องค์ประกอบทางเคมีที่มีฤทธิ์ต้านเซลล์มะเร็งของเปล้าใหญ่ *Croton oblongifolius* จากจังหวัดน่าน

นางสาวพรนิภา พาทา

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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CHEMICAL CONSTITUENTS WITH CYTOTOXICITY OF *Croton oblongifolius* FROM NAN PROVINCE

Miss Pornnipa Pata

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Thesis Title	CHEMICAL CONSTITUENTS WITH CYTOTOXICITY OF *Croton oblongifolius* FROM NAN PROVINCE
By	Miss. Pornnipa Pata
Field of Study	Chemistry
Thesis Advisor	Professor Sophon Roengsumran, Ph.D.

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

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พรนิภา พาทา : องค์ประกอบทางเคมีที่มีฤทธิ์ต้านเซลล์มะเร็งของเปล้าใหญ่ *Croton oblongifolius* จากจังหวัดน่าน (CHEMICAL CONSTITUENTS WITH CYTOTOXICITY OF *Croton oblongifolius* FROM NAN PROVINCE) อ. ที่ปรึกษา : ศ.ดร. โสภณ เริงสำราญ, 88 หน้า. ISBN 974-53-1498-6.

จากการศึกษาองค์ประกอบทางเคมีของเปลือกต้นเปล้าใหญ่ (*Croton oblongifolius* Roxb.) จากอำเภอเวียงสา จังหวัดน่าน สามารถสกัดแยกสารประกอบไดเทอร์พีนอยด์ใหม่ 2 ชนิดคือ 3-hydroxycleistantha-13(17),15-diene (**1**) และ 3,4-*seco*-cleistantha-4(18),13(17),15-trien-3-oic acid (**2**) และได้สังเคราะห์อนุพันธ์ 2 ชนิดจากสาร **2** โดยปฏิกิริยาอีพอกซิเดชัน คือ 3,4-*seco*-13,17-epoxycleistantha-4(18), 15-dien-3-oic acid (**3**) และ 3,4-*seco*-8,15-epoxypimara-4(18),15-dien-3-oic acid (**4**) ได้ทำการพิสูจน์โครงสร้างของสารใหม่โดยอาศัยข้อมูลทางสเปกโตรสโกปี ซึ่งได้แก่ IR, MS, 1D และ 2D NMR เทคนิค คือ COSY, NOESY, HMBC และ HMQC และนำสารทั้งหมดมาทดสอบฤทธิ์ในการยับยั้งเซลล์มะเร็งซึ่งได้แก่ SW620 (ลำไส้), BT474 (เต้านม), KATO-3 (กระเพาะอาหาร), HEP-G2 (ตับ) และ CHAGO (ปอด) พบว่าสาร **1** มีฤทธิ์ยับยั้งเซลล์มะเร็ง SW620 (ลำไส้), KATO-3 (กระเพาะอาหาร), HEP-G2 (ตับ) และ CHAGO (ปอด) โดยมีค่า IC_{50} เท่ากับ 0.5, 6.0, 6.1 และ 5.5 μg/mL ตามลำดับ สาร **2** มีฤทธิ์ยับยั้งเซลล์มะเร็งชนิด SW620 (ลำไส้) และ KATO-3 (กระเพาะอาหาร) เพียงเล็กน้อย โดยมีค่า IC_{50} เท่ากับ 8.6 และ 9.6 μg/mL ตามลำดับ ส่วนสาร **3** และ **4** ไม่มีฤทธิ์ในการยับยั้งเซลล์มะเร็งทั้ง 6 ชนิด

ภาควิชา..... เคมี..... ลายมือชื่อนิสิต.....
สาขาวิชา.....เคมี..... ลายมือชื่ออาจารย์ที่ปรึกษา.....
ปีการศึกษา.....2547..... ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

4672342123 : MAJOR CHEMISTRY

KEY WORD: *Croton oblongifolius* / cytotoxicity / diterpenenoid compound

PORNNIPA PATA : CHEMICAL CONSTITUENTS WITH CYTOTOXICITY OF *Croton oblongifolius* FROM NAN PROVINCE. THESIS ADVISOR : PROF. SOPHON ROENGSUMRAN, Ph.D. 88 pp. ISBN 974-53-1498-6.

Two novel diterpenoid compounds, 3-hydroxycleistantha-13(17),15-diene (**1**) and 3,4-*seco*-cleistantha-4(18),13(17),15-trien-3-oic acid (**2**) were isolated from the stem bark of *Croton oblongifolius* Roxb. from Amphur Veingsa, Nan Province. And two derivatives were modified by epoxidation of compound **2** to obtain 3,4-*seco*-13,17-epoxycleistantha-4(18), 15-dien-3-oic acid (**3**) and 3,4-*seco*-8,15-epoxypimara-4(18),15-dien-3-oic acid (**4**). The structure of new compounds were established by spectroscopic data (IR, MS, 1D, 2D NMR techniques including DEPT, COSY, NOESY, HMBC and HMQC) and they were tested for cytotoxicity against various human tumor cell lines SW620 (colon), BT474 (breast), KATO-3 (gastric), HEP-G2 (hepatoma) and CHAGO (lung). The result showed that compound **1** was active against SW620 (colon), KATO-3 (gastric), HEP-G2 (hepatoma) and CHAGO (lung) cell line with the IC_{50} values of 0.5, 6.0, 6.1 and 5.5 μg/mL, respectively. Compound **2** showed mild activities against SW620 (colon) and KATO-3 (gastric) cell line with IC_{50} values of 8.6 and 9.6 μg/mL, respectively. Compound **3** and **4** were inactive against all cell lines.

Department…….Chemistry……. Student's signature.....
Field of study…..Chemistry……. Advisor's signature.....
Academic year….2547…… Co-advisor's signature.....

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LIST OF ABBREVIATIONS

cm	centimeter
mm	millimeter
wt	weight
MHz	megahertz
TLC	thin layer chromatography
kg	kilogram
g	gram
MeOH	methanol
EtOAc	ethyl acetate
mg	milligram
mp	melting point
KBr	potassium bromide
ν_{max}	the reciprocating wavelength (IR spectrum)
λmax	the wavelength at maximum absorption (UV-VIS)
cm^{-1}	unit of wave number
s	strong (IR)
m	medium (IR)
w	weak (IR)
°C	degree Celsius
mL	milliter
R_f	rate of flow in chromatography
ppm	part per million
m/z	mass to charge ratio
δ	chemical shift
^{13}C-NMR	Carbon-13 Nuclear Magnetic Resonance
^{1}H-NMR	Proton Nuclear Magnetic Resonance
COSY	Correlated Spectroscopy
J	coupling constant
d	doublet (for NMR spectrum)
dd	double of doublet (for NMR spectrum)
MS	Mass Spectrometer

HMBC	Heteronuclear Multiple Bond Correlation
HSQC	Heteronuclear Single Quantum Correlation
M^+	molecular ion
NMR	Nuclear Magnetic Resonance
NOESY	Nuclear Overhauser Enhancement Spectroscopy
q	quartet (for NMR spectrum)
s	singlet (for NMR spectrum)
t	triplet (for NMR spectrum)

CHAPTER I

INTRODUCTION

Natural products, as the term implies, are those chemical compounds derived from living organisms, plants, animals, insects, and the study of natural products is the investigation of their structure, formation, use, and purpose in the organism. Drugs derived from natural products are usually secondary metabolites and their derivatives, and today those must be pure and highly characterized compounds. The natural products that were studied and used tended to be the compounds that occurred in the largest amounts, mostly in plants, and were most easily isolated in a pure, or sometimes not very pure, form by techniques such as simple distillation, steam distillation, or extraction with acid or base. From the Thai medicinal plant literature, plao-yai (*Croton oblongifolius* Roxb.) is often used with plao-noi, (*Croton sublyratus* Kurz.) as antipeptic ulcer drug. Therefore, its is very interesting to investigate chemical constituents of plao-yai for effective "lead" compound.

Plao Yai belongs to the Euphorbiaceae family [1]. It scientific name is *Croton oblongifolius* Roxb. In this family, there are 800 genera and 5000 species. Plao Yai was found in evergreen forests, deciduous forests and groves of brushwood, which is not more than 700 meters above sea level [2]. In Thailand, it is commonly called as Plao Yai (Central), Plao Luang (Northern)

Plao Yai is an interesting Thai medicinal plant because all of its parts are useful. For instance barks are used to inhibit chronic enlargements of livers and inhibit remittent fever, leaves can be used as a remedy to liver compliments, fruits and seeds are purgative and can be used to treat of snake bites, flowers are used to kill parasite, heartwood is a remedy of faint and roots are used to treat dysentery [3,4,5].

General Characterization of the Plants in the Genus Croton [6]

The genus Croton comprises of 700 species of trees or shrubs. Leaves are usually alternate with 2-glandular stipule at the base. Their flowers are solitary or clustered in the rachis of a terminal raceme and bracts are small. Male flowers contain

5-calyx, 5-petals. There are many stamens inserted on a hairy 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 opposite the sepals. There are three ovaries with solitary ovary 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 are 5.7-11.5 by 13.0-24.0 cm in size. The shape of leaf blade is oblong-lanceolate. Flowers are pale yellowish green and solitary in the axial of minute bracts on long erect racemes. The male flowers are locates in the upper part of the raceme and the females in the lower part. Male flowers are slender and have of pedicels the length of 4.0 mm. The calyx is more than 6.0 mm long and the 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 inflected in the bud and the lengths of the filaments are 3.0 mm. In female flowers, the pedicels are short and stout. Its sepals are more acute than in the male with densely ciliated margins. The diameter of the fruit is less than 1.3 cm, slightly 3-lobed and clothed with small orbicular scales. In each fruit, the number of seeds are eight which are 6.0 mm long rounded and quite smooth on the back.

The picture of stem bark, leaf, flower and fruit of *Croton oblongifolius* Roxb. are show in Figure.1

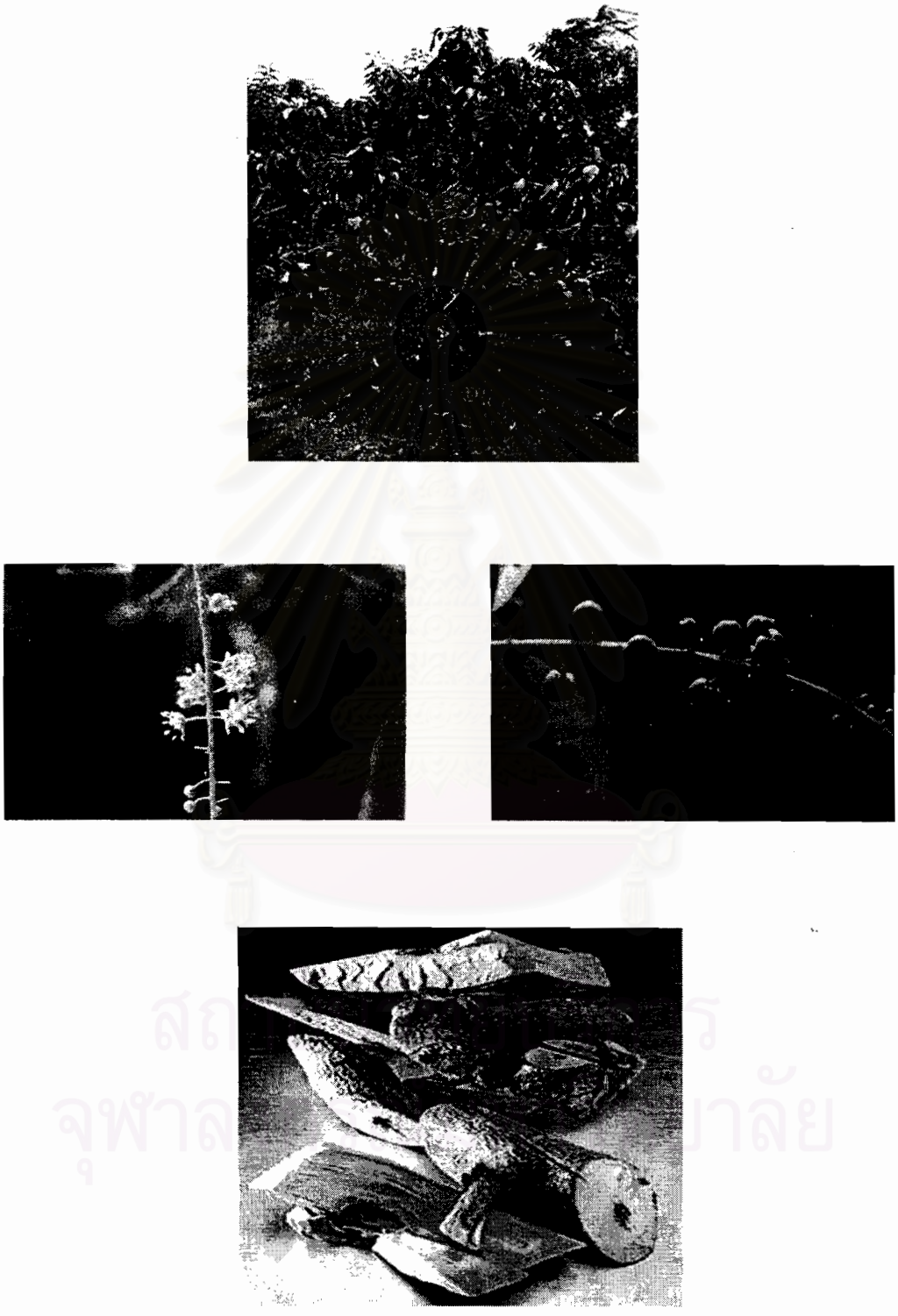


Figure 1. *Croton oblongifolius* Roxb.

From previous studies, *C. oblongifolius* Roxb. from different locations in Thailand give different diterpenoid compounds. According to the ^{1}H-NMR spectra of hexane crude extract from the stem barks of *C. oblongifolius* Roxb. from Amphoe Viengsa, NAN province, it indicated cleistanthane compounds. Not only different from the other locations but it also exhibited the cytotoxic activity. Thus, it is interesting to investigate the constituents and cytotoxic activity of these plant from Amphoe Viengsa, NAN province.

The objectives of this research are summarized as follows:

1. To extract and isolate the chemical constituents of the stem barks of *C. oblongifolius* Roxb. from Amphoe Veingsa, NAN province.
2. To elucidate the structure of the isolated substances.
3. To study cytotoxic activity of the isolated substances.

CHAPTER II

LITERATURE REVIEWS

2.1 The chemical constituents of *Croton oblongifolius* Roxb.

From the literature surveys, *C. oblongifolius* Roxb. have been widely studied and many diterpenoid compounds have been isolated and characterized.

In 1968, Rao, P. S., Sachdev, G. P., Seshadri, T. R., and Singh, H. B.[7] studied chemical constituents from the stem bark of *Croton oblongifolius* Roxb. They found a new diterpene alcohol, oblongifoliol.

In 1969, Aiyar, V. N., Rao, P. S., Sachdev, G. P., and Seshadri, T. R.[8] found deoxyoblongifoliol from the stem bark of *C. oblongifolius* Roxb.

In 1970, Aiyar, V. N. and Seshadri, T. R.[9]determined the structure of oblongifolic acid, the major diterpene acid component of the bark. It was assigned as isopimara-7(8),15-diene-19-oic acid.

In 1971, Aiyar, V. N. and Seshadri, T. R.[10] determine the structures of oblongifoliol and deoxyoblongifoliol again. Two components have been assigned their structure as *ent*-isopimara-7,15-diene-3β,19-diol and *ent*-isopimara-7,15-diene-3β-ol, respectively.

In the same year, they found three new minor components from the bark. One was *ent*-isopimara-7,15-diene, the second was 19-hydroxy-*ent*-isopimara-7,15-diene and the third was *ent*-isopimara-7,15-diene-19-aldeyde [11]. Moreover, Acetyl aleuritolic acid, 3β-acetoxy-olean-14(15)-ene-28-oic acid, has been obtained from the stem bark [12].

In 1972, Aiyar, V. N. and Seshadri, T. R. found two closely related furanoid diterpenes from the stem bark. One was *ent*-15,16-epoxy-3,11,13(16),14-clerodatetraen-19-oic acid or 11-dehydro(-)-hardwickiic acid and the second was (-)-hardwickiic acid [13]. They studied other parts of *Croton oblongifolius* Roxb. including the root-bark, wood, and leaves. Most compounds reported were isolated from the stem-bark in poorer yields, while the leaves gave only waxy materials [14].

In 1998, Roengsumran, S., *et. al*.[15] found two new cembranoids, one was crotocembraneic acid and the other was neocrotocembraneic acid.

In 1999, Roengsumran, S., *et. al*.[16] found new cembrane diterpene compound, neocrotocembranal from *C. oblongifolius* Roxb.

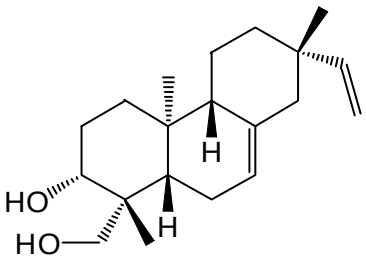
In the same year, Roengsumran, S., *et. al*.[17] found four new labdanes from *C. oblongifolius* Roxb. They were labda-7,12(*E*), 14-triene, the second was labda-7,12(*E*), 14-triene-17-al, the third was labda-7,12(*E*), 14-triene-17-ol and the fourth was labda-7,12(*E*), 14-triene-17-oic acid.

In 2001, Roengsumran, S., *et. al*.[18] found three new labdanes from *C. oblongifolius* Roxb. They were 2-acetoxy-3-hydroxy-labda-8(17), 3-acetoxy-2-labda-8(17),12(*E*),14-triene and 2,3-dihydroxy-labda-8(17),12(*E*),14-triene

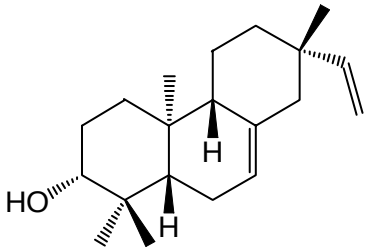
In 2002, Roengsumran, S., *et. al*.[19] found three compounds from *C. oblongifolius* Roxb. which were identified as crovatin, nidorellol and croblongifolin.

Table 1. The chemical constituents of *Croton oblongifolius* Roxb.

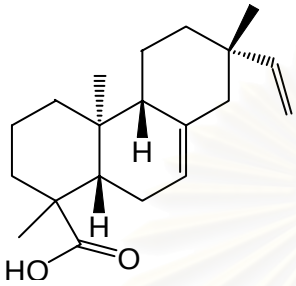
Substances	Location	References
Pimaranes		
Oblongifoliol	India	7
19-Deoxyoblongifoliol	India	8
3-Deoxyoblongifoliol	India	10
Oblongifolic acid	India	9
Isopimaranes		
ent-Isopimara-7,15-diene	India	11
19-hydroxy-ent-Isopimara-7,15-diene	India	11
ent-Isopimara-7,15-diene-19-aldehyde	India	11
Clerodanes		
(-)-Hardwickiic acid	india, Chonburi	13, 20
11-Dehydro-(-)-hardwickiic acid	India	13
Acetyl aleuritolic acid	India	12
Crovatin	Kanchanaburi	20
Cembranes		
Crotocembraneic acid	Petchaboon	15
Neocrotocembraneic acid	Petchaboon	15
Poilaneic acid	Chaingmai	20
Labdanes		
Labda-7,12(E),14-triene	Prachaubkhirikhan	17
Labda-7,12(E),14-triene-17-al	Prachaubkhirikhan	17
Labda-7,12(E),14-triene-17-ol	Prachaubkhirikhan	17
Labda-7,12(E),14-triene-17-oic acid	Prachaubkhirikhan	17
Halimanes		
Crotohalimaneic acid	Kanchanaburi	20
Benzoyl crotohalimaneic acid	Nakornratchasima	20



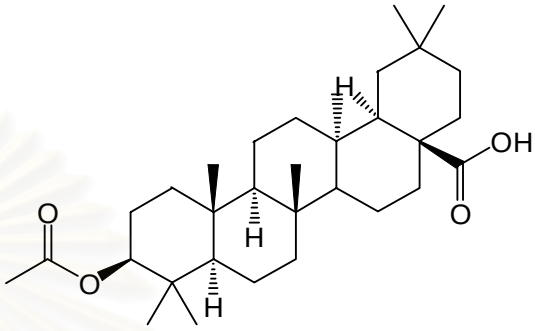
Oblongifoliol



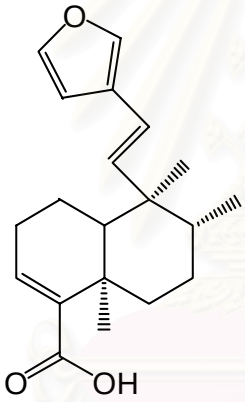
19-Deoxyoblongifoliol



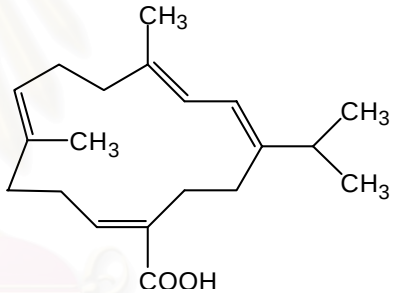
Oblongifolic acid



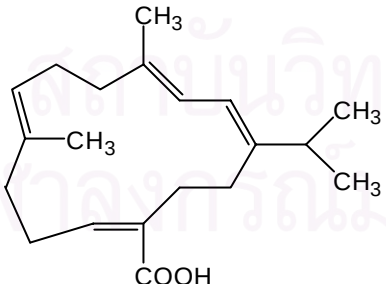
acetyl aleuritolic acid



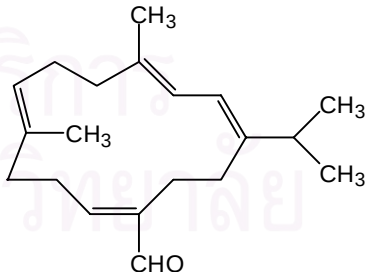
11-Dehydro-(-)-hardwickiic acid



crotocembraneic acid

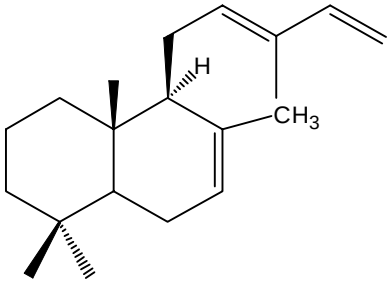


neocrotocembraneic acid

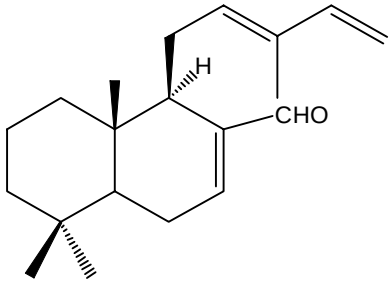


neocrotocembranal

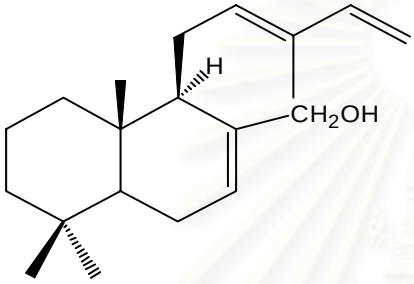
Figure 2. The structures of diterpenoid compounds from *C. oblongifolius* Roxb.



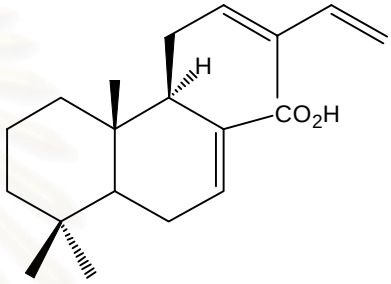
labda-7,12(*E*),14-triene



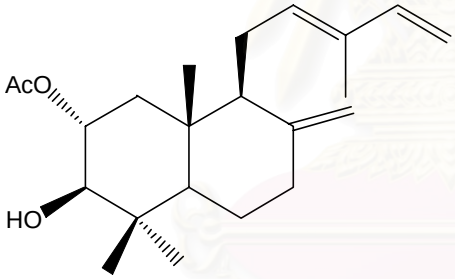
labda-7,12(*E*),14-triene-17-al



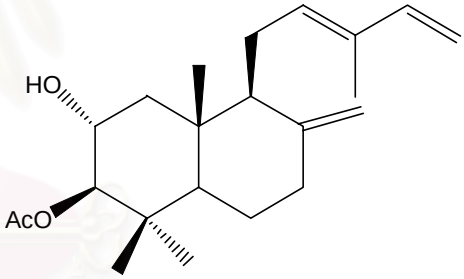
labda-7,12(*E*),14-triene-17-ol



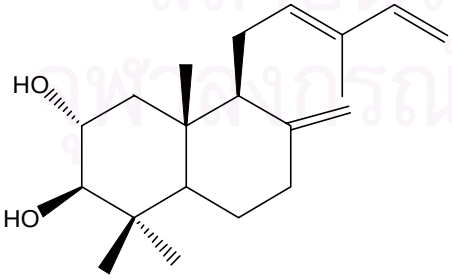
labda-7,12(*E*),14-triene-17-oic acid



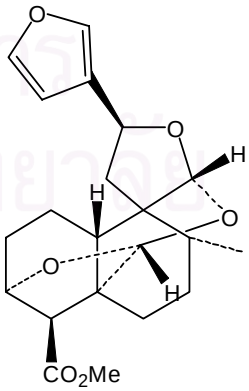
2-acetoxy-3-hydroxy-labda-8(17)



3-acetoxy-2-labda-8(17),12(*E*),14-triene



2,3-dihydroxy-labda-8(17),12(*E*),14-triene



crovatin

Figure 2. The structures of diterpenoid compounds from *C. oblongifolius* Roxb. (Continued)

2.2 The literature reviews of cleistanthane skeleton

In 1982, Craveiro, A.A., and Silveira, E.R. [21] found two Cleistanthane type diterpenes from *Croton sonderianus*, which were sonderianol and 3,4-seco-sonderianol.

In 1984, Pinto, A.C., *et. al*. [22] found new naphthalenic nor-cleistanthane diterpene from *Vellozia epidendroides* and *Vellozia phalocarpa*

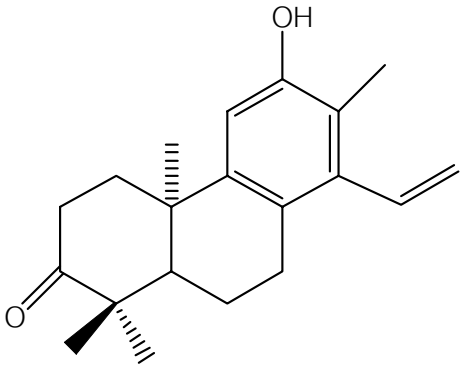
In 1984, Pinto, A.C., *et. al.* [23] found four cleistanthane diterpenes from several species of *Vellozia*, which were cleistantha-8,11,13-triene-3,7-dione, [5*S*, 7*S*, 10*R*]-7α,16; 7β,20-diepoxycleistantha-1,8,11,13-tetraen-3-one, [5*S*, 7*S*, 10*R*]-7α,16; 7β,20-diepoxycleistantha-8,11,13-trien-3-one and 3-oxo-cleistantha-8,11,13-trien-(16,7β)-olide.

In 1987, Pinto, A.C., *et. al*. [24] found three new cleistanthane diterpenes from *Vellozia flavicans*, which were (4*R*, 5*S*, 10*S*)-cleistantha-8,11,13-trien-19-ol, (4*R*, 5*S*, 10*S*)-cleistantha-8,11,13-trien-19-oic acid and (4*R*, 5*S*, 10*S*)-cleistantha-8,11,13-trien-19-al.

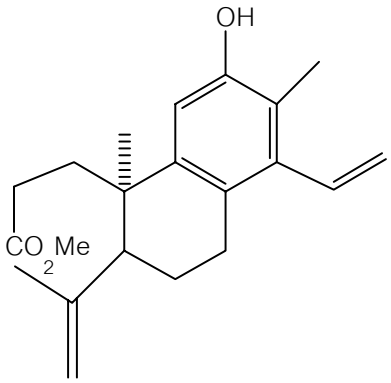
In 1988, Pinto, A.C., *et. al.* [25] found two new cleistanthane diterpenes from *Vellozia nivea*, which were 11-hydroxycleistantha-8,11,13-trien-7-one and 7,11-diketo-14α-hydroxy-cleistantha-8,12-diene.

In 1996, Ayer, W.A., and Khan, A.Q. [26] found three new cleistanthane type diterpenes from liquid cultures of a *Zythiostroma* species, which were zythiostromic acid A, zythiostromic acid B and zythiostromolid.

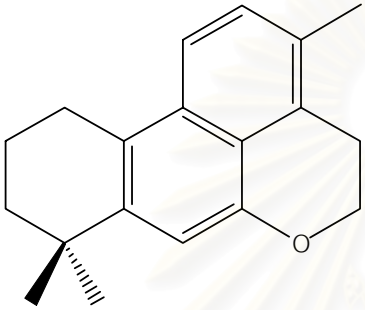
In 2000, Riehl, C.A., and Pinto, A.C. [27] found 8,11,13-cleistanthatrien-7-one-19,20β-olide from *Vellozia compacta*.



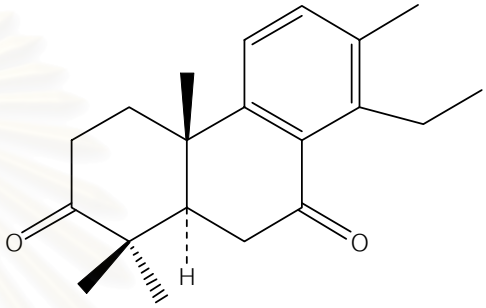
sonderianol



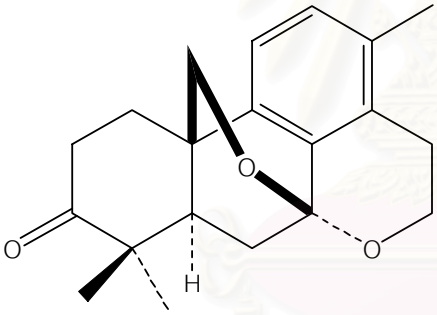
3,4-*seco*-sonderianol



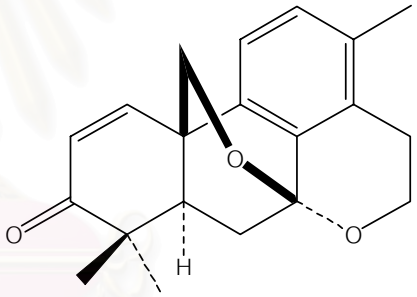
naphthalenic *nor*-cleistanthane diterpene



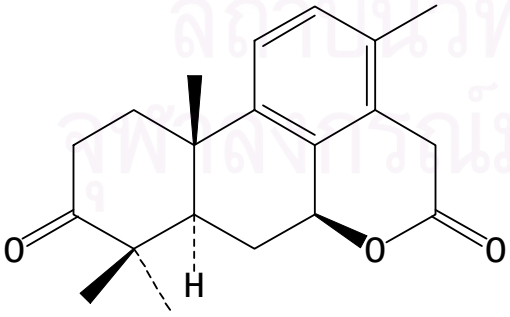
cleistantha-8,11,13-triene-3,7-dione



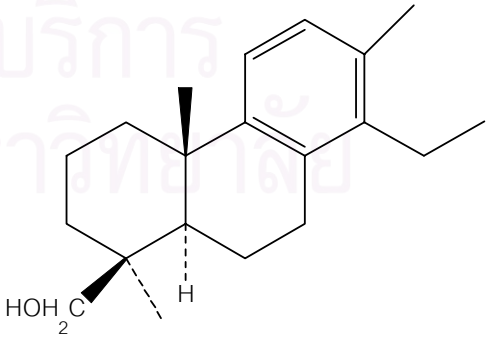
[5*S*, 7*S*, 10*R*]-7α,16; 7β,20-diepoxy-cleistantha-1,8,11,13-tetraen-3-one



[5*S*, 7*S*, 10*R*]-7α,16; 7β,20-diepoxy-cleistantha-8,11,13-trien-3-one

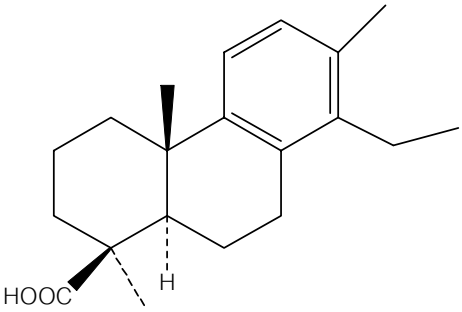


3-oxo-cleistantha-8,11,13-trien-(16,7β)-olide.

