สารออกฤทธิ์ทางชีวภาพของหญ้าค้อนกลอง *Sphaeranthus africanus* Linn. ตอนที่ 2

นางสาว พรพรรณ์ สุขปัญญาเลิศ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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BIOACTIVE COMPOUNDS OF *Sphaeranthus africanus* Linn. PART II

Miss Pornpan Sukpanyalert

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science in Chemistry

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By	Miss Pornpan Sukpanyalert
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……Dean of Faculty of Science
(Associate Professor Wanchai Phothiphichitr, Ph.D.)

Thesis committee

…….Chairman
(Professor Padet Sidisunthorn, Ph.D.)

……..Thesis Advisor
(Professor Udom Kokpol, Ph.D.)

……..Member
(Assistant Professor Warinthorn Chavasiri, Ph.D.)

……...Member
(Assistant Professor Preecha Lertpratchya, Ph.D)

พรพรรณ์ สุขปัญญาเลิศ:สารออกฤทธิ์ทางชีวภาพของหญ้าค้อนกลอง *Sphaeranthus africanus* Linn. ตอนที่2 (BIOACTIVE COMPOUNDS OF *Sphaeranthus africanus* Linn. Part II), อาจารย์ที่ปรึกษา ศ.ดร. อุดม ก๊กผล; 95 หน้า; ISBN 974-665-314-8

สิ่งสกัดเอทานอลของหญ้าค้อนกลองแสดงความเป็นพิษต่อเซลล์มะเร็งและให้ผลบวกต่อการทดสอบฤทธิ์การต้านอนุมูลอิสระ ในงานวิจัยนี้สามารถแยกสารได้ 7 ชนิด ได้แก่ stigmasterol, chrysophenol D, 3,7-dimethoxy-4′,5,6-trihydroxyflavone, chrysophenol C, 3α,5β-diangeloxoyloxy-7-hydroxycarvotanacetone, 1-angeloxoyloxy-3-[4′-angeloxoyloxy-3′-methoxy]-2-propene และ 1-angeloxoyloxy-3-[4′-isopentanoloxoxoyloxy-3′-methoxy]-2-propene สารประกอบชนิดสุดท้ายเป็นสารใหม่ที่ยังไม่เคยมีผู้รายงานมาก่อน ฟลาโวนอยด์ที่แยกได้ทั้งหมด คือ chrysophenol D, 3,7-dimethoxy-4′,5,6-trihydroxyflavone, chrysophenolC และ 3α,5β-diangeloxoyloxy-7-hydroxy carvotanacetone แสดงฤทธิ์ต้านอนุมูลอิสระ DPPH นอกจากนี้ 3α,5β-diangeloxoyloxy-7-hydroxy carvotanacetone และ chrysophenol D ยังแสดงความเป็นพิษอย่างจำเพาะเจาะจงกับต่อเซลล์มะเร็งกล่องเสียงอีกด้วย

ภาควิชา.....เคมี.....ลายมือชื่อนิสิต.....นางสาวพรพรรณ์ สุขปัญญาเลิศ.....

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PORNPAN SUKPANYALERT : BIOACTIVE COMPOUNDS OF *Sphaeranthus africanus* Linn. PART II
ADVISOR : PROFESSOR UDOM KOKPOL,Ph.D. 95 pp.
ISBN 974-665-314-8

The ethanolic crude extract of the whole plant of *Sphaeranthus africanus* Linn. exhibited cytotoxicity against various carcinoma cell lines and gave positive results for free radical scavenging activity test. Seven compounds were isolated, namely stigmasterol, chrysophenol D, 3,7-dimethoxy-4′,5,6-trihydroxyflavone, chrysophenol C, 3α,5β-diangeloxoyloxy-7-hydroxycarvotanacetone**,**1-angeloxoyloxy -3-[4′- angeloxoyloxy-3′-methoxy]-2-propene and 1-angeloxoyloxy-3-[4′-isopentano loxoyloxy-3′-methoxy]-2-propene. The last compound was found to be a new compound. All isolated flavonoids (chrysophenol D, 3,7-dimethoxy-4′,5,6-trihydroxy flavone, chrysophenol C) and 3α,5β-Diangeloxoyloxy-7-hydroxy carvotanacetone showed free radical scavenging activity against DPPH radical. 3α,5β-diangeloxo yloxy-7-hydroxy carvotanacetone and chrysophenol D also exhibited specifically cytotoxicity against KB cell.

Department…….Chemistry…….Student's signature…Miss Pornpan Sukpanyalert….
Field of study…….Chemistry… Advisor's signature….Prof. Dr. Udom Kokpol……...
Academic year……..2000……Co-Advisor's signature……...

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Special thank is given to her best friends and other foreign students in University of the Ryukyus for their friendship, comment, helpfulness, and cheerfulness.

Finally, the author would like to thank her parents and family in encouraging her to withstand ephemeral burden and despair.

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List of Abbreviations

br	broad
°C	degree Celsius
ca	calculation
cm^{-1}	unit of wavenumber
$CDCl_3$	deuterated chloroform
CH_2Cl_2	dichloromethane
dd	doublet of doublet
d	doublet (NMR)
DPPH	diphenyl picryl hydrazyl radical
EtOAc	ethyl acetate
Fig.	figure
g	gram
Hept	heptane
Hex	hexane
HPLC	high performance liquid chromatography
Hz	hertz
IR	infrared
J	coupling constant
kg	kilogram
LC_{50}	concentration of that 50% lethality
mg	milligram (s)
m	multiplet (NMR)
m	medium (IR)
mL	milliliter (s)
m.p.	melting point
MW	molecular weight
MeOH	methanol
NMR	nuclear magnetic resonance
ppm	part per million
q	quartet (NMR)
R_f	retardation factor

R_t	retention time
s	singlet (NMR)
s	strong (IR)
t	triplet (NMR)
td	triplet of doublet (NMR)
w	weak (IR)
w/w	weight by weight
δ	chemical shift
%	percent
μg	microgram (s)
μL	micro liter (s)

CHAPTER I
Introduction

Thailand is located in the tropical region of the world and agriculture is the major economic. Farmers have been continuously facing many problems such as climate, harmful insects, and weeds. Especially weed can make huge damage to agriculture for instance, diminishing quality and quantity of crops, blocking dam, and irrigation system, causing forest fire, making main plant die, including water pollution and in some case, irritating human or animals. In this sense, weeds are always the targets to be controlled. However, our investigation in Thailand revealed that many weed species have been utilized by indigenous people in various ways; as food, medicinal plants, animal feed, housing materials, materials for handicrafts and ornaments, agricultural use, and so on.

At the present time, there are many reports to affirm that some weeds constituted substances, which affected the growth of side-growing plants or other creatures. *Sphaeranthus indicus* Linn. contained 7-hydroxyfrullanolide, which can use for the antimicrobial agent and also this kind of weed has long been used in indigenous medicine in the treatment of septic gastric disorders.[1]

The popularity of utilizing weeds might be due to their fewer side effects, reasonable cost and greater availability. In addition, consuming local medicinal plants can reduce the import of synthetic drugs from foreign countries. However, the importance of the plants not only uses as food or medicine and also uses in agriculture and cosmetic industry as well.

Following the above mentioned outcome, natural product chemistry is one of the approaches to search for chemical constituents from natural materials and involves seeking their activities. The researchers who attended to natural product have the advantage in providing the basic information about the previous native people utilized, chemical constituents and bioactivities of the substances, which may have the wide utilization.

In term of natural product chemistry investigation, this research is focused on searching for bioactive compounds from the tropical weed. *Sphaeranthus africanus* Linn. was selected to study systematically.

S. africanus belonging to Sphaeranthus genus (Family Compositae)[2] wide spread in the tropical and subtropical areas of Africa, Asia, and Australia.[3] Among them about 40 species have been chemically studied.[4] Some species of this genus are widely used as a folk medicines for the treatment of skin infection, glandular swelling bronchitis, jaundice, and nervous depression, etc.[1]

In Thailand, two species, *S. africanus* Linn. (Yah khon klong) and *S. indicus* Linn. (Ka ra boon), are commonly found.[4] The former was selected for this research to investigate for bioactive compounds according to a promising preliminary screening test of ethanolic extract which revealed selective cytotoxicity against KB cell lines.

S. africanus is abundant in rice field, particularly in the central region of Thailand. Its life cycle is about 1 year. The botanical aspects of this plant can be summarized as follows.[5]

Stem: The height is 15-45 cm. The upper part is greenish and the lower parts are yellowish. The stem and branches are fully covered with hairs.

Leaves: The leaves are oval shaped, about 0.5-3.5 cm wide and 2-11 cm long.

Inflorescence: It is small, violet, and typically bisexual flowers.

Seed: Dried seed is brown and cylinder-shaped about 1 mm long.

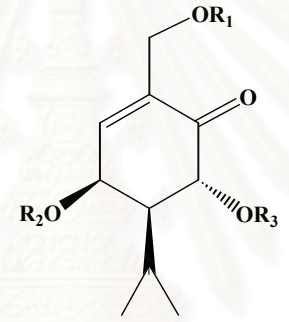
Figure 1.1 (1) habit, (2) the whole plant, (3) inflorescence, (4) seed of *S. africanus*

1.1 Chemical Constituents of *Sphaeranthus* Genus

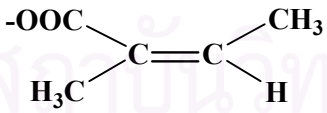
Among 40 species of genus *Sphaeranthus*, only 5 species *S. indicus, S. suaveolens, S. bullatus, S. kirkii, and S. africanus* have been chemically studied.[10] Over 50 compounds have been reported from these plants. These are mainly classified into four groups; monoterpenes, sesquiterpenes, flavonoids, and miscellaneous.

1.1.1 Monoterpenoids

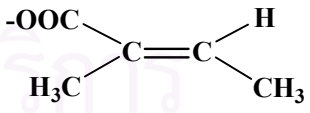
Monoterpenoids have been found in *S. indicus*, *S. bullatus, S. kirkii,* and *S. suaveolens*. They are mainly classified into two main groups, i.e., carvotanacetone derivatives such as compounds **1-7** and cyclic monoterpenes such as **8,9**.[6]



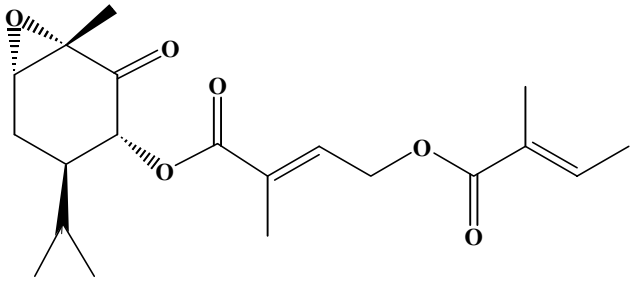
	(1)	(2)	(3)	(4)	(5)	(6)
R^1	Tigl	H	Tigl	Ac	H	H
R^2	Tigl	H	Tigl	Tigl	H	Ang
R^3	H	H	Ac	H	Tigl	Tigl



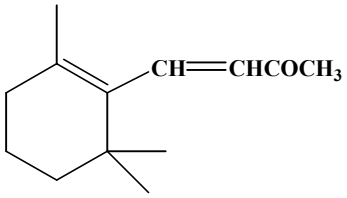
Angelic acid portion



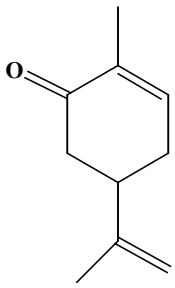
Tiglic acid portion



1α,2α-epoxy-5α(4-tigloyloxy-tigloyloxy)carvone (7)



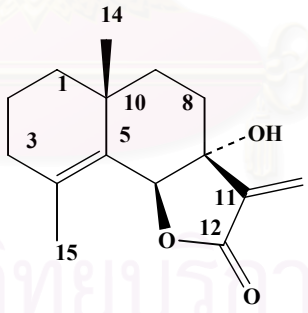
α–Ionone (8)



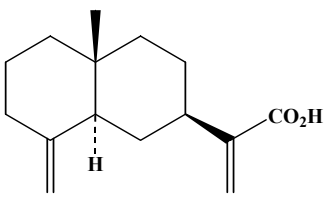
Carvone (9)

1.1.2 Sesquiterpenoids

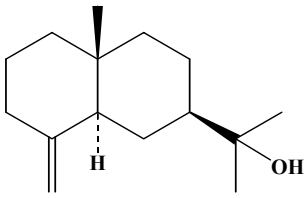
Numerous sesquiterpenoids have been reported from this genus.[7] Among them eudesmanolides, sesquiterpene lactones,[8] have often been found. Although eudesmanolides were found in many plants, 7-hydroxyeudesmanolides isolated from this genus are very rare in nature. Eudesmanolide and 7α-hydroxyeudesm-4-en-6-12-olide **(10)** was first reported in 1986 from genus *Sphaeranthus*.[9] This compound was particularly interesting since it showed pronounced cytotoxicity and antitumor activity against a number of human cancer cell lines.[10] In addition, a new sesquiterpene acid, 2-hydroxycostic acid **(11)**, along with known compounds β-eudesmol **(12)** and ilicic acid **(13)** have been isolated from *S. indicus*. Later on two new eudesmanolides **(14-15)** were reported by Supada *et al.*[11]



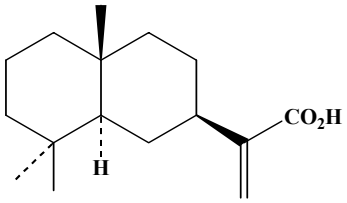
7α-hydroxyeudesm-4-en-6-12-olide (10)



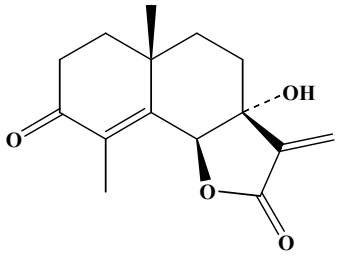
2-hydroxycostic acid (11)



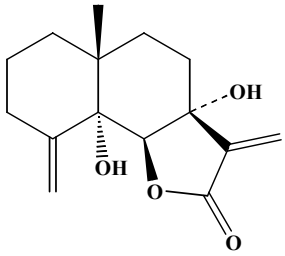
β-eudesmol (12)



ilicic acid (13)



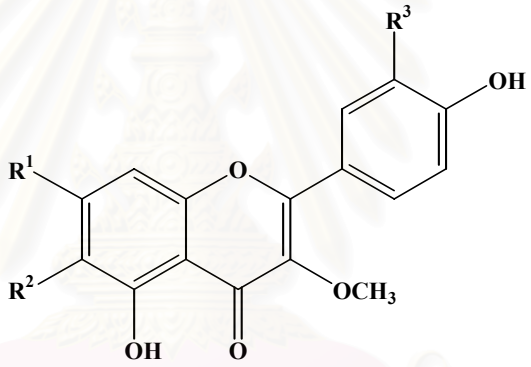
(14)



(15)

1.1.3 Flavonoids

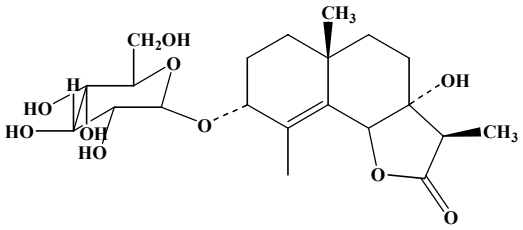
A number of flavonoids have been isolated from several plants in family Compositae including Sphaeranthus genus. Examples are flavonoids isolated from *S. africanus*[12]: quercetagetin-3,6,7-trimethyl ether **(16)**, quercetagetin-3,3',7-trimethyl ether **(17)**, quercetagetin-3,7-dimethyl ether **(18)**.



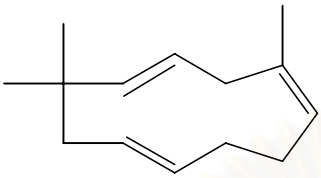
(16)	**(17)**	**(18)**
R^1 = OCH_3	**OCH_3**	**OCH_3**
R^2 = OCH_3	**OH**	**OH**
R^3 = OH	**OCH_3**	**OH**

1.1.4 Miscellaneous

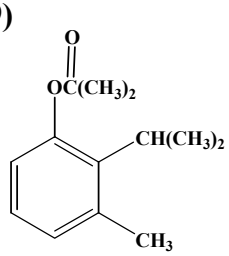
Examples of other types of compounds are given. A new sesquiterpene glycoside, sphaeranthanolides **(19)** has been isolated from the flowers of *S. indicus*. This compound exhibited immunostimulatory activity.[13] Further investigation of *S. bullatus* and *S. suaveolens* revealed the presence of α-humulene (**20)**, thymolisutyrate **(21).**[4]



sphaeranthanolides (19)



α-humulene (20)



thymolisutyrate (21)

1.2 Previous Research

1.2.1 Chemical Constituents and Biological Activities of *Sphaeranthus africanus* Linn.[12]

Mr. Panya Maneejak aimed at the search for rice growth inhibitor. The extraction procedure and the results of the research are as followed.

Scheme 1.1 The Extraction Procedure

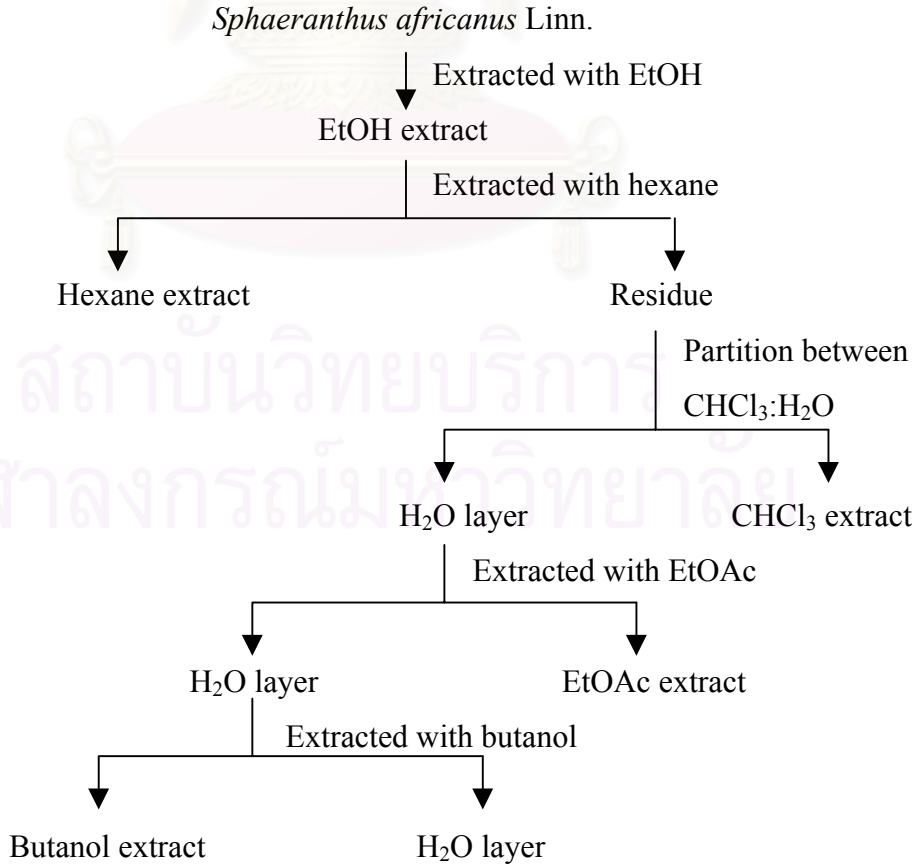


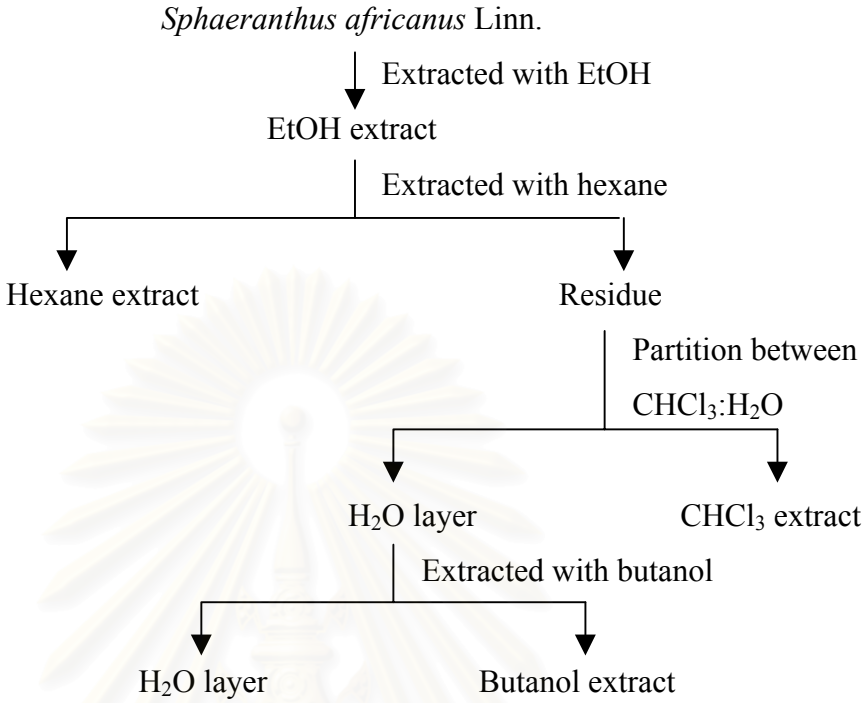
Table 1.1 The Result of Rice Growth Inhibition Activities.

Isolated Compounds	Eluent	Activities (%)	
		Root	Shoot
1. Mixture of long chain hydrocarbon (C_{26}-C_{34})	Hexane	-	-
2. Mixture of long chain ester	Hexane	62.92	73.69
3. Mixture of long chain alcohol (C_{23}, C_{25},C_{27}, C_{30}, C_{33})	Hexane	-	12.68
4. Friedelan-3β-ol	Hexane	Very small amount	
5. Mixture of long chain alcohol (C_{26}-C_{27}, C_{29}, C_{30}, C_{33})	Hexane	-	34.34
6. Stigmasterol	Hexane	18.79	7.79
7. Mixture of long chain acid (C_{19}-C_{25})	Hexane	97.70	98.77
8. Stigmasteryl-3-O-β-D-glucopyranoside	$CHCl_3$	31.49	0.44
9. 2-(3,4-dihydroxyphenyl)-5-hydroxy-3,6,7-trimethoxy-4*H*-1-benzopyran-4-one	$CHCl_3$	17.25	12.21
10. 2-(3-methoxy-4-hydroxyphenyl)-5,6-dihydroxy-3,7-dimethoxy-4*H*-1-benzopyran-4-one	$CHCl_3$	10.77	1.55
11. 2-(3,4-dihydroxyphenyl)-5,6-dihydroxy-3,7-dimethoxy-4*H*-1-benzopyran-4-one	EtOAc	42.77	35.22

1.2.2 Bioactive Compounds of *Sphaeranthus africanus* Linn.[14]

Miss Wimonpun Rungprom, she aimed for the purpose of bioactive compounds from this plant. This plant was selected from the result of the preliminary screening test. The ethanolic crude extracts of the whole plants of *S. africanus* revealed high cytotocixity activity against *Artemia salina* (brine shrimp) and six carcinoma cell lines. The extract was isolated as shown on the following scheme (Scheme 1.2).

Scheme 1.2 The Extraction Procedure



The isolated compounds were tested for brine shrimp lethality cytotoxicity activity and the results are shown in Table 1.2 and 1.3.

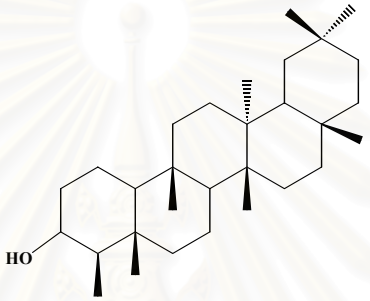
Table 1.2 The Result of Brine Shrimp Lethality Cytotoxicity Test of Reported Compounds

Isolated Compounds	LC_{50} (μg/mL)	Activity
Compound 1	180.62	Low activity
Compound 2	>1000	No activity
Compound 3	241.82	Low activity
Compound 4	12.34	Medium activity
Compound 5	11.45	Medium activity
Compound 6	145.61	Low activity
Compound 7	72.32	Medium activity
Compound 8	>1000	No activity

Table 1.3 The Percentage Inhibition of cAMP of Compounds 3, 6, 7, and 9

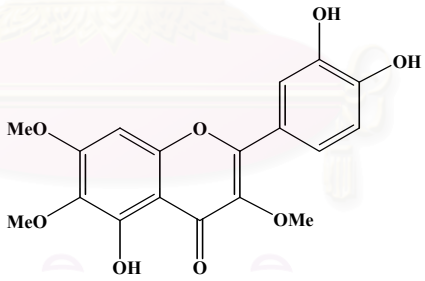
Isolated Compounds	% Inhibition
Compound 3	75.0
Compound 6	50.0
Compound 7	42.0
Compound 9	87.5

The Structure of Isolated Compounds.

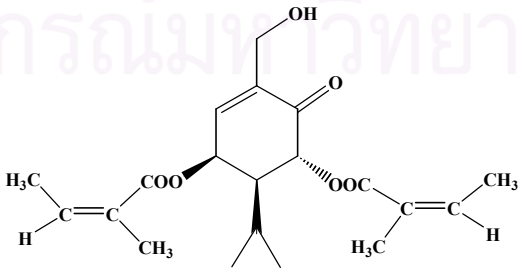


Compound 1 Friedelan-3β-ol

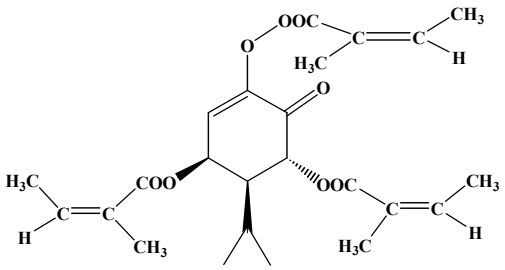
Compound 2 Mixture of stigmasterol and β-sitosterol



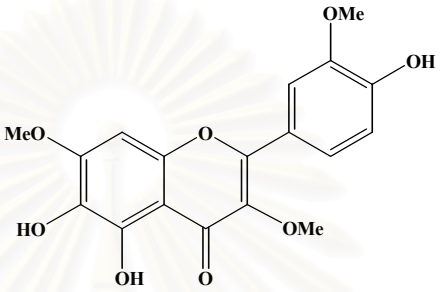
Compound 3 Quercetagentin-3,6,7-trimethyl ether



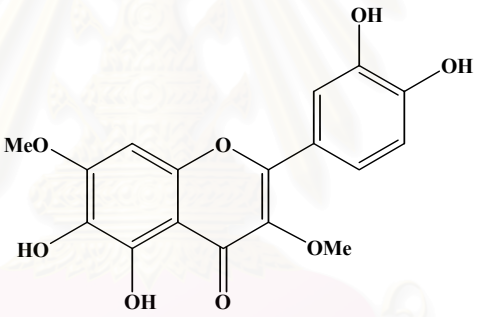
3α, 5β-diangeoloxoyloxy-7-hydroxycarvotanacetone



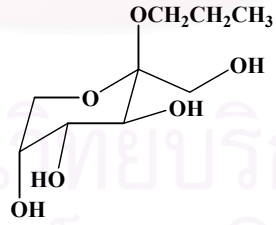
Compound 5 2,4α,6β-triangeoloxoyloxy-5-(sec-propyl)-2-cyclohexenone



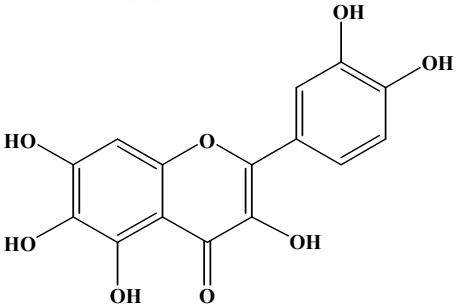
Compound 6 Chrysophenol-C



Compound 7 Quercetagentin-3,7-dimethyl ether



Compound 8 2-O-*n*-butyl-*β*-fructupyranose



Compound 9 Quercetin

1.3 The Goal of this Research

A preliminary screening of ethanol extract of the whole plant of *S. africanus* showed selective cytotoxicity against KB cells out of three carcinoma cell lines. Furthermore, previous researchers obtained some new compounds as well as known compounds exhibiting strong inhibition against cAMP and cytotoxicity against carcinoma cell lines. Therefore, it is worthwhile to continue the search for bioactive compounds from this plant. The goal of the present research is summarized as follows:

1. Extraction and isolation of the constituents from the whole plant of *S. africanus*.
2. Elucidation of the chemical structures of isolated compounds.
3. Search for active and useful compounds.

CHAPTER II
Experimental

2.1 Plant Material

The whole plant of *Sphaeranthus africanus* Linn. was collected at Kasetsart University, Bangkok, during January 1999. The voucher specimens (085404-87-154) have been deposited in the herbarium of the Royal Forest Department, Bangkok.

2.2 General Procedure

The ^{1}H and ^{13}C NMR spectra including 2D NMR spectra were recorded on a JEOL α– 500 and JEOL α–270 spectrometer. The NMR spectra were measured in $CDCl_3$ and DMSO-d_6 . TMS was used as internal standard.

The FT-IR spectra were recorded on a Fourier-Transform Infrared Spectrophotometer model Impact 410: solid samples were incorporated to potassium bromide to form a pellet.

HPLC separations were performed on a Hitachi L-6000 or Shimadzu LC-9A pump equipped with Waters Refractive Index Detector R401, and Hitachi L-4000 UV or Waters 486 UV detectors. Columns used were reversed-phase C-18 (10 x 250 mm, 5 μm, Capcell PAK), normal-phase silica (8 x 250 mm, 5 μm, Cosmosil 5SL) and NH_2 normal-phase (4.6 x 250 mm, 5 μm, UG 80).

