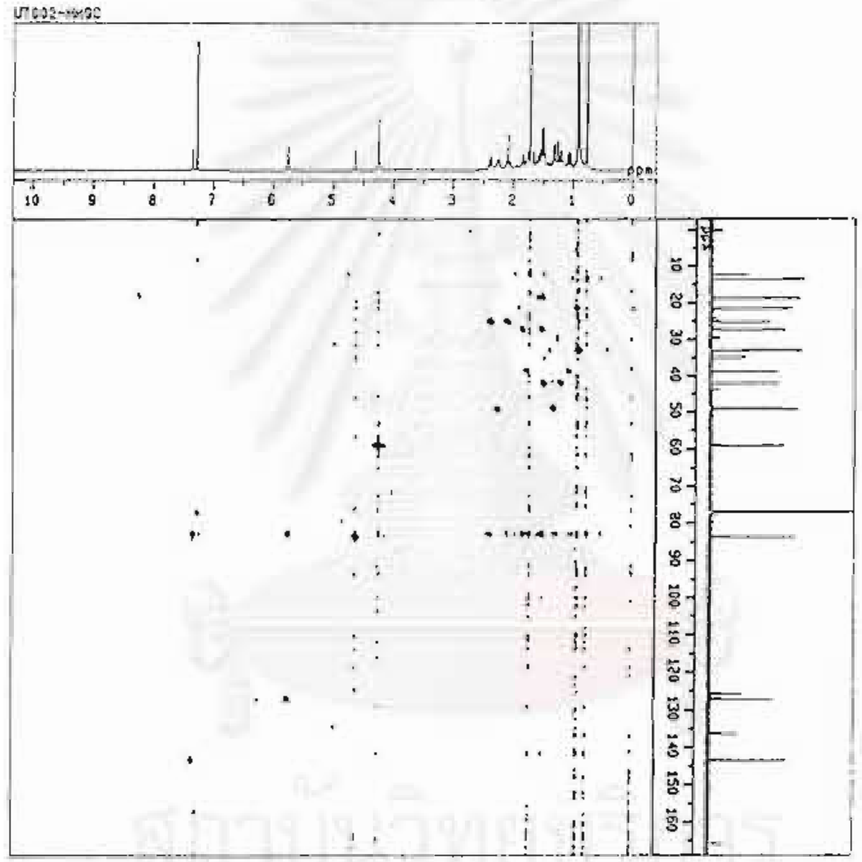

Figure 39. The HMQC-NMR spectrum of Compound 3

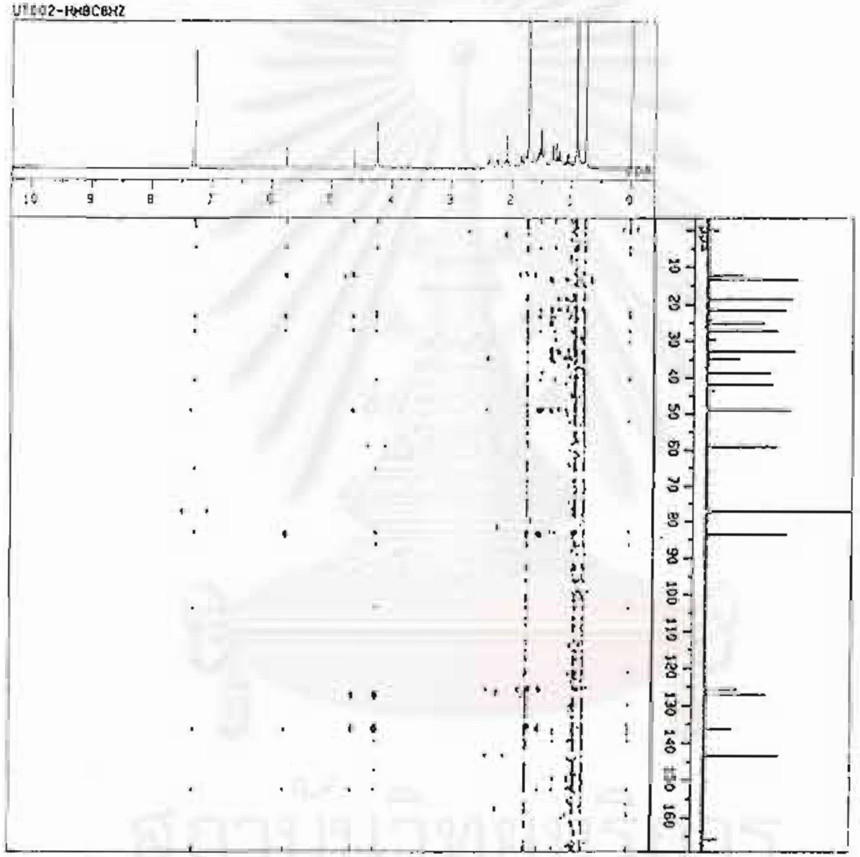

Figure 40. The HMBC-NMR spectrum of Compound 3

81

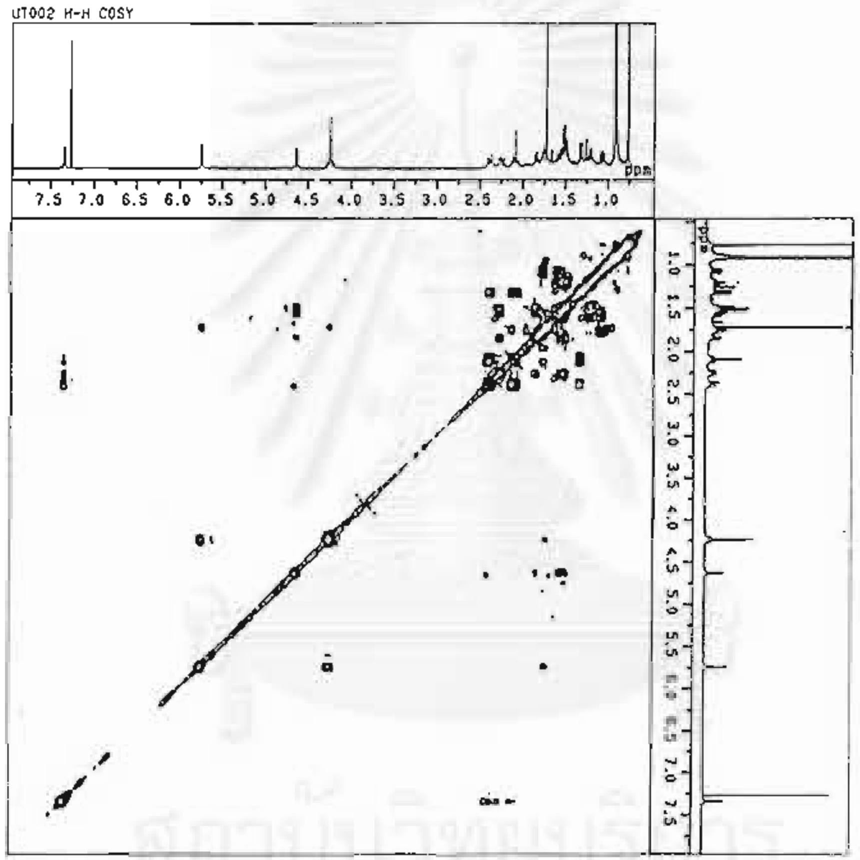

Figure 41. The COSY-NMR spectrum of Compound 3

82

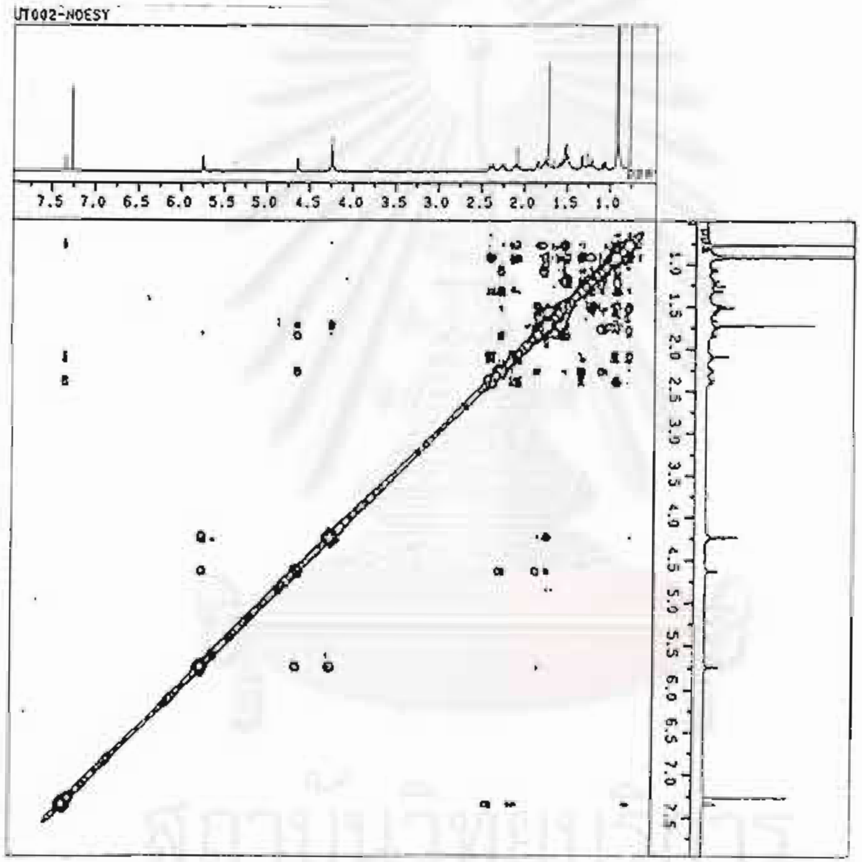

