CHAPTER 3
RESULTS AND DISCUSSION

3.1 Primary Bioassay Screening Results of Crude Extracts

3.1.1 A Microwell Cytotoxicity Assay using Brine Shrimp

In accordance with satisfied cytotoxic activity (LC_{50} 24.5 μg/ml) of methanolic extract against brine shrimp, the stems were extracted again with various solvents to afford 3 crude extracts. The cytotoxicity result of these crude extracts was showed in **Table 3.1**

Table 3.1 Brine Shrimp Cytotoxic Lethality Test of various crude extracts

Crude extracts	LC_{50} (μg/ml)	Bioactivity*
Dichloromethane	321.7	low activity
Ethyl acetate	56.2	medium activity
Methanol	418.8	low activity

*High activity (LC_{50} < 10 μg/ml)
Medium activity (LC_{50} < 100 μg/ml)
Low activity (LC_{50} < 1000 μg/ml)
No activity (LC_{50} > 1000 μg/ml)

3.1.2 Anticell Lines

The screening result of various crude extracts against cell lines was exhibited in **Table 3.2-3.6**

Table 3.2 The Result of Anticell lines of total (methanolic) crude extract various cell lines.

Cell lines	Concentration (μg/ml)	Inhibition (%)	Estimation
Bel-7402	1	-1.00	
	10	13.66	--
	100	42.19	
HCT-8	1	24.26	
	10	21.68	+
	100	79.57	
KB	1	49.39	
	10	50.15	++
	100	75.47	
BGC-823	1	24.48	
	10	29.08	+
	100	51.22	
HL-60	1	-24.26	
	10	17.93	--
	100	43.07	

Table 3.3 The Result of Anticell lines of Dichloromethane crude extract

Cell lines	Concentration (μg/ml)	Inhibition (%)	Estimation
Bel-7402	1	-6.25	
	10	0.05	+
	100	63.43	
HCT-8	1	20.71	
	10	16.45	+
	100	82.47	
KB	1	19.16	
	10	19.01	+
	100	76.57	
BGC-823	1	-4.28	
	10	7.55	--
	100	40.91	
HL-60	1	-11.98	
	10	6.04	+
	100	74.26	

Table 3.4 The Result of Anticell lines of Ethyl acetate crude extract

Cell lines	Concentration (μg/ml)	Inhibition (%)	Estimation
Bel-7402	1	-6.37	
	10	-1.20	--
	100	30.65	
HCT-8	1	15.10	
	10	19.07	+
	100	55.75	
KB	1	21.32	
	10	30.73	+
	100	52.80	
BGC-823	1	8.97	
	10	11.12	--
	100	24.79	
HL-60	1	-14.13	
	10	-2.53	+
	100	53.89	

Table 3.5 The Result of Anticell lines of Methanolic crude extract

Cell lines	Concentration (μg/ml)	Inhibition (%)	Estimation
Bel-7402	1	-4.13	
	10	1.54	--
	100	29.10	
HCT-8	1	22.45	
	10	16.36	--
	100	34.07	
KB	1	23.37	
	10	26.82	+
	100	62.31	
BGC-823	1	14.08	
	10	11.93	--
	100	21.42	
HL-60	1	36.06	
	10	49.09	+
	100	57.55	

Table 3.6 Summary the results of Anticell lines of various crude extracts

Crude extracts	Bel-7402	HCT-8	KB	BGC-823	HL-60
Total crude	--	+	++	+	--
Dichloromethane	+	+	+	--	+
Ethyl acetate	--	+	+	--	+
Methanol	--	--	+	--	+

Bel-7402 = Human Hepatocellular Carcinoma

HCT-8 = Human Colon Carcinoma

KB = Human Nasopharyngeal Carcinoma

BGC-823 = Human Gastric Carcinoma

HL-60 = Human Leukemia Carcinoma

3.1.3 Antioxidant Test

There were 2 methods used in antioxidant test. TLC autographic assay which were determined with DPPH radical or β-carotene (same result). The other one was O_2^- scavenging test in the XA/XOD system

Table 3.7 Antioxidant result from TLC autographic assay of various crude extracts.

Crude extracts	Antioxidant
Total crude	negative
Dichloromethane	negative
Ethyl acetate	positive
Methanol	negative

Table 3.8 Antioxidant result from O_2^{-1} scavenging test of various crude extracts.

Crude extracts	Dose (μg)	% NBT induction	% XOD inhibition	$\%O_2^{-1}$ Scavenging
Total crude	500	36.5	33.8	2.7
Dichloromethane	500	48.5	39.4	9.4
Ethyl acetate	500	84.2	48.9	35.3
Methanol	500	24.5	15.0	9.5

NBT = Nitrobluetetrazolium

XOD = Xanthine Oxidase

3.2 Properties and Structural Elucidation of Isolated Mixture and Compounds

3.2.1 Structural elucidation of Mixture A

The Mixture A, a white amorphous powder, was obtained from the dichloromethane crude extract which was fractionated by column chromatography over silica gel eluting with 1:1 CH_2Cl_2/hexane. It was purified several times by crystallization in a mixture of hexane and CH_2Cl_2, yielding 2.48×10^{-4} % (29 mg) of Mixture A (m.p. 81-83 °C).

The IR spectrum (**Figure 3.1**) indicated the characteristic absorption band of O-H stretching of hydroxyl group at 3600-3200 cm^{-1}. The absorption bands of C-H stretching of CH_3, CH_2 showed at 2900 and 2852 cm^{-1}. At 1470 and 1465 cm^{-1} were assigned C-H asymmetric bending of CH_3, CH_2. The absorption band at 1060 cm^{-1} was signal of C-O stretching of 1° ROH and $-(CH_2)_n-$ (730 and 720 cm^{-1}) were also observed. It could be determined that the white powder should be a mixture of saturated long chain aliphatic alcohols.

$$\mathbf{CH_3 - (CH_2)_n - CH_2OH}$$

Mixture of long chain aliphatic alcohols : Mixture **A**

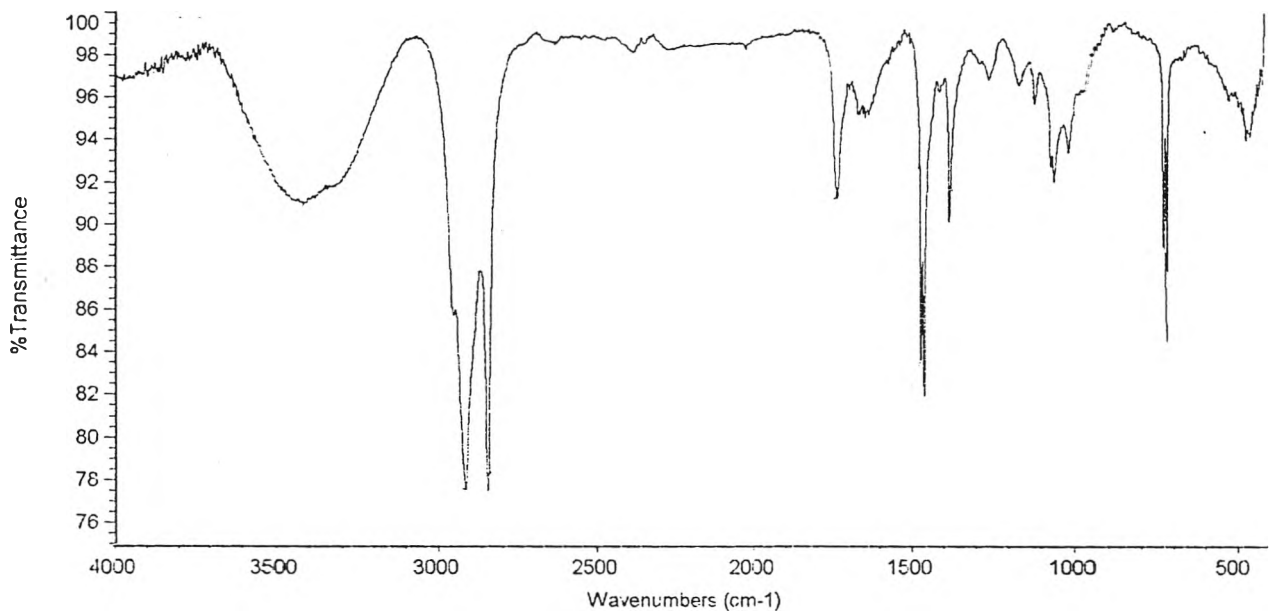


Figure 3.1 The IR spectrum of Mixture **A**

3.2.2 Structural elucidation of Mixture B

Almost 52 mg (yield 4.44×10^{-4} %) of the white powder (melting point 72-75 °C) was obtained from the dichloromethane crude extract which was eluted with 2:1 CH_2Cl_2/hexane from column chromatography. This product was purified several times by crystallization in hexane and CH_2Cl_2.

Table 3.9 The IR absorption band assignments of Mixture **B**

Wave number (cm^{-1})	Intensity	Tentative assignment
3500-2500	strong	O-H stretching vibration of R-COOH
2900, 2850	strong	C-H stretching vibration of $-CH_3$, $-CH_2-$
1700	moderate	C=O stretching vibration of R-COOH
1465	moderate	C-H bending vibration of $-CH_3$, $-CH_2-$
1430, 1300	weak	C-O stretching vibration and O-H bending vibration of R-COOH
920	weak	O-H stretching vibration out of plane
730,720	moderate	C-H rocking mode of $-(CH_2)_n-$

From IR spectrum (**Figure 3.2**), there are absorption bands of carboxylic group displayed at 3500-2500, 1700, 1430,1300 and 920 cm^{-1} and methylene group $-(CH_2)_n-$ indicated at 730 and 720 cm^{-1}

Owing to slightly low melting point and characteristic absorption bands of carboxylic acid and straight chain hydrocarbon, Mixture **B** was tentatively ascribable to a mixture of long chain carboxylic acids.

$$CH_3-(CH_2)_n-CH_2COOH$$

Mixture of long chain carboxylic acids: Mixture **B**

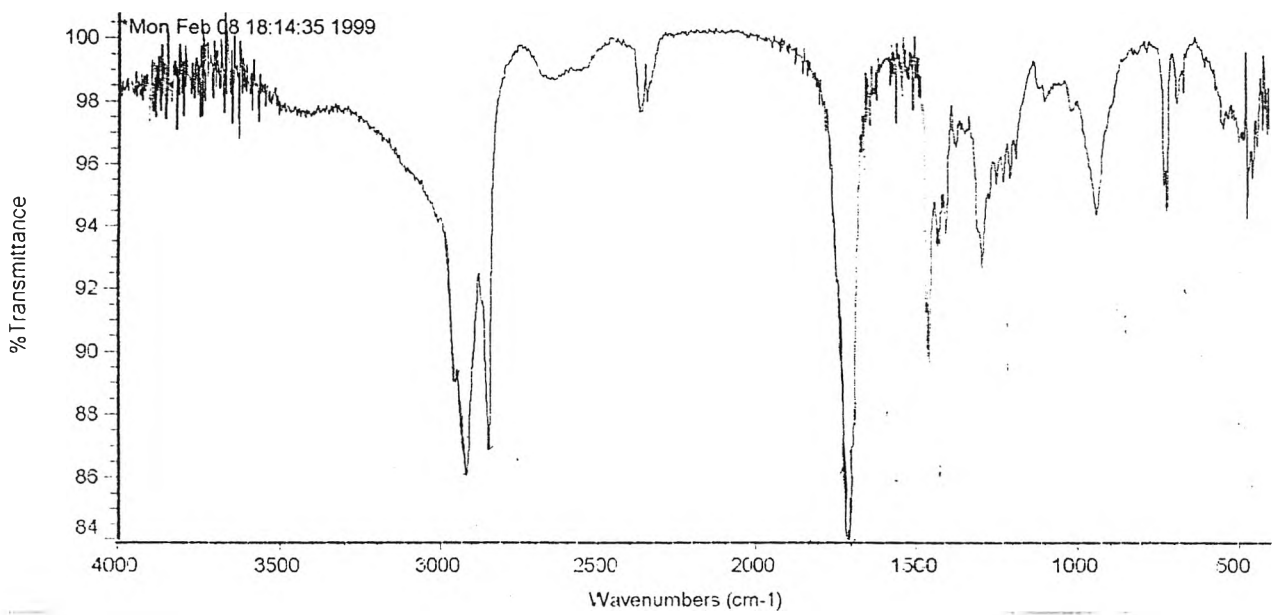
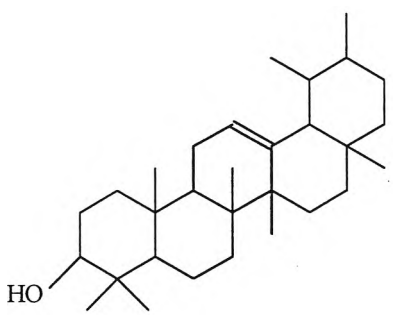


Figure 3.2 The IR spectrum of Mixture **B**

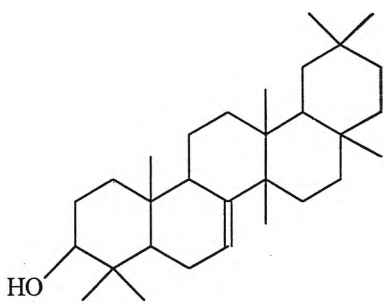
3.2.3 Structural elucidation of Mixture C

The pale yellow wax obtained from dichloromethane crude extract by eluting with 7:3 CH_2Cl_2/hexane was recrystalized in 1:7 CH_2Cl_2/hexane to afford 5.2 g (yield 4.44×10^{-2} %) of white needle. Its broad range of melting point, 200-205 °C, could be indicated that it was a mixture. The IR spectrum (**Figure 3.4**) showed strong absorption bands at 2930, 1456 cm^{-1} (-CH_3, -CH_2-) and the presence of hydroxyl group at 3600-3200 cm^{-1}

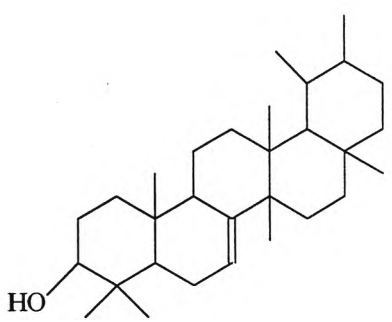
The GC-MS (**Figure 3.5**) gave three peaks with different retention times. Although each of which revealed the same molecular ion peak at m/z 426 in the mass spectrum (**Figure 3.5a, 3.5b, 3.5c**), their fragmentation patterns were different to each other. So the Mixture **C** consist of 3 compounds (**Figure 3.3**) which were elucidated as pentacyclic triterpenoid [earlier reported from bark of *G.multiflorum* (India)][23] , α-Amyrin, Multiflorenol and Bauerenol. The mass fragmentation patterns[39,40,41] were presented in **Scheme 3.1 - 3.3**.



α-Amyrin



Multiflorenol



Bauerenol

Figure 3.3 Structure of Three Compounds in Mixture **C**

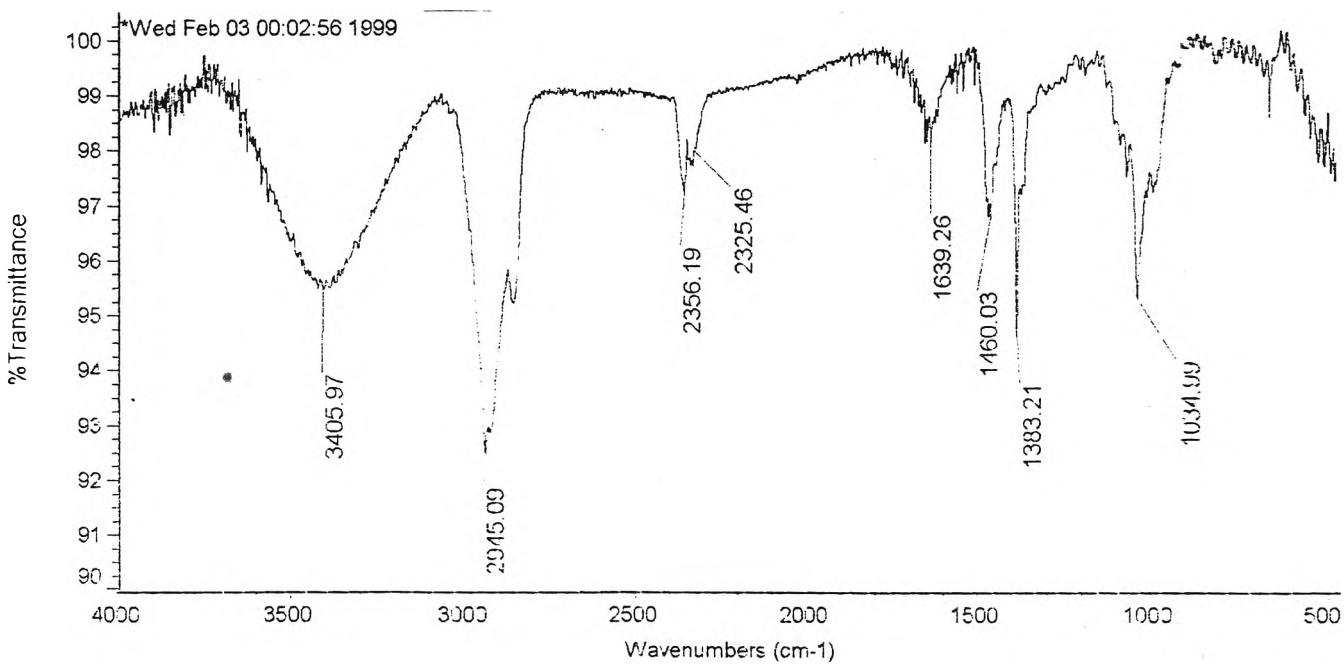


Figure 3.4 The IR spectrum of Mixture **C**

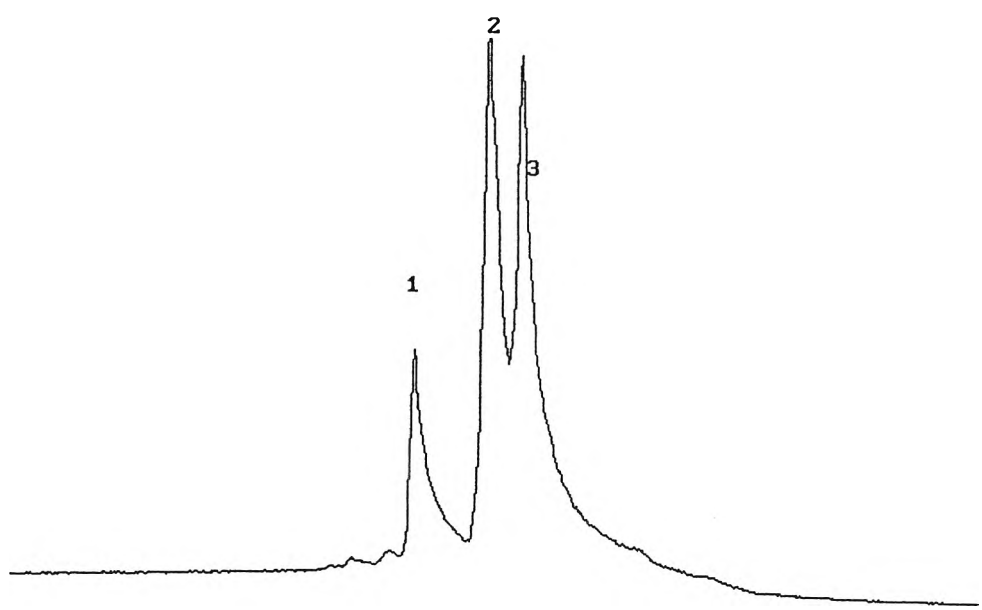


Figure 3.5 The GC chromatograms of Mixture **C**

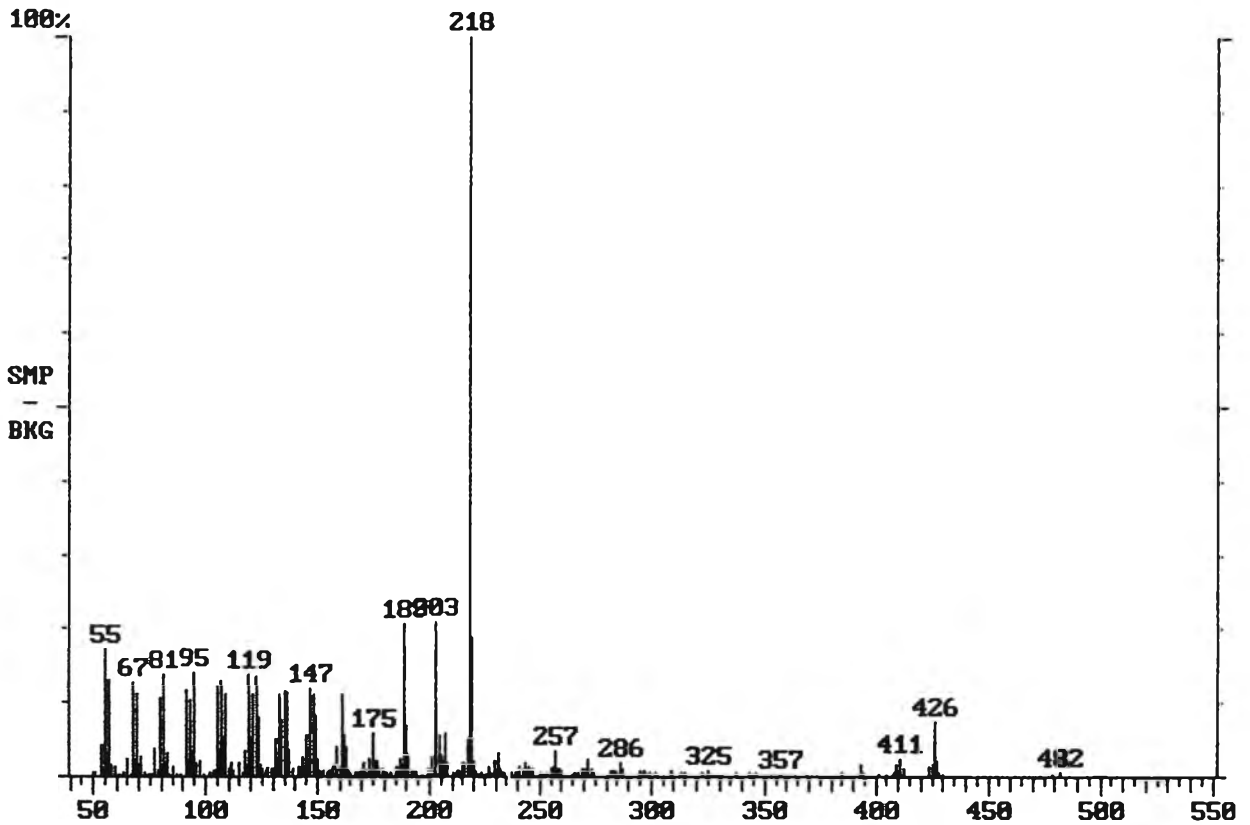
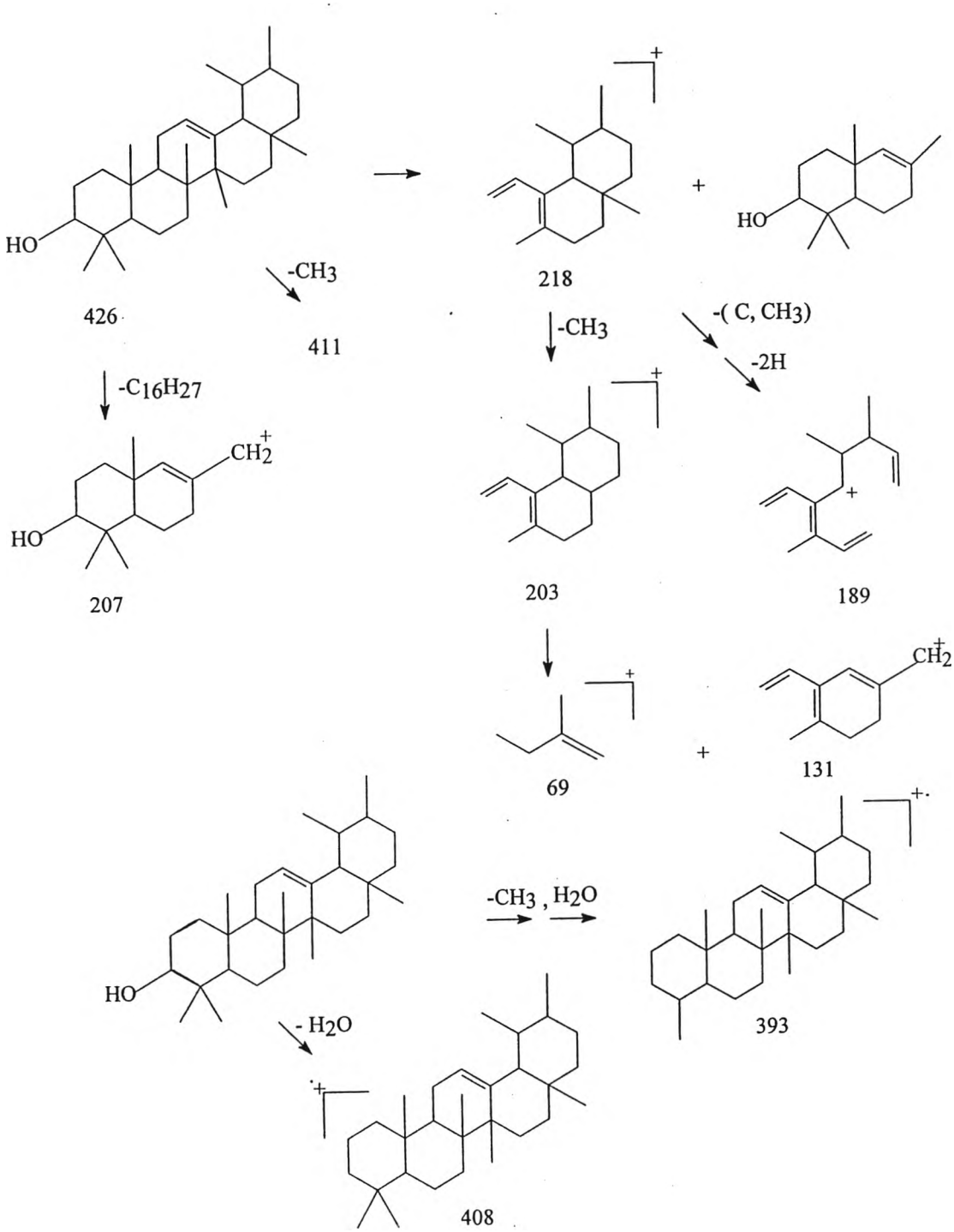


Figure 3.5a The Mass spectrum of Mixture **C-1** : α-Amyrin



Scheme 3.1 The Mass Fragementation pattern of α-Amyrin[39]

