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สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

CHEMICAL CONSTITUENTS OF *CURCUMA MANGGA* RHIZOME

Miss Kunlanat Makboon

A Thesis Submitted in Partial Fulfillment of the Requirements

for the Degree of Master of Science in Pharmacy

Department of Pharmacognosy

Faculty of Pharmaceutical Sciences

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Academic Year 2000

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สถาบันวิทยบริการ
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Thesis Title	Chemical Constituents of *Curcuma mangga* rhizome
By	Miss Kunlanat Makboon
Department	Pharmacognosy
Thesis Advisor	Associate Professor Nijsiri Ruangrungsi, Ph.D.

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

……..Dean of the Faculty of Pharmaceutical Sciences

(Associate Professor Sunibhond Pummangura, Ph.D.)

Thesis committee

……Chairman

(Associate Professor Sumphan Wongseripipatana, Ph.D.)

……Thesis Advisor

(Associate Professor Nijsiri Ruangrungsi, Ph.D.)

……Member

(Associate Professor Ekarin Saifah, Ph.D.)

……Member

(Associate Professor Wandee Gritsanapan, Ph.D.)

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กุลนาถ มากบุญ : องค์ประกอบทางเคมีของเหง้าขมิ้นขาว (CHEMICAL CONSTITUENTS OF *CURCUMA MANGGA* RHIZOME) อาจารย์ที่ปรึกษา : รศ. ดร. นิจศิริ เรืองรังษี 137 pp. ISBN 974-346-433-6

การศึกษาองค์ประกอบทางเคมีของเหง้าขมิ้นขาวทั้งในส่วนที่ได้จากการสกัดแยกโดยวิธีทางโครมาโทกราฟีและส่วนที่ได้จากการกลั่นน้ำมันระเหยพบว่า ส่วนที่ได้จากการสกัดแยกโดยวิธีทางโครมาโทกราฟีสามารถแยกองค์ประกอบทางเคมีจากสิ่งสกัดได้ 3 ชนิด ซึ่งเมื่อวิเคราะห์โครงสร้างด้วยวิธีทางสเปคโตรสโคปี พบว่ามีสารใหม่ 1 ชนิดในกลุ่มของ labdane diterpene คือ 15-ethoxy-8(17),12-labdadien-15,16-olide และสารอีก 2 ชนิด คือ สารในกลุ่ม norlabdane diterpene คือ 15,16-bisnorlabda-8(17),11-dien-13-one และของผสมในกลุ่ม steroid คือ β-sitosterol และ stigmasterol อนึ่งได้ทำการแก้ไขการกำหนดค่า chemical shift ของคาร์บอนและโปรตอนของ 15,16-bisnorlabda-8(17),11-dien-13-one ให้ถูกต้องและสมบูรณ์ยิ่งขึ้น สำหรับน้ำมันระเหยซึ่งวิเคราะห์หาองค์ประกอบด้วยเครื่อง GC/MS พบองค์ประกอบทางเคมี 30 ชนิด ประกอบด้วยสารกลุ่ม monoterpene ในปริมาณสูงสุดถึงร้อยละ 97.46 ซึ่งสารที่พบมากที่สุด คือ myrcene (ร้อยละ84.61) และ β-phellandrene (ร้อยละ6.63), *trans*-ocimene (ร้อยละ3.85) ตามลำดับ นอกจากนี้ได้ทำการศึกษาฤทธิ์ในการต้านจุลชีพของน้ำมันระเหย พบฤทธิ์ในการฆ่าเชื้อแบคทีเรียและเชื้อรา โดยเฉพาะ *Staphylococcus aureus* และ *Microsporum gypseum*

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

ภาควิชาเภสัชเวท ลายมือชื่อนิสิต.....

สาขาเภสัชเวท ลายมือชื่ออาจารย์ที่ปรึกษา.....

ปีการศึกษา 2543

##4176551233 : MAJOR PHARMACOGNOSY
KEY WORD : *CURCUMA MANGGA*/ LABDANE-TYPE DITERPENE/ NORLABDANE-TYPE DITERPENE/ ESSTENTIAL OIL/ ANTIMICROBIAL ACTIVITY
KUNLANAT MAKBOON: CHEMICAL CONSTITUENTS OF *CURCUMA MANGGA* RHIZOME. THESIS ADVISOR: ASSOCIATE PROFESSOR NIJSIRI RUANGRUNGSI Ph.D., 137 pp. ISBN 974-346-433-6

The investigation of chemical constituents of *Curcuma mangga* Valeton & van Zyp (Zingiberaceae) was performed on both non-volatile and volatile parts. Chromatographic separation led to the isolation of 3 compounds from non volatile part. The structure determination was accomplished by the aids of spectroscopic means and the previous data of related compound. A structure of new labdane type diterpene, 15-ethoxy-8(17),12-labdadien-15,16-olide was deduced through extensive 1D and 2D NMR studies. A norlabdane-type diterpene, 15,16-bisnorlabda-8(17),11-dien-13-one was determined whilst the complete revision of ^{1}H and ^{13}C assignments were attained. Moreover, β-sitosterol and stigmasterol was detected as a mixture of steroidal compounds. The volatile constituents hydrodistilled from the fresh rhizome was analysed by GC/MS. At least 30 components were determined. The major compound was monoterpene group which was accounting for 97.46% and myrcene (84.61%) appeared to be the major component followed by β-phellandrene (6.63%) and *trans*-ocimene(3.85%). From antimicrobial activity examination, the essential oil exhibited broad spectrum against microorganism with strong cidal effect on *Staphylococus aureus* and *Microsporum gypseum*.

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Department Pharmacognosy Student's signature…….

Field of study Pharmacognosy Advisor's signature…….

Academic year 2000

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LIST OF ABBREVIATIONS

AOAC	=	Association of official analytic chemists
c	=	Concentration
°C	=	Degree Celsius
CD	=	Circular Dichroism
$CDCl_3$	=	Deuterated chloroform
cm	=	Centimeter
COLOC	=	Correlation spectroscopy via Long-range Coupling
^{13}C NMR	=	Carbon-13 nuclear magnetic resonance
COSY	=	Correlation spectroscopy
d	=	Doublet (for NMR spectra)
dd	=	Doublet of doublets (for NMR spectra)
DEPT	=	Distortionless Enhancement by Polarization Transfer
DMSO	=	Deuterated dimethylsulfoxide
δ	=	Chemical shift
EIMS	=	Electron Impact Mass Spectrum
EtOAc	=	Ethylacetate
g	=	Gram
GC-MS	=	Gas chromatography-Mass spectrometry
μg	=	Microgram
h	=	Hour
HETCOR	=	Heteronuclear Chemical Shift Correlation

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^{1}H NMR	=	Proton nuclear magnetic resonance
HMBC	=	^{1}H-detected Heteronuclear Multiple Bond Coherence
H_2O	=	Water
HRFABMS	=	High Resolution Fast Atom Bombardment Mass Spectrum
Hz	=	Hertz
IR	=	Infrared spectrum
J	=	Coupling constant
Kg	=	Kilogram
L	=	Liter
μl	=	Microliter
λmax	=	Wavelength at maximal absorption
ε	=	Molar absorptivity
M^+	=	Molecular ion
m	=	Multiplet (for NMR spectra)
MeOH	=	Methanol
mg	=	Milligram
MHz	=	MegaHertz
min	=	Minute
ml	=	Milliliter
m/z	=	Mass to charge ratio
nm	=	Nanometer
NMR	=	Nuclear magnetic resonance
No.	=	Number

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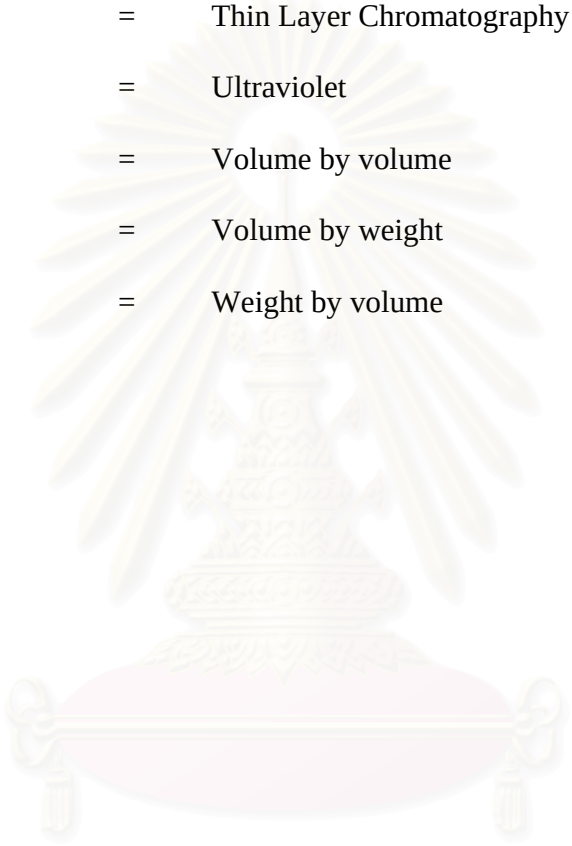
NOE	=	Nuclear Overhauser Effect
ppm	=	Part per million
νmax	=	Wave number at maximal absorption
s	=	Singlet (for NMR spectra)
TLC	=	Thin Layer Chromatography
UV	=	Ultraviolet
v/v	=	Volume by volume
v/w	=	Volume by weight
w/v	=	Weight by volume



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CHAPTER I

INTRODUCTION

The plants in the genus *Curcuma* is one of the genera of the family Zingiberaceae. The plants in this family are mainly confined to Old World tropics, with center of distribution in Indo-Malaya (Purseglove, 1983).

The botanical aspects of this genus are stemless herbs with tuberous rootstocks bearing sessile and long stipulate tubers. Leaves usually oblong, often very large. Flowers in dense compound spikes, vernal or aestival, and preceding, or autumnal and contemporaneous with the leaves, crowned by a coma of enlarged coloured bracts, lower bracts ovate, membranous, enclosing several bracteolate fugacious flowers which open in succession. Calyx short, cylindric, minutely toothed. Corolla tube fennel-shaped, corolla lobes usually ovate or oblong, the upper longer and somewhat concave. Stamen 1 perfect; filament short; anthers not crested, with contiguous cell spurred at base; lateral steminodes oblong, petaloid, connate with the filament. Lip orbicular, with a deflexed tip. Ovary 3-celled; ovules numerous on axile placentas; style filiform, stigma 2-lipped, the lips ciliate. Fruit a tardily dehiscent globose membranous 3-valved capsule. Seeds ovoid or oblong, usually arillalte. The rhizome is aromatic, stomachic, and carminative. (Kirtikar, Basu and An,1981)

There are more than 100 species of *Curcuma* in the world, however, in Thailand there are about 33 species as follows. (Tem Smitinand, 1980; Thawatchai Santisuk, 1996)

Curcuma aeruginosa Roxb.

: ว่านมหาเมฆ Waan mahaamek (Central)

Curcuma albiflora Thw.

Curcuma alismatifolia Gagnep.

: ขมิ้นโคก Khamin khok (Loei)

Curcuma amada Roxb.

Curcuma amarissima Roscoe.

: ขมิ้นขม Khamin khom (Northern)

Curcuma angustifolia Roxb.

Curcuma aromatica Salisb.

: ว่านนางคำ Waan naang kham

Curcuma attenuata Wall.

Curcuma aurantica van Zijp

Curcuma brog Val.

Curcuma cochinchinensis Gagnep.

Curcuma comosa Roxb.

: ว่านชักมดลูก Waanchak motluuk (Central)

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Curcuma ecomata Craib.

Curcuma elata Roxb.

Curcuma gracillima Gagnep.

Curcuma gracillima var. elatior Gagnep.

Curcuma harmandii Gagnep.

Curcuma longa L.

(*Curcuma domestica* Valeton*)*

: ขมิ้น Khamin (Central), ขมิ้นแกง Khamin kaeng, ขมิ้นหยอก Khamin yok, ขมิ้นหัว Khamin hua (Chiang Mai), ขมิ้นชัน Khamin chan (Central, Peninsular), ขี้มิ้น Kheemin, หมิ้น Min (Peninsular), ตายอ Taa-yo (Karen-Kamphaeng Phet), สะยอ Sa-yo (Karen-mae Hong Son), Turmeric

Curcuma mangga Valeton & van Zyp

: ขมิ้นขาว Khaminkhao (Central)

Curcuma oligantha Trimen aff.

Curcuma paviflora Wall.

Curcuma petiolata Roxb.

Curcuma rocoena Wall.

: ขมิ้นแดง Khamin daeng (Mae hong Son)

Curcuma sessilis Gage.

: กาเตียว Kaa tieo (Loei), จวด Chuat (Chumphon, Songkhla), อาวแดง Aao daeng (Northern)

Curcuma singularis Gagnep.

Curcuma sparganifolia Gagnep.

: กระเจียวบัว Krachieo bua (Central)

Curcuma stenochila Gagnep.

Curcuma thorelii Gagnep.

Curcuma thichosanta Gagnep.

Curcuma viridiflora Roxb.

Curcuma xanthorrhiza Roxb.

Curcuma zedoaria (Berg) Rosc.

: ขมิ้นอ้อย Khamin oi (Central), ขมิ้นขึ้น Khamin khuen (Northern), ละเมียด La-miat (Khmer), Zedoary

Curcuma zerumbret Roxb.

: แฮ้วดำ Haeo dam (Chiang Mai)

For *Curcuma mangga*, it is distributed in Bengal, North-eastern India, Malay Peninsular, West peninsular-Archipelago, Java and Thailand. *Curcuma mangga* has many common names up to each region such as Temu pauch :in Java, Temu mangga; Temu lalab :in Sundanese and Kaminkhao :in Thailand. The name *Curcuma mangga* is derived from a mango-like aroma of its rhizome. And also, its vernacular name “pauch” (Temu

pauch) is the name for one of Mangiferas. By the reason of the mango-like aroma of its rhizome, it is commonly called “mango ginger”. (Kirtikar *et al.*, 1981; Wong *et al.*, 1999)

This mango ginger is a perenial herb with freshy rhizomes. Rootstock large; sessile tubers thick, cylindric or ellipsoid, pale yellow inside. Leaves long-petiolate, in tufts, the blade 30-40 by 7.5-12.5 cm, oblong-lanceolate, acute or acuminate, narrowed to the base, glabrous and green on both sides; petioles as long as the leaf-blade (30-45 cm). Flowers in autumnal spikes 7.5-15 by 3.8-5 cm in the center of the tuft of leaves; peduncle 15 cm long or more; flowering bracts 2.5 cm long, greenish white; bracts of the coma longer and narrower, tinged with pink or red. Calyx nearly 13 mm long, obtusely 3-toothed. Corolla white or very pale yellow; tube about 2.5 cm long; lobes oblong acute. Lip semielliptic, yellow, 3-lobed, the middle lobe emarginate. (Kirtikar *et al*., 1981)

The information about the use of the mango ginger as traditional medicine has never been found, however, it has been used as dye, seasoning for food and vegetable in Thai cuisine. (Purseglove, 1983; Lemmens and Wulijarni-Soetjipto, 1991)

Turning to a biological activity issue, only antitumor and cytotoxic activities of *C. mangga* rhizome were reported. (Itokawa *et al.*, 1990; Murakami *et al.*, 1993)

Little has been known for the phytochemical study on *C. mangga*. Herein, the author reported the study on volatile and non volatile constituents of Thai *C. mangga* rhizome. Furthermore, the antimicrobial activities of the essential oil of this mango ginger rhizome were also conducted. Consequently, the present study was undertaken in order to expand our knowledge of the constituents of this medicinally interesting genus.

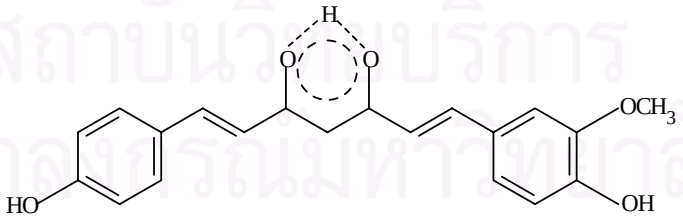
Figure 1 ***Curcuma mangga*** **Valeton & van Zyp (Zingiberaceae)**

CHAPTER II

HISTORICAL

1. Chemical Constituents of *Curcuma mangga*

There was no reports on the phytochemical study on this mango ginger, however, the TLC-densitometric method showed that *C. mangga* contained demethoxycurcumin. (Supinya Tewtrakul, 1993) In addition, the essential oil of the rhizomes of *C. mangga* analyzed by capillary GC and GC/MS showed that the essential oil mainly consisted of monoterpenes and sesquiterpenes. The major components were myrcene (78.6%), (E)-β-ocimene (5.1%), β-pinene (3.7%) and α-pinene (2.9%) as described in Table 1(Wong *et al*., 1999)



Demethoxycurcumin

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Table 1 Chemical constituents of essential oil from *Curcuma mangga*

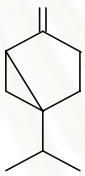
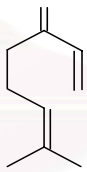
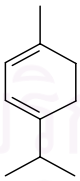
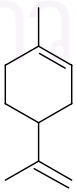
Chemical group	Compound	Structure	Percentage[a]
Monoterpene	α-pinene		2.9
	β-pinene		3.7
	sabinene		*tr*
	myrcene		78.6
	α-terpinene		0.1
	limonene		0.7

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Table 1 (Continued)

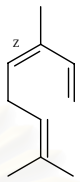
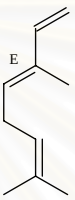
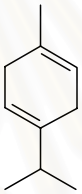
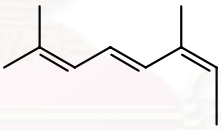
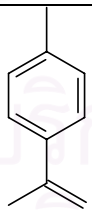
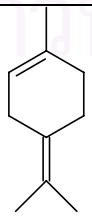
Chemical group	Compound	Structure	Percentage[a]
	(z)-β-ocimene		0.4
	(E)-β-ocimene		5.1
	γ-terpinene		0.1
	alloocimene		*tr*
	p-cymene		*tr*
	terpinolene		0.1

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Table 1 (Continued)

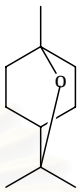
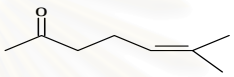
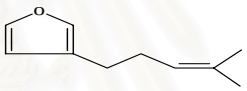
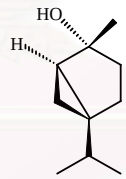
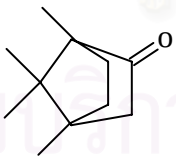
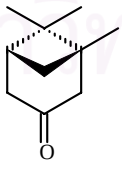
Chemical group	Compound	Structure	Percentage[a]
Oxygenated monoterpene	1,8 cineol		0.2
	6-methyl-5-hepten-2-one		*tr*
	perillene		0.4
	trans-sabinene hydrate		0.1
	camphor		0.1
	pinocamphone		0.1

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Table 1 (Continued)

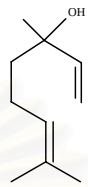
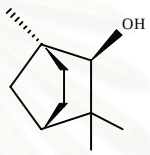
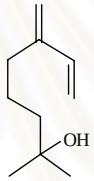
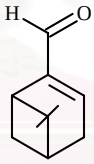
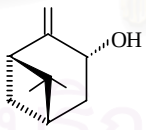
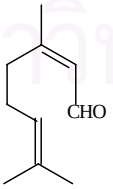
Chemical group	Compound	Structure	Percentage[a]
	linalool		0.4
	α-fenchol		0.1
	myrcenol		*tr*
	myrtenal		*tr*
	trans-pinocarveol		0.1
	neral		*tr*

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Table 1 (Continued)

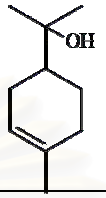
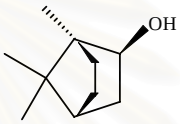
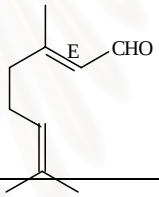
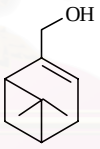
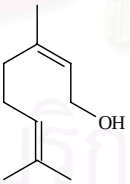
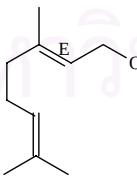
Chemical group	Compound	Structure	Percentage[a]
	α-terpineol		0.8
	borneol		0.1
	geranial		*tr*
	myrtenol		0.2
	nerol		*tr*
	geraniol		*tr*

Table 1 (Continued)

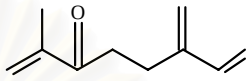
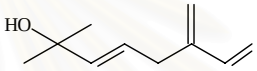
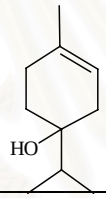
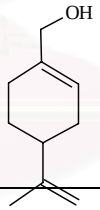
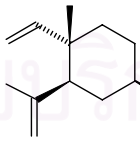
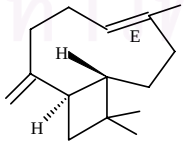
Chemical group	Compound	Structure	Percentage[a]
	2-methyl-6-methylene-1,7-octadiene-3-one		0.1
	2-methyl-6-methylene-3,7-octadien-2-ol		0.1
	terpinen-4-ol		0.5
	perillyl alcohol		0.1
Sesquiterpene	β-elemene		*tr*
	β-caryophyllene		0.9

Table 1 (Continued)

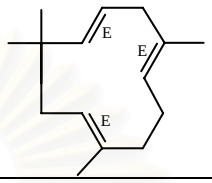
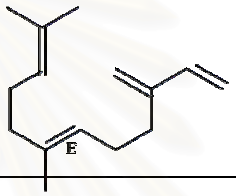
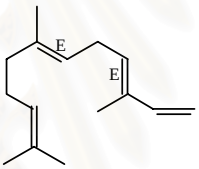
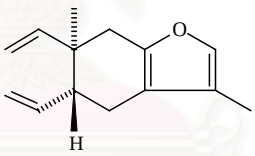
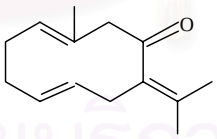
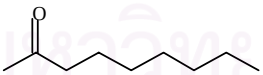
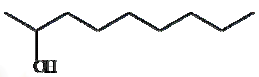
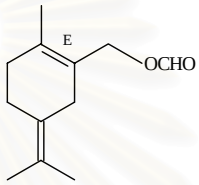
Chemical group	Compound	Structure	Percentage[a]
	α-humulene		0.1
	(E)- β-farnesene		*tr*
	(E,E)- α-farnesene		0.1
	curzerene		*tr*
	germacrone		*tr*
Miscellaneous	2-nonanone		0.1

Table 1 (Continued)

Chemical group	Compound	Structure	Percentage[a]
	2-nonanol		0.1
	geranyl formate		*tr*

[a] Percentage of total FID area

tr = trace (<0.05%)

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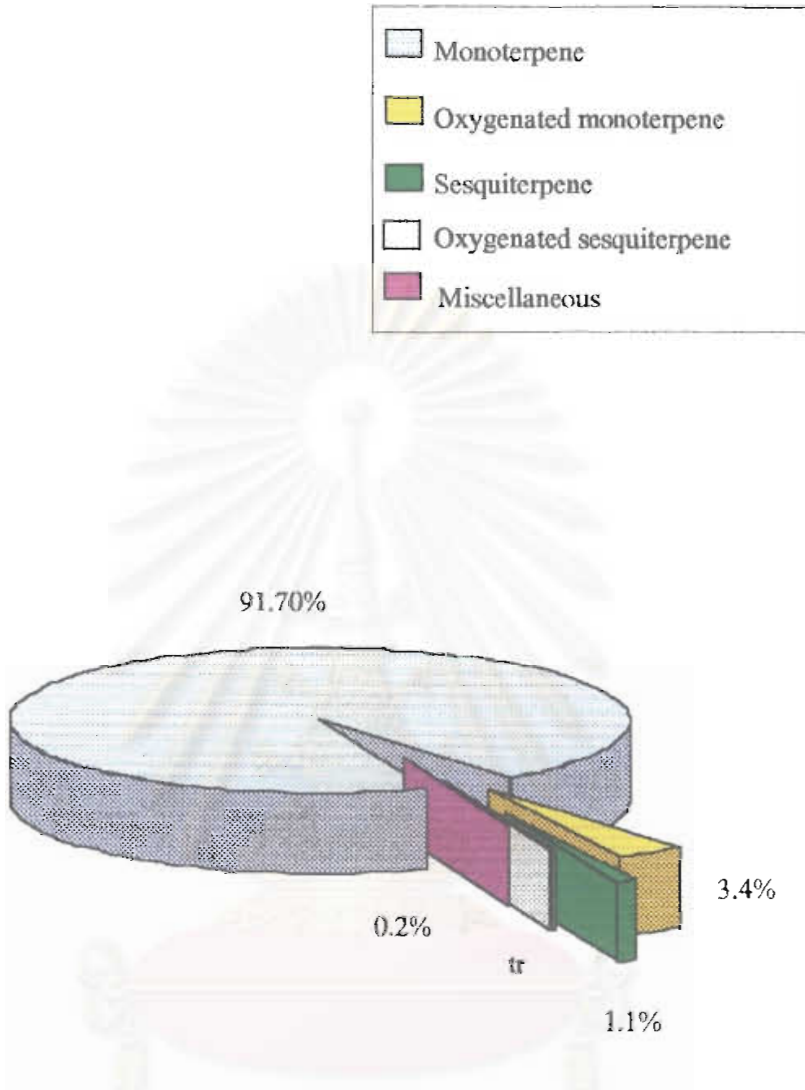
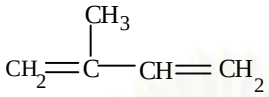


Figure 2 Composition of Malaysian *Curcuma mangga* Essential Oil

(Wong *et al.*, 1999)

2. Chemistry and distribution of some terpenoids

Terpenoids are constructed from isoprene (2-methylbutadiene) units.



Terpenoids are widespread in microorganisms, plants, and animals. The terpenes still contain the original carbon skeleton of the isoprene units. In other groups of isoprenoids the skeleton is altered by addition or loss of carbon atoms. The isoterpenoids are categorized as given in Table 2 according to the number of isoprene units used as building block.

Table 2 Secondary products derived from activated isoprene

Number of isoprene units	Group of compounds	Representative
1	Hemiterpenes	Isoprene, 3,3dimethylallylalcohol, isopentenol, isoamyl alcohol, tuliposides
2	Monoterpenes	Constituents of essential oils, e.g., geraniol, menthol, and thymol

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Table 1 (Continued)

Number of isoprene units	Group of compounds	Representative
3	Sesquiterpenes	Constituents of essential oils, e.g., farnesol, and bisabolol, and of sesquiterpene lactone, e.g., matricin, abscisic acid, juvenile hormone, sirenin
4	Diterpenes	Constituents of resins, e.g., abietic acid, gibberellins, phytol, vitamin A, croctrin
5	Sesterterpenes	Constituents of unsaponiable lipid extracts, waxes, ect.; ophiobilin, ircinin, variabilin
6	Triterpene	Squalene, steroids, pentacyclic triterpenes
7	Tetraterpenes	Carotenes, xanthophylls
>8	Polyterpenes	Rubber, gutta, solanesol, spadicol, dolichols, betulaprenols, ficaprenols

The basic structure of isoprenoids may be modified by the introduction of a wide variety of chemical groups, by isomerization, shift of double bonds, methyl groups, ect. Hence, a bewildering number of chemical structure arises. In addition, compounds derived from other biogenic pathways may contain isoprene residues. (Martin, 1990)

2.1 Monoterpenes (C_{10})

2.1.1 Introduction to monoterpenoids

These substance constitute a fairly large family of alcohols, hydrocarbons, ketones, ect., which are important components of the essential oil obtained from the leaves, root, and bark of various plants. They have pleasant odours, and many are of some industrial importance, though not necessarily as pure chemical individuals.

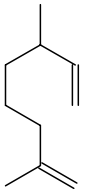
2.1.2 Classification of monoterpenoids (Ikan, 1991)

The monoterpenoids may be divided into three classes having zero, one, or two rings; further subdivisions are made on the basis of carbon skeleton.

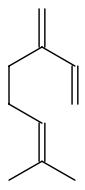
2.1.2.1 Acyclic monoterpenoids

Acyclic monoterpenoids are comparatively few in number but include some of the most important isolates in perfumery.

Among the important hydrocarbons are ocimene and myrcene.

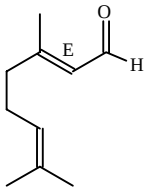


ocimene

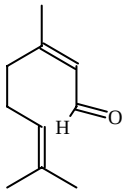


myrcene

Aldehydes

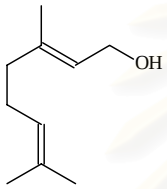


geranial

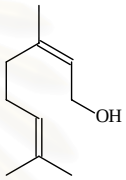


neral

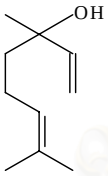
Alcohols



geraniol



nerol

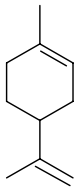


linalool

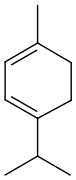
2.1.2.2 Monocyclic monoterpenoids

The great majority of these substances have *p*-menthane carbon skeleton. In addition there are a small group of methylated cyclohexanes and another small miscellaneous group including some derivatives of cyclopentane.

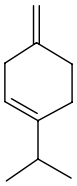
Important hydrocarbons are: (Ikan, 1991)



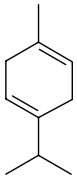
α-limonene



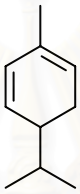
α-terpinene



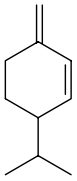
β-terpinene



γ-terpinene

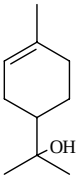


α-phellandrene

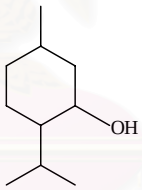


β-phellandrene

Alcohols



α-terpineol



menthol

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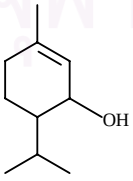
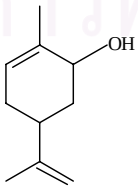
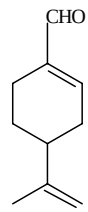


piperitol

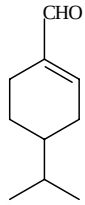


carveol

Aldehydes

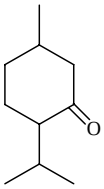


perillaldehyde

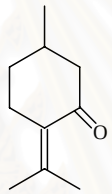


phellandral

Ketones

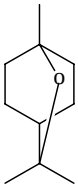


menthone

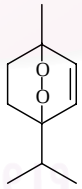


pulegone

Oxides



1,8 cineol



ascaridole

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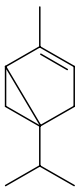
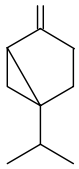
2.1.2.3 Bicyclic monoterpenoids

The bicyclic monoterpenoids may be divided into five groups.

Thujane group



α-thujene



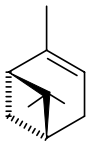
sabinene

Carane group

car-3-ene

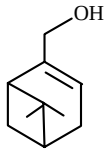
car-4-ene

Pinane group



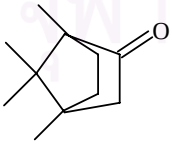
α-pinene

β-pinene

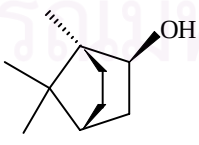


myrtenol

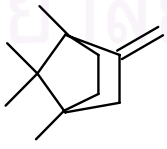
Camphene group



camphor



borneol



camphene

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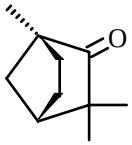
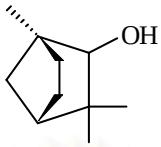
Fenchane



fenchone



fenchyl alcohol

2.2 Sesquiterpenoids (C_{15})

2.2.1 Intrduction to sesquiterpenoids

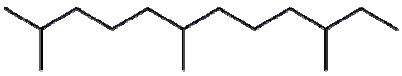
Although the study of the sesquiterpenoids was begun in the early years of the nineteenth century, it was only after about 1920 that serious progress to be made. Whilst Wallach, 1887 believed that the sesquiterpenoids, like the monoterpenoids, were built up from isoprene nuclei.

The sesquiterpenes are from the higher-boiling fraction of the essential oils. They are formed by the union of three isoprene units. Sesquiterpenes are unsaturated compounds and may be acyclic, monocyclic, bicyclic, and tricyclic. (Ikan, 1991)

2.2.2 Classification of sesquiterpenes

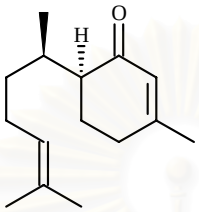
There are 14 types of sesquiterpene as follows.

Simple farnesene sesquiterpene



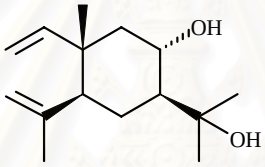
Farnessane

Bisabolane



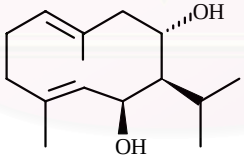
1-Bisabolone

Elemane



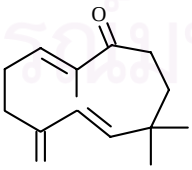
8-Hydroxyelemol

Germacrane



1(10),4-Germacradiene-6,8-diol

Humulane



γ-Humulen-9-one

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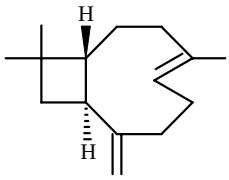
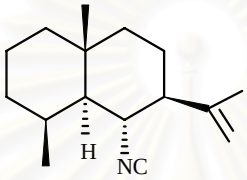
Caryophillane



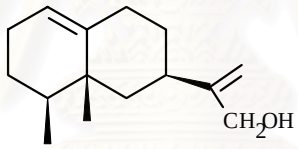
3(15),6-Caryophylladiene

Eudesmane



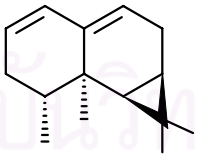
Acanthellin 1

Eremophilane



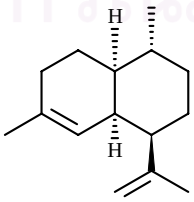
12-Hydroxyeremophilene

Aristolane



1,9 Aristoladiene

Cadinane



4,11-Amorphadiene

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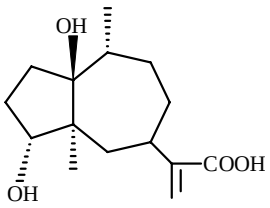
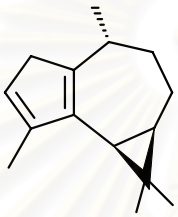
Pseudoguiane



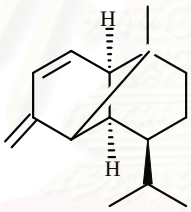
1,4-Dihydroxy-11(13)-pseudoguaien-12-oic acid

Aromadendrane



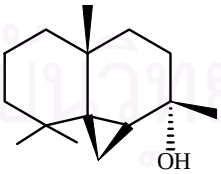
1(5),3-Aromadendradiene

Copaane



2,4(15)-Copaadiene

Thujopsane



7-Thujopsanol

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2.3 Diterpenoids (C_{20})

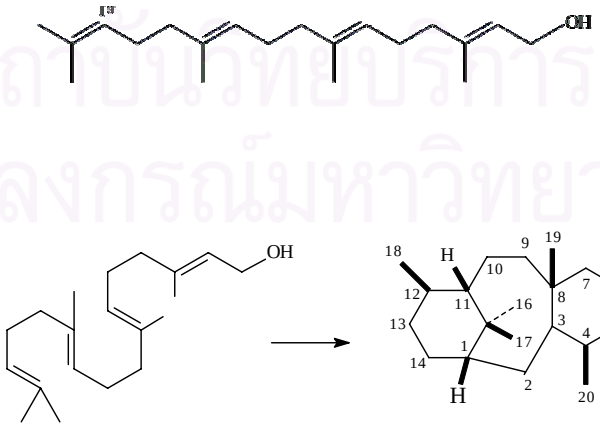
2.3.1 Introduction to diterpenoids

The diterpenes are C_{20} compounds biogenetically derived from geranylgeranyl pyrophosphate. The notable feature of diterpene structures is the fascinating variation encountered in their skeletons and the occurrence in nature of both normal and antipodal stereochemical series.