2.3 Chemicals

Merck silica gel 60H was used for open column chromatography. Analytical thin layer chromatography was performed on precoated TLC plates; Merck Kieselgel 60 F_{254}, HPTLC RP18 WF_{254}, and NH_2 F_{254}. Prep-TLC was also performed on Merck Kieselgel 60 F_{254} plates. All solvents used in this research were purified prior to use by standard methodology except for reagent grade ones.

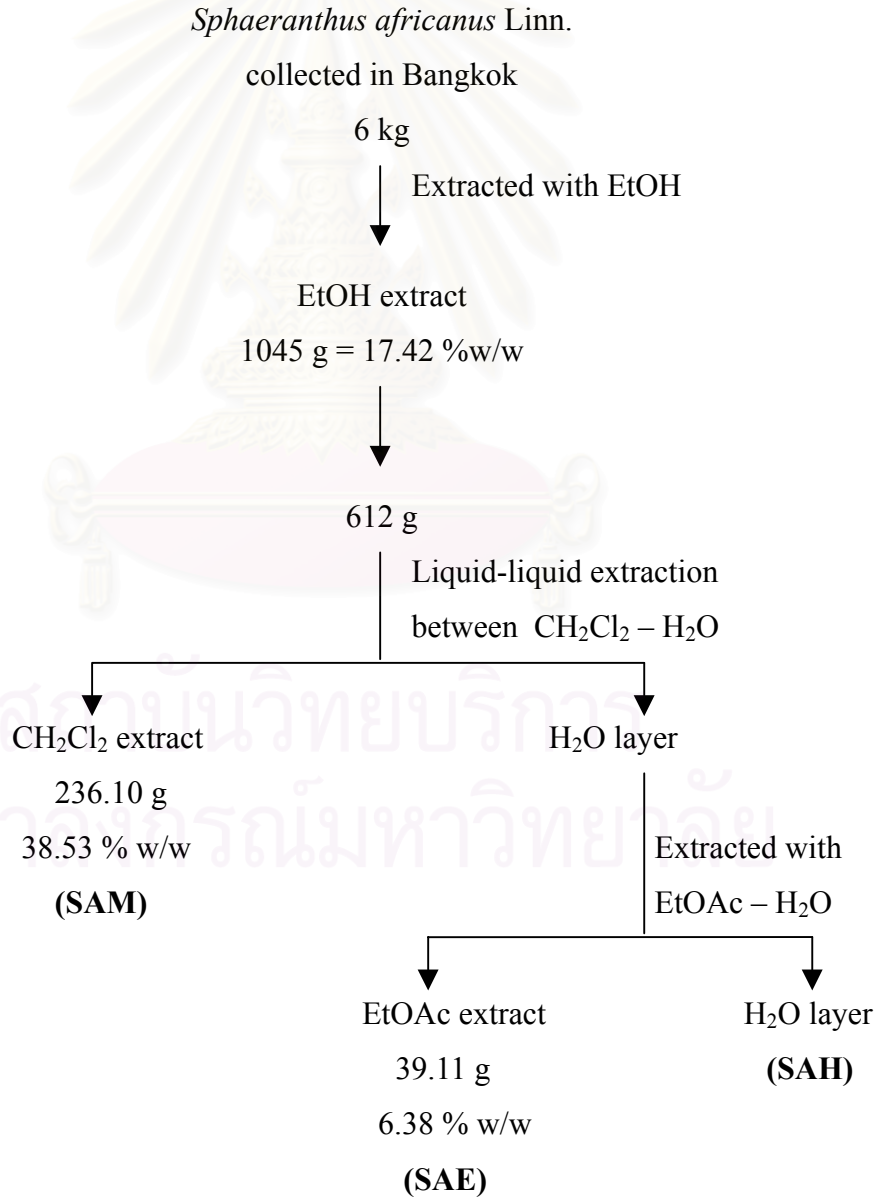
2.4 Extraction

A sample of the air-dried whole plant was cut into coarse pieces. The sample (approximately 6 kg) was extracted by soaking in ethanol for three days at room

temperature. The extraction was repeated for several times until the color of the last extract was clear. The solution was filtered, and the solvent was evaporated, yielding the ethanolic crude extract (1045 g).

A part (612 g) of the extract was partitioned between dichloromethane and water (volume ratio 1:3) to gain a dichloromethane soluble fraction and water-soluble fraction. The water-soluble fraction was further extracted with ethyl acetate to obtain an ethyl acetate extract. The procedure is shown in Scheme 2.1.

Scheme 2.1 Extraction Scheme of *Sphaeranthus africanus*



2.5 Assay

The following assays were performed for the isolation of active compounds from *S. africanus*, which might possibly be used for agricultural and/or medicinal purposes.

2.5.1 The Inhibitory Effect Against Carcinoma Cell Lines

Several crude extracts of *S. africanus* were preliminary screened by the MTT assay. This method used three carcinoma cell lines: human Nasopharyngeal carcinoma cell line (KB), human leukemia carcinoma cell line (HL-60), human colon carcinoma cell line (HCT-8). Beijing Medicinal School, Beijing, China, performed these experiments.

2.5.2 Free Radical Scavenging Action Against DPPH Radical

A sample was dissolved in ethanol or dimethylsulfoxide (DMSO), (ca. 3 mg/ mL or 1.5 mM) and further diluted to seven stages with 50% ethanol-water. The sample solution (100 μL) was added 50 μL of DPPH in ethanol (750 μM) to give 150 μL of 250 μM DPPH solution with 66.7% of the initial sample concentration. The solution was allowed to stand for 20 min at ambient temperature, then a microplate reader (Automated Microplate Reader Elx800, BIO-TEX Instruments, INC.) measured the optical density at 515 nm (OD515). The OD_{515} values obtained in the non-scavenger run and blank (50% ethanol-water) were regarded as 100% and 0% activities of DPPH, respectively. The OD_{515} values (duplicate) obtained for each sample was plotted against the sample concentration, and the EC_{50} was obtained from the graph.

CHAPTER III

Results and Discussion

3.1 Extraction for the Whole Plant of *Sphaeranthus africanus*

According to Scheme 2.1 the whole plant of *S. africanus* was extracted to give three crude extracts, SAM (CH_2Cl_2 extract), SAE (EtOAc extract), and SAH (H_2O layer).

3.2 Biological Activities of the Crude Extracts

3.2.1 Cytotoxicity of the EtOH Extract

Preliminary cytotoxicity test of the ethanol extract was performed against three carcinoma cell lines as shown in Table 3.1. The extract showed selective activity against KB cells.

Table 3.1 Cytotoxicity Test for the EtOH extract of *S. africanus*

Cell lines	Inhibition (%)			Estimation
	at 1 μg/mL	at 10 μg/mL	at 10 0μg/mL	
HL-60	-12.83	-14.22	8.13	-
KB	22.49	53.19	63.33	++
HCT-8	-23.23	-15.10	45.84	-

HL-60 = Human leukemia carcinoma cell line
KB = Human nasopharynxgal carcinoma cell line
HCT-8 = Human colon carcinoma cell line

- = No activity
+ = Weak activity
++ = Strong activity
+++ = Very strong activity

3.2.2 Activity of the CH_2Cl_2 and the EtOAc Extracts

Cytotoxicity of the SAM and the SAE (Table 3.2) and free-radical scavenging activity of the SAM, SAE, and SAH (Table 3.3) are tested. Both the SAM and SAE showed significant cytotoxicity against HL-60 and KB, but not HCT-8. The SAH showed only weak free-radical scavenging activity, whereas the SAE showed very strong and the SAM showed strong activities.

Table 3.2 Cytotoxicity Test for SAM and SAE

Cell lines	Samples	Inhibition (%)			Estimation
		at 1 μg/mL	at 10 μg/mL	at 100 μg/mL	
HL-60	SAM	-6.37	0.55	87.07	+
	SAE	4.62	26.96	86.33	+
KB	SAM	15.73	17.44	58.11	+
	SAE	33.12	54.53	66.37	++
HCT-8	SAM	-39.14	-35.14	9.38	--
	SAE	-36.46	0.26	44.95	--

Table 3.3 Free Radical Scavenging Action of SAM, SAE, and SAH to DPPH Radical Activity

Sample	EC_{50} ($EC_{125\mu M}$ DPPH) mg/mL
SAM	0.095
SAE	0.01
SAH	0.50

EC_{50} < 0.1 mg/mL = Strong

EC_{50} < 0.01 mg/mL = Very strong

3.3 Separation of Dichloromethane Extract (SAM) and Ethyl Acetate Extract (SAE)

The SAM and SAE, which revealed significant cytotoxicity against various carcinoma cell lines and free radical scavenging activity, were selected for investigation.

3.3.1 Separation of Dichloromethane Extract (SAM)

The dichloromethane extract (SAM) was separated by SiO_2 vacuum liquid chromatography (VLC). The dichloromethane solution of the extract (235.10 g) was mixed with silica gel (500 g). After removal of the solvent, the silica gel was put in a column, and eluted with Hex-CH_2Cl_2-EtOAc-MeOH by increasing polarity of solvent. Each fraction (about 1,000 mL) was collected and concentrated to a small volume and then checked by TLC. The fractions containing the same components were combined. The results of separation and combination are shown in Table 3.4. Cytotoxicity against KB cell (MTT method) and free-radical scavenging activity of each fraction are shown in Table 3.6 and 3.7, respectively.

Table 3.4 Sample Code, Property, and weight of VLC fractions of SAM

Eluent	Code	Property	Weight (g)
100% Hexane	SAM1	yellow viscous	7.28
25% CH_2Cl_2 in Hexane	SAM2	dark brown oil	28.29
50% CH_2Cl_2 in Hexane	SAM3	dark brown oil	18.48
75% CH_2Cl_2 in Hexane	SAM4	dark brown oil	35.71
100% CH_2Cl_2	SAM5	dark brown oil	7.27
25% EtOAc in CH_2Cl_2	SAM6	dark brown solid	9.11
50% EtOAc in CH_2Cl_2	SAM7	dark brown oil	6.31
75% EtOAc in CH_2Cl_2	SAM8	dark brown oil	6.57
100% EtOAc	SAM9	dark brown solid	5.31
25% MeOH in EtOAc	SAM10	dark brown oil	49.05
50% MeOH in EtOAc	SAM11	dark brown oil	7.66
75% MeOH in EtOAc	SAM12	dark brown oil	2.69

3.3.2 Separation of Ethyl Acetate Extract (SAE)

The ethyl acetate extract (SAE) was separated by open column chromatography (OCC). The extract (37.38 g) was dissolved in EtOAc and mixed with silica gel (40 g). After removal of the solvent, it was put on a top of a SiO_2 column, and eluted with gradient solvent system (Hex-CH_2Cl_2-EtOAc-MeOH). Each fraction (about 500 mL) was collected and the solvent was removed. According to a TLC analysis, the fractions containing the same components were combined. The results are shown in Table 3.5.

Table 3.5 Sample Code, Property, and Weight of OCC Fractions of SAE

Elutent	Code	Property	Weight (g)
10% EtOAc in Hexane	SAE1	yellow viscous oil	0.04
15% EtOAc in Hexane	SAE2	yellow viscous oil	0.02
30% EtOAc in Hexane	SAE3	yellow solid amorphous	0.15
50% EtOAc in Hexane	SAE4	yellow solid amorphous	1.13
60% EtOAc in Hexane	SAE5	yellow solid amorphous	3.11
70% EtOAc in Hexane	SAE6	black tar	4.40
80% EtOAc in Hexane	SAE7	black tar	2.55
90% EtOAc in Hexane	SAE8	black tar	4.12
100% EtOAc	SAE9	black tar	4.74
10% MeOH in EtOAc	SAE10	black tar	2.26
20% MeOH in EtOAc	SAE11	black tar	3.73
30% MeOH in EtOAc	SAAE12	black tar	1.73
50% MeOH in EtOAc	SAE13	black tar	1.40
70% MeOH in EtOAc	SAE14	black tar	0.87
100% MeOH in EtOAc	SAE15	black tar	0.83

Table 3.6 Cytotoxicity of SAM, SAE, and SAH fraction against KB cell

Sample	Dose (μg/mL)			
	10	**3**	**1**	**0.3**
SAM	50.5	-	-	-
SAE	15.3	-	-	-
SAH	12.6	-	-	-
SAM1	11.0	-	-	-
SAM2	25.7	-	-	-
SAM3	95.1	31.2	20.6	14.0
SAM4	93.0	40.9	28.3	19.6
SAM5	85.5	36.6	23.2	13.2
SAM6	32.7	-	-	-
SAM7	24.2	-	-	-
SAM8	27.2	-	-	-
SAM9	11.7	-	-	-
SAM10	16.0	-	-	-
SAM11	32.9	-	-	-
SAM12	18.6	-	-	-

- = no activity

Table3.7 Free Radical Scavenging Action of SAM, SAE, and SAH Fraction to DPPH Radical Activity

Sample	EC_{50} ($EC_{125\mu M}$ DPPH)
SAM	0.095
SAE	0.010
SAH	0.500
SAM1	-
SAM2	-
SAM3	1.840
SAM4	0.219
SAM5	0.079
SAM6	0.034
SAM7	0.050
SAM8	0.069
SAM9	0.127
SAM10	0.102
SAM11	0.194
SAM12	0.142

EC_{50} < 0.1 mg/mL = strong

< 0.01 mg/mL = very strong

From Table 3.6, sub-fractions SAM3, SAM 4, and SAM 5 are shown interesting cytotoxicity against KB cell more than other fractions. In case of free radical scavenging action against DPPH radical activity, the result is shown in Table 3.7. The SAM 5, SAM 6, SAM 7, and SAM 8 are shown strong activity but other fractions are exhibited weak activity.

3.4 Purification, Properties and Structural Elucidation of Organic Compounds Isolated from *S. africanus*

3.4.1 Purification, properties and structural elucidation of Compound 1

This compound was isolated from dichloromethane extract. After elution with 25 % dichloromethane in hexane, dark brown oil was gained. The dark brown oil was subjected to separation on silica gel (VLC; Si 60) with stepwise gradient elution (heptane-CH_2Cl_2-EtOAc-MeOH) to give thirteen fractions. Fraction 4 was further separated by open column chromatography (OCC) on silica gel. The ninth fraction was recrystallized from methanol to give white needles (m.p. 163-164°C, 73.3 mg, 0.2 % w/w of dichloromethane extract). It showed a single spot with R_f value of 0.55 in heptane:EtOAc (3:2) solvent system.

Its IR spectrum (Fig 3.1) showed the absorption band of a hydroxy group at 3400 - 3300 cm^{-1} and C-O stretching vibration at 1056 and 1032 cm^{-1}. In addition, the band at 1650 cm^{-1} revealed the presence of C=C functional group; the stretching and bending vibrations of $-CH_3$ and $-CH_2-$ were detected at 2960 - 2860 and 1460 cm^{-1}, respectively.

The ^1H NMR spectrum (Fig 3.2) showed important signals at δ 5.01 and 5.34 ppm compatible with -CH=CH- and -C=CH-, respectively. Hydroxy proton was detected at 3.48 ppm (1H, s). In addition, the ^{13}C NMR spectrum (Fig 3.3) exhibited total twenty nine carbon signals: at δ 140.7 and 121.7 ppm ascribing for the olefinic carbons, the signals at δ 138.3 and 129.3 ppm consistent with olefinic carbons and the signal at δ 71.8 ppm coinciding with the carbon that connected with a hydroxy group.

The ^1H and ^{13}C NMR data indicated a steroidal structure for compound 1. It was identified as stigmasterol by comparison of the ^{13}C NMR data with those reported in Table 3.8.[15]

According to the physical properties and spectral evidences, it could be concluded that compound 1 was stigmasterol. The structure of this compound is shown in Fig. 3.4.

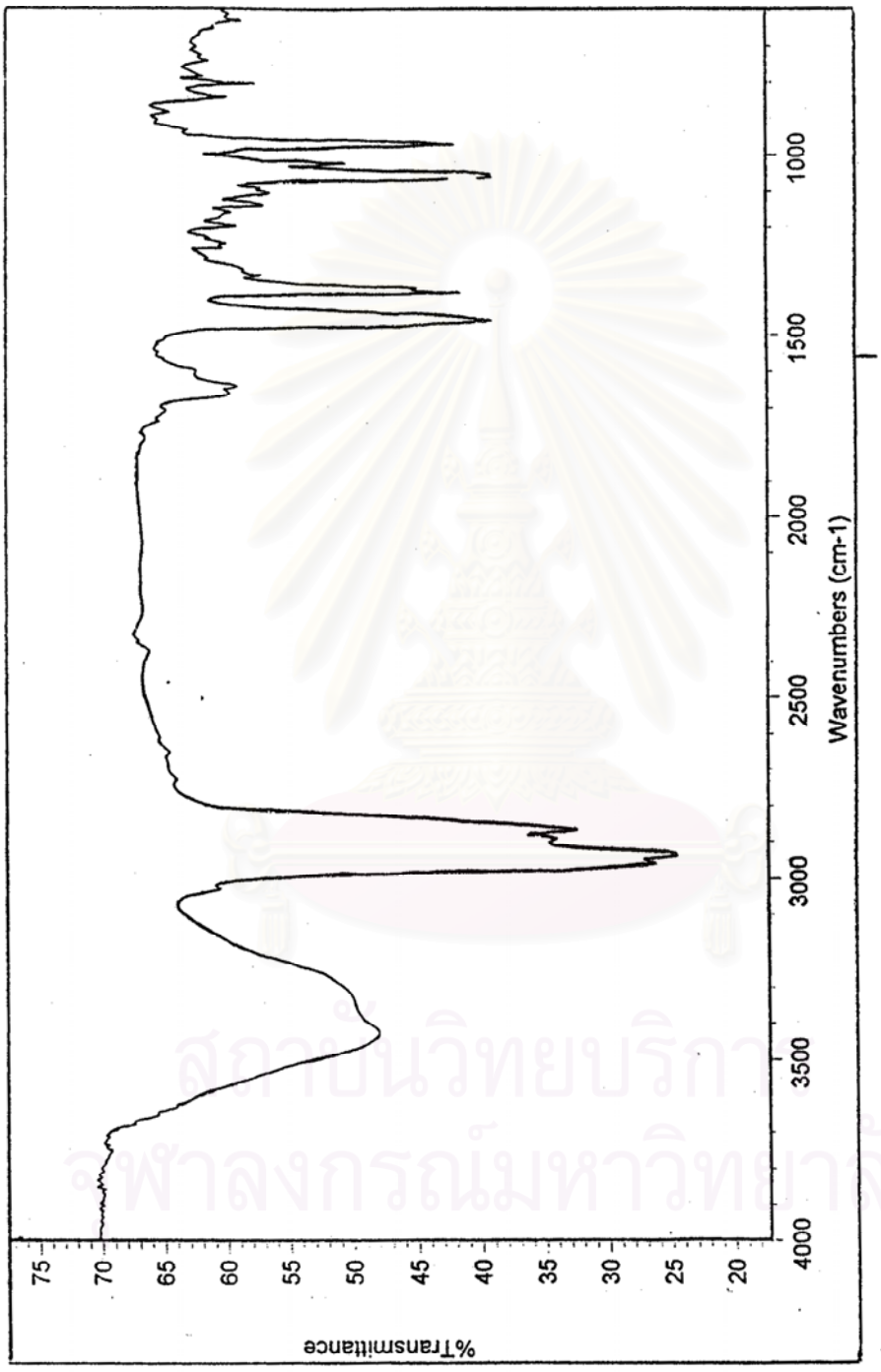


Fig 3.1 The IR Spectrum of Compound 1

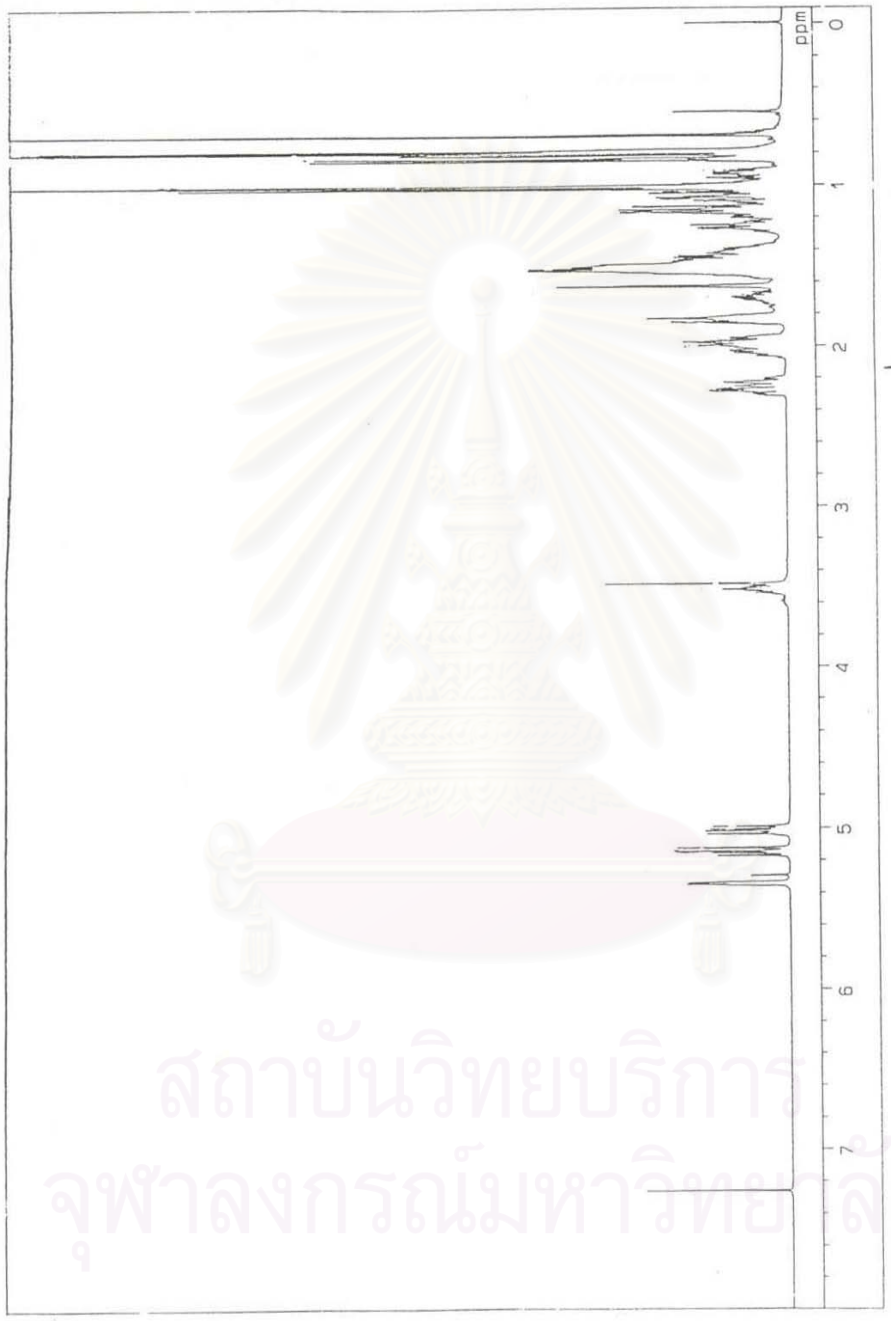


Fig 3.2 The ^{1}H NMR Spectrum of Compound 1

Fig 3.3 The ^{13}C NMR Spectrum of Compound 1

Table 3.8 The ^{13}C NMR Assignment of Stigmasterol and Compound 1

Position	Stigmasterol, Hz($CDCl_3$) [15]	Compound 1, Hz($CDCl_3$)
1	37.3	37.3
2	31.7	31.7
3	71.8	71.8
4	42.3	42.3
5	140.8	140.7
6	121.7	121.7
7	31.9	31.9
8	31.9	31.9
9	50.2	50.2
10	36.6	36.5
11	21.1	21.1
12	39.7	39.7
13	42.3	42.2
14	56.9	56.9
15	24.4	24.4
16	28.8	28.9
17	56.1	56.0
18	12.1	12.0
19	19.4	19.4
20	40.5	40.5
21	21.0	21.1
22	138.3	138.3
23	129.4	129.3
24	51.3	51.2
25	31.9	31.9
26	19.0	19.0
27	21.2	21.1
28	25.4	25.4
29	12.4	12.3

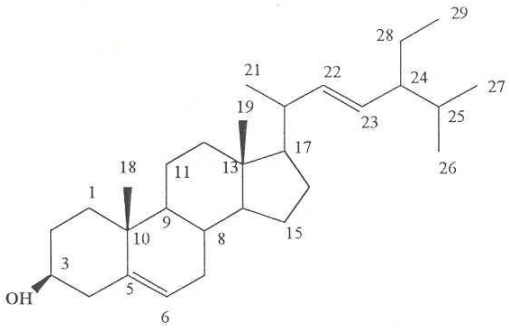


Fig. 3.4 The Structure of Compound 1 (Stigmasterol)

3.4.2 Purification, Properties and Structural Elucidation of Compound 2

Compound 2 was obtained from dichloromethane extract by eluting with 25% dichloromethane in hexane. This fraction was subsequently separated by OCC with hexane-EtOAc-MeOH, OCC (Si-60) with hexane-EtOAc, and preparative thin layer chromatography (Si-60, hexane-EtOAc, 1:1) to give three fractions. The third fraction was separated by reversed phase HPLC (MeOH:H_2O, 7:3) to give compound 2 (retention time = 46 minutes) and compound 3 (retention time = 50 minutes) as a minor compound. Compound 2 is colorless oil (3.4 mg, 1.45 $x10^{-3}$ % w/w of dichloromethane extract). It showed one spot on TLC with R_f value of 0.37 in MeOH:H_2O (7:1) as a solvent system.

The IR spectrum (Fig 3.5) of compound 2 showed the absorption band of an α,β-unsaturated carbonyl of ester at 1700 - 1770 cm^{-1}. The characteristic absorption peak due to an aromatic moiety was observed at 1600 - 1400 cm^{-1}.

The molecular formula of compound 2 (Fig 3.6) was established by high-resolution mass spectroscopy, which revealed a molecular ion peak at m/z 344, calculated for $C_{20}H_{24}O_5$. Other fragmentation and fragmentation pattern displayed in scheme 3.1.

The UV spectrum of this compound (Fig 3.7) showed three maximal absorption wavelengths at 290 ($\varepsilon = 4.7 \times 10^2$), 257 ($\varepsilon = 1.6 \times 10^2$), and 215 ($\varepsilon = 3.4 \times 10^2$) nm, respectively.

The ^1H NMR spectrum (Fig 3.8) composed of a signal for aromatic protons

at δ 6.98 (d (L,S) *J*=1.8, 7.9 Hz) position 6′, δ 7.0 (d (L) *J*= 8.2 Hz) position 5′, and δ 7.01 (d (S) *J*=1.8) position 2′. Furthermore, the characteristic signals of a trans -Ar-CH=CH-CH_2OCOR fragment were visible as one double triplet at δ 6.26 (*J* = 6.4, 15.6) and doublet at δ 6.64 (*J*= 15.8), and a double doublet at δ 4.81 (*J* = 1.5, 6.4). Two non equivalent 2-methylbut-2-enolate gave rise to two double quartets (3H each) at δ 2.07 (*J* = 1.5, 7.0) and 2.06 (*J* = 1.7, 9.5), one quintet (3H) at δ 1.92 (*J* = 1.6), one singlet (3H) at δ 2.05, one quartet of quartet (1H) at δ 6.07 (*J* = 1.5, 7.0) and one board quartet (1H) at δ 6.20 (*J* = 6.7). Finally, one methoxy group was assigned for a singlet signal at δ 3.84 ppm.

The ^{13}C NMR spectrum (Fig 3.9) was consisted with this structural assignment as it showed two carbonyl signals at δ 167.8 and 165.9. Furthermore, six aromatic carbons at δ 135.2, 119.4, 123.0, 139.7, 151.3, and 110.3 ppm, two methine carbons at δ 123.7 and 133.4 ppm and the presence of one methoxy group at δ 55.9 ppm were also observed. Finally, two 2-methyl but-2-enolate residues gave four methine carbons at δ 138.2, 127.8, 123.2, and 139.9, and four methyl groups at δ 15.8, 20.6, 15.9, and 20.7 ppm.

Nonetheless, the ^{13}C NMR spectrum exhibited signals of 2-methyl but-2-enolate that might be angelate (Z-isomer) and tiglate (E-isomer).[16] The conjugated ester group caused an upfield shift (δ=6.07 and 6.20 ppm) of neighboring proton more pronounce for angelate more than for tiglate.

In the case if geometries of double bonds in angelate portion were supported by COSY (Fig 3.10) and HMBC (Fig 3.11), that led to the structure of compound 2, a coniferyl alcohol derivative originally isolated from *Blumea lacera*.[17]

This structure was confirmed by ^{1}H NMR and mass spectra comparison with those reported. The proton-proton connectivity, the proton-carbon connectivity, and the structure of compound 2 are showed in Fig. 3.12, 3.13, and 3.14, respectively.

