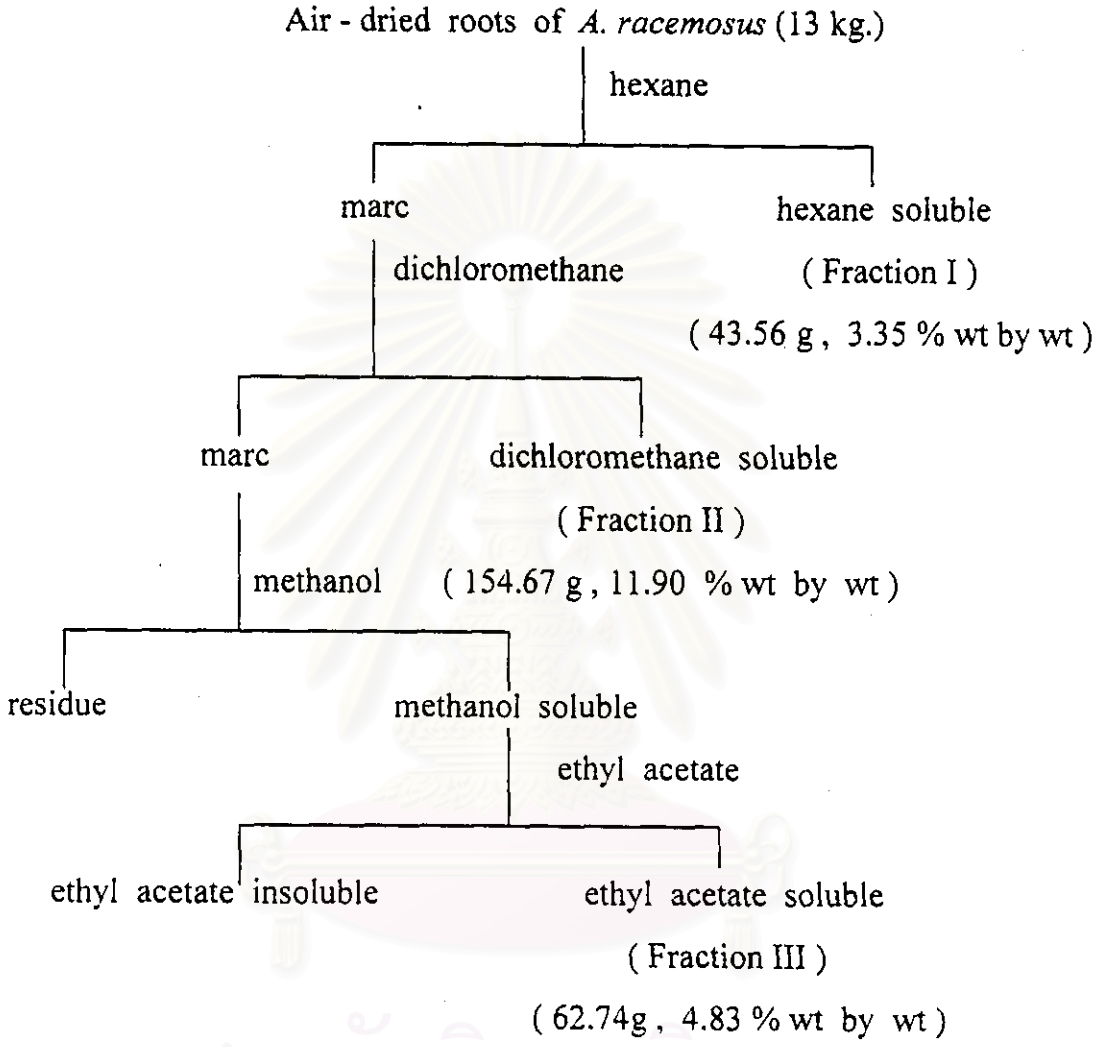
CHAPTER III

RESULTS AND DISCUSSION

3.1 Extraction results

The air-dried , coarsely powdered roots of *A . racemosus* (13 kg.) were extracted with hexane , dichloromethane , methanol and ethyl acetate respectively , which was presented in Chapter II . Fraction I , the hexane crude extract , was obtained as a yellowish - brown material (43.56 g, 3.35 % wt by wt of dried roots) while Fraction II , the dichloromethane crude extract , was obtained as a light brown material.(154.67 g , 11.90 % wt by wt of dried roots) Fraction III , a sticky pale brown material , was obtained by partition of the methanol soluble part with ethyl acetate (62.74 g , 4.83 % wt by wt of dried roots). The procedure and results of extraction are shown in Scheme 2.

Scheme 2 **The procedure and results of extraction of the roots of *A. racemosus***



3.2 Preliminary bioassay results of the inhibitory effect for tumor cell lines

Table 3 The results of the Preliminary screening of the methanol crude extract.

cell line	the methanol crude extract of the roots of *A. racemosus* $IC_{50} \geq$ (μg/ml)
Human Nasopharyngeal Carcinoma(KB)	10
Human Carcinoma of the Stomach	1
Human Leukemia (HL-60)	1
Human Mammary Cancer	1
K562	-
Human Carcinoma of Esophagus	10
Human Pulmonary Carcinoma	-

The methanol crude extract of the roots of *A. racemosus* showed inhibitory effects for 3 cell lines : Human Carcinoma of Stomach , Human Leukemia (HL-60) and Human Mammary Cancer , $IC_{50} \geq 1$ μg/ml.

3.3 Separation of Fraction I

The hexane crude extract , 43.56 g , was separated by open column chromatography . After the column was packed with silica gel as absorbent , the crude mixture, which was mixed with some silica gel , was added to the top of column. The column was eluted with an increasing gradient of dichloromethane in hexane , then methanol in dichloromethane . Each fraction was concentrated to a small volume and monitored by TLC . The fractions which had the same components were combined .

Table 4 The results of separation of Fraction I

Eluents	Fraction No.	Remarks	weight (g)
hexane	1-5	white wax (compound 1)	0.3736
5% CH_2Cl_2 in hexane	6-8	yellow oil	0.2865
10% CH_2Cl_2 in hexane	9-17	white ppt. in yellow oil (compound 2)	0.724
15% CH_2Cl_2 in hexane	18-20	yellow oil	0.2411
20% CH_2Cl_2 in hexane	21-25	white solid in yellow oil (compound 3)	0.9551
30% CH_2Cl_2 in hexane	26-36	white solid in orange oil	0.3652
40% CH_2Cl_2 in hexane	37-38	white solid in brown oil	0.2412
45% CH_2Cl_2 in hexane	39-44	white solid in yellow oil	0.6323
50% CH_2Cl_2 in hexane	45-51	white needle crystal in yellow oil (compound 4)	1.2545
60% CH_2Cl_2 in hexane	52-56	white plate in green oil (compound 4)	0.4585

Table 4 (cont.)

Eluents	Fraction No.	Remarks	weight (g)
70% CH_2Cl_2 in hexane	57-60	brown oil	0.5657
75% CH_2Cl_2 in hexane	61-62	greenish-brown oil	0.6788
80% CH_2Cl_2 in hexane	63-64	solid in reddish-brown oil (compound 5)	0.5850
90% CH_2Cl_2 in hexane	65-70	solid in brown oil (compound 5,6)	1.2435
100% CH_2Cl_2	71-74	white needle crystal in yellowish-brown oil (compound .6,7)	2.5563
2%MeOH in CH_2Cl_2	75-80	white needle crystal in yellow oil (compound 7)	2.1214
5%MeOH in CH_2Cl_2	81-87	pale brown oil	3.256
10%MeOH in CH_2Cl_2	88-92	brown oil	4.2351
20%MeOH in CH_2Cl_2	93-97	brown oil	0.8565
50%MeOH in CH_2Cl_2	98-100	dark brown oil	1.2566
100%MeOH	101-102	brown oil	1.3226

3.4 Separation of Fraction II

The dichloromethane crude extract , 154.67 g , was separated into fractions by column chromatography. The separation procedure and the eluents were the same as the separation of Fraction I. The results of separation and combination are indicated in Table 5.

Table 5 The results of the separation of Fraction II

Eluents	Fraction No.	Remarks	weight (g)
hexane	1-3	white wax (compound 1)	0.3228
10-15% CH_2Cl_2 in hexane	4-8	white ppt. in yellow oil (compound 1)	0.5121
15% CH_2Cl_2 in hexane	9-10	solid in orange oil (compound 2)	0.3266
20% CH_2Cl_2 in hexane	11-12	solid in yellow oil (compound 2)	0.6548
30% CH_2Cl_2 in hexane	13-18	yellow oil	0.3632
40% CH_2Cl_2 in hexane	19-20	white solid in orange oil (compound 3)	0.4585
45% CH_2Cl_2 in hexane	21-29	white solid in violet oil (compound 3)	0.7822
50% CH_2Cl_2 in hexane	30-33	white semi-solid in pale red oil (compound 4)	6.4550
60% CH_2Cl_2 in hexane	34-38	white needle crystal in yellow oil (compound 4)	3.6331
70% CH_2Cl_2 in hexane	39-54	solid in brown oil	4.5521

Table 5 (cont.)

Eluents	Fraction No.	Remarks	weight (g)
70-80%CH_2Cl_2 in hexane	55-61	solid in yellow oil	1.6567
90% CH_2Cl_2 in hexane	62-70	solid in light brown oil	0.6328
95% CH_2Cl_2 in hexane	71-72	light brown oil	0.4522
100% CH_2Cl_2	73-80	solid in yellowish-brown oil (compound 7)	1.2250
1% MeOH in CH_2Cl_2	81-85	solid in brown oil (compound 7)	1.3567
2% MeOH in CH_2Cl_2	86-90	solid in brown oil (compound 7)	2.6631
4% MeOH in CH_2Cl_2	91-96	solid in dark brown oil (compound 7)	2.7460
5-7% MeOH in CH_2Cl_2	97-100	light brown oil	0.8632
10% MeOH in CH_2Cl_2	101-106	white solid in brown oil (compound 8)	1.9638
15% MeOH in CH_2Cl_2	107-110	white solid in brown oil (compound 8)	0.9314
20% MeOH in CH_2Cl_2	111-114	white ppt. in brown oil (compound 8)	1.4060
25% MeOH in CH_2Cl_2	115-117	white ppt. in dark brown oil (compound 8)	0.8755
50% MeOH in CH_2Cl_2	118-120	dark brown oil	0.0978
75% MeOH in CH_2Cl_2	121-122	black tar	0.5561
100% MeOH	123-125	black tar	1.0023

3.5 The separation of the eluted fractions No. 39-54 , No. 55-61

TLC showed the constituents of fraction No. 39-54 and No. 55-61 were alike. Due to the many compounds that were visible under U.V. light, the mixture could be separated by chromototron . The chromatotron plate was eluted with hexane , ethyl acetate - hexane and ethyl acetate. While the mixture was eluted by solvents, the U.V. lamp with wavelength 254 nm was set above the plate. Each absorbtion band was collected into a fraction. After separation , the new fractions were checked by TLC. The results are shown in Table 6.

Table 6 The results of the separation of fraction 39-54 , 55-61.

Eluents	Fraction No.	Remarks	weight (g)
hexane	1	trace	0.0057
5-20% EtOAc in hexane	2	white semi-solid in yellow oil.(compound 4)	0.0812
30-40% EtOAc in hexane	3	white solid in green oil (compound 4)	0.5886
50-70% EtOAc in hexane	4-5	band 1 (compound 5)	0.1255
80% EtOAc in hexane	6	band 2 (compound 6)	0.1480
90% EtOAc in hexane	7	orange solution	0.0893
100% EtOAc	8	orange solution	0.0416

3.6 Separation of Fraction III

Column chromatography was used for the separation of the ethyl acetate crude extract . The column was eluted with an increasing gradiant of ethyl acetate in dichloromethane then methanol in ethyl acetate . The fractions were collected and the solvent were evaporated. Each fraction was checked by TLC and then combined.

Table 7 The results of the separation of Fraction III

Eluents	Fraction No.	Remarks	weight (g)
50% CH_2Cl_2 in hexane	1-2	trace	0.0248
60% CH_2Cl_2 in hexane	3-6	trace	0.0658
70% CH_2Cl_2 in hexane	7-8	yellow wax	0.0811
80% CH_2Cl_2 in hexane	9-10	yellow oil	0.2446
90% CH_2Cl_2 in hexane	11-14	white needle crystal in yellow oil (compound 4)	0.7040
100% CH_2Cl_2	15-20	white plate in green oil (compound 4)	0.6488
1-5%EtOAc in CH_2Cl_2	21-24	solid in orange oil (compound 4,5)	0.8425
10%EtOAc in CH_2Cl_2	25-29	white needle crystal in pale yellow oil (compound 6,7)	1.6691
20%EtOAc in CH_2Cl_2	30-34	white needle crystal in orange oil (compound 6,7)	0.7220
30%EtOAc in CH_2Cl_2	35-40	orange oil	0.5663

Table 7 (cont.)

Eluents	Fraction No.	Remarks	weight (g)
40%EtOAc in CH_2Cl_2	41-45	white solid in pale brown oil (compound 8)	0.8993
50-70%EtOAc in CH_2Cl_2	46-62	white solid in brown oil (compound 8)	1.6685
80%EtOAc in CH_2Cl_2	63-65	white solid in yellow oil (compound 9)	1.3225
90%EtOAc in CH_2Cl_2	66-68	white solid in brown oil (compound 9)	0.9661
100% EtOAc	69-72	brown oil	0.5210
5% MeOH in EtOAc	73-76	brown oil	0.8454
10% MeOH in EtOAc	77-80	brown oil	0.3667
20% MeOH in EtOAc	81-85	brown oil	0.6223
50% MeOH in EtOAc	86-90	brown tar	0.2357
70% MeOH in EtOAc	91-93	black tar	0.3769
100% MeOH	94-95	black tar	0.6928

3.7 Separation of the eluted fractions 25-34 (Table 7 , pp 27)

The combined fractions were also separated by chromatotron using the same procedure as for the separation of the eluted fractions No. 39-54 , 55-61 . The results are shown in Table 8 .

Table 8 The results of the separation of fraction 25-34.

Eluents	Fraction No.	Remarks	weight (g)
hexane	1	trace	0.0755
5-50% EtOAc in hexane	2-3	yellow oil	0.0328
60% EtOAc in hexane	4	solid in yellow oil (band 1 - compound 5)	0.0933
70% EtOAc in hexane	5	white solid in yellow oil (band 2 - compound 6)	0.1227
80-90% EtOAc in hexane	6	white solid in yellow oil (band 3 - compound 7)	0.8669
100% EtOAc	7	orange solution	0.3472

3.8 Purification , properties and structure elucidation of substances from *A. racemosus* .

3.8.1 Structure elucidation of Mixture 1

The white solid , Mixture 1 , was obtained from fraction 1-8 in the hexane crude extract separation. This product was purified by crystalization with a mixture of hexane - ethyl acetate , yielding 66.5 mg. (5.12×10^{-3} % wt by wt of dried roots) .The melting point of Mixture 1 was 46-48° C .

The IR spectrum of Mixture 1 showed absorption bands consistent with a long chain hydrocarbon. The strong band of C-H stretching was at < 3000 cm^{-1} which could be resolved as asymmetric stretching at 2919 cm^{-1} and symmetrical stretching at 2849 cm^{-1}. C-H Deformation bands due to C-H bending of methylene and methyl groups were observed at 1468 cm^{-1} and 1379 cm^{-1} respectively. The rocking vibration of a chain of methylene group was observed at 725 cm^{-1} . (Fig. 5)

Table 9 The IR absorption band assignments of Mixture 1.

vibration	wave number (cm^{-1})	intensity
C-H stretching of CH_2 , CH_3	2919 , 2849	strong
C-H bending of CH_2	1468	moderate
C-H bending of CH_3	1379	weak
CH_2 rocking in C-$(CH_2)_n$-C	725	moderate

Mixture 1 , was analyzed by gas chromatography. The results were compared with a standard mixture of long chain hydrocarbons containing 28, 29, 30, 31, 32, 33, 34 and 35 carbons. The chromatogram is shown in Figure 6.

Table 10 The retention time of standard long chain hydrocarbon compound compared with the retention time of Mixture 1.

No. of carbon	retention time of standard mixture	log retention time of standard mixture	retention time of Mixture 1.	log retention time of Mixture 1.
24	2.92	0.46	2.86	0.46
25	3.63	0.56	3.54	0.55
26	4.55	0.65	4.44	0.65
27	5.76	0.76	5.56	0.75
28	7.22	0.85	7.06	0.85
29	9.16	0.96	8.96	0.95
30	11.66	1.06	11.43	1.06
31	14.76	1.16	14.56	1.16
32	18.94	1.27	18.61	1.27
33	24.18	1.38	23.87	1.38

This comparison showed that Mixture 1 was a mixture of ten long chain hydrocarbons as shown in Table 11 .

CH_3-$(CH_2)_n$-CH_3 $\quad n = 24, 25, \ldots\ldots, 33$

Mixture 1

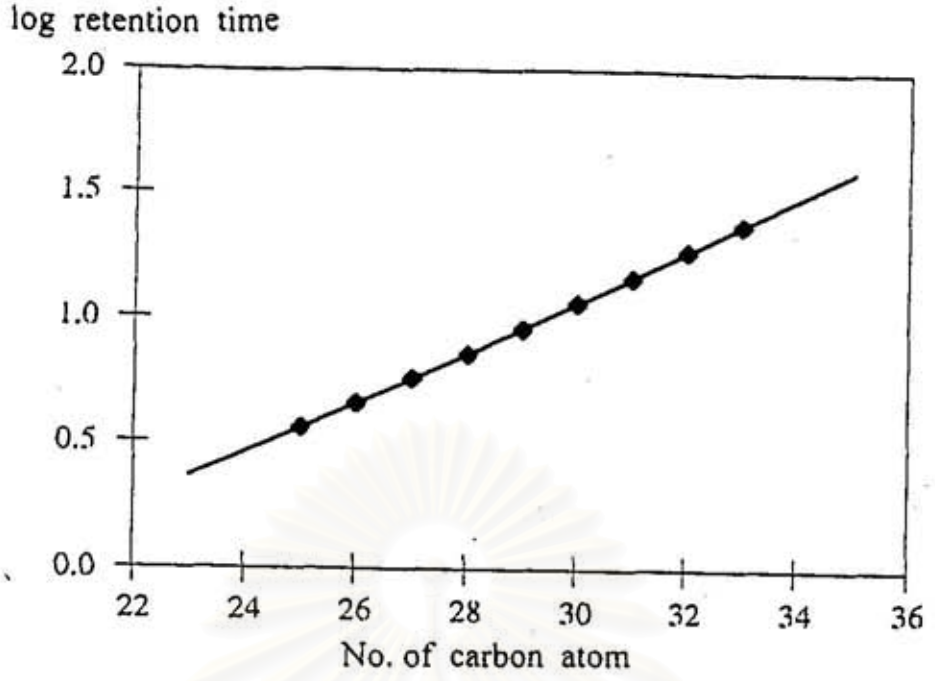


Figure 4 The correlation between log retention time and No. of carbon in a standard mixture of long chain hydrocarbons.

Table 11 Long chain hydrocarbons in Mixture 1.

Compound	Molecular Formula	Structural Formula	Molecular weight
tetracosane	$C_{24}H_{50}$	CH_3-$(CH_2)_{22}$-CH_3	338
pentacosane	$C_{25}H_{52}$	CH_3-$(CH_2)_{23}$-CH_3	352
hexacosane	$C_{26}H_{54}$	CH_3-$(CH_2)_{24}$-CH_3	366
heptacosane	$C_{27}H_{56}$	CH_3-$(CH_2)_{25}$-CH_3	380
octacosane	$C_{28}H_{58}$	CH_3-$(CH_2)_{26}$-CH_3	394
nonacosane	$C_{29}H_{60}$	CH_3-$(CH_2)_{27}$-CH_3	408
triacosane	$C_{30}H_{62}$	CH_3-$(CH_2)_{28}$-CH_3	422
bentriacontane	$C_{31}H_{64}$	CH_3-$(CH_2)_{29}$-CH_3	436
dotriacontane	$C_{32}H_{66}$	CH_3-$(CH_2)_{30}$-CH_3	450
tritriacontane	$C_{33}H_{68}$	CH_3-$(CH_2)_{31}$-CH_3	464

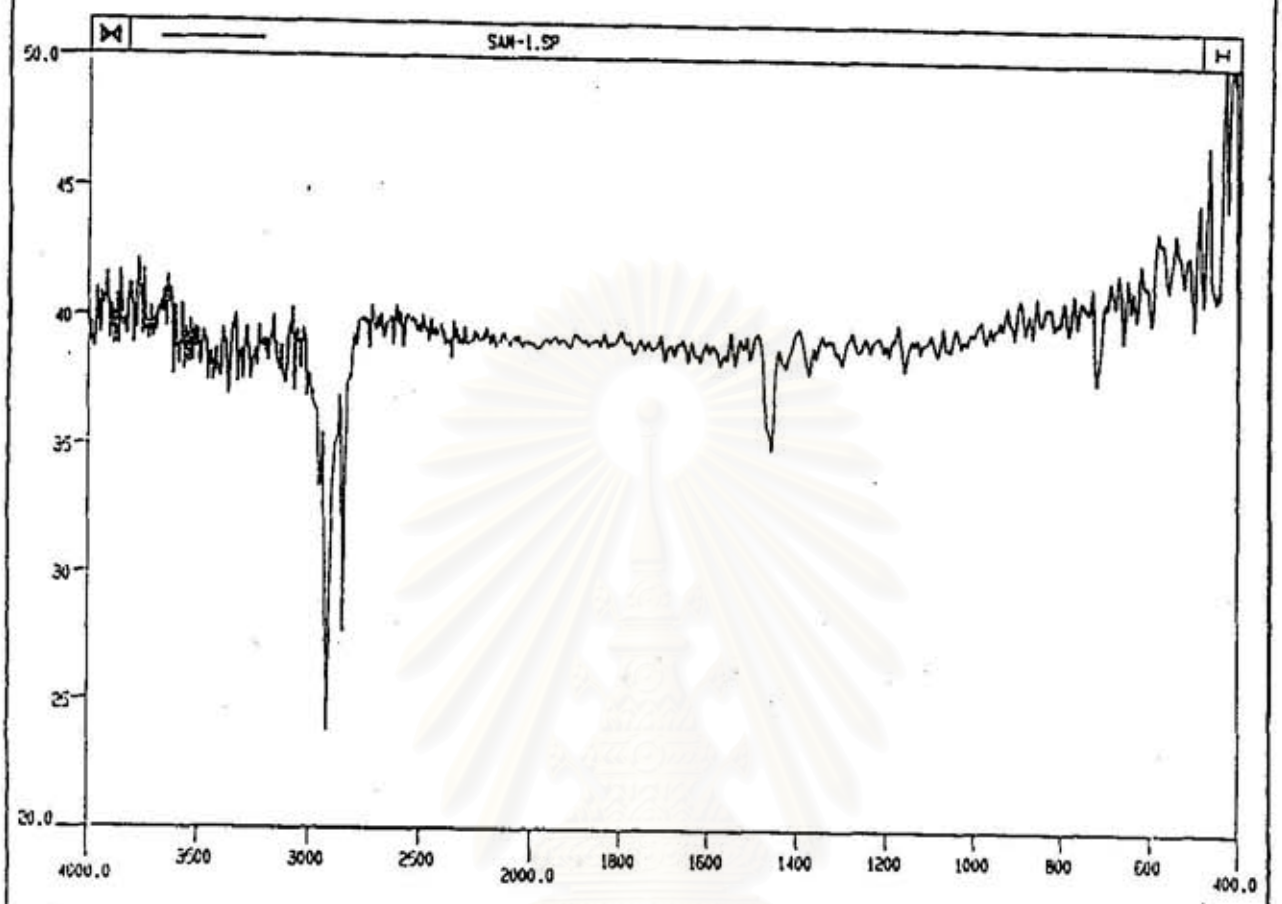


Figure 5 The IR spectrum of Mixture 1.

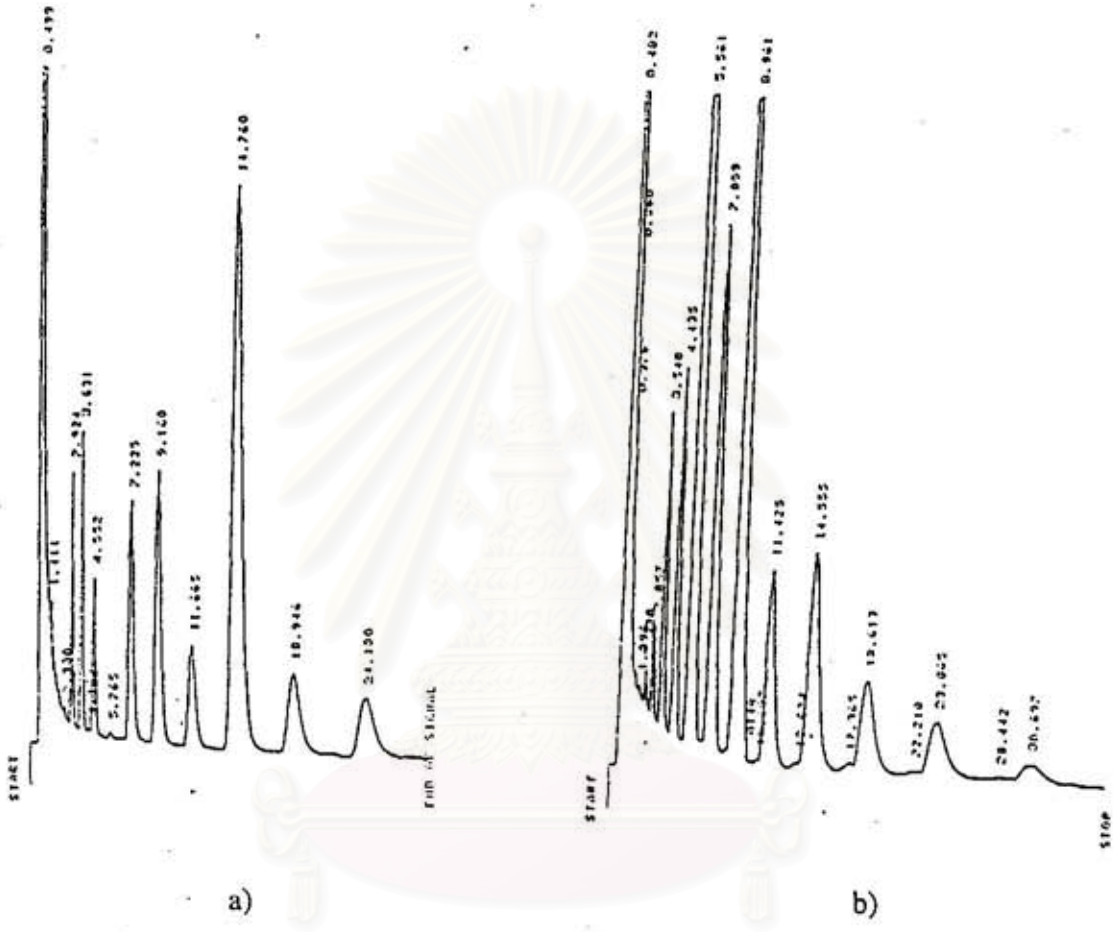


Figure 6 The GLC analysis results of

a) standard long chain hydrocarbon

b) Mixture 1

3.8.2 Structure Elucidation of Mixture 2.

Mixture 2 was an amorphous white solid in an orange oil ,obtained by column chromatography of the hexane crude extract (Table 3)The white solid was purified by crystalization from ethyl acetate , yielding 105.6 mg (8.12×10^{-3} % wt by wt of dried roots). The melting point was 72-74° C and R_f value was 0.78 [hexane: dichloromethane (20:1)] .Compound 2 was soluble in hexane and dichloromethane but not in methanol , ethanol and acetone.

The IR spectrum (Fig. 7) exhibited a strong absorption band at 1745 cm^{-1} , which is characteristic of an ester carbonyl group. The absorption band of hydrocarbons were also observed .

Table 12 The IR absorption band assignments of Mixture 2.

vibration	wave number (cm^{-1})	intensity
C-H stretching of $-CH_2-$, $-CH_3$	2920 , 2855	strong
C=O stretching of ester	1745	strong
C-H bending of $-CH_2-$, $-CH_3$	1470	moderate
C-O stretching	1180	moderate
CH_2 rocking in $C-(CH_2)_n-C$	725	weak

The IR results indicated that Mixture 2 was a mixture of long chain esters.

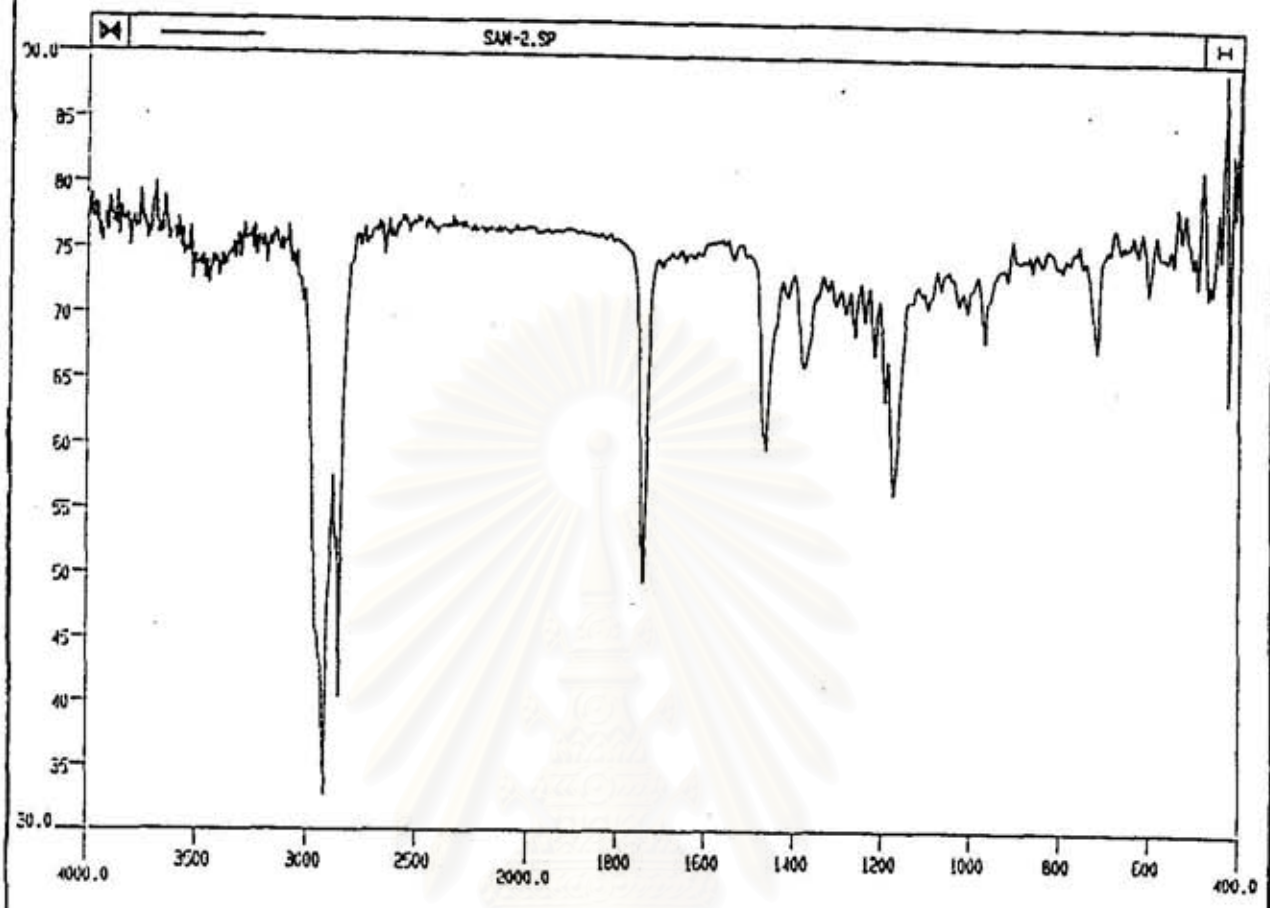


Figure 7 The IR spectrum of Mixture 2

3.8.3 Structure Elucidation of Mixture 3.

Mixture 3 was obtained as a white powder from hexane extract separation . This substance was purified by crystalized with acetone and yielding 140.4 mg. (1.08×10^{-2} % wt by wt of dried roots). R_f value of this compound was 0.62 [dichloromethane : methanol (9:1)] and the melting point was 76-78° C .

The IR spectrum displayed the band of a hydroxy group at 3500-3200 cm^{-1} , carbonyl group at 1705 cm^{-1} and long chain hydrocarbon at 2919 , 2850. The spectral data indicated this compound might be a long chain acid. (Fig.8)

Table 13 The IR absorption band assignments of Mixture 3

vibration	wave number (cm^{-1})	intensity
O-H stretching of carboxylic acid	3500 - 3200	weak , broad
C-H stretching of -CH_2 , -CH_3	2920 , 2850	strong
C=O stretching of carboxylic acid	1710	moderate
C-H bending of -CH_2 , -CH_3	1465 , 1435	moderate
C-O stretching	1305	moderate
CH_2 rocking in C-$(CH_2)_n$-C	725 , 690	weak

This compound was converted to a methyl ester by reacted with N-nitrosomethylurea [21] and analyzed by GC-MS. The Gas chromatogram (Fig. 9) and mass spectrum (Fig.10-13) were compared by library search and were found to match several in methyl esters as shown in Table 14.

Table 14 Number of carbon in methyl ester derivative of Mixture 3.

Compound	Structural formula	Retention time	m/z
Docosanoic acid , methyl ester	$C_{23}H_{46}O_2$	27.49	354
Tricosanoic acid , methyl ester	$C_{24}H_{48}O_2$	17.91	368
Tetracosanoic acid , methyl ester	$C_{25}H_{50}O_2$	14.57	382
Pentacosanoic acid , methyl ester	$C_{26}H_{52}O_2$	11.70	396
Hexacosanoic acid , methyl ester	$C_{27}H_{54}O_2$	9.98	410
Heptacosanoic acid , methyl ester	$C_{28}H_{56}O_2$	8.35	424
Octacosanoic acid , methyl ester	$C_{29}H_{58}O_2$	6.64	438
Triacontanoic acid , methyl ester	$C_{31}H_{62}O_2$	5.81	466

Physical and Chemical properties and spectral data indicated that Mixture 3 was the mixture of long chain acids. (Table 15)

Table 15 The mixture of long chain acids in Mixture 3.

Compound	Molecular formula	Structural formula	MW.
Docosanoic acid	$C_{22}H_{44}O_2$	CH_3-$(CH_2)_{19}$-CH_2-COOH	340
Tricosanoic acid	$C_{23}H_{46}O_2$	CH_3-$(CH_2)_{20}$-CH_2-COOH	354
Tetracosanoic acid	$C_{24}H_{48}O_2$	CH_3-$(CH_2)_{21}$-CH_2-COOH	368
Pentacosanoic acid	$C_{25}H_{50}O_2$	CH_3-$(CH_2)_{22}$-CH_2-COOH	382
Hexacosanoic acid	$C_{26}H_{52}O_2$	CH_3-$(CH_2)_{23}$-CH_2-COOH	396
Heptacosanoic acid	$C_{27}H_{54}O_2$	CH_3-$(CH_2)_{24}$-CH_2-COOH	410
Octacosanoic acid	$C_{28}H_{56}O_2$	CH_3-$(CH_2)_{25}$-CH_2-COOH	424
Triacontanoic acid	$C_{30}H_{60}O_2$	CH_3-$(CH_2)_{26}$-CH_2-COOH	438

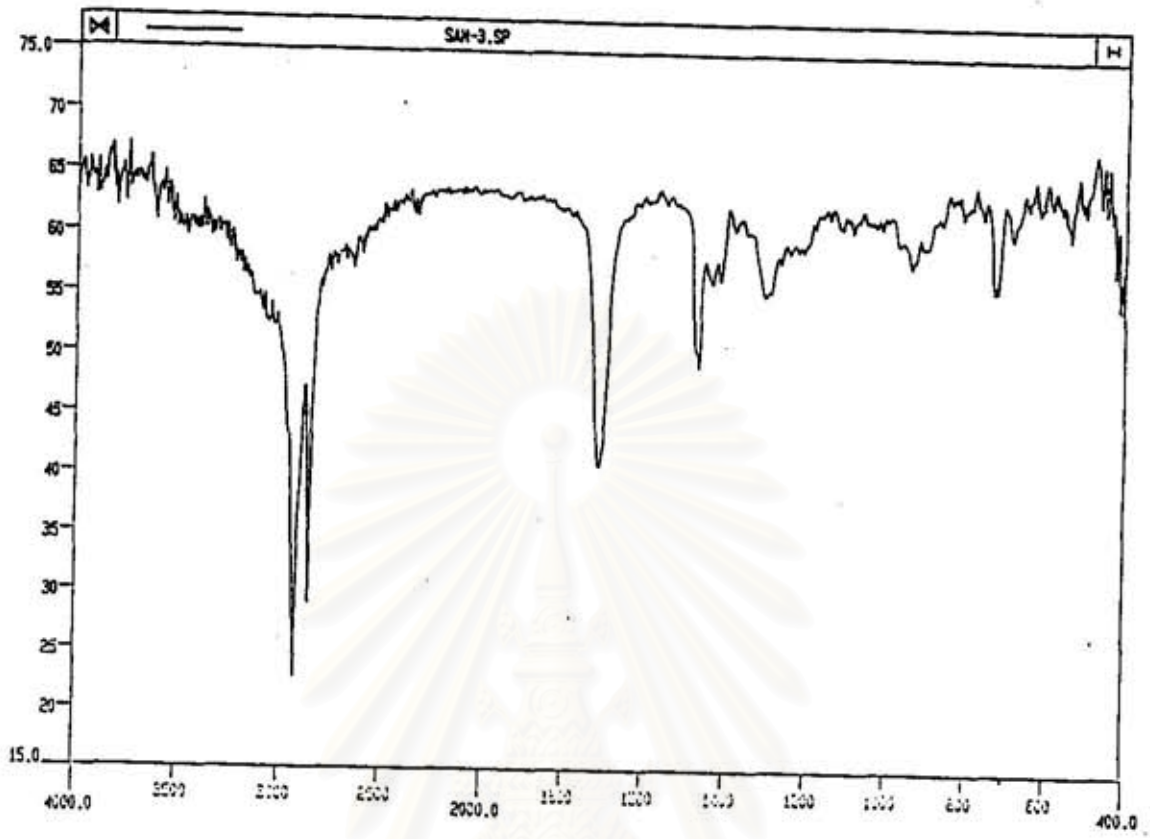


Figure 8 The IR Spectrum of Mixture 3

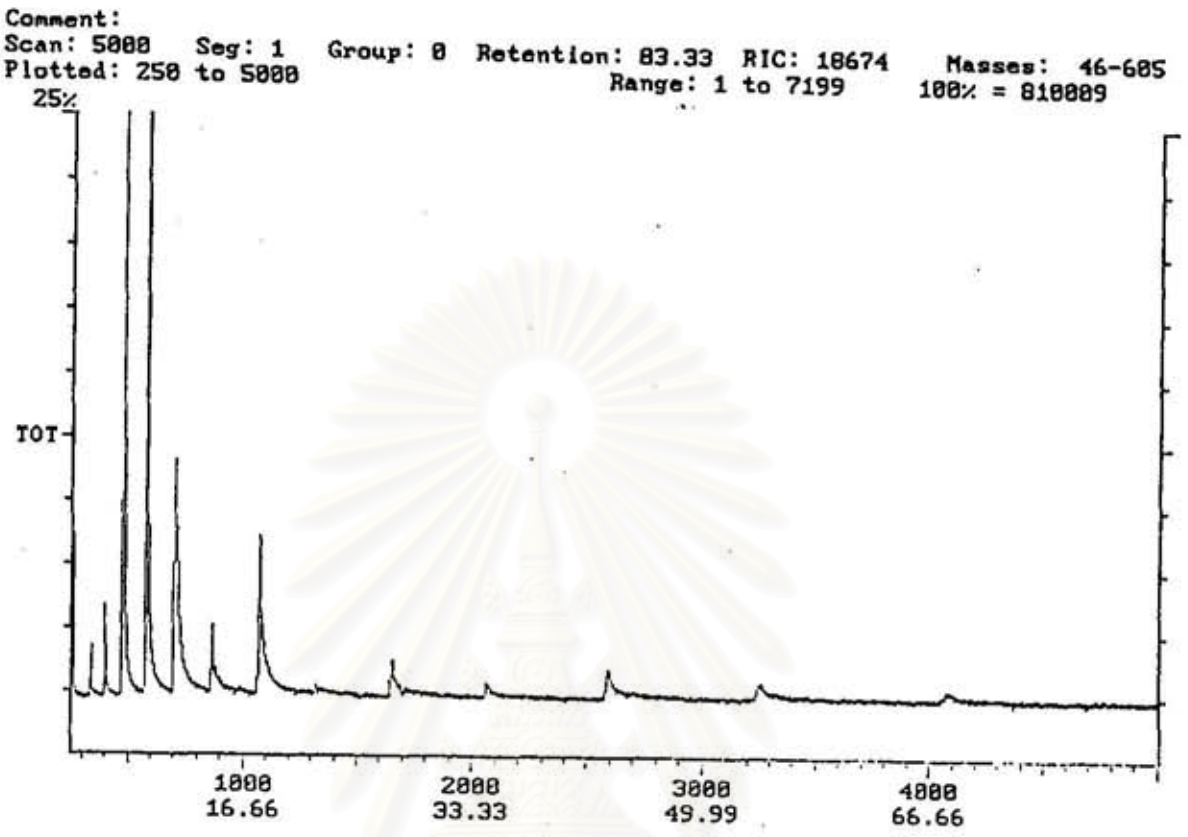


Figure 9 The GC analysis results of Mixture 3.

