องค์ประกอบทางเคมีและฤทธิ์ทางชีวภาพของเปลือกต้นเปล้าใหญ่ *Croton oblongifolius* Roxb. จากอำเภอด่านซ้าย จังหวัดเลย

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CHEMICAL CONSTITUENTS AND BIOLOGICAL ACTIVITY FROM THE STEM BARKS OF *Croton oblongifolius* Roxb. FROM AMPHOE DAN SAI, LOEI PROVINCE

Mr. Kullaphan Siriwat

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กุลพันธ์ ศิริวัฒน์ : องค์ประกอบทางเคมีและฤทธิ์ทางชีวภาพของเปลือกต้นเปล้าใหญ่ *Croton oblongifolius* Roxb. จากอำเภอด่านซ้าย จังหวัดเลย (CHEMIICAL CONSTITUENTS AND BIOLOGICAL ACTIVITY FROM THE STEM BARKS OF *Croton oblongifolius* Roxb FROM AMPHOE DAN SAI, LOEI PROVINCE) อาจารย์ที่ปรึกษา : รศ.ดร. อมร เพชรสม; 73 หน้า. ISBN 974-334-136-6

ในการศึกษาองค์ประกอบทางเคมีของเปลือกต้นเปล้าใหญ่ สามารถสกัดแยกสารประกอบไดเทอร์พีนอยด์ชนิดใหม่ คือ cleistantha-4(18),13(17),15-trien-3-oic acid (**1**) จากสิ่งสกัดเฮกเซนและสิ่งสกัดเอทธิลอะซิเตต เตรียมอนุพันธุ์ 3 ชนิดจากสาร **1** คือ methyl cleistantha-4(18),13(17),15-trien-3-oate (**2**), cleistantha-4(18),13(17),15-trien-3-ol (**3**) และ cleistantha-4(18),12,15-trien-3-oic acid (**4**) ได้ทำการพิสูจน์โครงสร้างของสารใหม่โดยอาศัยข้อมูลทางสเปกโตรสโกปี ซึ่งได้แก่ IR, MS, 1D และ 2D NMR เทคนิคคือ DEPT, COSY, NOESY, HMBC และ HMQC นำสารประกอบทั้งหมดมาทดสอบการยับยั้งเซลมะเร็งในหลอดทดลองกับเซลมะเร็ง BT474 (เต้านม), HEP-G2 (ตับ), SW620 (ลำไส้), CHAGO (ปอด) และ KATO-3 (กระเพาะอาหาร) พบว่า สาร **2** มีฤทธิ์ยับยั้งเซลมะเร็ง HEP-G2 (ตับ), SW620 (ลำไส้), CHAGO (ปอด) และ KATO-3 (กระเพาะอาหาร) มีค่า IC_{50} เป็น 10.0, 9.2, 9.7 และ 8.4 μg/ml ตามลำดับ สาร **3** มีฤทธิ์ยับยั้งเซลมะเร็ง BT474 (เต้านม), HEP-G2 (ตับ), SW620 (ลำไส้), CHAGO (ปอด) และ KATO-3 (กระเพาะอาหาร) โดยมีค่า IC_{50} เท่ากับ 8.3, 5.8, 4.3, 4.9 และ 5.0 μg/ml ตามลำดับ

ภาควิชา .....เคมี.....
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Kullaphan Siriwat : CHEMICAL CONSTITUENTS AND BIOLOGICAL ACTIVITY FROM THE STEM BARKS OF *Croton oblongifolius* Roxb FROM AMPHOE DAN SAI, LOEI PROVINCE. THESIS ADVISOR : ASSO. PROF. AMORN PETSOM, Ph.D. 73 pp. ISBN 974-334-136-6

In the investigation of chemical constituents of the stem barks *of Croton oblongifolius* Roxb., a new diterpenoids compound, cleistantha-4(18),13(17),15-trien-3-oic acid (**1**) was isolated from crude hexane and crude ethyl acetate. Three derivatives of compound **1** was synthesized, such as: methyl cleistantha-4(18),13 (17),15-trien-3-oate (**2**), cleistantha-4(18),13(17),15-trien-3-ol (**3**) and cleistantha-4 (18),12,15-trien-3-oic acid (**4**). The structure of the new compounds were established by spectroscopic data (IR, MS spectra, 1D and 2D NMR techniques including DEPT, COSY, NOESY, HMBC and HMQC). All of compounds were tested for cytotoxicity against various human tumor cell lines (BT474 (breast), HEP-G2 (hepatoma), SW620 (colon), CHAGO (lung), and KATO-3 (gastric)). Compound **2** was active against Hep-G2 cell line, SW620 cell line, CHAGO cell line and KATO-3 cell line, *in vitro*, with IC_{50} value of 10.0, 9.2, 9.7 and 8.4 μg/ml, respectively. Compound **3** was active against BT474 cell line, Hep-G2 cell line, SW620 cell line, CHAGO cell line and KATO-3 cell line, *in vitro*, with IC_{50} value of 8.3, 5.8, 4.3, 4.9 and 5.0 μg/ml, respectively.

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LIST OF SCHEMES

LIST OF ABBREVIATIONS

δ	Chemical shift
C.	Croton
cm^{-1}	reciprocal centimeter (unit of wave number)
ppm	part per million
d	doublet (NMR)
dd	double doublet
dt	double triplet
m	multiplet (NMR)
s	singlet (NMR)
t	triplet (NMR)
br	broad
s	strong
m	medium
w	weak
M^+	molecular ion
m/z	mass to charge ratio
ν_{max}	the reciprocating wavelength (IR)
λ_{max}	the wavelength at maximum absorption (UV)
ml	milliliter (s)
mg	milligram
Hz	Hertz
TMS	Tetramethylsilane
DEPT	Distortionless Enhancement by Polarization Transfer
HMQC	Heteronuclear Multiple Quantum Correlation
HMBC	Heteronuclear Multiple Bond Correlation
COSY	Correlated Spectroscopy
NOESY	Nuclear Overhauser Enhancement Spectroscopy

CHAPTER I

INTRODUCTION

Croton (or "Plao") [1] is a plant in family Euphorbiaceae, which have many species widely distributed in Thailand. Many species are useful as folk medicine. For example, Plao Nam Ngoen *(C. cascarilloides Raeusch.)* can be used as an antifebrile, Plao Lueat (*C. robutus* Kurz.) can be used as an antianemic agent, Plao Noi (*C. sublyratus* Kurz.) can be used as antiulceric agent.[2]

Croton oblongifolius Roxb. is one of a very interesting Thai medicinal plant because it is believed that all parts of the plant can be used as drugs. The leaves can be used as a tonic, the flowers are used as a teniacide, the fruits are used to treat dysmenorrhea, the seeds are used as a purgative, the barks are used to treat dyspepsia, and the roots are used to treat dysentery [3]. Moreover this plant is widely distributed through out Thailand climatic tolerant.

1.1 Botanical aspects of *Croton oblongifolius* Roxb.

Croton oblongifolius Roxb. is a medium sized deciduous tree in the Euphorbiaceae family. There are about 700 species in this family. In Thailand, it is commonly called Plao Yai (central) or Plao Luang (Northern). It is distributed throughout forests or shrubs below 700 meters above sea level. Its calyx and ovary are clothed with minute orbicular silvery scales. Leaves are 5.6-12.0 by 13.0-24.0 cm in size. The shape of leaf blade is oblong-lanceolate. Its flowers are pale yellowish green and solitary in the axials of minute bracts on long erect racemes. The male flowers are located in the upper part of the raceme and the females in the lower part. The male flowers are slender and have the length of pedicels of 4.0 mm. The calyx is more than 6.0 mm. long and segments are woolly. The twelve stamens are inflexed in bud and the length of filaments is 3.0 mm. In female flowers, the pedicels are short and stout. Its sepals are more acute than in the male with densely ciliated margins. The diameter of the fruit is less than 1.3 cm., slightly 3-lobed and clothed with small orbicular and quite smooth on the back [4,5]. The picture of stem, leaf, flowers and fruits of *Croton oblongifolius* Roxb. are shown in Fig.1.

Fig. 1 *Croton oblongifolius* Roxb.

1.2 Significance of the Problem

A large variety of diterpenoids compounds were found in *Croton oblongifolius* Roxb. and some compound have been shown to inhibit the growth of cancer cells[17]. Therefore, *C. oblongifolius* is a good source of large variety of diterpenoid compounds. To continue our investigation of *C. oblongifolius* Roxb. we took plant specimen from Amphur Dan Zai, Loei province. The NMR screening of crude hexane extract pointed out that this specimen contained difference diterpenoids which have been found previously. Therefore, it is of our interest to study chemical components as well as their biological activities in this plant specimen.

1.3 The objectives of this research

The objectives of this research can be summarized as follow:

1. To extract, isolate, and purify the organic constituents of the stem bark of *C. oblongifolius* Roxb. from Amphur Dan Zai, Loei province.
2. To identify the structural formula of the isolated substances.
3. To investigate the biological activity of the compounds that obtained.

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.

2.1.1 Foreign research

In 1968, Rao, P. S., Sachdev, G. P., Seshadri, T. R., and Singh, H. B.[6] studied chemical constituents from the bark of *Croton oblongifolius* Roxb. They found a new diterpene alcohol, oblongifoliol together with β-sitosterol.

In 1969, Aiyar, V. N., Rao, P. S., Sachdev, G. P., and Seshadri, T. R.[7] found deoxyoblongifoliol from the stem bark of *C. oblongifolius* Roxb.

In 1970, Aiyar, V. N. and Seshadfri, T. R.[8] studied 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. determined 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 [9]. 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-aldehyde [10]. Moreover, Acetyl aleuritolic acid, 3β-acetoxy-olean-14(15)-ene-28-oic acid, has been obtained from the bark [11].

In 1972, Aiyar, V. N. and Seshadri, T. R. found two closely related furanoid diterpenes from the 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 [12]. 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 [13].

2.1.2 Region research

In 1998, Roengsumran, S., et. al. [14] found two new cembranoids, one was crotocembraneic acid and the other was neocrotocembraneic acid.

In 1998, Roengsumran, S., et. al. [15] found four 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 1999, Roengsumran, S., et. al. [16]. They found a new neocrotocembranal from *C. oblongifolius* Roxb. In the same year Singtothong, P. reported eleven diterpenoids from stem-bark of *C. oblongifolius* from various sources and fourteen diterpenoids by chemical modification. The eleven compounds were: crotocembraneic acid, neocrotocembraneic acid, neocrotocembranal, crovatin, poilaneic acid, isokolavenol, crotohalimaneic acid, benzoyl crotohalimaneic acid, crotohalimoneic acid, hardwickiic acid and nidorellol. The fourteen modification compounds were methyl crotocembraneate, crotocembraneol, crotocembraneal, methyl neocrotocembraneate, neocrotocembraneol, neocrotocembraneal, methyl poilaneate, poilaneol, poilaneal, isokolavenal, isokolavenoic acid, methyl isokolavenoate, methyl crotohalimaneate and methyl crotohalimoneate [17].

In 2000, Kuptiyanuwat, N. [18] reported three new labdane diterpenoids and one from chemical modification from. stem-bark of *C. oblongifolius* Roxb. from Amphur Wang Sa Pung, Loei Province. The new labdanes were: 2-acetoxy-labda-8 (17), 12(E), 14-triene-3-ol, 3-acetoxy-labda-8(17), 12(E), 14-triene-2-ol, labda-8(17), 12(E), 14-triene-2, 3-diol. The modified labdane was 2, 3-diacetoxy-labda-8(17), 12 (E), and 14-triene. Baiagern, S. [19] reported three diterpenoids from stem-bark of *C. oblongifolius* Roxb. from Amphur Muang, Udonthani Province and three diterpenoids by chemical modification. Tanwattanakun, T. reported six diterpenoids from stem-bark of *C. oblongifolius* Roxb. from Amphur Muang, Uttaradit Province [20].

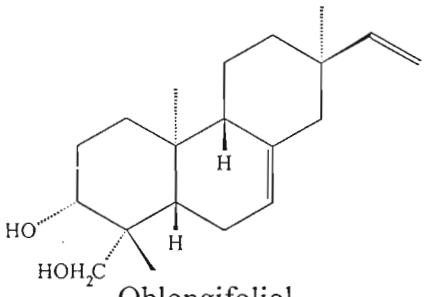
All diterpenoid compounds of *C. oblongifolius* Roxb. can be classified into 6 group of diterpenoids, which was shown in the following table.

Table 1. The Chemical Constituents of *Croton oblongifolius* Roxb.

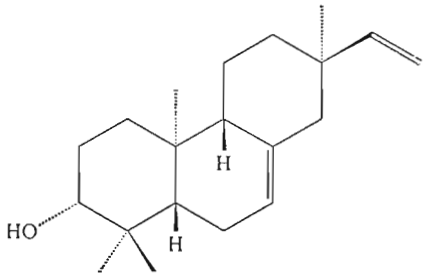
Plant parts	Substances	References
	Pimaranes	
Bark & Wood	Oblongifoliol	6
Bark & Wood	19-Deoxyoblongifoliol	7
Bark & Wood	3-Deoxyoblongifoliol	9
Bark & Wood	Oblongifolic acid	7,8
	Isopimaranes	
Bark & Wood	*ent*-Isopimara-7,15-diene	9
Bark & Wood	*ent*-Isopimara-7,15-diene-19-aldehyde	9
Bark	19-Hydroxy-*ent*-isopimara-7,15-diene	9
	Clerodanes	
Bark & Wood	(-)-Hardwickiic acid	10,17
Bark & Wood	11-Dehydro-(-)-hardwickiic acid	12
Wood	Acetyl aleuritolic acid	11
Bark	Crovatin	17
Bark	Isokolavenol	17
Modify	Isokolavenal	17
Modify	Isokolavenoic acid	17
Modify	Methyl isokolavenoate	17
	Cembranes	
Bark	Crotocembraneic acid	14,17
Bark	Neocrotocembraneic acid	14,17
Bark	Neocrotocembranal	16,17
Bark	Poilaneic acid	17
Modify	Methyl crotocembraneate	17
Modify	Crotocembraneol	17
Modify	Crotocembraneal	17
Modify	Methyl neocrotocembraneate	17
Modify	Neocrotocembraneol	17

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

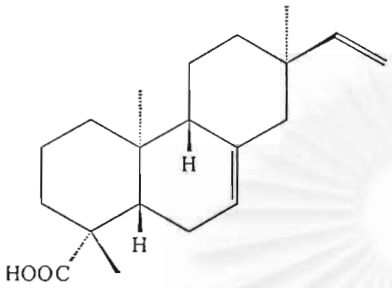
Plant parts	Substances	References
	Cembranes (Continued)	
Modify	Methyl poilaneate	17
Modify	Poilaneol	17
Modify	Poilaneal	17
	Labdanes	
Bark	Labda-7,12(E),14-triene	15
Bark	Labda-7,12(E),14-triene-17-al	15
Bark	Labda-7,12(E),14-triene-17-ol	15
Bark	Labda-7,12(E),14-triene-17-oic acid	15
	Halimanes	
Bark	Crotohalimaneic acid	17
Bark	Benzoyl crotohalimaneic acid	17
Bark	Crotohalimoneic acid	17
Modify	Methyl crotohalimaneate	17
Modify	Methyl crotohalimoneate	17



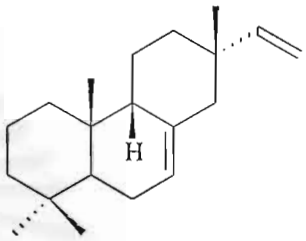
Oblongifoliol



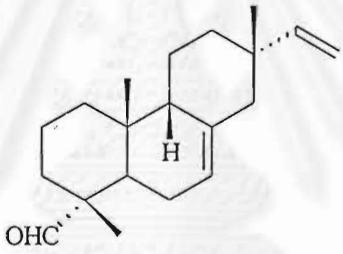
19-Deoxyoblongifoliol



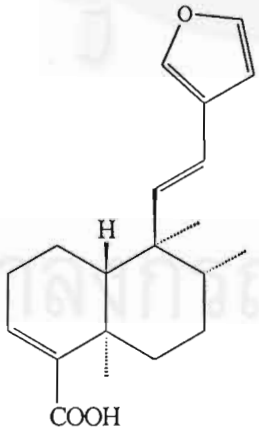
Oblongifolic acid



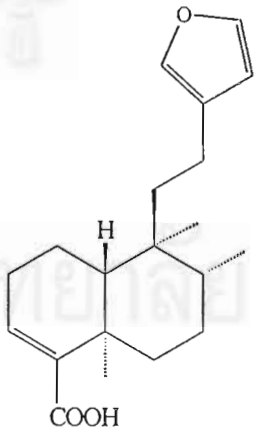
ent-Isopimara-7,15-diene



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

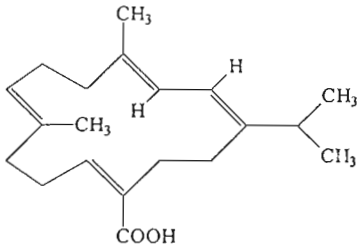


11-Dehydro-(-)-hardwickiic acid

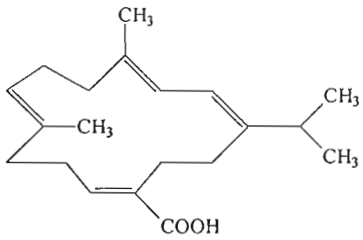


(-)-Hardwickiic acid

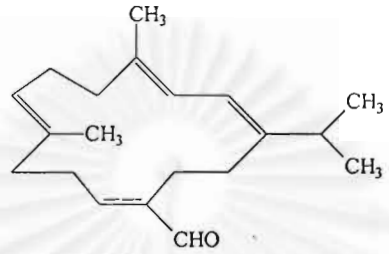
Fig. 2 The structures of diterpenoid compounds from *C. oblongifolius* Roxb.



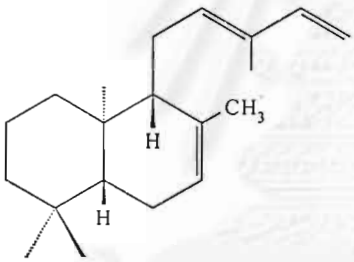
Crotocembraneic acid



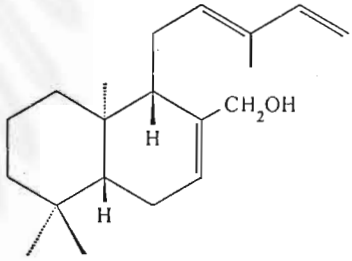
Neocrotocembraneic acid



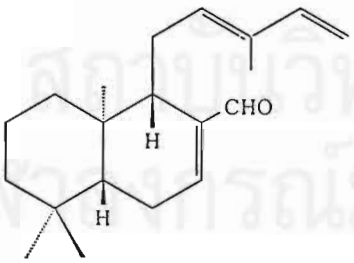
Neocrotocembranal



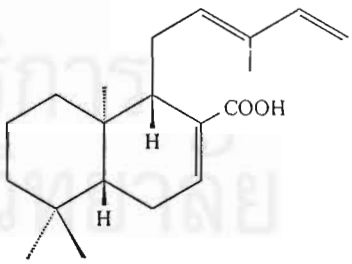
Labda-7,12(*E*),14-triene



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



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



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

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

2.2 The literature reviews of cleistanthane skeleton

From literature surveys, cleistanthane diterpenoids have been found in *Cleistanthus schlechteri* since 1971. The cleistanthane diterpenoids compounds found from 1971 to date are reported in the following examples.

In 1971, McGarry, E.J., et. al. [21] studied chemical constituents from heartwood of *Cleistanthus schlechteri.* They found a new diterpenoids skeleton namely cleistanthane. Major component has been assigned the structure as 8,11,13,15-cleistanthatethaene-2, 3,12-triol (Cleistanthol).

In 1978, Pinchin, R., et. al. [22] found two novel cleistanthanes from *Vellozai flavicans*. They found veadeirol and veadeiroic acid which was modified to methyl veadeiroate.

In 1982, Craveiro, A.A., and Silveira, E.R. [23] found two Cleistanthane type diterpenes from *Croton sonderianus,* which were sonderianol and 3,4-*seco*-sonderianol.

In 1982, Bohlmann, F., et. al. [24] found twelve cleistanthanes from roots of *Brickellia eupatoriedes.*

In 1985, Dunlop, R.W., found cleistanthane hydrocarbon in *Amphibolis aantartica.* [25]

In 1987, Prakash O., et. al. [26] had confirmed stereostructure of auricularic acid, isolated from *Pogostemon auricularis*. In the same year, Pinto, A.C., et. al. [27] isolated three new cleistanthanes from *Vellozai flavicans*. The first was (4R, 5R, 10S)-cleistantha-8, 11,13-trien-19-ol, the second was (4R, 5S, 10S)-cleistantha-8, 11,13-trien-19-oic acid, and the last one was (4r, 5s, 10s)-cleistantha-8, 11,13-trien-19-al.

In 1988, Pinto, A.C., et. al. [28] isolated two cleistanthanes skeleton from *Vellozai nivea.* One of both was 11-hydroxycleistantha-8,11,13-trien-7-one and the another one was 7,11-diketo-14α-hydroxy-cleistantha-8,12-diene. In the same year, Hussaini, F.A., et. al. [29] found three novel cleistanthane diterpenoids from *Pogostemon auricularis*. They have been isolated from whole plant, and characterized as cleistanth-13, 15-dien-18-oic acid and 7-hydroxy- and 7-acetoxycleistanth-13, 15-dien-18-oic acids.

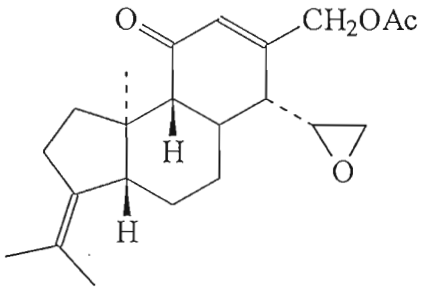
In 1989, Abad A., et. al. [30] synthesized cleistantha-13, 15-dien-18-oic acid, that an auricularic acid epimer.

In 1991, Pinto, A.C., et. al. [31] isolated two cleistanthanes skeleton from *Vellozai declinans*. One of both was 20-hydroxycleistantha-8,11,13-trien-7-one and the another one was 20-carboxaldehyde-cleistantha-8,11,13-trien-7-one.

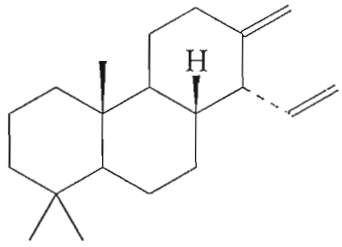
In 1992, Pinto, A.C., et. al. [32] isolated twelve cleistanthanes skeleton from *Vellozai declinans,* two of them have been reported. The ten compounds were: 7,16-epoxy-20-nor-5, 7,9,11,13-cleistanthapentaen-3-one, 20-hydroxy-8, 11,13-cleistanthatrien-7-one, 20-caboxaldehyde-8, 11,13-cleistanthatrien-7-one, 7,16-epoxy-20-nor-5, 7,9,11,13-cleistanthapentaen-3-one, 7,16-epoxy-20-nor-5, 7,9,11,13-cleistanthapentaene, (5S, 10S)-8,11,13-cleistanthatriene-7-one, (5R, 7S, 10R)-7α, 16,7β, 20-diepoxycleistantha-1, 8,11,13-tetraen-3-one, (5R, 7S, 10R)-7α, 16,7β, 20-diepoxycleistantha-8, 11,13-trien-3-one, 8,11,13-cleistanthatriene, 6,8,11,13-cleistanthatetraene, (5S,7S,10S)-7β-hydroxy-8,11,13-cleisttanthatriene, and (3S,5S,7S,10R)-3β-hydroxy-)-7α,16,7β,20-diepoxycleistantha-8,11,13-trien.