They are mainly of plant and fungal origin and usually occur as mixtures of closely related compounds. They include the resin acids and gibberellins. The following correlation chart shows the main diterpene skeletons according to the classification recommended by Rowe *et al*. (Nakanishi *et al*, 1974)

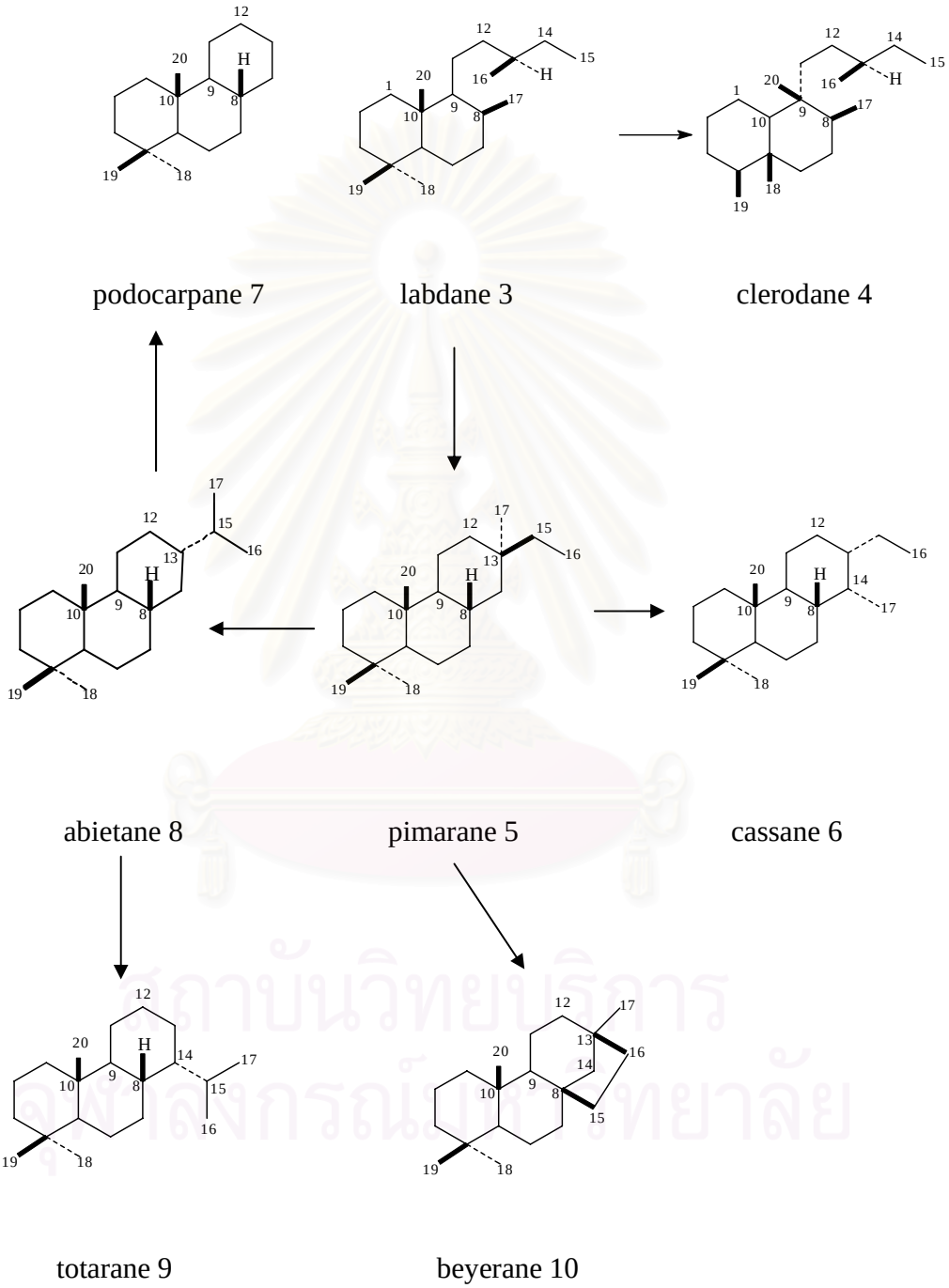
Scheme 1 Correlation of the main diterpene skeletons

This skeletons marked with * occur exclusively or almost exclusively in their antipodal forms in nature.



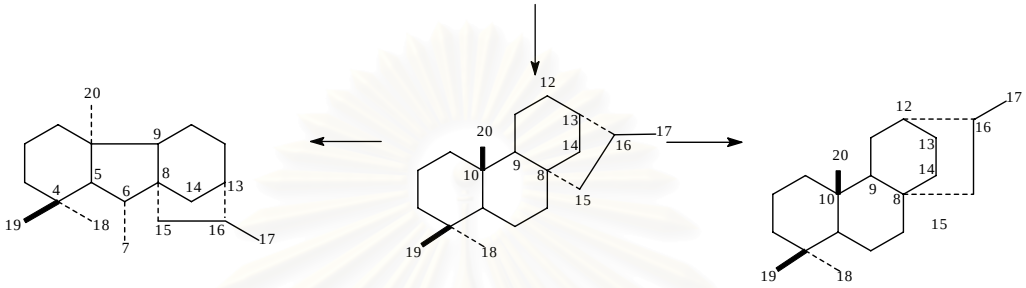
geranylgeraniol 1b taxane 2

Scheme 1 Correlation of main diterpene skeletons (Continued)



Scheme 1 Correlation of main diterpene skeletons (Continued)

from beyerane 10

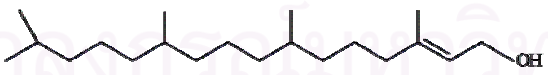


gibberellane* 13 kaurane* 11 atisane* 12

2.3.2. Classification of diterpenes (Ikan, 1991)

There are 4 types of diterpene as follows.

Acyclic diterpene



Phytol

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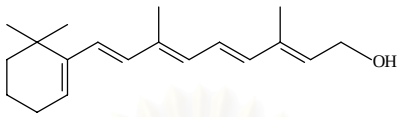
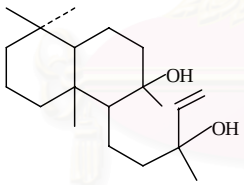
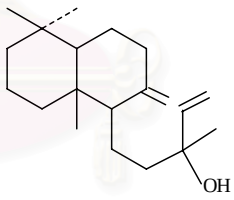
Monocyclic diterpene



Vitamin A_1

Bicyclic diterpenes





Sclareol Manool

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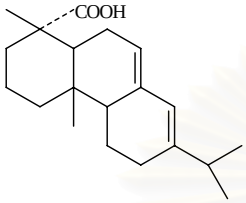
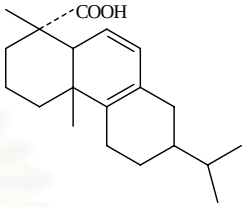
Tricyclic diterpenes



Abietic acid



Levopimaric acid

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CHAPTER III

EXPERIMENTAL

1. Source of Plant Material

The rhizome of *Curcuma mangga* Valeton & van Zyp was purchased from Pakklong Talad Market, Thailand in July 1998. Authentication of the plant material has been achieved by Prof. Puangpen Siriruksa, Department of Biology, Faculty of Sciences, Prince of Songkla University at Hat Yai, Songkla, Thailand.

2. Chromatographic Techniques

2.1 Analytical Thin-Layer Chromatography (TLC)

Technique	: One dimension, assending
Adsorbent	: Silica gel 60 F_{254} (E. Merck) precoated plate
Layer thickness	: 0.2 mm.
Distance	: 6 cm.
Temperature	: Laboratory temperature (30-35 °C)
Detection	: 1. Ultraviolet light at wavelengths of 254 and 365 nm
	2. Anisaldehyde-sulphuric acid reagent and heated at

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100°C for 5-10 min

2.2 Column Chromatography

2.2.1 Quick Column Chromatography

Adsorbent : Silica gel 60 (No.7734) particle size 0.063-0.200 nm (70-230 mesh ASTM) (E. Merck)

Packing method : Dry packing

Sample loading : The sample was dissolved in a small amount of organic solvent, mixed with a small quantity of adsorbent, triturated, dried and then placed gently on the top of the column.

Detection : Fractions were examined by TLC observing under UV light at the wavelengths of 254 and 365 nm after that sprayed with anisaldehyde-sulphuric reagent and heated at 100 °C for 5-10 min.

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2.2.2 Flash Column Chromatography

Adsorbent : Silica gel 60 (No.9385) particle size 0.040-0.063 nm (230-400 mesh ASTM) (E. Merck)

Packing method : Wet packing

Sample loading : The sample was dissolved in a small amount of eluent and then applied gently on the top of the column.

Detection : Fractions were examined following the description in section 2.2.1

2.2.3 Gel Filtration Chromatography

Gel filter : Sephadex LH 20 (Pharmacia)

Packing method : Gel was suspended in the eluant and left standing to swell for 24 hours prior to used. It was then poured into the column and allowed to set tightly.

Sample loading : The sample was dissolved in a small amount of eluant and applied on the top of the column.

Detection : Fractions were examined following the description in section 2.2.1

2.3 Spectroscopy Detection

2.3.1 Ultraviolet (UV) Absorption Spectra

UV (in methanol) spectra were obtained on a Shimadzu UV-160A UV/vis spectrophotometer (Pharmaceutical Research Instrument Center, Faculty of Pharmaceutical Sciences, Chulalongkorn University).

2.3.2 Infrared (IR) Absorption Spectra

IR spectra (KBr disc and film) were recorded on a Perkin Elmer Spectrum 2000 FT-IR spectrometer (Pharmaceutical Research Instrument Center, Faculty of Pharmaceutical Sciences, Chulalongkorn University) and a perkin Elmer FT-IR 1760X spectrometer (Sciencetific and Technological Research Equipment Center, Chulalongkorn University).

2.3.3 Mass Spectra

Electron Impact mass spectra (EIMS) were measured on a Fison Micromass VG Platform II mass spectrometer (Pharmaceutical Research Instrument Center, Faculty of Pharmaceutical Sciences, Chulalongkorn University) and FINNIGAN MAT INCOS 50 mass spectrometer (Faculty of Sciences, Mahidol University). High Resolution Fast Atom Bombardment mass spectrum (HRFABMS) was measured with Hitachi RMU-7M mass spectrometer (Instrumental Center, Chiba University).

2.3.4 Proton and Carbon-13 Nuclear Magnetic Resonance (^{1}H and ^{13}C-NMR) Spectra

^{1}H NMR (300 MHz) and ^{13}C NMR (75 MHz) spectra were obtained with a Bruker Advance DPX-300 FT-NMR spectrometer (Faculty of Pharmaceutical Sciences, Chulalongkorn University).

^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) spectra were obtained with a JOEL JMN-A 500 NMR spectrometer (Sciencetific and Technological Research Equipment Center, Chulalongkorn University).

Solvent for NMR spectra was deuterated chloroform (chloroform-*d*). Chemical shifts were reported in ppm scale using the chemical shift of the solvent as the reference signal.

2.4 Physical Properties

2.4.1 Melting Point

Melting point was obtained on a Fisher/Johns melting point apparatus (Department of Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Chulalongkorn University).

2.4.2 Optical Rotation

Optical rotations were measured on a Perkin Elmer 341 polarimeter (Pharmaceutical Research Center, Faculty of Pharmaceutical Sciences, Chulalongkorn University).

2.4.3 Circular Dichroic Spectrum

Circular Dichroic spectrum was measured on JASCO J-715 CD spectrometer (Institute of Molecular Biology and Genetics, Mahidol University).

2.5 Solvents

Throughout this work, all organic solvents were of commercial grade and were redistilled prior to use. However, in the study of the chemical constituents of the essentail oil, AR grade solvents were used with GC-MS.

3. Extraction and Isolation

3.1 Extraction

The fresh rhizomes of *Curcuma mangga* (30 kg) were sliced in small pieces and dried in hot air oven at temperature 50°C. The dried rhizomes (2.2 kg) were powdered and marcerated with 95% ethanol (1x500 ml) for 3 days. After marceration, the extract was filtered in order to separate the marc from the extraction. Then the filtrates were pooled and evaporated under reduced pressure at temperature not exeeding 40°C to yield an ethanol extract. The marc was remarcerated with 95% ethanol 3 times (3x500 ml) and conducted the same methods as described. The combination of each ethanol extracts were accomplished. The ethanol extract was partitioned with hexane (15 L) and the obtained extract was evaporated under reduced pressure to yield the hexane extract (45 g, 2.05% based on dried weight of rhizomes). Later, the residue

from hexane extraction was partitioned with chloroform (16 L) and the obtained extract was evaporated under reduced pressure to yield the chloroform extract.(53 g, 2.41% based on dried weight of rhizomes).

3.2 Isolation of CM I, Mangga IV and Mangga I

The 45 g of the hexane extract was dissolved in a small amount of hexane, triturated with silica gel 60 (No.7734) and dried under vacuum, then fractionated by quick column chromatography using a sintered glass filter column of silica gel 60 (No. 7734). Elution was performed in a polarity gradient manner with hexane and dichloromethane. The ratios and volumes of solvents used in this quick column chromatography are summarized in Table 3.

Table 3 The ratios and volumes of solvents for quick column chromatography of hexane extract of *Curcuma mangga*

Fraction	Ratio(%) of hexane : dichloromethane	Volume of solvent(ml)
A. 1-5	100:0	2000
B. 6-16	98:2	4400
C. 17-19	95:5	1200
D. 20-21	90:10	800
E. 22-24	80:20	1200

Table 3 The ratios and volumes of solvents for quick column chromatography of hexane extract of *Curcuma mangga* (Continued)

Fraction	Ratio(%) of hexane : dichloromethane	Volume of solvent(ml)
F. 25-28	70:30	1600
G. 29-33	50:50	2000
H. 34-36	30:70	1200
I. 37-40	0:100	1600

The eluates were examined by TLC using 2% dichloromethane in hexane as developing solvent. Fractions (40 fractions) with similar chromatographic pattern were combined to yield 9 fractions as shown in Table 2.

Fractions 6-16 were pooled and dried (5.1615 g) to yield fraction B. Fraction B was divided into 2 portions: J (2.6 g) and K (2.5615 g). Each was separated by gel filtration using Sephadex LH 20 (100 g, 2.5x80 cm) that was swollen in hexane : dichloromethane : methanol 2:5:1 (24 h) prior to use and also as eluant.

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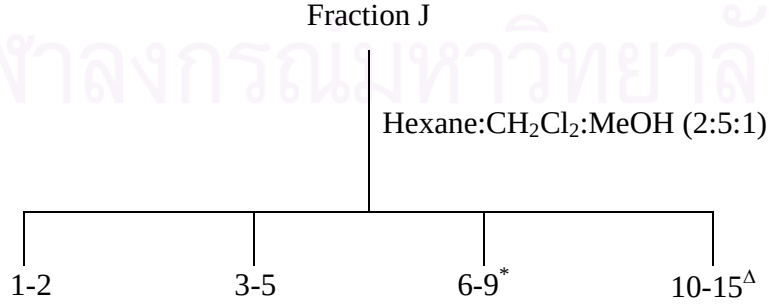
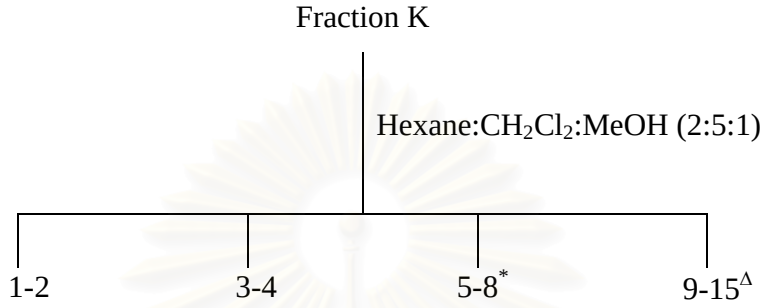
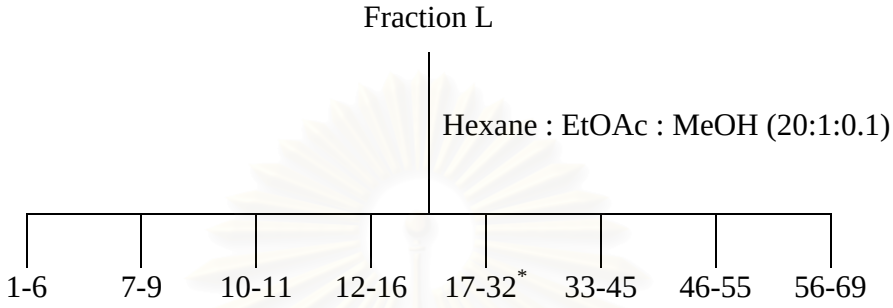




Colourless needles (**CM I**) were obtained from fractions 10-15$^{\Delta}$(from Fraction J) and fractions 9-15$^{\Delta}$ (from Fraction K) when recrystallized with methanol. TLC chromatogram showed no spot under UV light at 254 and 365 nm, but it gave violet spot when sprayed with anisaldehyde-sulphuric acid reagent and heated at 100°C for 5 min (R_f 0.3, 2% CH_2Cl_2 in hexane). These fractions were a mixture of β-sitosterol and stigmasterol (200 mg) (0.01% based on dried weight of rhizomes).

Owing to the chromatographic pattern of fractions 6-9* (from Fraction J) and fractions 5-8* (from Fraction K) were similar to each other when examined by TLC using 2% dichloromethane in hexane, they were combined and dried under reduced pressure to yield fraction L (4.1257 g). This fraction was sticky, transparent and light yellow.

Then, fraction L (4.1257 g) was separated on a column using silica gel 60 (9382) as the adsorbent and eluted with hexane: ethylacetate: methanol 20: 1: 0.1



Fractions with the same chromatographic patern examined by TLC using hexane: ethylacetate: methanol (15:1:0.1) were combined. Fractions 17-32* (from Fraction L) were pooled and dried to yield fraction M (2.1250 g). This fraction was sticky, transparent and light yellow. Fraction M was rechromatographed using silica gel 60 (9382) as the adsorbent and eluted with hexane: ethylacetate 25:1. After fraction 38, the column was washed with hexane: ethylacetate 20:1 to yield fractions 39-45.

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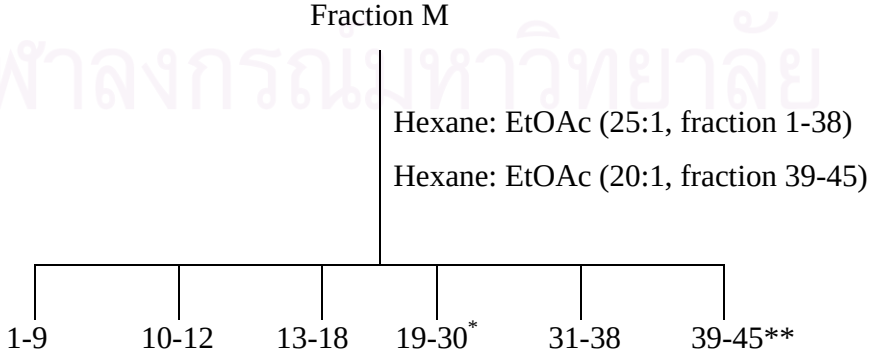
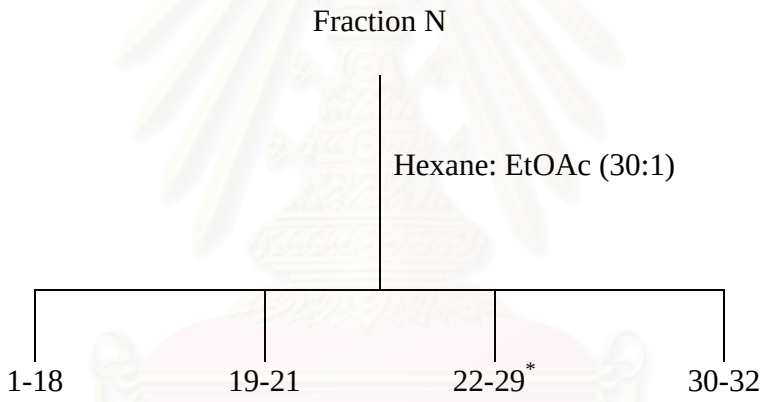
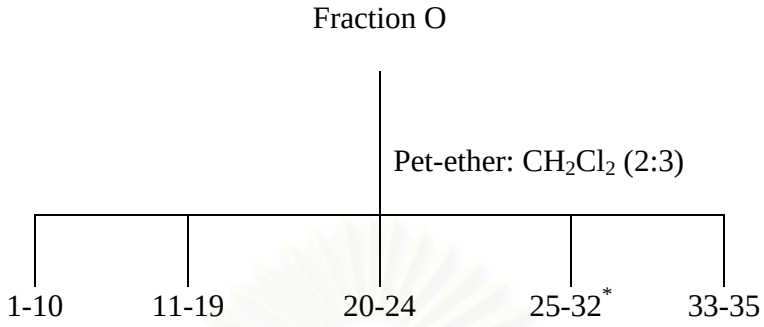


Fractions with the same chromatographic pattern examined by TLC using hexane: ethylacetate: methanol 15:1:0.1 were combined. Fractions 19-30* (from Fraction M) were pooled and dried to yield fraction N (253 mg). The fraction N was white solid. Fractions 39-45** were also pooled and dried to yield fraction O (1.4125 g). Fraction O was transparent, sticky oil. The fraction N and O were rechromatographed using silica gel 60 (9382) as the adsorbent. For fraction N, hexane: ethylacetate 30:1 was used as an eluant and fraction O was eluted with pet-ether: dichloromethane 2:3.



The TLC chromatogram of fractions 22-29* (from Fraction N)showed only one dark spot under UV light at 254 nm, R_f 0.39 (hexane: ethylacetate 15:1) and gave blue-violet spot when sprayed with anisaldehyde-sulphuric acid reagent. Evaporation of fractions 22-29 under reduced pressure gave 10 mg of **Mangga IV** as white solid (0.0005% based on dried weight of rhizomes). White needles obtained after dissolved Mangga IV with small amount of hexane and recrystallized with ethylacetate.



The TLC chromatogram of fractions 25-32* (from Fraction O) showed only one dark spot under UV light at 254 nm, R_f 0.26 (hexane: ethylacetate 15:1) and gave red-violet spot when sprayed with anisaldehyde-sulphuric acid reagent. Evaporation of fractions 25-32* under reduced pressure gave 30 mg of **Mangga I** as transparent oil (0.0015% based on dried weight of rhizomes).

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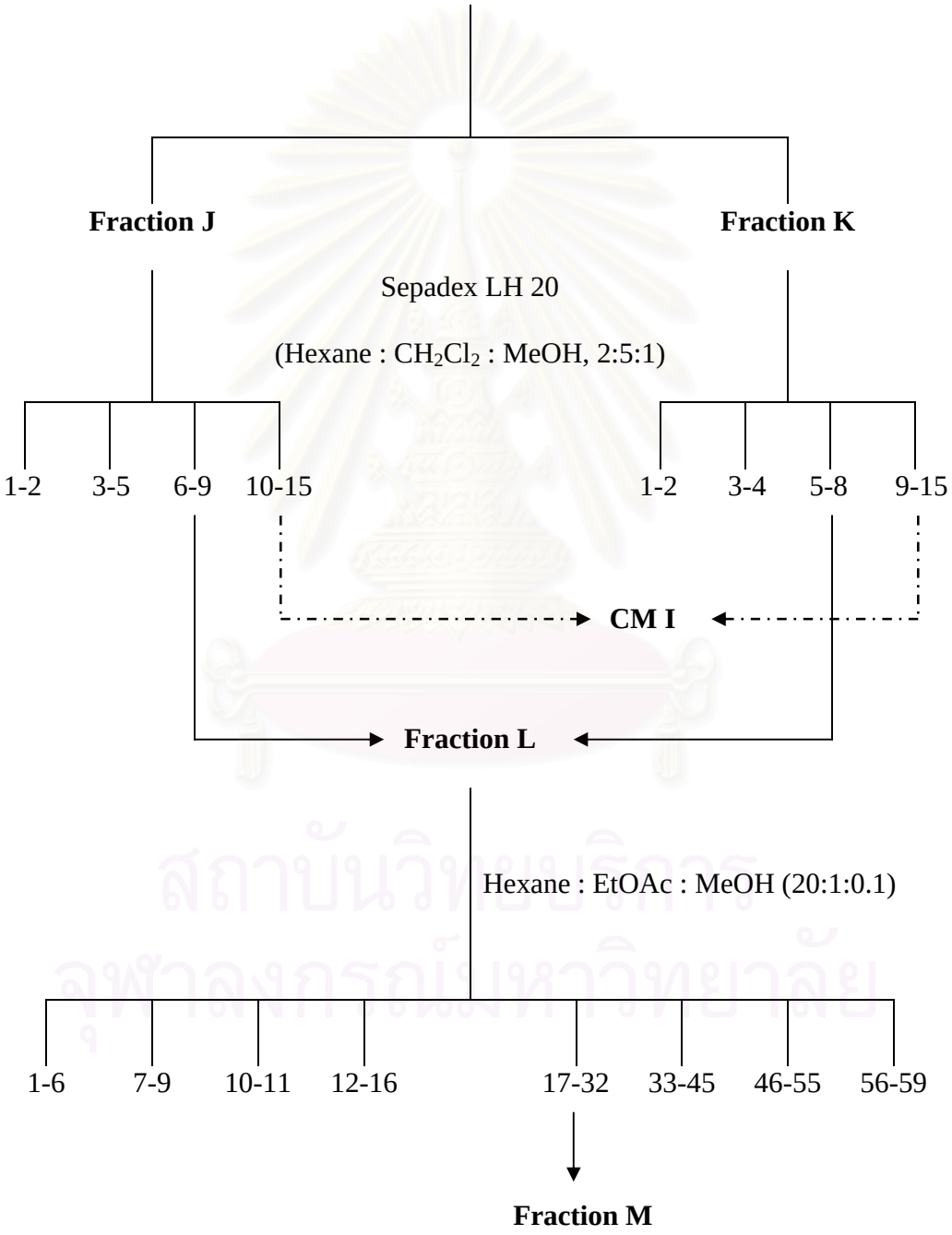
Scheme 2 Isolation of CM I, Mangga I V and Mangga I



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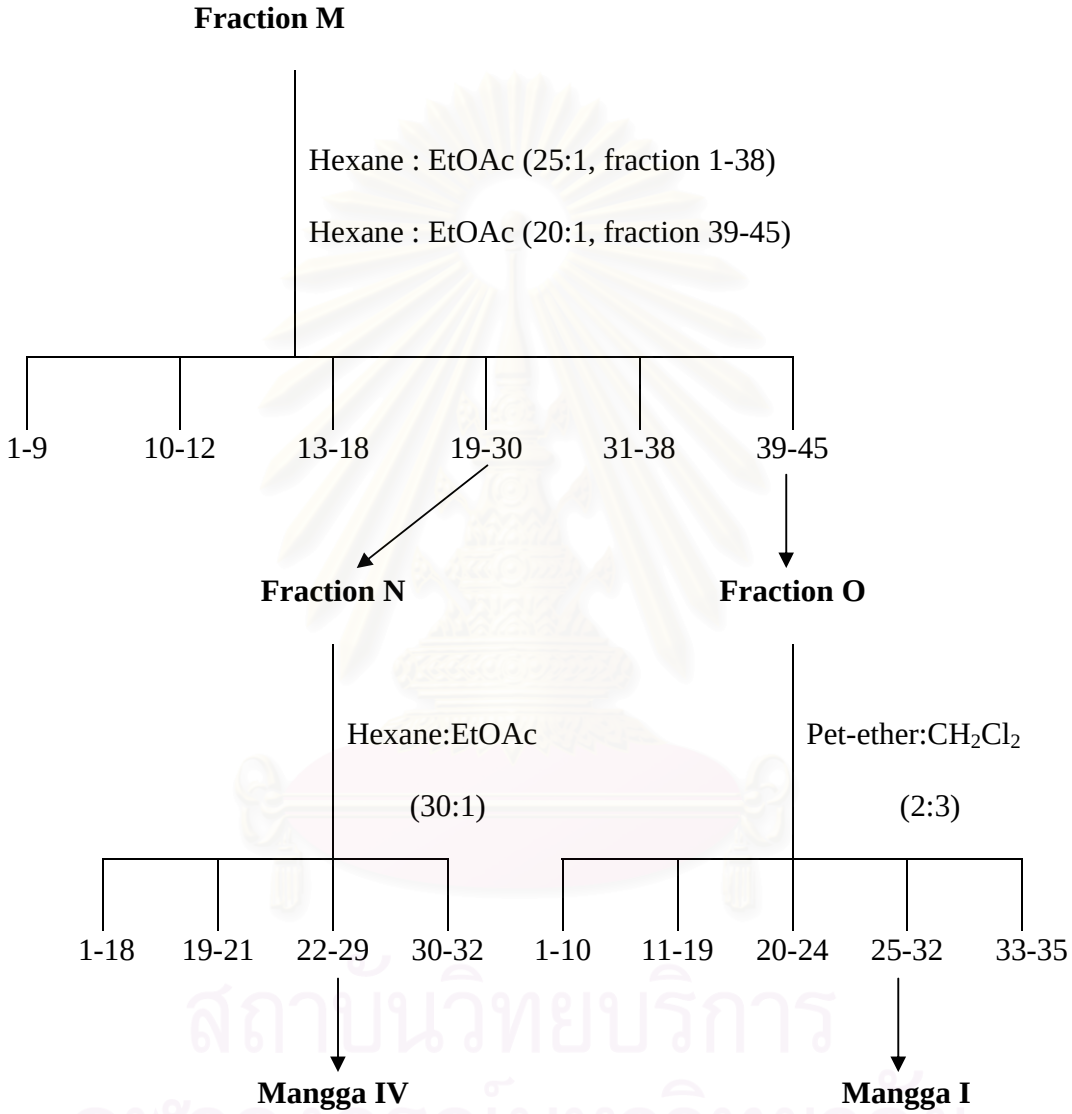
Scheme 2 (Continued)



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4. Physical and Spectral Data of Isolated Compounds

4.1 Isolate CM I

Isolate CM I was obtained as colourless needles (200 mg). It was soluble in chloroform.

EIMS : *m/z* (% relative intensity); Figure 7

414 (M^+, 90), 412 (M^+, 8), 399 (25), 396 (33), 381 (20), 329 (37), 303 (35), 273 (25), 255 (28), 231 (20), 213 (37), 161 (38), 159 (45), 145 (61), 133 (48), 119 (58), 95 (97), 81 (80), 55 (100)

^{13}CNMR : δ ppm, 75 MHz, in $CDCl_3$; Figure 9

See Table 4 .

4.2 Compound Mangga IV

Compound Mangga IV was obtained as white needles (10 mg). It was soluble in chloroform.

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Melting Point : 139-141 °C

$[\alpha]^{20}_D$: +4.58 (c = 0.3 g/100 ml)

EIMS : *m/z* (% relative intensity); Figure 12

260 (M^+, 65), 245 (15), 217 (35), 202 (15), 149 (54), 137 (68), 121 (100), 109 (54), 81 (89), 43 (96)

UV : λ_{max} nm (log ε), in methanol; Figure 10

220 (5.74), 210 (5.59)

IR : ν_{max} cm^{-1}, Film; Figure 11

2931, 2841, 1662, 1458, 1361, 1258, 998

^{1}H NMR : δ ppm, 500 MHz, in $CDCl_3$; Figure 13-14

0.82 (3H, s, H-19), 0.87 (6H, s, H-18, H-20), 1.00 (1H, H-1), 1.08 (1H, *dd*, *J*=12.51, 2.75 Hz, H-5) 1.17 (1H, m, H-3), 1.35 (2H, m, H-6, H-1) 1.40 (1H, m, H-2), 1.49 (1H,m, H-2), 1.70 (1H, m, H-6), 2.05 (1H, m, H-7), 2.25 (3H, s, H-14), 2.43 (1H, m, H-7), 2.45 (1H, *d*, *J*=10.45 Hz, H-9), 4.39 (1H, *d*, *J*=1.83 Hz, H-17), 4.77 (1H, *d*, *J*=1.83 Hz, H-17), 6.05 (1H, *d*, *J*=15.87 Hz, H-12), 6.85 (1H, *dd*, *J*=10.45, 15.87 Hz, H-11)

^{13}C NMR : δ ppm, 125 MHz, in $CDCl_3$; Figure 15

15.09 (q, C-20), 18.98 (t, C-2), 21.90 (q, C-19), 23.22 (t, C-6), 27.20 (q, C-14), 29.67 (s, C-4), 33.52 (q, C-18), 40.86 (t, C-1), 39.31 (s, C-10), 36.60 (t, C-7), 42.07 (t, C-3), 54.45 (d, C-5), 60.78 (d, C-9) 108.59 (t, C-17), 133.58 (d, C-12) 146.67 (d, C-11) 148.61 (d, C-8), 198.15 (s, C-13)

4.3 Compound Mangga I

Compound Mangga I was obtained as colorless oil (30 mg). It was soluble in chloroform and methanol.

HRFABMS	: *m/z* (%relative intensity) ; Figure 27
	347 ($[M+H]^+$, 20), 301 (100), 255 (15), 191 (15)
$[\alpha]^{20}_D$	: +33.1 (*c* =0.21 g/100ml, in chloroform)
UV	: λ_{max} nm (log ε), in $CDCL_3$ Figure 24
	210 (5.82), 230 (6.08)
IR	: ν_{max} cm^{-1}, liquid film; Figure 25
^{1}H NMR	: δ_{ppm}, 500 MHz, in $CDCL_3$; see Figure 29-30 and Table 7
^{13}C NMR	: δ_{ppm}, 125 MHz, in $CDCL_3$; see Figure 31and Table 7

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5. Essential oil content and composition

5.1 Essential oil content determination

Essential oil was determined by the method described in the Official Methods of Analysis of the Association of Official Analytical Chemist (AOAC, method 962.17) (Helrich, 1990). One hundred and fifty grams of material was put into a 1000 ml round bottom flask. The tridistilled water was added into the flask in order to reach half full of the volume. The flask was connected to the apparatus for the determination of volatile oil (Figure 3). The material was distilled for at least 2 hours until no additional oil volume was increased. After cooling, the oil volume was measured, calculated and expressed as milliliter of the oil per one hundred grams of the rhizome. Then the essential oil was collected and stored at 4°C until it was analyzed for it chemical composition by GC-MS.

5.2 Gas chromatography-mass spectrometry

Gas chromatography-mass spectrometry (GC-MS) was used in order to identify the composition of the essential oil of *Curcuma mangga* rhizome. The essential oil was diluted with methanol to the ratio of 1:100 before being injected into GC-MS system. The condition of GC-MS was described below. The spectra were recorded and compared with the terpene library programe (Adam, 1995).

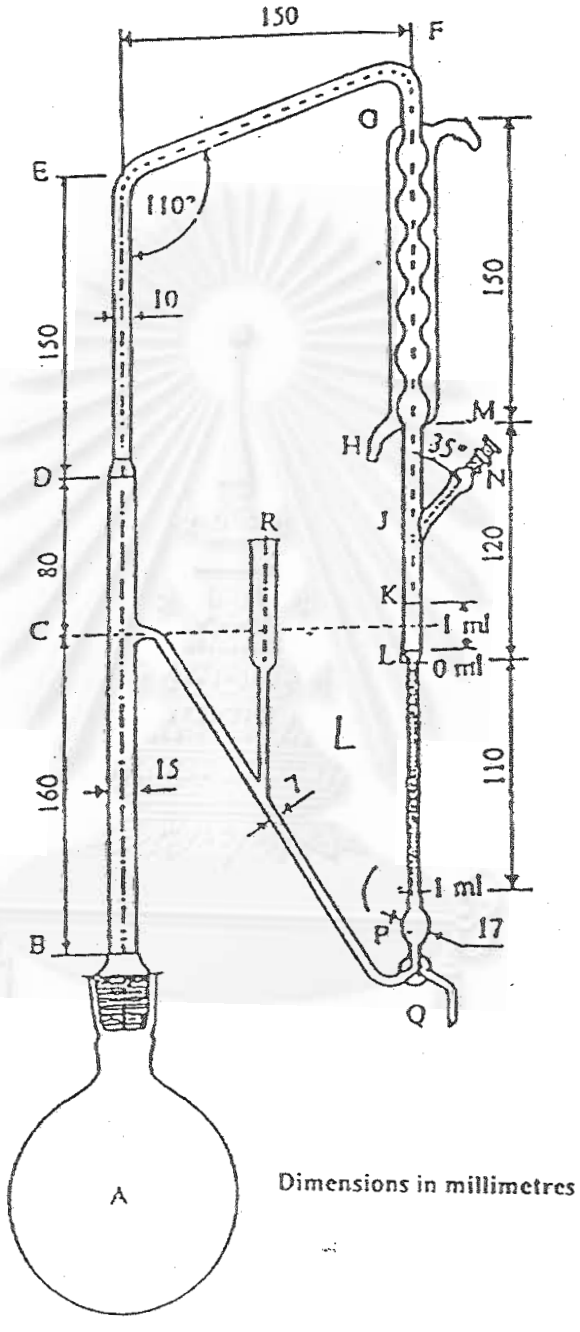

Figure 3 Apparatus for volatile oil determination

GC-MS condition

Instrument model	: Varian Saturn 3
Column	: fused silica capillary column (30x0.25 mm i.d) coated with DB-5 (95% dimethyl 5% diphenyl polysiloxane) (J&W), film thickness 0.25 μm
Column programming	: 60-180 °C rate 3 °C/min
Injector temperature	: 210 °C
Helium carrier gas	: 1 ml/min
Split ratio	: 1:50
Accelerating voltage	: 70 volts
Sample size	: 0.4 μl
Solvent	: methanol

6. Determination of antimicrobial activities of essential oils

6.1 Agar diffusion assay (Barry,1991)

6.1.1 Preparation of sample

The essential oil was diluted to a final concentration of 10% in 0.1% sterile Tween 80.

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6.1.2 Preparation of the inoculum

The bacterial strains used were as follows:

-*Staphylococcus aureus* ATCC 29213

-*Enterococcus faecalis* ATCC29212

-*Bacillus subtilis* ATCC 6633

-*Escherichia coli* ATCC25922

-*Pseudomonas aeruginosa* ATCC 27853

Preparations of bacterial inocula was done according to the standard method. Each bacterial strain was cultured overnight on trypticase soy agar (TSA) plate at 37°C. Four well isolated colonies of the overnight grown culture were inoculated into a 5 ml trypticase soy broth (TSB) and incubated at 37°C for 2-3 hours. The turbidity of inoculum was adjusted with sterile broth to match a 0.5 turbidity standard of McFarland No.1.