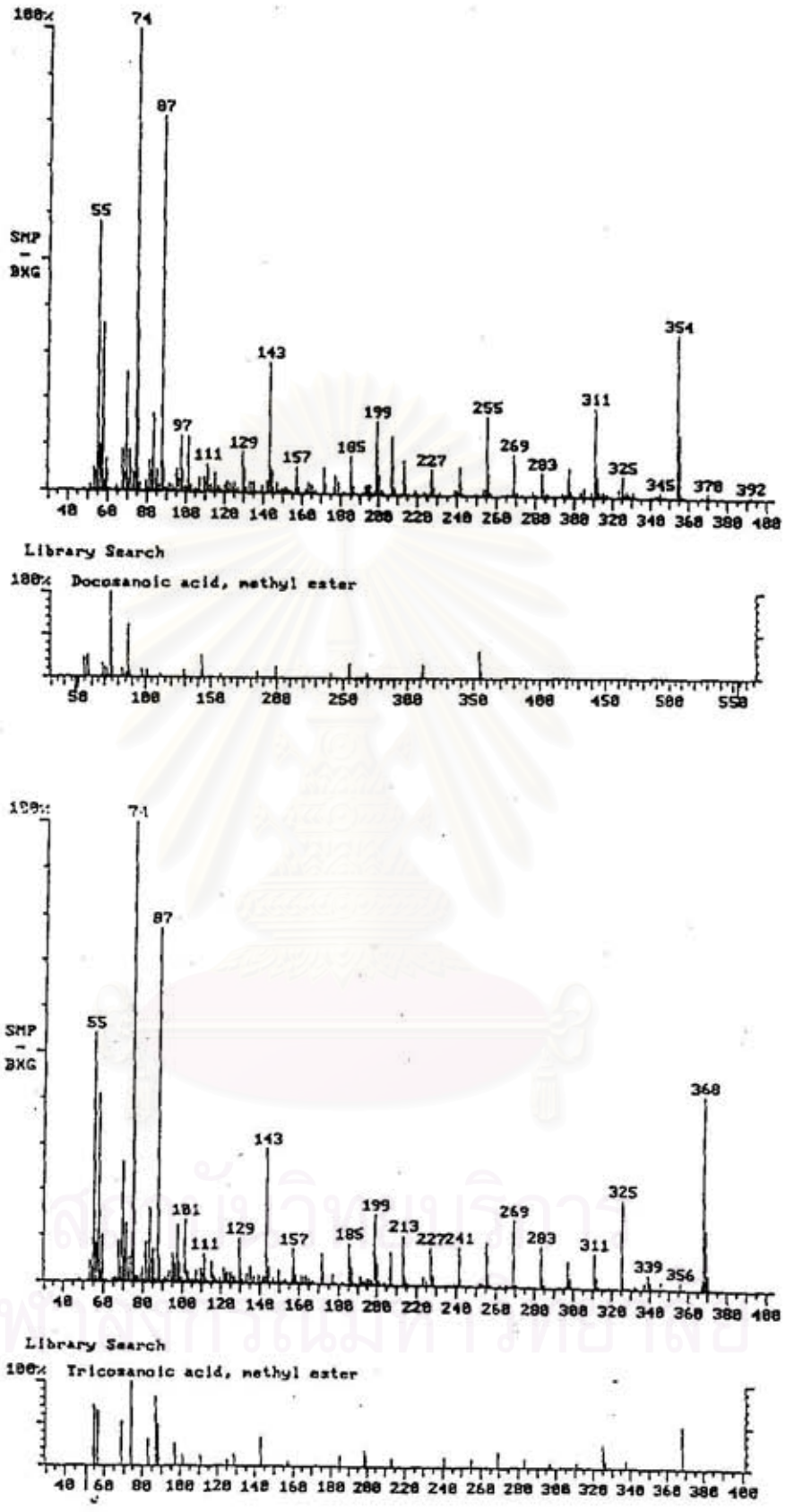


Figure 10 The mass spectrum of a component in Mixture 3.

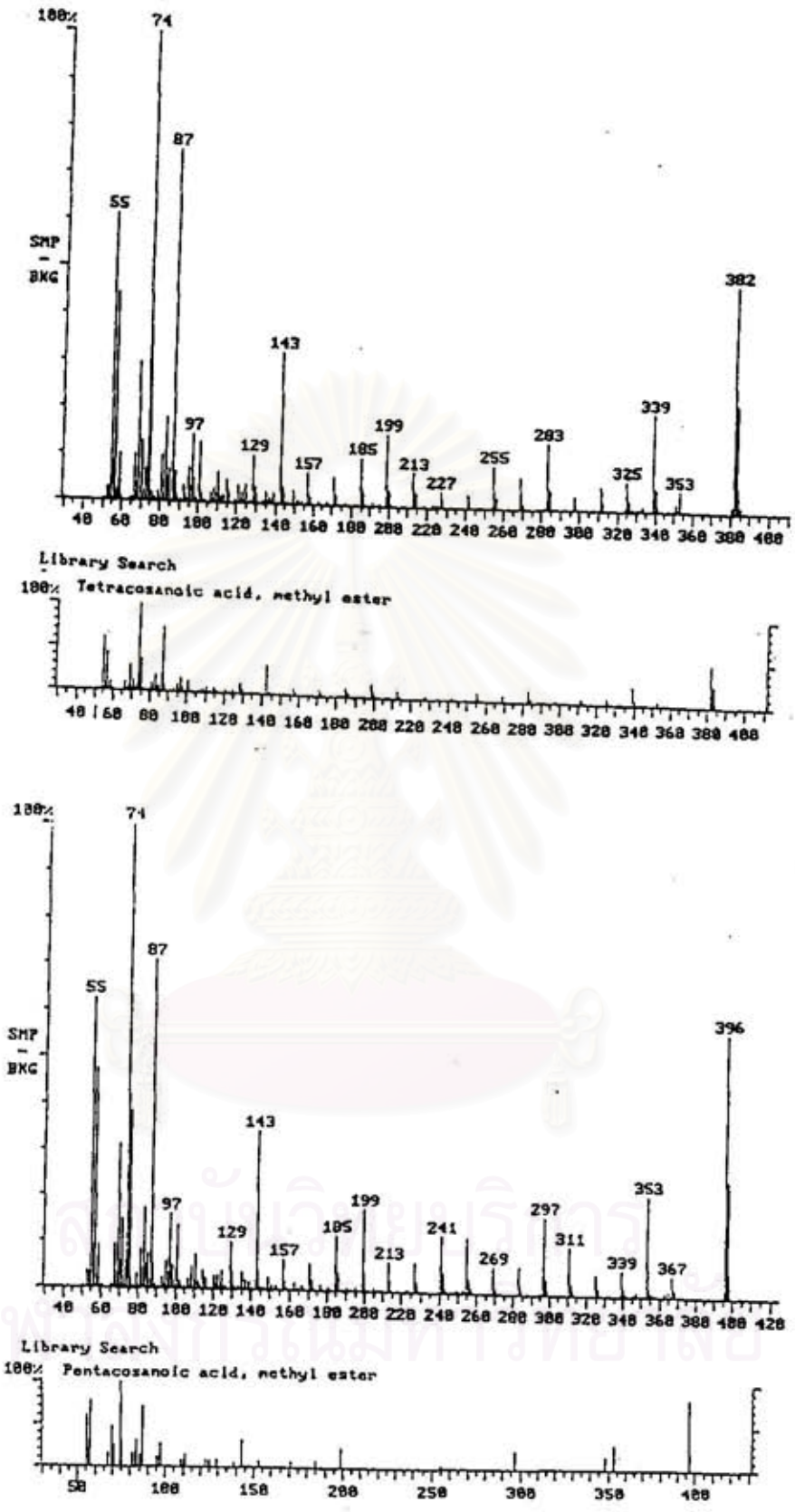


Figure 11 The mass spectrum of a component in Mixture 3.

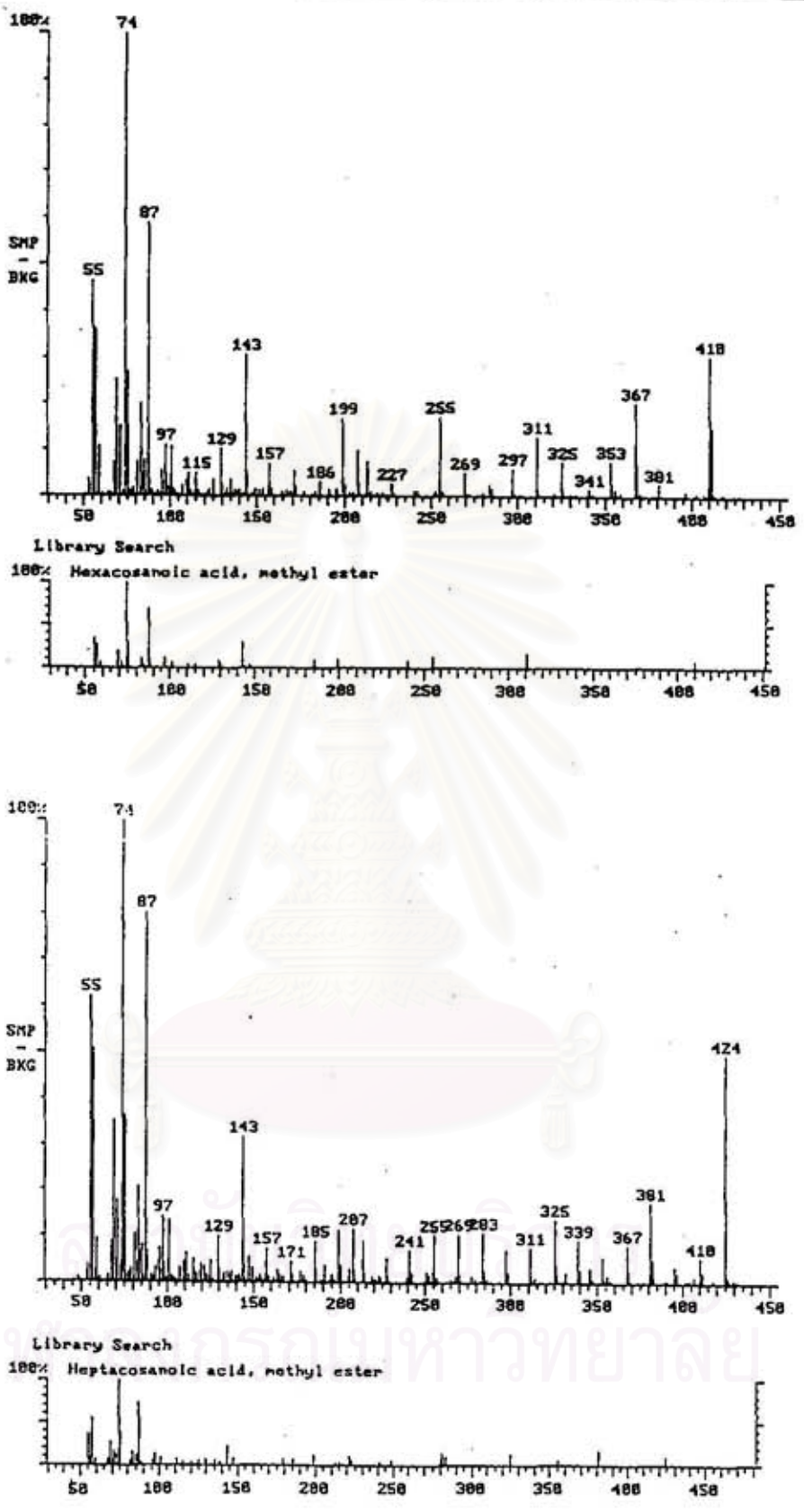


Figure 12 The mass spectrum of a component in Mixture 3.

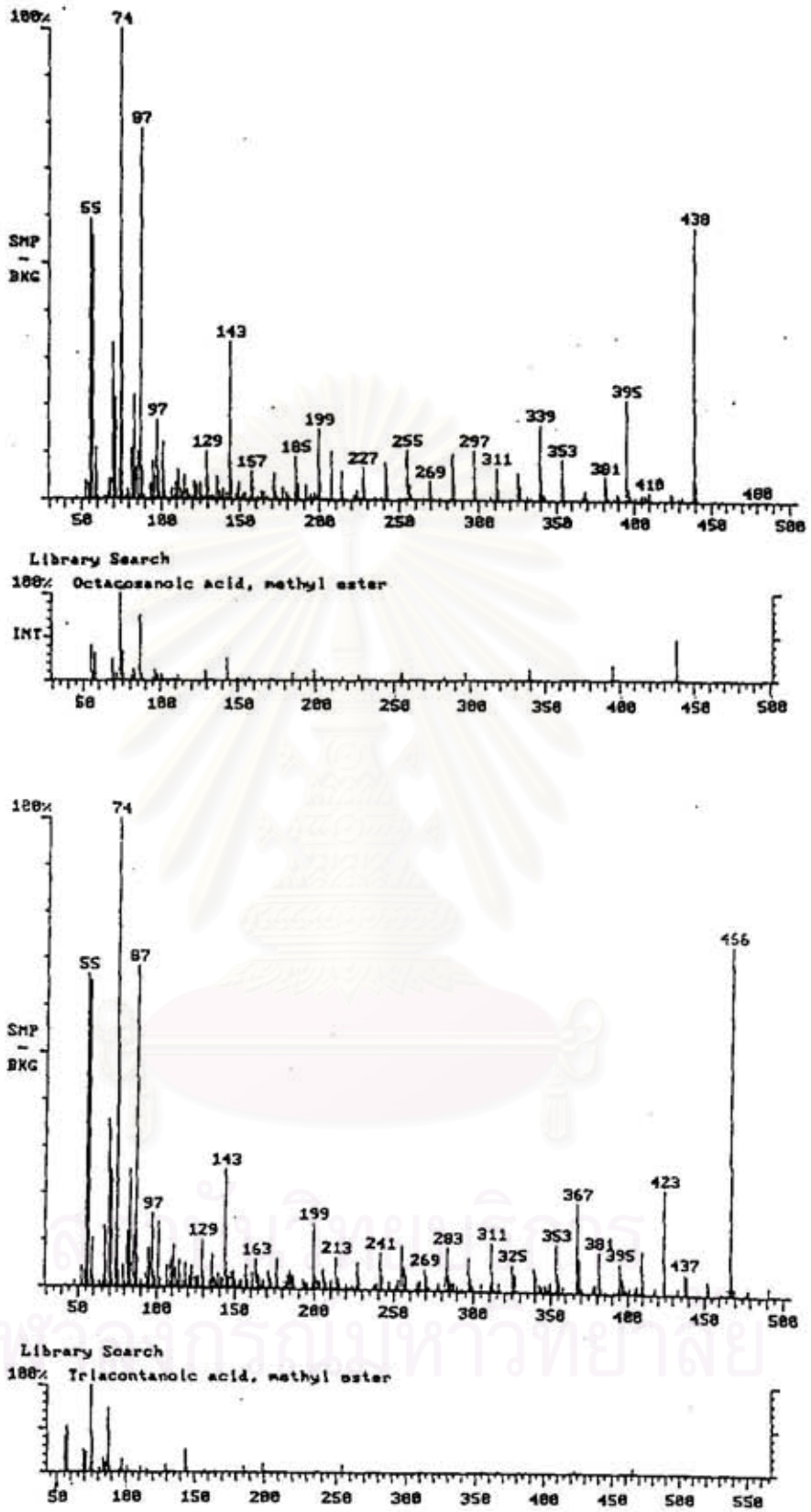


Figure 13 The mass spectrum of a component in Mixture 3.

3.8.4 Sructure elucidation of Compound 4.

Compound 4 was white needle crystals in a yellow oil, which was obtained from both the hexane and dichloromethane extracts. This compound was crystalized from hexane and yielded 1.32 g of the white needles (1.02×10^{-2} % % wt by wt of dried roots). The R_f value was 0.65 [hexane: dichloromethane (3:2)] , mp. 163-164° C . This compound was tested with Libermann- Burchard reagent and showed a blue colour, which is characteristic of the presence of a steroidal structure.

The IR absorption band at 3600-3300 cm^{-1} suggested the presense of hydroxy group (-OH) . Absorption bands due to a disubstituted alkene at 969 cm^{-1} and trisubstituted alkenes at 838 and 802 cm^{-1} were observed. (Fig.14)

The EI mass spectrum showed an [M^+] peak at m/z 412 and peaks of relatively high relative abundance at m/z 394 , 300 , 273 , 255 and 213. The molecular ion peak [M^+] at m/z 412 is consistent with a molecular weight of $C_{29}H_{48}O$. (Fig. 15)

The ^{1}H-NMR spectrum of compound 4 showed signals for -CH_3 , -CH_2- and -CH of steroid at δ 0.50-2.50 , hydroxy group at δ 3.50 , and the proton in -CH=CH- at δ 5.00 and 5.22 .(Fig. 16)

The ^{13}C NMR spectrum and DEPT 135 and 90 spectra showed that this compound contained 11 tertiary carbons , 8 methylene carbons , 6 methyl carbons and 4 quaternary carbons. (Fig. 17-19)

The chemical tests , IR spectrum and NMR spectra showed that Compound 4 could be a steroidal compound having a hydroxyl group and a double bond . These results were consistant with the formular of this compound as $C_{29}H_{48}O$.

Table 16 The IR absorbtion band assignments of Compound 4.

vibration	wave number (cm^{-1})	intensity
O-H stretching of R-OH	3600-3300	moderate
C-H stretching of CH_2 , CH_3	2937-2867	strong
C-H stretching of alkene	1645	weak
C-H bending of $-CH_2-$, $-CH_3$	1459-1377	moderate
C-O stretching and OH bending	1058	moderate
C-H out of plane bending - vibration of trans configuration	969	weak
C-H out of plane bending - vibration	838 , 802	weak

Gas chromatography was used to compare Compound 4 with a standard mixture of steroids : cholesterol , campesterol , stigmasterol and β-sitosterol . The retention time of the standard steroids were 13.06 , 17.61 , 18.76 and 21.00 The retention time of this compound was 18.21 , which indicated that this compound was stigmasterol (Fig.20).

The ^{13}C NMR spectrum of compound 4 was compared with that of stigmasterol [22] to confirm the structure. (Table 17)

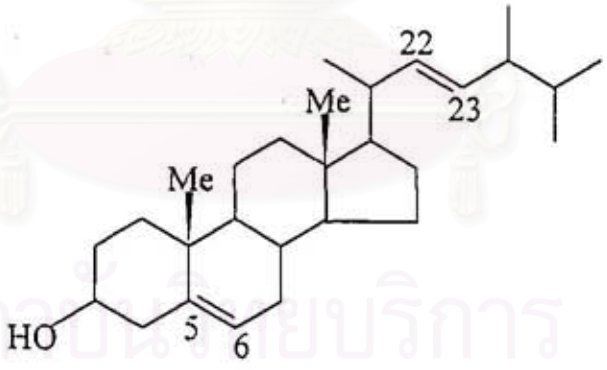
Table 17 ^{13}C-NMR spectrum of Compound 4 compared with stigmasterol [22] (50 MHz , $CDCl_3$)

position	chemical shift (δ ppm)	
	stigmasterol	compound 4
1	37.4	37.4
2	31.7	31.8
3	71.8	71.8
4	42.4	42.4
5	140.0	140.7
6	121.7	121.5
7	31.9	32.1
8	31.9	32.1
9	50.3	50.1
10	36.6	36.8
11	21.1	21.3
12	39.8	39.8
13	42.4	42.4
14	57.0	57.0
15	24.4	24.5
16	28.9	29.1
17	56.0	55.9
18	12.2	12.4
19	19.4	19.4
20	40.5	40.8
21	21.1	21.3

Table 17 (cont.)

position	chemical shift (δ ppm)	
	stigmasterol	compound 4
22	138.4	138.4
23	129.4	129.2
24	51.3	51.2
25	31.9	32.1
26	19.0	19.0
27	21.1	21.3
28	25.4	25.6
29	12.0	12.0

All of these results indicated that Compound 4 was stigmasterol.



stigmasterol

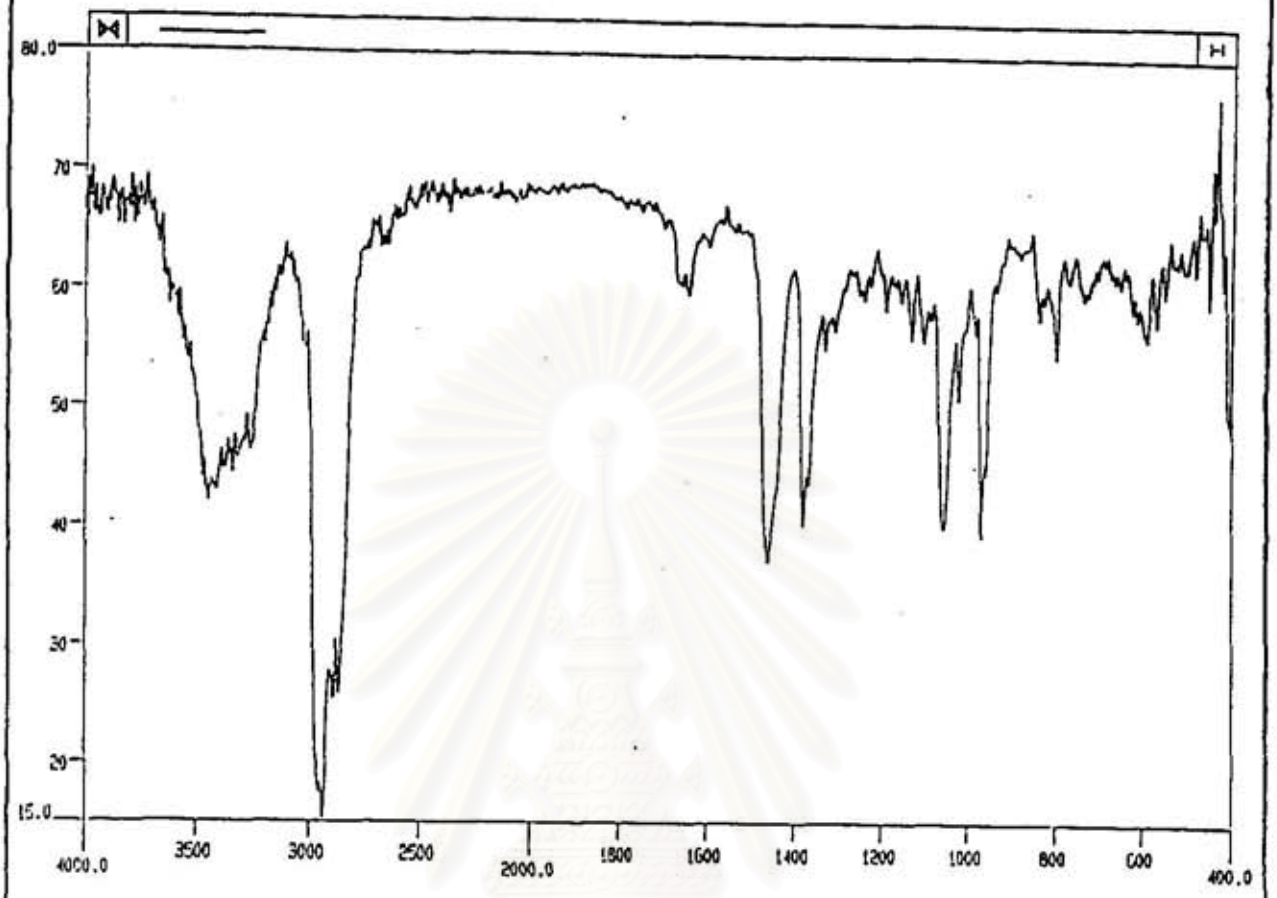


Figure 14 The IR spectrum of Compound 4

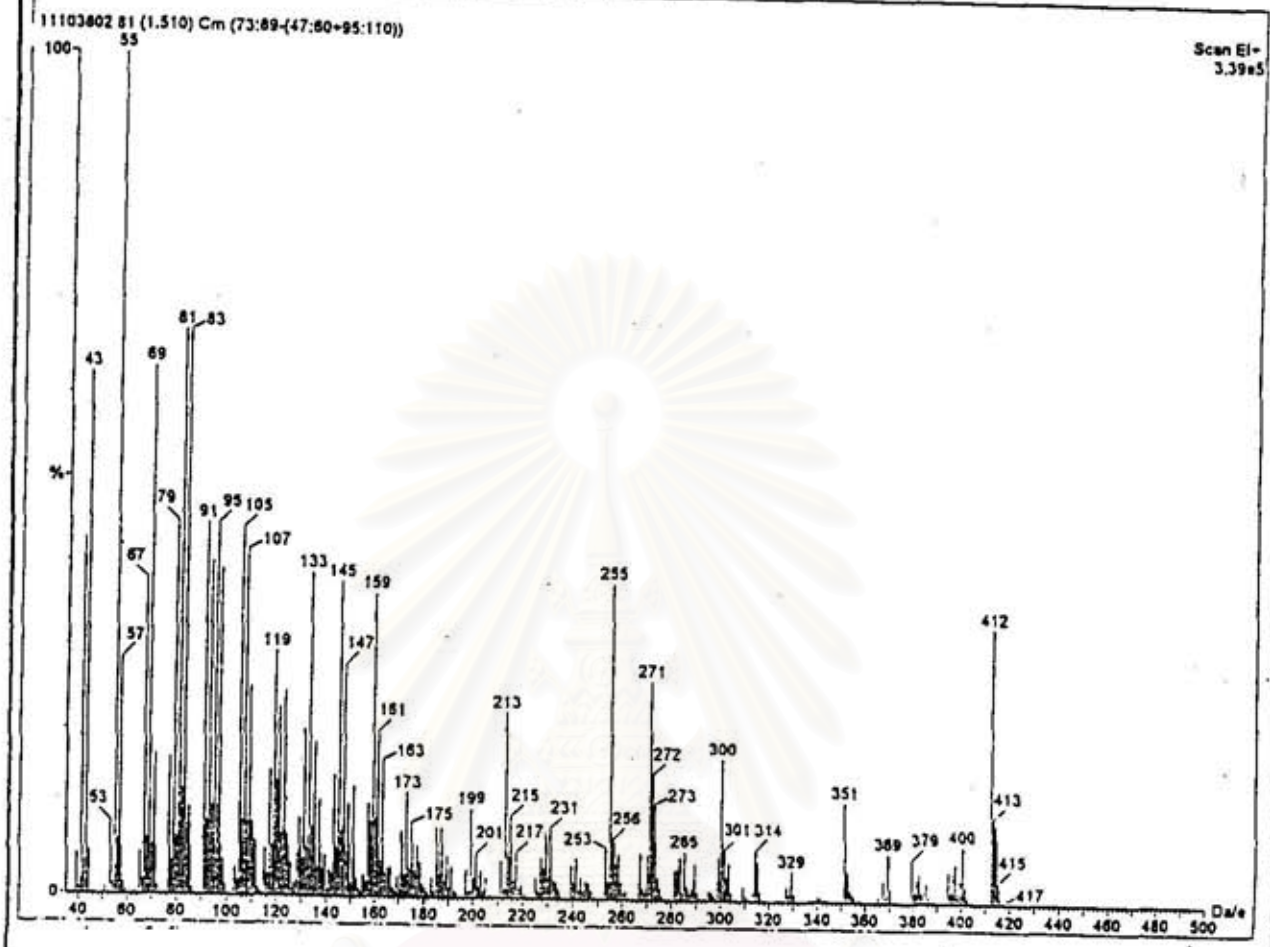


Figure 15 The mass spectrum of Compound 4

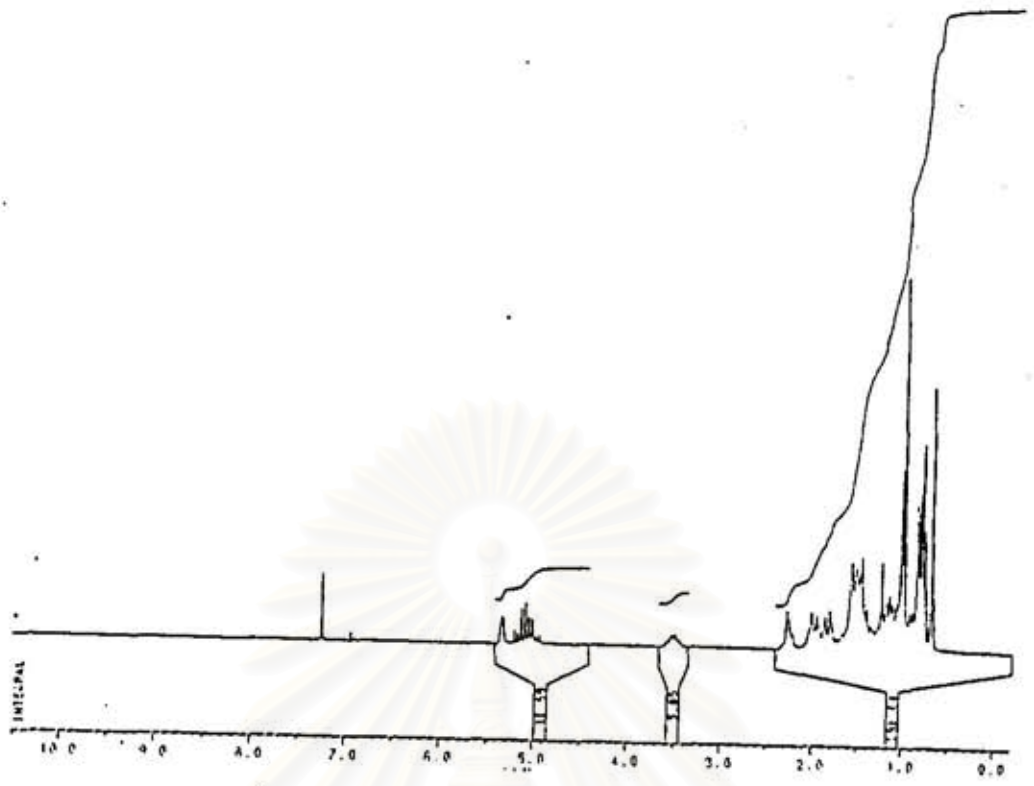


Figure 16 The ^{1}H NMR spectrum of Compound 4

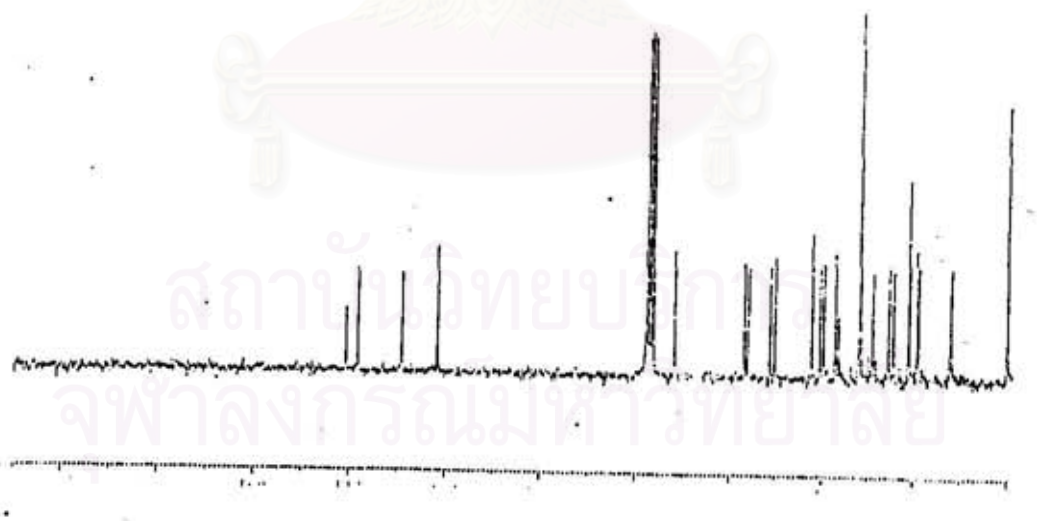


Figure 17 The ^{13}C NMR spectrum of Compound 4

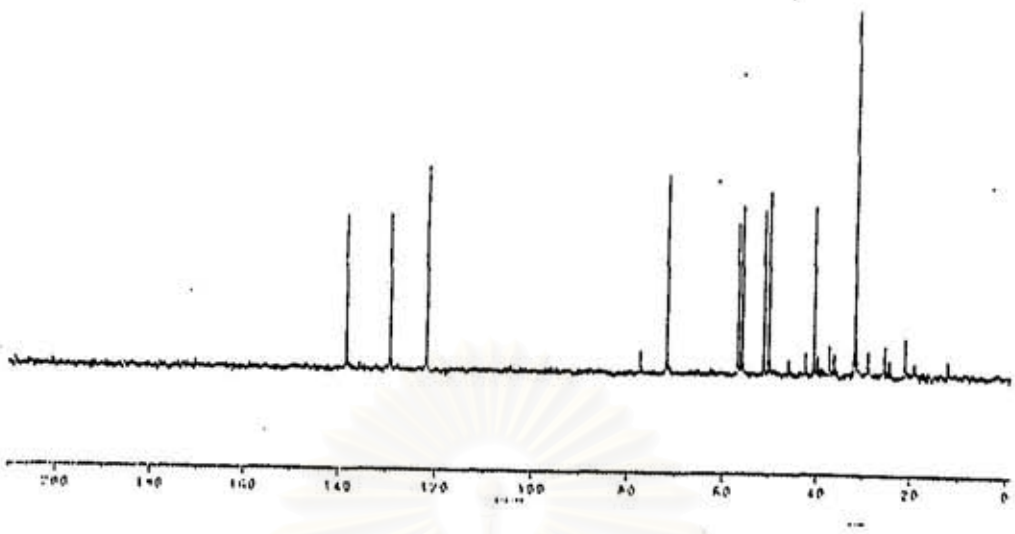


Figure 18 The DEPT 90 ^{13}C NMR spectrum of Compound 4

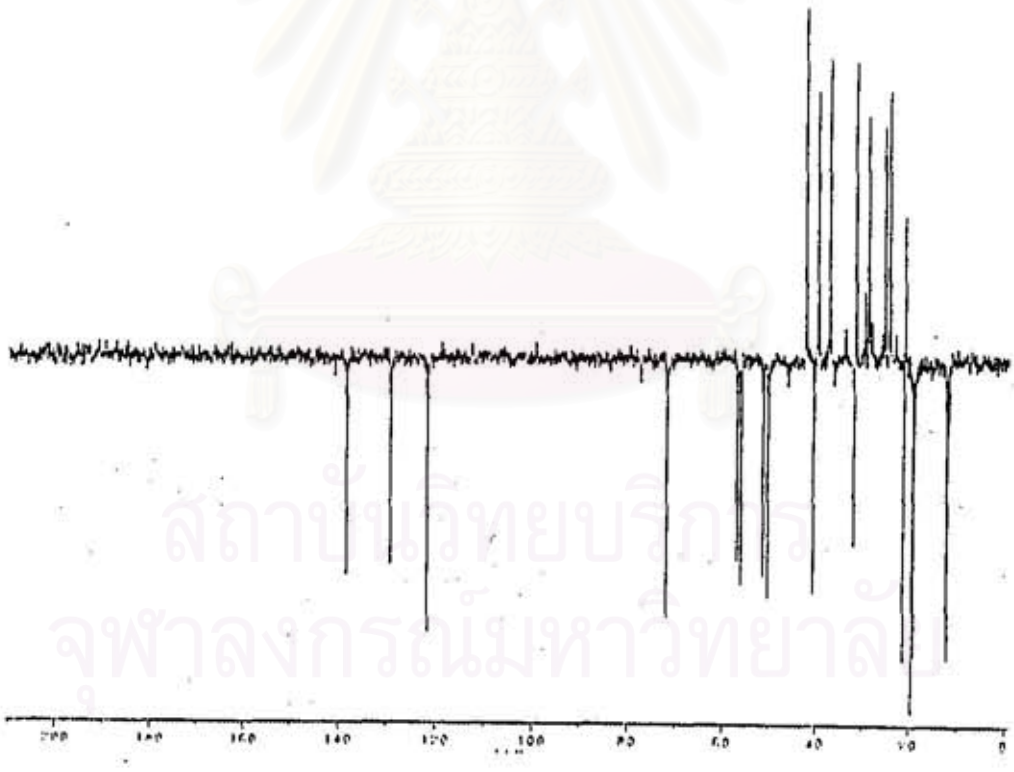


Figure 19 The DEPT 135 ^{13}C NMR spectrum of Compound 4

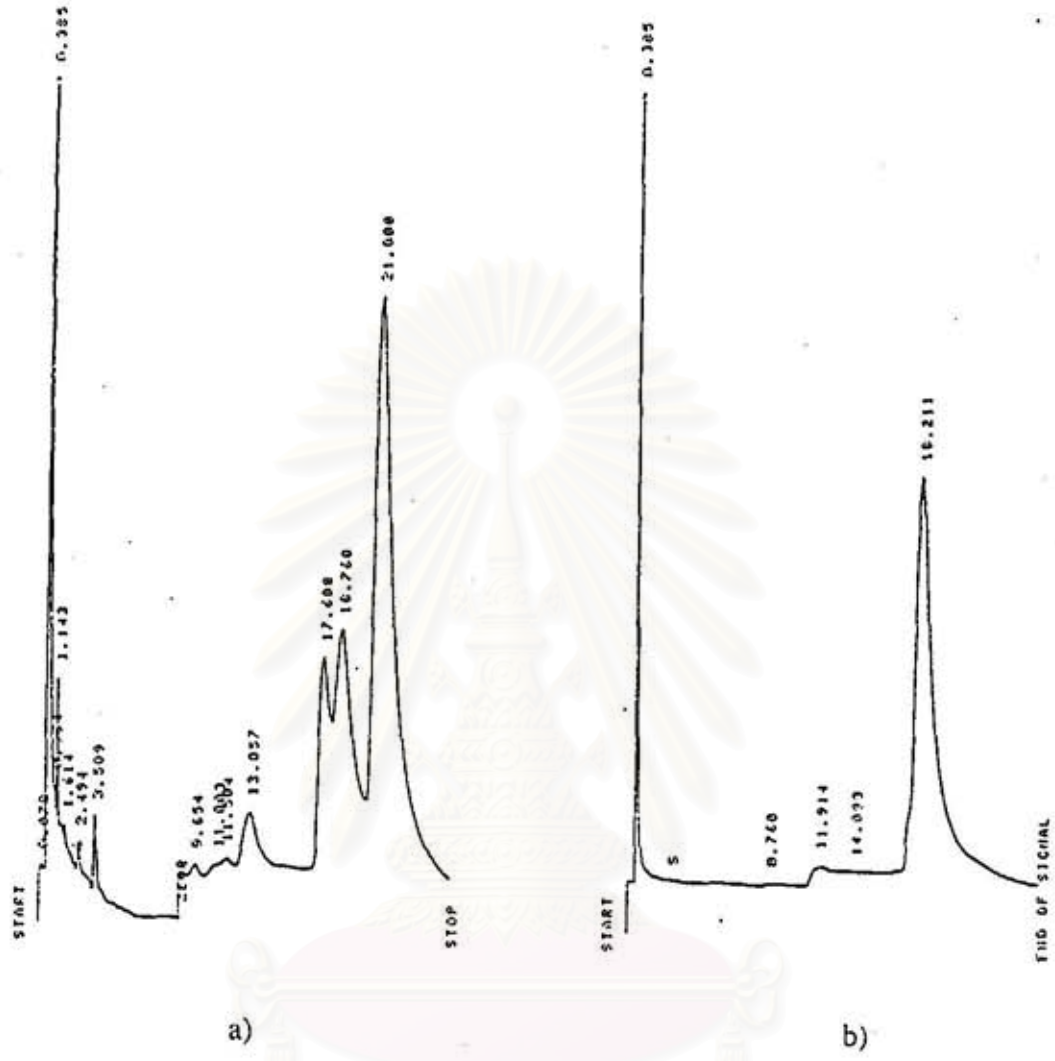


Figure 20 The GLC analysis results of

a) standard steroid ; cholesterol , campesterol , stigmasterol , β-sitosterol

b) Compound 4

3.8.5 Structure Elucidation of compound 5.

Compound 5 was isolated by silica gel chromatography of the crude dichloromethane extract and further purified by chromatotron and recrystalization (hexane:dichloromethane). This compound , mp. 181-183° C , was obtained as cubic crystals , 152.6 mg. (1.17 x 10^{-2} % wt by wt of dried roots). The R_f value was 0.54 [hexane: dichloromethane (1:4)]

The IR spectrum showed a broad absorption band of a hydroxy group at 3600-3200 cm^{-1}, the C-O stretching band at 1050 cm^{-1} and aromatic C=C bands around 1600 $cm.^{-1}$ (Fig.21)

The mass spectrum showed a molecular ion peak at M^+ 286 and other fragments at m/z 271 (M^+-CH_3) , 239 (M^+-C_2H_7O) , 253 (M^+-CH_5O) and 211 (M^+-$C_3H_7O_2$) . (Fig.22)

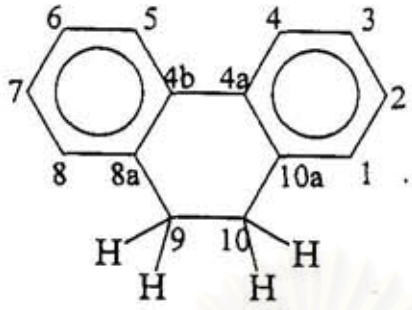
The ^1H NMR spectrum of compound 5 showed signals for ortho aromatic protons at δ 6.84 [1H , d , J = 8.6 Hz] and 7.86 [1H , d , J = 8.6 Hz] , another aromatic proton at δ 6.40 [1H ,s] , two phenolic hydroxy groups at δ 4.79 and 5.62 , two aromatic methoxy groups at δ 3.78 and 3.81 [each 3H , s] , one methyl group at δ 2.17 [3H , s] and two benzylic methylene protons at δ 2.69 and 2.76 [each 2 H , m] . (Fig.23-24)

The ^{13}C NMR spectrum exhibited 17 signals .DEPT 90 and 135 experiments showed one methyl carbon at δ 11.2 , two methylene carbons at δ 22.2 and 25.7 , two methoxy carbons at δ 55.6 and 61.4 , three tertiary carbons at δ 98.1, 112.4 and 125.1 and nine quaternary carbons at δ 112.7 , 117.0, 126.4, 130.6 , 139.4 , 143.3 , 146.6 , 152.8 and 155.5 . (Fig.25-26)

These results showed that this compound has 17 carbons , 18 protons , 2 oxygens in methoxy groups and 2 oxygens in phenolic hydroxy groups. The molecular ion peak at m/z 286 was consistent with a molecular weight of $C_{17}H_{18}O_4$, which confirmed that two hydroxy groups were present in this compound . The molecular formula showed a degree of unsaturation of nine which indicated the possibility of two aromatic rings and one cyclic structure or one double bond . Dihydrophenanthrenes and stilbenes are among the common natural product structures with these characteristics.