In 1995, Pinto, A.C., et. al. [33] isolated three cleistanthanes skeleton from *Vellozai flavicans*. The first was 8,11,13-cleistanthatrien-7-one-17-oic acid, the second was 8,11,13-cleistanthatrien-7-one-19-oic acid, and the last was 8,11,13-cleistanthatrien-17, 19-dioic acid.

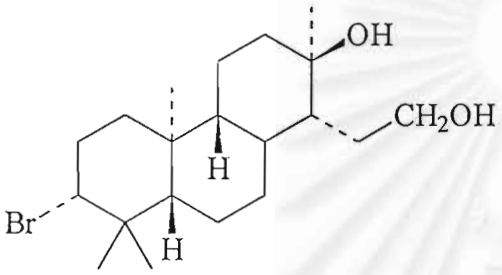
In 1996, Ayer, W.A. and Khan, A.Q. [34] found three new cleistanthane type diterpenes from liquid cultures of a *Zythiostroma* sp., a fungal associated with aspen. The three were zythiostromic acid A, zythiostromic acid B and zythiostromolid.



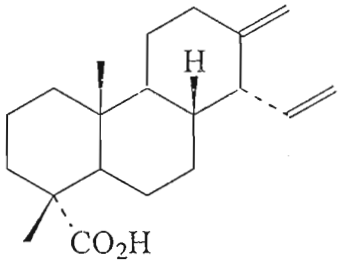
17-Acetoxy-15,16-epoxy-12-isocleistanthen-11-one



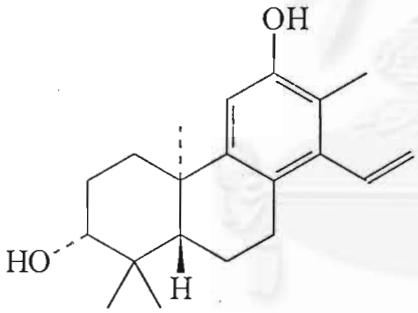
13,15-Cleistanthadiene



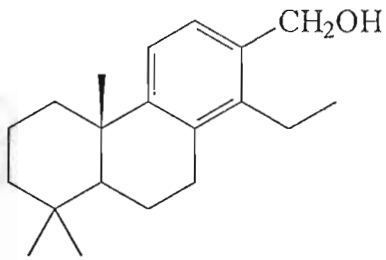
3-Bromo-13,16-cleistanthanediol



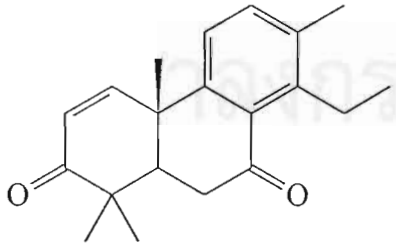
13,15-Cleistanthadien-18-oic acid



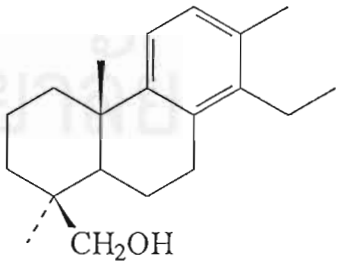
8,11,13,15-Cleistanthaene-3,12-diol



8,11,13-Cleistanthatrien-17-ol

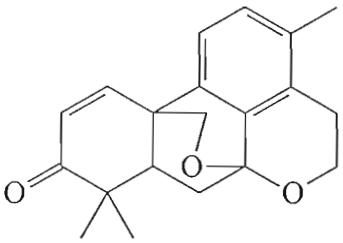


1,8,11,13-Cleistanthatetraene-3,7-dione

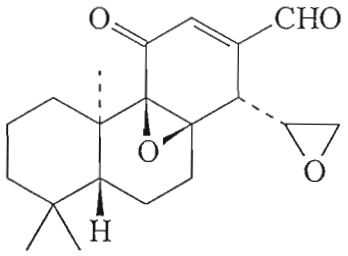


8,11,13-Cleistanthatrien-19-ol

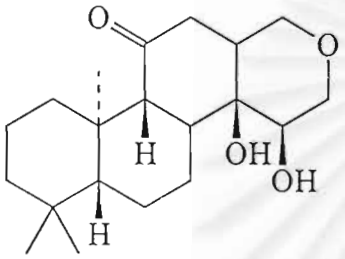
Fig. 3 The example of known cleistanthane skeleton.



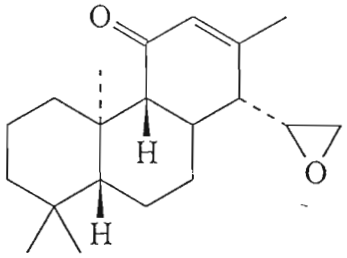
7,16:7,20-Diepoxy-1,8,11,13-cleistanthaen-3-one



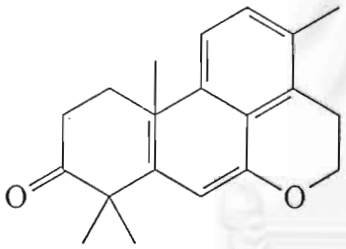
8,9:15,16-Diepoxy-11-oxo-12-cleistanthen-17-al



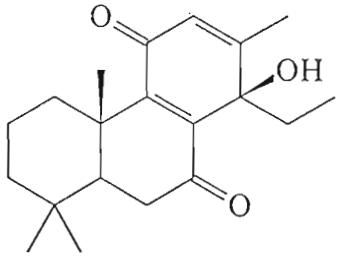
14,15-Dihydrooxy-16,17-epoxy-12-cleistanthen-11-one



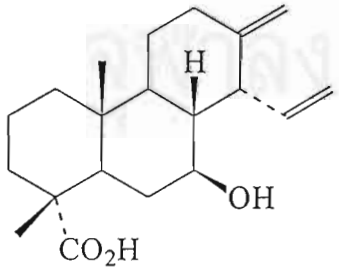
15,16-Epoxy-12-cleistanthen-11-one



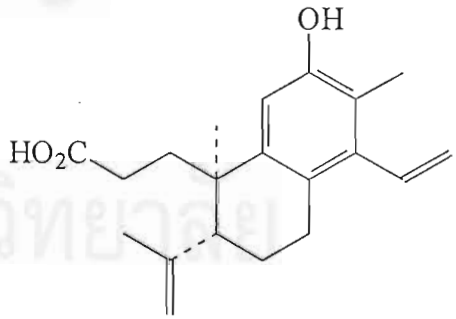
7,16-Epoxy-20-nor-5,6,8,13-cleistanthepentaen-3-one



14-Hydroxy-8,12-cleistanthsdiene-7,11-dione

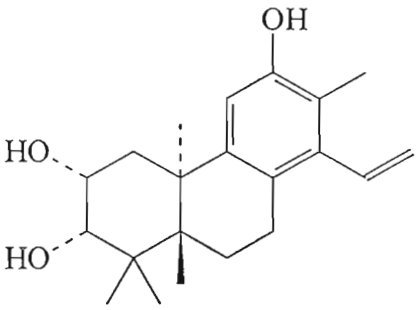


7-Hydroxy-13,15-cleistanthadien-18-oic acid

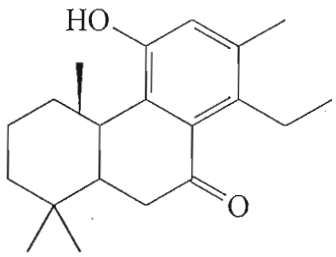


12-Hydroxy-3,4-seco-4,8,11,13,15-cleistantrapentaen-3-oic acid

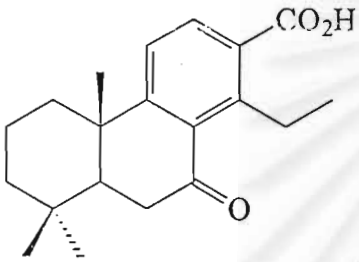
Fig. 3 The example of known cleistanthane skeleton. (Continued)



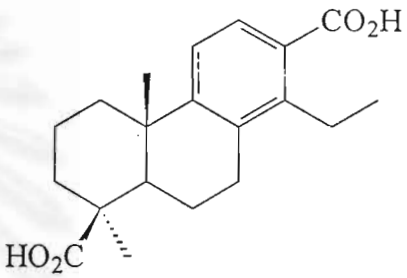
8,11,13,15-Cleistanthatetraene-2,3,12-triol



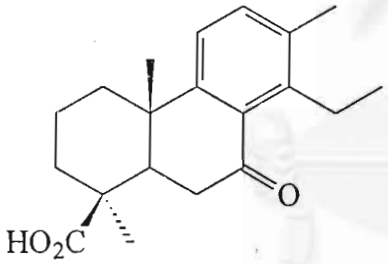
12-Hydroxy-8,11,13-cleistanthatrien-7-one



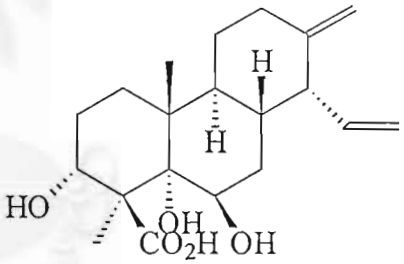
8,11,13-Cleistanthatrien-7-one-17-oic acid



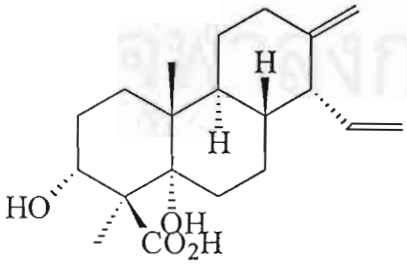
8,11,13-Cleistanthatrien-17,19-dioic acid



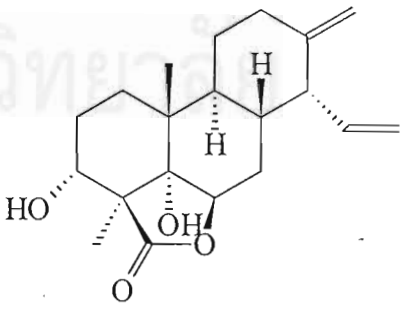
8,11,13-Cleistanthatrien-7-one-19-oic acid



Zythiostromic acid A

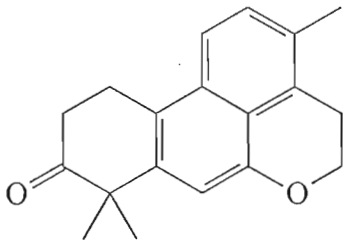


Zythiostromic acid B

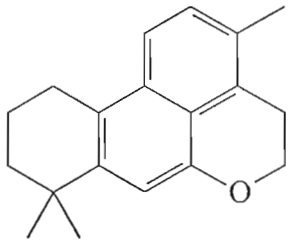


Zythiostromolid

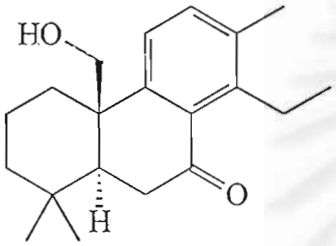
Fig. 3 The example of known cleistanthane skeleton. (Continued)



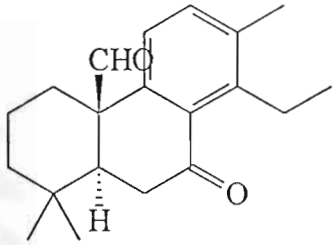
7,16-epoxy-20-nor-5, 7,9,11,13-cleistanthapentaen-3-one



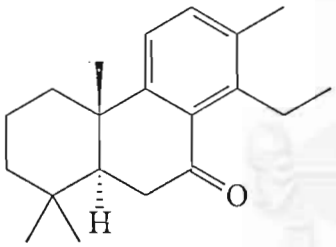
7,16-epoxy-20-nor-5, 7,9,11,13-cleistanthapentaene



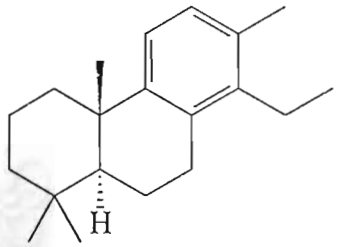
20-caboxaldehyde-8, 11,13-cleistanthatrien-7-one



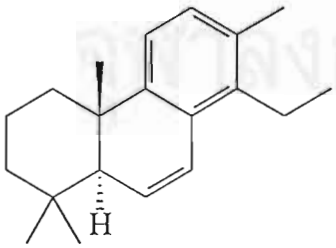
7,16-epoxy-20-nor-5, 7,9,11,13-cleistanthapentaen-3-one



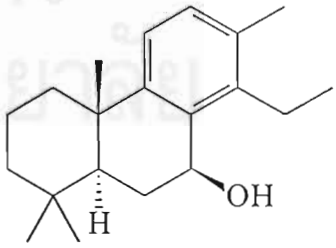
(5S, 10S)-8,11,13-cleistanthatriene-7-one



8,11,13-cleistanthatriene



6,8,11,13-cleistanthatetraene



(5S,7S,10S)-7β-hydroxy-8,11,13-cleisttanthatriene

Fig. 3 The example of known cleistanthane skeleton. (Continued)

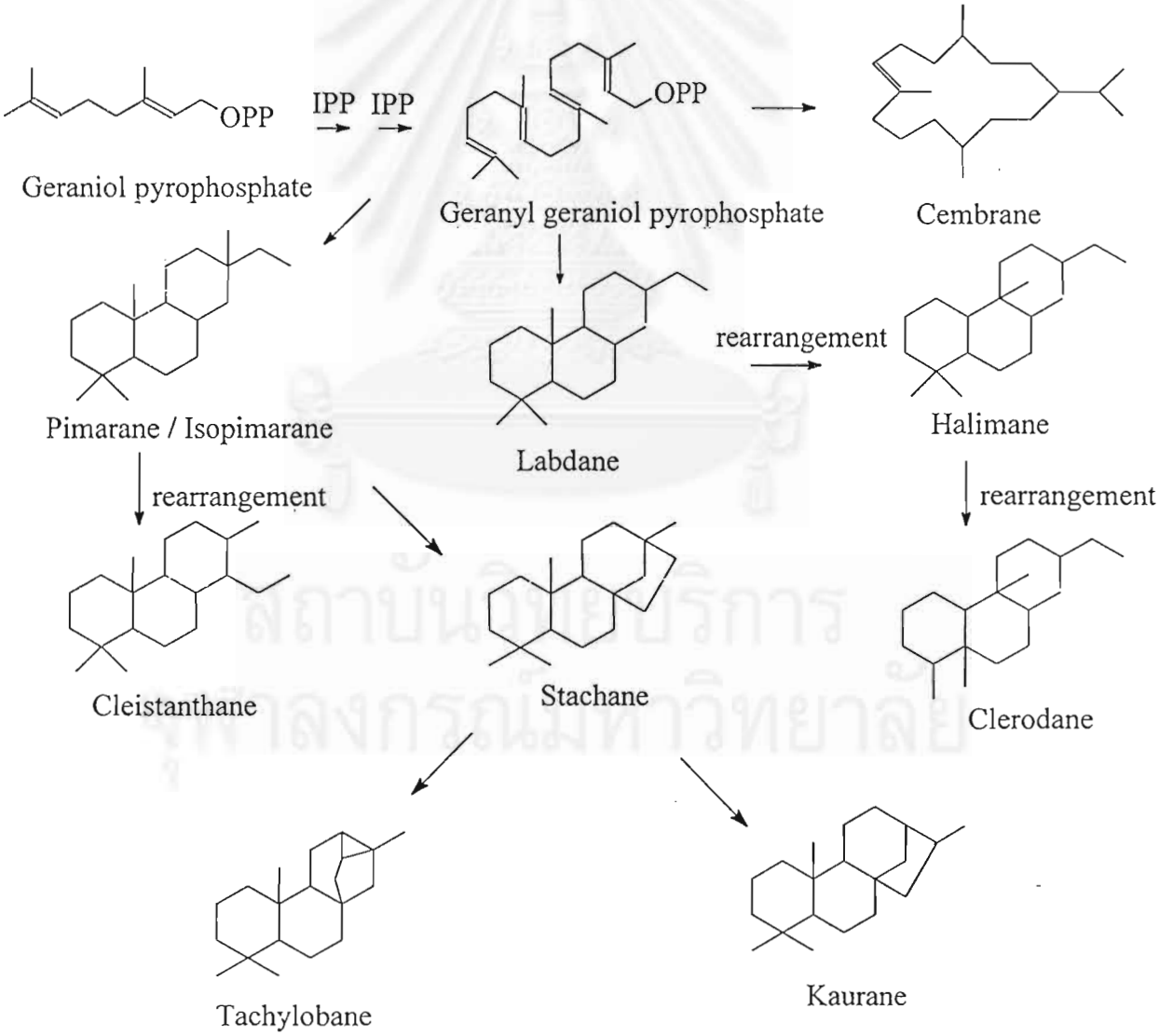
2.3 Biological activity review of cleistanthane

In 1988 Hussaini, F.A., et. al. report 7-acetoxycleistanth-13, 15-diene-18-oic acid was inhibited 10, 19 and 69% response of spasmogens at 2.5, 5 and 10 μg/ml respectively, on guinea pig ileum [29].

In 1996, Ayer, W.A. and Khan, A.Q. [34] reported zythiostromic acid A, zythiostromic acid B, and zythiostromolide showed no activity at 1000 ppm against *O. crassivaginatum* on agar culture plates, using 1 cm impregnated discs.

2.4 Biogenetic pathway of cleistanthane

Cleistanthane diterpenes are secondary metabolites uncommon in nature [36]. It was rearranged from pimarane. Biogenetic pathway is shown below.



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

CHAPTER III

EXPERIMENTS

3.1 Instruments and Equipments

1) Fourier Transform Infrared Spectrophotometer (FT-IR)

The FT-IR spectra were recorded on a Perkin-Elmer Model 1760X Fourier Transform Infrared Spectrophotometer. Solid samples were generally examined by incorporating the sample with potassium bromide (KBr) to form a pellet. Spectra of liquid samples were recorded as thinfilm on a sodium chloride (NaCl) cell.

2) Nuclear Magnetic Resonance Spectrometer (NMR)

The ^{1}H-NMR and ^{13}C-NMR spectra were recorded on a Bruker Model ACF 200 Spectrometer operated at 200.13 MHz. for ^{1}H and 50.32 MHz. for ^{13}C-nuclei. The 500 MHz. spectra and specialized NMR experiments were record on a JNM 500 MHz. The chemical shift was assigned in ppm unit and internally referenced with the residual protonated solvent ($CDCl_3$, δ=7.24 ppm.).

3) Mass Spectrometry (MS)

The mass spectra were recorded on a Fisons Instrument Mass Spectrometer Model Trio 2000 in EI mode at 70 eV.

4) Elemental Analysis

Elemental Analysis were measured on Perkin Elmer PE2400 SERIES II (CHN/O ANALYSER). Silica gel (Merck Dieselgel 60 and silica TLC plates (Si gel 60 F_{254}) were purchased from Merck Company.

5) UV-VIS spectrometry

UV-VIS spectra were recorded on a Hewlett Packare 8452A diode array spectrophotometer in EtOH.

3.2 Chemical Reagents

3.2.1 Solvents

All solvents used in this research such as hexane, dichloromethane (CH_2Cl_2), chloroform ($CHCl_3$), ethyl acetate (EtOAc) and methanol (MeOH) were commercial grade and were purified prior to use by distillation. The reagent grade solvents were used for recrystallization.

3.2.2 Other chemicals

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

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

3.) Merck's silica gel 60G ART.1.07731.1000 and $60GF_{254}$ ART. 1.07730.1000 were applied as adsorbent for preparative TLC.

4.) Merck's TLC aluminium sheet, silica gel 60F 254 precoated 25 sheets, 20x20 cm^2, layer 0.2 mm. was used to identify the identical fractions.

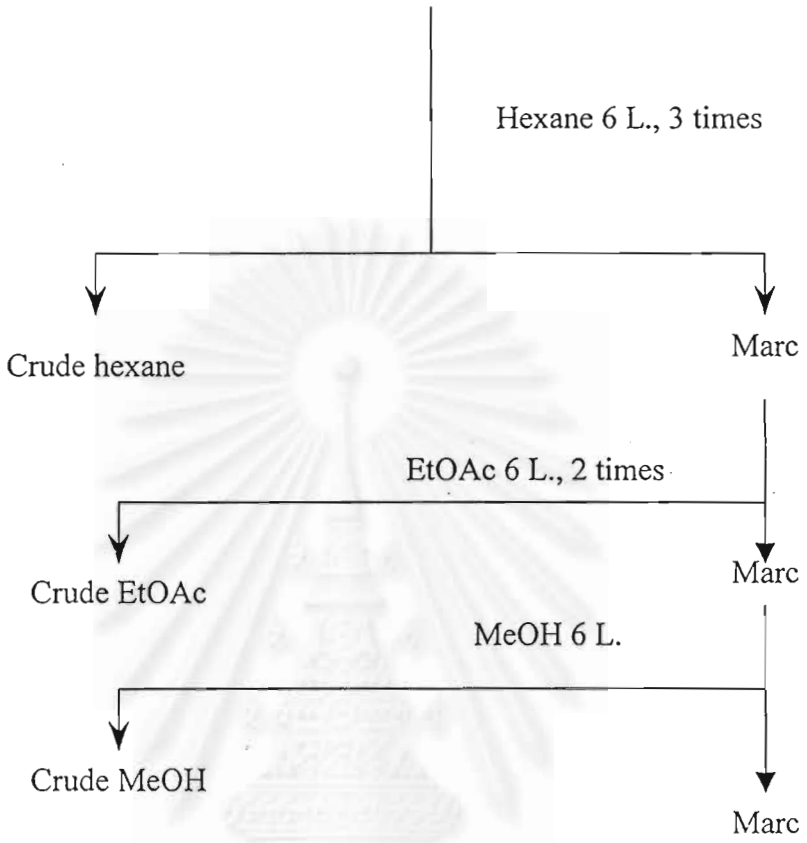
5.) Sephadex LH-20 was used as a stationary phase for column chromatography. The gel filter was dispersed in the eluent and left standing for gel initiation for about 24 hr. before use.

3.3 The plant material

The plant material of *C. oblongifolius* Roxb. was collected from Amphur Dan zai Loei province, Thailand in October 1997. The plant specimen was compared against voucher specimen no. BKF 084729 deposited in the herbarium of the Royal Forest Department of Thailand.

3.4 Separation of extract from *C. oblongifolius* Roxb.

Plant samples were air-dried and milled. The specimen (5.1 kg) was soaked in 6 liters hexane for 3 times at room temperature. The solution was filtered and evaporated by rotary vacuum evaporator to remove the solvent. The residue was re-extracted with 6 liters ethyl acetate for 2 times (solution was colorless) at room temperature. The solvent was evaporated and residue was re-extract with 6 liters methanol. The extraction procedures are shown in Scheme 1.



Scheme 1 Extraction procedure of the stem bark of *C. oblongifolius*

3.5 Biological activity test

3.5.1 Cytotoxicity test

Cytotoxicity test was carried out at the Institute of Biotechnology and Genetic Engineering by Mrs. Songchan Puthong using the following protocol. Bioassay of cytotoxic activity against human tumor cell culture *in vitro* was performed by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) colorimetric method [37,38]. In principle, the viable cell number / well is directly proportional to the production of formazan, which following solubilization, can be measured spectrophotometrically.

