องค์ประกอบทางเคมีและฤทธิ์ทางชีวภาพของเปลือกต้นเปล้าใหญ่ (*Croton oblongifolius* Roxb.) จากอำเภอเมือง จังหวัดอุตรดิตถ์

นางสาวธัญลักษณ์ ตันวัฒนกุล

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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CHEMICAL CONSTITUENTS AND THEIR BIOLOGICAL ACTIVITY OF STEM BARKS OF *Croton oblongifolius* Roxb. FROM AMPHOE MUANG, UTTARADIT PROVINCE

Miss. Thanyaluk Tanwattanakun

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ธัญลักษณ์ ตันวัฒนกุล: องค์ประกอบทางเคมีและฤทธิ์ทางชีวภาพของเปลือกต้นเปล้าใหญ่(*Croton oblongifolius* Roxb.)จากอำเภอเมือง จังหวัดอุตรดิตถ์ CHEMICAL CONSTITUENTS AND THEIR BIOLOGICAL ACTIVITY OF STEM BARKS OF *Croton oblongifolius* Roxb. FROM AMPHOE MUANG, UTTARADIT PROVINCE อ.ที่ปรึกษา: รศ.ดร. อมร เพชรสม ; 112 หน้า. ISBN 974-334-015-7

ในการศึกษาองค์ประกอบทางเคมีของเปลือกต้นเปล้าใหญ่ สามารถสกัดแยกสารประกอบไดเทอร์พีนอยด์ใหม่ 2 ชนิด คือ (2*E*,7*E*,11*E*)-1-isopropyl-1,4-dihydroxy-4,8-dimethylcyclotetradeca-2,7,11-triene-12-carboxylic acid กับ Methyl-15,16-epoxy-12-oxo-3,13(16),14-clerodatriene-20,19-olide-17-oate และสารประกอบไดเทอร์พีนอยด์ที่เคยพบมาแล้ว 4 ชนิด คือ (-)-Pimara-9(11),15-diene-19-oic acid, Crotocembraneic acid, Neocrotocembraneic acid, (-)-Pimara-9(11),15-diene-19-ol และได้ทำการพิสูจน์โครงสร้างใหม่นี้โดยอาศัยข้อมูลทางสเปกโตร-สโกปี ซึ่งได้แก่ IR, MS, 1D และ 2D NMR เทคนิค ซึ่งได้แก่ DEPT, COSY, NOESY, HMBC และ HMQC พร้อมกันนั้นได้มีการทดสอบฤทธิ์ทางชีวภาพของสารประกอบทั้งหมด โดยทดสอบกับเซลล์ในหลอดทดลอง 6 ชนิด ได้แก่ เซลล์เนื้อเยื่อปกติ (HS27), เซลล์มะเร็งกระเพาะอาหาร (KATO-3), เซลล์มะเร็งทรวงอก (BT474), เซลล์มะเร็งปอด (CHAGO), เซลล์มะเร็งลำไส้ใหญ่ (SW 620) และ เซลล์มะเร็งตับ (HEP-G2) ซึ่งสาร (-)-Pimara-9(11),15-diene-19-ol มีฤทธิ์ในการยับยั้ง เซลล์มะเร็งกระเพาะอาหาร (KATO-3), เซลล์มะเร็งปอด (CHAGO), เซลล์มะเร็งลำไส้ใหญ่ (SW 620) และ เซลล์มะเร็งตับ (HEP-G2) โดยมีค่า IC_{50} เท่ากับ 6.5, 6.1, 5.9 และ 6.7 μg/ml ตามลำดับ

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

สาขาวิชา.....เคมีอินทรีย์..... ลายมือชื่ออาจารย์ที่ปรึกษา.....

ปีการศึกษา.....2542.....

THANYALUK TANWATTANAKUN : CHEMICAL CONSTITUENTS AND THEIR BIOLOGICAL ACTIVITY OF STEM BARKS OF *Croton oblongifolius* Roxb. FROM AMPHOE MUANG, UTTARADIT PROVINCE. THESIS ADVISOR : ASSO. PROF. AMORN PETSOM, Ph.D. 112 pp.
ISBN 974-334-015-7

In the investigation of chemical constituents of the stem barks of *Croton oblongifolius* Roxb., 2 new diterpenoid compounds, (2*E*,7*E*,11*E*)-1-isopropyl-1,4-dihydroxy-4,8-dimethylcyclotetradeca-2,7,11-triene-12-carboxylic acid, Methyl-15,16-epoxy-12-oxo-3,13(16),14-clerodatriene-20,19-olide-17-oate and 4 known diterpenoids, (-)-Pimara-9(11),15-diene-19-oic acid, Crotocembraneic acid, Neocrotocembraneic acid, (-)-Pimara-9(11),15-diene-19-ol were isolated . The structure of these compounds were established by spectroscopic data such as IR, MS spectra, 1D and 2D techniques including DEPT, COSY, NOESY, HMBC and HMQC. All of compounds were tested biological activity against a panel of six cell lines including fibroblast (HS27), gastric carcinoma (KATO-3), breast carcinoma (BT474), lung carcinoma (CHAGO), colon carcinoma (SW 620), hepato carcinoma (HEP-G2). (-)-Pimara-9(11),15-diene-19-ol showed cytotoxic activity against the gastric carcinoma (KATO-3), lung carcinoma (CHAGO), colon carcinoma (SW 620) and hepato carcinoma (HEP-G2), *in vitro*, with IC_{50} values of 6.5, 6.1, 5.9 and 6.7 μg/ml, respectively.

ภาควิชา.....เคมี.....
สาขาวิชา.....เคมีอินทรีย์.....
ปีการศึกษา.....2542.....

ลายมือชื่อนิสิต.....
ลายมือชื่ออาจารย์ที่ปรึกษา.....

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Finally, she wishes to record my sincere gratitude to my parents and friends for their understanding and encouragement throughout the entire course of study.

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LIST OF 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
COSY	=	Correlated Spectroscopy
cm	=	Centimeter
^{13}C-NMR	=	Carbon-13 nuclear magnetic resonance
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
δ	=	Chemical Shift
EI MS	=	Electron Impact Mass Spectrum
EtOAc	=	Ethyl acetate
g	=	Gram
HMQC	=	Heteronuclear Multiple Quantum Correlation
HMBC	=	Heteronuclear Multiple Bond Correlation
^{1}H-NMR	=	Proton nuclear magnetic resonance
Hz	=	Hertz
in.	=	Inch
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
MS	=	Mass spectrometry
No.	=	Number
NMR	=	Nuclear Magnetic Resonance
NOESY	=	Nuclear Overhauser Enhancement Spectroscopy
ppm	=	Part per million
q	=	Quartet (for NMR spectra)
R_f	=	rate of flow in chromatography
s	=	Singlet (for NMR spectra)
t	=	Triplet (for NMR spectra)
TLC	=	Thin layer Chromatography
TMS	=	Tetramethylsilane

CHAPTER I

INTRODUCTION

Medicinal plants are one of the natural products evolved from man's desperate attempt to conquer of physical suffering, coupled with overwhelming desire for an eternal life. There is a worldwide trend towards the use of drugs of natural origin since they are believed to posses less harmful side effects than synthetic drugs. There has also been an effort to develop medicinal plants in order to make them be safe and effective drugs such as the development of an antipeptic ulcer drug from plao-noi (*Croton sublyratus* Kurz.). From the Thai medicinal plant literature, plao-yai (*Croton oblongifolius* Roxb.) was often used with plao-noi.

Plao Yai belongs to the Euphorbiaceae family[1]. The scientific name of Plao Yai is *Croton oblongifolius* Roxb. This plant is and intersting Thai medicinal plant because of it is believed that all parts can be used as a tonic, the flower are used as a teniacide, the fruits are used to treat dysmenorrhea, the seeds are used as a purgative, the roots are used to treat dysentery and the barks are used to treat dyspepsia. Moreover, the hot water extract of the bark of *Croton oblongifolius* Roxb. can be used as an antipyretic, myalgia, arthralgia and treatment of hepatitis.[2]

From the information, the stem barks of *Croton oblongifolius* Roxb. can be used as drug and the previous studies in chemical constituents of the stem barks of *Croton oblongifolius* Roxb. have been found some biologically active compounds. From screening test, ^{1}H-^{13}C NMR spectrum of hexane crude extract from the stem barks of *Croton oblongifolius* Roxb. from Amphur Muang, Uttaradit Province had differented from ^{1}H-^{13}C NMR spectrum of hexane crude extract from the stem barks of *Croton oblongifolius* Roxb. from another province suggested that there are interested compounds in this plant. Therefore it was decided to re-investigate diterpenoid compounds of the stem barks of *Croton oblongifolius* Roxb. from Amphur Muang, Uttaradit Province.

Thus, the objective of this research will be summarized as follows :

1. To extract and isolate the diterponoid compounds of the stem barks of *Croton oblongifolius* Roxb. from Amphur Muang, Uttaradit Province.
2. To identify the structural formula of the isolated substances.
3. To test biological activity of the isolated substances.

CHAPTER II

LITERATURE REVIEWS

General characterization of the plants in the Genus Croton[3].

The genus Croton comprises 700 species of trees or shurbs. Leaves are usually alternate with 2-glandular stipule at the base. Their flowers are solitary or clustered in the rhachis of a terminaaal raceme and bracts are small. Male flowers contain 5-calyx, 5-petals. There are many stamens inserted on a hsiry receptacle. In female flowers, sepals are usually more ovate than the male, petals are smaller than the sepals or missing and disk annular of 4-6 glands are oppsite the sepals. There are three ovary with solitary ovule in each cell. Seeds are smooth, albumen copious and broad cotyledons.

General characterization of *Croton oblongifolius* Roxb.[4].

Croton oblongifolius Roxb. is a medium sized tree. Its calyx and ovary are clothed with minute orbicular silvery scales. Leaves simple, alternate, oblong, elliptic- oblong, ovate or lanceolate, 5-10 cm wide,9-30 cm long.Young leave is brownish. Inflorescence in terminal raceme or panicle, unisexual, monoecious or dioecious. Flowers are pale geenish yellow and solitary in the axials of minute bracts on long erect racemes. The male flowers locate in the upper part of the raceme and the females in the lower part. Male flowers are slender and have the length of pedicels of 4.0 mm. Calyx is more than 6.0 mm long and segments are ovate, obtuse and more than 2.5 mm long. Petals are 3.0 mm long, elliptic-lanceolate and woolly. The twelve stamens are inflexed in bud and the length of filaments are 3.0 mm. Infemals flowers, the pedicels are short and stout. Its sepals are more acute than in the male with densely ciliated margins. Diameter of fruit is less than 1.3 cm, slightly 3-lobed and clothed with small orbicular scales In each fruit, the mumber of seeds are eight which are 6.0 mm long rounded and quite smooth on the back.

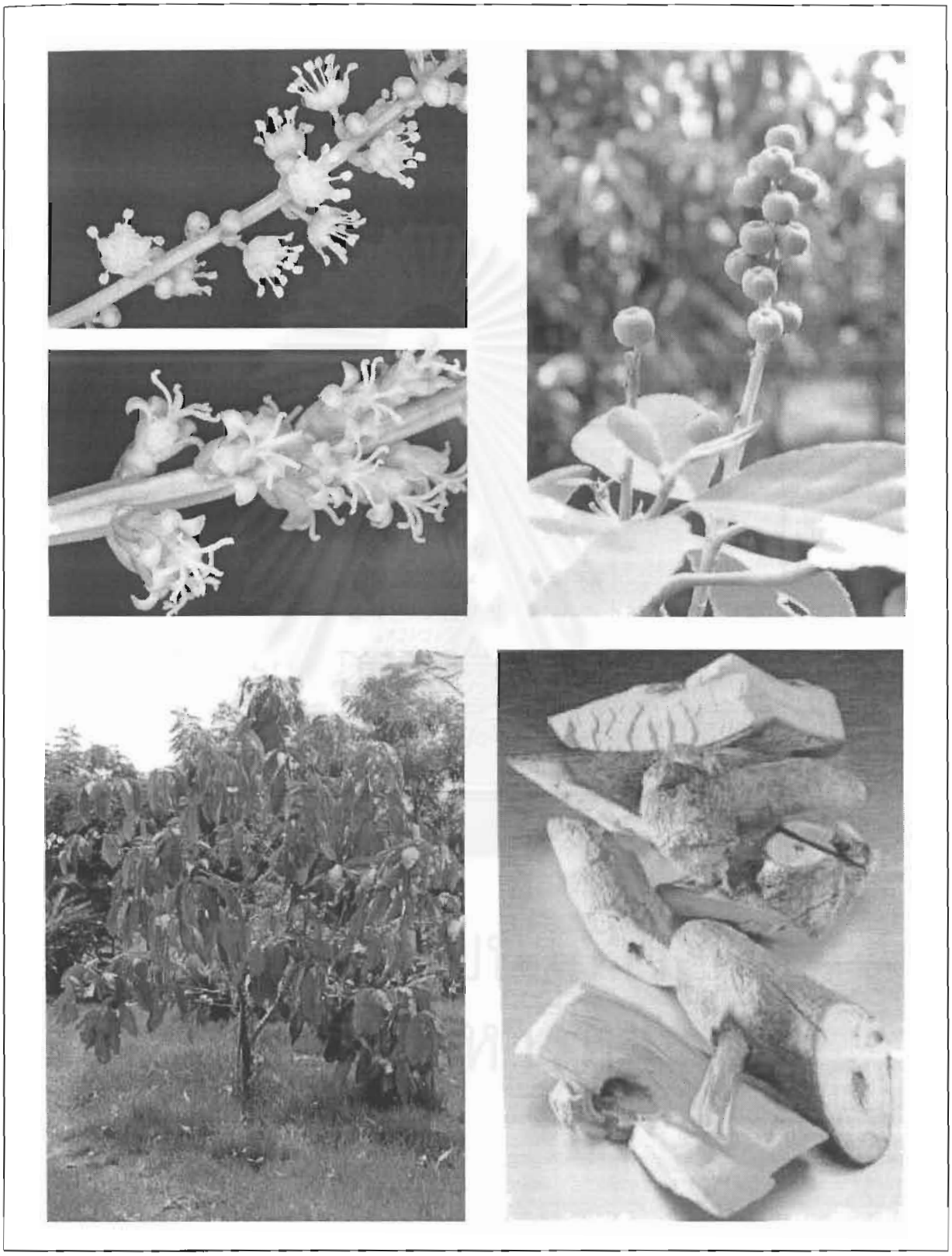


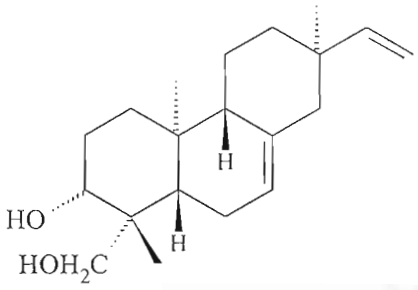
Figure 1 *Croton oblongifolius* Roxb.

The picture of stem-barks, leaf, flower and fruit of *Croton oblongifolius* Roxb. are shown in Fig. 1[5].

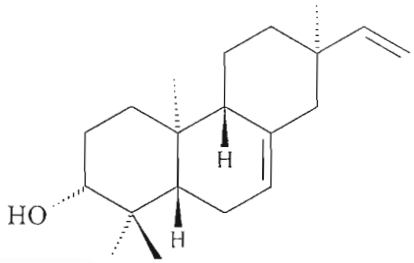
Previous studies of diterpenoid compounds from *Croton oblongifolius* Roxb.

From the literature surveys, *Croton oblongifolius* Roxb. have been widely studied and many diterpenoid compounds have been isolated and characterized in table below.

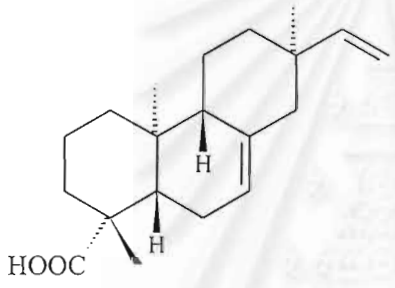
Plant parts	Crude Extract	Substaneces	References
Stem barks	Hexane	Oblongifoliol	[6]
		19-Deoxyoblingifoliol	[7]
		Oblongifolic acid	[8]
		ent-Isopimara-7,15-diene	[9]
		ent-Isopimara-7,15-diene-19-al	[9]
		11-Dehydro(-)-hardwickiic acid	[10]
		(-)-Hardwickiic acid	[10]
		Crotocembraneic acid	[11]
		neo-Crotocembraneic acid	[11]
		Labda-7,12(*E*),14-triene	[12]
		Labda-7,12(*E*),14-triene-17-al	[12]
		Labda-7,12(*E*),14-triene-17-ol	[12]
		Labda-7,12(*E*),14-triene-17-oic acid	[12]



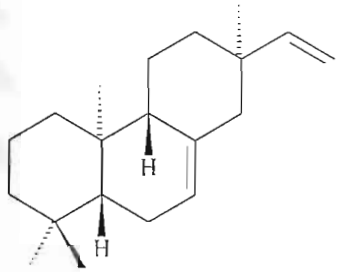
Oblongifoliol



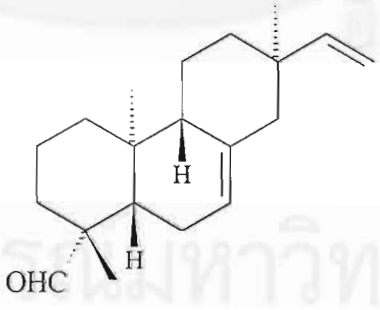
19- Deoxyoblongifoliol



Oblongifolic acid

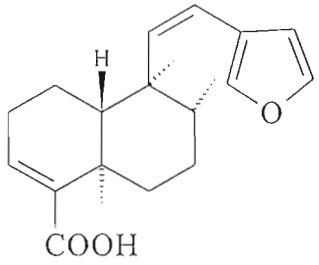


ent-Isopimara-7,15-diene

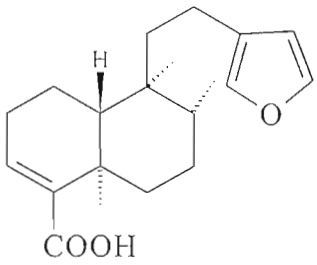


ent-Isopimara-7,15-diene-19-al

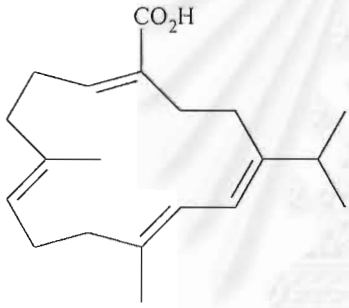
Figure 2 The structure of the diterpenoid compounds from *Croton oblongifolius* Roxb.



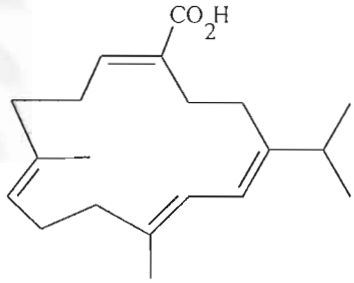
11-Dehydro(-)-hardwickiic acid



(-)-Hardwickiic acid

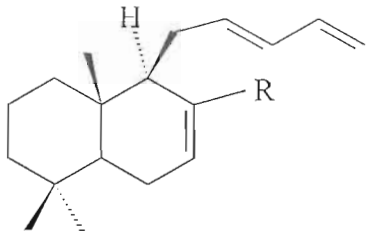


Crotocembraneic acid



Neocrotocembraneic acid

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



R = CH_3 = Labda-7,12(*E*),14-triene

R = CHO = Labda-7,12(*E*),14-triene-17-al

R = CH_2OH = Labda-7,12(*E*),14-triene-17-ol

R = CO_2H = Labda-7,12(*E*),14-triene-17-oic acid

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

CHAPTER III

EXPERIMENT

General experimental procedures.

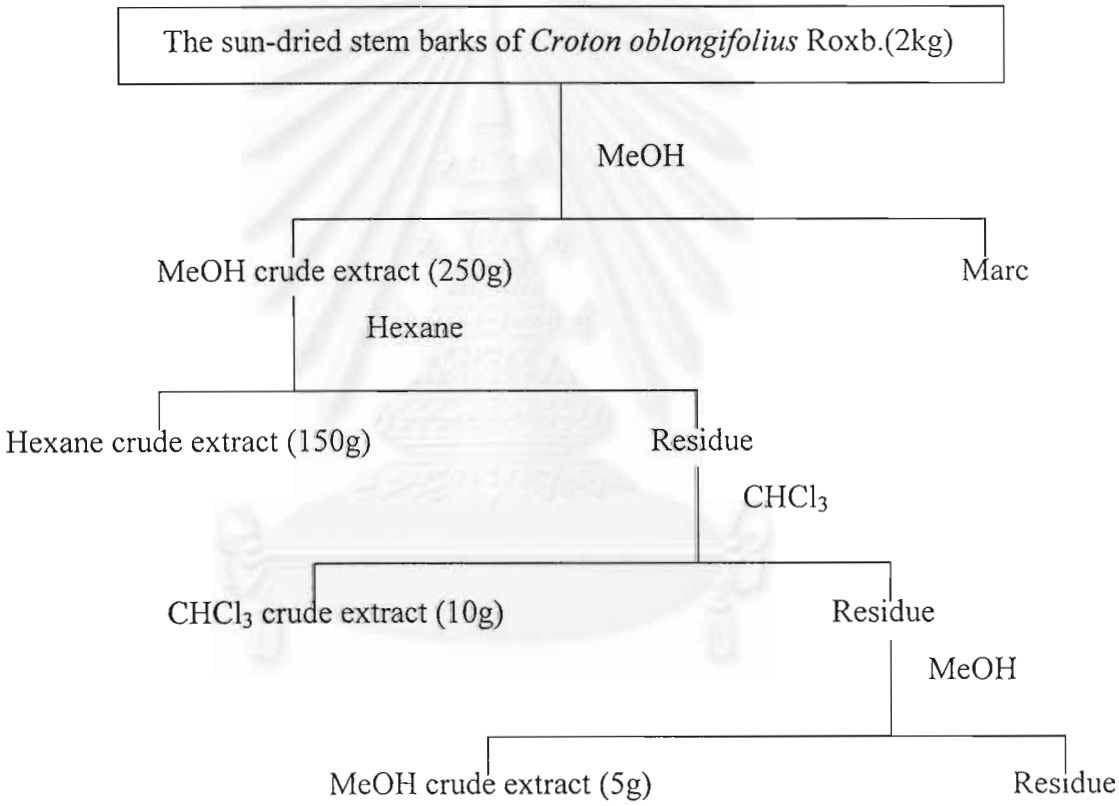
All solvents were distilled prior to use. UV-VIS spectra were recorded on a Hewlett Packard 8452A diode array spectrophotometer in $CDCl_3$. IR spectra were obtained on a Perkin Elmer Model 1760x Fourier Transform Infrared Spectrophotometer. Spectra of solid samples were recorded as KBr pellets and liquid 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. ^{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 reference.The COSY, NOESY HMQC and HMBC experiments were performed on the JEOL JNM-A500 Spectrometer. Elemental Analysies 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 plant material of *Croton oblongifolius* used in this study was collected from Amphur Muang, Uttradit province, Thailand in April 1998. The plant specimen was compared against voucher specimen no. BKF 084729 deposited in the herbarium of the Royal Forest Department of Thailand.

Extraction and isolation.

The powdered, sun-dried stem barks (2kg) of *Croton oblongifolius* Roxb. was repeatedly extracted with methanol. The methanol extract was filtered and evaporated under reduced pressure to obtain a dark-red gummy residue which was repeatedly reextracted with hexane, chloroform and methanol respectively. The extraction procedures are shown in scheme 1.



scheme 1 The extraction of the stem barks of *Croton oblongifolius* Roxb.

Isolation of crude extract of *Croton oblongifolius* Roxb.

Separation of hexane crude extract.

The hexane crude extract was obtained as a yellowish green oil (150g, 7.5 %wt by wt) after evaporation. The crude hexane extract (150g) 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 result of separation of hexane crude extract gave compounds 1-6 shown in Table 1.

Separation of chloroform crude extract.

Concentrated chloroform crude extract (10g, 0.5 % wt by wt) was separated on Silica gel 70-230 mesh ASTM using column chromatography technique. The column was eluted with hexane, hexane-chloroform, chloroform, chloroform-methanol, respectively. The eluted fraction was collected at about 250 ml each and evaporated to about 20 ml.

The ^{1}H-^{13}C NMR spectral data of chloroform crude extract were similar to hexane crude extract.

Separation of Methanol Crude Extract.

The methanol crude extract (5g, 0.25 % wt by wt) was gummy residues and insoluble in all solvent.Therefore, the methanol crude extract was not separated by column chromatography technique.

The weight of the crude extract is shown in Table 1.

Table 1 The weight of the crude extract

Extract	Weight (g)	% wt by wt
Hexane	150	7.5
$CHCl_3$	10	0.5
MeOH	5	0.25

Purification and properties of the compounds eluted from Column chromatography of hexane crude extract.

Purification and properties of Compound 1

Compound 1 was eluted with 15% $CHCl_3$ in 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 colorless needle-crystals (3.25g, 0.16 % wt by wt), $[\alpha]_D^{20}$-49.3($CHCl_3$, *c* 1.0), R_f ; 0.32 (1% MeOH-$CHCl_3$), m.p.135-136°C, UV λ_{max} ($CHCl_3$) 206.5sh (logε 3.68), EA; Found C 79.41, H 9.99% Calc. C 79.42, H 9.99%.

FT-IR spectrum (KBr) (Fig.16) ν_{max} (cm^{-1}) : 3400-2600(w), 2976(h), 2935(h), 2873(h), 1696(h), 1634(m)

^{1}H-NMR spectrum ($CDCl_3$, 500MHz) (Fig.17) δ (ppm) : 5.82(1H, dd), 5.40 (1H, m), 4.93(1H, dd), 4.87(1H, dd), 2.32(1H, m), 2.25(1H, d),2.19(1H, m), 2.06(1H, m), 1.95(1H, m), 1.92(1H, m), 1.81(1H, m), 1.76(1H, m), 1.74(1H, m), 1.66(1H, dd), 1.49(1H, m), 1.45(1H, m), 1.29(1H, d), 1.25(3H, s), 1.23(1H, m), 1.05(1H, m), 1.03 (1H, m), 0.99(3H, s), 0.96(3H, s)

^{13}C-NMR spectrum ($CDCl_3$, 125MHz) (Fig.18) δ (ppm) : 184.8(s), 150.2(d), 149.9(s), 116.6(d), 109.1(t), 48.0(d), 44.2(s), 41.9(t), 41.8(t), 38.4(s), 38.1(t), 37.5(t), 34.8(s), 28.7(d), 28.5(q), 27.8(t), 22.4(q), 22.2(q), 20.3(t), 18.9(t).

m/z (EI) (rel int.) (Fig.20) : 302[M^+](21), 287(100), 241(20), 234(13), 189(40), 173(47), 161(19), 159(30), 147(32), 133(37), 121(24), 119(57), 105(35), 93(20)

Purification and properties of Compound 2

Compound 2 was eluted with 10% $CHCl_3$ in hexane. Similar fraction were combined and the solvents were removed by rotary evaporation and futher purified by column chromatography (Merck's silica gel Art. 1.09385.1000). This compound is soluble in hexane, dichloromethane, chloroform ,ethyl acetate ,diethyl ether and methanol.

Compound 2 is a white solid (200 mg, 0.01 % wt by wt), R_f ; 0.13 (100% chloroform), m.p 110-111 °C, UV λ_{max} ($CHCl_3$) 249sh (logε 4.04)

FT-IR spectrum (KBr) (Fig.25) ν_{max} (cm^{-1}) : 3400-3000(w), 2960 and 2924(h), 1690(h), 1640(m)

^{1}H-NMR spectrum ($CDCl_3$, 200MHz) (Fig.26) δ (ppm) : 5.90-6.03(3H, m), 5.10(1H, t), 2.70(2H, q), 2.41(4H, m), 2.33(1H, m), 2.20(2H, d), 2.15(4H, m), 1.73 (3H, s), 1.54(3H, s), 1.04(6H, d)

^{13}C-NMR spectrum ($CDCl_3$, 50.25MHz) (Fig.27) δ (ppm) : 174.1(s), 146.9(s), 146.3(d), 135.2(s), 134.0(s), 130.9(s), 125.6(s), 121.6(d), 118.7(d), 39.2(t), 38.5(t), 33.8(d), 33.6(t), 28.7(t), 26.4(t), 25.1(t), 22.1(2q), 17.0(q), 15.8(q)

m/z (EI) (rel int.) (Fig.26) : 302[M^+](85), 152(100), 136(95), 121(96), 93(96)

Purification and properties of Compound 3

Compound 3 was eluted with 20%$CHCl_3$ in hexane. Similar fractions were combined and the solvents were removed by rotary evaporation and purified by column chromatography (Merck's silica gel Art. 1.09385.1000). This compound is soluble in hexane, dichloromethane, chloroform ,ethyl acetate ,diethyl ether ,methanol.

Compound 3 was a white solid (145mg, 0.007 % wt by wt), R_f ; 0.5 (100% chloroform), m.p 127-128 °C, UV λ_{max} ($CHCl_3$) 243sh (logε 3.95)

FT-IR spectrum (KBr) (Fig.29) ν_{max} (cm^{-1}) : 3400-3050(w), 2960 and 2930(h) 1684(h), 1635(m)

^{1}H-NMR spectrum ($CDCl_3$, 200MHz) (Fig.30) δ (ppm) : 6.89(1H, d), 6.01(1H, d), 5.91(1H, d), 5.14(1H, t), 2.36-2.39(5H, m), 2.15-2.26(8H, m), 1.71(3H, s), 1.68 (3H, s), 1.05(H, d)

^{13}C-NMR spectrum ($CDCl_3$, 50.25MHz) (Fig.31) δ (ppm) : 173.5(s), 146.5 (s),145.7(d), 135.5(s), 134.8(s), 132.0(s), 127.8(d), 120.0(d), 118.6(d), 38.5(t), 37.7(t), 34.6(d), 30.5(t), 29.1(t), 26.7(t), 24.7(t), 22.1(2q), 17.9(q), 17.4(q)

m/z (EI) (rel int.) (Fig.32) : 302[M^+](100), 152(30), 136(98), 121(98), 93(98)

Purification and properties of Compound 4

Compound 4 was obtained from 80% $CHCl_3$ in hexane fractions on silica gel column chromatography (Merck's silica gel Art. 1.09385.1000). This compound is soluble in chloroform, ether, ethyl acetate, methanol.

Compound 4 was white solid (80 mg, 0.004 % wt by wt), $[\alpha]_D^{20}$ –0.5 ($CHCl_3$, *c* 1.0), R_f ; 0.40 (1% MeOH-$CHCl_3$), m.p. 79-80 °C, UV λ_{max} ($CHCl_3$) 205.5sh (logε 3.82),EA; Found C 70.26, H 9.61% Calc. C 70.27, H 9.61%.