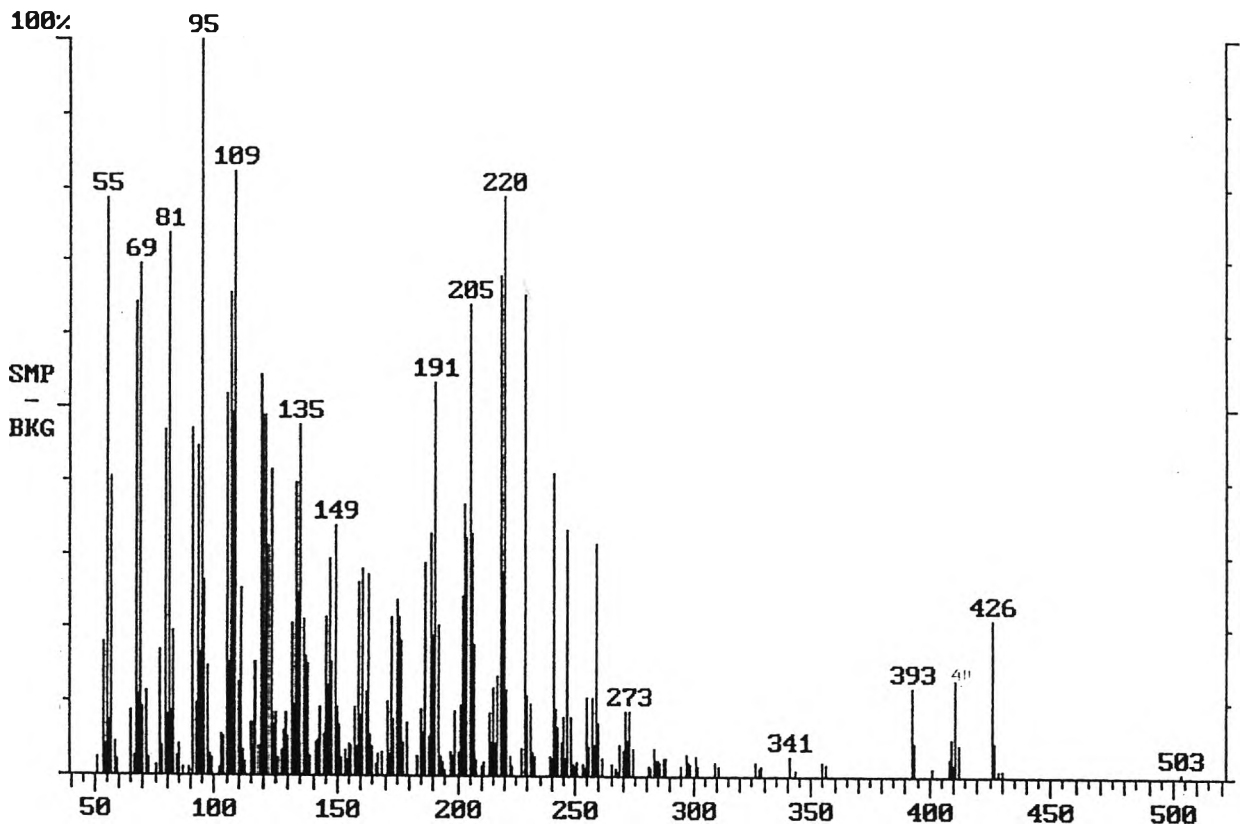
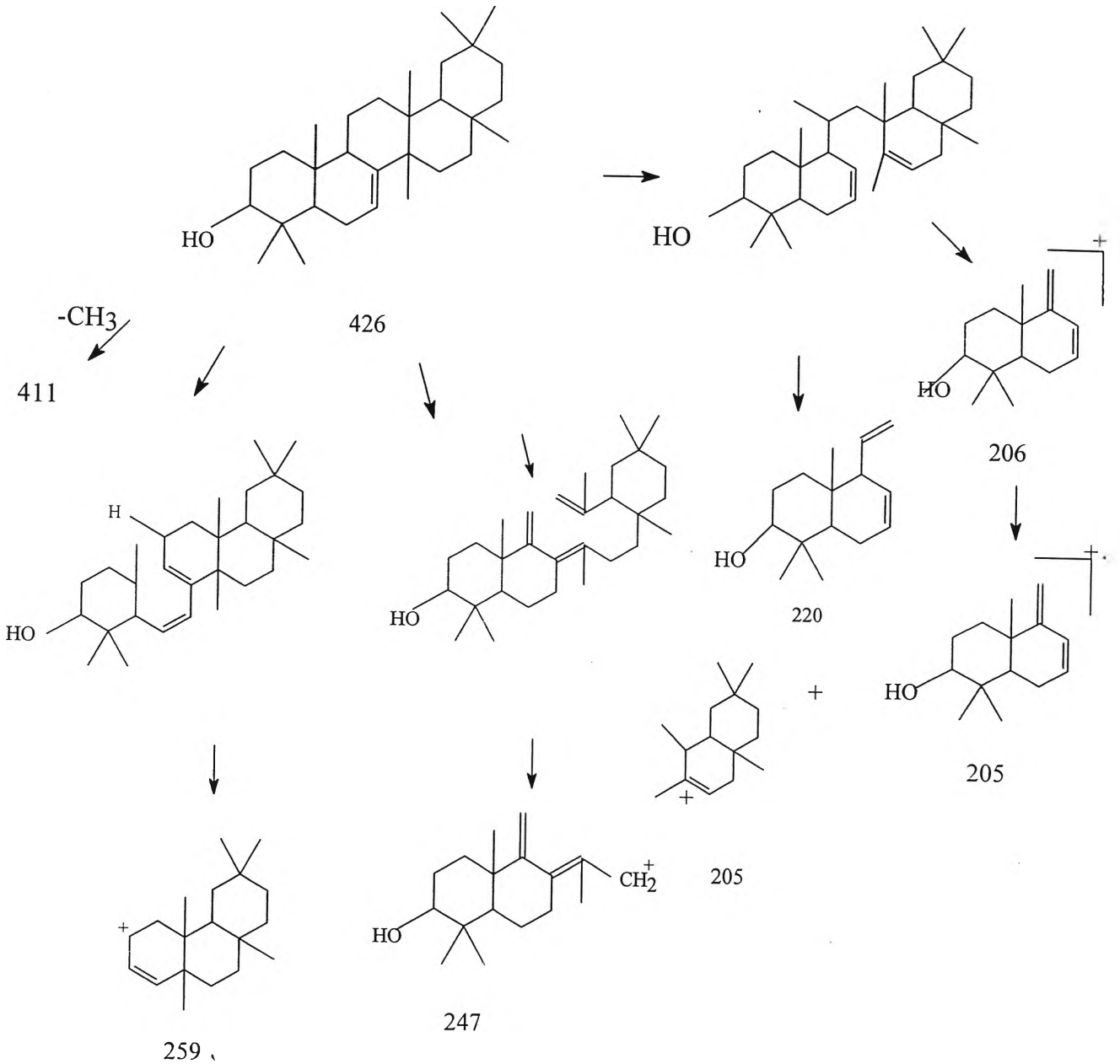


Figure 3.5b The Mass spectrum of Mixture **C-2** : Multiflorenol



Scheme 3.2 The Mass Fragementation pattern of Multiflorenol [40,41]

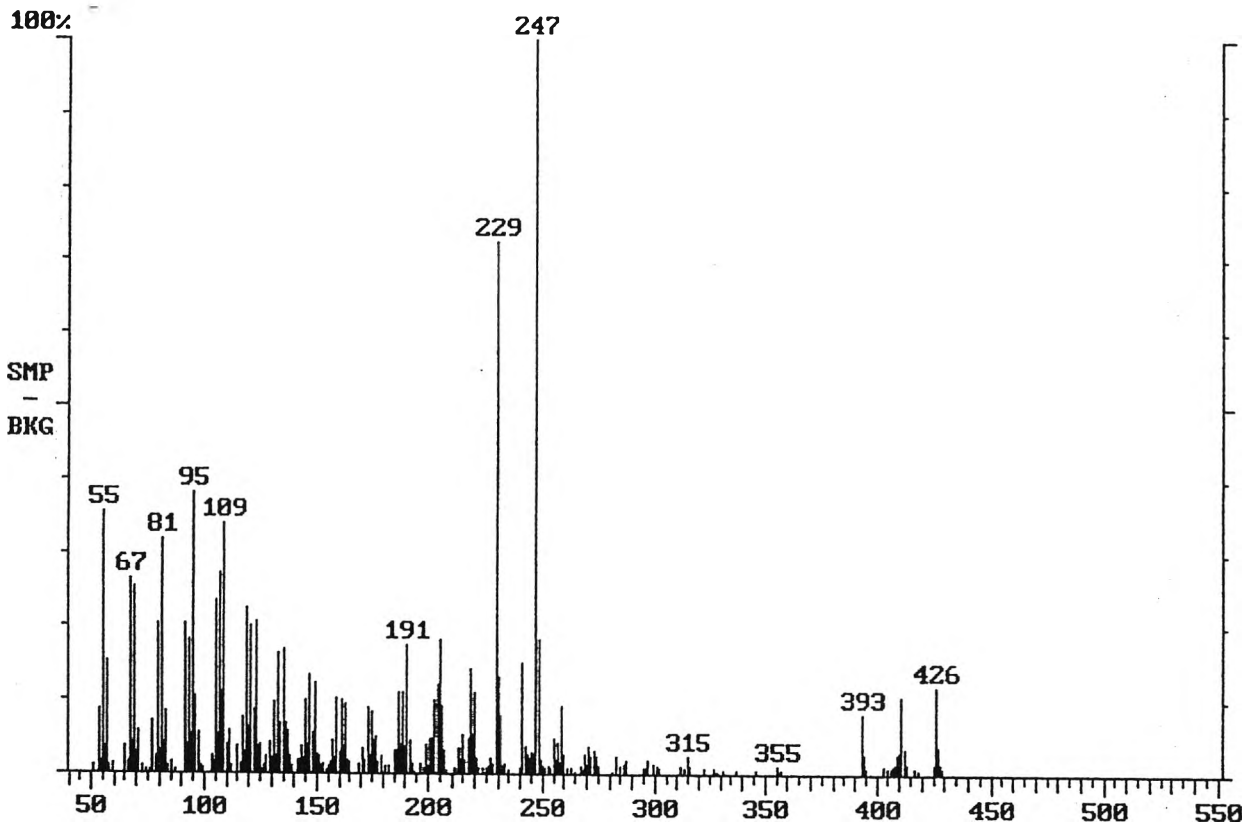
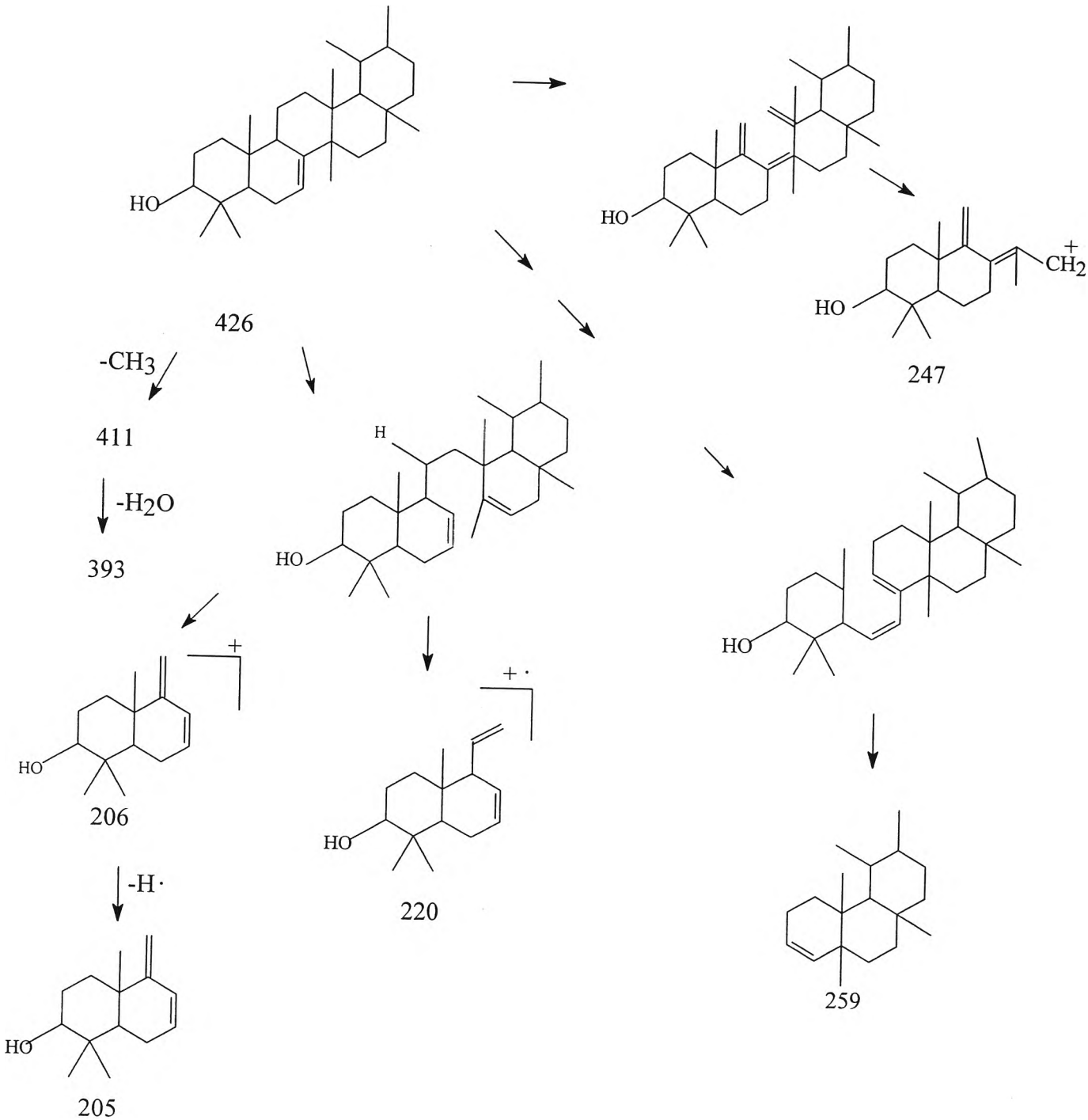


Figure 3.5c The Mass spectrum of Mixture **C-3** : Bauerenol



Scheme 3.3 The Mass Fragementation pattern of Bauerenol [42]

3.2.4 Structural elucidation of Mixture D

Mixture **D** was obtained as a yellow oil (yield 7.74×10^{-4} %). Its IR spectrum, **Figure.3.6**, indicated the presence of one or more ester groups (C=O stretching vibration band at 1743 cm^{-1} and C-O stretching vibration band of ester group at 1170 cm^{-1}). The C-H stretching vibration bands of an aliphatic compound was observed at 2926 and 2855 cm^{-1}. The absorption band at 1363 cm^{-1} corresponded to C-H symmetric bending vibration mode of CH_3 group. In addition, the absorption band at 724 cm^{-1} might possibly be indicated the presence of one or more saturated long chain of $(-CH_2-)_n$.

Table 3.10 The IR absorption band assignments of Mixture **D.**

Wave number (cm^{-1})	Intensity	Tentative assignment
2926,2855	strong	C-H stretching vibration of CH_3-, -CH_2-
1743	strong	C=O stretching vibration of ester
1462	moderate	C-H bending vibration of –CH_2- , CH_3-
1435,1363	moderate	C-H symmetric bending vibration of CH_3-
1172	moderate	C-O stretching vibration of ester
724	moderate	C-H rocking mode of –CH_2-

The spectral evidences above indicate that Mixture **D** is composed of a mixture of long chain esters.

$$CH_3\text{-}(CH_2)_n\text{-}CH_2COOR$$

Mixture of long chain esters: Mixture **D**

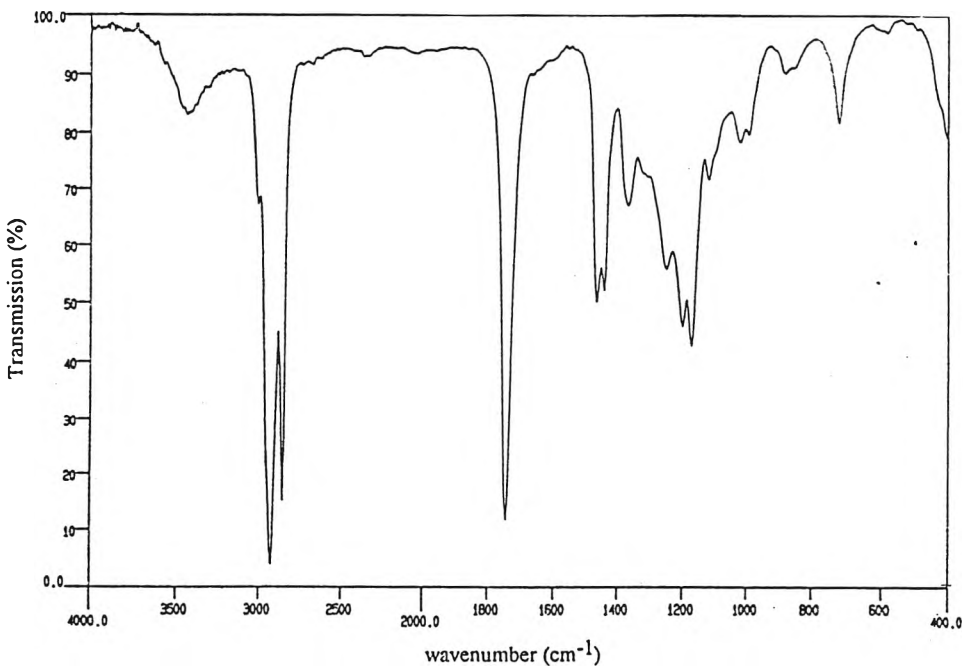


Figure 3.6 The IR spectrum of Mixture **D**

3.2.5 Structural elucidation of Compound 1

Compound **1** was bright white needle crystals in a yellow oil which was separated by column chromatography of dichloromethane extract. This compound was recrystallized by a mixture of dichloromethane and hexane several times to obtain approximately 0.85 g (yield 7.26×10^{-3} %). Its melting point was 163-164°C and R_f value was 0.65 (3:2 hexane – chloroform, silica gel).

The IR spectrum and its absorption bands assignment were shown in **Figure 3.7** and **Table 3.11**.

Table 3.11 The IR absorption band assignment of Compound **1**

Wave number (cm^{-1})	Intensity	Tentative assignment
3650-3300	strong	O-H stretching vibration of R-OH
2937,2866	strong	C-H stretching vibration of CH_3- ,-CH_2-
1641	weak	C=C stretching vibration of alkene
1460	strong	C-H bending vibration of CH_3- ,-CH_2-
1381,1370	moderate	C-H symmetric bending vibration of CH_3-
1157	moderate	C-O stretching vibration and O-H bending vibration of R-OH
970	weak	C-H out of plane bending vibration of trans configuration

From IR spectrum, there were absorption bands of alcoholic functional group at 3712-3146 cm^{-1} and unsaturation at 1641, 970 and 810 cm^{-1}

When Compound **1** was analyzed by GLC technique and compared its chromatogram with the mixture of three standard steroids (**Figure 3.8**) such as campesterol , stigmasterol and β-sitosterol. The retention time of this chromatogram (**Figure 3.9**) indicated that Compound **1** was stigmasterol. Retention time of Compound **1** and three standard steroids are shown in **Table 3.12.**

Table 3.12 Retention times of Compound **1** and standard steroids.

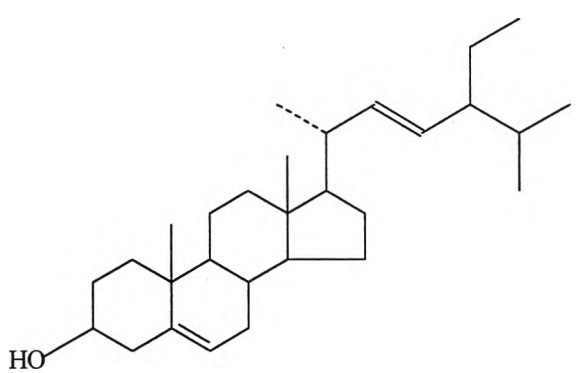
Steroid	R_t of standard steroid	R_t of Compound **1**
Campesterol	18.57	-
Stigmasterol	19.73	19.42
β-sitosterol	22.03	-

The ^{1}H NMR spectrum of Compound **1** showed signals of -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 5.01 and 5.22.(**Figure 3.10**)

The ^{13}C NMR spectrum (**Figure 3.11**) of Compound **1** appeared 29 peaks signal of carbon in CH_3- , CH_2- , CH- , C at 12.04-56.86 ppm exhibited steroid skeleton. The signal of carbon at 71.80 revealed carbon linked to hydroxy group. The signal of carbon in CH=C , CH=C , -CH=CH- and –CH=CH- patterns were indicated at 121.6 ,140.6 ,129.2 , and 138.2 ppm ,respectively. When Compound **1** was analyzed by DEPT 135 and 90 spectra showed that this compound contained 11 tertiary carbons , 8 methylene carbons , 6 methyl carbons and 4 quaternary carbons. (**Figure 3.12**).

The carbon chemical shifts of Compound **1** was compared with an authentic of stigmasterol [43,44] to confirm its structure. (**Table 3.13**)

All of these results indicated that Compound **1** was stigmasterol.



stigmasterol

Table 3.13 The Carbon chemical shifts of Stigmasterol and Compound **1**

Position	Stigmasterol	Compound 1
1	37.4	37.2
2	31.7	31.5
3	71.8	71.7
4	42.4	42.2
5	140.0	140.6
6	121.7	121.6
7	31.9	31.8
8	31.9	31.8
9	50.3	50.0
10	36.6	36.4
11	21.1	21.1
12	39.8	39.7
13	42.4	42.2
14	57.0	56.7
15	24.4	24.2
16	28.9	29.0
17	56.0	56.0
18	12.2	12.2
19	19.4	19.3
20	40.5	40.4
21	21.1	21.1
22	138.4	138.2
23	129.4	129.2
24	51.3	51.1
25	31.9	31.8
26	19.0	19.0
27	21.1	21.1
28	25.4	25.3
29	12.0	11.9

