ฤทธิ์การยับยั้งไซคลิกเอเอ็มพีฟอสโฟไดเอสเทอเรสของเปลือกต้นเปล้าใหญ่ *Croton oblongifolius* Roxb. จากอำเภอท่าอุเทน จังหวัดนครพนม

นางสาว อารีย์ สวนดง

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CYCLIC AMP PHOSPHODIESTERASE INHIBITORY ACTIVITY OF STEM BARK OF *Croton oblongifolius* Roxb. FROM AMPHOE THA-UTHEN, NAKHON PHANOM PROVINCE

Miss Aree Suandong

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Thesis Title	**CYCLIC AMP PHOSPHODIESTERASE INHIBITORY ACTIVITY OF STEM BARK OF *Croton oblongifolius* Roxb. FROM AMPHOE THA-UTHEN, NAKHONPHANOM PROVINCE**
By	Miss Aree Suandong
Field of Study	Biotechnology
Thesis Advisor	Associate Professor Amorn Petsom, Ph.D.
Thesis Co-advisor	Assistant Professor Nattaya Ngamrojnavanich. Ph.D.

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

Pipat Karntiang
Deputy Dean for Administrative Affairs
Acting Dean, Faculty of Science

(Associate Professor Pipat Karntiang, Ph.D.)

Thesis Committee

..... Chairman

(Associate Professor Soplion Roengsumran, Ph.D.)

..... Thesis Advisor

(Associate Professor Amorn Petsom, Ph.D.)

Nattaya Ngamrojnavanich
..... Thesis Co-advisor

(Assistant Professor Nattaya Ngamrojnavanich, Ph.D.)

Chaiyo Chaichantipyuth
..... Member

(Associate Professor Chaiyo Chaichantipyuth, M.Sc. in Pharm.)

Polkit Sangvanich
..... Member

(Polkit Sangvanich, Ph.D.)

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ได้ทำการสกัดเปลือกต้นเปล้าใหญ่ (*Croton oblongifolius* Roxb.) จากอำเภอท่าอุเทน จังหวัดนครพนม ด้วยตัวทำละลายเฮกเซนและเอทิลแอซิเทต นำส่วนสกัดที่ได้มาแยกองค์ประกอบทางเคมีโดยอาศัยเทคนิคคอลัมน์โครมาโทกราฟี และหาสูตรโครงสร้างของสารที่แยกได้ด้วยเทคนิคทางสเปกโตรสโกปี พบว่าในส่วนสกัดเฮกเซนและเอธิลอะซิเตต สามารถแยกสารบริสุทธิ์ได้ 4 ชนิด และของผสมสเตอรอยด์ไกลโคไซด์ได้ 1 ชนิด คือ Ledol (**1**), 5-hydroxy-3,7,4′-trimethoxyflavone (**2**), 5-hydroxy- 3,7,3′,4′-tetramethoxyflavone (**3**), Pterodoltriol D (**4**) และ campesteryl–3-*O*-β-D-glucopyranoside, stigmasteryl-3-*O*-β-D-glucopyranoside, และ β-sitosteryl–3-*O*-β-D-glucopyranoside (**5**) นำสารที่แยกได้มาทดสอบฤทธิ์การยับยั้งไซคลิกเอเอ็มพีฟอสโฟไดเอสเทอเรสด้วยวิธีมาลาไคท์กรีนพบว่าสารประกอบ **1, 2** และ **3** มีฤทธิ์ยับยั้งไซคลิกเอเอ็มพีฟอสโฟไดเอสเทอเรส

หลักสูตร...เทคโนโลยีทางชีวภาพ..... **ลายมือชื่อนิสิต**.....

สาขาวิชา. เทคโนโลยีทางชีวภาพ.... **ลายมือชื่ออาจารย์ที่ปรึกษา**.....

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AREE SUANDONG : CYCLIC AMP PHOSPHODIESTERASE INHIBITORY ACTIVITY OF STEM BARK OF *Croton oblongifolius* Roxb. FROM AMPHOE THA-UTHEN, NAKHONPHANOM PROVINCE. THESIS ADVISOR : ASSOC. PROF. AMORN PETSOM, Ph.D., THESIS COADVISOR : ASSIST. PROF. NATTAYA NGAMROJNAVANICH, Ph.D. 103 pp. ISBN 974-17-0315-5

The stem bark of *Croton oblongifolius* Roxb. from Nakhonphanom province was extracted with hexane and ethyl acetate. The crude extracts were separated using column chromatography and the structures of isolated components were elucidated using spectroscopic techniques. The crude extract gave ledol (**1**), 5-hydroxy-3,7,4′-trimethoxyflavone (**2**) and 5-hydroxy-3,7,3′,4′-tetramethoxyflavone (**3**), Pterodoltriol D (**4**) and campesteryl-3-*O*-β-D-glucopyranoside, stigmasteryl-3-*O*-β-D-glucopyranoside, and β-sitosteryl-3-*O*-β-D-glucopyranoside (**5**). Each compound was tested for inhibitory activity on cyclic AMP phosphodiesterase following the bioassay using malachite green method. The results showed that the compound **1, 2** and **3** showed high inhibitory activity.

Program..... Biotechnology.... **Student's signature**.....Aree Suandong.....

Field of study..... Biotechnology.....**Advisor's signature**.....

Academic year.....2001..... **Co-advisor's signature**.....Nattaya Ngamrojnavanich

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LIST OF ABBREVIATIONS

br	=	broad (for NMR, IR spectra)
c	=	concentration
^{o}C	=	degree Celsius
$CDCl_3$	=	deuterated chloroform
$CHCl_3$	=	chloroform
EtOAc	=	ethyl acetate
MeOH	=	methanol
cm	=	centimeter
^{13}C-NMR	=	carbon-13 nuclear magnetic resonance
d	=	doublet (for NMR spectra)
dd	=	doublet of doublet (for NMR spectra)
ddd	=	doublet of doublet of doublet (for NMR spectra)
DEPT	=	Distortionless Enhancement by Polarization Transfer
DMSO	=	dimethyl sulfoxide
δ	=	Chemical Shift
EI MS	=	Electron Impact Mass Spectrum
g	=	gram
Kg	=	kilogram
^{1}H-NMR	=	proton nuclear magnetic resonance
Hz	=	Hertz
IR	=	infrared spectrum
J	=	coupling constant
L	=	liter
M^+	=	molecular ion
mg	=	milligram
MHz	=	megahertz
ml	=	milliliter
mm	=	millimeter
m.p.	=	melting point
M	=	molar
m/z	=	mass to charge ratio

LIST OF ABBREVIATIONS (Cont.)

M.W.	=	molecular weight
MS	=	mass spectrometry
No.	=	number
NMR	=	Nuclear Magnetic Resonance
ppm	=	part per million
q	=	quartet (for NMR spectra)
s	=	singlet (for NMR spectra)
t	=	triplet (for NMR spectra)
TLC	=	thin layer chromatography
wt	=	weight
R_f	=	retention factor in chromatography
g/l	=	gram/liter
µl	=	microliter

CHAPTER I

INTRODUCTION

A large number of plants in Thailand have been used as traditional medicine for a long time. Human has used plants in various ways such as foods, feeds and medicines. In the past, medicinal plants have been the primary treatment in the health care system. At present, they also play an important role in modern medicine, because they are available in modern form, have good efficiency, inexpensive and believed to possess less side effect than synthetic drugs.

One way to screen for the biological activity of medicinal plants is the inhibition of enzymes, for example, cyclic AMP phosphodiesterase. The inhibition of cyclic adenosine monophosphate (cAMP) phosphodiesterase results in the increase of intracellular levels of cAMP. The cAMP has central role in regulating the function of airway smooth muscle, inflammatory cells and immune cells.[1] In some diseases, the low level of cAMP is associated with the decreased in the sensitivity of adenylyl cyclase stimulation. The most feasible approach to correct for the cAMP deficiency under these circumstances is the inhibition of phosphodiesterase.[2] Examples of these conditions are hypertension [3], secretion [4], asthma [5], allergic [6], inflammatory disease [7] and platelet aggregation.[8]

In 1981 Nikaido and co-worker [9] reported the results of screening cAMP phosphodiesterase inhibitors in medicinal plants. Of 222 samples tested 22 showed reproducible inhibition. In 1996 Chairungsrilerd and co-worker [10] found that mangostanol, and α- and γ-mangostin showed moderate inhibitory effects on cAMP phosphodiesterase.

In term of natural product research, this thesis is aimed to investigate for the chemical constituents of *Croton oblongifolius* Roxb. from Amphur Tha-Uthen, Nakhonphanom province, and to test the isolated compounds for their inhibitory activity on cAMP phosphodiesterase.

The purposes of this research

1. Extraction, isolation, and characterization of chemical constituents of stem bark of *C. oblongifolius* Roxb.
2. Examination of the inhibitory activity on cyclic AMP phosphodiesterase of the crude extract from stem bark of *C. oblongifolius* Roxb.
3. Examination of the inhibitory activity on cyclic AMP phosphodiesterase of the isolated compounds.

CHAPTER II

LITERATURE REVIEWS

2.1 Botanical Description of *Croton oblongifolius* Roxb.

Croton oblongifolius Roxb. is a plant in the Euphorbiaceae family, in the *Croton* genus, Commonly known as Plao Yai (Central part), Plao Luang (Northern part) Poh (Kamphaeng Phet) Khwa-wuu (Kanchanaburi), Saa-kuu-wa (Karen, Mae Hong Son) and Haa-yoeng (Shan, Mae Hong Son).[11]

The plant is a medium sized deciduous tree. It is widely distributed throughout Thailand. The calyx and ovary are clothed with minute orbicular silvery scales. The leaves fall between 5.6-12.0 cm by 13.0-24.0 cm in size. The leaf blade is oblong-lanceolate shaped. The flowers are 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 racemen 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.[12] The pictures of *C. oblongifolius* Roxb. are shown in Fig. 1.

C. oblongifolius Roxb. is one of the interesting Thai medicinal plants because it is believed that all parts of the plants can be used as drugs. Its leaves are used as a tonic, and the flowers are used as a teniacide, and the fruits are used to treat dysmenorrhea, The seeds are used as a purgative. The bark is used to treat dyspepsia, and the roots are used as dysentery.[13] Moreover, this plant has been used in combination with *C. sublyratus* to treat gastric ulcers and gastric cancers.[14]

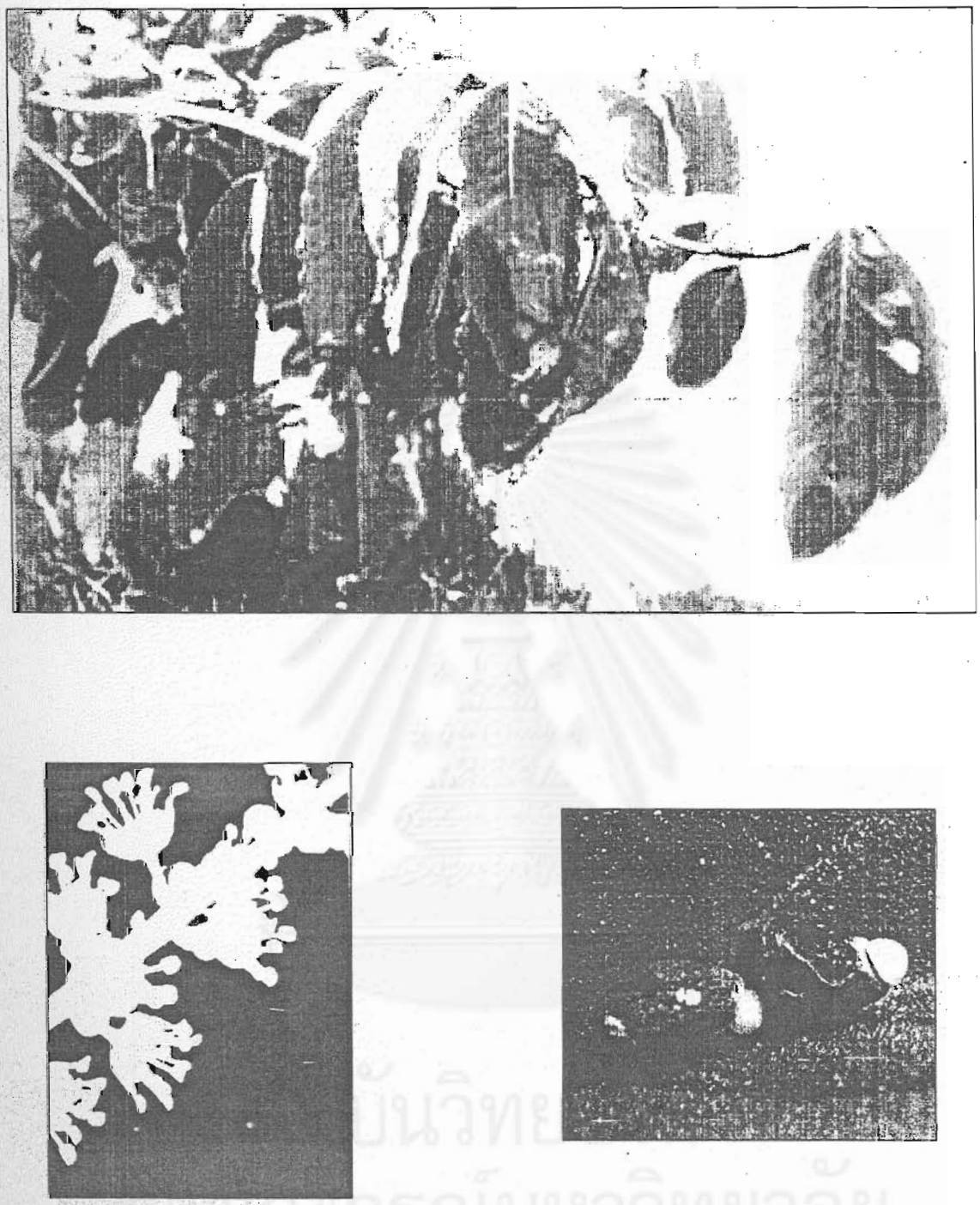


Figure 1 *Croton oblongifolius* Roxb.

2.2 Chemical constituents of *Croton oblongifolius* Roxb.

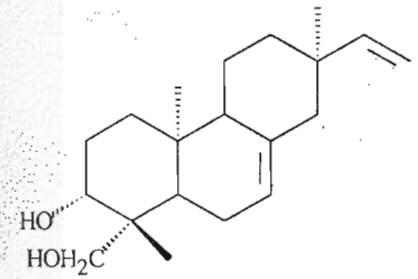
The diterpenoid compounds found in *C. oblongifolius* Roxb. are presented in Table 1 and Figure 2.[13-24]

Table 1 Chemical constituents of *C. oblongifolius* Roxb.

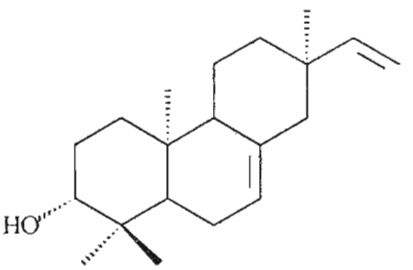
Plant parts	Chemical compounds	Reference
bark	Oblongifoliol	[15]
	19-deoxyoblongifoliol	[16]
	isopimara-7 (8),15-diene-19-oic acid	[17]
	ent-isopimara-7,15-diene-3β-19-diol	[18]
	ent-isopimara-7,15-diene-3β-ol	[18]
	ent-isopimara-7,15-diene	[19]
	19-hydroxy-*ent*-isopimara-7,15-diene	[19]
	ent-isopimara-7,15-diene-19-aldehyde	[19]
	acetylaleuritolic acid	[20]
	3β-acetoxy-olean-14 (15)- 28-oic acid	[20]
	ent-15,16-epoxy-3,11,13(16),14-cleroda-tetraen-19-oic acid	[21]
	11-dehydro(–)-hardwickiic acid	[21]
	crotocembraneic acid	[13]
	neocrotocembraneic acid	[13]
	neocrotoncembranal	[24]
	labda-7,12 (*E*), 14-triene	[23]
	labda-7,12 (*E*), 14-triene-17-ol	[23]
	labda-7,12 (*E*), 14-triene-17-al	[23]
	labda-7,12 (*E*), 14-triene-17-oic acid 2-acetoxy-3-hydroxy-8 (17), (*E*)-14-triene	[14]
	3-acetoxy-2-hydroxy-labda-8 (7),12 (*E*)-14-triene	[14]
	2,3-dihydroxy-labda-8 (17),12 (*E*),14-triene	[14]

Table 1 Continued

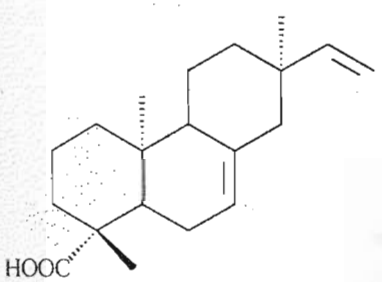
Plant parts	Chemical compounds	Reference
wood	oblongifoliol	[15]
	19-deoxyoblongifoliol	[16]
	oblongifolic acid	[17]
	ent-isopimara 7,15-diene	[18]
	3-deoxyoblongifoliol	[18]
	ent-isopimara-7,15-diene-19-aldehyde	[18]
	(-)-hardwickiic acid	[19]
	acetylaleuritolic acid	[20]
	11-dehydro-(-)-hardwickiic acid	[21]
leaves	waxy materials	[22]



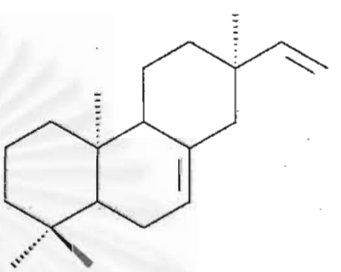
oblongifoliol [15]



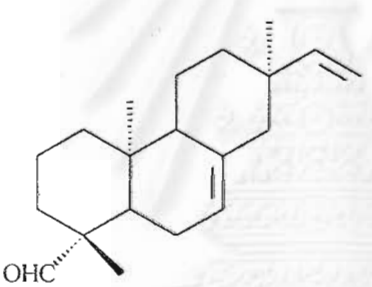
19-deoxyoblongifoliol [16]



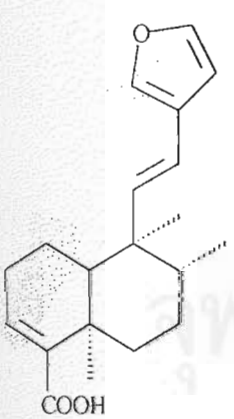
oblongifolic acid [17]



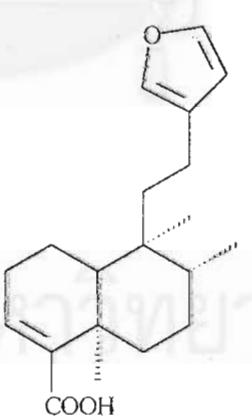
ent-isopimara-7, 15-diene [18]



ent-isopimara-7, 15-diene-19-aldehyde [18]

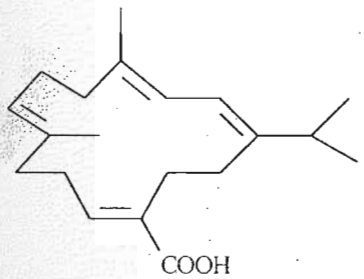


11-dehydro(-)-hardwickiic acid [21]

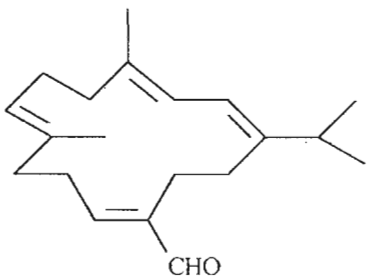


(–)-hardwickiic acid [19]

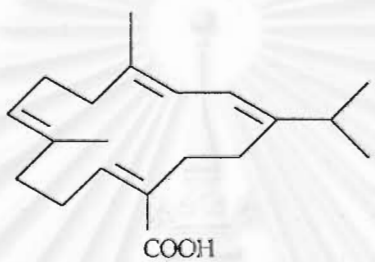
Figure 2 Structures of isolated compounds from *Croton oblongifolius* Roxb.



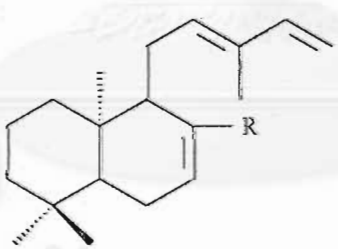
neocrotocembraneic acid [13]



neocrotocembranal [24]

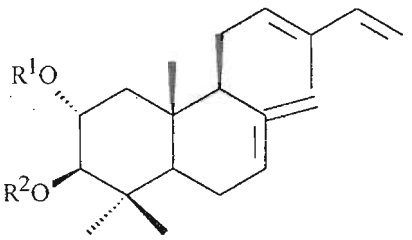


crotocembraneic acid [13]



R=CH_3	labda-7,12(*E*),14-triene [23]
=CH_2OH	labda-7,12(*E*),14-triene-17-ol [23]
=CHO	labda-7,12(*E*),14-triene-17-al [23]
=CO_2H	labda-7,12(*E*),14-triene-17-oic acid [23]

Figure 2 Continued



R^1	R^2	
CH_3CO	H	2-acetoxy-labda-8 (17), 12(*E*), 14-triene-3-ol [14]
H	CH_3CO	3-acetoxy-labda-8 (17), 12(*E*), 14-triene-2-ol [14]
H	H	labda-8 (17), 12(*E*), 14-triene-2, 3-diol [14]

Figure 2 Continued

Although chemical constituents from *C. oblongifolius* have been reported for long time, new compounds are still discovered, and some of them give interesting bioactivity. In addition, the chemical components from the stem bark of *C. oblongifolius* from various locations are different. For example, *C. oblongifolius* from India, is mainly categorized into the isopimarane and clerodane groups. While *C. oblongifolius* from Thailand (Amphur Vicheinburi, Petchaboon province) was found to have cembranoid diterpene skeleton.[24, 25] On the other hand, specimen from Amphur Pranburi, Prachuabkirikhan province gave labdane diterpenoids.[26] Therefore it was decided to investigate the chemical constituents of the stem bark of *C. oblongifolius* from Amphoe Tha–Uthen, Nakhonphanom province, Thailand.

This research deals with the sesquiterpene compounds and flavonoid compounds isolated from *C. oblongifolius*, Nakhonphanom province. The hexane crude extract and ethyl acetate crude extract were separated by a mean of column chromatography and purified by proper methods such as recrystallization. The structure elucidation was carried out by spectroscopic techniques. Finally, inhibitory activity on cyclic adenosine monophosphate (cAMP) phosphodiesterase of isolated compounds from the plants was studied.

2.3 Chemical constituents of *Croton* genus

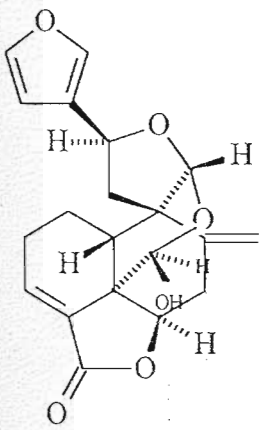
Literature surveys of plants belonging to the *Croton* genus revealed that many organic compounds have been isolated. The various types of organic substances reported are shown in Table 2 and some of their structures are shown in Fig. 3.

Table 2 Chemical constituents of *Croton* genus

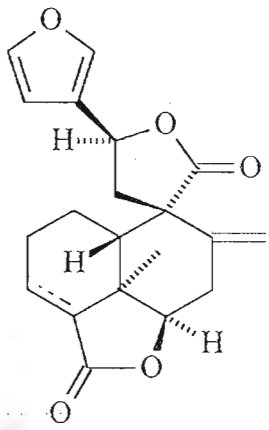
Scientific Name	Plant parts	Chemical compounds	Reference
Croton californicus	whole	(−)-hardwickiic acid	[26]
	plants	1-triacontanol	[26]
C. sublyratus	bark	plaunol A, B, C, D and E	[27]
		ent-3α-hydroxy-13-epimanool	[28]
		ent-16*β*, 17-dihydroxykaurane	[28]
C. poilanei Gagnep.	bark	poilaneic acid	[29]
		(*1R,2E,4Z,7E,11Z*)-12-carboxl -1-	[29]
		isopropyl	[29]
		4,8-dimethyl cyclotetradecate	[29]
		traene	[29]
C. sonderianus	heart wood	sonderianin	[30]
		coumarin, scopoletin	[30]
	roots	hardwickiic acid	[31]
		12-hydroxyhardwickic acid	[32]
		sonderianial	[32]
		(-)-hardwickic acid	[33]
		3,4-secotrachylobanoic acid	[33]

Table 2 Continued

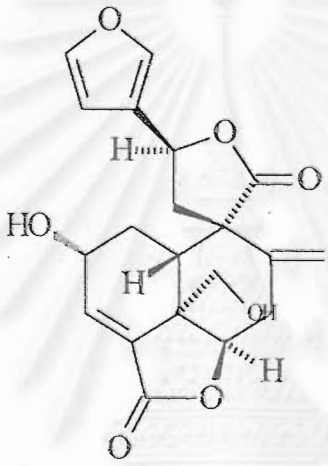
Scientific Name	Plant parts	Chemical compounds	Reference
C. lechleri	sap	1,3,5-trimethoxy benzene	[34]
		2,4,6-trimethoxy phenol	[34]
		3,4-dimethoxy phenol	[34]
		3,4-dimethoxy benzyl alcohol	[34]
		4-hydroxy phenethyl alcohol	[34]
		alcohol acetate	[34]
		sitosterol	[34]
		Sitosterol-β-D-glucopyranoside	[34]
		β-sitostenone	[34]
	bark	crolechinol	[34]
		crolechinic acid	[34]
C. cajucara	bark	*t*-dehydrocrotonin	[35]
	leaves	cajucarinolide	[35]
	bark	triterpene acetyl aleuritolic acid	[36]
	bark	*t*-cajucarin B	[37]
		sacacarin	[37]
		t-crotonin	[38]
		cajucarin B	[39]
	stem bark	*trans*-dehydrocrotonin (DCTN)	[37]
	leaves	kaempferol 3,4′,7-trimethyl ether	[40]
	leaves	3,7-dimethyl ether	[40]



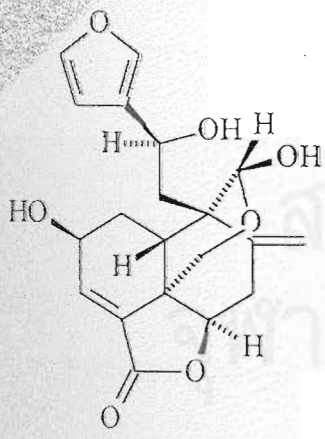
plaunol A [27]



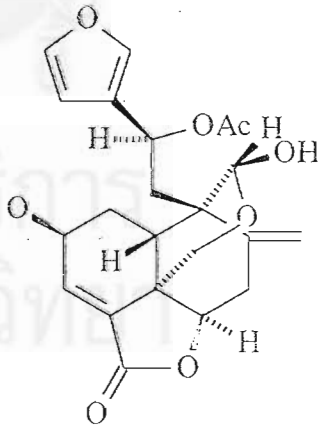
plaunol B [27]



plaunol C [27]

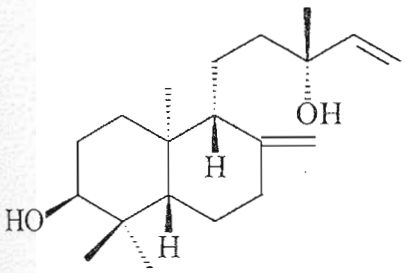


plaunol D [27]

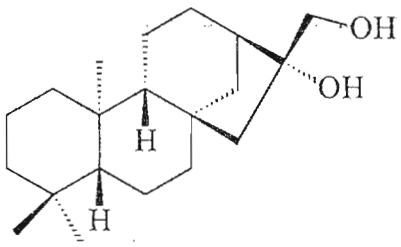


plaunol E [27]

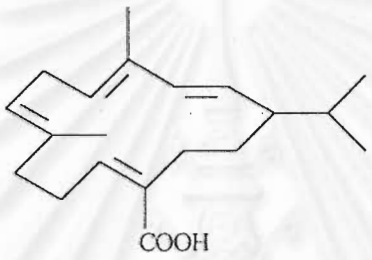
Figure 3 Structures of isolated compounds from *Croton* genus



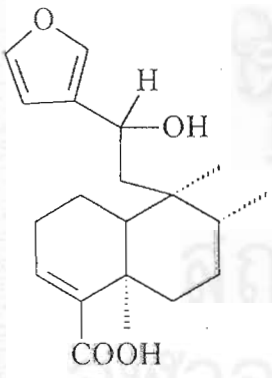
ent-3α-hydroxy-13-epimanool [28]



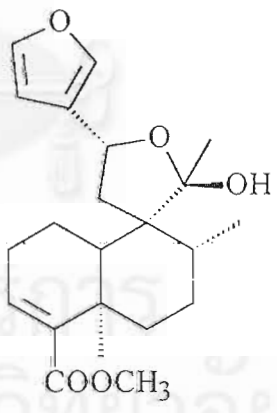
ent-16β, 17-dihydroxykaurane [28]



poilaneic acid [29]

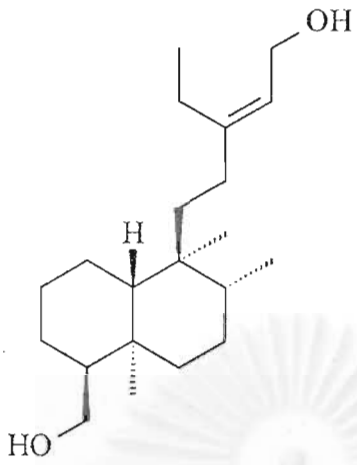


12-hydroxyhardwickic acid [32]

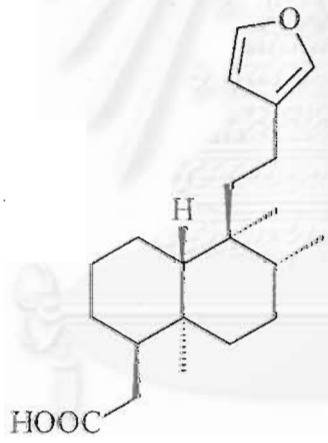


sonderianial [32]

Figure 3 Continued



crolechinol [34]



crolechinic acid [34]

Figure 3 Continued

2.4 Biological activity on cyclic AMP phosphodiesterase

In 1960, Sutherland and Rall [41] were the first to describe the role of cyclic AMP as a ''second messenger'', mediating the response of cells to a variety of hormones and neurotransmitters. Sutherland and his coworkers subsequently demonstrated the importance of cyclic AMP in the regulation of a variety of metabolic processes, including cardiac and smooth muscle contractility, glycogenolysis, platelet aggregation, secretion, and lipolysis. The physiological importance of cyclic GMP, on the other hand, remains largely a mystery, and early suggestions that cyclic GMP acts in opposition to cyclic AMP have in many cases been discounted.[42]

Cyclic nucleotide phosphodiesterases (PDEs, E. C. 3. 1. 4. 17) which degrade the second messengers cyclic 3′,5′-adenosine monophosphate (cyclic AMP) and cyclic 3′,5′-guanosine monophosphate (cyclic GMP) to 5′-AMP and 5′-GMP, nucleotides which are unable to activate the protein kinase cascade. As second messengers, cyclic AMP and cyclic GMP play key roles in the functional responses of cells to many hormones and neurotransmitters. The cyclic AMP cascade is depicted in Fig. 4.