The fungi strains used were as follows:

-*Candida albicans* ATCC 10231

-*Microsporum gypseum* (clinical isolate)

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Candida albicans ATCC 10231 was grown on Sabouraud dextrose agar (SDA) slant at 30°C for 24 hours. The inoculum was prepared by suspending the culture in sterile normal saline solution and turbidity of the inoculum was adjusted to match a 0.5 turbidity standard of McFarland No 1.

Spores of *Microsporum gypseum* grown on SDA slant at 30°C for 4 days were washed from slant culture with sterile 0.05% Tween 80 . The turbidity of the spore suspension was adjusted to match 0.5 turbidity standard of McFarland No 1.

6.1.3 Preparation of test plates

- For testing bacteria:

Mueller Hinton agar (MHA) was melted and allowed to cool at 45-50°C in a water bath. The 25 ml of the melted agar medium was dispensed into sterile glass petri dishes, with internal diameters of 9 cm, to yield a uniform depth of 4 mm. The agar was allowed to harden on a flat level surface. The plates were dried for 1 hour at 37°C.

- For testing fungi:

Sabouraud dextrose agar (SDA) was used and prepared as described above.

6.1.4 Inoculation of agar plates

A sterile cotton swab was dipped in each inoculum and the excess was removed by rotating the swab several times against the inside wall of the tube above the fluid level. The entire surfaces of the MHA plate and the SDA plate for testing bacteria and fungi, respectively, were inoculated by streaking with the swab for 3 times and each time was rotated 60 degree.

6.1.5 Assay procedure

A 50 µl of each 10 % oil sample or diluent (0.1 % sterile Tween 80) was delivered to each hole (6 mm diameter) in the inoculated medium. This was done in triplicate. After maintaining at room temperature for 1 h, bacterial and fungal plates were incubated at 37°C overnight and 30°C for 48-72 h, respectively. The oil samples showing inhibition zone were examined further for their minimal inhibitory concentration.

6.2 Determination of minimal inhibitory concentration (MIC)

Determination of the MIC of essential oil was by the broth microdilution test (Barry, 1991; Murry *et al*., 1995; Espinel-Ingroff and Pfaller, 1995).

6.2.1 Prepation of the inoculum

- For testing bacteria

The inoculum was prepared following the description in section 7.1.2. The turbidity of the 0.5 McFarland turbidity standard No.1 provides approximately 1x 10^8 CFU (colony forming unit)/ml. The inoculum was further diluted to 1:100 in Mueller Hinton broth.

- For testing fungi

The inoculum was prepared following the description in section 7.1.2. This produced a fungal suspension containing 1x 10^6 to 5x10^6 organisms per ml. The inoculum was further diluted to 1:100 in Sabouraud dextrose broth.

6.2.2 Preparation of the essential oil dilutions (Mann and Markham, 1998)

- For testing bacteria:

The oil sample was mixed with equal volume of 0.5% Tween 80 and diluted with MHB containing 0.1% agar in a two-fold dilution to give the concentrations ranging from 10% to 0.039% v/v.

- For testing fungi:

Sabouraud dextrose broth (SDB) was used instead of Mueller Hinton broth (MHB) and prepared as above.

6.2.3 Assay procedure

A 50 µl volume of each concentration of the essential oil was dispensed to the corresponding well of sterile multiwell microdilution plate (96-Flat-shaped wells). Another incubating the tray at 37°C for 24 h, the lowest concentration of oil sample that showed growth inhibition was considered as the MIC. The determination was done in duplicate. The corresponding concentrations of oil in diluent were used as

turbidity control. Inhibitiory effect of oil was examined by measurement of culture turbidity in each well using microplate reader (Bio-Rad, model 450).

The minimal bactericidal and fungicidal concentration (MBC and MFC) were determined by subculturing (on TSA and SDA, respectively) approximately 0.1 ml from each negative well for MBC and from the positive growth control too for MFC, incubated at 37°C overnight and at 30°C for 48 h or until growth of the subcultures from the growth control is apparent for MBC and MFC, respectively. The MBC and MFC were defined as the lowest concentration of antimicrobial agent from which subculture were negative.

7. Evaluation of cytotoxic potential

Cells were typically grown to 60%-70% confluence; the medium was then changed and the cells were used for test procedures one day later. In each case, 96 well tissue culture plates were used. Test samples were initially dissolved in DMSO and then diluted 10 fold with H_2O. Serial dilutions were performed using 10% aqueous DMSO as the solvent, and 10 μl were added to the wells. In general, five concentration were tested (in triplicate), and preliminary studies were conducted to determine test concentrations that were above and below the ED_{50} of the samples. Control groups were also added in which 10 μl of 10% DMSO were added to wells. After the plates were prepared, cells were removed from the tissue culture flasks by treatment with trypsin, enumerated, and diluted with fresh media. Various quantities of cells (in 190 μl of media) were then added to the 96-well plates, and incubations were performed for various periods of time, as

follows [cell number; incubation time (d)]: BC1 ($10x10^4$; 3), LU1 ($5x10^4$; 3), KB ($5x10^4$; 3), KB-V_1 ($6x10^4$; 3), and LNCap ($6.5x10^4$; 3). All incubations were performed at 37°C in CO_2 incubator with the plates capped in the normal fashion.

After incubation preriod, cells were fixed to the plastic substratum by the addition of 50 μl of cold 50% aqueous trichloroacetic acid. The plates were incubated at 4 °C for 1 h, was with tap H_2O (x4), and air dried. The trichloroacetic acid-fixed cells were stained by the addition of 0.4% sulfohodamine B (w/v) dissolved in 1% HOAc (30 min). Free sulfohodamine B solution was then removed by washing with 1% aqueous HOAc (x4). The plate were then air-dried, and the bound dye was solubilized by the addition of 10 mM unbuffer Tris base, pH 10 (200 μl). The plates were placed on the shaker for 5 min, and the absorption was determined at 515 nm using an ELISA plate reader. In each case a zero-day control was performed by adding an equivalent number of cells to serveral wells of the 96-well plates and incubating at 37 °C for a period of 10 min. The cells were then fixed with trichloroacetic acid and processed as described above.

Finally, the absorption values obtained with each of the treatment procedures were averaged, and the average value obtained with the zero day control was substracted. These values were then expressed as a percentage, relative to the solvent-traeted control incubations, and ED_{50} values were calculated using non linear regression analysis (percent survival versus concentration). These experimental conditions were established in preliminary studies wherein it was shown (a) there is at least a 7-fold increase in cell number relative to the amount of cells added to the plates at time zero, (b) the resulting absorption values were in a range to assure reading accuracy (i.e., <1.4 A_{515}

units), and (c) the cell number attained during the incubation period did not reach a plateau region on the growth curve (Likhitwitayawuid *et al*., 1993).

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CHAPTER IV

RESULT AND DISCSSION

The dried rhizomes of *Curcuma mangga* Valeton & van Zyp were extracted with 95% ethanol. The ethanol extract was partitioned respectively with hexane and chloroform to yield hexane extract and chloroform extract. Hexane extract was then separated by several chromatograpic techniques to afford three compounds (CM I, Mangga IV and Mangga I).

The structure determinations of all the isolates were performed by interpretation of their UV, IR, NMR, MS and CD data, and then confirmed by comparison with literature values.

1. Structure Determination of Isolated Compounds

1.1 Structure Determination of Isolate CM I

Isolate CM I was obtained as colourless needles. Two molecular ions at *m/z* 412 and 414 were observed in EIMS (Figure 7). Isolate CM I was identified as a mixture of β-sitosterol and stigmasterol by comparison of its ^{1}H and ^{13}C data with reported values (Khalil and Idler, 1980; Iribarren and Pomilio, 1985; Heupel *et al.*, 1978).

In the ^{1}H NMR spectrum (Figure 8a and 8b), the signals at δ 5.03 (0.08 H, *dd*, *J*= 15.2, 8.4 Hz), 5.17 (0.08 H, *dd*, *J*= 15.2, 8.4 Hz) and 5.63 (1 H, *d*, *J*= 4.8

Hz) were assigned to H-22 and H-23 of stigmasterol and H-6 of β-sitosterol and stigmasterol. The integration steps of H-6, H-22 and H-23 were approximately in the ratios of 1:0.08:0.08. Therefore, it could be deduced that CM I was a mixture of β-sitosterol and stigmasterol in the ratio of 23:2.

The ^{13}C NMR spectrum (Figure 9) of CM I displayed 46 signals comparison of these data with reported values of β-sitosterol and stigmasterol (Wright *et al.*, 1978) was shown in Table 4.

Table 4 ^{13}C NMR spectral data of β-sitosterol, stigmasterol, (NMR 300 MHz, in $CDCl_3$) and isolate CM I (in $CDCl_3$)

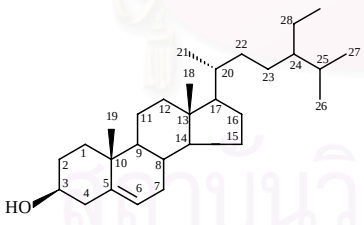
Carbon	Chemical shift (ppm)		
	β-Sitosterol	Stigmasterol	CM I
1	37.31	37.31	37.35
2	31.57	31.67	31.77
3	71.69	71.81	71.82
4	42.45	42.35	42.38,42.30
5	140.76	140.80	140.59
6	121.59	121.69	121.60

Table 4 ^{13}C NMR spectral data of β-sitosterol, stigmasterol, (NMR 300 MHz, in $CDCl_3$) and isolate CM I (in $CDCl_3$) (Continued)

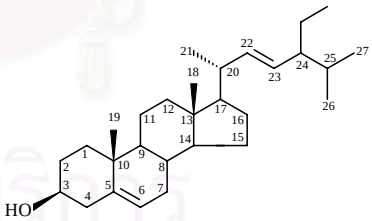
Carbon	Chemical shift (ppm)		
	β-Sitosterol	Stigmasterol	CM I
7	31.92	31.94	32.01
8	31.92	31.94	32.01
9	50.17	50.20	50.20
10	36.51	36.56	36.60
11	21.11	21.11	21.21
12	39.81	39.74	39.86,39.76
13	42.33	42.35	42.38
14	56.71	56.91	56.81,56.91
15	24.32	24.39	24.43
16	28.26	28.96	28.36,29.03
17	56.11	56.06	56.10,56.03
18	11.87	12.07	12.01,12.13
19	19.40	19.42	19.53
20	36.17	40.54	36.24,40.57
21	18.82	21.11	19.02,21.21
22	33.95	138.37	34.05,138.51

Table 4 ^{13}C NMR spectral data of β-sitosterol, stigmasterol, (NMR 300 MHz, in $CDCl_3$) and isolate CM I (in $CDCl_3$) (Continued)

Carbon	Chemical shift (ppm)		
	β-Sitosterol	Stigmasterol	CM I
23	26.13	129.32	26.19,129.14
24	45.85	51.29	45.91,51.29
25	29.18	31.94	29.27,32.01
26	19.84	21.26	19.95,21.34
27	19.04	19.02	19.17,19.12
28	23.09	25.44	23.19,25.52
29	12.32	12.27	12.13



β-Sitosterol

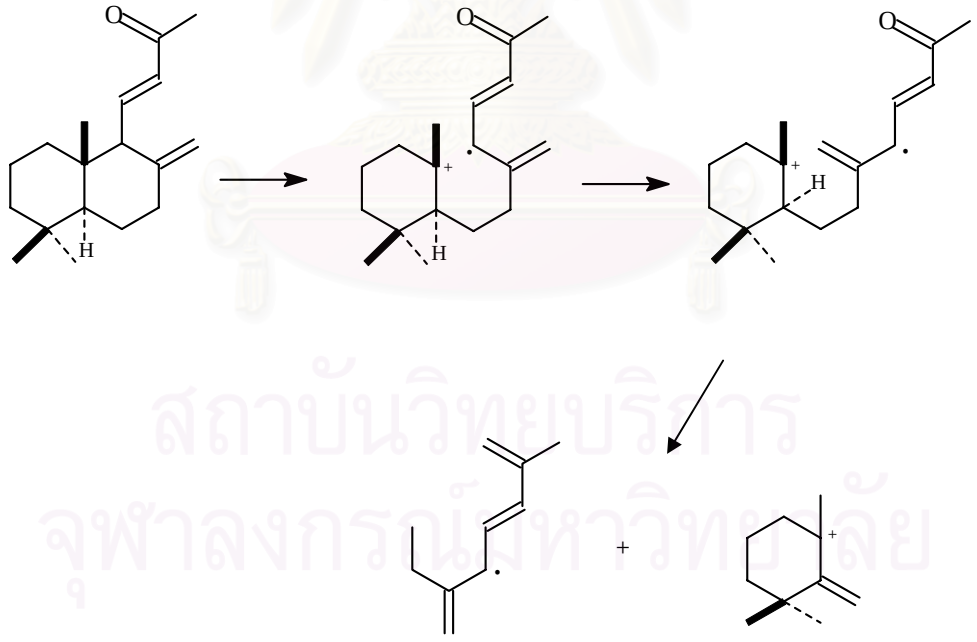


Stigmasterol

1.2 Structure Determination and Revision Assignment of Compound Mangga IV

Compound Mangga IV was obtained as white crystal. The UV spectrum (Figure 10) showed a maximal absorption at λ_{max}210 and 220 nm corresponding to an α,β-unsaturated ketone. The IR spectrum exhibited the peaks of an exomethylene at 3082 cm^{-1}, 1669 cm^{-1} and 998 cm^{-1}. (Figure 11)

The EI mass spectrum (Figure 12) revealed a molecular ion peak at *m/z* 260, consistent with the molecular formula $C_{18}H_{28}O$. It showed the major peaks at *m/z* 137 and 121 (Itokawa *et al.*,1988).



m/z 137

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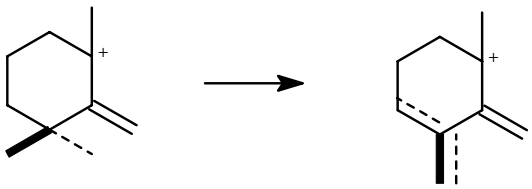


m/z 121

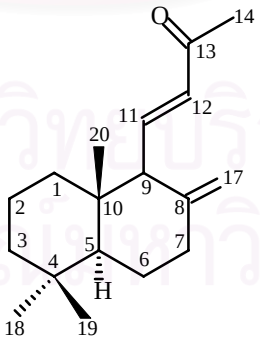
The ^{1}H NMR spectrum (Figure 13-14) showed exomethylene signals at δ 4.39 (1H, *d, J*=1.83 Hz) and δ 4.77 (1H, *d, J*=1.83 Hz) which were assigned to Ha-17 and Hb-17. There are four methyl signals. One is the methyl group which connected to carbonyl group appeared at the most down field signal among the methyl groups at δ 2.25 (3H) and assigned to H-14, the others were methyl groups at δ 0.82 (3H) and δ 0.87 (6H) which were later assigned to H-19 and H-18, H-20 respectively. Two *trans* olefenic proton signals were observed at δ 6.05 (1H, *d, J*=15.87 Hz) and assigned to H-12, the other was observed at δ 6.85 Hz (1H, *dd, J*=10.45, 15.87 Hz) and assigned to H-11.

In ^{13}C NMR (Figure 15), Mangga IV exhibited seventeen signals, but from the molecular formula, the structure should possess eighteen carbon atoms. This suggested that there must be one pair of carbon with equivalent chemical shift at δ 33.6 which could be assigned to C-4 and C-18. The spectra of DEPT 135 and DEPT 90 spectra (Figure 16) represented four methyl, six methylene, four methine and four quaternary carbons. The most downfield carbon signal was assigned to the C-13 carbonyl carbon. From HMQC spectrum (Figure 17-19) all protonated carbons were assigned. All the methylene carbons consisted of unequivalent protons. (Table 5)

The HMBC spectrum (Figure 20-23) clearly showed correlations of long-range H-C coupling. All quaternary carbons of Mangga IV were assigned. The most upfield methyl carbon was assigned to C-20 while the methyl carbon at δ 27.20 ppm was assigned to C-14 which was the carbon connected to carbonyl carbon. The other methyl carbons were assigned to C-18 at δ 33.52 ppm and C-19 at δ 21.90 ppm, respectively. (Table 5)

Compound mangga IV was identified as 15,16-bisnorlabda-8(17),11-dien-13-one. It was first isolated from *Alpinia speciosa* (Zingiberaceae) (Itokawa, Morita, and Mihashi, 1980) and fully characterization of this compound has not been done since 1980. Thus in the present investigation, complete assignment of both protons and carbons were reported through the use of extensive one and two dimensional NMR. (Table 6)

Detailed ^{1}H NMR and ^{13}C NMR data were revised and reported for the first time for this rare norditerpene skeleton in Table 6.



15,16-Bisnorlabda-8(17),11-dien-13-one

Table 5 ^{1}H and ^{13}C spectral data of compound Mangga IV (in $CDCl_3$) with long-range correlations observed in HMBC spectral

No.	δ_H (ppm)	multiplicity	δ_c (ppm)	HMBC (correlation with proton)
1	H_a=1.00 H_b=1.35	m	40.86(t)	H-20
2	H_a=1.40 H_b=1.49	m m	18.98(t)	H_a-1, H_a-3
3	H_a=1.17 H_b=1.40	m m	42.07(t)	H-18
4	-	-	33.6(s)	H-5* and H-19*
5	1.08	m	54.45(d)	H_b-3, H-18, H-19 and H-20
6	H_a=1.35 H_b=1.70	m	23.22(t)	H-5* and H_a-7*
7	H_a=2.05 H_b=2.43	m	36.60(t)	H_a-17 and H_b-17
8	-	-	148.61(s)	H_b-6, H_a–7*and H-9*,
9	2.45	*d*, *J*=10.45	60.78(d)	H_b-7, H-12, H-20, H_a-17 and H_b-17

Table 5 ^{1}H and ^{13}C spectral data of compound Mangga IV (in $CDCl_3$) with long-range correlations observed in HMBC spectral (Continued)

No.	δ_H (ppm)	multiplicity	δ_c (ppm)	HMBC (correlation with proton)
10	-		39.31(s)	H-20* and H-5*
11	6.85	*dd*, *J*=15.87, 10.45	146.67(s)	H-9*
12	6.05	*d*, *J*=15.87	133.58(d)	H-9
13	-	-	198.15(s)	H-11, H-12* and H-14*
14	2.25 (3H)	s	27.20(q)	H-12
15	-	-	-	-
16	-	-	-	-
17	H_a=4.39 H_b=4.77	*d*, *J*=1.83 *d*, *J*=1.83	108.59 (t)	H_a-7 and H-9
18	0.87(6H)	s	33.52(q)	H-5 and H-19
19	0.82(3H)	s	21.90(q)	H-5 and H-18
20	0.87(6H)	s	15.09(q)	H_a-1, H-5 and H-9

***** Two-bond coupling

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Table 6 Comparison of ^{1}H and ^{13}C chemical shifts of 15,16-bisnorlabda-8(17),11-dien-13-one between Itokawa*, et al*., 1980 and thesis experiment

No	NMR data of Itokawa,*et al*., 1980			NMR data of thesis experiment		
	δ_c (ppm)	δ_H (ppm)	Multiplicity (Hz)	δ_c (ppm)	δ_H (ppm)	multiplicity (Hz)
1	**36.60**(t)[a]	-	-	**40.86**(t)	**H_a=1.00** **H_b=1.35**	**m**
2	19.00(t)	-	-	18.98(t)	**H_a=1.40** **H_b=1.49**	**m** **m**
3	42.00(t)	-	-	42.07(t)	**H_a=1.17** **H_b=1.40**	**m** **m**
4	33.60(s)	-	-	33.60(s)	-	-
5	54.40(d)	-	-	54.45(d)	**1.08**	**m**
6	23.22(t)	-	-	23.22(t)	**H_a=1.35** **H_b=1.70**	**m**
7	**40.90**(t)[a]	-	-	**36.60**(t)	**H_a=2.05** **H_b=2.43**	**m**
8	148.50(s)	-	-	148.61(s)	-	-
9	60.80(d)	2.40	*d, J*=10.	60.78(d)	2.45	*d, J*=10.45

Table 6 Comparison of ^{1}H and ^{13}C chemical shifts of 15,16-bisnorlabda-8(17),11-dien-13-one between Itokawa *et al*., 1980 and thesis experiment (Continued)

No	NMR data of Itokawa *et al*., 1980			NMR data of thesis experiment		
	δ_c (ppm)	δ_H (ppm)	Multiplicity (Hz)	δ_c (ppm)	δ_H (ppm)	multiplicity (Hz)
10	39.30(s)	-	-	39.31(s)	-	-
11	146.50(d)	6.80	*dd, J*=16,10	146.67(s)	6.85	*dd, J*=15.87,10.45
12	133.50(d)	6.02	*d, J*=16	133.58(d)	6.05	*d, J*=15.87
13	197.90(s)	-	-	198.15(s)	-	-
14	27.20(q)	2.23 (3H)	s	27.20(q)	2.25 (3H)	s
15	-	-	-	-	-	-
16	-	-	-	-	-	-
17	108.60(t)	H_a=4.20 H_b=4.80	*d, J*=2 *d, J*=2	108.59 (t)	H_a=4.39 H_b=4.77	*d, J*=1.83 *d, J*=1.83
18	33.60(q)	**0.92(3H), 0.94(6H)**	s	33.52(q)	**0.87(6H)**	s
19	21.90(q)		s	21.90(q)	**0.82(3H)**	s
20	15.10(q)		s	15.09(q)	**0.87(6H)**	s

[a] Assignments are interchangeable

1.3 Structure Determination of Compound Mangga I

Compound Mangga I was obtained as translucent oil. The UV spectrum (Figure 24) showed a maximal absorption at λ_{max}210 and λ_{max}230 nm. The IR spectrum exhibited the peaks of an exomethylene at 3082 cm^{-1}, 1644 cm^{-1} and 950 cm^{-1} and the peaks of α,β-unsaturated γ-lactone at 1764 $cm^{-1}$and 1678 cm^{-1}. (Figure 25)

The EI mass spectrum (Figure 26) showed a molecular ion peak at *m/z* 346 corresponding to the molecular formula $C_{22}H_{34}O_3$ as well as supporting data of HRFABMS that exhibited a quasi-molecular ion peak at *m/z* 347.2554 $[M+H]^+$ (calcd. 347.2586). (Figure 27-28) It revealed the major peaks of naphthalenyl nucleus at *m/z* 137 and *m/z* 121. Compound Mangga I was identified as 15-ethoxy-8(17),12-labdadien-15,16-olide which was a new labdane-type diterpene.

m/z 137 *m/z* 121

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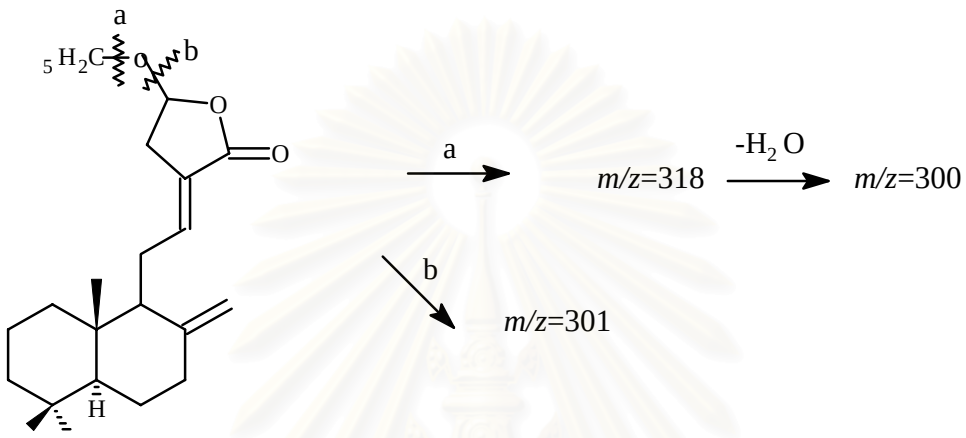
In addition to the fragmentation of the lactonic moiety, it was also detected at the peaks *m/z* 301 and *m/z* 300. (Itokawa *et al*.,1988)



The ^{1}H NMR (Figure 29-30) showed exomethylene protons at δ 4.35 (1H, *d*, *J*=1.23 Hz) and δ 4.83 (1H, *d*, *J*=1.23 Hz) assigned to H_a-17 and H_b-17, three methyl groups in naphthalenyl nucleus resonated at δ 0.69 (H_3-20), δ 0.79 (H_3-19) and δ 0.86 (H_3-18). One methyl group in ethoxy side chain resonated at δ 1.20 (H_3-2′) which was the most low field among all of the methyl groups as well as the signal of methylene group connecting to oxygen in ethoxy side chain displayed at δ 3.60 and δ 3.90. The proton signal at δ 6.70 was assigned as olefinic proton of H-12 while the lactonic methine of H-15 resonated at δ 5.53. The other methylene protons were unequivalent protons as shown in Table 7.

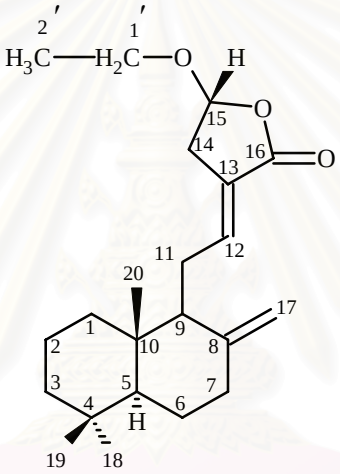
The ^{13}C NMR (Figure 31) of Mangga I exhibited twenty one signals. From the molecular formula, the structure should possess twenty two carbon

atoms. This suggested that there must be one pair of carbon with equivalent chemical shift at δ 33.5 which could be assigned to C-4 and C-18. The spectra of DEPT 90 and 135 (Figure 33-34) revealed four methyl, nine methylene, four methine and five quaternary carbons. The most low field carbon signal was assigned to the C-16 which was the carbonyl carbon in lactone ring. From HMQC spectrum (Figure 36-37) all protonated carbons could be assigned. Comparison of the spectral data above with the previously reported values of labdane-type diterpenes from *Hedychium coronarium* (Zingiberaceae) (Itokawa *et al.,* 1988) implied that compound Mangga I could be a labdane-type diterpene.

The HMBC spectrum (Figure 38-39) exhibited correlations of long-range H-C coupling of C-15 with H_a-1′ (δ 3.60) and H_b-1′ (δ 3.90) confirmed the location of the ethoxy group at C-15. The correlations of C-16 and C-14 with H-12 confirmed the position of olefinic proton of H-12. The correlation of both H-18 with C-3 and H-19 with C-5 indicated two methyl groups at C-4 while the correlations of C-20 with H-1 and H-5 confirmed terminal methyl group at C-10. The other correlations were exhibited in Table 7.

From NOE difference spectra (Figure 40-45), irradiation of H-14 (δ 2.67) enhanced the intensity of H-11 (δ 2.17) instead of H-12 (δ 6.70), thus the conformation of lactone ring should be as shown below. Moreover, the exomethylene proton at δ 4.35 showed NOE interaction with H-11 (δ 2.17). There was no NOE interaction between H-20 (δ 0.69) and H-5 (δ 1.09) supporting that H-20 (δ 0.69) was opposite to H-5 (δ 1.09) while H-19 (δ 0.79) was at the same position of H-20 (δ 0.69). Moreover, H-18 (δ 0.86) was at the same position of H-5 (δ 1.09). There were confirmed

by NOE interaction between H-20 (δ 0.69) with H-19 (δ 0.79) and H-18 (δ 0.86) with H-5. According to the naphthalenyl nucleus was similar to 15,16 bisnorlabda 8(17), 11-dien-13-one that was already reported, therefore the position of H-20 (δ 0.69) and H-5 (δ 1.09) was identical to previous report. All the unequivalent protons were already assigned as equatorial proton (H_e) and axial proton (H_a) as shown in Table 7.



15-Ethoxy-8(17),12-labdadien-15,16-olide

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^{13}C NMR exhibited 8 pairs of carbons which were not the chiral carbons and high temperature (Figure 46a and 46b) ^{13}C NMR showed changeable values in chemical shift, therefore it should be inferred that Mangga I had more than one conformers. From computer modeling, the most stable conformer was the model of Figure 4 with the minimized energy of 50.21 k cal/mole.

From CD spectrum (Figure 47), it showed positive cotton effect around 250 nm that was identical to coronarin C (Itokawa *et al*, 1988). The absolute configuration of lactonic methine was established to be *R* , thus the lactonic methine of Mangga I should be established as *R* .

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Table 7 ^{1}H and ^{13}C NMR spectral data of compound Mangga I (in $CDCl_3$) with long-range correlations observed in HMBC spectra

position	δ_H (multiplicity, *J* in Hz)	δ_C (ppm)	HMBC (correlation with proton)
1	H_a = 1.66 (m) H_e = 1.06 (m)	39.2[a]	H_a-3
2	H_a = 1.55 (m) H_e = 1.47 (m)	19.2	H_e-1, H_e-3* and H-18
3	H_a = 1.38 (m) H_e = 1.16 (m)	41.9	H-18 and H-19
4	-	33.5	H-5*, H_a-3*, and H_e-3*
5	1.09	55.3[a]	H-19, H-18, H_a-6* and H_a-7
6	H_a = 1.30 (m) H_e = 1.70 (m)	24.0	H-5
7	H_a = 2.36 (m) H_e = 1.96 (m)	37.7	H_a-17 and H_b-17
8	-	147.8[a]	H_a-7 * and H_e-7*
9	1.84	56.1	H_a-17, H_b-17, H_a-7, H_e-7, H_e-1 and H_a-11*

Table 7 (Continued)

position	δ_H (multiplicity, *J* in Hz)	δ_C (ppm)	HMBC (correlation with proton)
10	-	39.4	H_a-2, H_e-2, H_a-1*, H_e-1*, H-9*,H-5* and H-20*
11	H_a = 2.17 (m) H_b = 2.30 (m)	25.4	-
12	6.70	143.0[a]	H_a-14, H_b-14 and H_a-11*
13	-	124.0[a]	H-15
14	H_a = 2.67 (m) H_b = 2.97 (m)	32.9	H-12
15	5.53	101.0	H_a-1′, H_b-1′ and H_b-14
16	-	169.7	H-12
17	H_a = 4.35 (1H, *d*, *J*=1.23) H_b = 4.83 (1H, *d*, *J*=1.23)	107.0[a]	-
18	0.86(3H, s)	33.5	H-5, H_a-3, and H_e-3
19	0.79(3H, s)	21.7	H_a-3, H_e-3, H-5 and H-18
20	0.69(3H, s)	14.3[a]	H-5, H_e-1
1′	H_a = 3.60 (m) H_b = 3.90 (m)	65.2[a]	H-15 and H-2′
2′	1.20(3H) (t)	14.9	H_a-1′and H_b-1′

***** Two-bond coupling, [a] A pair of carbon

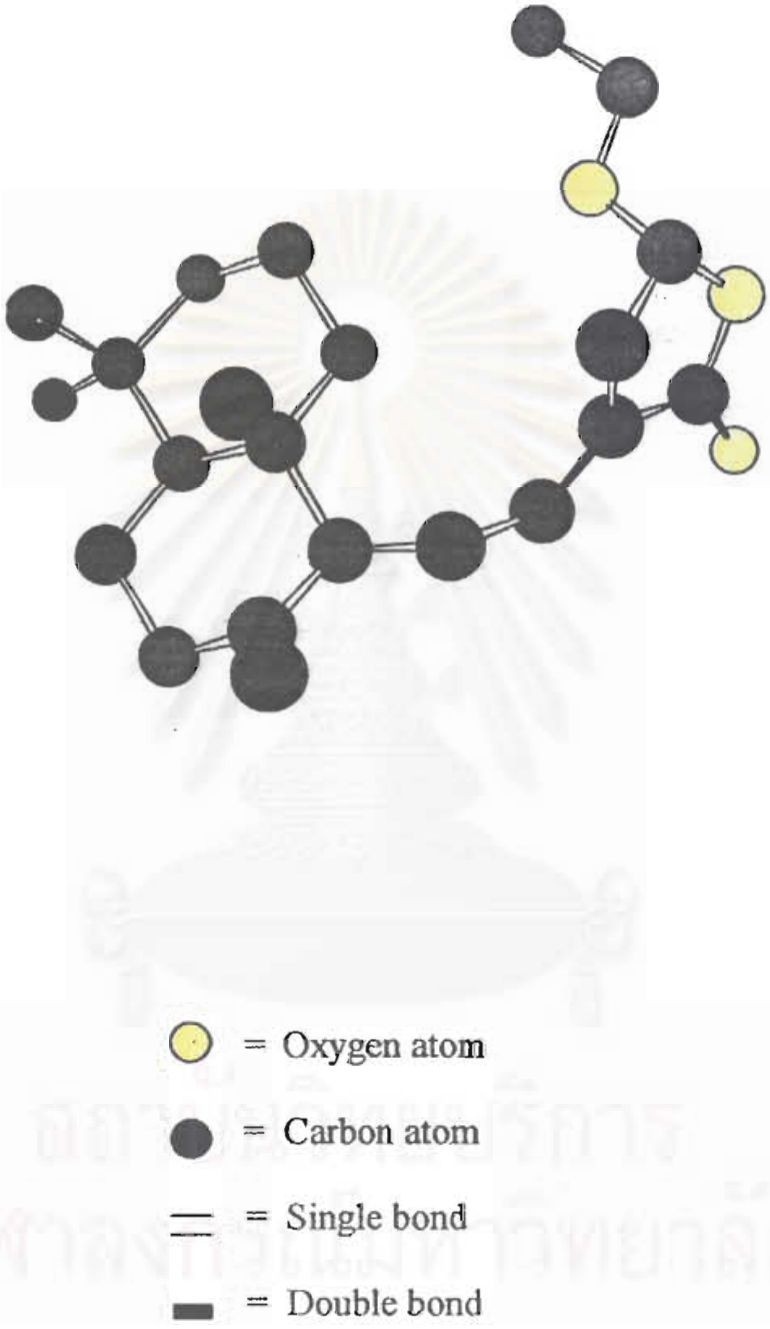

Figure 4 **Computer modeling of Mangga I structure**

2. Essential oil composition of *Curcuma mangga* rhizome

The essential oil of *Curcuma mangga* rhizomes was isolated by hydrodistillation. The oil yield was 0.12 % (v/w) of the fresh weight. The analysis of the essential oil by GC/MS showed 30 peaks well separated from each other (Figure 27). These peaks were identified as 12 monoterpenes, 6 oxygenated monoterpenes, 4 sesquiterpenes, 7 oxygenated sesquiterpenesterpene and 1 miscellaneous (Table 7). Among these, myrcene (84.61%) appeared to be the major component, followed by β-phellandrene (6.63%) and *trans*-ocimene (3.85%).

In term of relative amount, the monoterpenes appeared to be the major terpenoid group, accounting for 97.46% of the essential oil. Oxygenated sesquiterpenes and oxygenated monoterpenes were present in lesser amount at 0.98% and 0.36%, respectively (Figure 5).

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Table 8 Essential oil composition of *Curcuma mangga* rhizome

Peak no.	Compound	% Area
	Monoterpene	
1	α-thujene	*tr*
2	tricyclene	1.10
3	α-pinene	0.24
4	3-carene	0.19
5	β-phellandrene	6.63
6	myrcene	84.61
7	terpinolene	*tr*
8	*o*-cymene	*tr*
9	limonene	0.35
11	*cis*-ocimene	0.49
12	*trans*-ocimene	3.85
14	γ-terpinene	*tr*
	Oxygenated monoterpene	
10	1,8-cineol	0.36
13	*cis*-myrtanol	*tr*
18	perillaldehyde	*tr*
19	camphor	*tr*

Table 8 Essential oil composition of *Curcuma mangga* rhizome (Continued)

Peak no.	Compound	% Area
20	borneol	*tr*
21	4-terpineol	0.09
	Sesquiterpene	
15	δ-elemene	0.06
23	β-caryophyllene	0.51
24	α-humulene	0.05
26	β-selinene	0.09
	Oxygenated sesquiterpene	
16	β-eudesmol	0.20
17	*trans*-nerolidol	0.31
25	3-thujopsanone	*tr*
27	caryophyllene oxide	0.06
28	3-iso-thujopsanone	0.14
29	5-cedranone	*tr*
30	epi-13-manool	0.27
	Miscellaneous	
22	α-terpinyl acetate	0.10

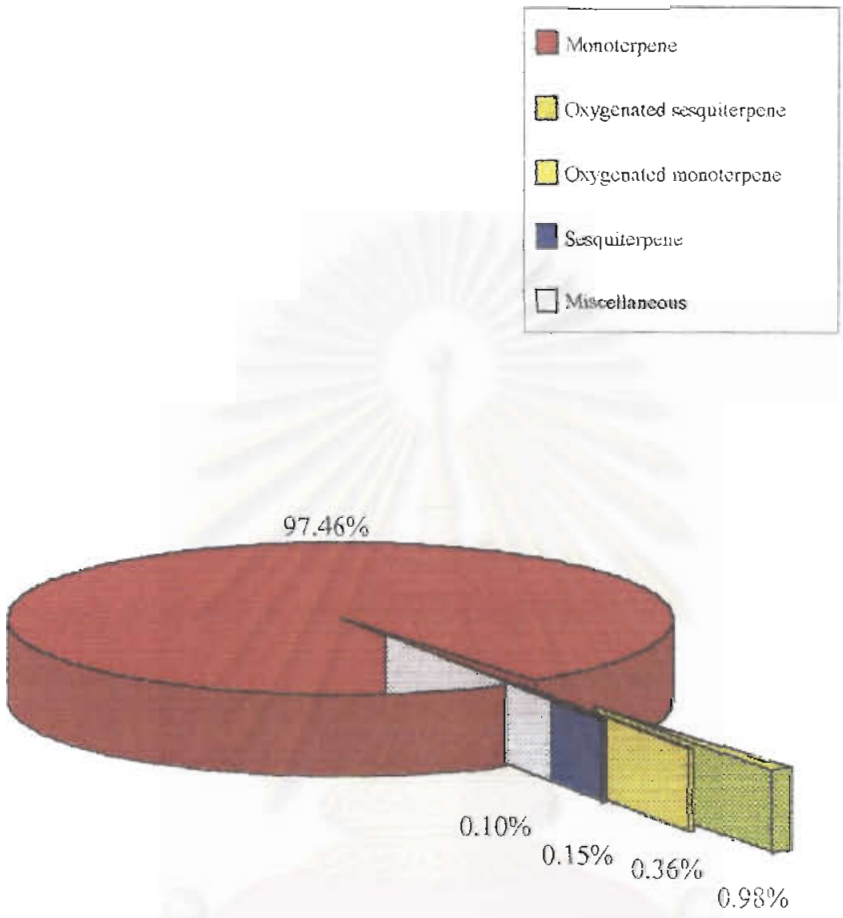

Figure 5 Composition of Thai *Curcuma mangga* Essential Oil

Comparison with the chemical constituents of essential oil from *Curcuma mangga* rhizomes (Wong *et al*., 1999) in Chapter II, 30 components were distributed in Thai specimen whilst 44 components were in Malaysian specimen by GC/MS analyses. Myrcene was the major component of both Thai and Malaysian *C. mangga*, but it was different in quantity. Myrcene in Thai *C. mangga* oil was present in greater amount, at 84.61% followed by β-phellandrene (6.63%) and *trans*-ocimene (3.85%). For Malaysian *C. mangga* oil, the amount of myrcene was 78.6% followed by (E)-β-ocimene (5.1%) and β-pinene (3.7%).