FT-IR spectrum (KBr) (Fig.33) ν_{max} (cm^{-1}) : 3500-3410(w), 2965 and 2920(h), 1684(h), 1625(h)

^{1}H-NMR spectrum ($CDCl_3$, 500MHz) (Fig.34) δ (ppm) :6.83(1H, m), 5.54 (1H, d), 5.39(1H, d), 4.72(1H, t), 2.38(1H, m), 2.29(1H, q), 2.20(1H, m), 2.16(1H, m), 2.19(1H, m), 2.13(1H, m), 2.01(1H, t), 1.85(1H, m), 1.79(1H, m), 1.78(1H, m), 1.67 (1H, m), 1.53(3H, d), 1.31(3H, s), 0.95(6H, dd)

^{13}C-NMR spectrum ($CDCl_3$, 125 MHz) (Fig.35) δ (ppm) : 167.8(s), 145.9(d), 138.0(d), 132.5(s), 127.5(d), 126.0(d), 125.7(s), 85.85(s), 72.9(s), 42.3(t),37.5(d), 36.4 (t), 27.3(t), 26.9(q), 25.8(t), 23.0(t), 20.6(t), 17.3(q), 17.1(q), 16.7(q)

m/z (EI) (rel int.) (Fig.36) : 336[M^+](2), 300(34), 285(11), 272(21), 257(26), 215(18), 213(30), 187(34), 171(32), 159(36), 147(47), 134(30), 119(53), 117(30), 105 (52), 85(71)

Purification and properties of Compound 5

Compound 5 was eluted with 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 5 is a white solid (130mg, 0.006 % wt by wt), $[\alpha]_D^{20}$ -55.3($CHCl_3$, *c* 1.0), R_f ; 0.45 (100% $CHCl_3$), m.p.73-74°C, UV λ_{max} ($CHCl_3$) 207.5sh (logε 3.60), EA; Found C 83.26, H 11.19% Calc. C 83.27, H 11.18%.

FT-IR spectrum (KBr) (Fig.42) ν_{max} (cm^{-1}) : 3500-3300(w), 2976(h), 2935(h), 2873(h), 1720(h), 1650(m)

^{1}H-NMR spectrum ($CDCl_3$, 500MHz) (Fig.43) δ (ppm) : 5.79(1H, dd), 5.33 (1H, m), 4.90(1H, dd), 4.83(1H, dd), 3.82(1H, d), 3.50(1H, d),2.28(1H, m), 2.00(1H, m), 1.88(1H, m), 1.84(1H, m), 1.82(1H, m), 1.75(1H, m),1.64(1H, m), 1.56(1H, s), 1.53(1H, m), 1.48(1H, m), 1.41(1H, m), 1.36(1H, ddd), 1.20(1H, m), 1.16(1H, m), 1.01(3H, s), 0.99(1H, m), 0.95(3H, s), 0.94(3H, s), 0.90(3H, s)

^{13}C-NMR spectrum ($CDCl_3$, 125MHz) (Fig.44) δ (ppm) : 151.3(s), 150.3(d), 115.8(d), 109.0(t), 64.9(t), 46.3((d), 41.6(t), 41.1(t), 38.4(s), 37.7(s), 37.6(t), 35.5(t), 34.8(s), 28.9(d), 26.8(t), 26.5(q), 26.0(q), 22.4(q), 19.1(t), 18.0(t)

m/z (EI) (rel int.) (Fig.46) : 288[M^+](15), 273(43), 258(14), 234(4), 189(30), 173(27), 161(38), 159(26), 147(40), 133(52), 121(55), 119(74), 105(100), 91(100)

Purification and properties of Compound 6

Compound 6 was eluted with 40% $CHCl_3$ in 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 6 is a white solid (391mg, 0.02 % wt by wt), $[\alpha]_D^{20}$ -62.8 ($CHCl_3$, *c* 1.0), R_f ; 0.50 (100% $CHCl_3$), m.p.108-109 °C, UV λ_{max} ($CHCl_3$) 205sh (logε 4.11), EA; Found C 67.71, H 6.52% Calc. C 67.73, H 6.50%.

FT-IR spectrum (KBr) (Fig.51) ν_{max} (cm^{-1}) : 3012(h), 2935(h), 2873(h), 1731 (h), 1772(h), 1675(m), 1199(m), 1163(m)

^{1}H-NMR spectrum ($CDCl_3$, 500MHz) (Fig.52) δ (ppm) : 8.00(1H, dd), 7.42 (1H, t), 6.74(1H, dd), 6.72(1H, dd), 4.33(1H, d), 3.93(1H, dd), 3.60(3H, s), 3.21(1H, dd), 3.04(1H, d),2.83(1H, d), 2.72(1H, dd), 2.29(1H, m), 2.21(1H, m), 2.05(1H, m), 1.99(1H, m), 1.87(2H, m), 1.64(1H, m), 1.35(1H, dd), 0.82(3H, s)

^{13}C-NMR spectrum ($CDCl_3$, 125MHz) (Fig.53) δ (ppm) : 193.7(s), 169.1(s), 174.0(s), 147.0(d), 144.3(d), 137.8(s), 136.3(d), 128.8(s), 108.5(d), 71.4(t), 51.4(q), 48.7(d), 46.6(d), 46.5(t), 45.1(s), 39.6(d), 33.2(t), 27.3(t), 22.1(t), 20.1(t), 19.2(q)

m/z (EI) (rel int.) (Fig.55) : 372[M^+](46), 354(14), 340(32), 312(12), 263(41), 245(83), 231(65), 217(24), 202(25), 172(28), 157(49), 145(63), 115(30), 110(100)

Biological evaluation

Cytotoxicity test

Bioassay of cytotoxic activity against human tumor cell *in vitro* was performed by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) colorimetric method [13-15]. In principle, the viable cell number / well is directly proportional to the production of formazan, which following solubilization, can be measured spectrophotometrically.

CHAPTER IV

RESULTS AND DISCUSSION

The hexane crude extract (150g, 7.5% wt by wt) was selected for separation by column chromatography. The result of separation is shown in Table 2.

Table 2 The results of separation of hexane crude extract by column chromatography.

compounds	physical appearance	% wt by wt
1	Colorless needle-crystals	0.16
2	white solid	0.01
3	white solid	0.007
4	white solid	0.004
5	white solid	0.006
6	colorless needle-crystals	0.02

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

1.Structure elucidation of Compound 1

Compound 1 was eluted with 15% $CHCl_3$ in 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 a colorless needle-crystals (3.25g, 0.16 % wt by wt), $[\alpha]_D^{20}$-49.3($CHCl_3$, *c* 1.0), R_f ; 0.32 (1% MeOH-$CHCl_3$), m.p.135-136°C.

The IR spectrum of compound 1 (Fig.16) showed the presence of a carboxylic group according to the broad absorbtion band between 3400 to 2600 cm^{-1} and the strong absorption band at 1696 cm^{-1} due to the carboxylic acid carbonyl.

Table 3 The IR absorption bands assignment of compound 1.

Wavenumber (cm^{-1})	intensity	Tentative assignment
3400-2600	broad	O-H stretching vibration of acid
2976,2935,2873	strong	C-H stretching vibration of $-CH_2$,-CH_3
1696	strong	C=O stretching vibration of acid
1634	medium	C =C stretching vibration

The ^{1}H-NMR spectrum (Fig. 17) showed an ABX pattern at the olefinic region (δ 4.87-5.82 ppm.) which was assigned to a monosubstituted double bond and closely resembled pimaradiene-type diterpenes. The multiplet at 5.40 ppm. was attributed to the proton of a trisubstituted double bond.

The double bond position was confirmed by ^{13}C-NMR data (Fig. 18, Table 4) and the presence of carboxylic moiety was supported by ^{13}C resonance at δ_C 184.8 ppm.

The DEPT-90 and DEPT-135 ^{13}C-NMR (Fig. 19) indicated this compound possesed twenty carbon atoms and thirty protoms. Assuming the compound may contain only carbon, proton and oxygen atoms. Thus, its molecular ion was established as $C_{20}H_{30}O_2$ which was confirmed by observing molecular formola at *m/z* 302 (Fig.20) and indicated that 6 DBE.

The spectroscopic data of compound 1 were consistent with (-)-pimara-9(11),15-diene-19-oic acid (m.p.135-136 °C) which was previously isolated from *Acanthopanax koreanum* in 1982 [16]. The ^{13}C-NMR agreed well with those reported for (-)-pimara-9(11),15-diene-19-oic acid (Table 4).

Table 4 ^{13}C-NMR spectral data of compound 1 compared with (-)-pimara-9(11),15-diene-19-oic acid.

Carbon No.	(-)-pimara-9(11),15-diene-19-oic acid	Compound 1
1	41.9(t)	41.9(t)
2	18.9(t)	18.9(t)
3	38.0(t)	38.1(t)
4	44.2(s)	44.2(s)
5	48.0(d)	48.0(d)
6	20.3(t)	20.3(t)
7	27.7(1t)	27.8(t)
8	28.6(d)	28.7(d)
9	149.8(d)	149.9(d)
10	38.4(s)	38.4(s)
11	116.5(d)	116.6(d)
12	37.4(t)	37.5(t)
13	34.8(s)	34.8(s)
14	418(t)	41.8(t)
15	150.1(d)	150.2(d)
16	109.1(t)	109.1(t)
17	22.2(q)	22.2(q)
18	28.5(q)	28.5(q)
19	185.0(s)	184.8(s)
20	22.3(q)	22.4(q)

The information from 2D-NMR techniques, HMQC correlations (Fig.21), HMBC correlations (Fig.22), COSY correlations (Fig.23), NOESY correlations (Fig.24) were used to assist the interpretation the structure of compound 1.

The long-range C-H correlations by HMBC sprectrum were summerized in figure 3

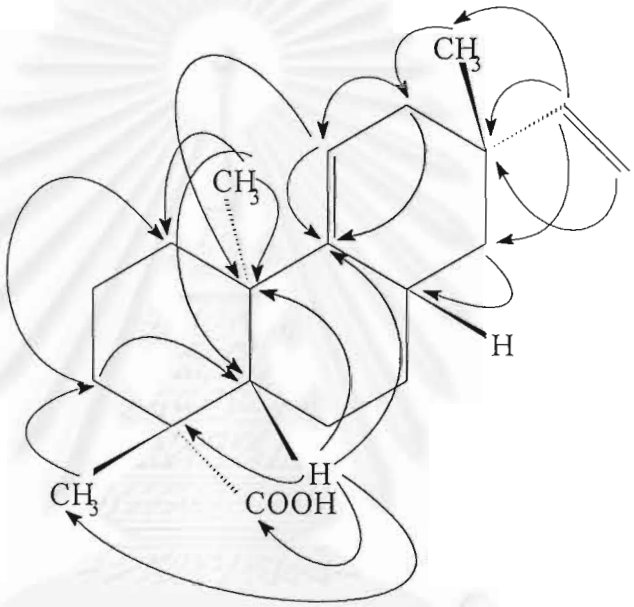


Figure 3 The HMBC correlations of compound 1.

The stereochemistry of compound 1 was confirmed by NOESY correlations, Key NOE correlations in compound 1 are shown in figure 4.

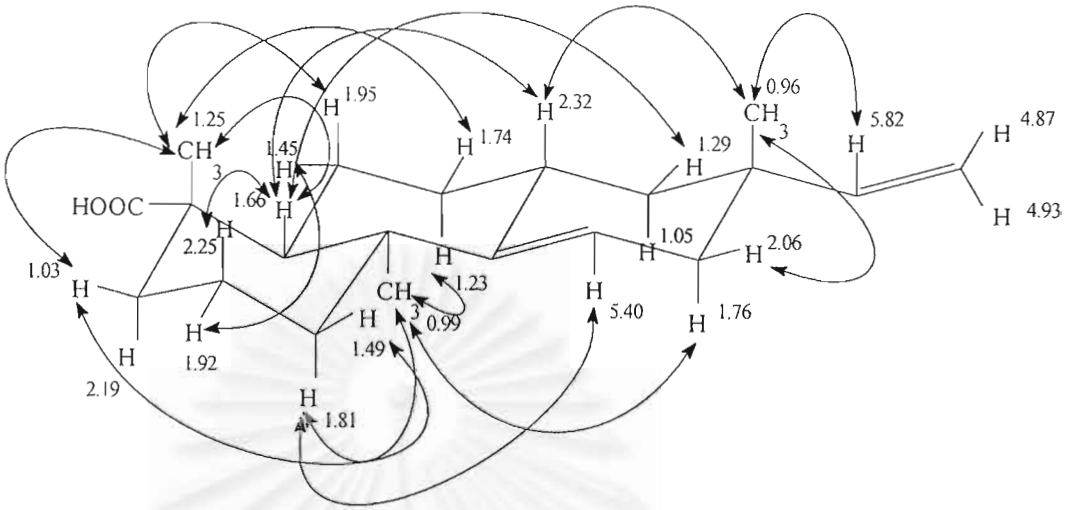


Figure 4 The NOESY correlations of compound 1.

From the data above, it can be concluded that compound 1 was (-)-pimara-9 (11),15-diene-19-oic acid or acanthoic acid and the structure of compound 1 was shown below.

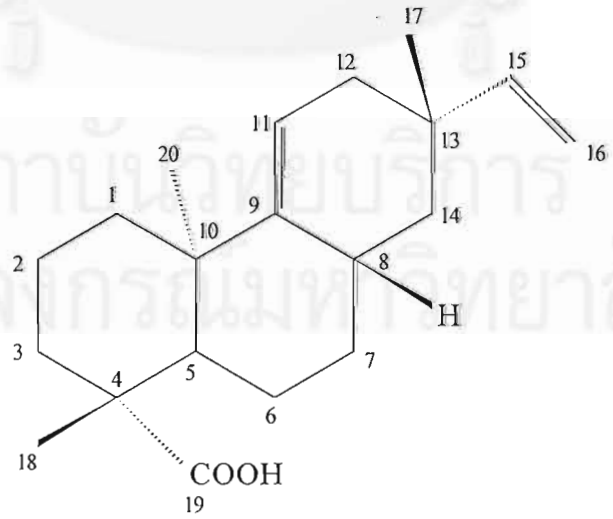


Figure 5 The structure of compound 1

From literlature, (-)-pimara-9(11),15-diene-19-oic acid gave promising results as antibacterial [17], anti-inflammatory agent [18], antifibrosis effect [18] and antioxidant [18]. Therefor, this compound exhibited ralatively interesting activity.

2. Structure elucidation of Compound 2

Compound 2 was eluted with 10% $CHCl_3$ in hexane. Similar fraction were combined and the solvents were removed by rotary evaporation and futher purified by column chromatography (Merck's silica gel Art. 1.09385.1000). It is a white solid (200 mg, 0.01 % wt by wt), R_f ; 0.13 (100% chloroform), m.p 110-111 °C.

The IR spectrum of compound 2 (Fig.25) was summarized in Table 5.

Table 5 The IR absorption bands assignment of compound 2.

Wavenumber (cm^{-1})	intensity	Tentative assignment
3400-3000	broad	O-H stretching vibration of acid
2960-2924	strong	C-H stretching vibration of $-CH_2$,-CH_3
1690	strong	C=O stretching vibration of acid
1640	medium	C =C stretching vibration

The ^{1}H-NMR spectrum (Fig.26) indicated that compound 2 possessed an isopropyl group (δ 1.04, 6H, d), one olefinic methyl groups attached to double bonds (δ1.54, 3H, s and 1.73, 3H, s) and four olefinic protons (δ 5.10, 1H, t and 5.85-6.04, 3H, m).

The ^{13}C-NMR spectrum (Fig.27) suggested the presence of olefinic carbons according to the signal at 146.9(s), 146.3(d),135.2(s), 134.0(s), 130.9(s), 125.6(d), 121.6(d), 118.7(d) ppm. The signal at 174.1 ppm. Should be the carbonyl group of carboxylic acid. There were 11 sp^3 carbon signals at 39.2(t), 38.5(t), 33.8(d), 33.6(t), 28.7(t), 26.4(t), 25.1(t), 22.1(2q), 17.0(q) and 15.8(q) ppm.

Its molecular fomula was established as $C_{20}H_{30}O_2$ which was confirm by observing molecular ion at *m/z* 302 (Fig.28).

To confirm the structure of this compound, the ^{13}C-NMR chemical shift were compared with literature [19] suggested that this compound might consist of a cembranoid structure which was a 14-membered-ring diterpene skeleton. The structure of crotocembraneic acid a seemed to fit all the number and type of bonds presented in compound 2 (Table 6).

Table 6 ^{13}C-NMR spectral data of compound 2 compared with Crotocembraneic acid.

Crotocembraneic acid	Compound 2
15.8(q)	15.8(q)
17.0(q)	17.0(q)
22.1(2q)	22.1(2q)
25.1(t)	25.1(t)
26.4(t)	26.4(t)
28.7(t)	28.7(t)
33.6(t)	33.6(t)
33.8(d)	33.8(d)
38.6(t)	38.5(t)
39.2(t)	39.2(t)
118.7(d)	118.7(d)
121.6(d)	121.6(d)
125.7(d)	125.6(d)
130.9(s)	130.9(s)
134.0(s)	134.0(s)
135.2(s)	135.2(s)
146.3(d)	146.3(d)
146.9(s)	146.9(s)
174.1(s)	174.1(s)

From the Data above, it can be concluded that compound 2 was Crotocembraneic acid and the structure of compound 2 can be shown below.

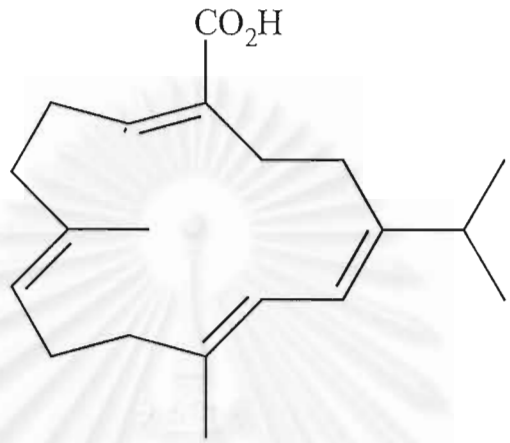


Figure 6 The structure of compound 2

3. Structure Elucidation of Compound 3

Compound 3 was eluted with 20%$CHCl_3$ in hexane. Similar fractions were combined and the solvents were removed by rotary evaporation and purified by column chromatography (Merck's silica gel Art. 1.09385.1000).It was a white solid (145mg, 0.007 % wt by wt), R_f ; 0.5 (100% chloroform), m.p 127-128 °C.

The IR spectrum of compound 3 (Fig.29) was summarized in Table 7.

Table 7 The IR absorption bands assignment of compound 3.

Wavenumber (cm^{-1})	Intensity	Tentative assignment
3400-3000	Broad	O-H stretching vibration of acid
2960-2930	strong	C-H stretching vibration of $-CH_2$,-CH_3
1684	strong	C=O stretching vibration of acid
1635	medium	C =C stretching vibration

The ^{1}H-NMR spectrum (Fig.30) indicated that compound 3 possessed an isopropyl group (δ 1.05, 6H, d), one olefinic methyl groups attached to double bonds (δ1.71, 3H, s and 1.68, 3H, s) and four olefinic proton (δ 6.89, 1H, t); (6.01, 1H, d); (5.91, 1H, d); (5.14, 1H, t).

The ^{13}C-NMR spectrum (Fig.31) suggested the presence of olefinic carbons according to the signal at 146.5(s), 145.7(d), 135.5(s), 134.8(s), 132.0(d), 127.8(d), 120.0(d) and 118.6(d) ppm. The signal at 173.5 ppm. should be the carbonyl group of carboxylic acid. There were 11 sp^3 carbon signals at 38.5(t).37.7(t), 34.6(d), 30.5(t), 29.1(t), 26.7(t), 24.7(t), 22.1(2q), 17.9(q), 17.4(q) ppm.

Its molecular fomula was established as $C_{20}H_{30}O_2$ which was confirm by observing molecular ion at *m/z* 302 (Fig.32).

To confirm the structure of this compound, the ^{13}C-NMR chemical shift were compareed with literature [20] suggested that this compound might consist of a cembranoid structure, 14-membered-ring diterpene skeleton. The structure of Neocrotocembraneic acid a seemed to fit all the number and type of bonds and presented in compound 3 (Table 8).

Table 8 ^{13}C-NMR spectral data of compound 3 compared with Neocrotocembraneic acid.

Neocrotocembraneic acid	Compound 3
17.4(q)	17.4(q)
17.9(q)	17.9(q)
22.1(2q)	22.1(2q)
24.7(t)	24.7(t)
26.7(t)	26.7(t)
29.1(t)	29.1(t)
30.5(t)	30.5(t)
34.6(d)	34.6(d)
37.7(t)	37.7(t)
38.5(t)	38.5(t)
118.6(d)	118.6(d)
120.0(d)	120.0d)
127.8(d)	127.8(d)
132.1(s)	132.0(s)
134.8(s)	134.8(s)
135.5(s)	135.5(s)
145.7(d)	145.7(d)
146.5(s)	146.5(s)
173.5(s)	173.5(s)

From the Data above, it can be concluded that compound 3 was Neocrotocembraneic acid and the structure of compound 3 was shown below.

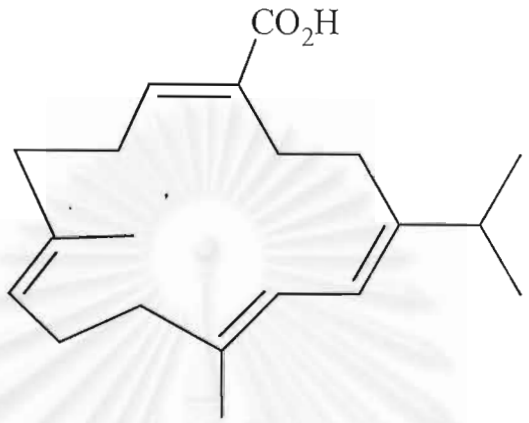


Figure 7 The structure of compound 3

4. Structure elucidation of Compound 4

Compound 4 was obtained from 80% $CHCl_3$ in hexane fractions on silica gel column chromatography (Merck's silica gel Art. 1.09385.1000). It was white solid (80 mg, 0.004 % wt by wt), $[\alpha]_D^{20}$ –0.5 ($CHCl_3$, *c* 1.0), R_f ; 0.40 (1% MeOH-$CHCl_3$), m.p. 79-80 °C.

The IR spectrum of compound 4 (Fig.33) showed the presence of a hydroxy group at 3500-3410 cm^{-1}, the carboxylic acid carbonyl group at 1684 cm^{-1}.

Table 9 The IR absorption bands assignment of compound 4.

Wavenumber (cm^{-1})	intensity	Tentative assignment
3500-3410	broad	O-H stretching vibration of alcohol
2965-2920	strong	C-H stretching vibration of $-CH_2$,-CH_3
1684	strong	C=O stretching vibration of acid
1625	medium	C =C stretching vibration

The ^{1}H-NMR spectrum (Fig.34) indicated that compound 4 possessed an isopropyl group (δ 0.95 ppm.), one olefinic methyl groups attached to double bonds (1.53 ppm.), one methyl group attached to C-OH (1.31 ppm.) and four olefinic proton (6.83, 5.54, 5.39, 4.72 ppm.).

The ^{13}C-NMR spectrum (Fig.35) suggested the presence of olefinic carbon according to the signal at 145.9, 138.0, 132.5, 126.0, 125.7 ppm. The signal at 169.8 ppm. should be the carbonyl group of carboxylic acid. There were two sp^3 carbons at 85.8 and 72.9 ppm. which have hydroxy group attached to carbon.

The DEPT-90 and DEPT-135 ^{13}C-NMR (Fig.36) indicated this compound possesses twenty carbon atoms and thirty two protons. Assuming the compound may contain only carbon, proton and oxygen atoms. Thus, its molecular formula was established as $C_{20}H_{32}O_4$ which was confirmed by observing molecular ion at *m/z* 336 (Fig.37).

The ^{13}C-NMR data revealed that the molecule possessed three double bonds. However, the DBE according to the molecular formula $C_{20}H_{32}O_4$ was 5 thus this compound must consist of a ring in addition to the 3 double bonds and a carbonyl group. Comparison of the characteristic ^{1}H and ^{13}C-NMR in addition to the number of ring and double bonds required with those in the literature [12] suggested that this compound might possessed a cembranoid structure which is a 14-membered-ring diterpene skeleton.The structure of Neocrotocembraneic acid had seemed to fit all the number and type of bonds and presented in compound 4 (Table 10). Therefore, compound 4 should be derivative of neocrotocembraneic acid.

Table 10 ^{13}C-NMR spectral data of compound 4 compared with Neocrotocembraneic acid.

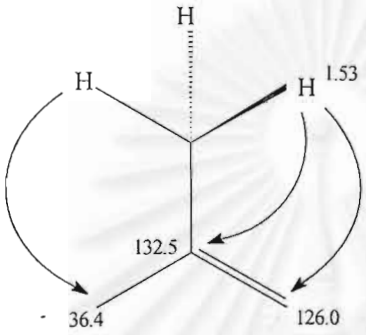
Neo-Crotocembraneic acid	Compound 4
17.4(q)	16.7(q)
17.9(q)	17.1(q)
22.1(q)	17.3(q)
22.1(q)	20.6(t)
24.7(t)	23.0(t)
26.7(t)	25.8(t)
29.1(t)	26.9(q)
30.5(t)	27.3(t)
34.6(d)	36.4(t)
37.7(t)	37.5(d)
38.5(t)	42.3(t)
118.6(d)	72.9(s)
120.0(d)	85.8(s)
127.8(d)	125.7(s)
132.1(s)	126.0(d)
134.8(s)	127.5(d)
135.5(s)	132.5(s)
145.7(d)	138.0(d)
146.5(s)	145.9(d)
173.5(s)	167.8(s)

Two dimensional NMR techniques were used for assisting the stucture assignment. The protons directly attached to carbon of the compound 4 were assigned by HMQC spectra (Fig.38) as show in Table 11.

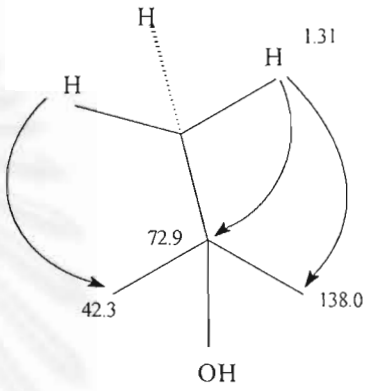
Table 11 The HMQC spectral data of compound 4.

^{13}C-NMR (ppm.)	^{1}H-NMR (ppm.), coupling constant (Hz)
16.7q	0.95(3H, dd, J=5.79,6.71)
17.1q	1.53(3H, d, J=0.92)
17.3q	0.95(3H, dd, J=5.79,6.71)
20.6t	2.19(1H, m), 2.28(1H, m)
23.0t	1.78(1H, m), 2.20(1H, m)
25.8t	2.13(1H, m), 2.29(1H, q, J=9.76)
26.9q	1.31(3H, s)
27.3t	1.79(1H, m), 1.93(1H, dd, J=6.41,13.89)
36.4t	2.01(1H, t, J=12.82), 2.16(1H, m)
37.5d	1.85(1H, m)
42.3t	1.67(1H, m), 1.80(1H, m)
72.9s	-
85.8s	-
125.7s	-
126.0d	4.72(1H, t, J=5.19)
127.5d	5.39(1H, d, J=16.18)
132.5s	-
138.0d	5.54(1H, d, J=16.17)
145.9d	6.83(1H, m)
167.8s	-

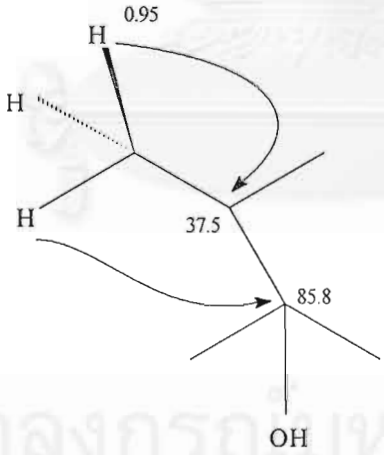
Crucial long-range ^{1}H-^{13}C correlation as established by an HMBC experiment (Fig.39) as shown below: H (1.53 ppm.) with C (132.5 ppm.), CH (126.0 ppm.), and CH_2 (36.4 ppm.) (scheme 2); H (1.31 ppm.) with C (72.9 ppm), CH (138.0 ppm.) and CH_2 (42.3 ppm.) (scheme 3); H (0.95 ppm.) with CH (37.5 ppm.) and C (85.8 ppm.) (scheme 4); H (1.85 ppm.) with CH_3 (17.3 ppm.), C (85.8 ppm.) (scheme 5).



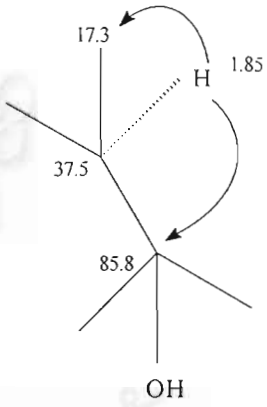
scheme 2



scheme 3

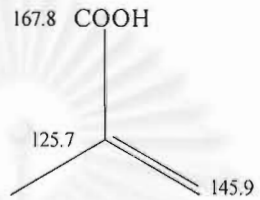


scheme 4



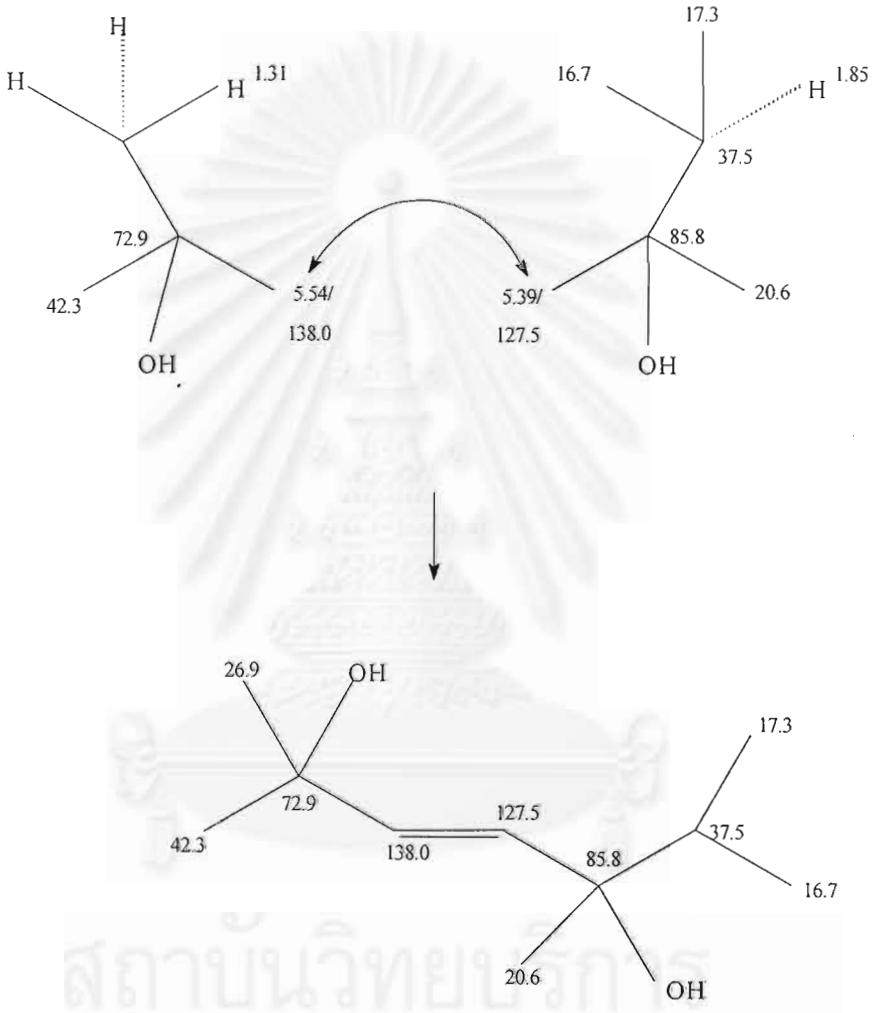
scheme 5

Because of the disappearance of carboxylic proton, long-rang correlation between this proton and carbons nearby were not observed but as there is only one position left for it to attach, which is the quarternary olefinic carbon at δ 125.7 ppm. (scheme 6).