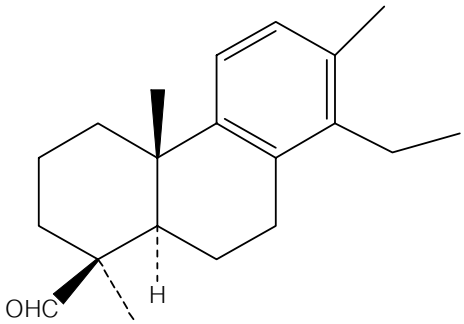


(4*R*, 5*S*, 10*S*)-cleistantha-8,11,13-trien-19-ol

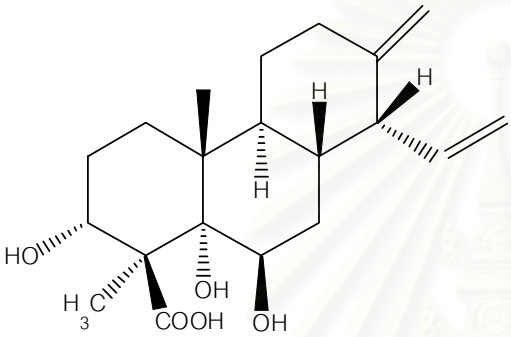
Figure 3. The sample of known cleistanthane skeleton.



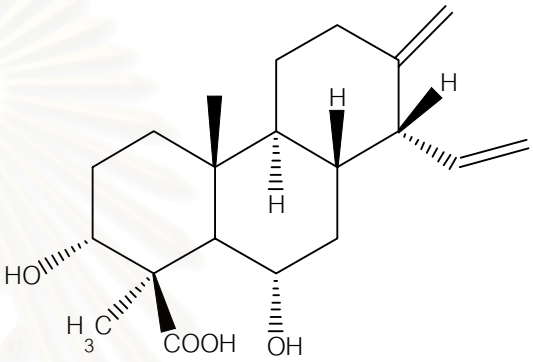
(4*R*, 5*S*, 10*S*)-cleistantha-8,11,13-trien-19-oic acid



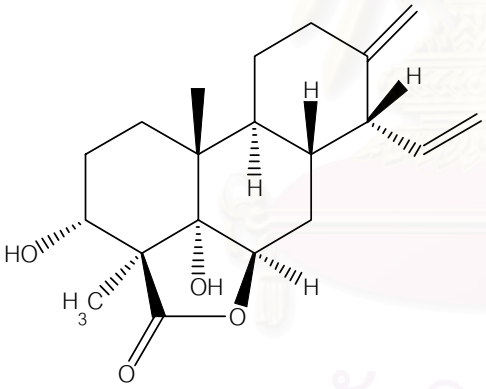
(4*R*, 5*S*, 10*S*)-cleistantha-8,11,13-trien-19-al



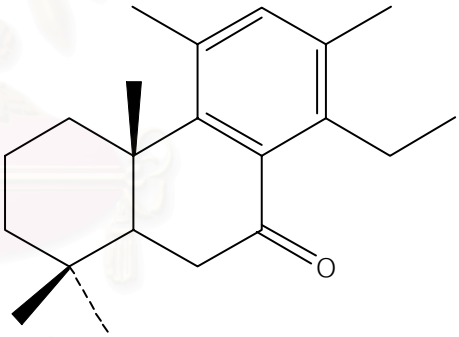
zythiostromic acid A



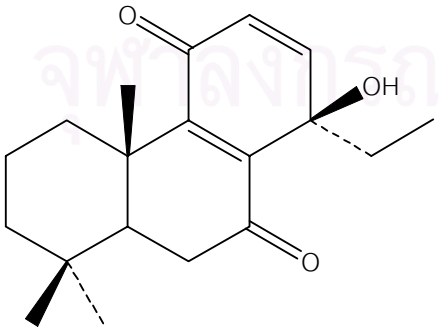
zythiostromic acid B



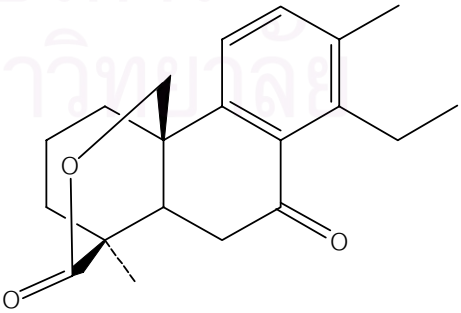
Zythiostromolid



11-hydroxycleistantha-8,11,13-trien-7-one



7,11-diketo-14α-hydroxy-cleistantha-8,12-diene



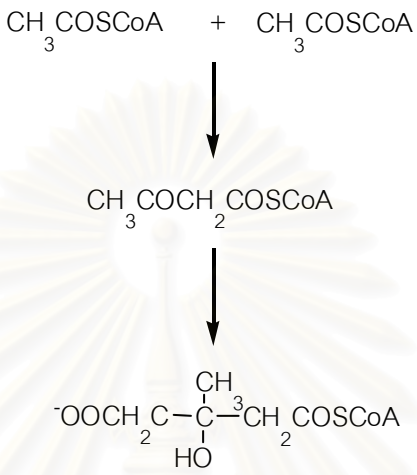
8,11,13-cleistanthatrien-7-one-19,20β-olide

Figure 3. The sample of known cleistanthane skeleton. (Continued)

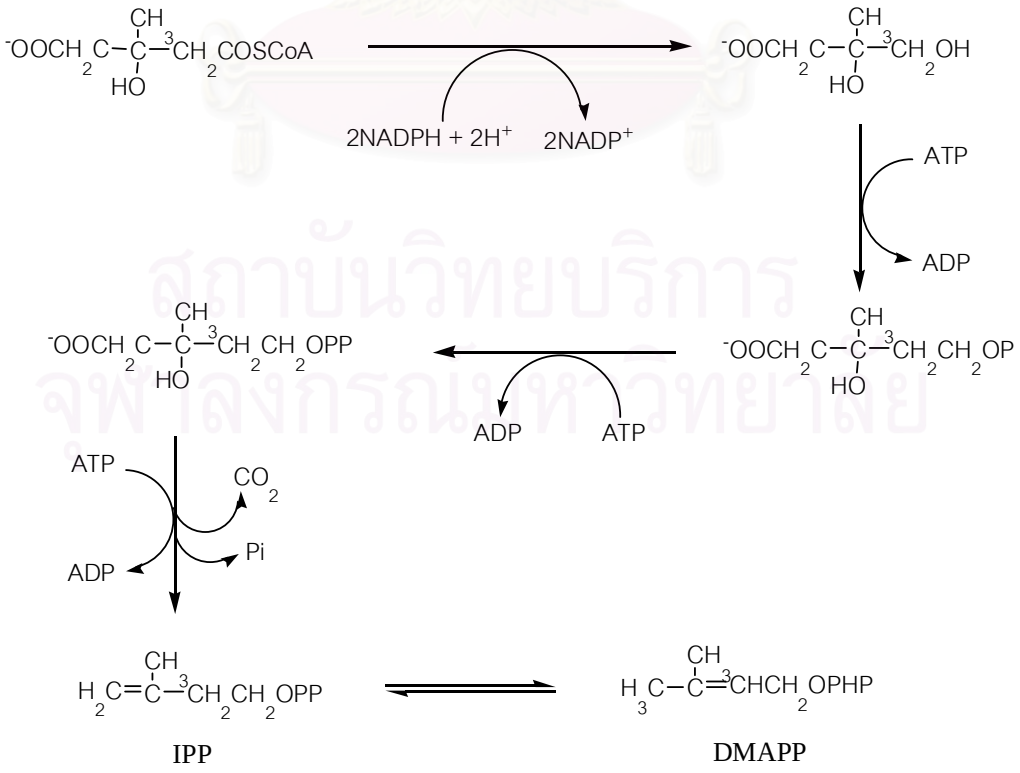
2.3 Biosynthesis of diterpenoid compounds

Biosynthesis of diterpenoid compounds is show in Scheme 1-3 [28]

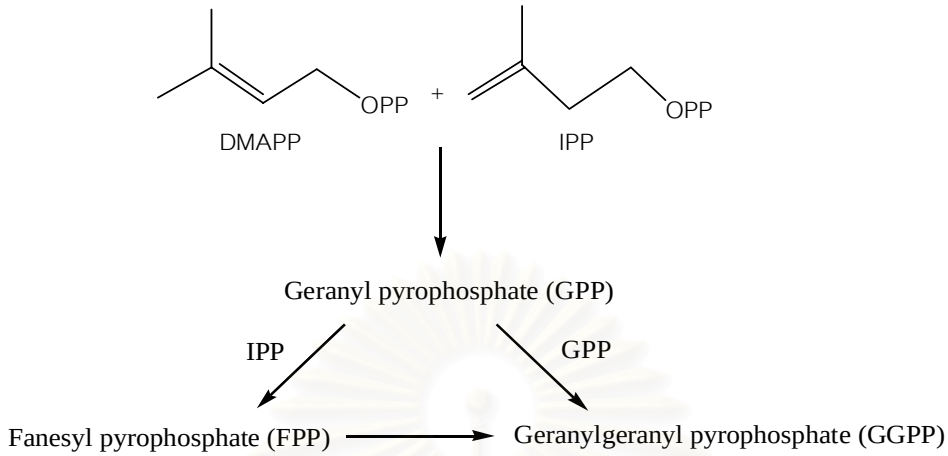
Scheme 1. Biosynthesis of (s)-3-hydroxy-3-methylglutaroyl coenzyme A



Scheme 2. Origin of the isopentenyl-pyrophosphate (IPP) and dimethyl allylpyrophosphate (DMAPP) units



Scheme 3. Assembly of Isoprenes



The geranylgeranyl pyrophosphate can cyclization gives many diterpenoid compounds such as labdane, clerodane, cleistanthane etc. In *C. oblongifolius* Roxb. can found several diterpenoid compounds.

2.4 Biogenetic pathway of cleistanthane

Cleistanthane diterpenes are secondary metabolites uncommon in nature [29]. It was rearranged from pimarane. Biogenetic pathway is show below.

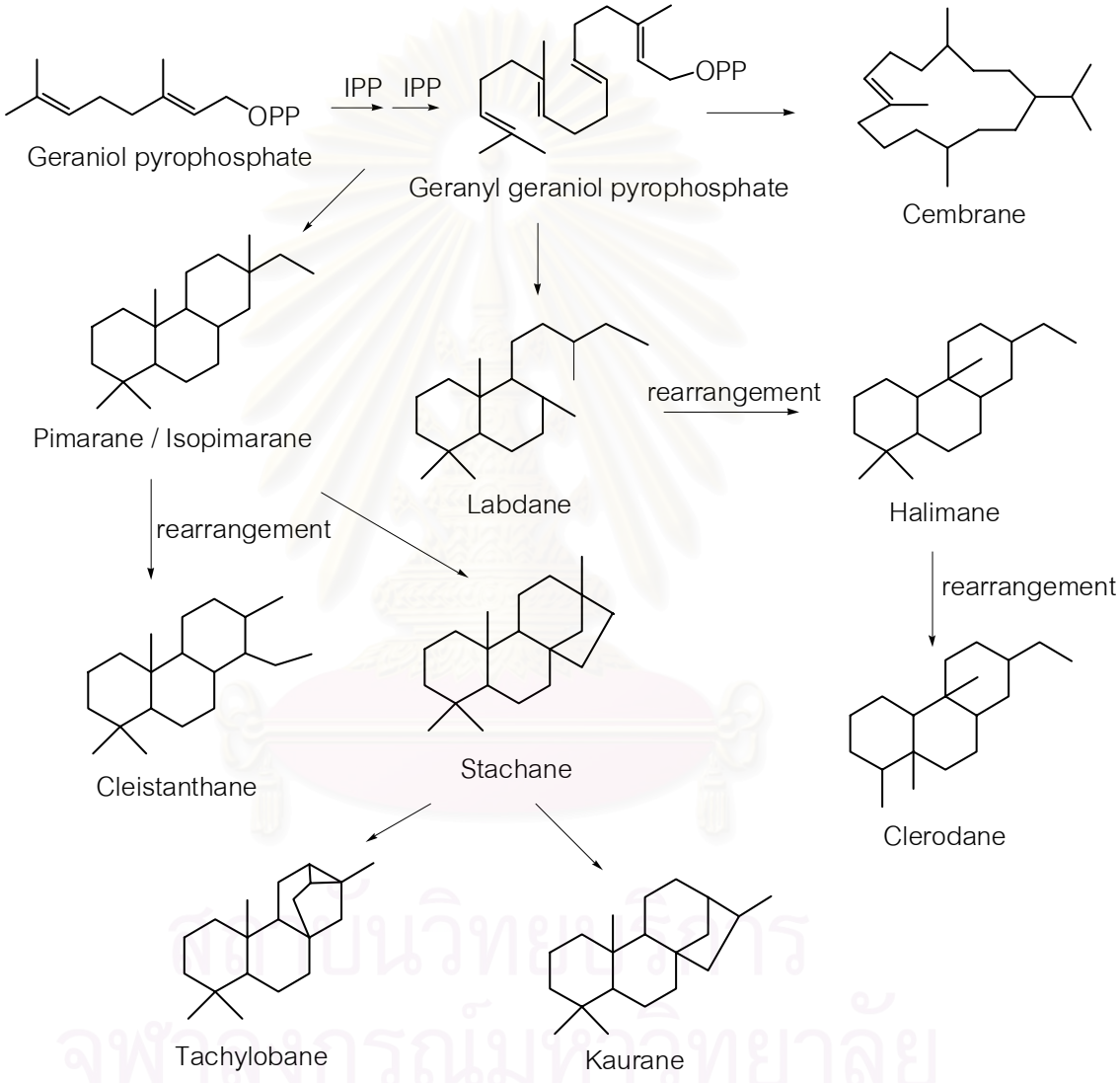


Figure 4. Biogenetic pathway of diterpenoids in *C. oblongifolius* Roxb.

CHAPTER III

EXPERIMENTS

3.1 Plant materials

The stem barks of *Croton oblongifolius* Roxb. were collected from Amphoe Viengsa, Nan province, Thailand, in november, 2003. Botanical identication was achieved through comparison with a voucher specimen No. BKF 084792 in the herbalium collection of the Royal Forest Department of Thailand.

3.2 Chemical reagents

3.2.1 Solvents

All commercial grade solvents which used in this research such as hexane, chloroform, ethyl acetate and methanol, were purified by distillation prior to use.

3.2.2 Other chemicals

1. Merck's silica gel 60 G Art. 7734 (70-230 mesh ASTM) and 9385 (230-400 mesh ASTM) were used as adsorbents for normal column chromatography and flash column chromatography.

2. Merck's TLC aluminum sheets, siliga gel $^{60}F_{254}$ precoated 25 sheets, 20x20 cm^2, layer thickness 0.2 mm were used for TLC analysis.

3. TLC spots were visualized with a UV lamp (254 and 365 nm).

3.3 Instruments and equipments

3.3.1 Melting point apparatus

The melting points were recorded on a Fisher - Johns melting point apparatus.

3.3.2 Rotary Evaporator

The Buchi rotary evaporator was used for the rapid removal of large amounts of volatile solvents.

3.3.3 Optical Rotation

The optical rotation values were measured by a Perkin - Elmer 341 polarimeter.

3.3.4 Elemental Analysis (EA)

The EA values were measured by a Perkin Elmer PE 2400 Series II (CHN/O Analyzer).

3.3.5 Ultraviolet - visible Spectrophotometer (UV-VIS)

The UV - VIS spectra were recorded on a Hewlett Packard 8452A diode array spectrophotometer in chloroform and methanol.

3.3.6 Fourier Transform - Infrared Spectrophotometer (FT- IR)

The FT-IR spectra were recorded on a Nicolet Impact 410 spectrophotometer.

Spectra of solid samples were recorded as KBr pellets.

3.3.7 Nuclear Magnetic Resonance Spectrometer (NMR)

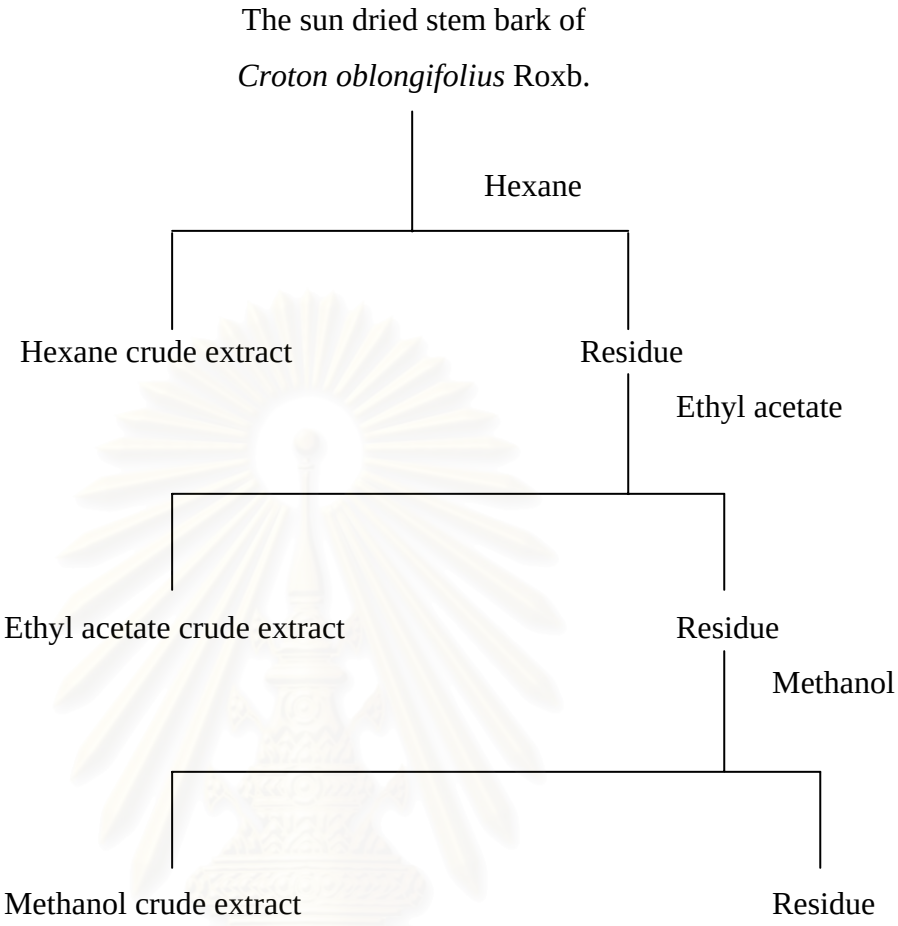
The ^{1}H and ^{13}C Nuclear Magnetic Resonance Spectra were recorded at 400 and 100 MHz, respectively, on a Varian Model Mercury 400 MHz in deuterated chloroform ($CDCl_3$), dimethylsulfoxide (DMSO) and water (D_20)

3.3.8 Mass Spectrometer (MS)

The mass spectra were acquired by a MALDI/TOF Mass Spectrometer Biflex Bruker Germany

3.4 Extraction and Isolation

The powdered, sun-dried stem barks (0.5 kg) of *C.oblongifolius* Roxb. were soakd with hexane (2 x 5 liters), ethyl acetate (2 x 5 liters) and methanol (2 x 5 liters), respectively. The first two extracts were evaporated under reduced pressure until dry, yielding hexane crude extracts as yellow green oil (15 g) and ethyl acetate crude extracts as yellow oil (25 g). The methanolic extract also evaporated under reduced pressure to obtain dark-red gummy residue (20 g). The extraction procedures are shown in Scheme 4.



Scheme 4. The extract procedure of the stem bark of *C.oblongifolius* Roxb.

3.5 Isolation of the chemical constituents from the stem barks of *Croton oblongifolius* Roxb.

3.5.1 Separation of hexane crude extract

The hexane crude extract was obtained as a yellowish green oil (15 g). The crude extract (15 g) was fractionated using silica gel column chromatography and by initially eluting it with hexane. The polarity of eluent was gradually increased from a low portion of ethyl acetate in hexane to 100% ethyl acetate. The volume of eluting solvent was approximately 500 ml and its was evaporated to about 30 ml. Fractions with similar components were combined together according to the TLC profile. The result of separation was shown in Table 2.

Table 2. The result from column chromatography of hexane crude extract.

Eluents	Fraction No.	Appearance	Weight (g.)
100% Hexane	1-10	Yellow viscous liquid	0.33
10% EtOAc in Hex	11-24	Yellow viscous liquid	0.94
20% EtOAc in Hex	25-42	Yellow viscous liquid (containing compound **1**)	2.06
30% EtOAc in Hex	43-57	Yellow viscous liquid (containing compound **2**)	5.33
50% EtOAc in Hex	58-74	Yellow viscous liquid	1.34
70% EtOAc in Hex	75-81	Yellow viscous liquid	0.53
100% EtOAc in Hex	82-85	Brown viscous liquid	0.98
5% MeOH in EtOAc	86-89	Dark brown gummy	2.45

3.5.2 Separation of ethyl acetate crude extract

The ethyl acetate crude extract (25 g) was separated by column chromatography. The column was eluted with hexane, hexane-ethyl acetate, ethyl acetate and ethyl acetate-methanol, respectively. The similar fractions were combined together according to the TLC profile. The result of separation was shown in Table 3.

Table 3. The result from column chromatography of ethyl acetate crude extract.

Eluents	Fraction No.	Appearance	Weight (g.)
100% Hexane	1-37	Yellow viscous liquid	0.23
20% EtOAc in Hex	38-43	Yellow viscous liquid	1.38
30% EtOAc in Hex	44-77	Yellow viscous liquid (containing compound **2**)	3.22
40% EtOAc in Hex	78-85	Yellow viscous liquid (containing compound **2**)	4.53
50% EtOAc in Hex	86-93	Yellow viscous liquid	3.38
70% EtOAc in Hex	94-113	Yellow viscous liquid	2.48
100% EtOAc	121-133	Brown viscous liquid	2.84
5% MeOH in EtOAc	134-141	Dark brown gummy	2.55

3.5.3 Separation of methanol crude extract

The methanol crude extract (20 g) was gummy residue that insoluble in all solvents, Therefore, it was not separate by column chromatrography.

3.6 Purification and properties of compounds from hexane crude extract.

3.6.1 Purification and properties of compound 1

The compound **1** was eluted with 20% ethyl acetate in hexane. The solvent was removed by rotary evaporation and the residue was purified by silica gel column chromatography (Merck's silica gel Art. 1.09385.1000). It was soluble in chloroform, ethyl acetate, methanol and ethanol.

Compound **1** is a white solid (200 mg, 0.04%), $[\alpha]_D^{25}$ +11.06 (EtOAc; *c* 0.32), R*f* = 0.42 (20% EtOAc in hexane), mp. 102-104°C, UV λ_{max}(MeOH) 213sh (log ε 2.89)

FT-IR spectrum (KBr) (Fig.15, Table 4) ν_{max} (cm^{-1}) 3400-3200 (OH), 2931, 1627 (C=C), 1047 (C-O).

^{1}H-NMR spectrum ($CDCl_3$, 400 MHz) (Fig.17, Table 5) δ (ppm) : 6.05(1H, dd d), 5.07(1H, dd), 5.06(1H, dd), 4.69(1H, s), 4.61(1H, s), 3.25(1H, dd), 2.85(1H, dd), 2.20(2H, m), 1.81(1H, m), 1.77(1H, m), 1.67(1H, m), 1.66(1H, m), 1.56(1H, m), 1.54 (1H, m), 1.53(1H, m), 1.38(1H, m), 1.28(1H, m), 1.10(1H, m), 1.10(1H, m), 1.08(1H, m), 0.91(3H, s), 0.84(3H, s), 0.83(3H, s), 0.82(1H, m).

^{13}C-NMR spectrum ($CDCl_3$,100 MHz)(Fig.18, Table 5) δ(ppm) : 152.4(s), 137.7(d), 115.9(t), 106.4(t), 79.1(d), 54.6(d), 54.3(d), 49.2(d), 40.5(d), 38.9(s), 37.6 (t), 36.9(s), 32.1(t), 31.3(t), 28.4(q), 27.5(t), 27.0(t), 21.3(t), 15.8(q), 14.0(q).

MS (Fig. 16) *m/z* : 289 $[M+H]^+$

3.6.2 Purification and properties of compound 2

The compound 2 was eluted with 30% ethyl acetate in hexane. The solvent was removed by rotary evaporation and the residue was purified by silica gel column chromatography (Merck's silica gel Art. 1.09385.1000). It was soluble in chloroform, ethyl acetate, methanol and ethanol.

Compound **2** is a colorless oil (600 mg, 0.12 %), $[\alpha]_D^{25}$ +9.36 (EtOAc; *c* 0.26), R*f* = 0.40 (30% EtOAc in hexane), UV λ_{max} (MeOH) 217 sh (log ε 3.40).

FT-IR spectrum (KBr) (Fig. 23, Table 7) ν_{max} (cm^{-1}) 3400-2400 (OH), 2854, 2931, 1704 (C=O), 1627 (C=C).

^{1}H-NMR spectrum ($CDCl_3$, 400 MHz) (Fig. 25,Table 8) δ (ppm) : 6.06

(1H, dt), 5.08(1H, dd), 5.06(1H, dd), 4.92(1H, d), 4.71(1H, d), 4.71(1H, d), 4.63(1H, d), 2.87(1H, dd), 2.46(1H, m), 2.34(1H, m), 2.24(2H, m), 2.00(1H, dd), 1.78(3H, s), 1.76(1H, m), 1.71(1H, m), 1.69(2H, m), 1.61(1H, m), 1.50(1H, m), 1.48(1H, m), 1.30 (1H, m), 1.28(1H, m), 1.21(1H, m), 0.87(3H, s).

^{13}C-NMR spectrum ($CDCl_3$, 100 MHz) (Fig. 26, Table 8) δ (ppm) : 181.0(s), 151.9(s), 148.4(s), 137.3(d),116.2(t), 113.8(t), 106.8(t), 54.7(d), 50.7(d), 41.0(d), 40.4 (d), 39.0(s), 32.1(t), 31.5(t), 31.3(t), 28.1(t), 27.6(t), 27.4(t), 23.9(q), 16.8(q).

MS (Fig. 24) *m/z* : 303 $[M+H]^+$

3.7 Purification and properties of compounds from ethyl acetate crude extract.

The spectral data [^{1}H and ^{13}C NMR spectra] of ethyl acetate crude extract were similar to hexane crude extract. Purification by column chromatography given compound **2** (1.41 g, 0.28 %)

3.8 The epoxidation reaction of compound 2

Solution of *m*CPBA (230 mg of 70% *m*CPBA) in CH_2Cl_2(5 mL) was added compound **2** (235 mg 0.7773 mmol). The resulting solution was then stirred for 5 h at room temperature. The reaction mixture was washed with saturated sodium carbonate solution (3 times) and water (3 times). The organic layer was dried (Na_2SO_4) and evaporated to yield 120 mg of compound **3** and 30 mg of compound **4**.

Compound **3** is a colorless oil (120 mg, 50% yield), $[\alpha]_D^{25}$ +9.72 (EtOAc; *c* 1.83), R*f* = 0.25 (40% EtOAc in hexane), UV λ_{max} (MeOH) 211 sh (log ε 6.65).

FT-IR spectrum (KBr) (Fig. 31, Table 10) ν_{max} (cm^{-1}) $IR_{\nu max}$ 3130-3000 (OH), 2862, 2935, 1699 (C=O), 1632 (C=C), 1293 (C-O) cm^{-1}

^{1}H-NMR spectrum ($CDCl_3$, 400 MHz) (Fig. 33, Table 11) δ (ppm) : 5.94(1H, ddd), 5.15(1H, dd), 5.07(1H, dd), 4.90(1H, s), 4.72(1H, s), 2.65(2H, dd), 2.47(1H, m), 2.32(1H, m), 2.06(1H, m), 2.00(1H, dd), 1.90(1H, m), 1.78(3H, s), 1.75(1H, m), 1.71 (1H, m), 1.69(2H, m), 1.68(1H, m), 1.50(1H, m), 1.46(1H, m), 1.43(1H, m), 1.29(1H, m), 1.23(1H, m), 1.20(1H, m), 0.94(3H, s).

^{13}C-NMR spectrum ($CDCl_3$, 100 MHz) (Fig. 34, Table 11) δ (ppm) : 180.1(s), 147.4(s), 135.5(d), 118.3(t),113.7(t), 61.5(s), 53.9(t), 53.0(d), 50.8(d), 40.2(d),39.0(s), 36.6(d), 32.1(t), 31.1(t), 29.3(t), 28.0(t), 27.5(t), 23.9(q), 23.0(t), 16.8(q).

MS (Fig. 32) *m/z* : 341 $[M+Na]^+$

Compound **4** is a colorless oil (50 mg, 21% yield), $[\alpha]_D^{25}$ -1.56 (EtOAc; *c* 2.60), R*f* = 0.4 (40% EtOAc in hexane), UV λ_{max} (MeOH) 222 sh (log ε 3.54).

FT-IR spectrum (KBr) (Fig. 80, Table 13) ν_{max} (cm^{-1}) IR_{vmax} 3000 (OH), 2926, 2865, 1699 (C=O), 1632 (C=C), 1283 (C-O) cm^{-1}

^{1}H-NMR spectrum ($CDCl_3$, 400 MHz) (Fig. 82, Table 14) δ (ppm) : 5.91(1H, dd), 5.08(1H, d), 5.05(1H, d), 4.94(1H, s), 4.78(1H, s), 2.59(1H, s), 2.26(1H, m), 2.49 (1H, m), 2.19(1H, dd), 2.06(1H, m), 1.93(1H, m), 1.82(3H, s), 1.70(1H, m), 1.60(1H, m), 1.53(1H, m), 1.42(1H, m), 1.31(1H, m), 1.26(2H, m), 1.14(3H, s), 1.02(3H, s).

^{13}C-NMR spectrum ($CDCl_3$, 100 MHz) (Fig. 83, Table 14) δ (ppm) : 178.7(s), 146.9(s), 146.8(d), 114.0(t),112.0(t), 63.7(d), 61.4(s), 50.6(d), 43.3(d), 40.3(s), 35.6 (s), 34.9(t), 34.7(t), 32.1(t), 28.0(t), 25.6(t), 23.6(q), 22.0(q), 18.3(q), 16.4(t).

MS (Fig. 81) *m/z* : 319 $[M+H]^+$

3.9 Biological evaluation

Each compound was tested for cytotoxic activity towards 6 cell lines which contain HEP-G2 (hepatoma), SW620 (colon), Chago (lung), Kato-3 (gastric) and BT-474 (breast), *in vitro* was performed by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] colorimetric method.[30,31]

CHAPTER IV

RESULT AND DISCUSSION

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

4.1.1 Structure elucidation of compound 1

The IR spectrum of compound **1** (Fig. 15) was summarized in Table 4.