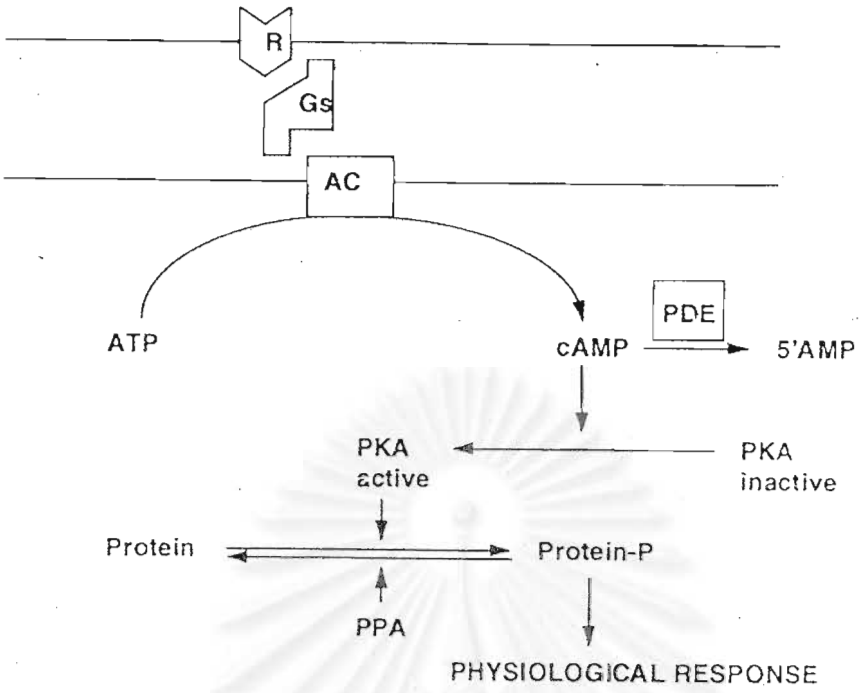


Figure 4 Cyclic AMP cascade

The cyclic AMP cascade illustrated the role of cyclic AMP PDE. Hormones, neurotransmitters, mediators, etc. bind to specific receptors (R) coupled to adenylate cyclase (AC) through a stimulatory G-protein (Gs). AC catalyzes the conversion of ATP to cyclic AMP by the catalytic action of adenyl cyclase which binds to the regulatory subunit of cyclic AMP-dependent protein kinase (PKA), so uncoupling it from the catalytic moiety. The activated PKA phosphorylates interact with intracellular protein substrates which alters their activities. This results in an altered cellular response, such as smooth muscle relaxation or suppression of inflammatory cell functions. The action of cyclic AMP is abrogated by cyclic AMP phosphodiesterase (PDE) which hydrolyses the 3'-phosphodiester bound to cyclic AMP and converting it to the inactive 5'-AMP. Protein phosphatases (PPA) act to dephosphorylate proteins and counteract the action of cyclic AMP.[43]

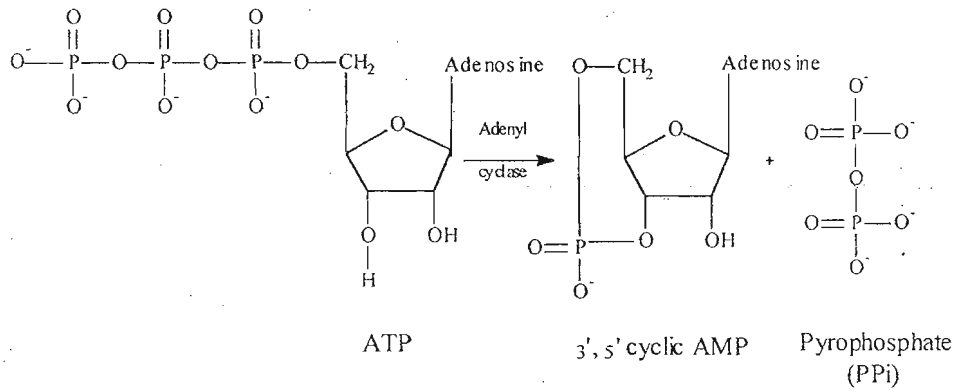
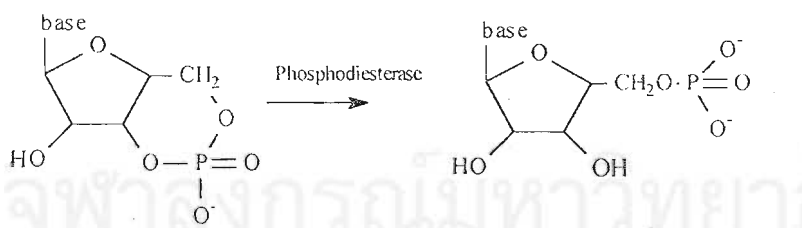


Figure 5 Formation of cyclic AMP

Seven cyclic nucleotide PDE families have been identified by Michaeli *et al.*[45] The families are designated as the Roman numberals I, II, III, IV, V, VI and VII, corresponding to Ca^{2+}/calmodulin stimulated-, cyclic GMP-stimulated-, cyclic GMP-inhibited-, cyclic AMP specific-, cyclic GMP-specific-, photoreceptor- and rolipram insensitive (cyclic AMP-specific)-PDEs, respectively.[44]

Phosphodiesterase catalyzes the apparently nonreversible hydrolysis of the 3′-bond in the cyclic nucleotides to produce their non-cyclic 5′-mono phosphate derivatives (Fig. 6). Phosphodiesterase catalyzed hydrolysis is the major physiological pathway for the termination of the intracellular effects of the cyclic nucleotides. There is not much support for other pathways for the degradation of cyclic AMP.



Cyclic nucleoside 3′,5′-monophosphate

nucleoside-5′-monophosphate

Figure 6 Phosphodiesterase catalyzes the cyclic nucleotide to noncyclic nucleotide

Phoshodiesterases occur widely in biological systems. They are present in nearly all mammalian tissues with the possible exceptions of red blood cells and isolated rat adrenal cells. These two cellular systems provide excellent models for studying cyclic nucleotide synthesis uncomplicated by the interference of phosphodiesterases. These enzymes have been found also in bacteria, yeast, insects, higher plants and several marine organisms. In general, they are found wherever the cyclic nucleotides exist. Phospho diesterase activity is detected early in embryonic development, increases up to maturity, seems to decrease with age, and varies in activity with the cell cycle.[2]

The development of novel phosphodiesterase (PDEs) inhibitors is of major interest. PDEs play a critical role in various biological processes by hydrolyzing the key second messengers adenosine and guanosine 3′,5′-cyclic monophosphate nucleotides (cAMP and cGMP, respectively) to the corresponding 5′-monophosphate nucleotides. Therefore, inhibition of PDE activity produces an increase of AMP and GMP intracellular levels that activates specific protein phosphorylation pathways involved in a variety of functional responses. Modulation of the intracellular second messenger cyclic 3′,5′-adenosine monophosphate (cAMP) by phosphodiesterase type 4 (PDE4) inhibitors represents a promising new approach for the treatment of chronic inflammatory diseases such as asthma, COPD, and rheumatoid arthritis.[46] Some of the cAMP phosphodiesterase inhibitors also have inhibitory activity on platelet aggregation. A cAMP phosphodiesterase inhibition test was recently introduced for the research of novel cardiotonics.[47]

The development of novel phosphodiesterase inhibitors is of major interest. Inhibition of phosphodiesterase activity has been reported for many different classes of compounds. These include the prototypical nonselective inhibitors papaverine, theophylline and 3-isobutyl-1-methylxanthine (IBMX).[42] The structures of these compounds are shown in Fig. 7.

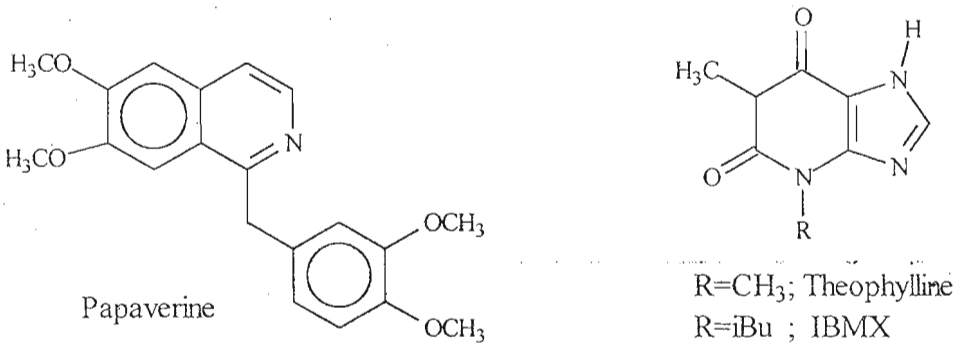


Figure 7 Some phosphodiesterase inhibitors

A literature surveys of cAMP phosphodiesterase inhibition have been widely studies. The various types of compounds are shown in Table 3 and some of their structures are shown in Fig. 8.

Table 3 Inhibitiory activity on cAMP phosphodiesterase of some compounds

Samples	IC_{50} (10^{-5}M)	References
1. Mangostanol from *Garcinia mangostana*		[10]
Mangostanol	4.7	
α-mangostin	2.4	
γ-mangostin	5.0	
2. Flavonoid from Licorice roots		[48]
Isoliquiritigenin-4'-*O*-apioglucoside	171	
Isoliquiritigenin	18	
Liquiritigenin	108	
Glabridin	8.2	
Licoricidin	4.9	
Licoarylcoumarin	1.0	
Glycycoumarin	0.7	
Glycyrol	4.4	
Licoricone	2.3	
papaverine	3.0	
3. Cerveratrum alkaloids from bulbs of *Fritillaria persica*		[49]
Compound 1 ($C_{27}H_{49}NO_2$)	24.7	
Compound 2 ($C_{27}H_{45}NO_2$)	8.8	
Compound 3 ($C_{33}H_{55}NO_7$)	12.7	
Compound 4 ($C_{27}H_{45}NO_2$)	>500	
Compound 5 ($C_{33}H_{55}NO_7$)	18.3	

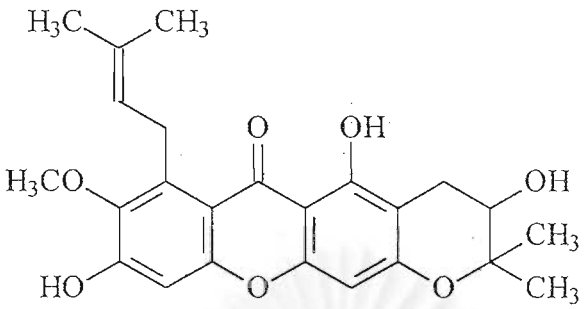
Table 3 Continued

Samples	IC_{50} (10^{-5}M)	References
4. Steroidal alkaloids from the bulbs of *Fritillaria persica*		[50]
Compound 2 ($C_{27}H_{41}NO_3$)	10.6	
Compound 3 ($C_{33}H_{51}NO_8$)	67.9	
Compound 4 ($C_{27}H_{41}NO_3$)	21.4	
Compound 5 ($C_{33}H_{51}NO_8$)	17.1	
5. Steroidal saponins from the rhizomes of *Smilax sieboldii*		[51]
Compound 1 ($C_{44}H_{70}NO_{18}$)	8.3	
Compound 2 ($C_{38}H_{60}NO_{13}$)	3.4	
Compound 6 ($C_{44}H_{72}NO_{17}$)	3.2	
6. Steroidal saponins from *Smilax riparia* and *S. china*		[52]
Compound 1 ($C_{39}H_{64}O_{12}$)	10.2	
Compound 2 ($C_{45}H_{74}O_{17}$)	5.5	
Compound 3 ($C_{51}H_{82}O_{21}$)	4.7	
Compound 4 ($C_{52}H_{86}O_{22}$)	29.4	
Compound 5 ($C_{45}H_{72}O_{16}$)	33.3	
Compound 6 ($C_{45}H_{72}O_{17}$)	9.3	

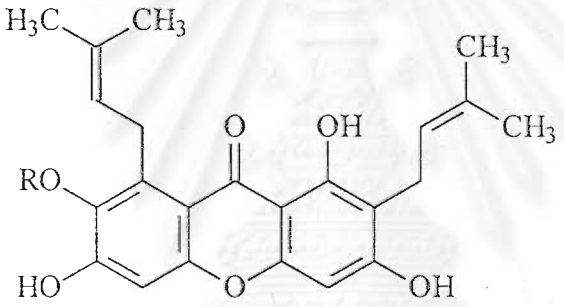
Table 3 Continued

Samples	IC_{50} (10^{-5}M)	References
7. Steroidal saponins from the tubers of *Dichelostemma multiflorum*		[53]
Compound 1 ($C_{40}H_{64}O_{13}$)	20.6	
Compound 1[b]	76.2	
Compound 2 ($C_{46}H_{74}O_{17}$)	11.8	
Compound 2[a]	10.0	
Compound 3	12.3	
Compound 4	11.4	
Compound 5 ($C_{50}H_{82}O_{22}$)	15.4	
Papaverine (positive control)	3.0	

1. Mangostanol from *Garcinia mangostana*



Mangostanol [10]

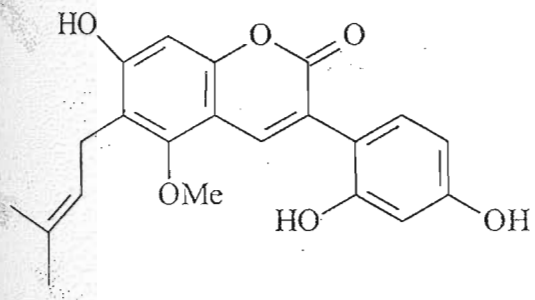


R=CH_3; α-mangostin [10]

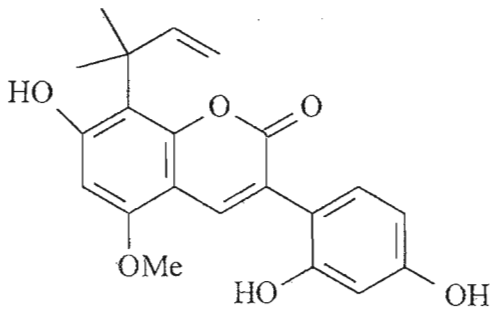
R=H; γ-mangostin [10]

Figure 8 Structure of compounds that have inhibitory activity on cAMP phosphodiesterase

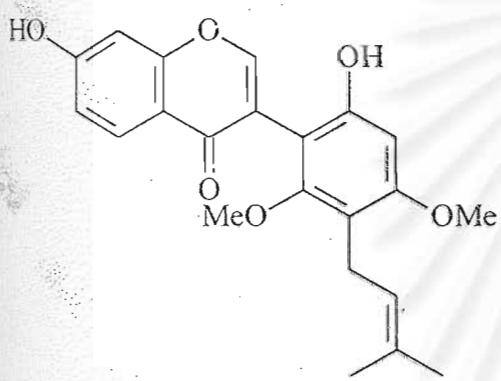
2. Flavonoid from Licorice roots



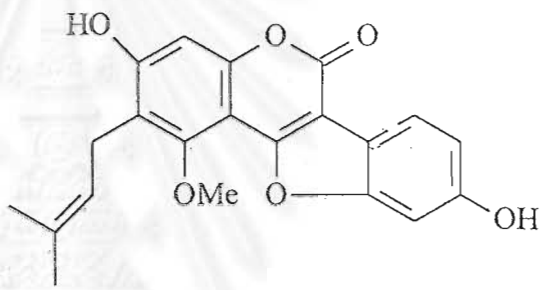
Glycycoumarin [48]



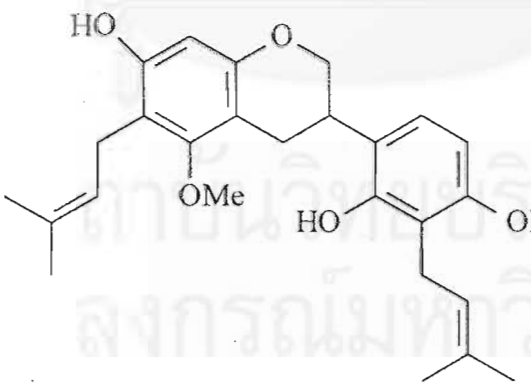
Licoarylcoumarin [48]



Licoricone [48]



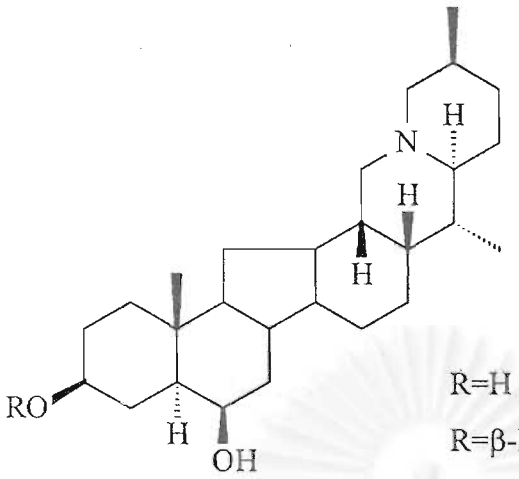
Glycyrol [48]



Licoricidin [48]

Figure 8 Continued

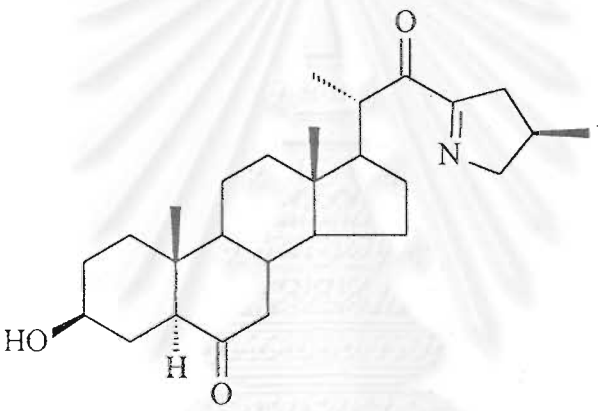
3. Cerveratrum alkaloids from bulbs of *Fritillaria persica*



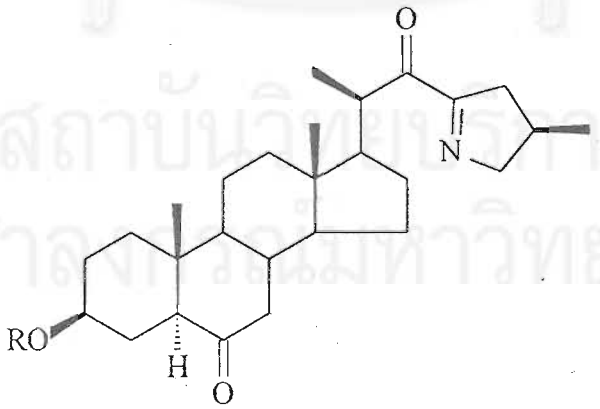
R=H ; Compound 2 [49]

R=β-D-Glc*p* ; Compound 3 [49]

4. Steroidal alkaloids from the bulbs of *Fritillaria persica*



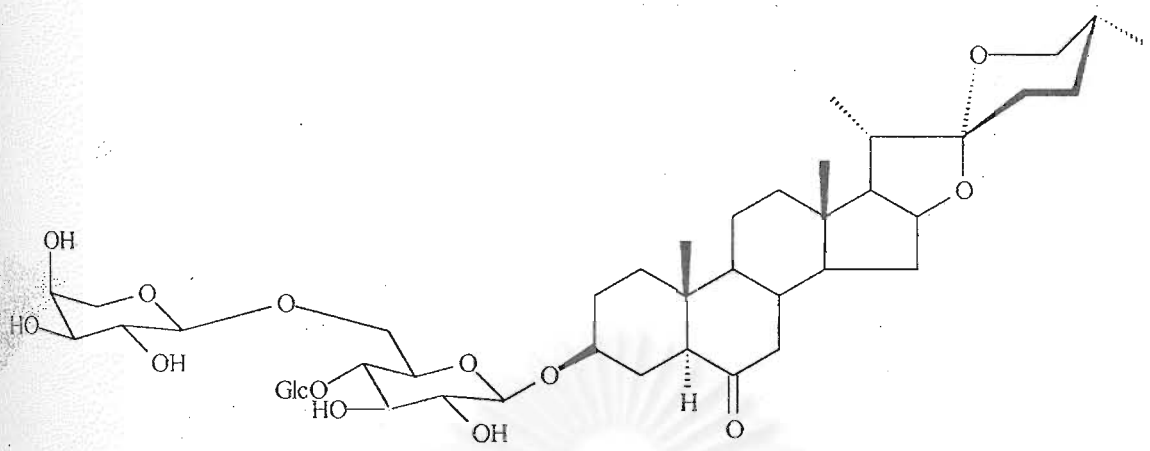
Compound 2 ($C_{27}H_{41}NO_3$) [50]



R= R=β-D-Glc*p*; Compound 5 ($C_{33}H_{51}NO_8$) [50]

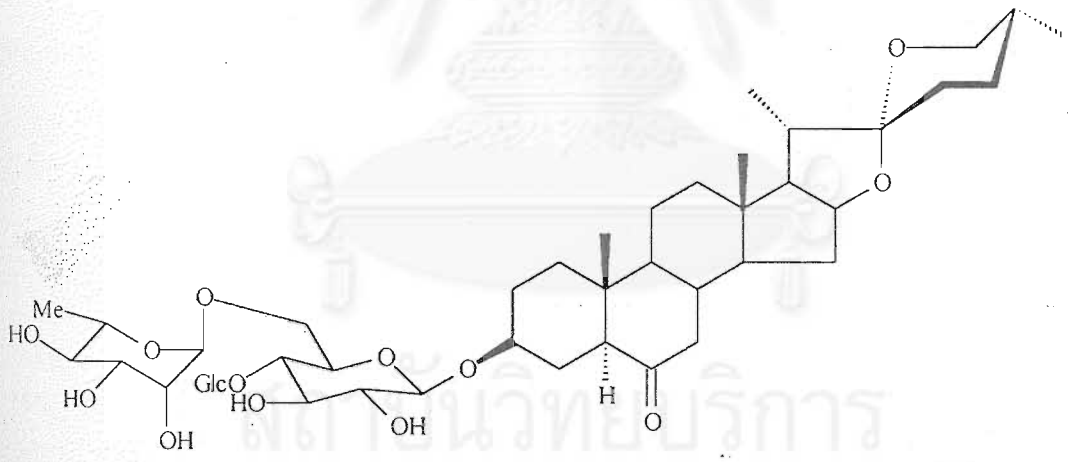
Figure 8 Continued

5. Steroidal saponins from the rhizomes of *Smilax sieboldii*



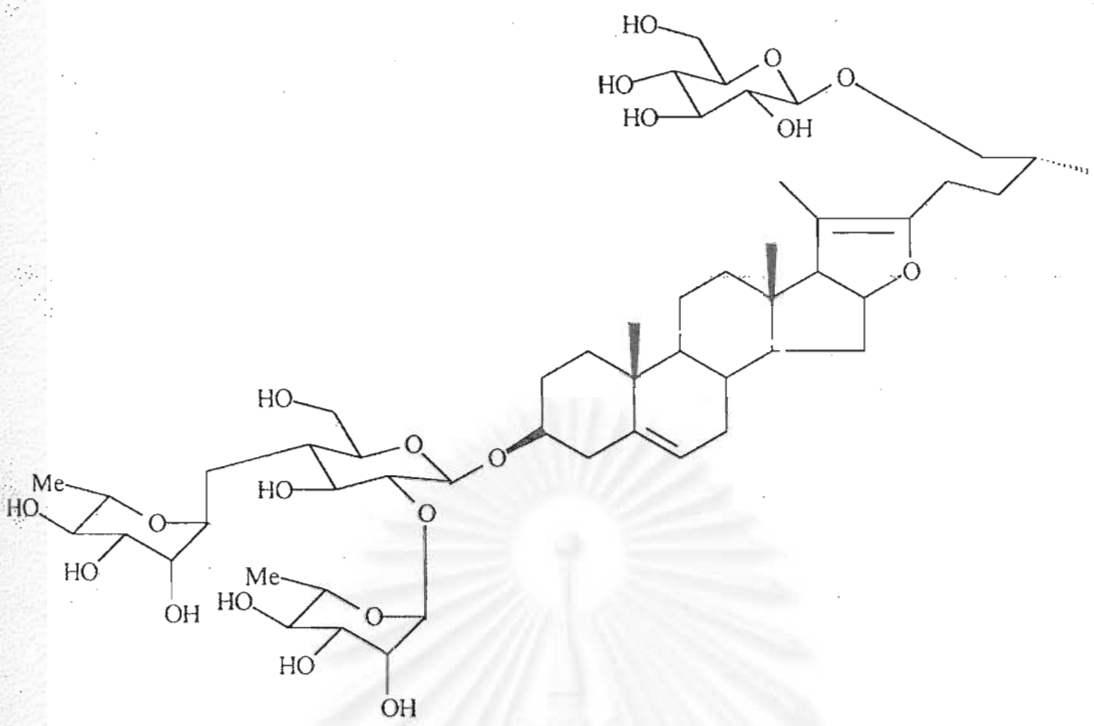
Compound 2 ($C_{38}H_{60}NO_{13}$) [51]

6. Steroidal saponins from *Smilax riparia* and *S. china*



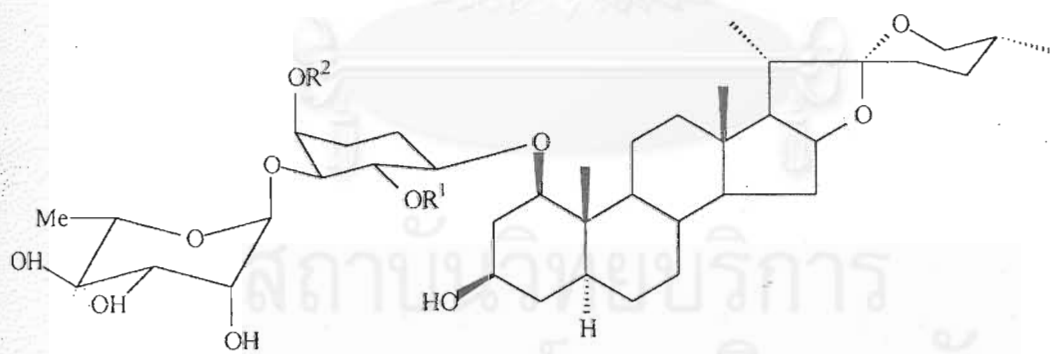
Compound 2 ($C_{45}H_{74}O_{17}$) [52]

Figure 8 Continued



Compound 3 ($C_{51}H_{82}O_{21}$) [52]

7. Steroidal saponins from the tubers of *Dichelostemma multiflorum*



R^1= -L-Rhap; R^2=Ac: Compound 2 [53]
R^1= -L-Rhap; R^2=H: Compound 2a

Figure 8 Continued

CHAPTER III

EXPERIMENTAL

3.1 Plant Material

The stem bark of *C. oblongifolius* Roxb. used in this study was collected from Amphur Tha-Uthen, Nakhonphanom Province, Thailand, in 1999, Botanical identification was achieved through comparison with a voucher specimen No. BKF 84729 in the herbarium collection of the Royal Forest Department, Ministry of Agriculture and Cooperatives, Bangkok, Thailand.