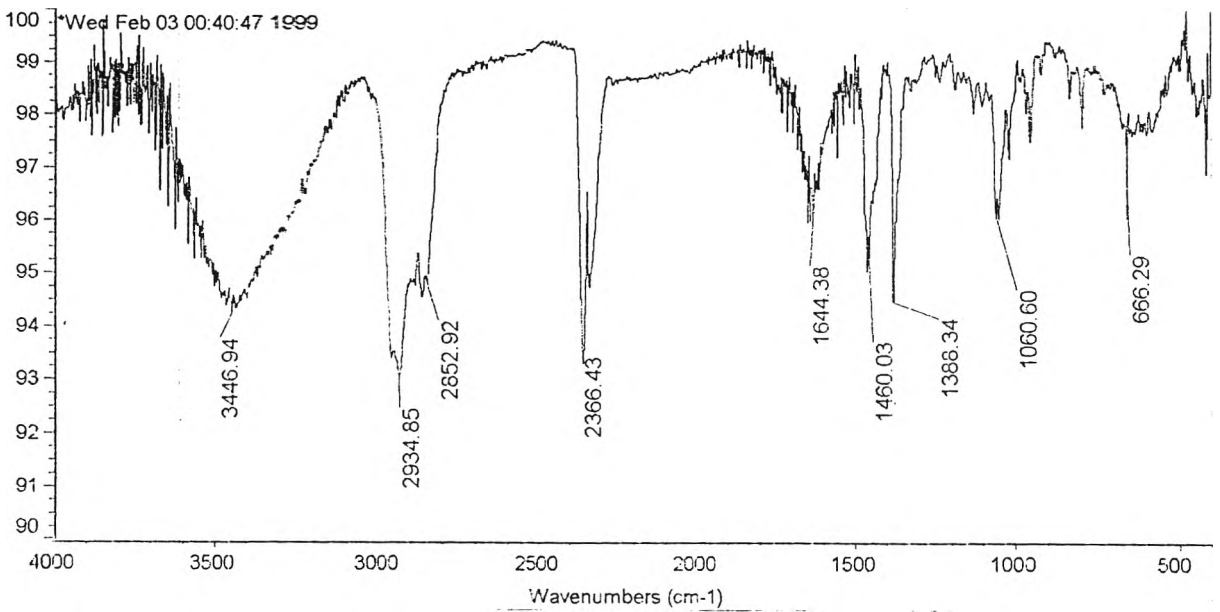


Figure 3.7 The IR spectrum of Compound **1**

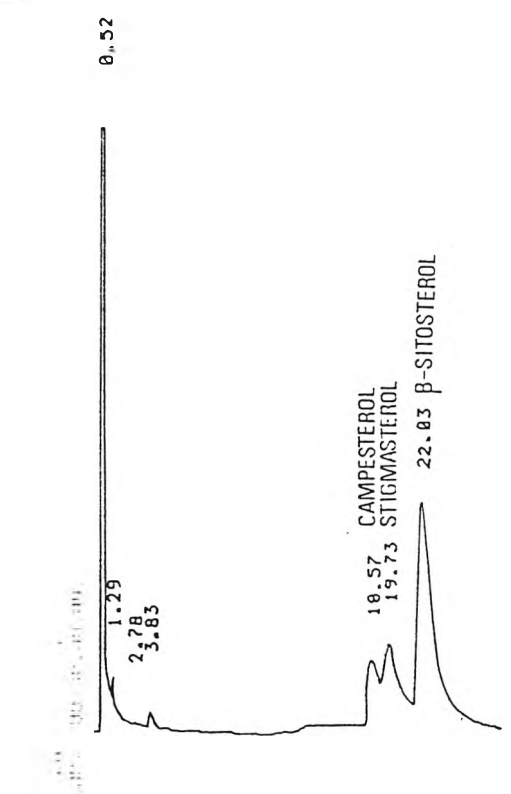


Figure 3. 8 The GC chromatograms of three standard steroids

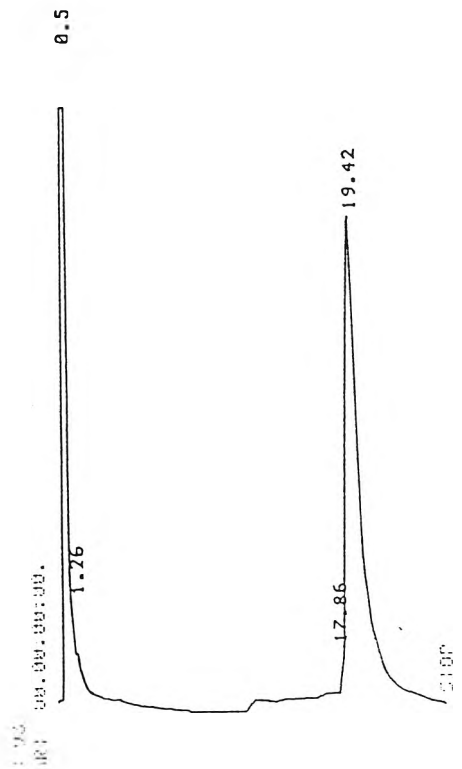


Figure 3.9 The GC chromatograms of Compound **1**

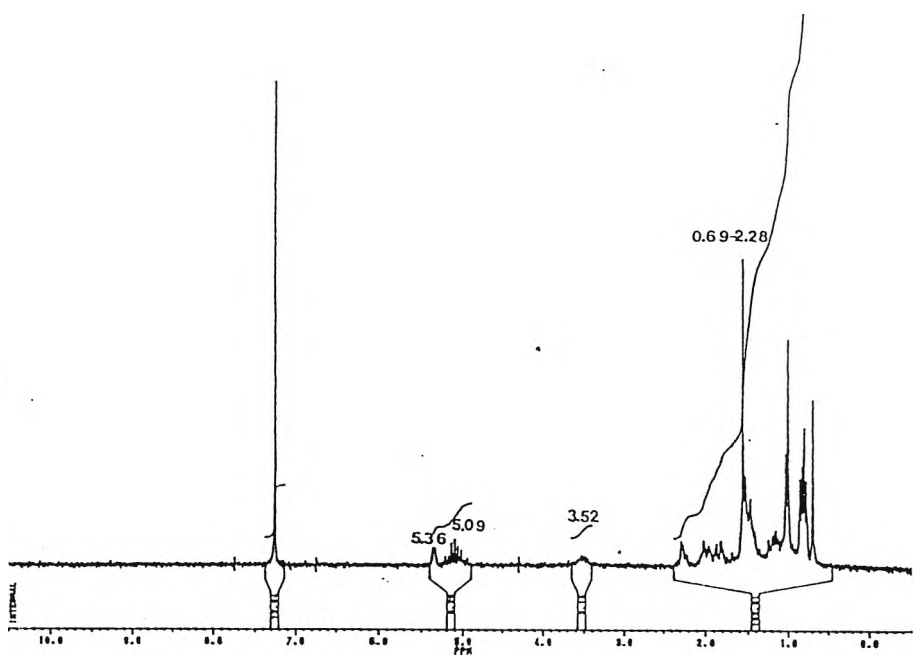


Figure 3.10 The ^{1}H NMR spectrum of Compound **1**

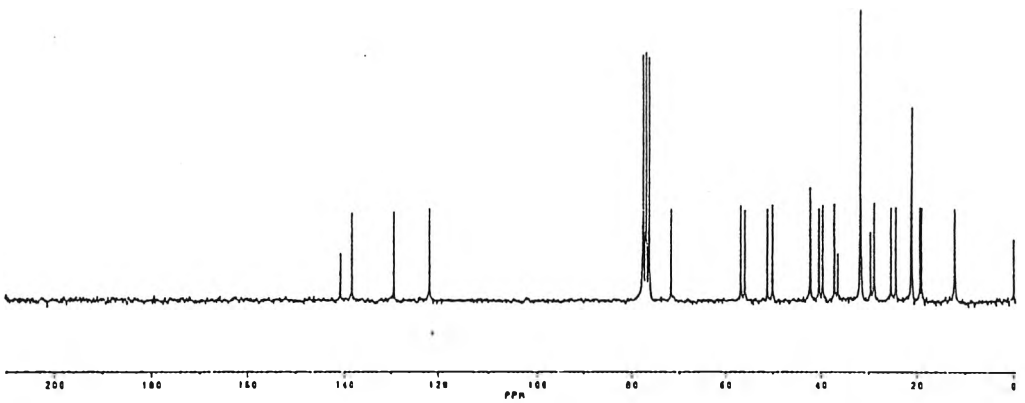


Figure 3.11 The ^{13}C NMR spectrum of Compound **1**

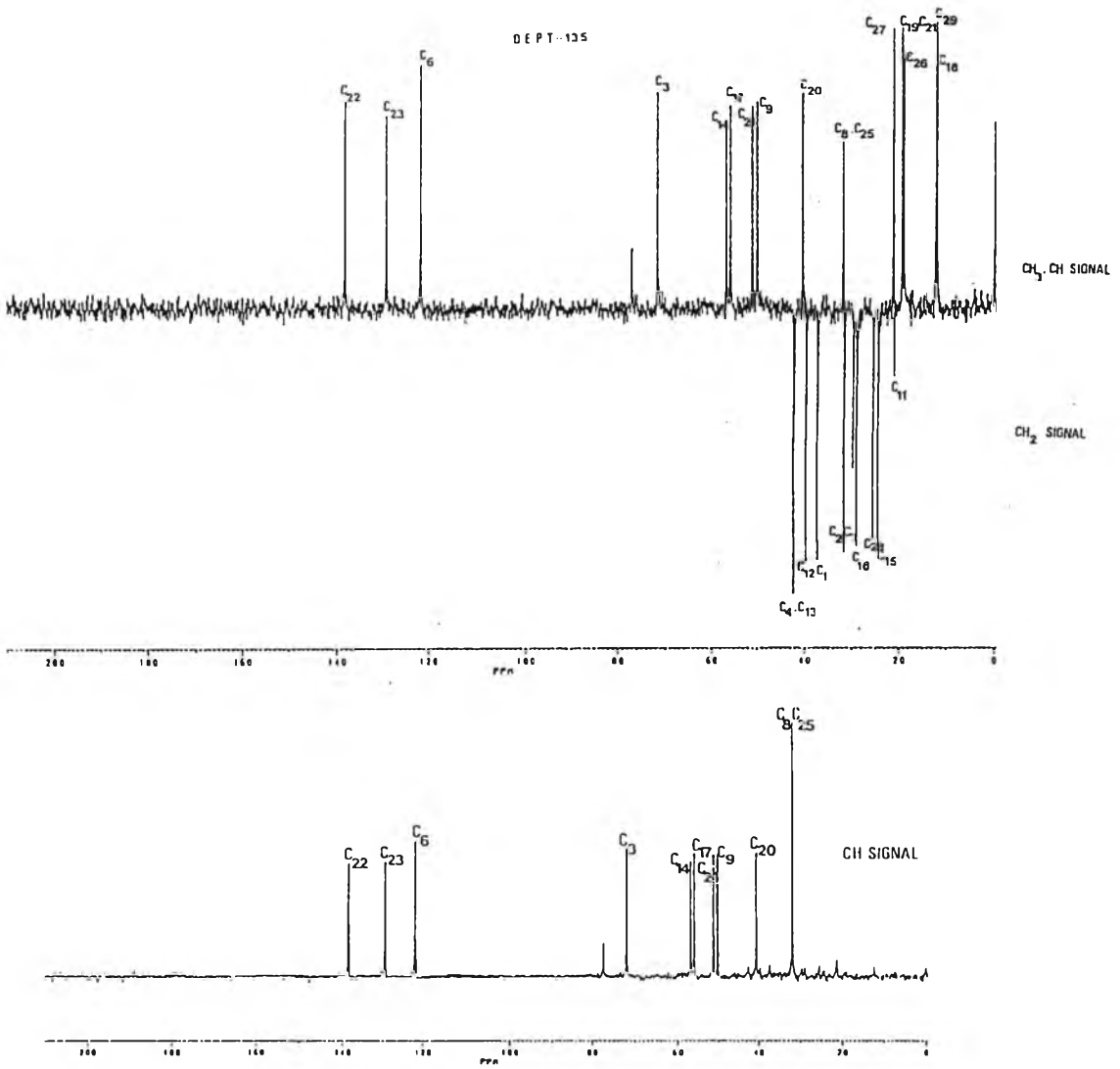


Figure 3.12 The DEPT 90 and 135 spectra of Compound **1**

3.2.6 Structural elucidation of Compound 2

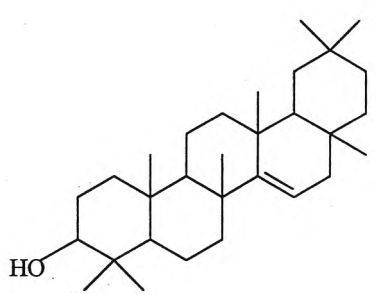
Compound **2** (weight 12.4 mg, yield 1.06 × 10^{-4} %) was isolated from dichloromethane crude extract by eluting with 25% CH_2Cl_2 in hexane, a yellow powder was formed. This Compound was tested with Liebermann-Burchard reagent and showed a red color, which is the characteristic of the presence of a triterpenoid structure.

The Mass spectrum (**Figure 3.13**) of Compound **2** showed a molecular ion peak at m/z 409 $[M\text{-}OH]^+$.

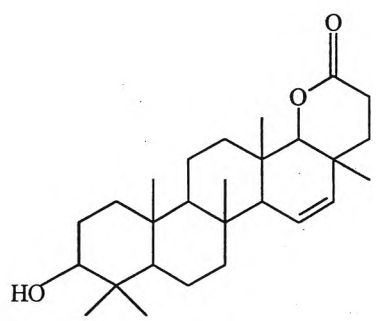
The ^1H NMR spectrum (**Figure 3.14**) displayed signals at 0.75 to 1.75 (36H, m) indicated the presence of methyl group, methylene and methine protons of triterpene skeleton.

The ^{13}C NMR spectrum showed carbon signals which looked closely resemble to those of certain triterpenoid structure, the spectra of ^{13}C NMR and DEPT 90, 135 of Compound **2** (**Figure 3.15** and **3.16**). The assignments of main structure were established by comparison of carbon chemical shifte of taraxerol (pentacyclic triterpenoid), **Table 3.14**. However, Compound **2** had one carbon connected to oxygen at δ 75.3 ppm, one carbonyl group at δ 173.6 ppm which indicated characteristic of lactone ring[46] . The other carbon connected to oxygen of hydroxy group at 80.6 and two olefinic tertiary carbons at 128.9, 131.6 ppm. This compound needed more spectroscopic data for further structure elucidation.

Thus, the possible structure of Compound **2** should be a pentacyclic triterpenoid.



Taraxerol[53]



Possible structure of Compound **2**

Table 3.14 The Carbon chemical shifts of Taraxerol[53] and Compound **2**

Position of Carbon	Chemical shift (ppm)	
	Taraxerol	Compound **2**
1	38.7	38.8
2	27.3	28.4
3	79.1	80.6
4	38.8	38.8
5	55.6	55.6
6	18.9	18.4
7	33.2	32.6
8	39.1	40.1
9	49.4	49.3
10	37.6	37.7
11	17.6	17.4
12	36.8	36.8
13	37.8	37.8
14	158.2	49.3
15	117.0	128.9
16	33.8	131.6
17	35.8	35.6
18	48.9	47.9
19	41.4	173.6
20	28.8	34.9
21	35.2	37.8
22	37.8	27.9
23	28.0	16.0
24	15.5	16.1
25	15.5	26.5
26	26.0	29.6
27	29.7	29.7
28	29.9	-
29	33.4	-
30	21.4	-

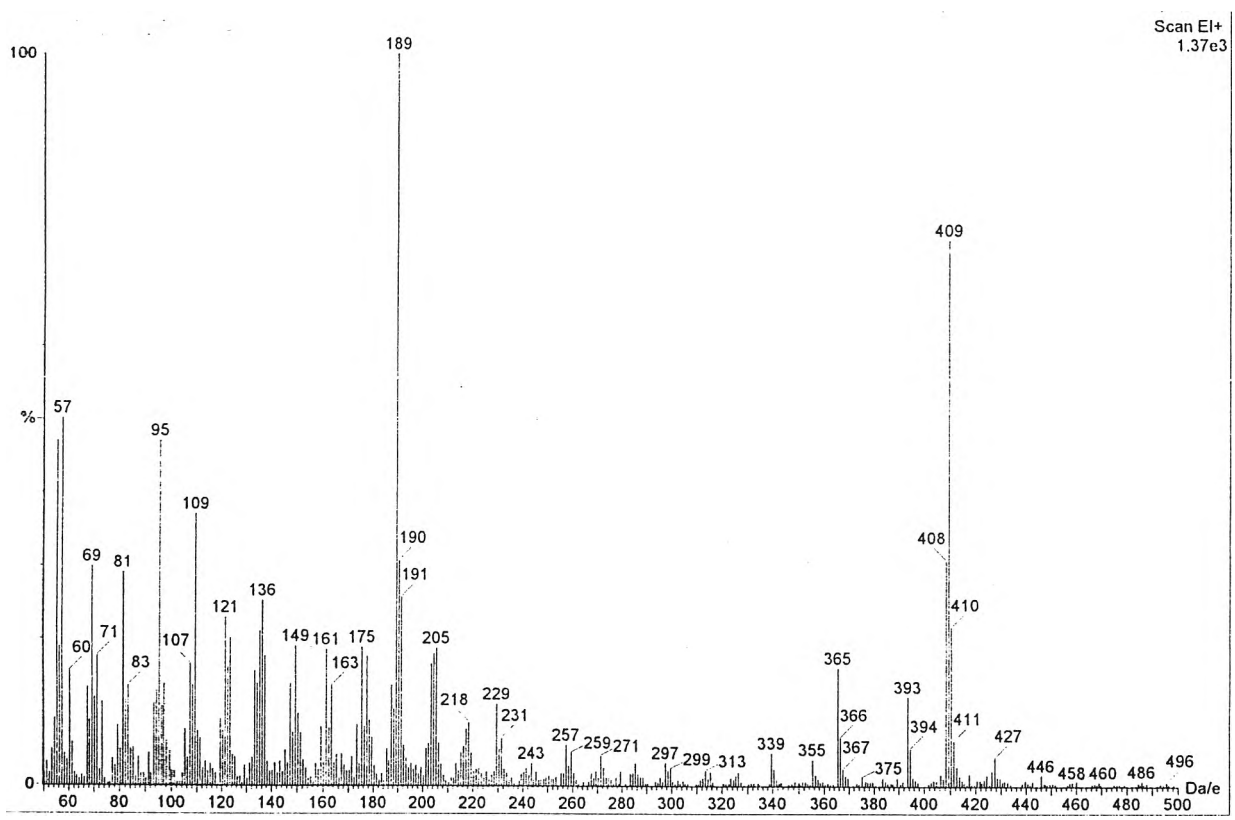


Figure 3.13 The Mass spectrum of Compound **2**

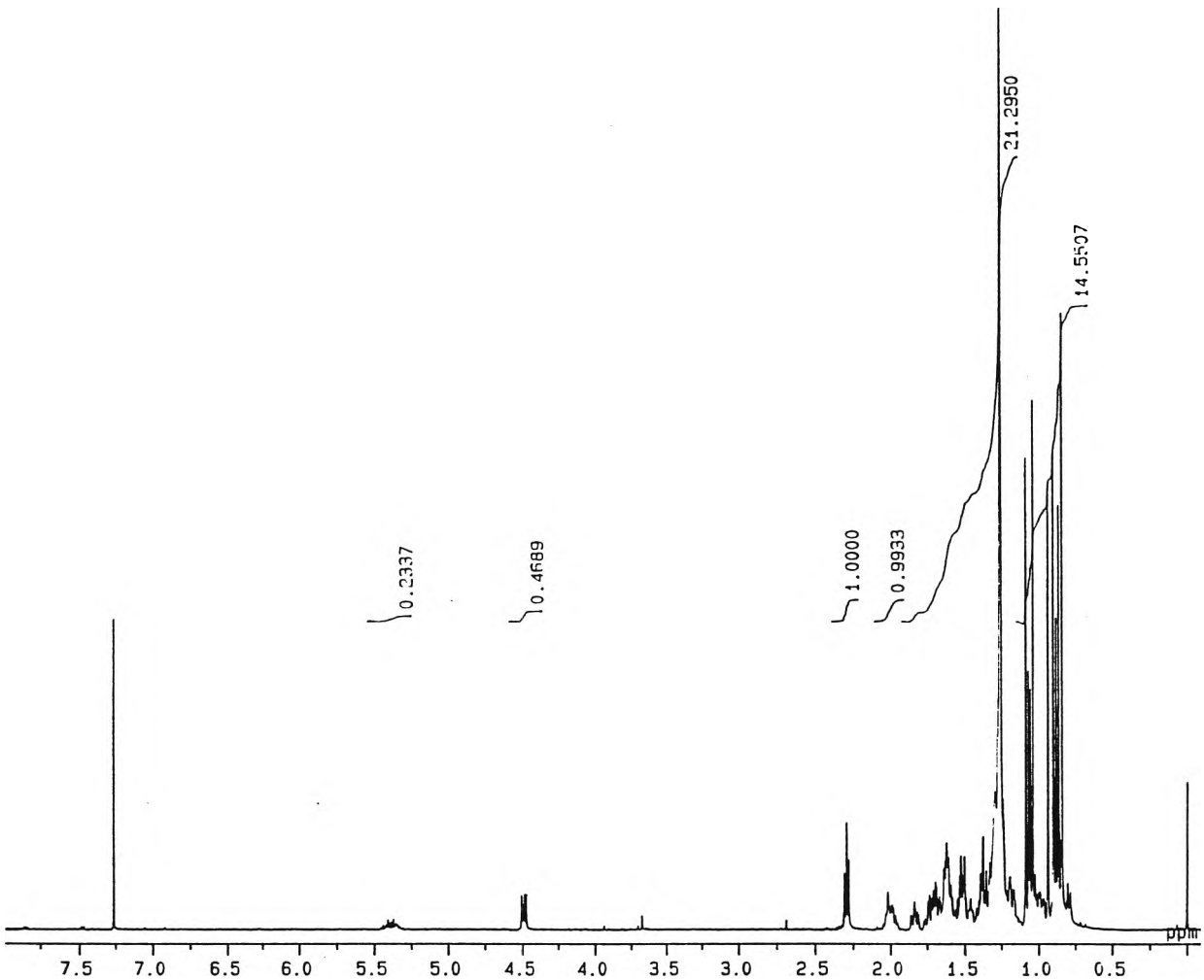


Figure 3.14 The ^{1}H NMR spectrum of Compound **2**

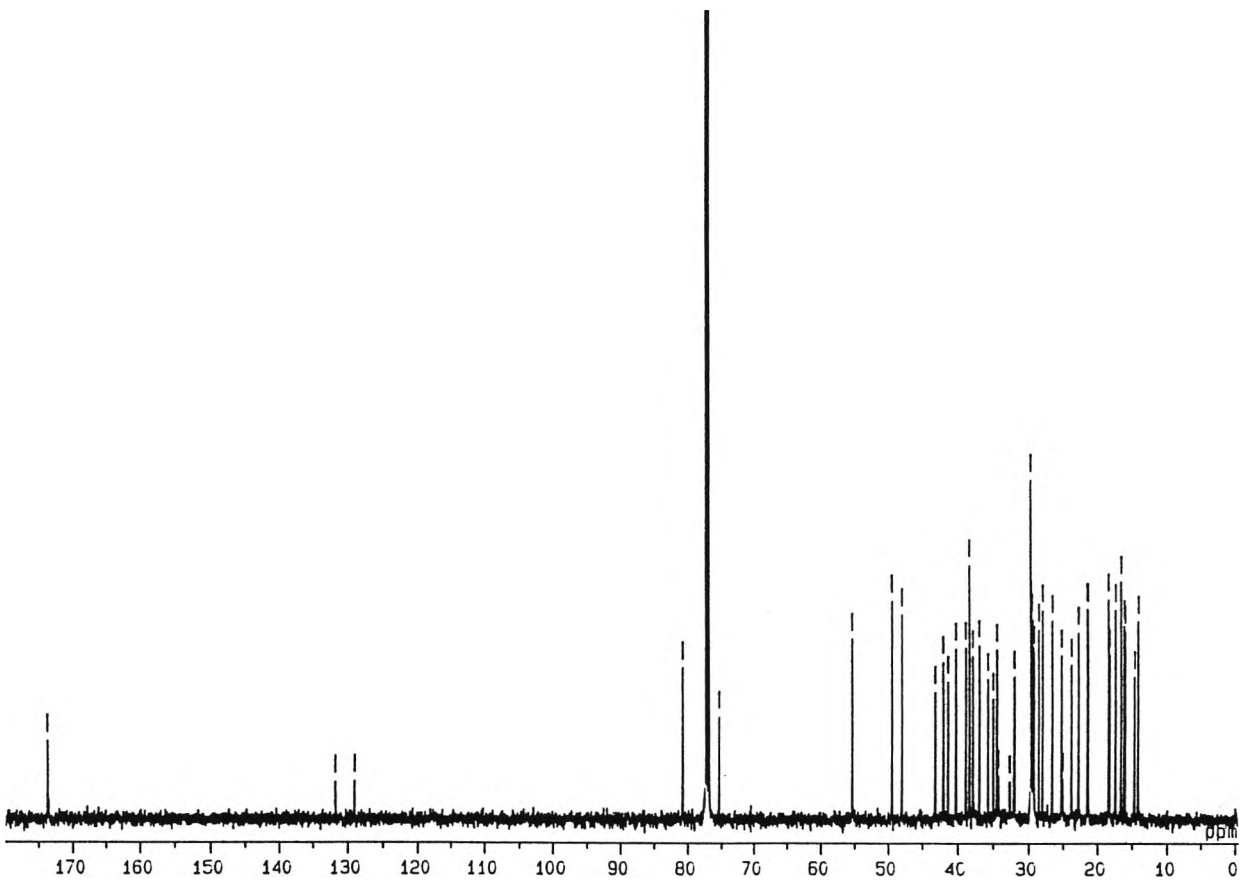


Figure 3.15 The ^{13}C NMR spectrum of Compound **2**

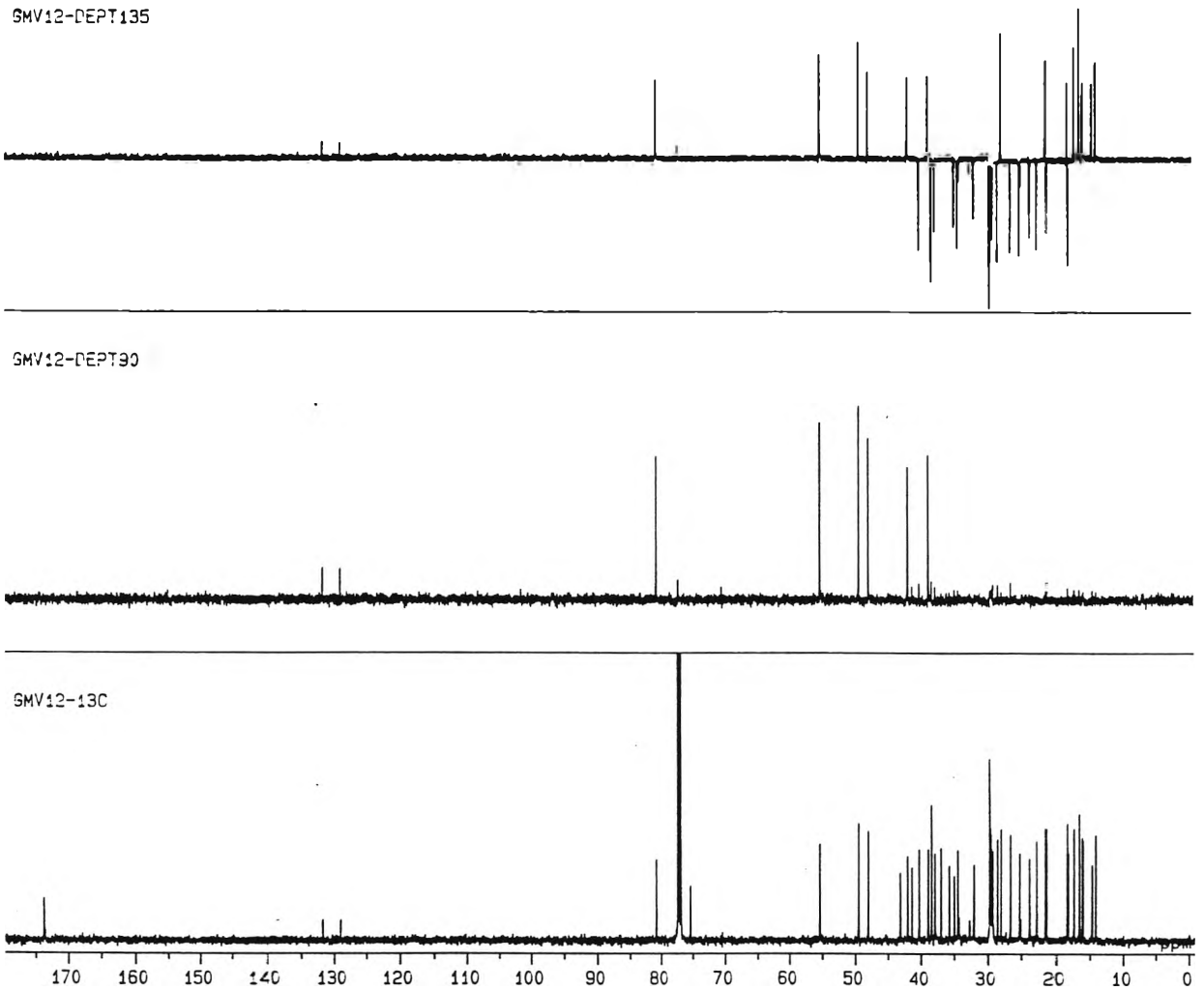


Figure 3.16 The DEPT 90 and 135 spectra of Compound **2**

3.2.7 Structural elucidation of Compound 3

Compound **3** was obtained as a pale yellow solid from dichloromethane crude extract by eluting with 1:4 EtOAc/CH_2Cl_2 was purified by PTLC to afford 4.6 mg (yield 3.93 $\times 10^{-5}$ %).

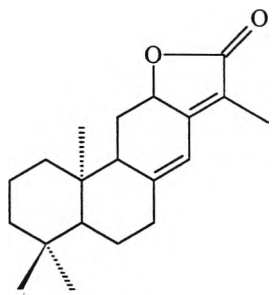
The ^{1}H NMR spectrum (**Figure 3.19**) showed an important signal at 6.26 ppm attributed to the signal of proton of an oxygenated carbon, revealed signal for $-CH_3$, -CH_2- and –CH of cyclic structure at δ 0.7 – 2.5 and proton of –CH= at 4.88 ppm.

The ^{13}C NMR spectrum (**Figure 3.20**) indicated signals for four tertiary methyl groups at δ_c 8.3, 16.8, 21.8 and 33.9, signals of oxygenated carbon at δ_C 76.0, 75.8 ppm, the double bond at 113.9, 116.2, 152.3 and 156.3 and the ketonic at 206.9 ppm. Furthermore, this compound was assigned the structure relate to jolkinolide E [45]. The comparative ^{13}C NMR shifts were presented in **Table 3.15**.

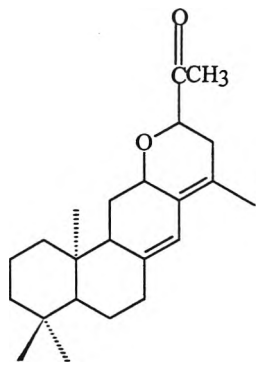
The DEPT 90 and 135 spectra (**Figure 3.21**) indicated that this compound contained five methyl carbons, seven methylene carbons, four tertiary carbons and six quaternary carbons which its structure corresponded to Jolkinolide E but not showed δ at 175.4 of lactone ring and had signal of an oxygenated methylene carbon at 75.8 ppm. The molecular formula of Compound **3** was assigned as $C_{23}H_{34}O_2$ from the NMR and mass spectra (m/z 342) in **Figure 3.22**. The ion peak at m/z 300 $[M-42]^+$ in the mass spectrum was consistent with the loss of a $-COCH_3$ side chain. The comparison of the mass fragmentation patterns of Compound **3** with Jolkinolide E, the Compound **3** displayed main fragmentation patterns at m/z 300, 285, 219, 203, 177, 164, 137, 123, 105, 91, 81, 69 and 55 similar to Jolkinolide E.

Moreover, from the HMBC and HMQC spectra (**Figure 3.23-3.26**) indicated that structure of Compound **3** was the most likely with Jolkinolide E because it showed the possible correlation of long range ^{1}H-^{13}C (**Figure 3.18**). The possible structure and carbon assignment can be deduced from the information of ^{1}H, ^{13}C NMR, HMBC, HMQC and Mass spectra and shown below (**Figure 3.17**).

Thus, the structure of Compound **3** was elucidated as illustrated and named helioscopinolide M as a new compound.