The human tumor cell line was harvested from exponential-phase maintenance cultures (T-75 cm^2 flask), counted by trypan blue exclusion, and

dispensed within replicate 96-well culture plates in 100-µl volumes using a repeating pipette. Following a 24-h incubation at 37°C, 5% CO_2, 100% relative humidity,100 µl of culture medium, culture medium containing sample was dispensed within appropriate wells (control group, N = 6; each sample treatment group, N = 3). Peripheral wells of each plate (lacking cells) were utilized for sample blank (N = 2) and medium / tetrazolium reagent blank (N = 6) "background" determinations. Culture plates were then incubated for 4 days prior to the additions of tetrazolium reagent. MTT stock solution was prepared as follows: 5 mg MTT / ml PBS was sterile and filtered with 0.45 -µm filtered units. MTT working solution was prepared just prior to culture application by diluting MTT stock solution 1 : 5 (v/v) in prewarmed standard culture medium. MTT working solution (50 µl) was added to each culture well resulting in 50 µg MTT/ 250 µl total medium volume) and cultures were incubated at 37°C for 4 to 24 h depending upon individual cell line requirements. Following incubation cell monolayers and formazan were inspected microscopically: Culture plates containing suspension lines or any detached cells were centrifuged at low speed for 5 min. All 10-20 µl of culture medium supernatant was removed from wells by slow aspiration through a blunt 18-guage needle and replaced with 150 µl of DMSO using a pipette. Following through formazan solubilization, the absorbance of each well was measured using a microculture plate reader at 540 nm (single wavelength, calibration factor = 1.00)

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

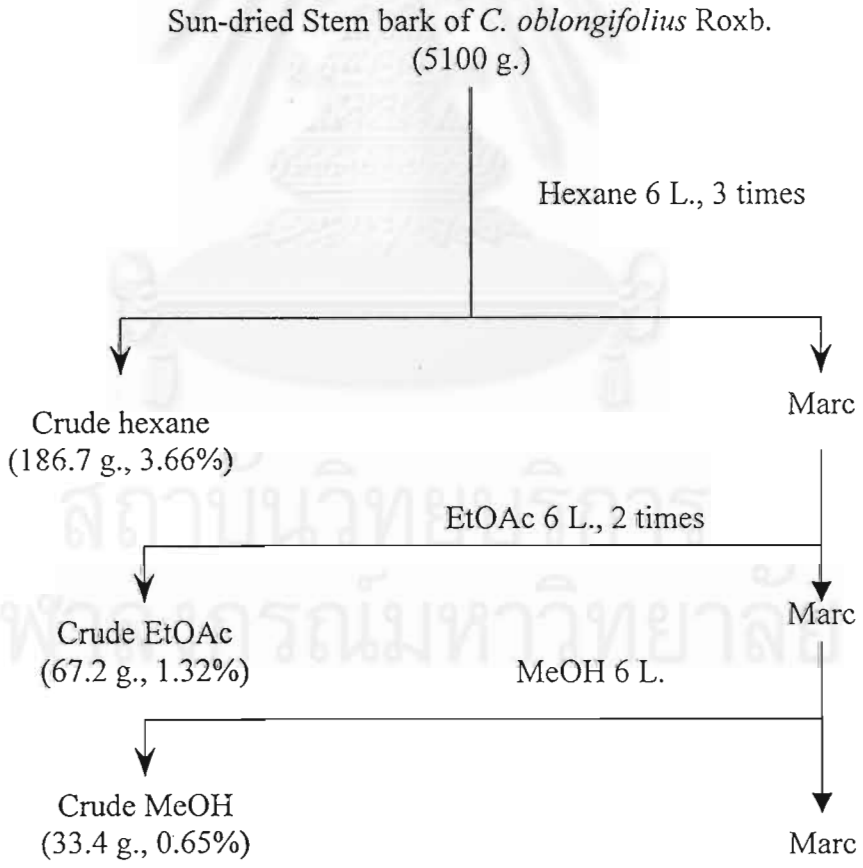
Samples were also tested for cytotoxic activity towards 6 cell lines, which contain HS27 (fibroblast), HEP-G2 (hepatoma), SW 620 (colon), Chago (lung), Kato-3 (gastric) and BT 474 (breast) following the experimental method of bioassay of cytotoxic activity.

CHAPTER IV

RESULTS AND DISCUSSION

4.1 The Results of extraction process

Plant samples were air-dried and milled (5.1 kg.). The specimen was soaked in 6 liters hexane for 3 times at room temperature. The solution was filtered and evaporated by rotary vacuum evaporator to remove the solvent. The extract was obtained as a yellowish brown oil (186.7g., 3.66%). The residue was re-extracted with 6 liters ethyl acetate for 2 times (solution was colorless) at room temperature. After removing solvent, crude ethyl acetate extract (67.2 g., 1.32%). The residue was re-extract with 6 liter methanol, filtered and evaporated, to give methanol crude extract (33.4 g., 0.65%). The extraction procedures are shown in Scheme 2.



Scheme 2 Results of extraction of the stem bark of *C. oblongifolius* Roxb.

4.2 The Results of Separation

4.2.1 Separation of hexane crude extract

The hexane extract (50 g.) was subjected to column chromatography (silica gel, 700 g.) using eluents of increasing polarity from hexane to chloroform. The results from the separation of hexane crude extract were tabulated in Table 2.

Table 2 The results form separation of hexane crude extract

Eluents	Fraction No.	Appearance	Weight (g.)
100% Hexane	1	Colorless liquid	6.24
100% Hexane	2 – 4	Yellow liquid	1.13
10% $CHCl_3$ in Hexane	5 – 13	Yellow viscous liquid	2.71
20% $CHCl_3$ in Hexane	14 – 24	Yellow viscous liquid	4.62
30% $CHCl_3$ in Hexane	25 – 36	Yellow viscous liquid (Containing compound 1)	11.32
50% $CHCl_3$ in Hexane	37 –42	Yellow viscous liquid (Containing mixture 2)	5.26
70% $CHCl_3$ in Hexane	43 – 48	Yellow viscous liquid	2.03
100% $CHCl_3$	49 – 55	Brown viscous liquid	0.74
1% MeOH in $CHCl_3$	56 – 60	Brown viscous liquid	0.92
5% MeOH in $CHCl_3$	61 – 65	Dark brown gummy	2.07
10% MeOH in $CHCl_3$	66 – 70	Dark brown gummy	2.39
20% MeOH in $CHCl_3$	71 – 74	Dark brown gummy	4.36

The fraction-containing compound 1 was subjected to column chromatography (silica gel, 80 g.) using eluents of 10% EtOAc/Hexane to give compound 1 (2.1g, 0.15% dry wt.) as semi-solid. The next fraction was further purified by recolumn using eluents of 10% EtOAc/Hexane, to obtain white needle crystal of mixture 2 (0.56 g., 0.04% dry wt.).

4.2.2 Separation of ethyl acetate crude extract

The extract was subjected to column chromatography (silica gel, 1000 g.) using eluents of increasing polarity from hexane to chloroform. The collected fractions gave TLC pattern and NMR spectra similar to those obtained from hexane crude extract. The results from the separation of crude ethyl acetate extract were tabulated in table 3.

Table 3 The results form separation of ethyl acetate crude extract

Eluents	Fraction No.	Appearance	Weight (g.)
10% $CHCl_3$ in Hexane	1 – 5	Yellow viscous liquid	3.21
20% $CHCl_3$ in Hexane	6 – 11	Yellow viscous liquid	4.77
30% $CHCl_3$ in Hexane	12 – 19	Yellow viscous liquid (Containing compound 1)	13.26
50% $CHCl_3$ in Hexane	20 – 23	Yellow viscous liquid (Containing mixture 2)	6.10
70% $CHCl_3$ in Hexane	24 – 28	Yellow viscous liquid	4.95
100% $CHCl_3$	29 – 32	Brown viscous liquid	5.03
1% MeOH in $CHCl_3$	33 – 37	Brown viscous liquid	6.22
5% MeOH in $CHCl_3$	38 – 41	Dark brown gummy	6.51
10% MeOH in $CHCl_3$	42 – 47	Dark brown gummy	4.86
20% MeOH in $CHCl_3$	48 – 55	Dark brown gummy	1.94

4.2.3 Separation of methanol crude extract

The methanol crude extract (33.4 g., 0.65%) was chromatographed on Sephadex LH-20 and eludes with $CHCl_3$: MeOH (1:1) and 25 ml of each fraction was collected, then analyzed by TLC. Similar fractions were combined found that Sephadex LH-20 did not separate methanol crude extract because it had high polarity.

4.3 Characterization of isolated compounds

4.3.1 Properties of Compound 1

Compound 1 is a colorless semi-solid, $[\alpha]_D^{20}$: +4.31° ($CHCl_3$, c1.0); Found C, 79.42; H, 9.93. $C_{20}H_{30}O_2$ requires: C 79.42; H, 10.0%; UV ($CHCl_3$)) λ_{max} (ε) : 241.5 (1971); IR (film) ν_{max} = 3078, 2930, 2863, 1706, 1655, 1634, 1450, 1414 cm^{-1}.

EI-MS *m/z* (%): 302[M^+], 287(45), 259(55), 201(87), 145(72), 145(86), 91 (100), 41(78).

^{1}H-NMR ($CDCl_3$, 500 MHz): δ = 6.00 (ddd, 16.18, 10.38, 8.85 Hz, 1 H,H-15), 5.02(m, 1 H, H-16), 5.00(s, 1 H, H-16), 4.85(br.s, 1 H, H-18), 4.65(br.s, 2 H, H-17),4.56(br.s, 1 H, H-17), 2.82(dd, 8.85, 4.58 Hz, 1 H, H-14), 2.40(ddd, 10.68, 7.02, 7.02 Hz, 1 H, H-2), 2.30(ddd, 10.68, 7.02, 7.02 Hz, 1 H, H-2), 2.17(m, 2 H, H-12), 1.95(dd, 12.82, 2.75 Hz, 1 H, H-5), 1.73(s, 3 H, H-19), 1.72(m, 2 H, H-11), 1.70(m, 1 H, H-6), 1.64(m, 2 H, H-1), 1.56(dddd,16.17, 12.51, 4.27, 4.27, 1 H, H-8), 1.42(m, 1 H, H-7), 1.26(m, 1 H, H-9), 1.24(m, 1 H, H-7), 0.82(s, 3 H, H-20).

^{13}C-NMR. ($CDCl_3$, 125 MHz): δ = 180.8(s, C-3), 151.8(s, C-13), 147.4(s, C-4), 137.3(d, C-15), 116.1(t, C-16), 113.6(t, C-18), 106.7(t, C-17), 54.6(d, C-14), 50.7 (d, C-5), 41.0(d, C-9), 40.3(d, C-8), 38.9(s, C-10), 32.1(t, C-1), 31.4(t, C-7), 31.3(t, C-12), 28.1(t, C-2), 27.5(t, C-6), 27.3(s, C-11), 23.8(q, C-19), 16.7(q, C-20).

4.3.2 Properties of Mixture 2

Mixture 2 was bright white needle like crystals, m.p. 139-142 °C. ; IR(KBr) ν_{max} = 3440, 2937, 2868, 1641, 1464, 1381, 1059, 802 cm^{-1}. ; EI-MS *m/z* (%): 414 [M^+, $C_{29}H_{50}O$], 412[M^+, $C_{29}H_{48}O$], 396(25), 329(29), 273(35), 271(36), 255(68), 213 (57), 83(100).

4.4 Structure elucidation

4.4.1 Structure elucidation of compound 1

The IR spectrum of compound 1 is shown in Fig. 8 and the absorption peaks were assigned as in Table 4. Its IR spectrum showed important absorption bands at 2400 – 3500 cm^{-1} (O-H stretching vibration of carboxylic acid), 2930 and 2863 cm^{-1} (C-H stretching vibration), 1706 cm^{-1} (C=O stretching vibration of carbonyl group), and 1655 and 1634 cm^{-1} (C=C stretching vibration of olefin).

Table 4. The IR absorption band assignment of compound 1

Wave number (cm^{-1})	Intensity	Tentative assignment
2400 – 3500	Broad	O-H stretching vibration of carboxylic acid
2930 , 2863	Medium	C-H stretching vibration of CH_3- , -CH_2-
1706	Strong	C=O stretching vibration of carbonyl group
1655, 1634	Weak	C=C stretching vibration of olefin

The ^{1}H-NMR spectrum (Fig. 9, Table 5) of compound 1 indicated that it possesses seven olefinic protons (δ 4.56,4.65,4.65,4.85,5.00,5.02, and 6.00 ppm.) and two methyl group (δ 0.82 and 1.73 ppm.).

The ^{13}C-NMR, DEPT-90, and DEPT-135 spectrum (Fig. 10, 11 Table 5) showed 20 signals. Six signals of olefinic carbons appeared at δ 106.7, 113.6, 116.1, 137.3, 147.4, and 151.8 ppm. The signal at 180.8 ppm should be the carboxylic group. There were thirteen sp^3 carbon signals at δ 16.7(q), 23.8(q), 27.3(t), 27.5(t), 28.1(t), 31.3(t), 31.4(t), 32.1(t), 38.9(s), 40.3(d), 41.0(d), 50.7(d), and 54.6(d) ppm.

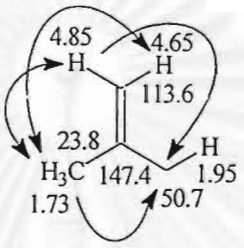
Compound 1 showed a molecular ion with m/z 302 ($C_{20}H_{30}O_2$) (Fig. 16) that indicated DBE of 6. The information from 2D-NMR technique, HMQC correlations (Fig. 12), COSY correlations (Fig. 13), NOESY correlations (Fig. 14) and HMBC correlations (Fig. 15) were used to assist the interpretion the structure of compound 1.

Two-dimensional NMR techniques were used to assist the structure assignment. The protons directly attached to carbon in compound 1 were assigned by HMQC spectra.

Table 5. ^{1}H-, ^{13}C-NMR and 2D long range ^{1}H-^{13}C correlation in the HMBC spectrum data of compound 1

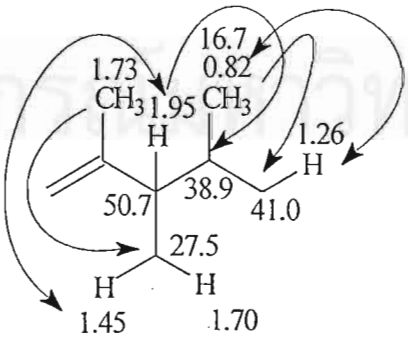
Position	^{1}H-NMR	^{13}NMR	Correlated hydrogen
1	1.64 (2H, m)	32.1 t	H-2, H-20
2	1.64 (2H, m)	28.1 t	H-1
3	-	180.8 s	H-2
4	-	147.4 s	H-5, H-19, H-20
5	1.95 (1H, dd, J = 12.82, 2.75 Hz)	50.7 d	H-1, H-19, H-20
6	1.45 (1H, m)	27.5 t	H-7, H-20
	1.70 (1H, m)		
7	1.24 (1H, m)	31.4 t	H-18
	1.42 (1H, m)		
8	1.56 (1H, dddd, J = 16.7, 12.51, 4.27, 4.27 Hz)	40.3 d	H-14
9	1.26 (1H, m)	41.0 d	H-1, H-8, H-20
10	-	38.9 s	H-1, H-8, H-20
11	1.72 (2H, m)	27.3 t	H-9
12	2.17 (2H, m)	31.3 t	H-17
13	-	151.8 s	H-12, H-14
14	2.82 (1H, dd, J = 8.85, 4.58 Hz)	54.6 d	H-17
15	6.00 (1H, dddd, J = 16.7, 12.51, 4.27, 4.27 Hz)	137.3 d	H-14, H-16
16	5.00 (1H, bs, s)	116.1 t	H-14
	5.02 (1H, m)		
17	4.56 (1H, m)	106.7 t	H-14
	4.65 (1H, m)		
18	4.65 (1H, m)	113.6 t	H-19, H-20
	4.85 (1H, bs, s)		
19	1.73 (3H, s)	23.8 q	H-5, H-18
20	0.82 (3H, s)	16.7 q	H-1, H-5

Crucial long-rang $^{1}H - {}^{13}C$ correlations were obtained by HMBC correlations (Fig. 5), the methyl proton at 1.73 ppm was coupled with carbon at 50.7 and 113.6 ppm and the proton at 4.85 was coupled with carbon at 50.7 ppm too. The COSY spectrum (Fig. 6) showed that the proton at 4.65 ppm was coupled with the proton at 1.73 ppm. The NOESY spectrum (Fig. 7) showed that the proton at 1.73 ppm was coupled with the proton at 4.85 ppm. Therefore, partial structure of compound 1 was obtained as shown in Scheme 3.



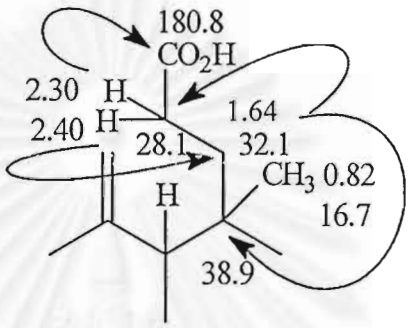
Scheme 3

The HMBC spectrum showed that the proton at 1.73 ppm was coupled with the carbon at 27.5 ppm, the proton at 1.95 ppm was coupled with carbon at 38.9 and 41.0 ppm, and the methyl proton (0.82 ppm) was couple with carbon at 50.7 and 41.0 ppm. The COSY spectrum showed the proton at 1.95 ppm was coupled with proton at 1.45 ppm and the proton at 0.82 ppm was coupled with proton at 1.26 and 1.45 ppm. The NOESY spectrum showed the proton at 1.95 ppm was coupled with the proton at 0.82 and 1.45 ppm. Therefore, partial structure of compound 1 was obtained as shown in Scheme 4.



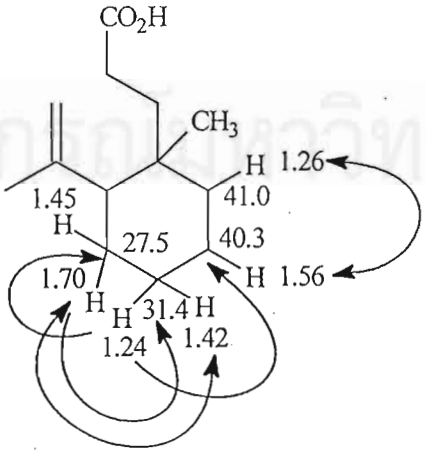
Scheme 4

The HMBC spectrum showed that the proton at 1.64 ppm was coupled with the carbon at 38.9 and 28.1 ppm, the proton at 2.30 ppm was coupled with carbon at 180.8 ppm, and the proton at 2.40 ppm was couple with carbon at 32.1 ppm. The COSY spectrum showed the proton at 1.64 ppm was coupled with proton at 1.95, 2.30, and 2.40 ppm. The NOESY spectrum showed the proton at 0.82 ppm was coupled with the proton at 1.64 ppm (Scheme 5).



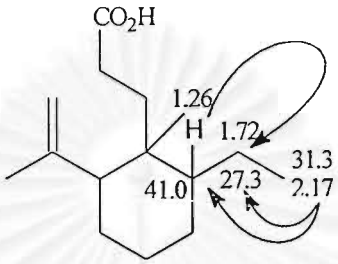
Scheme 5

The HMBC spectrum showed that the proton at 1.24 ppm was coupled with the carbon at 27.5 and 40.3 ppm, the proton at 1.70 ppm was coupled with carbon at 31.4 ppm. The COSY spectrum showed the proton at 1.26 ppm was coupled with proton at 1.56 ppm and the proton at 1.42 was coupled with proton at 1.70 ppm NOESY spectrum showed the proton at 1.24 ppm was coupled with the proton at 1.45 ppm (Scheme 6).



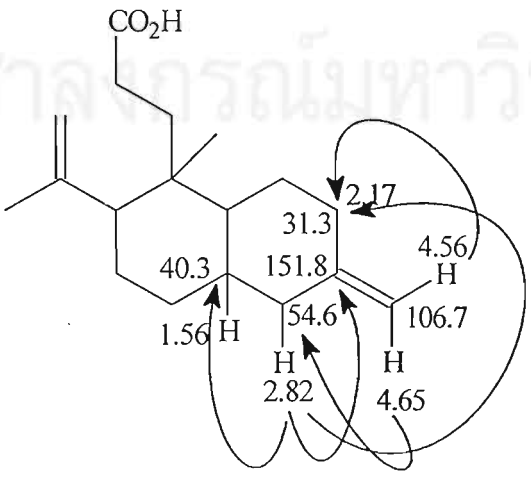
Scheme 6

The proton at 1.26 ppm was coupled with carbon at 27.3 ppm, the proton at 1.72 ppm was coupled with carbon at 41.0 ppm, and the proton at 2.17 ppm was coupled with carbon at 27.3 and 41.0 ppm according to HMBC spectrum. The proton at 2.17 ppm was coupled with proton at 1.26 and 1.72 ppm according to NOESY spectrum (Scheme 7).



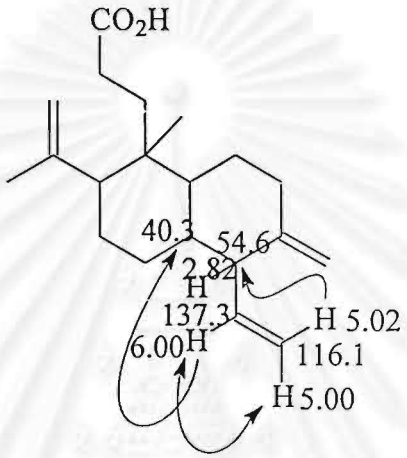
Scheme 7

The proton at 2.17 ppm was coupled with carbon at 151.8 ppm, the proton at 2.82 ppm was coupled with carbon at 31.3, 40.3 and 151.8 ppm, the proton at 4.56 ppm was coupled with carbon at 31.3 ppm and the proton at 4.65 ppm was coupled with carbon at 54.6 ppm according to HMBC spectrum. The proton at 2.17 ppm was coupled with proton at 4.56 ppm, and the proton at 1.56 ppm was coupled with proton at 2.82 ppm according to COSY spectrum. The proton at 1.56 ppm was coupled with proton at 2.82 and 4.65 ppm, and the proton at 2.17 ppm was coupled with proton at 4.56 ppm according to NOESY spectrum (Scheme 8).



Scheme 8

The proton at 2.82 ppm was coupled with carbon at 137.3 ppm, the proton at 5.02 ppm was coupled with carbon at 54.6 ppm, and the proton at 6.00 ppm was coupled with carbon at 40.3 ppm according to HMBC spectrum. The proton at 2.82 ppm was coupled with proton at 5.02 and 6.00 ppm, and the proton at 5.00 ppm was coupled with proton at 6.00 ppm according to COSY spectrum. The NOESY spectrum showed that proton at 5.00 ppm was coupled with proton at 6.00 ppm (Scheme 9).



Scheme9

Thus, the structure of compound 1 was proposed to be cleistantha-4(18), 13 (17), 15-triene-3-oic acid as shown in Figure 4. The long range C – H correlations by HMBC, COSY correlations and NOESY correlation spectrum were summarized in figure 5, 6 and 7 respectively.

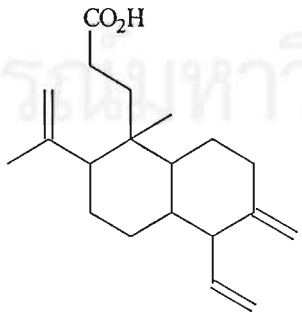
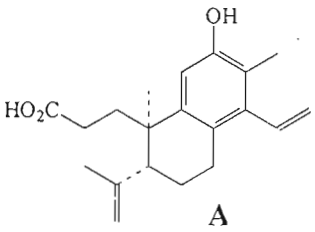


Figure 4

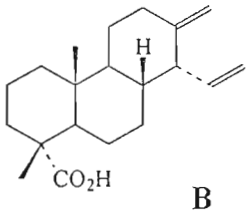
The structure scarch in the literature revealed that compound 1 was a novel compound. The most closely related structures were 12-hydroxy-3, 4-*seco*-4, 8, 11, 13, 15-cleistanthapentaen-3-oic acid (3, 4-*seco*-sanderianol) (A) [20] and auricularic acid (B) [23] because both compounds have partial structure 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 4.