3.2 Instruments and Equipments

3.2.1 Rotary Evaporator

The Buchi rotary evaporator model N-1 was used for the rapid removal of a large amounts of volatile solvents.

3.2.2 Fourier Transform Infrared Spectrometer (FT-IR)

The FT-IR spectra were recorded on a Nicolet Impact 410 Fourier Transform Infrared Spectrometer. Spectra of solid samples were recorded by incorporating the sample into a pellet of potassium bromide and spectra of liquid samples were recorded as thin films (NaCl cells).

3.2.3 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-NMR and at 50.32 MHz for ^{13}C-NMR.

The chemical shifts in δ (ppm) were referenced to the signal from the residual proton in deuterated solvents. Assignments of ^{13}C-NMR spectra were assisted by Distortionless Enhancement by Polarization transfer (DEPT).

3.2.4 Mass Spectrometer (MS)

The mass spectra were recorded on a Fisons Instruments Mass Spectrometer Model Trio 2000 GC-MS in Electron Impact (EI) mode at 70 eV.

3.2.5 Melting Point

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

3.2.6 Ultraviolet-Visible spectrometer (UV-Vis)

UV-VIS spectra were recorded on a Hewlett Peckard 8452A with diode array spectrophotometer in MeOH.

3.2.7 Optical Rotation

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

3.2.8 X-ray Diffractrometer

The x-ray diffractrometer were obtained on a SIEMEN SMART diffractrometer at Department of Physics, Faculty of Science and Technology, Thammasat University.

3.2.9 Microtiterplate Reader

The microtiterplate reader for biological activity test was carried out using a Awareness 3200 titreplate reader.

3.3 Solvents and Chromatographic Media

3.3.1 Solvents

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

3.3.2 Chromatographic Media

3.3.2.1 Merck' s silica gel 60 G Art. 7734.1000 (70-230 mesh ASTM) and 9385 (230-400 mesh ASTM) were used as adsorbents for normal column chromatogarphy and flash column chromatography.

3.3.2.2 Merck' s TLC aluminium sheets, silica gel 60 F254, 20 × 20 cm^3, layer 0.2 mm were used to identify the identical fractions.

3.4 The cAMP phosphodiesterase assay

3.4.1 cAMP phosphodiesterase (bovine heart) from Sigma Chemical Company, U.S.A.

3.4.2 5′-nucleotidase (*Crotualus atrox* venom) from Sigma Chemical Company, U.S.A.

3.4.3 Adenosine 3′,5′-monophosphate (c-AMP) sodium salt from Sigma Chemical Company, U.S.A.

3.4.4 Malachite green or Brilliant green ($C_{27}H_{34}N_2S$) from Tokyo Kasei Kogyo, Japan.

3.4.5 Polyvinyl alcohol from Metro Company Limited.

3.4.6 Ammonium molybdate from E. Merck Company, Germany.

3.4.7 Sodium citrate dihydrate from E. Merck Company, Germany.

3.4.8 Sodium dihydrogen phosphate monohydrate from E. Merck Company, Germany.

3.4.9 Tris (hydroxymethyl)-aminomethane from E. Merck Company, Germany.

3.4.10 Theophylline (1,3-dimethylxanthine) anhydrous from Sigma Chemical Company, U.S.A.

3.5 Extraction and isolation of stem bark of *croton oblongifolius* Roxb. from Amphoe Tha-Uthen, Nakhonphanom province

The powdered, sun-dried stem bark of *C. oblongifolius* Roxb. (4.5 kg) from Amphoe Tha-Uthen, Nakhonphanom province was soaked in hexane (12 liters) for 6-7 days at room temperature for 3 times. The solution was filtered, and evaporated under reduced pressure to obtain hexane extract of 154.72 g (2.38 % wt by wt) as a yellowish green oil. The residue was re-extracted with ethyl acetate (8 liters) for 3 times at room temperature. The filtered ethyl acetate solution was evaporated to afford the ethyl acetate extract of 162.96 g (3.62 % wt. by wt.) as a dark–red gummy and residue was re-extracted again with methanol (8 liters) until the eluted solution was clear. The combined methanol

solution was concentrated using a rotary evaporator under reduced pressure to give a methanol extract of 170.65 g (3.79 % wt by wt) as a dark-brown gummy.

3.6 Separation of the chemical constituents of the *C. oblongifolius* Roxb.

3.6.1 Separation of hexane crude extract

The hexane crude extract (120 g) was separated by Column chromatography using silica gel Art 7734 as stationary phase and hexane-ethyl acetate as gradient eluant in a stepwise fashion. Approximately 50 ml of eluant was collected for each fraction and concentrated by using a rotary evaporator. Each fraction was monitored by TLC and the identical fractions were combined.

3.6.2 Separation of ethyl acetate crude extract

The ethyl acetate crude extract (120 g) was separated by silica gel column Chromatography using Merck Silica gel 60 (Art 7734.1000, 70-230 mesh ASTM) as stationary phase and hexane-ethyl acetate as gradient eluant in a stepwise fashion.

After the eluate was collected to approximately 50 ml for each fraction, eluted solution was monitored by TLC. The fractions containing similar components were combined and then concentrated using a rotary evaporator to a final volume of 30 ml.

The methanol crude extract was obtained as a dark-brown gummy (170.65 g). This extract was not carried out for further separation because it was insoluble in all organic solvents use in this experiment.

3.7 Cyclic AMP phosphodiesterase assay

The original method for measuring the activity of adenosine 3′,5′-monophosphate phosphodiesterase (adenosine 3′,5′-monophosphate 5′-phosphate phosphohydrolase, E.C.3.1.4.17) (phosphodiesterase) as described by Buther and Sutherland in 1962 has remained essentially unchanged over past several years.[49] Chan, K. M,. and *et al.*, 1986 reported a simple and rapid colourimetric assay for measuring the high affinity Ca^{2+}-ATPase activity in subcellular fractions. A one step addition of a malachite green/ molybdate/polyvinyl alcohol reagent to the assay mixture at the end of the incubation period is all that is required for the spectrophotometric quantification of the phosphomolybdate malachite green complex.[50]

This thesis describes a method for determining phosphodiesterase activity by the malachite green method, which is highly sensitive to inorganic phosphate. The amount of inorganic phosphate liberated from the reaction of adenosine 5′-monophosphate and 5′-nucleotidase was measured. Figure 9 illustrates the quantitative determination of cyclic AMP phosphodiesterase activity by the malachite green method.

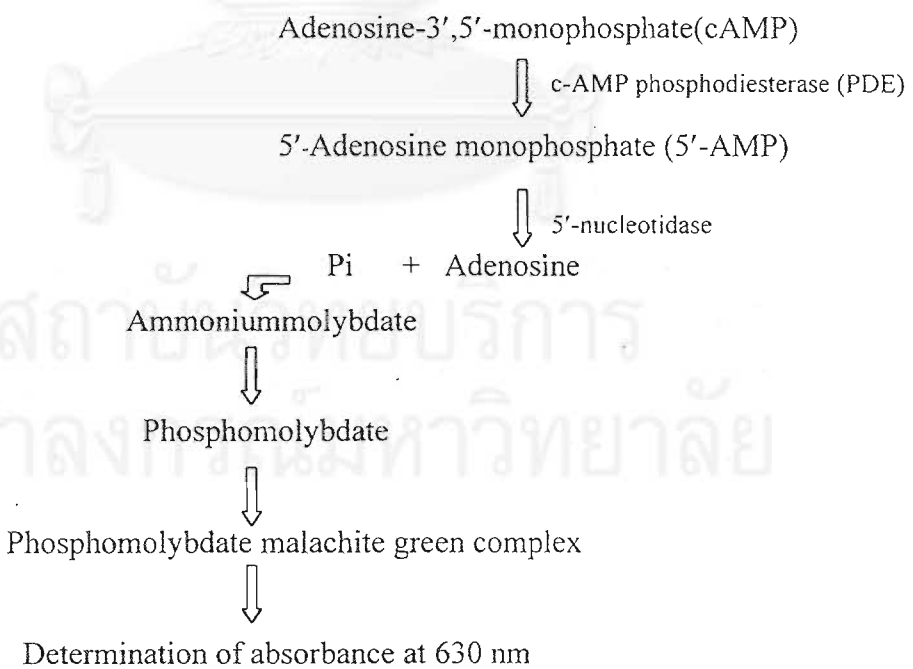


Figure 9 Quantitative determination of cAMP phosphodiesterase activity by malachite green method.

3.7.1 The procedure of PDE assay

The PDE assay of all compounds were carried out using bovine heart enzyme (PDE) and *Crotualus atrox* venom (5'-nucleotidase). The standard assay mixture contained, in a final volume of 100 µl, 0.5 M Tris-HCl buffer (pH 7.4) with or without 10 µl of enzyme solution. The stock solution of the sample compound in DMSO (dimethylsulfoxide) was added to the assay mixture to give 1 mM, 0.1 mM and 0.01 mM final concentrations of compounds. Cyclic AMP (10 mM) was added and incubation was carried out for 15 min at 37°C. The reaction was stoped by using 25% Na-citrate solution. Liberated orthop phosphate (Pi) was determined by the method of Chan and et al. [50] One unit of specific activity is defined as the liberation of 1 µmol of inorganic phosphate per mg protein per min. The bioassays were performed in five times replication on microtitre plates (96 wells) which contained 420 µl of mixture per well. Control was the PDE enzyme containing DMSO in equivalent amounts as the samples. Theophylline was used as a positive control. All reagents were prepared freshly and distilled water was used in making these reagents.

CHAPTER IV

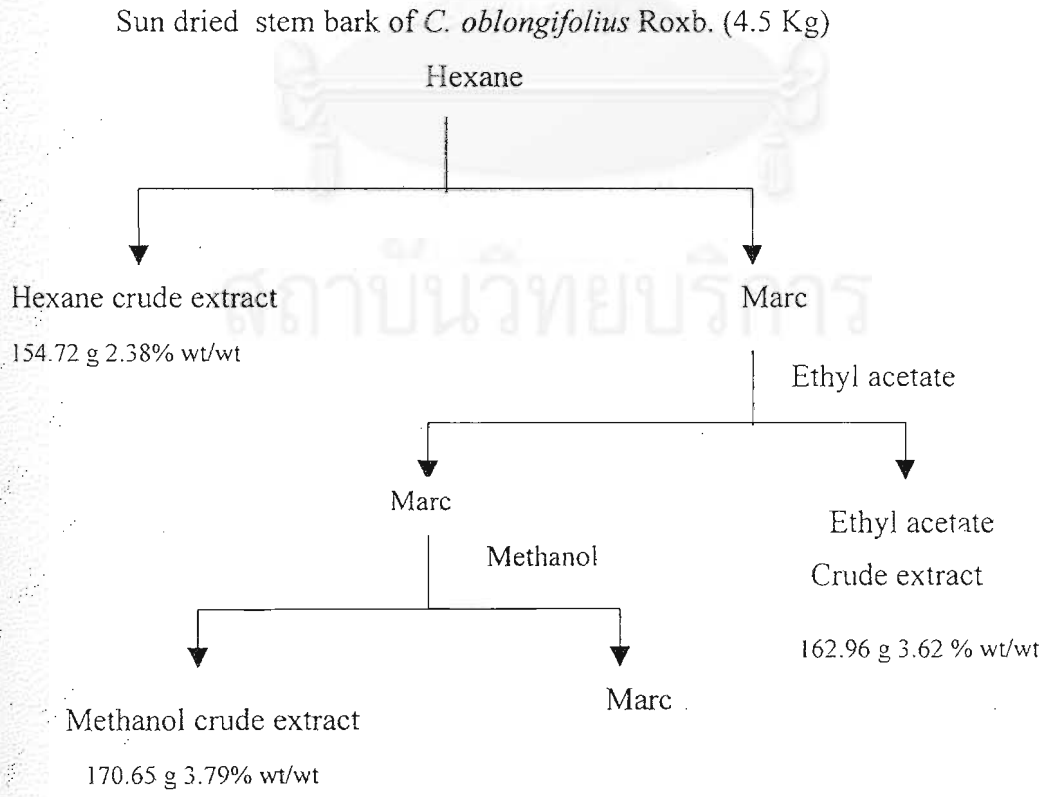
RESULTS AND DISCUSSION

The stem bark of *C. oblongifolius* was extracted by hexane, ethyl acetate and methanol to obtain three different crude extracts as shown in Table 4 and the procedure of the extraction is shown in scheme 1.

Table 4 Various extracts of the stem bark of *C. oblongifolius* Roxb.

Solvent extract	Appearance	Weight	%wt/wt of starting material
Hexane	yellowish green oil	154.72	2.38
Ethyl acetate	dark-red gummy	162.96	3.62
Methanol	dark-brown gummy	170.65	3.79

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



4.1 Isolation of chemical constituents of *C. oblongifolius* Roxb.

4.1.1 Separation of hexane crude extract

The hexane crude extract (120 g) was separated by column chromatography using hexane-ethyl acetate as gradient eluant in a stepwise fashion. The results of the separation of the hexane crude extract are presented in Table 5.

Table 5 Results of separation of hexane crude extract by column chromatography

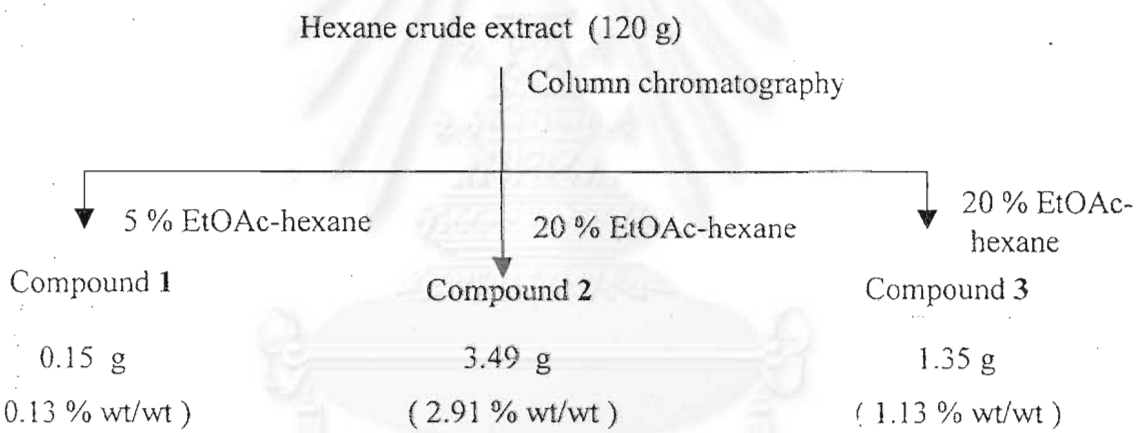
Eluent	Fraction No	Remark	Weight (g)
Hexane	1-50	Orange oil	1.76
	51-63	Yellow oil	1.76
	64-75	Yellow oil	1.55
	76-94	Yellow oil	1.64
5% EtOAc-hexane	95-105	Yellowish oil	1.74
	106-125	Dark yellow oil	1.56
	109-111	Semi solid in orange oil	1.05
10% EtOAc-hexane	112-157	Orange oil	1.04
	158-165	Yellow crystal in yellow oil	1.57
	166-230	Dark brown oil	2.34
20% EtOAc-hexane	231-237	Yellow crystal in brown oil	3.39
	238-260	Yellow solid in brown oil	1.46
	261-275	Brown oil	2.58
30% EtOAc-hexane	276-301	Brown oil	2.12
	302-324	Yellow solid in brown oil	1.58
	325-370	Brown oil	1.85
40% EtOAc-hexane	371-381	Dark yellow oil	1.68
	382-427	Dark brown oil	2.07
50% EtOAc-hexane	428-442	Dark brown oil	0.10
	443-479	Dark brown oil	1.29
60% EtOAc-hexane	480-511	Dark brown oil	0.03

Table 5 Continued

Eluent	Fraction No	Remark	Weight (g)
70%EtOAc-hexane	512-534	Dark brown oil	0.09
80%EtOAc-hexane	535-612	Dark brown oil	0.05
90% EtOAc-hexane	613-685	Dark brown tar	2.30
100%EtOAc	686-710	Dark brown tar	1.42
10% EtOAc–MeOH	711-730	Dark brown tar	1.10

The procedures of compound **1-3** from hexane crude extract are shown in scheme 2.

Scheme 2 Isolation of compound **1-3** from hexane crude extract



4.1.2 Separation of ethyl acetate crude extract

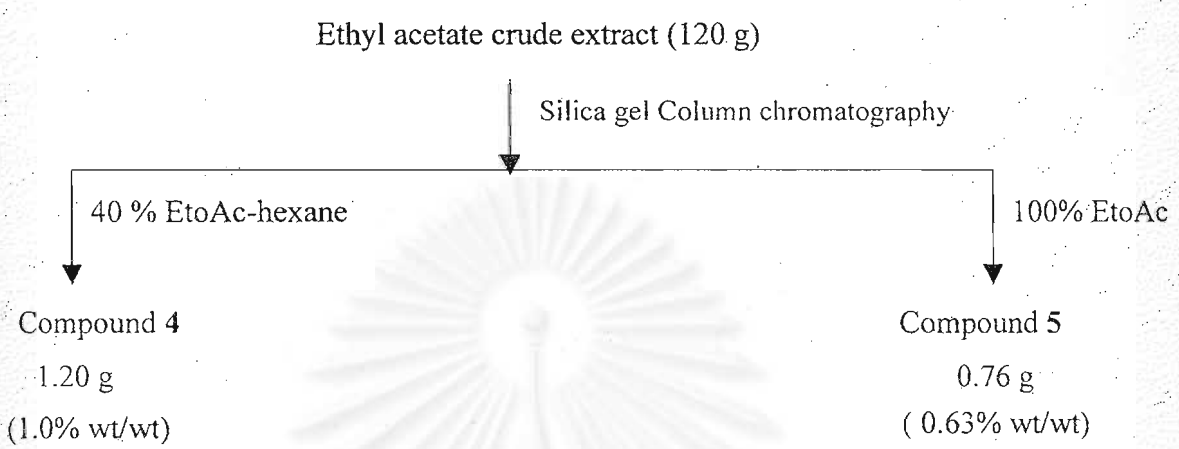
The ethyl acetate crud extract (120 g) was separated by column chromatography using hexane-ethyl acetate gradient in a stepwise fashion. The results from the separation of ethyl acetate crud extract are shown in Table 6.

Table 6 Result of the separation of ethyl acetate crude extract

Eluent	Fraction No	Remark	Weight (g)
Hexane	1-93	Yellow oil	0.29
5% EtOAc-hexane	94-118	Semi solid in yellow oil	1.36
	119-148	Dark yellow oil	0.52
10% EtOAc-hexane	149-169	Red-brown oil	0.65
	170-195	Yellow oil	4.60
	196-212	Semi solid in yellow oil	2.78
20% EtOAc-hexane	213-258	Semi solid in yellow oil	1.25
	259-271	Semi solid in yellow oil	0.91
	272-281	Semi solid in yellow oil	0.71
	282-294	Semi solid in yellow oil	3.14
30% EtOAc-hexane	295-328	Amorphous solid in yellow oil	5.82
	329-380	Brown oil	4.64
40% EtOAc-hexane	381-443	Brown oil	3.81
	444-489	White crystal in yellow oil	7.23
50% EtOAc-hexane	490-544	White crystal in yellow oil	2.81
60% EtOAc-hexane	545-763	Dark brown oil	4.44
70% EtOAc-hexane	764-837	Dark brown oil	2.53
80% EtOAc-hexane	838-869	Brown oil	1.85
90% EtOAc-hexane	870-903	Dark brown oil	1.24
100% EtOAc	904-1020	Dark brown oil	2.47
10% EtOAc–MeOH	1021-1024	White solid in brown oil	1.82
	1025-1050	Dark brown tar	2.95

The isolation of compounds **4-5** from ethyl acetate crude extract is shown in scheme 3.

Scheme 3 Isolation of compounds **4-5** from ethyl acetate crude extract



The hexane and the ethyl acetate crude extracts of the stem bark of *C. oblongifolius* Roxb. were separated by silica gel column chromatography to obtained five compounds. These compounds are shown in Table 7.

Table 7 Results of the separation of hexane and ethyl acetate crude extract of *C. oblongifolius* Roxb. by column chromatography

Compounds	Physical appearance	Weight (g)
1	Colourless crystal	0.15
2	Bright yellow needles	3.49
3	Yellow crystal	1.35
4	White needle crytal	1.20
Mixture **5**	White amorphous powder	0.76

4.2 Purification and properties of the compounds from *Croton oblongifolius* Roxb.

4.2.1 Purification and properties of compound 1

Compound **1**, a colorless crystals (0.15 g, 0.13 %yield from crude hexane and 0.003 %yield from starting material). The following properties were determined: m.p. 84-85 °C, $[\alpha]_D^{20}$–4.6 (0.315 g/100 ml in $CHCl_3$), UV$\lambda_{max}^{CHCl_3}$ nm (logε):206(2.57) ; EI-MS *m/z* found 222, Calcd for $C_{15}H_{26}O$. The R_f value of this compound was 0.63 (0.15 %EtOAc–hexane, SiO_2). Compound **1** was soluble in hexane, ethyl acetate, ethanol, chloroform, methanol and DMSO.

FI-IR spectrum (KBr)(Fig.19):ν_{max} (cm^{-1}):3332(m), 2937(s), 2865(s), 1470(m), 1376(m), 1107(m), 988(w), 939(w), and 888(w).

^{1}H-NMR spectrum (200.13 MHz, $CDCl_3$) (Fig. 20) δ (ppm):2.30 (1H, s), 2.06 (1H, td, *J*=10, 8 Hz), 1.01-1.61 (9H, m), 1.17-1.34 (1H, m), 1.12 (3H, s), 1.02 (3H, s), 0.96 (3H, s), 0.92 (3H, d, *J*=6Hz), 0.69-0.76 (1H, m), and 0.31 (1H, dd, *J*=10 Hz)

^{13}C-NMR spectrum (50.32 MHz, $CDCl_3$) (Fig. 21) δ (ppm): 74.59 (s), 53.75 (d), 40.76 (d), 39.18 (t), 38.41 (d), 30.78 (t), 30.49 (q), 28.64 (q), 25.0 (d), 24.61 (t), 23.37 (d), 20.82 (t), 19.71 (s), 15.98 (q), and 15.40 (q).

EI-MS spectrum *m/z*:(Fig. 23), 222 [M^+](15), 204 [M^+-H_2O](24), 189(20), 161 (41), 147(30), 122(100), 109(90), 81(75), 69(83), and 55(48).

4.2.2 Purification and properties compound 2

Compound **2**, a bright yellow needle, was recrystallized from chloroform several times to give 3.49 g (2.91 %yield from crude hexane extract and 0.08 %yield from starting material), m.p. 126-128°C, $[\alpha]_D^{20}$–3.7 (0.365 g/100 ml in $CHCl_3$), UV$\lambda_{max}^{CHCl_3}$ nm (logε):270(4.37); EI-MS *m/z* found 328 and calc. for $C_{18}H_{16}O_6$. The R_f value of this compound was 0.58 (25 % EtOAc-hexane, SiO_2). Compound **2** was soluble in chloroform, DMSO, ethanol, and methanol.

FT-IR spectrum (KBr)(Fig. 24):ν_{max} (cm^{-1}):3600-3200(br, w), 3089(w), 2946(m), 2844(m), 1656(s), 1600(s), 1344(s), 1179(s), 941(w), and 831(s).

^{1}H-NMR spectrum (200.13 MHz, $CDCl_3$)(Fig. 25) δ (ppm):12.63 (1H, s), 8.04 (2H, d, *J*=8 Hz), 6.98 (2H, d, *J*=8 Hz), 6.41 (1H, d, *J*=2 Hz), 6.31 (1H. d, *J*=2 Hz), 3.87 (3H, s), 3.84 (3H, s), and 3.83 (3H, s).

^{13}C-NMR spectrum (50.23 MHz, $CDCl_3$)(Fig. 26) δ (ppm):178.75(s), 165.37(s), 161.58(s), 161.52(s), 156.71(s), 155.94(s), 138.81(s), 130.14(d), 122.75(s), 144.03(d), 106.01(s), 97.81(d), 92.12(d), 60.13(q), 55.79(q), and 55.43(q).

EI-MS spectrum *m/z* (Fig. 28):328 [M^+](90), 313 [M^+-OCH_3](6), 285(38), 150 (30), 135(31), 119(15), and 78(13).

4.2.3 Purification and properties of compound 3

Compound **3,** a yellow solid, was recrystallized from chloroform several times to give a yellow crystal, 1.35 g (1.13 % yield from crude ethyl acetate and 0.03 % yield from starting material) with m. p. of 134–135°C. $[\alpha]_D^{20}$ +1.9 (0.333 g/100 ml in $CHCl_3$), UV$\lambda_{max}^{CHCl_3}$ nm (logε):256(4.21);EI-MS *m/z* found 358, calcd. for $C_{19}H_{18}O_7$. The R_f value of this compound was 0.35 (30% EtOAc-hexane, SiO_2). Compound **3** was soluble in chloroform, ethyl acetate, methanol and hot DMSD.

FT-IR spectrum (KBr)(Fig. 29), ν_{max} (cm^{-1}):3400 –3200 (br,w), 3016, 2944, 2837 (m), 1663 (s), 1600 (m), 1515 (m), 1327 (m), 1130 (s), 1009 (m), 948 , 908 (s), 852 (s), and 807 (s)

^{1}H-NMR spectrum (200.13 MHz, $CDCl_3$)(Fig. 30) δ (ppm):12.62 (1H, s), 7.64-7.72 (2H, m), 6.95 (1H, d, *J*=6 Hz), 6.40 (1H, d, *J*=2 Hz), 6.31 (1H, d, *J*=2 Hz), 3.95 (3H, s), 3.94 (3H, s), 3.85 (3H, s), and 3.84 (3H, s).

^{13}C-NMR (50.23 MHz, $CDCl_3$)(Fig. 31) δ(ppm):178.71 (s), 165.42 (s), 161.98 (s), 156.69 (s), 155.80 (s), 151.35 (s), 148.73 (s), 138.95 (s), 122.89 (s), 122.16 (d), 111.22 (d), 110.82 (d), 105.99 (s), 97.81 (d), 92.18 (d), 60.17 (q), 56.04 (q), 55.98 (q), and 55.80 (q).

EI-MS spectrum *m/z* (Fig. 33):358 [M^+](100), 343 [M^+-CH_3](40), 315 [M^+-CH_3CO] (35), 165(50), 149(20), and 119(12).

4.2.4 Purification and properties of compound 4

Compound **4**, a white crystal, (1.2 g, 1.0 %yield from crude ethyl acetate and 0.027% yield from starting material), was obtained from silica gel column chromatography with m.p. 180–181°C $[\alpha]_D^{20}$–4.2 (0.353 g/100 ml in MeOH), UVλ_{max}^{MeOH}nm (logε):226 (1.10); EI-MS *m/z* found 256, the molecular formular was determined to be $C_{15}H_{28}O_3$. The R_f value was 0.43 in 40%EtOAc-hexane system (SiO_2). This compound was soluble in methanol and DMSO.

FT-IR spectrum (KBr)(Fig. 34), ν_{max} (cm^{-1}) showed absorbtion bands at 3400–3100(br, s), 2937(s), 1466(m), 1385, 1340(m), 1148(m), 1085, 1044(m), 984(w), and 913 (w).

^{1}H-NMR spectrum (200.13 MHz, $CDCl_3$)(Fig. 35) δ (ppm):5.7 (1H, d, *J*=2 Hz), 5.54 (1H, s), 4.44 (1H, d, *J*=6 Hz), 4.13 (2H, d, *J*=10 Hz), 2.49 (1H, s), 1.89-2.01 (1H, m), 1.39-1.54 ((1H, d, *J*=2 Hz), 14H, m), 1.18(3H, s), 1.03 (3H, d, *J*=6 Hz), 0.86 (3H, d, *J*=8 Hz), and 0.8 (3H, s)

^{13}C-NMR spectrum (50.23 MHz, $CDCl_3$)(Fig. 36) δ (ppm):155.91(s), 77.79(d), 71.87(d), 71.77(s), 49.65(d), 46.42(d), 39.97(t), 35.64(t), 28.02(t), 24.77(q), 24.51(d), 23.49 (q), 22.35(q), 22.22(t), and 13.94(q).

EI-MS spectrum *m/z* (Fig. 38):256 [M^+], 238 [M^+-H_2O](24), 223(18), 220 [M^+-H_2O](16), 195(15), 147(30), 177(16), 153(35), 123(43), 101(100), 95(50), 81(54), and 55 (48).

4.2.5 Purification and properties of mixture 5

Mixture **5**, a white powder, (75.5 mg, 0.63 % yield and 0.017% yield from starting material) was obtained from crude ethyl acetate, m.p. 270–271. $[\alpha]_D^{20}$–37.5 (0.380 g/100 ml in DMSO), UVλ_{max}^{DMSO}nm (logε):210 (1.10) ; EI-MS *m/z* found 396. The R_f value of this compound was 0.9 (20% EtOH-hexane, SiO_2), This compound was soluble in hot DMSO.

FT-IR spectrum (KBr)(Fig. 39), ν_{max} (cm^{-1}) showed sbsorption band at 3600–3200 (br, m), 2933, 2867(s), 1129(w), 1466, 1376, 1278(m), 1075, 1024 (m), 798 (w), and 667 (w).