(a) (b)

Further more , two methylene protons at δ 2.69 [2H , m] and 2.76 [2H , m] showed the presence of adjacent methylene carbons in this molecule. This is typical of the H_2-9 and H_2-10 of 9,10 dihydrophenanthrene derivatives. The signals of these four benzylic protons were multiplets. This result suggested that the protons which were attached to C-9 and C-10 were not identical. From these data , Compound 5 was presumed to be a 9,10 dihydrophenanthrene derivative having two methoxy groups , two hydroxy groups and one methyl group.



Substituents

- OCH_3 (2)
- OH (2)
- CH_3 (1)

The basic skeleton of 9,10 - dihydrophenanthrenes

The ^1H NMR spectral data also revealed the presence of ortho aromatic protons at δ 6.84 [1H , d , J = 8.6 Hz] and 7.86 [1H , d , J = 8.6 Hz] in this molecule.

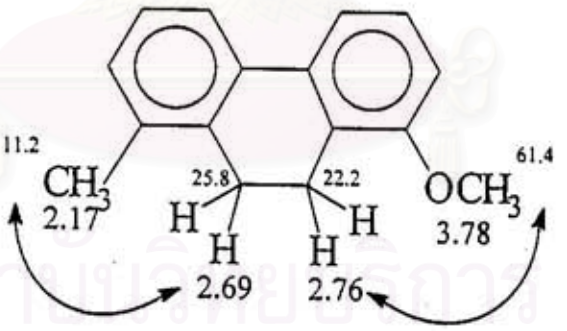
Two dimensional NMR techniques were used to provide further information . One Bond Correlation (HMQC) data revealed that the proton at δ 7.86 was attached to the carbon at 125.1 ppm. , the proton at δ6.84 was attached to the carbon at 112.4 ppm. and the proton at δ 6.40 was attached to the carbon at 98.1 ppm. The proton of the methoxyl group at δ 3.81 was attached to the carbon at 55.6 ppm. and δ3.78 was attached to the carbon of the methoxy group at 61.4ppm. The methylene proton at δ 2.76 was attached to the carbon at 22.2 ppm. and δ 2.62 was attached to the carbon at 25.7 ppm.. The proton of the methyl group at δ 2.17 was attached to the carbon at 11.2 ppm. (Fig.27-29)

Table 18 ^{1}H and ^{13}C NMR spectral data of Compound 5 (500/125 MHz , $CDCl_3$)

Position	ppm.	Attached proton
C-5	155.5	-
C-7	152.8	-
C-4	146.7	-
C-1	143.3	-
C-8a	139.4	-
C-10a	130.6	-
C-4b	126.4	-
C-2	125.1	7.86(d) J = 8.6 Hz
C-4a	117.0	-
C-8	112.7	-
C-3	112.0	6.84(d) J = 8.9 Hz
C-6	98.1	6.40(s)
methoxy group at C-1	61.4	3.78(s)
methoxy group at C-7	55.6	3.81(s)
C-9	25.7	2.69(m)
C-10	22.2	2.76(m)
methyl group	11.2	2.17(s)
hydroxy group at C-4	-	4.79(s)
hydroxy group at C-5	-	5.62(s)

NOE difference experiments were used to obtain additional information. Irradiation of the proton at δ 6.84 caused enhancement of the signal at δ 7.86 while irradiation of the proton at δ 2.69 caused enhancement of the signal at δ 2.17 in the methyl group at C-8. NOEs between the proton at δ 6.84 and proton at δ 7.86 , the proton at δ 2.76 and the proton in methoxy group at δ 3.78 were seen . When the proton in the methyl group at δ 2.17 was irradiated , an enhancement of the signal at δ 2.69 were observed. Irradiation of the proton at δ 6.40 caused enhancement of the signal at δ 3.81 and 5.62 and irradiation of the proton at δ 5.62 caused enhancement of the signal at δ 4.79. (Fig. 30-39)

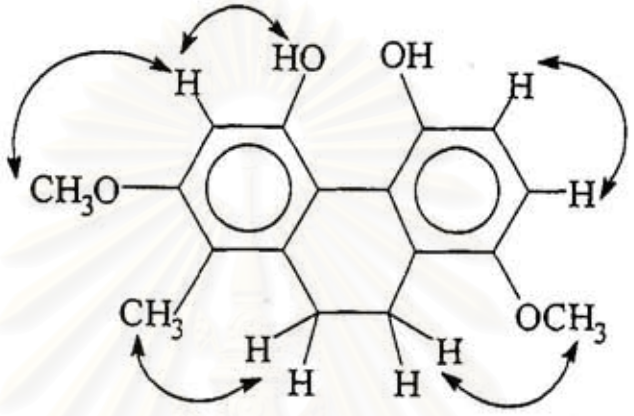
The NOEs data indicated that the methyl group was near the methylene protons at δ 2.69 while the other methylene protons at δ 2.76 was near the methoxy group at δ 3.78 .



Moreover , these data confirmed that the proton at δ 6.84 [1H , d , J = 8.6 Hz] was near the proton at δ 7.86 [1H , d , J = 8.6 Hz]. The proton at δ 6.40 [1H ,s] was situated between the methoxy group at δ 3.81 and the

hydroxy group at δ 5.62. When the proton in other hydroxy group was irradiated, the signals for both hydroxyls and residual water were saturated.

This molecular structure is in agreement with the NOE data as follow.

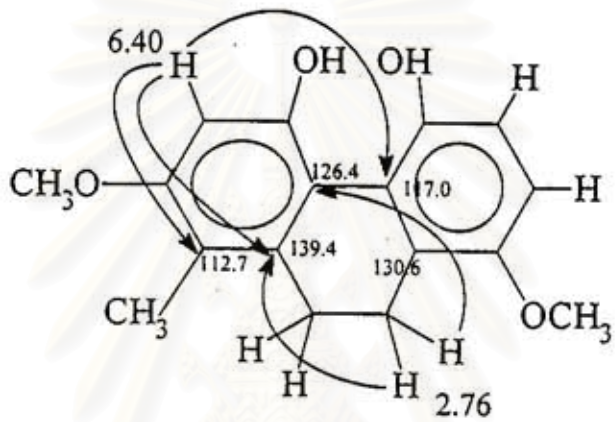


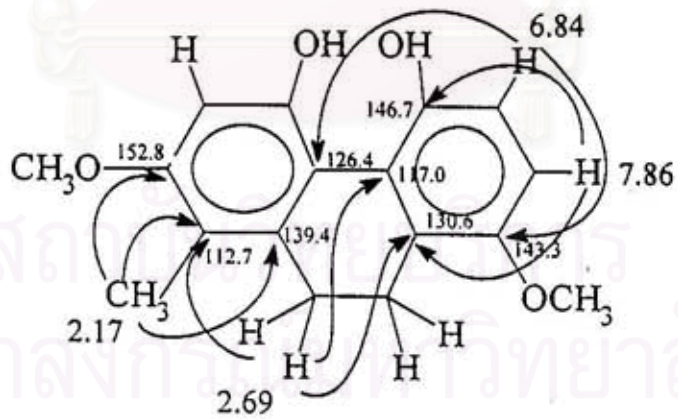
Most significant interactions observed in NOEs of Compound 5.

In the H - C long range coupling spectrum obtained by HMBC (Heteronuclear multiple bond correlation), the aromatic proton at δ 7.86 showed cross peaks with the carbons at δ 130.6 (C-10a) , 146.7 (C-4) , and 117.0 (C-4a) , the proton at δ6.8 showed cross peaks with the carbons at δ 143.3 (C-1),146.7 (C-4) , and 126.4 (C-4b) , the proton at δ 6.40 showed cross peaks with the carbons at δ112.7 (C-8) , 155.5 (C-5) , 152.8 (C-7) and 116.0 (C-4a). The methylene proton at δ 2.76 showed long-range correlations with carbons at δ 139.4(C-8a), 143.3 (C-1), 130.4(C-10a) and 126.3(C-4b) , the proton at δ 2.62 was related to carbons at δ 112.7 (C-8), 130.6 (C-10a) , 116.0 (C-4a) and 139.4(C-8a) . The proton of the methyl group at δ 2.17 showed

cross peaks with the carbons at δ 152.7(C-7) , 139.4 (C-8a) , 126.4 (C-4b) and 112.7 (C-8). (Fig. 40-44)

The HMBC data confirmed the possible structure suggested following the NOE experiments.





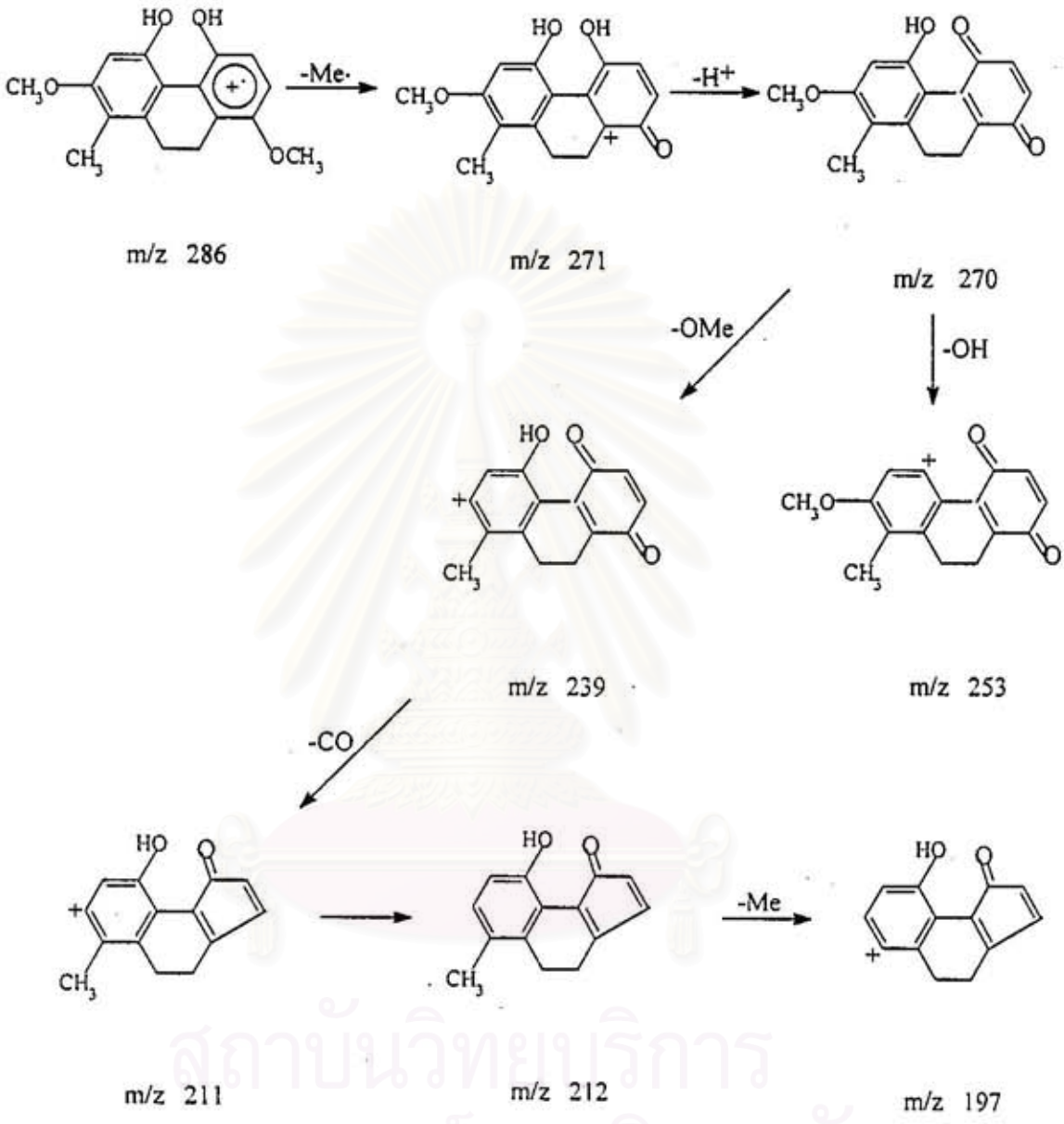
Most significant correlations observed in HMBC of Compound 5.

NOE between the proton at δ 6.40 and the proton in methoxy group at δ 3. 81 was not seen . This result showed that the O-CH_3 bond in the methoxy group which can rotate in space , prefered to be near the proton at δ 6.40 rather than the methyl group because of the steric hindrance of the methyl group. NOE between the proton at δ 7.86 and the hydrogen in methoxy group at δ 3.78 were also not seen. This result suggested that the O-CH_3 bond in the methoxy group rotated close to the methylene proton at δ 2.76.

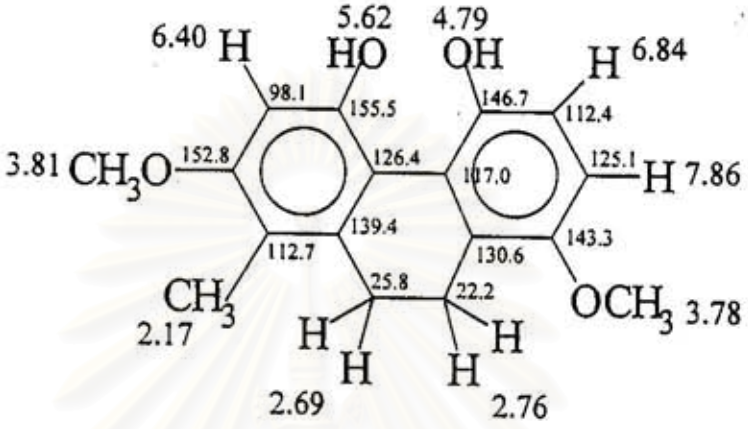
Table 19 One bond and multiple bond correlation of Compound 5.

Proton (ppm)	one bond correlations Attached carbon	multiple bond correlations Attached carbon
7.86	125.1	130.6,146.7,117.0(w)
6.84	112.0	143.3,126.4,146.7(w)
6.40	98.06	112.7,116.0,152.8,155.5(w)
3.78	61.36	-
3.81	55.55	-
2.76	25.72	126.4,130.6,139.4,143.3(w)
2.69	22.17	116.0,130.6,139.4,112.7(w)
2.17	11.18	112.7,139.4,152.7,126.4(w)

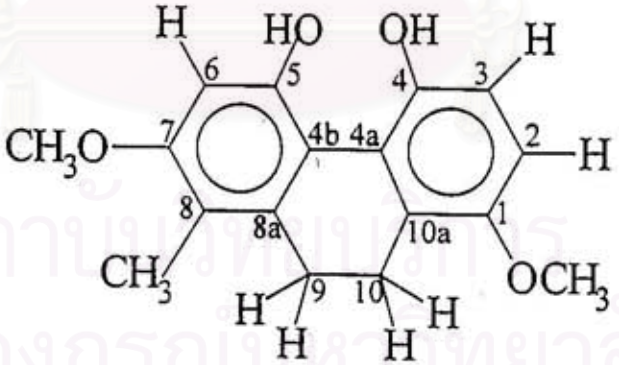
The spectral data is consistent with the identification of compound 5 as **4,5 - dihydroxy - 1,7 - dimethoxy - 8 - methyl - 9,10 - dihydro phenanthrene.**



Scheme 3 The possible mass fragmentation patterns of Compound 5 [23]

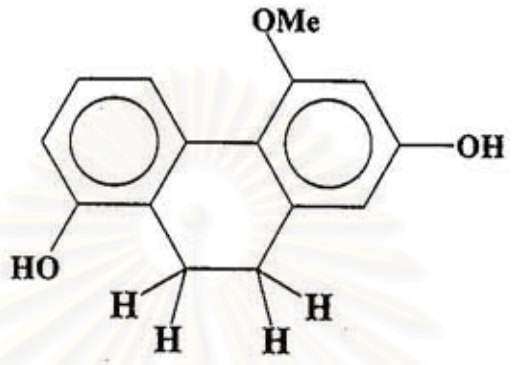


Compound 5.



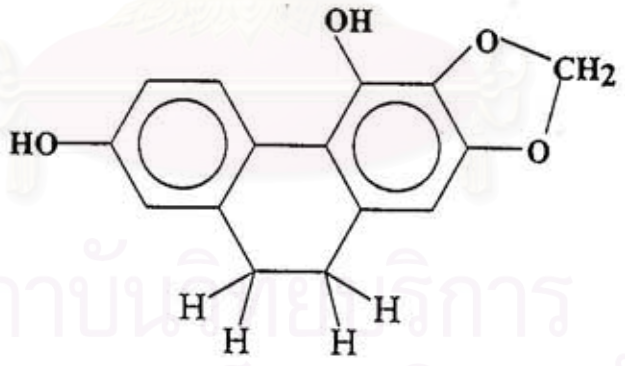
4,5 - dihydroxy - 1,7 - dimethoxy - 8 - methyl - 9,10 - dihydro phenanthrene

In recent years , not many reports of 9,10-dihydrophenanthrene derivetive were made. Most of them were discovered in orchid species. 1,7-Dihydroxy-5-methoxy- 9,10-dihydrophenanthrene was isolated from the rhizome of the orchid *Epipactis palustris*. [24]



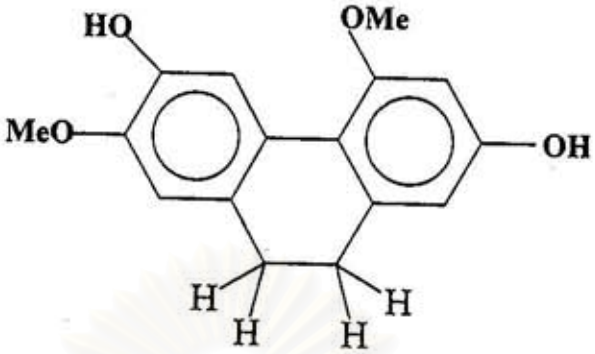
1,7-dihydroxy-5-methoxy- 9,10-dihydrophenanthrene

The air-dried whole plant of the orchid *Cirrhopetalum andersonil* , contained new stabenoids which was shown to be 4,7-dihydroxy-2,3-methylenedioxy-9,10-dihydrophenanthrene . [25]



4,7-dihydroxy-2,3-methylenedioxy-9,10-dihydrophenanthrene

The other 9,10-dihydrophenanthrene derivetive was isolated from the whole plant of the orchid , *Coelogyne flacida* , which was established as 2,6-dihydroxy-4,7-dimethoxy-9,10-dihydrophenanthrene . [26]



2,6-dihydroxy-4,7-dimethoxy-9,10-dihydrophenanthrene

Compound 5 , 4,5 - dihydroxy - 1,7 - dimethoxy - 8 - methyl - 9,10 -dihydro phenanthrene , which having 5 substituents is different from earlier reports. A computerized search indicated that this is the first report of this compound. It appears to be unusual due to the extent and nature of its substitution pattern.