In term of relative amount, the major group of Thai specimen was monoterpenes (97.46%), followed by oxygenated sesquiterpenes (0.98%) and oxygenated monoterpenes (0.36%) respectively whereas Malaysian specimen yielded monoterpenes (91.70%) as the major group of terpenoid, followed by oxygenated monoterpenes (3.4%) and sesquiterpenes (1.1%) respectively. (Figure 6)

The difference in both type and quantity of Thai *C. mangga* oil and Malaysian *C. mangga* oil depended upon age, locality, season and ect., of *C. mangga* in each country, but they shared in common in the major component as described above.

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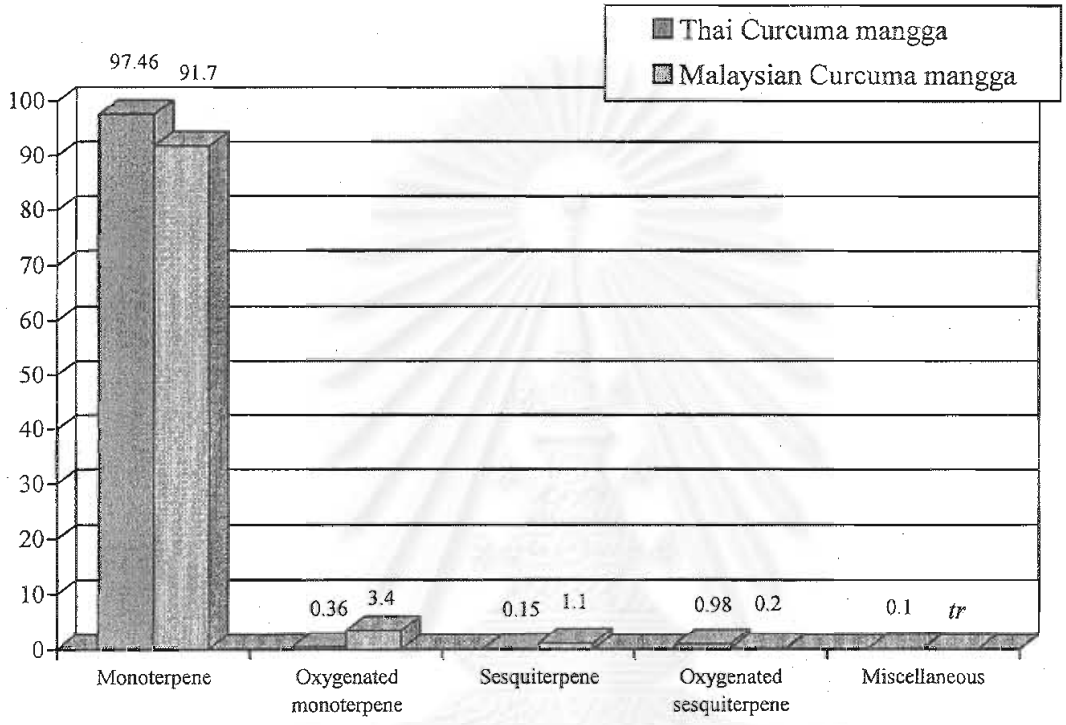

Figure 6 Comparison of Chemical Group of Thai and Malaysian *Curcuma mangga* Essential Oil

3. Antimicrobial Activity of the Essential Oil from the Rhizome of *C. mangga*

Antimicrobial activity of the essential oil from *C. mangga* was shown in Table 9 and 10. The activity was rather broad spectrum because it exhibited the activity against *S. aureus, E. faecalis, B. subtilis, P. aeruginosa* and *M. gypseum*. According to MIC, MBC and MFC, this essential oil displayed strong activity against *S. aureus* and *M. gypseum* as well as the antimicrobial activity of *C. mangga* oil was cidal effect.

Table 9 Antimicrobial activity of the essential oils (10% oil in 0.1% Tween80) of *C. mangga*

Type of microorganism	Inhibition zone (mm)*
Staphylococcus aureus ATCC 29213	8.53
Streptococcus faecalis ATCC 29212	12.48
Bacillus subtilis ATCC 6633	9.33
Escherichia coli ATCC 25922	0
Pseudomonas aeruginosa ATCC 27853	11.50
Candida albicans ATCC 10231	0
Microsporum gypseum (clinical isolate)	17.10

*Average from the duplicate results.

Table 10 MIC[a], MBC[b], and MFC[c] values of the essential oil (10% oil in 0.1% Tween80) of *C. mangga*

Type of microorganism	Type of test		
	MIC (%)	MBC (%)	MFC (%)
Staphylococcus aureus ATCC 29213	0.156	0.156	
Streptococcus faecalis ATCC 29212	5.0	5.0	
Bacillus subtilis ATCC 6633	5.0	5.0	
Escherichia coli ATCC 25922	ND[d]	ND[d]	
Pseudomonas aeruginosa ATCC 27853	≥ 5[e]	≥ 5[e]	
Candida albicans ATCC 10231	ND[d]		ND[d]
Microsporum gypseum (clinical isolate)	0.3125		0.3125

[a]Minimal inhibitory concentratiion.

[b]Minimal bactericidal concentration.

[c]Minimal fungicidal concentration.

[d]Not determined.

[e]2.5-5.0 % of DMSO showed some effects on the bacterial growth.

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4. Cytotoxic Activity of Compound Mangga I

Cytotoxic evaluation of Mangga I was found that it exhibited moderate activity on KB and LNCaP cell lines as shown in Table 11.

Table 11 Evaluation of the cytotoxic potential of compound Mangga I

Cell line	Cytotoxic activity ED_{50} (μg/ml)
Human Breast Cancer (BC1)	>20
Human Lung Cancer (Lu1)	>20
Human Colon Cancer (Col2)	>20
Human Epidermoid Carcinoma (KB)	9.7
Vinblastine-resistant KB with Vinblastine (KB-V(+VLB))	>20
Vinblastine-resistant KB without Vinblastine (KB-V(-VLB))	>20
Hormone Dependent Human Prostate Cancer (LNCaP)	11.4

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CHAPTER V

CONCLUSION

The investigation of non volatile part of *Curcuma mangga* Valeton & van Zyp rhizome, led to the isolation of two compounds. A new labdane-type diterpene with moderate cytotoxic activity on KB and LNCap cell lines was identified as 15-ethoxy-8(17),12-labdadien-15,16-olide and the other was a norlabdane-type diterpene, 15,16-bisnorlabda-8(17),11-dien-13-one that complete revision of ^{1}H and ^{13}C assignment was carried out. Additionally, the presence of two steroids viz β-sitosterol and stigmasterol was detected. The volatile part of fresh rhizome was hydrodistilled and analysed by GC/MS. It revealed the major components of myrcene (84.61%), β-phellandrene (6.63%) and *trans*-ocimene (3.85%), respectively. In term of relative amount, the monoterpene appeared to be major group accounting for 97.16% followed by oxygenated sesquiterpene (0.98%) and oxygenated monoterpene (0.36%). For antimicrobial activity study, the volatile oil of *C. mangga* rhizome exhibited broad spectrum against *S. aureus, E. faecalis, B. subtilis, P. aeruginosa* and *M. gypseum*. Moreover, from MIC, MBC and MFC, the essential oil displayed strong cidal effect on *S. aureus* and *M. gypseum*.

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APPENDIX

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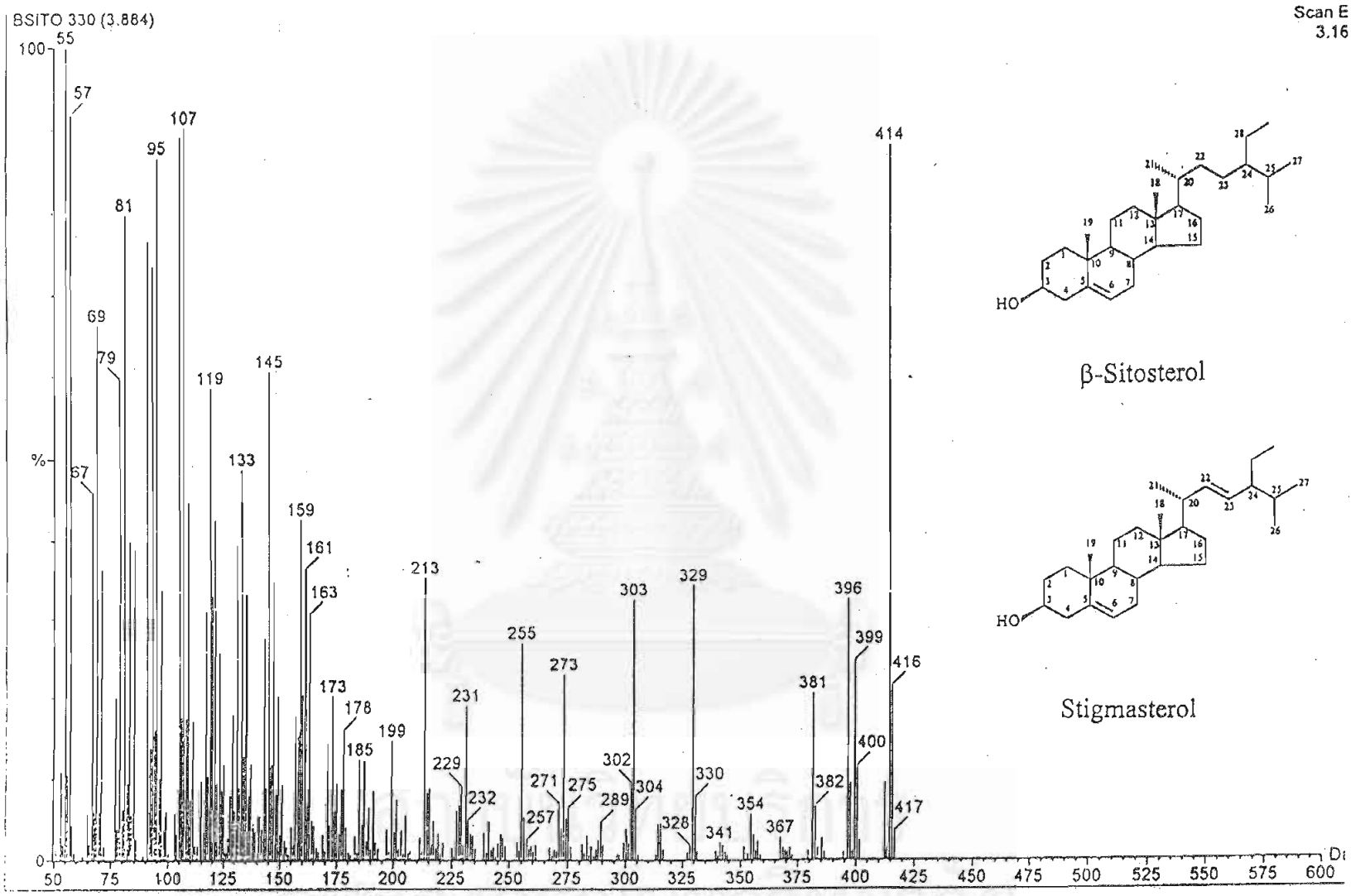
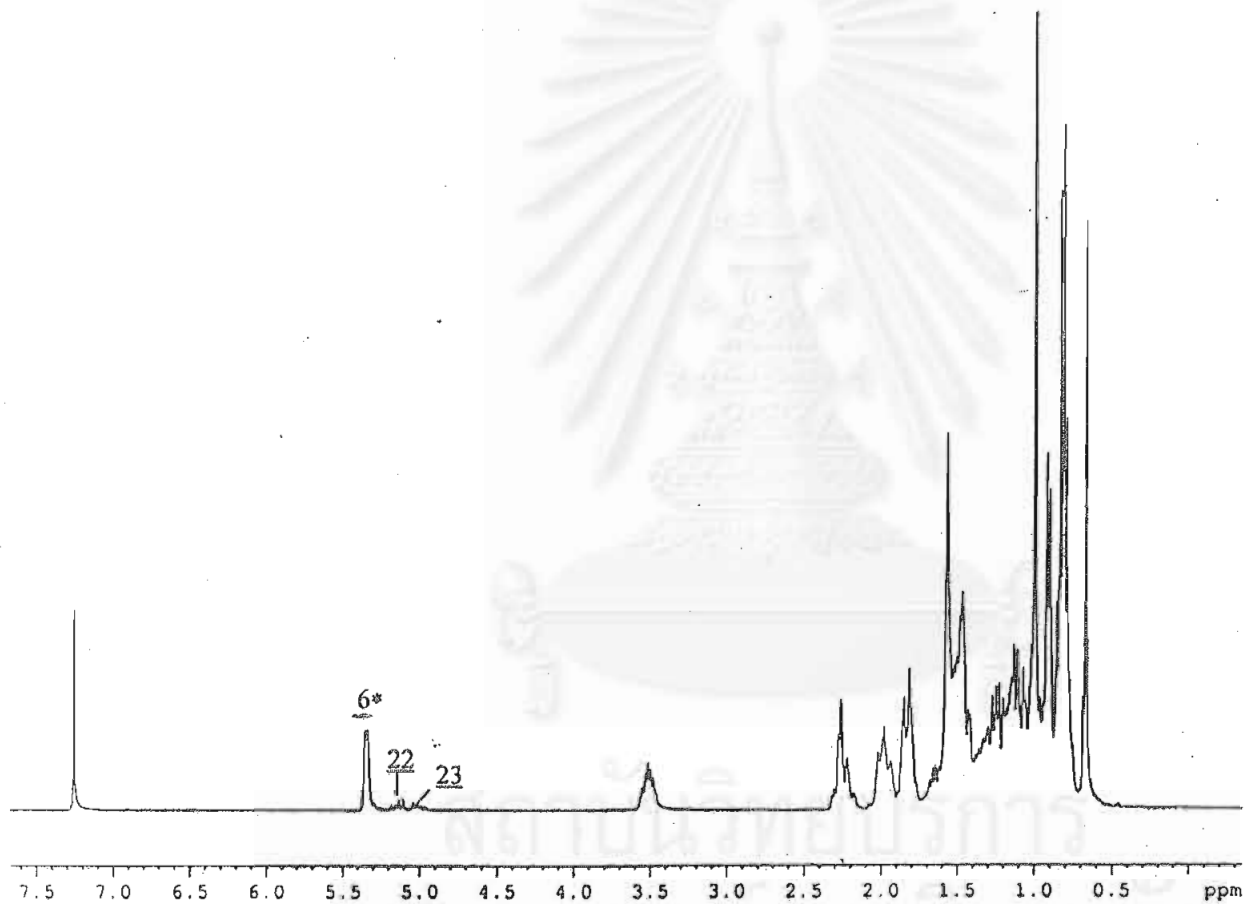
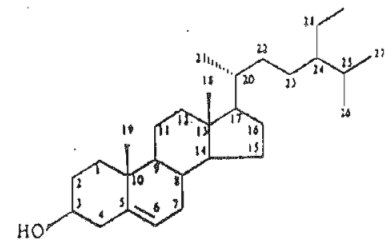
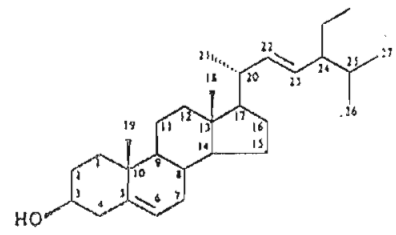


Figure 7 EI mass spectrum of compound CM I



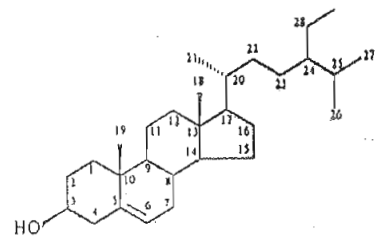


*β-Sitosterol



– Stigmasterol

Figure 8a 300 MHz ^{1}H NMR spectrum of compound CM I (in $CDCl_3$) (expanded from 4.9-5.4 ppm)



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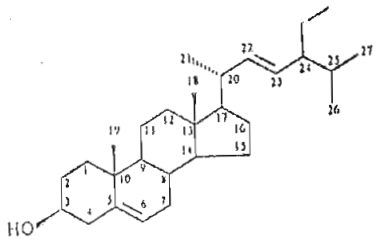

Stigmasterol

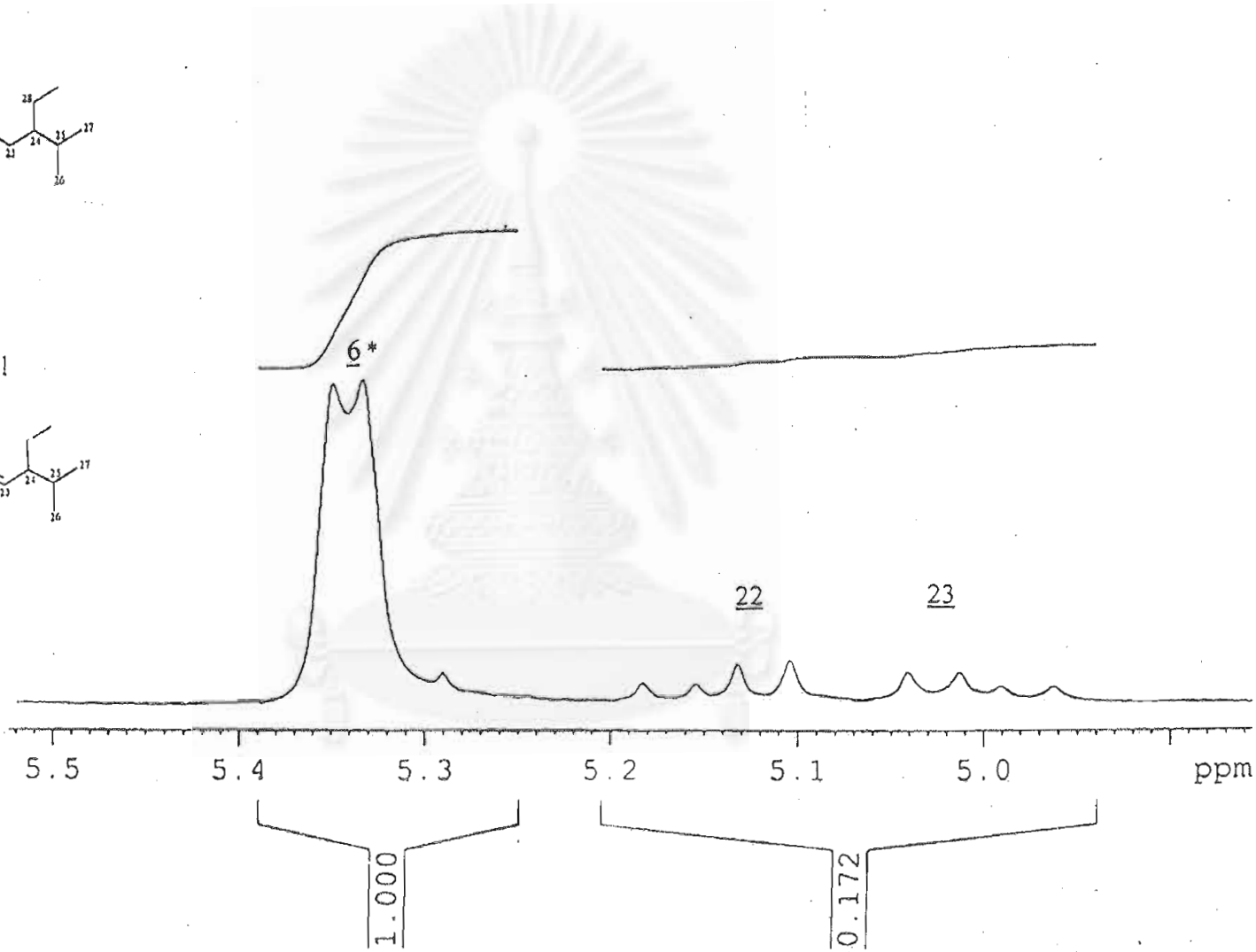

Figure 8b 300 MHz ^{1}H NMR spectrum of compound CM I (in $CDCl_3$.)

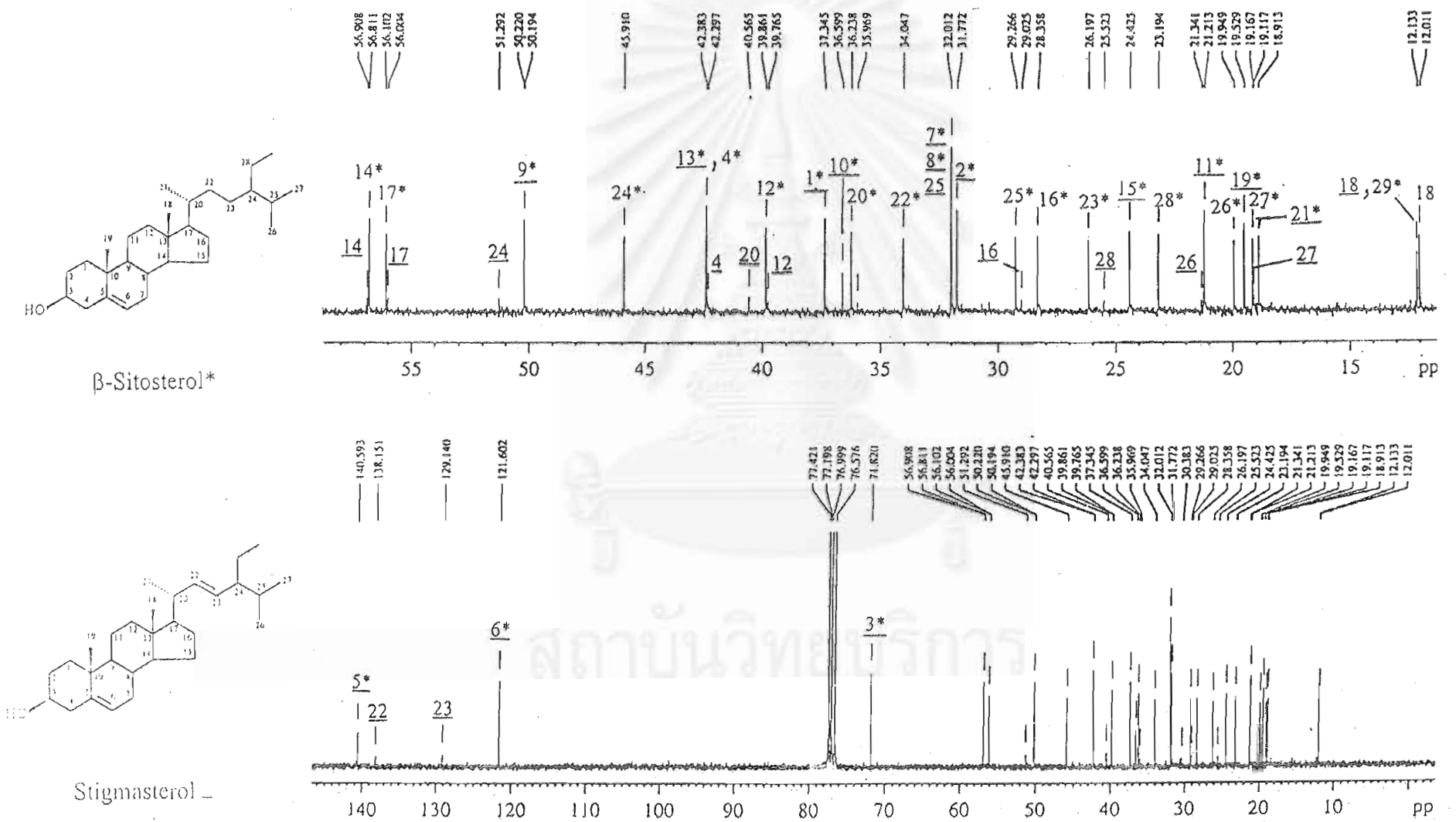

Figure 9 75 MHz ^{13}C NMR spectrum of compound CM I (in $CDCl_3$)

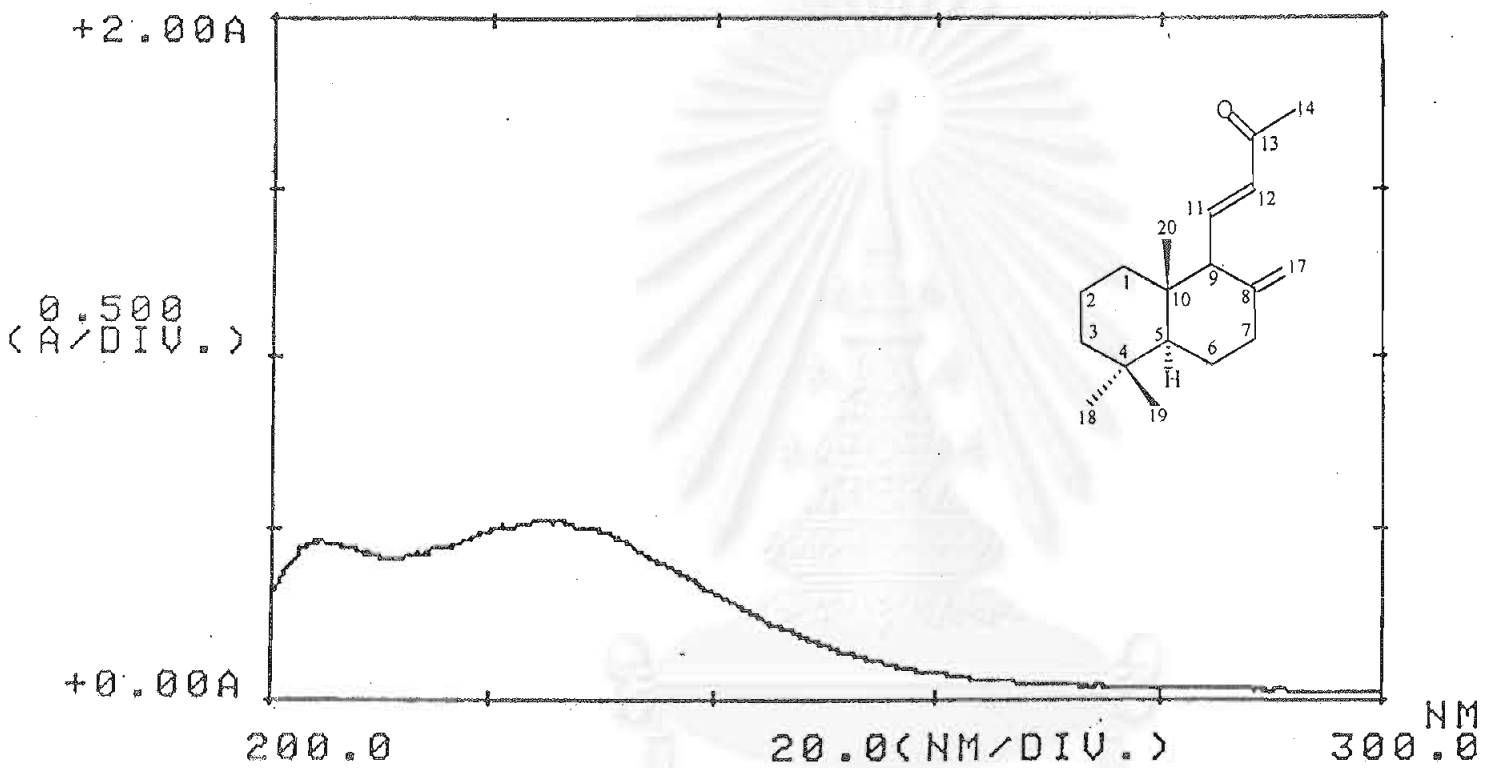

Figure 10 UV spectrum of compound Mangga IV (in methanol)

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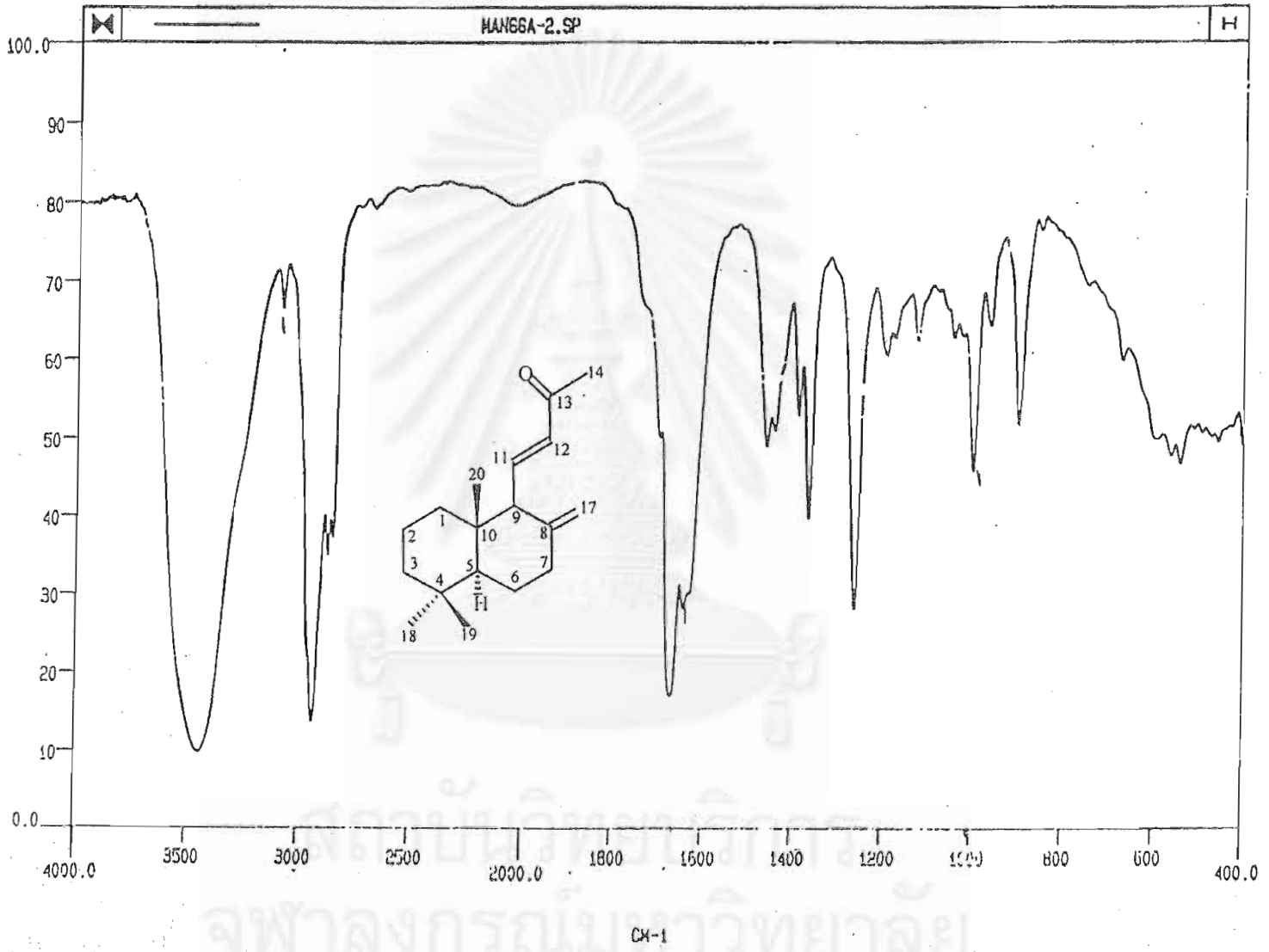

Figure 11 IR spectrum of compound Mangga IV (KBr disc)

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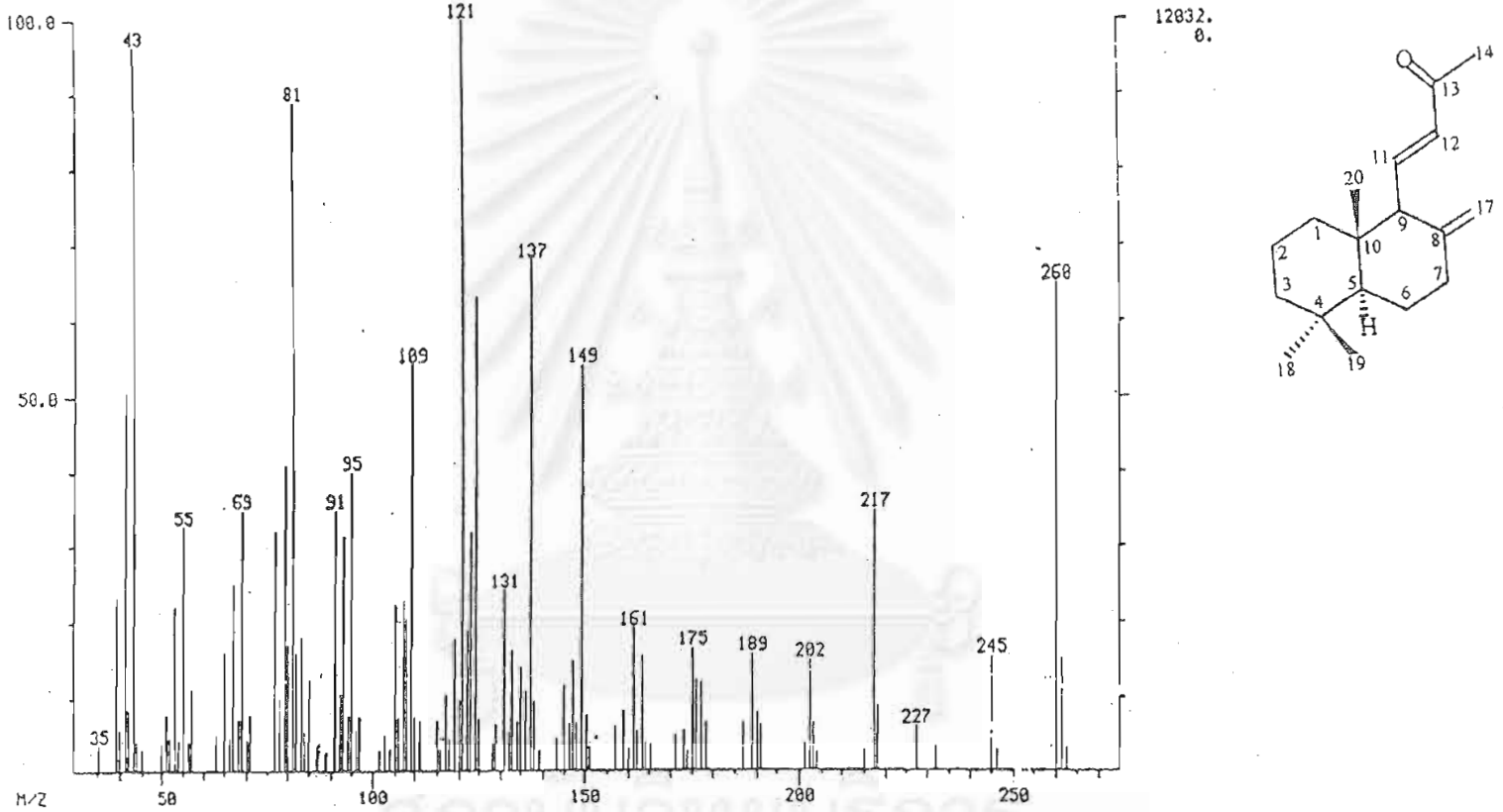