^{1}H-NMR spectrum (200.13 MHz, $CDCl_3$)(Fig. 40) δ (ppm):5.3 (1H, s), 4.9 (4H, s), 4.48 (1H, t), 4.21 (1H, d; *J*=4 Hz), 2.87-3.04 (m), 2.49 (4H, s), and 0.64-2.37 (m).

^{13}C-NMR spectrum (50.23 MHz, $CDCl_3$)(Fig. 41) δ (ppm):140.4, 138.1, 128.5, 121.2, 100.7, 76.9, 76.7, 73.4, 70.0, and 61.0.

EI-MS spectrum *m/z* (Fig. 42):396 [M^+](50), 383 [M^+-CH_3](10), 255 (28), 213 (22), 145(45), 107(54), 95(78), 83(90), 69(82), and 57(65).

4.3 Structure elucidation of the isolated compounds from the stem bark of *Croton oblongifolius* Roxb.

4.3.1 Structure elucidation of compound 1

Compound **1** was isolated from hexane crude extract with 5% EtOAc-hexane to obtain colourless crystals, m.p. 84-85 °C. The structure of compound **1** was elucidated by using spectroscopic tecniques.

IR-spectrum of compound **1** is shown in Fig. 19. The spectrum showed important absorption band at 3332 cm^{-1} (O-H stretching vibration of alcohol), 2937,2865 cm^{-1} (C-H stretching vibration of $-CH_3$,$-CH_2$), 1470, 1376 cm^{-1} ($-CH_2$, $-CH_3$ bending) and 1107 cm^{-1} (-C-O stretching vibration).

The ^{1}H-NMR spectrum in $CDCl_3$ (Fig. 20) of compound **1** revealed four methyl groups at 0.92 (3H, d, J=6 Hz), 0.96 (3H, s), 1.02 (3H, s) and 1.12 (3H, s) one hydroxy group at 2.30 (1H, s) and three aromatic protons at 1.16-2.10 ppm and 1.19-1.34 ppm, respectively.

The ^{13}C-NMR spectrum (Fig. 21) indicated 15 signals. Moreover, DEPT 90 and DEPT 135 experiments (Fig. 22) showed four methyl carbons at 15.40, 15.98, 28.64, and 30.44 ppm, five saturated carbons at 23.37, 25.00, 38.41, 40.76, and 53.75 ppm, four methylene carbons at 20.82, 24.61, 30.78, and 39.18 ppm, which indicated that the carbon signals at 19.71 and 74.59 ppm, were quarternary carbons.

The mass spectrum (Fig. 23) showed a molecular ion peak at *m/z* 222 (15 %). The ion at *m/z* 204 (24%) is M-H_2O.

The above data indicated that compound **1** has 15 carbons, 26 protons and one hydroxy group. The molecular ion at *m/z* 222 indicated that the molecular formula was $C_{15}H_{26}O$ corresponding to a degree of unsaturation of three. Compound **1** must consist of three rings and one hydroxy group. These data indicated that compound **1** was ledol which is a known compound, by comparison of its ^{1}H-NMR and ^{13}C-NMR which those reported [51] which was closely matched as shown in Table 8.

Table 8 ^{1}H-NMR and ^{13}C-NMR spectral data of compound **1** and ledol

Carbon position	Chemical shifts (ppm)			
	^{1}H-NMR		^{13}C-NMR	
	Compound **1**	Ledol	Compound**1**	Ledol
1	2.06, td (10,8)	2.09, td (9.5,6.5)	53.75d	53.79d
2	1.93, m	1.90,m	24.61t	24.63t
3	1.28, m	1.30, m	30.78t	30.80t
4	1.96, m	1.99,dsxt(10.5,6.5)	38.41d	38.44d
5	1.79, m	1.78, dt (10.5, 6.5)	40.76d	40.79d
6	0.31,ddd (8.2,10)	0.33, dd (10.5, 9)	23.37d	23.41d
7	0.73, m	0.72, ddd (11,9,6)	25.00d	25.03d
8	1.82, m	1.83, m	20.82t	20.30t
9	1.24, m	1.86, m	39.18t	39.21t
10	-	-	74.59s	74.59s
11	-	-	19.17s	19.19s
12	0.96, s	0.98, s	15.40q	15.41q
13	1.02, s	1.04, s	28.64q	28.66q
14	1.12, s	1.14, s	30.49q	30.52q
15	0.92, d (6.7)	0.94, d (7)	15.98q	15.99q
OH	2.90, s	2.92, s	-	-

dsxt= doublet of sextets.

This compound has been firstly found in liverwort *Lepicolea ochroleucaa* [52] and fruits of *Piper clusii* [53] . Moreover, it has been first reported in *C. oblongifolius* Roxb. The structure of compound **1** is shown in Fig. 10.

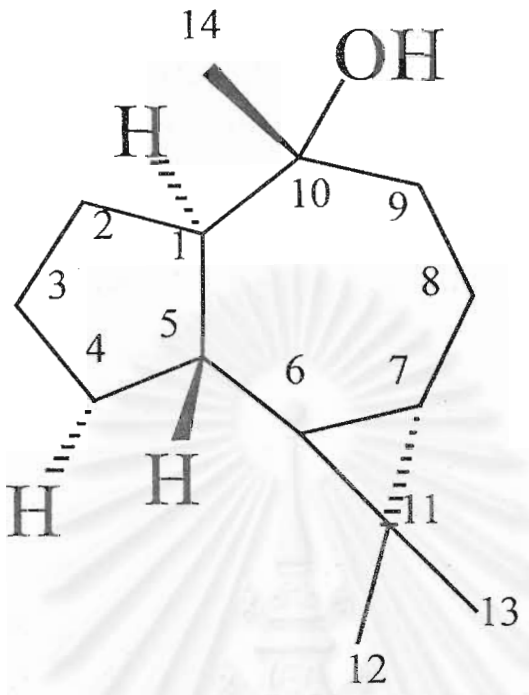


Figure 10 Structure of compound **1**

4.3.2 Structural elucidation of compound 2

Compound **2** was isolated from hexane crude extract with 10% EtOAc-hexane and from ethyl acetate crude extract eluted with 10 %EtOAc-hexane to afford bright yellow needles, m.p. 126–128 °C. The structure of compound **2** was elucidated by using spectroscopic tecniques. The data of this compounds are shown below.

IR-spectrum of compound **2** is shown in Fig. 24. The spectrum showed important absorption band at 3400-3200 cm^{-1} (O-H stretching vibration), 3089, 2946, and 2843 cm^{-1} (C-H stretching vibration of $-CH_3$, $-CH_2$), 1656 cm^{-1} (C=O stretching vibration of conjugated ketone), 1600, 1585, and 1497 cm^{-1} (C=C stretching vibration of aromatic), 1344 cm^{-1} (C-H bending vibration of $-CH_3$, $-CH_2$), 1179 cm^{-1} (C-O stretching vibration asymmetric of C-O-C) and 877, 831 cm^{-1} (=C-H out of plane bending of aromatic).

The ^{1}H-NMR spectrum in $CDCl_3$ of compound **2** (Fig. 25) showed three methoxy groups at 3.83 (3H, s), 3.84 (3H, s), and 3.87 (3H, s) one hydroxy group at 12.63 (1H, s) ppm and two aromatic protons with a meta-relationship at 6.98 (2H, d, J=10 Hz) and 8.04 (2H, d, J=8 Hz), another aromatic protons at 6.31 (1H, d, J=2 Hz) and 6.41 (1H, d, J=2 Hz) ppm.

The ^{13}C-NMR spectrum showed 18 signals (Fig. 26). Additionaly, DEPT 90 and DEPT 135 experiments (Fig. 27) showed three methyl carbons at 55.43, 55.79, and 60.13 ppm, six tertiary carbons at 92.12, 97.81, 114.03 (2C) and 130.14 (2C) ppm, nine quarternary carbons at 106.01, 122.75, 138.81, 155.94, 156.71, 161.58, 161.52, 165.37 and 178.75 ppm, respectively, but no methylene carbons. One carbonyl carbon of a conjugated ketone appeared at 178.75 ppm.

The mass spectrum (Fig. 28) showed a molecular ion peak at *m/z* 328 (90%). The ion at *m/z* 313 (6%) was derived from the loss of methoxy group.

The above result showed that compound **2** has a molecular weight of 328 and contained 18 carbons, 16 protons, three oxygens in three methoxy group and one oxygen in one hydroxy groups. The proposed molecular formula should be $C_{18}H_{16}O_6$. From molecular formula, degree of unsaturation of compound **2** is 11, which suggested that compound **2** may consist of a flavone skeleton as shown below:

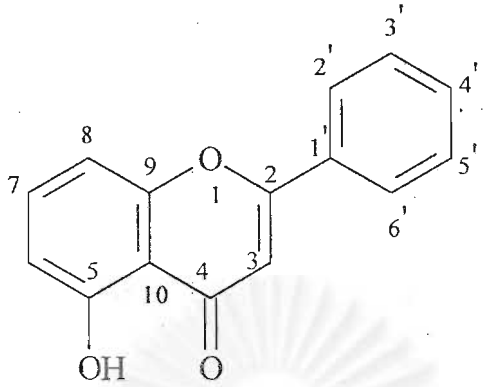


Figure 11 Basic skeleton of flavone

The ^{1}H-NMR and ^{13}C-NMR data showed the presence of five substituents; three methoxy groups at 3.83 (3H, s), 3.84 (3H, s) and 3.87 (3H, s), one hydroxy group at 12.63 (1H, s) ppm. The hydroxy group was unusually deshieded, which could result from H-bonding with the carbonyl carbon at the position C-4.

The ^{1}H-NMR and ^{13}C-NMR data indicated that compound **2** could be flavonoid. From previous report [54] the compound **2** exhibited the ^{1}H-NMR chemical shifts similar to those of 5-hydroxy-3,7,4′-trimethoxyflavone.

The ^{1}H-NMR chemical shifts of compound **2** and 5-hydroxy-3,7,4′-trimethoxy flavone are compared in Table 9.

Table 9 ^{1}H-NMR spectral data of compound **2** and 5-hydroxy-3,7,4′-trimethoxyflavone

Carbon position	^{1}H-NMR chemical shifts (ppm)	
	5-hydroxy -3,7,4′-trimethoxyflavone	Compound **2**
3	3.84, s	3.84, s
5	10.32, s	12.63, s
6	6.33, d (*J*=2.5 Hz)	6.31, d (*J*=2 Hz)
7	3.84, s	3.83, s
8	6.42, d (*J*=2.5 Hz)	6.41, d (*J*=2.5 Hz)
2′	8.04, d (*J*=9 Hz)	8.04, d (*J*=8 Hz)
3′	6.98, d (*J*=9 Hz)	6.98, d (*J*=8 Hz)
4′	3.87, s	3.87, s
5′	6.98, d (*J*=9 Hz)	6.98, d (*J*=8 Hz)
6′	8.04, d (*J*=9 Hz)	8.04, d (*J*=8 Hz)

The proposed structure of compound **2** are shown below.

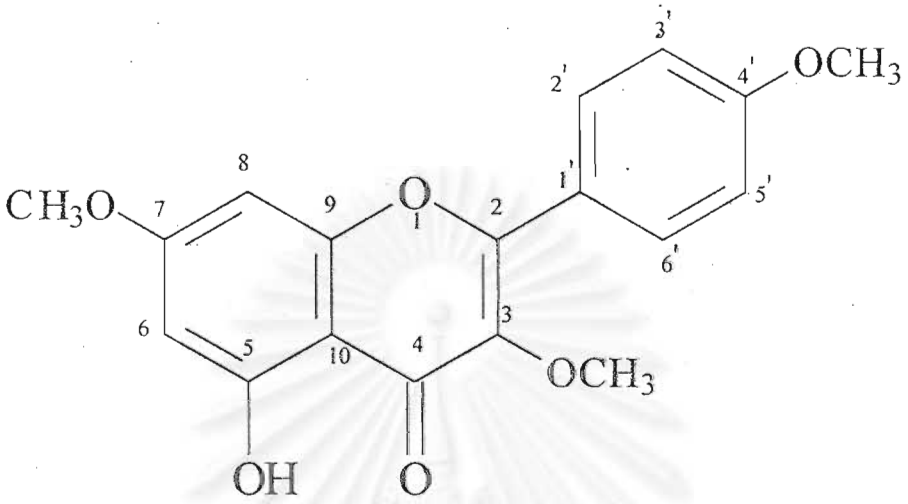


Figure 12 Structure of compound **2**

This compound has been reported previously in the stems of *Boesenbergia pandurata* Schi. [54] This is the first report of 5-hydroxy-3,7,4′-trimethoxyflavone in *C. oblongifolius* Roxb.

4.3.3 Structural elucidation of compound 3

Compound **3** was isolated from hexane crude extract with 20%EtOAc-hexane to afford yellow crystal, m.p. 134-135°C. The structure of compound **3** was elucidated by using spectroscopic tecniques. The data of this compounds are shown below.

IR-pectrum of compound **3** is shown in Fig. 29. The spectrum showed important absorption band at 3400-3200 cm^{-1} (O-H stretching vibration of alcohol), 3016, 2944, and 2837 cm^{-1} (C-H stretching vibration of $-CH_3$, $-CH_2$), 1663 cm^{-1} (C=O stretching vibration of conjugated ketone), 1600, 1515, and 1425 cm^{-1} (C=C stretching vibration of aromatic), 1327 cm^{-1} (C-H bending vibration of $-CH_3$, $-CH_2$), 1130 cm^{-1} (C-O stretching vibration asymmetric of C-O-C), 1009 cm^{-1} (C-O stretching vibration symmetric of C-O-C) and 908, 852 cm^{-1} (=C-H out of plane bending of aromatic).

The ^{1}H-NMR spectrum in $CDCl_3$ of compound **3** (Fig. 30) showed four methoxy groups at 3.95 (3H, s), 3.94 (3H, s), 3.85 (3H, s) and 3.84 (3H, s) one hydroxy group at 12.62 (1H, s) ppm and two aromatic protons whit a meta-relationship at 6.95 (1H, d, J=6 Hz) and 7.64-7.72 (2H, m), and another aromatic proton at 6.40 (1H, d, J=2 Hz) and 6.31 (1H, d, J=2 Hz) ppm.

The ^{13}C-NMR spectrum showed 19 signals (Fig.31). Moreover, DEPT 90 and DEPT 135 experiments (Fig.32) showed four methyl carbons at 55.81, 55.98, 56.04 and 60.18 ppm, five tertiary carbons at 92.18, 97.81, 110.82, 111.18 and 122.16 ppm, ten quarternary carbons at 105.99, 122.89, 136.95, 148.73, 151.35, 155.81, 156.69, 161.98, 165.42 and 178.71 ppm, respectively, but no methylene carbons. One carbonyl carbon of a conjugated ketone appeared at 178.71 ppm.

The mass spectrum (Fig. 33) showed a molecular ion peak of *m/z* 358 (100%) as the base peak. The ion of *m/z* 343 (40%) was derived from the loss of methyl group.

The above data showed that compound **3** has a molecular weight of 358 and contained 19 carbons, 18 protons, four oxygens in four methoxy group and one oxygen in one hydroxy groups. Compound **3** was assigned $C_{19}H_{18}O_7$ as the molecular formula corresponding to a degree of unsaturation of 11 which indicated that compound **3** possesses a flavone skeleton. This data indicated that compound **3** was 5-hydroxy-3,7,3',4'-tetramethoxyflavone which is a known compound, by comparison of its ^{1}H-NMR which those reported [55] which was closely matched as shown in Table 10.

Table 10 ^{1}H-NMR spectral data of compound **3** and 5-hydroxy-3,7,3′,4′-tetramethoxyflavone

Carbon position	^{1}H-NMR chemical shifts (ppm)	
	5-hydroxy -3,7,3′,4′-tetramethoxyflavone	Compound **3**
3	3.89, s	3.85, s
5	12.64, s	12.62, s
6	6.35, d (*J*=2.5 Hz)	6.31, d (*J*=2 Hz)
7	3.89, s	3.84, s
8	6.45, d (*J*=2.5 Hz)	6.40, d (*J*=2 Hz)
2′	7.69-7.85, m	7.64-7.72, m
3′	3.98, s	3.95, s
4′	3.98, s	3.94, s
5′	7.0, d (*J*=9.5 Hz)	6.95, d (*J*=6 Hz)
6′	7.69-7.85, m	7.64-7.72, m

The structure of compound **3** is shown below.

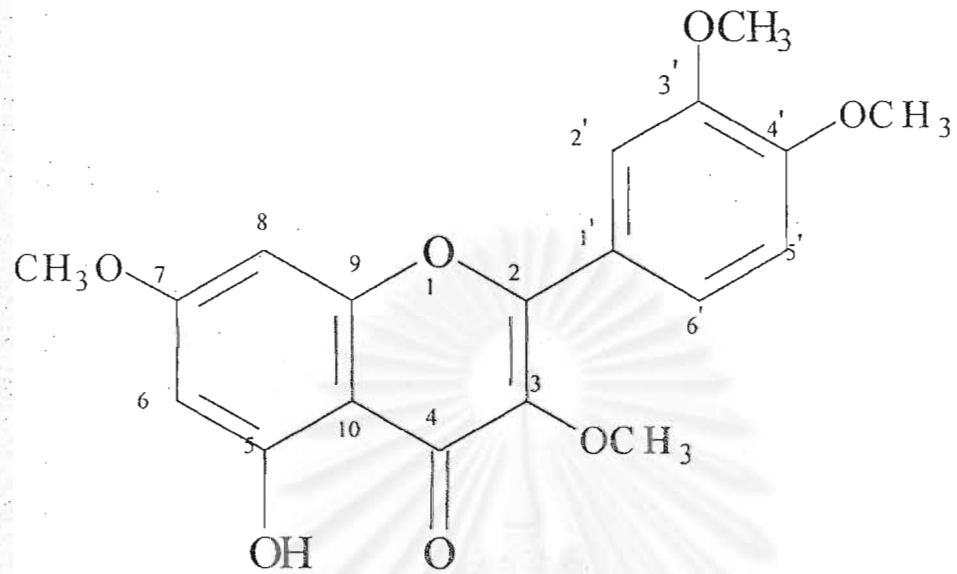


Figure 13 Structure of compound **3**

This compound has been reported previously in the stems of *Aframomum giganteum* K. Schum. [55] This is the first report of 5-hydroxy-3,7,3′,4′-tetramethoxyflavone in *C. oblongifolius* Roxb.

4.3.4 Structure elucidation of compound 4

Compound **4** was isolated from ethylacetate crude extract with 40% EtOAc-hexane and was recrystalyzed to afford white needle crystals, m.p. 181-182°C. The structure of compound **4** was elucidated by using spectroscopic tecniques. The data of this compounds are shown below.

IR-spectrum of compound **4** is shown in Fig. 34. The spectrum showed important absorption bands at 3400-3100 cm^{-1} (O-H stretching vibration of alcohol), 2936, 2910 cm^{-1} (C-H stretching vibration of $-CH_3$, $-CH_2$), 1466 cm^{-1} (C=C stretching vibration) 1385 and 1340 cm^{-1} (C-H bending vibration of $-CH_2$, $-CH_3$), 1085 and 1045 cm^{-1} (C-O stretching vibration).

The ^{1}H-NMR spectrum in DMSO (Fig.35) of compound **4** exhibited two methyl doublets at 1.02, and 0.86 (6H, d, J=8) and two methyl singlets at 1.17 and 0.80 (6H)

The ^{13}C-NMR spectrum (Fig. 36) showed 15 signals, DEPT 90 and DEPT 135 experiments (Fig. 37) showed four methyl carbons at 13.94, 22.35, 23.49, and 24.77 ppm, four methylene carbons at 22.22, 28.02, 35.64, and 39.97 ppm, five methine carbons at 24.51, 46.42, 49.65, 71.87 and 77.79 ppm which indicated that the carbon signals at 71.77 and 155.91 ppm were quarternary carbons.

The mass spectrum (Fig. 38) showed a molecular ion peak of *m/z* 256 as the base peak. The ion of *m/z* 238 (24%) and 220 (16%) were due to loss of H_2O .The ion of *m/z* 195 (15%) was due to loss of CH_3CO.

The above data indicated that compound **4** has 15 carbons, 26 protons and one hydroxy group. The result from mass spectrum suggested that the molecular formula of compound **4** should be $C_{15}H_{28}O_3$. Additionaly, the double bond equalivalent is two, thus compound **4** must consist of two rings and three hydroxy group. These data indicated that compound **4** was pterodontriol D by comparison of its ^{13}C-NMR which those reported in the literature [56] which are shown in Table 11.

Table 11 ^{13}C-NMR spectral data of compound **4** and Pterodontriol D

Carbon position	^{13}C-NMR chemical shifts (ppm)	
	Compound **4**	Pterodontriol D
1	77.8	79.3
2	71.9	29.4
3	71.8	36.7
4	49.7	72.7
5	46.4	47.9
6	40.7	73.1
7	40.3	51.2
8	35.6	23.4
9	28.0	41.4
10	24.8	41.5
11	24.0	25.9
12	23.5	24.5
13	22.4	25.1
14	22.2	22.6
15	13.9	14.7

Furthermore, the structure of compound 4 has been confirmed using X-ray diffraction. The single crystal has been obtained in ethyl acetate-methanol (1:3) as the solvent. It was the frist report of X-ray crystallographic analysis but some report have been made of its derivative which is Pterodontriol D.[56] The ORTEP structure of these compound is shown in Fig. 14 and the X-ray diffraction informations are shown in Table 12 and 13

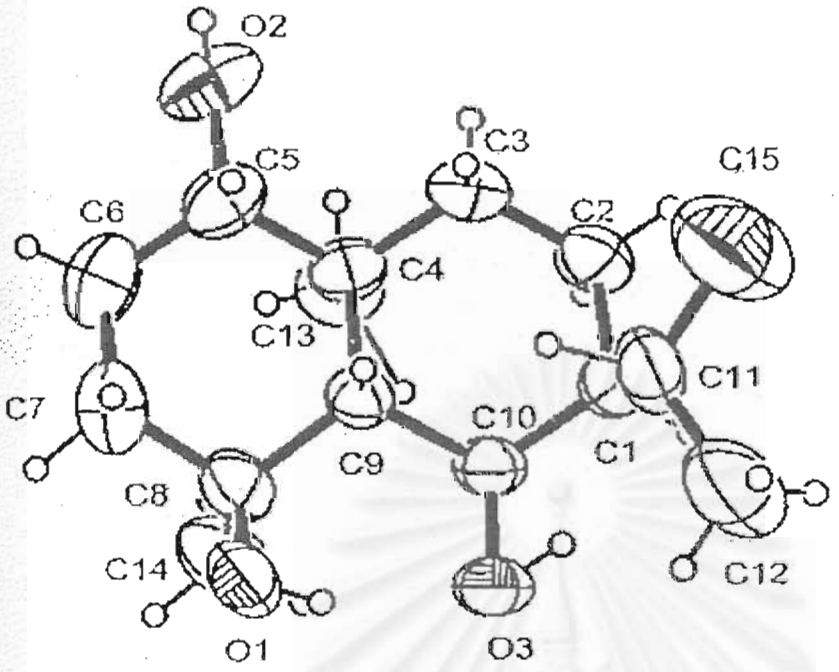


Figure 14 ORTEP structure of compound **4**

Table 12 Crystal data and structure refinement for compound **4**

Empirical formula	$C_{15}H_{28}O_3$	
Formula weight	256	
Temperature	293(2) K	
Wavelength	0.71073 A	
Crystal system, space group	trilinic, P(-1)	
Unit cell dimentions	a=6.30740 (10) A	alpha=60.4990 (10)
	b=14.3112 (2) A	beta=87.4051 (10)
	c=14.46990 (10) A	gramma=88.5230 (10)
Volume	1135.63 (3) A^{o3}	
Z, Calculated density	3, 1.423 Mg/ A^{o3}	
Absorption coefficient	0.098 mm^{-1}	
F(000)	516	
Theta range for data collecttion	1.62 to 30.45 deg.	
Limiting indices	$-8\leq h\leq 8$, $-19\leq k\leq 15$, $-20\leq 1\leq 20$	
Reflections collected/unique	8431/7177 [R(int) = 0.0122]	
Completeness to theta = 30.45	89.3%	
Refinement method	Full-matric least-squares on F^2	
Data/restraints/parameters	7177/3/667	
Goodness-of-fit on F^2	1.003	
Final R indices [I>2 sigma (I)]	R1 = 0.0598, wR2 = 0.1709	
R indices (all date)	R1 = 0.0716, wR2 = 0.1880	
Absolute structure parameter	0.6 (10)	
Largest diff, peak and hold	0.260 and –0.262 $e.A^{-3}$	

Table 13 Bond angles (deg.) for compound **4**

Angles	(deg.)	Angles	(deg.)
C (1)-C (11)	1.539(5)	C (13)-C (4)-C(9)	114.9(2)
C (1)-C (2)	1.540(4)	C (14A)-C (4A)-C(5A)	109.3(3)
C (1)-C (10)	1.550(4)	C (14A)-C (4A)-C(3A)	109.1(3)
C (1A)-C (2A)	1.533(5)	C (5A)-C (4A)-C(3A)	109.0(2)
C (1A)-C (11A)	1.558(7)	C (14A)-C (4A)-C(9A)	115.4(2)
C (1A)-C (10A)	1.554(5)	C (5A)-C (4A)-C(9A)	107.2(2)
C (1B)-C (2B)	1.528(5)	C (3A)-C (4A)-C(9A)	106.7(2)
C (1B)-C (10B)	1.563(5)	C (5B)-C (4B)-C(3B)	109.1(2)
C (1B)-C (11B)	1.572(6)	C (5B)-C (4B)-C(14B)	109.5(2)
C (2)-C (3)	1.523(5)	C (3B)-C (4B)-C(14B)	108.7(3)
C (2A)-C (3A)	1.531(5)	C (5B)-C (4B)-C(9B)	107.4(2)
C (2B)-C (3B)	1.526(5)	C (3B)-C (4B)-C(9B)	106.9(2)
C (3)-C (4)	1.535(4)	C (14B)-C (4B)-C(9B)	115.2(2)
C (4)-C (13)	1.539(4)	O (2)-C (5)-C(6)	111.1(3)
C (4)-C (9)	1.568(3)	O (2)-C (5)-C(4)	110.3(3)
C (4A)-C (14A)	1.538(4)	C (6)-C (5)-C(4)	112.2(3)
C (4A)-C (5A)	1.542(4)	O (2A)-C (5A)-C(6A)	111.8(3)
C (4A)-C (9A)	1.564(4)	O (2A)-C (5A)-C(4A)	110.1(3)
C (4B)-C (5B)	1.536(4)	C (6A)-C (5A)-C(4A)	111.7(3)
C (4B)-C (14B)	1.547(4)	O (2B)-C (5B)-C(6B)	111.2(3)
C (4B)-C (9B)	1.568(3)	O (2B)-C (5B)-C(4B)	109.5(3)
C (5)-C (2)	1.437(4)	C (6B)-C (5B)-C(4B)	112.6(3)
C (5)-C (6)	1.517(6)	C (5)-C (6)-C(7)	111.0(3)
C (5A)-C (2A)	1.435(4)	C (5A)-C (6A)-C(7A)	110.8(3)
C (5A)-C (6A)	1.517(5)	C (7B)-C (6B)-C(5B)	110.2(3)
C (5B)-C (2B)	1.451(4)	C (6)-C (7)-C(8)	114.5(3)
C (5B)-C (6B)	1.521(5)	C (6A)-C (7A)-C(8A)	115.3(3)
C (6)-C (7)	1.530(6)	C (6B)-C (7B)-C(8B)	115.4(3)
C (6A)-C (7A)	1.521(6)	O (1)-C (8)-C(14)	108.0(3)
C (6B)-C (7B)	1.517(6)	O (1)-C (8)-C(7)	103.1(3)
C (7)-C (8)	1.535(5)	C (14)-C (8)-C(7)	113.1(3)
C (7A)-C (8A)	1.538(6)	O (1)-C (8)-C(9)	108.3(2)
C (7B)-C (8B)	1.519(5)	C (14)-C (8)-C(9)	113.9(3)
C (8)-C (1)	1.459(4)	C (7)-C (8)-C(9)	109.7(3)
C (8)-C (14)	1.526(5)	O (1A)-C (8A)-C(16A)	108.3(3)
C (8)-C (9)	1.557(4)	O (1A)-C (8A)-C(7A)	103.8(3)