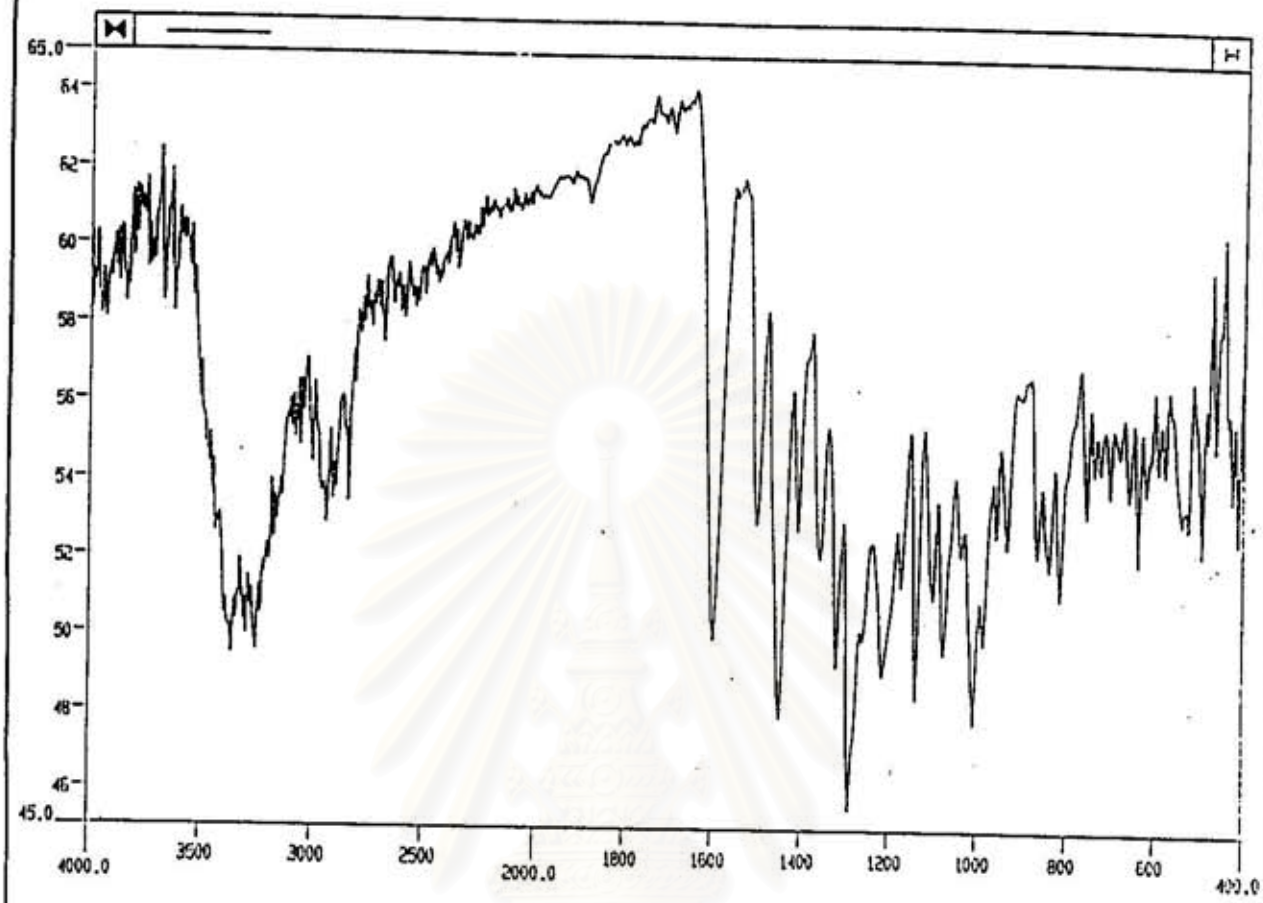


Figure 21 The IR spectrum of Compound 5

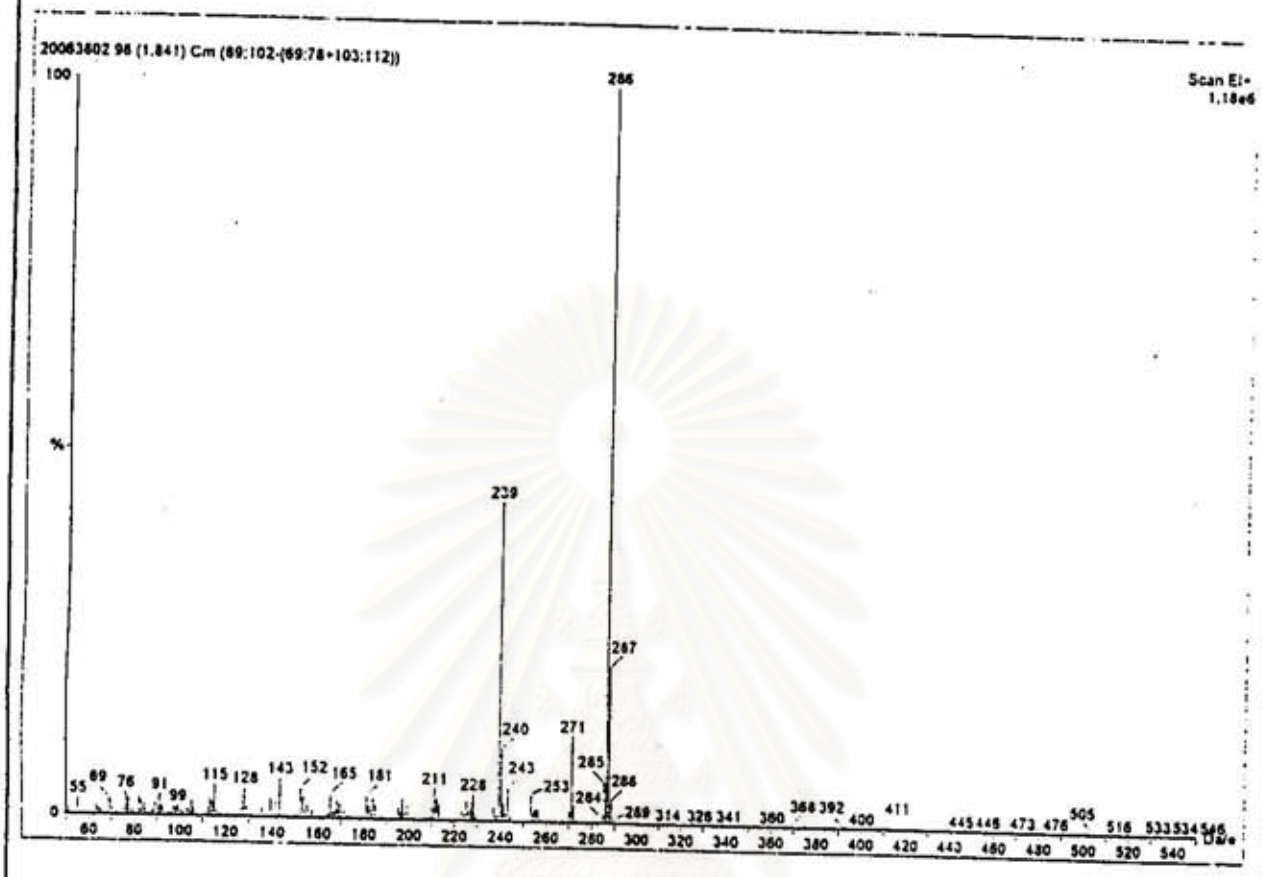


Figure 22 The mass spectrum of Compound 5

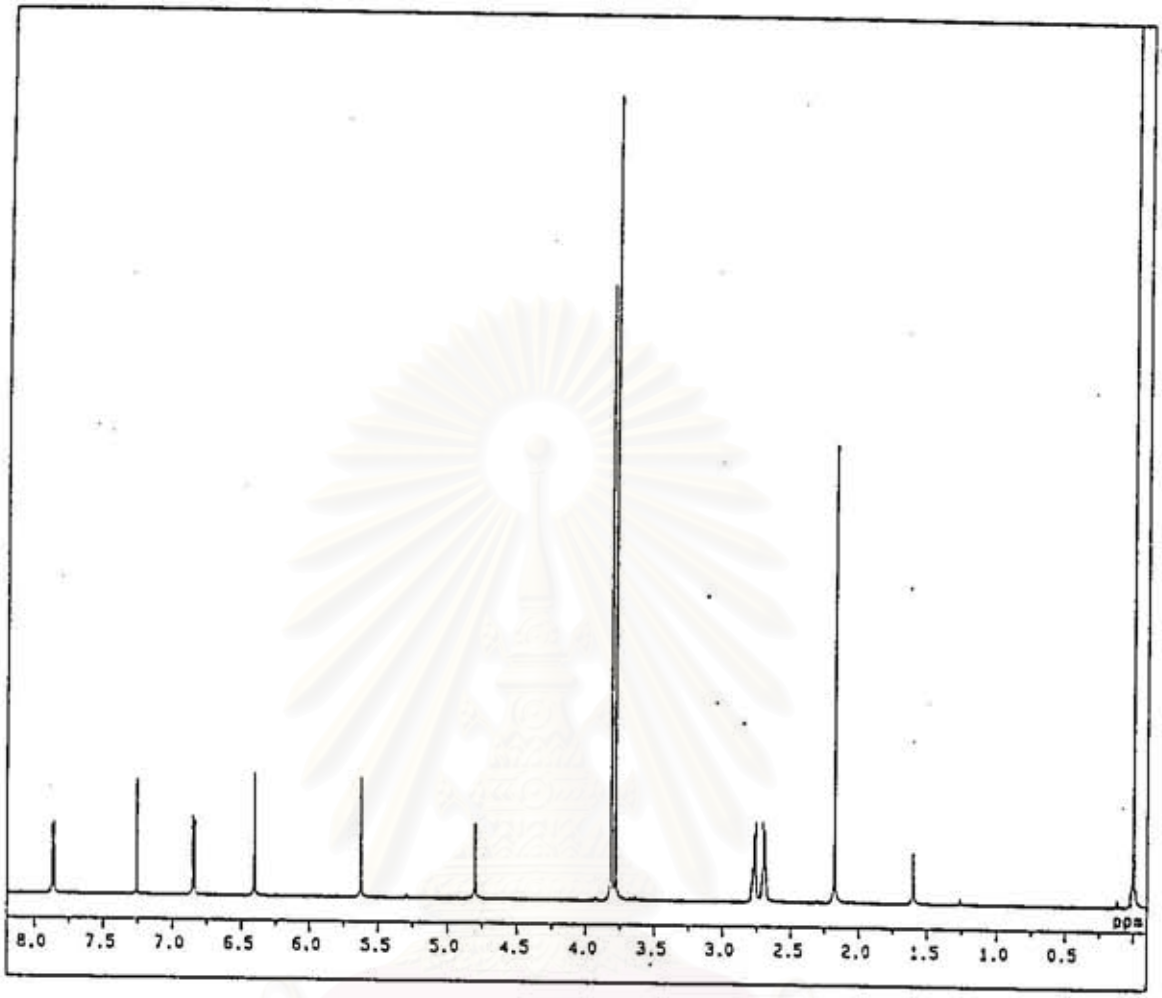


Figure 23 The ^{1}H NMR spectrum of Compound 5

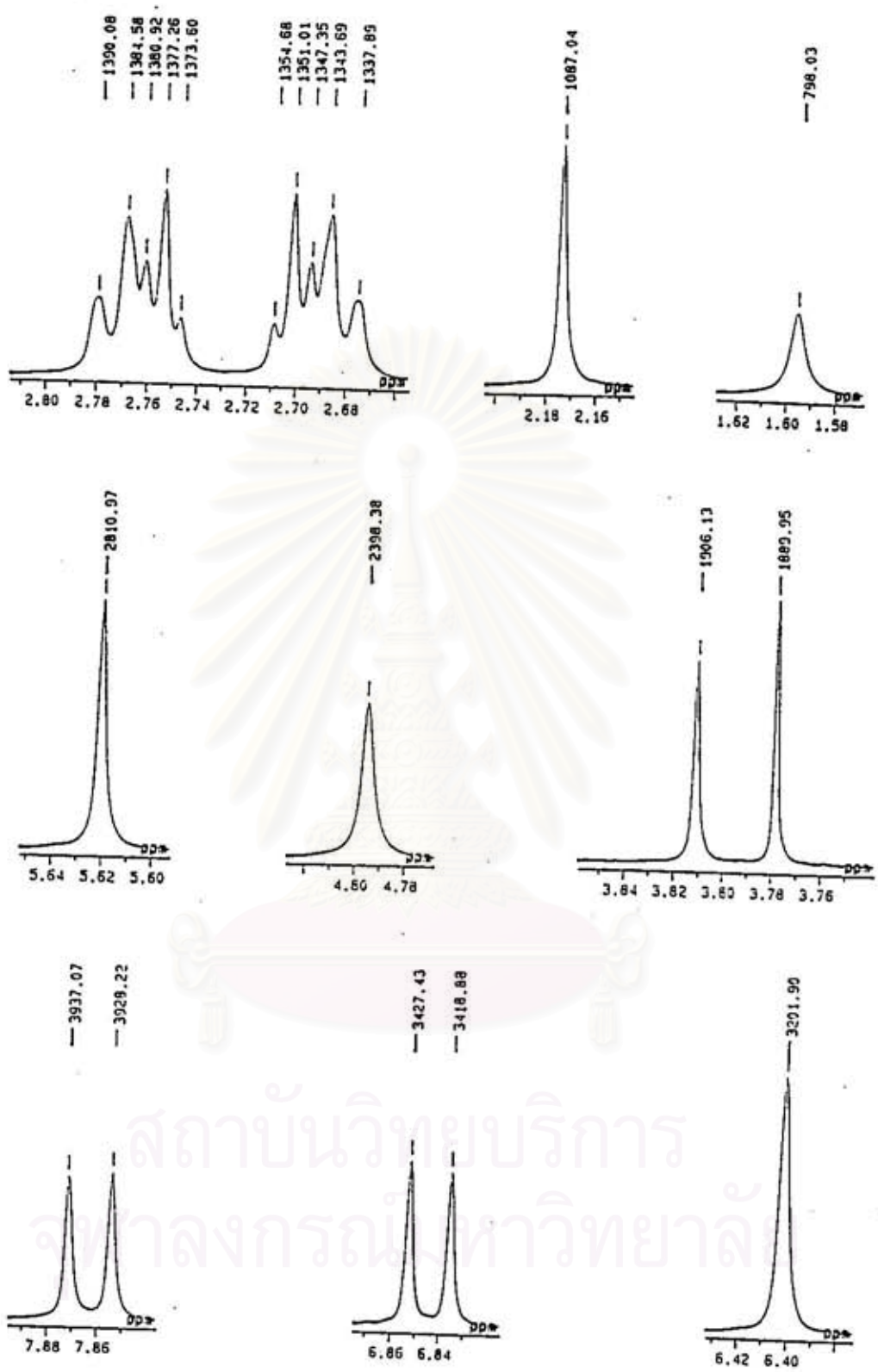


Figure 24 The expansion of ^{1}H NMR Spectrum of Compound 5

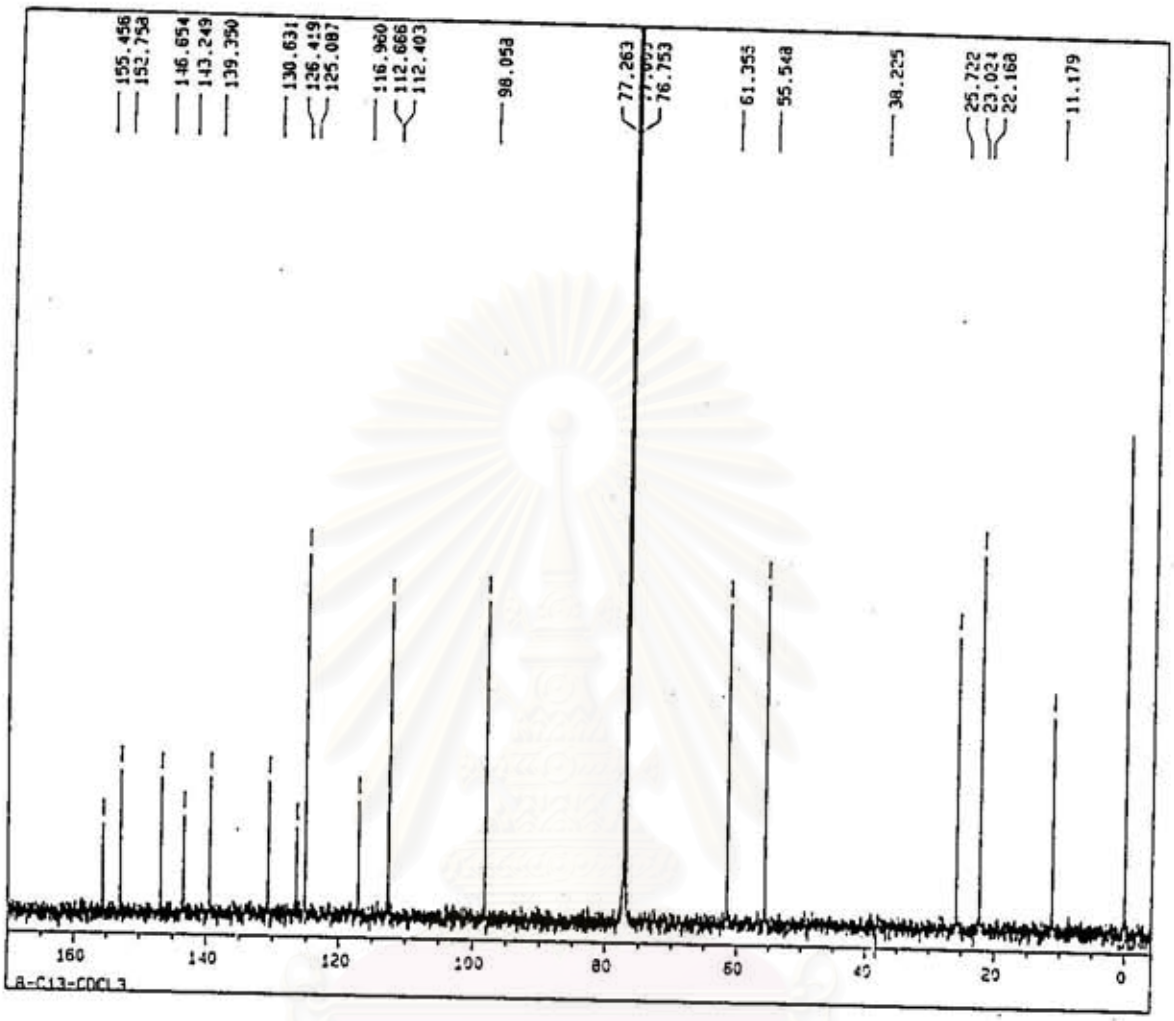


Figure 25 The ^{13}C NMR spectrum of Compound 5

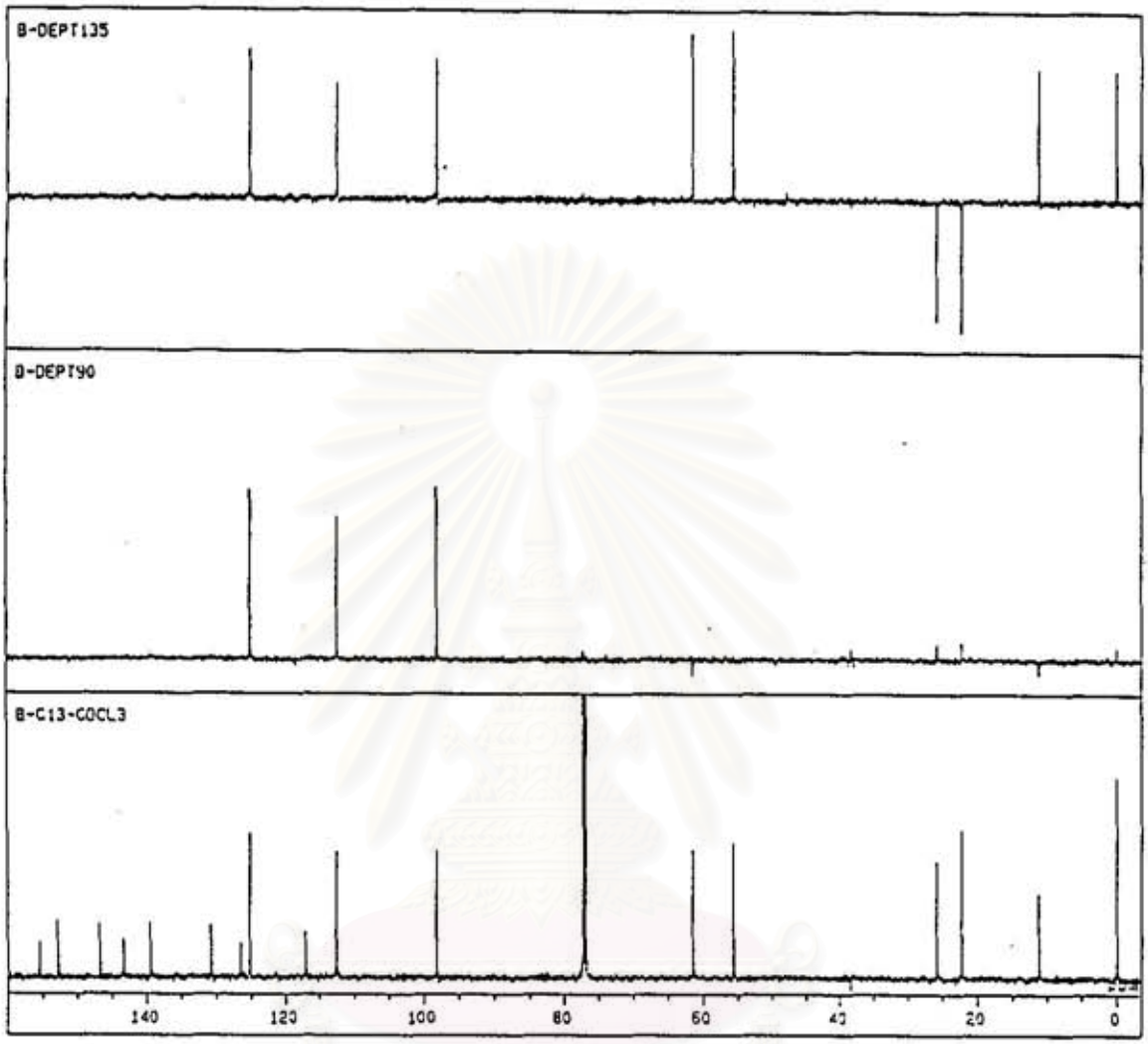


Figure 26 The DEPT 90 , 135 - ^{13}C NMR spectrum of Compound 5

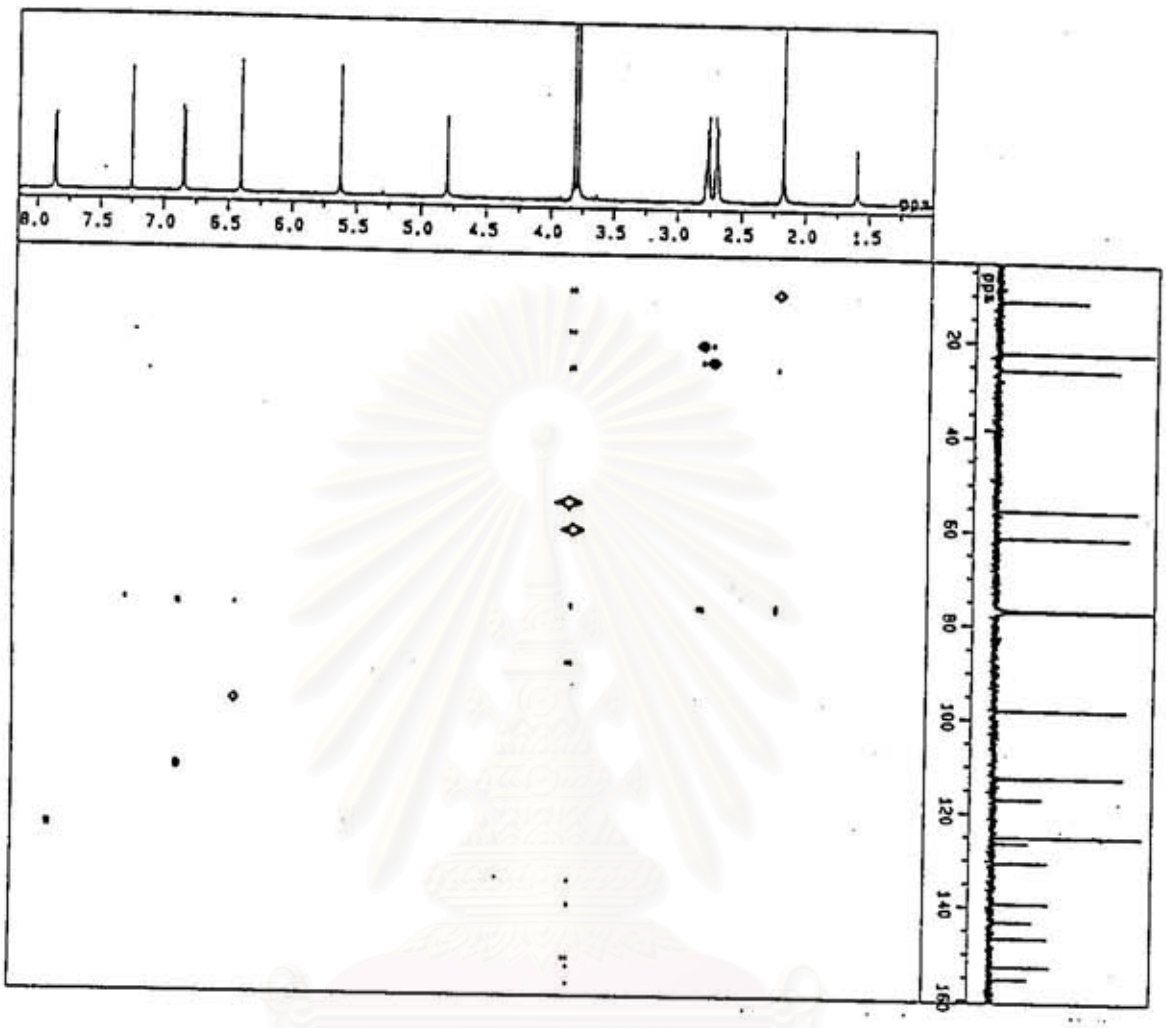


Figure 27 The HMQC spectrum of Compound 5

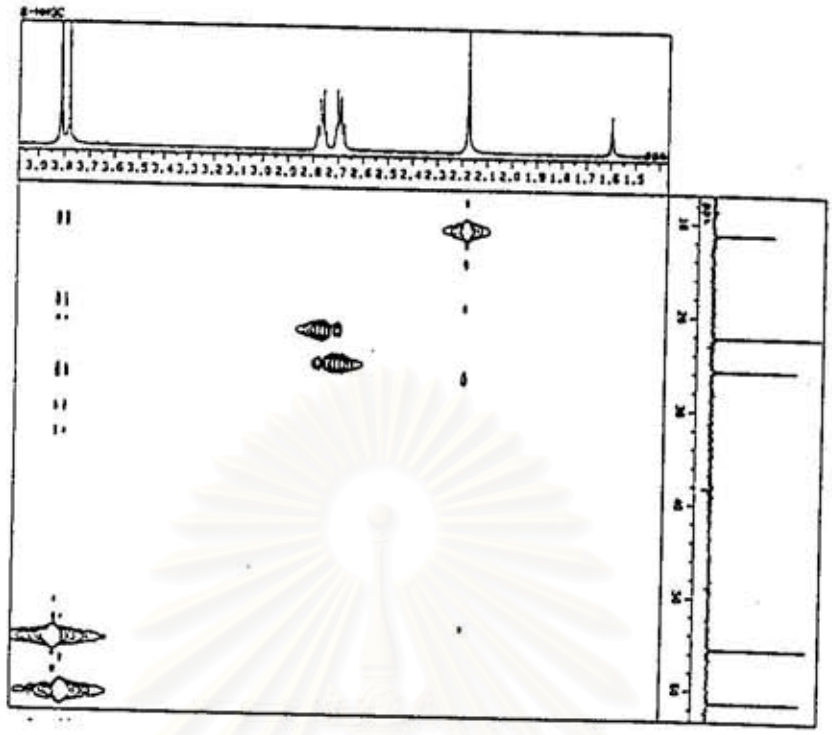


Figure 28 The expansions of HMQC spectrum of Compound 5

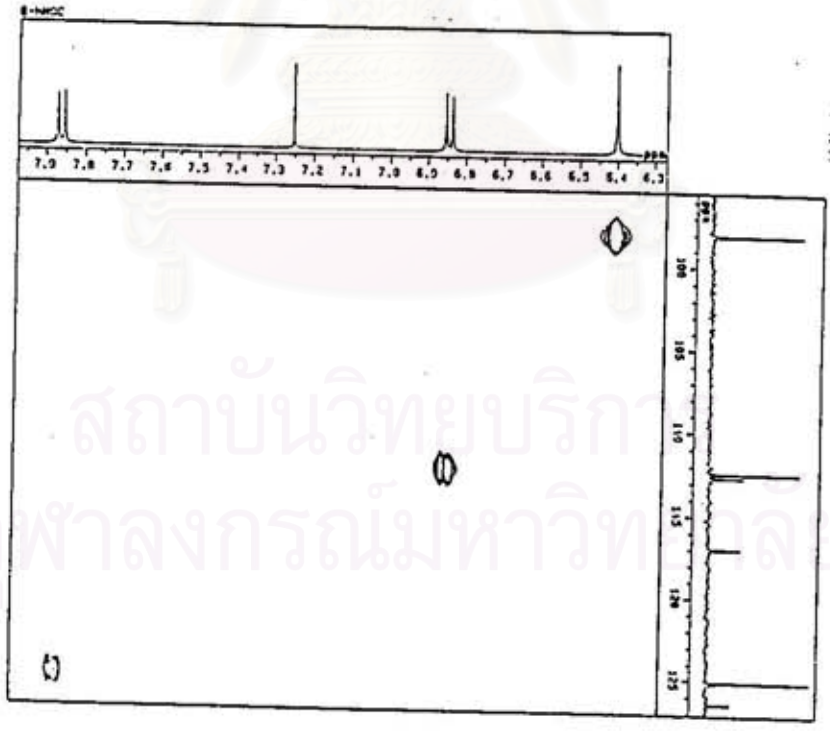


Figure 29 The expansions of HMQC Spectrum of Compound 5

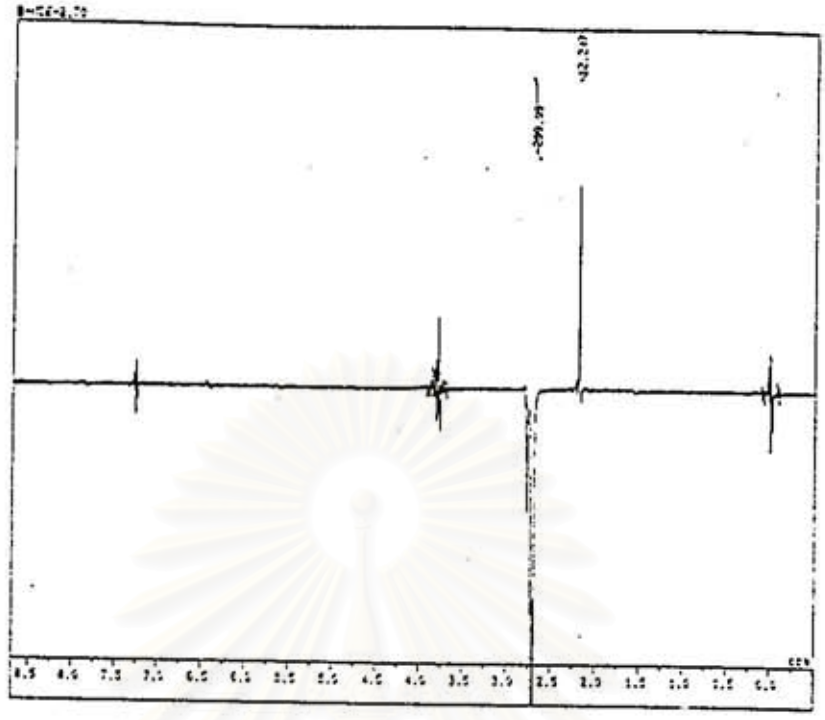


Figure 30 The NOE difference spectrum of Compound 5
(irradiate at δ 2.69 ppm.)

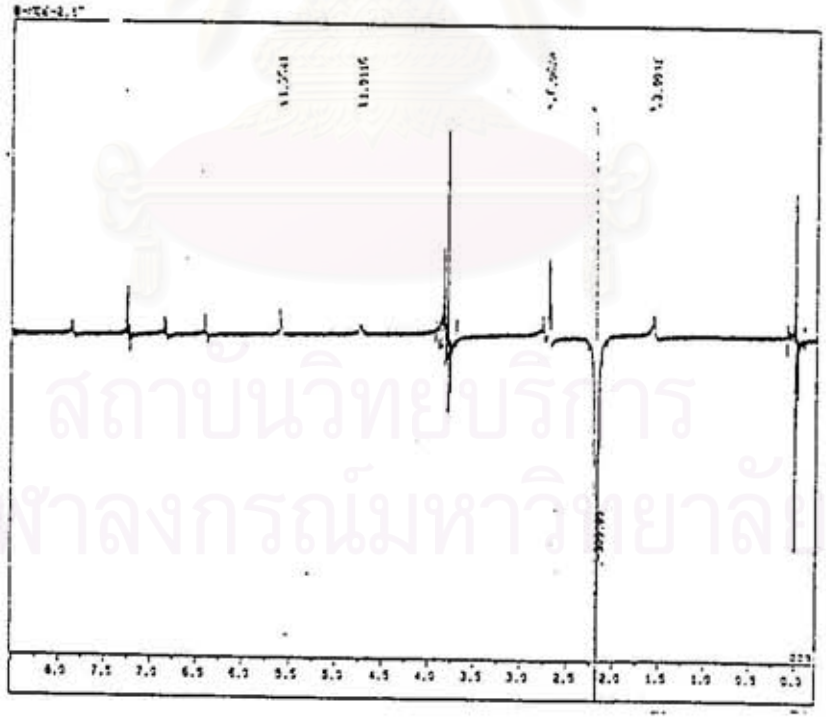


Figure 31 The NOE difference spectrum of Compound 5
(irradiate at δ 2.17 ppm.)

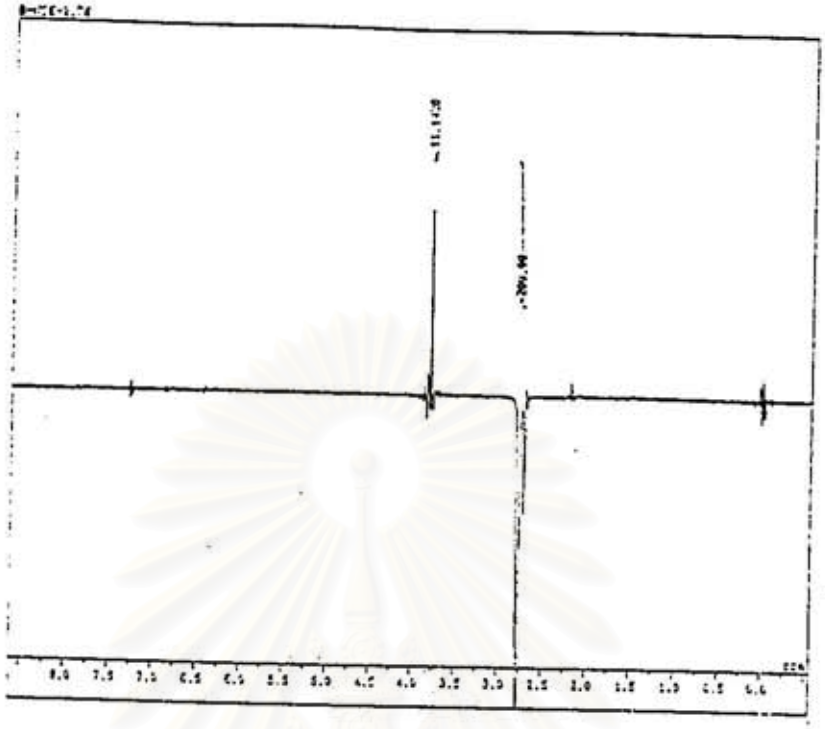


Figure 32 The NOE difference spectrum of Compound 5
(irradiate at δ 2.76 ppm.)

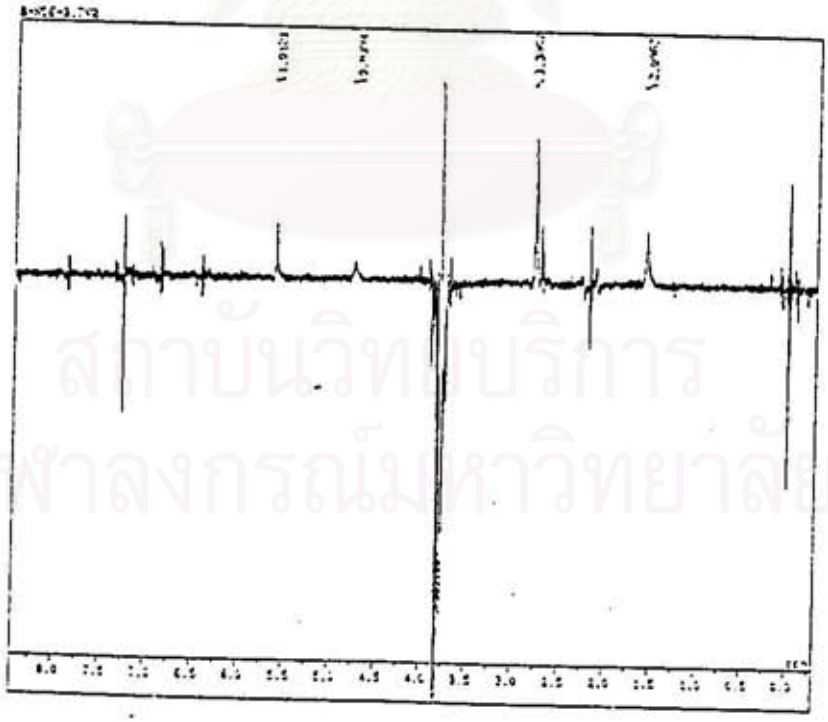


Figure 33 The NOE difference spectrum of Compound 5
(irradiate at δ 3.78 ppm.)

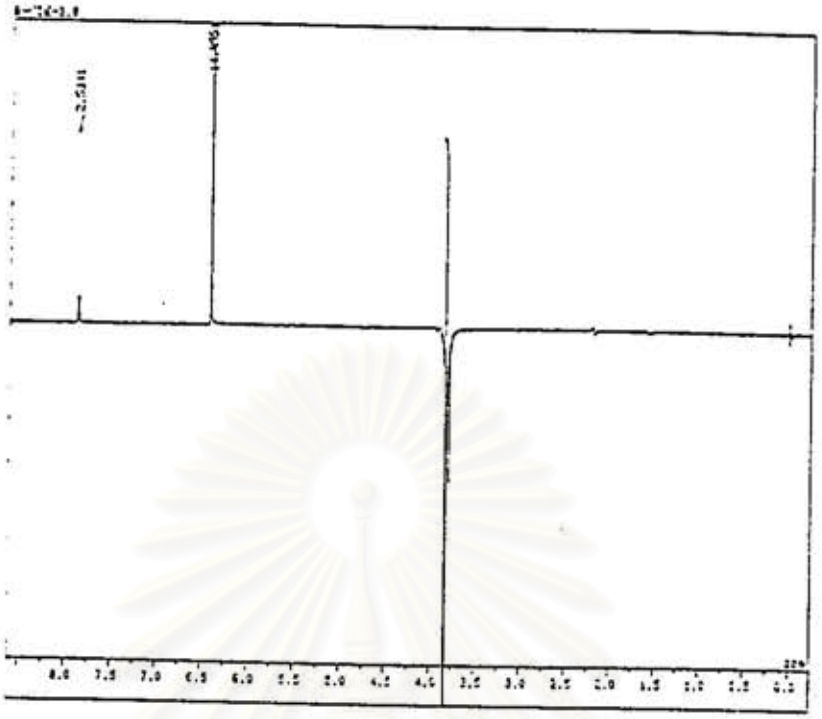


Figure 34 The NOE difference spectrum of Compound 5
(irradiate at δ 3.81 ppm.)

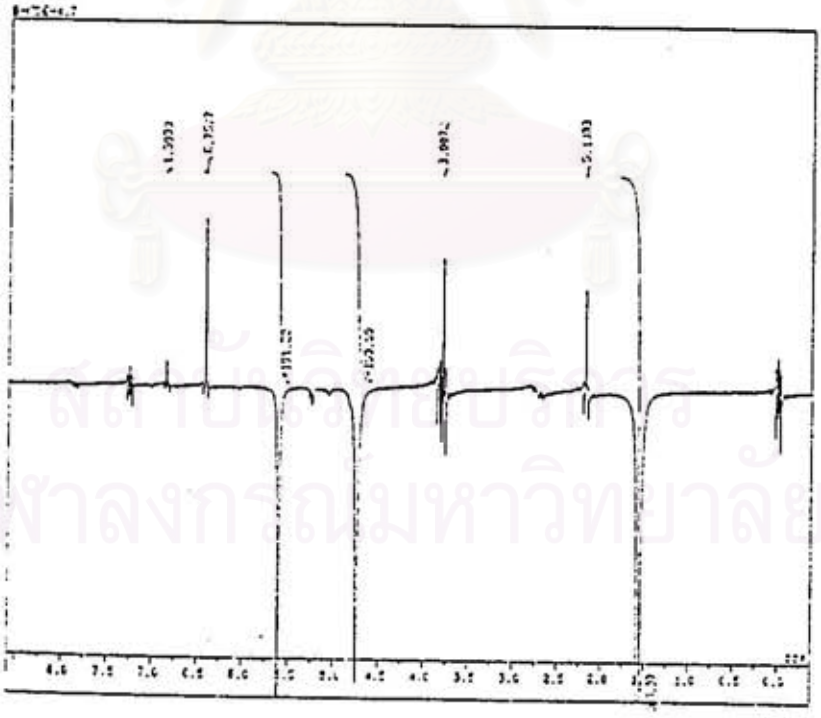


Figure 35 The NOE difference spectrum of Compound 5
(irradiate at δ 4.79 ppm.)

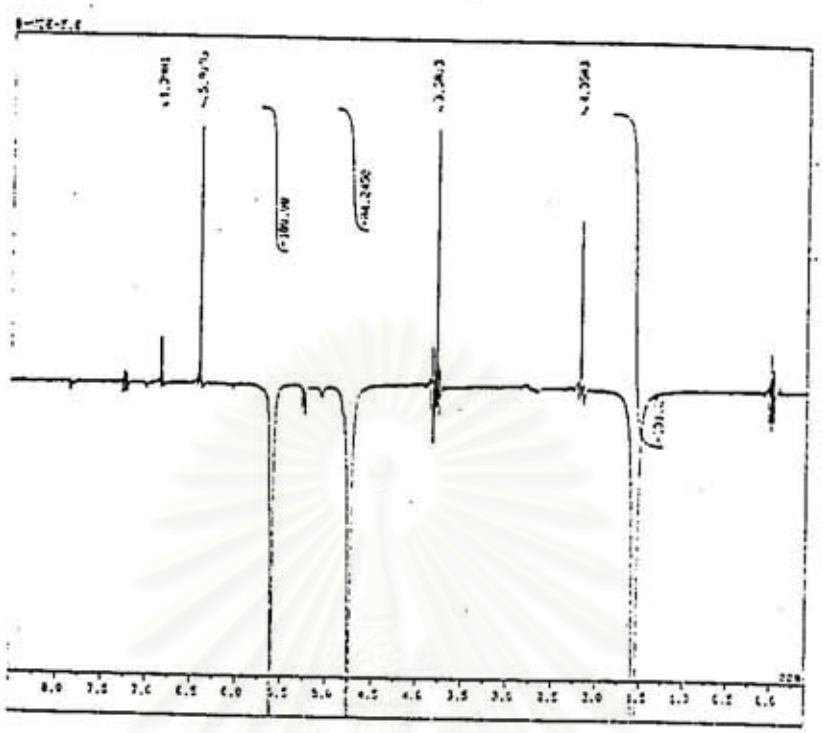


Figure 36 The NOE difference spectrum of Compound 5 (irradiate at δ 5.62 ppm.)

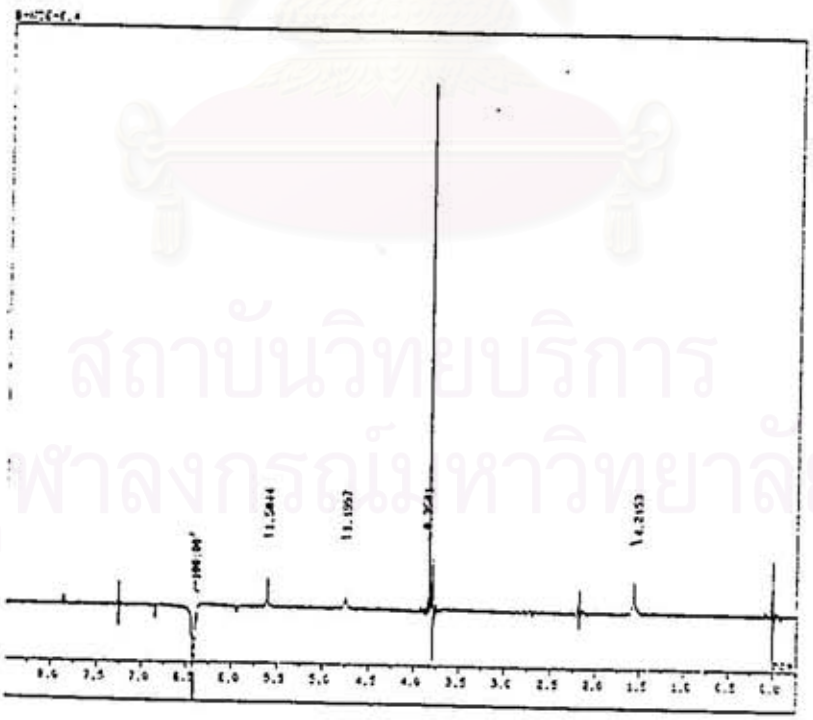


Figure 37 The NOE difference spectrum of Compound 5 (irradiate at δ 6.40 ppm.)

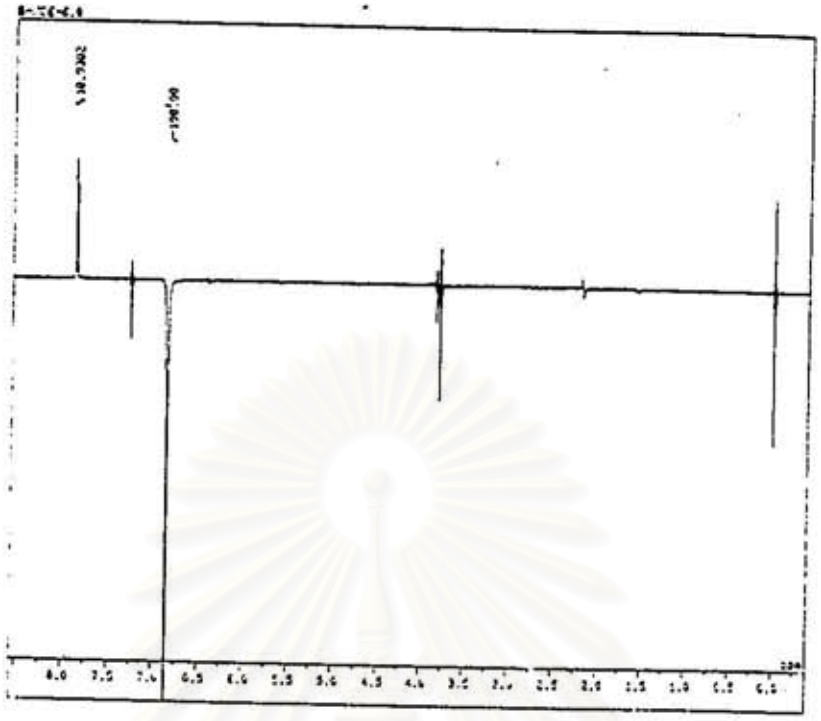


Figure 38 The NOE difference spectrum of Compound 5
(irradiate at δ 6.84 ppm.)

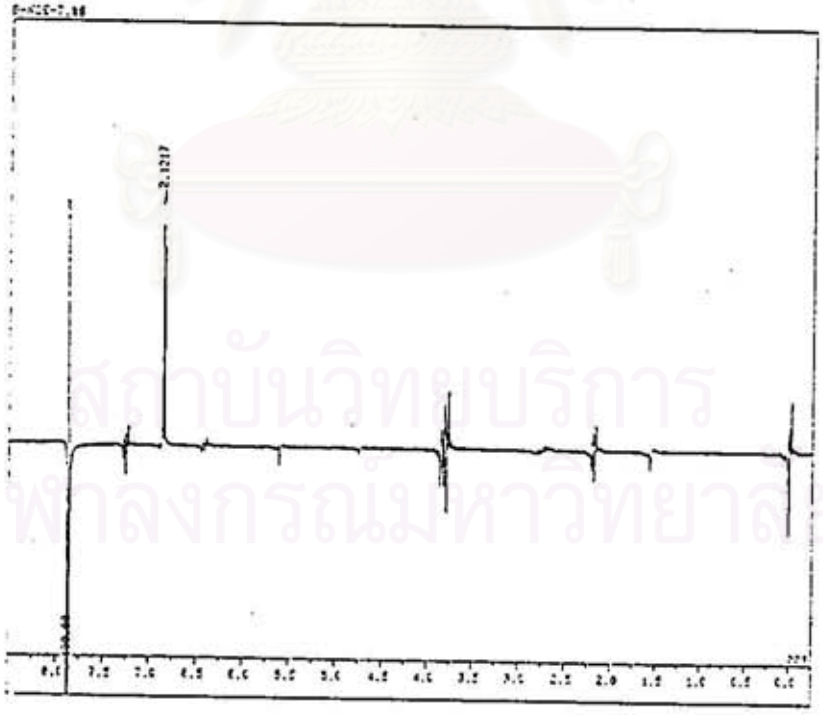


Figure 39 The NOE difference spectrum of Compound 5
(irradiate at δ 7.86 ppm.)