Jolkinolide E



Compound **3**

Table 3.15 The Carbon chemical shifts of Jolkinolide E[45] and Compound **3**

Position of Carbon	Chemical shift (ppm)	
	Jolkinolide E	Compound 3
1	39.7	39.7
2	19.1	19.1
3	41.9	41.9
4	33.6	33.6
5	55.3	55.3
6	23.9	23.9
7	37.2	37.2
8	156.3	156.3
9	51.9	51.9
10	41.6	41.6
11	27.5	27.6
12	76.1	76.0
13	152.3	152.3
14	113.9	113.9
15	116.2	116.2
16	175.4	29.7
17	8.3	75.8
18	33.9	206.9
19	21.8	30.9
20	16.8	8.3
21	-	33.8
22	-	21.8
23	-	16.8

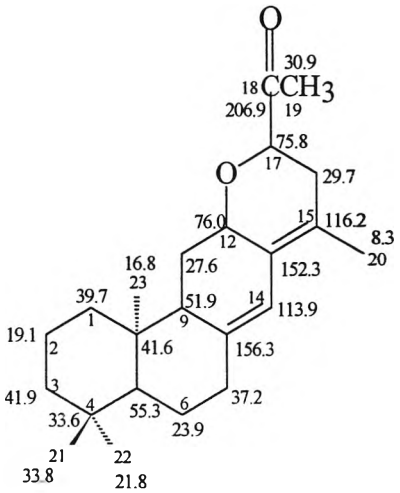


Figure 3.17 Carbon assignments of Compound **3**

Table 3.16 The some carbon and attached proton determined by one bond correlation in HMQC spectra

Carbon	Chemical shift Attached proton
27.6	2.57
29.7	1.25
21.8, 33.8	0.80 – 0.95
37.2	2.50
39.7	1.90
51.9	2.19
75.8, 76.0	4.85