Table 13 Continued

Angles	(deg.)	Angles	(deg.)
C (8A)-C (1A)	1.447(4)	C (16A)-C (8A)-C(7A)	112.9(3)
C (8A)-C (16A)	1.532(5)	O (1A)-C (8A)-C(9A)	108.0(2)
C (8A)-C (9A)	1.556(4)	C (16A)-C (8A)-C(9A)	113.9(3)
C (8B)-C (3B)	1.449(4)	C (7A)-C (8A)-C(9A)	109.4(3)
C (8B)-C (16B)	1.538(5)	O (3B)-C (8B)-C(7B)	104.1(3)
C (8B)-C (9B)	1.557(3)	O (3B)-C (8B)-C(16B)	107.6(3)
C (9)-C (10)	1.545(3)	C (7B)-C (8B)-C(16B)	113.3(3)
C (9A)-C (10A)	1.551(4)	O (3B)-C (8B)-C(9B)	108.2(2)
C (9B)-C (10B)	1.542(4)	C (16B)-C (8B)-C(9B)	113.1(3)
C (10)-C (3)	1.449(3)	C (10)-C (9)-C(8)	114.0(2)
C (10A)-C (3A)	1.437(4)	C (10)-C (9)-C(4)	111.05(19)
C (10B)-C (1B)	1.447(3)	C (8)-C (9)-C(4)	115.8(2)
C (11)-C (4)	1.522(6)	C (10A)-C (9A)-C(8A)	113.9(2)
C (11)-C (12)	1.537(7)	C (10A)-C (9A)-C(4A)	111.4(2)
C (11A)-C (4A)	1.501(8)	C (8A)-C (9A)-C(4A)	115.7(2)
C (11A)-C (12A)	1.569(9)	C (10B)-C (9B)-C(8B)	115.0(2)
C (11B)-C (4B)	1.533(7)	C (10B)-C (9B)-C(4B)	111.1(2)
C (11B)-C (12B)	1.556(7)	C (8B)-C (9B)-C(4B)	115.4(2)
C (11)-C (1)-C(2)	114.9(3)	O (3)-C (10)-C(9)	107.9(2)
C (2)-C (1)-C(10)	107.6(3)	O (3)-C (10)-C(1)	111.2(2)
C (2A)-C (1A)-C(11A)	115.1(4)	C (9)-C (10)-C(1)	112.7(2)
C (2A)-C (1A)-C(10A)	107.8(3)	O (3A)-C (10A)-C(9A)	108.3(2)
C (11A)-C (1A)-C(10A)	113.1(3)	O (3A)-C (10A)-C(1A)	111.1(3)
C (2B)-C (1B)-C(11B)	107.8(3)	C (9A)-C (10A)-C(1A)	112.7(2)
C (10B)-C (1B)-C(11B)	115.9(4)	O (1B)-C (10B)-C(9B)	108.3(2)
C (3)-C (2)-C(1)	112.4(3)	O (1B)-C (10B)-C(1B)	109.8(3)
C (11)-C (1)-C(2)	112.2(2)	C (9B)-C (10B)-C(1B)	112.9(2)
C (3A)-C (2A)-C(1A)	112.5(3)	O (4)-C (11)-C(1)	115.2(3)
C (3B)-C (2B)-C(1B)	113.8(3)	O (4)-C (11)-C(12)	105.3(4)
C (2)-C (3)-C(4)	112.3(2)	C (1)-C (11)-C(12)	111.4(5)
C (2A)-C (3A)-C(4A)	112.6(3)	O (4A)-C (11A)-C(1A)	116.0(4)
C (2B)-C (3B)-C(4B)	113.1(3)	O (4A)-C (11A)-(12A)	107.5(4)
C (3)-C (4)-C(5)	109.3(2)	C (1A)-C (11A)-C(12A)	108.5(6)
C (3)-C (4)-C(13)	108.6(3)	O (4B)-C (11B)-C(12B)	106.7(4)
C (5)-C (4)-C(13)	110.1(3)	O (4B)-C (11B)-C(1B)	114.8(4)
C (3)-C (4)-C(9)	106.9(2)	C (12B)-C (11B)-C(1B)	108.6(5)
C (5)-C (4)-C(9)	106.9(2)		

The structure of compound **4** is shown below

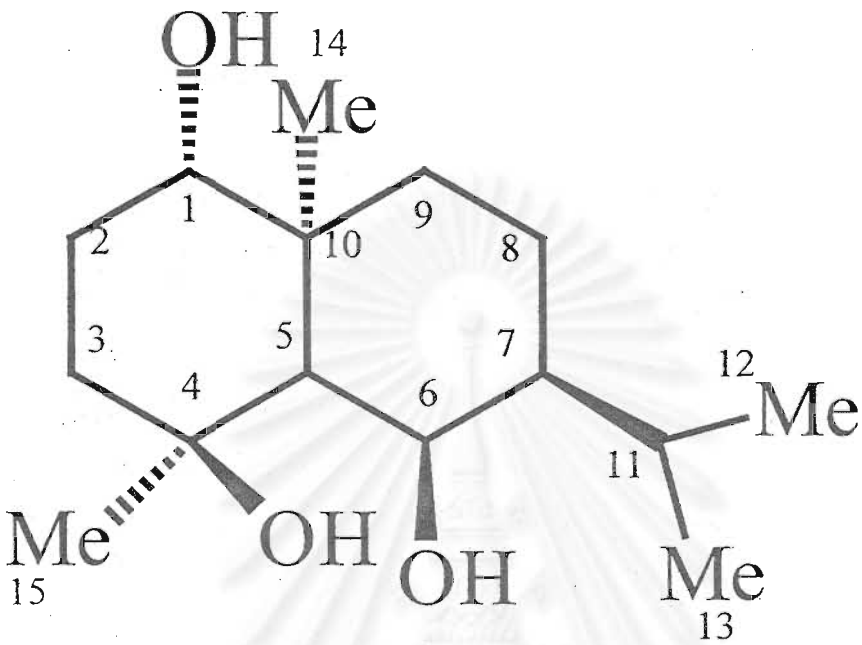


Figure 15 Structure of compound **4**

From x-ray data indicated compound **4** has the steriochemistry diferent from that reported in the literature [56] at C-1. Additionaly, the optical rotation data of compound **4** is -4.2 but pterodontriol D is +0.4. Therefore, compound **4** is a new isomer of pterodontriol D.

4.3.5 Structure elucidation of mixture 5

Mixture **5** is white amorphous solid, m.p. 273-275°C. The structure of mixture **5** was elucidated by using spectroscopic techniques. The data of this compounds are shown below.

IR-spectrum of mixture **5** is shown in Fig. 39. The spectrum showed important absorption bands at 3600-3200 cm^{-1} (O-H stretching vibration of alcohol), 2933, 2867 cm^{-1} (C-H stretching vibration of $-CH_3$, $-CH_2$), 1729 cm^{-1} (C=C stretching vibration of olefin) 1385 and 1340 cm^{-1} (C-H bending vibration of $-CH_2$, $-CH_3$), 1075–1024 cm^{-1} (C-O stretching vibration of OH group of sugar) and 886 cm^{-1} (C-H bending vibration of anomeric axial proton of β-sugar) and 798, 667 cm^{-1} (C-H out of plane bending vibration of trisubstituted vinyl).

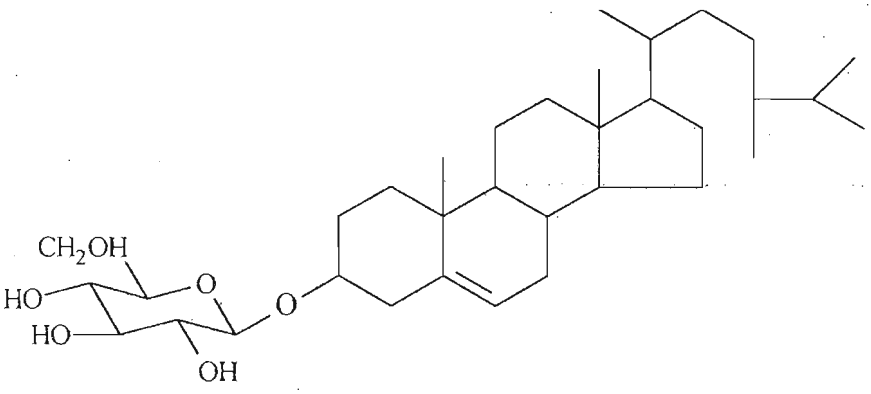
The ^{1}H-NMR spectrum in DMSO (Fig. 40) of mixture **5** showed the signals at 0.64-2.37 ppm, which were the signals of methyl, methylene, and methine groups of steroids ($-CH_3$, $-CH_2$, -CH respectively). The multiplet signals at 2.87-3.04 ppm were assigned to the protons of a sugar. The proton on carbon which is attached to sugar (-CH-O-sugar) appeared as the multiplet signal at 4.21 ppm. and the signals at 4.48 ppm belonged to the anomeric proton. The multiplet signal at 4.90 ppm was assigned as disubstituted vinyl protons (-CH=CH-). The last signal at 5.30 ppm was the signal of trisubstituted vinyl proton (-CH=C-).

The ^{13}C-NMR spectrum (Fig. 41) showed carbon signals at 11.6-56.1 ppm which were the signals of CH_3,CH_2,CH of steroid. The olefinic carbon signals were observed at 121.2, 128.5, 138.1 and 140.4 ppm, while the signal at 61.0,70.0,73.4,76.7,76.9 and 100.7 ppm were characteristic for a glycoside. The ^{13}C-NMR spectrum of the aglycone correspounded to those of a mixture of campesterol, stigmasterol and β-sitosterol. ^{13}C-NMR spectrum of mixture **5** in the region of 60-100 ppm suggested that it is probably glucose by comparison with those in the literature [57] and the data are shown in Table 14.

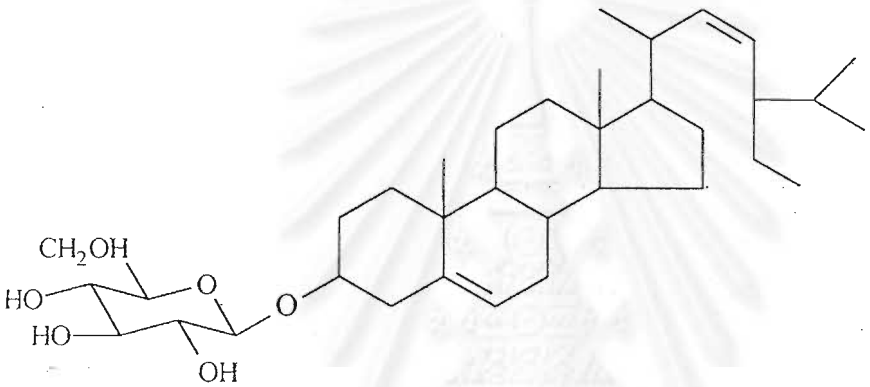
Table 14 Partial ^{13}C-NMR spectrum of sugar in mixture **5** with steroid 3-*O*-β-D-glucopyranoside

Carbon position	Mixture **5**	Steroid-3-*O*-β -D-glucopyranoside
C-1	100.69	100.74
C-2	73.4	73.43
C-3	76.92	76.93
C-4	70.04	70.06
C-5	76.65	76.69
C-6	61.03	61.05

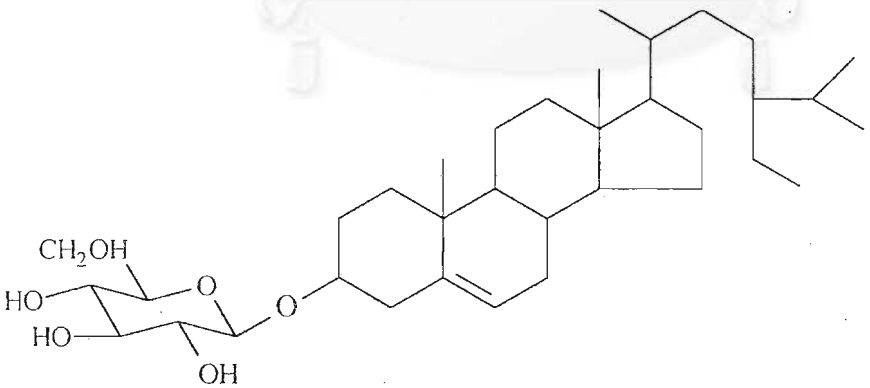
The mass spectrum (Fig. 42) showed the molecular ion peaks of campesterol, stigmasterol, and β-sitosterol of *m/z* 412 ($C_{29}H_{48}O$) and 414 ($C_{29}H_{50}O$), respectively. The fragmentation ion mass spectrum pattern of this mixture indicated that it was a mixture of steroids. The above results of ^{1}H-NMR, ^{13}C-NMR, EI-MS spectra and a literature search indicated that mixture **5** was a mixture of steroid glucoside; campesteryl-3-*O*-β-D-glucopyranoside, stigmasteryl-3-*O*-β-D-glucopyranoside, and β-sitosteryl-3-*O*-β-D-glucopyranoside. The structure of mixture **5** is shown in Fig. 45.



campesteryl-3-*O*-β-D-glucopyranoside



stigmasteryl-3-*O*-β-D-glucopyranoside



β-sitosteryl-3-*O*-β-D-glucopyranoside

Figure 16 Structure of mixture 5

4.4 Inhibitory activities crude extracts on cAMP phosphodiesterase

The *in vitro* activity of the three crude extracts from *C. oblongifolius* were tested for their cAMP phosphodiesterase inhibition by using malachite green method. The results are shown in Table 15.

Table 15 Inhibitory activities of three crude extracts from *C. oblongifolius* Roxb.

Sample	Concentration (μg)	OD*	Specific activity**	%Inhibition
DMSO	-	303.50	0.213	0
Theophylline	5	361.20	0.253	0
	50	33.67	0.024	88.73
	500	16.00	0.011	94.84
Hexane crude extract	5	160.67	53.05	46.95
	50	157.75	52.11	47.89
	500	132	43.66	56.34
Ethylacetate crude extract	5	212.50	69.95	30.05
	50	132.00	43.66	56.34
	500	115.25	38.03	61.97
Methanol crude extract	5	112.33	37.09	62.91
	50	71.40	23.47	76.53
	500	62.00	20.19	79.81

*OD (Optical density)= ODsample-ODblank

**Specific activity=$[(0.2 \times OD)/OD_{Pi}] \times 15/200$

From the data in Table 15. The IC_{50} values of three crude extract were determined by graph plotting between concentration of sample (x-axis) and % Inhibition (y-axis). To determine IC_{50} value, a perpendicular line was drown from the y-axis at the % inhibition value of 50 to the x-axis as shown in Fig. 17.

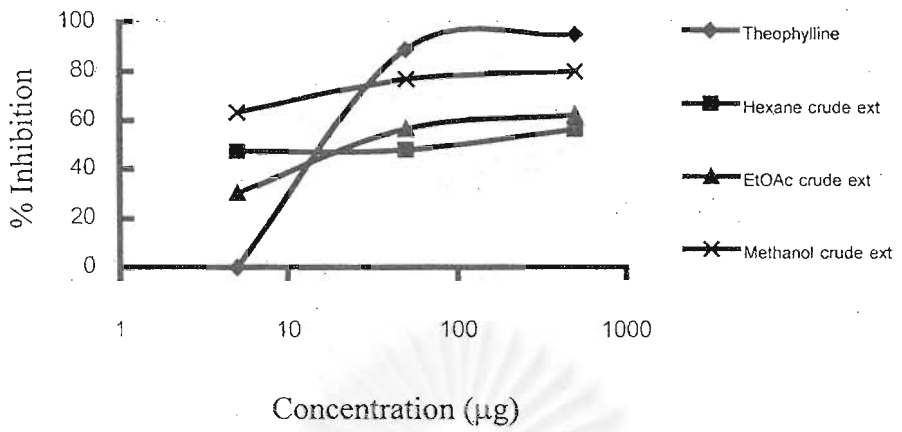


Figure 17 Effects of crude extracts on cAMP phosphodiesterase

The result indicated that three crude extracts of *C. oblongifolius* showed significant inhibitory on cAMP phosphodiesterase. The ethyl acetate and methanol crude extract showed high inhibitory activities. The IC_{50} of hexane, ethyl acetate, and methanol crude extracts are 250, 30 and less than 5 μg, repectively. Methanol crude extract was insoluble in all organic solvents. Consequently it was not suiTable to separate and purify using silica-gel column chromatography. Hexane and ethyl acetate crude extract were selected and purified by using silica-gel column chromatography with hexane-ethyl acetate gradient solvent system to give four compounds and one mixture. The inhibitory activity of compounds is shown in Table 16 and the IC_{50} of compounds is shown in Fig. 18.

Table 16 Inhibitory activities of compound **1-5** from *C. oblongifolius* on cyclic AMP phosphodiesterase

Sample	Concentration (μg)	OD*	Specific activity**	%Inhibition
DMSO	-	295.30	0.103	0
Theophylline	10^{-5}	248.82	0.09	15.55
	10^{-4}	197.34	0.07	33.01
	10^{-3}	82.94	0.03	71.84
Ledol (**1**)	10^{-5}	177.32	0.06	39.5
	10^{-4}	154.44	0.05	47.37
	10^{-3}	117.26	0.04	60.53
5-hydroxy-3,7,4′-trimethoxyflavone (**2**)	10^{-5}	185.90	0.07	36.89
	10^{-4}	154.44	0.05	47.57
	10^{-3}	28.60	0.01	90.29
5-hydroxy-3,7,3′,4′-tetramethoxyflavone (**3**)	10^{-5}	160.16	0.06	45.63
	10^{-4}	122.98	0.04	58.25
	10^{-3}	114.4	0.04	61.17
(-)Pterodontriol D (**4**)	10^{-5}	217.36	0.08	26.21
	10^{-4}	188.76	0.07	35.92
	10^{-3}	183.04	0.06	37.06
Glucopyranoside (**5**)	10^{-5}	254.54	0.09	13.59
	10^{-4}	243.10	0.08	17.48
	10^{-3}	185.9	0.07	36.89

*OD (Optical density)= ODsample-ODblank

**Specific activity=$[(0.2 \times OD)/OD_{Pi}] \times 15/200$

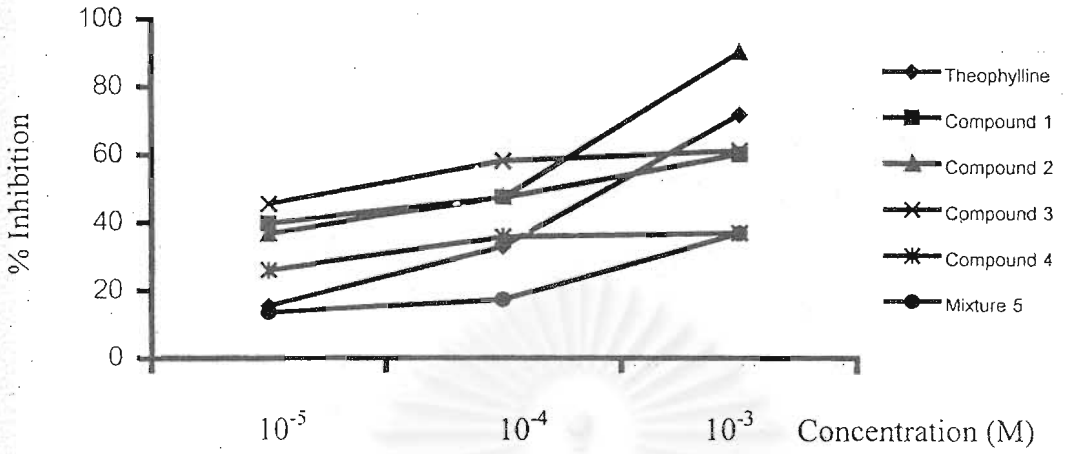


Figure 18 Inhibitory activities of compounds **1-5** on cAMP phosphodiesterase

The IC_{50} values (Fig. 18) of theophylline and isolated compounds are summarized in Table 17.

Table 17 IC_{50} values of compounds **1-5** and positive control

Sample	IC_{50} (mM)
Theophylline	0.5
Ledol (**1**)	0.4
5-hydroxy-3,7,4′-trimethoxyflavone (**2**)	0.1
5-hydroxy-3,7,3′,4′-tetramethoxyflavone (**3**)	0.04
(-)Pterodontriol D (**4**)	>1
Glucopyranoside (**5**)	>1

The Table 17 indicated that ledol (**1**), 5-hydroxy-3,7,4′-trimethoxyflavone (**2**) and 5-hydroxy-3,7,3′,4′-tetramethoxy (**3**) exhibited high inhibitory activity against cAMP phosphodiesterase, with IC_{50} of 0.40, 0.10 and 0.04 mM, repectively. These results

indicated that ledol (**1**), 5-hydroxy-3,7,4′-trimethoxyflavone (**2**) and 5-hydroxy-3,7,3′,4′-tetramethoxyflavone (**3**) can stimulated central nervous system. Therefore these compounds are potential candidates to be developed as new drugs in the future.

CHAPTER V

CONCLUSION

The powdered, sun-dried stem of *C. oblongifolius* Roxb. from Amphoe Tha-Uthen, Nakhonphanom province was extracted with hexane, ethyl acetate and methanol, respectively. Three different crude extracts and their bioactivities are shown in Table 18.

Table 18 Three different crude extracts and their IC_{50} values

Sample	Starting material (% wt/wt)	IC_{50} (μg/ml)
Hexane crude extract	2.38	250
Ethyl acetate crude extract	3.62	30
Methanol crude extract	3.79	<5

Hexane crude extracted was separated using silica-gel column chromatography with hexane-ethyl acetate gradient system to obtained ledol (compound **1**), 5-hydroxy-3,7,4′-trimethoxyflavone (compound **2**) and 5-hydroxy-3,7,3′,4′-tetramethoxyflavone (compound **3**).

Ethyl acetate crude extracted of *C. oblongifolius* was separated on silica-gel column chromatography. To obtain a sesquiterpene (compound **4**) and a mixture of three steroid glycosides: campesteryl-3-*O*-β-D-glucopyranoside, stigmasteryl-3-*O*-β-D-glucopyranoside and β-sitosteryl-3-*O*-β-D-glucopyranoside (compound **5**).

All isolated compounds from hexane and ethyl acetate crude extracts and the IC_{50} values of these compounds are shown in Table 19.

Compounds **1-5** were tested for the inhibition of adenosine 3′,5′-cAMP monophosphate phosphodiesterase (PDE) and found that only compounds 1-3 were active with the IC_{50} at 0.4, 0.1 and 0.04 mM, respectively.

Table 19 Isolated compounds from the stem bark of *C. oblongifolius* and inhibitory activity on cAMP phosphodiesterase

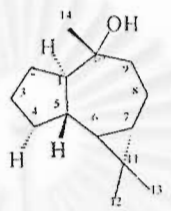
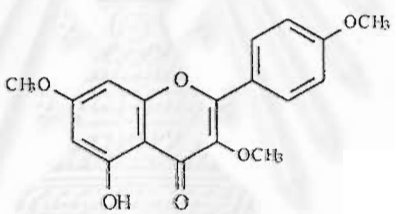
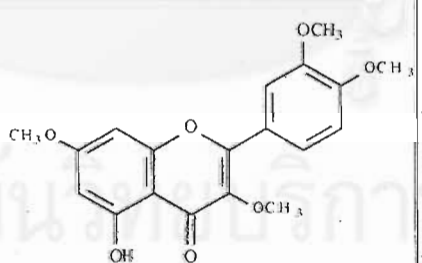
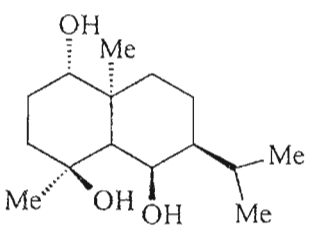
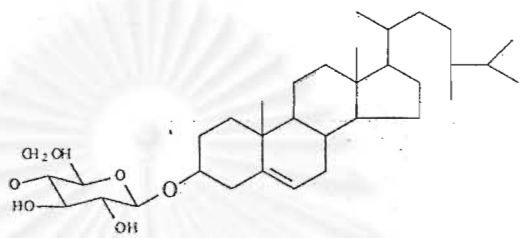
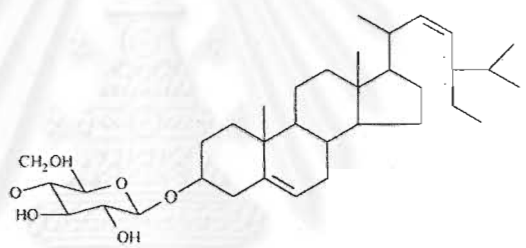
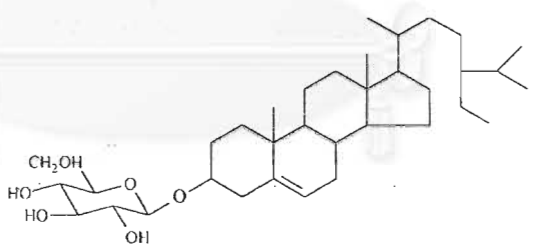
Compound No	Name of compounds	Structure	% wt/wt of crude extract	IC_{50} (mM)
1	ledol		0.13	0.4
2	5- hydroxy -3,7,4'-trimethoxyflavone		2.91	0.1
3	5-hydroxy–3,7,3',4'-tetramethoxyflavone		1.13	0.04
4	(-) Pterodontriol D		1.0	>1

Table 19 Continued

Compound No	Name of compounds	Structure	% wt/wt of crude extract	IC_{50} (mM)
5	campesteryl -3-*O*-β-D-glucopyranoside		0.63	>1
	stigmasteryl - 3 - *O* - β - D - glucopyranoside			
	β-sitosteryl-3-*O*-β-D-glucopy ranoside.			