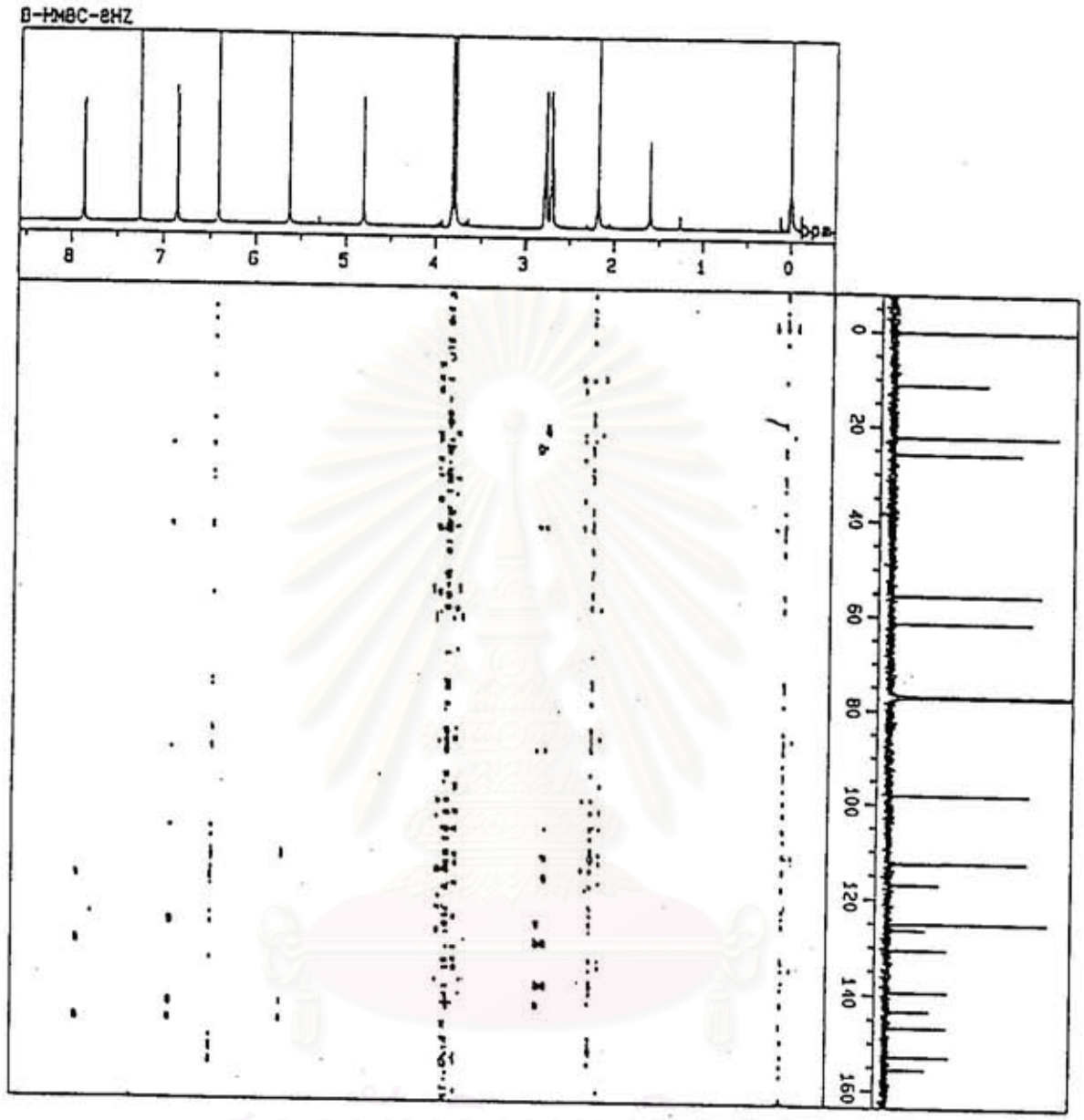


Figure 40 The HMBC spectrum of Compound 5

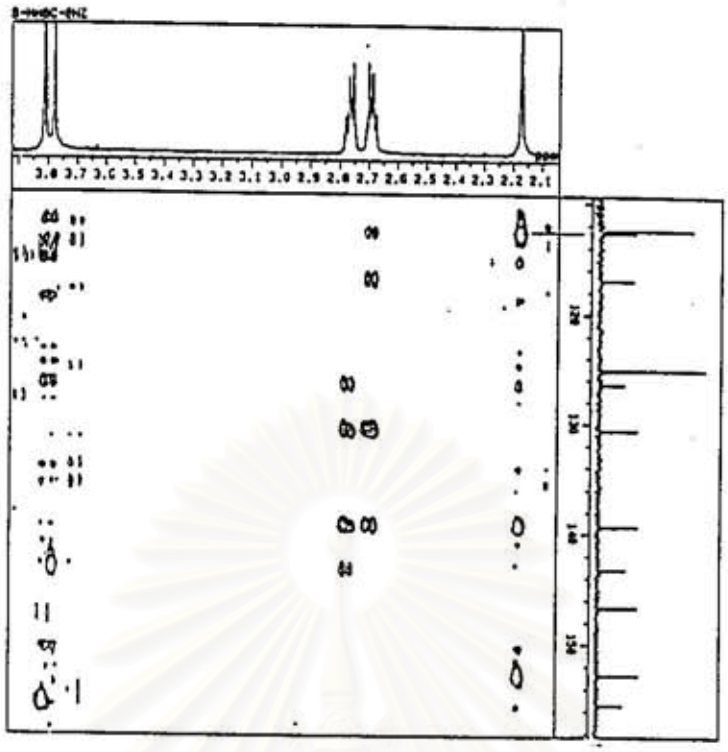


Figure 41 The expansions of HMBC Spectrum of Compound 5

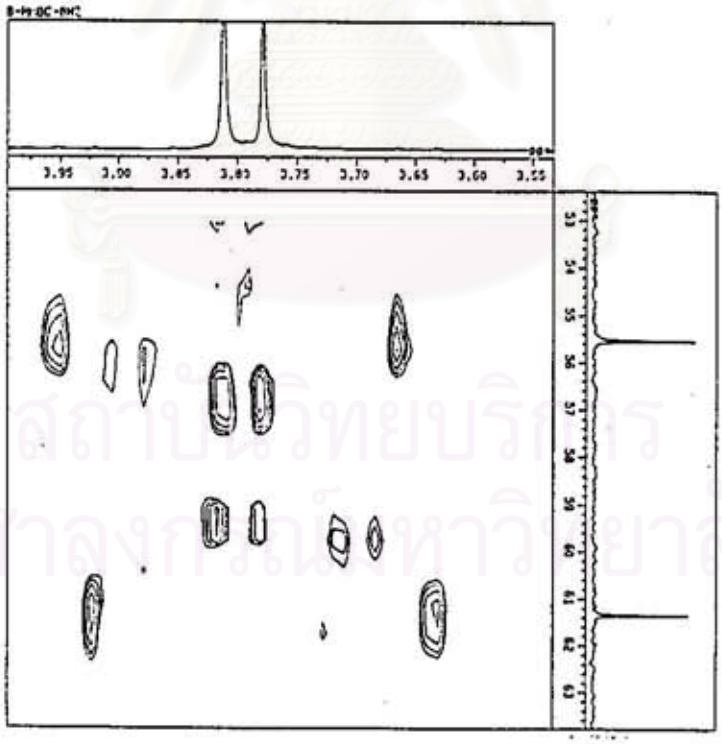


Figure 42 The expansions of HMBC Spectrum of Compound 5

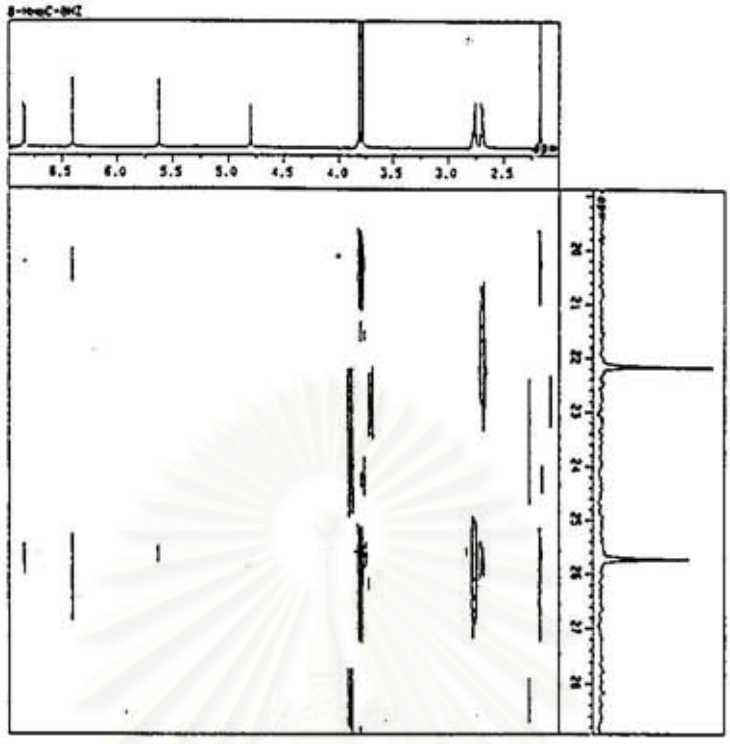


Figure 43 The expansions of HMBC Spectrum of Compound 5

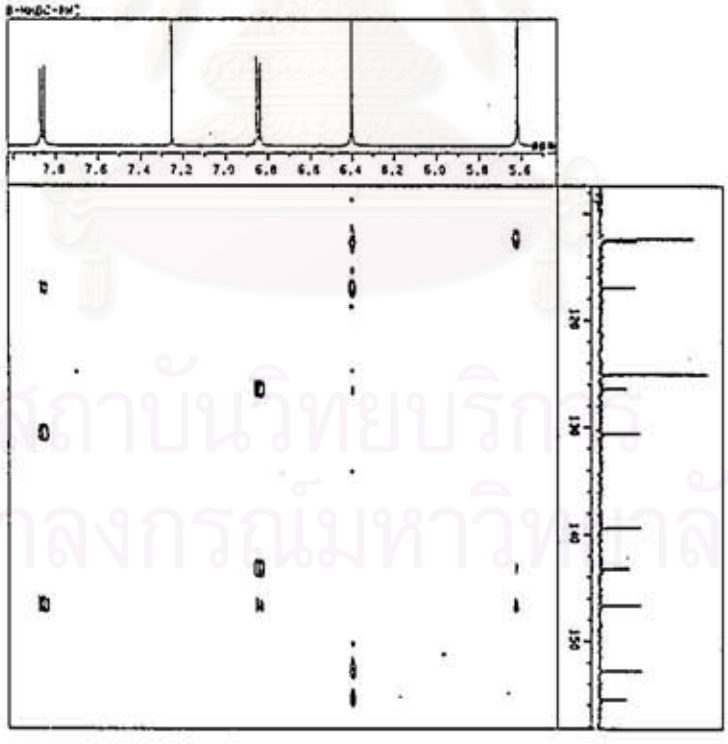


Figure 44 The expansions of HMBC Spectrum of Compound 5

3.8.6 Structure Elucidation of compound 6.

Compound 6 was isolated using gel chromatography and purified with the chromatotron. This compound was obtained as a white powder, 146.8 mg. (1.13×10^{-2} % wt by wt of dried roots), from the second band of the chromatotron plate .The melting point was 150-151°C. The R_f value was 0.45 [hexane:dichloromethane (4:1)].

The infrared spectrum showed the presence of a hydroxy group at 3500-3100 cm^{-1} and an aromatic ring at 1600 cm^{-1} (Fig.45)

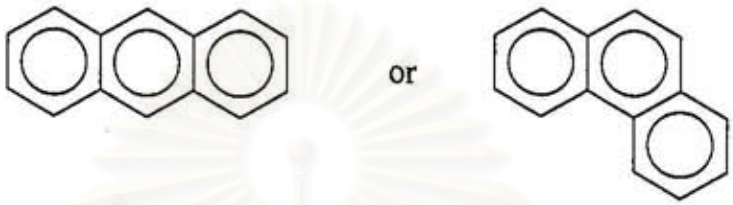
The molecular ion was observed at m/z 284 and other fragments were at m/z 269 (M^+-CH_3), 267 (M^+-OH), 241 (M^+-C_2H_3O), 225 (M^+-C_3H_7O) and 197 (M^+-$C_4H_7O_2$). (Fig.46)

The ^1H NMR spectrum gave ten signals: five aromatic protons at δ 6.64 [1H , dd , J = 6.1 , 2.2 Hz], 6.88 [1H , s], 6.91 [1H , s], 7.14 [1H, d, J = 6.1 Hz] , 7.15 [1H , d , J = 2.2 Hz], two phenolic hydroxy groups at δ 4.84 (s) and 5.18 (s), one aromatic methoxy group at δ 3.87 [3H , s] and two methyl groups at δ 2.39 [3H, s] and 2.18 [3H , s]. (Fig.47-48)

The ^{13}C NMR spectrum and DEPT - 90 ,135 experiments showed 17 signals as follows ; two methyl carbons (8.5 and 13.1), one methoxy group (55.8), five tertiary carbons (101.7 , 103.3 , 104.3 , 107.9 and 124.8) and nine quaternary carbons (112.4 , 114.5 , 118.4 , 128.4 , 149.0 , 153.0 , 154.9 , 156.0 and 156.1) . The multiplicity of the aromatic protons was confirmed by Homonuclear decoupling experiments. (Fig.49-50)

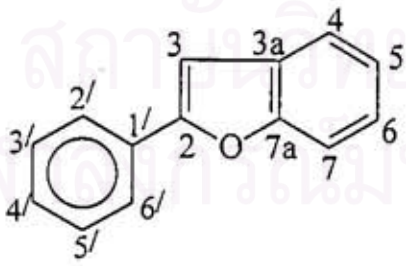
These data. indicated that Compound 6 has 17 carbons and 16 protons. The presence of two hydroxy groups and one hydroxy group suggested that this compound has at least three oxygen atoms in the molecule . The

molecular ion at m/z 284 indicated the molecular formula was $C_{17}H_{16}O_4$, corresponding to a degree of unsaturation of ten. This result implied that this compound has the posibility of 3 aromatic rings as in phenanthrene or anthracene.



anthracene or phenanthrene

However , the molecular formula suggested that this compound has another oxygen in the structure . The other possibility was an aryl benzofuran . There are many naturally occuring 2-aryl benzofurans , as well as the corresponding 2,3-dihydro derivatives. [28 , 29 , 30] Hence , this structure was postulated as the nucleus of Compound 6. This idea was supported that the basic skeleton of Compound 6 is benzofuran derivative with an aryl group having 2 hydroxy groups , 1 methoxy group and 2 methyl groups.



Substituents

- OH_1 (2)
- OCH_3 (1)
- CH_3 (2)

The basic skeleton of Compound 6

Moreover , the ^{1}H NMR and the homonuclear decoupling data showed the presence of the two ortho-coupling protons at δ 6.64 [1H , dd , J = 6.1 , 2.2 Hz] and 7.14 [1H , d , J= 6.1 Hz]. This results also showed the proton at δ 6.64 was coupled with the other proton in meta position.

For further information , 2D-HMQC spectra was used to correlated the ^{1}H and ^{13}C shifts as shows in Table 20. The spectra showed that the proton at δ 2.18 was attached to the methyl group at C-2′ the proton at δ 2.39 was attached to the methyl group at C-4′ , the proton at δ 3.87 was attached to the methoxy group at C-5′ , the proton at δ 6.88 was attached to the carbon at 101.7 (C-3) , the proton at δ 6.91 was attached to the carbon at 103.3 (C-6′) , the proton at δ 7.15 was attached to the carbon at 124.8 (C-7) , the proton at δ 6.64 was attached to the carbon at 107.9 (C-5) and the proton at δ 7.14 was attached to the carbon at 104.3 (C-4). (Fig. 51-53)

Table 20 ^{1}H and ^{13}C NMR spectral data of Compound 6 (500/125 Mhz , $CDCl_3$)

carbon	ppm.	Attached proton
C-2	154.9	-
C-3	101.7	6.88(s)
C-3a	118.4	-
C-4	104.3	7.14(d) J = 6.1 Hz
C-5	107.9	6.64(dd) J = 6.1 , 2.2 Hz
C-6	149.0	-
C-7	124.8	7.15(d) J = 2.2 Hz
C-7a	156.1	-
C-1′	128.4	-
C-2′	112.4	-
C-3′	153.0	-
C-4′	114.5	-
C-5′	156.0	-
C-6′	103.3	6.91(s)
methoxy group at C-5′	55.8	3.87(s)
methyl group at C-2′	13.1	2.39(s)
methyl group at C-4′	8.5	2.18(s)
hydroxy group at C-6	-	4.84(s)
hydroxy group at C-3′	-	5.18(s)

The locations of the substituents of the benzofuran were determined by an HMBC spectrum and NOEs.

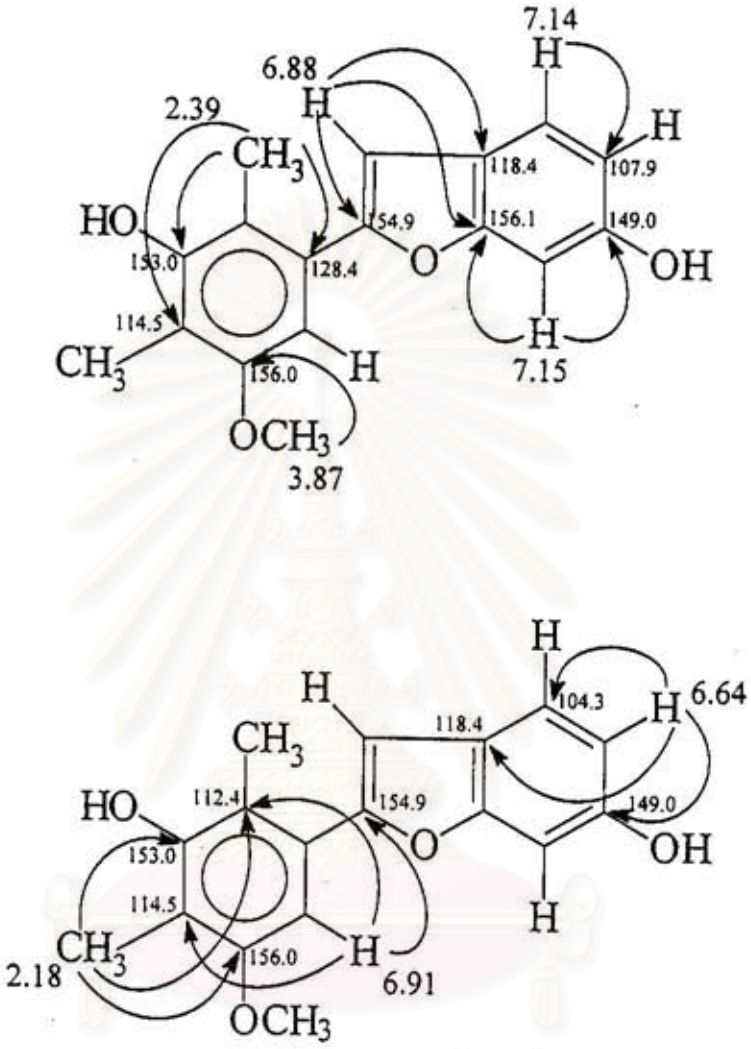
NOE difference spectroscopy revealed that irradiation of the proton at δ 6.88 caused enhancement of the signal δ 2.39 and irradiation of the proton in the methyl group at δ 2.39 caused enhancement of the signal δ 5.18 and 6.88. Irradiation of the proton at δ 6.91 caused enhancement of the signal δ 3.87 while irradiation of the proton at δ 7.14 caused enhancement of the signal δ 6.64. (Fig.54-63)

These data implied that the proton at δ 6.91 was near the methoxy group. The methyl group at δ 2.39 was near the proton at δ 6.88 and the hydroxy group at δ 5.18. The proton at δ 6.64 was near the proton at δ 7.14 which corresponding to two ortho coupled protons.

Once again, irradiation of either hydroxy peak resulted saturation of both signals, and that of water.

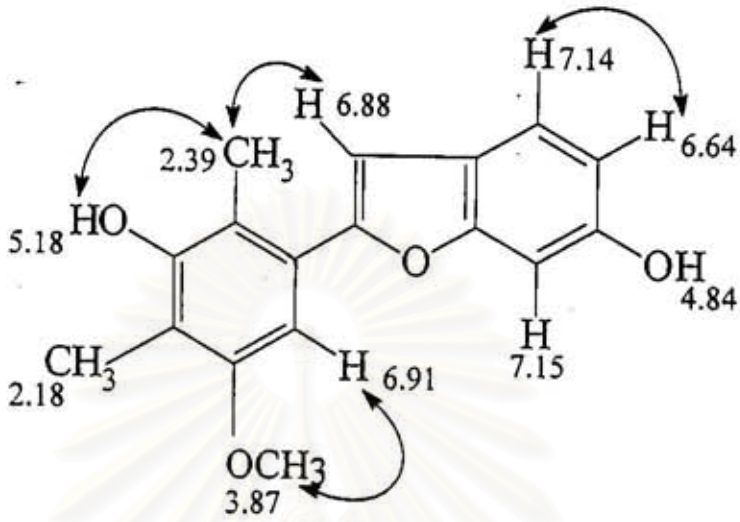
The Long-range correlations (HMBC) were seen between the proton at δ 2.18 and C-2′ (112.4), C-3′ (153.0) and C -5′(156.0); the proton at δ 2.39 and C-4′ (114.5), C-1′(128.4) and C-3′ (153.0); the proton of the methoxy group at δ 3.87 and C-5′ (156.0). The proton at δ 6.64 shows long-range correlations with C-7(104.3), C-3a (118.4) and C-6(149.0) while the proton at δ 6.88 was correlated with C-3a(118.4), C-2(154.9) and C-7a (156.1). The proton at δ 6.91 coupled to C-2′ (112.4), C-4′(114.5) and C-2 (154.9) while the proton at δ 7.14 correlated with C-5 (107.9) and the proton at δ 7.15 show long-range correlations with C-6 (149.0) and C-7a (156.1). (Fig.64-67)

The structure could be put forward based on the HMBC data the following. The proton at δ 6.88, which was believed to be the proton at C-3, was correlated with all of the carbons in furan ring.



Most significant correlations observed in HMBC of Compound 6.

This structure of Compound was consistent with the NOE data.



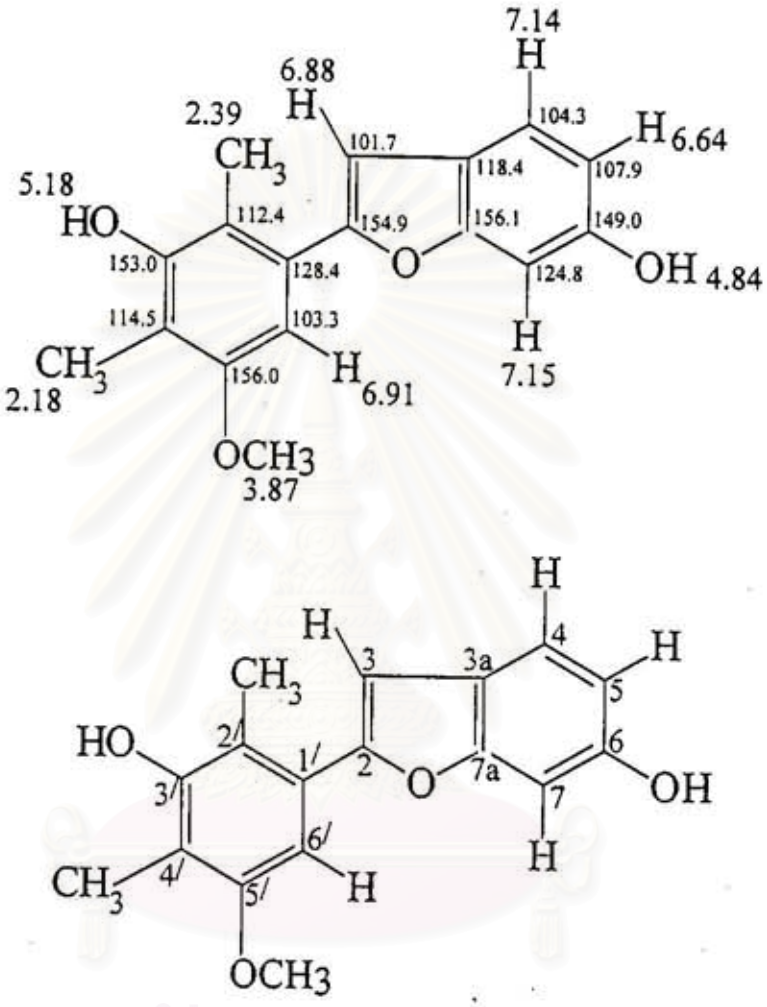
Most significant correlations observed in NOEs of Compound 6.

The NOE between the protons of the methyl group at δ 3.87 and the other neighbering group , the methyl group at δ 2.18 , was not seen because of the steric effect of the methyl group. This effect caused the O-CH_3 bond to rotate putting the CH_3 group close to the proton at δ 6.91. An NOE , have wan observed.

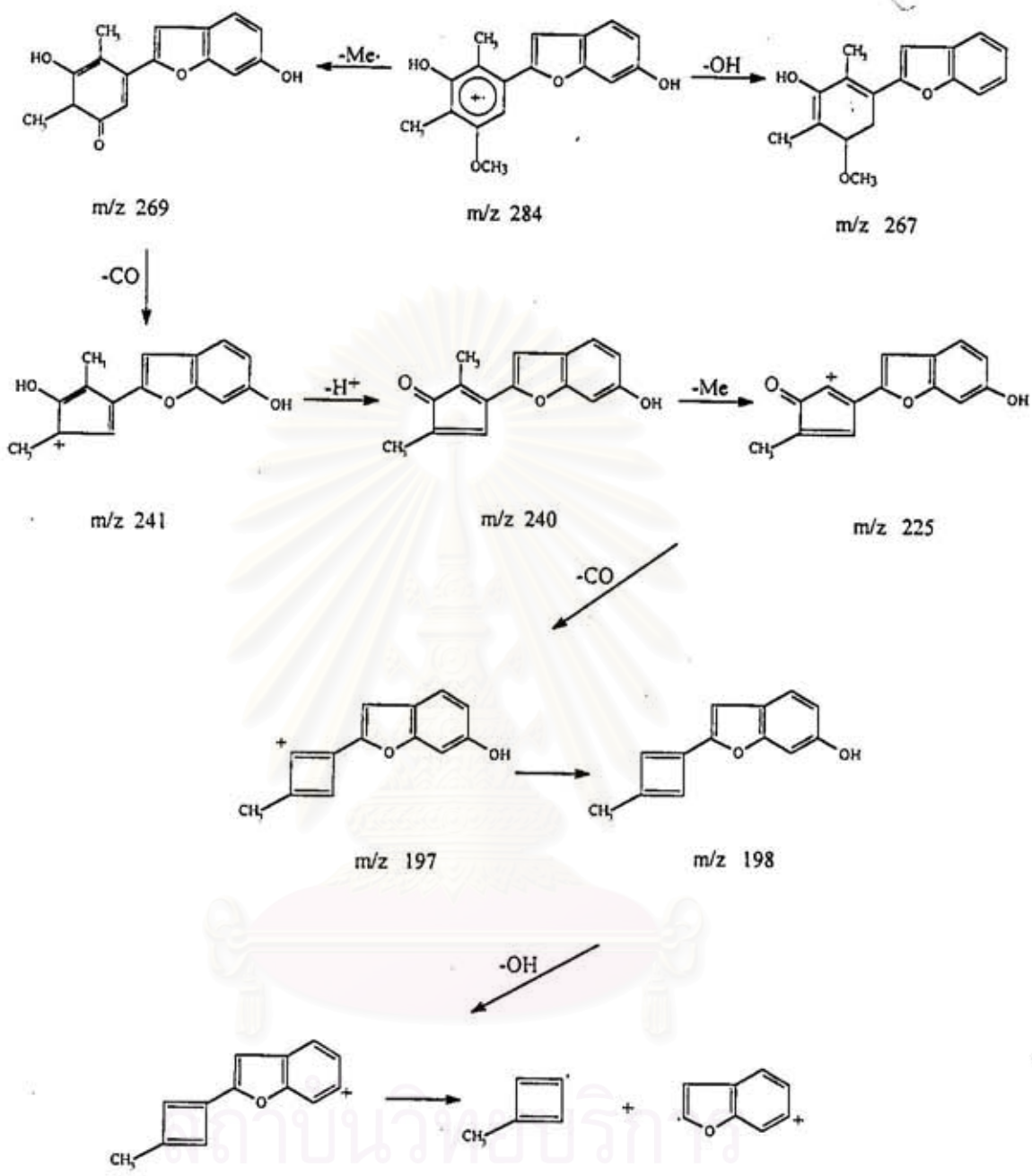
Table 21 One bond and multiple bond correlation of compound 6.

Proton (ppm.)	one bond correlations Attached proton	multiple bond correlations Attached proton
6.88	101.7	118.4 , 154.9 , 156.1
7.15	124.8	149.0 , 156.1
6.64	107.9	104.3 , 118.4 , 149.0
7.14	104.3	107.9
6.91	103.3	112.4 , 114.5 , 154.9
3.87	55.7	156.0
2.39	13.1	114.5 , 128.4 , 153.0
2.18	8.5	112.4 , 153.0 , 156.0
4.84	-	-
5.18	-	-

From these results , we could assign the ^{1}H and ^{13}C NMR signals as follow .

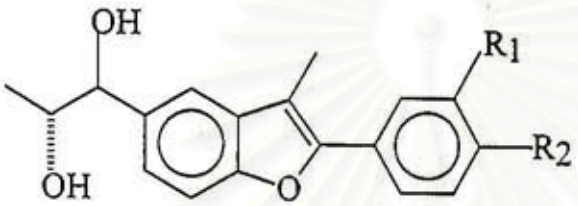


Compound 6 was assigned as **6-hydroxy-2-(3′-hydroxy-5′-methoxy-2′,4′-dimethyl phenyl)-benzofuran.** To our knowledge , this compound has not been previously reported .



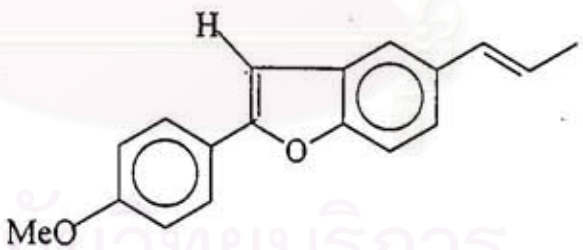
Scheme 4 The possible mass fragmentation patterns of Compound 6 [27]

There are many examples of benzofuran derivatives with an aryl group at C-2. Three new polar eupomatenoids were isolated from the bark of *Caryodaphnosis tondinensis* as 5 - (erythro-1,2-dihydroxy-propyl)-2-(4-hydroxyphenyl)-3-methylbenzo[b]furan (1) , 5-(erythro-1,2-dihydroxypropyl) -3-methyl -2- (3,4- methylenedioxyphenyl) benzo [b] furan (2) and 5-(erythro-1,2-dihydroxypropyl)-2-(4-hydroxy-3-methoxyphenyl)-3-methylbenzo[b]furan(3).[28]



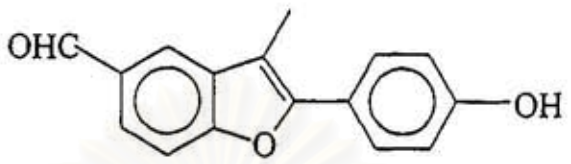
1. $R_1 = H$, $R_2 = OH$
2. $R_1 + R_2 = OCH_2O$
3. $R_1 = OMe$, $R_2 = OH$

Hans *et al* have investigated the roots of *Krameric grayi* and found the unknown nor-neolignan, 2-(4-methoxy phenyl)-5-((E)-1-propenyl) benzofuran. twelve known benzofuran derivatives were also found in this species. [29]



2-(4-methoxy phenyl)-5-((E)-1-propenyl)benzofuran

An other new benzofuran derivative was isolated from the bark of *Caryodaphnosis tondinensis* and was found to be 2-(4-hydroxyphenyl) -3-methylbenzo[b]furan -5- carbaldehyde . [30]



2-(4-hydroxyphenyl) -3- methylbenzo[b]furan -5- carbaldehyde

The compound isolated from *A. racemosus* , 6-hydroxy-2-(3′-hydroxy-5′-methoxy-2′,4′-dimethyl phenyl) benzofuran , is a benzofuran derivative having an aryl group at C-2 , but with the location of the substituents being different to other benzofurans.