Table 4. The IR absorption band assignment of compound **1**

Wave number (cm^{-1})	Intensity	Tentative assignment
3400 - 3200	Broad	O-H stretching vibration of alcohol
2931	Strong	C-H stretching vibration of CH_3-,CH_2-
1627	Weak	C=C stretching vibration of olefin
1047	Strong	C-O stretching vibration

The ^{1}H-NMR spectrum (Fig. 17, Table 5) of compound **1** showed three methyl groups attaching to quaternary carbons (0.91, 0.84 and 0.83 ppm) and five olefinic protons (6.05, 5.07, 5.06, 4.69 and 4.61 ppm)

The ^{13}C-NMR spectrum (Fig. 18, Table 5) showed 20 lines. Four signals of olefinic carbons appeared at 152.4, 137.7, 115.9 and 106.4 ppm. The signals at 14.0, 15.8 and 28.4 ppm indicated the signal of methyl carbon.

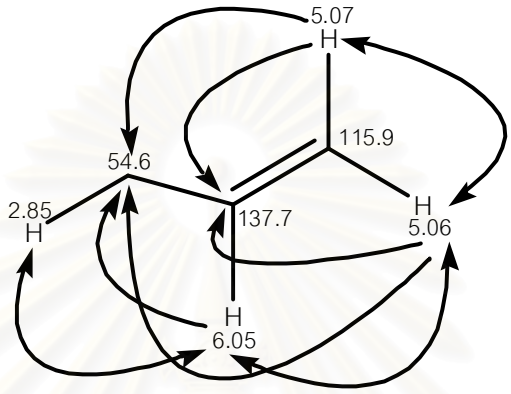
Compound **1** showed a molecular ion with *m/z* 288 ($C_{20}H_{32}O$) which indicated DBE of 5. The information from 2D-NMR techniques, COSY correlations (Fig. 20, Table 5), HMQC correlations (Fig. 19, Table 5), NOESY correlations (Fig. 21) and HMBC correlations (Fig. 22, Table 5) were used to assist the structure assignment of compound **1**.

Table 5. The HMQC, HMBC and COSY spectral data of compound **1**

Position	δ_C[a]	δ_H	HMBC	COSY
1	37.6(t)	1.10 (1Ha, m)	C-9, C-20, C-3	H-2, H-1b
		1.77 (1Hb, m)	C-3, C-2	H-2, H-1a
2	27.5(t)	1.56 (1Ha, m)	C-3	H-3, H-1
		1.67 (1Hb, m)	C-3, C-10	H-3, H-1
3	79.1(d)	3.25 (1H, dd, *J*=11.6, 4.4 Hz)	C-4, C-19, C-18	H-2
4	38.9(s)	-		
5	54.3(d)	0.82 (1H, m)	C-3, C-1, C-9, C-18	H-6
6	21.3(t)	1.38 (1H, m)	C-7	H-5, H-7, H-6b
		1.66 (1H , m)	C-8, C-7, C-3, C-1	H-6a, H-7, H-5
7	32.1(t)	1.28 (1H, m)	C-14, C-8, C-6	H-6, H-8
		1.54 (1H, m)	C-5, C-9, C-14	H-6, H-8
8	40.5(d)	1.53 (1H,m)	C-6	H-14, H-7, H-9
9	49.2(d)	1.08 (1H,m)	C-1	H-11, H-8
10	36.9(s)	-		
11	27.0(t)	1.10 (1Ha, m)	C-8, C-12	H-12, H-11b, H-9
		1.81 (1Hb,m)	C-13, C-8	H-12, H-11a, H-9
12	31.3(t)	2.20 (2H, m)	C-13,C-17,C-9,C-11	H-17a, H-11
13	152.4(s)	-		
14	54.6(d)	2.85 (1H, dd, *J*=9.2, 4.4 Hz)	C-13,C-15,C-16,C-17,	H-15, H-8
			C-9, C-8, C-7, C-12	H-16, H-14
15	137.7(d)	6.05 (1H,ddd, *J*=9.6, 9.6, 19.6 Hz)	C-14	H-15
16	115.9(t)	5.06 (1Ha, dd, *J*=9.6, 2.0 Hz)	C-15, C-14	H-15
		5.07 (1Hb, dd, *J*=20.0, 2.0 Hz)	C-15, C-14	H-17b, H-12
17	106.4(t)	4.61 (1Ha, s)	C-14, C-12	H-17a
		4.69 (1Hb, s)	C-14, C-13, C-12	
18	15.8(q)	0.84 (3H, s)	C-3, C-5, C-4, C-19	
19	28.4(q)	0.91 (3H, s)	C-3, C-5, C-4, C-18	
20	14.0(q)	0.83 (3H, s)	C-1, C-5, C-9, C-10	

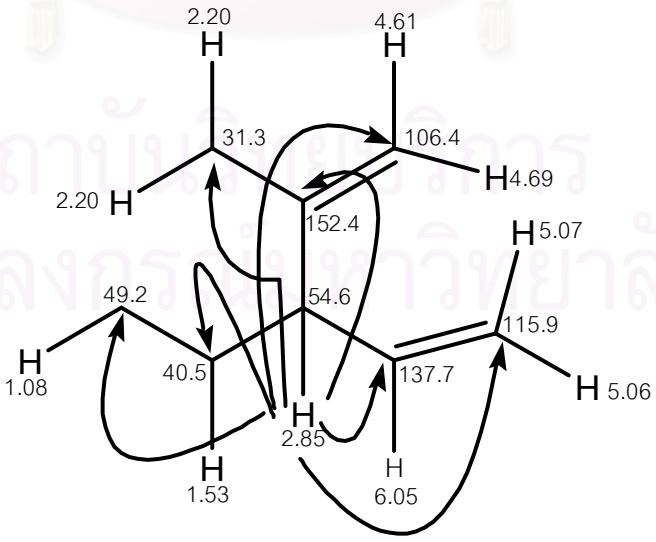
[a]Carbon type determined by DEPT experiments : s = singlet, d = doublet, t = triplet, q = quartet

Crucial long-range ^{1}H-^{13}C correlations were obtained by HMBC correlations (Fig. 22), the proton at 5.07 and 5.06 ppm were coupled with carbon at 137.7 and 54.3 ppm and the proton at 6.05 ppm was coupled with carbon at 54.6 ppm too. The COSY spectrum (Fig.20) showed that the proton at 6.05 ppm were coupled with proton at 5.07 and 5.06 ppm and with the proton at 2.85 ppm (see Scheme 5).



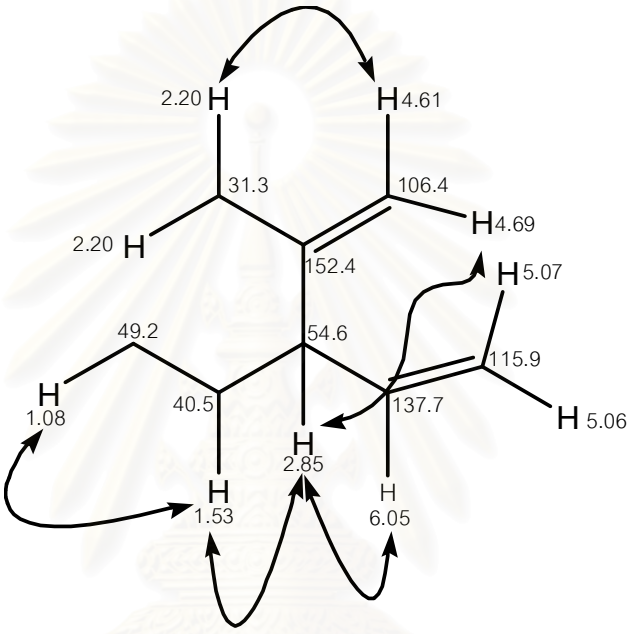
Scheme 5

The HMBC spectrum showed that the proton at 2.85 ppm was coupled with the carbon at 152.4, 137.7, 115.9, 106.4, 49.2, 40.5 and 31.3 ppm (see Scheme 6).



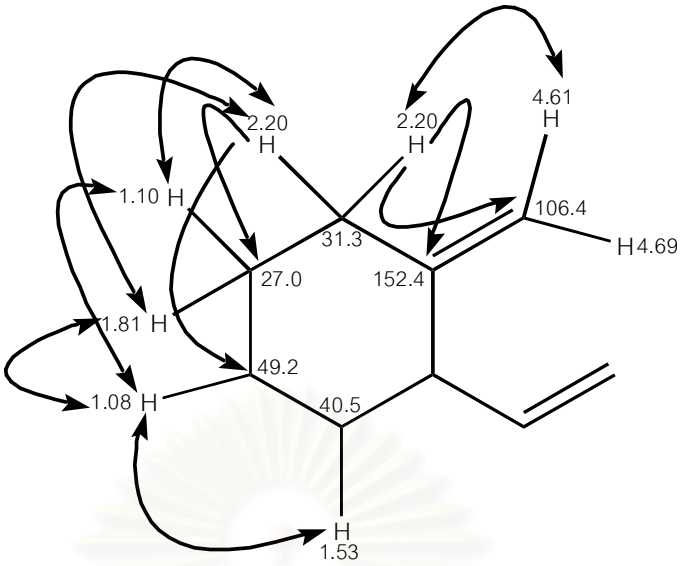
Scheme 6

The COSY spectrum showed that the proton at 2.85 ppm was coupled with proton at 6.05 and 1.53 ppm, the proton at 1.53 ppm was coupled with proton at 2.85 and 1.08 ppm and the proton at 2.20 ppm was coupled with proton at 4.61 ppm. The NOESY spectrum showed the proton at 2.85 ppm was coupled with the proton at 4.69 ppm and the proton at 2.20 ppm was coupled with the proton at 4.61 ppm. Therefore, partial structure of compound **1** was obtained as shown in Scheme 7.



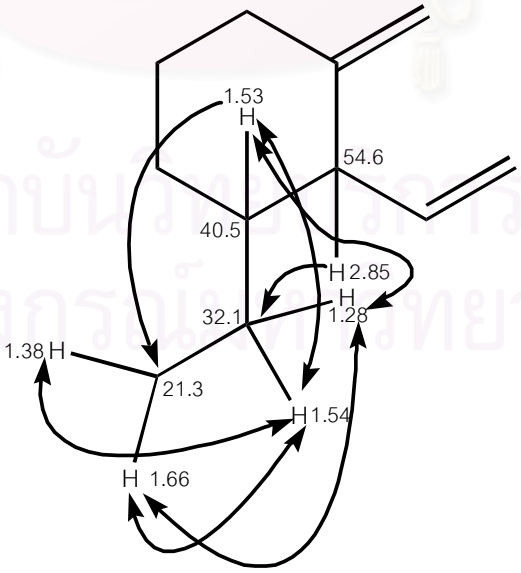
Scheme 7

The HMBC spectrum showed that the proton at 2.20 ppm was coupled with the carbon at 152.4, 106.4, 49.2 and 27.0 ppm. The COSY spectrum showed that the proton at 2.20 ppm was coupled with proton at 4.61, 1.81 and 1.10 ppm and the proton at 1.08 ppm was coupled with proton at 1.81, 1.53 and 1.10 ppm (see Scheme 8).



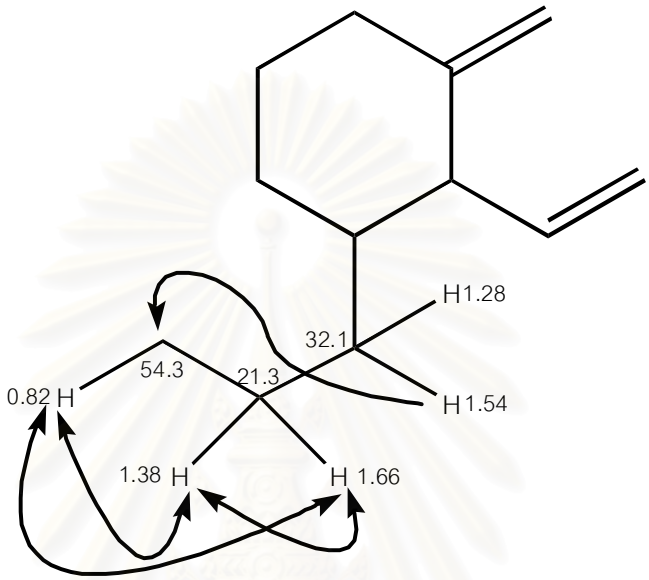
Scheme 8

The HMBC spectrum showed that the proton at 2.85 ppm was coupled with carbon at 32.1 ppm and the proton at 1.53 ppm was coupled with the carbon at 21.3 ppm. The COSY spectrum showed that the proton at 1.53 ppm was coupled with the proton at 1.54 and 1.28 ppm and the proton at 1.54 and 1.28 ppm were coupled with the proton at 1.53, 1.66 and 1.38 ppm (see Scheme 9).



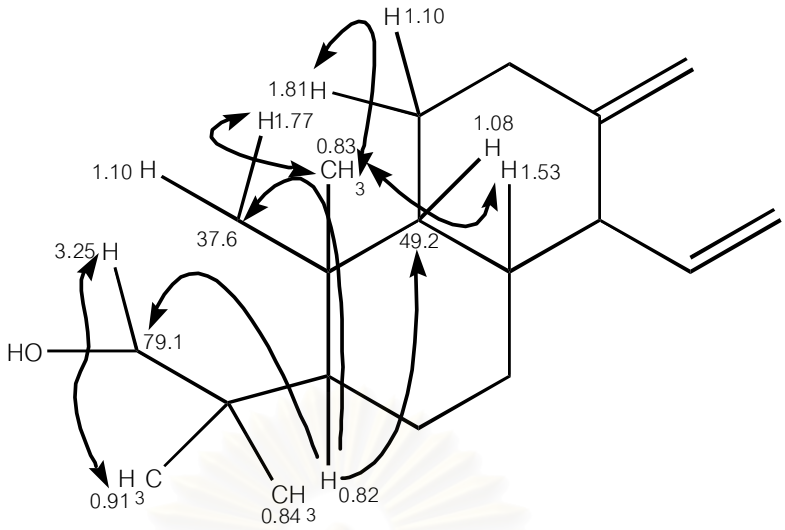
Scheme 9

The proton at 1.54 ppm was coupled with carbom at 54.3 ppm according to HMBC spectrum. The proton at 1.38 ppm was coupled with the proton at 1.66 and 0.82 ppm and the proton at 1.66 ppm was coupled with the proton at 1.38 and 0.82 ppm according to COSY spectrum (see Scheme 10).



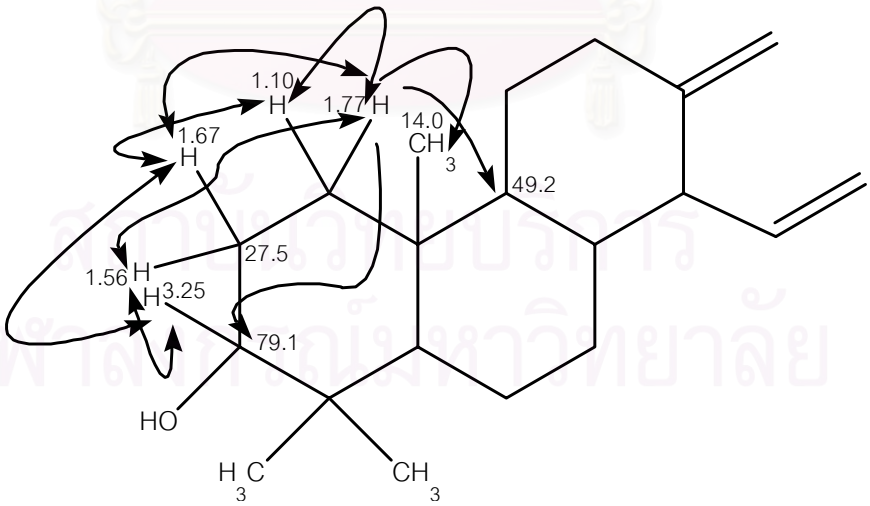
Scheme 10

The proton at 0.82 ppm was coupled with carbon at 79.1, 49.2 and 37.6 ppm according to HMBC spectrum. The NOESY spectrum showed the methyl proton at 0.83 ppm was coupled with the proton at 1.38 and 1.10 ppm, the methyl proton at 0.91 ppm was coupled with the 3.25 ppm and the proton at 1.08 ppm was coupled with the proton at 0.82 ppm (see Scheme 11).



Scheme 11

The proton at 1.77 ppm was coupled with carbon at 79.1 and 27.5 ppm and the proton at 1.10 ppm was coupled with carbon at 79.1, 49.2 and 14.0 ppm according to HMBC spectrum. The proton at 1.77 ppm was coupled with proton at 1.67, 1.56 and 1.10 ppm, the proton at 1.10 ppm coupled with proton at 1.67, 1.56 and 1.77 ppm and the proton at 3.25 ppm was coupled with proton at 1.67 and 1.56 ppm according to COSY spectrum (see Scheme 12).



Scheme 12

Thus, the structure of compound **1** was proposed to be 3-hydroxycleistantha-13(17),15-diene as show in Figure 5. The long range C-H correlation by HMBC and COSY correlation spectrum were summarized in Figure 6 and 7, respectively.

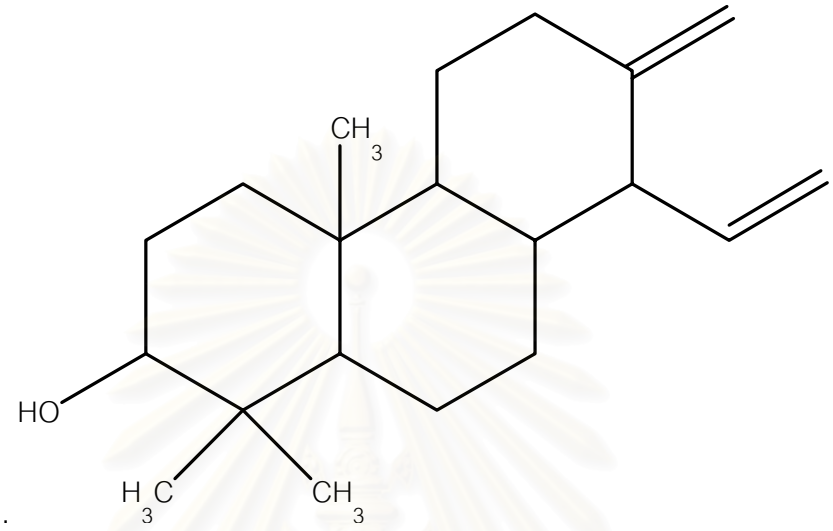


Figure 5. The structure of compound **1**

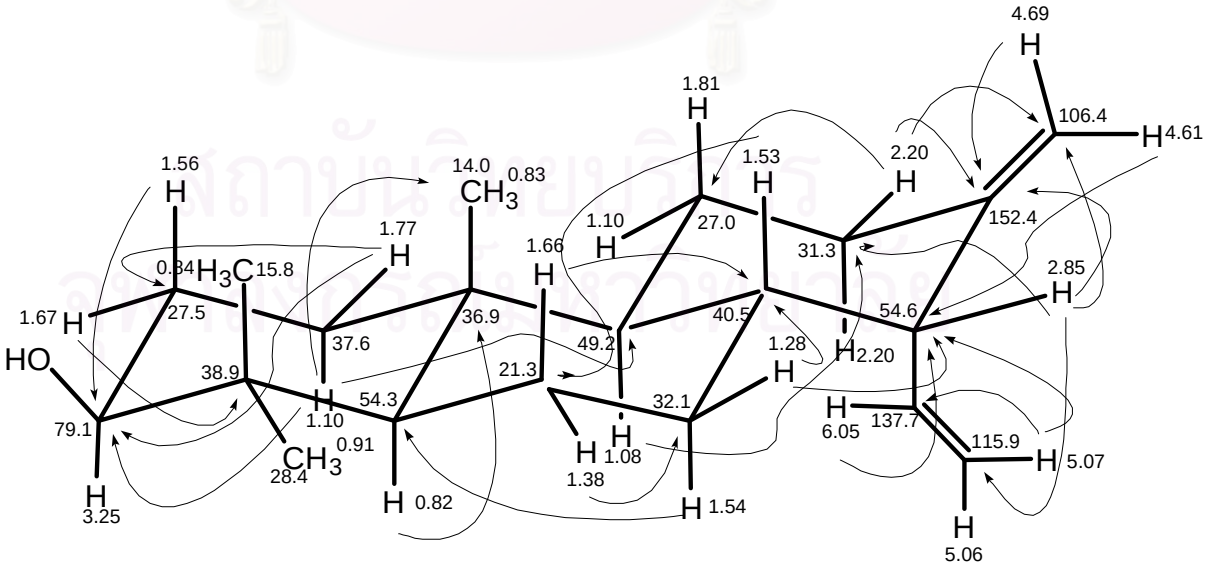


Figure 6. The HMBC correlations of compound **1**

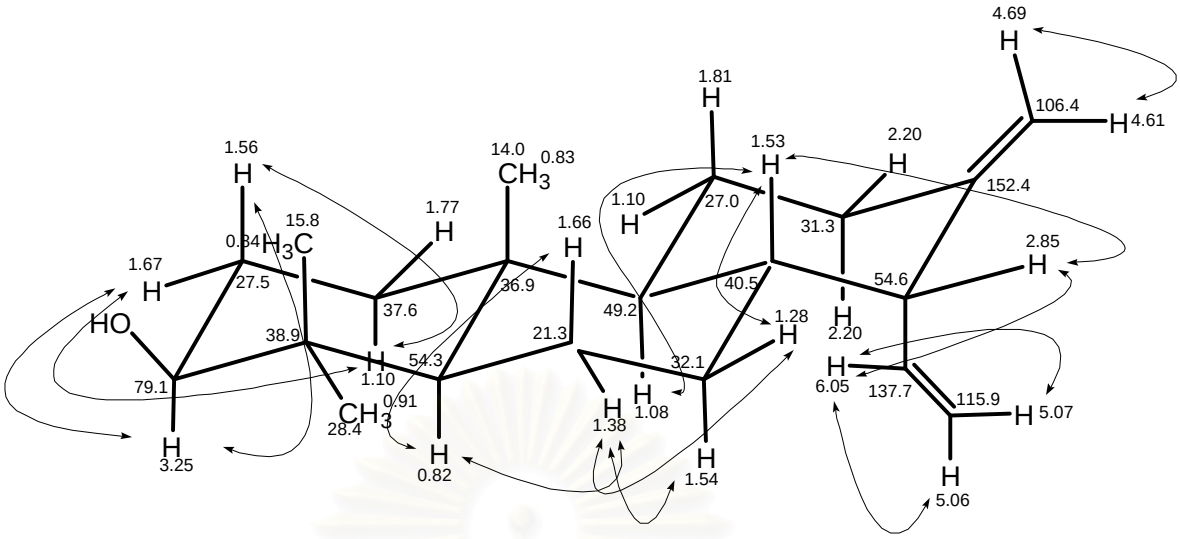
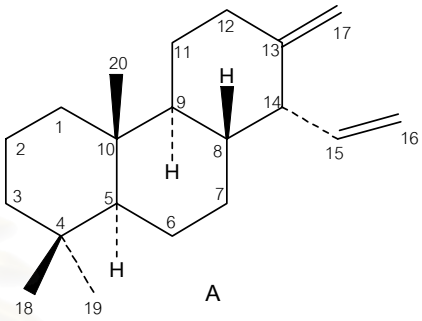


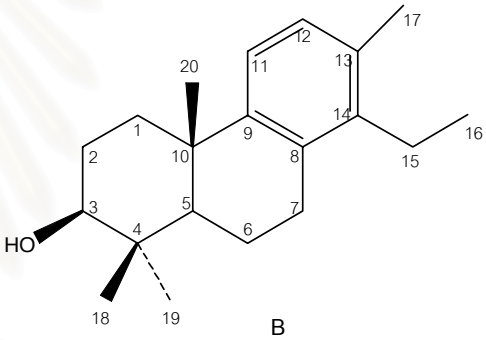
Figure 7. The COSY correlations of compound **1**

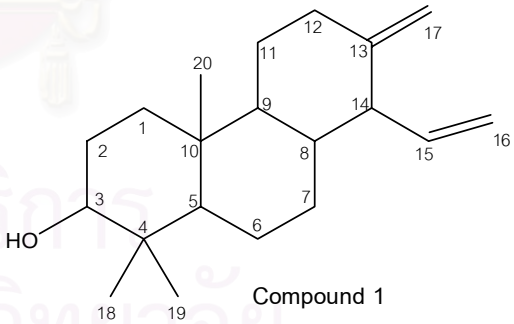
The structure search in the literature revealed that compound **1** was a novel compound. The most closely related structures were (5α,8β,9α,10β)-4,4,10-trimethylphenanthrene (A) [32] and 8,11,13-cleistanthatrien-3β-ol (B) [33] because both compounds have partial structure similar to compound 1 (Table 6). From ^{13}C-NMR chemical shift of compound 1 compared with those of A and B, the structure of compound 1 was proposed as in Figure 5.

Table 6. ^{13}C-NMR chemical shift of compound **1** was compared to compound **A**, **B**

Position	Chemical shift of ^{13}C-NMR		
	Compound 1	A	B
1	37.6	39.6	37.4
2	27.5	18.9	27.9
3	79.1	42.2	78.5
4	38.9	32.1	38.8
5	54.3	55.2	49.1
6	21.3	21.5	19.0
7	32.1	31.4	28.0
8	40.5	40.7	132.7
9	49.2	54.8	147.4
10	36.9	39.6	37.6
11	27.0	27.0	121.7
12	31.3	29.6	127.8
13	152.4	152.8	132.4
14	54.6	49.4	140.1
15	137.7	138.1	22.1
16	115.9	115.7	13.0
17	106.4	106.3	19.3
18	15.8	22.1	27.9
19	28.4	33.6	15.3
20	14.0	14.0	25.0







A = (5α,8β,9α,10β)-4,4,10-trimethylphenanthrene

B = 8,11,13-cleistanthatrien-3β-ol

The stereochemistry at the different chiral centres in the molecule was established by the coupling constants in the ^{1}H NMR spectrum and NOESY spectra. In the ^{1}H NMR spectrum of compound **1**, H-14 (2.85 ppm) showed a 9.2 Hz coupling with H-15 (6.05 ppm) and a 4.4 Hz coupling with H-8 (1.53 ppm), suggesting that H-14 is equatorial. This assignment is confirmed by NOESY spectra, the long range ^{1}H-^{1}H correlation of H-14 with H-17b (4.69 ppm). Morever, the β-equatorial orientation of the hydroxyl group at C-3 was assigned by NOESY spectrum, the proton at C-3 (3.25 ppm) was correlation with the proton at 0.82 ppm. So, relative stereochemistry of compound **1** was determined on the basis of NOESY spectra, key NOESY correlations in compound **1** are shown in Figure 8.

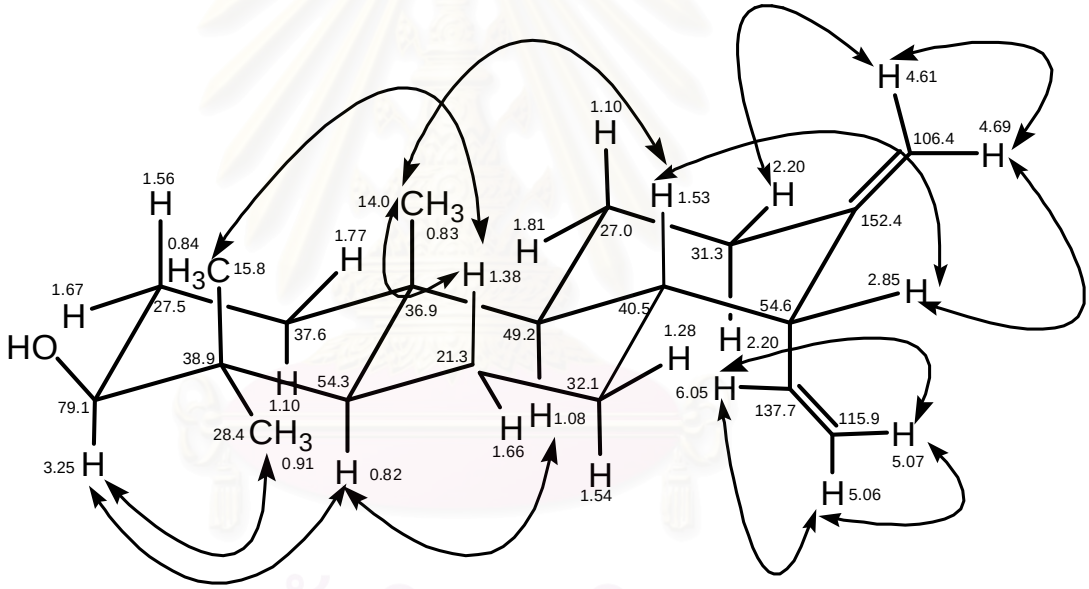


Figure 8. The NOESY correlations of compound **1**

4.1.2 Structure elucidation of compound 2

The IR spectrum of compound **2** (Fig. 23) was summarized in Table 7.

Table 7. The IR absorption band assignment of compound **2**

Wave number (cm^{-1})	Intensity	Tentative assignment
3400 - 2400	Broad	O-H stretching vibration of carboxylic acid
2931, 2854	Strong	C-H stretching vibration of CH_3-, -CH_2-
1704	Strong	C=O stretching vibration of carbonyl group
1627	Medium	C=C stretching vibration of olefin

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. 28, Table 8), HMQC correlations (Fig. 27, Table 8), NOESY correlations (Fig. 29) and HMBC correlations (Fig. 30, Table 8) were used to assist the structure assignment of compound **2**.

The ^{1}H-NMR spectrum (Fig. 25, Table 8) of compound **2** revealed the presence of two terminal methylene groups [two olefinic protons at δ 4.63 (1H, d, *J* = 0.89 Hz) and δ 4.71 (1H, d, *J* = 0.89 Hz) and two olefinic protons at δ 4.71 (1H, br, s) and δ 4.92 (1H, br, s)]. The ^{1}H NMR also showed the presence of vinyl group from the signals at δ 6.06 (1H, dd, *J* = 16.4 and 9.2 Hz), δ 5.06 (1H, dd, *J* = 10.8 and 2.2 Hz) and δ 5.08 (1H, dd, *J* = 16.4 and 2.2 Hz) and two methyl groups at δ 1.78 (3H, s) and δ 0.87 (3H, s)

The ^{13}C-NMR spectrum (Fig. 26, Table 8) and HSQC spectrum of compound **2** showed 20 lines. The spectrum illustrated the signal of a carbonyl carbon of carboxylic group at 181.0 ppm and the olefinic carbon signals at 106.8, 113.8, 116.2, 137.3, 148.4 and 151.9 ppm. The signals at 39.0 ppm indicated the signal of quaternary carbon. Moreover, the signals indicated the four methine carbons at 40.4, 41.0, 50.7 and 54.7 ppm, six methylene carbons at 27.4, 27.6, 28.1, 31.3, 31.5 and 32.1 ppm and methyl group at 16.8 and 23.9 ppm.