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APPENDIX

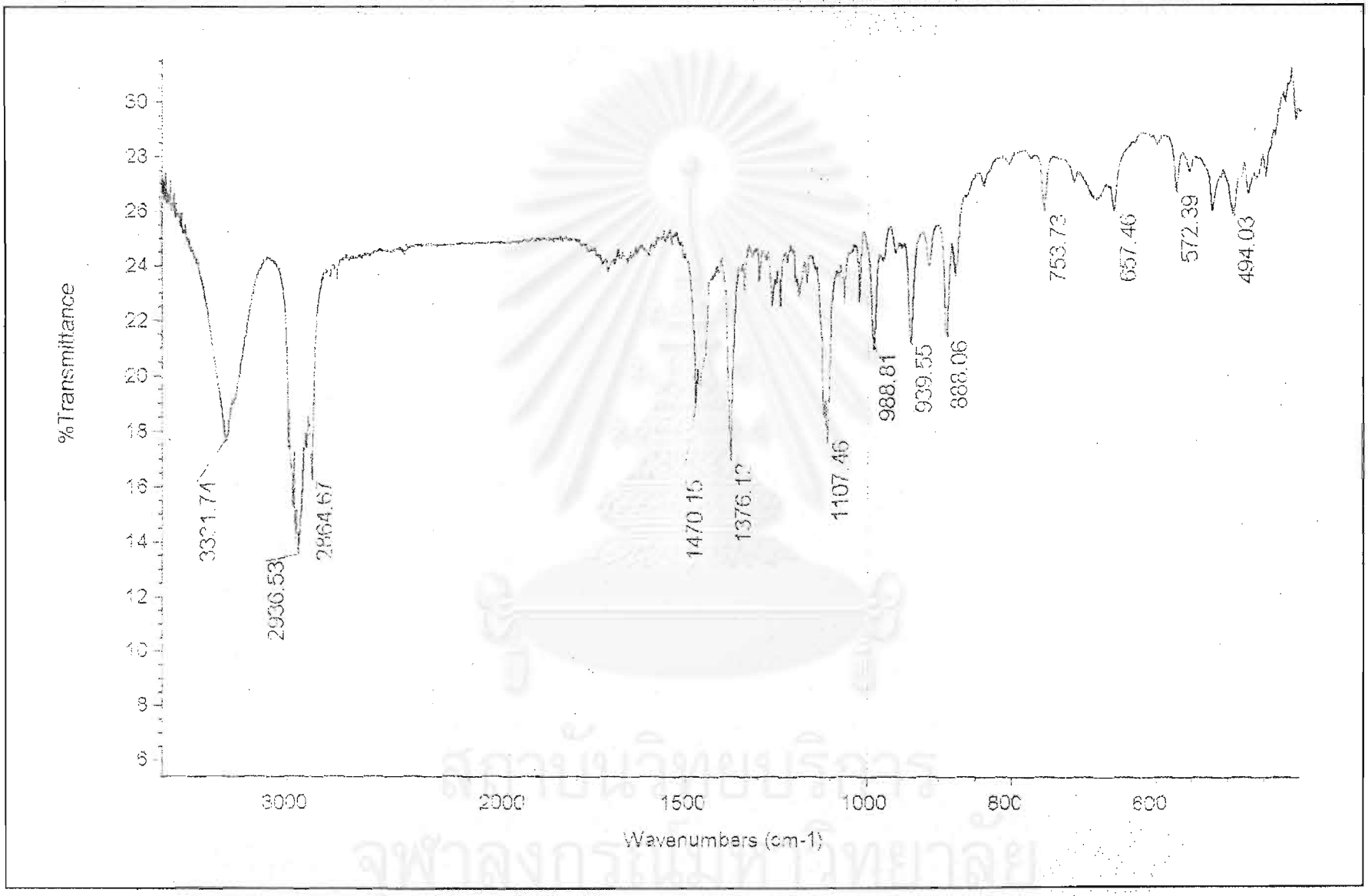

Figure 19 IR spectrum of compound **1**

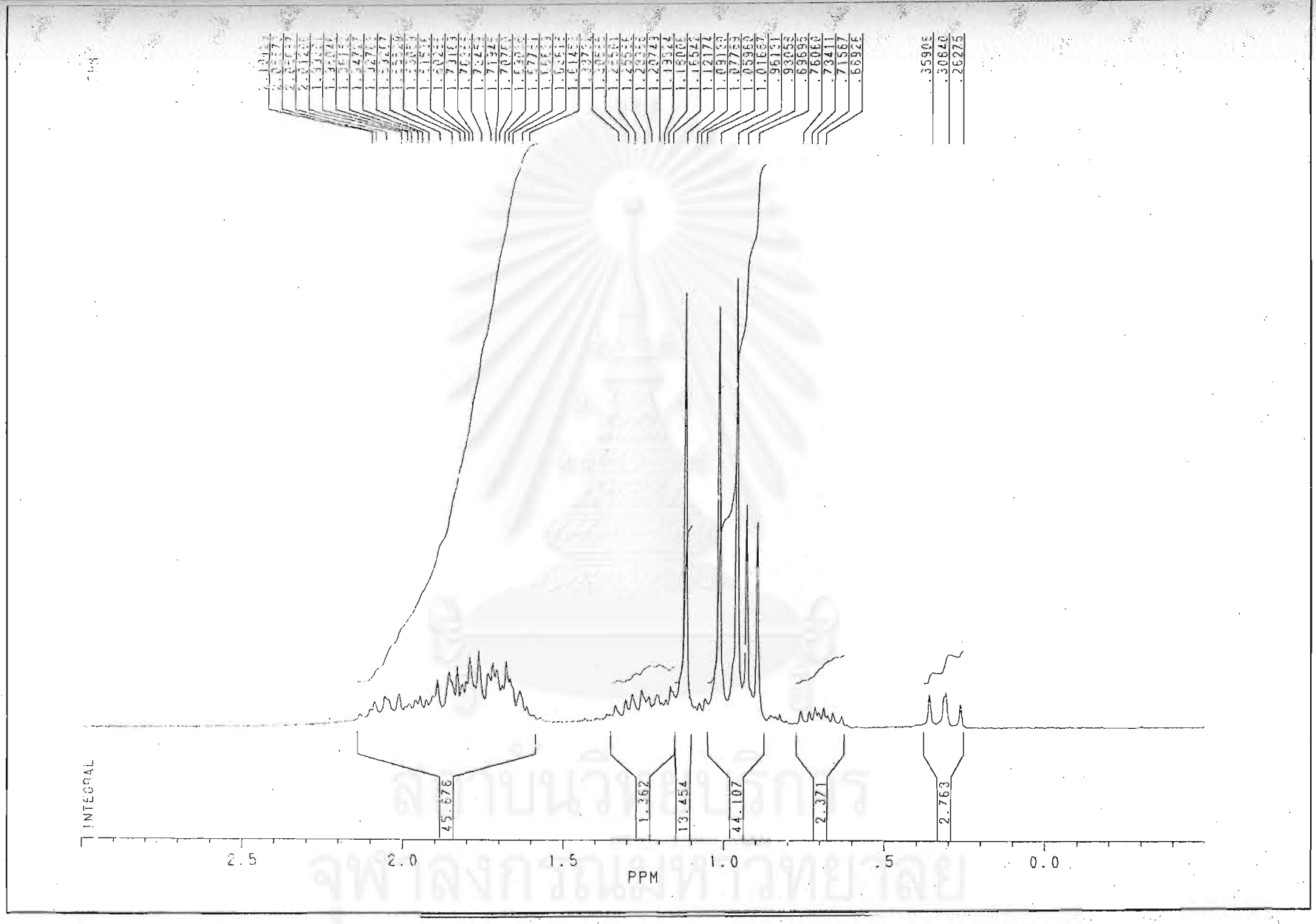

Figure 20 ^{1}H-NMR spectrum of compound **1**

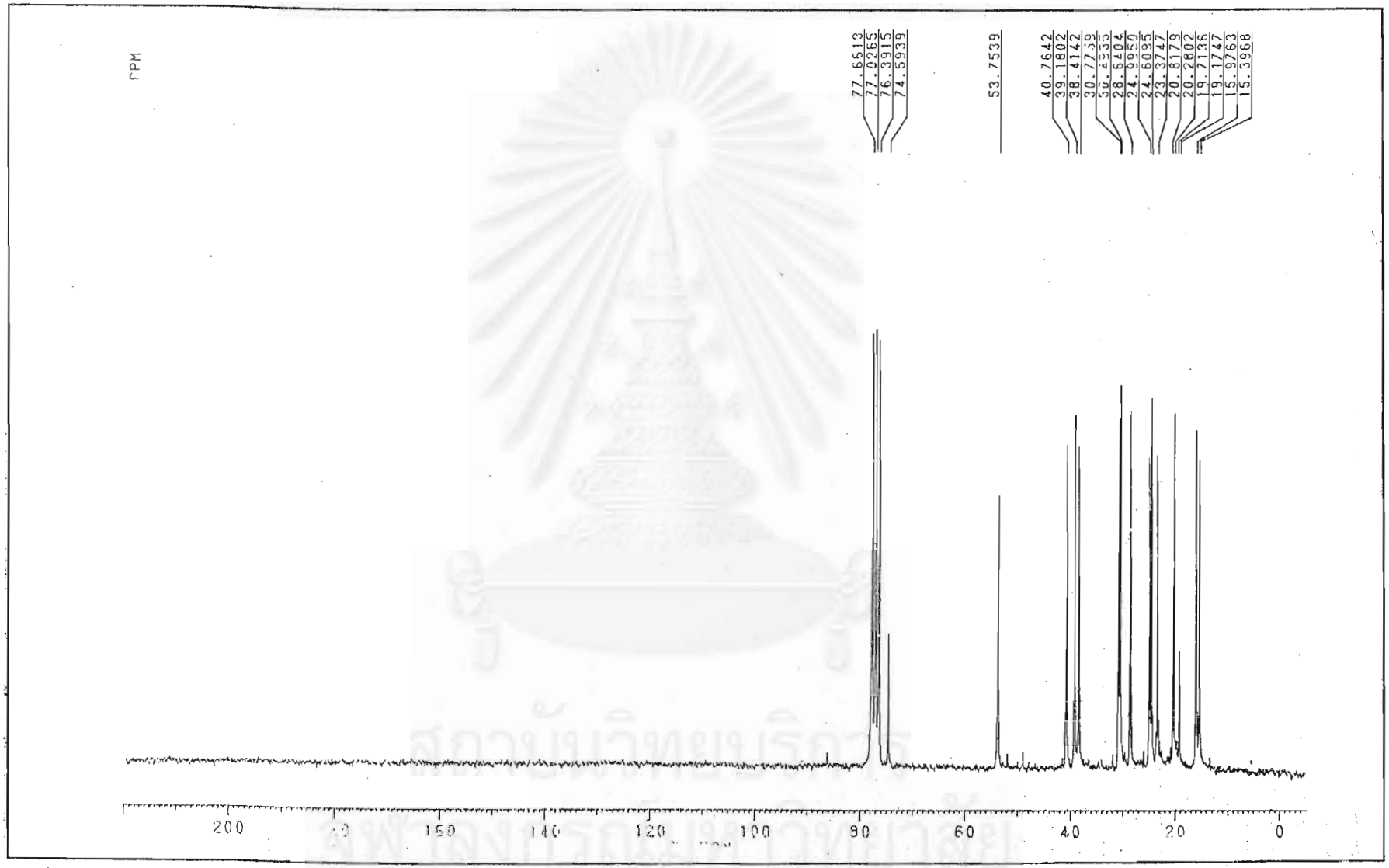

Figure 21 ^{13}C-NMR spectrum of compound **1**

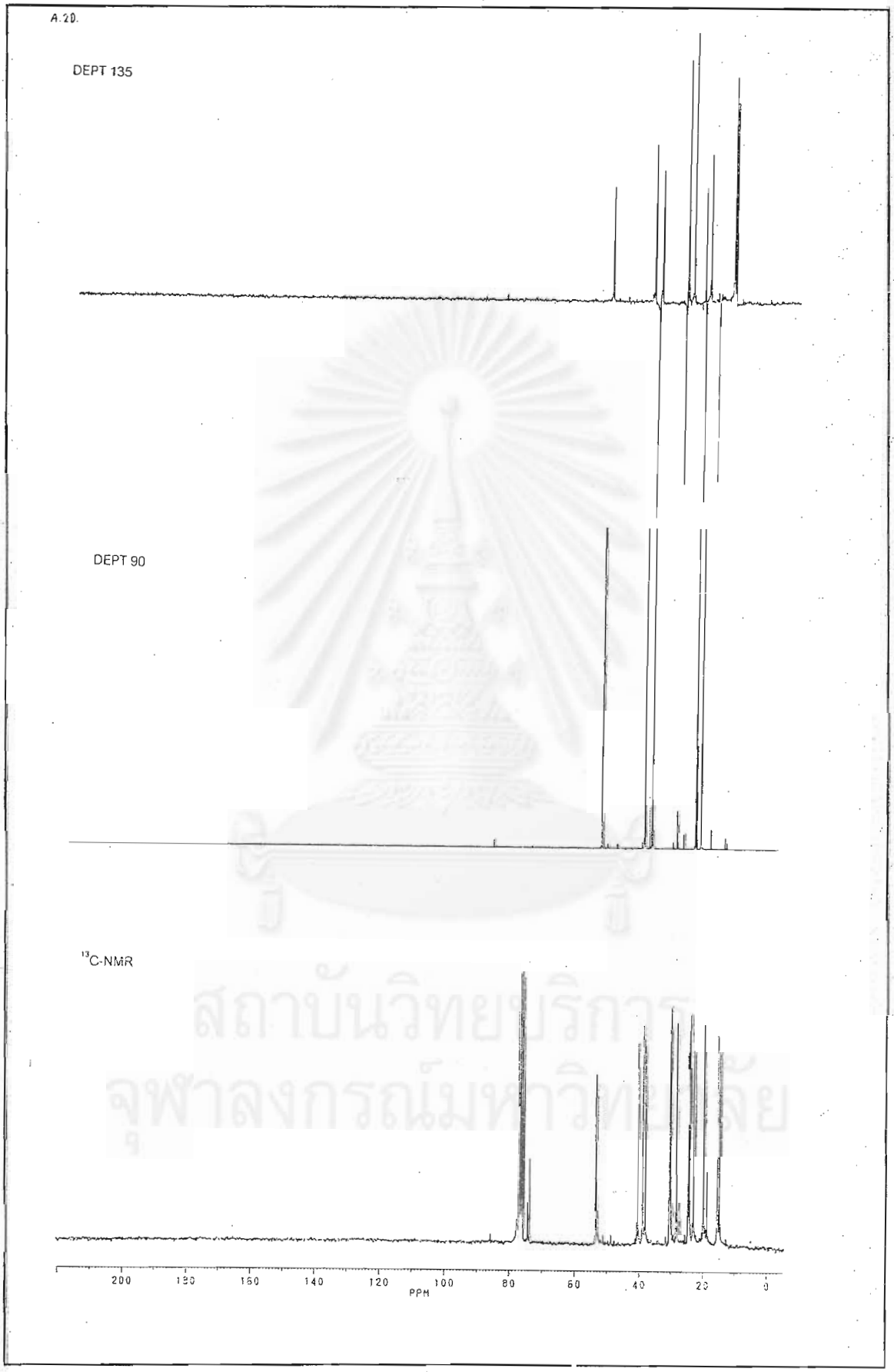

Figure 22 DEPT-135,90 and ^{13}C-NMR spectrum of compound **1**

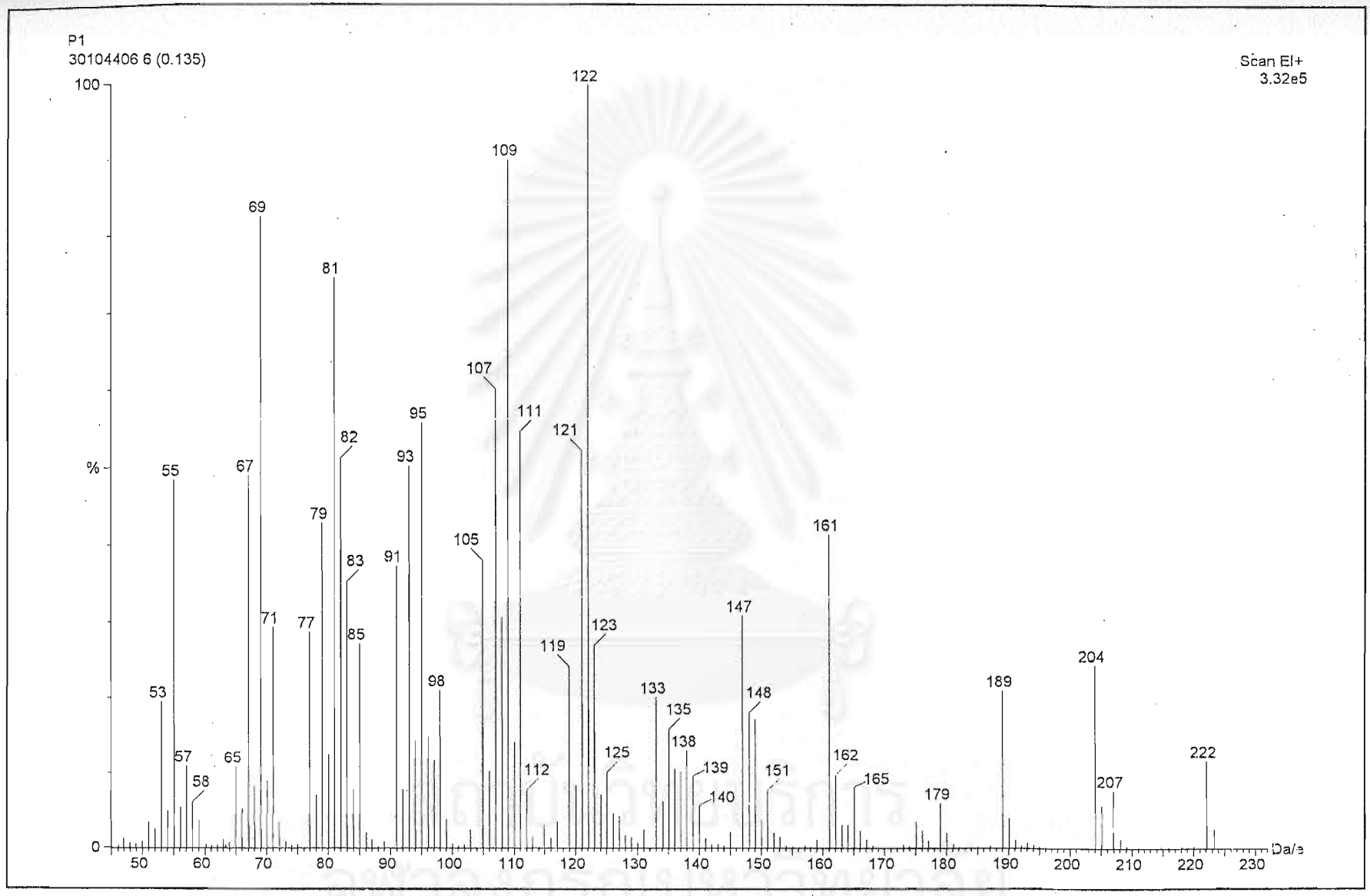

Figure 23 EIMS spectrum of compound **1**

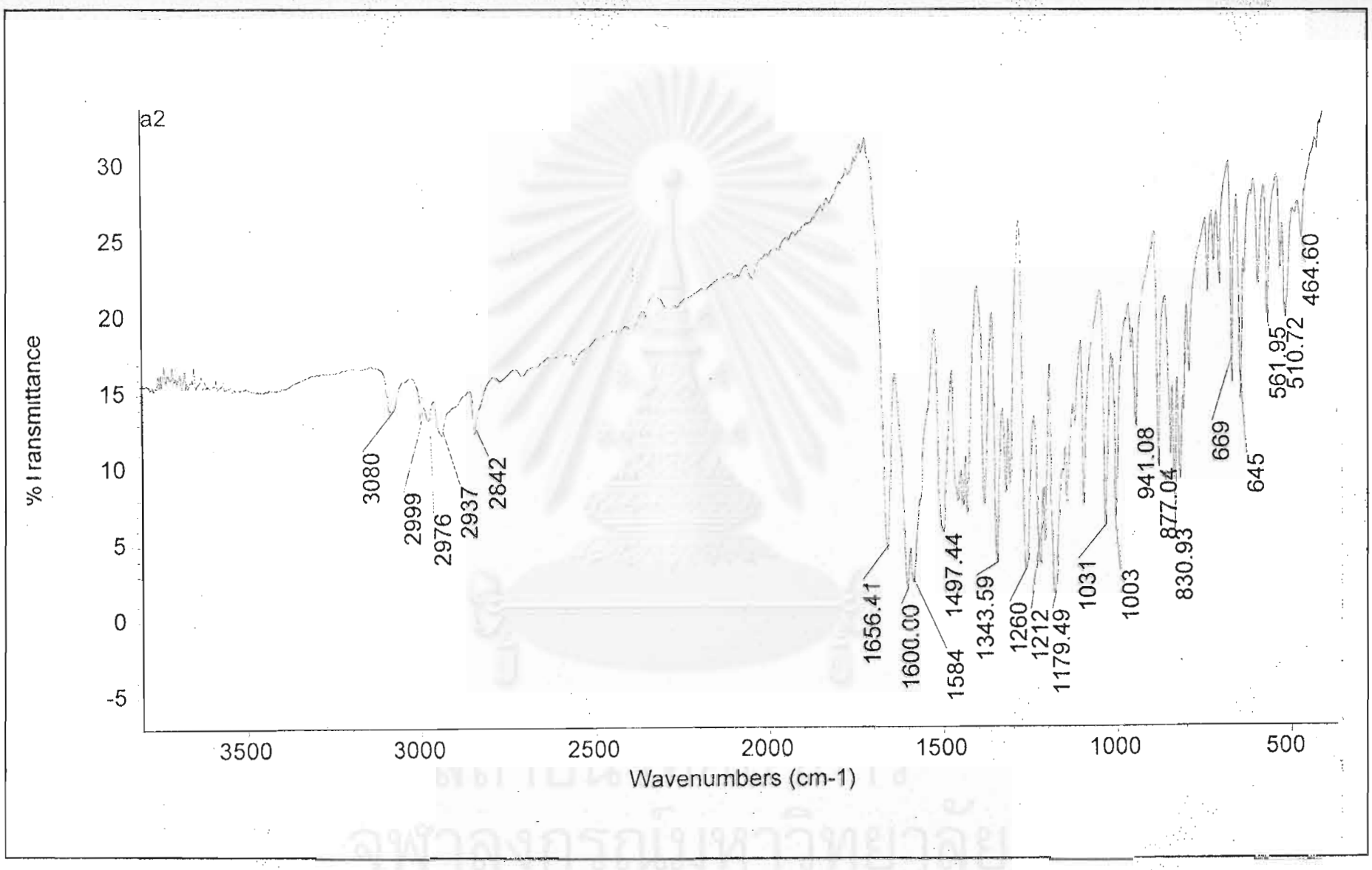

Figure 24 IR-spectrum of compound **2**

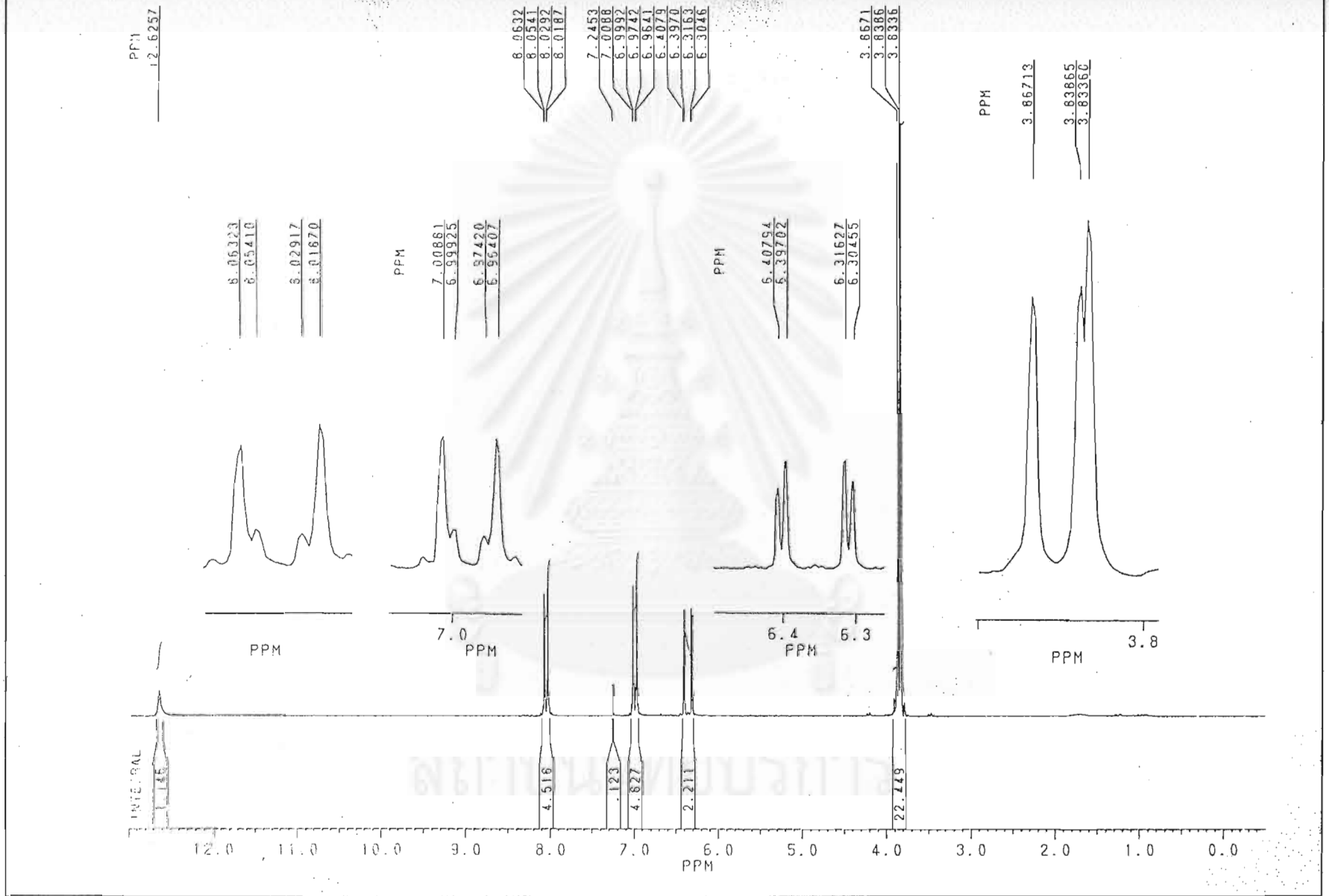

Figure 25 ^{1}H-NMR spectrum of compound **2**

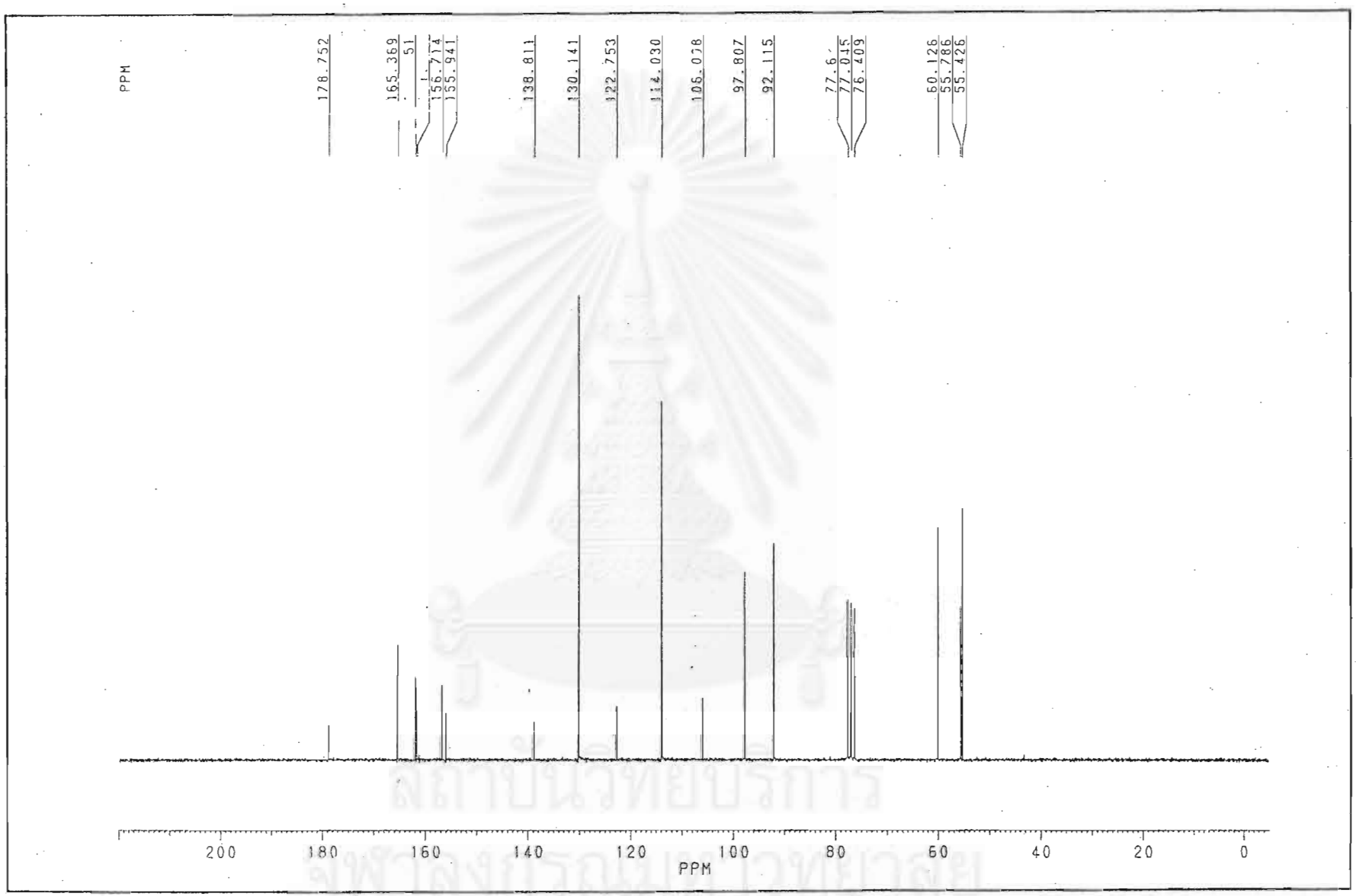

Figure 26 ^{13}C-NMR spectrum of compound **2**

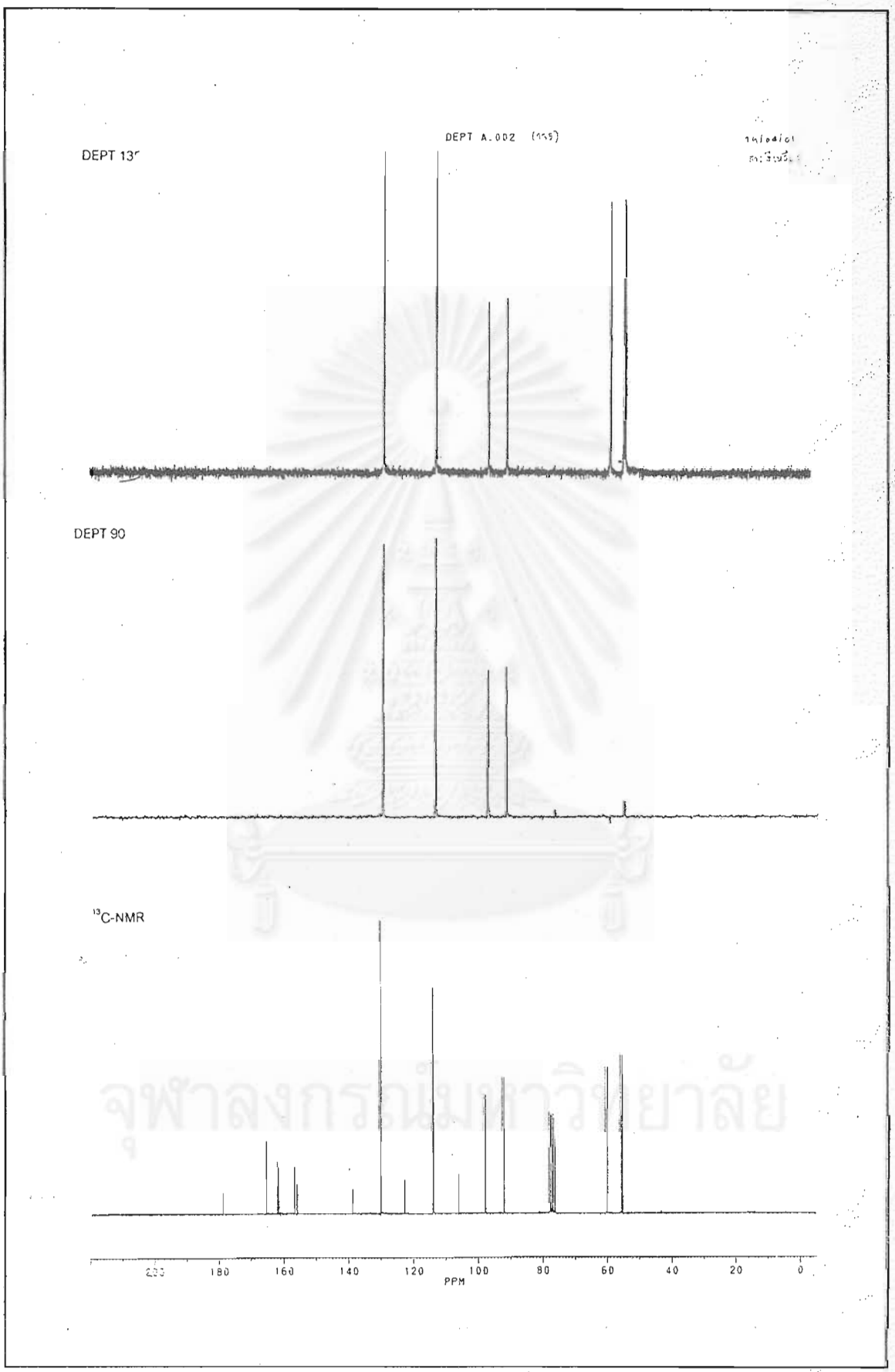

Figure 27 DEPT-135, 90 and ^{13}C-NMR spectrum of compound **2**

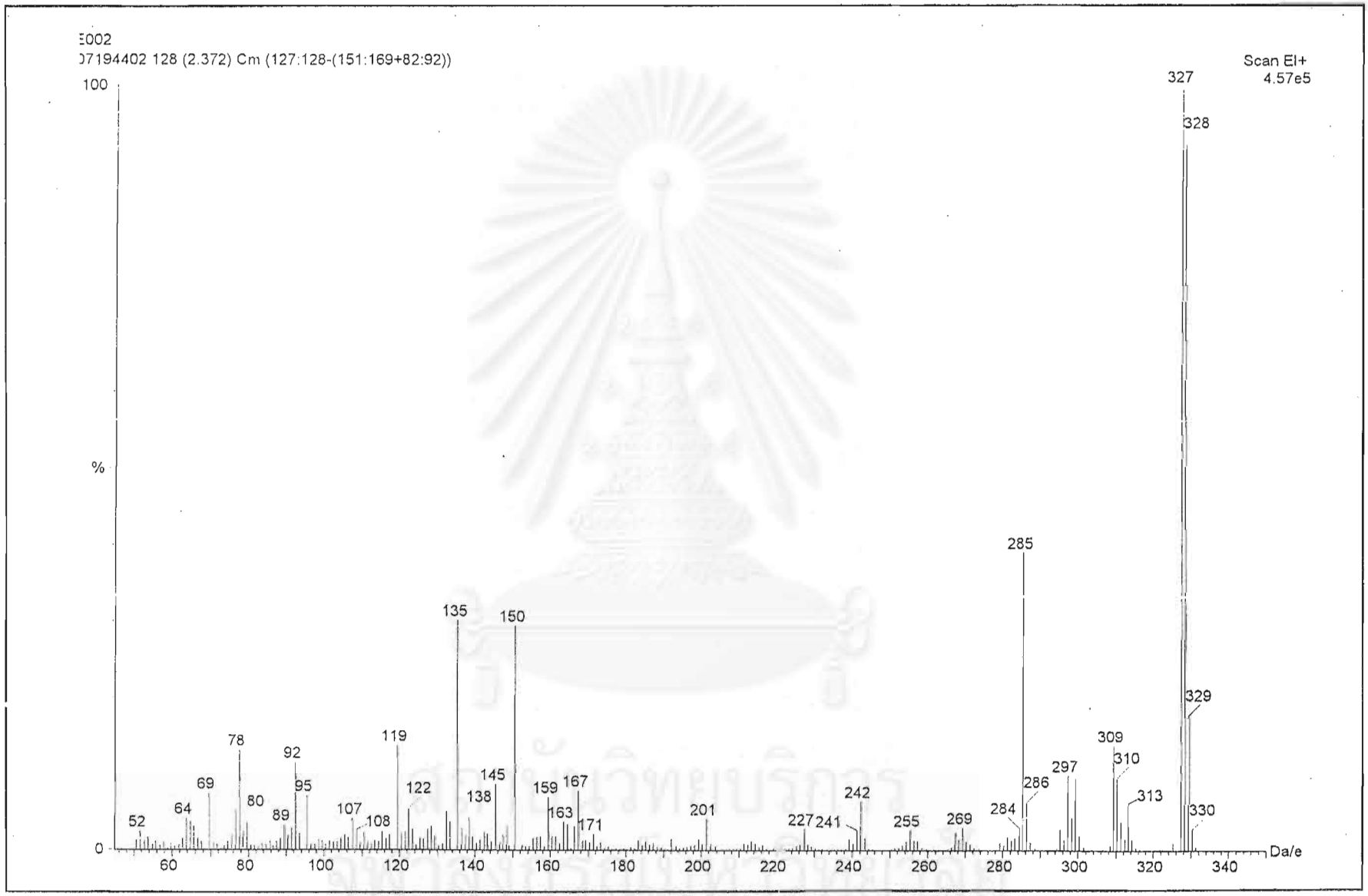