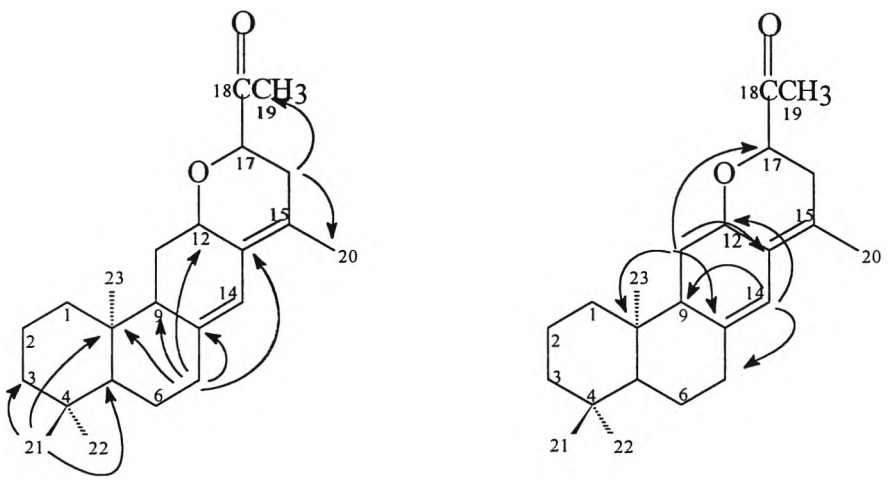


Figure 3.18 Long range ^{1}H - ^{13}C coupling as detected in HMBC spectra

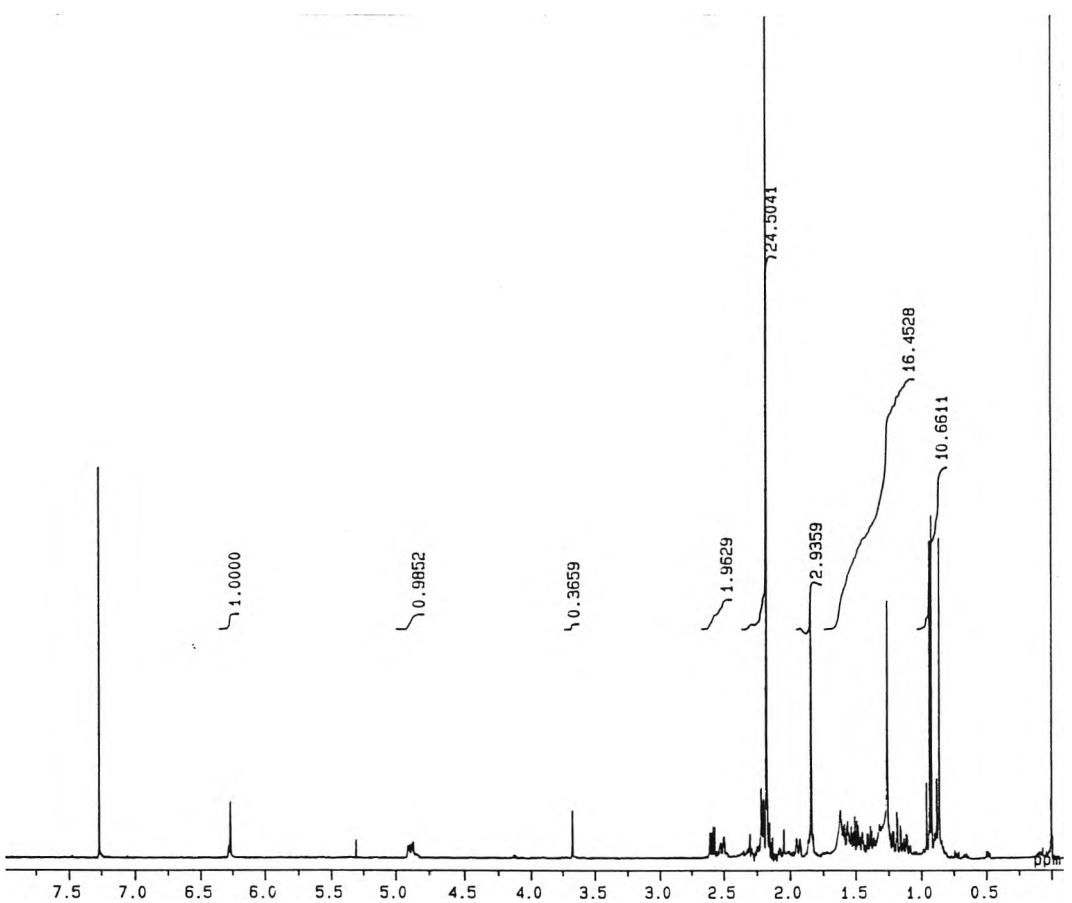


Figure 3.19 The ^{1}H NMR spectrum of Compound **3**

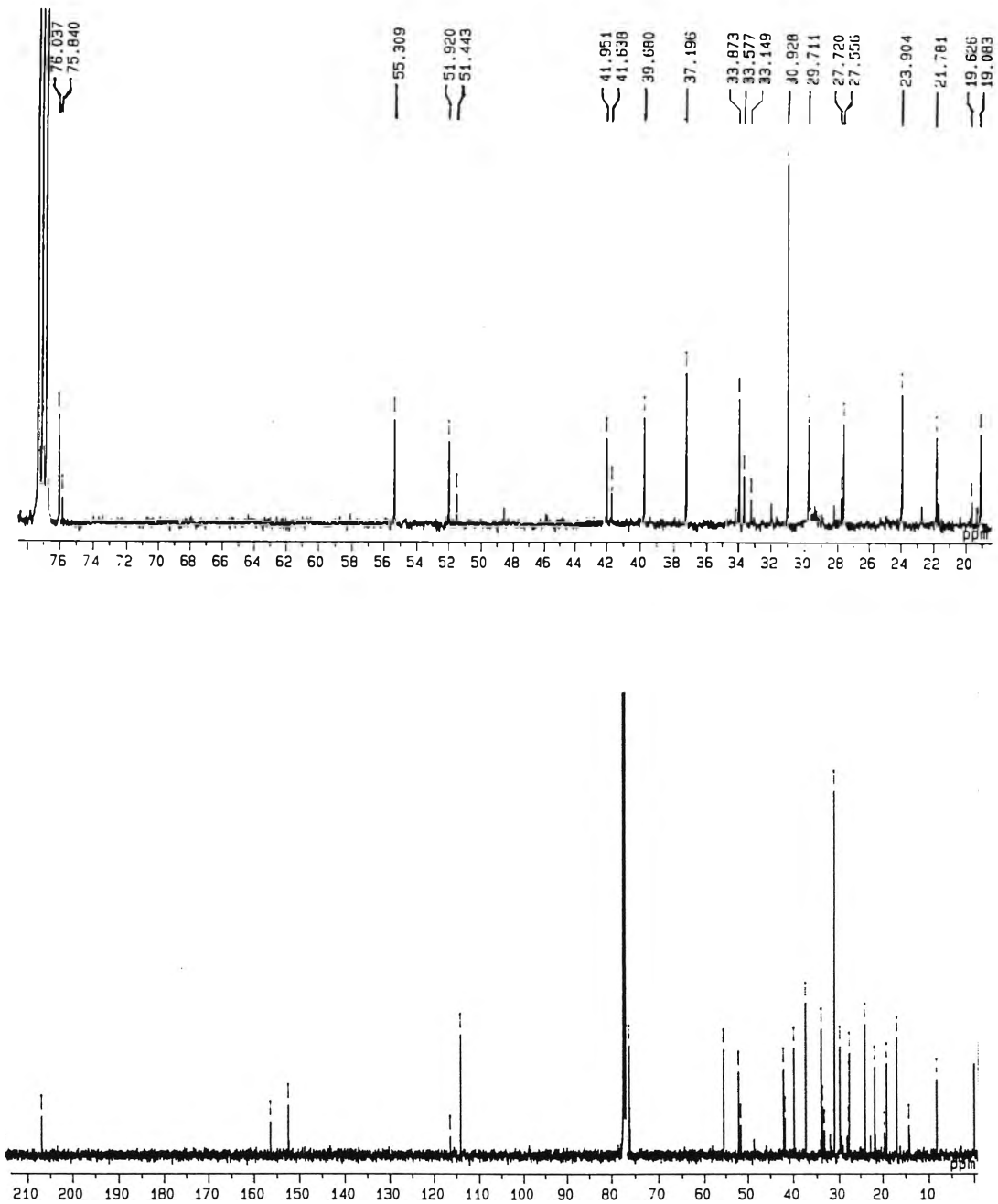


Figure 3.20 The ^{13}C NMR spectrum of Compound **3**

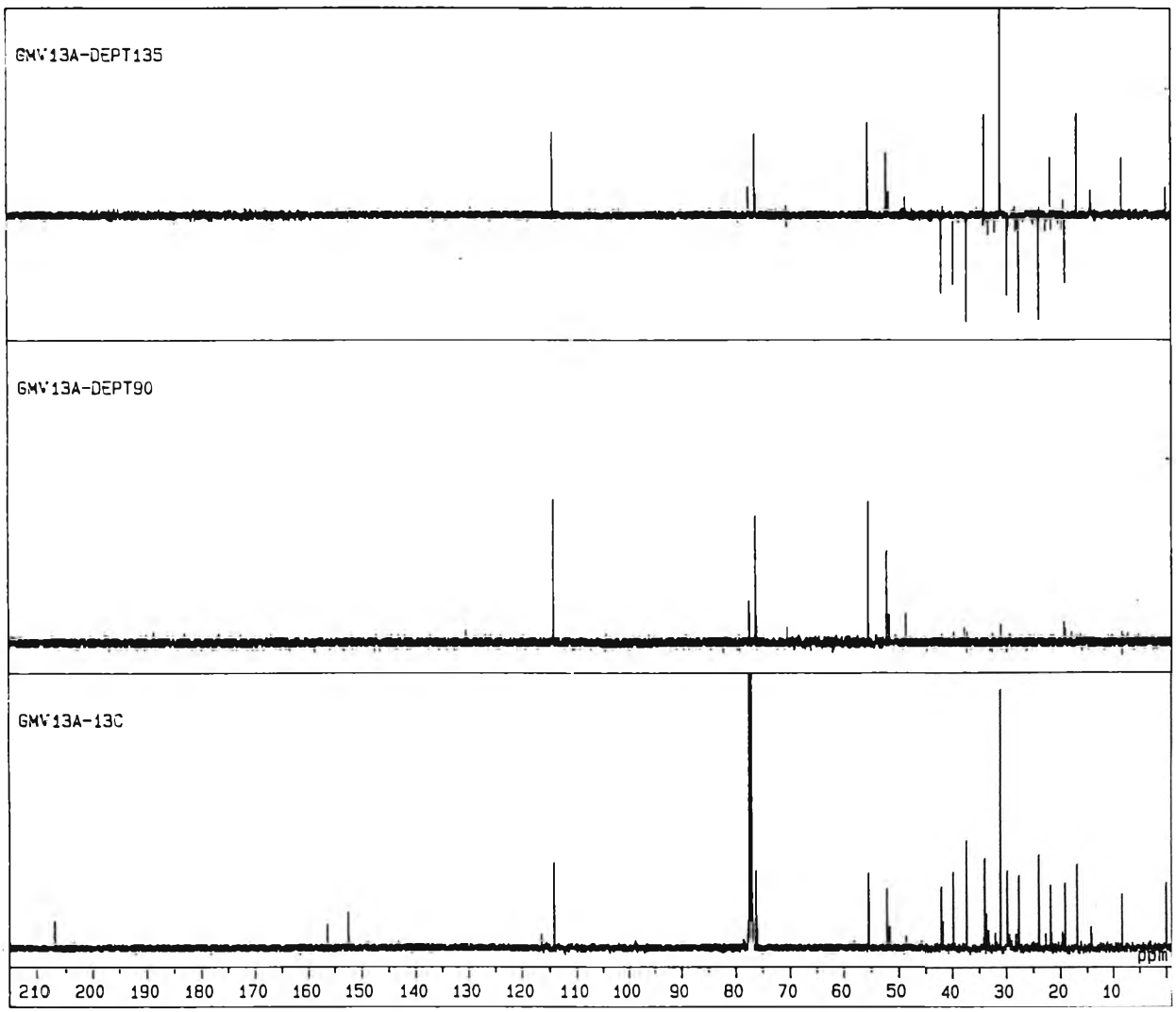


Figure 3.21 The DEPT 90, 135 spectra of Compound **3**

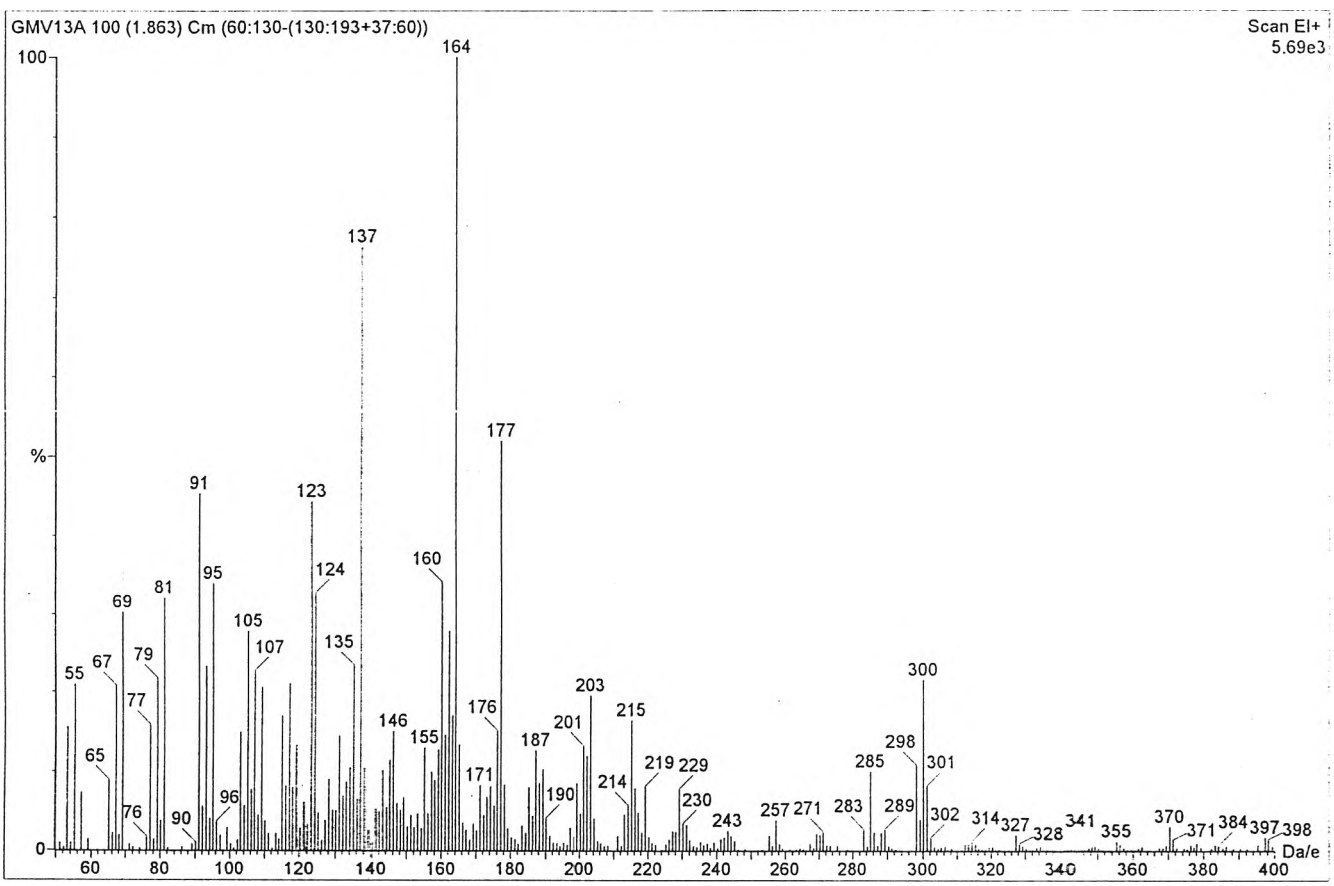


Figure 3.22 The Mass spectrum of Compound **3**

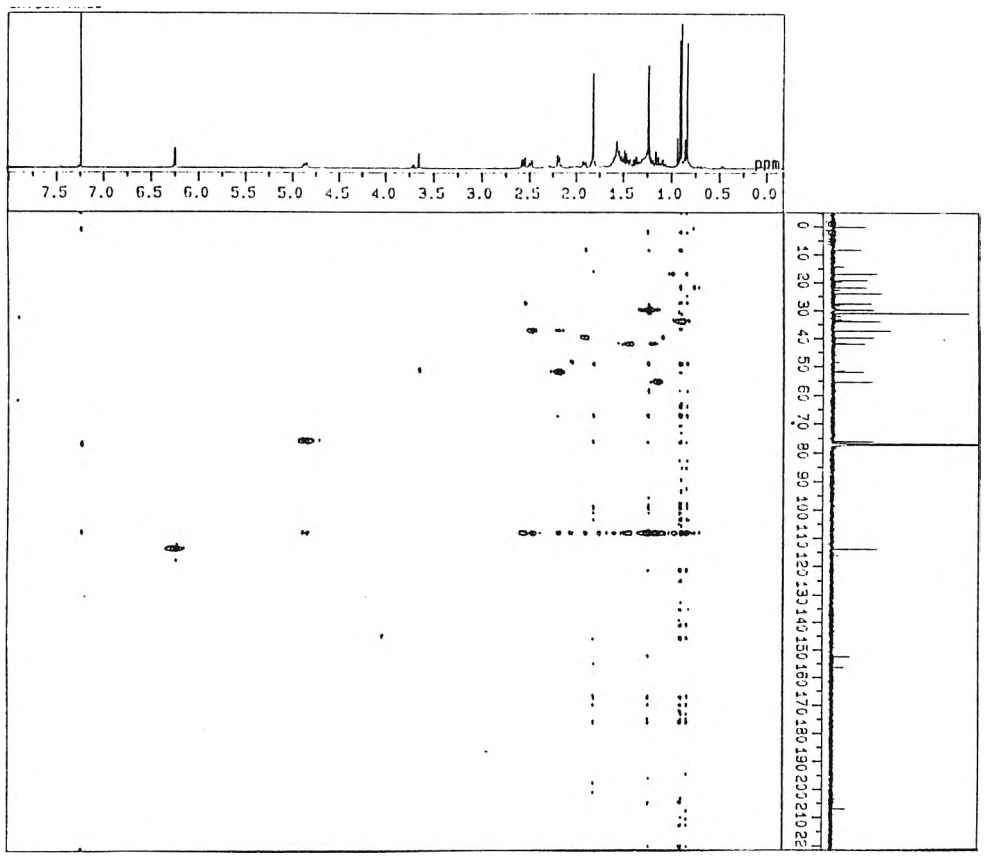


Figure 3.23 The HMQC spectrum of Compound **3**

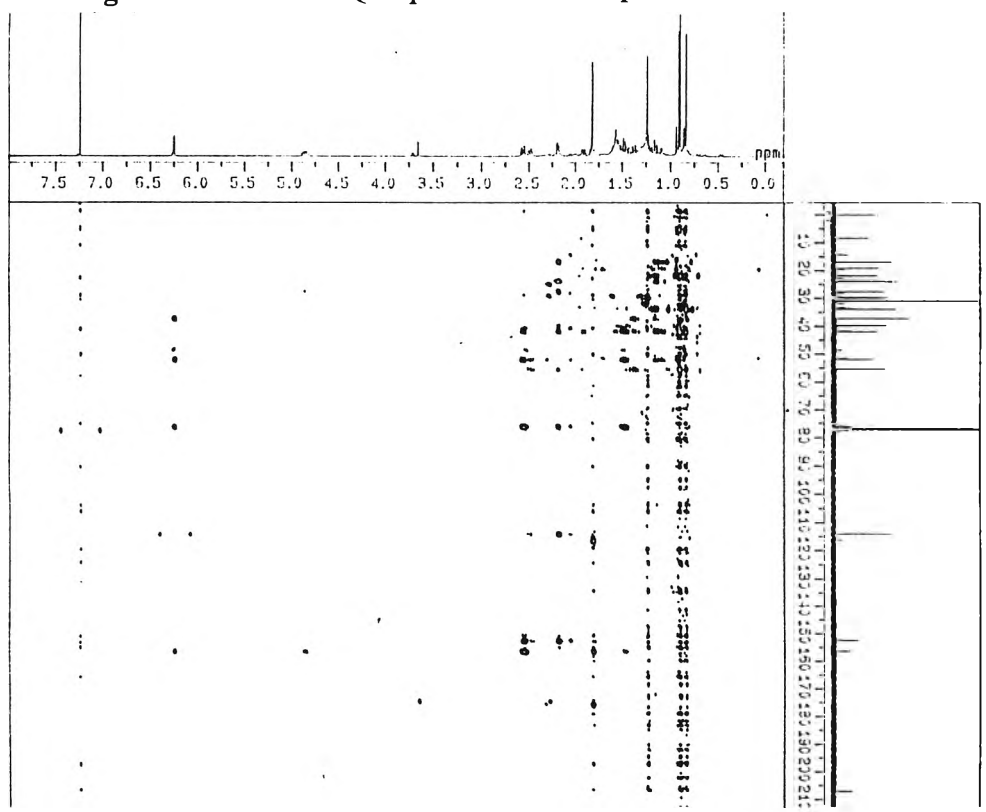


Figure 3.24 The HMBC spectrum of Compound **3**

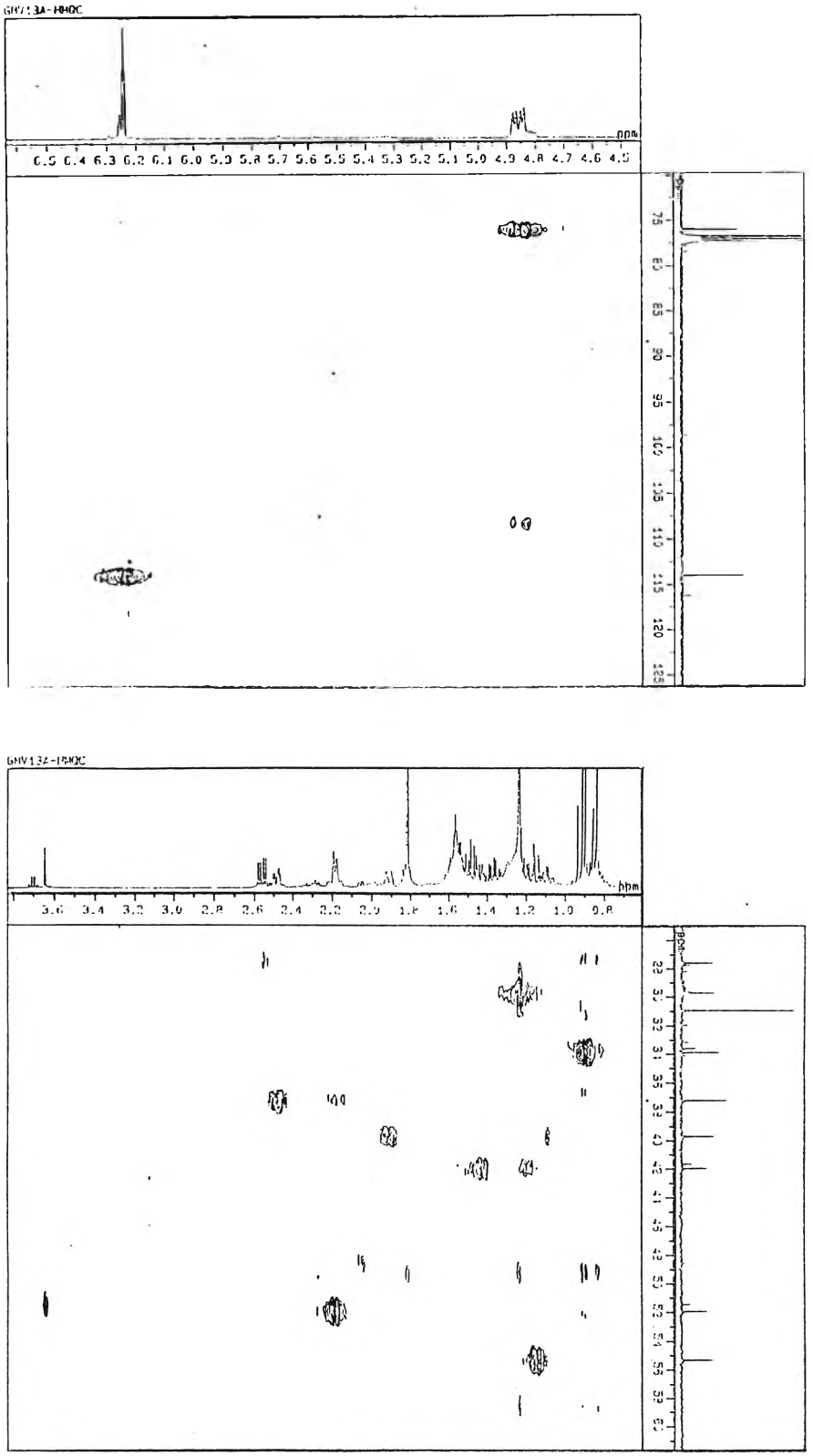


Figure 3.25 The expanded HMQC spectrum of Compound **3**

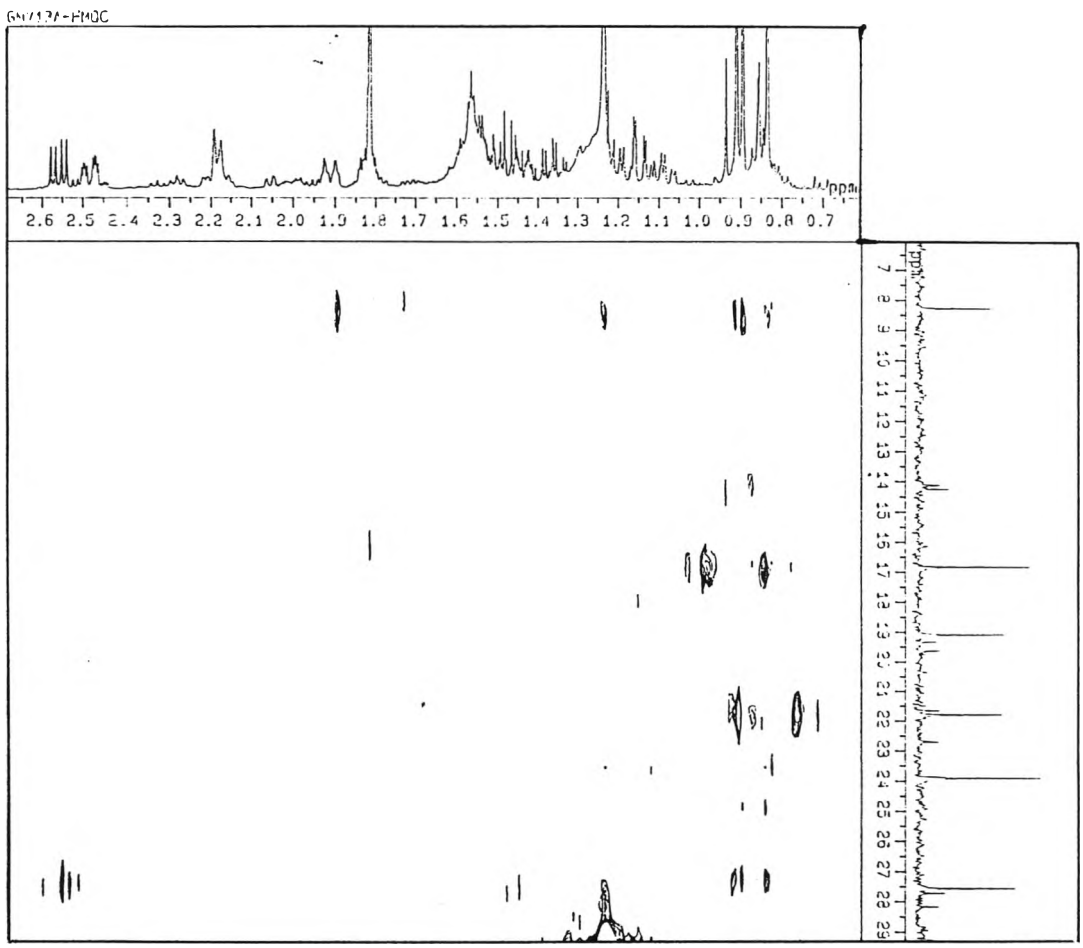


Figure 3.25 (cont.) The expanded HMQC spectrum of Compound **3**

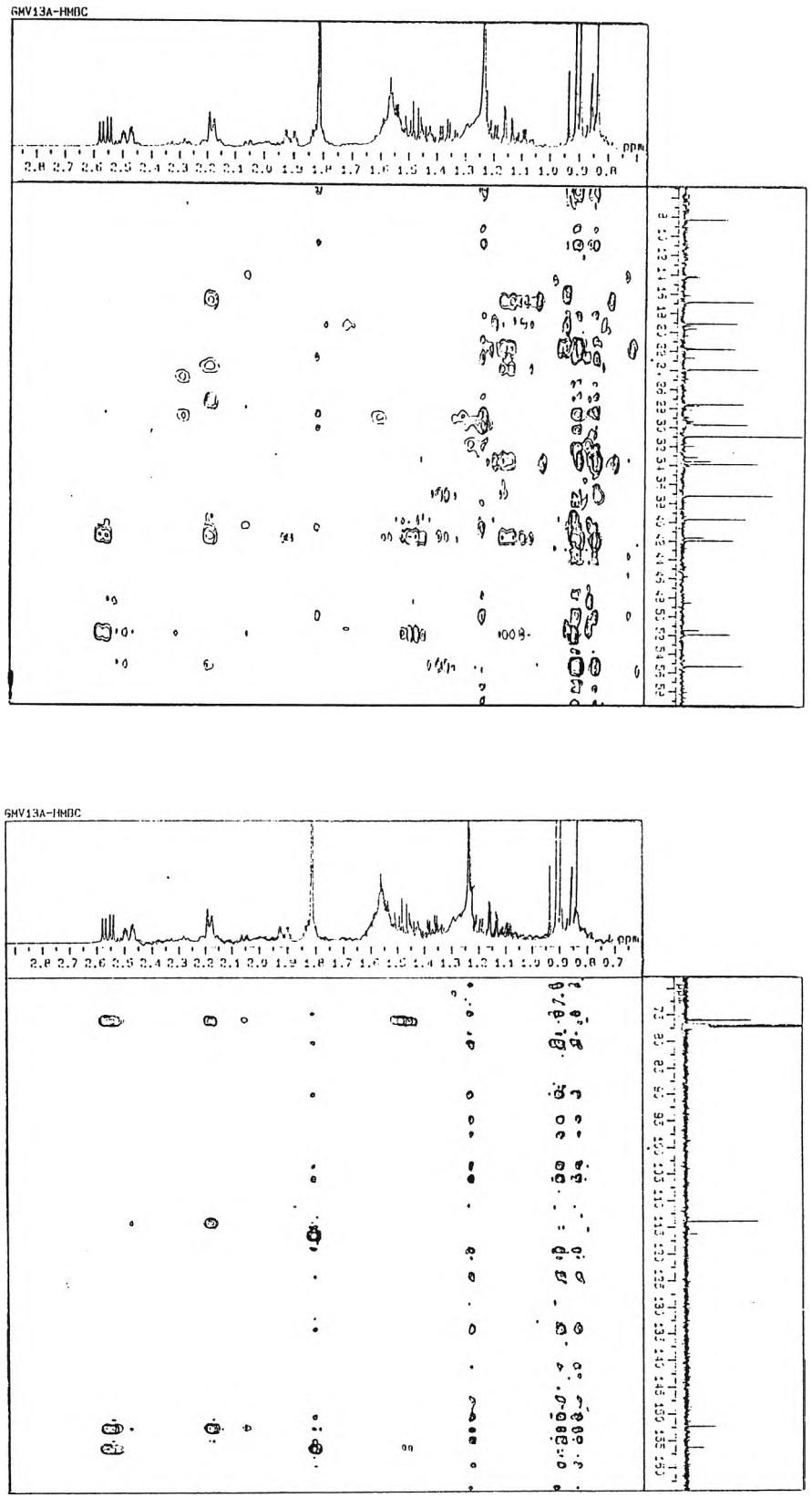


Figure 3.26 The expanded HMBC spectrum of Compound **3**

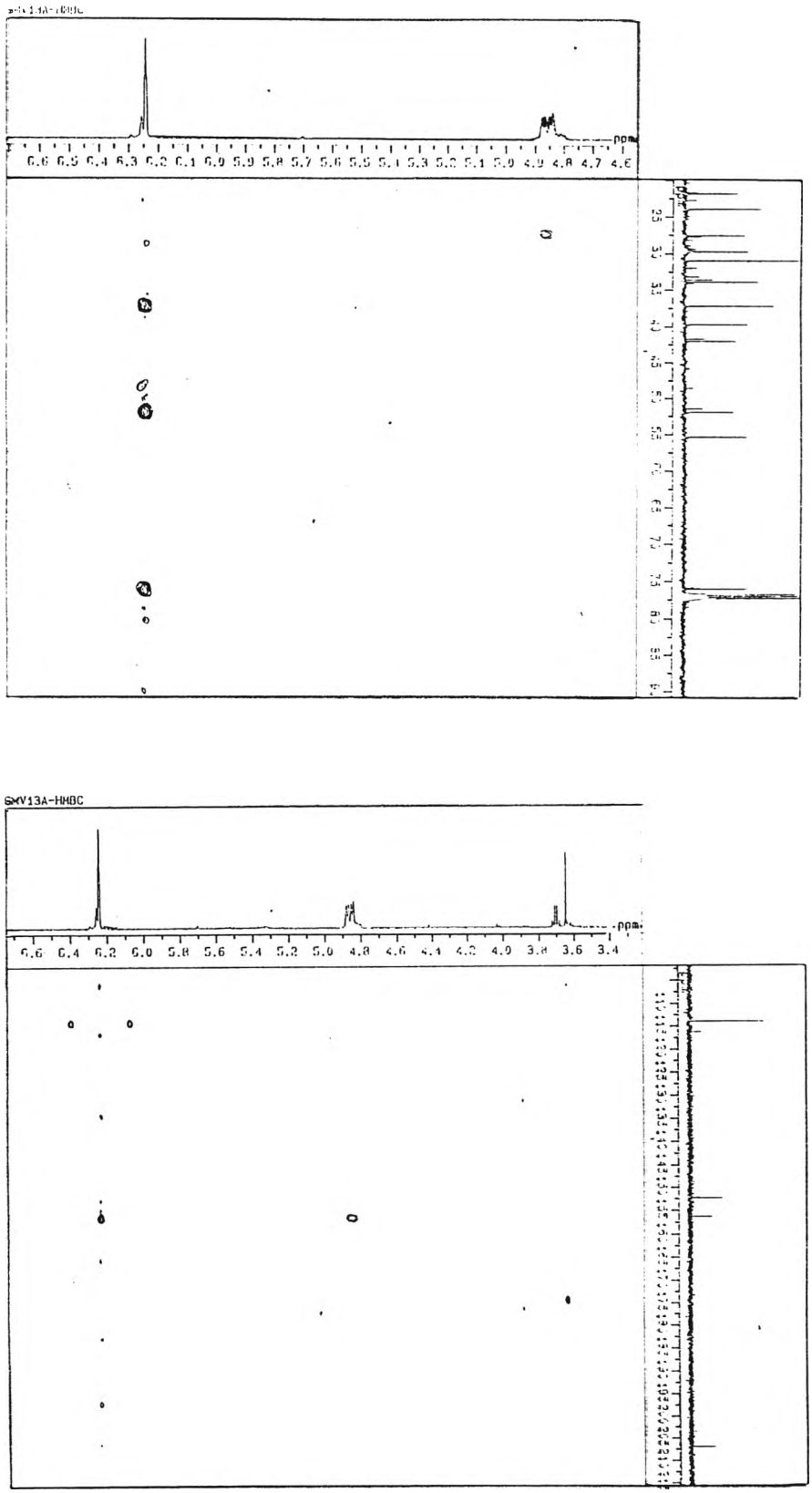


Figure 3.26 (cont.) The expanded HMBC spectrum of Compound 3

3.2.8 Structural elucidation of Compound 4

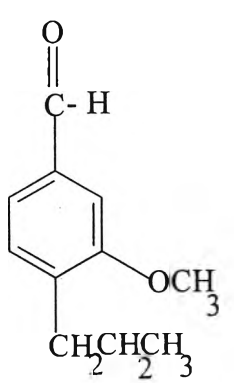
Compound **4**, a yellow solid, was obtained from dichloromethane crude extract by eluting with 25% EtOAc in CH_2Cl_2. This fraction was further purified by PTLC, yielding 3.41×10^{-5} % (weight 4.0 mg) of Compound **4**.

The ^1H NMR spectrum (**Figure 3.27**) and signal integration indicated important proton signals of at 9.83 (1H, s) of aldehyde. The signals of three aromatic protons at 7.43 (1H, dd, *J*= 7.2, 1.8 Hz and 1H, d, *J*=1.5 Hz), 7.04 (1H, d *J*=8.5 Hz) indicated 3,4 – disubstituted benzaldehyde pattern. The methoxy protons at 3.97 (3H, s) and propyl protons at 0.9-1.5 ppm were also observed.

The ^{13}C NMR (**Figure 3.28**) the DEPT 90 and 135 spectra (**Figure 3.29**) exhibited carbon signal of aldehydic at δ 190.8 and those of aromatic moiety at δ (ppm): 151.6 (C-3), 147.5 (C-4), 129.9 (C-1), 127.5 (C-6), 114.3 (C-2) and 108.7 (C-5). The signals of methoxy and aliphatic carbon ($-CH_2-CH_2-CH_3$) showed at 56.1, 29.6, 22.7, 14.1 ppm, respectively. Compound **4** had four quaternary carbons, two methyl and two methylene carbons.

The Mass spectrum of Compound **4** (**Figure 3.30**) showed a molecular ion peak at m/z 178 ($C_{11}H_{14}O_2$) and the fragmentation pattern was summarized: 178 $[M]^+$, 153 $[M-OCH_3]^+$, 149 $[M-CHO]^+$,135 $[M-C_3H_7]^+$, 97, 81, 69, 58, 43, 28.

Compound **4**, therefore, should be 3-methoxy-4-propylbenzaldehyde. The structure of Compound **4** is shown below.



Compound 4 : 3-methoxy-4-propylbenzaldehyde

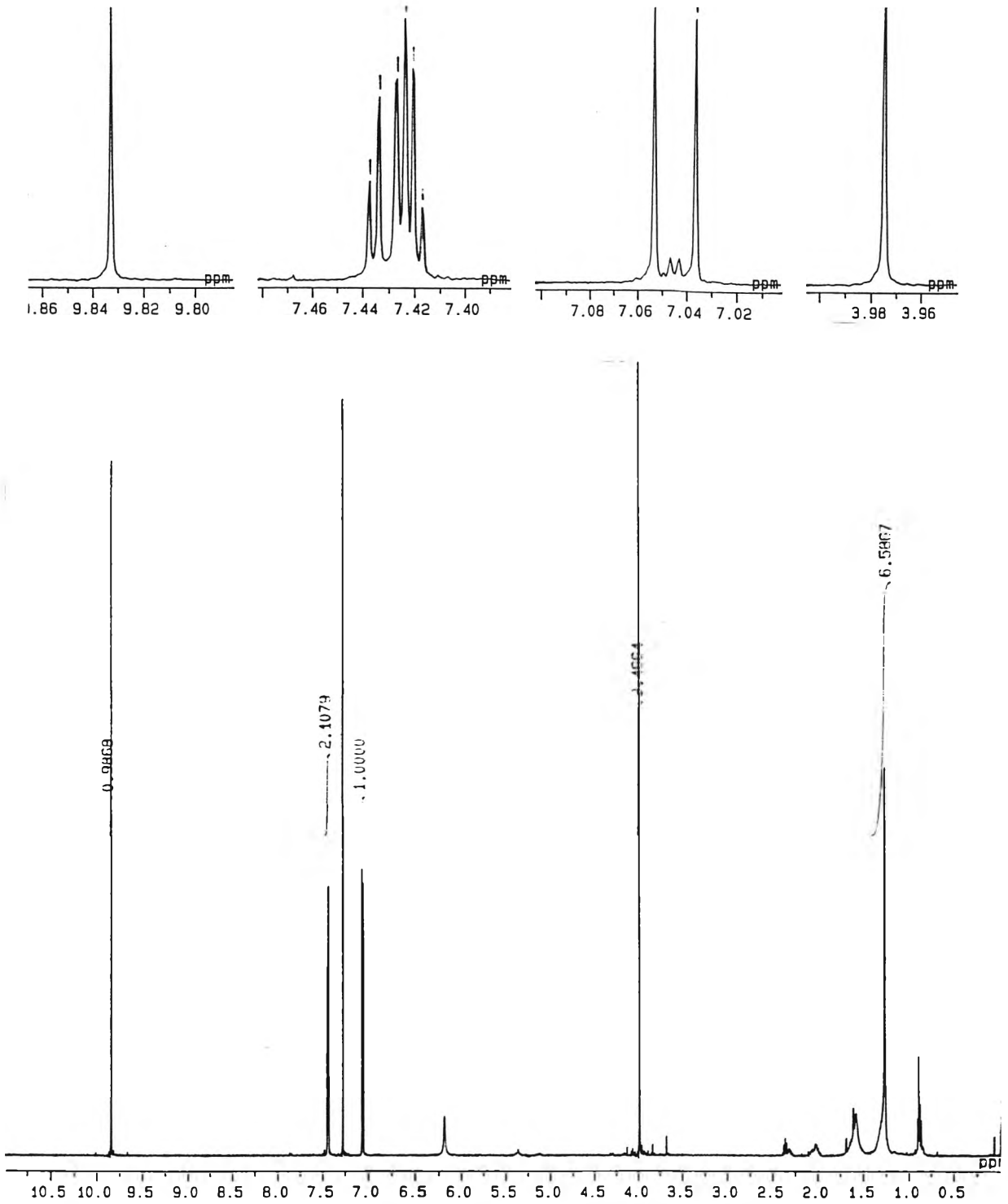


Figure 3.27 The ^{1}H NMR spectrum of Compound **4**

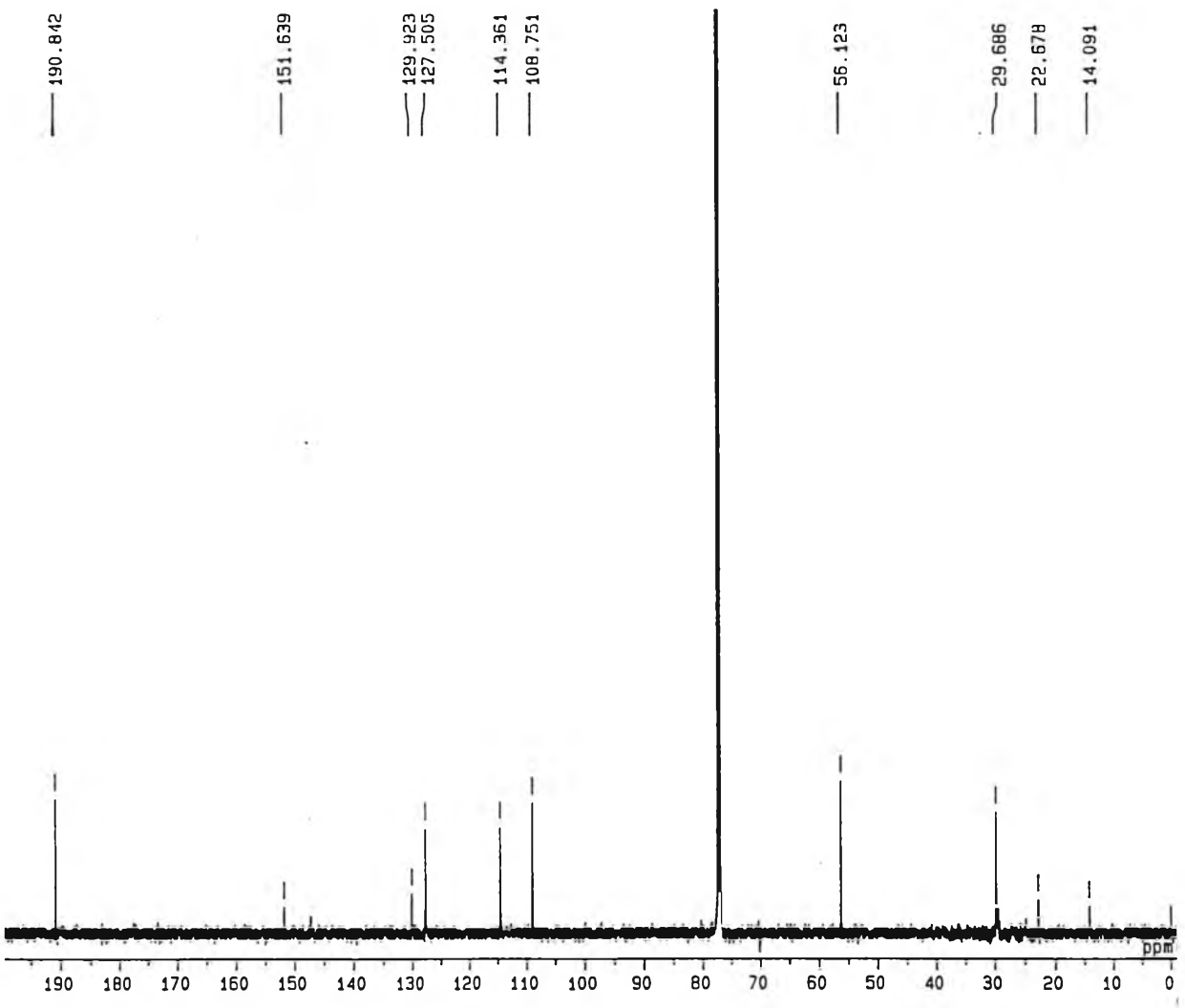


Figure 3.28 The ^{13}C NMR spectrum of Compound **4**

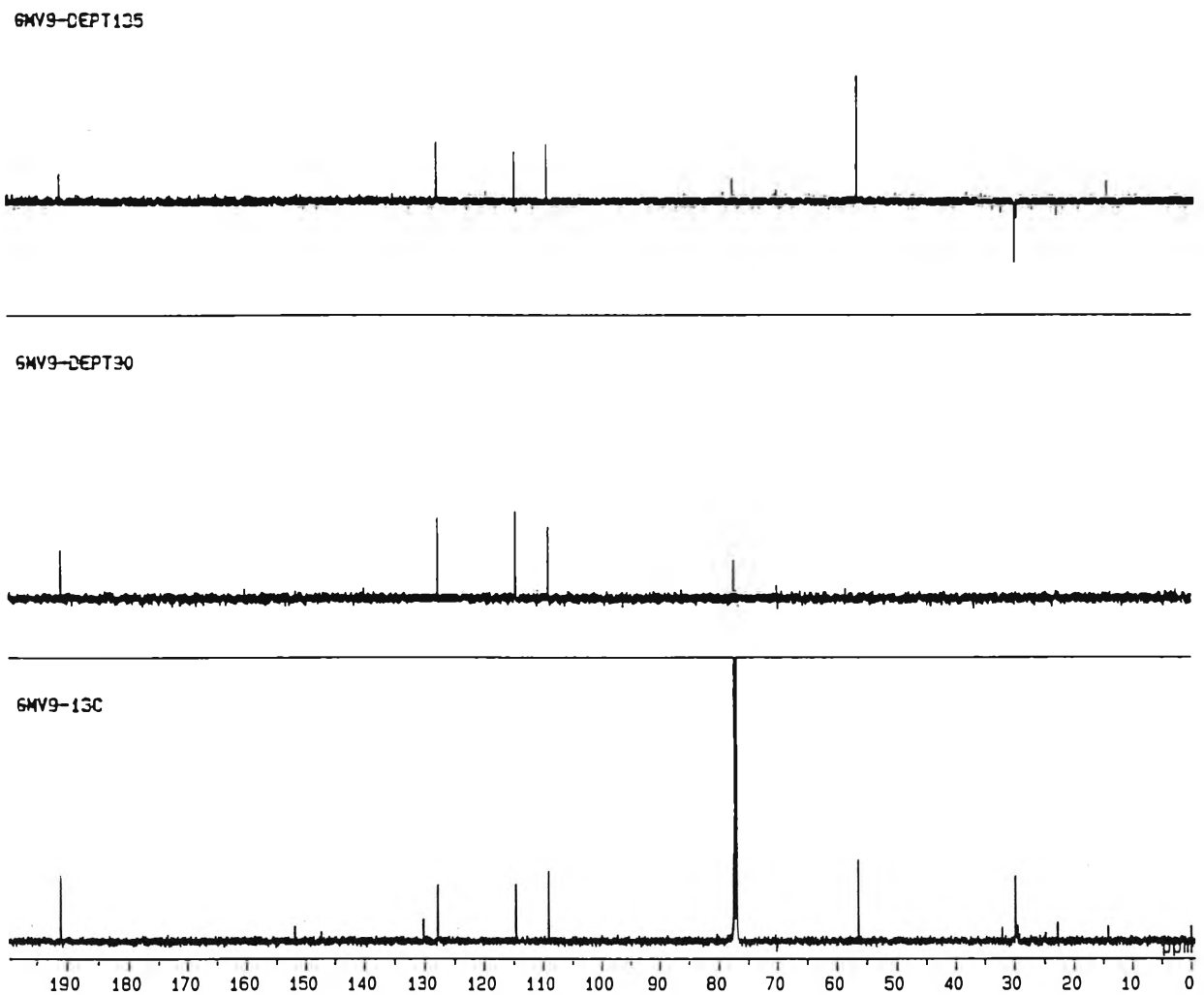


Figure 3.29 The DEPT 90 and 135 spectra of Compound **4**

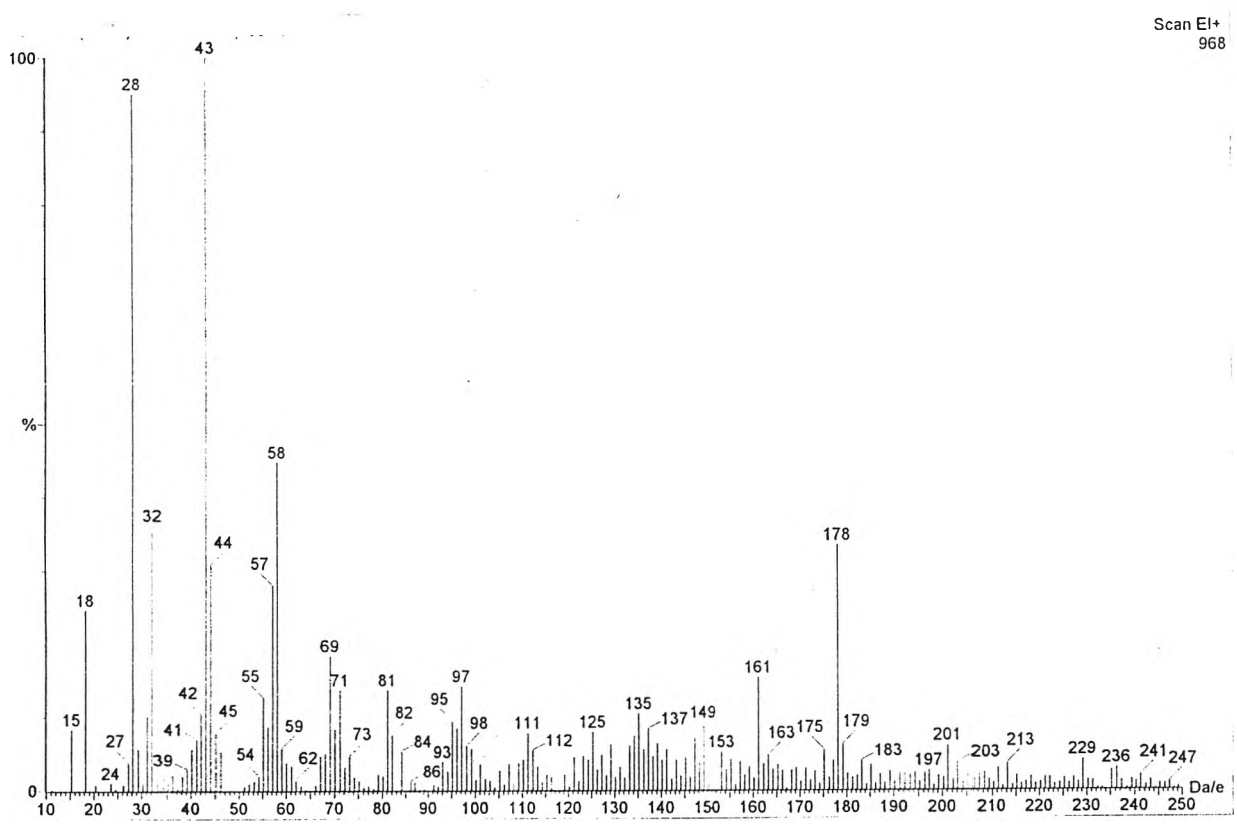


Figure 3.30 The Mass spectrum of Compound **4**

3.2.9 Structural elucidation of Compound 5

Compound **5,** a bright crystal of melting point 197 °C, was obtained from dichloromethane crude extract. Several eluted fractions with 20% EtOAc in CH_2Cl_2 were united and purified by recrystallization in a mixture of chloroform and methanol, yielding 2.99×10^{-3} % (weight 350.3 mg) of compound **5** with R_f 0.58 [EtOAc:hexane (1:1); silica gel].

The IR spectrum (**Figure 3.31**) showed three absorption bands (1762, 1705, 1680 cm^{-1}) of C=O stretching of conjugated ketone and a band of unsaturated hydrocarbon.

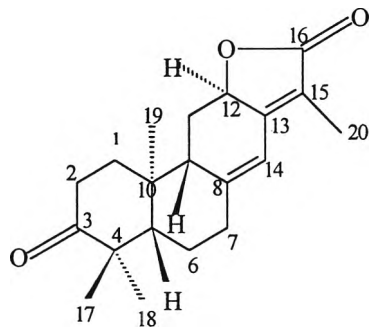
The molecular formula of Compound **5** was established by NMR spectral data and mass spectrum (**Figure 3.32**) which revealed a molecular ion peak at m/z 314.

The ^1H NMR spectrum (**Figure 3.33**) displayed signals at 1.07-1.82 ppm, a typical region of methyl and methylene protons and at δ 6.35 ppm of an olefinic proton.