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

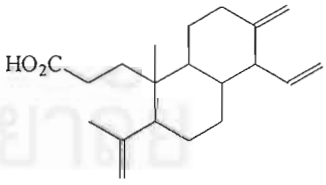
Position	Chemical shift of ^{13}C-NMR		
	Compound 1	A	B
1	32.1 t	34.8 t	38.0 t
2	28.1 t	28.6 t	19.5 t
3	180.8 s	175.3 s	39.7 t
4	147.4 s	141.3 s	43.9 s
5	50.7 d	46.6 d	56.4 d
6	27.5 t	24.9 t	23.2 t
7	31.4 t	29.6 t	32.7 t
8	40.3 d	127.0 s	40.9 d
9	41.0 d	139.1 s	48.9 d
10	38.9 s	41.2 s	37.7 s
11	27.3 t	111.6 d	27.4 t
12	31.3 t	152.6 s	31.5 t
13	151.8 s	120.0 s	152.2 s
14	54.6 d	146.9 s	54.7 d
15	137.3 d	135.5 d	137.7 d
16	116.1 t	119.6 d	115.7 t
17	106.7 t	12.9 q	106.5 t
18	113.6 t	114.3 t	16.6 q
19	23.8 q	27.9 q	184.0 s
20	16.7 q	51.7 q	14.2 q



A



B



Compound 1

A = 3, 4-*Seco*-sanderianol

B = Auricularic acid

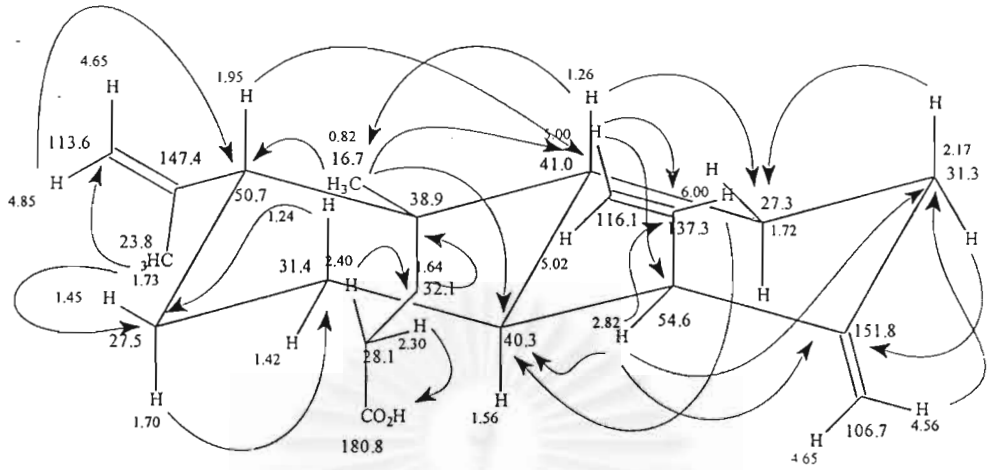


Fig. 5 The HMBC correlation of compound 1

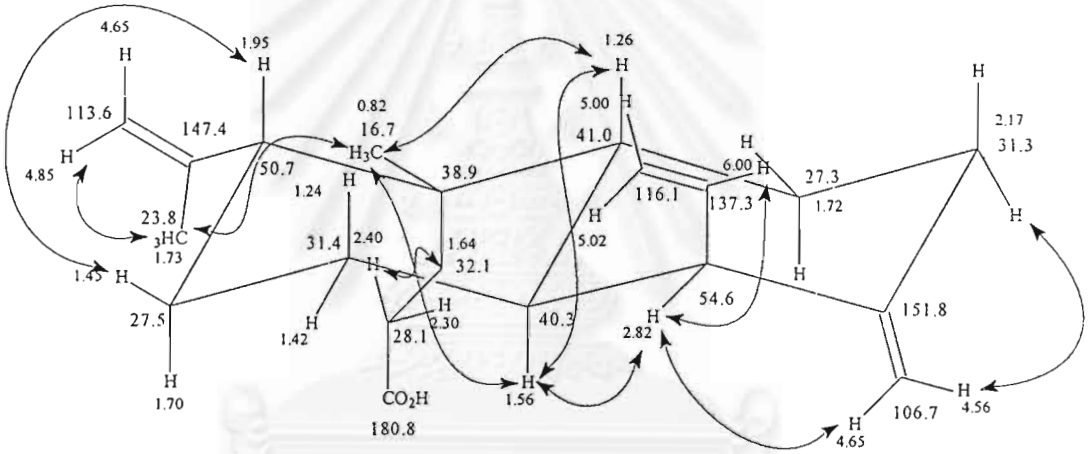


Fig. 6 The COSY correlation of compound 1

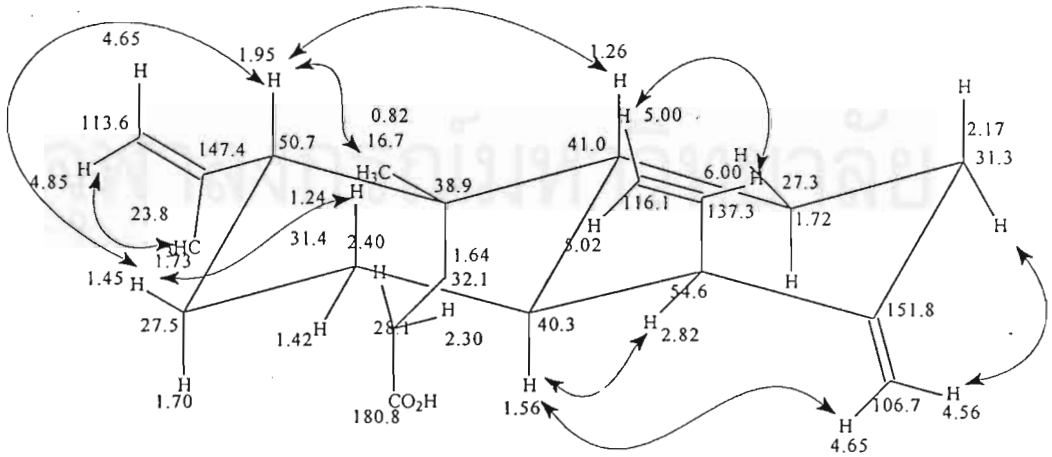


Fig. 7 The NOESY correlation of compound 1

Comparison of ^{13}C-NMR spectrum of compound 1 and 3, 4-*seco*-sanderianol suggested that the compound 1 contain the opening skeleton cleistanthane like 3, 4-seco-sanderianol. Comparison with the spectral data of compound 1 and auricularic acid suggested that the stereochemistry of the methyl (C20) at chiral center (C10) is different and this confirmed by NOESY correlations (Fig. 7). If the stereochemistry of methyl (C20) had similar structure to auricularic acid, NOESY correlations must showed coupling of the proton at H8 (1.56 ppm) with the protons of methyl (H20), but no such coupling was observed. It was very interesting to note that compound 1 was the second example known so far of cleistanthane with opened ring A. Moreover, it was the second report of cleistanthane in genus Croton. The discovery of cleistanthane skeleton in *C. oblongifolius* Roxb. made this plant a valuable source of different diterpenoid compounds. In addition, the biogenetic pathway shown before was almost completed.

4.4.2 Structure elucidation of mixture 2

Mixture 2 was bright white needle like crystals, m.p. 139-142 °C. The R_f value was 0.45 using 50% chloroform in hexane.

The IR spectrum of mixture 2 is shown in Fig. 17 and the absorption peaks were assigned as in Table 7. Its IR spectrum showed important absorption bands at 3200 – 3700 cm^{-1} (O-H stretching vibration of alcohol), 2937 and 2868 cm^{-1} (C-H stretching vibration), 1670 - 1630 cm^{-1} (C=C stretching vibration of olefin), 1461 and 1381 cm^{-1} (C-H bending vibration of CH_3- , -CH_2-), 1059 cm^{-1} (C-O stretching vibration), 802 cm^{-1} (C-H out of plane bending vibration).

Table 7. The IR absorption band assignment of mixture 2

Wave number (cm^{-1})	Intensity	Tentative assignment
3200 – 3700	Broad	O-H stretching vibration of alcohol
2937 , 2868	Strong	C-H stretching vibration of CH_3- , -CH_2-
1641	Weak	C=C stretching vibration of olefin
1461 , 1381	Medium	C-H bending vibration of CH_3- , -CH_2-
1059	Medium	C-O stretching vibration
802	Medium	C-H out of plane bending vibration

The ^{1}H-NMR spectrum (Fig. 18) of mixture 2 showed the signals at 0.68-2.30 ppm. which were the signals of angular methyl, methylene and methine groups of steroids. The proton of hydroxyl group was shown at 3.50 ppm. The protons at 5.09, 5.32 ppm. were the signals of vinyl protons.

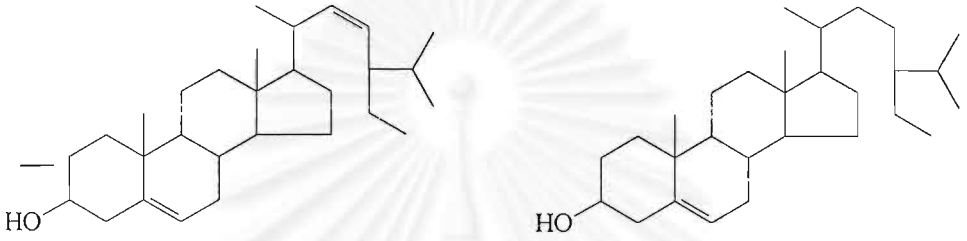
The ^{13}C NMR spectrum (Fig. 19) exhibited the olefinic carbon signals at 121.63, 129.20, 138.23 and 140.68 ppm. The carbon signal at 71.7 ppm exhibited the C-OH of steroid. According to the information of mixture 2, it was suggested that mixture 2 could be steroid. To confirm the structure, ^{13}C NMR spectrum of a mixture 2 was compared to stigmasterol and β–sitosterol as shown in Table 8 [35].

Table 8 ^{13}C-NMR chemical shift of β-Sitosterol, Stigmasterol and Mixture 2

Position	Chemical shift (ppm.)		
	β-Sitosterol	Stigmasterol	Mixture 2
1	37.1	37.4	37.3
2	31.8	31.7	31.6
3	71.9	71.8	71.7
4	42.4	42.4	42.3
5	140.9	140.0	140.8
6	121.8	121.7	121.6
—7	32.0	31.9	31.9
8	32.0	31.9	31.9
9	50.3	50.3	50.2
10	36.6	36.6	36.5
11	21.1	21.1	21.1
12	39.9	39.8	39.7
13	42.4	42.4	42.3
14	56.8	57.0	56.8
15	24.3	24.4	24.3
16	28.2	28.9	28.2 , 28.9
17	56.2	56.0	56.0
18	11.9	12.2	11.8, 12.2
19	19.4	19.4	19.4
20	36.2	40.5	36.2 , 40.4
21	19.1	21.1	19.0 , 21.1
22	34.0	138.4	34.0 , 138.3
23	29.3	129.4	29.2 , 129.3
24	50.3	51.3	50.2
25	26.2	31.9	26.1 , 31.9
26	18.8	19.0	19.0
27	19.8	21.1	19.8 , 21.2
28	23.1	25.4	23.1 , 25.4
29	11.9	12.0	11.8 , 12.0

The EI mass spectrum of mixture 2 (Fig. 20) showed important fragmentation ion peaks at m/z 414 ($C_{29}H_{50}O$) and 412 ($C_{29}H_{48}O$) and other fragmentation ion peaks at m/z 396, 273, 255 and 213.

From all of the data (IR, NMR and Mass spectrum), it was concluded that mixture 2 was a mixture of stigmasterol ($C_{29}H_{50}O$, MW = 414) and β-sitosterol ($C_{29}H_{48}O$, MW = 412). The structures of these steroids are shown as below.

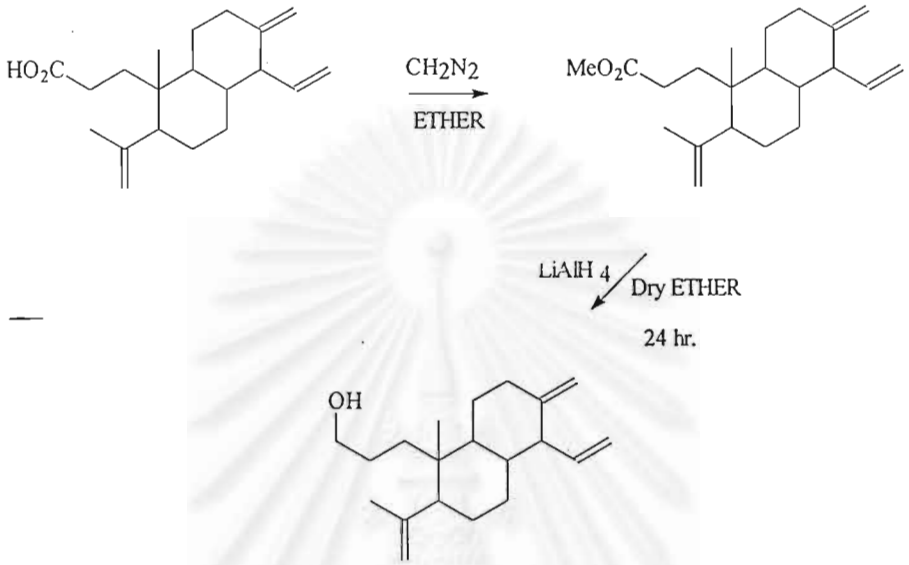


β-Sitosterol Stigmasterol

4.5 Modification of compound 1

4.5.1 Modification functional group

The pathway of modification of compound 1 is shown in scheme 10



Scheme 10 Modification of compound 1

4.5.1.1 Methylation of compound 1

The compound 1 (500 mg) was methylated with diazomethane in diethyl ether to give compound 1a (512 mg, 97.9% yield), as viscous transparent oil, $[\alpha]_D^{20}$: +4.27° ($CHCl_3$, c1.0) ; UV ($CHCl_3$) $\lambda_{max}(\varepsilon)$: 239.8 (1856) ; EA : Found C 79.70%; H,10.16%. Calc. C 79.70%, H 10.19%.

The IR spectrum of compound 1a is shown in Fig.21 and the absorption peaks were assigned as in Table 9. Its IR spectrum showed important absorption bands at 2929, and 2868 cm^{-1} (C-H stretching vibration), 1731 cm^{-1} (C=O stretching vibration of carbonyl group of ester), and 1639, and 1439 cm^{-1} (C=C stretching vibration of olefin), and 1173 cm^{-1} (C-O stretching vibration of ester).

The ^{1}H-NMR spectrum (Fig. 22) of compound 1a indicated that it possesses seven olefinic protons (δ 4.47,4.55,4.55,4.78,4.90,4.95, and 5.90 ppm.), one methyl ester group (δ 3.55 ppm.) and two methyl group (δ 0.82 and 1.73 ppm.).

Table 9 The IR absorption bands assignment of compound 1a

Wave number (cm^{-1})	Intensity	Tentative assignment
2929, 2868	Medium	C-H stretching vibration of CH_3- , -CH_2-
1731	Strong	C=O stretching vibration of carbonyl group
1639, 1439	Weak	C=C stretching vibration of olefin
1173	Medium	C-O stretching vibration of ester

The ^{13}C-NMR spectrum (Fig. 23) showed 21 signals. Six signals of olefinic carbons appeared at δ 106.7, 113.5, 116.0, 137.2, 147.3, and 151.6 ppm. The signal at 174.2 ppm should be the ester group. There were thirteen sp^3 carbon signals at δ 16.6 (q), 23.9(q), 27.3(t), 27.6(t), 27.9(t), 31.3(t), 31.4(t), 32.3(t), 38.9(s), 40.3(d), 40.9(d), 50.5(d), and 54.6(d) ppm. and one methyl ester at δ 51.4 ppm.

Its molecular formula was established as $C_{21}H_{32}O_2$, which was confirmed by observing a molecular ion at *m/z* 316 (Fig. 24).

4.5.1.2 Reduction of compound 1a

The compound 1a (480 mg) was reduced with lithium aluminium hydride in diethyl ether to give compound 1b (402 mg, 92.1% yield), as a viscous transparent oil, $[\alpha]_D^{20}$: +3.12° ($CHCl_3$, c1.0) ; UV ($CHCl_3$) $\lambda_{max}(\varepsilon)$: 2243.4 (1128) ; EA : Found C 83.21%; H,11.17%. Calc. C 83.86%, H 10.56%.

The IR spectrum of compound 1b is shown in Fig 25 and the absorption peaks were assigned as in Table 10. Its IR spectrum showed important absorption bands at 3359 cm^{-1} (O-H stretching vibration of alcohol), 2929 and 2868 cm^{-1} (C-H stretching vibration), 1634, and 1444 cm^{-1} (C=C stretching vibration of olefin), and 1060 cm^{-1} (C-O stretching vibration of primary alcohol).

The ^{1}H-NMR spectrum (Fig. 26) of compound 1a indicated that it possesses seven olefinic protons (δ 4.49,4.58,4.58,4.77,4.85,4.90, and 6.00 ppm.), methylene alcohol (δ 3.50 ppm.) and two methyl group (δ 0.78 and 1.70 ppm.).

Table 10 The IR absorption band assignment of compound 1b

Wave number (cm^{-1})	Intensity	Tentative assignment
3359	Broad	O-H stretching vibration of alcohol
2929, 2868	Strong	C-H stretching vibration of CH_3- , -CH_2-
1634, 1444	Weak	C=C stretching vibration of olefin
1060	Medium	C-O stretching vibration of primary alcohol

The ^{13}C-NMR spectrum (Fig. 27) showed 21 signals. Six signals of olefinic carbons appeared at δ 106.5, 113.1, 115.9, 137.5, 147.9, and 152.0 ppm. There were sp^3 thirteen carbon signals at δ 17.7(q), 23.9(q), 25.8(t), 27.3(t), 27.7(t), 31.4(t), 31.5 (t), 33.4(t), 38.8(s), 40.3(d), 40.9(d), 50.5(d), and 54.7(d) ppm. and one methylene carbon of alcohol at δ 63.2 ppm.

Its molecular formula was established as $C_{20}H_{32}O$, which was confirmed by observing molecular ion at *m/z* 288 (Fig. 28).

4.5.2 Acid catalyzed rearrangement

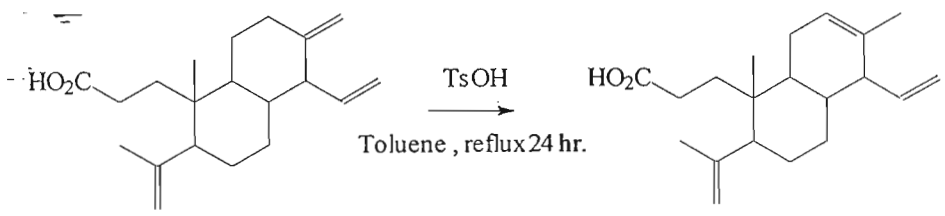
Compound 1 (4.77 g) was modified by acid catalyst reaction in order to obtain other diterpenoids with potential biological activity. The product was a mixture of closely related structures in which compound 3 was isolated in low yield as major product (375mg, 7.86% yield).

The ^{13}C-NMR, DEPPT-90, DEPT-135 (Fig.30,31 Table 12) of compound 3 gave 20 signals. Six signals of olefinic carbon at δ 147.5, 138.1, 134.2, 121.1, 116.1, and 113.6 ppm. The signal at δ 180.8 ppm. should be the carbonyl of carboxylic acid. There were thirteen sp3 carbon signals at δ 51.0(d), 50.2(d), 38.7(s), 36.6(d), 34.9(d), 32.2(t), 31.0(t), 28.2(t), 27.4(t), 24.8(t), 23.8(q), 21.9(q), and 16.4(q) ppm. The comparison of NMR-data of Compound 3 with those of compound 1, suggested that it could be a rearrangement product (scheme 11).

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

Table 11 ^{13}C-NMR chemical shift of Compound 1, Compound 1a and Compound 1b

Position	**Chemical shift (ppm.)**		
	Compound 1	Compound 1a	Compound 1b
1	32.1 t	32.3 t	31.5 t
2	28.1 t	27.9 t	27.7 t
3	180.8 s	174.2 s	63.2 t
4	147.4 s	147.3 s	147.9 s
5	50.7 d	50.5 d	50.5 d
6	27.5 t	27.6 t	27.3 t
7	31.4 t	31.4 t	31.5 t
8	40.3 d	40.3 d	40.3 d
9	41.0 d	40.9 d	40.9 d
10	38.9 s	38.9 s	38.8 s
11	27.3 t	27.3 t	27.3 t
12	31.3 t	31.3 t	31.4 t
13	151.8 s	151.6 s	152.0 s
14	54.6 d	54.6 d	54.7 d
15	137.3 d	137.2 d	137.5 d
16	116.1 t	116.0 t	115.9 t
17	106.7 t	106.7 t	106.5 t
18	113.6 t	113.5 t	113.1 t
19	23.8 q	23.9 q	23.9 q
20	16.7 q	16.6 q	17.1 q
OMe		51.4 q	



Scheme 11 Acid catalyzed rearrangement of compound 1

Table 12 ^{13}C-NMR compound 3 compared with compound 1

Position	Compound 1	Compound 3
1	32.1 (t)	32.2 (t)
2	28.1 (t)	28.2 (t)
3	180.8 (s)	180.8 (s)
4	147.4 (s)	147.5 (s)
5	50.7 (d)	50.2 (d)
6	27.5 (t)	27.4 (t)
7	31.4 (t)	31.0 (t)
8	40.3 (d)	34.9 (d)
9	41.0 (d)	36.6 (d)
10	38.9 (s)	38.7 (s)
11	27.3 (t)	24.8 (t)
12	31.3 (t)	121.1 (d)
13	151.8 (s)	134.2 (s)
14	54.6 (d)	51.0 (d)
15	137.3 (d)	138.1 (d)
16	116.1 (t)	116.1 (t)
17	106.7 (t)	21.9 (q)
18	113.6 (t)	113.6 (t)
19	23.8 (q)	23.8 (q)
20	16.7 (q)	16.4 (q)

4.6 Result of biological activity test

The *in vitro* activity of some compounds from *C. oblongifolius* against 6 cell lines, for example, HS 27 (fibroblast), HEP-G2 (hepatoma), SW620 (colon), CHAGO (lung), KATO-3 (gastric), BT474 (breast) cancer was reported in Table 13.

Table 13 Cytotoxic activity against cell line of some compounds from *Croton oblongifolius* Roxb.

Compound	IC_{50} (μg/ml)					
	HS 27 (fibroblast)	BT474 (breast)	HEP-G2 (hepatoma)	SW620 (colon)	CHAGO (lung)	KATO-3 (gastric)
1	> 10	> 10	10	> 10	> 10	> 10
1a	> 10	> 10	10	9.2	9.7	8.4
1b	7.8	8.3	5.8	4.3	4.9	5.0
3	> 10	10	10	> 10	> 10	10

IC_{50} was the minimum concentration of 50% inhibitory activity.

The results from table 13 showed that, compound 1b markedly exhibited cytotoxicity activity against all cell line in this test, and compound 1a showed activity with SW 620, Chago and Kato-3 but compound 1 not. Therefore, these cleistanthane best active when functional group was alcohol, methyl ester better than carboxylic acid.