Figure 28 EIMS spectrum of compound **2**

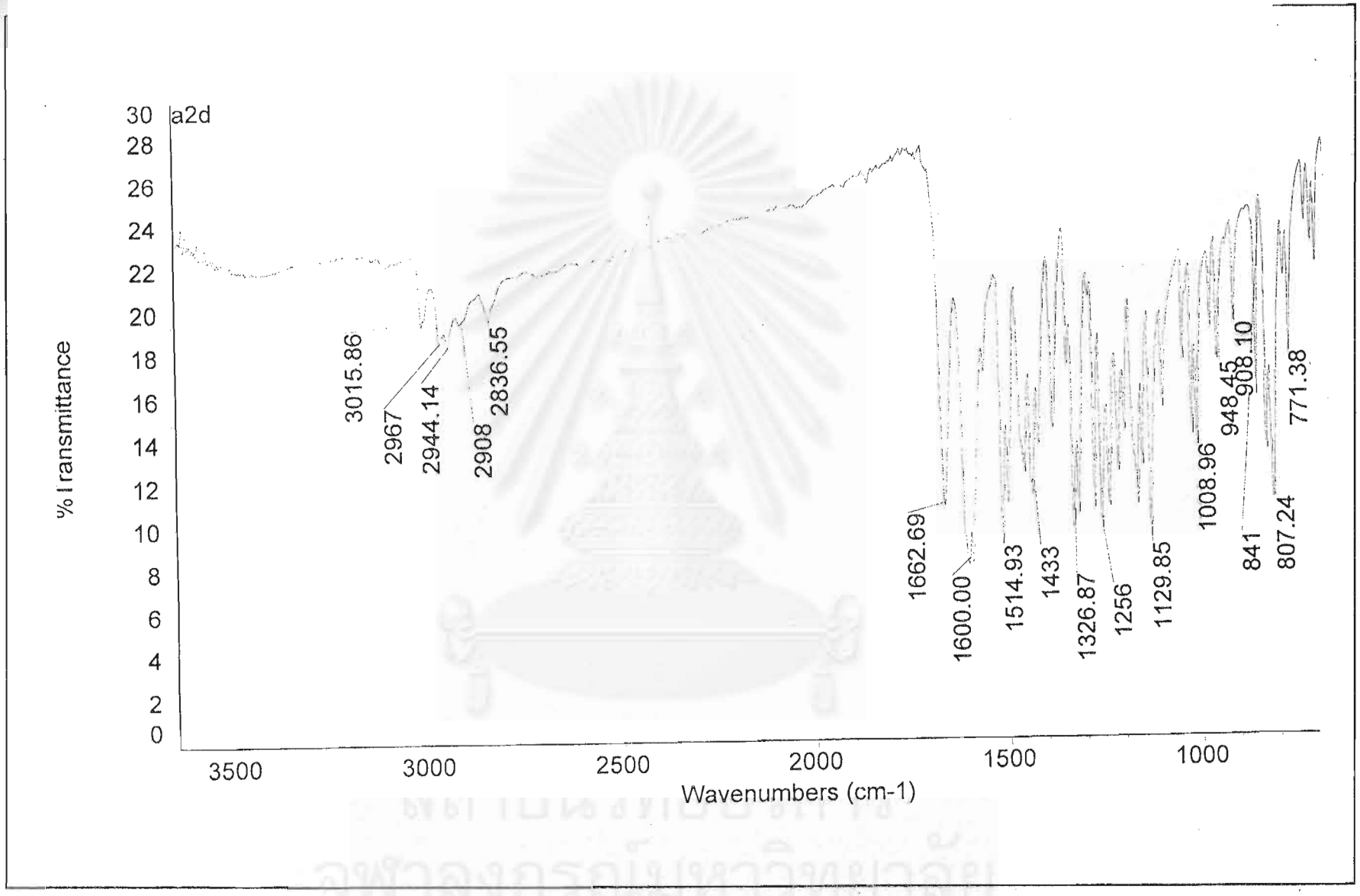

Figure 29 IR-spectrum of compound **3**

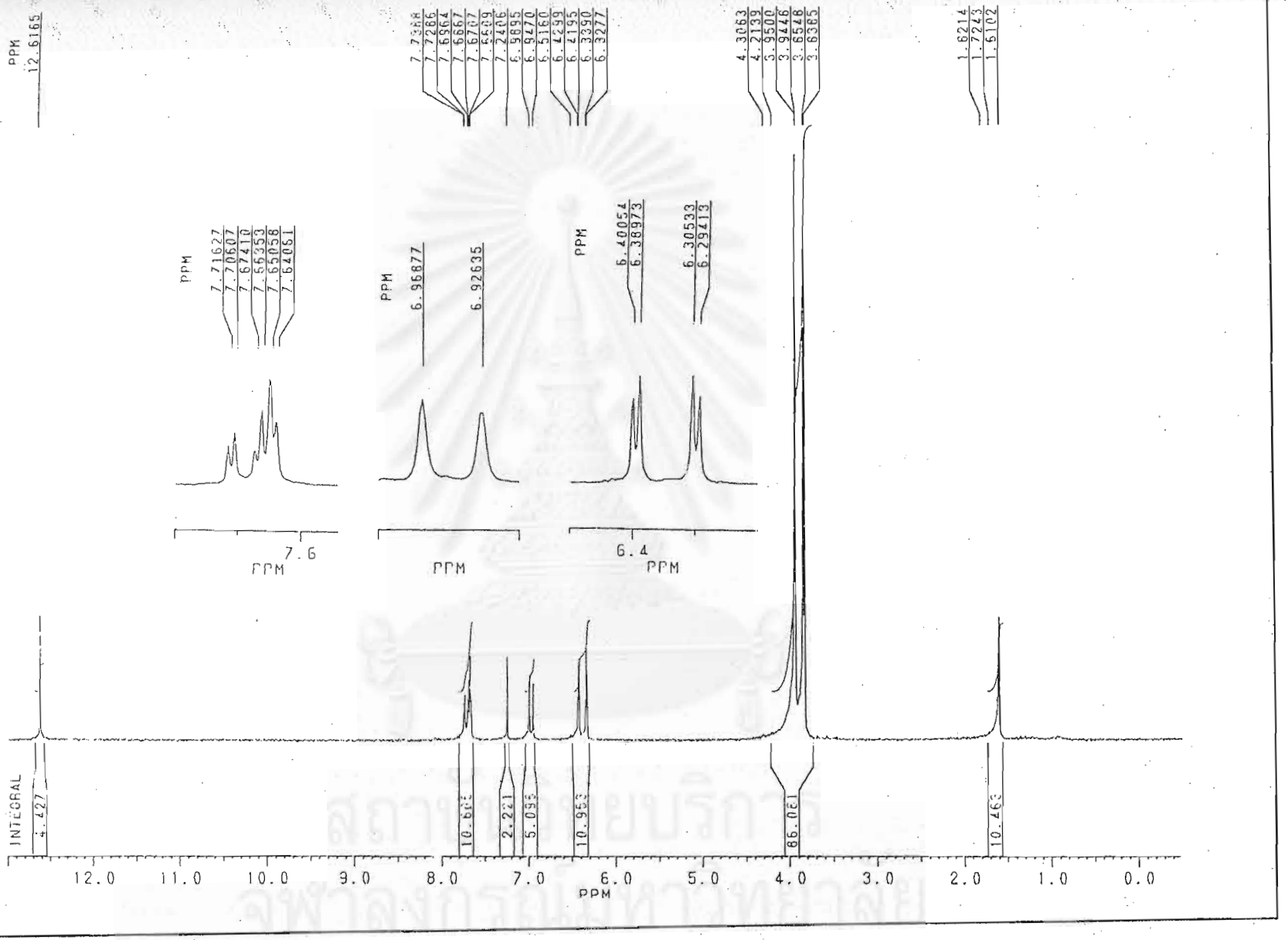

Figure 30 ^{1}H-NMR spectrum of compound **3**

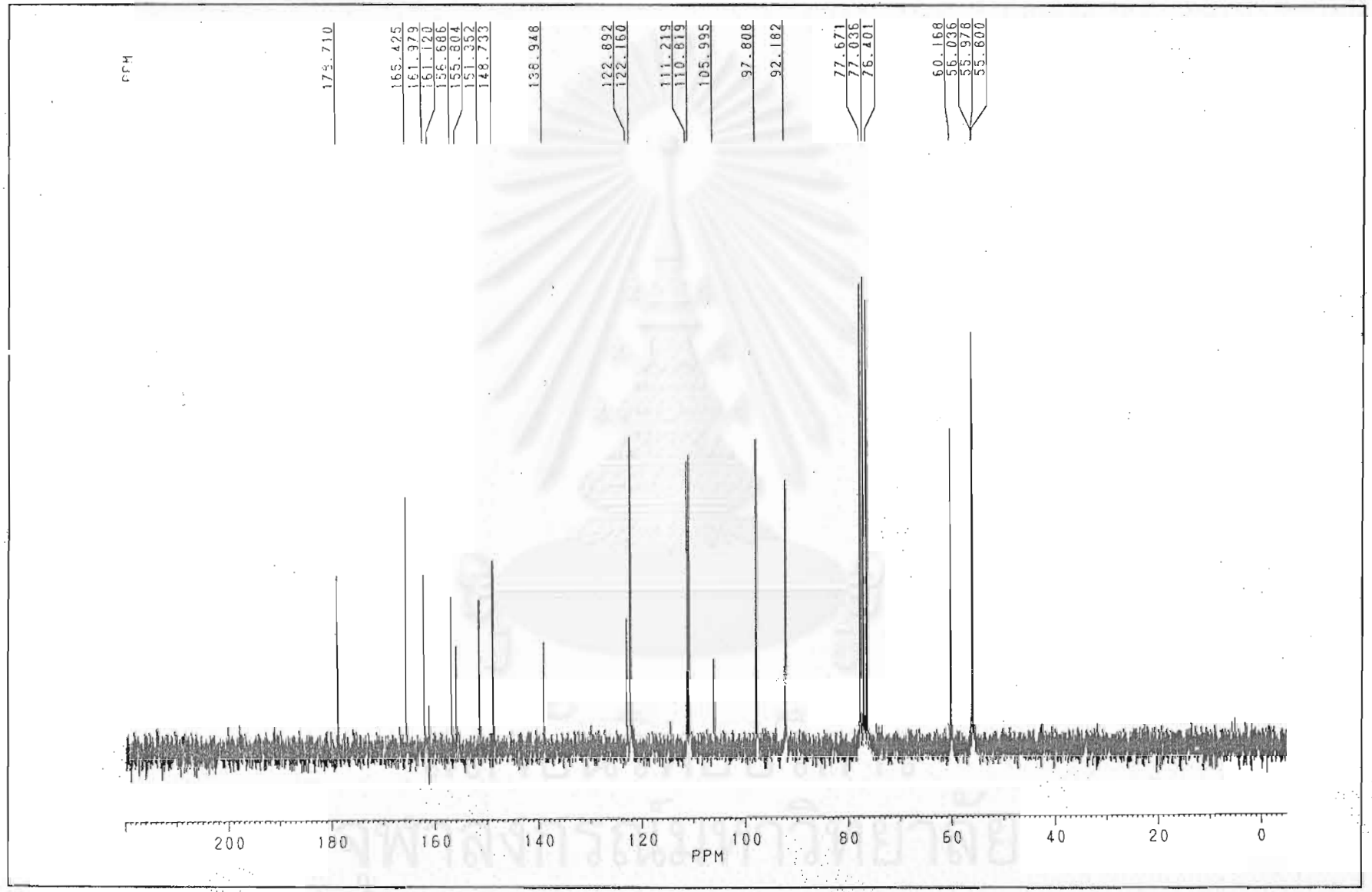

Figure 31 ^{13}C-NMR spectrum of compound **3**

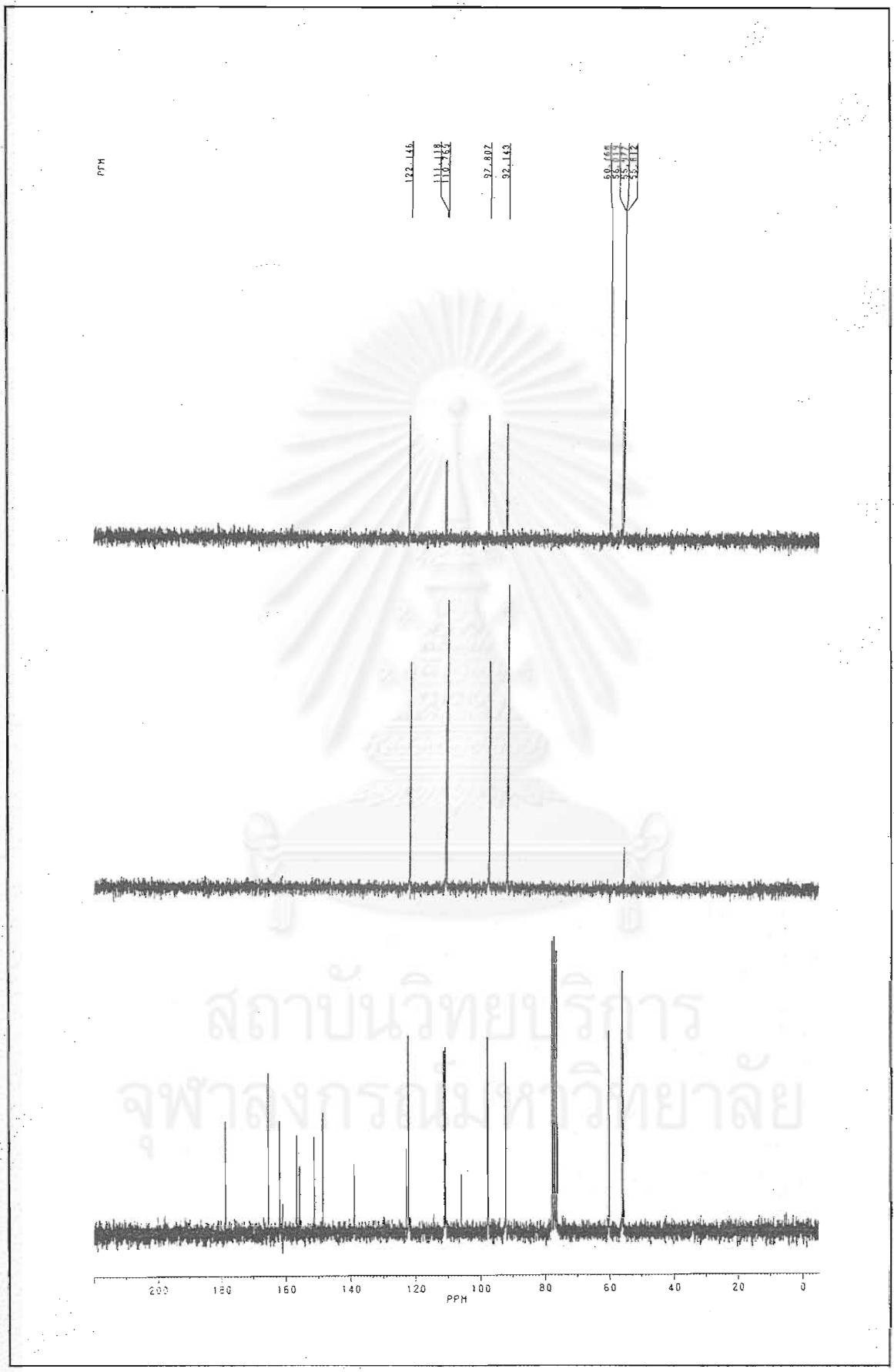

Figure 32 DEPT-135, 90 and ^{13}C-NMR spectrum of compound **3**

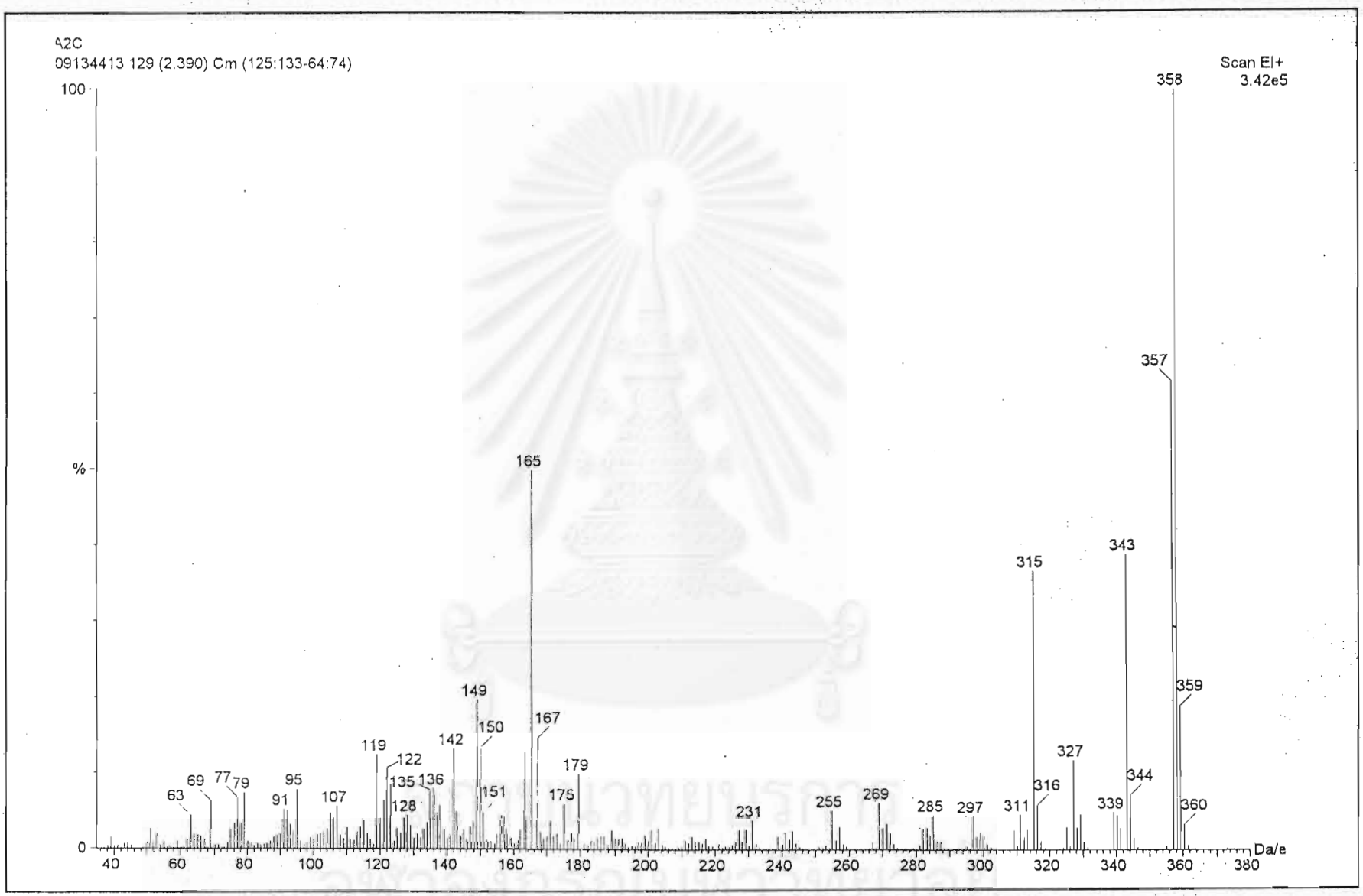

Figure 33 EIMS spectrum of compound **3**

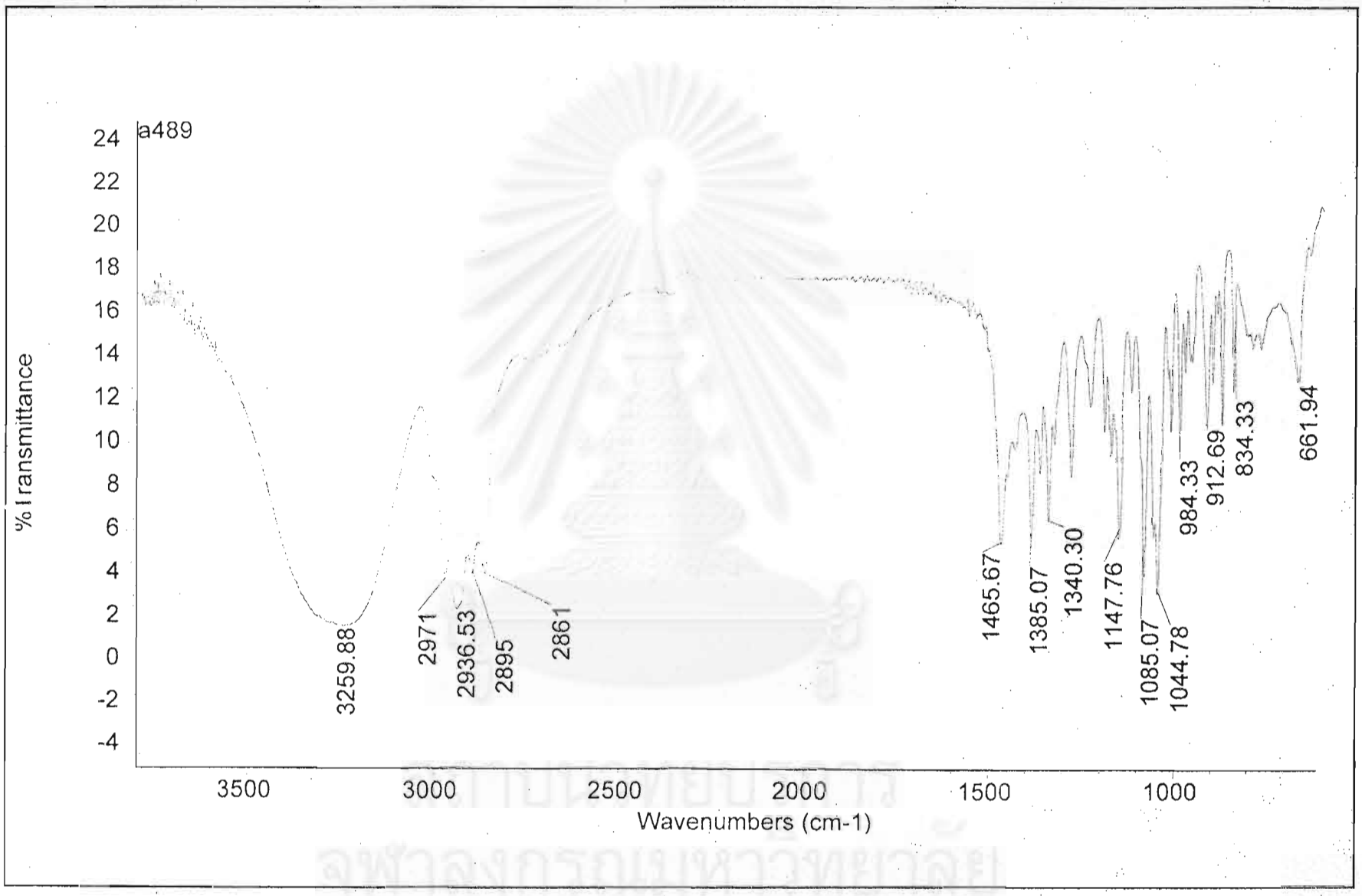

Figure 34 IR-spectrum of compound 4

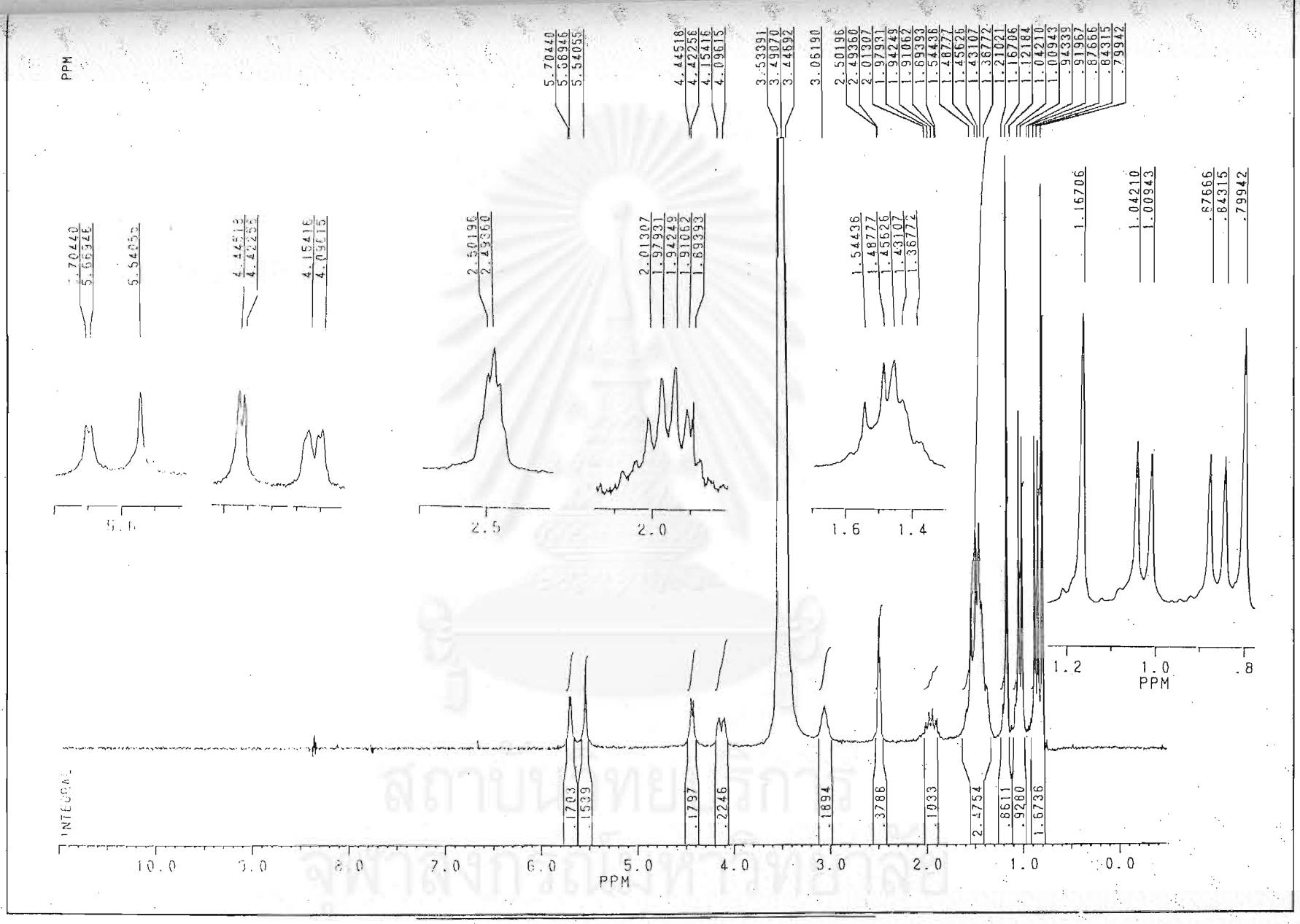

Figure 35 ^{1}H-NMR spectrum of compound **4**

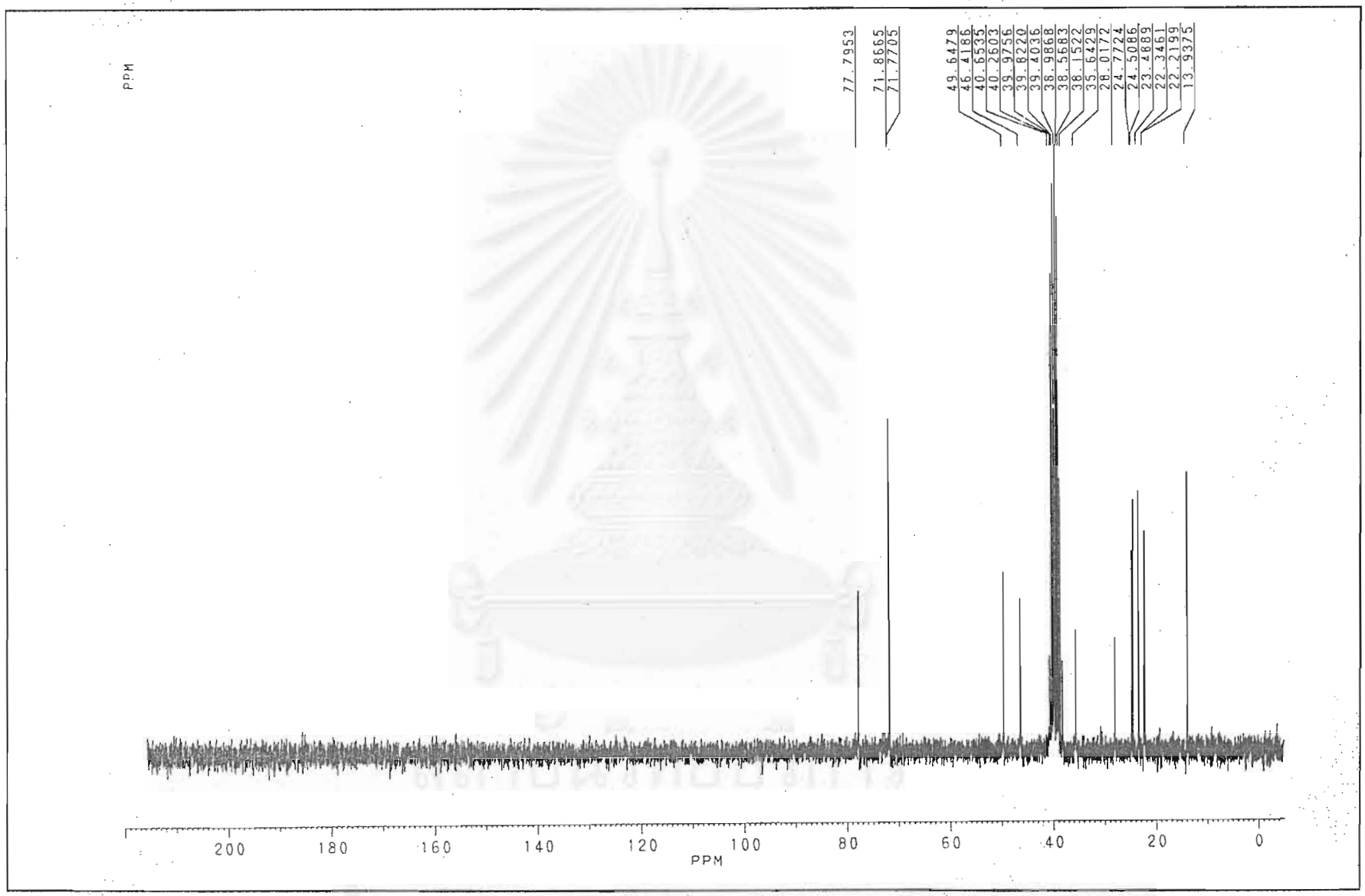

Figure 36 ^{13}C-NMR spectrum of compound **4**

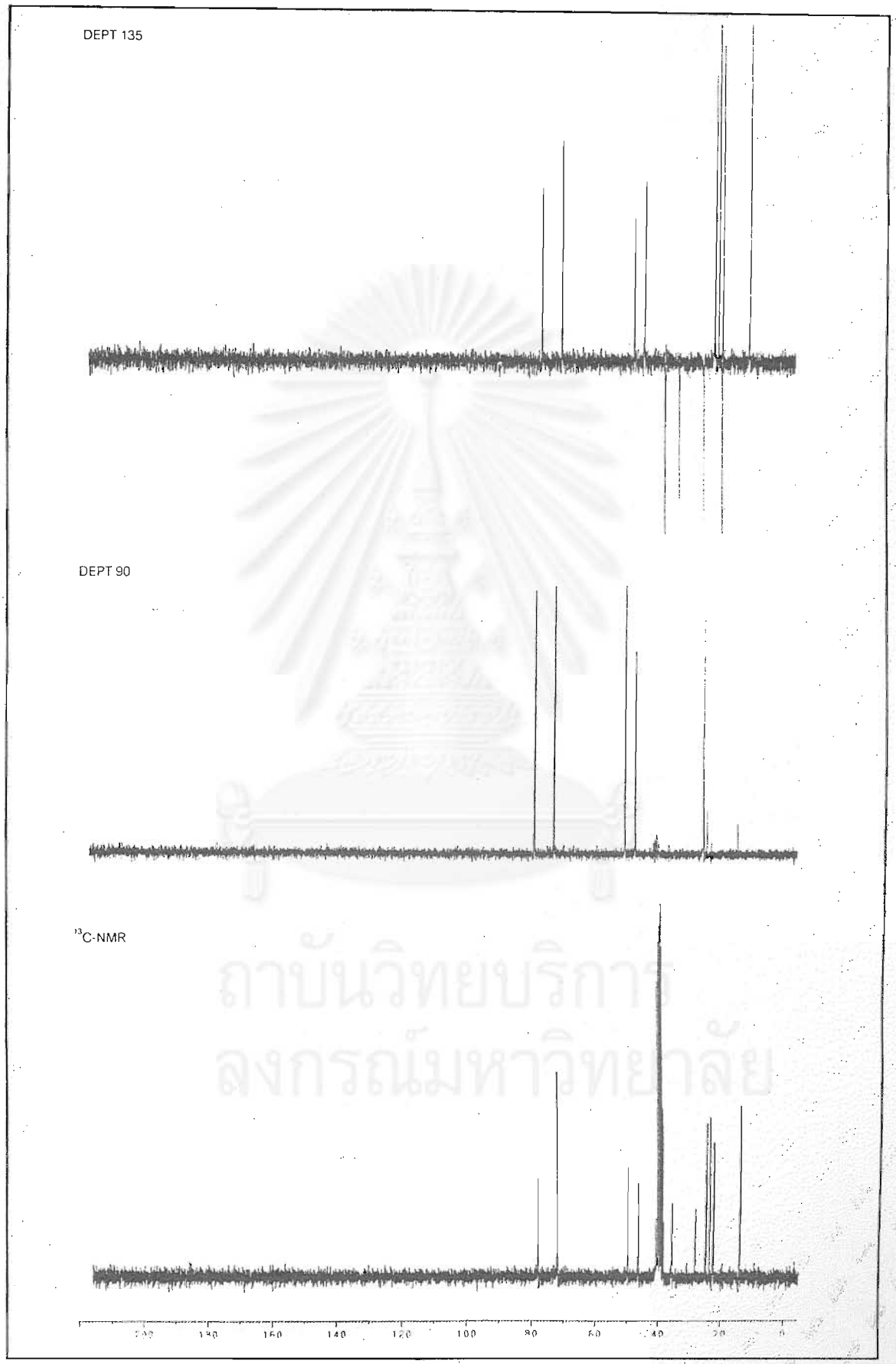

Figure 37 DEPT-135,90 and ^{13}C-NMR spectrum of compound **4**

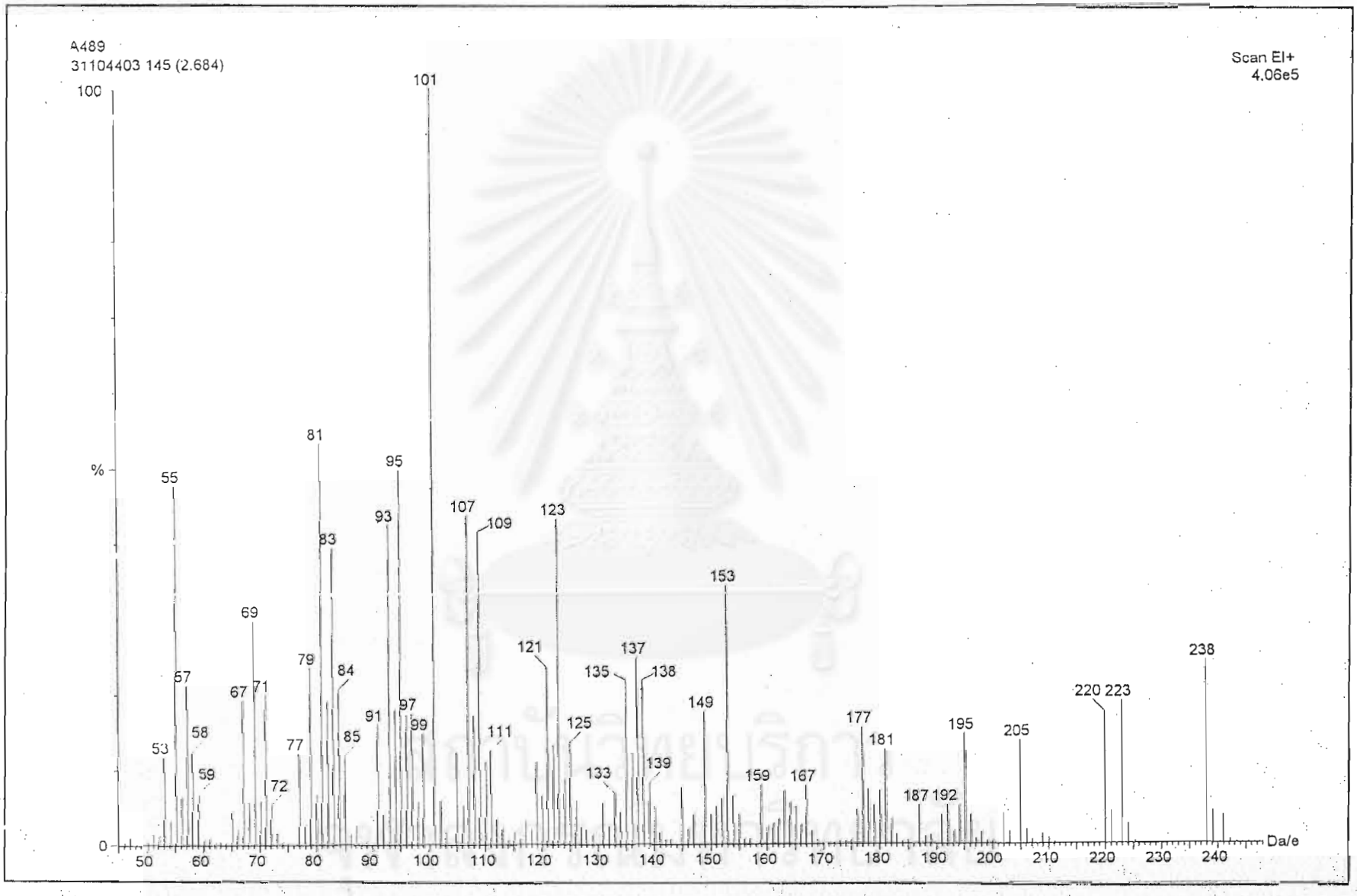