The ^{13}C NMR, DEPT 90, 135 spectra of this compound (**Figure 3.34** and **3.35**) indicated twenty carbons signals : four methyl carbons at δ 8.3, 16.2, 21.7 and 26.5 ppm, five methylene carbons at δ 24.5, 27.7, 34.3, 36.5 and 37.5 ppm , four tertiary carbons at δ 50.6, 54.7, 75.6 and 114.7 ppm, quaternary carbons at 40.9, 47.5 ppm, a carbonyl carbon at δ 215.5 ppm, a lactone carbonyl carbon at 175.0 ppm, together with three more olefinic carbons at δ 117.0, 150.1 and 155.5 ppm.

The evidence from IR, MS, ^1H and ^{13}C NMR spectrum of Compound **5** suggested that its structure was likely to be a diterpenoid lactone which corresponded to molecular formula $\mathbf{C_{20}H_{26}O_3}$ (m/z 314). Compound **5** was deduced to be helioscopinolide E owing to their exactly resemble NMR spectra.

The Carbon chemical shifts of Compound **5** were compared to those of helioscopinolide E [46] in **Table 3.17** .



Helioscopinolide E

Table 3.17 The Carbon chemical shifts of Helioscopinolide E[46] and Compound **5**

Position of Carbon	Chemical shift (ppm)	
	Helioscopinolide E	Compound **5**
1	37.4	37.3
2	34.4	34.3
3	215.6	215.5
4	47.5	47.5
5	54.8	54.7
6	24.6	24.5
7	36.6	36.5
8	150.2	150.1
9	50.7	50.6
10	40.9	40.9
11	27.8	27.7
12	75.6	75.6
13	155.5	155.5
14	114.8	114.7
15	117.1	117.0
16	175.1	175.0
17	26.5	26.5
18	21.8	21.7
19	16.2	16.2
20	8.3	8.3

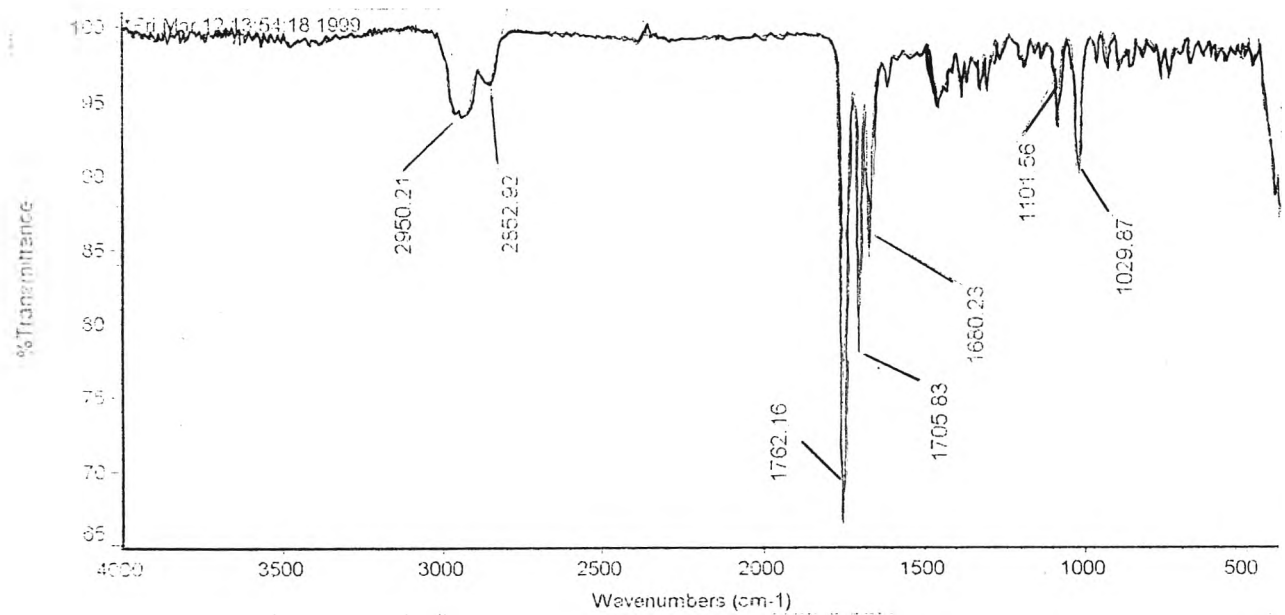


Figure 3.31 The IR spectrum of Compound **5**

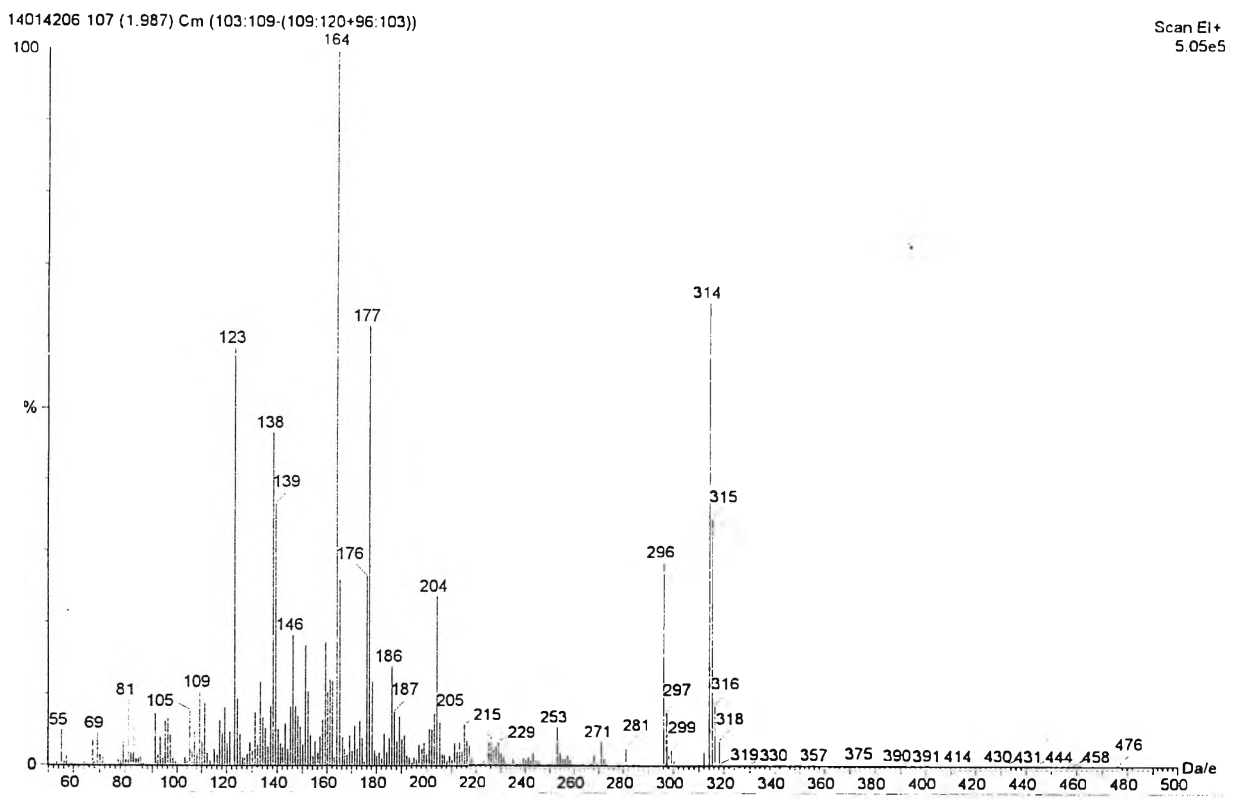


Figure 3.32 The Mass spectrum of Compound **5**

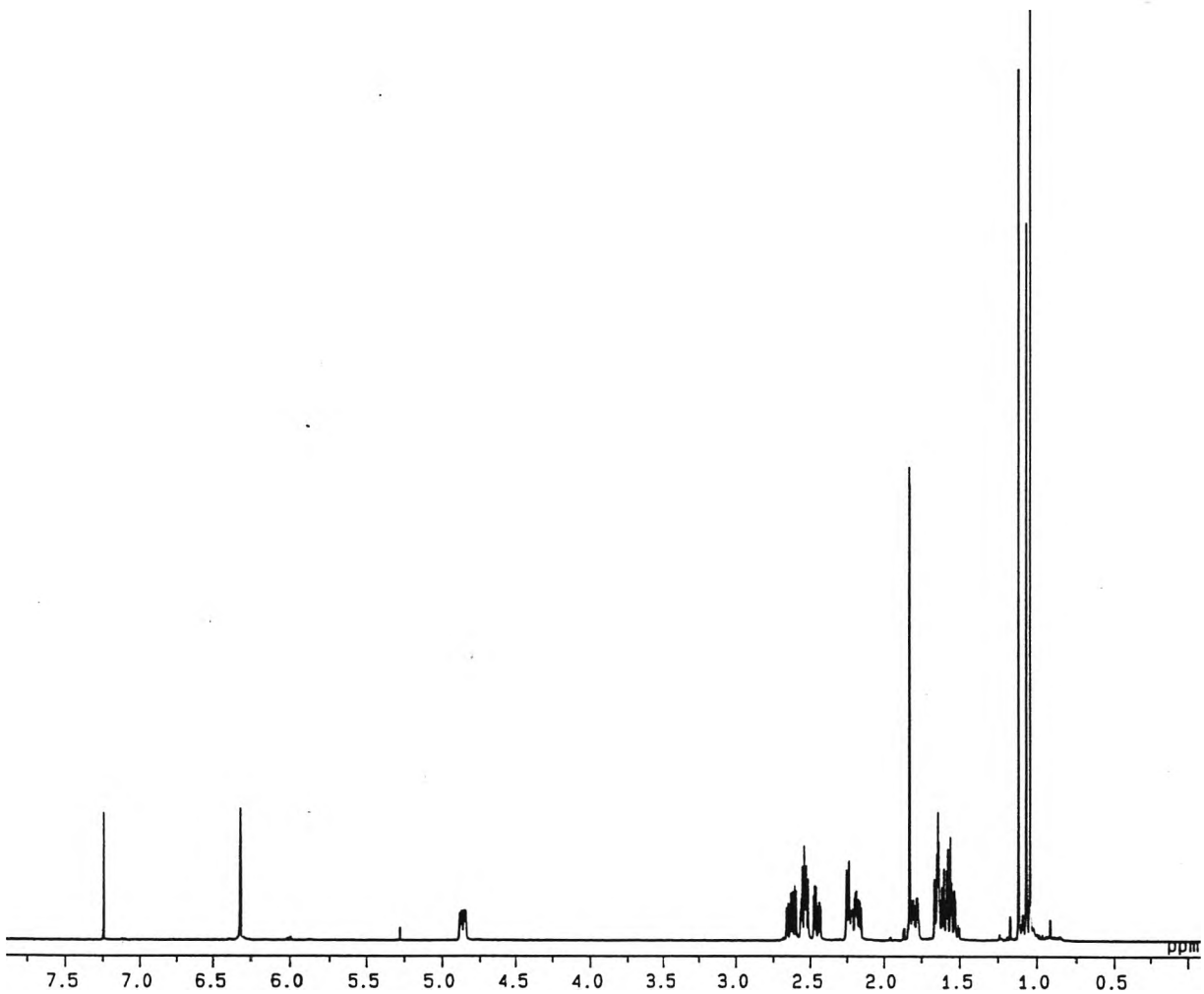


Figure 3.33 The ^{1}H NMR spectrum of Compound **5**

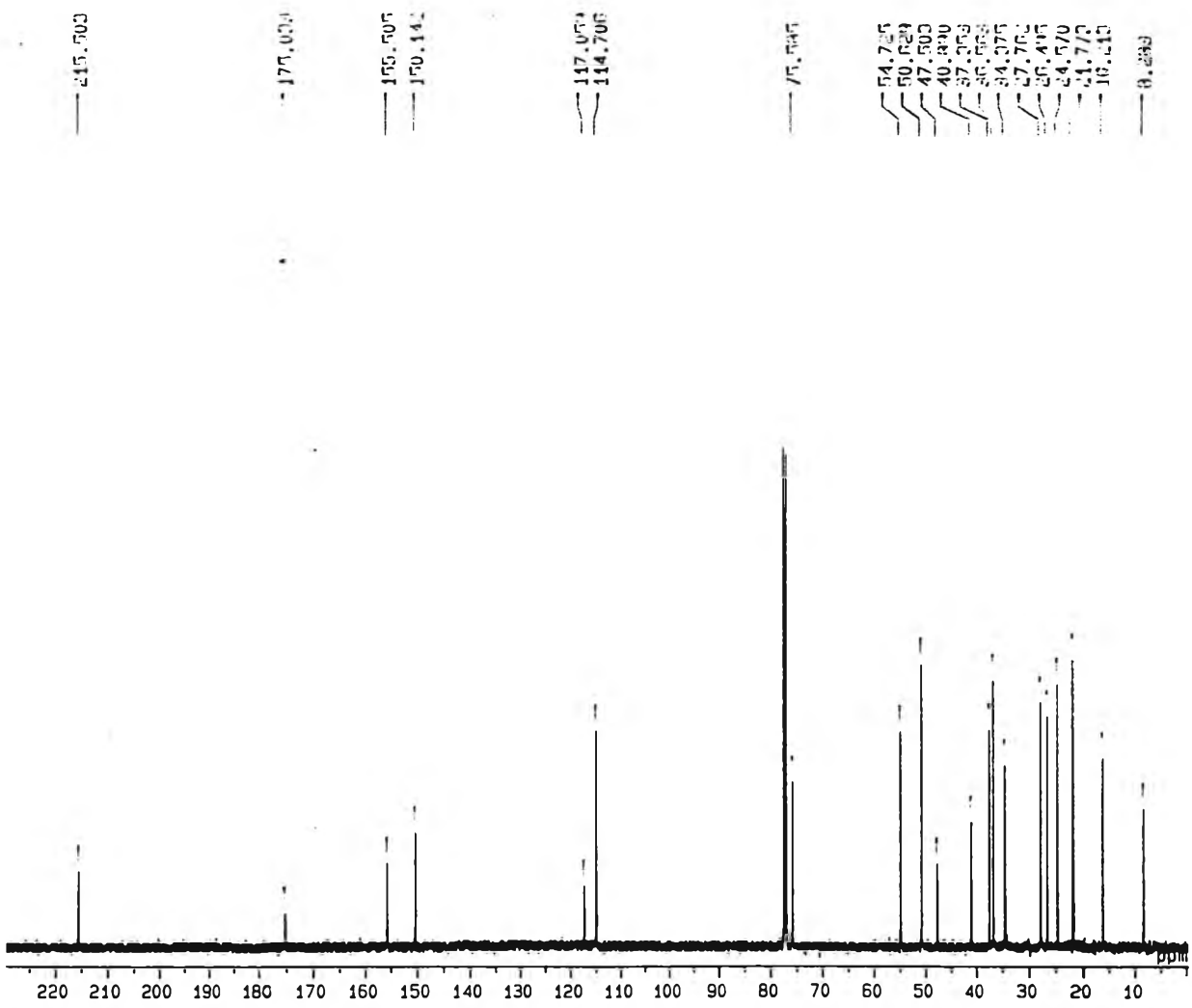


Figure 3.34 The ^{13}C NMR spectrum of Compound **5**

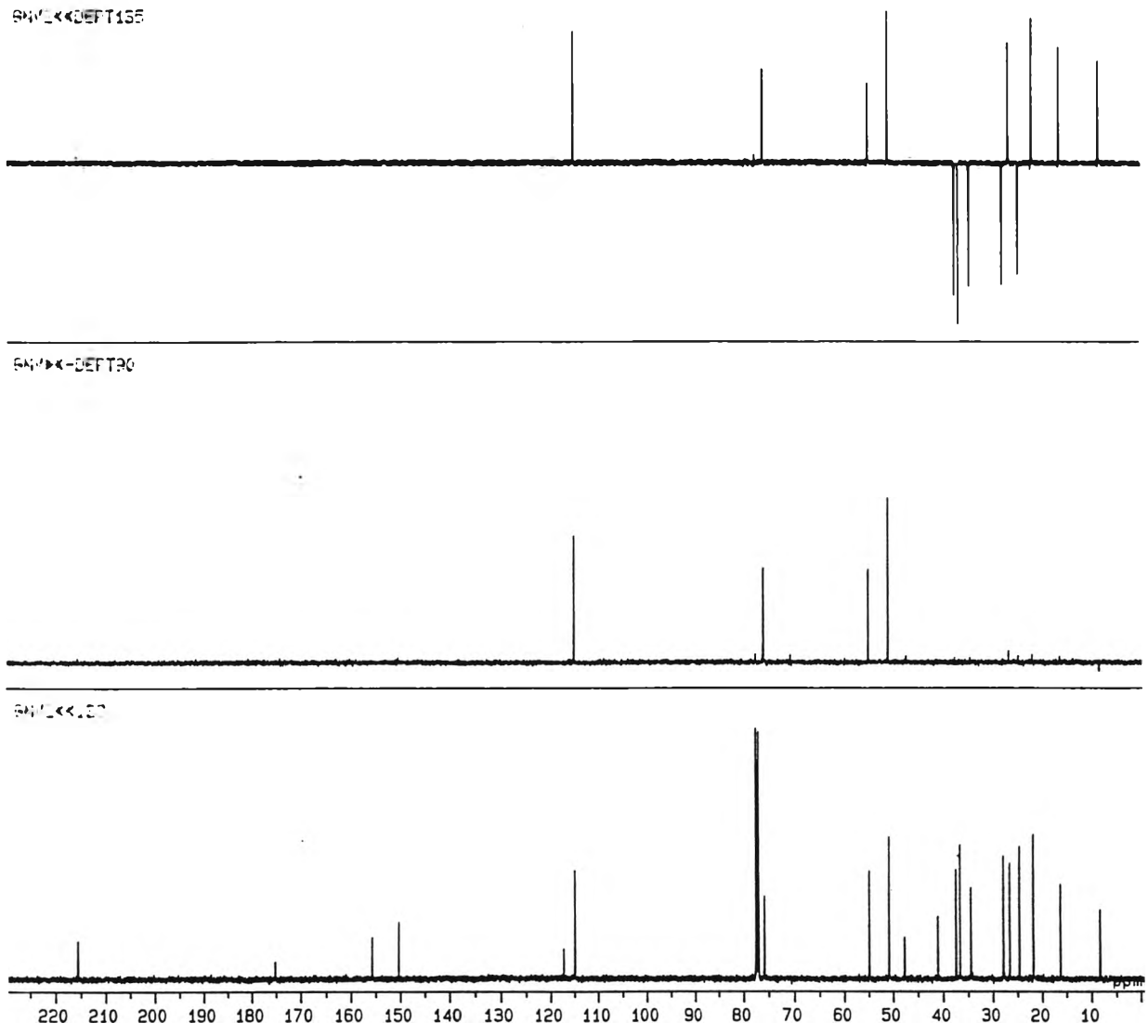


Figure 3.35 The DEPT 90 and 135 spectra of Compound **5**

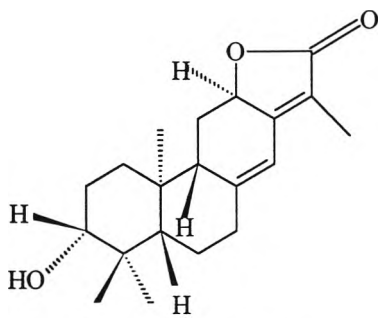
3.2.10 Structural elucidation of Compound 6

Compound **6** (4.3 mg; yield 3.67×10^{-5} %) was yielded from dichloromethane crude extract by eluting with 100% CH_2Cl_2 to 20% EtOAc-CH_2Cl_2 . After being purified by preparative TLC (developed with 40% EtOAc in hexane), a green crystal was obtained.

A number of characteristic features can be identified by the ^1H and ^{13}C NMR spectra (**Figure 3.36** and **3.37**): three methyl groups (δ_H 0.84, 0.94, 1.05 ; δ_C 15.6, 16.7, 28.7) bounded to the two quaternary carbons (δ_C 39.1, 41.2) and one methyl group (δ_H 1.84, δ_C 8.3) bonded to an olefinic carbon ; an isolated olefinic hydrogen (δ_H 6.29, δ_C 114.2) ; a CHOH group (δ_C 78.6). In the down field region of the ^{13}C NMR spectrum, three more olefinic carbons can be found at δ 116.6, 151.4 and 156.0 , together with a characteristic peak of lactone at δ 175.3. The DEPT 90 and 135 spectra (**Figure 3.38**) showed signals of four methyl, five methylene, five tertiary and six quaternary carbons, which were similar to Compound **5**.

An accordance to spectral data indicated that structure of Compound **5** and Compound **6** were similar to each other. However, the structure of Compound **6** differed from Compound **5** only carbon at position 3. This position of Compound **6** connected to hydroxy group (δ_C 78.6) while Compound **5** attached to carbonyl group. Resulting in slight shift of nearby carbon signals C-2 (δ 27.6) and C-4 (δ 39.1), Compound **6** was ascribable to helioscopinolide A, a reduced from of helioscopinolide E. From mass spectrum (**Figure 3.39**), Compound **6** had a molecular weight of 316 daltons and molecular composition of $\mathbf{C_{20}H_{28}O_3}$.

The carbon chemical shifts of Compound **6** and helioscopinolide A[46] were displayed in **Table 3.18**. The carbon chemical shifts of Compound **6** was identical to helioscopinolide A.



helioscopinolide A

Table 3.18 The Carbon chemical shifts of Helioscopinolide A[46] and Compound **6**

Position of Carbon	Chemical shift (ppm)	
	Helioscopinolide A	Compound 6
1	37.4	37.4
2	27.6	27.6
3	78.6	78.6
4	39.1	39.1
5	54.4	54.4
6	23.4	23.4
7	36.9	36.9
8	151.4	151.4
9	51.5	51.6
10	41.2	41.2
11	27.5	27.5
12	75.8	75.8
13	156.0	156.0
14	114.2	114.2
15	116.6	116.6
16	175.3	175.3
17	28.6	28.7
18	15.5	15.6
19	16.6	16.7
20	8.2	8.3