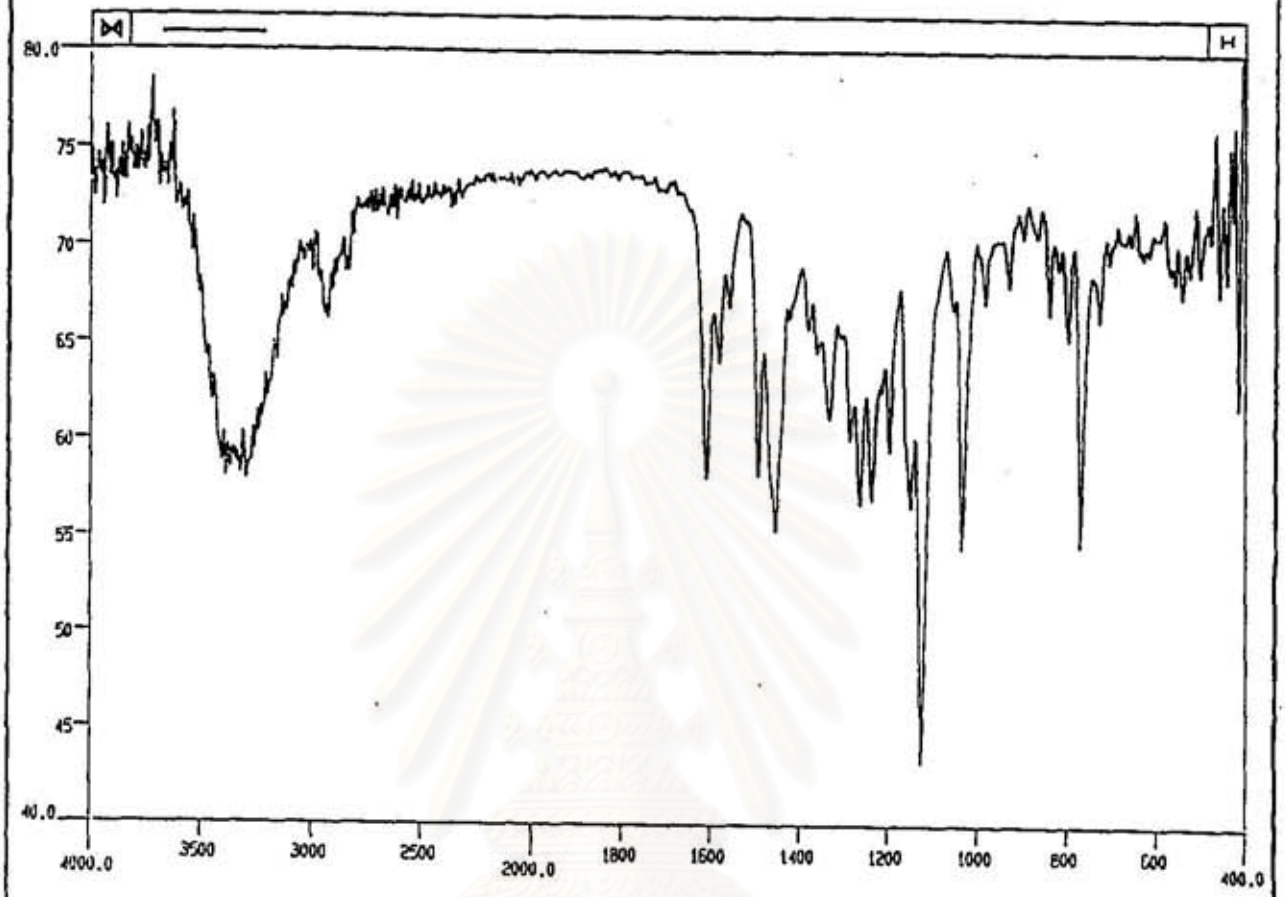


Figure 45. The IR spectrum of Compound 6

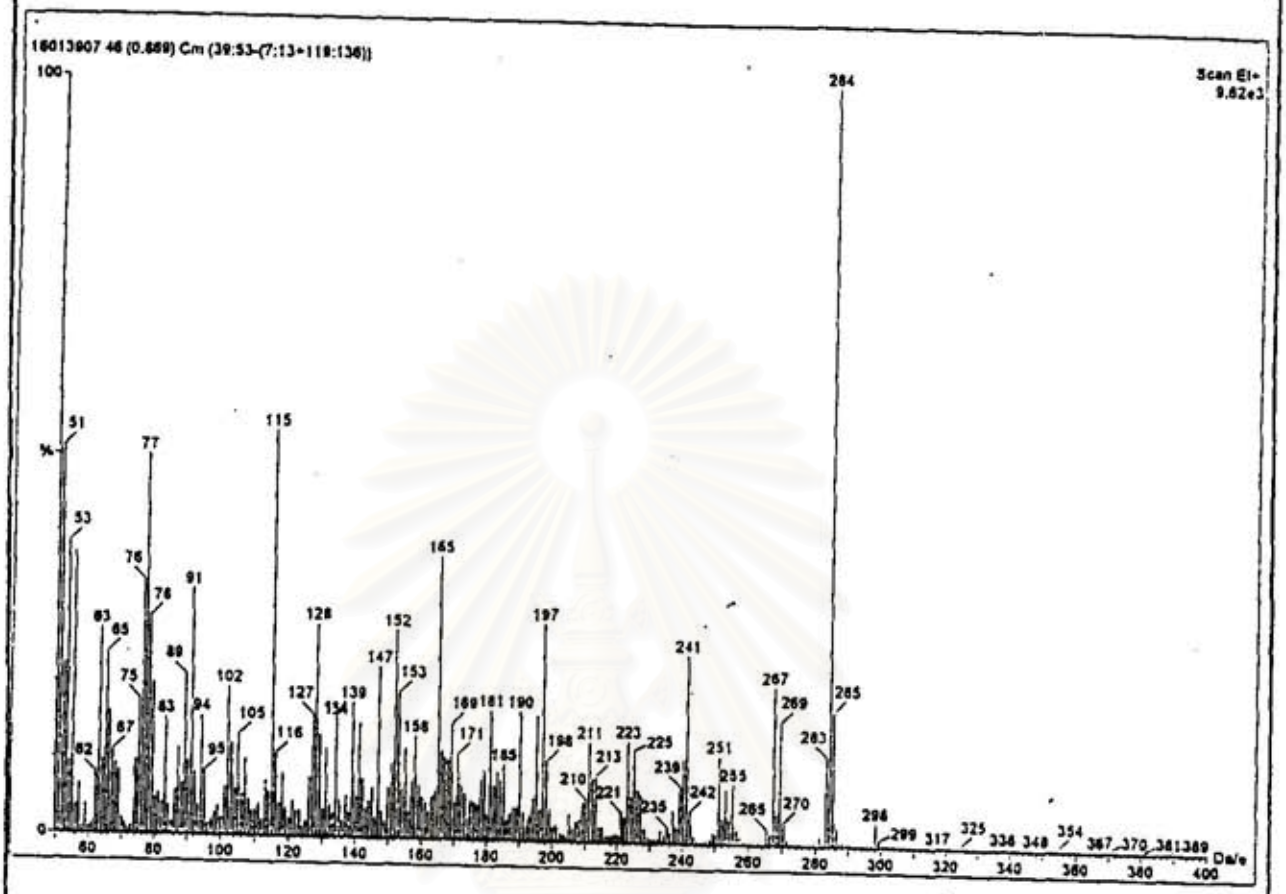


Figure 46 The mass spectrum of Compound 6

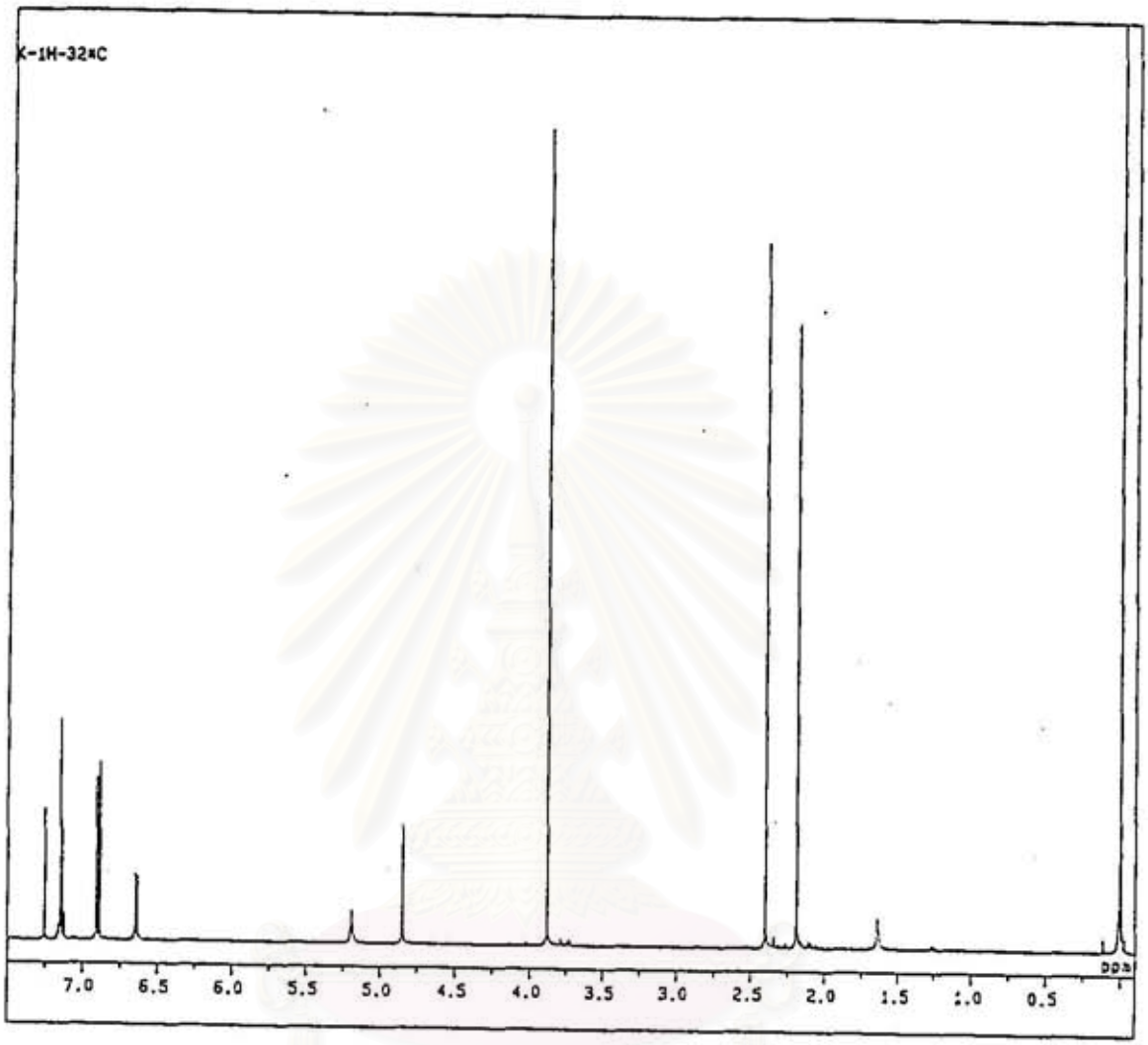


Figure 47 The ^{1}H NMR spectrum of Compound 6

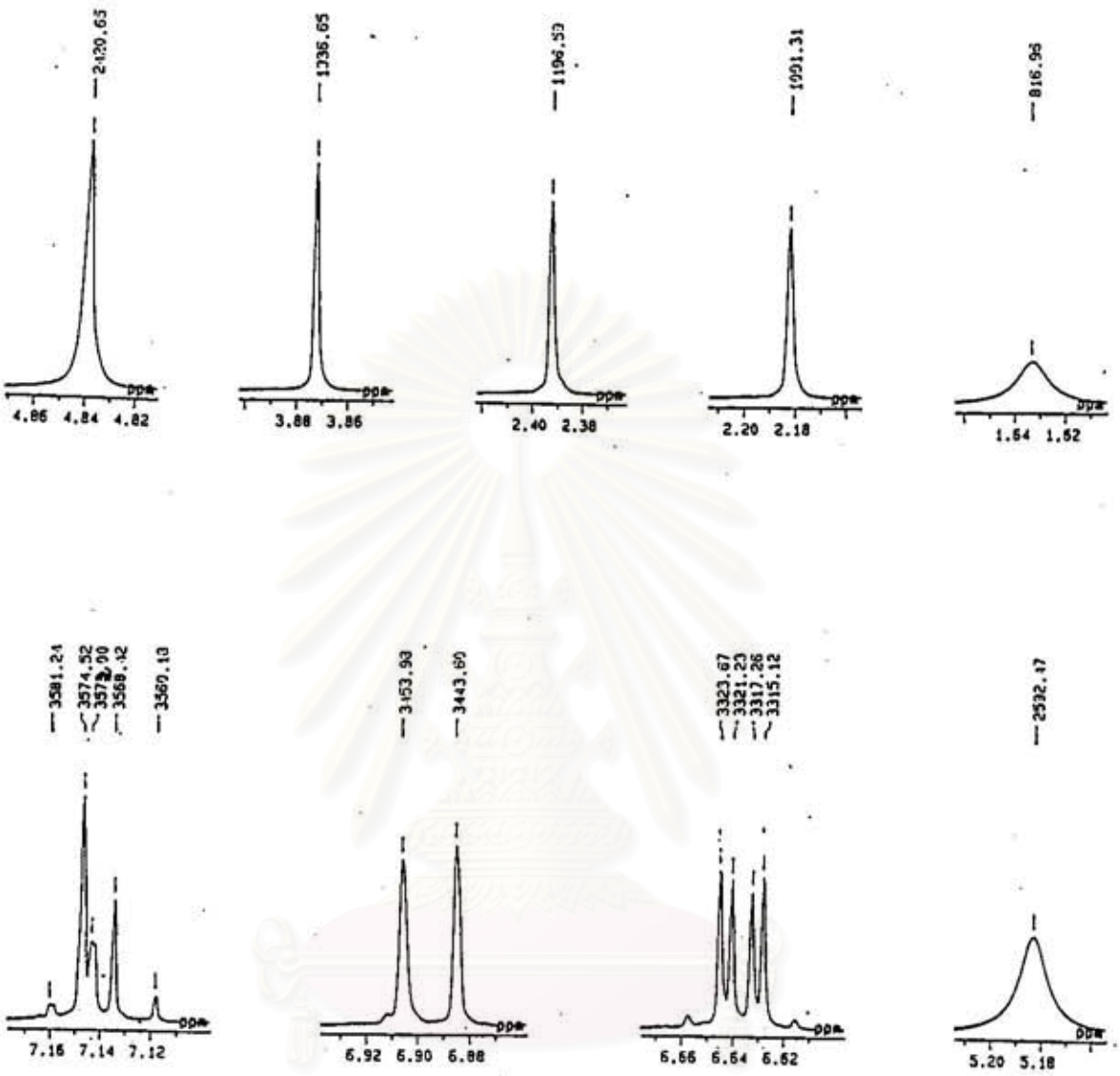


Figure 48 The expansions of ^{1}H NMR Spectrum of Compound 6

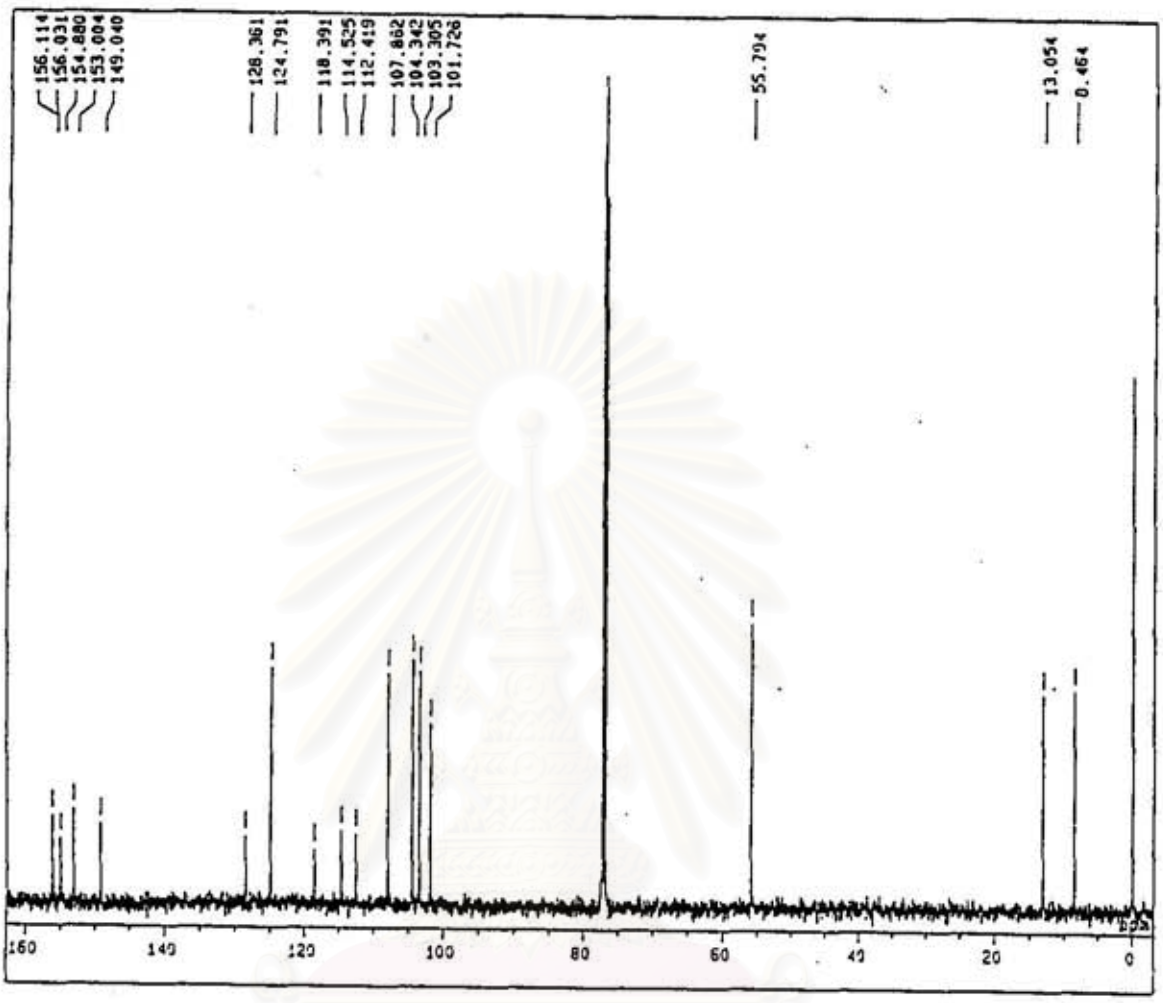


Figure 49 The ^{13}C NMR spectrum of Compound 6

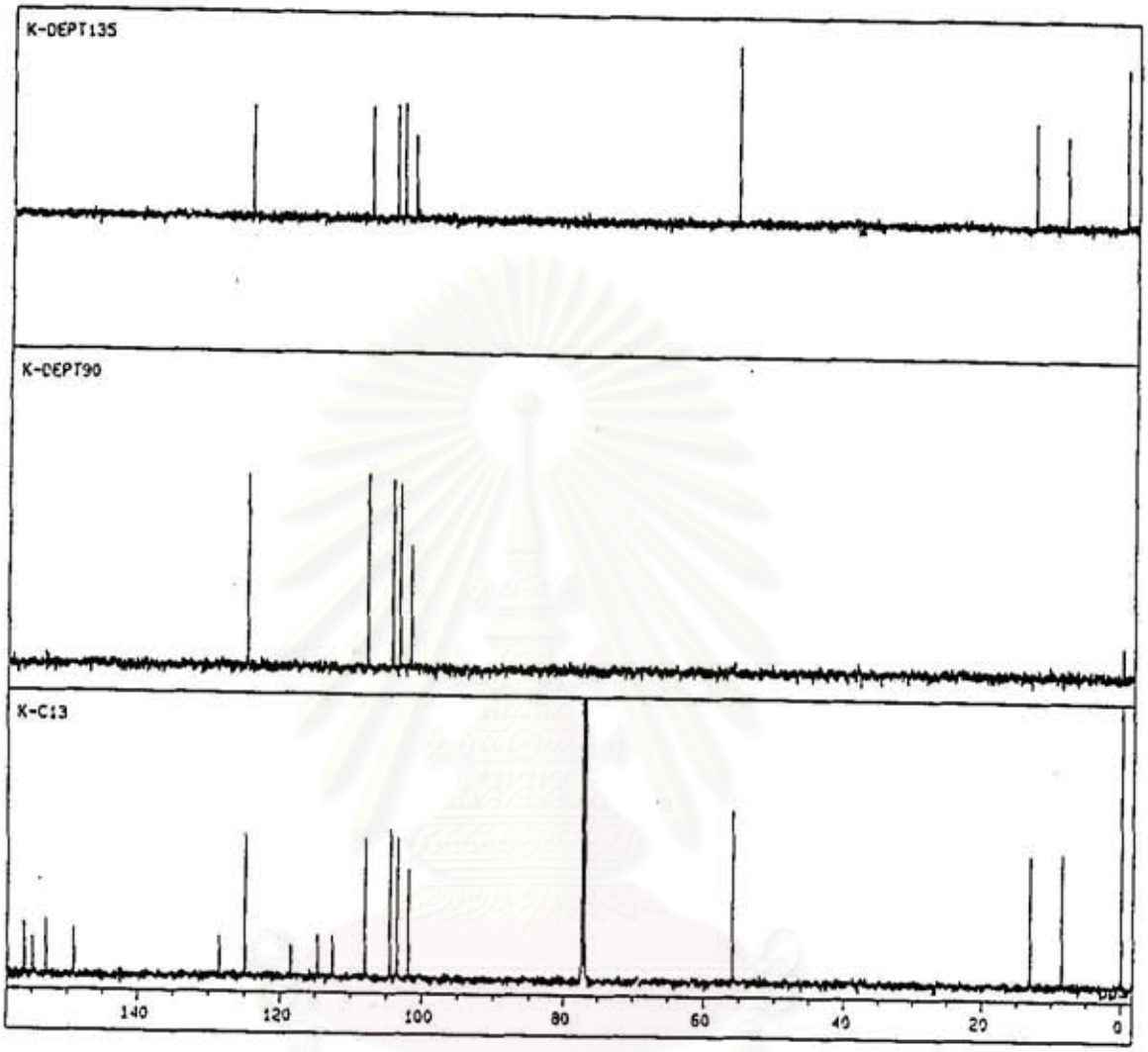


Figure 50 The DEPT 90 , 135 - ^{13}C NMR spectrum of Compound 6

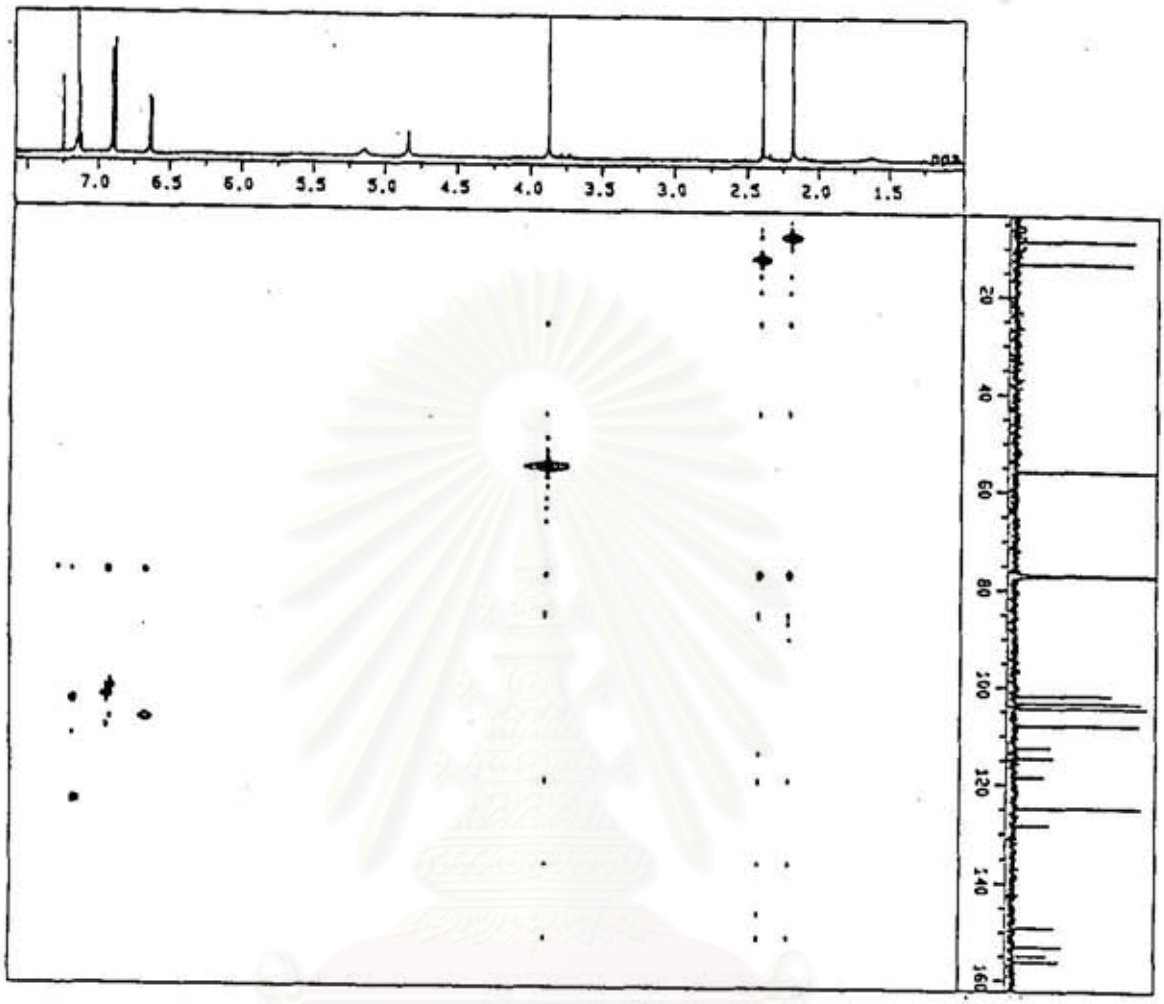


Figure 51 The HMQC spectrum of Compound 6

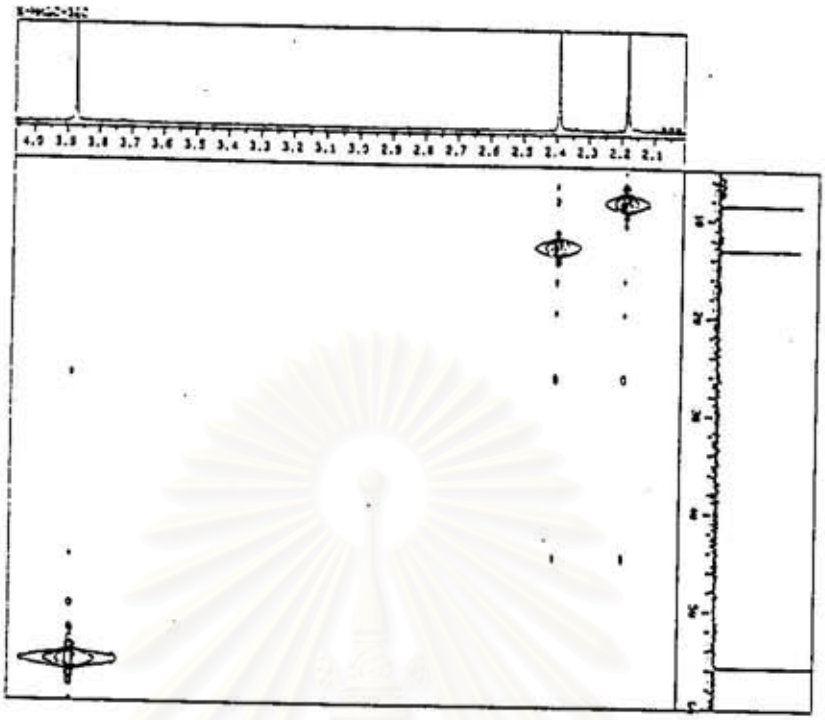


Figure 52 The expansions of HMQC Spectrum of Compound 6

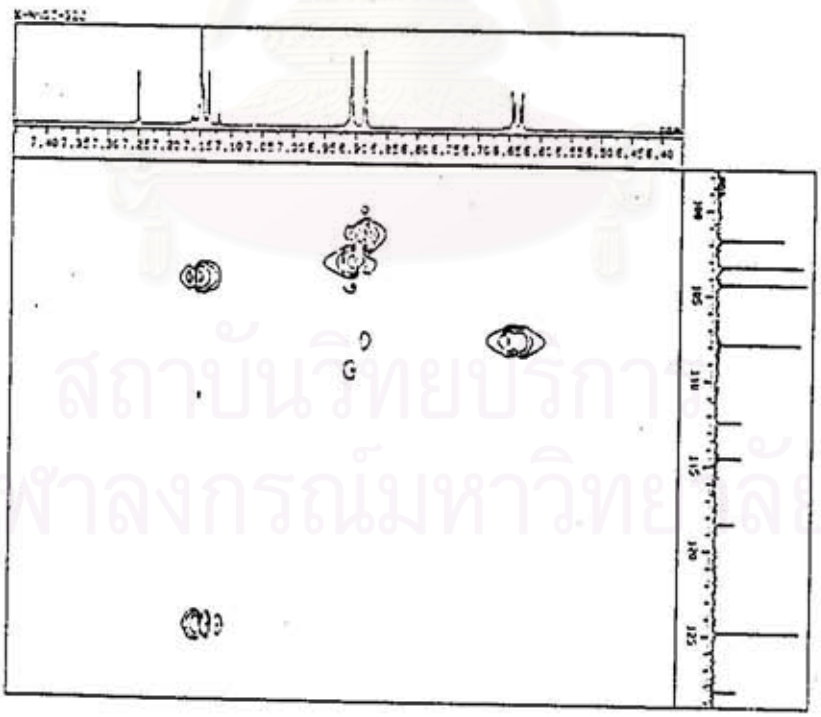


Figure 53 The expansions of HMQC Spectrum of Compound 6

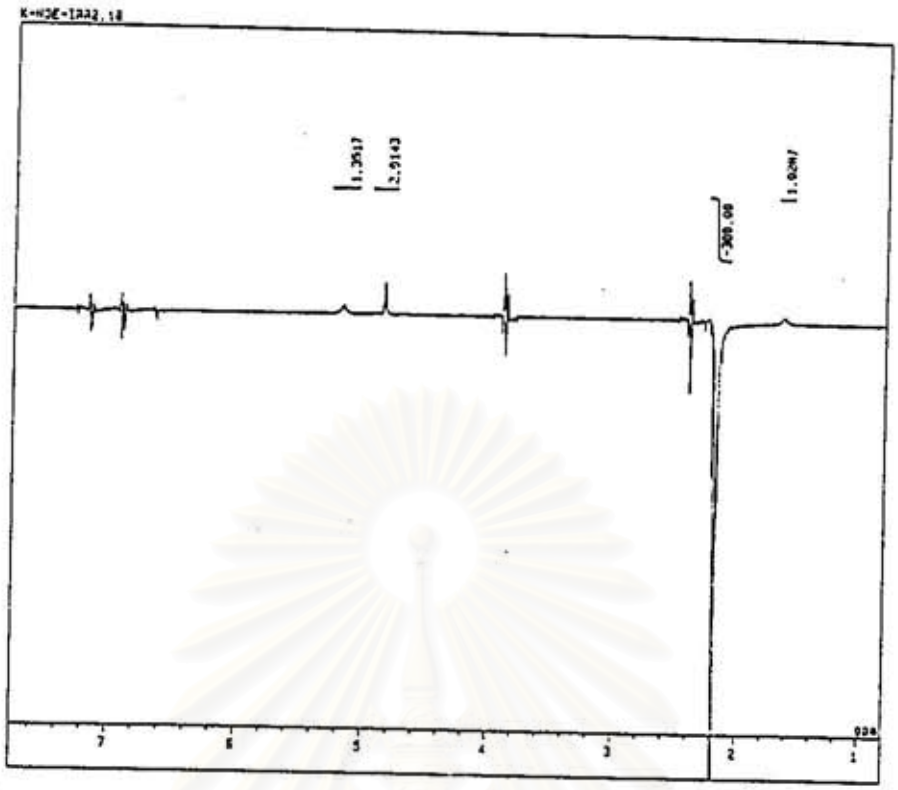


Figure 54 The NOE difference spectrum of Compound 6 (irradiate at δ 2.18 ppm.)

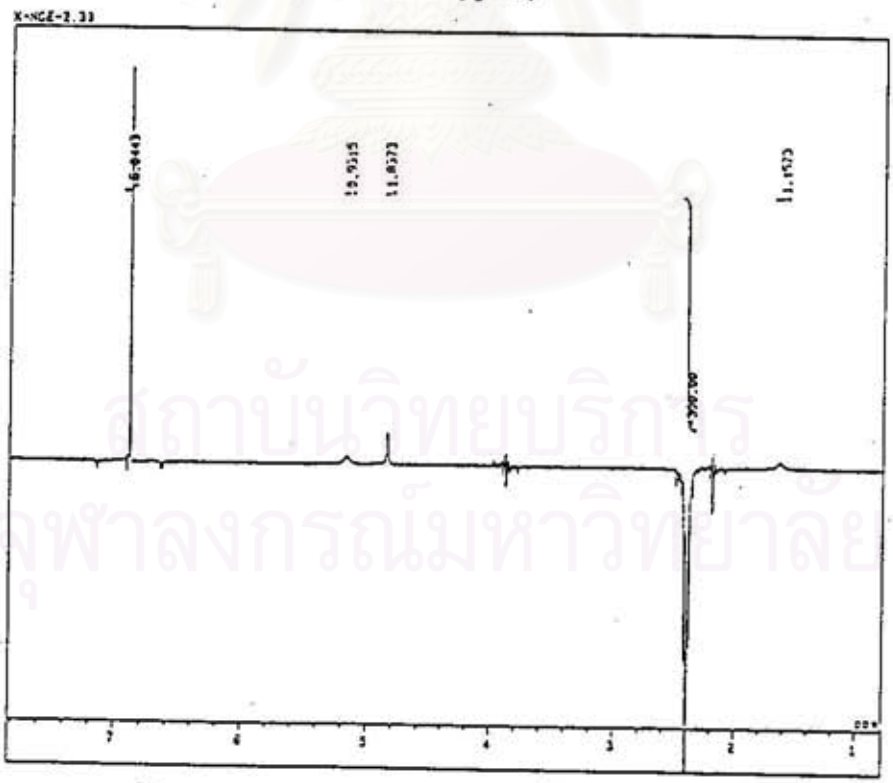


Figure 55 The NOE difference spectrum of Compound 6 (irradiate at δ 2.39 ppm.)

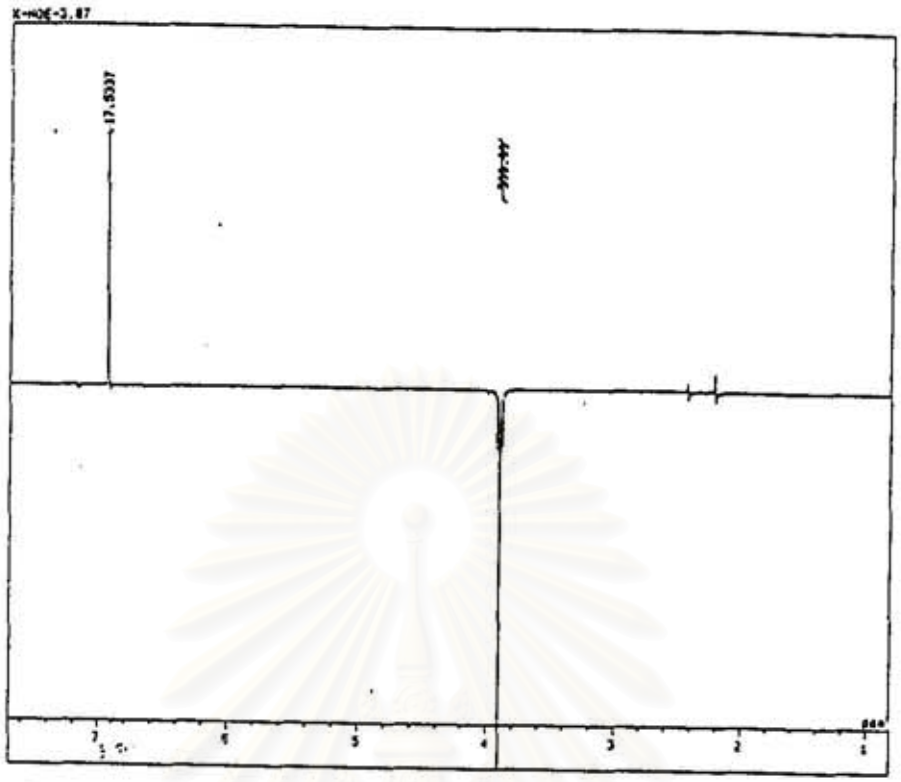


Figure 56 The NOE difference spectrum of Compound 6 (irradiate at δ 3.87 ppm.)

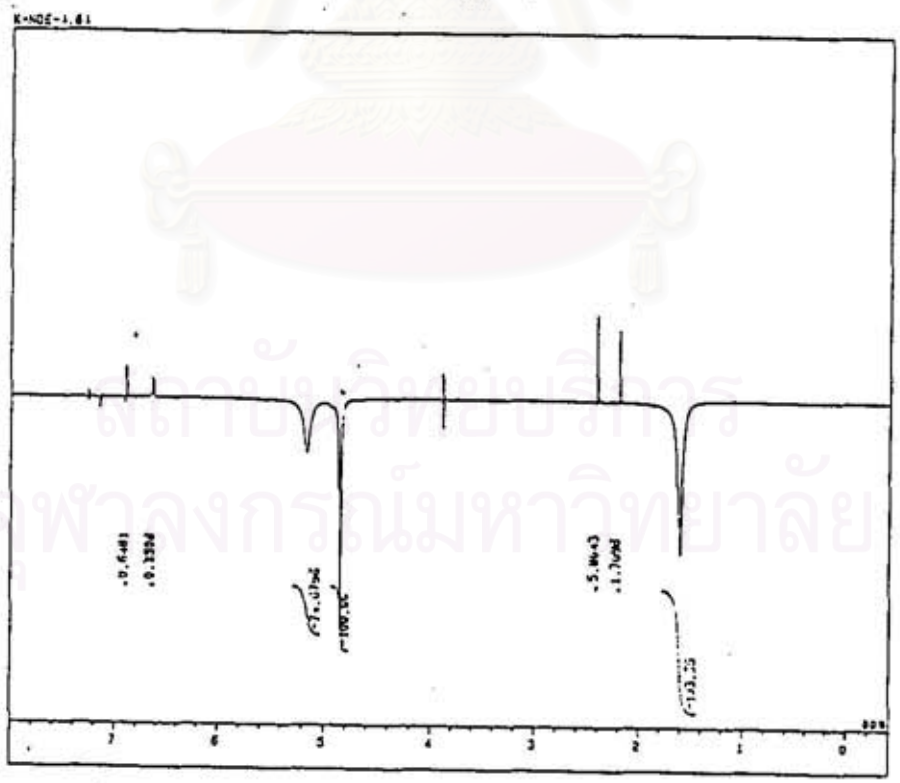


Figure 57 The NOE difference spectrum of Compound 6 (irradiate at δ 4.84 ppm.)

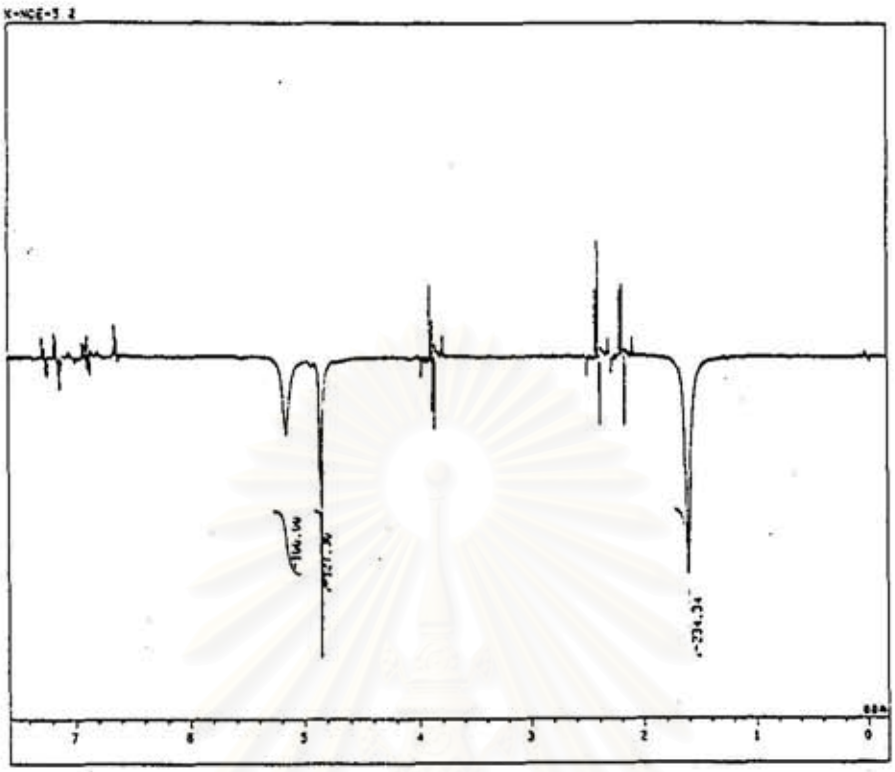


Figure 58 The NOE difference spectrum of Compound 6 (irradiate at δ 5.18 ppm.)

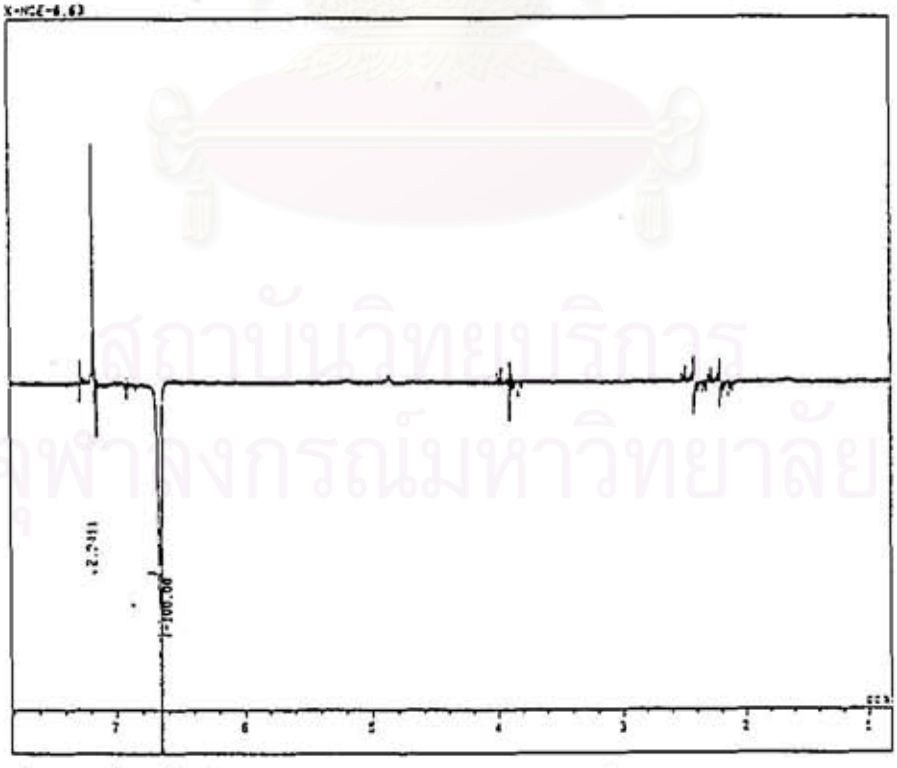


Figure 59 The NOE difference spectrum of Compound 6 (irradiate at δ 6.64 ppm.)

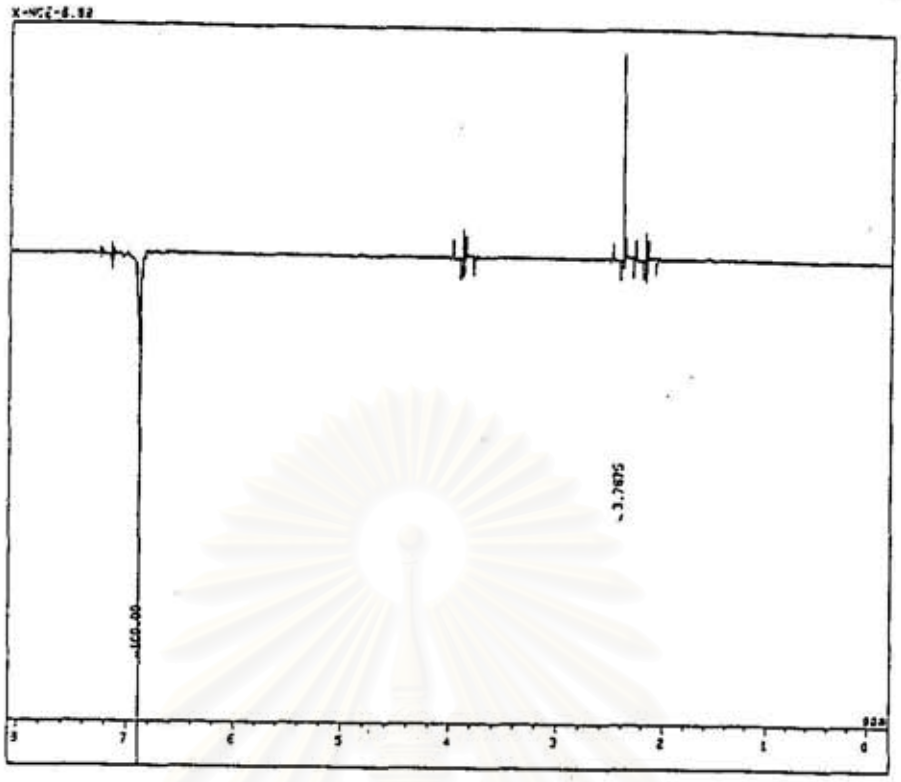


Figure 60 The NOE difference spectrum of Compound 6 (irradiate at δ 6.88 ppm.)

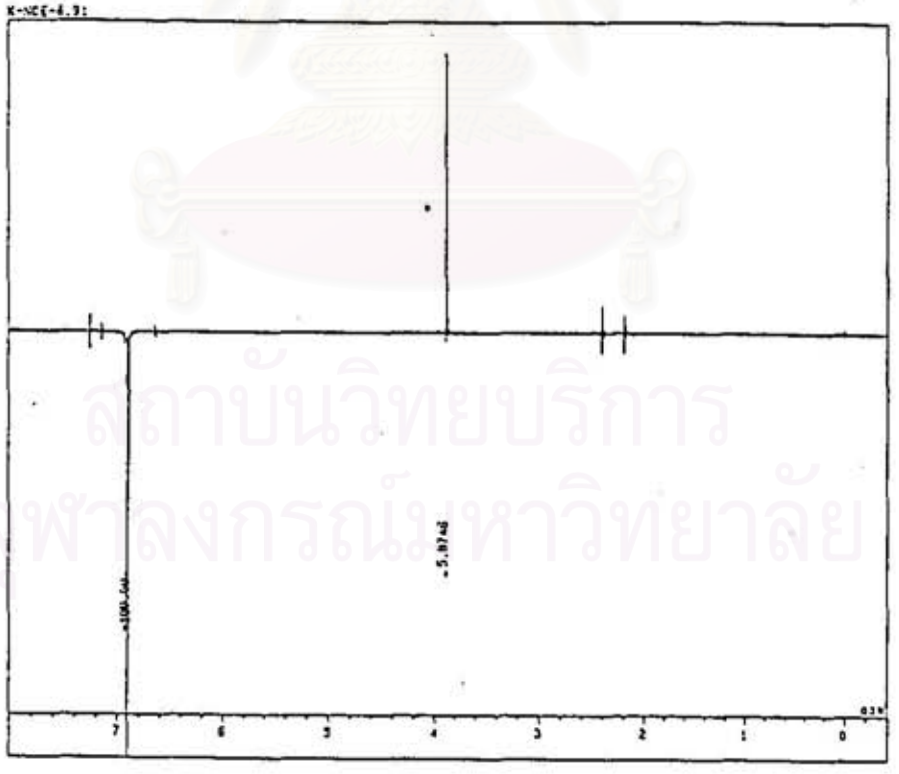


Figure 61 The NOE difference spectrum of Compound 6 (irradiate at δ 6.91 ppm.)

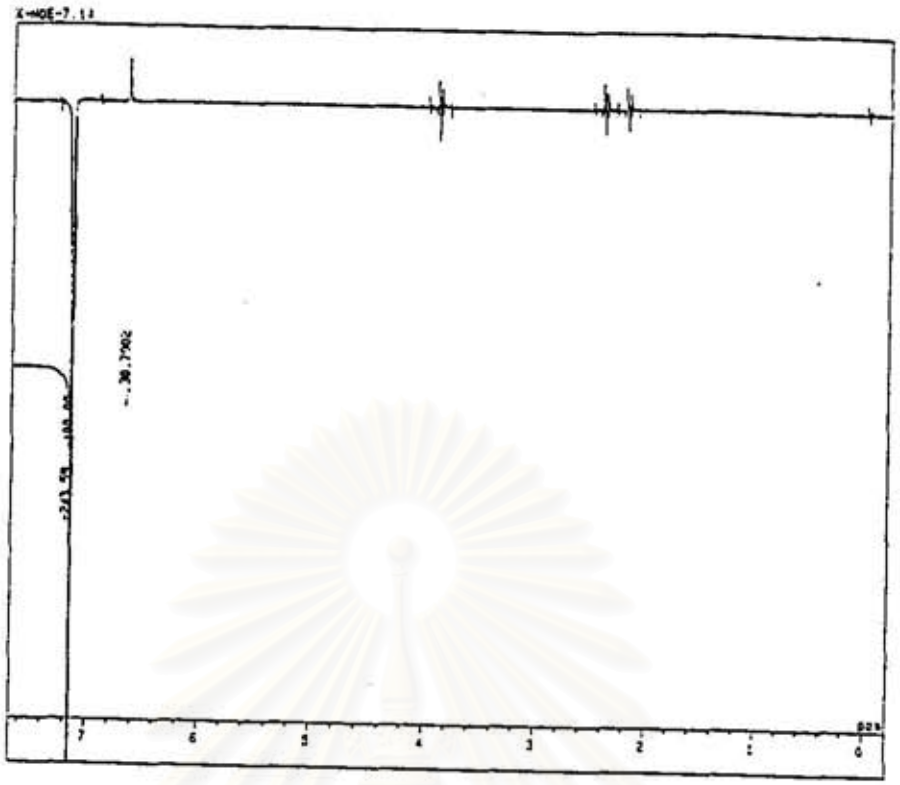


Figure 62 The NOE difference spectrum of Compound 6 (irradiate at δ 7.13 ppm.)