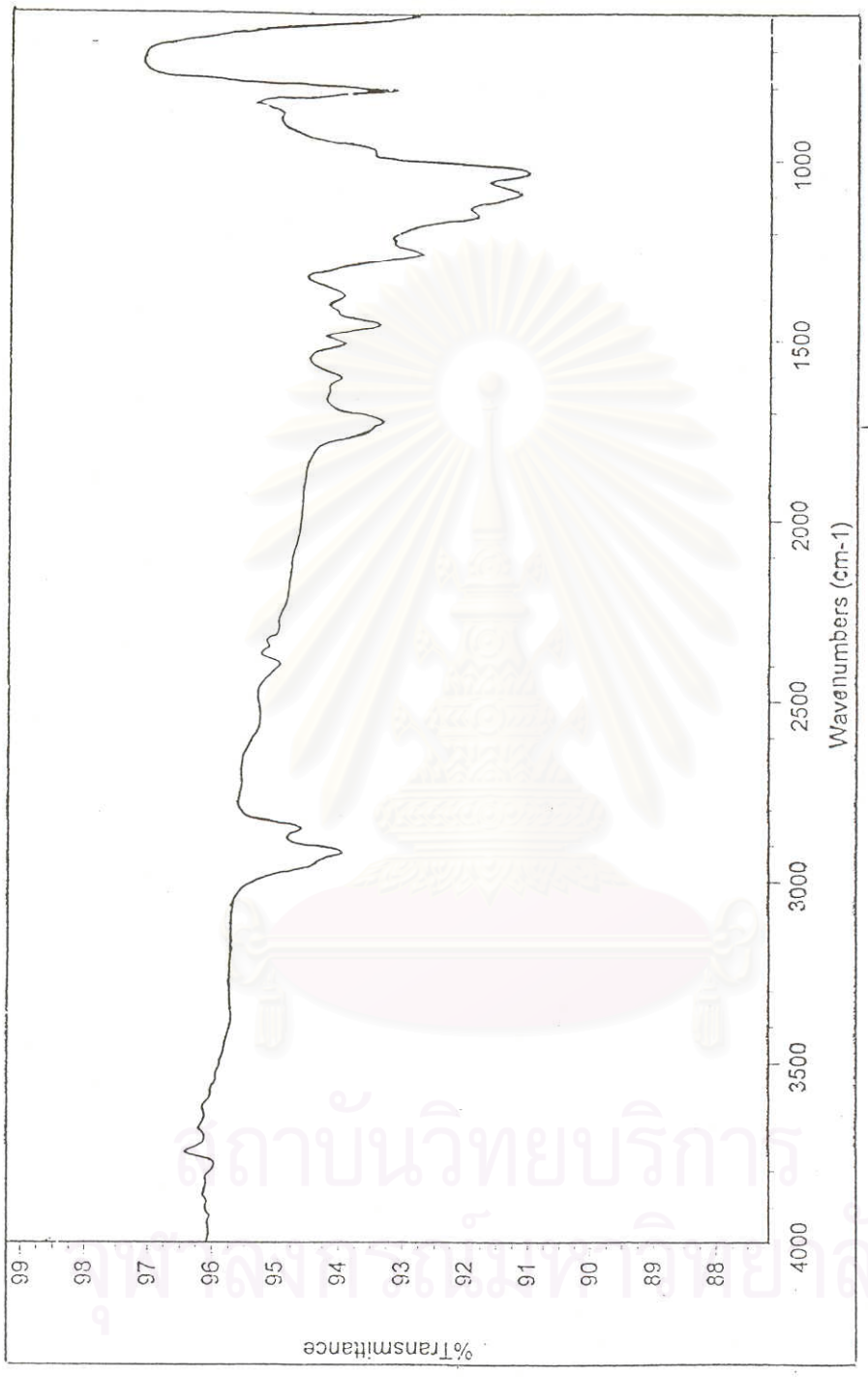


Fig 3.5 The IR Spectrum of Compound 2

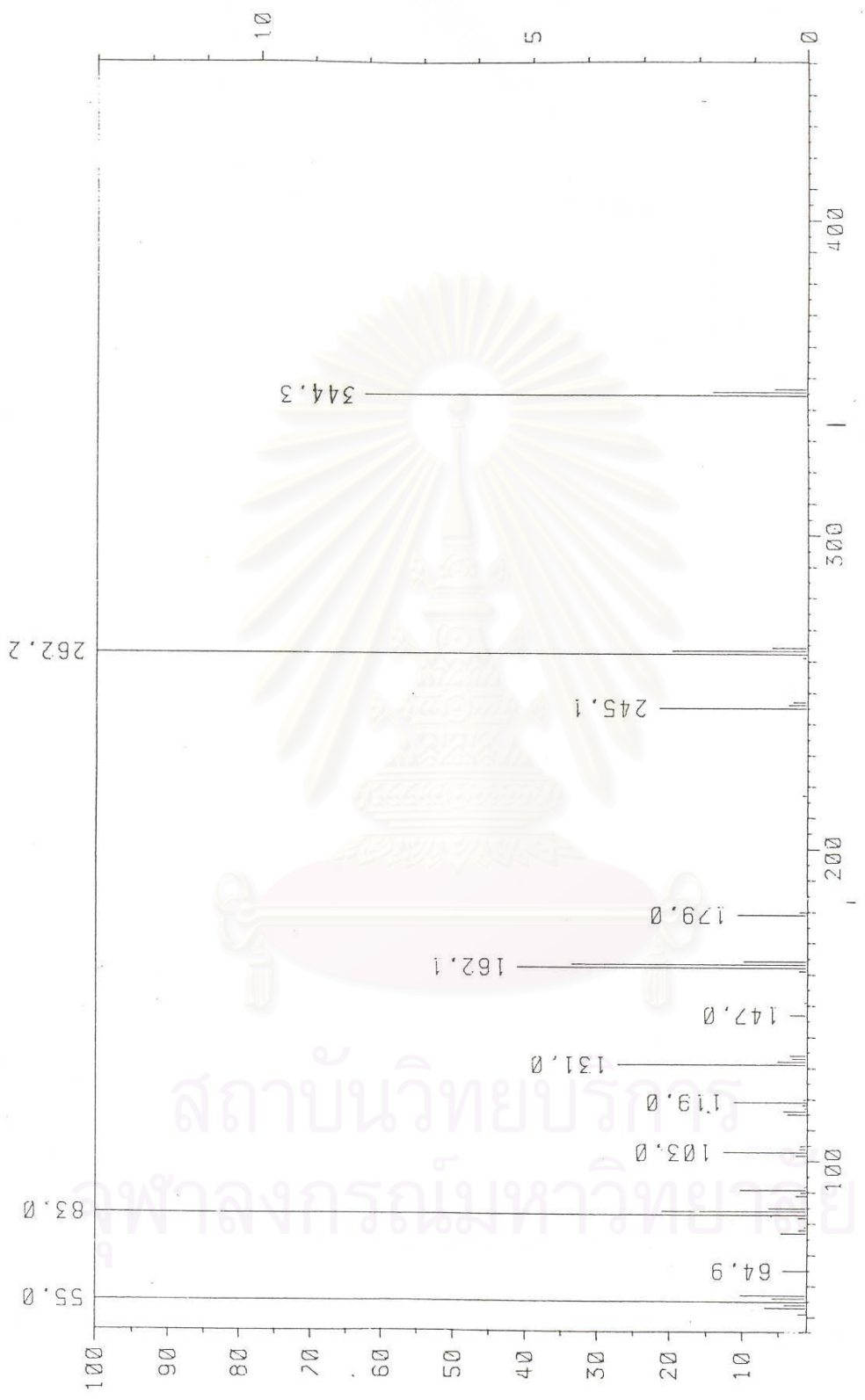


Fig 3.6 The mass Spectrum of Compound 2

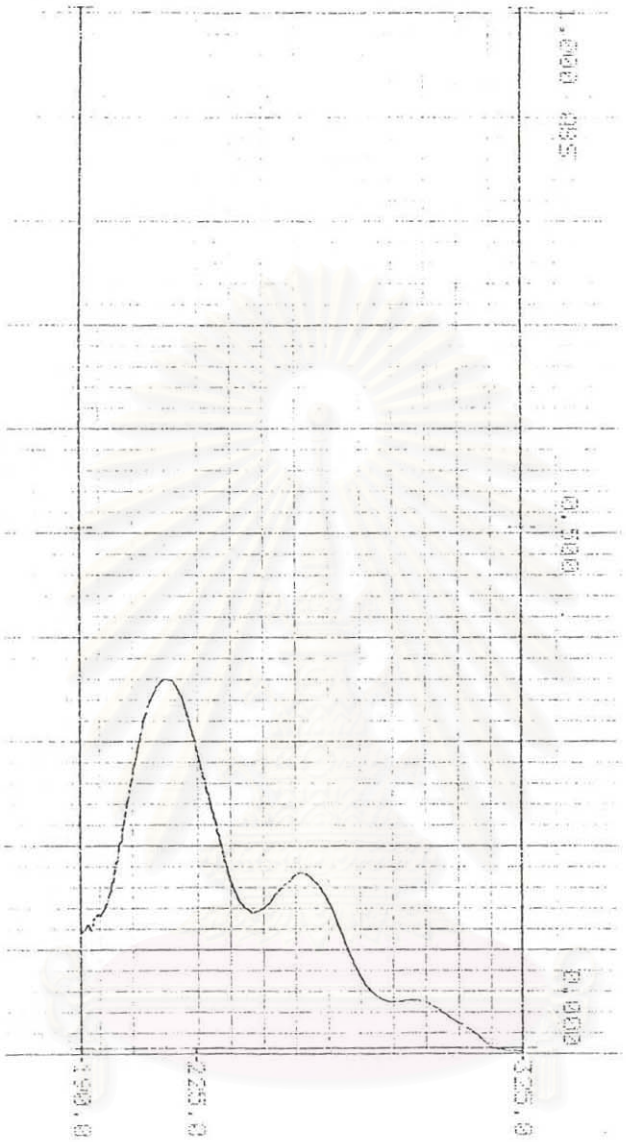


Fig 3.7 The UV Spectrum of Compound 2

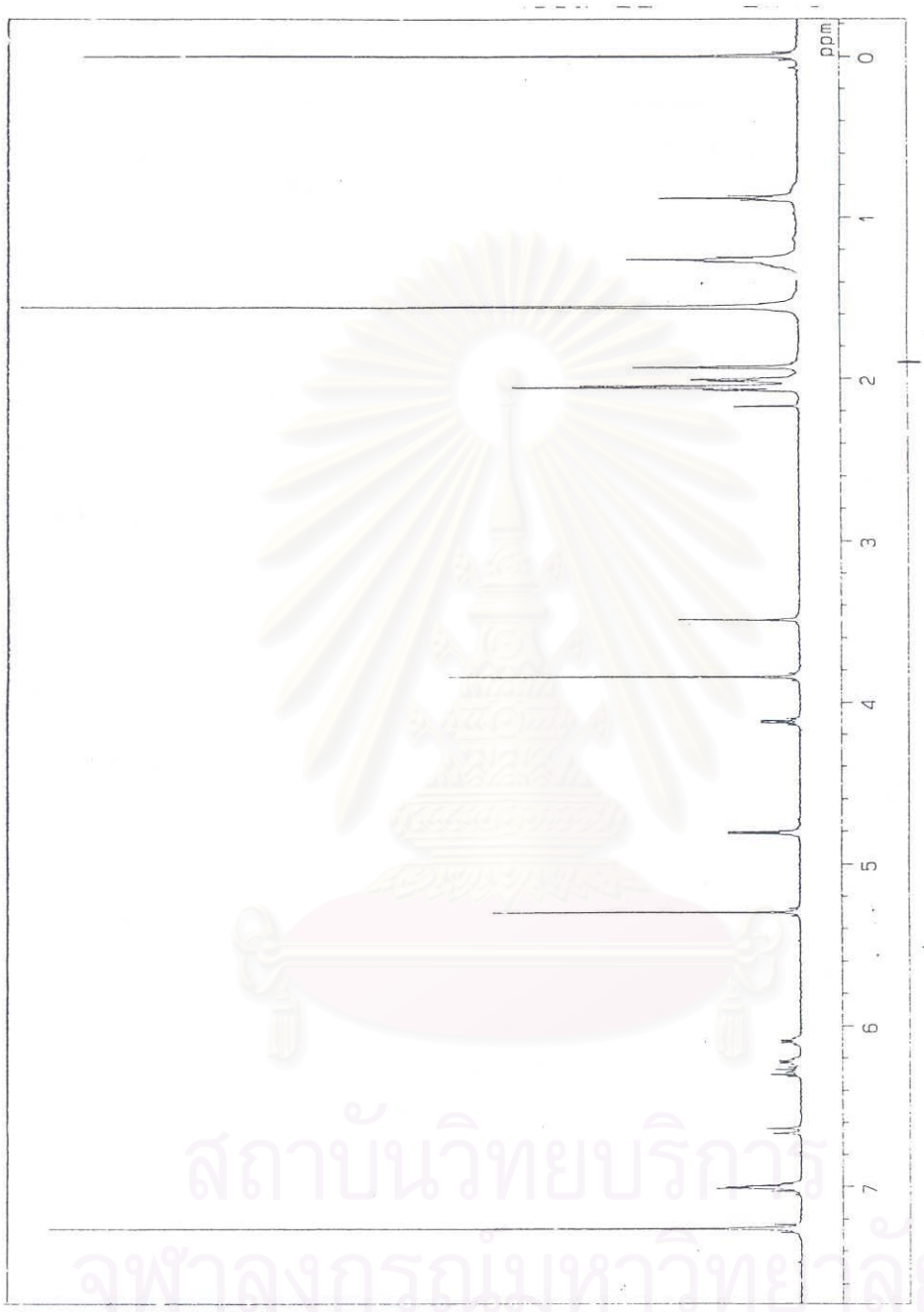


Fig 3.8 The ^{1}H NMR Spectrum of Compound 2

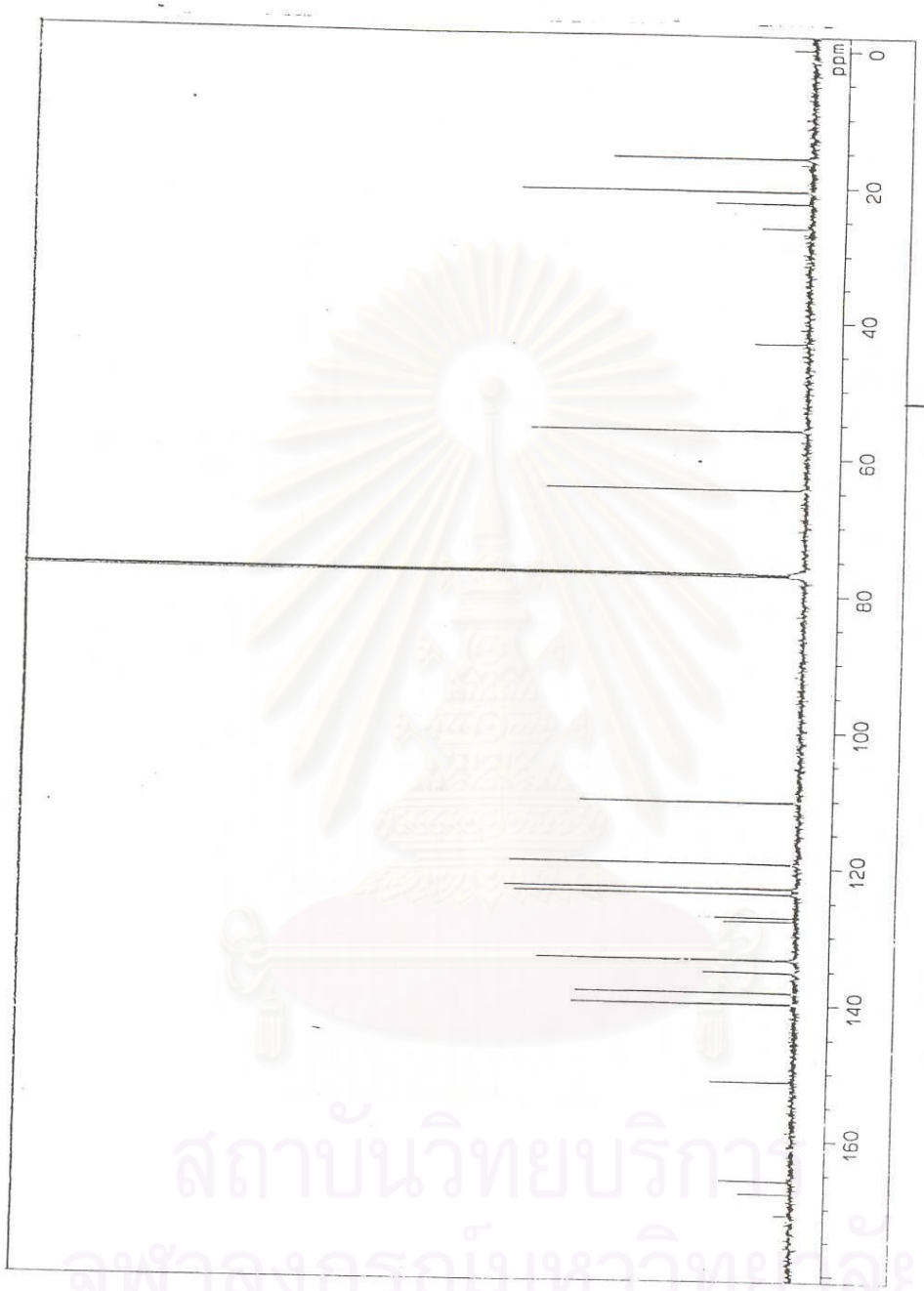


Fig 3.9 The ^{13}C NMR Spectrum of Compound 2

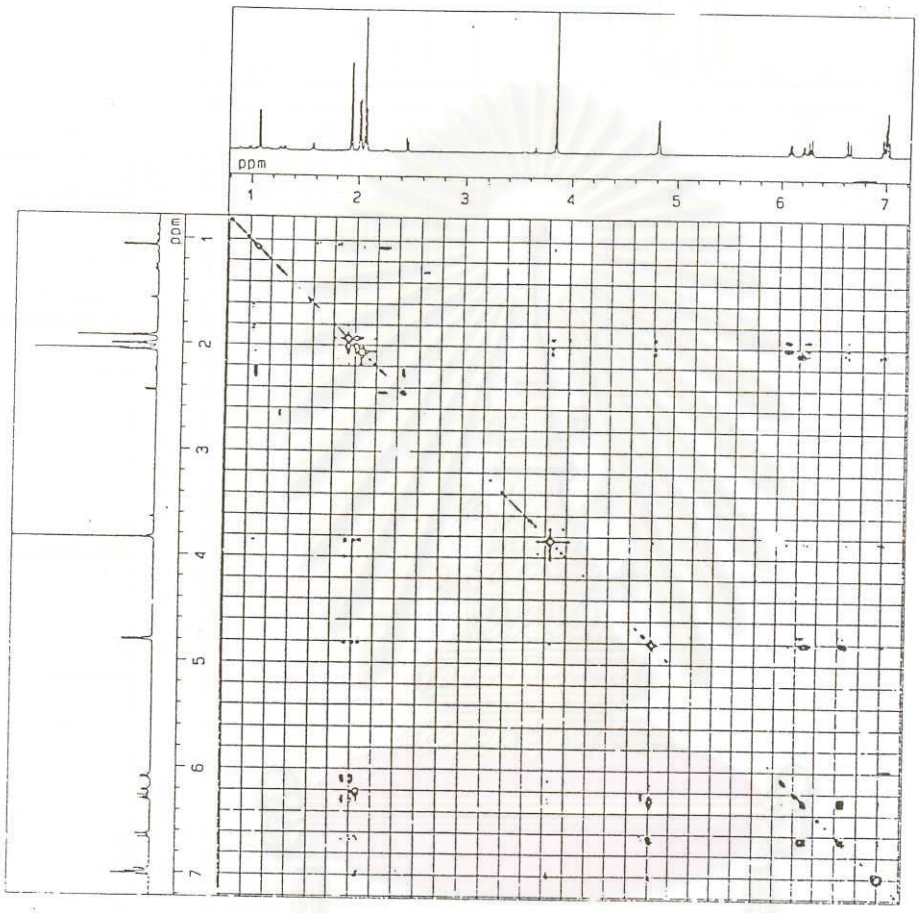


Fig 3.10 The COSY Spectrum of Compound 2

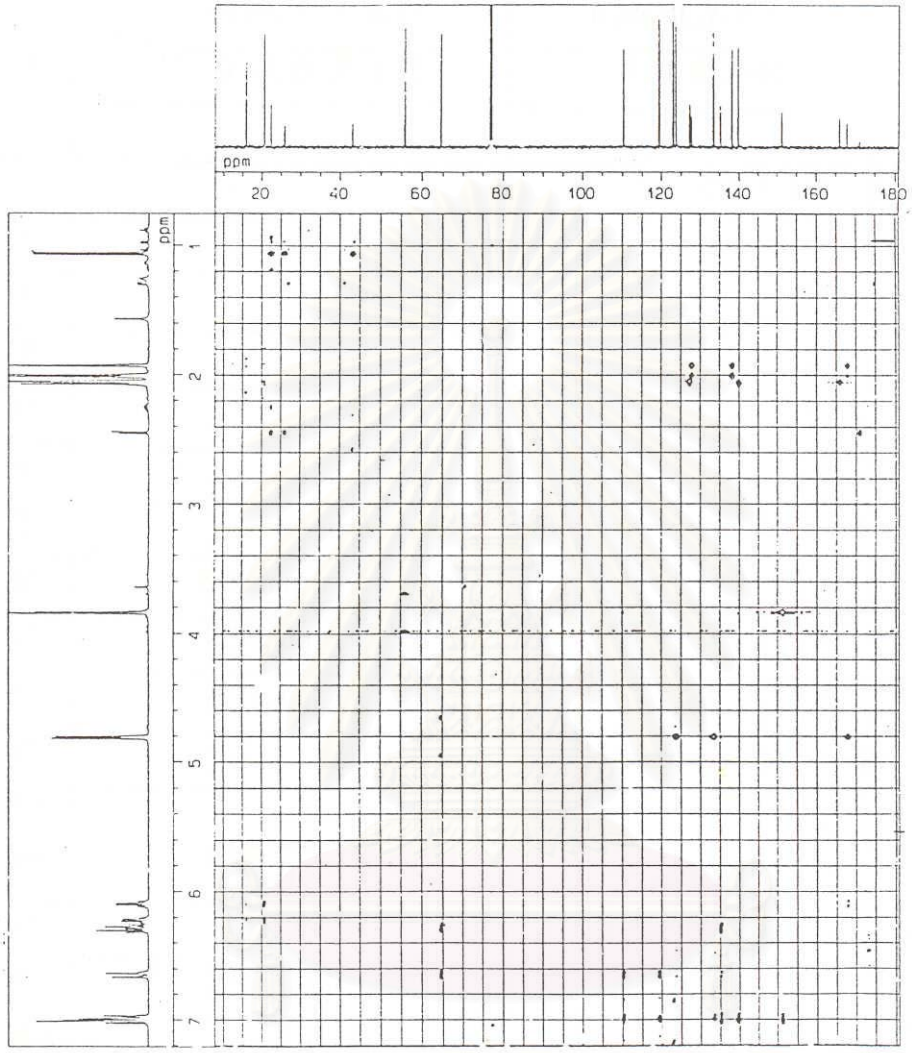


Fig 3.11 The HMBC Spectrum of Compound 2

Table 3.9 ^{1}H NMR Data of Coniferyl Alcohol Derivative and the Compound 2

Position	Coniferyl alcohol Derivative ($CDCl_3$)	Compound 2 ($CDCl_3$)	
	^{1}H, mult, *J*(Hz)	^{1}H, mult, *J*(Hz)	^{13}C,Hz
1	4.72, dd, *J*=1,6	4.81, dd, *J*=1.5,6.4	64.6
2	6.29, dt, *J*=6,15	6.26, dt, *J*=6.4,15.6	123.7
3	6.59, dt, *J*=1,15	6.64, d, *J*=15.8	133.4
1'			135.2
2'		7.01, d(S), *J*=1.8	110.3
3'			151.3
4'			139.7
5'		7.02, d(L), *J*=8.2	123.0
6'		6.98, d(L,S), *J*= 1.8,7.9	119.4
1''			165.9
2''			123.2
3''	1.90, dq, *J*=1.5,1.5	2.05, s	20.7
4''	1.99, dq, *J*=1.5,6	2.06, dq, *J* = 1.7, 9.5	15.9
5''	6.71, qq, *J*=1.5,6	6.20, br q, *J* = 6.7	139.9
1'''			167.8
2'''			127.8
3'''	6.03, qq, *J*=1.5,6	6.07, qq, *J*=1.5,7.0	138.2
4'''	2.00, m	1.92, quint, *J*=1.6	20.6
5'''	2.03, dq, *J*=1.5,6	2.07, dq, *J*=1.5,7.0	15.8
-OMe	3.81, s	3.84, s	55.9

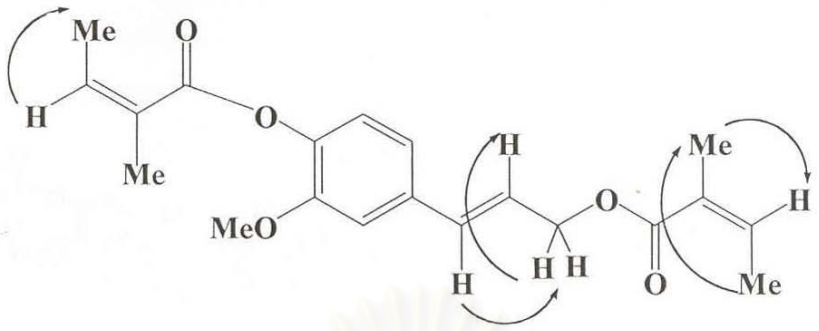


Fig. 3.12 The COSY Connectivity for Compound 2

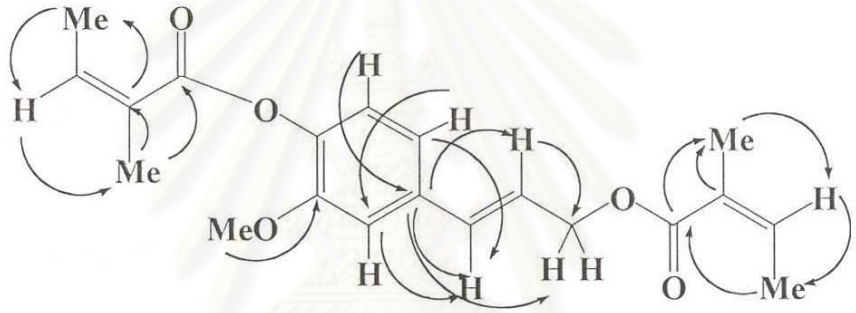


Fig. 3.13 The HMBC Connectivity for Compound2

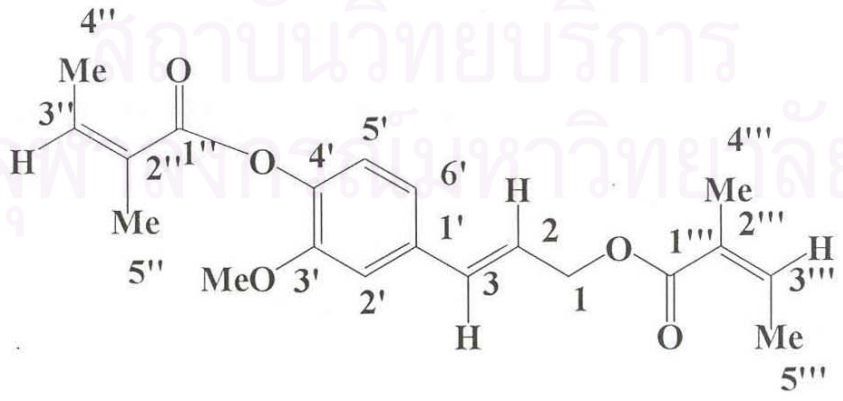
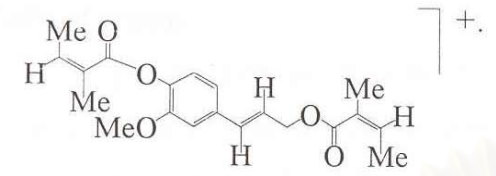


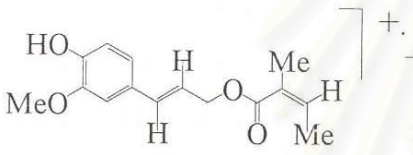
Fig. 3.14 The Structure of Compound 2

1-Angeloxoyloxy-3-[4′-angeloxoyloxy-3′-methoxy]-2-propene

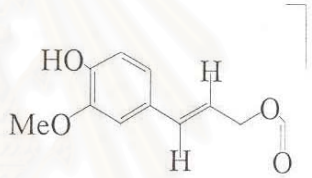
Scheme 3.1 The possible mass fragmentation of Compound 2



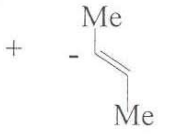
$C_{20}H_{24}O_5$ M^+ 344



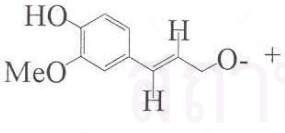
$C_{15}H_{18}O_4$ M^+ 262



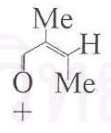
$C_{10}H_{10}O_2$ M^+ 162



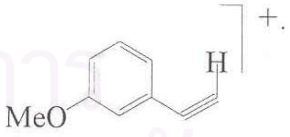
C_4H_7 M^+ 55



$C_{10}H_{11}O_3$ M^+ 179



C_5H_7O M^+ 83



C_9H_7O M^+ 131

3.4.2 Purification, properties and structural elucidation of Compound 3

Compcund 3 is colorless oil (2.0 mg, 8.51 x 10^{-4} % w/w of dichloromethane extract). It exhibited one spot on TLC with R_f value of 0.35 in MeOH:H_2O (7:1) as a solvent system.

The IR spectrum of compound 3 (Fig 3.15) displayed the absorption band due to α,β-unsaturated carbonyl of ester group at 1770 - 1700 cm^{-1}. In addition, the absorption peaks at 1600 - 1400 cm^{-1}suggested the presence of aromatic moiety.

The UV spectrum (Fig 3.16) showed absorption wavelength at 290 (ε = 1.8 x 10^3), 255 (ε = 7.1 x 10^3), and 215 (ε = 1.3 x 10^4) nm.

The mass spectrum (Fig 3.17) showed the molecular peak at m/z 346, which is consistent with a molecular weight of $C_{20}H_{26}O_5$. Other fragmentation and fragmentation pattern displayed in scheme 3.2.

The ^{1}H NMR (Fig 3.18) showed signals of aromatic protons at δ 6.97 (2H, br s) and 6.99 (1H, br s). One methoxy group which was detected at δ 3.84 (3H, s). In addition the methine protons were found at δ 2.45 (1H,d *J*=7) and 2.25 (1H,m).

The ^{13}C NMR (Fig 3.19) exhibited aromatic carbons at δ 138.2, 119.3, 122.9, 133.3, 151.2, and 110.2 ppm, two carbonyl esters at δ 167.1 and 171.0 ppm. The methoxy group was detected at δ 55.9 ppm. The ^{1}H and ^{13}C NMR data of this compound are shown in the Table 3.10.