Figure 42. The NOESY-NMR spectrum of Compound 3

83

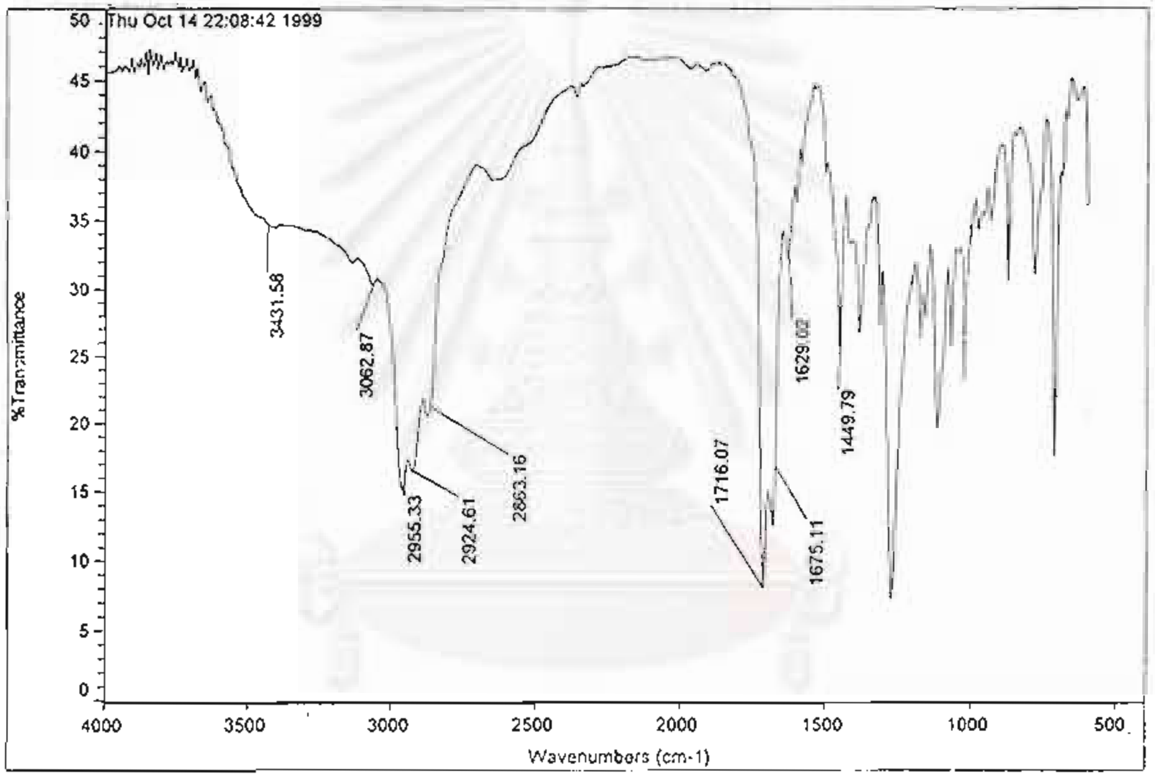

Figure 43. The IR spectrum of Compound 4

84

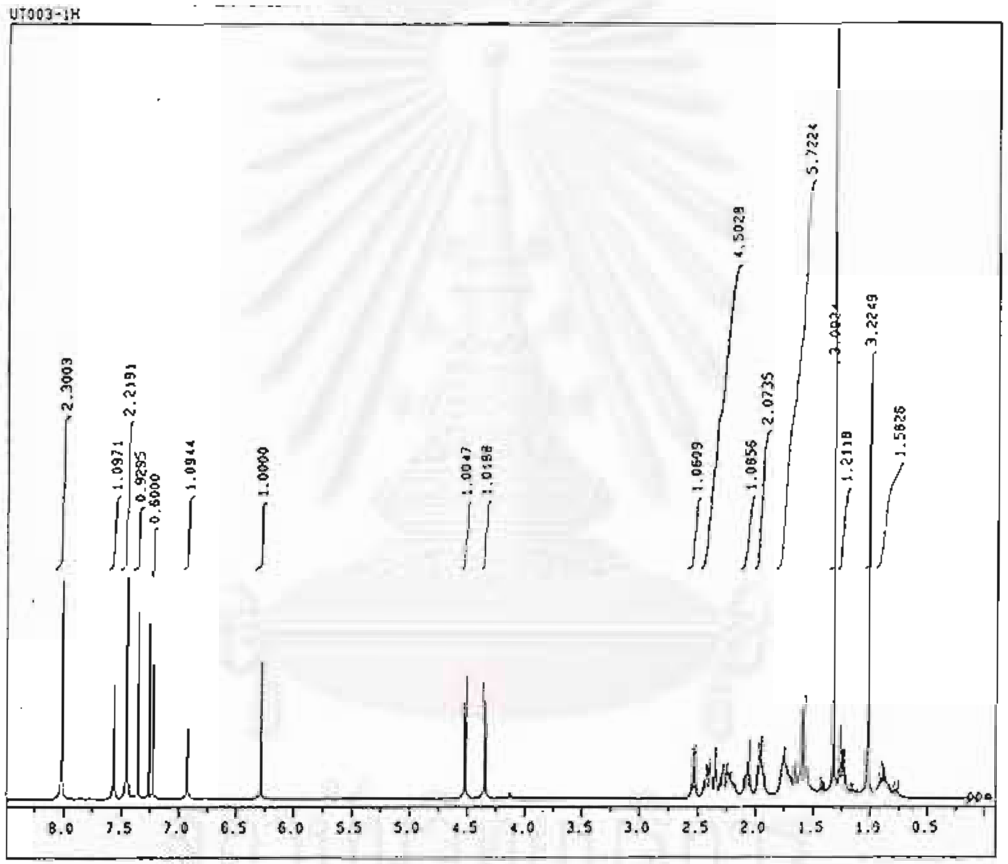

Figure 44. The ^{1}H-NMR spectrum of Compound 4

85

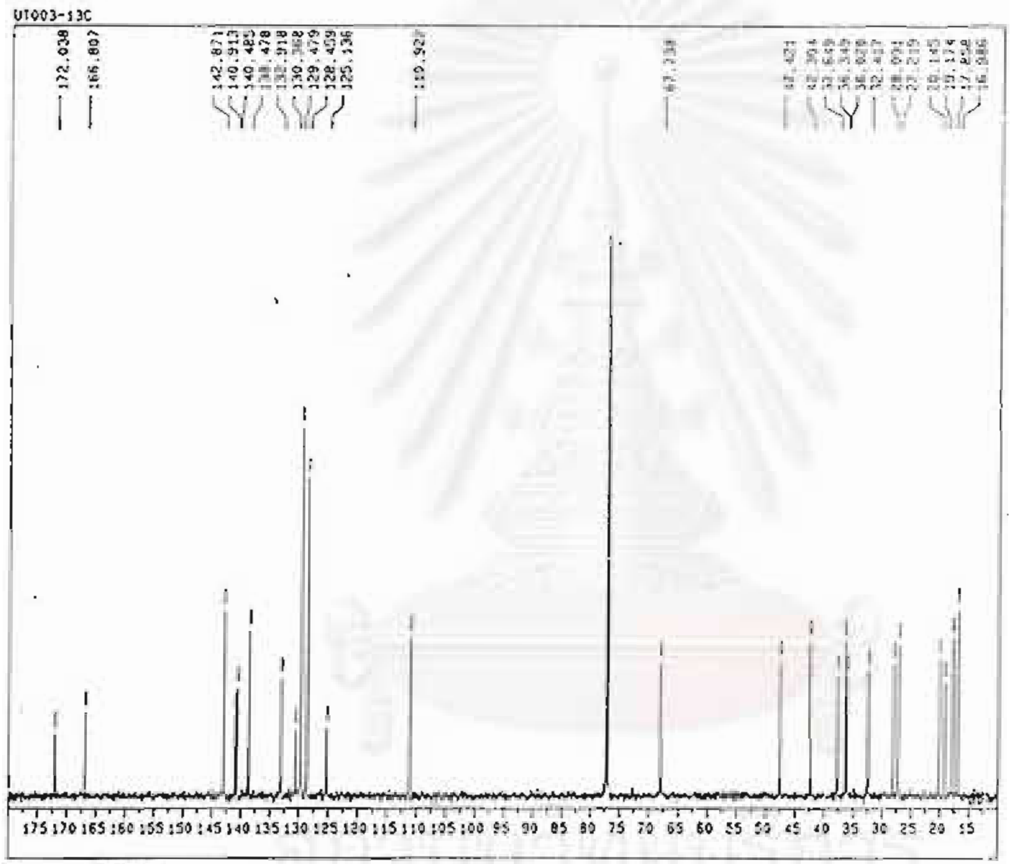