CHAPTER V

CONCLUSION

From previous studied, chemical constituents of *Croton oblongifolius* Roxb. in Thailand were different. All diterpenoid compounds of *Croton oblongifolius* Roxb. can be classified into 6 groups as pimarane, isopimarane, clerodane, cembrane, labdane and halimane. From this research, the chemical constituent in the stem bark of *Croton oblongifolius* Roxb. form Amphur Dan Sai, Loei province was found cleistanthane diterpenoids, cleistantha-4, 13(17), 15-triene-3-oic acid (**1**) as a major product (2.10 g, 0.15%). It represented the second report of opened-ring A of cleistanthane skeleton. The derivatives of this compound were synthesized, such as: methyl cleistantha-4, 13(17), 15-triene-3-oate (**1a**), cleistantha-4, 13(17), 15-triene-3-ol (**1b**) and cleistantha-4, 12, 15-triene-3-oic acid (**3**). Mixture of stigmasterol and β-sitosterol (**2**) was also found in crude hexane extract (0.56 g, 0.04%).

All cleistanthanes were tested for cytotoxicity against various human tumor cell lines (BT474 (breast), HEP-G2 (hepatoma), SW620 (colon), CHAGO (lung), and KATO-3 (gastric)). Compound **1** was active against HEP-G2 cell line, *in vitro*, with IC_{50} value of 10.0 μg/ml. Compound **1a** was active against HEP-G2 cell line, SW620 cell line, CHAGO cell line and KATO-3 cell line, *in vitro*, with IC_{50} value of 10.0, 9.2, 9.7 and 8.4 μg/ml, respectively. Compound **1b** was active against BT474 cell line, HEP-G2 cell line, SW620 cell line, CHAGO cell line and KATO-3 cell line, *in vitro*, with IC_{50} value of 8.3, 5.8, 4.3, 4.9 and 5.0 μg/ml, respectively. Compound **3** was active against BT474 cell line, HEP-G2 cell line and KATO-3 cell line, *in vitro*, with IC_{50} value of 10.0, 10.0 and 10.0 μg/ml.

Suggestion for future work

Complete identification of diterpenoids from various sources of *C. oblongifolius* Roxb in Thailand should be continued in order to identify potential sources of various interesting chemicals.

Structure modification as well as the chemistry of cleistanthane should be explored because chemistry of cleistanthane is not so well known.