The proton and carbon connectivity was studied by HMQC and supported by the COSY and HMBC cross peaks as indicated in Fig 3.20 and Fig 3.21, respectively. By analysis of the NMR data it was suggested that compound 3 is a derivative of compound 2 where C-2" double bond is saturated. The geometries of the remaining double bonds were determined by NOESY experiment (Fig 3.22) and confirmed by comparison with those of compound 2. The portion of 2-methyl butanolic acid in compound 3 is confirmed by NMR comparison with the known compound 3a (Table 3.11).[18]

According to the above data compound 3 was named 1-Angeloxoyloxy-3-[4'-isopentanoloxoyloxy-3'-methoxy]-2-propene. However, this compound was analogue of coniferyl alcohol. Computational searching suggested that there was no report of the isolation of this compound; consequently, it was a new compound.

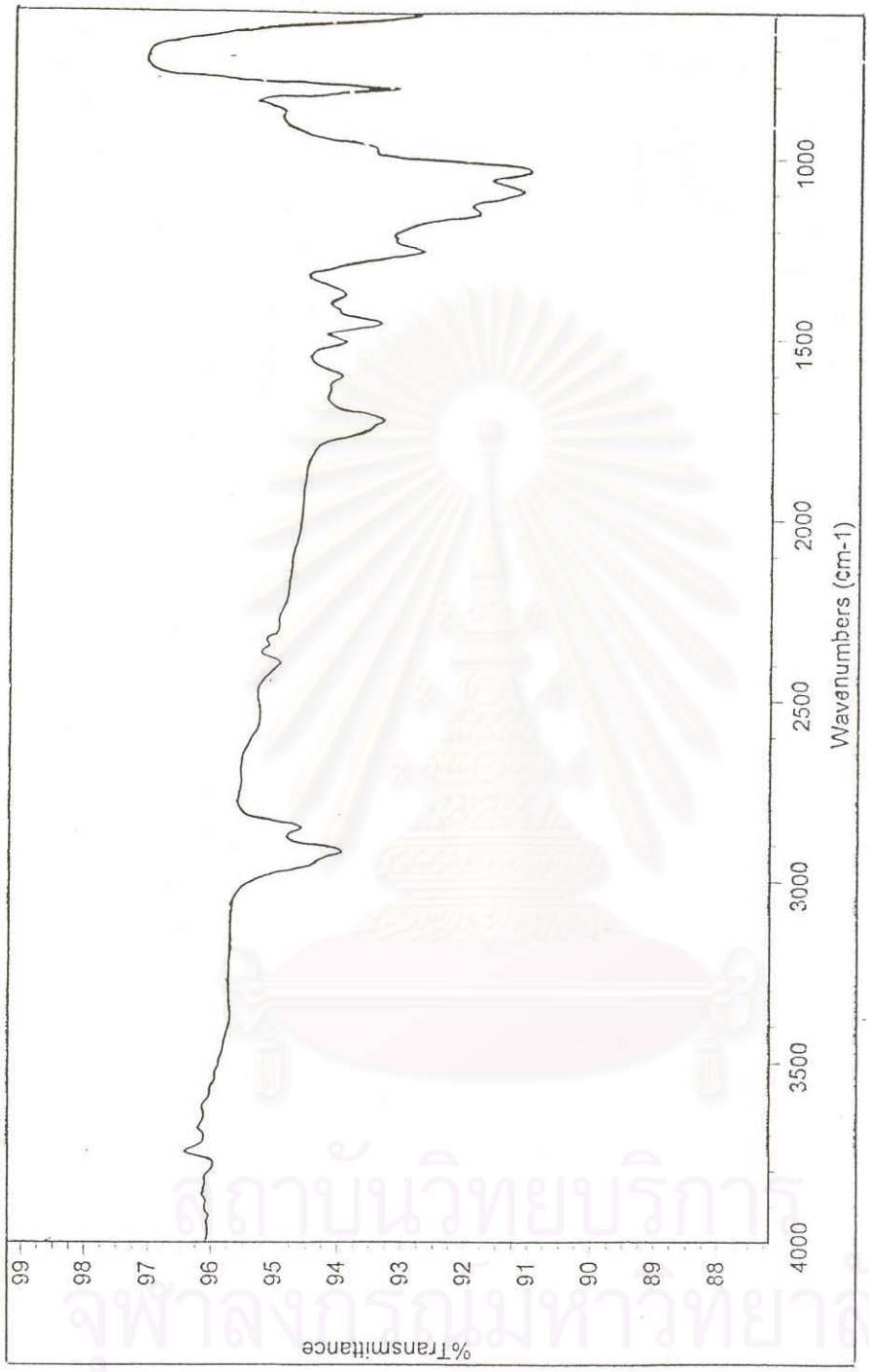


Fig 3.15 The IR Spectrum of Compound 3

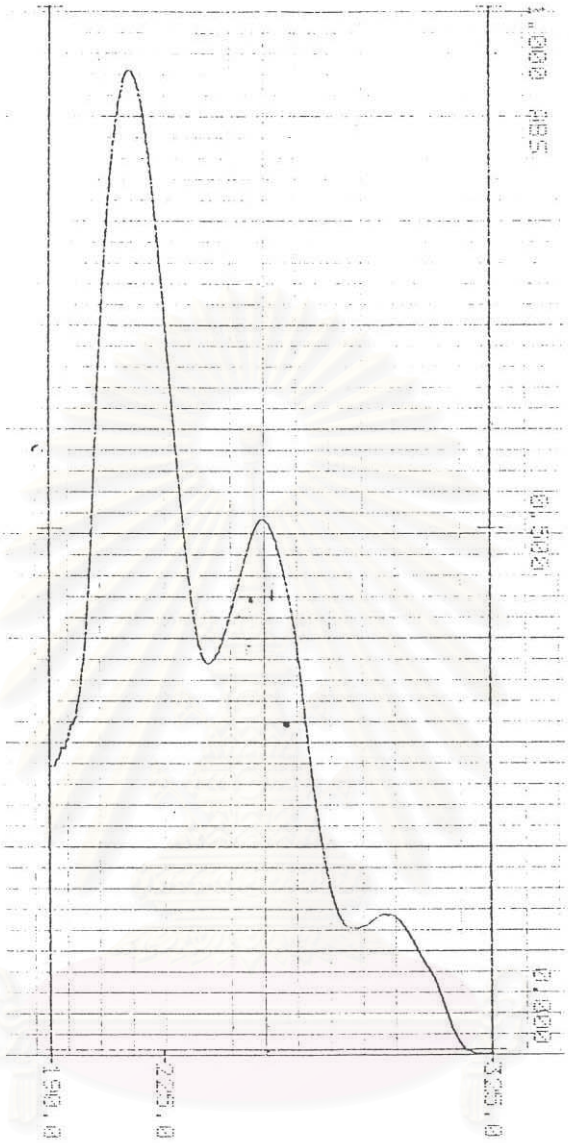


Fig 3.16 The UV Spectrum of Compound 3

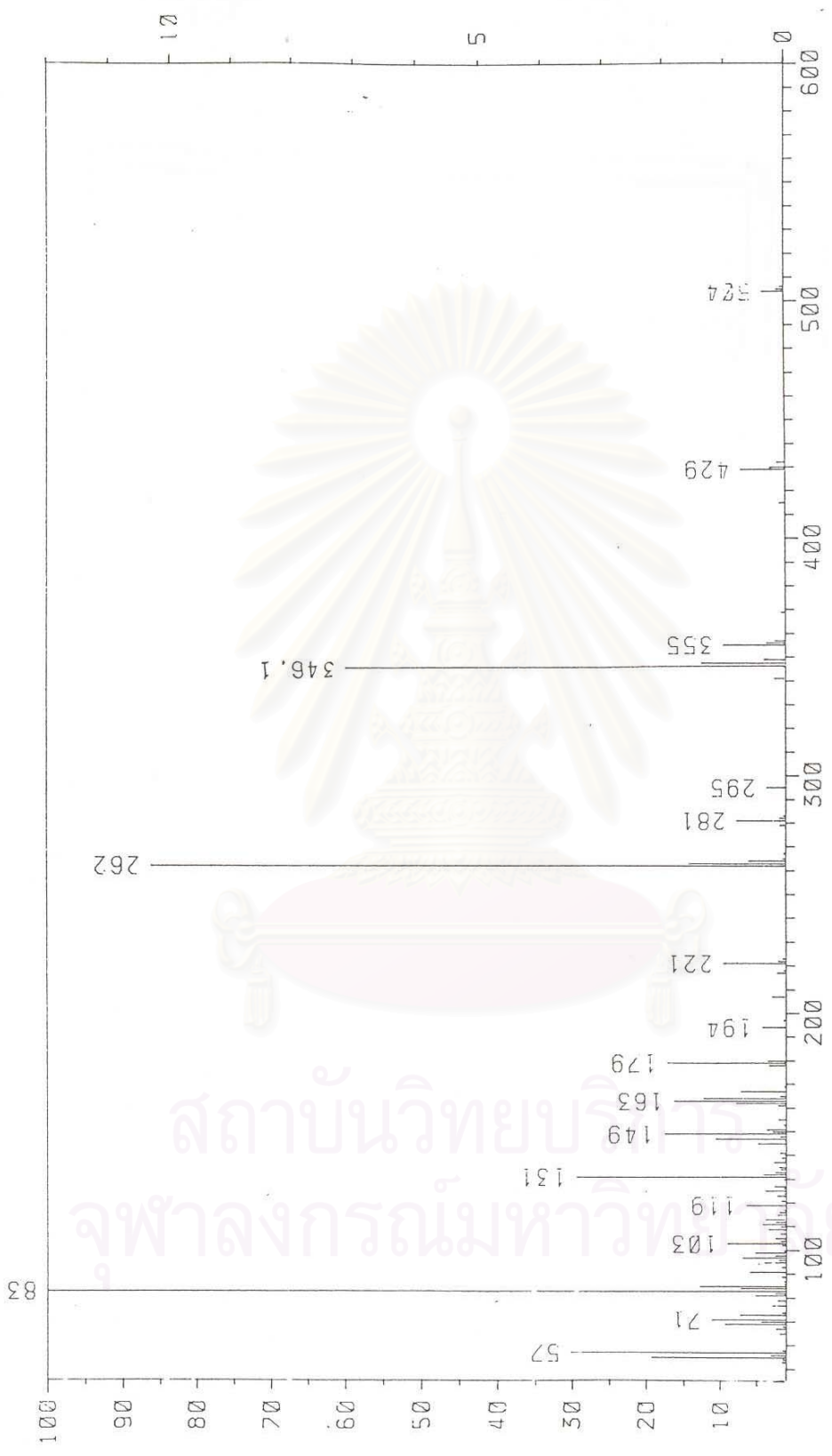


Fig 3.17 The mass Spectrum of Compound 3

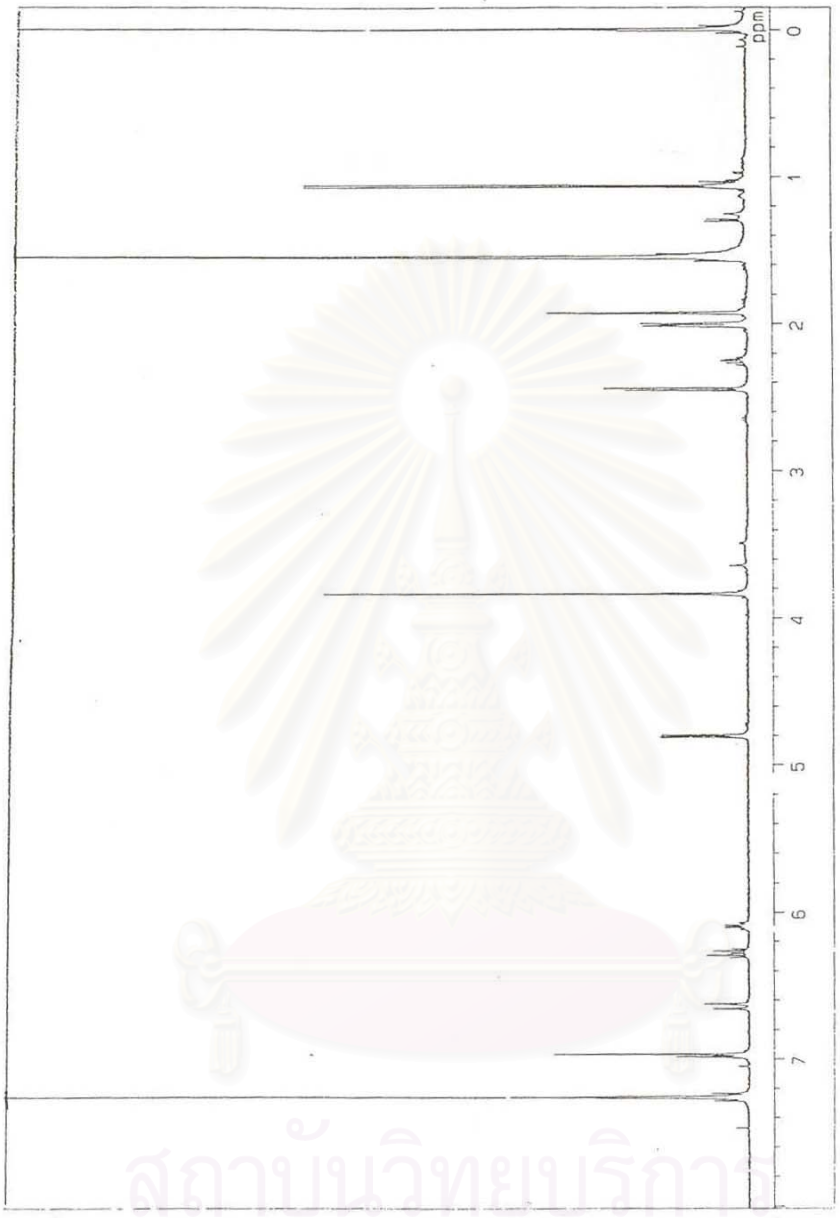


Fig 3.18 The ^{1}H NMR Spectrum of Compound 3

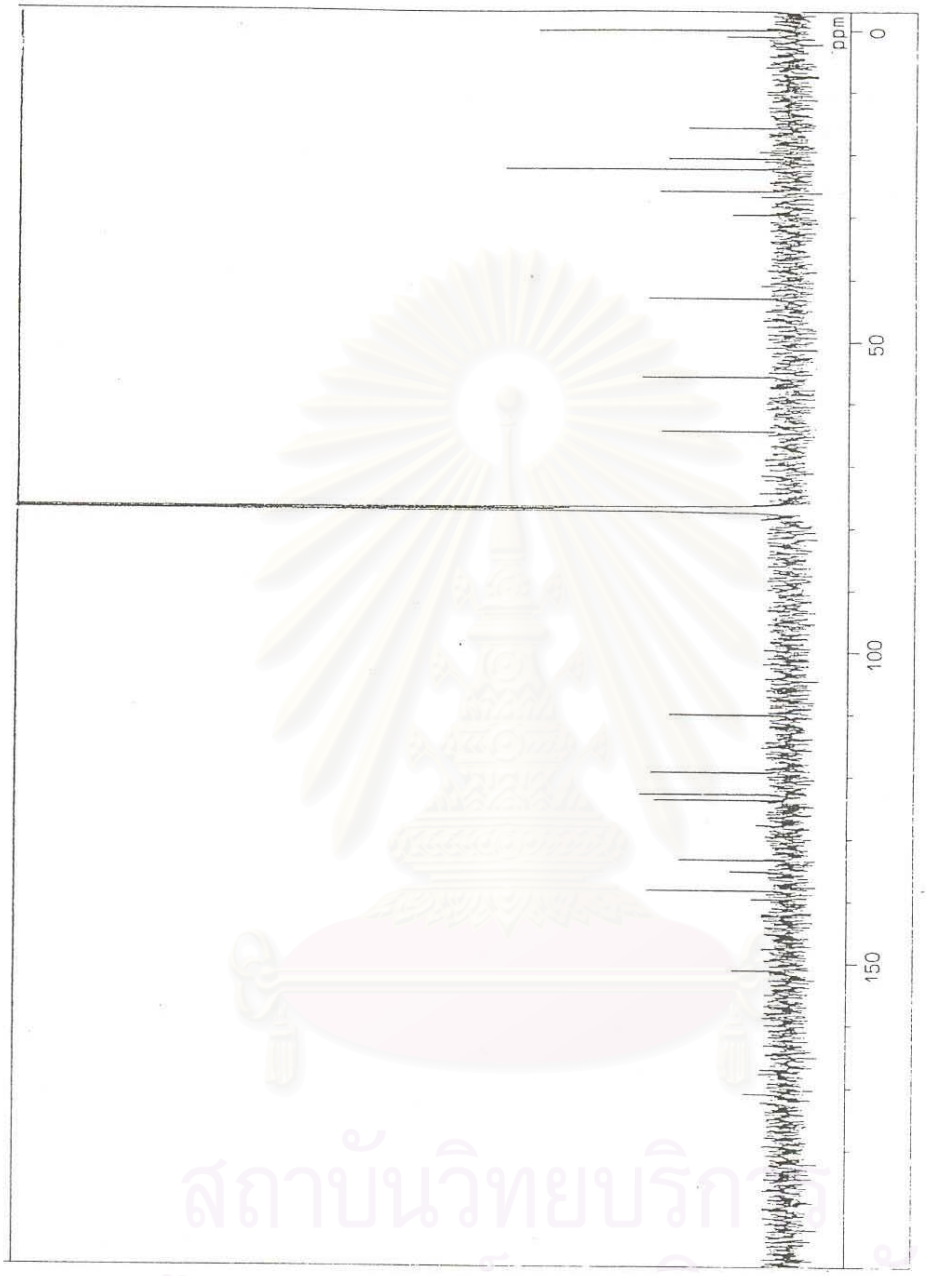


Fig 3.19 The ^{13}C NMR Spectrum of Compound 3

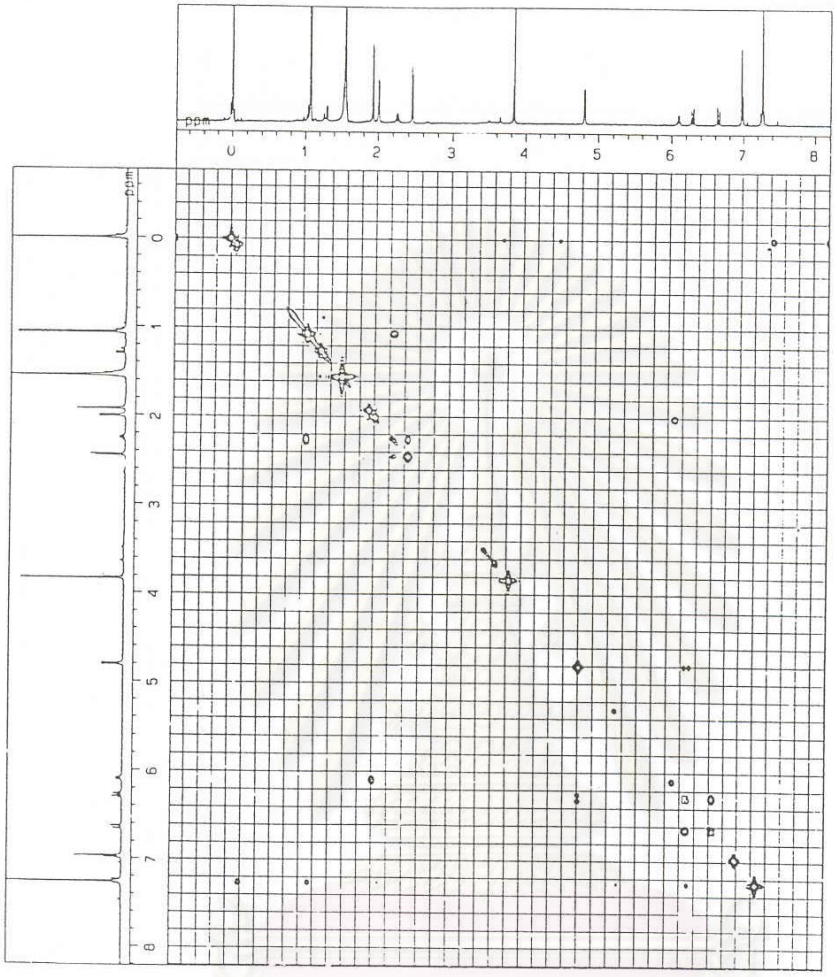


Fig 3.20 The COSY Spectrum of Compound 3

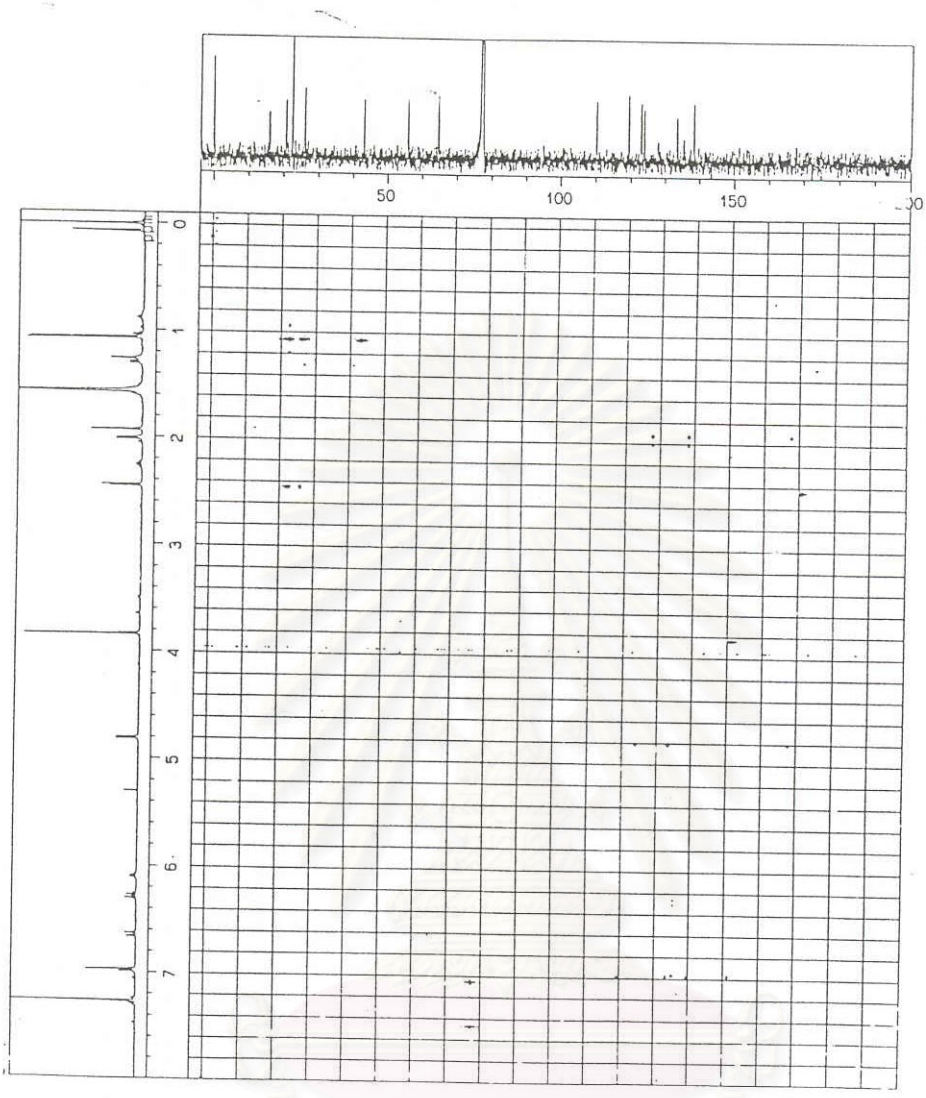


Fig 3.21 The HMBC Spectrum of Compound 3

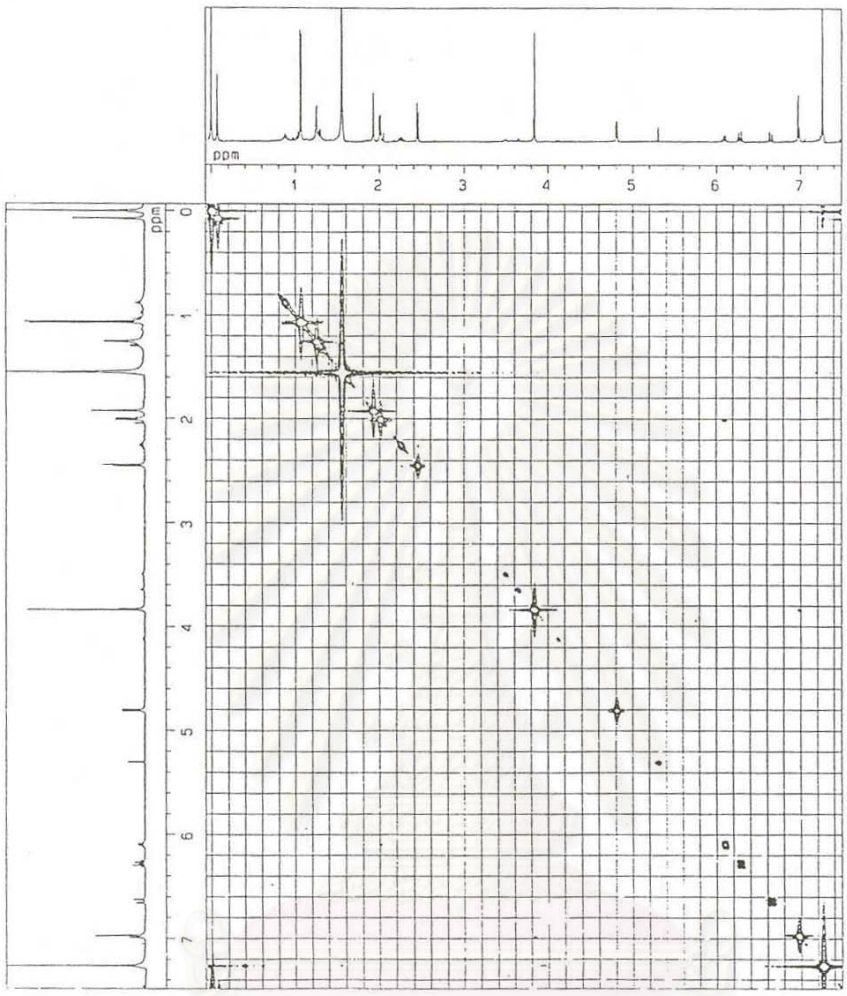


Fig 3.22 The NOESY Spectrum of Compound 3

Table 3.10 The ^{1}H and ^{13}C NMR data of Compound 3

Position	Compound 3 ($CDCl_3$)	
	^{1}H, mult, *J*(Hz)	^{13}C, Hz
1	4.81, dd, *J*=1.5,6.5	64.5
2	6.28, td, *J*=6.5,16	123.8
3	6.64, br d, *J*=16	135.2
1'		138.2
2'	6.99, br s	110.2
3'		151.2
4'		133.3
5'	6.97, br s	122.9
6'	6.97, br s	119.3
1"		171.0
2"	2.45, d, *J*=7	43.0
3"	2.25, m	25.9
4"	1.07, d, *J*=6.5	22.3
5"	1.07, d, *J*=6.5	22.3
1"'		167.1
2"'		127.8
3"'	6.09, qq, *J*=0.5,7	139.6
4"'	1.92, dq, *J*=0.5,1.5	20.6
5"'	2.01, qq, *J*=1.5,7.5	15.8
-OMe	3.84, s	55.9

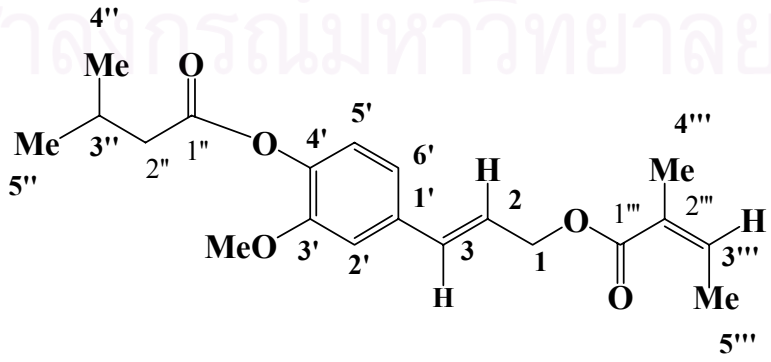


Fig. 3.23 Structure of Compound 3

Table 3.11 The ^{1}H and ^{13}C NMR Data of Compound 3a

Position	Sinapyl alcohol diisovalerate ($CDCl_3$)	
	^{1}H, mult, *J*(Hz)	^{13}C, Hz
1	4.71, dd, *J*=6.4,0.9	64.6 t
2	6.22, dt, *J*=16,6.4	123.7 d
3	6.50, dt, *J*=16,6.4	134.0 d
1'		128.8 s
2'	6.61, s	103.4 d
3'		152.3 s
4'		134.6 s
5'		152.3 s
6'	6.61, s	103.4 d
1"		170.7 s
2"	2.45, d, *J*=7.5	43.0 t
3"	2.2, m	25.7
4"	1.06, d, *J*=6.5	22.3 q
5"	1.06, d, *J*=6.5	22.3 q
1"'		172.9 s
2"'	2.23, d, *J*=7.5	43.4 t
3"'	2.2, m	26.04
4"'	0.97, d, *J*=6.5	22.44 q
5"'	0.97, d, *J*=6.5	22.44 q
-OMe	3.9, s	56.1 q
-OMe	3.9, s	56.1 q