Figure 12 EI mass spectrum of compound Mangga IV

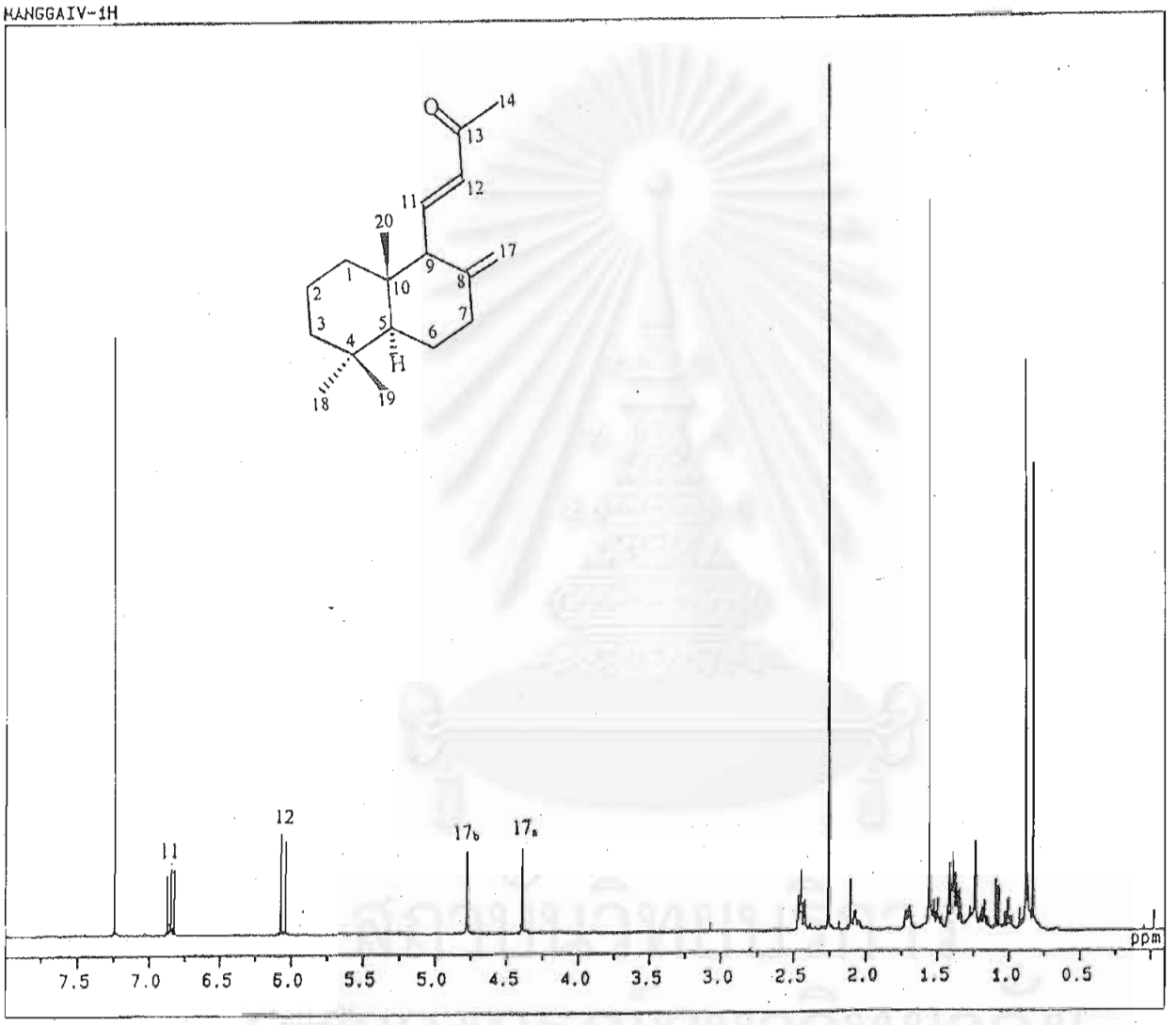
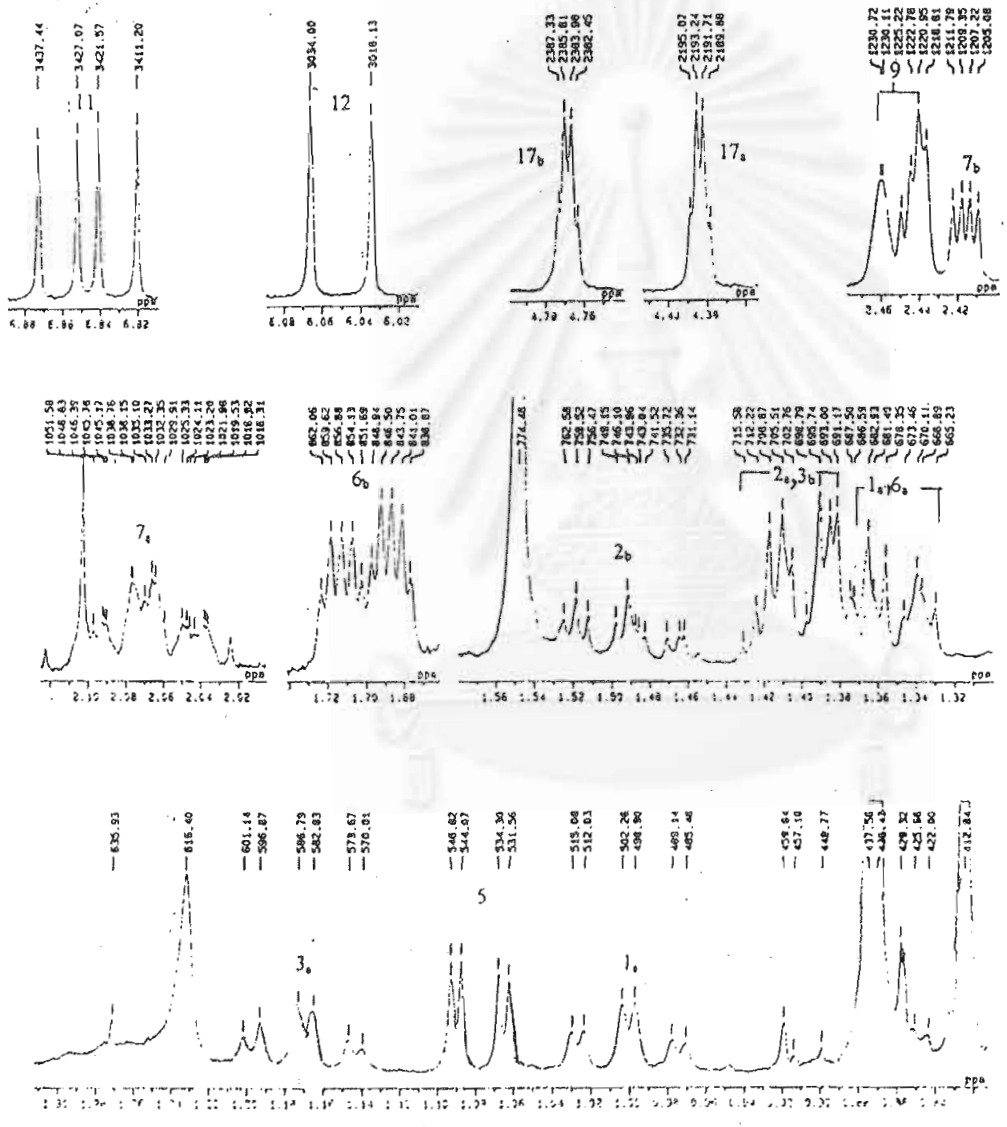


Figure 13 500 MHz ^{1}H NMR spectrum of compound Mangga IV (in $CDCl_3$.)



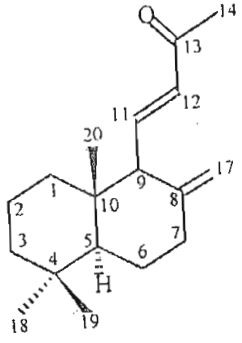


Figure 14 500 MHz ^{1}H NMR spectrum of compound Mangga IV (in $CDCl_3$) (expanded)

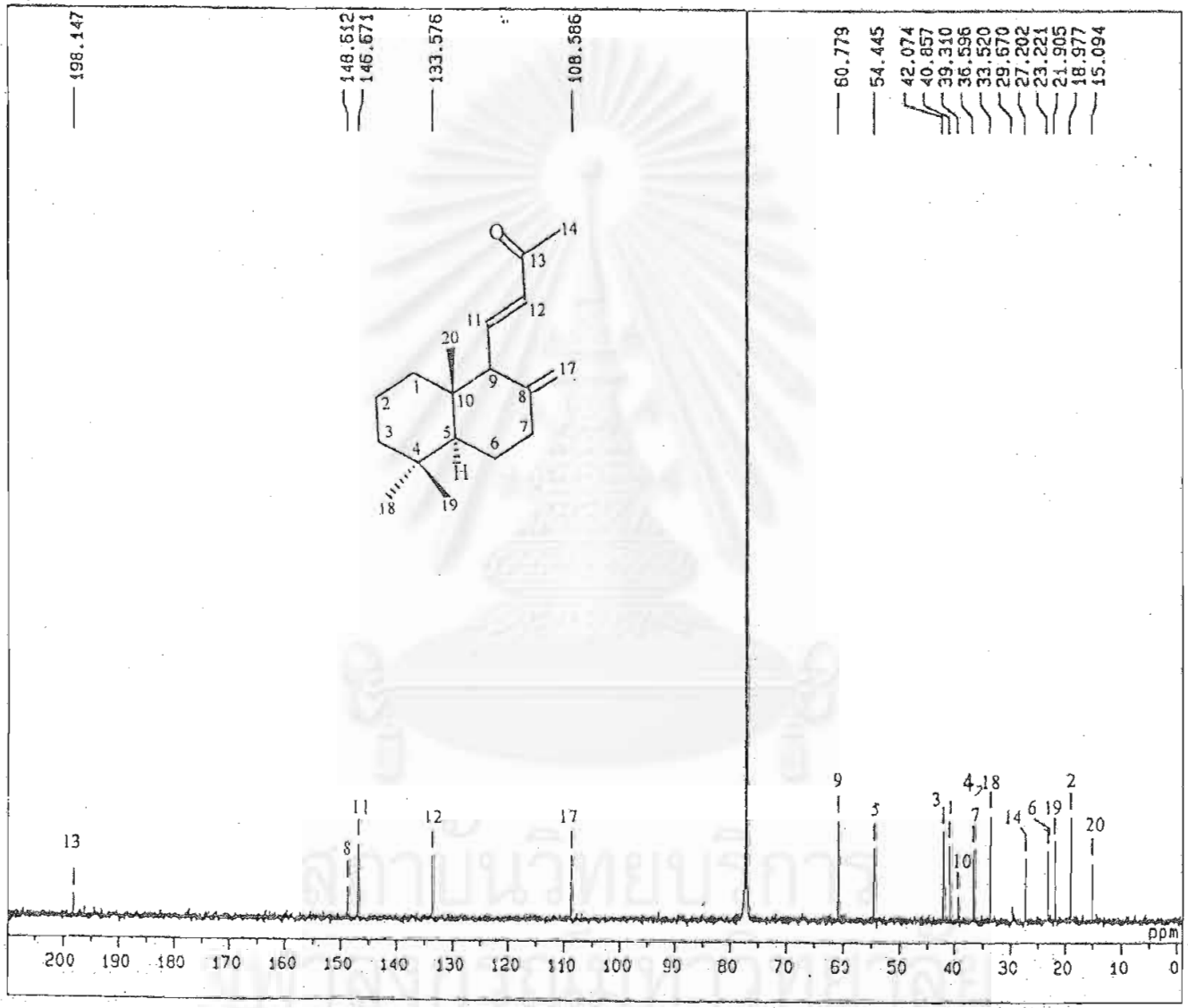
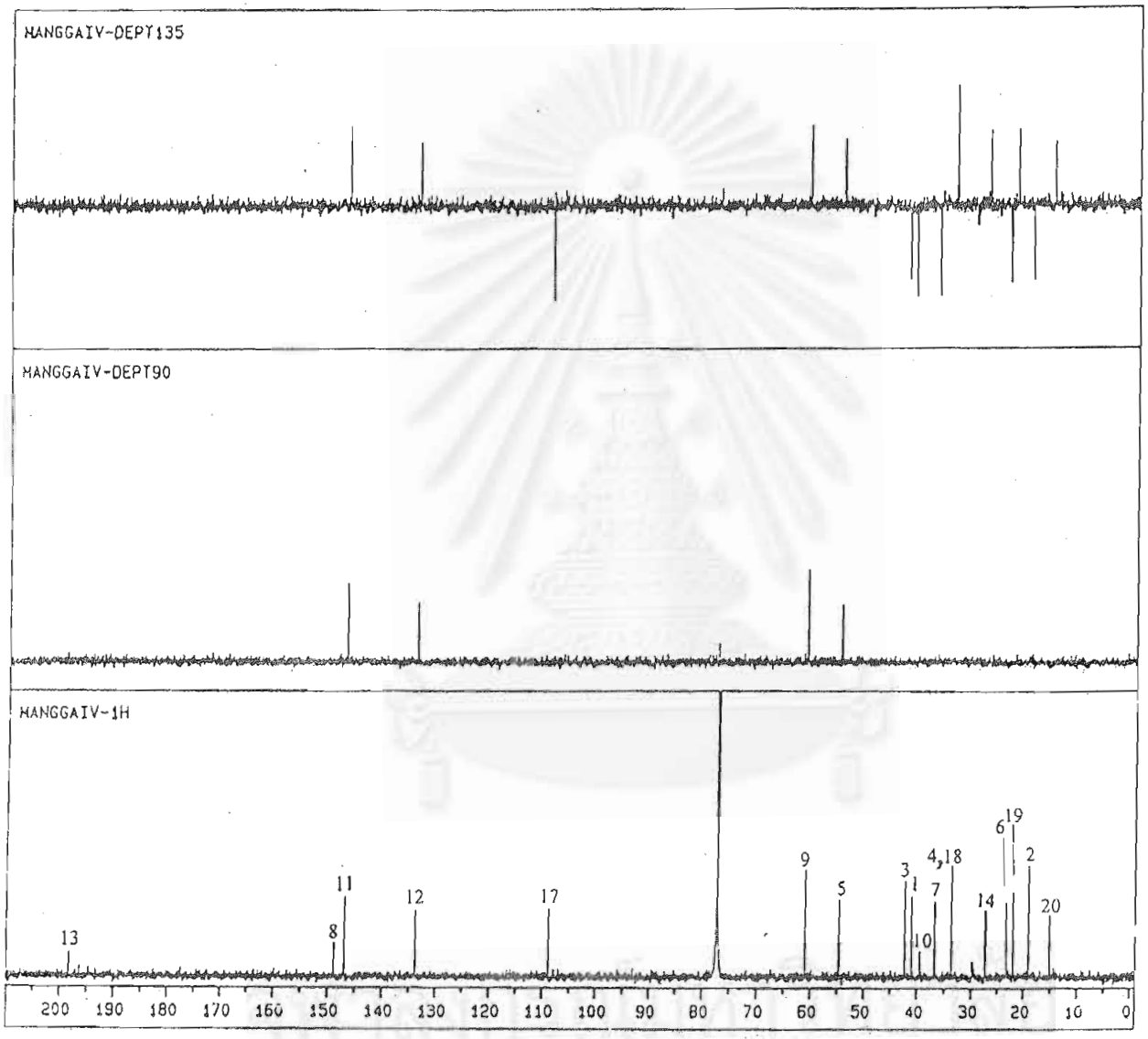


Figure 15 125 MHz ^{13}C NMR spectrum of compound Mangga IV (in $CDCl_3$)



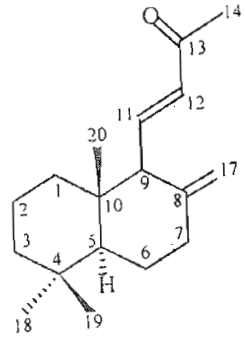


Figure 16 125 MHz ^{13}C NMR, DEPT 90 and DEPT 135 spectra of compound Mangga IV (in $CDCl_3$)

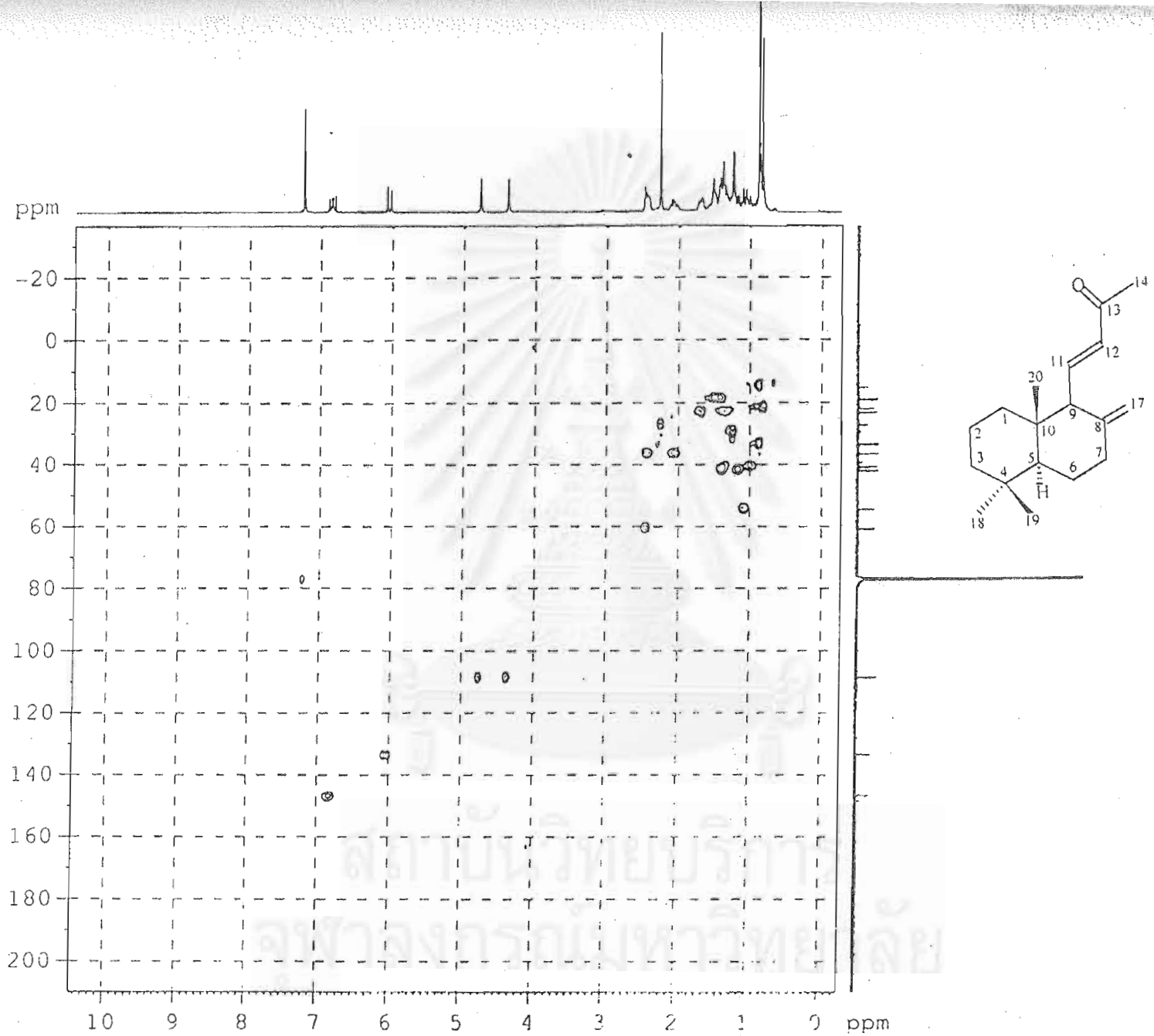


Figure 17 HMQC spectrum of compound Mangga IV (in $CDCl_3$)

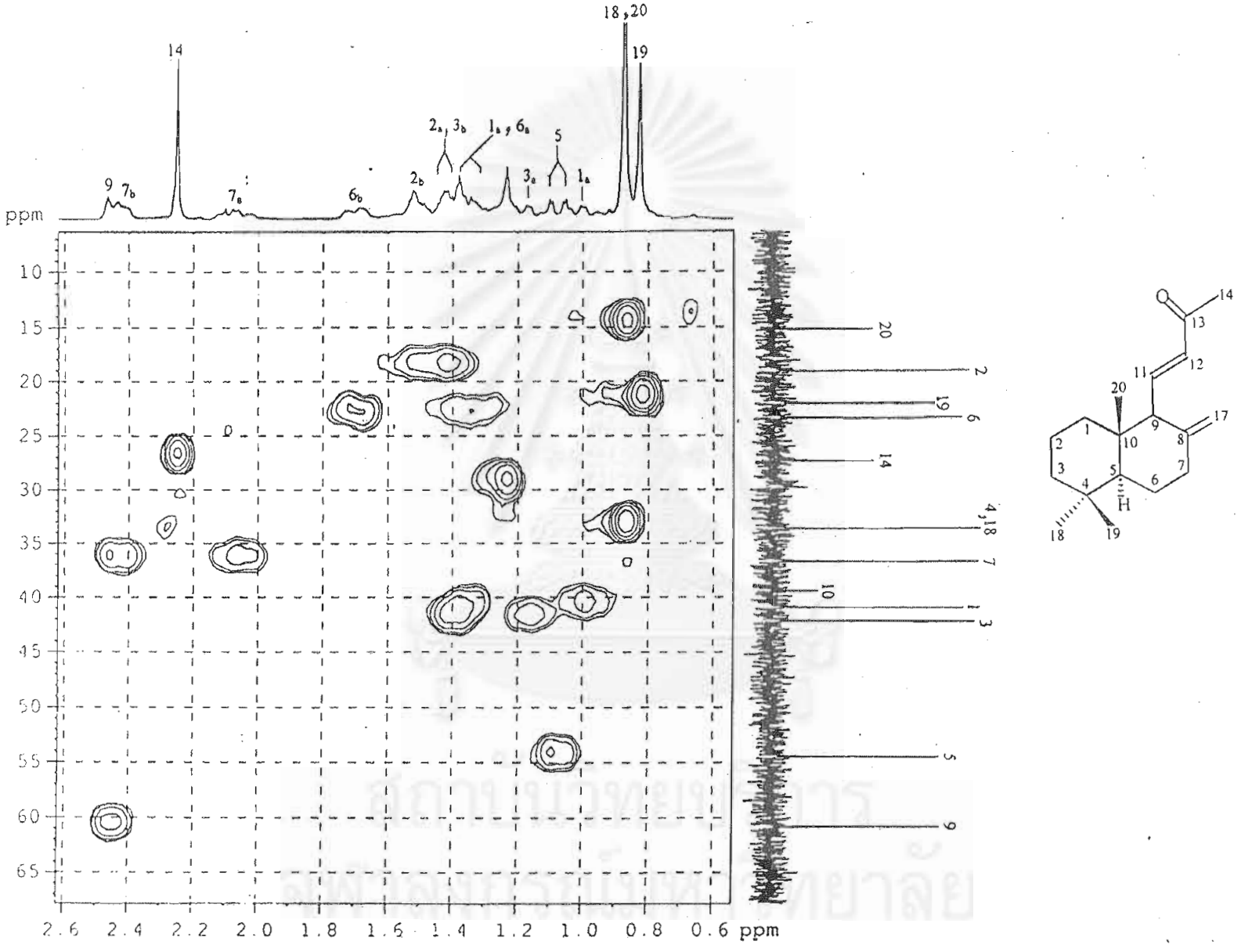


Figure 18 HMQC spectrum (partially expanded: δ_H 0.6-2.6 ppm, δ_C 10-65 ppm) of compound Mangga IV (in $CDCl_3$)

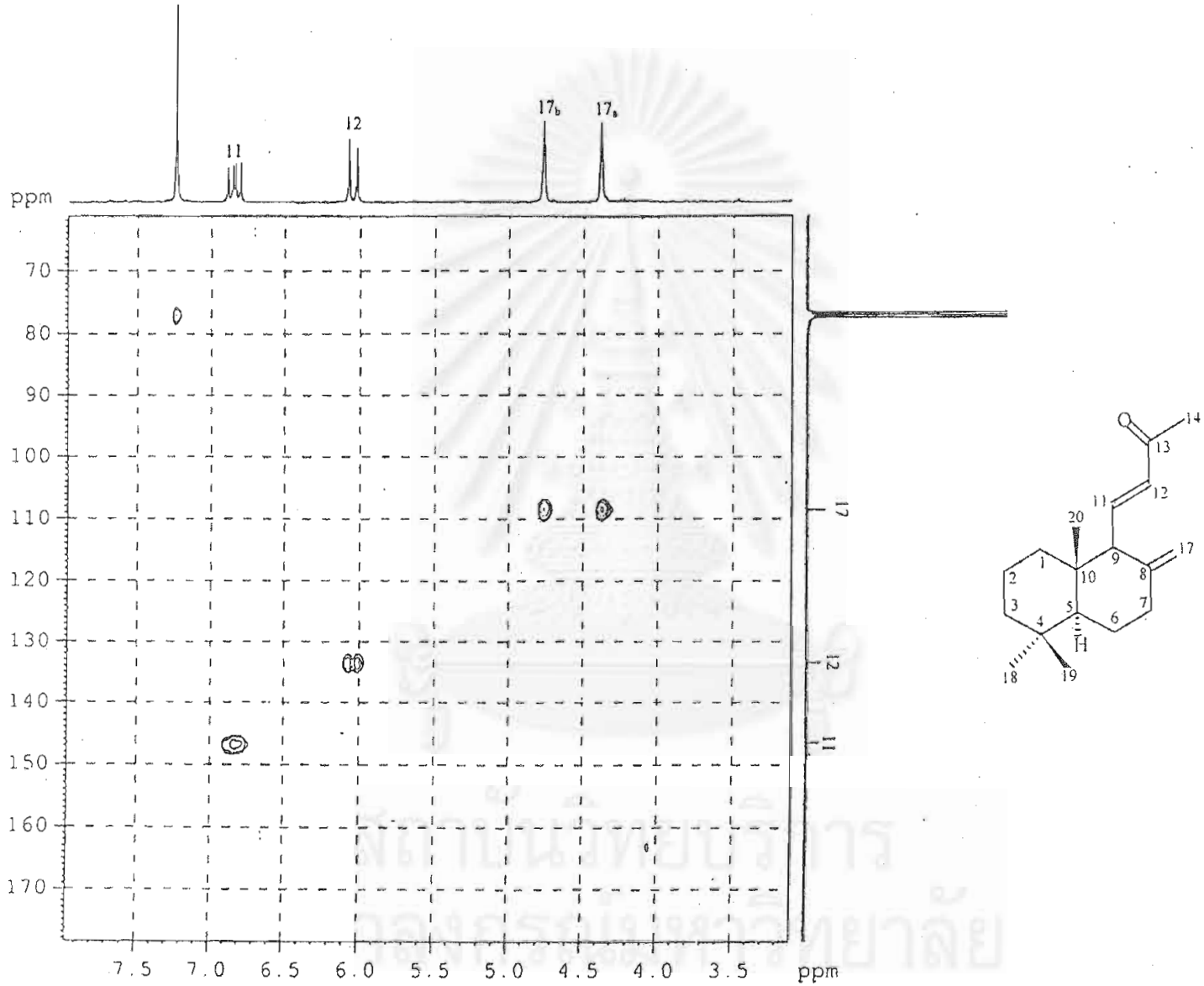


Figure 19 HMQC spectrum (partially expanded: δ_H 3.5-7.5 ppm, δ_C 70-160 ppm) of compound Mangga IV (in $CDCl_3$)

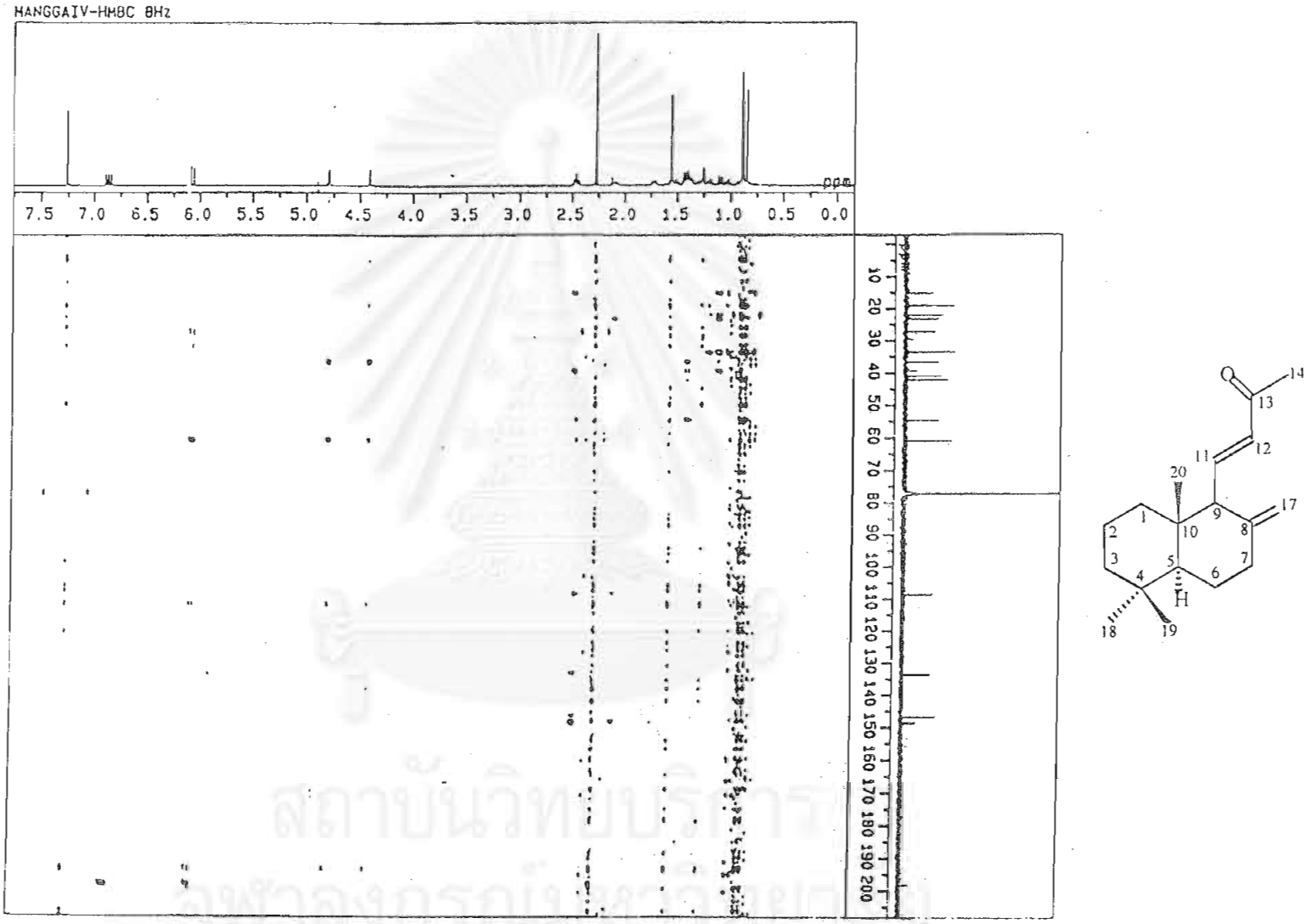


Figure 20 HMBC spectrum of compound Mangga IV (in $CDCl_3$)

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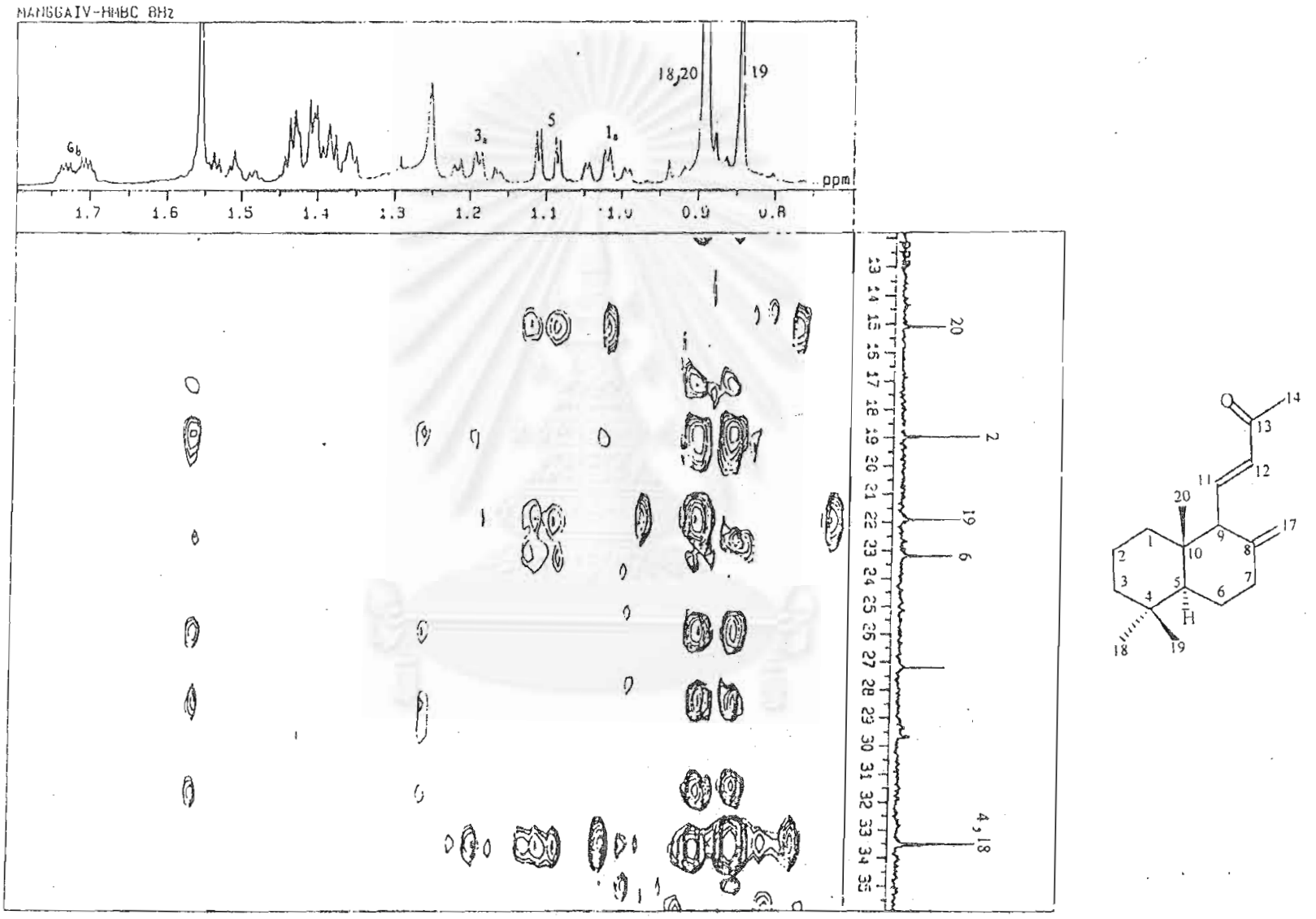
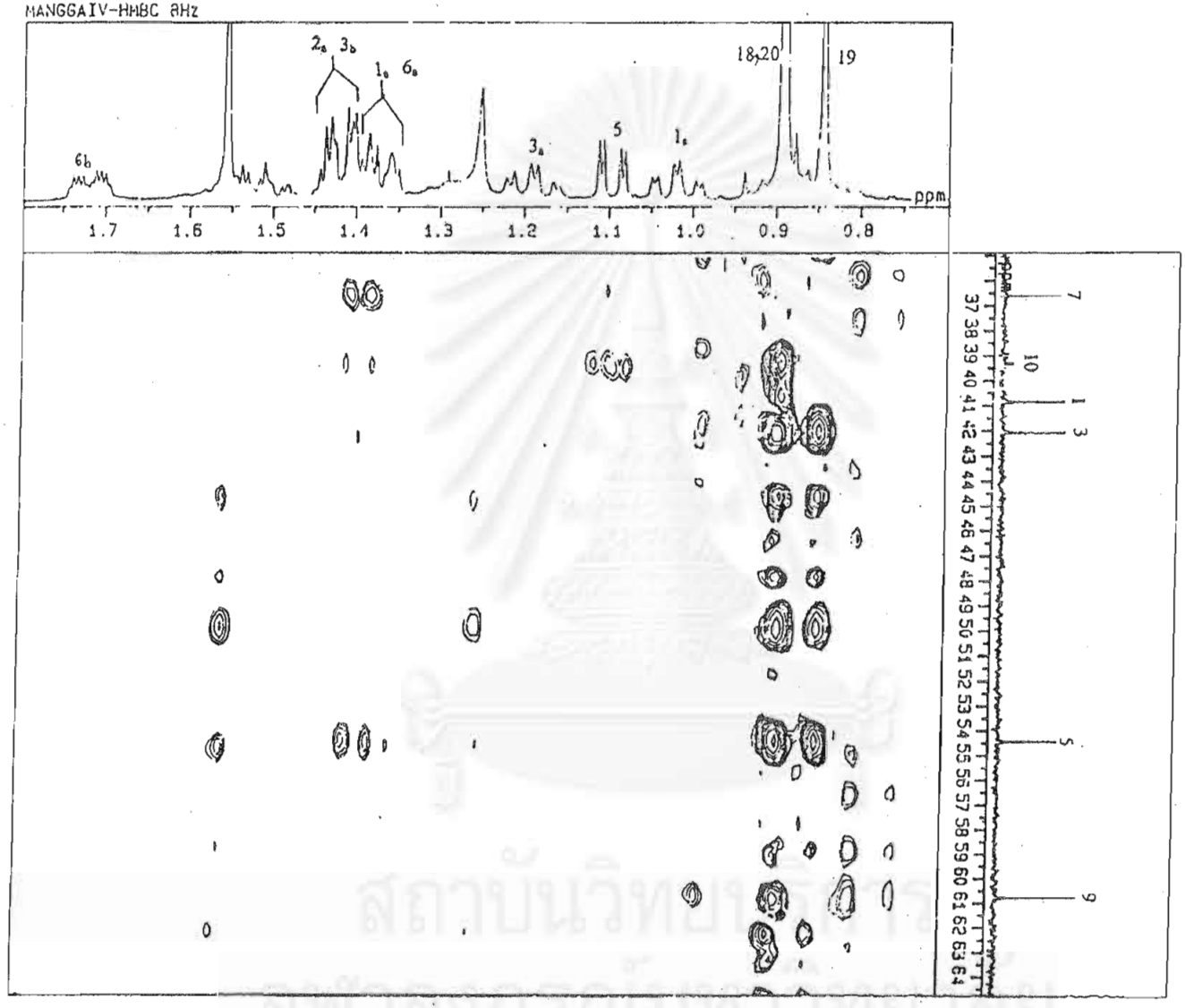