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APPENDIX

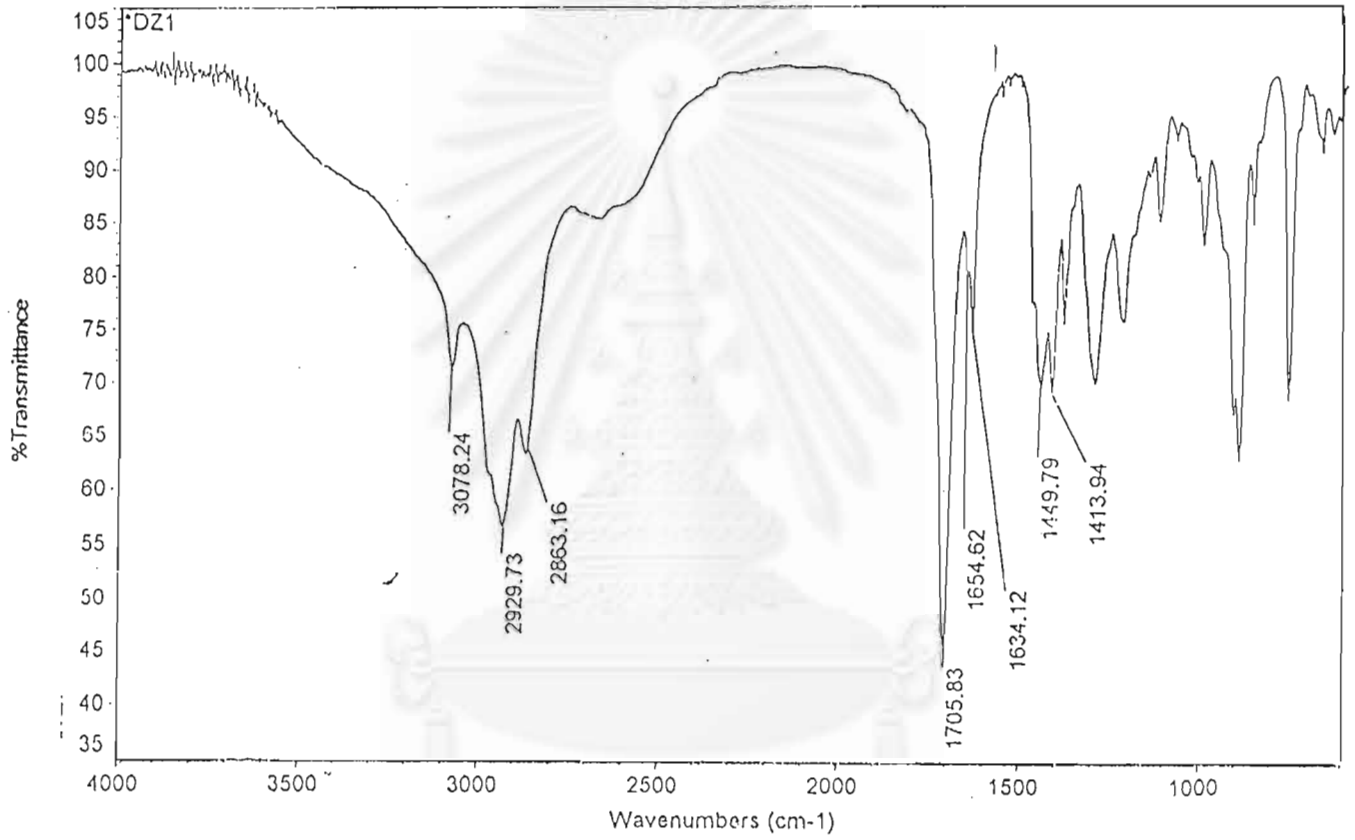

Figure 8 The IR sectrum of compound 1

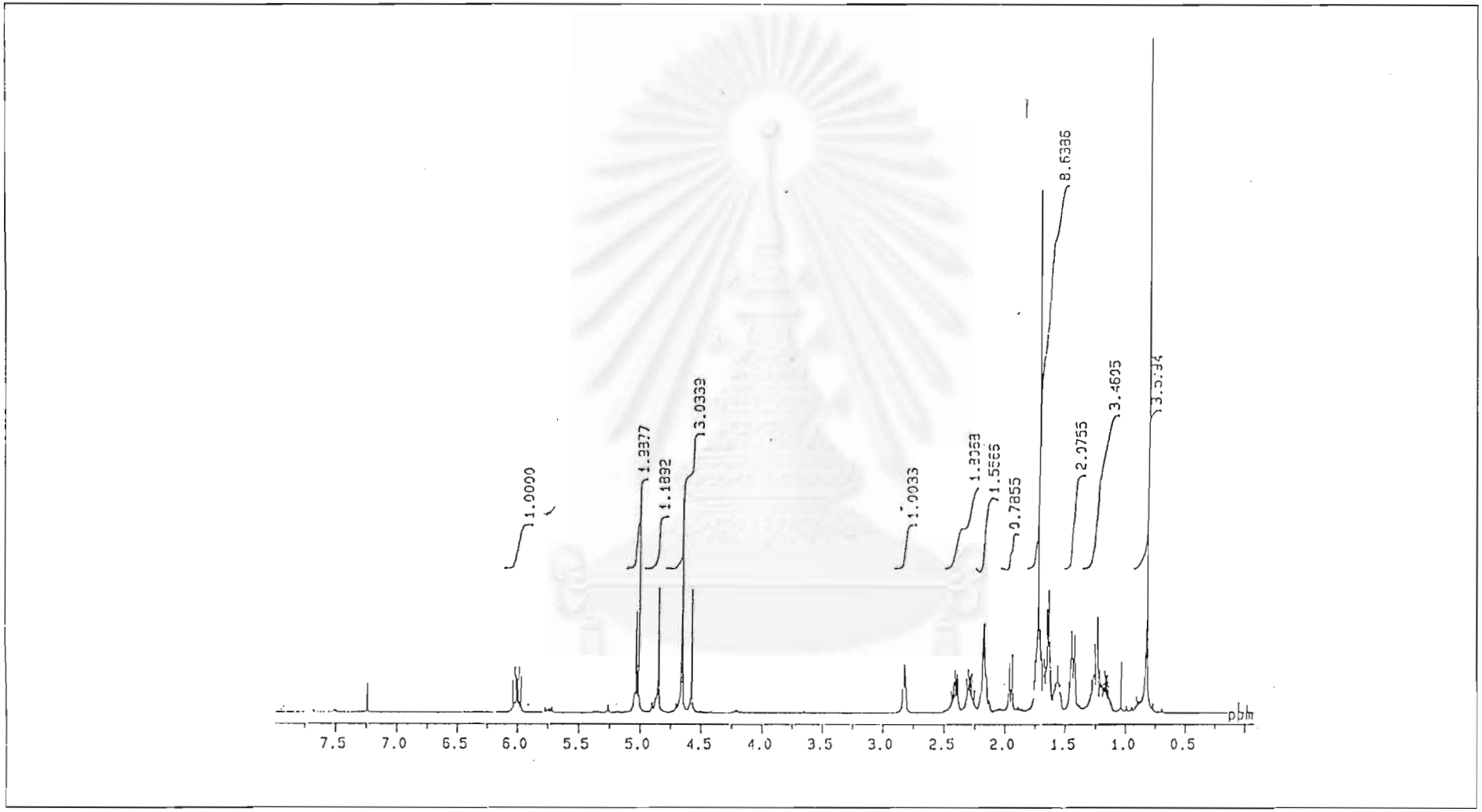

Figure 9 The ^{1}H-NMR setrum of compound 1

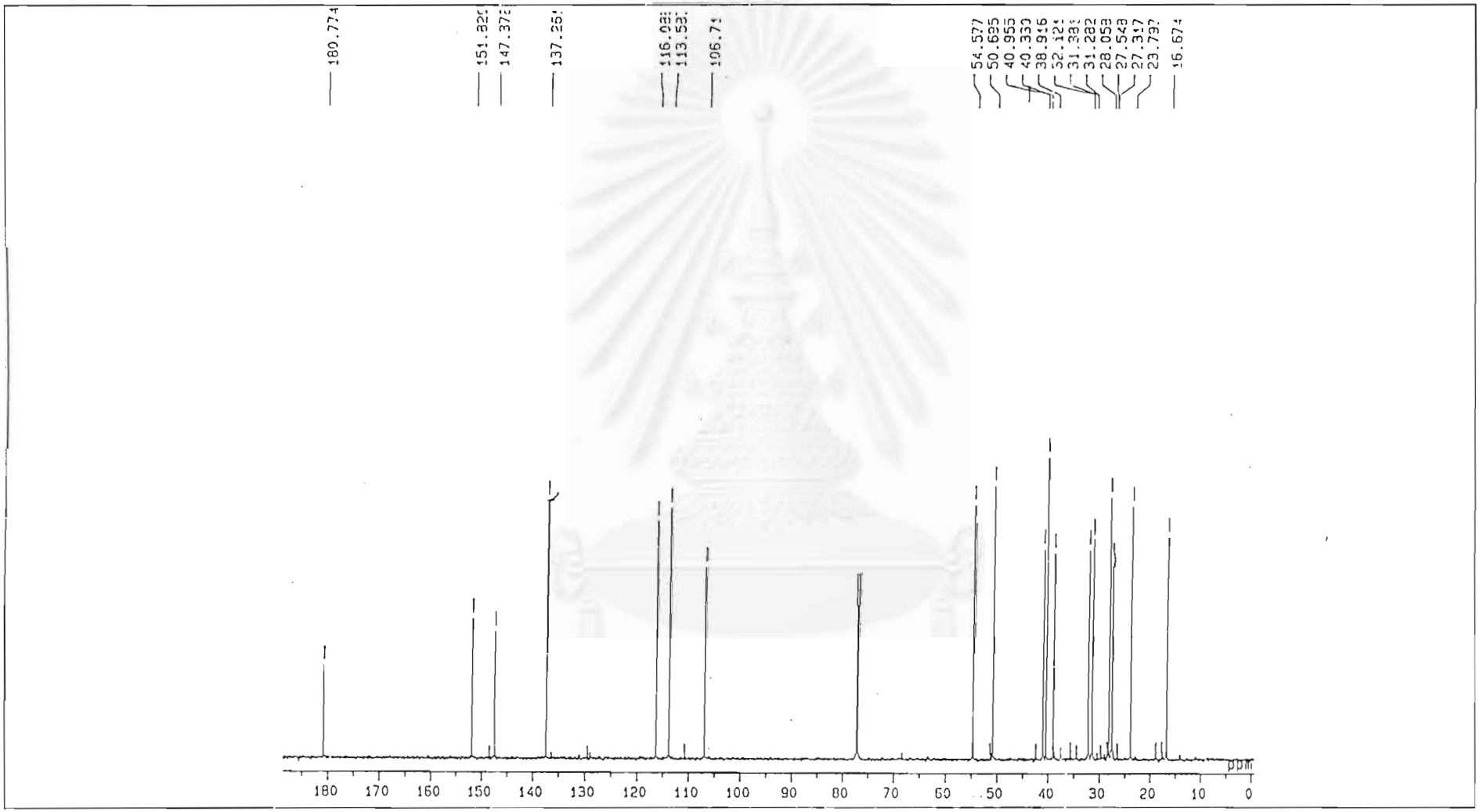

Figure 10 The ^{13}C-NMR setrum of compound 1

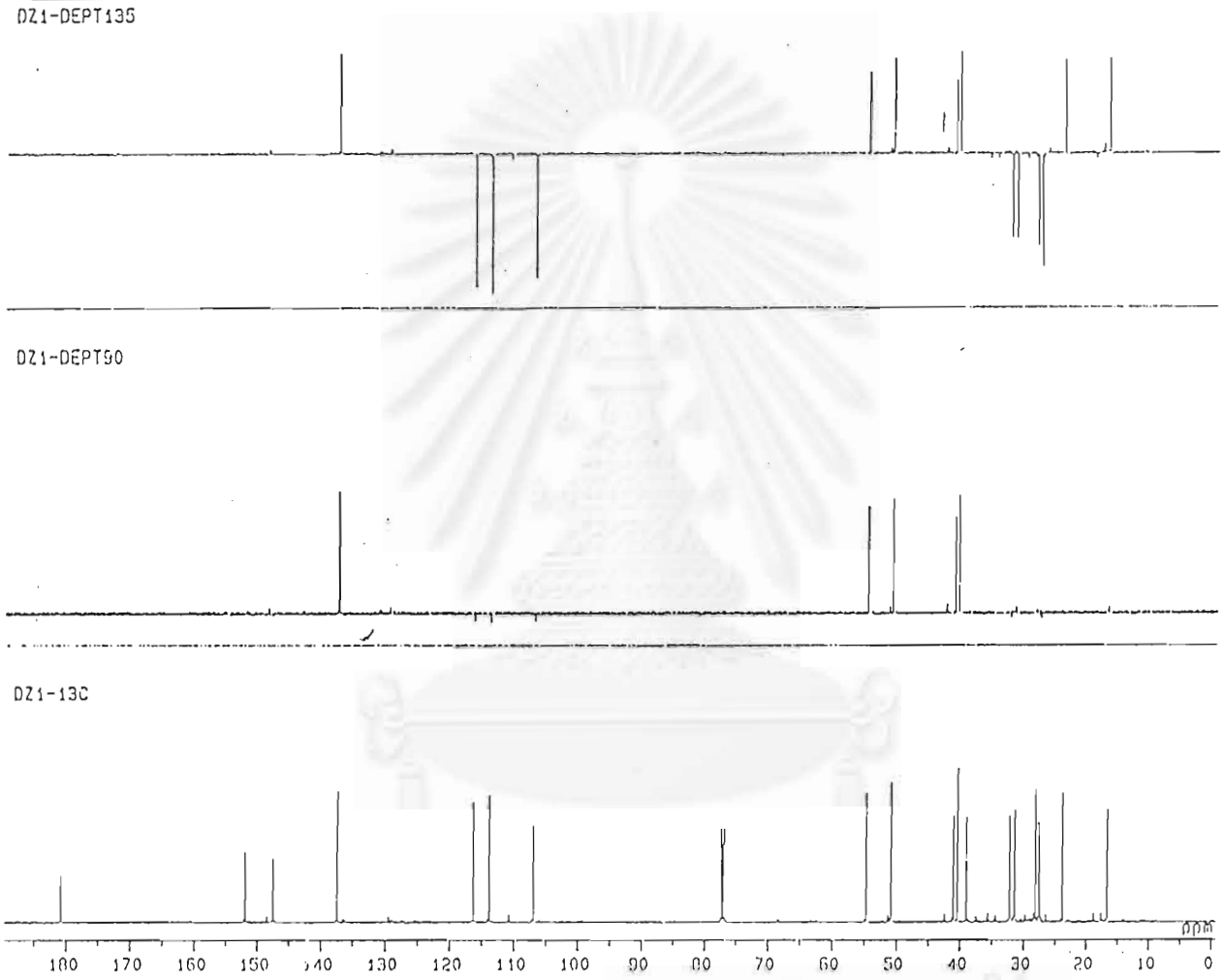

Figure 11 The DEPT-90 and DET-135, ^{13}C-NMR sectrum of compound 1

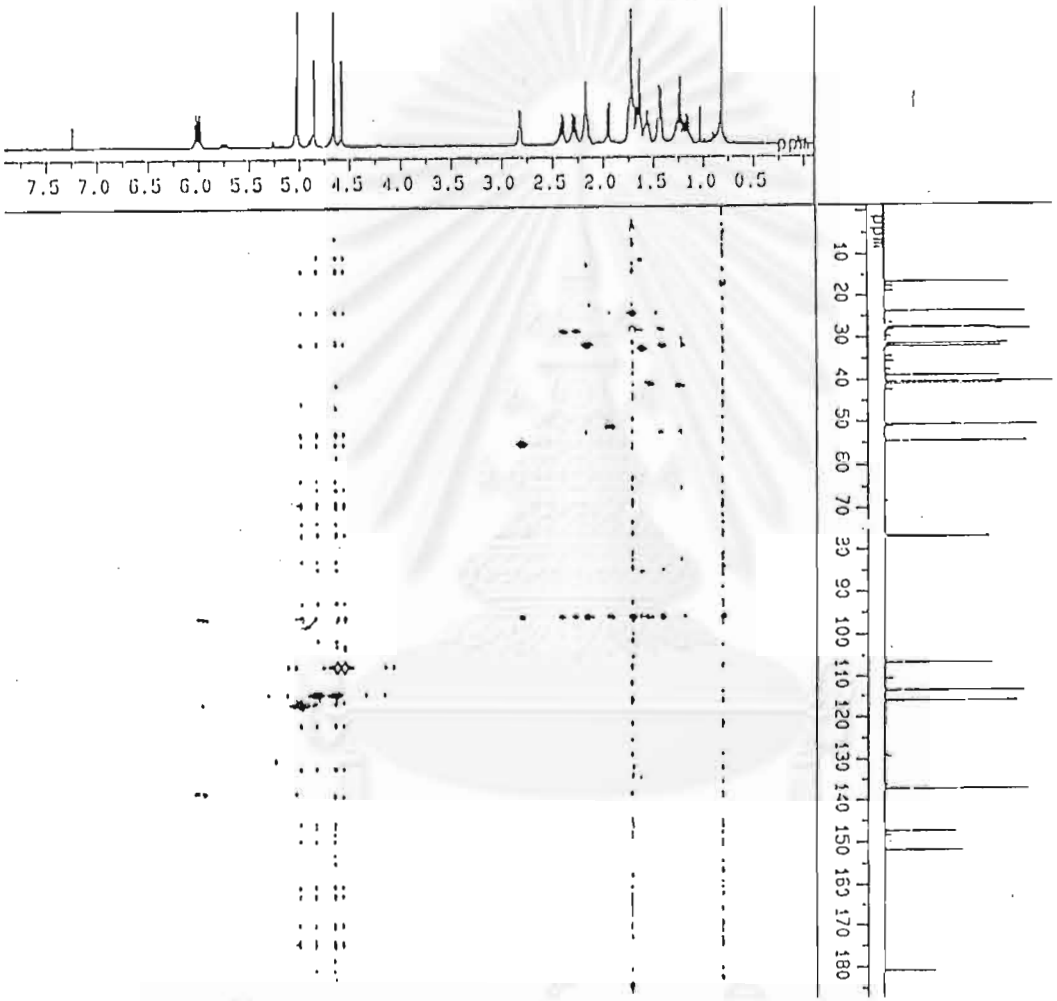

Figure 12 The HMQC-NMR sectrum of comound 1

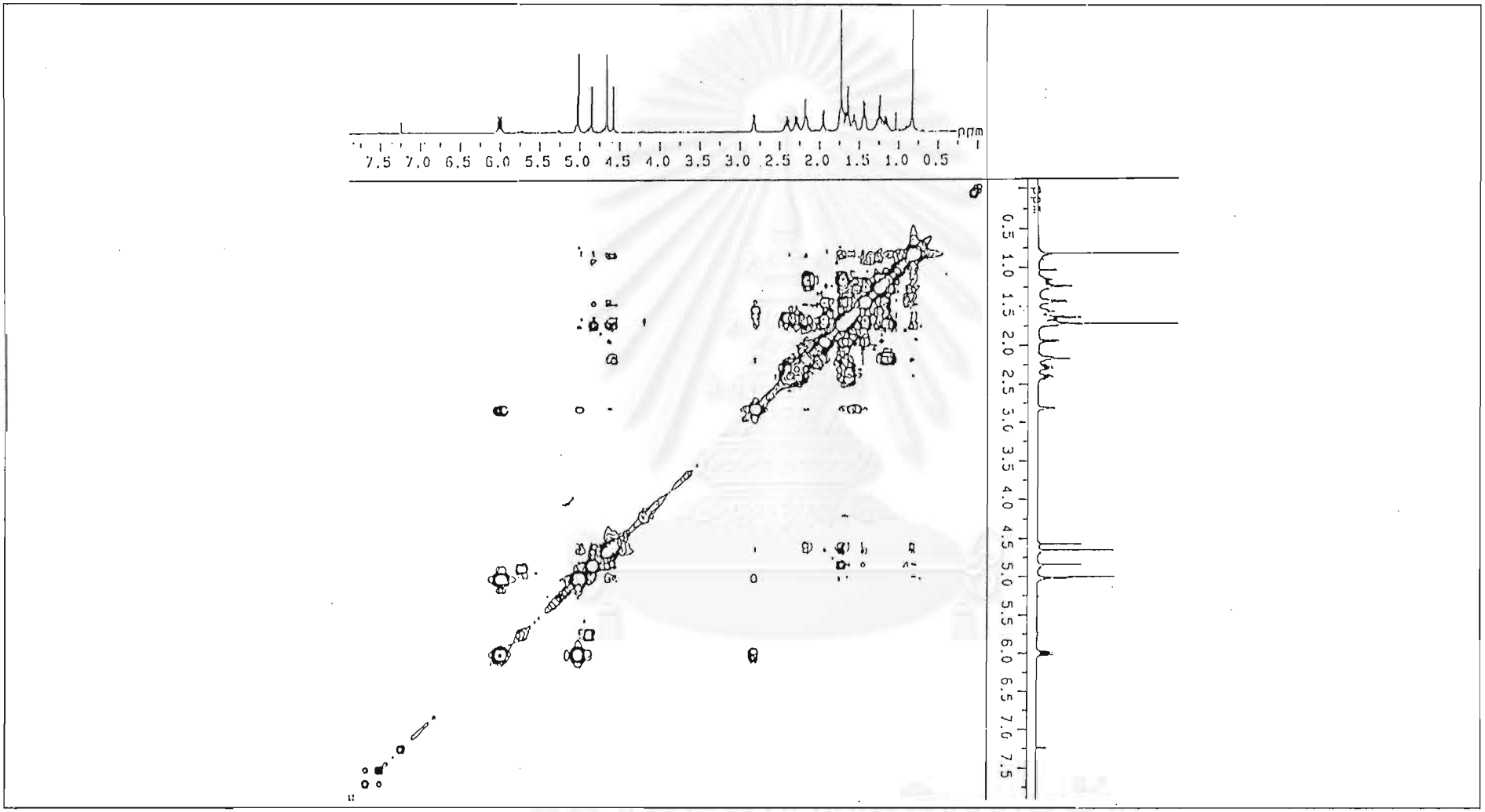

Figure 13 The COSY-NMR sectrum of compound 1

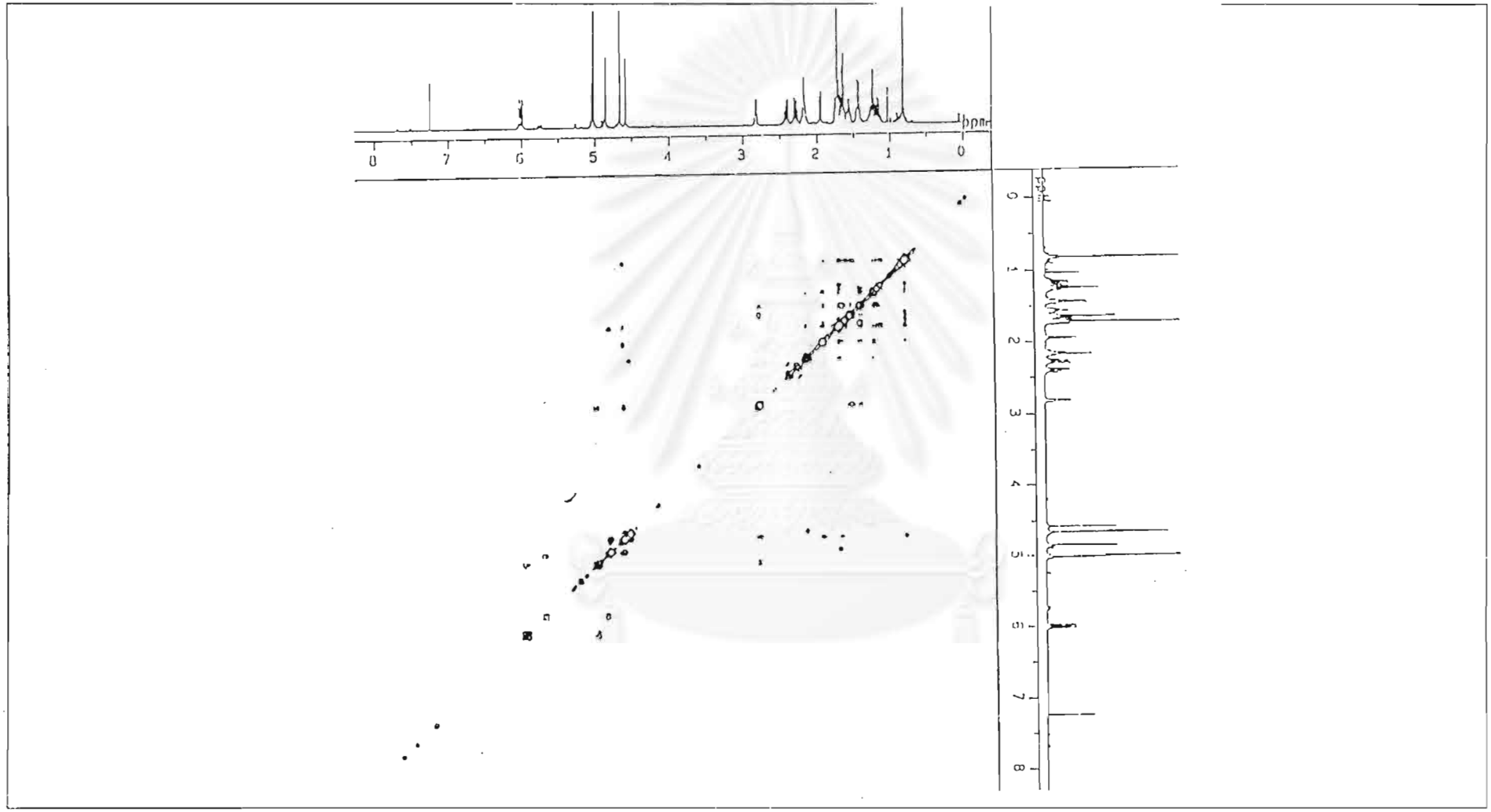

Figure 14 The NOESY-NMR sectrtum of compound 1

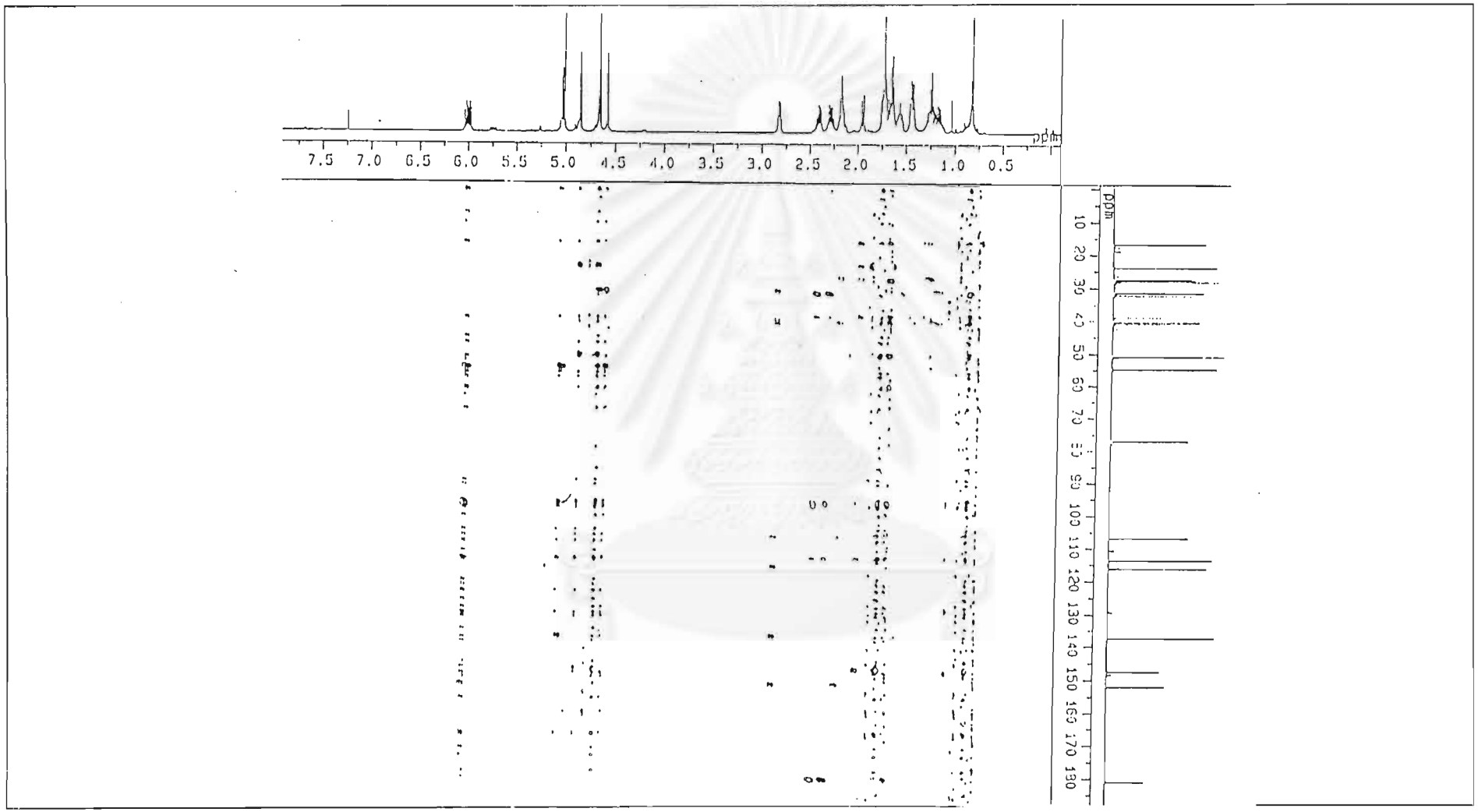

Figure 15 The HMBC-NMR sectrum of compound 1

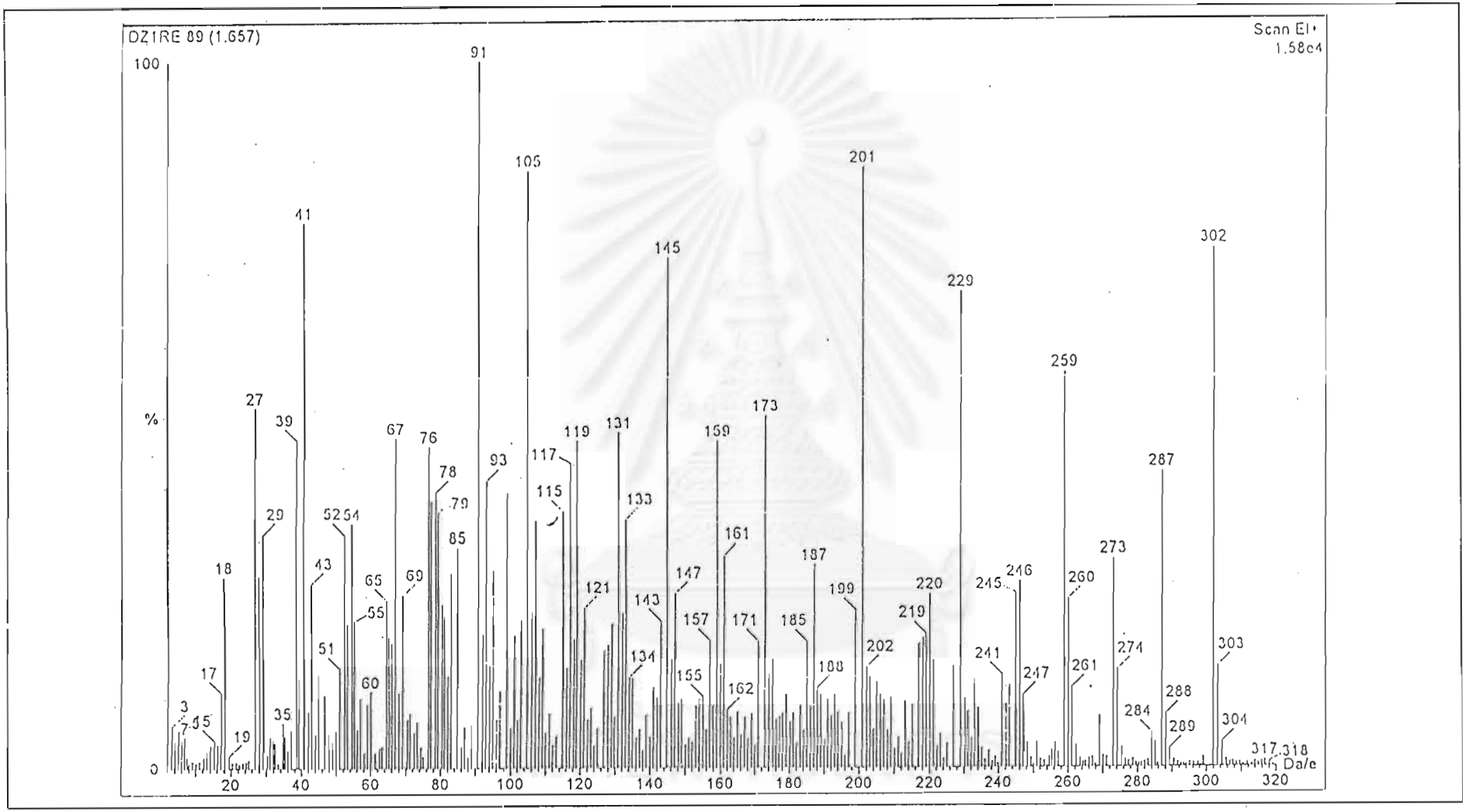

Figure 16 The EI-MS sectrum of compound 1