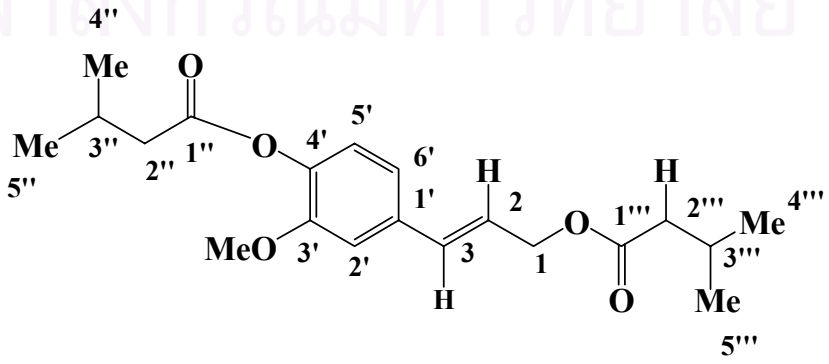


Fig. 3.24 Structure of Compound 3a

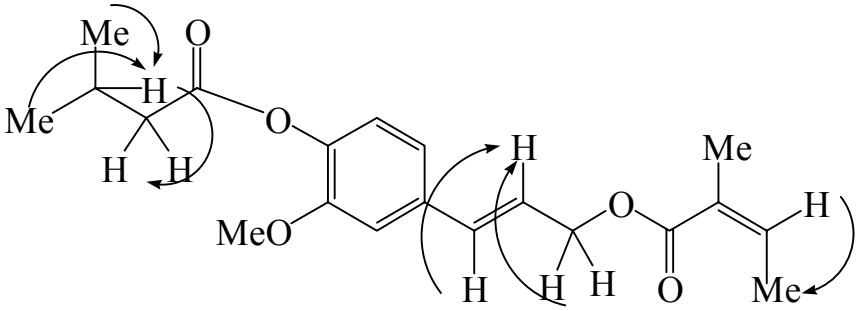


Fig. 3.25 The COSY Connectivity of Compound 3

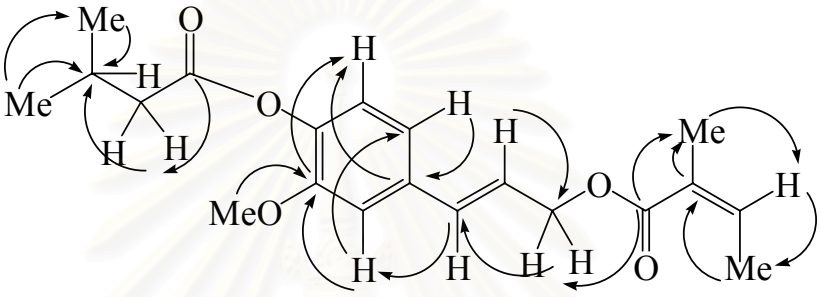


Fig. 3.26 The HMBC Connectivity of Compound 3

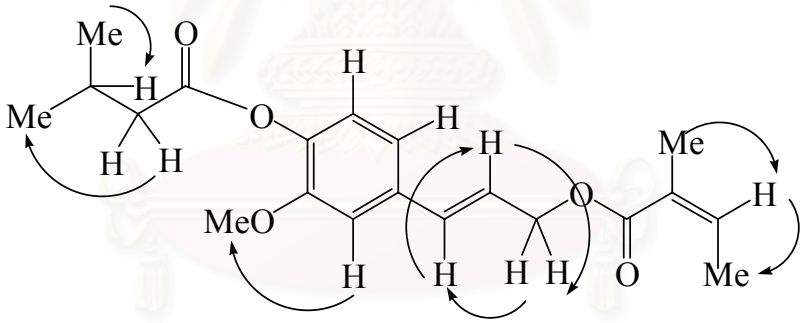


Fig. 3.27 The NOESY Connectivity of Compound 3

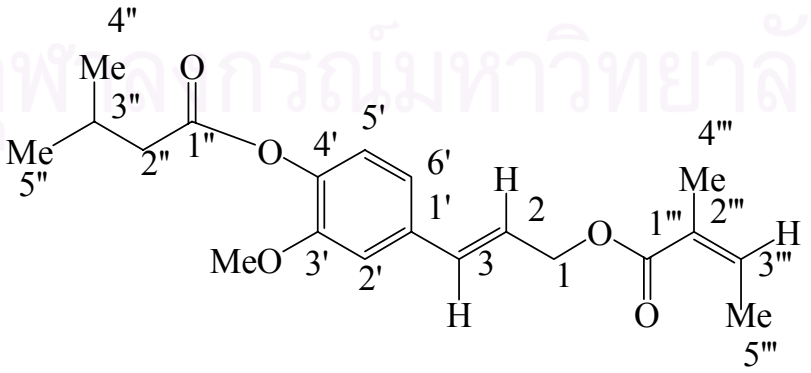
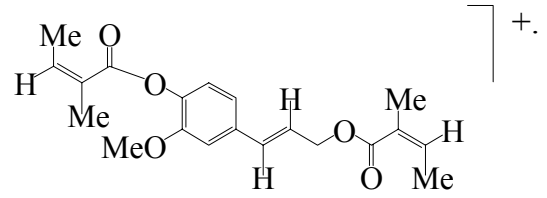


Fig. 3.28 The Structure of Compound 3

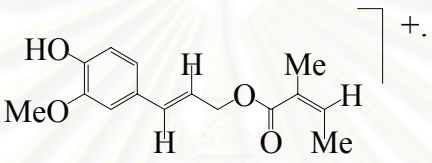
1-Angeloxoyloxy-3-[4'-isopentanoloxoyloxy-3'-methoxy]-2-propene

Scheme 3.2 The Possible Mass Fragmentation of Compound 3

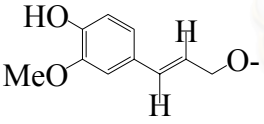


$C_{20}H_{26}O_5$ M^+ 346

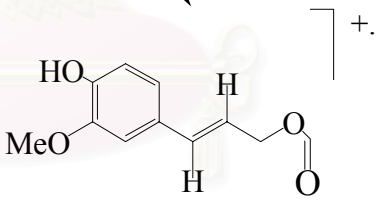
C_4H_9 +
M^+ 57



$C_{15}H_{18}O_4$ M^+ 262

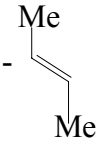


$C_{10}H_{11}O_3$ M^+ 179



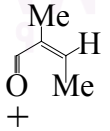
$C_{10}H_{10}O_2$ M^+ 162

+

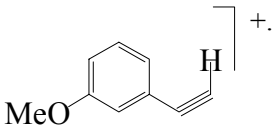


C_4H_7 M^+ 55

+



C_5H_7O M^+ 83



C_9H_7O M^+ 131

3.4.4 Purification, Properties and Structural Elucidation of Compound 4

Compound 4 was isolated from dichloromethane extract by eluting with 50% dichloromethane in hexane. This fraction was further purified by VLC (Si-60) with stepwise gradient elution (heptane-CH_2Cl_2-EtOAc-MeOH) to give 7 fractions. Chlorophyll was removed from fraction 4 by VLC (MCI GEL CHP 20P) eluting with MeOH and then with EtOAc. The MeOH fraction was separated by OCC (Si-60) with increasing polarity of the eluents (hexane-CH_2Cl_2-MeOH) to give four fractions. Fraction 2 was purified by preparative TLC (Si-60; hexane:EtOAc (3:2)) to give this compound. Compound 4 was colorless oil 9.2 mg (3.91×10^{-3} % w/w of dichloromethane extract). Its R_f value was 0.65 (hexane:EtOAc 3:2).

The IR spectrum of this compound (Fig 3.29) showed an intense band of OH stretching at 3530 cm^{-1}, the characteristic band of α,β-unsaturated ester ($C=CCO_2R$) at 1710 cm^{-1} and α,β-unsaturated ketone (C=CC=O) at 1650 cm^{-1}.

The UV absorption was measured (Fig 3.30) and showed the maximum wavelength at 225 nm ($\varepsilon = 2.6 \times 10^4$). This compound has optical rotation activity of $[\alpha]_D^{29}$ -227.75 (c 0.4833, $CHCl_3$).

The mass spectrum (Fig 3.31) revealed a molecular ion peak at m/z 364, calculated for $C_{20}H_{28}O_6$. The loss of one part of angelate and two molecules of angelate obtained peaks at m/z 265 and 164, respectively. Other fragmentation peaks were found at m/z 83 $[C_4H_7CO]^+$, the characteristic of cyclic ketone and at m/z 55 $[83\text{-}CO]^+$. The possible mass fragmentation pattern[6] of this compound was shown in scheme 3.3.

The ^1H NMR spectrum (Fig 3.32) indicated important proton signals at δ (ppm); 7.04 (1H, dt, *J*=1, 6 Hz), 6.15 (1H, dq, *J*=1.5,7 Hz), 6.18 (1H,dq, *J*=1.5,7 Hz), 5.84 (1H, d, *J*=12.5 Hz), 5.76 (1H, dd, *J*=3.5,5.5 Hz), 4.29 (1H, br d, *J*=14 Hz), 4.37 (1H, br d, *J*=14 Hz), 2.51 (1H, dddd, *J*=3.5, 7.0, 12.5 Hz), 2.07 (1H, dd, *J*=2.5 ,7.0 Hz), 1.92 (3H, q, *J*=1.5 Hz), 1.97 (3H,q, *J*=1.5 Hz), 1.05 (3H, d, *J*=7.0 Hz), 1.00 (3H, d, *J*=7 Hz), and 2.03 (2H, dq, *J*=1.5, 6.0 Hz)

The ^{13}C NMR spectrum (Fig 3.33) showed a ketonic carbon (δ 195.3), ester carbons (δ 167.0 and 166.7), methine carbons (δ 73.0, 66.8, 46.4, and 27.7), methylene carbon (δ 60.7), methyl carbons (δ 19.9, 19.6, 16.0, and 15.8), tertiary carbon (δ 138.9, 140.4, and 139.4) and quarternary carbons (δ 139.1, 126.9, and 129.2) The ^1H and ^{13}C NMR data are shown in Table 3.12.

Generally, most carvotanacetones had hydroxy groups at C-3, C-5, and C-7 which were always esterified by most common acids such as tiglic acid, angelic acid, and acetic acid. In compound 4 the substituents in these positions were confirmed by COSY ans HMBC spectrum showed in Fig 3.34 and Fig 3.35, respectively. It was indicated that the substituents on C-3 and C-5 are angelate and on C-7 is a hydroxy group.

Relative stereochemistry of three methines at δ 5.76 (H-3), 2.51 (H-4), and 5.74 (H-5) was deduced from their coupling constant. Owing to the greater coupling constant of $^{3}J_{4,5}$ (12.5 Hz), H-4 and H-5 were arranged in the opposite direction, while H-3 and H-4 were oriented in the same plane. The ^{1}H-^{1}H COSY spectrum allowed the assignment of all proton signals while the ^{13}C NMR signals were assigned by HMQC and HMBC experiments. The ^{1}H-^{1}H COSY connectivity, ^{1}H-^{13}C HMBC connectivity are showed in Fig 3.36 and Fig 3.37, respectively.

All above data allowed us to assign compound 4 as 3α–5β–diangeloxoyloxy-7-hydroxy carvotanacetone and confirmed with the reference.[15,20] The structure of this compound is exhibited in Fig 3.38.