Figure 45. The ^{13}C-NMR spectrum of Compound 4

86

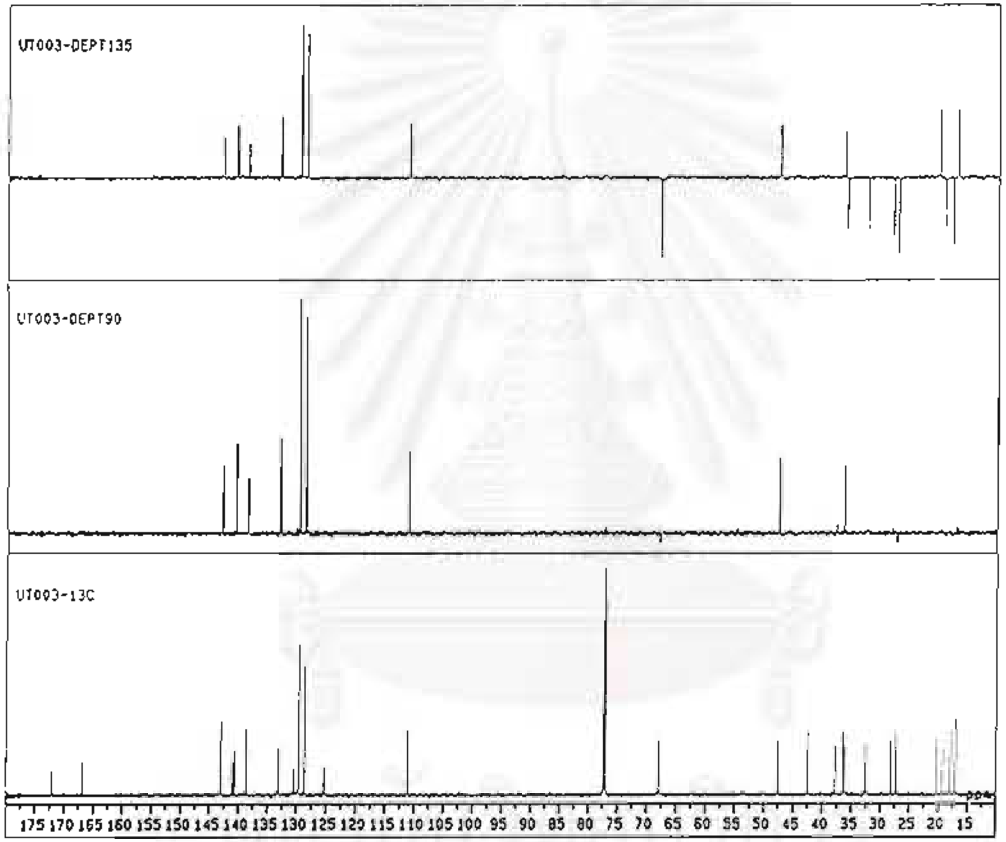

Figure 46. DEPT-135, 90 ^{13}C-NMR of Compound 4

87

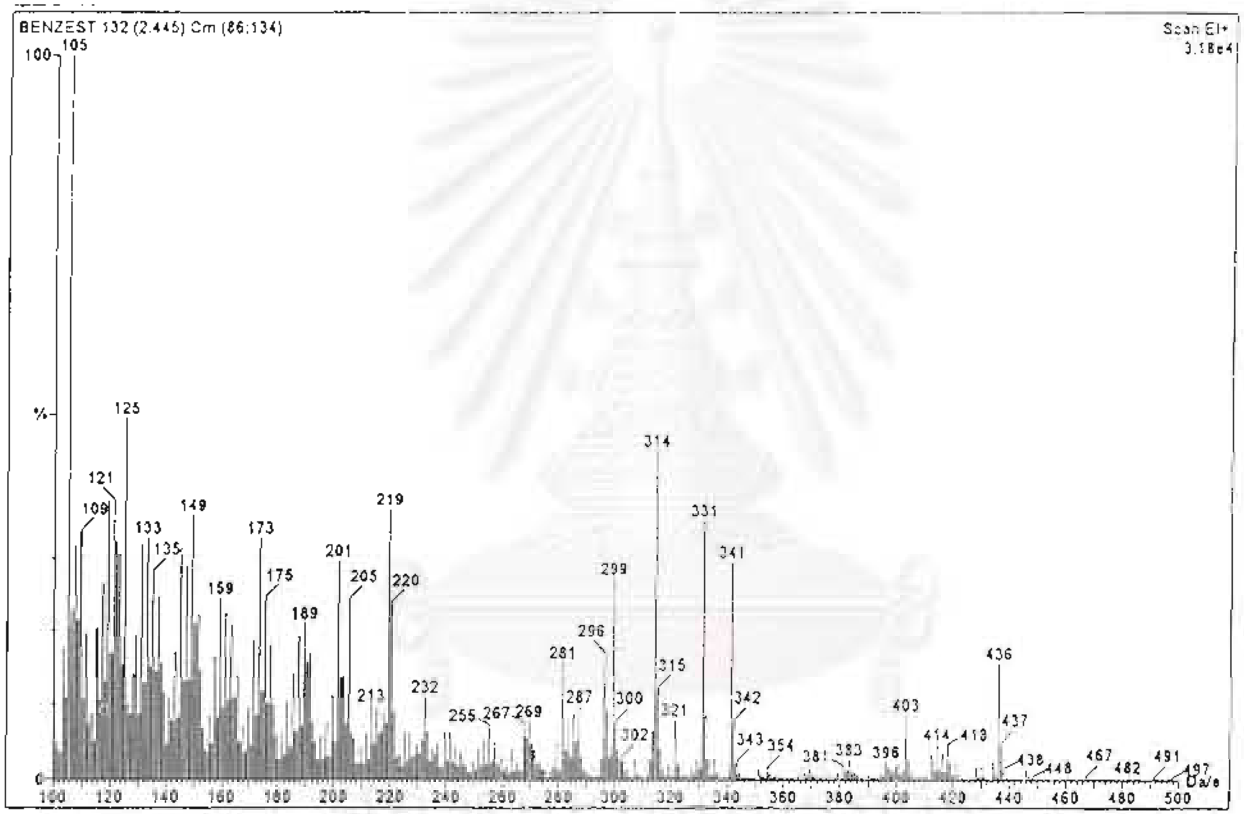

Figure 47. The EI MS spectrum of Compound 4

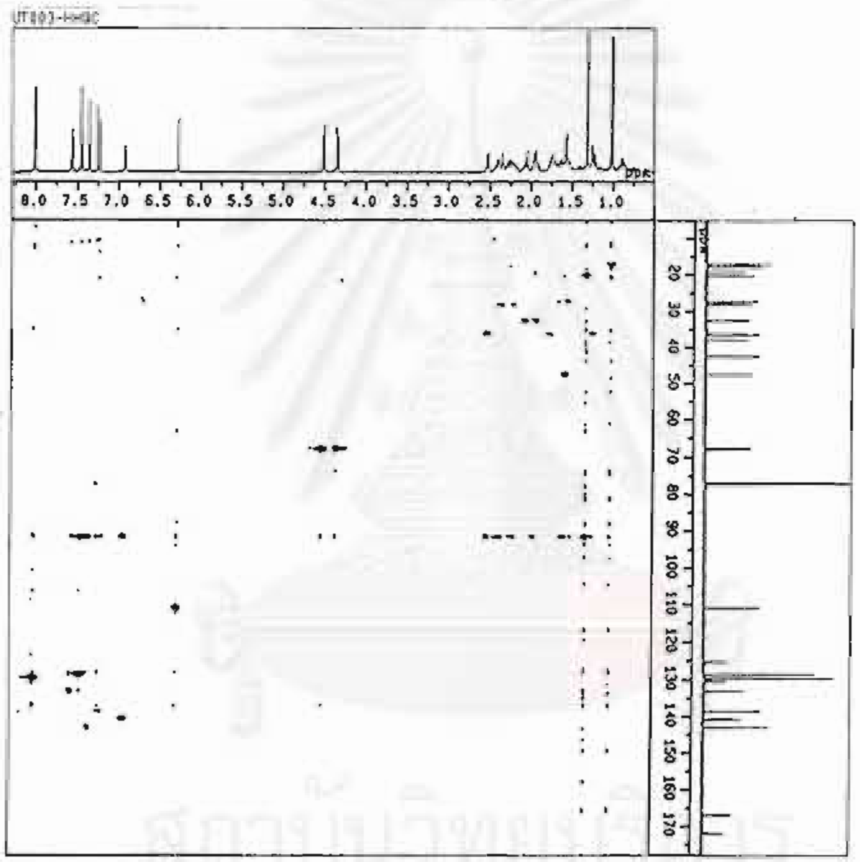

Figure 48. The HMQC-NMR spectrum of Compound 4