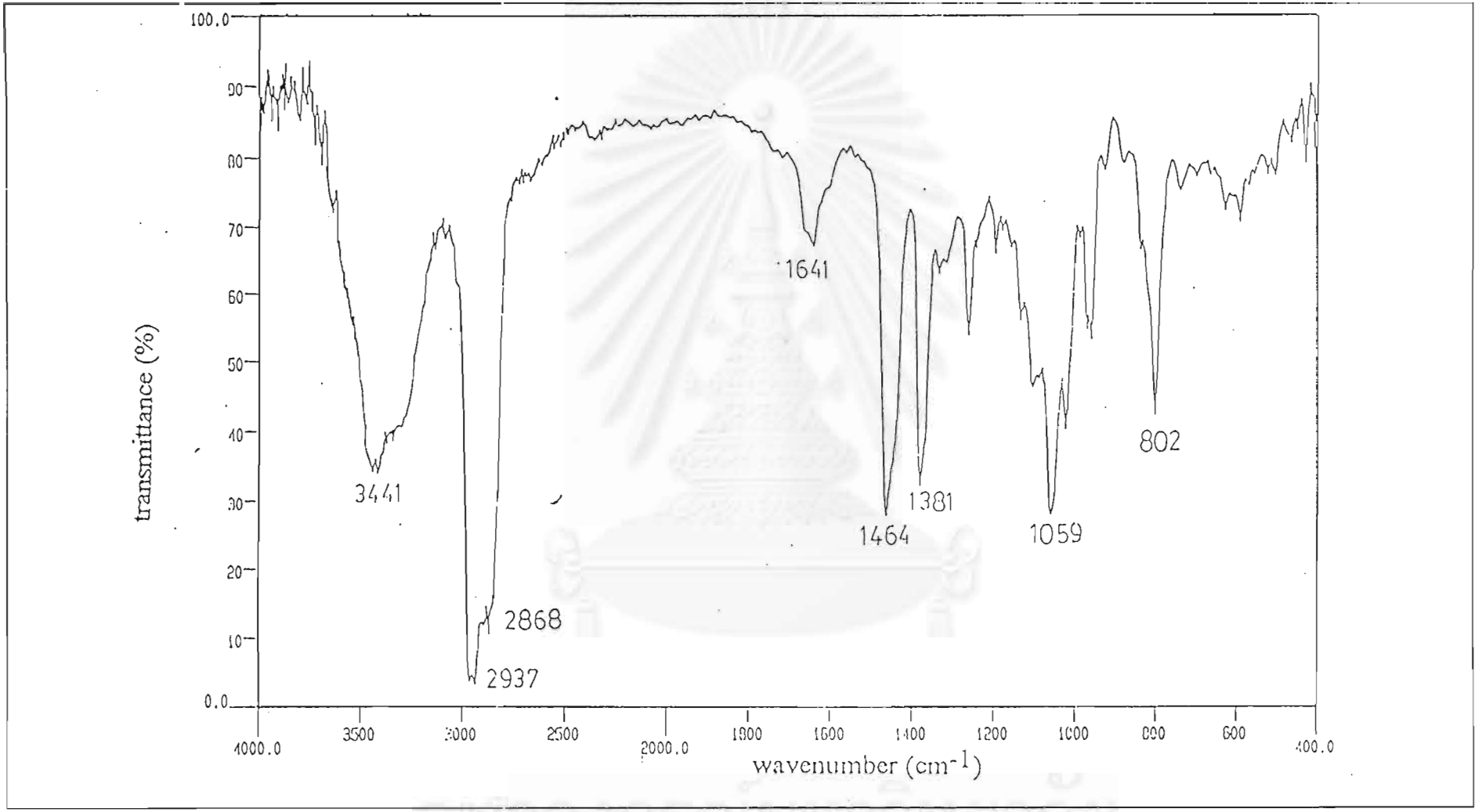

Figure 17 The IR sectrum of mixture 2

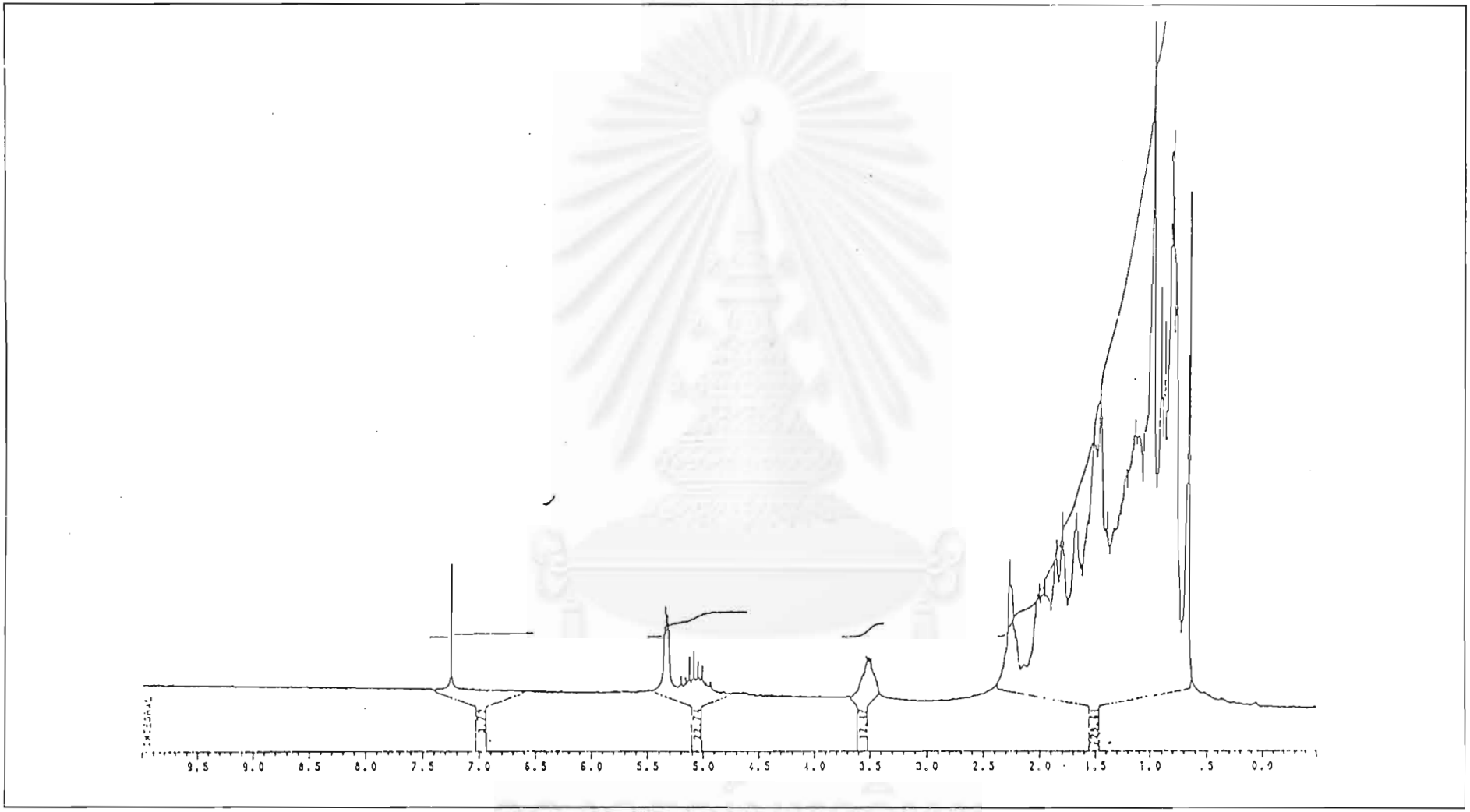

Figure 18 The ^{1}H-NMR setrum of mixture 2

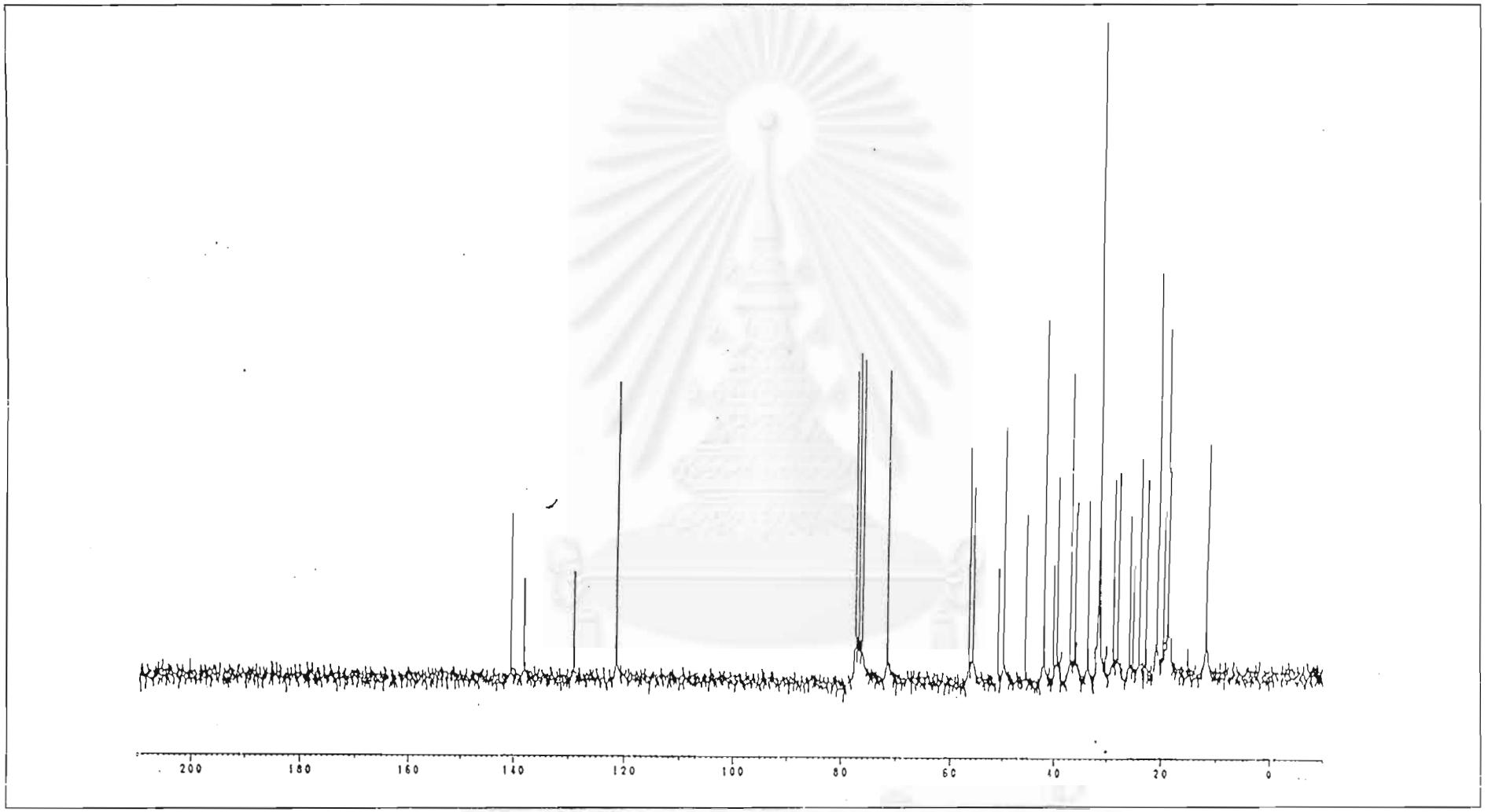

Figure 19 The ^{13}C-NMR setrum of mixture 2

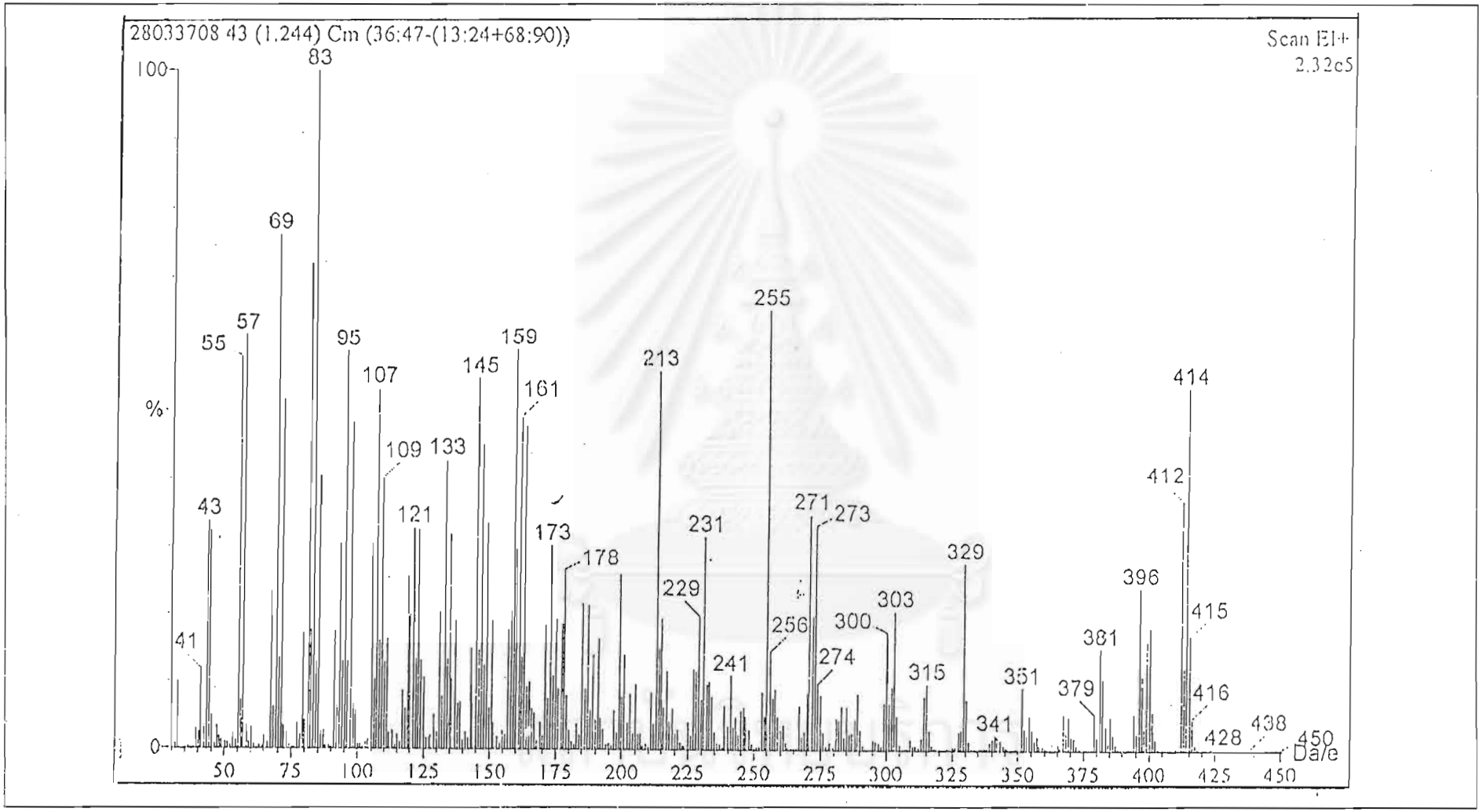

Figure 20 The EI-MS sectrum of mixture 2

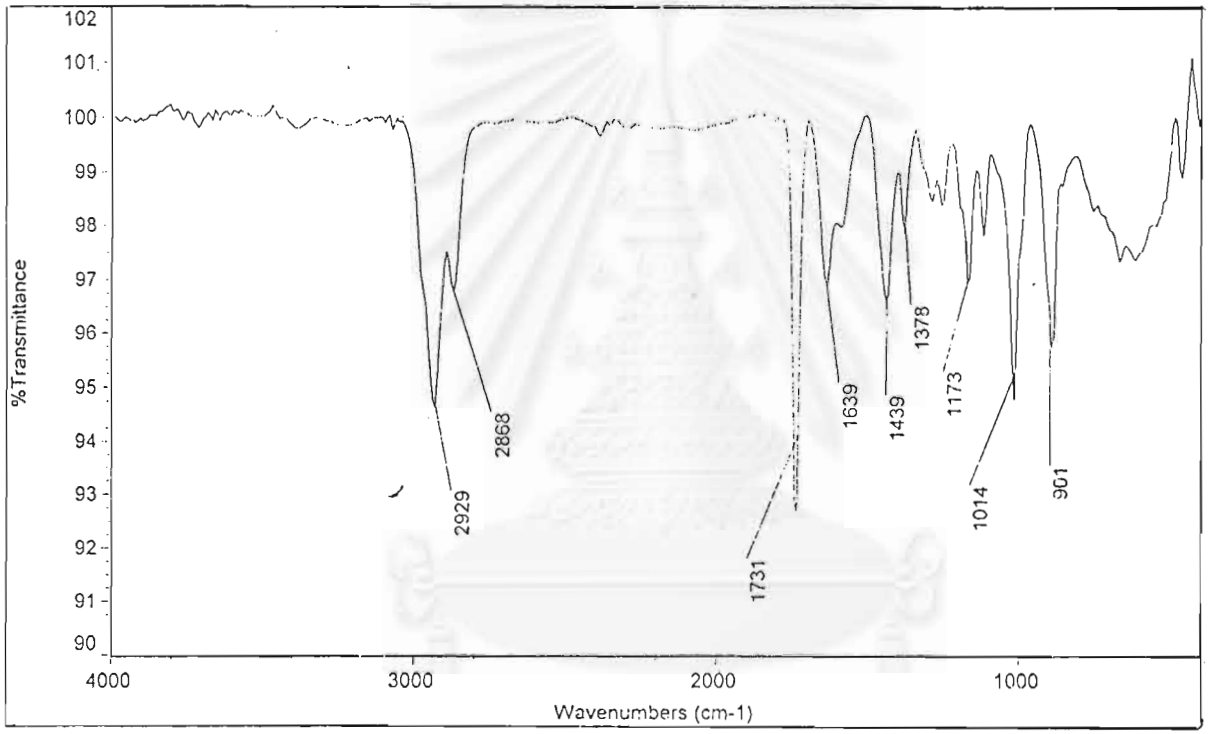

Figure 21 The IR sectrum of compound 1a

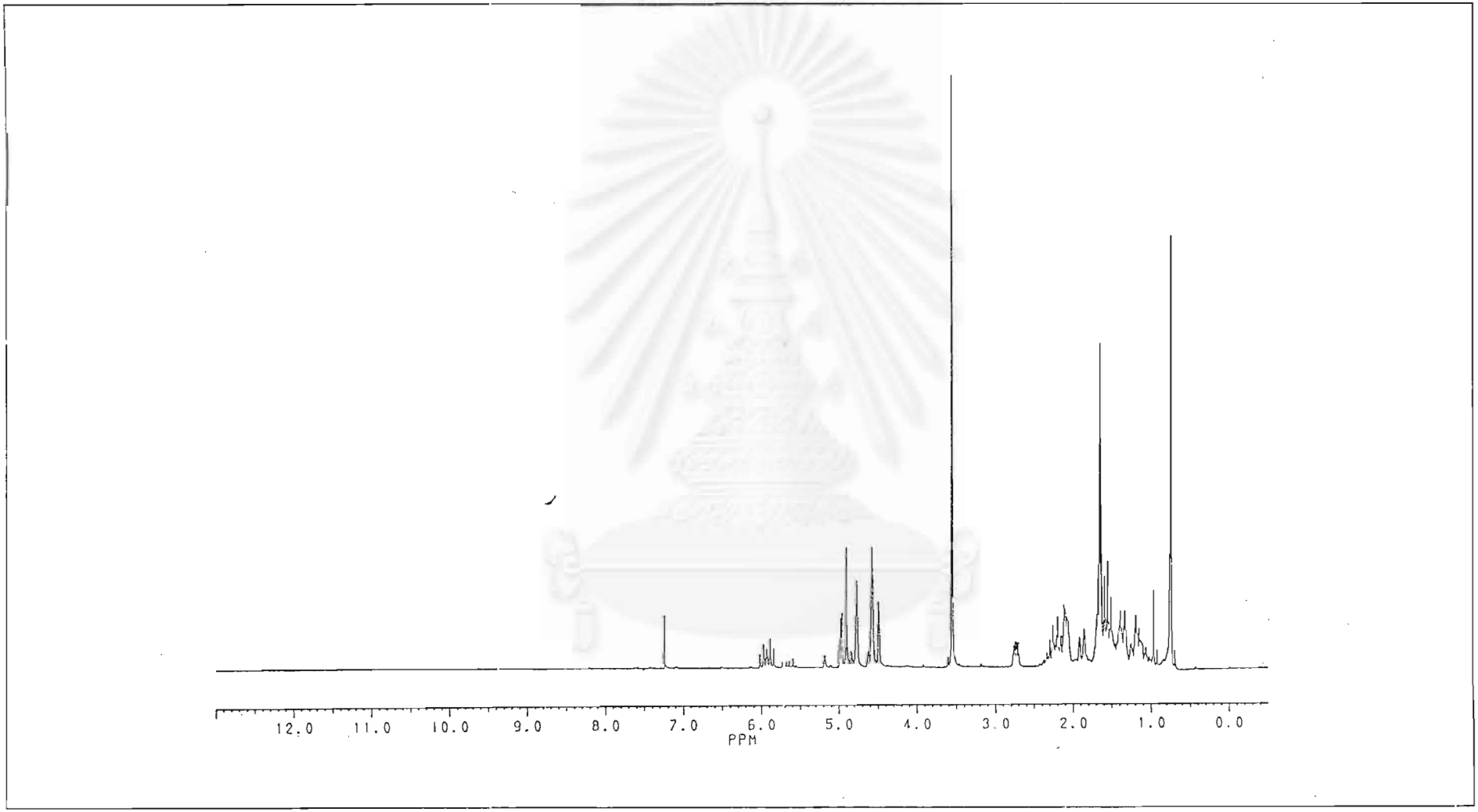

Figure 22 The ^{1}H-NMR setrum of compound 1a

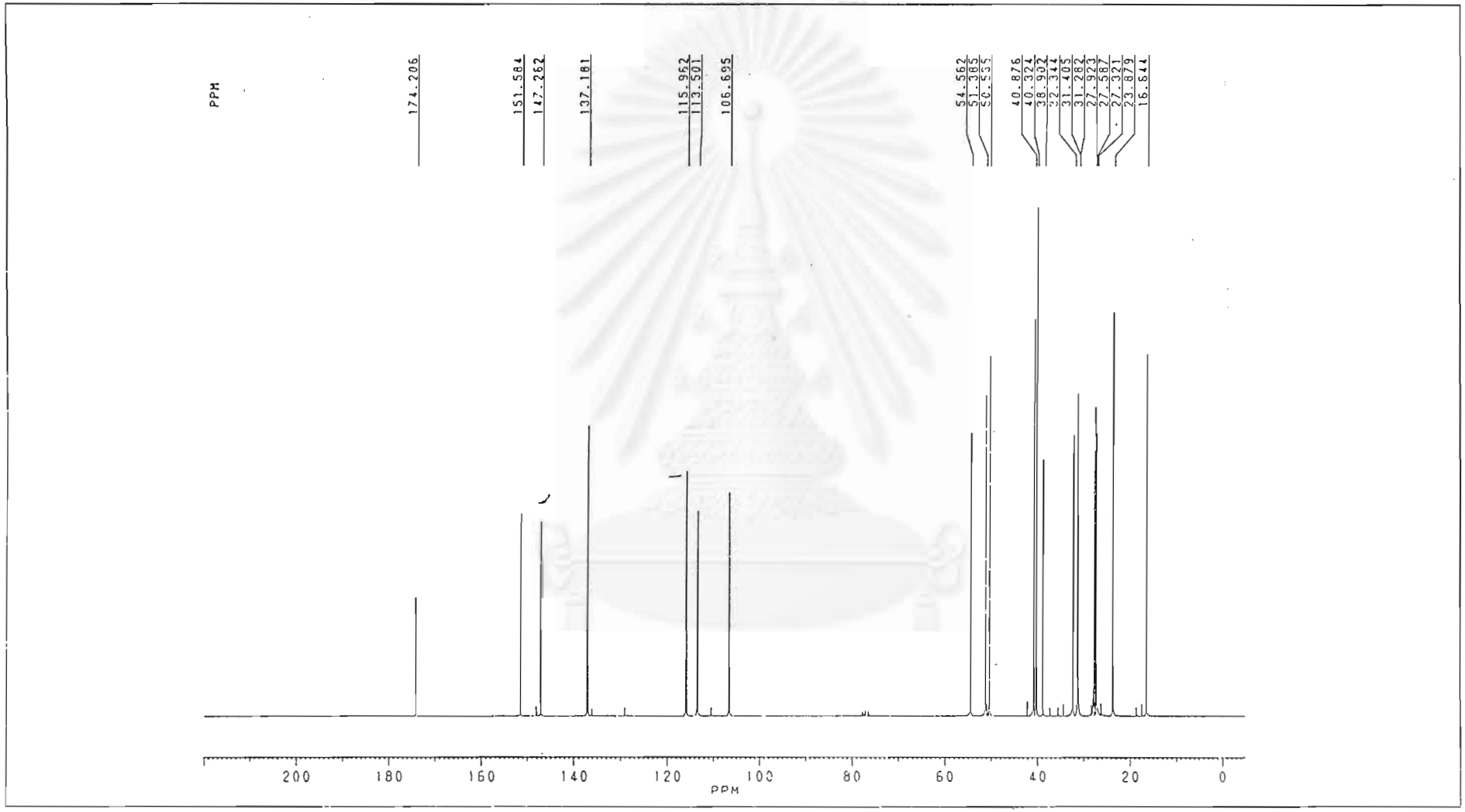

Figure 23 The ^{13}C-NMR setrum of compound 1a

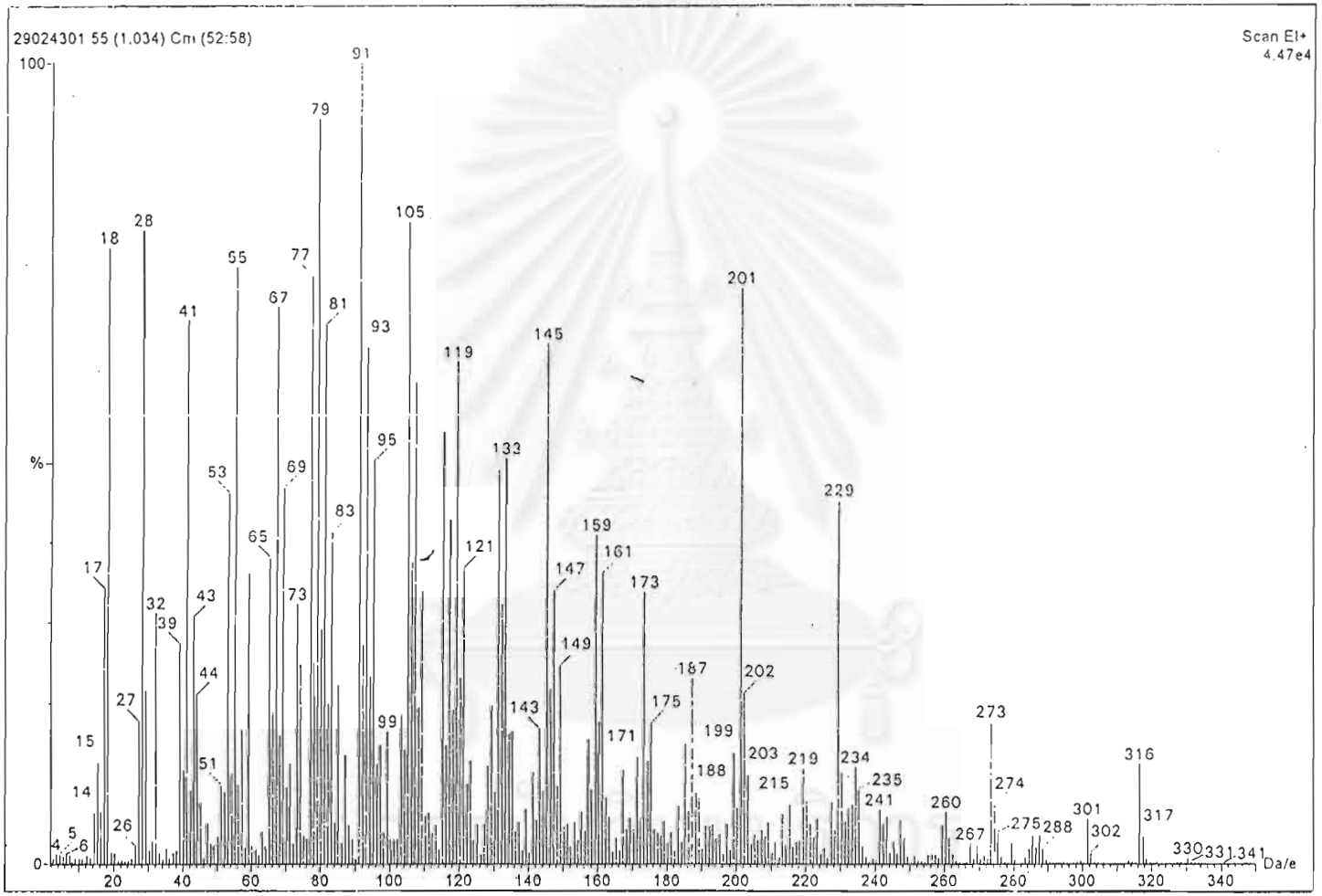

Figure 24 The EI-MS sectrum of compound 1a

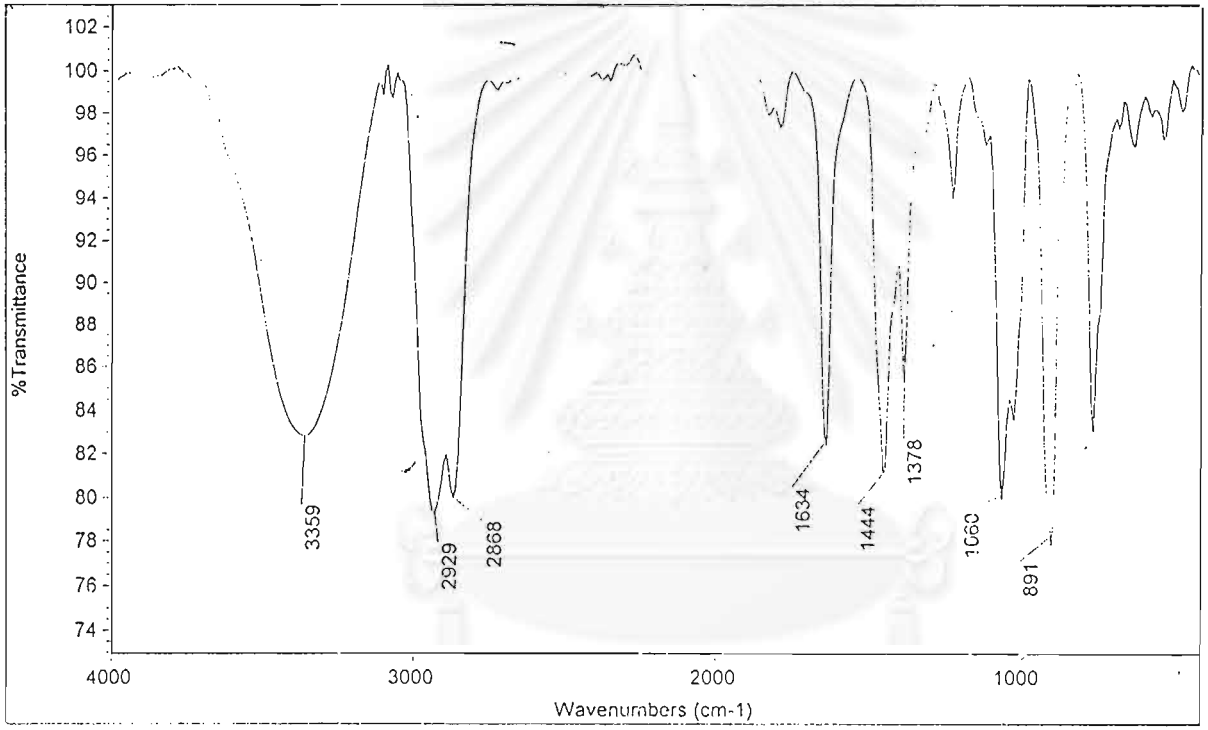

Figure 25 The IR sectrum of compound 1b

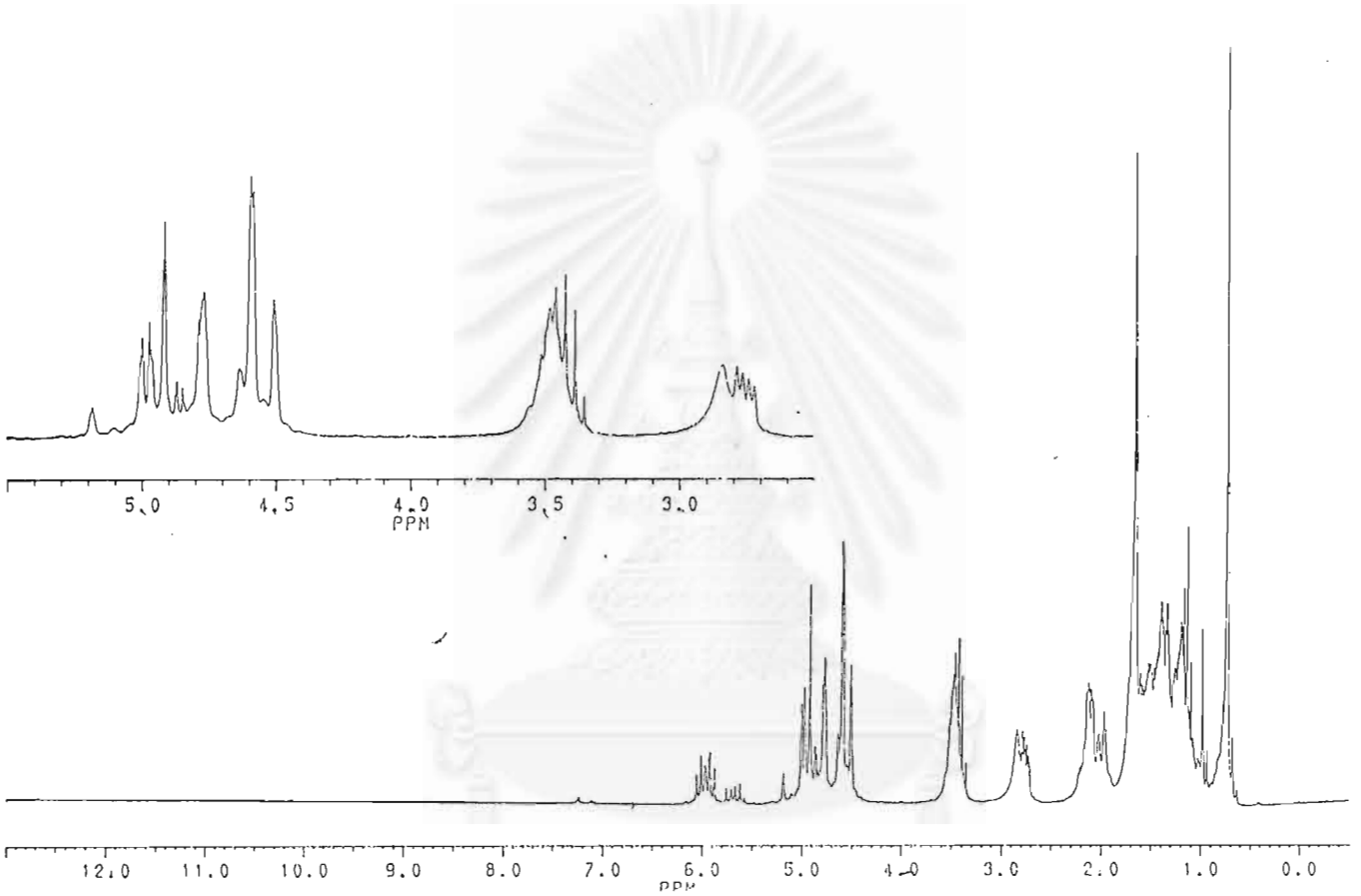