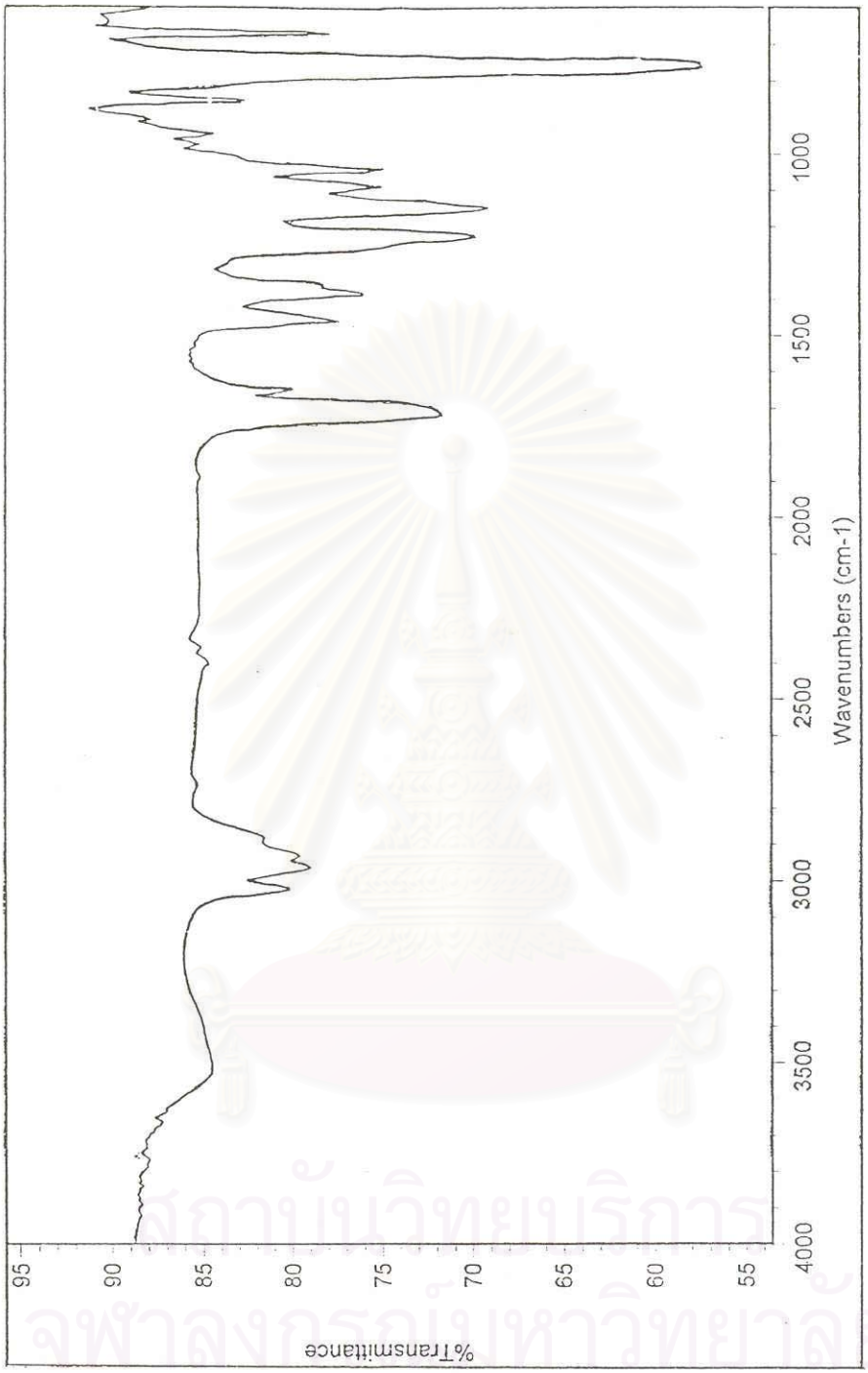


Fig 3.29 The IR Spectrum of Compound 4

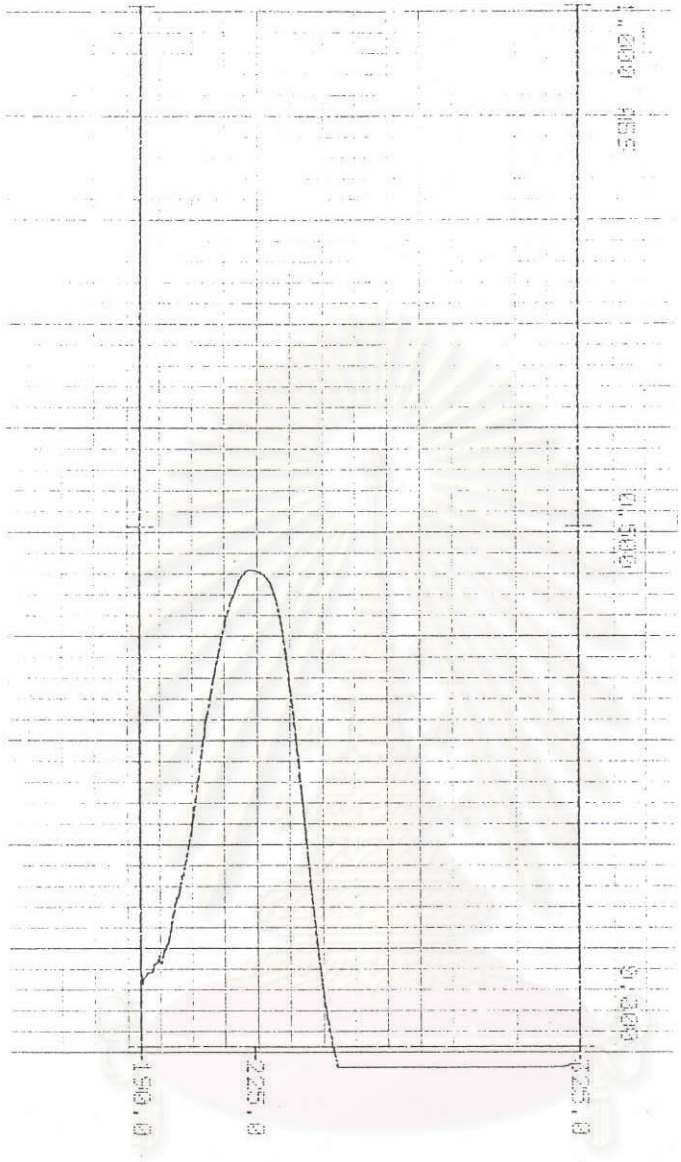


Fig 3.30 The UV Spectrum of Compound 4

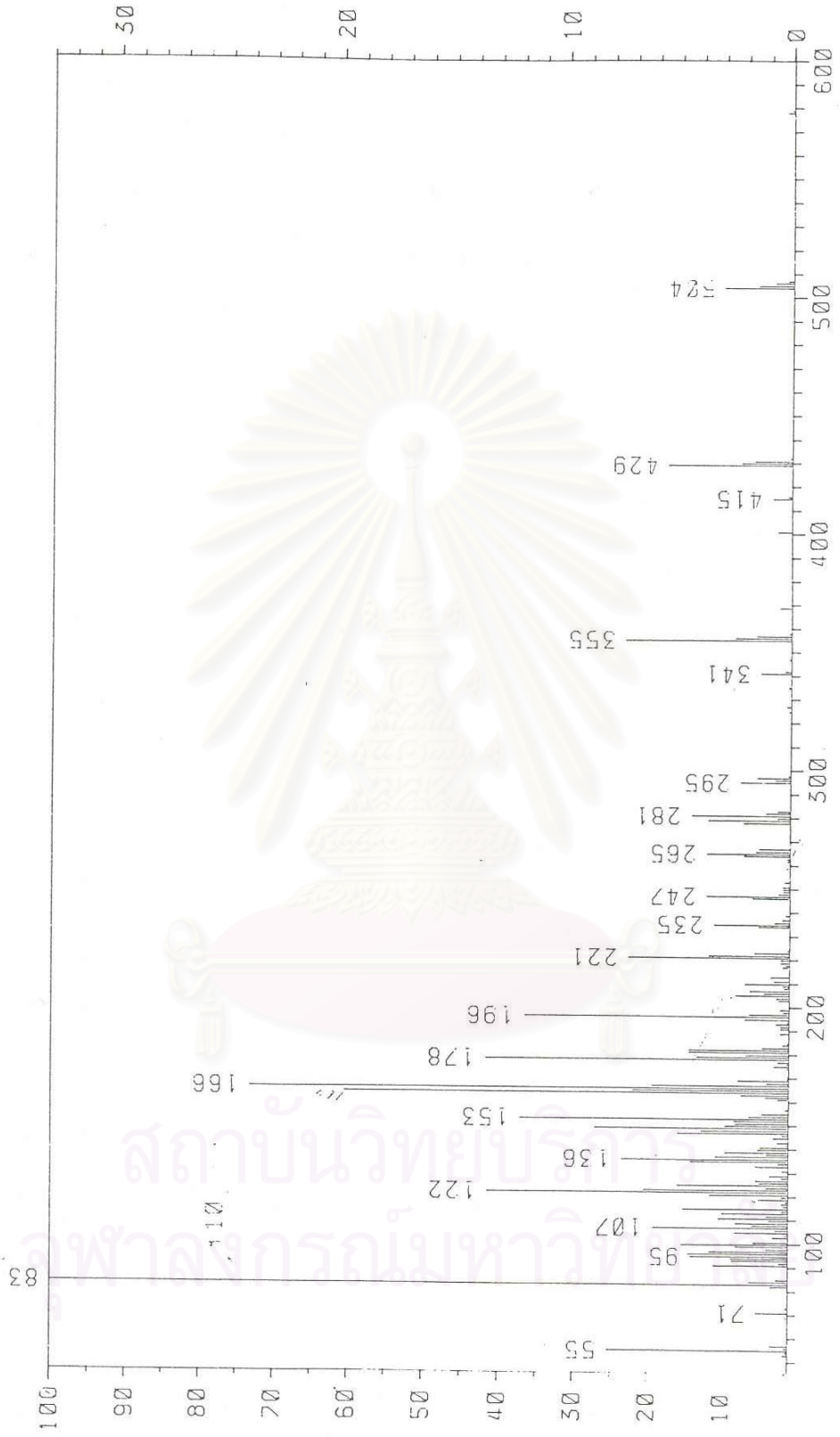


Fig 3.31 The mass Spectrum of Compound 4

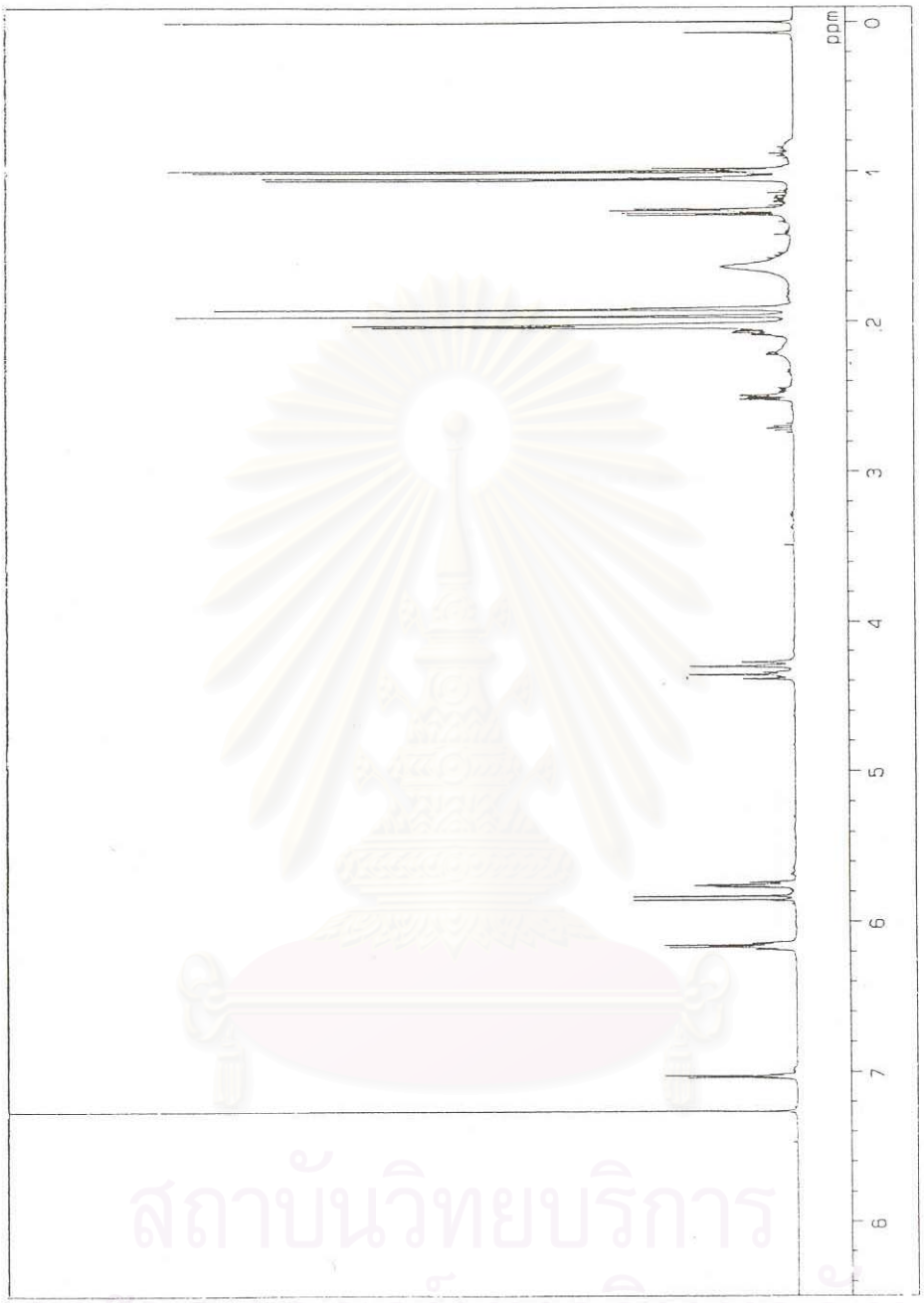


Fig 3.32 The ^{1}H NMR Spectrum of Compound 4

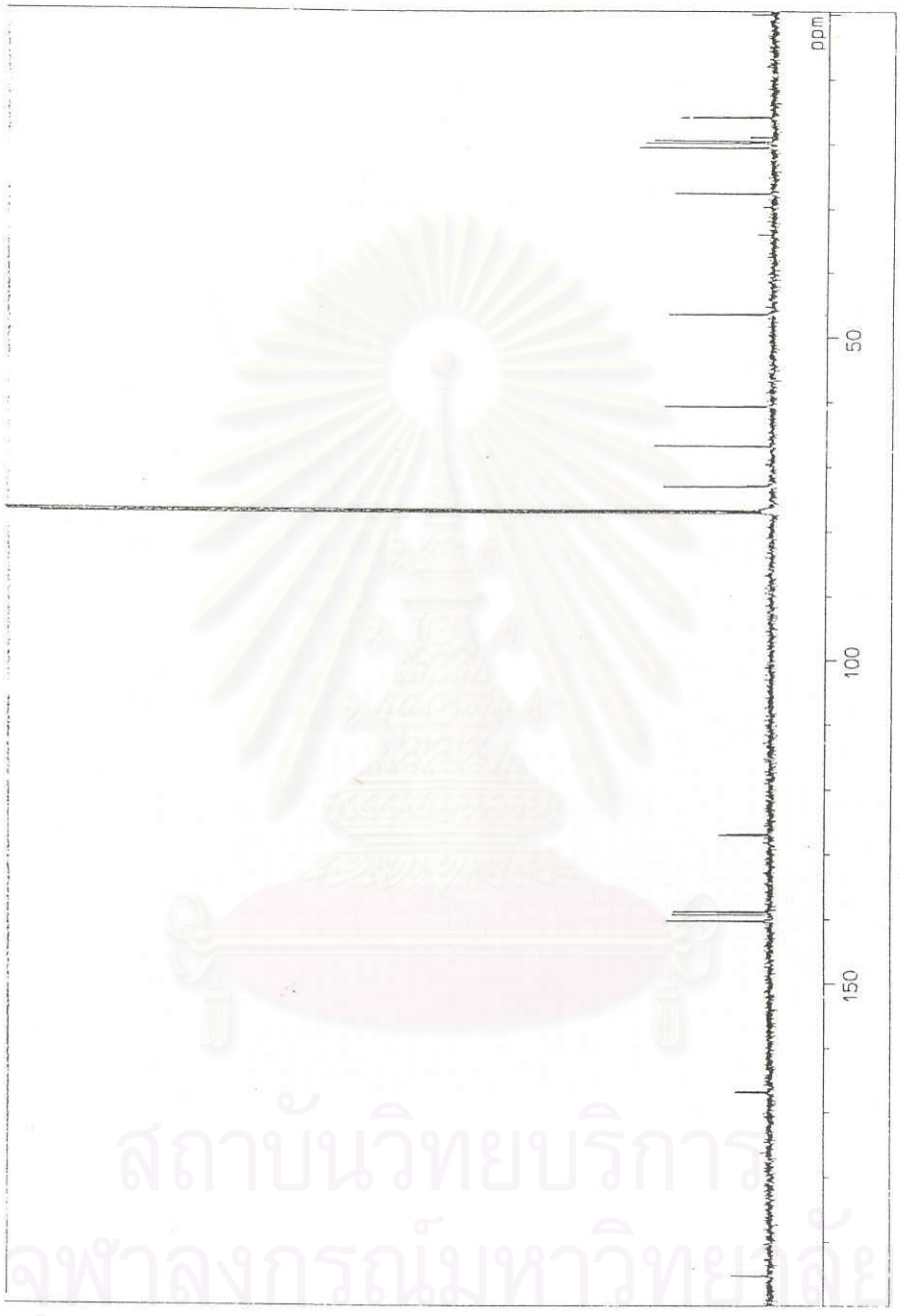


Fig 3.33 The ^{13}C NMR Spectrum of Compound 4

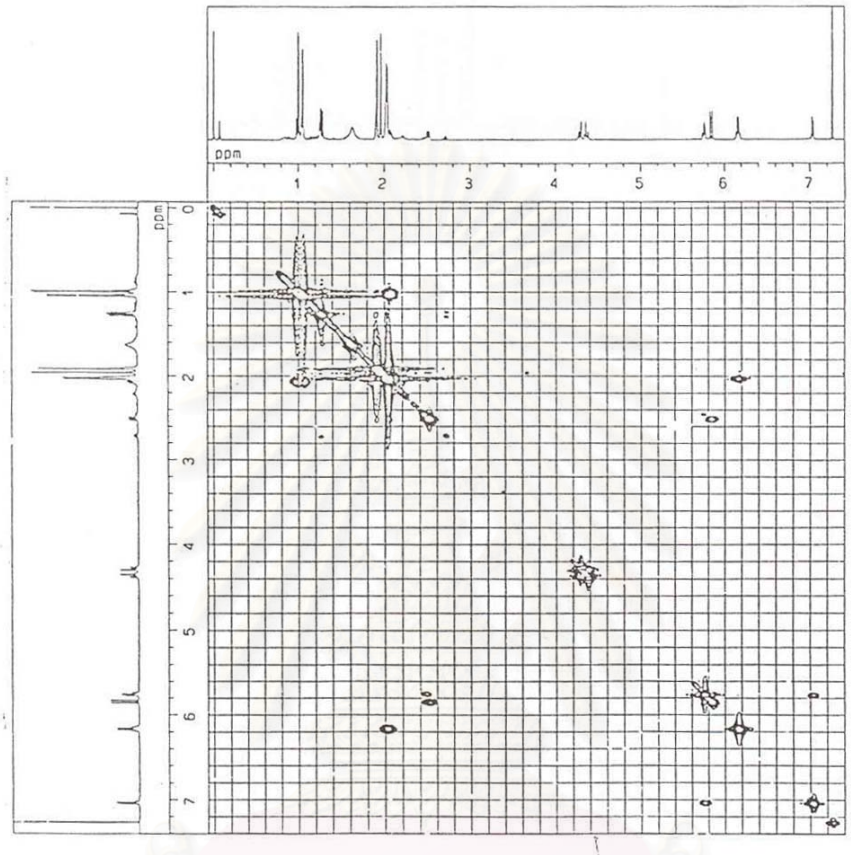


Fig 3.34 The COSY Spectrum of Compound 4

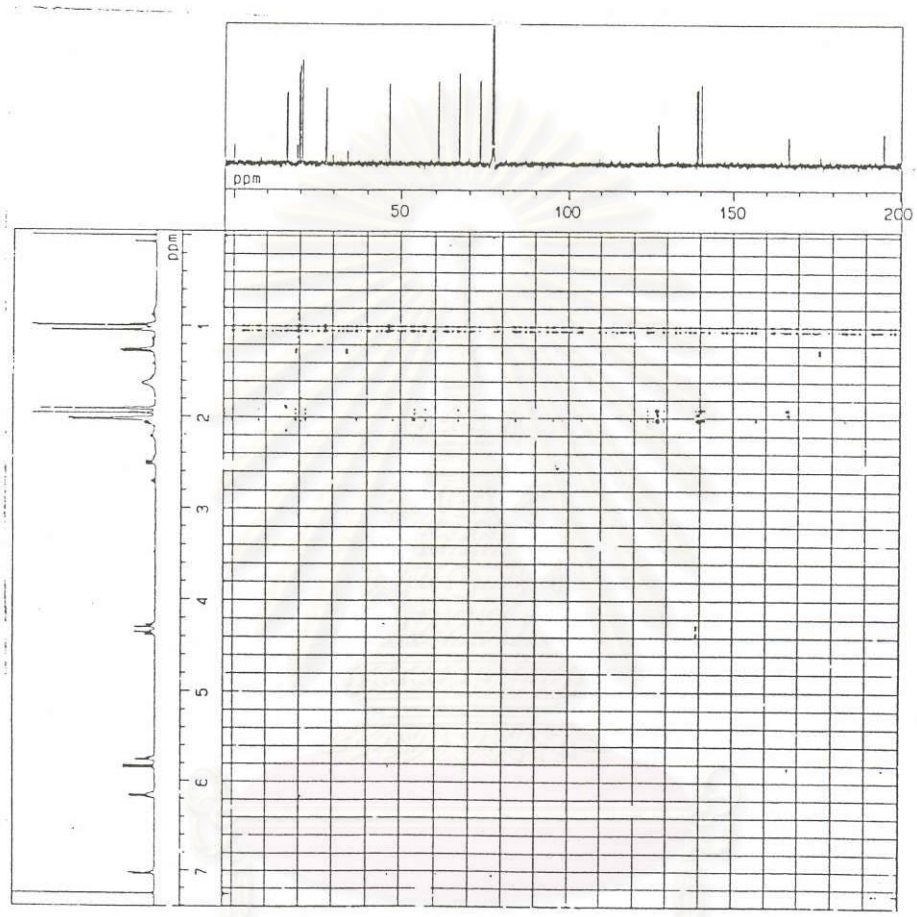


Fig 3.35 The HMBC Spectrum of Compound 4

Table 3.12 The ^{1}H and ^{13}C NMR Data of Compound 4

Position	Compound 4 ($CDCl_3$)	
	^{1}H, mult, *J*(Hz)	^{13}C, Hz
1		139.1
2	7.04, dt, *J=1,6*	138.9
3	5.76, dd, *J=3.5, 5.5*	66.8
4	2.51, dddd, *J=3.5,5.5*	46.4
5	5.84, d, *J=12.5*	73.0
6		195.3
7	4.29, br d, *J=14* 4.37, br d, *J=14*	60.0
8	2.07, dd, *J=2.5, 7*	27.7
9	1.00, d, *J=7*	19.6
10	1.05, d, *J=7*	19.9
1'		166.7
2'		126.9
3'	6.15, dq, *J=1.5, 7*	140.4
4'	2.03, dq, *J=1.5, 6*	15.8
5'	1.92, q, *J=1.5*	20.6
1"		167.0
2"		127.2
3"	6.18, dq, *J=1.5, 7*	139.4
4"	2.03, dq, *J=1.5, 6*	16.0
5"	1.97, q, *J=1.5*	20.5

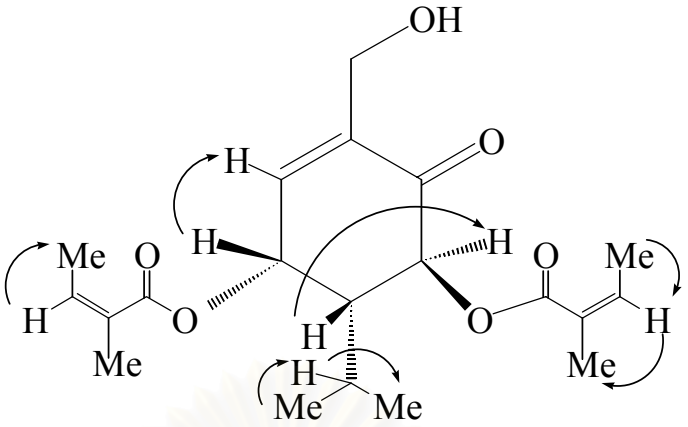


Fig. 3.36 The COSY Connectivity of Compound 4

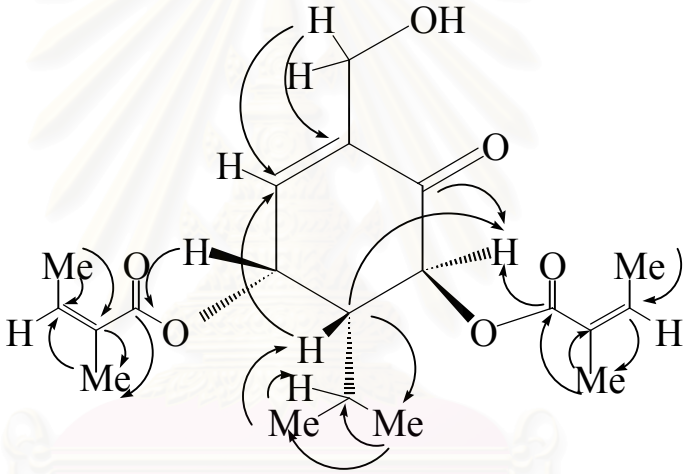


Fig. 3.37 The HMBC Connectivity of Compound 4

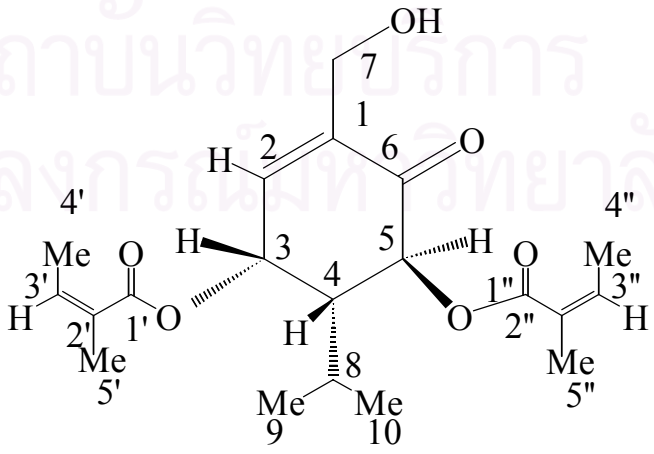
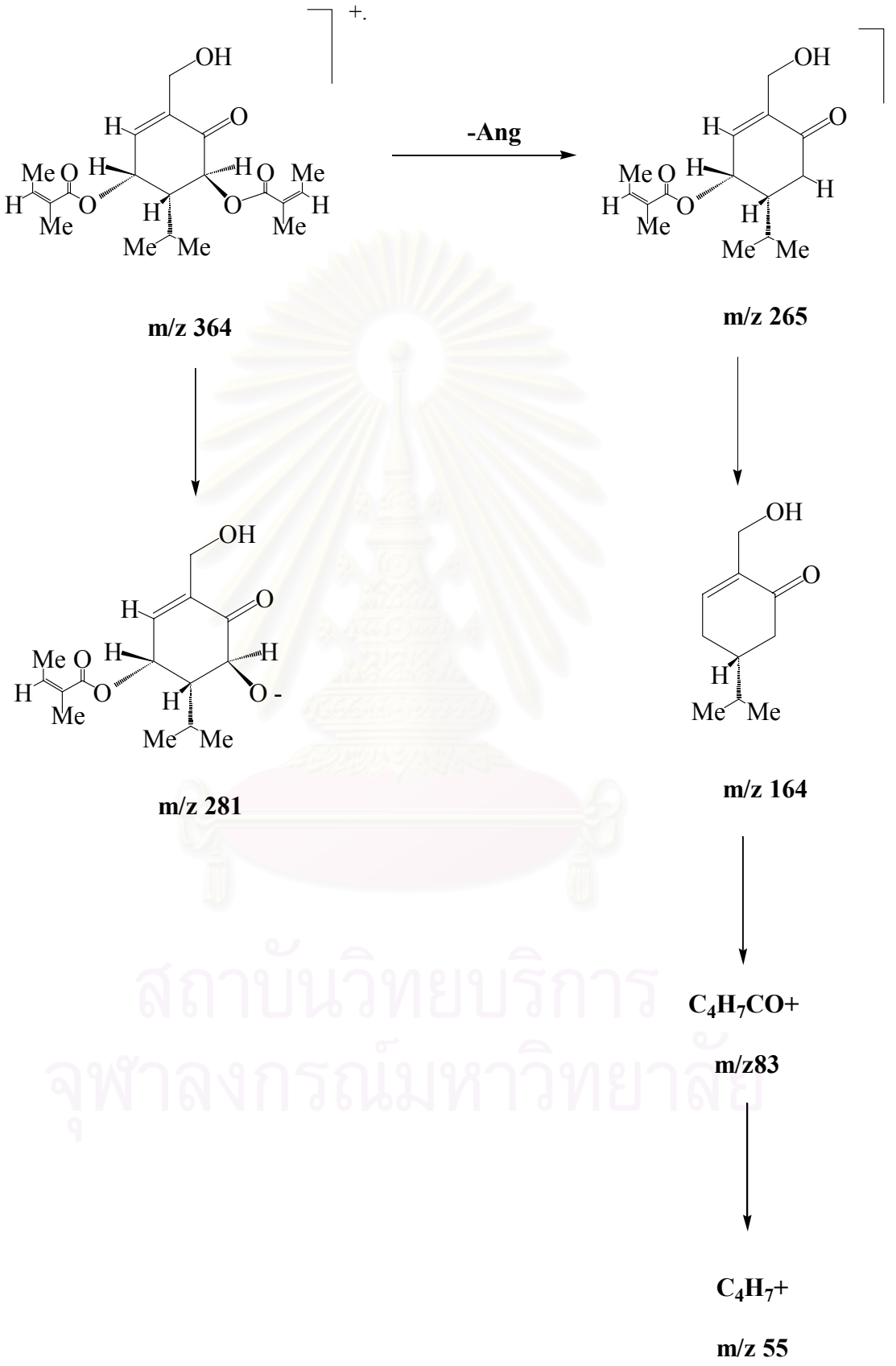


Fig. 3.38 The Structure of Compound 4

3α,5β-Diangeloxoyloxy-7-hydroxycarvotanacetone

Scheme 3.3 The Possible Mass Fragmentation of Compound 4



3.4.5 Purification, Properties and Structural Elucidation of Compound 5

Compound 5 (75.8 mg, 3.22 x 10^{-2} % w/w of dichloromethane extract), yellow needles and m.p. = 214-215°C, was isolated from dichloromethane extract by eluting with 100% dichloromethane . Chlorophyll was removed from this fraction by VLC (MCI GEL CHP 20P) using MeOH and EtOAc as the eluents. MeOH fraction was separated by OCC (Si-60) with increasing polarity of eluent (hexane-EtOAc-MeOH) to give eleven fractions. Fraction 5 and 6 were recrystallised from CH_2Cl_2:MeOH (1:1) to obtain this compound.

The IR spectrum (Fig 3.39) exhibited an absorption band due to OH stretching vibration of hydroxy group at 3400 - 3300 cm^{-1}. The absorption band at 1660 cm^{-1} which corresponded to the C=O stretching vibration of , possibly an α,β-unsaturated ester were observed. In addition, the absorption peak at 1600 - 1400 cm^{-1} suggested the presence of aromatic moiety.

The molecular formula of compound 5 was established by low-resolution mass spectroscopy (Fig 3.40) which revealed a molecular ion peak at m/z 360. Other fragmentation and fragmentation pattern displayed in scheme 3.4.

The ^{1}H NMR spectrum (Fig 3.41) and ^{13}C NMR spectrum (Fig 3.42) were measured in DMSO-*d6*. Then the data was investigated and compared with the reference[12] as shown in the Table 3.13.

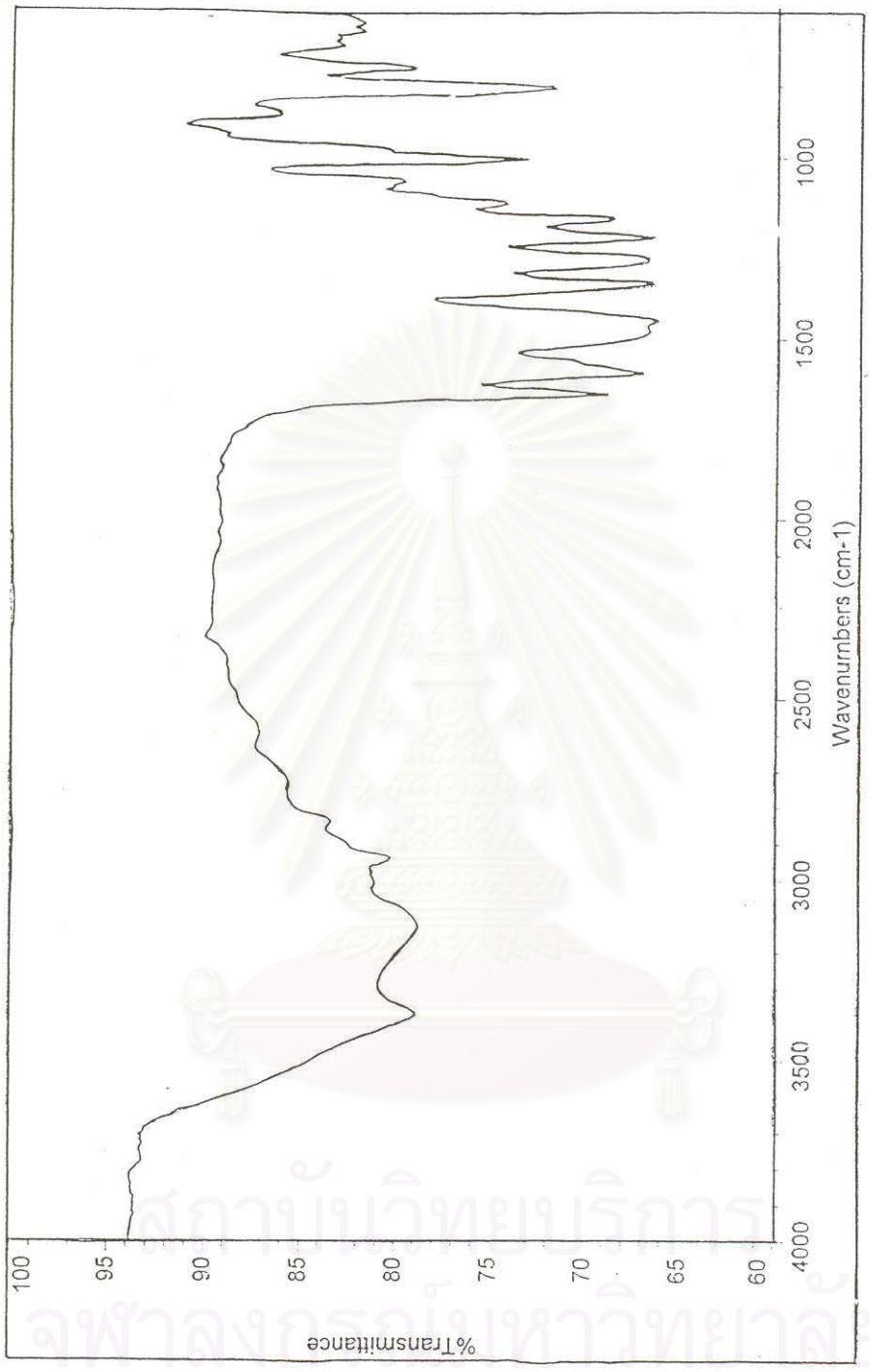


Fig 3.39 The IR Spectrum of Compound 5

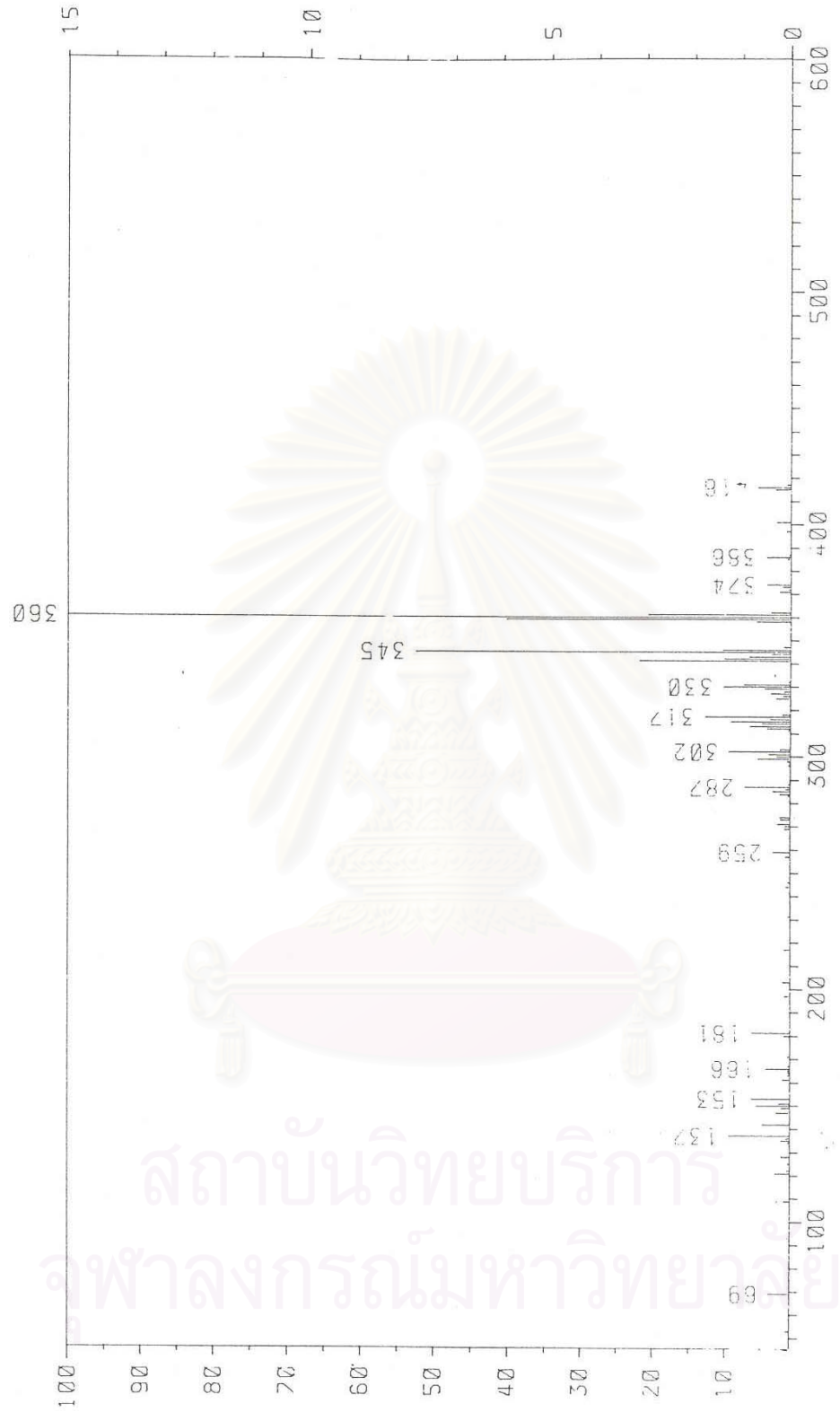


Fig 3.40 The mass Spectrum of Compound 5

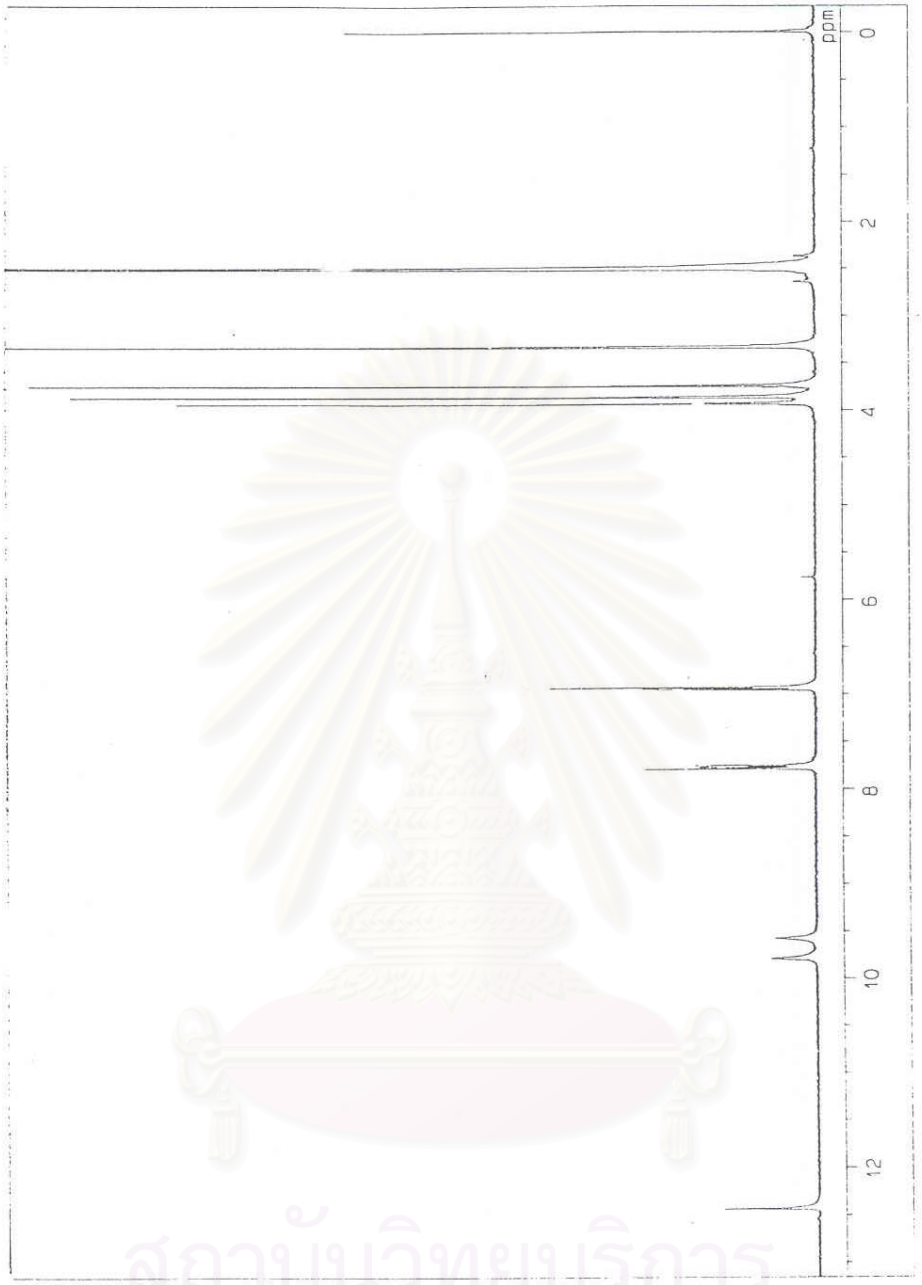


Fig 3.41 The ^{1}H NMR Spectrum of Compound 5

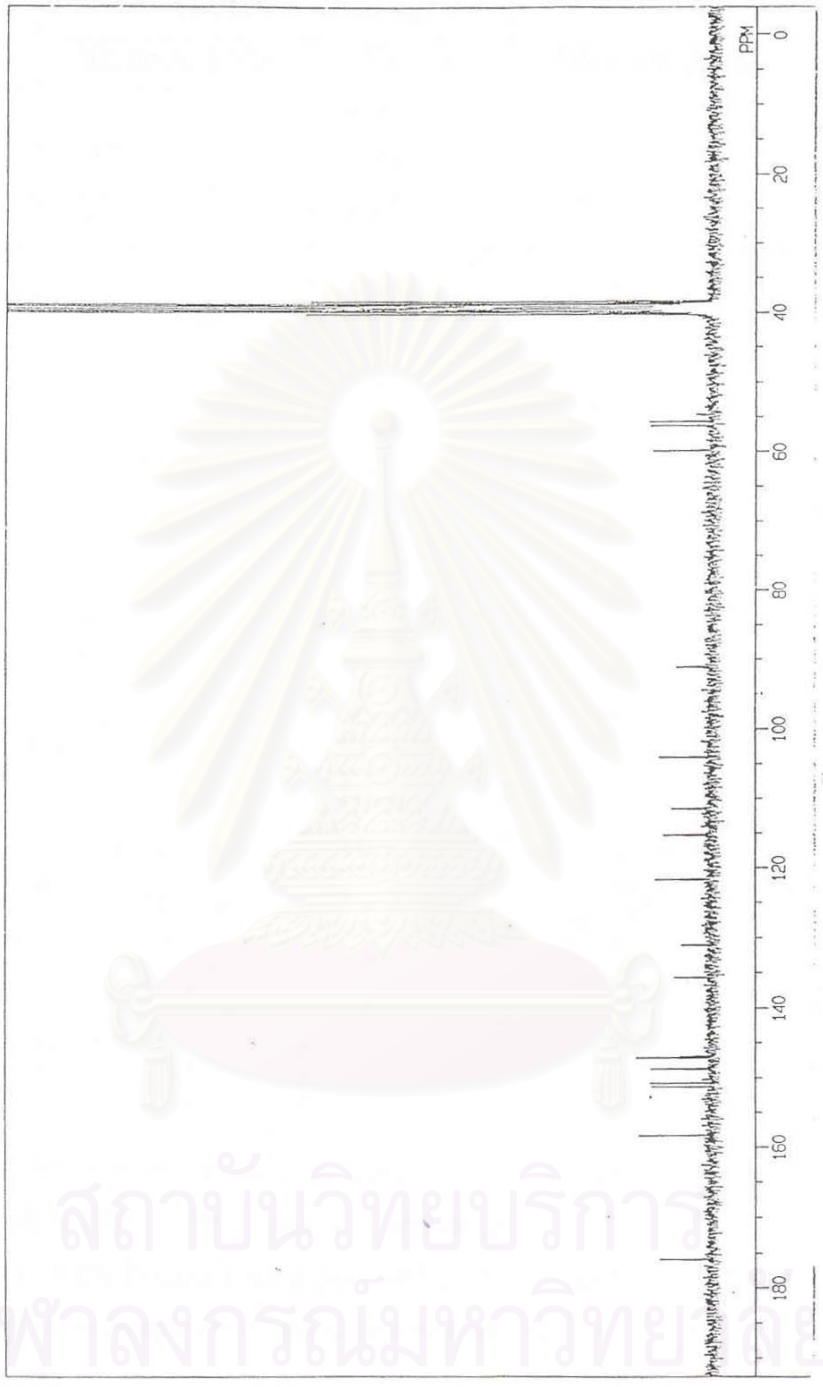


Fig 3.42 The ^{13}C NMR Spectrum of Compound 5

Table 3.13 The ^{1}H and ^{13}C NMR Data of Chrysophenol D and Compound 5

Position	Chrysophenol D (DMSO-d_6+$CDCl_3$)		Compound 5 (DMSO-d_6)	
	^{1}H, mult, *J(Hz)*	^{13}C, Hz	^{1}H, mult, *J(Hz)*	^{13}C, Hz
2		155.9		156.0
3		137.6		137.7
4		178.1		178.2
5		151.7		151.7
5-OH	12.63, s		12.63, br s	
6		131.5		131.6
7		158.5		158.6
8	6.81, s	91.1	6.84, s	91.3
9		151.7		151.7
10		105.5		105.5
1`		120.8		120.7
2`	7.58, s	115.7	7.57, d, J = 2.5	115.5
3`		145.2		145.3
4`		148.8		148.9
5`	6.89, d, J = 9	115.6	6.88, d, J = 8.5	115.7
6`	7.47, d, J = 9	120.5	7.47, dd, J = 2.5,8.4	120.6
-OMe	3.71, s	56.3	3.70, s	56.5
-OMe	3.77, s	59.6	3.77, s	59.6
-OMe	3.81, s	60.0	3.89, s	60.1

All the above data suggested that compound 5 is concluded to be Chrysophenol D. The proton and carbon assignments of this compound were achieved on the basis of comparison with previous report. The structure, the ^{1}H and ^{13}C NMR data assignment of this compound are exhibited in Fig 3.43, 3.44, and 3.45, respectively.