89

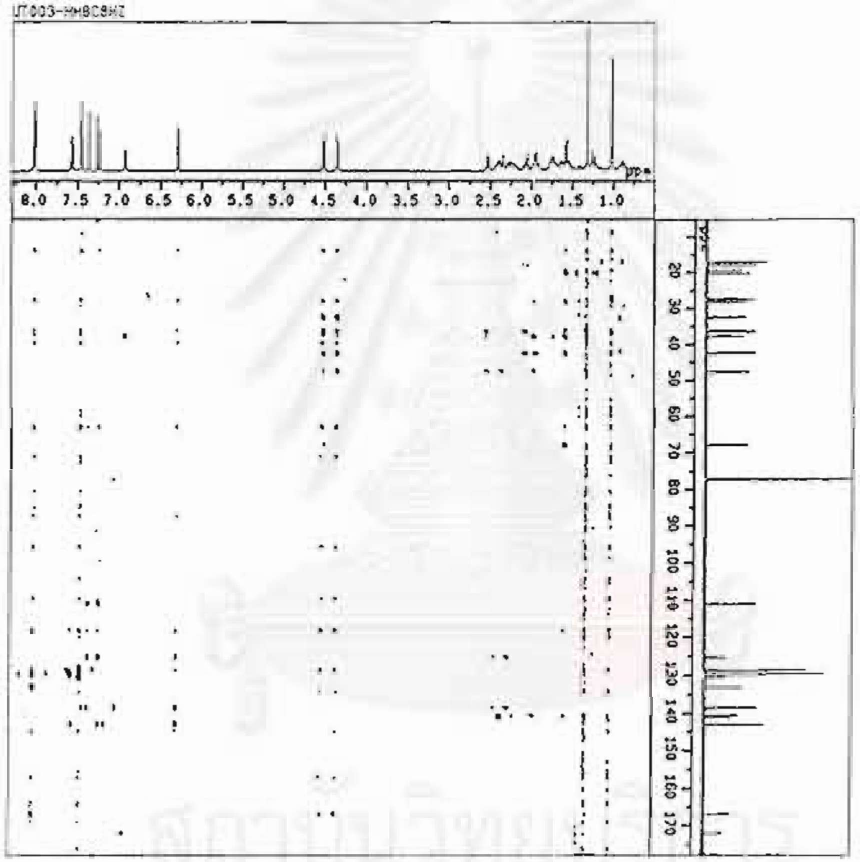

Figure 49. The HMBC-NMR spectrum of Compound 4

90

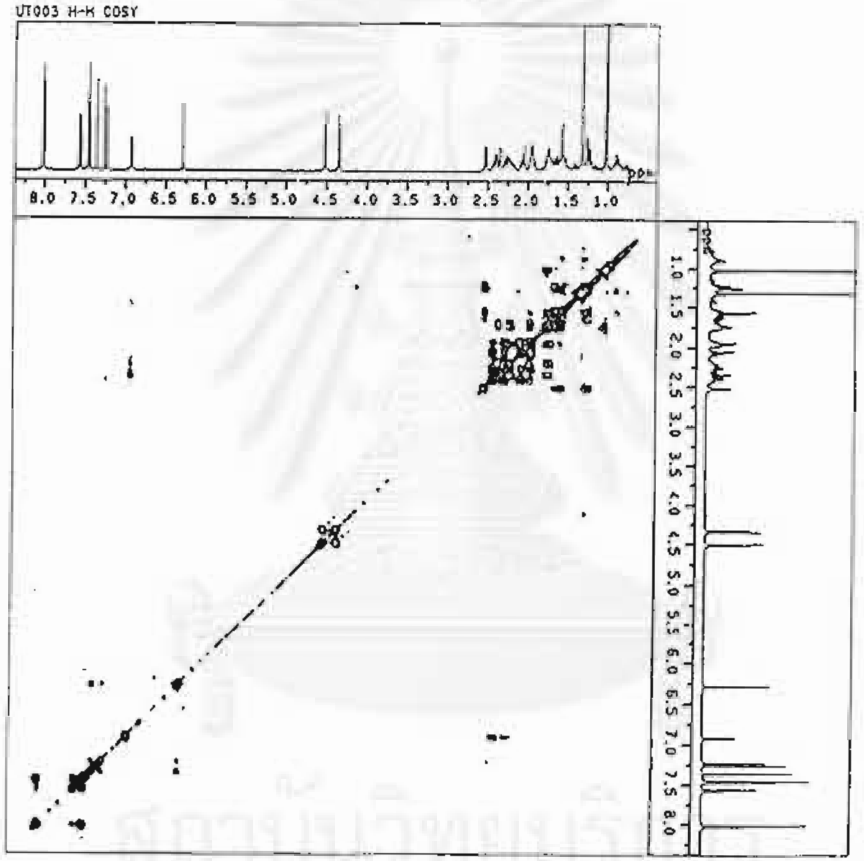

Figure 50. The COSY-NMR spectrum of Compound 4

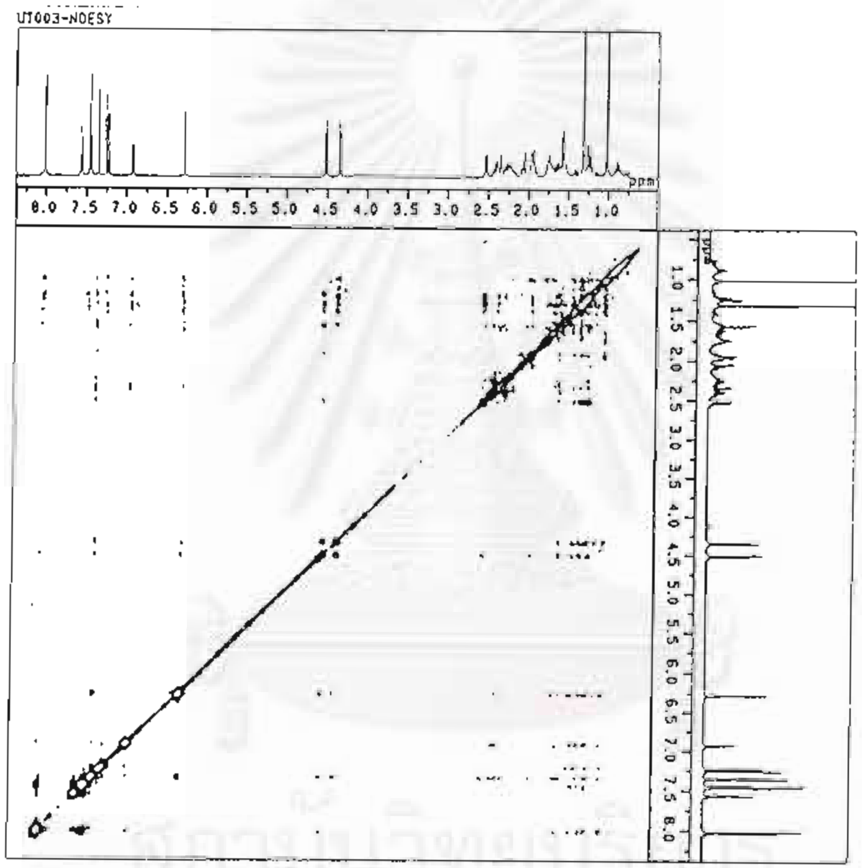

Figure 51. The NOESY-NMR spectrum of Compound 4