Table 8. The HMQC, HMBC and COSY spectral data of compound **2**

Position	δ_C[a]	δ_H	COSY	HMBC
1	32.1(t)	1.69 (2H, m)	H-2	C-3, C-5, C-10,C-2,C-20
2	28.1(t)	2.46 (1Ha, m)	H-1	C-3,C-10,C-1
		2.34 (1Hb, m)	H-1	C-3, C-10, C-1
3	181.0(s)	-	-	-
4	148.4(s)	-	-	-
5	50.7(d)	2.0 (1H, dd, *J*=12.8,2.4 Hz)	H-6	C-4, C-18,C-10,C-1, C-7,C-6,C-19,C-20
6	27.6(t)	1.48 (1Ha, m)	H-5, H-7	C-5,C-8,C-10
		1.71 (1Hb, m)	H-5, H-7	C-5, C-9,C-10, C-20
7	31.5(t)	1.28 (1Ha, m)	H-6, H-8	C-5, C-14, C-8
		1.50 (1Hb, m)	H-6, H-8	C-5, C-14, C-9
8	40.4(d)	1.61 (1H, m)	H-7, H-9, H-14	C-15,C-14,C-9, C-6
9	41.0(d)	1.30 (1H, m)	H-8, H-11	C-1,C-11,C-20
10	39.0(s)	-	-	-
11	27.4(t)	1.21 (1Ha, m)	H-9, H-12	C-9, C-12
		1.76 (1Hb, m)	H-9, H-12	C-8, C-10, C-12
12	31.3(t)	2.24 (2H, m)	H-11, H-17a	C-13, C-9, C-11, C-17
13	151.9(s)	-	-	-
14	54.7(d)	2.87 (1H, dd, *J*=8.8, 4.4 Hz)	H-8, H-15	C-13,C-15, C-16,C-17, C-9, C-8, C-7, C-12
15	137.3(d)	6.06 (1H,dt, *J*=16.4, 9.2 Hz)	H-16, H-14	C-13,C-14,C-8
16	116.2(t)	5.06 (1Ha, dd, *J*=10.8, 2.2 Hz)	H-15, H-16b	C-15, C-14
		5.08 (1Hb, dd, *J*=6.4, 2.2 Hz)	H-15, H-16a	C-15, C-14
17	106.8(t)	4.63 (1Ha, d, *J*=0.89 Hz)	H-12, H-17b	C-13, C-14, C-12, C-15
		4.71 (1Hb, d, *J*=0.89 Hz)	H-17a	C-13, C-14, C-12, C-15
18	113.8(t)	4.71 (1Ha, s)	H-18b, H-19	C-5, C-4, C-19
		4.92 (1Hb, s)	H-18a, H-19	C-5, C-4, C-19
19	23.9(q)	1.78 (3H, s)	H-18, H-20	C-4, C-18, C-5
20	16.8(q)	0.87 (3H, s)	H-19	C-1, C-5, C-9, C-10

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

By comparison of spectral data of **2** with those of **1** (Table 9). Including a detailed analysis of 2D NMR spectra, it seemed structure **2** was quite similar to of **1**, except that the ring A of **2** was broken at C-3 - C-4 and C-3 became a carboxylic group. The ^{1}H and ^{13}C NMR and 2D NMR spectra data supported a cleistanthane-type diterpenoid structure for compound **2**.

The relative configuration for all the five asymmetric centers of compound **2** was determined on the basis of NOESY spectra. The NOESY revealed interactions between H-8 (1.61 ppm) and Me-20 (0.87 ppm), H-9 (1.30 ppm) and H-7b (1.50 ppm), H-14 (2.87 ppm) and H-17b (4.71 ppm), and H-5 (2.0 ppm) and H-6b (1.71 ppm)

Thus, the structure of compound **2** was proposed to be 3,4-*seco*-cleistantha-4(18),13(17),15-trien-3-oic acid. The ^{13}C-NMR chemical shift of compound **2** was compared with that of compound **1** to confirm the structure (Table 9). The structure of compound **2** is shown in Figure 9. The long rang C-H correlations by HMBC, COSY correlations and NOESY correlation spectrum were summarized in Figure 10, 11 and 12, respectively.

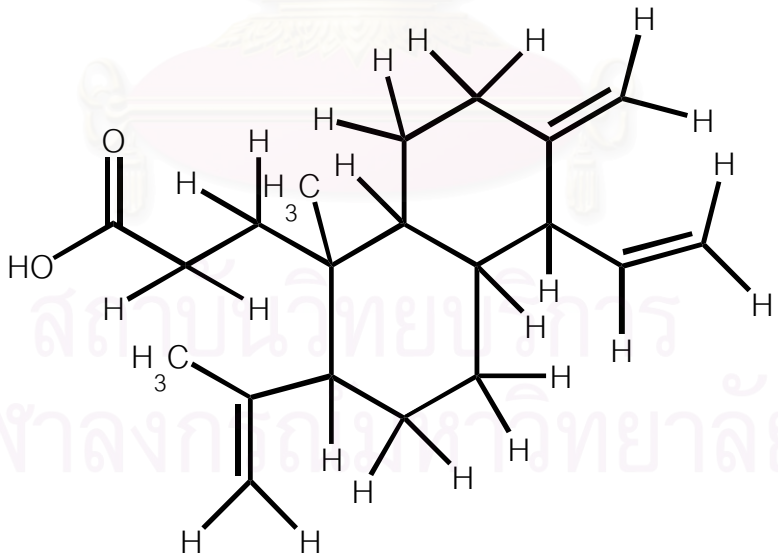
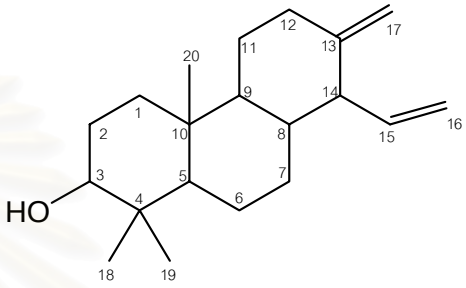


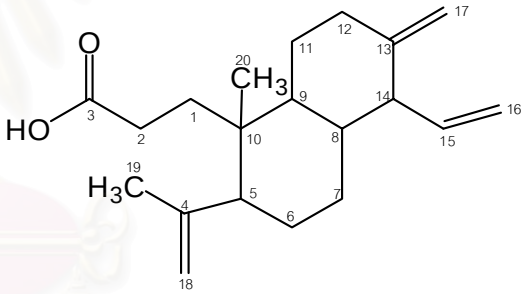
Figure 9. The structure of compound **2**

Table 9. ^{13}C-NMR chemical shift of compound **2** was compared to compound **1**

Position	Chemical shift of ^{13}C-NMR	
	Compound **2**	Compound **1**
1	32.1	37.6
2	28.1	27.5
3	181.0	79.1
4	148.4	38.9
5	50.7	54.3
6	27.6	21.3
7	31.5	32.1
8	40.4	40.5
9	41.0	49.2
10	39.0	36.9
11	27.4	27.0
12	31.3	31.3
13	151.9	152.4
14	54.7	54.6
15	137.3	137.7
16	116.2	115.9
17	106.8	106.4
18	113.8	15.8
19	23.9	28.4
20	16.8	14.0



Compound 1



Compound 2

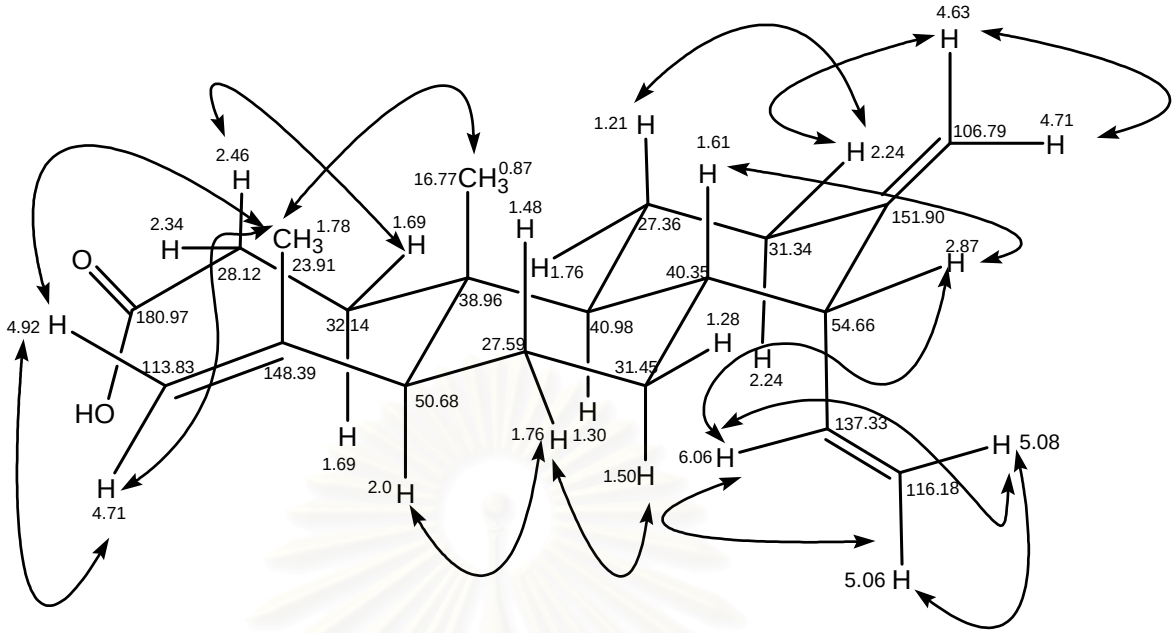


Figure 10. The COSY correlations of compound **2**

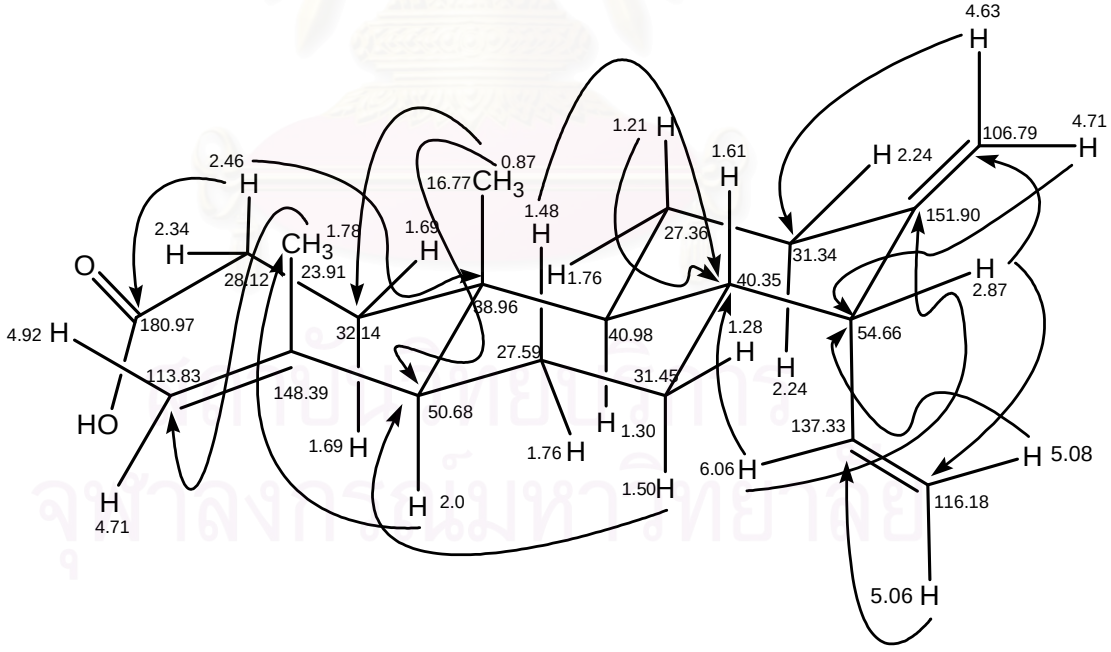


Figure 11. The HMBC correlations of compound **2**

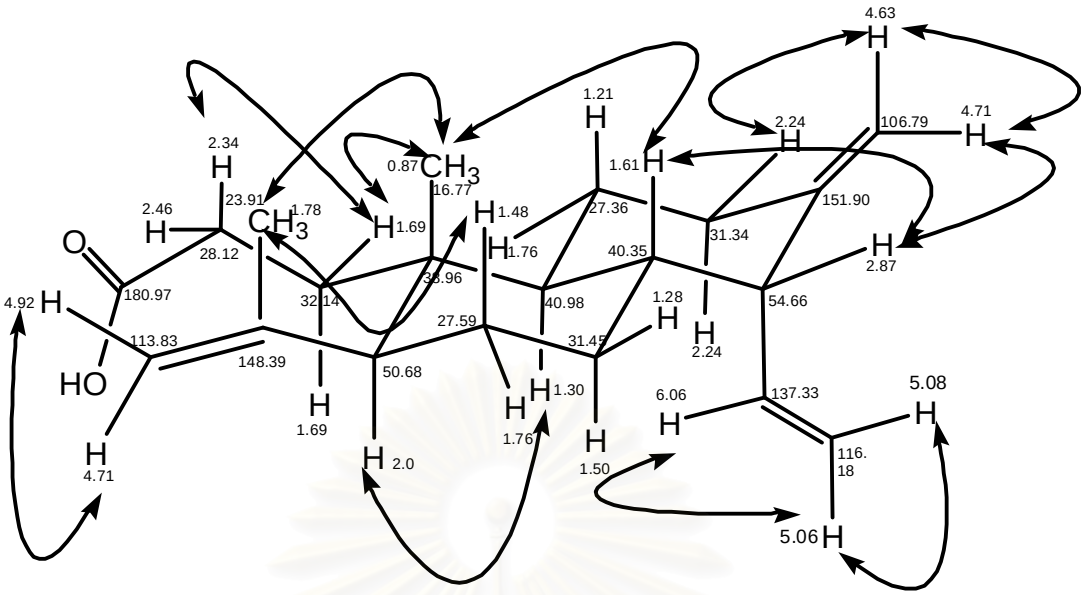


Figure 12. The NOESY correlations of compound **2**

4.2 Epoxidation reaction of compound 2

Compound **3** and compound **4** could be synthesized by epoxidation of compound **2**. The reaction between compound **2** (1.0 equiv) and *m*CPBA (1.2 equiv) was carried out in dichloromethane at room temperature for 5 hour to afforded quantitative yield of compound **3** and **4**.

4.2.1 Structure elucidation of compound 3

The spectroscopic data clearly confirmed the structure of compound **3**. The IR spectrum of compound **3** (Fig. 31) was summarized in Table 10.

Table 10. The IR absorption band assignment of compound **3**

Wave number (cm^{-1})	Intensity	Tentative assignment
3130 -3000	Broad	O-H stretching vibration of carboxylic acid
2935, 2862	Strong	C-H stretching vibration of CH_3-, -CH_2-
1699	Strong	C=O stretching vibration of carbonyl group
1632	Medium	C=C stretching vibration of olefin
1293	Medium	C-O stretching vibration

Compound **3** showed a molecular ion with *m/z* 341 ($C_{20}H_{30}O_3$) which indicated DBE of 6. The information from 2D-NMR techniques, COSY correlations (Fig. 36, Table 11), HMQC correlations (Fig. 35, Table 11), NOESY correlations (Fig. 37, Table 11) and HMBC correlations (Fig. 38, Table 11) were used to assist the structure assignment of compound **3**.

Table 11. The HMQC, HMBC and COSY spectral data of compound **3**.

Position	δ_C[a]	δ_H	COSY	HMBC
1	32.1(t)	1.69 (2H, m)	H-2	C-2, C-3, C-5,C-9,C-20
2	28.0(t)	2.32 (1Ha, m)	H-2b, H-1	C-1, C-3, C-10
		2.47 (1Hb, m)	H-2a, H-1	C-1, C-3, C-10
3	180.1(s)	-	-	-
4	147.4(s)	-	-	-
5	50.7(d)	2.00 (1H, dd, *J*=12.4,2.8 Hz)	H-6, H-19	C-4, C-10, C-18, C-19, C-20
6	27.5(t)	1.46 (1Ha, m)	H-5, H-7	C-5, C-8
		1.75 (1Hb, m)	H-5, H-7	C-5, C-7, C-8
7	31.1(t)	1.23 (1Ha, m)	H-6, H-8	C-6, C-8, C-9, C-14
		1.43 (1Hb, m)	H-6, H-8	C-9
8	36.6(d)	1.90 (1H, m)	H-7, H-9	C-7, C-9, C-14, C-15
9	40.2(d)	1.29 (1H, m)	H-8	C-10, C-11, C-20
10	39.0(s)	-	-	-
11	29.3(t)	1.20 (1Ha, m)	H-12	C-13
		2.06 (1Hb, m)	H-12	C-12
12	23.0(t)	1.50 (1Ha, m)	H-11	C-9, C-11
		1.68 (1Hb, m)	H-11	C-9, C-11
13	61.5(s)	-	-	-
14	53.0(d)	1.71 (1H, m)	H-8, H-15	C-9, C-13, C-15, C-16, C-17
15	135.5(d)	5.94 (1H, ddd, *J*=20, 10, 10 Hz)	H-14, H-16	C-8, C-13, C-14
16	118.3(t)	5.07 (1Ha, dd, *J*=10.4,2.0 Hz)	H-15, H-16b	C-13, C-14
		5.15 (1Hb, dd, *J*=17.2,1.6 Hz)	H-15, H-16a	C-13, C-14
17	53.9(t)	2.64 (1H, d, *J*=4.4 Hz)	-	C-8, C-11, C-13, C-14
		2.67 (1H, d, *J*=4.8 Hz)	-	C-11, C-12, C-13, C-14
18	113.7(t)	4.72 (1Ha, s)	H-18b, H-19	C-4, C-5, C-19
		4.90 (1Hb, s)	H-18a, H-19	C-4, C-5, C-19
19	23.9(q)	1.78 (3H, s)	H-18, H-20	C-4,C-5,C-18
20	16.8(q)	0.94 (3H, s)	H-18b, H-19	C-1,C-2,C-4,C-5, C-9,C-10

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

The ^{1}H-NMR spectrum (Fig. 33, Table 11) of compound **3** showed a methyl group attaching to quaternary carbon (0.94 ppm) and an olefinic methyl group (1.78 ppm) and five olefinic protons (4.72, 4.90, 5.07, 5.15 and 5.94 ppm)

The ^{13}C-NMR spectrum (Fig. 34, Table 11) and HSQC spectrum of compound **3** showed 20 lines. The spectrum illustrated the signal of a carbonyl carbon of carboxylic group at 180.1 ppm and the olefinic carbon signals at 113.7, 118.3, 135.5 and 147.4 ppm. The signals at 39.0 ppm indicated the signal of quaternary carbon. Moreover, the signals indicated the four methine carbons at 36.6, 40.2, 50.8 and 53.0 ppm, six methylene carbons at 23.0, 27.5, 28.0, 29.3, 31.1 and 32.1 ppm and a methyl group at 16.8 ppm.

In comparison of ^{1}H-NMR spectrum of compound **3** with that of compound **2**, the signals of olefinic proton at 4.63 and 4.71 ppm of compound **2** disappeared in ^{1}H-NMR spectrum of compound **3**. The signal of methylene proton at 2.65 ppm was increased in the spectrum of compound **3**. Moreover, the signals of methylene proton and methine proton at 2.24, 2.24 and 2.87 ppm shifted to 1.50, 1.68 and 17.1 ppm, respectively.

In comparison of ^{13}C-NMR spectrum of compound **3** with that of compound **2** (Table 12), the signals of olefinic carbon at 106.8 and 151.9 ppm of compound **2** disappeared in ^{13}C-NMR spectrum of compound **3** and the signals of methylene carbon at 53.9 ppm and quaternary carbon at 61.5 ppm were observed.

From the spectroscopic data, it can be concluded that compound **3** was 3,4-*seco*-13,17-epoxycleistantha-4(18),15-dien-3-oic acid. The structure of compound **3** was shown in Figure 13. The NOESY correlation spectrum were summarized in Figure 14.

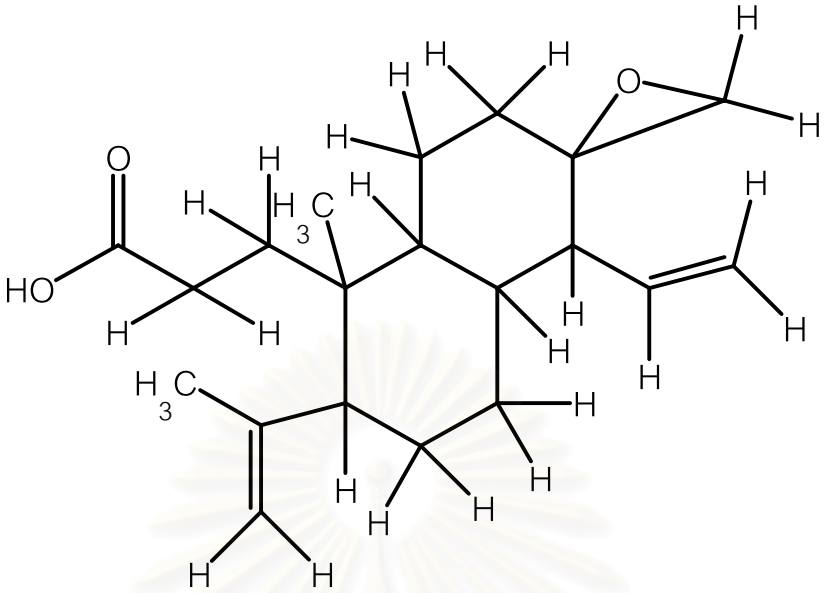


Figure 13. The structure of compound **3**

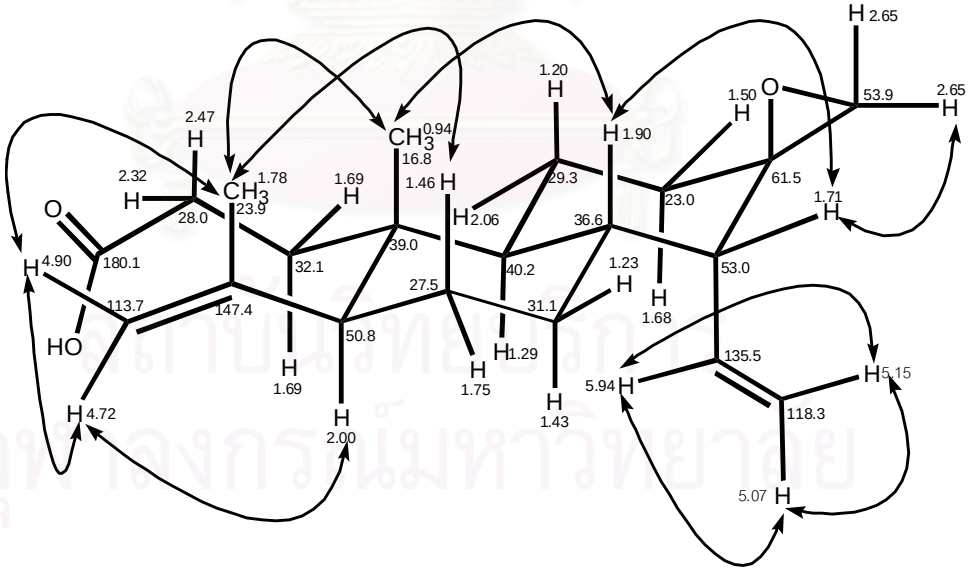
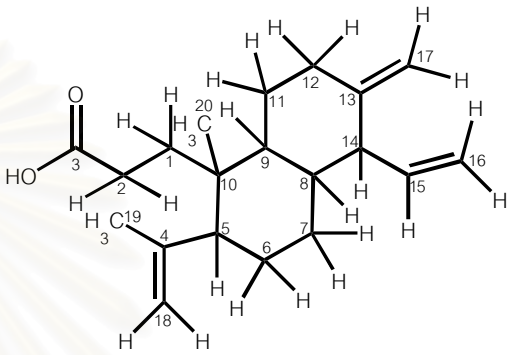


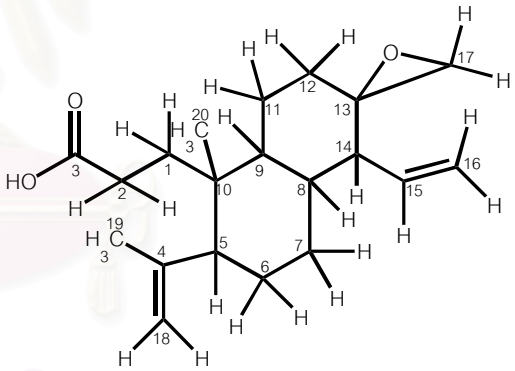
Figure 14. The NOESY correlations of compound **3**

Table 12. ^{13}C-NMR chemical shift of compound **2** was compared to compound **3**

Position	Chemical shift of ^{13}C-NMR	
	Compound **2**	Compound **3**
1	32.1	32.1
2	28.1	28.0
3	181.0	180.1
4	148.4	147.4
5	50.7	50.7
6	27.6	27.5
7	31.5	31.1
8	40.4	36.6
9	41.0	40.2
10	39.0	39.0
11	27.4	29.3
12	31.3	23.0
13	151.9	61.5
14	54.7	53.0
15	137.3	135.5
16	116.2	118.3
17	106.8	53.9
18	113.8	113.7
19	23.9	23.9
20	16.8	16.8



Compound 2



Compound 3

4.2.2 Structure elucidation of compound 4

The IR spectrum of compound **4** (Fig. 80) was summarized in Table 13.

Table 13. The IR absorption band assignment of compound **4**

Wave number (cm^{-1})	Intensity	Tentative assignment
3000	Broad	O-H stretching vibration of carboxylic acid
2926, 2865	Strong	C-H stretching vibration of CH_3-, -CH_2-
1699	Strong	C=O stretching vibration of carbonyl group
1632	Medium	C=C stretching vibration of olefin
1283	Medium	C-O stretching vibration

Compound **4** showed a molecular ion with *m/z* 318 ($C_{20}H_{30}O_3$) which indicated DBE of 6. The information from 2D-NMR techniques, COSY correlations (Fig. 85, Table 14), HMQC correlations (Fig. 84, Table 14), NOESY correlations (Fig. 86) and HMBC correlations (Fig. 87, Table 14) were used to assist the structure assignment of compound **4**.

Table 14. The HMQC, HMBC and COSY spectral data of compound **4**.

Position	δ_C[a]	δ_H	COSY	HMBC
1	32.1(t)	1.70 (2H, m)	H-2	C-2, C-3, C-5,C-9,C-20
2	28.0(t)	2.26 (1Ha, m)	H-2b, H-1	C-1, C-3
		2.49 (1Hb, m)	H-2a, H-1	C-1, C-3
3	178.7(s)	-	-	-
4	146.9(s)	-	-	-
5	50.6(d)	2.19 (1H, dd, *J*=12.4,2.4 Hz)	H-6	C-4, C-6, C-10, C-18, C-19, C-20
6	25.6(t)	1.53 (1Ha, m)	H-5, H-7	C-5
		2.06 (1Hb, m)	H-5, H-7	C-5, C-7
7	34.7(t)	1.31 (1Ha, m)	H-6	C-4, C-5
		1.93 (1Hb, m)	H-6	C-6
8	61.4(s)	-	-	-
9	43.3(d)	1.60 (1H, m)	H-11	C-8, C-10, C-20
10	40.3(s)	-	-	-
11	16.4(s)	1.26 (2H, m)	H-12	C-8, C-9, C-13, C-17
12	34.9(t)	1.42 (2H, m)	H-11	C-9, C-13, C-14, C-17
13	35.6(s)	-	-	-
14	63.7(d)	2.59 (1H, s)		C-8, C-13, C-15
15	146.8(d)	5.91 (1H, dd, *J*=17.6, 10.4 Hz)	H-16	C-13, C-14, C-17
16	112.0(t)	5.05 (1Ha, d, *J*=2.8 Hz)	H-15	C-13, C-15
		5.08 (1Hb, d, *J*=2.4 Hz)	H-15	C-13, C-15
17	22.0(q)	1.14 (3H, s)		C-13, C-14, C-15
18	114.0(t)	4.78 (1Ha, s)	H-18b, H-19	C-5, C-19
		4.94 (1Hb, s)	H-18a, H-19	C-5, C-19
19	23.6(q)	1.82 (3H, s)	H-18	C-4,C-5,C-18
20	18.3(q)	1.02 (3H, s)		C-1,C-2, C-5, C-9, C-10

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

The ^{1}H-NMR spectrum (Fig. 43, Table 14) of compound **4** showed two methyl group attaching to quaternary carbon (1.02 and 1.14 ppm) and an olefinic methyl group (1.82 ppm) and five olefinic protons (4.78, 4.94, 5.05, 5.08 and 5.91 ppm)

The ^{13}C-NMR spectrum (Fig. 44, Table 14) and HSQC spectrum of compound **4** showed 20 lines. The spectrum illustrated the signal of a carbonyl carbon of carboxylic group at 178.7 ppm and the olefinic carbon signals at 112.0, 114.0, 146.8 and 146.9 ppm. The signals at 35.6 and 40.3 ppm indicated the signal of quaternary carbon. Moreover, the signals indicated the three methine carbons at 43.3, 50.6 and 63.7 ppm, six methylene carbons at 16.4, 25.6, 28.0, 32.1, 34.7 and 34.9 ppm and methyl group at 18.3, 22.0 and 23.6 ppm.

In comparison of ^{1}H-NMR spectrum of compound **4** with that of compound **2**, the signals of olefinic proton at 4.63 and 4.71 ppm and methine proton at 1.61 ppm of compound **2** disappeared in ^{1}H-NMR spectrum of compound **4**. The signal of methyl proton at 1.14 ppm was increased in the spectrum of compound **4**. Moreover, the signal of proton at 2.24 and 2.87 ppm shifted to 1.42 and 2.59 ppm, respectively.

In comparison of ^{13}C-NMR spectrum of compound **4** with that of compound **2** (Table 15), the signals of olefinic carbon at 106.8 and 151.9 ppm and methine carbon at 40.4 ppm of compound **2** disappeared in ^{13}C-NMR spectrum of compound **4.** The signals of methyl carbon at 22.0 ppm and quaternary carbon at 61.4 ppm were observed. Moreover, the signal of methine carbon at 54.7 ppm shifted to 63.7 ppm.

From the spectroscopic data, it can be concluded that compound **4** was 3,4-*seco*-8,14-epoxypimara-4(18),15-dien-3-oic acid. The structure of compound **4** was shown in Figure 15. The NOESY correlation spectrum were summarized in Figure 16.