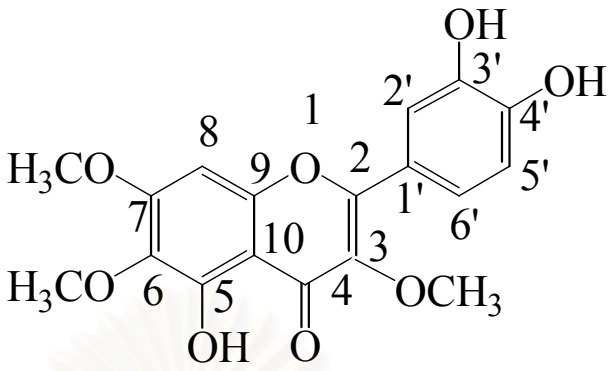


Fig. 3.43 The Structure of Compound 5 Chrysophenol D

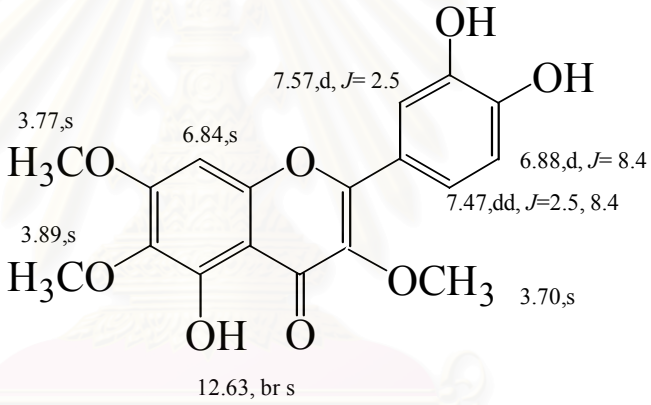


Fig. 3.44 The Proton Assignment of Compound 5

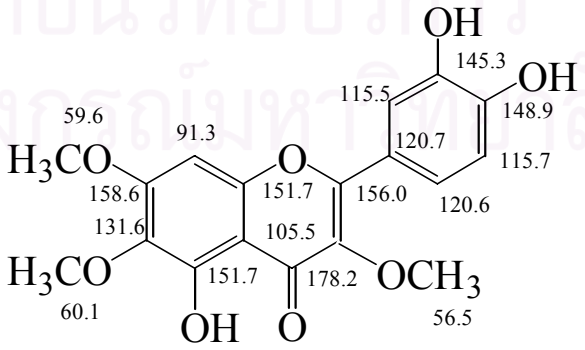
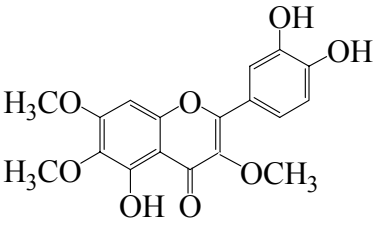
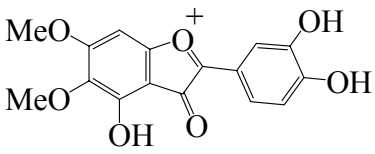


Fig. 3.45 The Carbon Assignment of Compound 5

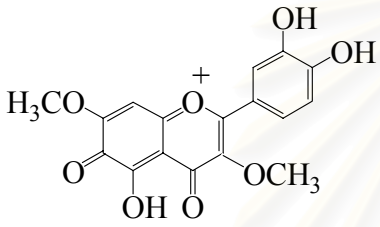
Scheme 3.4 The Possible Mass Fragmentation of Compound 5



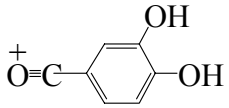
$C_{18}H_{16}O_8$ m/z 360



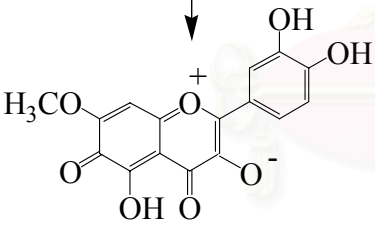
$C_{16}H_{13}O_7$ m/z 317



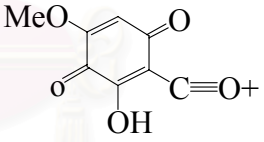
$C_{17}H_{13}O_8$ m/z 345



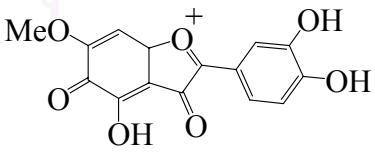
$C_7H_5O_3$ m/z 137



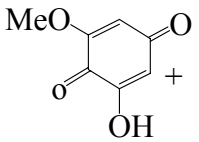
$C_{16}H_{11}O_8$ m/z 330



$C_8H_5O_5$ m/z 181



$C_{15}H_{10}O_7$ m/z 302



$C_7H_5O_4$ m/z 153

3.4.5 Purification, Properties and Structural Elucidation of Compound 6

Ethyl acetate extract was isolated by OCC (Si-60) eluted with step gradient solvent system (hexane-EtOAc-MeOH). The 30% EtOAc in hexane fraction was purified by recrystallisation from CH_2Cl_2:MeOH (1:1) to give compound 6 and compound 7. Compound 6 is yellow needle, 47.9 mg (0.13 % w/w of EtOAc extract) m.p. = 279°C.

The IR spectrum (Fig 3.46) of compound 6 showed the absorption band of a hydroxy group at 3500 - 3200 cm^{-1}. The strong absorption band at 1654 cm^{-1} revealed the presence of α,β-unsaturated carbonyl and the characteristic absorption peak due to an aromatic moiety was observed at 1600 - 1400 cm^{-1}.

The molecular formula of compound 6 (Fig 3.47) was determined as $C_{17}H_{14}O_7$ by low-resolution mass spectrometry (M^+) m/z 330. The fragmentation pattern displayed in scheme 3.5.

The ^1H and ^{13}C NMR data (Fig 3.48 and Fig 3.49) was investigated and compared with those of the references[21,22] as shown in Table 3.14. Compound 6 was identified to be 3,7-dimethoxy-4',5,6-trihydroxy flavone as they exhibited the same NMR data.

Fig 3.50 displayed the structure of compound 6 and the proton and carbon assignment of this compound are showed in Fig 3.51 and 3.52.

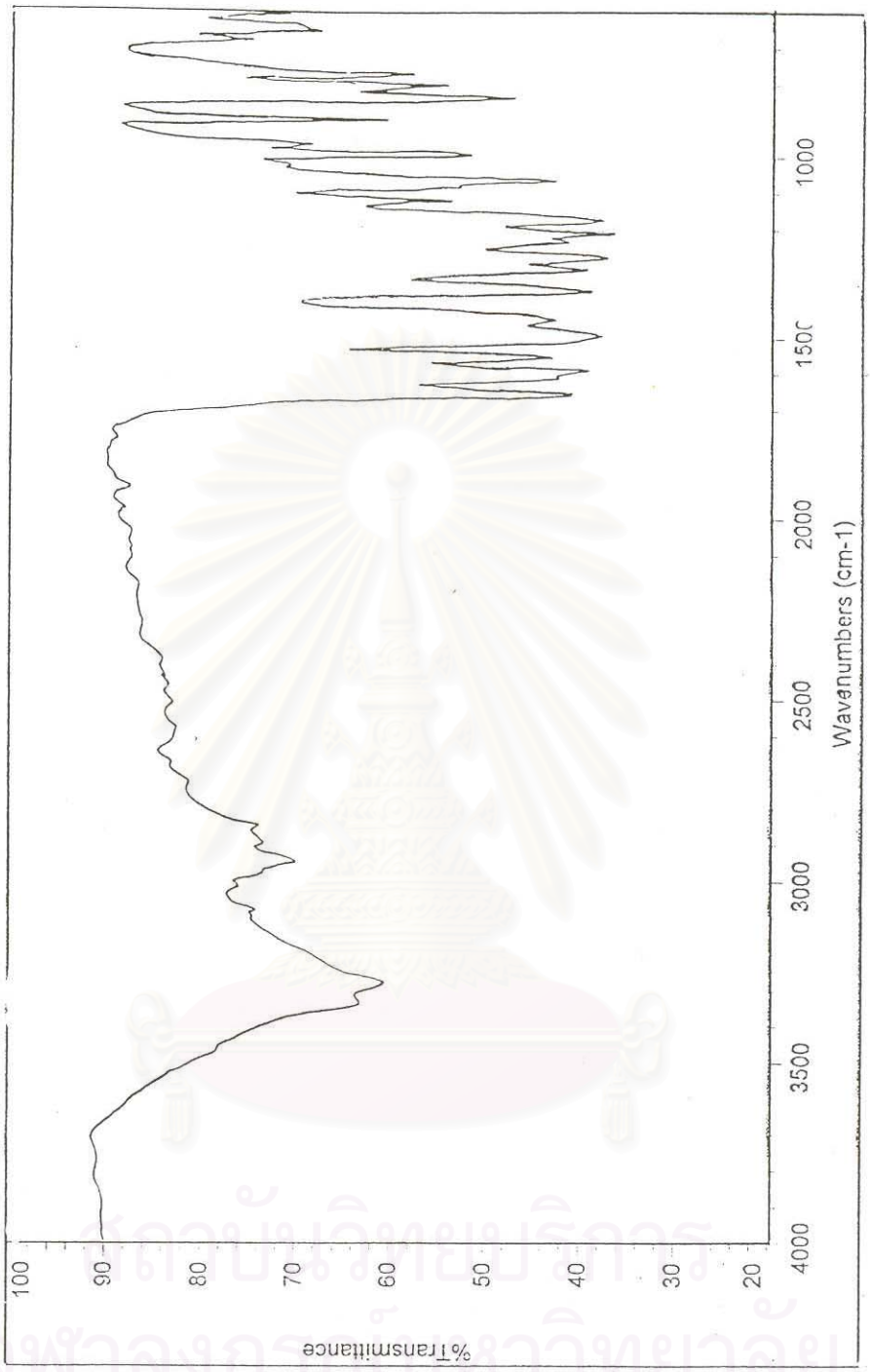


Fig 3.46 The IR Spectrum of Compound 6

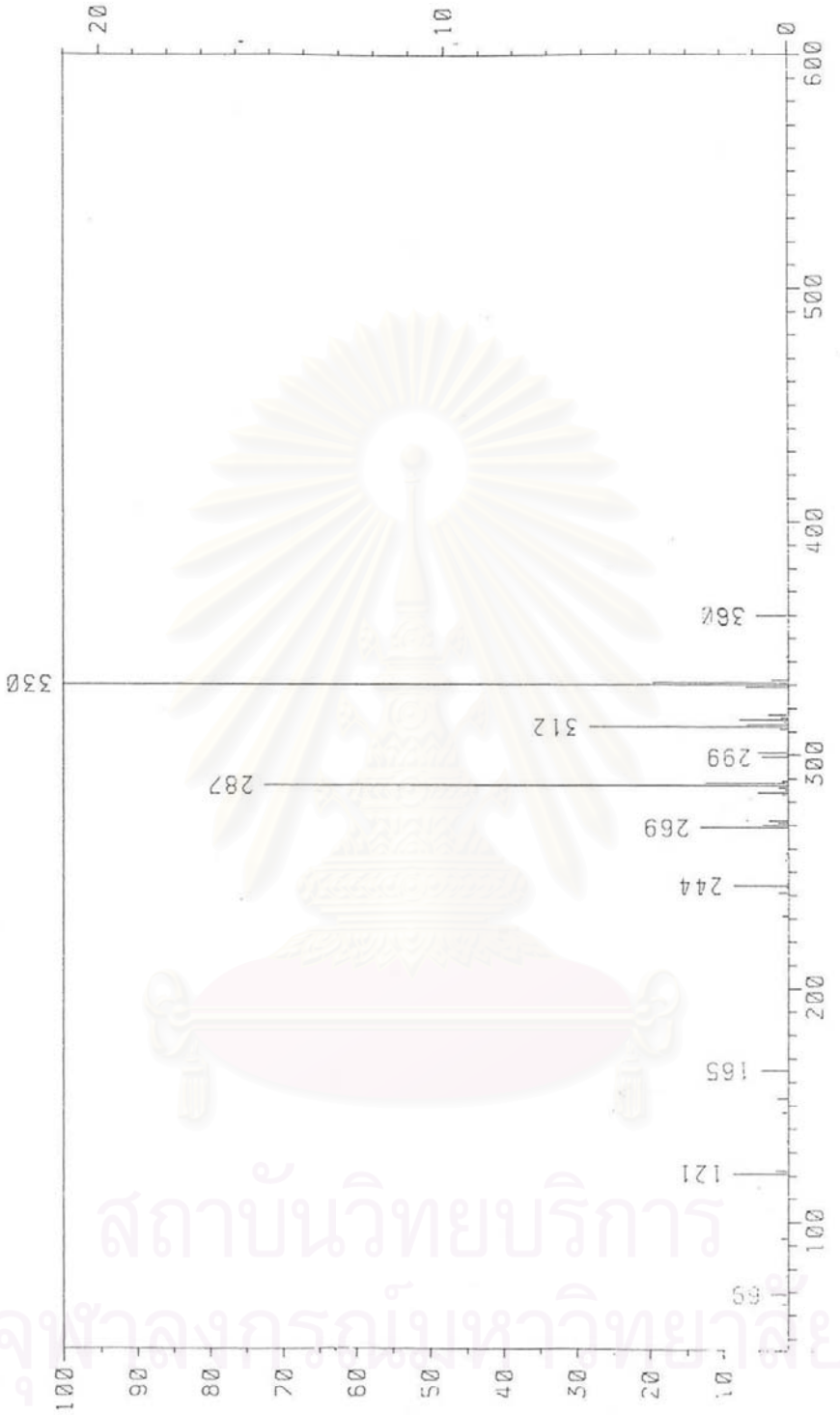


Fig 3.47 The mass Spectrum of Compound 6

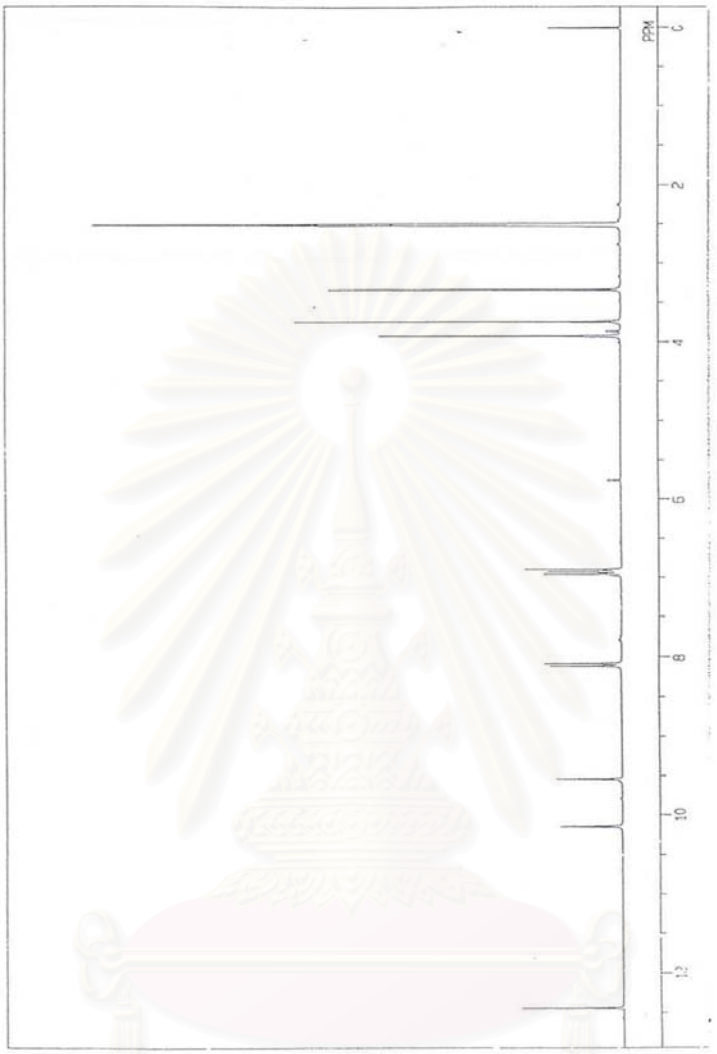


Fig 3.48 The ^{1}H NMR Spectrum of Compound 6

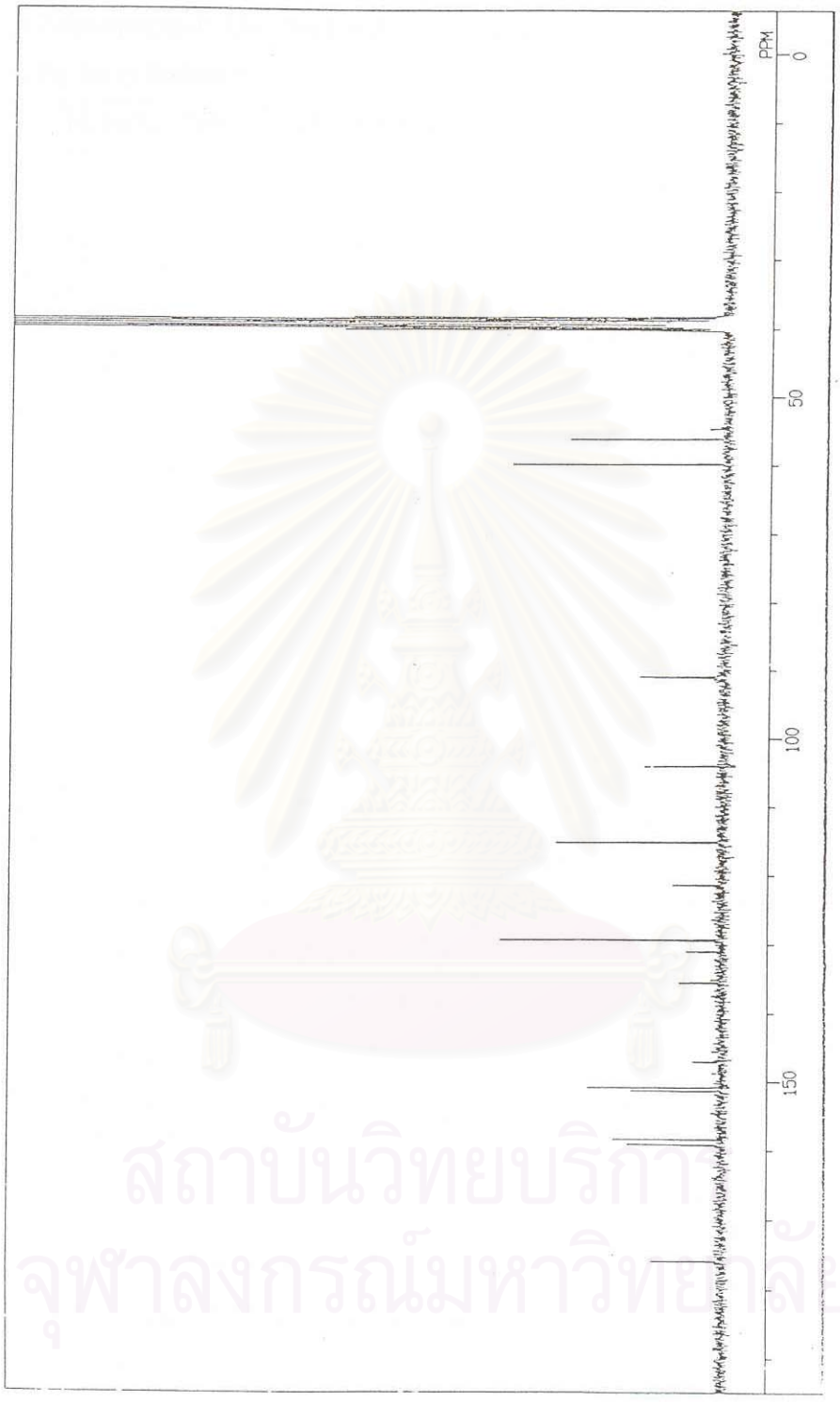


Fig 3.49 The ^{13}C NMR Spectrum of Compound 6

Table 3.14 The ^{1}H and ^{13}C NMR Data of Compound 6, 3,7-dimethoxy-4',5-trihydroxy flavone, and Herbacetin-3,7-dimethoxy

#	**3,7-dimethoxy-4',5,6-trihydroxy flavone***	**Herbacetin-3,7-dimethoxy***		**Compound 6#**	
	^{1}H, mult,*J*(Hz)	^{1}H, mult,*J*(Hz)	^{13}C	^{1}H, mult,*J*(Hz)	^{13}C
2			155.8		158.3
3			137.4		135.5
4			178.4		175.9
5			152.7		151.3
5-OH	12.45, s	12.21, s		12.45, s	
6			95.4		131
6-OH	9.60, s	6.57, s		9.55, s	
7			154		150.8
8	6.85 1H s	8.82 -OH s	126.1	6.90 1H s	91
9			143.7		147.1
10			104.4		104.1
1`			120.8		121.4
2`	7.97 m, *Jo*=9, *Jm*=2	8.03 1H d, *J* = 8.8	130.3	8.10 1H d, *J* = 8.9	129.3
3`	6.95 m, *Jo*=9, *Jm*=2	6.97 1H d, *J* = 8.8	115.6	6.94 1H d, *J* = 8.9	115.2
4`			160.2		159.1
4`-OH	10.10, s	10.28, s		10.15, s	
5`	6.95 m, Jo=9, *Jm*=2	6.97 1H d, *J* = 8.8	115.6	6.94 1H d, *J* = 8.9	115.2
6`	7.97 m, Jo=9, *Jm*=2	8.03 1H d, *J* = 8.8	130.3	8.10 1H d, *J* = 8.9	129.3
OMe	3.80 3H S	3.80 3H S	59.6	3.75 3H s	59.8
OMe	3.92 3H S	3.91 3H S	56.4	3.93 3H s	56.2

Note :

3.□.□ Solvent for NMR data is DMSO-d_6 + $CDCl_3$

Solvent for NMR data is DMSO-d_6

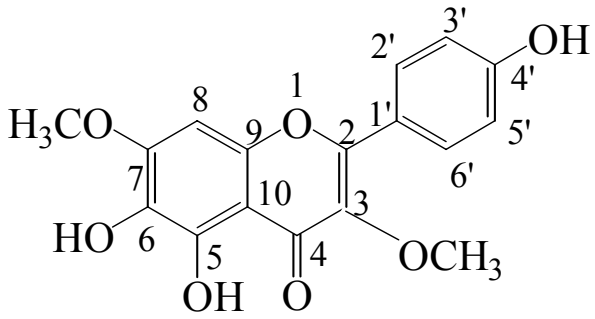


Fig. 3.50 The Structure of Compound 6

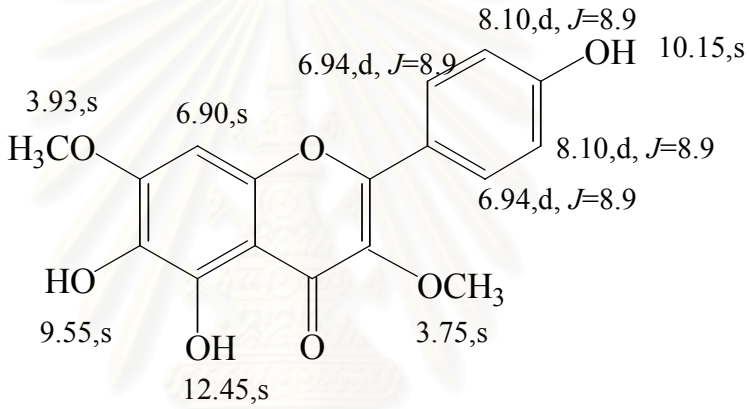


Fig. 3.51 The Proton Assignment of Compound 6

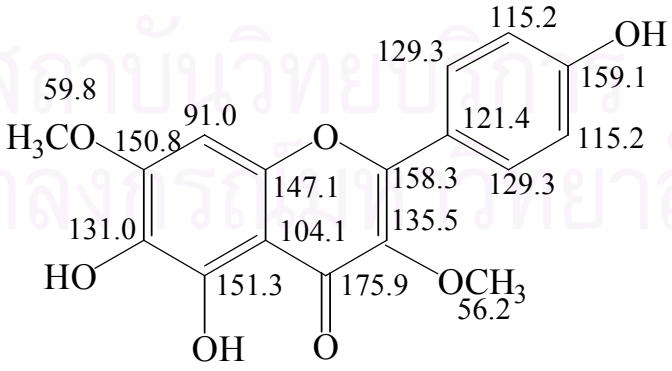
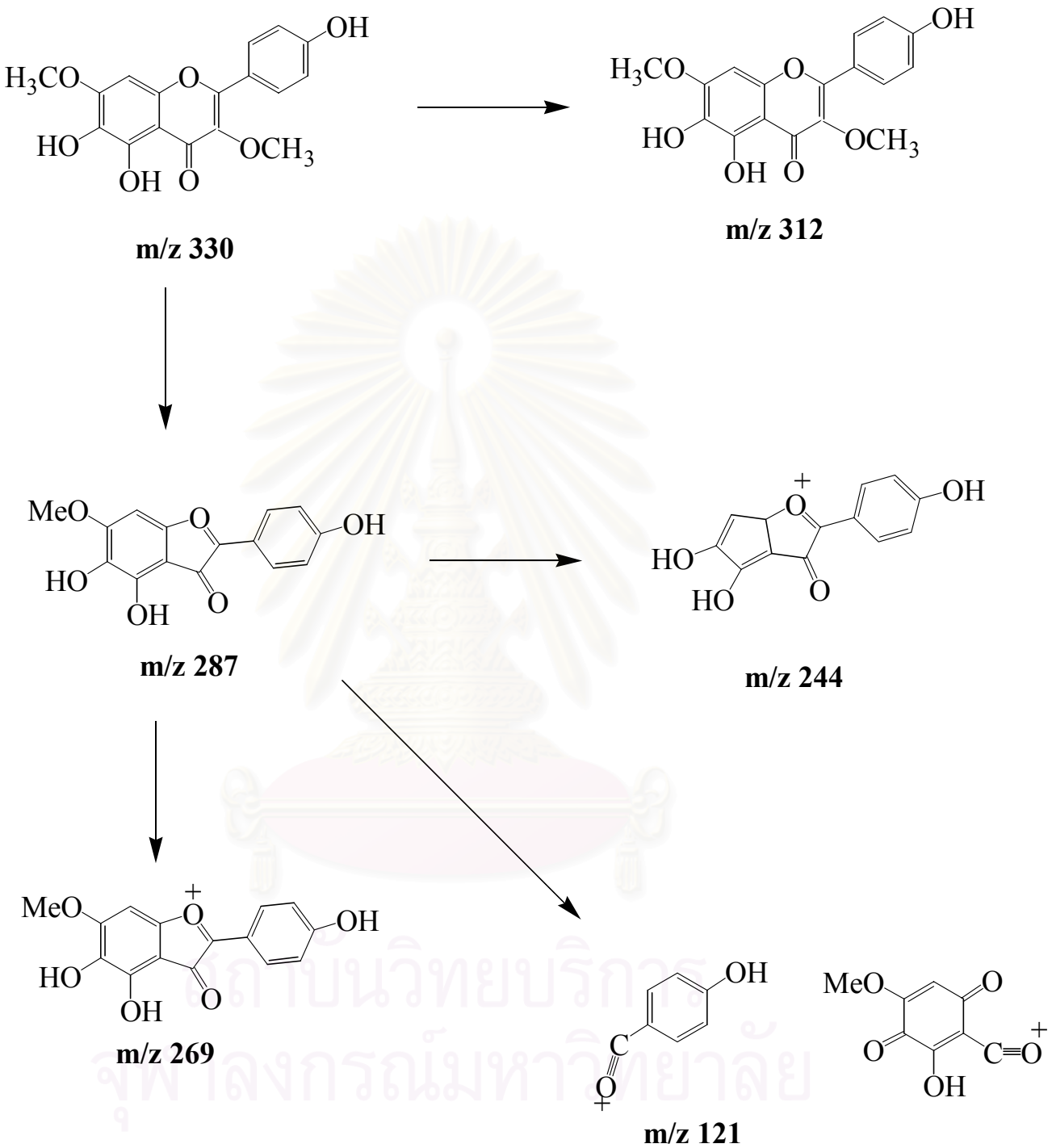


Fig. 3.52 The Carbon Assignment of Compound 6

Scheme 3.6 The Possible Mass Fragmentation of Compound 6



3.4.7 Purification, Properties and Structural Elucidation of Compound 7

Compound 7 (49.5 mg, 0.13 % w/w of ethyl acetate extract) was obtained as yellow amorphous substance. It melted at 217-218°C.

The IR spectrum (Fig 3.53) of this compound showed the existence of hydroxy group and carbonyl group at 3500 - 3200 cm^{-1} and 1649 cm^{-1}, respectively.

The molecular formula of compound was confirmed by the molecular peak at m/z 360, displayed in molecular mass spectrum (Fig 3.54) and fragmentation pattern exhibited in scheme 3.7.

The ^{1}H and ^{13}C NMR data were compared with that reported[12] as displayed in Table 3.15. The proton and carbon NMR spectrum were exhibited in Fig 3.55 and Fig 3.56. Both ^{1}H and ^{13}C NMR spectral data of compound 7 was closely resembled to those of Chrysophenol C. The structure, the proton, and the carbon assignment of this compound are exhibited in Fig 3.57, 3.58, and 3.59, respectively.