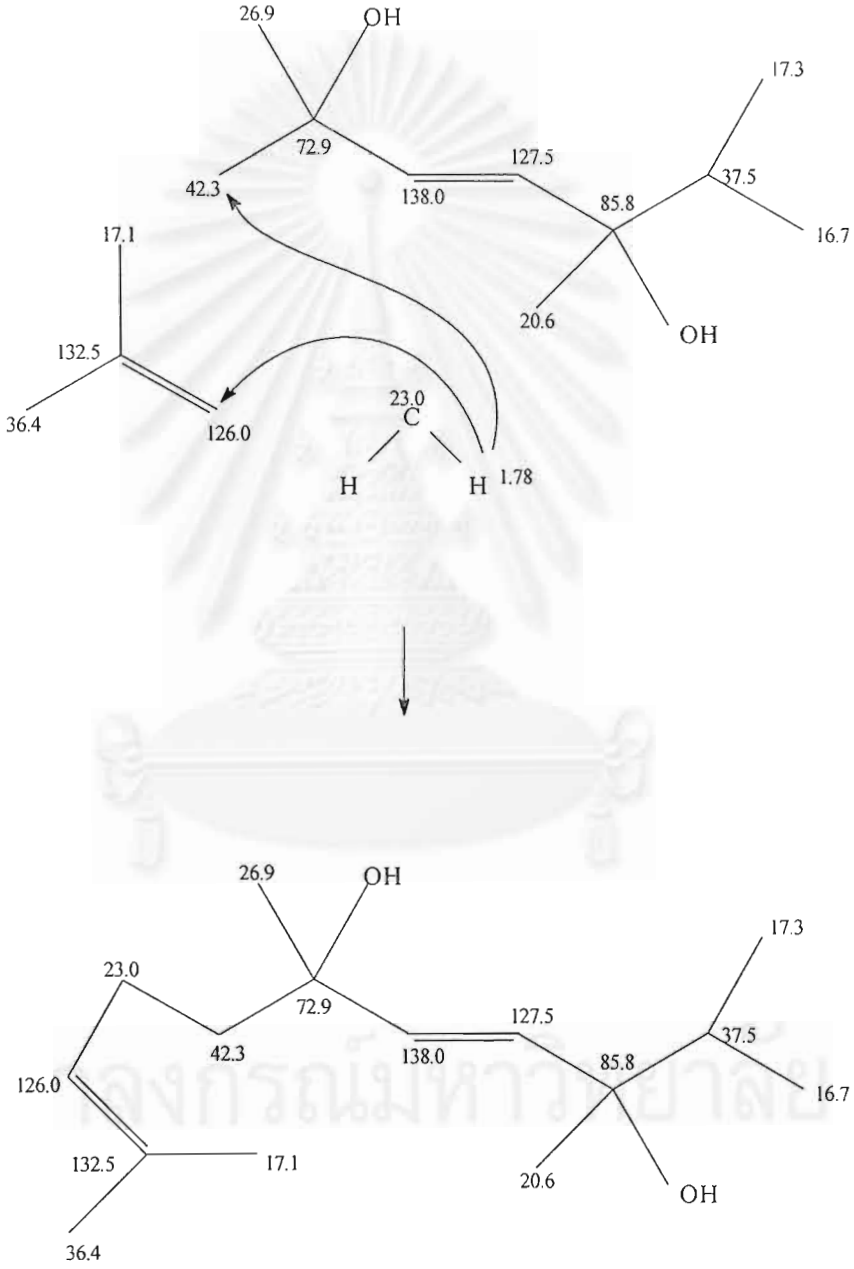
scheme 6

The COSY spectrum (Fig.40) established the one bond correlation between the proton at 5.54 (δ_C 138.0) and 5.39 ppm. (δ_C 127.5). Therefore, partial structure was obtained as follow (scheme 7).



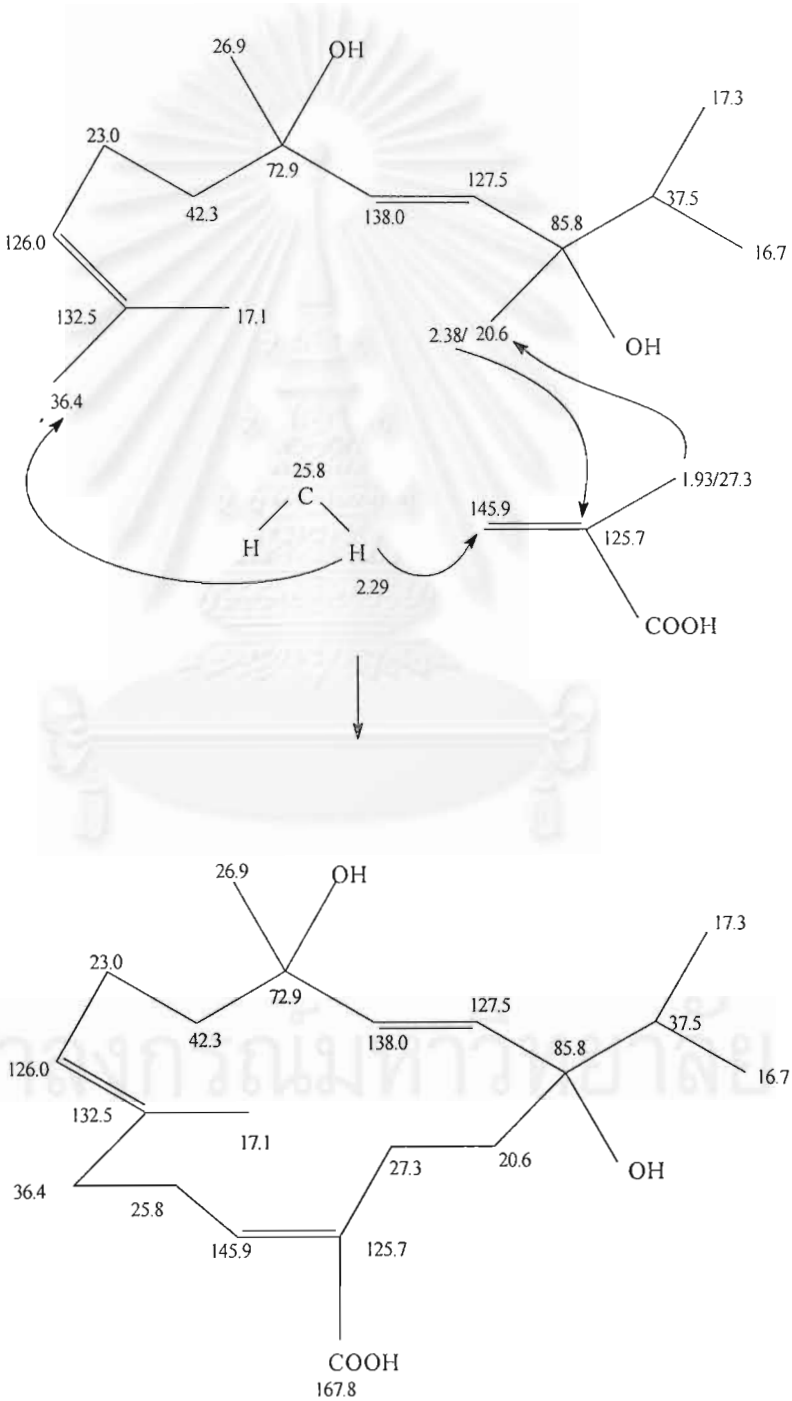
scheme 7

The CH_2 protons at 1.78 (δ_C = 23.0) ppm. also showed long-range correlation with CH_2 (42.3 ppm.) and CH (126.0 ppm.) in the HMBC sprectrum (scheme 8).



scheme 8

The protons at 2.38(δ_C = 20.6) ppm. showed long-range correlation with carbon at 125.7 ppm. and the proton at 1.93(δ_C 27.3) ppm. showed long-range correlation with CH_2 (20.6 ppm.). In addition, the protons at 2.29(δ_C 25.8) ppm. was correlated to CH(145.9 ppm.) and CH_2 (36.4 ppm.).After connecting all possible fragments together, the structure of compound 4 must structure as follow (scheme 9).



scheme 9

The long-range C-H correlation by HMBC sprectrum were summarized in Table 12 and schematically shown as follow (Fig. 8).

Table 12 ^{13}C-NMR and 2D Long-range ^{1}H - ^{13}C correlations in the HMBC spectra of compound 4.

Carbon	δ_C	Correlated
C-1	85.8 s	H-2, H-3, H-15, H-16
C-2	127.5 d	H-3, H-5
C-3	138.0 d	H-2, H-5, H-15, H-18
C-4	72.9 s	H-2, H-4, H-18
C-5	42.3 t	H-2, H-3, H-6, H-7, H-18
C-6	23.0 t	H-5, H-7
C-7	126.0 d	H-5, H-6, H-9, H-19
C-8	132.5 s	H-9, H-19
C-9	36.4 t	H-2, H-3, H-7, H-10, H-19
C-10	25.8 t	-
C-11	145.9 d	H-9, H-10
C-12	125.7 s	H-10, H-13, H-14
C-13	27.3 t	H-2, H-15
C-14	20.6 t	H-13
C-15	37.5 d	H-16
C-16	17.3 q	H-15
C-17	16.7 q	H-15
C-18	26.9 q	H-3, H-5
C-19	17.1 q	H-5, H-7, H-9
COOH	167.8 s	H-16, H-17, H-18

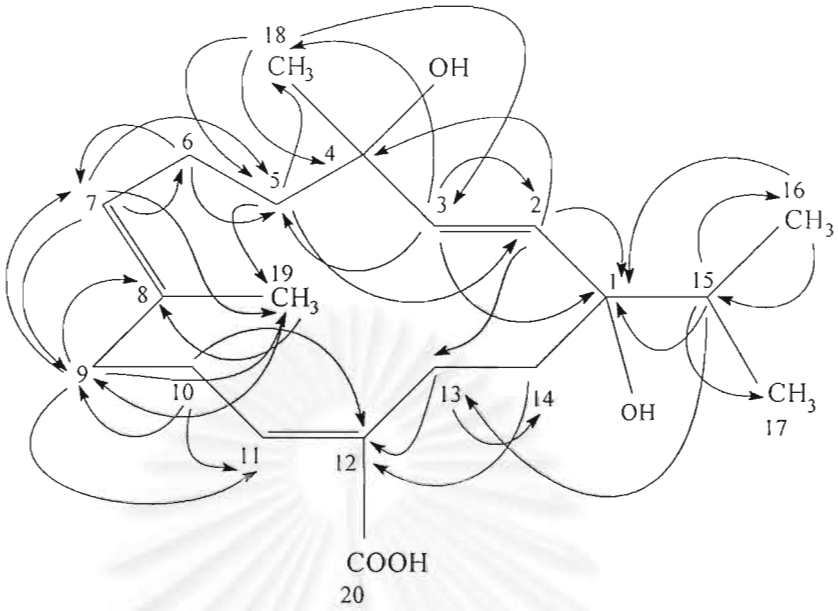


Figure 8 The HMBC correlations of compound 4.

Relative Stereochemistry at the C_1 and C_{14} of the main structure of compound 4 could not be conclusively by modelling and NOESY correlation (Fig.41). Threrfore this remains to be solved, probably by x-ray crystlallography.

From the Data above, it can be concluded that compound 4 was (2*E*,7*E*,11*E*)-1-isopropyl-1,4-dihydroxy-4,8-dimethylcyclotetradeca-2,7,11-triene-12-carboxylic acid. The same compound have been previously isolated from the stem barks of *Croton oblongifolius* Roxb. from Pethchaboon Province, Thailand by Khanitha Pudhom in 1997 although it has not been fully characterized at that time.The structure of this compound that can propose as shown in figure 9.

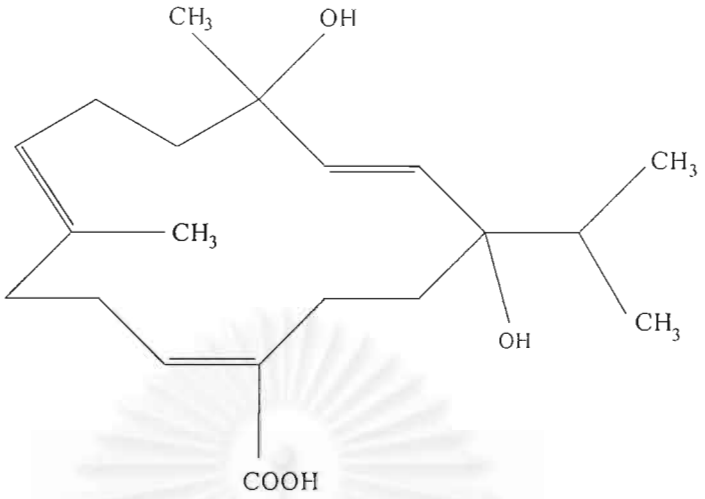


Figure 9 The structure of compound 4

Recently another related cembranoid diterpene has been isolated from *Croton joufra* [21] and the structure has been identified as 1-isopropyl -4,8-dimethylcyclotetradeca-1,4, 8-triol-2*E*,6*Z*,11*E*-triene-12-carboxylic acid was similar to that of compound 4.

5. Structure elucidation of compound 5

Compound 5 was eluted with 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 a white solid (130mg, 0.006 % wt by wt), $[\alpha]_D^{20}$ -55.3 ($CHCl_3$, *c* 1.0), R_f ; 0.45 (100% $CHCl_3$), m.p.73-74°C.

The IR spectrum of compound 5 (Fig.42) showed the presence of a hydroxy group band between 3500-3300 cm^{-1}.

Table 13 The IR absorption bands assignment of compound 5.

Wavenumber (cm^{-1})	intensity	Tentative assignment
3500-3300	broad	O-H stretching vibration of alcohol
3083,2976,2935,2873	strong	C-H stretching vibration of $-CH_2$,$-CH_3$
1720,1650	medium	C =C stretching vibration

The ^{1}H-NMR spectrum (Fig. 43) showed an ABX pattern at the olefinic region (δ 4.83-5.79 ppm.) which was assigned to a monosubstituted double bond and closely resembled pimaradiene-type diterpenes. The multiplet at 5.33 ppm. was attributed to the proton of a trisubstituted double bond.

The double bond position was confirmed by ^{13}C-NMR data (Fig.44, Table 14). Analysis of the spectral data of compound 5 suggest it was a diterpenoid closely related to compound 1. Thus, when the ^{1}H-^{13}C-NMR spectra was compared to those of compound 1, there was and appearance of the C-19 methylene group signal of compound 1 (^{1}H-NMR, δ 3.50 and 3.82 ppm.; ^{13}C-NMR, δ 64.9 ppm.) and the disappearance of a carboxyl group (^{13}C-NMR, δ 184.8 ppm.).

The DEPT-90 and DEPT-135 ^{13}C-NMR (Fig.45) indicated this compound posses twenty carbon atoms and thirty two protons. Assuming the compound may contain only carbon, proton and oxygen atoms. Thus, its molecular formular was established as $C_{20}H_{32}O$ which was confirmed by observing molecular ion at *m/z* 288 (Fig.46) which indicated that 5 DBE.

The spectroscopic data of compound 5 were consistent with (-)-pimara-9(11),15-diene-19-ol (m.p.73-74°C) which was isolated from *Acanthopanax koreanum* in 1982 [16]. The ^{13}C-NMR agreed well with those reported for (-)-pimara-9(11),15-diene-19-ol (Table 14).

Table 14 ^{13}C-NMR spectral data of compound 5 compared with (-)-pimara-9(11),15-diene-19-ol.

Carbon No.	(-)-pimara-9(11),15-diene-19-ol	Compound 5
1	41.1(t)	41.1(t)
2	18.0(t)	18.0(t)
3	35.4(t)	35.5(t)
4	38.3(s)	38.4(s)
5	46.3(d)	46.3(d)
6	19.1(t)	19.1(t)
7	26.8(t)	26.8(t)
8	28.9(d)	28.9(d)
9	151.2(d)	151.3(d)
10	37.9(s)	37.7(s)
11	115.7(d)	115.8(d)
12	37.6(t)	37.6(t)
13	34.8(s)	34.8(s)
14	41.6(t)	41.6(t)
15	150.2(d)	150.3(d)
16	109.0(t)	109.0(t)
17	22.4(q)	22.4(q)
18	26.5(t)	26.5(q)
19	64.7(t)	64.9(t)
20	26.0(q)	26.0(q)

The information from 2D-NMR techniques, HMQC correlations (Fig.47), HMBC correlations (Fig.48), COSY correlations (Fig.49), NOESY correlations (Fig.50) were used to assist the interpretation the structure of compound 5.

The long-range C-H correlations by HMBC sprectrum were summarized in figure 10

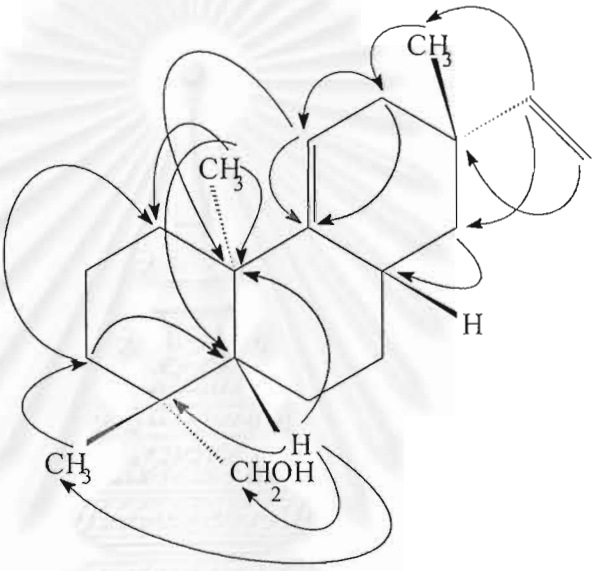


Figure 10 The HMBC correlations of compound 5.

The stereochemistry of compound 5 was confirmed by NOESY correlations, Key NOE correlations in compound 5 are shown in figure 11

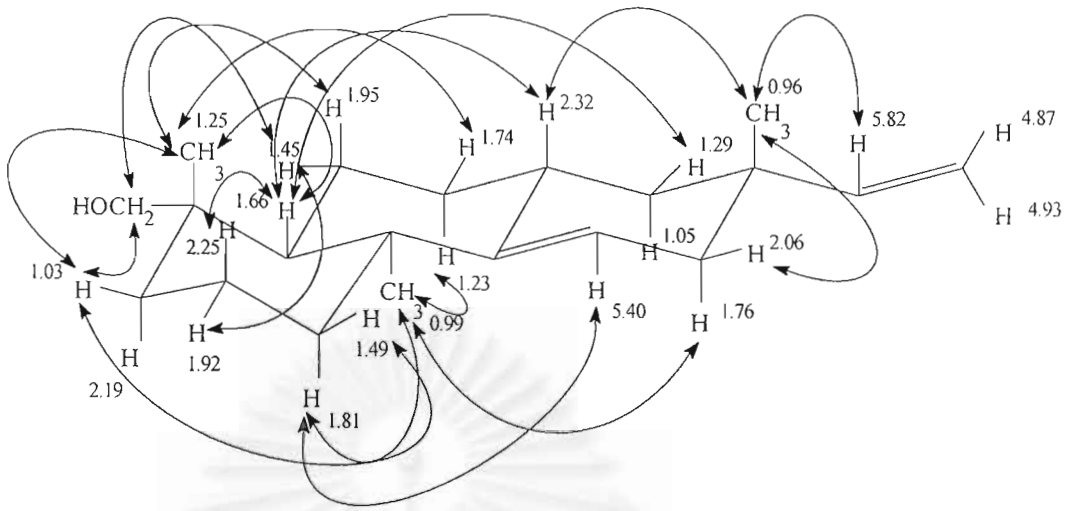


Figure 11 The NOESY correlations of compound 5

From the data above, it can be concluded that compound 5 was (-)-pimara-9 (11),15-diene-19-ol and the structure of compound 5 can be shown below.

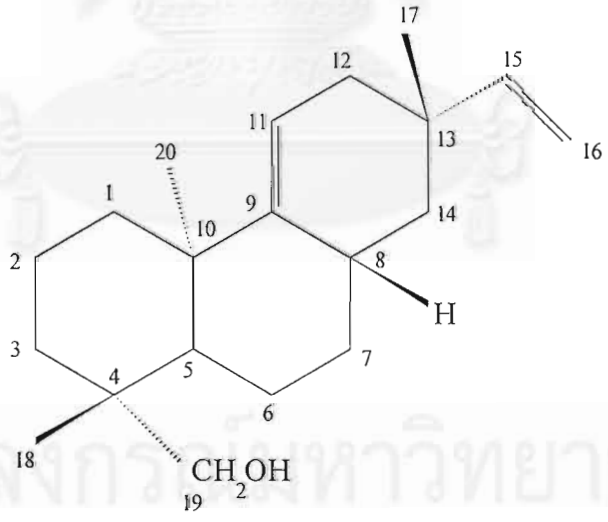


Figure 12 The structure of compound 5

6. Structure Elucidation of compound 6

Compound 6 was eluted with 40% $CHCl_3$ in 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 a white solid (391mg, 0.02 % wt by wt), $[\alpha]_D^{20}$-62.8 ($CHCl_3$, *c* 1.0), R_f ; 0.50 (100% $CHCl_3$), m.p.108-109 °C.

The IR spectrum of compound 6 (Fig.51) revealed the presence of a β-substituted furan ring at 1560, 1510, and 880 cm^{-1}.

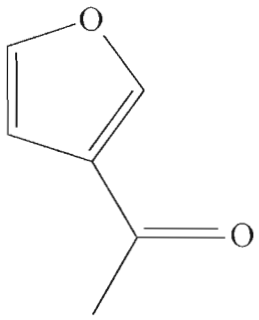
Table 15 The IR absorption bands assignment of compound 6.

Wavenumber (cm^{-1})	Intensity	Tentative assignment
3012,2935,2873	Strong	C-H stretching vibration of $-CH_2$,-CH_3
1731,1772	Strong	C=O stretching vibration
1675	medium	C=C stretching vibration
1199,1163	medium	C-O stretching vibration

The ^{1}H-NMR spectrum (Fig.52) showed the pattern of a β-monosubstituted furan at 8.00, 7.42 and 6.42 ppm. which agreed with ^{13}C-NMR absorption at 147.0, 144.3 and 108.5, respectively.

From ^{13}C-NMR spectrum (Fig.53), DEPT-90 and DEPT-135 ^{13}C-NMR spectrum (Fig.54), there were twenty one carbon atoms and twenty four protons. This compound probably contained carbon, hydrogen and oxygen atoms. The molecular fomular, $C_{21}H_{24}O_6$, was determined from its mass spectrum (Fig.55) which showed the molecular ion at *m/z* 372 and indicated DBE of 10.

The prominent ion at *m/z* 263(M-109) indicated that the compound 6 probably contained a furano-carbonyl side chain.



a furano-carbonyl side chain

The information from 2D-NMR tetchniques, HMQC correlations (Fig.56, Table16), HMBC correlations (Fig.57, Table 17), COSY correlation (Fig.58, Table 18), were used to assist the interpretion the structure of compound 6.

Table 16 The HMQC spectral data of compound 6.

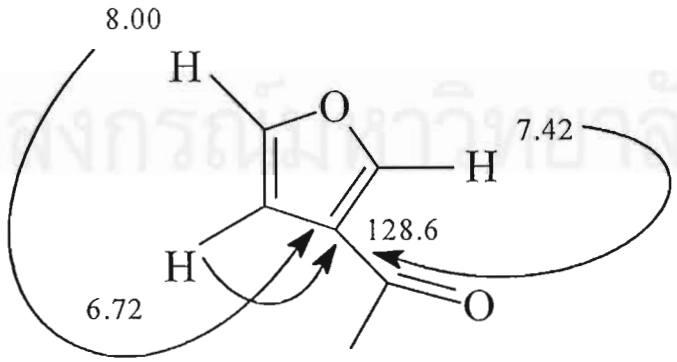
^{13}C-NMR (ppm.)	^{1}H-NMR (ppm.),coupling constant (Hz)
19.2q	0.82(3H, s)
20.1t	1.64(1H, m), 1.87(1H, m)
22.1t	2.05(1H, m), 1.87(1H, m)
27.3t	2.21(1H, m), 2.29(1H, m)
33.2t	1.35(1H, dd, J=2.14,3.97), 1.99(1H, m)
39.6d	-
45.1s	-
46.5t	2.83(1H, d, J=17.7), 3.04(1H, d, J=17.7)
46.6d	2.72(1H, dd, J=1.22,12.51)
48.7d	3.21(1H, dd, J=4.28,12.82)
51.4q	3.60(3H, s)
71.4t	3.93(1H, dd, J=2.14,8.24), 4.33(1H, d, J=8.24)
108.5d	6.72(1H, dd, J=0.61,1.83)

Table 16 The HMQC spectral data of compound 6. (continued)

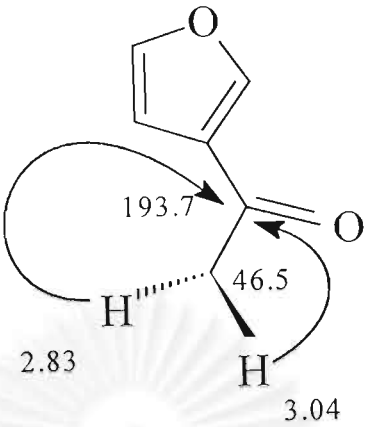
^{13}C-NMR (ppm.)	^{1}H-NMR (ppm.),coupling constant (Hz)
128.6s	-
136.3d	6.74(1H, dd, J=2.44,7.32)
137.8s	-
144.3d	7.42(1H, t, J=1.53)
147.0d	8.00(1H, dd, J=0.92,1.53)
169.1s	-
174.0s	-
193.7s	-

The long-range ^{1}H-^{13}C correlation were determined by HMBC experiment (Fig.57, Table 17). The singlet carbon (128.6 ppm.) showed correlation with three protons (8.00, 7.42, 6.72 ppm.) (scheme 10). Furthermore, the protons at 2.83, 3.04 ppm.(δ_C 46.5) showed correlation with carbon at 193.7 ppm. (scheme 11).

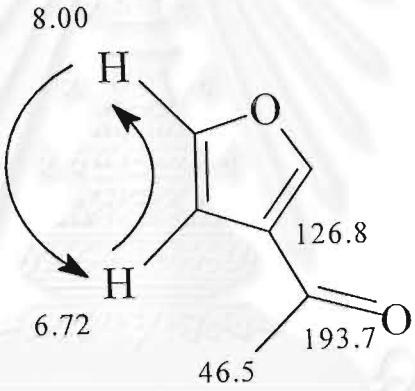
The COSY spectrum (Fig.58) of compound 6 showed the connectivity with δ 8.00 and 6.72 ppm. (scheme 12). From the scheme 9-11 the positions of a furano-carbonyl side chain could be assigned as in scheme 13.



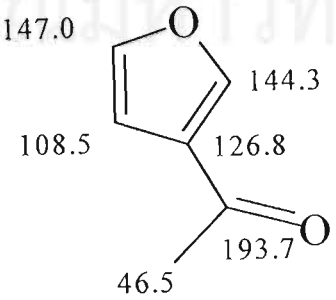
scheme 10



scheme 11

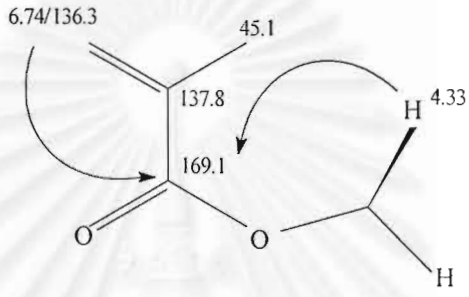


scheme 12

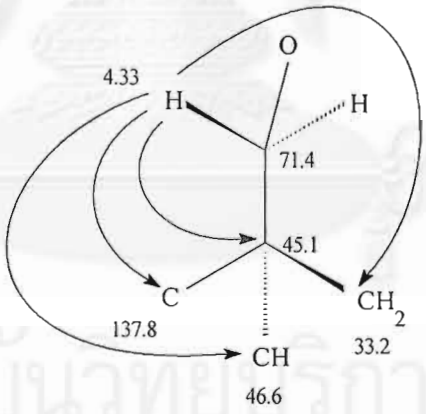


scheme 13

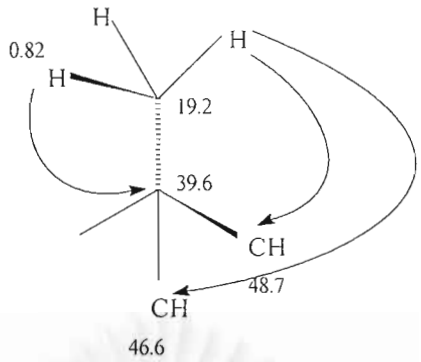
Crucial long-range ^{1}H-^{13}C correlation were : H (6.74 ppm.) and H (4.33 ppm.) with C (169.1 ppm.) (scheme 14); H (4.33 ppm.) with C (137.8 ppm.), CH (46.6 ppm.), C (45.1 ppm.) and CH_2 (33.2 ppm.) (scheme 15); H (0.82 ppm.) with C (39.6 ppm.), CH (46.6 ppm.), CH (48.7 ppm.) (scheme 16); H (3.21 ppm.) and H (3.60 ppm.) with C (174 ppm.) (scheme 17).