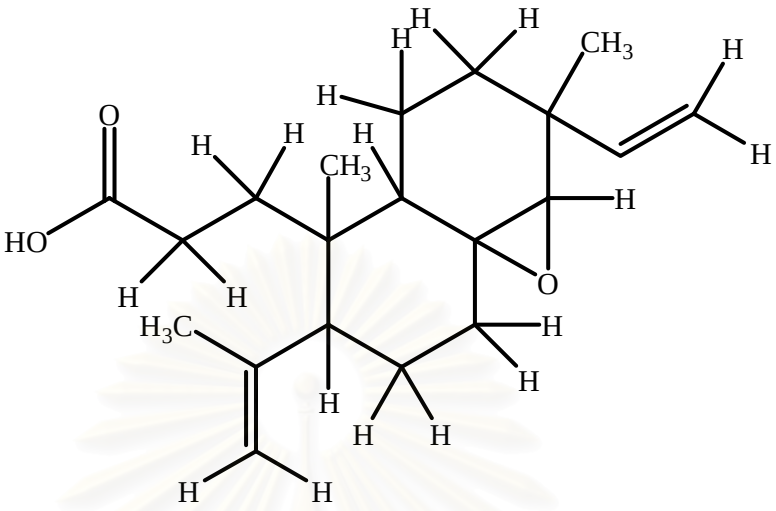


Figure 15. The structure of compound **4**

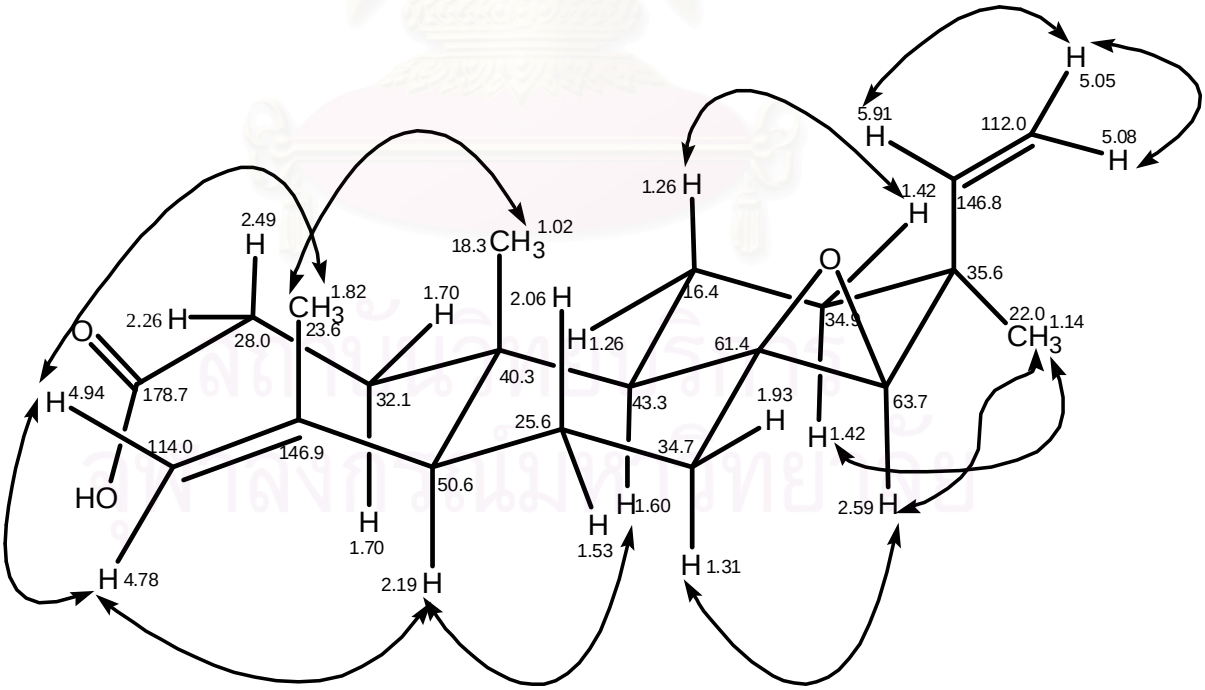
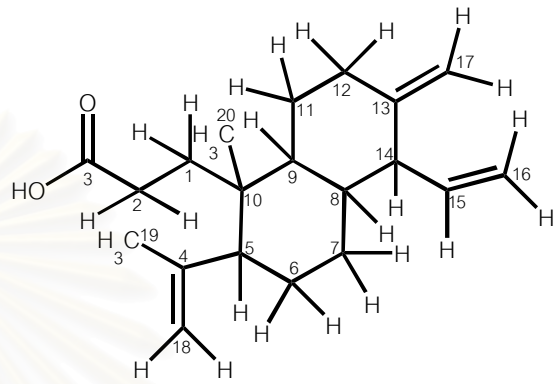


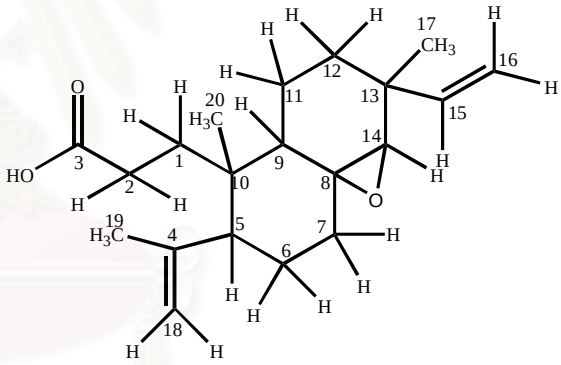
Figure 16. The NOESY correlations of compound **4**

Table 15. ^{13}C-NMR chemical shift of compound **2** was compared to compound **4**

Position	Chemical shift of ^{13}C-NMR	
	Compound **2**	Compound **4**
1	32.1	32.1
2	28.1	28.0
3	181.0	178.7
4	148.4	146.9
5	50.7	50.6
6	27.6	25.6
7	31.5	34.7
8	40.4	61.4
9	41.0	43.3
10	39.0	40.3
11	27.4	16.4
12	31.3	34.9
13	151.9	35.6
14	54.7	63.7
15	137.3	146.8
16	116.2	112.0
17	106.8	22.0
18	113.8	114.0
19	23.9	23.6
20	16.8	18.3



Compound 2



Compound 4

4.3 Biological activity

Compound **1**-**4** were tested for their cytotoxicity against human tumor cell lines. Compound **1** showed non-specific strong cytotoxicity against human colon adenocarcinoma (SW 620), lung carcinoma (CHAGO), human gastric carcinoma (KATO-3) and human liver hepatoblastoma (HEP-G2) at 0.5, 5.5, 6.0 and 6.1 μg/ml, respectively. Compound **2** showed weak activity against human colon adenocarcinoma (SW 620) and human gastric carcinoma (KATO-3) at 8.6 and 9.6 μg/ml. Compound **3** and **4** were inactive against all cell lines (> 10 μg/ml). Therefore, these cleistanthane best active when functional group was alcohol better than carboxylic acid and epoxide. Cytotoxicity data of compound **1**-**4** was show in Table 13.

Table 16. Cytotoxicity data of compound **1**-**4**[a]

Compound	Cell lines[b]				
	KATO-3	BT474	HEP-G2	SW 620	CHAGO
1	6.0	>10	6.1	0.5	5.5
2	9.6	>10	10	8.6	>10
3	>10	>10	>10	>10	>10
4	>10	>10	>10	>10	>10

[a]Results are expresses as IC_{50} values (μg/mL), [b]KATO-3 : human gastric carcinoma ATCC No. HTB 103; SW 620 : human colon adenocarcinoma ATTC No. CCL227; BT474 : human breast ductol carcinoma ATCC No. HTB20; HEP-G2 : human liver hepatoblastoma ATCC No. HB 8065; CHAGO : human undifferentiated lung carcinoma.

CHAPTER V

CONCLUSION

In previous studies the chemical constituent of *Croton oblongifolius* Roxb. from various parts of Thailand were investigated and several diterpenoid compounds were reported, for example, cembranes, labdane, halimane and clerodane. From this research, the chemical constituent in the stem bark of *Croton oblongifolius* Roxb. from Amphur Viengsa, Nan province was found to contain two new cleistanthane diterpenoids, 3-hydroxycleistantha-13(17),15-diene (**1**) (200 mg, 0.04%) and 3,4-*seco*-cleistantha-4(18),13(17),15-trien-3-oic acid (**2**) (2 g, 0.4%). Compound **3** and **4** were obtained from epoxidation of compound **2**, which were assigned as 3,4-*seco*-13,17-epoxycleistantha-4(18),15-dien-3-oic acid (**3**) and 3,4-*seco*-8,14-epoxypimara-4(18),15-dien-3-oic acid (**4)**.

Compound **1-4** were tested for their cytotoxicity against human tumor cell lines BT474 (breast), HEP-G2 (hepatoma), SW620 (colon), CHAGO (lung), and KATO-3 (gastric). Compound **1** showed non-specific stronger activity against human colon adenocarcinoma (SW 620), lung carcinoma (CHAGO), human gastric carcinoma (KATO-3) and human liver hepatoblastoma (HEP-G2) at 0.5, 5.5, 6.0 and 6.1 μg/ml, respectively. Compound **2** showed weak activity against human colon adenocarcinoma (SW 620) and human gastric carcinoma (KATO-3) at 8.6 and 9.6 μg/ml. Compound **3** and **4** were inactive against all cell lines (> 10 μg/ml).