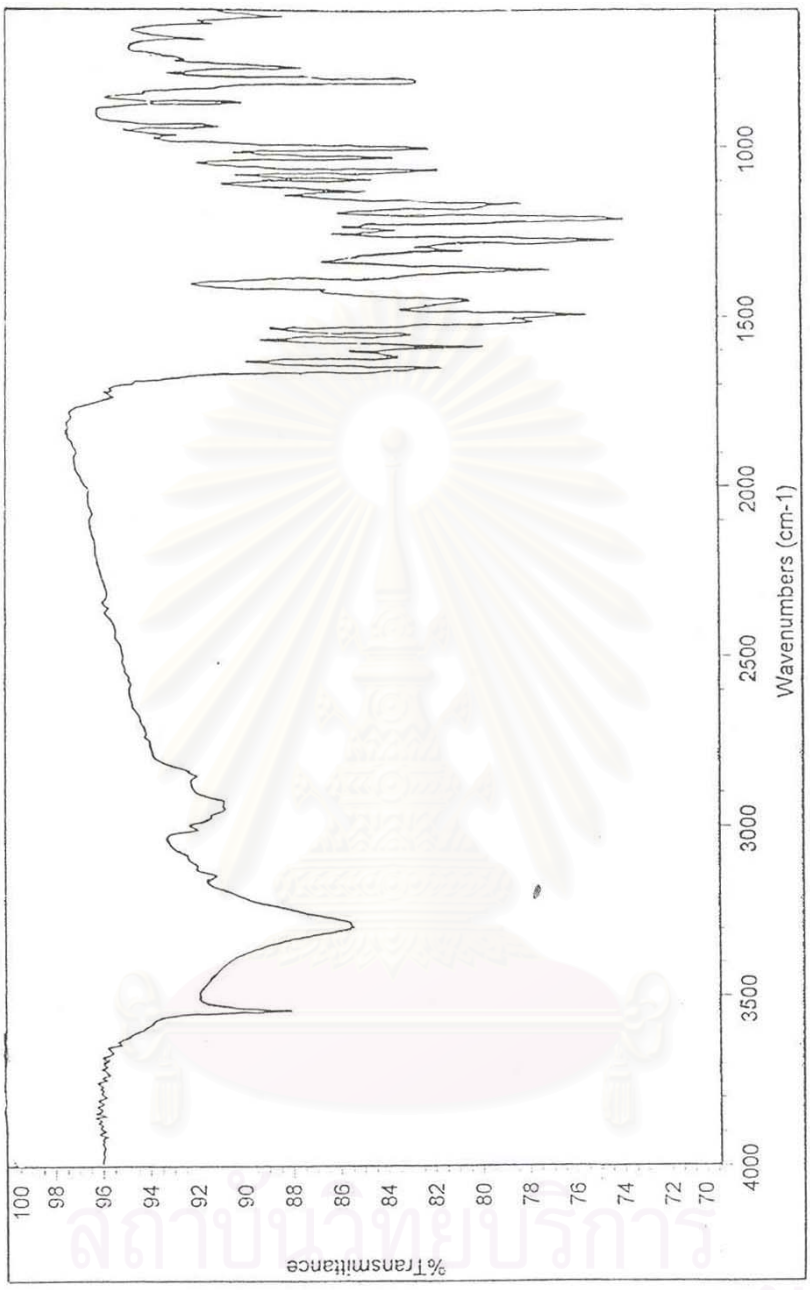


Fig 3.53 The IR Spectrum of Compound 7

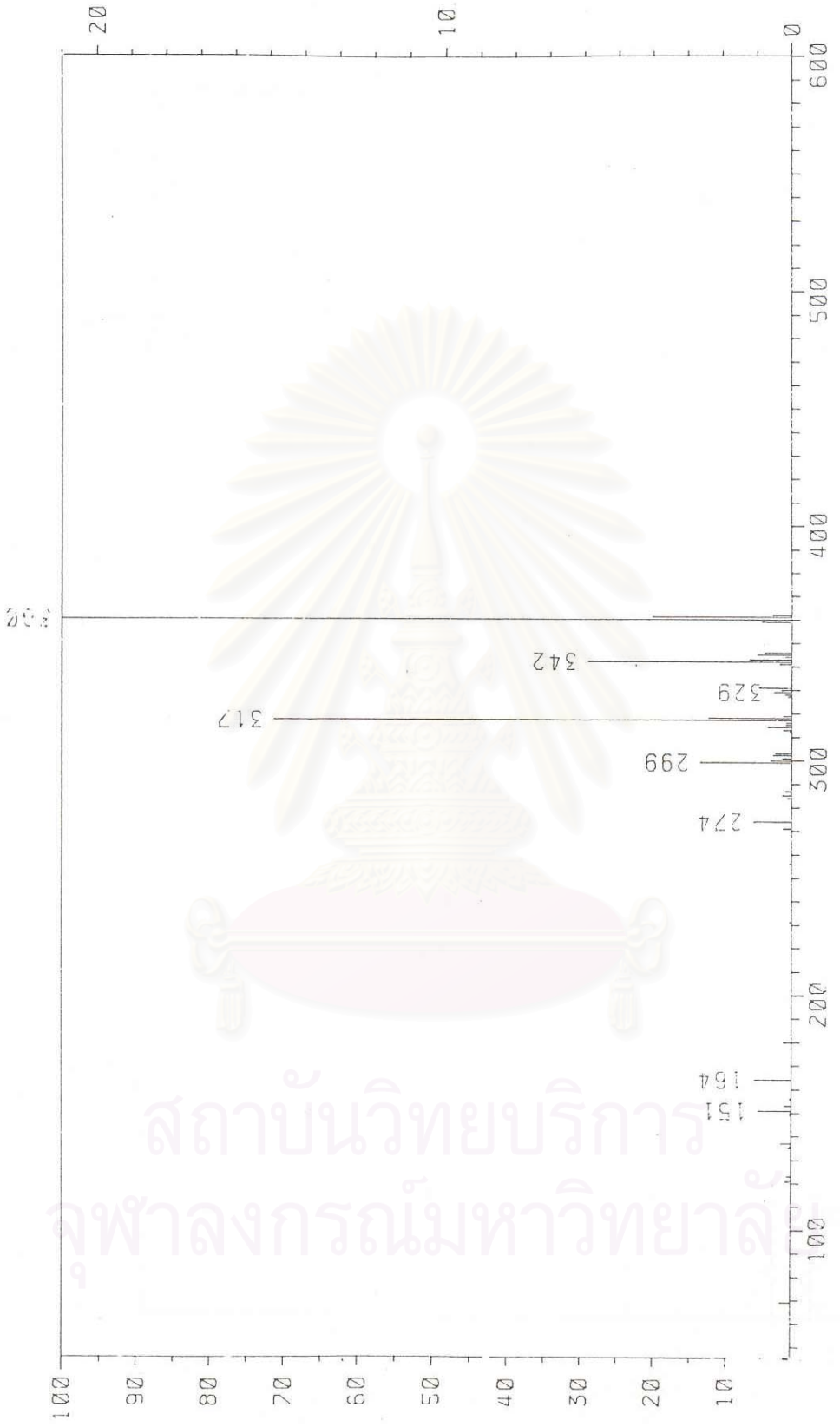


Fig 3.54 The mass Spectrum of Compound 7

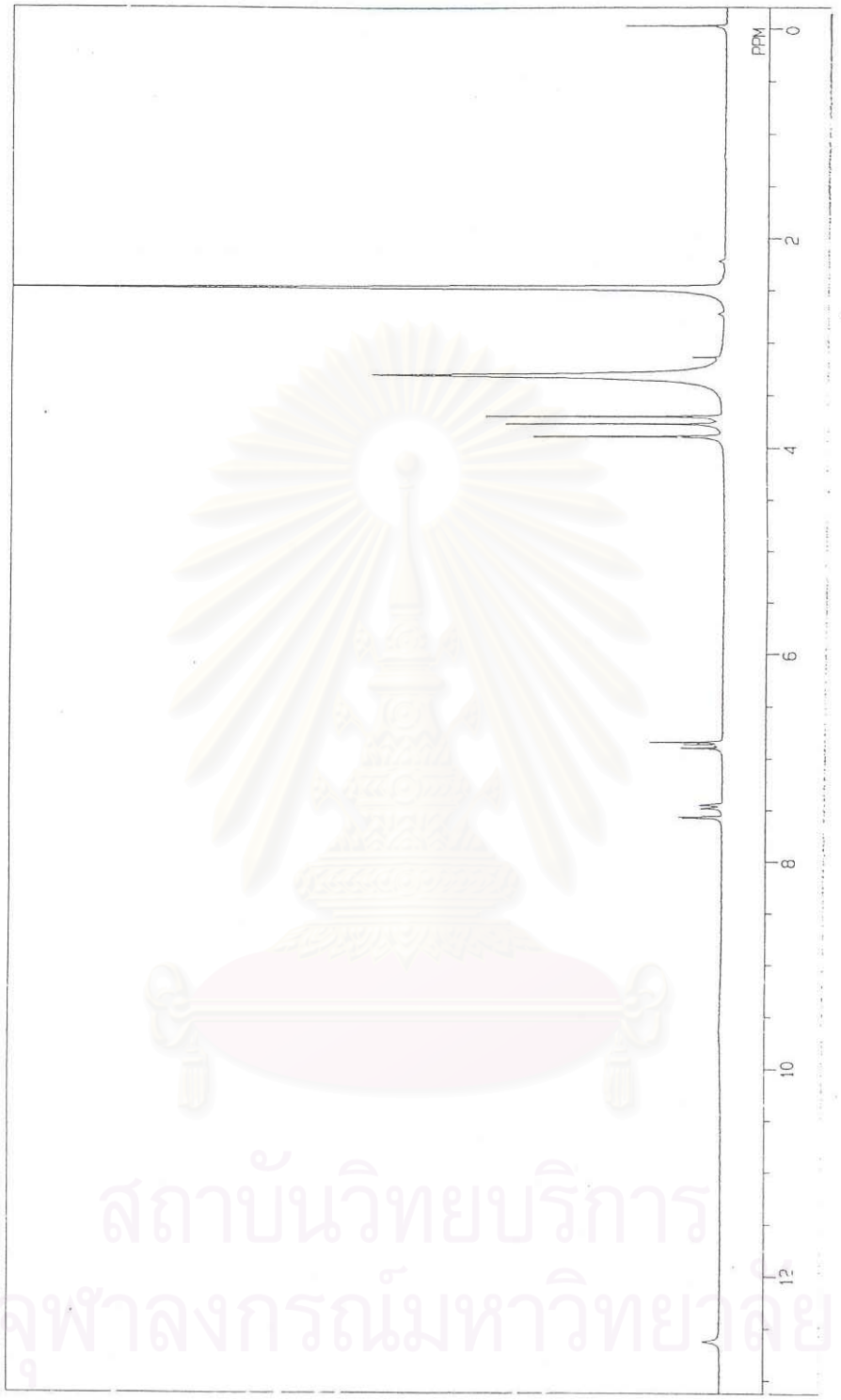


Fig 3.55The ^{1}H NMR Spectrum of Compound 7

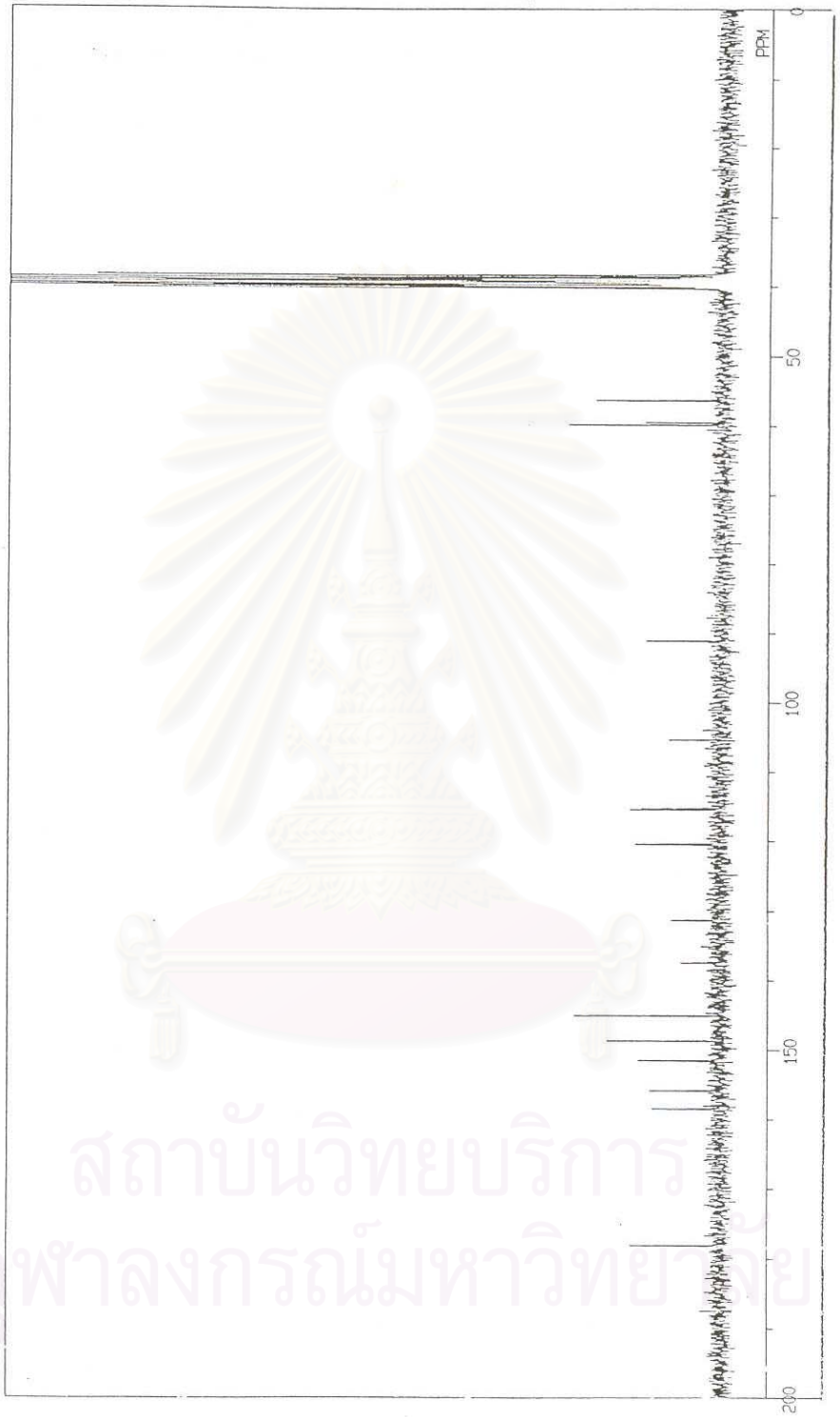


Fig 3.56 The ^{13}C NMR Spectrum of Compound 7

Table 3.15 The ^{1}H and ^{13}C NMR Data of quercetagetin-3,3`,7-trimethoxy ether and Compound 7

#	Chrysophenol C (DMSO-d_6)		Compound 7 (DMSO-d_6)	
	^{1}H, mult, J(Hz)	^{13}C, Hz	^{1}H, mult, J(Hz)	^{13}C, Hz
2		151.6		151.3
3		135.9		135.7
4		176.1		175.9
5		151.0		150.8
5-OH	12.43, s		12.44, br s	
6		131.3		131.1
6-OH	9.96, s		9.58, br s	
7		158.6		158.3
8	6.88, s	91.3	6.93, s	91.1
9		149.0		148.8
10		104.3		104.1
1`		121.9		121.7
2`	7.77, s	111.8	7.79, d, J = 2.5	111.5
3`		147.4		147.2
4`		147.1		146.9
4`-OH	9.77, s		9.79, br s	
5`	6.94, d, J = 8	115.6	6.95, d, J = 8.5	115.3
6`	7.72, d, J = 8	121.9	7.76, dd, J = 2.5,10	121.7
-OMe	3.73, s	60.1	3.74, s	59.8
-OMe	3.85, s	56.5	3.86, s	56.2
-OMe	3.91, s	55.9	3.93, s	55.6

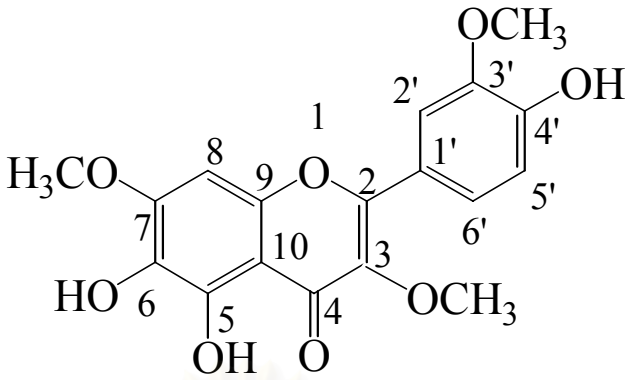


Fig. 3.57 The Structure of Compound 7

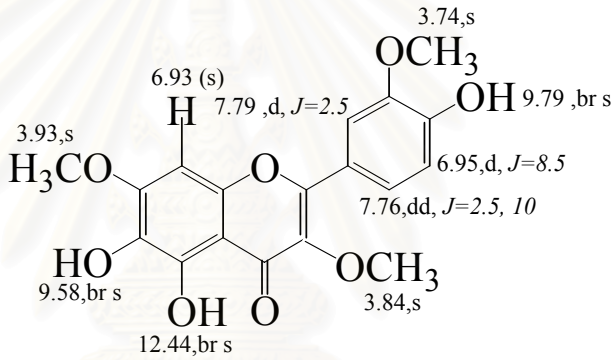


Fig. 3.58 The Proton Assignment of Compound 7

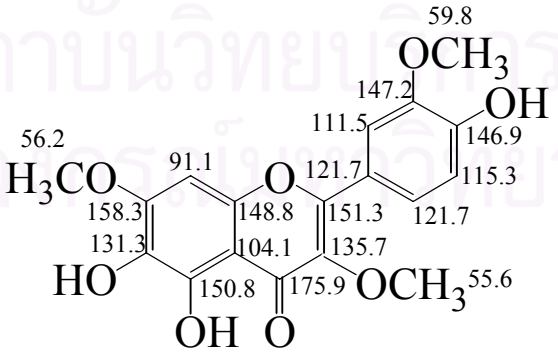
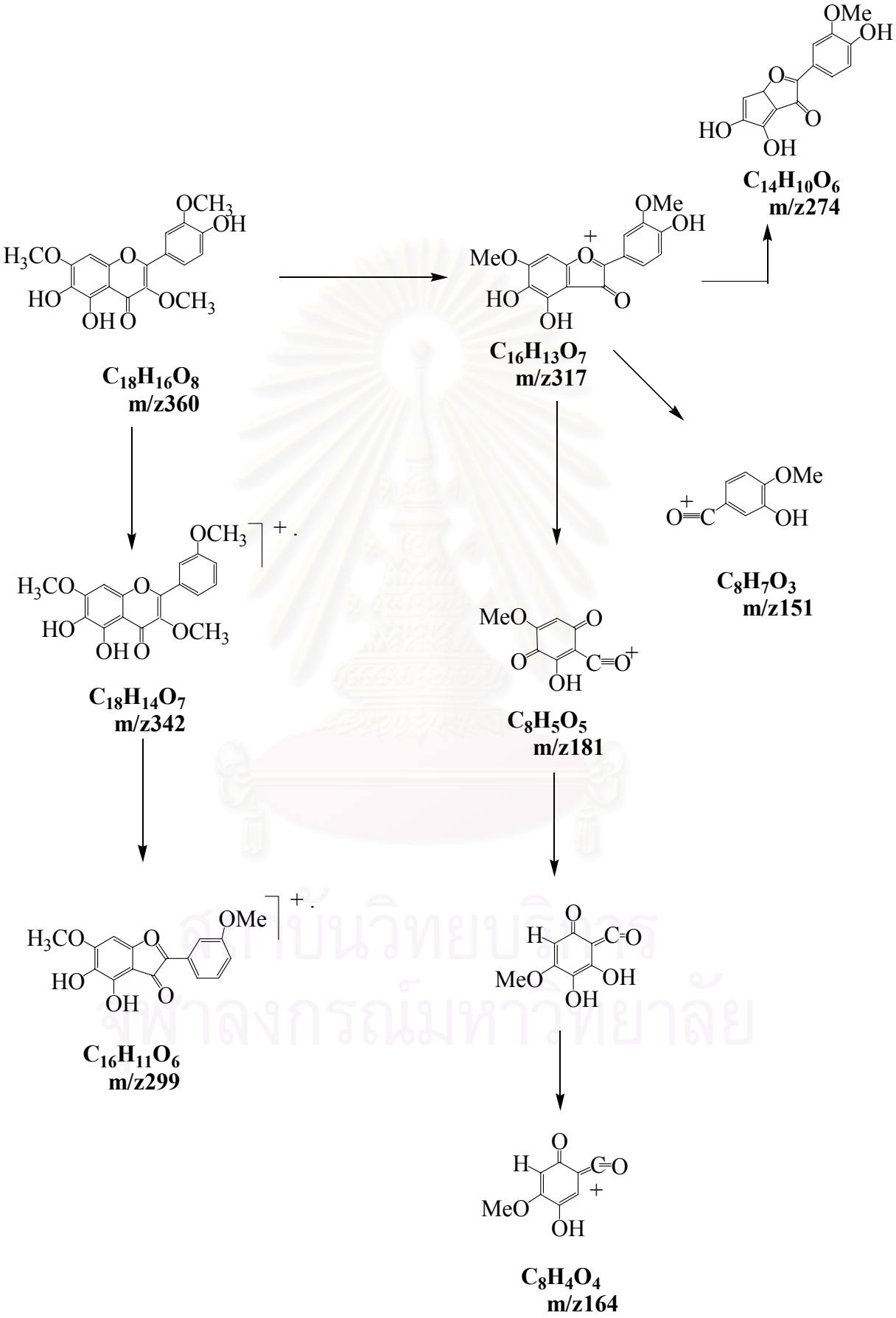


Fig. 3.59 The Carbon Assignment of Compound 7

Scheme 3.7 The Possible Mass Fragmentation of Compound 7



3.5 The Result of Activities of Isolated Compounds

3.5.1 The Cytotoxicity Against KB Cell of Isolated Compounds

According to preliminary cytotoxicity screening test, dichloromethane and ethyl acetate extracts showed significant cytotoxicity against human leukemia carcinoma cell line (HL-60) and human nasopharyngeal carcinoma cell line (KB). Especially ethyl acetate extract exhibited strong cytotoxicity against KB cell line. The results of cytotoxicity testing are ahown in Table 3.16.

Table 3.16 Cytotoxicity Against KB Cell of Isolated Compounds

Samples	% Inhibition / Dose (μg/mL)			
	10	**3**	**1**	**0.3**
Compound 1	24.4	-	-	-
Compound 2	47.4	-	-	-
Compound 3	34.0	-	-	-
Compound 4	**96.4**	**94.4**	**48.4**	**28.5**
Compound 5	**42.8**	**34.0**	**20.3**	**13.2**
Compound 6	11.0	-	-	-
Compound 7	37.5	-	-	-

From above data, compound 4 and compound 5 revealed strong and medium cytotoxicity against KB cell line, respectively.

- = No activity

3.5.2 The Free Radical Scavenging Action to DPPH Radical Activity

Isolated compounds were tested for free radical scavenging activity. The results are showed in Table 3.17.

Table 3.17 The Free Radical Scavenging action to DPPH Radical Activity

Samples	EC_{50} ($EC_{125\ \mu M}$ DPPH) mM/mL
Compound 1	-
Compound 2	-
Compound 3	-
Compound 4	0.870
Compound 5	0.030
Compound 6	0.060
Compound 7	0.040
Catechin	0.105

EC_{50} < 0.1 mM/mL = strong

< 0.01 mM/mL = very strong

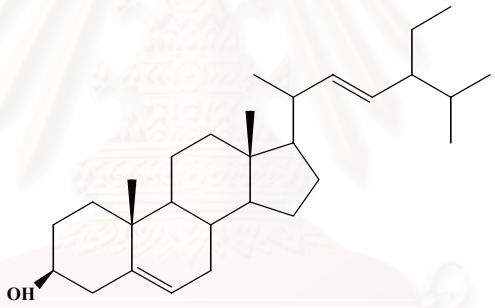
In this method, catechin was selected as the standard. From this table compounds 1, 2, and 3 did not show the activities. Compound 4 showed weaker activity than catechin, while, compounds 5, 6, and 7 showed strong activity. After compared with standard, all flavonoids exhibited stronger activity than catechin.

CHAPTER IV
Conclusion

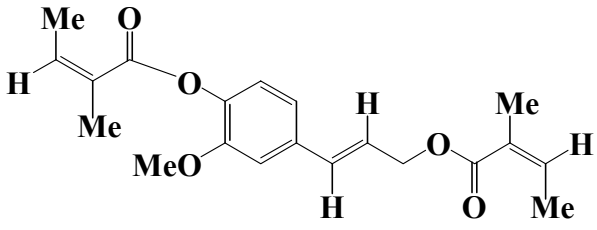
In this research, seven compounds were isolated from bioactive fractions of *S. africanus*. Their structures were characterized by spectroscopic study, and cytotoxicity and free radical scavenging action have been examined. The isolated compounds are summarized as follows.

Compound 1; Stigmasterol

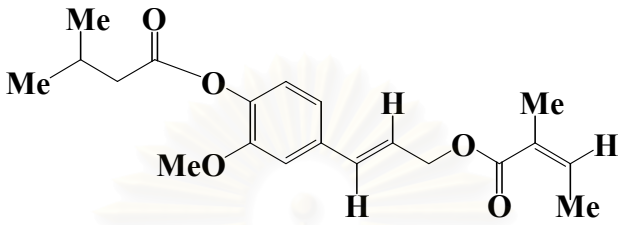
(73.3 mg, 1.20 x 10^{-2} % w/w of ethanol extract, 1.22 x 10^{-3} % w/w of dry plant)



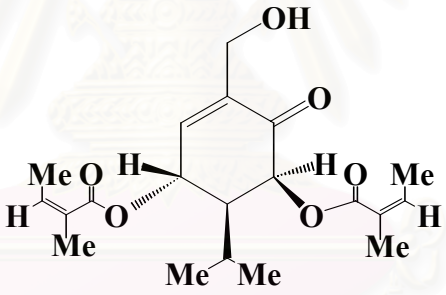
Compound 2; 1-angeloxoyloxy-3-[4′-angeloxoyloy-3′-methoxy]-2-Propene (3.4 mg, 5.56 x 10^{-4} % w/w of ethanol extract, 5.67 x 10^{-5} % w/w of dry plant)



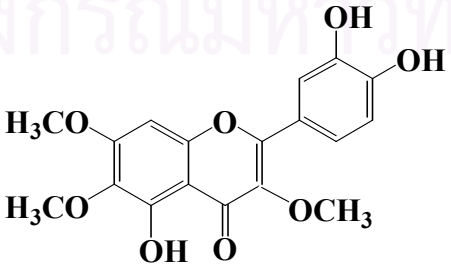
Compound 3; 1-angeloxoyloxy-3-[4′-isopentanoloxoyloxy-3′-methoxy]-2-Propene (2.0 mg, 3.27 x 10^{-4} w/w of ethanol extract, 3.33 x 10^{-5} % w/w of dry plant)



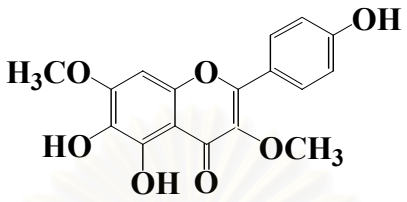
Compound 4; 3α,5β-diangeoloxoyloxy-7-hydroxycarvotanacetone (9.2 mg, 1.50 x 10^{-3} w/w of ethanol extract, 1.53 x 10^{-4} % w/w of dry plant)



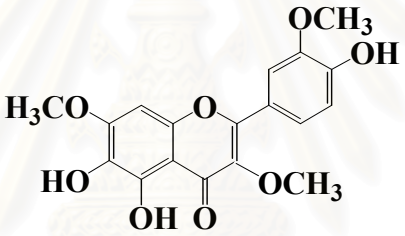
Compound 5; Chrysophenol D (75.8 mg, 1.24 x 10^{-2} % w/w of ethanol extract, 1.26 x 10^{-3} % w/w of dry plant)



Compound 6; 3,7-dimethoxy-4′,5,6-trihydroxy flavone (49.7 mg, 8.12 x 10^{-3} % w/w of ethanol extract, 8.28 x 10^{-4} % w/w of dry plant)



Compound 7; Chrysophenol C (49.5 mg, 8.09 x 10^{-3} w/w of ethanol extract, 8.25 x 10^{-4})



All the isolated substances, carvotanacetone derivative (**Compound 4**) and chrysophenol D (**Compound 5**) have significantly revealed 96.4 % and 42.8 % inhibition at 10 μg/mL against KB cell line. In the case of free radical scavenging action to DPPH radical activity, carvotanacetone (**Compound 4**) shown weak activity (EC_{50} = 0.870 mM/mL. Where as, chrysophenol D (**Compound 5**), 3,7-dimethoxy-4',5,6- trihydroxy flavone (**Compound 6**), and chrysophenol C (**Compound 7**) exhibited (EC_{50} = 0.03, 0.06, and 0.04 mM/mL, respectively) stronger activity than catechin (commercial standard, EC_{50} = 0.105 mM/mL). As above, the carvotanacetone derivative and flavonoid derivatives have displayed the significant cytotoxicity against KB carcinoma cell line, and they have also shown high antoxidation activity. Therefor in the future may use this result to modify the useful substances or medicines.

Proposal for future work

It could be clearly seen that various biologically active compounds could be isolated from the active fractions of whole plant of *S.africanus*. Even though, the uses of human carcinoma cell lines cytotoxicity lethality and free radical scavenging activity against DPPH assay are the preliminary indication among well known bioassays, their results were firmly reliable and showed good tendency towards studying on more sophisticated bioassays such as anticancer in pharmaceutical aspects or in agricultural aspects. Therefore, further studies on those mentioned bioassays should be conducted.

1-Angeloxoyloxy-3-[4′-isopentanoloxoyloxy-3′-methoxy]-2-propene is a new compound but exhibited not very strong activities. Although the naturally relative amount of this compound was tiny, it may be synthesized from 4-hydroxy isoeugenol.[22] After that we can synthesize its derivatives and study about structure activity relationship.

Another aspect that would make whole plant used research fulfill is the chemotaxonomy study on chemical constituents of other parts of *S. africanus* and also various parts of another species in Thailand such as *S. indicus*.

The outcome obtained from this research supported a promising concept of the fully utilization and biologically active compound searching from ideal and potential natural resources of Thai weeds.

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Curriculum Vitae

Miss Pornpan Sukpanyalert was born on August 16, 1976 in Ayutthaya, Thailand. She graduated with Bachelor Degree of Science, major in Chemistry from Chulalongkorn University. She was supported by a research from the Association International Education, Japan (AIEJ), University of the Ryukyus during October 1999 to August 2000 and from Natural Products Research Unit of Department of Chemistry, Chulalongkorn University. Her present address is 846/77 Pracha-Utid 44, Bangmod, Tungkru, Bangkok 10140.