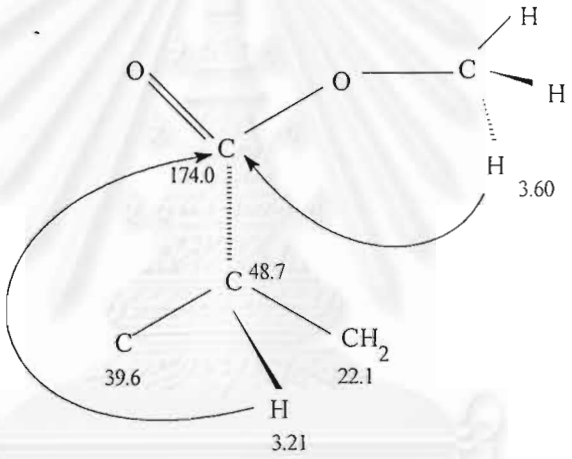
scheme 14



scheme 15

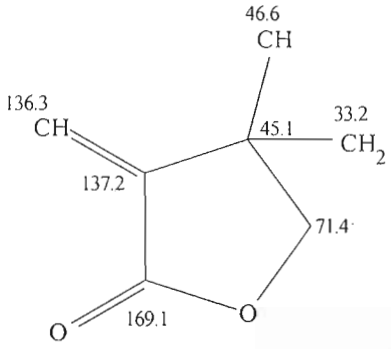


scheme 16

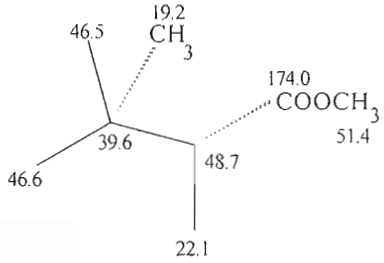


scheme 17

The structure in scheme 14 and scheme 15 shared a common carbon atom resonating at 137.8 ppm. Thus the two structures could be joined as shown in scheme 18. Furthermore, the structure in scheme 16 and 17 shared the same two carbon atoms at 39.6 and 48.7 ppm. The combined structure was shown in scheme 19.

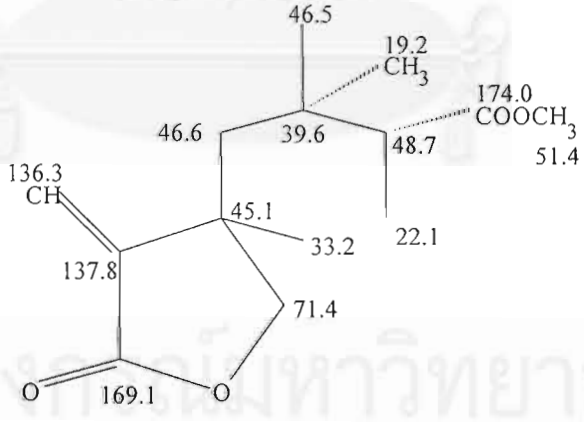


scheme 18



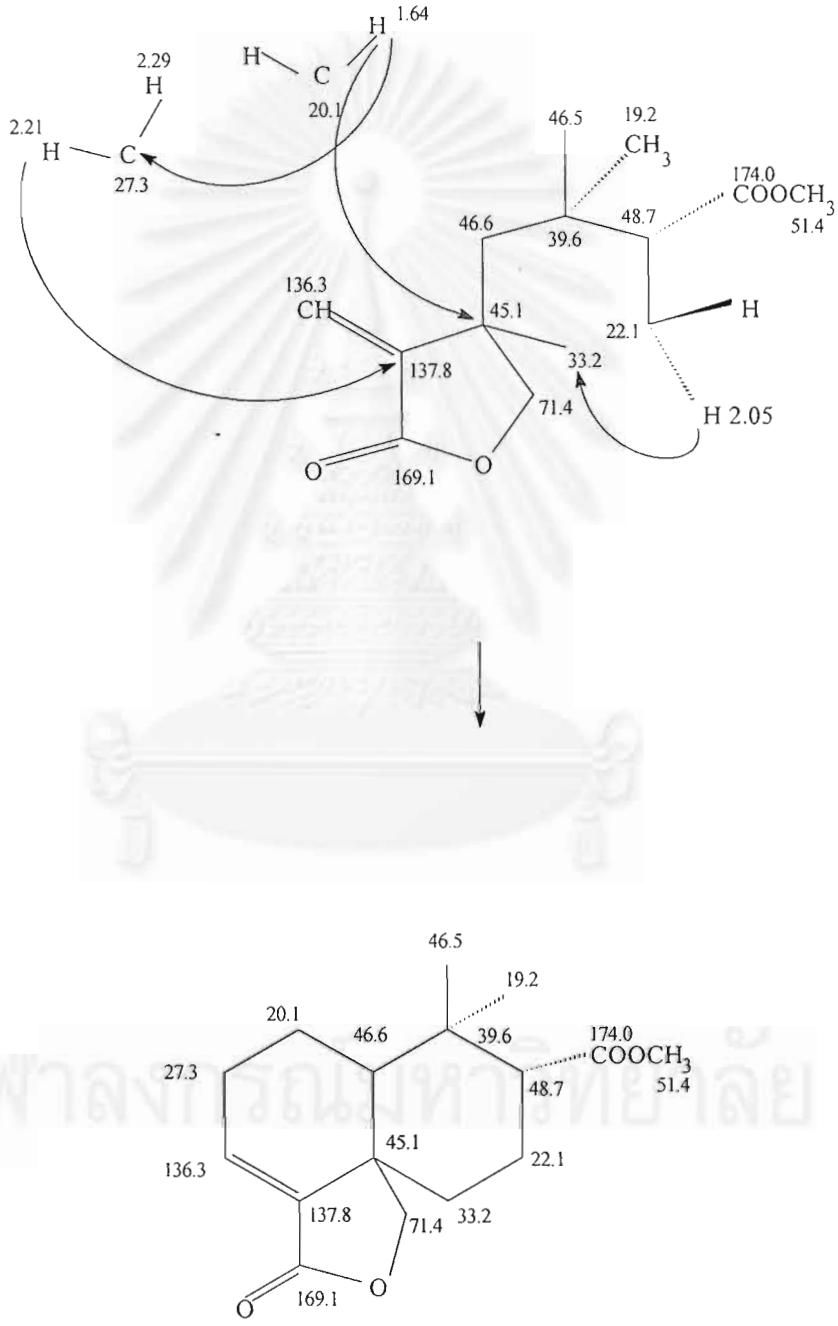
scheme 19

The structure in scheme 17 and scheme 18 could be further joined via the common carbon atom resonating at δ 46.6 ppm. Then, the combined structure was shown below (scheme 20).



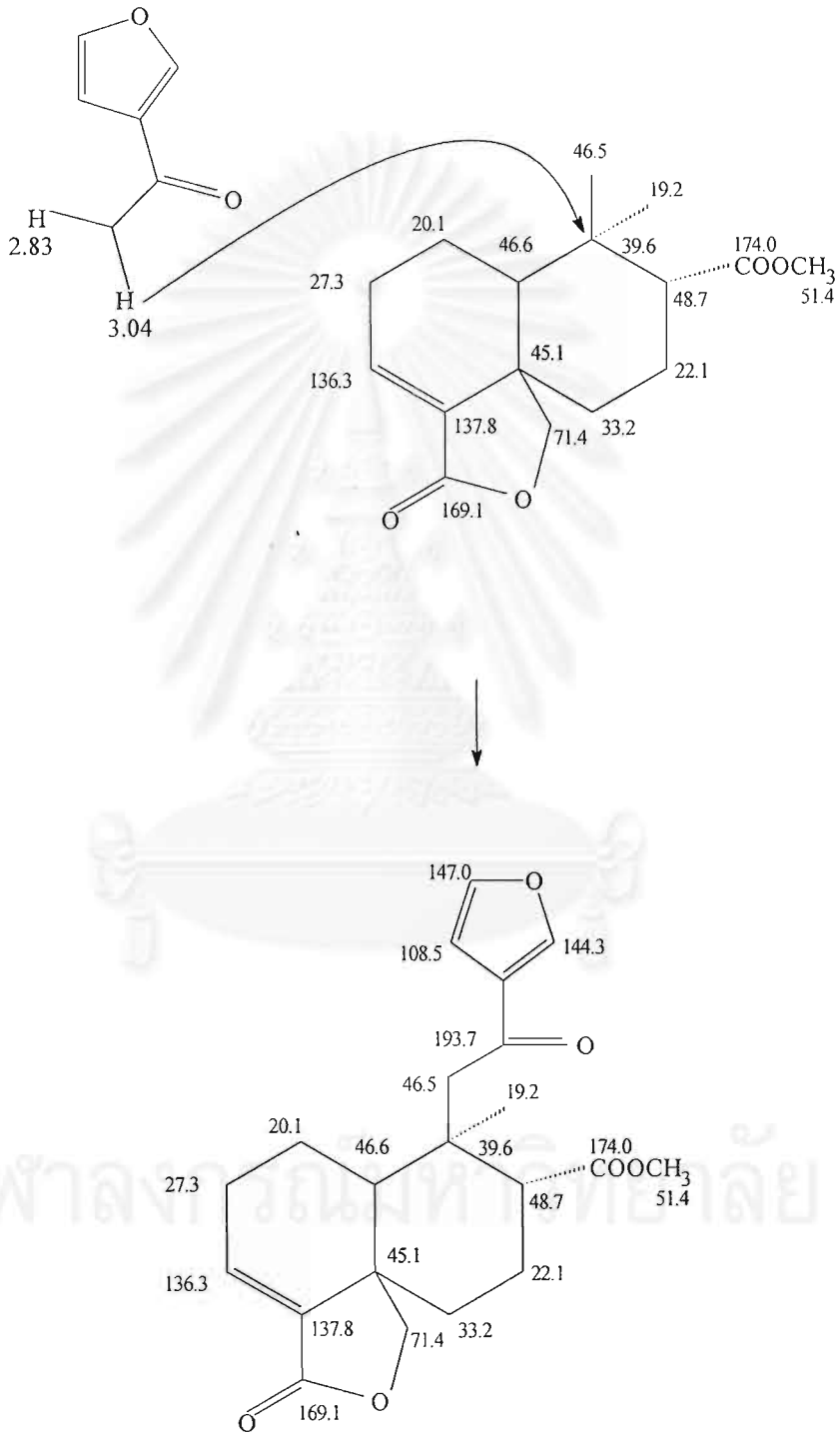
scheme 20

From the HMBC spectra (Fig.57), the protons at 2.21, 2.29 ppm. showed long-range correlations with C (137.8 ppm.), the proton at 1.64 ppm. with a quarternary C (45.1 ppm.) and the proton at 2.05 ppm with CH_2 (33.2 ppm.) as shown below (scheme 21).



scheme 21

The proton at 2.83 and 3.04 ppm. in a furano-carbonyl side chain showed long-range correlation with C (39.6 ppm.) (scheme 22).



scheme 22

By the HMBC spectra (Fig.57), the position of the substituents of compound 6 could be deduced as follow (Fig.13).

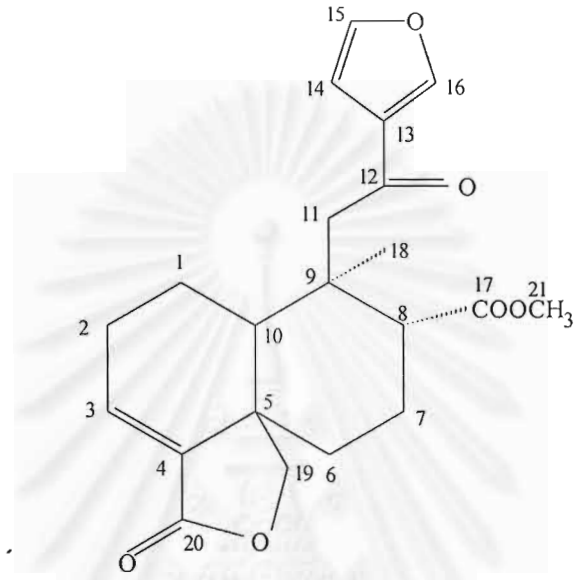


Figure 13 The position of the substituents of compound 6

Table 17 ^{13}C-NMR and 2D Long-range ^{1}H-^{13}C correlations in the HMBC spectra of compound 6.

Carbon	δ_C	Correlated
C-1	20.1 t	H-2, H-3, H-10
C-2	27.3 t	H-1, H-3, H-10
C-3	136.3 d	H-1, H-2
C-4	137.8 s	H-2, H-19
C-5	45.1 s	H-1, H-3, H-6, H-10, H-19
C-6	33.2 t	H-7, H-10, H-19
C-7	22.1 t	H-6, H-8
C-8	48.7 d	H-6, H-7, H-11, H-18
C-9	39.6 d	H-1, H-8, H-10, H-11, H-18
C-10	46.6 d	H-2, H-6, H-7, H-11, H-18
C-11	46.5 t	H-8, H-18
C-12	193.7 s	H-11
C-13	128.6 s	H-14, H-15, H-16
C-14	108.5 d	H-15, H-16
C-15	147.0 d	H-14, H-16
C-16	144.3 d	H-14, H-15
C-17	174.0 s	H-8, H-21
C-18	19.2 q	H-8, H-10, H-11
C-19	71.4 t	H-10
C-20	169.1 s	H-3, H-19
C-21	51.4 q	-

Table 18 COSY and NOSY spectral data of compound 6

Proton	COSY	NOESY
H-1	H-2	H-2, H-10, H-19
H-2	H-3	H-1, H-3, H-10
H-3	H-2	H-2
-	-	-
-	-	-
H-6	H-7	H-8, H-10
H-7	H-6	H-8, H-18, H-19
H-8	H-7	H-6, H-7, H-10
-	-	-
H-10	-	H-1, H-2, H-6, H-8
H-11	-	H-1
-	-	-
-	-	-
H-14	H-15, H-16	H-15, H-16, H-21
H-15	H-14, H-16	H-11, H-14, H-16, H-21
H-16	H-14, H-15	H-14, H-15
-	-	-
H-18	-	H-7, H-19
H-19	-	H-1, H-6, H-7, H-18
-	-	-
H-21	-	H-14, H-15

Stereochemistry of compound 6 could be confirmed by NOESY correlation (Fig.59, Table 18). The relative stereochemistry of compound 6 was determided on the basis of NOESY spectra and key NOE correlation in compound 6 are shown in figure 14.

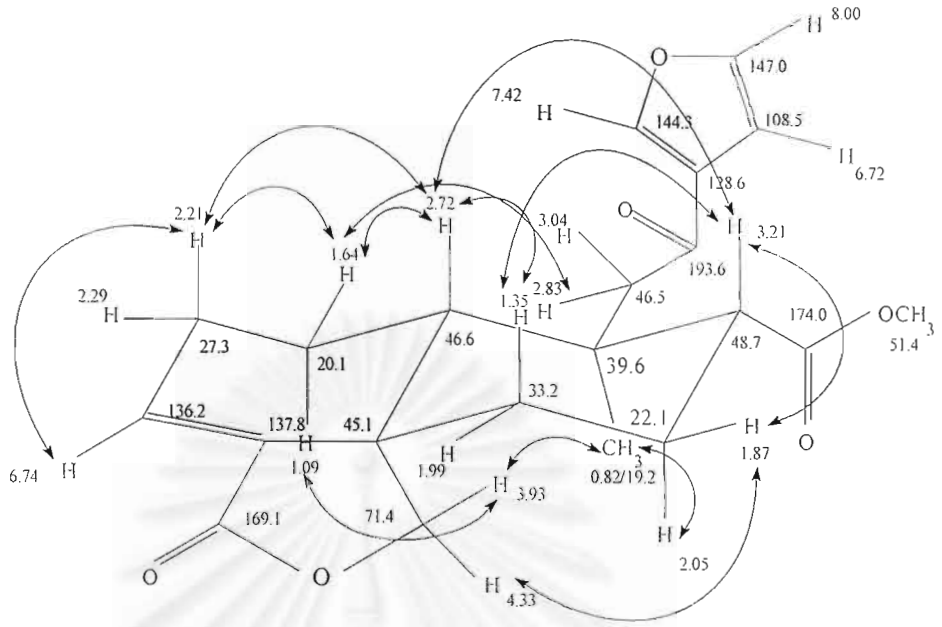


Figure 14 The NOESY correlations of compound 6

From the Data above, it can be concluded that compound 6 was new clerodane diterpene compound, Methyl-15,16-epoxy-12-oxo-3,13(16),14-clerodatriene-20,19-olide-17-oate.The structure of compound 6 can be shown below.

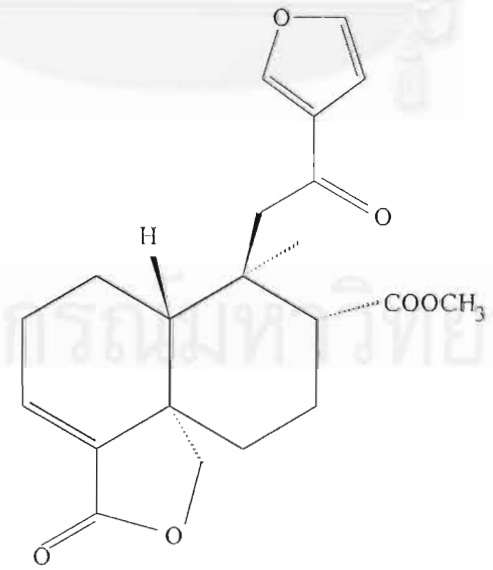
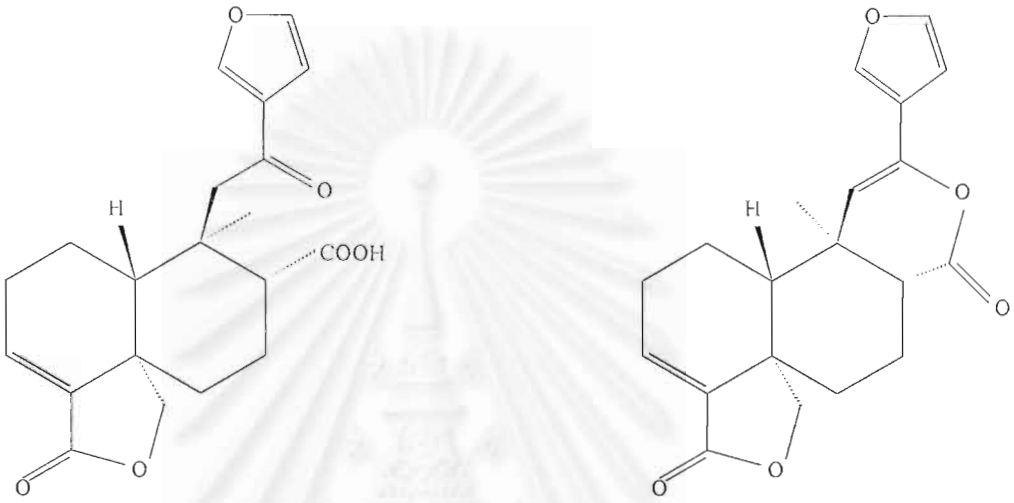


Figure 15 The structure of compound 6

However, it is difficult to confirm that compound 6 is really the constituent of *Croton oblongifolius* Roxb. or it is an artifact from methanolysis of precursors such as 6a and 6b.



6a 6b

Fig. 16 The substrates of compound 6

An insect antifeedant clerodane, Tanabalin have been recenly isolated from the dried flowers of a Brazilian medicinal plant *Tanacetum balsamita* [22].

Comparison of the structure of Tanabalin (Fig 17) with that of compound 6, indicated that this compound differed from compound 6 in having an acetoxy group in place of the carbonyl group of compound 6 at C-11 and a methyl group inplace of the methyl ester group of compound 6 at C-17.

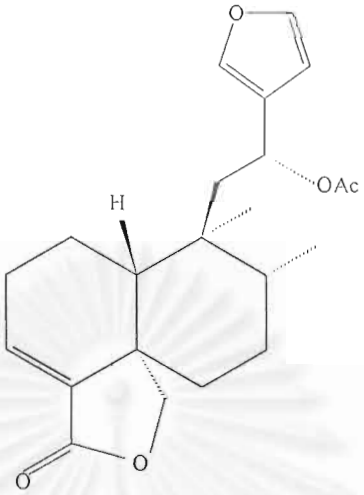
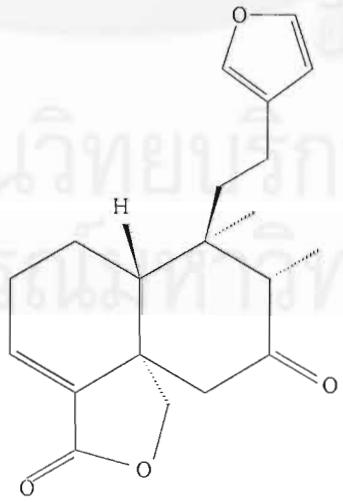


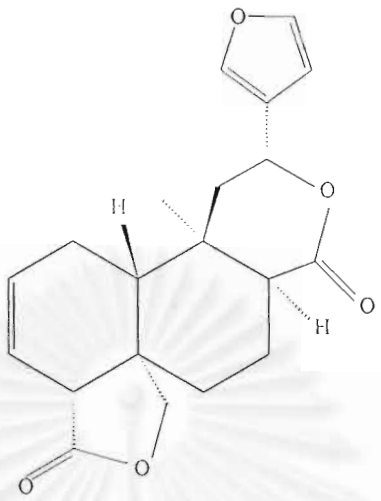
Fig. 17 The structure of Tanabalin.

There are some other known compounds which have similar the structures to that of compound 6 such as 15,16-Epoxy-7-oxo-3,13(16),14-clerodatriene-18,19-olide [23], 15,16-Epoxy-2,13(16),14-clerodatriene-17,12:18,19-diolide [24], 15,16-Epoxy-3,13(16),14-clerodatriene-17,12:18,19-diolide[25], 15,16-Epoxy-12-hydroxy-3,13 (16),14-clerodatriene-18,19-olide [26].

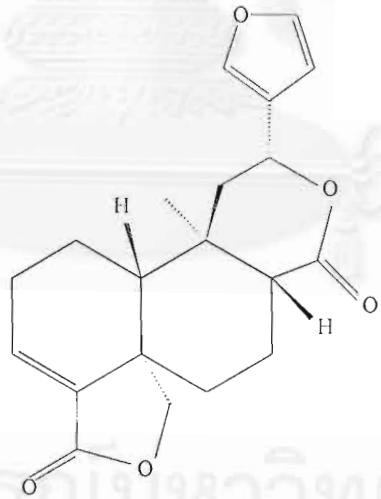


15,16-Epoxy-7-oxo-3,13(16),14-clerodatriene-18,19-olide

Fig. 18 The structures were similar to that of compound 6.

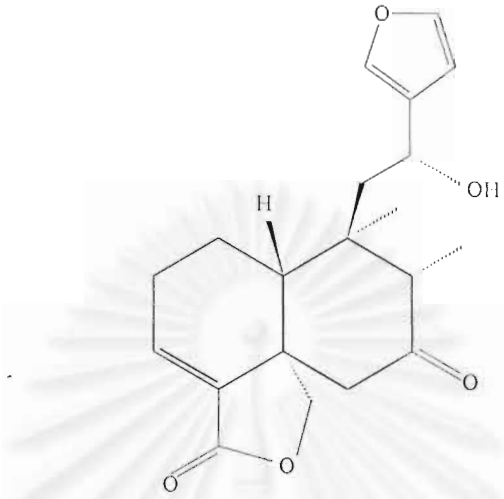


15,16-Epoxy-2,13(16),14-clerodatriene-17,12:18,19-diolide



15,16-Epoxy-3,13(16),14-clerodatriene-17,12:18,19-diolide

Fig. 18 The structures were similar to that of compound 6. (continued)



15,16-Epoxy-12-hydroxy-3,13(16),14-clerodatriene-18,19-olide

Fig. 18 The structures were similar to that of compound 6. (continued)

7. Result of biological activity test

Cytotoxic activity against cell lines

Compound 1-6 (10 μg/ml) were tested the *in vitro* activity against 6 cell lines such as fibroblast (HS27), gastric carcinoma (KATO-3), breast carcinoma (BT474), lung carcinoma (CHAGO), colon carcinoma (SW 620), hepato carcinoma (HEP-G2).

The cytotoxic activity of all compounds from *Croton oblongifolius* Roxb. against 6 cell lines were reported in Table 19.

Table 19 Cytotoxic activity against 6 cell line of compounds 1-6 from *Croton oblongifolius* Roxb.

Compound	% Survival of cell line					
	HS27 (fibroblast)	KATO (gastric)	BT 474 (breast)	CHAGO (lung)	SW 620 (colon)	HEP-G2 (hepatoma)
1	116	77	117	85	95	95
2	120	85	112	100	108	92
3	127	81	90	95	97	83
4	110	78	93	99	111	68
5	89	16	43	66	62	14
6	119	79	89	105	93	79

All compounds showed cytotoxic activity against 6 cell lines. Moreover, compound 5 which consisted of an alcohol group, showed remarkable cytotoxic activity against all cell lines tested. The cytotoxicity data of this compound 5 as shown in Table 20

Table 20 Cytotoxicity data of compound 5

Compound	IC_{50} (□μg/ml) for cell lines					
	HS27 (fibroblast)	KATO (gastric)	BT 474 (breast)	CHAGO (lung)	SW 620 (colon)	Hep-G2 (hepatoma)
5	7.4	6.5	>10	6.1	5.9	6.7

From Table 19 and Table 20, compound 5 showed moderated cytotoxic activity against 6 cell lines and exhibited cytotoxic activity against the gastric carcinoma (KATO-3), lung carcinoma (CHAGO), colon carcinoma (SW 620) and hepato carcinoma (HEP-G2), *in vitro*, with IC_{50} values of 6.5, 6.1, 5.9 and 6.7 μg/ml, respectively.

CHAPTER V

CONCLUSION

In the course of research work, the stem barks of *Croton oblongifolius* Roxb. from Amphur Muang, Uttaradit Povince were investigated for their chemical constituents and their biological activity. The concentrated methanolic extract of *Croton oblongifolius* Roxb. stem bark was re-extracted with hexane. The hexane crude extract was saparated on silica gel column chromatography using hexane-chloroform gradient system to obtain six compounds, two of new diterpenoids, (2*E*,7*E*,11*E*)-1-isopropyl-1,4-dihydroxy-4,8-dimethylcyclotetradeca -2,7,11-triene-12-carboxylic acid and Methyl-15,16-epoxy-12-oxo-3,13(16),14-clerodatriene-20,19-olide-17-oate and four known diterpenoids, (-)-Pimara-9(11),15-diene-19-oic acid, Crotocembraneic acid, Neocrotocembraneic acid and (-)-Pimara-9(11),15-diene-19-ol.

All isolated substrances were summarized in Table 18.

Table 21 Isolated substances from the stem barks of *Croton oblongifolius* Roxb.

Compound	Name of compound	% wt by wt
1	(-)-Pimara-9(11),15-diene-19-oic acid	0.16
2	Crotocembraneic acid	0.01
3	Neocrotocembraneic acid	0.007
4	(2*E*,7*E*,11*E*)-1-Isopropyl-1,4-dihydroxy-4,8-dimethylcyclotetradeca -2,7,11-triene-12-carboxylic acid	0.004
5	(-)-Pimara-9(11),15-diene-19-ol	0.006
6	Methyl-15,16-epoxy-12-oxo-3,13(16),14-clerodatriene-20,19-olide-17-oate	0.02

The isolated compounds showned cytotoxicity against 6 cell lines such as fibrolast (HS27), gastric carcinoma (KATO-3), breast carcinoma (BT474), lung carcinoma (CHAGO), colon carcinoma (SW 620), hepato carcinoma (HEP- G2) Moreover, compound 5, (-)-Pimara-9(11),15-diene-19-ol, which consisted of an alcohol group, showed moderated cytotoxic activity against 6 cell lines and exhibited cytotoxic activity against the gastric carcinoma (KATO-3), lung carcinoma (CHAGO), colon carcinoma (SW 620) and hepato carcinoma (HEP-G2), *in vitro*, with IC_{50} values of 6.5, 6.1, 5.9 and 6.7 μg/ml, respectively.

Proposal for the future work

The discivery of compounds belonging to *Croton oblongifolius* Roxb. would be interesting for future investigation. The hexane crude extract of the stem bark of this plant have been interested because there were isolated some compounds that shown their activity from this crude extract. So, it is interesting to in vestigate bioactive compounds which have not been isolated from this crude extract. In addition, the future work should be investigate the relative stereochemistry of compound 4 which lead to confirmed the new structure compound to be additional information about organic compounds. The absolute structure of compound 4 was determined by X-ray crystallography together with modified Mosher's method. In the aspect of searching for chemical constituents, the hexane crude extract might be investigate which may lead to discovery of new structure compounds.