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APPENDICES

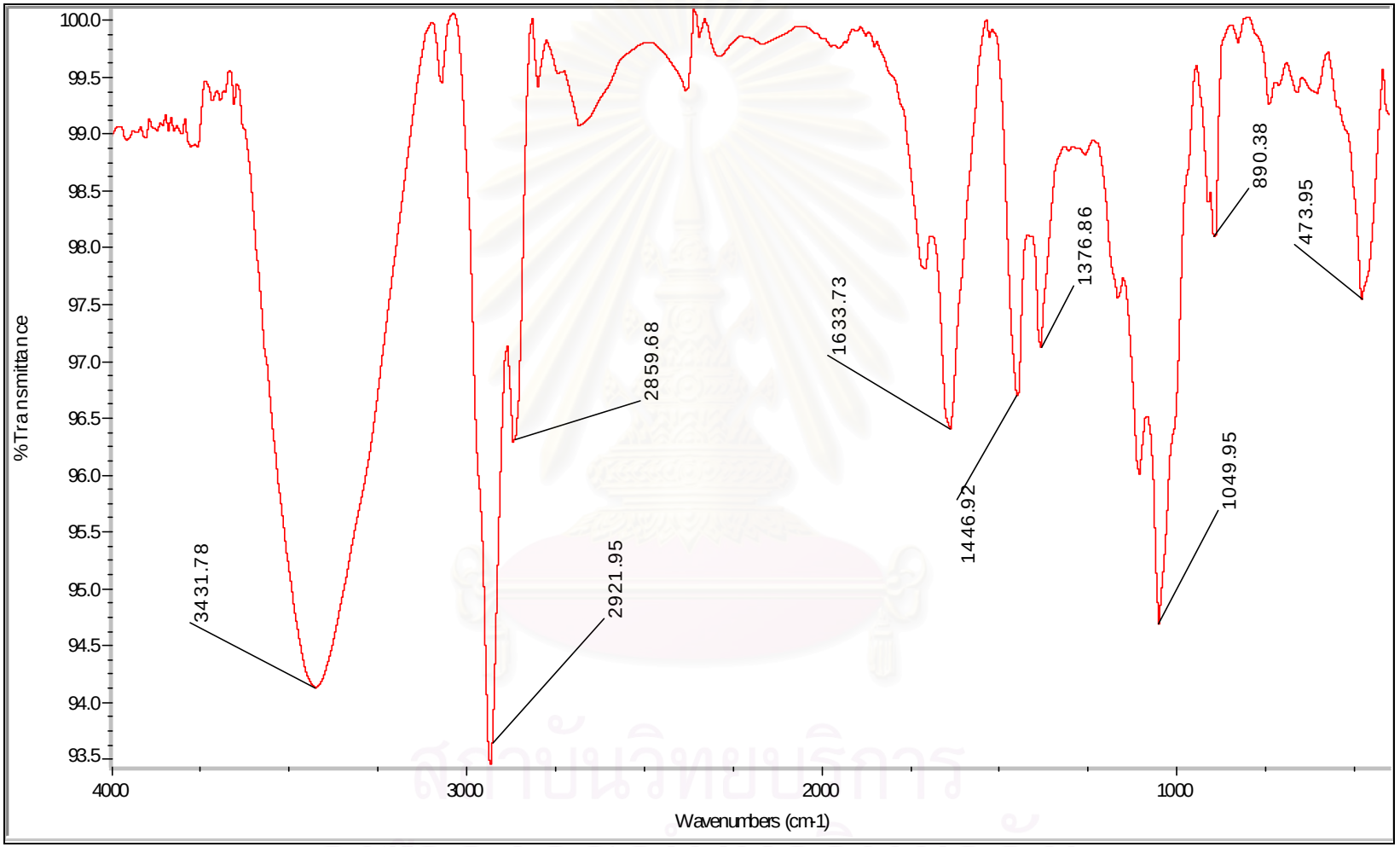

Figure 17. IR spectrum of compound **1**

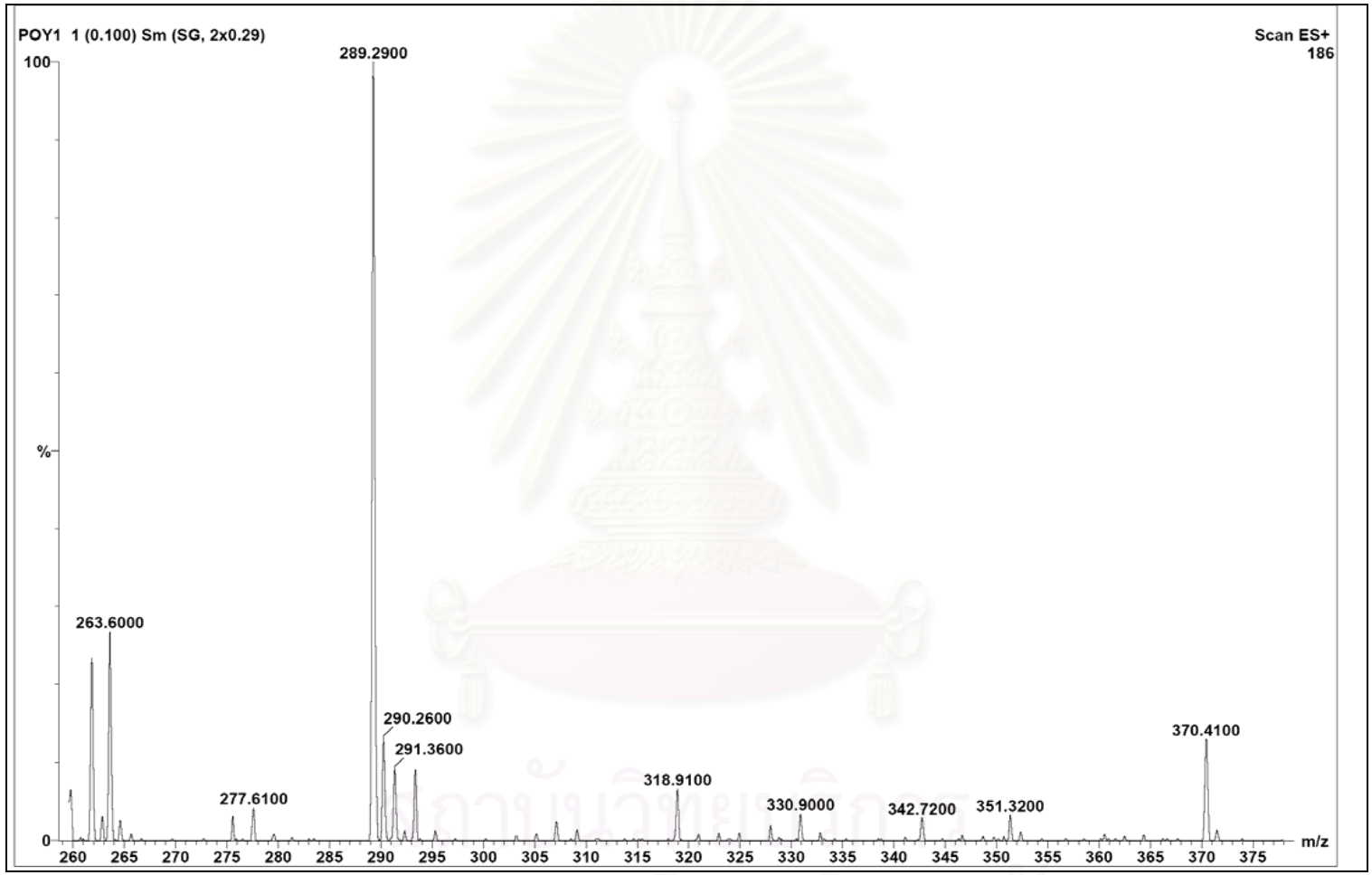

Figure 18. Mass spectrum of compound **1**

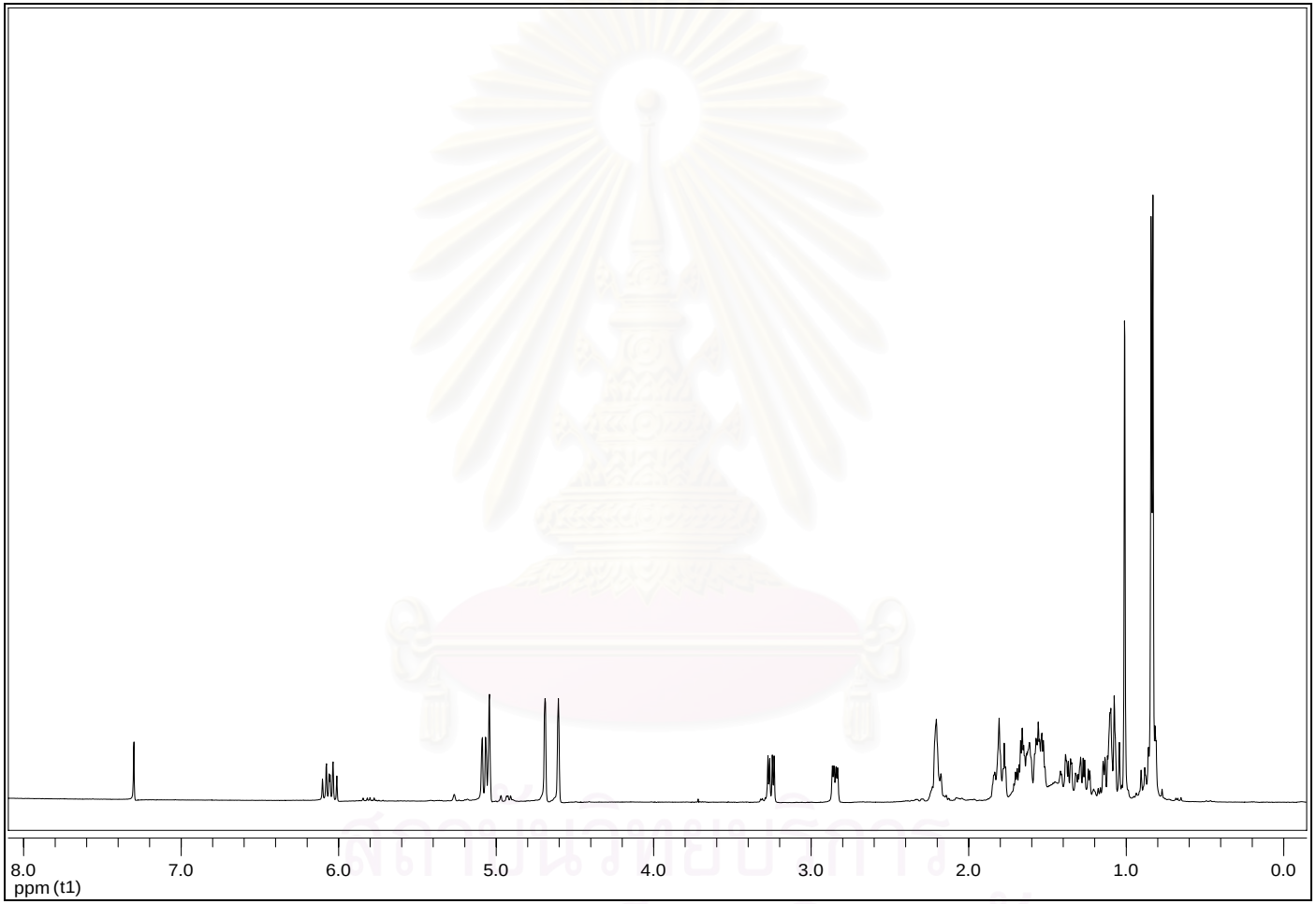

Figure 19. ^{1}H-NMR spectrum of compound **1**

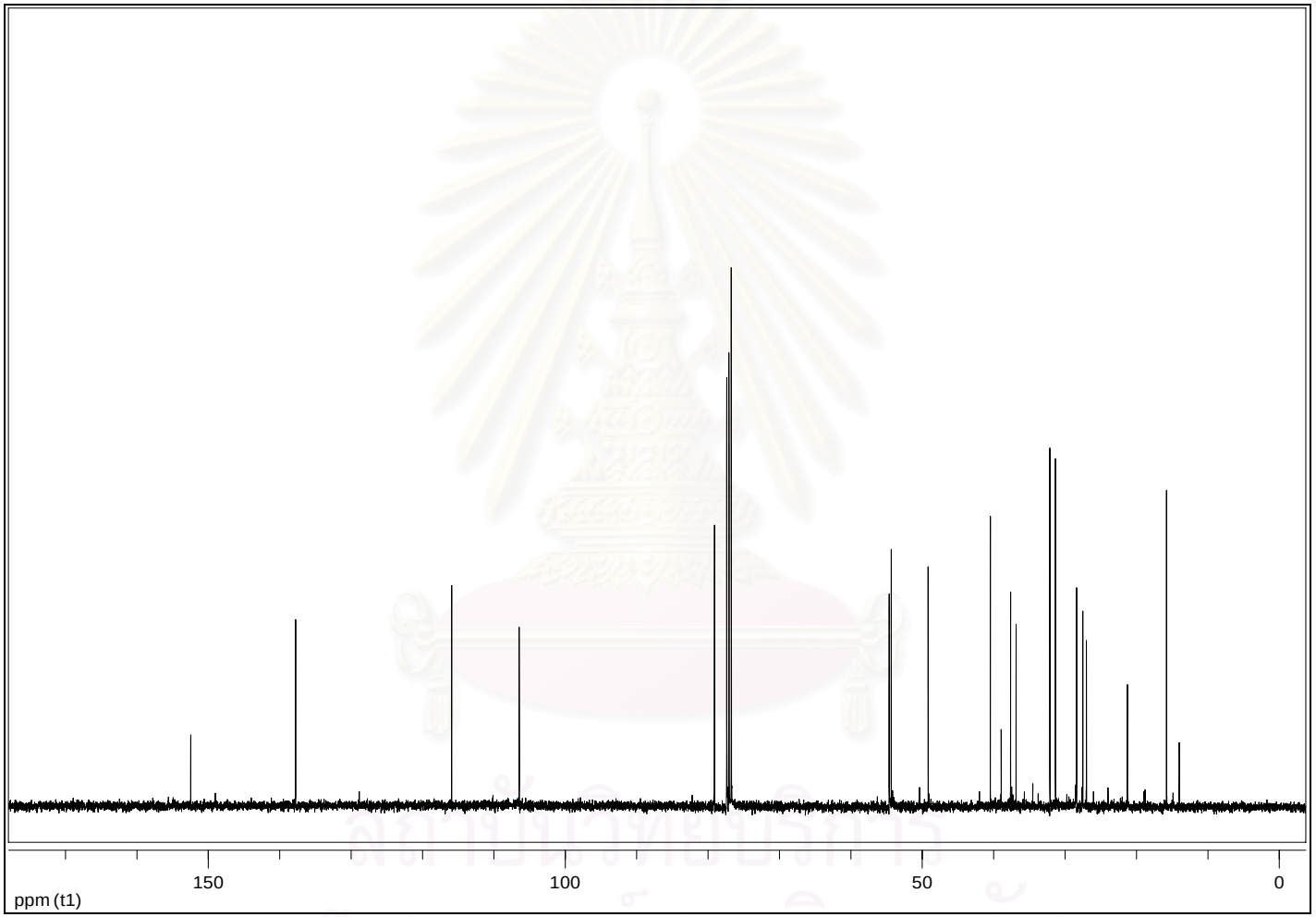

Figure 20. ^{13}C-NMR spectrum of compound **1**

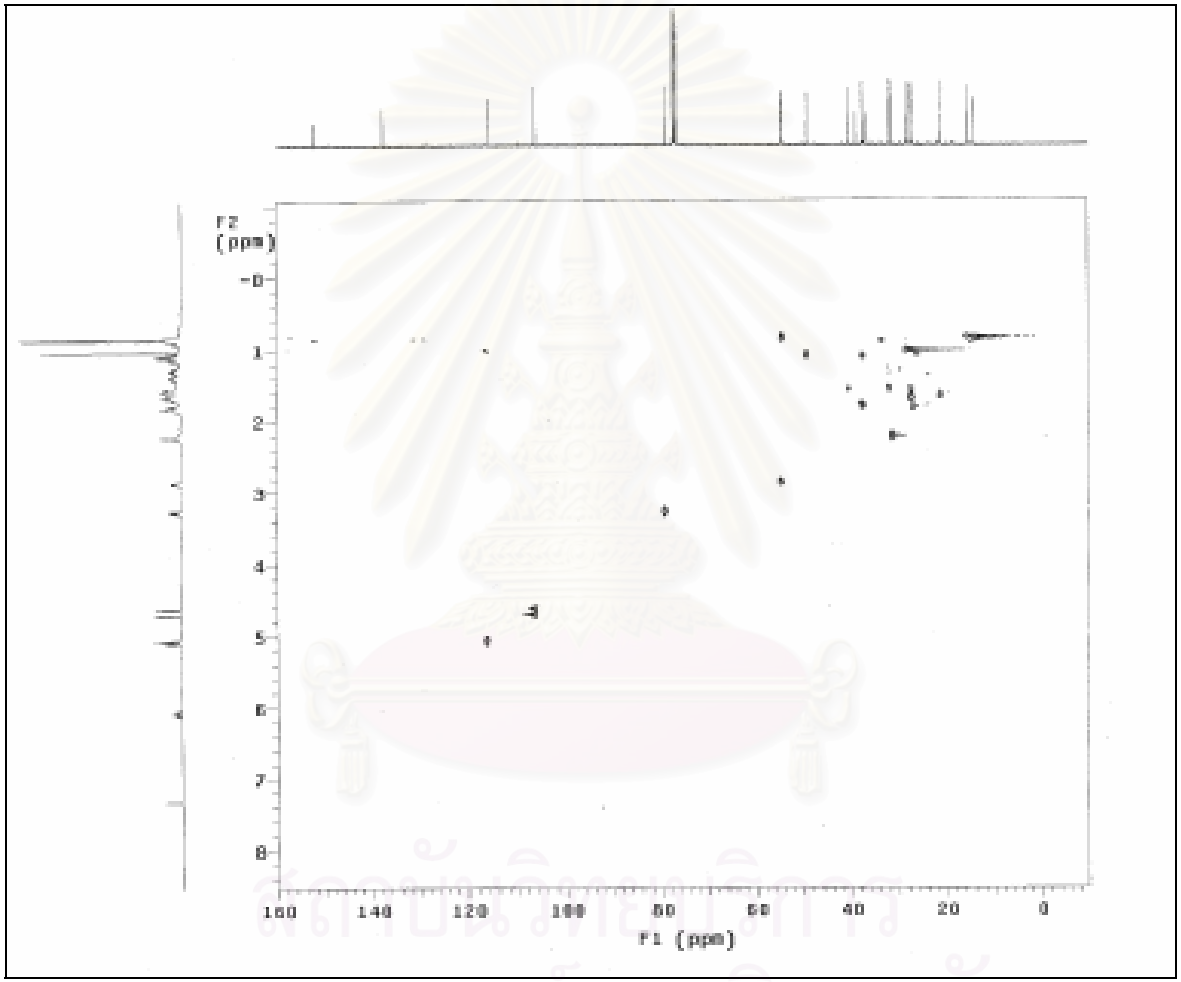

Figure 21. HSQC spectrum of compound **1**

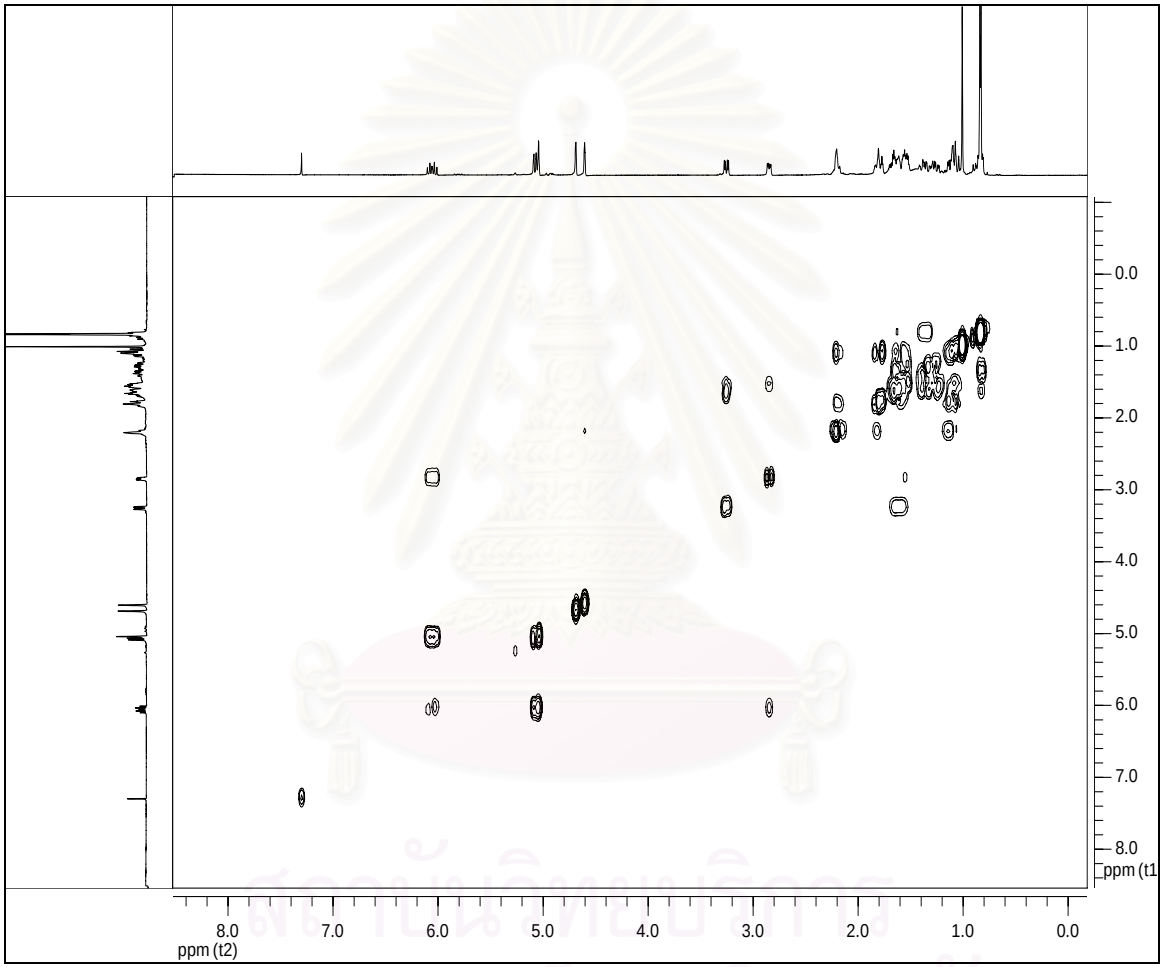

Figure 22. COSY spectrum of compound **1**

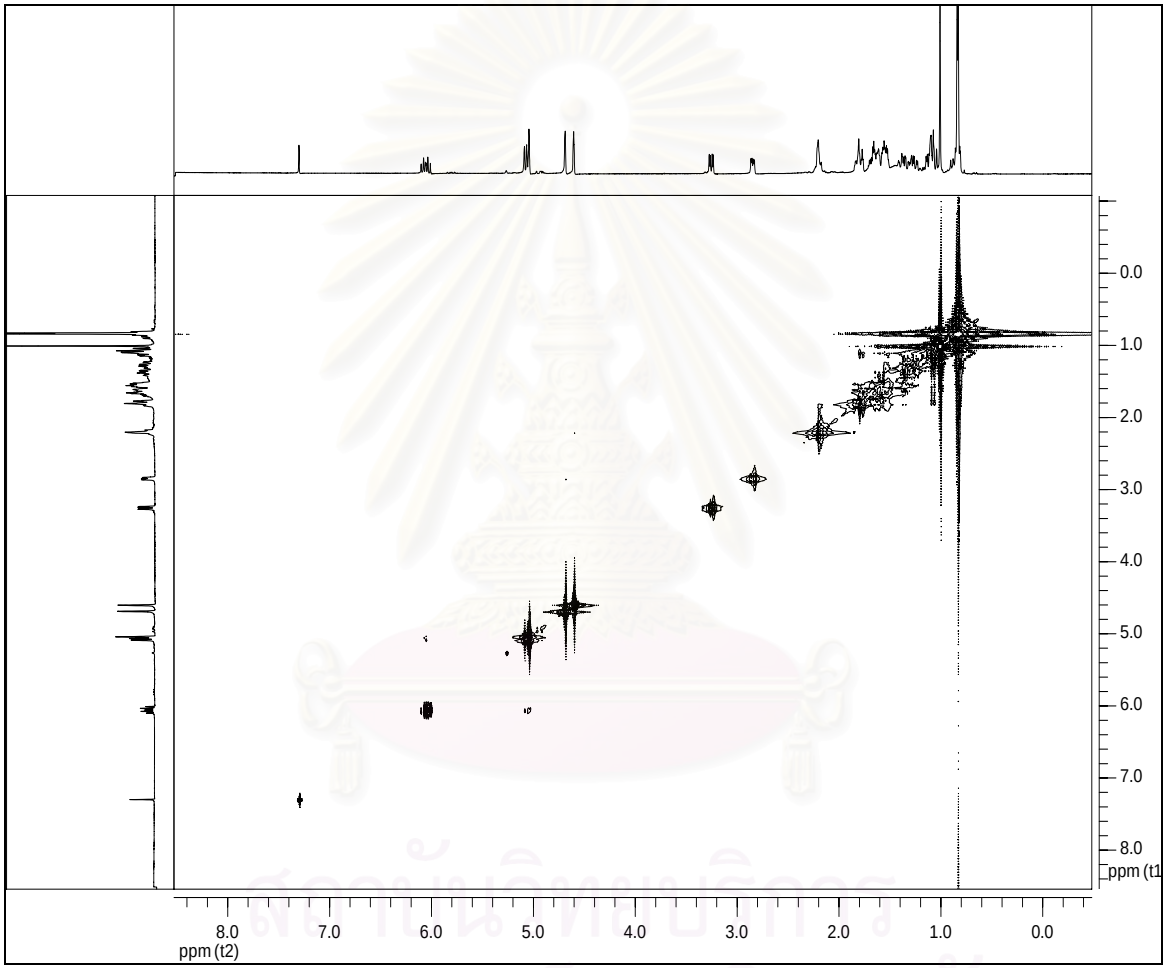

Figure 23. NOESY spectrum of compound **1**

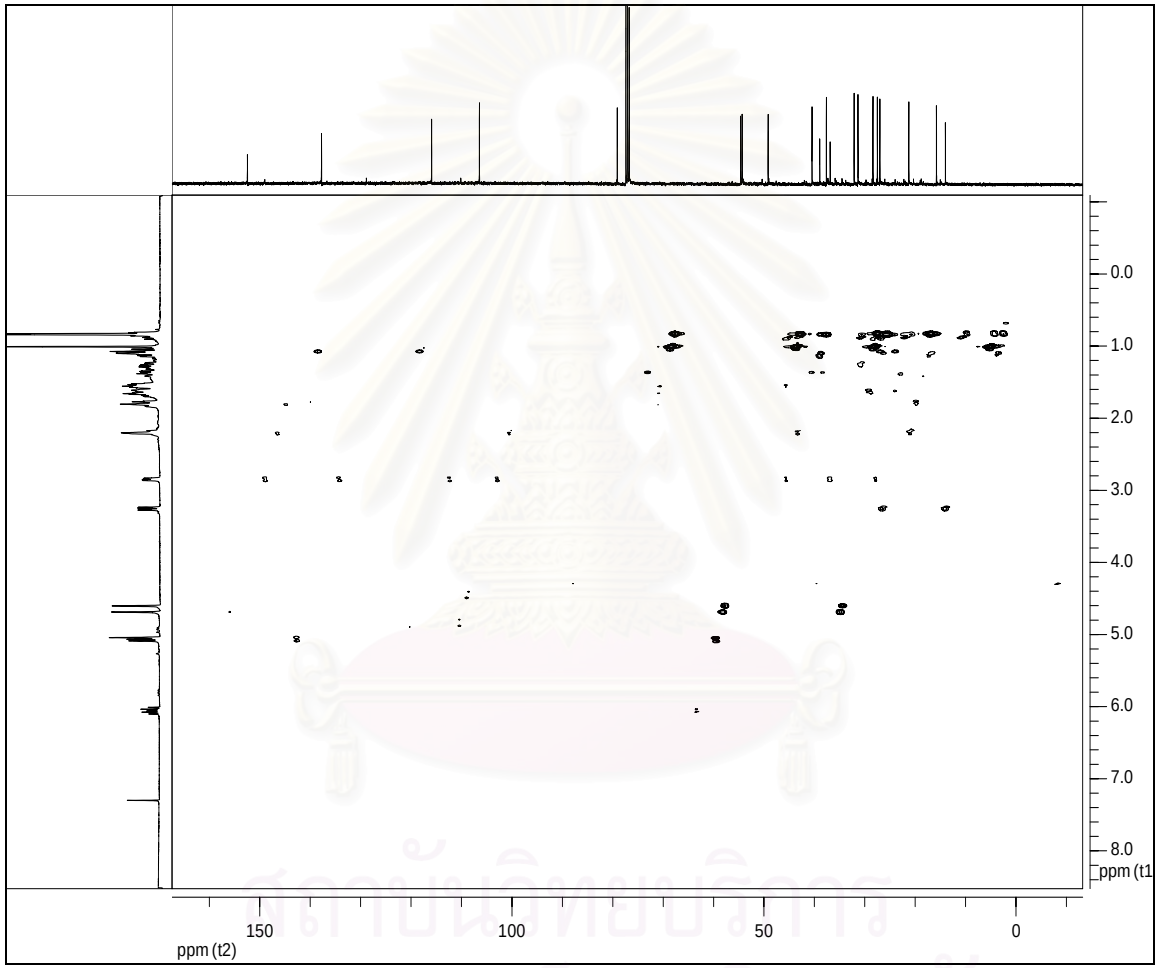

Figure 24. HMBC spectrum of compound **1**

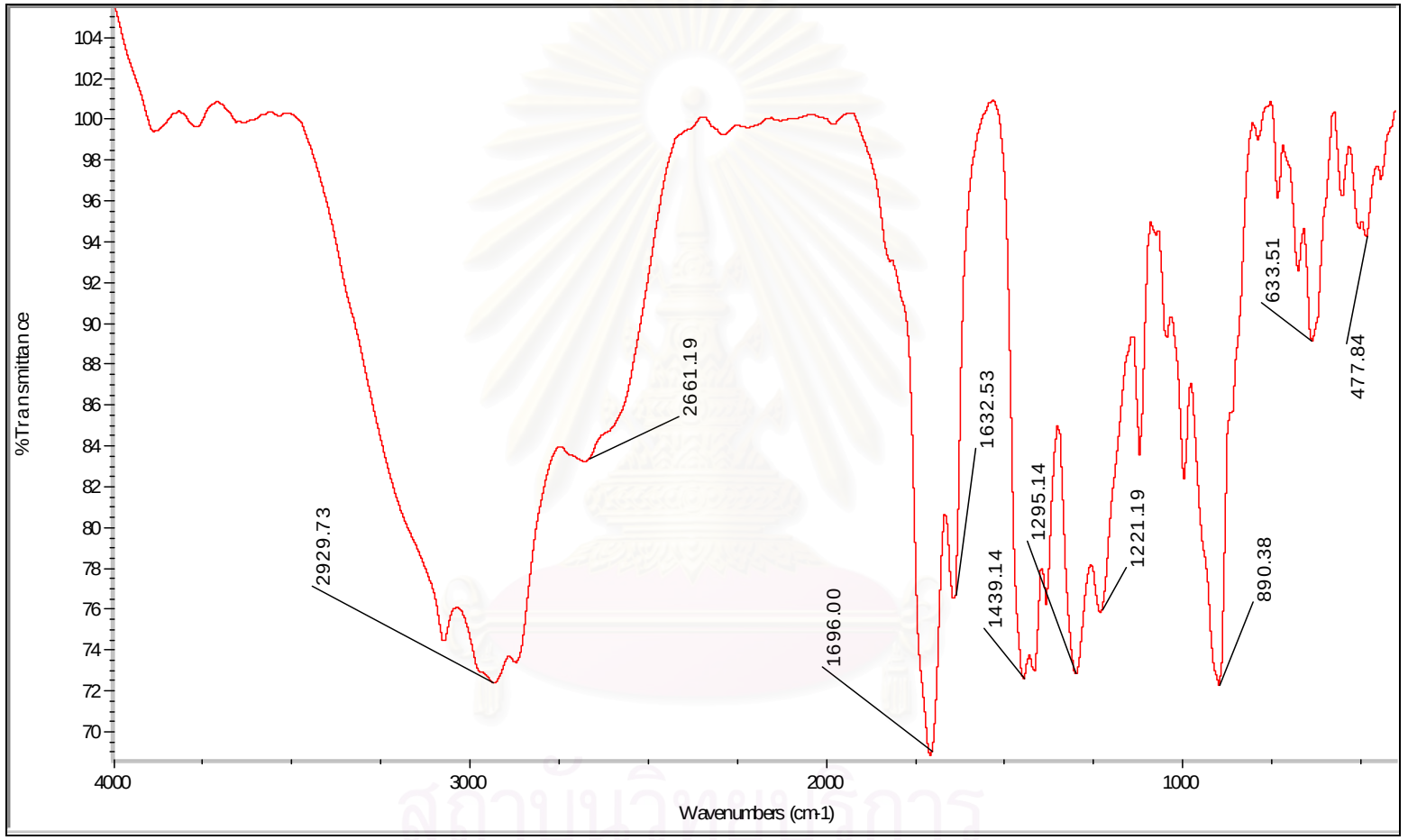

Figure 25. IR spectrum of compound **2**

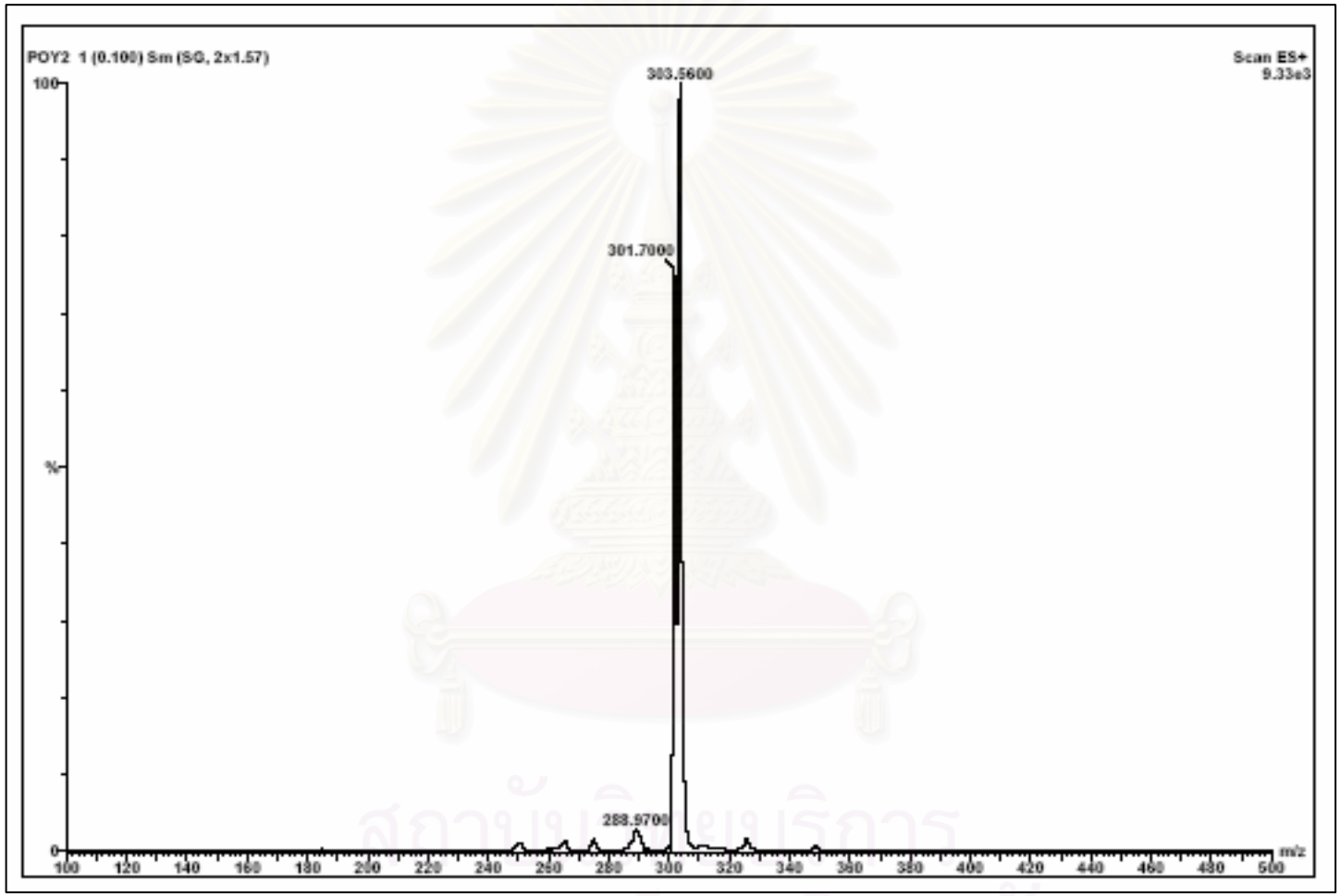

Figure 26. Mass spectrum of compound **2**

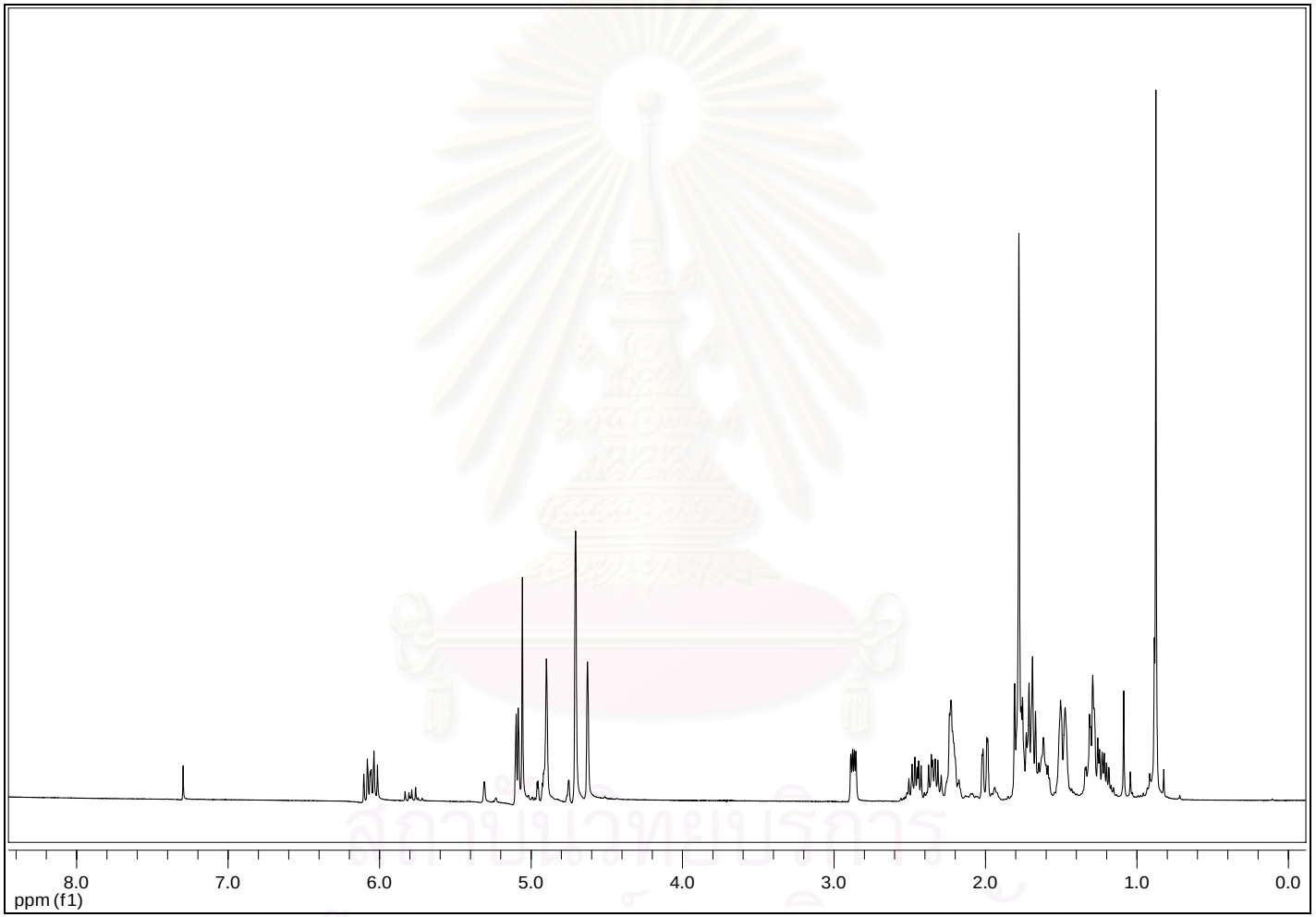

Figure 27. ^{1}H-NMR spectrum of compound **2**

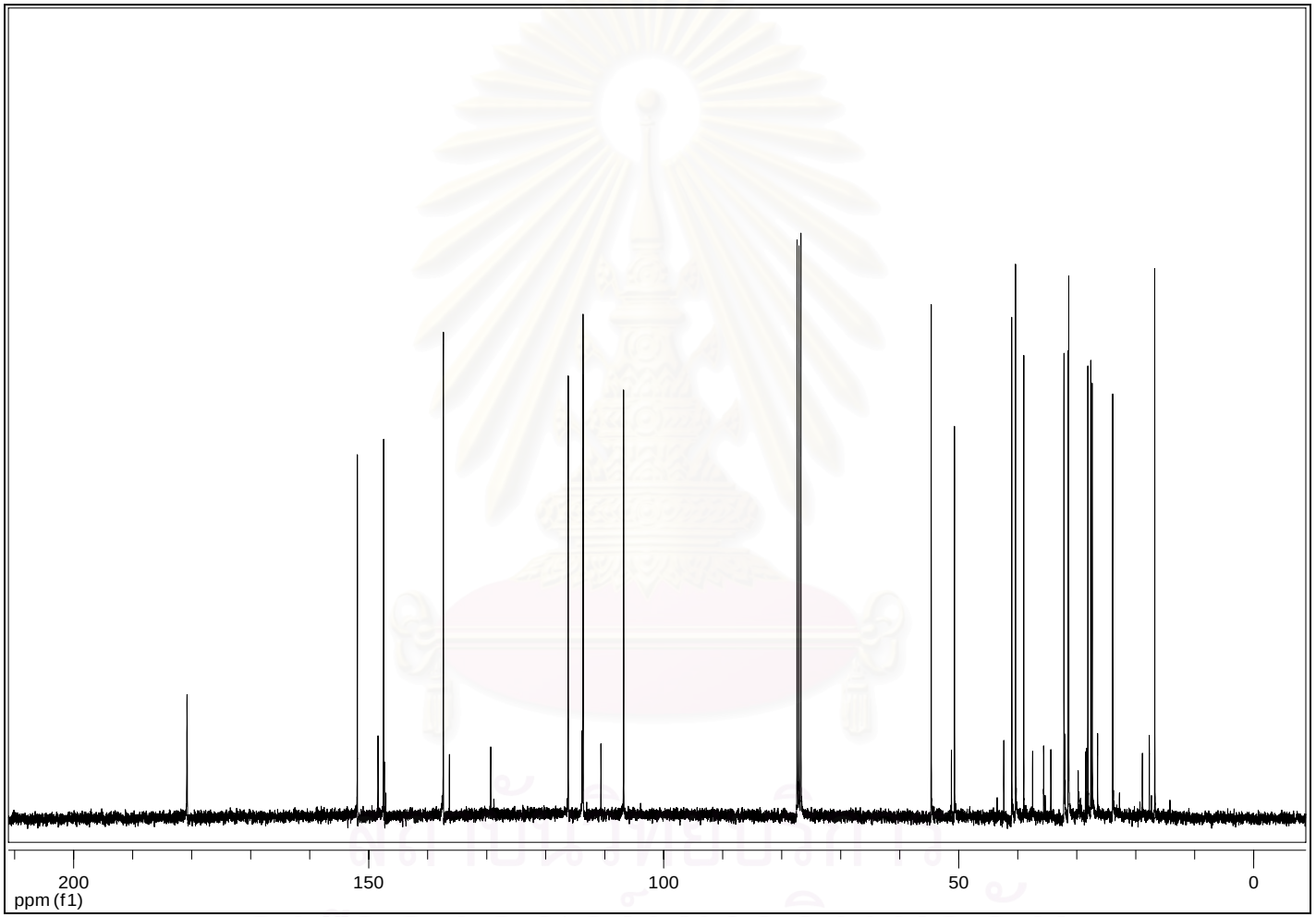