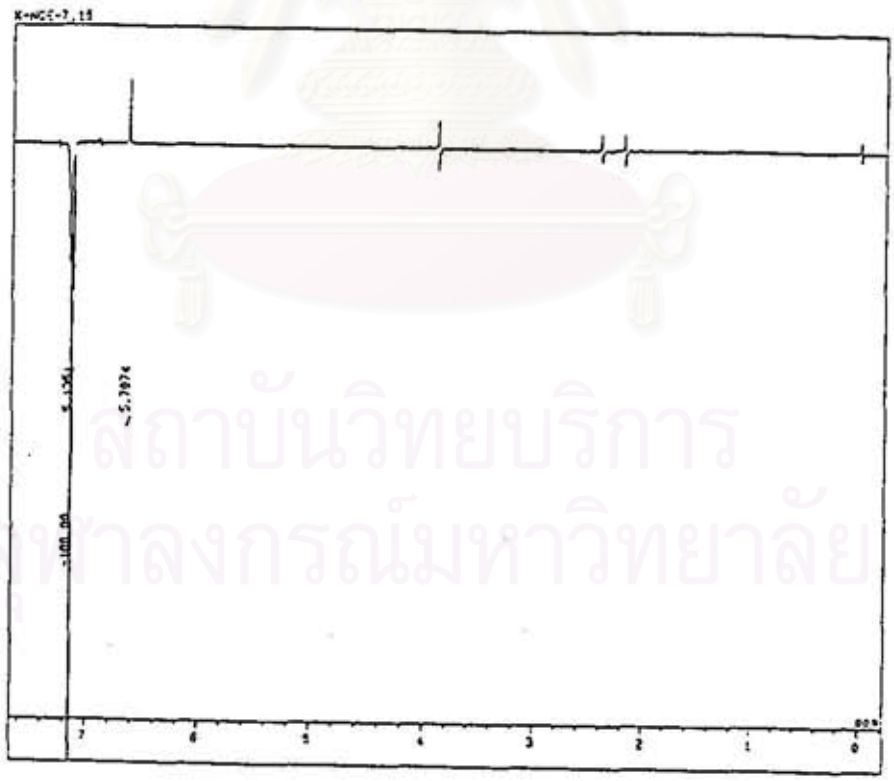


Figure 63 The NOE difference spectrum of Compound 6 (irradiate at δ 7.15 ppm.)

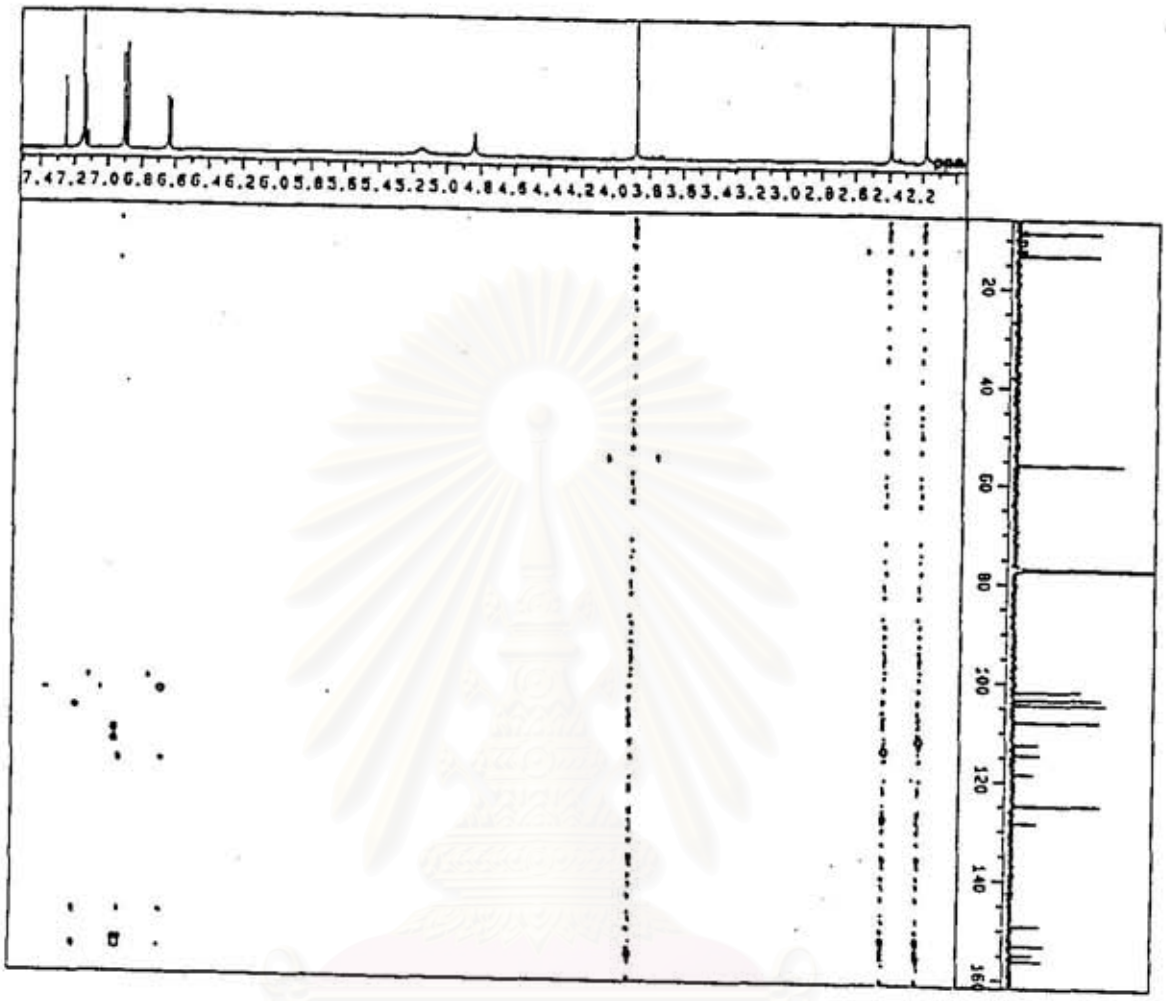


Figure 64 The HMBC spectrum of Compound 6

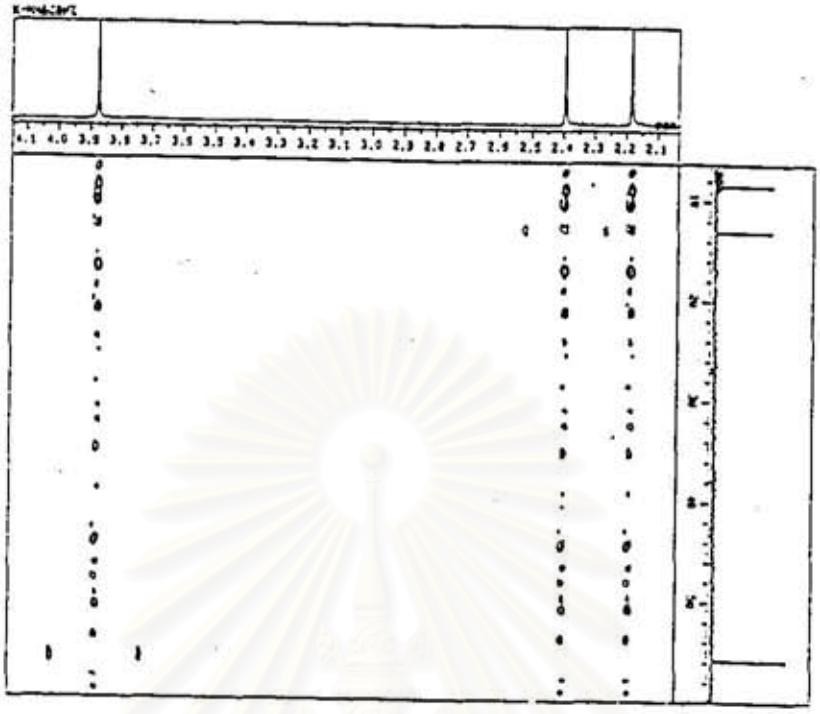


Figure 65 The expansions of HMBC Spectrum of Compound 6

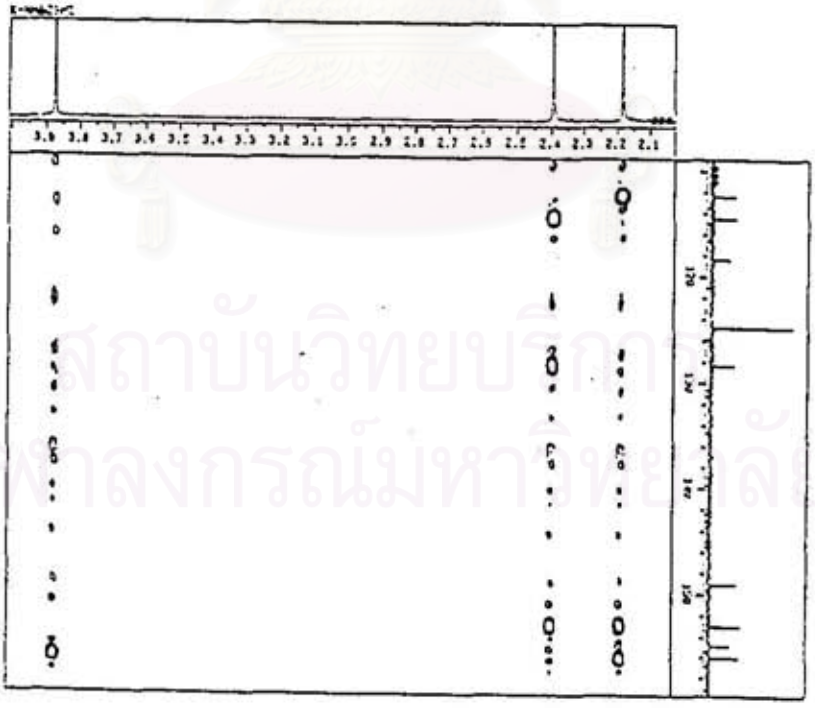


Figure 66 The expansions of HMBC Spectrum of Compound 6

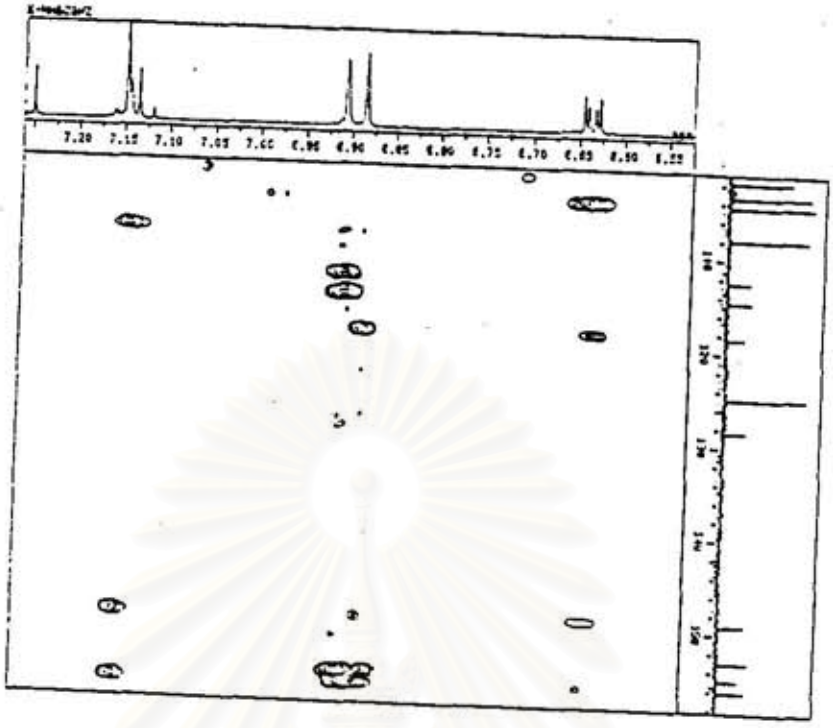


Figure 67 The expansions of HMBC spectrum of Compound 6

3.8.7 Structure Elucidation of compound 7.

Compound 7 was isolated as a major compound , 3.56 g. ($2.74x10^{-1}$ % wt by wt of dried roots) , of this plant from Fractions II and III . It was positive to Maeyer's , Valser's , Wagner's , Dragendorff's , Kraut's and Marme's reagents. This compound crystallized as colourless prisms , mp 190-191° C , and its R_f value was 0.48 [hexane: dichloromethane (3:2)] . From the results of the chemical reactions , compound 7 was thought to be an alkaloid.

The IR absorption bands at 1770 , 1630 and 1020 cm^{-1} indicated the presence of an unsaturated lactone structure in the molecule. (Fig.68) Mass spectrometry indicated $M^+ = 385$. The mass spectrum showed fragments at 369 , 355 , 235 and 207. (Fig.69)

The ^1H NMR spectrum showed signal at δ 2.87 [1 H , d , J=5.8 Hz] , δ 3.55[1H , t , J = 4.3 Hz], δ 4.22 [1H , t , J = 1.5 Hz] , two olefinic protons at δ 5.53 [1 H , dt , J = 15.6 , 1.52 Hz] and δ 5.80 [1H , dt , J = 15.6 , 6.4 Hz] , two adjacent protons at C-11, C-12 between δ 1.94-1.98 and 2.98-3.04 [each 1H , m] , methylene protons between δ 1.78-1.86 , δ 3.07-3.16 ,δ 1.87-1.93 and 2.08 [each 2H , m] , three methyl protons at δ 1.00 [3H , t , J = 7.5 Hz] , 1.39 [3H, d , J = 6.7 Hz], 2.07 [3H , s] and one methoxy group of C-18 at δ 4.15 [3H , s]. (Fig.70)

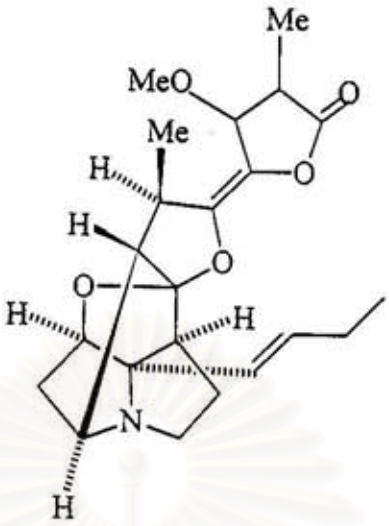
The ^{13}C NMR , DEPT 90 and 135 spectrum showed signals of 22 carbons as follows : three methyl groups at δ 83.2 (C-8), 98.5 (C-15) and 18.2 (C-17) , one methoxy group at δ 58.8 (C-18) , four methylene carbons at δ 25.3 (C-22) , 26.7 (C-2) ,32.8 (C-6) and 47.5 (C-3) , seven tertiary carbons at δ 34.5 (C-11) , 47.4 (C-12) , 51.0 (C-1) , 60.9 (C-5), 80.5(C-7), 126.2 (C-20) and 133.5

(C-21) , and seven quaternary carbons at δ 83.2 (C-8), 98.5 (C-15) , 112.6 (C-9) , 127.8 (C-13) , 148.3 (C-10) ,162.7 (C-14) and 169.6 (C-16) . (Fig.71-73)

One bond correlation (HMQC) data showed that the proton at δ 2.87 was attached to C-1 , the proton between δ 1.78-1.86 was attached to C-2 , the proton between δ 3.07-3.16 was attached to C-3 , the proton at δ 3.55 was attached to C-5 , the proton between δ 1.87-1.93 was attached to C-6 , the proton at δ 4.22 was attached to C-7 , the proton between δ 2.98-3.04 was attached to C-11 , the proton between δ 1.94-1.98 was attached to C-12 , the proton at δ 1.39 was attached to C-17 and the proton of methoxy group at δ 4.15 was attached to C-18 . (Fig.74)

The above results indicated that Compound 7 was an alkaloid with a lactone ring , double bonds , three methyl groups and one methoxy group were present in the structure . Hence , this compound had a molecular formula of $C_{22} H_{27} NO_5$.

This data is consistent with Compound 7 being Asparagamine A , a pyrrolizidine alkaloid recently isolated from this plant. [6.7] The ^{1}H and ^{13}C NMR closely matched those reported.



Asparagamine A.

Table 22 ^{1}H and ^{13}C NMR spectral data of compound 7 (500/125 MHz , $CDCl_3$) compared with the Literature. [7]

Position	^{13}C (Lit.)	^{13}C	^{1}H
C-1	51.1	51.0	2.87(d) J = 5.8 Hz
C-2	26.8	26.7	1.78-1.86 (m)
C-3	47.9	47.5	3.07-3.16 (m)
N	-	-	-
C-5	60.7	60.9	3.55 (t) J = 4.3 Hz
C-6	32.8	32.8	1.87-1.93 (m)
C-7	80.6	80.5	4.22 (t) J = 1.5 Hz
C-8	82.9	83.2	-
C-9	112.7	112.6	-
C-10	148.3	148.3	-
C-11	34.5	34.5	2.98-3.04 (m)
C-12	47.5	47.4	1.94-1.98 (d) J = 12.2 Hz
C-13	127.7	127.8	-
C-14	162.8	162.7	-
C-15	98.3	98.5	-
C-16	169.7	169.6	-
C-17	18.2	18.2	1.39 (d) J = 6.7 Hz
C-18	58.8	58.8	4.15 (s)
C-19	9.0	9.1	2.07 (s)
C-20	126.4	126.2	5.53 (dt) J = 15.6 ,1.5 Hz
C-21	133.2	133.5	5.80 (dt) J = 15.6 , 6.4 Hz
C-22	25.2	25.3	2.08(s)
C-23	13.4	13.4	1.00 (t) J = 7.5 Hz

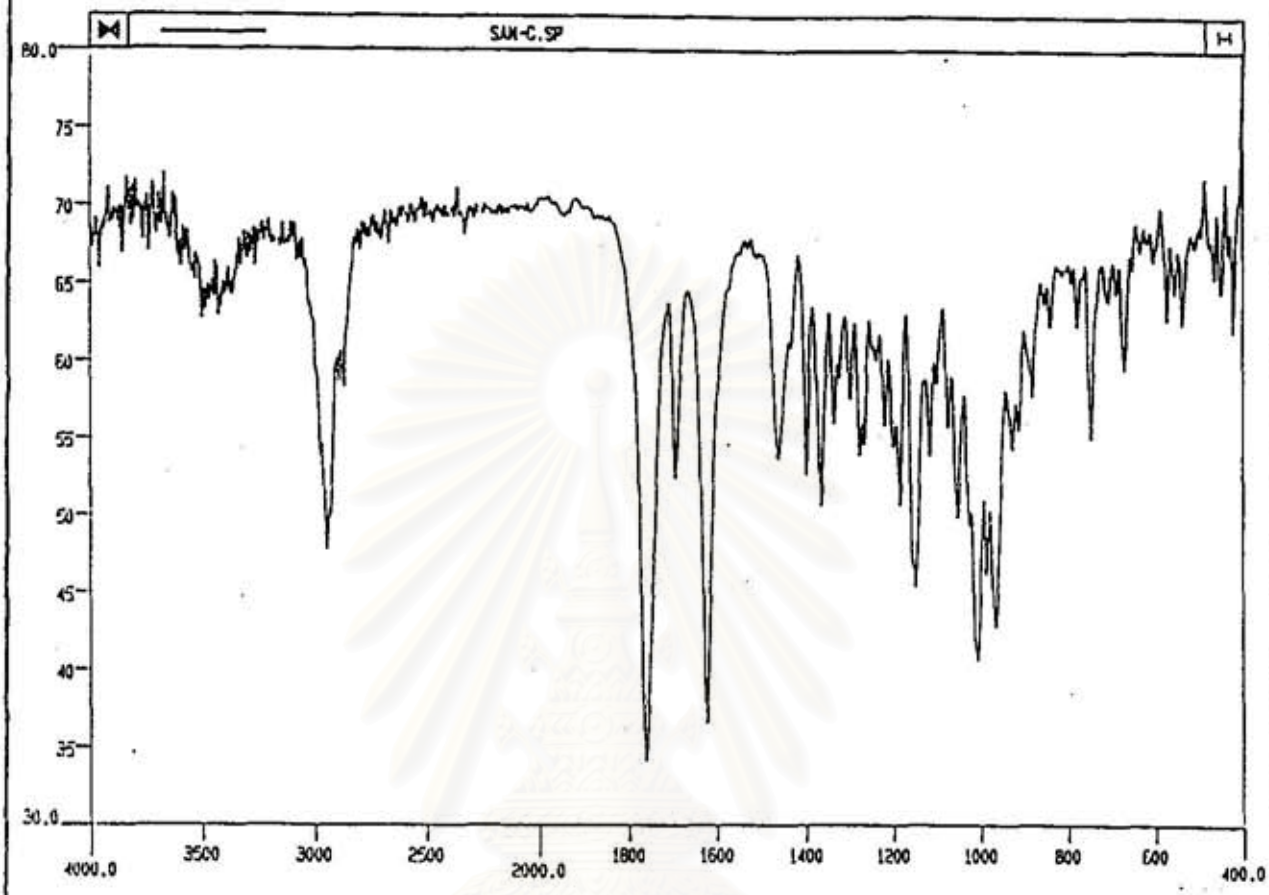


Figure 68 The IR spectrum of Compound 7

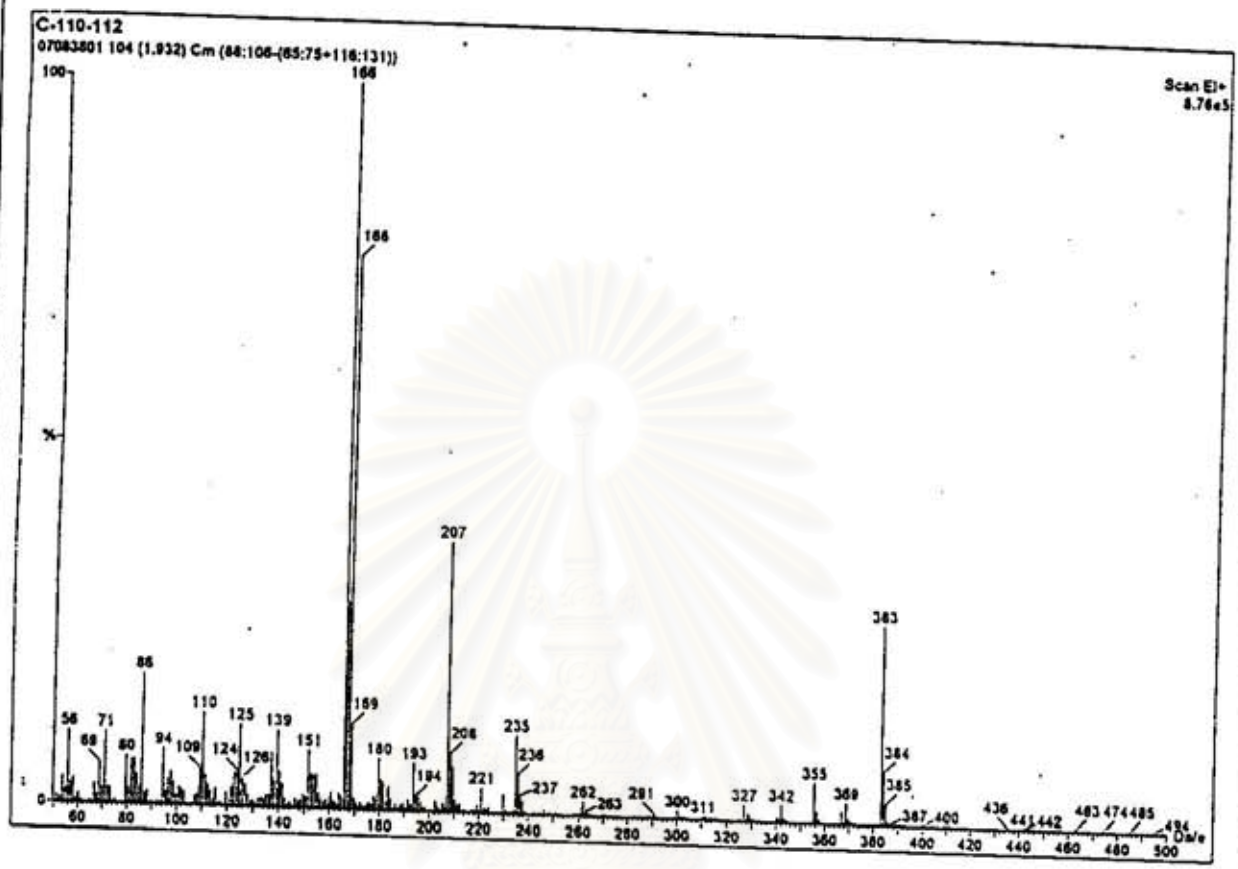


Figure 69 The mass spectrum of compound 7

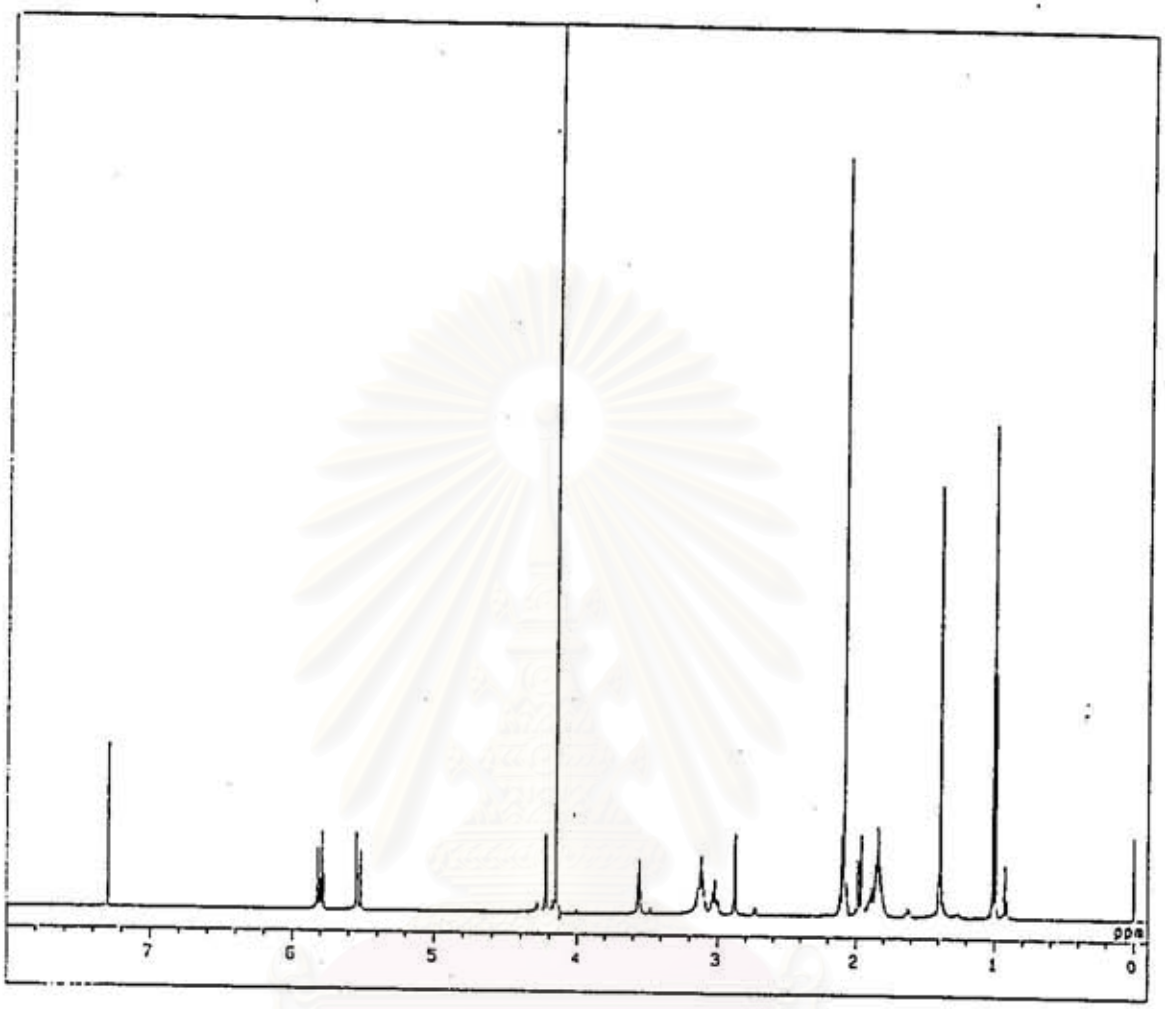


Figure 70 The ^{1}H NMR spectrum of Compound 7

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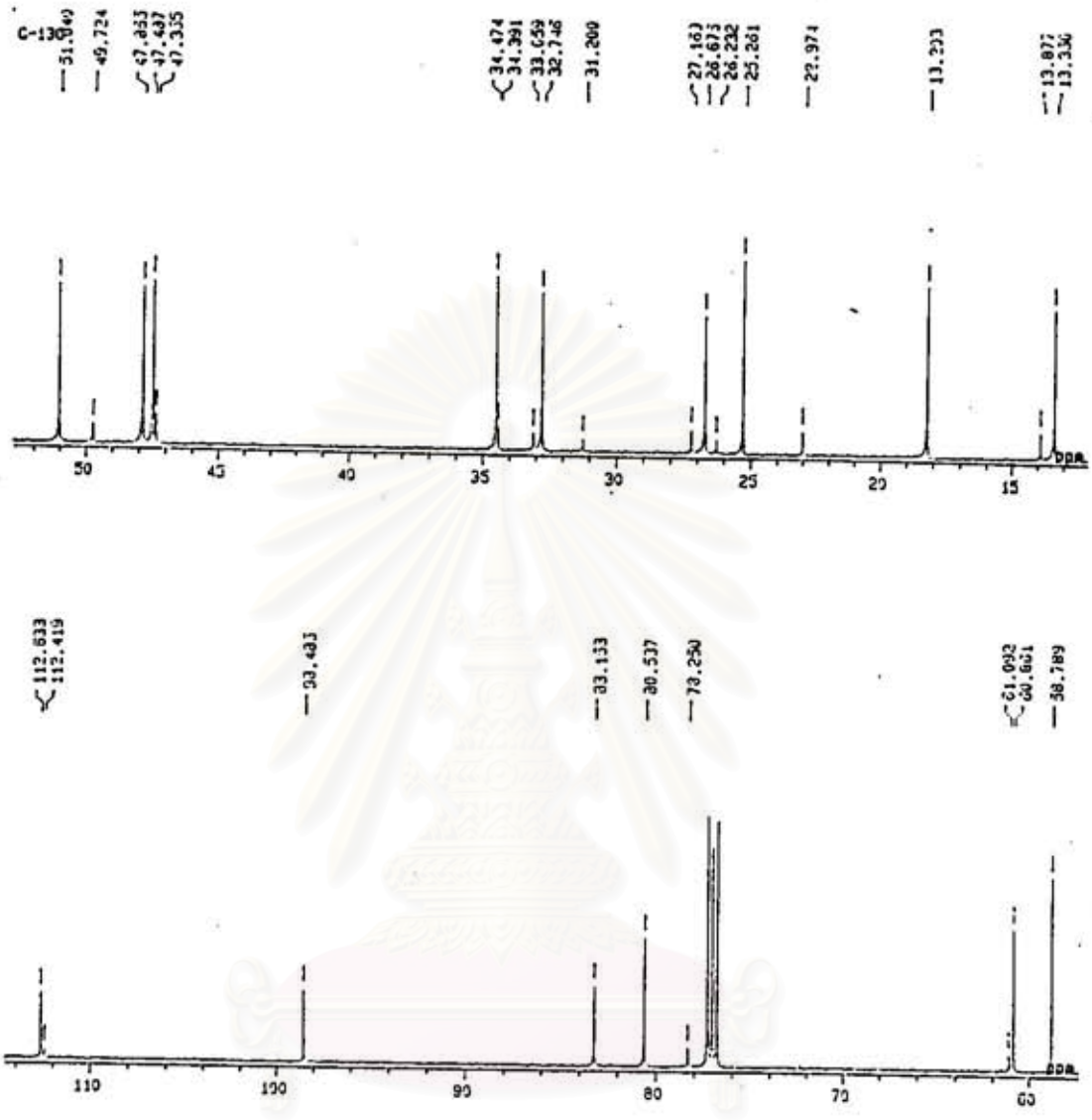


Figure 72 The expansions of ^{13}C NMR Spectrum of Compound 7

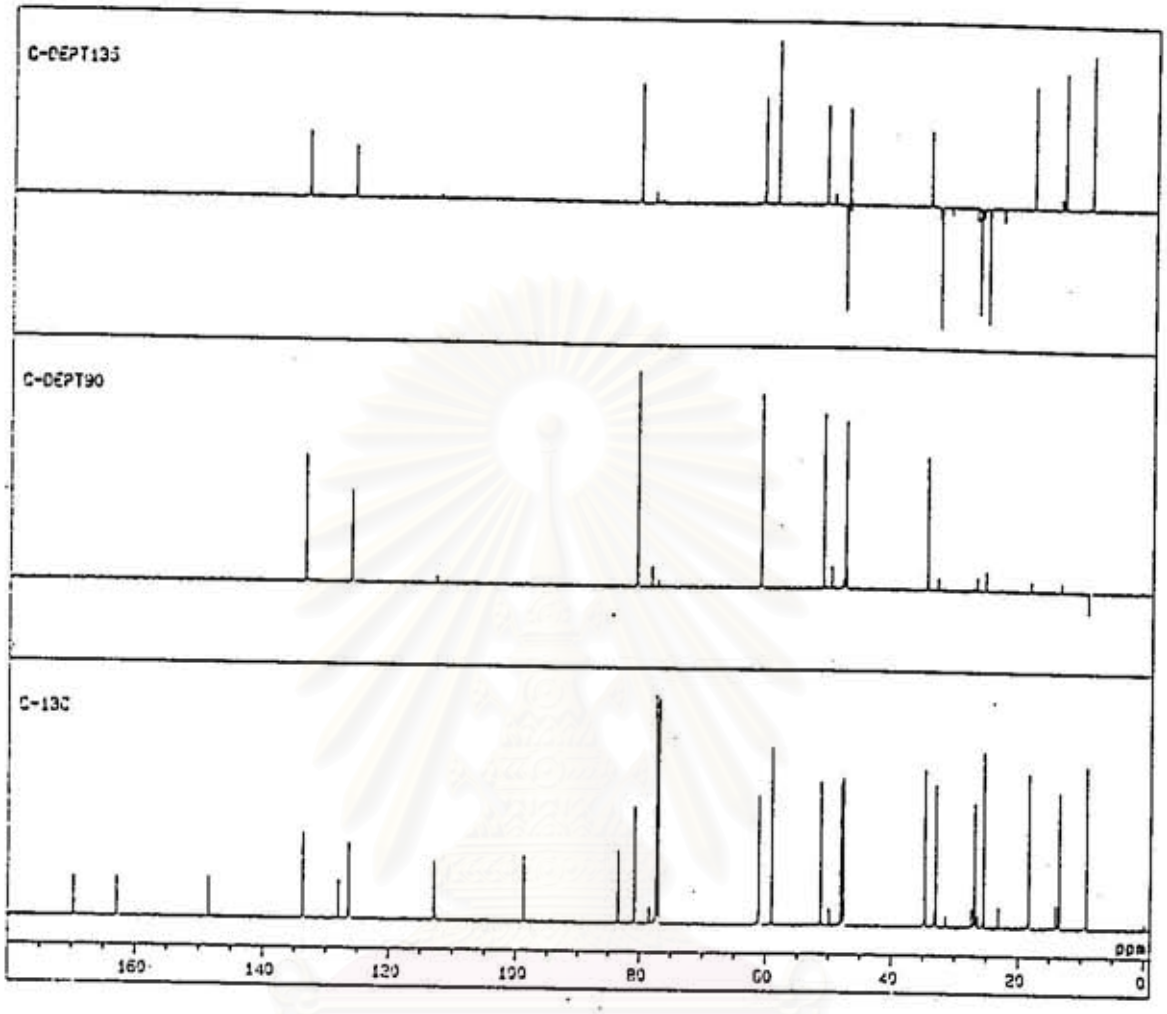


Figure 73 The DEPT 90 , 135 - ^{13}C NMR spectrum of Compound 7

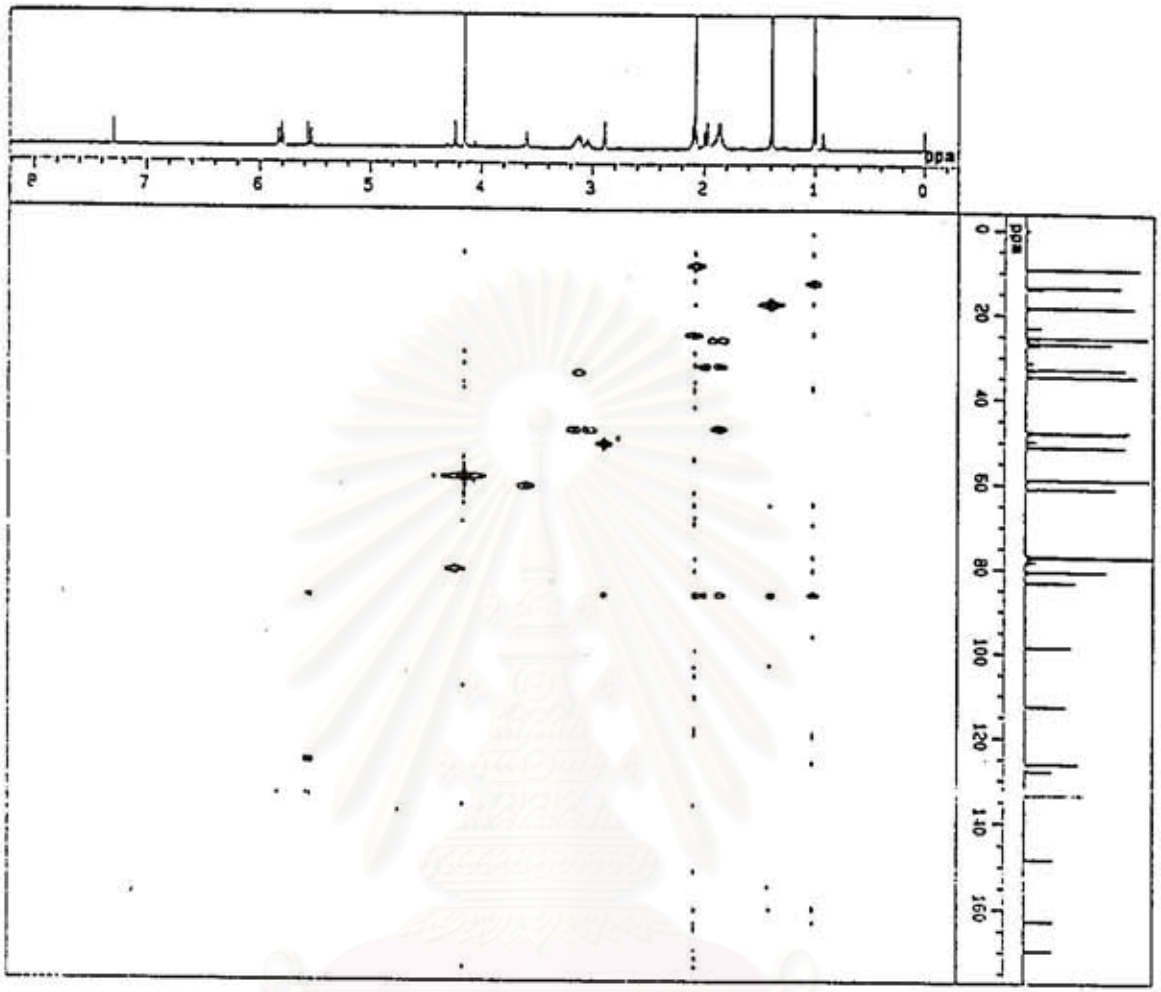


Figure 74 The HMQC spectrum of Compound 7.

3.8.8 Biological Activity of Asparagamine A.

Bioassay of Asparagamine A. was carried out for anti-oxytocin activity which induced uterus muscle contraction , *in vivo* . The activity of this compound was investigated at doses of 5 and 10 mg / 0.2 ml / rat as shown in Table 23.

Table 23 Oxytocin-activity of Asparagamine A. in wistar rats.

Treatment	Parturitient time				delayed parturition (hr.)
	P_{21}-P_{22} 16.00 pm-6.00 am.	P_{22} 10.00 am.	P_{22} 13.00 pm.-15.00 pm.	P_{22} 15.00 pm.-18.00 pm.	
control	13	-	-	-	-
5 mg.	7	-	-	-	-
10 mg.	-	2	-	-	4
	-	-	6	-	7-9
	-	-	-	11	9-12

This data showed that Asparagamine A., an anti-oxytocin agent , caused the delay of parturition in pregnant rats in a dose of 10 mg / 0.2 ml / rat.