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APPENDIX

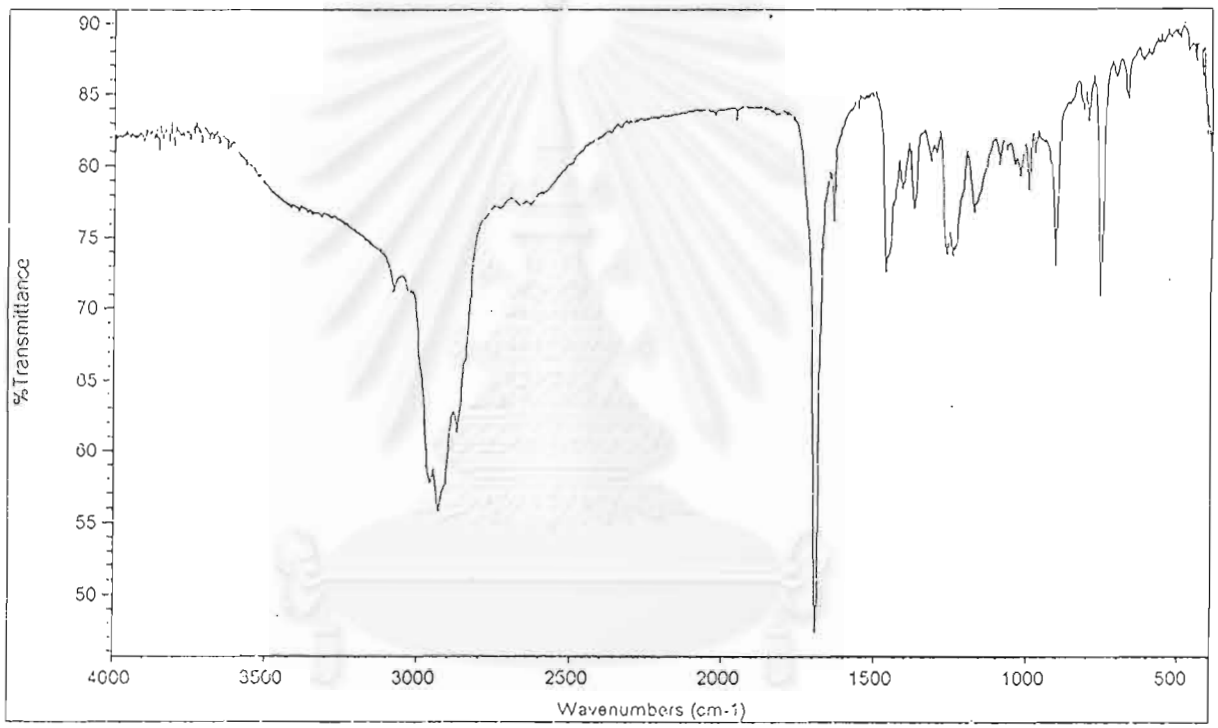

Figure 19 The IR spectrum of compound 1

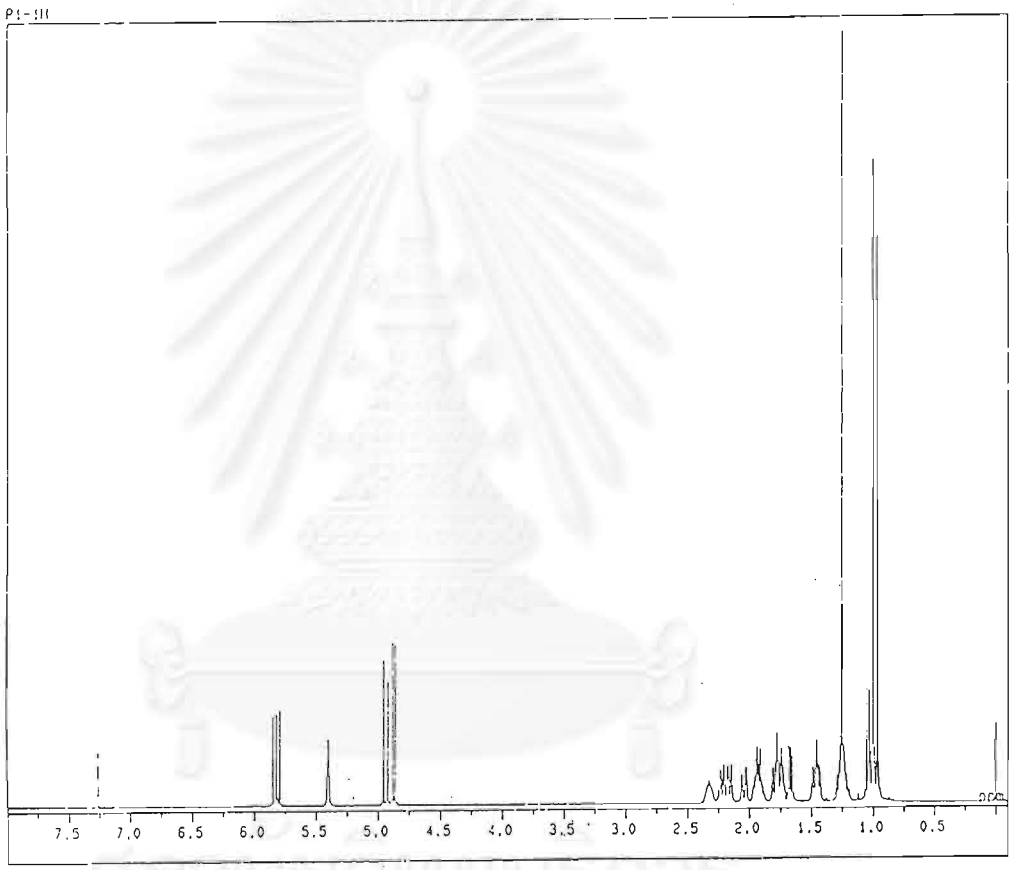

Figure 20 The ^{1}H-NMR spectrum of compound 1

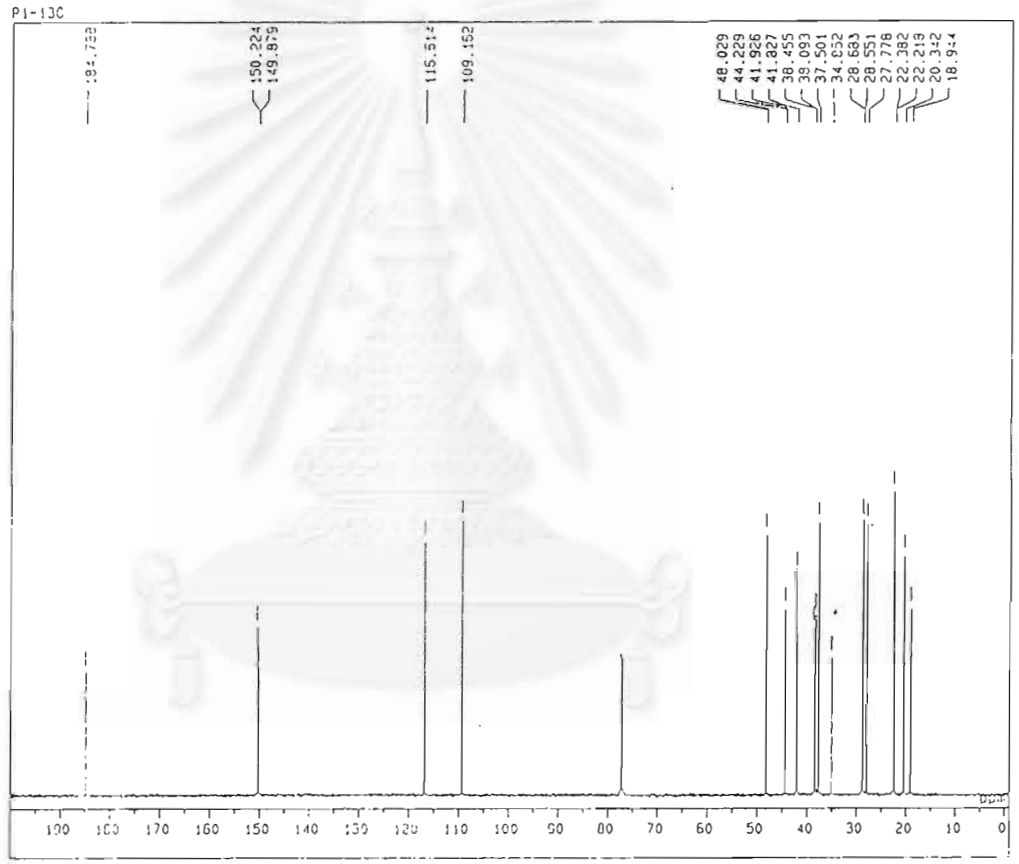

Figure 21 The ^{13}C-NMR spectrum of compound 1

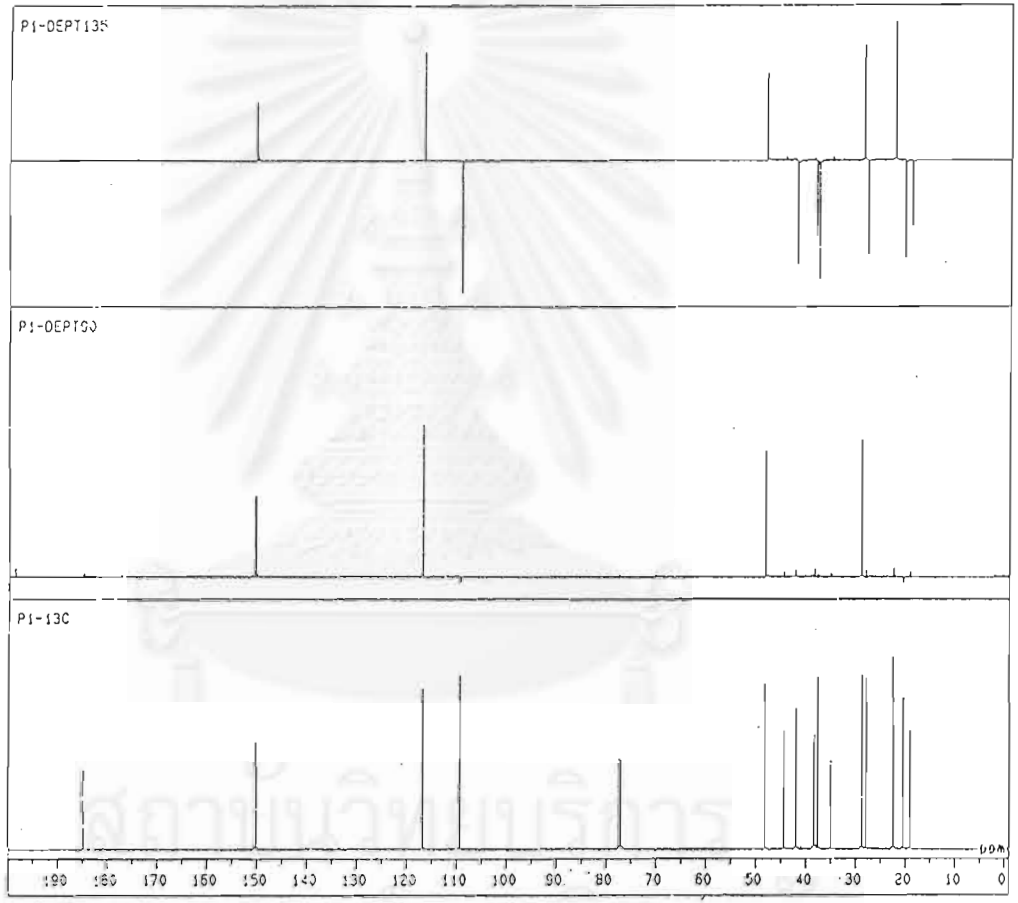

Figure 22 DEPT-135, 90 ^{13}C-NMR spectrum of compound 1

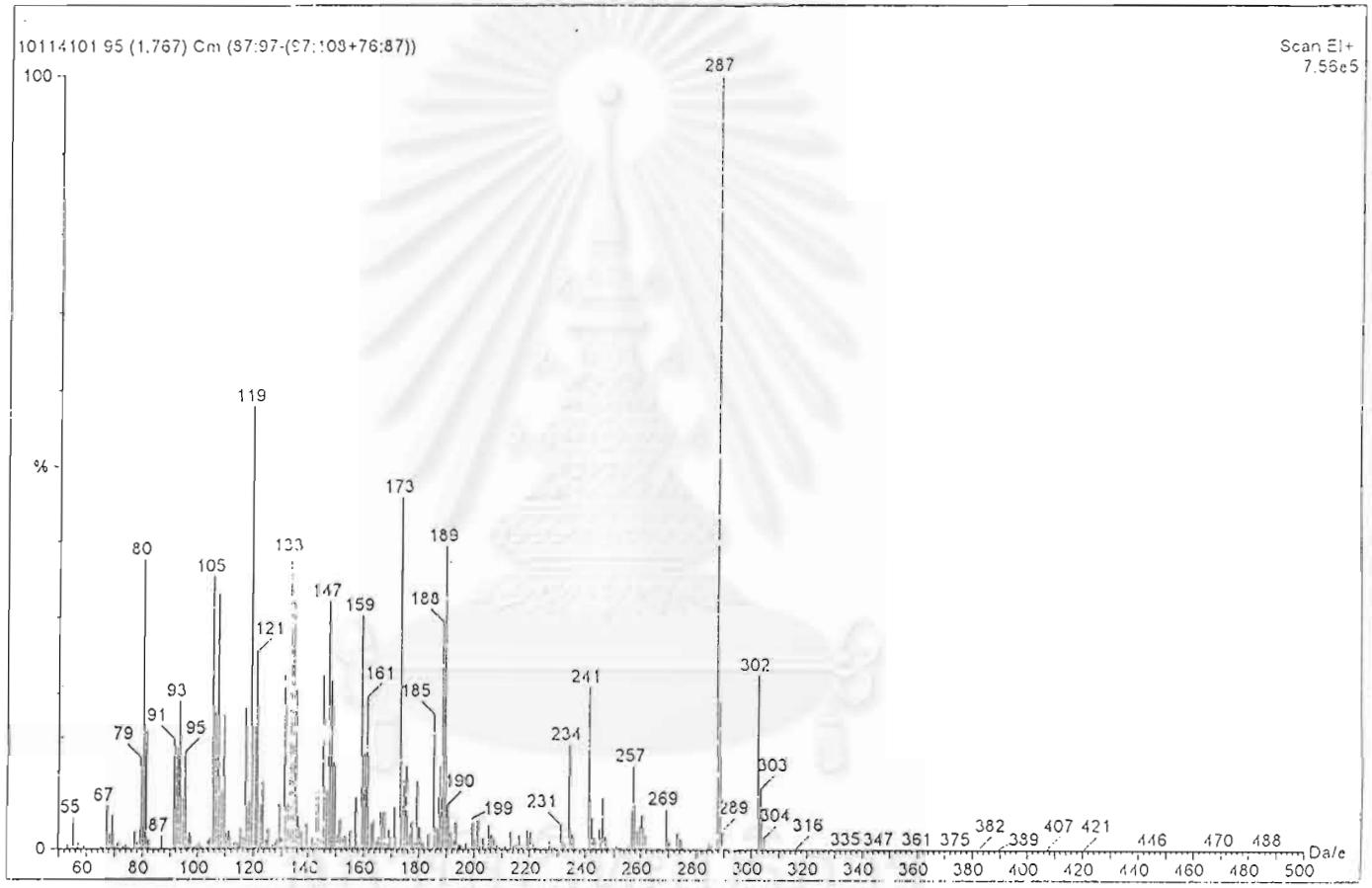

Figure 23 The EI MS spectrum of compound 1

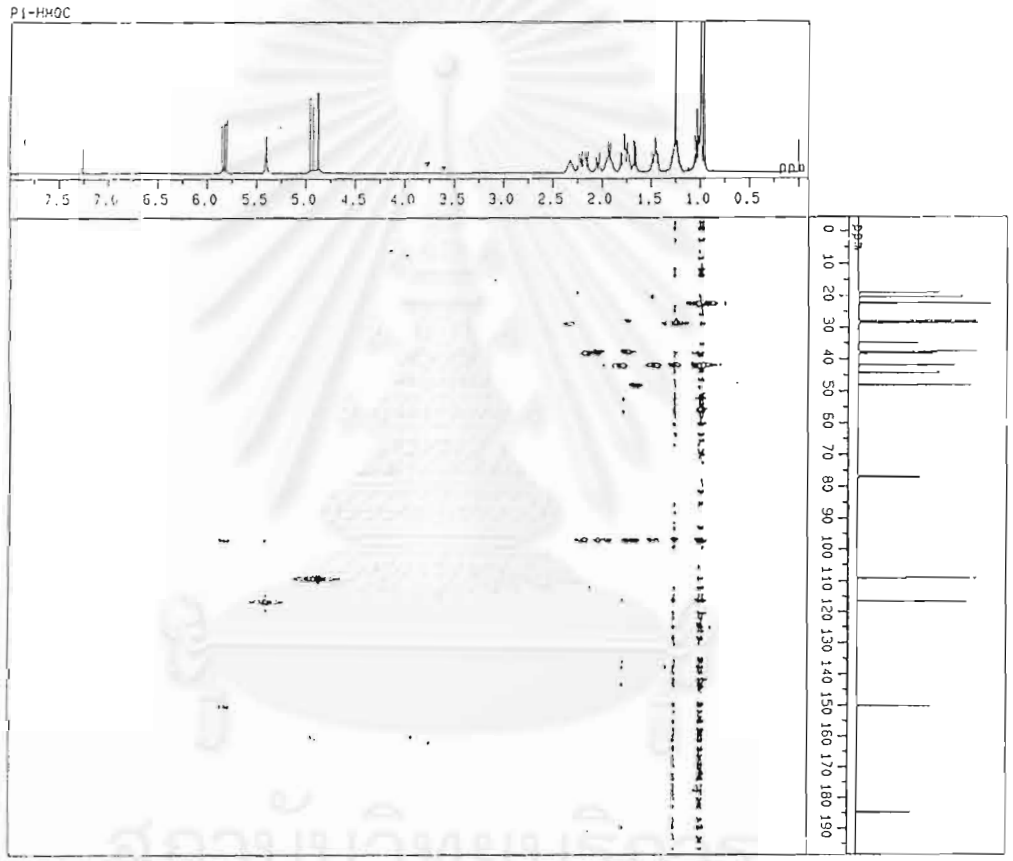

Figure 24 The HMQC-NMR spectrum of compound 1

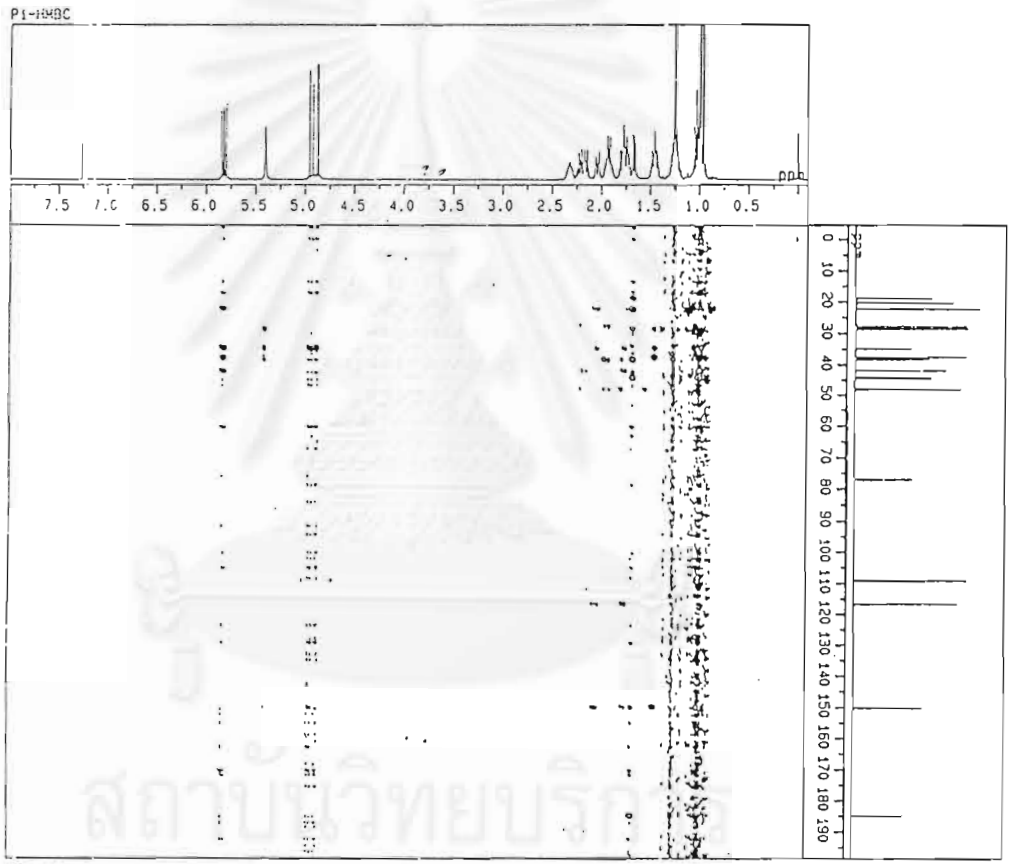

Figure 25 The HMBC-NMR spectrum of compound 1

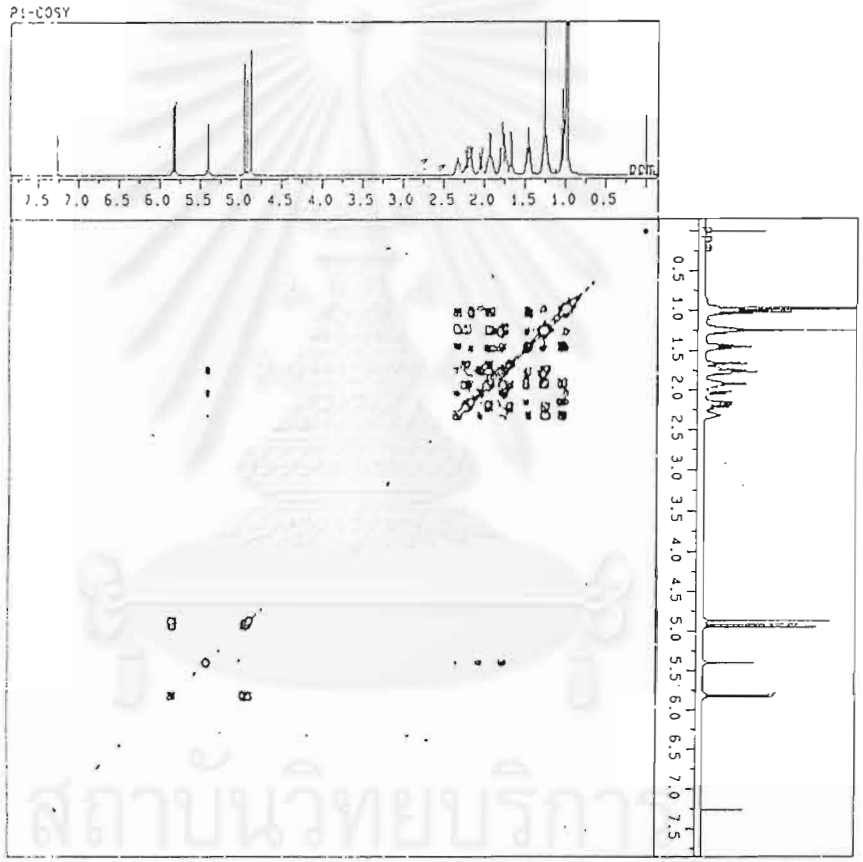

Figure 26 The COSY-NMR spectrum of compound 1

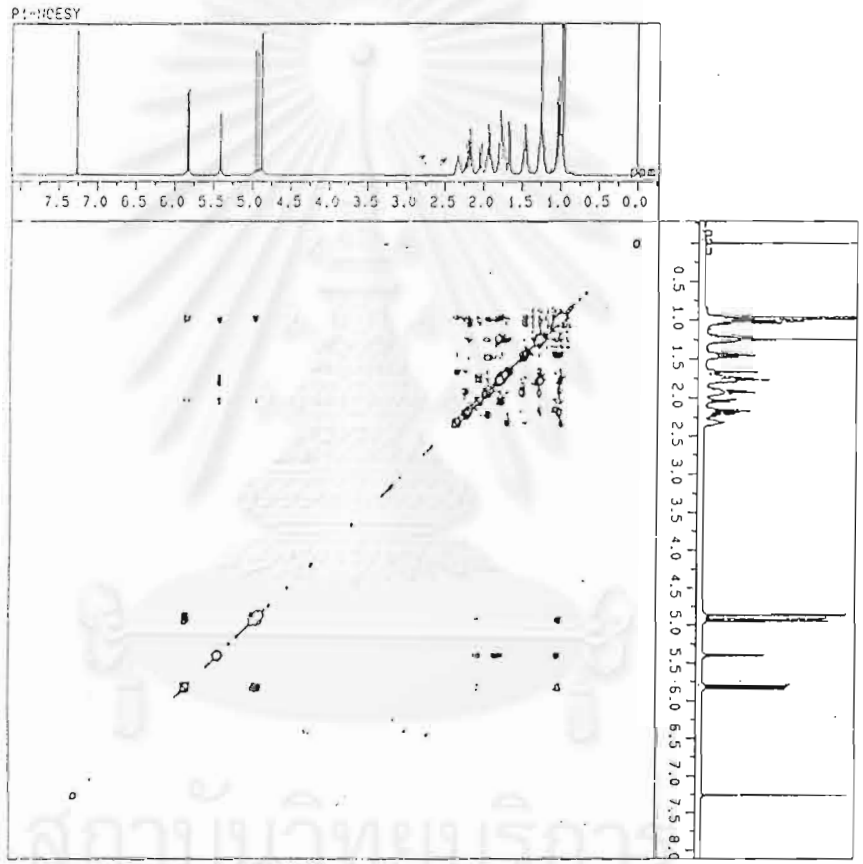

Figure 27 The NOESY-NMR spectrum of compound 1

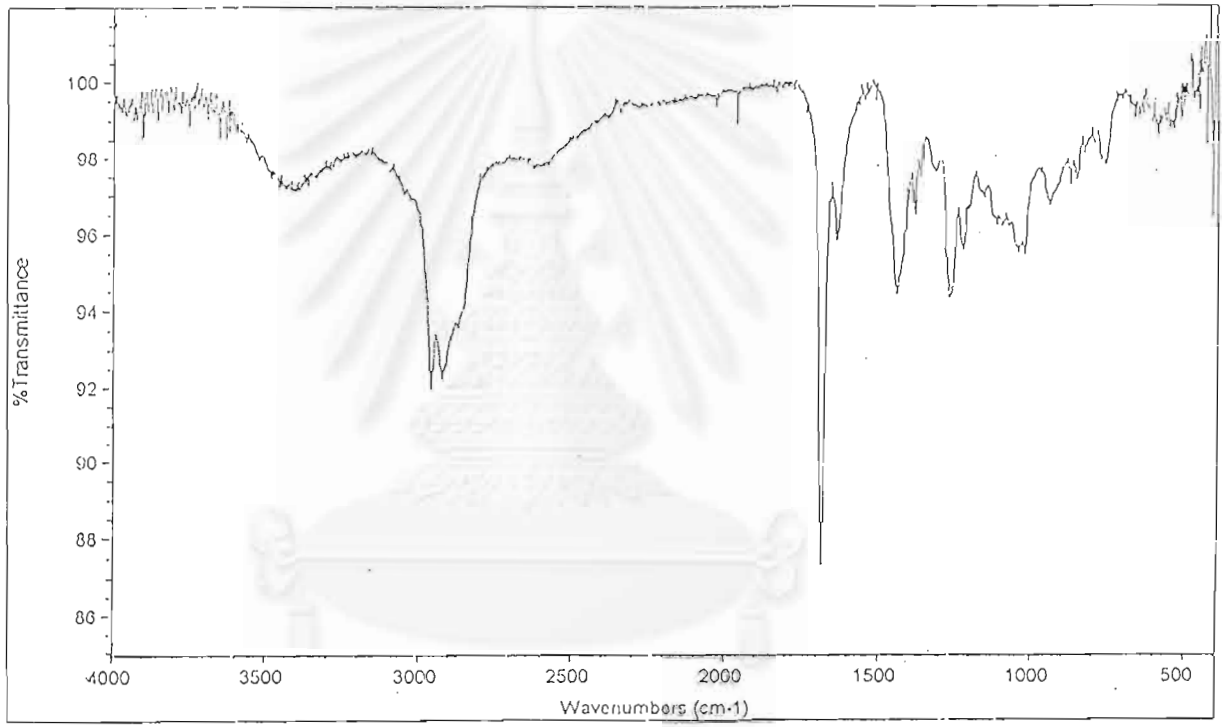

Figure 28 The IR spectrum of compound 2

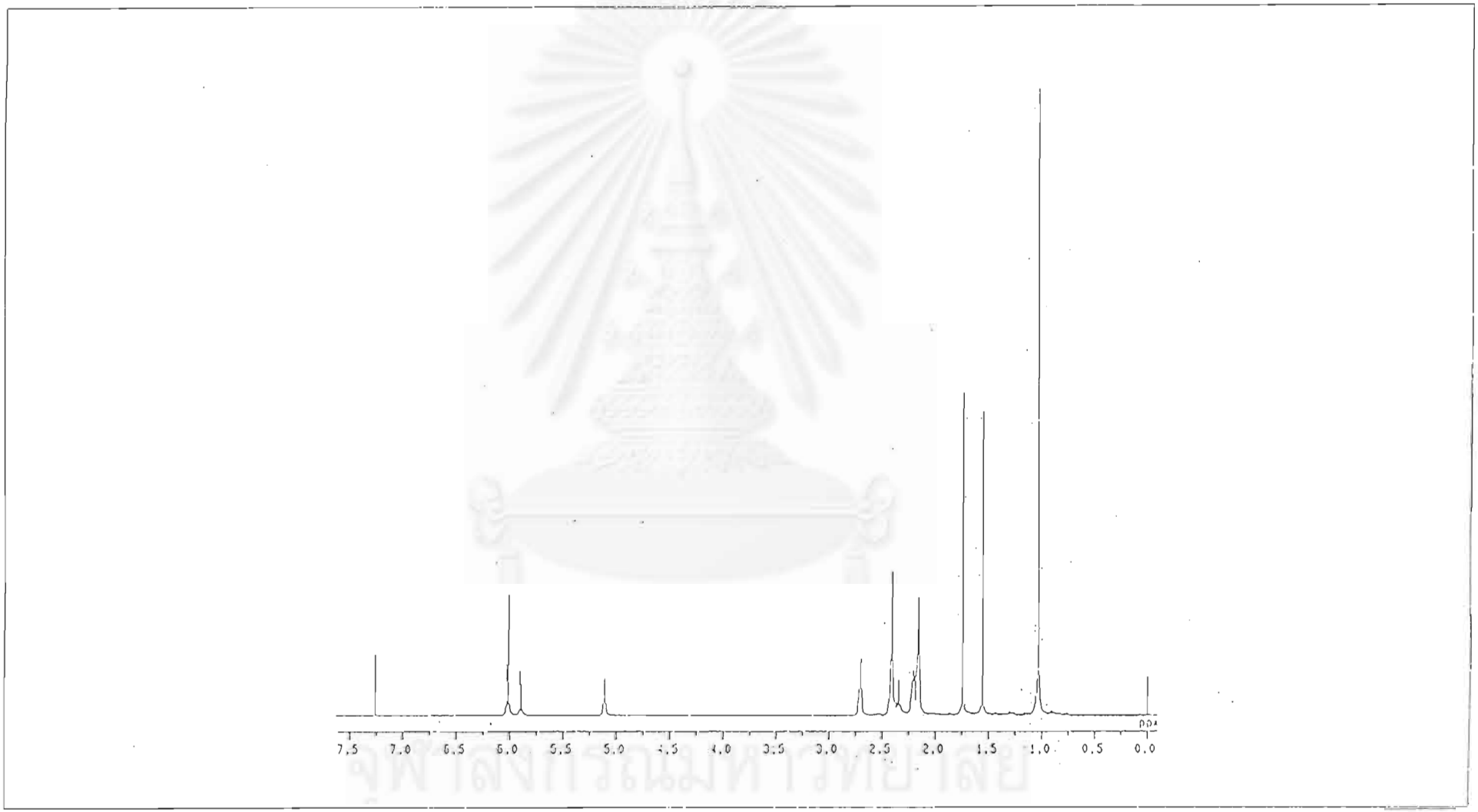

Figure 29 The ^{1}H-NMR spectrum of compound 2