Figure 21 HMBC spectrum (partially expanded: δ_H 0.8-1.7 ppm, δ_C 14-35 ppm) of compound Mangga IV (in $CDCl_3$)



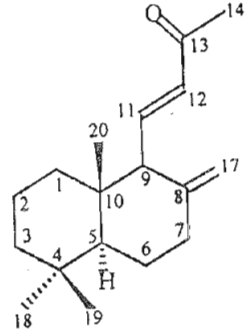
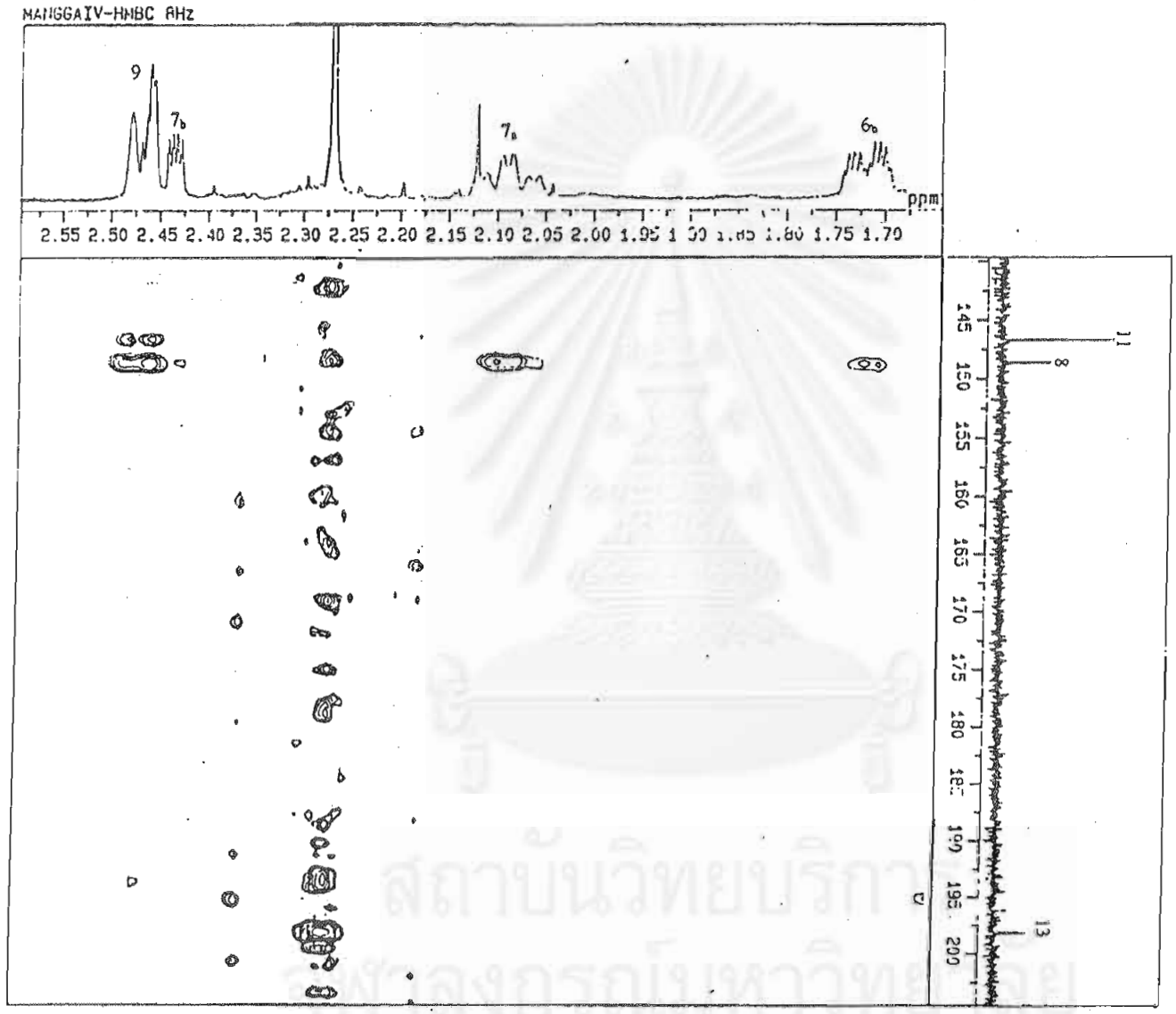


Figure 22 HMBC spectrum (partially expanded: δ_H 0.8-1.7 ppm, δ_C 37-62 ppm) of compound Mangga IV (in $CDCl_3$)



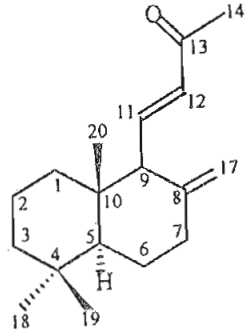
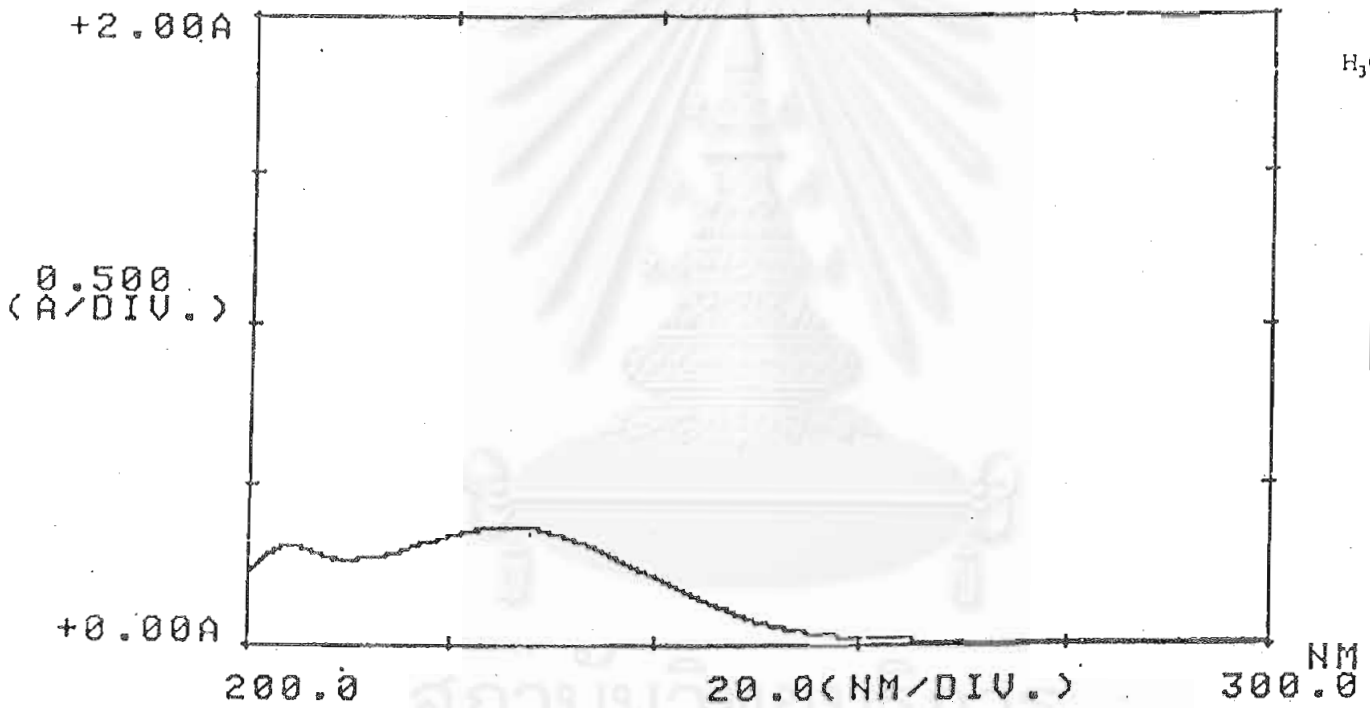


Figure 23 HMBC spectrum (partially expanded: δ_H 1.7-2.5 ppm, δ_C 145-200 ppm) of compound Mangga IV (in $CDCl_3$)



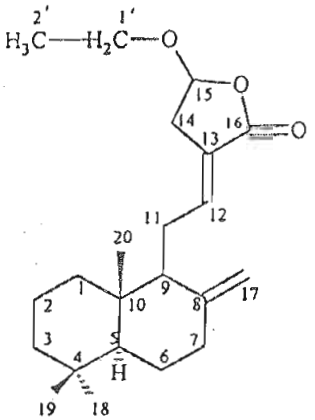


Figure 24 UV spectrum of compound Mangga I (in methanol)

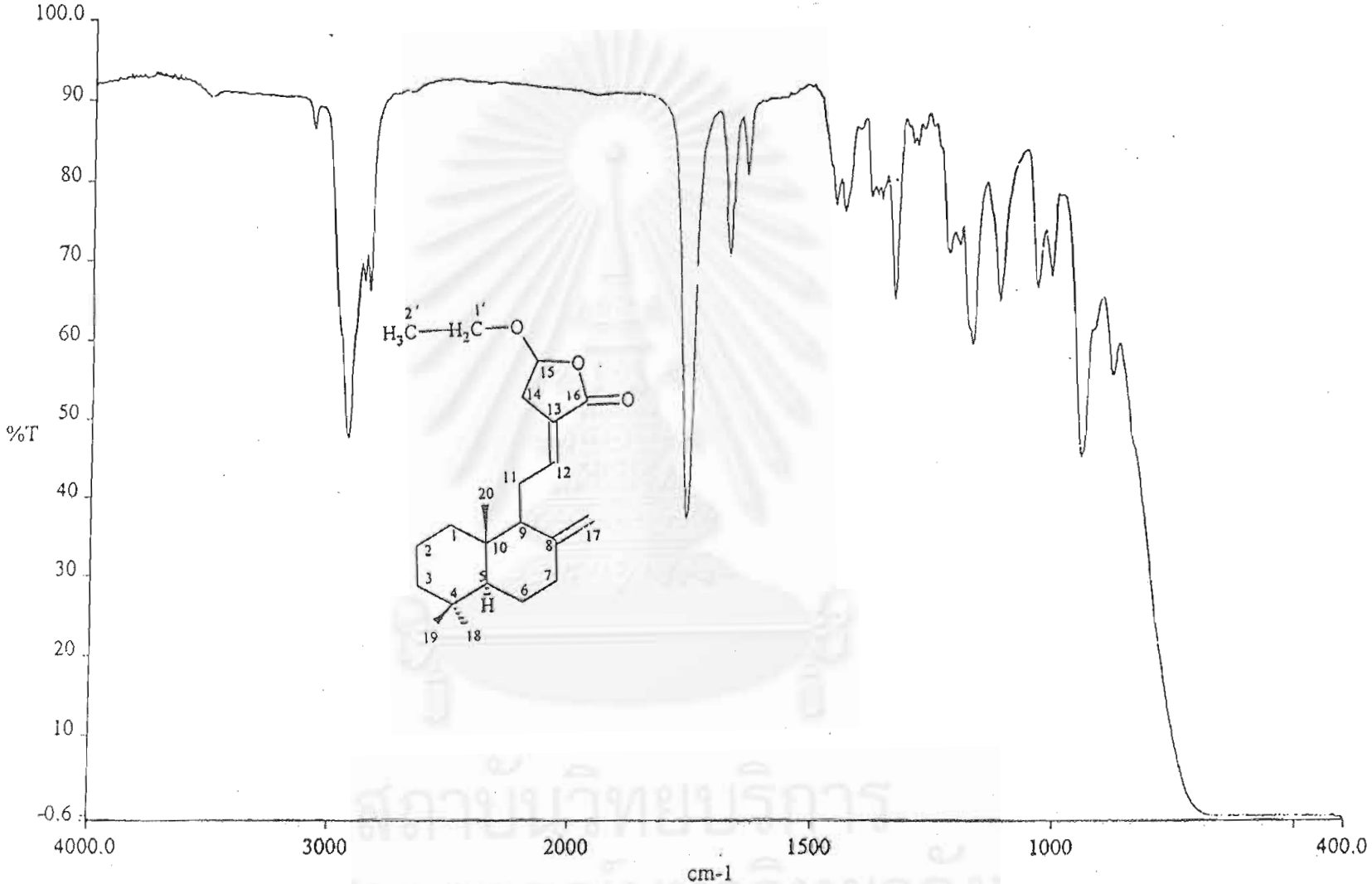


Figure 25 IR spectrum of compound Mangga I (Film)

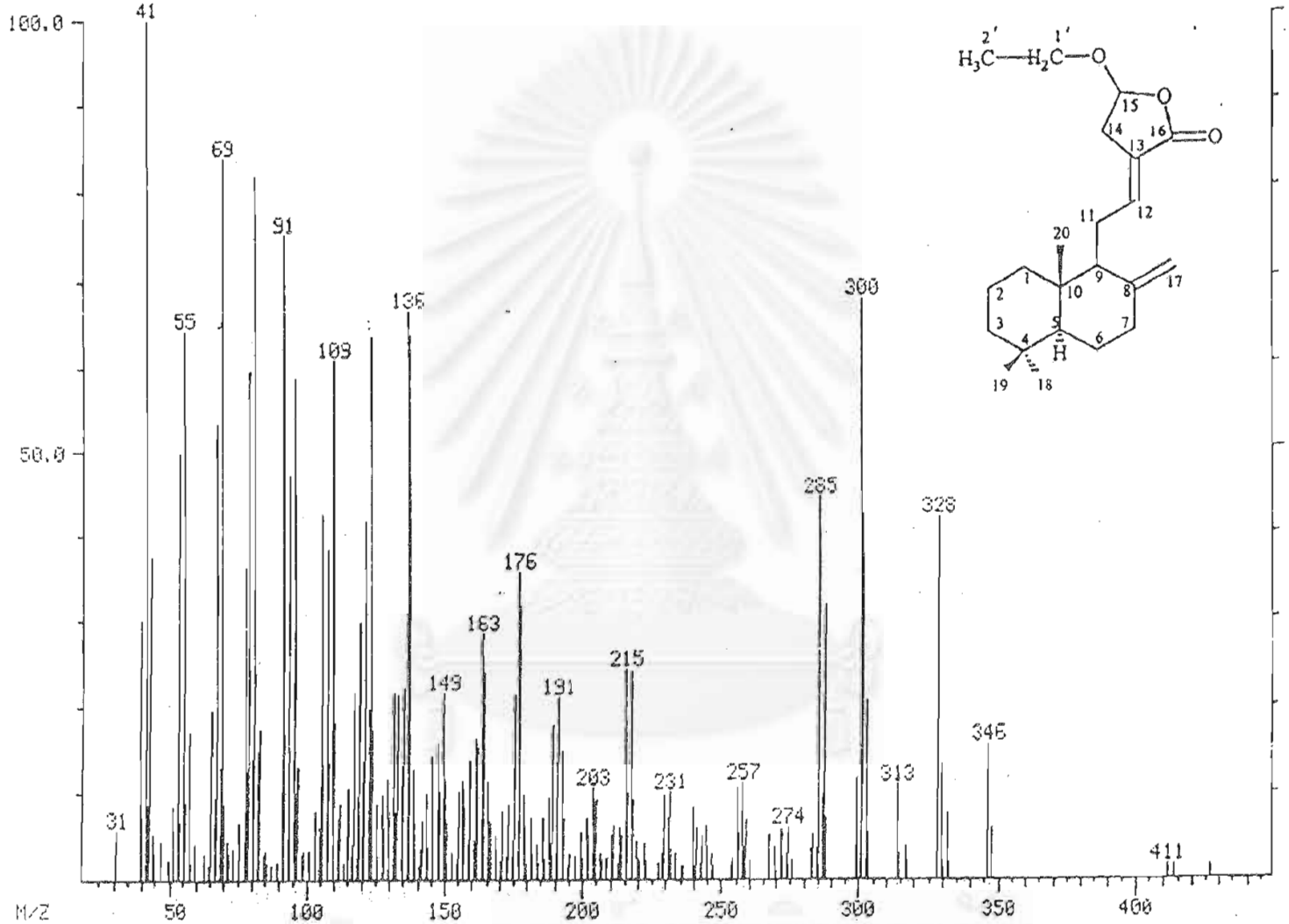


Figure 26 EI mass spectrum of compound Mangga I

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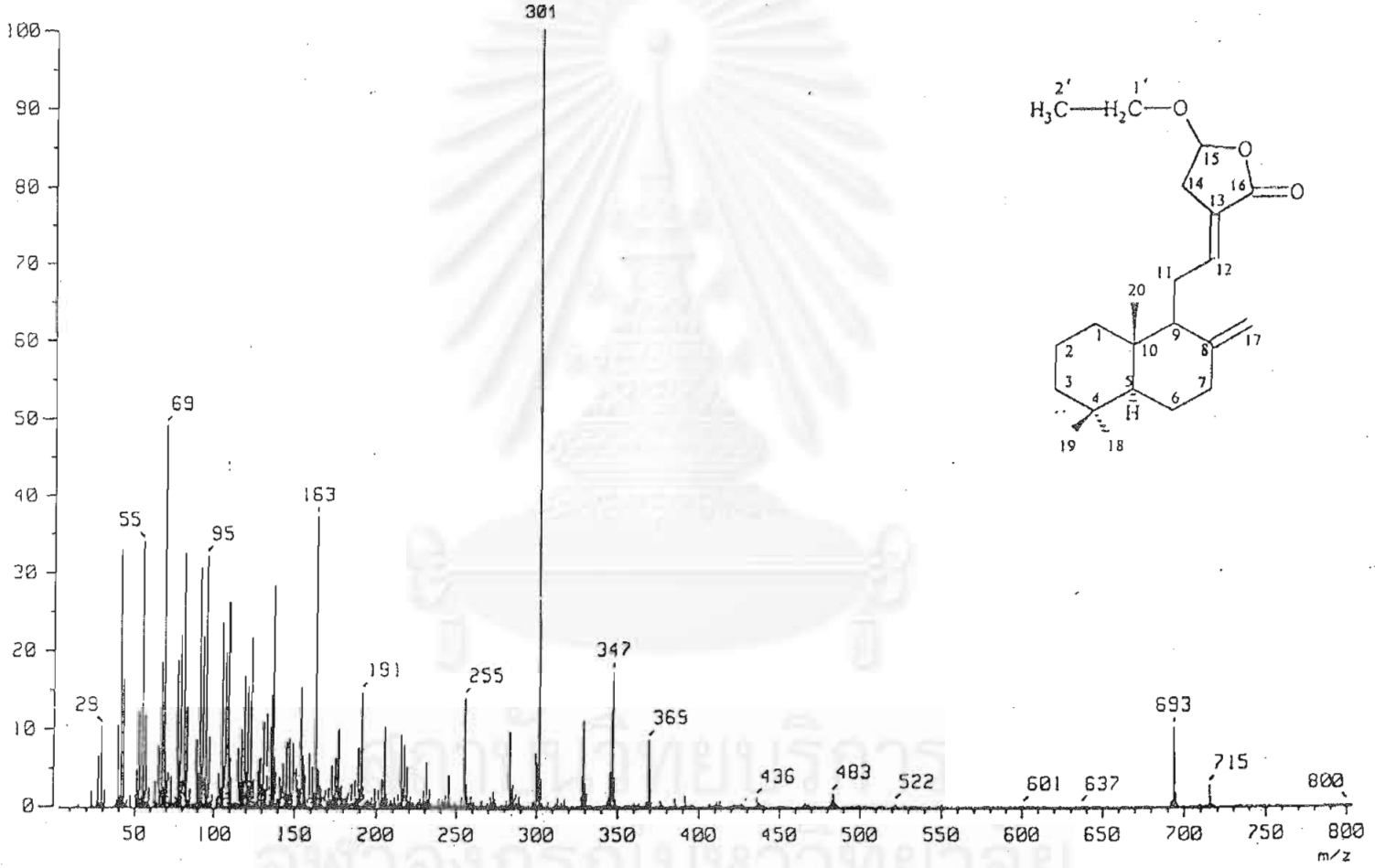


Figure 27 HRFAB mass spectrum of compound Mangga I

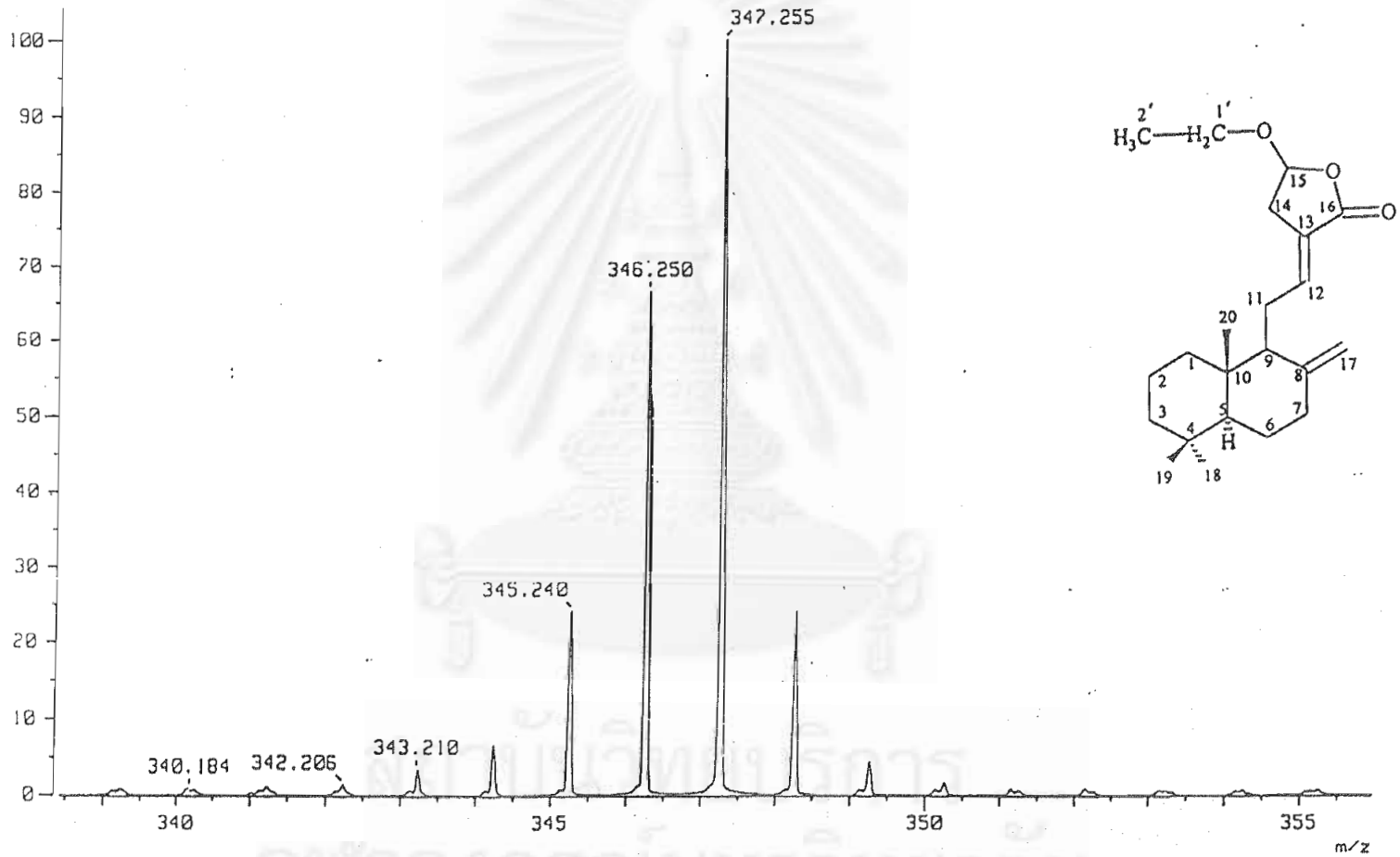


Figure 28 HRFAB mass spectrum of compound Mangga I (expanded)

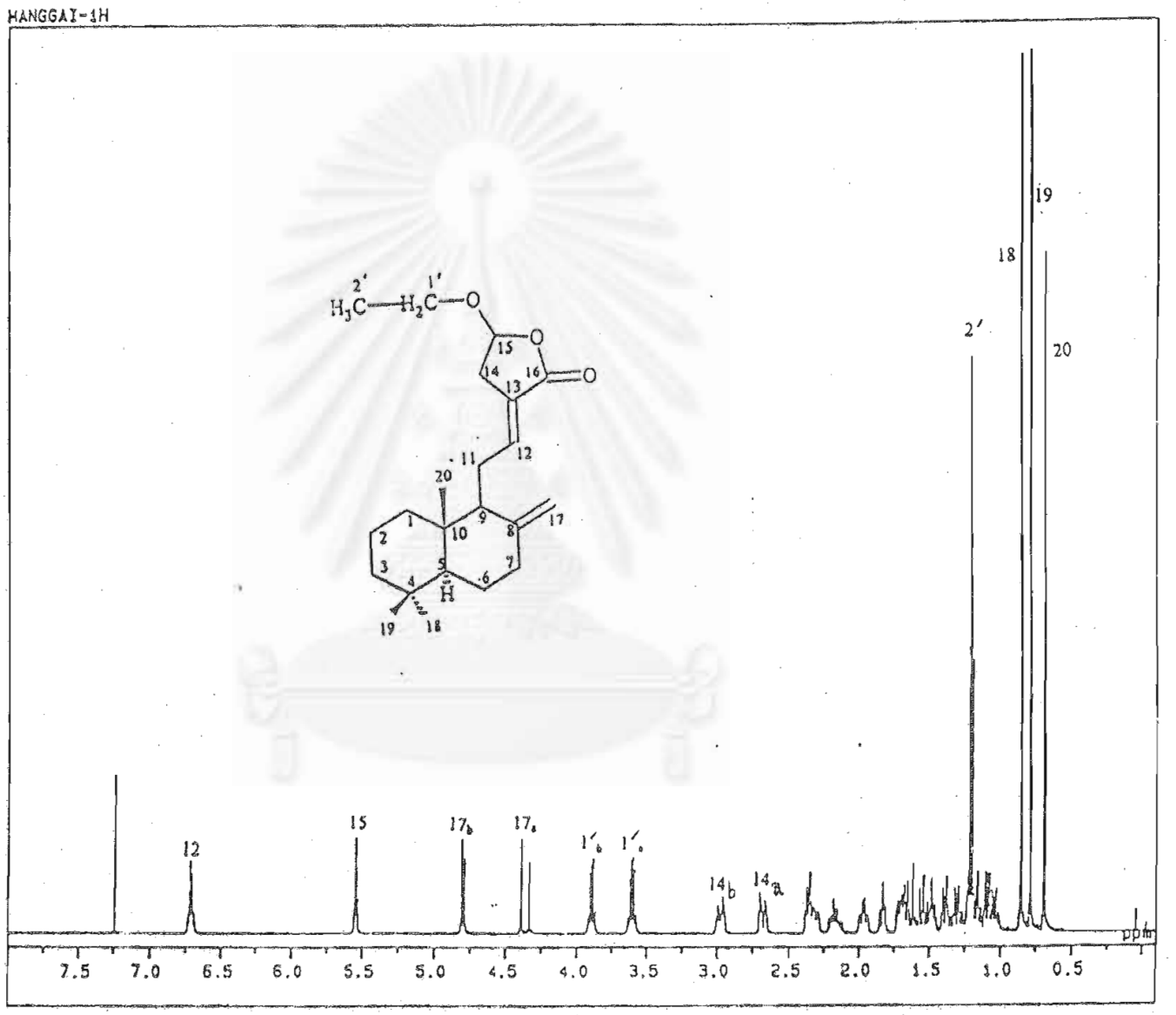
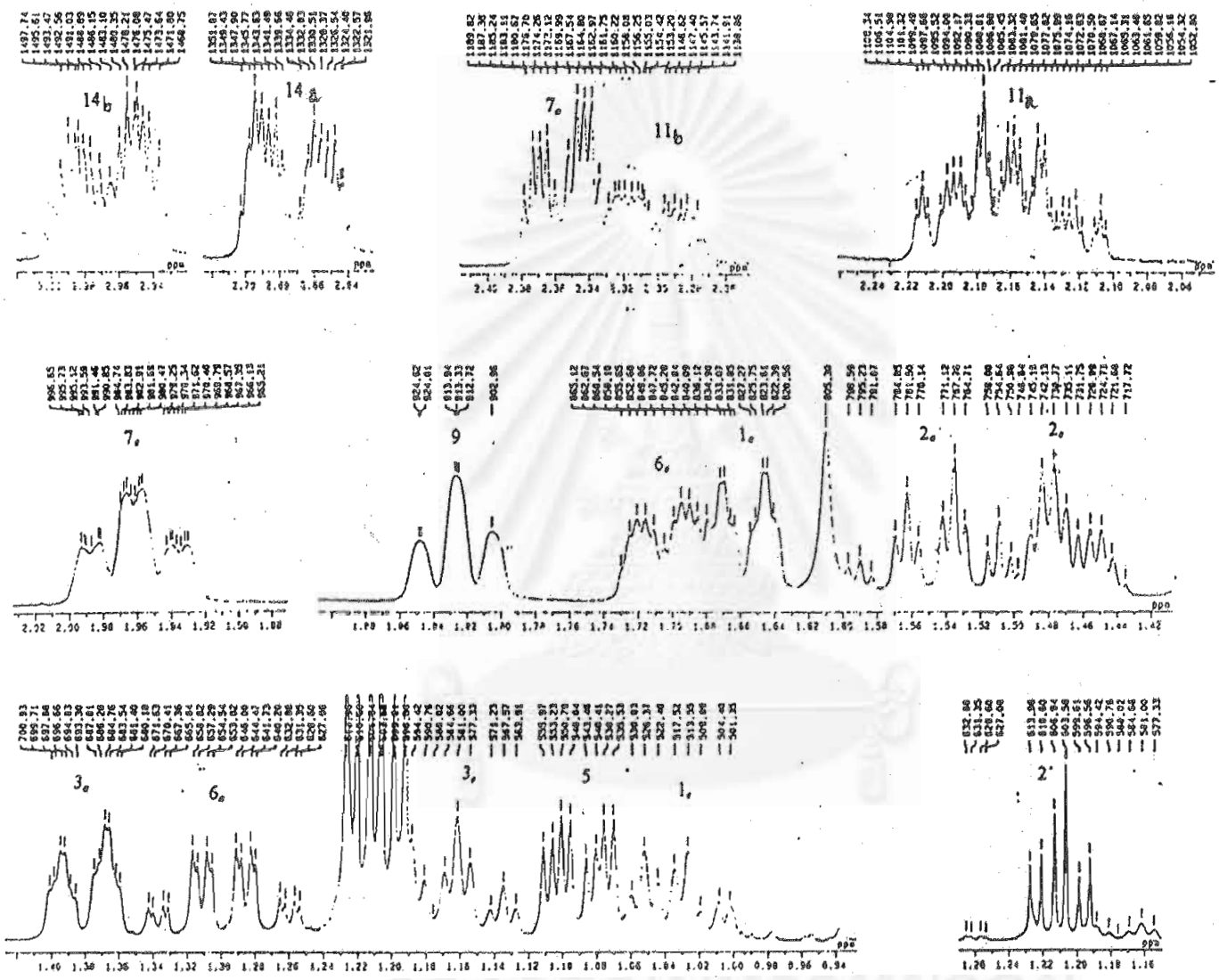


Figure 29 500 MHz ^{1}H NMR spectrum of compound Mangga I (in $CDCl_3$.)