Figure 38 EIMS spectrum of compound **4**

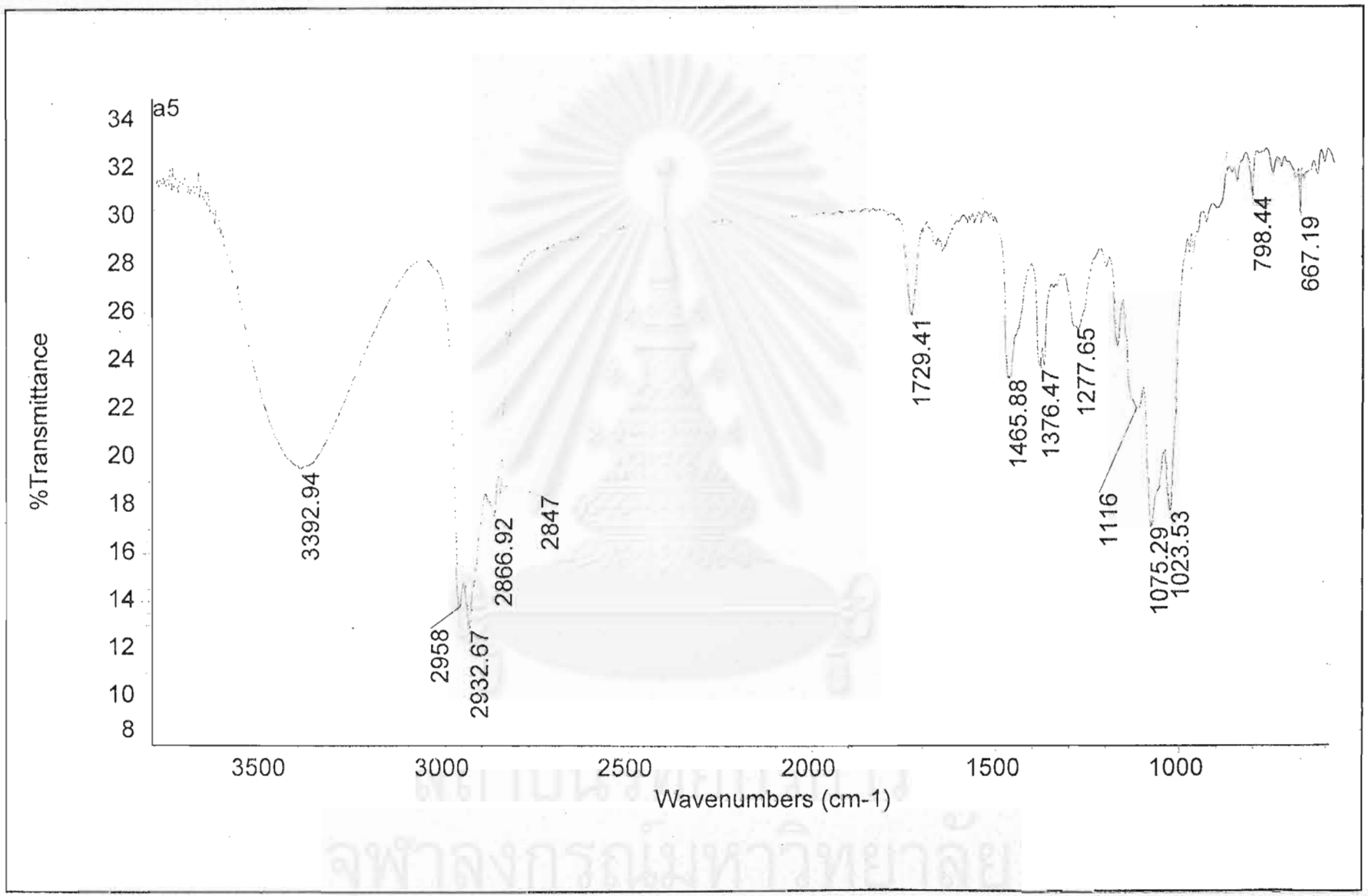

Figure 39 IR-spectrum of mixture 5

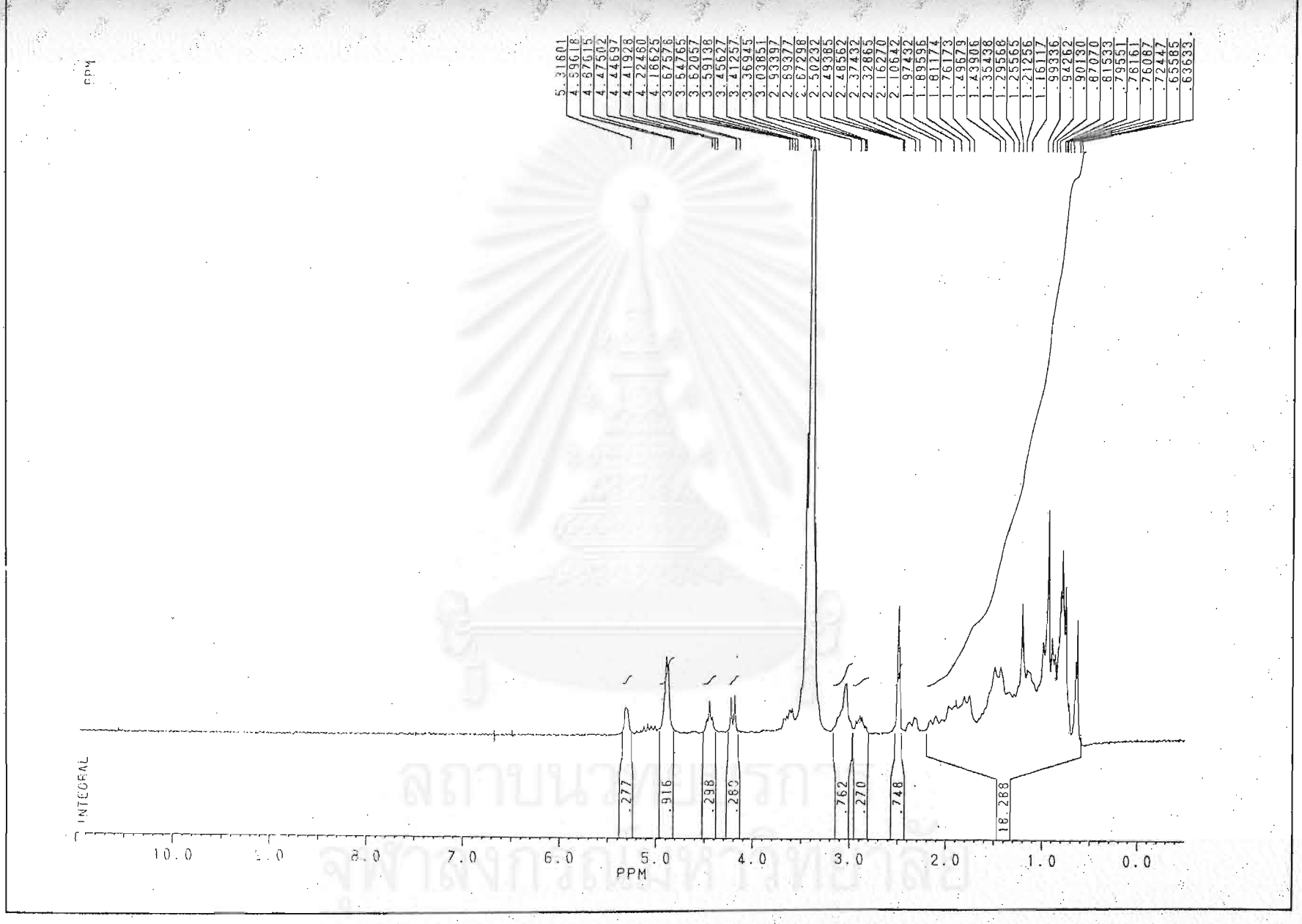

Figure 40 ^{1}H-NMR spectrum of mixture **5**

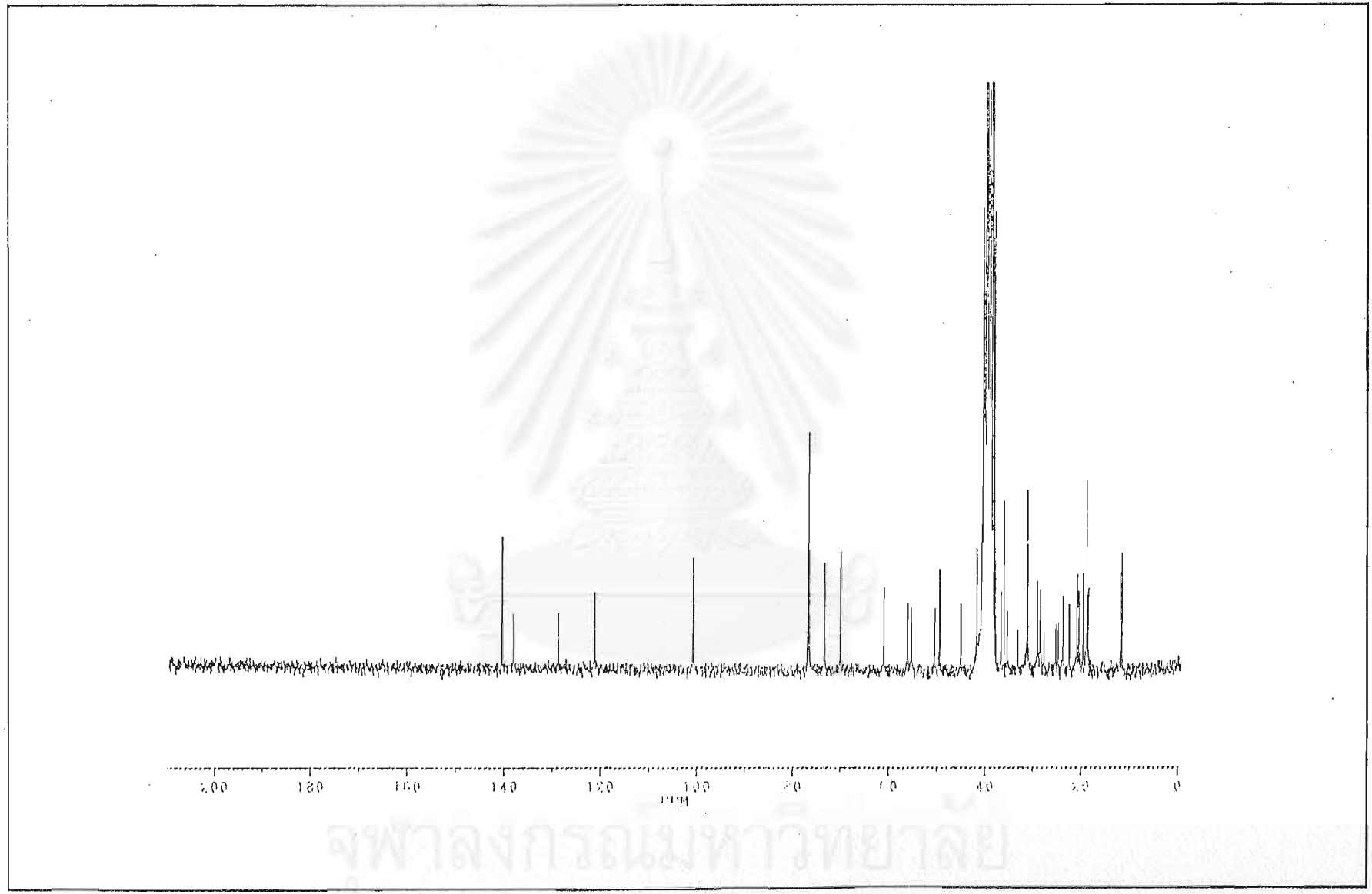

Figure 41 ^{13}C-NMR spectrum of mixture 5

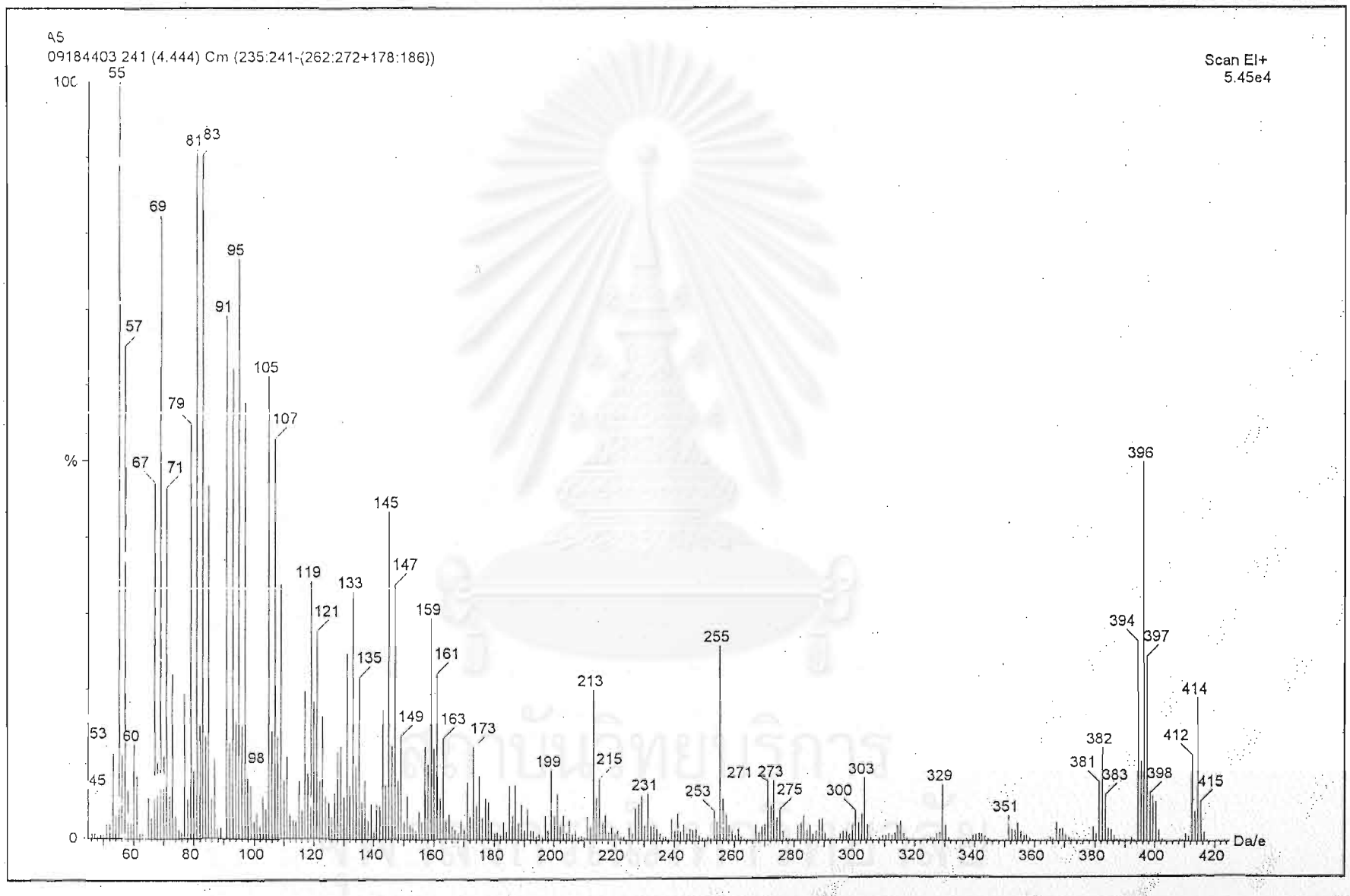

Figure 42 EIMS spectrum of mixture 5

VITA

Miss Aree Suandong was born on January 8, 1977 in Nakhonratchasima province. She graduated with Bachelor Degree of Science in Biology at Chulalongkorn University in 1999. In the same year, she was admitted into a Master Degree program in biotechnology at Chulalongkorn University. During her study toward the Master's degree; she received financial support from Science Program in Biotechnology, Faculty of Science and the Graduate School, Chulalongkorn University and the Royal Bangkok Sport Club. She completed her M. Sc. degree in 2002.