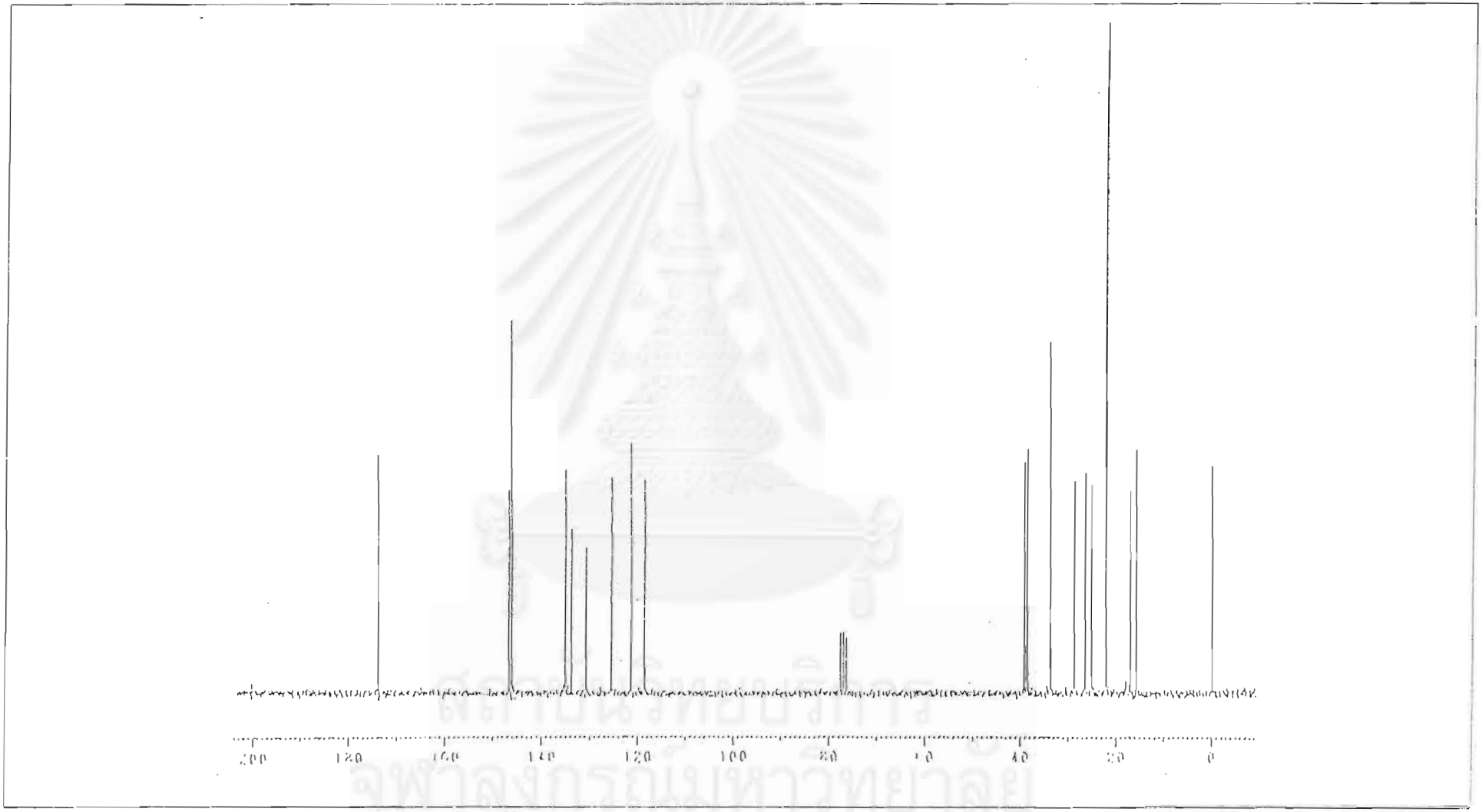

Figure 30 The ^{13}C-NMR spectrum of compound 2

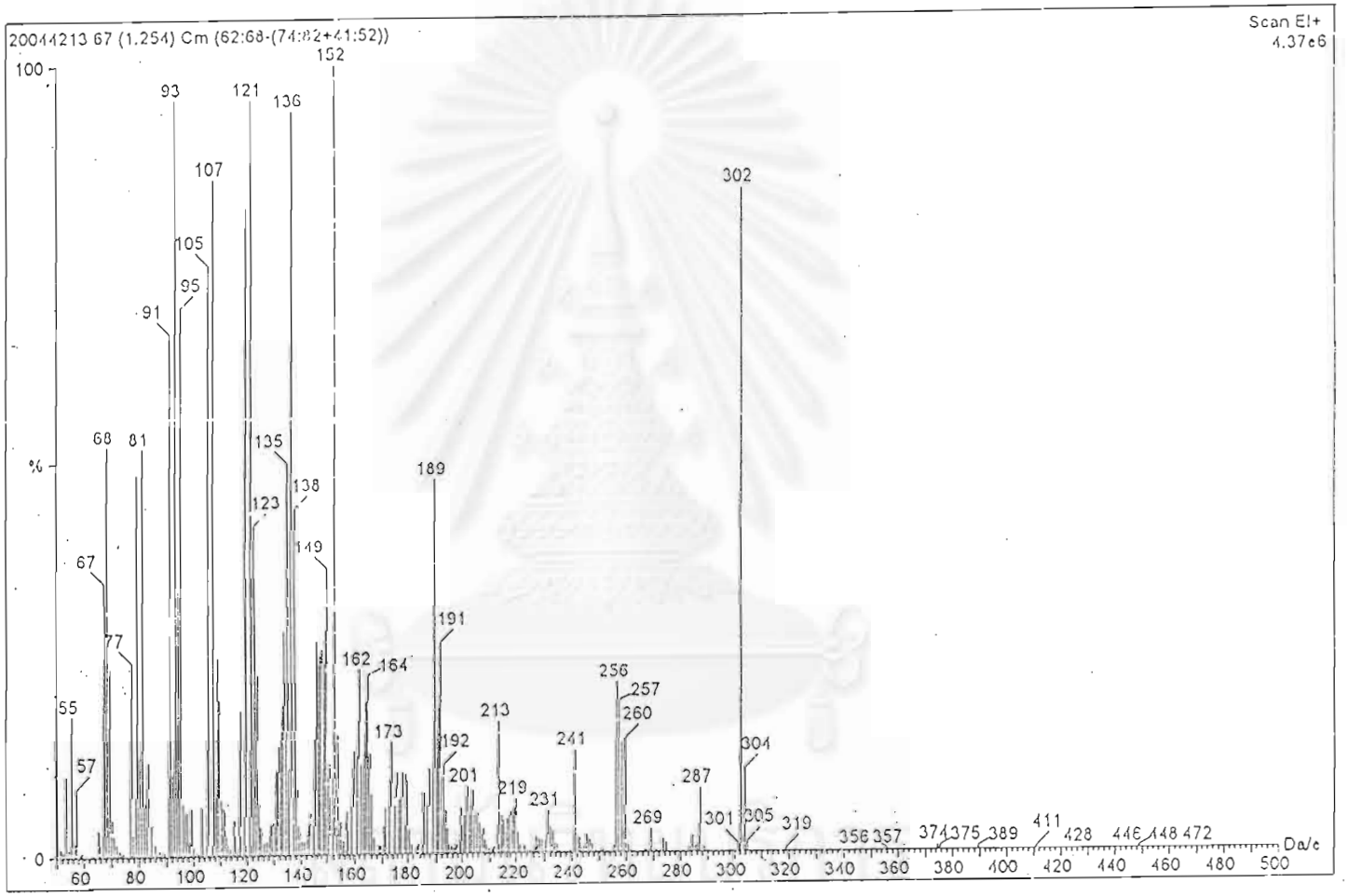

Figure 31 The EI MS spectrum of compound 2

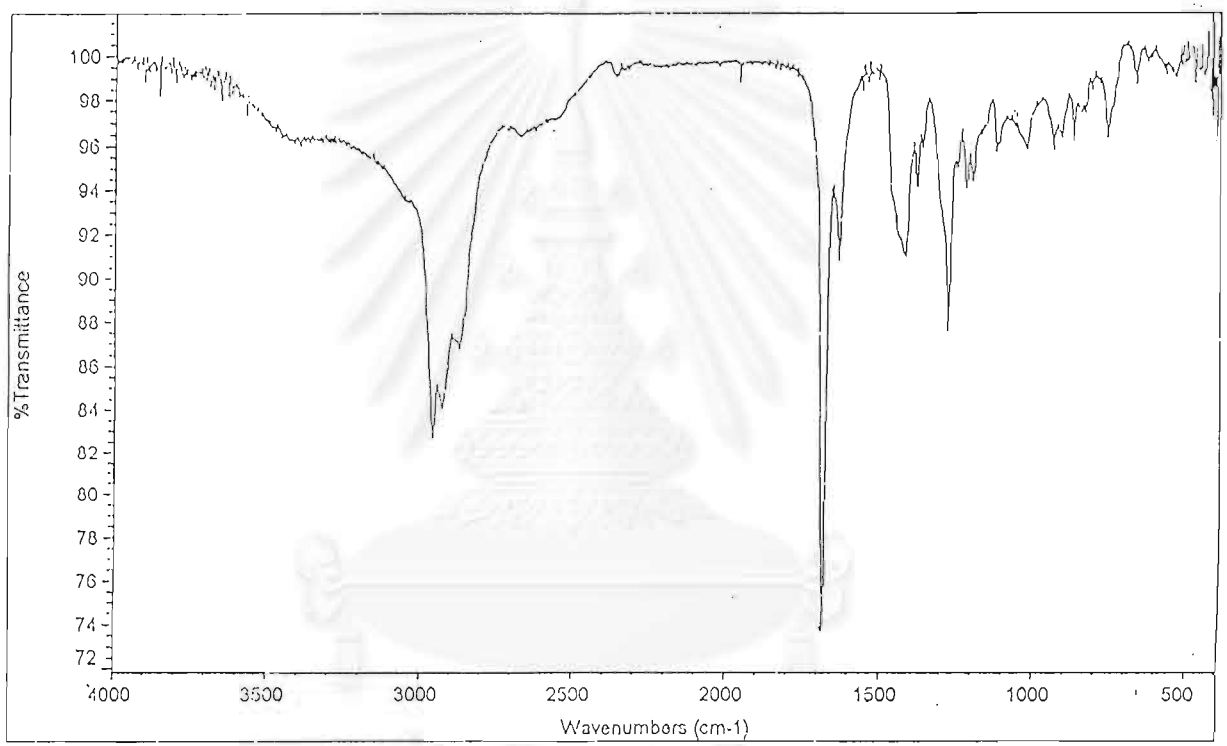

Figure 32 The IR spectrum of compound 3

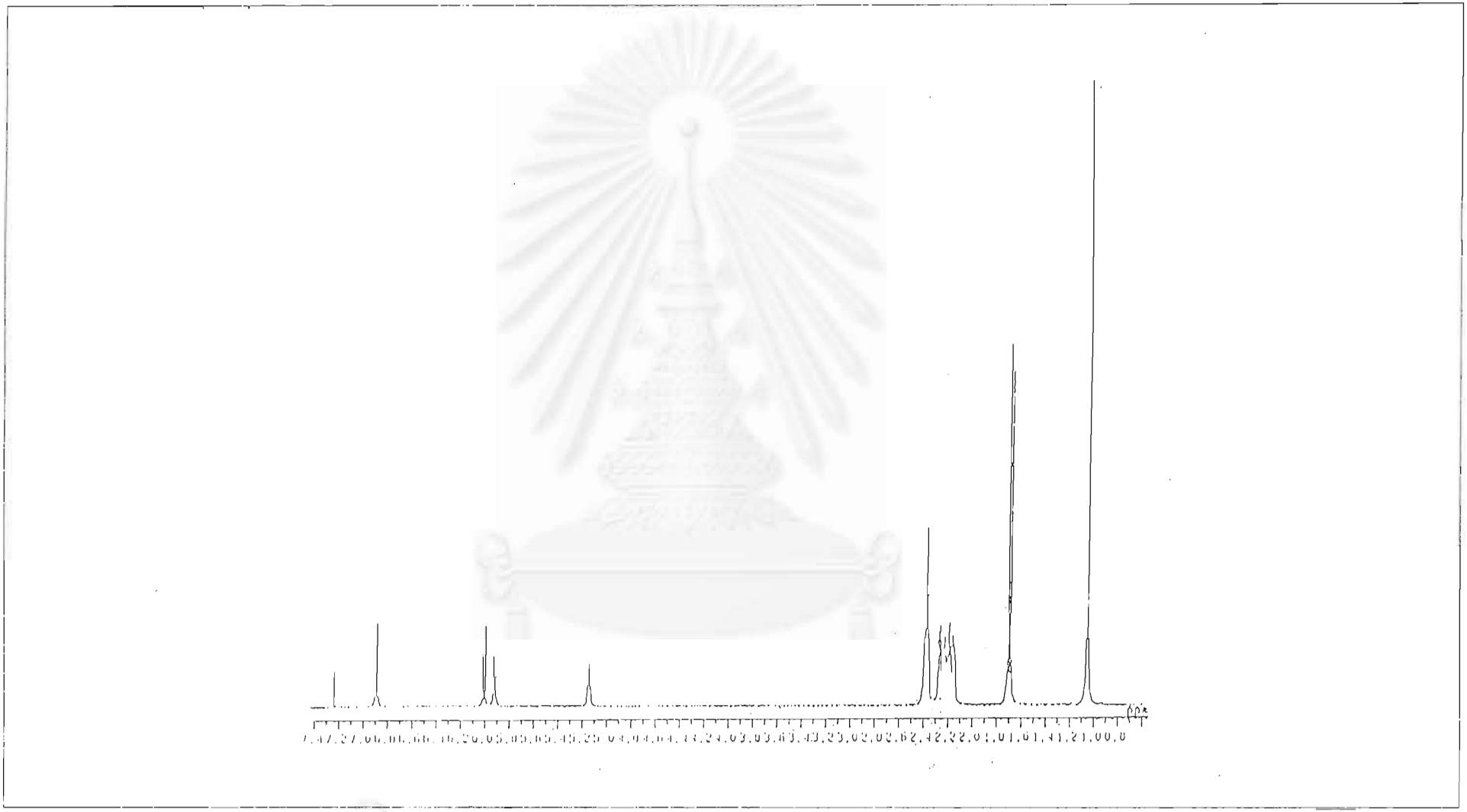

Figure 33 The ^{1}H-NMR spectrum of compound 3

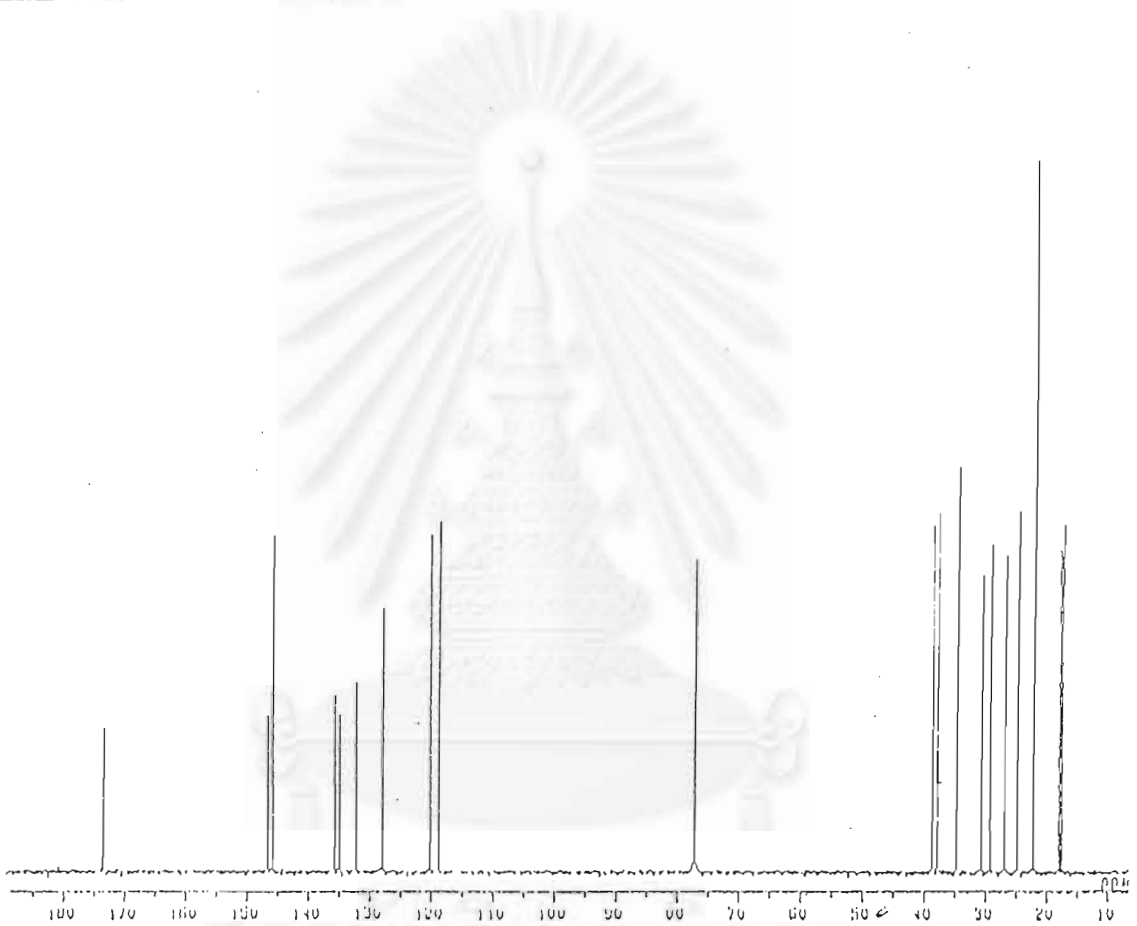

Figure 34 The ^{13}C-NMR spectrum of compound 3

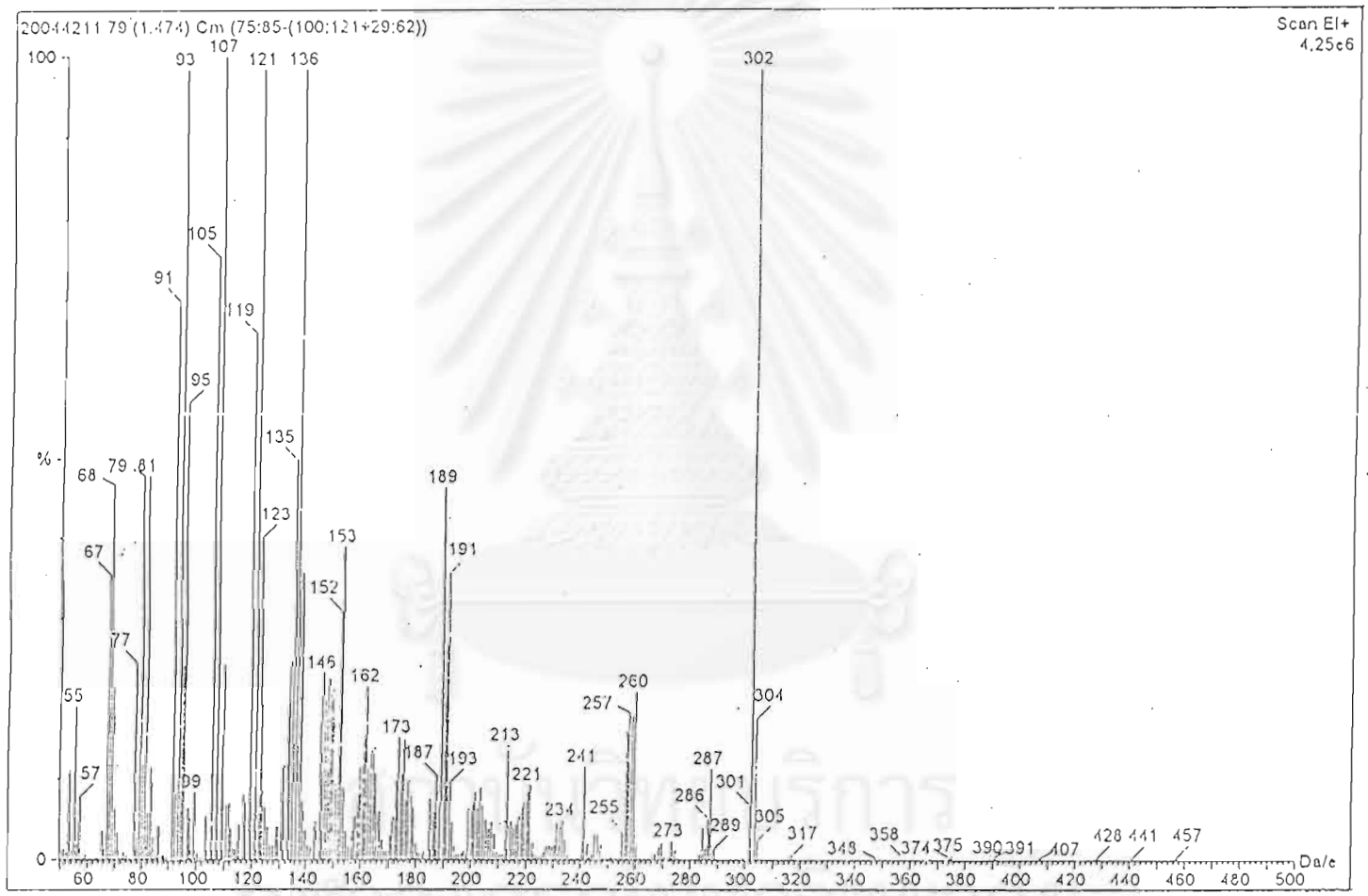

Figure 35 The EI MS spectrum of compound 3

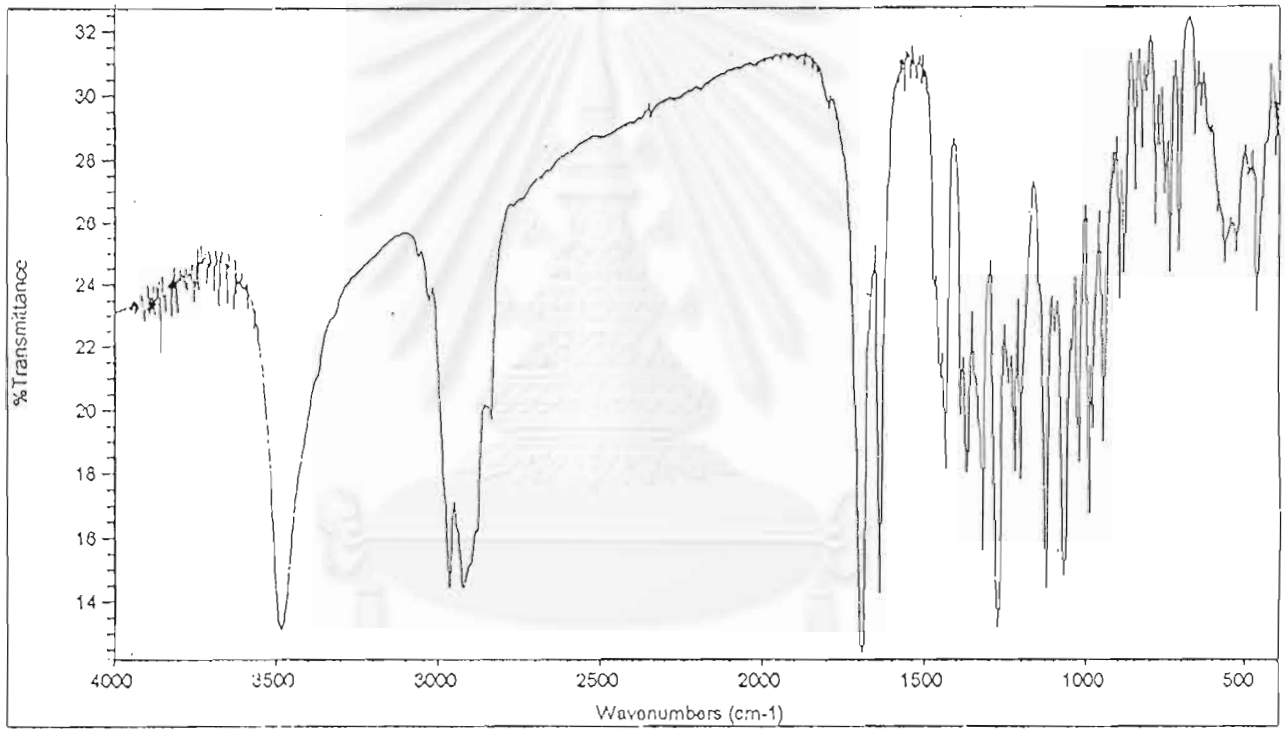

Figure 36 The IR spectrum of compound 4

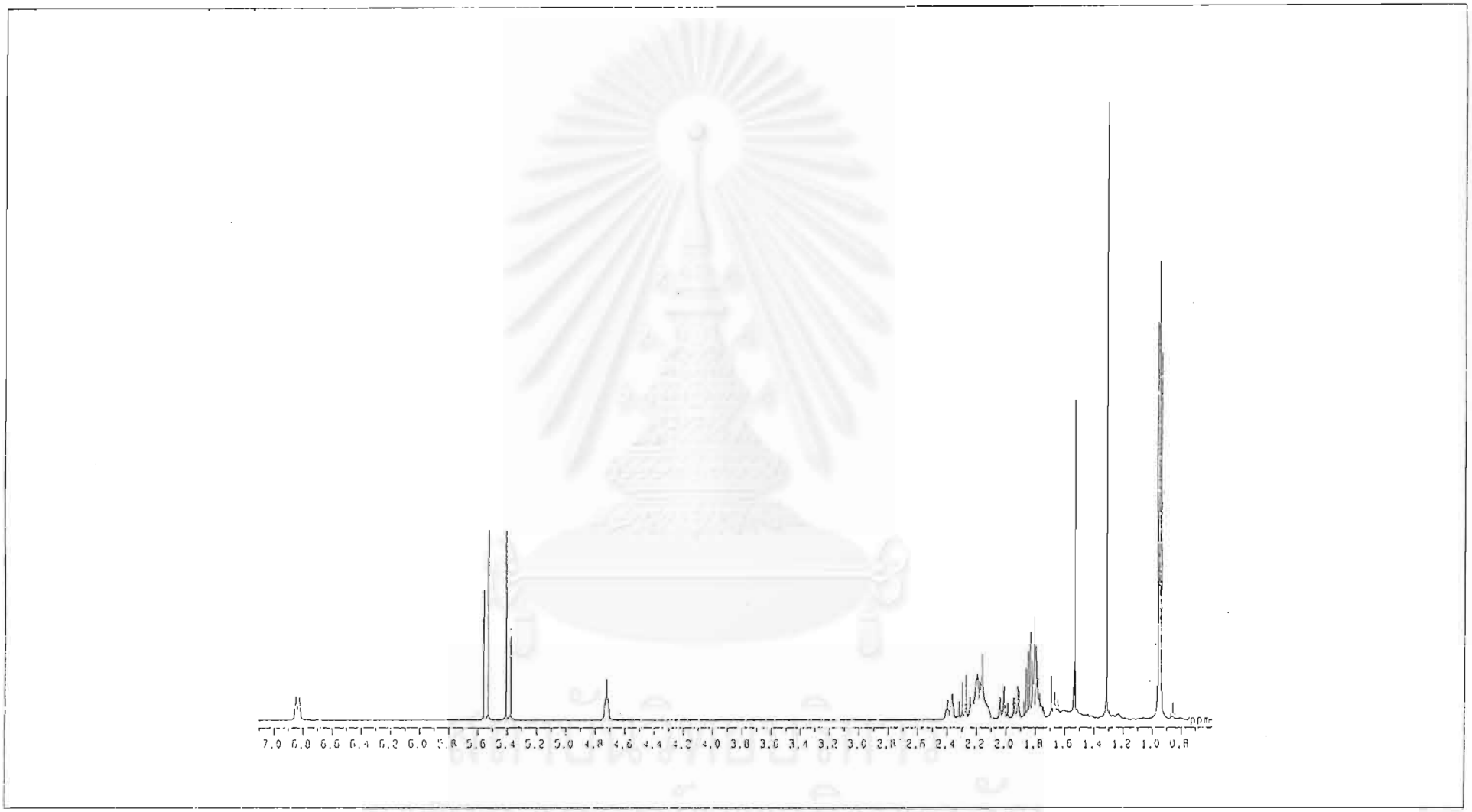

Figure 37 The ^{1}H-NMR spectrum of compound 4

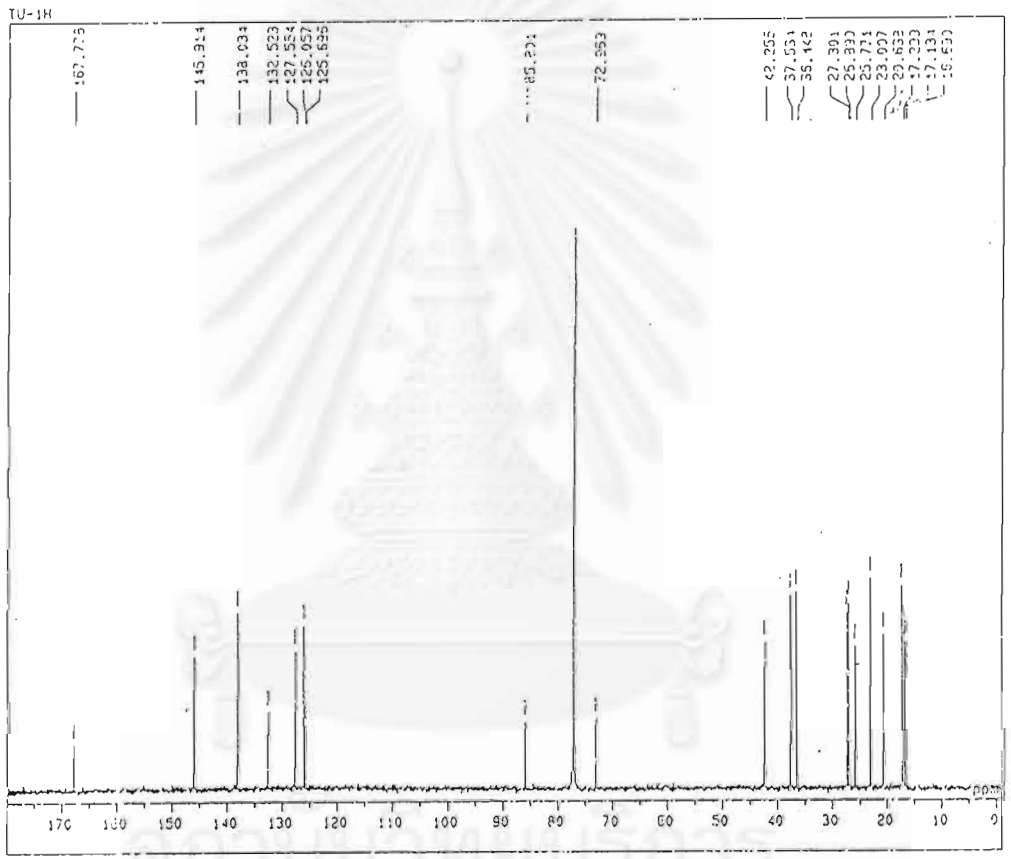

Figure 38 The ^{13}C-NMR spectrum of compound 4

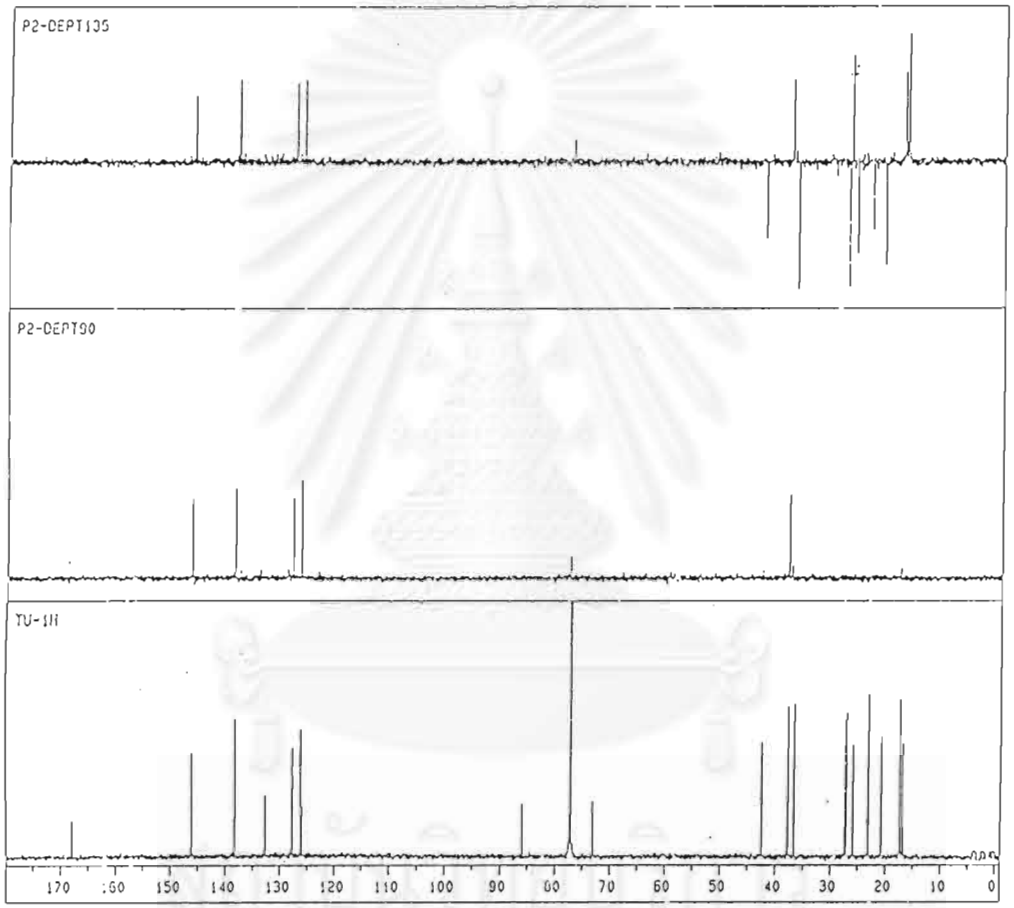

Figure 39 DEPT-135, 90 ^{13}C-NMR spectrum of compound 4

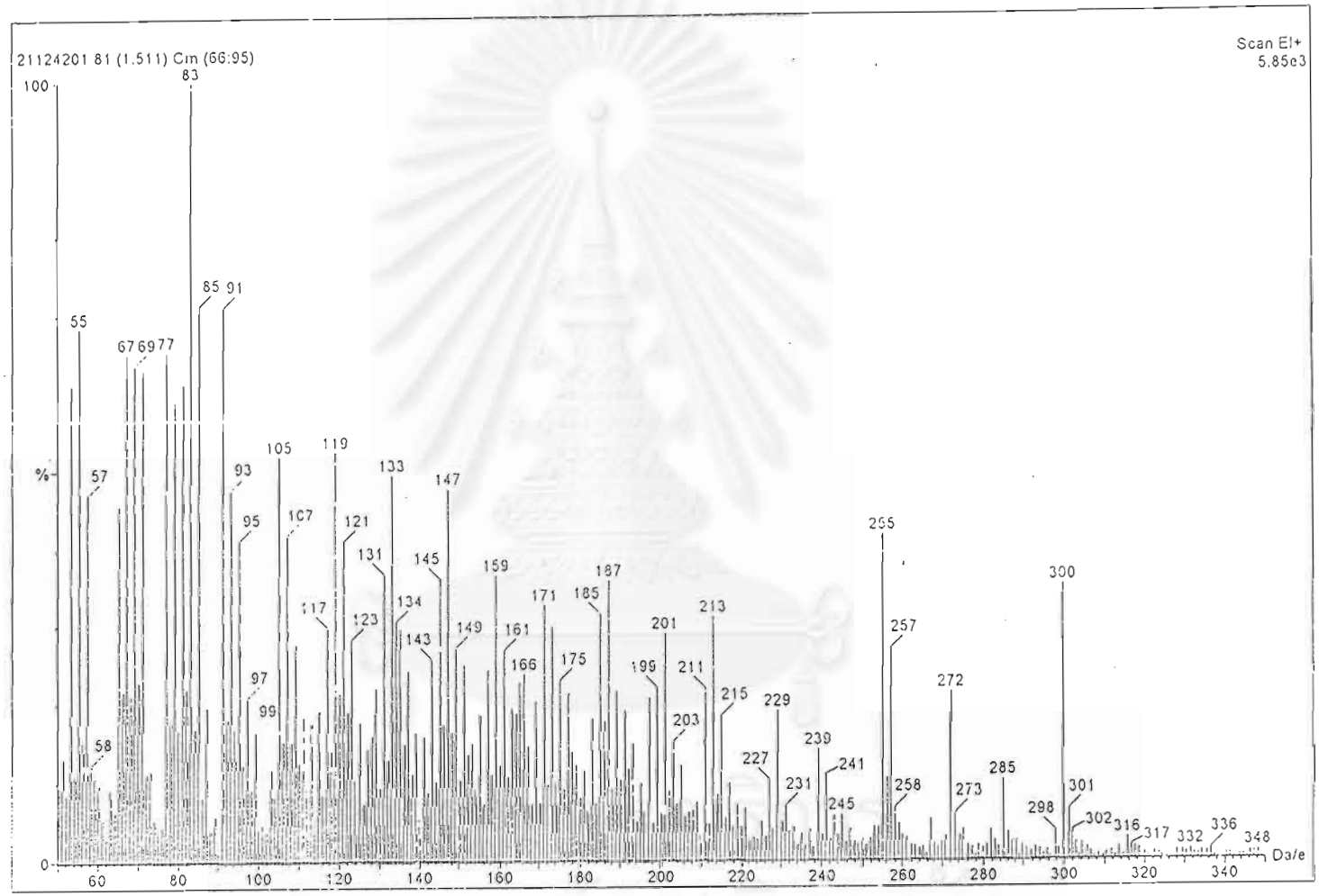

Figure 40 The EI MS spectrum of compound 4

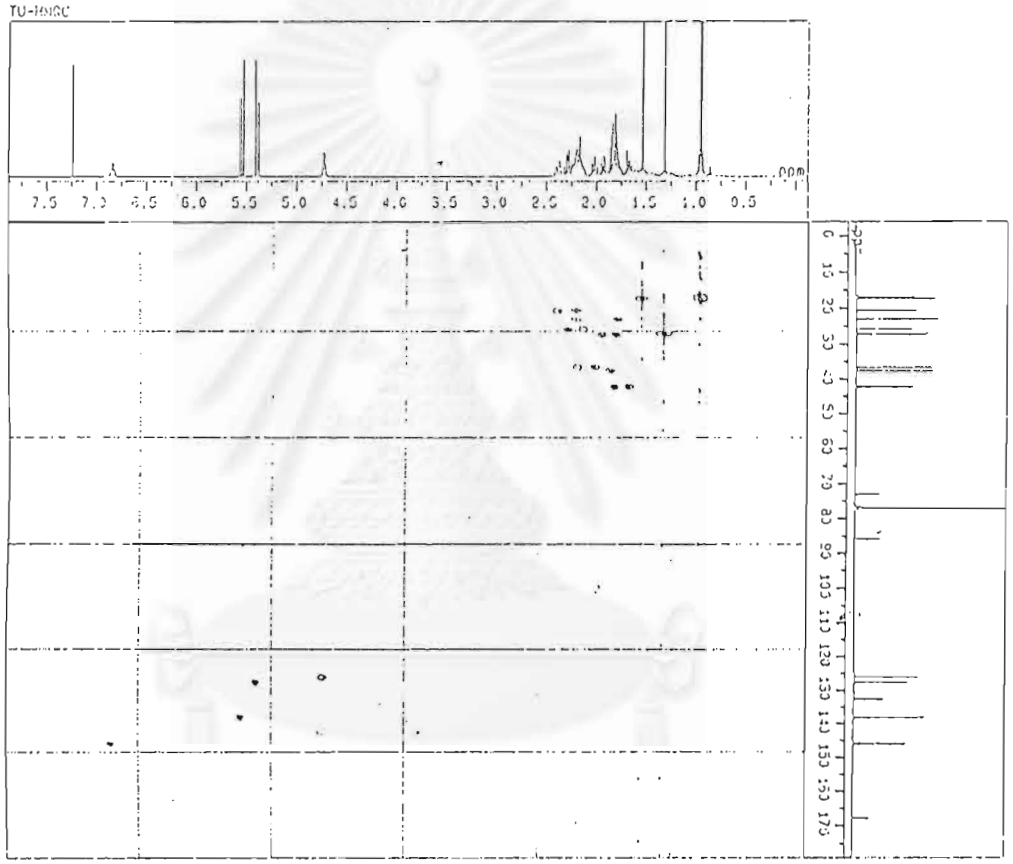