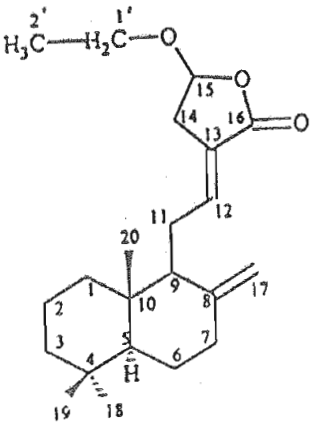


Figure 30 500 MHz ^{1}H NMR spectrum of compound Mangga I (in $CDCl_3$) (expanded)

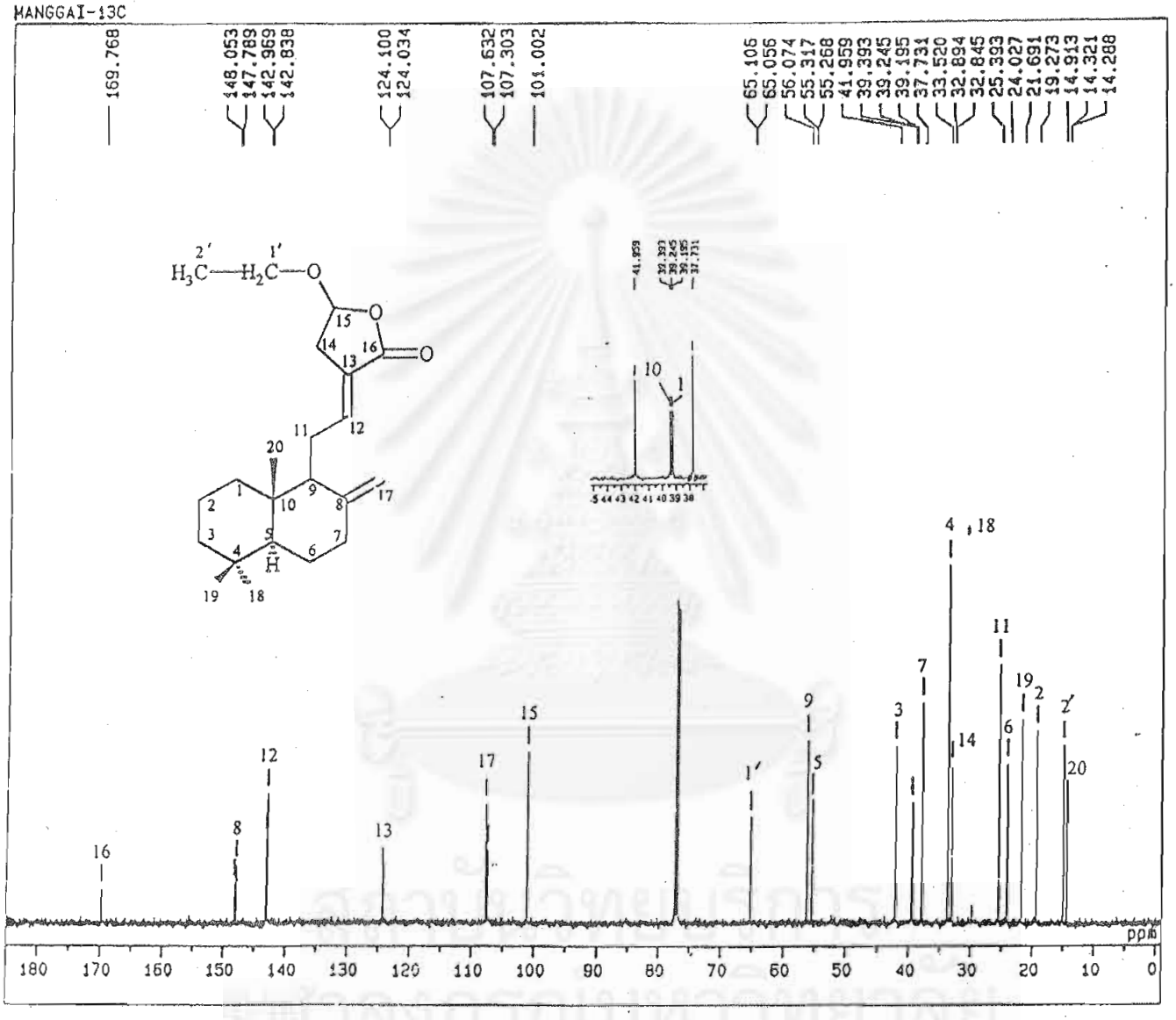
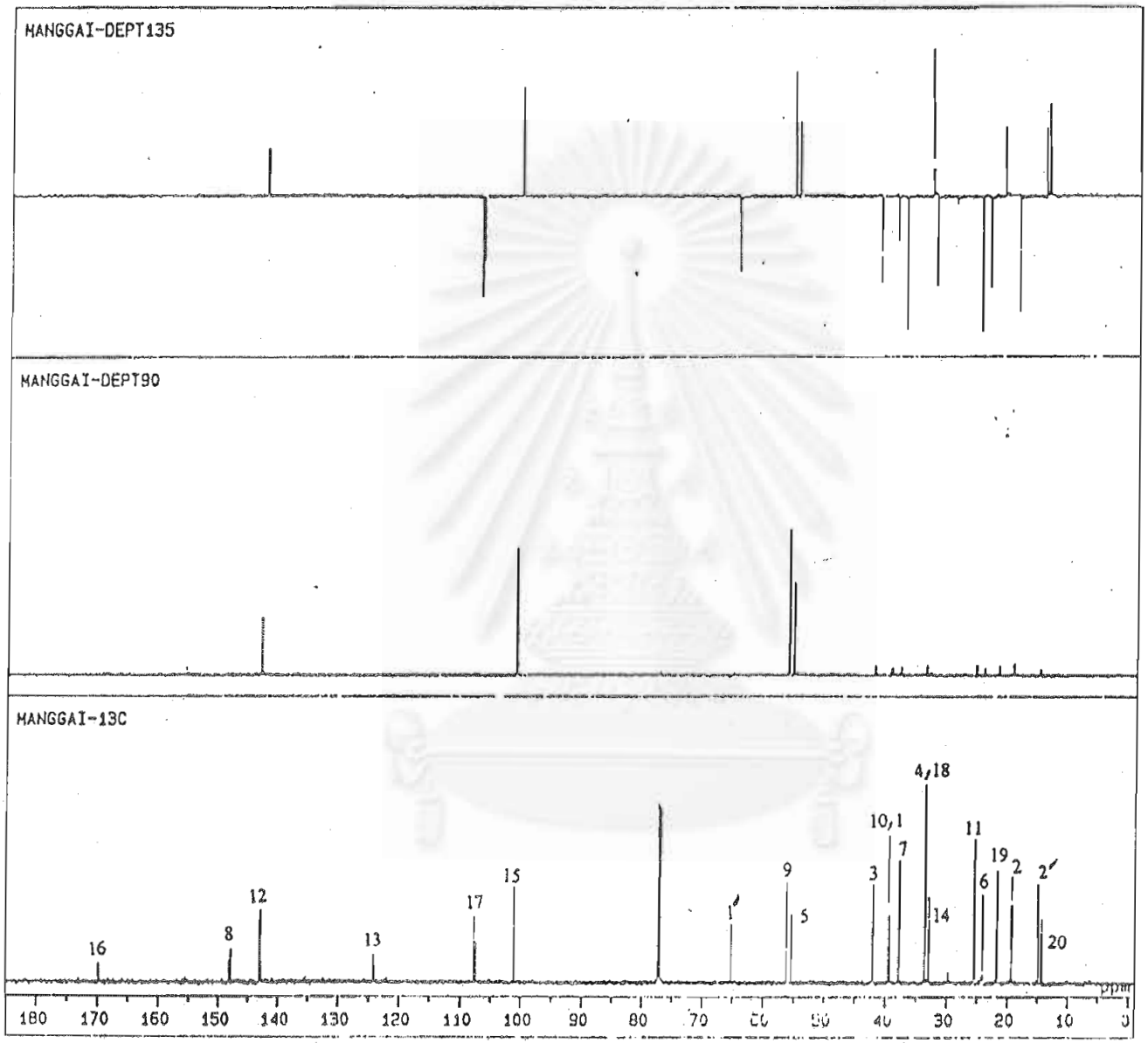


Figure 31 125 MHz ^{13}C NMR of compound Mangga I (in $CDCl_3$)



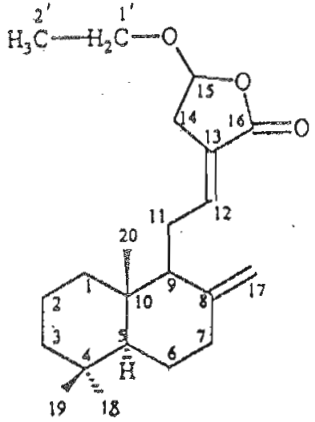
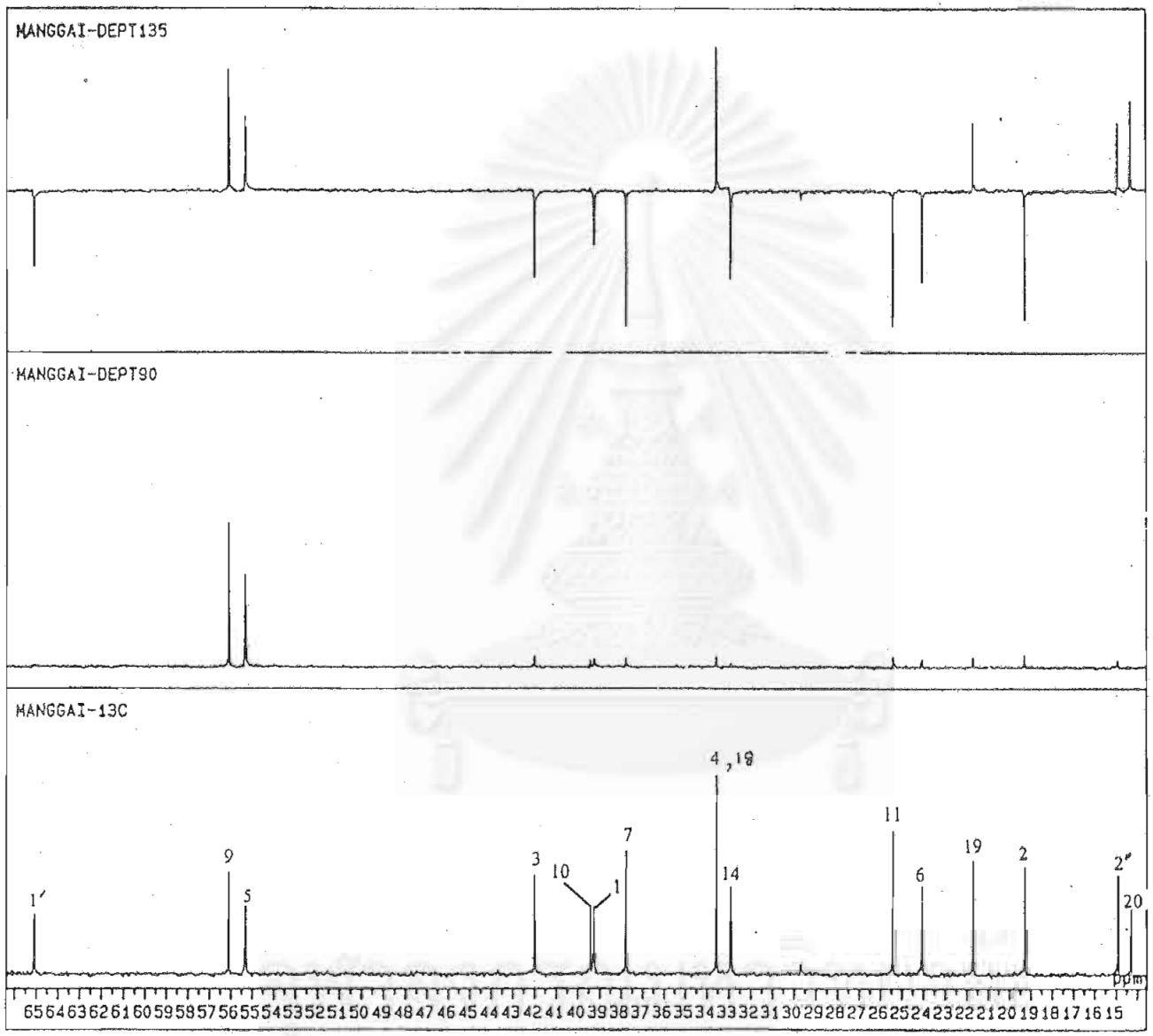


Figure 32 125 MHz ^{13}C NMR, DEPT 90 and DEPT 135 spectra of compound Mangga I (in $CDCl_3$)



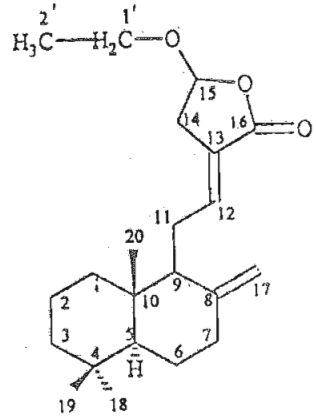
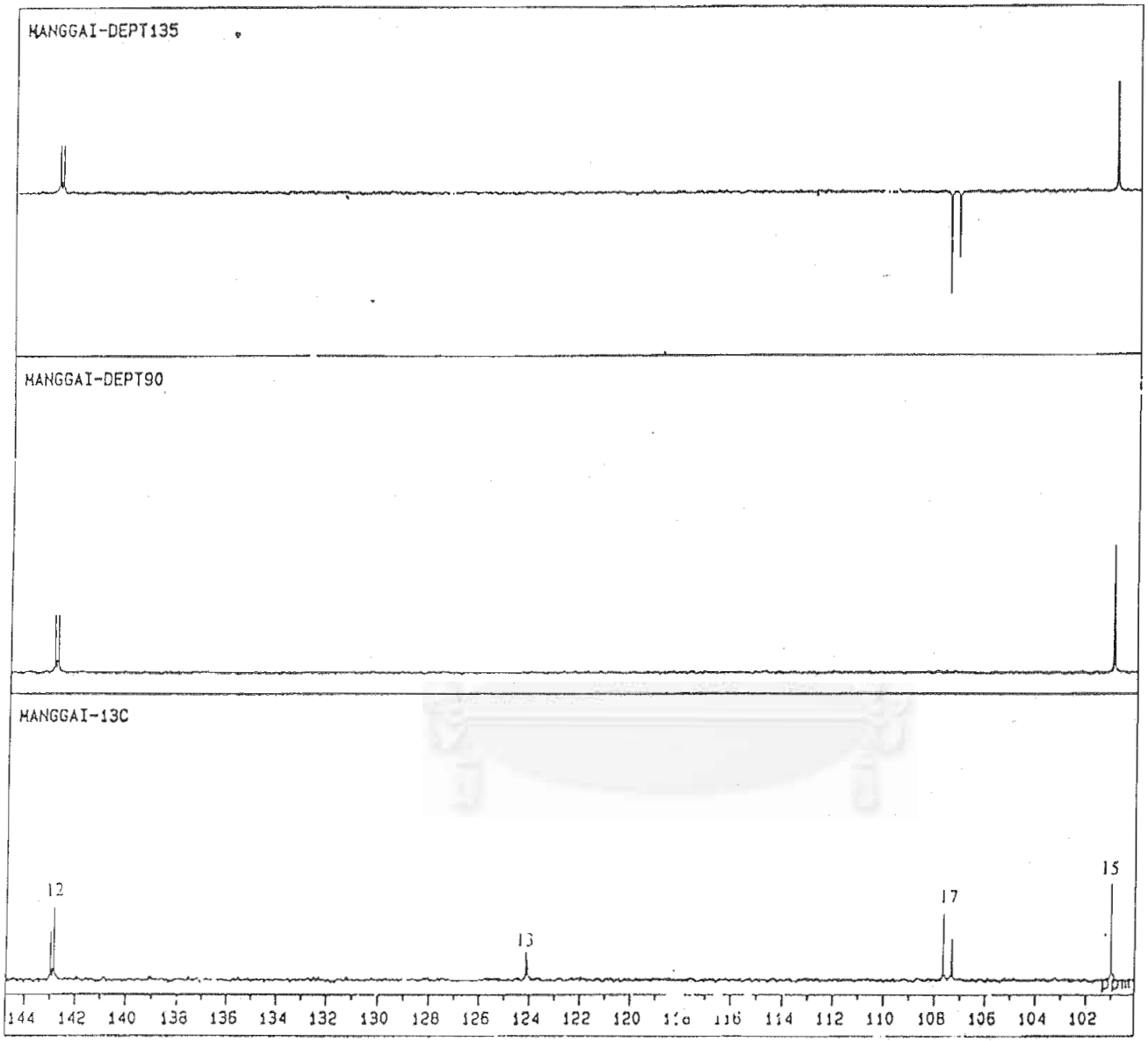


Figure 33 125 MHz ^{13}C NMR, DEPT 90 and DEPT 135 spectra (partially expanded: δ_C 14-66 ppm) of



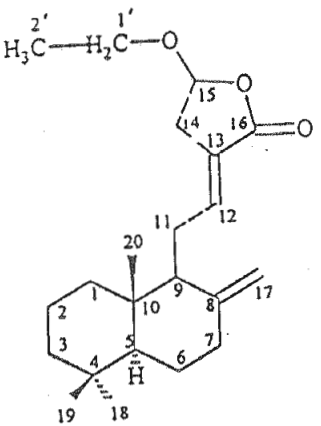
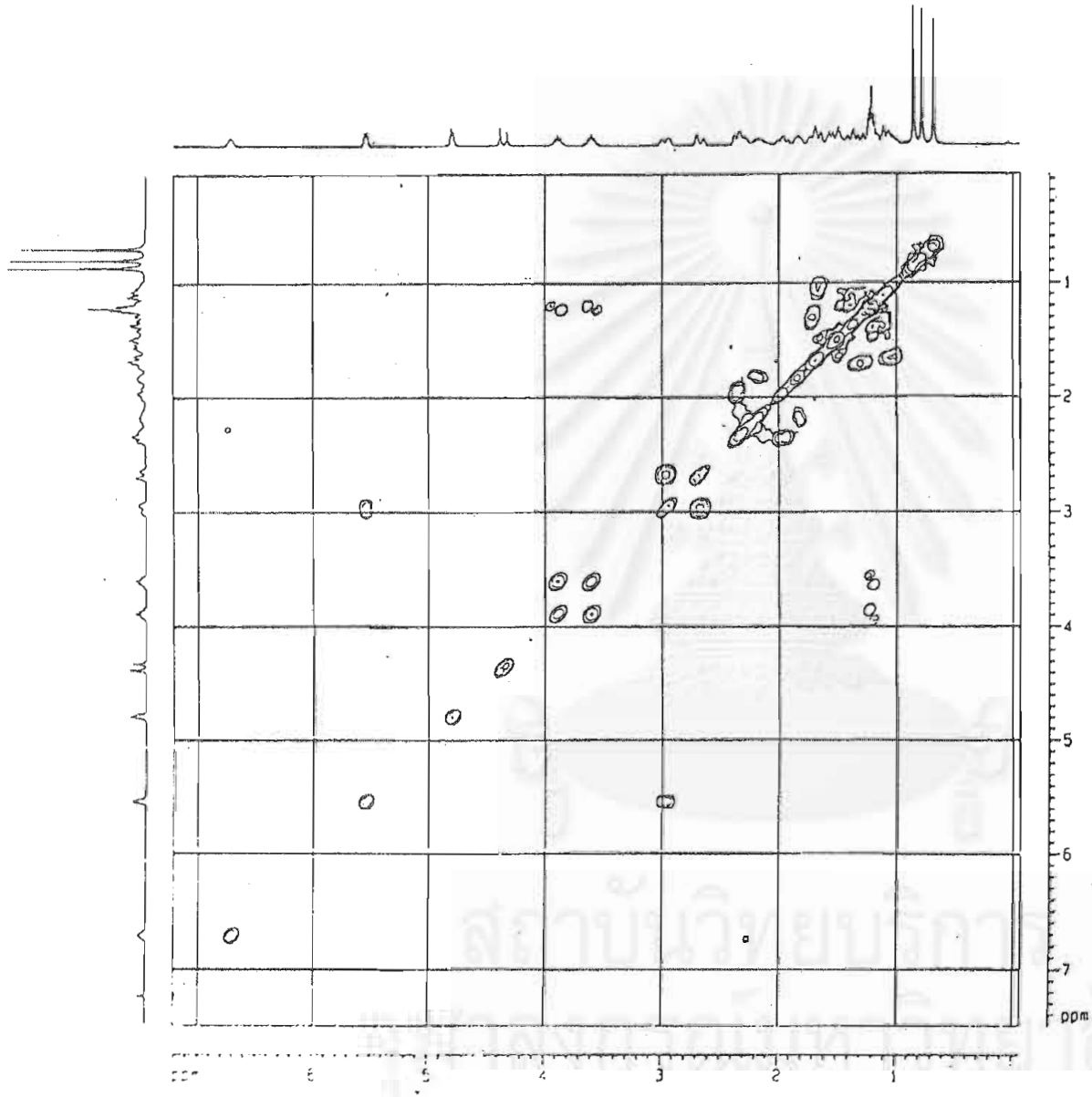


Figure 34 125 MHz ^{13}C NMR, DEPT 90 and DEPT 135 spectra (partially expanded: δ_C 102-143 ppm) of compound Mangga I (in $CDCl_3$)



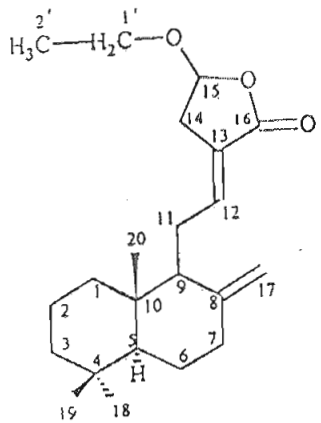
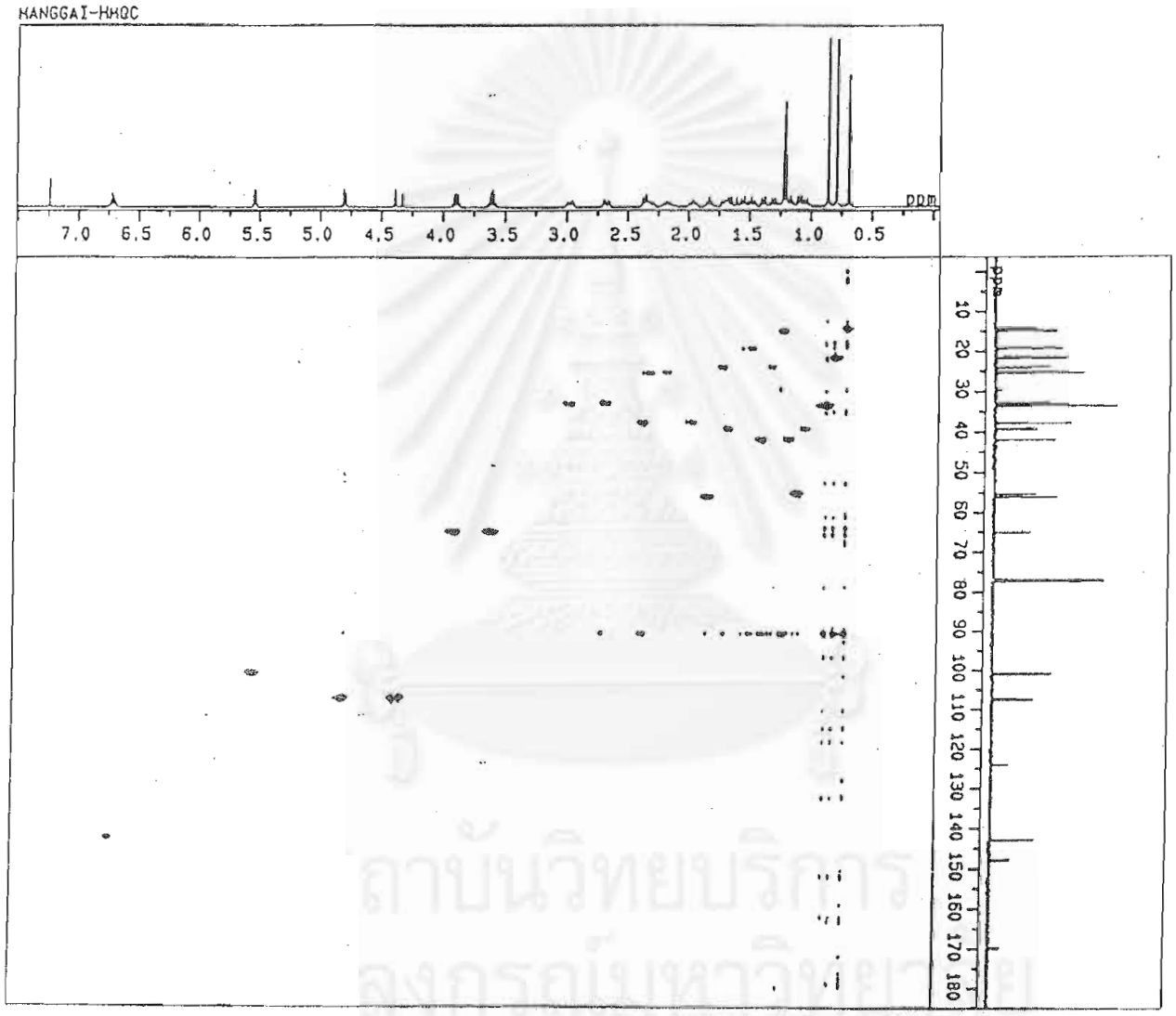


Figure 35 ^{1}H-^{1}H COSY spectrum of compound Mangga I (in $CDCl_3$)



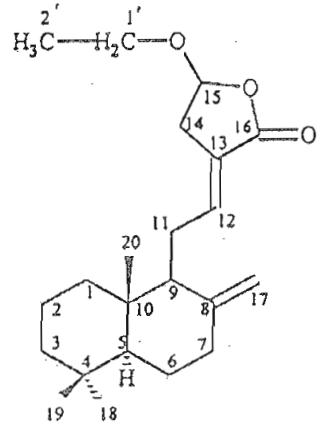


Figure 36 HMQC spectrum of compound Mangga I (in $CDCl_3$)

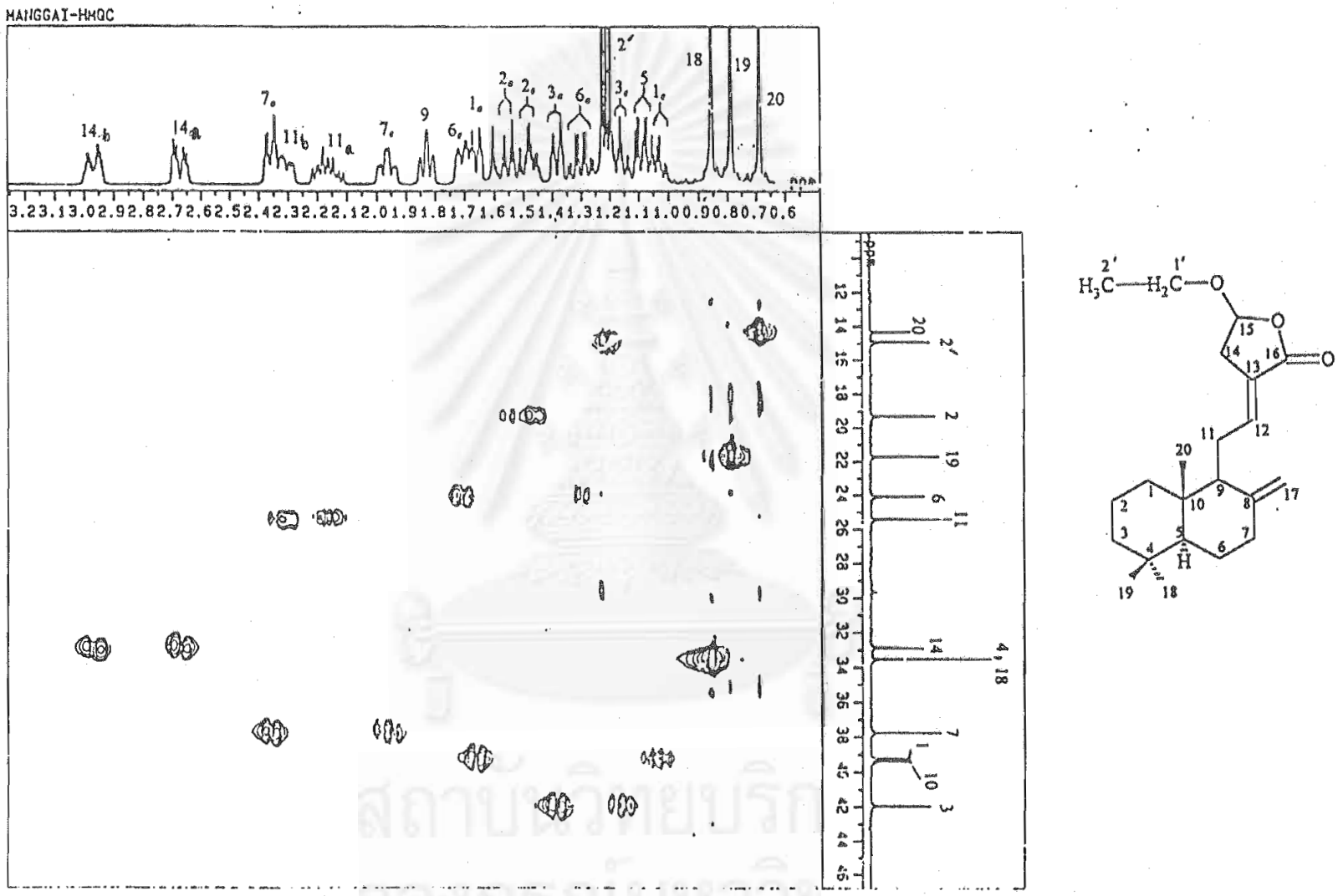
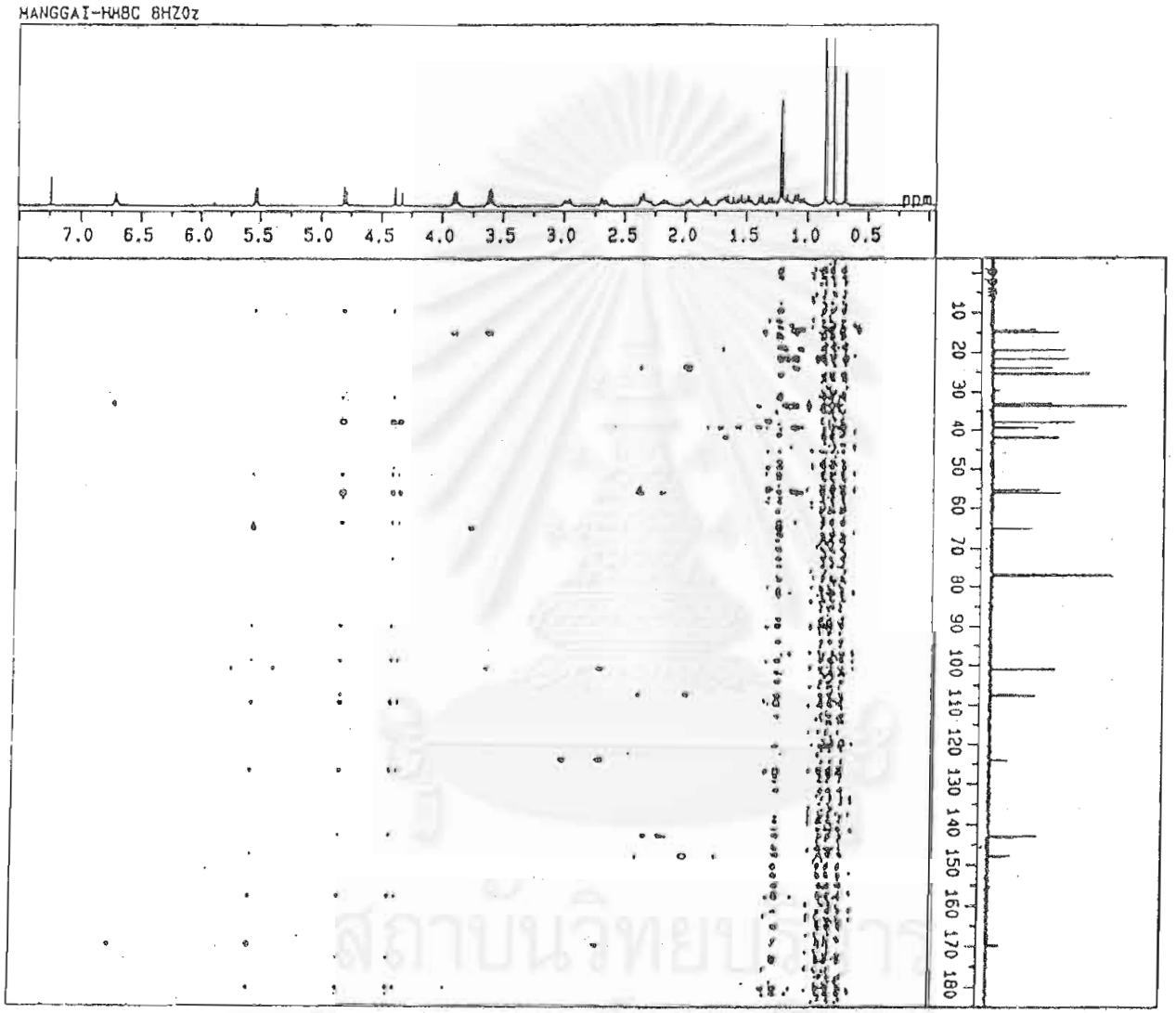


Figure 37 HMQC spectrum (partially expanded: δ_H 0.6-3.13 ppm, δ_C 14-45ppm) of compound Mangga I (in $CDCl_3$)



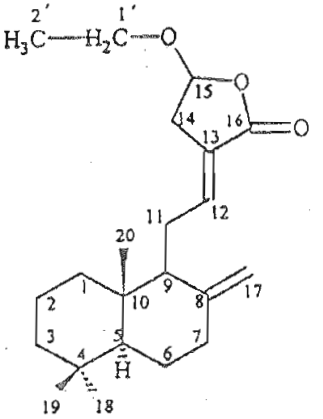


Figure 38 HMBC spectrum of compound Mangga I (in $CDCl_3$)

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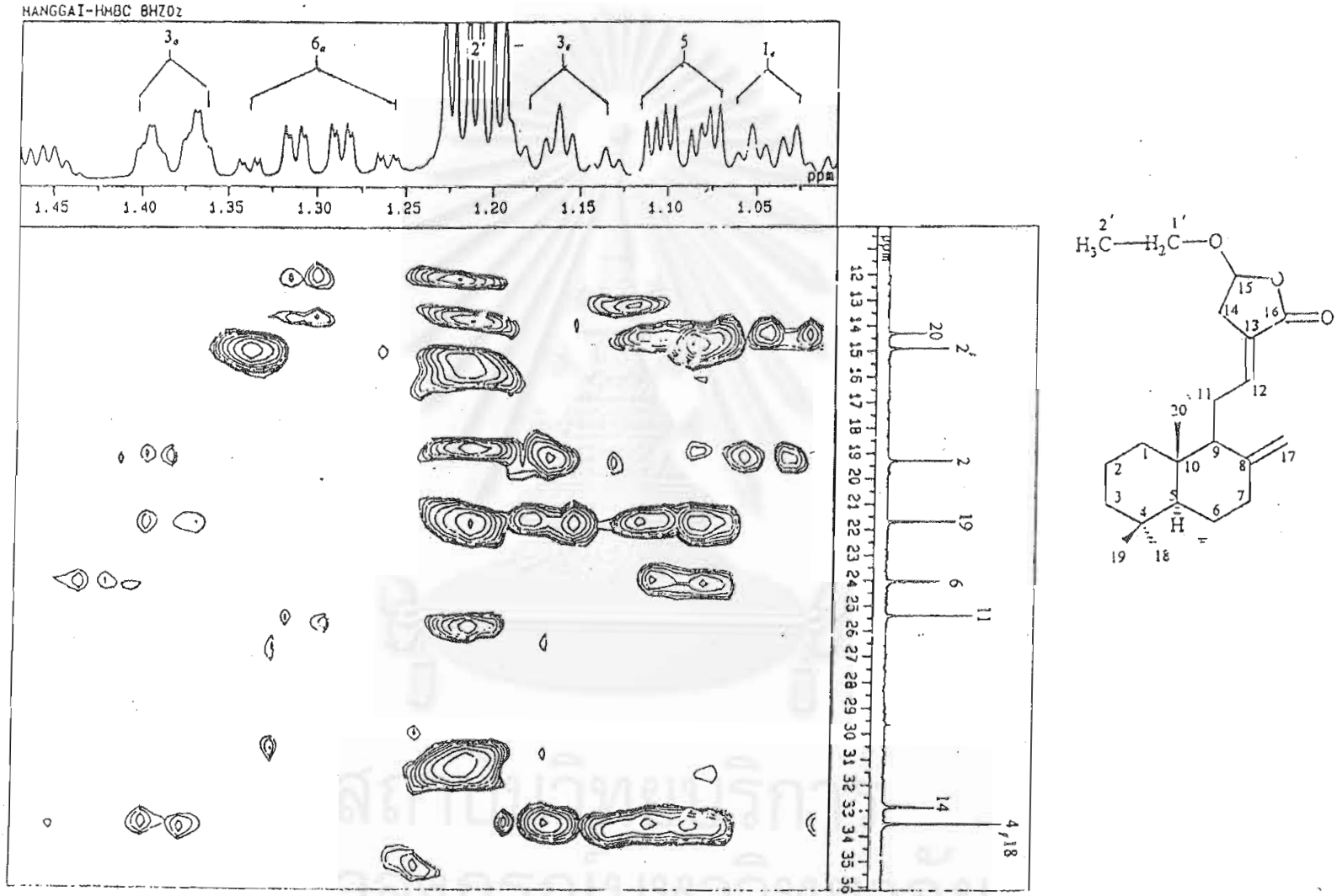


Figure 39 HMBC spectrum (partially expanded: δ_H 1.00-1.45 ppm, δ_C 14-34 ppm) of compound Mangga I (in $CDCl_3$)

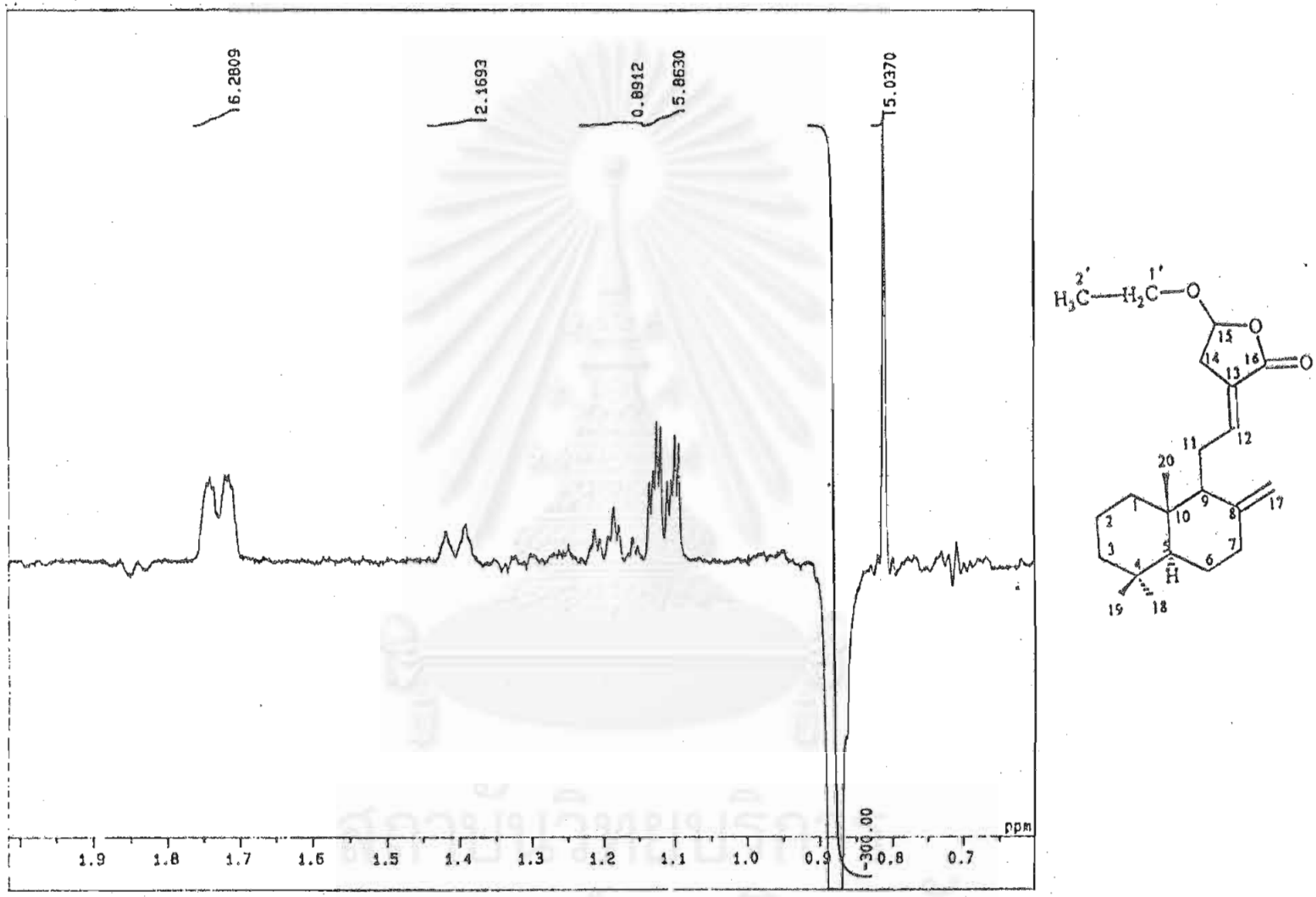
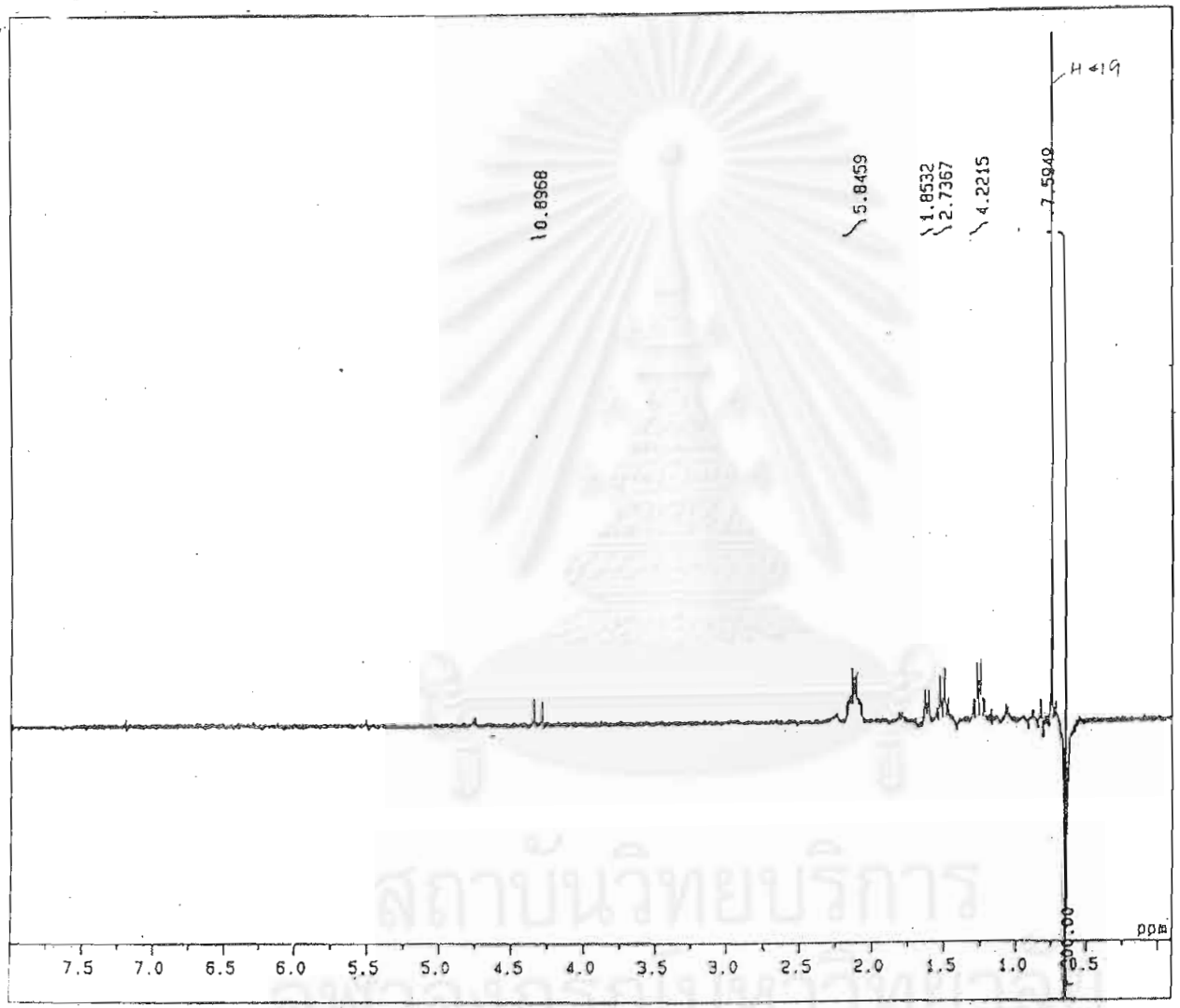