Figure 28. ^{13}C-NMR spectrum of compound **2**

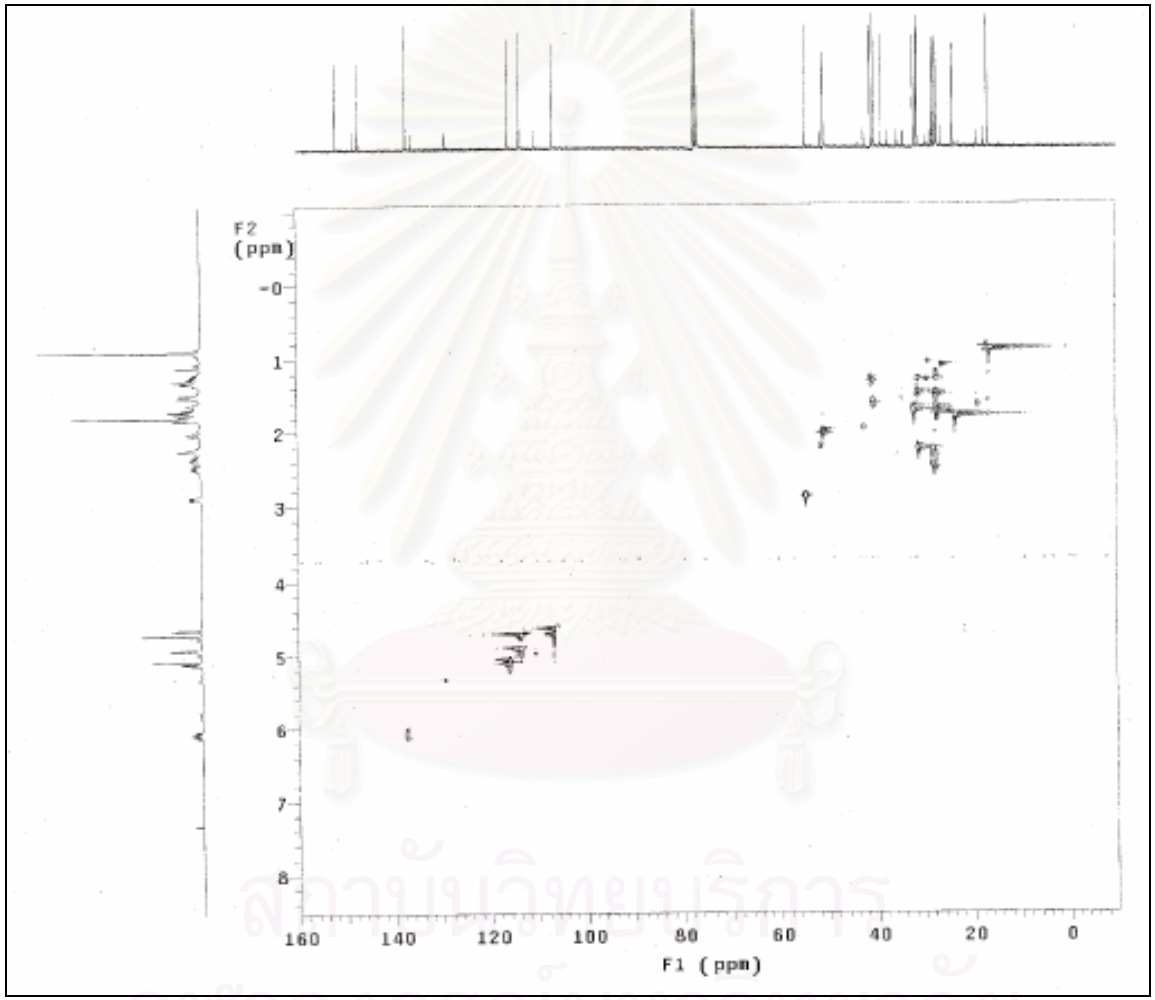

Figure 29. HSQC spectrum of compound **2**

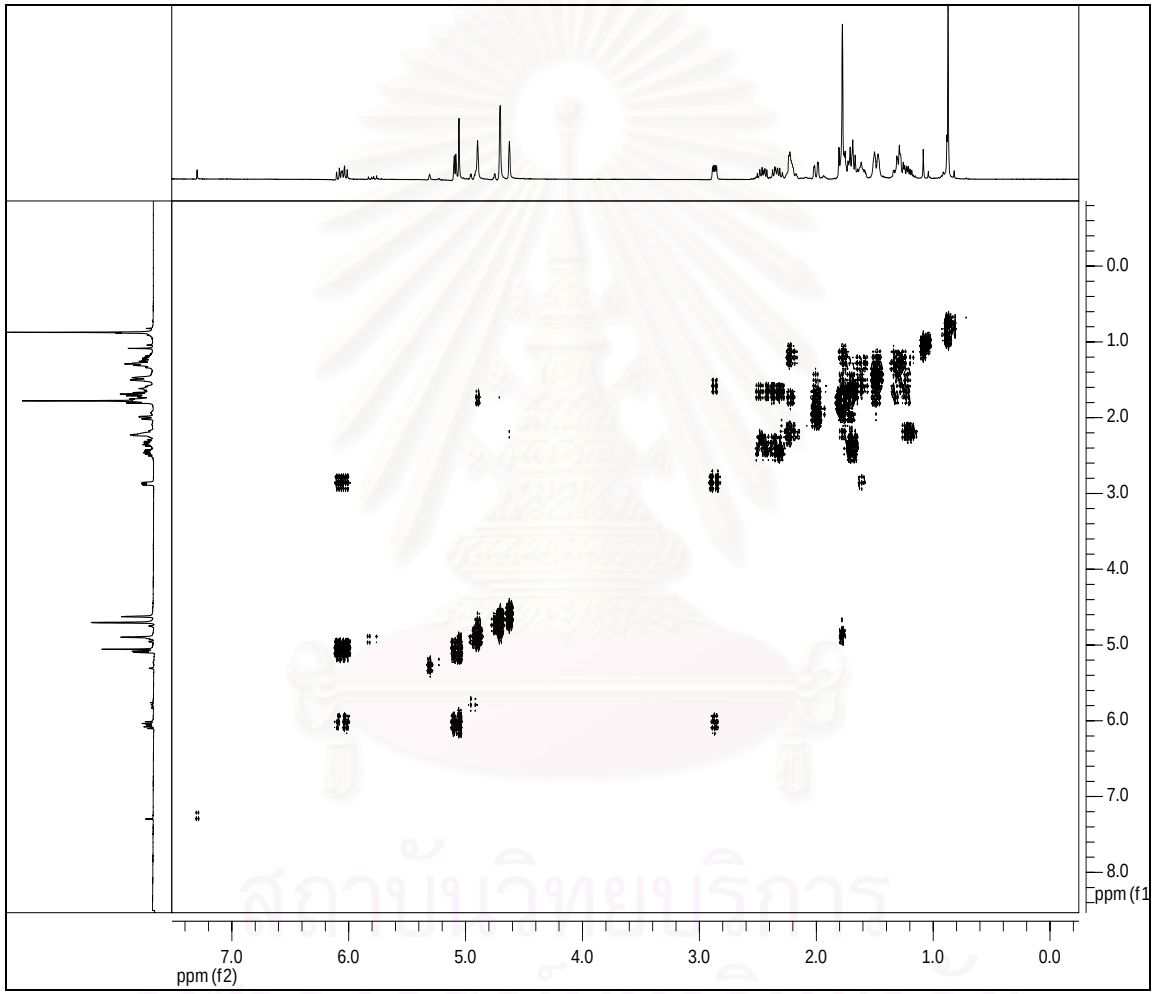

Figure 30. COSY spectrum of compound **2**

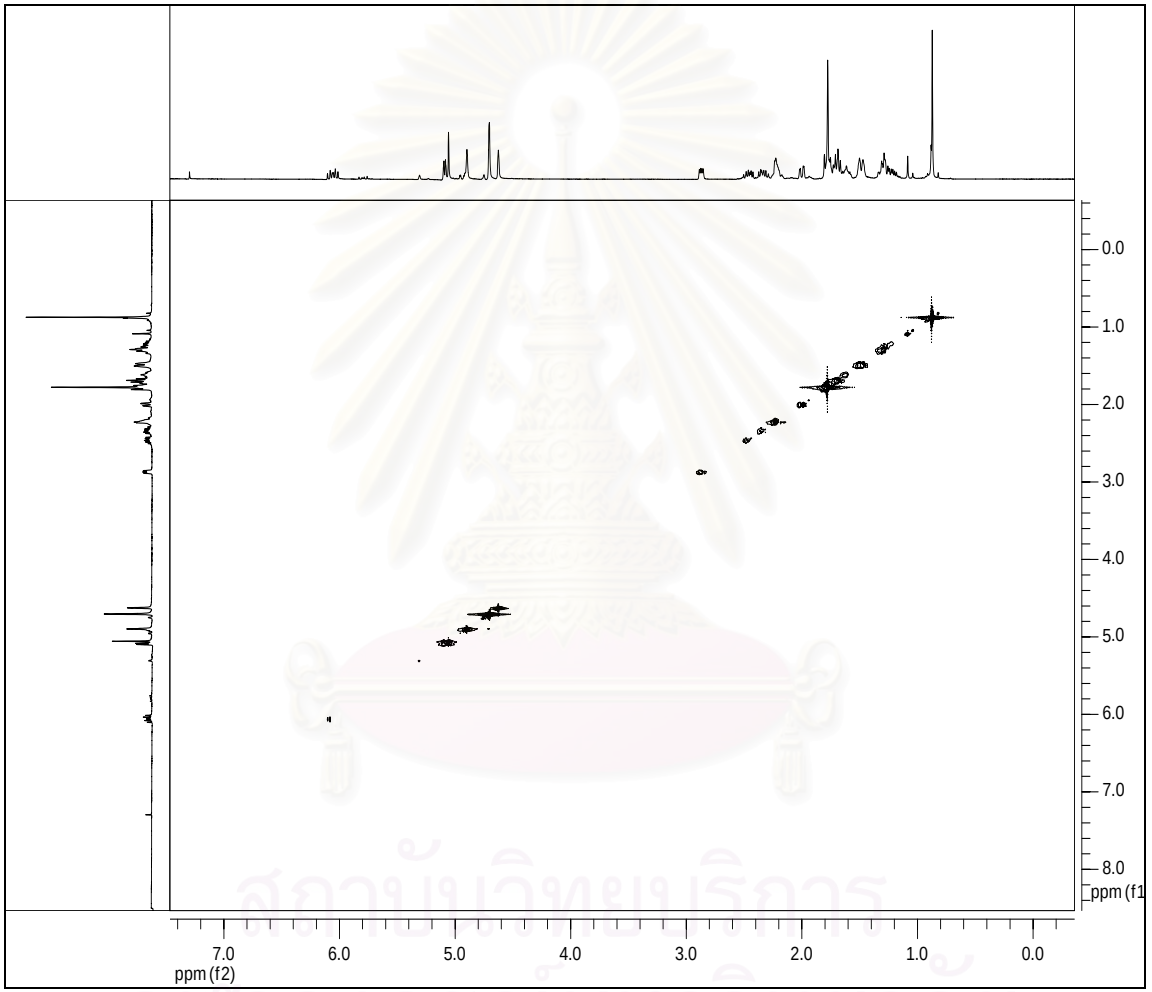

Figure 31. NOESY spectrum of compound **2**

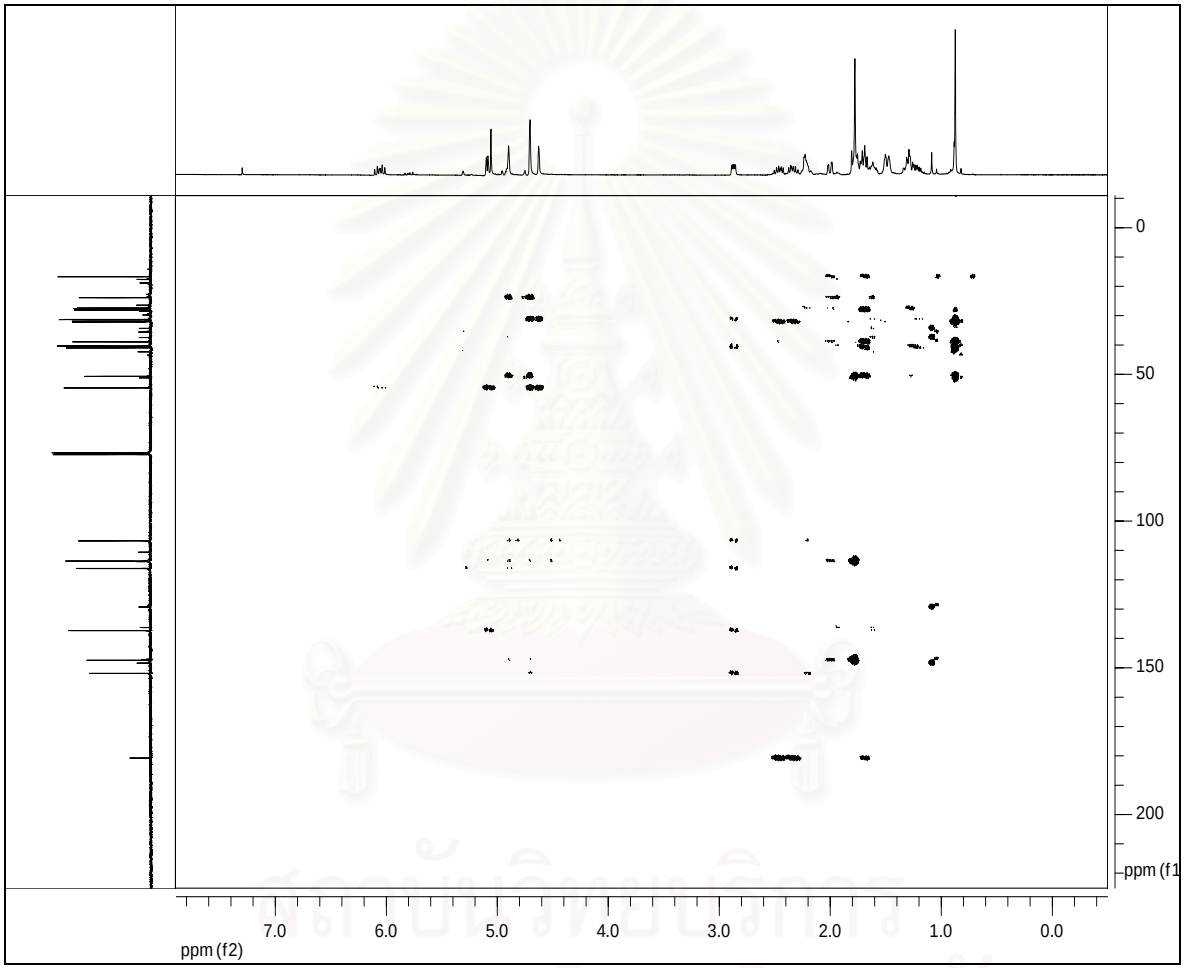

Figure 32. HMBC spectrum of compound **2**

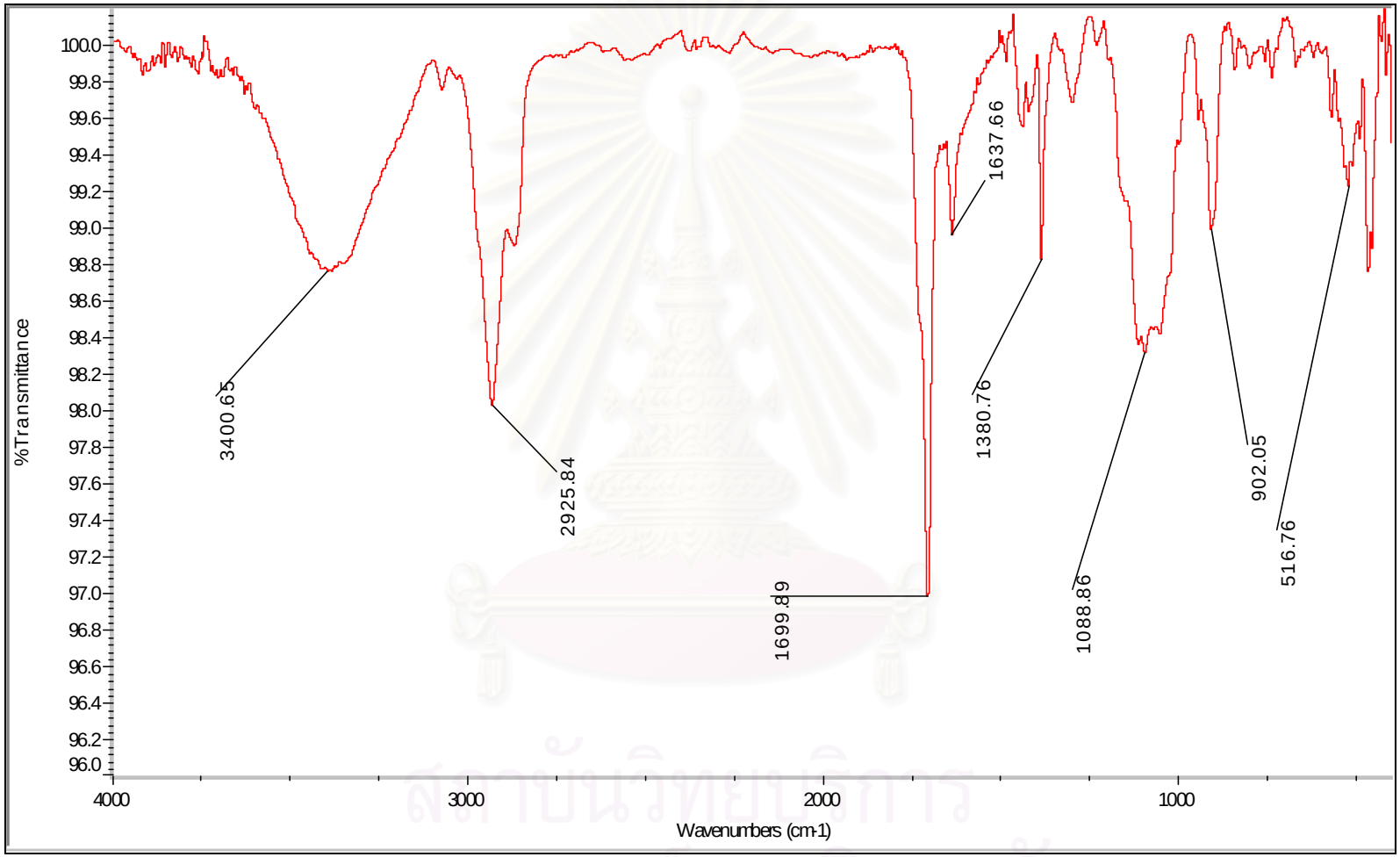

Figure 33. IR spectrum of compound **3**

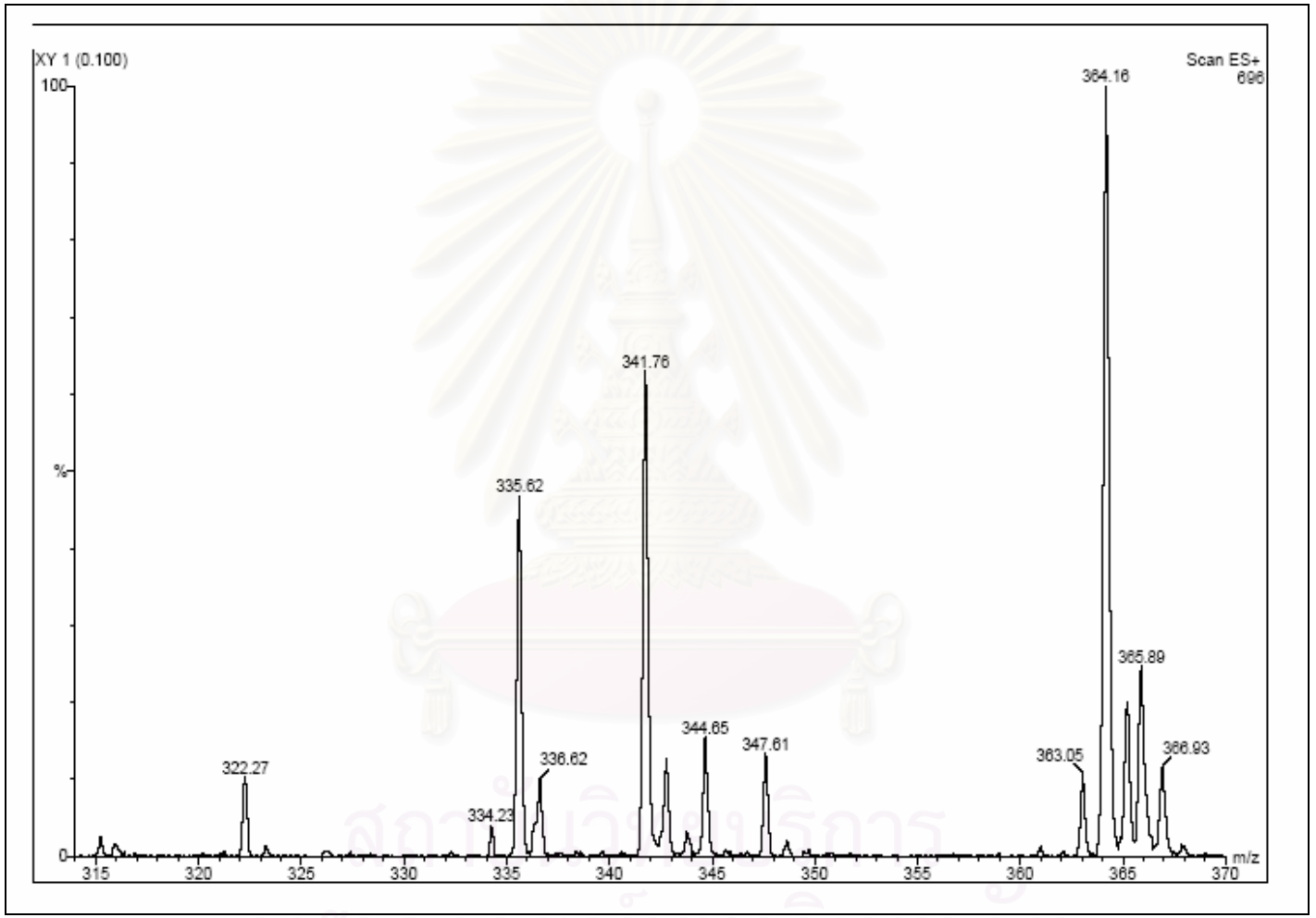

Figure 34. Mass spectrum of compound **3**

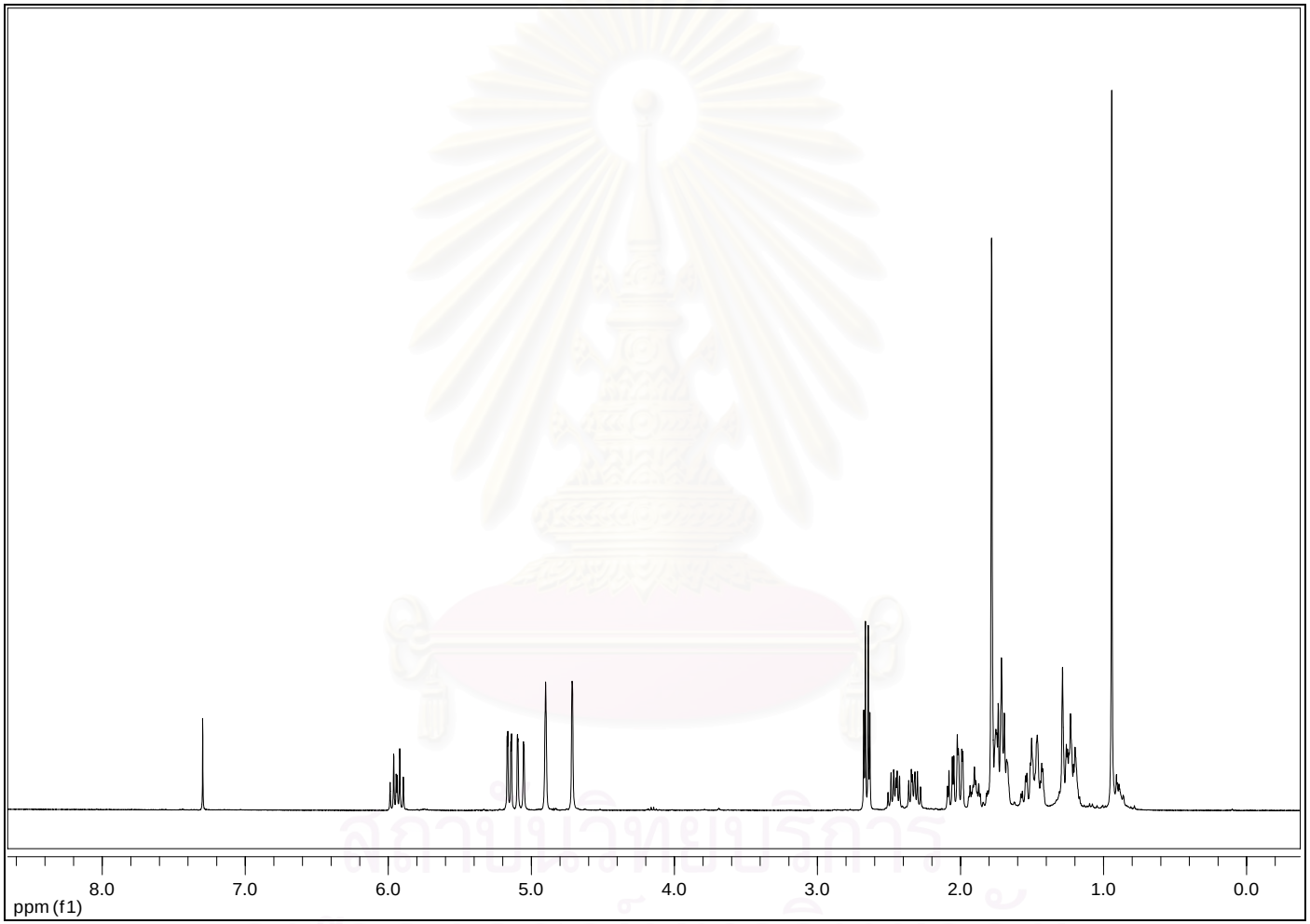

Figure 35. ^{1}H-NMR spectrum of compound **3**

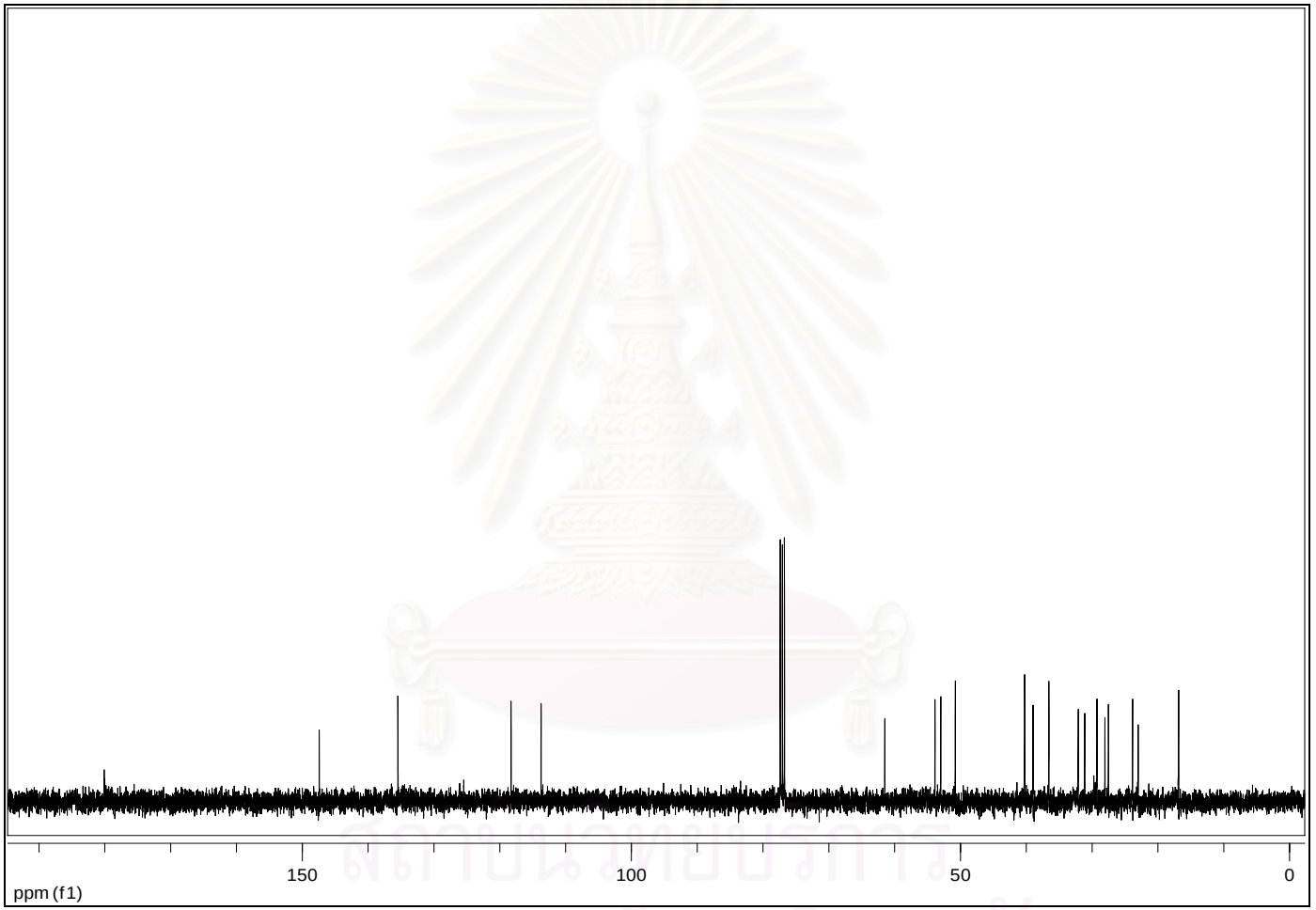

Figure 36. ^{13}C-NMR spectrum of compound **3**

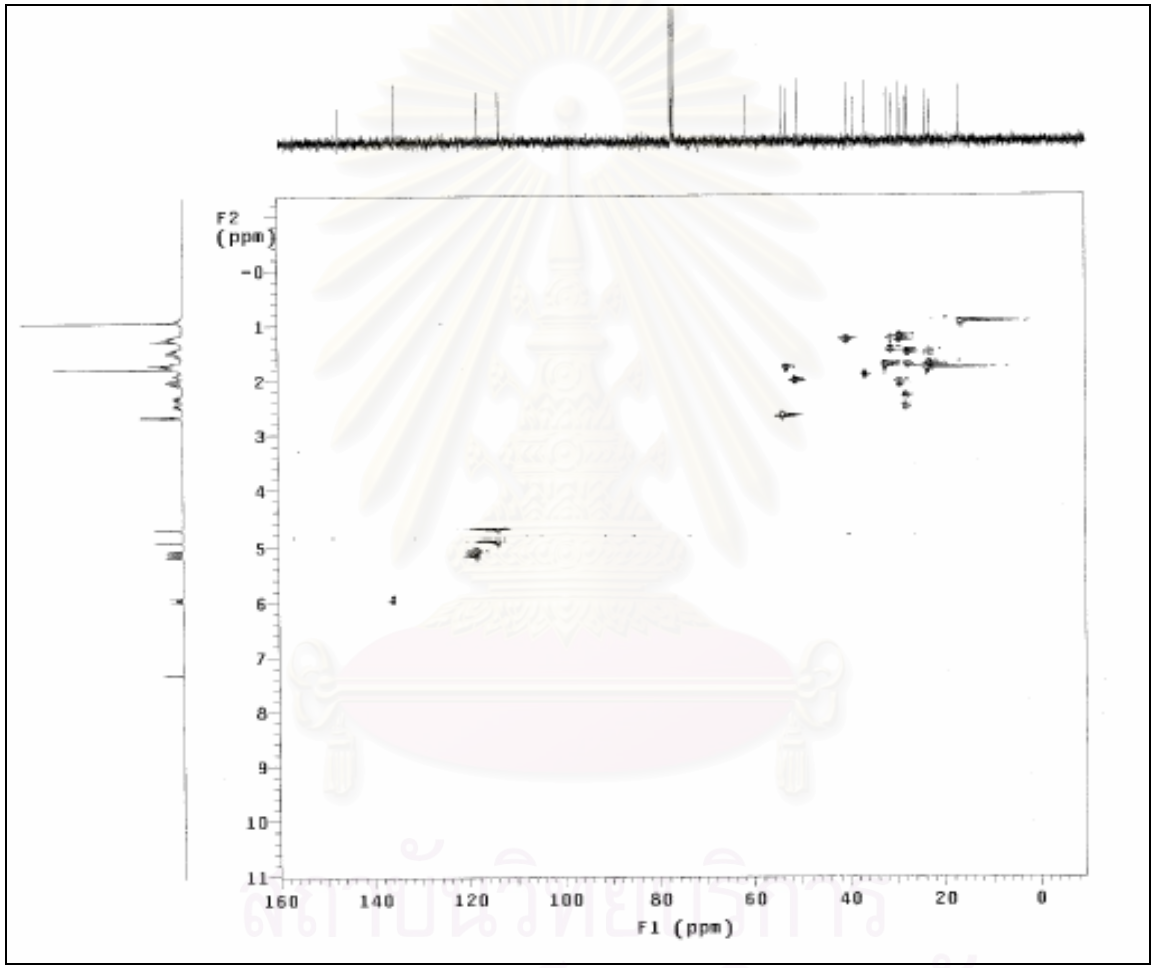

Figure 37. HSQC spectrum of compound **3**

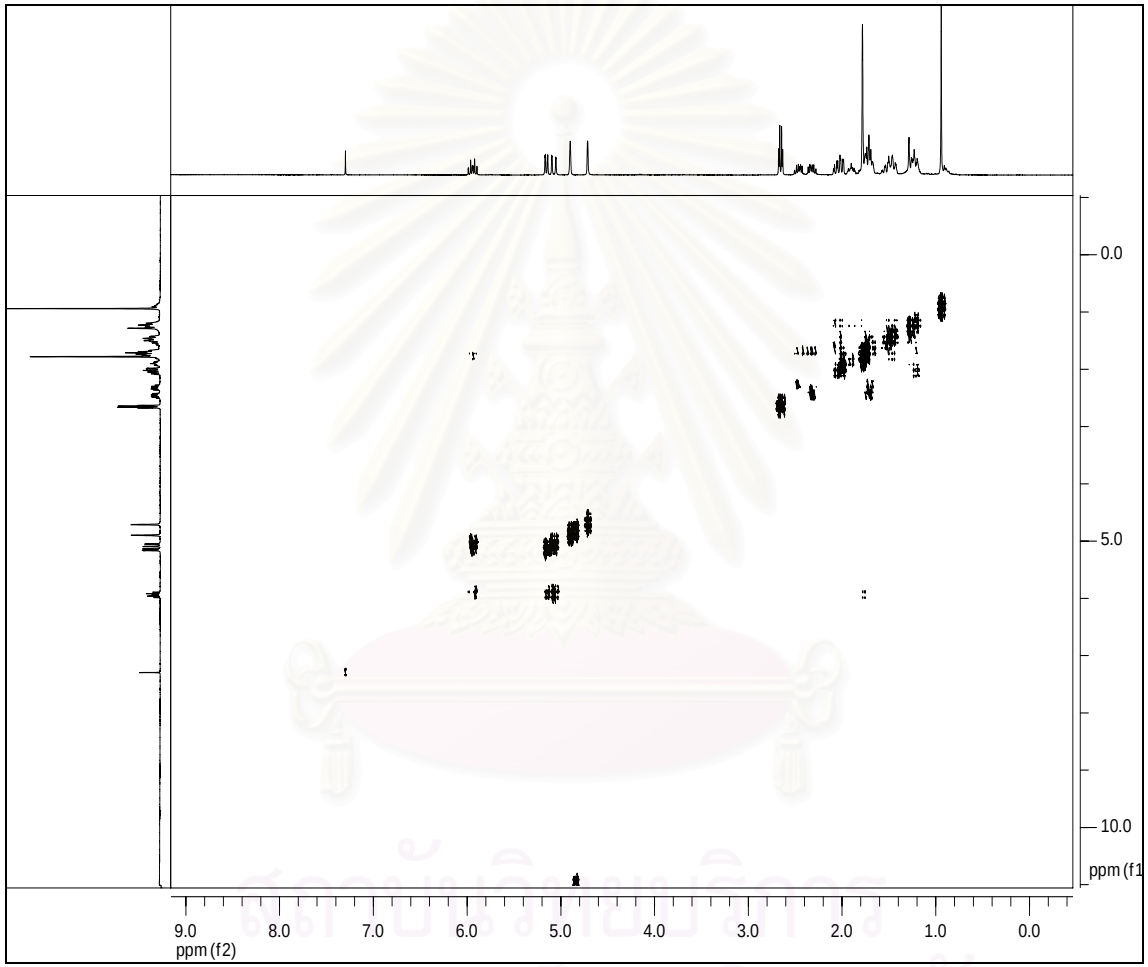

Figure 38. COSY spectrum of compound **3**

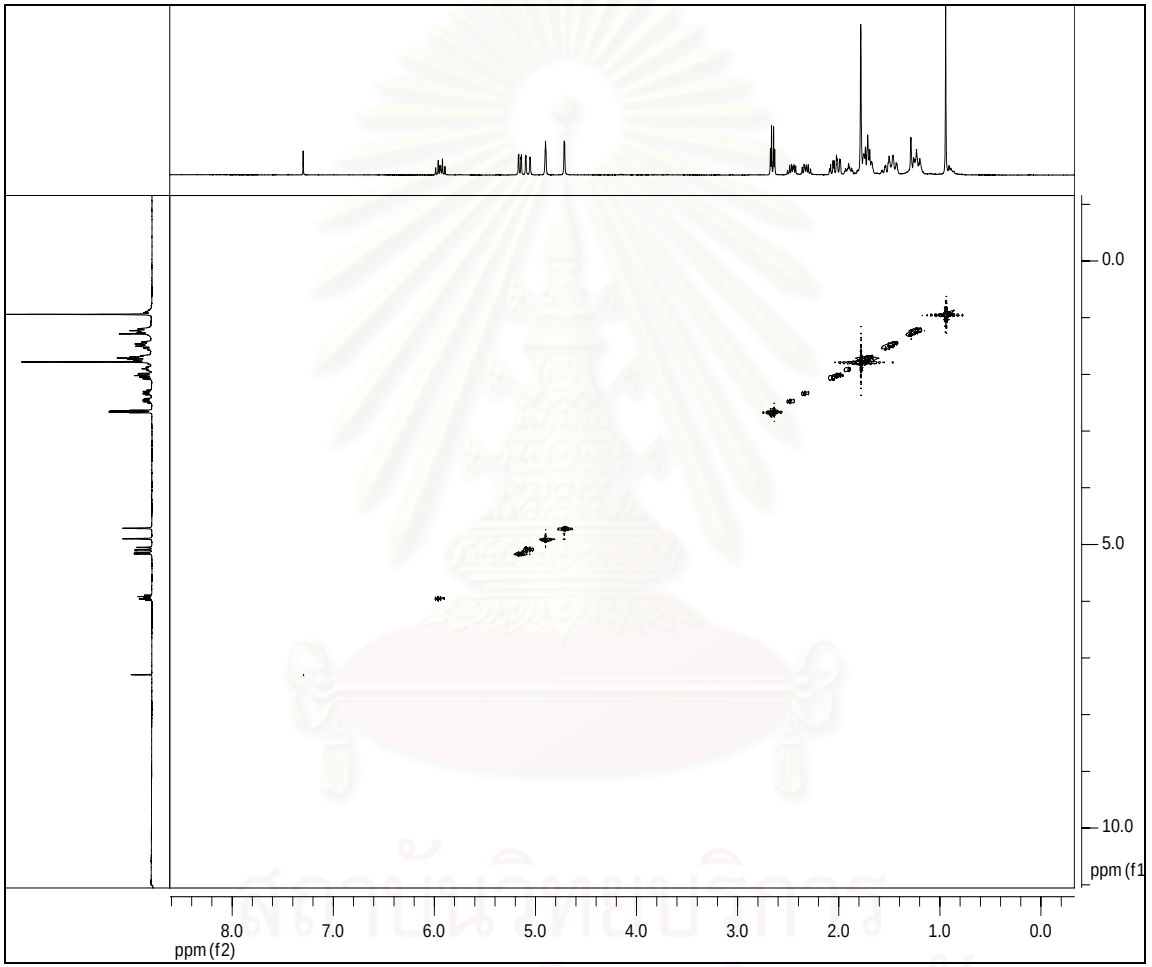

Figure 39. NOESY spectrum of compound **3**

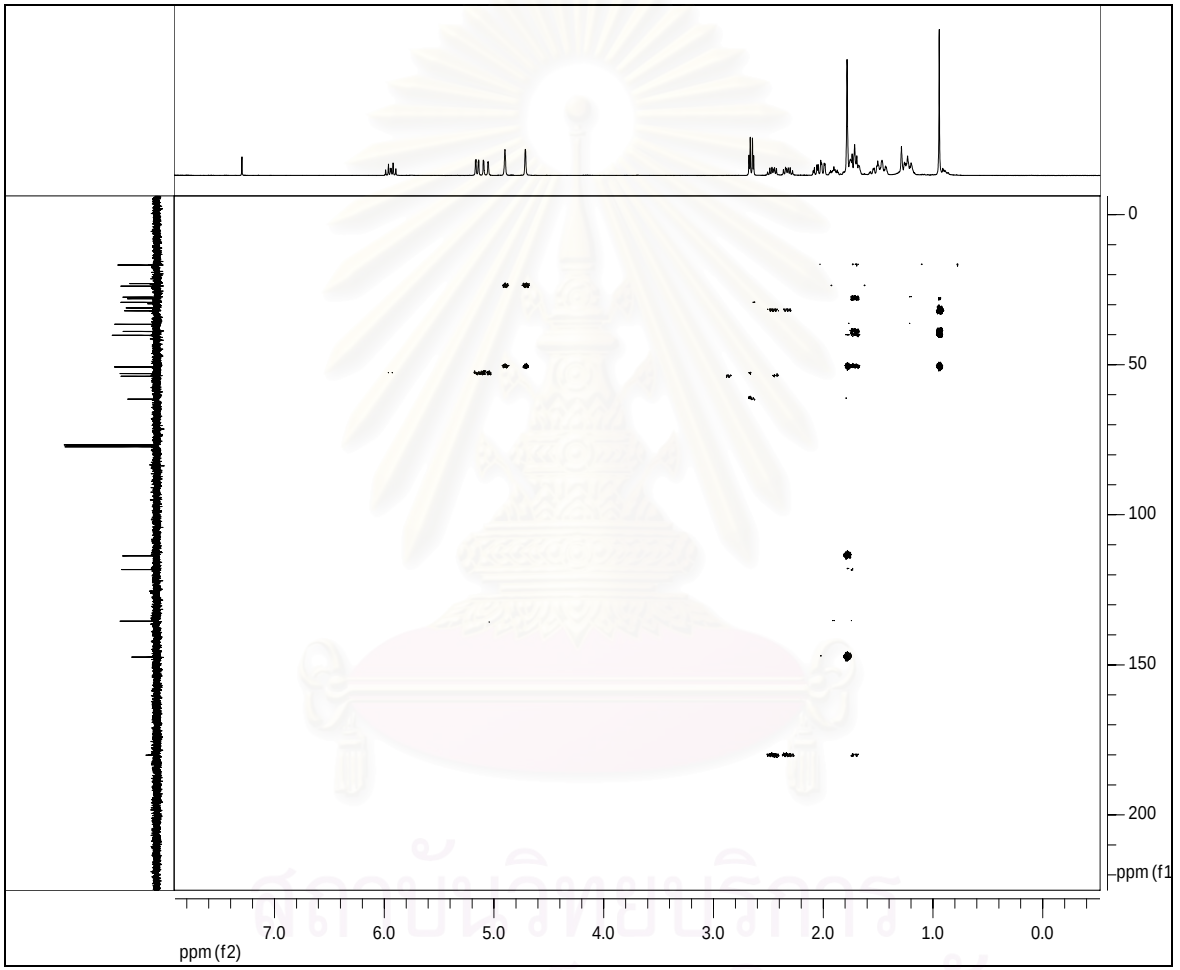

Figure 40. HMBC spectrum of compound **3**