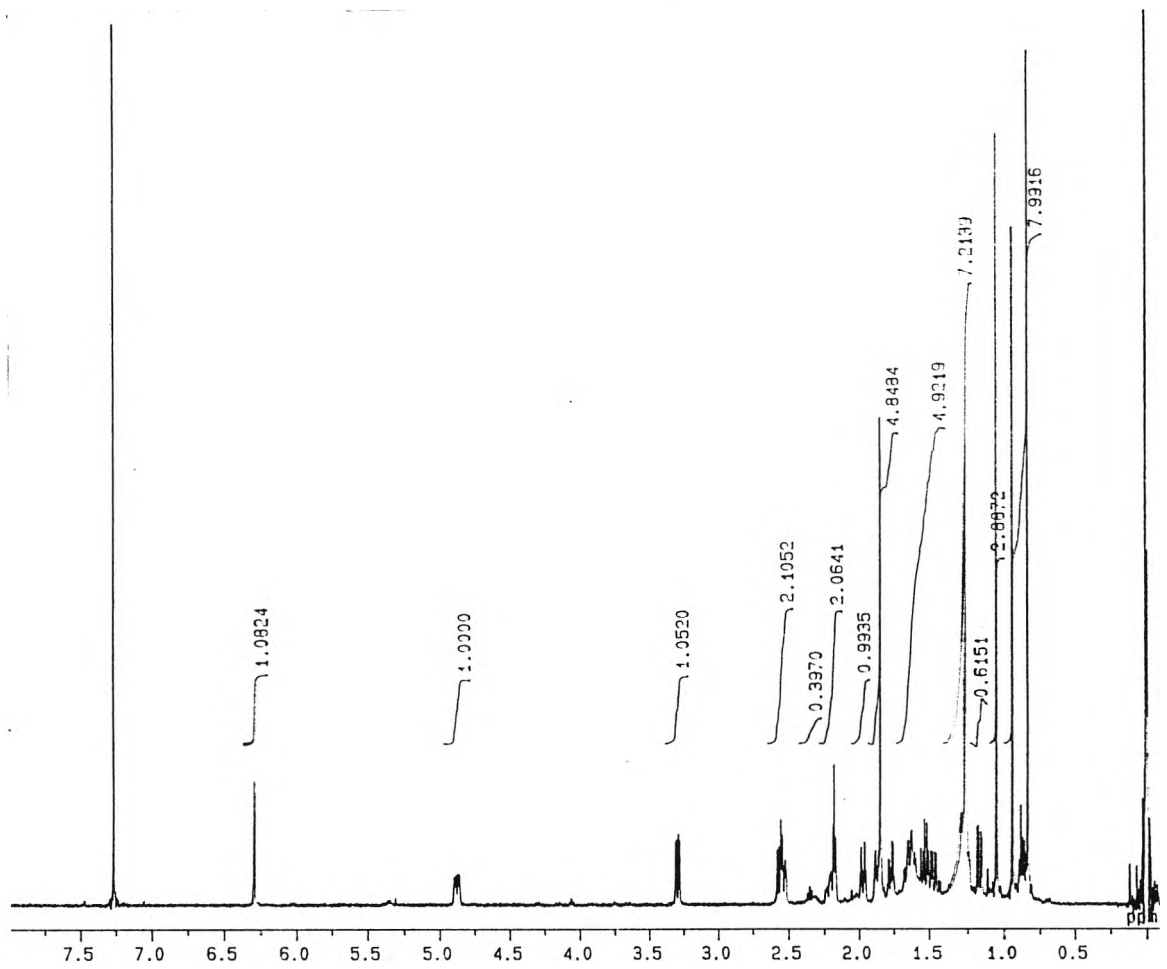


Figure 3.36 The ^{1}H NMR spectrum of Compound **6**

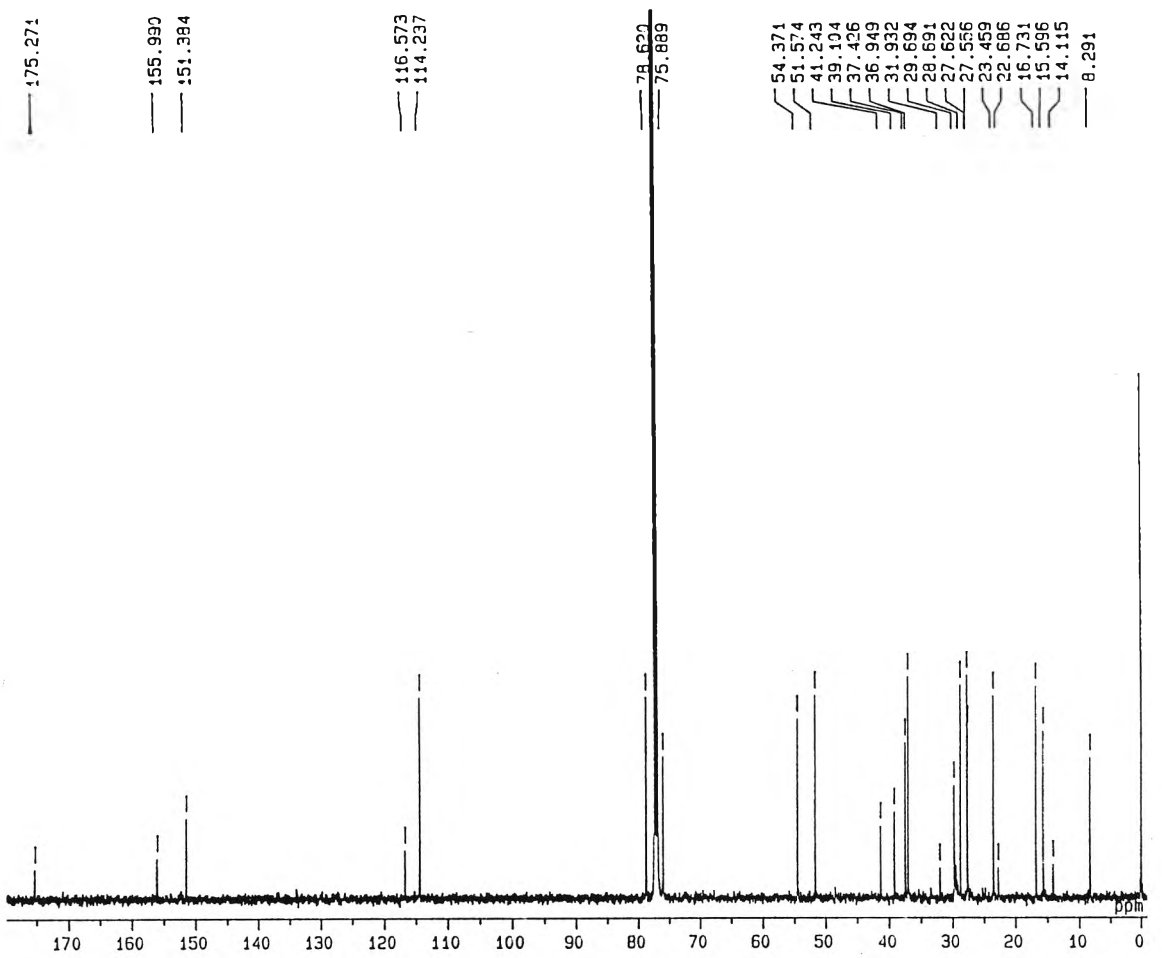


Figure 3.37 The ^{13}C NMR spectrum of Compound **6**

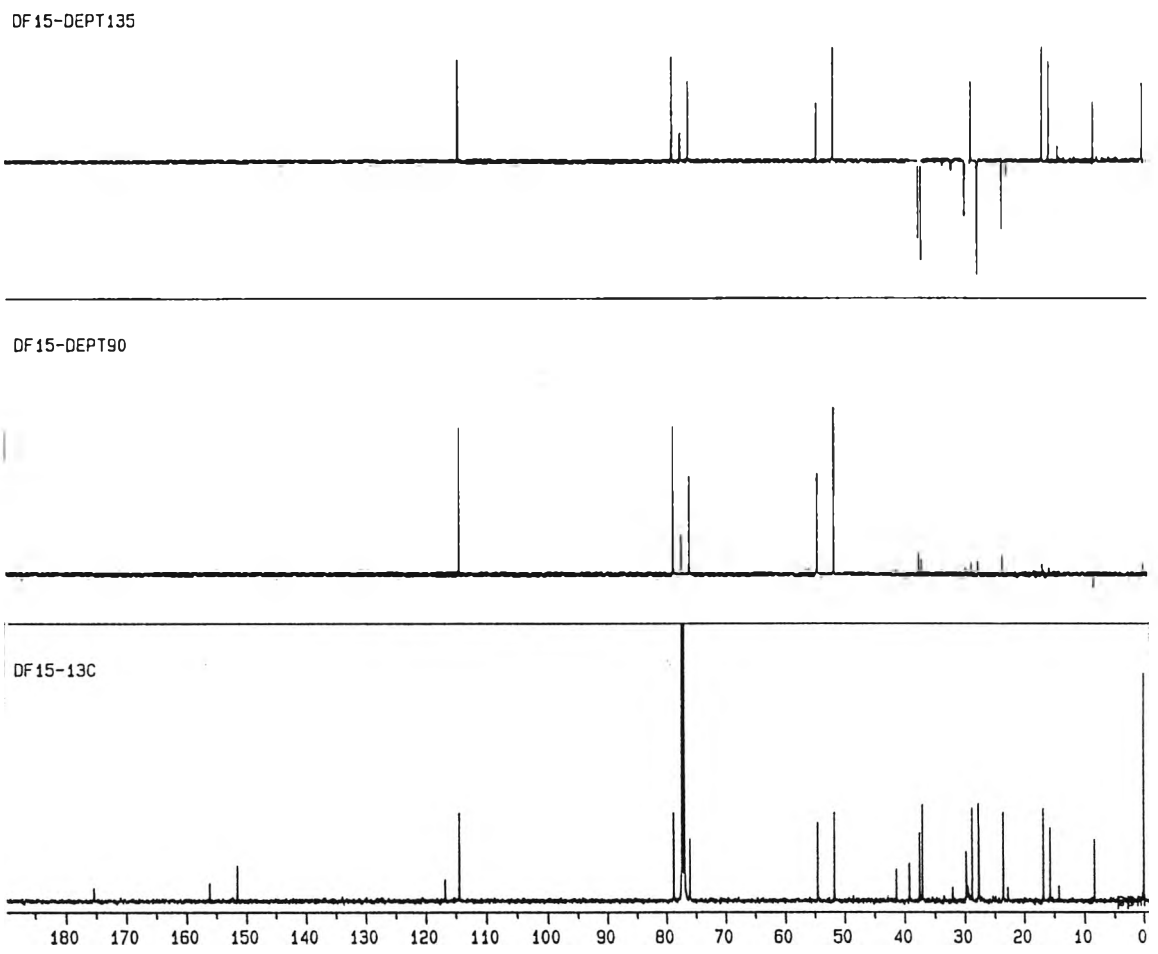


Figure 3.38 The DEPT 90 and 135 spectra of Compound **6**

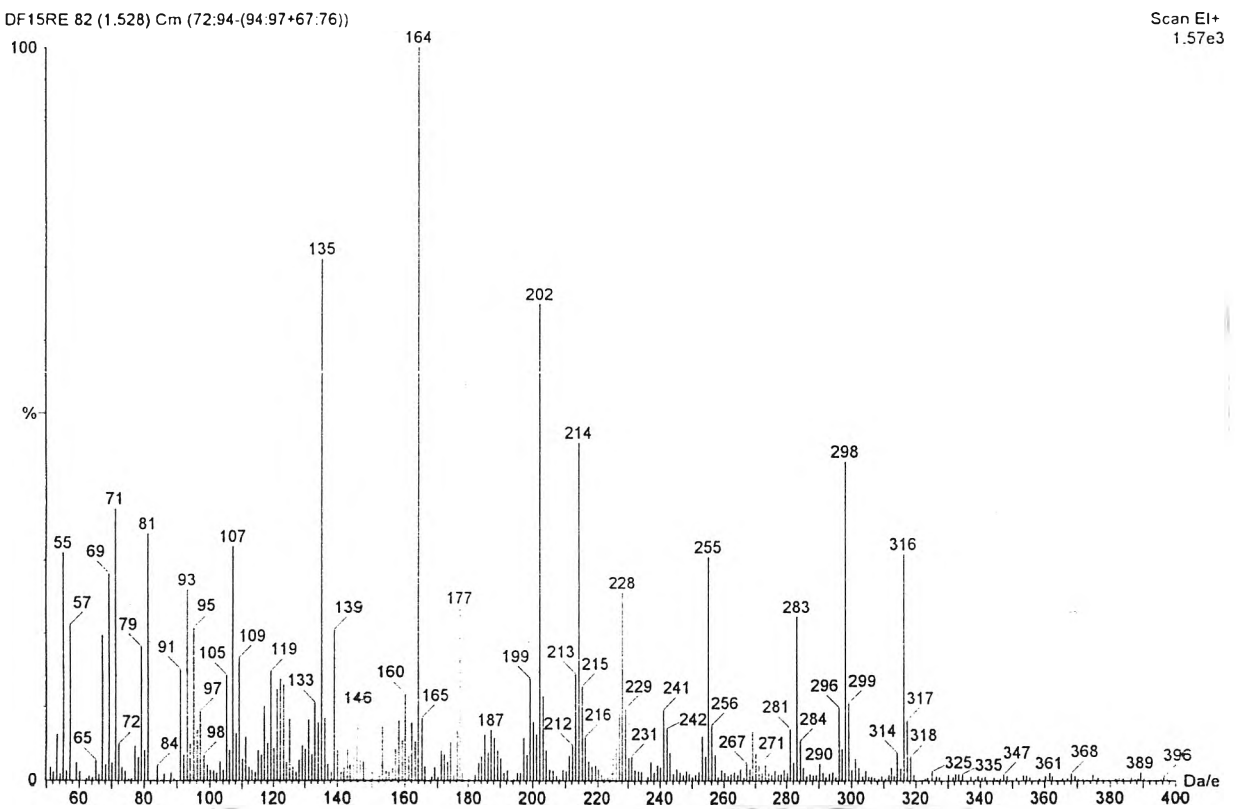


Figure 3.39 The Mass spectrum of Compound **6**

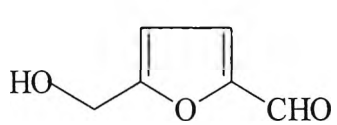
3.2.11 Structural elucidation of Compound 7

Compound 7 was orange viscous oil, which was obtained from ethyl acetate crude extract. This compound was eluted with 20% EtOAc in hexane and further purified by chromatotron, yielding 1.03×10^{-4} % (12.1 mg) of Compound **7**. It preformed a red spot with DNPH dipping reagent [R_f = 0.65 (1:4 hexane-EtOAc,silica gel)]. This suggested the presence of a carbonyl group and the IR spectrum (**Figure 3.40**) further specified this carbonyl would be an aldehyde and the characteristic C-H stretching at 2940 and 2840 cm^{-1}. In addition, there were absorption bands of hydroxy (3375 cm^{-1}) and furan ring (1028 cm^{-1}).

The ^1H NMR spectrum (**Figure 3.41** and **3.42**) also exhibited signals corresponded to those of already assigned carbons such as aldehyde and methylene protons resonated at δ 9.57 (1H, s) and 4.69 (2H, s), respectively. The signals of furano protons resonated at δ 6.49 (1H, d, J = 3.7 Hz) and 7.19 (1H, d, J = 3.4 Hz). Magnitude of coupling constants, 3.4 -3.7 Hz, resulted from the coupling of H-3 and H-4. Then, the disubstituted furan mentioned above was 2,5- disubstituted one. [49,50]

The ^{13}C NMR and DEPT 90, 135 spectra (**Figure 3.43 and 3.44**) showed the most downfield tertiary carbon of an aldehyde at δ 177.6 and methylene carbon which directly attached to oxygen atom at δ 57.6. Then, there were two substituent groups attached to furan ring, one group was certainly aldehyde and the another should be hydroxymethyl group ($HOCH_2$-). The four carbon signals at 110.0, 122.7, 152.4 and 160.6 ppm belonged to furan skeleton.

From the comparison of physical properties and all spectroscopic data with an authentic sample[51] allowed to assign Compound **7** as 5–hydroxymethyl furfuraldehyde or 5-HMF.



Compound 7 : 5-hydroxy methyl furfuraldehyde

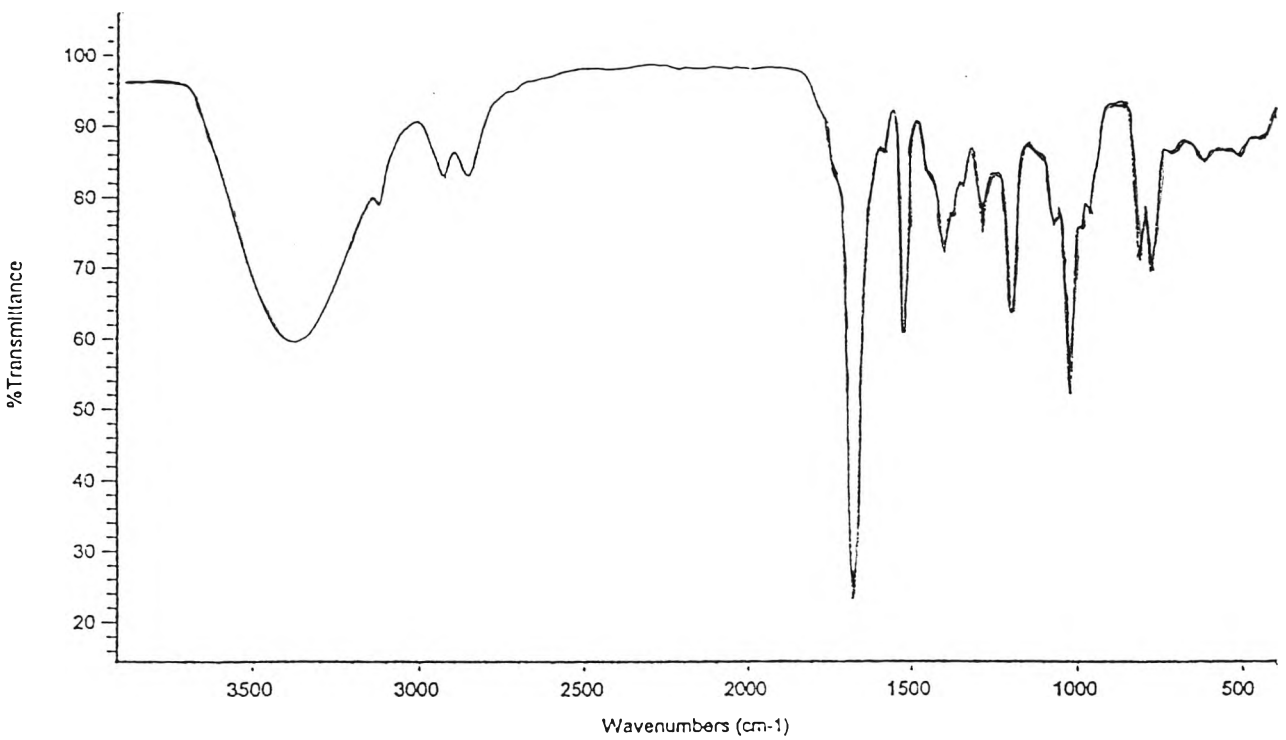


Figure 3.40 The IR spectrum of Compound **7**

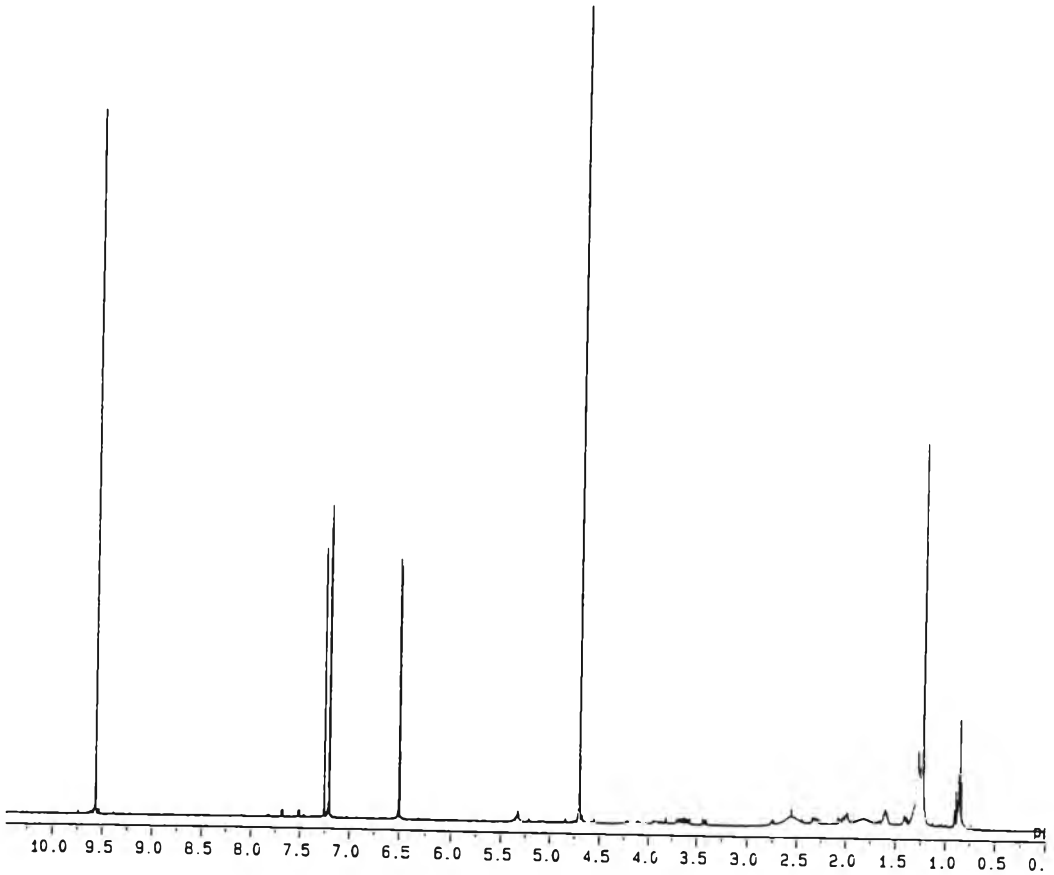


Figure 3.41 The ^{1}H NMR spectrum of Compound 7

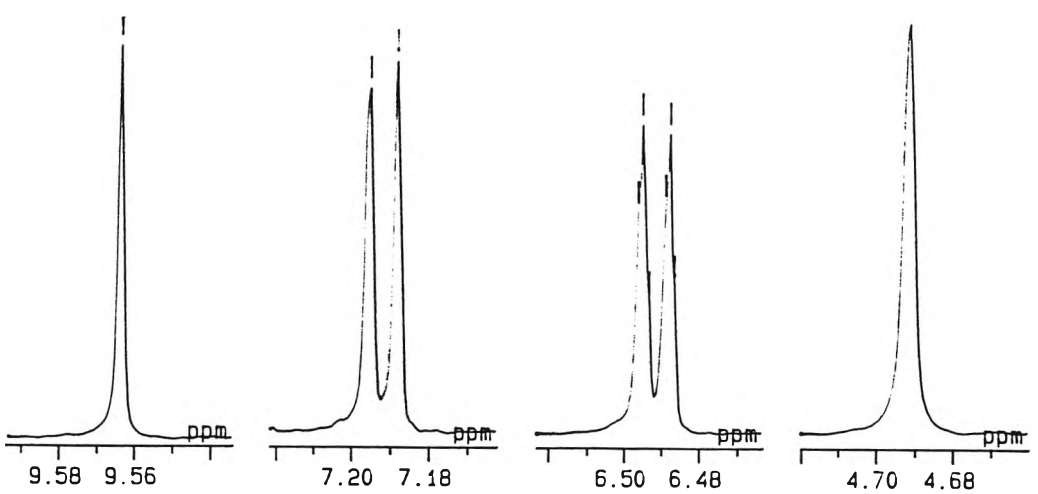


Figure 3.42 The Expansion of ^{1}H NMR spectra of Compound 7

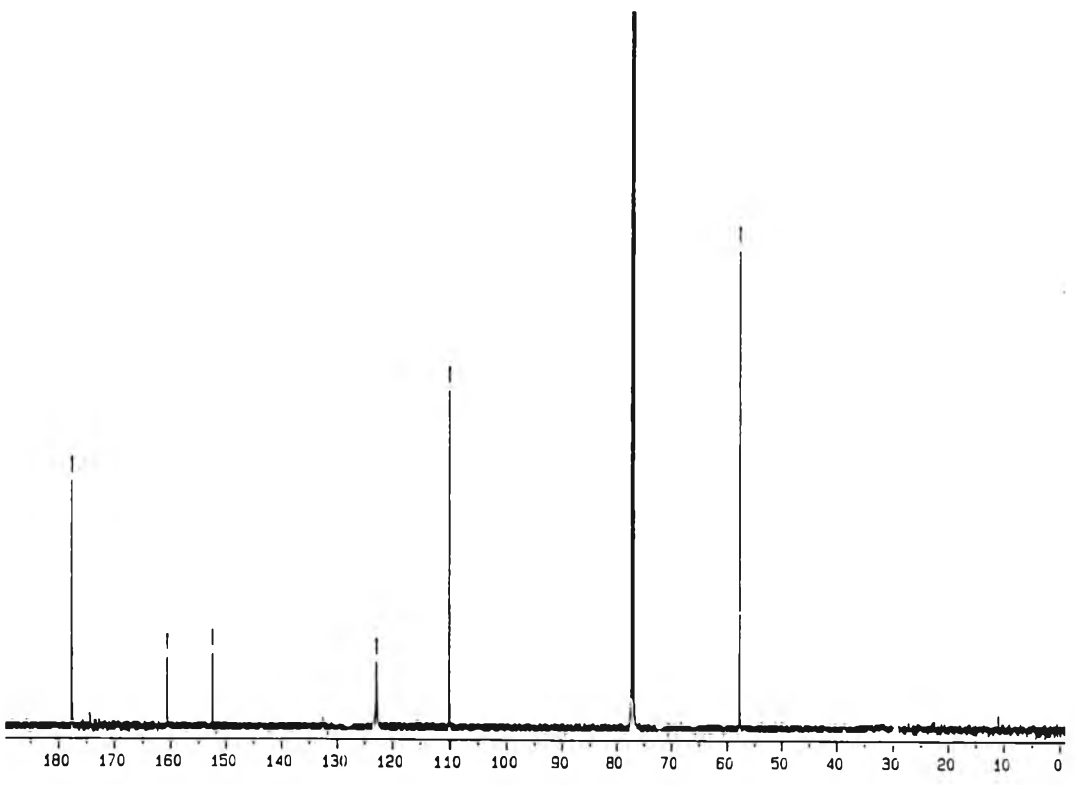


Figure 3.43 The ^{13}C NMR spectrum of Compound **7**

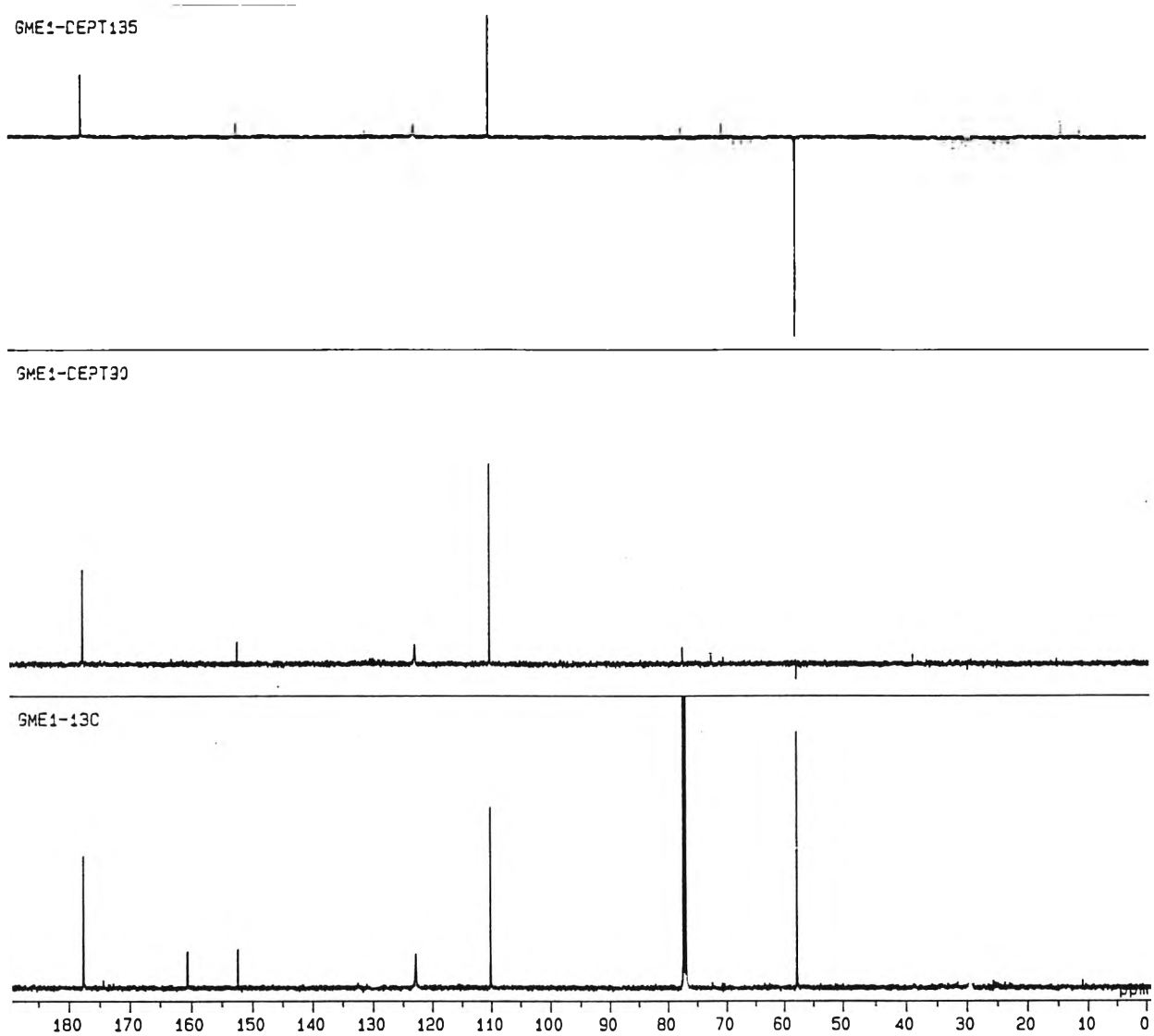


Figure 3.44 The DEPT 90 and 135 spectra of Compound **7**

3.2.12 Structural elucidation of Compound 8

Yellow needle crystal, yield 5.04×10^{-5} % (5.9 mg) of Compound **8** was obtained from EtOAc crude extract which was fractionated by column chromatography over silica gel eluting with 1:4 EtOAc/hexane. It was purified by PTLC (developed with 50% EtOAc in hexane). Compound **8** displayed bright blue fluorescence under UV lamp (365 nm) which suggested that it might be 7-hydroxycoumarins or 6, 7 – dialkoxycoumarins.[52]

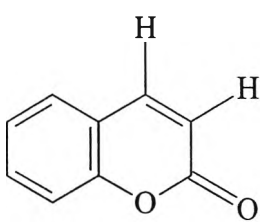
The IR spectrum (**Figure 3.45**) indicated the characteristic absorption band of O-H streching of hydroxy group at 3600-3200 cm^{-1}.

Molecular peak (**Figure 3.46**) at m/z 192 corresponded to a molecular formular $C_{10}H_8O_4$. The ion peak at m/z 177 suggested the presence of methyl group that might be a part of methoxy one.

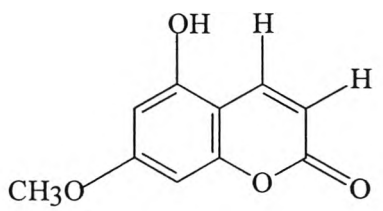
As the result of ^1H NMR spectrum (**Figure 3.47**), there were signals of protons at δ 3.96 (3H, s, $-OCH_3$), 6.16 (broad, OH), 6.27 (1H, d, *J*=9.5 Hz), 6.84(1H, s), 6.92 (1H, s), and 7.60 (1H, d, *J*=9.5 Hz).

From ^{13}C NMR and DEPT spectra (**Figure 3.48** and **3.49**), it showed one methoxy carbon at δ 56.4 ppm, four methine carbons at δ 103.2, 107.5, 113.4 and 143.3 ppm, four quaternary carbons at δ 111.5, 144.0, 149.7 and 150.3 including one carbonyl carbon at δ 161.4 ppm

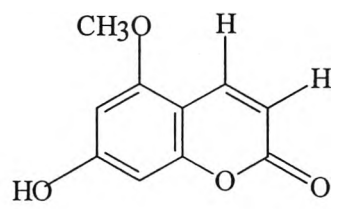
Other than the UV active results, there are the evidences to conclude that Compound **8** was the coumarin compound, such as the two doublet signals, at 6.27 and 7.60 ppm, with identical coupling constants (*J*=9.46 Hz) and carbonyl carbon, adjacent to O-heteroatom at δ 161.2 ppm, which the main structure of this compound is shown below.