Table 24 The percent of delayed group in a dose of 10 mg / 0.2 ml / rat.

delayed parturition time (hr.) (dose 10 mg / 0.2 / rat)	percent of delayed group (%)
4	10.53
7-9	31.58
9-12	57.90

Asparagamine A., which is a major component of *A. racemosus*, has been tested for anti-oxytocin activity of rat diestrus uterus, *in vitro*.[7] In this study, Asparagamine A. also showed this activity *in vivo*, at doses of 10 mg / 0.2 ml / rat. This result supports the earlier report which suggested that Asparagamine A. is a significant portion of the anti-abortifacient activity of this Ayurvedic crude drug.

3.8.9 Structure elucidation of compound 8.

Compound 8 , 108.5 mg (8.32×10^{-3} % wt by wt of dried roots) of a white amorphous solid , was obtained from the dichloromethane crude extract and the ethyl acetate crude extract. This compound was recrystalized from chloroform and methanol. Its melting point was 255-258° C . R_f value was 0.58 [dichloromethane : methanol (1:5)].The colour test gave a green-blue colour with Liebermann-Burchard's reagent , which indicated the presence of a steroidal nucleus in this molecule.

The IR spectrum showed a broad band of hydroxy group (-OH) at 3440 cm^{-1} , the C-O stretching vibrations of glycosidic linkage at 1080-1035 cm^{-1} and an anomeric axial C-H deformation of β-sugar at 890 cm^{-1}. (Fig.75)

The results of colour test and IR spectrum indicated that Compound 8 might be a steroid glycoside.

The mass spectrum did not give the molecular ion peak (M^+) due to fragmentation of molecule. The spectrum revealed the dominant fragmentation ion peak at 412 , and other fragments at 394 , 255 and 213. This is similar to the fragmentation pattern of stigmasterol. (Fig.76)

The ^1H NMR spectrum showed signals which corresponded to a steroid glycoside. The signal at δ 0.50-2.50 ppm corresponds to a methyl and methylene protons of a steroid while the signals at δ 5.00-5.22 ppm. corresponds to alkene protons. A doublet at δ 4.30 ppm (1H , d , J = 8.2 Hz) was present for the anomeric protons of β-D-glucose and the other sugar moiety showed a multiplet between δ 4.50-5.00 ppm (Fig.77)

The ^{13}C NMR spectrum of compound 8 was compared with stigmasterol [22] and glucose [31] which obtained from hydrolysis of glycoside to confirmed the structure. (Fig.78)

Table 25 ^{13}C NMR spectrum of Compound 8 (selected signals) compared with standard stigmasterol [22].

position	chemical shift (δ ppm)	
	stigmasterol	compound 8
C-1	37.4	37.4
C-2	31.7	31.8
C-3	71.8	71.8
C-4	42.4	42.4
C-5	140.0	140.7
C-6	121.7	121.5
C-7	31.9	32.1
C-8	31.9	32.1
C-9	50.3	50.1
C-10	36.6	36.8
C-11	21.1	21.3
C-12	39.8	39.8
C-13	42.4	42.4
C-14	57.0	57.0
C-15	24.4	24.5
C-16	28.9	29.1
C-17	56.0	55.9

Table 25 (cont.)

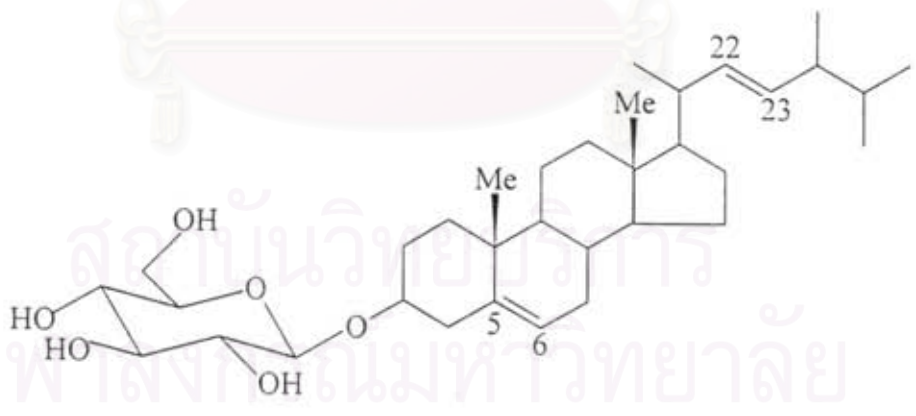
position	chemical shift (δ ppm)	
	stigmasterol	compound 8
C-18	12.2	12.4
C-19	19.4	19.4
C-20	40.5	40.8
C-21	21.1	21.3
C-22	138.4	138.4
C-23	129.4	129.2
C-24	51.3	51.2
C-25	31.9	32.1
C-26	19.0	19.0
C-27	21.1	21.3
C-28	25.4	25.6
C-29	12.0	12.0

In addition , the ^{13}C NMR spectrum showed signals between δ 61.5-77.5 ppm , corresponding to the signals of glucose. The ^{13}C NMR spectrum of compound 8 was compared with that of this sugar to confirm the structure. (Table 26)

Table 26 ^{13}C NMR spectrum of Compound 8 (selected signals) compared with standard glucose.[31]

position	chemical shift (δ ppm)	
	glucose	compound 8
G1	100.7	100.5
G2	73.4	73.5
G3	76.9	77.6
G4	70.1	70.5
G5	76.7	77.5
G6	61.1	61.5

All of that results and literature comparison suggested that Compound 8 is stigmasteryl-β-D-glucopyranoside.



Stigmasteryl-3-O-β-D-glucopyranoside

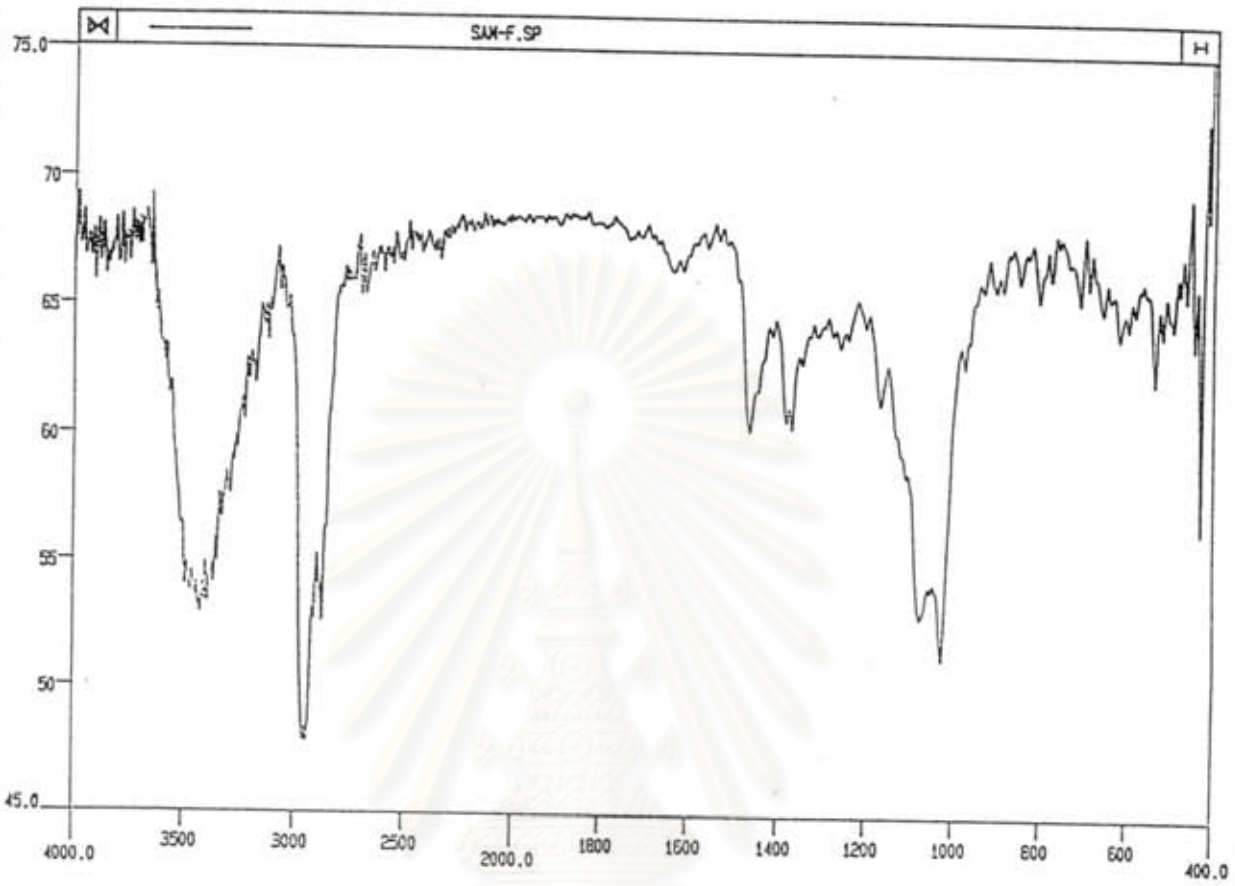


Figure 75 The IR spectrum of Compound 8

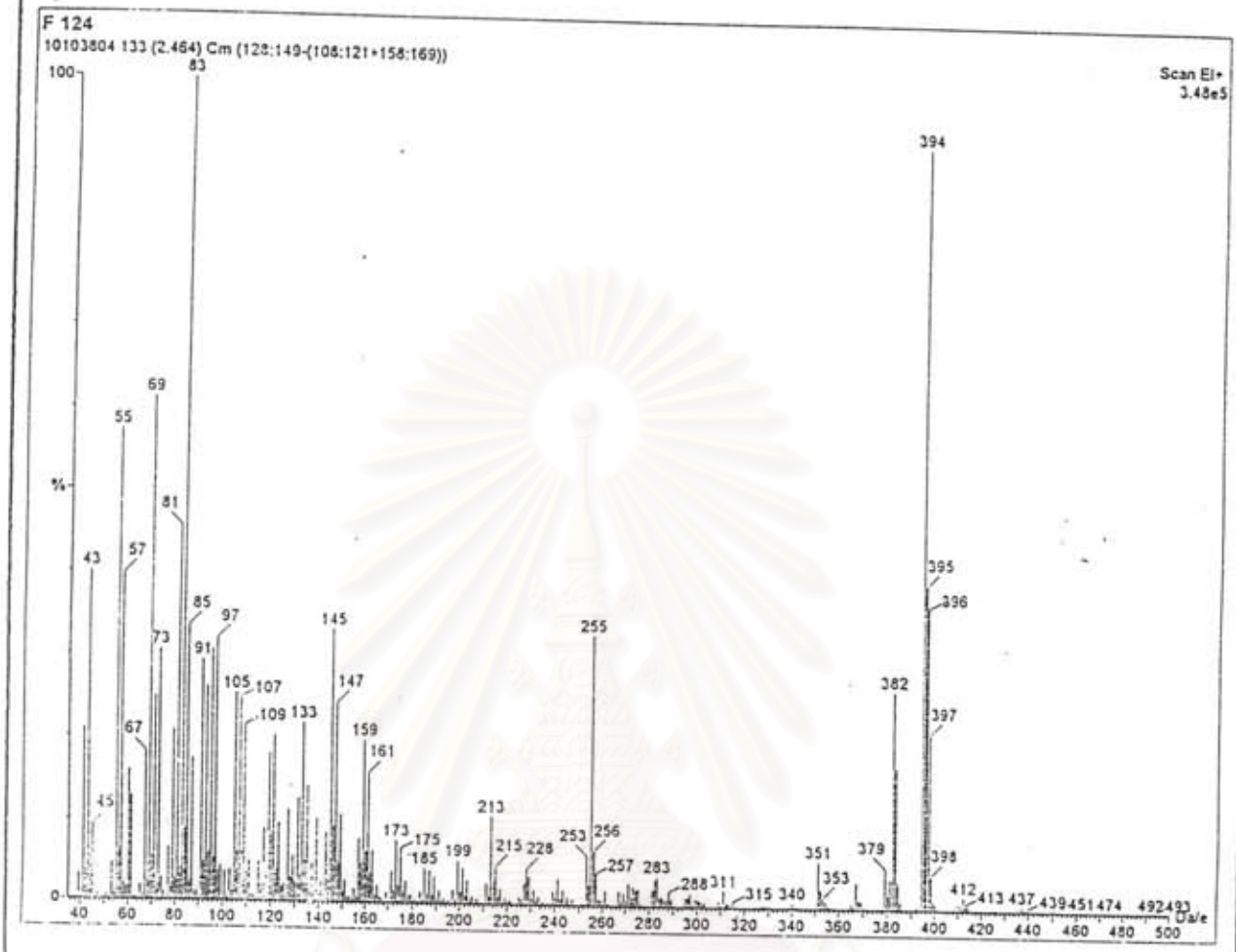


Figure 76 The mass spectrum of Compound 8

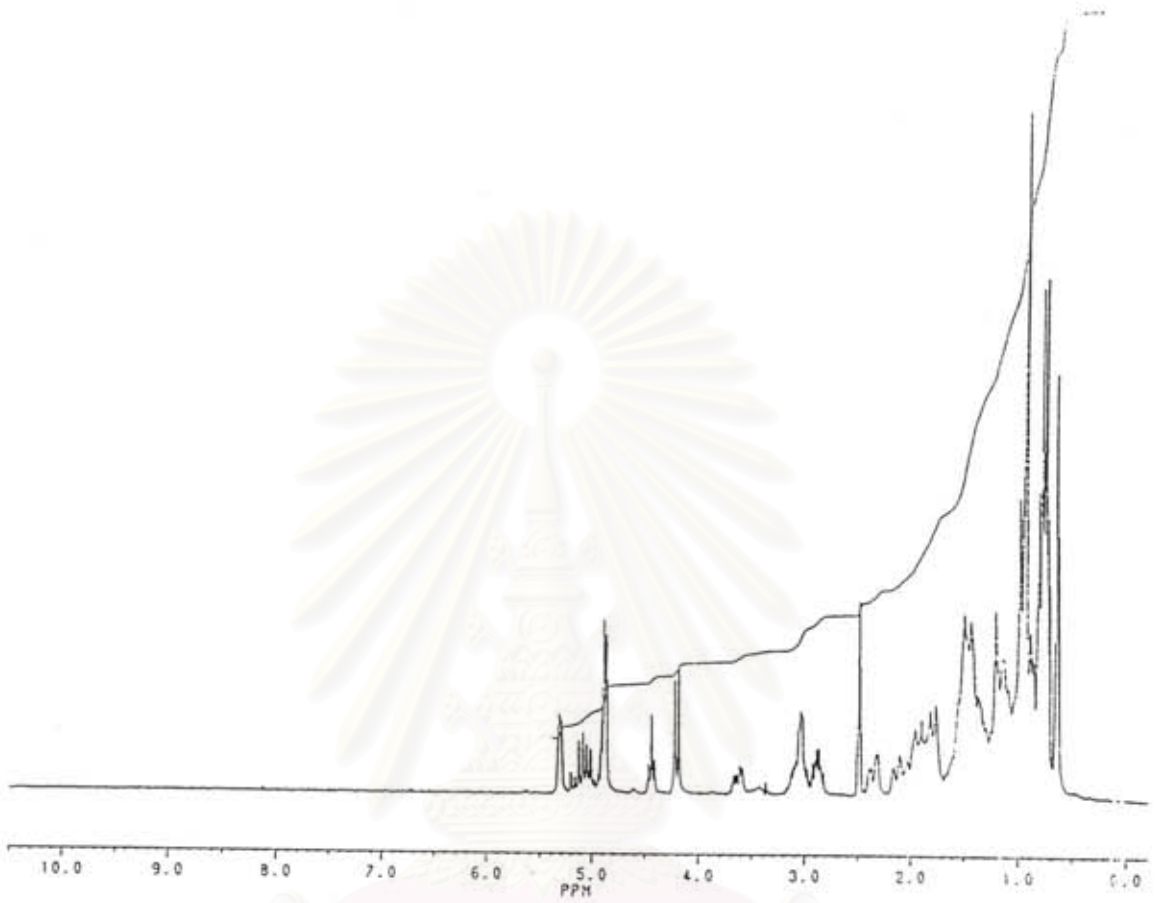


Figure 77 The ^{1}H NMR spectrum of Compound 8

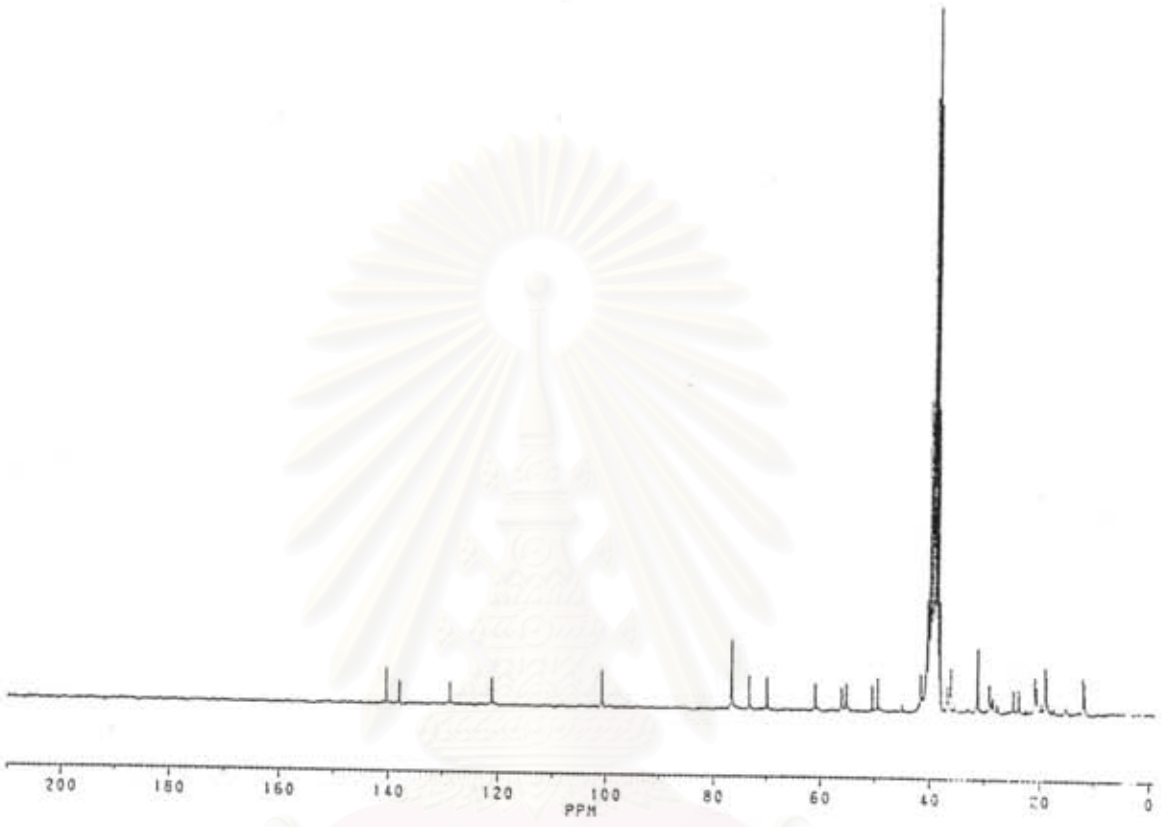


Figure 78 The ^{13}C NMR spectrum of Compound 8

3.8.10 Structure elucidation of compound 9.

Compound 9 , a white powder , was collected from the ethyl acetate crude extract and was crystalized from methanol. This compound was weighted on 37.54 mg. (2.89×10^{-3} % wt by wt of dried roots) Its melting point was 185-190 ° C . R_f value was 0.28 [dichloromethane : methanol : H_2O (13:7:2)].

The IR spectrum showed a broad band of a hydroxy group (-OH) at 3420 cm^{-1} , the C-O stretching vibrations of glycosidic linkage at 1080-1030 cm^{-1} and an anomeric axial C-H deformation of a β-sugar at 890 cm^{-1}. (Fig.79)

The mass spectrum did not give the molecular ion peak (M^+) because of the fragmentation of the molecule . The spectrum showed the dominant fragmentation ion peak at 416 , likely to be due to cleavage of the glycoside linkage. This peak would therefore be the aglycone, which should be sarsasapogenin. ($C_{27}H_{44}O_3$) (Fig.80)

The ^1H NMR spectrum indicated the presence of four methyl signals of a steroid at δ 0.65-2.50 ppm. A broadened doublet at δ 4.30 (1H , d , J = 8.2 Hz) ppm. was present for the anomeric protons of β-D-glucose. (Fig.81)

The ^{13}C NMR spectrum of Compound 9, showing 27 nonsaccharide carbons which was compared with that of sarsasapogenin to confirm the structure of aglycone. (Table 27) (Fig.82)

Moreover , The signals of methyl carbon at δ 18 ppm. and the broadened multiplet proton between δ 4.50-5.00 ppm. suggested that the other sugar was α-rhamnose.

Table 27 ^{13}C NMR spectrum of Compound 9 (selected signals) compared with sarsasapogenin [32].

position	chemical shift (δ ppm)	
	sarsasapogenin	compound 9
C-1	29.9	29.9
C-2	27.8	27.8
C-3	67.0	67.8
C-4	33.6	33.6
C-5	36.5	36.5
C-6	26.6	26.6
C-7	26.6	26.6
C-8	35.2	35.4
C-9	40.3	40.3
C-10	35.3	35.5
C-11	20.9	20.5
C-12	39.9	39.9
C-13	40.6	40.6
C-14	56.4	56.5
C-15	31.7	31.5
C-16	80.9	80.9
C-17	62.1	62.2
C-18	16.5	16.3
C-19	23.9	23.9
C-20	42.1	42.0

Table 27 (cont.)

position	chemical shift (δ ppm)	
	sarsasapogenin	compound 9
C-21	14.3	14.5
C-22	109.5	109.5
C-23	27.1	27.1
C-24	25.8	25.5
C-25	26.0	26.0
C-26	65.0	66.5
C-27	16.1	15.9

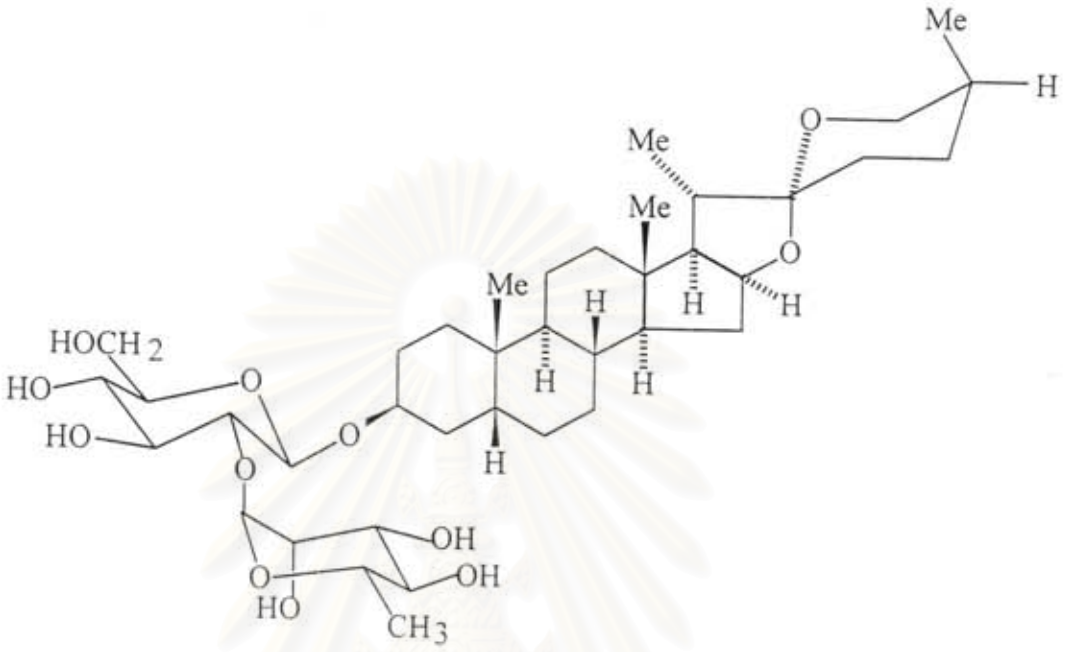
These results indicated that Compound 9 should be a sarsasapogenin glycoside with β-glucose and α-rhamnose in the molecule .The ^{13}C NMR spectrum showed signals between δ 65.5-77 ppm. signals which corresponded to the signals of these sugars. The signals in the ^{13}C NMR spectrum were compared with the spectra of rhamnose and glucose of 3-O-[α-L-rhamnopyranosyl -(1→ 2)-O-β-D- glucopyranosyl] diosgenin in literature [33].

Table 28 ^{13}CNMR spectrum of Compound 9 (sugar carbon region) compared with glucose and rhamnose [33].

position	chemical shift (δ ppm.)		
	glucose	rhamnose	Compound 9
G-1	100.4	-	100.0
G-2	79.7	-	79.0
G-3	78.0	-	77.5
G-4	71.9	-	71.5
G-5	77.9	-	77.0
G-6	62.7	-	62.5
R-1	-	102.1	103.5
R-2	-	72.7	72.0
R-3	-	72.9	72.5
R-4	-	73.8	74.0
R-5	-	69.6	69.5
R-6	-	18.9	18.0

The ^{13}C NMR indicated the signals of the two anomeric sugar carbons occurring at δ 100 and 103.5 ppm. Table 28 showed the Glucose G-2 resonance were shifted down field 5.6 ppm from the normal position of an unsubstituted G-2 shift. This result indicated that the rhamnose was attached to the G-2 position of the glucose . Thus , the terminal sugar was α-rhamnose.

The above results indicated that the possible structure of Compound 9 is 3-O-[α-L - rhamnopyranosyl -(1→ 2)-O-β-D- glucopyranosyl] sarsasapogenin .



3-O-[α-L-rhamnopyranosyl-(1→2)-O-β-D-glucopyranosyl] sarsasapogenin

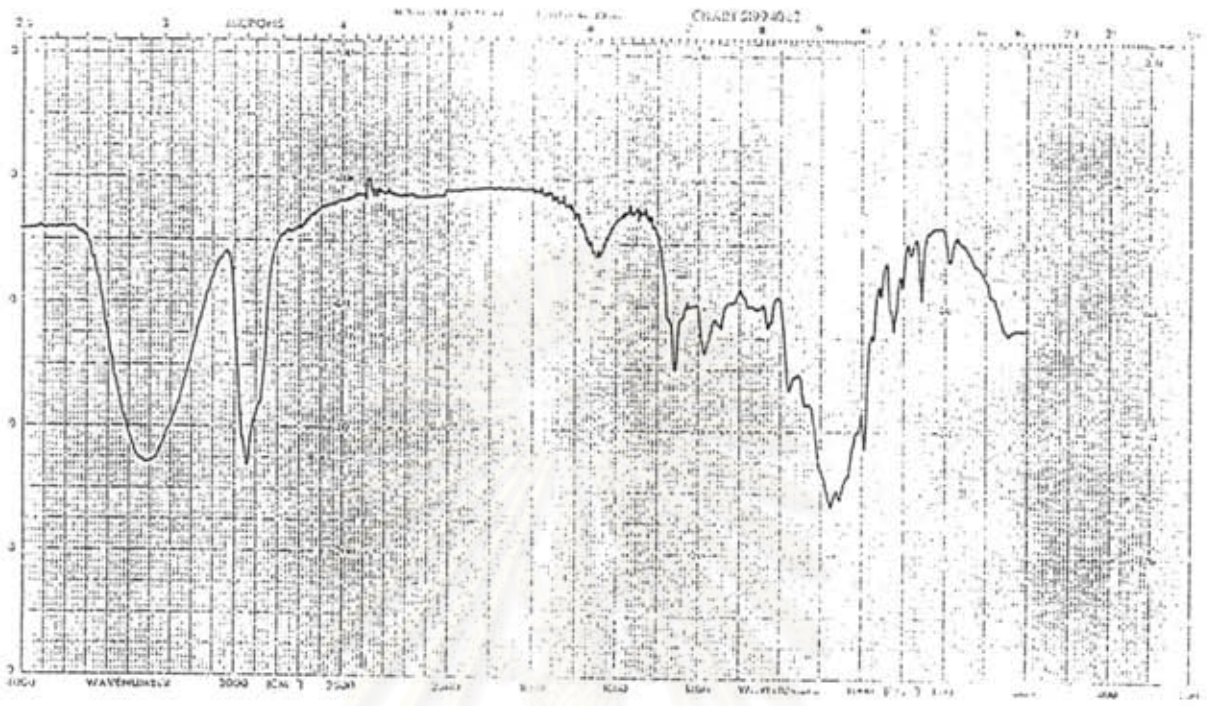


Figure 79 The IR spectrum of Compound 9

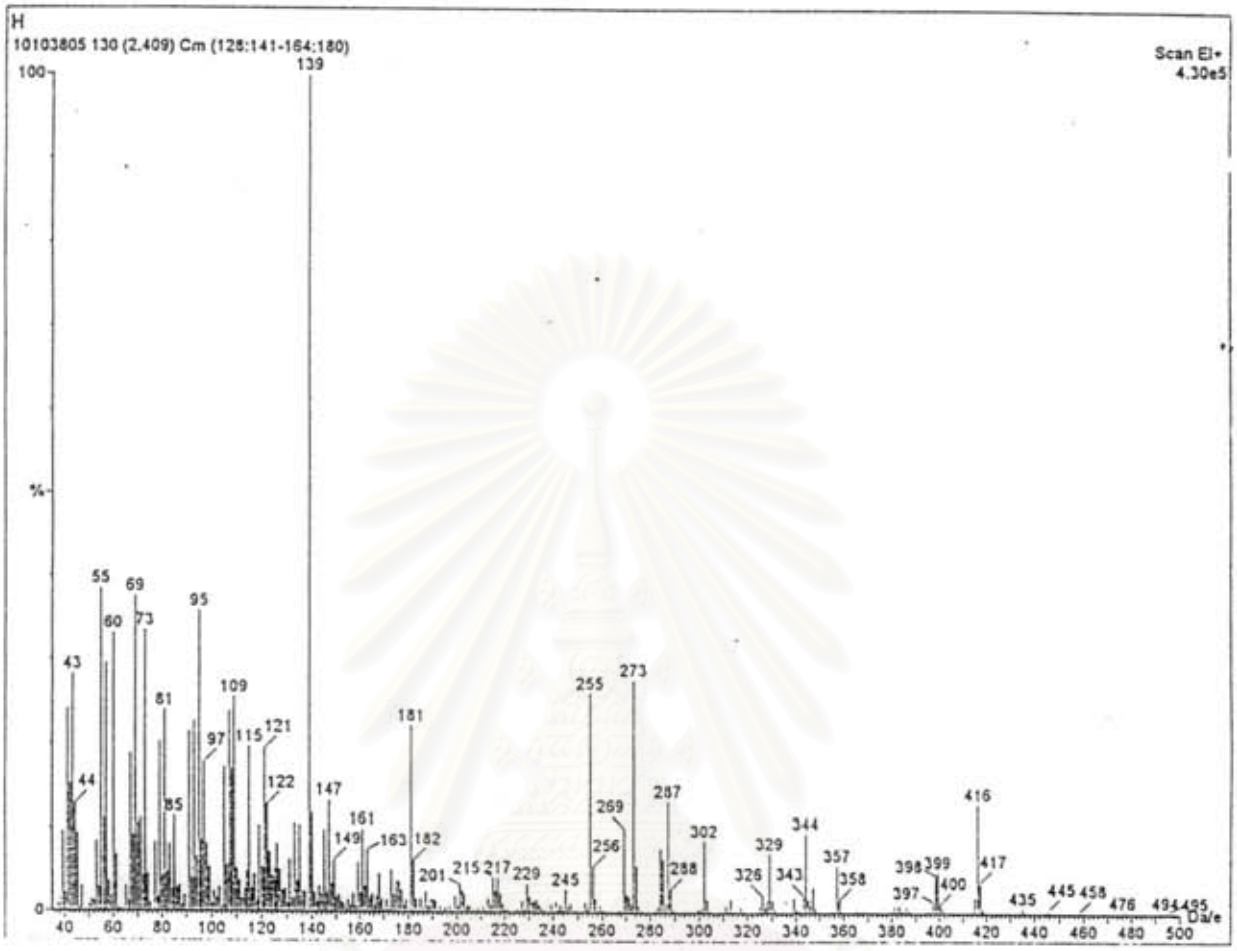


Figure 80 The mass spectrum of Compound 9

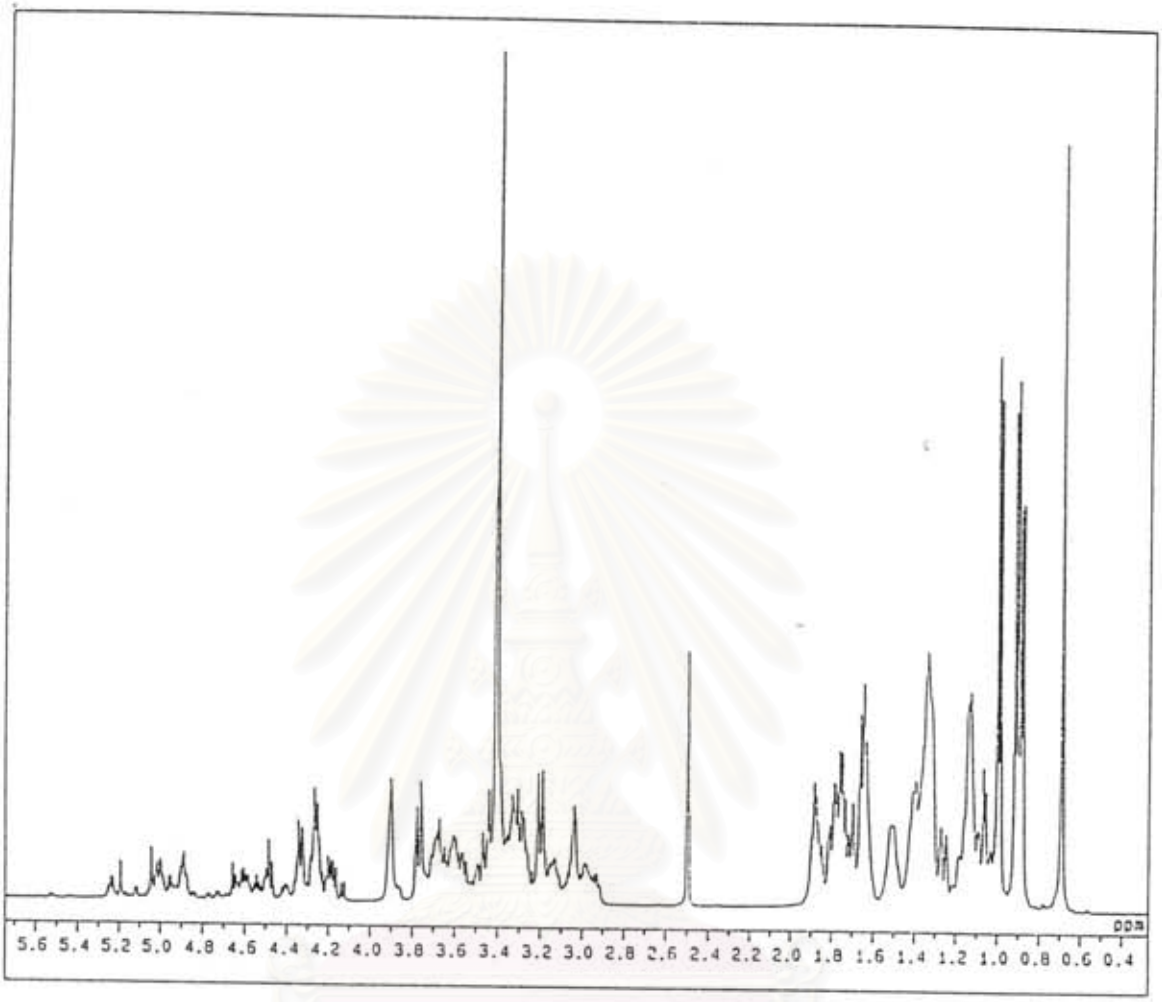


Figure 81 The ^{1}H - NMR Spectrum of Compound 9

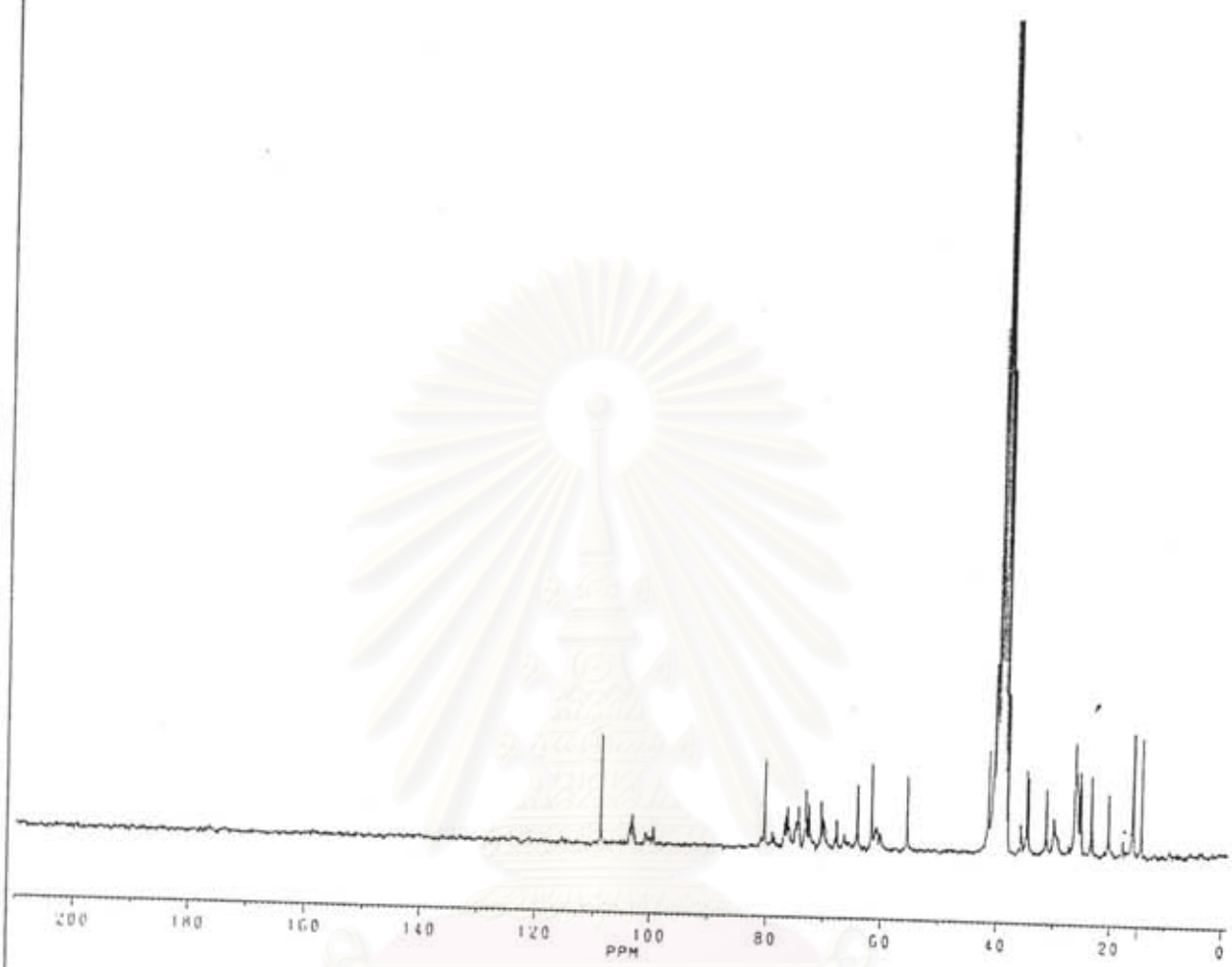


Figure 82 The ^{13}C - NMR Spectrum of Compound 9