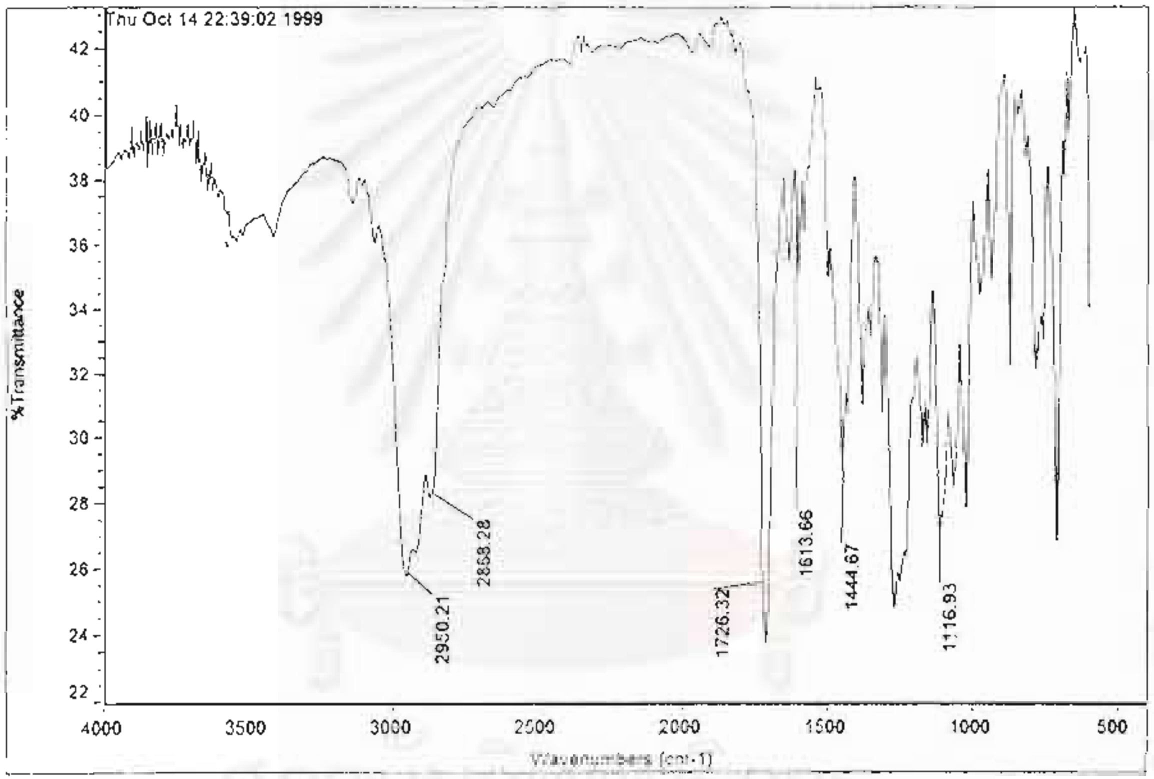

Figure 52. The IR spectrum of Compound 4a

93

94

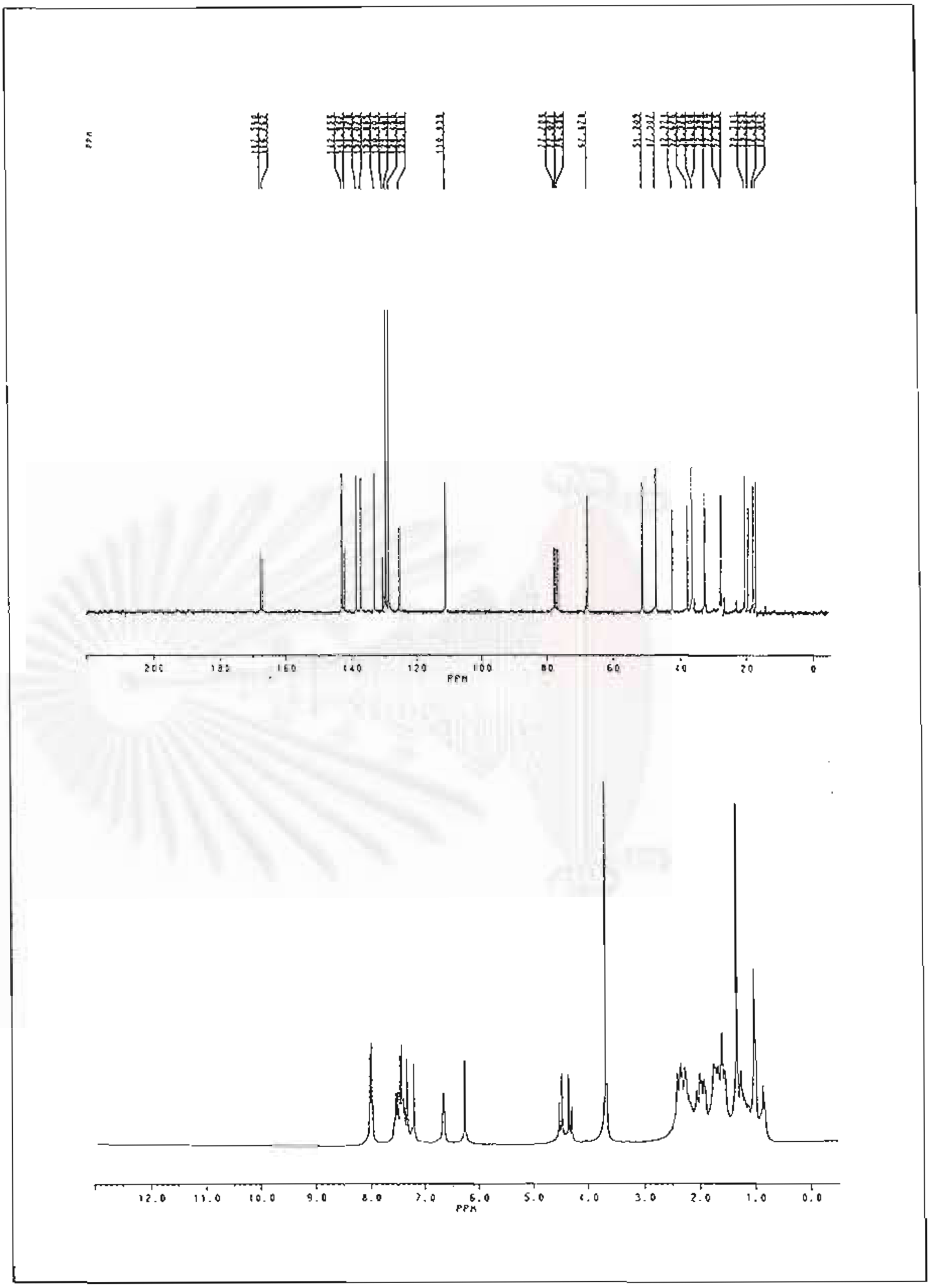

Figure 53. The ^{1}H-NMR and ^{13}C-NMR of Compound 4a

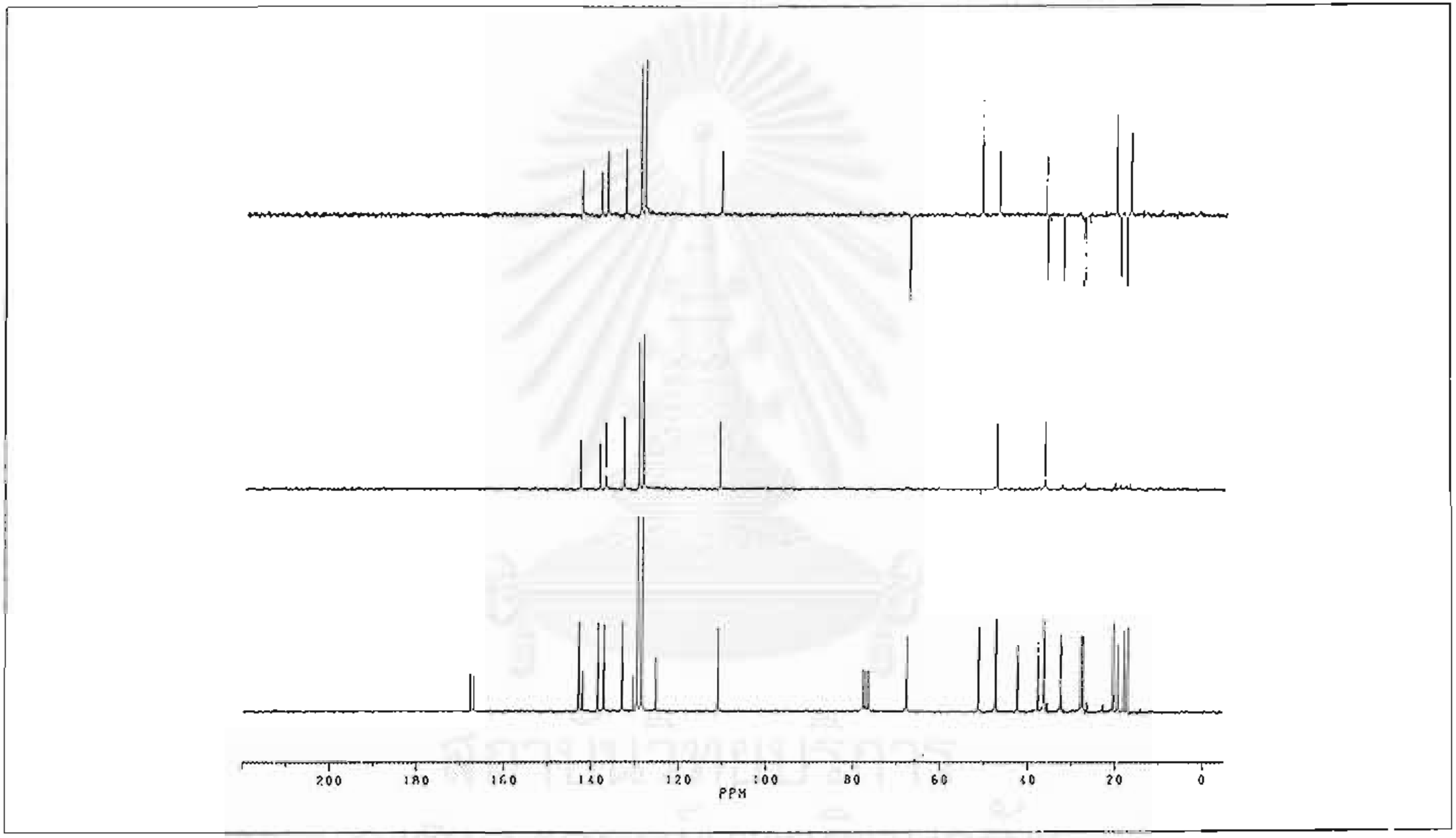

Figure 54. DEPT-135, 90 ^{13}C-NMR of Compound 4a

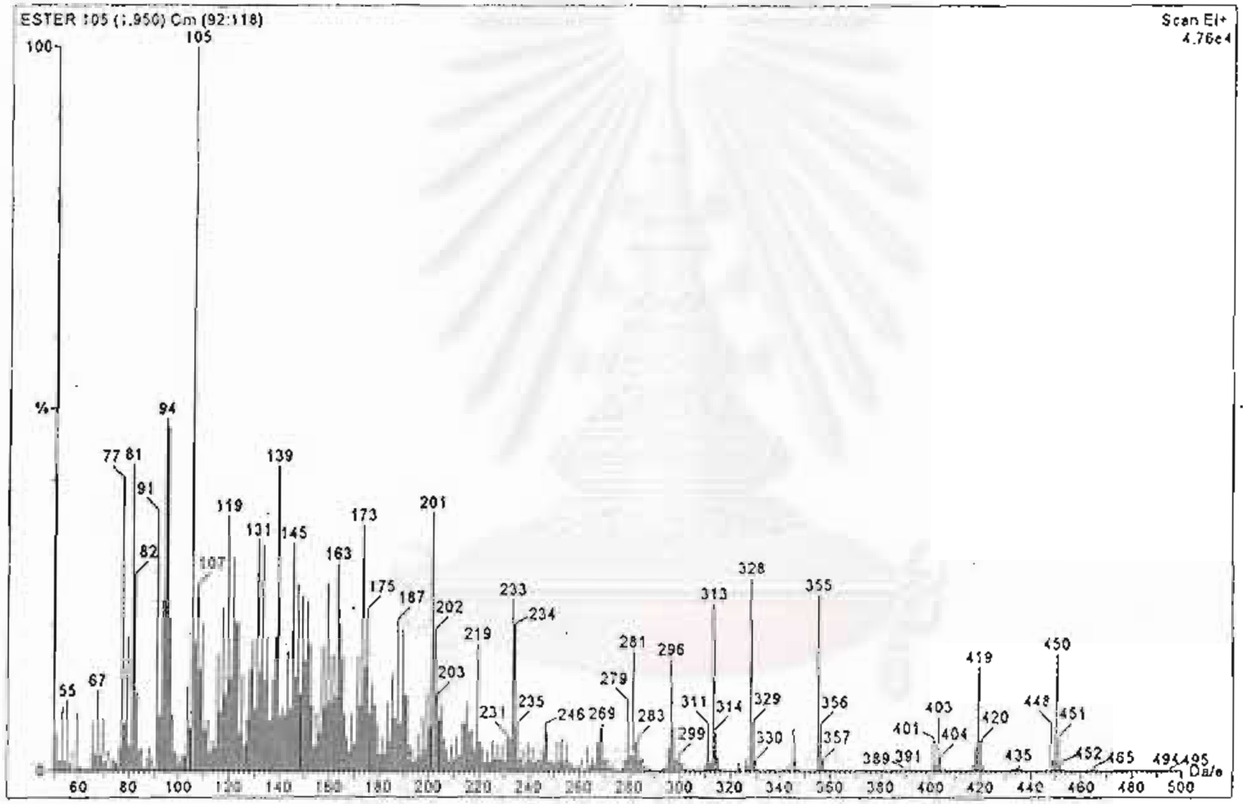