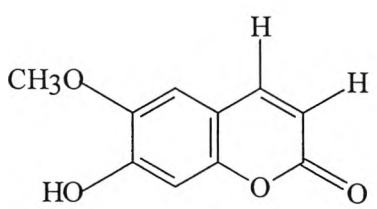
Additional methoxy group can be found in the ^{1}H and ^{13}C NMR spectra and hydroxy group can be observed in the IR spectrum, according with two substituents on the benzene moiety of the coumarin ring. The possible structure of this compound can be deduced as follow:



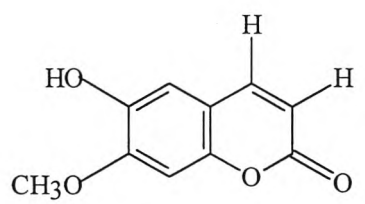
(a)



(b)



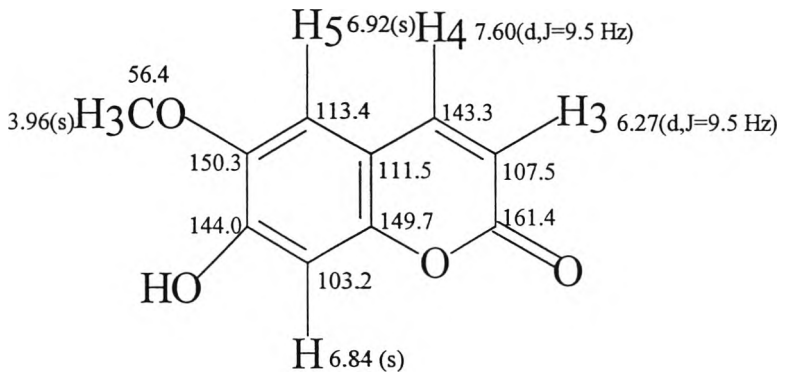
(c)



(d)

NOE difference (**Figure 3.50**) was a useful technique to determine which one was Compound **8.** Irradiation of the aromatic proton at δ 6.92 resulted in enhancement of H-4 at δ 7.60 ppm. This provided clear evidence that the irradiated proton belonged to H-5 ; therefore, structure(a) and (b) was canceled. Methoxy proton at δ 3.96 enhanced by the irradiation of H-5 could be ascribed to 6- OCH_3. Thus, the structure of Compound **8** should be the structure (c). Once again, irradiation of 6-OCH_3 led to merely one enhancement of H-5, indicated C-7 was then replaced by a hydroxy group. Similarly, irradiation of an aromatic proton at δ 6.84 caused no signal to rise. This unambiguously confirmed the presence of H-8 (δ 6.84). Mainly based upon spectral data, particularly NOE technique, Compound **8** was verified as 7-hydroxy-6-methoxycoumarin or scopoletin.

Complete proton and carbon assignments is shown below.



Compound **8** : **7-Hydroxy-6-methoxycoumarin (Scopoletin)**

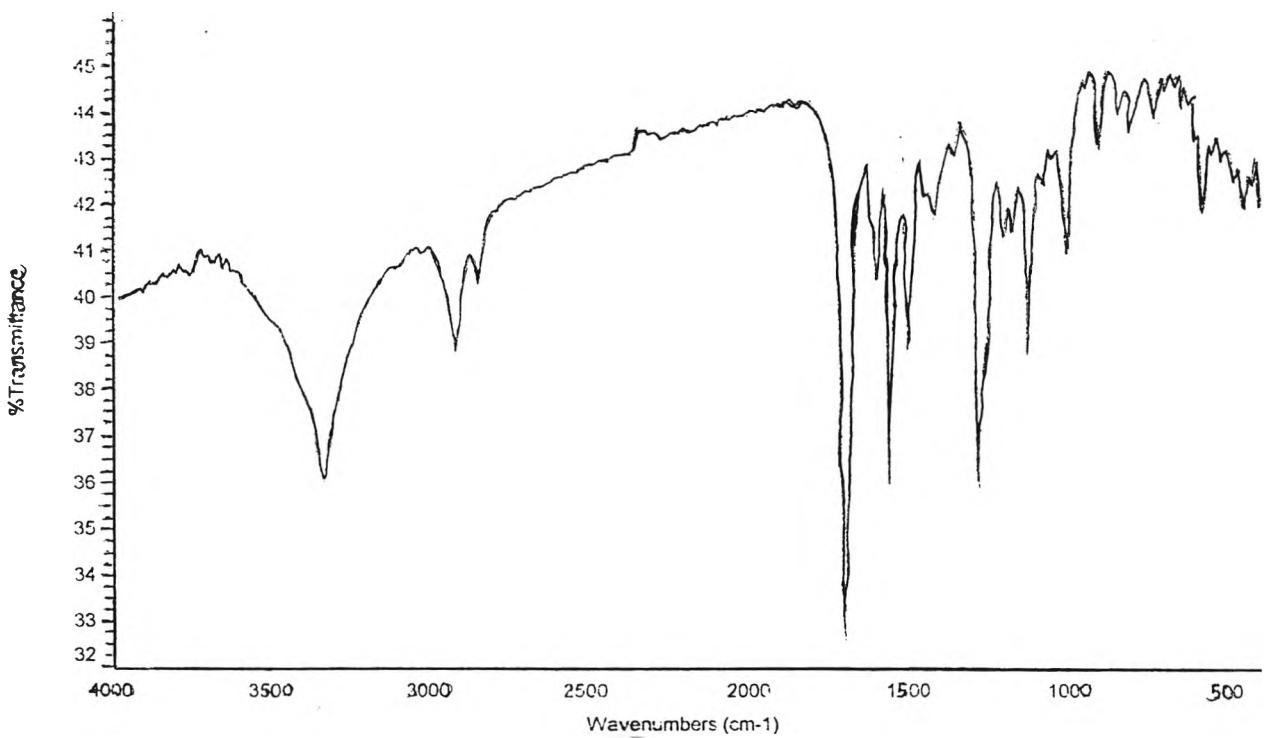


Figure 3.45 The IR spectrum of Compound **8**

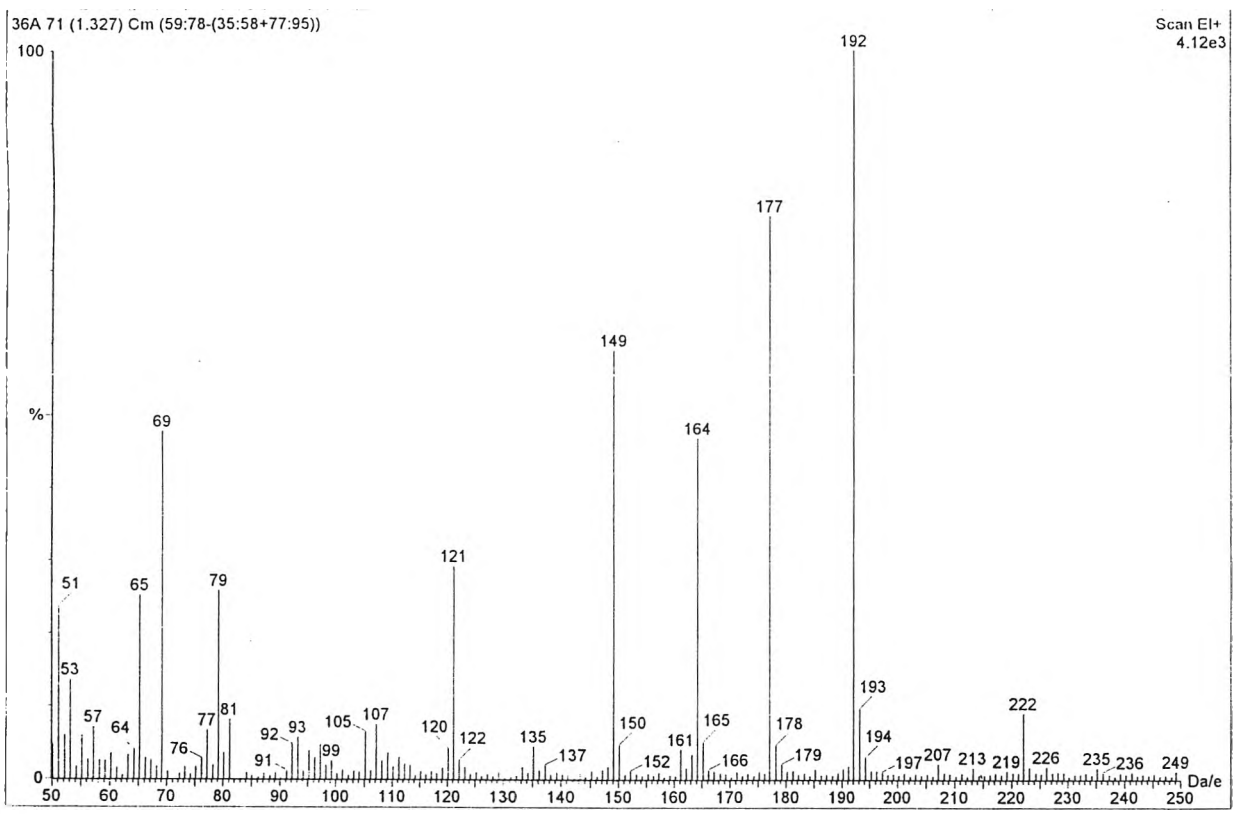


Figure 3.46 The Mass spectrum of Compound **8**

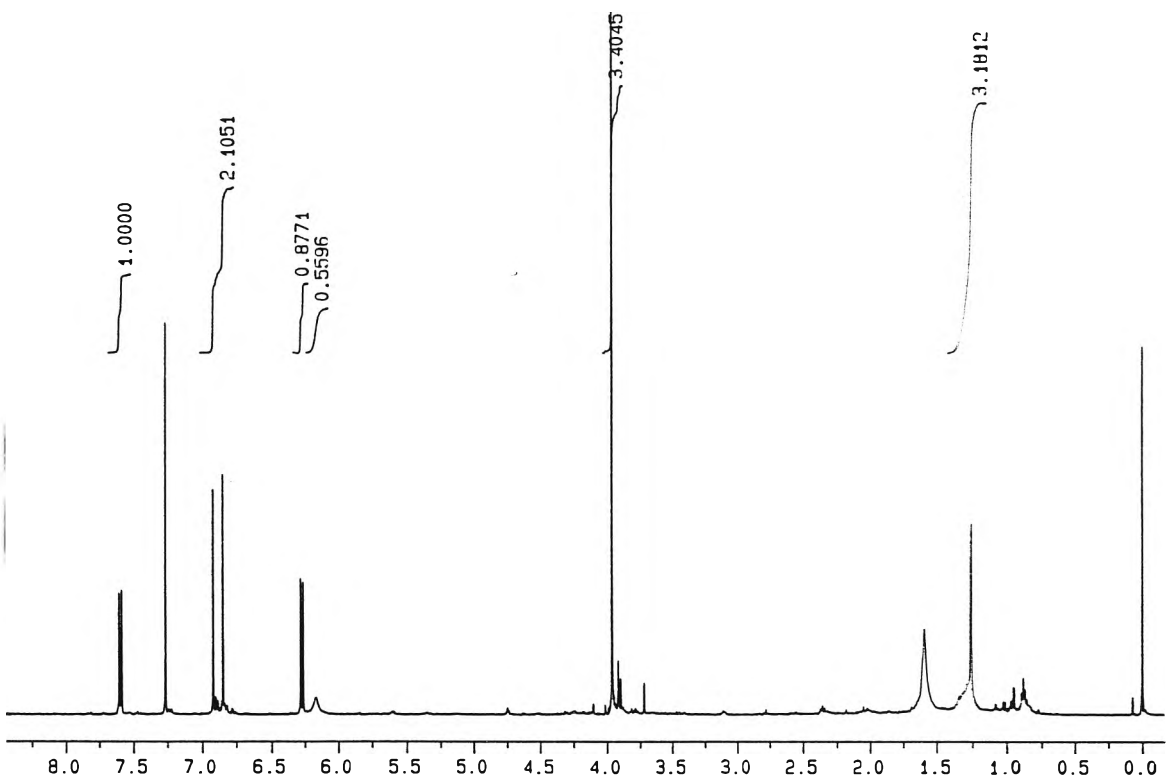


Figure 3.47 The ^{1}H NMR spectrum of Compound **8**

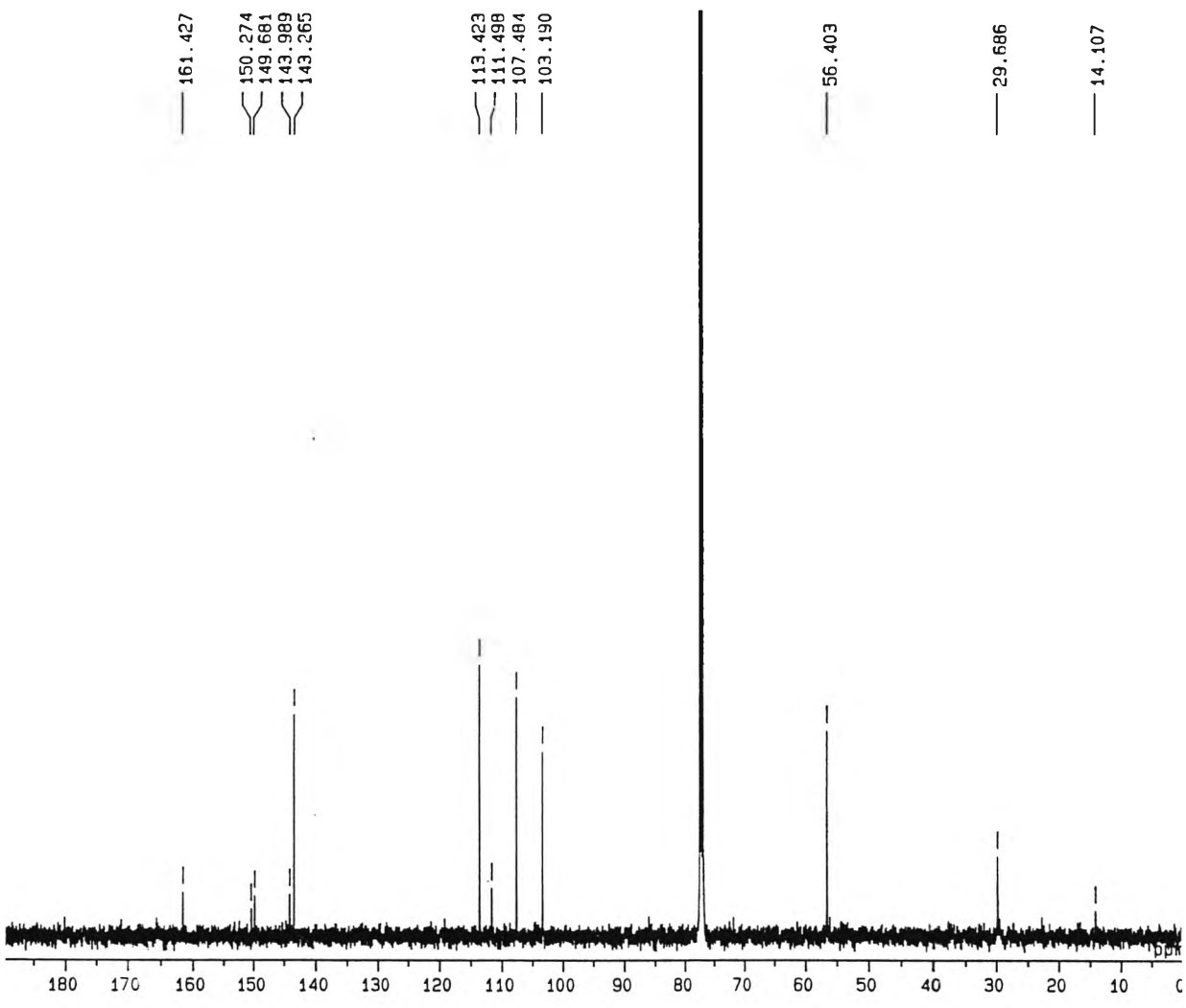


Figure 3.48 The ^{13}C NMR spectrum of Compound **8**

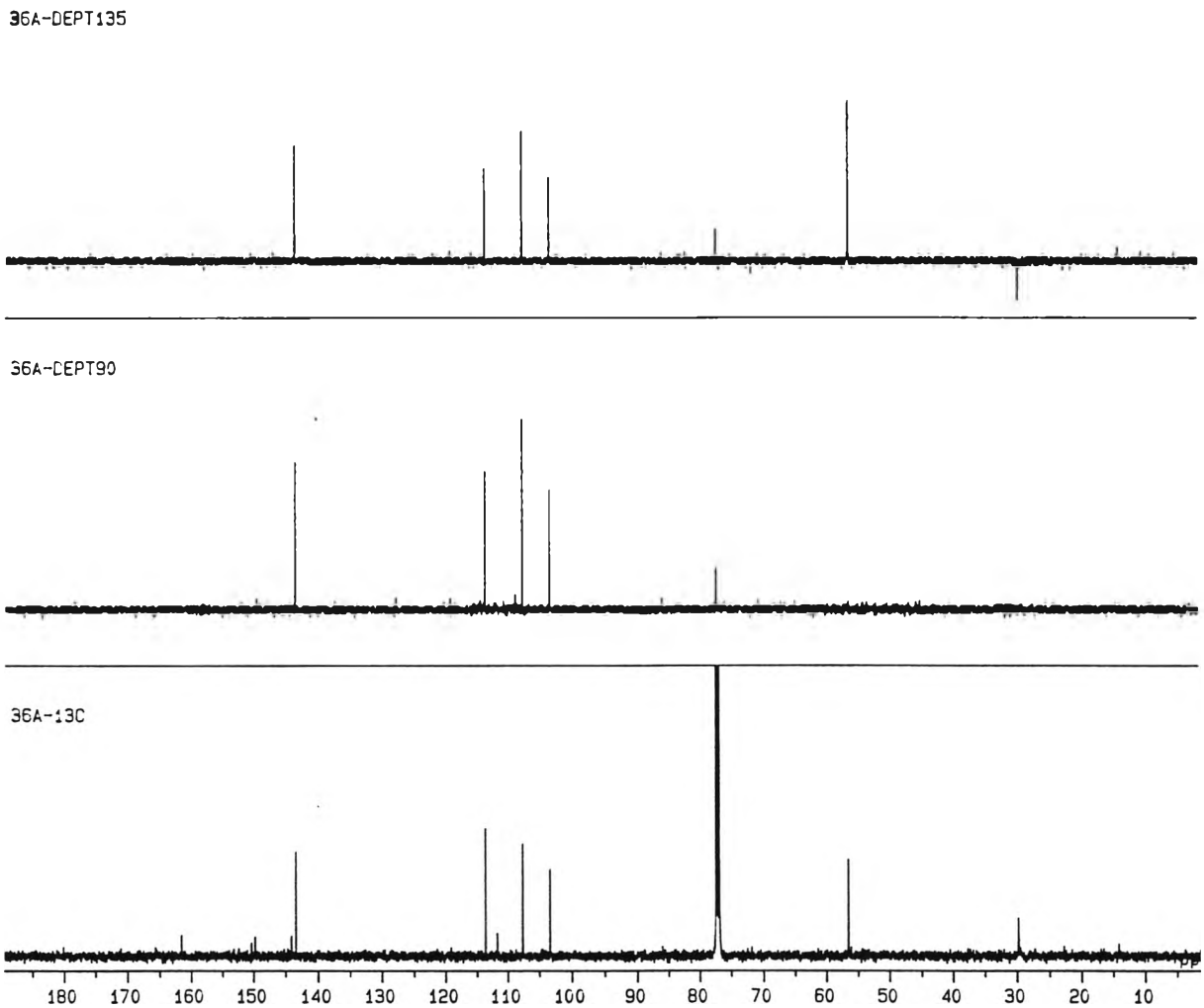
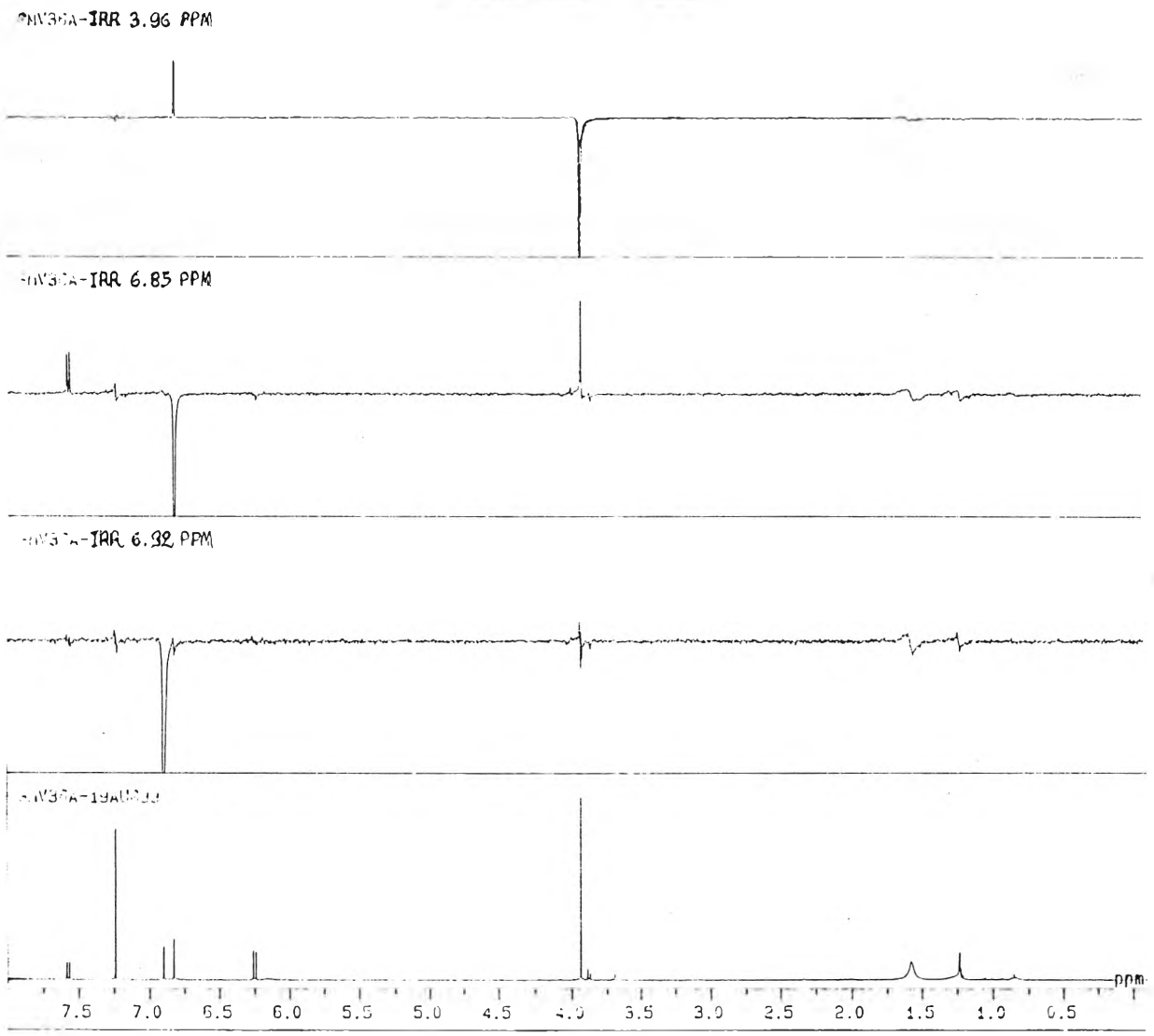


Figure 3.49 The DEPT 90 and 135 spectra of Compound **8**



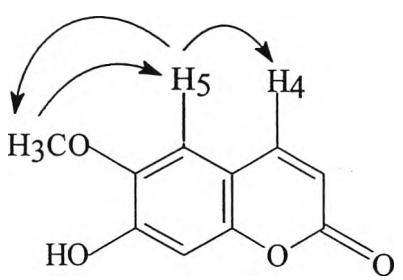


Figure 3.50 NOE Difference spectra of Compound **8**

3.3 The Results of Biological Activites of Isolated Compounds

3.3.1 A Microwell Cytotoxicity Test Result of Isolated Compounds

According to the preliminary cytotoxicity screening test, Dichloromethane and Ethyl acetate revealed medium activity against brine shrimp (*Artemia salina*). Thus, both of them were selected for further investigation of bioactive compounds. The isolated compound were tested the cytotoxic against brine shrimp. The results of test displayed in **Table 3.19**

Table 3.19 The Result of Brine Shrimp Cytotoxicity Lethality Test of Isolated Compounds

Isolated Compound	LC_{50} (µg / ml)	Bioactivity
Compound 2	38.33	medium activity
Compound 5	591.19	low activity
Compound 7	39.28	medium activity
Compound 8	13.31	medium activity

From **Table 3.19**, Compound **2, 7** and **8** showed the moderate cytotoxic lethality on brine shrimp.

3.3.2 Antioxidant Result of Isolated Compounds

After developed TLC with appropiate solvent, was sprayed with two standard reagents : DDPH for free radical scavenging activity and β-carotene for β-carotene bleaching activity.

Only Compound **8** showed the positive results both free radical scavenging and β-carotene bleaching activity.

Table 3.20 The Result of Antioxidant Test of Isolated Compounds

Isolated Compound	**Dose**	**%NBT induction**	**%XOD inhibition**	**%O^{-}_{2} scavenging**
Compound 1	500 μg	12.03	NE	12.03
Compound 2	50 μg	NE	NE	NE
	10 μg	NE	NE	NE
Compound 3	500 μg	NE	NE	NE
Compound 5	50 μg	2.8	12.8	NE
	10 μg	NE	NE	NE
Compound 8	500 μg	63.16	74.90	NE

NBT = Nitrobluetetrazolium

XOD = Xanthine Oxidase

NE = No Effect (Negative result)

3.3.3 Biological Activity Studies of Isolated Compounds

The isolation of the dichloromethane and ethyl acetate crude extracts obtained four triterpenoids : Bauerenol, α-Amyrin, Multiflorenol and Triterpenoid I (triterpene lactone), three diterpenoids : Helioscopinolide A, E (diterpene lactone) and Helioscopinolide M (new compound), along with 5-hydroxymethyl furfuraldehyde, 3-methoxy-4-propylbenzaldehyde,7-hydroxy-6-methoxycoumarin (scopoletin) and stigmasterol.

Compound **1** (Stigmasterol) did not exhibit interesting activities on brine shrimp and antioxidant test.

Compound **2**, the bioassay results showed the medium cytotoxicity on brine shrimp (LC_{50} 38.33 μg/ml) and had no antioxidant activity. Because of the small amount of this compound further elucidation of the structure was not possible.

Compound **3** and **4** were obtained in a very small amount, therefore further investigation for biological activities were not carried out.

Compound **5** (helioscopinolide E) did not exhibit significant cytotoxic against brine shrimp(*Artemia salina* Linn.) and the negative results were obtained in both antioxidant activity tests. From literature surveys, helioscopinolide E were obtained from *Euphorbia helioscopia*[45] and produced in trace amounts by wile type cell cultures of *Euphorbia calyptrata*[46] . Besides, this compound possessed a significantly mild and short depressant effect on the CNS[47].

Compound **6** was reported to be isolated from *Euphorbia helioscopia*[45] and both root and cell cultures of *Euphorbia calyptrata*[46] . When, helioscopinolide A were investigated and found to have an opposite excitatory effect on the CNS[47]. However, due to the small amount of this compound, further investigation for biological activities were not carried out.

Compound **7** was identified as 5-hydroxymethyl furfuraldehyde (5-HMF)[48]. From literature surveys, 5-HMF has been obtained from several plants and their pharmacological activities was interesting[49,50]. It was used as an anthelmintic against *Clenorchia sinensis* (Chinese liver fluks) and used as sunburn-prevention containing cosmetics reacted with monohydroxy-1, 4-naphthoquinones. Furthermore, it show the interesting activity on antibacterial activity[50].

Compound **8** exhibited significant medium cytotoxic lethality on brine shrimp (LC_{50} 13.31 μg/ml) and the positive result in both antioxidant activity tests, which might lead to being antioxidant substance. Nevertheless, this compound should be further tested for specific antioxidative bioassay and anticell lines activity.

Because of the small amount of most compounds, further investigation of biological activities were not carried out completely.