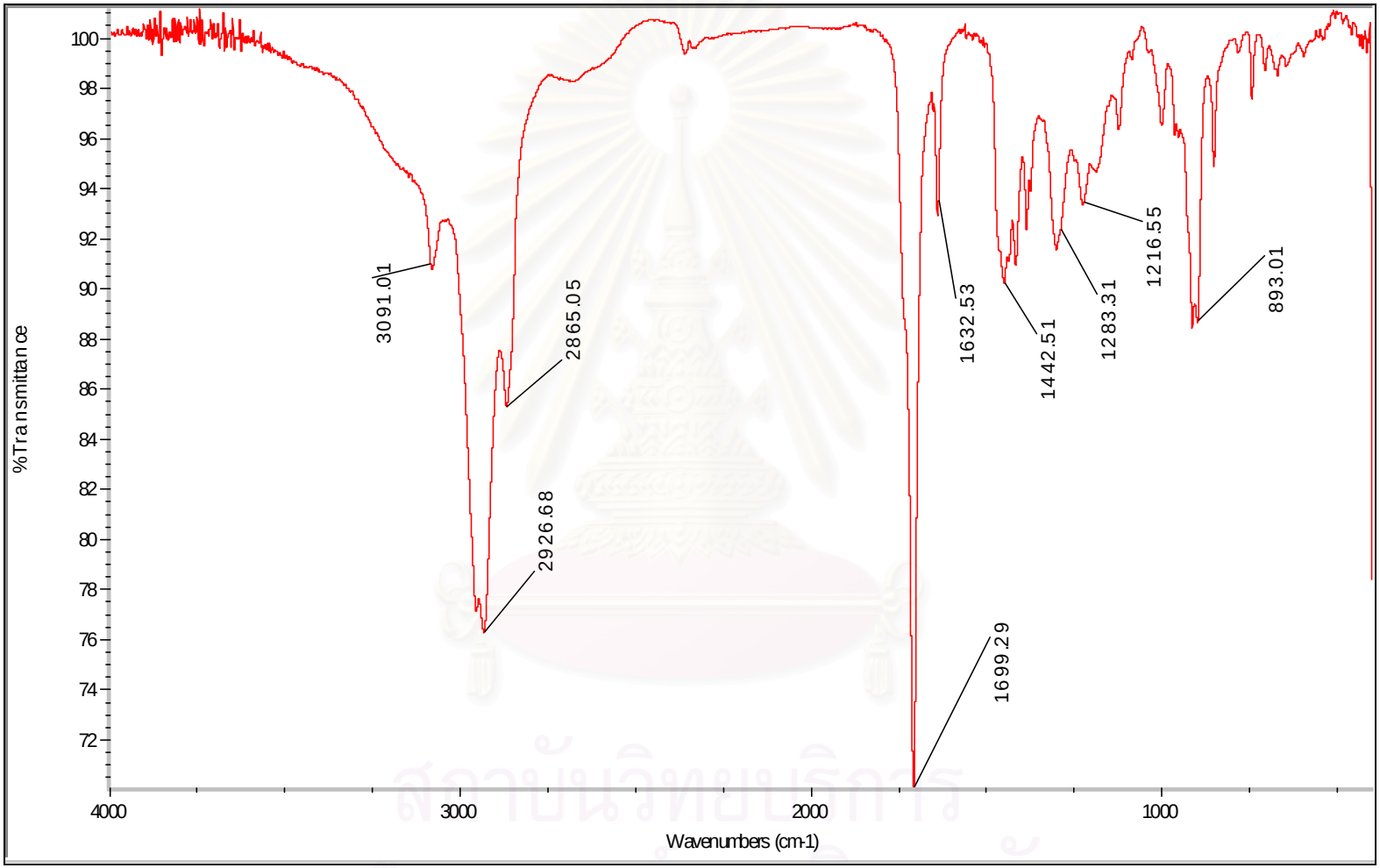

Figure 41. IR spectrum of compound **4**

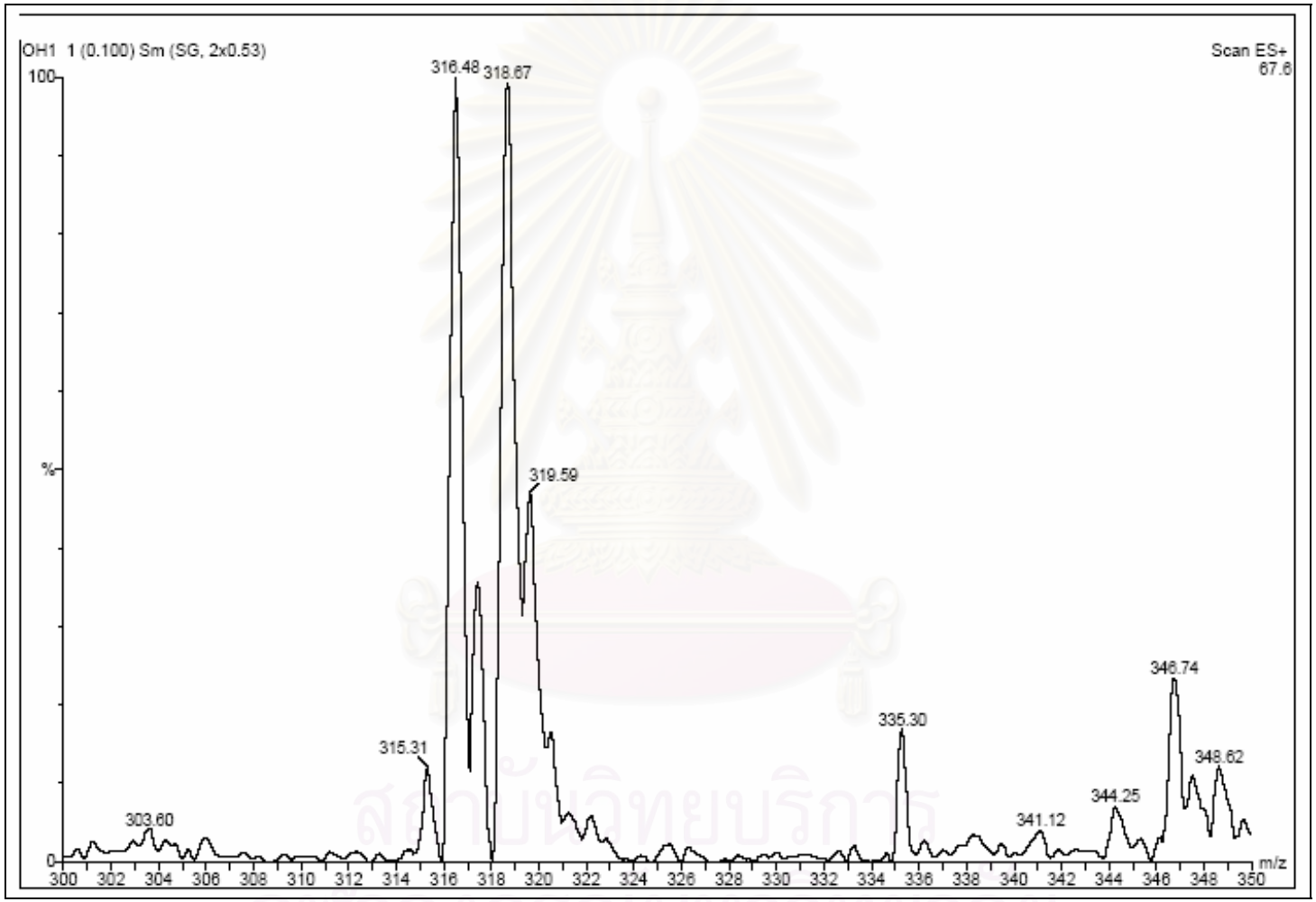

Figure 42. Mass spectrum of compound **4**

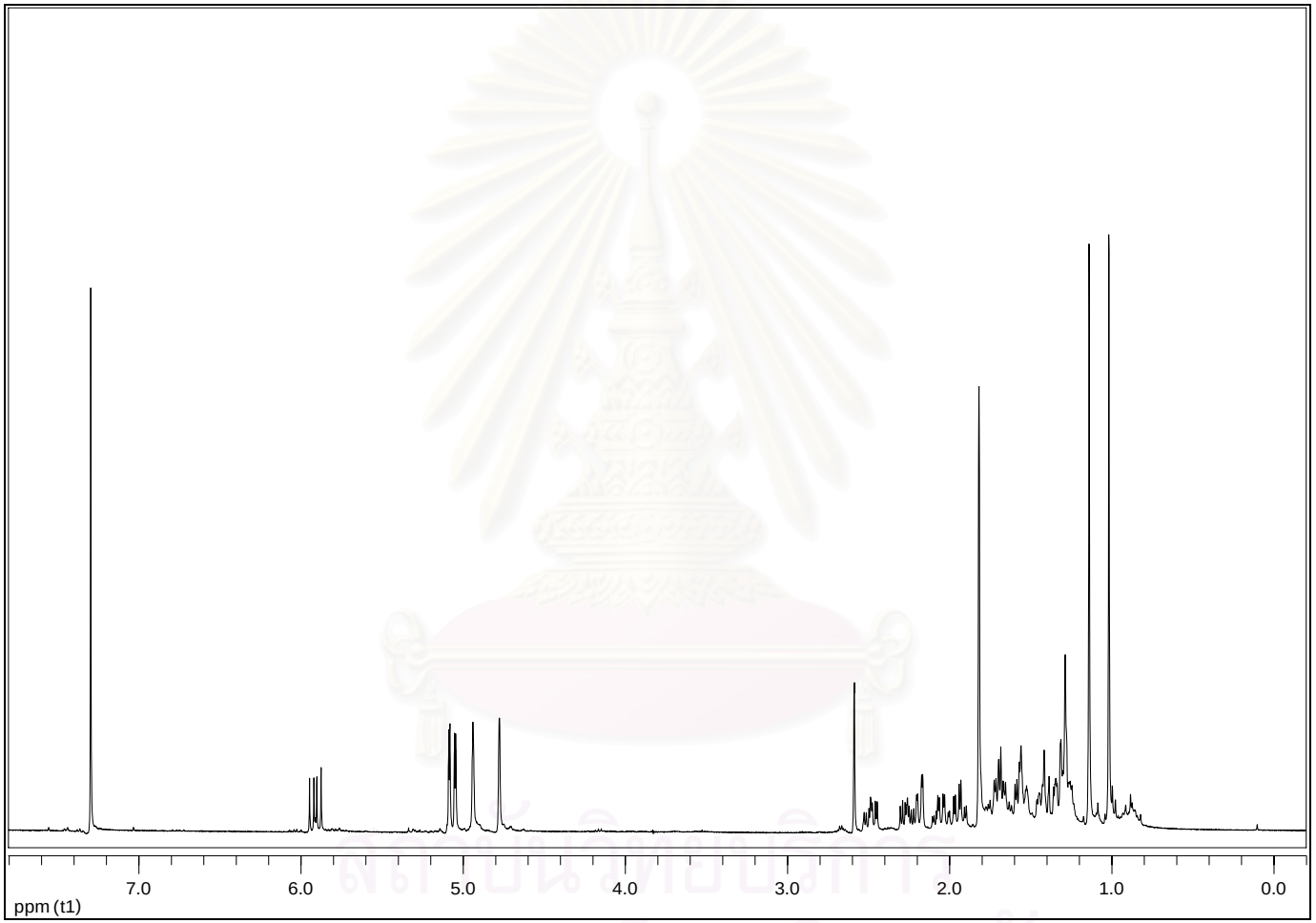

Figure 43. ^{1}H-NMR spectrum of compound **4**

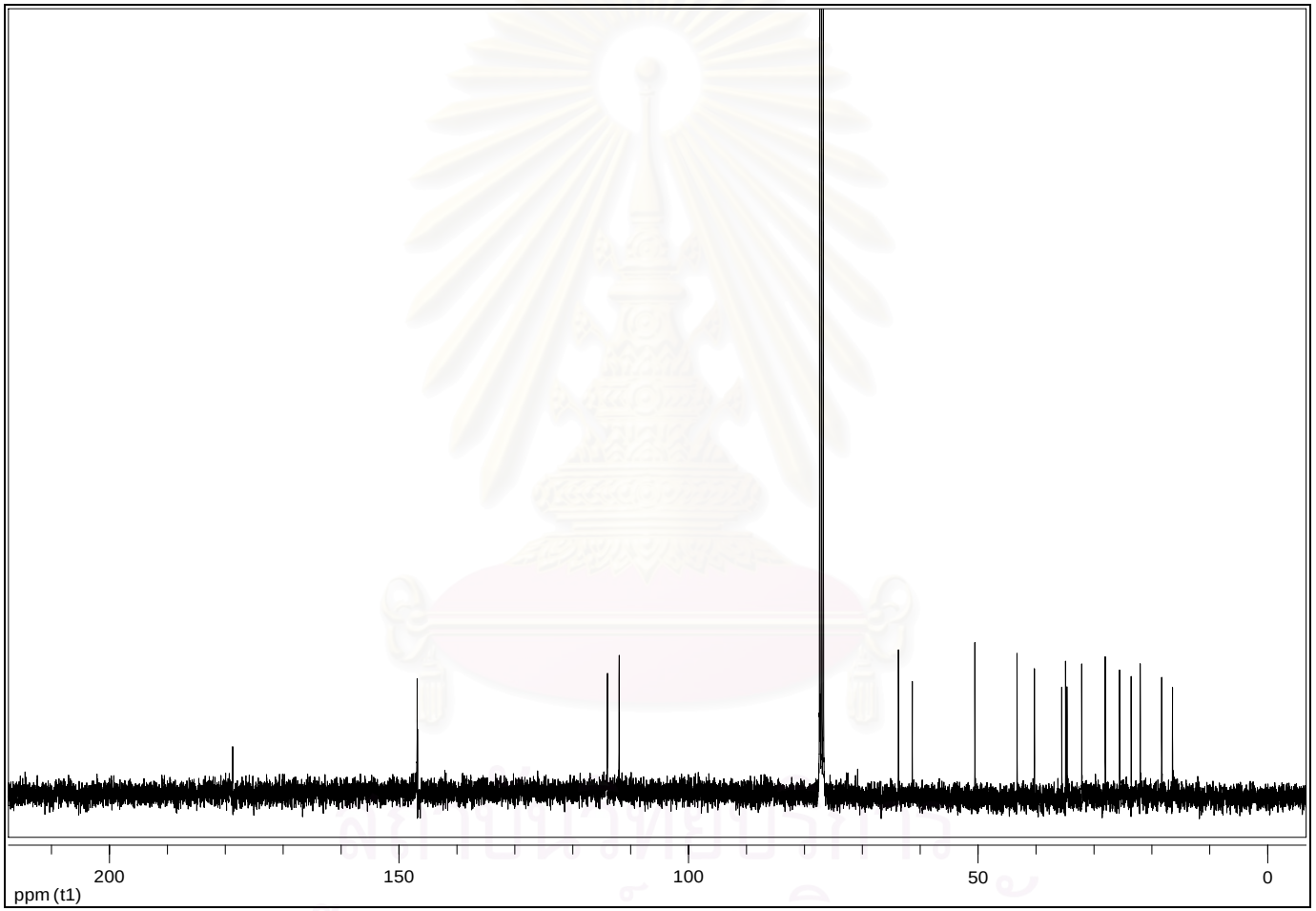

Figure 44. ^{13}C-NMR spectrum of compound **4**

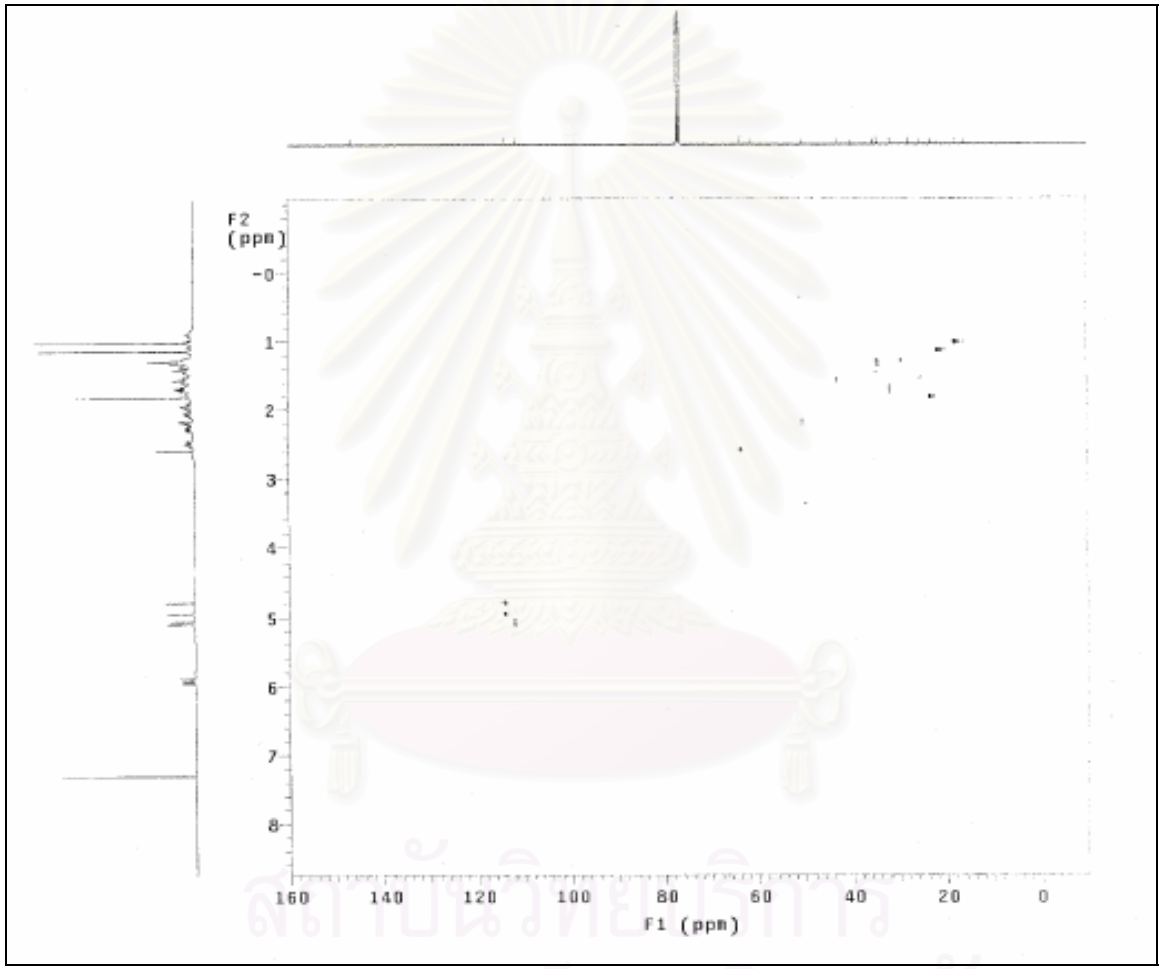

Figure 45. HSQC spectrum of compound **4**

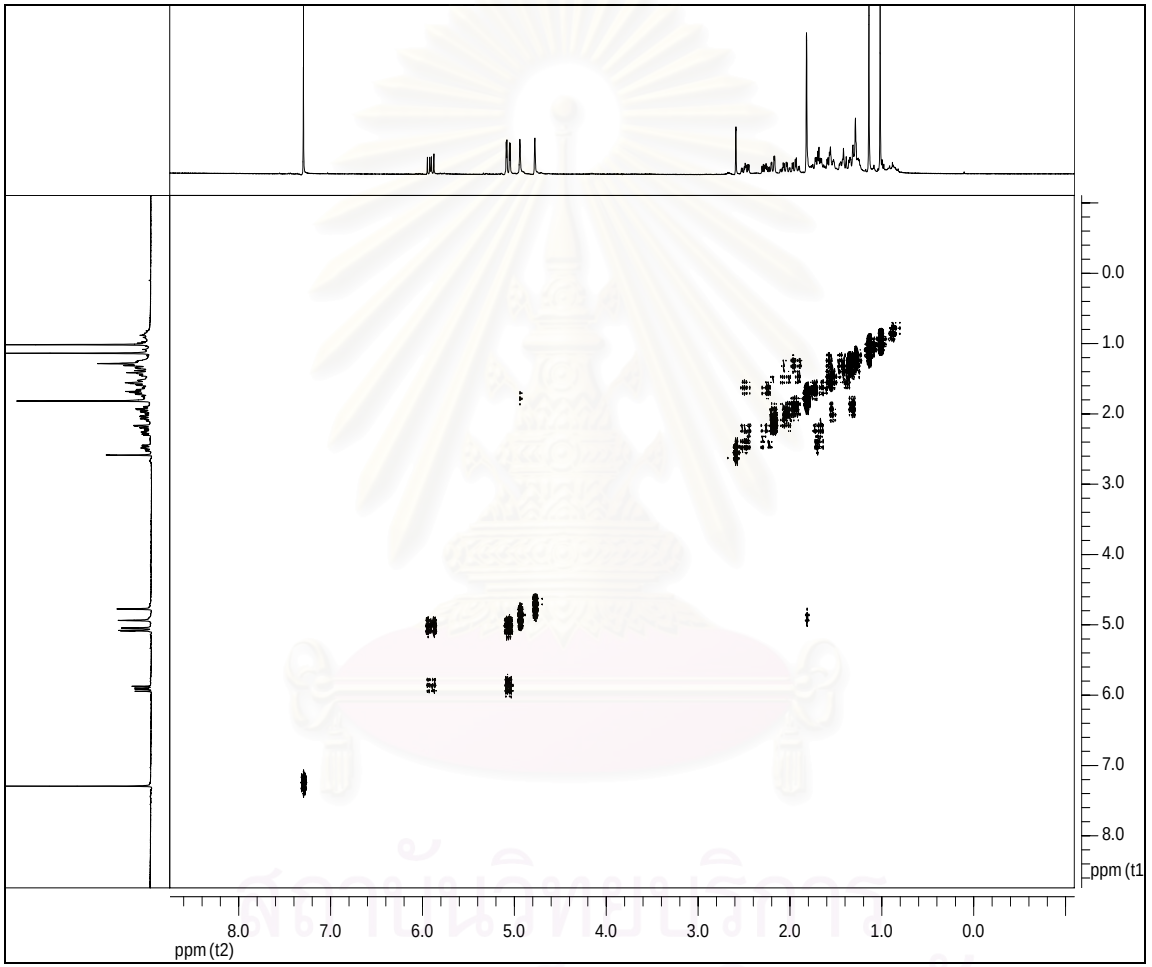

Figure 46. COSY spectrum of compound **4**

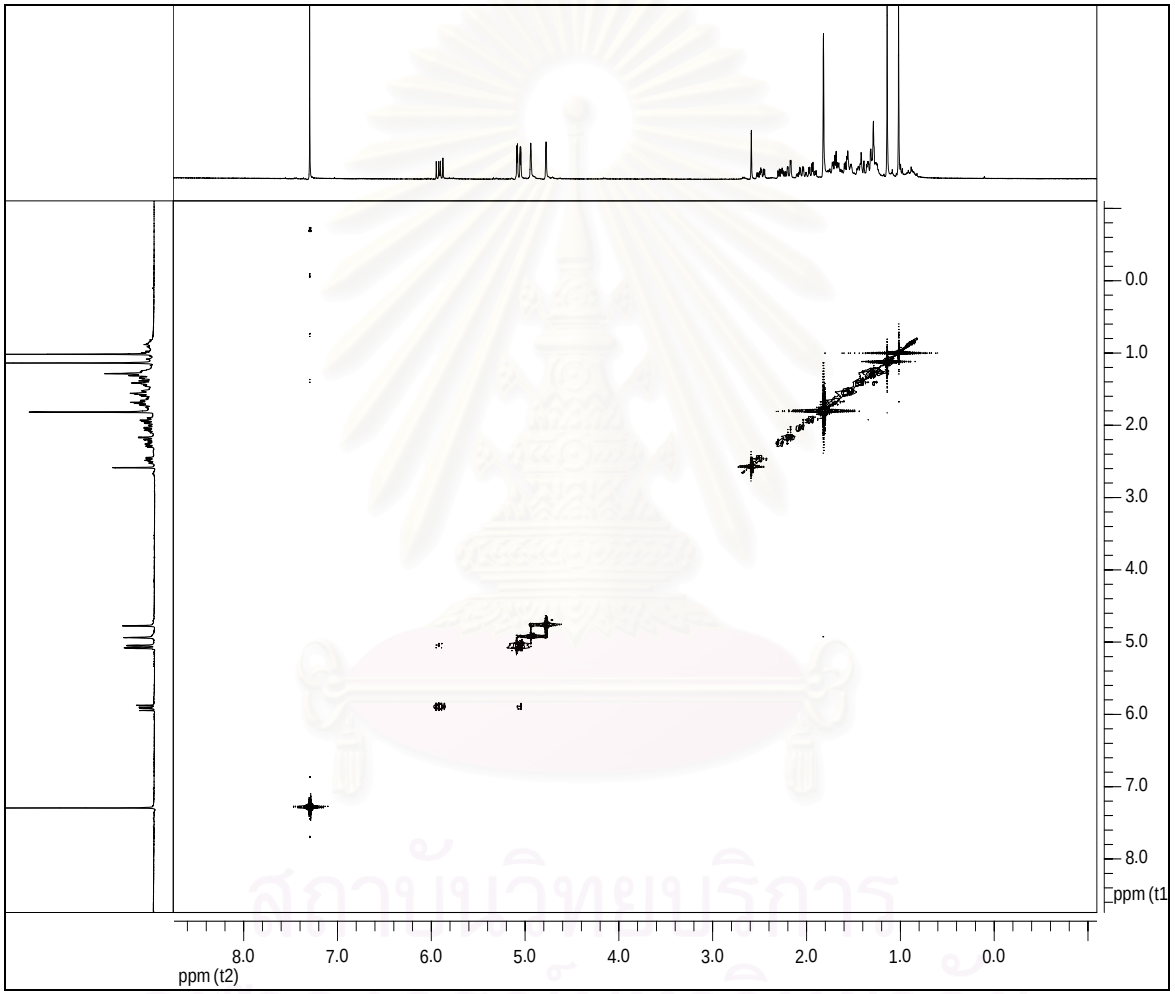

Figure 47. NOESY spectrum of compound **4**

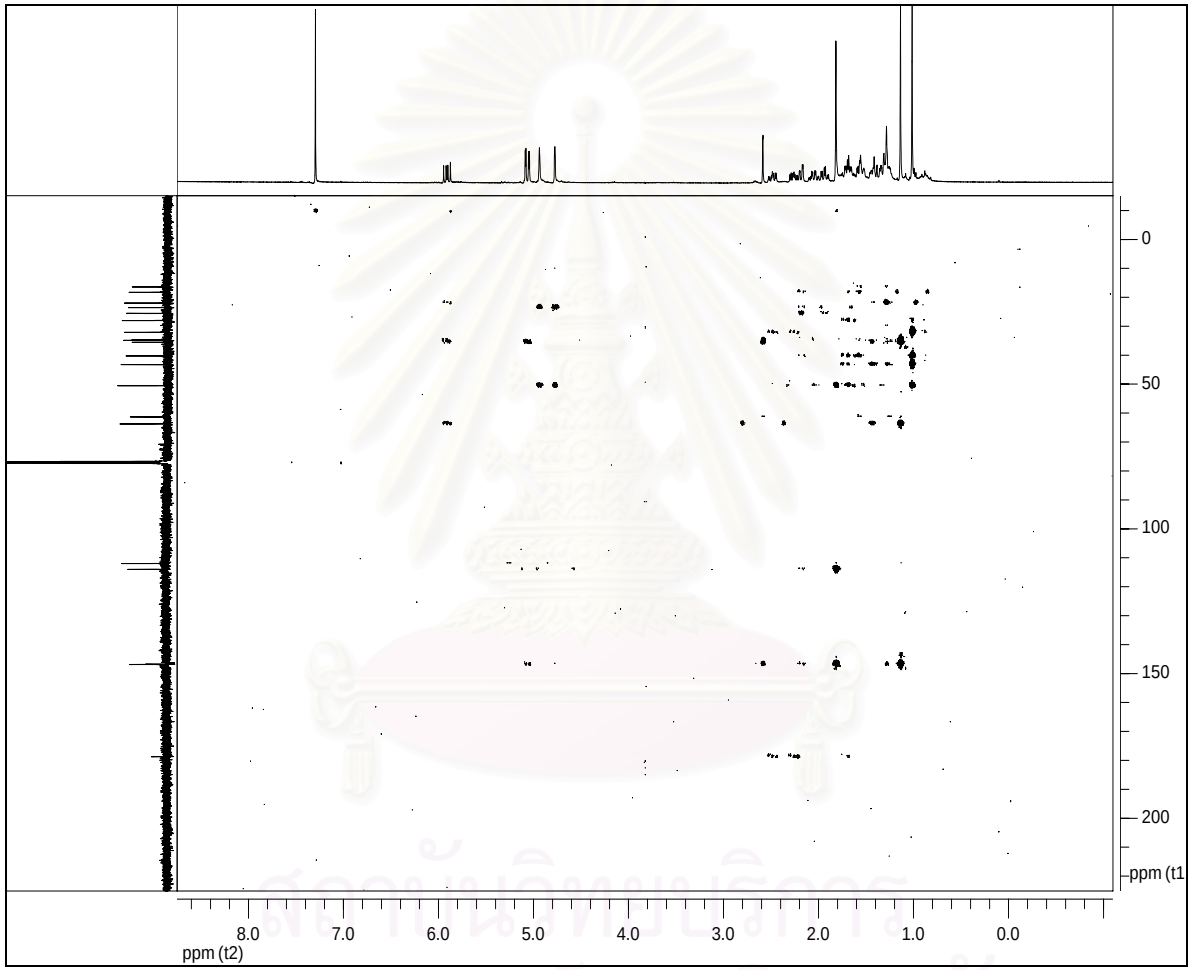

Figure 48. HMBC spectrum of compound **4**

VITAE

Miss Pornnipa Pata was born on July 22, 1981 in Chonburi Province, Thailand. She graduated with a Bachelor Degree of Science in Chemistry from Burapha University in 2002. In the same year, she was admitted into a Master Degree program in organic chemistry at Department of Chemistry, Faculty of Science Chulalongkorn University and completed the program in 2005.