Figure 26 The ^{1}H-NMR setrum of compound 1b

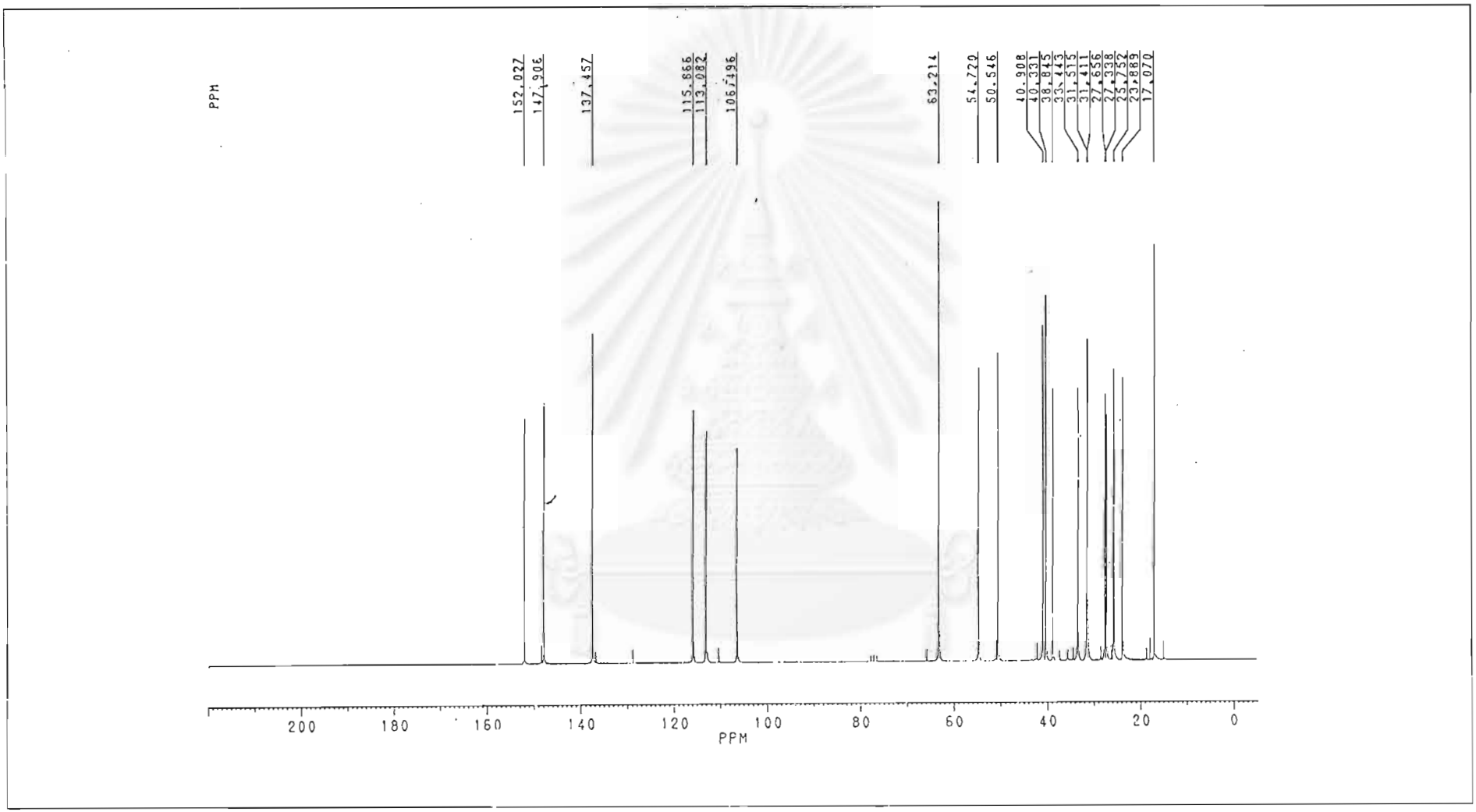

Figure 27 The ^{13}C-NMR setrum of compound 1b

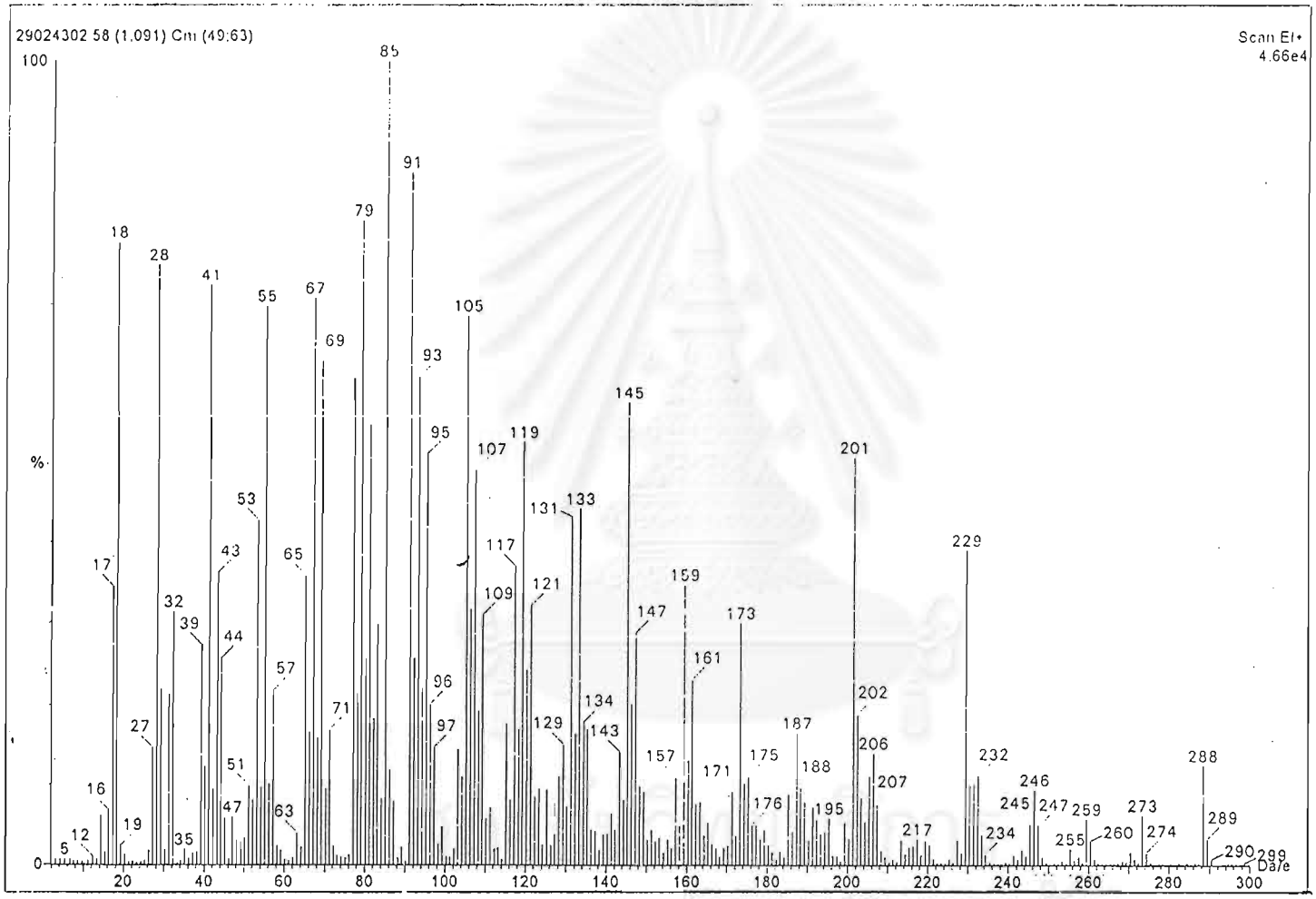

Figure 28 The EI-MS sectrum of compound 1b

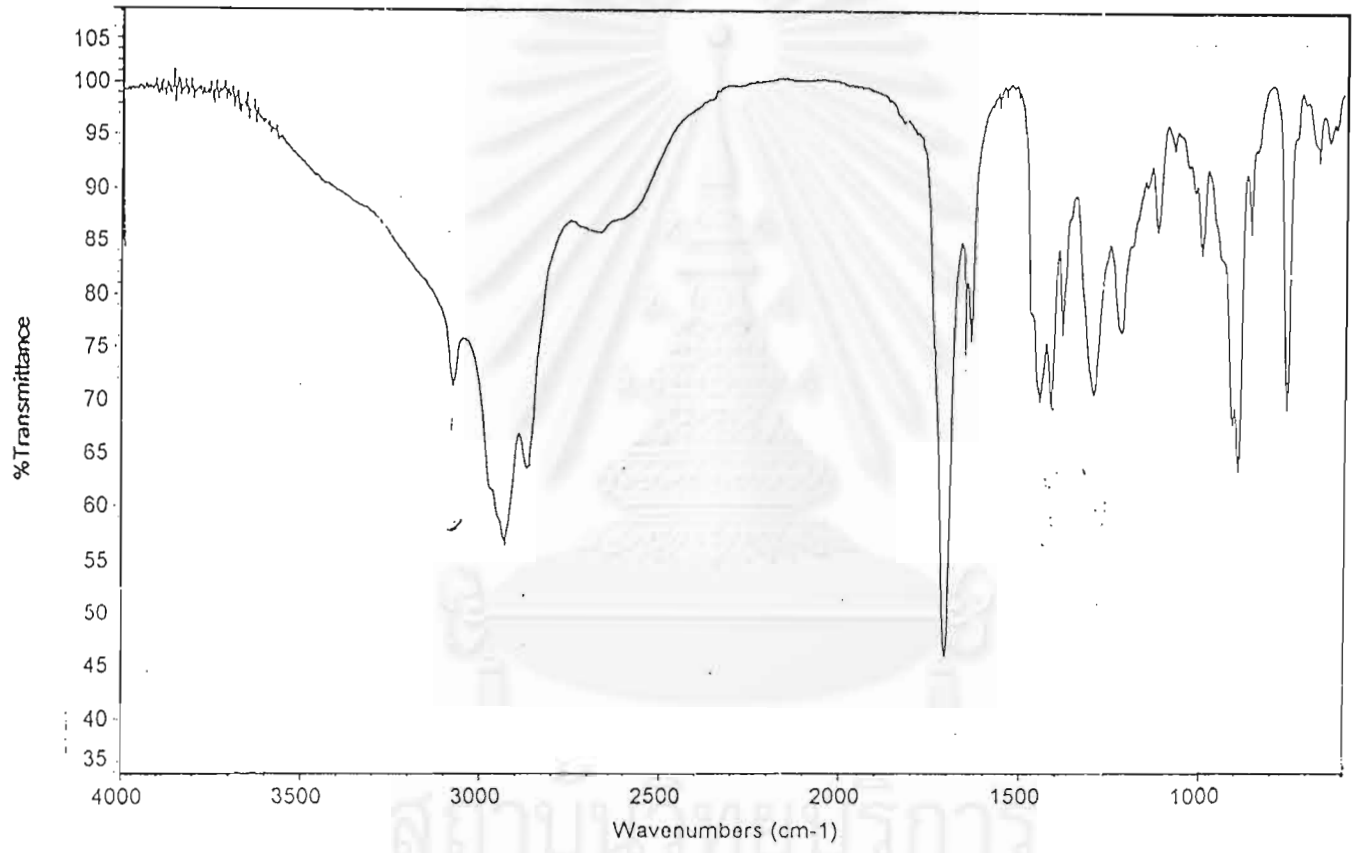

Figure 29 The IR sectrum of compound 3

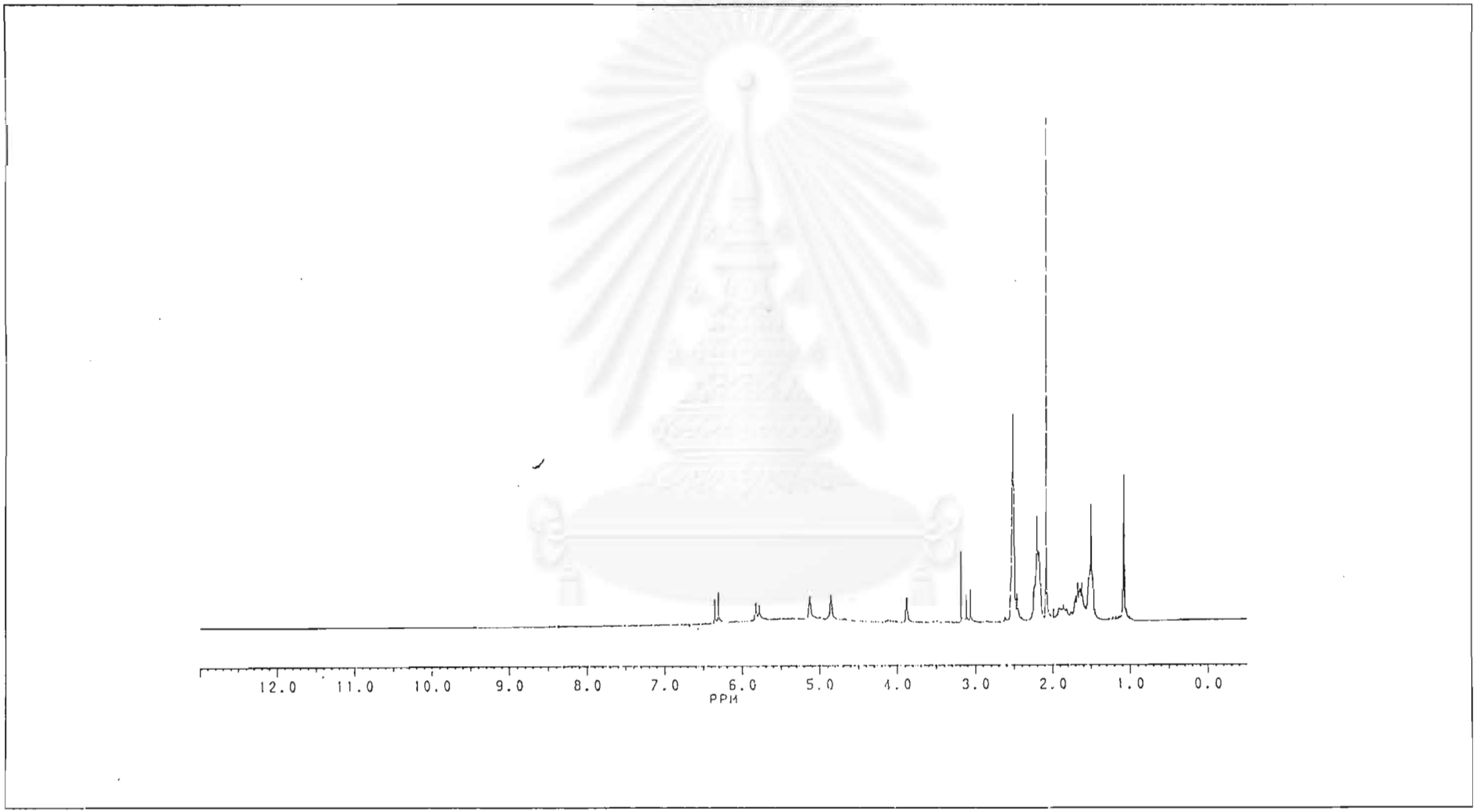

Figure 30 The ^{1}H-NMR setrum of compound 3

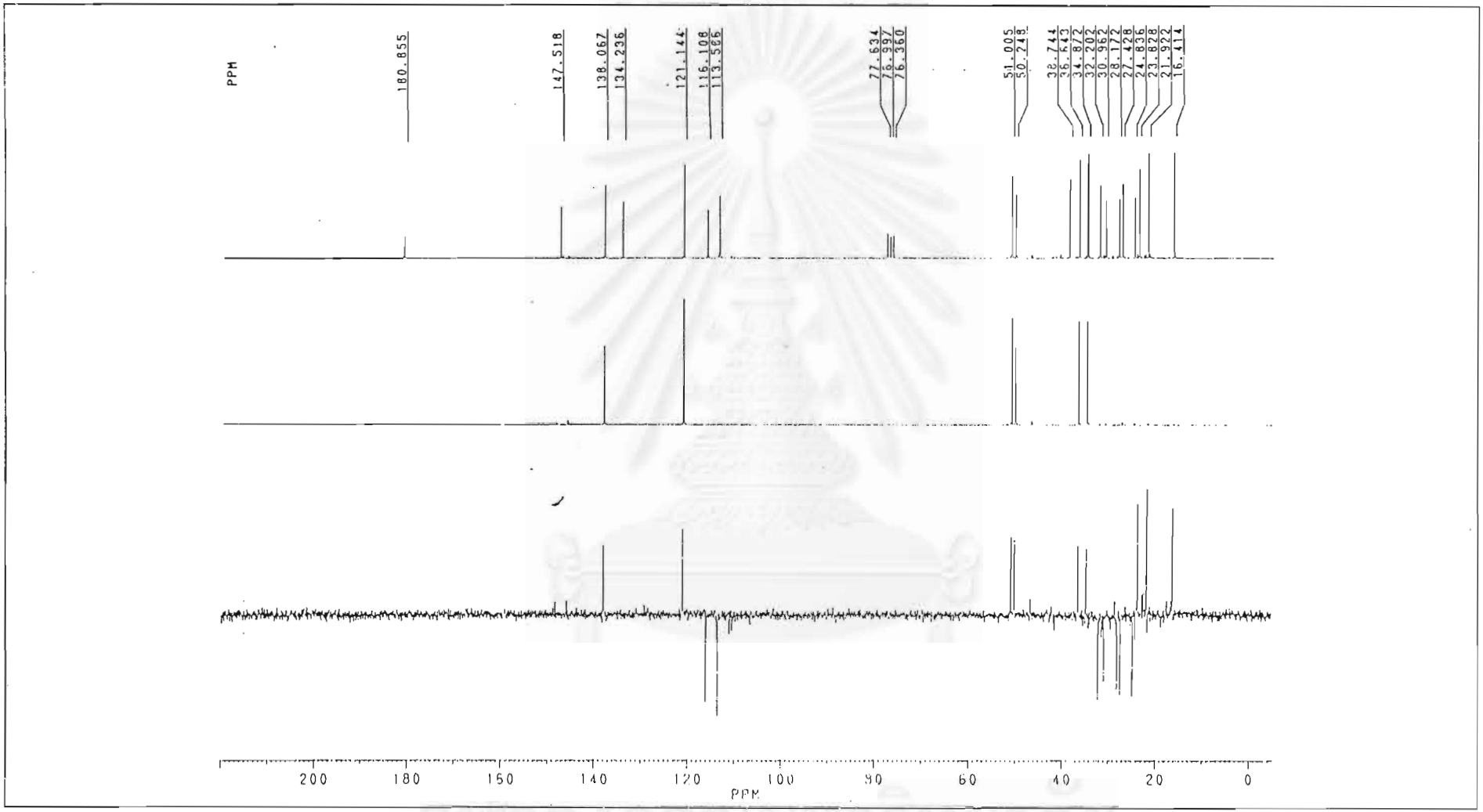

Figure 31 The ^{13}C-NMR setrum of compound 3

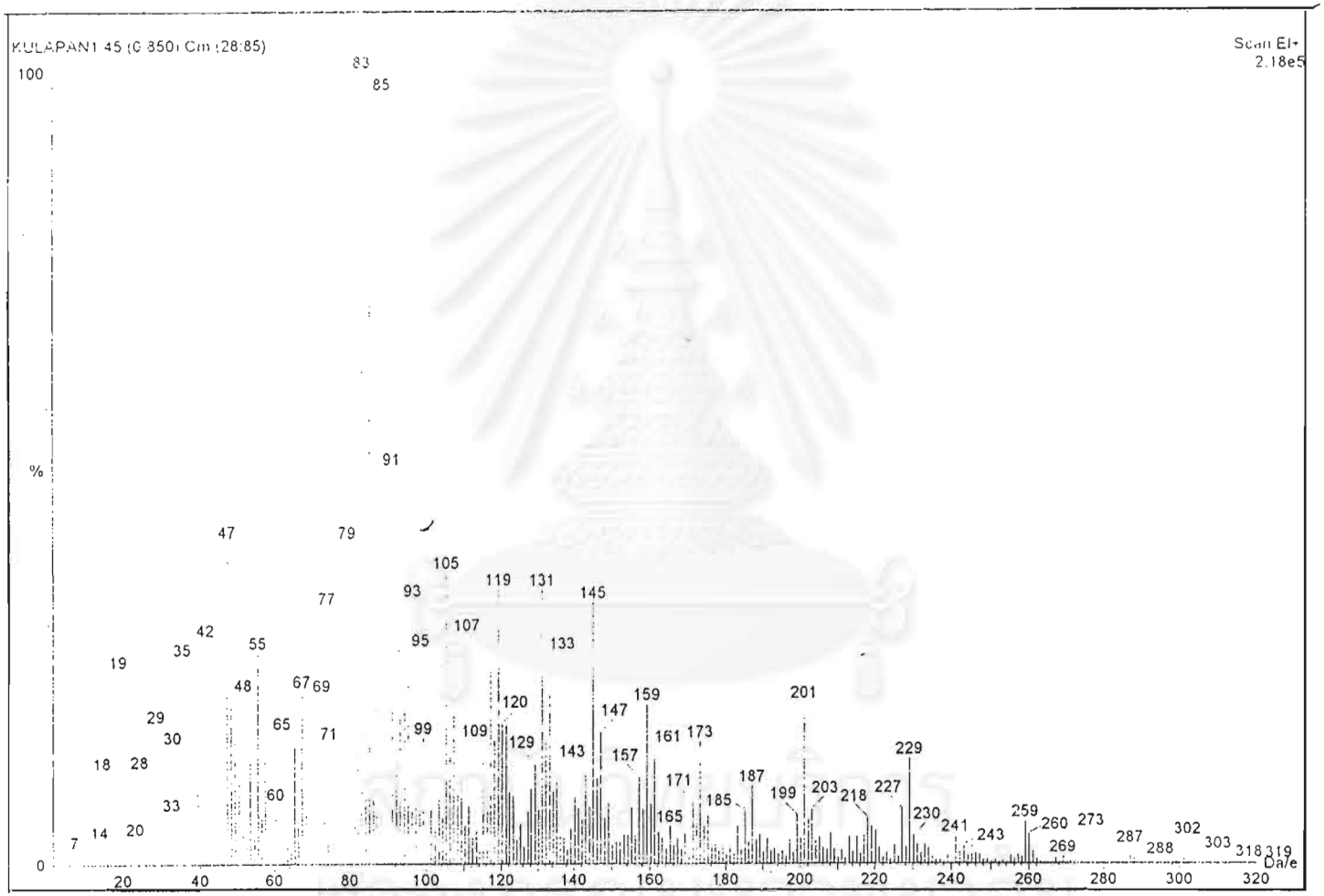

Figure 32 The EI-MS sectrum of compound 3

BIOGRAPHY

Mr. Kullaphan Siriwat was born on April 20, 1973 in Bangkok, Thailand. He graduated with a Bachelor Degree of Science in General Science (Environmental Science) from Chulalongkorn University in 1997, and he has been studying for a Master Degree of Science in Organic Chemistry since 1999.