Figure 41 The HMQC-NMR spectrum of compound 4

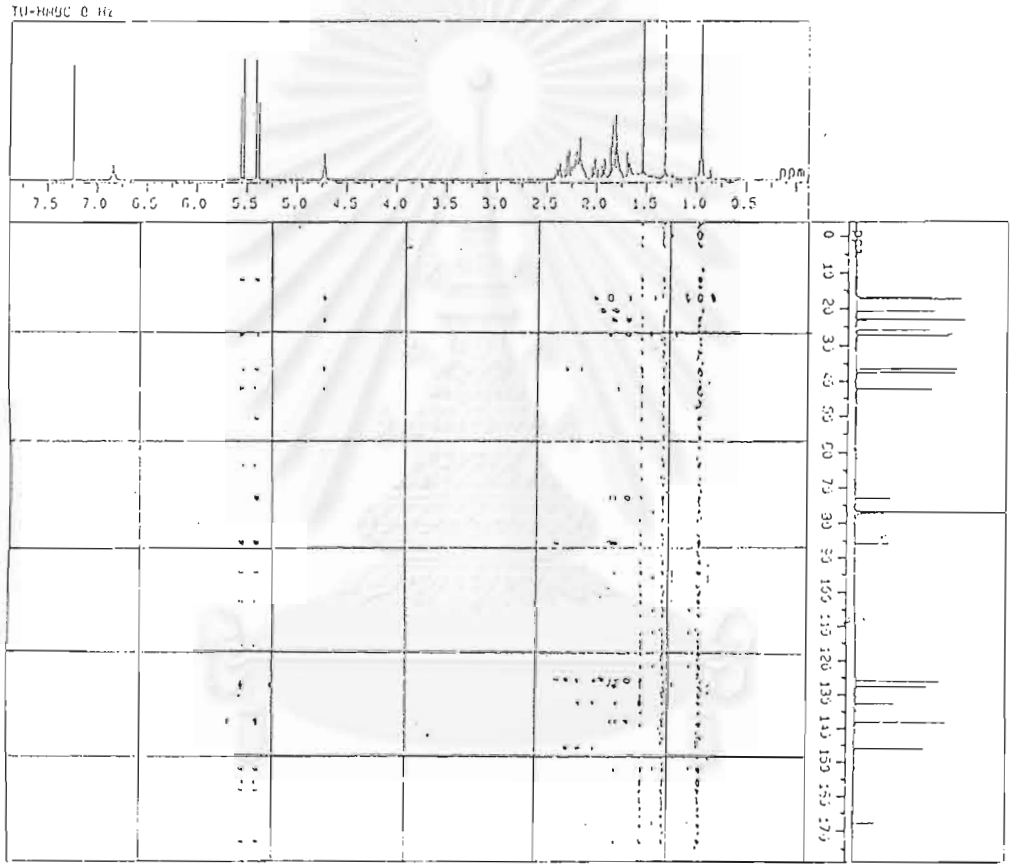

Figure 42 The HMBC-NMR spectrum of compound 4

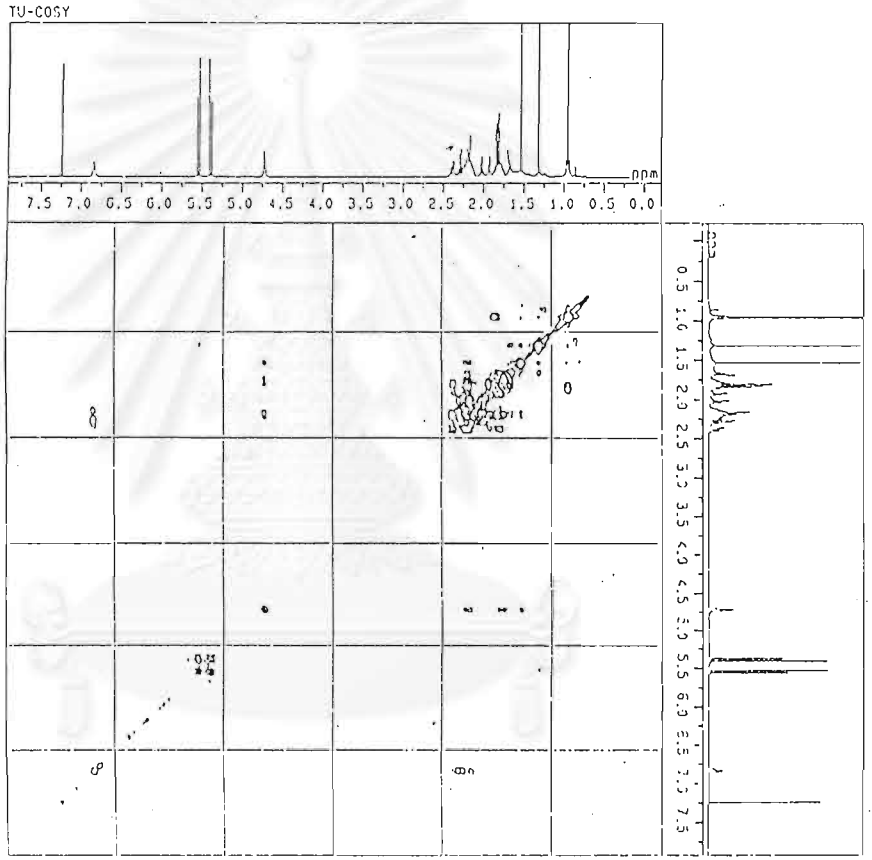

Figure 43 The COSY-NMR spectrum of compound 4

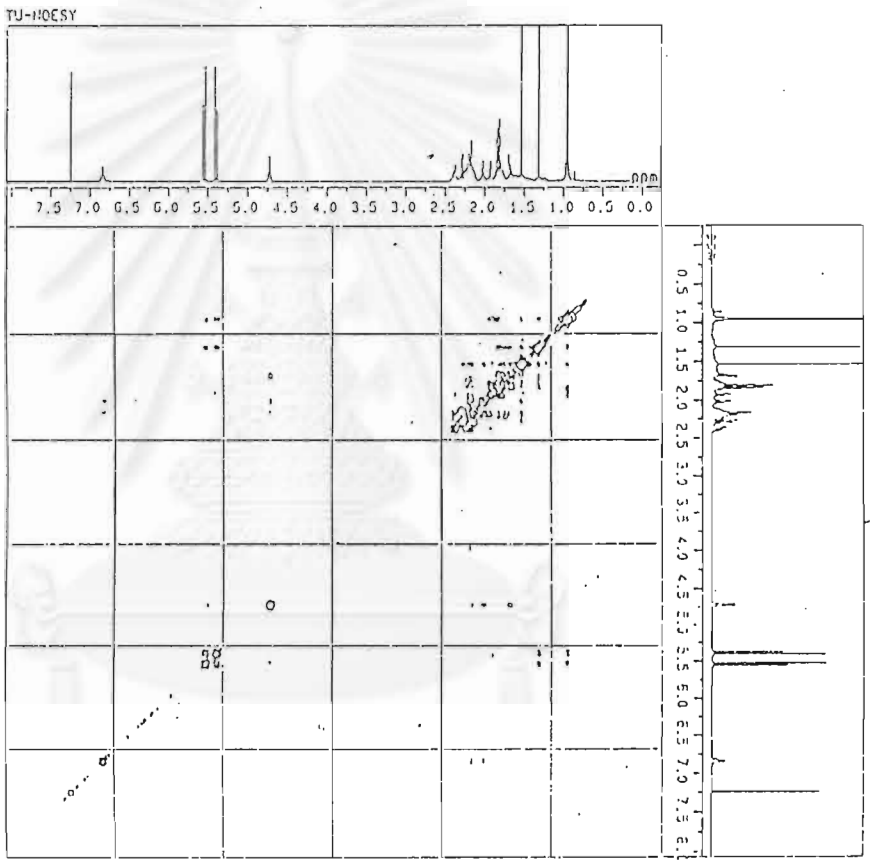

Figure 44 The NOESY-NMR spectrum of compound 4

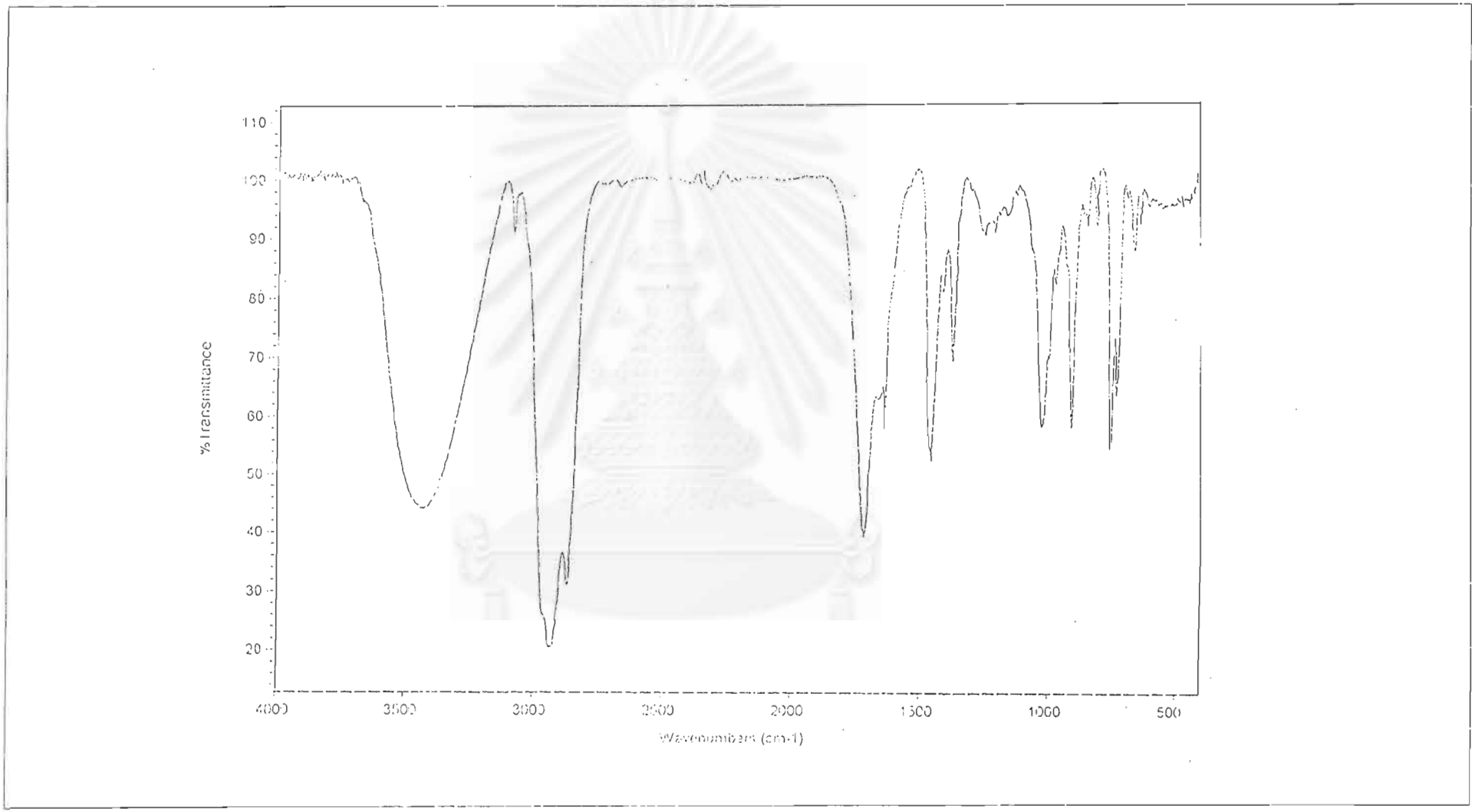

Figure 45 The IR spectrum of compound 5

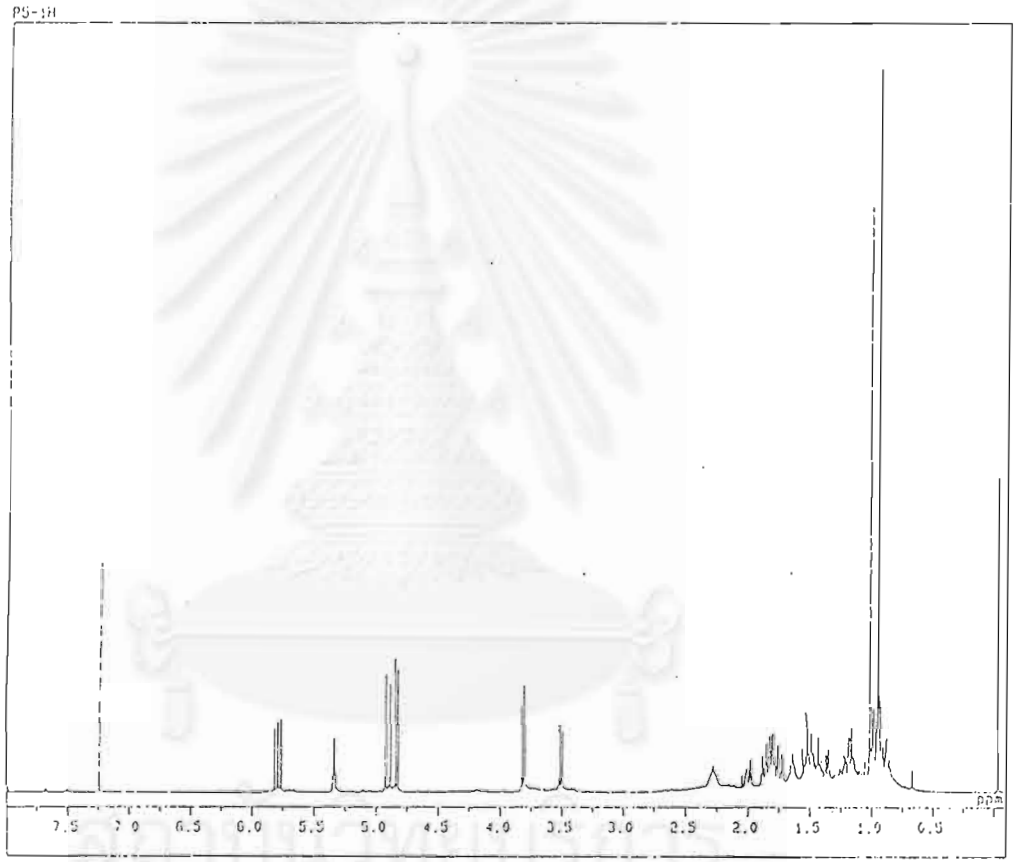

Figure 46 The ^{1}H-NMR spectrum of compound 5

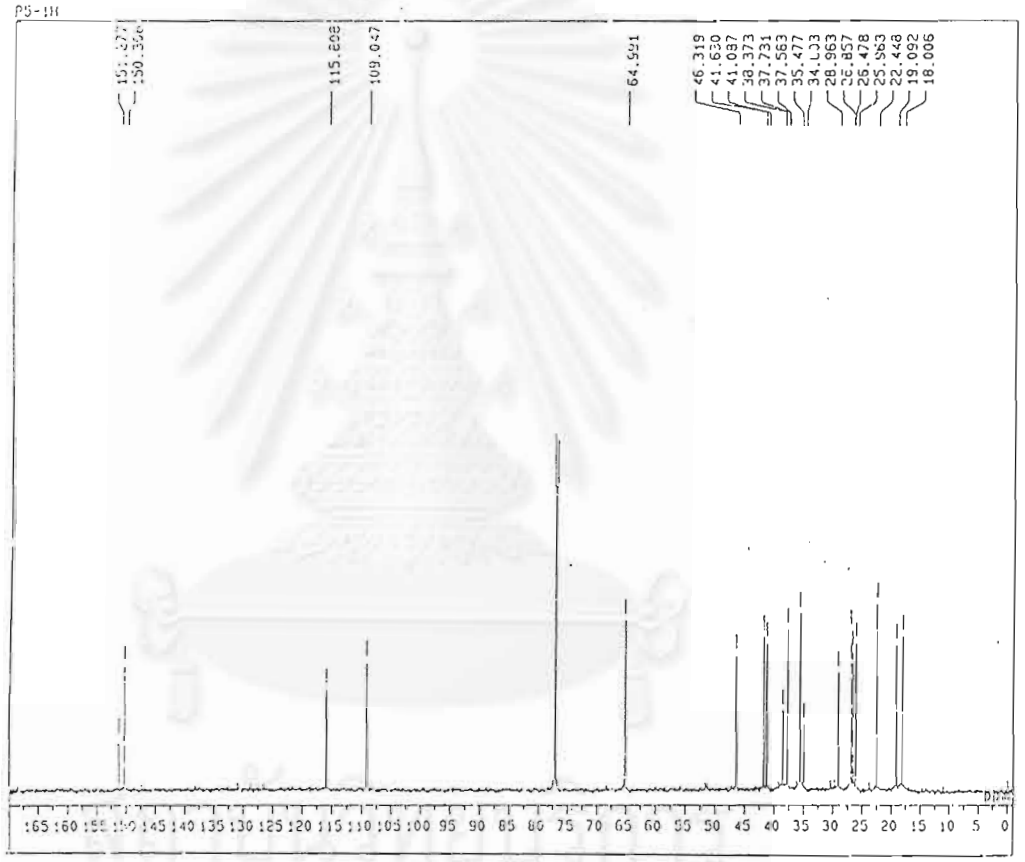

Figure 47 The ^{13}C-NMR spectrum of compound 5

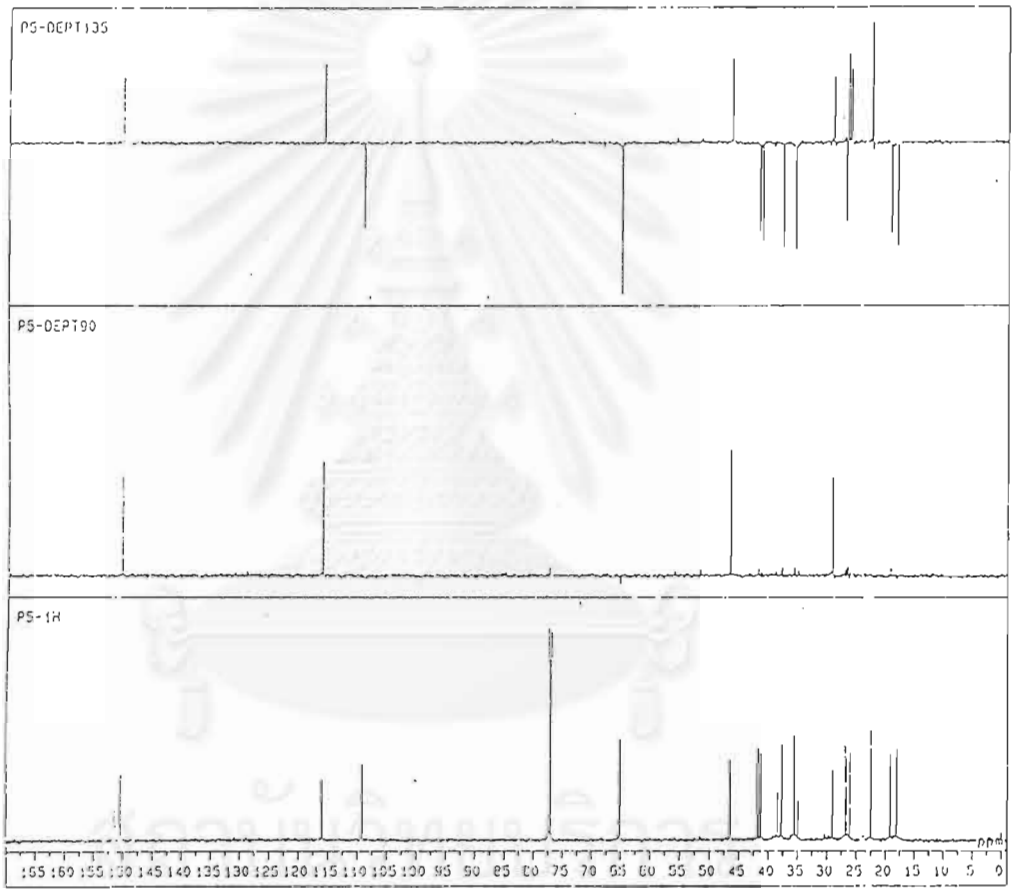

Figure 48 DEPT-135, 90 ^{13}C-NMR spectrum of compound 5

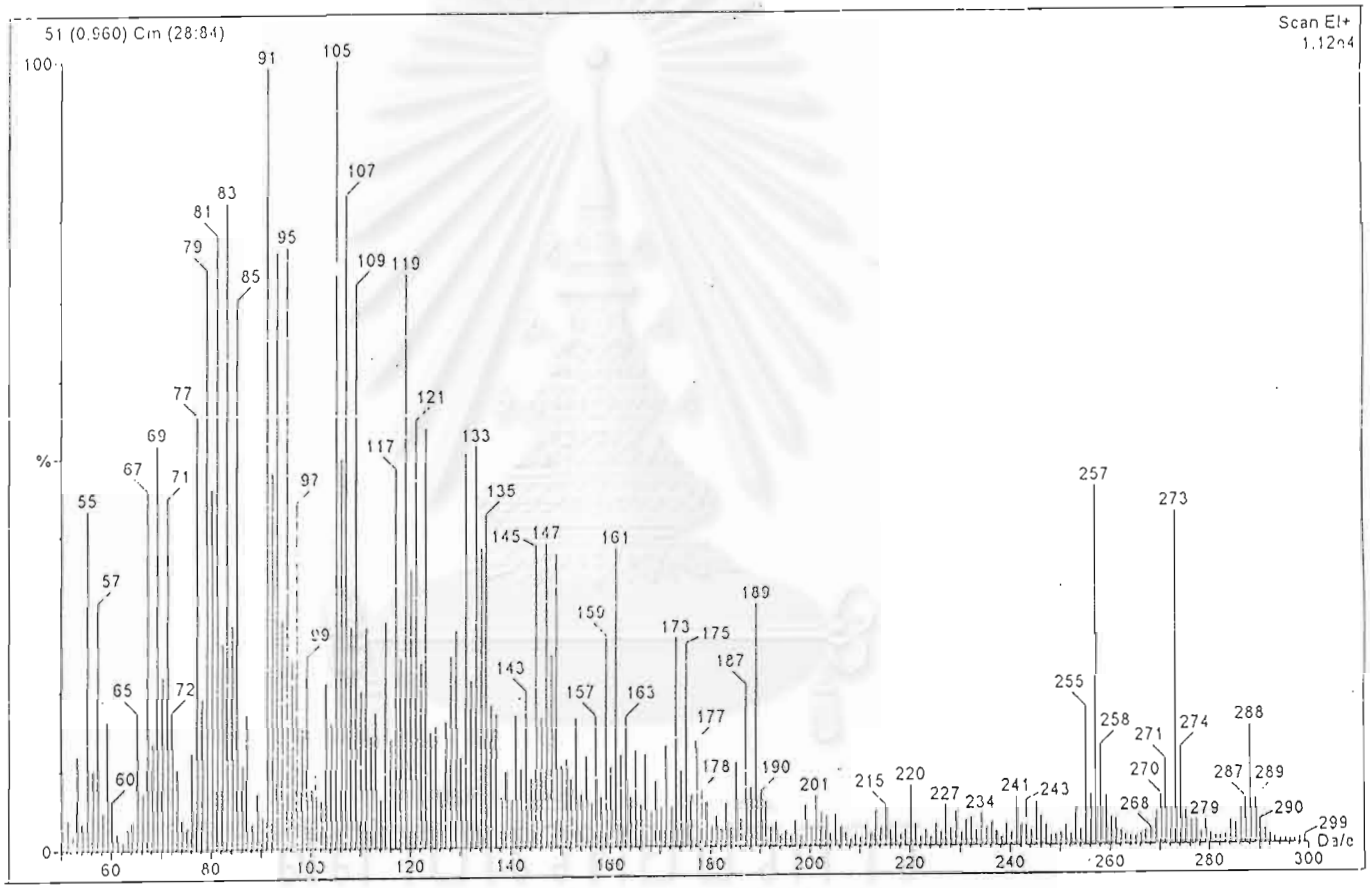

Figure 49 The EI MS spectrum of compound 5

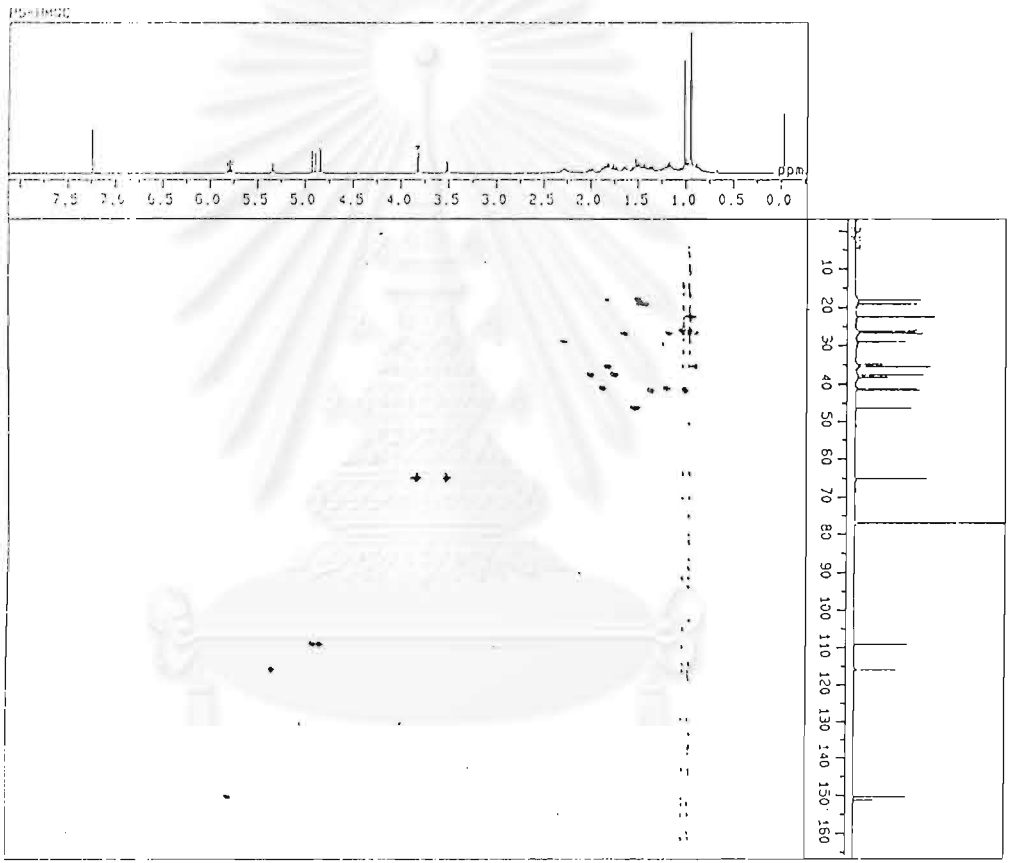

Figure 50 The HMQC-NMR spectrum of compound 5

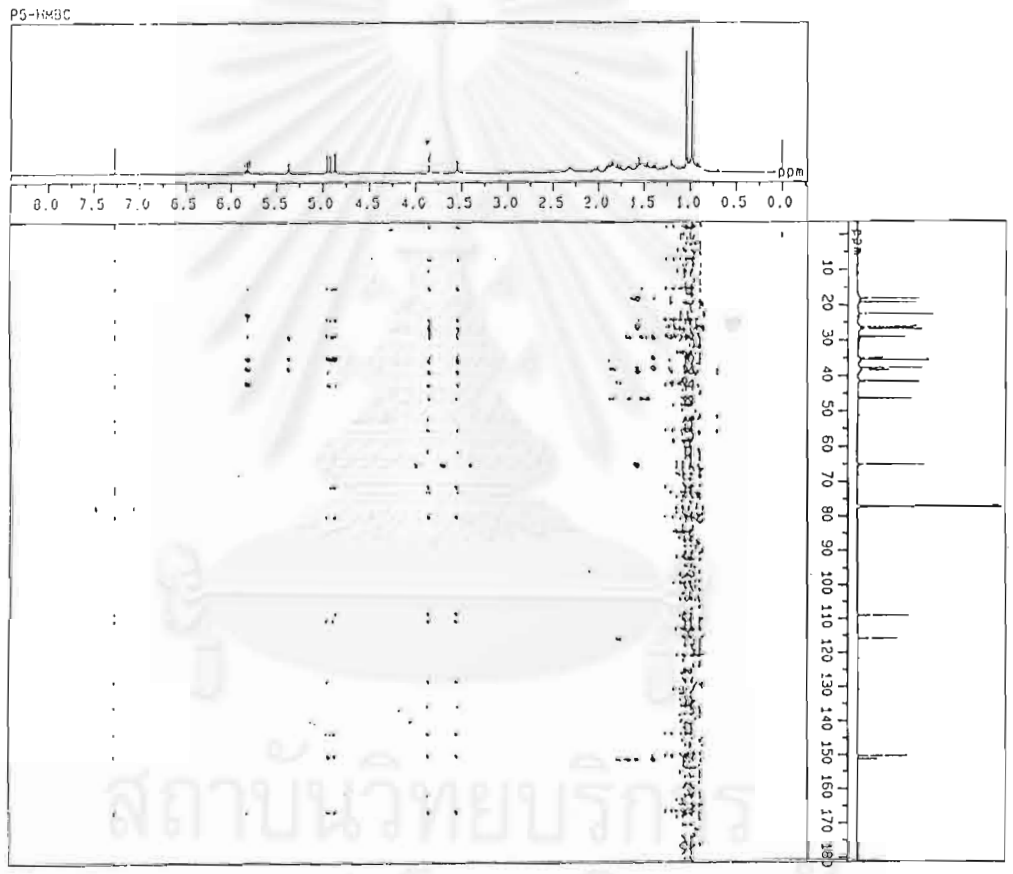

Figure 51 The HMBC-NMR spectrum of compound 5

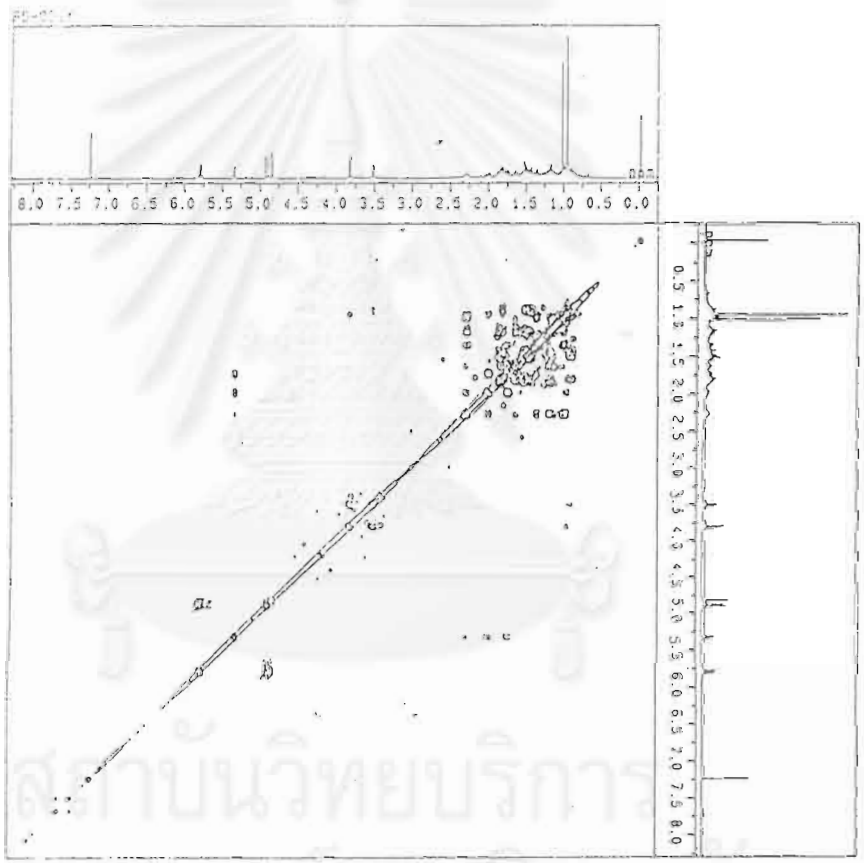