Figure 55. The EI MS spectrum of Compound 4a

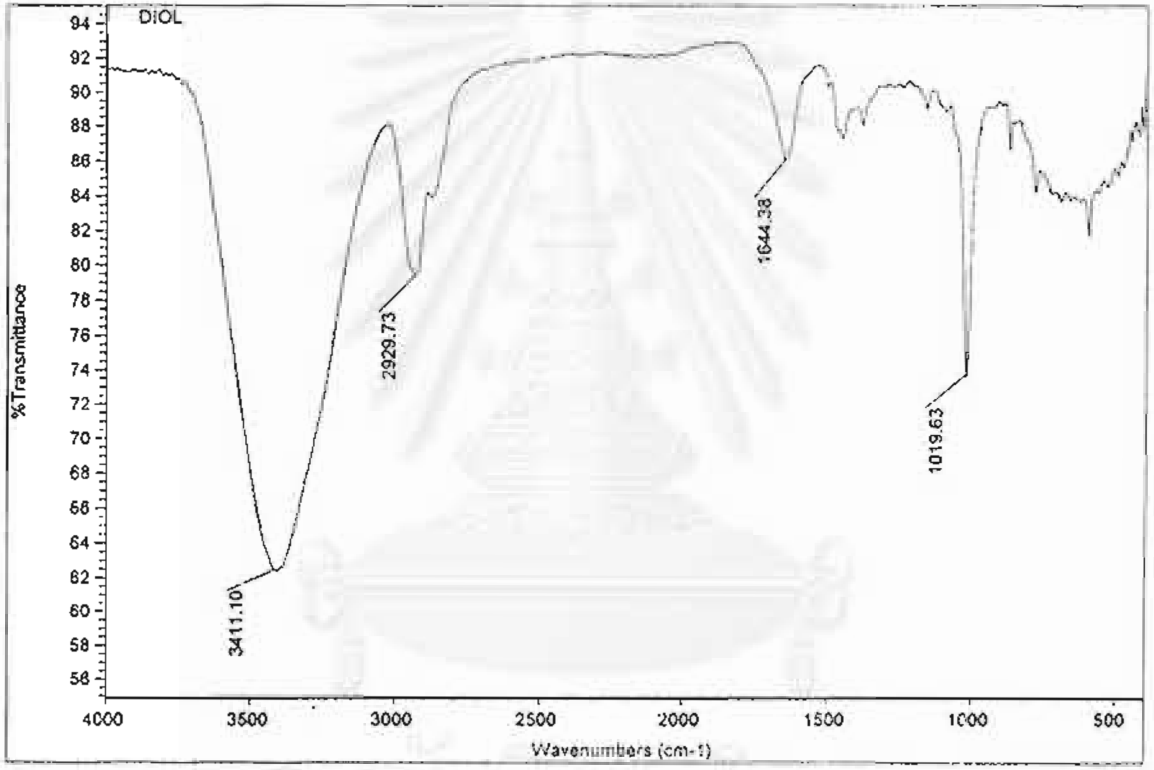

Figure 56. The IR spectrum of Compound 4b

97

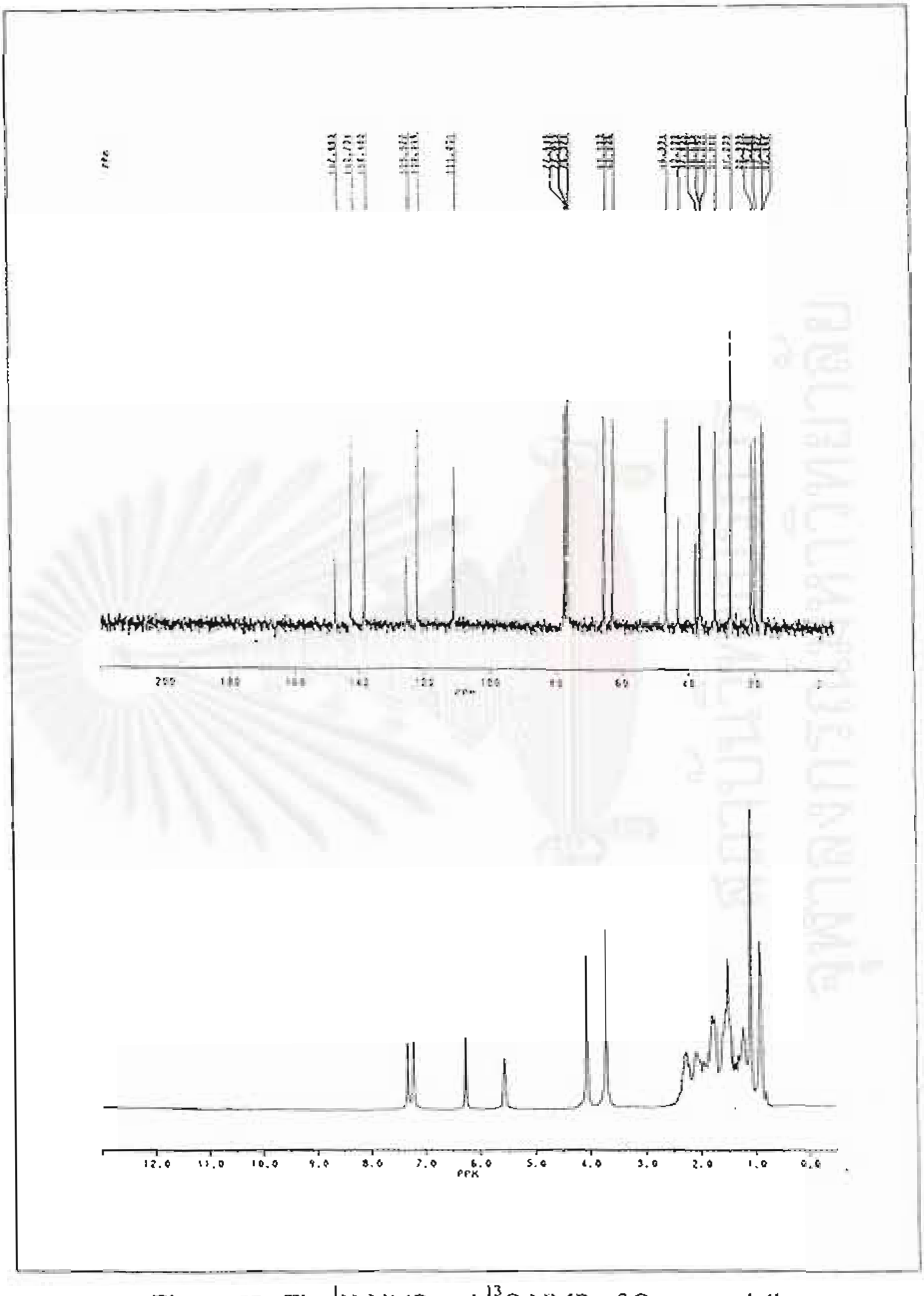

Figure 57. The [1]H-NMR and [13]C-NMR of Compound 4b

98

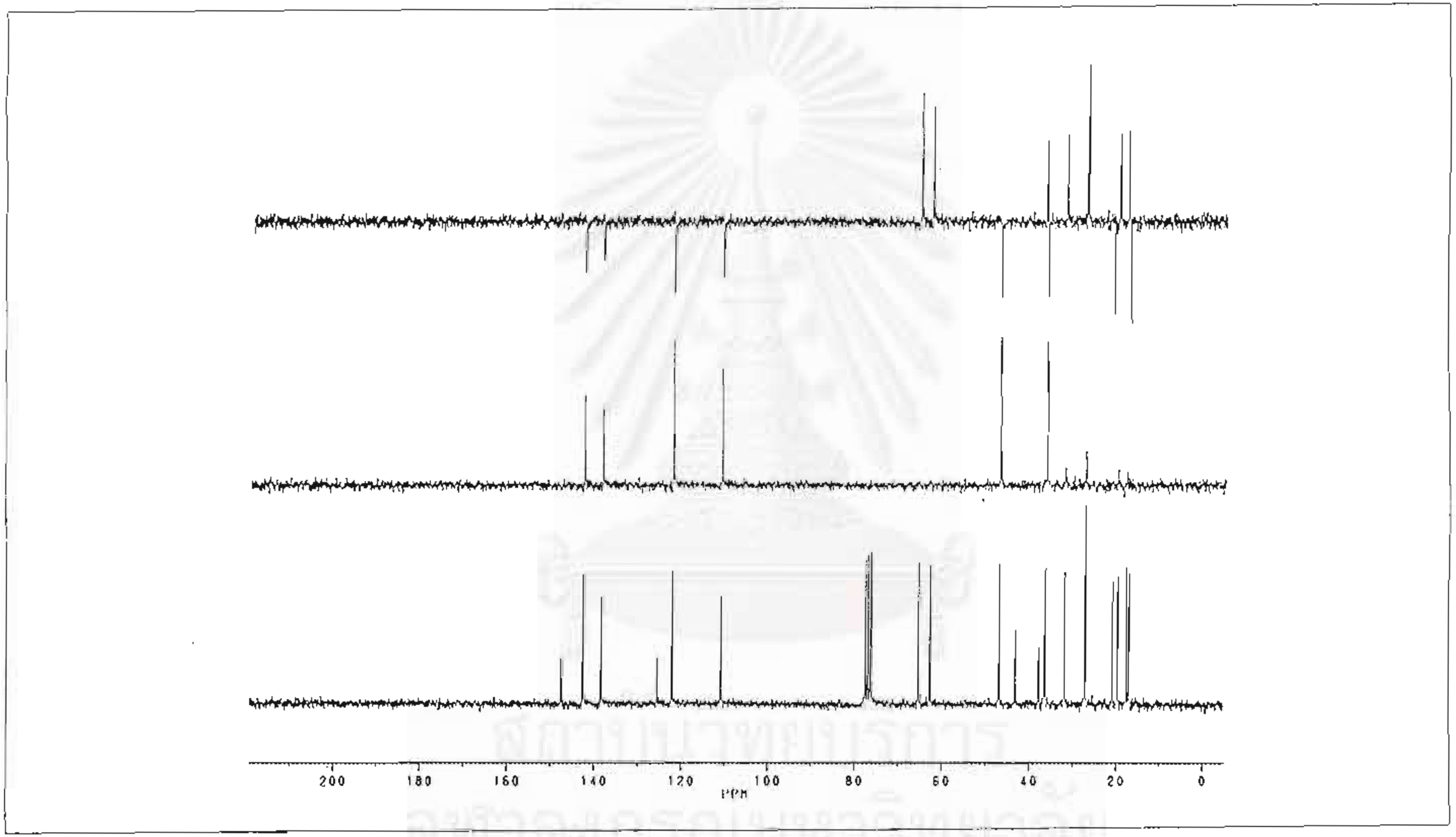

Figure 58. DEPT-135, 90 ^{13}C-NMR of Compound 4b