Figure 40 ^{1}H NOE difference spectra of compound Mangga I (in $CDCl_3$) (irradiated at δ_H 0.86 ppm)



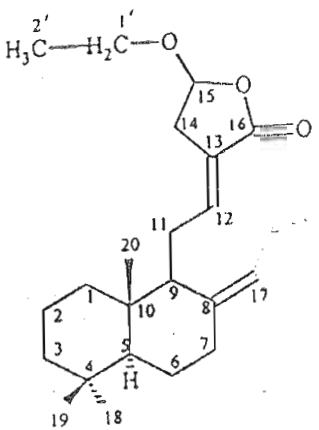
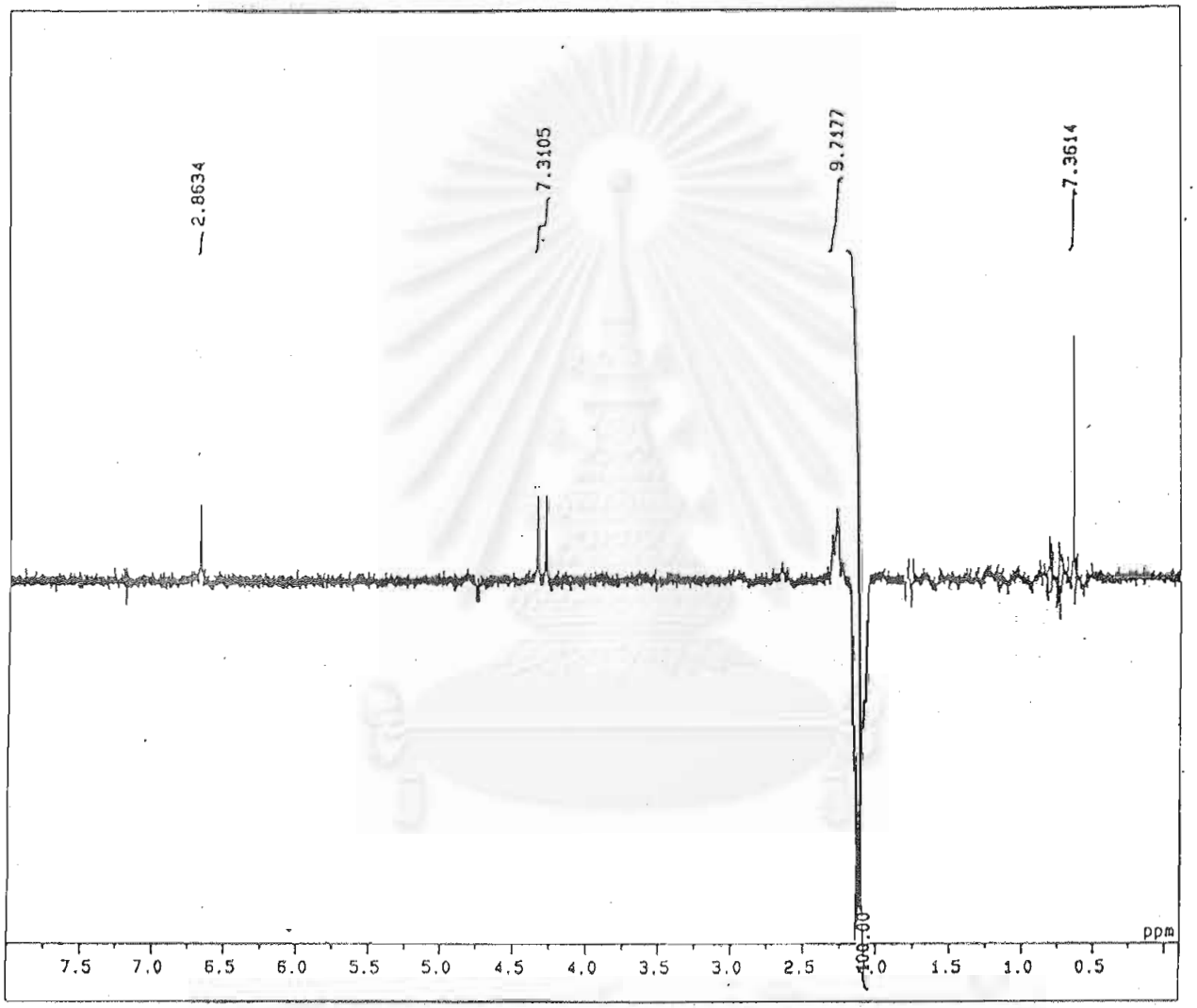


Figure 41 ^{1}H NOE difference spectra of compound Mangga I (in $CDCl_3$) irradiated at δ_H 0.69 ppm



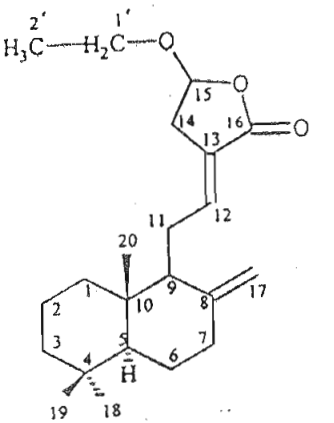
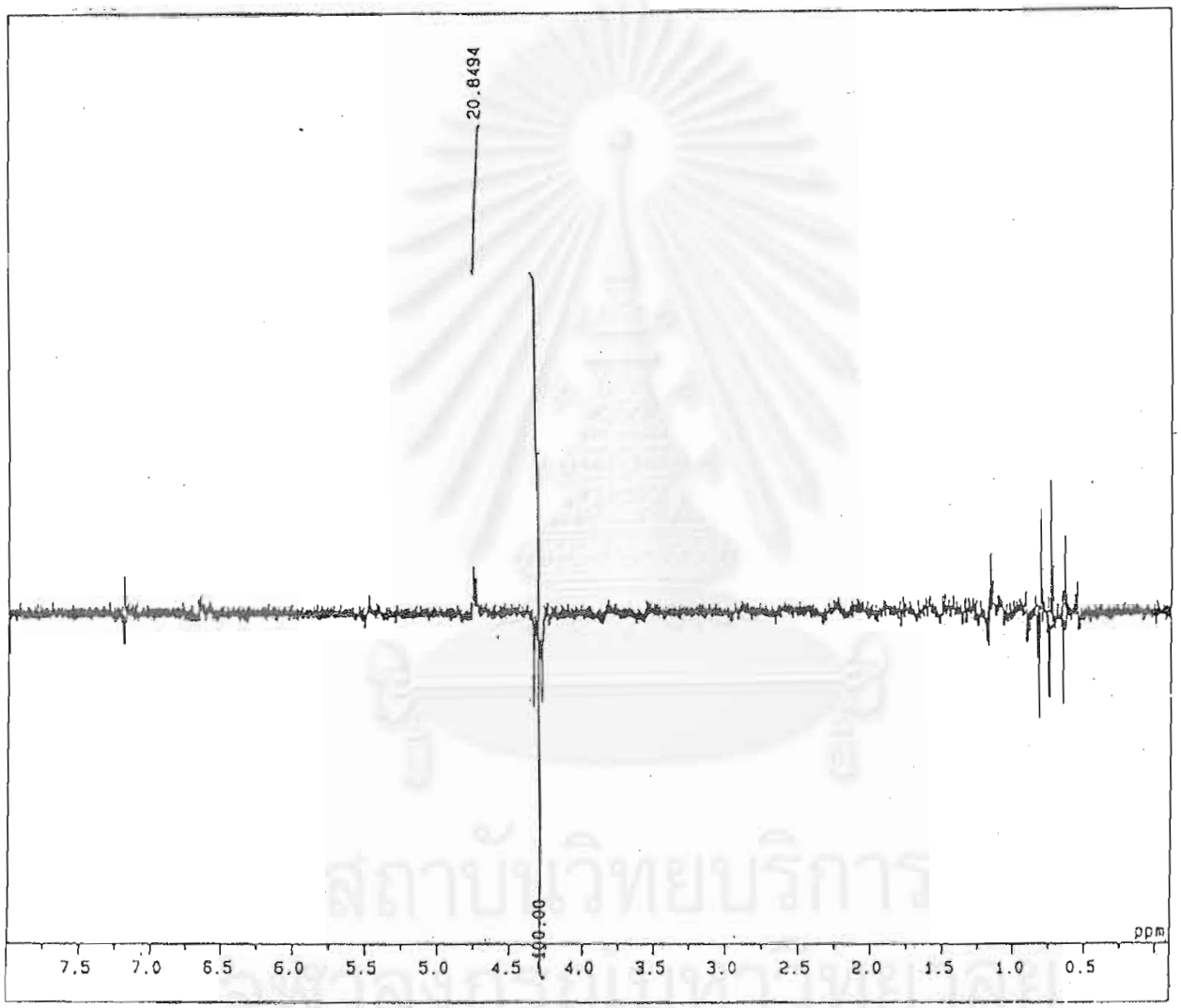


Figure 42 ^{1}H NOE difference spectrum of compound Mangga I (in $CDCl_3$) irradiated at δ_H 2.17 ppm



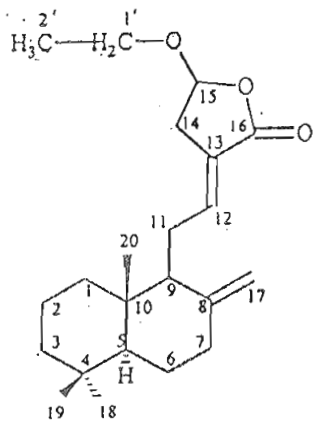
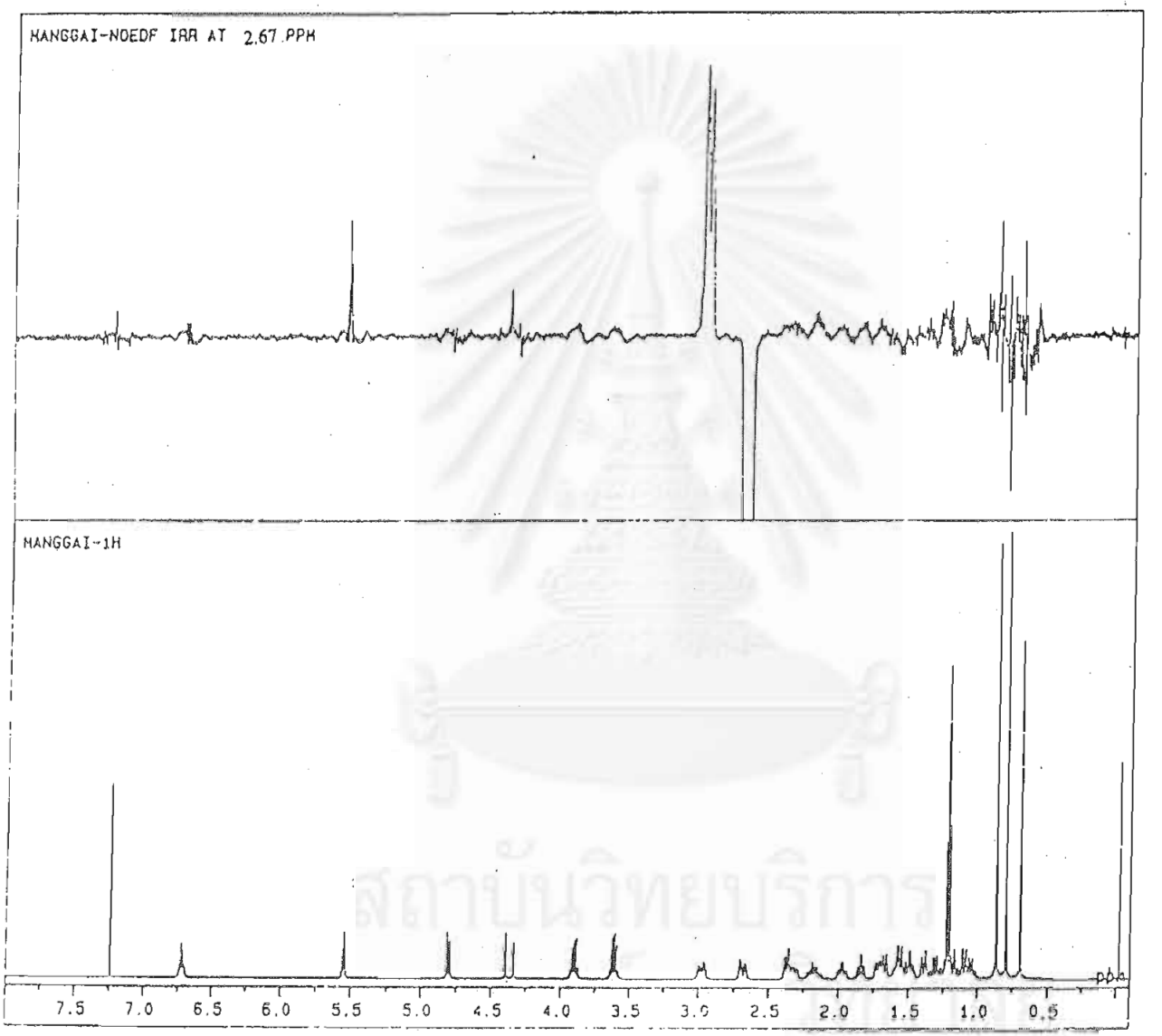


Figure 43 ^{1}H NOE difference spectrum of compound Mangga I (in $CDCl_3$) irradiated at δ_H 4.35 ppm



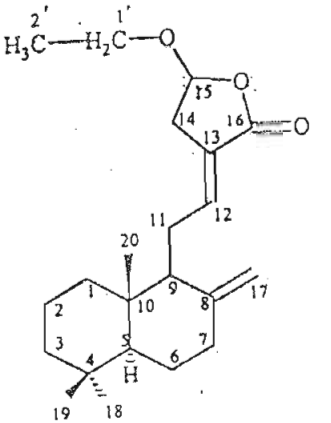
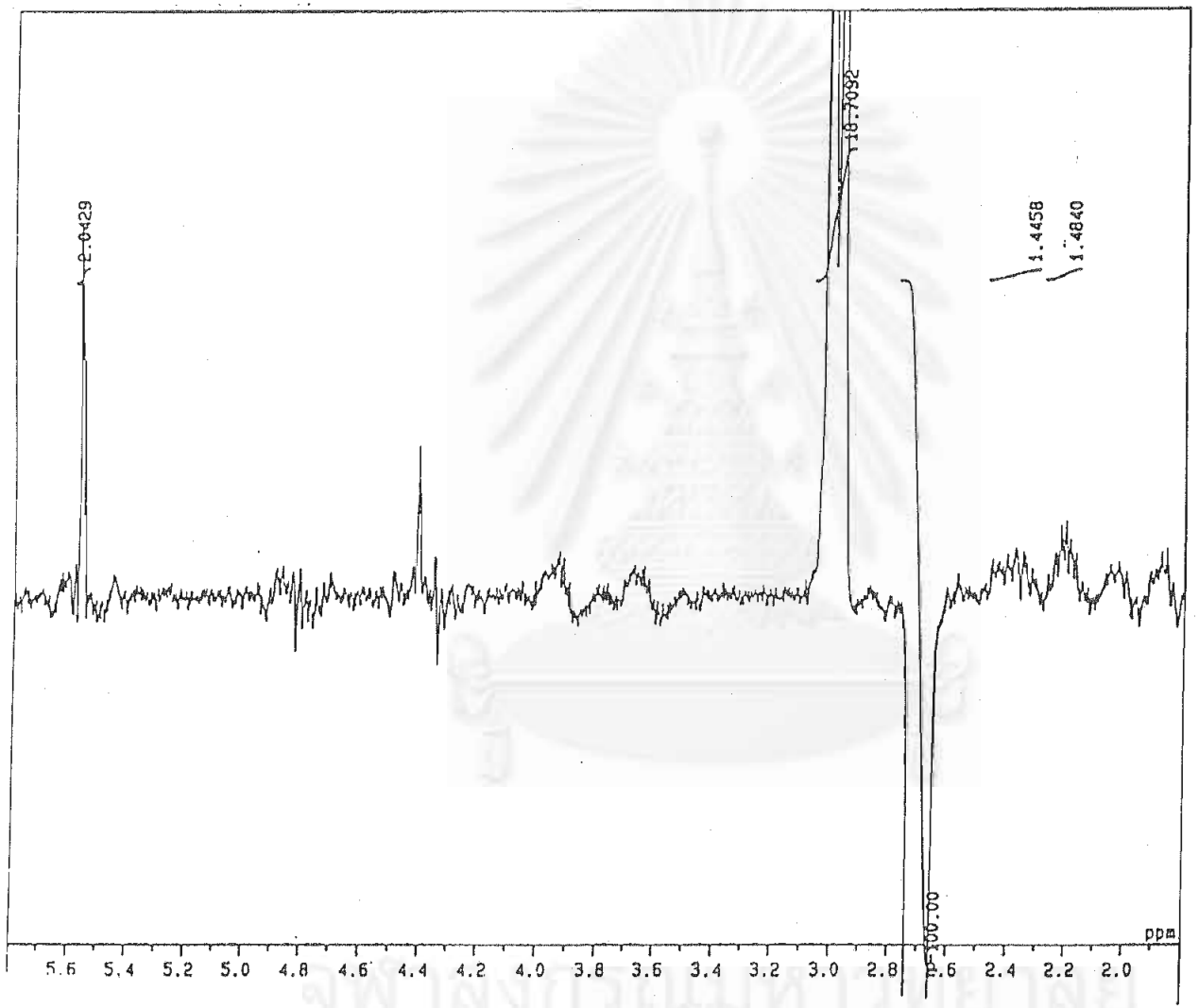


Figure 44 ^{1}H NOE difference spectrum of compound Mangga I (in $CDCl_3$) irradiated at δ_H 2.67 ppm



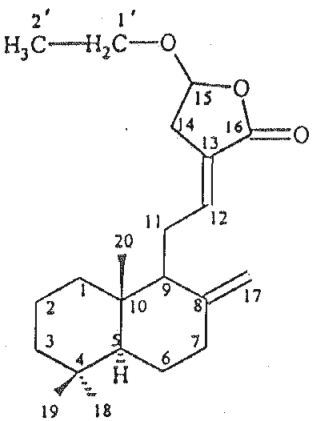
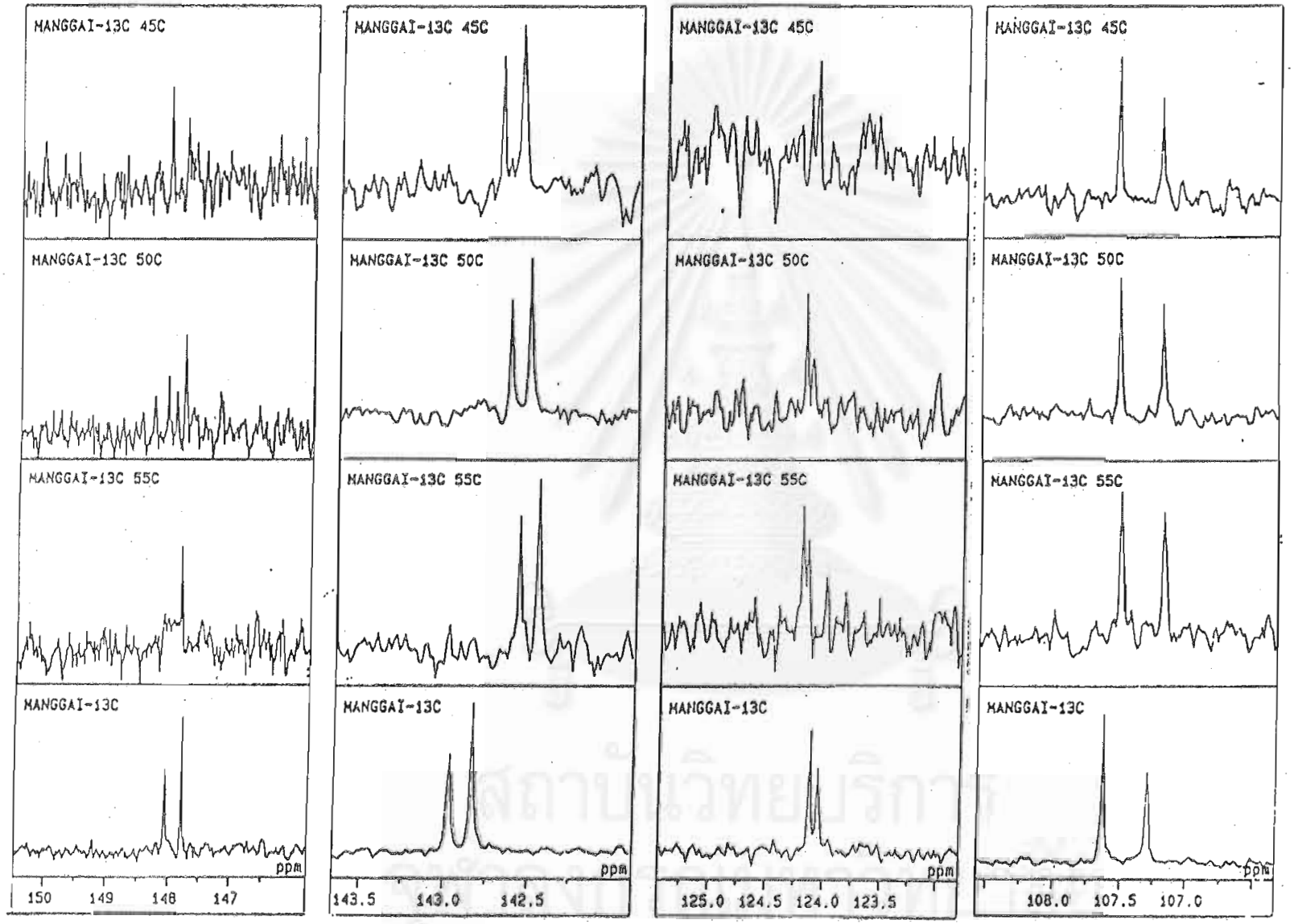


Figure 45 ^{1}H NOE difference spectra of compound Mangga I (in $CDCl_3$) irradiated at δ_H 2.67 ppm



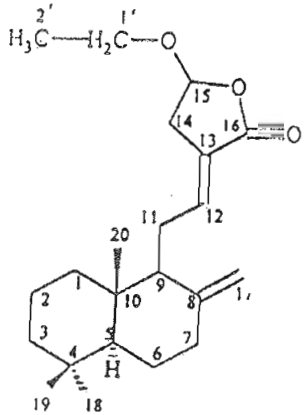
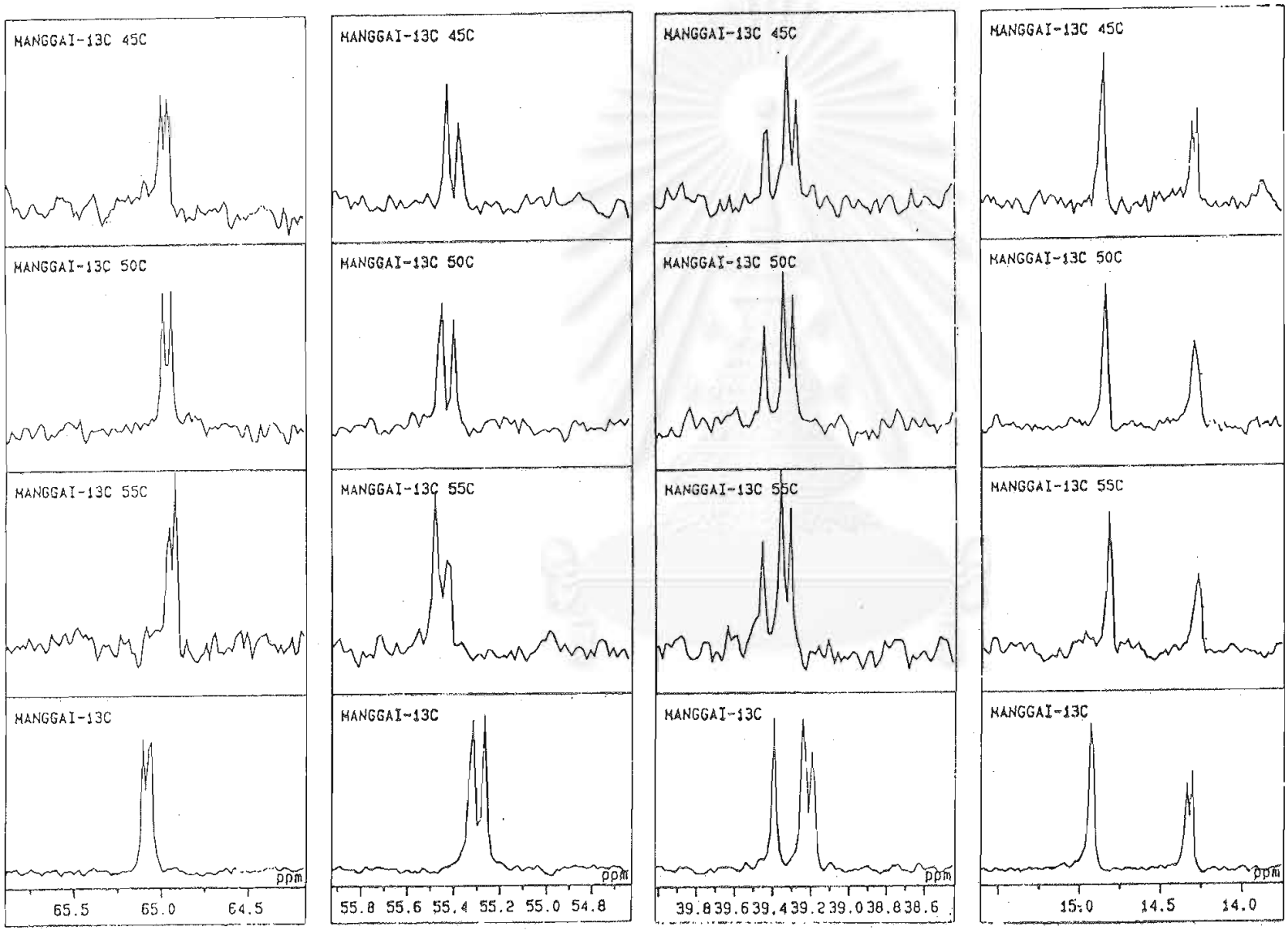


Figure 46a 125 MHz ^{13}C NMR at 45 °C, 50°C and 55 °C



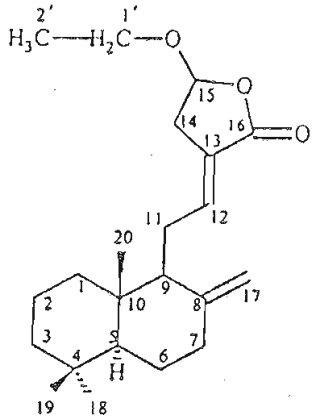
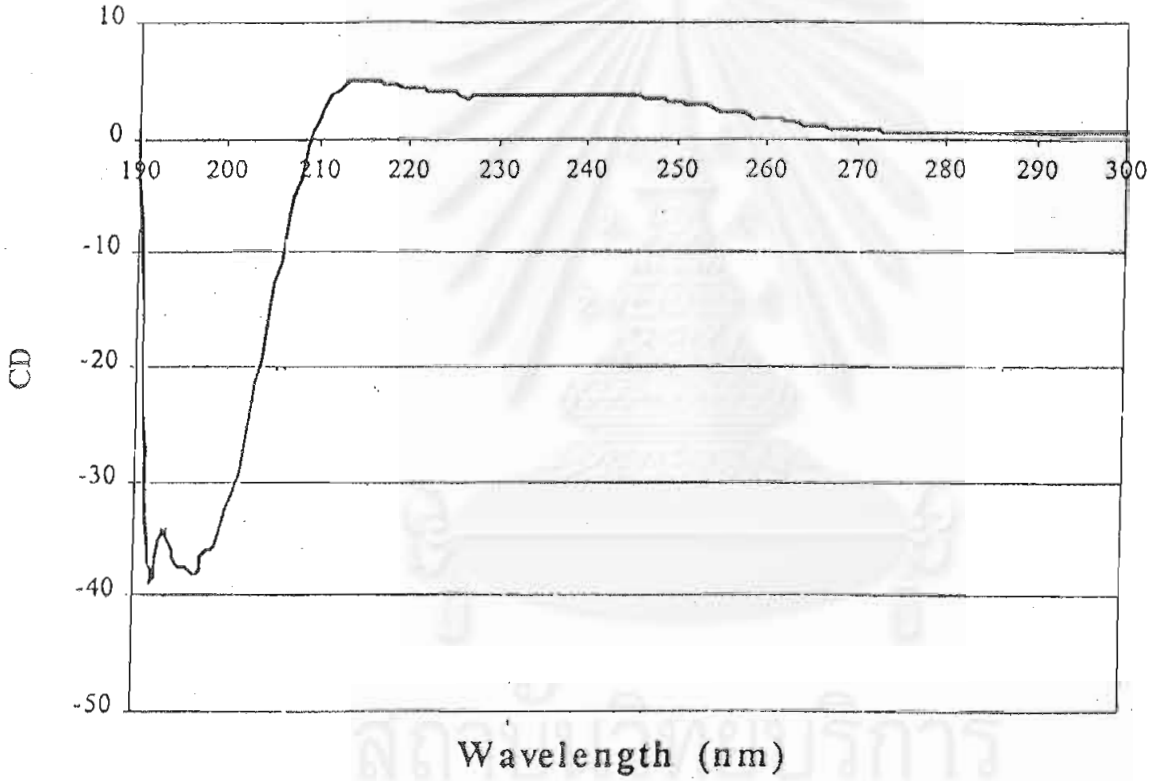


Figure 44b 125 MHz ^{13}C NMR at 45 °C, 50 °C and 55 °C



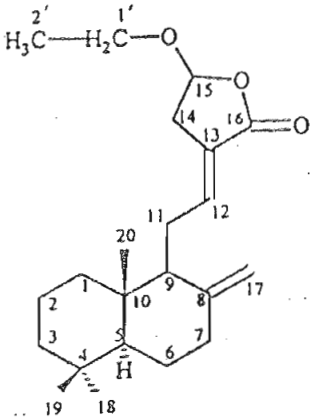


Figure 47 CD spectrum of compound Mangga I (in MeOH)

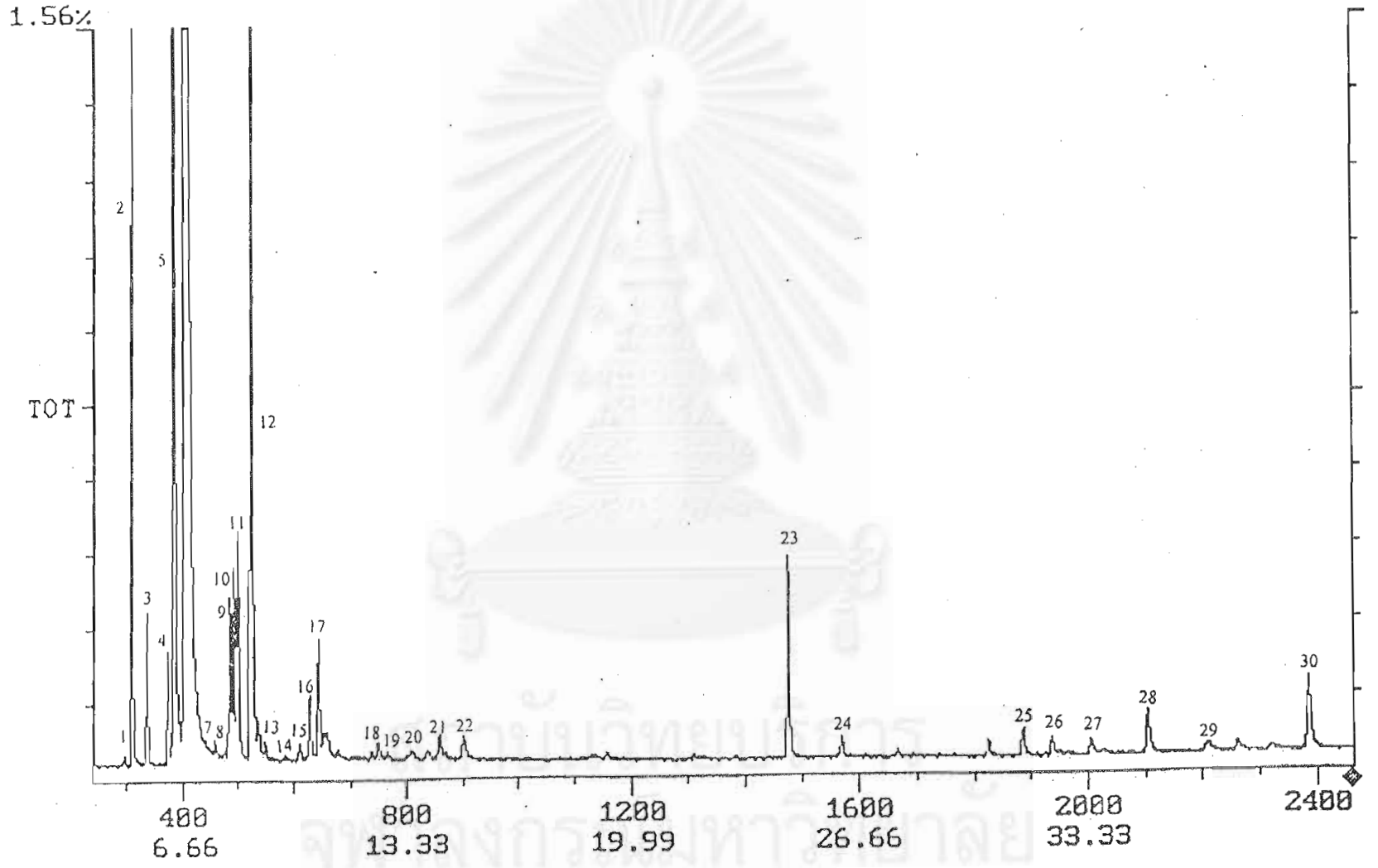


Figure 48 GC chromatogram of the essential oil from *Curcuma mangga* rhizome

VITA

Miss Kunlanat Makboon was born on 25 th June, 1972 in Nakhonsawan, Thailand. She received her bachelor's degree of Science in Pharmacy in 1995 from the Faculty of Pharmacy, Mahidol University, Bangkok, Thailand. At present, she is a pharmacist at Singburi Hospital, Singburi, Thailand.

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