Figure 52 The COSY-NMR spectrum of compound 5

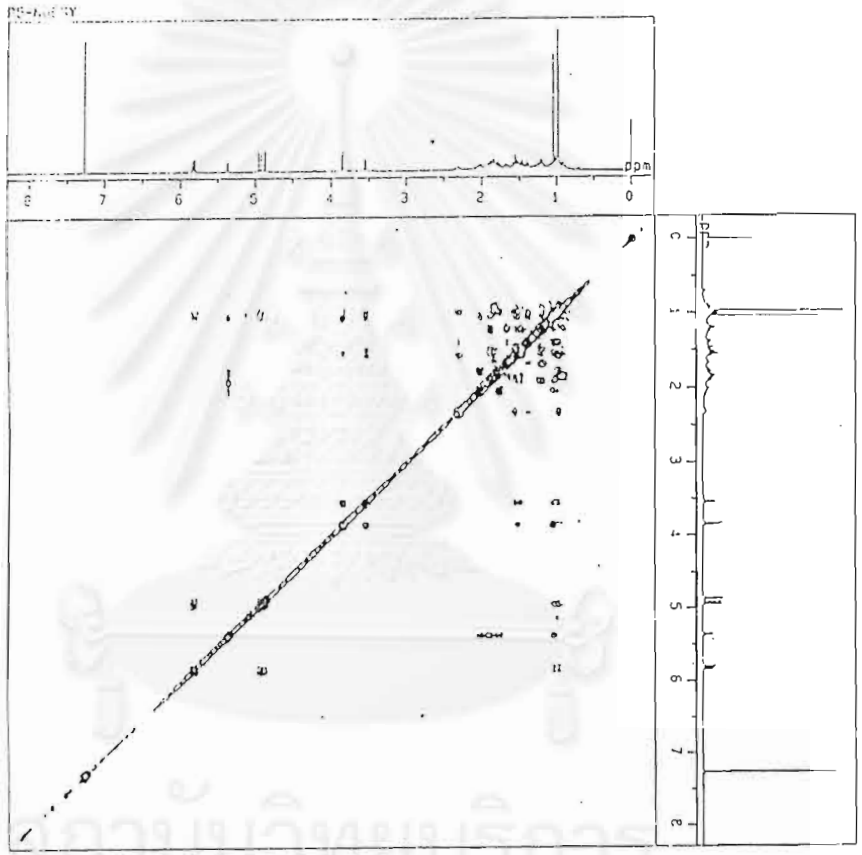

Figure 53 The NOESY-NMR spectrum of compound 5

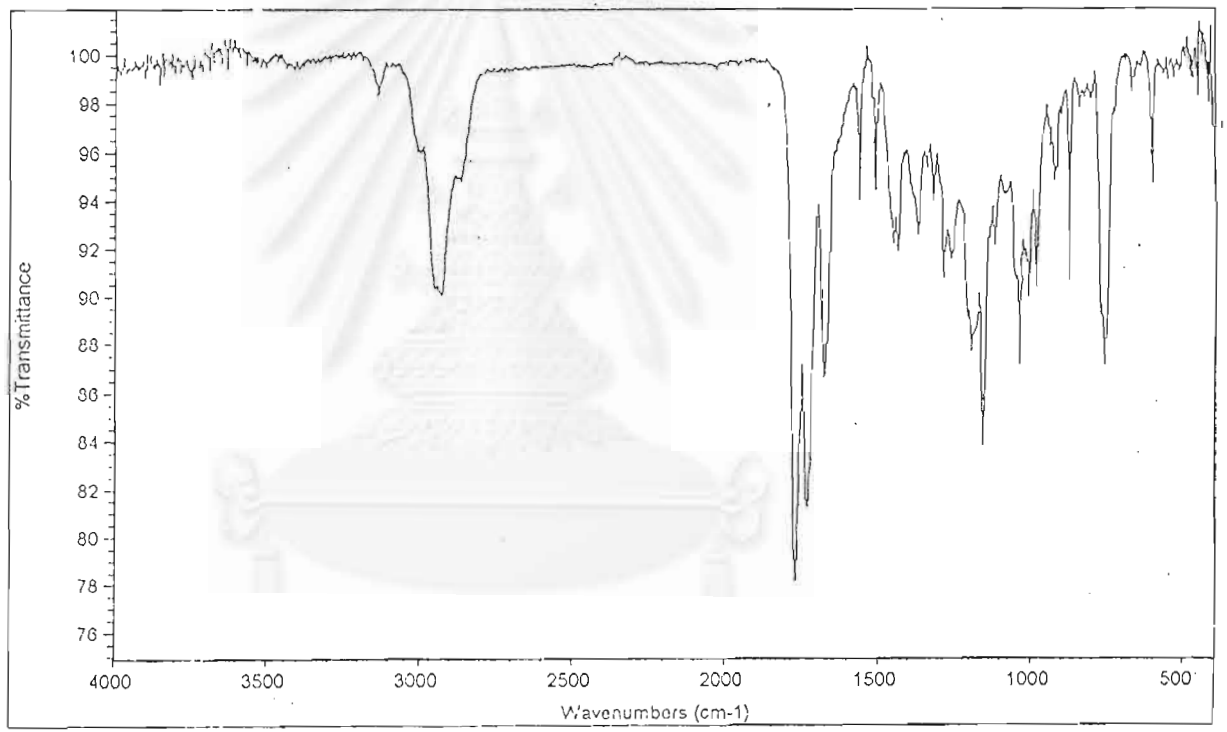

Figure 54 The IR spectrum of compound 6

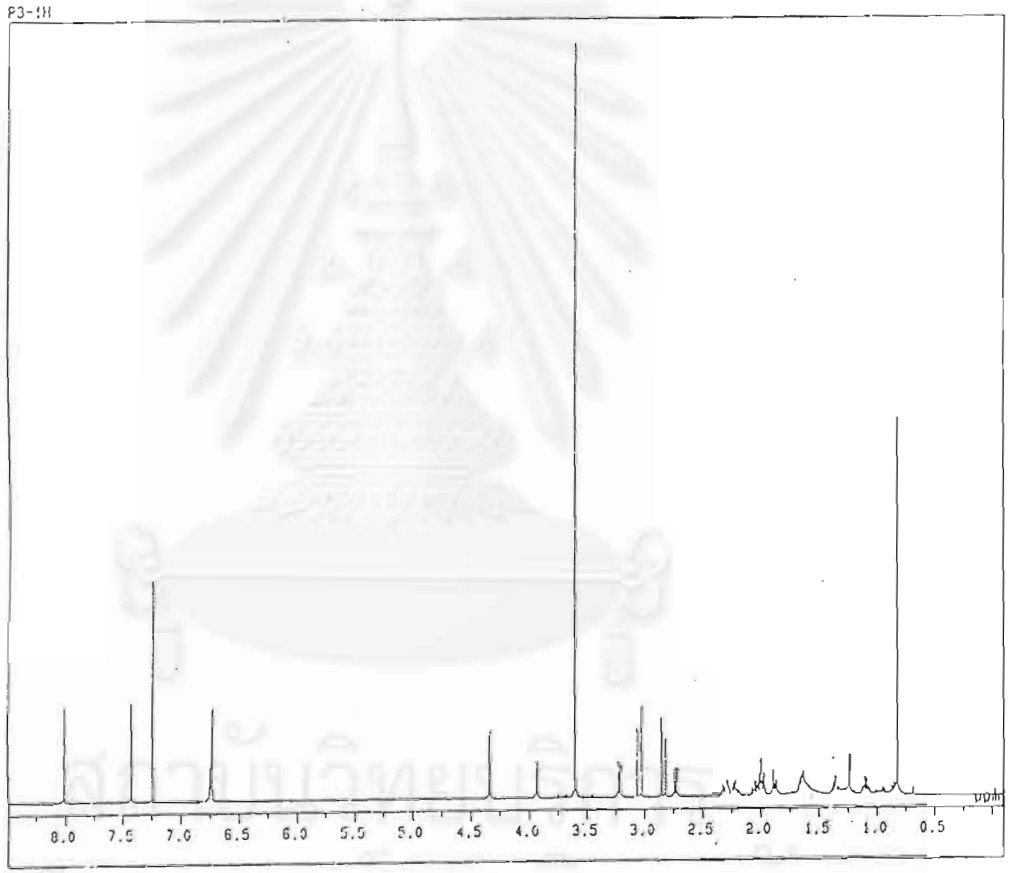

Figure 55 The ^{1}H-NMR spectrum of compound 6

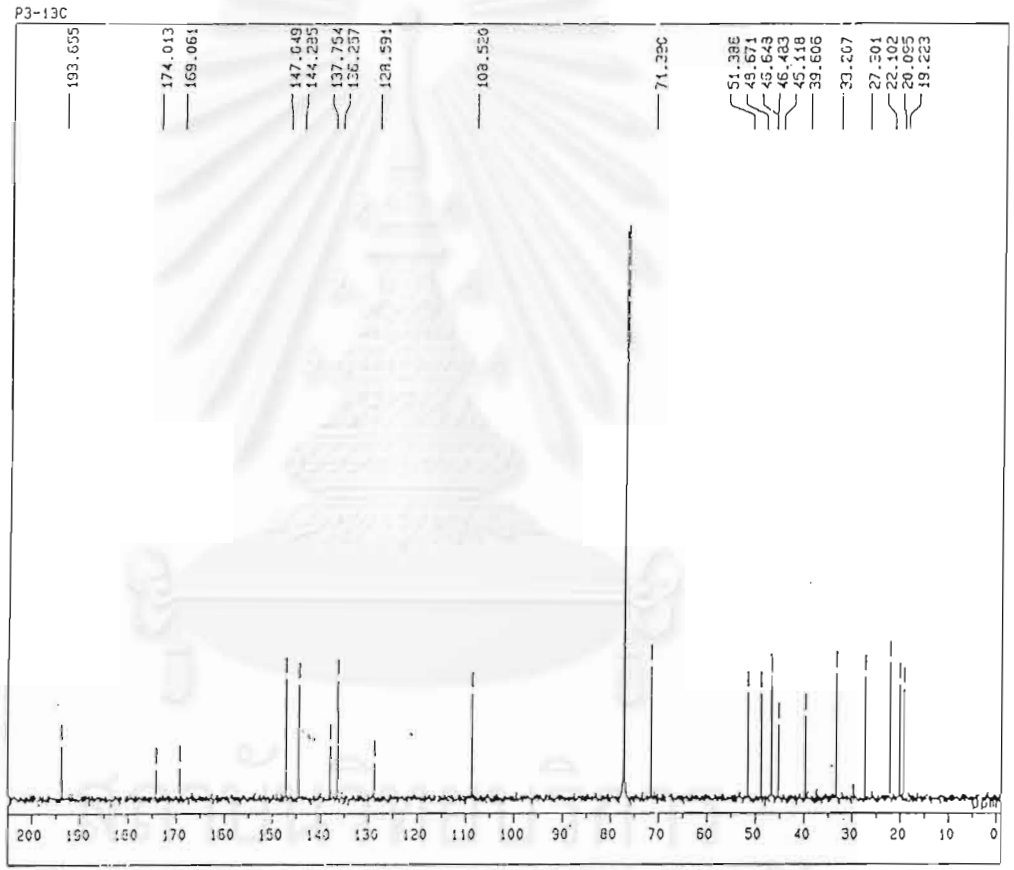

Figure 56 The ^{13}C-NMR spectrum of compound 6

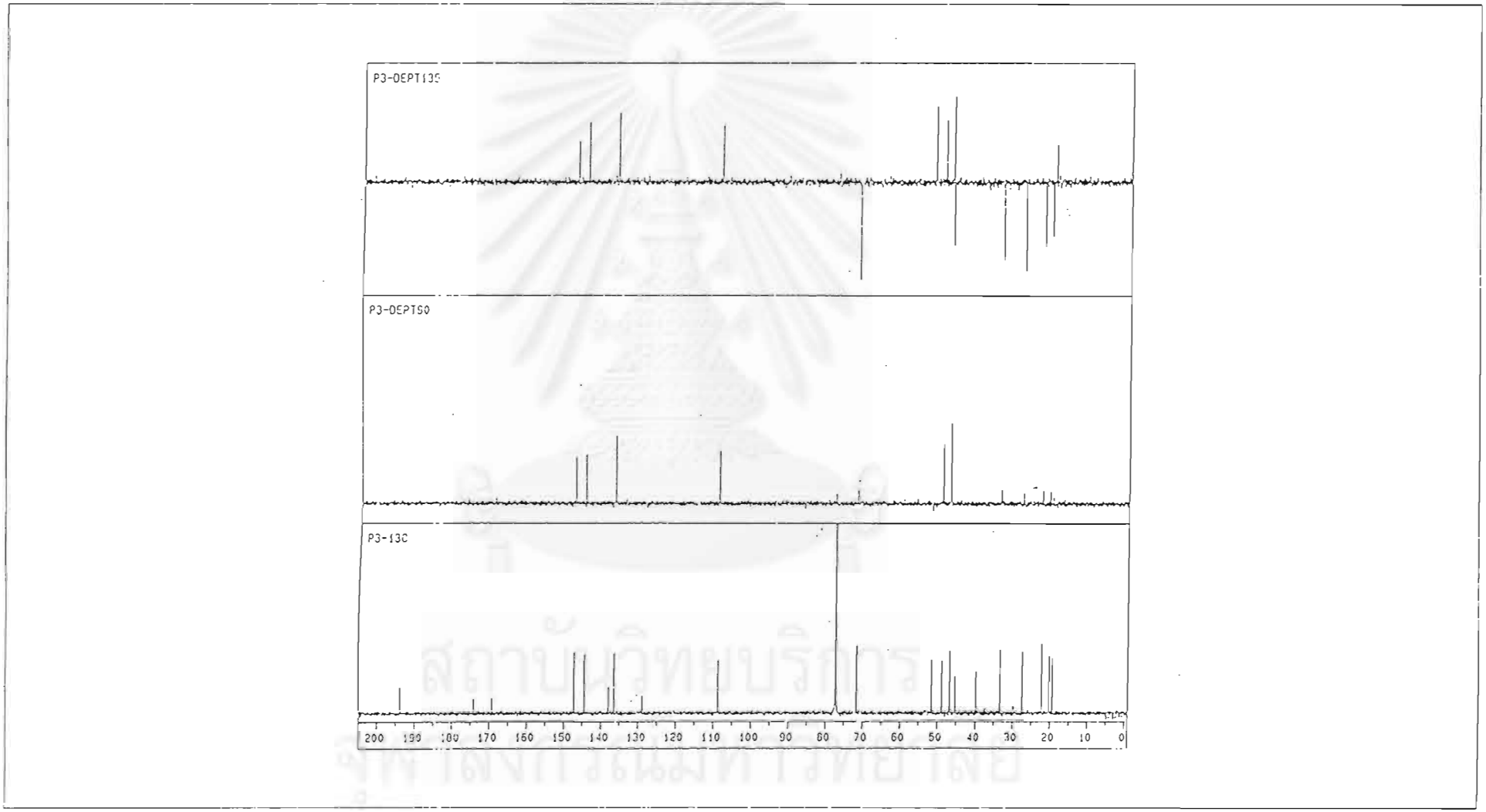

Figure 57 DEPT-135, 90 ^{13}C-NMR spectrum of compound 6

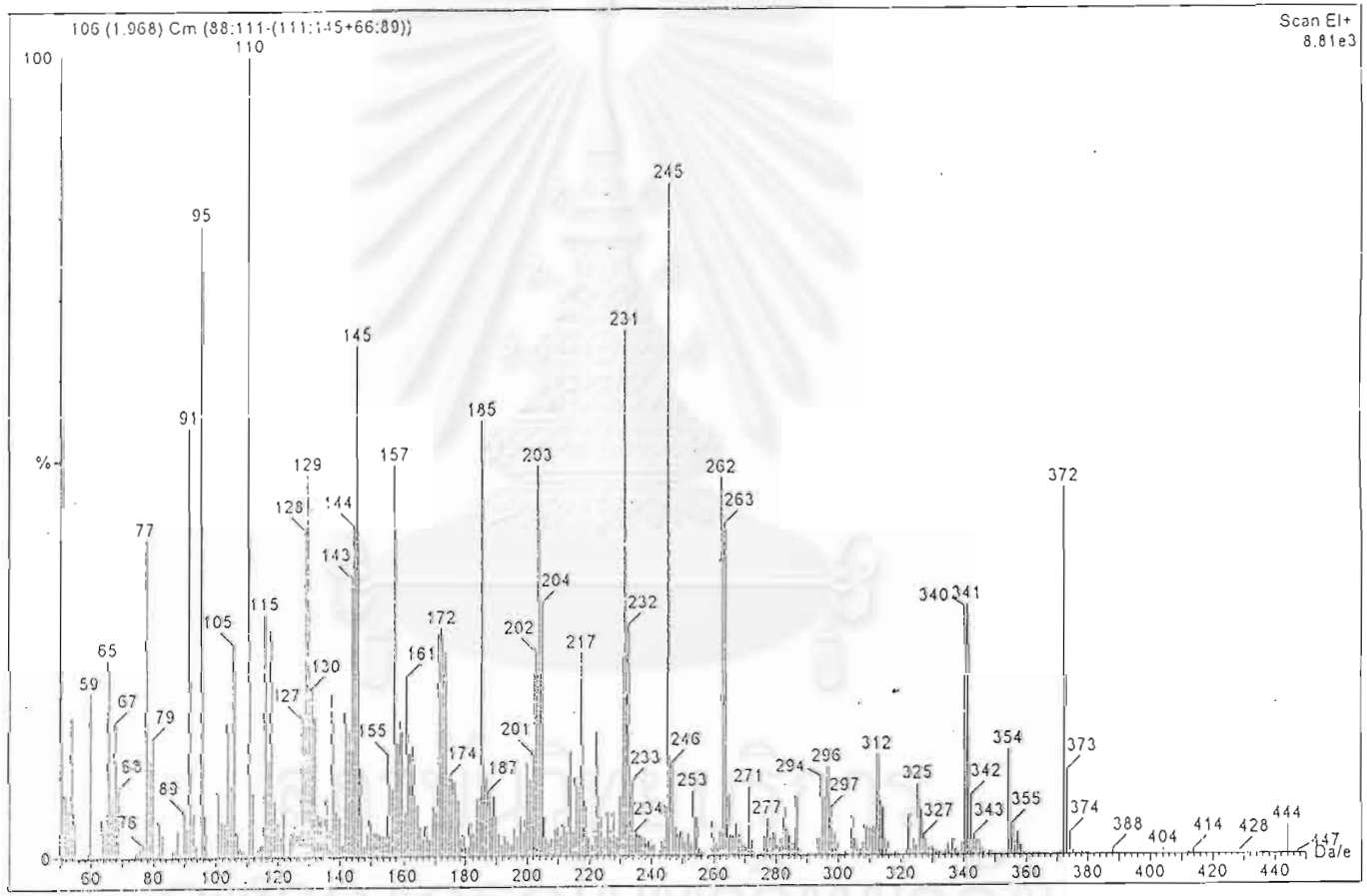

Figure 58 The EI MS spectrum of compound 6

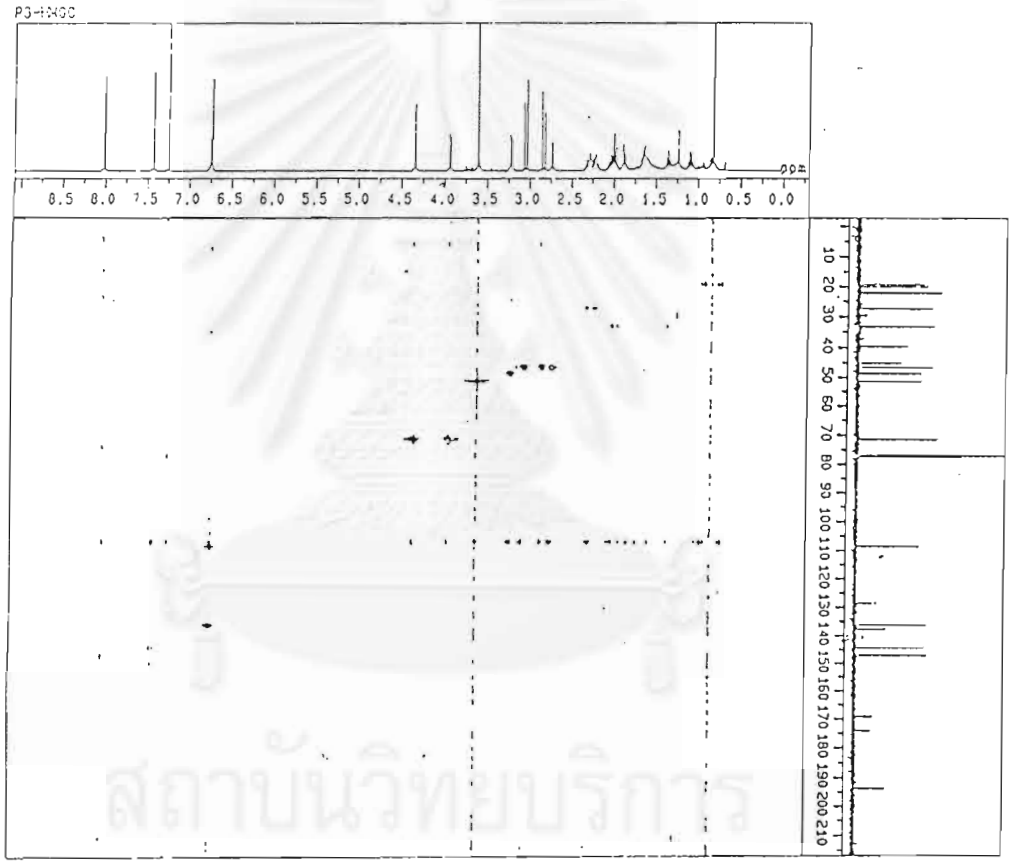

Figure 59 The HMQC-NMR spectrum of compound 6

VITA

Miss. Thanyaluk tanwattanakun was born on November 24, 1975 in Chonburi province, Thailand. She graduated with Bachelor Degree of Science of Chemistry at Chulalongkorn University in 1996. 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 UDC scholarship and Depertment of Chemistry Faculty of Science, Chulalongkorn University in 1997 and 1999, respectively.