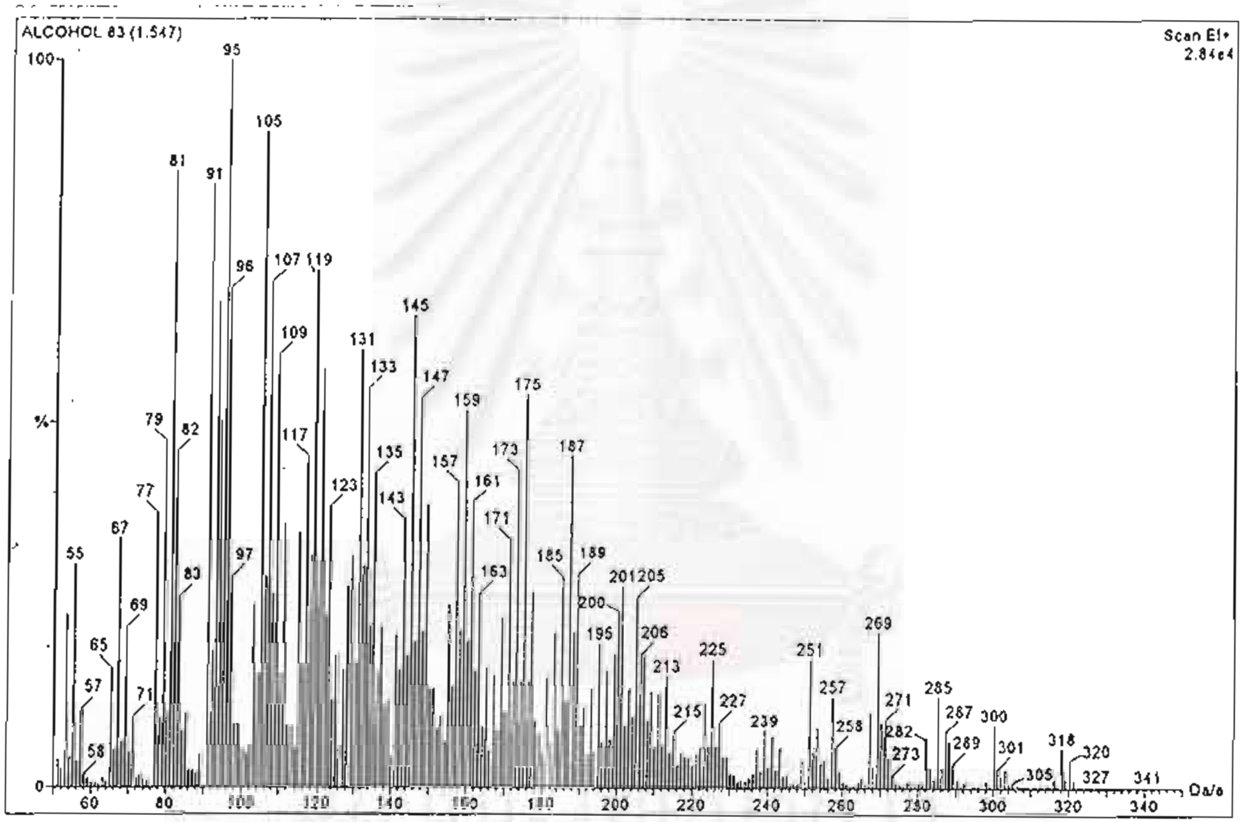

Figure 59. The EI MS spectrum of Compound 4b

100

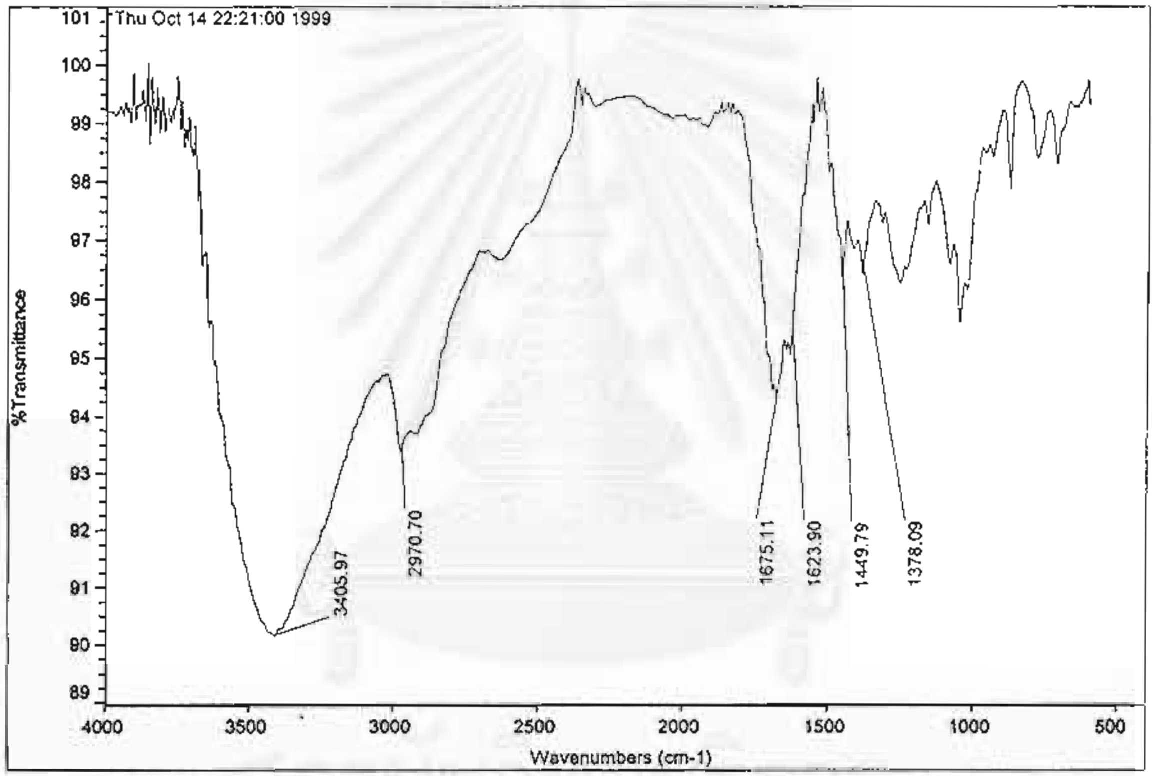

Figure 60. The IR spectrum of Compound 4c

101

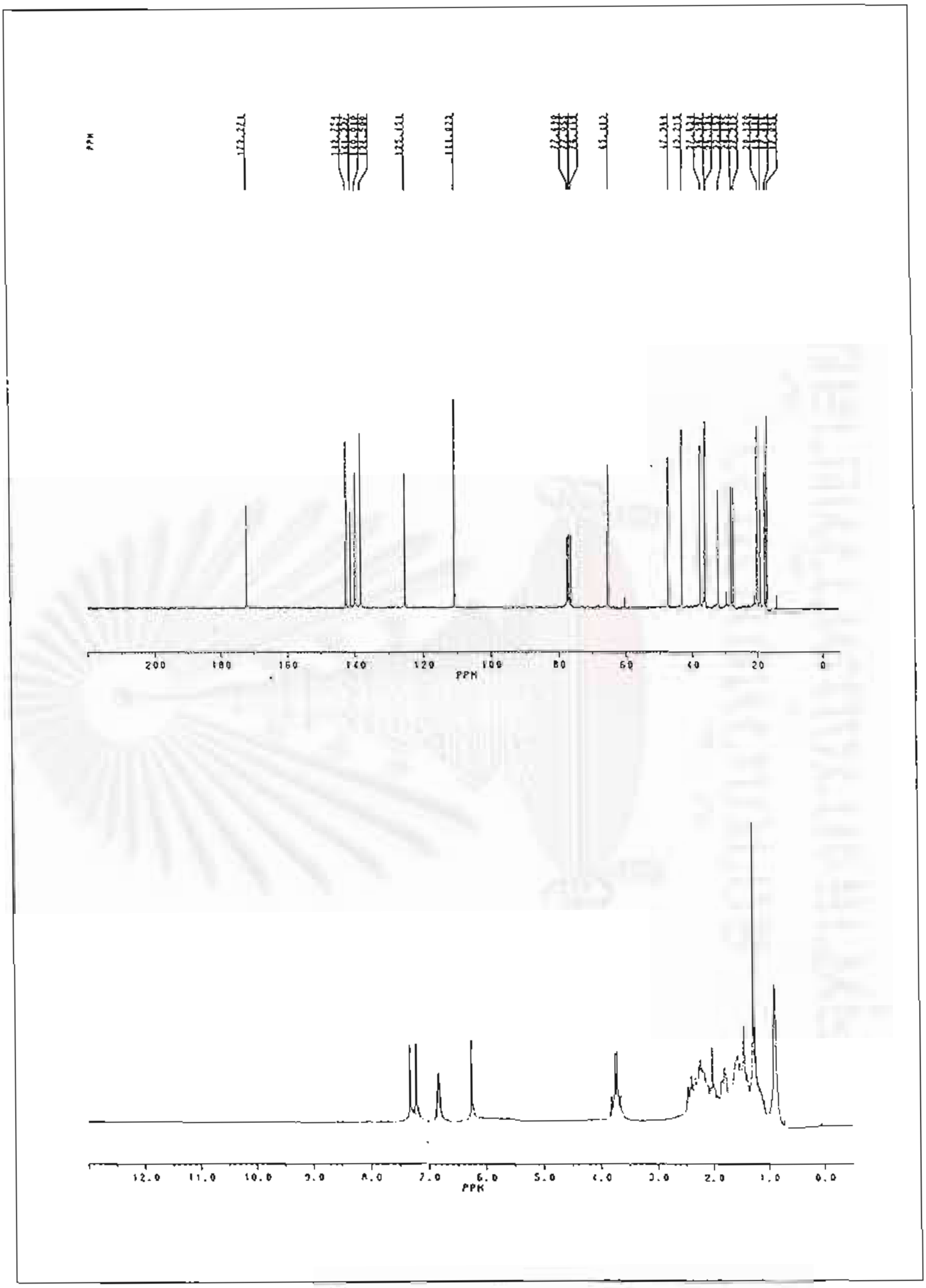

Figure 61. The ^{1}H-NMR and ^{13}C-NMR of Compound <u>4c</u>

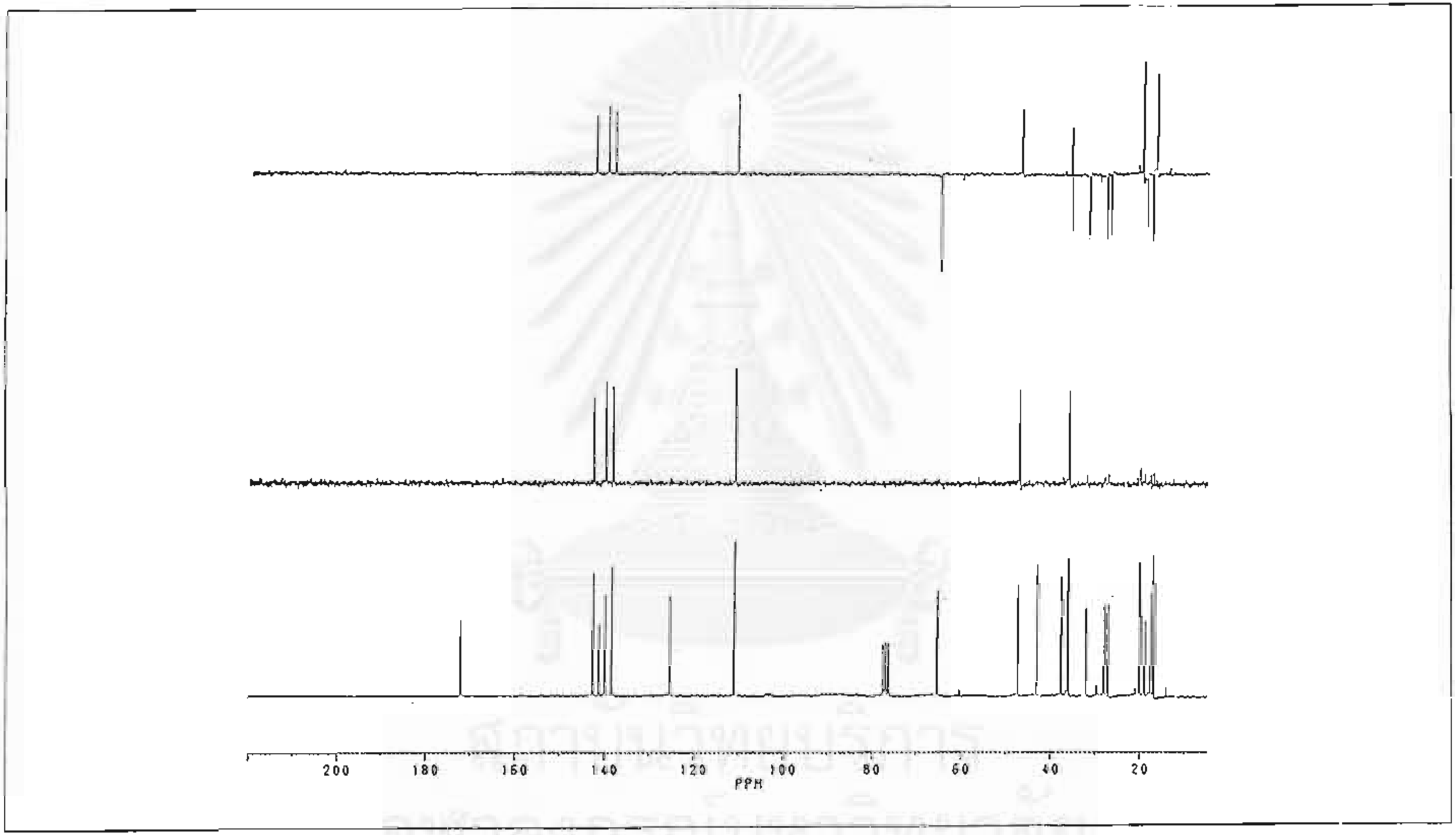

Figure 62. DEPT-135, 90 ^{13}C-NMR of Compound 4c

103

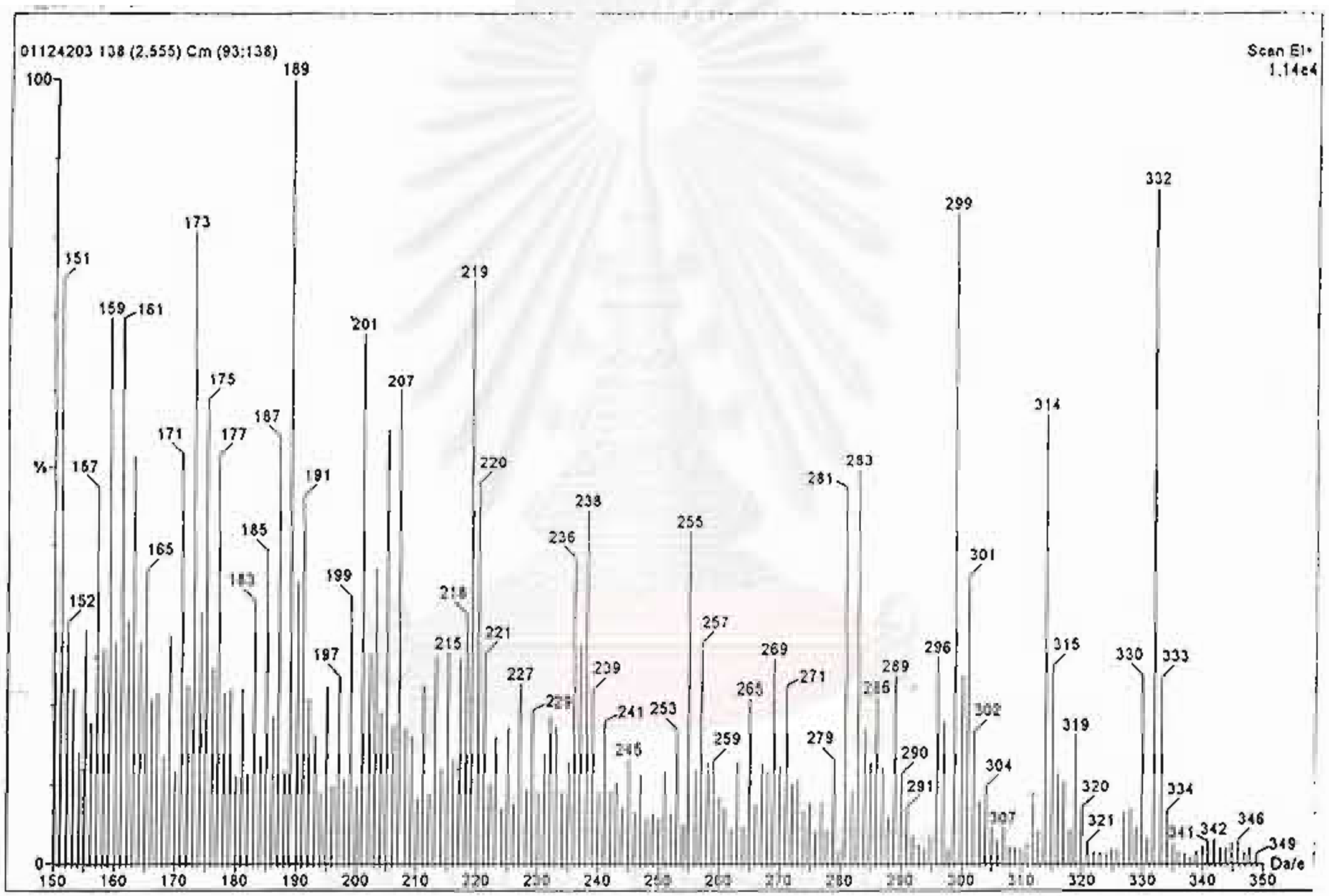

Figure 63. The EI MS spectrum of Compound 4c

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VITA

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